

Instytut Chemii Bioorganicznej
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**Badanie właściwości przeciwnowotworowych kwasów
nukleinowych i ich pochodnych**

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wykonana pod kierunkiem
dr hab. Katarzyny Rolle, prof. ICHB PAN
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STRESZCZENIE

Skuteczna terapia chorób nowotworowych jest obecnie jednym z najpoważniejszych wyzwań współczesnej medycyny. Z tego też powodu, poszukiwanie nowych rozwiązań terapeutycznych skutkujących zwiększeniem selektywności i aktywności substancji używanych w terapii nowotworów jest jednym z priorytetów współczesnej onkologii.

Celem badań podjętych w ramach niniejszej pracy doktorskiej było pogłębienie wiedzy na temat nowych strategii terapeutycznych chorób nowotworowych, szczególnie glejaka wielopostaciowego (GBM) poprzez identyfikację i charakterystykę kluczowych dla rozwoju nowotworu cząsteczek o potencjalnym znaczeniu diagnostycznym, prognostycznym i terapeutycznym, jak również opracowanie i charakterystykę narzędzi i podejść o potencjale terapeutycznym.

W ramach niniejszej rozprawy doktorskiej powstała biblioteka związków niskocząsteczkowych składająca się z 250 unikalnych struktur chemicznych (analogi nukleozydów i nukleotydów, pronukleotydy zwitterjonowe, fluorowane α -aminofosfoniany), które następnie scharakteryzowano pod względem właściwości fizykochemicznych oraz aktywności biologicznej. Szeroko zakrojona skala analizy, jak również wysoka jakość otrzymanych wyników, pozwoliła wyselekcjonować 11 związków o wysokiej i selektywnej aktywności względem GBM, których zastosowanie oraz wyjaśnienie mechanizmu działania będzie kontynuowane w perspektywie dalszego rozwoju naukowego.

RNA podobnie jak białka tworzy różne struktury przestrzenne, które mogą stanowić cel terapeutyczny dla niskocząsteczkowych ligandów. Podczas realizacji drugiej części pracy, analizowano dojrzałe miRNA pod względem ich struktury I- i II-rzędowej w celu przyszłego wykorzystania już scharakteryzowanych i wyselekcjonowanych związków do obniżenia ekspresji onkogennych miRNA biorących udział w progresji nowotworu. Przeprowadzona analiza sekwencji nukleotydowych 2042 dojrzałych miRNA wykazała bezpośrednią zależność pomiędzy sekwencją miRNA, a rodzajem regulowanych przez nie docelowych mRNA. Przedstawiona charakterystyka dojrzałych miRNA może mieć kluczowe znaczenie dla wyboru związków niskocząsteczkowych o charakterze terapeutycznym oraz może wpłynąć na selekcję związków do dalszego testowania w modelach *in vitro* i *in vivo* już na etapie projektowania strategii terapeutycznej.

W ramach kolejnego projektu, skupiono się na zwiększeniu efektywności dostarczania dwuniciowego RNA (dsRNA) o sekwencji homologicznej do tenascyny-C (ATN-RNA) w terapii guzów mózgu. Jest to białko macierzy zewnątrzkomórkowej, mające udział w adhezji, inwazyjności oraz migracji komórkowej. W wyniku przeprowadzonych badań opracowano nowy system dostarczania ATN-RNA do komórek GBM w celu obniżenia ekspresji tenascyny-C, który wykorzystuje dobrze scharakteryzowane nanocząstki magnetyczne pokryte polietylenoiminą. Zarówno same nanocząstki, jak i ich kompleksy z ATN-RNA nie wykazują toksyczności oraz nie wywołują odpowiedzi immunologicznej w linii komórkowej GBM.

Dodatkowym wynikiem prowadzonych w ramach niniejszej rozprawy doktorskiej było opracowanie testu do badania mechanizmu fosforylacji rybonukleozydów purynowych przez kinazę adenozynową, który może zostać wykorzystany w dalszych badaniach przesiewowych w celu poszukiwania nowych inhibitorów kinazy adenozynowej.

Wyniki uzyskane w ramach mojej pracy doktorskiej zostały opublikowane w siedmiu recenzowanych publikacjach eksperymentalnych oraz jednej przeglądowej.

SUMMARY

Effective cancer therapy is one of the most serious challenges in modern medicine. For this reason, the search for new therapeutic solutions resulting in increased selectivity and activity of cancer drugs is of the highest priority.

Based on the identification and characterization of key molecules important to the development of cancer this PhD thesis work was aimed at developing diagnostic, prognostic and therapeutic strategies, as well as the characterization of tools and approaches with therapeutic potential. The outcome is new knowledge for therapeutic strategies to treat human cancers, in particular, glioblastoma multiforme (GBM).

A chemical library of small-molecules was created consisting of 250 unique chemical structures (nucleoside and nucleotide analogues, zwitterionic pronucleotides, fluorinated α -aminophosphonates), which were further characterized in terms of physicochemical properties and biological activity. Eleven compounds were identified with high and selective activity against GBM, whose application and explanation of the mechanism of action will be continued from the perspective of further scientific development.

In another section of this thesis, mature miRNAs were analysed for their primary and secondary structures and their potential in regulating cancer cell function. The analysis of 2042 mature miRNA nucleotide sequences showed a direct relationship between the miRNA sequence and the type of target mRNA they regulate. The wide analysis of mature miRNA sequences was high-throughput and overcomes uncertainties in selecting the most appropriate molecules which are at the beginning of therapeutic strategy design. In the future, already characterized and selected compounds can be explored to reduce the expression of oncogenic miRNAs involved in tumour progression.

Further analyses were focused on increasing the efficiency of double-stranded RNA (dsRNA) delivery with homological sequences to the mRNA of tenascin-C (named ATN-RNA) for the treatment of brain tumours. Tenascin-C is an extracellular matrix protein that is involved in cancer cell adhesion, invasion, and migration. We have successfully demonstrated a new dsRNA delivery system that harnesses well-characterized magnetic nanoparticles coated with polyethyleneimine to effectively silence expression of tenascin-C in GBM. Both mere nanoparticles and their complexes with dsRNA do not show toxicity and do not provoke undesired immune responses in GBM cell line.

Lastly, was an additional result of the doctoral project. An enzymatic assay was developed to test the mechanism of phosphorylation of purine ribonucleosides by adenosine kinase. This assay can be used in further screening in search of new adenosine kinase inhibitors.

The results presented in this doctoral thesis have been published in seven peer-reviewed experimental articles and one review.

OPIS WYNIKÓW PRACY DOKTORSKIEJ

WPROWADZENIE

Zgodnie ze statystykami Światowej Organizacji Zdrowia (ang. *World Health Organization*, WHO) choroby nowotworowe są drugą po chorobach układu krążenia przyczyną zgonów na świecie. Trend ten utrzymuje się już od wielu lat. Współczesne analizy oraz obserwacje wskazują, że w kategorii struktury zachorowań i zgonów obecna dekada nie będzie już jednak należeć do chorób układu sercowo-krążeniowego, pierwszą pozycję na tej liście zajmą nowotwory złośliwe.

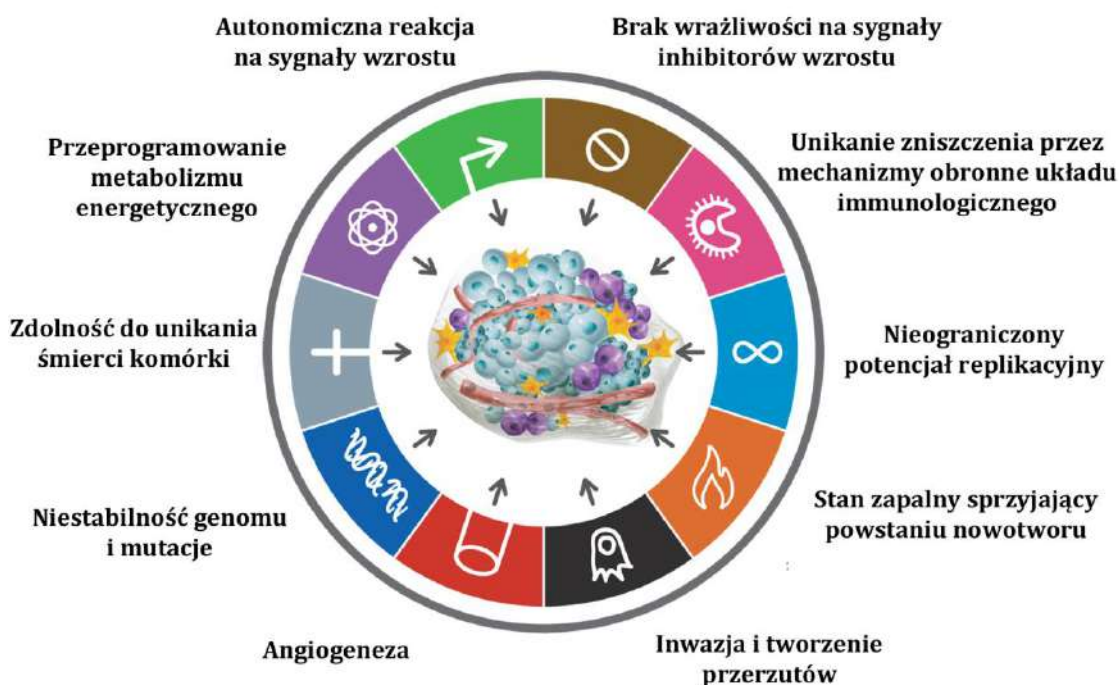
Nowotwory ośrodkowego układu nerwowego (OUN) stanowią zaledwie 2% wszystkich zarejestrowanych przypadków guzów pierwotnych. Pomimo tak niskiej częstotliwości występowania, odpowiedzialne są jednak za 7% zgonów wynikających z zapadalności na chorobę nowotworową przed 70 rokiem życia (Vigneswaran, 2015). Pierwotne nowotwory glejowe (glejaki) są najczęściej występującymi guzami w OUN. Odpowiadają za powstanie ponad 32% wszystkich guzów mózgu (Jovcevska, 2013). Zmienione nowotworowo komórki glejowe dają początek guzom glejowym, z których najbardziej złośliwym, opornym na leczenie i źle rokującym jest glejak wielopostaciowy (ang. *glioblastoma multiforme*, GBM). Rokowanie u pacjentów z GBM jest złe, średni czas przeżycia wynosi 7-12 miesięcy, a tylko około 3-5% chorych przeżywa ponad 3 lata (Krex, 2007). Pod względem histologicznym GBM charakteryzuje się występowaniem słabo zróżnicowanych neoplastycznych komórek astrocytarnych, komórkowej i jądrowej atypii, zakrzepicy naczyniowej, intensywnej aktywności mitotycznej, angiogenezy, ograniczonej apoptozy oraz ognisk martwicy (Adamson, 2009). Skuteczne leczenie złośliwych guzów jest trudne do opracowania z szeregu powodów wynikających z deregulacji wielu szlaków komórkowych (Alifieris, 2015), występowania populacji komórek infiltrujących, które bardzo trudno jest usunąć chirurgicznie oraz obecności bariery krew-mózg (ang. *blood-brain barrier*, BBB) często uniemożliwiającej skuteczne docieranie leków do komórek glejaka (Alphandery, 2018). Ponadto, wczesna diagnoza GBM, która mogłaby poprawić skuteczność leczenia, umożliwiając usunięcie guzów o małych rozmiarach, nie jest przeprowadzana rutynowo. Pomimo wielu wysiłków guzy te są nadal śmiertelne i chociaż GBM rzadko daje przerzuty poza ośrodkowy układ nerwowy (ok. 0,5% przypadków), są bardzo inwazyjne i niszczą przylegające tkanki, co przyczynia się do szybkiego postępu choroby, niskiej jakości życia oraz ostatecznie do bardzo wysokiej śmiertelności (Azizi, 1998; Kleihues, 2002). Brak wrażliwości glejaka wielopostaciowego na konwencjonalne metody leczenia oraz wyjątkowo

skomplikowane podłoże molekularne tego nowotworu skłaniają do poszukiwania nowych cząsteczek terapeutycznych, jak również nowych metod terapii (Verhaak, 2010)

Chociaż leżące u podstaw procesu nowotworzenia szlaki komórkowe i molekularne mogą się różnić, efekt jest jednak taki sam. W roku 2000, po wieloletnich badaniach, Hanahan i Weinberg ze Szwajcarskiego Instytutu Badań Eksperymentalnych nad Rakiem (Swiss Institute for Experimental Cancer Research, ISREC) zdefiniowali sześć cech charakterystycznych dla większości nowotworów. Zauważyli, że do kancerogenezy niezbędne są: (1) nabycie przez komórki zdolności autonomicznego wytwarzania czynników wzrostu, (2) brak wrażliwości na czynniki indukujące apoptozę, (3) niewrażliwość na inhibitory wzrostu, (4) zdolność do angiogenezy, (5) nieograniczony potencjał replikacyjny oraz (6) naciekanie i tworzenie przerzutów (Hanahan, 2000). Po jedenastu latach zmodyfikowali swoją koncepcję włączając do niej dwie dodatkowe cechy umożliwiające nabycie sześciu cech opisanych wcześniej, tj. (7) niestabilność genomu oraz (8) stan zapalny sprzyjający powstawaniu nowotworów (Rycina 1). Ponadto, w swojej pracy przedstawili dwie tzw. cechy potencjalne: (9) przeprogramowanie metabolizmu energetycznego komórek nowotworowych oraz (10) zdolność unikania niszczenia ich przez mechanizmy obronne układu immunologicznego (Hanahan, 2011). Chociaż istnieją dowody znaczącej roli tych dwóch ostatnich z wymienionych procesów w kancerogenezie, wciąż określa się je jako cechy potencjalne, ponieważ ich powiązanie z wymienionymi wcześniej, już ustalonymi ośmioma cechami charakterystycznymi dla procesu nowotworzenia wymaga dalszych badań (Luengo, 2017; Vander Heiden, 2011; Vander Heiden, 2017). Dzięki cechom (1-8) komórki nowotworowe stają się komórkami autonomicznymi oraz mogą tworzyć nowe relacje z niektórymi komórkami prawidłowymi, tworzącymi swoiste mikrośrodowisko nowotworowe umożliwiające progresję nowotworową (Joyce, 2005). Z przedstawionych powyżej podstawowych informacji dotyczących cech nowotworu wynika wiele istotnych klinicznie zagadnień, które mogą wytyczać mapę drogową rozwoju nowych czynników terapeutycznych w leczeniu nowotworów (Bailon-Moscoso, 2014).

Poszukiwanie nowych terapii glejaka wielopostaciowego w dużej mierze koncentruje się na celach molekularnych odkrytych dzięki szeroko zakrojonej analizie genomowej, takiej jak Atlas Genomu Nowotworów (ang. *The Cancer Genome Atlas*, TCGA). Znamionną cechą GBM są anomalie molekularne zaburzające m.in. (1) szlaki sygnałowe zaangażowane w regulację wzrostu tj. EGFR/PDGFR/FGFR/MAPK/PI3K, (2) cykl komórkowy, (3) autofagię, (4) naprawę DNA i apoptozę, (5) regulację angiogenezy oraz (6) punkty kontrolne odpowiedzi immunologicznej (Zhou, 2019). Niestety, spersonalizowane terapie

ukierunkowane na te zmiany genomowe nie odniosły jeszcze sukcesu klinicznego, być może z powodu intensywnej heterogenności wewnątrznowotworowej charakteryzującej GBM (Qazi, 2017).



Rycina 1. Dziesięć znamion charakterystycznych dla komórek nowotworowych. Opracowanie graficzne ryciny – mgr inż. Mateusz Starosta, na podstawie (Hanahan, 2011).

Jak już wspomniano, GBM stanowi archetypowy przykład heterogennego nowotworu złośliwego, dzięki obecności komórek macierzystych glejaka (ang. *glioblastoma stem cells*, GSC), które są źródłem pełnej hierarchii zróżnicowanych komórek guza (ang. *differentiated glioblastoma cells*, DGC) wynikającej z ich wysokiego potencjału do różnicowania się i zdolności do podziałów (Patel, 2014). Znaczenie kliniczne GSC potwierdza ich odporność na konwencjonalną chemioterapię i radioterapię w porównaniu z DGC (S. Bao, 2006; Liu, 2006). Rola GSC nie ogranicza się jednak do inicjacji i progresji nowotworu. Komórki macierzyste glejaka uczestniczą także w angiogenezie, inwazji oraz supresji układu immunologicznego, napędzając agresywny fenotyp GBM (Cheng, 2013). GSC są w stanie kreować dogodne dla swojego rozwoju warunki mikrośrodowiska m in. poprzez przeprogramowanie metabolizmu energetycznego (X. Wang, 2019). Cecha ta jest wynikiem bezpośredniej i/lub pośredniej aktywacji onkogennej (np. amplifikacja MYC, delecja PTEN,

mutacja TP53) mającej na celu zapewnienie komórkom dostatecznie dużej ilości składników odżywczych i energii do nieustannej proliferacji (Ward, 2012). W przypadku GBM mutacje genetyczne i zmiany metaboliczne oddziałują bilateralnie (DeBerardinis, 2016; Venneti, 2017). Onkogenne ścieżki sygnałowe, w tym kinazy 3-fosfatydylinozytolu (PI3K) i receptora naskórkowego czynnika wzrostu (EGFR) promują glikolizę beztlenową, znaną jako efekt Warburga (Warburg, 1956), aby umożliwić szybką proliferację i progresję komórek nowotworowych (Lunt, 2011). Z drugiej strony, przeprogramowanie metaboliczne moduluje epigenom i globalną ekspresję genów, np. mutacje dehydrogenazy izocytrynianowej (IDH) w glejakach prowadzą do wytworzenia onko-metabolitu - 2-hydroksyglutaranu, powodującego globalną hipermetylację histonów i DNA w celu promowania onkogenezy (Lu, 2012; Luengo, 2017; Turcan, 2012). Mechanizmy prawidłowego podziału komórek wymagają ciągłego dostarczania nukleotydów do syntezy DNA i RNA. Nukleotydy są syntetyzowane z amfibolicznych związków pośrednich, pochodzących z produktów metabolizmu komórkowego (synteza *de novo*) lub przez ponowne wykorzystanie wolnych, istniejących już zasad (szlak rezerwowy; ang. *salvage pathway*). W przeciwieństwie do komórek nieproliferujących, komórki proliferujące, takie jak komórki układu odpornościowego czy komórki nowotworowe, są predysponowane do korzystania ze szlaku syntezy *de novo* (Lane, 2015; Tong, 2009).

Termin „magiczna kula” (ang. „*magic bullet*”), wymyślony przez bakteriologa Paula Ehrlicha pod koniec XIX wieku, pierwotnie opisywał substancję chemiczną zdolną do specyficznego niszczenia mikroorganizmów. W poszukiwaniu takiego „magicznego pocisku” na początku dwudziestego stulecia Ehrlich rozpoczął prawdopodobnie pierwszy program przesiewowy, którego celem było znalezienie leku na syfilis. Jego koncepcja celowanej terapii została następnie rozszerzona o leczenie nowotworów i została z powodzeniem zastosowana do opracowania innowacyjnych strategii terapeutycznych o bardziej specyficznych mechanizmach działania niż konwencjonalne środki chemioterapeutyczne (Chabner, 2005). Takie terapie celowane molekularnie obejmują związki niskocząsteczkowe, przeciwciała monoklonalne (mAb), peptydomimetyki oraz oligonukleotydy antysensowne (Sawyers, 2004). Postęp w zrozumieniu roli nieprawidłowych szlaków sygnałowych w różnych typach komórek nowotworowych umożliwił wytypowanie wielu regulatorów metabolizmu i progresji nowotworów jako obiecujących celów terapeutycznych (Hanahan, 2000). Białkowe modulatory przekazywania sygnałów w komórkach o aktywności kinaz tyrozynowych są dobrym celem terapeutycznym dla związków niskocząsteczkowych działających poprzez wiązanie i blokowanie domeny

odpowiedzialnej za wiązanie ATP, zapobiegając aktywacji enzymu (Baselga, 2006). Druga grupa celów reprezentowana jest przez antygeny znajdujące się na powierzchni komórek, które są specyficzne dla danego nowotworu i mogą być rozpoznawane przez przeciwciała monoklonalne (Carter, 2006). Obecnie główny obszar zainteresowania badań i rozwoju w branży opracowywania nowych leków onkologicznych koncentruje się na niskocząsteczkowych inhibitorach, ze względu na zdolność do osiągnięcia przez nie celów wewnątrzkomórkowych oraz większą wygodę pacjenta dzięki ich dużej biodostępności po podaniu doustnym, wynikającej z małej masy cząsteczkowej (około 500 Da). W przypadku przeciwciał, które są dużymi białkami o masie zbliżonej do 150 kDa wymagane jest podanie dożylne (Imai, 2006). Duża masa cząsteczkowa przeciwciał monoklonalnych jest przyczyną ich nieefektywnego dostarczania do tkanki mózgowej z powodu bariery krew-mózg, dlatego też aplikacja terapeutycznych przeciwciał w przypadku nowotworów mózgu wymaga dostarczenia śródnowotworowego (Butowski, 2005). W odróżnieniu od przeciwciał, związki niskocząsteczkowe mogą przenikać do cytoplazmy i dlatego można je tak modyfikować, aby docierały do dowolnego celu terapeutycznego niezależnie od jego lokalizacji komórkowej (Dancey, 2003). Przeciwnie, ze względu na niezdolność do pokonywania barier komórkowych przez przeciwciała monoklonalne, ich zastosowanie jest ograniczone tylko do cząsteczek ulegających ekspresji na powierzchni błony komórkowej (Carter, 2006). Inhibitory niskocząsteczkowe są ogólnie uważane za mniej specyficzne niż przeciwciała monoklonalne, ponieważ ich działanie wiąże się z ryzykiem zwiększonej toksyczności (Huang, 2004). Wynika ona ze zdolności związków do jednoczesnego hamowania kilku szlaków sygnałowych przy klinicznie akceptowalnym stężeniu leku w osoczu (Xia, 2005). Ich niższa specyficzność może być jednak paradoksalnie korzystna w podejściu terapeutycznym. Takie kooperacyjne działanie na kilka cząsteczek jednocześnie, może umożliwiać szersze spektrum działania przeciwnowotworowego i poprawiać skuteczność leczenia. Ponadto, wystąpienie lekooporności jest mniej prawdopodobne. Dotychczas, w przypadku przeciwciał monoklonalnych takiej synergistycznej reaktywności krzyżowej nie stwierdzono (Imai, 2006). W odróżnieniu od związków niskocząsteczkowych, ekspozycja na każde białko, nawet terapeutyczne może okazać się również potencjalnie immunogenna. Taki problem nie znika nawet przy zastosowaniu chimerycznych lub humanizowanych, czy nawet ludzkich przeciwciał monoklonalnych (Imai, 2006).

Rozwój leków opartych na związkach niskocząsteczkowych wiąże się z wyjątkowymi wyzwaniem, takimi jak decyzje dotyczące chemicznej optymalizacji struktury wiodącej, uzyskania odpowiedniej selektywności względem innych podobnych celów molekularnych,

zachowania właściwości fizykochemicznych pozwalających na doustne stosowanie przy dobrej farmakokinytyce czy też, poszukiwania cząsteczek przeciwko niebiałkowym, mniej podatnym na terapię celom, takim jak onkogenne RNA.

CEL PRACY

Ogólnym celem badań podjętych w ramach mojej pracy doktorskiej było pogłębienie wiedzy na temat nowych strategii terapeutycznych chorób nowotworowych, szczególnie glejaka wielopostaciowego (GBM) poprzez identyfikację i charakterystykę kluczowych dla rozwoju nowotworu cząsteczek o potencjalnym znaczeniu diagnostycznym, prognostycznym i terapeutycznym, jak również opracowanie i charakterystykę narzędzi i podejść o potencjale terapeutycznym.

Związki niskocząsteczkowe o znanej budowie i określonych właściwościach fizykochemicznych mają często lepiej określoną aktywność biologiczną i profil farmakokinetyczny niż leki biologiczne. Łatwość pozyskiwania takich związków i modyfikacji ich struktury podczas syntezy chemicznej, przekłada się na możliwość dostosowywania cząsteczek do potrzeb badacza i optymalizacji ich działania. W związku z powyższym, w pierwszym nurcie badań, które prowadziłem, skupiłem się na charakterystyce i ocenie aktywności przeciwnowotworowej związków niskocząsteczkowych, analogów nukleozydów i nukleotydów, z grupy tzw. proleków (pronukleotydów) o zwiększonej selektywności w stosunku do komórek nowotworowych, mających na celu blokowanie podwyższonej aktywności białek odpowiedzialnych za wzmożoną proliferację komórek nowotworowych [**publikacje 1 - 5**].

Charakterystyka i ocena biologiczna związków niskocząsteczkowych prowadzi nie tylko do selekcji związków aktywnych biologicznie, ale również dostarcza wiedzy dotyczącej cytotoksyczności oraz wpływu na zdolność do proliferacji i migracji komórek. Otwiera to drogę do dalszych badań nad mechanizmem działania tych związków i chociaż koncentrują się one głównie na białkowych celach terapeutycznych, to możliwość celowania w RNA za pomocą takich związków ma ogromny potencjał. RNA podobnie jak DNA może kodować informację oraz podobnie jak białka tworzyć różne struktury przestrzenne, które mogą stanowić cel terapeutyczny. W związku z powyższym, w drugim nurcie moich badań skupiłem się na analizie dojrzałych miRNA pod względem ich struktury I- i II-rzędowej w celu przyszłego wykorzystania już scharakteryzowanych i wyselekcjonowanych związków niskocząsteczkowych do obniżenia ekspresji onkogennych miRNA, ponieważ badania profilowania ekspresji RNA dowodzą, iż cząsteczki te biorą udział w powstawaniu i progresji nowotworu [**publikacja 6**].

Skuteczność obecnie istniejących terapii przeciwnowotworowych może ulec podniesieniu poprzez zwiększenie efektywności dostarczania nie tylko chemioterapeutyków,

ale również molekularnych czynników terapeutycznych, takich jak siRNA, dsRNA czy rybozymów. Dlatego, też w trzecim nurcie badań skupiłem się na wykorzystaniu mRNA tenascyny-C jako cząsteczki docelowej, a następnie opracowaniu nowej i precyzyjnej metody dostarczania dwuniciowego RNA jako specyficznego i efektywnego narzędzia molekularnego w potencjalnej terapii [**publikacja 7**].

Przedostatnia część mojej pracy obejmuje badania aktywności biologicznej nowych fluorowanych, fosfonianowych analogów fenyloalaniny. W momencie podjęcia przeze mnie tej tematyki badawczej, w literaturze nie było badań odnoszących się do fluorowanych α -aminofosfonianów jako potencjalnych cząsteczek o aktywności przeciwnowotworowej względem komórek glejaka. Jest to bardzo ciekawa grupa związków, która może charakteryzować się aktywnością inhibicyjną wobec wybranych grup enzymów ulegających nadekspresji w nowotworowych komórkach macierzystych [**publikacja 8**].

Dodatkowym celem badań prowadzonych w ramach niniejszej pracy doktorskiej było opracowanie i zastosowanie enzymatycznie-sprzężonego testu spektrofotometryczno-fluorymetrycznego, służącego do zbadania katalizowanej kinazą adenozynową fosforylacji nukleozydów purynowych, będących potencjalnymi pronukleotydami aktywowanymi bezpośrednio w tkance nowotworowej wykazującej nadekspresję kinazy adenozynowej.

MATERIAŁY

Materiałem wykorzystywanym w prowadzonych przeze mnie eksperymentach były linie komórkowe prawidłowe i nowotworowe (Tabela 1). W zależności od rodzaju linii komórkowej stosowano następujące media hodowlane: DMEM, EMEM lub RPMI-1640 suplementowane 10% FBS oraz 1% mieszaniną antybiotyku i antymykotyku. Dodatkowo do pożywek: RPMI-1640 oraz DMEM (Sigma) dodawano 1% mieszaninę witamin. Hodowle prowadzono w 10ml odpowiedniej pożywki w atmosferze nasyconej parą wodną z dodatkiem 5% CO₂, utrzymując temperaturę 37°C. Pożywkę zmieniano co 48 – 72 godziny oraz dokonywano mikroskopowej kontroli wzrostu komórek.

Tabela 1. Warunki hodowli komórkowych.

Linia komórkowa	Model nowotworu	Hodowla komórkowa	Medium hodowlane
<i>T98G</i>	Glejak wielopostaciowy	Adherentna	EMEM + 10% FBS (ATCC)
<i>U-87 MG</i>	Glejak wielopostaciowy	Adherentna	EMEM +10% FBS (ATCC)
<i>U-118 MG</i>	Glejak wielopostaciowy	Adherentna	DMEM + 10% FBS (ATCC)
<i>HepG2</i>	Rak wątroby	Adherentna	DMEM + 10% FBS (Sigma)
<i>Caco-2</i>	Rak jelita grubego	Adherentna	DMEM + 10% FBS (Sigma)
<i>HeLa</i>	Rak szyjki macicy	Adherentna	RPMI + 10% FBS (Sigma)
<i>T-47D</i>	Rak piersi	Adherentna	RPMI + 10% FBS (Sigma)
<i>MRC-5</i>	Fibroblasty	Adherentna	EMEM + 10% FBS (Sigma)
<i>HaCaT</i>	Keratynocyty	Adherentna	DMEM + 10% FBS (Sigma)

Biblioteka badanych związków

W badaniach wykorzystano 208 nowych analogów nukleozydowych i nukleotydowych zsyntetyzowanych w Zakładzie Chemii Kwasów Nukleinowych Instytutu Chemii Bioorganicznej PAN przez zespół prof. dr hab. Adama Kraszewskiego, 30 związków z serii dimerów nukleozydowych zsyntetyzowanych przez zespół prof. dr hab. Jerzego Boryskiego oraz 12 nowych fluorowanych, fosfonianowych analogów fenyloalaniny zsyntetyzowanych przez zespół dr hab. Donaty Pluskoty-Karwatki z Zakładu Syntezy i Struktury Związków Organicznych UAM Poznań. Dwuniciowy RNA (dsRNA) o sekwencji homologicznej do tenascyny-C (ATN-RNA) został otrzymany w Zakładzie Neuroonkologii Molekularnej Instytutu Chemii Bioorganicznej PAN według wcześniej opracowanego w Instytucie Chemii Bioorganicznej PAN protokołu (Rolle, 2010; Zukiel, 2006). Praktyczne zastosowanie ATN-RNA obejmuje terapię eksperymentalną pacjentów ze zdiagnozowanym glejakiem wielopostaciowym WHO IV, która prowadzona była w oparciu o zgodę Komisji Bioetycznej UM w Poznaniu jako uzupełnienie standardowych procedur medycznych. Sekwencja dsRNA, sposób otrzymania preparatu (formulacja) oraz podanie są chronione patentem amerykańskim (No. 8,946,400 B2). Magnetyczne nanocząstki zostały przygotowane według standardowej metody współstrąceniowej w Centrum NanoBioMedycznym UAM Poznań przez zespół prof. dr hab. Stefana Jurgi (Mrówczyński, 2016).

METODY

Ocena stabilności związków w medium hodowlanym oraz surowicy ludzkiej (HS) i bydlęcej (FBS) metodą wysokosprawnej chromatografii cieczowej (HPLC)

Stabilność badanych nukleozydów i nukleotydów oceniana była przy użyciu systemu Thermo Scientific DIONEX UltiMate 3000 wyposażonego w detektor UV-DAD (detekcję prowadzono przy długości fali $\lambda=256\text{nm}$). Do medium hodowlanego (RPMI-1640) zawierającego 10% FBS (1) oraz 100% ludzkiej surowicy (2) dodawano 2 mM roztwory badanych związków i podgrzewano w termobloku do temperatury 37°C . Rozdział chromatograficzny przeprowadzono na kolumnie Lichrospher RP-18 endcapped ($5.0\text{ }\mu\text{m}$, $4.6\text{ mm} \times 250\text{ mm}$) przy przepływie $1,5\text{ ml/min}$ (objętość nastrzyku wynosiła $20\text{ }\mu\text{l}$). W celu wymycia badanych związków faza ruchoma składała się z $0,01\text{M}$ octanu trietyloamoniowego o $\text{pH } 7,4$ (faza ruchoma A) oraz $0,01\text{ M}$ octanu trietyloamoniowego o $\text{pH } 7,4$ i acetonitrylu w stosunku $1:4\text{ (v/v)}$ (faza ruchoma B). Rozdział prowadzono w gradiencie liniowym fazy ruchomej. W czasie $t=0$: A-100%, B-0%; w 5 minucie analizy skład fazy ruchomej zmieniano liniowo do B-100%, A-0%; taki skład fazy ruchomej utrzymywano przez następne 30 minut, a na końcu przemywano przez 5 minut fazą A-100%, B-0%; aż do zakończenia pomiaru.

Test MTT

Ustalenie toksyczności badanych związków zostało przeprowadzone w oparciu o test MTT. W tym celu komórki zostały wysiane na 96-dołkowe płytki w ilości 10000 komórek/dołek. Po 24 godzinnej inkubacji wzrastające stężenia badanych związków zostały podane na płytki (6 dołków na stężenie), a komórki poddano 48-godzinnej inkubacji. Roztwory wyjściowe zostały przygotowane w DMSO, a następnie rozcieńczone medium hodowlanym, tak aby stężenie DMSO nie przekroczyło $0,2\%$. Komórki w medium hodowlanym zostały przyjęte jako kontrole. Po 48 godzinach został przeprowadzony test MTT, polegający na dodaniu barwnika MTT ($0,5\text{ mg/ml}$) w medium hodowlanym i godzinnej inkubacji. Po tym czasie zmierzono absorbancję powstałego formazanu przy długości fali 490 nm , za pomocą czytnika płytek wielodołkowych Synergy2. Wyniki, jako średnia z minimum 3 powtórzeń zostały przedstawione w formie wartości IC_{50} .

Test SRB

Sulforodamina B (SRB) jest anionowym barwnikiem, który wiąże się z aminokwasami białek komórkowych. Oznaczanie aktywności cytotoksycznej w tym teście prowadzi się na podstawie pomiaru ilości białka komórkowego. Komórki zostały wysiane na 96-dołkowe płytki w ilości 10000 komórek/dołek. Po 24 godzinnej inkubacji wzrastające stężenia badanych związków zostały podane na płytki (6 dołków na stężenie), a komórki poddano 72-godzinnej inkubacji. Roztwory zostały przygotowane w sposób analogiczny jak w teście MTT. Po 72 godzinach został przeprowadzony test SRB, polegający na utrwaleniu komórek za pomocą 10% kwasu trichlorooctowego (TCA), a następnie wybarwieniu za pomocą 0,057% roztworu SRB rozpuszczonego w 1% kwasie octowym. Związany barwnik rozpuszczono poprzez dodanie 10 mM TRISu. Absorpcję roztworów oznaczono spektrofotometrycznie przy długości fali 510 nm, za pomocą czytnika płytek wielodołkowych Synergy2. Wyniki, jako średnia z minimum 3 powtórzeń zostały przedstawione w formie wartości IC₅₀.

Test klonogenności

W celu ustalenia tzw. toksyczności przewlekłej badanych związków, komórki zostały wysiane na 6-dołkowe płytki w ilości 500 komórek/dołek. Po 24 godzinach wzrastające stężenia badanych związków zostały podane na płytki (3-6 dołków na stężenie). Roztwory zostały przygotowane w sposób analogiczny jak w teście MTT. Po siedmiodniowej inkubacji powstałe kolonie zostały utrwalone 70% roztworem etanolu, wybarwione 0,05% roztworem fioletu krystalicznego, a następnie zliczone. Wyniki, jako średnia z minimum 3 powtórzeń zostały przedstawione w formie % wartości kontroli.

Test rodzaju śmierci komórkowej

Procesy apoptozy oraz nekrozy są najlepiej poznane wśród rodzajów śmierci komórkowej. Przy użyciu cytometru przepływowego Guava easyCyte rozróżniono subpopulacje komórek w fazie wczesnej i późnej apoptozy oraz nekrozy. Komórki zostały wysiane na 6-dołkowe płytki w ilości $2,5 \times 10^5$ komórek/dołek. Po 24 godzinach wzrastające stężenia badanych związków zostały podane na płytki (3 dołki na stężenie), a komórki

poddano 24- i 48-godzinnej inkubacji. Przy użyciu białka aneksyny V sprzężonego z izotiocyanianem fluoresceiny (FITC) znakowano komórki, w których fosfatydyloseryna wchodząca w skład cytoplazmatycznej części błony komórkowej przeszła na zewnętrzną stronę dwuwarstwy lipidowej. Jako drugiego barwnika użyto 7-aminoaktynomycyny D (7-AAD), który wnikać przez nieszczelną błonę uszkodzonych komórek wskazuje nekrozę bądź późną apoptozę.

Ocena wzrostu liczby komórek w czasie rzeczywistym

Ocena wzrostu komórek w czasie rzeczywistym odbywała się w systemie xCELLigence, z zastosowaniem aparatu RTCA DP (ACEA Biosciences), który monitoruje przyrost komórek na podstawie zmian impedancji mikroelektrod umieszczonych w podłożu płytki hodowlanej. Komórki zostały wysiane na 16-dołkowe płytki E-Plate w ilości 10000 komórek/dołek. Po 24 godzinnej inkubacji przeprowadzano transfekcję komórek wzrastającymi stężeniami badanych związków. Proliferację komórek monitorowano przez kolejne 72 godziny. Analizę wyników prowadzono używając oprogramowania systemu xCELLigence – RTCA Software.

Cytometryczna analiza faz cyklu komórkowego

Analizę faz cyklu komórkowego przeprowadzono przy użyciu barwnika jodku propidyny (PI), który przenika przez błonę komórkową uszkodzonych komórek i na zasadzie interkalacji łączy się z dwuniciowym DNA. Wyniki badań dostarczają informacji o ilości komórek w poszczególnych fazach cyklu komórkowego (G_0/G_1 , S, G_2/M). W tym celu, komórki zostały wysiane na 6-dołkowe płytki w ilości $2,5 \times 10^5$ komórek/dołek. Po 24 godzinach wzrastające stężenia badanych związków zostały podane na płytki (3 dołki na stężenie), a komórki poddano 24-godzinnej inkubacji. W celu utrwalenia komórek osad komórkowy zawieszano w 70% etanolu i inkubowano przez noc w 4°C. Aby oczyścić próbę z RNA do osadu dodano RNazę A (100 $\mu\text{g}/\mu\text{l}$, Sigma Aldrich) i całość inkubowano 30 minut w temperaturze pokojowej. W celu wybarwienia DNA osad komórkowy zawieszono w roztworze PI (50 $\mu\text{g}/\text{ml}$, Sigma Aldrich) i inkubowano godzinę w temperaturze 4°C. Oceny cyklu komórkowego dokonano stosując cytometr przepływowy BD FACS Calibur (Becton

Dickinson). Do analizy procentowego udziału poszczególnych faz cyklu komórkowego wykorzystano oprogramowanie ModFit LT (Verity Software House).

Ocena migracji komórkowej w czasie rzeczywistym

Migracja komórek została zbadana w czasie rzeczywistym za pomocą systemu xCELLigence. Eksperyment przeprowadzono na składających się z dwóch komór, 16-dołkowych płytkach CIM-Plate. Dołki obu składowych płytki oddziela od siebie porowata, politereftalenowa membrana. Naniesione na nią złote mikroelektrody zbierają informacje o zachodzących podczas migracji komórek zmianach w impedancji elektrycznej. Komórki zostały wysiane na górną część 16-dołkowych płytek CIM-Plate w ilości 10000 komórek/dołek. Po 24 godzinnej inkubacji przeprowadzano transfekcję komórek wzrastającymi stężeniami badanych związków. Migrację komórek monitorowano przez kolejne 72 godziny. Analizę wyników prowadzono używając oprogramowanie systemu xCELLigence – RTCA Software.

Ocena migracji komórkowej metodą „wound healing assay”

Jako drugiej metody do zobrazowania potencjału migracyjnego komórek używano testu zabliźniania rany (ang. *wound healing assay*). Eksperyment przeprowadzano w dwudołkowych insertach silikonowych do testów migracji (ibidi) umieszczonych w płytkach 6-dołkowych. Do każdego dołka wysiewano po 2×10^4 komórek w 70 μ l medium hodowlanego na dołek. Po 24 godzinnej inkubacji usuwano silikonowy insert uzyskując efekt blizny. Następnie dodawano wzrastające stężenia badanych związków w 2 ml medium hodowlanego. Od tego momentu przez 72 godziny w odstępach 12 godzinnych wykonywano zdjęcia hodowli obserwowanej pod mikroskopem fluorescencyjnym. Analizę stopnia zabliźnienia poszczególnych „ran” przeprowadzano komputerowo wykorzystując program TScratch (CSElab).

Analiza zależności pomiędzy strukturą i parametrami fizykochemicznymi, a aktywnością biologiczną związków

Obliczeniami metodą *in silico* wyznaczono niektóre parametry fizykochemiczne determinujące biodostępność. Osiem parametrów lipofilowości (LogP) obliczono przy użyciu serwera Virtual Computational Chemistry Laboratory (Tetko, 2005). Kalkulacje polarnego (PSA) i apolarnego (aPSA) pola powierzchni cząsteczek wykonano za pomocą programu VEGA ZZ (<http://www.vegazz.net>) (Pedretti, 2002). Do obliczeń absorpcji w przewodzie pokarmowym (Caco-2) oraz parametrów strukturalnych (HBD, HBA, MW) użyto aplikacji preADMET (<http://preadmet.bmdrc.org>).

Analiza statystyczna

Analizę statystyczną wyników wykonano przy użyciu programu statystycznego Prism 5.01 (GraphPad Software) posługując się testem analizy wariancji dla powtórzonych pomiarów (ang. *repeated measures ANOVA*) oraz testem Newman-Keuls jako post-hoc. Porównania krzywych w funkcji czasu dokonano za pomocą wieloczynnikowej analizy wariancji (ang. *two-way ANOVA*). Wyniki przedstawiono jako wartości średnie $\pm$ standardowy błąd średniej (SEM). Za istotne statystycznie przyjęto różnice z wartością współczynnika $P < 0,05$. Wykresy przeżywalności pacjentów tworzone były według metody Kaplana-Meiera, a w analizach statystycznych w tym przypadku wykorzystano testy log-rank.

SKRÓTOWY OPIS PUBLIKACJI WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ

1. Projektowanie i badanie mechanizmu działania przeciwnowotworowego nowych analogów nukleozydów i nukleotydów

Analogi nukleozydów stanowią zasadniczą grupę stosowanych klinicznie leków przeciwwirusowych i przeciwnowotworowych. Należą one do grupy proleków, gdyż same są nieaktywne i dopiero w organizmie człowieka ulegają przemianom do formy aktywnej, którą są ich 5'-trifosforany (Jordheim, 2013). Dla wielu modyfikowanych nukleozydów pierwszy etap fosforylacji jest etapem decydującym o szybkości całego procesu, przez co bezpośrednio wpływa na aktywność biologiczną i efektywność terapeutyczną tej grupy związków (Rautio, 2008). Wprowadzenie analogów nukleozydów do komórki w postaci 5'-monofosforanów jest niekorzystne, ponieważ w warunkach fizjologicznych ($\text{pH}=7,0-7,4$) monofosforany występują jako aniony, co utrudnia ich przenikanie przez ujemnie naładowaną błonę komórkową. Również obecne we krwi i na powierzchni komórki fosfohydrolazy metabolizują je do odpowiednich nukleozydów (Wagner, 2000). Jednym ze sposobów zwiększania skuteczności terapii jest projektowanie pronukleotydów, mających za zadanie ominięcie pierwszego etapu aktywacji enzymatycznej. Ich znaną cechą w porównaniu z dotychczas stosowanymi analogami nukleozydów jest to, że ich bioaktywacja do aktywnych 5'-trifosforanów nie wymaga zaangażowania komórkowych kinaz nukleozydowych. Zaopatrzone w odpowiednie grupy ochronne przenikają przez błonę cytoplazmatyczną i są w stanie osiągnąć wewnątrzkomórkowe cele molekularne. Korzyściami w przypadku stosowania pochodnych analogów nukleozydowych jako proleków w terapii przeciwnowotworowej są m.in.: ułatwiony transport przez błony komórkowe oraz barierę krew-mózg, zredukowana toksyczność i zwiększony czas półtrwania w osoczu, co w rezultacie poprawia efektywność ich działania (Jordheim, 2013; Rautio, 2008).

Metody obliczeniowe SAR (ang. *structure activity relationship*) należą do standardowych narzędzi wczesnej fazy analiz właściwości fizykochemicznych kandydatów na nowe leki. Obecnie duże znaczenie we wczesnym okresie badań nad nowymi cząsteczkami terapeutycznymi mają metody obliczeniowe pozwalające symulować procesy farmakokinetyczne przyszłego leku. Metody te opracowywane są na podstawie baz danych właściwości fizykochemicznych związków już znanych (Grabowski, 2008). Badania szeregu zależności wspólnych dla cząsteczek terapeutycznych doprowadziły do sformułowania przez

Lipińskiego reguły pięciu (Lipinski, 2001). Związek, który spełnia regułę powinien cechować się masą cząsteczkową mniejszą od 500 Da, współczynnikiem rozdziału oktanol/woda LogP o wartości <5 . Aby związek cechował się odpowiednią biodostępnością powinien posiadać nie więcej niż 5 donorów wiązań (HBD) oraz nie więcej niż 10 akceptorów (HBA) tych wiązań.

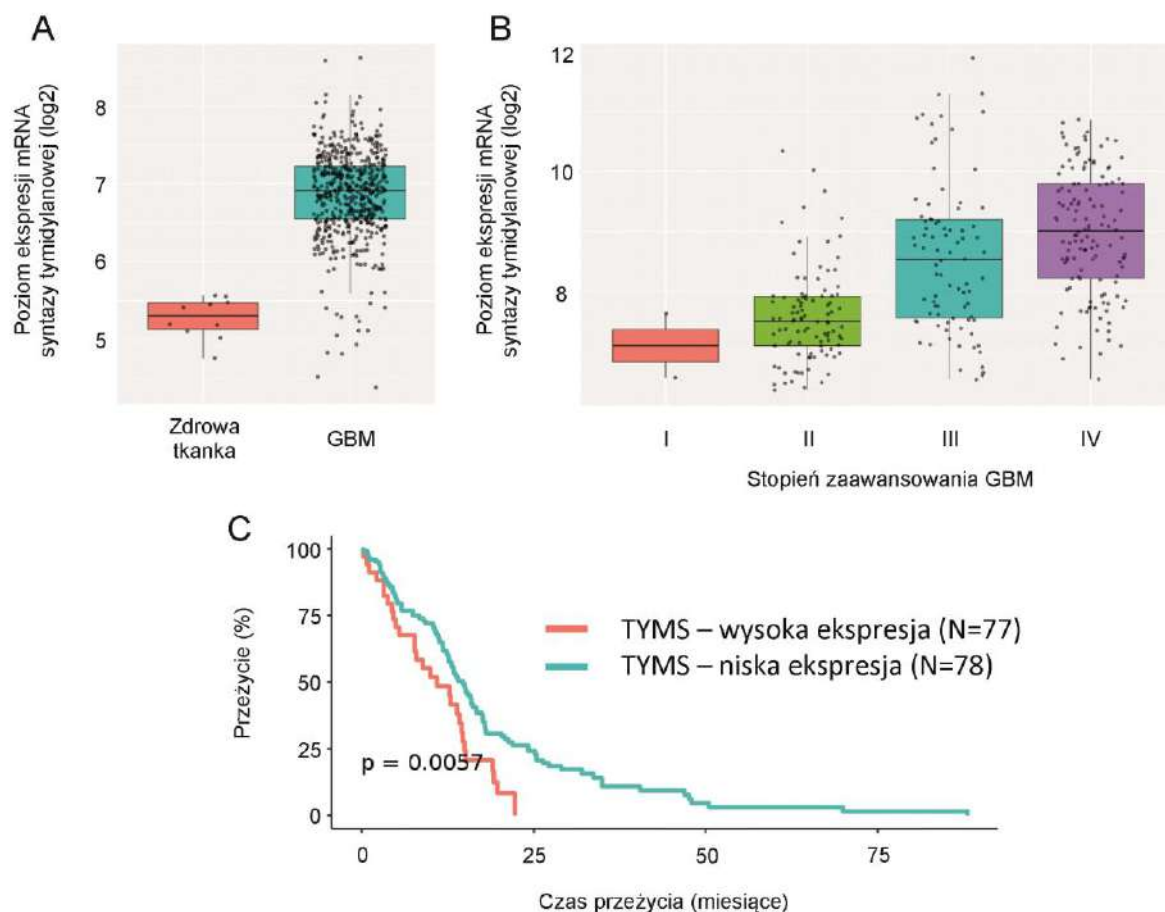
Współczynnik podziału między wodę i *n*-oktanol – LogP (logarytm z wartości rozdziału pomiędzy obie fazy) jest deskryptorem powszechnie używanym w badaniach SAR i najczęściej stosowanym parametrem lipofilowości. Wskaźnik ten wpływa na wszystkie trzy fazy działania leku: farmaceutyczną (forma i sposób uwalniania leku), farmakokinetyczną (losy leku w ustroju – absorpcja, dystrybucja, metabolizm i eliminacja) oraz fazę farmakodynamiczną (oddziaływanie z receptorem) (Mannhold, 2008). Jednakże największy wpływ na fazę farmakokinetyczną wywiera lipofilowość, bowiem parametr ten decyduje o transporcie leku przez błony biologiczne, a więc o procesie dystrybucji leku. Przyjmuje się, iż związki których LogP jest mniejsze od jedności, mogą być charakteryzowane jako hydrofilowe, a więc takie, które w niewielkim stopniu przechodzą przez błony komórkowe drogą biernej dyfuzji. Związki dla których $1 < \text{LogP} < 3$, charakteryzują się średnią lipofilowością i mogą swobodnie migrować przez błony komórkowe. Natomiast związki, których $\text{LogP} > 3$, charakteryzują się wysoką lipofilowością i niską biodostępnością. Powodem jest wiązanie się do błon komórkowych i utrudniona dystrybucja w środowisku hydrofilowym (Grabowski, 2008).

Bardzo użytecznym wskaźnikiem w przewidywaniu absorpcji związku jest powierzchnia polarna (ang. *polar surface area*, PSA) – definiowana jako ta część powierzchni dostępnej dla rozpuszczalnika, która wiąże się z obecnością akceptorów wiązania wodorowego lub donorów wiązania wodorowego (Waterbeemd, 2006). Na podstawie analizy SAR leków przyjmowanych doustnie, potwierdzono, że związki o dużej powierzchni polarnej ($>140 \text{ \AA}^2$) cechują się niską biodostępnością związaną z niską dystrybucją i możliwością przenikania przez błony komórkowe. Z kolei cząsteczki o małej powierzchni polarnej ($<60\text{--}70 \text{ \AA}^2$) są praktycznie w 100% absorbowane na drodze dyfuzji biernej (Palm, 1997). Parametr PSA uznawany jest za dobry wskaźnik absorpcji jelitowej, przenikalności przez błony, w tym także przez barierę krew-mózg (Grabowski, 2008; Kelder, 1999; Palm, 1997).

W ramach projektu badawczego Narodowego Centrum Nauki pod tytułem „Nowa generacja pro-nukleotydów antynowotworowych” przebadalem serię 208 nowych związków nukleozydowych i nukleotydowych. Związki te zsyntetyzowano w Zespole Prof. Adama Kraszewskiego z Zakładu Chemii Kwasów Nukleinowych IChB PAN. Badania nad

właściwościami przeciwnowotworowymi i mechanizmem indukowanej przez te związki toksyczności w wybranych liniach komórek nowotworowych ze szczególnym uwzględnieniem glejaka wielopostaciowego stały się przedmiotem badań mojej rozprawy doktorskiej.

W pierwszej z omawianych prac, tj. „*New 3'-O-aromatic acyl-5-fluoro-2'-deoxyuridine derivatives as potential anticancer agents*” (Szymanska-Michalak, 2016) [publikacja 1], opisałem wyniki badań serii aromatycznych i alifatycznych 3'-O-estrów 5-fluoro-2'-deoksyurydyny. Fluoropirymidyny reprezentują najczęściej zalecaną na świecie grupę leków przeciwnowotworowych. Koncepcja ich użycia pojawiła się w późnych latach pięćdziesiątych (Focher, 2001), a w roku 1970 Amerykańska Agencja Żywności i Leków (ang. *Food and Drug Administration*, FDA) zaaprobowала 5'-fluoro-2'-deoksyurydynę (FdU) jako lek stosowany u chorych z nowotworami (Parker, 2009). Aktywność cytostatyczna 5-FdU wynika ze zmniejszenia stężenia 5'-trifosforanu tymidyny (TTP) w wyniku hamowania syntazy tymidylanowej (TYMS). Aby uzyskać tę aktywność, FdU musi ulec fosforylacji do 5-fluoro-2'-deoksyurydyno-5'-fosforanu (FdUMP), który jest swoistym inhibitorem enzymu (Bosch, 1958). Niestety, wydajność transformacji FdU do FdUMP w komórce jest hamowana przez hydrolizę wiązania glikozydowego nukleozydu przez fosforylaze tymidyny (TP), która uwalnia 5-fluorouracyl (5-FU). Aby odzyskać aktywność przeciwnowotworową, 5-FU musi przejść ponownie przez cały cykl biosyntezy, od zasady poprzez FdU do FdUMP, co prowadzi do zmniejszenia działania przeciwnowotworowego oryginalnego leku - FdU (Chaudhuri, 1958). Ze względu na niską biodostępność, 5-fluorouracyl oraz 5-fluoro-2'-deoksyurydyna podawane są w postaci wlewów dożylnych, które są obciążone ryzykiem zakażenia oraz powikłaniami zakrzepowymi. W związku z powyższym, nasze badania koncentrowały się na zaprojektowaniu nowych pronukleotydowych pochodnych FdU o dużej dostępności biologicznej nakierowanych na komórki glejaka. Przeprowadzona przeze mnie analiza danych pochodzących z dużych projektów badania genomów nowotworowych (TCGA, REMBRANDT) dostępna w portalu GlioVis (Bowman, 2017) wykazała, że syntaza tymidylanowa jest genem ulegającym wysokiej ekspresji w GBM. Ponadto, wzrost ekspresji TYMS skorelowany jest ze stopniem złośliwości nowotworu oraz skutkuje obniżoną przeżywalnością pacjentów (Rycina 2).



Rycina 2. Poziom ekspresji mRNA syntazy tymidylanowej (TYMS) w GBM w porównaniu do zdrowej tkanki (A). Poziom ekspresji TYMS w GBM w zależności od stopnia zaawansowania nowotworu (B). Krzywa przeżycia pacjentów GBM z wysoką i niską ekspresją TYMS (C).

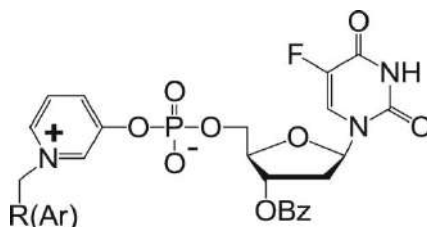
W ramach serii aromatycznych i alifatycznych 3'-O-estrów 5-fluoro-2'-deoksyurydyny poza związkiem macierzystym (**FdU**) otrzymaliśmy jedenaście związków (**7a-k**) [Table 1 w (Szymanska-Michalak, 2016)] oraz trzy di- i tri- estry kwasów karboksylowych (**11**, **12**, **14**) [Table 2 w (Szymanska-Michalak, 2016)]. Struktury badanych związków nie są przypadkowe i tak w serii acylowych pochodnych 5-fluoro-2'-deoksyurydyny o strukturalnie odmiennych grupach aromatycznych (**7a-g**) decydowały spodziewana kinetyka rozpadu i lipofilowość, a w przypadku związków (**7h-k**) dochodziła dodatkowa aktywność przeciwnowotworowa wnoszona przez podstawnik acylowy (kwas acetylosalicylowy, kwas liponowy, kwas dichlorooctowy, lonidamina). Właściwości cytotoksyczne związków badałem na trzech liniach komórek glejaka wielopostaciowego (T98G, U-118 MG, U-87 MG), komórkach raka piersi (T-47D), raka szyjki macicy (HeLa) powszechnie akceptowanych jako referencyjne komórki nowotworowe, raka jelita grubego (Caco-2) jako standardu zdolności absorpcji leku do krwi oraz na komórkach prawidłowych MRC-5. Uzyskane wartości parametru IC_{50} zostały

przedstawione w [Table 3 w (Szymanska-Michalak, 2016)]. Takie zestawienie otrzymanych wyników umożliwia porównanie toksyczności badanych związków wobec komórek nowotworowych jak i prawidłowych. W wyborze najmniej toksycznych związków spośród najbardziej aktywnych biologicznie zastosowano wskaźnik selektywności *in vitro* (ang. *selectivity index*, SI). Jest on definiowany jako stosunek dawki toksycznej do terapeutycznej ($SI = IC_{50} \text{ komórek prawidłowych} / IC_{50} \text{ komórek nowotworowych}$). Analiza ta pozwala określić selektywność działania badanych związków w stosunku do określonego typu komórek (Badisa, 2009). Pierwsza seria (**7a-g**) badanych związków składała się z acylowych pochodnych 5-fluoro-2'-deoksyurydyny o strukturalnie odmiennych grupach aromatycznych. Mimo niższych wartości IC_{50} niż w przypadku cząsteczki macierzystej (**FdU**) nie wyodrębniono z tej grupy związku o korzystnym indeksie selektywności. Biorąc pod uwagę charakter fizykochemiczny grup 3'-O-acylowych tych związków, uzasadnione wydaje się twierdzenie, że obserwowaną wyższą cytotoksyczność (**7a-g** vs **FdU**) można przypisać ich wyższej lipofilowości, która ułatwia penetrację błon komórkowych [jak wynika z parametrów logP, PSA i aPSA w Table 1 w (Szymanska-Michalak, 2016)]. W serii pochodnych o podwójnej aktywności biologicznej (**7h-k**) tylko 3'-O-(2-acetylosalicylan) 5-fluoro-2'-deoksyurydyny (**7h**) okazał się związkiem o pożądanach wartościach IC_{50} i wysokiej selektywności względem komórek nowotworowych. Metabolizm związku (**7h**) prowadzi do powstania FdU oraz kwasu acetylosalicylowego, dlatego zaobserwowane zjawisko jest zgodne z odkryciem, że oba związki mogą działać synergistycznie względem komórek nowotworów złośliwych, pozostając bez większego wpływu na komórki prawidłowe (Li, 2013; Spitz, 2009). Trzecia seria związków (**11**, **12**, **14**) obejmująca di- i tri- estry aromatycznych kwasów karboksylowych mimo niekorzystnych parametrów farmakokinetycznych (ujemne wartości LogP świadczące o charakterze hydrofilnym oraz wysokie wartości parametru PSA predysponujące do niskiej biodostępności) charakteryzowała się działaniem antynowotworowym zbliżonym do związku macierzystego (**FdU**). Otrzymane związki były badane pod względem trwałości i dróg rozpadu w ekstraktach komórkowych i w warunkach hodowli komórkowych tj. w medium hodowlanym RPMI-1640 oraz medium hodowlanym RPMI-1640 wzbogaconym płodową surowicą bydlęcą (9:1, v/v). W przypadku pełnych ekstraktów komórkowych, badania trwałości były wyjątkowo trudne ze względu na balast białek i kwasów nukleinowych. Otrzymane wyniki pozwalały wyłącznie na bardzo przybliżoną ocenę trwałości związków i z tych względów badanie trwałości ograniczono do środowiska hodowli komórkowych. Metodą regresji liniowej obliczono stężenia związków i ich metabolitów na podstawie

całkowania powierzchni pików uzyskanych za pomocą analizy HPLC. Ponieważ czyste RPMI-1640 jest wolne od aktywności enzymatycznej, czasy półtrwania ($t_{1/2}$) w tym medium odzwierciedlają podatność badanych związków na degradację chemiczną (hydrolizę), natomiast w RPMI-1640/FBS (9:1, v/v) oraz ludzkim osoczu (ang. *human blood plasma*, HBP) w których oczekuje się aktywności enzymatycznej (np. karboksyesteraz) odzwierciedlona jest zarówno stabilność chemiczna, jak i enzymatyczna. Jak wynika z danych w [Table 1 w (Szymanska-Michalak, 2016)] wszystkie analizowane związki (**7a-g**) były raczej stabilne w badanych warunkach reakcji i do komórek mogły wnikać jako nienaruszone cząsteczki. Z kolei, stabilność pochodnych o podwójnej aktywności biologicznej (**7h-k**) była różna. Związek (**7i**) był bardzo stabilny we wszystkich ośrodkach ($t_{1/2}>240$ h), podczas gdy (**7k**) w tych samych warunkach ulegał szybkiej hydrolizie ($t_{1/2}<0,1$ h). W przypadku di- i tri-estrów aromatycznych kwasów karboksylowych byliśmy w stanie ustalić $t_{1/2}$ tylko dla związku (**12**) z powodu ograniczonej rozpuszczalności (**11** i **14**). Znacząco krótszy okres półtrwania (**12**) w RPMI-1640/FBS wskazuje na udział enzymatycznej hydrolizy. Ponadto hydroliza drugiego estru karboksylowego związku (**12**) była znacznie dłuższa niż pierwszego [Supplementary data w (Szymanska-Michalak, 2016)]. Jest to zgodne z cechą karboksyesteraz, które wykazują niższą reaktywność względem polarnych lub naładowanych substratów (Peyrottes, 2004). Ze względu na możliwość tworzenia przez badane związki mikro-struktur (np. mikromicel), które mogłyby wpływać na transport dokomórkowy dla modelowego, amfifilowego związku 5-fluoro-3'-O-benzoilo-2'-deoksyurydyny zostały przeprowadzone badania metodą dynamicznego rozproszenia światła (ang. *dynamic light scattering*, DLS) w Instytucie Katalizy i Fizykochemii Powierzchni PAN w Krakowie. Wykazały one, że tworzenie takich struktur jest możliwe jedynie przy bardzo wysokich, niefizjologicznych stężeniach.

Podsumowując, spośród przebadanych aromatycznych i alifatycznych 3'-O-estrów 5-fluoro-2'-deoksyurydyny najbardziej obiecująca wydaje się pochodna acylowa (**7h**) o podwójnej aktywności przeciwnowotworowej, która uwalnia w sposób kontrolowany nie tylko znany nukleozyd 5-fluoro-2'-deoksyurydynę, ale także dodatkowy aktywny metabolit (kwas acetylosalicylowy) wzmacniający działanie terapeutyczne FdU. Obiecujące wskaźniki terapeutyczne wykazują, także niektóre di- i tri-estry aromatycznych kwasów karboksylowych (**11** i **12**). Ponadto, powyższe badania koncentrowały się także na poszukiwaniach najbardziej odpowiednich pochodnych nukleozydowych, które miały być substratami do syntezy i badań biologicznych pronukleotydów antynowotworowych, ukierunkowanych na glejaka wielopostaciowego.

W kolejnym etapie prac eksperymentalnych prowadzonych w ramach projektu „Nowa generacja pro-nukleotydów antynowotworowych” oceniłem działanie przeciwnowotworowe pronukleotydów FdU posiadających ugrupowania naładowane zarówno dodatnio jak i ujemnie, maskujących fosforan (Rycina 3). Projektując pronukleotydy z serii „jonów obojnych” (tzw. pronukleotydy zwitterjonowe) FdUMP spodziewaliśmy się, oprócz potencjalnych korzyści terapeutycznych, wzrostu rozpuszczalności w płynach fizjologicznych oraz selektywności działania wynikającej z osłabienia sił wiązania lipid-lipid w błonach komórek nowotworowych o zaburzonych relacjach lipidów i ich interakcjach z białkami błonowymi (Fahs, 2014). Wyniki badań opisaliśmy w pracy pt. „*New antiglioma zwitterionic pronucleotides with an FdUMP framework*” (Szymanska-Michalak, 2018) [publikacja 2].



Rycina 3. Struktura chemiczna zwitterjonowych pronukleotydów FdUMP.

Dla powyższych pronukleotydów, poza związkiem macierzystym (**FdU**), otrzymaliśmy szesnaście związków (**4a-m**, **5a**, **5c-d**) [Table 2 w (Szymanska-Michalak, 2018)]. W większości przypadków badane pronukleotydy typu 4 (**4a-m**) posiadają resztę 3'-O-benzoilo-5-fluoro-2'-deoksyurydyn-5'-ylu w części nukleozydowej, co samo w sobie czyni je aktywnymi wobec GBM (Framski, 2016). Dla porównania oceniono także aktywność analogów nukleotydów z wolną grupą 3'-OH (**5a**, **5c-d**). Z wyjątkiem związków (**4a-b**) wspólnym motywem strukturalnym badanych fosfodiesterów (**4c-m**) i (**5c-d**) jest obecność aminy alifatycznej lub aromatycznej, która wraz z resztą fosforanową może tworzyć struktury jonów obojnych (**4c-d**) (Kolodziej, 2015) lub trwale zwitterjony (**4e-m**). Związki (**4h-i**) zostały zaprojektowane jako analogi nukleotydowe leku przeciwbakteryjnego o potencjalnych właściwościach przeciwnowotworowych, bromku 12-metakryloyloksydodecylo-pirydyniowego (ang. *12-methacryloyloxydodecyl pyridinium bromide*, MDBP) (Fahs, 2014). Z podobnych powodów, do fosfodiesterów (**4j-k**) dołączono części znanych związków cytostatycznych, miltefozyny (Dorlo, 2012) i peryfozyny (Gills, 2009). Właściwości cytotoksyczne związków badałem na trzech liniach komórek glejaków (T98G, U-118 MG, U-

87 MG), komórkach raka piersi (T-47D), raka szyjki macicy (HeLa), raka jelita grubego (Caco-2) oraz na komórkach prawidłowych MRC-5. Uzyskane wartości parametru IC_{50} oraz SI zostały przedstawione w [Table 2 w (Szymanska-Michalak, 2018)]. Zarówno proste pronukleotydy anionowe (**4a-b**) jak i zwitterjonowe (**4c-f**) wykazują wysoką aktywność przeciwnowotworową w liniach komórkowych glejaków. Jednak porównanie par analogów FdUMP (**4a** vs **4c**) oraz (**4b** vs **4f**) jednoznacznie wskazuje na wyższe indeksy selektywności fosfodiestrów zwitterjonowych (**4c, f**). Kolejne związki z serii jonów obojnych (**4g-i**) również wykazują wysoką aktywność cytostatyczną względem komórek glejakowych (wartości IC_{50} głównie w zakresie nM), jednocześnie będąc mniej szkodliwymi dla komórek prawidłowych MRC-5. Selektywną aktywność biologiczną w stosunku do komórek nowotworowych, można częściowo przypisać interakcji związków (**4c, 4f-i**) z błoną komórek glejaka, która prowadzi do ich zwiększonego pobierania. Pronukleotydy (**4j-m**) zawierające zwitterjonowe reszty alifatyczno-fosforanowo-amonowe, charakteryzują się brakiem znaczącej selektywnej aktywności przeciwnowotworowej (**4k, m**) oraz zwiększoną toksycznością wobec komórek prawidłowych (**4j, l**). Możliwym powodem tego może być fakt, że błony komórkowe różnych linii komórkowych mogą być selektywnie penetrowane przez zwitterjonowe pronukleotydy, ze względu na specyficzne oddziaływania między jonami obojnymi, a składnikami błony komórkowej. Pronukleotydy typu 5 (**5a, c-d**) z wolną grupą 3'-OH wykazują aktywność przeciwnowotworową, ale w większości przypadków brakuje im selektywności działania względem komórek nienowotworowych. Jedynie związek (**5c**) jest wysoce cytotoksyczny, ale tylko względem komórek T98G (SI=7). Jednak w przypadku pozostałych linii glejakowych (U-118 MG i U-87 MG) brakuje mu selektywnej aktywności (SI<1). Najkorzystniejszym parametrem indeksu selektywności spośród wszystkich badanych pronukleotydów charakteryzują się związki (**4c, f-i**). Ich znaczne wartości SI (w zakresie 12-66) wynikają z wysokiej aktywności cytotoksycznej ($IC_{50}=0,32-1,57 \mu M$) dla linii komórkowych glejaka i niskiej toksyczności ($IC_{50}=8,6-38,5 \mu M$) względem komórek prawidłowych. Dodatkowo wyznaczyliśmy aktywność cytotoksyczną fenoli (**2c, 2f-i**), które stanowią części związków typu 4. Stwierdziliśmy, że wartości IC_{50} tych fenoli w stosunku do linii komórek glejaków T98G, U-118 MG i U-87 MG mieszczą się w zakresie 55,61–500 μM [Supplementary data w (Szymanska-Michalak, 2018)], a zatem ich powstawanie jako potencjalnych metabolitów nie odpowiada za obserwowaną wysoką aktywność przeciwnowotworową pronukleotydów (**4c, 4f-i**). Podobnie jak w przypadku nukleozydów serii aromatycznych i alifatycznych 3'-O-estrów 5-fluoro-2'-deoksyurydyny (Szymanska-Michalak, 2016), otrzymane związki z serii pronukleotydów były badane pod

względem trwałości i dróg rozpadu w warunkach hodowli komórkowych (RPMI/FBS) oraz ludzkiej surowicy (ang. *human serum*, HS) [Table 1 w (Szymanska-Michalak, 2018)]. W medium hodowlanym RPMI-1640/FBS (9:1, v/v) rozkład badanych fosfodiestrów serii 4 i 5 był podobny i prowadził w pierwszej kolejności do utworzenia nukleotydu 8 (seria 4) oraz nukleotydu 10 (seria 5) [Scheme 2 w (Szymanska-Michalak, 2018)]. W przypadku czystej pożywki RPMI-1640, wszystkie fosfodiestry typu 4 i 5 pozostały niezmienione przez kilka dni. Stąd obserwowany rozkład można przypisać aktywności enzymatycznej fosfodiesteraz, które są obecne w surowicy ludzkiej i bydlęcej (Gosselin, 1993; Puech, 1993). Trzy z badanych związków (**4b**, **k**, **m**) były bardzo stabilne zarówno w medium hodowlanym jak i ludzkiej surowicy ($t_{1/2} > 240$ h) i jest to konsekwencja zawaad sterycznych nadawanych przez grupy maskujące fosforan (**4k**, **m**) oraz lipofilowości reszty fosfoestrowej nonylofenylu, która wytwarza powłokę hydrofobową, ograniczającą dostęp cząsteczek wody do centrum grupy fosforanowej (**4b**). Okresy połowicznej przemiany ($t_{1/2}$) fosfodiestrów posiadających grupę 3'-O-benzoilową (**4a**, **c-d**) były 3–4 razy krótsze niż w przypadku ich odpowiedników z wolną grupą 3'-OH (**5a**, **c**, **d**). W związku z faktem, iż hydroliza grupy 3'-O-benzoilowej z nukleotydu 8 oraz nukleozydu 9 przebiegała bardzo powoli, można wnioskować, że głównym związkiem odpowiedzialnym za działanie przeciwnowotworowe badanych pronukleotydów jest nukleotyd 8, a w mniejszym stopniu nukleozyd 9. Okresy połowicznej przemiany ($t_{1/2}$) wszystkich fosfodiestrów wynosiły kilka godzin, co zapewniało wystarczający czas na ekspozycję traktowanych komórek przy dostępie niezmienionych pronukleotydów. Stąd też można wnioskować, że oddysocjowanie kationowych grup ochronnych i uwolnienie FdUMP zachodziło we wnętrzu komórek.

Podsumowując, pięć z szesnastu związków (**4c**, **4f-i**) wykazywało znaczącą aktywność przeciwnowotworową i wysoką selektywność działania (SI=12–66). Jest to prawdopodobnie związane z faktem, że zwitterjonowe pronukleotydy mogą działać w podobny sposób jak zwitterjonowe fosfolipidy (Fahs, 2014), osłabiając wiązania lipid-lipid poprzez zaburzenie ładunku błony komórkowej, czyniąc tym samym komórkę przepuszczalną dla cząsteczek terapeutycznych.

Rosnącym zainteresowaniem cieszą się pochodne nukleozydów posiadające zamiast kanonicznej zasady azotowej ugrupowanie 1,2,3-triazolu (Efthymiou, 2012). Powodem zainteresowania tą grupą związków jest łatwość syntezy z zastosowaniem tzw. chemii „click” dzięki 1,3-dipolarnej cykloaddycji Huisgena (Kolb, 2001). Modyfikacje z użyciem chemii „click” we fragmencie zasady azotowej nukleozydu przeprowadza się w celu znakowania

fluoroforowego dla obrazowania molekularnego (Gramlich, 2008; Østergaard, 2010), formowania połączeń między niciami oligonukleotydów (El-Sagheer, 2009; Jacobsen, 2010; Xiong, 2012), czy do wytwarzania połączeń z cząsteczkami transportującymi w terapiach z użyciem kwasów nukleinowych (Seela, 2010). Niewiele natomiast jest prac dotyczących zmian naturalnej struktury szkieletu oligonukleotydowego (Krishna, 2012). Polianionowy charakter wynikający z obecności łącznika fosfodiesterowego ogranicza zastosowanie analogów oligonukleotydów jako substancji terapeutycznych (Bumcrot, 2006; Watts, 2008). W publikacji „*Synthesis and biological assay of new 2'-deoxyuridine dimers containing a 1,2,3-triazole linker. Part I*” (Michalska, 2019) [publikacja 3] skoncentrowałem się na wykorzystaniu jako potencjalnych chemioterapeutyków nowych dimerów 2'-deoksyurydyny z innymi nukleozydami pirymidynowymi zawierających układ 1,2,3-triazolowy w części fosforanowo-cukrowej. Przesłanką dla projektowania tego typu związków jest to, że uzyskiwany układ 1,2,3-triazolowy jest bioizosteryczny z wiązaniami peptydowymi i fosforanowymi. Jako substytut dla wiązania fosfodiesterowego układ ten w warunkach fizjologicznych jest elektrycznie obojętny. Powinno to sprzyjać aktywności biologicznej koniugatów wobec istotnych procesów enzymatycznych zachodzących w komórce. Ocenę cytotoksyczności sześciu dimerów nukleozydowych, związki oznaczone w [Table 1 w (Michalska, 2019)] jako nr (3a–c, 4a–c), pięciu nukleozydowych składników otrzymanych dimerów (związki 1a–c, 2a–b) oraz nukleozydu referencyjnego – 5-fluoro-2'-deoksyurydyny (5-FdU) wyznaczyłem z wykorzystaniem testu SRB wobec pięciu typów komórek nowotworowych: HeLa (komórki raka szyjki macicy), HepG2 (komórki raka wątroby), T98G, U-118 MG, U-87 MG (komórki glejaków), a także wobec komórek prawidłowych MRC-5 (fibroblasty). Uzyskane wyniki przedstawiłem w [Table 1 w (Michalska, 2019)]. Niestety, badane związki (1a–c, 2b, 3a–c, 4a–c), poza jednym wyjątkiem (2a), nie wykazują cytotoksyczności wobec komórek nowotworowych jak i prawidłowych, czego nie można powiedzieć o stosowanym referencyjnie cytostatyku – 5-fluoro-2'-deoksyurydynie (5-FdU). Wartości parametru IC₅₀ dla badanych cząsteczek są wyższe od 100 µM. Związek referencyjny wykazuje silną toksyczność zarówno wobec komórek nowotworowych, jak i prawidłowych. Jako jedyna spośród tych dwunastu związków, propargilowa pochodna 2'-deoksyurydyny (związek 2a) wykazuje aktywność cytotoksyczną, ale jest ona nieselektywna w stosunku do komórek nowotworowych i prawidłowych. Wartości IC₅₀ dla tego analogu wyznaczone na komórkach nowotworowych mieszczą się w zakresie 5,85 – 47,46 µM, a dla komórek prawidłowych wynoszą 32,91 µM. W świetle wyników analizy SRB wykazującej, że dimery nukleozydowe zawierające układ 1,2,3-triazolowy są nietoksyczne sugeruje się,

że za brak aktywności biologicznej może odpowiadać brak hydrolizy połączenia triazolowego i zbyt duża trwałość koniugatów, co uniemożliwia pokonywanie barier komórkowych. Ocenę stabilności sześciu dimerów nukleozydowych (**3a–c**, **4a–c**) w medium hodowlanym suplementowanym płodową surowicą bydlęcą (9:1, v/v) przeprowadziłem metodą HPLC. Badania wykazały, że wszystkie dimery pirymidynowe zawierające układ 1,2,3-triazolowy imitujący wiązanie fosfodiesterowe (**3a–c**, **4a–c**) wykazują się bardzo wysoką stabilnością, w przypadku których, po 120 godzinach inkubacji w 37°C, pozostało w medium z surowicą jeszcze ponad 90% stężenia wyjściowego. Obliczone *in silico* parametry fizykochemiczne [Table 2 w (Michalska, 2019)] wszystkich związków z wyjątkiem propargilowej pochodnej 2'-deoksyurydyny (**2a**) wskazują, że średnie wartości LogP mieszczą się w przedziale -2,08 do 1,01. Masy cząsteczkowe powyżej 500 Da dimerów nukleozydowych (**3a–c**, **4a–c**) oraz ujemne wartości LogP wskazujące na hydrofilowy charakter związków mogą sugerować trudności w ich przenikaniu i transporcie dokońrkowym. Na podstawie wyznaczonego współczynnika przenikalności tP_{app} przez błonę komórkową hodowli Caco-2 oraz danych literaturowych (Yazdanian, 1998), dimery nukleozydowe zawierające układ 1,2,3-triazolowy w części fosforanowo-cukrowej można zaklasyfikować do słabo absorbowanych z przewodu pokarmowego ($tP_{app} < 70$ nm/s). Wysokie wartości pola powierzchni polarnej (PSA) mieszczące się w przedziale 125,5–187,9 Å² wskazują więc na niską biodostępność badanych koniugatów wynikającą z niskiej dystrybucji i ograniczonej przenikalności przez błony komórkowe i barierę krew-mózg (Clark, 1999a, 1999b). Analizując parametry fizykochemiczne jedynej aktywnej biologicznie cząsteczki, tj. propargilowej pochodnej 2'-deoksyurydyny (**2a**) można zauważyć, że średnia wartość LogP dla tego związku wynosi 2,02, masa cząsteczkowa 380,18 Da (<500 Da), a pole powierzchni polarnej 51,0 Å². Spełnia ona także, zgodnie z regułą pięciu Lipińskiego (Lipinski, 2001), kryterium ilości akceptorów i donorów wiązań wodorowych (HBD, HBA). Oznacza to, że związek ten w porównaniu z nieaktywnymi dimerami cechuje się wyższą lipofilowością oraz małą powierzchnią polarną (<60 Å²), co praktycznie czyni go wysoce przenikalnym przez błony komórkowe, w tym także przez barierę krew-mózg (Kelder, 1999; Palm, 1997).

Reasumując, wyniki badań *in vitro* poparte obliczeniami *in silico*, wykazały, że brak aktywności biologicznej dimerów nukleozydowych zawierających układ 1,2,3-triazolowy w części fosforanowo-cukrowej wynika z trwałości i stabilności w warunkach fizjologicznych układu triazolowego, który rzutuje na niską biodostępność takich koniugatów.

W badaniach opisanych w publikacji „*Searching for anti-glioma activity. Ribonucleoside analogues with modifications in nucleobase and sugar moieties*” (Framski, 2016) [publikacja 4] określiłem cytotoksyczność analogów rybonukleozydów o różnych cechach strukturalnych i konformacyjnych z wykorzystaniem testu MTT wobec dwóch typów komórek nowotworowych: HeLa (komórki raka szyjki macicy) i T98G (glejak wielopostaciowy), a także wobec komórek prawidłowych MRC-5 (fibroblasty). Uzyskane wartości parametru IC_{50} zostały przedstawione w [Table 1 w (Framski, 2016)]. Eksperymenty przesiewowe wykazały, że większość rybonukleozydów purynowych, związki oznaczone w [Table 1 w (Framski, 2016)] jako nr 1–9 (z wyjątkiem nukleozydu 6-metylopuryny, związek nr 4) nie były cytotoksyczne wobec badanych komórek prawidłowych (MRC-5). Nukleozyd 6-metylopuryny (4) znany jest ze swojej silnej aktywności przeciw wirusowi opryszczki pospolitej (*herpes simplex virus*, HHV-1) oraz nieselektywnej aktywności cytotoksycznej względem komórek nowotworowych (Montgomery, 1968; Wu, 2010). W naszych badaniach wykazywał również (związek nr 4) wysoką cytotoksyczność ($IC_{50} < 1 \mu M$) zarówno wobec komórek nowotworowych glejaka T98G jak i raka szyjki macicy HeLa, ale jednocześnie był toksyczny wobec komórek prawidłowych linii MRC-5 ($IC_{50} = 3,3 \mu M$). Szczególnie aktywnymi związkami w tej podgrupie rybonukleozydów okazały się związki oznaczone jako nr (3) oraz nr (5). Naturalny 7-regioizomer guanozyny (5) oraz nukleozyd 6-chloropuryny (3) wykazywały wysoką i selektywną cytotoksyczność wobec komórek T98G przy czym nie były toksyczne dla prawidłowych fibroblastów (MRC-5). Wartość parametru IC_{50} dla tych związków mieści się w zakresie 1-10 μM . Dla porównania, wartość parametru IC_{50} dla temozolomidu (związek stosowany w terapii glejaków) wyznaczonego dla komórek T98G mieści się w zakresie kilku - kilkuset μM (Barciszewska, 2015). Pierścień pirymidyny, oprócz tego, że jest elementem struktury naturalnych nukleozydów stanowi ważny farmakofor obdarzony właściwościami farmakologicznymi (Radi, 2009). Wśród syntetycznych pochodnych zasad pirymidynowych zarejestrowanych jako produkty lecznicze, możemy wyróżnić 5-fluorouracyl i 5-azacytydynę (leki przeciwnowotworowe) oraz pochodne kwasu barbiturowego, które są środkami nasennymi i uspokajającymi. W podgrupie rybonukleozydów pirymidynowych przeanalizowałem cytydynę (10) i 5-azacytydynę (11) jako związki referencyjne oraz dwa rybonukleozydy o nietypowej konformacji *syn* wokół wiązania glikozydowego, mianowicie kwas 1-(β -D-rybofuranozylo) cyjanurowy (12) i 6-metylouracydynę (13) (Felczak, 1996; Khaled, 2004). 5-azacytydyna (Vidaza[®]), która jest zatwierdzona do leczenia zespołu mielodysplastycznego (ang. myelodysplastic syndrome, MDS) jest analogiem cytydyny inhibitującym metylotransferazę DNA, w której węgiel C5

w pierścieniu heterocyklicznym został zastąpiony przez azot (Wu, 2010). Możliwa rola metylacji DNA w terapii przeciwnowotworowej guzów mózgu skłoniła nas do włączenia 5-azacytydyny do naszych badań przesiewowych (Barciszewska, 2014; Christman, 2002). Spośród tych czterech związków tylko 5-azacytydyna (**11**) wykazywała aktywność cytotoksyczną, ale była ona nieselektywna w stosunku do komórek nowotworowych i prawidłowych. Nieco lepszą selektywność wobec komórek glejaka T98G wykazywał nukleozyd kwasu cyjanurowego (**12**), ale wskaźnik SI był nadal niski (SI=4). Kolejną grupą analogów rybonukleozydów, którą przeanalizowaliśmy była klasa związków zachowujących szkielet naturalnych rybonukleozydów, ale mających podstawnik β -metylowy w pozycji C2' ugrupowania D-rybozy. Ten rozgałęziony element strukturalny 2'-C-metylowy przekształca naturalne rybonukleozydy w cząsteczki o silnym i szerokim spektrum działania przeciwwirusowego (Benzaria, 2007; Walton, 1969). Do naszych badań wybraliśmy 2'-C- β -metylo-D-cytydynę (**14**), 5-aza-2'-C- β -metylo-D-cytydynę (**15**), 5-fluoro-2'-C- β -metylo-D-cytydynę (**16**) oraz 2'-C- β -metylo-D-guanozynę (**17**). Tylko pochodna cytydyny (**14**) wykazywała umiarkowaną, nieselektywną cytotoksyczność ($IC_{50} > 50 \mu M$), podczas gdy reszta związków z tej grupy była zasadniczo nietoksyczna. Porównując ją z grupą analogów pirymidynowych wydaje się oczywiste, że obecność grupy 2'-C-metylowej całkowicie znosiła cytotoksyczność 5-azacytydyny (**15** vs **11**), ale zauważalnie zwiększała cytotoksyczność cytydyny (**14** vs **10**). Bicykliczne analogi nukleozydów pirymidynowych (ang. *bicyclic pyrimidine nucleoside analogues*, BCNAs) to związki powstałe przez wprowadzenie dodatkowego heterocyklicznego pierścienia skondensowanego z pierścieniem pirymidynowym. Analogi nukleozydów, które posiadają w swojej strukturze pierścienie furano-[2,3-*d*]pirymidynowe oraz pirolo-[2,3-*d*]pirymidynowe wykazują właściwości fluorescencyjne oraz aktywność przeciwko wirusowi ospy wietrznej i półpaśca (*varicella zoster virus*, VZV) (Carangio, 2001; McGuigan, 2001). Natomiast aktywność BCNAs jako związków przeciwnowotworowych jest dotychczas słabo poznana. Badaniami aktywności biologicznej objęliśmy trzy lipofilowe bicykliczne pirolopirymidynowe analogi nukleozydów (**18-20**), trzy niepodstawione bicykliczne furanopirymidynowe analogi nukleozydów (**21-23**) oraz trzy lipofilowe bicykliczne furanopirymidynowe analogi nukleozydów (**24-26**). Chociaż większość badanych związków z tej podgrupy nie była cytotoksyczna lub wykazywała umiarkowaną i nieselektywną cytotoksyczność, to lipofilowa pochodna 6-oktylopirolu (**18**) wyraźnie wyróżniała się wysoką cytotoksycznością wobec komórek glejaka linii T98G ($IC_{50} = 0,74 \mu M$) oraz wysokim wskaźnikiem selektywności (SI=173).

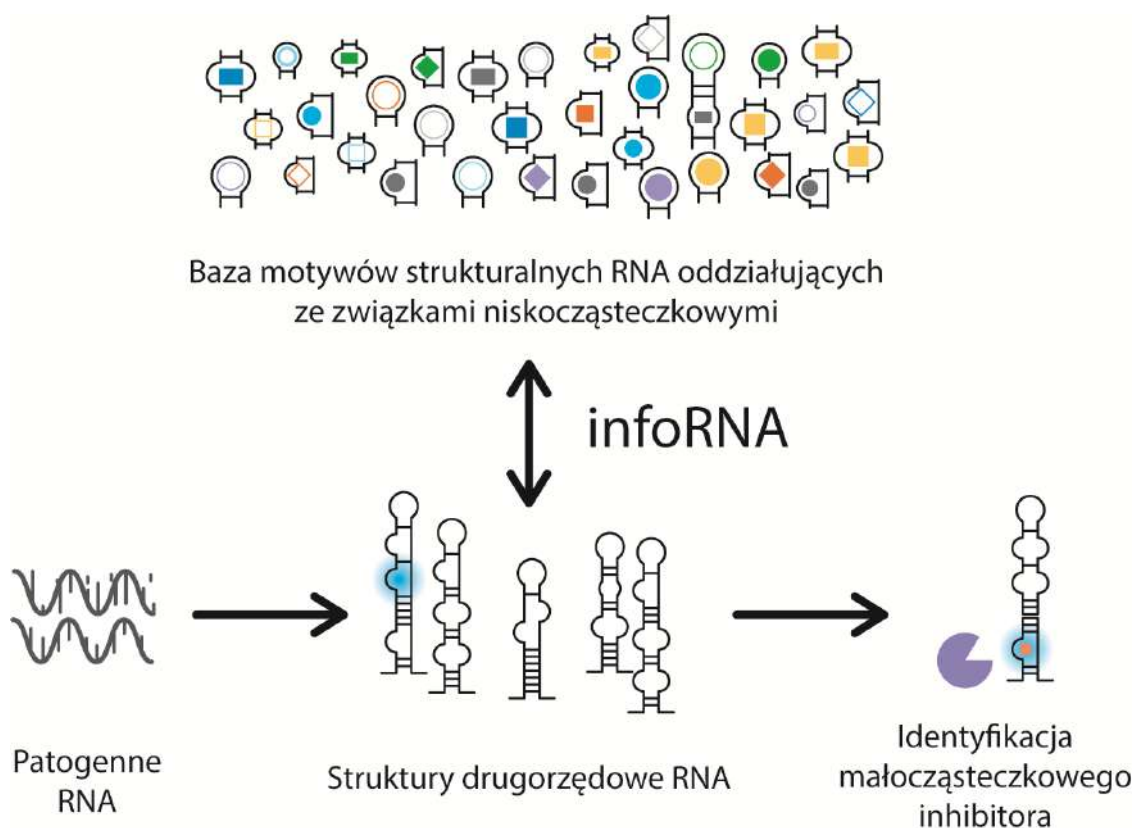
Podsumowując, przetestowaliśmy wybrane związki z naszej biblioteki analogów rybonukleozydów pod kątem właściwości przeciwnowotworowych względem komórek glejaka wielopostaciowego. W przypadku trzech związków (3), (5), (18) wykazaliśmy wysoką i selektywną cytotoksyczność wobec komórek linii T98G. Związki te mogą stanowić struktury wiodące do dalszych syntez nowych analogów o wysokiej aktywności biologicznej względem komórek nowotworowych.

Satysfakcjonujące wyniki badań z wykorzystaniem analogów nukleozydów i nukleotydów stały się dla mnie inspiracją i zachętą do poszukiwania i charakterystyki nowych cząsteczek o potencjale terapeutycznym. W nurcie tych poszukiwań, stwierdziłem, iż w literaturze brakuje szerokiego przeglądu dotyczącego właściwości biologicznych naturalnie występujących pochodnych adeniny. Zgromadzoną wiedzę podsumowałem w artykule przeglądowym pt. **„Aktywność biologiczna N^6 -furfuryloadenozyny” (Wawrzyniak, 2019) [publikacja 5]**. W pracy tej podsumowałem właściwości biologiczne cytokinin - małowcząsteczkowych związków pochodzenia naturalnego, pochodnych adeniny z co najmniej jednym podstawnikiem w pozycji N^6 . Moje szczególne zainteresowanie koncentrowało się na zagadnieniach dotyczących występowania, metabolizmu oraz aktywności biologicznej N^6 -furfuryloadeniny (kinetyny) i jej rybozydu – N^6 -furfuryloadenozyny (rybozyd kinetyny). W wyniku przeprowadzonego przeglądu literatury, zgromadziłem doniesienia naukowe dotyczące działania cytoprotekcyjnego i antystarzeniowego kinetyny oraz przeciwnowotworowego rybozydu kinetyny zarówno w warunkach *in vitro*, jak i *in vivo*. Kinetyna jest ważnym komponentem ścieżki metabolicznej, która umożliwia komórce pozbycie się nadmiaru wolnych rodników. Wpływa na wzrost i kształt komórek, tempo ich wzrostu oraz strukturę cytoszkieletu. Z kolei rybozyd kinetyny w zależności od stężenia, długości czasu leczenia i modelu doświadczalnego prowadzi do blokady postępu cyklu komórkowego oraz indukcji apoptozy poprzez obniżenie endogennego ATP i zaburzenie potencjału mitochondrialnego. Poza właściwościami biologicznymi, wskazałem potencjał aplikacyjny tych cząsteczek, nie podlegających dotąd zastosowaniom terapeutycznym.

2. Wykorzystanie miRNA jako cząsteczek docelowych w terapii nowotworów

Cząsteczki RNA, obok DNA i białek, mają kluczowe znaczenie dla procesów biochemicznych zachodzących w komórkach wszystkich organizmów i są niezbędnym elementem w przesyłaniu informacji oraz regulacji genów. Duże znaczenie niekodujących RNA (ncRNA) w chorobach nowotworowych predysponuje je jako cel terapeutyczny dla związków niskocząsteczkowych. Dostępne w piśmiennictwie dane wskazują, że małe cząsteczki mogą się wiązać i modulować funkcję RNA ulegających powtórzeniom (Arambula, 2009; Ofori, 2012), miRNA (Bose, 2012) oraz wirusowych RNA (Parsons, 2009; Stelzer, 2011). Przekonanie wielu badaczy, że RNA jest niedostępnym celem terapeutycznym dla leków nie ułatwia poszukiwania i opracowywania bioaktywnych związków, które mogłyby być na ten zamiar ukierunkowane. Kluczem do identyfikacji skutecznych i selektywnych względem RNA terapeutyków jest dokładna charakterystyka struktury RNA, a w szczególności motywów strukturalnych, które stanowią preferowane miejsce wiązania związków niskocząsteczkowych o wysokim stopniu powinowactwa i specyficzności (Velagapudi, 2014). Ograniczeniem poszukiwania cząsteczek nakierowanych na elementy strukturalne RNA może być jego polianionowy charakter, który sprzyja niespecyficznemu wiązaniu małych cząsteczek o dodatnim ładunku (Disney, 2016). Ponadto, za wyjątkiem rybosomalnych RNA (rRNA), które stanowią 80-90% wszystkich komórkowych RNA, większość RNA ulega ekspresji na niskim poziomie, co może powodować znaczne ograniczenie dostępności celu dla małych ligandów (Johnson, 1977). Mimo tych ograniczeń, celowanie w cząsteczki RNA może być bardzo korzystne. Podobnie jak białka, RNA w wyniku fałdowania tworzy elementy strukturalne takie jak, pętle, wybrzuszenia, motywy spinki czy pseudowęzły do których mogłyby wiązać się związki niskocząsteczkowe. Selektywność takiego działania można zwiększyć, biorąc pod uwagę fakt, że nie wszystkie miejsca wiązania małych cząsteczek są miejscami funkcjonalnymi, co oznacza, że mimo występowania danego motywu w wielu transkryptach, wiązanie tylko z jednym z nich może powodować efekt biologiczny (Disney, 2016). Inną ważną zaletą postrzegania RNA jako celu terapeutycznego jest istnienie algorytmów przewidujących strukturę drugorzędową na podstawie sekwencji nukleotydów oraz metod wspomagających projektowanie małowiązących ligandów wiążących się do RNA (Mathews, 2014). Jednym z takich narzędzi, które ułatwia poszukiwanie niskocząsteczkowych farmakoforów obdarzonych właściwościami farmakologicznymi i mogących wiązać się do motywów strukturalnych RNA

jest infoRNA (Disney, 2016; Velagapudi, 2014). Na rycinie 4 przedstawiono zasadę działania tego narzędzia.



Rycina 4. Wspomagane przez infoRNA projektowanie związków niskocząsteczkowych ukierunkowanych na strukturę RNA. Użytkownik identyfikuje RNA będący przedmiotem zainteresowania i generuje jego strukturę drugorzędową. Następnie infoRNA wyodrębnia motywy strukturalne z docelowego RNA i porównuje je z bazą danych w celu wytypowania najlepszych struktur wiodących dla zaprojektowania cząsteczek terapeutycznych. Opracowanie graficzne ryciny – mgr inż. Mateusz Starosta, na podstawie (Disney, 2016).

Przeprowadzone wcześniej badania biblioteki związków niskocząsteczkowych obejmującej ponad 200 struktur chemicznych doprowadziły do wyselekcjonowania dużej populacji aktywnych cząsteczek o potencjale cytostatycznym. Analizę taką można określić jako przesiewową, ponieważ otwiera ona drogę do dalszych badań nad mechanizmem działania tych związków i chociaż koncentrują się one głównie na białkowych celach terapeutycznych, to możliwość celowania w RNA za pomocą takich związków ma ogromny potencjał, który zamierzam wykorzystać w moich dalszych badaniach. Wykorzystanie onkogennych miRNA jako celu terapeutycznego dla małych cząsteczek w pierwszej

kolejności wymaga poznania różnych aspektów oddziaływania niekodujących RNA zawierających wysoce ustrukturyzowane elementy. Dla zrozumienia funkcji miRNA ważna jest więc nie tylko identyfikacja docelowych cząsteczek mRNA czy badanie prekursorów miRNA. Ze względu na brak jasnych danych wskazujących na specyficzność działania miRNA, poza powszechnie przyjętym mechanizmem regulacji w oparciu o rejon źródłowy, uznaliśmy, iż klasa miRNA wymaga głębszej charakterystyki.

W związku z powyższym, w celu poznania i zrozumienia mechanizmów regulacji potranskrypcyjnej z udziałem miRNA wykonaliśmy szeroką i szczegółową analizę dojrzałych miRNA pod względem ich struktury I- i II-rzędowej, a wyniki badań zostały opisane w „*The sequence and structure determine the function of mature human miRNAs*” (Rolle, 2016) [publikacja 6]. Moja rola w niniejszym projekcie polegała na przeprowadzeniu analiz sekwencji nukleotydowych 2042 dojrzałych miRNA oraz tych zawierających pojedyncze powtórzenia (ang. *simple sequence repeats*, SSRs). Analizy przeprowadziłem z wykorzystaniem skryptów w języku Python (v.2.7.3) napisanych przez mgr Jarosława Jeleniewicza oraz oprogramowania SSRIT (*Simple Sequence Repeat Identification Tool*) do identyfikacji i lokalizacji pojedynczych powtórzeń w zadanych sekwencjach miRNA (<http://www.gramene.org/db/searches/ssrtool>). Na podstawie analizy składu nukleotydowego stwierdziłem, iż miRNA bogate są przede wszystkim w guanozynę i uracyl, który dodatkowo zajmuje zwykle pierwsze i ostatnie miejsce w łańcuchu nukleotydowym. Wykazałem, iż trzy pierwsze nukleotydy przy końcu 5' miRNA to UAA, a rejon źródłowy (2-8nt) zaangażowany bezpośrednio w oddziaływanie z docelowym mRNA jest bogaty w uracyl i adeninę. Obecność termodynamicznie słabego oddziaływania pomiędzy miRNA a docelowym mRNA, sugeruje działanie dodatkowych czynników stabilizacyjnych, co skłoniło nas do szczegółowej analizy struktury drugorzędowej dojrzałych miRNA.

Na podstawie przeprowadzonej metaanalizy literatury w kontekście funkcjonalnych aspektów obecności motywów strukturalnych i sekwencyjnych przeanalizowaliśmy dostępne w miRBase dojrzałe miRNA pod kątem obecności: (a) motywu UGUGU – znany ze zdolności aktywowania odpowiedzi interferonowej; (b) powtórzeń GU – znacząca przewaga tych dinukleotydów w sekwencjach miRNA; (c) zawartości puryn oraz pirymidyn. Wykorzystując narzędzia bioinformatyczne, w oparciu o wskazane kryteria, zaproponowaliśmy listę 50 miRNA zawierających motyw indukcji interferonu, 23 miRNA bogate w powtórzenia GU (3 lub więcej powtórzeń) oraz 141 miRNA bogatych w puryny i 187 miRNA bogatych w pirymidyny. Konsekwencją analizy sekwencji dojrzałych miRNA była obszerna charakterystyka szlaków, procesów biologicznych oraz białek związanych

z poszczególnymi charakteryzowanymi miRNA. Wykazaliśmy, iż grupa miRNA charakteryzująca się obecnością motywu UGUGU aktywuje docelowe mRNA związane ze szlakami indukcji interferonu lub z procesami nowotworzenia. Z kolei, miRNA bogate w sekwencje powtórzone GU są zaangażowane głównie w procesy neurogenezy, podczas gdy miRNA obfitujące w puryny lub pirymidyny odpowiadają za regulację procesów transportu i/lub degradacji RNA.

Podsumowując, w wyniku przeprowadzonej analizy wykazaliśmy bezpośrednią zależność pomiędzy sekwencją miRNA, a rodzajem regulowanych przez nie docelowych mRNA. Przedstawiona charakterystyka dojrzałych miRNA może mieć kluczowe znaczenie dla wyboru związków niskocząsteczkowych o charakterze terapeutycznym oraz może wpłynąć na selekcję związków do dalszego testowania w modelach *in vitro* i *in vivo* już na etapie projektowania strategii terapeutycznej.

3. Wykorzystanie i dostarczanie dwuniciowego RNA jako specyficznego i efektywnego narzędzia molekularnego

W ramach opracowania nowego podejścia terapeutycznego opartego na efektywnej genoterapii z wykorzystaniem nanotechnologii, zaangażowany byłem w realizację projektu badawczego Narodowego Centrum Nauki pod tytułem „*Nowe, wielozadaniowe nanocząstki w skojarzonej geno- i fototerapii*”, którego celem jest synteza nowych, wielozadaniowych nanocząstek opartych na rdzeniu nieorganicznym i polimerowym dla dostarczania dwuniciowego RNA (dsRNA) o sekwencji homologicznej do tenascyny-C (TNC) do komórek glejaka wielopostaciowego. W wyniku realizacji tego projektu, przeprowadziłem badania funkcjonalne na linii komórkowej glejaka U-118 MG po traktowaniu nanocząstkami magnetycznymi (magnetyt, Fe₃O₄) otoczonymi polietylenoiminą (PEI) skoniugowanymi z dsRNA. Wyniki badań zostały opisane w pracy pt. **“*Nano-Mediated Delivery of Double-Stranded RNA for Gene Therapy of Glioblastoma Multiforme*”** (Grabowska, 2019) [publikacja 7]. Guzy glejowe wykazują szereg markerów powierzchniowych, które mogą być wykorzystane w leczeniu tych nowotworów. Jednym z nich jest tenascyna-C, białko macierzy zewnątrzkomórkowej będące glikoproteiną mającą udział w adhezji, inwazyjności i migracji tychże guzów (Orend, 2006; Pas, 2006). Opracowana przez zespół prof. dr hab. Jana Barciszewskiego z Instytutu Chemii Bioorganicznej PAN wraz z grupą prof. dr hab. Stanisława Nowaka z Kliniki Neurochirurgii Uniwersytetu Medycznego im. Karola

Marcinkowskiego w Poznaniu innowacyjna metoda zastosowania terapii genowej polegającej na wprowadzeniu do komórek glejowych u pacjentów dwuniciowego RNA o sekwencji homologicznej do tenascyny-C (ATN-RNA) pozwoliła na obniżenie ekspresji tenascyny-C, zahamowanie wzrostu guza w rejonie podania preparatu oraz opóźnienie wznowy nowotworu. Ponadto, zastosowana terapia genowa nazwana interwencją interferencyjnym RNA (iRNAi) doprowadziła do poprawy komfortu oraz przedłużenia życia pacjentów (Rolle, 2010; Zukiel, 2006). Pomimo niezwykle obiecujących wyników terapii eksperymentalnej, pamiętać należy iż prowadzona ona była w oparciu o podawanie terapeutycznego bezpośrednio w miejsce usuniętego guza w trakcie operacji neurochirurgicznej. Jedną z głównych trudności terapii genowej glejaka pozostaje jednak problem dostarczania terapeutycznego do wnętrza komórek docelowych w aplikacji systemicznej oraz brak stabilności niemodyfikowanych kwasów nukleinowych w płynach ustrojowych pacjentów (Al-Dosari, 2009). Stąd, zaprojektowanie skutecznej terapii kwasami nukleinowymi wymaga opracowania systemu nośnika, który powinien zapewniać skuteczne i precyzyjne dostarczanie leku do wnętrza komórek oraz ochronę przed degradacją enzymatyczną, nie wpływając na właściwości fizykochemiczne samego leku (Williford, 2014). Dzięki nowoczesnej nanotechnologii możliwe jest otrzymywanie nowych zaawansowanych nanonośników pozwalających na precyzyjne wprowadzenie hydrofobowych i ujemnie naładowanych „ładunków” terapeutycznych (kwasy nukleinowe, siRNA, DNA) do komórek, ale także umożliwiających diagnostykę oraz łączenie kilku terapii w celu uzyskania synergistycznego efektu terapeutycznego (G. Bao, 2013). Ostatnie doniesienia pokazują, że magnetyczne nanocząstki mogą skutecznie wprowadzać siRNA przeciwko surwiwinie do komórek glejowych, jednak efekt terapeutyczny jest niezbyt wysoki (Xueqin Wang, 2015). W związku z powyższym, celem pracy była synteza i charakterystyka nanocząstek magnetycznych pokrytych polietylenoiminą (Mag@PEI) skoniugowanych z ATN-RNA oraz ocena funkcjonalna w komórkach glejaka wielopostaciowego.

W pierwszym etapie zaplanowanych badań, przeprowadziłem analizę cytotoksyczności nanocząstek Mag@PEI bez dwuniciowego RNA o sekwencji homologicznej do tenascyny-C (ATN-RNA). W wyniku przeprowadzonej oceny cytotoksyczności Mag@PEI za pomocą testu SRB wykazałem, że nanocząstki nie są toksyczne w analizowanym zakresie stężeń wynoszącym 0–1300 ng/ml. Kluczowe dla projektu było potwierdzenie wiązania dwuniciowego RNA do nanocząstek. Na obrazie żelu agarozowego widać wyraźnie brak prążka od ATN-RNA przy stosunku masowym Mag@PEI do ATN-RNA 3:1, co świadczy o jego wiązaniu do materiału [Fig 4 (Grabowska, 2019)]. Następnie w celu zbadania

skuteczności dostarczania ATN-RNA na nanonośnikach Mag@PEI oceniłem szybkość proliferacji komórek nowotworowych. W tym celu komórki U-118 MG traktowałem Mag@PEI oraz Lipofectamine 2000 (kontrola, nośnik kationowy rutynowo stosowany w transfekcji komórek) w połączeniu z ATN-RNA, a następnie mierzyłem tempo proliferacji komórek w czasie rzeczywistym w systemie xCELLigence. Przeprowadzone testy wykazały zależne od stężenia ATN-RNA obniżenie tempa proliferacji o 40-61% dla nośnika Lipofectamine 2000 z największym spadkiem proliferacji przy stężeniu 100 nM ATN-RNA [Fig 10B (Grabowska, 2019)]. W przypadku nośnika Mag@PEI w pierwszych 24 godzinach zaobserwowaliśmy odwrotny efekt, tj. wzrost proliferacji o 10-28% również najskuteczniejsze stężenie ATN-RNA wynosiło 100 nM [Fig 10A (Grabowska, 2019)]. Warto zauważyć, że 25 nM i 50 nM stężenie ATN-RNA było już wystarczającym dla wydajnych zmian w proliferacji komórek U-118 MG. Po 48 i 72 godzinach od transfekcji ponownie zaobserwowaliśmy hamowanie proliferacji, odpowiednio o 7-45%. To pozwoliło nam założyć, że najskuteczniejsze dostarczanie ATN-RNA osiągnęte jest po pierwszych 24 godzinach od transfekcji. Pozwala to zatem sugerować, że istotny spadek szybkości proliferacji zarówno dla Lipofectamine 2000, jak i dla Mag@PEI następuje po 48 godzinach. Natomiast po 72 godzinach od transfekcji obserwowany jest raczej efekt toksyczny aniżeli działanie ATN-RNA. Następnie w przeprowadzonych testach migracji w czasie rzeczywistym, zaobserwowałem, że obniżenie ekspresji tenascyny-C przez ATN-RNA dostarczanym na nośniku Mag@PEI znacząco zaburzyło migrację komórek U-118 MG [Fig 11A (Grabowska, 2019)]. Wykazałem, że komórki U-118 MG transfekowane ATN-RNA miały najniższą ruchliwość zaczynając od 12 godzin po transfekcji. Stwierdziłem, że ATN-RNA na nośniku Mag@PEI opóźnia migrację komórek U-118 MG o $5,76 \pm 1,02$ (10 nM); $30,06 \pm 5,78$ (25 nM); $31,46 \pm 3,41$ (50 nM) i $20,03 \pm 2,65$ godzin (100 nM). Stąd można zakładać, że najskuteczniejsze stężenie ATN-RNA wpływające na potencjał migracji komórek wynosi 50 nM, podczas gdy dwa razy wyższe stężenie prowadzi do zwiększonej umieralności komórek.

Aby zbadać, czy rzeczywiście materiał Mag@PEI wnika do komórek glejaka wielopostaciowego przeprowadzono badania nad internalizacją nanocząstek z wykorzystaniem konfokalnej mikroskopii laserowej bazując na intensywności fluorescencji barwnika ATTO550 połączonego z Mag@PEI. Zdjęcia z mikroskopii konfokalnej dla komórek inkubowanych z nanocząstkami pozwoliły na potwierdzenie hipotezy o wnikaniu materiału do komórek glejaka wielopostaciowego [Fig 5 (Grabowska, 2019)].

Niezwykle ważnym aspektem prowadzonych badań było sprawdzenie, czy kompleksy dsRNA z materiałem Mag@PEI nie powodują odpowiedzi immunologicznej. W tym celu przeprowadzono analizę ekspresji genów OAS1 (syntetaza 2',5'-oligoadenylnowa 1), OAS3 (syntetaza 2',5'-oligoadenylnowa 3), RIG1 (receptor RIG-I), TLR3 (Toll-like receptor 3) oraz INF γ (interferon gamma). Uzyskane dane wykazały, że pod wpływem kompleksu ATN-RNA z Mag@PEI zwiększeniu ekspresji ulegają tylko geny TL3 oraz INF γ , jednak ekspresja względem kontroli nie różniła się dwukrotnie (fold > ± 2) [patrz Fig 8 (Grabowska, 2019)].

Podsumowując, w wyniku przeprowadzonych badań zaproponowaliśmy nowy system dostarczania dsRNA (ATN-RNA) do komórek glejaka wielopostaciowego w celu obniżenia ekspresji tenascyny-C, który wykorzystuje dobrze scharakteryzowane nanocząsteczki magnetyczne pokryte polietylenoiminą. Zarówno same nanocząstki, jak i ich kompleksy z dsRNA nie wykazują toksyczności oraz nie wywołują niepożądaną odpowiedzi immunologicznej w linii komórkowej U-118 MG.

4. Badanie aktywności biologicznej analogów strukturalnych aminokwasów

Jestem również współautorem pracy eksperymentalnej pt. „*Synthesis, structural studies and biological properties of some phosphonoperfluoro-phenylalanine derivatives formed by S_NAr reactions*” (Kwiczak-Yiğitbaşı, 2019) [publikacja 8]. Mój udział w powyższej publikacji, która została włączona do niniejszej rozprawy, jest wynikiem współpracy naszego zespołu z prof. UAM dr hab. Donatą Pluskotą-Karwatką (Zakład Syntezy i Struktury Związków Organicznych, UAM Poznań) i prof. dr hab. Marcinem Hoffmannem (Pracownia Chemii Kwantowej, UAM Poznań), w ramach której byłem odpowiedzialny za przeprowadzenie badań farmakodynamicznych *in vitro* w komórkach glejaka oraz wyznaczenie parametrów farmakokinetycznych na podstawie obliczeń *in silico* fluorowanych α -aminofosfonianów. Kwasy α -aminofosfonowe i ich estry stanowią licznie reprezentowaną klasę związków chemicznych, stanowiących analogi naturalnie występujących aminokwasów karboksylowych. Zdolność do selektywnej inhibicji różnego rodzaju enzymów przekłada się na ich wysoką i różnokierunkową aktywność biologiczną. Znajdują one zastosowanie jako antybiotyki, leki przeciwnowotworowe, leki antywirusowe, leki antypasożytnicze, regulatory wzrostu roślin, fungicydy, pestycydy czy też herbicydy (Kafarski, 2001; Orsini, 2010). Jednak do tej pory, nie ma badań odnoszących się do fluorowanych α -aminofosfonianów jako potencjalnych cząsteczek o aktywności przeciwnowotworowej względem komórek glejaków.

W toku realizowanych prac badawczych zsyntetyzowano bibliotekę fluorofosfonianowych analogów fenyloalaniny, poszerzając klasę związków o potencjalnej aktywności biologicznej [Table 7 w (Kwiczak-Yiğitbaşı, 2019)]. Obecność perfluorowanego pierścienia sprawiła, że α -aminofosfonian (**1a**) poddano regioselektywnym reakcjom aromatycznej substytucji nukleofilowej [Scheme 1 w (Kwiczak-Yiğitbaşı, 2019)] z nukleofilami, takimi jak, tiole, aminy, fenole, w wyniku których otrzymano szereg *para* podstawionych w pierścieniu aromatycznych pochodnych [**2a-s**, Table 1 w (Kwiczak-Yiğitbaşı, 2019)]. Dla dwóch związków (**2k**) i (**2l**) wykonano badania rentgenograficzne metodą dyfrakcji promieni rentgenowskich na monokryształach. W stanie stałym cząsteczki te różnią się konformacją, (**2k**) (pochodna zawierająca grupę metyloaminową) przyjmuje konformację z prawie prostopadłym układem obu pierścieni aromatycznych, podczas gdy (**2l**) (pochodna zawierająca grupę propyloaminową) preferuje wydłużoną konformację z pierścieniami aromatycznymi rozmieszczonymi prawie równolegle [Fig. 1 i Fig. 2 w (Kwiczak-Yiğitbaşı, 2019)]. Co ciekawe, struktura krystaliczna związku macierzystego (**1a**) również przyjmuje konformację z równoległe zorientowanymi pierścieniami aromatycznymi. Oznacza to, że wprowadzenie podstawnika w pozycję *para* w fluorowanym pierścieniu aromatycznym, może, ale nie musi, zmieniać konformacji uzyskanej cząsteczki w porównaniu ze strukturą związku macierzystego. Ze względu na różną konfigurację atomów azotu w anilinie, dwie cząsteczki o strukturze krystalicznej (**2k**) stanowią parę diastereoizomerów. Zjawisko to badano za pomocą obliczeń kwantowo-chemicznych. Wyniki uzyskane na poziomie teorii WB97XD/6-31+G(d) dla izolowanej cząsteczki wskazują na stereoizomer *R,S,R* jako energetycznie korzystniejszy, natomiast dla cząsteczki w ośrodku ciągłym naśladującym roztwór chloroformu na stereoizomer *R,R,R*. Wyniki te potwierdzają różnorodność strukturalną stereoizomerów, a ich współistnienie obserwowane w strukturze krystalicznej jest najprawdopodobniej spowodowane interakcjami międzycząsteczkowymi.

Ocenę cytotoksyczności (1) sześciu fosfonianowych analogów fluorowanej fenyloalaniny, związki oznaczone w [Table 7 w (Kwiczak-Yiğitbaşı, 2019)] jako (**1a-f**), (2) sześciu fosfonianowych analogów fluorowanej fenyloalaniny otrzymanych na drodze reakcji aromatycznej substytucji nukleofilowej (związki **2b-r**) oraz (3) związku referencyjnego – 5-fluoro-2'-deoksyurydyny (**5-FdU**) wyznaczyłem z wykorzystaniem testu MTT wobec dwóch typów komórek nowotworowych glejaka wielopostaciowego: T98G i U-118 MG, a także wobec komórek prawidłowych HaCaT (keratynocyty). Wyniki badań, wykazały, że wszystkie badane związki prezentowały aktywność cytostatyczną w obu liniach komórek glejaka. Większość z badanych związków wykazywała wyższą aktywność w stosunku do linii

komórkowej T98G. Tylko pochodne (**1d**) i (**1e**) oraz w niewielkim stopniu (**2d**) były aktywniejsze w glejaku linii U-118 MG. Przeważająca ilość pochodnych otrzymanych na drodze reakcji aromatycznej substytucji nukleofilowej (**2b-f**) była bardziej cytotoksyczna względem obu linii komórek glejaka wielopostaciowego niż macierzysty α -aminofosfonian (**1a**). Wyjątek stanowiły związki (**2j**) i (**2r**). Oznacza to, że wprowadzenie tiofenoli, ale nie alifatycznych tioli czy fenoli w pozycję *para* fluorowanego pierścienia fenyłowego cząsteczki (**1a**) może zwiększać aktywność cytostatyczną względem komórek glejaka. Przedstawione w [Table 7 w (Kwiczak-Yiğitbaşı, 2019)] wartości IC₅₀ pokazują, że dla najaktywniejszych związków (**2b**), (**2d**), (**2e**) i (**2f**), również podstawniki w ugrupowaniu tiofenolowym miały wpływ na aktywność cytotoksyczną. Wszystkie te podstawniki należą do grupy elektrodonorowej i można wywnioskować, że mniej nukleofilowy charakter grupy -CH₃ niż grup -OCH₃ i -NH₂, powoduje wyższą aktywność pochodnej (**2b**) w porównaniu z związkami (**2d-f**). Analiza wartości IC₅₀ uzyskanych dla α -aminofosfonianów (**1a-f**) wskazuje, że liczba podstawników fluorowych w jednym pierścieniu aromatycznym, a także obecność i charakter podstawników w drugim pierścieniu przyczyniają się do działania przeciwnowotworowego wykazywanego przez te związki. Pochodne (**1b**) i (**1c**) zawierające w swoich cząsteczkach jeden atom fluoru i jeden atom chloru, prezentują bardzo podobną aktywność cytotoksyczną przeciw obu liniom nowotworowym, co sugeruje, że pozycja atomu fluoru nie ma większego wpływu na aktywność biologiczną. Porównanie aktywności tych związków z aktywnością pochodnej (**1e**) wskazuje, że podstawnik chloru może stanowić ważny czynnik przyczyniający się do zwiększenia aktywności przeciwnowotworowej. Dodatkowo, zweryfikowałem aktywność antyproliferacyjną fluorowanych α -aminofosfonianów w teście klonogennym z zastosowaniem linii komórkowej T98G. Podobnie, pochodne otrzymane na drodze reakcji aromatycznej substytucji nukleofilowej były znacznie aktywniejsze, aniżeli niepodstawione α -aminofosfoniany. Przeżywalność i zdolność do kolejnych podziałów komórek T98G traktowanych 20 μ M stężeniami związków (**2d**), (**2e**) i (**2f**) wynosiła odpowiednio 85%, 30% i 10% w porównaniu z komórkami kontrolnymi. Dla wyższego stężenia 50 μ M, przeżywalność była jeszcze mniejsza i wynosiła odpowiednio 75%, 15% i 2% [Fig. 6 w (Kwiczak-Yiğitbaşı, 2019)]. W celu scharakteryzowania parametrów fizykochemicznych badanych związków przeprowadziłem kompleksową analizę *in silico*, która pozwoliła wykazać potencjalny wpływ modyfikacji na procesy farmakokinetyczne. Obliczone deskryptory dla wszystkich pochodnych [Table 6 w (Kwiczak-Yiğitbaşı, 2019)] wskazują na dobre wchłanianie w przewodzie pokarmowym oraz wysoką przenikalność przez błony komórkowe i barierę krew-mózg. Ponadto, wszystkie pochodne spełniają kryteria

zawarte w regule pięciu Lipińskiego (Lipinski, 2001).

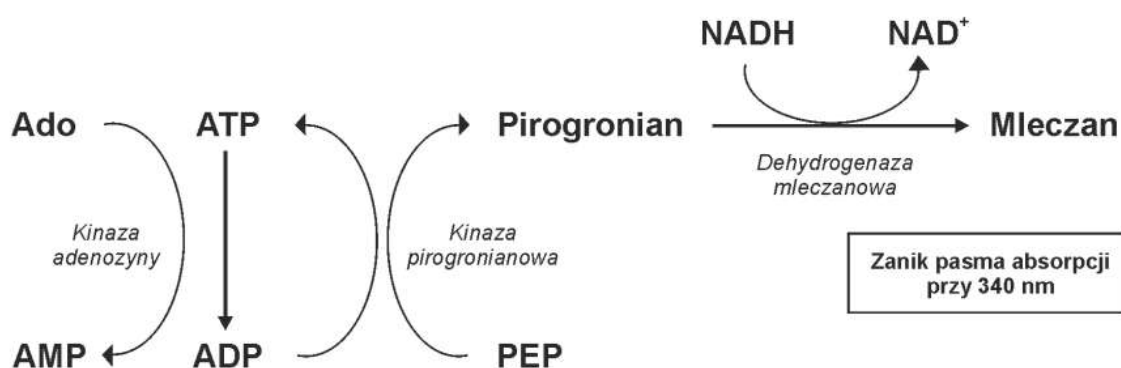
Reasumując, wyniki testów aktywności biologicznej wykazały, że fluorowane pochodne α -aminokwasów, zwłaszcza te utworzone przez wprowadzenie ugrupowania tiofenolowego w pozycję *para* fluorowanego pierścienia aromatycznego, wykazują się znacznie wyższą aktywność przeciwnowotworową wobec komórek glejaków wielopostaciowych (T98G i U118 MG) niż związek macierzysty (**1a**). Pozwala to wnioskować, że analogi α -aminofosfonianowe oparte o strukturę perfluorofenyloalaniny mogą stanowić podstawę do opracowania nowych cząsteczek o aktywności przeciwdziałającej rozwojowi glejaków.

5. Wybrane inne projekty badawcze

Poza badaniami przedstawionymi w jednotematycznym cyklu publikacji wchodzących w skład rozprawy doktorskiej obszar moich zainteresowań naukowych dotyczył między innymi badania mechanizmu fosforylacji rybonukleozydów purynowych przez kinazę adenozynową (ADK). ADK katalizuje reakcję fosforylacji adenozyny do AMP przy udziale drugiego substratu, jakim jest Mg/ATP^{2-} . Obecnie trwają intensywne poszukiwanie proleków aktywowanych bezpośrednio w komórce nowotworowej. Koncepcja ta oparta jest na spostrzeżeniu, że w komórkach neoplastycznych często dochodzi do zwiększonej ekspresji charakterystycznych dla danego typu nowotworu enzymów, które nie są obecne w komórkach prawidłowych lub ich ekspresja jest ograniczona (Zawilska, 2013). Poszukiwane są więc cząsteczki, które mogą ulegać aktywacji w komórkach nowotworowych za sprawą specyficznie występujących w nich enzymów, a jednym z nich ulegających nadekspresji w guzach mózgu jest kinaza adenozyny (Lee, 2008). W badaniach klinicznych znajduje się tricykliczna pochodna adenozyny (Triciribine) fosforylowana przez ADK do aktywnej postaci, która jest inhibitorem kinazy białkowej B. Związek ten zaproponowano jako chemioterapeutyk do spersonalizowanej terapii przeciwnowotworowej opartej o profilowanie ekspresji ADK u chorego (Shedden, 2003).

Badania fosforylacji rybonukleozydów purynowych prowadziłem w ramach projektu naukowego, którego byłem kierownikiem. Grant dla młodych naukowców i doktorantów Instytutu Chemii Bioorganicznej PAN pt. „Aktywność przeciwnowotworowa modyfikowanych nukleozydów purynowych i pirymidynowych w wybranych liniach komórek nowotworowych” (Nr 25/GM/2017) finansowany był z Ministerstwa Nauki i Szkolnictwa Wyższego. Celem badań było (1) opracowanie

enzymatycznie-sprężonego testu spektrofotometryczno-fluorymetrycznego dla kinazy adenozynowej prowadzonego w sposób ciągły, a następnie (2) zbadanie katalizowanej kinazą adenozynową fosforylacji pochodnych adenozyny podstawionych w pozycji N^6 będących potencjalnymi pronukleotydami aktywowanymi bezpośrednio w tkance nowotworowej wykazującej nadekspresję kinazy adenozynowej (np. glejak wielopostaciowy). Pierwszy etap pracy obejmował przygotowanie enzymatycznie-sprężonego testu spektrofotometryczno-fluorymetrycznego dla kinazy adenozynowej (Rycina 5).

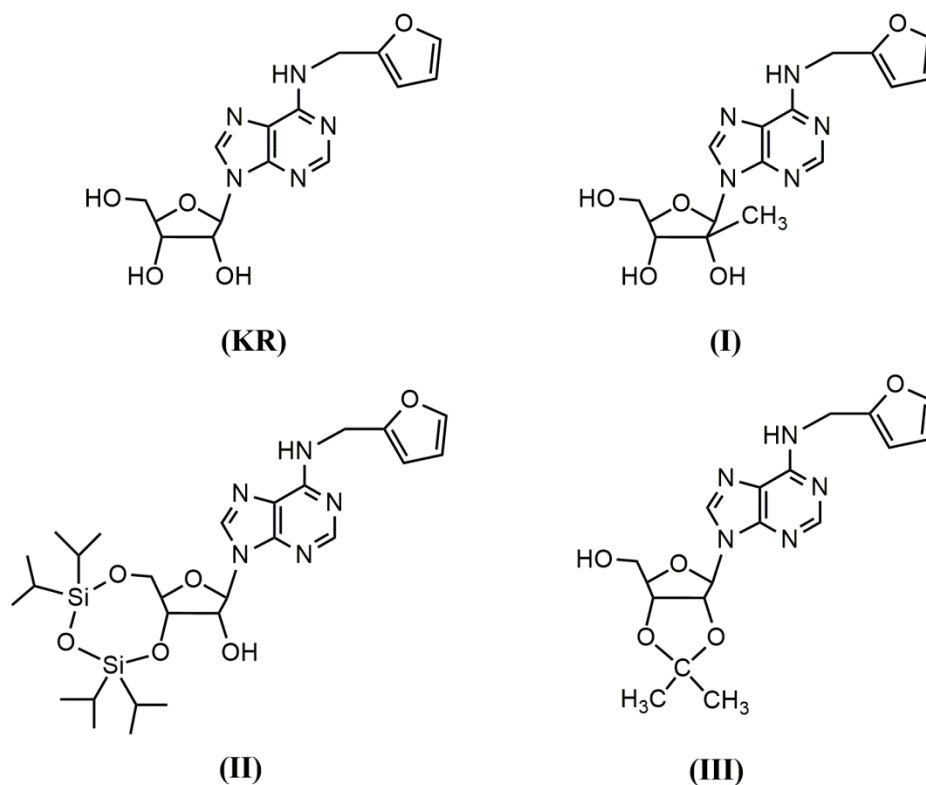


Rycina 5. Schemat enzymatycznie-sprężonego testu spektrofotometrycznego dla ADK. Ado; adenozyna (przykładowy substrat reakcji fosforylacji) jest przekształcana do AMP, a PEP: fosfoenolopirogronian do pirogronianu. Redukcji pirogronianu do mleczanu towarzyszy zanik pasma absorpcji przy 340 nm. NADH: zredukowany dinukleotyd nikotynoamido-adeninowy, NAD^+ : utleniony dinukleotyd nikotynoamidoadeninowy.

W reakcji fosforylacji modyfikowanego rybonukleozydu katalizowanego przez ADK przy udziale ATP powstaje ufosforylowany nukleozyd oraz ADP, który może ponownie przekształcić się w ATP, przejmując fosforan od wysokoenergetycznego fosfoenolopirogronianu (PEP) w reakcji katalizowanej przez kinazę pirogronianową (PK). Następnie powstający pirogronian ulega redukcji do mleczanu przy udziale dehydrogenazy mleczanowej (LDH), podczas której NADH utlenia się do NAD^+ . Przebieg reakcji monitorowano (1) spektrofotometrycznie mierząc zmiany absorbancji w czasie reakcji (forma zredukowana NADH posiada dodatkowe pasmo absorpcji przy 340 nm) oraz (2) spektrofluorymetrycznie ($\lambda_{ex}=355$ nm; $\lambda_{em}=460$ nm).

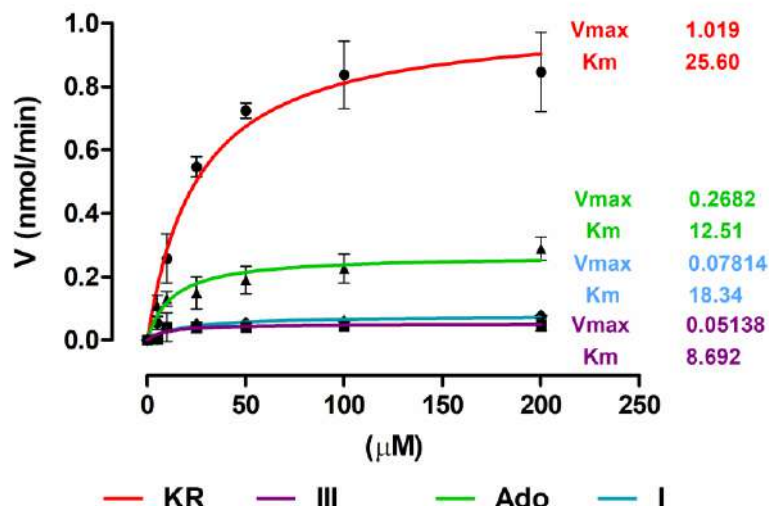
W drugim etapie wdrożyłem test do badania mechanizmu fosforylacji rybonukleozydów przez kinazę adenozynową. Analiza obejmowała rybozyd kinetyny (N^6 -furfuryloadenozyna, KR) i jego pochodne modyfikowane w części cukrowej: 3',5'-O-(tetraizopropylodisiloksano-

1,3-diylo)- N^6 -furfuryloadenozynę (II); 2'- C -metylo N^6 -furfuryloadenozynę (I); 2',3'- O -izopropylideno- N^6 -furfuryloadenozynę (III) oraz natywny nukleozyd – adenozynę, Ado (Rycina 6). Obecność powstałego mononukleotydu rybozydu kinetyny została potwierdzona rozdziałem chromatograficznym (technika HPLC).



Rycina 6. Struktury chemiczne badanych związków.

Uzyskana wartość K_m dla naturalnego substratu ADK - adenozyny wynosi $12,51 \mu\text{M}$ i jest identyczna z deklaracją producenta enzymu - Novocib ($11,4 \mu\text{M}$). Powinowactwo modyfikowanych rybonukleozydów: rybozydu kinetyny oraz pochodnych I i III do kinazy adenozynowej jest podobne do adenozyny. Jednak szybkość reakcji fosforylacji rybozydu kinetyny ($V_{\max}=1,019 \text{ nmol/min}$) jest 3,8 razy szybsza niż adenozyny ($V_{\max}=0,268 \text{ nmol/min}$). Szybkość fosforylacji 2'- C -metylo N^6 -furfuryloadenozyny ($V_{\max}=0,078 \text{ nmol/min}$) jest 3,5 razy mniejsza niż adenozyny, co jest spowodowane obecnością grupy 2'- C -metylowej. Modyfikacja części cukrowej adenozyny obniża wydajność fosforylacji 2-5 krotnie stąd tak niskie wartości $V_{\max}$ dla pochodnych I i III (Rycina 7) (Wawrzyniak, manuskrypt w przygotowaniu).

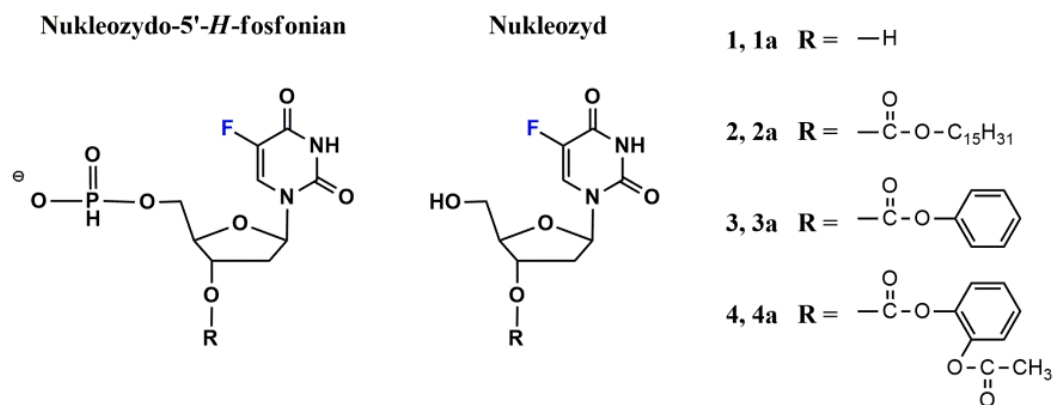


Rycina 7. Kinetyka fosforylacji nukleozydów purynowych katalizowana przez kinazę adenozynową.

Dostępne w piśmiennictwie dane wskazują, że kinaza adenozynowa posiada szeroką specyficzność substratową, rozpoznaje bowiem pochodne adenozyny podstawione w zasadzie purynowej w pozycji C²-, N⁶-, N⁷- oraz C⁸- (Bennett, 1975). Modyfikacja części cukrowej jest dobrze tolerowana, ale obniża wydajność fosforylacji 2-5 krotnie. Jedynym wymogiem jaki substrat ADK musi spełniać jest obecność zasady purynowej oraz grupy 2'-OH rybozy, która jest niezbędna do aktywności tego enzymu. Ponadto grupa 2'-OH musi być w konformacji trans w stosunku do pierścienia purynowego (Bennett, 1975). Związki takie jak: α -adenozyna czy β -D-arabinozylo-8-azaadenina nie są fosforylowane przez ADK ze względu na konformację cis grupy 2'-hydroksylowej względem zasady purynowej (Bennett, 1975). Jedną z bezpośrednich konsekwencji szerokiej specyficzności substratowej ADK wobec nukleozydów jest aktywacja dużej liczby antymetabolitów.

W ramach drugiego grantu dla młodych naukowców i doktorantów Instytutu Chemii Bioorganicznej PAN, którego byłem kierownikiem pt. „5'-H-fosfoniany 5-fluoro-2'-deoksyurydyny jako nowe proleki o potencjale przeciwnowotworowym – badania w modelach *in vitro* i *in vivo*, farmakokinetycznych *in silico* oraz stabilności związków” (Nr 36/SN/2018), prowadziłem badania z zakresu wstępnej oceny przedklinicznej pronukleotydów 5-fluoro-2'-deoksyurydyny z serii H-fosfonianów. Nukleozydo-5'-H-fosfoniany, które opracowałem mogą mimikować fosforany w komórce przy prawdopodobnie lepszej penetracji błony komórkowej z uwagi na formę jednoujemnego anionu

niż odpowiadające im dwuujemne monofosforany. Głównym celem działania tych związków jest hamowanie syntazy tymidylanowej (TYMS), która jest jedynym źródłem syntezy tymidylanu (TMP) *de novo* w komórce. Wyniki moich wcześniejszych badań (Rycina 2) wskazują, że TYMS może być potencjalnie nowym celem terapeutycznym w złośliwych guzach mózgu. Zwiększona ekspresja tego enzymu w GBM koreluje z obniżoną przeżywalnością pacjentów ($p=0,0057$). Nukleozydowe pochodne 5-fluoro-2'-deoksyurydyny i ich 5'-*H*-fosfoniany (Rycina 8) będące obiektem badań, zostały opracowane na podstawie wcześniejszych badań własnych (Szymanska-Michalak, 2016; Szymanska-Michalak, 2018) i zsyntezowane przez dr Agnieszkę Szymańską-Michalak z Zakładu Chemii Kwasów Nukleinowych Instytutu Chemii Bioorganicznej PAN.



Rycina 8. Struktury chemiczne badanych związków. Seria proleków obejmowała 5'-*H*-fosfoniany 3'-*O*-estrów aromatycznych i alifatycznych 5-fluoro-2'-deoksyurydyny (1-4). Aktywność pronukleotydów została porównana z odpowiadającymi im nukleozydami (związki 1a-4a).

Pierwszy etap pracy obejmował wyznaczenie wartości IC₅₀ oraz wykonania obliczeń farmakokinetycznych metodami *in silico*. Badania przeprowadzono na fibroblastach (MRC-5), komórkach glejaka wielopostaciowego (T98G, U-118 MG, A-172, U-87 MG), raka szyjki macicy (HeLa) oraz nowotworu piersi (T-47D). Wzory badanych związków przedstawiono na rycinie 8. Wyniki aktywności biologicznej oraz parametrów fizykochemicznych 5'-*H*-fosfonianów 5-fluoro-2'-deoksyurydyny i odpowiadających im nukleozydom przedstawiono w tabeli 2.

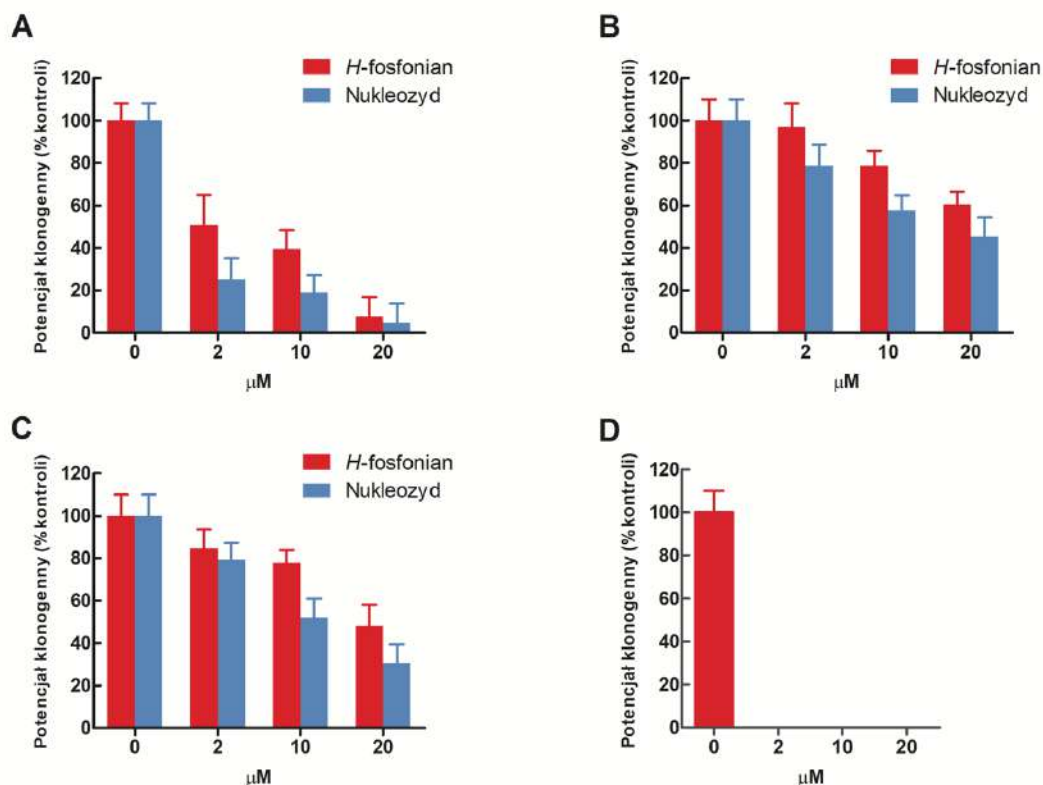
Tabela 2. Wartości IC_{50} (μM) oraz wybrane parametry właściwości fizykochemicznych badanych związków.

	1	1a	2	2a	3	3a	4	4a
<i>IC₅₀ (μM)</i>								
MRC-5	11,43	25,53	5,94	2,64	20,83	1,48	61,72	50,24
T98G	0,97	5,57	0,15	3,91	1,33	0,44	30,40	9,69
U-118 MG	4,61	23,40	4,12	18,89	4,48	0,26	--	8,79
A-172	1,01	5,38	0,67	--	0,81	--	4,09	--
U-87 MG	5,53	10,37	0,34	5,06	1,13	0,53	11,18	3,58
HeLa	0,93	5,31	0,33	2,67	3,09	0,57	55,34	2,75
T-47D	6,04	13,64	3,98	--	15,43	7,52	--	15,04
<i>Lipofilność</i>								
LogP	-5,26	-1,28	1,17	5,43	-4,09	0,72	-4,41	0,54
<i>Powierzchnia</i>								
PSA ($\text{\AA}^2$)	212,4	153,7	171,4	133,8	174,1	88,7	179,5	86,0
aPSA ($\text{\AA}^2$)	95,9	119,3	375,2	388,6	195,1	123,7	211,5	128,9
<i>Struktura</i>								
HBD	2	3	1	2	1	2	1	2
HBA	9	7	10	8	10	8	12	10
MW	309,17	246,19	547,58	484,61	413,27	350,3	471,31	408,33
<i>Absorpcja</i>								
tP _{app} (nm/s)	0,79	20,62	4,26	31,99	1,42	19,74	1,28	18,73

LogP, współczynnik rozdziału oktanol/woda; PSA, polarna powierzchnia cząsteczki; aPSA, apolarna powierzchnia cząsteczki; HBD, donory wiązań wodorowych; HBA, akceptory wiązań wodorowych; MW, masa cząsteczkowa; tP_{app}, przepuszczalność błon komórkowych Caco-2

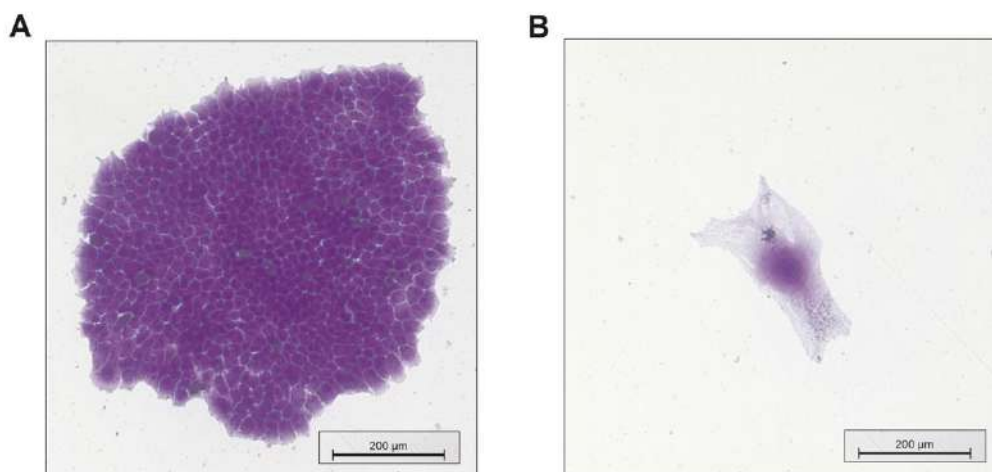
Wartości IC_{50} dla 5'-H-fosfonianów 5-FdU i odpowiadających im nukleozydów wyznaczone na sześciu liniach nowotworowych mieszają się w zakresie 0,15 - 55,34 μM . Wszystkie nukleozydo-5'-H-fosfoniany (**1**, **2**, **3**, **4**) charakteryzują się wysokim i selektywnym działaniem antyproliferacyjnym względem komórek GBM (T98G, U-118 MG, A-172, U-87 MG). Szczególnie aktywnymi związkami w tej grupie okazały się 5'-H-fosfonian-3'-palmitoilo 5-FdU (**2**) oraz 5'-H-fosfonian 5-FdU z wolną grupą 3'-OH (**1**), które były skuteczniejsze niż nukleozydy macierzyste z których się wywodzą (**2a**, **1a** odpowiednio). W przypadku 5'-H-fosfonianu-3'-palmitoilo 5-FdU, może to wynikać z optymalnych parametrów fizykochemicznych predysponujących do pokonywania barier komórkowych. Nukleozyd macierzysty (**2a**) był zbyt lipofilny ($\text{LogP} > 5$), co utrudniało jego rozpuszczanie. Z kolei w parze 3'-benzoilo 5-FdU (**3** vs **3a**) oraz 3'-salicyloilo 5-FdU (**4** vs **4a**), macierzyste nukleozydy (**3a**, **4a**) były aktywniejsze niż nukleozydo-5'-H-fosfoniany (**3**, **4**) i jest to związane z niską powierzchnią polarną cząsteczek i bardziej lipofilowym charakterem ($\text{LogP} \sim 1$). W drugim etapie oceniono wpływ badanych związków na tworzenie kolonii

komórkowych przy użyciu testu klonogenego w komórkach glejaka wielopostaciowego linii T98G. Wyniki przedstawiono na rycinie 9.



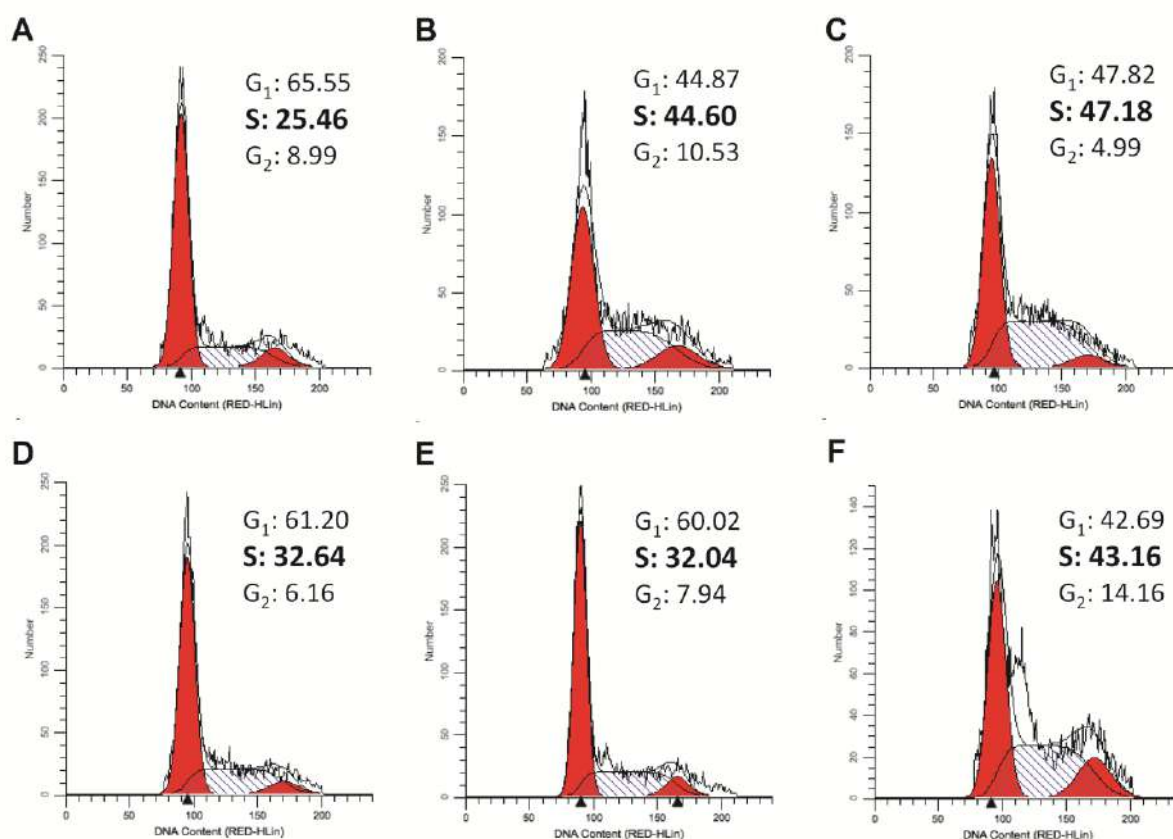
Rycina 9. Wpływ analizowanych związków na potencjał klonogeniczny komórek T98G. 5'-H-fosfonian 5-FdU vs 5-FdU (A). 5'-H-fosfonian-3'-benzoilo 5-FdU vs 3'-benzoilo 5-FdU (B). 5'-H-fosfonian-3'-salicyloilo 5-FdU vs 3'-salicyloilo 5-FdU (C). 5'-H-fosfonian-3'-palmitoilo 5-FdU (D).

W teście tym wszystkie badane związki hamowały potencjał klonogeniczny komórek glejakowych w zakresie niskich stężeń mikromolarnych (2-20 μM). W serii aromatycznych 3'-O-estrów 5-fluoro-2'-deoksyurydyny podobnie jak w testach cytotoksyczności, wyższą aktywność wykazywały nukleozydy niż 5'-H-fosfoniany nukleozydów. Odwrotna zależność była obserwowana w przypadku 5-fluoro-2'-deoksyurydyny, gdzie aktywniejszym związkiem był 5'-H-fosfonian 5-fluoro-2'-deoksyurydyny. Z kolei najaktywniejszą cząsteczką okazał się 5'-H-fosfonian-3'-palmitoilo 5-FdU, który już przy stężeniu 2 μM powodował praktycznie 100% zahamowanie proliferacji komórek T98G. Dalsze badania na jeszcze niższych stężeniach tego związku (0,1 μM) doprowadziły do obserwacji powstania gigantycznych komórek z całkowicie zahamowanym potencjałem do tworzenia kolonii (Rycina 10).



Rycina 10. Wpływ 5'-H-fosfonianu 3'-palmitoilo 5-FdU na zdolność klonogenną komórek glejakowych linii T98G. Kolonia komórek kontrolnych nie traktowanych związkami niskocząsteczkowymi składająca się z około 500 komórek (A). Gigantyczna komórka GBM z zahamowanym potencjałem klonogennym (B).

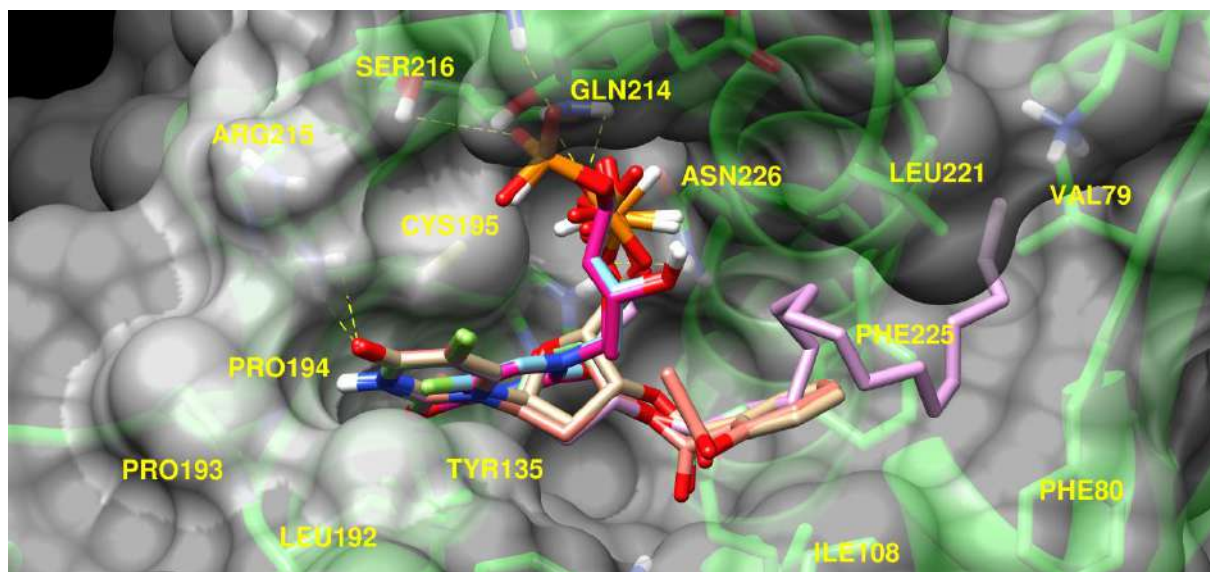
Głównym mechanizmem działania fluoropirymidyn, zarówno 5-fluoro-2'-deoksyurydyny jak i jej pronukleotydów, jest hamowanie syntazy tymidylanowej (TYMS) w wyniku którego następuje zmniejszenie poziomu dTTP i zahamowanie replikacji DNA co prowadzi do zatrzymania cyklu komórkowego w fazie S (Longley, 2003). W celu sprawdzenia, czy 5'-H-fosfoniany 5-FdU wykazują podobny mechanizm działania jak związek macierzysty, określiłem ich wpływ na cykl komórkowy. W rezultacie otrzymałem dane potwierdzające hamowanie cyklu komórkowego w fazie S przez wszystkie nukleozydo-5'-H-fosfoniany (Rycina 11). Wzrost odsetka komórek pozostających w fazie S wynosił od 7% w przypadku 5'-H-fosfonianów; -3'-benzoilo 5-FdU oraz -3'-salicyloilo 5-FdU, aż do 22% dla 5'-H-fosfonianu-5-FdU. Z kolei dla związku macierzystego (5-FdU) wartość ta wynosiła 19%.



Rycina 11. Wpływ 5-FdU oraz nukleozydo-5'-H-fosfonianów na cykl komórkowy. Komórki kontrolne nie traktowane związkami (A), 5-FdU (B), 5'-H-fosfonian 5-FdU (C), 5'-H-fosfonian-3'-benzoilo 5-FdU (D), 5'-H-fosfonian-3'-salicyloilo 5-FdU (E), 5'-H-fosfonian-3'-palmitoilo 5-FdU (F).

Również, badania dotyczące dokowania 5'-H-fosfonianów 5-FdU do centrum aktywnego syntazy tymidylanowej wykazały bardzo podobny sposób wiązania tych związków jak naturalnego inhibitora tego enzymu 5'-monofosforanu 5-fluoro-2'-deoksyurydyny (5-FdUMP). Energie wiązania 5'-H-fosfonianu 3'-benzoilo 5-FdU oraz 5'-H-fosfonianu 3'-salicyloilo 5-FdU wynoszą odpowiednio -8,5 oraz -8,2 kcal/mol. 5'-H-fosfonian 5-FdU wykazuje pewne przesunięcie względem pozycji zajmowanej przez powyższe dwa ligandy, co może być związane z brakiem podstawnika hydrofobowego w pozycji 3'-OH. Prawdopodobnie ze względu na brak łańcucha hydrofobowego kotwiczącego ligand, ma on najwyższą energię wiązania -6,3 kcal/mol. W przypadku 5-FdUMP wynosi ona -6,4 kcal/mol. Dla 5'-H-fosfonianu-3'-palmitoilo 5-FdU najlepszy energetycznie model ma energię wiązania -7,5 kcal/mol, co spowodowane jest interakcją długiego podstawnika palmitynowego wewnątrz „kieszeni wiążącej”. Jednak trudność w upakowaniu wewnątrz

i lokalizacja fragmentu palmitynowego w pobliżu naładowanych aminokwasów wpływa negatywnie na energię wiązania (Rycina 12).



Rycina 12. Dokowanie 5-FdUMP oraz 5'-H-fosfonianów 5-FdU do centrum aktywnego syntazy tymidylanowej. Współpraca z dr inż. Karol Kamel.

W świetle powyższych danych podjąłem się weryfikacji aktywności przeciwnowotworowej 5'-H-fosfonianu-3'-palmitoilo 5-FdU w układzie eksperymentalnym *in vivo*. Będąc w zgodzie z zasadą 3R dotyczącej badań toksykologicznych oraz mówiącej o redukcji, zastępowaniu i doskonaleniu metod doświadczalnych prowadzonych na zwierzętach, badania *in vivo* przeprowadziłem na wyplawku *Schmidtea mediterranea*. *Planarian sp.* zyskują popularność jako organizm w badaniach związanych ze starzeniem, regeneracją i komórkami macierzystymi. Bezkęgowce te mają duży odsetek komórek macierzystych, które są jedynymi dzielącymi się komórkami w tym organizmie, co jest zaletą w poznawaniu szlaków komórkowych, różnicowania się i wzrostu komórek, ale także procesów związanych z nowotworzeniem (Grohme, 2018). Ze względu na dotychczasowy brak udokumentowanych badań wpływu związków niskocząsteczkowych na wyplawki *Schmidtea mediterranea* sugerowałem się dobrze znanymi protokołami stosowanymi przy wyciszaniu RNA dostosowując dawkę, sposób przygotowania oraz metodę dostarczenia preparatu. Do iniekcji przygotowano 15 robaków, a seria nakłuć obejmowała trzy iniekcje przez trzy dni (w sumie 300 nl roztworu związku). Cztery godziny po ostatniej serii iniekcji wyplawki fragmentowano na trzy odcinki (głowy, brzuszny, ogonowy). Obserwacje prowadzono przez minimum dwa tygodnie do momentu pojawienia się fenotypów.

Ekspozycja na 5'-*H*-fosfonian-3'-palmitoilo 5-FdU wydłużyła czas regeneracji wypławków, pojawiły się osobniki, które w miejscu zranienia wykształciły dwie głowy oraz u których doszło do zaburzenia orientacji linii środka obserwowanej jako fenotyp cyklopa. Taki zespół cech obserwowany jest głównie przy interferencji RNA białek Slit-ROBO, które wyznaczają linię środka zwierzęcia (Reddien, 2005). Wstępne obserwacje wskazują, że związek ten wykazuje działanie podobne do inhibitorów acetylotransferaz, które blokują białka z rodziny PAT (ang. *protein acyltransferases*). Są one odpowiedzialne za palmitoilację czyli modyfikację białek polegającą na dołączeniu do cząsteczki białka reszty kwasu palmitynowego. Główne białka szlaku sygnalizacyjnego Wnt i Slit ulegają modyfikacji posttranslacyjnej przez palmitoilację N-końcowej reszty cysteiny, co umożliwia im wiązanie z błoną komórkową i jest niezbędne do aktywności sygnałowej (Goetz, 2002). Wyniki te wraz z obserwacją gigantycznych komórek wskazują na nowy mechanizm działania, którego wyjaśnieniem będę zajmował się w perspektywie dalszego rozwoju naukowego.



Rycina 13. Fenotypy wypławków, które poddano nanoiniekcji 5'-*H*-fosfonianem-3'-palmitoilo 5-FdU czternaście dni od przeprowadzonej fragmentacji. Współpraca z dr Patrick Perrigue oraz lic. Daria Olszewska.

6. Podsumowanie

W trakcie realizacji pracy doktorskiej powstała biblioteka związków niskocząsteczkowych licząca 250 unikalnych struktur chemicznych. Każdy związek scharakteryzowałem pod względem właściwości fizykochemicznych oraz aktywności biologicznej w kilku liniach nowotworowych, a szczególnie w komórkach glejaka wielopostaciowego. Uzyskane wyniki posłużyły do wyselekcjonowania 11 związków o wysokiej i selektywnej aktywności względem GBM i mogą one posłużyć do dalszego rozwijania strategii racjonalnego projektowania nowych związków, charakteryzujących się jeszcze lepszą aktywnością przeciwnowotworową oraz selektywnością.

Przeprowadzona przeze mnie analiza danych pochodzących z dużych projektów badania genomów nowotworowych (TCGA, REMBRANDT) wykazała, że syntaza tymidylanowa może być nowym celem terapeutycznym w złośliwych guzach mózgu, a zaprojektowane inhibitory tego białka z serii nukleozydo-5'-*H*-fosfonianów okazały się skutecznym narzędziem o potencjale terapeutycznym.

W celu pełniejszego wykorzystania potencjału wstępnie scharakteryzowanych pod kątem aktywności przeciwnowotworowej związków niskocząsteczkowych wykonałem analizę dojrzałych miRNA pod względem ich struktury I- i II-rzędowej, aby móc w przyszłości wykorzystać te cząsteczki do obniżenia onkogennych miRNA. Przeprowadzona analiza wykazała bezpośrednią zależność pomiędzy sekwencją miRNA, a rodzajem regulowanych przez nie docelowych mRNA.

Wykorzystując najnowsze zdobycze nanotechnologii uzyskaliśmy zwiększenie efektywności dostarczania terapeutycznego dsRNA (ATN-RNA) do komórek glejaka wielopostaciowego wykorzystując magnetyczne nanocząstki pokryte polietylenoiminą.

Ponadto, w ramach projektów badawczych, których byłem kierownikiem opracowałem test do badania mechanizmu fosforylacji rybonukleozydów purynowych przez kinazę adenozynową, który może zostać wdrożony do programu wysokoprzepustowych badań przesiewowych w konsorcjach typu EU-OPENSREEN do poszukiwania nowych inhibitorów kinazy adenozynowej.

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Projekty badawcze

Tytuł: 5'-H-fosfoniany 5-fluoro-2'-deoksyurydyny jako nowe proleki o potencjale przeciwnowotworowym – badania w modelach *in vitro* i *in vivo*, farmakokinetycznych *in silico* oraz stabilności związków.

Nr projektu: 36/SN/2018

Kierownik: Dariusz Wawrzyniak

Grant MNiSW dla młodych naukowców i doktorantów Instytutu Chemii Bioorganicznej PAN

Charakter udziału: **kierownik i wykonawca**

Tytuł: Funkcjonalna interakcja pomiędzy kolistymi RNA (circRNA), a macierzą zewnątrzkomórkową (ECM) - znaczenie dla komórek macierzystych nowotworu (GSC) oraz przejścia nabłonkowo-mezenchymalnego (EMT) w guzach mózgu.

Nr projektu: NCN 2017/25/B/NZ3/02173

Kierownik: dr hab. Katarzyna Rolle

Charakter udziału: **wykonawca**

Tytuł: Nowe, wielozadaniowe nanocząstki w skojarzonej geno- i fototerapii.

Nr projektu: NCN 2016/21/B/ST8/00477

Kierownik: prof. dr hab. Stefan Jurga

Charakter udziału: **wykonawca**

Tytuł: Demetylaza histonów JMJD3 jako epigenetyczny regulator procesów senescencji oraz nowotworzenia.

Nr projektu: NCN 2016/21/D/NZ3/00641

Kierownik: dr Patrick McDevitt Perrigue

Charakter udziału: **wykonawca**

Tytuł: Aktywność przeciwnowotworowa modyfikowanych nukleozydów purynowych i pirymidynowych w wybranych liniach komórek nowotworowych.

Nr projektu: 25/GM/2017

Kierownik: Dariusz Wawrzyniak

Grant MNiSW dla młodych naukowców i doktorantów Instytutu Chemii Bioorganicznej PAN

Charakter udziału: **kierownik i wykonawca**

Tytuł: Nowa generacja pro-nukleotydów antynowotworowych.

Nr projektu: NCN 2011/01/B/NZ4/04936

Kierownik: prof. dr hab. Jacek Stawiński

Charakter udziału: **główny wykonawca**

Tytuł: **Rybozymy anty-miRNA jako potencjalne terapeutyki w leczeniu guzów mózgu u ludzi.**

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New 3'-O-aromatic acyl-5-fluoro-2'-deoxyuridine derivatives as potential anticancer agents



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ABSTRACT

New aromatic and aliphatic 3'-O-acyl-5-fluoro-2'-deoxyuridine derivatives were synthesized and evaluated as candidates for prodrugs against various cancer cell lines. As the most promising candidate for antimalignant therapeutics was found a dual-acting acyl derivative **7h**, which apparently released not only the known anticancer nucleoside, 5-fluoro-2'-deoxyuridine (FdU), but also an additional active metabolite, acetylsalicylic acid, reinforcing thus therapeutic effect of FdU. Promising therapeutic indices showed also some aromatic dicarboxylic acids derivatives decorated with FdU esters (**11** and **12**).

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1. Introduction

5-Fluoro-2'-deoxyuridine (FdU) as an antimetabolite has been discovered nearly 60 years ago [1] and after approval by FDA in 1970, it has been widely used in anticancer therapy [2]. Cytostatic activity of FdU is due to depletion of thymidine 5'-triphosphate (TTP) concentration as a result of thymidylate synthase inhibition. To acquire this activity, FdU has to be first phosphorylated to 5-fluoro-2'-deoxyuridine-5'-phosphate (FdUMP) that is a true and the specific enzyme inhibitor [3]. Unfortunately, efficiency of the FdU transformation into FdUMP in cell is hampered by a simultaneous cleavage of the glycosyl bond of the nucleoside by thymidine phosphorylase that releases nucleobase 5-fluorouracil (FUra). To regain its anticancer activity FUra has to go again through the whole biosynthetic cycle from nucleobase via FdU to FdUMP, and this diminished anticancer potency of the original FdU drug [4].

Although clinically valuable and effective, FdU discloses off

target side-effects in the gastrointestinal tract and bone marrow that may pose problems during therapy [5–7]. In addition, due to low oral absorption, FdU requires expensive and often harmful intravenous infusion [5]. To improve bioavailability of FdU, the prodrug approach was recently explored for its delivery [8–10], notably by using 3', 5'-O-di-acyl and 3-N, 3', 5'-O-acyl esters, that in several instances improved anticancer potency and therapeutic value of FdU. The most important feature of these prodrugs was an ability to modulate their conversion into a biologically active drug in physiological milieu (via chemical and enzymatic hydrolysis [11,12]), solubility and physicochemical properties in aqueous environment, and cellular uptake (cellular membrane interaction and transmembrane transport). Recent papers have shown [10,13] that simple acyl esters of FdU possess the presupposed features in terms of lipophilicity, resistance to chemical hydrolysis, and variation in susceptibility to enzymatic hydrolysis. Although none of the newly synthesized compounds delivered outstanding anticancer properties, several valuable conclusions could be drawn from those studies. The most interesting observation was that lipophilicity, commonly accepted to be responsible for the transmembrane transport of a compound, although important, can't be correlated directly with the observed cytostatic activity. Despite of

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intensive studies on FdU acyl esters [8,11,12,14–16], none of the 3'-O- or 5'-O-acyl-FdU derivatives found application in anticancer therapy yet. However, several trends in modulation of cytostatic activity of acyl-FdU as function of the acyl group used could be distinguished. In general, presence of an acyl group usually increases total lipophilicity of the compound and often improves its IC₅₀, and thus can be used for tuning stability in physiological-like media (RPMI/FBS). The same seems to be true for amino acid derivatives of FdU [10,13,17] but in these cases, active intracellular transport emerged as an additional factor strongly affecting cytostatic activity. It should be also mentioned that 3'-carboxylic ester of 5-fluoro-2'-deoxyuridine bearing tumor-homing cyclic peptides CNGRC [18] or plasminogen activator inhibitor of type II (PAI-2) [19] disclose very selective cytostatic activity when targeting the tumor marker APN/CD13 of HT-1080 tumor cell lines and urokinase over-expressing malignant cells.

Inspired by the studies on 3'-O-retinoyl-5-fluoro-2'-deoxyuridine [20,21] as a dual-acting anticancer nucleosidic compound, we designed 3'-O-acyl-FdU bearing esters of carboxylic acids, that by simple chemical or carboxylesterase-assisted hydrolysis can generate additional active metabolites, e.g. lipoic acid [22–24], acetylsalicylic acid [25–29], and indazole-3-carboxylic acid derivative (lonidamine [30,31]) reinforcing thus therapeutic effect of FdU. Modulation of FdU anticancer potency of the compounds designed herein was empowered by a set of aromatic multi carboxylic acid esterified with FdU that supposedly may serve as an effective vehicle delivering in a form of one conjugated molecule more than one of cytostatic metabolite.

2. Results and discussion

2.1. Chemistry

2.1.1. 3'-O-aromatic acyls-5-fluoro-2'-deoxyuridines

The starting material for the synthesis of all 3'-O-acyl-5-fluoro-2'-deoxyuridines of type **7** (Scheme 1) was 5'-O-(4,4'-dimethoxytrityl)-5-fluoro-2'-deoxyuridine, obtained by a classical Khorana's procedure [32,33]. The choice of methods for the introduction of acyl groups into the 3'-O-position of 5-fluoro-2'-deoxyuridine based exclusively on simplicity of the procedure and commercial availability of the acylating reagents. Thus, 3'-O-acylated nucleosides **7a–h** bearing aromatic acyls were obtained by acylation of suitably protected 5-fluoro-2'-deoxyuridine derivative **1** with acyl chlorides **2**. To this end 5'-O-dimethoxytrityl-5-fluoro-2'-deoxyuridine **1** was dissolved in pyridine and treated with the respective acylating agent of type **2** (2 M equiv.) until the nucleosidic substrate reacted completely (ca 2 h at room temp.). Inspection of the reaction mixtures by TLC analysis usually revealed the presence of two acylated compounds, 3'-O-monoacyl nucleoside of type **5** and, most likely, 3'-O, 3-N-bisacyl nucleoside of type **6** (¹H and ¹³C NMR spectroscopy analysis).

Formation of the bisacylated by-products **6** was studied in detail using benzoyl chloride **2a** as an acylating agent in pyridine [34]. The structure of the isolated **6a** was unambiguously assigned as a 3'-O, 3-N-bisacyl derivative on the basis of comparative ¹³C NMR spectroscopic analysis of compounds **5a**, **6a** and **7a** [35,36]. The use of a stoichiometric amount of acylating reagent **2a** reduced significantly formation of the undesired N-acylation products, but at the same time a considerable portion of the starting nucleoside **1** remained unreacted.

Considering economy of the procedure and rather high costs of 5-fluoro-2'-deoxyuridine, we chose an approach in which the use of higher excess of benzoyl chloride (2 M equiv.) resulted in complete consumption of the starting nucleoside **1**, and then the formed N-acylated by-products **6** were attempted to convert into

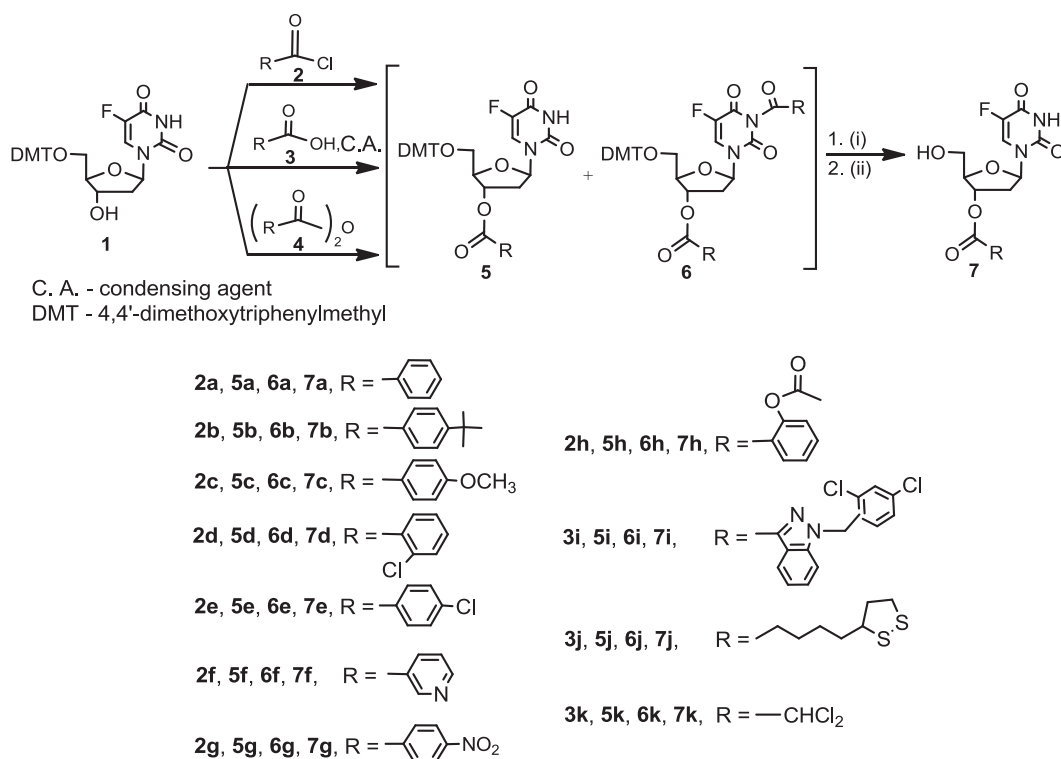
the desired products **5**. To this end, the crude reaction mixture containing products **5a** and **6a** was treated for 30 min with 3 M excess of morpholine in methylene chloride (DCM). It was rewarding to observe that under these conditions 3-N-benzoyl group was removed selectively from bisacylated product **6a** without affecting 3'-O-benzoyl ester group in **5a**. This procedure was found to be also effective for other N-acylated products **6a–g** having different aromatic acyl groups, and allowed to obtain the desired 3'-O-acyl-5-fluoro-2'-deoxyuridines **7a–g** in satisfactory overall yields (>70%, vide Experimental) [37,38].

During synthesis of **7h**, however, somewhat unexpectedly acylation of nucleoside **1** with 2-acetylsalicyl chloride **2h** in pyridine afforded several products (TLC analysis). Treatment of such reaction mixture with morpholine (to remove possible N-acyl groups), followed by 80% acetic acid aq. (to remove the 5'-O-DMT group) produced two compounds (ratio ca 1:1), which after purification were identified (¹H, ¹³C NMR, and HRMS analyses) as target product **7h** and 3'-O-(2-acetylsalicyl)salicyl derivative **8** (Fig. 1). To explain formation of compound **8** we considered two scenarios, with a key role played by pyridine. In the first one, the initially formed product **5h** was assumed to undergo a pyridine-catalyzed transacylation of the acetyl group in the 2-acetylsalicyl moiety by excess of acyl chloride **2h** to produce **8** [39], and in the second one, a similar phenomenon (a pyridine-catalyzed transacylation of the acetyl group) could occur in 2-acetylsalicyl chloride **2h** itself, prior to acylation of nucleoside **1** [40]. Since the second scenario appeared to be more likely, in order to avoid formation of salicyl-salicylated products of type **8** the reactions of **1** with **2h** was carried out in methylene chloride (DCM), in the absence of pyridine. The acylation was slow (completion overnight) and at the end of the reaction, the TLC analysis revealed, apart from 3'-O-acylated-, and 3'-O,3-N-bisacylated products, **5h** and **6h**, formation of some side products due to instability of the DMT group under the reaction conditions. To remedy this additional problem we added to the reaction mixture 2,6-lutidine as moderately strong (pK_a 6.60) [41] non-nucleophilic base (6 M equiv.). This secured fast (3 h), clean, and quantitative conversion of nucleoside **1** in the reaction with **2h** into 2-acetylsalicylated products **5h** and **6h**. These, after a successive treatment with morpholine and 80% acetic acid, afforded after purification the desired 3'-O-(2-acetylsalicyl)-5-fluoro-2'-deoxyuridine **7h** in 68% yield. The only disadvantage of this approach was a tedious procedure for the removal of lutidine before the deprotection of the DMT group (at least five extractions with phosphoric buffer pH 4.0). This inconvenience could be alleviated by using stronger, non-nucleophilic base, namely diisopropylethylamine (DIPEA, pK_a 11.44) [42] (3 M equiv.) and this resulted also in higher isolated yield of **7h** (79%).

2.1.2. Double-barrelled 3'-O-acyl-5-fluoro-2'-deoxyuridines

Compound **7h** was first in the series of double barrelled compounds (**7h–k**) bearing additional to FdU pharmacophore such as lonidamine, lipoic acid, and dichloroacetic acid. With the exception of dichloroacetyl derivative **7k**, for the synthesis of the other compounds we chose an approach in which dimethoxytritylated fluorodeoxyuridine **1** was acylated with the corresponding carboxylic acid (**3i** and **3j** respectively) activated with N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) in the presence of 4-(dimethylamino)pyridine (DMAP) (Scheme 1). The choice of EDC over other carbodiimides [e.g. N,N'-dicyclohexylcarbodiimide [43] (DCC), N,N'-diisopropylcarbodiimide [44] (DIC) or unsymmetrical N-cyclohexyl-N'-isopropylcarbodiimide [45]] as carboxylic acids activator was based on its proven efficacy in similar reactions of lonidamine with various carbohydrates, trouble-free work-up, and simple isolation procedure of the final products [46].

In a typical procedure 5'-protected nucleoside **1** and the desired



Reagents and conditions: (i) morpholine, 3 molar equiv.; (ii) 80% acetic acid aq.

Scheme 1. Synthesis of 3'-O-acyl 5-fluoro-2'-deoxyuridines.

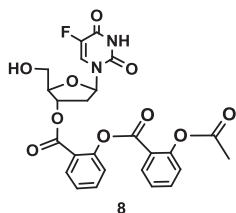


Fig. 1. A side product formed during acylation of **1** with 2-acetylsalicyl chloride.

carboxylic acid **3** (1.2 M equiv.) were dissolved in acetonitrile and treated with EDC (1.7 M equiv.) in the presence of DMAP (2.2 M equiv.) at room temperature. After 20 h (usually overnight) the reactions were complete and TLC analysis of the reaction mixtures usually revealed presence of a major product of higher R_f value (acylated nucleosides of type **5**). After evaporation of acetonitrile and removal of the DMT group with 80% acetic acid, the final products **7i,j** were isolated by the silica gel column chromatography (yields 64–88%). In none of the instances the formation of possible *N*-acylated products (vide supra) was observed, apparently, due to lower acylating potency of the in situ activated carboxylic acid vs acyl chlorides.

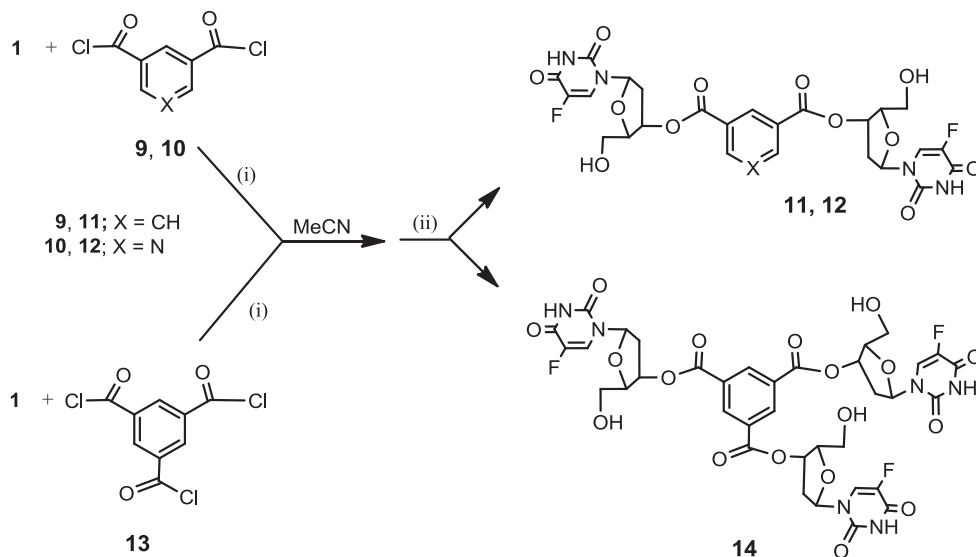
For the synthesis of dichloroacetyl derivative **7k**, the last in the series of double barrelled compounds, dichloroacetic anhydride **4** (2 M excess) was used as an acylating agent. The reaction was carried out in pyridine in the presence of DMAP (2.5 M equiv.) to afford 3'-O-acylated nucleoside **5k** (TLC analysis), which upon treatment with 80% acetic acid aq., furnished 3'-O-dichloroacetyl-5-fluoro-2'-deoxyuridine **7k**. After standard work-up and the silica gel column purification, the final product **7k** was obtained in high

yield (72%). No *N*-acylation of the nucleobase was observed under the reaction conditions.

2.1.3. Multi carboxylic aromatic acids esterified with 3'-O-5-fluoro-2'-deoxyuridine

As a conceptual extension of gathering anticancer potency in one molecule, we have synthesized and studied 5-fluoro-2'-deoxyuridine esters of di- and tricarboxylic aromatic acids i.e. isophthalic acid, pyridine 3,5-dicarboxylic acid and benzene-1,3,5-tricarboxylic acid (compounds **11**, **12** and **14** respectively, [Scheme 2](#)).

Di-(5-fluoro-2'-deoxyuridin-3'-yl) benzene-1,3-dicarboxylate and di-(5-fluoro-2'-deoxyuridin-3'-yl) pyridine-3,5-dicarboxylate **11** and **12** respectively, and tri-(5-fluoro-2'-deoxyuridin-3'-yl)-benzene-1,3,5-tricarboxylate **14**, were obtained in the reaction of dicarboxylic acid chlorides **9** or **10** or benzene-1,3,5-tricarboxylic acid chloride **13** with a slight excess of 5'-O-dimethoxytrityl-5-fluoro-2'-deoxyuridine **1** (1.05 M equiv. per one acid chloride function) in the presence of excess of DMAP (2.5 M equiv. per one acid chloride function) ([Scheme 2](#)). The reactions were carried out in MeCN and the molar ratio of reagents used ensured completion of the reaction in 2 h. After excess of DMAP was shed off the reaction mixture was treated with acetic acid (80% aq.) to obtain the unprotected compound. Such a two-step one-pot approach was possible to apply since both reactions i.e. esterification of **9**, **10** and **13** as well as dimethoxytrityl group removal, proceeded practically quantitatively. After acetic acid was evaporated, final products **11**, **12** and **14** were isolated by the silica gel column chromatography using a stepwise gradient (0–20% v/v) of propan-2-ol in dichloromethane. Pure products were solidified by freeze-drying from benzene/methanol and were obtained as white powders in high



Reagents and conditions: (i) DMAP 7.5 equiv., in MeCN. (ii) 80 % AcOH aq., 30 min.

Scheme 2. Synthesis of FdU esterified aromatic di- and tricarboxylic acids.

yields (**11**, 76%; **12**, 72%, **14**, 68%). Their structure were unambiguously confirmed with ^1H , ^{13}C NMR and HRMS analysis.

2.2. Physicochemical and chemical data

To evaluate drug-likeness of chemical compounds at the early stages of a drug discovery process, several descriptors have been devised. For example, Lipinski et al., proposed “rule of 5” [47–50], M. Congreve et al., “rule of 3” [51], and A. Rayna et al., a set of rules for prediction of oral drug-likeness [52]. At the moment these “rules” are unquestionably useful in introductory considerations of drug candidate's structure but are constantly discussed and many modifications and extensions were postulated [53–64]. In our studies we chose a common set of physicochemical and chemical parameters, which would provide the most useful information on structure relation activity (SAR) and biological potency of the studied compounds. Additionally, because our compounds contain aromatic rings we also included aromatic ring count which appeared to be an important parameter defining aqueous solubility, lipophilicity, or protein binding [65]. The collective physicochemical data for all the compounds investigated in this paper are listed in Tables 1 and 2.

2.2.1. Drug likeness

Except of multicarboxylic acids derivatives **11**, **12** and **14** (vide infra) the compounds investigated in this paper obey, within acceptable tolerance, all the criteria of the Lipinski drug-likeness “rule of 5” [47] (Table 1). In majority of cases physicochemical parameters of compounds **7a–k** argue against the “rule of 3” [51] but this disagreement seems to be rather apparent because this rule is dedicated to leads and not to initial screenings presented herein. The other essential for cellular up-take and blood–brain barrier penetration parameter, namely polar surface area (PSA), in most of the cases do not exceed value of $90 \text{ \AA}^2$ and should favour effective cellular internalisation of a given molecule [66]. Considering analysis by T. J. Ritchie and J. F. Macdonald [65] two aromatic rings present in compounds **7a–h** should not affect their drug developability. Thus, all physicochemical parameters of compounds **7a–k** are within limits of values for high drug-likeness of potential drug candidates.

Considering drug-likeness parameters alone i.e., M.W., $\log P^a$, PSA, HBD, HBA, tPapp, similar conclusions can be drawn for double barrelled FdU derivatives **7i–k** (Scheme 1, Table 1), although in these instances the SAR might not be as straightforward as for compounds **7a–h**. For more complex and conceptually different molecules **11**, **12** and **14** (Scheme 2, Table 2) which do not obey standard the Lipinski “rule of 5” [47–50], (MW > 500, higher number of HBD and particularly HBA), drug-likeness prediction needs probably other type of evaluation regime (for instance, kinetics of liberation of biologically active FdU, vide infra).

2.2.2. Stability in cell culture media

By checking stability of compounds **7a–h** in neat RPMI, RPMI/FBS 9:1 (v/v), and human blood plasma (HBP) one can get rough information what can happen with the investigated compounds under physiological conditions. Using linear regression method, the concentrations of substrates **7a–h** and their metabolites were calculated on the basis of integration of peaks area obtained by HPLC analysis. As the neat RPMI is free from enzymatic activities, half-life times in this media reflect susceptibility of the investigated compounds to chemical degradation (hydrolysis), while in RPMI/FBS, and particularly in HBP, in which enzymatic activities are expected (e.g. carboxyesterases), both chemical and enzymatic factors can be responsible for stability. As it is apparent from data in Table 1, all the analysed compounds were rather stable under the investigated reaction conditions and this could be correlated with pK_a values of the respective carboxylic acids [67,68] (the stronger the acid, the less stable the ester). A potential carboxyesterases activity expected for RPMI/FBS and HBP had no pronounced effect along the series **7a–h**. Similar $t_{1/2}$ values in RPMI, RPMI/FBS, and HBP supports the assumption that in these media contribution of enzymatic transformation of **7a–h** is not essential. In addition, considering high half-times it might be presumed that during 48 h the experimental cells are exposed mainly to unchanged compounds **7a–h** what should facilitate an effective cellular up-take of the intact drug molecules.

Stabilities in cell culture media of potentially dual-acting compounds **7i–k** derived from FdU, bearing lonidaminyl, lipoyl and dichloroacetyl moieties (Scheme 1) differed significantly. The FdU-lonidaminyl conjugate **7i** was stable in all the investigated media

Table 1Selected physicochemical data of 3'-O-aryl acyl-5-fluoro-2'-deoxyuridines **7a-k**.

Cpd	FdU	7a	7b	7c	7d	7e	7f	7g	7h	7i	7j	7k
R	—											—CHCl ₂
LogP ^a	−1.28	0.72	2.27	0.61	1.21	1.25	−0.45	0.64	0.54	3.24	1.45	0.06
PSA [Å ²]	153.7	88.7	82.7	78.1	83.4	84.7	92.8	143.6	86.0	78.1	143.9	153.7
aPSA [Å ²]	119.3	123.7	134.7	136.2	129.0	125.3	113.8	87.3	128.9	167.9	78.4	160.8
HBD	3	2	2	2	2	2	2	2	2	2	2	2
HBA	7	8	8	9	8	8	9	11	10	10	8	8
MW	246.19	350.30	406.41	380.33	384.74	384.74	351.29	395.30	408.33	549.34	434.50	357.12
tP _{app} (nm/s)	20.62	19.74	21.42	18.49	20.47	18.37	19.65	11.38	18.73	20.68	14.72	20.11
t _{1/2} [h] RPMI		>240	>240	>240	77	82.5	21.5	12.7	21.4	>240	72.2	<0.1
t _{1/2} [h] RPMI/FBS		228	>240	>240	81.6	144.4	23	28.8	41.3	>240	105	<0.1
t _{1/2} [h] HBP		144	>240	>240	105	58.5	105	15	21.8	>240	14.5	<0.1
No of Ar	0	2	2	2	2	2	2	2	2	4	0	0

LogP^a – ALOGPS 2.1 software (vide Experimental). PSA – polar surface area, HBD – hydrogen bonds donors, HBA – hydrogen bonds acceptors, tP_{app} – apparent theoretical permeability; RPMI – RPMI 1640 media; FBS – foetal bovine serum, HBP – human blood plasma, MRC-5 – normal human foetal lung fibroblast, SI – IC₅₀ non-tumor cell line/IC₅₀ tumor cell line.

Table 2^aSelected physicochemical and pharmacokinetic data of multicarboxylic acid esters decorated with 3'-O-5-fluoro-2'-deoxyuridine, **11**, **12** and **14**.

Cpds	FdU	11	12	14
Structure				
t _{1/2} RPMI		ND	6.3 h	ND
t _{1/2} RPMI/FBS		ND	2.8 h	ND
t _{1/2} BP		ND	5.2 h	ND
LogP	−1.28	−0.46	−1.64	−1.61
PSA (Å ²)	153.7	107.9	111.3	105.8
aPSA (Å ²)	119.3	159.5	155.8	203.9
HBD	3	4	4	6
HBA	7	16	17	24
MW	246.19	622.49	623.47	894.68
Absorption				
tP _{app} (nm/s)	20.62	17.25	17.33	15.25
IC ₅₀ [μM]/SI				
HeLa	5.31/4	2.57/17	8.18/10	7.68/4
Caco-2	12.85/2	>100/<1	>100/<1	31.16/1
T-47D	5.61/4	3.83/12	14.73/6	8.17/4
T98G	5.57/4	17.97/2	6.45/13	7.03/4
U-118 MG	23.40/1	31.16/1	18.86/4	29.71/1
U-87 MG	10.37/2	26.03/1	10.63/8	10.49/3
MRC-5	22.46	44.15	84.50	30.74

^a Abbreviations as in Table 1.

($t_{1/2} > 240$ h), while 3'-O-dichloroacetyl-FdU **7k** under the same conditions hydrolysed readily ($t_{1/2} < 0.1$ h). Lipoyl-FdU **7j** conjugate showed moderate stability in neat RPMI (chemical stability), but in the RPMI/FBS and HBP media, which contain enzymatic activity, $t_{1/2}$ value of **7j** changed somewhat unpredictable, possibly as a result of different substrate affinity towards enzyme present in these media (Table 1).

It seems that stability of FdU multicarboxylates in cell culture media as well as in the cell might be crucial for their cytostatic activity because the active part after hydrolysis of multiester is free nucleoside – FdU. It might be presumed then, that their activity should correlate straightforward with unleashing of FdU. Unfortunately, we were able to measure half-time only for diFdU ester of 3,5-dicarboxy pyridinic acid **12** because of solubility of compound **11** and **14** in aqueous environment (RPMI or HBP) was well below 2 mM and was insufficient for monitoring of their metabolism by HPLC under our standard analytical conditions (initial concentration 2 mM). Stability of compound **12** in the discussed media

followed the order RPMI > HBP > RPMI/FBS (Table 2). This stability order points to involvement of enzymatic hydrolysis of the investigated compounds. In addition, what potentially might be beneficial for pharmacodynamics of compounds of type **12** is that hydrolysis of the second carboxylic ester was much slower even in RPMI/FBS (see Supplementary data). This is in agreement with known features of carboxyesterases which disclose much lower reactivity towards polar or charged substrates [69].

Although stability of compounds **11** and **14** could not be measured, it is likely that all rules governing their kinetics of hydrolysis remain similar as those for compound **12** described above.

2.2.3. Hydrodynamic diameters

Since most of the compounds studied herein are amphiphilic and consist of a polar part i.e. 5-fluorouracil-1-yl and 2'-deoxyribose residue at one side, and a lipophilic aromatic substituent at the 3'-position of nucleoside at the other one, one can suspect formation of microstructures (e.g. micromicelles) in aqueous

environment. This might reinforced their drug-likeness and facilitate the cellular and/or intestinal up-take [70,71]. For this reason we attempted to measure hydrodynamic diameters using a dynamic light scattering (DLS) method, expecting that if any microstructures will be formed they should be detected by this method. The obtained results for **7a** indicated that this type of compounds may form stable, DLS-detectable aggregates of size close to 200–400 nm. After dilution by pure water to the final concentration in the range of 1–0.25 mg/mL, the values of hydrodynamic diameter remains unchanged, what may suggests that the investigated compounds under the experimental conditions did not form micelle-like structures.

2.3. In vitro cytostatic activity

Glioblastoma multiforme (GBM) is the most common, most aggressive, and lethal type of primary brain tumor. Current therapies for GBM (surgical resection, radiotherapy and chemotherapy) are weakly successful [72] with five-year survival rates less than 5% (according to The Central Brain Tumor Registry of the United States, 2012). Therefore, novel effective therapeutic agents for treating GBM are urgently needed. It has been shown recently [73] that the anticancer drugs from distinct pharmacological classes (other than temozolomide – widely used drug in GBM treatment) exert inhibitory effects on GBM cell growth and invasion.

Few years ago Barciszewski et al., from our institute, reported on a successful application of a sequence specific iRNA in brain tumor therapy [74,75]. Since FdU was hardly explored as drug against GBM, in this studies we focused on low molecular weight, novel FdU derivatives **7a–7k**, **11**, **12** and **14** which were evaluated for their antiproliferative activity against six human cancer cell lines, including three glioma cell lines i.e. T98G, U-118 MG, U-87 MG. Similar investigations were performed also on the cervical (HeLa), breast (T-47D) and colon (Caco-2) cancer cell lines because of slightly different reasons. HeLa cells were used as commonly accepted standard of human malignant cells, T47D cells were used because 5-fluorouracil (FU) is often one of the components in breast cancer multi-drug therapy [76,77], and Caco-2 cells were used for preliminary evaluation of intestinal up-take potency of the examined compounds when administrated orally [78]. Finally, non-cancerous lung fibroblasts (MRC-5) were used as the reference for estimation of therapeutic value of the examined compounds for malignant vs normal cells. The results of antiproliferative activity of FdU derivatives **7a–k**, **11**, **12** and **14** are listed in Table 3 and Table 2, respectively.

2.3.1. 3'-O-acyl- FdU derivatives of aromatic carboxylic acids

The obtained results indicate that, with a few exceptions, all 3'-

O-acyl-5-fluoro-2'-deoxyuridines **7a–h** are more cytotoxic than the parent FdU in the investigated cancer cell lines and in the reference normal human foetal lung fibroblasts (MRC-5) (Table 3). Considering character of the 3'-O-acyl groups, it seems reasonable to postulate that the observed higher antimalignant potency of **7a–h** vs FdU might be attributed to their higher lipophilicity (as is apparent from the corresponding logP, PSA and aPSA values, Table 1), that should facilitate cell membrane penetration and cellular up-take.

Regardless of a higher cytotoxicity (IC₅₀) of **7a–g** in all examined carcinoma cells, their potential therapeutic value as measured by a selectivity index (SI), is lower than that of the parent FdU (in most of the cases SI < 1) and thus do not justify their further development as potential anticancer drugs. The only exception in this subseries of the aromatic acyl derivatives, was a double barrelled 3'-O-(2-acetylsalicyl)-5-fluoro-2'-deoxyuridine (**7h**) whose cytotoxicity in each cancer cell lines investigated was distinctly higher than FdU (in some cases well above ten times) with superior selectivity indices (SI), e.g. for HeLa and Caco-2 cell lines (SI 18 and 33 respectively), and for the series of glioma cell lines T98G, U-118 MG and U-87MG (SI 5, 6 and 14 respectively). Considering that apart from FdU, the other metabolite generated from **7h** is aspirin or salicylic acid, the observed phenomenon is in line with the finding that both compounds can induce growth inhibition of various malignancies while leaving normal cells unaffected [25–27,29]. The pronounced differences in SI values of **7a–g** vs **7h** were rather unexpected but it might be speculated that kinetics of internalization and intracellular metabolism of **7h** towards biologically active FdU and aspirin or salicylic acid is so far distinct from the analogous decomposition of **7a–g** that may result in much higher selectivity of growth inhibition of cancer cells vs normal cells (MRC-5).

2.3.2. Double barrelled FdU derivatives

In comparison to the parent FdU, with a few exceptions (i.e. **7a**, **7f** and Caco-2 cells, **7a**, **7d**, **7f**, **7k** and T-47D cells, **7h** and T98G cell line, **7d**, **7e** and U-118MG cells), all investigated compounds disclosed higher (up to 90 times, vide U-118G cells and compound **7a**) cytostatic activity in cancer cell lines but with exception of **7h**, also clearly higher cytotoxicity for referential normal MRC-5 cells (Table 3). This makes application of these compounds rather doubtful in cancer therapy. Unfortunately, this tentative conclusion concerns also to compounds **7i–k** armed with two type of anticancer activities (FdU and second metabolite lonidamine, lipoic acid, dichloroacetic acid, respectively). Although some of them showed antiproliferating activity but none of them disclosed anticancer potency advantageous over the parent FdU.

However, selectivity indices SI of 3'-O-benzoyl- and 3'-O-

Table 3

^aCytostatic and SI values [IC₅₀ (μM)/SI] of 3'-O-aryl acyl-5-fluoro-2'-deoxyuridines **7a–k**.

Cpd	FdU	7a	7b	7c	7d	7e	7f	7g	7h	7i	7j	7k
R	—											
HeLa	5.31/4	0.57/3	0.39/<1	0.55/1	6.46/<1	1.75/<1	0.94/4	0.87/1	2.75/18	1.12/4	0.86/1	6.95/<1
Caco-2	12.85/2	36.96/<1	0.18/<1	0.38/2	1.26/2	0.42/1	70.55/<1	2.12/<1	1.53/33	1.03/4	6.92/<1	12.23/<1
T-47D	5.61/4	7.52/<1	2.98/<1	2.06/<1	13.77/<1	0.68/1	16.35/<1	1.06/1	15.04/3	4.41/1	7.23/<1	7.02/<1
T98G	5.57/4	0.44/<3	1.11/<1	0.39/2	1.50/2	0.67/1	0.66/<1	0.40/3	9.69/5	3.97/1	0.33/2	5.09/<1
U-118 MG	23.40/1	0.26/<6	3.88/<1	19.26/<1	41.35/<1	46.27/<1	5.48/<1	0.65/2	8.79/6	9.21/1	6.19/<1	2.71/1
U-87 MG	10.37/2	0.53/<3	0.34/<1	0.52/1	0.64/4	0.42/1	13.35/<1	0.88/1	3.58/14	5.65/1	0.56/1	n.d.*
MRC-5	22.46	1.48	0.07	0.66	2.46	0.60	3.30	1.14	50.24	4.15	0.59	3.75

*n.d. — not determined.

^a Abbreviations as in Table 1.

acetylsalicyl derivatives of FdU (**7a** and **7h** respectively), stand out from the others due to their SI parameter for GMB cell lines that are in each case exceeding those of FdU (Table 3). It is worthy to notice that in the case of **7a**, favorably values of SI parameters were due to its high cytostatic activity (low IC₅₀) while in the case of compound **7h** the high SI values were related to its low toxicity towards non-malignant MRC-5 cells. It is also worth to notice that **7h** disclosed highest therapeutic potency in Caco-2 and HeLa cells (SI = 33 and 18, respectively, Table 3) what makes this compound therapeutically promising in fighting malignancy of other tissues. To lesser extend similar conclusions concern also compound **7g**.

Some comments deserve results of experiments performed on Caco-2 cells in which with exception of compounds **7a** and **7f** all other investigated compounds showed much higher anticancer activity (IC₅₀ values in the range 0.18–6.92 µM) than FdU (IC₅₀ = 12.85 µM). Considering that monolayer Caco-2 cells are widely used as a *in vitro* model for prediction of drug absorption in humans [79–81], it might be concluded that compounds **7b–e** and **7g–i** should be well absorbed from intestinal fluid. Derivatives **7a** and **7f** proved to be poorly active in Caco-2 cells (IC₅₀ 36.96 and 70.55 µM respectively) and their low antiproliferative activity may suggests that they are substrates for P-glycoprotein, a protein belonging to the ATP-binding cassette (ABC) transporters superfamily, that has clinical relevance due to its role in drug metabolism and multidrug resistance (MDR). The last partially argues against calculated *t*_{app} value because all of the compounds including **7a** and **7f** (19.74 nm/s and 19.65 nm/s respectively) belong to class of medium permeability [82] with *t*_{app} in the range of 11.38 nm/s (for **7g**) and 21.42 nm/s (for **7b**). Apparently, low antiproliferative potency of **7a** and **7f** in Caco-2 cells was an effect of unrecognized yet intracellular and not outer membrane “molecular” events.

Considering therapeutic potency of the examined derivatives of FdU **7a–h** and the double barreled **7i–k** (Table 3) in fighting breast cancer cell line T-47D, their parameters IC₅₀ and SI clearly point out that these compounds (with exception of **7h**) did not exceed therapeutic potency of FdU. Although antiproliferative activities (IC₅₀) of **7b**, **7c**, **7e**, **7g**, **7i** were higher than that of FdU but at the same time their cytotoxicity towards non-malignant cells was also higher (Table 3), the SI values for these compounds did not exceed 1 (except for **7h**, SI = 3).

2.3.3. FdU esters of multi carboxylic acids

Biological activity found for FdU esters of multi carboxylic acids **11**, **12** and **14** are somehow slightly difficult to rationalise. In comparison to FdU, all these compounds disclosed comparable antiproliferative activity but at the same time were less toxic (up to four times, compound **12**) for normal MRC-5 cells (Table 2). As a result their SI value in several cases were essentially improved as compare to those of FdU. For example, di-(5-fluoro-2'-deoxyuridin-3'-yl)-3,5-dicarboxypyridinylate **12** showed higher therapeutic potency in nearly all the examined cell lines (with the exception of Caco-2 cells) and the SI exceeded even six times (for T98G cells) that of FdU. This apparent increase in value of SI parameter of compound **12** vs FdU, might points to this compound as a good candidate for a new antimalignant pro-drug. As such conclusion might be premature, the results obtained for dicarboxylate **12** should certainly warrant further studies on development of anticancer compounds based on FdU skeleton. The second noteworthy in this series compound is di-(5-fluoro-2'-deoxyuridin-3'-yl)-1,3-dicarboxybenzoate **11** that showed much higher antiproliferative potency than FdU in two cell lines, i.e. HeLa (four times) and breast cancer T-47D cells (three times). The last finding can be of interest because none of the investigated herein compounds disclosed advantageous over the parent FdU therapeutic potency in this cell line. Finally, it was slightly surprising that tri-(5-fluoro-2'-

deoxyuridin-3'-yl)-1,3,5-tricarboxybenzoate **14** did not showed any advantages over 5-fluoro-2'-deoxyuridine in all of the investigated cell lines.

3. Conclusions

We have synthesised new aromatic and aliphatic 3'-O-acyl-5-fluoro-2'-deoxyuridine derivatives **7a–k**, **11**, **12** and **14** as potential anticancer agents. The chemistry involved acylation of 5'-O-dimethoxytrityl-5-fluoro-2'-deoxyuridine with various acylating reagents (acyl chlorides, carboxylic acid anhydrides) or reagent systems (carboxylic acid in combination with condensing agents) that afforded the corresponding 3'-O-acylated FdU derivatives in good yield. In some cases the formation of *O,N*-bisacylated nucleosides was observed, but this problem was remedied by a selective removal of the *N*-acyl groups by a short treatment with morpholine.

For evaluation of antimalignant potency of the synthesised compounds three lines of glioblastoma cells (T98G, U-118 MG and U-87 MG), HeLa cells, breast cancer cells (T-47D), and Caco-2 cells were chose. The first series of the investigated compounds, **7a–g**, consisted of FdU acylated with electronically and structurally different aromatic groups, and did not provide any significant improvements in terms of SI over the parent FdU. In contrast to this, for the double-barrelled 3'-O-(2-acetylsalicyl)-5-fluoro-2'-deoxyuridine **7h**, the therapeutic indices were notably higher than those of FdU (except for breast cancer cells, T-47D). Compound **7i–k**, bearing potentially active additional metabolites (lipoic acid, **7i**; lonidamine, **7j**; dichloroacetic acid, **7k**) turned out to be disappointing as anticancer agents in the investigated cell lines.

Finally, in the third series of compounds investigated, multi-FdU esters of di- and tri-carboxylic aromatic acids i.e. **11**, **12** and **14**, di-FdU 1,3-dicarboxybenzoate **11** appeared to be active in two cell lines (HeLa and breast cancer cells T-47D), with the SI indices ca three times higher than that of FdU. Compound **12** had therapeutic parameters evidently superior over the parent nucleoside (FdU) in glioblastoma cell lines T98G, U-118 MG and U-87 MG, mainly due to its much lower toxicity. The IC₅₀ and SI parameters of benzenetricarboxylic acid derivative **14** in all the investigated cell lines were similar to those of FdU.

Although not impressive in terms of biological activity, some of the investigated compounds (for instance **7h** and **12**) can constitute a launch pad for further studies on double-barrelled and multi loaded antimalignant potential drugs.

4. Experimental

4.1. Material and methods

¹H, ¹³C NMR spectra were recorded on Bruker Avance II 400 or 500 MHz machines. Mass spectra were recorded with the ESI technique with negative or positive ionization with accuracy below 5 ppm. Amount of water in anhydrous solvents was controlled using Karl Fischer coulometric titration (Metrohm 684 KF coulometer). Thin-layer chromatography was performed on Merck silica gel 60F₂₅₄ plates and visualized with UV. For column chromatography Kieselgel 60 Merck was used. HPLC analyses were performed on a Nucleosil 100-5C18 column (5.0 µm, 4.6 mm × 150 mm) using and Waters Breeze HPLC systems with A + B solvent systems (A, 0.01 M aqueous triethylammonium acetate pH 7.4; B, A/acetonitrile, 1:4, v/v) at 35 °C, flow rate 1.5 mL/min; events: 5 min A 100%, linear gradient of B 0–100% in 20 min, 5 min B 100% and A 100% 10 min wash. Lonidamine was obtained as described previously by I.G. George et al. [83]. Aromatic di-carboxylic acid dichlorides were obtained according to R. Martin et al. [84]. Hydrodynamic

diameters were measured with dynamic light scattering (DLS) method using Zetasizer Nano ZS Malvern. Samples were prepared in DMSO at the following concentrations: 7 mg/mL, 2 mg/mL and 1 mg/mL. Next, the samples were diluted by pure water and the hydrodynamic size of compounds was measured using the glass cuvette. To check stability of prepared solutions the measurements were performed in intervals of time during 12 h.

4.1.1. General procedure for the synthesis of 3'-O-acyl-5-fluoro-2'-deoxyuridines of type 7

Method A (for compounds 7a-h). 5'-O-dimethoxytrityl-5-fluoro-2'-deoxyuridine **1** (1 mmol) was rendered anhydrous by evaporation of the added pyridine and then dissolved in pyridine (10 mL) (or in DCM with 3 M equiv. excess of DIPEA for compound **7 h**). To this, the respective acyl chloride of type **2** (2 mmol) was added, and the reaction mixture was left for 2–3 h at room temp yielding two acylated compounds of type **5** and **6**. Then the solvent was evaporated, the crude mixture dissolved in DCM (10 mL) was treated for 30 min with 3 M excess of morpholine. After this time the resulting mixture was washed twice with water (2 × 5 mL), the organic layer separated, evaporated and treated with 80% acetic acid aq. (5 mL) for 30 min. After deprotection with AcOH, the mixtures were concentrated to an oil under reduced pressure. 3'-O-acyl-5-fluoro-2'-deoxyuridines **7** were isolated by a silica gel 60 column chromatography using a stepwise gradient (0–10%) of propan-2-ol in methylene chloride. The fractions containing pure products were collected and evaporated yielding non-hygroscopic foams. After freeze-drying from benzene, compounds **7** were obtained as amorphous solids.

Method B (for compounds 7i-k). 5'-O-dimethoxytrityl-5-fluoro-2'-deoxyuridine **1** (1 mmol) and the respective carboxylic acids **3** (1.2 mmol) were rendered anhydrous by the evaporation of the added pyridine and then were dissolved in acetonitrile (10 mL) and treated by EDC (1.7 mmol) in the presence of DMAP (2.2 mmol) at room temperature. After 20 h the reaction was complete (TLC analysis), acetonitrile was evaporated, and the residue was treated with 80% acetic acid aq., (5 mL) for 30 min. Further work-up as for Method A.

Method C (for compound 7l). 5'-O-dimethoxytrityl-5-fluoro-2'-deoxyuridine **1** (1 mmol) was rendered anhydrous by the evaporation of the added pyridine and then dissolved in pyridine (10 mL). To this, 2 M excess of dichloroacetic anhydride **4** in the presence of 2.5 M equiv. of DMAP was added. After 4 h the reaction was complete (TLC analysis), solvent was evaporated, and the residue was treated with 80% acetic acid (5 mL). Further work-up as for Method A.

3'-O-benzoyl-5-fluoro-2'-deoxyuridine (7a). White solid; yield 0.29 g, 83%; RP HPLC Rt 13.96 min; ¹H NMR (400 MHz, DMSO-d₆): δ_H 11.88 (s, 1H, NH), 8.27 (d, 1H, J_{6-F5} = 7.2 Hz, H-6), 8.03–8.01 (m, 2H, o-H of Ar), 7.71–7.67 (m, 1H, p-H of Ar), 7.58–7.54 (m, 2H, m-H of Ar), 6.24 (t, J = 6.6 Hz, 1H, H-1'), 5.48 (d, J = 5.6 Hz, 1H, H-3'), 4.20–4.21 (m, 1H, H-4'), 3.70–3.74 (m, 2H, H-5', H-5''), 2.40–2.46 (m, 2H, H-2', H-2'') ppm; ¹³C NMR (100 MHz, DMSO-d₆): δ_C 165.65 (C=O), 157.40 (d, J = 26 Hz, C-4), 149.47 (C-2), 141.53 (d, J = 230 Hz, C-5), 134.03, 129.75, 129.20 (C of Ar), 124.93 (d, J = 34 Hz, C-6), 85.33 (C-4'), 84.95 (C-1'), 76.07 (C-3'), 61.74 (C-5'), 37.43 (C-2') ppm; HRMS (ESI): calcd for C₁₆H₁₄N₂O₆F [M-H]⁻: 349.08359, found: 349.08459.

3'-O-(4-tert-butylbenzoyl)-5-fluoro-2'-deoxyuridine (7b). White solid; yield 0.30 g, 73%; RP HPLC Rt 17.56 min; ¹H NMR (400 MHz, DMSO-d₆): δ_H 11.89 (s, 1H, NH), 8.26 (d, 1H, J_{6-F5} = 7.2 Hz, H-6), 7.94

(d, J = 8.4 Hz, 2H, o-H of Ar), 7.57 (d, J = 8.4 Hz, 2H, m-H of Ar), 6.26 (t, J = 6.8 Hz, 1H, H-1'), 5.48–5.49 (m, 1H, H-3'), 4.20–4.21 (m, 1H, H-4'), 3.71–3.72 (m, 2H, H-5', H-5''), 2.39–2.43 (m, 2H, H-2', H-2''), 1.31 (s, 9H, CH₃ of t-Bu) ppm; ¹³C NMR (100 MHz, DMSO-d₆): δ_C 165.56 (C=O), 157.40 (d, J = 26 Hz, C-4), 157.12 (C of Ar), 149.47 (C-2), 140.53 (d, J = 230 Hz, C-5), 129.68, 127.045 & 126.013 (C of Ar), 124.91 (d, J = 34 Hz, C-6), 85.35 (C-4'), 84.93 (C-1'), 75.84 (C-3'), 61.74 (C-5'), 37.47 (C-2'), 35.28 [C(CH₃)₃], 31.19 [C(CH₃)₃] ppm; HRMS (ESI): calcd for C₂₀H₂₂N₂O₆F [M-H]⁻: 405.14619, found: 405.14736.

3'-O-(4-methoxybenzoyl)-5-fluoro-2'-deoxyuridine (7c). White solid; yield 0.28 g, 75%; RP HPLC Rt 14.14 min; ¹H NMR (400 MHz, DMSO-d₆): δ_H 11.88 (s, 1H, NH), 8.26 (d, J_{6-F5} = 7.2 Hz, 1H, H-6), 7.96 (d, J = 8.8 Hz, 2H, o-H of Ph), 7.06 (d, J = 8.8 Hz, 2H, m-H of Ar), 6.26 (t, J = 6.8 Hz, 1H, H-1'), 5.43–5.45 (m, 1H, H-3'), 4.17–4.18 (m, 1H, H-4'), 3.84 (s, 3H, OCH₃), 3.70–3.73 (m, 2H, H-5', H-5''), 2.38–2.43 (m, 2H, H-2', H-2'') ppm; ¹³C NMR (100 MHz, DMSO-d₆): δ_C 165.31 (C=O), 163.81 (C of Ar), 157.40 (d, J = 26 Hz, C-4), 149.47 (C-2), 140.52 (d, J = 230 Hz, C-5), 131.91 (C of Ar), 124.92 (d, J = 34 Hz, C-6), 121.90, 114.48 (C of Ar), 85.40 (C-4'), 84.94 (C-1'), 75.67 (C-3'), 61.73 (C-5'), 55.73 (OCH₃), 37.27 (C-2') ppm; HRMS (ESI): calcd for C₁₇H₁₆N₂O₇F [M-H]⁻: 379.09415, found: 379.09524.

3'-O-(2-chlorobenzoyl)-5-fluoro-2'-deoxyuridine (7d). White solid; yield 0.33 g, 85%; RP HPLC Rt 14.52 min; ¹H NMR (400 MHz, DMSO-d₆): δ_H 11.88 (s, 1H, NH), 8.25 (d, 1H, J_{6-F5} = 7.2 Hz, H-6), 7.88–7.91 (m, 1H, o-H of Ar), 7.59–7.62 (m, 2H, m-H of Ar), 7.48–7.52 (m, 1H, p-H of Ar), 6.23–6.26 (m, 1H, H-1'), 5.49–5.51 (m, 1H, H-3'), 4.19–4.21 (m, 1H, H-4'), 3.71–3.73 (m, 2H, H-5', H-5''), 2.41–2.45 (m, 2H, H-2', H-2'') ppm; ¹³C NMR (100 MHz, DMSO-d₆): δ_C 164.90 (C=O), 157.42 (d, J = 27 Hz, C-4), 149.47 (C-2), 141.54 (d, 230 Hz, C-5), 133.94, 132.36, 131.80, 131.27, 129.98, 127.89 (C of Ar), 124.91 (d, J = 35 Hz, C-6), 85.16 (C-4'), 84.94 (C-1'), 76.73 (C-3'), 61.73 (C-5'), 37.27 (C-2') ppm; HRMS (ESI): calcd for C₁₆H₁₃N₂O₆ClF [M-H]⁻: 383.04462, found: 383.04573.

3'-O-(4-chlorobenzoyl)-5-fluoro-2'-deoxyuridine (7e). White solid; yield 0.29 g, 75%; RP HPLC Rt 15.32 min; ¹H NMR (400 MHz, DMSO-d₆): δ_H 11.88 (s, 1H, NH), 8.26 (d, 1H, J_{6-F5} = 7.2 Hz, H-6), 8.02 (d, J = 8.4 Hz, 2H, o-H of Ar), 7.62 (d, J = 8.4 Hz, 2H, m-H of Ar), 6.27 (t, J = 7.2 Hz, 1H, H-1'), 5.47–5.48 (m, 1H, H-3'), 4.19–4.21 (m, 1H, H-4'), 3.66–3.76 (m, 2H, H-5', H-5''), 2.32–2.50 (m, 2H, H-2', H-2''); ¹³C NMR (100 MHz, DMSO-d₆): δ_C 164.43 (C=O), 157.13 (d, J = 26 Hz, C-4), 149.47 (C-2), 140.11 (d, J = 229 Hz, C-5), 138.53, 131.24, 128.96, 128.21 (C of Ar), 124.17 (d, J = 34 Hz, C-6), 84.86 (C-4'), 84.52 (C-1'), 75.97 (C-3'), 61.32 (C-5'), 36.96 (C-2') ppm; HRMS (ESI): calcd for C₁₆H₁₃N₂O₆ClF [M-H]⁻: 383.04407, found: 383.04562.

3'-O-(nicotinoyl)-5-fluoro-2'-deoxyuridine (7f). White solid; yield 0.27 g, 78%; RP HPLC Rt 11.75 min; ¹H NMR (400 MHz, DMSO-d₆): δ_H 11.88 (s, 1H, NH), 9.15–9.16 (m, 1H, o-H of Ar), 8.83–8.85 (m, 1H, p-H of Ar), 8.34–8.37 (m, 1H, o-H of Ar), 8.26 (d, 1H, J_{6-F5} = 7.2 Hz, H-6), 7.58–7.61 (m, 1H, m-H of Ar), 6.29 (t, J = 7.2 Hz, 1H, H-1'), 5.50–5.51 (m, 1H, H-3'), 4.24 (br s, 1H, H-4'), 3.68–3.77 (m, 2H, H-5', H-5''), 2.39–2.44 (m, 2H, H-2', H-2'') ppm; ¹³C NMR (125 MHz, DMSO-d₆): δ_C 164.77 (C=O), 157.47 (d, J = 27 Hz, C-4), 154.34, 150.70 (C of Ar), 149.53 (C-2), 141.59 (d, J = 228 Hz, C-5), 137.56, 125.91 (C of Ar), 125.03 (d, J = 34 Hz, C-6), 124.39 (C of Ar), 85.24 (C-4'), 85.03 (C-1'), 76.55 (C-3'), 61.78 (C-5'), 37.4 (C-2') ppm; HRMS (ESI): calcd for C₁₅H₁₃N₃O₆F [M-H]⁻: 350.07884, found: 350.07959.

3'-O-(4-nitrobenzoyl)-5-fluoro-2'-deoxyuridine (7g). Yellow solid; yield 0.28 g, 71%; RP HPLC Rt 15.54 min; ¹H NMR (400 MHz, DMSO-

d₆): δ_{H} 11.89 (s, 1H, NH), 8.35–8.38 (m, 2H, o-H of Ar), 8.24–8.28 (m, 3H, m-H of Ar and H-6), 6.28–6.32 (m, 1H, H-1'), 5.52–5.53 (m, 1H, H-3'), 4.25 (br s, 1H, H-4'), 3.68–3.78 (m, 2H, H-5', H-5''), 2.40–2.55 (m, 2H, H-2', H-2'') ppm; ^{13}C NMR (125 MHz, DMSO-d₆): δ_{C} 164.30 (C=O), 157.46 (d, J = 27 Hz, C-4), 150.84 (C of Ar), 149.55 (C-2), 141.61 (d, J = 230 Hz, C-5), 135.35, 131.38 (C of Ar), 125.49 (d, J = 34 Hz, C-6), 124.34 (C of Ar), 85.24 (C-4'), 85.01 (C-1'), 77.08 (C-3'), 61.82 (C-5'), 37.38 (C-2') ppm; HRMS (ESI): calcd for C₁₆H₁₃N₃O₈F [M-H]⁻: 394.06867, found: 394.06964.

3'-O-(2-acetylsalicyl)-5-fluoro-2'-deoxyuridine (7h). White solid; yield 0.32 g, 79%; RP HPLC Rt 14.59 min; ^1H NMR (400 MHz, DMSO-d₆): δ_{H} 11.88 (s, 1H, NH), 8.26 (d, 1H, J_{6-F5} = 7.2 Hz, H-6), 8.01 (d, 1H, J = 8.0 Hz o-H of Ar), 7.71 (t, 1H, J = 8.0 Hz p-H of Ar), 7.44 (m, J = 7.8 Hz, 1H, m-H of Ar), 7.28 (d, J = 8.0 Hz, 1H, m-H of Ar), 6.24 (t, J = 7.6 Hz, 1H, H-1'), 5.45 (br s, 1H, H-3'), 4.13 (br s, 1H, H-4'), 3.70 (m, 2H, H-5', H-5''), 2.38–2.41 (m, 2H, H-2', H-2''), 2.31 (s, 3H, CH₃) ppm; ^{13}C NMR (100 MHz, DMSO-d₆): δ_{C} 169.60 (C=O), 164.07 (C=O), 157.48 (d, J = 26 Hz, C-4), 150.39 (C of Ar), 149.54 (C-2), 140.59 (d, J = 228 Hz, C-5), 135.07, 131.95, 128.68, 126.85, 129.98, 127.89 (C of Ar), 124.66 (d, J = 35 Hz, C-6), 124.52, 123.28 (C of Ar), 85.26 (C-4'), 84.94 (C-1'), 76.33 (C-3'), 61.76 (C-5'), 37.35 (C-2') ppm; HRMS (ESI): calcd for C₁₈H₁₆N₂O₈F [M-H]⁻: 407.08907, found: 407.09018.

3'-O-[1-(2,4-dichlorobenzyl)-1H-indazole-3-carbonyl]-5-fluoro-2'-deoxyuridine (7i). White solid; yield 0.35 g, 64%; RP HPLC Rt 20.97 min; ^1H NMR (400 MHz, DMSO-d₆): δ_{H} 11.90 (s, 1H, NH), 8.29 (d, 1H, J_{6-F5} = 7.2 Hz, H-6), 8.12 (d, 1H, J = 8.0 Hz, H of Ar), 7.81 (d, 1H, J = 8.8 Hz, H of Ar), 7.64 (d, 1H, J = 2.0 Hz, H of Ar), 7.52 (t, 1H, J = 7.8 Hz, H of Ar), 7.39 (t, J = 7.4 Hz, 1H, H of Ar), 7.35 (dd, J = 8.4 Hz, J = 2.0 Hz, 1H, H of Ar), 6.92 (d, J = 8.4 Hz, 1H, H of Ar), 6.30 (t, J = 6.4 Hz, 1H, H-1'), 5.88 (s, 2H, CH₂), 5.59–5.61 (m, 1H, H-3'), 4.29 (br s, 1H, H-4'), 3.72–3.80 (m, 2H, H-5', H-5''), 2.50–2.57 (m, 2H, H-2', H-2''); ^{13}C NMR (100 MHz, DMSO-d₆): δ_{C} 161.13 (C=O), 157.02 (d, J = 27 Hz, C-4), 149.10 (C-2), 140.89 (C of LND), 140.15 (d, 229.5 Hz, C-5), 134.50, 133.47, 133.19, 133.00, 130.76, 129.08, 127.76, 127.35 (C of LND), 124.52 (d, J = 35 Hz, C-6), 123.68, 122.89, 121.37, 110.86 (C of LND), 84.94 (C-4'), 84.58 (C-1'), 75.59 (C-3'), 61.34 (C-5'), 49.98 (C of LND), 37.10 (C-2'); HRMS (ESI): calcd for C₂₄H₁₈N₄O₆Cl₂F [M-H]⁺: 547.05874, found: 547.06033.

3'-O-[(R)-5-(1,2-Dithiolan-3-yl)-pentanoyl]-5-fluoro-2'-deoxyuridine (7j). Yellow solid; yield 0.38 g, 88%; RP HPLC Rt 17.83 min; ^1H NMR (400 MHz, DMSO-d₆): δ_{H} 11.87 (s, 1H, NH), 8.21 (d, 1H, J_{6-F5} = 7.2 Hz, H-6), 6.15 (t, J = 7.6 Hz, 1H, H-1'), 5.31 (t, J = 5.2 Hz, 1H, H-3'), 4.00 (br s, 1H, H-4'), 3.62–3.65 [m, 3H, H-5', H-5'', H of lipoic acid (LA)], 3.08–3.22 (m, 2H, H of LA), 2.31–2.45 (m, 3H, H-2', H-2'' & H of LA), 2.22–2.30 (m, 2H, H of LA), 1.34–1.91 (m, 7H, H of LA) ppm; ^{13}C NMR (100 MHz, DMSO-d₆): δ_{C} 172.44 (C=O), 156.97 (d, J = 26 Hz, C-4), 149.02 (C-2), 140.08 (d, J = 229 Hz, C-5), 124.44 (d, J = 34 Hz, C-6), 84.94 (C-4'), 84.40 (C-1'), 74.60 (C-3'), 61.24 (C-5'), 56.00, 40.17, 38.08 (C of LA), 36.88 (C-2'), 34.76, 33.23, 28.00, 24.04 (C of LA) ppm; HRMS (ESI): calcd for C₁₇H₂₂N₂S₂O₆F [M-H]⁻: 433.08978, found: 433.09117.

3'-O-dichloroacetyl-5-fluoro-2'-deoxyuridine (7k). Yellow solid; yield 0.26 g, 72%; RP HPLC Rt 13.88 min; ^1H NMR (400 MHz, DMSO-d₆): δ_{H} 11.89 (s, 1H, NH), 8.20 (d, 1H, J_{6-F5} = 7.2 Hz, H-6), 6.92 (s, 1H, CH), 6.18 (t, J = 7.6 Hz, 1H, H-1'), 5.38–5.40 (m, 1H, H-3'), 4.08 (br s, 1H, H-4'), 3.66 (t, J = 4 Hz, 2H, H-5', H-5''), 2.33–2.42 (m, 2H, H-2', H-2''); ^{13}C NMR (100 MHz, DMSO-d₆): δ_{C} 163.94 (C=O), 156.96 (d, J = 26 Hz, C-4), 149.04 (C-2), 140.12 (d, J = 229 Hz, C-5), 124.39 (d, J = 34 Hz, C-6), 84.28 (C-4' & C-1'), 78.38 (C-3'), 66.34 (CH), 61.21 (C-5'), 36.39 (C-2') ppm; HRMS (ESI): calcd for C₁₁H₁₀N₂Cl₂O₆F [M-H]⁻: 354.98999, found: 354.99103.

4.1.2. General procedure for the synthesis of 3'-O-esters of 5-fluoro-2'-deoxyuridines with aromatic multi carboxylic acids

Nucleoside **1** (1.05 equiv. per one acid chloride function) was rendered anhydrous by evaporation with dry pyridine and dissolved in acetonitrile (10 mL/1 mmol of nucleoside **1**). To this solution DMAP (2.5 equiv per one acid chloride function) and the respective carboxylic acid chloride (1 mmol) were added and the reaction mixture was left for 2 h at room temperature (TLC analysis). The reaction was quenched by addition of water (1 mL) and the solution concentrated to a viscous oil. The residue was dissolved in methylene chloride (10 mL/1 mmol) and washed three times with water (3 × 5 mL), the organic layer separated, and after drying (Na₂SO₄ anhyd.) it was evaporated under vacuum. After dimethoxytrityl group was removed (80% acetic acid, 5 mL, 30 min) and the final product was isolated by a silica gel 60 column chromatography using a stepwise gradient 0 → 20% (v/v) propane-2-ol in dichloromethane. The fractions containing pure products were combined, evaporated and solidified by freeze-drying from benzene/methanol.

Di-(5-fluoro-2'-deoxyuridin-3'-yl)-benzene-1,3-dicarboxylate (11). White solid 0.47 g, yield: 76%; RP HPLC Rt 13.97 min; ^1H NMR (400 MHz, DMSO-d₆): δ_{H} 11.87 (s, 2H, NH), 8.53 (t, J = 2 Hz, 2H, H of Ar), 8.30 (dd, J = 1.8 Hz, J = 7.8 Hz, 4H, H of Ar), 8.27 (d, J_{6-F5} = 7.2 Hz, 2H, H-6), 7.78 (t, J = 2 Hz, 1H, H of Ar), 6.26–6.30 (m, 2H, H-1'), 5.40 (t, J = 5.2 Hz, 2H, H-3'), 4.21–4.24 (m, 2H, H-4'), 3.68–3.73 (m, 4H, H-5', H-5''), 2.38–2.53 (m, 4H, H-2', H-2''); ^{13}C NMR (100 MHz, DMSO-d₆): δ_{C} 164.46 (C=O), 157.01 (d, J = 26 Hz, C-4), 149.07 (C-2), 140.12 (d, J = 229 Hz, C-5), 134.13, 134.01, 130.02, 129.62 (C of Ar), 124.49, 124.44 (2xd, J = 34 Hz, C-6), 84.78 (C-4'), 84.51 (C-1'), 76.17 (C-3'), 61.32 (C-5'), 36.93 (C-2') ppm; HRMS (ESI): calcd for C₂₆H₂₃N₄O₁₂F₂ [M-H]⁻: 621.12805 found: 621.12904.

Di-(5-fluoro-2'-deoxyuridin-3'-yl)-pyridine-3,5-dicarboxylate (12). White solid; yield 0.45 g, 72%; RP HPLC Rt 12.68 min; ^1H NMR (400 MHz, DMSO-d₆): δ_{H} 11.87 (s, 2H, NH), 9.38 (d, J = 2 Hz, 2H, o-H of Ar), 8.72 (t, 1H, J = 2 Hz, 1H, p-H of Ar), 8.28 (d, 2H, J_{6-F5} = 6.8 Hz, H-6), 6.29–6.33 (m, 2H, H-1'), 5.39 (t, J = 5.0 Hz, 2H, H-3'), 4.28–4.29 (m, 2H, H-4'), 3.69–3.78 (m, 4H, H-5', H-5''), 2.40–2.58 (m, 4H, H-2', H-2''); ^{13}C NMR (125 MHz, DMSO-d₆): δ_{C} 163.59 (C=O), 157.07 (d, J = 26 Hz, C-4), 153.99 (C of Ar), 149.12 (C-2), 140.17 (d, J = 229 Hz, C-5), 137.42, 125.70 (C of Ar), 124.57 (d, J = 34 Hz, C-6), 84.69 (C-4'), 84.60 (C-1'), 76.65 (C-3'), 61.35 (C-5'), 36.92 (C-2') ppm; HRMS (ESI): calcd for C₂₅H₂₃N₅O₁₂F₂ [M-H]⁻: 622.12330, found: 622.12475.

Tri-(5-fluoro-2'-deoxyuridin-3'-yl)-benzene-1,3,5-tricarboxylate (14). White solid; yield 0.61 g, 68%; RP HPLC Rt 13.52 min; ^1H NMR (400 MHz, DMSO-d₆): δ_{H} 11.86 (s, 3H, NH), 8.72 (s, 3H, H of Ar), 8.26 (d, J_{6-F5} = 7.2 Hz, 2H, H-6), 6.29 (t, J = 6.6 Hz, 3H, H-1'), 5.54–5.56 (m, 3H, H-3'), 4.24–4.29 (m, 3H, H-4'), 3.58–3.60 (m, 6H, H-5', H-5''), 2.40–2.58 (m, 6H, H-2', H-2''); ^{13}C NMR (100 MHz, DMSO-d₆): δ_{C} 163.73 (C=O), 157.18 (d, J = 25.75 Hz, C-4), 149.21 (C-2), 140.16 (d, J = 229 Hz, C-5), 133.93, 130.94, 128.30 (C of Ar), 124.44 (d, J = 35 Hz, C-6), 84.61 (C-4'), 84.49 (C-1'), 76.70 (C-3'), 61.31 (C-5'), 36.84 (C-2') ppm; HRMS (ESI): calcd for C₃₆H₃₂N₆O₁₈F₃ [M-H]⁻: 893.17197, found: 893.17464.

3'-O-[2-(2-acetyl-salicyl)-salicyl]-5-fluoro-2'-deoxyuridine (8). White solid; ^1H NMR (400 MHz, DMSO-d₆): δ_{H} 11.87 (s, 1H, NH), 8.23 (dd, J = 1.6 Hz, J = 8 Hz, 1H, H of Ar), 8.19 (d, J_{6-F5} = 6.8 Hz, 1H, H-6), 8.06 (dd, J = 1.6 Hz, J = 7.6 Hz, 1H, H of Ar), 7.75–7.81 (m, 2H, H of Ar), 7.51 (t, J = 7.6 Hz, 2H, H of Ar), 7.28–7.37 (m, 2H, H of Ar), 6.11 (t, J = 6.4 Hz, 1H, H-1'), 5.37–5.38 (m, 1H, H-3'), 3.99 (br s, 1H, H-4'),

3.60 (m, 2H, H-5', H-5''), 2.22–2.31 (m, 2H, H-2', H-2''), 2.21 (s, 3H, CH₃) ppm; ¹³C NMR (125 MHz, DMSO-d₆): δ_C 169.07 (C=O), 163.44, (C=O), 162.22 (C=O), 156.95 (d, J = 26 Hz, C-4), 150.77, 149.52 (C of Ar), 148.93 (C-2), 140.04 (d, J = 230 Hz, C-5), 135.27, 134.75, 131.93, 131.54, 126.72, 126.50 (C of Ar), 124.46 (d, J = 34 Hz, C-6), 122.94, 121.67 (C of Ar), 84.53 (C-4'), 84.35 (C-1'), 75.66 (C-3'), 61.07 (C-5'), 36.87 (C-2'), 20.64 (CH₃) ppm; HRMS (ESI): calcd for C₂₅H₂₁N₂O₁₀FNa [M+Na]⁺: 551.1072, found: 551.1091.

5'-O-(4,4'-dimethoxytrityl)-3'-O-benzoyl-5-fluoro-2'-deoxyuridine (**5a**). ¹H NMR (400 MHz, DMSO-d₆): δ_H 11.93 (s, 1H, NH), 7.98–8.01 (m, 3H, H-6, o-H of Bz), 7.67–7.71 (m, 1H, p-H of Bz), 7.53–7.57 (m, 2H, m-H of Bz), 7.39–7.41 (m, 2H, H of DMTr), 7.20–7.30 (m, 7H, H of DMTr), 6.86 (d, J = 8.8 Hz, 4H, H of DMTr), 6.26 (t, J = 6.8 Hz, 1H, H-1'), 5.51 (t, J = 3.2 Hz, 1H, H-3'), 4.26–4.27 (m, 1H, H-4'), 3.72 (s, 6H, OCH₃), 3.28–3.48 (m, H-5', H-5''), 2.53–2.64 (m, 2H, H-2', H-2'') ppm; ¹³C NMR (100 MHz, DMSO-d₆): δ_C 165.58 (C=O), 158.56 (C of DMTr), 157.40 (d, J = 26 Hz, C-4), 149.40 (C-2), 145.05 (C of DMTr), 140.55 (d, J = 230 Hz, C-5), 135.72, 135.61 (C of DMTr), 134.04 (C of Bz), 130.11 (C of DMTr), 130.09, 129.63 (C of Bz), 128.39, 128.01, 127.19 (C of DMTr), 125.01 (d, J = 34 Hz, C-6), 113.64 (C of DMTr), 86.46 (C-4'), 85.03 (C-1'), 83.22 (C of DMTr), 75.13 (C-3'), 66.76 (OCH₃), 64.03 (C-5'), 36.86 (C-2') ppm.

5'-O-(4,4'-dimethoxytrityl)-3'-O-benzoyl-5-fluoro-3-N-benzoyl-2'-deoxyuridine (**6a**). ¹H NMR (400 MHz, DMSO-d₆): δ_H 8.24 (d, J_{6-F5} = 6.8 Hz, 1H, H-6), 8.12 (d, J = 7.6 Hz, 2H, o-H of N-Bz), 7.99 (d, J = 7.2 Hz, 2H, o-H of O-Bz), 7.81 (t, J = 7.3 Hz, 1H, p-H of N-Bz), 7.68 (t, J = 7.3 Hz, 1H, p-H of O-Bz), 7.61 (t, J = 7.1 Hz, 2H, m-H of N-Bz), 7.54 (t, J = 7.1 Hz, 2H, m-H of O-Bz), 7.41–7.43 (m, 2H, H of DMTr), 7.21–7.30 (m, 7H, H of DMTr), 6.88 (d, J = 7.92 Hz, 4H, H of DMTr), 6.26–6.27 (m, 1H, H-1'), 5.54 (br s, 1H, H-3'), 4.3 (br s, 1H, H-4'), 3.72 (s, 6H, OCH₃), 3.47–3.61 (m, H-5', H-5''), 2.60–2.76 (m, 2H, H-2', H-2'') ppm; ¹³C NMR (100 MHz, DMSO-d₆): δ_C 168.49 (C of Bz), 165.57 (C=O), 158.57 (C of DMTr), 156.28 (d, J = 26 Hz, C-4), 147.99 (C-2), 145.03 (C of DMTr), 140.22 (d, J = 230 Hz, C-5), 136.33 (C of Bz), 135.71, 135.59 (C of DMTr), 134.06 (C of Bz), 131.16, 131.08 (C of Bz), 130.12 (C of DMTr), 129.93, 129.93, 129.60, 129.17 (C of Bz), 128.32, 128.03, 127.21 (C of DMTr), 126.14 (d, J = 34 Hz, C-6), 113.67 (C of DMTr), 86.55 (C-4'), 85.67 (C-1'), 83.43 (C of DMTr), 74.71 (C-3'), 66.76 (OCH₃), 63.84 (C-5'), 37.06 (C-2') ppm.

4.2. Biological assays

4.2.1. Cell line and culture conditions

GBM cell lines (T98G, U-118 MG, U-87 MG), HeLa (cervical cancer cell line), T-47D (breast cancer cell line), Caco-2 (colon cancer cell line), and non-cancerous lung fibroblast cell line (MRC-5) were purchased from ATCC (Manassas, USA). All cell lines were from human origin. HeLa and T-47D were cultured in RPMI 1640 medium. Caco-2 and U-118 MG were cultured in DMEM medium. T98G, U-87 MG and MRC-5 were cultured in EMEM medium. Each medium was supplemented with 10% fetal bovine serum (FBS) and 10 mg/mL antibiotics (penicillin and streptomycin). Cells were cultured at 37 °C with 5% CO₂ in humidified air. Cell media (RPMI 1640, DMEM, EMEM), Human Blood Plasma (HBP) were obtained from Sigma–Aldrich Chemie GmbH (Steinheim, Germany) and ATCC. Cell concentrations in culture were adjusted to allow for exponential growth.

4.2.2. Cell viability/proliferation assay

Cell viability/proliferation was evaluated by a dye staining method using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT). The protocol was adapted from the literature methods [85]. The monolayer cell culture was trypsinized

and counted. To each well of the 96-well plate, 100 μL of the diluted cell suspension (1 × 10⁴ cells) was added. After 24 h, when a partial monolayer was formed, 100 μL of fresh medium with different compound concentrations (7.81, 15.625, 31.25, 62.5, 125, 250, 500 and 1000 μg/mL) were added to the cells. After 48 h, the supernatant was washed out and 100 μL of MTT solution in medium (final concentration 0.5 mg/mL) were added to each well for 2 h. After the incubation time was complete, unreacted dye was removed by aspiration. The formazan crystals were dissolved in 100 μL/well DMSO and measured spectrophotometrically in a multi-well Synergy2 plate reader (BioTek Instruments, USA) at a test wavelength of 492 nm and a reference wavelength of 690 nm. The results were calculated as an IC₅₀ (inhibitory concentration 50) – the IC₅₀ corresponds to the concentration of tested compound that inhibits cell viability/proliferation by 50%. Results are presented as mean of at least three independent experiments.

4.2.3. In silico pharmacokinetic prediction

Calculations of pharmacokinetic profile descriptors of the synthesized compounds were performed by various software solutions accessible on-line. The transformation of the stoichiometric formulas of the compounds into a SMILES code (Simplified Molecular Input Line Entry System) was carried out by ChemBioDraw Ultra version 12.0 program (Cambridge Software). The SMILES code was applied to calculate logP values with ALOGPS 2.1 software [86] (mean value obtained from ALOGPs, AC_logP, miLogP, ALOGP, MLOGP, LogKOWWIN, XLOGP2, XLOGP3 methods), PSA (topological polar surface area) and aPSA (apolar surface area) values. PSA and aPSA descriptors were calculated using the VEGA ZZ program [87]. The pharmacokinetic profile was evaluated according to the Lipinski “rule of five” [48] by using Molinspiration application (<http://www.molinspiration.com>), which includes also analyses of molecular weight (MW), number of hydrogen-bond acceptors (HBA) and number of hydrogen-bond donors (HBD). The Caco-2 prediction model based on descriptors generated by preADMET (<http://preadmet.bmdrc.org>) was used to compute Caco-2 apparent permeability (tP_{app}). In this model a number of hydrogen bond donors and three molecular surface area properties determine membrane permeability of compounds.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at <http://dx.doi.org/10.1016/j.ejmech.2016.03.010>. These data include MOL files and InChIKeys of the most important compounds described in this article.

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 - [39] This hypothesis was verified negatively in experiment in which 3'-O-salicylated **5h** dissolved in neat pyridine was allowed to react with 1 molar equiv. of **2h**. After 12 h the reaction mixture was treated with morpholine and then the DMT protecting group was removed with 80% acetic acid. Inspection of the reaction mixture with TLC analysis did not show the presence of compound **8**.
 - [40] This scenario was checked by keeping O-acetylsalicyl chloride **2h** in neat pyridine-d5 and recording periodically the ¹H NMR spectra of the reaction mixture. After 10 min in the region of chemical shifts δ_H 2.3 – 2.5 ppm typical for resonance frequency of the methyl group of the acetyl moiety in **2h**, several additional signals appeared, and their number and intensities were increasing in time. After 3 h intensity of the signal of the methyl group of **2h** became comparable to those of the new signals (data not shown). This seemed to indicate that O-acetylsalicyl chloride **2h** when left in pyridine underwent deacetylation and produced dimeric (and most likely also polymeric) acylating species, which in the reaction with nucleoside **1** could produce O-salicyl-salicylated 5-fluoro-2'-deoxyuridine **8** as a predominant product.
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MATERIAŁY UZUPEŁNIAJĄCE DO PUBLIKACJI

Szymańska-Michalak A. i wsp., Eur. J. Med. Chem. 2016

Supplementary material –¹H, ¹³C NMR spectra, and RP HPLC chromatograms

New 3'-O-aromatic acyl-5-fluoro-2'-deoxyuridine derivatives as potential anticancer agents

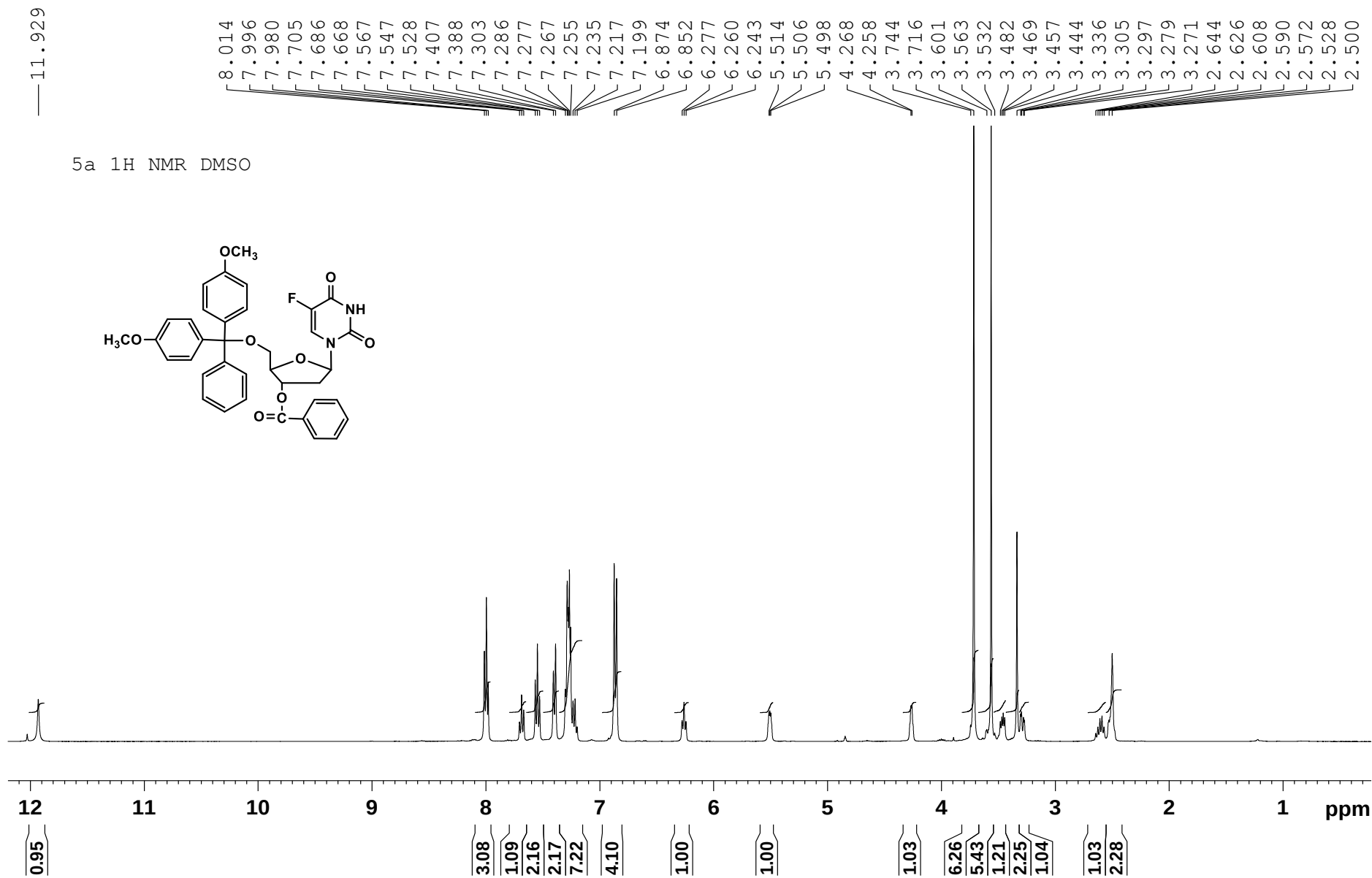
Agnieszka Szymańska-Michalak^a, Dariusz Wawrzyniak^a, Grzegorz Framski^a, Marta Kujda^b, Paulina Zgoła^a, Jacek Stawiński^a, Jan Barciszewski^a, Jerzy Boryski^a, and Adam Kraszewski^{a*}

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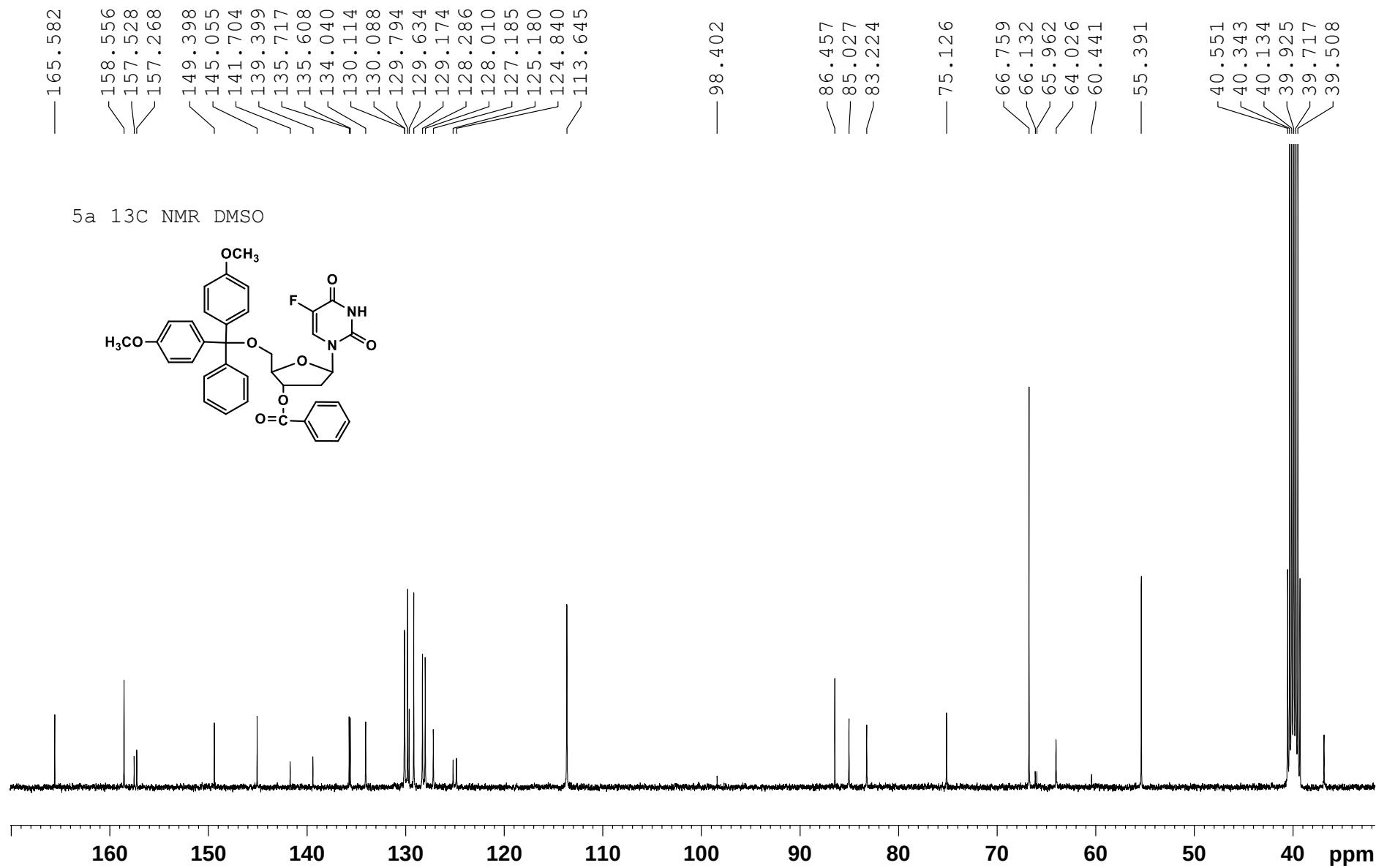
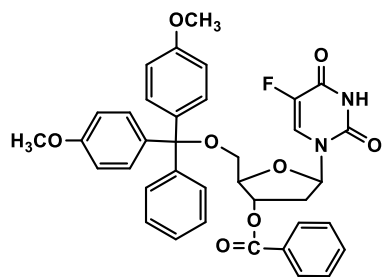
^bJerzy Haber Institute of Catalysis and Surface Chemistry, Polish Academy of Sciences, Niezapominajek 8, 30-239 Cracow, Poland

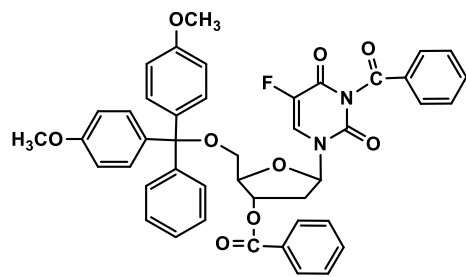
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5'-O-(4,4'-dimethoxytrityl)-3'-O-benzoyl-5-fluoro-2'-deoxyuridine 5a	S2
5'-O-(4,4'-dimethoxytrityl)-3'-O-benzoyl-5-fluoro-3-N-benzoyl-2'-deoxyuridine 6a	S4
3'-acyl 5-fluoro-2'-deoxyuridines 7a	S6
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3'-acyl 5-fluoro-2'-deoxyuridines 7c	S12
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3'-acyl 5-fluoro-2'-deoxyuridines 7j	S33
3'-acyl 5-fluoro-2'-deoxyuridines 7k	S36

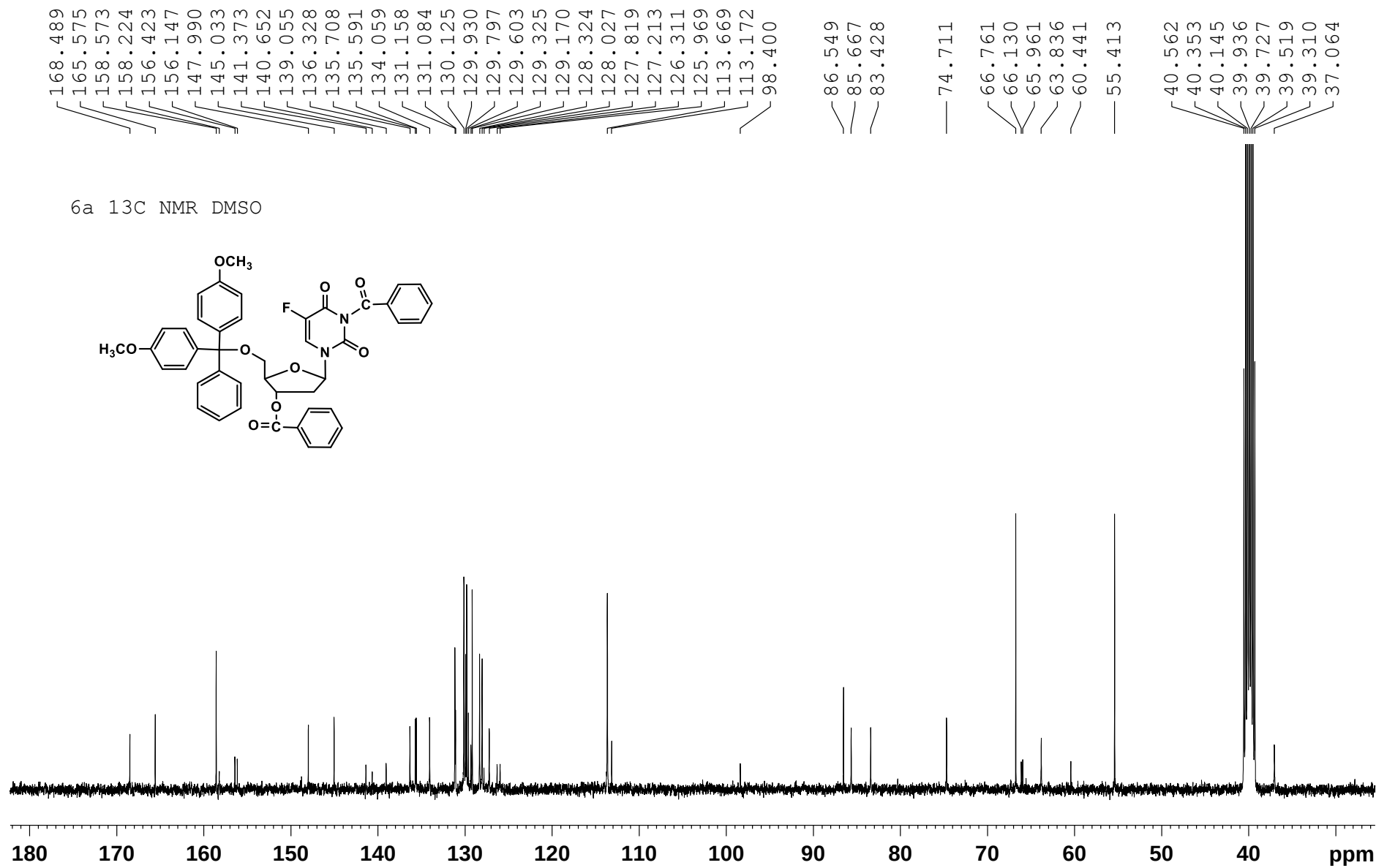


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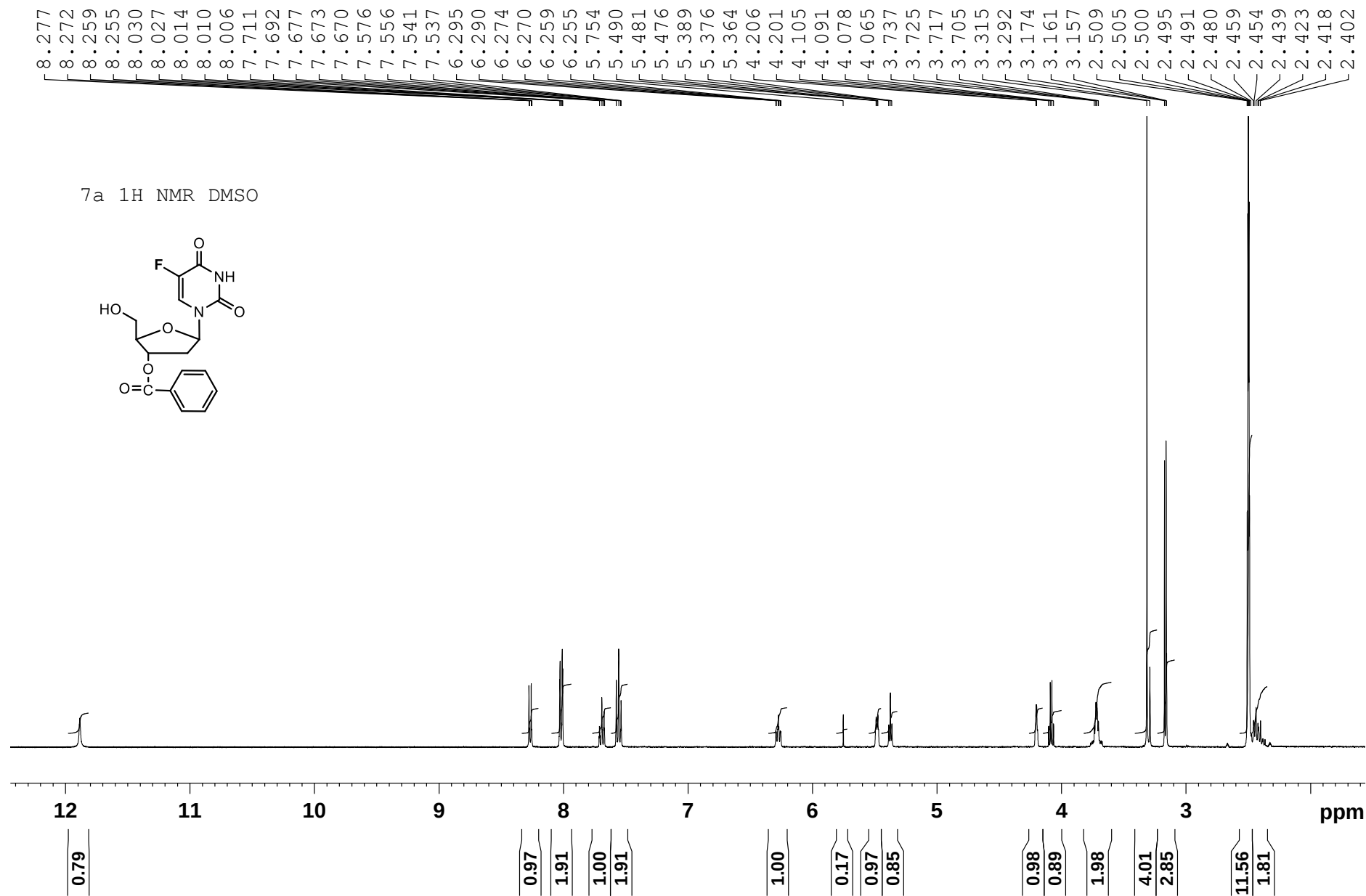
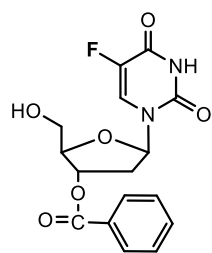




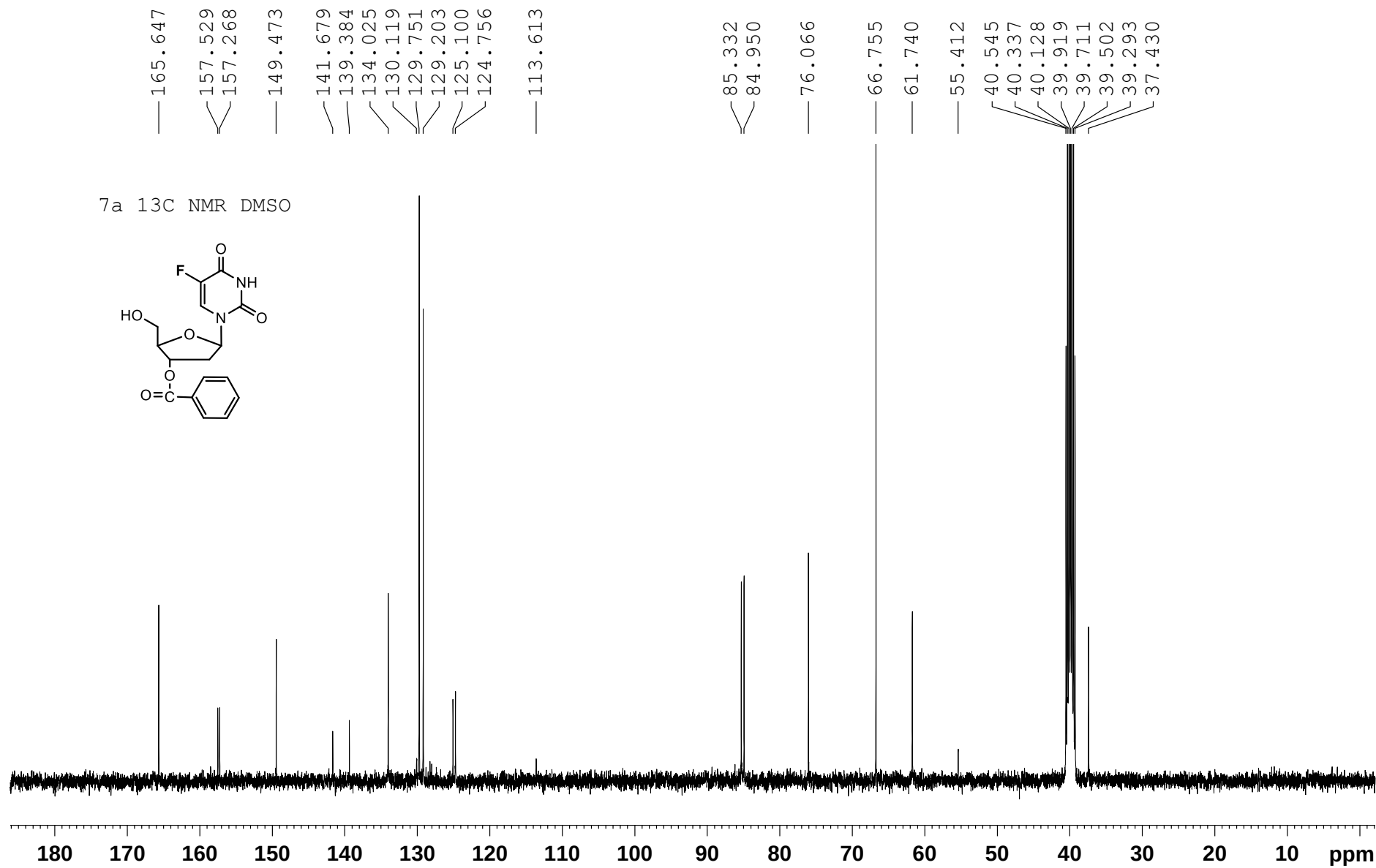
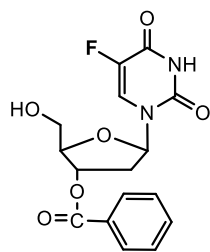
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7a ¹H NMR DMSO

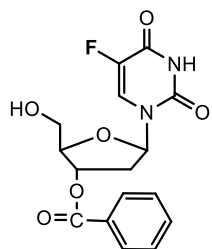


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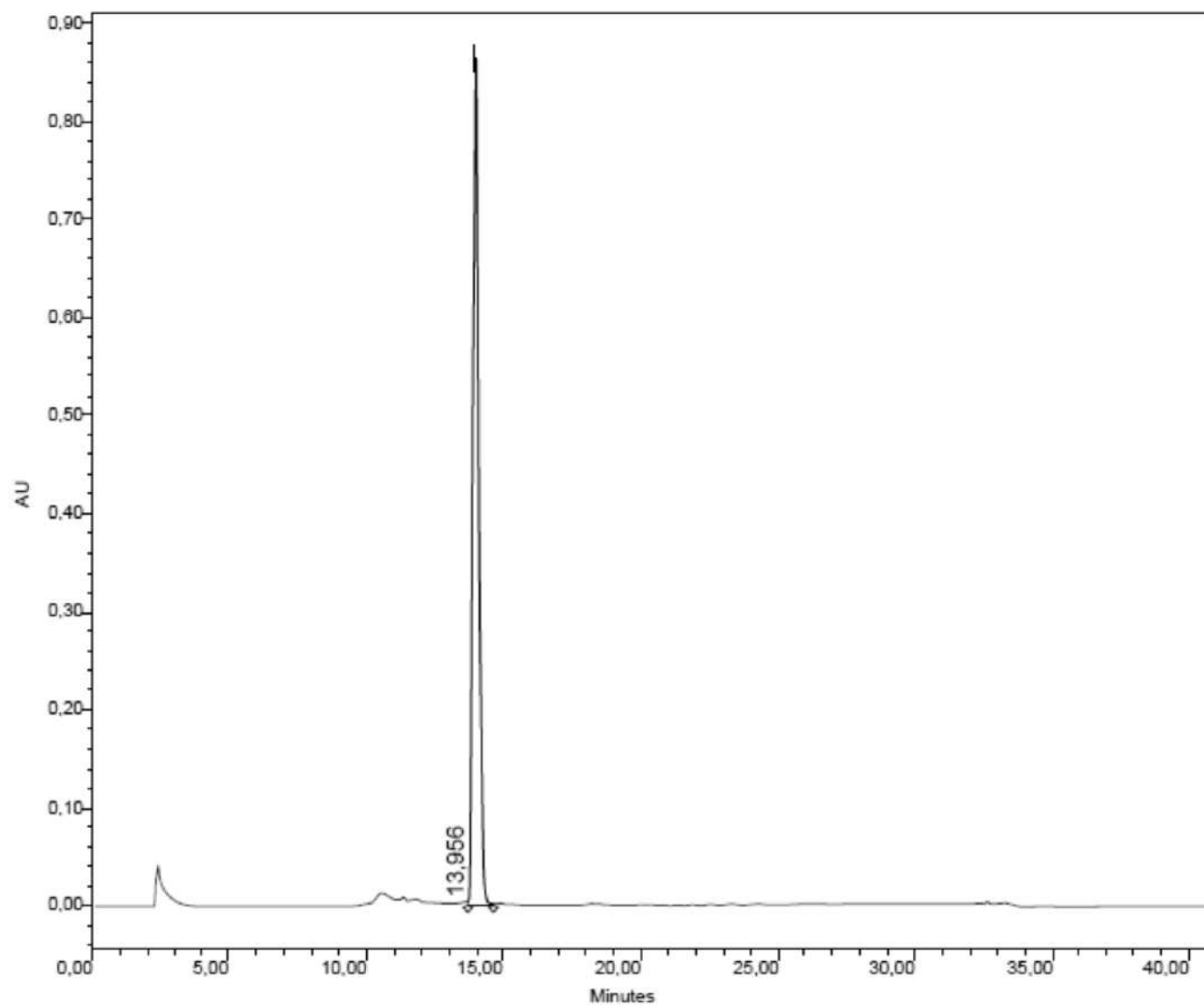
Chromatogram

7a

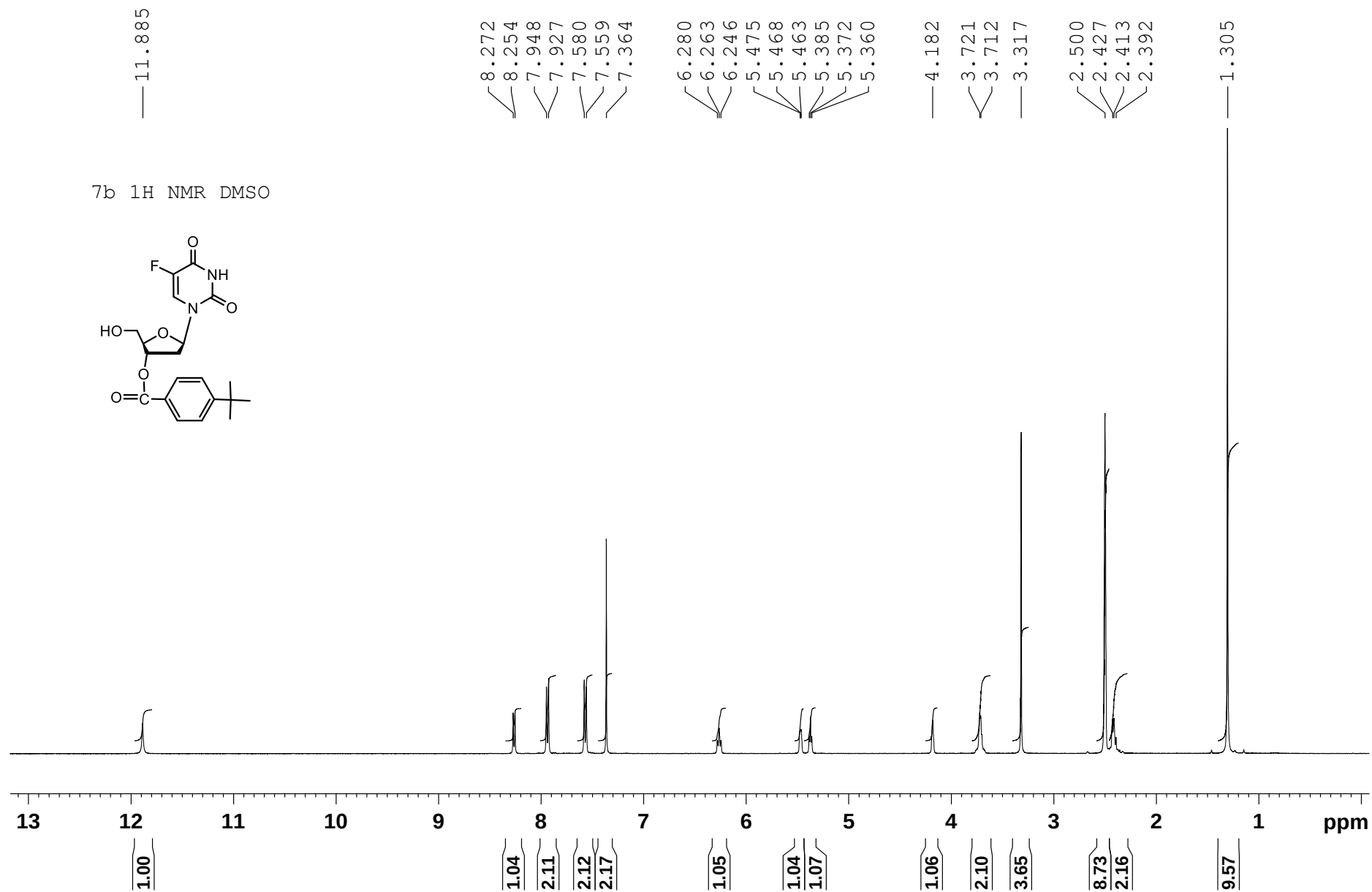
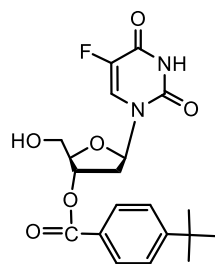


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(5.0 μm , 4.6 mm $\times$ 150 mm)

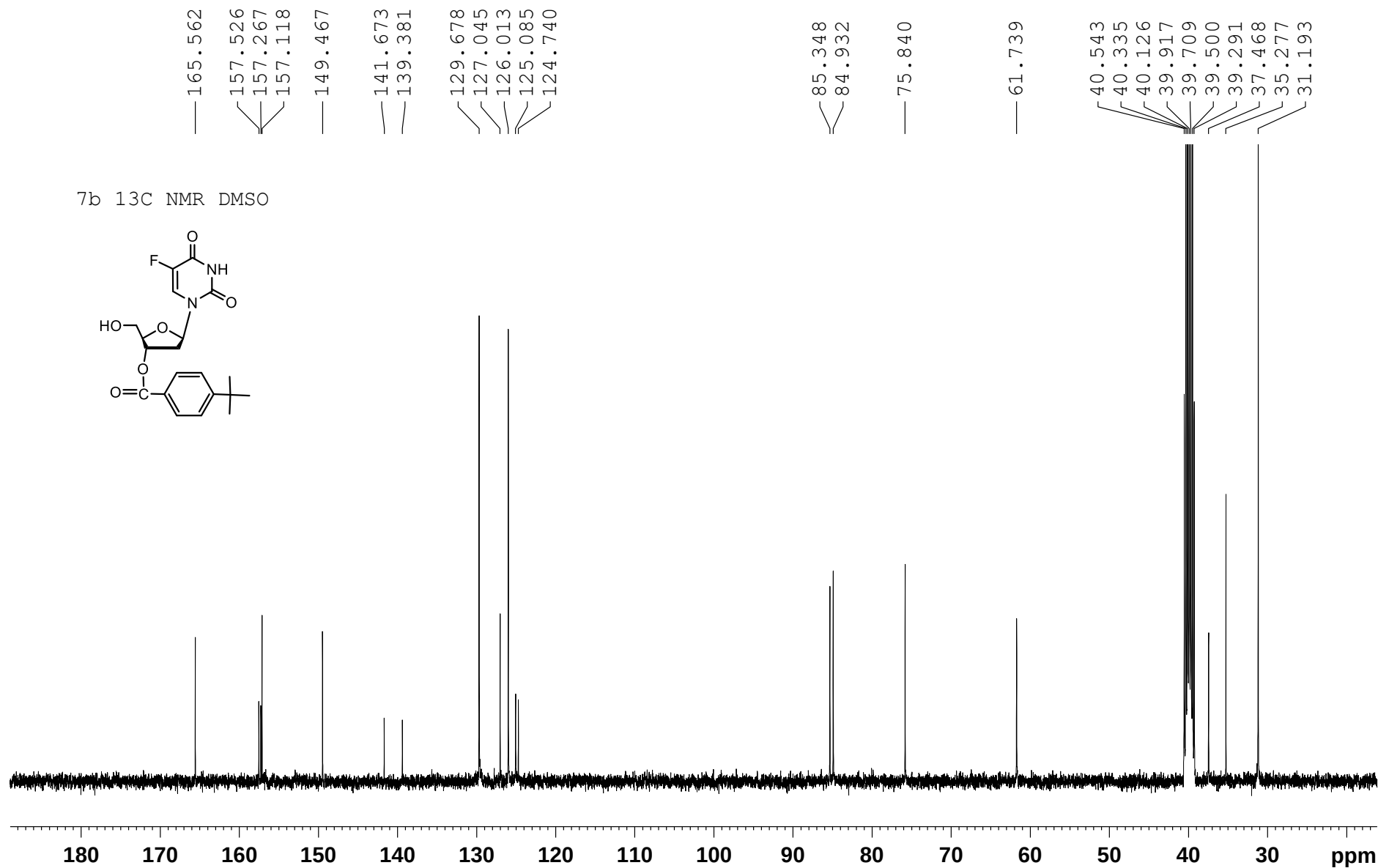
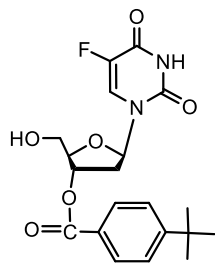
Waters Breeze HPLC
systems with A + B solvent
(A, 0.01 M aqueous
triethylammonium acetate
pH 7.4; B, A / acetonitrile,
1: 4, v/v) at 35°C, flow rate
1.5 mL/min; events: 5 min A
100%, 20 min B 0–100%,
5 min B 100% and 10 min A
100%.



7b ¹H NMR DMSO

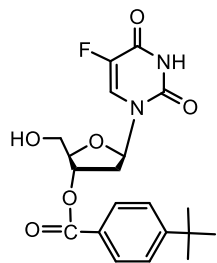


7b ¹³C NMR DMSO



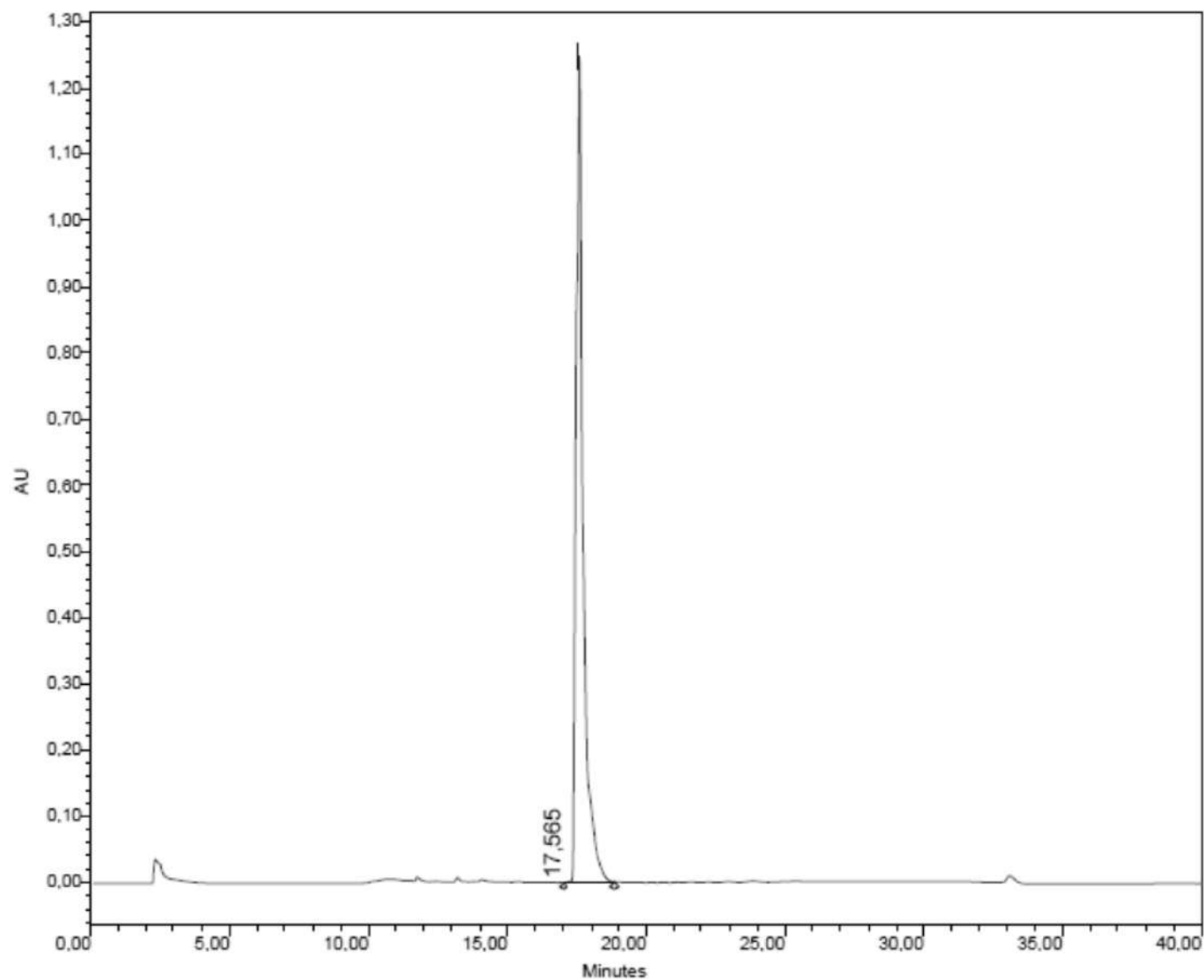
Chromatogram

7b



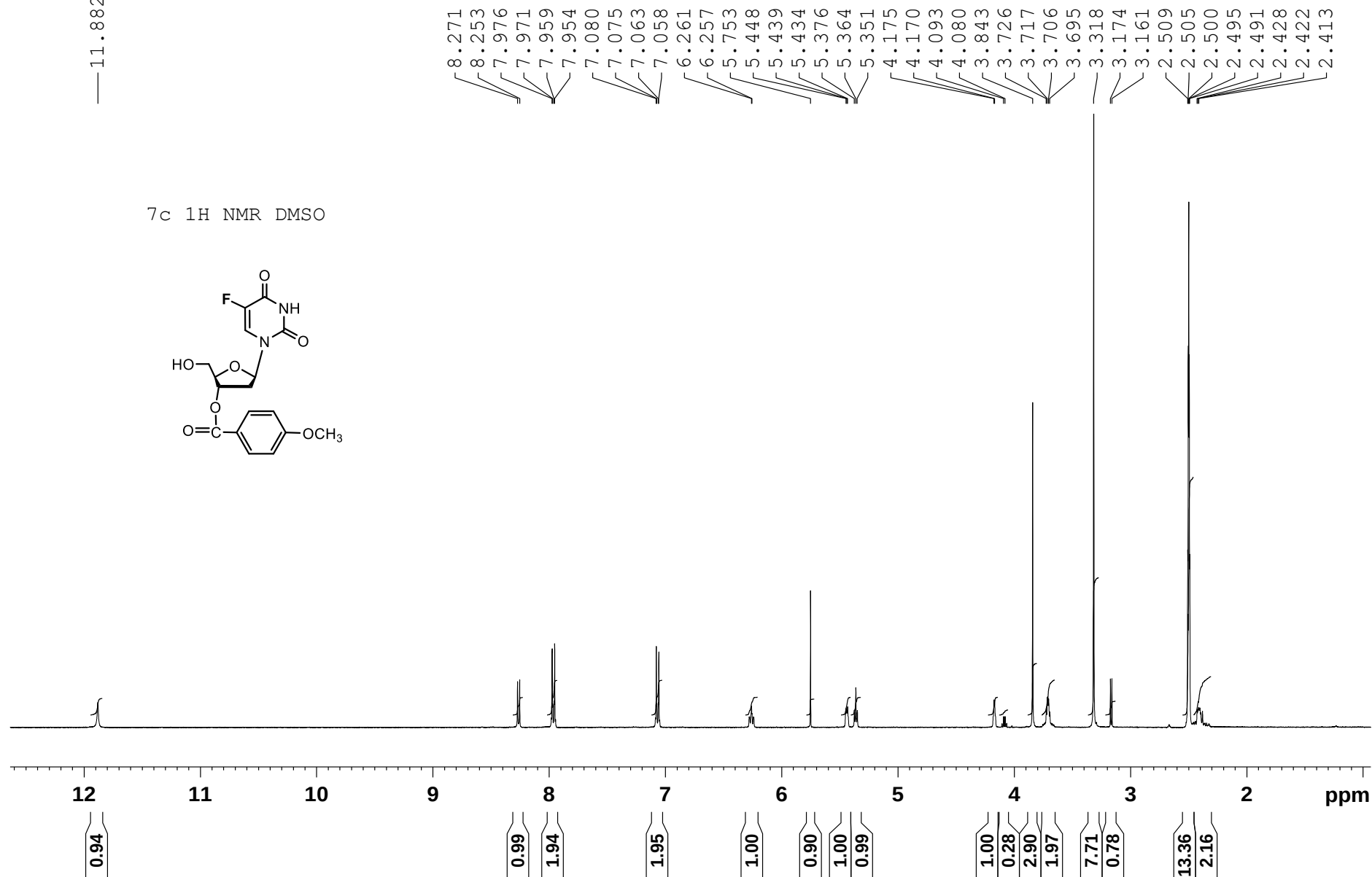
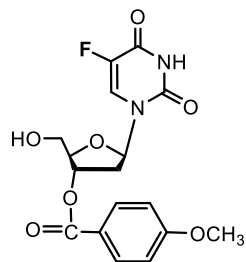
Nucleosil 100-5C18
(5.0 μm , 4.6 mm $\times$ 150 mm)

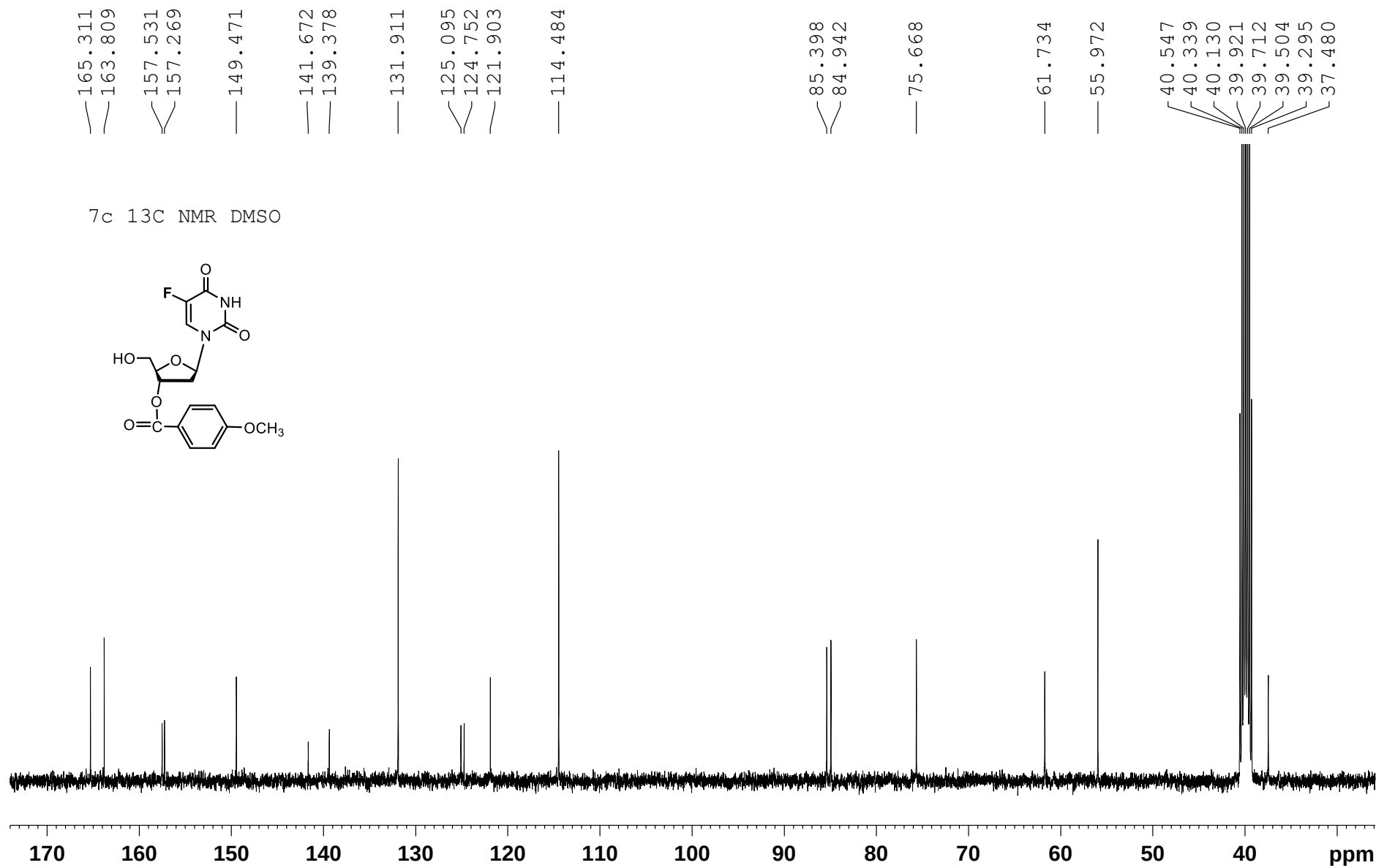
Waters Breeze HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium
acetate pH 7.4; B, A /
acetonitrile, 1: 4, v/v) at 35°C,
flow rate 1.5 mL/min; events:
5 min A 100%, 20 min B
0–100%, 5 min B 100% and 10
min A 100%.



—11.882

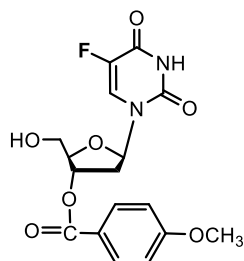
7c 1H NMR DMSO





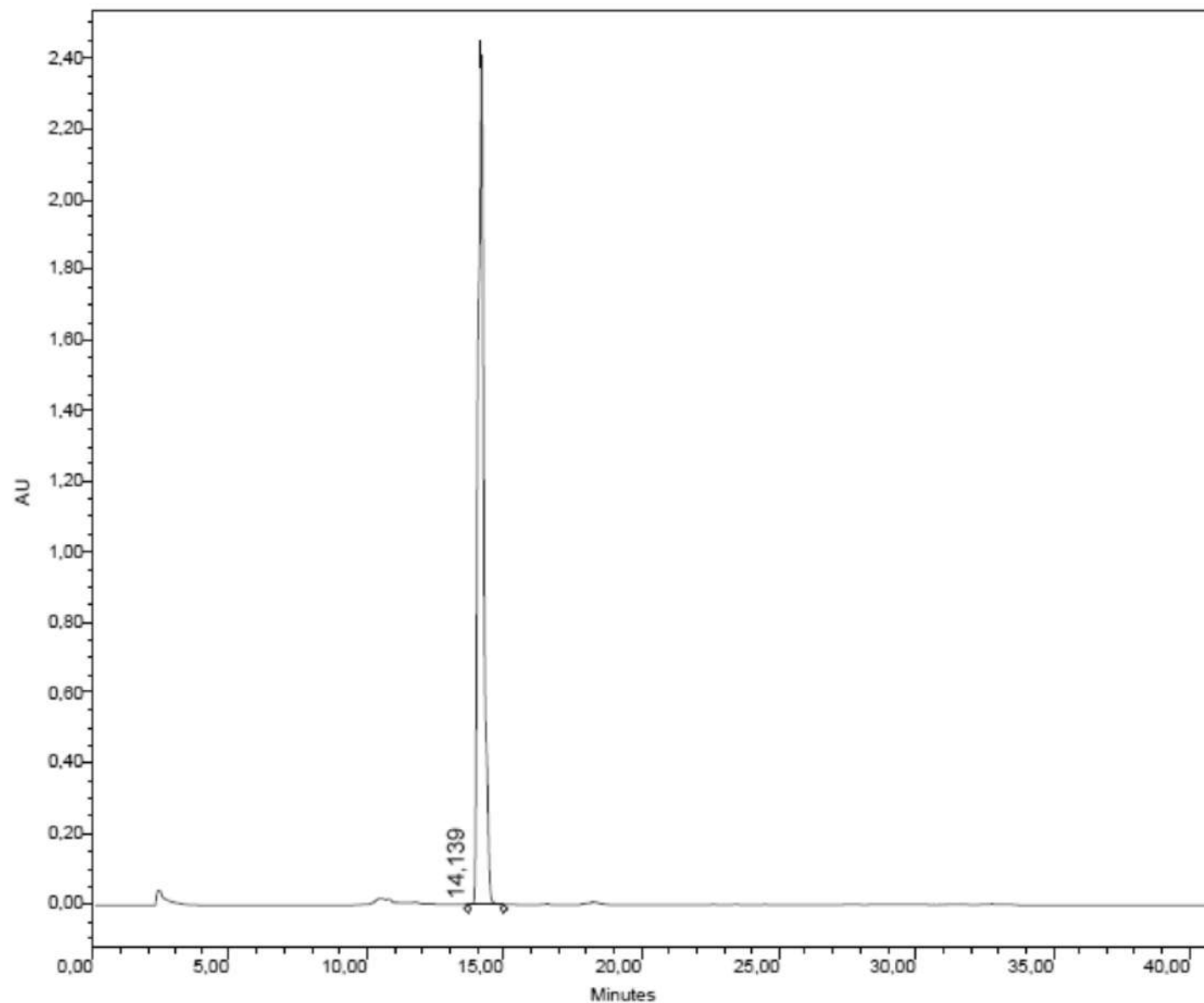
Chromatogram

7c

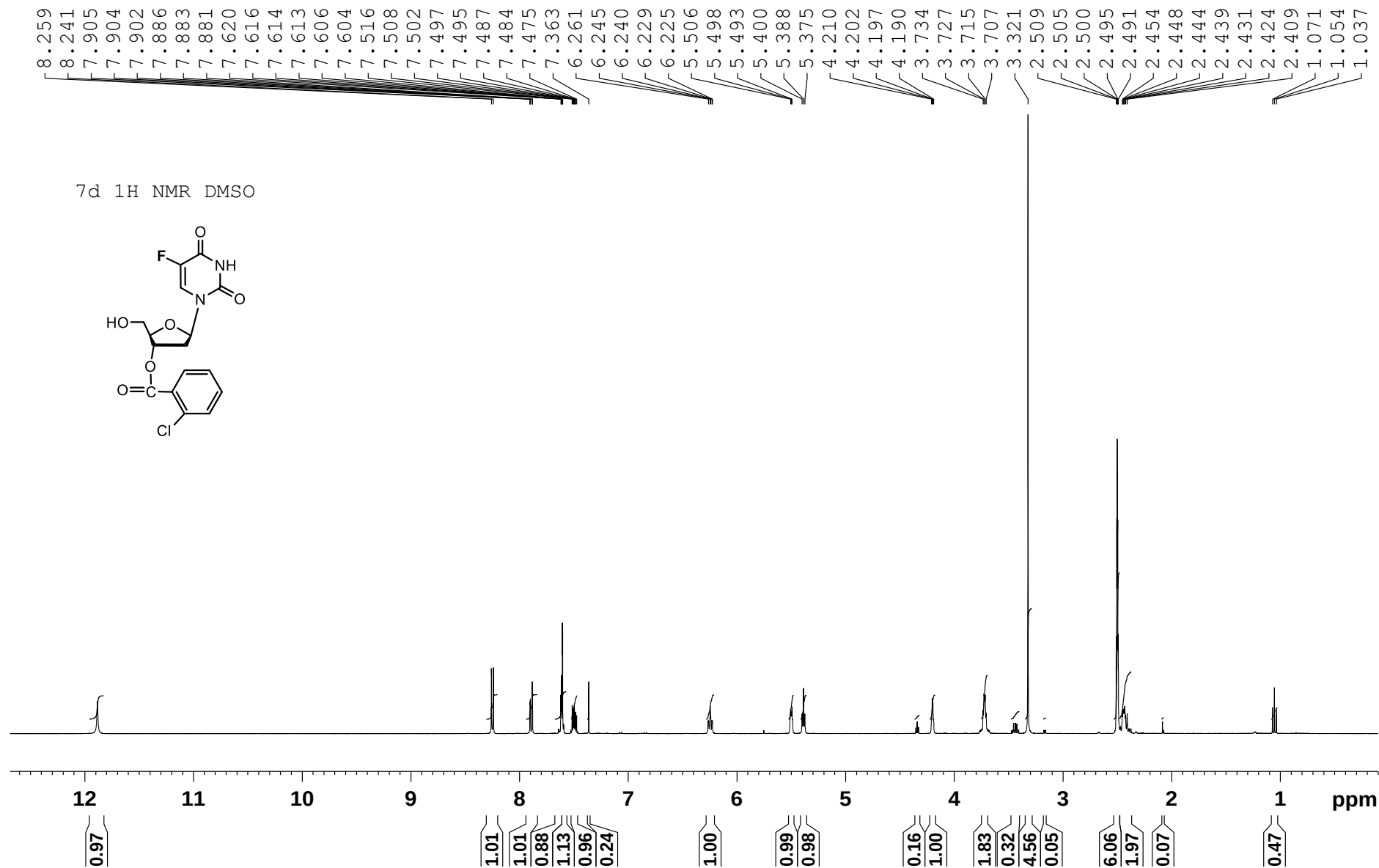
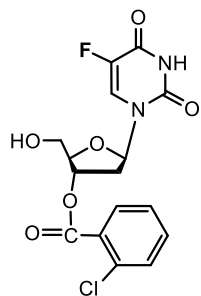


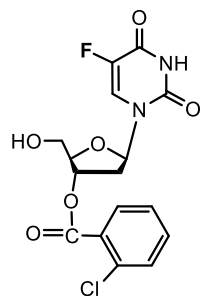
Nucleosil 100-5C18
(5.0 μm , 4.6 mm $\times$ 150 mm)

Waters Breeze HPLC systems with A + B solvent (A, 0.01 M aqueous triethylammonium acetate pH 7.4; B, A / acetonitrile, 1: 4, v/v) at 35°C, flow rate 1.5 mL/min; events: 5 min A 100%, 20 min B 0–100%, 5 min B 100% and 10 min A 100%.

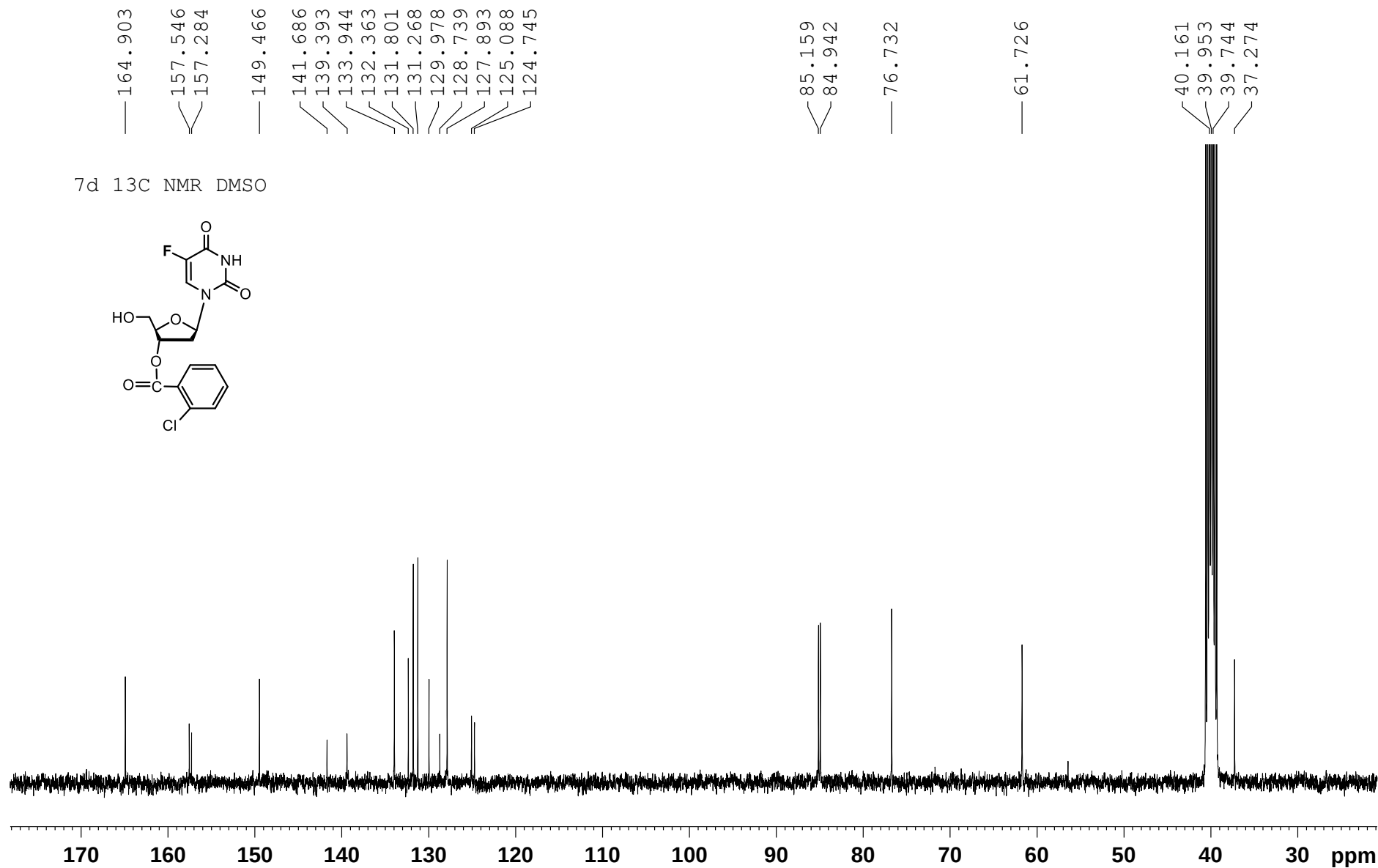


7d ¹H NMR DMSO



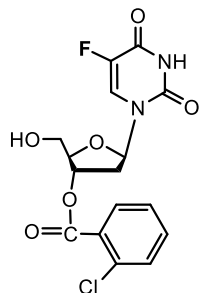


7d ¹³C NMR DMSO



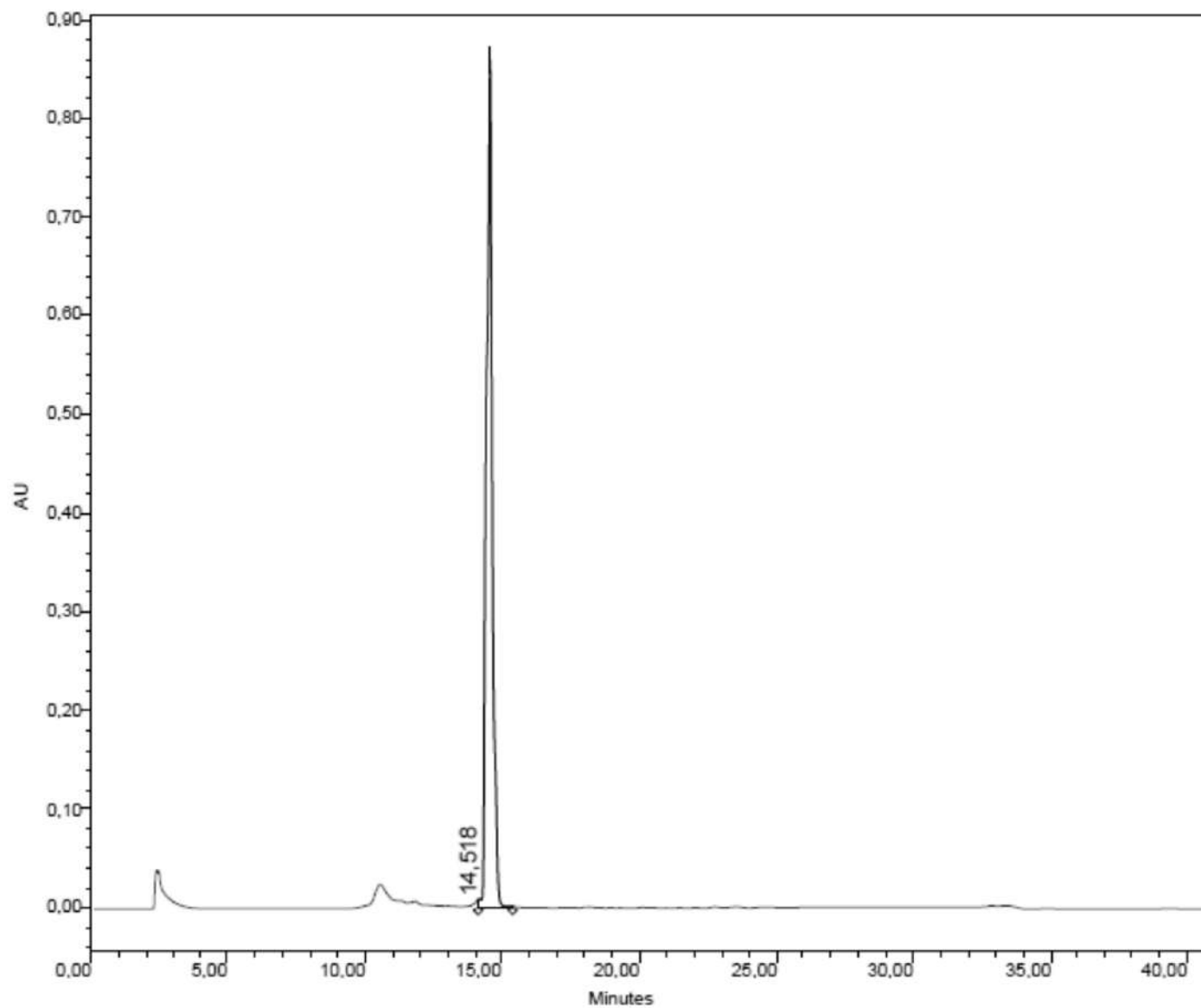
Chromatogram

7d

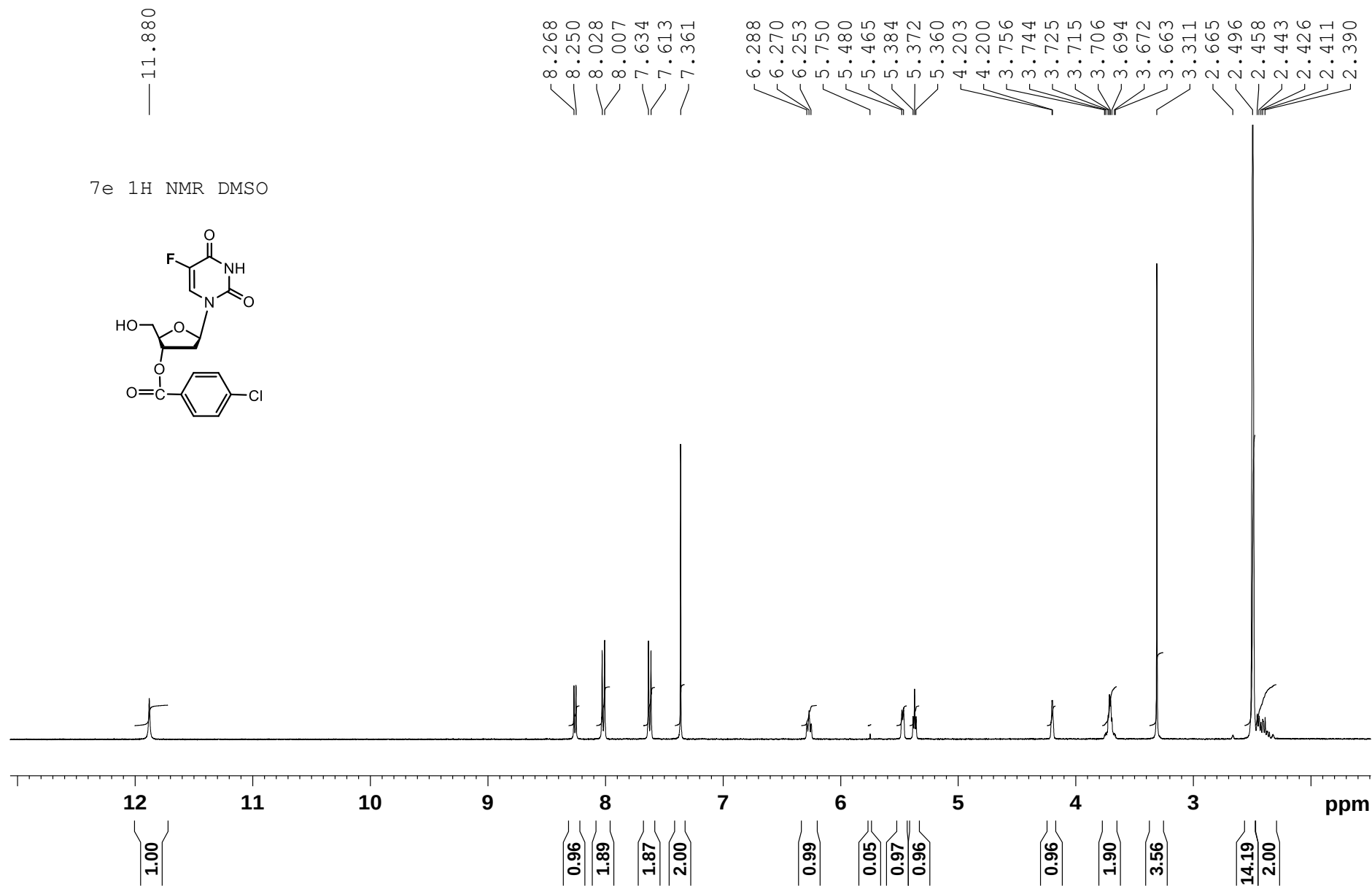
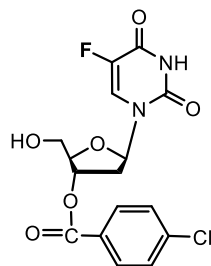


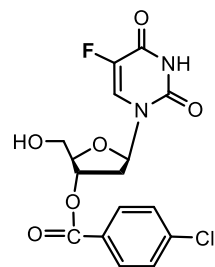
Nucleosil 100-5C18
(5.0 μm , 4.6 mm $\times$ 150 mm)

Waters Breeze HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium
acetate pH 7.4; B, A /
acetonitrile, 1: 4, v/v) at 35°C,
flow rate 1.5 mL/min; events:
5 min A 100%, 20 min B
0–100%, 5 min B 100% and 10
min A 100%.

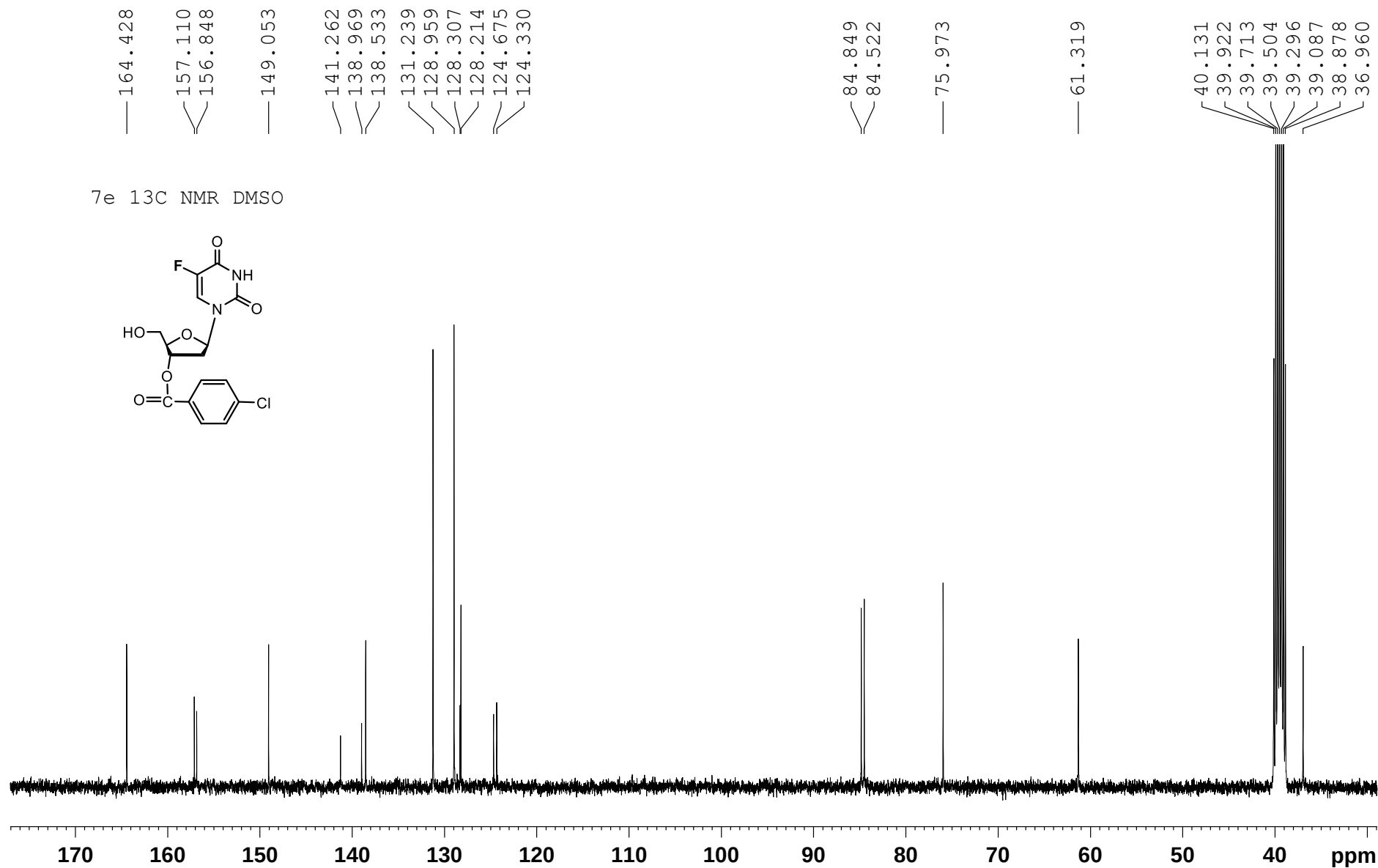


7e ¹H NMR DMSO



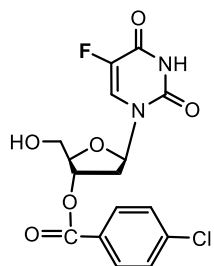


7e ¹³C NMR DMSO



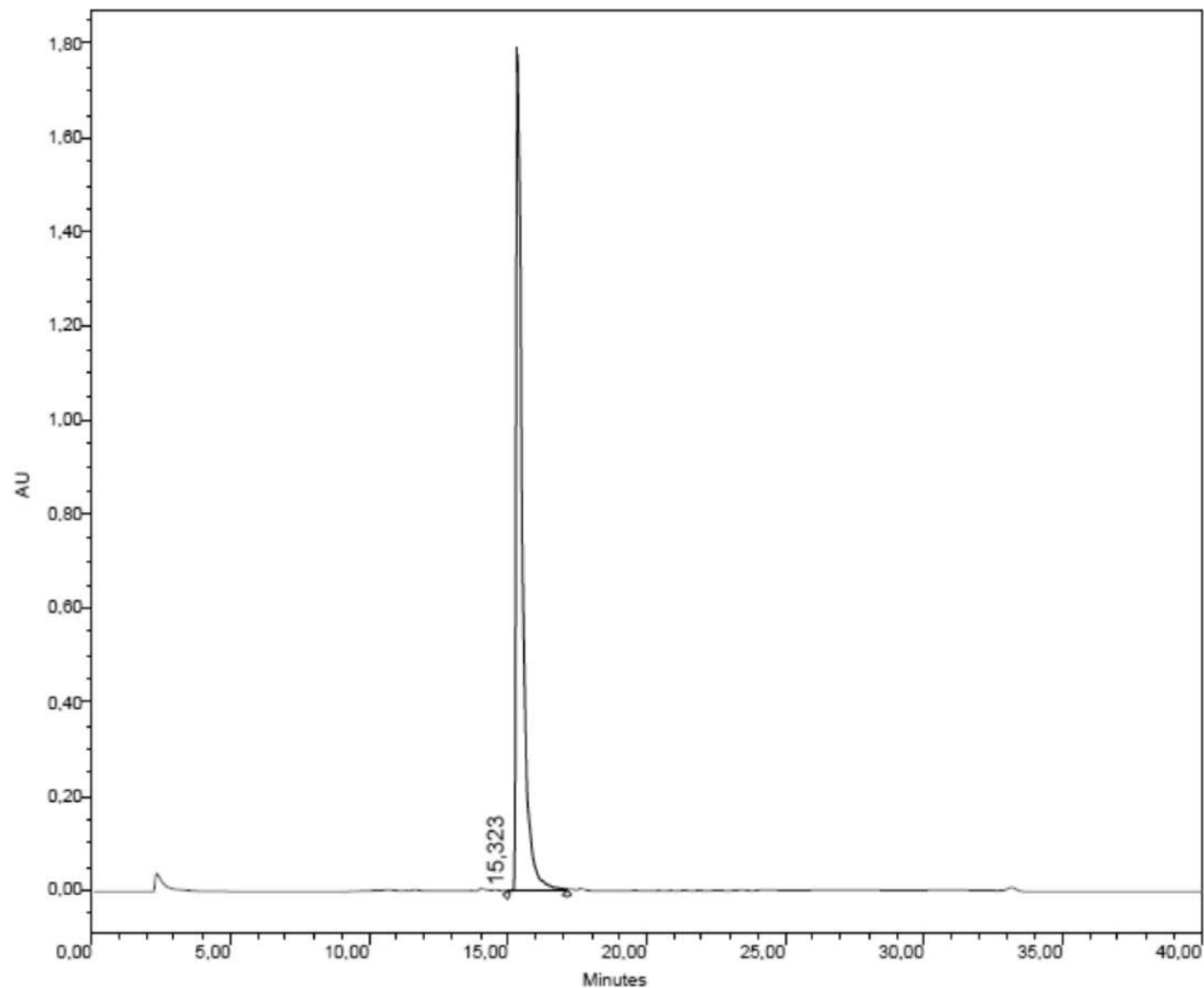
Chromatogram

7e



Nucleosil 100-5C18
(5.0 μm , 4.6 mm $\times$ 150 mm)

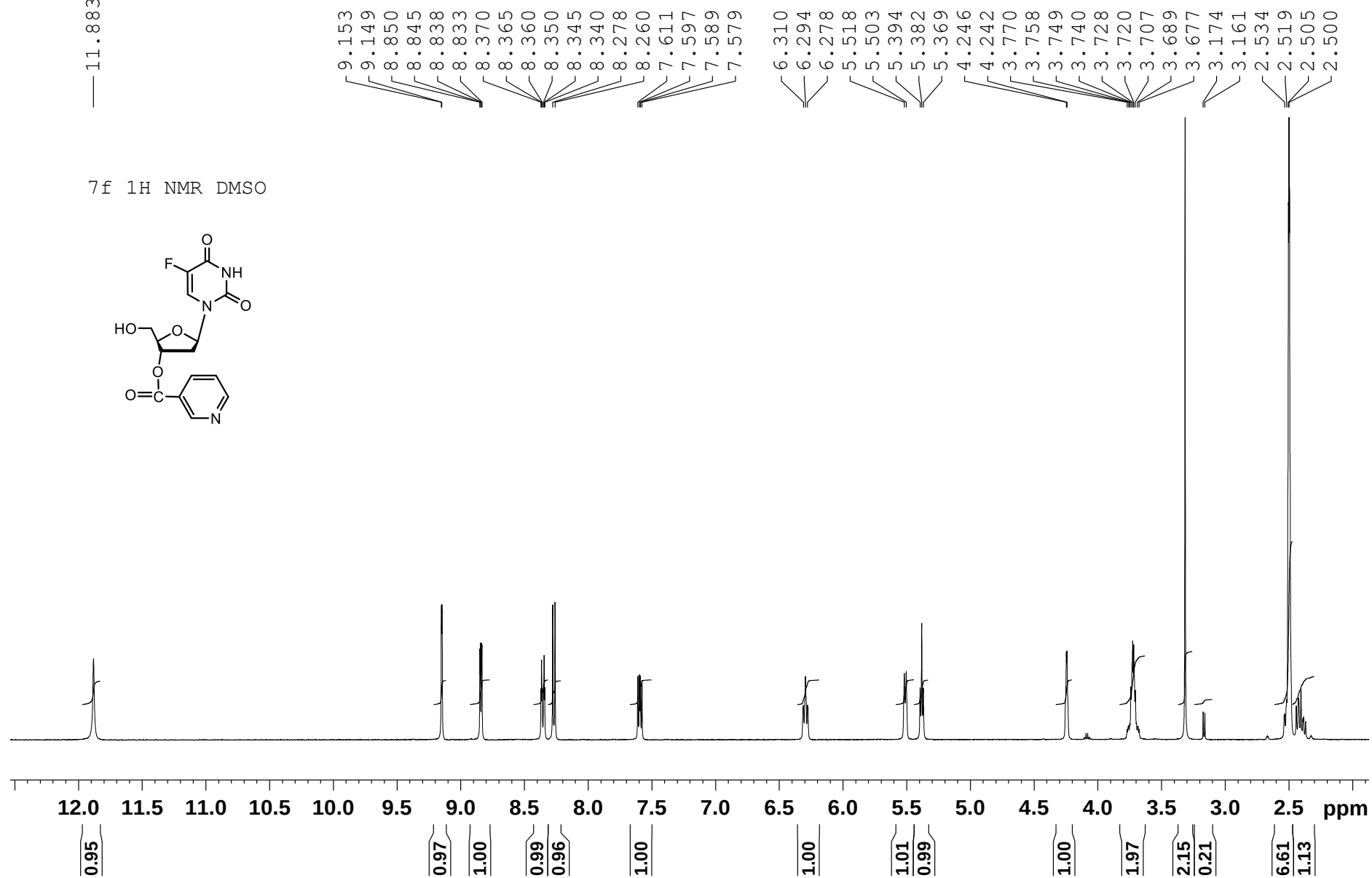
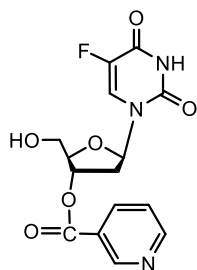
Waters Breeze HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium
acetate pH 7.4; B, A /
acetonitrile, 1: 4, v/v) at 35°C,
flow rate 1.5 mL/min; events:
5 min A 100%, 20 min B
100%, 5 min B 100% and 10
min A 100%.

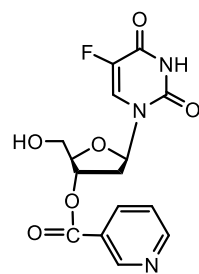


0-

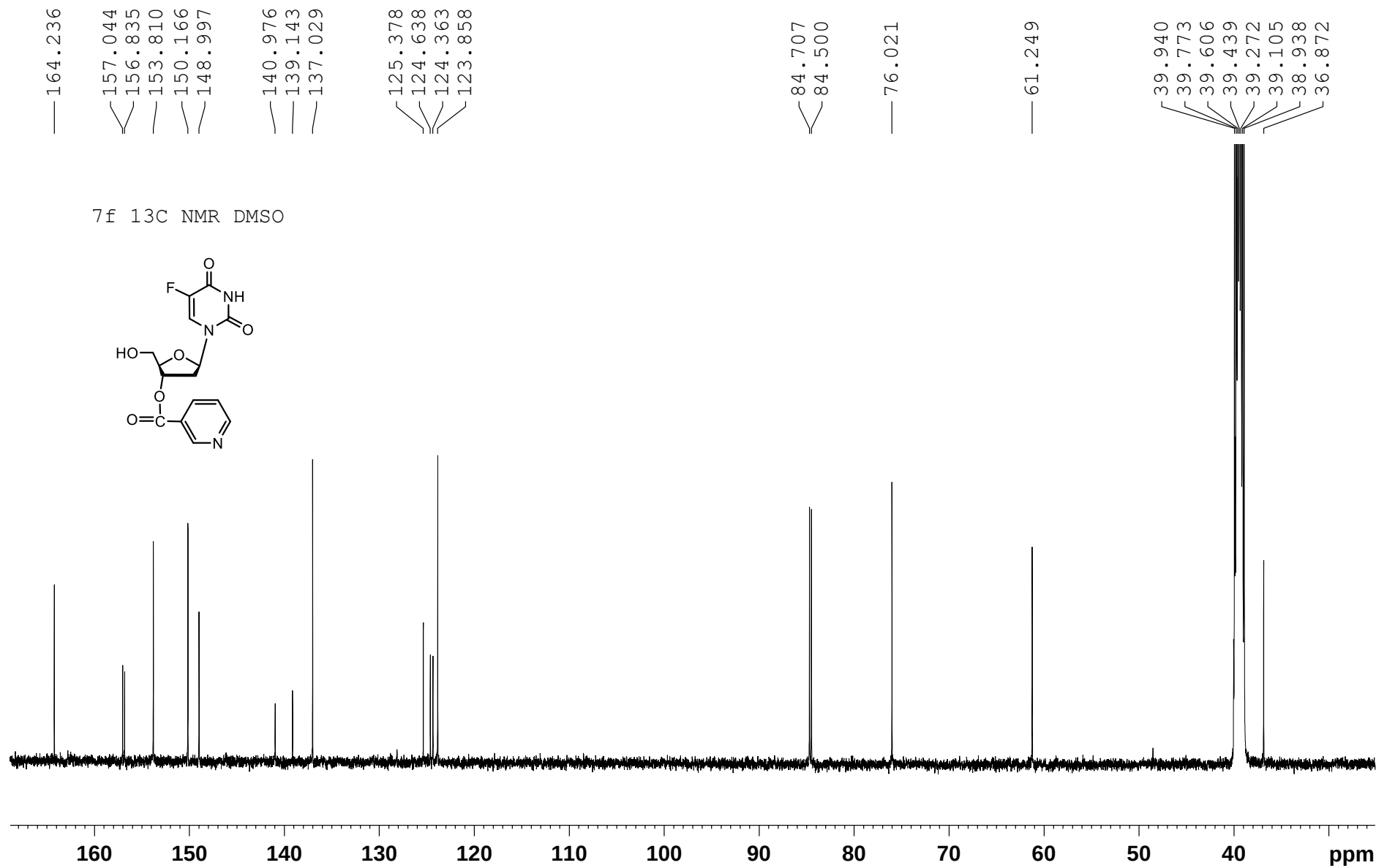
—11.883

7f ¹H NMR DMSO



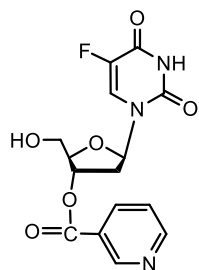


7f ¹³C NMR DMSO



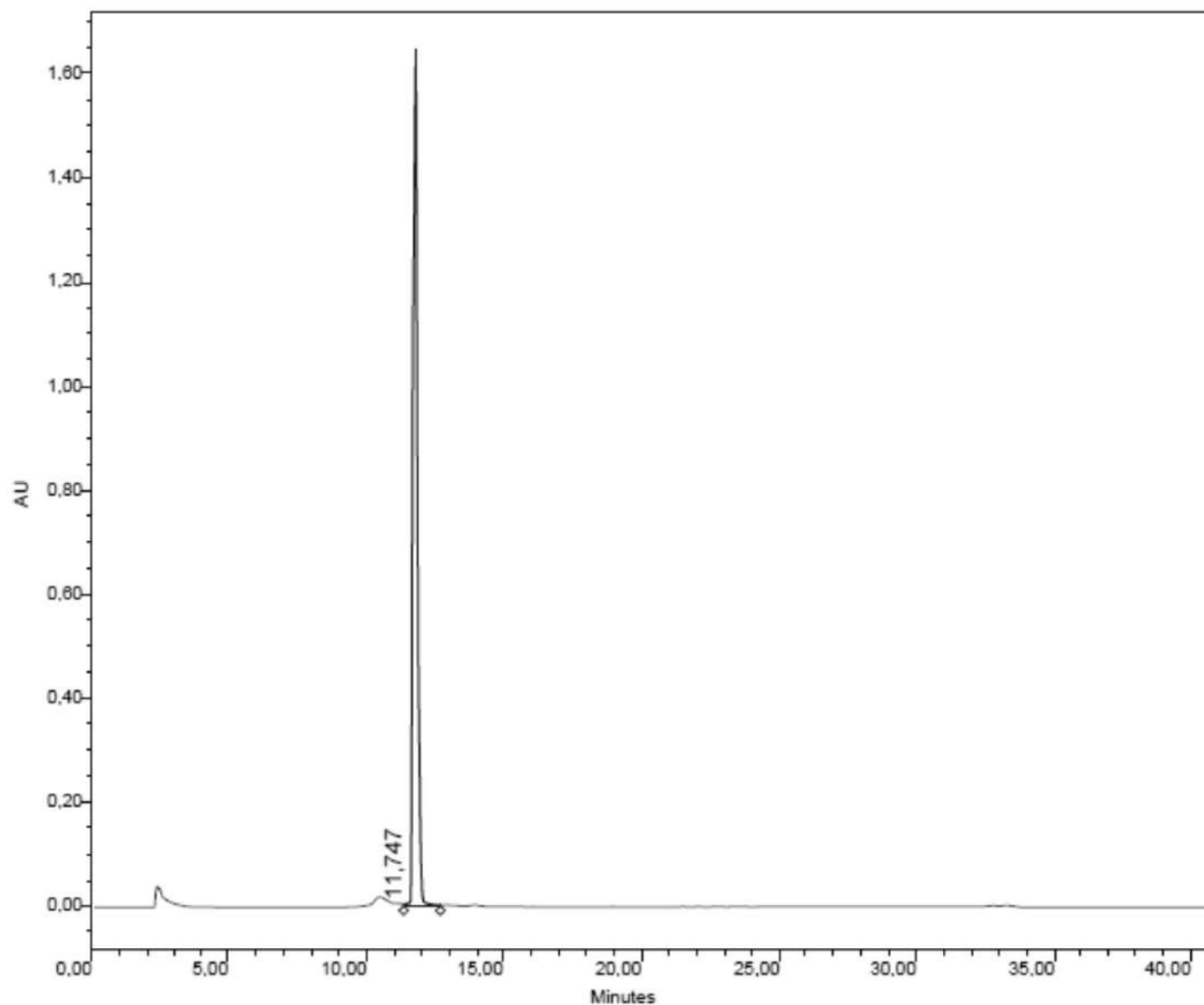
Chromatogram

7f

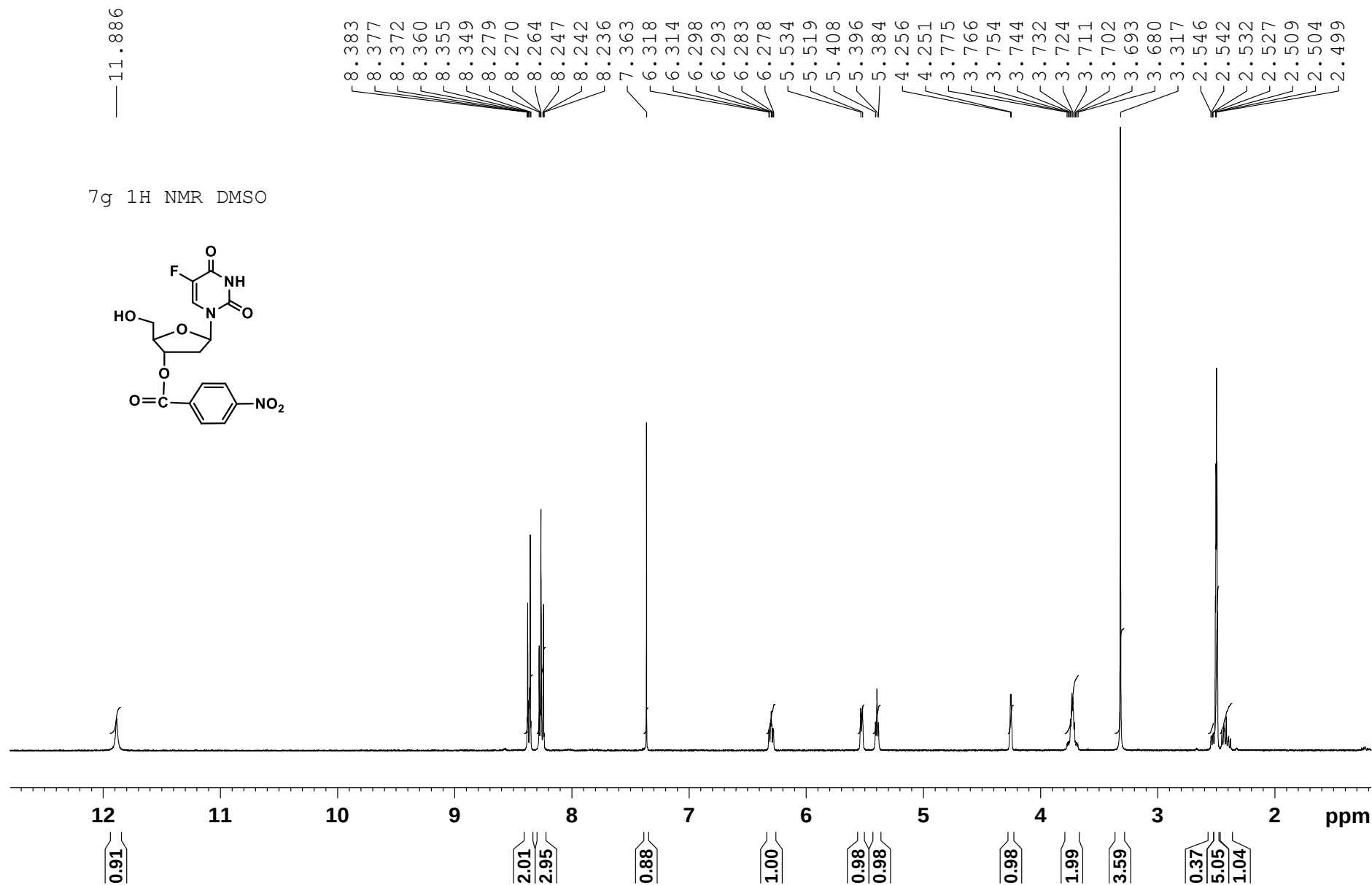
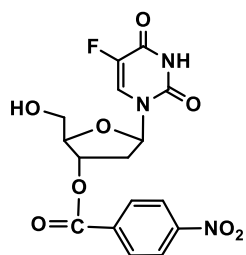


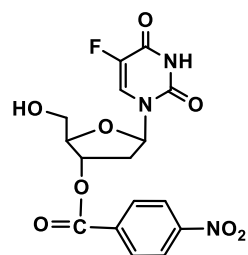
Nucleosil 100-5C18
(5.0 μm , 4.6 mm $\times$ 150 mm)

Waters Breeze HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium
acetate pH 7.4; B, A /
acetonitrile, 1: 4, v/v) at 35°C,
flow rate 1.5 mL/min; events:
5 min A 100%, 20 min B
0–100%, 5 min B 100% and 10
min A 100%.

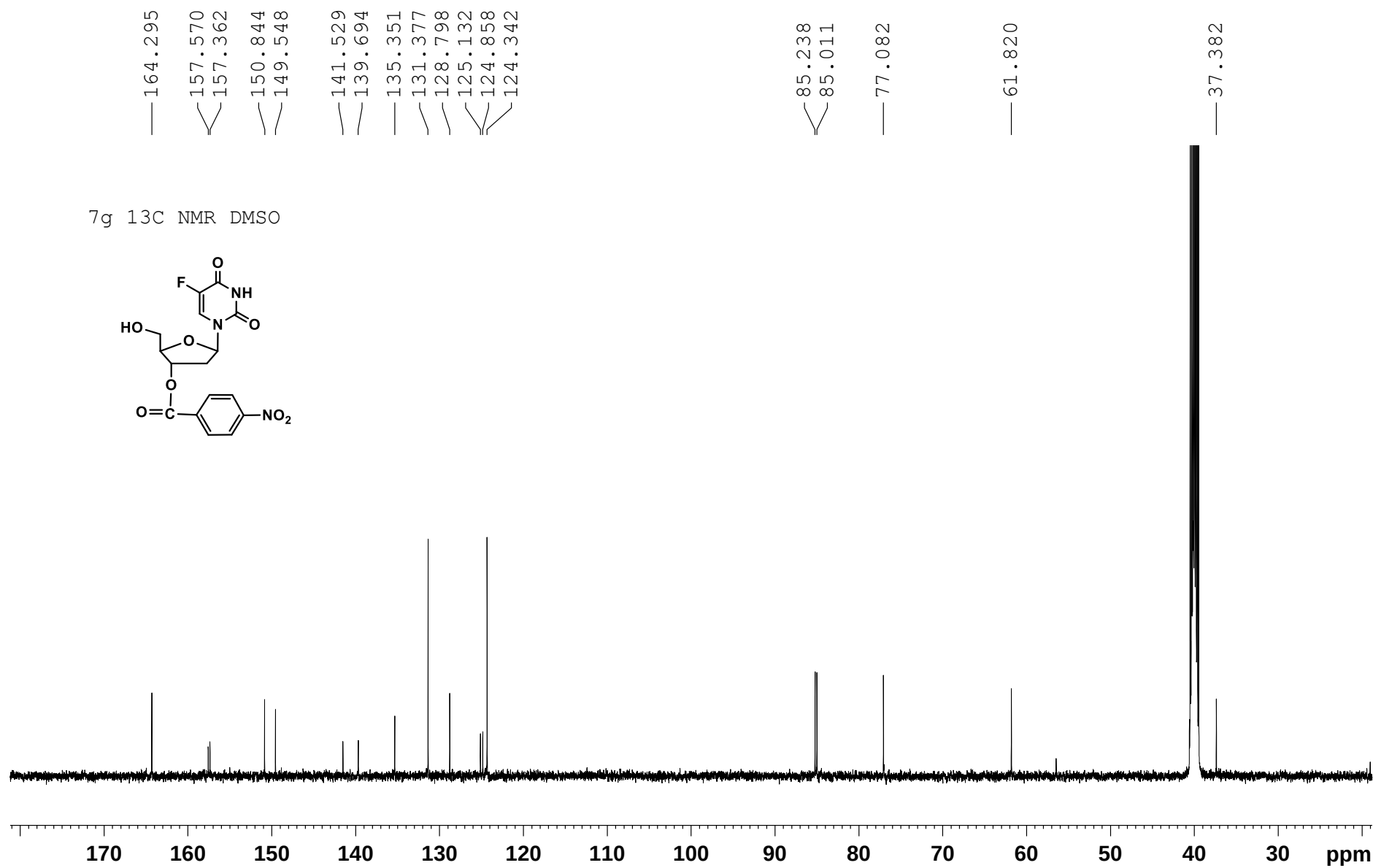


7g ¹H NMR DMSO



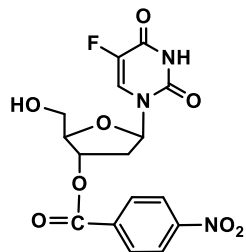


7g ¹³C NMR DMSO



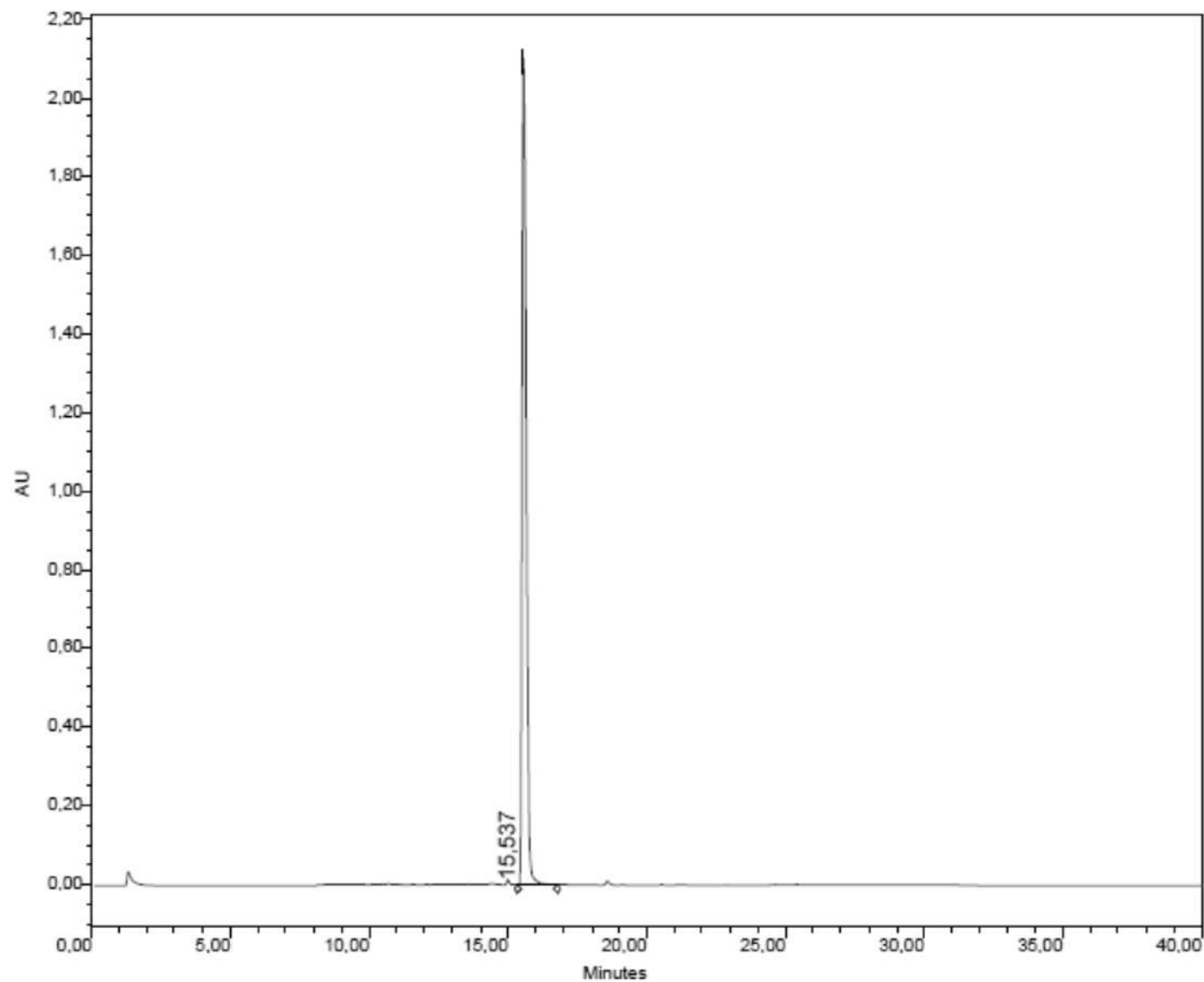
Chromatogram

7g

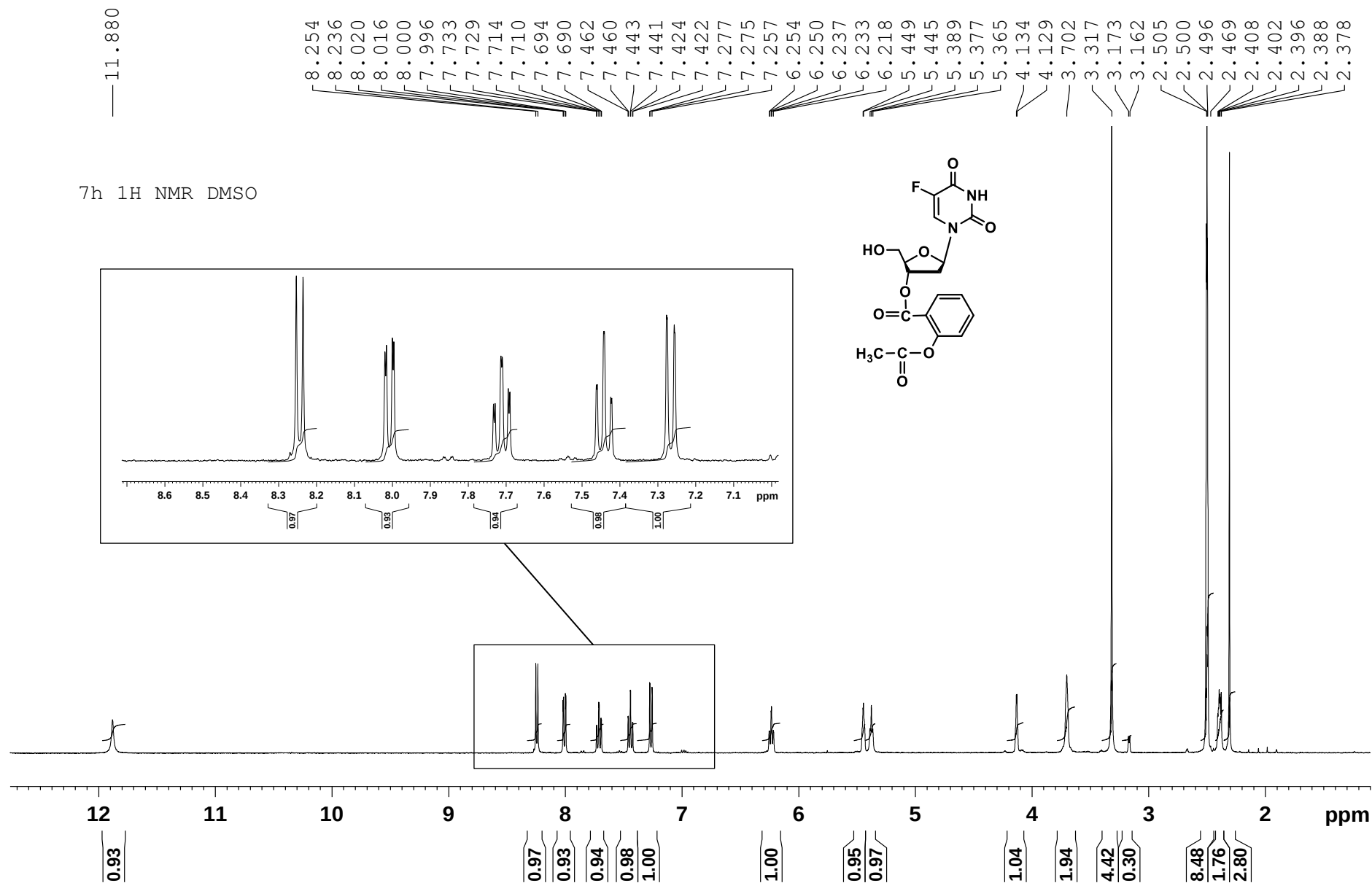


Nucleosil 100-5C18
(5.0 μm , 4.6 mm $\times$ 150 mm)

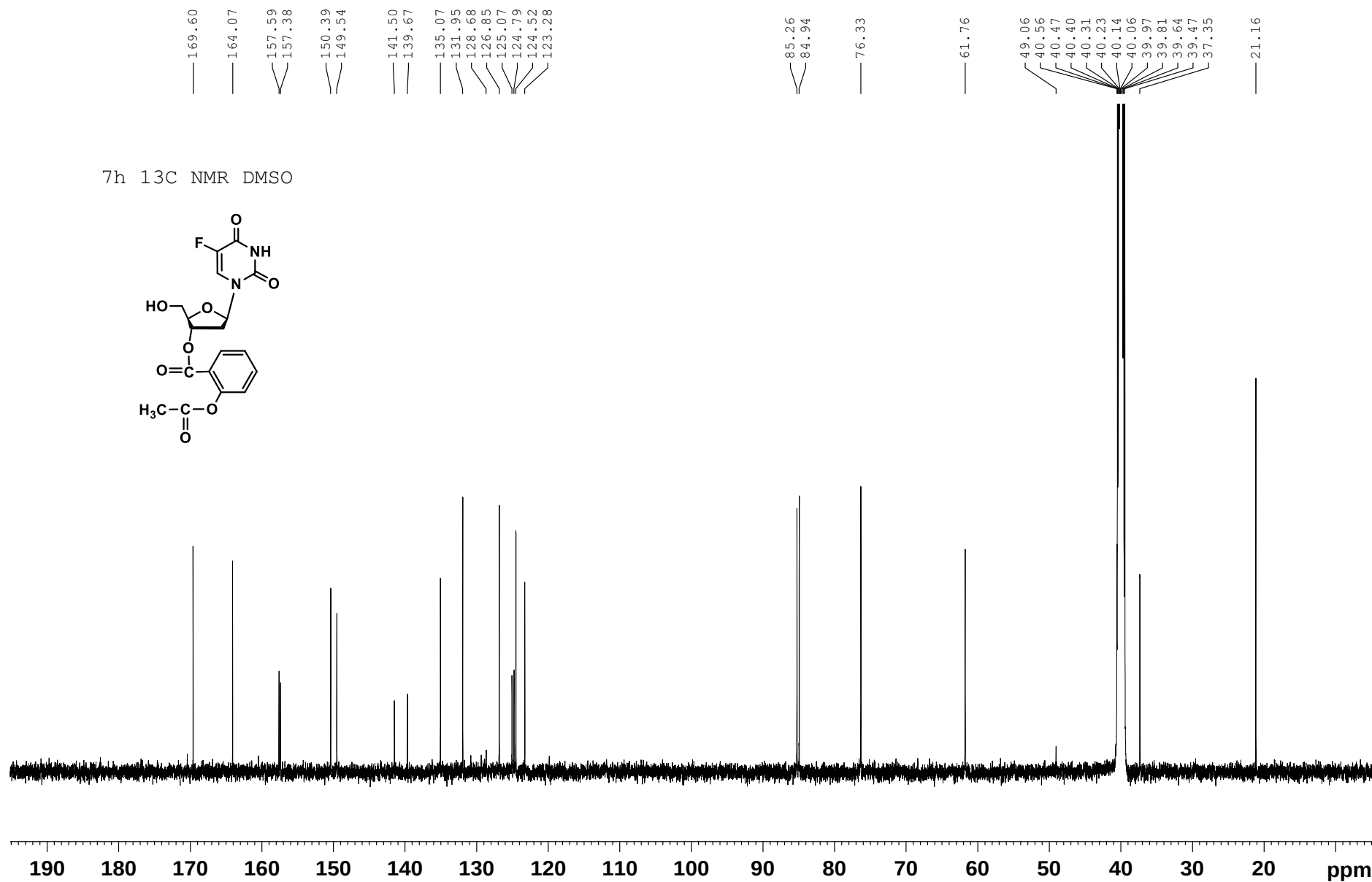
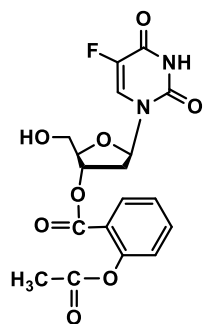
Waters Breeze HPLC systems
with A + B solvent (A, 0.01
M aqueous triethylammonium
acetate pH 7.4; B, A /
acetonitrile, 1: 4, v/v) at
35°C, flow rate 1.5 mL/min;
events: 5 min A 100%, 20
min B 0–100%, 5 min B
100% and 10 min A 100%.



7h ¹H NMR DMSO

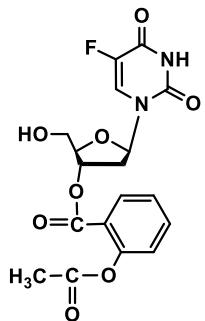


7h ¹³C NMR DMSO



Chromatogram

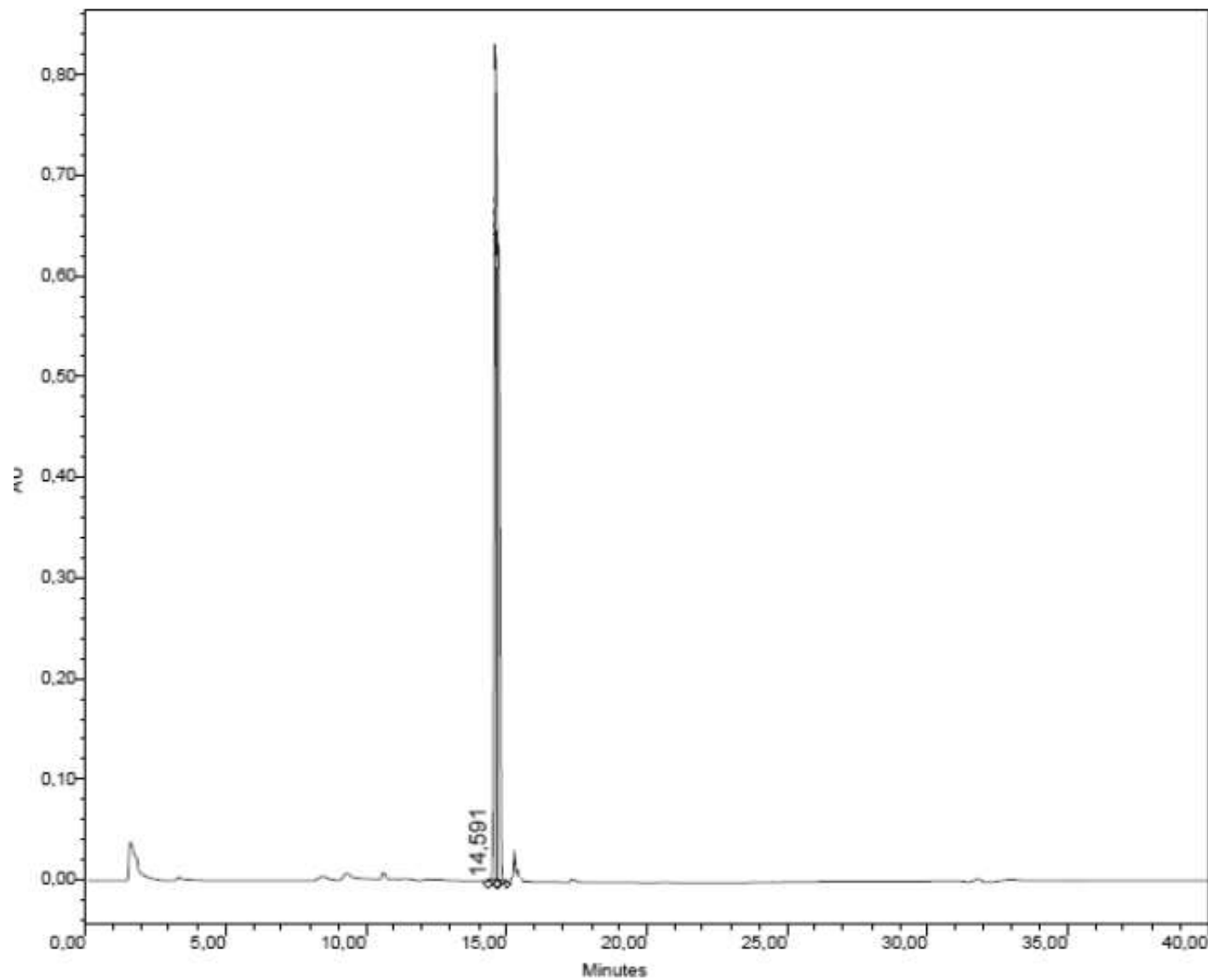
7h



Nucleosil 100-5C18

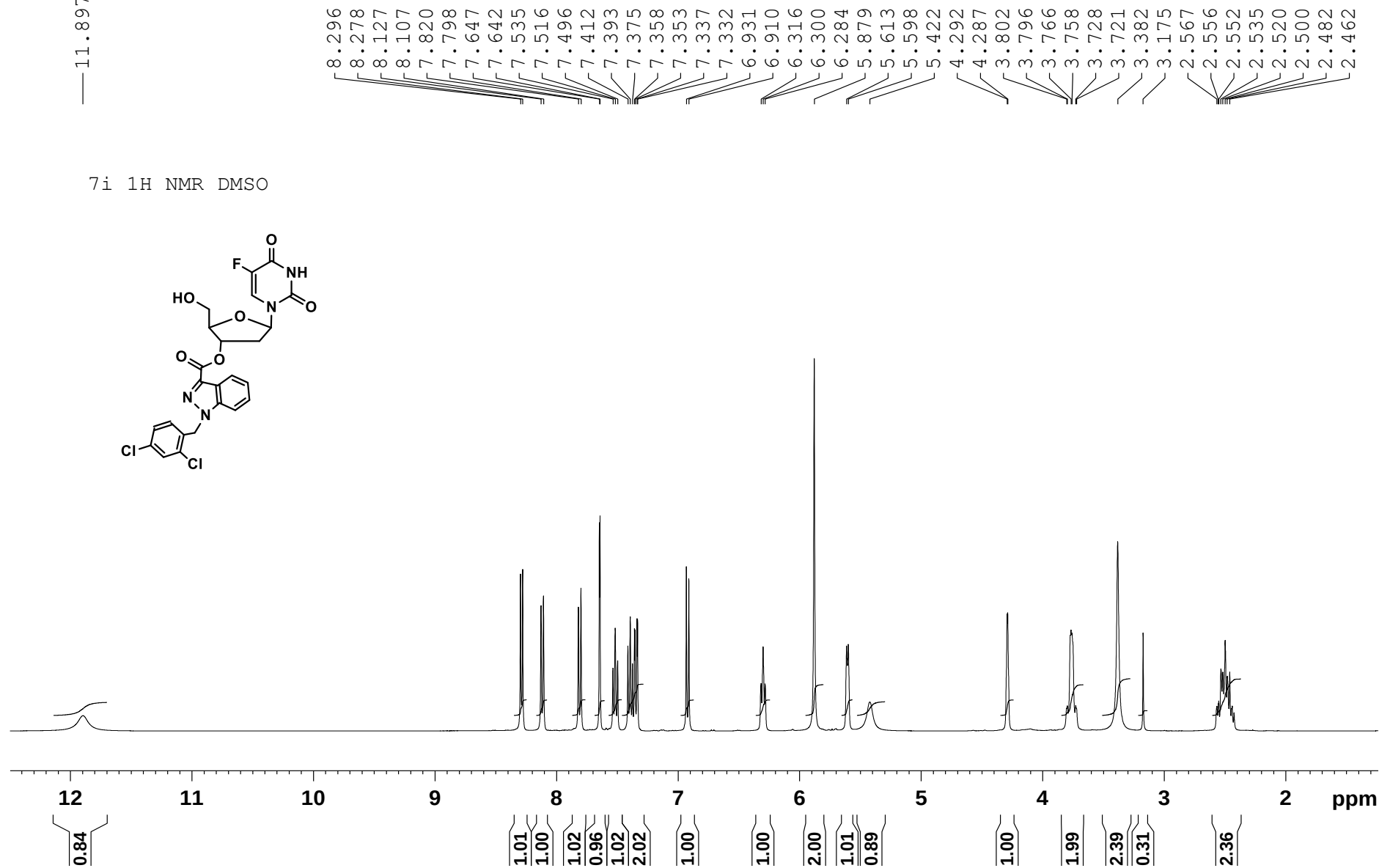
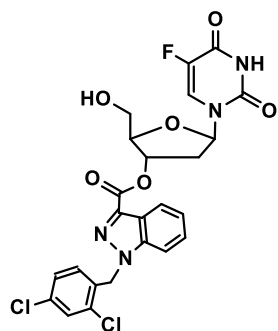
(5.0 µm, 4.6 mm × 150 mm)

Waters Breeze HPLC systems with A + B solvent (A, 0.01 M aqueous triethylammonium acetate pH 7.4; B, A / acetonitrile, 1: 4, v/v) at 35°C, flow rate 1.5 mL/min; events: 5 min A 100%, 20 min B 0–100%, 5 min B 100% and 10 min A 100%.



—11.897

7i 1H NMR DMSO



— 161.127
 < 157.154
 < 156.894
 — 149.097
 < 141.301
 < 140.892
 < 139.006
 < 134.499
 < 133.467
 < 133.192
 < 133.002
 < 130.763
 — 129.076
 < 127.764
 < 127.353
 < 124.693
 < 124.350
 < 123.684
 < 122.891
 < 121.369
 < 110.855

< 84.941
 < 84.582

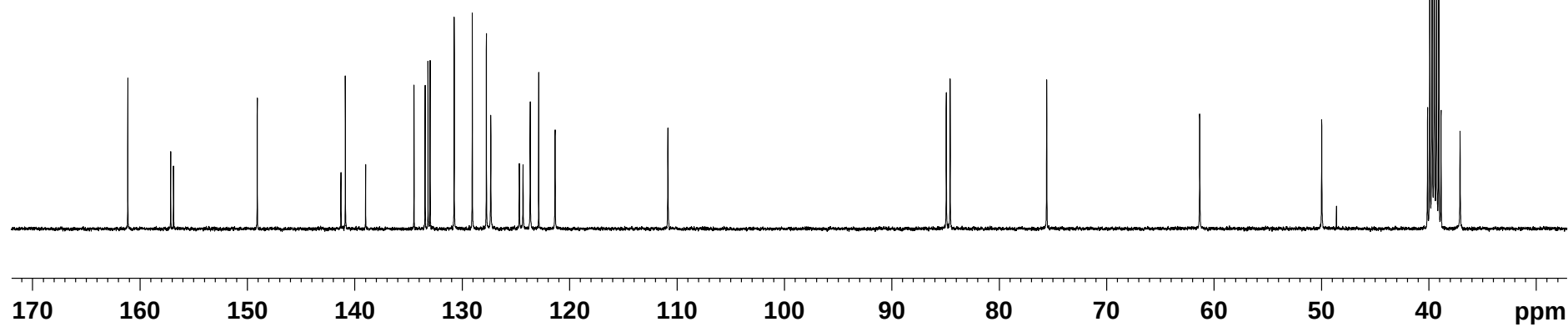
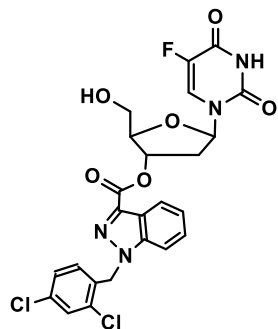
— 75.592

— 61.343

< 49.983
 < 48.614

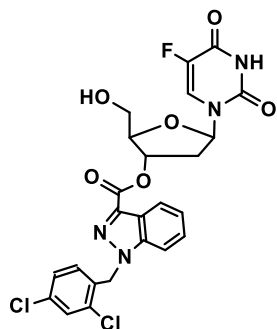
— 39.501
 — 37.100

7i ¹³C NMR DMSO



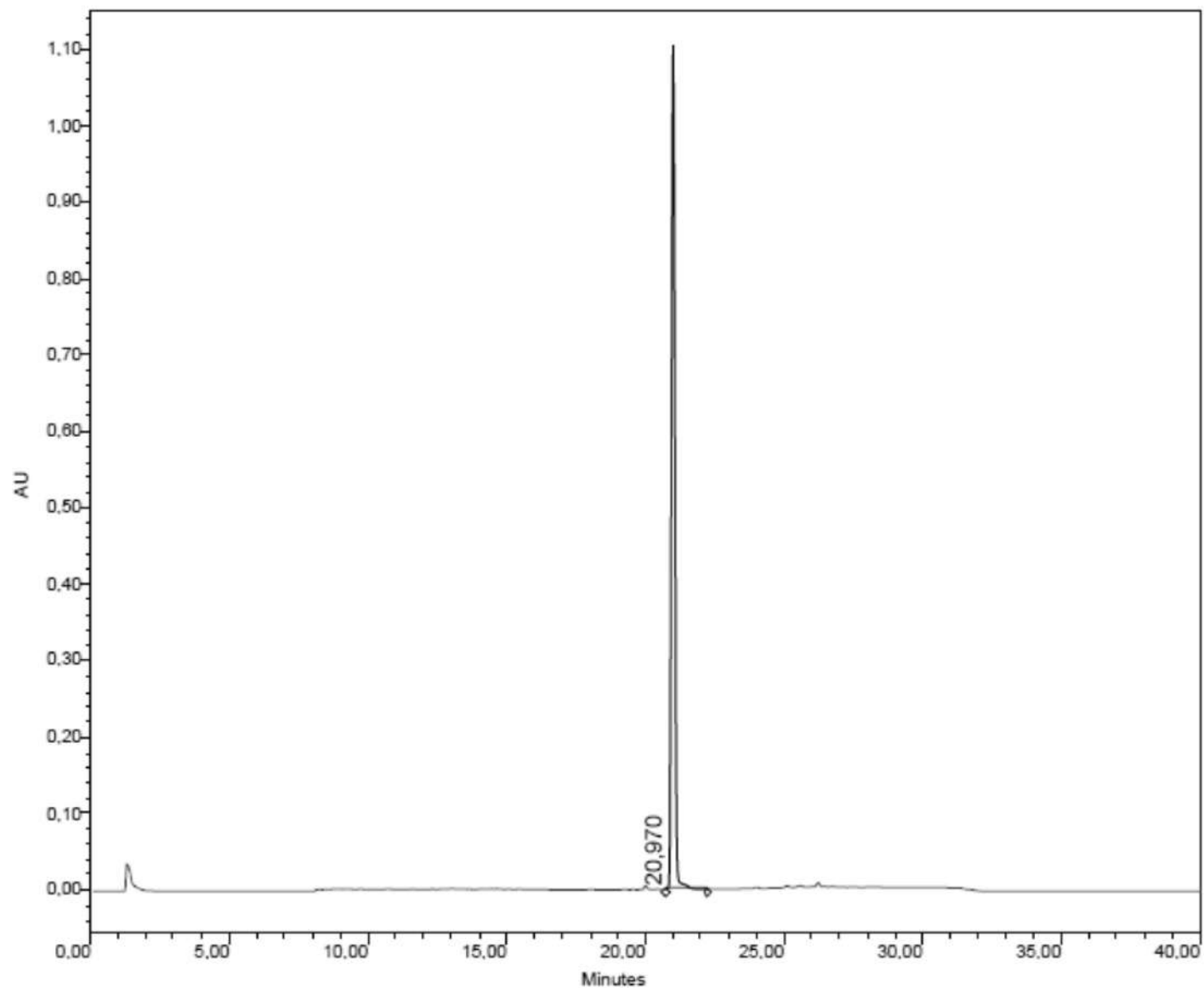
Chromatogram

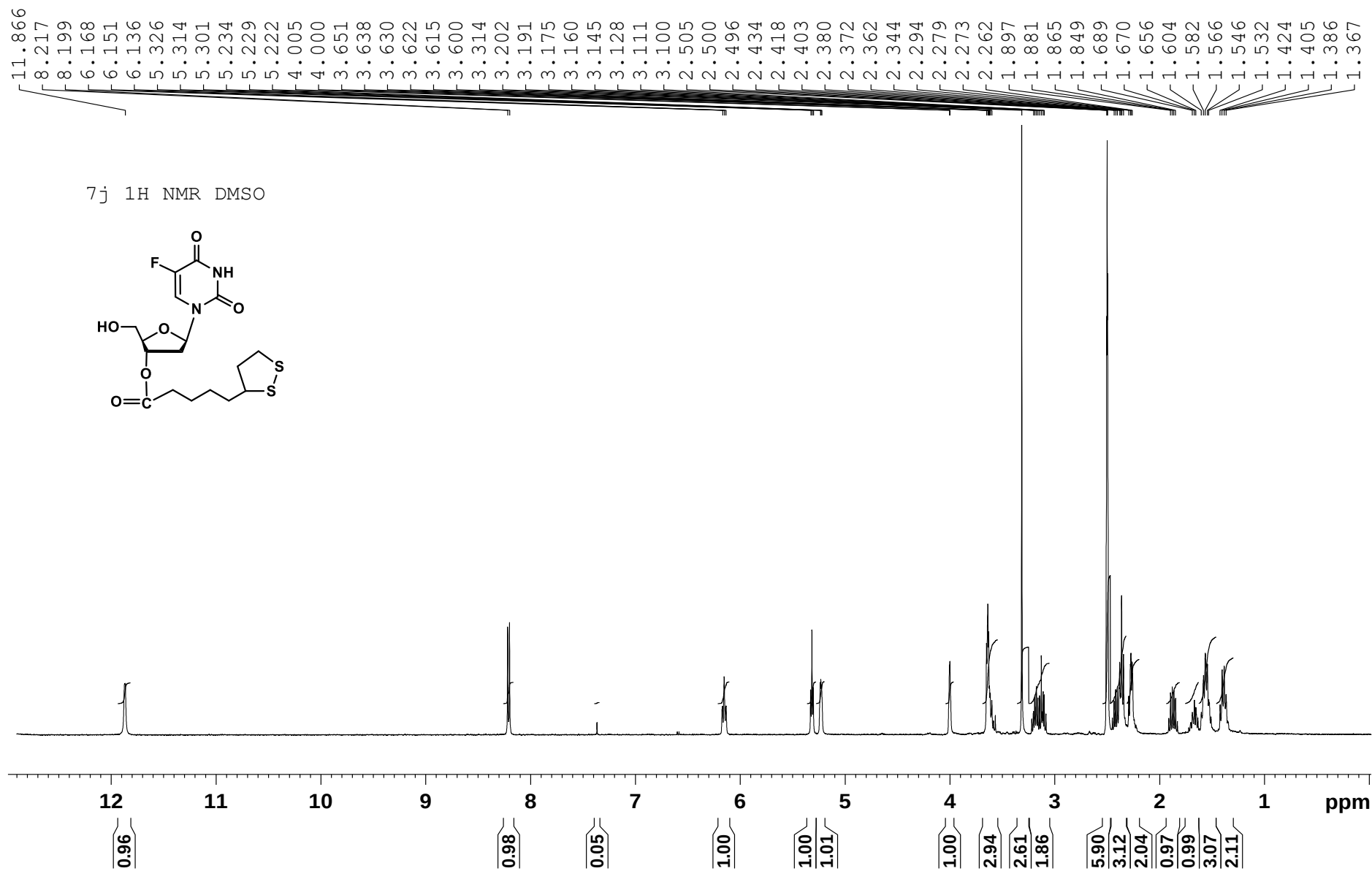
7i

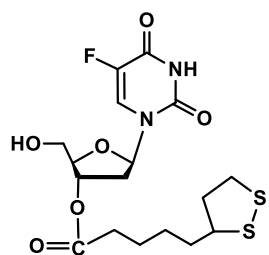


Nucleosil 100-5C18
(5.0 μm , 4.6 mm $\times$ 150 mm)

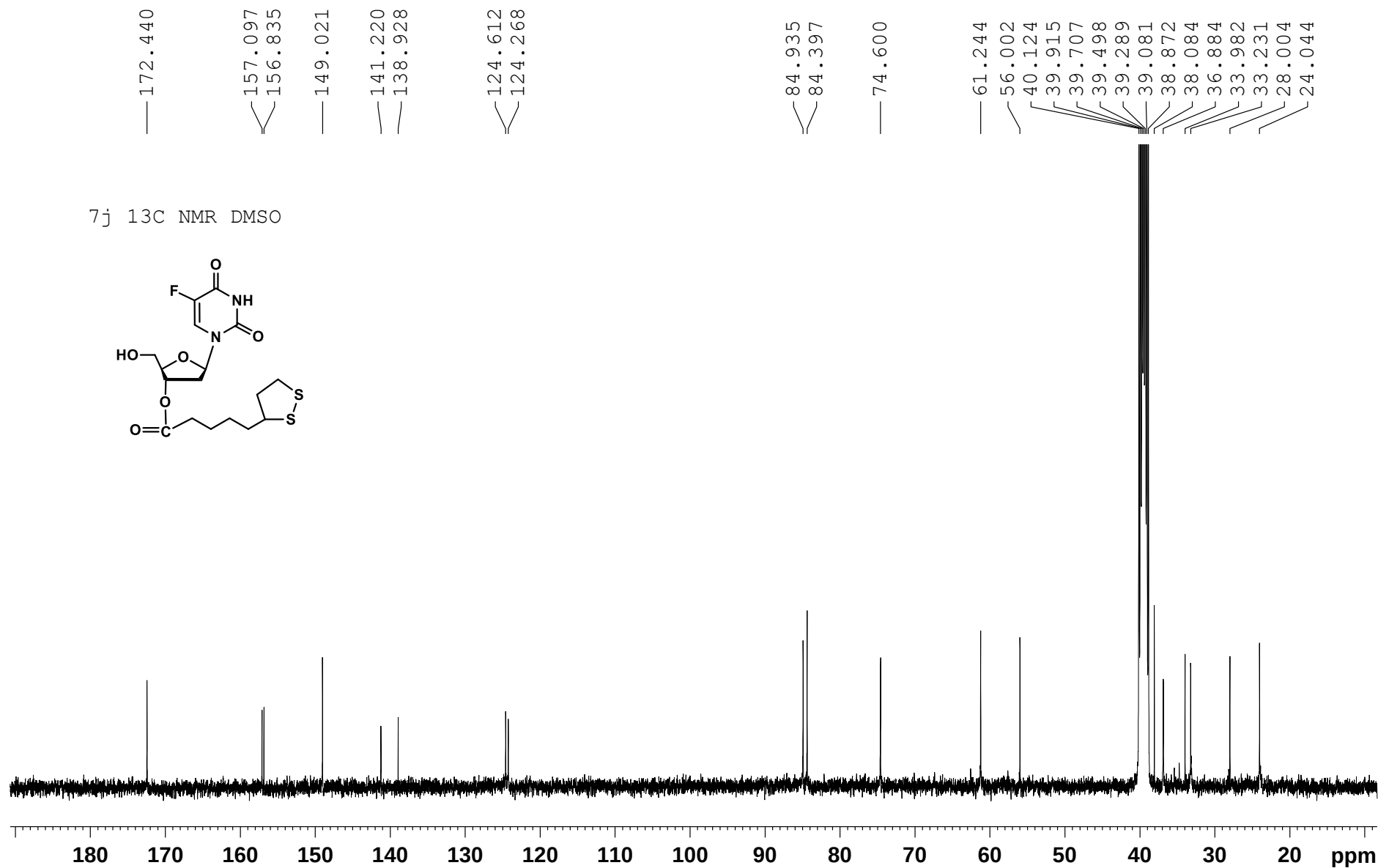
Waters Breeze HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium
acetate pH 7.4; B, A /
acetonitrile, 1: 4, v/v) at 35°C,
flow rate 1.5 mL/min; events:
5 min A 100%, 20 min B
0–100%, 5 min B 100% and 10
min A 100%.





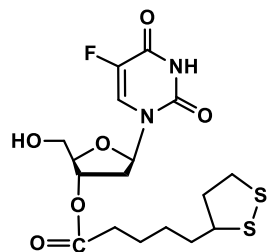


7j ¹³C NMR DMSO



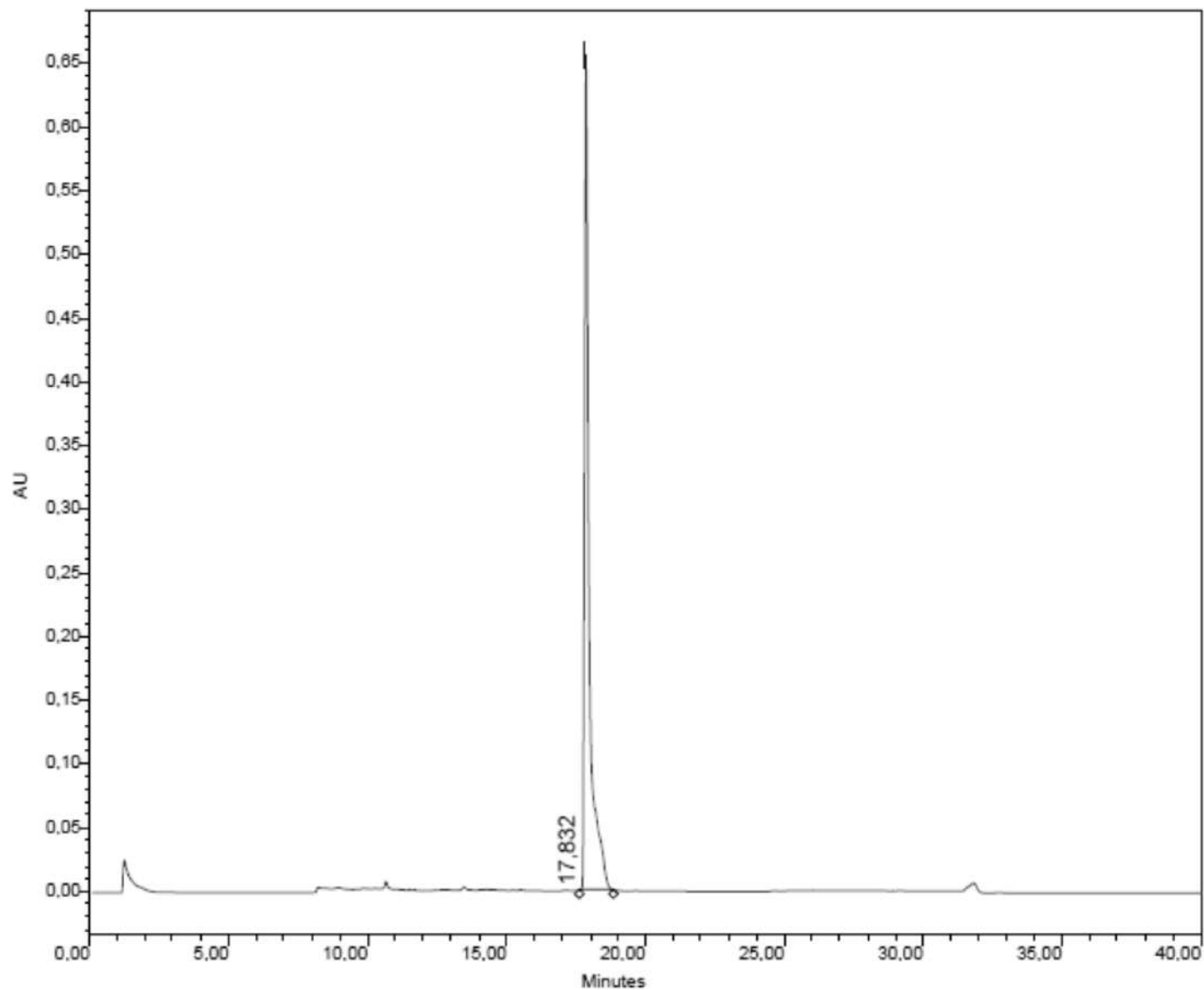
Chromatogram

7j

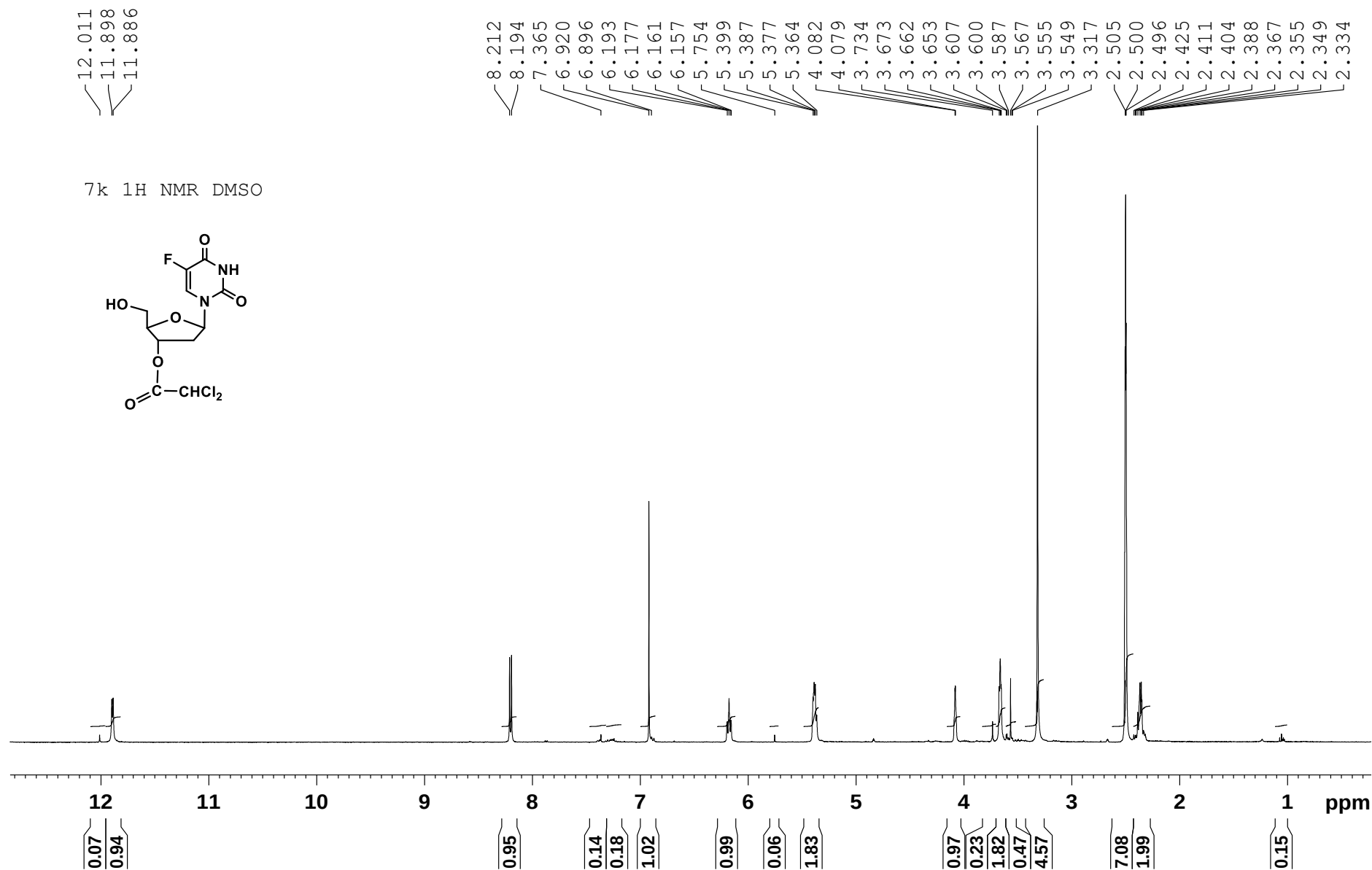
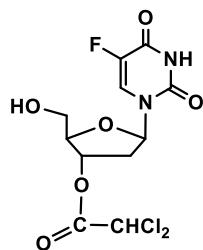


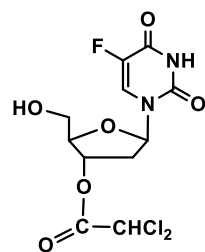
Nucleosil 100-5C18
(5.0 µm, 4.6 mm × 150 mm)

Waters Breeze HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium
acetate pH 7.4; B, A /
acetonitrile, 1: 4, v/v) at 35°C,
flow rate 1.5 mL/min; events:
min A 100%, 20 min B 0–100%,
5 min B 100% and 10 min A
100%.

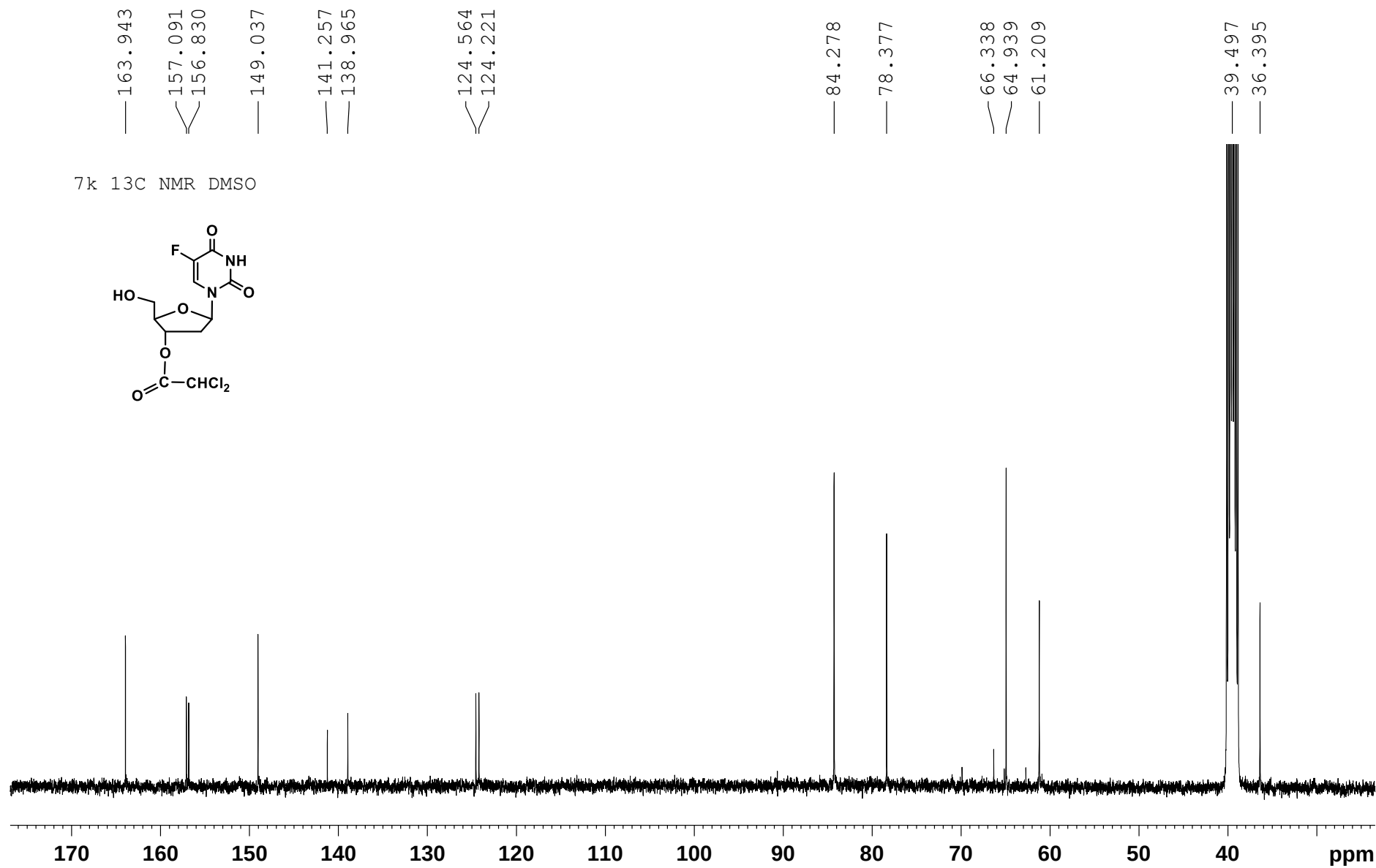


7k ¹H NMR DMSO



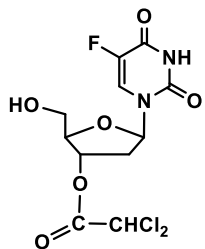


7k ¹³C NMR DMSO



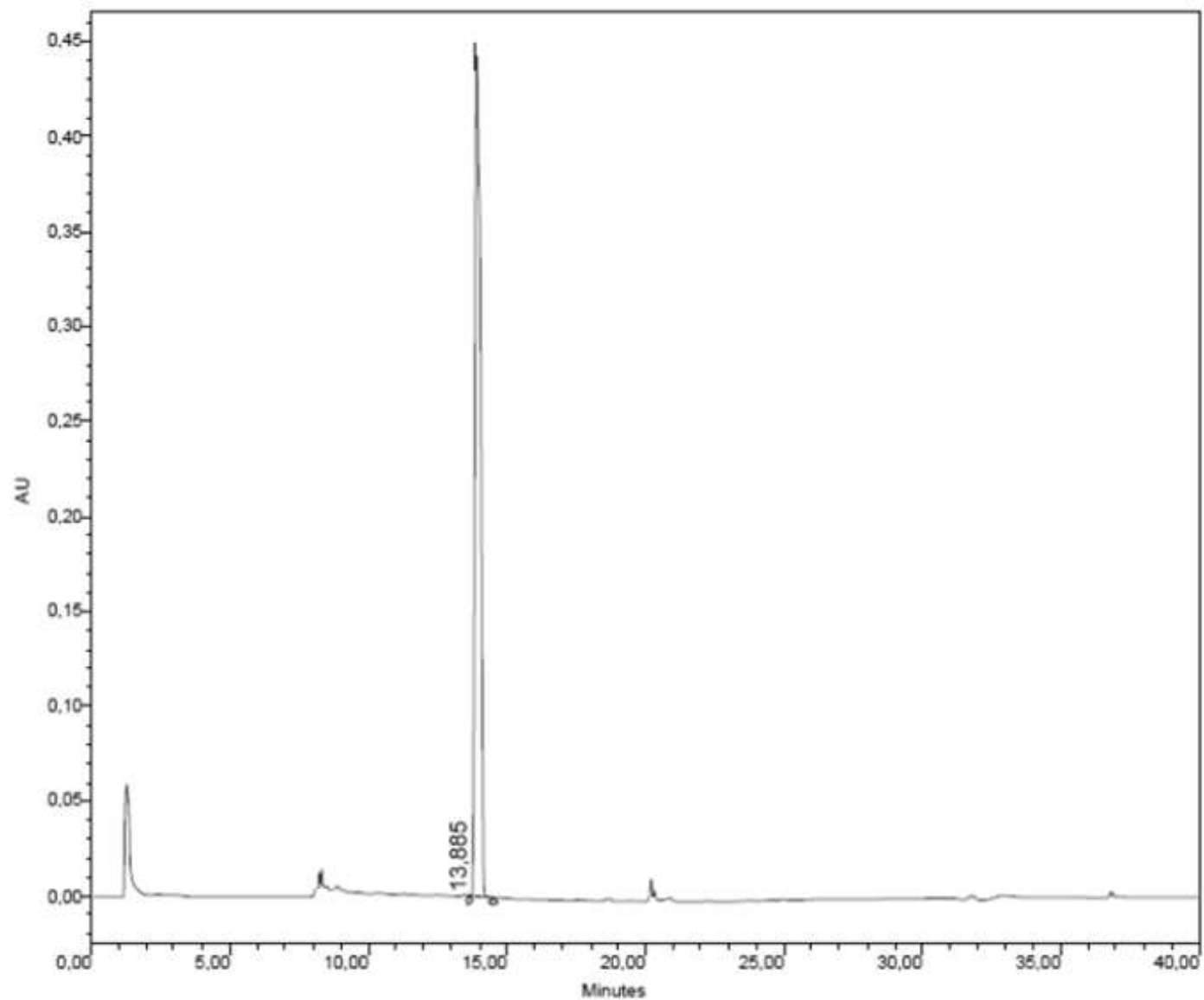
Chromatogram

7k



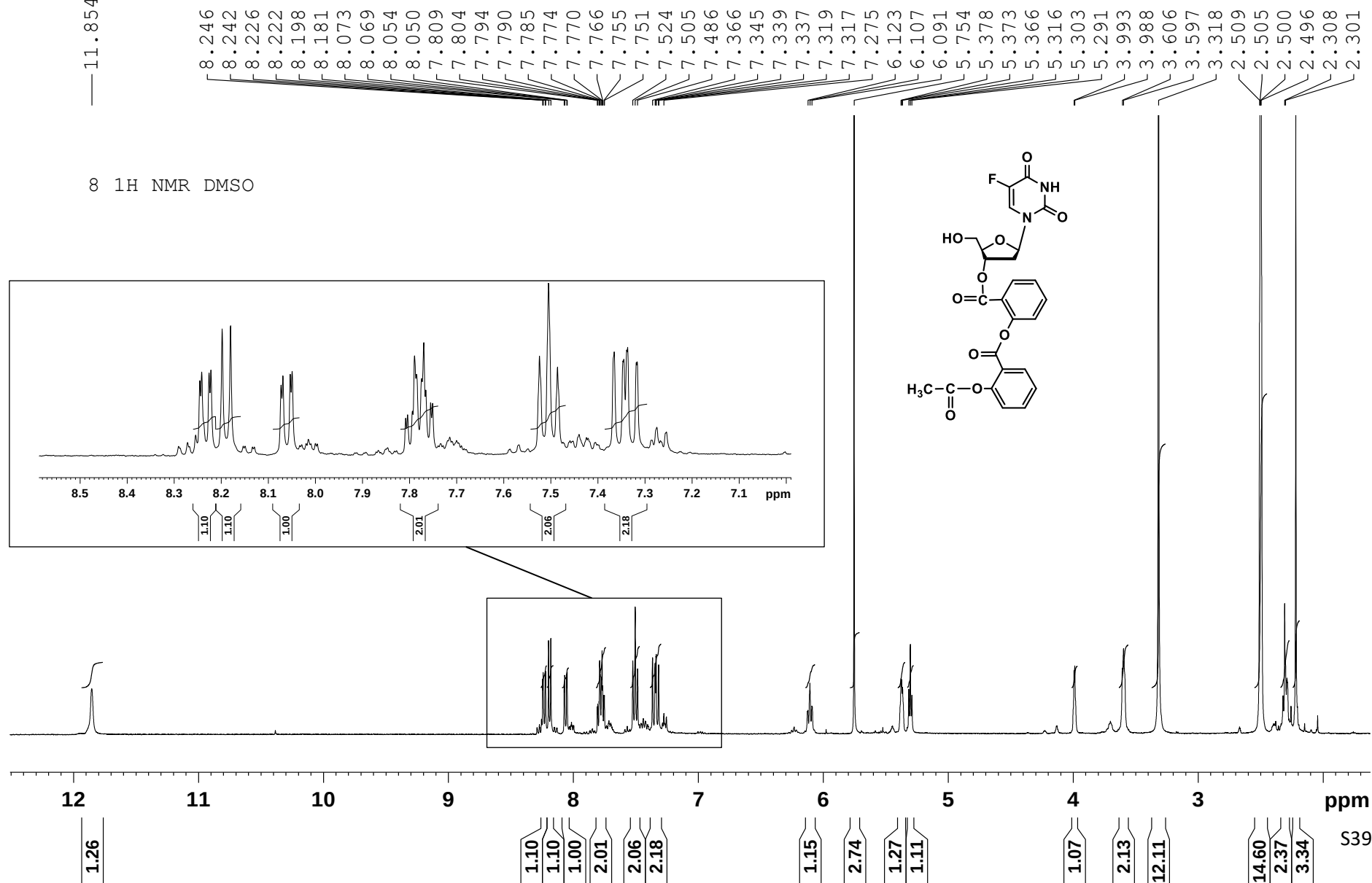
Nucleosil 100-5C18
(5.0 μm , 4.6 mm $\times$ 150 mm)

Waters Breeze HPLC systems with A + B solvent (A, 0.01 M aqueous triethylammonium acetate pH 7.4; B, A / acetonitrile, 1: 4, v/v) at 35°C, flow rate 1.5 mL/min; events: 5 min A 100%, 20 min B 0–100%, 5 min B 100% and 10 min A 100%.

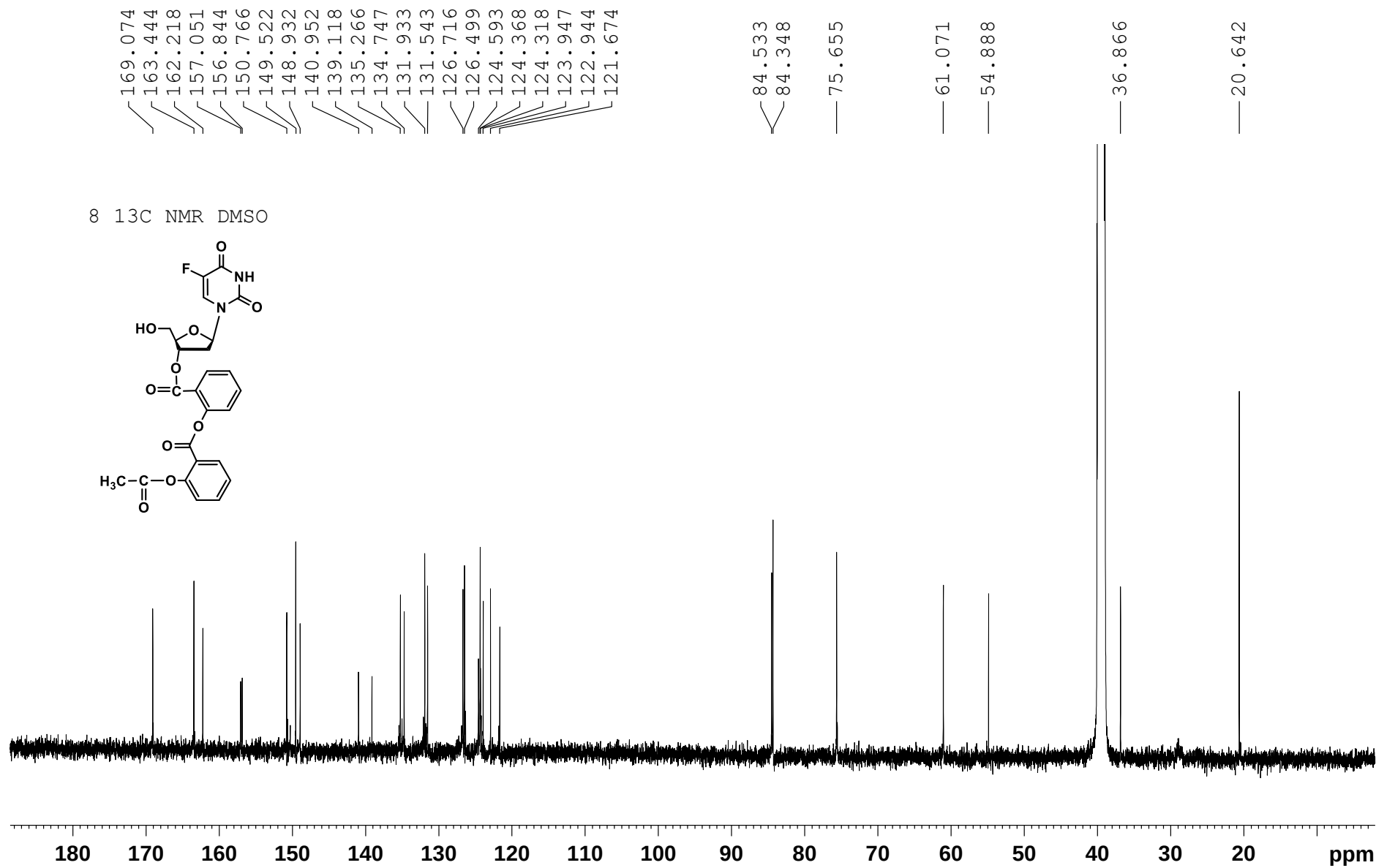
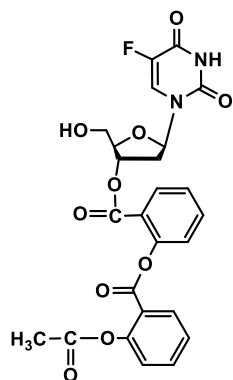


—11.854

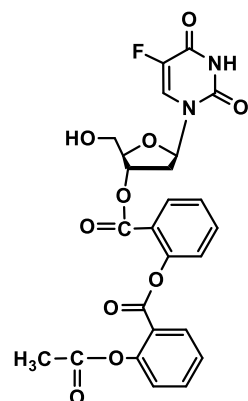
8 ¹H NMR DMSO



8 ¹³C NMR DMSO

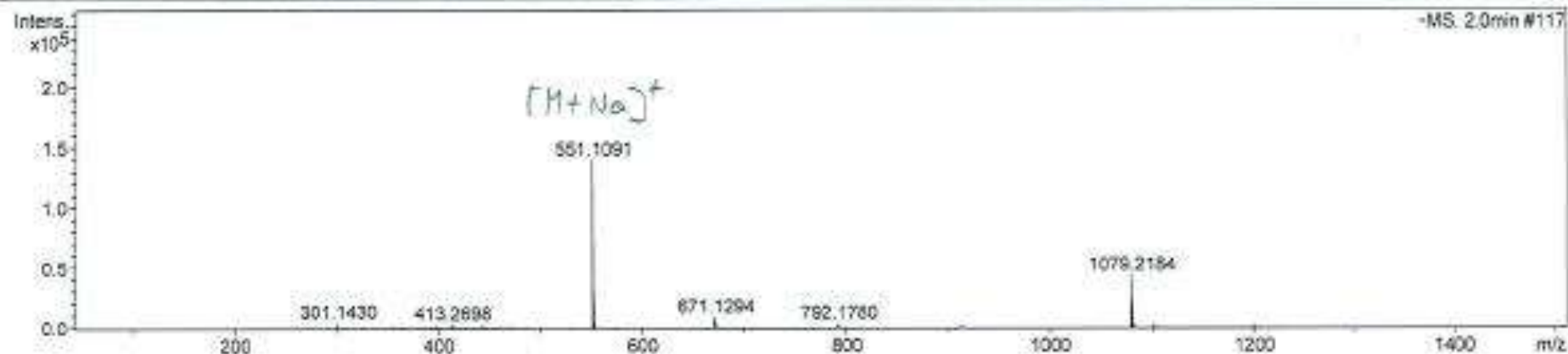


Mass Spectrum 8

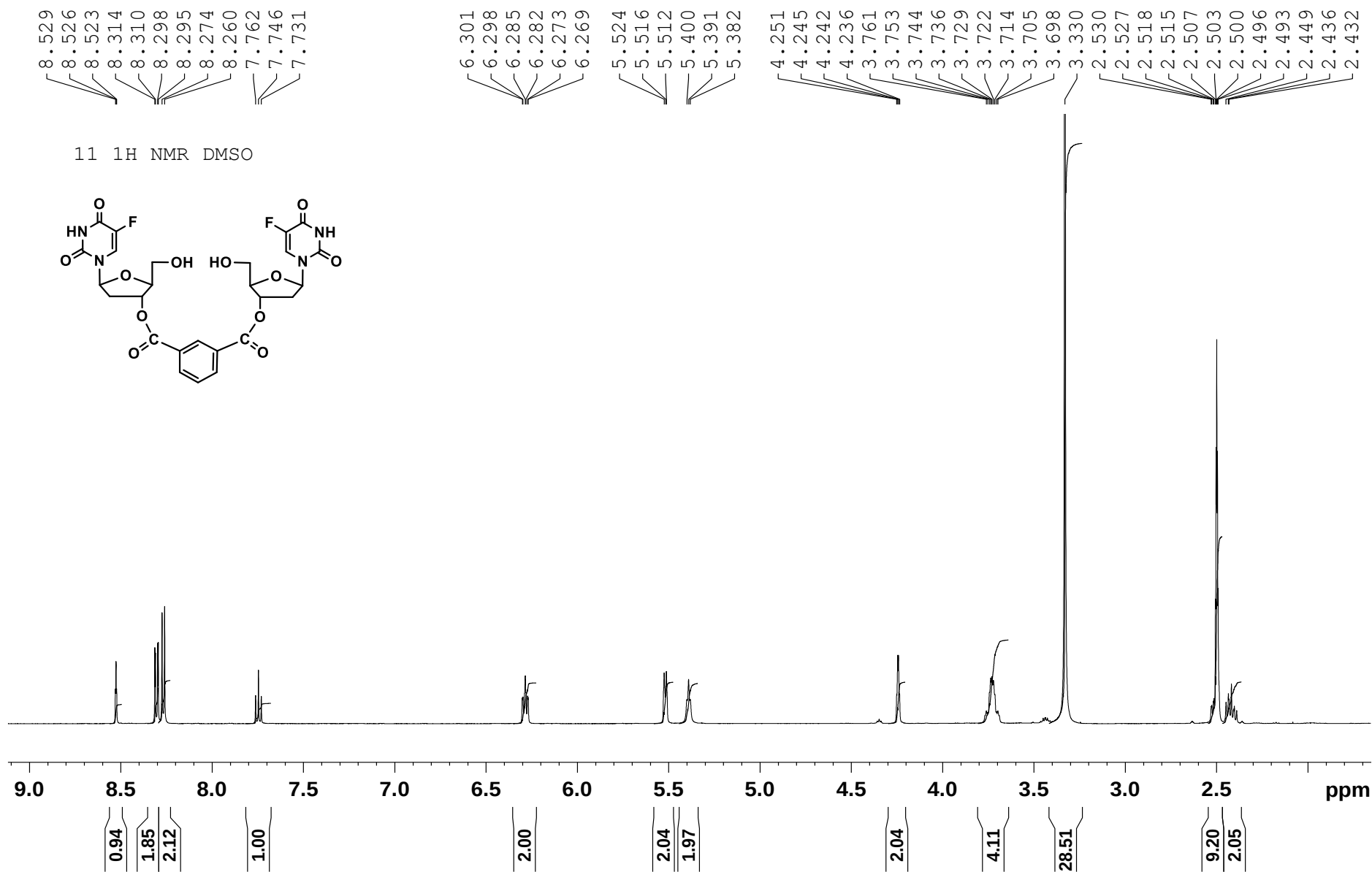


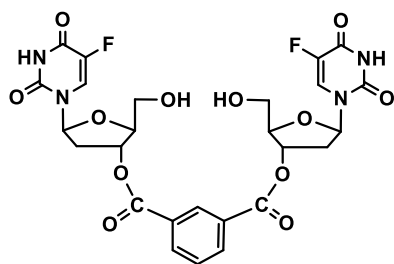
Acquisition Parameter

Source Type	Nano ESI Online	Ion Polarity	Positive	Set Nebulizer	2.0 Bar
Focus	Not active	Set Capillary	4000 V	Set Dry Heater	240 °C
Scan Begin	50 m/z	Set End Plate Offset	0 V	Set Dry Gas	3.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	120.0 Vpp	Set Divert Valve	Waste

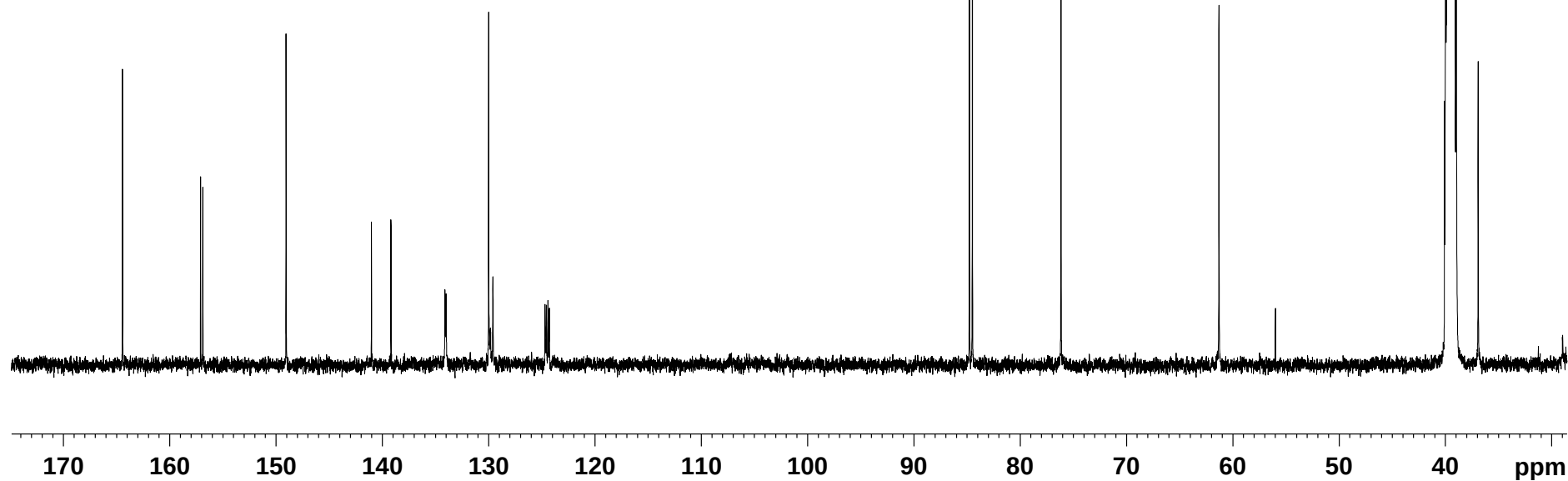


Meas. m/z	#	Formula	m/z	err [ppm]	Mean err [ppm]	rdB	N-R	e ⁻	mSig	Std I	Std Mea n m/z	Std I VarNo rm	Std m/z Diff	Std Comb Dev
551.1091	1	C 25 H 21 F N 2 Na O 10	551.1072	-3.4	-2.6	15.5	ok	even	24.8	51.5	1.7	18.0	2.1	842.7





11 ¹³C NMR DMSO



— 164.458
 < 157.111
 < 156.903
 — 149.070
 < 141.036
 — 139.201
 < 134.132
 < 134.010
 < 130.018
 < 129.615
 < 124.714
 < 124.575
 < 124.438
 < 124.303

< 84.784
 < 84.514

— 76.172

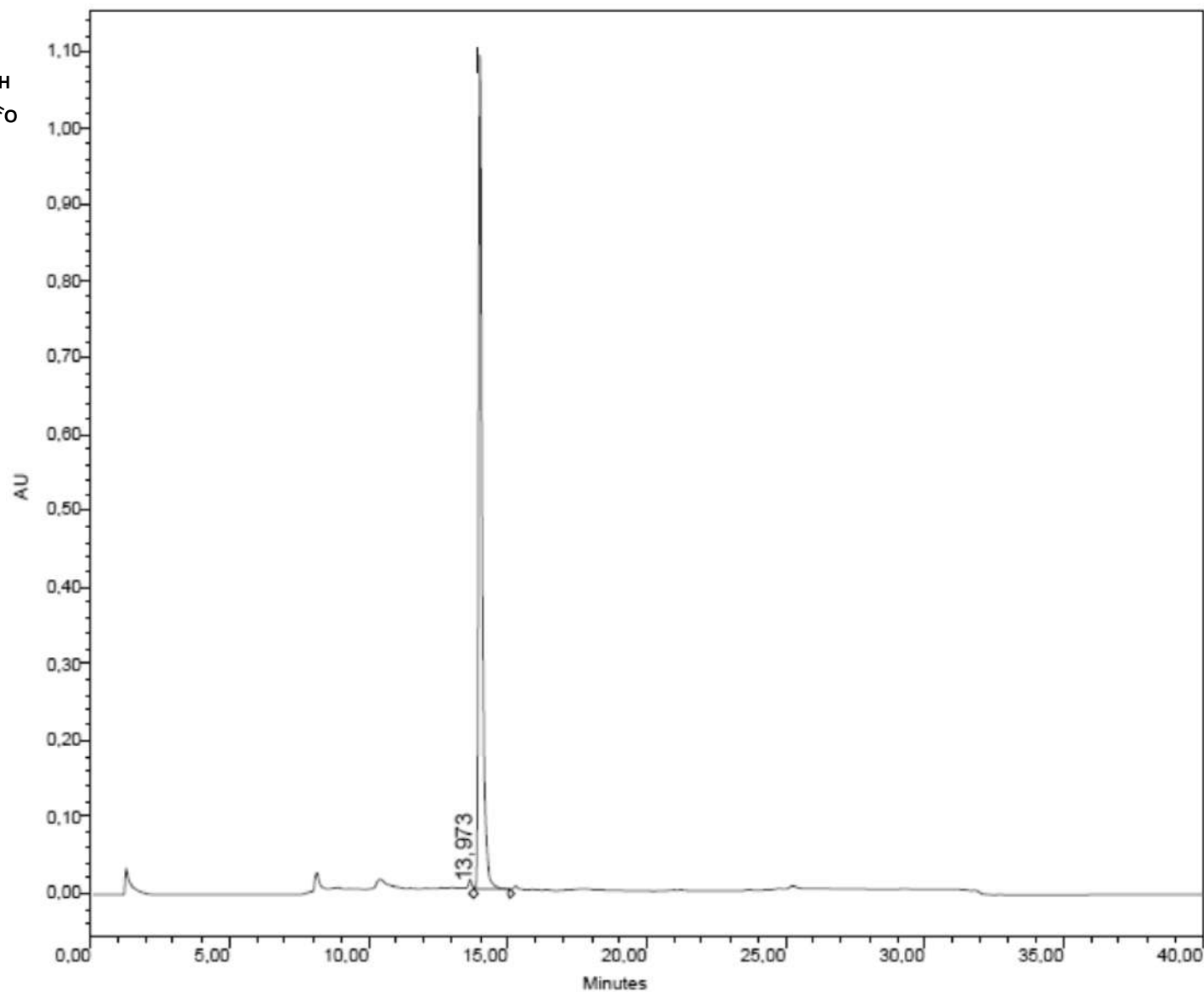
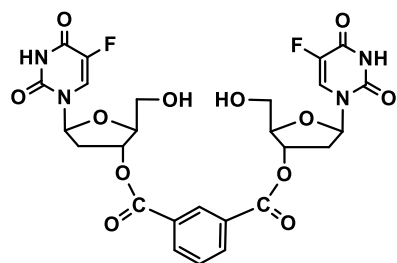
— 61.319

— 56.003

< 39.664
 < 39.497
 < 39.330
 < 36.930

Chromatogram

11



Nucleosil 100-5C18
(5.0 μm , 4.6 mm $\times$ 150 mm)

Waters Breeze HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium
acetate pH 7.4; B, A /
acetonitrile, 1: 4, v/v) at 35°C,
flow rate 1.5 mL/min; events:
5 min A 100%, 20 min B
0–100%, 5 min B 100% and 10
min A 100%.

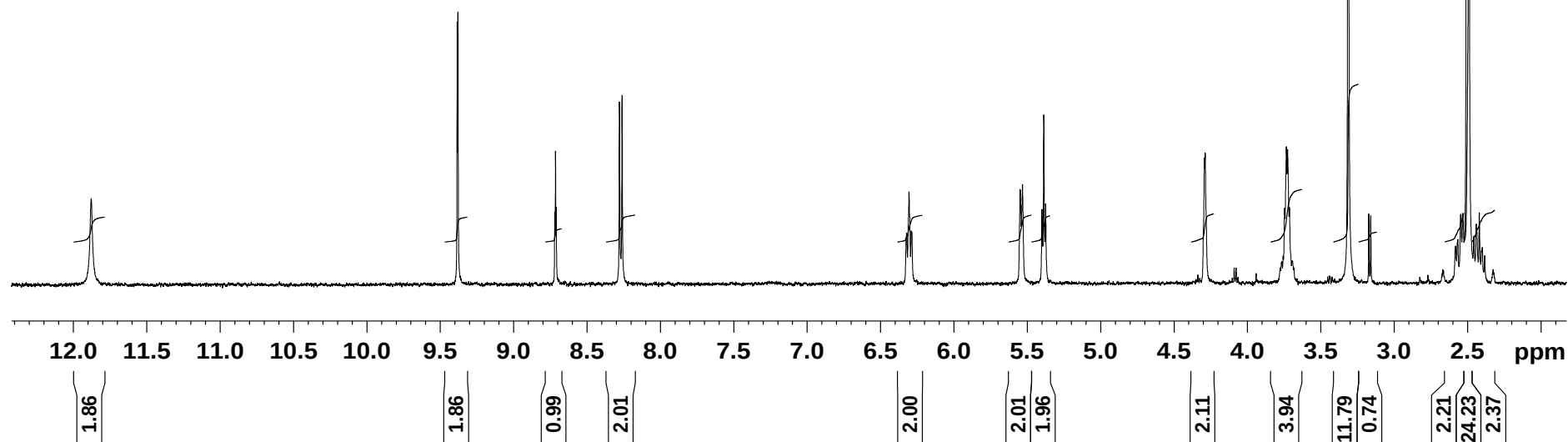
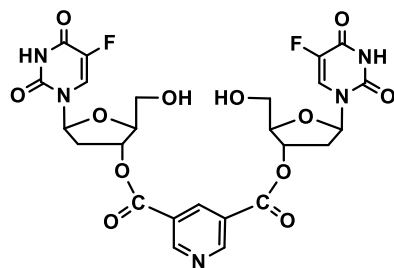
—11.879

9.386
9.381

8.721
8.716
8.711
8.280
8.262

6.323
6.307
6.291
5.549
5.534
5.401
5.388
5.376
4.294
4.289
3.757
3.748
3.736
3.727
3.714
3.454
3.437
3.424
3.406
3.315
3.174
3.161
2.826
2.772
2.669
2.581

12 ¹H NMR DMSO



— 163.593
 — 157.174
 — 156.965
 — 153.987
 — 149.118

— 141.084
 — 139.250
 — 137.424

— 125.703
 — 124.711
 — 124.436

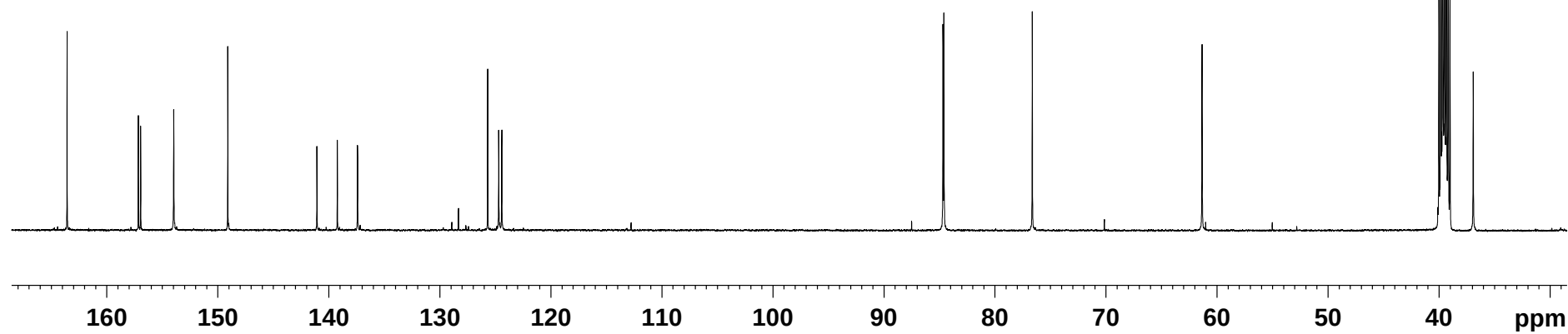
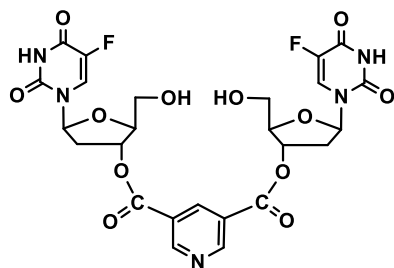
— 84.691
 — 84.603

— 76.649

— 61.352

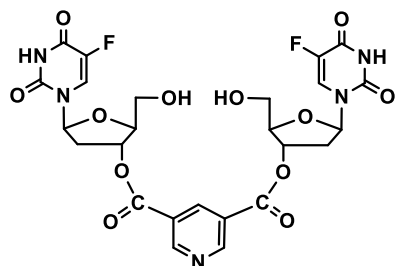
— 39.682
 — 39.515
 — 39.348
 — 36.922

12 ¹³C NMR DMSO



Chromatogram

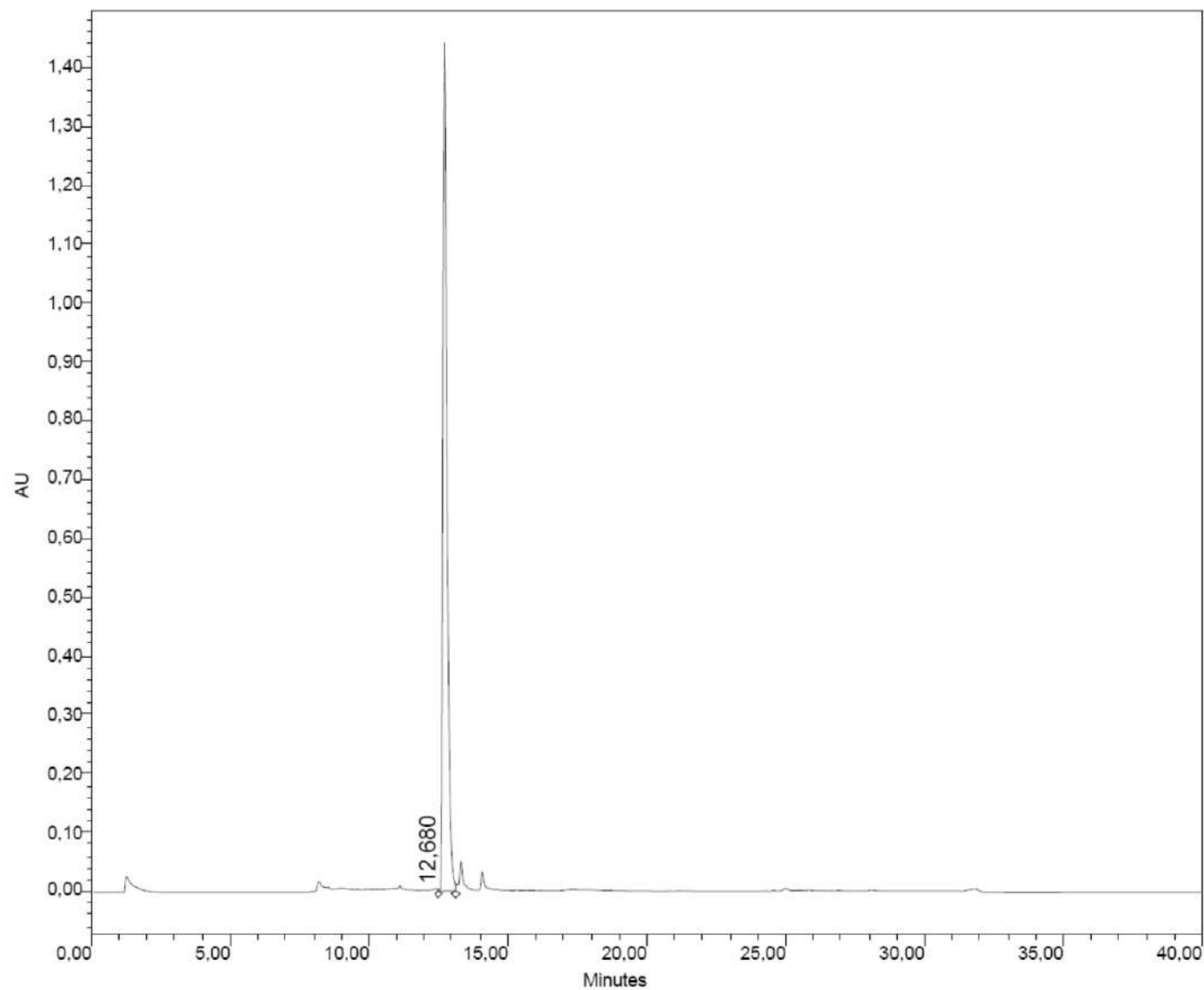
12



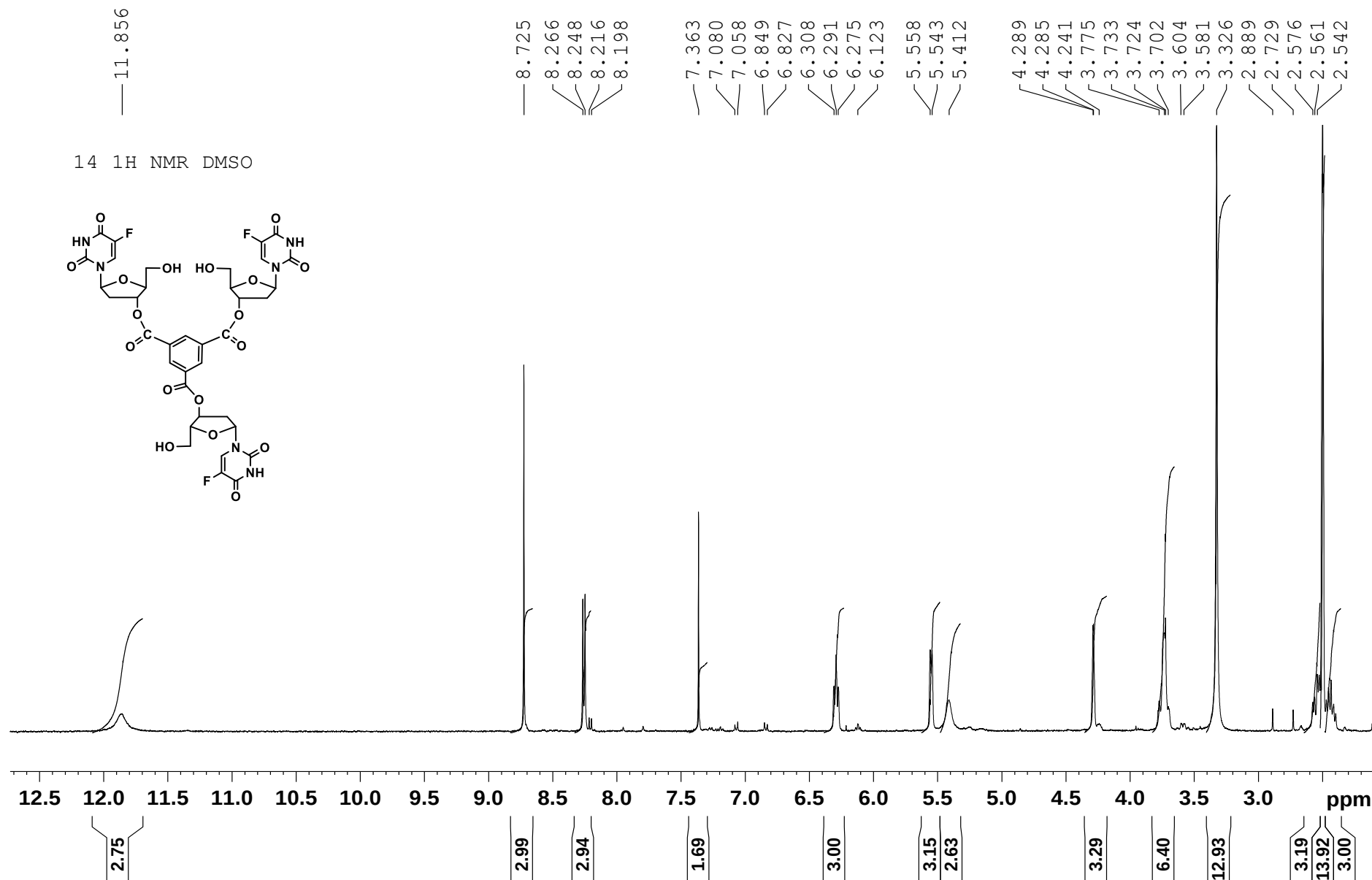
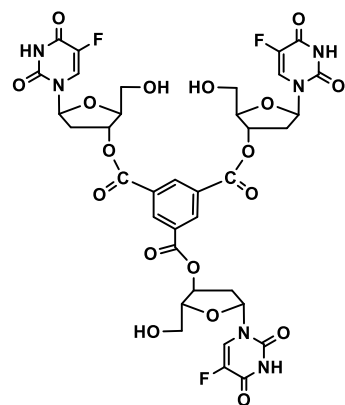
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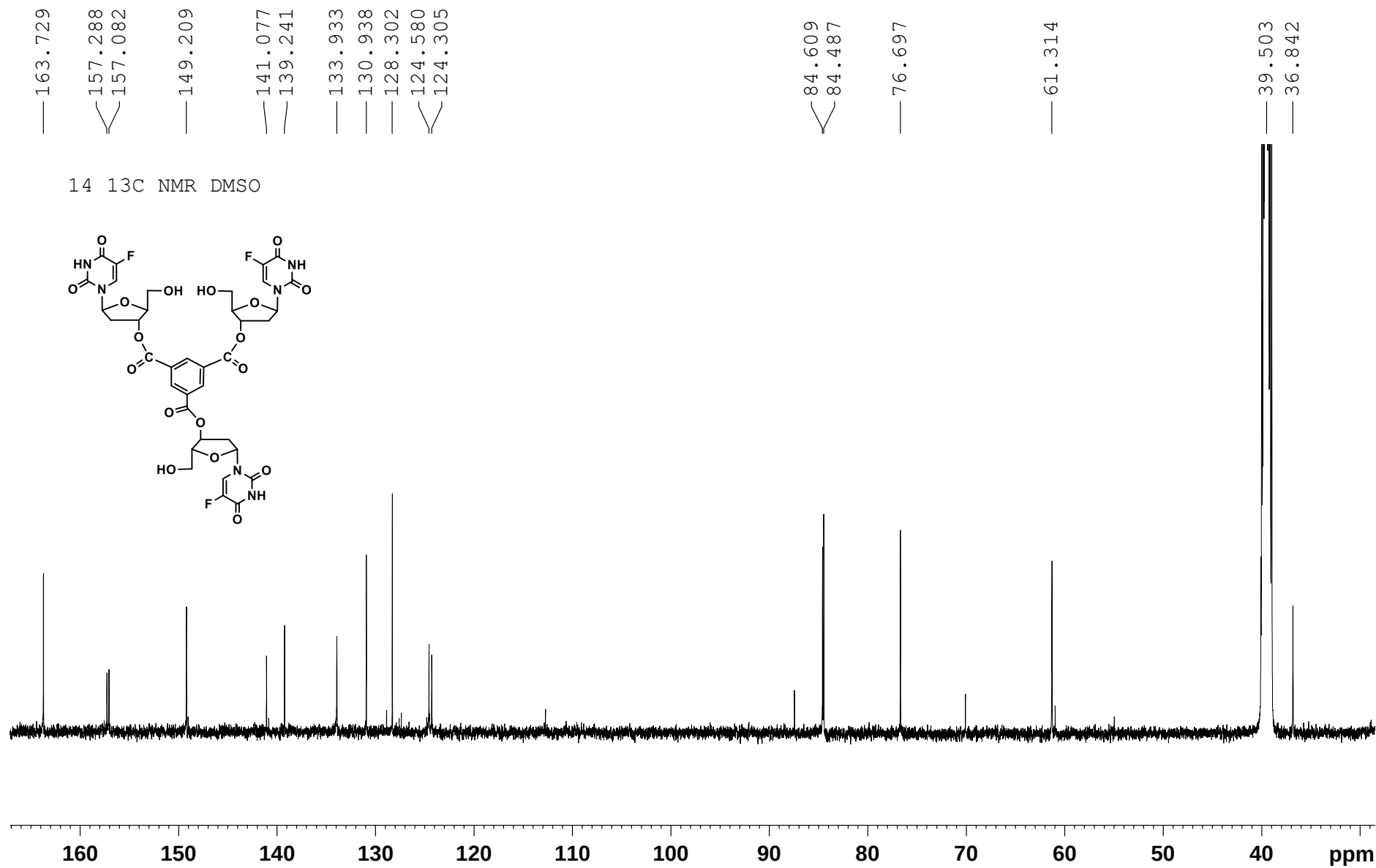
(5.0 μm , 4.6 mm $\times$ 150 mm)

Waters Breeze HPLC systems with A + B solvent (A, 0.01 M aqueous triethylammonium acetate pH 7.4; B, A / acetonitrile, 1: 4, v/v) at 35°C, flow rate 1.5 mL/min; events: 5 min A 100%, 20 min B 0–100%, 5 min B 100% and 10 min A 100%.



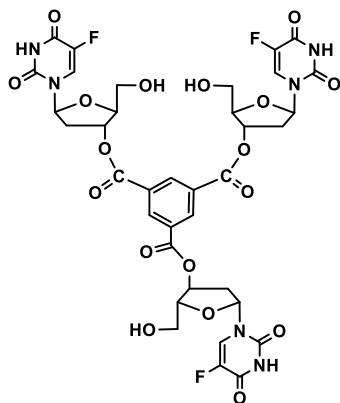
14 ¹H NMR DMSO





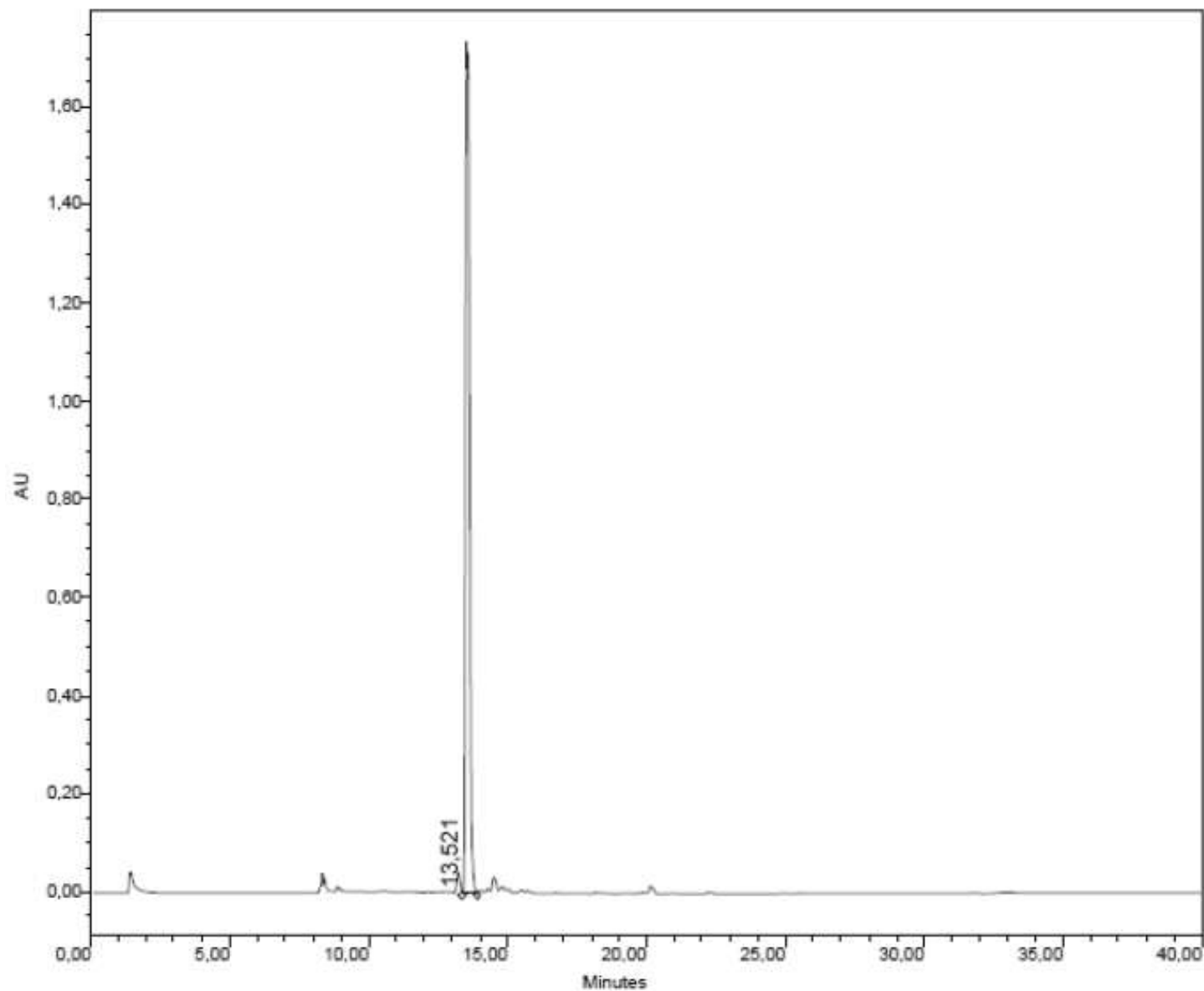
Chromatogram

14



Nucleosil 100-5C18
(5.0 μm , 4.6 mm $\times$ 150 mm)

Waters Breeze HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium
acetate pH 7.4; B, A /
acetonitrile, 1: 4, v/v) at 35°C,
flow rate 1.5 mL/min; events:
5 min A 100%, 20 min B
0–100%, 5 min B 100% and 10
min A 100%.



2

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Research paper

New anti-glioma zwitterionic pronucleotides with an FdUMP framework

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ABSTRACT

We have designed and synthesized new 5-fluoro-2'-deoxyuridine 5'-phosphate pronucleotides which can function as potential agents against the *glioblastoma multiforme* tumor. Their anti-malignant potency has been tested against T98G, U-118 MG, U-87 MG gliomas, HeLa, and Caco-2 cancer cell lines, using MRC-5 healthy cells as a reference. Five of the sixteen compounds (**4c**, **4f-i**) exhibited significant anti-cancer potency and high selectivity indices (SI 12–66). It is likely that these zwitterionic pronucleotides may function in a similar manner to zwitterionic phospholipids, by inducing cell membrane charge disorder, making the cell permeable to bioactive agents. The most promising therapeutic pronucleotides **4c**, **4f-h**, have high intestinal-blood uptake potency (Caco-2 cell line), and may be considered as potential, orally administrated, anticancer drugs.

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1. Introduction

Glioblastoma multiforme (GBM) still remains one of the most lethal types of brain tumor, and medicine is far from finding a solution to overcome this [1]. Contemporary therapies against GBM (surgical resection, radiotherapy and chemotherapy) are hardly successful [1], with five-year survival rates lower than 5% (according to the Central Brain Tumor Registry of the United States, 2012). Therefore, novel efficient therapeutic agents for treatment of GBM are urgently needed. The examples of new approaches to combating glioblastoma encompass application of a sequence-specific RNAi in brain tumor therapy [2,3] and the use of new analogues of 5-fluoro-2'-deoxyuridine 5'-phosphate (FdUMP) that were designed as pronucleotides (vide infra). The choice of FdUMP as a leading compound was based on findings indicating that (i) thymidylate synthetase (TS) is overexpressed in glioblastoma cells [4], and that (ii) FdUMP proves to function as a true and specific inhibitor of this enzyme, with the K_m value of three orders of magnitude lower than that of the natural substrate, dUMP

(2.2×10^{-8} vs 3.7×10^{-5} , respectively) [5]. Since FdUMP, as a dianionic molecule, is not able to penetrate the cellular membrane [6,7], multiple efforts were made to deliver it into the cell in a less charged, masked form, e.g., as uncharged phosphotriesters [8–10], lipophilic phosphodiester [8], dinucleoside phosphates [8], carboxylic-phosphate mixed anhydrides [8], or amino acid phosphoramidates [8,11–20]. Irrespectively of the masking system, all of the above-mentioned compounds were designed as prodrugs (pronucleotides) which should release FdUMP, the true drug, after chemical or enzyme-assisted conversion within the cell. However, other studies on FdUMP pronucleotides [21–27] and other type of biologically active compounds (for instance, phospholipids [28–34]), proved that the presence of a charge in combination with specific structural fragments, e.g., lipophilic or zwitterionic elements [27,35–37], did not prevent cellular internalization of the compounds and in consequence expression of their biological activity.

In this paper, we described the synthesis of new FdU-based pronucleotides carrying zwitterionic phosphate masking systems (Fig. 1, abbreviations as in Scheme 1), and evaluated their anti-proliferative potency on several glioma cell lines. By choosing charged zwitterionic pronucleotides, we expected, besides of potential therapeutic advantages, an increase of solubility in

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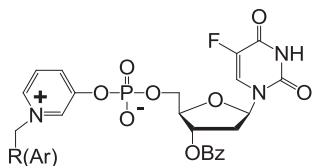


Fig. 1. Zwitterionic FdUMP pronucleotides.

physiological media. Additionally, we hoped that cellular membrane charge disorder, caused by ionic compounds, including zwitterions [38,39], can be different in healthy and malignant cells, and be beneficial with regard to combating the neoplasm.

2. Results and discussion

2.1. The rationale behind the choice of FdUMP derivatives

We focused our attention on the FdU 5'-phosphodiester derivatives, that should meet the criteria typical of pronucleotides. In most cases, the investigated pronucleotides of type **4** (Scheme 1) have 3'-O-benzoyl-5-fluoro-2'-deoxyuridin-5'-yl residue in the nucleoside part, that *per se* demonstrates advantageous antigioma potency [40]. For comparison, nucleotide analogues **5a,c,d** with free 3'-OH function were also evaluated. With exception of compounds **4a** and **4b**, a common structural motif of the examined nucleoside phosphodiester **4c-m** and **5c-d** is an aliphatic or aromatic amine that together with a phosphate residue can form a zwitterionic structure (e.g. **4c** and **4d** [37]) or are permanent

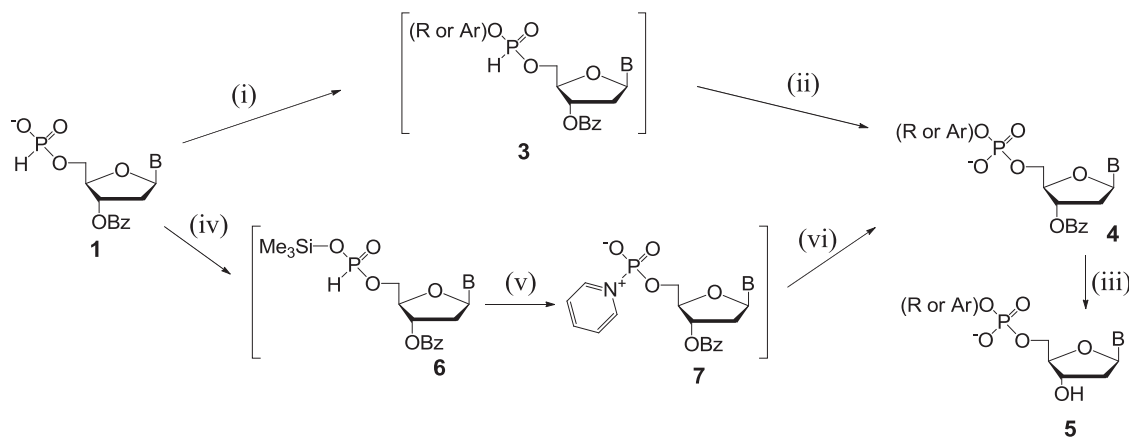
zwitterions (**4e-m**). Phosphodiester **4e-m**, in addition to a zwitterionic part, have also different lipophilic handles that may further modulate their ability of cellular membrane penetration. Compounds **4h** and **4i** were designed as nucleotide analogues of the known biocidal, membrane interactive drug, 12-methacryloyloxydodecylpyridinium bromide [38]. For similar reasons, phosphodiester **4j** and **4k** were appended with 2-*N,N,N*-trimethylethanaminium or *N,N*-dimethylpiperidinium zwitterionic residues that are part of the known cytostatic compounds, e.g. miltefosine [36] and perifosine [35], respectively. In the studied compounds, the aryl and alkyl groups were selected as lipophilic parts of pronucleotides, in order to find out which type of the group will turn out to be more beneficial for antigioma activity, and thus worthy of further development.

2.2. Chemistry

2.2.1. Synthesis of aryl(alkyl) nucleoside 5'-phosphate diesters of type **4** and **5**

A route via *H*-phosphonate diesters of type **3** as intermediates.

For the purpose of the synthesis of aryl nucleoside phosphate diesters, we used an approach that was previously developed in our laboratory [41]. Consequently, nucleoside 5'-*H*-phosphonates of type **1** were coupled with phenols **2a-d** or alkanols **2j-m**, with the aid of diphenyl chlorophosphate (Scheme 1). The reactions were carried out in CH₂Cl₂/pyridine 9: 1 (v/v) to avoid double activation of the *H*-phosphonate component [42]. Formation of the respective *H*-phosphonate diesters of type **3** as well as their oxidation with iodine towards phosphodiester **4**, proceeded smoothly, practically quantitatively (as judged from ³¹P NMR spectra), and could be



B = 5-fluorouracil-1-yl

Bz = benzoyl;

2a, 3a, 4a, 5a; Ar = phenyl;

2b, 3b, 4b; Ar = 4-nonylphenyl

2c, 3c, 4c, 5c; Ar = pyridin-3-yl;

2d, 3d, 4d, 5d; Ar = quinolin-6-yl

2e, 4e; Ar = *N*-methylpyridinium-3-yl;

2f, 4f; Ar = *N*-decylpyridinium-3-yl;

2g, 4g; Ar = *N*-benzylpyridinium-3-yl;

2h, 4h; Ar = *N*-(2-ethoxy-2-oxoethyl)pyridinium-3-yl;

2i, 4i; Ar = *N*-(2-ethoxy-2-oxoethyl)quinolinium-6-yl;

2j, 3j, 4j; R = 2-(*N,N,N*-trimethylaminium)ethyl;

2k, 3k, 4k; R = *N,N*-dimethylpiperidinium-4-yl;

2l, 3l, 4l; R = 2-(*N*-benzyl-*N,N*-dimethylaminium)ethyl;

2m, 3m, 4m; R = *N*-benzyltropanium-3-yl;

Scheme 1. Synthesis of FdUMP pronucleotides of type **4** and **5**. (i) 1 mmol of **1** in 10 mL of CH₂Cl₂/pyridine 9: 1 (v/v), ArOH or ROH, **2**, 1.5 mmol, diphenylchlorophosphate 1.5 mmol, 5 min, r. t.; (ii) 2 mmol of I₂ in 5 mL pyridine, after 10 s H₂O > 10 mmol, 5 min; (iii) 40% of MeNH₂ aq., rt, overnight; (iv) 1 mmol of **1** in 10 mL of acetonitrile, trimethylsilyl chloride, 2 mmol and Et₃N, 2 mmol, < 3 min; (v) 2 mmol of I₂ in 5 mL pyridine, < 3 min; (vi) 2.5 mmol of ArOH **2**.

performed as one-pot reactions. The final products **4a–d** and **4j–m**, were then purified by the silica gel column chromatography, and obtained in high yields (vide Experimental section).

A route via pyridiniumphosphoramidate of type **7** as an intermediate.

The attempted synthesis of compounds **4e–i**, bearing aryls **2e–i** in the phosphate moiety via intermediate **3** (Scheme 1), turned out to be unsuccessful, most likely due to similar nucleophilicity and the leaving group ability of phenols **2e–i** vs other nucleophiles present in the reaction mixture. Considering the thermodynamic stability of the betaine form of nucleoside phosphate diesters **4e–i**, we attempted to perform their synthesis using intermediacy of pyridiniumphosphoramidate of type **7** [43–45] (Scheme 1). To this end, nucleoside 5'-*H*-phosphonate of type **1**, after silylation with TMSCl (species **6**), was oxidized with I_2 in anhydrous pyridine, and converted almost quantitatively (^{31}P NMR spectra) into nucleoside pyridiniumphosphoramidate **7**. This proved to be sufficiently reactive upon treatment with phenols **2e–i**, to produce the corresponding phosphodiester **4e–i** (ca. 5 min). The whole reaction sequence **1**–**6**–**7**–**4** can be monitored with the ^{31}P NMR spectroscopy (**1**, δ_P 2.96 ppm, $^1J_{HP}$ 618.8 Hz, $^3J_{HP}$ 6.5 Hz; **6**, δ_P –1.19 ppm, $^1J_{HP}$ 691.7 Hz; **7**, δ_P –5.19 ppm, $^3J_{HP}$ 7.3 Hz; data for **4e–i**, see Experimental section). It showed quantitative conversion of *H*-phosphonate **1** into the trimethylsilyl *H*-phosphonate diester **6**, followed by its complete oxidation to pyridinium intermediate **7**, and its esterification with phenols **2e–i**. Moreover, this synthetic route could be executed with a one-pot process that resulted in high overall yields (60%–80%) of the final products **4e–i**.

2.2.2. Synthesis of the sugar unprotected aryl nucleoside phosphodiester **5a** and **5c–d**

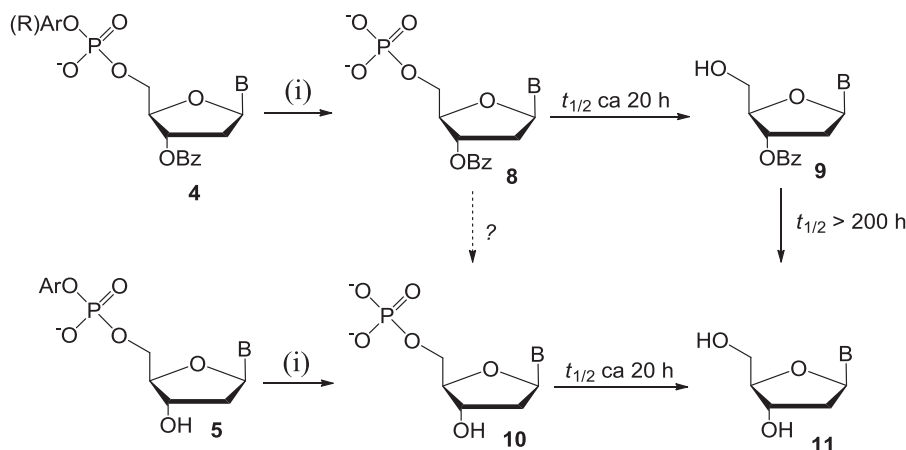
Compounds **5a** and **5c–d** were obtained from the corresponding aryl 3'-*O*-benzoyl-5-fluoro-2'-deoxyuridine 5'-phosphate **4a** and **4c–d** (vide supra) by overnight treatment with 40% aqueous solution of $MeNH_2$, at ambient temperature. Products **5a** and **5c–d** were isolated as white amorphous solids in virtually quantitative yields.

2.3. Degradation of FdUMP derived nucleotide analogues of type **4** and **5** in cell culture media (RPMI/FBS) and human serum (HS)

In cell culture media (RPMI/FBS 9: 1, v/v), the decomposition of the examined phosphodiester **4** or **5** was similar and produced the respective nucleoside 5'-phosphate monoesters of type **8** or nucleotide **10** as the initial products (Scheme 2). The half times of hydrolysis in RPMI/FBS and human serum (HS)

varied (Table 1) but these differences seemed not be essential in the context of the observed anticancer activity of the investigated compounds (vide infra and Table 2). Three of the examined compounds, namely **4b** with 4-nonylphenyl, **4k** with *N,N*-dimethylpiperidinium-4-yl and **4m** with *N*-benzyltropinium-3-yl phosphoester groups, were stable ($t_{1/2} \gg 120$ h) under the investigated conditions. Therefore, one may hypothesize that stability of **4k** and **4m** is a consequence of steric hindrance imparted by the phosphate masking groups. In the case of nucleotide derivative **4b**, its resistance to hydrolysis might stem from lipophilicity of the phosphoester nonylphenyl residue that generate hydrophobic sphere which limits access of water molecules to the phosphorus center. The rate of hydrolysis of phosphodiester possessing the 3'-*O*-benzoyl group (**4a,c,d**) was 3–4 times higher than that of their 3'-OH counterparts (**5a,c,d**) ($t_{1/2}$ 6.6 h, 6.7 h, 7.7 h vs 27.5 h, 16.7 h, 25.7 h, respectively). In neat RPMI, all phosphodiester of type **4** and **5** remained unchanged for several days (data not shown), hence the observed decomposition (vide supra) can be attributed to phosphodiesterase activity of FBS or HS. Considering the fact that enzymatic activities present in the cell culture media (from FBS) are ca. 10–50 times lower than those found in cells extracts [46,47], one may postulate that for the examined compounds, the qualitatively similar, but kinetically different decomposition paths might occur in the cell.

When analyzing the decomposition paths of phosphodiester of type **4** and **5**, several tentative conclusions can be drawn. (i) In RPMI/FBS 9: 1 (v/v), the primary and the major decomposition path is the hydrolysis of phosphodiester **4** or **5**, that released the nucleotides of type **8** or **10**, respectively. (ii) The rates of conversion varied, depending mainly on the type of phosphate masking groups [e.g. 4-nonylphenyl (**4b**), *N,N*-dimethylpiperidinium-4-yl (**4k**), and *N*-benzyltropinium-3-yl (**4m**) ($t_{1/2} > 120$ h, respectively) vs most of other compounds that were hydrolyzed faster]. Because the $t_{1/2}$ of the examined compounds was higher than few hours, this should secure sufficient exposition time of the treated cells to the unchanged pronucleotides. (iii) In most cases, kinetically similar or slightly favored over phosphomonoesters formation was a consecutive, FBS phosphatase aided, dephosphorylation of nucleotide **8** as well as **10** that precluded accumulation of FdUMP under the investigated conditions. (iv) Since the removal of 3'-*O*-benzoyl group from nucleotide **8** and nucleoside **9** proceeded very slowly, the major player in the anti-proliferative activity (vide infra) should be nucleotide **8**, and to a lesser extent nucleoside **9**.



Scheme 2. The observed paths of decay of nucleoside phosphodiester **4** and **5**, in RPMI/FBS (9: 1 v/v) media. (i) RPMI/FBS (9: 1 v/v), 37 °C. Abbreviation as in Scheme 1.

Table 1
Selected physicochemical data for FdU pronucleotides of type **4** and **5**.

Cpd	MW	aPSA [$\text{\AA}^2$]	PSA [$\text{\AA}^2$]	aPSA/PSA	HBD	HBA	LogP	$t_{1/2}$ [h] RPMI/FBS	$t_{1/2}$ [h] HS
4a	505.37	297.2	126.1	2.4	1	11	1.36	6.6	5.1
4b	632.62	254.7	79.3	3.2	2	11	5.66	>120	>120
4c	507.37	201.2	74.1	2.7	2	12	0.23	6.7	17.8
4d	557.343	320.8	130.9	2.5	2	12	1.1	7.7	16.5
4e	521.39	208.6	72	2.9	1	12	−0.41	14.8	37.3
4f	647.64	231.7	62.6	3.7	1	12	3.29	16.5	8.3
4g	597.49	235.5	61.3	3.8	1	12	0.9	10.8	14.4
4h	593.46	217.6	70.6	3.1	1	14	−0.21	8.6 ^a (1.1)	1.4
4i	643.52	240.7	68.6	3.5	1	14	0.82	15.6 ^a (2.0)	5.9
4j	515.43	187.9	72	2.6	1	12	−1.68	20.0	115.5
4k	541.47	218.4	56.7	3.9	1	12	−1.31	>120	>120
4l	591.53	202.8	72	2.8	1	12	−0.35	54.0	96.3
4m	629.58	242.3	67.7	3.6	1	12	0.72	>120	>120
5a	401.26	154.2	85.9	1.8	2	10	−0.72	28.0	52.3
5c	403.25	147.5	90.7	1.6	3	11	−1.74	16.7	19.9
5d	453.32	173.6	86.6	2.0	3	11	−0.64	26.0	26.2

PSA - polar surface area, aPSA - apolar surface area, HBD - hydrogen bond donors, HBA - hydrogen bond acceptors, LogP - calculated with ALOGPS 2.1 software (vide Experimental); RPMI - RPMI 1640 media; FBS - foetal bovine serum; HS - human serum; SI - IC_{50} non-tumor cell line/ IC_{50} tumor cell line.

^a Half-life time calculated on the basis of cumulative concentration of metabolites of known anticancer activity (see in the text). The $t_{1/2}$ values in parentheses, were calculated on the basis of the disappearance of a respective substrate.

2.4. Drug likeness

The most common criteria used for preliminary evaluation of drug likeness of a compound encompass the Lipinski's "rule of 5" (Ro5) [48–50]. In this respect, the physicochemical parameters of the examined compounds match the aforementioned rule only to a certain extent (Table 1). Specifically, (i) most of the investigated compounds only slightly exceeded the molecular weight criteria ($\text{MW} < 500$), (ii) all of them have more than 10, but less than 14 hydrogen bond acceptors (HBA), (iii) the calculated polar surface area (PSA) of the studied compounds was found to fall in the range of 60–130 $\text{\AA}^2$, and only two of them exceeded the values that favor cellular uptake ($\text{PSA} < 90 \text{\AA}^2$) [51]. The above parametric values are not surprising when it comes to pronucleotides. In order to acquire appropriate stability and/or lipophilicity (for instance diaryl phosphotriester [52] or SATE pronucleotides [53]) a nucleotide skeleton is usually equipped with masking group(s), at the expense of an increased molecular weight. It seems that the criteria applied in the case of pronucleotides are more complex than those of Ro5 or Ro3, and e.g., rotatable bonds [54–56], cell or blood-brain membrane permeability [57–59] will become important for drug likeness evaluation.

2.5. In vitro cytostatic activity

For the purposes of these studies we chose T98G, U-118 MG, U-87 MG glioblastoma multiforme cells as the main targets, and noncancerous lung fibroblast (MRC-5) as referential healthy cells. Additionally, we screened our compounds against two other malignant cell lines, namely cervical cancer cell line (HeLa) and human breast tumor (T-47D). To evaluate intestinal uptake potency [60,61] of the examined pronucleotides of type **4** and **5**, Caco-2 cell line was used.

In the standard tests (vide infra, Experimental section), compounds **4a** and **4c** exhibited similar cytostatic activity (IC_{50} ca. 1 μM) in glioblastoma cell lines (T98G, U-118 MG and U-87 MG), but **4c** appeared to be much less toxic for MRC-5 cells. This resulted in a much higher selectivity index of **4c**, in comparison to **4a** (Table 2). Both compounds showed also similar, high activity in Caco-2 cells. These results, together with nearly identical stability of **4a** and **4c** in cell culture media (Table 1) might suggest that pyridiniumphosphate zwitterionic system in compound **4c** is more

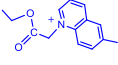
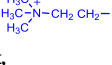
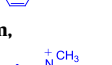
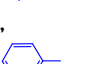
effective in terms of penetration of glioma cells, although LogP value would seem to favor phenyl phosphoester **4a** in this respect.

A similar correlation is also evident while comparing another pair of FdUMP analogues, namely **4b** and **4f**, that bear highly lipophilic nonylphenyl and decylpyridyl phosphoesters. In this group, the zwitterionic compound **4f** was found to be significantly more cytotoxic for all glioma cells. Other zwitterionic compounds **4g**, **4h** and **4i**, also showed high cytostatic activity (IC_{50} , mostly in nM range) for glioma cells (Table 2), but were much less harmful towards the referential, healthy MRC-5 cells. It can be assumed that the observed biocidal selectivity for glioma cell vs healthy ones, can be attributed, at least partly, to the different mode of interactions of compounds **4c**, **4f**–**4i** with glioma cells membrane, that facilitate their intracellular uptake. Compounds **4j**–**4m**, bearing aliphatic ammonium-phosphate zwitterionic residues, neither show appreciable anti-proliferative selectivity (**4k**, **4m**), nor were more toxic for non-cancerous cells MRC-5 (**4j**, **4l**). A possible reason for this might be the fact that cell membranes of different cell lines can be selectively penetrated by zwitterionic pronucleotides, due to specific interaction between their zwitterion parts and cellular membrane components.

Considering the selectivity indices (SI), compounds **4c**, **4f**–**i** clearly outstood other ones. Their high SI values (in the range of 12–66) derived from their high biocidal activity ($\text{IC}_{50} = 0.32$ – $1.57 \mu\text{M}$) for glioma cell lines and low toxicity ($\text{IC}_{50} = 8.6$ – $38.5 \mu\text{M}$) for the healthy MRC-5 cells. It is noteworthy that compounds **4d** and **4e** were similarly biocidal for glioma cells as **4c** and **4f**–**g**, but were also toxic towards referential healthy MRC-5 cells (Table 2). This makes compounds **4d** and **4e** less promising candidates in terms of developing drugs against glioma, and indicates that apparently modest structural changes can essentially influence biological features of a compound. It is also noteworthy that compounds **4c** and **4f**–**g**, besides having outstanding anti-glioma potency, turned out to be effective cytostatics for Caco-2 cells as well, implying a potential possibility for oral administration of such compounds. Nucleotide analogues **4j**–**m**, bearing aminoalkyl-phosphate-based permanent zwitterions were less effective towards glioma cells.

While analyzing cytostatic parameters (Table 2) of the examined compounds within the glioma cell lines, some interesting differences can be observed. For example, within glioma cell lines T98G, U-118 MG and U-87 MG, the most pronounced differences in IC_{50}

Table 2
Cytotoxicity (IC₅₀) and selectivity index (SI^a) values of FdU pronucleotides of type **4** and **5**.

Cpd No, Ar, R	MRC-5	HeLa	T-47D	T98G	SI ^a	U-118 MG	SI ^a	U-87 MG	SI ^a	Caco-2
	IC ₅₀ [μM]	IC ₅₀ [μM]	IC ₅₀ [μM]	IC ₅₀ [μM]		IC ₅₀ [μM]		IC ₅₀ [μM]		IC ₅₀ [μM]
4a , 	1.27 ± 0.3 ^a	5.86 ± 0.5	6.88 ± 0.6	0.81 ± 0.09	2	0.97 ± 0.07	1	0.80 ± 0.05	2	1.02 ± 0.08
4b , 	39.9 ± 2.9	40.13 ± 3.1	26.66 ± 3.4	6.74 ± 0.9	6	10.97 ± 1.4	4	6.73 ± 0.5	6	20.41 ± 3.1
4c , 	15.6 ± 0.9	6.90 ± 1.0	14.38 ± 1.9	1.30 ± 0.08	12	0.92 ± 0.07	17	0.71 ± 0.04	22	2.50 ± 0.1
4d , 	1.30 ± 0.1	7.30 ± 0.9	7.14 ± 0.6	1.30 ± 0.1	1	0.79 ± 0.09	2	0.88 ± 0.07	1	2.46 ± 0.3
4e , 	5.75 ± 0.7	1.26 ± 0.2	3.91 ± 0.5	3.48 ± 0.2	2	3.15 ± 0.5	2	0.59 ± 0.1	10	1.38 ± 0.2
4f , 	14.1 ± 1.0	1.20 ± 0.2	8.73 ± 1.1	0.40 ± 0.07	35	0.32 ± 0.04	44	0.67 ± 0.09	21	12.88 ± 1.9
4g , 	38.5 ± 4.5	0.53 ± 0.04	3.43 ± 0.4	2.60 ± 0.3	15	1.47 ± 0.2	26	0.66 ± 0.1	58	0.43 ± 0.06
4h , 	8.60 ± 0.9	6.10 ± 0.4	12.65 ± 1.5	1.60 ± 0.2	5	1.54 ± 0.1	6	0.58 ± 0.09	15	2.62 ± 0.3
4i , 	34.9 ± 1.7	0.60 ± 0.04	21.58 ± 1.5	13.00 ± 1.0	3	0.76 ± 0.05	46	0.53 ± 0.03	66	28.88 ± 2.1
4j , 	1.30 ± 0.2	3.60 ± 0.5	39.62 ± 2.4	24.70 ± 1.7	<1	7.54 ± 1.0	<1	1.79 ± 0.2	<1	8.12 ± 0.7
4k , 	8.30 ± 1.1	27.20 ± 1.8	11.88 ± 1.6	6.70 ± 1.1	1	4.42 ± 0.3	2	1.28 ± 0.2	7	15.70 ± 1.1
4l , 	0.60 ± 0.1	1.50 ± 0.2	33.03 ± 2.9	2.00 ± 0.3	<1	3.69 ± 0.5	<1	0.61 ± 0.07	1	11.72 ± 1.4
4m , 	62.50 ± 5.5	>80	>80	49.9 ± 2.5	1	66.28 ± 3.1	1	35.94 ± 1.9	2	>80
5a , 	36.70 ± 2.6	15.20 ± 2.0	13.36 ± 1.8	32.2 ± 1.9	1	14.56 ± 1.7	3	33.68 ± 2.8	1	26.55 ± 2.0
5c , 	8.50 ± 0.6	0.70 ± 0.09	9.35 ± 1.0	1.20 ± 0.1	7	27.49 ± 2.2	<1	39.82 ± 2.5	<1	3.39 ± 0.4
5d , 	1.30 ± 0.1	7.30 ± 0.9	8.11 ± 1.1	1.90 ± 0.2	<1	7.07 ± 0.9	<1	10.66 ± 1.5	<1	1.4 ± 0.2

^a SI = IC₅₀ MRC-5 cell line/IC₅₀ of glioma cell lines, rounded to the nearest integral value.

values were found for compounds **4i** and **5c**. Pronucleotide **4i** was highly toxic towards U-118 MG and U-87 MG cells, while ca 25 times less toxic towards T98G cells. Compound **5c**, on the other hand, showed opposite selectivity and revealed high activity in T98G glioma cells, in comparison to U-118 MG and U-87 MG cells.

Furthermore, pronucleotide **4g** showed uniform, high anticancer potency (IC₅₀) for all the investigated cancer cell lines (including HeLa and Caco-2), and was only slightly toxic towards referential healthy cells (MRC-5). Compound **4i**, despite its high anti-proliferative activity in three cancer cell lines (HeLa, U-118 MG

and U-87 MG), indicated rather low activity in Caco-2 cells, which might limit its potential for therapeutic application, due to possible problems when it comes to oral administration. All the above-mentioned features can be of importance when selecting compounds as potential leads for GMB therapy.

To sum up this part, by comparing therapeutic indices of nucleotide analogues **4c** and **4f-i** to those of the parent FdU (Table 2), it is clear that all of them exhibit advantageous anti-proliferative activity (IC₅₀) and therapeutic potency (SI) vs FdU [62]. Additionally, we have examined the antiglioma activity of phenols **2c**, **2f-2i** that were parts of these compounds. We found that IC₅₀ values of these phenols against T98G, U-118 and U-87 glioma cell lines were in the range of 55.61 μ M – >500 μ M (vide Supplementary data), and thus their formation as potential metabolites cannot contribute to the observed high activity of pronucleotides **4c**, **4f-4i**.

3. Conclusions

In conclusion, we reported herein the synthesis of new FdUMP analogues designed as antiglioma pronucleotides. A substrate for the synthesis of all the investigated compounds was an easily accessible nucleoside 5'-H-phosphonate **1**, that was efficiently converted via two reaction pathways into the target compounds of type **4** and **5**. The choice of a synthetic approach depended on phenols or alkanols used, and for those of low nucleophilicity, intermediacy of pyridiniumphosphoramidate **7** was required. To ensure easy access to the designed compounds (short time and high yields), convenient one-pot protocols were developed. Among the investigated compounds, the nucleotide analogues bearing pyridinium (**4f**, **4g** and **4h**) or quinolinium (**4i**) permanent, cationic phosphate masking group, revealed the best anti-proliferating properties. Comparison of anticancer potency of simple anionic and zwitterionic phosphodiester (**4a** vs **4c** and **4b** vs **4f**, respectively) unambiguously pointed to zwitterionic pronucleotides as more advantageous ones. It is likely that zwitterionic nucleotide analogues might act similarly as zwitterionic phospholipids [38], by inducing cell membrane charge disorder and making cell permissive towards the drug. Our results suggest that the described pronucleotides can enter the cell and, after being correctly processed, reach the assumed target, thymidylate synthase (TS). Taking into account the fact that TS is overexpressed in glioblastoma [4,63] cells, and that FdUMP is a true inhibitor of this enzyme, further search of effective, FdUMP-based antiglioma pronucleotides, bearing zwitterionic auxiliary system, is already in progress at our laboratory.

4. Experimental

4.1. Material and methods

¹H, ¹³C, ³¹P NMR spectra were recorded on Bruker Avance II 400, 500 or 700 MHz machines. Mass spectra were recorded with the ESI technique, with negative or positive ionization mode with an accuracy below 5 ppm. The amount of water in anhydrous solvents was controlled using Karl Fischer coulometric titration (Metrohm 684 KF coulometer). Thin-layer chromatography was performed on Merck silica gel 60F₂₅₄ plates, and visualized with UV. For column chromatography, Kieselgel 60 Merck was used. HPLC analyses were performed on a Lichrospher RP-18 endcapped (5.0 μ m, 4.6 mm $\times$ 250 mm), using Thermo Scientific™ HPLC systems with A + B solvent systems (A, 0.01 M aqueous triethylammonium acetate pH 7; B, A/acetoneitrile, 1: 4, v/v) at 35 °C, flow rate 1.5 mL/min; events: 5 min A 100%, linear gradient of B 0–100% in 20 min, 5 min B 100% and A 100% 15 min wash. Purity of the obtained

Table 3

³¹P NMR data of products (in DMSO, unless stated otherwise).

Cpd	δ_P [ppm]	J_{HP} [Hz]	Cpd.	δ_P [ppm]	J_{HP} [Hz]
4a	−5.50	6.48 (t)	4i	−5.70	6.48 (t)
4b	−7.54	6.48 (t)	4j	−1.22	6.48 (quint)
4c	−5.43	6.48 (t)	4k	−1.06	6.48 (q)
4d	−5.37	7.29 (t)	4l	−0.60	6.48 (quint)
4e	−5.38	6.48 (t)	4m	−1.31	6.48 (q)
4f	−5.95	6.48 (t)	5a	−5.59	6.48 (t)
4g	−5.35	6.48 (t)	5c	−5.14	6.48 (t)
4h	−5.38	6.48 (t)	5d	−5.11	6.48 (t)

compounds was higher than 97% (HPLC chromatograms are enclosed in the Supplementary data). Nucleoside 5'-H-phosphonate **1** was synthesized from 3'-O-benzoyl-5-fluoro-2'-deoxyuridine and ammonium fluoren-9-yl H-phosphonate as described earlier [64]. ³¹P NMR data of products (in DMSO, unless stated otherwise) are listed in Table 3 (vide infra).

4.1.1. A general procedure for the synthesis of 3'-O-benzoyl-5-fluoro-2'-deoxyuridin-5'-yl aryl(alkyl) phosphates of type **4**. Route via H-phosphonate diesters of type **3** as intermediates (for compounds **4a-4d** and **4j-4m**)

3'-O-Benzoyl-5-fluoro-2'-deoxyfluorouridin-5'-yl H-phosphonate **1** (1 mmol) and respective phenols **2a-d** or alkanols **2j-m** (1.5 mmol) were rendered anhydrous, by the evaporation of the added pyridine, and then dissolved in 10 mL of dichloromethane, containing 10% (v/v) of pyridine. For this purpose, diphenyl chlorophosphate (1.5 mmol) was added, and after the formation of H-phosphonate diesters of type **3** (5 min, ³¹P NMR) iodine (2 mmol) in pyridine (1 mL), and after 10 s water (>10 mmol) was also added. After 5 min, the excess of iodine was decomposed with the added ethanethiol, and the mixture was concentrated to an oil under reduced pressure. Aryl(alkyl) 3'-O-benzoyl-5-fluoro-2'-deoxyuridine 5'-phosphodiester of type **4** were isolated by a silica gel 60 column chromatography, using a 0–50% gradient of MeOH in toluene. Fractions containing pure products were collected and evaporated yielding amorphous solids.

4.1.1.1. 3'-O-benzoyl-5-fluoro-2'-deoxyuridin-5'-yl phenyl phosphate (4a). White solid; yield: 92%; RP HPLC Rt 17.395 min; ¹H NMR (400 MHz, DMSO-d₆): δ_H 11.76 (br s, 1H of NH), 8.18 (br s, 1H of H-6), 7.99 (d, J = 7.2 Hz, 2H of Ar), 7.69 (t, J = 7.4 Hz, 1H of Ar), 7.55 (t, J = 7.6 Hz, 2H of Ar), 7.17 (br s, 4H of Ar), 6.93 (br s, 1H of Ar), 6.24–6.26 (m, 1H, H-1'), 5.45 (br s, 1H, H-3'), 4.27 (br s, 1H, H-4'), 3.99–4.10 (m, 2H, H-5', H-5''), 2.39 (br s, 2H, H-2', H-2'') ppm; ¹³C NMR (125 MHz, DMSO-d₆): δ_C 165.08 (C=O), 157.18 (d, J = 26.25 Hz, C-4), 153.82 (C of Ar), 149.25 (C-2), 140.14 (d, J = 231.25 Hz, C-5), 133.59, 129.30, 128.74 (C of Ar), 124.50 (d, J = 33.75 Hz, C-6), 121.75, 119.74 (C of Ar), 84.44 (C-1'), 83.26 (C-4'), 75.94 (C-3'), 64.93 (d, J = 6 Hz, C-5'), 36.34 (C-2') ppm; HRMS (ESI): calcd for [C₂₂H₁₉N₂O₉F][−]: 505.0818, found: 505.0799.

4.1.1.2. 3'-O-benzoyl-5-fluoro-2'-deoxyuridin-5'-yl pyridin-3-yl phosphate (4c). White solid; yield: 89%; RP HPLC Rt 16.148 min; ¹H NMR (400 MHz, DMSO-d₆): δ_H 11.88 (br s, 1H, NH), 8.38 (br s, 1H of Ar), 8.13–8.26 (m, 2H of Ar), 8.00 (d, J_{6-F5} = 7.0 Hz, H-6), 7.53–7.70 (m, 4H of Ar), 7.21–7.25 (m, 1H of Ar), 6.25–6.28 (m, 1H, H-1'), 5.46 (br s, 1H, H-3'), 4.28 (br s, 1H, H-4'), 4.02–4.11 (m, 2H, H-5', H-5''), 2.39–2.42 (m, 2H, H-2', H-2''), ppm; ¹³C NMR (100 MHz, DMSO-d₆): δ_C 165.14 (C=O), 157.37 (d, J = 26 Hz, C-4), 150.38 (C of Ar), 149.41 (C-2), 142.96, 142.02 (d, J = 5 Hz, C of Ar), 140.22 (d, J = 231 Hz, C-5), 133.64, 129.34, 129.26, 128.79 (C of Ar), 126.73 (d, J = 4 Hz, C of Ar), 124.40 (d, J = 34 Hz, C-6), 123.64 (C of Ar), 84.51 (C-

1'), 83.13 (d, $J = 7$ Hz, C-4'), 75.78 (C-3'), 65.19 (d, $J = 5$ Hz, C-5'), 36.34 (C-2') ppm; HRMS (ESI): calcd for $[C_{21}H_{18}N_3O_9FP]^-$: 506.0770, found: 505.0768.

4.1.1.3. 3'-O-Benzoyl-5-fluoro-2'-deoxyuridin-5'-yl quinolin-6-yl phosphate (4d). Yellow solid; yield: 88%; RP HPLC Rt 16.812 min; 1H NMR (400 MHz, DMSO- d_6): δ_H 8.72–8.73 (m, 1H of Ar), 8.21 (d, 1H, $J_{6-F5} = 6.8$ Hz, H-6), 8.14 (d, $J = 8.0$ Hz, 1H of Ar), 7.98–7.99 (m, 2H of Ar), 7.86 (d, $J = 9.2$ Hz, 1H of Ar), 7.66–7.69 (m, 2H of Ar), 7.59–7.62 (m, 1H of Ar), 7.53 (t, $J = 7.8$ Hz, 2H of Ar), 7.39–7.43 (m, 1H of Ar), 6.27 (t, $J = 6.4$ Hz, 1H, H-1'), 5.50 (br s, 1H, H-3'), 4.29 (br s, 1H, H-4'), 4.03–4.17 (m, 2H, H-5', H-5''), 2.36–2.47 (m, 2H, H-2', H-2'') ppm; ^{13}C NMR (100 MHz, DMSO- d_6): δ_C 165.10 (C=O), 157.30 (d, $J = 25$ Hz, C-4), 151.88 (d, $J = 6$ Hz), 149.39 (C of Ar), 148.34 (C-2), 144.14 (C of Ar), 140.18 (d, $J = 231$ Hz, C-5), 134.86, 133.58, 129.58, 129.31, 129.27, 128.76, 128.52 (C of Ar), 124.84 (d, $J = 5$ Hz, C of Ar), 124.49 (d, $J = 34$ Hz, C-6), 121.27 (C of Ar), 114.61 (d, $J = 5$ Hz, C of Ar), 84.46 (C-1'), 83.30 (d, $J = 6$ Hz, C-4'), 75.94 (C-3'), 65.07 (d, $J = 5$ Hz, C-5'), 36.38 (C-2') ppm; HRMS (ESI): calcd for $[C_{25}H_{20}N_3O_9FP]^-$: 556.0927, found: 556.0925.

4.1.1.4. 3'-O-Benzoyl-5-fluoro-2'-deoxyuridin-5'-yl 2-(N,N,N-trimethylaminium)ethyl phosphate (4j). White solid; yield: 72%; RP HPLC Rt 15.505 min; 1H NMR (400 MHz, DMSO- d_6): δ_H 8.14 (d, 1H, $J_{6-F5} = 7.2$ Hz, H-6), 8.00–8.03 (m, 2H of Ar), 7.67–7.71 (m, 1H of Ar), 7.54–7.71 (m, 2H of Ar), 6.31–6.35 (m, 1H, H-1'), 5.52 (d, $J = 5.2$ Hz, 1H, H-3'), 4.26 (br s, 1H, H-4'), 4.09 (br s, 2H, CH₂), 3.89–4.03 (m, 2H, H-5', H-5'') 3.53 (t, $J = 4.8$ Hz, 2H, CH₂), 3.14 (s, 9H, CH₃), 2.35–2.46 (m, 2H, H-2', H-2'') ppm; ^{13}C NMR (100 MHz, DMSO- d_6): δ_C 165.22 (C=O), 159.17 (d, $J = 24$ Hz, C-4), 150.90 (C-2), 140.72 (d, $J = 232$ Hz, C-5), 133.63, 129.35, 128.83 (C of Ar), 123.74 (d, $J = 35$ Hz, C-6), 84.45 (C-1'), 83.34 (d, $J = 7$ Hz, C-4'), 76.27 (C-3'), 65.51 (C-5'), 64.74 (d, $J = 5$ Hz, CH₂), 58.33 (d, $J = 5$ Hz, CH₂), 53.12 (CH₃), 36.47 (C-2') ppm; HRMS (ESI): calcd for $[C_{21}H_{27}N_3O_9FPNa]^+$: 538.1361, found: 538.1382.

4.1.1.5. 3'-O-Benzoyl-5-fluoro-2'-deoxyuridin-5'-yl N,N-dimethylpiperidin-4-yl phosphate (4k). White solid; yield: 89%; RP HPLC Rt 15.530 min; 1H NMR (400 MHz, DMSO- d_6): δ_H 11.84 (br s, 1H, NH), 8.37 (d, 1H, $J_{6-F5} = 7.2$ Hz, H-6), 8.00–8.02 (m, 1H of Ar), 7.69 (t, $J = 7.4$ Hz, 1H of Ar), 7.56 (t, $J = 7.6$ Hz, 2H of Ar), 6.31 (t, $J = 6.8$ Hz, 1H, H-1'), 5.56 (d, $J = 4.8$ Hz, 1H, H-3'), 4.27 (br s, 1H, H-4'), 4.21–4.22 (m, 1H, CH), 4.01–4.04 (m, 2H, H-5', H-5''), 3.32–3.49 (m, 4H, CH₂), 3.11 (s, 3H, CH₃), 3.06 (s, 3H, CH₃), 2.41–2.47 (m, 2H, H-2', H-2''), 2.03–2.09 (m, 2H, CH₂), 1.90–1.93 (m, 2H, CH₂) ppm; ^{13}C NMR (100 MHz, DMSO- d_6): δ_C 165.20 (C=O), 157.04 (d, $J = 29$ Hz, C-4), 149.20 (C-2), 140.18 (d, $J = 230$ Hz, C-5), 133.63, 129.34, 128.81 (C of Ar), 123.84 (d, $J = 34$ Hz, C-6), 90.68, 84.47 (C-1'), 83.78 (d, $J = 7$ Hz, C-4'), 76.27 (C-3'), 65.16 (C-5'), 58.25 (CH₂), 52.17, 49.33 (CH₂), 36.54 (C-2'), 21.14 (CH₃) ppm; HRMS (ESI): calcd for $[C_{23}H_{30}N_3O_9FP]^+$: 542.1698, found: 542.1672.

4.1.1.6. 3'-O-Benzoyl-5-fluoro-2'-deoxyuridin-5'-yl 2-(N-benzyl-N,N-dimethylaminium)ethyl phosphate (4l). White solid; yield: 78%; RP HPLC Rt 17.198 min; 1H NMR (400 MHz, DMSO- d_6): δ_H 11.90 (s, 1H, NH), 8.33 (d, $J_{6-F5} = 7.2$ Hz, 1H, H-6), 8.00–8.02 (m, 2H of Ar), 7.4 (t, $J = 7.4$ Hz, 1H of Ar), 7.48–7.71 (m, 7H of Ar), 6.30 (m, 1H, H-1'), 5.54 (s, 1H, H-3'), 4.63 (br s, 2H, CH₂), 4.30 (br s, 1H, H-4'), 4.19 (br s, 2H, CH₂), 3.99–4.07 (m, 2H, H-5', H-5''), 3.52 (t, $J = 4.6$ Hz, 2H, CH₂), 3.05 (s, 6H, CH₃), 2.44–2.46 (m, 2H, H-2', H-2'') ppm; ^{13}C NMR (100 MHz, DMSO- d_6): δ_C 165.17 (C=O), 156.98 (d, $J = 26$ Hz, C-4), 149.15 (C-2), 140.17 (d, $J = 230$ Hz, C-5), 133.62, 133.11, 130.22, 129.32, 129.29, 128.83, 128.78, 128.05 (C of Ar), 124.74 (d, $J = 34$ Hz, C-6), 84.98 (C-1'), 84.01 (d, $J = 7$ Hz, C-4'), 76.48 (C-3'), 67.91 (C-5'), 65.26 (d, $J = 5$ Hz, CH₂), 63.97 (d, $J = 6$ Hz, CH₂), 58.81 (d, $J = 5$ Hz,

CH₂), 50.24 (CH₃), 36.94 (C-2') ppm; HRMS (ESI): calcd for $[C_{27}H_{31}N_3O_9FPNa]^+$: 614.1674, found: 614.1690.

4.1.1.7. 3'-O-Benzoyl-5-fluoro-2'-deoxyuridin-5'-yl (3-endo)-8-benzyl-8-methyl-8-azabicyclo[3.2.1]octan-3-yl phosphate (4m). White solid; yield: 82%; RP HPLC Rt 17.678 min; 1H NMR (400 MHz, DMSO- d_6): δ_H 8.30 (d, 1H, $J_{6-F5} = 7.2$ Hz, H-6), 8.00–8.02 (m, 2H of Bz), 7.67–7.71 (m, 1H of Ar), 7.48–7.57 (m, 7H of Ar), 7.32–7.34 (m, 1H of Ar), 6.29–6.32 (m, 1H, H-1'), 5.54 (d, $J = 5.2$ Hz, 1H, H-3'), 4.45 (s, 2H, CH₂), 4.31–4.35 (m, 1H, H-4'), 4.28 (s, 1H of tropine), 3.87–4.04 (m, 2H, H-5', H-5''), 3.79 (br s, 2H of tropine), 3.29 (s, 3H, CH₃), 3.05 (s, 6H, CH₃), 2.52–2.58 (m, 2H of tropine), 2.34–2.46 (m, 4H, H-2', H-2'', H of tropine), 2.07–2.14 (m, 2H of tropine) ppm; ^{13}C NMR (100 MHz, DMSO- d_6): δ_C 165.25 (C=O), 157.26 (d, $J = 26$ Hz, C-4), 149.37 (C-2), 140.25 (d, $J = 231$ Hz, C-5), 133.68, 132.95, 130.20, 129.38, 128.86, 128.36, 128.78, 128.26 (C of Ar), 124.77 (d, $J = 34$ Hz, C-6), 84.52 (C-4'), 83.74 (d, $J = 7$ Hz, C-1'), 76.28 (C-3'), 65.72, 65.69 (C of tropine), 64.55 (C-5'), 63.04 (d, $J = 5$ Hz, CH₂), 62.83 (C of tropine), 39.29 (CH₃), 36.55 (C-2'), 33.37, 33.34 (C of tropine), 24.71 (C of tropine) ppm; HRMS (ESI): calcd for $[C_{31}H_{36}N_3O_9FP]^+$: 644.2168, found: 644.2176.

4.1.2. A general procedure for the synthesis of 3'-O-Benzoyl-5-fluoro-2'-deoxyuridin-5'-yl aryl(alkyl) phosphates of type 4. Route via pyridiniumphosphoramidate of type 7 as intermediates (for compounds 4e–4i)

3'-O-Benzoyl-2'-deoxy-5-fluorouridin-5'-yl H-phosphonate **1** (1 mmol) was rendered to turn anhydrous, by the evaporation of the added pyridine, and then dissolved in 10 mL of acetonitrile, in the presence of Et₃N (2 mmol), and finally treated with trimethylsilyl chloride (2 mmol). After silylation (^{31}P NMR spectra), species of type **6** ($\delta_P -1.19$ ppm, $^1J_{HP} = 691.7$ Hz) was oxidized with iodine (2 mmol) in pyridine (1 mL), to produce nucleoside pyridinium-phosphoramidate of type **7** ($\delta_P -5.19$ ppm, $^3J_{HP} = 7.3$ Hz). This reaction mixture was added to an anhydrous phenols **2e–i** (2.5 mmol), and yielded phosphodiester **4e–4i**. The excess of iodine was decomposed with the added ethanethiol, and the mixture was concentrated to an oil under reduced pressure. Crude phosphodiester of type **4** were applied to a silica-gel column, and eluted with 0–50% gradient of MeOH in toluene. Fractions containing pure products were collected and evaporated, yielding amorphous solids.

4.1.2.1. 3'-O-Benzoyl-5-fluoro-2'-deoxyuridin-5'-yl N-methylpyridinium-3-yl phosphate (4e). White solid; yield: 79%; RP HPLC Rt 15.70 min; 1H NMR (400 MHz, DMSO- d_6): δ_H 11.88 (s, 1H, NH), 8.86 (s, AH of Ar), 8.59 (d, $J = 5.6$ Hz, 1H of Ar), 8.39 (dd, $J = 6.8$ Hz, $J = 2.0$ Hz, 1H of Ar), 7.94–8.01 (m, 4H of H-6, H of Ar), 7.67–7.71 (m, 1H of Ar), 7.54–7.57 (m, 1H of Ar), 6.26–6.30 (m, 1H, H-1'), 5.43 (br s, 1H, H-3'), 4.29 (s, 3H of CH₃), 4.27 (br s, 1H, H-4'), 4.00–4.11 (m, 2H, H-5', H-5''), 2.38–2.40 (m, 2H, H-2', H-2'') ppm; ^{13}C NMR (125 MHz, DMSO- d_6): δ_C 165.16 (C=O), 158.29 (d, $J = 23$ Hz, C-4), 153.41 (d, $J = 5$ Hz, C of Ar), 150.17 (C-2), 140.43 (d, $J = 232$ Hz, C-5), 139.5, 137.50 (d, $J = 6.25$ Hz, C of Ar), 135.12 (d, $J = 3.75$ Hz, C of Ar), 133.66, 129.35, 129.25, 128.81, 127.76 (C of Ar), 123.92 (d, $J = 35$ Hz, C-6), 84.49 (C-4'), 82.87 (d, $J = 7.5$ Hz, C-1'), 75.62 (C-3'), 65.46 (d, $J = 6.25$ Hz, C-5'), 47.93 (CH₃), 36.25 (C-2') ppm; HRMS (ESI): calcd for $[C_{22}H_{21}N_3O_9P Na]^+$: 544.0892, found: 544.0878.

4.1.2.2. 3'-O-Benzoyl-5-fluoro-2'-deoxyuridin-5'-yl N-decylpyridinium-3-yl phosphate (4f). Bright yellow solid; yield: 77%; RP HPLC Rt 23.248 min; 1H NMR (400 MHz, DMSO- d_6): δ_H 8.95 (s, 1H of 3-Py), 8.71 (d, $J = 6.0$ Hz, 1H of Ar), 8.43 (d, $J = 8.4$ Hz, 1H of Ar), 8.10 (d, $J_{6-F5} = 6.8$ Hz, 1H, H-6), 7.98–8.02 (m, 3H of Ar), 7.69 (t, $J = 7.4$ Hz, 1H of Ar), 7.55 (t, $J = 7.8$ Hz, 1H of Ar), 6.27 (t, $J = 6.8$ Hz,

1H, H-1'), 5.43 (br s, 1H, H-3'), 4.53 (t, $J = 7.4$ Hz, 2H, CH₂), 4.29 (br s, 1H, H-4'), 4.03–4.12 (m, 2H, H-5', H-5''), 2.40–2.42 (m, 2H, H-2', H-2''), 1.86 (br s, 2H, CH₂), 1.21–1.25 (m, 14H, CH₂), 0.84 (t, $J = 6.8$ Hz, 3H, CH₃) ppm; ¹³C NMR (100 MHz, DMSO-d₆): δ_c 165.11 (C=O), 157.04 (d, $J = 25$ Hz, C-4), 153.71 (d, $J = 5$ Hz, C of Ar), 149.17 (C-2), 140.14 (d, $J = 231$ Hz, C-5), 137.83, 136.61 (d, $J = 6$ Hz), 135.58 (d, $J = 3$ Hz), 133.64, 129.21, 128.76, 128.08 (C of Ar), 124.40 (d, $J = 34$ Hz, C-6), 84.48 (C-1'), 83.08 (d, $J = 7$ Hz, C-1'), 75.57 (C-3'), 65.18 (d, $J = 6$ Hz, CH₂), 60.87 (C-5'), 36.32 (C-2'), [31.23, 30.74, 28.84, 28.74, 28.61, 28.35, 25.36, 20.05 (CH₂)], 13.92 (CH₃) ppm; HRMS (ESI): calcd for [C₃₁H₃₉N₃O₉FPNa]⁺: 670.2300, found: 670.2306.

4.1.2.3. 3'-O-benzoyl-5-fluoro-2'-deoxyuridin-5'-yl N-benzylpyridinium-3-yl phosphate (4g). White solid; yield: 78%; RP HPLC Rt 17.788 min; ¹H NMR (400 MHz, DMSO-d₆): δ_H 9.10 (s, 1H of Ar), 8.82 (d, $J = 6.0$ Hz, 1H of Ar), 8.43 (dd, $J = 8.4$ Hz, $J = 2$ Hz, 1H of Ar), 8.11 (d, 1H, $J_{6-F5} = 6.8$ Hz, H-6), 7.99–8.05 (m, 3H of Ar), 7.69 (t, $J = 7.4$ Hz, 1H of Ar), 7.50–7.58 (m, 4H of Ar), 7.40–7.44 (m, 3H of Ar), 6.25 (t, $J = 6.6$ Hz, 1H, H-1'), 5.80 (s, 2H of CH₂), 5.40–5.42 (m, 1H, H-3'), 4.33 (br s, 1H, H-4'), 3.98–4.11 (m, 2H, H-5', H-5''), 2.39–2.43 (m, 2H, H-2', H-2''), ppm; ¹³C NMR (100 MHz, DMSO-d₆): δ_c 165.60 (C=O), 157.40 (d, $J = 26$ Hz, C-4), 154.40 (d, $J = 5$ Hz), 149.55 (C-2), 140.08 (d, $J = 231$ Hz, C-5), 137.86 (C of Ar), 136.60 (d, $J = 7$ Hz), 135.66 (d, $J = 4$ Hz), 134.17, 133.64, 129.33, 129.28, 129.22, 129.14, 128.78, 128.63, 128.53 (C of Ar), 124.44 (d, $J = 34$ Hz, C-6), 84.95 (C-1'), 83.56 (d, $J = 7$ Hz, C-4'), 76.03 (C-3'), 66.82 (CH₂), 65.92 (d, $J = 6$ Hz, C-5'), 36.81 (C-2') ppm; HRMS (ESI): calcd for [C₂₈H₂₅N₃O₉FPNa]⁺: 620.1205, found: 620.1221.

4.1.2.4. 3'-O-benzoyl-5-fluoro-2'-deoxyuridin-5'-yl (N-carboethoxymethyl)pyridinium-3-yl phosphate (4h). White solid; yield: 72%; RP HPLC Rt 16.837 min; ¹H NMR (400 MHz, DMSO-d₆): δ_H 11.86 (br s, 1H, NH), 8.95 (s, 1H of Ar), 8.67 (d, $J = 6.0$ Hz, 1H of Ar), 8.66 (dd, $J = 8.8$ Hz, $J = 1.6$ Hz, 1H of Ar), 8.16 (d, 1H, $J_{6-F5} = 6.8$ Hz, H-6), 8.08–8.11 (m, 3H of Ar), 7.80 (dd, $J = 8.4$ Hz, $J = 1.2$ Hz, 2H of Ar), 7.69 (t, $J = 7.4$ Hz, 1H of Ar), 7.55 (t, $J = 7.6$ Hz, 1H of Ar), 6.26 (t, $J = 6.6$ Hz, 1H, H-1'), 5.61 (s, 2H of CH₂), 5.44–5.45 (m, 1H, H-3'), 4.30 (br s, 1H, H-4'), 4.23 (q, $J = 7.0$ Hz, 2H, CH₂), 4.05–4.10 (m, 2H, H-5', H-5''), 2.33–2.50 (m, 2H, H-2', H-2''), 1.25 (t, $J = 7.0$ Hz, 3H, CH₃) ppm; ¹³C NMR (100 MHz, DMSO-d₆): δ_c 166.23 (C=O), 165.13 (C=O), 156.96 (d, $J = 27$ Hz, C-4), 153.51 (d, $J = 5$ Hz), 149.09 (C-2), 139.44 (C of Ar), 140.13 (d, $J = 231$ Hz, C-5), 138.76 (d, $J = 6$ Hz, C of Ar), 136.73, 133.64, 129.35, 129.25, 128.79, 127.92 (C of Ar), 124.51 (d, $J = 34$ Hz, C-6), 84.50 (C-1'), 83.08 (d, $J = 7$ Hz, C-4'), 75.59 (C-3'), 65.47 (C-5'), 62.25 (CH₂), 60.34 (CH₂), 36.33 (C-2'), 13.89 (CH₃) ppm; HRMS (ESI): calcd for [C₂₅H₂₆N₃O₁₁FPNa]⁺: 616.1103, found: 616.1123.

4.1.2.5. 3'-O-benzoyl-5-fluoro-2'-deoxyuridin-5'-yl (N-carboethoxymethyl)quinolinium-6-yl phosphate (4i). Orange solid; yield: 58%; RP HPLC Rt 16.942 min; ¹H NMR (400 MHz, DMSO-d₆): δ_H 11.86 (br s, 1H, NH), 9.31 (d, $J = 6.0$ Hz, 1H of Ar), 9.22 (d, $J = 8.4$ Hz, 1H of 6-HQ), 8.32–8.36 (m, 1H of Ar), 8.08–8.21 (m, 4H, H-6, 6-HQ), 7.97 (d, $J = 7.2$ Hz, 2H of Ar), 7.68 (t, $J = 7.4$ Hz, 1H of Ar), 7.54 (t, $J = 8.0$ Hz, 1H of Ar), 6.26 (t, $J = 6.6$ Hz, 1H, H-1'), 6.07 (s, 2H of CH₂), 5.47 (br s, 1H, H-3'), 4.30 (br s, 1H, H-4'), 4.22 (q, $J = 7.0$ Hz, 2H, CH₂), 4.01–4.15 (m, 2H, H-5', H-5''), 2.39–2.43 (m, 2H, H-2', H-2''), 1.23 (t, $J = 7.0$ Hz, 3H, CH₃) ppm; ¹³C NMR (100 MHz, DMSO-d₆): δ_c 166.02 (C=O), 165.05 (C=O), 156.98 (d, $J = 26$ Hz, C-4), 154.70 (d, $J = 6$ Hz, C of Ar), 149.07 (C-2), 148.54, 147.46 (C of Ar), 140.08 (d, $J = 231$ Hz, C-5), 134.03, 133.59, 130.89, 129.29, 129.22, 129.08, 128.75 (C of Ar), 121.51 (d, $J = 34$ Hz, C-6), 121.92, 119.97, 116.4 (C of Ar), 84.46 (C-1'), 83.24 (d, $J = 7$ Hz, C-4'), 75.79 (d, C-3'), 65.21 (d, $J = 6$ Hz, C-5'), 62.36 (CH₂), 57.39 (CH₂), 36.36 (C-2'), 13.87 (CH₃) ppm; HRMS (ESI):

calcd for [C₂₉H₂₇N₃O₁₁FPNa]⁺: 666.1259, found: 666.1280.

4.1.3. A general procedure for the synthesis of 5-fluoro-2'-deoxyuridin-5'-yl aryl phosphates of type 5

3'-O-Benzoyl-5-fluoro-2'-deoxyuridin-5'-yl aryl phosphates **4a**, **4c** and **4d** were dissolved in 40% aqueous solution of MeNH₂ (0.1 mmol/1 mL) and stirred overnight at ambient temperature. By this time, the reaction was complete (TLC), the solvent evaporated and the residue was dissolved in water, and then washed twice with methylene chloride. The aqueous layer was separated, evaporated and final products were obtained after passage of the reaction mixture through silica gel column using 0–50% gradient of MeOH in toluene. Fractions containing pure products were collected and evaporated, yielding amorphous solids.

4.1.3.1. 5-Fluoro-2'-deoxyuridin-5'-yl phenyl phosphate (5a). White solid; yield: 83%; RP HPLC Rt 17.395 min; ¹H NMR (400 MHz, DMSO-d₆): δ_H 7.98 (d, $J_{6-F5} = 6.8$ Hz, 1H, H-6), 7.14–7.20 (m, 4H of Ar), 6.94 (t, $J = 7.2$ Hz, 1H of Ar), 6.16 (t, $J = 6.2$ Hz, 1H, H-1'), 4.21 (br s, 1H, H-4'), 3.82–3.92 (m, 3H, H-4', H-5', H-5''), 2.02–2.06 (m, 2H, H-2', H-2'') ppm; ¹³C NMR (100 MHz, DMSO-d₆): δ_c 158.91 (d, $J = 24$ Hz, C-4), 154.48 (d, $J = 6$ Hz), 150.68 (C-2), 140.76 (d, $J = 231$ Hz, C-5), 128.79 (C of Ar), 124.65 (d, $J = 34$ Hz, C-6), 121.08, 120.25 (C of Ar), 86.48 (d, $J = 8$ Hz, C-4'), 84.94 (C-1'), 71.41 (C-3'), 65.51 (d, $J = 5$ Hz, C-5'), 23.78 (C-2') ppm; HRMS (ESI): calcd for [C₁₅H₁₅N₂O₈FP][−]: 401.0556, found: 401.0554.

4.1.3.2. 5-Fluoro-2'-deoxyuridin-5'-yl pyridin-3-yl phosphate (5c). White solid; yield: 76%; RP HPLC Rt 12.563 min; ¹H NMR (400 MHz, DMSO-d₆): δ_H 11.91, (br s, 1H, NH), 8.34 (d, $J = 2.8$ Hz, 1H of Ar), 8.16 (dd, $J = 4.8$ Hz, $J = 1.2$ Hz, 1H of Ar), 8.03 (d, $J_{6-F5} = 6.8$ Hz, 1H, H-6), 7.58–7.60 (m, 1H of Ar), 7.22–7.25 (m, 1H of Ar), 6.12–6.16 (m, 1H, H-1'), 4.21 (br s, 1H, H-3'), 3.86–3.90 (m, 3H, H-4', H-5', H-5''), 2.00–2.12 (m, 2H, H-2', H-2''), ppm; ¹³C NMR (100 MHz, DMSO-d₆): δ_c 157.12 (d, $J = 25$ Hz, C-4), 151.83 (d, $J = 6$ Hz), 149.82 (C-2), 143.13, 142.49, 142.43 (C of Ar), 140.52 (d, $J = 231$ Hz, C-5), 127.14, 127.10 (C of Ar), 124.99 (d, $J = 34$ Hz, C-6), 124.29, 124.05 (C of Ar), 86.47 (d, $J = 7$ Hz, C-4'), 84.96 (C-1'), 71.28 (C-3'), 65.61 (d, $J = 5$ Hz, C-5'), 25.95 (C-2') ppm; HRMS (ESI): calcd for [C₁₄H₁₄N₃O₈FP][−]: 402.0508, found: 402.0523.

4.1.3.3. 5-Fluoro-2'-deoxyuridin-5'-yl quinolin-6-yl phosphate (5d). Orange solid; yield: 63%; RP HPLC Rt 13.510 min; ¹H NMR (400 MHz, D₂O): δ_H 11.80 (br s, NH), 8.73 (dd, $J = 4.8$ Hz, $J = 1.6$ Hz, 1H of Ar), 8.17 (d, $J = 8.0$ Hz, 1H of Ar), 8.06 (d, $J = 6.8$ Hz, 1H of H-6), 7.87 (d, $J = 8.8$ Hz, 1H of Ar), 7.67 (s, 1H of Ar), 7.58 (dd, $J = 9.2$ Hz, $J = 2.4$ Hz, 1H of Ar), 7.42 (dd, $J = 8.4$ Hz, $J = 4.0$ Hz, 1H of Ar), 6.14 (t, $J = 6.2$ Hz, 1H, H-1'), 5.39 (br s, 1H, OH), 4.22 (br s, 1H, H-3'), 3.87–3.96 (m, H-4', H-5', H-5''), 1.98–2.13 (m, 2H, H-2', H-2'') ppm; ¹³C NMR (100 MHz, DMSO-d₆): δ_c 157.54 (d, $J = 25$ Hz, C-4), 151.90 (d, $J = 6$ Hz), 149.49, 148.34 (C-2), 144.13 (C of Ar), 140.08 (d, $J = 231$ Hz, C-5), 134.88, 129.54, 128.54, 124.92 (C of Ar), 124.89 (d, $J = 34$ Hz, C-6), 121.30, 114.62 (C of Ar), 86.06 (d, $J = 7$ Hz, C-4'), 84.49 (C-4'), 70.86 (C-3'), 65.16 (d, $J = 5$ Hz, C-5'), 39.32 (C-2') ppm; HRMS (ESI): calcd for [C₁₈H₁₆N₃O₈FP][−]: 452.0665, found: 452.0676.

4.1.4. Measurement of decomposition half time ($t_{1/2}$) for compounds 4 and 5 in RPMI, RPMI/FBS 9: 1 (v/v) and HS

Stability of phosphodiester **4** and **5** was examined in cell culture media RPMI, RPMI/FBS 9: 1 (v/v) and human sera (HS). To this end, 2 mM solution of each compound in the aforementioned media, containing 5% (v/v) DMSO, were prepared and then incubated at 37 °C. 20 μ L samples were analysed with HPLC in respective time intervals. The peaks of substrate and decomposition products were identified by spiking with authentic samples, and

their amount was measured by integrating the peaks' areas. Microcal Origin v. 3.5 programme was used for calculation of $t_{1/2}$. The results are listed in Table 1.

4.2. Biological assays

4.2.1. Cell lines and culture conditions

GBM cell lines (T98G, U-118 MG, U-87 MG), HeLa (cervical cancer cell line), T-47D (breast cancer cell line), Caco-2 (colon cancer cell line), and non-cancerous lung fibroblast cell line (MRC-5) were purchased from ATCC (Manassas, USA). All cell lines were of human origin. HeLa and T-47D were cultured in RPMI 1640 medium. Caco-2 and U-118 MG were cultured in DMEM medium. T98G, U-87 MG and MRC-5 were cultured in EMEM medium. Each medium was supplemented with 10% fetal bovine serum (FBS) and 10 mg/mL of antibiotics (penicillin and streptomycin). Cells were cultured at 37 °C with 5% CO₂ in humidified air. Cell media (RPMI 1640, DMEM, EMEM) and Human Blood Plasma (HBP) were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and ATCC. Cell concentrations in the culture were adjusted to allow for their exponential growth.

4.2.2. Cell viability/proliferation assay

Cell viability/proliferation was evaluated with a dye-staining method, using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) [65]. The monolayer cell culture was trypsinized and computed. Subsequently, 100 μ L of the diluted cell suspension (1×10^4 cells) was added to each well of the 96-well plate. After 24 h, when a partial monolayer was formed, 100 μ L of fresh medium with different compound concentrations (7.81, 15.625, 31.25, 62.5, 125, 250, 500 and 1000 μ g/mL) were added to the cells. After 48 h, the supernatant was washed out and 100 μ L of MTT solution in medium (final concentration – 0.5 mg/mL) was added to each well for 2 h. After the incubation time passed, unreacted dye was removed through aspiration. The formazan crystals were dissolved in 100 μ L/well DMSO, and measured spectrophotometrically in a multi-well Synergy2 plate reader (BioTek Instruments, USA) at a test wavelength of 492 nm and a reference wavelength of 690 nm. The results were calculated, resulting in IC₅₀ (inhibitory concentration 50) – the IC₅₀ corresponds to the concentration of the tested compound that inhibits cell viability/proliferation by 50%. Results were presented in the form of at least three independent experiments.

4.2.3. In silico pharmacokinetic prediction

Calculations of pharmacokinetic profile descriptors of the synthesized compounds were performed with various software packages, accessible on-line. The transformation of the stoichiometric formulas of the compounds into a SMILES code (Simplified Molecular Input Line Entry System) was carried out with ChemBioDraw Ultra version 12.0 program (Cambridge Software). The SMILES code was applied to calculate the logP values (octanol/water partition coefficient), in eight variants (ALOGPs, AC_logP, miLogP, ALOGP, MLOGP, LogKOWWIN, XLOGP2, XLOGP3), PSA (topological polar surface area) and aPSA (apolar surface area). The LogP values were calculated with ALOGPS 2.1 software (<http://www.vcclab.org/lab/alogps>) [66]. PSA and aPSA descriptors were calculated using the VEGA ZZ program (<http://www.vegazz.net>) [67]. The pharmacokinetic profiles were also evaluated according to the Lipinski's "rule of five" [48–50], using Molinspiration application (<http://www.molinspiration.com>), which analyses molecular weight (MW), number of hydrogen-bond acceptors (HBA) and number of hydrogen-bond donors (HBD). The Caco-2 prediction model, based on descriptors generated by preADMET (<http://preadmet.bmdrc.org>), was used to compute Caco-2 apparent

permeability (tP_{app}), for the tested compounds. In this model, the number of hydrogen bond donors and three molecular surface area properties determine the membrane permeability of compounds.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.ejmech.2017.12.070>.

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MATERIAŁY UZUPEŁNIAJĄCE DO PUBLIKACJI

Szymańska-Michalak A. i wsp., Eur. J. Med. Chem. 2018

Supplementary material – ¹H, ¹³C, ³¹P NMR spectra, and RP HPLC chromatograms and cytotoxicity (IC₅₀) of chosen phenols

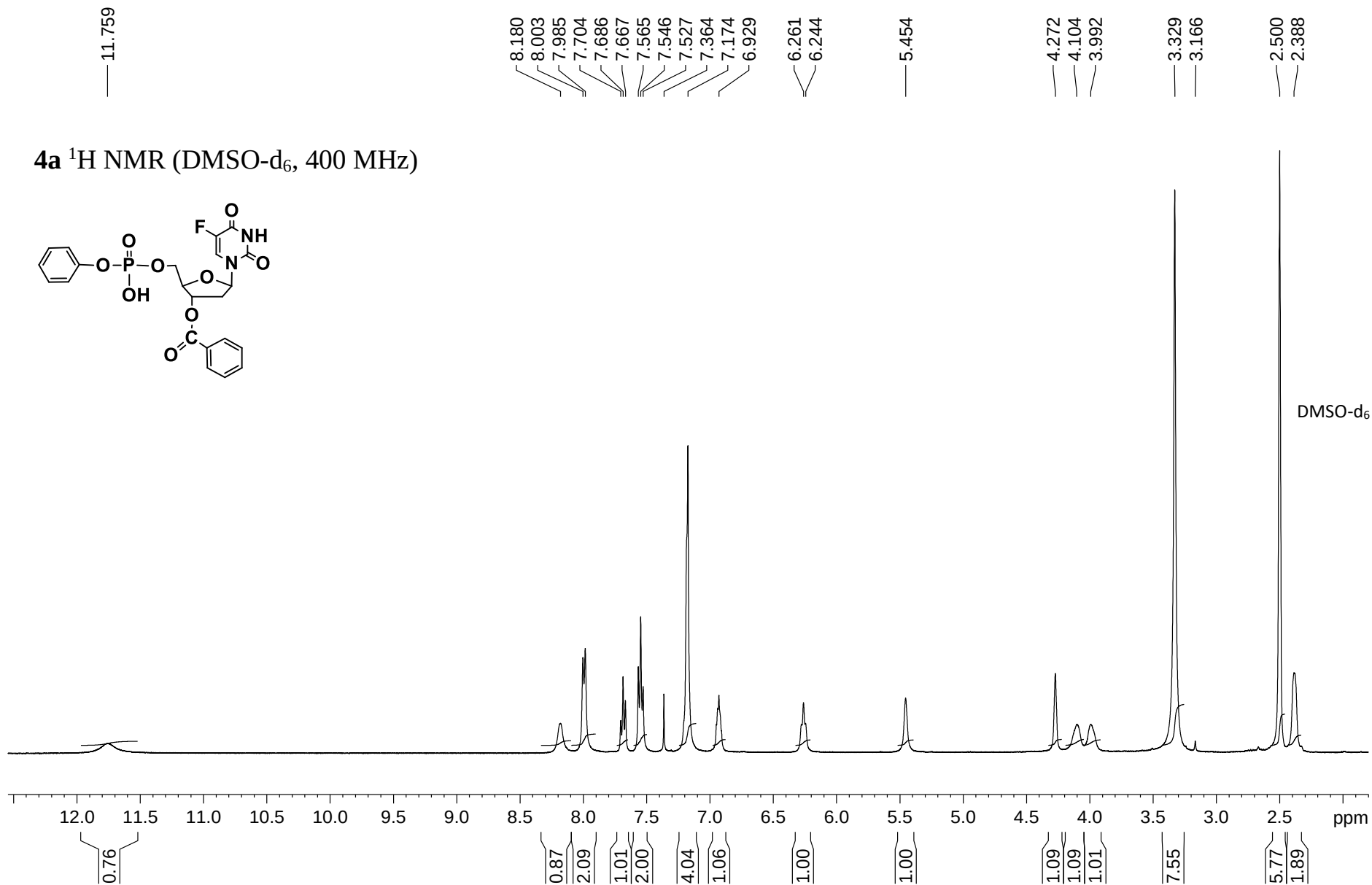
New anti glioma zwitterionic pronucleotides with an FdUMP framework

Agnieszka Szymañska-Michalak, Dariusz Wawrzyniak, Grzegorz Framski, Jacek Stawiñski, Jan Barciszewski and Adam Kraszewski

Institute of Bioorganic Chemistry, Polish Academy of Sciences, Noskowskiego 12/14, 61-704, Poznañ, Poland

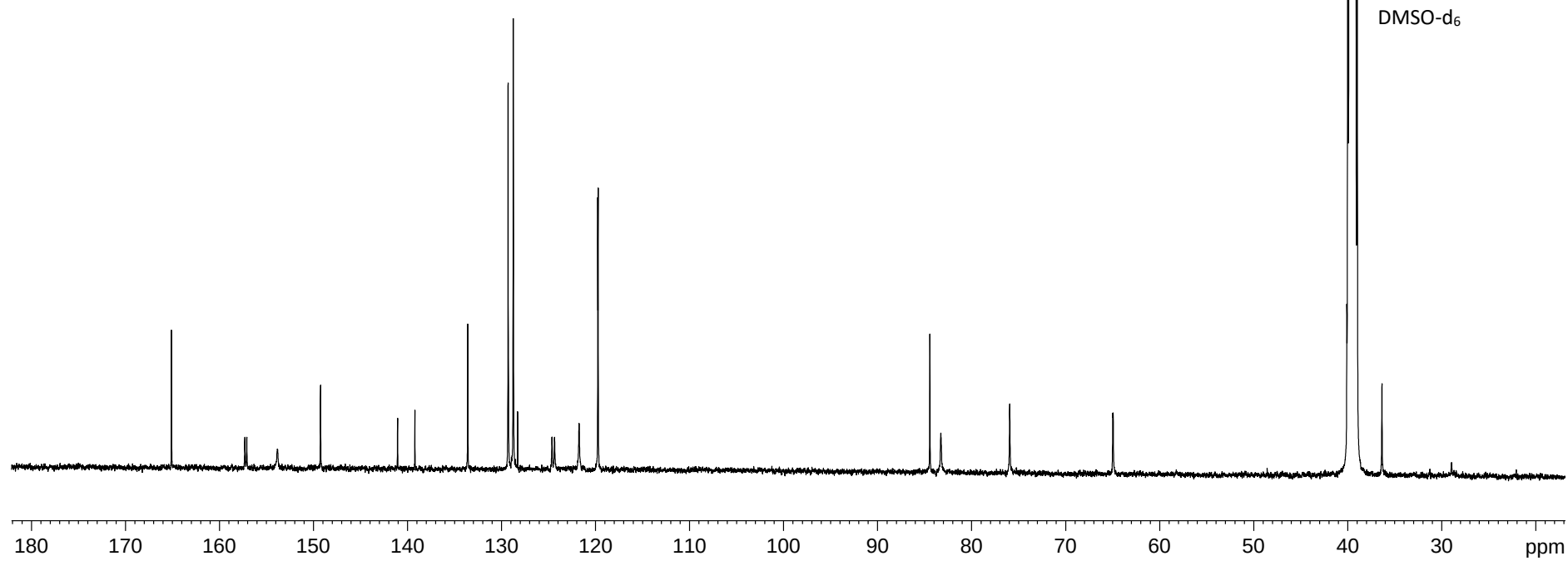
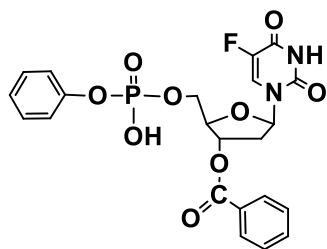
Contents

3'-O-Benzoyl-5-fluoro-2'-deoxyuridin-5'-yl phenyl phosphate (4a).....	S2
3'-O-Benzoyl-5-fluoro-2'-deoxyuridin-5'-yl 4-nonylphenyl phosphate (4b).....	S6
3'-O-Benzoyl-5-fluoro-2'-deoxyuridin-5'-yl pyridin-3-yl phosphate (4c).....	S10
3'-O-Benzoyl-5-fluoro-2'-deoxyuridin-5'-yl quinolin-6-yl phosphate (4d).....	S14
3'-O-Benzoyl-5-fluoro-2'-deoxyuridin-5'-yl N-methylpyridinium-3-yl phosphate (4e).....	S18
3'-O-Benzoyl-5-fluoro-2'-deoxyuridin-5'-yl N-decylpyridinium-3-yl phosphate (4f).....	S22
3'-O-Benzoyl-5-fluoro-2'-deoxyuridin-5'-yl N-benzylpyridinium-3-yl phosphate (4g).....	S26
3'-O-Benzoyl-5-fluoro-2'-deoxyuridin-5'-yl (N-carboethoxymethyl)pyridinium-3-yl phosphate (4h).....	S30
3'-O-Benzoyl-5-fluoro-2'-deoxyuridin-5'-yl (N-carboethoxymethyl)quinolinium-6-yl phosphate (4i).....	S34
3'-O-Benzoyl-5-fluoro-2'-deoxyuridin-5'-yl 2-(N,N,N-trimethylaminium)ethyl phosphate (4j).....	S38
3'-O-Benzoyl-5-fluoro-2'-deoxyuridin-5'-yl N,N-dimethylpiperidinium-4-yl phosphate (4k).....	S42
3'-O-Benzoyl-5-fluoro-2'-deoxyuridin-5'-yl 2-(N-benzyl-N,N-dimethylaminium)ethyl phosphate (4l).....	S46
3'-O-Benzoyl-5-fluoro-2'-deoxyuridin-5'-yl (3-endo)-8-benzyl-8-methyl-8-azabicyclo[3.2.1]octan-3-yl phosphate (4m).....	S50
5-Fluoro-2'-deoxyuridin-5'-yl phenyl phosphate (5a).....	S54
5-Fluoro-2'-deoxyuridin-5'-yl pyridin-3-yl phosphate (5c).....	S58
5-Fluoro-2'-deoxyuridin-5'-yl quinolin-6-yl phosphate (5d).....	S62
Table 1. Cytotoxicity (IC ₅₀) of chosen phenols of type 2	S66

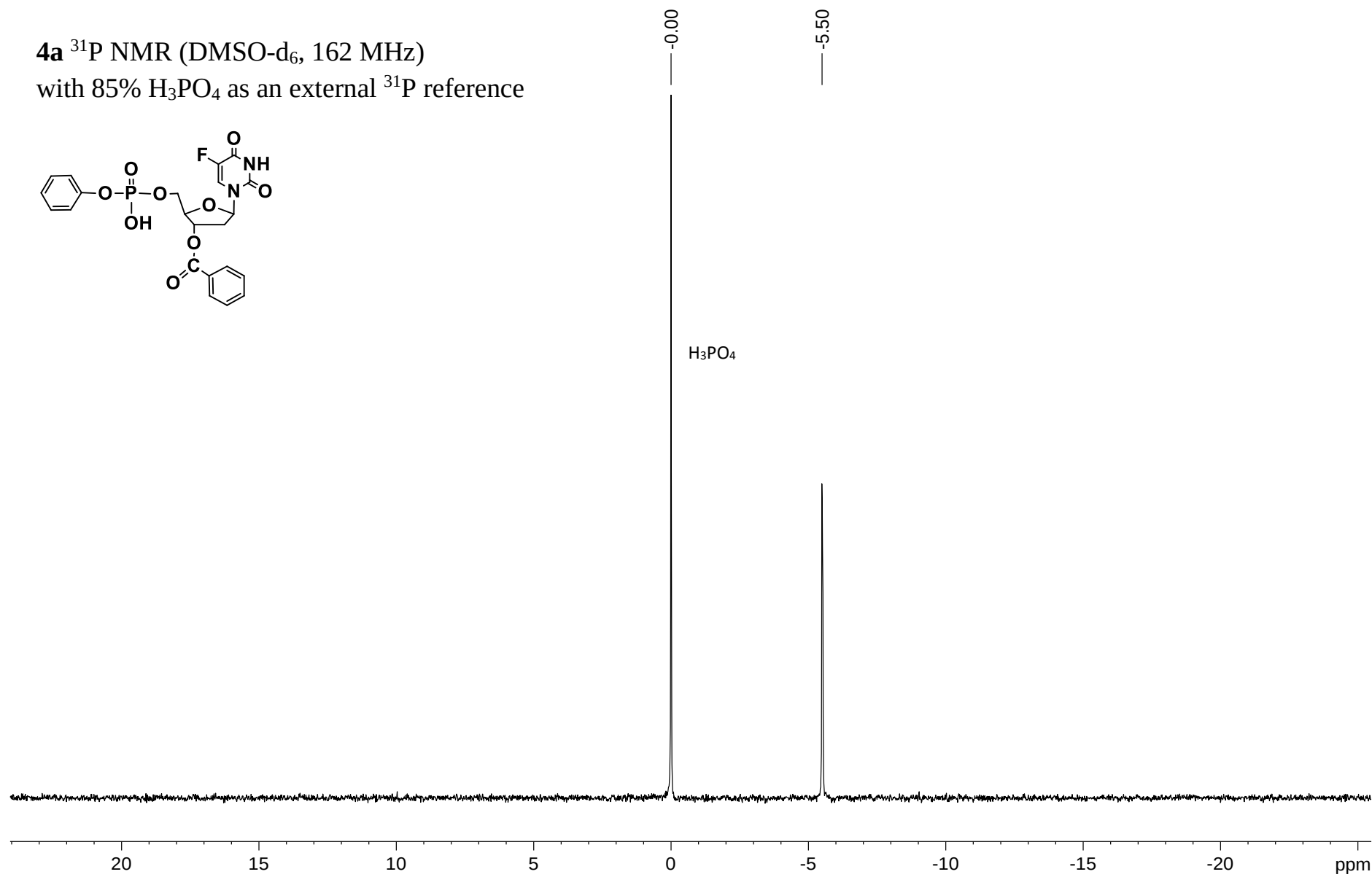
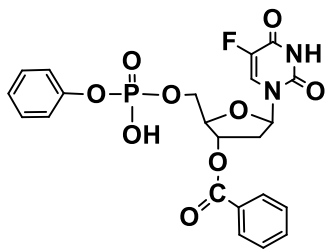




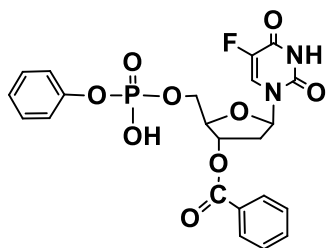
4a ^{13}C NMR (DMSO- d_6 , 125 MHz)



4a ^{31}P NMR (DMSO- d_6 , 162 MHz)
with 85% H_3PO_4 as an external ^{31}P reference

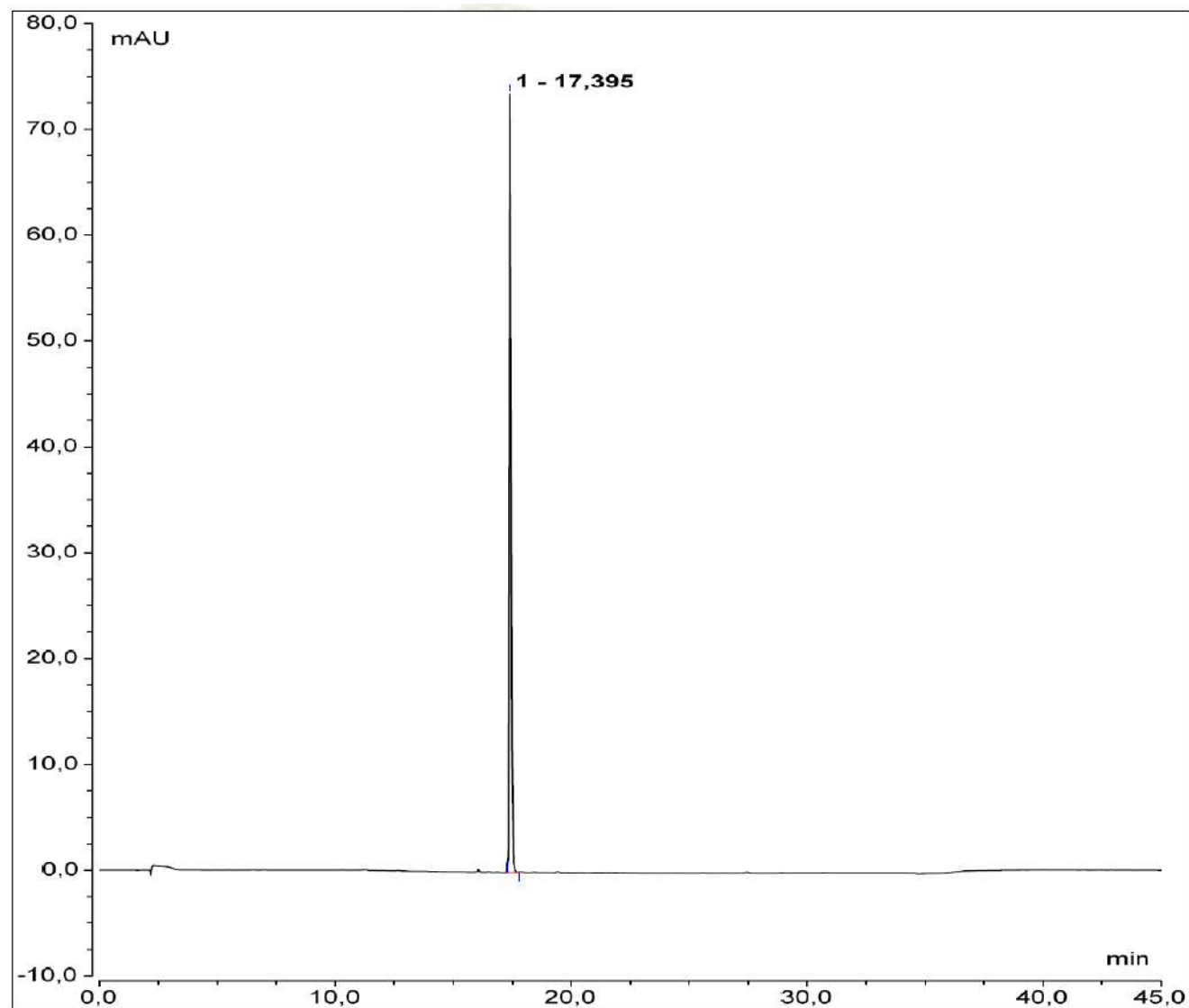


Chromatogram 4a



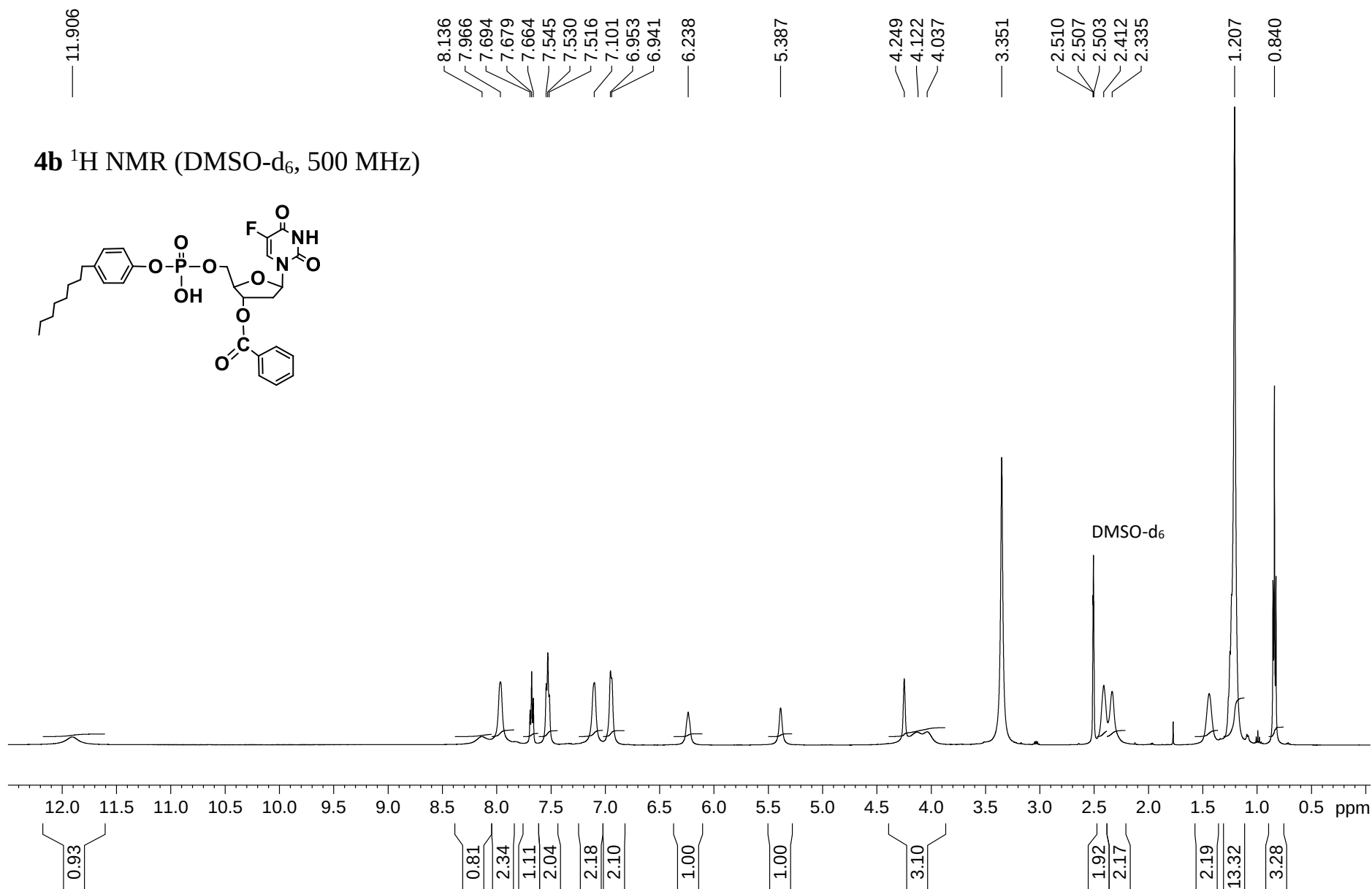
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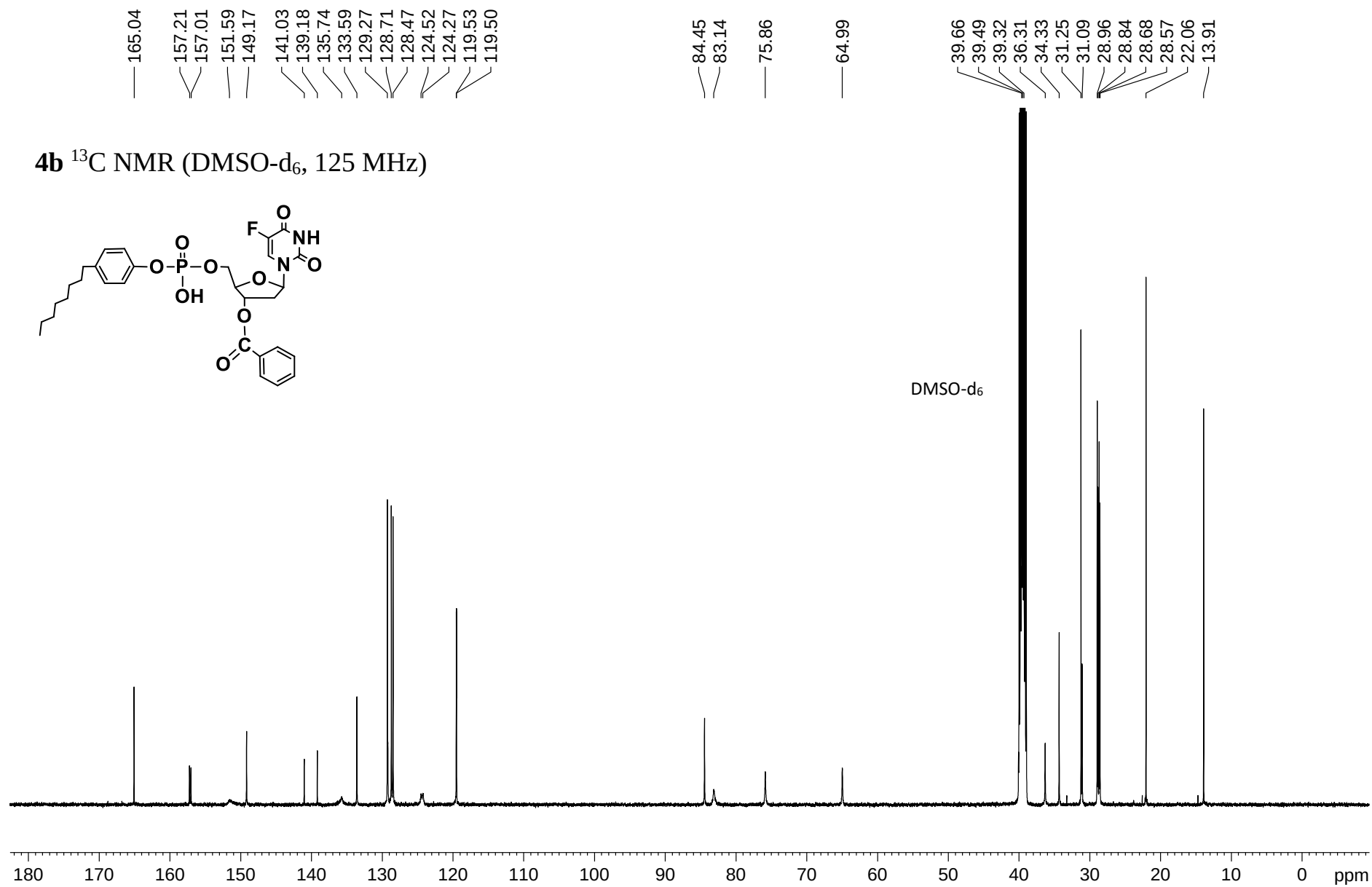
Thermo Scientific HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium acetate
pH 7; B, A / acetonitrile, 1: 4, v/v)
at 35°C, flow rate 1.5 mL/min;
events: 5 min A 100%, 20 min
B 0–100%, 5 min B 100% and
15 min A 100%.



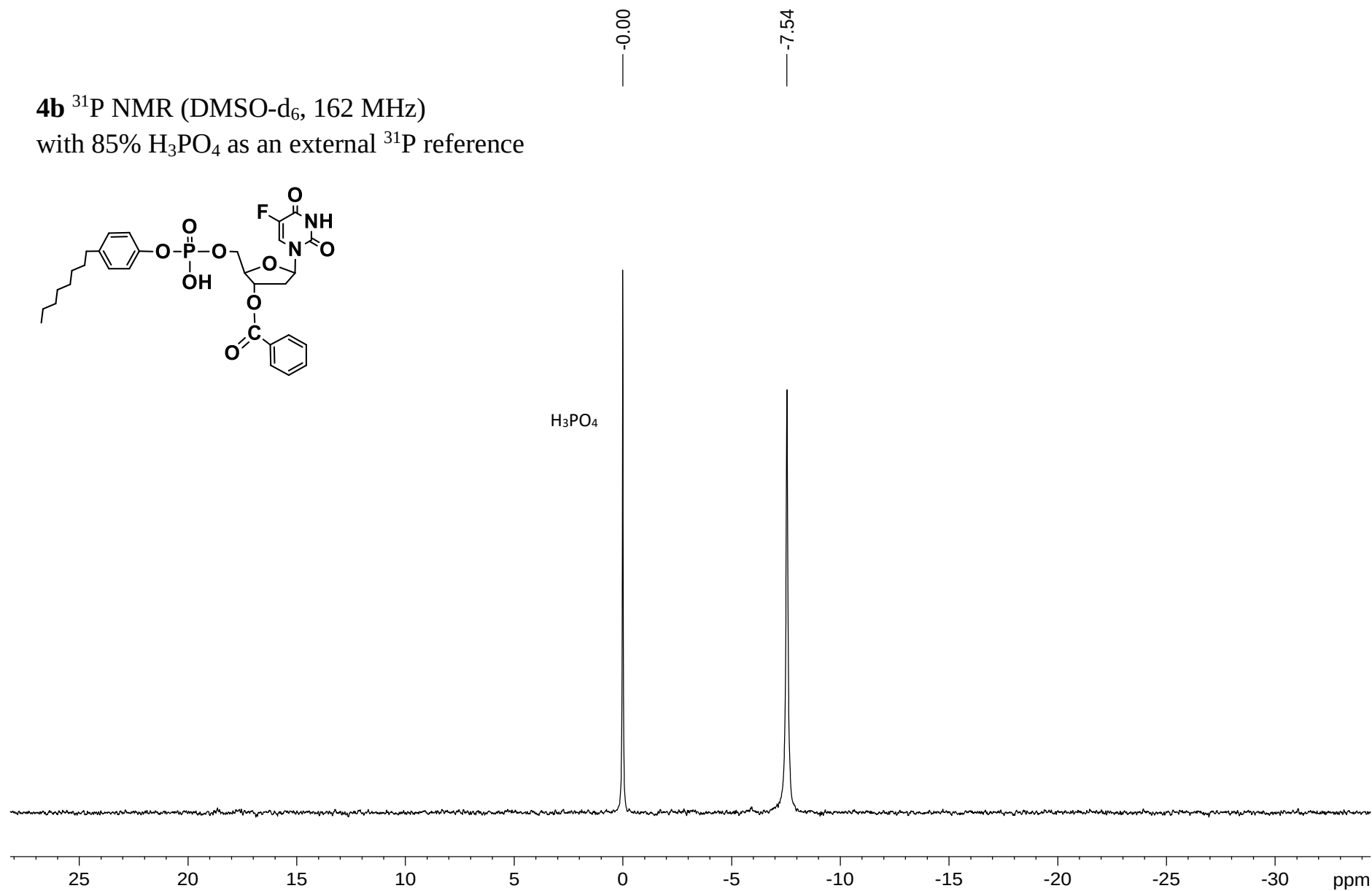
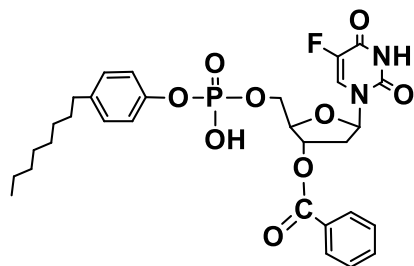
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Chromeleon (c) Dionex
Version 7.2.3.7553

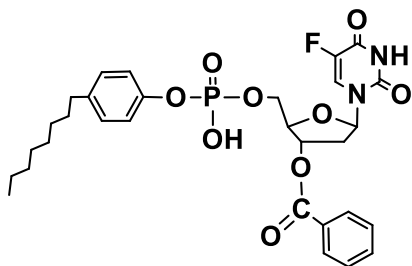




4b ^{31}P NMR (DMSO- d_6 , 162 MHz)
with 85% H_3PO_4 as an external ^{31}P reference

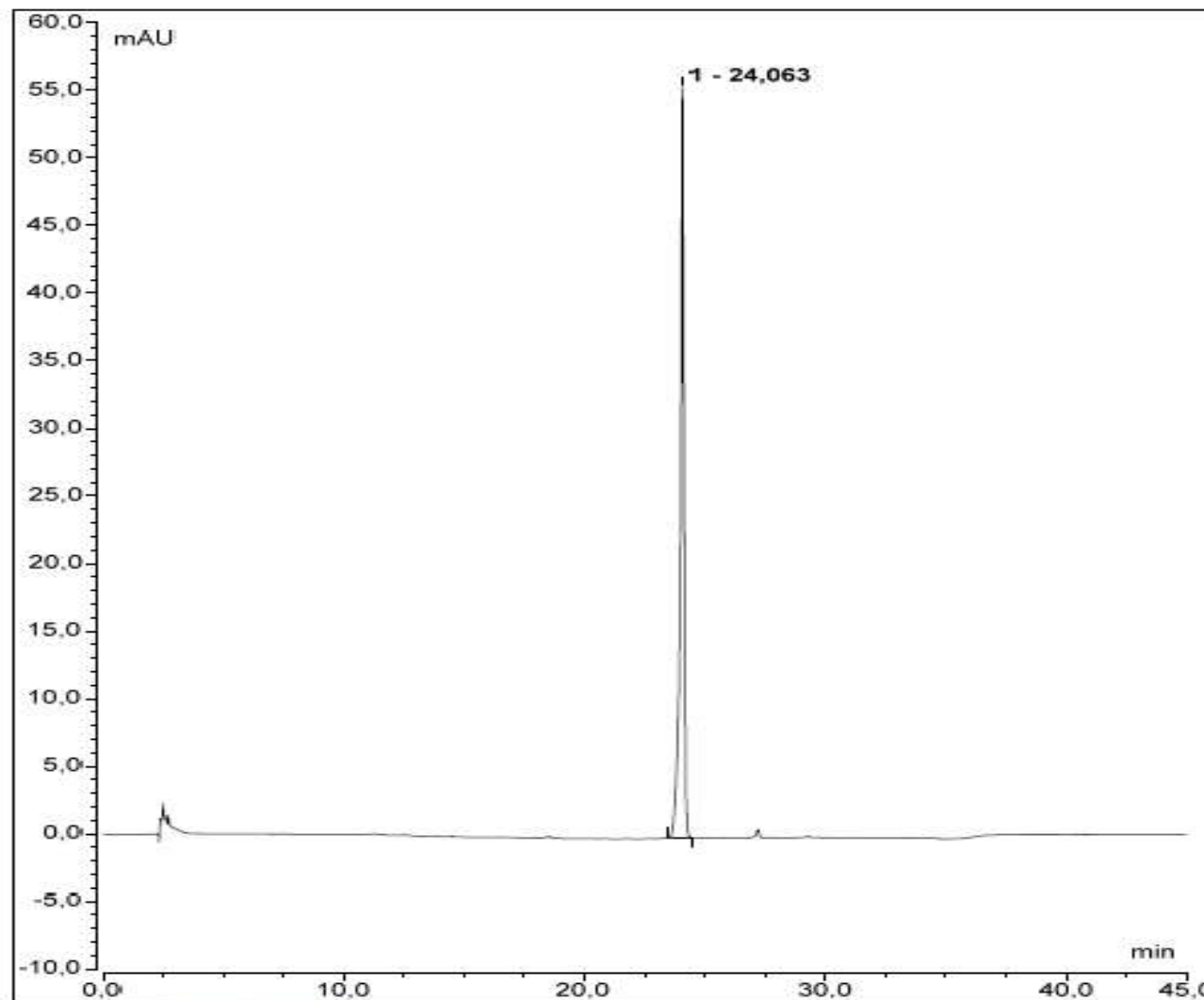


Chromatogram 4b



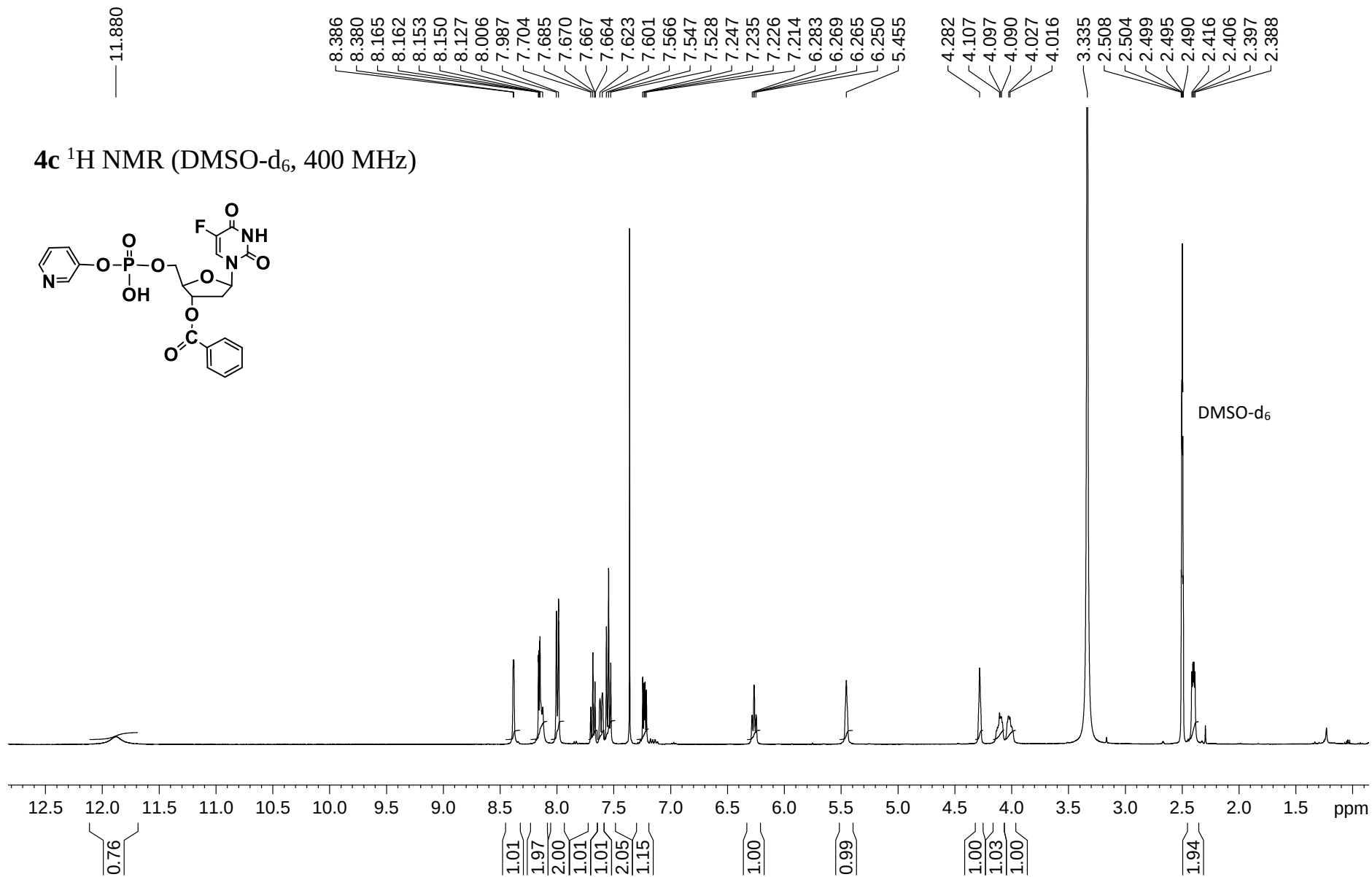
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(5.0 μm , 4.6 mm $\times$ 150 mm)

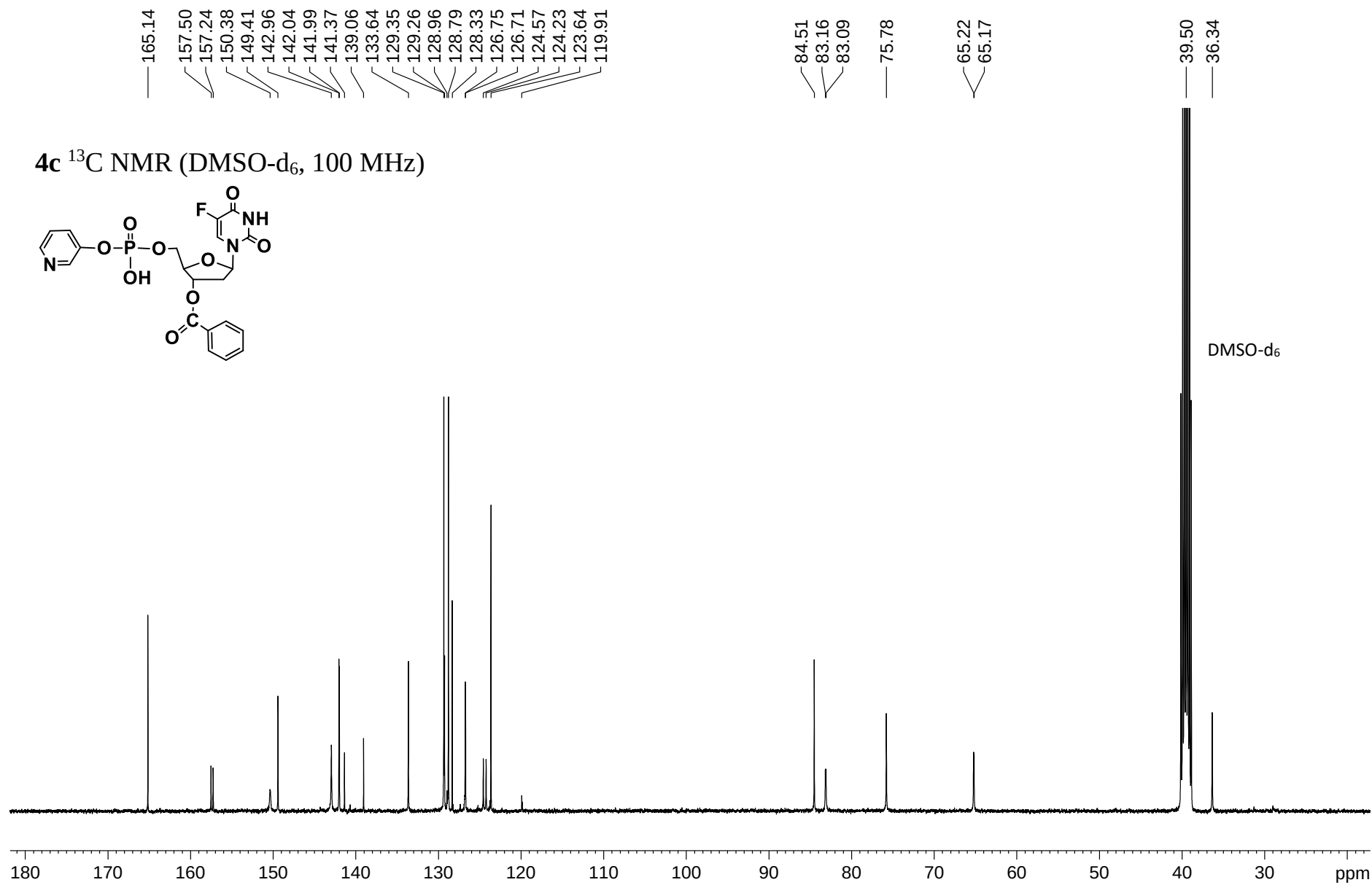
Thermo Scientific HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium acetate
pH 7; B, A / acetonitrile, 1: 4, v/v)
at 35°C, flow rate 1.5 mL/min;
events: 5 min A 100%, 20 min
B 0–100%, 5 min B 100% and
15 min A 100%.



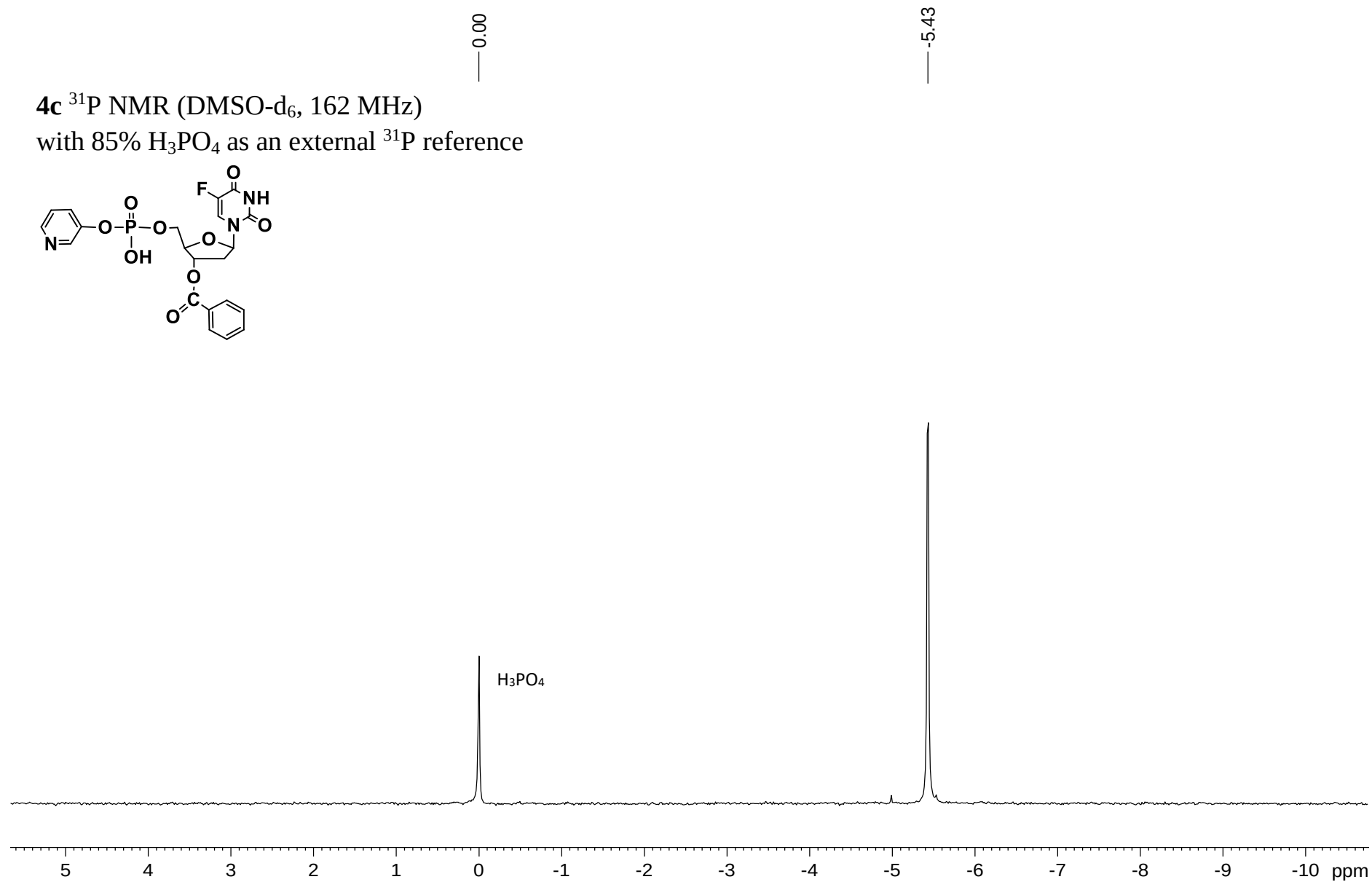
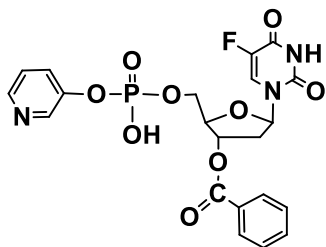
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Chromaleon (c) Dionex
Version 7.2.3.7553

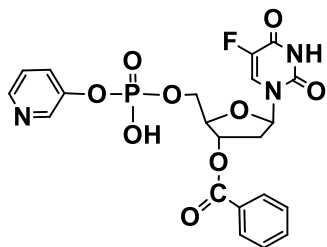




4c ^{31}P NMR (DMSO- d_6 , 162 MHz)
with 85% H_3PO_4 as an external ^{31}P reference

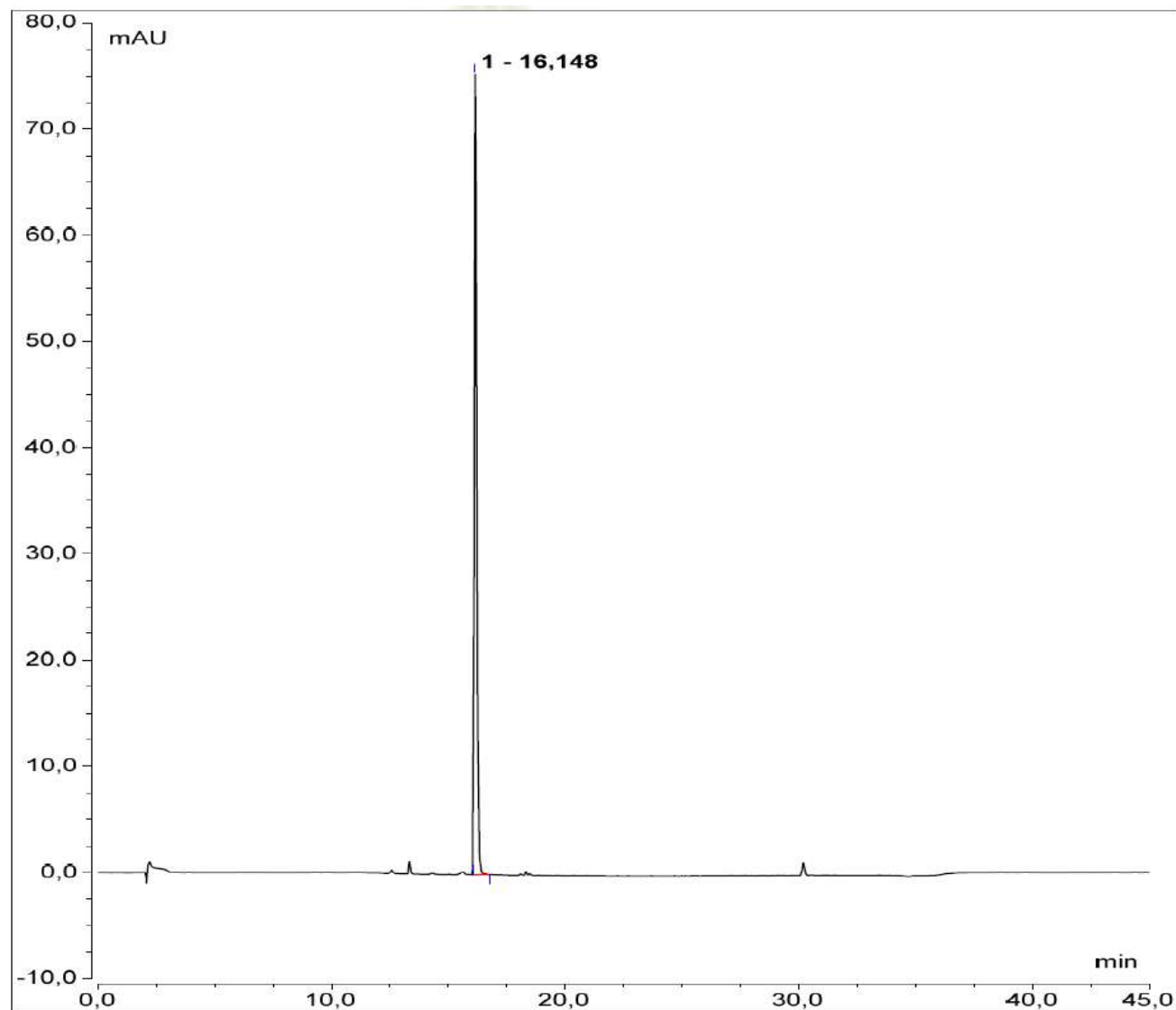


Chromatogram 4c



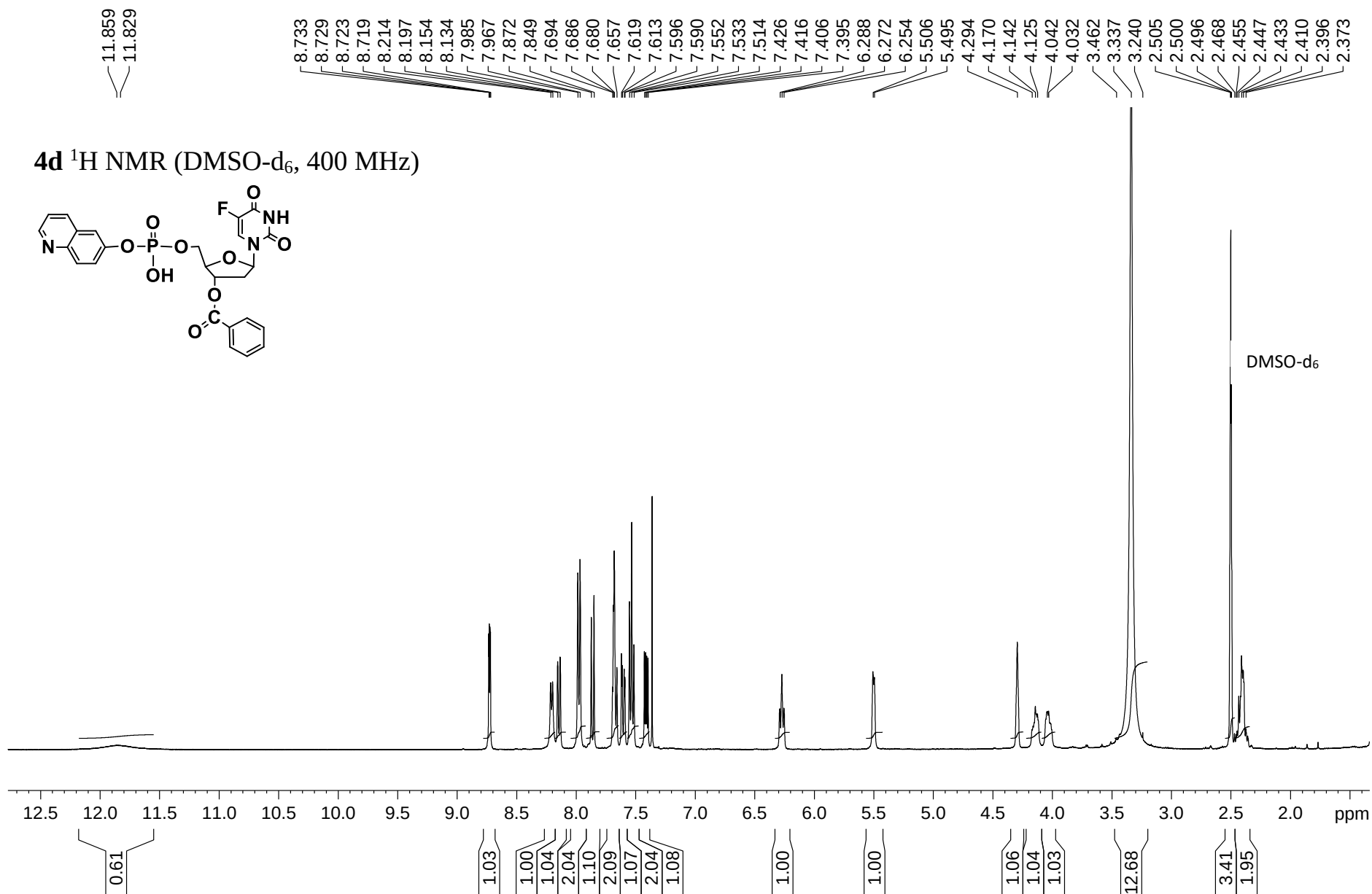
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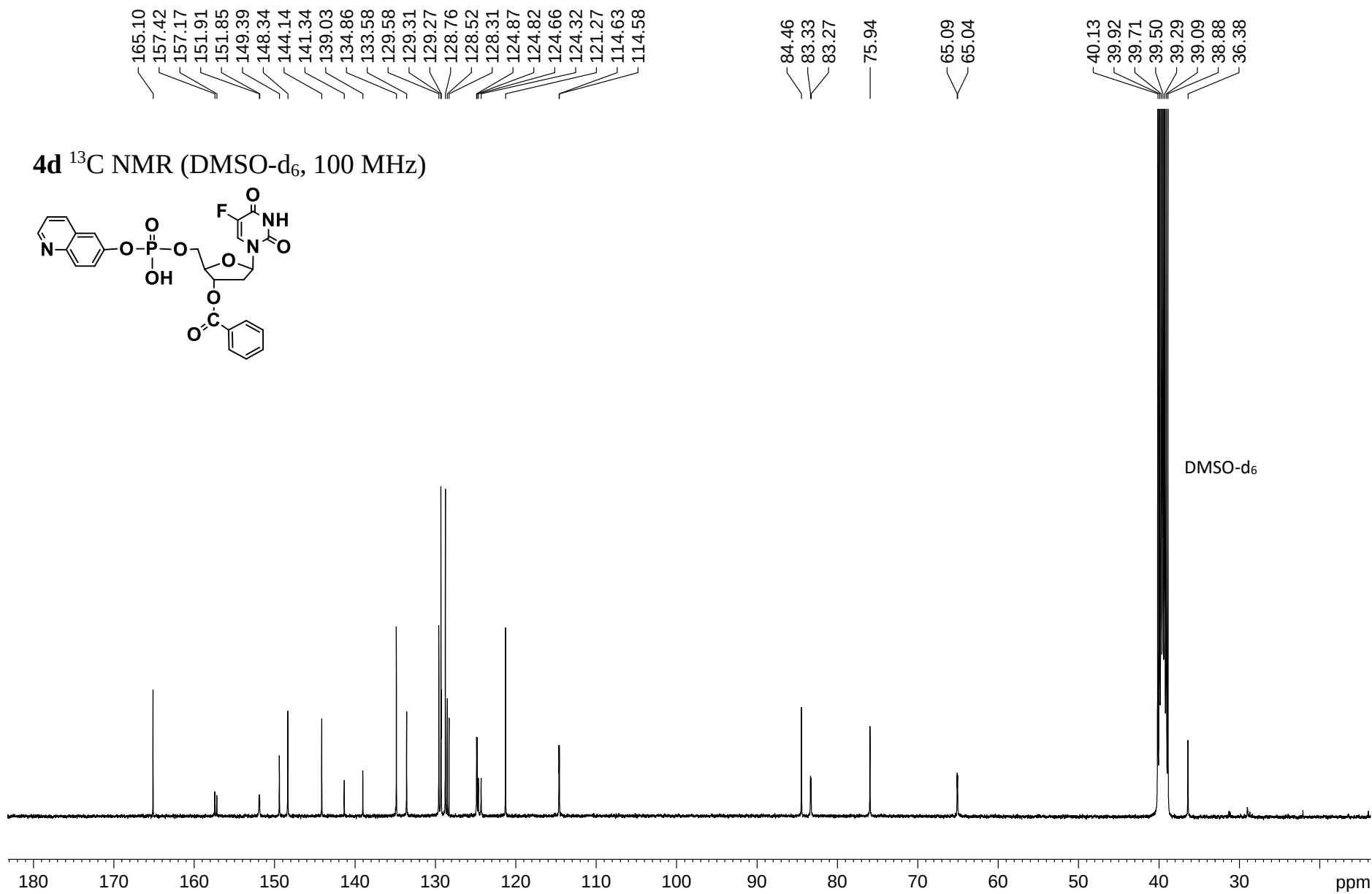
Thermo Scientific HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium acetate
pH 7; B, A / acetonitrile, 1: 4, v/v)
at 35°C, flow rate 1.5 mL/min;
events: 5 min A 100%, 20 min
B 0–100%, 5 min B 100% and
15 min A 100%.



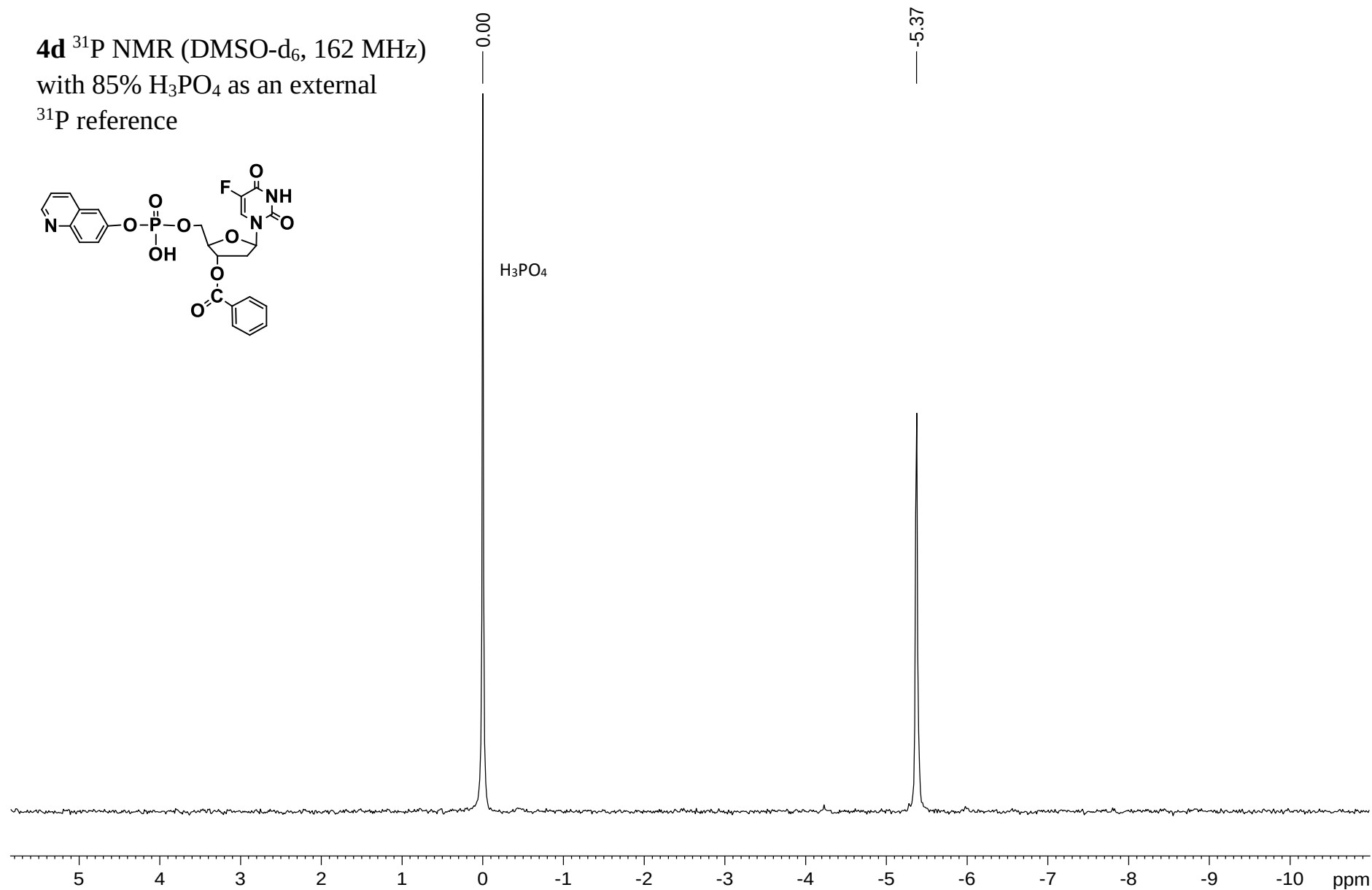
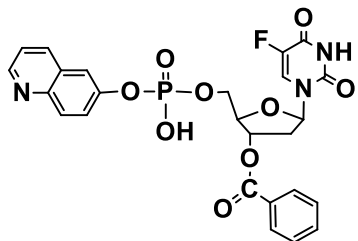
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Chromeleon (c) Dionex
Version 7.2.3.7553



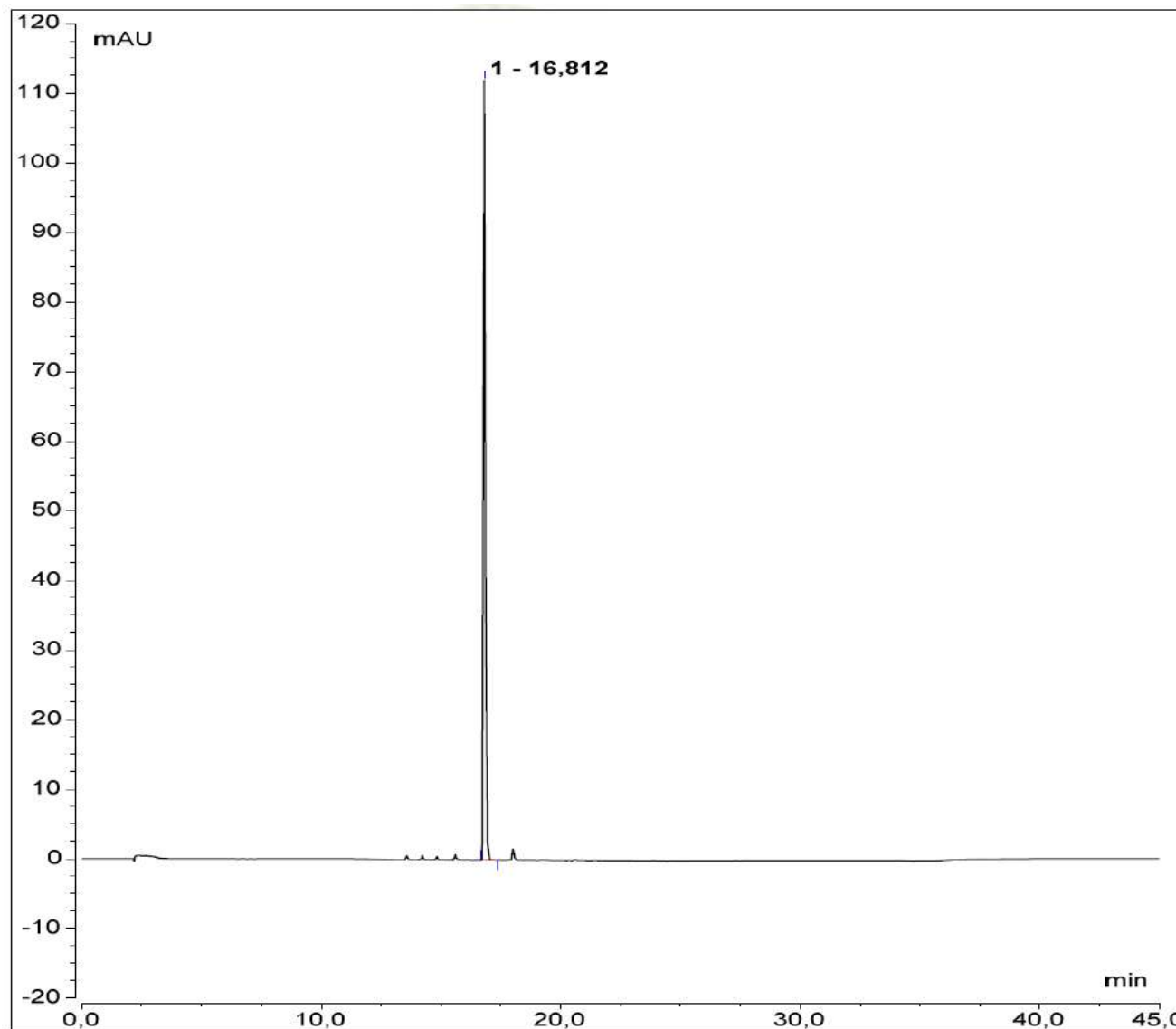


4d ^{31}P NMR (DMSO- d_6 , 162 MHz)
with 85% H_3PO_4 as an external
 ^{31}P reference

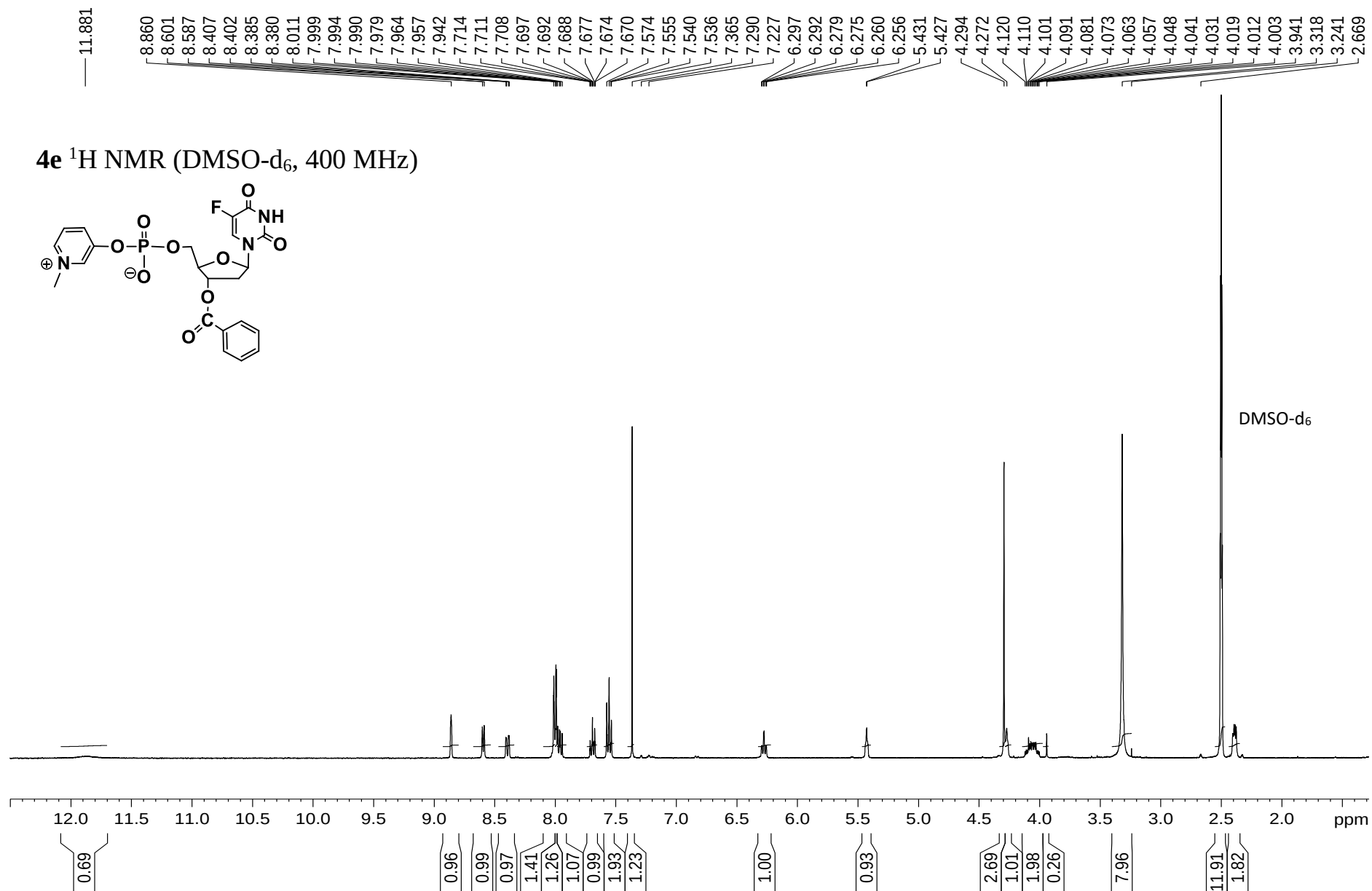


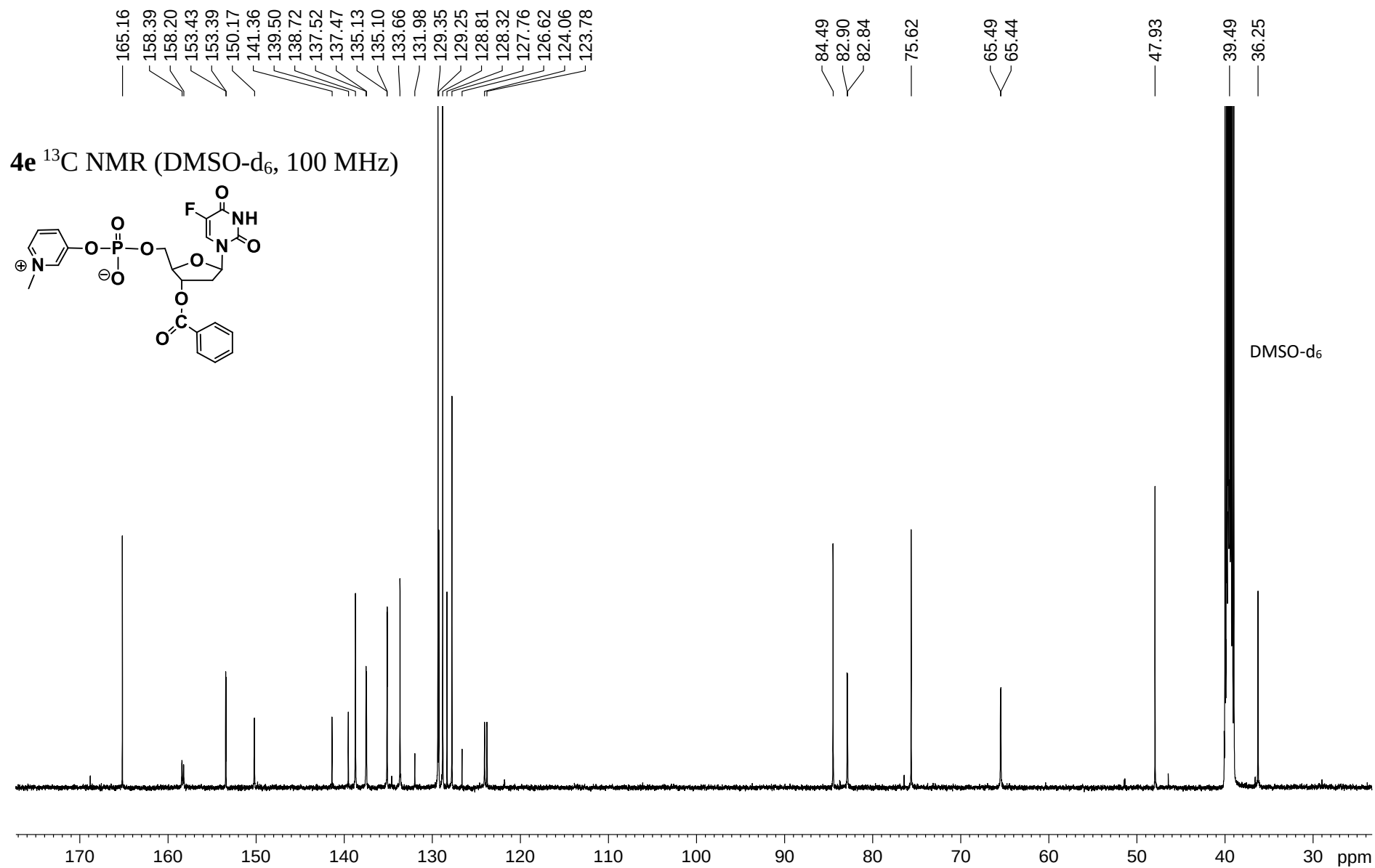
O=P([O-])([O-])OC[C@H]1O[C@@H](C(=O)c2ccccc2)[C@H](N2C(=O)NC(=O)N2C(F)=O)[C@H](O1)c3ccc4ncnc4c3

Thermo Scientific HPLC systems with A + B solvent (A, 0.01 M aqueous triethylammonium acetate pH 7; B, A / acetonitrile, 1: 4, v/v) at 35°C, flow rate 1.5 mL/min; events: 5 min A 100%, 20 min B 0–100%, 5 min B 100% and 15 min A 100%.

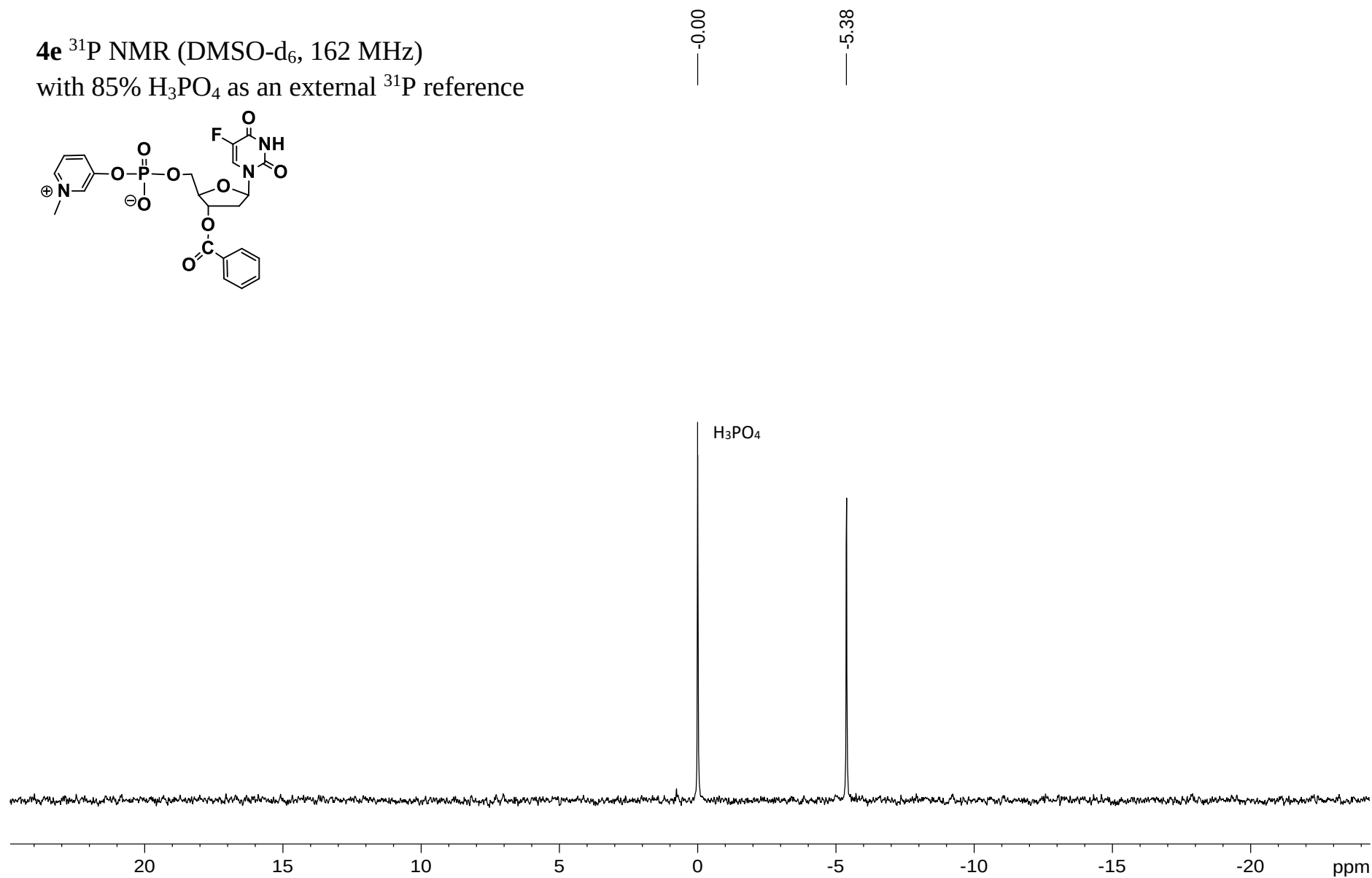
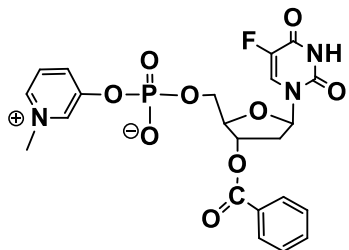


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Version 7.2.3.7553

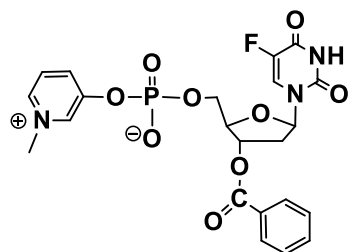




4e ^{31}P NMR (DMSO- d_6 , 162 MHz)
with 85% H_3PO_4 as an external ^{31}P reference

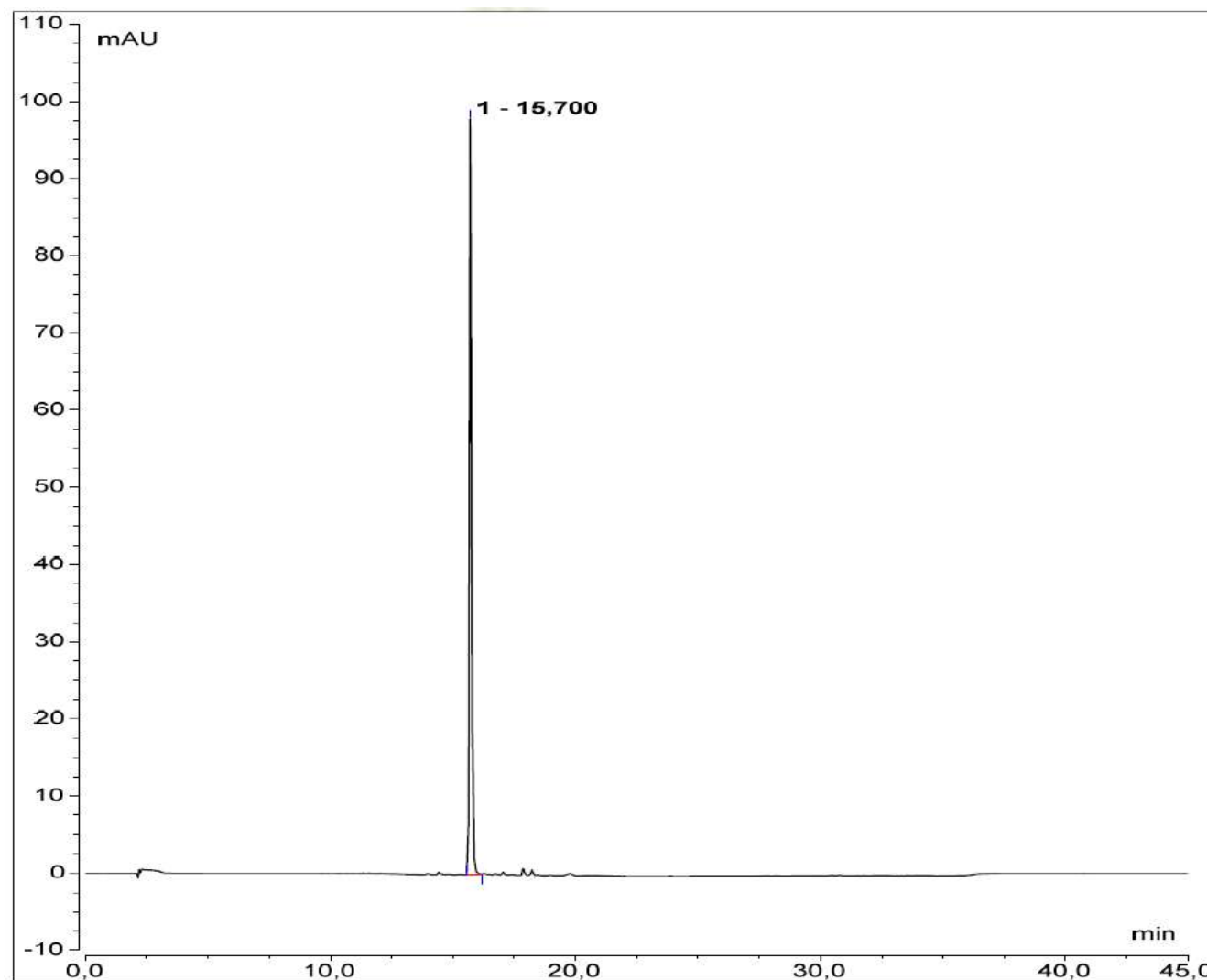


Chromatogram 4e



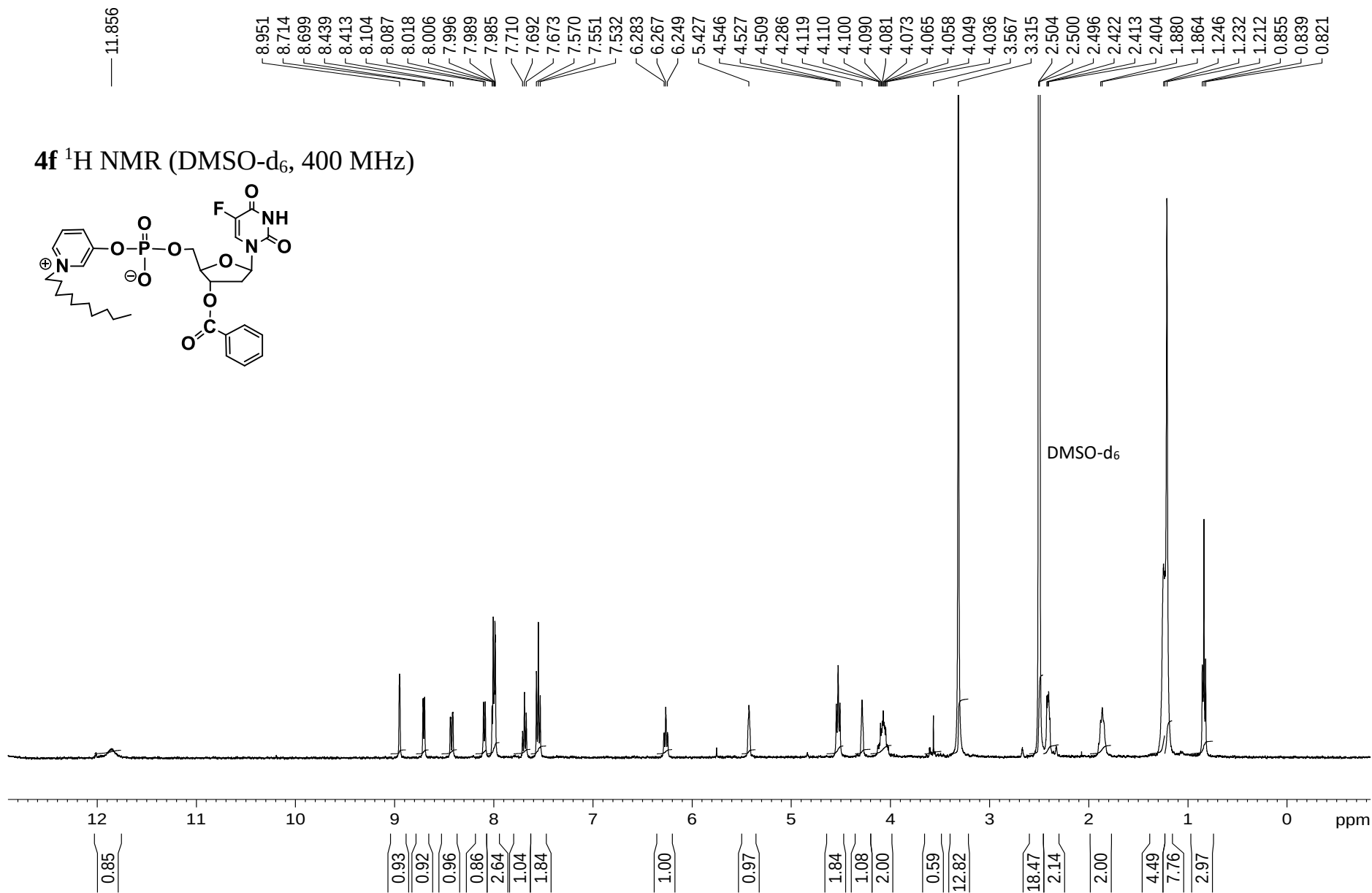
Lichrospher RP-18 endcapped
(5.0 μm , 4.6 mm $\times$ 150 mm)

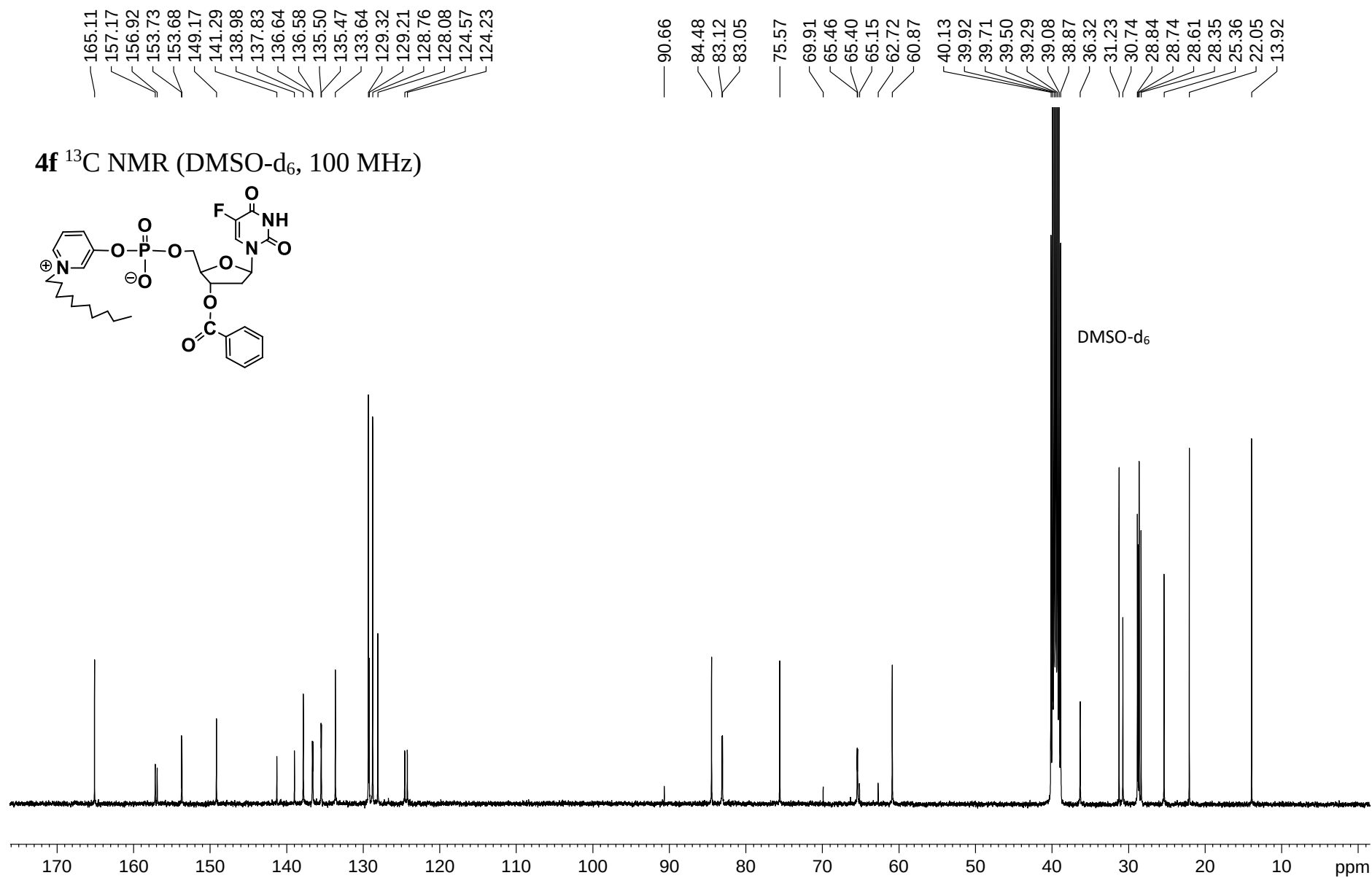
Thermo Scientific HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium acetate
pH 7; B, A / acetonitrile, 1: 4, v/v)
at 35°C, flow rate 1.5 mL/min;
events: 5 min A 100%, 20 min
B 0–100%, 5 min B 100% and
15 min A 100%.



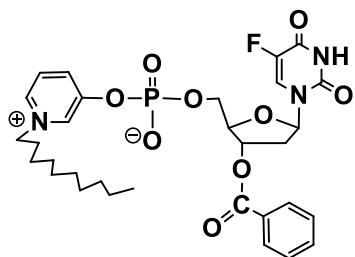
Default/Chromatogram

Chromeleon (c) Dionex
Version 7.2.3.7553



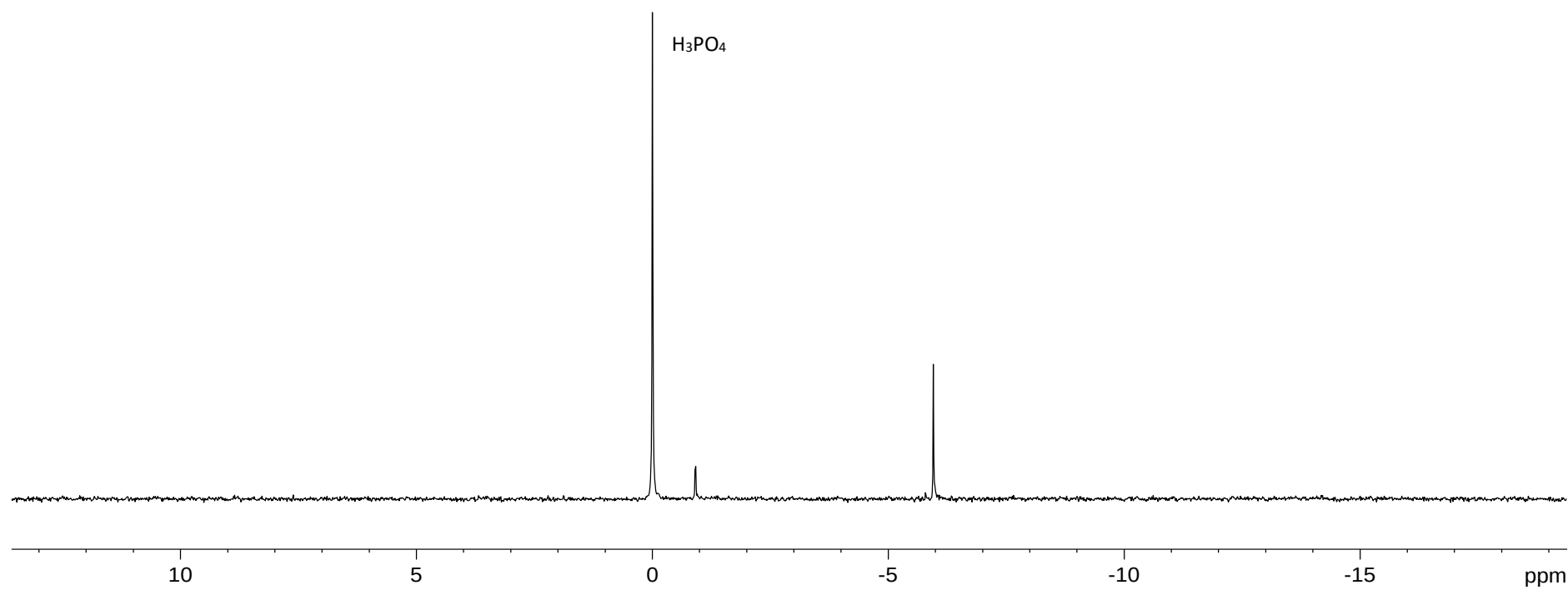


4f ^{31}P NMR (DMSO- d_6 , 162 MHz)
with 85% H_3PO_4 as an external ^{31}P reference

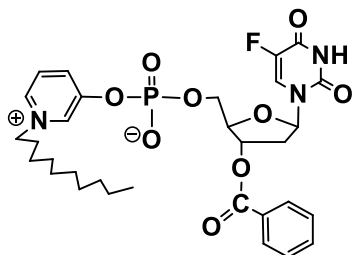


— 0.00

— -5.95

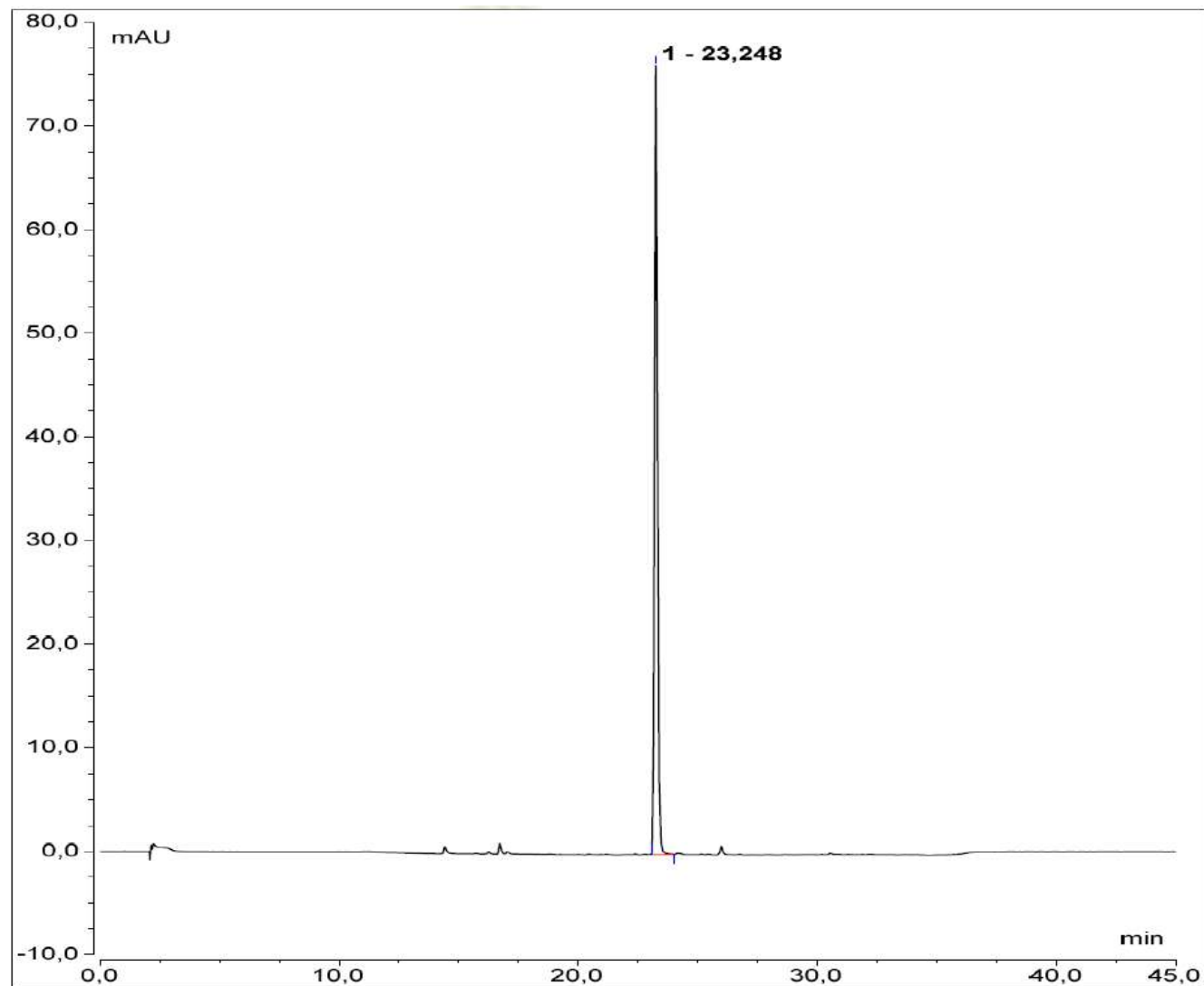


Chromatogram 4f



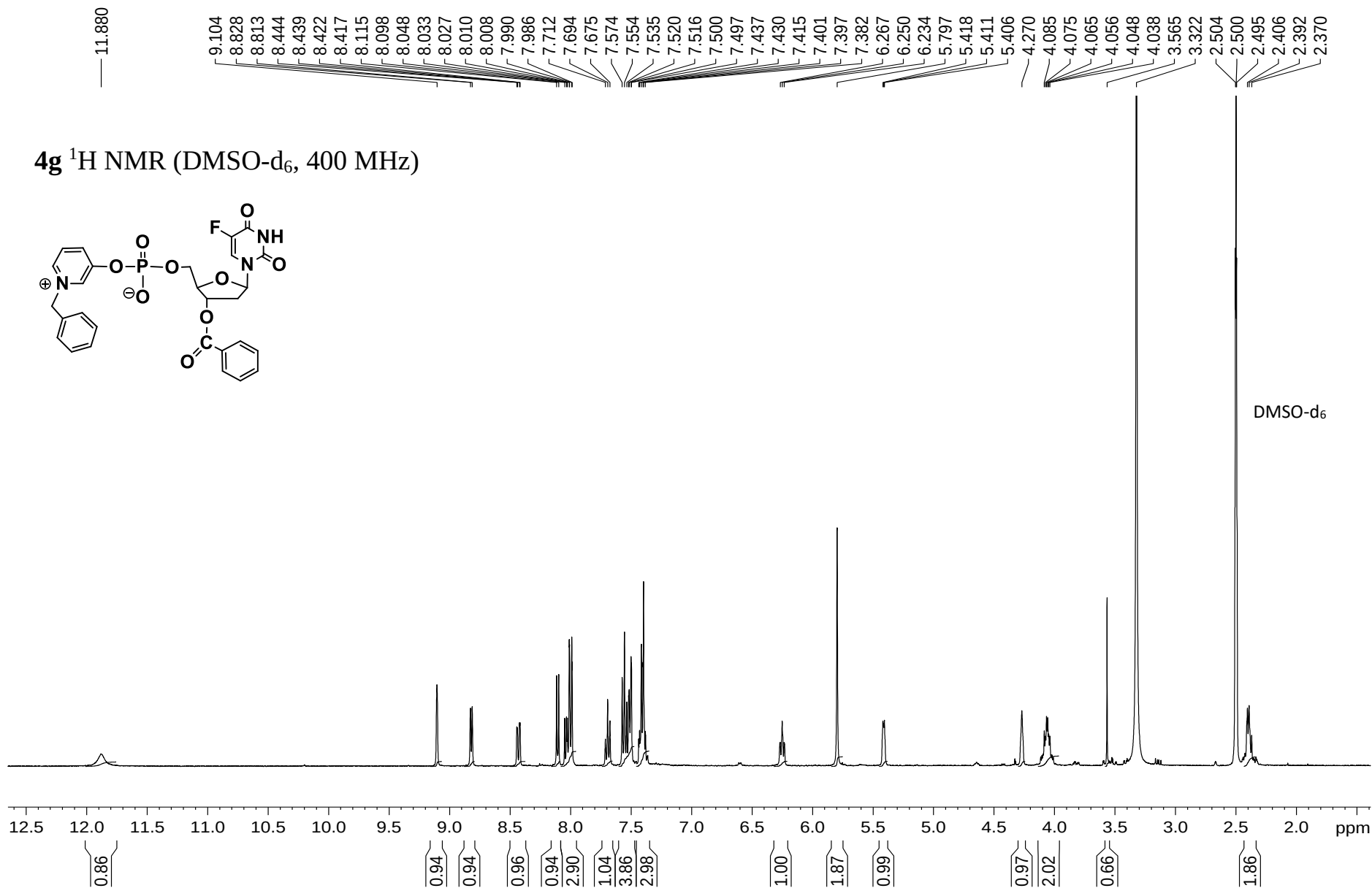
Lichrospher RP-18 endcapped
(5.0 μm , 4.6 mm $\times$ 150 mm)

Thermo Scientific HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium acetate
pH 7; B, A / acetonitrile, 1: 4, v/v)
at 35°C, flow rate 1.5 mL/min;
events: 5 min A 100%, 20 min
B 0–100%, 5 min B 100% and
15 min A 100%.

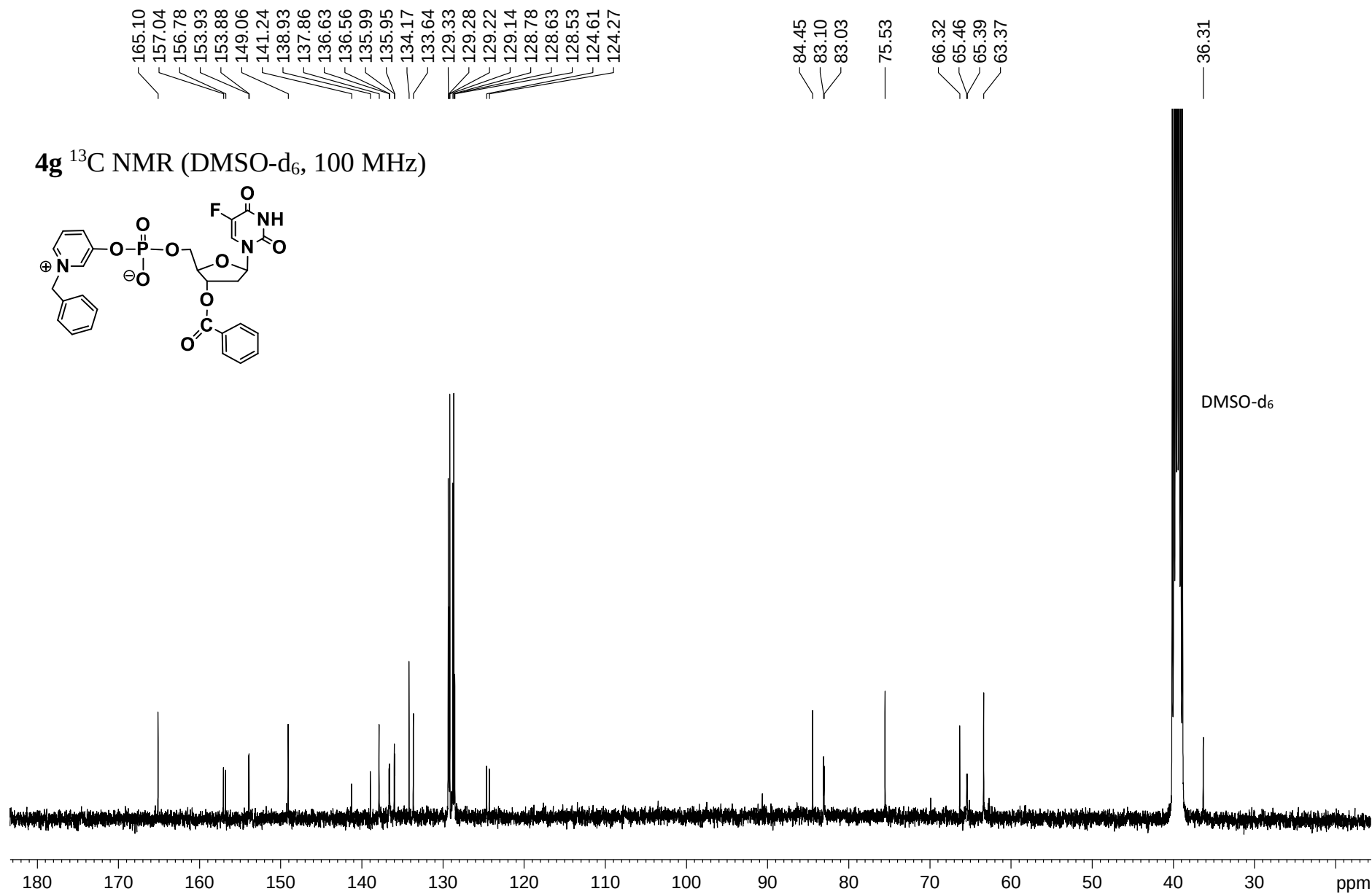
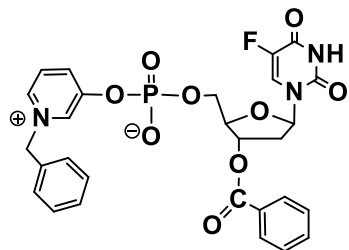


Default/Chromatogram

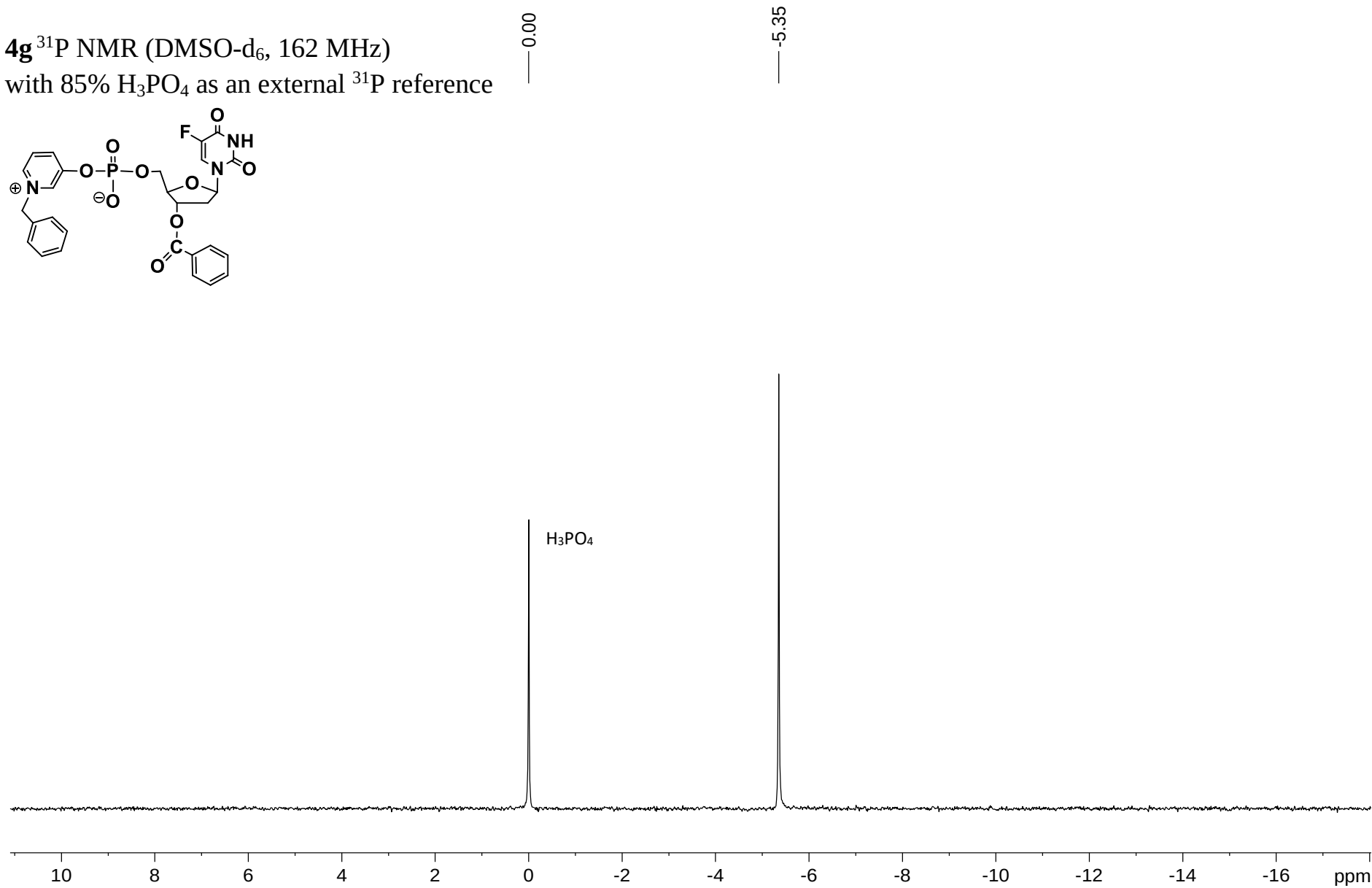
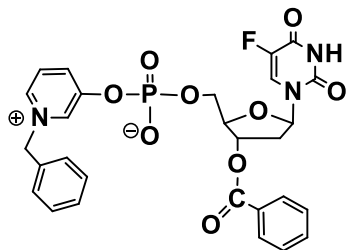
Chromeleon (c) Dionex
Version 7.2.3.7553



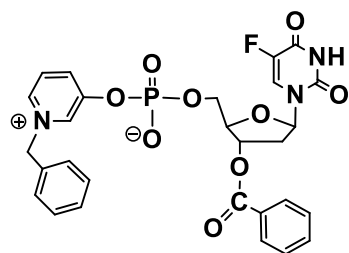
4g ^{13}C NMR (DMSO- d_6 , 100 MHz)



4g ^{31}P NMR (DMSO- d_6 , 162 MHz)
with 85% H_3PO_4 as an external ^{31}P reference

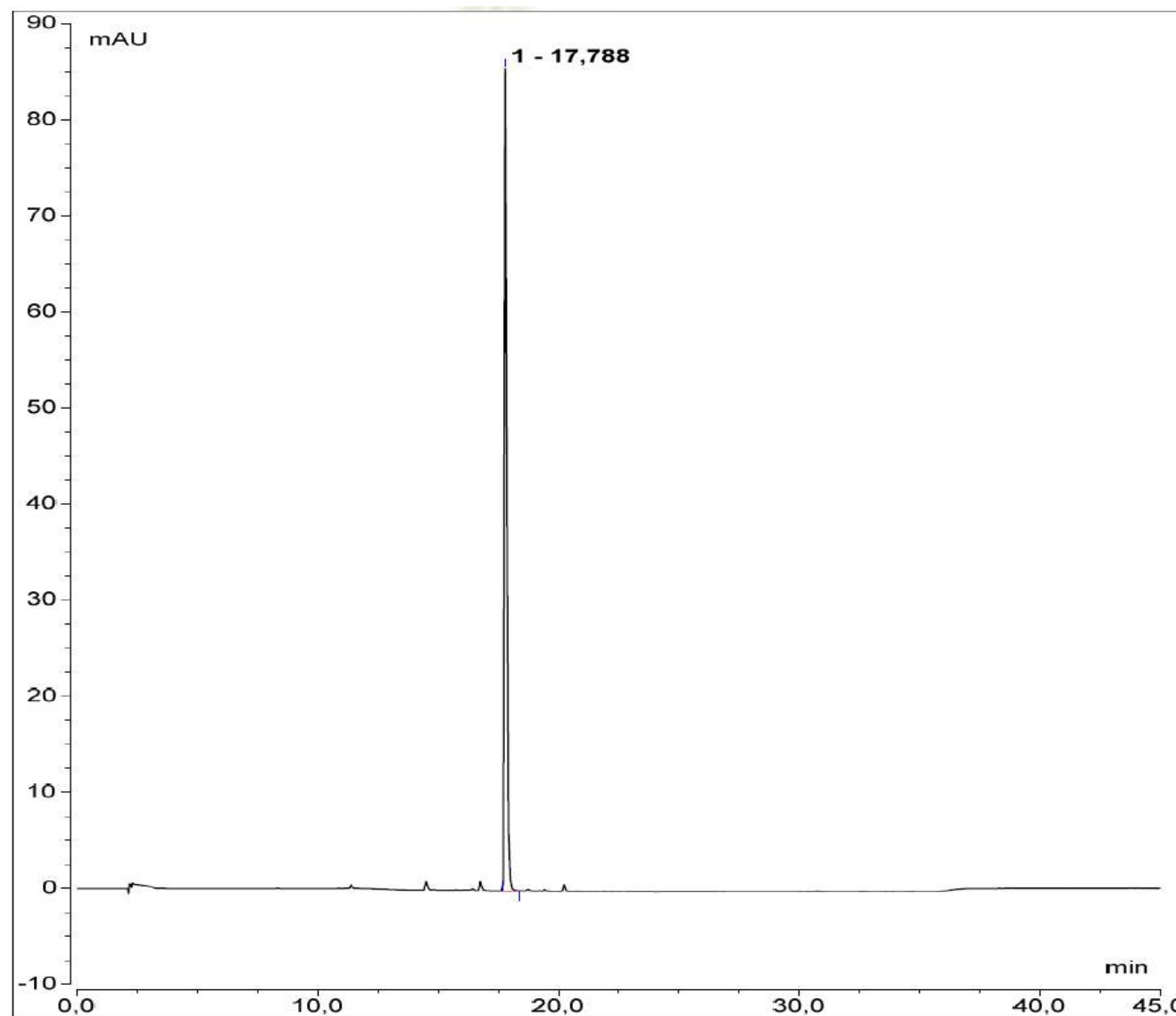


Chromatogram 4g



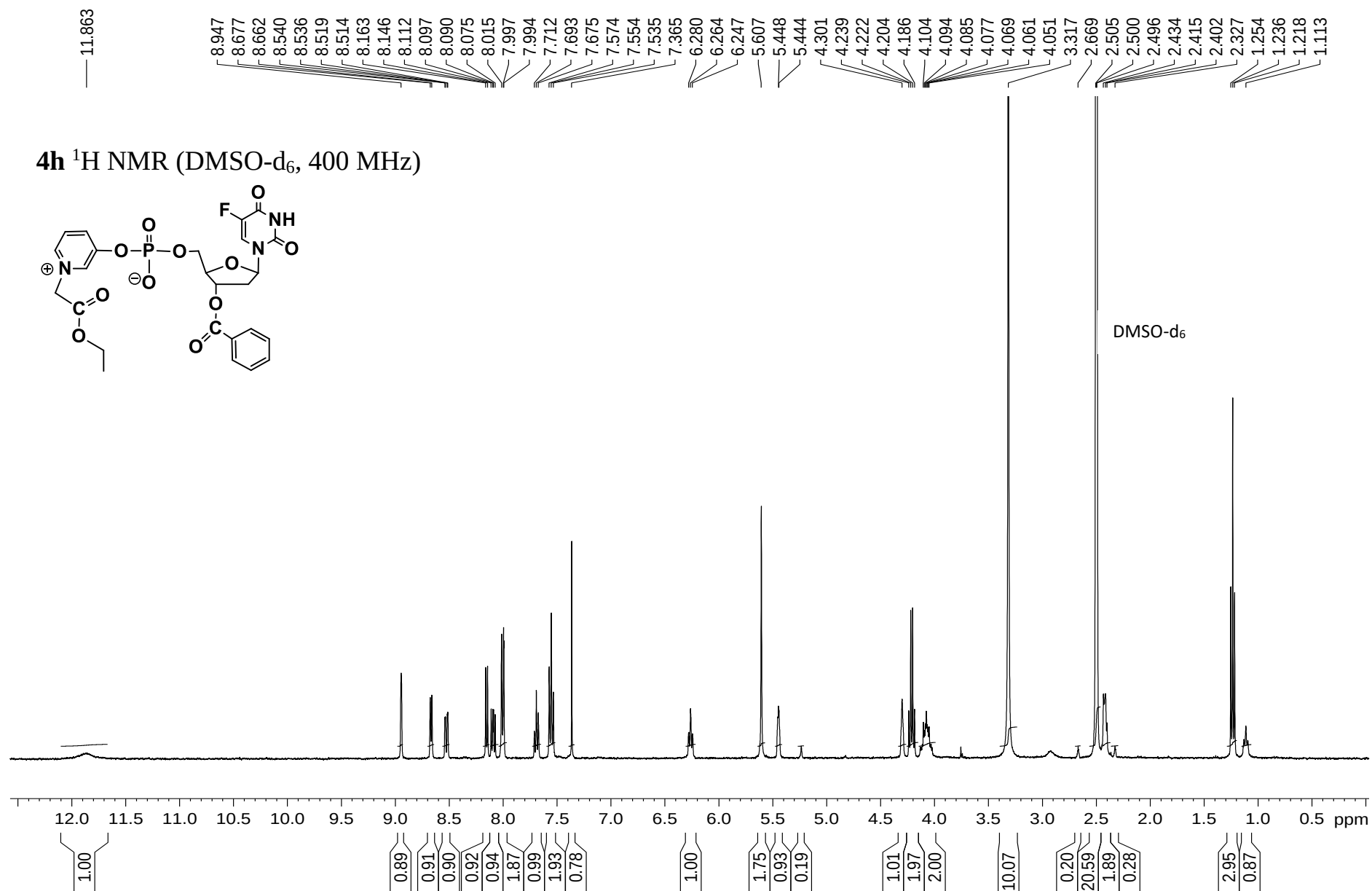
Lichrospher RP-18 endcapped
(5.0 μm , 4.6 mm $\times$ 150 mm)

Thermo Scientific HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium acetate
pH 7; B, A / acetonitrile, 1: 4, v/v)
at 35°C, flow rate 1.5 mL/min;
events: 5 min A 100%, 20 min
B 0–100%, 5 min B 100% and
15 min A 100%.

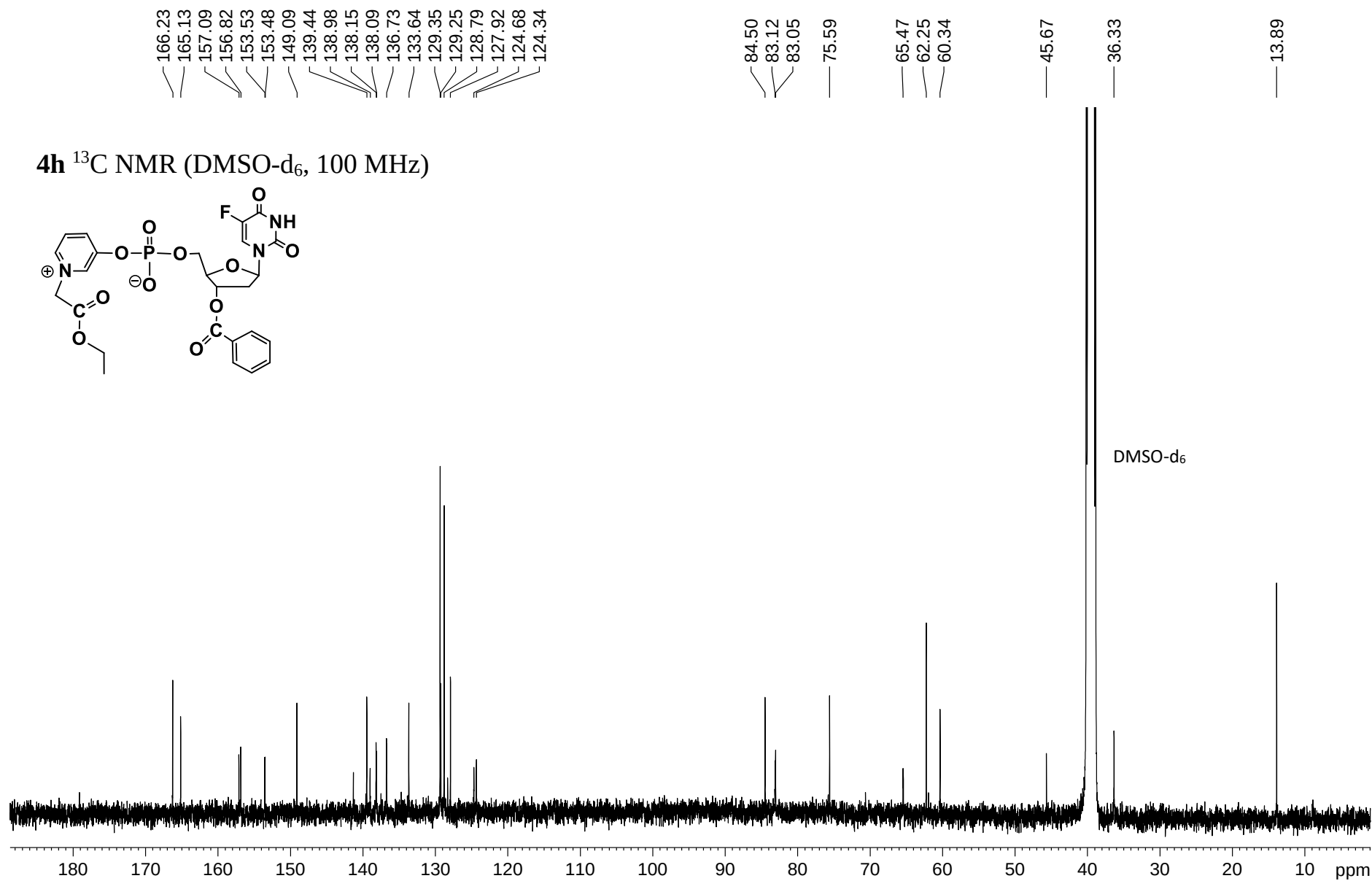
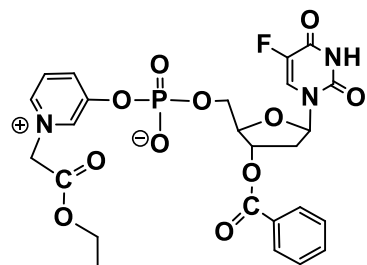


Default/Chromatogram

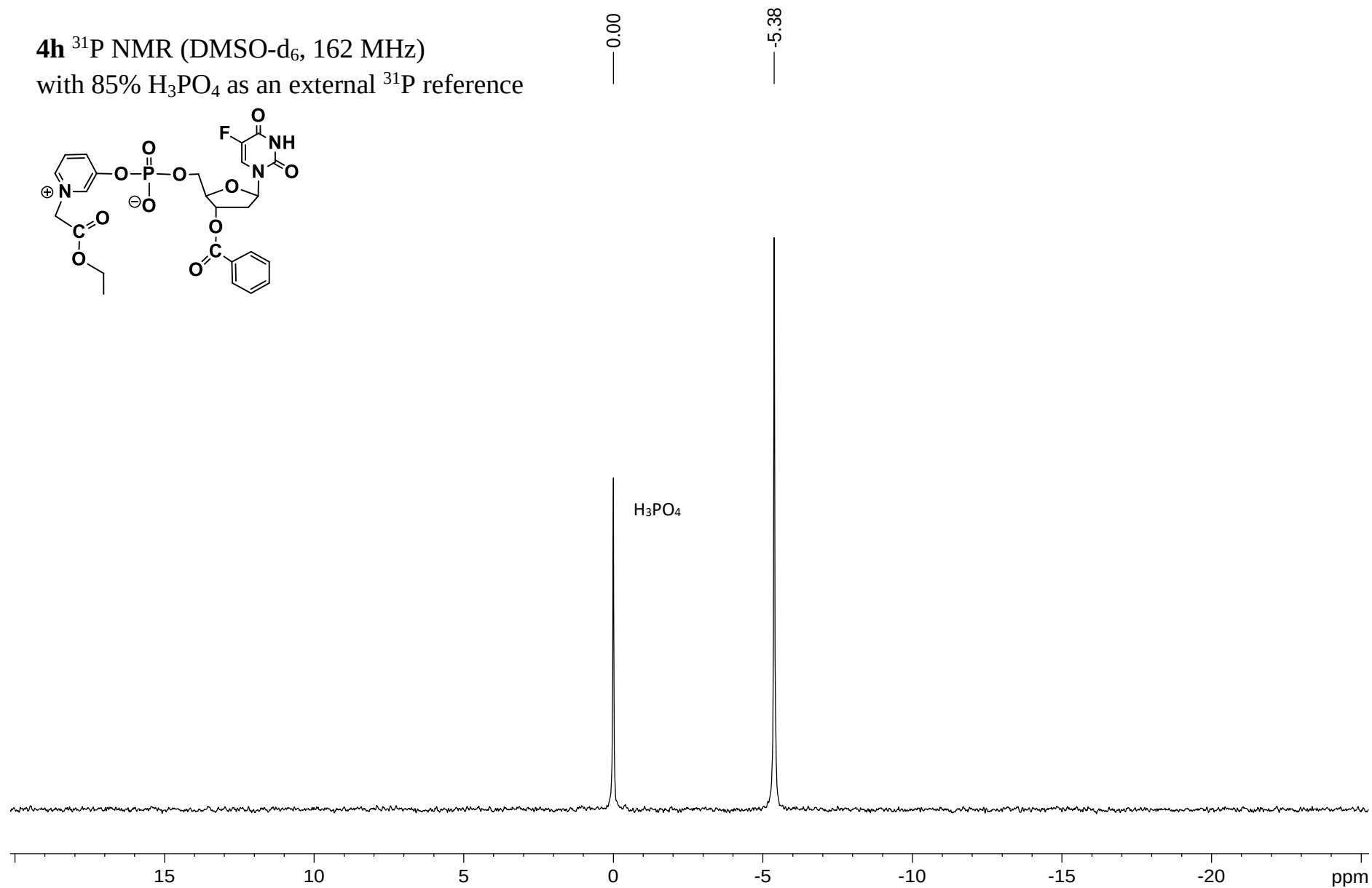
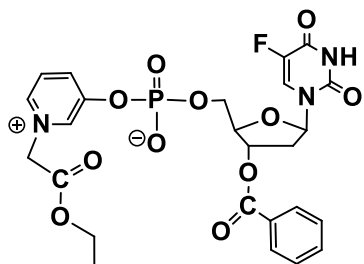
Chromeleon (c) Dionex
Version 7.2.3.7553



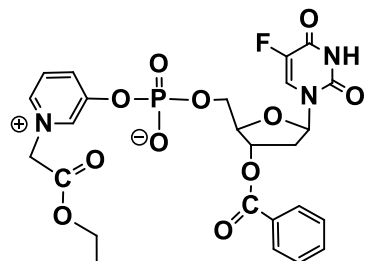
4h ^{13}C NMR (DMSO- d_6 , 100 MHz)



4h ^{31}P NMR (DMSO- d_6 , 162 MHz)
with 85% H_3PO_4 as an external ^{31}P reference

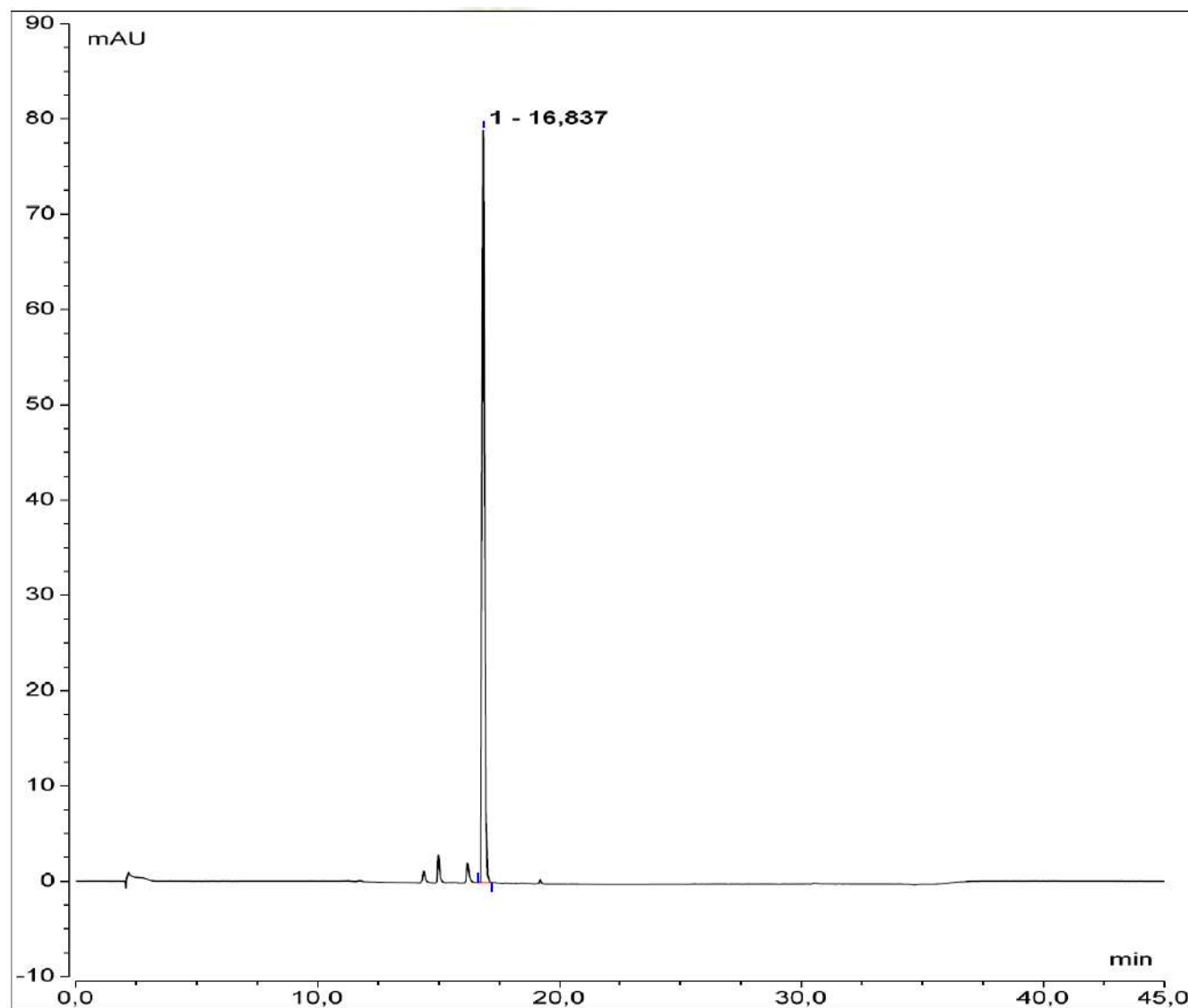


Chromatogram 4h



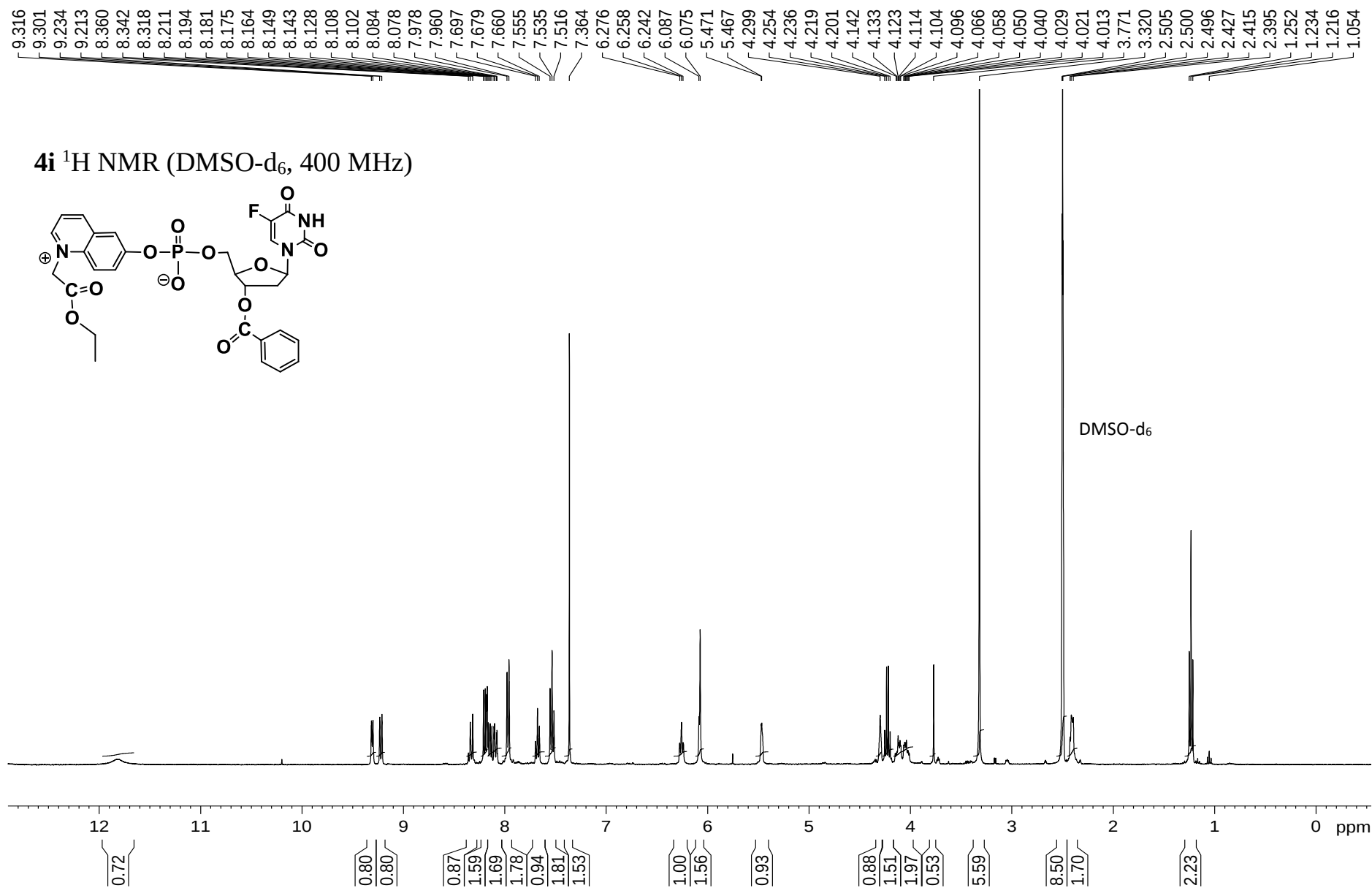
Lichrospher RP-18 endcapped
(5.0 μm , 4.6 mm $\times$ 150 mm)

Thermo Scientific HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium acetate
pH 7; B, A / acetonitrile, 1: 4, v/v)
at 35°C, flow rate 1.5 mL/min;
events: 5 min A 100%, 20 min
B 0–100%, 5 min B 100% and
15 min A 100%.



Default/Chromatogram

Chromeleon (c) Dionex
Version 7.2.3.7553



166.52
166.02
165.05
157.05
156.79
154.73
154.67
149.07
148.54
147.46
141.23
138.92
134.03
133.59
130.89
130.82
129.29
129.22
129.08
128.75
128.30
124.72
124.38
121.92
119.97
116.40
116.35

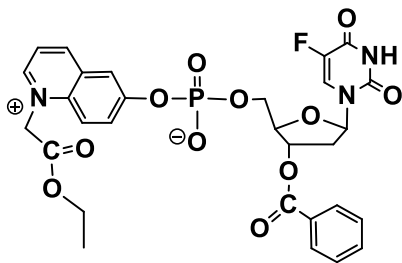
84.46
83.27
83.20
75.79

65.24
65.18
63.26
62.36
57.39
53.19

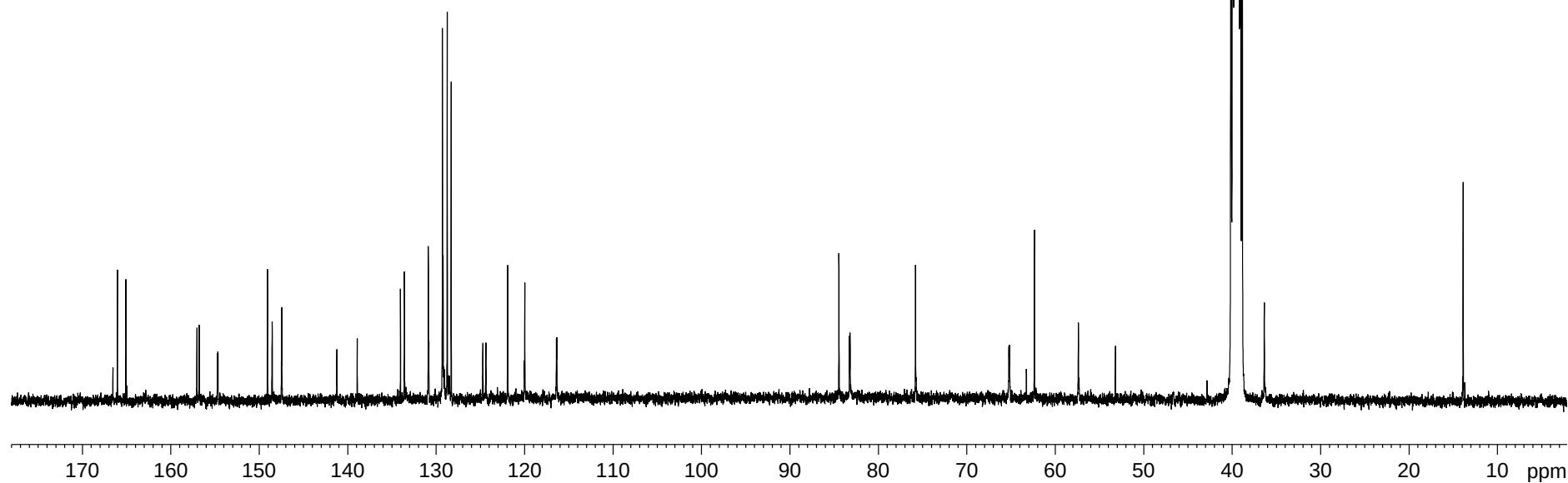
42.86
39.50
36.36

13.87

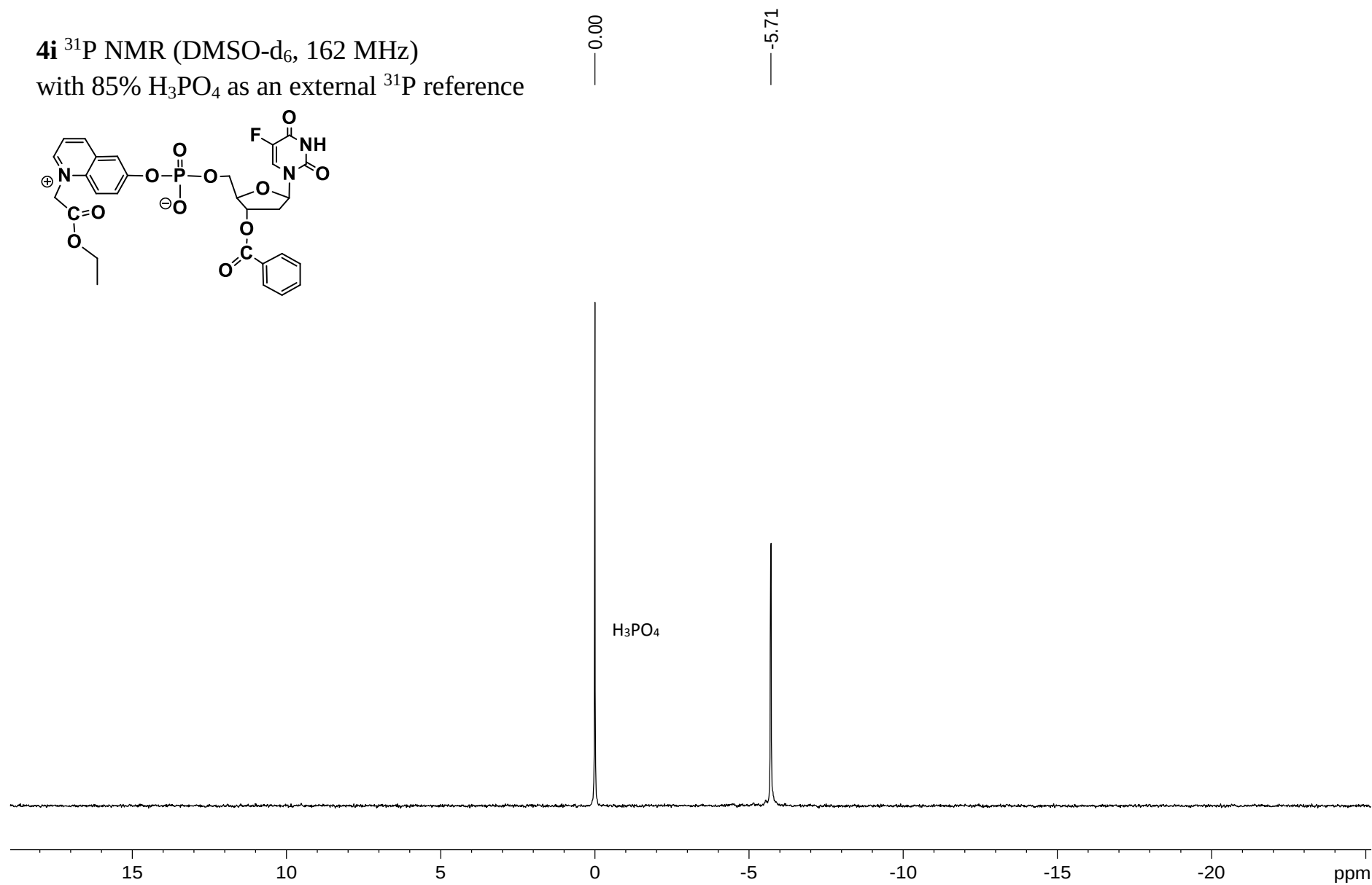
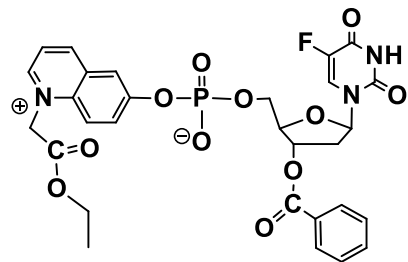
4i ^{13}C NMR (DMSO- d_6 , 100 MHz)



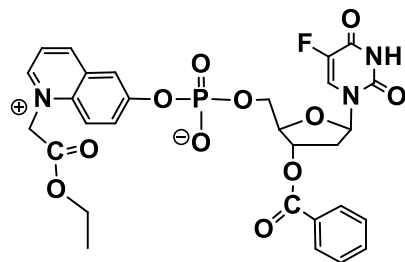
DMSO- d_6



4i ^{31}P NMR (DMSO- d_6 , 162 MHz)
with 85% H_3PO_4 as an external ^{31}P reference

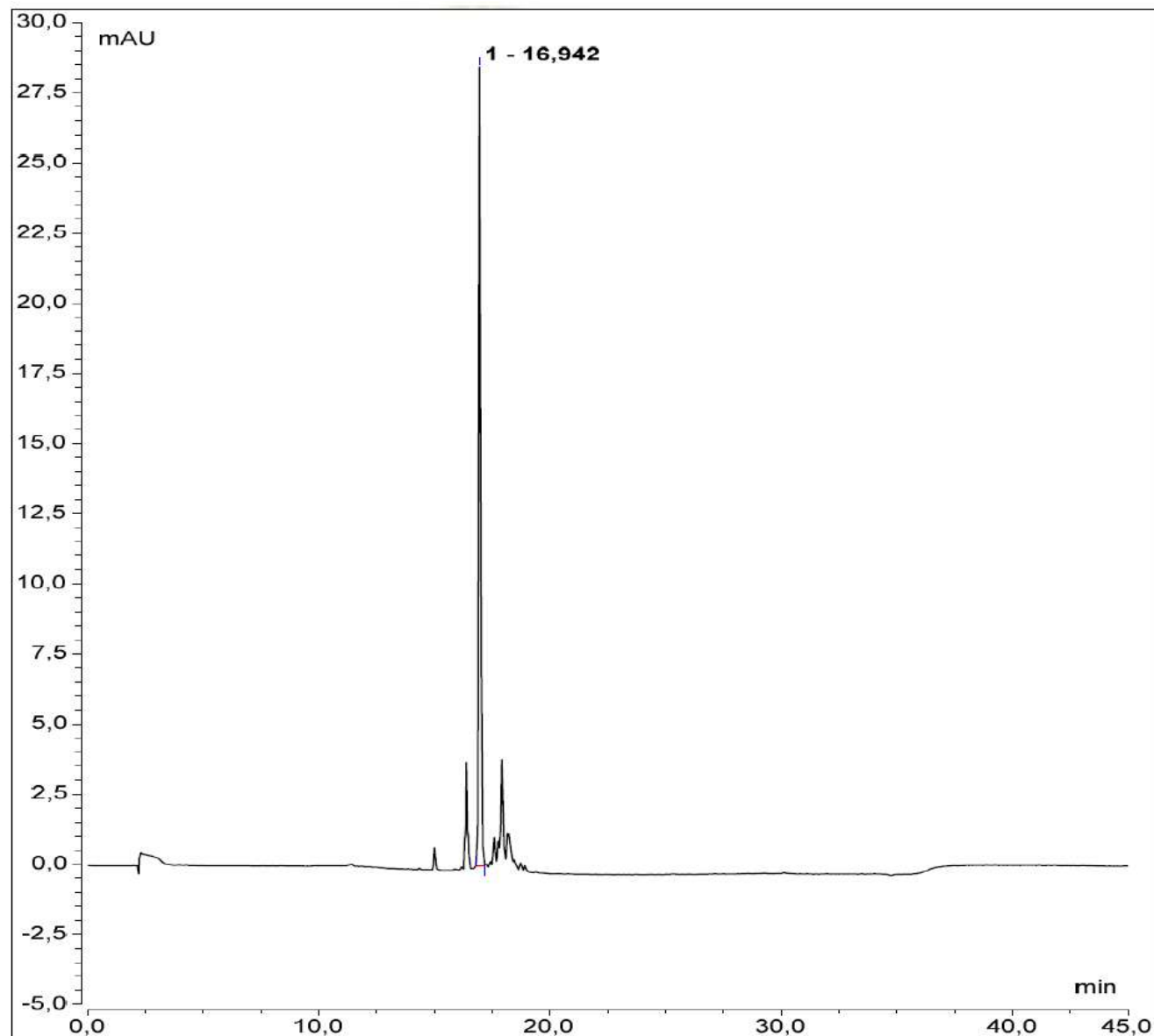


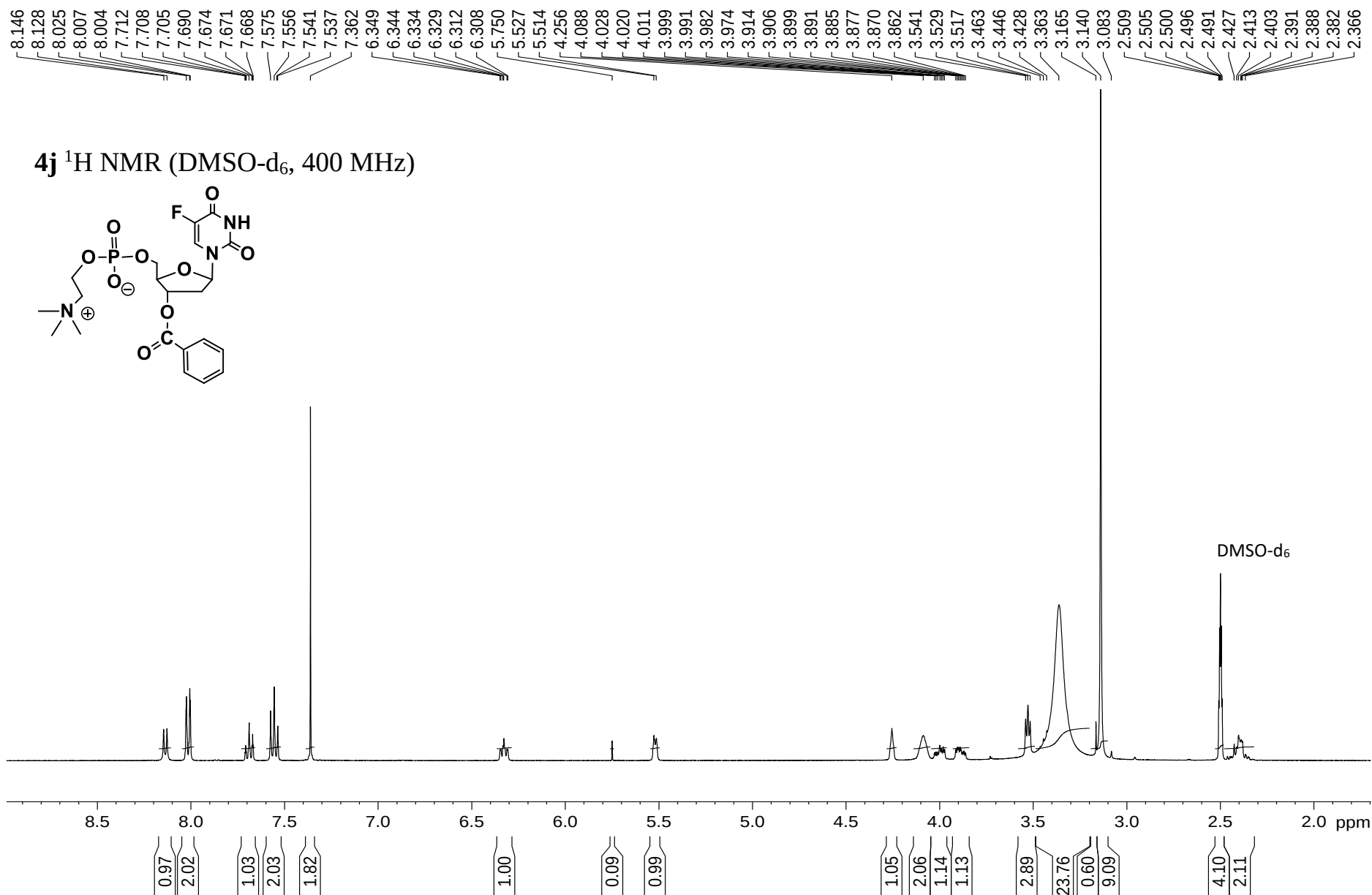
Chromatogram 4i

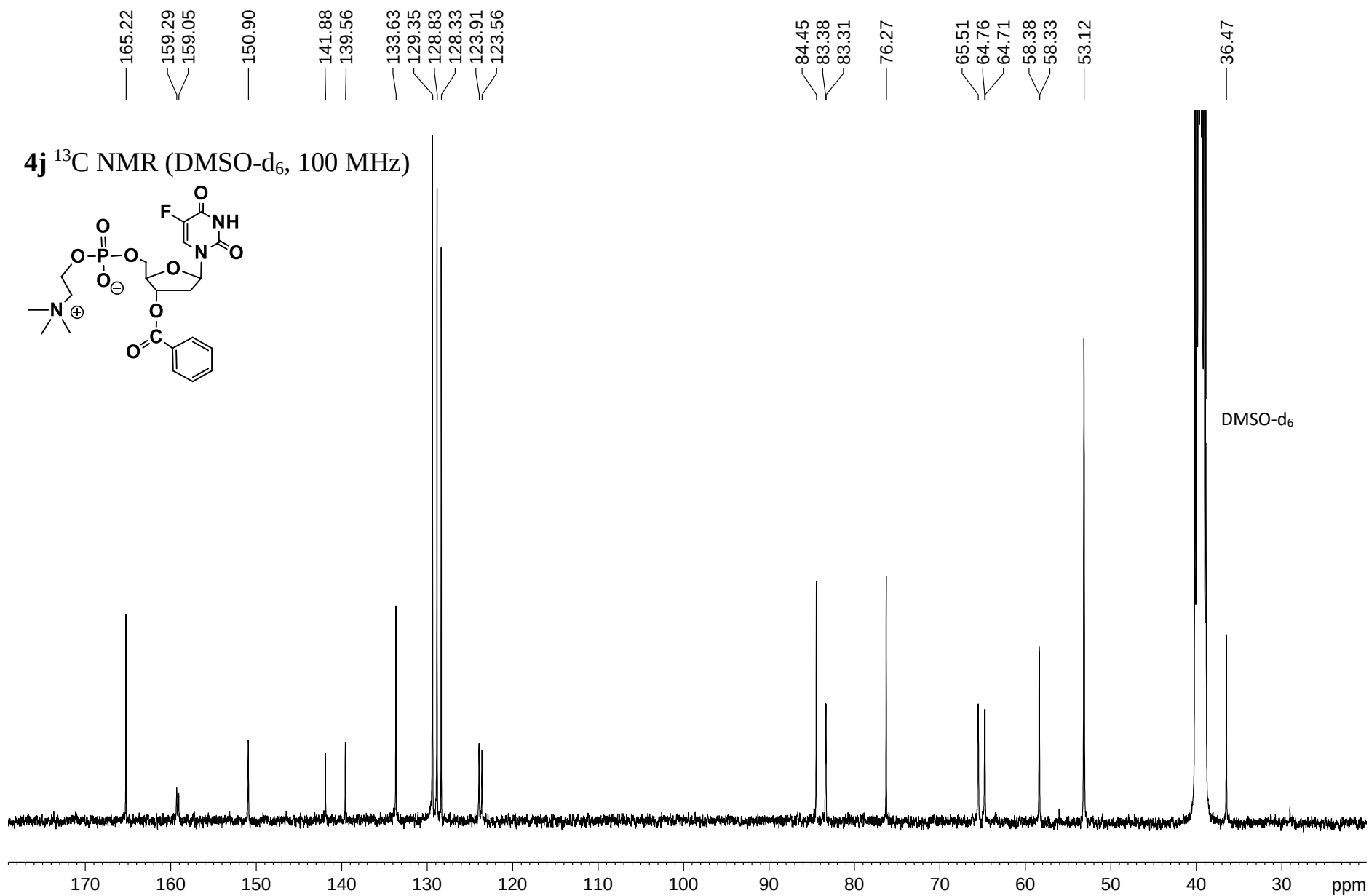


Lichrospher RP-18 endcapped
(5.0 μm , 4.6 mm $\times$ 150 mm)

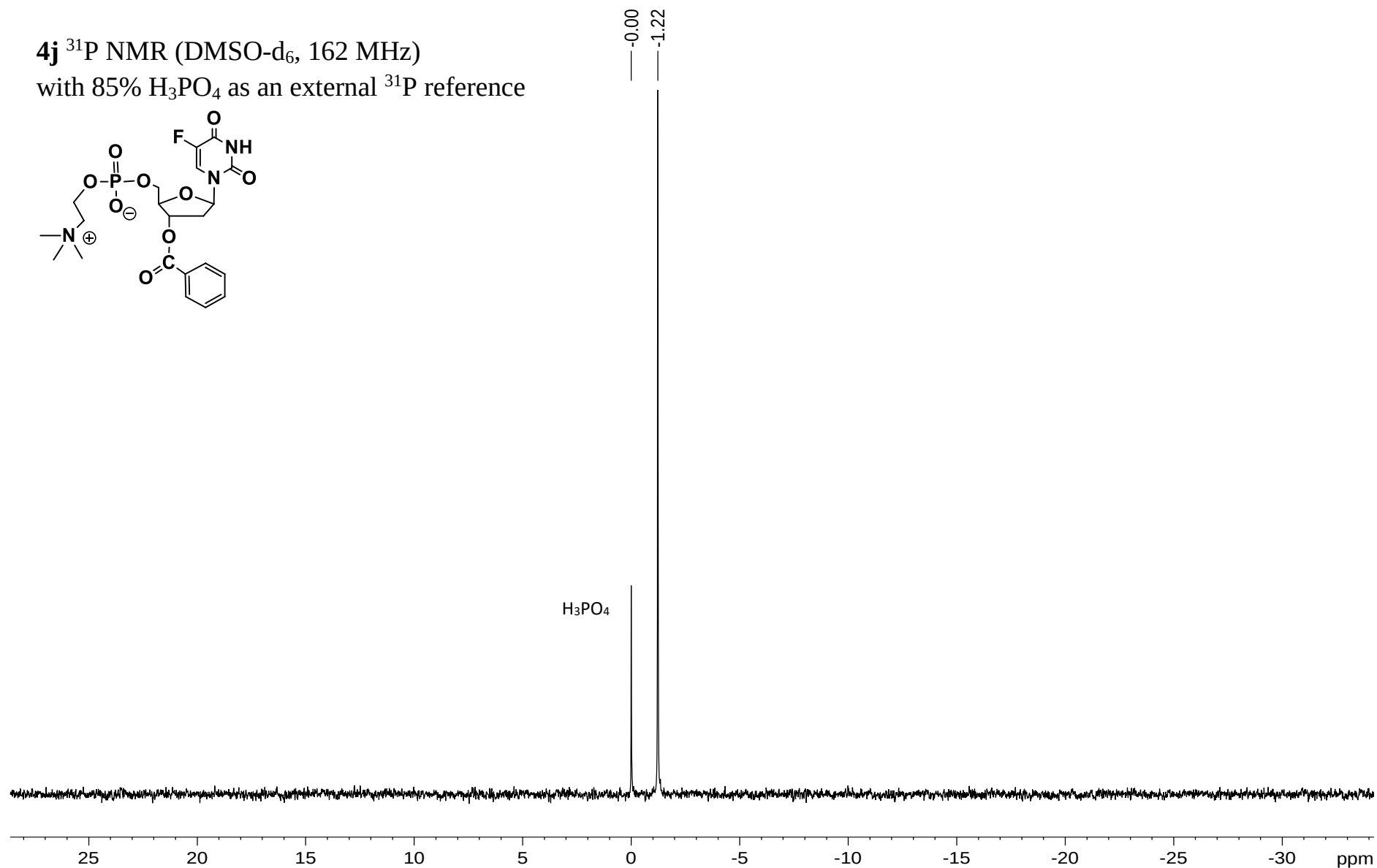
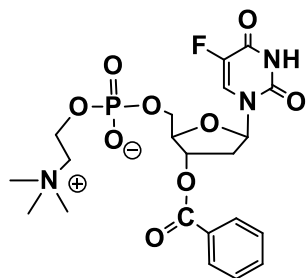
Thermo Scientific HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium acetate
pH 7; B, A / acetonitrile, 1: 4, v/v)
at 35°C, flow rate 1.5 mL/min;
events: 5 min A 100%, 20 min
B 0–100%, 5 min B 100% and
15 min A 100%.



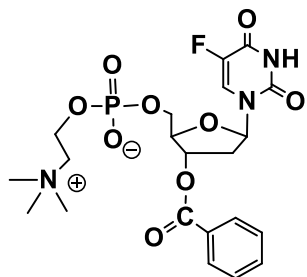




4j ^{31}P NMR (DMSO- d_6 , 162 MHz)
with 85% H_3PO_4 as an external ^{31}P reference

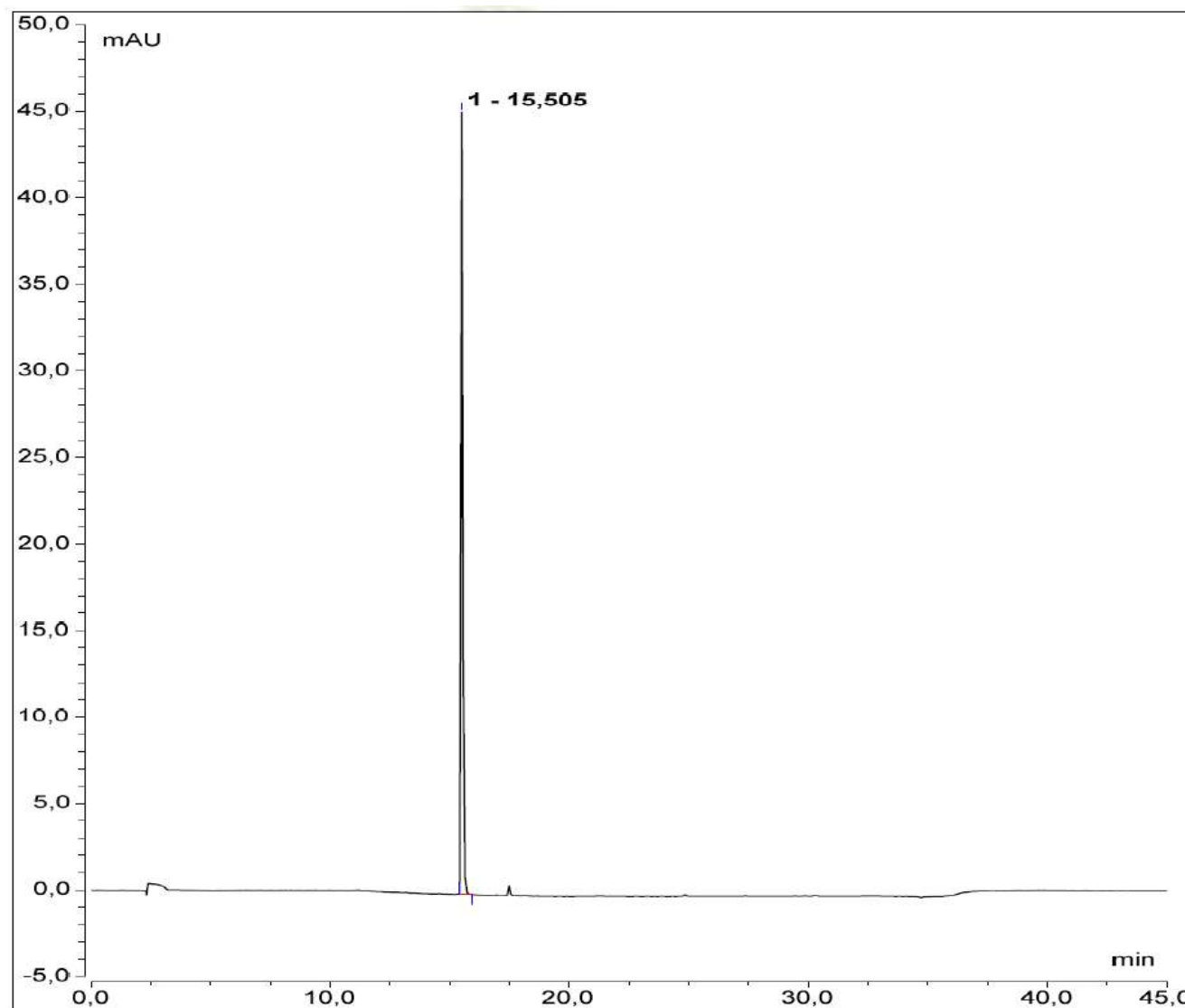


Chromatogram 4j



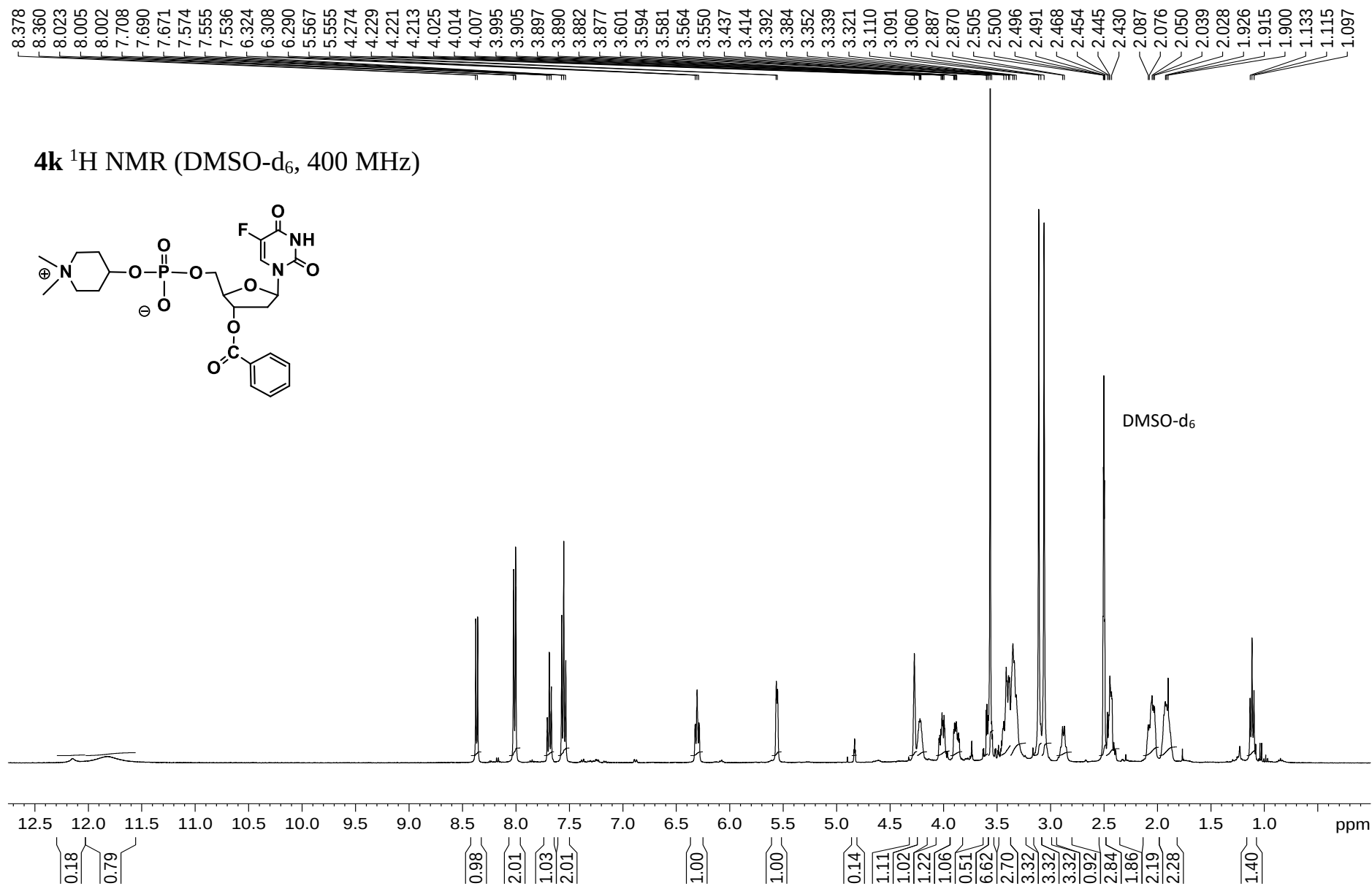
Lichrospher RP-18 endcapped
(5.0 μm , 4.6 mm $\times$ 150 mm)

Thermo Scientific HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium acetate
pH 7; B, A / acetonitrile, 1: 4, v/v)
at 35°C, flow rate 1.5 mL/min;
events: 5 min A 100%, 20 min
B 0–100%, 5 min B 100% and
15 min A 100%.



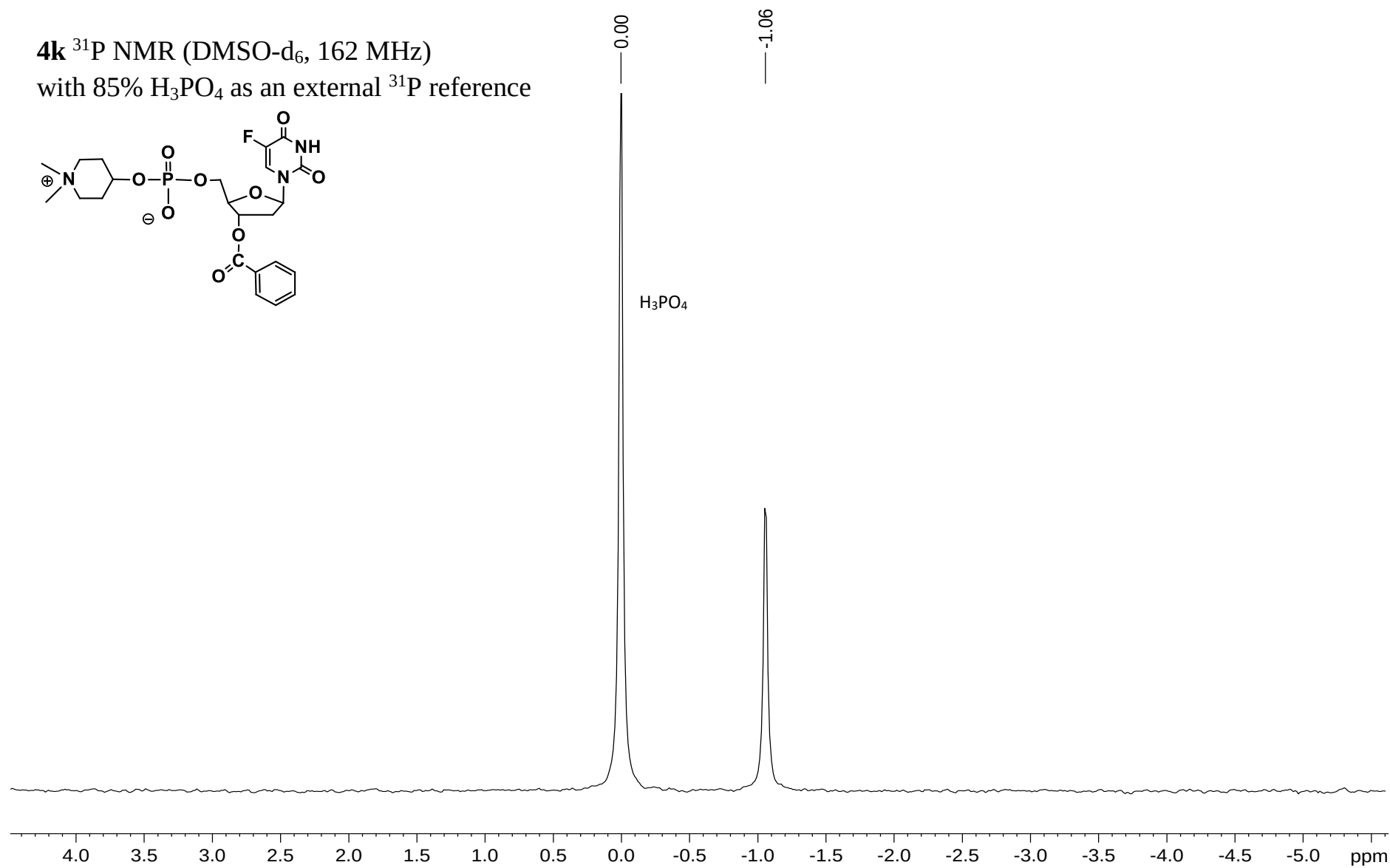
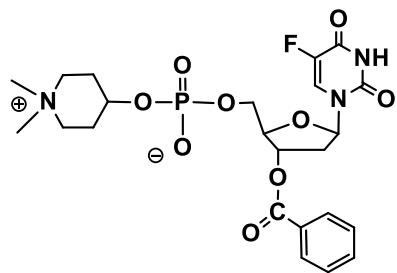
Default/Chromatogram

Chromeleon (c) Dionex
Version 7.2.3.7553

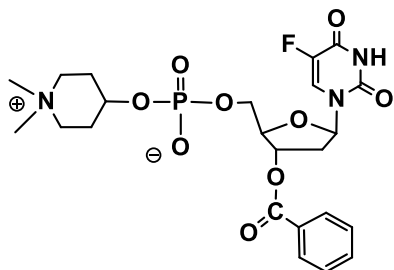




4k ^{31}P NMR (DMSO- d_6 , 162 MHz)
with 85% H_3PO_4 as an external ^{31}P reference

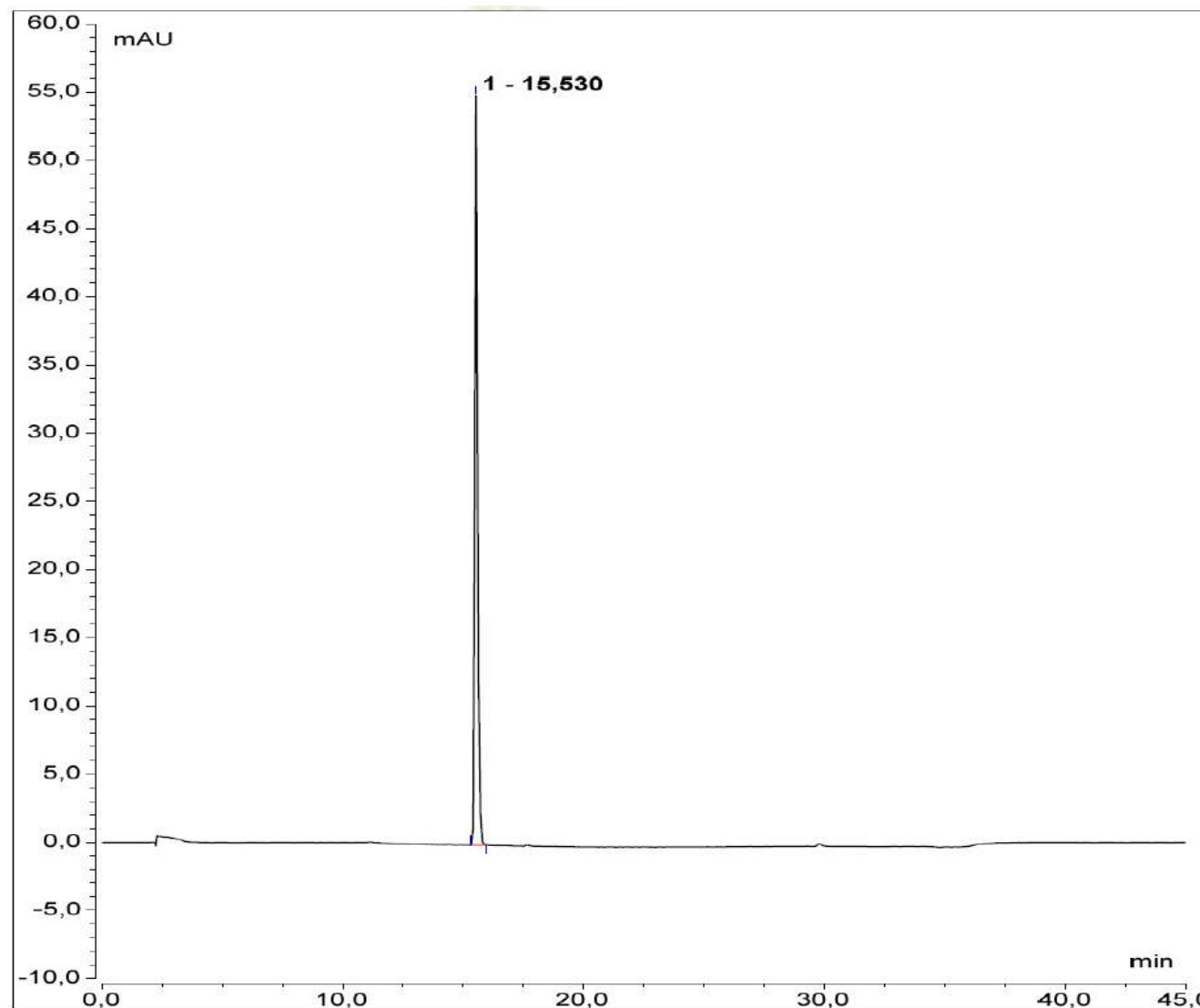


Cromatogram 4k



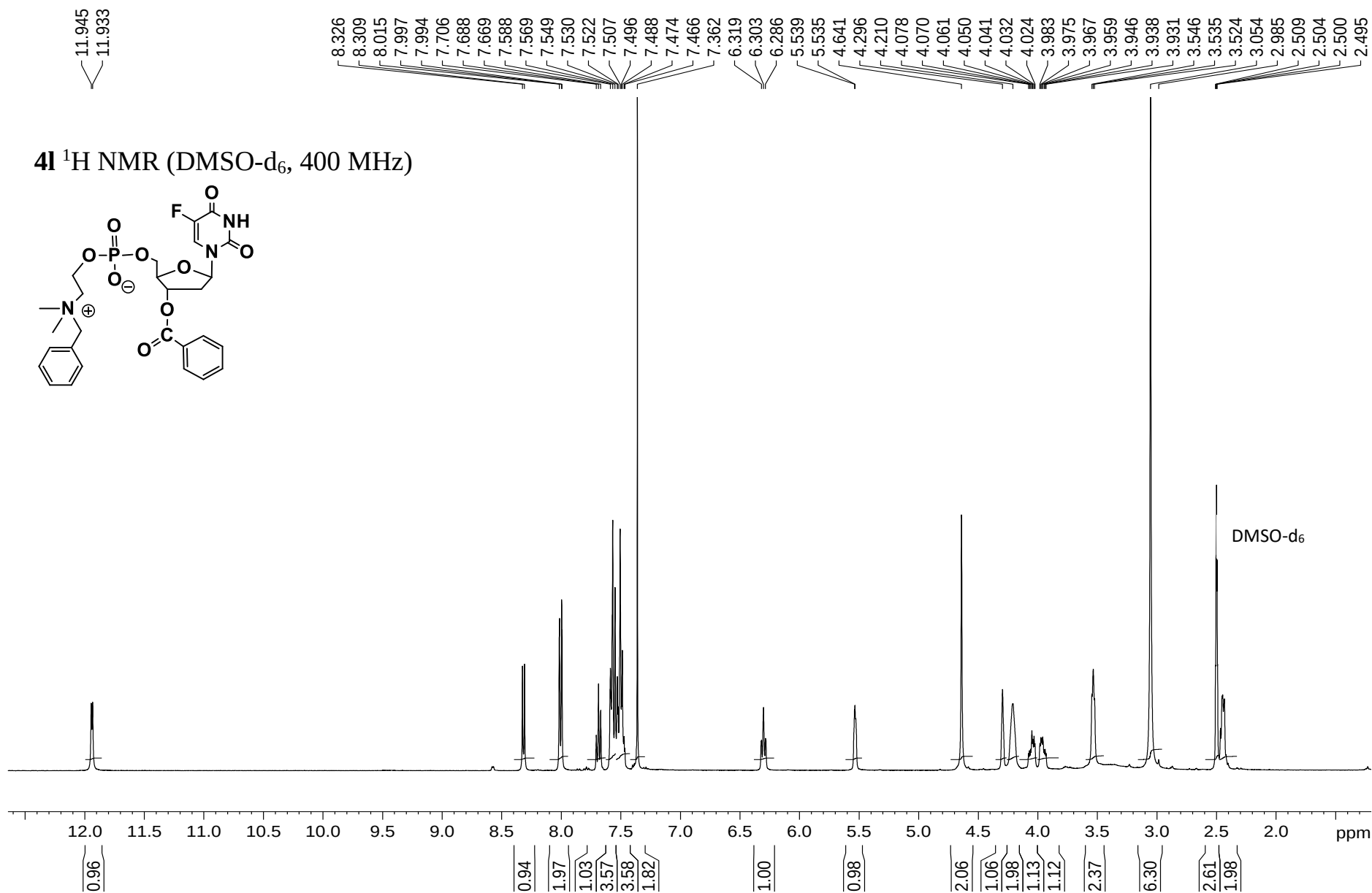
Lichrospher RP-18 endcapped
(5.0 μm , 4.6 mm $\times$ 150 mm)

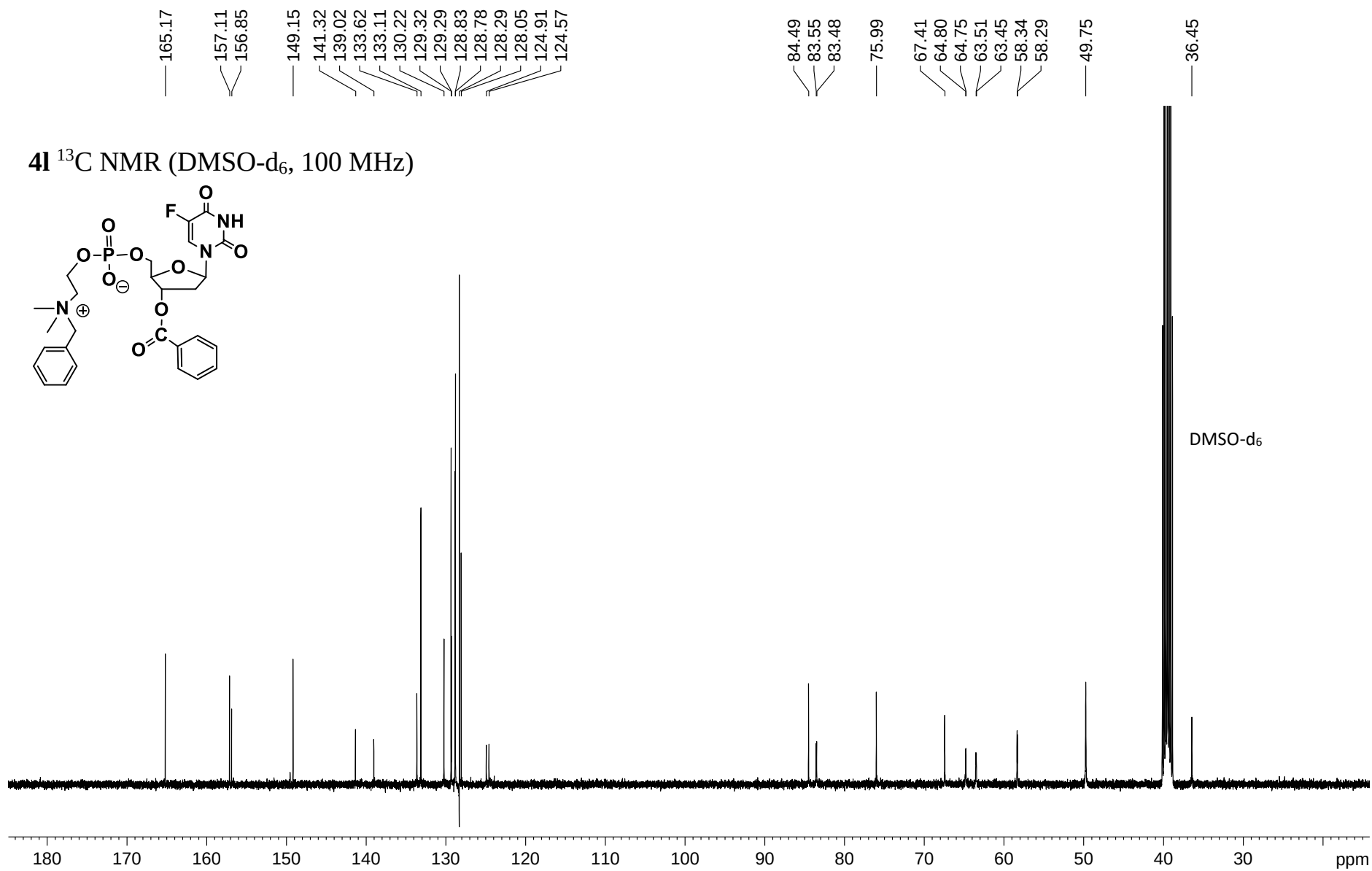
Thermo Scientific HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium acetate
pH 7; B, A / acetonitrile, 1: 4, v/v)
at 35°C, flow rate 1.5 mL/min;
events: 5 min A 100%, 20 min
B 0–100%, 5 min B 100% and
15 min A 100%.



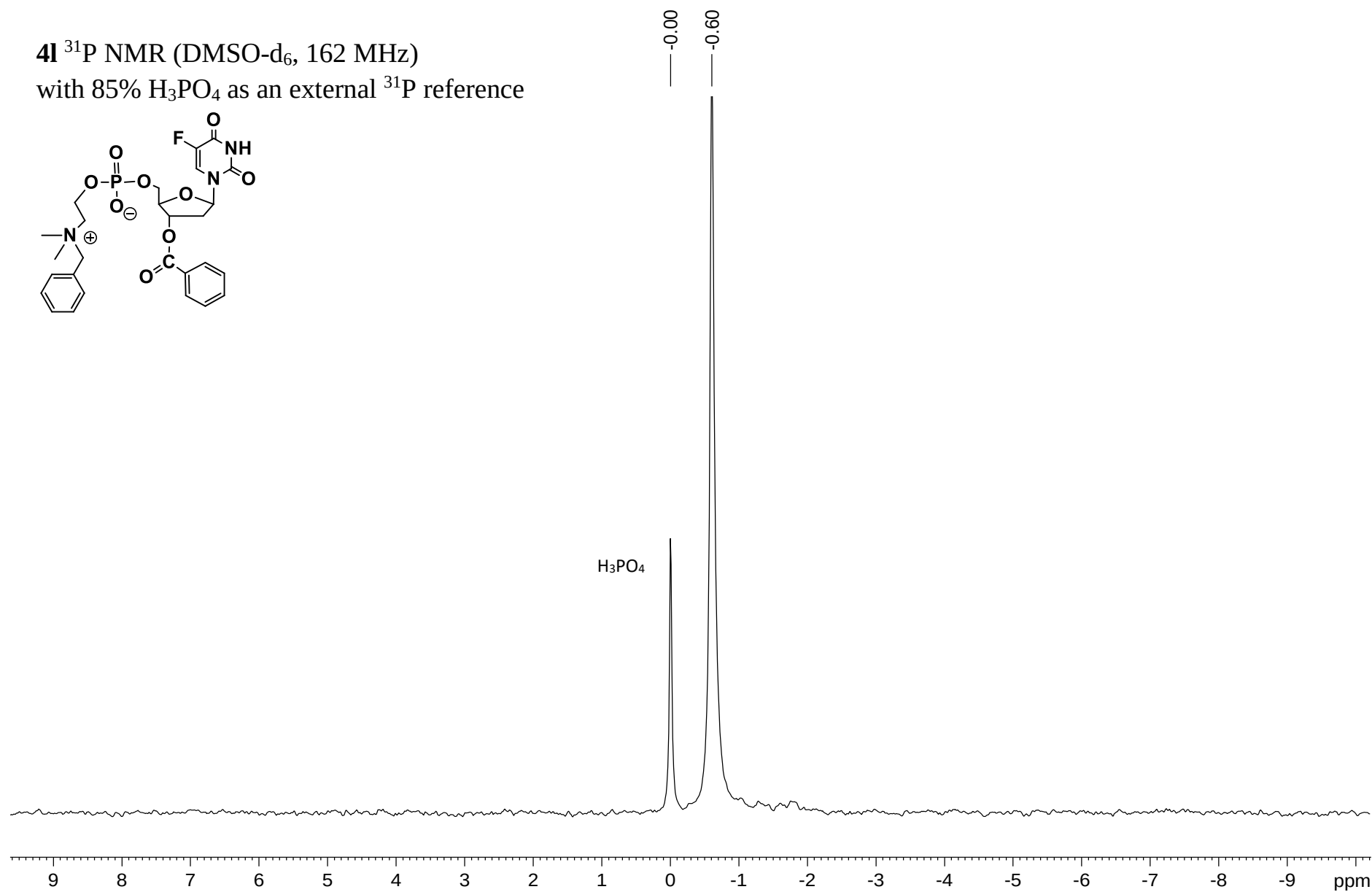
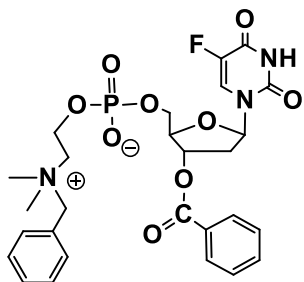
Default/Chromatogram

Chromeleon (c) Dionex
Version 7.2.3.7553

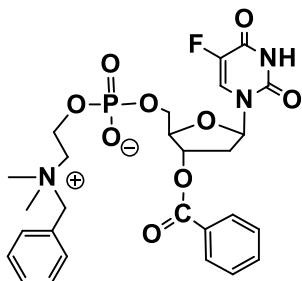




4l ^{31}P NMR (DMSO- d_6 , 162 MHz)
with 85% H_3PO_4 as an external ^{31}P reference

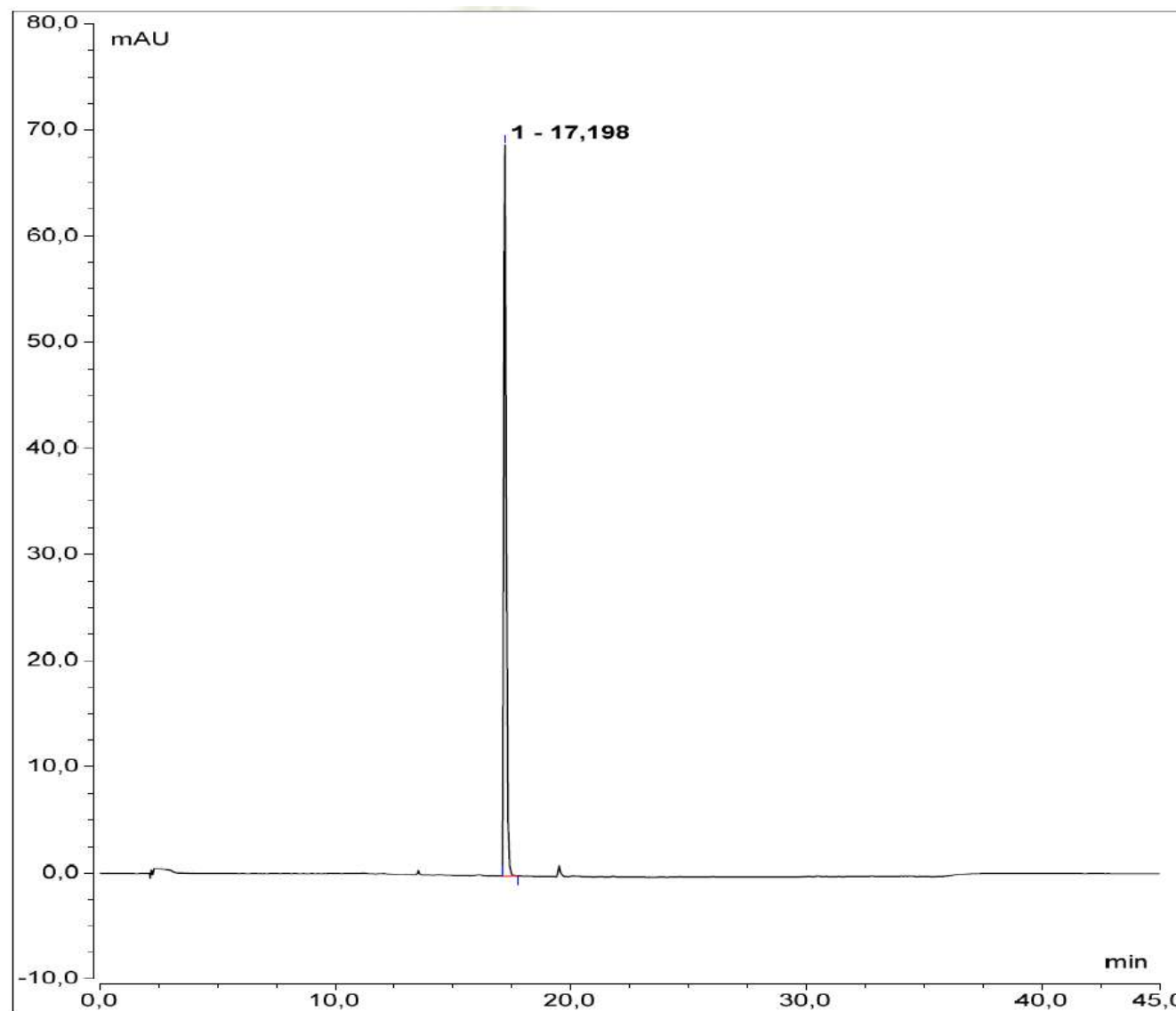


Chromatogram 4I



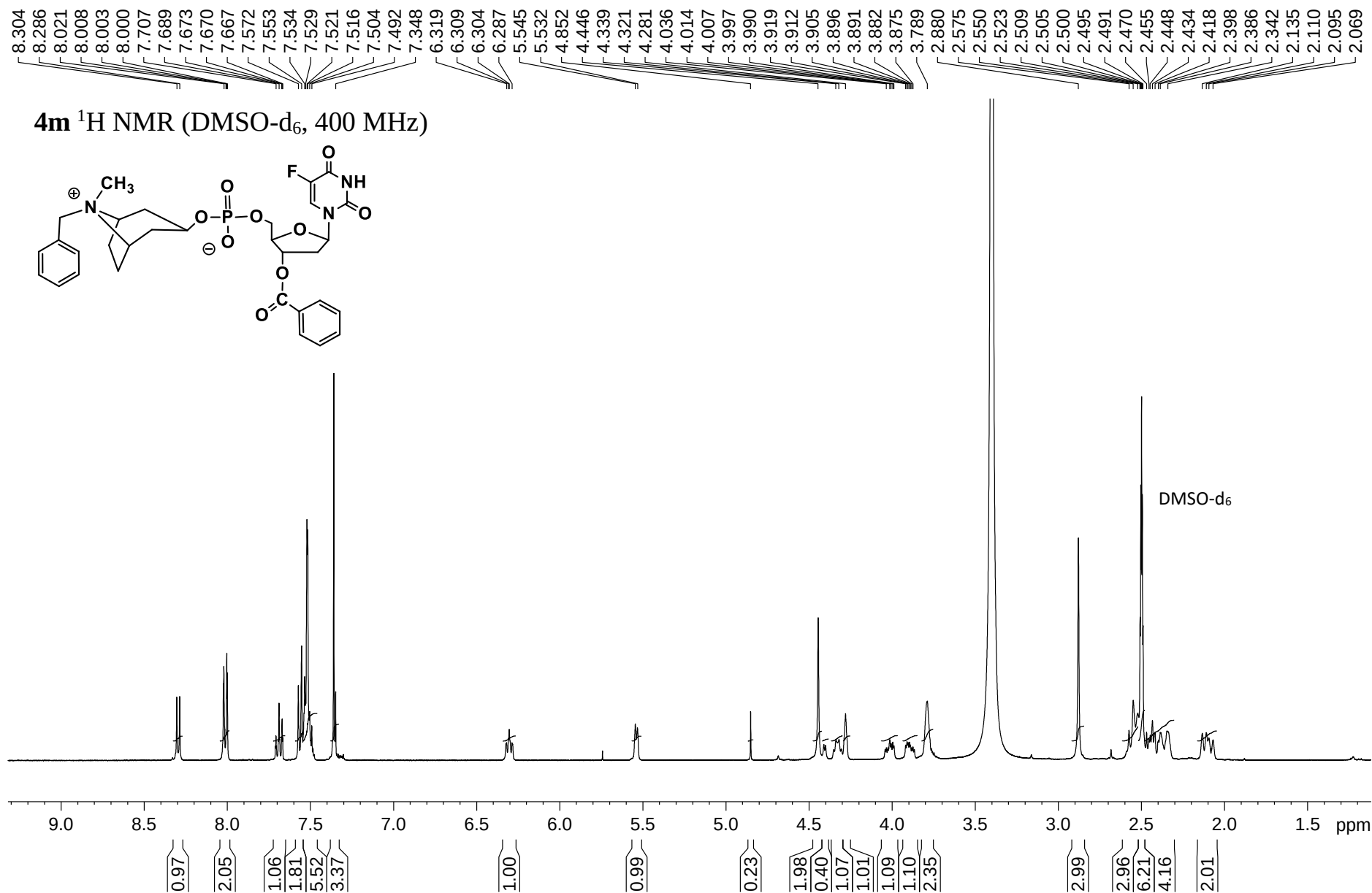
Lichrospher RP-18 endcapped
(5.0 μm , 4.6 mm $\times$ 150 mm)

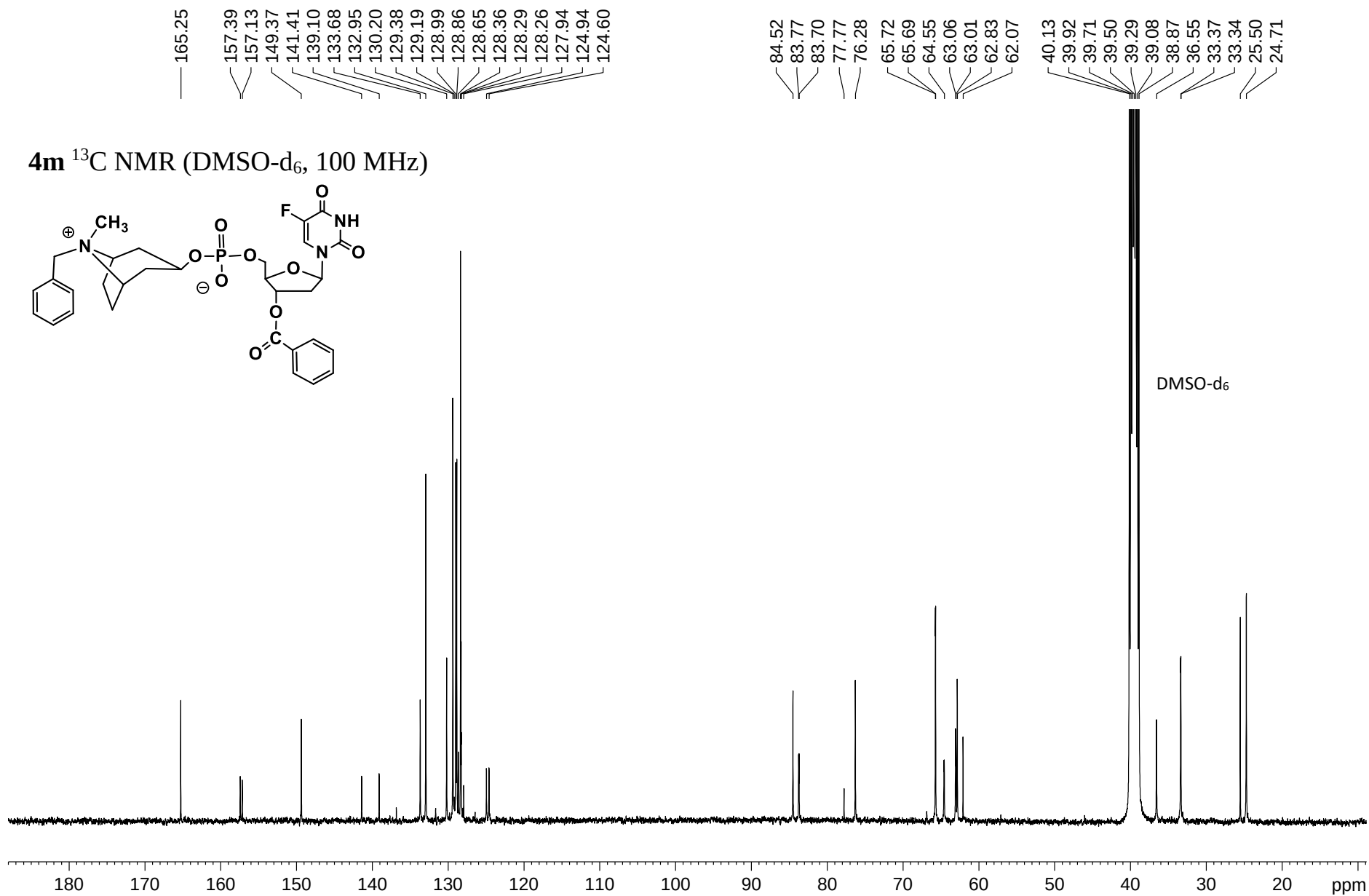
Thermo Scientific HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium acetate
pH 7; B, A / acetonitrile, 1: 4, v/v)
at 35°C, flow rate 1.5 mL/min;
events: 5 min A 100%, 20 min
B 0–100%, 5 min B 100% and
15 min A 100%.



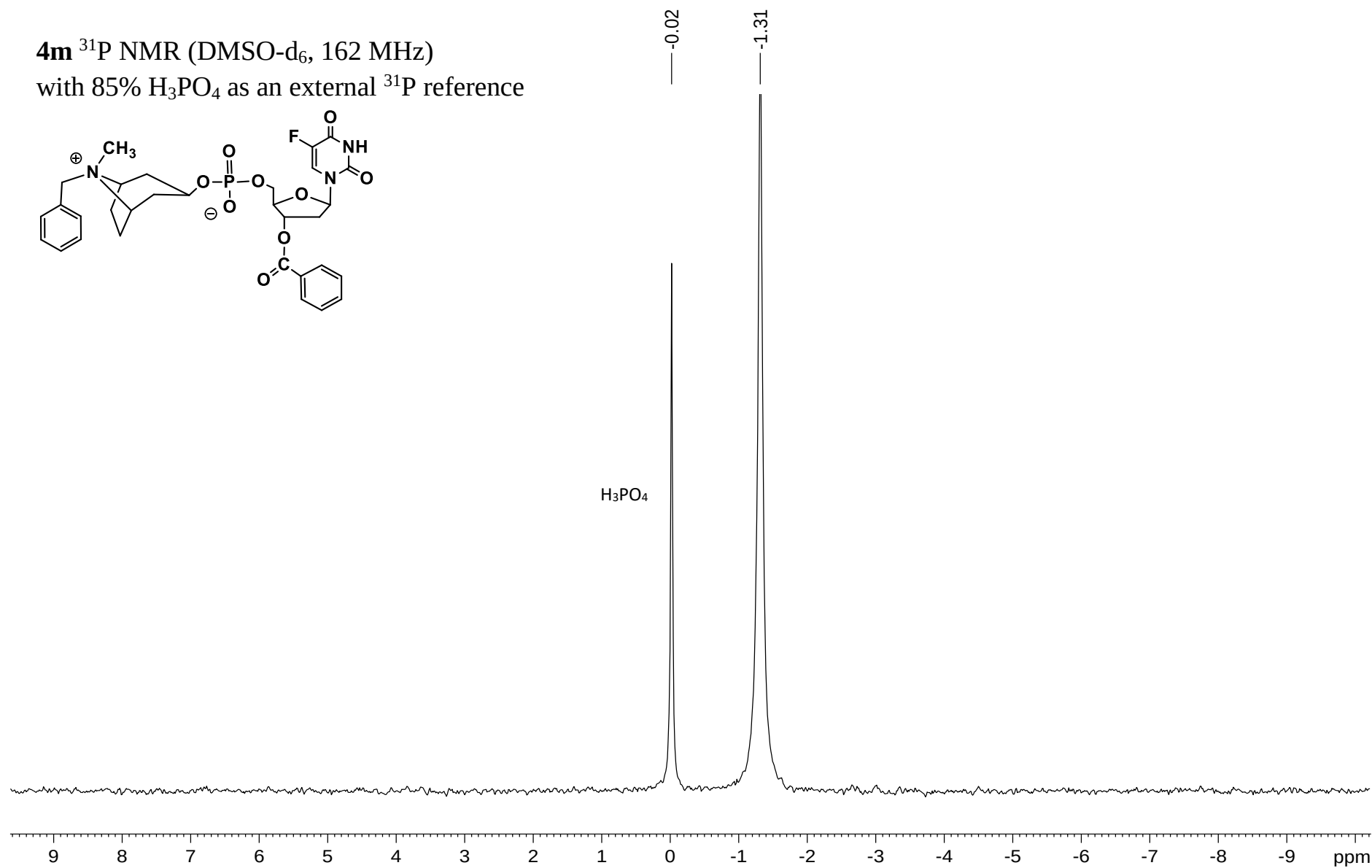
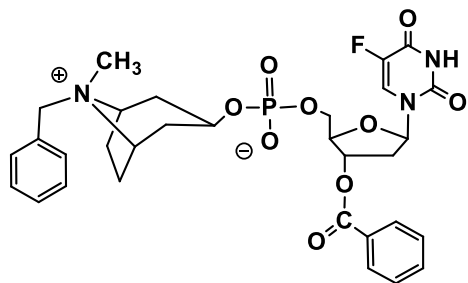
Default/Chromatogram

Chromeleon (c) Dionex
Version 7.2.3.7553

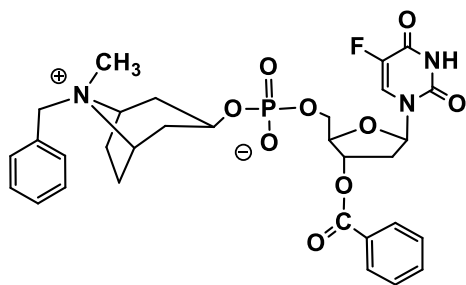




4m ^{31}P NMR (DMSO- d_6 , 162 MHz)
with 85% H_3PO_4 as an external ^{31}P reference

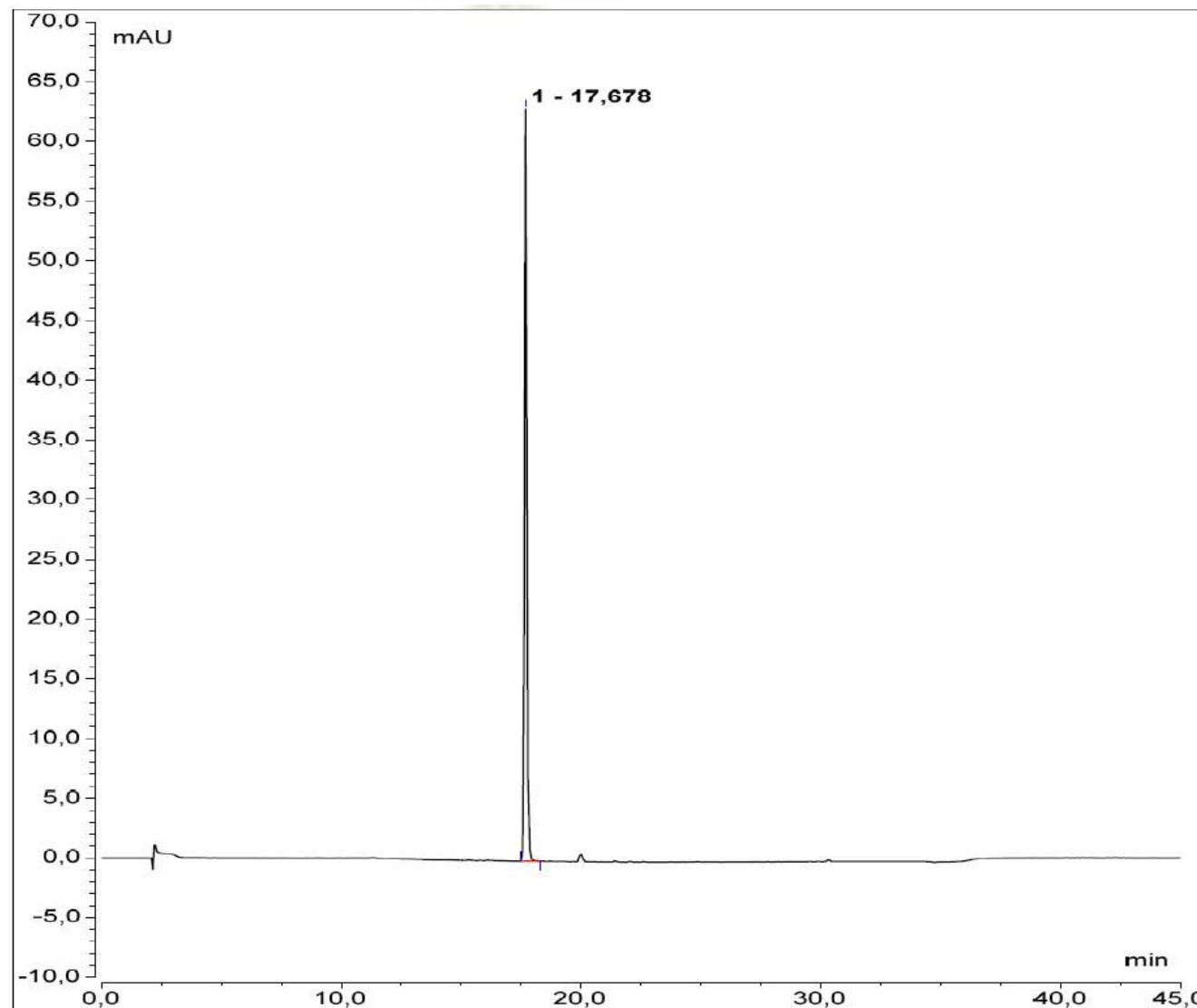


Chromatogram 4m



Lichrospher RP-18 endcapped
(5.0 μm , 4.6 mm $\times$ 150 mm)

Thermo Scientific HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium acetate
pH 7; B, A / acetonitrile, 1: 4, v/v)
at 35°C, flow rate 1.5 mL/min;
events: 5 min A 100%, 20 min
B 0–100%, 5 min B 100% and
15 min A 100%.



Default/Chromatogram

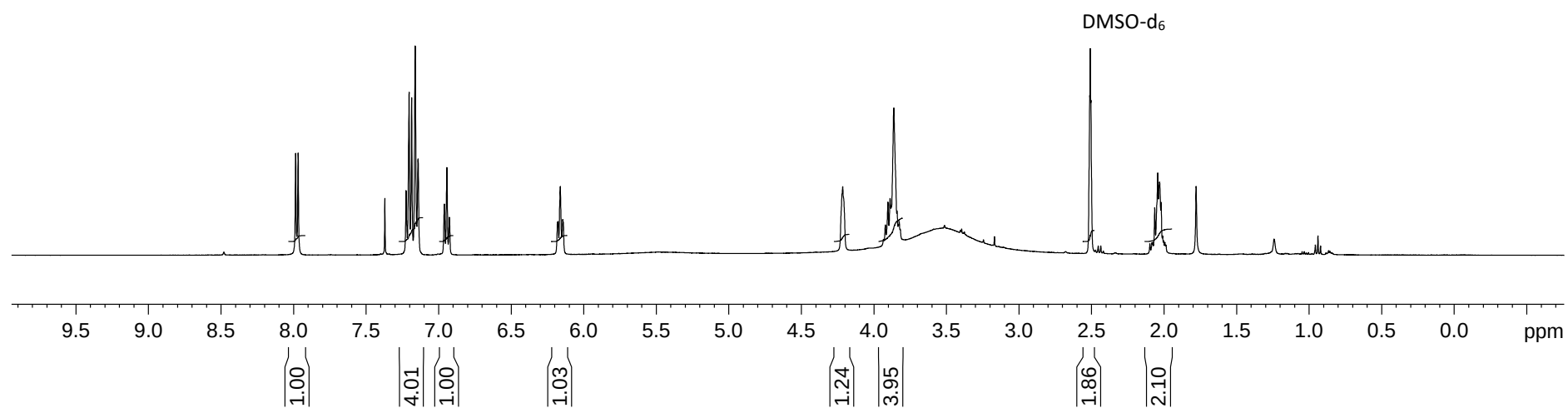
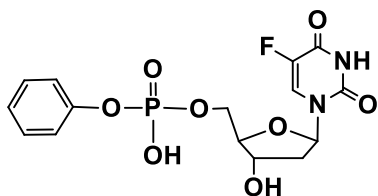
Chromeleon (c) Dionex
Version 7.2.3.7553

7.986
7.969
7.202
7.184
7.160
7.141
6.960
6.942
6.924
6.180
6.176
6.160
6.145

4.215
3.889
3.863

2.513
2.509
2.505
2.064
2.044
2.031
2.021

5a ^1H NMR (DMSO- d_6 , 400 MHz)



158.55
158.35
154.04
153.99
150.22

141.21
139.36

128.73
128.32
124.31
124.04
121.61
119.78
119.74

86.04
85.98
84.47

70.94

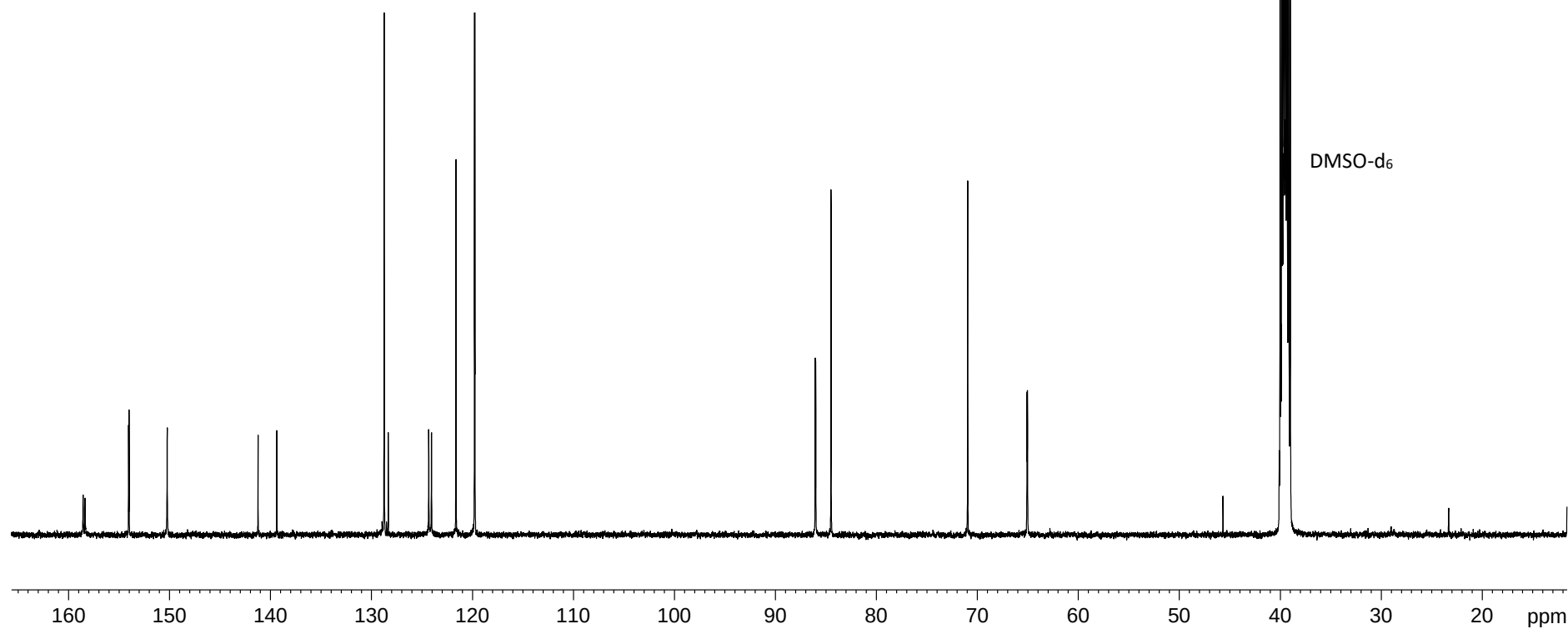
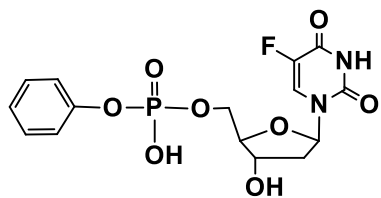
65.06
65.02

45.66

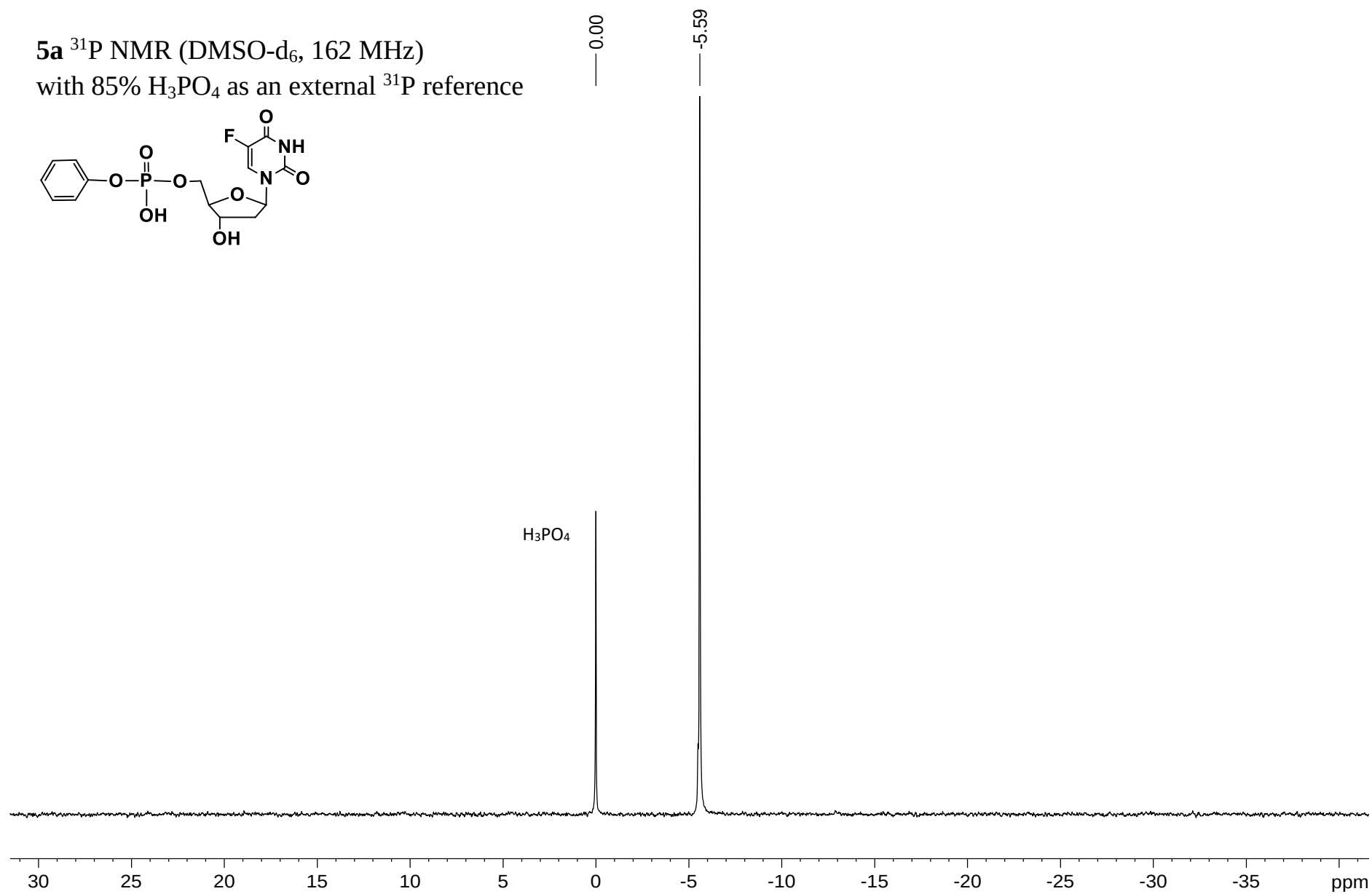
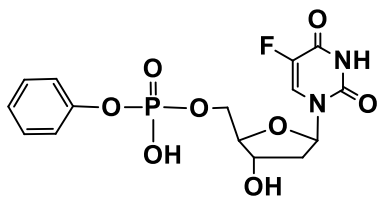
39.66
39.50
39.33

23.31

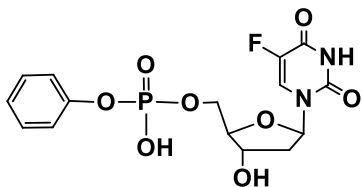
5a ^{13}C NMR (DMSO- d_6 , 100 MHz)



5a ^{31}P NMR (DMSO- d_6 , 162 MHz)
with 85% H_3PO_4 as an external ^{31}P reference

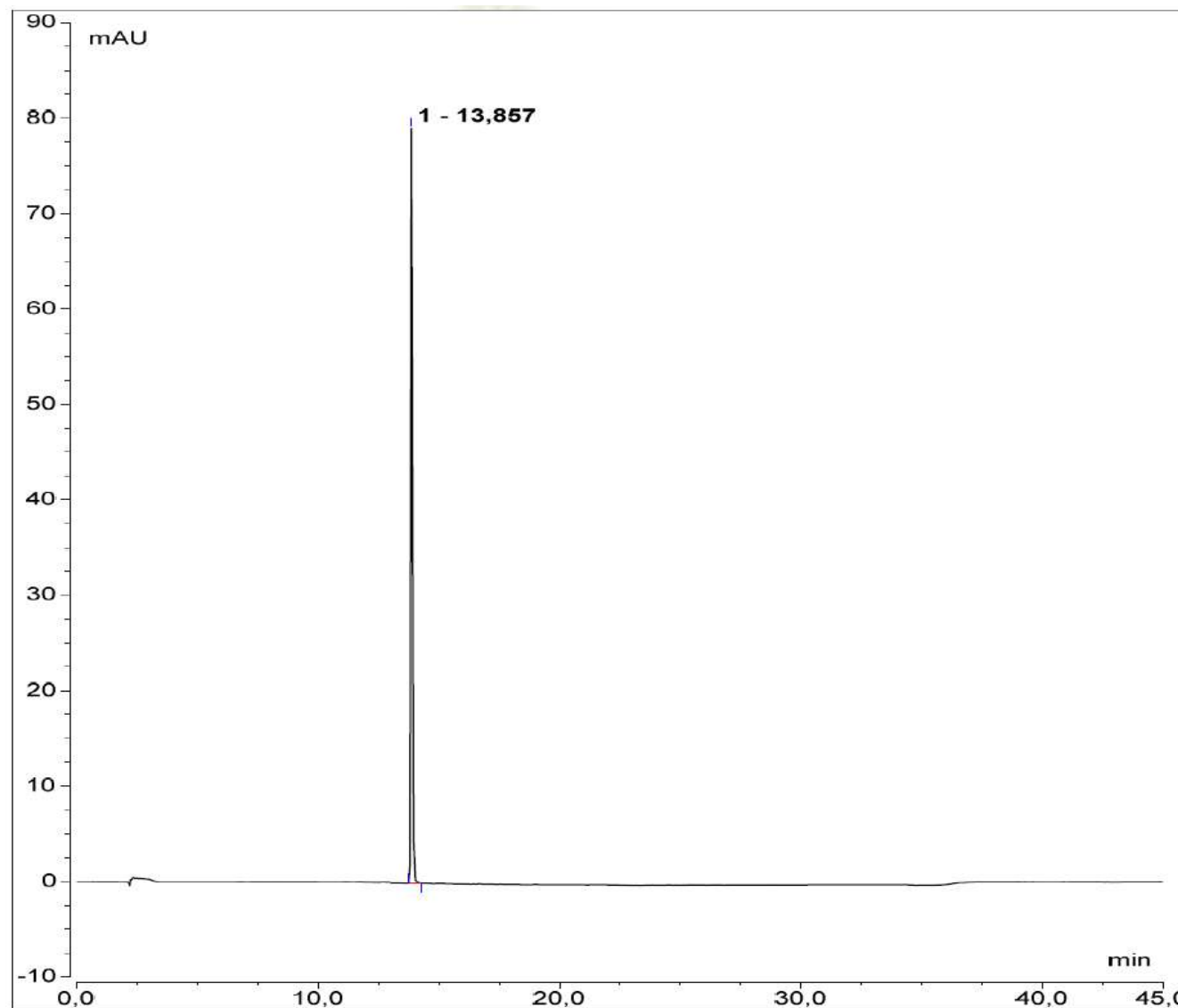


Chromatogram 5a



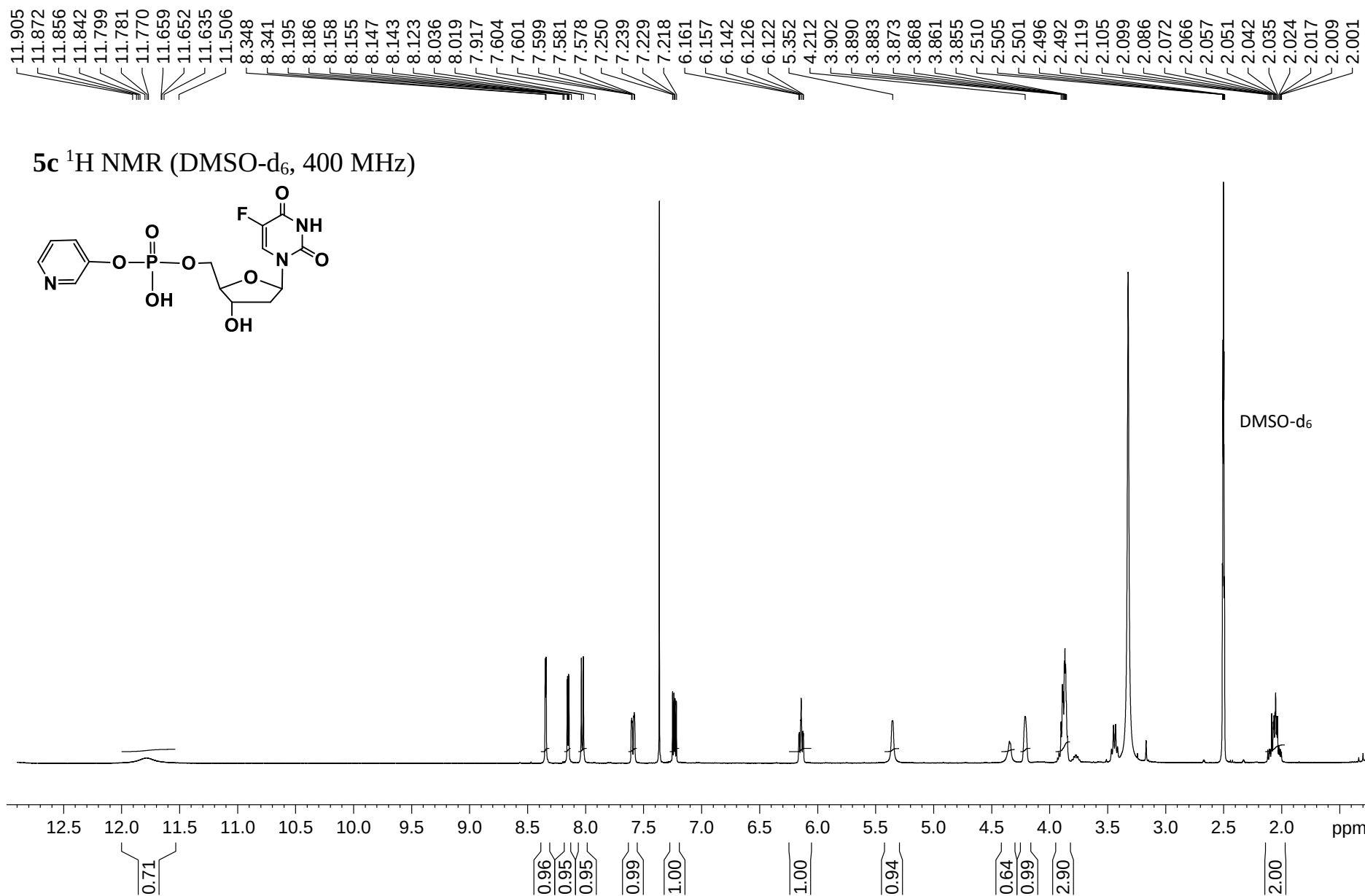
Lichrospher RP-18 endcapped
(5.0 μm , 4.6 mm $\times$ 150 mm)

Thermo Scientific HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium acetate
pH 7; B, A / acetonitrile, 1: 4, v/v)
at 35°C, flow rate 1.5 mL/min;
events: 5 min A 100%, 20 min
B 0–100%, 5 min B 100% and
15 min A 100%.

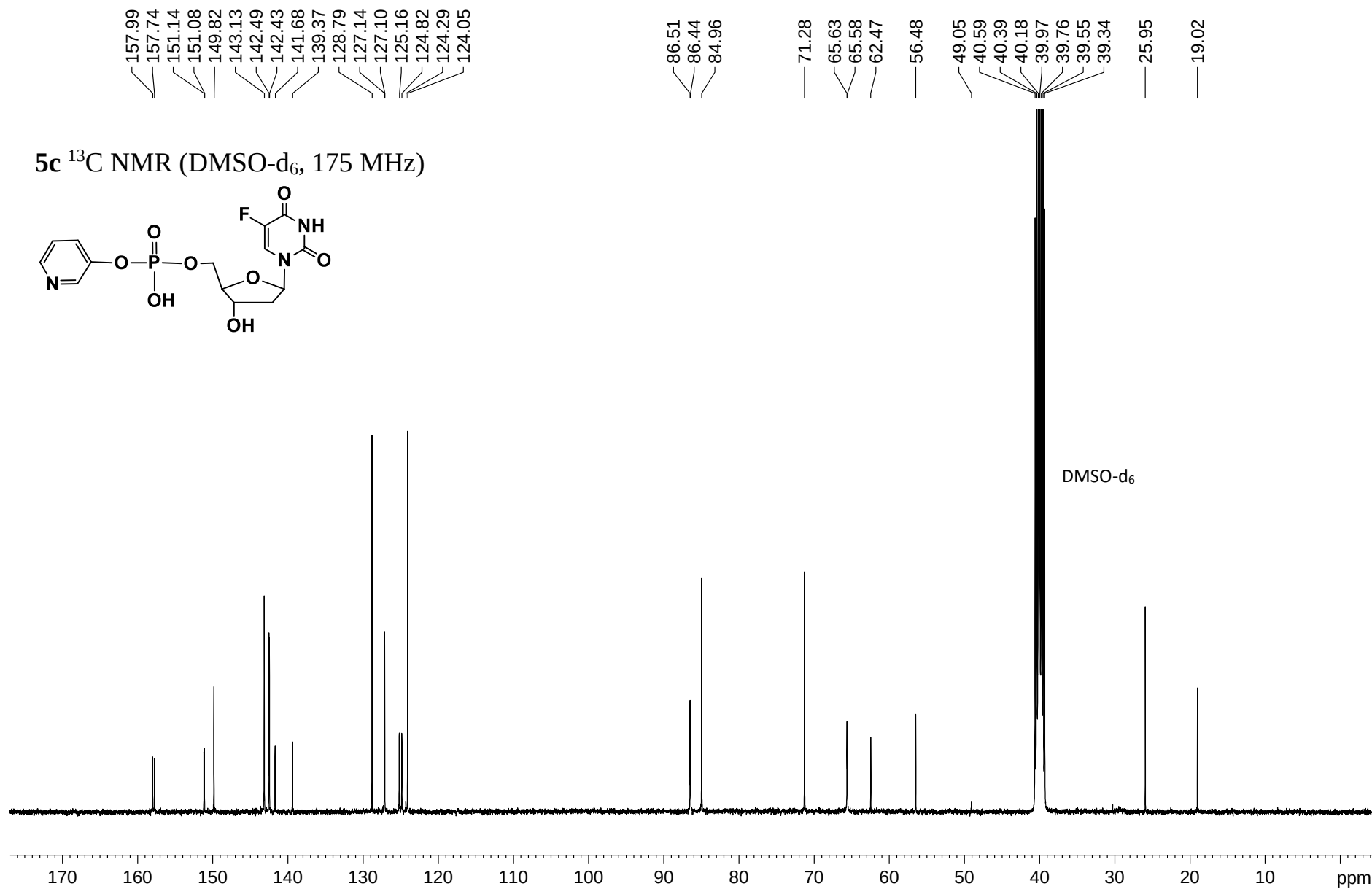
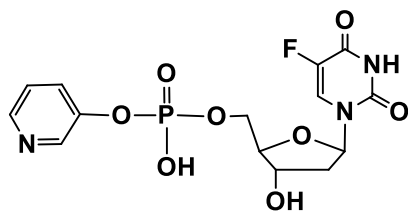


Default/Chromatogram

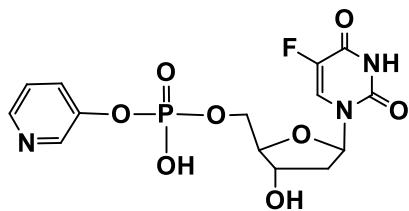
Chromeleon (c) Dionex
Version 7.2.3.7553



5c ^{13}C NMR (DMSO- d_6 , 175 MHz)

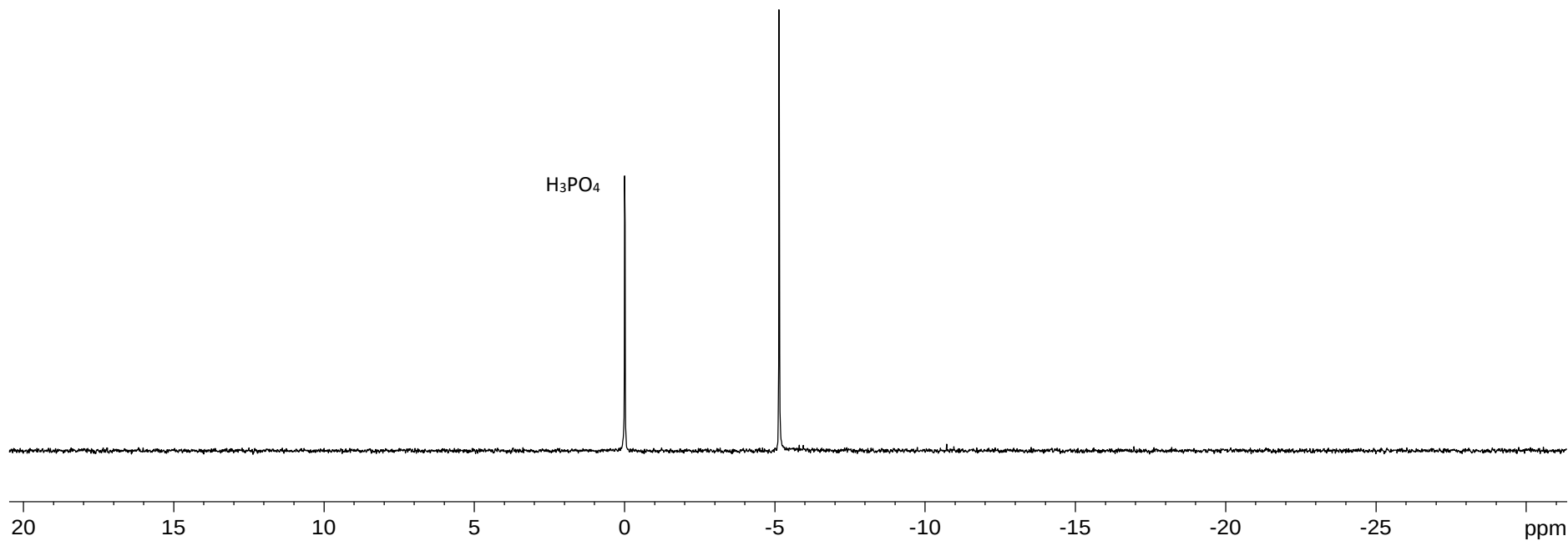


5c ^{31}P NMR (DMSO- d_6 , 162 MHz)
with 85% H_3PO_4 as an external ^{31}P reference

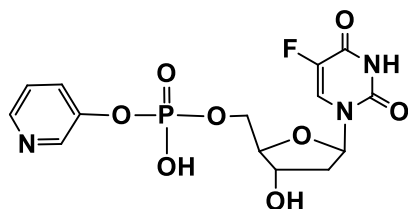


— -0.01

— -5.14

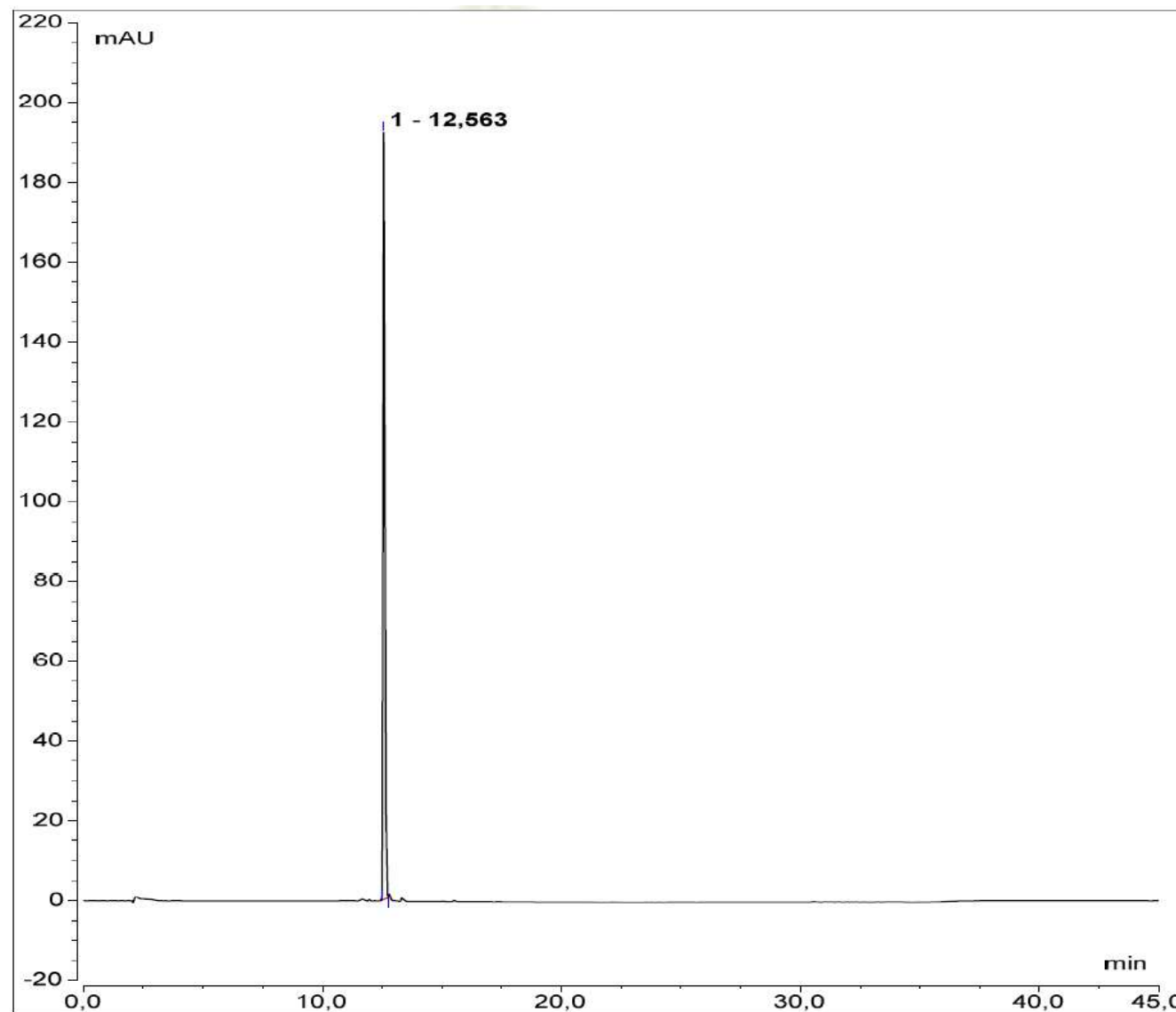


Chromatogram 5c



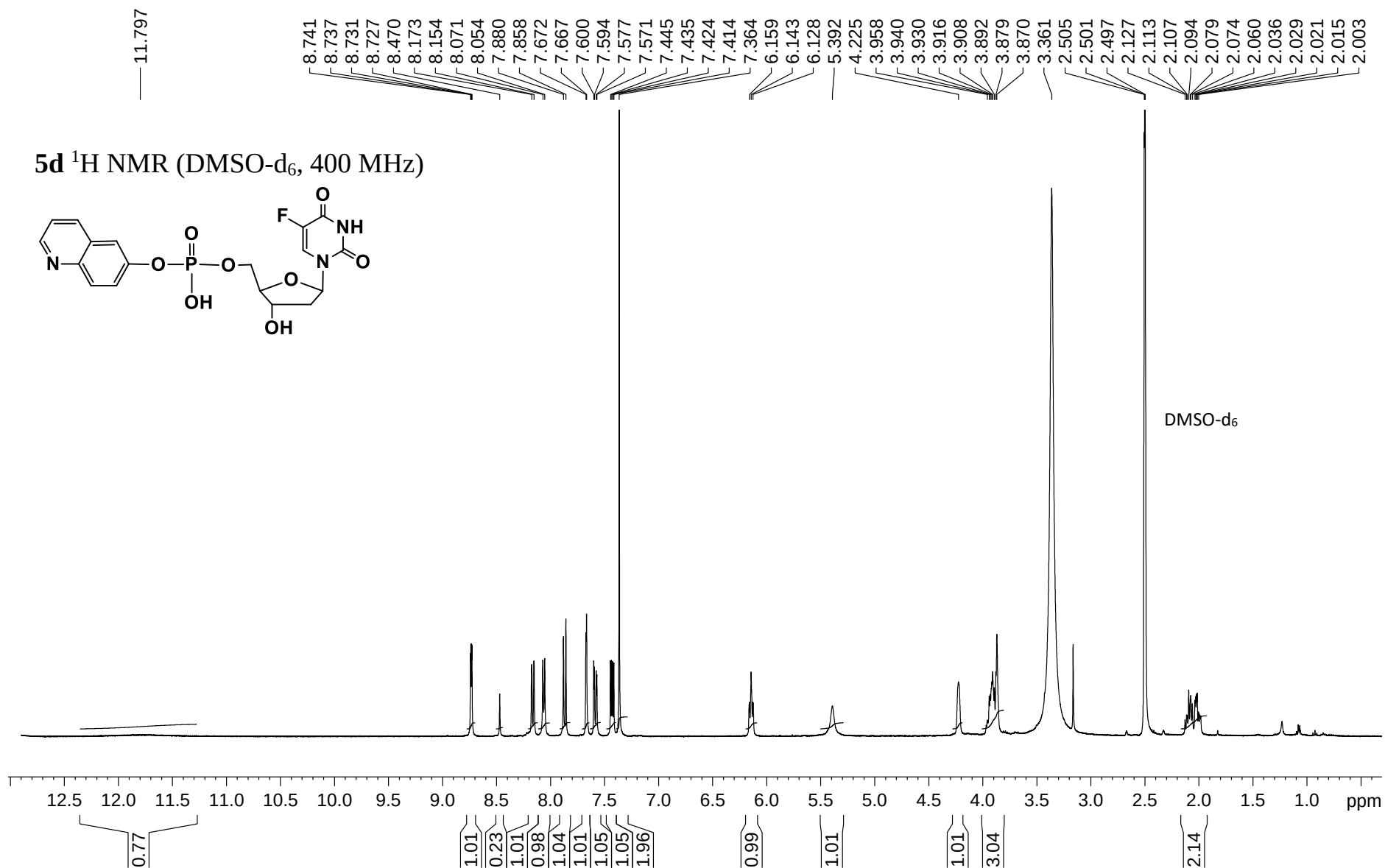
Lichrospher RP-18 endcapped
(5.0 μm , 4.6 mm $\times$ 150 mm)

Thermo Scientific HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium acetate
pH 7; B, A / acetonitrile, 1: 4, v/v)
at 35°C, flow rate 1.5 mL/min;
events: 5 min A 100%, 20 min
B 0–100%, 5 min B 100% and
15 min A 100%.



Default/Chromatogram

Chromeleon (c) Dionex
Version 7.2.3.7553



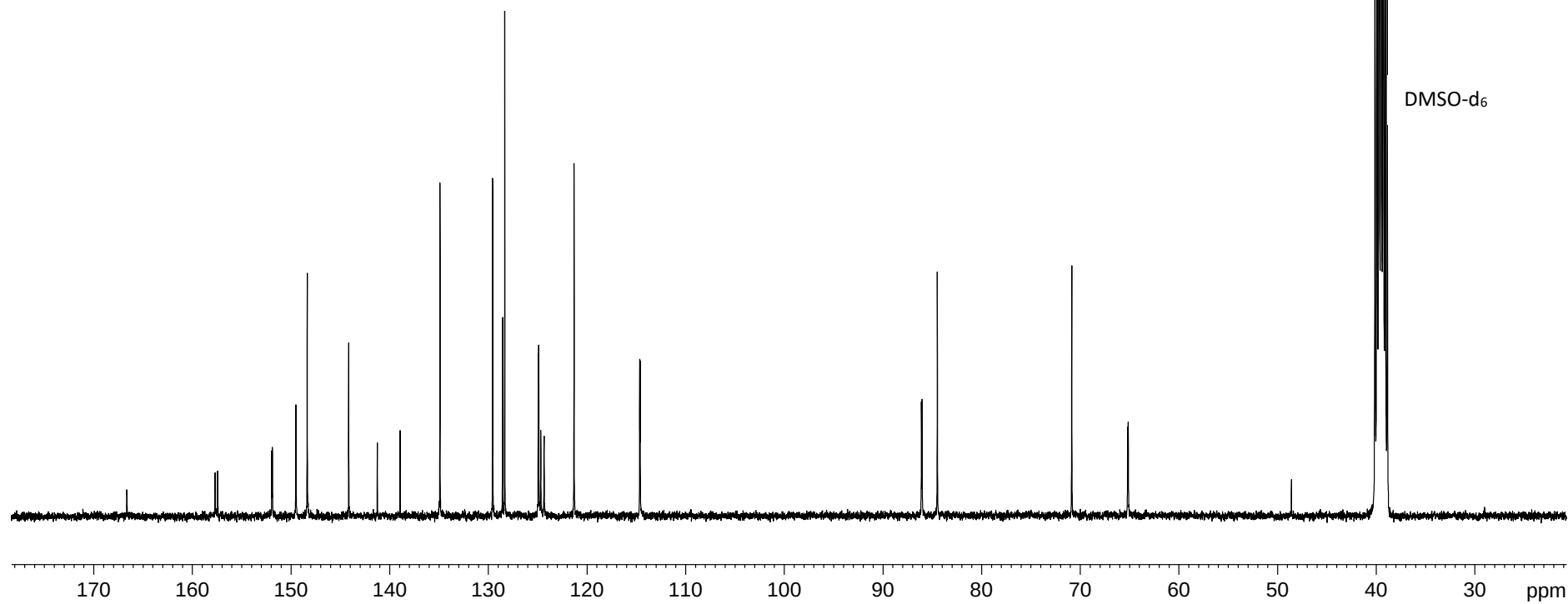
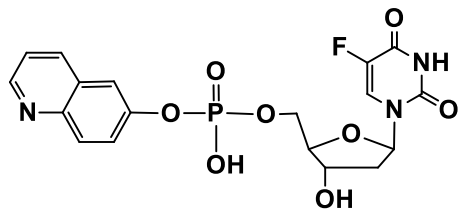
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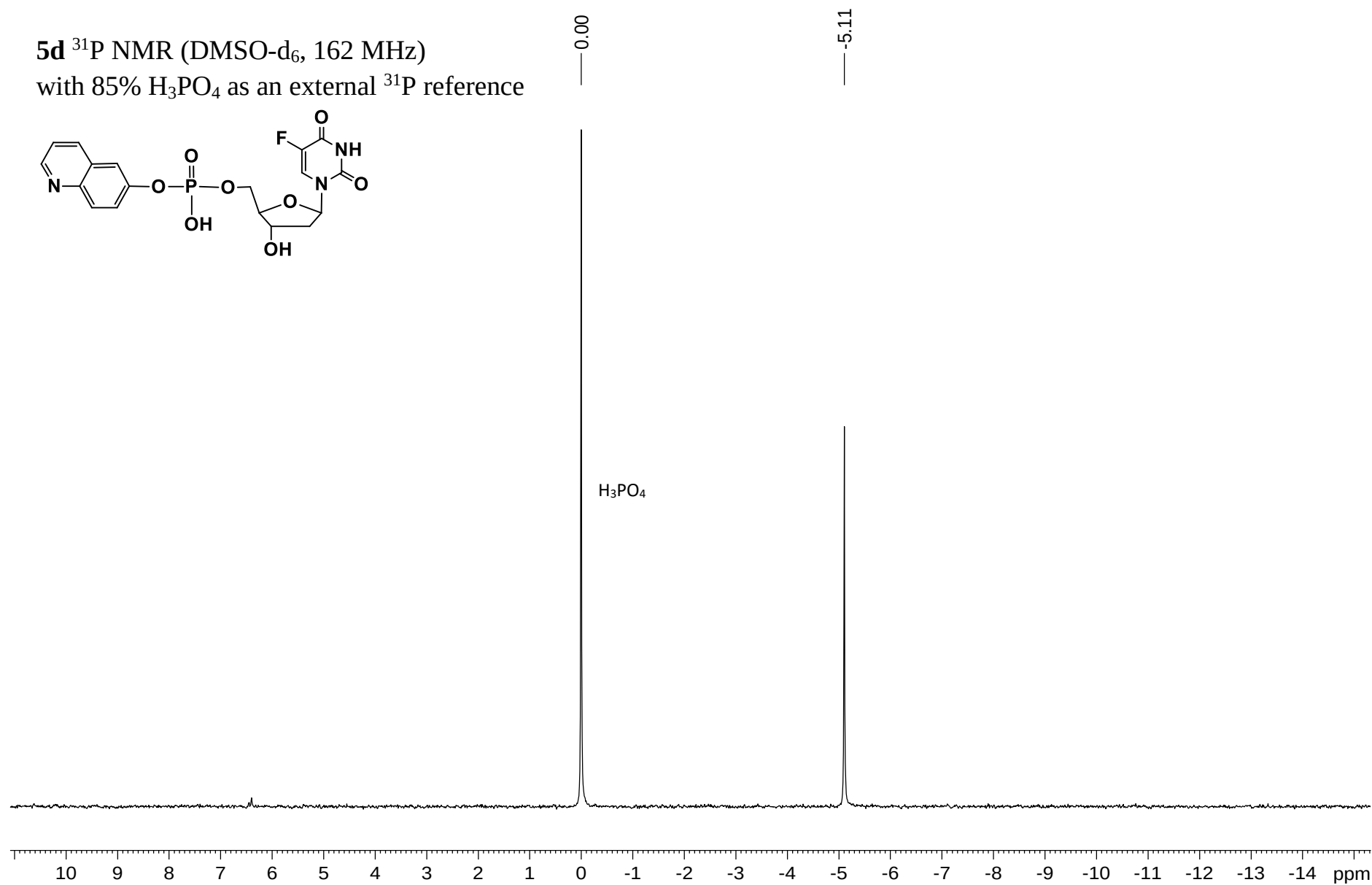
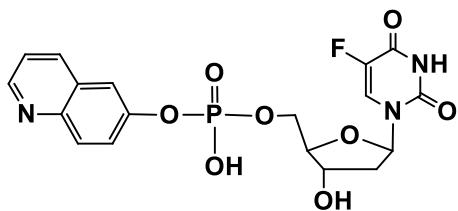
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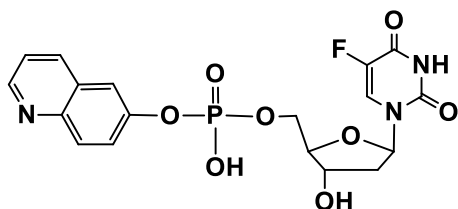
5d ^{13}C NMR (DMSO- d_6 , 100 MHz)



5d ^{31}P NMR (DMSO- d_6 , 162 MHz)
with 85% H_3PO_4 as an external ^{31}P reference

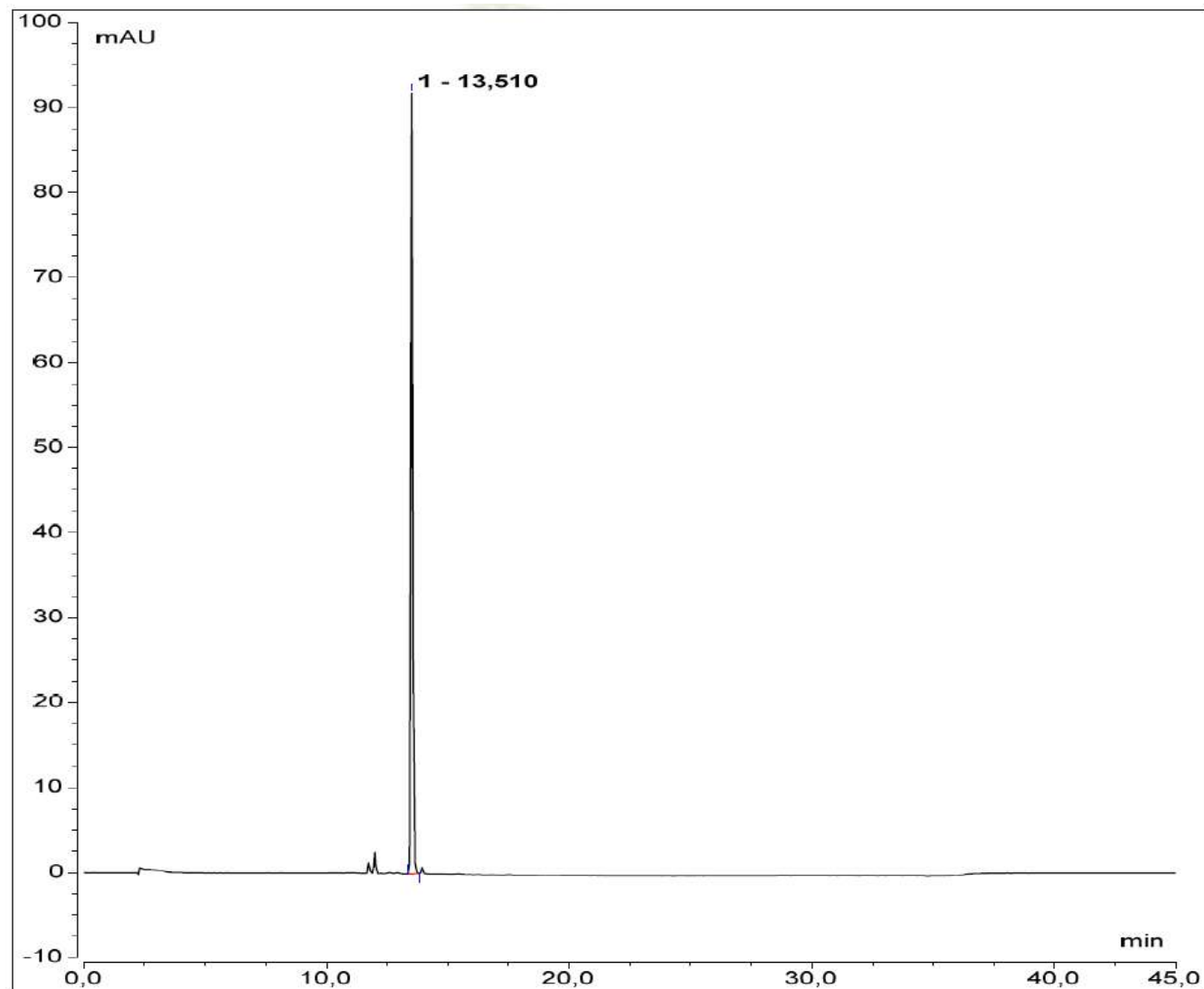


Cromatogram 5d



Lichrospher RP-18 endcapped
(5.0 μm , 4.6 mm $\times$ 150 mm)

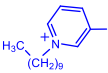
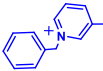
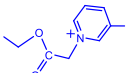
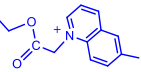
Thermo Scientific HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium acetate
pH 7; B, A / acetonitrile, 1: 4, v/v)
at 35°C, flow rate 1.5 mL/min;
events: 5 min A 100%, 20 min
B 0–100%, 5 min B 100% and
15 min A 100%.



Default/Chromatogram

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Table 1. Cytotoxicity (IC₅₀) of chosen phenols of type 2

Cpd No, Ar	MRC-5	T98G	U-118 MG	U-87 MG
	IC ₅₀ [μM]	IC ₅₀ [μM]	IC ₅₀ [μM]	IC ₅₀ [μM]
2c, 	>500	>500	>500	>500
2f, 	126.40 ± 5.8	55.61 ± 7.2	125.10 ± 6.1	158.10 ± 9.8
2g, 	>200	>200	>200	>200
2h, 	>200	>200	>200	>200
2i, 	162.40 ± 10.1	>200	>200	>200

3

Michalska L, Wawrzyniak D, Szymańska-Michalak A, Barciszewski J, Boryski J, Baraniak D

“Synthesis and biological assay of new 2'-deoxyuridine dimers containing a 1,2,3-triazole linker. Part I”

Nucleosides, Nucleotides and Nucleic Acids, 2019, 3:218-235



Synthesis and biological assay of new 2'-deoxyuridine dimers containing a 1,2,3-triazole linker. Part I

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ABSTRACT

We describe a simple method for the synthesis of modified dinucleosides containing pyrimidine nucleoside analogues (2'-deoxyuridine, thymidine and 5-fluoro-2'-deoxyuridine). Six different dimers with a 1,2,3-triazole linkage were obtained by azide-alkyne 1,3-dipolar cycloaddition (click reaction), starting from propargylated 2'-deoxyuridine and 5'-azido-nucleoside derivatives. Their cytotoxic activity was tested in five human cancer cell lines: cervical (HeLa), high grade gliomas (U-118 MG, U-87 MG, T98G), liver (HepG2), and normal human fibroblast cell line (MRC-5) using the sulforhodamine B (SRB) assay. The experiment showed that the obtained dimers with a 1,2,3-triazole moiety were very stable compounds, also in the physiological-like media, and had no anticancer activity.

ARTICLE HISTORY

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2'-deoxyuridine; floxuridine; nucleoside dimers derivatives; heterodinucleotides; click chemistry; 1,3-dipolar cycloaddition; 1,2,3-triazoles; cancer therapy; cytotoxic activity; human cancer cell lines

1. Introduction

Nucleosides analogues are important group of compounds because of their variety of pharmacological applications in the medicine.^[1,2] Therefore, scientists widely explore this area of medicinal chemistry to find new biologically active structures.

In this short paper we present some new nucleoside dimers analogues containing 2'-deoxyuridine (dU) and its simple analogues. 2'-Deoxyuridine is a naturally occurring, endogenous nucleoside, whose role is basically reduced to be a building element of deoxyribonucleic acids. Interestingly, the derivatives of 2'-deoxyuridine, especially those containing a substituent at the position 5, exhibit valuable biological properties and many of them have found clinical applications (Figure 1).^[3,4]

For instance, 5-iodo-2'-deoxyuridine (5-IdU), was the first nucleoside analogue introduced into medicine (it is used in keratitis therapy induced by HSV-1 since 1962).^[5] From a chemical viewpoint, 5-iodo derivative is a key substrate in the synthesis of different 5-substituted 2'-deoxyuridine

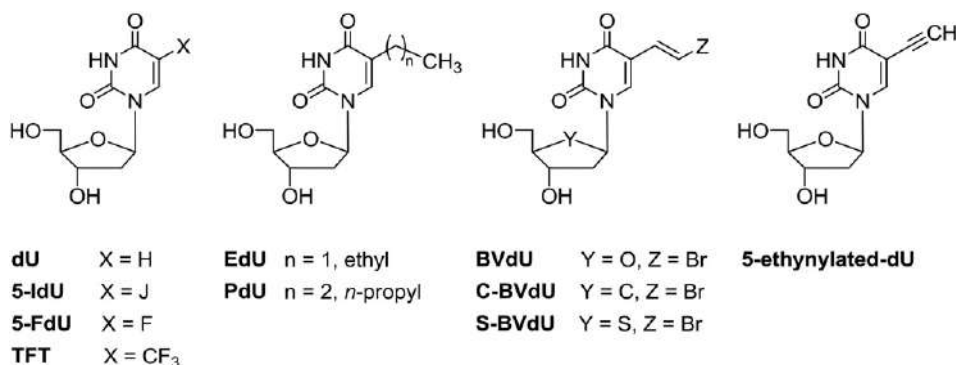


Figure 1. 2'-Deoxyuridine and its simple derivatives of medicinal application.

derivatives, among others – 5-trifluoromethyl-2'-deoxyuridine (TFT). Both, 5-IdU and TFT, are antiviral drugs used as DNA polymerase inhibitors (anti-herpesvirus compounds).^[6] In this group of the halogen derivatives, very important role plays 5-fluoro-2'-deoxyuridine (5-FdU) – a potent inhibitor of thymidylate synthetase (TS) that shows a broad spectrum of action in the cancer therapy.^[7,8]

Other useful pyrimidine analogues of 2'-deoxyuridine (with a short alkyl substituent) include 5-ethyl-2'-deoxyuridine (EdU)^[9] and 5-*n*-propyl-2'-deoxyuridine (PdU)^[10], which are antiviral drugs of high effectiveness against herpes simplex virus.

Derivatives with an unsaturated substituent are represented by (*E*)-5-(2-bromovinyl)-2'-deoxyuridine (BVdU), 1-[3-hydroxy-4-(hydroxymethyl)-cyclopentyl]-5-(2-bromovinyl)-2,4-(1*H*,3*H*)-pyrimidinedione (carbocyclic BVdU or C-BVdU) and (*E*)-5-(2-bromovinyl)-2'-deoxy-4'-thiouridine (S-BVdU).^[11,12] BVdU is a compound that exhibits the highest antiviral activity at nanomolar concentrations ($IC_{50} = 0.008 \mu\text{g/mL}$, $SI > 25\ 000$) and it is active against herpes simplex virus type 1 (HSV-1) and varicella zoster virus (VZV).^[13,14] C-BVdU, the carbocyclic nucleoside, is a very stable compound, because it is not a substrate for phosphorylases and hydrolases that normally cleave the *N*-glycosidic bond between the nucleobase and furanose ring of nucleosides. As a result, it has an increased chemical and metabolic stability. This compound possesses activity against HSV-1 and VZV, too.^[15] In turn, the sulfur derivative – S-BVdU exhibits antiherpes activity comparable with BVdU. However, this compound is resistant to glycosidic bond cleavage by mammalian phosphorylases, what is a major advantage compared to the cleavage-susceptible parent compound BVdU.^[16] The stability of S-BVdU against nucleoside phosphorylase and its reduced toxicity *in vivo* makes it a better antiviral agent than BVdU.^[17] Moreover, S-BVdU shows also an exquisite cytostatic effect, which makes it a promising candidate for the combined gene/chemotherapy treatment of cancer.

And the last of the described representatives – 5-ethynyl-2'-deoxyuridine is an anticancer drug.^[18] It is a potent inhibitor especially of human breast cancer cells (MCF-7) proliferation with the cytotoxic activity IC_{50} (μM) = 0.4 ± 0.3 . This means that the activity is exceeding or matching the IC_{50} of cisplatin or 5-fluorouracil.

Examples of 2'-deoxynucleoside derivatives with a triazole substituent, that have antitumor activity, are described by Raić-Malić et al.^[19] However, the most of 2'-deoxynucleoside derivatives described in the literature are compounds of antiviral activity. In majority of cases their anticancer activity has not been studied. And that was our goal in this work.

Thymidylate synthase (TS) is a critical cellular target for cancer chemotherapeutics, particularly for the fluoropyrimidine agents. Thus, there is a need to develop agents directed to selective inhibition of this enzyme. Therefore, we put the emphasis on pyrimidine derivatives with different arrangements of fluorine atom. In our work, the other non-fluorinated dimers were used as references in order to follow possible changes in the structure–activity relationship resulting from the presence of 5-fluorine substituent.

Taking this into account, we decided to explore the click chemistry in search for new potential chemotherapeutics.^[20–22] Our goal was the synthesis of novel analogues of dinucleotides, in which the phosphodiester bond would be replaced by a 1,2,3-triazole linker.^[23,24] We applied here the click reaction using 3'-O-propargylated 2'-deoxyuridine and the appropriated 5'-azido-nucleoside derivatives as substrates. The second part of our research was to check the structure – activity relationship and the impact of substituents with different chemical character but of a similar size.

We have explored the behavior of our six dinucleosides analogues and the relevant substrates in two biological assays. The potential molecular target was thymidylate synthase (TS) which is inhibited by 5-fluorouracil (5-FUra) derivatives. 5-Fluoro-2'-deoxyuridine (5-FdU) is a substrate for TS, hence the sense of search for new compounds inactivating this enzyme. We described anticancer activities of the dimers and their stability in the cell culture media (RPMI/FBS) and human serum (HS). For the cell growth inhibition assays, we tested all of our compounds against five human cancer cell lines of various origins – HeLa, U-118 MG, U-87 MG, T98G and HepG2 – and use the sulforhodamine B screening assay to determine their degrees of cancer growth inhibition.

2. Results and discussion

2.1. Chemistry

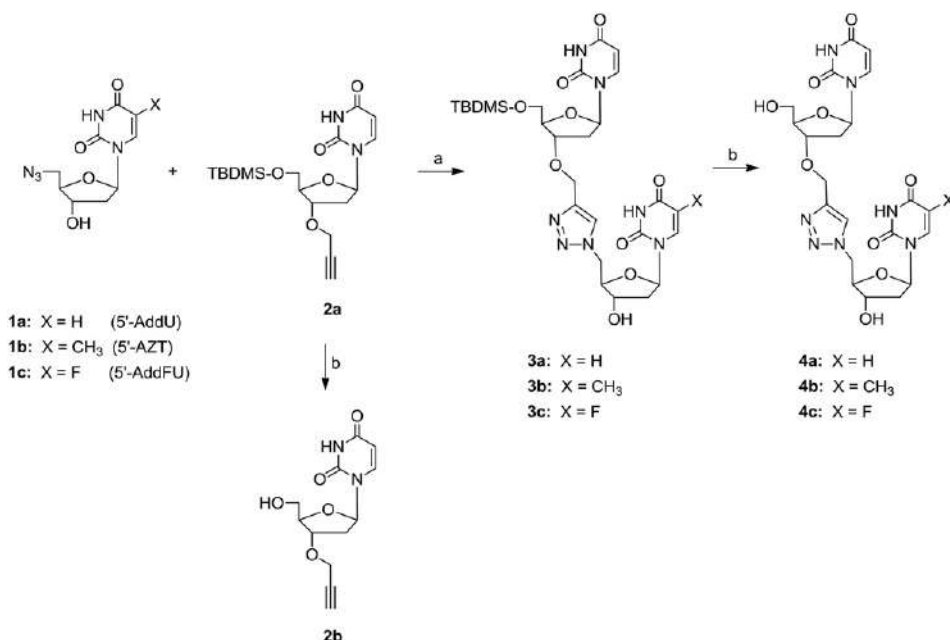
The purity and structures of the obtained compounds were characterized using NMR and ESI MS methods. The 1H , ^{13}C and ^{19}F NMR signals were assigned using one- and two-dimensional (1H – 1H COSY, 1H – ^{13}C HSQC,

^1H – ^{13}C HMBC) spectra. The NMR and ESI MS spectra of all conjugates are included in the [Supplementary material](#).

The synthesis of a series of nucleosides dimers analogues **3a–c** and **4a–c** ([Scheme 1](#)) was performed as the Cu(I)-catalyzed Huisgen reaction under classic Sharpless conditions for click chemistry.^[20,21] To synthesize the first series of compounds **3a–c**, we took advantage of the 1,3-dipolar cycloaddition by coupling 5'-azidonucleosides analogues, namely: 5'-azido-2',5'-dideoxyuridine (**1a**, 5'-AddU), 5'-azido-5'-deoxythymidine (**1b**, 5'-AZT) and 5'-azido-2',5'-dideoxy-5-fluorouridine (**1c**, 5'-AddFU) with the corresponding 3'-O-propargyl derivative **2a** using again the procedure involving copper(I) cations generated *in situ* from copper(II) sulfate and sodium ascorbate (NaAsc) in THF–water medium. Next, the silylated conjugates **3a–c** were deprotected with ammonium fluoride in absolute methanol, which lead to the second series of nucleosides dimers analogues – 1,4-disubstituted 1,2,3-triazoles (**4a–c**) ([Scheme 1](#)).

There is also another possibility to obtain dimers of the type **4a–c**, namely by using the unblocked – 3'-O-propargyl 2'-deoxyuridine (**2b**) as a substrate and coupling with the proper 5'-azidonucleoside.

The 5'-azido-nucleosides analogues: 5'-azido-2',5'-dideoxyuridine (**1a**, 5'-AddU), 5'-azido-5'-deoxythymidine (**1b**, 5'-AZT) and 5'-azido-2',5'-dideoxy-5-fluorouridine (**1c**, 5'-AddFU) were synthesized using the method by Nyiles et al.^[25] ([Scheme 2](#)). In this procedure the substrate underwent a selective



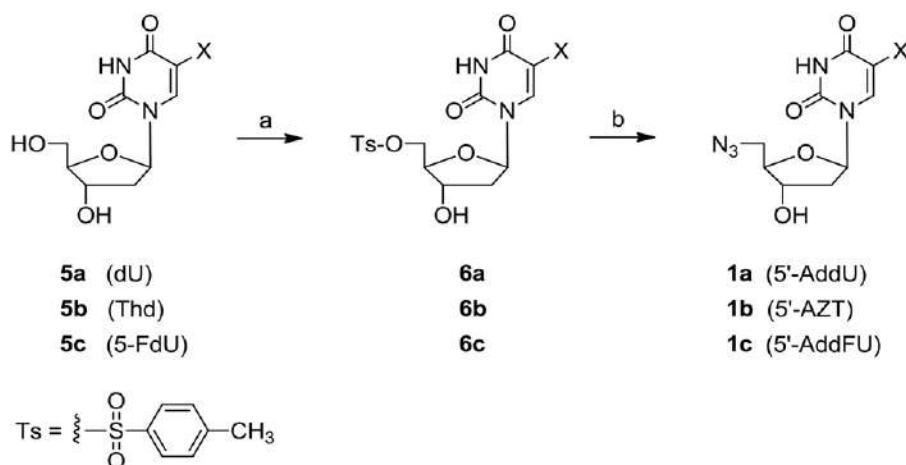
Scheme 1. Coupling of 5'-azido-nucleosides and 3'-O-propargyl-2'-deoxyuridine *via* click chemistry. Reagents and conditions: (a) 1M CuSO₄ (0.4 eq), NaAsc (0.8 eq), THF–H₂O (3:1, v/v), rt, 24 h; (b) NH₄F (5 eqs), abs. MeOH, reflux, 4 h.

tosylation reaction in the 5'-position of the pyrimidine nucleoside, what resulted in the transformation of the hydroxyl group into the good leaving O-tosyl group. Next, the obtained intermediates – 5'-O-tosyl derivatives (**6a–c**) were treated with sodium or lithium azide in dimethylformamid in order to displace the tosyl group with an azide. After the azidation reaction the required 5'-azido-nucleosides were obtained (**1a–c**).

The ethynyl derivatives, which are represented by two propargylated analogues of dU (**2a**, **2b**) with the substituent at 3'-position, were obtained according to the recently published procedure.^[26] The synthetic method for nucleoside propargylation consisted of two steps. The first one involved the generation of hydroxyl anion at the 3'-position of 5'-O-(*tert*-butyldimethylsilyl)-2'-deoxyuridine using sodium hydride in tetrahydrofuran under argon atmosphere for 30 minutes. The preparation of 3'-O-propargyl-5'-O-(*tert*-butyldimethylsilyl)-2'-deoxyuridine was done by addition of the propargyl bromide as 80% solution in toluene. The reaction mixture was stirred for 12 hours. Removal of the TBDMS protecting group was relatively simple. It was done by utilizing the reactivity of the fluoride ion towards the *tert*-butyldimethylsilyl moiety, what resulted in obtaining the desired 3'-O-propargyl-2'-deoxyuridine (**2b**) (Scheme 1).

2.2. Biology (Cytostatic activity and stability in physiological-like media)

Glioblastoma multiforme (GBM) is a very aggressive primary brain tumor, and affected patients survive approximately a year after diagnosis.^[27] The high invasiveness of glioma cells results from their remarkable ability to infiltrate healthy brain tissue and migrate within the central nervous system, which makes them intangible targets for effective surgical resection



Scheme 2. Synthesis of 5'-azido-2',5'-dideoxyuridine nucleosides. Reagents and conditions: (a) TsCl (1.3 eq), Py, 0–5°C, 24 h; (b) NaN₃ (1.8 eq), DMF, 100°C, 3 h.

and radiotherapy.^[28] Therefore, novel therapeutic compounds for treating GBM cells are urgently needed. Since nucleoside analogs were hardly explored as therapeutic agents against GBM,^[29] in this studies we focused on novel triazole dimers of 2'-deoxyuridine with other pyrimidine nucleosides, which were evaluated for their cytotoxic activity in five human cancer cell lines: cervical (HeLa), high grade gliomas (U-118 MG, U-87 MG, T98G), liver (HepG2) and normal human lung fibroblast cell line (MRC-5) employing the sulforhodamine B (SRB) assay.^[30] The results were compared with the activity of 5-FdU, the known anticancer agent, as a reference drug. The cytotoxicity of the tested compounds was expressed by median growth inhibitory concentration (IC_{50}) which corresponds to the concentration of tested compound that inhibits cell viability by 50% after 72 hours exposure to tested compounds. The screening results are given in Table 1. Unfortunately, dimers with a 1,2,3-triazole linkage (**3a–4c**), did not show anticancer activity. The IC_{50} values of these compounds for all cells were above 100 μ M. Apparently, no anticancer activities of these compounds was an effect of lack of hydrolysis and diffusion across a lipid bilayer cell membrane. Regarding the series of nucleoside analogues **1a–2b**, an anticancer activity was displayed only by **2a**. Other compounds of this group showed no activity. Interestingly, compound **2b** was found potent cytotoxic agent ($IC_{50} = 13.3 \mu$ M) only in HepG2 cancer cell line.

All six dimers (**3a–4c**) remained unchanged for over 5 days in RPMI/FBS (9:1, v/v) and HS (RP HPLC analysis, data not shown). This confirms high enzymatic resistance of compounds with a 1,2,3-triazole linkage as opposed to natural phosphodiesteres. It can be assumed that the examined compounds may not cross cellular membranes and exert biological effect in cancer cells.

2.3. Physicochemical data and drug-likeness

To predict the potential of nucleosides analogues as potential drugs, some descriptors of their pharmacokinetic profile were determined. Parameters

Table 1. *In vitro* cytotoxic activity (IC_{50} , μ M)^a of 5-FdU and compounds **1a–4c**.

Compd	HeLa	HepG2	T98G	U-118 MG	U-87 MG	MRC-5
1a	>100	>100	>100	>100	>100	>100
1b	>100	>100	>100	>100	>100	>100
1c	>100	>100	>100	>100	>100	>100
2a	19.93 $\pm$ 2.61	5.85 $\pm$ 0.52	47.46 $\pm$ 8.42	25.73 $\pm$ 10.93	44.94 $\pm$ 7.99	32.91 $\pm$ 17.76
2b	>100	13.30 $\pm$ 2.57	>100	>100	>100	>100
3a	>100	>100	>100	>100	>100	>100
3b	>100	>100	>100	>100	>100	>100
3c	>100	>100	>100	>100	>100	>100
4a	>100	>100	>100	>100	>100	>100
4b	>100	>100	>100	>100	>100	>100
4c	>100	>100	>100	>100	>100	>100
5-FdU	2.87 $\pm$ 0.78	37.86 $\pm$ 6.69	17.81 $\pm$ 7.03	11.88 $\pm$ 4.31	18.46 $\pm$ 9.62	14.06 $\pm$ 6.34

^a IC_{50} – is the compound concentration required to inhibit cell growth by 50%.

such as the molecular polar surface area (PSA)^[31], the lipophilic character (LogP)^[32], the ability to be absorbed through the intestinal tract to the blood (Caco-2 cell permeability, tP_{app}) and the verification of Lipinski's "rule of five"^[33] were calculated in order to analyze their drug likeness for a potential oral used as anti-glioma agents. The calculated values of *in silico* screening (Table 2), indicate low to moderate octanol/water log P coefficients (LogP) for all the compounds. The low lipophilic character (<1.0) and molecular weights higher than 500 g/mol calculated for modified dinucleosides (**3a–4c**), may suggest difficulties in their transport through the cell membranes. The Caco-2 permeability rates described by tP_{app} parameter (4–70 nm/s) indicate that the absorption through the intestinal tract to the blood is limited.^[34] Also the compounds with polar molecular surface area equal or greater than 140 Å² should have poor absorption ability.^[35] Taken together these data indicate that the modified nucleoside dimers with a 1,2,3-triazole linkage do not meet most of the drug-like criteria and can't be considered as drug candidates. Only compound **2a** meets the drug-likeness criteria and thus can be considered as a potential drug candidate. Molecular weight lower than 500 g/mol, moderate lipophilic nature, no violation of the Lipinski's "rule of five" and small polar molecular surface area (<60 Å²) indicate that the absorption through the intestinal track to blood and brain penetration is possible.^[36]

3. Experimental protocols

3.1. General chemistry methods

¹H and ¹³C NMR spectra were recorded on a Bruker 400 and 500 spectrometers operating at 400 or 500 MHz and 100 or 125 MHz, respectively.

Table 2. Selected physicochemical data of 5-FdU and compounds **1a–4c**.

Compd	MW	PSA ^a [Å ²]	aPSA ^b [Å ²]	HBD ^c	HBA ^d	LogP ^e	tP_{app} [nm/s] ^f	Violations of „rule of five“
1a	253.21	160.4	125.2	2	9	−1.17	17.16	0
1b	267.24	153.1	144.6	2	9	−0.8	18.29	0
1c	271.21	156.4	136.9	2	9	−0.88	14.28	0
2a	380.18	51.0	278.6	1	7	2.02	23.92	0
2b	266.09	106.0	173.7	2	7	−0.79	13.90	0
3a	633.73	132.8	319.6	3	16	0.68	5.48	2
3b	647.75	125.5	338.9	3	16	1.01	6.85	2
3c	651.71	128.8	331.3	3	16	0.91	5.12	2
4a	519.46	187.9	214.7	4	16	−2.08	16.26	2
4b	533.49	180.6	234.1	4	16	−1.72	16.85	2
4c	537.45	183.9	226.4	4	16	−1.84	16.46	2
5-FdU	246.19	129.2	136.9	3	7	−1.28	20.62	0

^aPSA – polar surface area.

^baPSA – a polar surface area.

^cHBD – hydrogen bonds donors.

^dHBA – hydrogen bonds acceptors.

^elogP – calculated with ALOGPS 2.1 software (wide Experimental).

^f tP_{app} – apparent theoretical permeability.

^{19}F NMR spectra were recorded on a Bruker 500 spectrometer at 470 MHz. The chemical shifts were reported in ppm (δ scale). Mass spectra were recorded using ESI-MS QqToF Bruker mass spectrometer. Thin-layer chromatography (TLC) was carried out on Merck precoated 60 F₂₅₄ silica gel plates, while column chromatography on Merck silica gel 60H (40–63 μm). HPLC analyses were performed on a Lichrospher RP-18 endcapped (5.0 μm , 4.6 mm $\times$ 250 mm) using Thermo ScientificTM HPLC systems with A + B solvent systems (A, 0.01 M aqueous triethylammonium acetate pH 7; B, A/acetonitrile, 1:4, v/v) at 35 °C, flow rate 1.5 mL/min; events: 5 min A 100%, linear gradient of B 0–100% in 20 min, 5 min B 100% and A 100% 15 min wash. The substrate for the synthesis of compound **2a** (3'-O-propargyl-5'-O-(*tert*-butyldimethylsilyl)-2'-deoxyuridine) was synthesized using well-known silylation procedure described in the literature.^[26] Chemical reagents were purchased from Acros Organics, Alfa Aesar, Carbosynth and Sigma-Aldrich.

3.2. Chemical synthesis

3.2.1. General procedure for synthesis of 5'-azidonucleosides

3.2.1.1. Synthesis of 5'-O-tosyl-nucleosides (6a–c). An appropriate nucleoside (**5a–c**; 4 mmol, 1 eq) was dissolved in anhydrous pyridine (Py, 40 mL) under argon atmosphere. After the substrate was dissolved, the flask with the solution was placed in an ice bath and cooled. Then tosyl chloride (1.3 eq, Ts-Cl) was added and stirred until it was completely dissolved. The reaction flask was placed in a refrigerator for 24 hours and progress of the reaction was checked by the thin-layer chromatography (TLC) using chloroform – methanol (10:1, v/v) as a solvent system. The reaction mixture was quenched with methanol and then evaporated to dryness. The crude product was dissolved in chloroform and extracted with saturated aqueous solution of NaHCO_3 . The organic layer was evaporated to dryness and purified by silica gel column chromatography using methylene chloride – methanol (2 $\rightarrow$ 10 $\rightarrow$ 15%) as an eluent to give pure products **6a–c** (white solids, yield ca. 70%).

3.2.1.2. Synthesis of 5'-azido-nucleosides (1a–c). Sodium azide (NaN_3 , 5.18 mmol, 1.8 eq) was added to a solution of 5'-O-tosyl-nucleoside (**6a–c**; 2.88 mmol, 1 eq) in dimethylformamid (DMF, 30 mL). The mixture was stirred under reflux at 100 °C for 4 hours. Then the reaction was checked by thin-layer chromatography (TLC) using the chloroform – methanol (10:1, v/v) as a solvent system. The reaction mixture was evaporated to dryness and purified by silica gel column chromatography, using methylene chloride – methanol (5%) as an eluent to give pure products **1a–c**.

3.2.1.3. 5'-Azido-2',5'-dideoxyuridine (1a, 5'-AddU). White solid; Yield: 72%; ^1H NMR (400 MHz, DMSO- d_6) δ : 2.12 (1H, m, H-2''), 2.23 (1H, m, H-2'), 3.55 (2H, observed d, $J=5.2$ Hz, H-5'/5''), 3.85 (1H, observed dd, $J=9.0/5.0$ Hz, H-4'), 4.18 (1H, m, H-3'), 5.42 (1H, br. s, OH), 5.66 (1H, dd, $J=8.0/4.0$ Hz, H-5), 6.18 (1H, t, $J=6.0$ Hz, H-1'), 7.67 (1H, d, $J=8.0$ Hz, H-6), 11.33 (1H, s, NH); ^{13}C NMR (125 MHz, DMSO- d_6) δ : 38.24 (C-2'), 51.60 (C-5'), 70.60 (C-3'), 84.16 (C-1'), 84.60 (C-4'), 102.08 (C-5), 140.70 (C-6), 150.41 (C-2), 162.99 (C-4); HRMS (ESI-) calcd for $\text{C}_9\text{H}_{10}\text{N}_5\text{O}_4$ $[\text{M}-\text{H}]^-$ 252.0733, found 252.0742, calcd for $\text{C}_9\text{H}_{11}\text{N}_5\text{O}_4\text{Cl}$ $[\text{M}+\text{Cl}]^-$ 288.0410, found 288.0511.

3.2.1.4. 5'-Azido-5'-deoxythymidine (1b, 5'-AZT). White solid; Yield: 99%; ^1H NMR (400 MHz, DMSO- d_6) δ : 1.79 (3H, d, $J=0.8$ Hz, 5- CH_3), 2.08 (1H, m, H-2''), 2.25 (1H, m, H-2'), 3.56 (2H, d, $J=5.6$ Hz, H-5'/5''), 3.84 (1H, m, H-4'), 4.19 (1H, m, H-3'), 5.40 (1H, br. s, 3'-OH), 6.20 (1H, t, $J=7.0$ Hz, H-1'), 7.49 (1H, d, $J=1.2$ Hz, H-6), 11.32 (1H, s, NH); ^{13}C NMR (100 MHz, DMSO- d_6) δ : 12.08 (5- CH_3), 38.04 (C-2'), 51.66 (C-5'), 70.70 (C-3'), 83.82 (C-1'), 84.52 (C-4'), 109.78 (C-5), 136.05 (C-6), 150.47 (C-2), 163.65 (C-4); HRMS (ESI-) calcd for $\text{C}_{10}\text{H}_{12}\text{N}_5\text{O}_4$ $[\text{M}-\text{H}]^-$ 266.0889, found 266.0900.

3.2.1.5. 5'-Azido-2',5'-dideoxy-5-fluorouridine (1c, 5'-AddFU). White solid; Yield: 85%; ^1H NMR (400 MHz, DMSO- d_6) δ : 2.10 (1H, m, H-2''), 2.25 (1H, m, H-2'), 3.52–3.63 (2H, m, H-5'/5''), 3.84 (1H, m, H-4'), 4.17 (1H, m, H-3'), 5.42 (1H, d, $J=4.0$ Hz, 3'-OH), 6.16 (1H, td, $J=6.9/1.8$ Hz, H-1'), 7.97 (1H, d, $J=6.8$ Hz, H-6), 11.88 (1H, s, NH); ^{13}C NMR (100 MHz, DMSO- d_6) δ : 38.03 (C-2'), 51.56 (C-5'), 70.53 (C-3'), 84.48 (C-4'), 84.68 (C-1'), 124.72/125.06 (d, $J=34$ Hz, C-6), 138.92/141.22 (d, $J=230$ Hz, C-5), 149.05 (C-2), 157.09/156.83 (d, $J=24$ Hz, C-4); ^{19}F NMR (376 MHz, DMSO- d_6) δ : -91.21 (1F, d, $J=7.06$ Hz, F-5); HRMS (ESI-) calcd for $\text{C}_9\text{H}_9\text{FN}_5\text{O}_4$ $[\text{M}-\text{H}]^-$ 270.0639, found 270.0638, calcd for $\text{C}_9\text{H}_9\text{FN}_5\text{O}_4\text{Cl}$ $[\text{M}+\text{Cl}]^-$ 306.0405, found 306.0399.

3.2.2. Synthesis of propargylated 2'-deoxyuridine derivatives

3.2.2.1. Synthesis of 3'-O-propargyl-5'-O-(tert-butyltrimethylsilyl)-2'-deoxyuridine (2a). 5'-O-(tert-butyltrimethylsilyl)-2'-deoxyuridine (1 g, 1 eq) was dissolved in anhydrous tetrahydrofuran (THF, 10 mL) and sodium hydride (60% NaH in mineral oil, 0.58 g, 5 eqs) was added in small portions. The mixture was stirred under argon atmosphere at room temperature for 40 minutes. After that propargyl bromide (80% solution $\text{HC}\equiv\text{CCH}_2\text{Br}$ in toluene, 0.85 mL, 2.7 eqs) was added and the reaction mixture was stirred for 12 hours. The reaction was quenched with methanol and the reaction progress was checked by

thin-layer chromatography (TLC) using two solvent systems: 1) chloroform – methanol (95:5, v/v) and 2) *n*-hexane – ethyl acetate (2:1, v/v). The reaction mixture was evaporated to dryness and the product was isolated by silica gel chromatography in chloroform – methanol, then finally purified by chromatography in *n*-hexane – ethyl acetate (2:1, v/v).

White solid; Yield: 80%; ^1H NMR (500 MHz, DMSO- d_6) δ : 0.09 (6H, s, Si(CH₃)₂), 0.89 (9H, s, C(CH₃)₃), 2.12 (1H, m, H-2'), 2.34 (1H, m, H-2''), 3.47 (1H, t, J = 2.2 Hz, $\equiv\text{CH}$), 3.72–3.80 (2H, m, H-5'/5''), 4.01 (1H, m, H-4'), 4.22 (2H, s, OCH₂), 4.24 (1H, m, H-3'), 5.61 (1H, d, J = 8.0 Hz, H-5), 6.08 (1H, dd, J = 6.0/8.0 Hz, H-1'), 7.74 (1H, d, J = 5.0 Hz, H-6), 11.35 (1H, s, NH); ^{13}C NMR (125 MHz, DMSO- d_6) δ : -5.63 (Si(CH₃)₂), -5.55 (Si(CH₃)₂), 17.91 (C(CH₃)₃), 25.73 (C(CH₃)₃), 36.36 (C-2'), 55.82 (OCH₂), 63.04 (C-5'), 77.37 (C $\equiv$ CH), 78.05 (C-3'), 79.98 (C $\equiv$ CH), 84.05 (C-4'), 84.33 (C-1'), 101.75 (C-5), 139.98 (C-6), 150.29 (C-2), 162.96 (C-4); HRMS (ESI-) calcd for C₁₈H₂₇N₂O₅Si [M-H]⁻ 379.1689, found 379.1564.

3.2.2.2. Synthesis of 3'-O-propargyl-2'-deoxyuridine (2b). 3'-O-propargyl-5'-O-(*tert*-butyldimethylsilyl)-2'-deoxyuridine (**2a**, 0.50 g, 1.31 mmol, 1 eq) was placed in a round bottom flask and dissolved in anhydrous methanol (25 mL) under argon atmosphere. Then ammonium fluoride (NH₄F, 0.24 g, 6.57 mmol, 5 eqs) was added and the reaction was carried out on an oil bath under reflux for 4 hours. The reaction mixture was evaporated to dryness and the product was purified by silica gel column chromatography, using chloroform – methanol (2→10%) as an eluent, then finally purified by chromatography in *n*-hexane – ethyl acetate (2:1, v/v).

White solid; Yield: 85%; ^1H NMR (500 MHz, DMSO- d_6) δ : 2.12 (1H, m, H-2'), 2.31 (1H, m, H-2''), 3.48 (1H, t, J = 2.4 Hz, $\equiv\text{CH}$), 3.58 (2H, t, J = 4.4 Hz, H-5'/5''), 3.97 (1H, m, H-4'), 4.22 (2H, dd, J = 0.8/1.6 Hz, OCH₂), 4.25 (1H, m, H-3'), 5.12 (1H, t, J = 5.0 Hz, 5'-OH), 5.66 (1H, d, J = 8.4 Hz, H-5), 6.09 (1H, dd, J = 5.6/8.4 Hz, H-1'), 7.86 (1H, d, J = 8.0 Hz, H-6), 11.32 (1H, s, NH); ^{13}C NMR (125 MHz, DMSO- d_6) δ : 36.29 (C-2'), 55.84 (OCH₂), 61.41 (C-5'), 77.27 (C $\equiv$ CH), 78.63 (C-3'), 80.20 (C $\equiv$ CH), 84.60 (C-4'), 84.14 (C-1'), 101.93 (C-5), 140.36 (C-6), 150.41 (C-2), 163.04 (C-4); HRMS (ESI-) calcd for C₁₂H₁₃N₂O₅ [M-H]⁻ 265.0824, found 265.0806, calcd for C₁₂H₁₄N₂O₅Cl [M + Cl]⁻ 301.0591, found 301.0561.

3.2.3. General procedure for synthesis of nucleosides dimers

3.2.3.1. Dimers of the type 3a–c. Nucleoside azide (5'-AddU, 5'-AZT or FAddU, 1 mmol) and an equimolar amount of 3'-O-propargyl derivative (1 mmol) were placed in a round-bottomed flask. The substrates were dissolved in THF-H₂O (3:1, v/v, 4 mL) and stirred at room temperature until dissolved completely. Subsequently, sodium ascorbate (80 mol%) was added.

The mixture was stirred until a homogenous solution was obtained. Finally, 1M CuSO₄ solution (40 mol%) was added and the reaction mixture was vigorously stirred at room temperature for 24 hours. When the reaction was complete, the solvent was removed using a rotary evaporator, and the compound was purified by silica gel chromatography using a methylene chloride – methanol mixture (5→10%, v/v) as the eluent. Following the chromatographic purification, products **3a–c** were obtained in 74–97% yield.

Compound 3a:

White solid; Yield: 75%; ¹H NMR (400 MHz, DMSO-*d*₆) δ: 0.05 (6H, d, *J* = 2.4 Hz, Si(CH₃)₂), 0.85 (9H, s, C(CH₃)₃), 2.13–2.23 (3H, m, H-2'A, H-2''B, H-2'B), 2.34 (1H, m, H-2''A), 3.73 (2H, ddd, *J* = 21.7/11.3/3.7 Hz, H-5'/5''A), 4.02 (1H, m, H-4'A), 4.07 (1H, m, H-4'B), 4.16 (1H, m, H-3'A), 4.26 (1H, m, H-3'B), 4.57 (2H, s, OCH₂), 4.62 (2H, dd, *J* = 14.2/4.2 Hz, H-5'/5''B), 5.50 (1H, d, *J* = 4.4 Hz, 3'-OH), 5.60 (1H, d, *J* = 8.0 Hz, H-5B), 5.63 (1H, d, *J* = 8.0 Hz, H-5A), 6.09 (1H, dd, *J* = 4.0/8.0 Hz, H-1'A), 6.14 (1H, t, *J* = 6.8 Hz, H-1'B), 7.58 (1H, d, *J* = 8.4 Hz, H-6A), 7.74 (1H, d, *J* = 8.0 Hz, H-6B), 8.07 (1H, s, H-triazole), 11.32 (2H, s, NH); ¹³C NMR (125 MHz, DMSO-*d*₆) δ: 162.98 (C4B), 162.96 (C4A), 150.34 (C2B), 150.30 (C2A), 143.64 (C4 triazole), 140.80 (C6B), 140.02 (C6A), 124.68 (C5 triazole), 101.71 (C5B), 102.05 (C5A), 84.41 (C1'B), 84.39 (C1'A), 84.21 (C4'B), 84.25 (C4'A), 78.55 (C3'A), 70.75 (C3'B), 63.12 (C5'A), 61.68 (OCH₂), 51.30 (C5'B), 38.06 (C2'B), 36.74 (C2'A), 25.71 (C(CH₃)₃), 17.89 (C(CH₃)₃), -5.60 (CH₃Si), -5.67 (CH₃Si); HRMS (ESI-) calcd for C₂₇H₃₈N₇O₉Si [M-H]⁻ 632.2500, found 632.2518, calcd for C₂₇H₃₉N₇O₉SiCl [M + Cl]⁻ 668.2267, found 668.2284.

Compound 3b:

White solid; Yield: 97%; ¹H NMR (400 MHz, DMSO-*d*₆) δ: 0.05 (6H, d, *J* = 2.0 Hz, Si(CH₃)₂), 0.85 (9H, s, C(CH₃)₃), 1.79 (3H, d, *J* = 0.8 Hz, 5-CH₃), 2.06–2.14 (2H, m, H-2'A, H-2''B), 2.17 (1H, m, H-2'B), 2.32 (1H, m, H-2''A), 3.72 (2H, ddd, *J* = 21.8/11.2/3.8 Hz, H-5'/5''A), 4.01 (1H, m, H-4'A), 4.06 (1H, m, H-4'B), 4.16 (1H, m, H-3'A), 4.27 (1H, m, H-3'B), 4.57 (2H, s, OCH₂), 4.68–4.73 (2H, m, H-5'/5''B), 5.49 (1H, d, *J* = 4.4 Hz, 3'-OH), 5.59 (1H, d, *J* = 8.4 Hz, H-5A), 6.09 (1H, dd, *J* = 8.0/6.0 Hz, H-1'A), 6.16 (1H, t, *J* = 7.0 Hz, H-1'B), 7.34 (1H, d, *J* = 0.8 Hz, H-6B), 7.74 (1H, d, *J* = 8.0 Hz, H-6A), 8.08 (1H, s, H-triazole), 11.30 (1H, s, NH B), 11.33 (1H, s, NH A); ¹³C NMR (125 MHz, DMSO-*d*₆) δ: 163.66 (C4B), 163.02 (C4A), 150.41 (C2B), 150.35 (C2A), 143.70 (C4 triazole), 140.05 (C6A), 136.07 (C6B), 124.73 (C5 triazole), 109.86 (C5B), 101.76 (C5A), 84.41 (C1'A), 84.28 (C4'A), 84.03 (C1'B), 83.99 (C4'B), 78.61 (C3'A), 70.76 (C3'B), 63.17 (C5'A), 61.75 (OCH₂), 51.24 (C5'B), 37.93 (C2'B), 36.75 (C2'A), 25.74 (C(CH₃)₃), 17.93 (C(CH₃)₃), 12.08 (⁵CH₃B), -5.56 (CH₃Si), -5.63 (CH₃Si); HRMS (ESI-) calcd for C₂₈H₄₀N₇O₉Si [M-H]⁻ 646.2657, found 646.2667.

Compound 3c:

White solid; Yield: 74%; ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ : 0.05 (6H, d, $J=2.0$ Hz, $\text{Si}(\text{CH}_3)_2$), 0.85 (9H, s, $\text{C}(\text{CH}_3)_3$), 1.98–2.15 (2H, m, H-2'A, H-2''B), 2.22 (1H, m, H-2'B), 2.33 (1H, m, H-2''A), 3.74 (2H, ddd, $J=21.6/11.4/3.6$ Hz, H-5'/5''A), 4.02 (1H, m, H-4'A), 4.07 (1H, m, H-4'B), 4.16 (1H, m, H-3'A), 4.25 (1H, m, H-3'B), 4.57 (2H, s, OCH_2), 4.63–4.72 (2H, m, H-5'/5''B), 5.50 (1H, d, $J=4.4$ Hz, 3'-OH), 5.59 (1H, d, $J=8.0$ Hz, H-5A), 6.10 (1H, m, H-1'A), 6.14 (1H, m, H-1'B), 7.74 (1H, d, $J=8.0$ Hz, H-6A), 7.93 (1H, d, $J=7.2$ Hz, H-6B), 8.10 (1H, s, H-triazole), 11.33 (1H, s, NH A), 11.85 (1H, s, NH B); ^{13}C NMR (125 MHz, $\text{DMSO}-d_6$) δ : 162.97 (C4A), 157.09/156.88 (d, $J=26$ Hz, C4B), 150.30 (C2A), 149.01 (C2B), 143.67 (C4 triazole), 139.21/141.05 (d, $J=230$ Hz, C5B), 140.02 (C6A), 124.85/125.12 (d, $J=34$ Hz, C6B), 124.63 (C5 triazole), 101.72 (C5A), 84.63 (C1'B), 84.16 (C1'A), 84.03 (C4'B), 83.99 (C4'A), 78.53 (C3'A), 70.64 (C3'B), 63.11 (C5'A), 61.68 (OCH_2), 51.20 (C5'B), 37.93 (C2'B), 36.72 (C2'A), 25.90 ($\text{C}(\text{CH}_3)_3$), 17.89 ($\text{C}(\text{CH}_3)_3$), -5.61 (CH_3Si), -5.68 (CH_3Si); ^{19}F NMR (no dec) (470 MHz, $\text{DMSO}-d_6$) δ : -91.07 (1F, d, $J=6.7$ Hz, F-5); ^{19}F NMR (dec) (470 MHz, $\text{DMSO}-d_6$) δ : -91.07 (1F, s, F-5); HRMS (ESI-) calcd for $\text{C}_{27}\text{H}_{37}\text{FN}_7\text{O}_9\text{Si}$ $[\text{M}-\text{H}]^-$ 650.2406, found 650.2391, calcd for $\text{C}_{27}\text{H}_{38}\text{FN}_7\text{O}_9\text{SiCl}$ $[\text{M}+\text{Cl}]^-$ 686.2173, found 686.2182.

3.2.3.2. Dimers of the type 4a-c. Ammonium fluoride (10 eqs) was added to a stirred solution of silylated dimer (**3a-c**; 1 mmol) in anhydrous MeOH (4 mL) and the reactants were refluxed for 4 h. After that time, the silica gel was added to the reaction mixture and it was evaporated under reduced pressure. The residue was applied on top of a silica gel column and chromatographed using a mixture methylene chloride – methanol (10→15%) as the eluent, to afford products **4a-c**.

Compound 4a:

White solid; Yield: 48%; ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ : 2.12–2.40 (3H, m, H-2'A, H-2''B, H-2'B), 2.30 (1H, m, H-2''A), 3.57 (2H, s, H-5'/5''A), 3.99 (1H, m, H-4'A), 4.09 (1H, m, H-4'B), 4.22 (1H, m, H-3'A), 4.26 (1H, m, H-3'B), 4.58 (2H, s, OCH_2), 4.61–4.73 (2H, m, H-5'/5''B), 5.10 (1H, t, $J=5.0$ Hz, 5'-OH), 5.52 (1H, d, $J=4.4$ Hz, 3'-OH), 5.62–5.66 (2H, m, H-5A, H-5B), 6.10 (1H, dd, $J=8.2/5.8$ Hz, H-1'A), 6.16 (1H, t, $J=6.8$ Hz, H-1'B), 7.56 (1H, d, $J=8.0$ Hz, H-6B), 7.86 (1H, d, $J=8.0$ Hz, H-6A), 8.10 (1H, s, H-triazole), 11.32 (2H, s, NH); ^{13}C NMR (125 MHz, $\text{DMSO}-d_6$) δ : 163.05 (C4A), 162.98 (C4B), 150.44 (C2B), 150.37 (C2A), 143.79 (C4 triazole), 140.82 (C6A), 140.42 (C6B), 124.69 (C5 triazole), 102.06 (C5B), 101.92 (C5A), 84.77 (C4'A), 84.46 (C1'B), 84.20 (C4'B), 84.20 (C1'A), 79.04 (C3'A), 70.75 (C3'B), 61.71 (OCH_2), 61.47 (C5'A), 51.27 (C5'B), 38.03 (C2'B), 36.58 (C2'A); HRMS (ESI-) calcd for $\text{C}_{21}\text{H}_{24}\text{N}_7\text{O}_9$

$[M-H]^-$ 518.1636, found 518.1625, calcd for $C_{21}H_{25}N_7O_9Cl$ $[M+Cl]^-$ 554.1402, found 554.1386.

Compound 4b:

White solid; Yield: 50%; 1H NMR (400 MHz, DMSO- d_6) δ : 1.77 (3H, d, $J=0.8$ Hz, 5- CH_3), 2.04–2.14 (2H, m, H-2''B, H-2'A), 2.17 (1H, m, H-2'B), 2.28 (1H, m, H-2''A), 3.53 (2H, s, H-5'/5''A), 3.95 (1H, m, H-4'A), 4.06 (1H, m, H-4'B), 4.18 (1H, m, H-3'A), 4.25 (1H, m, H-3'B), 4.55 (2H, s, OCH_2), 4.59–4.71 (2H, m, H-5'/5''B), 5.07 (1H, t, $J=5.0$ Hz, 5'-OH), 5.47 (1H, d, $J=4.4$ Hz, 3'-OH), 5.62 (1H, d, $J=8.0$ Hz, H-5A), 6.08 (1H, dd, $J=8.2/5.8$ Hz, H-1'A), 6.14 (1H, t, $J=7.0$ Hz, H-1'B), 7.32 (1H, d, $J=1.2$ Hz, H-6B), 7.83 (1H, d, $J=8.0$ Hz, H-6A), 8.08 (1H, s, H-triazole), 11.32 (2H, s, NH); ^{13}C NMR (125 MHz, DMSO- d_6) δ : 163.64 (C4B), 163.05 (C4A), 150.42 (C2B), 150.38 (C2A), 143.80 (C4 triazole), 140.41 (C6A), 136.05 (C6B), 124.70 (C5 triazole), 109.84 (C5B), 101.91 (C5A), 84.76 (C4'A), 84.17 (C1'A), 84.01 (C1'B), 83.94 (C4'B), 79.06 (C3'A), 70.72 (C3'B), 61.73 (OCH_2), 61.46 (C5'A), 51.15 (C5'B), 37.85 (C2'B), 36.57 (C2'A), 12.05 (5CH_3B); HRMS (ESI-) calcd for $C_{22}H_{26}N_7O_9$ $[M-H]^-$ 532.1792, found 532.1785, calcd for $C_{22}H_{27}N_7O_9Cl$ $[M+Cl]^-$ 568.1559, found 568.1549.

Compound 4c:

White solid; Yield: 86%; 1H NMR (400 MHz, DMSO- d_6) δ : 2.07–2.13 (2H, m, H-2'A, H-2''B), 2.22 (1H, m, H-2'B), 2.29 (1H, m, H-2''A), 3.54 (2H, s, H-5'/5''A), 3.96 (1H, m, H-4'A), 4.06 (1H, m, H-4'B), 4.19 (1H, m, H-3'A), 4.24 (1H, m, H-3'B), 4.55 (2H, s, OCH_2), 4.61–4.72 (2H, m, H-5'/5''B), 5.08 (1H, t, $J=5.0$ Hz, 5'-OH), 5.50 (1H, d, $J=4.4$ Hz, 3'-OH), 5.63 (1H, d, $J=8.0$ Hz, H-5A), 6.08 (1H, m, H-1'A), 6.11 (H-1'B), 7.83 (1H, d, $J=8.0$ Hz, H-6A), 7.91 (1H, d, $J=6.8$ Hz, H-6B), 8.10 (1H, s, H-triazole), 11.29 (1H, s, NH A), 11.83 (1H, s, NH B); ^{13}C NMR (125 MHz, DMSO- d_6) δ : 163.06 (C4A), 157.07/156.87 (d, $J=25$ Hz, C4B), 150.43 (C2A), 149.00 (C2B), 143.82 (C4 triazole), 139.20/141.04 (d, $J=230$ Hz, C5B), 140.42 (C6A), 124.88/125.15 (d, $J=34$ Hz, C6B), 124.64 (C5 triazole), 101.91 (C5A), 84.76 (C4'A), 84.67 (C1'B), 84.26 (C4'B), 84.18 (C1'A), 79.03 (C3'A), 70.63 (C3'B), 61.72 (OCH_2), 61.46 (C5'A), 51.20 (C5'B), 37.91 (C2'B), 36.58 (C2'A); ^{19}F NMR (no dec) (470 MHz, DMSO- d_6) δ : -91.09 (1F, d, $J=6.7$ Hz, F-5); ^{19}F NMR (dec) (470 MHz, DMSO- d_6) δ : -91.09 (1F, s, F-5); HRMS (ESI-) calcd for $C_{21}H_{23}FN_7O_9$ $[M-H]^-$ 536.1541, found 536.1540, calcd for $C_{21}H_{24}FN_7O_9Cl$ $[M+Cl]^-$ 572.1308, found 572.1306.

3.3. General biological methods

3.3.1. Cell line and culture conditions

GBM cell lines (U-118 MG, U-87 MG, T98G), HeLa (cervical cancer cell line), HepG2 (liver cancer cell line), and non-cancerous lung fibroblast cell

line (MRC-5) were purchased from ATCC (Manassas, USA). All cell lines are of human origin. HeLa cells were cultured in RPMI 1640 medium. U-118 MG cells were cultured in DMEM medium. HepG2, U-87 MG, T98G and MRC-5 cells were cultured in EMEM medium. Each medium was supplemented with 10% fetal bovine serum (FBS Biowest S1810-500 is not heat inactivated) and 10 mg/mL antibiotics (penicillin and streptomycin). Cells were cultured at 37 °C with 5% CO₂ in humidified air. Cell media and other chemicals were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and ATCC. Cell concentrations in culture were adjusted to allow for exponential growth.

3.3.2. *In vitro* cytotoxicity assay

The protein-staining sulforhodamine B (SRB, Sigma–Aldrich) microculture colorimetric assay, developed by the National Cancer Institute (USA) for *in vitro* antitumor screening was used in this study, to estimate the cell number by providing a sensitive index of total cellular protein content, being linear to cell density.^[33] The monolayer cell culture was trypsinized and counted. To each well of the 96-well plate, 100 µL of the diluted cell suspension (1×10^4 cells) was added. After 24 hours, when a partial monolayer was formed, 100 µL of fresh medium with different compound concentrations (7.81, 15.625, 31.25, 62.5, 125, 250, 500 and 1000 µg/mL) were added to the wells. The cells were exposed to compounds for 72 h at 37 °C in a humidified atmosphere (90% RH) containing 5% CO₂. After that, 100 µL of 10% trichloroacetic acid was added to the wells and the plates were incubated for 1 h at 4 °C. The plates were then washed out with the distilled water to remove traces of medium and next dried by the air. The air-dried plates were stained with 100 µL of 0.057% sulforhodamine B (prepared in 1% acetic acid) and kept for 30 min at room temperature. The unbound dye was removed by washing five times with 1% acetic acid and then the plates were air dried overnight. The protein-bound dye was dissolved in 200 µL of 10 mM unbuffered Tris base (pH 10.5) for optical density determination at 510 nm. All cytotoxicity experiments were performed three times. Cell survival was measured as the percentage absorbance compared to the control (non-treated cells). The results were calculated as an IC₅₀ (inhibitory concentration 50) – the IC₅₀ corresponds to the concentration of tested compound that inhibits cell viability by 50%.

3.3.3. *In silico* pharmacokinetic prediction

Calculations of pharmacokinetic profile descriptors of synthesized compounds were performed by various software solutions accessible on-line. The transformation of the stoichiometric formulas of the compounds into a

SMILES code (Simplified Molecular Input Line Entry System) was carried out by ChemBioDraw Ultra version 12.0 program (Cambridge Software). The SMILES code was applied to calculate logP values (octanol/water partition coefficient) in eight variants (ALOGPs, AC_logP, miLogP, ALOGP, MLOGP, LogKOWWIN, XLOGP2, XLOGP3), PSA (topological polar surface area) and aPSA (apolar surface area). The logP values were calculated by ALOGPS 2.1 software (<http://www.vcclab.org/lab/alogs>).^[34] PSA and aPSA descriptors were calculated using the VEGA ZZ program (<http://www.vegazz.net>).^[35] The pharmacokinetic profile was also evaluated according to the Lipinski's "rule of five"^[30] by using Molinspiration application (<http://www.molinspiration.com>), which analyses molecular weight (MW), number of hydrogen-bond acceptors (HBA) and number of hydrogen-bond donors (HBD). The Caco-2 prediction model based on descriptors generated by preADMET (<http://preadmet.bmdrc.org>) was used to compute Caco-2 apparent permeability (tP_{app}) for the tested compounds. In this model a number of hydrogen bond donors and three molecular surface area properties determine membrane permeability of compounds.

4. Conclusions

An efficient synthesis of novel 1,2,3-triazole derivatives of 2'-deoxyuridine dimers *via* a copper(I)-catalyzed 1,3-dipolar cycloaddition reaction is presented. The 1,2,3-triazole derivatives of pyrimidine nucleosides dimers are regioselectively obtained in good yields under mild conditions using $\text{CuSO}_4 \times 5\text{H}_2\text{O}$ and sodium ascorbate as a catalyst system, and THF/ H_2O (3:1, v/v) as a solvent system. To synthesize the desired compounds, we have taken advantage of the Huisgen cycloaddition by coupling 5'-azidonucleosides analogues and 5'-O-propargylated nucleosides of 2'-deoxyuridine to give the dimers **3a–c**, which subsequently could be deblocked with ammonium fluoride in absolute methanol to afford the second group of dimers **4a–c**. All the obtained compounds **1–4** have been examined for their cytotoxic activity in five human cancer cell lines: HeLa (cervical), U-118MG, U-87MG and T98G (high grade gliomas), HepG2 (liver). Some biological activity possess the propargylated nucleoside **2a** (in particular in HepG2 cells), where it was six times more active ($\text{IC}_{50} = 5.85 \mu\text{M}$) than 5-FdU. Interestingly, compound **2b** was found to be cytotoxic potent ($\text{IC}_{50} = 13.3 \mu\text{M}$) only in HepG2 cells, where it is almost three times more active ($\text{IC}_{50} = 13.30 \mu\text{M}$) than the parent nucleoside. The experiments have shown that the obtained nucleoside dimers with a 1,2,3-triazole linkage were very stable compounds in the physiological-like media and had no significant anticancer activity. This lack of activity is probably due to the fact that such large and polar compounds cannot diffuse across a lipid

bilayer. The modified dinucleosides also do not meet the criteria of the Lipinski drug-likeness “rule of five”. But it is worth to notice, that in nucleic acid based-drugs there are many exceptions for the Lipinski rules.^[37]

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Synthesis and biological assay of new 2'-deoxyuridine dimers containing a 1,2,3-triazole linker. Part I

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1. General chemistry methods

^1H and ^{13}C NMR spectra were recorded on a Bruker 400 and 500 spectrometers operating at 400 or 500 MHz and 100 or 125 MHz, respectively. ^{19}F NMR spectra were recorded on a Bruker 500 spectrometer at 470 MHz. The chemical shifts were reported in ppm (δ scale). Mass spectra were recorded using ESI-MS QqToF Bruker mass spectrometer. Thin-layer chromatography (TLC) was carried out on Merck precoated 60 F₂₅₄ silica gel plates, while column chromatography on Merck silica gel 60H (40–63 μm). HPLC analyses were performed on a Lichrospher RP-18 endcapped (5.0 μm , 4.6 mm $\times$ 250 mm) using Thermo Scientific™ HPLC systems with A+B solvent systems (A, 0.01 M aqueous triethylammonium acetate pH 7; B, A/acetonitrile, 1:4, v/v) at 35°C, flow rate 1.5 mL/min; events: 5 min A 100%, linear gradient of B 0–100% in 20 min, 5 min B 100% and A 100% 15 min wash. Chemical reagents were purchased from Acros Organics, Alfa Aesar, Carbosynth and Sigma-Aldrich.

2. Chemical synthesis

2.1. General procedure for synthesis of 5'-azidonucleosides

Synthesis of 5'-O-tosyl-nucleosides (6a-c)

An appropriate nucleoside (**5a-c**; 4 mmol, 1 eq) was dissolved in anhydrous pyridine (Py, 40 mL) under argon atmosphere. After the substrate was dissolved, the flask with the solution was placed in an ice bath and cooled. Then tosyl chloride (1.3 eq, Ts-Cl) was added and stirred until it was completely dissolved. The reaction flask was placed in a refrigerator for 24 hours and progress of the reaction was checked by the thin-layer chromatography (TLC) using chloroform – methanol (10:1, v/v) as a solvent system. The reaction mixture was quenched with methanol and then evaporated to dryness. The crude product was dissolved in chloroform and extracted with saturated aqueous solution of NaHCO_3 . The organic layer was evaporated to dryness and purified by silica gel column chromatography using methylene chloride – methanol (2→10→15%) as an eluent to give pure products **6a-c** (white solids, yield ca. 70%).

Synthesis of 5'-azido-nucleosides (**1a-c**)

Sodium azide (NaN_3 , 5.18 mmol, 1.8 eqs) was added to a solution of 5'-*O*-tosyl-nucleoside (**6a-c**; 2.88 mmol, 1 eq) in dimethylformamid (DMF, 30 mL). The mixture was stirred under reflux at 100°C for 4 hours. Then the reaction was checked by thin-layer chromatography (TLC) using the chloroform – methanol (10:1, v/v) as a solvent system. The reaction mixture was evaporated to dryness and purified by silica gel column chromatography, using methylene chloride – methanol (5%) as an eluent to give pure products **1a-c**.

5'-Azido-2',5'-dideoxyuridine (**1a**, 5'-AddU)

White solid; Yield: 72%; ^1H NMR (400 MHz, DMSO-d_6) δ : 2.12 (m, H-2''), 2.23 (m, H-2'), 3.55 (2H, d, $J = 4.0$ Hz, H-5'/5''), 3.85 (1H, q, $J = 8.0/4.0$ Hz, H-4'), 4.18 (1H, m, H-3'), 5.42 (1H, br. s, OH), 5.66 (1H, dd, $J = 8.0/4.0$ Hz, H-5), 6.18 (1H, t, $J = 6.0$ Hz, H-1'), 7.67 (1H, d, $J = 8.0$ Hz, H-6), 11.33 (1H, s, NH); ^{13}C NMR (125 MHz, DMSO-d_6) δ : 38.24 (C-2'), 51.60 (C-5'), 70.60 (C-3'), 84.16 (C-1'), 84.60 (C-4'), 102.08 (C-5), 140.70 (C-6), 150.41 (C-2), 162.99 (C-4); HRMS (ESI-) calcd for $\text{C}_9\text{H}_{10}\text{N}_5\text{O}_4$ $[\text{M-H}]^-$ 252.0733, found 252.0742, calcd for $\text{C}_9\text{H}_{11}\text{N}_5\text{O}_4\text{Cl}$ $[\text{M+Cl}]^-$ 288.0410, found 288.0511.

5'-Azido-5'-deoxythymidine (**1b**, 5'-AZT)

White solid; Yield: 99%; ^1H NMR (400 MHz, DMSO-d_6) δ : 1.79 (3H, d, $J = 0.8$ Hz, 5- CH_3), 2.08 (1H, m, H-2''), 2.25 (1H, m, H-2'), 3.56 (2H, d, $J = 5.6$ Hz, H-5'/5''), 3.84 (1H, m, H-4'), 4.19 (1H, m, H-3'), 5.40 (1H, br. s, 3'-OH), 6.20 (1H, t, $J = 7.0$ Hz, H-1'), 7.49 (1H, d, $J = 1.2$ Hz, H-6), 11.32 (1H, s, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ : 12.08 (5- CH_3), 38.04 (C-2'), 51.66 (C-5'), 70.70 (C-3'), 83.82 (C-1'), 84.52 (C-4'), 109.78 (C-5), 136.05 (C-6), 150.47 (C-2), 163.65 (C-4); HRMS (ESI-) calcd for $\text{C}_{10}\text{H}_{12}\text{N}_5\text{O}_4$ $[\text{M-H}]^-$ 266.0889, found 266.0900.

5'-Azido-2',5'-dideoxy-5-fluorouridine (**1c**, 5'-AddFU)

White solid; Yield: 85%; ^1H NMR (400 MHz, DMSO-d_6) δ : 2.10 (1H, m, H-2''), 2.25 (1H, m, H-2'), 3.56 (2H, m, H-5'/5''), 3.84 (1H, m, H-4'), 4.17 (1H, m, H-3'), 5.42 (1H, d, $J = 4.0$ Hz, 3'-OH), 6.16 (1H, td, $J = 6.9/1.8$ Hz, H-1'), 7.97 (1H, d, $J = 6.8$ Hz, H-6), 11.88 (1H, s, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ : 38.03 (C-2'), 51.56 (C-5'), 70.53 (C-3'), 84.48 (C-4'), 84.68 (C-1'), 124.72/125.06 (d, $J = 34$ Hz, C-6), 138.92/141.22 (d, $J = 230$ Hz, C-5), 149.05 (C-2), 157.09/156.83 (d, $J = 24$ Hz, C-4); ^{19}F NMR (376 MHz, DMSO-d_6) δ : -91.21 (1F, d, $J = 7.06$ Hz, F-5); HRMS (ESI-) calcd for $\text{C}_9\text{H}_9\text{FN}_5\text{O}_4$ $[\text{M-H}]^-$ 270.0639, found 270.0638, calcd for $\text{C}_9\text{H}_9\text{FN}_5\text{O}_4\text{Cl}$ $[\text{M+Cl}]^-$ 306.0405, found 306.0399.

2.2. Synthesis of propargylated 2'-deoxyuridine derivatives

2.2.1. Synthesis of 3'-O-propargyl-5'-O-(tert-butyldimethylsilyl)-2'-deoxyuridine (**2a**)

5'-O-(tert-butyldimethylsilyl)-2'-deoxyuridine (1 g, 1 eq) was dissolved in anhydrous tetrahydrofuran (THF, 10 mL) and sodium hydride (60% NaH in mineral oil, 0.58 g, 5 eqs) was added in small portions. The mixture was stirred under argon atmosphere at room temperature for 40 minutes. After that propargyl bromide (80% solution HC≡CCH₂Br in toluene, 0.85 mL, 2.7 eqs) was added and the reaction mixture was stirred for 12 hours. The reaction was quenched with methanol and the reaction progress was checked by thin-layer chromatography (TLC) using two solvent systems: 1) chloroform – methanol (95:5, v/v) and 2) *n*-hexane – ethyl acetate (2:1, v/v). The reaction mixture was evaporated to dryness and the product was isolated by silica gel chromatography in chloroform – methanol, then finally purified by chromatography in *n*-hexane – ethyl acetate (2:1, v/v).

White solid; Yield: 80%; ¹H NMR (500 MHz, DMSO-*d*₆) δ: 0.09 (6H, s, Si(CH₃)₂), 0.89 (9H, s, C(CH₃)₃), 2.12 (1H, m, H-2'), 2.34 (1H, m, H-2''), 3.47 (1H, t, J = 2.2 Hz, ≡CH), 3.75 (2H, m, H-5'/5''), 4.01 (1H, m, H-4'), 4.22 (2H, s, OCH₂), 4.24 (1H, m, H-3'), 5.61 (1H, d, J = 8.0 Hz, H-5), 6.08 (1H, dd, J = 6.0/8.0 Hz, H-1'), 7.74 (1H, d, J = 5.0 Hz, H-6), 11.35 (1H, s, NH); ¹³C NMR (125 MHz, DMSO-*d*₆) δ: -5.63 (Si(CH₃)₂), -5.55 (Si(CH₃)₂), 17.91 (C(CH₃)₃), 25.73 (C(CH₃)₃), 36.36 (C-2'), 55.82 (OCH₂), 63.04 (C-5'), 77.37 (C≡CH), 78.05 (C-3'), 79.98 (C≡CH), 84.05 (C-4'), 84.33 (C-1'), 101.75 (C-5), 139.98 (C-6), 150.29 (C-2), 162.96 (C-4); HRMS (ESI-) calcd for C₁₈H₂₇N₂O₅Si [M-H]⁻ 379.1689, found 379.1564.

2.2.2. Synthesis of 3'-O-propargyl-2'-deoxyuridine (**2b**)

3'-O-propargyl-5'-O-(tert-butyldimethylsilyl)-2'-deoxyuridine (**2a**, 0.50 g, 1.31 mmol, 1 eq) was placed in a round bottom flask and dissolved in anhydrous methanol (25 mL) under argon atmosphere. Then ammonium fluoride (NH₄F, 0.24 g, 6.57 mmol, 5 eqs) was added and the reaction was carried out on an oil bath under reflux for 4 hours. The reaction mixture was evaporated to dryness and the product was purified by silica gel column chromatography, using chloroform – methanol (2→10%) as an eluent, then finally purified by chromatography in *n*-hexane – ethyl acetate (2:1, v/v).

White solid; Yield: 85%; ¹H NMR (500 MHz, DMSO-*d*₆) δ: 2.12 (1H, m, H-2'), 2.31 (1H, m, H-2''), 3.48 (1H, t, J = 2.4 Hz, ≡CH), 3.58 (2H, t, J = 4.4 Hz, H-5'/5''), 3.97 (1H, m, H-4'), 4.22 (2H, dd, J = 0.8/1.6 Hz, OCH₂), 4.25 (1H, m, H-3'), 5.12 (1H, t, J = 5.0 Hz, 5'-OH), 5.66 (1H, d, J = 8.4 Hz, H-5), 6.09 (1H, dd, J = 5.6/8.4 Hz, H-1'), 7.86 (1H, d, J = 8.0 Hz, H-6), 11.32 (1H, s, NH); ¹³C NMR (125 MHz, DMSO-*d*₆) δ: 36.29 (C-2'), 55.84 (OCH₂), 61.41 (C-5'), 77.27 (C≡CH), 78.63 (C-3'), 80.20 (C≡CH), 84.60 (C-4'), 84.14 (C-1'), 101.93 (C-5), 140.36 (C-6), 150.41

(C-2), 163.04 (C-4); HRMS (ESI⁻) calcd for C₁₂H₁₃N₂O₅ [M-H]⁻ 265.0824, found 265.0806, calcd for C₁₂H₁₄N₂O₅Cl [M+Cl]⁻ 301.0591, found 301.0561.

2.3. General procedure for synthesis of nucleosides dimers

2.3.1. Dimers of the type **3a-c**

Nucleoside azide (5'-AddU, 5'-AZT or FAddU, 1 mmol) and an equimolar amount of 3'-*O*-propargyl derivative (1 mmol) were placed in a round-bottomed flask. The substrates were dissolved in THF-H₂O (3:1, v/v, 4 mL) and stirred using a magnetic stirrer at room temperature until dissolved completely. Subsequently, sodium ascorbate (80 mol%) was added. The mixture was stirred until a homogenous solution was obtained. Finally, 1M CuSO₄ solution (40 mol%) was added and the flask was closed with a glass stopper. The reaction mixture was vigorously stirred at room temperature for 24 hours. When the reaction was complete, the solvent was removed using a rotary evaporator, and the compound was purified by silica gel chromatography using a methylene chloride – methanol mixture (5→10%, v/v) as the eluent. Following the chromatographic purification, products **3a-c** were obtained in 74–97% yield.

Compound **3a**:

White solid; Yield: 75%; ¹H NMR (400 MHz, DMSO-*d*₆) δ: 0.05 (6H, d, J = 2.4 Hz, Si(CH₃)₂), 0.85 (9H, s, C(CH₃)₃), 2.13 (2H, m, H-2'A, H-2''B), 2.19 (1H, m, H-2'B), 2.34 (1H, m, H-2''A), 3.73 (2H, ddd, J = 21.7/11.3/3.7 Hz, H-5'/5''A), 4.02 (1H, m, H-4'A), 4.07 (1H, m, H-4'B), 4.16 (1H, m, H-3'A), 4.26 (1H, m, H-3'B), 4.57 (2H, s, OCH₂), 4.62 (2H, dd, J = 14.2/4.2 Hz, H-5'/5''B), 5.50 (1H, d, J = 4.4 Hz, 3'-OH), 5.60 (1H, d, J = 8.0 Hz, H-5B), 5.63 (1H, d, J = 8.0 Hz, H-5A), 6.09 (1H, dd, J = 4.0/8.0 Hz, H-1'A), 6.14 (1H, t, J = 6.8 Hz, H-1'B), 7.58 (1H, d, J = 8.4 Hz, H-6A), 7.74 (1H, d, J = 8.0 Hz, H-6B), 8.07 (1H, s, H-triazole), 11.32 (2H, s, NH); ¹³C NMR (125 MHz, DMSO-*d*₆) δ: 162.98 (C4B), 162.96 (C4A), 150.34 (C2B), 150.30 (C2A), 143.64 (C4 triazole), 140.80 (C6B), 140.02 (C6A), 124.68 (C5 triazole), 101.71 (C5B), 102.05 (C5A), 84.41 (C1'B), 84.39 (C1'A), 84.21 (C4'B), 84.25 (C4'A), 78.55 (C3'A), 70.75 (C3'B), 63.12 (C5'A), 61.68 (OCH₂), 51.30 (C5'B), 38.06 (C2'B), 36.74 (C2'A), 25.71 (C(CH₃)₃), 17.89 (C(CH₃)₃), -5.60 (CH₃Si), -5.67 (CH₃Si); HRMS (ESI⁻) calcd for C₂₇H₃₈N₇O₉Si [M-H]⁻ 632.2500, found 632.2518, calcd for C₂₇H₃₉N₇O₉SiCl [M+Cl]⁻ 668.2267, found 668.2284.

Compound **3b**:

White solid; Yield: 97%; ^1H NMR (400 MHz, DMSO- d_6) δ : 0.05 (6H, d, J = 2.0 Hz, $\text{Si}(\text{CH}_3)_2$), 0.85 (9H, s, $\text{C}(\text{CH}_3)_3$), 1.79 (3H, d, J = 0.8 Hz, 5- CH_3), 2.12 (2H, m, H-2'A, H-2''B), 2.17 (1H, m, H-2'B), 2.32 (1H, m, H-2''A), 3.72 (2H, ddd, J = 21.8/11.2/3.8 Hz, H-5'/5''A), 4.01 (1H, m, H-4'A), 4.06 (1H, m, H-4'B), 4.16 (1H, m, H-3'A), 4.27 (1H, m, H-3'B), 4.57 (2H, s, OCH_2), 4.65 (2H, m, H-5'/5''B), 5.49 (1H, d, J = 4.4 Hz, 3'-OH), 5.59 (1H, d, J = 8.4 Hz, H-5A), 6.09 (1H, dd, J = 8.0/6.0 Hz, H-1'A), 6.16 (1H, t, J = 7.0 Hz, H-1'B), 7.34 (1H, d, J = 0.8 Hz, H-6B), 7.74 (1H, d, J = 8.0 Hz, H-6A), 8.08 (1H, s, H-triazole), 11.30 (1H, s, NH B), 11.33 (1H, s, NH A); ^{13}C NMR (125 MHz, DMSO- d_6) δ : 163.66 (C4B), 163.02 (C4A), 150.41 (C2B), 150.35 (C2A), 143.70 (C4 triazole), 140.05 (C6A), 136.07 (C6B), 124.73 (C5 triazole), 109.86 (C5B), 101.76 (C5A), 84.41 (C1'A), 84.28 (C4'A), 84.03 (C1'B), 83.99 (C4'B), 78.61 (C3'A), 70.76 (C3'B), 63.17 (C5'A), 61.75 (OCH_2), 51.24 (C5'B), 37.93 (C2'B), 36.75 (C2'A), 25.74 ($\text{C}(\text{CH}_3)_3$), 17.93 ($\text{C}(\text{CH}_3)_3$), 12.08 ($^5\text{CH}_3\text{B}$), -5.56 (CH_3Si), -5.63 (CH_3Si); HRMS (ESI-) calcd for $\text{C}_{28}\text{H}_{40}\text{N}_7\text{O}_9\text{Si}$ $[\text{M}-\text{H}]^-$ 646.2657, found 646.2667.

Compound **3c**:

White solid; Yield: 74%; ^1H NMR (400 MHz, DMSO- d_6) δ : 0.05 (6H, d, J = 2.0 Hz, $\text{Si}(\text{CH}_3)_2$), 0.85 (9H, s, $\text{C}(\text{CH}_3)_3$), 2.13 (2H, m, H-2'A, H-2''B), 2.22 (1H, m, H-2'B), 2.33 (1H, m, H-2''A), 3.74 (2H, ddd, J = 21.6/11.4/3.6 Hz, H-5'/5''A), 4.02 (1H, m, H-4'A), 4.07 (1H, m, H-4'B), 4.16 (1H, m, H-3'A), 4.25 (1H, m, H-3'B), 4.57 (2H, s, OCH_2), 4.67 (2H, m, H-5'/5''B), 5.50 (1H, d, J = 4.4 Hz, 3'-OH), 5.59 (1H, d, J = 8.0 Hz, H-5A), 6.10 (1H, m, H-1'A), 6.14 (1H, m, H-1'B), 7.74 (1H, d, J = 8.0 Hz, H-6A), 7.93 (1H, d, J = 7.2 Hz, H-6B), 8.10 (1H, s, H-triazole), 11.33 (1H, s, NH A), 11.85 (1H, s, NH B); ^{13}C NMR (125 MHz, DMSO- d_6) δ : 162.97 (C4A), 157.09/156.88 (d, J = 26 Hz, C4B), 150.30 (C2A), 149.01 (C2B), 143.67 (C4 triazole), 139.21/141.05 (d, J = 230 Hz, C5B), 140.02 (C6A), 124.85/125.12 (d, J = 34 Hz, C6B), 124.63 (C5 triazole), 101.72 (C5A), 84.63 (C1'B), 84.16 (C1'A), 84.03 (C4'B), 83.99 (C4'A), 78.53 (C3'A), 70.64 (C3'B), 63.11 (C5'A), 61.68 (OCH_2), 51.20 (C5'B), 37.93 (C2'B), 36.72 (C2'A), 25.90 ($\text{C}(\text{CH}_3)_3$), 17.89 ($\text{C}(\text{CH}_3)_3$), -5.61 (CH_3Si), -5.68 (CH_3Si); ^{19}F NMR (no dec) (470 MHz, DMSO- d_6) δ : -166.73/-166.71 (1F, d, J = 9.4 Hz, F-5); ^{19}F NMR (dec) (470 MHz, DMSO- d_6) δ : -166.72 (1F, s, F-5); HRMS (ESI-) calcd for $\text{C}_{27}\text{H}_{37}\text{FN}_7\text{O}_9\text{Si}$ $[\text{M}-\text{H}]^-$ 650.2406, found 650.2391, calcd for $\text{C}_{27}\text{H}_{38}\text{FN}_7\text{O}_9\text{SiCl}$ $[\text{M}+\text{Cl}]^-$ 686.2173, found 686.2182.

2.3.2. Dimers of the type **4a-c**

Ammonium fluoride (10 eq) was added to a stirred solution of silylated dimer (**3a-c**; 1 mmol) in anhydrous MeOH (4 mL) and the reactants were refluxed for 4 h. After that time, the silica gel was added to the reaction mixture and it was evaporated under reduced pressure. The residue was applied on top of a silica gel column and chromatographed using a mixture methylene chloride – methanol (10→15%) as the eluent, to afford products **4a-c**.

Compound 4a:

White solid; Yield: 48%; ^1H NMR (400 MHz, DMSO- d_6) δ : 2.12 (2H, m, H-2'A, H-2''B), 2.19 (1H, m, H-2'B), 2.30 (1H, m, H-2''A), 3.57 (2H, m, H-5'/5''A), 3.99 (1H, m, H-4'A), 4.09 (1H, m, H-4'B), 4.22 (1H, m, H-3'A), 4.26 (1H, m, H-3'B), 4.58 (2H, s, OCH₂), 4.66 (2H, m, H-5'/5''B), 5.10 (1H, t, J = 5.0 Hz, 5'-OH), 5.52 (1H, d, J = 4.4 Hz, 3'-OH), 5.64 (2H, m, H-5A, H-5B), 6.10 (1H, dd, J = 8.2/5.8 Hz, H-1'A), 6.16 (1H, t, J = 6.8 Hz, H-1'B), 7.56 (1H, d, J = 8.0 Hz, H-6B), 7.86 (1H, d, J = 8.0 Hz, H-6A), 8.10 (1H, s, H-triazole), 11.32 (2H, s, NH); ^{13}C NMR (125 MHz, DMSO- d_6) δ : 163.05 (C4A), 162.98 (C4B), 150.44 (C2B), 150.37 (C2A), 143.79 (C4 triazole), 140.82 (C6A), 140.42 (C6B), 124.69 (C5 triazole), 102.06 (C5B), 101.92 (C5A), 84.77 (C4'A), 84.46 (C1'B), 84.20 (C4'B), 84.20 (C1'A), 79.04 (C3'A), 70.75 (C3'B), 61.71 (OCH₂), 61.47 (C5'A), 51.27 (C5'B), 38.03 (C2'B), 36.58 (C2'A); HRMS (ESI-) calcd for C₂₁H₂₄N₇O₉ [M-H]⁻ 518.1636, found 518.1625, calcd for C₂₁H₂₅N₇O₉Cl [M+Cl]⁻ 554.1402, found 554.1386.

Compound 4b:

White solid; Yield: 50%; ^1H NMR (400 MHz, DMSO- d_6) δ : 1.77 (3H, d, J = 0.8 Hz, 5-CH₃), 2.11 (2H, m, H-2''B, H-2'A), 2.17 (1H, m, H-2'B), 2.28 (1H, m, H-2''A), 3.53 (2H, m, H-5'/5''A), 3.95 (1H, m, H-4'A), 4.06 (1H, m, H-4'B), 4.18 (1H, m, H-3'A), 4.25 (1H, m, H-3'B), 4.55 (2H, s, OCH₂), 4.64 (2H, m, H-5'/5''B), 5.07 (1H, t, J = 5.0 Hz, 5'-OH), 5.47 (1H, d, J = 4.4 Hz, 3'-OH), 5.62 (1H, d, J = 8.0 Hz, H-5A), 6.08 (1H, dd, J = 8.2/5.8 Hz, H-1'A), 6.14 (1H, t, J = 7.0 Hz, H-1'B), 7.32 (1H, d, J = 1.2 Hz, H-6B), 7.83 (1H, d, J = 8.0 Hz, H-6A), 8.08 (1H, s, H-triazole), 11.32 (2H, s, NH); ^{13}C NMR (125 MHz, DMSO- d_6) δ : 163.64 (C4B), 163.05 (C4A), 150.42 (C2B), 150.38 (C2A), 143.80 (C4 triazole), 140.41 (C6A), 136.05 (C6B), 124.70 (C5 triazole), 109.84 (C5B), 101.91 (C5A), 84.76 (C4'A), 84.17 (C1'A), 84.01 (C1'B), 83.94 (C4'B), 79.06 (C3'A), 70.72 (C3'B), 61.73 (OCH₂), 61.46 (C5'A), 51.15 (C5'B), 37.85 (C2'B), 36.57 (C2'A), 12.05 (⁵CH₃B); HRMS (ESI-) calcd for C₂₂H₂₆N₇O₉ [M-H]⁻ 532.1792, found 532.1785, calcd for C₂₂H₂₇N₇O₉Cl [M+Cl]⁻ 568.1559, found 568.1549.

Compound 4c:

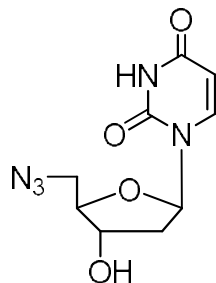
White solid; Yield: 86%; ^1H NMR (400 MHz, DMSO- d_6) δ : 2.12 (2H, m, H-2'A, H-2''B), 2.22 (1H, m, H-2'B), 2.29 (1H, m, H-2''A), 3.54 (2H, m, H-5'/5''A), 3.96 (1H, m, H-4'A), 4.06 (1H, m, H-4'B), 4.19 (1H, m, H-3'A), 4.24 (1H, m, H-3'B), 4.55 (2H, s, OCH₂), 4.65 (2H, m, H-5'/5''B), 5.08 (1H, t, J = 5.0 Hz, 5'-OH), 5.50 (1H, d, J = 4.4 Hz, 3'-OH), 5.63 (1H, d, J = 8.0 Hz, H-5A), 6.08 (1H, m, H-1'A), 6.11 (H-1'B), 7.83 (1H, d, J = 8.0 Hz, H-6A), 7.91 (1H, d, J = 6.8 Hz, H-6B), 8.10 (1H, s, H-triazole), 11.29 (1H, s, NH A), 11.83 (1H, s, NH B); ^{13}C NMR (125 MHz, DMSO- d_6) δ : 163.06 (C4A), 157.07/156.87 (d, J = 25 Hz, C4B), 150.43 (C2A), 149.00 (C2B), 143.82 (C4 triazole), 139.20/141.04 (d, J = 230 Hz, C5B), 140.42 (C6A), 124.88/125.15 (d, J = 34 Hz, C6B), 124.64 (C5 triazole), 101.91 (C5A), 84.76 (C4'A), 84.67 (C1'B), 84.26 (C4'B), 84.18 (C1'A), 79.03 (C3'A), 70.63 (C3'B), 61.72 (OCH₂), 61.46 (C5'A), 51.20 (C5'B), 37.91 (C2'B), 36.58 (C2'A); ^{19}F NMR (no dec)

(470 MHz, DMSO- d_6) δ : -166.75/-166.74 (1F, d, J = 4.7 Hz, F-5); ^{19}F NMR (dec) (470 MHz, DMSO- d_6) δ : -166.74 (1F, s, F-5); HRMS (ESI-) calcd for $\text{C}_{21}\text{H}_{23}\text{FN}_7\text{O}_9$ $[\text{M}-\text{H}]^-$ 536.1541, found 536.1540, calcd for $\text{C}_{21}\text{H}_{24}\text{FN}_7\text{O}_9\text{Cl}$ $[\text{M}+\text{Cl}]^-$ 572.1308, found 572.1306.

3. Characterization data

5'-Azido-2',5'-dideoxyuridine (1a, 5'-AddU)

Figure S1. Chromatogram



Lichrospher RP-18 endcapped
(5.0 μm , 4.6 mm x 150 mm)

Thermo Scientific HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium acetate
pH 7; B, A / acetonitrile, 1: 4, v/v)
at 35°C, flow rate 1.5 mL/min;
events: 5 min A 100%, 20 min
B 0–100%, 5 min B 100% and
15 min A 100%.

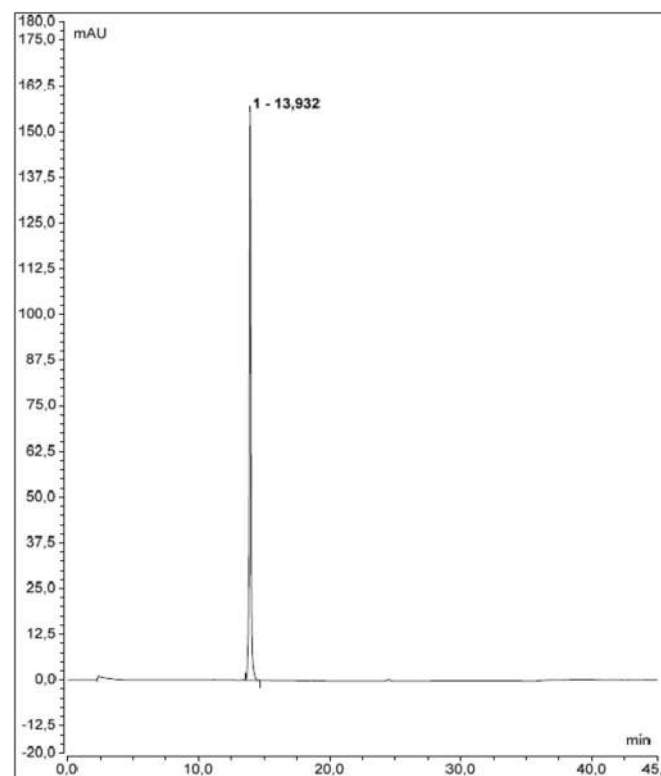


Figure S2. ^1H NMR spectrum
(DMSO- d_6 , 500 MHz)

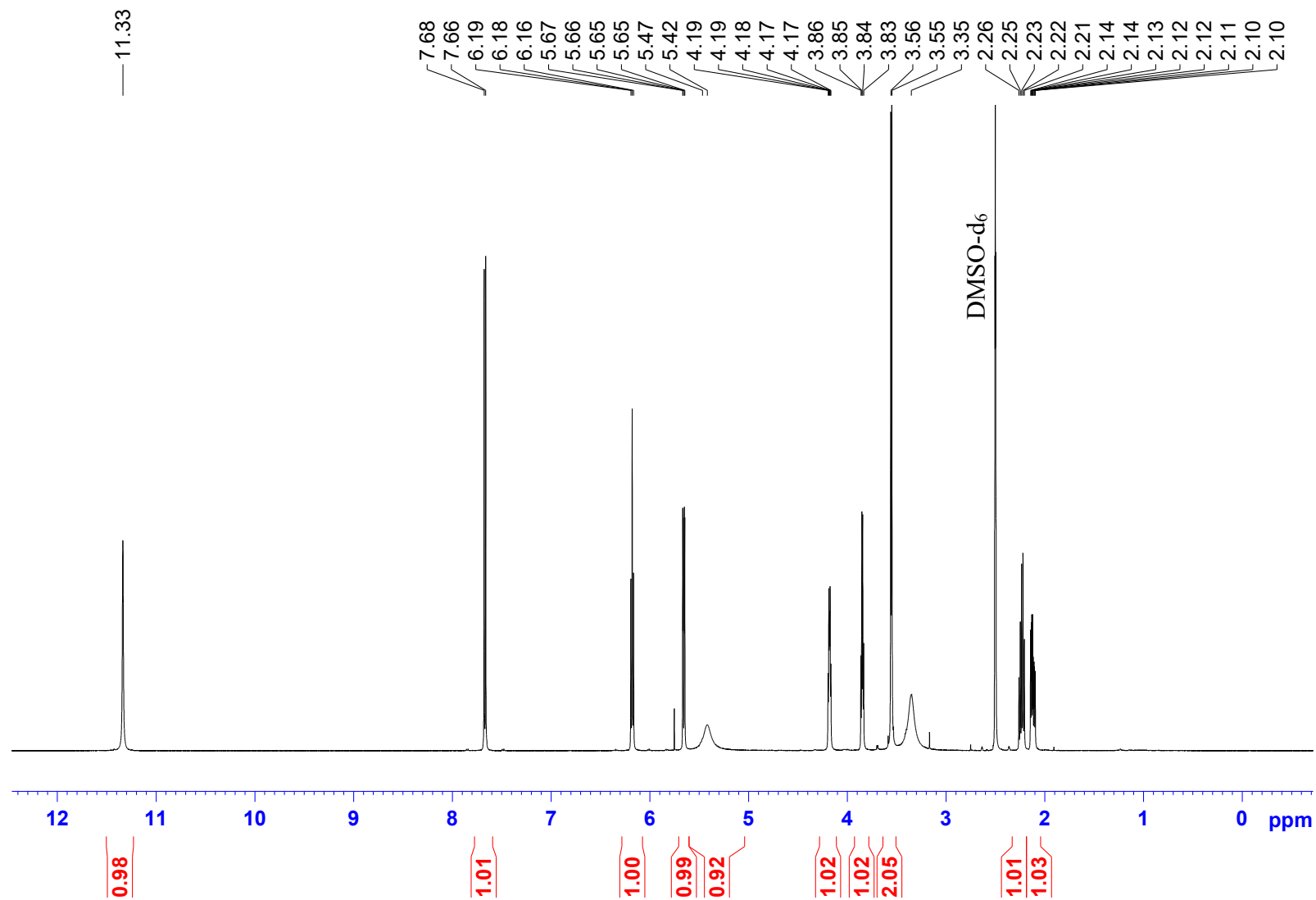
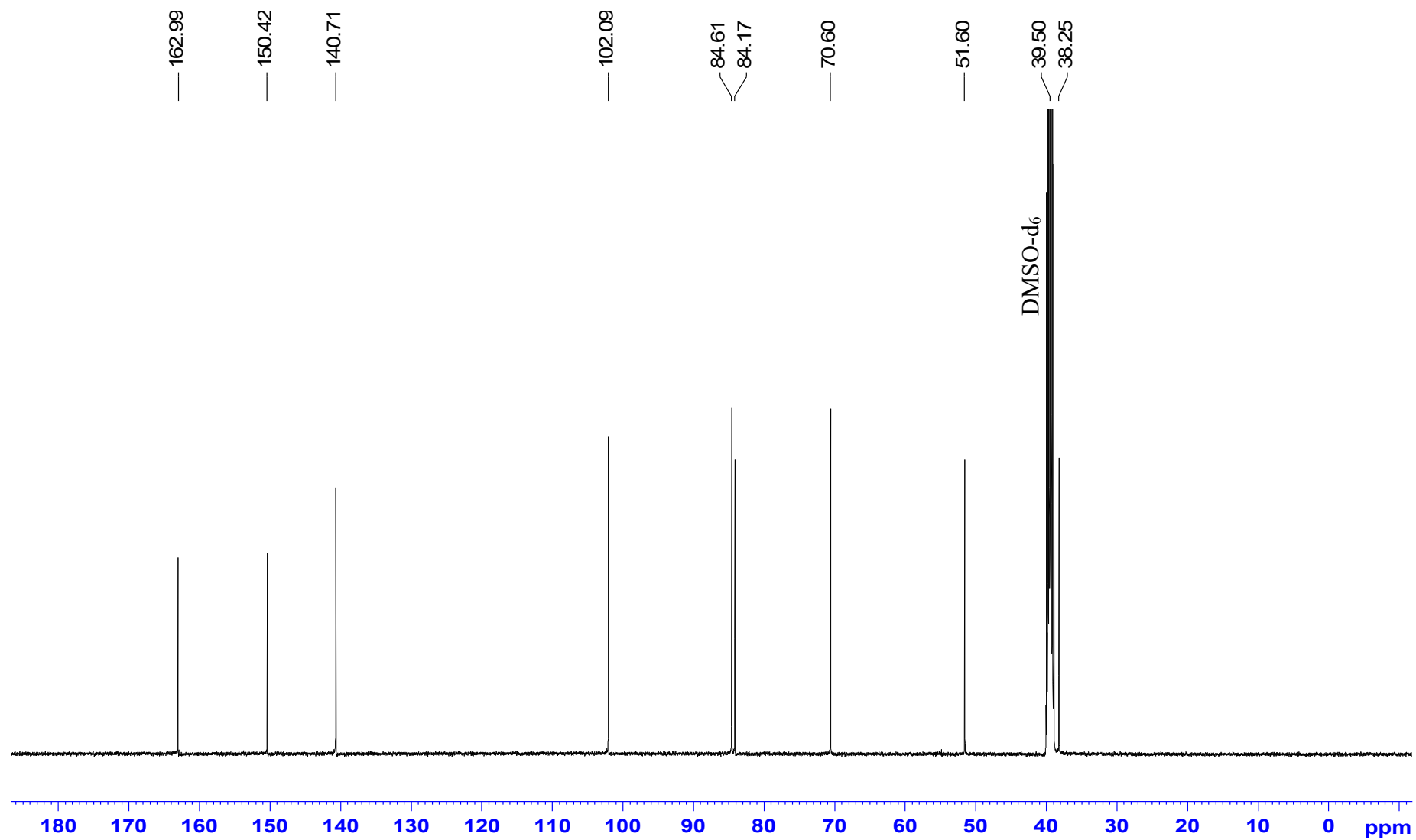
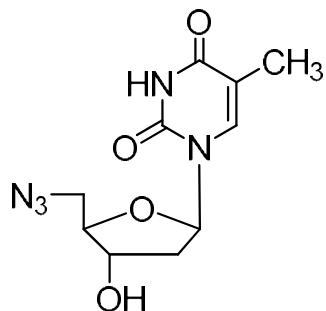


Figure S3. ^{13}C NMR spectrum
(DMSO- d_6 , 100 MHz)



5'-Azido-5'-deoxythymidine (1b, 5'-AZT)

Figure S4. Chromatogram



Lichrospher RP-18 endcapped
(5.0 μ m, 4.6 mm x 150 mm)

Thermo Scientific HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium acetate
pH 7; B, A / acetonitrile, 1: 4, v/v)
at 35°C, flow rate 1.5 mL/min;
events: 5 min A 100%, 20 min
B 0–100%, 5 min B 100% and
15 min A 100%.

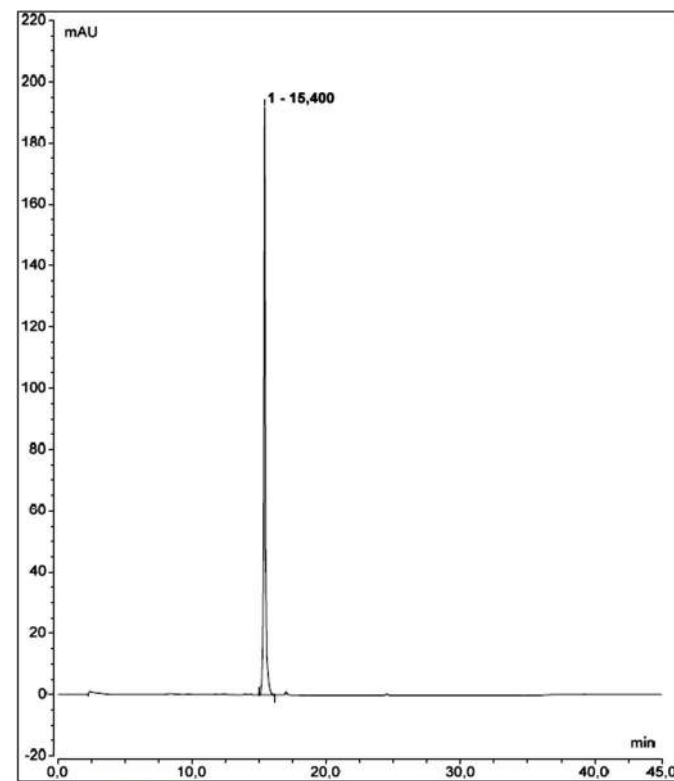


Figure S5. ^1H NMR spectrum
(DMSO- d_6 , 400 MHz)

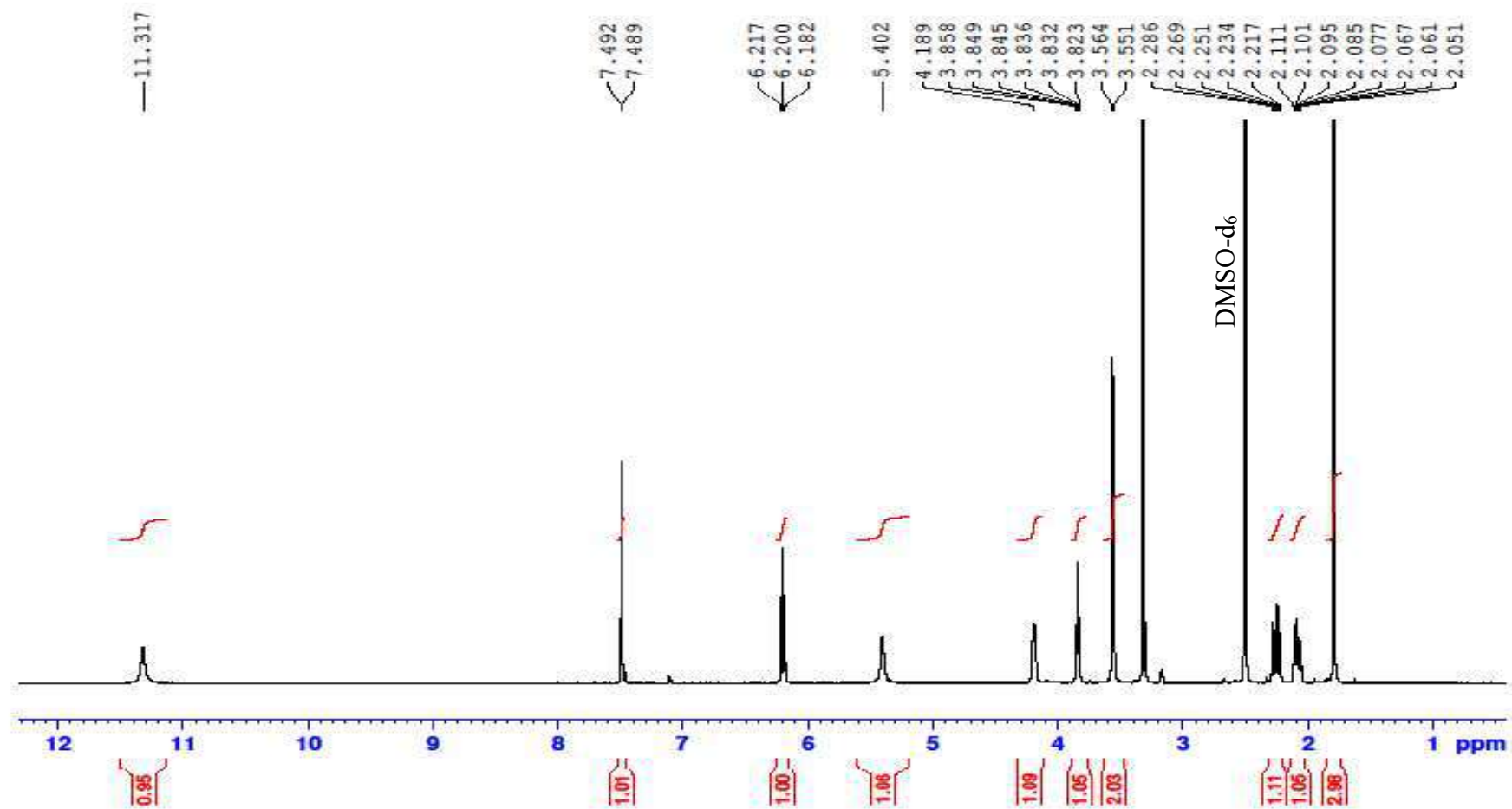
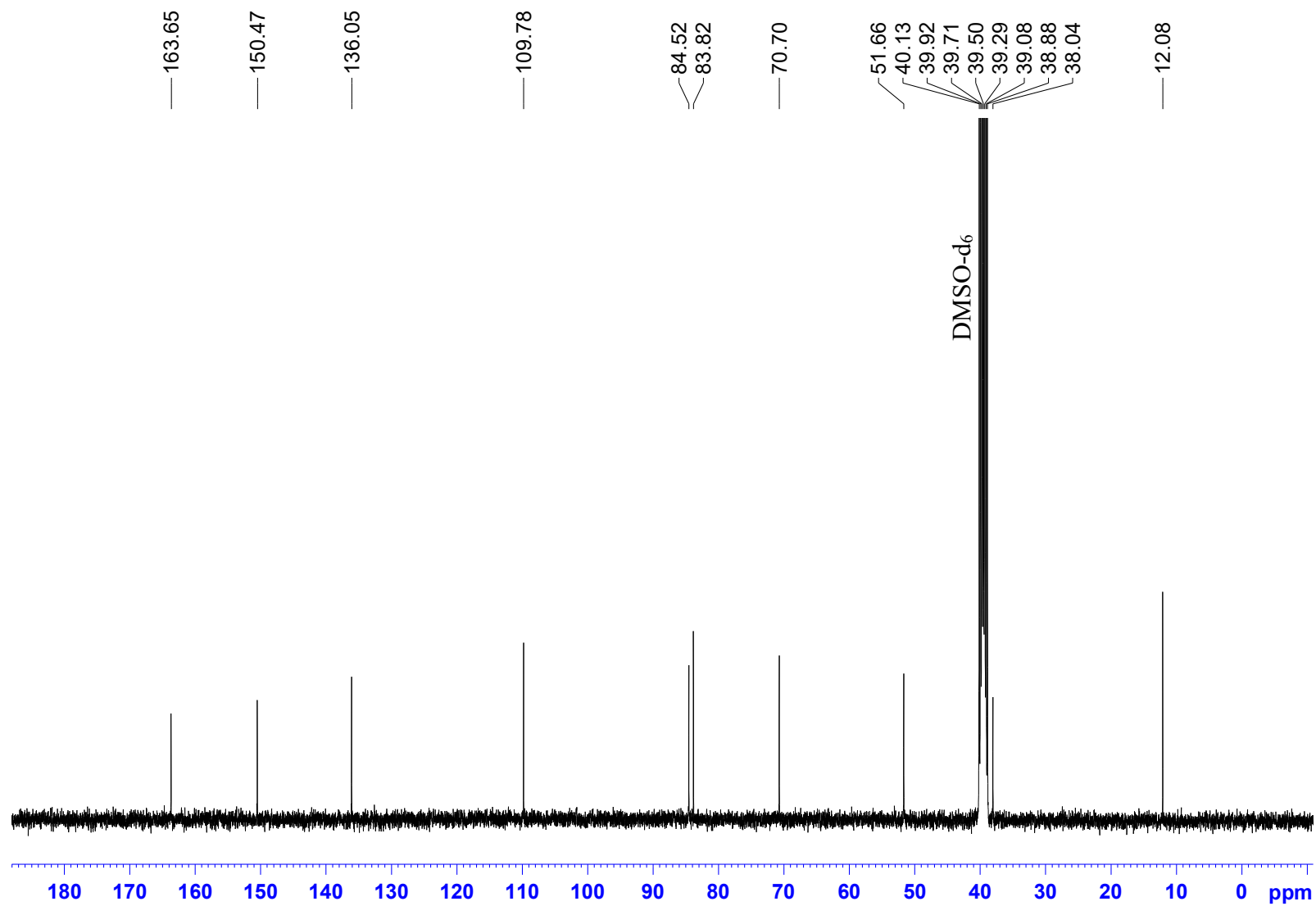
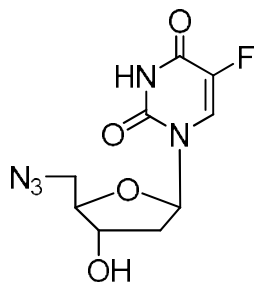


Figure S6. ^{13}C NMR spectrum
(DMSO- d_6 , 100 MHz)



5'-Azido-2',5'-dideoxy-5-fluorouridine (1c, 5'-AddFU)

Figure S7. Chromatogram



Lichrospher RP-18 endcapped
(5.0 μ m, 4.6 mm x 150 mm)

Thermo Scientific HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium acetate
pH 7; B, A / acetonitrile, 1: 4, v/v)
at 35°C, flow rate 1.5 mL/min;
events: 5 min A 100%, 20 min
B 0–100%, 5 min B 100% and
15 min A 100%.

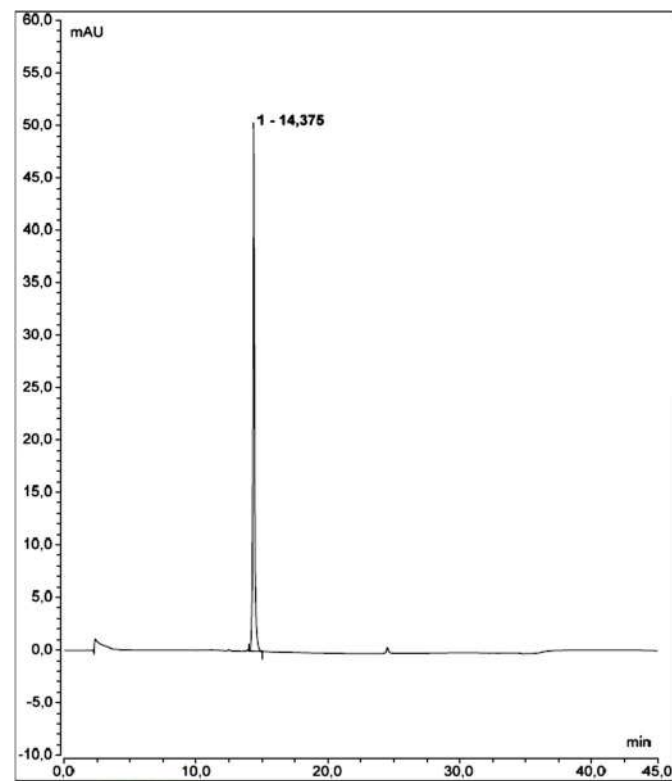


Figure S8. ^1H NMR spectrum
(DMSO- d_6 , 400 MHz)

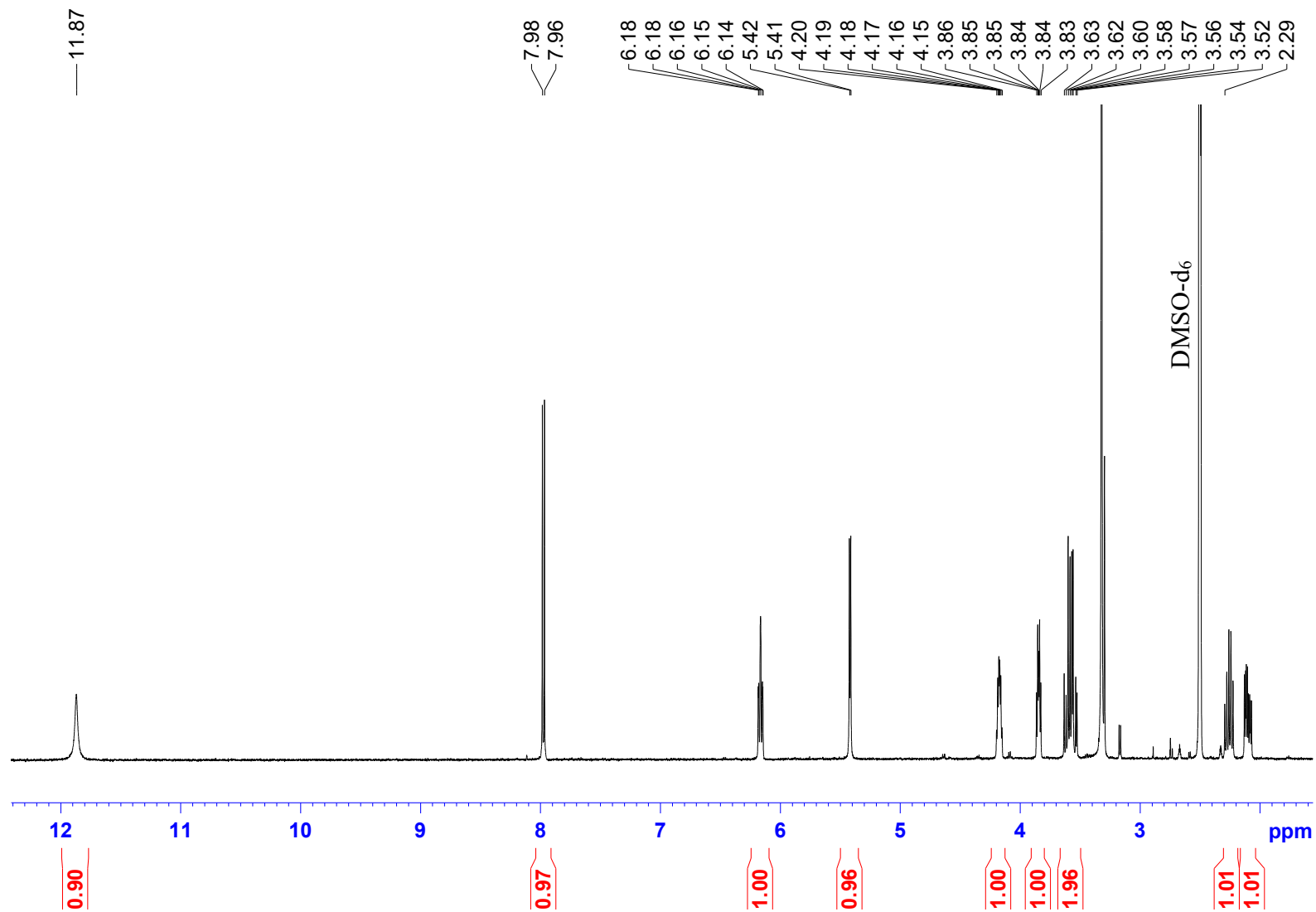


Figure S9. ^{13}C NMR spectrum
(DMSO- d_6 , 100 MHz)

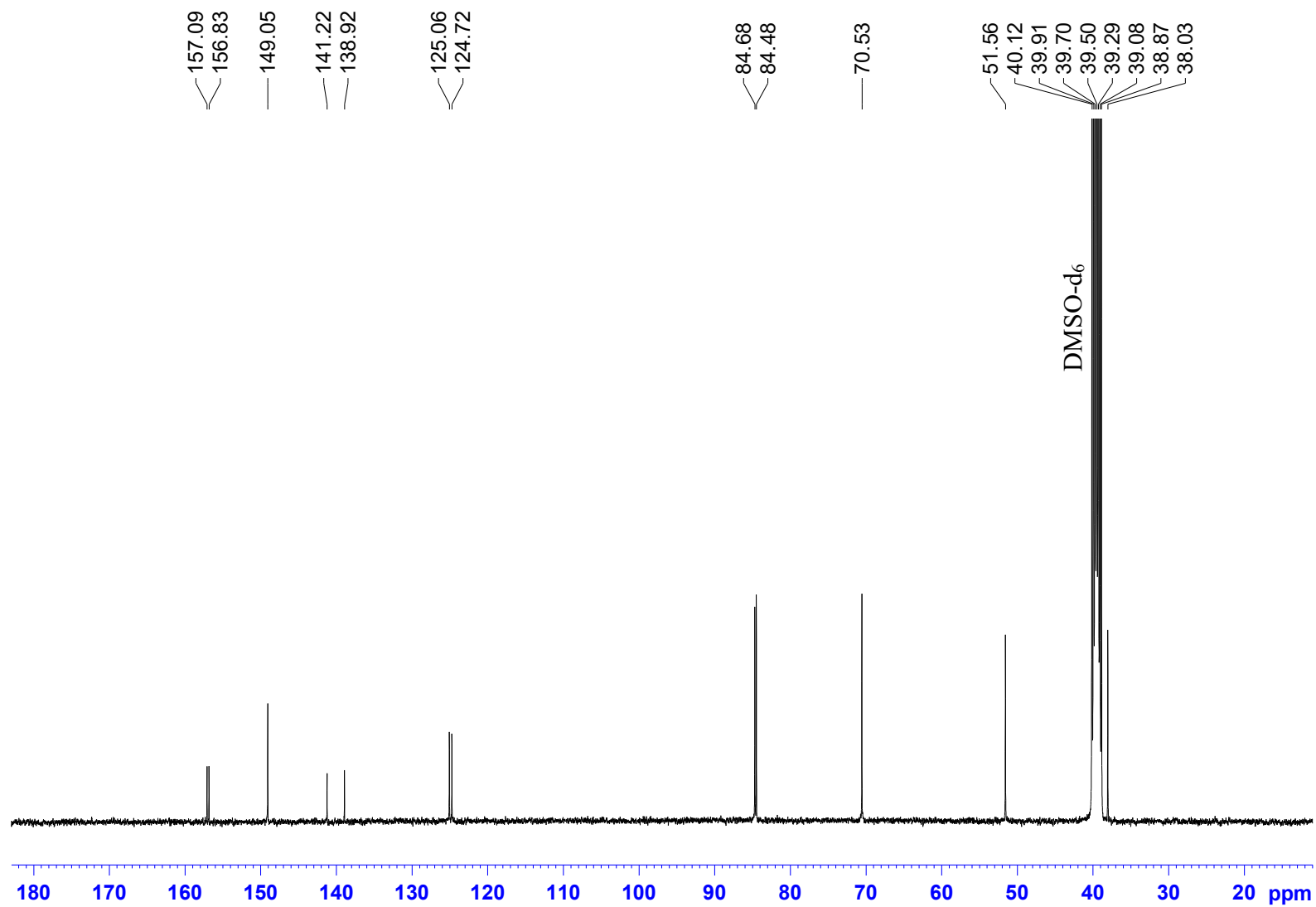
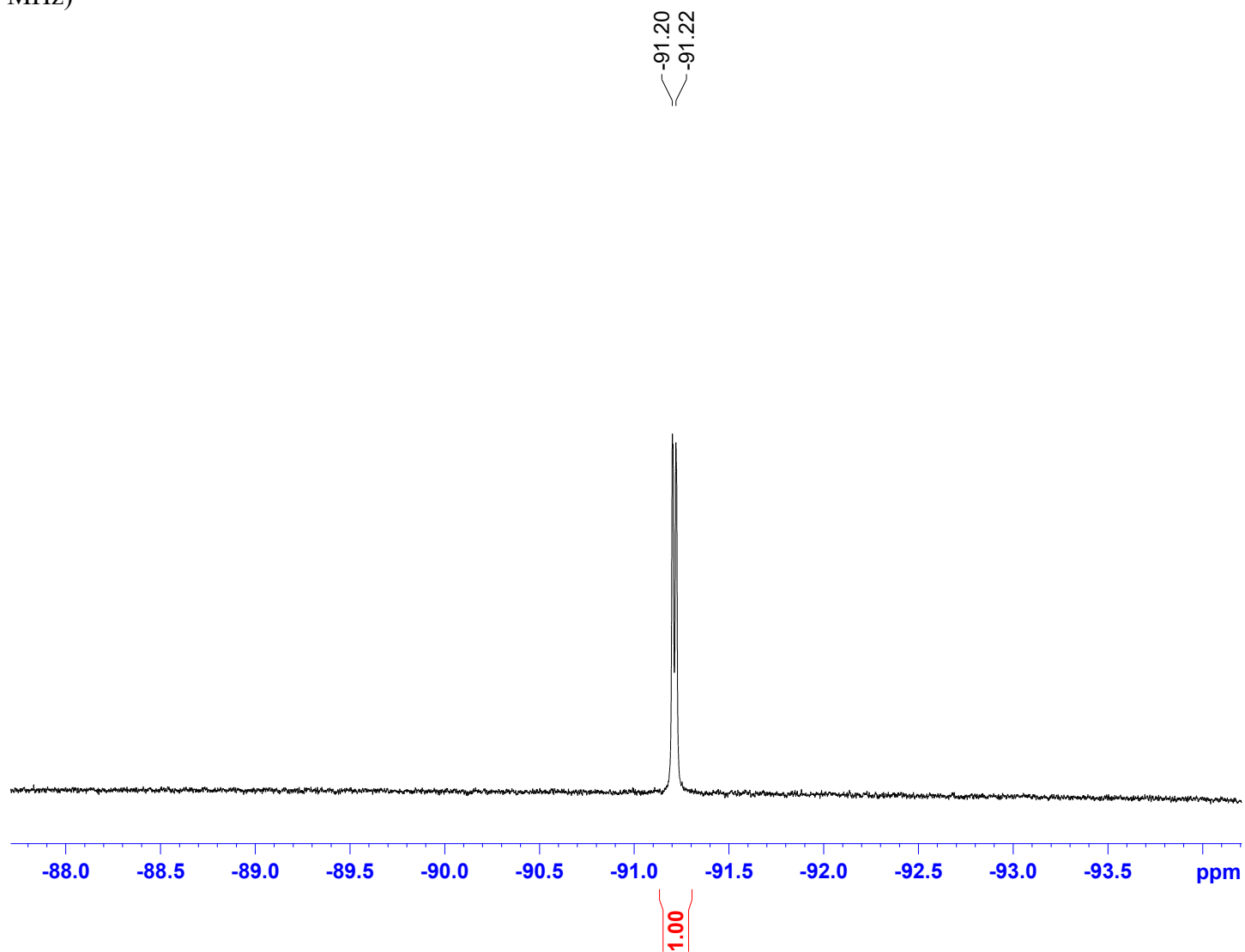
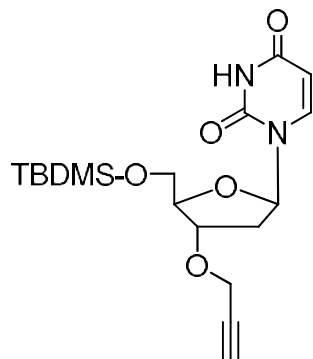


Figure S10. ^{19}F NMR spectrum
(DMSO- d_6 , 376 MHz)



3'-O-propargyl-5'-O-(tert-butyldimethylsilyl)-2'-deoxyuridine (2a)

Figure S11. Chromatogram



Lichrospher RP-18 endcapped
(5.0 μ m, 4.6 mm x 150 mm)

Thermo Scientific HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium acetate
pH 7; B, A / acetonitrile, 1: 4, v/v)
at 35°C, flow rate 1.5 mL/min;
events: 5 min A 100%, 20 min
B 0–100%, 5 min B 100% and
15 min A 100%.

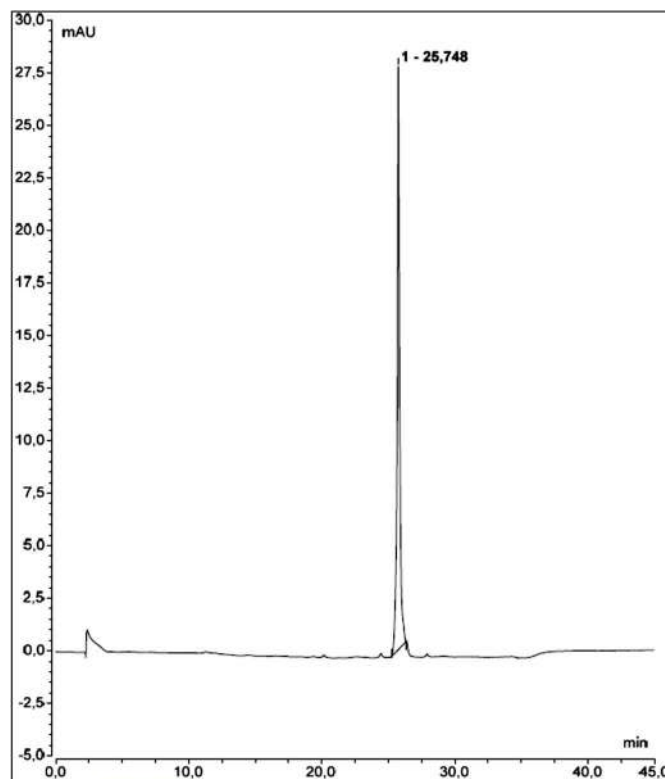


Figure S12. ^1H NMR spectrum
(DMSO- d_6 , 500 MHz)

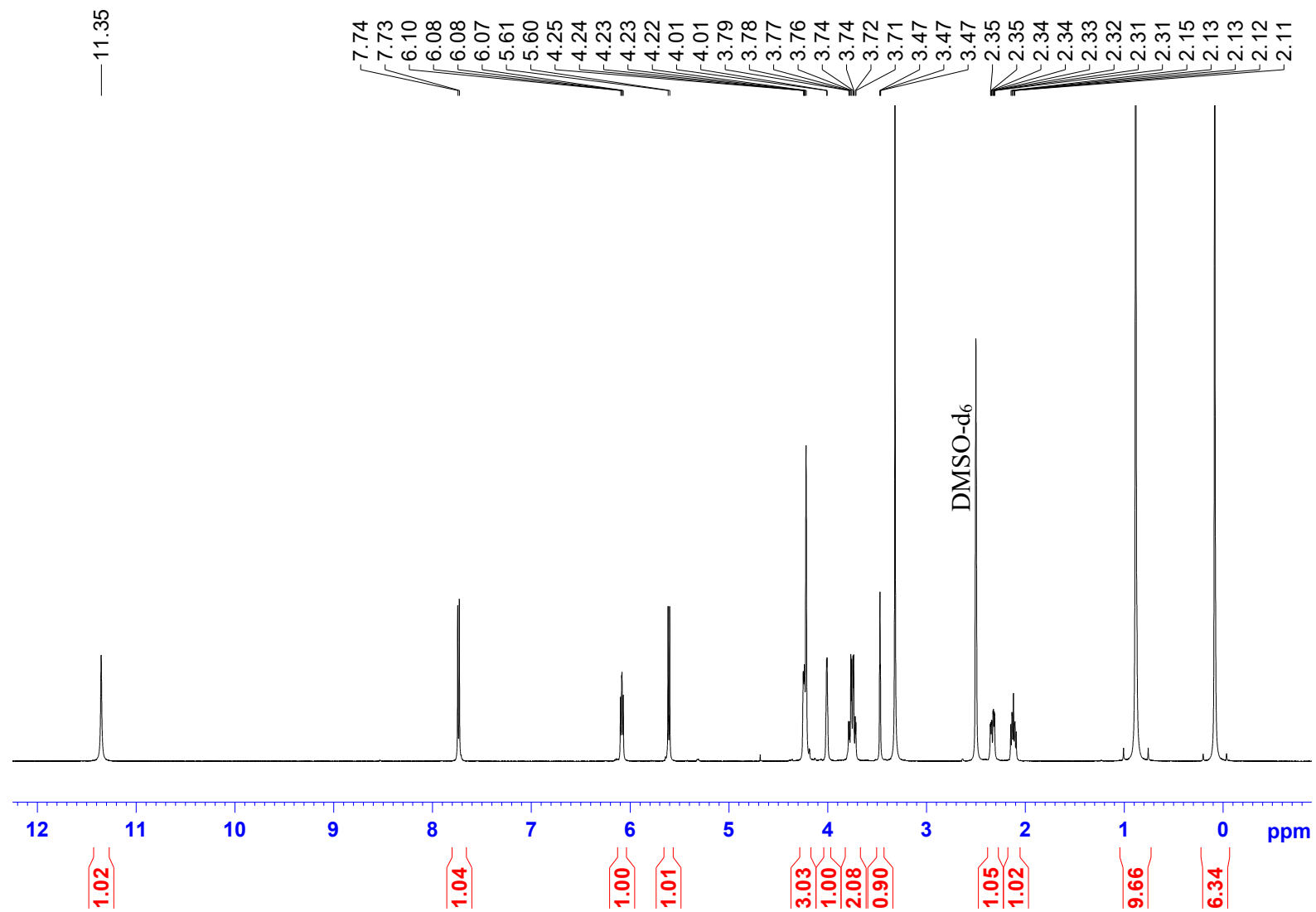
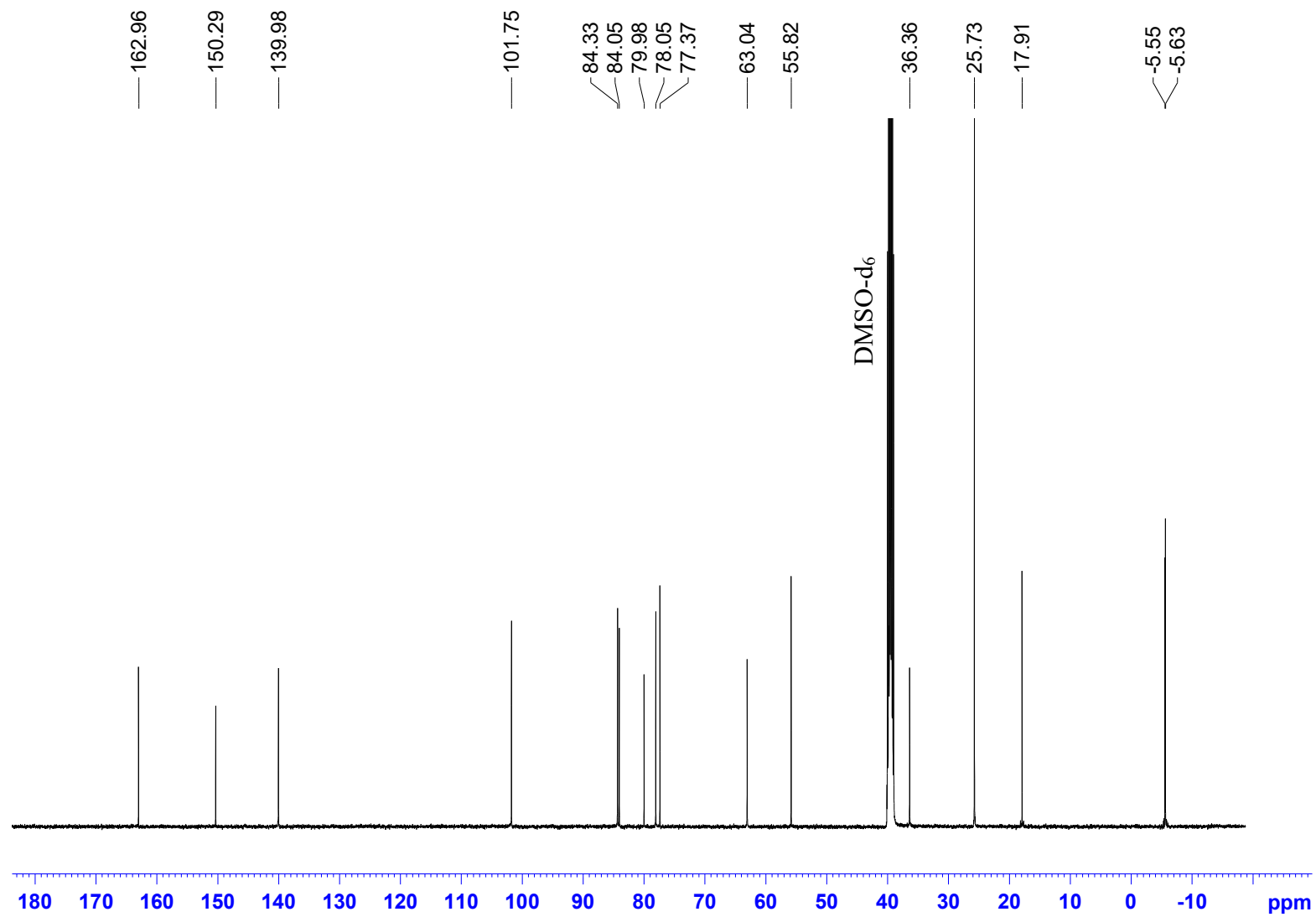
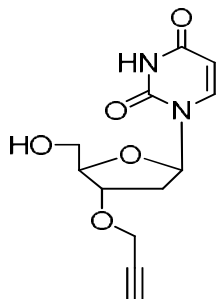


Figure S13. ^{13}C NMR spectrum
(DMSO- d_6 , 125 MHz)



3'-O-propargyl-2'-deoxyuridine (2b)

Figure S14. Chromatogram



Lichrospher RP-18 endcapped
(5.0 μm , 4.6 mm x 150 mm)

Thermo Scientific HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium acetate
pH 7; B, A / acetonitrile, 1: 4, v/v)
at 35°C, flow rate 1.5 mL/min;
events: 5 min A 100%, 20 min
B 0–100%, 5 min B 100% and
15 min A 100%.

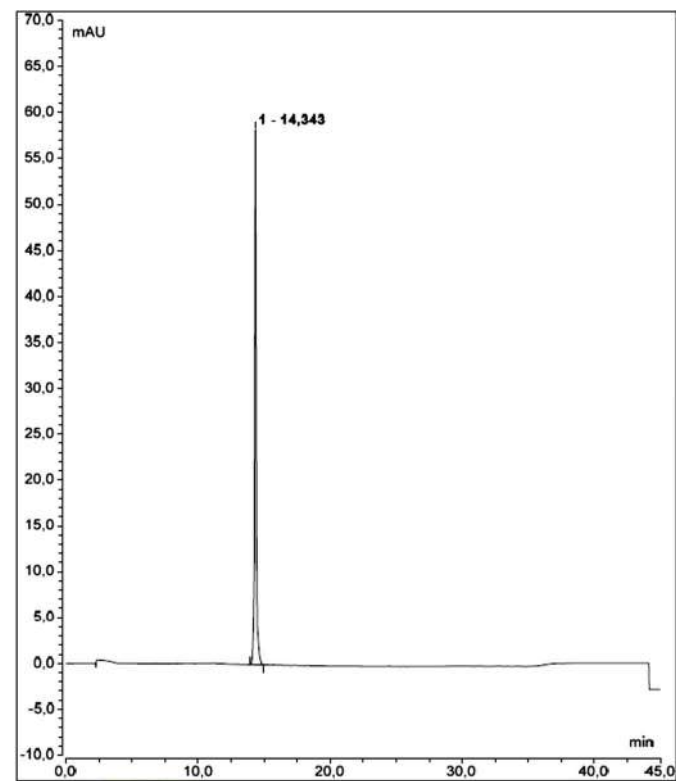


Figure S15. ^1H NMR spectrum
(DMSO- d_6 , 500 MHz)

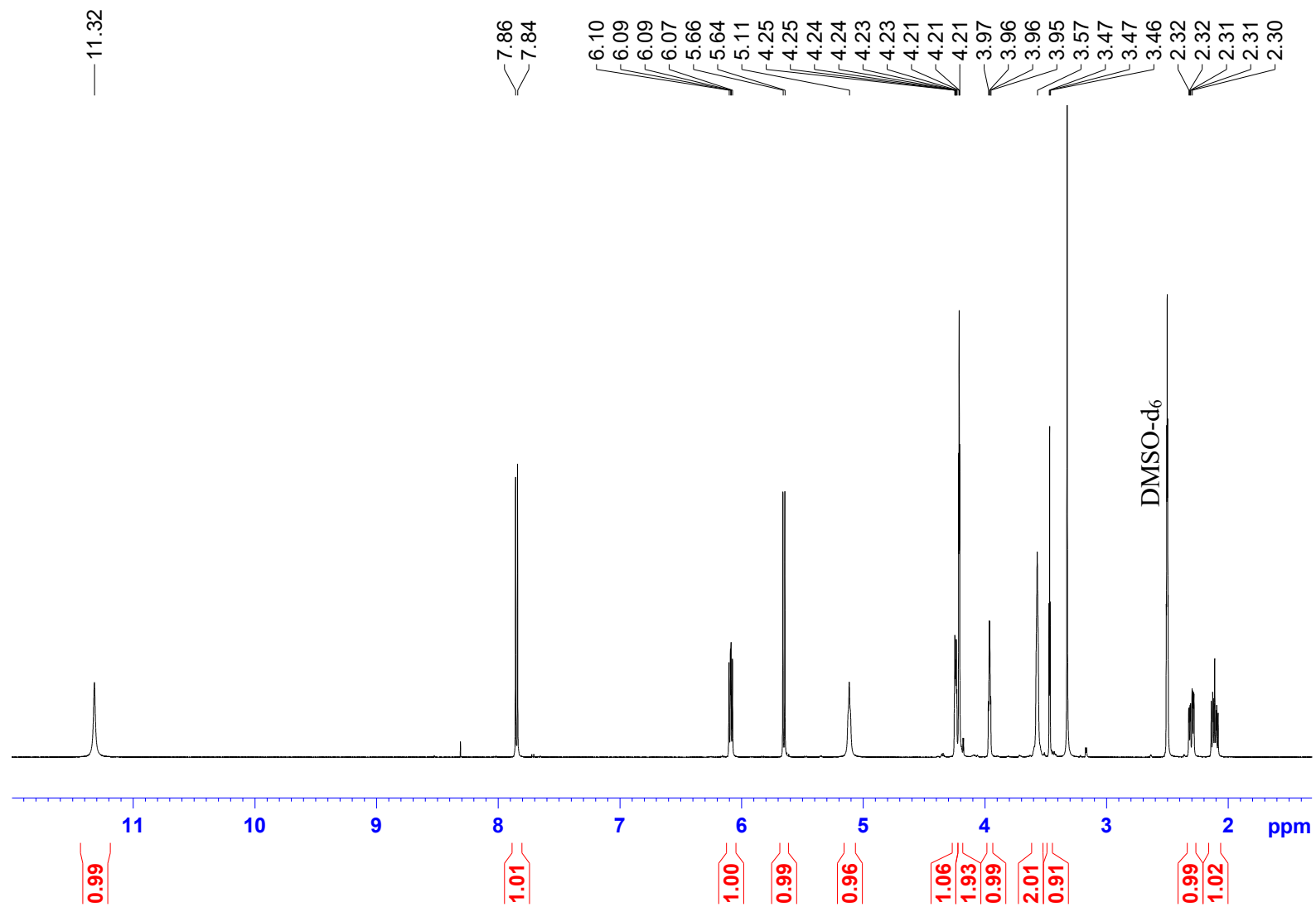
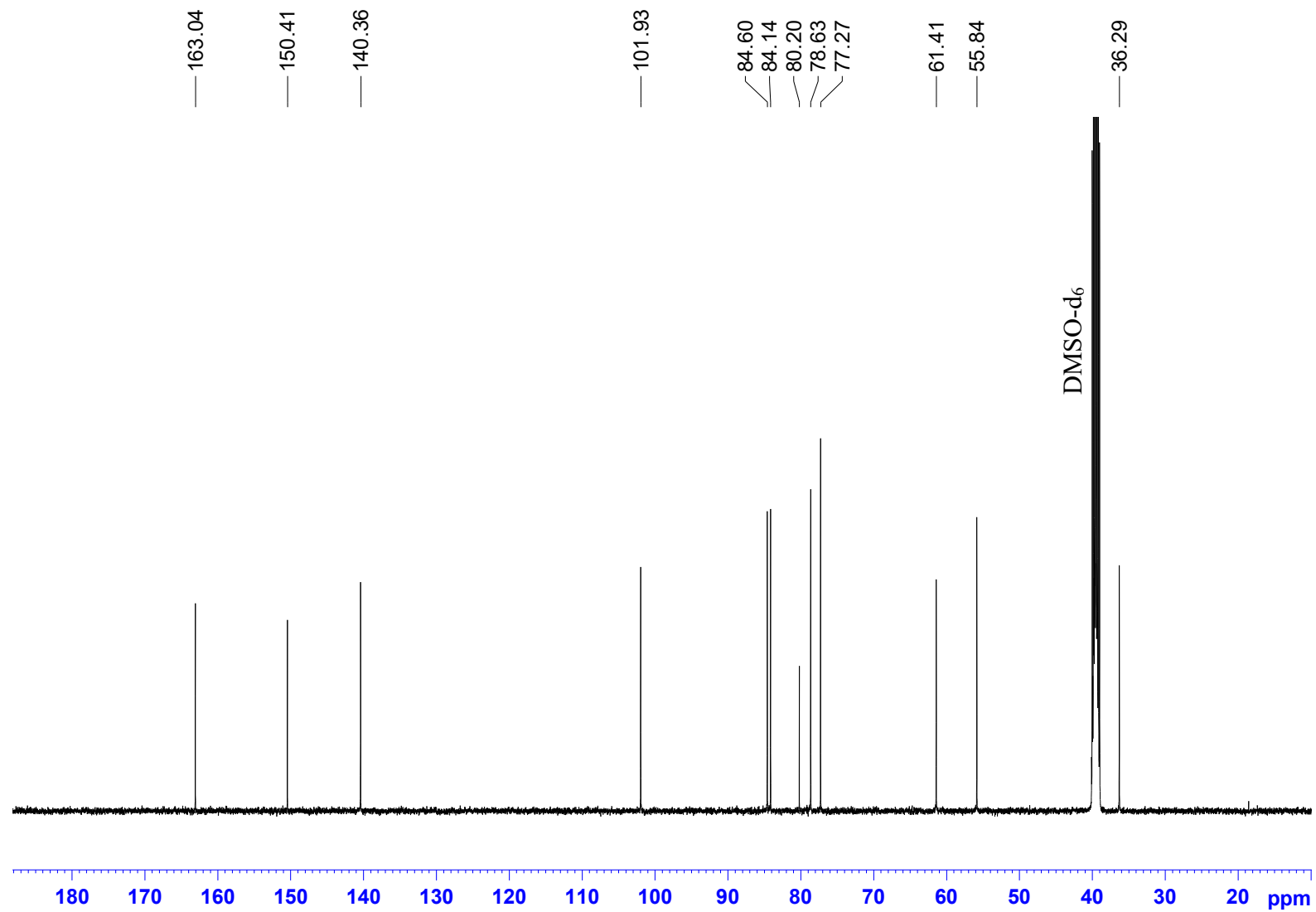


Figure S16. ^{13}C NMR spectrum
(DMSO- d_6 , 125 MHz)



Compound 3 a)

Figure S17. Chromatogram

Lichrospher RP-18 endcapped
(5.0 μm , 4.6 mm x 150 mm)

Thermo Scientific HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium acetate
pH 7; B, A / acetonitrile, 1: 4, v/v)
at 35°C, flow rate 1.5 mL/min;
events: 5 min A 100%, 20 min
B 0–100%, 5 min B 100% and
15 min A 100%.

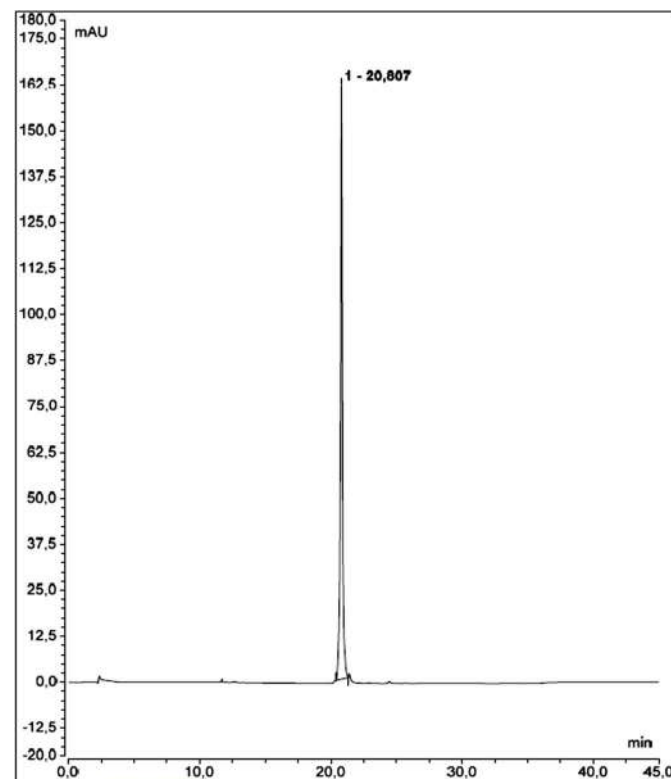


Figure S18. ^1H NMR spectrum
(DMSO- d_6 , 500 MHz)

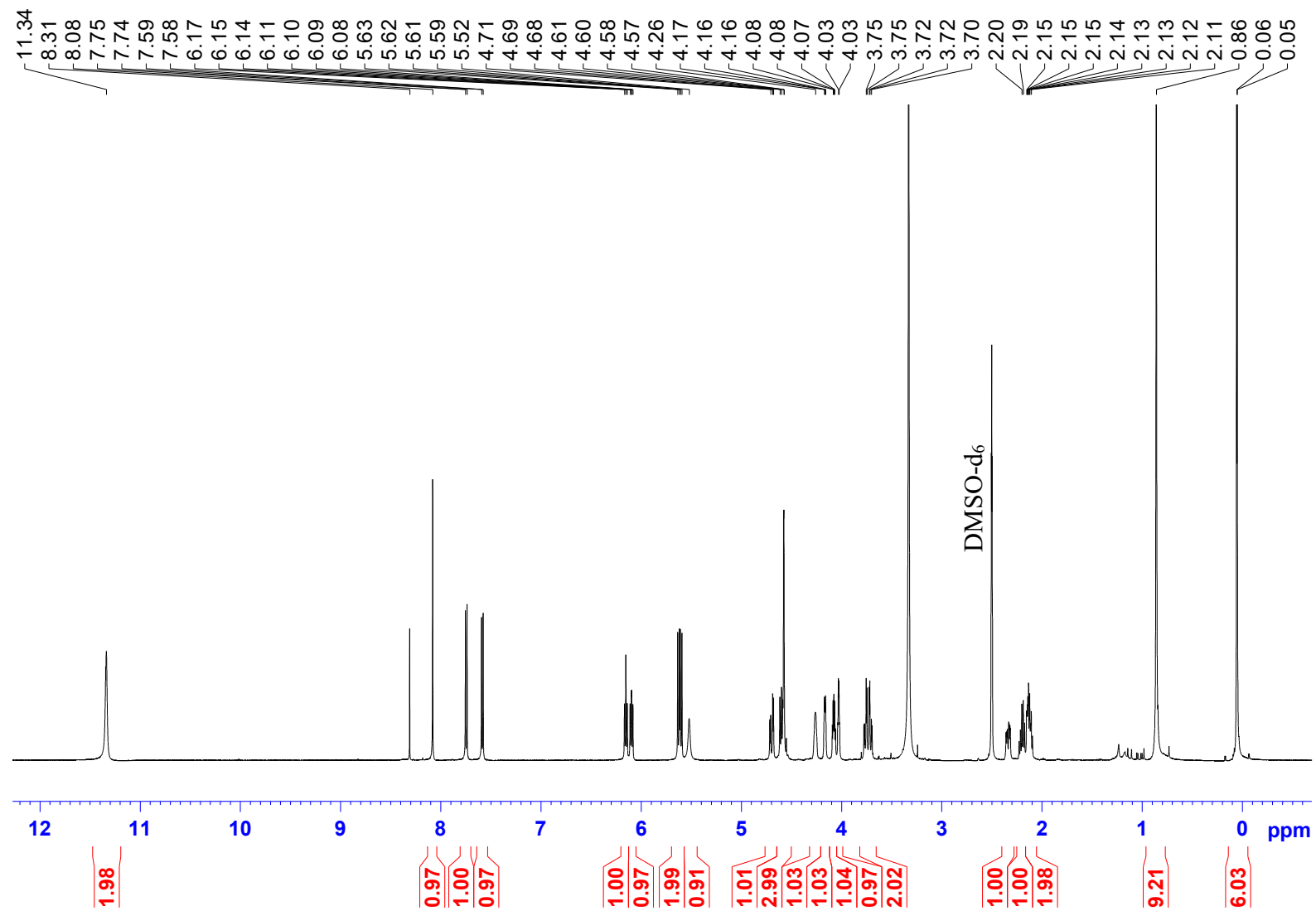
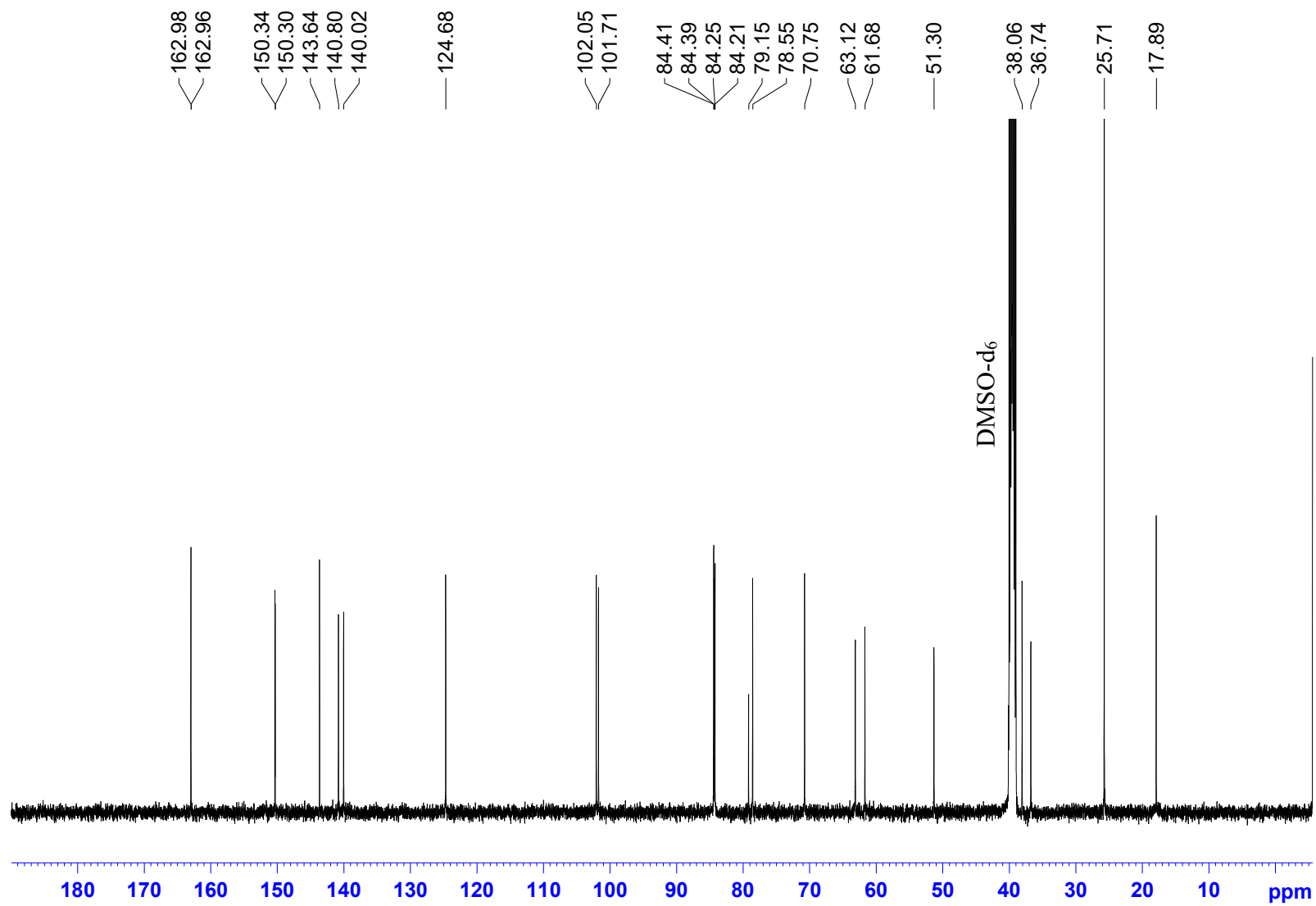


Figure S19. ^{13}C NMR spectrum
(DMSO- d_6 , 125 MHz)



Compound 3 b)

Figure S20. Chromatogram

Lichrospher RP-18 endcapped
(5.0 μm , 4.6 mm x 150 mm)

Thermo Scientific HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium acetate
pH 7; B, A / acetonitrile, 1: 4, v/v)
at 35°C, flow rate 1.5 mL/min;
events: 5 min A 100%, 20 min
B 0–100%, 5 min B 100% and
15 min A 100%.

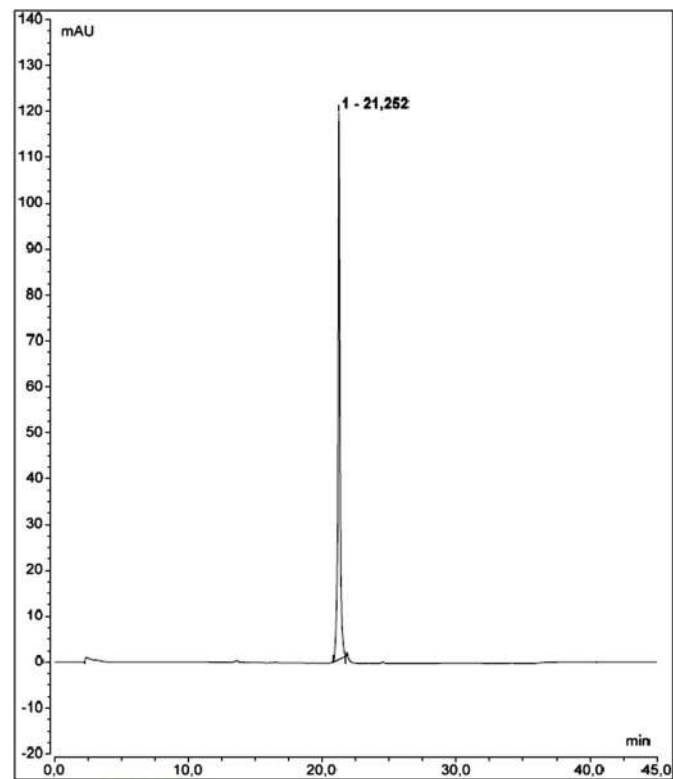


Figure S21. ^1H NMR spectrum
(DMSO- d_6 , 500 MHz)

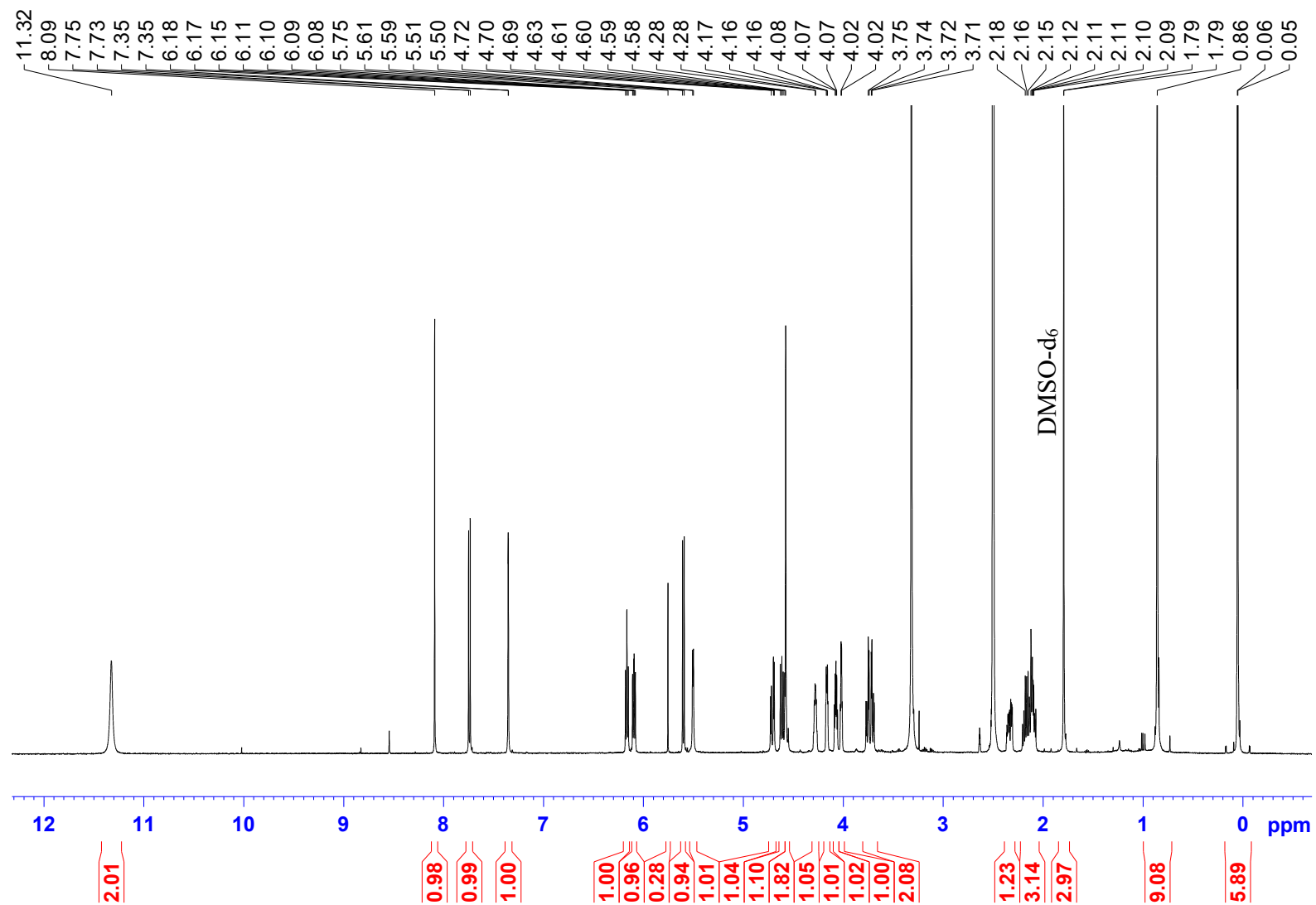
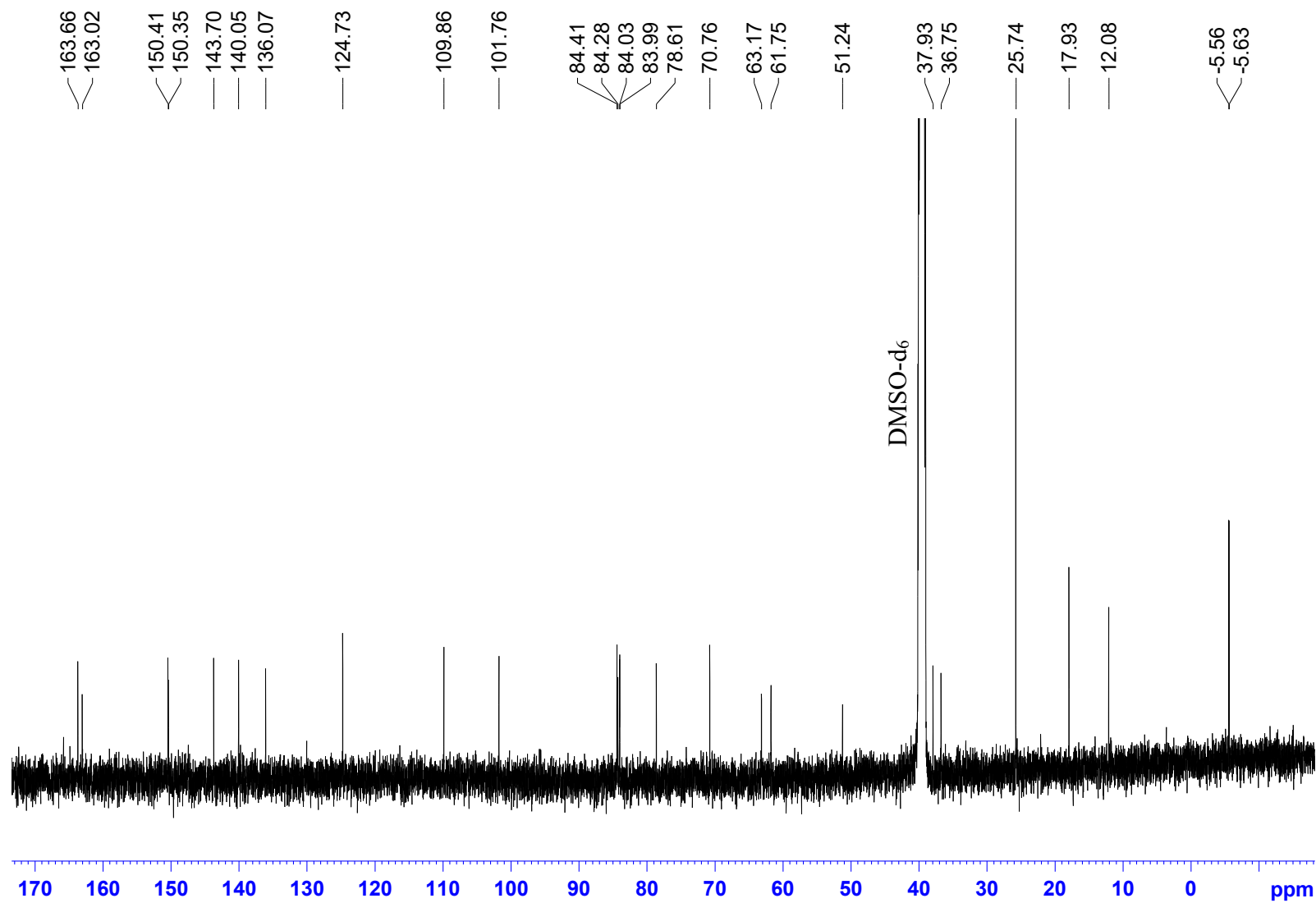


Figure S22. ^{13}C NMR spectrum
(DMSO- d_6 , 125 MHz)



Compound 3 c)

Figure S23. Chromatogram

Lichrospher RP-18 endcapped
(5.0 μm , 4.6 mm x 150 mm)

Thermo Scientific HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium acetate
pH 7; B, A / acetonitrile, 1: 4, v/v)
at 35°C, flow rate 1.5 mL/min;
events: 5 min A 100%, 20 min
B 0–100%, 5 min B 100% and
15 min A 100%.

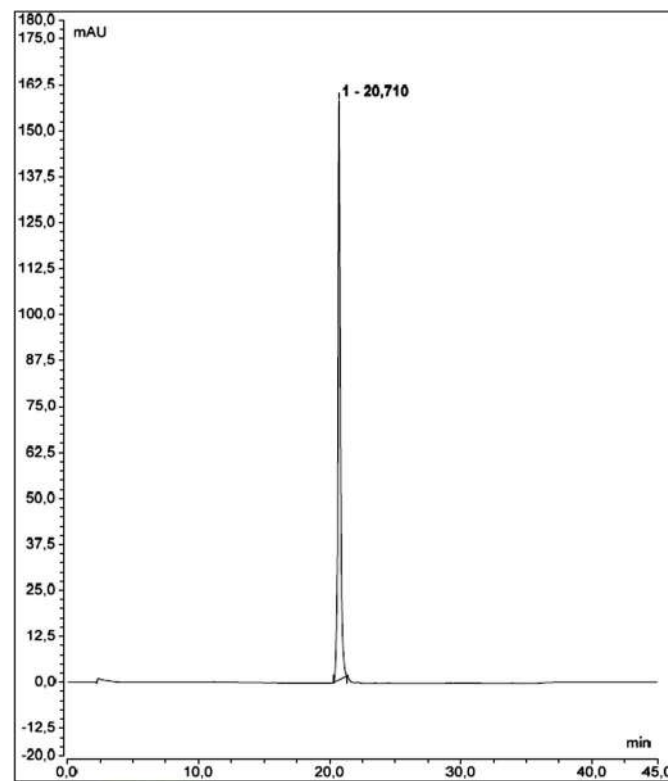


Figure S24. ^1H NMR spectrum
(DMSO- d_6 , 500 MHz)

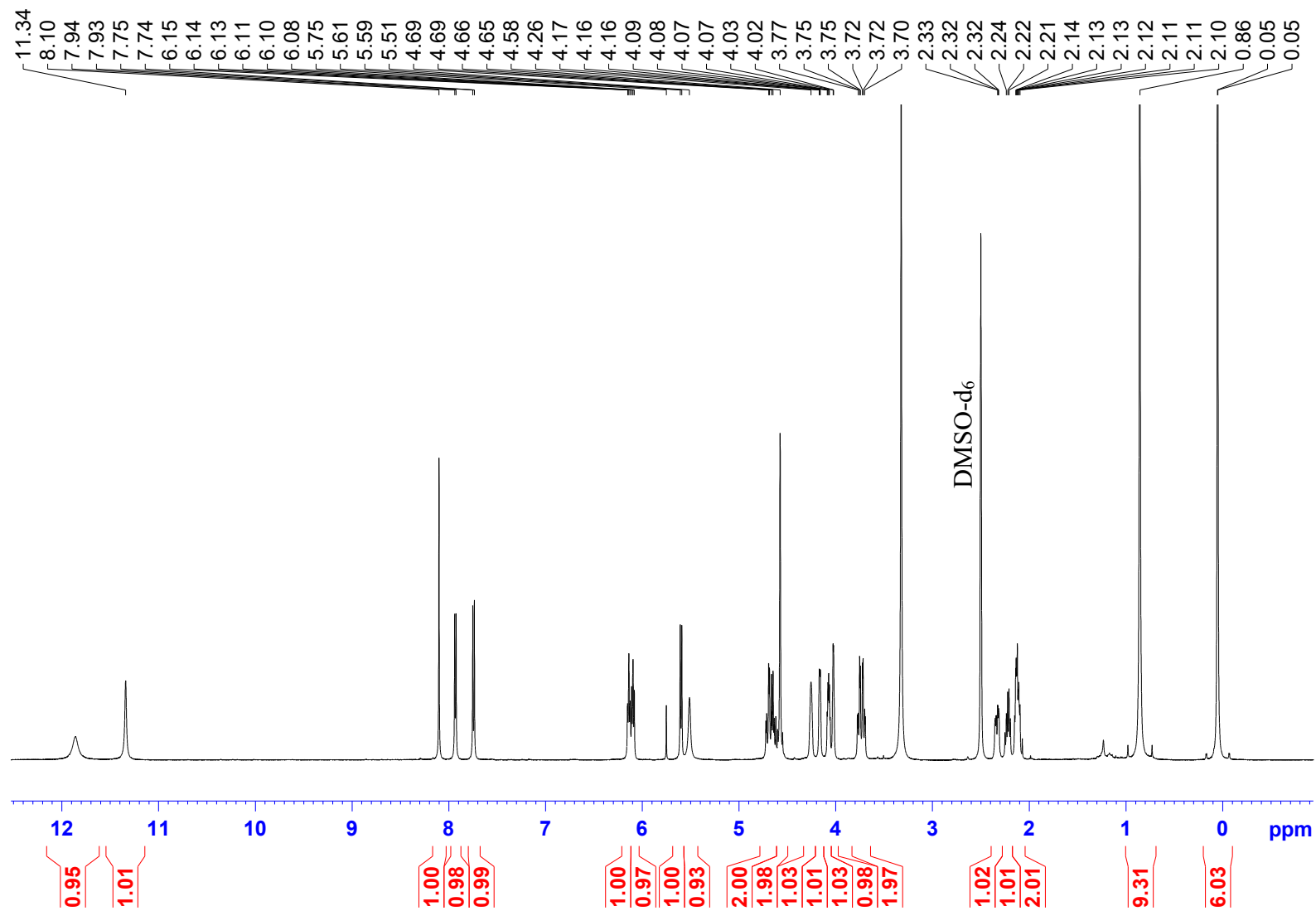


Figure S25. ^{13}C NMR spectrum
(DMSO- d_6 , 125 MHz)

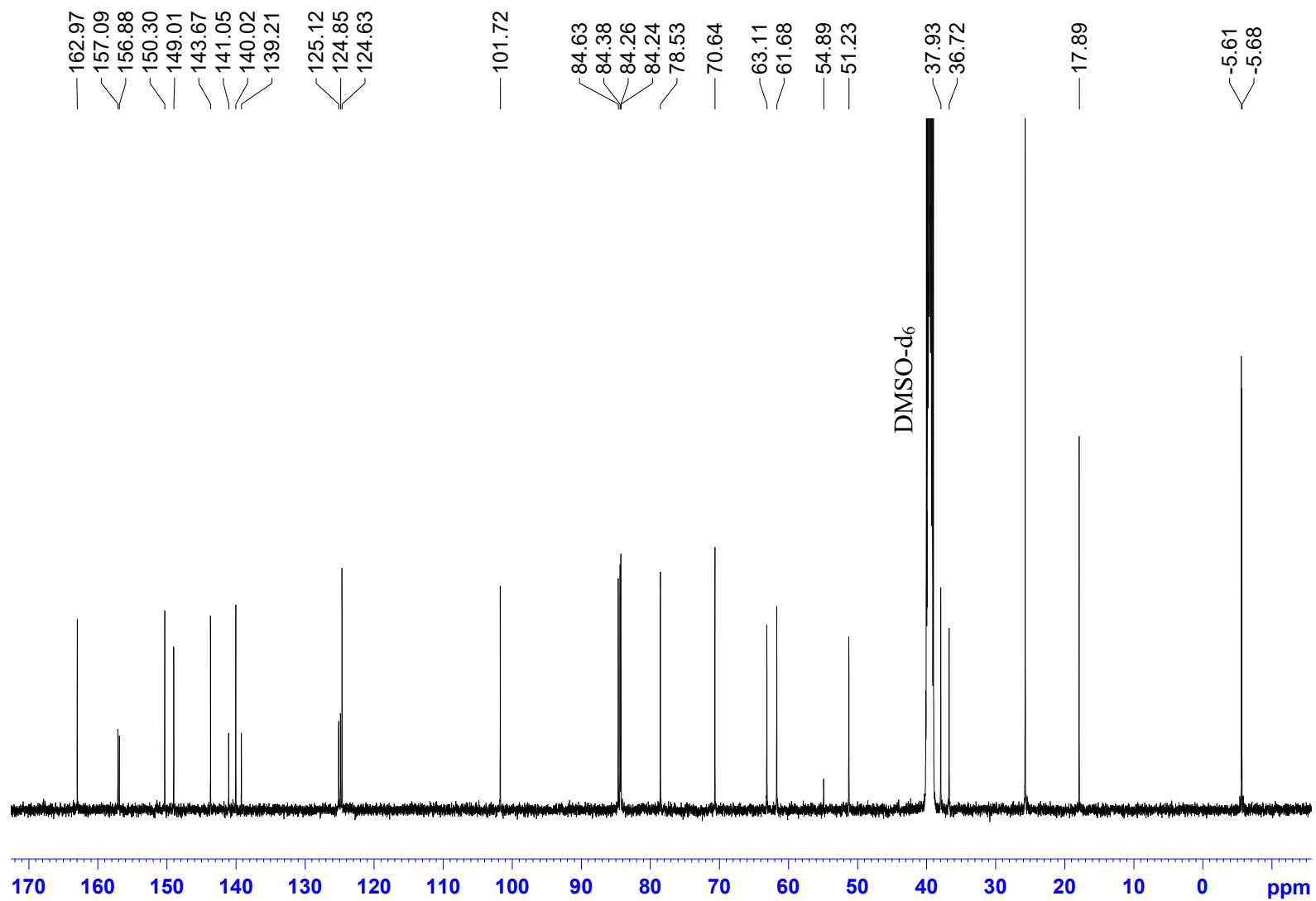
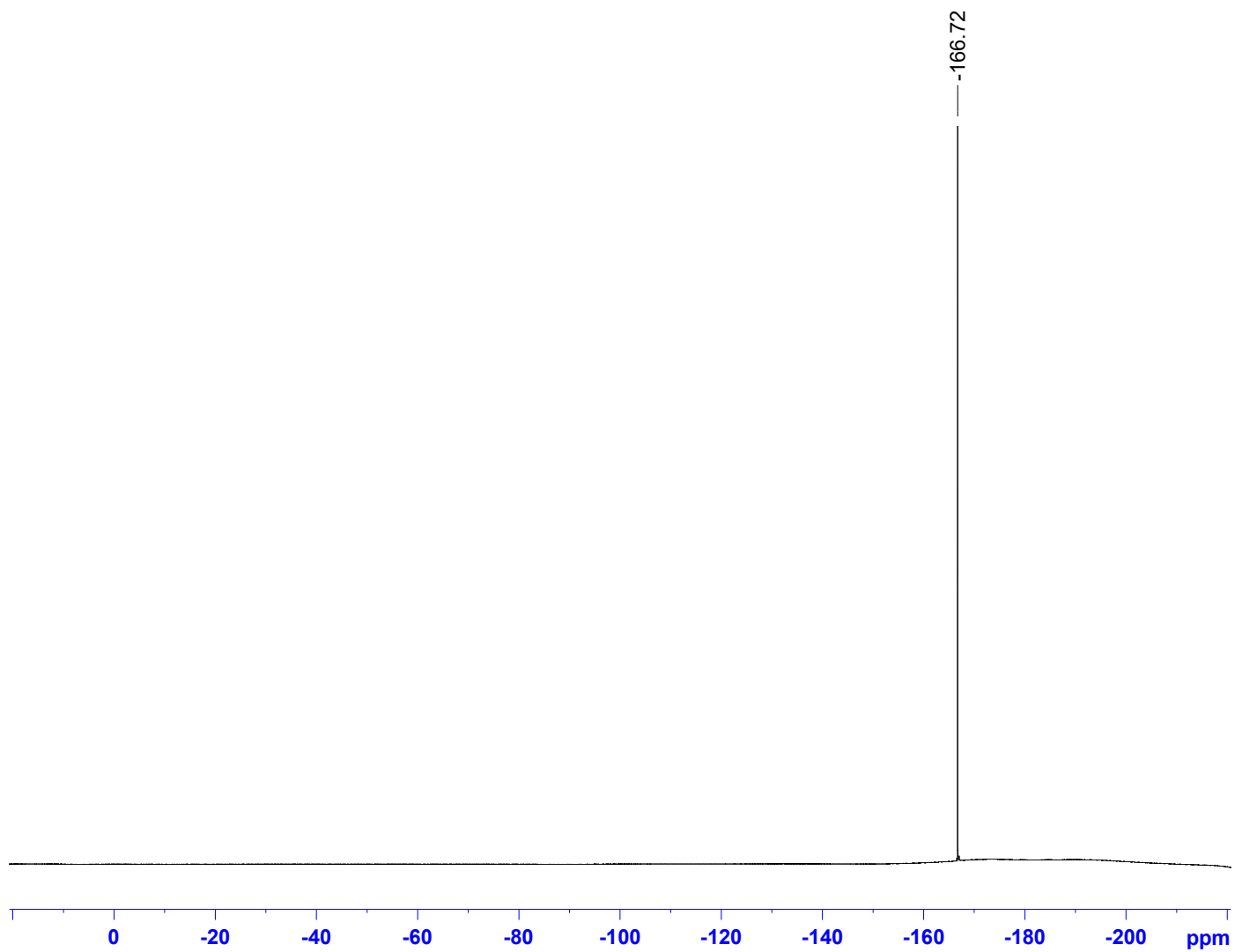


Figure S26. ^{19}F NMR spectrum (dec)
(DMSO- d_6 , 470 MHz)



Compound 4 a)

Figure S27. Chromatogram

Lichrospher RP-18 endcapped
(5.0 μm , 4.6 mm x 150 mm)

Thermo Scientific HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium acetate
pH 7; B, A / acetonitrile, 1: 4, v/v)
at 35°C, flow rate 1.5 mL/min;
events: 5 min A 100%, 20 min
B 0–100%, 5 min B 100% and
15 min A 100%.

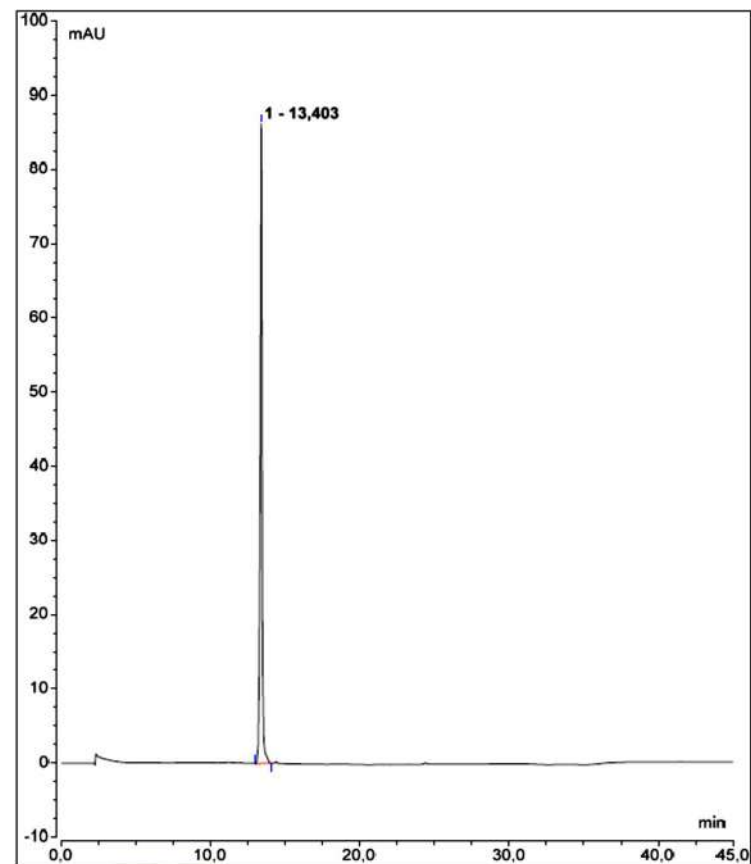


Figure S28. ^1H NMR spectrum
(DMSO- d_6 , 500 MHz)

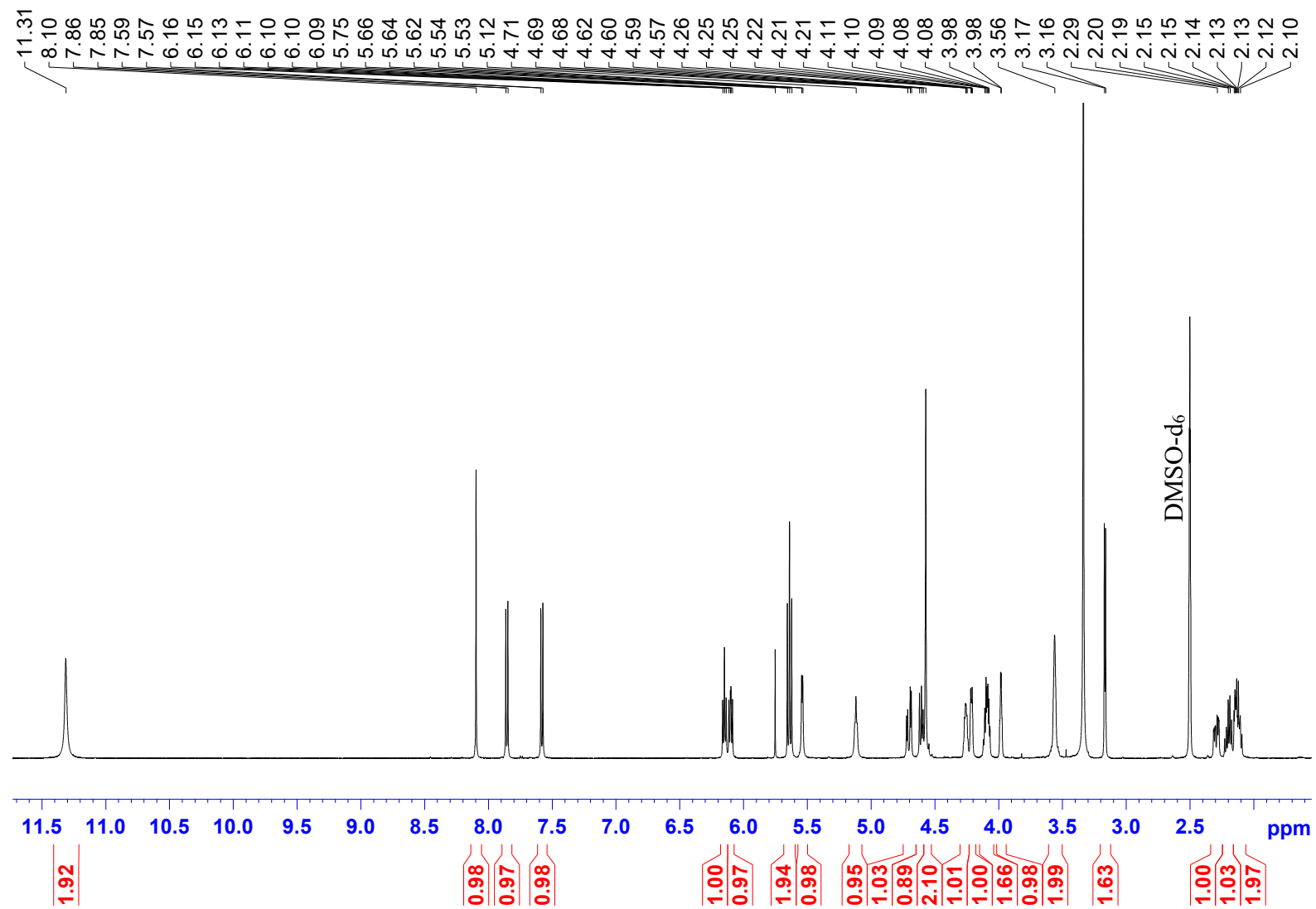
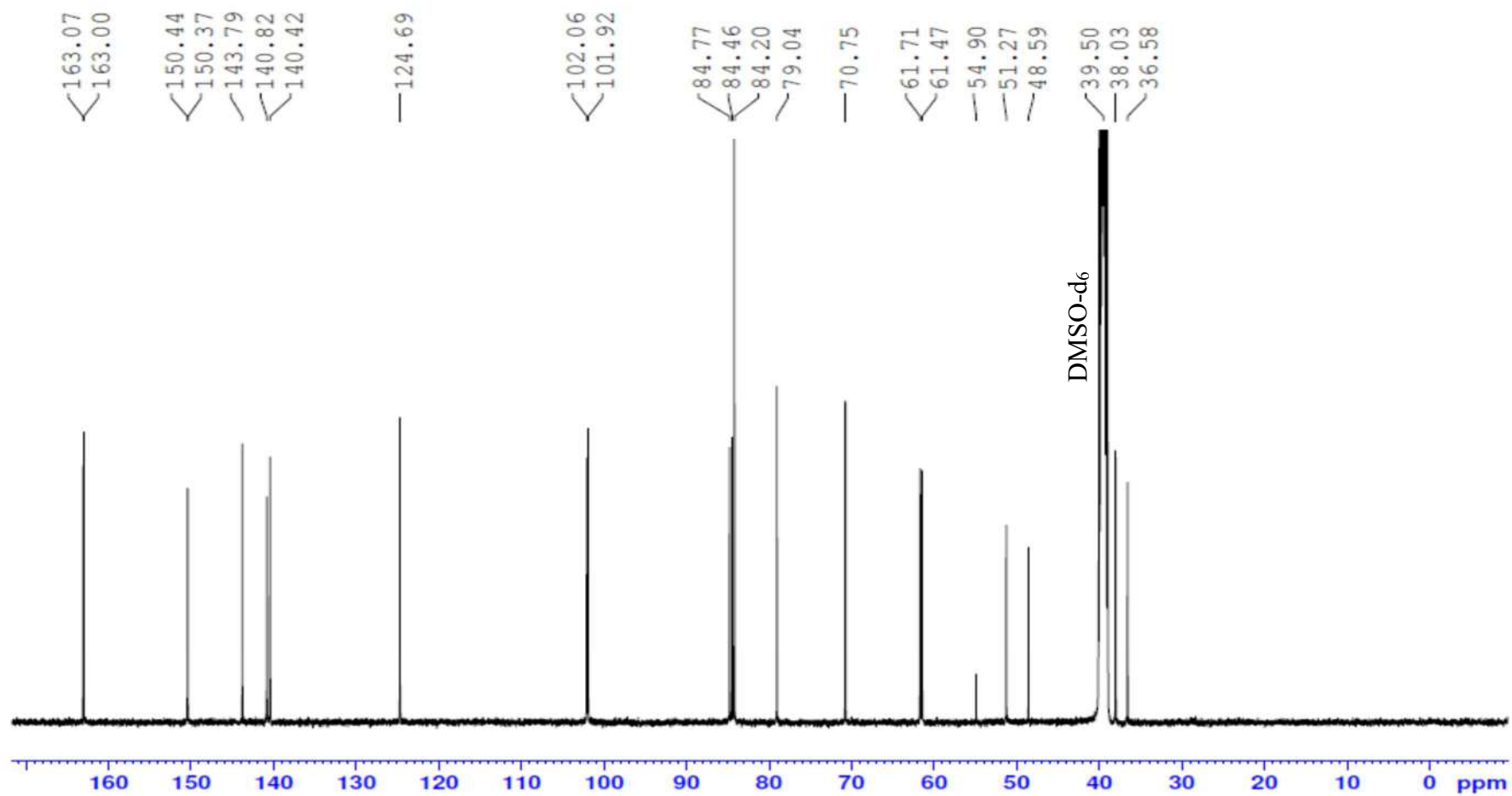


Figure S29. ^{13}C NMR spectrum
(DMSO- d_6 , 125 MHz)



Compound 4 b)

Figure S30. Chromatogram

Lichrospher RP-18 endcapped
(5.0 μm , 4.6 mm x 150 mm)

Thermo Scientific HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium acetate
pH 7; B, A / acetonitrile, 1: 4, v/v)
at 35°C, flow rate 1.5 mL/min;
events: 5 min A 100%, 20 min
B 0–100%, 5 min B 100% and
15 min A 100%.

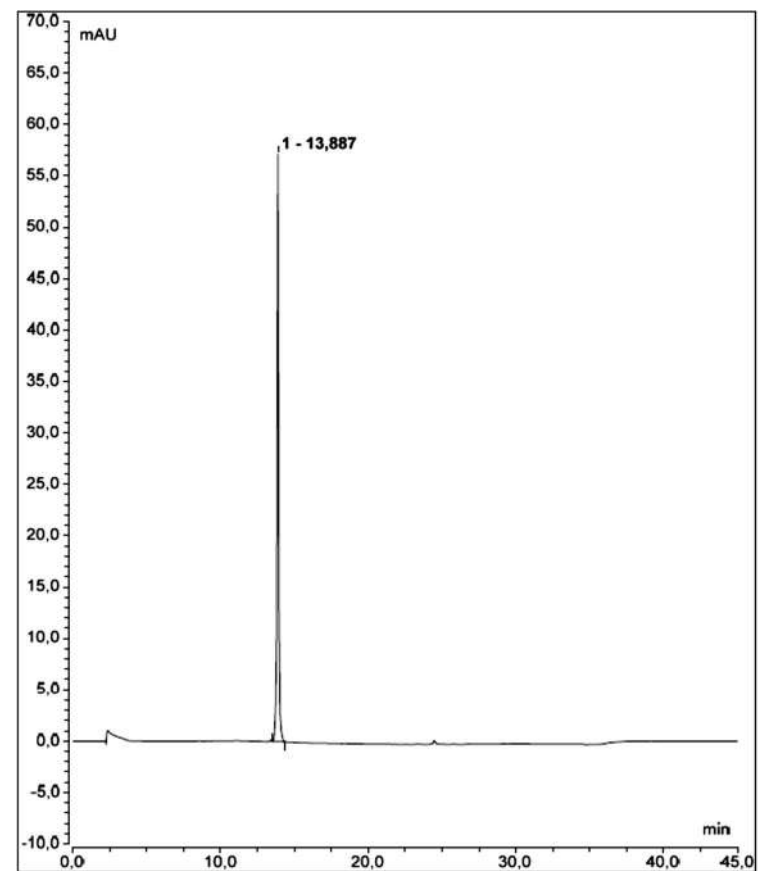


Figure S31. ^1H NMR spectrum
(DMSO- d_6 , 500 MHz)

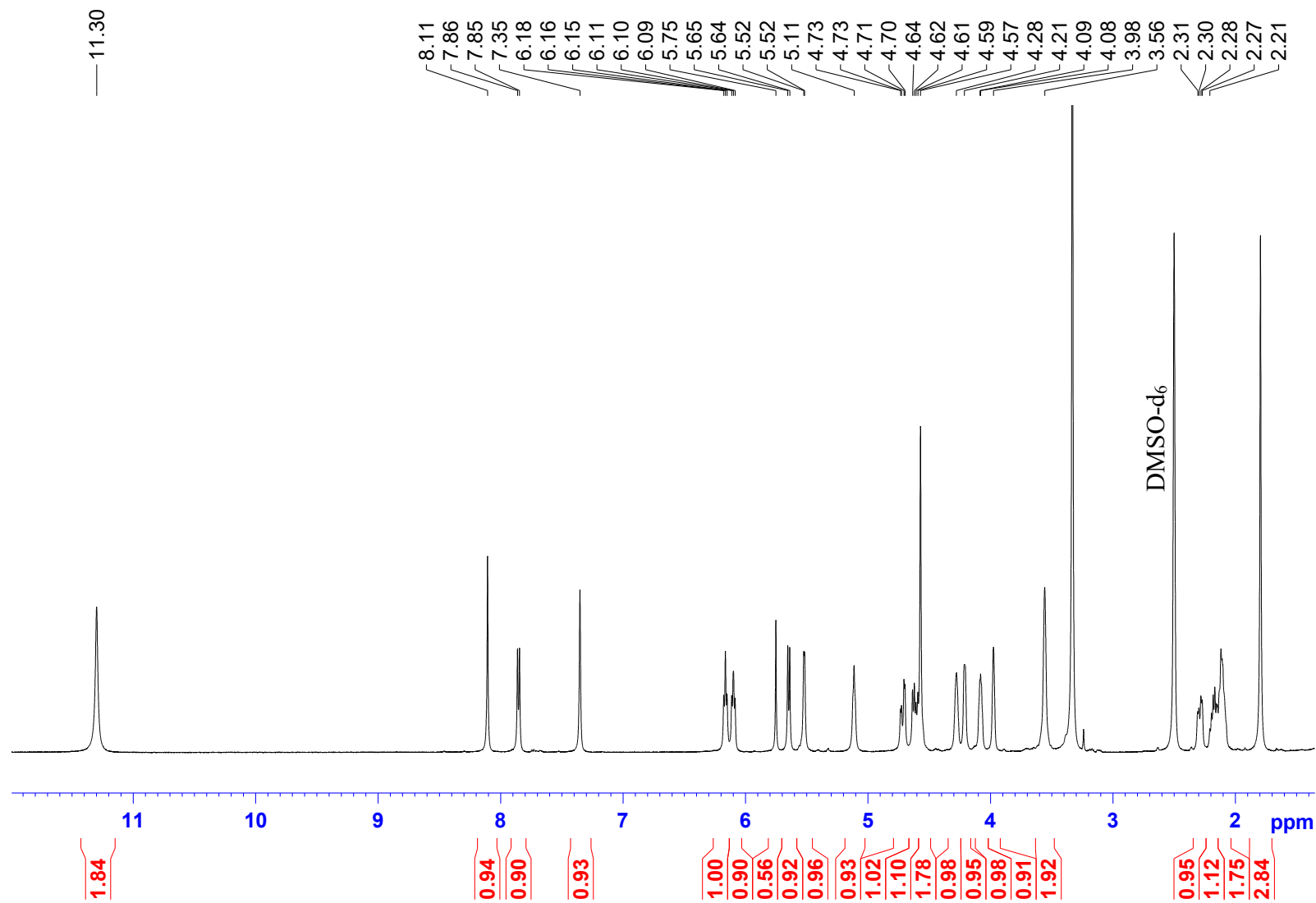
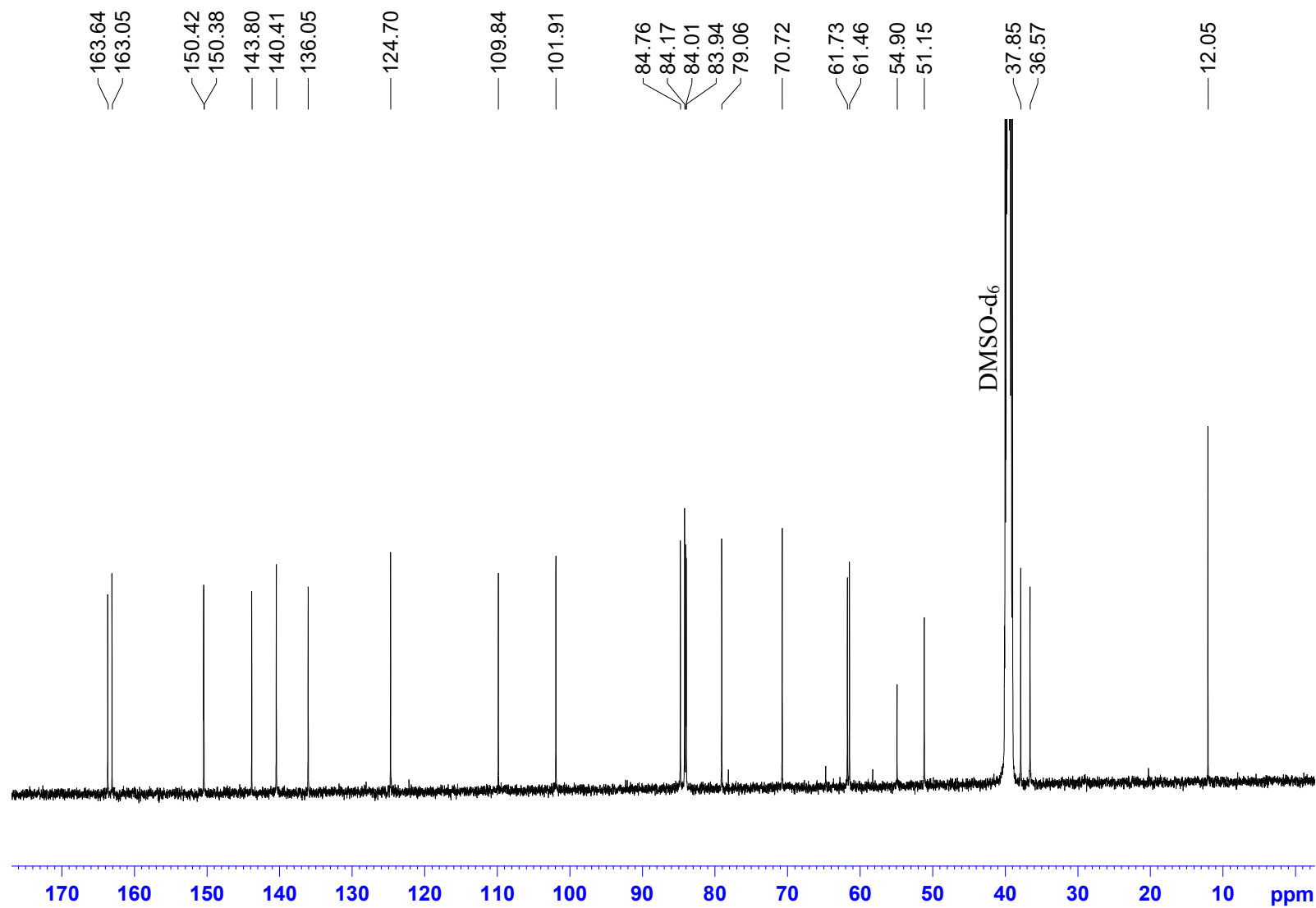


Figure S32. ^{13}C NMR spectrum
(DMSO- d_6 , 125 MHz)



Compound 4 c)

Figure S33. Chromatogram

Lichrospher RP-18 endcapped
(5.0 μm , 4.6 mm x 150 mm)

Thermo Scientific HPLC systems
with A + B solvent (A, 0.01 M
aqueous triethylammonium acetate
pH 7; B, A / acetonitrile, 1: 4, v/v)
at 35°C, flow rate 1.5 mL/min;
events: 5 min A 100%, 20 min
B 0–100%, 5 min B 100% and
15 min A 100%.

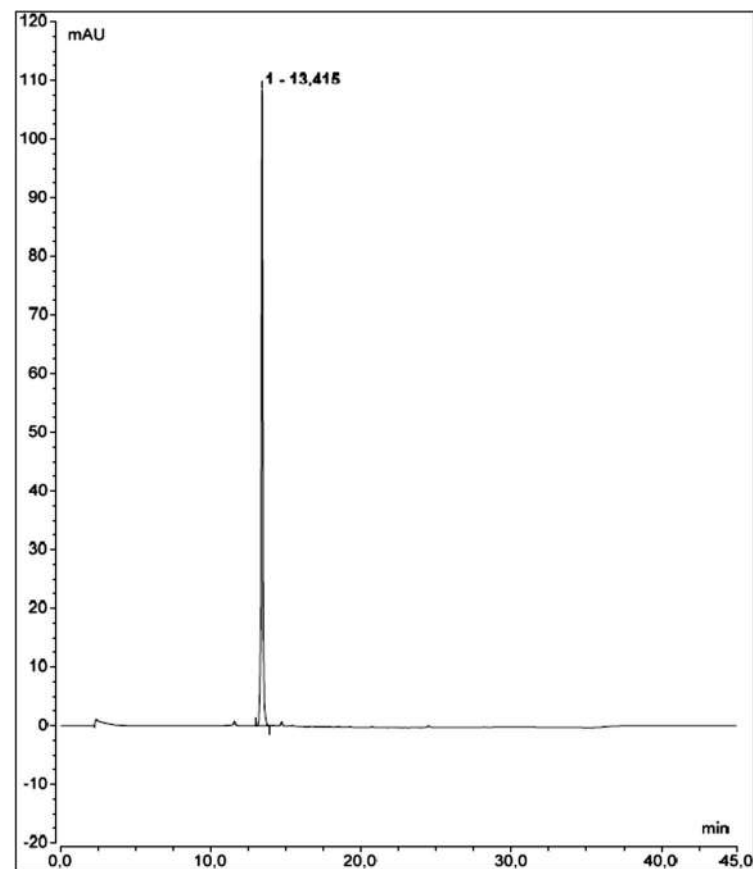


Figure S34. ^1H NMR spectrum
(DMSO- d_6 , 500 MHz)

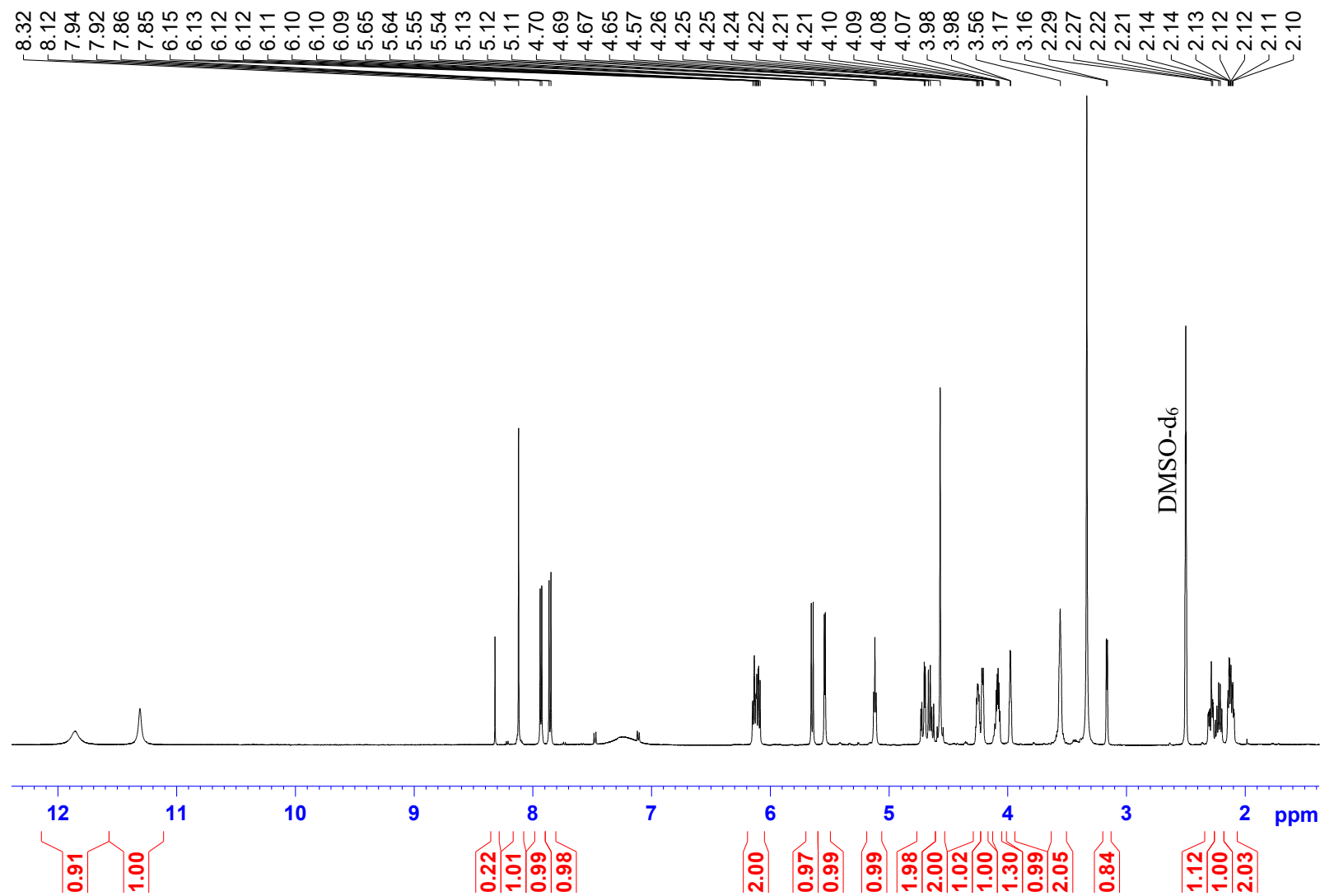


Figure S35. ^{13}C NMR spectrum
(DMSO- d_6 , 125 MHz)

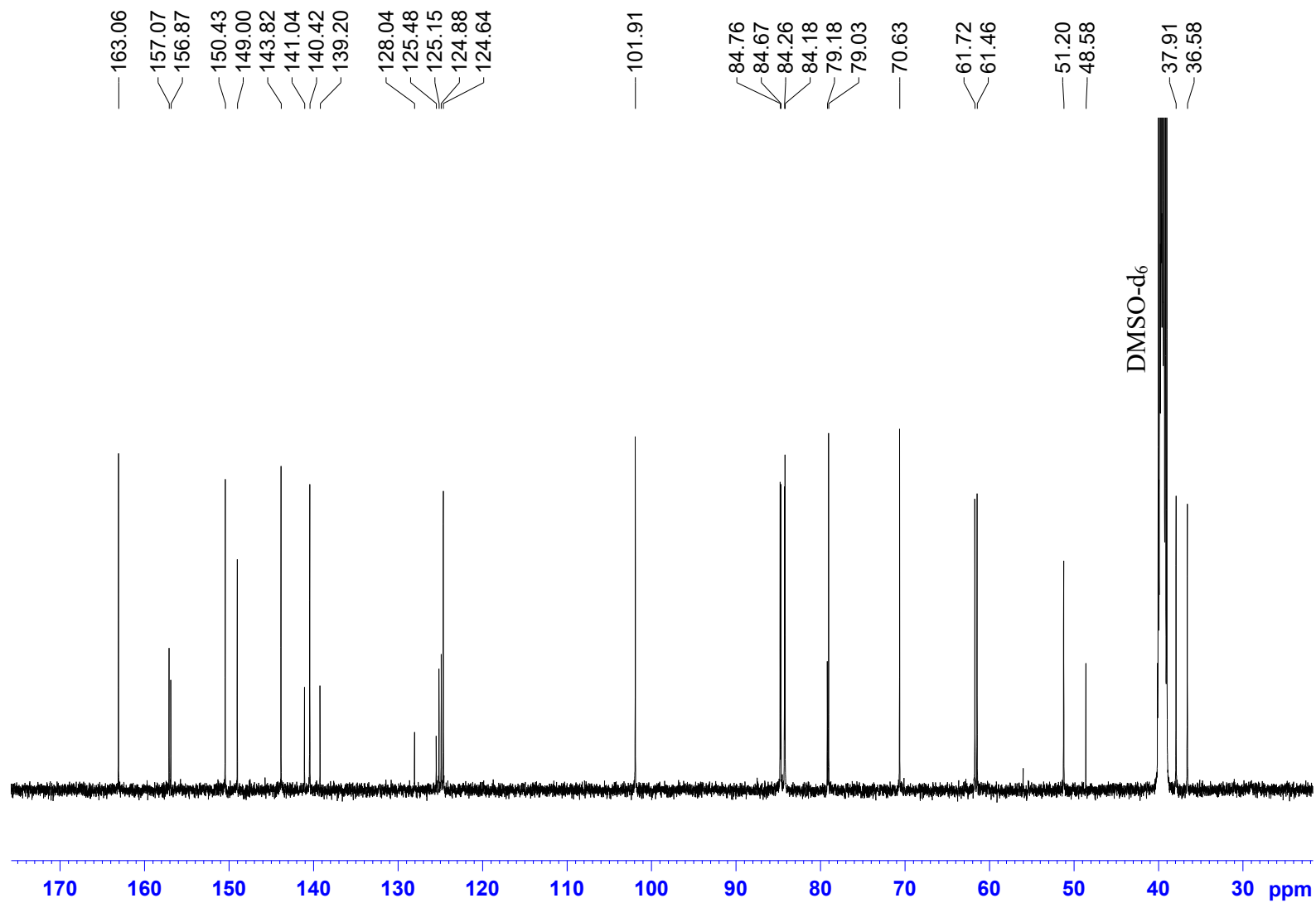
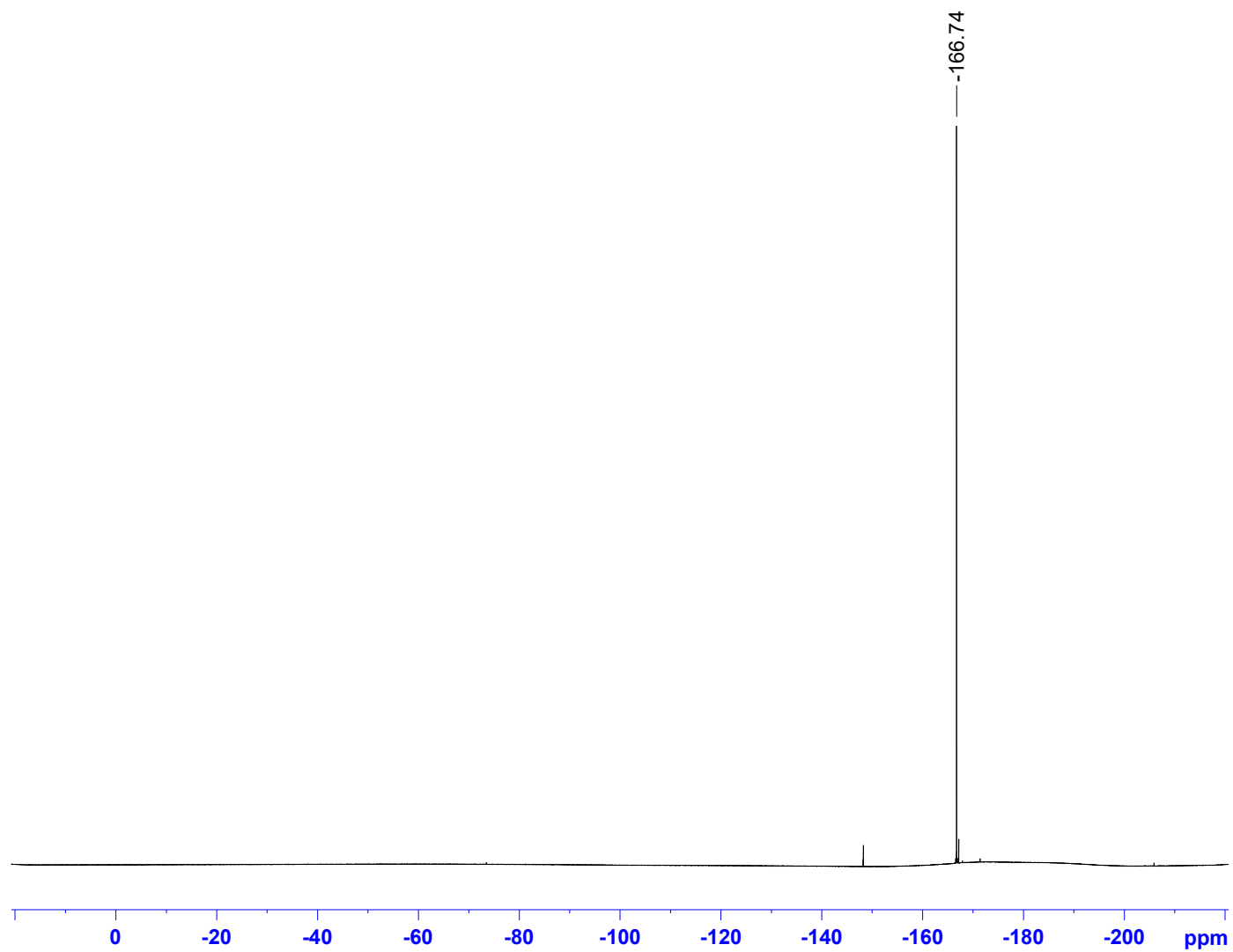


Figure S36. ^{19}F NMR spectrum (dec)
(DMSO- d_6 , 470 MHz)



4. General biological methods

4.1. Cell line and culture conditions

GBM cell lines (U-118 MG, U-87 MG, T98G), HeLa (cervical cancer cell line), HepG2 (liver cancer cell line), and non-cancerous lung fibroblast cell line (MRC-5) were purchased from ATCC (Manassas, USA). All cell lines are of human origin. HeLa cells were cultured in RPMI 1640 medium. U-118 MG cells were cultured in DMEM medium. HepG2, U-87 MG, T98G and MRC-5 cells were cultured in EMEM medium. Each medium was supplemented with 10% fetal bovine serum (FBS) and 10 mg/mL antibiotics (penicillin and streptomycin). Cells were cultured at 37°C with 5% CO₂ in humidified air. Cell media and other chemicals were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and ATCC. Cell concentrations in culture were adjusted to allow for exponential growth.

4.2. *In vitro* cytotoxicity assay

The protein-staining sulforhodamine B (SRB, Sigma–Aldrich) microculture colorimetric assay, developed by the National Cancer Institute (USA) for *in vitro* antitumor screening was used in this study, to estimate the cell number by providing a sensitive index of total cellular protein content, being linear to cell density [34]. The monolayer cell culture was trypsinized and counted. To each well of the 96-well plate, 100 µL of the diluted cell suspension (1×10^4 cells) was added. After 24 hours, when a partial monolayer was formed, 100 µL of fresh medium with different compound concentrations (7.81, 15.625, 31.25, 62.5, 125, 250, 500 and 1000 µg/mL) were added to the wells. The cells were exposed to compounds for 72 h at 37°C in a humidified atmosphere (90% RH) containing 5% CO₂. After that, 100 µL of 10% trichloroacetic acid was added to the wells and the plates were incubated for 1 h at 4°C. The plates were then washed out with the distilled water to remove traces of medium and next dried by the air. The air-dried plates were stained with 100 µL of 0.057% sulforhodamine B (prepared in 1% acetic acid) and kept for 30 min at room temperature. The unbound dye was removed by washing five times with 1% acetic acid and then the plates were air dried overnight. The protein-bound dye was dissolved in 200 µL of 10 mM unbuffered Tris base (pH 10.5) for optical density determination at 510 nm. All cytotoxicity experiments were performed three times. Cell survival was measured as the percentage absorbance compared to the control (non-treated cells). The results were calculated as an IC₅₀ (inhibitory concentration 50) – the IC₅₀ corresponds to the concentration of tested compound that inhibits cell viability by 50%.

4.3. *In silico* pharmacokinetic prediction

Calculations of pharmacokinetic profile descriptors of synthesized compounds were performed by various software solutions accessible on-line. The transformation of the stoichiometric formulas of the compounds into a SMILES code (Simplified Molecular Input Line Entry System) was carried out by ChemBioDraw Ultra version 12.0 program (Cambridge Software). The SMILES code was applied to calculate logP values (octanol/water partition coefficient) in eight variants (ALOGPs, AC_logP, miLogP, ALOGP, MLOGP, LogKOWWIN, XLOGP2, XLOGP3), PSA (topological polar surface area) and aPSA (apolar surface area). The logP values were calculated by ALOGPS 2.1 software (<http://www.vcclab.org/lab/alogps>) [35]. PSA and aPSA descriptors were calculated using the VEGA ZZ program (<http://www.vegazz.net>) [36]. The pharmacokinetic profile was also evaluated according to the Lipinski's "rule of five" [30] by using Molinspiration application (<http://www.molinspiration.com>), which analyses molecular weight (MW), number of hydrogen-bond acceptors (HBA) and number of hydrogen-bond donors (HBD). The Caco-2 prediction model based on descriptors generated by preADMET (<http://preadmet.bmdrc.org>) was used to compute Caco-2 apparent permeability (tP_{app}) for the tested compounds. In this model a number of hydrogen bond donors and three molecular surface area properties determine membrane permeability of compounds.

4.4. Physicochemical data and drug-likeness

Table 2. Selected physicochemical data of 5-FdU and compounds **1a–4c**

Compd	MW	PSA ^a [Å ²]	aPSA ^b [Å ²]	HBD ^c	HBA ^d	LogP ^e	tP _{app} [nm/s] ^f	Violations of „rule of five”
1a	253.21	160.4	125.2	2	9	-1.17	17.16	0
1b	267.24	153.1	144.6	2	9	-0.8	18.29	0
1c	271.21	156.4	136.9	2	9	-0.88	14.28	0
2a	380.18	51.0	278.6	1	7	2.02	23.92	0
2b	266.09	106.0	173.7	2	7	-0.79	13.90	0
3a	633.73	132.8	319.6	3	16	0.68	5.48	2
3b	647.75	125.5	338.9	3	16	1.01	6.85	2
3c	651.71	128.8	331.3	3	16	0.91	5.12	2
4a	519.46	187.9	214.7	4	16	-2.08	16.26	2
4b	533.49	180.6	234.1	4	16	-1.72	16.85	2
4c	537.45	183.9	226.4	4	16	-1.84	16.46	2
5-FdU	246.19	129.2	136.9	3	7	-1.28	20.62	0

^a PSA – polar surface area

^b aPSA – a polar surface area

^c HBD – hydrogen bonds donors

^d HBA – hydrogen bonds acceptors

^e logP – calculated with ALOGPS 2.1 software (wide Experimental)

^f tP_{app} – apparent theoretical permeability

4

Framski G, Wawrzyniak D, Jahnz-Wechmann Z, Szymańska-Michalak A, Kraszewski A,
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“Searching for anti-glioma activity. Ribonucleoside analogues with modifications in
nucleobase and sugar moieties”

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Searching for anti-glioma activity. Ribonucleoside analogues with modifications in nucleobase and sugar moieties

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Several ribonucleoside analogues with modifications in the nucleobase and sugar moiety have been screened for anti-glioma activity in the T98G glioma cell line using cervical (HeLa) cell line as reference human malignant cells, and lung fibroblast (MRC-5) cell line as non-cancerous reference cells. Among the investigated compounds, ribonucleosides containing 6-chloropurine (3), 7-guanine (5) and a pyrrolopyrimidine (18) as nucleobases, show promising anti-glioma activity with good selectivity indices, and can be considered as lead structures for further anti-cancer studies.

Key words: ribonucleoside analogues, anti-glioblastoma activity, anticancer ribonucleotides

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INTRODUCTION

Glioblastoma or grade IV astrocytoma, known also as glioblastoma multiforme (GBM), belongs to the most devastating brain cancers that is characterized by high malignancy and poor clinical outcome. This cancer invades the glial cells of the brain and often is malignant by the time of diagnosis. A conventional treatment of this type of cancer involves surgical resection, followed by chemotherapy and radiation therapy (Gallego, 2015). Unfortunately, this does little to improve life expectancy since the cancer usually recurs, and the length of survival after diagnosis is typically about 12 months.

Treatment of primary brain tumors is a very difficult task for several reasons. The most important are: (i) the tumor cells are resistant to conventional anticancer therapies, (ii) the brain is particularly prone to damages imparted by such interventions, (iii) the brain has a poor capacity to repair itself from the damage inflicted by chemical or radiation therapy, and finally, (iv) many drugs cannot surmount the blood-brain barrier to reach the targeted cancerous cells (Lawson *et al.*, 2007).

To overcome these inherent problems of the GBM anticancer therapy, alternative approaches were explored. The most prominent among them are those making use of the body's immune system to eradicate malignant cells (anticancer vaccine immunotherapies) (Xu *et al.*, 2014; Yang *et al.*, 2006), and those based on nucleic acid technologies (Catuogno *et al.*, 2012). Immunotherapy is appealing due to its potential of high tumor specificity, which is particularly important for brain cancers (Prins *et al.*, 2011). Some of the anti-GBM vaccines are at present in phase III clinical trials (NTC00045968) (Xu *et al.*, 2014). As to nucleic acids as potential therapeutics,

the most promising in anti-glioma therapy is a sequence-specific inhibition of RNA expressed in tumor cells by interference RNA intervention (iRNAi) (Catuogno *et al.*, 2012; Piwecka *et al.*, 2011; Rolle *et al.*, 2010). Preliminary results using this technique had shown a significant improvement in the overall survival without compromising the quality of life of the patients (Rolle *et al.*, 2010).

Apart from these, there are numerous experimental therapies investigated that involve targeting various molecules expressed in GBM in the form of inhibitors, alkylating agents, or antibodies (Agnihotri *et al.*, 2013; Chen *et al.*, 2014).

As part of our interest in the development of new anticancer agents, we recently investigated various 3'-O-acyl-5-fluoro-2'-deoxyuridine derivatives as potential drugs (Szymanska-Michalak *et al.*, 2016). Although some of them displayed higher cytotoxicity against GBM of the T98G cell line than the parent 5-fluoro-2'-deoxyuridine, their SI selectivity indices were rather poor (< 5). Since until now the only preferred therapy against GBM is surgery, followed by a chemistry supported radiation (notably, with temozolomide as a sensitizing agent) (Neidle & Thurston, 2005; Stupp *et al.*, 2005), we have been searching for compounds with increased selective toxicity that could act *per se* as anticancer agents against this type of brain tumor. In this paper we report our results on screening of several ribonucleoside analogues with diverse structural features as potential low molecular weight selective cytotoxic agent against GBM.

MATERIALS AND METHODS

Reagents. Adenosine (1), 6-chloro-9-(β -D-ribofuranosyl)purine (3), inosine (6), and cytidine (10) were of commercial grades obtained from Sigma. Other ribonucleosides used in this study were prepared according to the published procedures. Specifically, 7-(β -D-ribofuranosyl)adenine (2) and 1-(β -D-ribofuranosyl)adenine (7) were obtained as kinetic products in the ribosylation of adenine (Framski *et al.*, 2006); 6-methyl-9-(β -D-ribofuranosyl)purine (4) and 7-(β -D-ribofuranosyl)guanine (5), *via* transglycosylation of inosine (Boryski, 1998) and guanosine (Boryski, 2008), respectively; 1-(β -ribofuranosyl)indazole (8) and 2-(β -D-ribofuranosyl)indazole (9) *via* direct ribosylation of indazole (Boryski, 1995); 5-azacytidine (11), *via* coupling of the silylated 5-azacytosine with peracetylated ribofuranose accord-

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Abbreviations: GMB, glioblastoma multiforme; MRC-5, lung fibroblast cell; T98G, glioblastoma cell line

ing to the Vorbrüggen method (Vorbrüggen & Bennua, 1978); 1-(β -D-ribofuranosyl)cyanuric acid (**12**), from silylated cyanuric acid and 1-O-acetyl-2,3,5-tri-O-benzoyl-D-ribofuranose (Khaled *et al.*, 2004); 6-methyluridine (**13**), by Lewis acid catalyzed condensation of a silylated 6-methyl-4-methylthiouracyl with suitably protected ribofuranose (Felczak *et al.*, 1996); 2'-C- β -methyl-D-cytidine (**14**), 5-aza-2'-C- β -methyl-D-cytidine (**15**), 5-fluoro-2'-C- β -methyl-D-cytidine (**16**), and 2'-C- β -methyl-D-guanosine (**17**), were obtained by ribosylation of cytosine, its analogues, or guanine with 1,2,3,5-tetra-O-benzoyl-2'-C-methyl- β -D-ribofuranose (Fogt *et al.*, 2008). The series of furano[2,3-*d*]pyrimidine ribonucleoside derivatives (**18**, **19**, and **20**) were synthesized by the Pd-catalyzed cross-coupling of 5-iodouridine with appropriate alkyne derivatives (Jahnz-Wechmann *et al.*, 2015; McGuigan *et al.*, 2001; Tolstikov *et al.*, 1993), and the pyrrolo[2,3-*d*]pyrimidine ribonucleoside derivatives (**21-26**), by the ammonia treatment of the corresponding furanopyrimidine nucleoside precursors (Diez-Torrubia *et al.*, 2011; Januszczak *et al.*, 2009).

Biological assays. Cell line and culture conditions. Glioblastoma cell line (T98G), cervical cancer cell line (HeLa), and non-cancerous lung fibroblast cell line (MRC-5), were purchased from ATCC (Manassas, USA). All cell lines were of human origin. HeLa were cultured in RPMI 1640 medium, and T98G as well as MRC-5, were cultured in EMEM medium. Each medium was supplemented with 10% (v/v) foetal bovine serum (FBS) and 10 mg/mL antibiotics (penicillin and streptomycin). Cells were cultured at 37°C with 5% CO₂ in humidified air. Cell media (RPMI 1640 and EMEM) were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and ATCC. Cell concentrations in the culture were adjusted to allow for an exponential growth.

Cell viability/proliferation assays. The assays were performed essentially as described in our recent paper (Szymanska-Michalak *et al.*, 2016). Cell viability/proliferation was evaluated by a dye staining method using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT). The protocol was adapted from methods found in the literature (Xavier *et al.*, 2011). The monolayer cell culture was trypsinized and counted. To each well of the 96-well plate, 100 μ L of the diluted cell suspension (1×10^4 cells) was added. After 24 hours, when a partial monolayer was formed, 100 μ L of the fresh medium with different compound concentrations (7.81, 15.625, 31.25, 62.5, 125, 250, 500 and 1000 μ g/mL) were added to the cells. After 48 hours, the supernatant was washed out and 100 μ L of MTT solution in the medium (final concentration 0.5 mg/mL) were added to each well for 2 h. After the incubation time was complete, unreacted dye was removed by aspiration. The formazan crystals were dissolved in 100 μ L/well DMSO and measured spectrophotometrically in a multi-well Synergy2 plate reader (BioTek Instruments, USA) at a test wavelength of 492 nm and a reference wavelength of 690 nm. The half-maximal inhibitory concentrations (IC₅₀) were calculated by fitting experimental values to sigmoidal bell-shaped equation using GraphPad Prism v5.01 (GraphPad Software, Inc., USA). Results are presented as mean of at least three independent experiments.

RESULTS AND DISCUSSION

Since most studies until now show no benefit from the addition of chemotherapy in treatment of GBM

(Stupp *et al.*, 2005), we searched for low molecular weight compounds with increased selective cytotoxicity against GBM that could constitute a lead structure for further developments. To this end we selected four groups of ribonucleoside analogues with diverse structural and conformational features, and estimated their effect on the T98G glioblastoma cell line and HeLa as standard human malignant cells. The compounds investigated were either ribonucleoside analogues with known antiviral/anticancer properties or those being subjects of recent investigations in this laboratory. As a reference of non-cancerous cells, lung fibroblast (MRC-5) cells were used.

Purine nucleoside analogues

Adenosine receptors (ARs) emerged in the last three decades as important targets for drug development (Baraldi *et al.*, 2008). Apart from adenosine, the natural ligand for these receptors, other nucleosides, *e.g.* inosine, were found to be potent triggers of these receptors (Baraldi *et al.*, 2008; Gomez and Sitkovsky, 2003). Of particular interest in the context of the present study was the finding that activation of ARs is implicated in inhibition of tumor growth both *in vitro* and *in vivo* (Merighi *et al.*, 2003). As potential modulators of the ARs' activity, or other unknown biological targets, nine ribonucleosides with diverse purine base modifications were selected from our nucleoside library.

Anticancer drugs should not only have potent activity at low concentrations but also should exhibit a high degree of selectivity. Therefore, all studied ribonucleoside analogues were also tested for their anti-proliferative activity against non-cancerous lung fibroblasts (MRC-5). In the selection of the least toxic compounds among the most active ones, a special parameter, known as Selectivity Index (SI) was applied (Badisa *et al.*, 2009). The *in vitro* selectivity index of a drug is defined as the ratio of the toxic dose to the therapeutic dose ($SI = IC_{50} \text{ non-tumor cell line} / IC_{50} \text{ tumor cell line}$) and is a selectivity indicator of the tested compounds towards tumor cells.

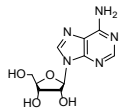
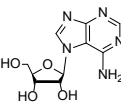
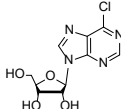
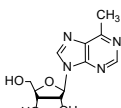
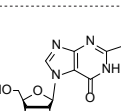
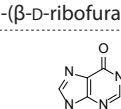
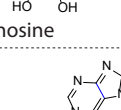
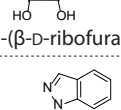
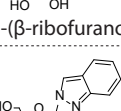
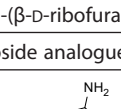
The screening experiments showed that most of the investigated ribonucleoside **1-9**, with a notable exception of 6-methyl-9-(β -D-ribofuranosyl)purine (**4**), were not cytotoxic against the non-cancerous MRC-5 cells (Table 1). 6-Methylpurine nucleoside **4**, that is known for its potent anti-herpes simplex virus 1 activity and non-selective cytotoxicity for cancerous cells (Montgomery and Hewson, 1968; Wu *et al.*, 2010), in our screening also displayed high toxicity against both, HeLa and T98G cell lines, with rather low selectivity indices relative to the non-cancerous cells (Table 1).

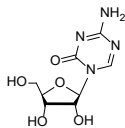
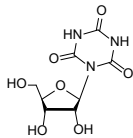
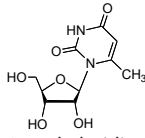
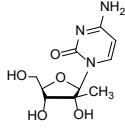
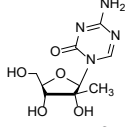
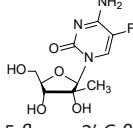
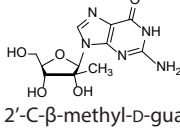
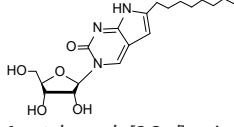
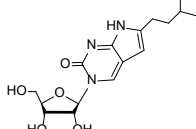
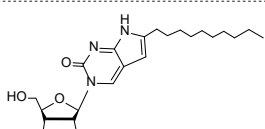
In this subgroup of the ribonucleosides investigated, a regioisomer of natural guanosine, 7-(β -D-ribofuranosyl) guanine (**5**), clearly stands out from the rest, with its high, selective cytotoxicity against glioblastoma ($IC_{50} = 1.6$) *vs* HeLa ($IC_{50} > 177$), and *vs* MRC-5 cell lines ($IC_{50} > 100$). These values translated into SI of 111 for HeLa/T98G, and SI = 64 for MRC-5/glioblastoma T98G cell lines. Also, 6-chloro-9-(β -D-ribofuranosyl)purine (**3**) displayed some selective cytotoxicity for glioblastoma *vs* MRC-5 cells, but the selectivity index was rather modest ($SI = 23$).

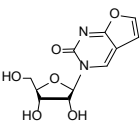
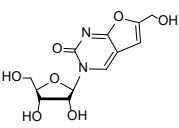
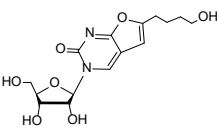
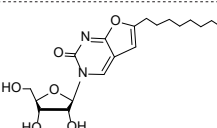
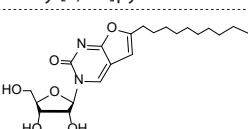
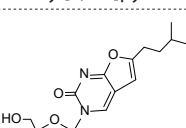
Pyrimidine nucleoside analogues

The pyrimidine ring, apart from being a part of natural nucleosides, constitutes an important pharmacophore endowed with drug like properties (Radi *et al.*, 2009), and when appended with a sugar moiety, may show a wide

Table 1. Cytotoxic activity of selected classes of ribonucleoside analogues*

Cmpd	Structure	IC ₅₀ [μM] (MTT)		
		HeLa	T98G	MRC-5
Purine nucleoside analogues				
1	 adenosine	>187 SI =1.0	110 SI =1.7	>187
2	 7-(β-D-ribofuranosyl)adenine	>187 SI =1.0	41 SI =4.6	>187
3	 6-chloro-9-(β-D-ribofuranosyl)purine	29.3 SI =5.8	7.32 SI =23	>170
4	 6-methyl-9-(β-D-ribofuranosyl)purine	0.65 SI =5.1	0.83 SI =4.0	3.3
5	 7-(β-D-ribofuranosyl)guanine	>177 SI =0.6	1.6 SI =63	>100
6	 inosine	>180 SI =1.0	95 SI =1.9	>180
7	 1-(β-D-ribofuranosyl)adenine	>180 SI =0.6	>180 SI =0.6	<100
8	 1-(β-ribofuranosyl)indazole	101 SI =2.0	58.1 SI =3.4	>199
9	 2-(β-D-ribofuranosyl)indazole	27.6 SI =1.5	52.8 SI =0.8	42.3
Pyrimidine nucleoside analogues				
10	 cytidine	130 SI =1.1	138 SI =1.0	138

11		15.89 SI =1.4	12.48 SI =1.8	22.65
12		> 191 SI =1.0	48 SI =4.0	> 191
13		>190 SI =1.0	138 SI =1.4	>190
Pyrimidine 2'-C-methyl nucleoside analogues				
14		60.91 SI =0.8	76.31 SI =0.7	49.62
15		>190 SI =1.0	>190 SI =1.0	>190
16		>180 SI =1.0	>180 SI =1.0	>180
17		>170 SI =1.0	>170 SI =1.0	>170
Pyrimidine bicyclo nucleoside analogues				
18		17.8 SI =7.3	0.75 SI =173	>130
19		9.79 SI =4.1	14.31 SI =2.8	40.18
20		18.58 SI =1.3	33.07 SI =0.7	23.64

21		48.6 SI = 2.9	187 SI = 0.8	143.3
22		>100 SI = 1.0	>100 SI = 1.0	>100
23		96.68 SI = 1.0	>100 SI = 1.0	>100
24		80.9 SI = 0.9	20.01 SI = 3.7	74.83
25		17.15 SI = 1.1	29.71 SI = 0.7	19.46
26		>100 SI = 1.0	>100 SI = 1.0	>100

*HeLa, cervical cancer cell line; T98G, glioblastoma cancer cell line; MCR-5, non-cancerous lung fibroblast cell line; IC_{50} , concentration of the compound that inhibits cells proliferation by 50%; SI, selectivity index; IC_{50} non-tumor cell line/ IC_{50} tumor cell line.

range of pharmacological activities (Walker *et al.*, 1979). For this study, we chose 5-azacytidine (**11**) and natural cytidine (**10**) as a reference, and two ribonucleosides with a non-typical syn conformation around the glycosidic bond, namely, 1-(β -D-ribofuranosyl)cyanuric acid (**12**) (Khaled *et al.*, 2004) and 6-methyluridine (**13**) (Felczak *et al.*, 1996).

5-Azacytidine, which is an US FDA approved drug (Vidaza, Pharmion; inhibitor of DNA methylation) for treatment of the myelodysplastic syndrome (MDS) (Issa *et al.*, 2005), is an analogue of cytidine in which carbon-5 in the heterocyclic ring is replaced by nitrogen. This changes the number and pattern of the hydrogen-acceptor centres when compared to cytidine **10**, and may be responsible for its known general cytotoxicity. A possible role of DNA methylation in cancer therapy (Christman, 2001) prompted us to include 5-azacytidine **11** in our screening experiments.

Among the four investigated pyrimidine ribonucleoside analogues (Table 1), only 5-azacytidine **11** was cytotoxic, but with practically no selectivity against cancerous and non-cancerous cell lines. Slightly better selective toxicity against T98G was displayed by cyanuric acid nucleoside **12**, but the SI index was still low (SI = 4).

Pyrimidine 2'-C-methylnucleoside analogues

This class of analogues retains the skeleton of natural ribonucleosides, but possesses a β -methyl substituent at the 2'-position of the D-ribose moiety. This 2'-C-methyl branching structural element (Walton *et al.*, 1969) transforms natural ribonucleosides into potent, broad-spectrum antiviral species (Benzaria *et al.*, 2007). For our studies, we selected 2'-C- β -methyl-D-cytidine (**14**), 5-aza-2'-C- β -methyl-D-cytidine (**15**), 5-fluoro-2'-C- β -methyl-D-cytidine (**16**), and 2'-C- β -methyl-D-guanosine (**17**).

As for toxicity of the investigated compounds **14-17**, only cytidine derivative **14** displayed a moderate, non-selective cytotoxicity ($IC_{50} > 50$, Table 1), while the rest was essentially non-toxic. From comparison with the group of pyrimidine ribonucleoside analogues (*vide supra*), it seems apparent that the presence of 2'-C-methyl group in the sugar moiety completely suppressed toxicity of 5-azacytidine (**15** *vs* **11**), but noticeably increased cytotoxicity of cytidine (**14** *vs* **10**).

Pyrimidine bicyclo nucleoside analogues

Addition of a furano or pyrrolo fused ring structure to pyrimidine nucleosides transformed these compounds into fluorescent, potent antiviral agents (Carangio *et al.*,

2001; McGuigan *et al.*, 2001). Properties of the bicyclic, furano-pyrimidine and pyrrolo-pyrimidine, pharmacophores have been investigated mainly for deoxyribonucleoside (Carangio *et al.*, 2001; McGuigan *et al.*, 2001), and to lesser extent for ribonucleoside (Jahnz-Wechmann *et al.*, 2015; Koh *et al.*, 2007), or dideoxy derivatives (McGuigan *et al.*, 2013).

Selected examples of this type of nucleoside analogues in our investigated series included: three lipophilic pyrrolo derivatives [6-octyl- (18), 6-(2-methylbutyl)- (19), and 6-decylpyrrolo [2,3-*d*]pyrimidine ribonucleosides (20)], unsubstituted furano[2,3-*d*]pyrimidine ribonucleoside 21, 6-hydroxymethylfurano[2,3-*d*]pyrimidine ribonucleoside 22, 6-(1-hydroxybutylfurano[2,3-*d*]pyrimidine ribonucleoside 23, and three lipophilic furano derivatives [6-octyl- (24), 6-decyl- (25), and 6-(2-methylbutylpyrrolo[2,3-*d*]pyrimidine ribonucleosides (26)].

Although most of the investigated compounds in this subgroup were not cytotoxic or displayed a moderate to low, not selective cytotoxicity, the 6-octylpyrrolo derivative 18 clearly stood out with its high selective cytotoxicity against the T98G glioblastoma cells ($IC_{50} = 0.74$), and high selectivity index, $IC_{50} \text{ MRC-5} / IC_{50} \text{ T98G}$, $SI = 173$.

It seems that the pyrrolo bicyclo nucleosides in this series are on average more cytotoxic than the furano derivatives, however, a pairwise comparison, *e.g.* 18 *vs* 24, 19 *vs* 26, 20 *vs* 26, does not support this notion.

CONCLUDING REMARKS

We have screened selected compounds from our ribonucleoside library to find nucleosides with an anti-glioma activity. Three of them, namely 6-chloro-9-(β -D-ribofuranosyl)purine (3), 7-(β -D-ribofuranosyl)guanine (5), and 6-octyl-furano[2,3-*d*]pyrimidine ribonucleoside (18) were found to have appreciable selective cytotoxicity against T98G glioblastoma when compared to non-cancerous MRC-5 cells, and also HeLa human cancer cell line, and deserve some further studies as possible lead structures for development of an anti-glioma drug.

With the growing understanding of molecular alterations and complexity found in gliomas (Bleeker *et al.*, 2012; Zhang *et al.*, 2012) it seems, however, that a single-agent therapy in treatment of this kind of cancer will be insufficient; rather, a multi drug combination treatment will be necessary. This calls for further searching for anti-glioma activity in structurally diverse classes of compounds that could selectively interact with therapeutically important molecular targets revealed by the GBM cancer.

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Wawrzyniak D, Rolle K, Barciszewski J
“Aktywność biologiczna N⁶-furfuryloadenozyny”
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STRESZCZENIE

Cytokiny stanowią jedną z grup hormonów roślinnych, które odgrywają ważną rolę we wzroście i rozwoju roślin. Wykazują różne efekty działania, szczególnie stymulują syntezę białek i uczestniczą w kontroli cyklu komórkowego. Pierwszą odkrytą cytokiną była N⁶-furfuryloadenina (kinetyna). Jest ona uważana za silny inhibitor procesów utleniania białek i kwasów nukleinowych w warunkach *in vitro* oraz *in vivo*. Zarówno kinetyna, jak i jej rybozyd (N⁶-furfuryloadenozyna, rybozyd kinetyny) jako naturalne związki występują w mleku kokosowym w stężeniu nanomolarnym. Rybozyd kinetyny selektywnie hamuje proliferację komórek nowotworowych oraz indukuje ich apoptozę. W tym artykule skupiono się na występowaniu rybozydu kinetyny, a przede wszystkim na jego metabolizmie oraz aktywności biologicznej.

WPROWADZENIE

Choroby nowotworowe są w Polsce i na świecie wraz z chorobami układu krążenia główną przyczyną przedwczesnych zgonów. Według danych epidemiologicznych z 2010 roku Polskiej Unii Onkologii i Centrum Onkologii w Warszawie prognozuje się odpowiednio wzrost zachorowań z 130 tys. do 160 tys. oraz wzrost śmiertelności z 85 tys. do 100 tys. rocznie [1]. Dane te obrazują skalę problemu i wskazują na konieczność poszukiwania nowych terapeutyków. Aktualne zainteresowania koncentrują się wokół nowych analogów obecnie stosowanych leków przeciwnowotworowych, małych związków chemicznych pochodzenia naturalnego oraz cząsteczek działających selektywnie na komórki nowotworowe i nie wpływających na komórki prawidłowe organizmu [2]. Wśród cząsteczek o wysokiej aktywności biologicznej i znacznym potencjale terapeutycznym znajdują się hormony roślinne (cytokiny), które są pochodnymi adeniny modyfikowanymi w pozycji N⁶ podstawnikami izoprenoidowymi bądź aromatycznymi.

Rolą cytokin u roślin jest regulowanie wzrostu i różnicowania komórek oraz indukowanie apoptozy [3]. Prowadzone badania mają na celu określenie ich aktywności w komórkach zwierzęcych oraz wykorzystania ich w ograniczeniu zaburzeń proliferacji i różnicowania komórek.

AKTYWNOŚĆ FARMAKOLOGICZNA ANALOGÓW NUKLEOZYDÓW

Modyfikowane nukleozydy purynowe i pirymidynowe stanowią bardzo różnorodną grupę związków terapeutycznych i są stosowane w leczeniu chorób nowotworowych oraz zakażeń wirusowych. Działają cytotoksycznie na komórki dzielące się oraz pozostające w fazie G₀/G₁ cyklu komórkowego, który składa się z interfazy (okres między podziałami) oraz właściwego podziału - mitozy. W interfazie wyróżnia się fazy G₁, S oraz G₂, a jej celem jest podwojenie materiału genetycznego niezbędnego dla prawidłowego przebiegu mitozy. W pierwszym etapie G₁ następuje wejście komórki w następny cykl replikacyjny i rozpoczęcie biosyntezy białek. W fazie S dochodzi do powielenia materiału genetycznego komórki i formowania chromosomów. W kolejnym etapie, G₂ następuje dalszy wzrost i synteza dodatkowych składników niezbędnych w mitozie, a także naprawa uszkodzeń DNA. Po zakończonym cyklu komórka może ponownie wejść w fazę G₁ i dokonać następnego podziału lub w G₀, która jest stanem zawieszenia aktywności podziałowej. Schematyczny przebieg cyklu komórkowego obrazuje rycina 1. Komórki w fazie syntezy DNA (S) i podziału (M) są podatne na działanie chemoterapeutyków, natomiast w stanie spoczynkowym (G₀, G₁ i G₂), wykazują oporność na aktywność wielu cytostatyków [4,5].

W komórkach proliferujących cytotoksyczność modyfikowanych nukleozydów obejmuje zahamowanie syntezy deoksyrybonukleotydów, inhibicję polimeraz DNA oraz indukowanie apoptozy na drodze zewnątrzpocho-

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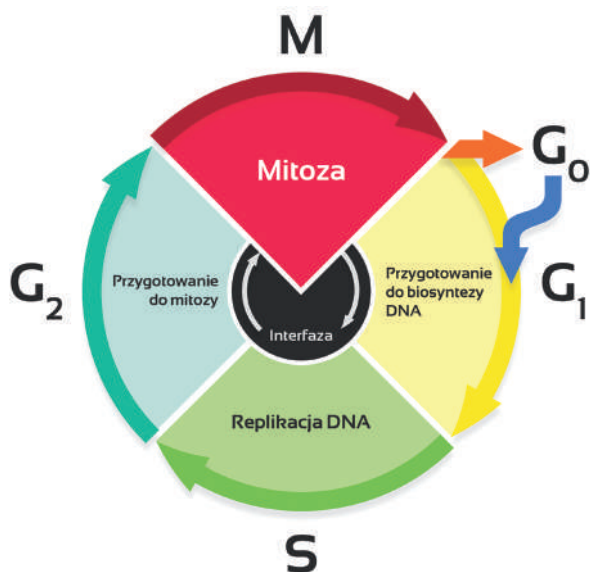
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Słowa kluczowe: cytokiny, rybozyd kinetyny, kinaza adenozy, nowotwory, stres oksydacyjny, szlaki sygnałowe

Wykaz skrótów: 8-oxo-dG – 8-oksyo-2'-deoksyguanozyna; ADA – deaminaza adenozy; AdK – kinaza adenozy; Ado – adenozy; BA – N⁶-benzyloadenina; BAR – N⁶-benzyloadenozyna; CDK – kinazy cyklinozależne; CKI – inhibitory kinaz cyklinozależnych; IC₅₀ – stężenie hamujące, dla którego wzrost i proliferacja komórek w hodowli zostają zahamowane w 50%; iP – N⁶-izopentenyloadenina; iPR – N⁶-izopentenyloadenozyna; K – kinetyna; KR – rybozyd kinetyny; LC-MS/MS – tandemowa spektrometria mas sprzężona z chromatografią ciecząową; NBTI – nitrobenzylotioinozyna; RFT – reaktywne formy tlenu.



Rycina 1. Schemat cyklu komórkowego.

lub komórkowych odpowiedzialnych za powielanie materiału genetycznego i wpływających na syntezę nukleotydów, które stanowią rdzeń cząstek wirusowych lub wchodzą w skład zmienionych genomowo komórek nowotworowych. Aby naturalny nukleozyd został włączony do syntetyzowanej nici DNA w komórce musi przejść trzystopniową fosforylację przy udziale kaskady wewnątrzkomórkowych kinaz. Podobnym przemianom metabolicznym podlegają wszystkie modyfikowane nukleozydy o działaniu przeciwnowotworowym, które dzięki właściwemu mechanizmowi molekularnemu są zdolne do indukowania programowanej śmierci komórki. Efektywność ich działania jest zróżnicowana i zależy od specyficzności enzymów metabolizujących oraz interakcji z białkami uczestniczącymi w procesach replikacji, naprawy i metylacji DNA.

AKTYWNOŚĆ PRZECIWNOWOTWOROWA ANALOGÓW NUKLEOZYDÓW

Modyfikowane nukleozydy i zasady azotowe jako leki przeciwnowotworowe były jedną z pierwszych grup cytostatyków stosowanych w onkologii. Wśród tych leków znalazły się pochodne purynowe oraz pirymidynowe, które znalazły zastosowanie zarówno w leczeniu nowotworów krwi jak i litych guzów nowotworowych. Historia badań nad nukleozydami i DNA rozpoczęła się już w 1847 r. od odkrycia pierwszego nukleotydu purynowego – 5'-fosforanu inozyny przez Justusa von Liebiga [6].

Analogi nukleozydowe purynowe, np. kladrybina, fludarabina, kłofarabina oraz pirymidynowe, np. cytarabina, 5-azacytydyna stosowane są w leczeniu układu krwiotwórczego między innymi w przewlekłej białaczce limfatycznej [7-10]. Natomiast pirymidynowy analog – gemcytabina jest obecnie stosowany w leczeniu nowotworów litych takich jak rak piersi, niedrobnokomórkowy rak płuc, a także rak pęcherza [11-13]. Analogi urydyny takie jak 5-fluoro-2'-deoksyurydyna, oraz 5-fluoro-5'-deoksyurydyna są stosowane w leczeniu rozsiaanych nowotworów przewodu pokarmowego, trzustki, prostaty, piersi i jajników [14,15]. Ponadto analogi pirymidynowe takie jak: 2',3'-dideoksycytydyna (zalcytabina), 3'-azi-

do-3'-deoksytymidyna (zydowudyna) oraz analogi inozyny np. 2',3'-dideoksyinozyna znalazły zastosowanie w terapii przeciwwirusowej [16-18]. Każdy z tych związków wykazuje zróżnicowaną aktywność w zależności od rodzaju schorzenia lub typu komórek. Dla przykładu, cytotoksyczna aktywność analogów puryny takich jak fludarabina i kladrybina w niedzieliących się komórkach wynika raczej z zaburzeń naprawy DNA niż z powodu terminacji replikacji czy wpływu na aktywność mitochondriów [19].

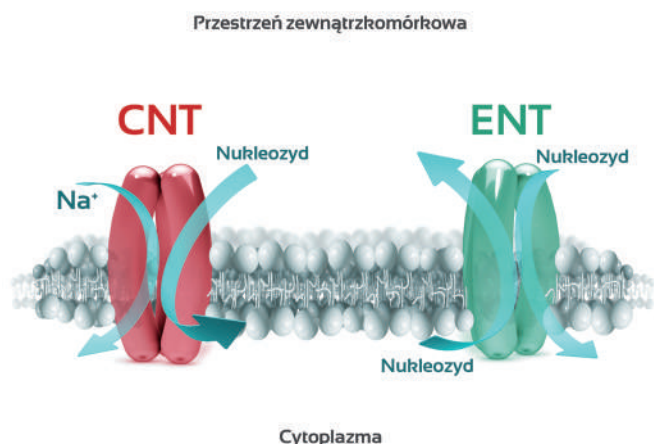
Wspólną cechą nukleozydów jest transport błonowy przy udziale białkowych transporterów, wewnątrzkomórkowa aktywacja oraz synteza nukleotydu [4]. Nukleozydy i ich analogi są związkami hydrofilowymi i aby przeniknąć do wnętrza komórki wymagają specyficznych białek transportujących. Istnieje coraz więcej dowodów, że liczebność oraz dystrybucja białkowych transporterów na powierzchni komórek jest przyczyną wrażliwości i specyficzności na analogi nukleozydów [20].

CHARAKTERYSTYKA SYSTEMÓW TRANSPORTOWYCH NUKLEOZYDÓW

Błony biologiczne (błony elementarne, błony cytoplazmatyczne, biomembrany) zbudowane są z dwuwarstwy białkowo-lipidowej i stanowią barierę przepuszczalności dla jonów i cząsteczek polarnych. Przepuszczalność jest możliwa dzięki obecności pomp i kanałów. Pompy transportują jony lub cząsteczki w kierunku wzrastającego stężenia, zużywając do tego energię w postaci wiązań wysokoenergetycznych ATP. Natomiast kanały umożliwiają szybki przepływ jonów w kierunku termodynamicznie korzystnym. Cząsteczki o charakterze hydrofilowym wymagają selektywnie działających transporterów, natomiast związki hydrofobowe przenikają na zasadzie dyfuzji [21].

Nukleozydy i ich analogi przechodzą przez błony plazmatyczne dzięki specyficznym białkom – transporterom nukleozydowym. Możemy wyróżnić transport bierny nośnikowy (ang. *sodium-independent equilibrative nucleoside transport*) lub transport drogą aktywnego symportu z jonami sodu (ang. *sodium-dependent equilibrative nucleoside transport*) [22, 23].

Pierwszy z nich, ENT (ang. *Equilibrative Nucleoside Transport system*) – transport Na^+ -niezależny, transportuje nukleozydy przez błonę komórkową w obu kierunkach na zasadzie dyfuzji ułatwionej zgodnie z gradientem stężeń (Ryc. 2). System ten dzielimy na dwa podtypy: *es* (ang. *equilibrative sensitive*) i *ei* (ang. *equilibrative insensitive*), w zależności od wrażliwości na inhibicję przez nitrobenzylotioinozynę (NBTI) [24, 25]. Zidentyfikowano cztery transportery należące do systemu Na^+ -niezależnego: ENT1, ENT2, ENT3 oraz ENT4 (Tab. 1). Transportery ENT1 i ENT3 zostały zaliczone do systemu *es* na podstawie wrażliwości na NBTI oraz ich selektywności substratowej. Natomiast ENT2, który nie jest hamowany przez ten inhibitor, należy do systemu *ei* [26]. Białka transportowe systemu ENT są szeroko rozpowszechnione w komórkach wielu typów i charakteryzują się niską selektywnością wobec przenoszonych cząsteczek [22]. Najlepiej poznane są transportery ENT1 i ENT2. Ludzkie białka ENT1 (hENT1) oraz ENT2 (hENT2), które są wrażliwe na NBTI, są ponadto hamowane przez związki



Rycina 2. Typy transporterów nukleozydowych. W systemie CNT nukleozydy są transportowane do wnętrza komórki w symporcie z jonem sodowym. Białko ENT transportują nukleozydy przez błonę komórkową w obu kierunkach zgodnie z gradientem stężeń.

rozszerzające naczynia takie jak: dipirydamol, dilazep i drafazylna [22,27].

W drugim systemie, CNT (ang. *Concentrative Nucleoside Transport system*), transport nukleozydów odbywa się tylko do wnętrza komórki w symporcie z jonem sodowym (Ryc. 2). W obrębie tego systemu wyszczególniono sześć funkcjonalnie różnych podsystemów (N1–N6), ze względu na różnice w przenoszonych substratach [24]. Dotąd poznano i opisano trzy białka transportowe systemu CNT oznaczane jako CNT1, CNT2 oraz CNT3. Transporter CNT1 jest białkiem swoście przenoszącym nukleozydy pirymidynowe, CNT2 jest specyficzny w stosunku do nukleozydów purynowych, natomiast transporter CNT3 transportuje zarówno nukleozydy pirymidynowe jak i purynowe [28–31]. Dane dotyczące poszczególnych systemów transportowych zostały zestawione w tabeli 1.

Poza rodziną transporterów ENT oraz CNT znane są inne białka transportowe, które przemieszczają zarówno nukleozydy jak i ich analogi z komórki bądź do jej wnętrza. Ze względu na mechanizmy wykorzystywane do transportu wyróżnia się następujące rodziny transporterów: MRP (ang. *multidrug resistance protein*), PEPT (ang. *peptide transporter*), OAT (ang. *organic anion transporter*) oraz OCT (ang. *organic cation transporter*) [32].

Tabela 1. Porównanie systemów transportujących nukleozydy.

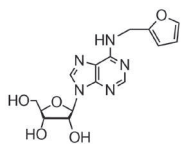
Systemy transportowe nukleozydów	Białko	Selektywność substratowa
es	ENT1	Nukleozydy purynowe, pirymidynowe
ei	ENT2	Nukleozydy purynowe, pirymidynowe i niektóre zasady
es	ENT3	Nukleozydy purynowe, pirymidynowe i zasady
?	ENT4	Nukleozydy purynowe
N1	CNT2	Nukleozydy purynowe, urydyna, formycyna B
N2	CNT1	Nukleozydy pirymidynowe
N3	CNT3	Nukleozydy purynowe, pirymidynowe
N4	–	Nukleozydy purynowe, pirymidynowe
N5	–	Analogi adenozyiny (formycyna B, fludarabina, kładrybina)
N6	–	Guanozyna

STRUKTURA CHEMICZNA CYTOKININ I ICH WYSTĘPOWANIE

Cytokiny stanowią jedną z głównych grup hormonów roślinnych. Stymulują procesy anaboliczne, cytokinezę, kariokinezę oraz tworzenie natywnej struktury białek. Ponadto hamują aktywność enzymów, które uczestniczą w degradacji kwasów nukleinowych i białek, przez co opóźniają procesy starzenia się komórek. Cytokiny są pochodnymi zasady purynowej – adeniny, w której przy N⁶ podstawiony jest najczęściej alifatyczny łańcuch izoprenowy lub podstawnik aromatyczny [33].

W roślinach najczęściej występują cytokiny z łańcuchem alifatycznym: *trans*-zeatyna, *cis*-zeatyna (N⁶-izopen-tenyloadenina), dihydrozeatyna i ich modyfikacje z podstawnikami metylowymi lub hydroksylowymi. Rzadziej spotykane są z podstawnikiem aromatycznym reprezentowanym przez N⁶-benzyloadeninę i jej hydroksylowane pochodne *m*- i *o*-topoliny. Związki te występują w mikro molarnych stężeniach w liściach topoli [34]. Do grupy syntetycznych analogów cytokin zaliczamy: N⁶-farnesyloadeninę, N⁶-geranyloadeninę oraz do 1996 r. N⁶-furfuryloadeninę (kinetynę), kiedy to Barciszewski i wsp. [35] potwierdzili jej występowanie w komercyjnie dostępnym i świeżo wyizolowanym DNA. Cytokiny występują w postaci kilku form metabolicznych: wolnych zasad, rybozydów oraz 5'-fosforanów rybozydów (rybotydów). Nukleotydy te występują w pętli antykodonu tRNA, fragmencie poliadenylowym mRNA oraz w rRNA, spełniając rolę regulacyjną w procesie translacji. Największą aktywność biologiczną wykazują cytokiny w formie wolnych zasad i połączeń rybozydowych [36].

Najlepiej poznaną cytokiną jest kinetyna, która po raz pierwszy została wyizolowana z DNA plemników śledzi w 1955 r. Jej występowanie potwierdzono, także w: komercyjnie dostępnym DNA, świeżo wyekstrahowanym z ludzkich komórek DNA oraz ekstraktach z komórek roślinnych i ludzkim moczu [35,37,38]. Kinetynę oraz jej rybozyd (Ryc. 3) również zidentyfikowano w płynnym endospermie młodych orzechów kokosowych (*Cocos nucifera* L.) za pomocą techniki LC-MS/MS. Średnie stężenia związków wynoszą odpowiednio 0,34 i 0,29 nM [39].



Rycina 3. Struktura rybozydu kinetyny (N⁶-furfuryladenozyny).

Kinetyna wykazuje właściwości antyoksydacyjne zarówno *in vitro* jak i *in vivo*. Chroni DNA komórek przed oksydacyjnymi uszkodzeniami, głównie guanozyny i gromadzeniem 8-oxo-dG [40]. Znane są jej właściwości spowalniania procesów starzenia się komórek roślinnych oraz ludzkich, m. in. fibroblastów [41–43]. Kinetyna w układzie bezkomórkowym i w wysokich stężeniach (500 nM i więcej) nie wykazuje aktywności przeciwutleniającej. W przeciwieństwie do tego, niskie dawki (stężenia do 100 nM) chronią keratynocyty Ha-CaT oraz promielocyty HL-60 przed stresem oksydacyjnym [44]. Kinetyna nie tylko wydłuża życie nicienia *Caenorhabditis elegans*, ale również zwiększa odporność na stres oksydacyjny i opóźnia proces starzenia się. Jest to działanie niezależne od czynnika transkrypcyjnego DAF-16, a zatem prawdopodobnie nie jest związane z szlakiem insulinopodobnego czynnika wzrostu. Wykazano również, że *C. elegans* przekształca kinetynę do rybozydu kinetyny oraz 5'-monofosforanu rybozydu kinetyny [45]. Kinetyna nie tylko odwraca alternatywny splicing pre-mRNA genu białkowego związanego z kinazą IκB (IKBKAP), który jest odpowiedzialny za tworzenie się rodzinnej dysautonomii (FD) w modelu komórkowym [46–48], ale również w warunkach *in vivo* [49,50]. Kinetyna podawana przez 8 dni 29 zdrowym nosicielom głównej mutacji FD w dawce 23,5 mg/kg/dzień zwiększała włączanie egzonu 20 z endogennego genu. Istniała liniowa zależność między poziomem kinetyny w osoczu, a odpowiadającą zmianą logarytmiczną w ekspresji mRNA IKBKAP [49].

ANTYPROLIFERACYJNA AKTYWNOŚĆ N⁶-POCHODNYCH ADENOZYN

Naturalnie występujące analogi nukleozydów takie jak: N⁶-izopentenyloadenozyna (iPR), rybozyd kinetyny (KR) oraz N⁶-benzyloadenozyna (BAR) wykazują inhibicję wzrostu i indukcję apoptozy w komórkach nowotworowych [51–57]. Cytokininy w formie wolnych zasad, takie jak: N⁶-izopentenyloadenina (iP), kinetyna (K) oraz N⁶-benzyloadenina (BA) hamują proliferację komórek białaczkowych NB4, ML-1, U937 oraz powodują różnicowanie ich w dojrzałe granulocyty [58]. Wyniki korelują między stymulującym działaniem cytokinin na różnicowanie komórek w roślinach, a mechanizmem ich działania w procesie różnicowania się komórek białaczki szpikowej [51]. Z kolei rybozydy cytokinin zmniejszają proliferację komórek białaczki mieloidalnej HL-60 oraz czerniaka M4Beu i B16 [59]. Cytokininy powodują różnicowanie komórek nowotworowych w zakresie wysokich stężeń (25–100 μM), podczas gdy ich rybozydy wywołują apoptozę komórek białaczkowych w stężeniach mikromolarnych [60]. KR, iPR, BAR obniżają zawartość wewnątrzkomórkowego ATP, indukują powstawanie zaburzeń w funkcjonowaniu mitochondriów oraz generują RFT, podczas gdy cytokininy (iP, K, BA) nie wykazują takiej aktywności. Wyznaczone wartości IC₅₀ na linii komórkowej HL-60 dla cytokinin oraz ich rybozydów jako pochodnych adeniny zostały przedstawione w tabeli 2.

Stwierdzono również, że apoptozie stymulowanej przez KR oraz BAR towarzyszy znaczący wzrost aktywności kaspazy 3 i 9. Uwolniony do cytoplazmy z przestrzeni międzylądowej mitochondriów cytochrom c, wiąże się z prokaspazami rozpoczynając proces kaskadowej aktywacji kaspaz, prowadzący do apoptozy komórki. W komórkach nowotworowych traktowanych KR oraz BAR zaobserwowano apoptozę w obrębie jądra komórkowego. Była ona skutkiem kondensacji chromatyny częściowo zależnej od kaspaz i dostępności ATP oraz tworzenia ciałek apoptotycznych i fragmentacji DNA zależnej od kaspaz. Wykazano, że cytotoksyczny efekt rybozydów cytokinin jest związany z wzrostem aktywności określonych kaspaz oraz dostępnością wewnątrzkomórkowego ATP [60,61].

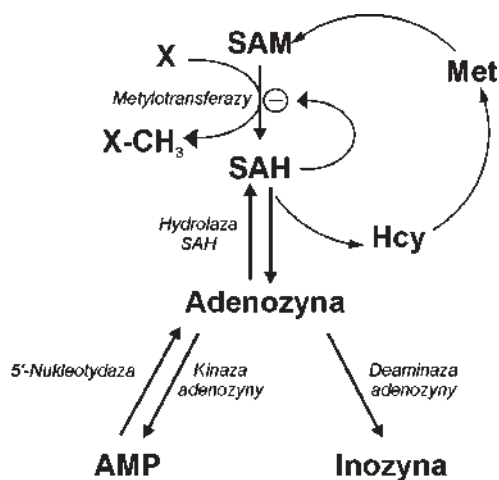
Badano wpływ N⁶-pochodnych adeniny i adenozyne na indukcję apoptozy w komórkach HL-60. Wykazano, że pochodne adenozyne oraz adeniny ulegają wewnątrz-komórkowej fosforylacji do monofosforanów. Analogi adenozyne były fosforylowane przez kinazę adenozyne (AdK). Z kolei pochodne adeniny były przetwarzane w analogiczne mononukleotydy za pomocą transferazy fosforybozylowej, której wydajność jest 50–100 razy mniejsza, aniżeli AdK. W związku z tym N⁶-pochodne adeniny okazały się niewystarczającym induktorem apoptozy. Natomiast zastosowanie inhibitorów AdK, które hamują reakcję fosforylacji N⁶-pochodnych adenozyne do odpowiednich monofosforanów zapobiegało procesowi apoptozy. Wynik ten wskazuje, że efekt wyzwalający apoptozę komórek za pomocą N⁶-pochodnych adenozyne związany jest z wewnątrzkomórkową aktywacją do odpowiednich mononukleotydów, których akumulacja prowadzi do indukcji zaprogramowanej śmierci komórki [62].

Kinaza adenozyne (AdK, EC 2.7.1.20) jest wewnątrzkomórkowym enzymem metabolizującym puryny (Ryc. 4), który odgrywa kluczową rolę w regulacji wewnątrz- i zewnątrzkomórkowego stężenia adenozyne (Ado). AdK katalizuje reakcję fosforylacji Ado do AMP przy udziale drugiego substratu, Mg/ATP²⁺. Ponadto AdK zaangażowana jest w procesy metylacji zachodzące w komórce. Niedobór AdK u transgenicznym myszy AdK^{-/-} prowadzi do ostrego stłuszczenia wątroby i wczesnej śmiertelności urodzeniowej [63]. U ssaków, w tym u ludzi, występują dwie izoformy kinazy adenozykowej. Rekombinowane białka obu izoform wykazują aktywność enzymatyczną oraz nie przejawiają żadnych różnic we właściwościach kinetycznych [64]. Sekwencje aminokwasów izoformy

Tabela 2. Efekt N⁶-pochodnych adeniny na przeżywalność komórek HL-60 [51].

Pochodne adeniny	IC ₅₀ (μM)
Puryna	58,4
Adenina	261,0
Izopentenyloadenina	47,6
Kinetyna	48,8
Benzyloadenina	67,6
Trans-zeatyna	516,0
Adenozyne	685,0
Izopentenyloadenozyna	0,972
Rybozyd kinetyny	0,981
Rybozyd benzyloadeniny	0,706

długiej (AdK-L) oraz krótkiej (AdK-S) są identyczne za wyjątkiem N-końca, gdzie ludzka AdK-L zawiera dodatkowe 20–21 aminokwasów [65]. Wcześniejsze badania wskazują, że lokalizacja AdK-L znajduje się w jądrze komórkowym, a AdK-S w cytoplazmie [66]. Obie izoformy AdK ulegają ekspresji w różnych tkankach. Najwyższy poziom ekspresji AdK występuje w wątrobie i zmniejsza się w nerkach, płucach, sercu, mózgu oraz mięśniach szkieletowych [64,65]. W warunkach fizjologicznych poziom Ado wynosi 10–100 nM [67]. Enzymem odpowiedzialnym za utrzymanie takiego stężenia poza AdK jest deaminaza adenozyńska (ADA), która katalizuje deaminację Ado do inozyny. Wiadomo też, że powinowactwo adenozy do AdK ($K_M=1 \mu\text{M}$) jest wyższe niż do ADA ($K_M=25 \mu\text{M} - 150 \mu\text{M}$) [68–69]. Biorąc pod uwagę fakt, że poziom endogennej Ado osiąga wartości nanomolarne, zdaje się, że główną ścieżką regulacji poziomu Ado jest fosforylacja przez AdK. Inną możliwą drogą inaktywacji Ado jest odwracalna reakcja katalizowana przez hydrolazę SAH, która prowadzi do utworzenia S-adenozylhomocysteiny (SAH) z adenozy i L-homocysteiny [70]. Kinaza adenozyńska posiada szeroką specyficzność substratową, rozpoznaje pochodne adenozy podstawione w zasadzie purynowej w pozycji C²-, N⁶-, N⁷- oraz C⁸- [71]. Modyfikacja części cukrowej jest dobrze tolerowana, ale obniża wydajność fosforylacji 2–5 krotnie. Jedynym wymogiem jaki substrat AdK musi spełniać jest obecność zasady purynowej oraz grupy 2'-OH rybozy, która jest niezbędna do aktywacji tego enzymu. Ponadto grupa 2'-OH musi być w konformacji *trans* w stosunku do pierścienia purynowego [71]. Związki takie jak: α -adenozy i β -D-arabinozylo-8-azaadenina nie są fosforylowane przez AdK ze względu na konformację *cis* grupy 2'-hydroksylowej względem zasady purynowej [71]. Jedną z bezpośrednich konsekwencji szerokiej specyficzności substratowej AdK wobec nukleozydów jest aktywacja dużej liczby antymetabolitów. Jednym z nich jest fludarabina, która po wnikięciu do komórki ulega fosforylacji i wywołuje efekt cytotoksyczny poprzez zaburzenie integralności DNA komórki [72]. Dowiedziono również, że selektywną cytotoksyczność komórek raka okrężnicy ludzkiej można osiągnąć w oparciu o nadekspresję białka AdK [73]. Co należy podkreślić, w badaniach klinicznych znajduje się nowa tricykliczna pochodna adenozy (Triciribine) fosforylowana przez AdK do aktywnej postaci, która jest inhibitorem kinazy białkowej B. Związek



Rycina 4. Metabolizm adenozy (opis w tekście); SAM, S-adenozylometionina; SAH, S-adenozylhomocysteina; Met, metionina; Hcy, homocysteina; X, akceptor grupy metylowej; X-CH₃, zmetylowany związek; na podstawie [63].

ten zaproponowano jako terapeutyk do spersonalizowanej terapii przeciwnowotworowej opartej o profilowanie ekspresji AdK u chorego [74].

WPŁYW RYBOZYDU KINETYNY NA CYKL KOMÓRKOWY I APOPTOZĘ

Pierwsze eksperymenty wykazujące zdolność hamowania proliferacji komórek nowotworowych i nienowotworowych przez KR zostały wykonane na początku drugiej połowy XX wieku na ludzkich kulturach komórkowych linii naskórka, raka sutka i jego przewlekłego zapalenia torbielowatego. Użyte stężenie związku okazało się skuteczne do zahamowania proliferacji komórek [75]. Stwierdzono również zatrzymanie proliferacji i indukcję apoptozy w roślinnych komórkach nowotworowych indukowanych przez zakażenie *Agrobacterium tumefaciens* [59].

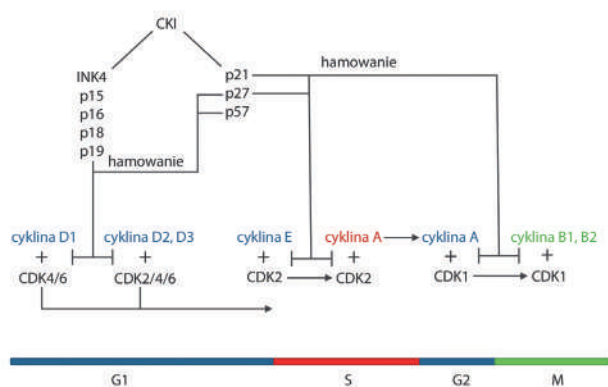
Zaobserwowano, że KR wpływa na przebieg cyklu komórkowego, jednakże wyniki badań różnią się w zależności od zastosowanego modelu doświadczalnego. Cheong i wsp. [53] wykazali w linii komórek raka wątrobowokomórkowego HepG2, że KR zatrzymuje cykl komórkowy w fazie G₂/M, poprzez obniżenie odsetka komórek w fazie G₀/G₁ i zwiększenie w G₂/M. Zatrzymaniu cyklu towarzyszyła zmiana morfologii oraz znaczny spadek proliferacji komórek w zależności od zastosowanej dawki (IC₅₀=8,33 mg/l). Pomimo, że nie stwierdzono aktywacji kaspazy-3, zdaniem autorów tłumienie przejścia z fazy G₁ do S cyklu komórkowego jest konsekwencją ingerencji związku w łańcuch transportu elektronów zachodzący w mitochondriach.

Wykazano ponadto, że KR indukuje cytotoksyczność zależną od dawki w komórkach raka okrężnicy HCT-15 dla których wartość IC₅₀ wynosi 2,5 μM . Przeżywalność komórek HCT-15 nie ulegała zmianie przy stężeniu mniejszym niż 0,5 μM , natomiast hamowanie proliferacji o 15%, 35% oraz 70% występowało po 4 dniowej inkubacji w obecności odpowiednio 0,5 μM , 1 μM i 25 μM stężeń KR. Następowaly zmiany morfologiczne typowe dla procesu apoptozy, tj. utrata przyczepności, zaokrąglenie i kurczenie się komórek oraz oderwanie od podłoża. Analiza cyklu komórkowego wykazała, że przy stężeniu 5 μM KR następowało zwiększenie odsetka komórek w fazie G₂/M i S oraz zmniejszenie w fazie G₀/G₁. Zahamowanie zdolności klonogennej komórek HCT-15 obserwowano po 2,5 μM ekspozycji na KR. Czas trwania testu wyniósł 14 dni [76].

Antyproliferacyjny i proapoptotyczny mechanizm działania KR związany jest z hamowaniem ekspresji genów kodujących cykliny [54]. Kinazy cyklinozależne (CDK) są regulatorami różnych faz cyklu komórkowego komórek eukariotycznych. Regulacja cyklu komórkowego i wzrost są ze sobą ściśle związane. Aktywacja CDK zachodzi przez przyłączenie cykliny wraz z fosforylacją reszty treoninowej przez kinazę aktywującą CDK (CAK). Niewłaściwa ekspresja lub mutacje w genach kodujących CDK, cykliny lub inhibitory CDK występują w wielu typach nowotworów.

Do tej pory zidentyfikowano szesnaście cyklina, jednak tylko niektóre biorą aktywny udział w przebiegu cyklu [77]. Cykliny D (D1, D2, D3) tworzą kompleksy z kinazami CDK4 i CDK6, odpowiedzialnymi za modulację przebiegu fazy G₁ oraz nie-

zbędnymi do zapoczątkowania replikacji DNA w fazie S poprzez fosforylację kluczowych dla cyklu komórkowego białek, np. Rb. Cyklina E oraz A łączy się z CDK2 w czasie przejścia między fazami G₁/S, a następnie w trakcie fazy S. Cykliny B wiążą się z CDK1 podczas fazy M. Ponadto aktywność CDK podlega hamowaniu przez inhibitory CDK (CKI). Wyróżnia się dwie grupy CKI. Rodzinę INK4 tworzą białka: p15, p16, p18 oraz p19, które wiążąc się z CDK4 i CDK6 uniemożliwiają ich połączenie z cyklinami D. Do rodziny Cip/Kip należą białka: p21, p27 oraz p57, które hamują aktywność CDK1 i CDK2 [77,78]. Złożoność zależności między czynnikami mającymi wpływ na przebieg cyklu komórkowego ilustruje rycina 5.



Rycina 5. Regulacja cyklu komórkowego w czasie jego trwania. Opis w tekście.

Tiedemann i wsp. [54] wykazali natomiast, że KR obniża poziom ekspresji cykliny D1 i D2 poprzez hamowanie *cis*- oraz *trans*-aktywacji genów CCND1 i CCND2 w pierwotnych hodowlach komórek szpiczaka mnogiego, co skutkuje aresztem cyklu komórkowego w fazie G₀/G₁. W zdrowych komórkach zachodzi koekspresja cyklin D1, D2 oraz D3, które zamiennie spełniają swoje funkcje tworząc kompleksy z CDK4 i CDK6 [79]. Z kolei komórki szpiczaka mnogiego, a także inne nowotwory układu krwiotwórczego wykazują nadekspresję tylko jednej z cyklin D. Zablockowanie ekspresji genów indywidualnych cyklin stanowi skuteczniejszy cel terapii przeciwnowotworowej aniżeli stosowanie inhibitorów CDK będących obecnie w trakcie badań klinicznych. Na poziomie molekularnym nowotwory te znacznie różnią się od zdrowych tkanek pod względem jakościowym i ilościowym w ekspresji genów dla cyklin D, natomiast w przypadku CDK różnica ta jest niewielka. W grupie szpiczaka mnogiego 54% nowotworów wykazuje nadekspresję genu cykliny D1 (CCND1), 48% nadekspresję genu cykliny D2 (CCND2), 3% nadekspresję genu cykliny D3 (CCND3) a 8% jednoczesną nadekspresję genów CCND1 oraz CCND2 [80]. Badania przeprowadzone na 12 liniach komórek białaczkowych charakteryzujących się znaczną nadekspresją cykliny D1 oraz D2, wykazały efekt antyproliferacyjny KR (IC₅₀ < 15 μM, 72 godziny) oraz spadek ilości cyklin D1 i D2 w ciągu 6 godzin od momentu inkubacji ze związkiem. Natomiast nie stwierdzono zmian w poziomie cykliny D3. Badając mechanizm wyciszenia ekspresji cyklin D1 i D2 wykazano, że KR blokuje *trans*- oraz *cis*-aktywację genów CCND2 i CCND1 w znacznej mierze indukowaną cAMP, poprzez nadekspresję represora transkrypcji należącego do rodziny białek CREM

(ang. *cAMP-response element modulator*). Ponadto KR skutecznie wycisza *trans*-aktywację genu CCND2 spowodowaną nadekspresją czynnika transkrypcyjnego MAF, który bezpośrednio łączy się z promotorem genu CCND2. Wynik ten sugeruje, że pod wpływem KR dochodzi do mobilizacji represorów transkrypcji dla promotora CCND2 lub zachodzi spadek kluczowych kofaktorów wymaganych do transkrypcji genu CCND2. Następnie sprawdzono czy wstrzymanie ekspresji cykliny D1 i D2 jest bezpośrednim efektem działania KR, czy może rezultatem aresztu cyklu komórkowego w fazie S, kiedy to fizjologicznie dochodzi do spadku ekspresji genów CCND1 i CCND2. W ciągu 20-godzinnej inkubacji, KR powodował zmniejszenie odsetka komórek w fazie S cyklu o 50-70% oraz zwiększenie w fazie G₀/G₁. Dane te sugerują, że mechanizm zatrzymania cyklu komórkowego wynika z bezpośredniego hamowania ekspresji genów cykliny D1 i D2. Cabello i wsp. [61] wykazali w linii komórek raka trzustki MIA Paca-2 oraz czerniaka złośliwego A375, że KR zwiększa ekspresję genu CDKN1A kodującego białko p21, które w odpowiedzi na stres cytotoksyczny hamuje cykl komórkowy w fazie G₀/G₁ oraz G₂/M. Znaczącą akumulację komórek MIA PaCa-2 i A375 obserwowano w fazie G₂/M w czasie 24-godzinnej hodowli. Wydłużenie czasu inkubacji do 72 godzin zwiększało odsetek komórek w fazie G₂/M i spadek w fazie S cyklu.

Choi i wsp. [55], a także Cabello i wsp. [61] badali zdolność rybozydu kinetyny do indukcji apoptozy poprzez mechanizm wewnątrzkomórkowy, wywołujący depolaryzację błony mitochondrialnej. Szlak wewnętrzny apoptozy – mitochondrialny ulega aktywacji na skutek stresu oksydacyjnego, uszkodzeń DNA oraz wzrostu tlenu azotu i stężenia jonów Ca²⁺ [81]. Do najbardziej poznanych czynników regulatorowych apoptozy, wpływających na zmiany przepuszczalności błony mitochondrialnej, należą białka z rodziny Bcl-2. W jej skład wchodzi białka proapoptotyczne i antyapoptotyczne, które działają antagonistycznie. Interakcja między czynnikami: antyapoptotycznym i proapoptotycznym stwarza podstawy modulowania programowanej śmierci komórki. Deregulacja białek Bcl-2 prowadzi do obumarcia komórek lub nadmiernej ich proliferacji, takiej jaka charakteryzuje choroby neurodegeneracyjne lub nowotwory. Pomimo tego, że mutacje w obrębie rodziny białek Bcl-2 występują rzadko, stwierdzono, że nadekspresja białek antyapoptotycznych Bcl-2 oraz Bcl-X_L jest zjawiskiem powszechnym w komórkach nowotworowych. Zablockowanie funkcji tych białek przez inhibitor niskocząsteczkowy ABT-737 wzmacnia apoptozę w ludzkich komórkach białaczkowych [82]. Ponadto terapia oligonukleotydami antysensownymi skierowanymi przeciwko mRNA Bcl-2 zwiększa odsetek komórek ulegających apoptozie [83].

Wykazano, że antyproliferacyjna aktywność KR jest związana z modulacją rodziny białek Bcl-2 oraz kaspazy-3. Eksperymenty przeprowadzono na dwóch liniach nowotworowych: ludzkim raku szyjki macicy (HeLa) i mysim czerniaku (B16F-10) oraz na dwóch liniach nienowotworowych: ludzkich fibroblastach skóry (CCL-116) i pierwotnych fibroblastach bydłych. W czasie 24-godzinnej inkubacji komórek HeLa i B16F-10 z KR (1 μM–1 mM) zaobserwowano zależny od stężenia efekt cytotoksyczny związku. W tym samym czasie nie zauważono istotnych zmian w przeżywalności fibroblastów obu linii. Barwienie aneksyną V i jodkiem propidyny potwierdziło działanie cytotoksyczne KR w komórkach HeLa

wynikające z indukcji procesu apoptozy. Wykazano zdolność KR do nadmiernej aktywacji PARP-1 (ang. *poly ADP-ribose polymerase*) oraz fragmentacji DNA. Ponadto barwienie jąder komórkowych barwnikiem Hoechst33258 uwidocznilo ich fragmentację, która jest charakterystycznym objawem zaprogramowanej śmierci komórki. W kolejnym etapie zbadano wpływ KR na funkcjonowanie mitochondriów. Wykazano, że w komórkach nowotworowych HeLa KR powodował mitochondrialne dysfunkcje poprzez stymulację proapoptycznych białek Bad i Bak oraz inhibicję antyapoptotycznych białek Bcl-2 i Bcl-X_L. Zaburzenie równowagi białek rodziny Bcl-2 skutkowało uwolnieniem cytochromu c oraz aktywacją kaspazy-3, przyczyniając się do śmierci komórek nowotworowych. W tym samym czasie w fibroblastach CCL-116 nie wykazano wzrostu aktywności kaspazy-3 oraz związanego z tym efektu cytotoksycznego. W celu potwierdzenia indukcji apoptozy zależnej od aktywacji kaspazy-3 zastosowano inhibitor kaspaz – zVAD-fmk pod wpływem, którego doszło do skutecznego zablokowania kaspazy-3, a śmiertelność komórek HeLa była statystycznie nieistotna [55]. Podobnie, KR w komórkach raka trzustki MIA PaCa-2 powoduje utratę potencjału błony mitochondrialnej, uwolnienie cytochromu c do cytoplazmy oraz aktywację kaspazy-3 [61].

Zaobserwowano, że KR wykazuje zdolność do szybkiego i znacznego obniżenia wewnątrzkomórkowego ATP i akumulacji reaktywnych form tlenu, co w konsekwencji prowadzi do stresu genotoksycznego i nadekspresji genów uszkadzających DNA (HMOX1, DDIT3, GADD45A) w komórkach raka trzustki MIA PaCa-2 oraz czerniaka złośliwego A375 [61].

Potwierdzono również, że KR hamuje wzrost czerniaka złośliwego w modelu *in vivo*. W tym celu posłużono się mysim modelem z wszczepionymi podskórnie komórkami czerniaka złośliwego. Bezpośrednie zastrzyki KR w okolice guza w dawce 40 mg/kg masy ciała doprowadziły do znaczącej redukcji masy nowotworowej. Podobny efekt uzyskano stosując dawkę 20 mg/kg dakarbazy – cytostatyku alkilującego używanego w leczeniu czerniaka. Wyniki potwierdzają selektywną skuteczność cytotoksyczną KR zarówno *in vitro* jak i *in vivo* [55].

Cytotoksyczność rybozydu kinetyny jest zależna od poziomu ekspresji kinazy adenozynowej (AdK) i znoszona przez zastosowanie jej inhibitorów. Wyciszenie ekspresji genu kinazy adenozynowej za pomocą RNAi w linii czerniaka złośliwego A375 objawiało się całkowitym zniesieniem antyproliferacyjnej aktywności KR. Podobny efekt obserwowano po zastosowaniu inhibitora nukleozydowego AdK – 5'-amino-5'-deoksyadenozyny (d'-AdA). W hodowli komórkowej raka trzustki MIA PaCa-2 blokowanie ekspresji genu kinazy skutkowało wysoką cytotoksycznością i tylko inhibicja białka za pomocą d'-AdA chroniła komórki przed proapoptotycznym działaniem KR [61].

PODSUMOWANIE

Jedno z podstawowych założeń przeciwnowotworowego działania rybozydu kinetyny wpisuje się w prowadzone obecnie intensywne poszukiwanie leków aktywowanych bezpośrednio w komórce nowotworowej. Koncepcja ta oparta jest na spostrzeżeniu, że w komórkach nowotworowych często

dochodzi do zwiększonej ekspresji charakterystycznych dla danego typu nowotworu enzymów, które nie są obecne w komórkach prawidłowych lub ich ekspresja jest ograniczona [84]. Poszukiwane są więc cząsteczki, które mogą ulegać aktywacji w komórkach nowotworowych za sprawą specyficznie występujących w nich enzymów. Poszczególne pochodne KR wskazują wyraźnie na drogę wewnątrzkomórkowego przekształcania związku do monofosforanu, który zostaje przekształcony w komórce nowotworowej do difosforanu, a następnie trifosforanu jako aktywna forma związku. W tym przypadku enzymem, który katalizuje pierwszy etap biotransformacji KR do aktywnego metabolitu – monofosforanu KR jest kinaza adenozynowa [76,85].

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Biological activity of N⁶-furfuryladenosine

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ABSTRACT

Cytokinins are a group of plant hormones which play an important role in plant growth and development. They produce various effects when applied to intact plants. They particularly stimulate protein synthesis and participate in cell cycle control. First discovered cytokinin was N⁶-furfuryladenine (kinetin). It is a strong inhibitor of proteins and nucleic acids oxidation *in vitro* and *in vivo*. Both kinetin and its ribosides (N⁶-furfuryladenosine, kinetin riboside) as natural compounds occur in the milk of coconuts on the nanomole level. Kinetin riboside selectively inhibits the proliferation of cancer cells and induce their apoptosis. This review focuses on the kinetin riboside occurrence, and primarily on its metabolism, and biological activity.

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“The sequence and structure determine the function of mature human miRNAs”

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RESEARCH ARTICLE

The Sequence and Structure Determine the Function of Mature Human miRNAs

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Abstract

Micro RNAs (miRNAs) (19–25 nucleotides in length) belong to the group of non-coding RNAs are the most abundant group of posttranscriptional regulators in multicellular organisms. They affect a gene expression by binding of fully or partially complementary sequences to the 3'-UTR of target mRNA. Furthermore, miRNAs present a mechanism by which genes with diverse functions on multiple pathways can be simultaneously regulated at the post-transcriptional level. However, little is known about the specific pathways through which miRNAs with specific sequence or structural motifs regulate the cellular processes. In this paper we showed the broad and deep characteristics of mature miRNAs according to their sequence and structural motifs. We investigated a distinct group of miRNAs characterized by the presence of specific sequence motifs, such as UGUGU, GU-repeats and purine/pyrimidine contents. Using computational function and pathway analysis of their targeted genes, we were able to observe the relevance of sequence and the type of targeted mRNAs. As the consequence of the sequence analysis we finally provide the comprehensive description of pathways, biological processes and proteins associated with the distinct group of characterized miRNAs. Here, we found that the specific group of miRNAs with UGUGU can activate the targets associated to the interferon induction pathway or pathways prominently observed during carcinogenesis. GU-rich miRNAs are prone to regulate mostly processes in neurogenesis, whereas purine/pyrimidine rich miRNAs could be involved rather in transport and/or degradation of RNAs. Additionally, we have also analyzed the simple sequence repeats (SSRs). Their variation within mature miRNAs might be critical for normal miRNA regular activity. Expansion or contraction of SSRs in mature miRNA might directly affect its mRNA interaction or even change the function of that distinct miRNA. Our results prove that due to the specific sequence features, these molecules can also be involved in well-defined cellular processes depending on their sequence contents. The pathway mapping and theoretical gene target identification allowed us to create a biological framework to show the relevance of the specific miRNAs in regulation the distinct type of targets.

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Introduction

Among all biological macromolecules, RNAs are one of the most structurally and functionally diverse molecular players within the cell. They fulfill many different functions such as coding and transferring genetic information, controlling various cellular processes, as well as catalyzing cleavage reactions, synthesis and ligations [1]. RNAs execute these roles through their variable and dynamic tertiary structures, which enable the RNA to interact specifically with themselves, with other RNAs, ligands and RNA-binding proteins [1]. miRNAs are a class of endogenous, non-coding small RNA, 19–25 nucleotides in length, that mediate the post-transcriptional regulation of mRNAs expression. They are evolutionary conserved, and control diverse biological processes as developmental timing, differentiation, cell proliferation, cancer or neurodegenerative disorders and apoptosis [2–9]. In the canonical pathway, the regulatory function of miRNAs is achieved primarily by binding target mRNAs at sites frequently located in the 3' untranslated region (UTR). However, miRNA binding sequences have been identified also in the coding region and in 5'UTR [10–11]. miRNA activity requires its incorporation in a RNA-induced silencing complex (RISC). Here, a target recognition relies mainly on near-perfect complementarity of the mRNA with the miRNA “seed region”, 6–8 nucleotide-long sequence at the 5' end of miRNA. Upon target binding, repression of gene expression is accomplished by suppressing translation, but some recent reports suggest that the major role of miRNAs is the reduction of target mRNA stability [2, 12–19]. The specificity of miRNA targeting in mammals is not restricted to the 5' and 3' ends of the seed region and Watson-Crick binding of the seed is not always sufficient for the repression of targets in mammals [20–21]. It has already been reported that the residues in positions 13–16 from the 5' end of the miRNAs are involved in stabilization of miRNA-mRNA interactions, especially when base-pairing in the 5' seed region is suboptimal [8, 20–22]. Unlike G and U residues, which can form wobble pairs (C/U or A/G), A residue can only pair with U residue of target mRNAs. It is known that sequence at 5' end of miRNA determines the preferential strand selection and/or rejection in maturation process, between two strands of the same precursor duplex [21]. Therefore, pronounced occurrence of U at the 5' end enhances the preference of the strand selection.

Thus, mature miRNA can control the expression of thousands of target mRNAs, and on the other hand, it is believed that a single mRNA is targeted by multiple miRNAs [20–23]. However, due to the current, incomplete understanding of the mechanism of miRNAs action beyond their biogenesis and target inhibition, the last process is somewhat unpredictable and often not as efficient as expected.

Recently, some non-canonical pathways of miRNA biogenesis have been proposed [24–28]. The analyses of data from next generation sequencing or microarrays, have revealed subclasses of miRNA species, derived from alternative biogenesis pathways. They only partially meet the classical definition, such as mirtrons—miRNAs derived from snoRNAs, from endogenous short-hairpin RNAs (endo-shRNAs) or tRNA precursors [27–28].

The broad analysis of the role of miRNA-target base-pairing in regulation by miRNAs has been shown that this interaction is essential for miRNA specificity in regulatory processes [22, 28]. Although the strength of the interaction is one of the essential factors that can alter the stability of a dsRNA duplex, the contribution of the base-pairing potency to miRNA-target duplex stability remains still unclear. There was also already shown that the sequence of the particular class of miRNA is important for mRNA repression, e.g. in *Arabidopsis* stress-responsive miRNAs were found to be G/C rich [29]. This nucleotide content increases the miRNA-target duplex stability, which accelerates the stress response.

miRNA-mediated gene regulation and mRNA expression are undoubtedly dependent on both nucleic acid conformation and target recognition. We assumed then, if base-pairing

potency could affect the functionality of the miRNA-mediated gene expression regulation, the full understanding of various biological functions of miRNA needs the knowledge about the sequence and structure of mRNA targets, miRNAs precursors but also of mature miRNAs is needed.

In this paper, we consistently studied across the mature human miRNAs: (i) the length distribution; (ii) characteristics of nucleotides content, their occupancy position, sequence-based motifs, like: UGUGU, GU-repeats and purine/pyrimidine contents; (iii) simple sequence repeats (SSRs). Additionally, we have also examined the structural tetranucleotide motifs (UUCG, GAAA, GCAA, GAGA, GUGA, GGAA, CUUG, UUUG) involved in stable hairpin formation and their propensity for regulation of specific group of targets. Using ModeRNA software, for the first time we have modeled the tertiary structure of mature miRNAs, suggesting that the miRNAs-target recognition can be not only sequence-, but also structure-related. Thus, in this paper we investigate the sequence features of mature miRNAs, convincing that the nature of these analysis can reveal the complexity of the miRNA molecular structures, what can give a hint for a genetic, including cancer, research. To predict the biological functions behind our sequence analysis results, we search for the corresponding pathways of the distinct group of miRNAs using DNA intelligent (DIANA) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses. Accordingly, with DIANA-miRPath software we performed a computational target prediction combined with pathway analysis. Apart from the pathways analysis, the gene ontology including biological processes was also taken into account. The validity of the KEGG pathway clustering approach is further supported by the additional analysis with PANTHER classification system, that provided also the list of different protein classes being involved in the distinct processes and pathways. Based on these analysis we have found a link between the sequence content and pathways, biological processes and proteins associated with the distinct group of characterized miRNAs.

Materials and Methods

Library construction and clustering

All metazoan and human miRNAs sequences, available on 17th October 2012, were downloaded from miRBASE version 19 in FASTA format (<ftp://ftp.sanger.ac.uk/pub/mirbase/19.0/>). The data were scanned for unique sequences of mature miRNAs. The molecules with identical sequences, but annotated under different names in the miRBASE, were removed. The cured library contained 2042 human miRNA sequences.

Analysis of miRNA nucleotide sequences and SSR analysis

The analysis was done with the use of Phyton language, version 2.7.3. For each of the analyses, different scripts were prepared ([S1 file](#)). Whole sequence analysis was conducted on a specific number of sequences, to a single one accuracy.

For calculation of the monorepeats in a single miRNA sequences, the regular expressions language was used.

Di-, tri-, tetra- and pentanucleotide repeats were identified and localized by the software SSRIT (<http://www.gramene.org/db/searches/ssrtool>).

Calculation SSR relative count

SSR relative count is the total repeats per miRNA on average. The calculated number of repeats were divided by the number of miRNAs is on each analyzed group (e.g. relative count for mononucleotide repeats $2326/2042 = 1.14$).

Functional analysis with DIANA miRPath

For the identification of the networks and pathways of the selected miRNAs we used DIANA miRPath (v2.0) software <http://diana.imis.athena-innovation.gr> [30]. The prediction algorithm DNA Intelligent Analysis (DIANA) DIANA-microT-CDS (v5.0) was used for the identification of potential target RNAs from each cluster. A core analysis was performed to recognition of the most relevant miRNA targets, canonical pathways, biological functions and physiological processes from the interactions provided by the DIANA database. The identification of all the significantly targeted by the selected miRNA pathways was performed in the mode “Union of pathways”. The enrichment analysis and calculation of the significance levels (p-values) for each selected miRNA individually was performed. Fisher’s meta-analysis method was applying for calculation a merged p-value for each pathway. For the analysis a *posteriori* approach was used, where the statistical results showed the probability that the surveyed pathway is significantly enriched with gene targets of at least one selected miRNA. The significance for all the miRNA-mRNA pairs in a pathway were performed and calculated, followed by the combination into a merged P-value for each pathway. The results are reported and visualized as heat maps, and the pathways are clustered based on significance levels.

Classification and pathway analysis with PANTHER System

The list of miRNAs with distinct sequence motif were subjected to TargetScanHuman program, release 7.0 (August 2015) analysis prior to obtain the full list of predicted targets for the specific group of miRNAs. The lists of targets were then uploaded to the PANTHER (protein annotation through evolutionary relationship) Classification System (<http://pantherdb.org>). By overrepresentation test the comparison of provided data set with a reference list in PANTHER database was performed. Ontology categories for over- and underrepresentation were determined statistically with Binominal distribution test.

miRNA sequence alignment

ClustalW command line version of the multiplatform sequence alignment was used for comparison of distinct miRNAs sequences and other RNAs with previously, experimentally confirmed hairpin structures.

Secondary structure analysis

Mfold program, version 3.5, was used to calculate the folding free energy in the conditions given by the program for all the sequences towards formation of hairpin structures (<http://mfold.bioinfo.rpi.edu>).

miRNAs structure modeling

ModeRNA program (<http://iimcb.genesilico.pl/moderna/>) for miRNAs 3D modeling based on the experimentally confirmed structures (templates) was used [31]. The structure for RNAs were downloaded from PDB database. At least 80% of similarity was taken as a threshold with the sequences alignment. The PyMOL programm was applied as a tool for figures generation.

Results

The nucleotide composition in mature human miRNAs

The analysis of a length heterogeneity of 2042 sequences of human mature miRNAs resulted that number of nucleotides is a discrete random variable, which ranges from 16–27. miRNAs

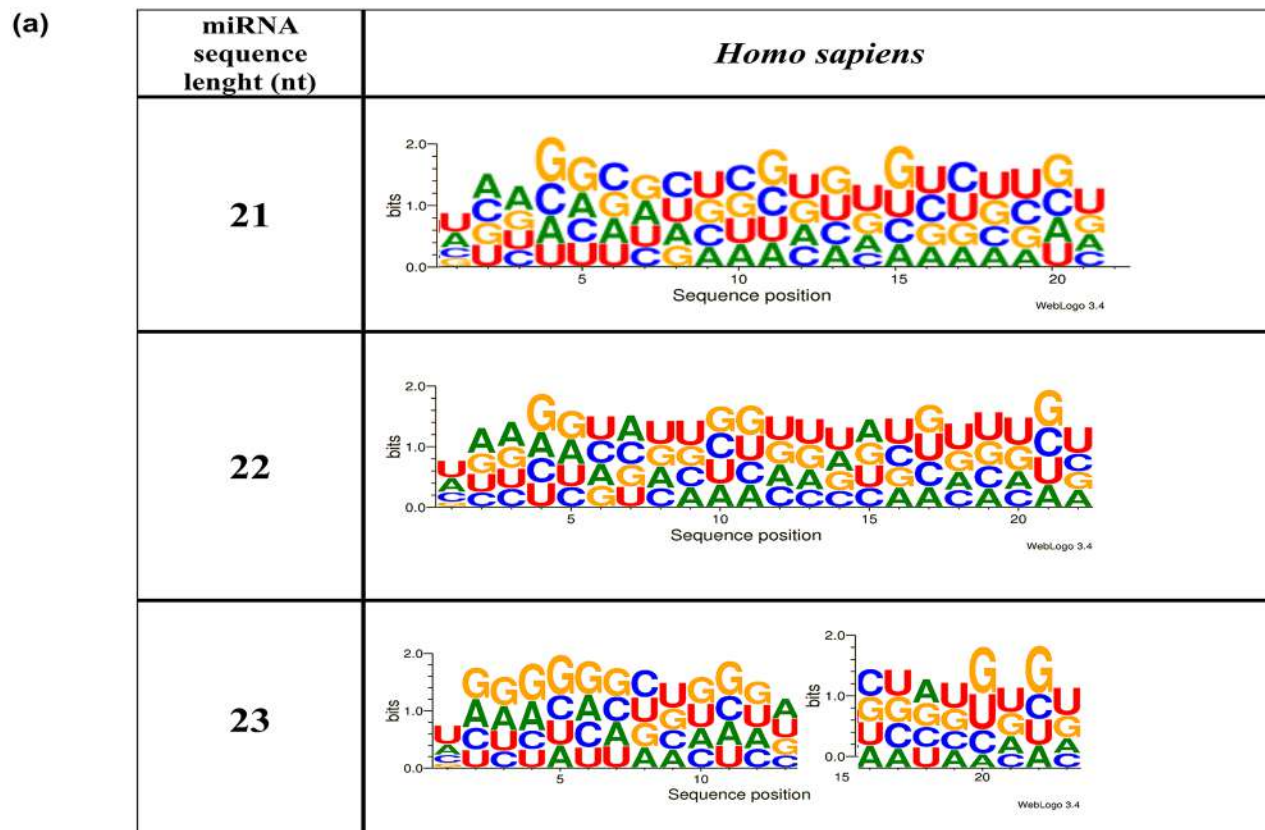
sequences with 21 (12%) and 23 (14%) nucleotides are most abundant, nevertheless miRNAs with 22 nucleotides in length are predominant (47%) (S1 Fig). These results corroborate with the recently published observations [32]. The sequence length analysis of human mature miRNAs showed that the length heterogeneity is related to some biological factors such as evolution conservations or miRNA's regulatory mechanism [32]. It has been also established that miRNAs that regulate cancer-associated targets (oncogenes/tumor suppressors) show stable lengths of 22 nucleotides, whereas longer miRNAs tend to regulate more genes [32].

Furthermore we analyzed the nucleotides content in human mature miRNAs, what showed inequality of base presence. It showed a prevalence of guanosine and uridine at 29 and 26%, and lower levels of adenosine (23%) and cytidine (22%), respectively. This observations is also consistent with the previously shown results [32]. Although the most abundant nucleotide in miRNAs is guanosine, the first position at 5' end is more frequently occupied by the uridine (35%) (Fig 1a and 1b). Furthermore, the second and third position are usually occupied by adenosine, making the 5' end (nucleotides position- 1–3) mostly UAA-rich. The 5' end for the most represented lengths of mature miRNAs: 21 and 22 nt long is UAA-rich, what is shown on Fig 1a. miRNAs with 23 nt in length at the 5' end show mostly UGG sequence. The seed sequence which is considered as position 2–8 is basically G-rich for 21 and 23 nt long miRNAs, whereas for 22 nt long- U/A rich.

However, one can notice, that A nucleotide at 2nd and 3rd positions is only slightly overrepresented than G. (Fig 1a and 1b). It has been also observed that the presence of A residue in the targeted mRNA, from position 11 of the 5' end of a miRNA, has a great impact on the improvement of the miRNAs binding to the specific target [20, 33]. This strengthen of the interaction is observed, although it was shown, that the exact base-pairing between the A residue and the nucleotide at position 1 of the 5' end of the miRNA is not necessary [20, 22]. The strongest representation of the U residue in the first and the last position of miRNAs sequence was observed not only for human, but also five other mammalian species, such as *Pongo pygmaeus*, *Pan troglodytes*, *Macaca mulatta*, *Mus musculus* and *Rattus norvegicus*. This observation suggests that both ends, more likely than just the 5' end, are also involved in target recognition [34].

Based on a single position analysis of the whole sequences, we have also looked at the purines or pyrimidines tracts in mature miRNAs. We assumed the threshold for purine/pyrimidine rich sequences over 70% for G, A and C,U, respectively. Based on that criterion we found 141 CU-rich and 187 AG-rich sequences. We found: miR-4290, miR-1281, miR-4716-5p, miR-483-3p and miR-877-3p containing almost only UC nucleotides and miR-6124, miR-483-5p, miR-4271, miR-4644, miR-4716-3p and miR-1234-5p purine-rich. In the Table 1 we showed only highly purine/pyrimidine rich sequences, where the level of the investigated nucleotides is over 90%.

The functional analysis of the cellular pathways analysis showed that the purine/pyrimidine-rich sequences show the involvement mostly in lysine degradation, RNA transport and spliceosome (Fig 2a, S1 Table). The search generated a rank-ordered list of KEGG and PANTHER pathways, issuing statistical significance based on p-values. Examining the pathways affected by the distinct group of miRNAs we highlighted pathways associated with different physiological processes connected to the specified group of miRNAs. The heat map indicates the high enrichment of the e.g. miR-483 in RNA degradation, miR-4271 in lysine degradation or miR-4716 in spliceosome (Fig 2b). The list of proteins involved in the pathways and processes associated with the purine/pyrimidine-rich miRNAs also corroborate with these results (S1 Table). The membrane trafficking regulatory, membrane traffic or nucleic acid binding proteins are the most common proteins being a main players in binding and RNA transport processes (S1b Table). Apart from the targeting the distinct group of mRNAs, polypyrimidine-tracts could allow possibly for the interaction of miRNAs with other proteins, such as PTB (polypyrimidine tract-binding protein), preventing the typical regulatory action of miRNAs



(b)

Sequence length (nt)/number of sequences	5' end first position			
	A	U	C	G
21/370	108 (29%)			
		132 (36%)		
			70 (19%)	
				60 (16%)
22/948	295 (31%)			
		351 (37%)		
			200 (21%)	
				102 (11%)
23/280	54 (19%)			
		125 (45%)		
			31 (11%)	
				70 (25%)

Fig 1. Nucleotide occupancy position in human mature miRNAs. A Web logo with the distinct nucleotides at all positions of the mature miRNA sequences. B. Nucleotides representation at the first position of miRNAs mature strand at 5' and 3' end, in context to their length. The number under the name of nucleotide (e.g. 108 for A) reflects 108 sequences with A as a first nucleoside at 5' end, and 27 at 3', respectively.

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Table 1. Pyrimidines- and purines-rich sequences of miRNAs. The table shows the content of the pyrimidines or purines nucleotides according to the sequence of miRNAs. The total contents of purines or pyrimidines of the miRNAs presented in the table is above 90%.

miRNA	Sequence	Pyrimidine/purine content [%]	Sequence lenght
Pyrimidines-rich sequences			
hsa-miR-4290	UGCCCUCCUUCUUCUCCUC	95	19
hsa-miR-1281	UCGCCUCCUCCUCCUCCC	94	17
hsa-miR-4716-5p	UCCAUGUUUCCUUCUCCUUCU	91	22
hsa-miR-483-3p	UCACUCCUCCUCCUCCGUCUU	90	21
hsa-miR-877-3p	UCCUCUUCUCCUCCUCCAG	90	21
Purines-rich sequences			
hsa-miR-6124	GGGAAAAGGAAGGGGGAGGA	100	20
hsa-miR-4271	GGGGGAAGAAAAGGUGGGG	95	22
hsa-miR-483-5p	AAGACGGGAGGAAAGAAGGGAG	95	22
hsa-miR-4644	UGGAGAGAGAAAAGAGACAGAAG	91	23
hsa-miR-4716-3p	AAGGGGAAGGAAACAUGGAGA	91	22
hsa-miR-1234-5p	GGGGGGGGGGGGGGGGGCCG	90	21

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[35]. On the other hand, such miRNAs can interact with Kozak sequence- purine-rich motif, similar to prokaryotic Shine-Dalgarno sequence [36–37]. Recently, the novel mechanism for post-transcriptional control of gene expression was shown [38]. It involves the formation of an intermolecular G-quadruplex between small RNA and mRNA, where a four stranded RNA structure is formed through direct RNA-RNA dimerization. This is hint to suppose, that the G-rich miRNAs could be also regulate the gene expression via this kind of mechanism.

Simple sequence repeats (SSR) analysis

Regardless the extensive research, there are only limited data about the nucleotide composition, especially in terms of the nucleotide repeats in mature miRNAs. This analysis could be beneficial, since the SSRs can be potentially used for genetic maps construction, as a genetic markers, but also in the linkage association research or phylogenetics and population genetics studies [39–44]. It was also shown, that SSRs could serve for a target genes involved in diseases, for evolution history research or finally, in the execution of the paternity tests [45–48].

Based on the nucleotide sequence analysis, we performed SSR monitoring. We searched in human miRNAs for the occurrence and nature of SSRs. The most important standard for the identification of SSRs in a given sequence is the definition of the minimum repeat number. The minimum repeat units in track is annotated as the valid SSR tract. In order to detect various repeats in mature miRNAs, and taking into account the length of the molecules, we considered minimum three repeats. Consequently, three minimum repeats were used for the survey of SSRs in pre-miRNA studies [49]. Among the analyzed mature miRNA sequences we were able to define: mono-, di-, tri-, tetra- and pentanucleotide repeats.

Mononucleotide repeats poly (U) and poly (G) were the predominant repeats in all of the analyzed mature miRNAs (Table 2). The longest mononucleotide repeats observed, was the 18 nucleotides SSR poly (G). For poly (U), (A) and (C) repeats we were able to find maximum 10 nucleotides long, both for “U” and “A” SSR and 7 for “C”, respectively. Dinucleotides were the second most common repeats in mature miRNAs (Table 2). We have found that GU/UG were also predominant for human miRNAs (Table 2). To get comprehensive view on specified miRNAs, we established the list GU/UG-rich miRNAs (Table 3). The less frequent, with almost the same relative count, were the AG/GA and AU/UA repeats (Table 2). CG/GC repeats in mature

(a)

Purine/pyrimidine-rich sequences					
miRNAs	KEGG Pathway	Pathway ID	# of Genes (union)	p-value (union)	# miRNAs involved in pathway
hsa-miR-4290	Lysine degradation	hsa00310	15	3.74e-10	5
hsa-miR-1281	RNA transport	hsa03013	17	6.30e-05	3
hsa-miR-4716-5p	Spliceosome	hsa03040	18	9.12e-05	2
hsa-miR-483-3p	PI3K-Akt-signaling pathway	hsa04151	25	0.000253	3
hsa-miR-877-3p	Transcriptional misregulation in cancer	hsa05202	19	0.000362	3
hsa-miR-6124	N-Glycan biosynthesis	hsa00510	2	0.000727	2
hsa-miR-4271	Other types of O-glycan biosynthesis	hsa00514	2	0.005192	2
hsa-miR-483-5p	p53 signaling pathway	hsa04115	12	0.0057423	3
hsa-miR-4644	RNA degradation	hsa03018	12	0.007102	2
hsa-miR-4716-3p	Prostate cancer	hsa05215	14	0.014886	4
hsa-miR-1234-5p					

(b)

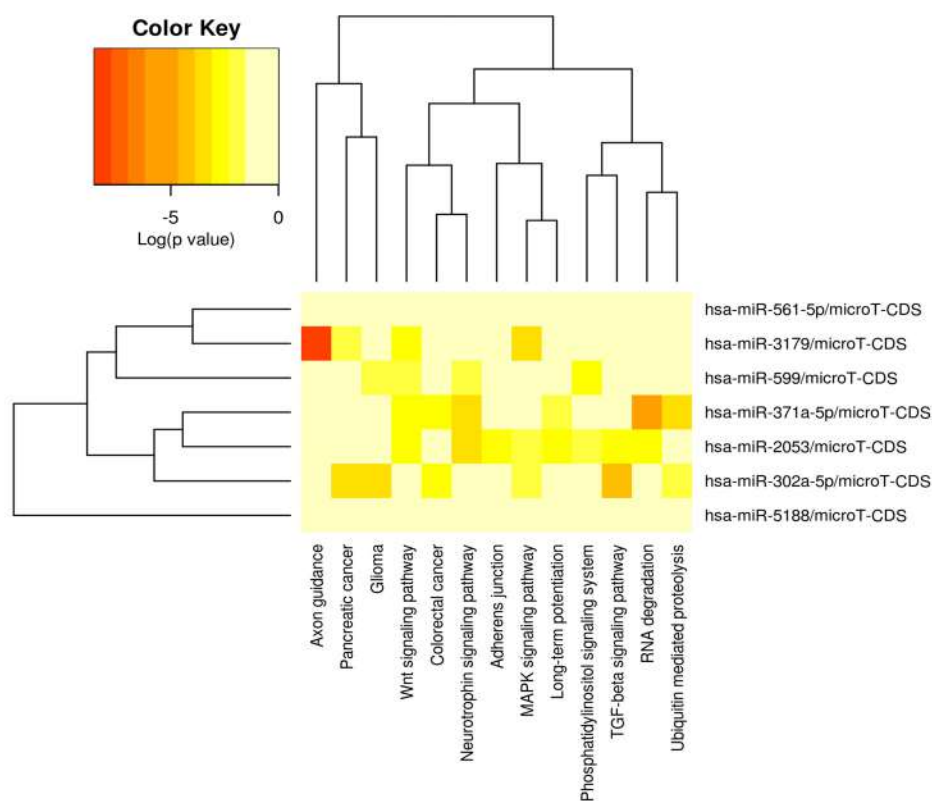


Fig 2. The KEGG pathway analysis for pyrimidine/purine (P/P)-rich miRNAs. A. The table illustrating the: list of P/P-rich miRNAs (first column); the IDs and KEGG pathways names (second and third column); the number of genes and P/P-rich miRNAs involved associated with the pathways (fourth and sixth column). P-value was given in fifth column as a result of statistical analysis. P-value threshold is considered 0.05. B. P/P-rich miRNAs in predicted pathway heat map. Significant miRNA-pathway interaction $p < 0.001$.

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Table 2. Occurrence and relative count of SSRs in mature miRNAs. Occurrence and relative count of all SSRs in mature miRNAs: mononucleotides, dinucleotides and the list of the longest tetra- and pentanucleotides.

SSRs	Mono	Di	Tri	Tetra	Penta	Total
Total numbers of repeat	2326 (1.14)	324 (0.16)	43 (0.02)	3 (0.001)	4 (0.002)	2700 (1.32)
Repeat type and number	A: 462 (0.23) U: 569 (0.28) G: 802 (0.40) C: 493 (0.24)	AG/GA: 66 (0.03) GU/UG: 83 (0.04) AC/CA: 45 (0.02) CU/UC: 67 (0.03) AU/UA: 47 (0.02) CG/GC: 16 (0.008)	See details in S2 Table	(AGGA) ₃	(UGGGC) ₄	2700 (1.32)

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miRNAs are relatively rare ([Table 2](#)). The most frequent dinucleotide repeat we have found within the sequence of has-miR-1277-5p-1, was (AU) motif, repeated 7 times.

Interestingly, most of the trinucleotide repeats contain base “U” and “G”, although it can be noticed that this type of SSR is less represented. AGU, GGU and UGU SSR were not observed in humans mature miRNAs sequences, whereas GUG motif was observed 6 times. The detailed data about trinucleotide repeats are given in [S2 Table](#). Mature human miRNAs with the longest trinucleotide repeat- (AGG)₄ is hsa-miR-4298-1.

Although, in the mature miRNAs tetranucleotide and pentanucleotide repeats were less expected to detect, however we were still able to find such SSRs type ([Table 2](#)). Interesting example of mature miRNA, with pentarepeats, is hsa-miR-3620-5p-1, in which we identified (UGGGC)₄ ([Table 2](#)).

Table 3. The list of GU-rich miRNAs. GU-tracts are marked in bold among the sequence.

miRNA	Sequence
miR-574-5p	UGAG UGUGUGUGUGUGUG AGUGUGUGU
miR-941	CACCCGGCUG UGUG GCACAUGUGC
miR-3149	UUU GU AUGGAUAUG UGUGUGUG AU
miR-1238-5p	GU GAGUGGGAGCCCCAG UGUGUG G
miR-545-3p	UCAGCAAACAUAUU UGUGUG GC
miR-2278	GAGAGCAG UGUGUGUG UGCCUGG
miR-3148	UGGAAAAAACUG UGUGUG GCUU
let-7b-5p	UGAG GUAGUGUGUGUGUG U
miR-493-3p	UGAAG GU CUACUG UGUG GCCAGG
miR-1180	UUUCCGGCUCGC UGUGUGUGUG
miR-539-5p	GGAGAAUUUAUCCUUG UGUGUG
miR-32-3p	CAAUUUA UGUGUGUGUG GAUAUUU
miR-206	UGGAU GU AAGGAAG UGUGUG GG
miR-1299	UUCUGGAAUUC UGUGUG AGGGA
miR-3911	UGUGUG GGAUCCUGGAGGAGGCA
miR-297	AUGUAU UGUGUG GCAUGUGCAUG
miR-610	UGAGCUAAA UGUGUG GCUGGGGA
miR-1228-5p	GU GGGCGGGGGCAG UGUGUGUG G
miR-595	GAA UGUGUG GCCUG UGUGUGUG CU
miR-4455	AGG UGUGUGUGUGUG UUUU
miR-3650	AG UGUGUGUGUG CU UGAGAGUCC
miR-147a	UGUGUGUG GGAUUGCUUCUGC
miR-660-3p	ACCUC UGUGUGUG GCAUGGAUUA

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Until now, there is only a single study focused on SSRs in short sequences, such as pre-miRNAs [49]. However, since miRNAs are functional molecules, we decided to monitor the SSRs in mature miRNAs. For this group of molecules, we have noticed the same tendency that has been already observed for pre-miRNAs. Among the majority of the analyzed miRNAs, poly (A/U) repeats were more frequent than poly (G/C) repeats. Having converted this data into genome level, this observation, both among the previous pre-miRNAs analysis and our mature miRNAs studies, is similar to primate genome [50]. Mononucleotide and dinucleotide repeats were significantly predominant, which was similar to pre-miRNAs and to that of introns, in which majority of SSRs were also mono- and dinucleotides, whereas tri-, tetra-, penta- and hexanucleotide repeats were relatively rare. It has been reported that (GT)_n is the most predominant dinucleotide repeat motif in animal and invertebrates. Additionally, similarly to pre-miRNAs sequences, the most abundant repeats were (GU)_n/(UG)_n as well. Thus, one could assume that SSRs, both in pre-miRNAs and miRNAs, might have a functional meaning. They can be considered for providing a molecular basis for organization of pre-miRNAs *in vivo* or rapid mature miRNAs maturation. SSRs changes within the mature miRNAs sequences, similarly to pre-miRNAs might have a critical impact on the normal miRNA regulation activity and the variations of SSRs in mature miRNAs can influence their direct mRNA target interaction or even alter the function of that distinct miRNA.

Additionally, our analysis showed that miRNAs enriched with the particular sequence motif are predicted to target different pathways. The pathways associated with GU-rich miRNAs as the most significant indicate a “dopaminergic synapse”, “lysine degradation” and “long-term potentiation”. The more intense red color in a heat map indicates higher probability that a specific pathway is significantly enriched with target genes for a certain miRNA (Fig 3). This analysis also revealed that the GU-rich miRNAs more likely are involved in the neurological processes such as “Opioid proopiomelanocortin pathway” or “Axon guidance mediated by netrin” (Fig 3, S3 Table). The list of the most important proteins involved in the processes with the participation of GU-rich miRNAs covers among others proteins of voltage-gated sodium channel, sodium channel, but also SNARE proteins, which the primary role is to mediate lysosome formation, particularly in the presynaptic membrane in neurons (S3 Table). Although AU-rich elements (AREs) are very abundant in the 3'UTRs of many different mammalian mRNAs with unstable structure, the presence and function of GU-rich elements (GREs) are still poorly understood. There was found that through genome-wide analysis at least 5% of human genes contain GREs in their 3'UTR. The functional over-representation of it is assigned for the genes involved in transcription, nucleic acid metabolism, developmental processes and neurogenesis [51]. Until now, there is no report showing the importance of the GU-rich miRNAs in specific processes. However, in our global sequence analysis of miRNAs, we demonstrate, that this type of miRNAs target also mRNAs involved in biological processes such as mRNA 3'-end processing, mRNA transcription or nervous system development (S3 Table). On the other hand, it was shown that GREs are the targets for at least one RNA-binding protein: CUG-binding protein 1 (CUG-BP1)—the member of the highly conserved CELF family of RNA-binding proteins that are post-transcriptional regulators of deadenylation, mRNA decay, translation and pre-mRNA processing [52–57]. It is possible CUG-BP1 could also bind directly GU-rich miRNAs and affect the protein expression. This needs, however, further investigation.

The identification of interferon induction motif (IIM)

Going deeper into the sequence analysis within the sequences of mature miRNAs, we identified 5'-UGUGU-3' motif, which is known for stimulation of innate immune response. We have found 50 of mature miRNAs within that motif (Table 4). That feature of the UGUGU sequence

(a)

GU-rich sequences					
miRNAs	KEGG Pathway	Pathway ID	# of Genes (union)	p-value (union)	# miRNAs involved in pathway
miR-4455	Dopaminergic synapse	hsa04728	51	1.17e-08	5
miR-3650	Lysine degradation	hsa00310	19	2.16e-08	9
miR-147a	Long-term potentiation	hsa04720	32	1.84e-07	7
miR-660-3p	Mucin type O-Glycan biosynthesis	hsa00512	14	2.23e-07	4
miR-595					
miR-1228-5p	Retrograde endocannabinoid signaling	hsa04723	38	5.74e-07	5
miR-610					
miR-297	Long-term depression	hsa04730	21	6.71e-07	7
miR-3911	Prostate cancer	hsa05215	41	1.51e-06	7
miR-1299	Gap junction	hsa04540	36	4.79e-06	7
miR-4700-5p	Glioma	hsa05214	31	5.87e-06	6
miR-206	Glutamatergic synapse	hsa04724	39	7.07e-06	7
miR-32-3p	Nicotine addiction	hsa05033	12	1.00e-05	6
miR-539-5p					
miR-1180	Neurotrophin signaling pathway	hsa04722	55	1.15e-05	9
miR-493-3p	GABAergic synapse	hsa04727	30	0.00023	5
miR-7b-5p	Cholinergic synapse	hsa04725	40	0.00041	4
miR-3148	Endometrial cancer	hsa05213	27	0.00051	6
miR-2278					
miR-545-3p					
miR-1238-5p					
miR-3149					
miR-941					
miR-574-5p					

(b)

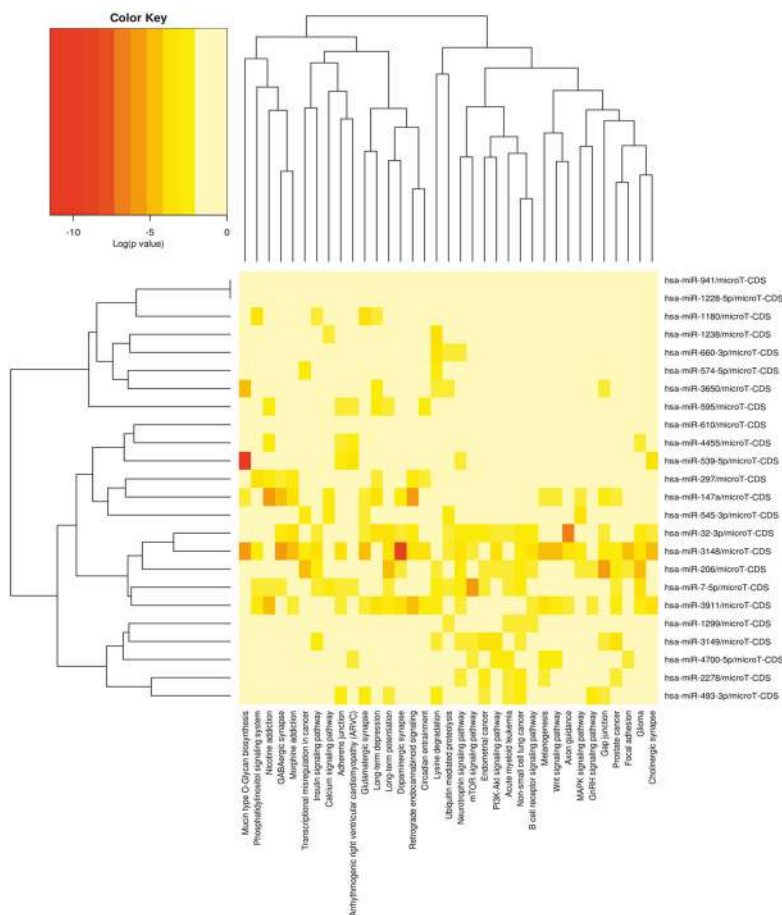


Fig 3. The KEGG pathway analysis for GU-rich miRNAs. A. The table illustrating the: list of GU-rich miRNAs (first column); the IDs and KEGG pathways names (second and third column); the number of genes and GU-rich miRNAs involved associated with the pathways (fourth and sixth column). P-value was given in fifth column as a result of statistical analysis. P-value threshold is considered 0.05. B. GU-rich miRNAs in predicted pathway heat map. Significant miRNA-pathway interaction $p < 0.001$.

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Table 4. Selected human miRNAs contained immunostimulatory motifs. The table presents the miRNA sequences with at least one UGUGU motif (highlighted in bold).

miRNA	Sequence (5'-3')	miRNA	Sequence (5'-3')
miR-3682-5p	CUACUUCUACC UGUGU UAUCAU	miR-539-5p	GGAGAAUUAUCCUUGG UGUGU
miR-2278	GAGAGCAG UGUGU GUUGCCUGG	miR-1180-3p	UUUCCGGCUCGCGUGGG UGUGU
miR-1238-5p	GUGAGUGGGAGCCCCAG UGUGU G	miR-450a-5p	UUUUGCGA UGUGU UCCUAAUUAU
miR-3149	UUGUAUGGAUA UGUGU GUGUAU	miR-493-3p	UGAAGGUCUAC UGUGU GCCAGG
let-7b-5p	UGAGGUAGUAGGU UGUGU GGUU	miR-4669	UGUGU CCGGGAAGUGGAGGAGG
miR-4455	AGGG UGUGU GUGUUUUU	miR-4753-3p	UUCUCUUUCUUUAGCCU UGUGU
miR-4717-5p	UAGGCCACAGCCACCCA UGUGU	miR-624-5p	UAGUACCAGUACCU UGUGU UCA
miR-573	CUGAAGUGA UGUGU AACUGAUCAG	miR-3657	UGUGU CCCAUUAUUGGUGAUU
miR-660-3p	ACCUC UGUGU GCAUGGAUUA	miR-3148	UGGAAAAACUGG UGUGU GCUU
miR-378a-5p	CUCUGACUCCAGGUCC UGUGU	miR-3177-5p	UGUGU ACACACGUGCCAGGCGCU
miR-599	GU UGUGU CAGUUUAUCAAAC	miR-581	UCU UGUGU UCUCUAGAUCAGU
miR-1226-3p	UCACCAGCCC UGUGU UCCCUAG	miR-642b-5p	GGUCCCUCCUCCAA UGUGU CU
miR-1270	CUGGAGAUUUGGAAGAGC UGUGU	miR-147a	G UGUGU GGAAUAGCUUCUGC
miR-610	UGAGCUAAA UGUGU GCUGGGA	miR-592	U UGUGU CAAUAUGCGAUGAUGU
miR-3911	UGUGU GGAUCCUGGAGGAGGCA	miR-545-3p	UCAGCAAACAUUUU UGUGU GC
miR-3650	AGG UGUGU CUGUAGAGUCC	miR-1228-5p	GUGGGCGGGGCGAGG UGUGU G
miR-4789-5p	GUAUACACCUGAUUA UGUGU AUG	iR-642a-5p	GUCCCUCCUCCAA UGUGU CUUG
miR-330-5p	UCUCUGGGCC UGUGU CUUAGGC	miR-3152-3p	UGUGU UAGAAUAGGGGCAUAA
miR-941	CACCCGGC UGUGU GCACAUGUGC	miR-32-3p	CAAUUUAG UGUGU GUGAUUUU
miR-597	UGUGU CACUCGAUGACCACUGU	miR-1299	UUCUGGAAU UGUGU GAGGGA
miR-5580-5p	UGCUGGCUCAUUUCAUA UGUGU	miR-297	AUGUA UGUGU GCAUGUGCAUG
miR-4700-5p	UCUGGGGAUGAGGACAG UGUGU	miR-187-3p	UCGUGUCU UGUGU UGCAGCCGG
miR-892a	CAC UGUGU CCUUUCUGCGUAG	miR-5010-3p	UUU UGUGU CUCCCAUCCCCAG
miR-206	UGGAAUGUAAGGAAG UGUGU GG	miR-574-5p	UGAG UGUGU GUGUGU UGUGU GAG UGUGU
miR-595	GAAGUGUGCCGUGG UGUGU C	miR-649	AAAC UGUGU UGUUAAGAGUC

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was first shown for siRNAs as extremely important, as it may cause variety of non-specific side effects, including stimulation of interferon and cytokine production, global shutdown of protein synthesis or nonspecific degradation of mRNAs [58–59]. The miRNA, containing interferon stimulation motifs, can induce itself one or more undesirable effects, such as proliferation blockage, differentiation or apoptosis of cancer cells, or it can even serve as potent immunomodulatory agent [58–59].

We have found, that apart from the potential of these group of miRNAs to possible direct activation of interferon response, they also target particular mRNAs involved in specific pathways and cellular processes. Analysis for UGUGU-rich miRNAs ranked at the top of the list following pathways: pathways in cancer, PI3K-Akt signaling, MAP-signaling and hedgehog signaling pathways (Fig 4, S4 Table). Our analysis showed that these type of miRNAs is involved among others in processes such as induction of apoptosis, immune system process, immune response or macrophage activation (S4 Table). Due to the KEGG and PANTHER analyses of miRNAs involvement in this type of biological processes, it appears, that also for IIM-rich miRNAs we could expect the direct recognition of the mRNAs involved in PI3K-Akt signaling and MAP signaling pathways (Fig 4a, S4 Table). The potential involvement of IIM-contained miRNAs in these pathways are also supported by the list of protein class provided by the PANTHER analysis (S4 Table). It was shown that positioning of this 5'-UGUGU-3' motif especially at the 5'- end of the sense strand of siRNAs results in a rapid and enhanced induction

(a)

UGUGU-rich sequences					
miRNAs	KEGG Pathway	Pathway ID	# of Genes (union)	p-value (union)	# miRNAs involved in pathway
miR-3682-5p, miR-2278, miR-1238-5p, miR-3149, let-7b-5p, miR-4455, miR-4717-5p, miR-573, miR-660-3p, miR-378a-5p, miR-599, miR-1226-3p, miR-1270, miR-610, miR-3911, miR-3650, miR-4789-5p, miR-330-5p, miR-941, miR-597, miR-5580-5p, miR-4700-5p, miR-892a, miR-206, miR-595, miR-539-5p, miR-1180-3p, miR-450a-5p, miR-493-3p, miR-4669, miR-4753-3p, miR-624-5p, miR-3657, miR-3148, miR-3177-5p, miR-581, miR-642b-5p, miR-147a, miR-592, miR-545-3p, miR-1228-5p, miR-642a-5p, miR-3152-3p, miR-32-3p, miR-1299, miR-297, miR-187-3p, miR-5010-3p, miR-574-5p, miR-649	Pathways in cancer PI3K-Akt signaling pathway MAP signaling pathway HTLV-I infection Regulation of actin cytoskeleton Focal adhesion Endocytosis Wnt signaling pathway Chemokine signaling pathway Axon guidance RNA transport Ubiquitin mediated proteolysis Dopaminergic synapse Glutamatergic synapse Calcium signaling pathway	hsa05200 hsa04151 hsa04010 hsa05166 hsa04810 hsa04510 hsa04144 hsa04310 hsa04062 hsa04360 hsa03013 hsa04120 hsa04728 hsa04724 hsa04070	186 183 138 137 124 123 107 99 100 80 89 85 82 72 93	2.79e-27 3.43e-25 1.06e-19 1.25e-19 1.77e-18 2.01e-17 5.99e-17 2.31e-15 1.94e-14 1.43e-13 4.09e-13 1.19e-12 1.80e-12 1.86e-12 1.86e-12	43 43 44 40 42 43 41 40 39 40 41 40 43 43 46

(b)

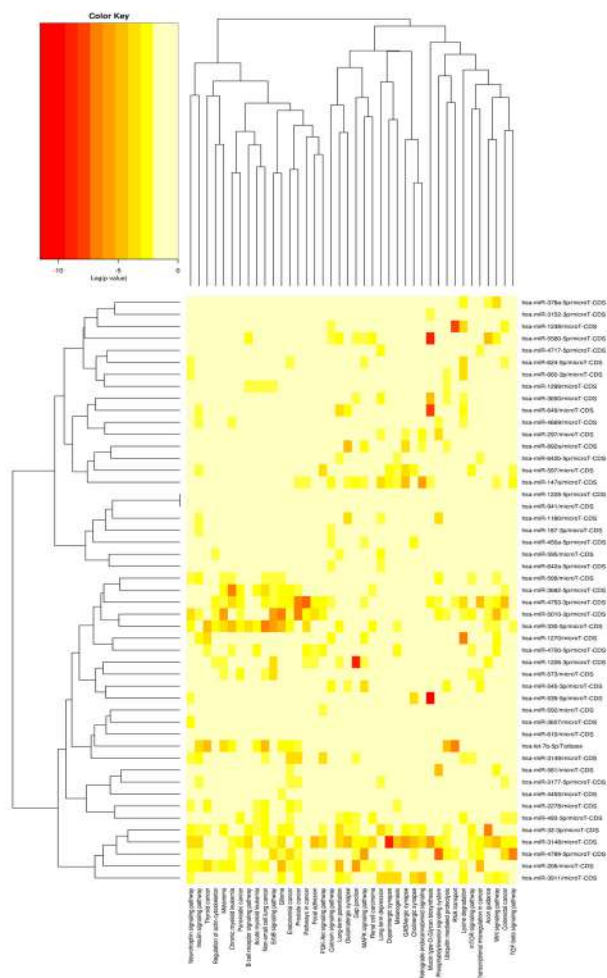


Fig 4. The KEGG pathway analysis for interferon induction motif contained-(IIM) miRNAs. A. The table illustrating the: list of IIM-contained miRNAs (first column); the IDs and KEGG pathways names (second and third column); the number of genes and IIM-rich miRNAs involved associated with the pathways (fourth and sixth column). P-value was given in fifth column as a result of statistical analysis. P-value threshold is considered 0.05. B. IIM-rich miRNAs in predicted pathway heat map. Significant miRNA-pathway interaction $p < 0.001$.

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of type I IFN. Rapid production of IFN β involves thus activation of signaling cascades governed by effectors that are intermediates in the JAK/STAT, mitogen-activated kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways [60–61].

This results indicates that IIM-rich miRNAs, similarly to the cancer-associated miRNAs (CA-miRNAs) are closely connected to cancer [62]. The one of the most prominently observed pathway for these class of IIM- miRNAs- pathways in cancer is highly represented by the gene targets for IIM-miRNAs. This kind of pathway clearly demonstrated the involvement into biological capabilities such as evading of apoptosis, block of differentiation, unlimited replication, increased angiogenesis, sustained ability for invasion and metastasis in malignant transformation [62]. Mitogen –activated protein kinase (MAPK) pathway functions also as integrating signals that affect proliferation, differentiation, survival and migration. The results indicates, that the IIM-rich miRNAs could also be a promising clinical targets for cancer through the MAPK pathway. The phosphatidylinositol 3-kinase(PI3K)-Akt signaling pathway is activated by many types of cellular stimuli or toxic agents and it is involved in regulation of basic physiological cellular functions such as transcription, translation, proliferation, growth or survival [63]. There was shown that serine/threonine kinase Akt/PKB plays significant role in this pathway. Moreover, a impaired activation of the PI3K-Akt pathway has been associated with the development of different types of diseases such as diabetes, mellitus, autoimmunity, and finally- cancer [64].

The characteristic of stem-loop hairpin structure of mature human miRNAs

It is already known that over 50% of the hairpins predicted for the human 16S and 23S rRNA have tetranucleotides loops [65]. The tetraloops are thought to fulfill a variety of functions, including recognition elements for interactions with proteins and other RNAs. They can regulate the activity of a biological system by shifting the equilibrium between alternate structures [66]. It was observed, with the use of the NMR methods that small stem-loops can exist, in solution, in equilibrium with duplex forms [67]. About 70% of these tetraloops have the consensus loop sequences (GNRA) or UNCG (where N = A,C,G or U; and R = A or G. It has been shown that RNA hairpins within these sequences form unusually stable loop conformation [65–67].

Table 5. Occurrence of tetranucleotides motifs connected with hairpin formation in human miRNA sequences. The all miRNAs with the ability to form secondary structure were subjected to the analysis. The searching was performed according to: first—motifs present in loop of the predicted hairpins, second: motifs present in the loop where at least 3 base pair were predicted in the stem and finally-where at least 3 base pair and additionally C-G as a closing pair in the stem were predicted.

Motif	Frequency		
	Sequences		
	Hairpin-formation	3 base-pairs in stem	3 base-pairs and C-G as a closing pair in stem
GGAA	25	21	8
CUUG	10	8	4
GCAA	13	10	2
GUGA	23	17	0
GAAA	19	17	3
GAGA	32	18	1
UUCG	5	5	2
UUUU	28	21	12
UUUG	24	22	6
Total	179	139	38

doi:10.1371/journal.pone.0151246.t005

With Mfold algorithm, we found 1431 (70%) sequences of human miRNAs, able to fold into the hairpin structure, whereas 588 sequences did not show such a propensity. The minimal free energy of these structures (ΔG) falls in the range from -0.1 to -11.1 kcal/mol, however, the most widely represented structures indicate the energy levels from -0.4 to -3.3 kcal/mol [68]. In order to gain better insight into the potential secondary structure of mature miRNAs, we have also analyzed a possibility of the hairpin stem-loop formation. Our calculation revealed that the most frequently motif occurred in miRNAs, is the four nucleotides motif (tetraloop), found in 433 sequences of mature RNAs. Other hairpin-loop of miRNA sequences are: 413, 283 and 114 for three-, five- and six-nucleotides loops, respectively.

Among tetraloops, we specified sequences: UUCG, GAAA, GCAA, GAGA, GUGA, GGAA, CUUG, UUUG—involved in the hairpin loop formation. Many sequences of mature miRNAs can fold exactly into the hairpin loop, with the nucleotides of interest located in the loop, containing minimum 3 base pairs in the stem additionally (Table 5).

We found that 179 miRNAs can form stable hairpin structure, with the 4-nt motifs (Table 5). The most represented motif in human miRNA sequences is GAGA, although GGAA, GAAA or UUUU motifs are also abundant. UUUU motif in the loop, together with the CG as a closing pair in the stem, is most widely represented among the mature human miRNAs (Table 5).

The observation that miRNA can fold into the secondary structure is consistent also with previous reports, that plant miRNA can form secondary structure with free energy of: -9.3 to +1.5 kcal/mol [69]. It directly supports the observation that given miRNAs differ dramatically in terms of the half-life. Thus, the knotty secondary structures of miRNAs makes them nucleases resistant, implicating their long surveillance in the cell. The higher order structure of miRNA can play a crucial role in conformational changes during miRNA-mRNA interactions. This could modulate the pairing and also explain the different degree of genetic regulation for the specific miRNA. The secondary structure could be also extremely important in the mechanism by which sequences for some miRNAs are selected, what can modulate its affinity with their mRNA targets. It can also provide the specificity of miRNAs-mRNA interaction what could be achieved by the presence of miRNA secondary structure, by precluding the possibility of binding of other miRNAs and genomic RNAs with complementary sequences.

Although the mature miRNAs are generally considered as single stranded, there are very few reports suggesting a self-complementarity in mature miRNAs, that has already been observed in more than 50% of mature miRNAs. They are prone to form hairpins and/or homo-duplexes in solution [38, 57–58]. NMR studies have shown that hsa-miR-520h mature strand can fold into hairpin structure or into self-complementary homo-duplex, in higher concentration [70].

Next, we used the ModeRNA program for modelling of miRNA 3D structure, based on templates of related molecule. To find tertiary structures, we searched the PDB database for experimentally confirmed (X-ray, NMR or CD), about 24 nucleotides long RNAs. We have found 168 sequences, which were aligned to human mature miRNA sequences. For further analysis, we only took into account sequences with at least 80% similarity, since only that level provides reliable, homology modeling. The alignment showed 35 miRNAs that were further processed with ModeRNA server (Fig 5a). Positions with identical nucleotides were fixed, whereas remaining positions were modeled by the program. The best matched results we obtained include miR-381-3p with 1R4H RNA, miR-4649-5p with 2KYE and miR-3677-5p with 1Q8N RNA (Fig 5b). Due to the homology modeling, ModeRNA analysis makes our calculation and secondary structure prediction more accurate and give strong support for miRNAs hairpin loop formation hypothesis.

Our CD, NMR and enzymatic probing data for some miRNAs also prove that miRNAs have the intrinsic potential to form secondary structure and that hairpin possibly is a prevailing form of miRNA in the cell [68].

miRNA/RNA structure (PDB)	Alignment	miRNA/RNA structure (PDB)	Alignment	miRNA/RNA structure (PDB)	Alignment
hsa-miR-4649-5p 2KYE:A	UGGGCGAGGGGUGGGCUCUCAGAG -----GAGAGUUGGGCUCUC----- *** * *****	hsa-miR-4725-3p 2K65:A	UGGGGAAGGCUCAGUGUCGGG -----CAGUGUC----- *****	hsa-miR-4656 1R4H:A	UGGGCUGAGGGCAGGAGGCCUCU -----GGGCA--AGCCC----- ***** ** *
hsa-miR-34b-5p 2K65:B	UAGGCAGUGUCAUAGCUGAUUG -----CAGUGUC----- *****	hsa-miR-4720-3p 2OJ7:A	UGCUUAAAGUUGAACCAAGUAU -GCU--GUUGU----- *** *****	hsa-miR-4799-3p 2K65:A	ACUGGCAUGCUGCAUUUAUAUA -----GGCA--CUG----- **** ** *
hsa-miR-381-3p 1R4H:A	UAUACAAGGCAAGCUCUCUGU -----GGGCAAGCCC----- ***** *	hsa-miR-4654 2K65:A	UGUGGGAUCUGGAGGCAUCUGG -----GGCA--CUG----- ***** ** *	hsa-miR-93-5p 2OJ7:A	CAAAGUCUGUUGCUGCAGGUAG -----GCUGUU--GU----- ***** **
hsa-miR-648 2K65:A	AAGUGUGCAGGGCAGCUGU -----GGCACUG-- *****	hsa-miR-635 2K65:A	ACUUGGGCAGUGAAACAAUGUCC -----GGCACUG----- *****	hsa-miR-216a-5p 2K65:A	UAAUCUCAGCUGGCAACUGUGA -----GGCA--CUG----- ***** ** *
hsa-miR-3677-5p 1R4H:A	CAGUGGCAGAGGCCUCGACAGU ---GGGCA-AGCCC----- ** * *****	hsa-miR-5195-5p 2K65:A	AACCCCUAAGGCAACUGGAUGG -----GGCA--CUG----- ***** ** *	hsa-miR-3064-5p 2OJ7:A	UCUGGCUGUUGUGGUGUGCAA ---GCUGUUGU----- *****
hsa-miR-27a-5p 2OJ7:A	AGGGCUUAGCUGCUUGUGAGCA -----GCUG--UUGU----- **** ** *	hsa-miR-597-5p 1K0S:A	UGUGUCACUCGAUGACCACUGU CUGUGU---UCGAU--CCAAG-- ***** ** *	hsa-miR-365b-5p 2K65:A	AGGGACUUUCAGGGGCAGCUGU -----GGCA--CUG----- ***** ** *
hsa-miR-4726-5p 1ZIG:A	AGGGCCAGAGGAGCCUGAGUGG -GGCCGAG--AGCCU----- ***** ** *	hsa-miR-33b-3p 1Q75:A	CAGUGCCUCGCGAGGCGAGCCC ---GGCUCU-CAGUG-AGCC-- * ** *****	hsa-miR-183-5p 2K65:A	UAUGGCACUGGUAGAAUACACU ---GGCACUG----- *****
hsa-miR-550b-3p 2K65:A	UCUUACUCCUCAGGCACUG -----GGCACUG----- *****	hsa-miR-4447 2OJ7:A	GGUGGGGCGUGUGUUU -----GCUGUUGU-- *****	hsa-miR-135b-3p 1R4H:A	AUGUAGGGCUAAAGGCCAUGGG -----GGGC--AAGCCC----- *** ** *
hsa-miR-4260 1R4H:A	CUUGGGGCAUGGAGUCCCA ---GGGCA--AGCCC-- ***** ** *	hsa-miR-199a-3p 2K65:B	ACAGUAGUCGCACAUGGUUA -CAGU--GUC----- ***** ** *	hsa-miR-5095 1AFX:A	UUACAGGCUGAACCACCGCG ---GGUGUGAAC--ACC--- * ** *****
hsa-miR-23b-5p 2K65:B	UGGGUUCUGGGCAGUGCAUUU -----GGCA--CUG----- **** ** *	hsa-miR-34a-5p 2K65:B	UGGCAGGUGCUUAGCUGGUUGU ---CAGUGUC----- *****	hsa-miR-296-3p 2KYE:A	GAGGGUUGGGUGAGGCUCCUC GAGAGUUGGG-----CUCUC- *** ** *
hsa-miR-3670 2K65:A	AGAGCUCACAGCUGUCCUUCUA -----CAG-UGUC----- *** ** *	hsa-miR-34a-5p 2OJ7:A	UGGCAGUGUCUUAGCUGGUUGU -----CGUG--UUGU----- **** ** *	hsa-miR-3177-3p 2K65:A	UGCACGGGACUGGGGACACGU -----GGCACUG----- *****
hsa-miR-4487 2F87:A	AGAGCUGGCUGAAGGGCAG -----GGCUGAAGGGCC----- *****	hsa-miR-6131 1ATW:A	GGCUGGUCAGAUGGGAGUG -GCU--CCAGAUGG-AGGC *** ** *		

STRUCTURE		miRNA	miR-3677-5p	miR-381-3p	miR-4649-5p
	PRIMARY		CAGUGGCCAGAGCCUGCAGUG	UAUACAAGGGCAAGCUCUCUGU	UGGCGAGGGGUGGGCUCUCAGAG
	SECONDARY		<pre> A G C G C C G A G C C G C C U G C A G C A U U 5' C-GCAGUG 3' </pre>	<pre> A A C G G C G U G C A U C A C C G 5' UAU A-U 3' </pre>	<pre> U G G G C G G C G U G C A U A U 5' UGGGCG-CAGAG 3' </pre>
	TERTIARY				

doi:10.1371/journal.pone.0151246.q005

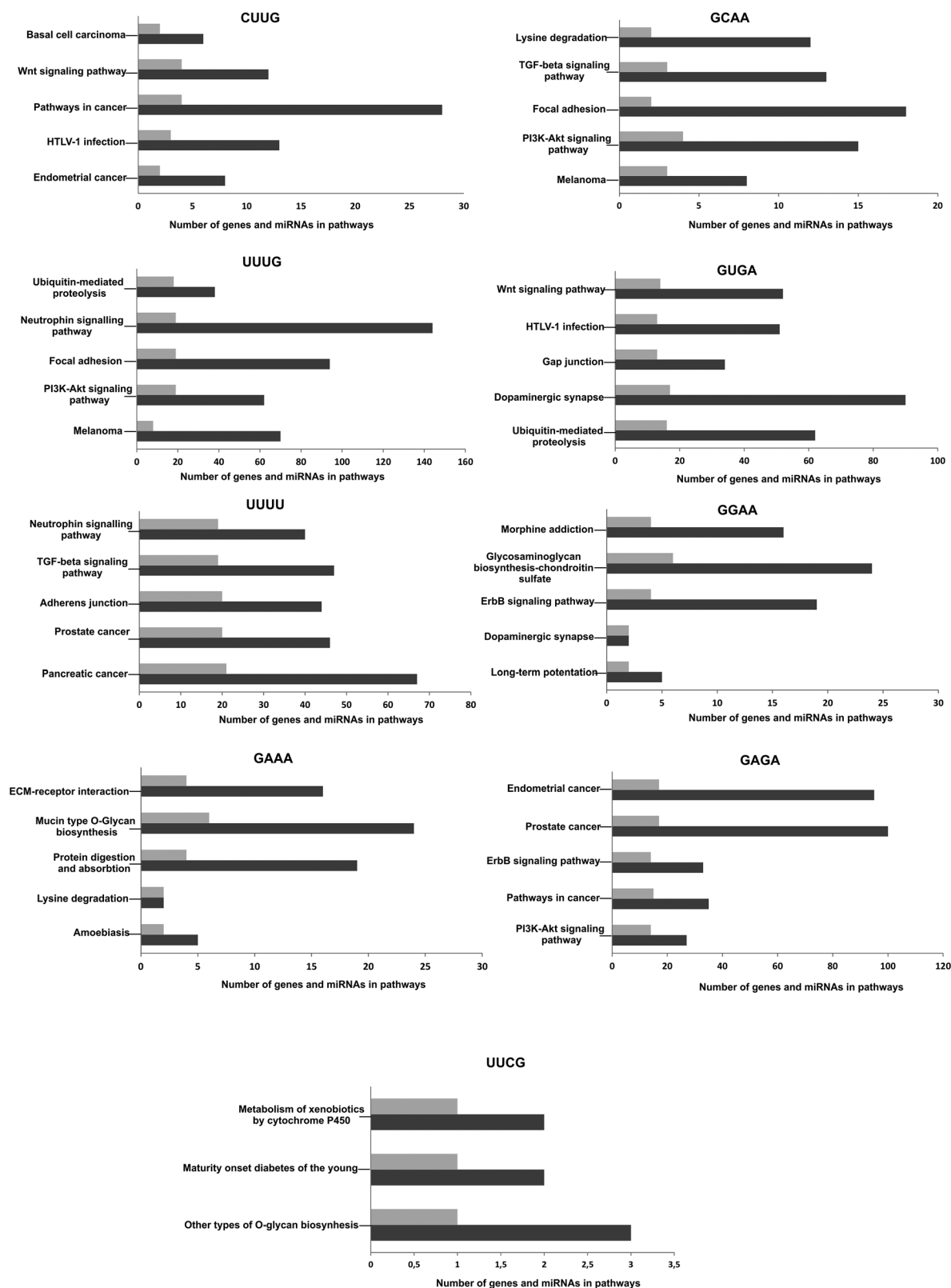


Fig 6. The major class of potential pathways associated with the miRNAs with the stable secondary structure and defined tetraloop. The number of genes connected to the pathways were marked with dark grey, with light grey- the number of miRNAs.

doi:10.1371/journal.pone.0151246.g006

The association of structured miRNAs and the cellular pathways

The pathway analysis revealed that miRNAs contained different sequences in the tetraloop also can cluster into specific group. This clustering approach revealed that miRNAs enriched in CUUG or GUGA sequence in the tetraloop are a regulators of the targets from Wnt signaling pathways. Neutrophin signaling pathway appears to be more associated with miRNA contained UUUG or UUUU motifs, whereas dopaminergic synapse pathways are more targeted by miRNAs with GUGA and GGAA motifs and PI3K-Akt signaling pathway by UUUG and GAGA-enriched miRNAs, respectively (Fig 6, S5 Table).

Conclusions

For a very long time, RNA was considered to be exclusively the carrier of genetic information, but now, the group of RNAs with regulatory function have evolved into the miRNAs, long-noncoding RNAs (lncRNAs), circular RNAs or miRNA-sponges [71].

Although, one miRNA can potentially regulate hundreds of different mRNAs, the majority of transcripts are still actively expressed and translated, which supports the existence of other mechanisms, that counteract miRNA regulation, to achieve homeostasis.

Expression of the gene families or several components of a particular signaling pathway are frequently regulated by miRNAs. The pathway analysis of potential targets based on the sequence, thus the structure of miRNAs could then enhance the probability of identification and verification of the relevant miRNA-target interactions.

Our observation strongly suggest that miRNA persistence is related to biological function, thus better characterization of miRNA structure, stability and associated regulatory mechanism should provide new avenues for the characterization of their biological function. We postulate that, due to the specific sequence features, these molecules can also be involved in very well defined cellular processes depending on theirs sequence contents. Moreover, the unique features encoded in the sequence and in the structure of mature miRNA can be a key to understand the mRNA target recognition.

Supporting Information

S1 Fig. The length heterogeneity of human mature miRNAs.

(DOC)

S1 File. ("mirna_an.py"). Python scripts used for miRNA nucleotide sequences and SSR analysis.

(PY)

S1 Table. The results from the analysis pyrimidines/purines-rich miRNAs using PANTHER classification system. The table presents: top 10 biological processes related to input miRNAs; most significant pathways derived from overrepresentation test and top 10 protein classes related to input miRNAs. +/- shows over—or underrepresentation. Second and third columns contain the number of genes in reference and input list, respectively. P-value threshold is considered 0.05.

(DOC)

S2 Table. Occurrence and relative count of trinucleotide repeats in mature miRNAs.

(DOC)

S3 Table. The results from the analysis of GU-rich miRNAs using PANTHER classification system. The table presents: top 10 biological processes related to GU-rich miRNAs; most significant pathways derived from overrepresentation test and top 10 protein classes related to

GU-rich miRNAs. +/- shows over—or underrepresentation. Second and third columns contain the number of genes in reference and input list, respectively. P-value threshold is considered 0.05.

(DOC)

S4 Table. The results from the analysis of interferon induction motif (IIM)- contained miRNAs using PANTHER classification system. The table presents: top 10 biological processes related to IIM motif-contained miRNAs; most significant pathways derived from overrepresentation test and top 10 protein classes related to IIM motif-contained miRNAs. +/- shows over—or underrepresentation. Second and third columns contain the number of genes in reference and input list, respectively. P-value threshold is considered 0.05.

(DOC)

S5 Table. Molecular KEGG pathways analysis for miRNAs with defined tetraloops in the secondary structure.

(DOC)

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Author Contributions

Conceived and designed the experiments: KR MP AB DW JJ MZB JB. Performed the experiments: KR MP AB DW JJ. Analyzed the data: KR MP AB DW JJ MZB JB. Contributed reagents/materials/analysis tools: KR MP AB DW JJ MZB JB. Wrote the paper: KR MP AB JB.

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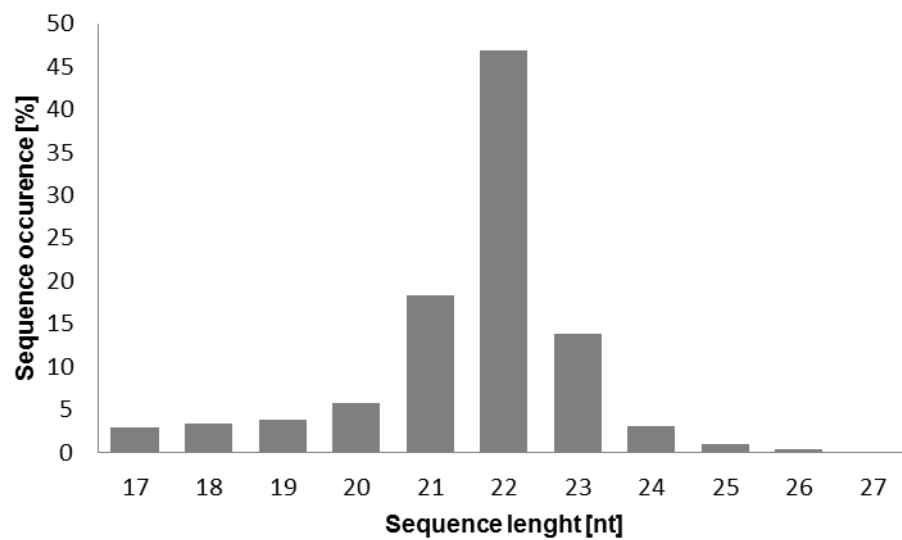
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MATERIAŁY UZUPEŁNIAJĄCE DO PUBLIKACJI

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S1 Fig



S1 Table

GO Biological process	<i>H.sapiens</i> (REF) #	Input #	Fold Enrichment	+/-	P-value
Locomotion	65	10	>5	+	1.20E-03
Neurotransmitter secretion	145	18	>5	+	6.03E-06
Synaptic transmission	331	24	3.02	+	5.77E-04
Nervous system development	823	52	2.63	+	9.35E-08
Neurological system process	1064	64	2.50	+	5.64E-09
Protein phosphorylation	603	35	2.42	+	5.05E-04
Organelle organization	571	33	2.41	+	1.08E-03
Ectoderm development	663	38	2.39	+	2.48E-04
System process	1296	66	2.12	+	2.16E-06
Cell-cell signaling	633	32	2.10	+	1.90E-02
Pathways					
PDGF signaling pathway	138	18	>5	+	2.01E-06
Ras pathway	79	10	>5	+	4.37E-03
Synaptic vesicle trafficking	31	8	>5	+	1.78E-04
Angiogenesis	154	18	4.87	+	1.02E-05
PANTHER protein class					
Basic helix-loop-helix transcription factor	92	10	4.52	+	2.01E-02
Membrane trafficking regulatory protein	119	12	4.20	+	8.54E-03
Membrane traffic protein	366	22	2.50	+	2.17E-02
Kinase	514	30	2.43	+	2.22E-03
Enzyme modulator	1346	62	1.92	+	1.80E-04
Transcription factor	1565	67	1.78	+	7.54E-04
Nucleic acid binding	2297	90	1.63	+	5.17E-04

S2 Table

Repaeat type	Number of repeats	Repaeat type	Number of repeats
ACA	0	GCU	3
ACC	0	GGA	6
AGA	3	GGC	6
AGG	4	GGU	0
AGU	0	GUG	6
AUG	0	GUU	0
AUU	0	UAC	0
CAG	0	UAU	0
CAU	0	UCC	0
CCU	3	UCG	0
CGU	0	UGA	3
CUC	3	UGC	0
CUG	0	UGG	0
CUU	0	UGU	0
GAG	0	UUC	0
GAU	3	UUG	0
GCC	3		
Total 43			

S3 Table

GO Biological process	H.sapiens (REF) #	Input #	Fold Enrichment	+/-	P-value
mRNA 3'-end processing	30	34	2.47	+	6.64E-04
Embryo development	150	134	1.95	+	4.33E-10
mRNA transcription	57	47	1.80	+	3.33E-02
Protein targeting	112	89	1.73	+	2.58E-04
Protein localization	116	89	1.67	+	9.86E-04
Negative regulation of apoptotic process	95	71	1.63	+	1.82E-02
Nervous system development	823	601	1.59	+	3.63E-25
Muscle organ development	288	200	1.51	+	4.30E-06
Cellular protein modification process	1317	909	1.50	+	7.50E-31
Catabolic process	407	277	1.48	+	6.27E-08
Synaptic transmission	331	215	1.41	+	1.50E-04
Pathways					
Opioid proopiomelanocortin pathway	20	24	2.61	+	5.08E-03
Metabotropic glutamate receptor group II pathway	34	40	2.56	+	2.65E-05
Opioid proenkephalin pathway	21	24	2.49	+	1.07E-02
Ionotropic	57	63	2.41	+	1.09E-07
PI3 kinase pathway	49	54	2.40	+	1.86E-06
Hedgehog signaling pathway	22	24	2.38	+	2.12E-02
Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade	33	36	2.38	+	5.57E-04
Axon guidance mediated by netrin	34	37	2.37	+	4.35E-04
p38 MAPK pathway	39	41	2.29	+	3.04E-04
5HT1 type receptor mediated signaling pathway	31	32	2.25	+	5.27E-03
PANTHER protein class					
Serine/threonine protein kinase receptor	19	24	2.75	+	3.01E-03
Protein kinase receptor	33	38	2.51	+	1.15E-04
SNARE protein	42	44	2.28	+	1.90E-04
Voltage-gated sodium channel	36	36	2.18	+	4.53E-03
Sodium channel	36	36	2.18	+	4.53E-03
Ubiquitin-protein ligase	189	163	1.88	+	2.91E-11
Protein kinase	373	224	1.73	+	1.81E-16
G- protein	211	168	1.73	+	5.77E-09
Small GTPase	133	104	1.70	+	6.65E-05
Basic helix-loop-helix transcription factor	92	71	1.68	+	6.48E-03

S4 Table

GO Biological process	H.sapiens (REF) #	Input #	Fold Enrichment	+/-	P-value
mRNA 3'-end processing	30	17	2.77	+	4.91E-02
Embryo development	150	73	2.38	+	1.11E-08
Protein targeting	112	48	2.10	+	6.51E-04
Protein phosphorylation	603	242	1.96	+	9.82E-20
mRNA splicing	183	69	1.84	+	4.75E-04
Angiogenesis	198	73	1.80	+	5.32E-04
Induction of apoptosis	129	47	1.78	+	3.86E-02
Nervous system development	823	290	1.72	+	4.05E-16
Immune system process	1391	224	1.69	+	1.59E-02
Immune response	518	61	1.58	+	2.60E-04
Macrophage activation	167	11	1.32	+	8.18E-04
Pathways					
PI3 kinase pathway	49	38	3.79	+	1.73E-09
Hedgehog signaling pathway	22	16	3.56	+	3.07E-03
Axon guidance mediated by semaphorins	22	15	3.33	+	1.12E-02
p38 MAPK pathway	39	26	3.26	+	4.97E-05
Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade	33	20	2.96	+	4.07E-03
Oxidative stress response	27	16	2.90	+	3.17E-02
Interferon-gamma signaling pathway	39	22	2.75	+	2.49E-02
EGF receptor signaling pathway	130	72	2.71	+	3.28E-11
FGF signaling pathway	123	68	2.70	+	1.62E-10
Ras pathway	79	34	2.10	+	1.07E-02
PANTHER protein class					
TGF-beta receptor	15	12	3.91	+	1.76E-02
mRNA polyadenylation factor	21	14	3.26	+	3.17E-02
Protein kinase receptor	33	18	2.67	+	4.72E-02
HMG box transcription factor	45	22	2.39	+	4.70E-02
Non -receptor serine/ threonine protein kinase	272	124	2.22	+	2.47E-13
Kinase modulator	194	86	2.17	+	2.05E-08
Protein kinase	373	165	2.16	+	7.80E-17
Chromatin/chromatin-binding protein	184	80	2.13	+	2.11E-07
Kinase inhibitor	73	31	2.08	+	3.58E-02
G-protein	211	78	1.81	+	2.06E-04

S5 Table

Motif in a loop	KEGG pathway(pathawy ID)	p-value (union)
CUUG	- Basal cell carcinoma (hsa05127) - Wnt signaling pathway (hsa04310) - Pathways in cancer (hsa05200) - HTLV-1 infection (hsa05166) - Endometrial cancer (hsa05123)	0.0002603625 0.0003335464 0.001969395 0.002748364 0.01658278
UUUG	- Ubiquitin-mediated proteolysis (hsa04120) - Neurotrophin signalling pathway (hsa04722) - Focal adhesion (hsa04510) - PI3K-Akt signaling pathway (hsa04151) - Melanoma (hsa05218)	1.09105E-33 6.568774E-28 6.665626E-27 1.049143E-24 1.164063E-18
GCAA	- Lysine degradation (hsa00310) - TGF-beta signaling pathway (hsa04350) - Focal adhesion (hsa04510) - Ubiquitin-mediated proteolysis (hsa04120) - Wnt signaling pathway (hsa04310)	3.156403E-09 2.637396E-06 0.0005666272 0.001157976 0.001927753
GUGA	- Wnt signaling pathway (hsa04310) - HTLV-1 infection (hsa05166) - Gap junction (hsa04540) - Dopaminergic synapse (hsa04728) - Ubiquitin-mediated proteolysis (hsa04120)	9.005047E-13 5.368155E-10 6.428566E-10 6.428566E-10 7.76398E-10
UUUU	- Neurotrophin signalling pathway (hsa04722) - TGF-beta signaling pathway (hsa04350) - Adherens junction (hsa04350) - Prostate cancer (hsa05215) - Pancreatic cancer (hsa05212)	9.533143E-35 9.885926E-28 1.509373E-25 1.270366E-24 1.286761E-22
GGAA	- Morphine addiction (hsa05032) - Glycosaminoglycan biosynthesis-chondroitin sulfate (hsa00532) - ErbB signaling pathway (hsa04012) - Dopaminergic synapse (hsa04728) - Long-term potentiation (hsa04720)	3.582207E-06 5.061091E-06 9.824829E-06 0.0002367772 0.001532743
GAAA	- ECM-receptor interaction (hsa04512) - Mucin type O-Glycan biosynthesis (hsa00512) - Protein digestion and absorption (hsa04974) - Lysine degradation (hsa00310) - Amoebiasis (hsa05146)	<1E-16 <1E-16 4.440892E-16 4.440892E-16 1.287859E-14
UUCG	- Metabolism of xenobiotics by cytochrome P450 (hsa00980) - Maturity onset diabetes of the young (hsa04950) - Other types of O-glycan biosynthesis (hsa00514)	4.778008E-09 0.00798776 0.04455185
GAGA	- Endometrial cancer (hsa05123) - Prostate cancer (hsa05215) - ErbB signaling pathway (hsa04012) - Pathways in cancer (hsa200) - PI3K-Akt signaling pathway (hsa04151)	5.004043E-25 8.307837E-17 1.206676E-15 2.729161E-15 1.464602E-14

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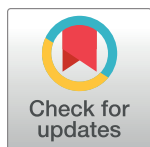
RESEARCH ARTICLE

Nano-mediated delivery of double-stranded RNA for gene therapy of glioblastoma multiforme

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Abstract

Glioblastoma multiforme (GBM) is the most common type of malignant gliomas, characterized by genetic instability, intratumoral histopathological variability and unpredictable clinical behavior. Disappointing results in the treatment of gliomas with surgery, radiation and chemotherapy have fueled a search for new therapeutic targets and treatment modalities. Here we report new approach towards RNA interference therapy of glioblastoma multiforme based on the magnetic nanoparticles delivery of the double-stranded RNA (dsRNA) with homologous sequences to mRNA of tenascin-C (TN-C), named ATN-RNA. The obtained nanocomposite consisted of polyethyleneimine (PEI) coated magnetic nanoparticles conjugated to the dsRNA show high efficiency in ATN-RNA delivery, resulting not only in significant TN-C expression level suppression, but also impairing the tumor cells migration. Moreover, synthesized nanomaterials show high contrast properties in magnetic resonance imaging (MRI) and low cytotoxicity combining with lack of induction of interferon response. We believe that the present work is a successful combination of effective, functional, non-immunostimulatory dsRNA delivery system based on magnetic nanoparticles with high potential for further application in GBM therapy.

Introduction

Although accounting for less than 2% of adult cancers, gliomas are the most common form of malignant primary brain tumor in adults.[1] Glioblastoma multiforme constitutes 25% of all malignant nervous system tumors and the median overall survival remains around 12–15 months, even after combination treatments of cytoreductive surgical resection, radiotherapy, and adjuvant oral chemotherapy with temozolomide[2,3,4,5] The recent medical treatment strategies have been progressing toward individualized therapy and many targeted drugs have

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been investigated, but the identification of molecular biomarkers in GBM as well as the novel drugs delivery strategies are still of considerable therapeutic importance.

Among recently developed new methods towards GBM treatment, a lot of attention has been drawn to gene therapy.[6,7,8] One of the strategy is RNA interference (RNAi), a general term referring to post-transcriptional gene silencing mediated by either degradation or translation arrest of target RNA. RNAi is triggered by gene silencing mechanism that is initiated with the introduction of dsRNA into a cell.[9] Specifically designed RNAi molecules can target mRNAs and initiate their degradation. We have previously reported that dsRNA synthesized by *in vitro* method can cleave the targeted mRNA and silenced the gene of interest expression.[10,11] We used double-stranded 164 nucleotides long double stranded RNA specific for tenascin-C (TN-C) mRNA. That agent, called ATN-RNA, induces RNAi pathway to degradation of TN-C, the extracellular matrix (ECM) protein which is highly overexpressed in brain tumor tissue. The technology was coined interference RNA intervention (iRNAi). With the approach ATN-RNA was administrated locally into the tumor's cavity during standard neuro-surgical procedures. Although the obtained clinical outcome from the experimental therapy has been found as the very promising, resulting with the improving of the quality of patients life, as well as the increasing of the overall survival, we are still far from the conclusion that the used approach can be the most effective GBM treatment. Lack of an effective delivery method for dsRNA and the instability of the nucleic acids during and/or after the delivery are still the major difficulties in gene silencing studies.

In the last decade, nanotechnology has been widely applied in synthesis of nanoscale materials with main focus on cancer therapy.[12,13,14] Especially a lot of work has been put on the development of new, multifunctional delivery systems which could overcome drawbacks of conventional treatment methods and improve existing therapies by selective enrichment of active substances in diseased tissue structures, an increase in bioavailability, the decrease of the active substance degradation and, above all, the reduction and/or avoidance of unwanted side effects.[15,16] Magnetic nanoparticles, mostly based on magnetite (Mag) have drawn so much considerable attention in the field of smart materials due to their unique advantages over other materials.[17,18] They are easy to produce and the synthesis cost is relatively small in comparison to other nanomaterials.[19] Moreover, they are physically and chemically stable, biocompatible and environmentally friendly. Therefore, they have been frequently used in gene therapy as non-viral gene carriers.[20] Another important point in the application of magnetic nanoparticles is the fact that they can be employed as contrast agents in MRI (Magnetic Resonance Imaging) what makes them a very powerful diagnostic tool.[21] Indeed, the magnetic nanoparticles have been successfully applied also in MRI of a brain tumor as well as in magnetic hyperthermia resulting in reducing of brain tumor mass.[22,23] Recently, they have been also used in gene therapy for delivery of siRNA against survivin but reported results showed moderate mortality of the cells.[24]

In the paper, we demonstrate application of magnetic nanoparticles as a multifunctional carrier for a double-stranded RNA with contrast properties in MRI. We used a previously reported ATN-RNA sequence shown to significantly reduce the expression level of TN-C. The conjugation of nanoparticles with ATN-RNA showed the more effective TN-C downregulation followed by the substantial impairment of the migration properties of cancer cells regarding the routinely used transfection agent. Furthermore, obtained nanocomposites and their complexes with ATN-RNA did not show the toxic properties and consequently, they do not stimulate the interferon induction and innate immune response. Therefore, our results shed new light on application of multifunctional magnetic nanoparticles in gene therapy of GBM utilizing RNAi approach and point promising candidate for further studies.

Materials and methods

All reagents and solvents were of reagent-grade quality. For all experiments, Milli-Q deionized water (resistivity $18 \text{ M}\Omega \times \text{cm}^{-1}$) was used.

Synthesis and labeling of Mag@PEI nanoparticles

Magnetic nanoparticles coated with PEI (Mag@PEI) were synthesized according to a previously reported protocol with small modifications. Briefly, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (Sigma-Aldrich) (135 mg, 0.5 mmol) were mixed with $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (Sigma-Aldrich) (50 mg, 0.25 mmol) in water (5 mL) and degassed with N_2 . The mixture was heated up to 80°C followed by addition of 1 mL of working solution (0.5 g of 25-kDa branched polyethyleneimine (PEI-25_{Br}), 250 μL of Capstone FS-65 fluorosurfactant (Du Pont) and 2.5 mL of NH_4OH dissolved in 10 mL of Milli-Q water) and constant heating was maintained for 120 min. After cooling down the mixture of nanoparticles was washed with water (2 x 150 mL) and collected by an external magnet. Finally, the NPs were redispersed in 10 mL of water. The Mag@PEI concentration was determined spectrophotometrically in terms of the iron content in an aqueous suspension of the stock nanomaterial by complexation with 1,10-phenanthroline (Sigma-Aldrich) as described previously.[25]

Nanoparticles conjugated with fluorescent dye ATTO 550 were prepared as described elsewhere [26] Briefly, 2 mL of Mag@PEI nanoparticle suspension in water (2.25 mg Fe/mL) was mixed with 490 μL 0.1 M borate buffer, pH 8.5, and 10 μL solution of ATTO 550 NHS-ester (Sigma-Aldrich) in DMSO (10 mg/mL). The resulting suspension was incubated o/n at room temperature and dialyzed against water using Slide-A-Lyzer Dialysis Cassette G2 (Thermo Fisher Scientific) with a cut-off at 3500 MW.

Physical characterization

For transmission electron microscopy (TEM) a small amount of the sample was placed on a copper measuring grid (Formvar/Carbon 200 Mesh made by TedPella (USA)) after 5 minutes of sonication in deionized water. Then, the sample was dried in a vacuum desiccator for 24 hours. TEM images were recorded on a JEM-1400 microscope made by JEOL (Japan) at an accelerating voltage of 120 kV. The ImageJ software (Bethesda, MD, USA) was used to process TEM micrographs in order to analyze the size of nanoparticles. Magnetic measurements were performed on SQUID magnetometer at 5 K and 300 K. FT-IR spectra were recorded on Bruker Tensor 27 spectrometer in KBr pellets. Multiple light scattering (MLS) measurements were performed on Turbiscan Lab produced by Formulacion SA in 10 mL vials. Zeta (ζ) potential measurements were carried out using Zetasizer Nano-ZS ZEN 3600 produced by Malvern Instruments Ltd (UK). The experimental setup for magnetic resonance imaging studies consisted of an Agilent 9.4 T MRI preclinical scanner equipped with a 120 mm gradient coil (2 mT/m/A) and 30 mm millipede coil. In order to perform a quantitative T_2 relaxation experiment (at 18°C) we employed MEMS protocol. Each echo is acquired after an excitation pulse with an increasing echo time. The parameters of the data acquisition were as follows: field of view 15 mm (FOV), matrix size 128×128 , Gaussian-shaped pulse with 2048 μs length. The echo time was set to 4 and 10 ms in two separate experiments. In total, 16 consecutive images with varied echo time were acquired for each sample. The initial analysis was performed in VnmrJ 4.2 revision software (Santa Clara, CA, USA). The raw relaxation data points were collected from an average intensity obtained from circular Region of Interests (ROIs) and then analyzed using Origin 8.5 software (Origin Lab, Northampton, MA, USA) using simple single exponential decay function. In order to prepare MRI agarose-based phantom, nanoparticles were suspended in a hot 2% agarose solution. The hot solution was finally transferred to 10 mm plastic vials and left for full agarose gelation.

ATN-RNA preparation

ATN-RNA was synthesized *in vitro* as it has been already published.[11] ATN DNA harboring plasmid was cleaved by EcoRI and HindIII restriction enzymes (Promega). The two strands of RNA were transcribed separately with T3 and T7 RNA polymerases from MEGAscript Transcription Kits (Ambion). Hybridization of RNA was performed in a buffer containing 20 mM Tris-HCl, pH 7.5 and 50 mM NaCl. The reaction was carried out for 3 min at 95°C, 30 min at 75°C and at the end slowly cooled down to the room temperature.

Sequence of ATN-RNA (US Patent US 8,946,400 B2)

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5' CAAGCGACAGAGUGGGUGAACGCCACCCUGCCAGAAGAGAACCAGCCAGUGGUGUU
UAACCACGUUUACAACAUCAAGCUGCCAGUGGGAUCCAGUGUUCGGUGGAUCUGGAGUCA
GCCAGUCCCCUCUUCUGGACCGGGCGGAAGUCUCGGGCGCU 3'

3' GUUCGCUGUCUCACCCACUUGCGGUGGGACGGUCUUCUCUUGGUCGGUCACCACAA
AUUGGUGCAAAUGUUGUAGUUCGACGGUCACCCUAGGGUCACAAGCCACCUAGACCUCAGU
CGGUCACCCUCUUCUGGACCGGGCGGAAGUCUCGGGCGCU 5'

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Preparation of Mag@PEI/ATN-RNA complexes

The binding ability of ATN-RNA to Mag@PEI was performed by the gel retardation assay and UV-Vis spectrophotometry. To prepare Mag@PEI/ATN-RNA complexes, 1 µg of ATN-RNA was mixed with Mag@PEI with a series of Fe weight ratios (1, 2, 3, 4, 5, 8, 10 Mag@PEI to ATN-RNA wt:wt ratio) in the nuclease-free water, and incubated for 30 min at RT, allowing for sufficient binding of dsRNA molecules with the Mag@PEI. The Mag@PEI/ATN-RNA complexes were loaded onto 1% agarose gel for electrophoresis in TAE buffer at a constant voltage of 70 V for 30 min to visualize the ATN-RNA bands using a Digital Imaging and Analysis System II (Serva). SimplySafe fluorescent stain was used for detecting RNA in agarose gel. Meanwhile, the formed Mag@PEI/RNA complexes suspension was centrifuged (10000 rpm, 10 min) and the prepared supernatant was analyzed spectrophotometrically (NanoDrop 2000, Thermo Scientific) at A260 nm to reveal ATN-RNA content.

Cell culture

The study was performed on a human U-118 MG cell line (ATTC) derived from a glioblastoma multiforme. Adherent cell growth was carried out in a Dulbecco's Modified Eagle Medium (DMEM, Gibco) High Glucose supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 1% penicillin-streptomycin antibiotic (Sigma-Aldrich). Cells were grown at 37°C in a 95% humidified chamber with 5% CO₂.

Transfection of GBM cells

ATN-RNA transfection was performed with Mag@PEI or Lipofectamine (Invitrogen) as a carrier. The procedure was carried out on the day-old cell culture seeded on 12 or 96-well plates in the supplemented medium. In a case of the 12-well plate, 150,000 cells were seeded in 1 mL of medium, for 96-well it was 10,000 cells in 200 µL. Once the cells have reached 75–90% confluency, the medium was removed, cells were washed with phosphate-buffered saline (PBS, Sigma-Aldrich) buffer and an unsupplemented culture medium in the amount reduced by the volume of the transfection mixture was added. Transfection complexes containing Mag@PEI nanoparticles were prepared by mixing Mag@PEI suspension (100 µg Fe/mL) with ATN-RNA solution (100 ng/µL) in serum and supplement-free DMEM with an iron-to-RNA ratio of 3:1 (w/w). The mixture was further incubated at RT for 30 min to allow the components to

assemble. After this time, the volumes of the prepared complexes corresponding to the proper ATN-RNA final concentration (10, 25, 50 and 100 nM) were transferred to the wells.

Lipofection was performed according to the manufacturer's recommendations. Two separate mixtures, OptiMEM (Gibco) with Lipofectamine and OptiMEM with ATN-RNA, were incubated for 5 min at the room temperature. After that, both solutions were combined. The prepared reaction mixture was incubated 20 min at the room temperature and then transferred to the appropriate wells. In addition, cells treated with Lipofectamine or Mag@PEI carriers only were used as controls.

Cellular uptake of transfection complexes

To track the magnetic complexes in the cell, ATN-RNA was fluorescently labelled with fluorescein, according to the manufacturer's protocol (Label IT Tracker Intracellular Nucleic Acid Localization Kits, Mirus Bio, USA). The final concentration of the fluorescein containing RNA was quantified on a spectrophotometer (NanoDrop 2000). The fluorescently labelled Mag@PEI nanoparticles were then used to assemble complexes with fluorescently labelled ATN-RNA, as described in the previous section. U-118 cells (2.5×10^4 cells/well) were plated onto an 8-well Nunc Lab-Tek Chamber Slide (Thermo Fisher Scientific) and cultured for 24 h. Next, 50 μ L of Mag@PEI/ATN-complexes (100 nM) was added and incubated with the cells at 37°C for 24 h.

For visualization under a confocal microscope, the cells were fixed with 4% formaldehyde (Sigma-Aldrich) in phosphate-buffered saline (PBS) for 15 min. The cell membranes were stained with ConcanavalinA-FITC (Life Technologies) at a concentration of 50 μ g/mL, the F-actin fibers were stained with Oregon Green 488 phalloidin (Molecular Probes) at a concentration of 165 nM and the cell nuclei were stained with Hoechst 33342 (Molecular Probes) at a concentration of 8 μ M. Cells were imaged using a confocal laser scanning microscope (Olympus FV1000, Japan). Image acquisition and analysis were performed with a 60x objective, a 1.4 oil immersion lens and FV10-ASW software (Olympus). Images of the Mag@PEI were visualized using 559 nm excitation and 570–590 nm emission filters, whereas ATN-RNA was visualized using 488 nm excitation and 495–545 nm emission filters. To visualize the cell membranes or cytoskeleton, 488 nm excitation and 495–545 nm emission filters were applied. The Hoechst fluorescence was detected using 405 nm excitation source and 425–475 nm emission filters. The 3D-scan of the sample was performed using Z-stack mode measurements and analyzed with Imaris software (Bitplane).

Cytotoxicity assays

The WST-1 cell proliferation assay, as well as fluorescent cell viability assay, were carried out to assess the cytotoxicity of the nanoparticles and complexes with ATN-RNA.

In WST-1 assay (Takara), U-118 cells were seeded at the density of 10,000 cells per well in the 96-well plate. After 24 hours, medium containing an increasing concentration of tested Mag@PEI/ATN-RNA complexes (in terms of RNA concentration) was added to each well and the cells were incubated for 24 h. After incubation, 10 μ L of the WST-1 Cell Proliferation Reagent was added to each well and incubated for 4 hours. After this time, the absorbance at 450 nm (reference wavelength 620 nm) was recorded against the background control, using a multiwell plate reader (Zenyth, Biochrom) and the cell viability was expressed as the respiration activity normalized to untreated cells.

In Live/Dead assay, the U-118 cells were seeded in black polystyrene 96-wells flat bottom plate with the transparent bottom (Greiner Bio-One GmbH) at densities of 10 000 cells per well. After 24 h, medium containing an increasing concentration of tested Mag@PEI/

ATN-RNA complexes (in terms of RNA concentration) was added to each well. Following 24 h exposure to the complexes, cells were incubated with 2 μ M calcein AM (Thermo Fisher Scientific), 2 μ M ethidium homodimer-1 (Thermo Fisher Scientific) and 8 μ M Hoechst 33342 containing PBS (100 μ L/well) during 30 minutes at 37°C. Finally, the cells were analyzed with the IN Cell Analyzer 2000 (GE Healthcare Life Sciences, UK). Viable cells were imaged using the FITC/FITC excitation/emission filters while for the dead cells the TexasRed/TexasRed ex/em filter combination was applied. DAPI/DAPI was applied to detect the Hoechst 33342 blue signal. A minimum of 20 fields was imaged per well with a 20x magnification. Analysis of the collected images was performed with the IN Cell Developer Toolbox software (GE Healthcare Life Sciences, UK) using in-house developed protocol. First, the total cell number was retrieved from the DAPI images by means of defining and counting the nuclei. Subsequently, the number of viable cells from the FITC images and the number of dead cells from the TexasRed images were determined.

Nucleic acid extraction and quantification

Total RNA was extracted from cell lines with TRIZOL solution (Invitrogen) according to the Chomczynski's procedure.[27] Obtained RNA was purified from DNA residues with the DNA-free DNA Removal Kit (Ambion) following the manufacturer's instructions. The procedure was completed by examining the quantity and quality of the resulting RNA solution. The concentration was measured spectrophotometrically at a wavelength of $\lambda = 260$ nm by Nano-Drop 2000. The degree of possible material degradation was verified by an electrophoretic separation in the 1% agarose gel. Purified RNA was a template for complementary DNA (cDNA) synthesis. Reverse transcription was performed using 500 ng of material with the Transcriptor High Fidelity cDNA Synthesis Kit (Roche) according to the attached procedure.

qRT-PCR

The quantitative reverse transcriptase real-time PCR (qRT-PCR) was performed with the LightCycler480 using LightCycler480 Probes Master and Universal Probe Library (UPL) Set for Human (Roche). Primers with probes were designed by the Universal Probe Library Assay Design Center (<https://qpcr.probefinder.com/organism.jsp>). Primers sequences are given in Table 1. The efficiency of primers was estimated on the standard curve with series of 2-fold dilutions. The qRT-PCR proceeded under the following conditions: an initial 5 min preincubation at 95°C, 45 cycles of denaturation in 95°C for 10 sec, annealing at 55°C for 30 sec and extension at 72°C for 10 sec. Hypoxanthine phosphoribosyltransferase (HPRT) was used as the endogenous control. The LightCycler480 Software release 1.5.1.62 allowed for an analysis basing on the E-method (Roche) expression level. All experiments were performed in triplicates.

Western blot analysis

Transfected cells were lysed by sonication. 150 μ g and 10 μ g of denaturated protein extracts were separated on 12% and 15% SDS-PAGE (SDS-polyacrylamide gel electrophoresis) for tenascin-C and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) detection respectively. Spectra Multicolor Broad Range Protein Ladder (Thermo Fisher Scientific) was used as the size marker. Subsequently the wet transfer onto PVDF membrane with 0.45 μ m pore size (GE Healthcare) was performed in transfer buffer (25 mM Tris base, 190 mM glycine, 20% methanol). The membranes were placed in the SNAP i.d. 2.0 apparatus (EMD Milipore), where it was blocked with a 0.5% solution of skimmed milk in PBS. For detection of tenascin-C the TNC polyclonal antibody (H-300, Santa Cruz) was used, while GAPDH was detected using monoclonal antibody (0411, Santa Cruz). Antibodies were diluted 1:500 in 3% BSA (Sigma-

Table 1. Primer sequences for qRT-PCR.

Primer Sequence 5' → 3'	UPL
RIG1_L GGCAAGTCCCGCTGTAAAC	42
RIG1_R TTGGTATCTCCTAATCGCAAAAG	
OAS1_L CATCCGCCTAGTCAAGCACT	87
OAS1_R CAGGAGCTCCAGGGCATAC	
OAS3_L TCCCATCAAAGTGATCAAGGT	41
OAS3_R ACGAGGTCGGCATCTGAG	
TLR3_L TGGATATCTTTGCCAATTCATCT	80
TLR3_R ATCTTCCAATTGCGTGAAAAC	
INTY_L GGCATTTTGAAGAATTGGAAAG	21
INTY_R TTGGATGCTCTGGTCATCTT	
GPX_L CAACCAGTTTGGGCATCAG	77
GPX_R TCTCGAAGAGCATGAAGTTGG	
TNC_L CCGGACCAAAAACCATCAGT	76
TNC_R GGGATTAATGTCGGAAATGGT	
HPRT_L TGACCTTGATTTATTTTGCATACC	73
HPRT_R CGAGCAAGACGTTTCAGTCCT	

<https://doi.org/10.1371/journal.pone.0213852.t001>

Aldrich). Membranes were incubated with the primary antibody for 10 min or overnight,. Afterwards, secondary anti-rabbit IgG and anti-mouse IgG antibodies conjugated with horse-radish peroxidase (HRP) (Sigma-Aldrich) were used. Detection of a protein was carried out with WesternBright Sirius Chemiluminescent Detection Kit (Advansta). Intensity of individual bands was analyzed qualitatively by Multi Gauge ver. 2.0 (Fujifilm).

Cell proliferation and migration assays

Real-time cell proliferation and migration were monitored by measuring changes in electrical impedance using xCELLigence RTCA DP system (ACEA Biosciences). In the first step, the background impedance of a culture medium in plates was measured. In proliferation experiments, the 16-well plate with incorporated sensor electrode arrays (E-plate) were seeded with 10,000 cells per well in a final volume of 200 μ L of medium. Next, the impedance was measured at 15-minute intervals for 72 h. The transfection was performed 24 h after cell seeding. For migration assays, the 16-well plate consisting of two chambers with a microporous polyethylene terephthalate (PET) membrane containing microfabricated gold electrode arrays on the bottom side of the membrane between them (CIM-plate) was used. 10,000 cells per well were seeded in an upper chamber in unsupplemented medium and moved towards a bottom chamber filled with supplemented medium. The impedance was measured at 15-minute intervals for 96 h. The seeded cells were transfected 24 h before experiment. Obtained cell index (CI) values were entered to the GraphPad Prism ver. 5.1 (GraphPad Software, Inc., La Jolla, CA, USA) software and used to calculate half maximal inhibitory concentrations (IC_{50}) of effecting cell proliferation. In case of transwell migration assay, obtained CI values from each experimental condition were plotted against time, fitted to four-parameter logistic non-linear regression model and the half-time of migration (half maximal effective time, ET_{50}) was calculated.

Scratch assay

Cell migration scratch assay was performed on 12-well plate, with 200,000 cells per well seeded in a supplemented medium. Cells were grown for 24 h under optimal growth conditions.

Next, the transfection was performed and the plate was incubated for another 24 h. After this time the medium was replaced and "wound" was created in the monolayer of cells covering the well. The scar effect was obtained by scraping cells in a straight line using a 200 μ L tip. From that moment, images of the cultured cells at 12 h intervals were taken for 48 h using a fluorescence microscope at 5x magnification. The analysis of the degree of scarring of individual "wounds" was carried out by computer using the Tscratch program (CSElab).

Statistical analysis

Experimental results were subjected to statistical evaluation using GraphPad Prism ver. 5.1. Values presented are an average of three biological along with three technical replicates as mean values $\pm$ standard error of the mean (SEM). Differences between mean values of tested samples and controls were compared with the analysis of variance (ANOVA) extended by Tukey or Bonferroni post-hoc tests. Statistically significant results were obtained at the level of: * for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$; no statistical significance for $p \geq 0.05$.

Results and discussion

Synthesis and characterization of nanoparticles

In the first step (Fig 1), the PEI coated magnetic nanoparticles (Mag@PEI) were prepared in a straightforward manner *via* previously reported procedure in one step protocol from iron III and II chlorides in the molar ratio 2:1 in the presence of polyethyleneimine and surfactant.[25]

The morphology of obtained nanocomposites was investigated by means of TEM. The particles were spherical in shape, however, some aggregations were observed (Fig 2A). The obtained average size of magnetic nanomaterials ranged between 8 to 12 nm. In order to confirm the successful coating with PEI, the FT-IR spectrum was recorded. The spectrum showed a peak at 585 cm^{-1} which was assigned Fe-O bond. Characteristic signals at 1080 cm^{-1} and 1330 cm^{-1} were attributed to C-N stretching vibration from the PEI. The peak observed at 1575 cm^{-1} corresponds to N-H bending vibration from amine moieties present in polyethyleneimine structure. Moreover, the signals from CH_2 groups were observed at 2820 cm^{-1} and 2950 cm^{-1} , respectively. Thus, FT-IR proved attachment of PEI at the surface of magnetic nanoparticles (Fig 2B).

Since PEI molecules bound to magnetite nanoparticles by electrostatic interaction and the negative charge on the surface of the particles is converted to positive charge, zeta potential of Mag@PEI NPs was measured. Performed studies revealed that zeta potential of Mag@PEI NPs was +40.6 mV what indicated the high colloidal stability of nanocomposites and additionally confirmed successful functionalization of magnetic nanoparticles (Fig 2D). Encouraged by the results obtained from zeta potential measurement we also investigated the colloidal stability of Mag@PEI NPs in real-time by Multiple Light Scattering (MLS) using Turbiscan apparatus.

This technique provides the information about TSI global index which is a sum of all destabilization processes occurring in the sample. However, one needs to take into account that the lower the TSI index, the more stable the sample. The investigation of kinetic destabilization for Mag@PEI NPs in water was monitored over a period of 24 h. The TSI index changed over time from 0.6 to 6.8 for 4 h and 24 h, respectively, what proved that the Mag@PEI NPs had high colloidal stability and sedimentation process occurred only in small extent (Figure A in S1 File). The magnetic properties of obtained materials were measured by means of SQUID. The sample exhibited superparamagnetic behavior, as evidenced by the lack of hysteresis loop and blocking temperature of 155 K. Obtained nanomaterial had high magnetic saturation above 40 emu/g at 300 K, which is important for its further guidance by external magnetic

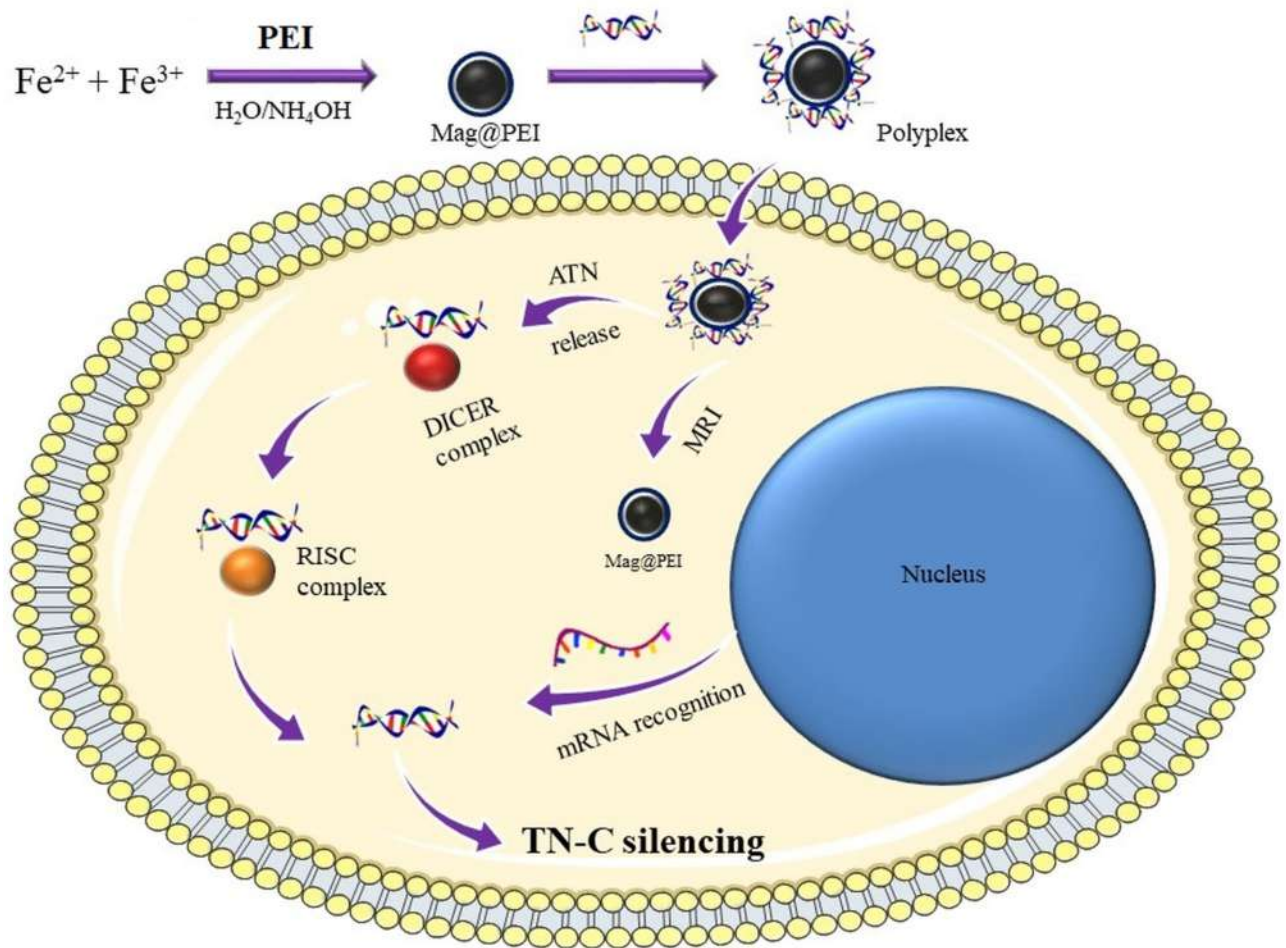


Fig 1. Schematic diagram of preparation of Mag@PEI /ATN-RNA complexes and its application in RNAi therapy of GBM cells.

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field and during magnetic separation. Moreover, the sample was easy handled by an external magnet (Fig 2C).

Furthermore, contrast properties of synthesized magnetic nanocarriers and their potential application in magnetic resonance imaging were assessed. In order to avoid drift of magnetic nanomaterial in the high magnetic field, the suspensions of nanoparticles in an agarose gel (2 mg/mL) were prepared, according to recently reported protocol.[28] The spin echo (MEMS) imaging results obtained for Mag@PEI NPs are shown in Fig 3. The relaxivity value measured for our nanocarrier was as high as $225 \text{ mM}^{-1}\text{s}^{-1}$ proving high contrast properties of synthesized nanoparticles. Moreover, this value is much higher than the relaxivity values reported for commercial contrast agents based on magnetic nanoparticles like Feridex ($120 \text{ mM}^{-1}\text{s}^{-1}$), Resovist ($186 \text{ mM}^{-1}\text{s}^{-1}$) and Combidex ($65 \text{ mM}^{-1}\text{s}^{-1}$).[29] Thus, our nanoparticles exhibited strong application potential in further MRI studies.

The characterized nanocarrier was further used to determine its binding capability towards ATN-RNA. In this experiment, ATN RNA was mixed with Mag@PEI NPs for 30 minutes at different weight ratios varied from 1:1 to 10:1. We expected that negatively charged ATN-RNA will interact with positively charged Mag@PEI NPs resulting in polyplexes formation. This type of structure is often postulated in literature when PEI is used as a carrier for nucleic acids

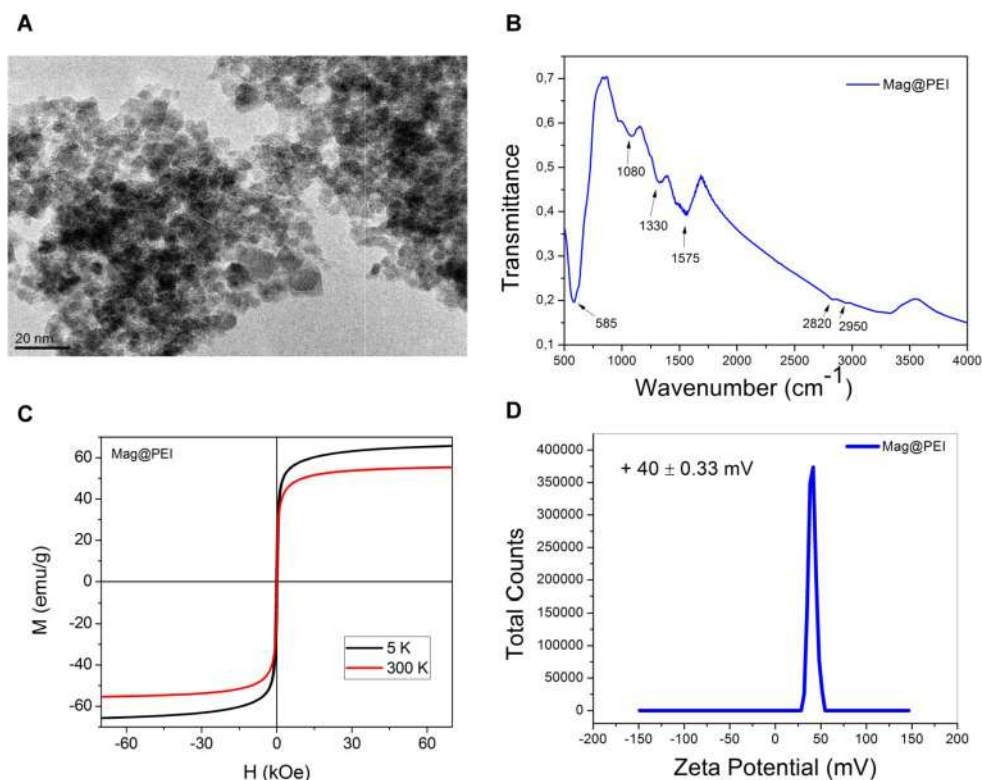


Fig 2. Characteristics of Mag@PEI. A. TEM picture of Mag@PEI NPs B. FTIR spectra of Mag@PEI NPs C. Hysteresis loop recorded for Mag@PEI NPs by means of SQUID D. Zeta potential of synthesized Mag@PEI NPs.

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delivery.[30] In the next step, obtained Mag@PEI/ATN-RNA complexes were submitted to agarose gel electrophoresis assay to visualize the linking of ATN-RNA to Mag@PEI NPs (Fig 4A). It is worth to highlight that the total amount of iron in the sample was first determined to

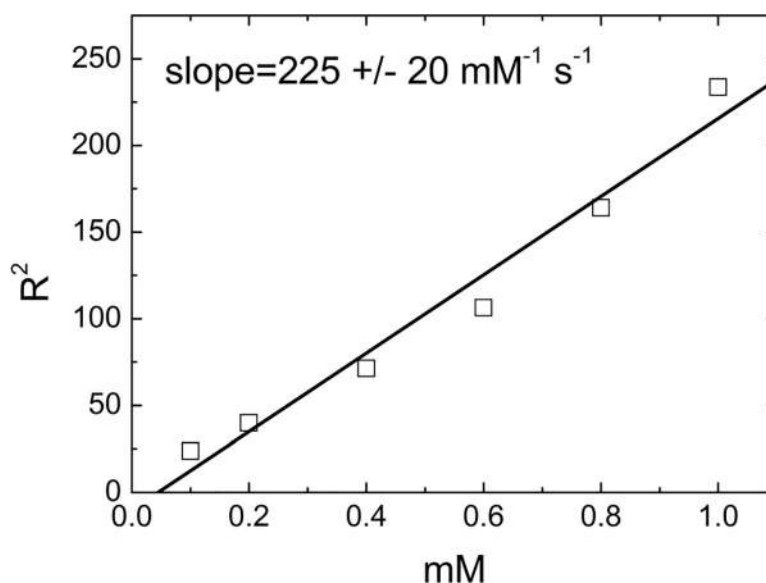


Fig 3. Contrast properties of Mag@PEI. Relaxation rates R^2 as well as relaxivity obtained from MRI experiment for Mag@PEI NPs in agarose gel 2 wt %.

<https://doi.org/10.1371/journal.pone.0213852.g003>

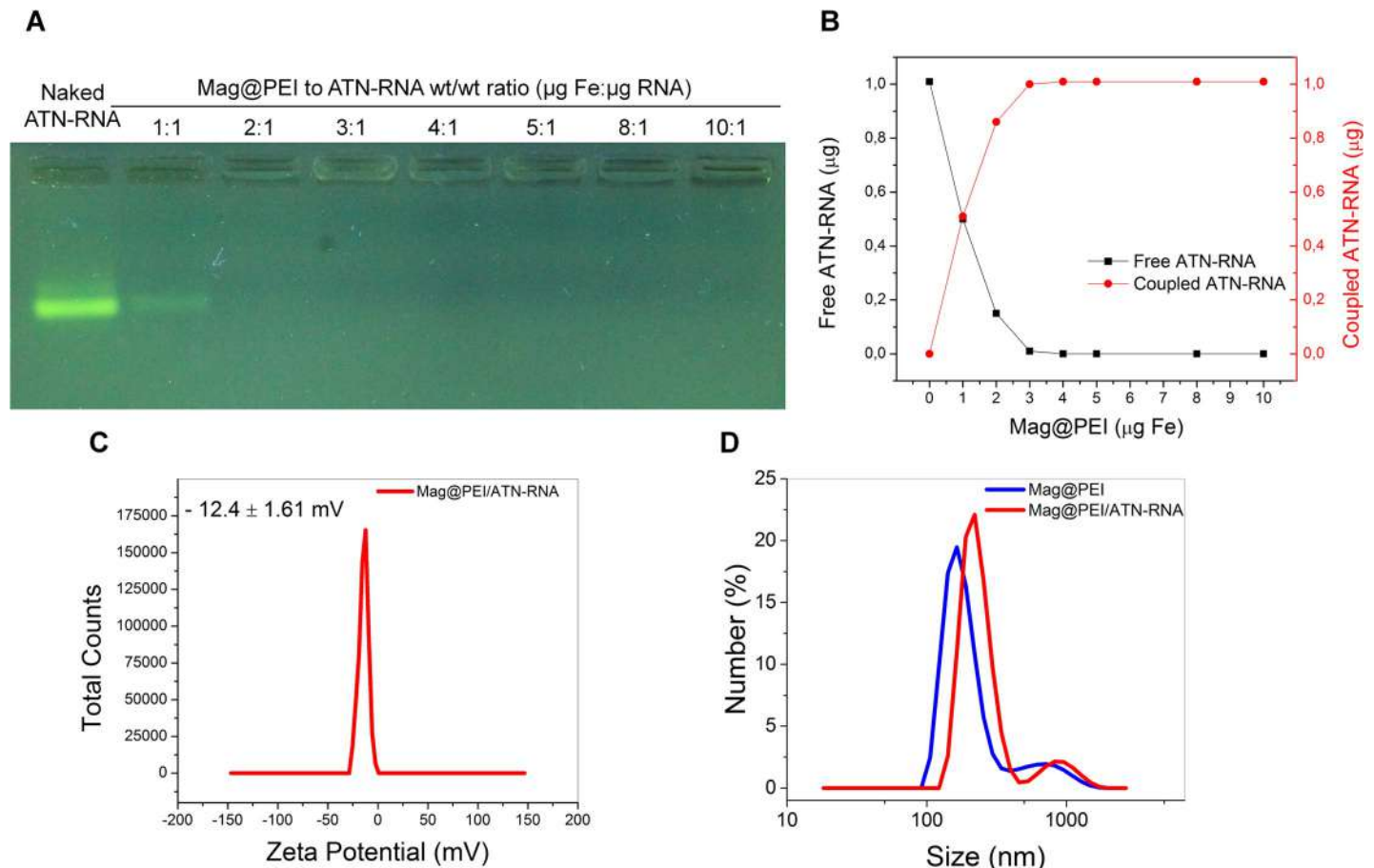


Fig 4. Binding of ATN-RNA to Mag@PEI NPs. A. Agarose gel electrophoresis of Mag@PEI/ATN-RNA complexes at the different mass ratio. B. Binding capability of Mag@PEI NPs towards ATN-RNA recorded using Nanodrop. C. Zeta potential of complexes obtained between Mag@PEI NPs and ATN-RNA at ratio 3:1. D. DLS size distribution for Mag@PEI NPs and Mag@PEI/ATN-RNA complexes at ratio 3:1.

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keep this value constant in order to repeat the experiments with different batches of nanoparticles which could slightly differ between each other. Analysis revealed that 2 weight equivalents were sufficient to bind almost all of the ATN-RNA used in the experiment. However, to get a deeper insight into this process, ATN-RNA concentration in the supernatant was investigated by UV-Vis spectroscopy. This technique has higher sensitivity than the gel electrophoresis. The data are presented in Fig 4B. UV-Vis experiments revealed that at Mag@PEI NPs to ATN-RNA ratio of 2:1, free ATN-RNA was still present in the supernatant and the loading was around 90%. However, at the ratio 3:1, the ATN-RNA was completely linked to Mag@PEI NPs. Furthermore, at this ratio, a change in zeta potential from +40 mV for Mag@PEI NPs to -12 mV for Mag@PEI/ATN-RNA complexes was observed (Fig 4C). This clearly proved that negatively charged ATN-RNA bind to the surfaces of Mag@PEI NPs.

The DLS measurements were performed to investigate the size of synthesized nanoparticles and prepared complexes in water. The Mag@PEI NPs had the hydrodynamic diameter of ~150 nm while the hydrodynamic diameter of Mag@PEI/ATN-RNA complexes increased slightly to around 200 nm after nucleic acid binding (Fig 4D).

The uptake of Mag@PEI/ATN-RNA complexes by U-118 cells was investigated by means of confocal microscopy. First, Mag@PEI nanoparticles were labelled with ATTO 550 dye. As illustrated in Fig 5, glioma cells could be successfully transfected with nanoparticles since the

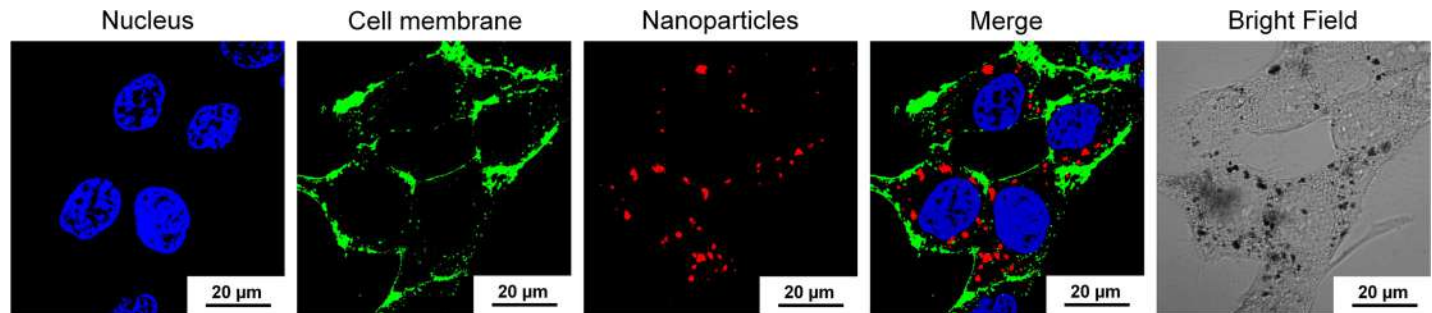


Fig 5. Cellular uptake of complexes containing fluorescently labelled Mag@PEI NPs into U-118 cells. The representatives of the colors are blue (Hoechst 33342) for nuclei, green (Concanavalin A-FITC) for cell membranes, and red (ATTO550) for Mag@PEI nanoparticles.

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red fluorescence from the dye was observed in the cytoplasm. Moreover, the analysis of the 3D reconstruction further confirmed their internalization capacity of obtained complexes. (S1 Video). The bright field images show the presence of black dots corresponding to the aggregated complexes containing fluorescently labelled Mag@PEI NPs.

A colocalization analysis was also performed to further demonstrate transfection of Mag@PEI/ATN-RNA complexes into glioblastoma cells. As shown in Fig 6, there is a visible colocalization between the ATTO 550 labelled Mag@PEI nanoparticles (red color) and the FITC labelled ATN-RNA (green color) in the cytoplasm suggesting high transfection efficiency of the synthesized nanoparticles.

Cytotoxicity of magnetic nanocarriers and complexes with ATN-RNA

Cytotoxicity of both Mag@PEI NPs and polyplexes bearing ATN-RNA on GBM U-118 cells were determined by two cytotoxicity tests, namely WST-1 and LIVE/DEAD assays. In the first step, the toxicity of Mag@PEI NPs without bounded ATN-RNA at the concentrations of NPs corresponding to the concentrations used in further transfection experiments (Figure B in S1 File). Mag@PEI carrier did not cause any significant toxic effect in the investigated concentration range and the data from both tests were consistent. Also, SRB test did not show any

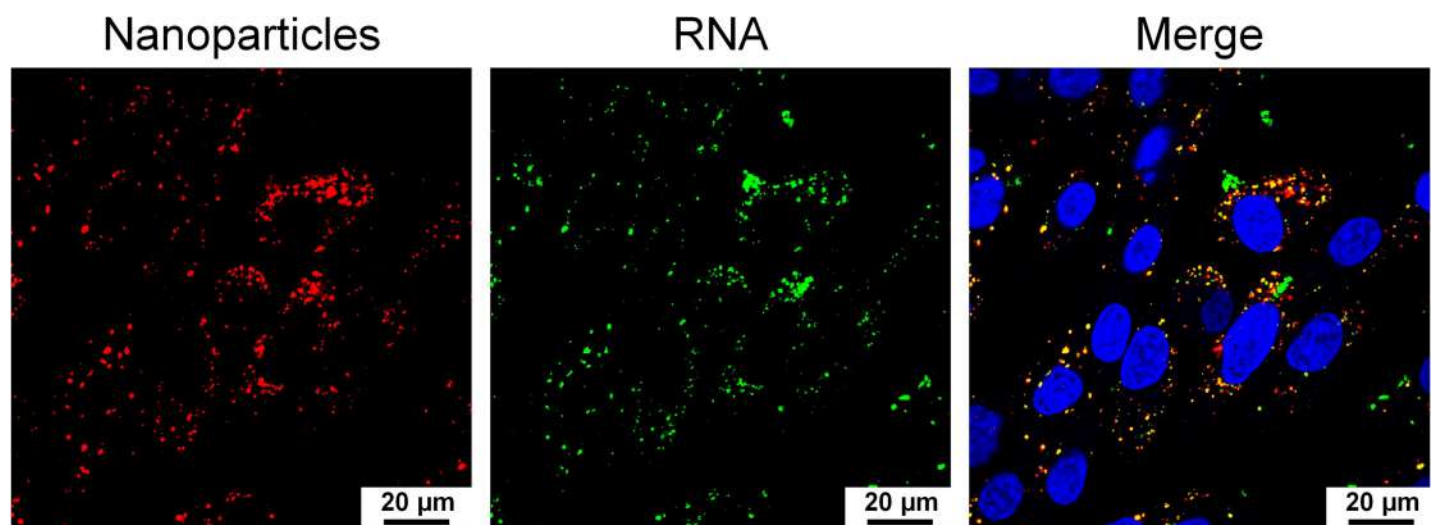


Fig 6. Colocalization of Mag@PEI NPs and ATN-RNA in U-118 cells. The representatives of the colours are blue (Hoechst 33342) for nuclei, green (FITC) for ATN-RNA, and red (ATTO550) for Mag@PEI nanoparticles.

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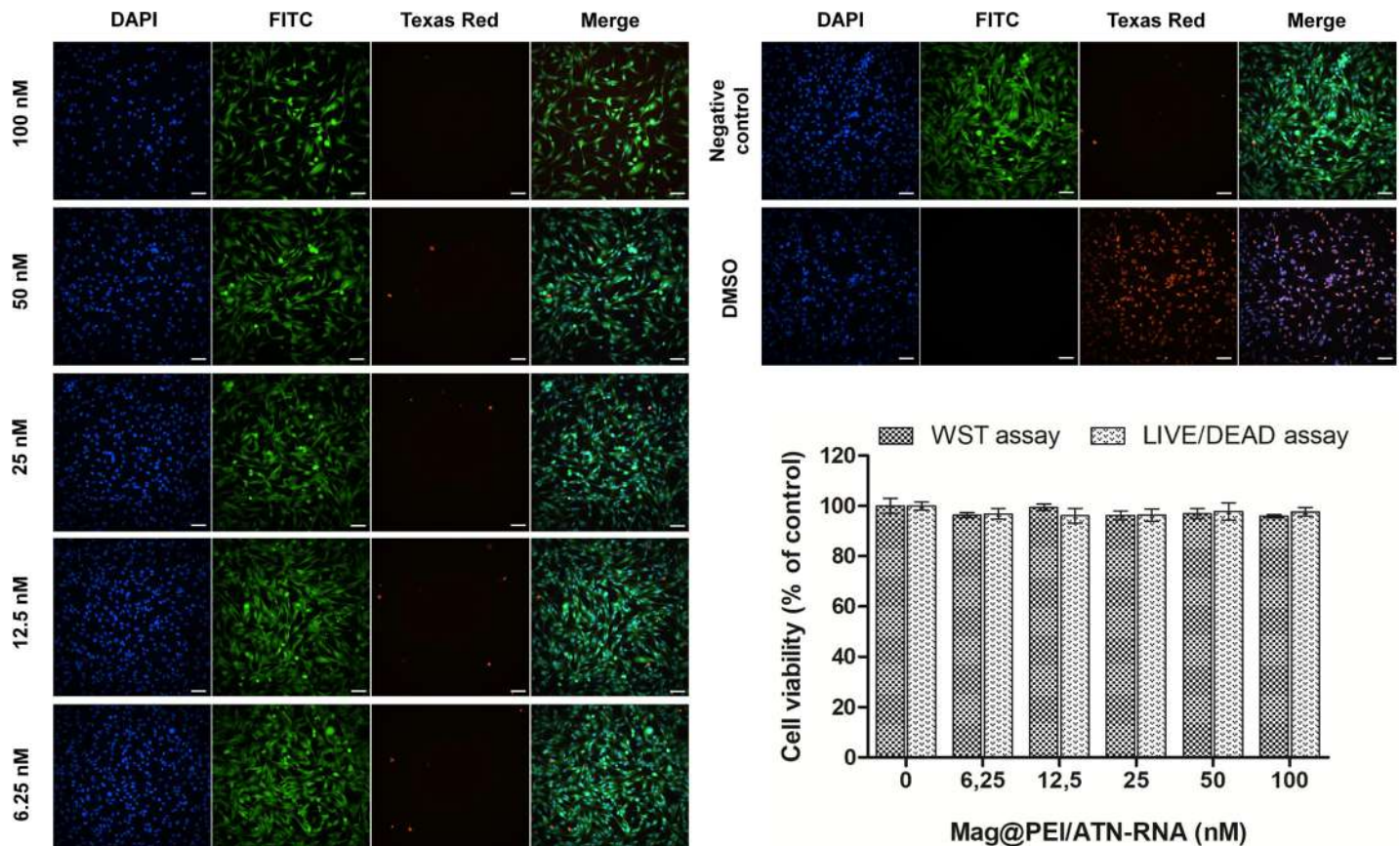


Fig 7. Representative high-content images of U-118 cells exposed to Mag@PEI/ATN-RNA complexes (6.25–100 nM). 10% DMSO was used as a positive control. Images were obtained using different filters to detect nuclei (DAPI), live cells (FITC), and dead cells (TexasRed). The scale bars denote 100 μ m. Cell viability of U118 cells exposed to Mag@PEI/ATN-RNA complexes (6.25–100 nM) for 24 h. The value at X-axis in Fig 7 corresponds to concentration of free ATN-RNA on the carrier.

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toxicity of our carrier (Figure C in S1 File). Subsequently, we assessed the cytotoxicity of Mag@PEI/ATN-RNA complexes (Fig 7). To keep the same range of concentrations as previously used in RNAi approach [10], we used the ATN-RNA concentration between 10 to 100 nM. Both cytotoxicity assays revealed high viability of glioblastoma cells in the investigated concentration range. So we could exclude undesired side effects coming from the nanoparticles.

Immune response

Taking into account the possible use of nanoparticles as the therapeutic tool in GBM treatment, the analysis of expression level of the genes involved in innate immune response was performed. We examined whether our nanoparticles delivery system would elicit an innate immune response *in vitro*. Undesired immune responses are an important consideration in gene therapy and in the development of gene delivery systems because the introduction of exogenous genes can activate the innate immune system of human cells to combat foreign gene or pathogen invasion.[31] For example, siRNA can provoke an immune response *via* its interactions with Toll-like receptors (TLRs) and trigger an interferon (IFN) response.[32] Additionally, systemically introduced lipid nanoparticles have been reported to induce an immune response in mice.[33] Thus, the potential immunostimulatory properties of a proposed gene delivery system are important. We characterized U-118 GBM cells for their innate

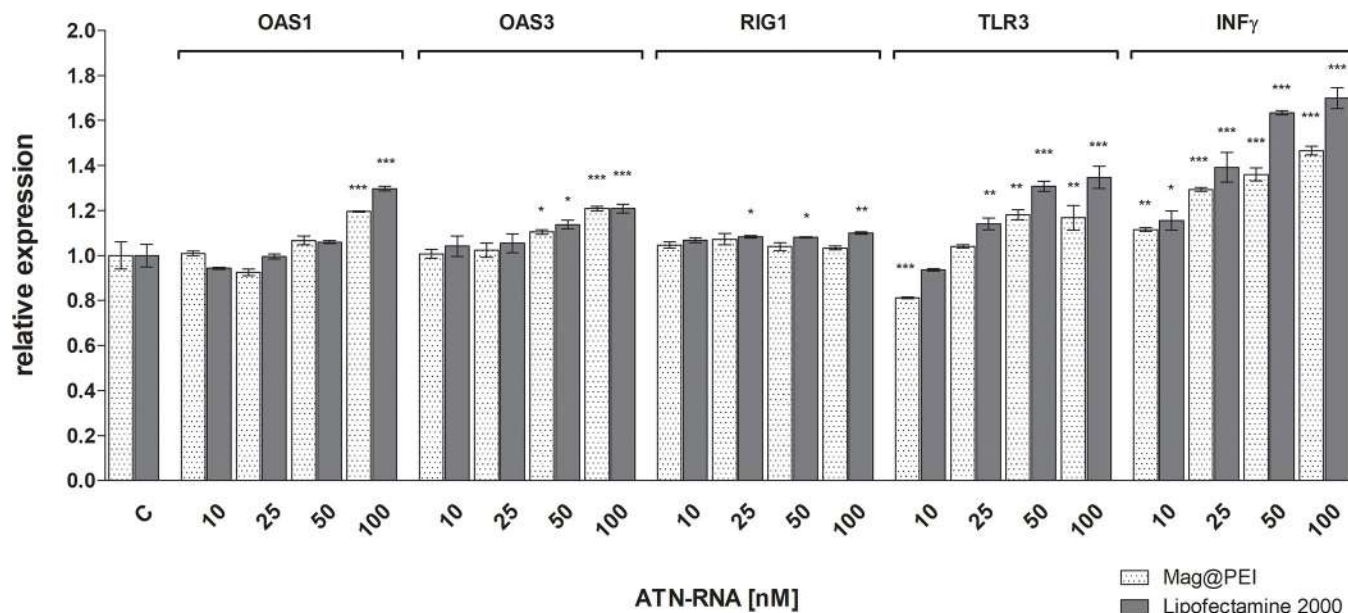


Fig 8. The expression level of immune response genes after lipo- and nano-mediated ATN-RNA delivery to U-118 cell line. The relative expression level of the expression of OAS1, OAS3, RIG1, INFγ and TLR3 established by qRT-PCR calculated with the $-\Delta\Delta C_p$ method. Statistical evaluation of ATN-RNA versus control (Clipo or Cnano, respectively) cells was performed using one-way ANOVA followed by Tukey's posthoc test. Significance value: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to untreated cells (Clipo or Cnano, respectively). Legend: Mag@PEI—nano-mediated ATN-RNA delivery; Lipofectamine—lipo-mediated ATN-RNA delivery.

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immune response to the nanoparticle complexes. We measured the expression of OAS1, OAS3, RIG1, TLR3 and INFγ using the qRT-PCR (Fig 8). The changes after ATN-RNA treatment were either not observed, as for the RIG1 gene or negligible, as for OAS3 gene. However, the highest concentration of ATN-RNA (50, 100 nM) caused the weak activation of interferon response of above mentioned genes, but this activation was slightly higher in case of lipofection when compared to magnetic nanocarrier—about 8% ($p < 0.01$ and $p < 0.005$, respectively). The higher activation of OAS 1 gene was measured both in case of lipofectamine and nanoparticles treatment, but it needs to be highlighted, that at 100nM ATN-RNA working concentration the expression level of that gene was about 30% higher for lipofection, what gives 10% increase in relation to the nanoparticles-mediated delivery ($p < 0.005$).

The highest activation with ATN-RNA both with lipofectamine and using Mag@PEI nano-carrier was observed for TLR3 and INFγ genes. The ATN-RNA delivered with lipofectamine caused the TLR3 gene expression activation at the level from 16% to 37%, starting from the 25 nM ATN-RNA concentration ($p < 0.005$). The Mag@PEI NPs used as the ATN-RNA carrier resulted in the lowest activation of the TLR3 gene expression with the observed 8–15% level, for 25–100 nM ATN-RNA concentrations ($p < 0.01$).

INFγ gene was significantly upregulated both by the lipofectamine and Mag@PEI NPs. However, with the same ATN-RNA concentration, the gene expression was elevated at the higher level by the lipofectamine-mediated ATN-RNA delivery. With the already lowest concentration, we observed 10–12% overexpression by the lipofection and Mag@PEI NPs ($p < 0.01$). However, starting from the 25 nM ATN-RNA concentrations we noticed the growing differences with the effect of lipofectamine versus Mag@PEI NPs. With this concentration, we observed the INFγ gene overexpression of about 27 and 42% for Mag@PEI NPs and lipofection, respectively. The highest concentrations caused—for 50 nM: 28 and 63%, for 100 nM: 44 and 75% in case of use of Mag@PEI NPs and lipofection, respectively.

RNA interference therapy

To achieve down-regulation of TN-C expression in U-118 glioblastoma cell line, lipofection along with Mag@PEI NPs with various concentration of ATN-RNA– 10, 25, 50 and 100 nM was performed. 24 hours after transfection the expression level of tenascin-C was examined by qRT-PCR analyses. The downregulation of TN-C mRNA expression was observed compared to controls treated with the transfection agent or with scrambled RNA. The level of TN-C was decreased from 2% at a concentration of 10 nM ATN-RNA to 34% for cells treated with 100 nM ATN-RNA in comparison to the untreated cells ($p < 0.005$) in case of samples where ATN-RNA was delivered with lipofectamine (Fig 9). At the same time, the above mentioned range of ATN-RNA concentration was delivered with Mag@PEI nanoparticles resulted in the significant drop of the TN-C expression level. The downregulation effect was visible already with the lowest concentration with the decrease at 20%. The highest concentrations: 25, 50 and 100 nM caused the TN-C downregulation about 43, 48 and 80%, respectively ($p < 0.005$) (Fig 9A).

The qRT-PCR analysis was also supported by the direct analysis of the protein expression level. The Western blot detection revealed again with the more significant drop of the TN-C expression caused by the nanoparticles-mediated ATN-RNA delivery. We observed already 40% decrease of the protein expression with the 10 nM ATN-RNA. The highest concentration used caused the dramatic drop of the protein expression measured as the 85% of the decrease. The lipofectamine mediated ATN-RNA delivery at the same time caused only about 40% decrease of the expression level with ATN-RNA concentrations 50 and 100 nM (Fig 9B). These observations were fully consistent with the obtained relative TN-C expression level measured by qRT-PCR.

These results clearly support the idea of nanoparticles mediated delivery in order to achieve the significant biological effect along with the lowest concentration of therapeutic agent. The Mag@PEI mediated ATN-RNA delivery was already efficient with the lowest- 10 nM concentration- 20% of TNC downregulation, while the lipofectamine at the same time was not able yet to deliver the RNA. During the whole experiment, we observed about 30–50% higher efficiency of nanomediated delivery compared to the lipofectamine-driven one.

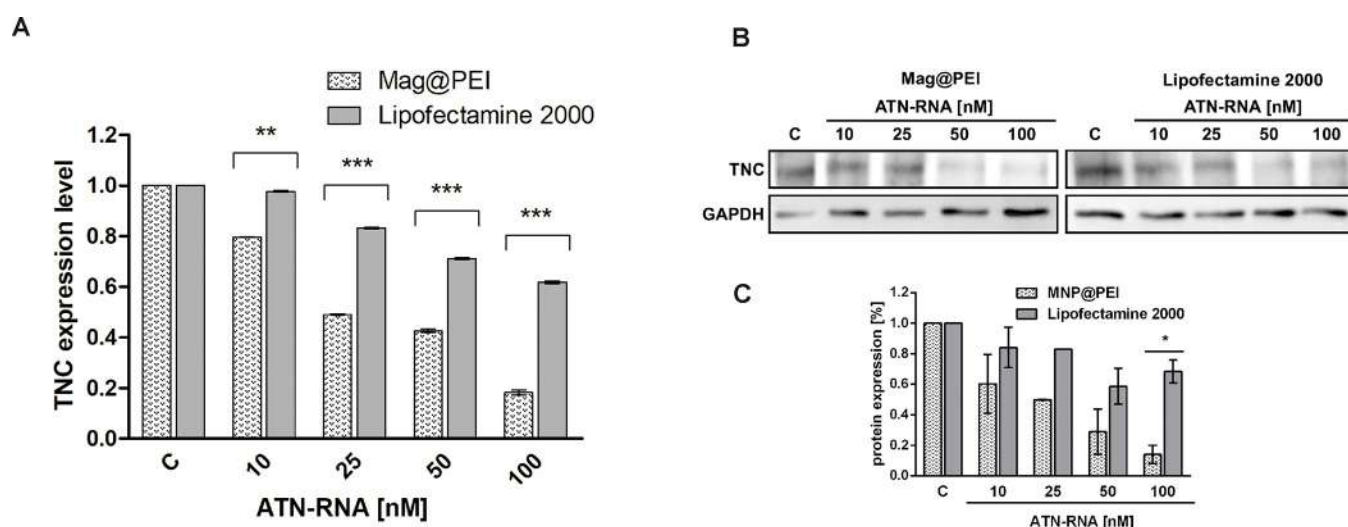


Fig 9. The expression level of TN-C after lipo- and nano-mediated ATN-RNA delivery to U-118 cell line. A. The relative expression level of the expression of TN-C established by qRT-PCR calculated with the $\Delta\Delta C_p$ method. B. The protein expression level measured by Western blot with densitometric analysis (C.) Statistical evaluation of ATN-RNA versus control (Clipo or Cnano, respectively) cells was performed using one-way ANOVA followed by Tukey's posthoc test. Significance value: ** $p < 0.01$, *** $p < 0.001$. Legend: Mag@PEI- nano-mediated ATN-RNA delivery; Lipofectamine- lipo mediated ATN-RNA delivery.

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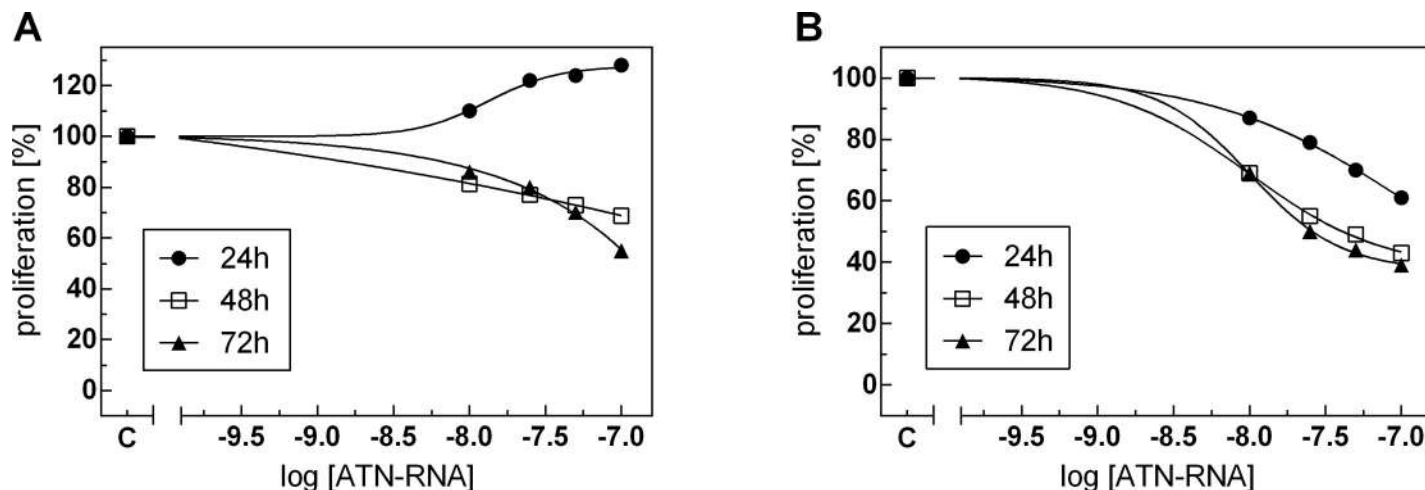


Fig 10. Anti-proliferative activity of ATN-RNA, after nano- (A) and lipo-mediated (B) delivery. Proliferation was monitored in real-time using the xCELLigence system. Differences between CI values for ATN-RNA treated and control cells were statistically evaluated using one-way ANOVA followed by Tukey's posthoc test. Dose-dependent effects of ATN-RNA on proliferation was evaluated using non-linear regression by fitting experimental values to sigmoidal, bell-shaped equation. Legend: Mag@PEI- nano-mediated ATN-RNA delivery; Lipofectamine—lipo mediated ATN-RNA delivery.

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Proliferation and migration of GBM cells

In order to investigate the delivery efficacy of the carriers, we measured the cells proliferation rate. U-118 glioblastoma cell line was treated with Mag@PEI NPs and Lipofectamine combined with ATN-RNA followed by the real-time cell proliferation assay with the xCELLigence system. The cell's ability for proliferation was measured for 72 h. We noticed ATN-RNA concentration-dependent decrease of proliferation rate both for Lipofectamine carrier with the significant decrease of proliferation with 100 nM–ATN-RNA concentration. The proliferation rate dropped for ATN-RNA delivered with the cationic carrier from 40–61% (Fig 10B). However, for Mag@PEI NPs we observed the opposite effect, resulting in the increase of proliferation—most effective concentration of ATN-RNA was 100 nM, with an increase from 10–28% after 24 h, respectively (Fig 10A). Noteworthy, 25 nM and 50 nM of ATN-RNA was already sufficient concentration for the efficient changes of GBM cells proliferation. After 48 and 72 h post transfection we observed again the inhibition of proliferation, from 7–45%, respectively. This allowed us to assume that the most effective delivery of ATN-RNA is achieved after first 24 h from transfection. We also would postulate that the significant drop of the proliferation rate both for Lipofectamine and for nanoparticles mediated delivery 48 and especially 72 h post transfection is more likely due to the simple death of the cells, rather than the ATN-RNA action.

To get more insight into the down-regulation of TN-C expression by ATN-RNA on the mobility of glioblastoma cancer cells, real-time measurements of migration was carried out. In this approach, U-118 cells were treated again with Mag@PEI NPs and Lipofectamine and ATN-RNA and allowed to migrate through microporous polyethylene terephthalate (PET) membrane towards chemoattractant. The number of cells that crossed the membrane was continuously recorded.

We found that down-regulation of TN-C expression by ATN-RNA delivered by Mag@PEI NPs significantly impaired the cell migration in GBM cells (Fig 11). The results were quantitatively assessed during 72 h of experiment and showed that U-118 cells transfected with ATN-RNA had the lowest motility beginning from 12 h post transfection. It was established that Mag@PEI NPs with ATN-RNA delayed the migration of U-118 cells by 5.76 ± 1.02 ,

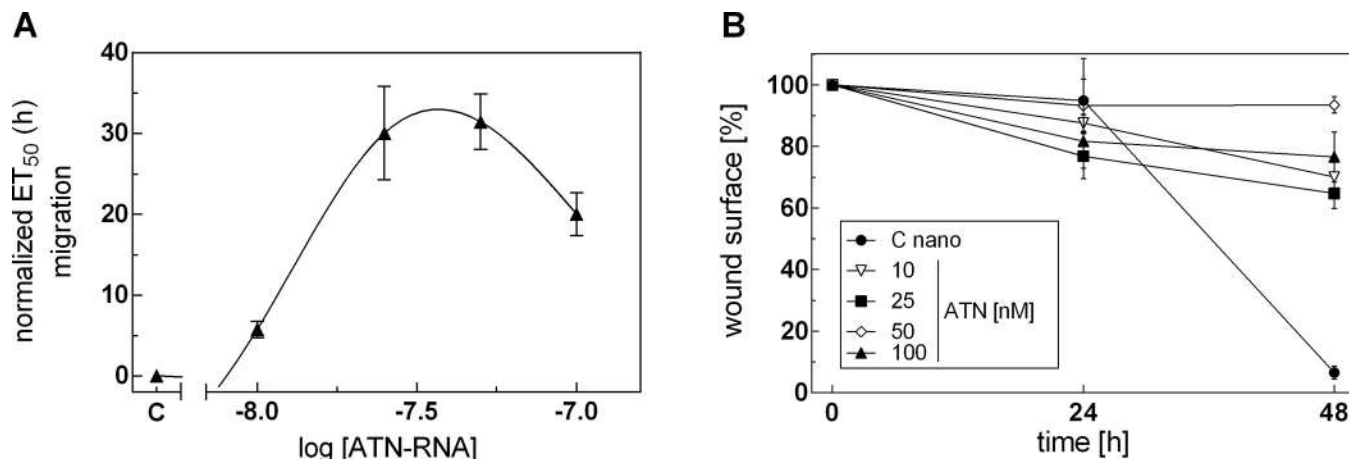


Fig 11. The effect of nano-mediated delivery of ATN-RNA on the migration processes. (A) Migration of U-118 GBM cancer cells was studied using the xCELLigence system. Serum-depleted cells were transfected with increasing concentrations of ATN-RNA (from 10 to 100 nM) or vehicle (Opti-MEM, C-control). Impedance (CI values) of each experimental condition was recorded over time, plotted against time, fitted to four-parameter logistic non-linear regression model and ET₅₀ was calculated for each ATN-RNA concentration to generate dose-response curves. The ET₅₀ value was normalized to the data obtained for cells treated with native Mag@PEI and plotted as normalized half maximal effective time (ET₅₀) of cell migration against ATN-RNA concentrations. (B) The scratch assay analysis. U-118 GBM cells were transfected with Mag@PEI/ATN-RNA complexes. Images were captured after 24 and 48 h (Figure D in S1 File). The rate of migration was measured by quantifying the total distance that the U-118 cells moved from the edge of the scratch toward the centre of the scratch.

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30.06 ± 5.78, 31.46 ± 3.41 and 20.03 ± 2.65 h with 10, 25, 50 and 100 nM concentration, respectively (Fig 11A). Notably, the most effective concentration influencing the migration potential of the cells is 50 nM, while 100 nM seems to cause the elevated cells death. One can notice the entirely different results observed for the ATN-RNA delivery driven by Lipofectamine. During the experiment, we were not able to detect the specific migration impairment, most probably due to the high toxicity of Lipofectamine. To get more insight into the problem, we performed also the scratch assay. Again, with the lipofectamine delivered ATN-RNA we were not able to quantify the existing wound since the transfection resulted in the cells death. Conversely, the results from nanoparticles-mediated ATN-RNA delivery in the wound-healing assay demonstrated that U-118 ATN-RNA treated cells migrate into the scratched area more slowly than the control cells- 18% after 24 and 24% after 48 h, respectively (Fig 11B).

Notably, the infection did not change substantially also the viability of the cells, what was observed in terms of lipofection (Figure D in S1 File) Thus, taking together the independent experiments proved the significant impairment of migration rate of the Mag@PEI/ATN-RNA treated U-118 cells, suggesting the therapeutic potential of the used nanocomposite. This extremely interesting observation could suggest either the gradual release of the dsRNA from the nanoparticles surface, resulting in the best effect already at the higher concentration or the highest stability of dsRNAs. At the same time, one can notice the significant migration impairment is already established with the 25 nM concentration. This could suggest that the nanoparticles-mediated delivery would use the lowest concentration of the therapeutic agent allowed for an efficient silencing of the tenascin-C. Observed migration delay as the results with the nanoparticles delivery is fully consistent with the tenascin-C expression level measured by qRT-PCR. The highest TN-C downregulation has functional consequences with the migration rate of U-118 glioblastoma cells.

Our functional studies with using both nano- and lipo- carriers showed the potential of nanoparticles with dsRNA delivery. ATN-RNA in our previous studies had a great impact on the migration and proliferation properties of cancer's cells resulted in the decrease of both of

these potentials. In the present study, however, we observed surprisingly the increase of the proliferation potential caused by the ATN-RNA delivery with nanoparticles. Since we observed the enhanced TN-C downregulation followed by the ATN-RNA delivery with nanoparticles, our results seem to be in concordance with the results in intracranial xenografts.[34]

The TN-C knockdown in the tumor microenvironment modulated the behavior of a tumor stromal cells, inhibited tumor invasion, and increased tumor cell proliferation. The combination of this phenotypic phenomenon is similar to the go-or-grow glioma growth phenotype found in human neoplasms, which represent characteristic, highly proliferative tumor cores and diffuse borders with a low proliferation rate.[34]

Conclusions

We have successfully demonstrated a new dsRNA delivery system that harnesses well- characterized magnetic nanoparticles coated with PEI to effectively silence expression of tenascin-C in glioblastoma multiforme cell line. Both mere nanoparticles and their complexes with dsRNA do not show toxicity and do not provoke undesired immune responses in U-118 GBM cell line. Additionally, we proved by confocal microscopy imaging, that they could be successfully internalized into glioblastoma cells. The most important thing is that synthesized nanocarrier was a more efficient tool in delivery ATN-RNA than routinely used Lipofectamine. In consequence, the gene therapy employing ATN was improved resulting in stronger silencing of TN-C, followed by the further diminishing of migration of glioblastoma cells. Finally, since the magnetic core provided high contrast properties in MRI, therefore, synthesized nanocarrier system can be considered as the robust theranostic nanoplatforams for simultaneous gene therapy and imaging. The present approach is then the first demonstration of an effective and safe dsRNA delivery based on multifunctional nanoplatforams. The used technology is an evidence of a promising nanoparticles-based shuttle with a high potential for further clinical use in GBM treatment.

Supporting information

S1 File. (Figure A) Destabilization kinetic of Mag@PEI recorder by Turbiscan apparatus over 24 h, (Figure B) Representative high-content images of U-118 cells exposed to Mag@PEI nanoparticles at various concentrations. 10% DMSO was used as a positive control. Images were obtained using different filters to detect nuclei (DAPI), live cells (FITC), and dead cells (TexasRed). The scale bars denote 100 μ m. Cell viability of U118 cells exposed to Mag@PEI, (Figure C) Cytotoxicity SRB of Mag@PEI for various nanoparticles concertation, (Figure D) Schratck test. (DOCX)

S1 Video. MNP@PEI_RNA_5well_3_3d. (AVI)

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MATERIAŁY UZUPEŁNIAJĄCE DO PUBLIKACJI

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Nano-Mediated Delivery of Double-Stranded RNA for Gene Therapy of Glioblastoma Multiforme

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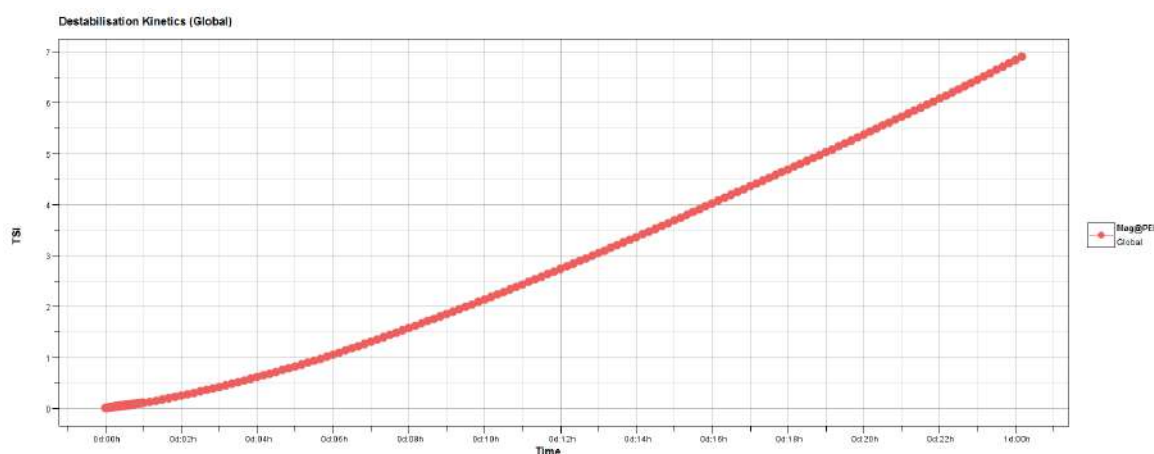


Figure A Destabilization kinetic of Mag@PEI recorder by Turbiscan apparatus over 24 h.

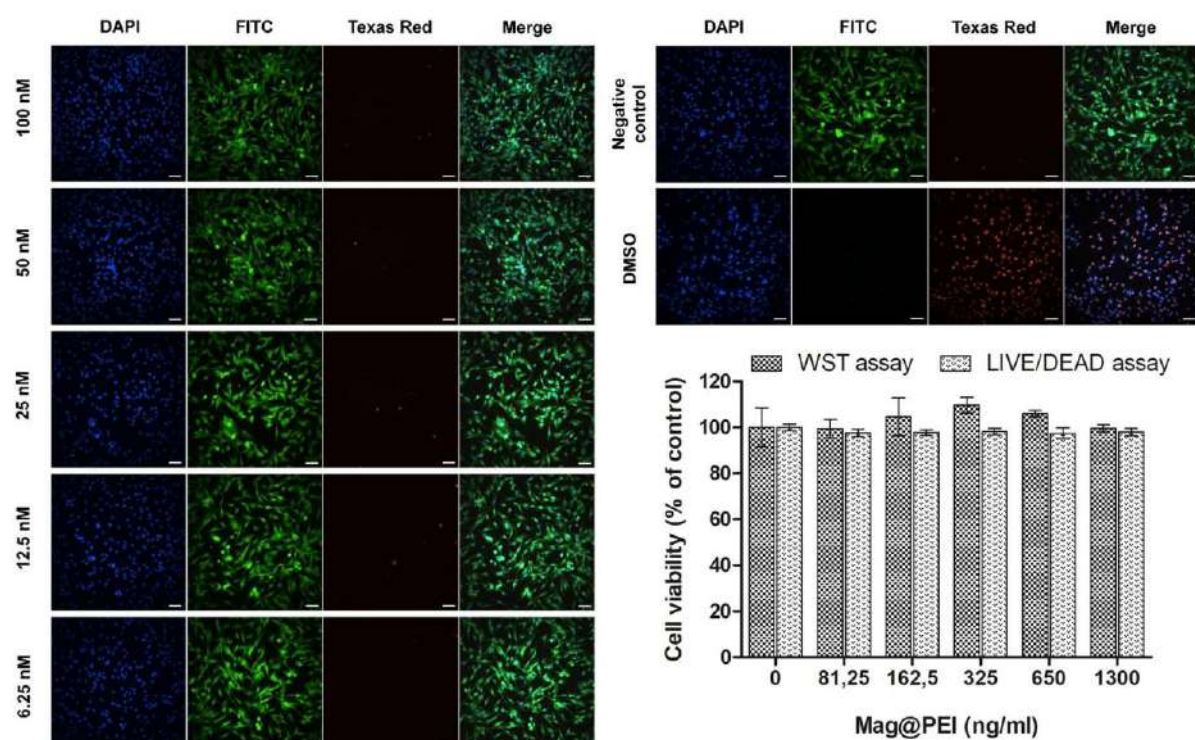


Figure B. Representative high-content images of U-118 cells exposed to Mag@PEI nanoparticles at various concentrations. 10% DMSO was used as a positive control. Images were obtained using different filters to detect nuclei (DAPI), live cells (FITC), and dead cells (TexasRed). The scale bars denote 100 μ m. Cell viability of U118 cells exposed to Mag@PEI.

SRB assay

The protein-staining sulforhodamine B (SRB, Sigma–Aldrich) microculture colorimetric assay, developed by the National Cancer Institute (USA) for in vitro antitumor screening was used in this study, to estimate the cell number by providing a sensitive index of

total cellular protein content, being linear to cell density. The monolayer cell culture was trypsinized and counted. To each well of the 96-well plate, 100 μ L of the diluted cell suspension (5,000 and 10,000 cells) was added. After 24 hours, when a partial monolayer was formed, 100 μ L of fresh medium with different Mag@PEI concentrations (81.25, 162.5, 325, 650 and 1300 ng/mL) were added to the wells. The cells were exposed to compounds for 24 h at 37°C in a humidified atmosphere (90% RH) containing 5% CO₂. After that, 100 μ L of 10% trichloroacetic acid was added to the wells and the plates were incubated for 1 h at 4°C. The plates were then washed out with the distilled water to remove traces of medium and next dried by the air. The air-dried plates were stained with 100 μ L of 0.057% sulforhodamine B (prepared in 1% acetic acid) and kept for 30 min at room temperature. The unbound dye was removed by washing five times with 1% acetic acid and then the plates were air-dried overnight. The protein-bound dye was dissolved in 200 μ L of 10 mM unbuffered Tris base (pH 10.5) for optical density determination at 510 nm. All experiments were performed in triplicates. Cell survival was measured as the percentage absorbance compared to the control (non-treated cells).

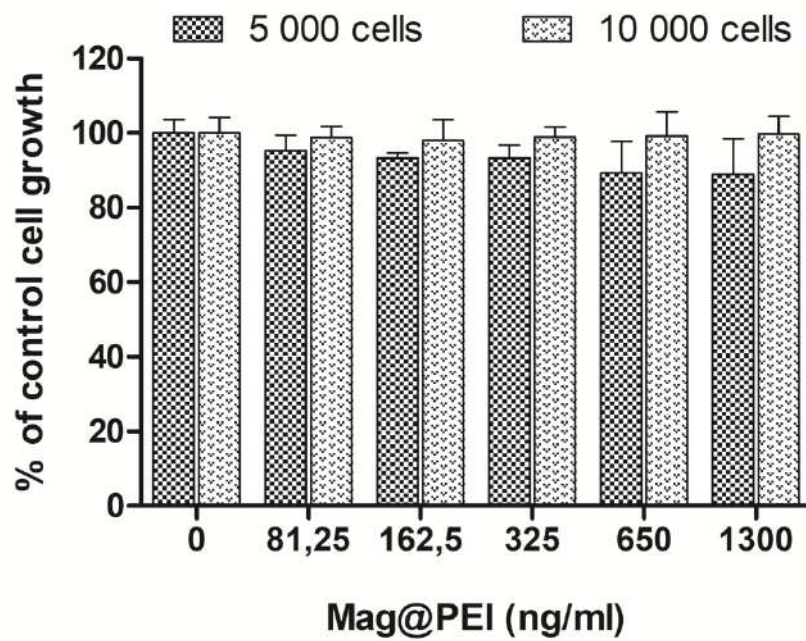


Figure C. Cytotoxicity SRB of Mag@PEI for various nanoparticles concentration.

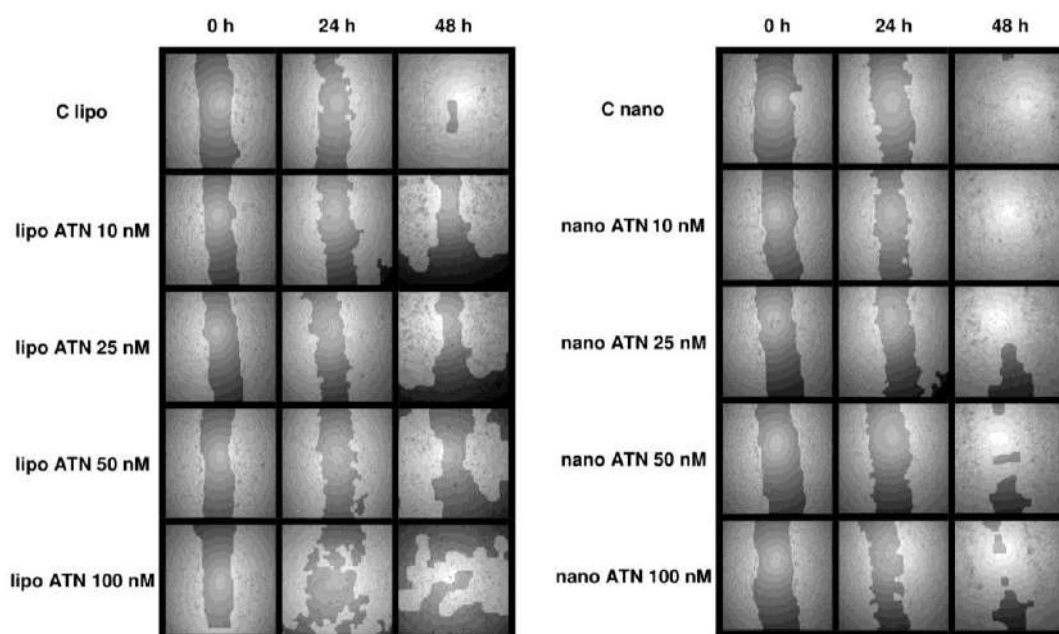


Figure D. Schratch test

8

Kwiczak- Yiğitbaşı J, Pirat J-C, Virieux D, Volle J-N, Janiak A, Hoffmann M, Mrzygłód J,
Wawrzyniak D, Barciszewski J, Pluskota-Karwatka D

“Synthesis, structural studies and biological properties of some phosphonoperfluoro-
phenylalanine derivatives formed by S_NAr reactions”

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Synthesis, structural studies and biological properties of some phosphono-perfluorophenylalanine derivatives formed by S_NAr reactions†‡

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Several novel phosphono-perfluorophenylalanine derivatives, as mimetics of phenylalanine, were synthesized by subjecting diethyl (2-(perfluorophenyl)-1-(phenylamino)ethyl)-phosphonate to S_NAr reactions with different types of nucleophiles such as thiols, amines and phenols. The structure of the products was confirmed using spectroscopic and spectrometric techniques. For two compounds X-ray single crystal diffraction analysis and DFT investigations were performed providing information in regard to the preferable conformation, hydrogen bonds and other interactions. The antiproliferative potency of some of the new phosphono-perfluorophenylalanine derivatives obtained as well as representatives of previously synthesized perfluorophenyl phosphonate analogues of phenylalanine was studied on selected glioma cell lines. Preliminary evaluation of the compounds drug likeness was examined with respect to Lipinski's and Veber's rules, and showed that they meet the criteria perfectly. MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay results demonstrated that the compounds exhibit moderate activity against the glioblastoma multiforme cell lines (T98G and U-118 MG). Moreover most of the studied S_NAr reaction products displayed significantly higher inhibitory activity against both cancer cell lines than the parent diethyl (2-(perfluorophenyl)-1-(phenylamino)ethyl) phosphonate.

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1. Introduction

Chemistry of α -aminophosphonates has played a significant role in the development of organophosphorus compounds, and still remains a field of great interest.^{1–3} As structural analogues of α -amino acids, α -aminophosphonates have found application in organic and medicinal chemistry,⁴ mainly due to their anticancer,^{5–9} antiviral¹⁰ and antibacterial^{11,12} activities. Moreover, phosphonates represent classical phosphate bioisosters, in which the labile O–P bond is replaced by an enzymatically

and chemically stable C–P bond. This change makes α -amino-phosphonates structurally similar to phosphate esters or anhydrides, yet increases their stability under physiological and chemical conditions.^{13,14} Also, the tetrahedral geometry on the phosphorus atom mimics the transition state of peptide hydrolysis thus, α -aminophosphonates can act as enzyme inhibitors.^{15–17}

Among α -aminophosphonates, the fluorinated ones constitute a particularly important group of compounds. The incorporation of fluorine atoms in the structure of α -aminophosphonates provides access to more lipophilic molecules, which often exhibit better biological activities than the parent compounds.^{18,19} What is more, the presence of fluorine and phosphorus atoms in the α -aminophosphonates structure gives us a chance to use ¹⁹F and ³¹P NMR spectroscopy to follow the location and to study the molecular interactions in biological systems.^{20,21}

Introduction of fluorine has also become an important strategy in protein biochemistry. Fluorinated amino acids serve as powerful tools for exploring polar π -interactions in proteins,^{22,23} and enabling novel recognition mechanisms for protein design.^{24–26} Heavily fluorinated aromatic residues are

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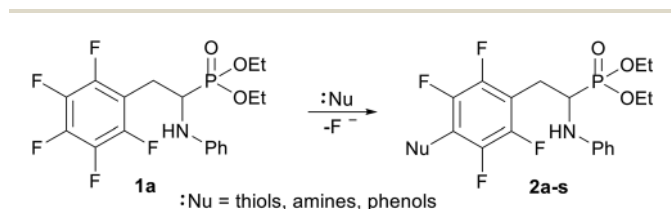
† Dedication to Professor Henryk Koroniak in honour of his 70th birthday.

‡ Electronic supplementary information (ESI) available. CCDC 1912530 and 1912531. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c9ra03982a

particularly desirable because multifluorination gives maximum electronic perturbation of aromatic rings.

Fluorinated aromatic rings may undergo nucleophilic aromatic substitution reactions (S_NAr). Due to higher electronegativity of carbon comparing to hydrogen, benzene exhibits a negative potential on the π face and a positive one around the periphery.²⁷ Fluorine as strongly electronegative element, reverses this distribution to give a negative potential on the periphery and a positive on the aromatic ring,²⁷ or, as theoretical studies suggest, localized more to carbon skeleton than the π -electrons cloud.^{28,29} Thus fluorination clearly enhances the rate of nucleophilic attack. In general, the reaction of pentafluorophenyl moiety is highly regioselective; nucleophile replaces almost exclusively the fluorine atom in the *para* position.²⁷ The S_NAr reactions found application for fluorinated amino acids syntheses. Regioselective nucleophilic addition-elimination reaction of pentafluorobenzyl moiety was the key step on the synthetic route to tetrafluorotyrosine.³⁰ This strategy was then extended to preparation of a series of *para*-substituted tetrafluorophenylalanines.³¹ Also cysteine arylation was achieved *via* S_NAr reactions.³² Reaction between cysteine thiolate and perfluoroaromatic molecules afforded exclusively 1,4-disubstituted products. Such approach enabled selective modification of cysteine residues in unprotected peptides.³²

As part of our investigations, we have recently reported a convenient synthetic method for the preparation of a series of phosphonate analogues of phenylglycine, homophenylalanine, and phenylalanine that differ in the number and position of fluorine atoms in the phenyl ring.^{33,34} These studies were supported by single-crystal X-ray diffraction analysis and quantum chemical calculations that provided information concerning the conformational preferences both in the solid and isolated states. Indeed, some of the obtained aminophosphonates underwent intramolecular S_NAr reactions yielding indolinyphosphonates as minor products.³³ Since S_NAr reaction is an attractive and effective way for modifying structure of fluorinated aromatic compounds, we decided to study the ability of the synthesized α -aminophosphonates to undergo such a transformation. It gave us a chance to obtain a library of diversely substituted α -aminophosphonates and opened a new perspectives to seek for original bioactive molecules. Therefore in this paper we describe the synthesis of *para* substituted derivatives of diethyl (2-(perfluorophenyl)-1-(phenylamino)ethyl)phosphonate **1a** (Scheme 1) as well as results of their X-ray and DFT studies. Structural variations on products were achieved by subjecting the phenylalanine analogue **1a** to reactions with various nucleophiles, *i.e.* thiols, amines and phenols (Scheme 1).



Scheme 1 Synthesis of **2a-s**.

Some of the phosphono-perfluorophenylalanine derivatives obtained together with representatives of previously synthesized³³ perfluorophenyl phosphonate analogues of phenylalanine were subjected to studies aimed at evaluation of the compounds antiproliferative potency on chosen glioblastoma multiforme (GBM) cell lines.

GBM is highly infiltrative tumour which displays extreme resistance to conventional radiotherapy and chemotherapy.³⁵ It contains self-renewing, tumorigenic cancer stem cells (CSCs) that contribute to tumour initiation and therapeutic resistance. These cells in malignant gliomas were called glioblastoma stem cells (GSCs).³⁶ Recent studies uncovered increased expression, aberrant localization and disturbed functions of certain cysteine cathepsins in GSCs.³⁷ Cathepsins B and L are overexpressed in glioma cells and responsible for glioblastoma cell invasion.^{38,39} The scientific literature shows ability of aminophosphonic acids and aminophosphonates to act as inhibitors of esterases,^{17,40} protease inhibitors⁴¹ and cathepsins. Therefore, targeting cathepsin activity by specific protease inhibitors in GBM is a logical consequence and might interfere with proliferation, and migration to avoid recurrence of glioblastoma.

2. Results and discussion

2.1 Synthesis

Diethyl (2-(perfluorophenyl)-1-(phenylamino)ethyl)phosphonate (**1a**, Scheme 1) was subjected to S_NAr reactions with thiols, amines and phenols resulted in the formation of *para*-substituted products (**2a-s**, Scheme 1, Table 1) with full regioselectivity.

Several conditions already described in the literature for these S_NAr reactions^{32,42-44} were applied to the reaction of **1a** with both hard and soft nucleophiles. Hard nucleophiles, such as hydroxide or alkoxides, had a clear tendency to attack the phosphonate ester while soft nucleophiles cleanly afforded the S_NAr reactions (Table 1).

Following the procedure developed by Spokoiny *et al.*,³² thiols reacted smoothly in DMF, in the presence of tris(hydroxymethyl)aminomethane (TRIS) at room temperature within short reaction time (2 h). In order to study the scope of the reaction, syntheses with different thiols were performed (Table 1, entries 1–10). Nucleophilic aromatic substitution of **1** with thiophenols (Table 1, entries 1–7) occurred in good to excellent yields (76–95%). Both EDG (Table 1, entries 2–6) and EWG (Table 1, entry 7) substituents did not affect the reaction yields in comparison to the reaction performed with unsubstituted thiophenol. Decylthiol was chosen to introduce long aliphatic chain into the structure (Table 1, entry 9). However, compound **2i** was obtained in a modest 37% yield. Introduction of other aliphatic thiols, such as benzyl mercaptan (Table 1, entry 8) and *N*-acetyl-L-cysteine methyl ester (Table 1, entry 10), resulted in better yields. Nevertheless, reactions performed with the aliphatic thiols occurred with lower yields than those with the aromatic ones. Formation of **2h-i** was monitored by ¹⁹F NMR analysis. ³¹P NMR spectra of crude material confirmed the full conversion of **1a**. However, lower yields which the reactions occurred with, were caused by formation of several minor by-



Table 1 Reaction conditions and yield of products formed in S_NAr reactions of **1a** with nucleophiles

Entry	Compound	Nucleophile	Conditions	Yield [%]
1	2a	C_6H_5SH	TRIS, DMF, rt, 2 h	87
2	2b	$4-CH_3C_6H_4SH$		89
3	2c	$3,5-(CH_3)_2C_6H_3SH$		81
4	2d	$4-CH_3OC_6H_4SH$		95
5	2e	$3-CH_3OC_6H_4SH$		84
6	2f	$3-NH_2C_6H_4SH$	DMSO, 80 °C, 3 h	76
7	2g	$4-BrC_6H_4SH$		86
8	2h	$C_6H_5CH_2SH$		65
9	2i	$CH_3(CH_2)_8CH_2SH$		37
10	2j	<i>N</i> -acetyl-L-cysteine methyl ester		71
11	2k	CH_3NH_2	K_2CO_3 , DMF, 80 °C, 24 h	71
12	2l	$CH_3(CH_2)_2NH_2$		74
13	2m	$CH_3(CH_2)_3NH_2$		60
14	2n	$CH_2=CHCH_2NH_2$		65
15	2o	$C_6H_5CH_2NH_2$		63
16	2p	C_6H_5OH		68
17	2q	$4-CH_3OC_6H_4OH$		48
18	2r	$4-ClC_6H_4OH$		56
19	2s	$3-NO_2C_6H_4OH$		48

products. This can be rationalized by the fact that aliphatic thiols are better nucleophiles in comparison to thiophenols thus, their reactivity is increased.

Reaction of **1a** with a large excess of alkylamine in DMSO after 3 hours of heating at 80 °C gave aryl aminophosphonates **2k–o** in yields ranging from 60 to 74% (Table 1, entries 11–15). Progress of the reactions was monitored by ^{19}F NMR, and after 3 hours a total conversion of the starting α -aminophosphonate was generally observed.

Different phenols were consecutively used as nucleophiles. The expected products were obtained in moderate yields (Table 1, entry 16–19). Following known procedure for this kind of reactions,^{42,43} the syntheses were performed in DMF in the presence of K_2CO_3 , at 80 °C for 24 hours. In comparison to alkoxides in which the negative charge is located only on the oxygen atom, phenoxides are much softer nucleophiles therefore, the reactions proceeded efficiently.

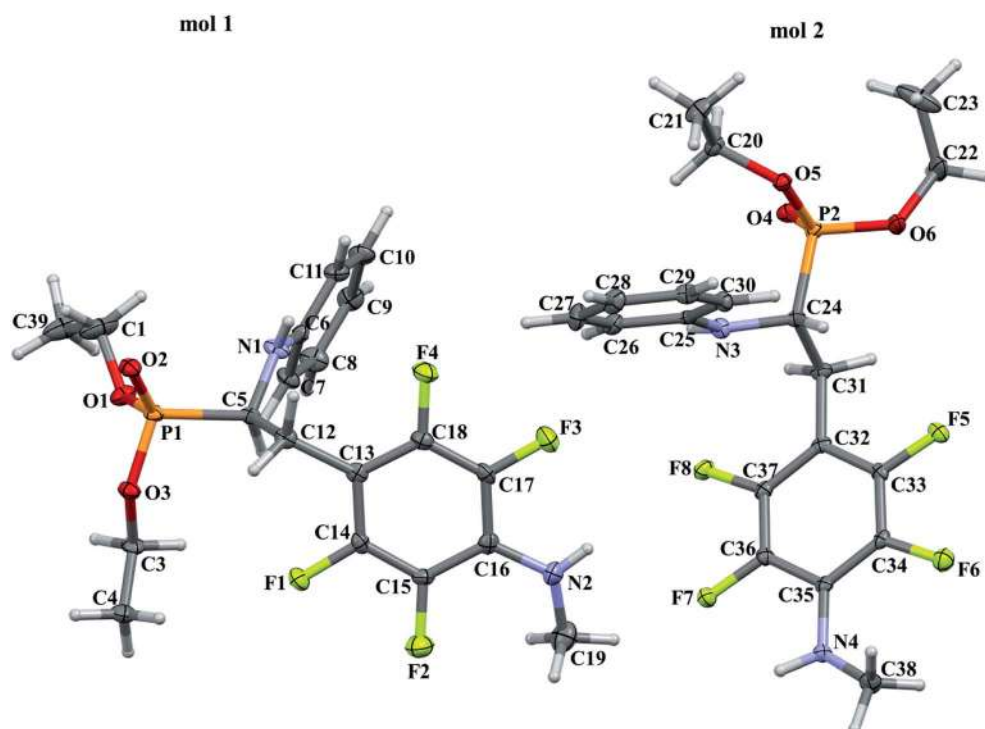


Fig. 1 A perspective view showing the asymmetric part of unit cell of **2k** together with the labelling atoms scheme. Ellipsoids are drawn at the 30% probability level, hydrogen atoms are represented by spheres of arbitrary radii.



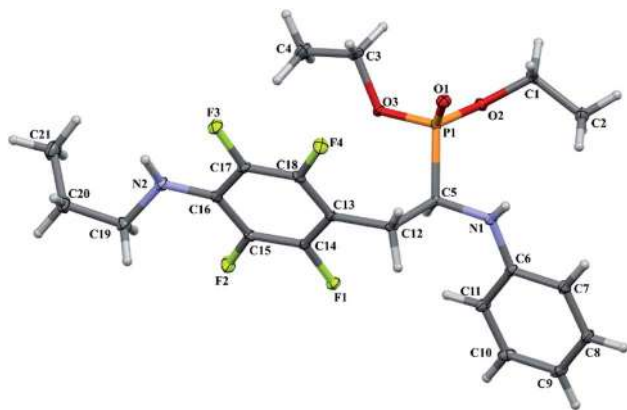


Fig. 2 A perspective view showing the asymmetric part of unit cell of **2l** together with the labelling atoms scheme. Ellipsoids are drawn at the 30% probability level, hydrogen atoms are represented by spheres of arbitrary radii.

2.2 X-ray and DFT studies

In order to comprehensively understand the structural features of compounds investigated we made attempts to grow their crystals by slow evaporation of toluene solutions under ambient conditions. This process resulted in obtaining crystals suitable for X-ray single crystal diffraction analysis only for **2k** and **2l**. In terms of chemistry, compounds **2k** and **2l** differ in the chain length of substituent in *para* position of the fluorinated ring; **2k** contains the methylamine group, while propylamine group is present in **2l**. Molecules of **2k** and **2l** are chiral, however, they are not enantiomerically pure since the racemic mixture of substrate (**1a**) was used for their synthesis. Thus, crystallization yielded single crystals containing both enantiomers. Since the nitrogen atoms included in the molecular structure of **2k** and **2l** are pyramidal in shape, *i.e.* are bonded to three different groups and possess lone pair of electrons, they constitute stereogenic centres. This, in turn, may affect the occurrence of diastereomers in the crystals.

Both compounds examined crystallize in the triclinic space group *P*-1. **2k** crystallizes with two molecules (mol 1 and mol 2) in the asymmetric unit that adopt a similar molecular conformation described by two torsion angles $\text{Csp}^2\text{-N-Csp}^3\text{-Csp}^3$ and $\text{Csp}^2\text{-Csp}^3\text{-Csp}^3\text{-N}$. The torsion angle values are $-131.3(3)^\circ$ and $-83.9(3)^\circ$ for mol 1, and $147.3(2)^\circ$ and $-68.9(3)^\circ$ for mol 2, while

the dihedral angle of two aromatic rings are $68.6(3)^\circ$ and $78.0(3)^\circ$ in mol 1 and 2, respectively. Adoption of very similar conformation (marked as a conformation B), by some phosphonates analogues of fluorophenylalanine was previously observed in our research group.³³ The main structural differences are displayed within the aliphatic diethyl phosphonate chains ($\text{Csp}^3\text{-Csp}^3\text{-O-P}$) where the chains adopt anti-periplanar-antiperiplanar conformation with the corresponding torsion angle values of $-171(3)$ and $165.7(2)^\circ$ for mol 1, while the synclinal-antiperiplanar conformation is characterized by the values of $65.5(4)$ and $176.1(2)^\circ$ for mol 2. Interestingly, the configuration of the aniline nitrogen atom in both symmetrically independent molecules shows the opposite stereochemistry *i.e.* *S* in mol 1 and *R* in mol 2; however, the methylamine moieties have the same *R* configuration. As a consequence these two molecules possess *R*(C5), *S*(N1), *R*(N2) and *R*(C24), *R*(N3), *R*(N4) configurations, respectively. Therefore they are diastereomers. Since the crystals of **2k** are centrosymmetric, two pairs of diastereomers coexist in the crystal structure. The pyramidal environment of N atom in methylamine group also affects significant deviation of this group from the tetrafluorophenyl plane; the $\text{Csp}^2\text{-Csp}^2\text{-N-Csp}^3$ torsion angle is $18.6(5)^\circ$ in mol 1 and $21.2(4)^\circ$ in mol 2. In contrast, **2l** crystallizes with one molecule in the asymmetric unit and adopts previously described conformation C (extended)³³ with the corresponding torsion angles of $80.3(2)^\circ$ and $-172.4(2)^\circ$. The anticlinal-antiperiplanar combination of the aliphatic diethyl phosphonate chains have torsion angles of $107.5(2)$ and $178.8(2)^\circ$. The propylamine group adopts a folded conformation with an $\text{N-Csp}^3\text{-Csp}^3\text{-Csp}^3$ torsion angle of $62.5(3)^\circ$. The stereochemistry of nitrogen atoms in this group is *S* while in the aniline moiety is *R*, hence the molecule in the asymmetric unit has a *R*(C5), *R*(N1), *S*(N2) configuration. The crystals investigated were chosen such that the *R* stereogenic centre at the carbon atom constitutes the asymmetric unit. The molecular structures of **2k** and **2l** are shown in Fig. 1 and 2.

The different configuration exhibited by the aniline nitrogen atoms in **2k**, prompted us to use quantum-chemical methods to study energy differences between the diastereomers. For this purpose all possible stereoisomers (*R,R,R*; *R,S,S*; *R,S,R*; *R,R,R*) were built based on the geometry of mol 1 derived from the crystal. The calculations were performed *in vacuo*, as well as using the polarisable continuum model (PCM) to take into account the

Table 2 Relative energies (ΔE (kcal mol⁻¹)^a, zero-point energy (ZPE (kcal mol⁻¹)^b, thermal correction to Gibbs free energy (TCG (kcal mol⁻¹)^c and percentage of populations (Pop) calculated for **2k** stereoisomers *in vacuo* and using PCM for chloroform at the WB97XD/6-31+G(d) level of theory. Not that the absolute configuration *R* is imposed at the C5 and C24 carbon atoms

Stereoisomer	ΔE <i>in vacuo</i>	ΔE in chloroform	ZPE	TCG	Pop <i>in vacuo</i>	Pop in chloroform
<i>R,S,R</i>	0.00	5.12	0.00	0.00	98.9	<1.0
<i>R,S,S</i>	2.35	5.25	0.15	0.50	1.0	<1.0
<i>R,R,S</i>	5.77	1.88	0.39	0.97	<1.0	20.2
<i>R,R,R</i>	5.90	0.00	0.78	2.00	<1.0	79.1

^a Relative energies calculated with respect to the lowest energy structure at WB97XD/6-31+G(d) level is equal to -1813.40587669 hartree. ^b Zero-point energy; the lowest value was equal to 0.412559 hartree. ^c Thermal correction to Gibbs free energy at 298 K; the lowest value was equal to 0.348198 hartree. The relative energy in chloroform was equal to -1813.41658767 hartree.



Table 3 Valence angles values at nitrogen atoms in crystal structure of **2k** (X-ray), and calculated at the WB97XD/6-31+G(d) level of theory (DFT). The numbering of valence angles are the same as numbering atoms scheme in Fig. 1. Not that the absolute configuration *R* is imposed at the C5 and C24 carbon atoms

The valence angle [°]	Stereomer					
	<i>R,S,R</i> DFT	X-ray	<i>R,R,R</i> DFT	X-ray	<i>R,R,S</i> DFT	<i>R,S,S</i> DFT
C6–N1–H1 or C25–N3–H3	112.3	116.7	113.6	115.2	114.2	113.1
C5–N1–H1 or C24–N3–H3	113.2	115.8	112.3	117.1	113.1	113.3
C6–N1–C5 or C25–N3–C24	126.1	125.4	123.1	124.9	123.5	126.2
Sum of the valence angles	351.6	357.9	349.0	357.2	350.8	352.6
C16–N2–H2 or C35–N4–H4	111.6	115.9	111.1	113.9	111.5	111.5
C19–N2–H2 or C38–N4–H4	113.4	116.9	112.8	113.2	113.2	113.3
C16–N2–C19 or C35–N4–C38	121.8	125.2	120.7	123.3	121.6	121.5
Sum of the valence angles	346.8	358.0	344.9	350.4	346.3	346.3

effect of chloroform solution as NMR spectra of **2k** were recorded for the compound sample dissolved in CDCl₃.

The results obtained support structural diversity of the possible stereoisomers. The results show that the *R,R,R* stereoisomer is energetically favourable at WB97XD/6-31+G(d) level of theory including PCM, while *in vacuo* the lowest relative energy is assigned to the *R,S,R* stereoisomer (Table 2).

These findings suggest a strong influence of surrounding medium on the energy preferences of stereoisomers (diastereoisomers). *In vacuo* the *R,S,R* stereoisomer constitutes over 98% in the

population of the calculated diastereoisomers (Table 2). Its population drops below 1% when chloroform solvent is taken into consideration *via* PCM approximation. In chloroform *R,R,R* stereoisomer seems to be predominant as it constitutes almost 80% in the population of diastereoisomers – a striking difference with its contribution (below 1%) *in vacuo*.

Analysis of the nitrogen atoms pyramidal environment in both the optimized and crystal structures of **2k** shows strong deviation from the ideal tetrahedral geometry, described by set of valence angles equal to 109.5°, towards the planar one. As

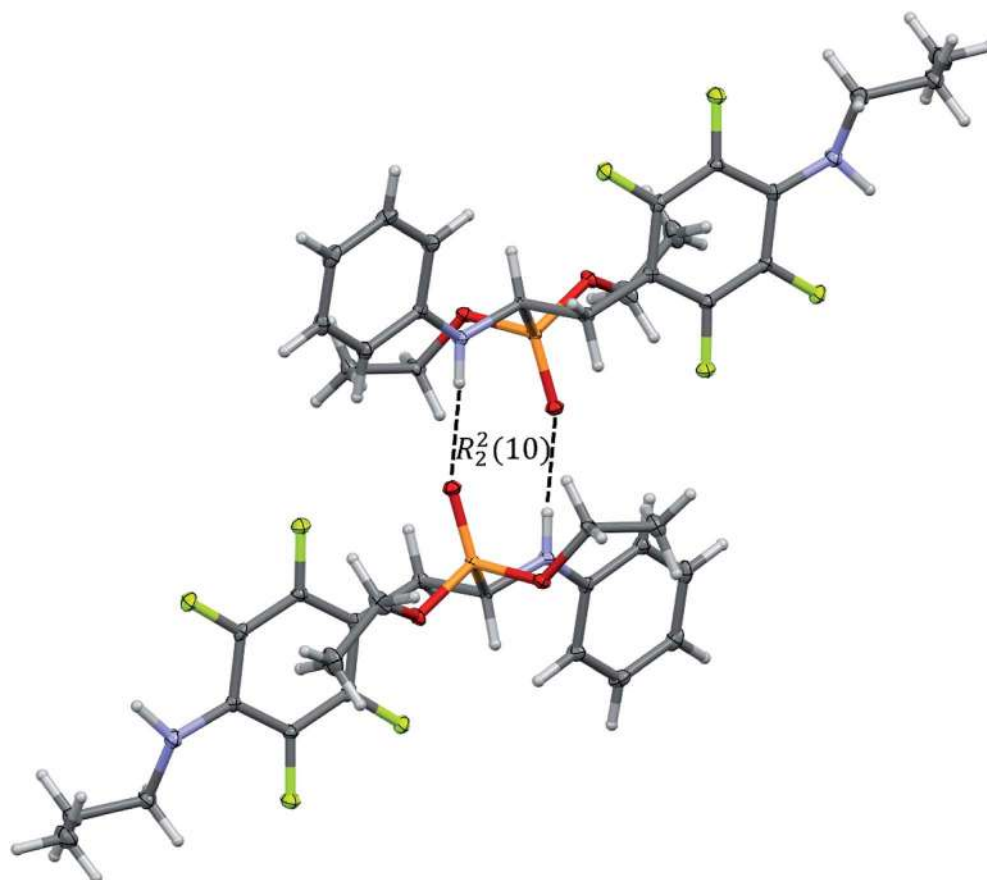


Fig. 3 Dimeric motif of N–H···O(=P) hydrogen bonds between two enantiomers of opposite stereochemistry in the crystals of **2l**. This motif is also observed in the crystals of **2k**.

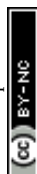


Table 4 Geometrical parameters describing the N–H...O interactions in the crystals of 2k and 2l

	D–H [Å]	H...A [Å]	D...A [Å]	D–H...A [°]	SYMM
2k					
N1–H1...O1	0.81(2)	2.16(2)	2.975(3)	177(2)	$[-x + 2, -y + 1, -z + 1]$
N3–H3...O4	0.86(2)	2.12(3)	2.964(2)	168(3)	$[-x + 1, -y + 1, -z]$
2l					
N1–H1...O1	0.85(3)	2.03(3)	2.871(3)	173(2)	$[-x + 1, -y + 2, -z + 1]$

seen in Table 3, the valence angles values in both structures are similar. For example, in the crystal structure the values of C6–N1–H1, C5–N1–H1 and C6–N1–C5 valence angles are equal to 116.7°, 115.8°, and 125.4°, in mol 1, and 115.2°, 117.1°, and 124.9° for C25–N3–H3, C24–N3–H3 and C24–N3–C25 in mol 2, while in the calculated *R,R,R* stereomer the corresponding valence angles adopt values of 113.6°, 112.3° and 123.1° (Table 3). The sum of the valence angles values in the crystal structure is equal to 357.9° and 357.2°, in mol 1 and mol 2, respectively, and 349° in the *R,R,R* stereomer calculated. All these values correspond to forms between the ideally planar (360°) and ideally tetrahedral (328.5°) geometry but closer to the planar one.

The bond order analysis showed that bond order for the bond between C6–N1 (nitrogen atom attached to the carbon atom in the phenyl ring) is *ca* 1.10. For the bond between C16–N2 (nitrogen atom attached to the carbon atom in the

perfluorinated phenyl ring) the bond order is *ca* 1.12, further explaining partially planar arrangement at the nitrogen atoms. Indeed the pyramidal shape at the N atom can invert its configuration in the structures investigated. The energy barrier of transition between the diastereomers (invertomers) was estimated in DFT calculations to *ca* 2.7 kcal mol^{−1} *in vacuo*. The temperature of coalescence (*T_c*) was approximated to *ca* 53 K in accordance to eqn (2) (Experimental section). The transition energy in chloroform differed from the value calculated *in vacuo* conditions, and was estimated to *ca* 2.5 kcal mol^{−1}. The *T_c* temperature was approximated to *ca* 50 K.

Our quantum chemical computational results refer to isolated molecule or a molecule in a polarisable continuum that model chloroform solution. On the other hand in the solid state the molecules can interact with the actual molecules in the crystal environment. Unfortunately, the attempt to perform calculations with PBC (Periodic Boundary Conditions) for such

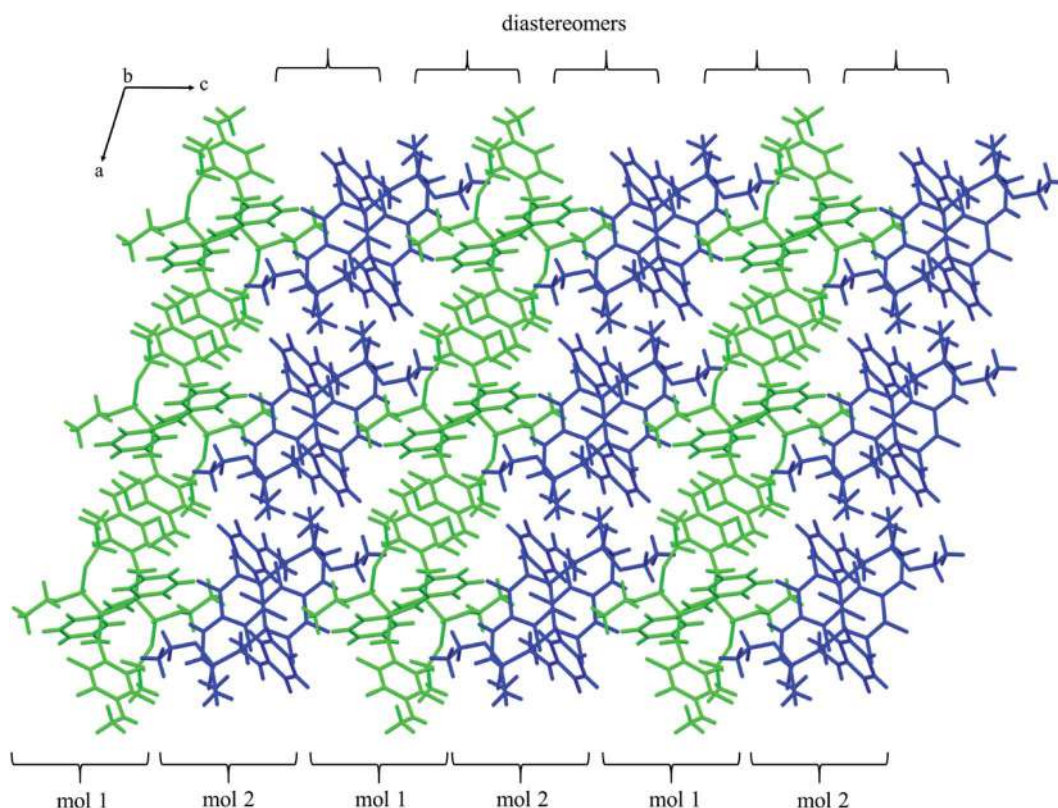


Fig. 4 Self-sorting of diastereomers on supramolecular level in the crystals of 2k. Two symmetry independent molecules that represent two diastereomers (mol 1 and mol 2) were distinguished by green and blue colors.



Table 5 Geometrical parameters for other types of intermolecular interactions that occurs in the crystals investigated

	D–H [Å]	H···A [Å]	D···A [Å]	D–H···A [°]	SYMM
2k					
C11–H11···F7	0.95	2.55(3)	3.333(4)	140(3)	$[x + 1, y, z]$
C22–H22A···F3	0.99	2.55(3)	3.387(4)	143(3)	$[-x + 1, -y + 1, -z]$
C26–H26···F3	0.95	2.42(2)	3.153(3)	134(3)	
N4–H4···O1	0.85(3)	2.29(4)	3.074(3)	154(3)	$[-x + 1, -y + 1, -z + 1]$
N2–H2···F7	0.79(3)	2.53(3)	3.017(3)	121(3)	
C22–H22A··· π	0.99	2.78(4)	3.486(5)	129(4)	$[-x + 1, -y + 1, -z]$
C22–H22B··· π	0.99	2.67(5)	3.529(6)	145(5)	$[-x + 1, -y + 1, -z]$
C28–H28···F5	0.95	2.67(3)	3.299(3)	125(3)	$[-x, -y, -z]$
2l					
C1–H1B···F1	0.99	2.59(3)	3.339(2)	133(2)	$[-x + 2, -y + 2, -z + 1]$
C5–H5···O2	1.00	2.62(2)	3.526(2)	152(2)	$[-x + 2, -y + 2, -z + 1]$
N2–H2···F3	0.98(3)	2.19(3)	3.159(2)	171(2)	$[-x + 1, -y + 1, -z]$

a large system resulted in failure due to restrictions on super-computer's resources.

In the crystal structures of **2k** and **2l** the principal interactions are N–H···O(=P) hydrogen bonds that hold two

enantiomers of opposite stereochemistry together around a centre of symmetry, as shown in Fig. 3. Geometrical parameters describing hydrogen bonds are listed in Table 4. It is worth

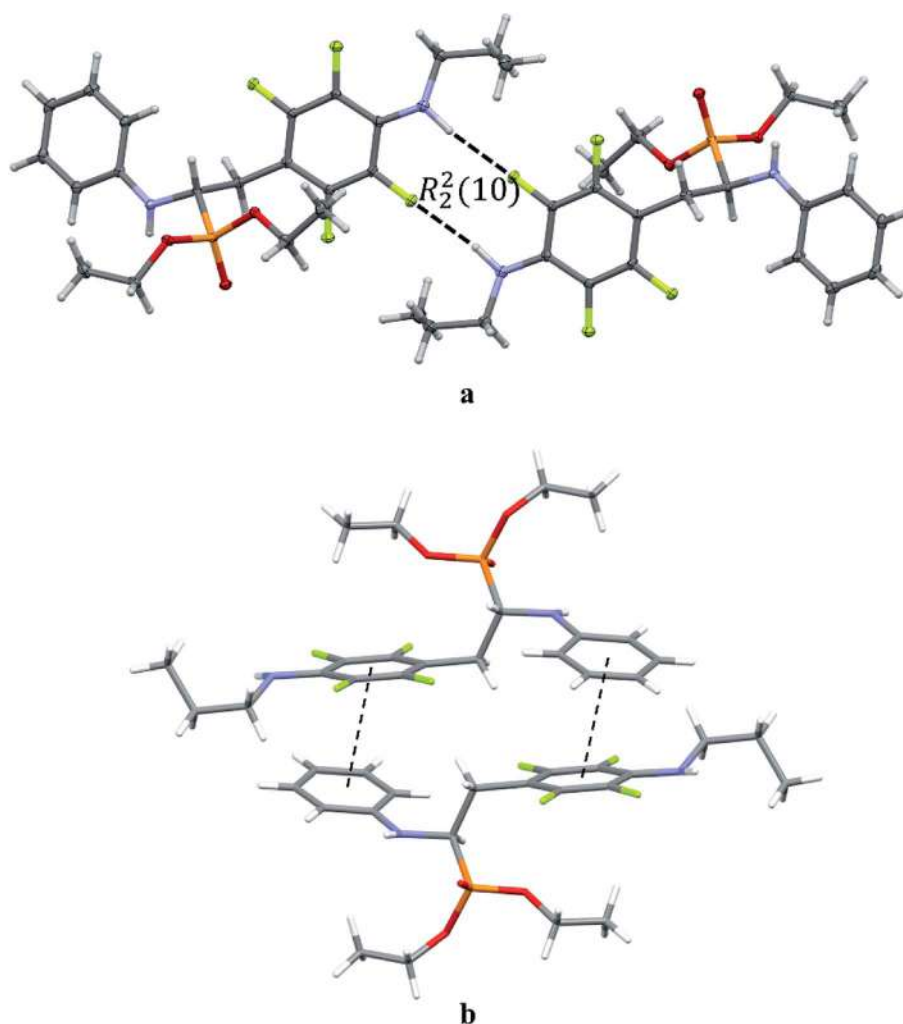


Fig. 5 Dimeric motif of N–H···F interactions (a) and $\pi\cdots\pi$ interactions between two enantiomers of opposite stereochemistry in the crystals of **2l** (b).



noting that in **2k** this motif occurs between enantiomers but not between diastereomers.

The self-sorting phenomenon of diastereomers at supramolecular level is observed in the crystals of **2k**. Since there are two diastereomers in the asymmetric unit, each of them forms separate layer perpendicular to *c* lattice direction containing pairs of enantiomers. The layers are stabilized by the previously mentioned N–H...O(=P) hydrogen bonds, which are supported by interactions involving π -electrons. The fluorinated rings of two neighbouring mol 1 oriented parallel to each other favours the formation of π ... π interactions between these moieties, while a tilted arrangement of fluorinated and non-fluorinated aromatic rings of two mol 2 leads to formation of H... π and H...F contacts (2.77(3) and 2.67(3) Å, respectively) that are equal or shorter than the sum of van der Waals radii of hydrogen and carbon atoms ($H_{vdW} = 1.2$ Å, $C_{vdW} = 1.7$ Å, the sum = 2.9 Å),⁴⁵ and hydrogen and fluorine atoms ($F_{vdW} = 1.47$ Å, the sum is 2.67 Å).⁴⁵ The geometrical parameters describing π ... π interactions are 3.319(3) and 0.88 Å for distance between the planes of aromatic rings along with the offset, respectively. The two layer types are alternately arranged in the crystal as shown in Fig. 4, and are related to each other through N–H...O(=P) hydrogen bonds in which the methylamine group acts as a donor of hydrogen bonds. The geometrical parameters of the hydrogen bond listed in Table 5 suggest that the interactions between diastereomers are slightly weaker than those between enantiomers in the layer. Moreover, the proximity of the aromatic rings and ester group belonging to diastereomers in two neighbouring layers promotes the formation of multiple H...F and H...F interactions and thus substantially affects the crystal structure stabilization.

The extended C molecular conformation of **2l** favours self-association forming dimers in which the two molecules interact *via* N–H...F hydrogen bonding and strong affects the formation of isolated π ... π intermolecular interactions between fluorinated and non-fluorinated aromatic rings as shown in Fig. 5. The interacting rings are tilted toward each other by 16° while the interplanar distance between the centres of gravity is

3.796 Å. The intermolecular interactions present in the crystals are reported in Table 5.

2.3 Biological evaluation

Six randomly chosen S_NAr reactions products, and also six of the fluorinated phosphonate analogues of phenylalanine, including **1a**, previously synthesized in our research group,³³ were subjected to biological studies. The first group of compounds contained mainly different thiophenols derivatives, while various α -aminophosphonates differing in number and position of fluorine substituents in one of the phenyl rings constituted the second group.

In the compounds molecules, apart from amino group, two additional pharmacophores are present; fluorine substituents and phosphonate group that replaced an amino acid carbonyl one. A considerable number of α -aminophosphonates is known to exhibit various biological activities including anticancer one. On the other hand, a lot of existing drugs, for example synthetic statins and 5-fluorouracil, contain fluorine. This shows a remarkable potential of fluorine in pharmaceutical chemistry and provide a source for drug discovery.¹⁸ Therefore combination of fluorine pharmacophore with amino and phosphonate group in molecules of target compounds was expected to have an impact on their bioactivity.

2.3.1 Drug likeness. With the aim to develop novel anti-proliferative therapeutics, which are orally bioavailable and focusing particularly on glioma treatment, human intestinal absorption and blood–brain barrier penetration of α -aminophosphonates were calculated. The structure-based prediction models depended on physicochemical and molecular properties of the compounds were performed with various computing software.

The most common criteria used for preliminary evaluation of drug likeness of a compound encompass the Lipinski's "rule of 5".^{46,47} In this respect, the physicochemical parameters of the examined compounds generally match the rule (Table 6).

According to Veber's rule, reduced molecular flexibility, as measured by the number of rotatable bonds, and low polar surface area or total hydrogen bond count (sum of donors and

Table 6 Selected physicochemical data for the studies α -aminophosphonates^a

Cpd	MW	aPSA [Å ²]	PSA [Å ²]	log <i>P</i>	HBD	HBA	RB	Caco-2 [nm s ⁻¹]	BB	MDCK [nm s ⁻¹]
1a	423.31	303.9	8.7	4.22	1	4	9	21.72	1.38	86.84
1b	385.80	294.6	13.5	4.27	1	4	9	21.79	1.30	102.26
1c	385.80	312.7	8.7	4.25	1	4	9	21.74	1.27	104.23
1d	383.37	295.7	13.5	4.18	1	4	9	21.72	1.26	116.53
1e	351.35	297.4	8.7	3.64	1	4	9	21.72	0.98	168.46
1f	387.34	283.1	13.5	3.95	1	4	9	21.72	1.00	109.96
2b	527.52	364.9	27	6.25	1	4	11	21.72	1.06	42.40
2d	543.52	363.8	35.3	5.75	1	5	12	21.72	0.18	12.35
2e	543.52	367.5	36	5.75	1	5	12	21.72	0.18	14.77
2f	528.51	325.4	52	5.18	3	5	11	21.70	0.19	3.94
2j	580.15	329.0	81.8	3.75	2	8	15	21.69	0.01	0.06
2r	531.87	361.8	13.4	5.92	1	5	11	21.73	3.06	44.20

^a MW – molecular weight, aPSA – apolar surface area, PSA – polar surface area, log *P* – an octanol–water partition coefficient calculated with ALOGPS 2.1, HBD – number of hydrogen bond donors, HBA – number of hydrogen bond acceptors, RB – rotatable bonds number.

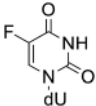


Table 7 Cytotoxicity (IC₅₀) values of the studies α -aminophosphonates

Compound	Compound structure	T98G IC ₅₀ [μ M]	U-118 MG IC ₅₀ [μ M]	HaCaT IC ₅₀ [μ M]
1a		40.4 $\pm$ 6.2	60.67 $\pm$ 8.9	56.8 $\pm$ 7.4
1b		73.5 $\pm$ 9.2	88.2 $\pm$ 7.5	>100
1c		67.9 $\pm$ 11.2	91.2 $\pm$ 15.5	>100
1d		71.3 $\pm$ 8.7	48.6 $\pm$ 10.4	67.9 $\pm$ 11.8
1e		106.4 $\pm$ 14.7	86.8 $\pm$ 7.2	61.8 $\pm$ 9.1
1f		25.1 $\pm$ 4.5	40.8 $\pm$ 8.4	33.2 $\pm$ 5.1
2b		14.5 $\pm$ 3.3	37.9 $\pm$ 2.5	26.5 $\pm$ 4.7
2d		36.8 $\pm$ 5.7	33.2 $\pm$ 7.3	19.8 $\pm$ 3.9
2e		20.4 $\pm$ 3.1	21.4 $\pm$ 2.7	10.1 $\pm$ 2.2
2f		25.5 $\pm$ 1.9	28.1 $\pm$ 3.6	18.2 $\pm$ 3.4
2j		82.7 $\pm$ 15.7	>100	>100
2r		70.3 $\pm$ 8.3	79.2 $\pm$ 6.4	48.4 $\pm$ 7.4



Table 7 (Contd.)

Compound	Compound structure	T98G IC ₅₀ [μM]	U-118 MG IC ₅₀ [μM]	HaCaT IC ₅₀ [μM]
5-FdU (5-fluoro-2'-deoxyuridine)		5.57 ± 0.9	23.40 ± 1.6	4.42 ± 1.1

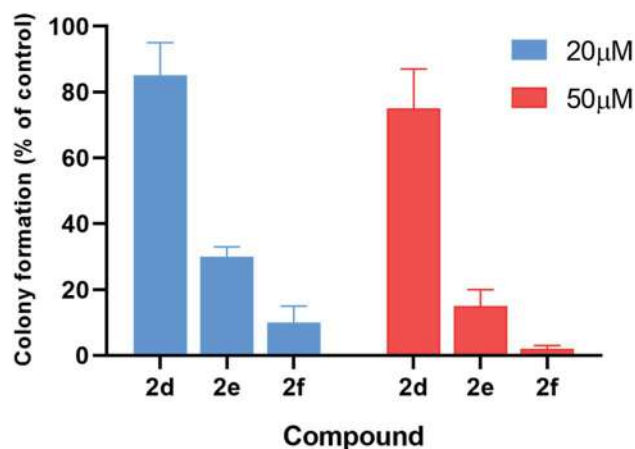


Fig. 6 Treatment with compounds **2d–f** significantly reduces the colony-forming ability of T98G cells as compared to untreated cells. Error bars display the standard deviation from at least three independent measurements.

acceptors) are found to be important predictors of good oral bioavailability, independent of molecular weight.⁴⁸ Veber's observations suggest that compounds which meet only the two criteria: 10 or fewer rotatable bonds and polar surface area equal to or less than 140 Å² (or 12 or fewer H-bond donors and acceptors) will have a high probability of good oral bioavailability. All of the examined compounds meet these criteria perfectly (Table 6).

Caco-2 cell permeability model classifies compounds into 3 classes of permeability (high, medium and low). Compounds with t_{Papp} below 4 nm s⁻¹ are classified as low permeable; compounds with t_{Papp} above 70 nm s⁻¹ are classified as high permeable and compounds with permeability values between 4–70 nm s⁻¹ are classified as medium.⁴⁹ For blood–brain barrier penetration we calculated the BB value which is defined as the ratio of the concentration of a drug in the brain and in the blood, measured at equilibrium ($\text{BB} = C_{\text{Brain}}/C_{\text{Blood}}$). Compounds with $\text{BB} > 2.0$ cross the blood–brain barrier readily (high absorption to CNS (central nervous system)) while molecules with $\text{BB} < 0.1$ are poorly distributed to the brain (low absorption to CNS).⁵⁰ Results show that all compounds are characterized by a good intestinal absorption (20–80%) and relatively high brain penetration, therefore making these compounds more druggable (Table 6).

2.3.2 In vitro cytostatic activity. Two glioblastoma multi-forme cell lines: T98G and U118 MG, and HaCaT as referential

healthy cells were chosen. For comparison, IC₅₀ values reflecting inhibitory activity of known anticancer drug 5-fluoro-2'-deoxyuridine in the chosen cell lines were included in Table 7.

As shown in this table, all compounds exhibited moderate cytostatic activity in the both glioblastoma cell lines. Most of the compounds showed higher activity against T98G cell line. Only **1d**, **1e** and to a small degree also **2d** occurred to be better inhibitors of U-118MG cell line. Most of the studied S_NAr reactions products displayed significantly higher inhibitory activity against both glioblastoma multi-forme cell lines than the parent α-aminophosphonate **1a**. This indicates that the introduction of thiophenols, but not aliphatic thiols nor phenols, to the *para* position in the fluorinated phenyl ring of **1a** molecule improve the anti-glioma activity. IC₅₀ values also demonstrate that for compounds **2b**, **2d**, **2e** and **2f**, the substituents in the thiophenol moiety had an influence on the cytotoxic activity (Table 7). All these substituents belong to EDG and it can be concluded that less electrodonating character of CH₃ group than of OCH₃ and NH₂ groups, results in higher activity of **2b** in comparison with **2d–2f**. Among the S_NAr reactions products studied, **2b** exhibiting the highest cytotoxic activity against T98G cell line and good selectivity can be considered as the best candidate for anti-glioma drug.

Analysis of IC₅₀ values for α-aminophosphonates **1a–1f**, indicates that number of fluorine substituents in one aromatic ring as well as presence and character of substituents in the second ring contribute to the anticancer activity displayed by these compounds. Compounds **1b** and **1c** containing one fluorine and one chlorine atoms, exhibit very similar inhibitory activity against both cancer cell lines indicating that position of this fluorine atom does not influence much the activity. Comparing these compounds with the **1e** demonstrates that chlorine substituent may constitute an important factor contributing to anticancer activity. Number of fluorine substituents also seems to have impact on the cytotoxic inhibition, though the influence is irregular.

The inhibitory effects of α-aminophosphonates in T98G cells were further assessed by performing clonogenic assays (Fig. 6). Compounds **2d–f** were also significantly more potent than other compounds in this assay. Treatment with 20 μM **2d–f** resulted in clonogenic survival of 85%, 30%, and 10%, respectively, compared to control cells. When we tested the effect of each compound at 50 μM, we found that **2d** reduces cancer cell clonogenic potential by 25%, **2e** by 85%, and **2f** by 98%.



3. Conclusions

Several diethyl (2-(perfluorophenyl)-1-(phenylamino)ethyl) phosphonate derivatives were synthesised by S_NAr reactions, and subjected to structural and biological studies. X-ray single crystal diffraction analysis performed for two of the obtained compounds (**2k** and **2l**) revealed their conformational preferences, self-sorting at supramolecular level and different conformation at the aniline nitrogen atoms. It was found that in the solid state, **2k** molecules adopt conformation with almost perpendicular arrangement of both aromatic rings, while molecule of **2l** prefers extended conformation with the aromatic rings arranged nearly parallel. Interestingly, in the crystals of parent **1a** its molecules also adopt conformation with parallel oriented rings. This indicates that introduction of substituent into *para* position in the fluorinated ring may or may not change the conformation of the resulted molecule comparing to the starting material.

Due to the different configuration at the aniline nitrogen atoms, two molecules in **2k** crystal structure constitute a pair of diastereomers. This phenomenon was studied with the use of quantum-chemical methods. Results obtained at WB97XD/6-31+G(d) level of theory for isolated molecule show energetic preference of *R,S,R* stereomer, while for a molecule in a polarisable continuum mimicking chloroform solution indicate the *R,R,R* stereomer as an energetically favourable. These results generally support the stereomers structural diversity. Coexistence of both stereomers observed in the crystal structure is most likely due to intermolecular interactions.

Structurally various α -aminophosphonates are known to exhibit activity against different cancer cell lines. However, to the best of our knowledge there is no studies referring to α -aminophosphonates as potential antiglioma agents. Malignant gliomas remain almost always fatal, and none of the current state of the art treatments can be regarded as effective.^{51,52} Synthesised by S_NAr reactions phosphono-perfluorophenylalanine derivatives perfectly meet the criteria of Lipinski's and Veber's rules. MTT assay results demonstrated that the compounds, particularly these formed by introduction of thiophenol moiety to *para* position in the fluorinated ring, exhibited significantly higher inhibitory activity against T98G and U118 MG cell lines than the parent **1a**. This enables to conclude that phosphono-perfluorophenylalanine core constitute promising scaffold for further studies focused on developing of new agents active against glioblastoma multiforme.

4. Experimental Section

4.1 Synthesis

4.1.1 General methods. Reagent grade chemicals were used. Solvents were dried over 4 Å molecular sieves. All moisture sensitive reactions were carried out under nitrogen atmosphere with dry, freshly distilled reagents when possible. All glassware was carefully dried under vacuum with a flameless heat gun. TLC was performed on Merck Kieselgel 60-F254 with EtOAc/cyclohexane as eluent, and products were detected by UV light (254 nm) and with a solution of potassium permanganate.

Merck Kieselgel 60 (230–400 mesh) was used for column chromatography. NMR spectra were recorded with instrument operating at 400 MHz (1H), 101 MHz (^{13}C), 377 (^{19}F) and 162 MHz (^{31}P). Chemical shifts (δ) are given in ppm and calibrated from residual signals of $CDCl_3$ (7.26 ppm), acetone- d_6 (2.09 ppm) for 1H NMR and $CDCl_3$ (77.16 ppm) and acetone- d_6 (29.84 ppm) for ^{13}C NMR. High resolution mass spectra were measured using electrospray ionization (ESI, positive-ion mode).

4.1.2 General procedure for the nucleophilic aromatic substitution of **1a with thiols.** To the mixture of thiol (0.24 mmol) and tris(hydroxymethyl)aminomethane (0.037 g, 0.30 mmol) in dry DMF (1 mL), a solution of **1a** (0.05 g, 0.12 mmol) in dry DMF (1 mL) was added under N_2 atmosphere. The mixture was stirred at room temperature for 2 h. Then solvent was removed under vacuum, and the crude was purified by column chromatography (CH_2Cl_2 /acetone 4 : 1, v/v).

4.1.2.1 Diethyl (1-(phenylamino)-2-(2,3,5,6-tetrafluoro-4-(phenylthio)phenyl)ethyl)phosphonate (2a**).** Pale yellow oil (48 mg, 87%). 1H NMR (400 MHz, acetone- d_6): δ = 7.33–7.28 (m, 3H, 3 CHar), 7.18–7.16 (m, 2H, 2CHar), 7.06–7.02 (m, 2H, 2CHar), 6.70 (d, J = 7.9 Hz, 2H, 2CHar), 6.60 (t, J = 7.3 Hz, 1H, CHar), 4.86–4.83 (m, 1H, NH) 4.16–4.05 (m, 5H, 2OCH₂, CH), 3.36–3.30 (m, 1H, ArCH₂), 3.24–3.14 (m, 1H, ArCH₂), 1.25 (t, J = 7.1 Hz, 3H, CH₃), 1.20 (t, J = 7.1 Hz, 3H, CH₃). ^{19}F NMR (377 MHz, acetone- d_6) δ = –135.96 to –136.06 (m, 2F), –142.90 to –142.99 (m, 2F). ^{31}P NMR (162 MHz, acetone- d_6) δ = 22.95 (s). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 148.05–145.45 (m, 2Car) 146.31 (d, J = 7.3 Hz, Car), 146.79–144.11 (m, 2Car) 133.39 (s, Car), 129.83 (s, 2CHar), 129.32–129.26 (m, 4 CHar) 127.50 (s, CHar), 118.77 (s, CHar), 118.25–117.89 (m, Car), 113.28 (s, 2CHar), 112.14–111.74 (m, Car), 63.73 (d, J = 7.0 Hz, OCH₂), 62.46 (d, J = 7.4 Hz, OCH₂), 50.46 (d, J = 158.3 Hz, CH), 25.16 (d, J = 7.4 Hz, ArCH₂), 16.56, 16.51, 16.45, and 16.39 (2CH₃). HRMS (ESI⁺) calcd for $C_{24}H_{25}F_4NO_3PS$ ($M + H$)⁺: 514.1229, found: 514.1230.

4.1.2.2 Diethyl (1-(phenylamino)-2-(2,3,5,6-tetrafluoro-4-(*m*-tolylthio)phenyl)ethyl)phosphonate (2b**).** Pale yellow solid (55 mg, 89%), mp = 103–105 °C. 1H NMR (400 MHz, acetone- d_6) δ = 7.20–7.10 (m, 4H, 4 CHar), 7.05–7.01 (m, 2H, 2CHar), 6.70 (d, J = 7.9 Hz, 1H, 2CHar), 6.60 (t, J = 7.3 Hz, 1H, CHar), 4.87–4.83 (m, 1H, NH), 4.13–4.04 (m, 5H, CH, 2OCH₂), 3.33–3.28 (m, 1H, ArCH₂), 3.22–3.13 (m, 1H, ArCH₂), 2.30 (s, 3H, ArCH₃), 1.24 (t, J = 7.1 Hz, 3H, CH₃), 1.19 (t, J = 7.1 Hz, 3H, CH₃). ^{19}F NMR (377 MHz, $CDCl_3$) δ = –134.05 (dd, J = 23.6, 11.5 Hz, 2F), –142.52 to –142.62 (m, 2F). ^{31}P NMR (162 MHz, acetone- d_6) δ = 23.01 (s). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 147.96–146.36 (m, 2Car), 146.77–144.05 (m, 2Car), 146.32 (d, J = 7.2 Hz, Car), 137.99 (s, Car), 130.83 (s, 2CHar), 130.11 (s, 2CHar), 129.62 (s, Car), 129.31 (s, 2CHar), 118.80 (s, CHar), 117.83–117.47 (m, Car), 113.36 (s, 2CHar), 112.87 (s, Car), 63.75 (d, J = 7.0 Hz, OCH₂), 62.48 (d, J = 7.4 Hz, OCH₂), 50.51 (d, J = 158.2 Hz, CH), 25.13 (d, J = 7.4 Hz, ArCH₂), 21.23 (s, ArCH₃), 16.59, 16.54, 16.48, and 16.42 (2CH₃). HRMS (ESI⁺) calcd for $C_{25}H_{27}F_4NO_3PS$ ($M + H$)⁺: 528.1385, found: 528.1387.

4.1.2.3 Diethyl (2-(4-((3,5-dimethylphenyl)thio)-2,3,5,6-tetrafluorophenyl)-1-(phenylamino)ethyl)phosphonate (2c**).** Pale yellow solid (52 mg, 81%). 1H NMR (400 MHz, acetone- d_6) δ =



7.05–7.01 (m, 2H, 2CHar), 6.91–6.89 (m, 3H, 3CHar), 6.72 (d, $J = 7.8$ Hz, 2H, 2CHar), 6.57 (t, $J = 7.3$ Hz, 1H, CHar), 4.89–4.86 (m, 1H, NH), 4.13–4.02 (m, 5H, CH, 2OCH₂), 3.33–3.30 (m, 1H, ArCH₂), 3.23–3.13 (m, 1H, ArCH₂), 2.83 (s, 6H, 2 ArCH₃), 1.23 (t, $J = 7.1$ Hz, 3H, CH₃), 1.17 (t, $J = 7.1$ Hz, 3H, CH₃). ¹⁹F NMR (377 MHz, CDCl₃) $\delta = -133.72$ to -133.81 (m, 2F), -142.38 (dd, $J = 24.4$, 11.9 Hz, 2F). ³¹P NMR (162 MHz, acetone-*d*₆) $\delta = 23.10$ (s). ¹³C NMR (101 MHz, CDCl₃) $\delta = 148.13$ – 145.45 (m, 2Car), 146.78–144.11 (m, 2Car) 146.30 (d, $J = 6.5$ Hz, Car), 139.06 (s, Car), 132.63 (s, 2Car), 129.69 (s, CHar) 129.31 (s, 2CHar), 128.05 (s, 2CHar), 118.87 (s, CHar), 118.13–117.64 (m, Car), 113.49 (s, 2CHar), 112.67–112.26 (m, Car), 63.69 (d, $J = 7.0$ Hz, OCH₂), 62.44 (d, $J = 7.4$ Hz, OCH₂), 50.60 (d, $J = 157.4$ Hz, CH), 25.09 (d, $J = 7.3$ Hz, ArCH₂), 21.28 (s, 2 ArCH₃), 16.56, 16.51, 16.44, and 16.38 (2CH₃). HRMS (ESI⁺) calcd for C₂₆H₂₉F₄NO₃PS (M + H)⁺: 542.1542, found: 542.1542.

4.1.2.4 Diethyl (1-(phenylamino)-2-(2,3,5,6-tetrafluoro-4-((methoxyphenyl)thio)phenyl)ethyl)phosphonate (2d). Pale yellow solid (61 mg, 95%), mp = 93–95 °C. ¹H NMR (400 MHz, acetone-*d*₆) $\delta = 7.30$ – 7.28 (m, 2H, 2CHar), 7.05–6.98 (m, 2H, 2CHar), 6.92–6.90 (m, 2H, 2CHar), 6.68 (d, $J = 7.8$ Hz, 2H, 2CHar), 6.58 (t, $J = 7.2$ Hz, 1H, CHar), 4.85–4.82 (m, 1H, NH), 4.11–4.06 (m, 5H, CH, 2OCH₂), 3.80 (s, 3H, ArCH₃), 3.31–3.27 (m, 1H, ArCH₂), 3.19–3.10 (m, 1H, ArCH₂), 1.23 (t, $J = 7.1$ Hz, 3H, CH₃), 1.17 (t, $J = 7.1$ Hz, 3H, CH₃). ¹⁹F NMR (377 MHz, CDCl₃) $\delta = -134.67$ (dd, $J = 24.0$, 11.8 Hz, 2F), -142.70 (dd, $J = 24.3$, 12.2 Hz, 2F). ³¹P NMR (162 MHz, acetone-*d*₆) $\delta = 23.04$ (s). ¹³C NMR (101 MHz, CDCl₃): $\delta = 159.95$ (s, Car), 147.79–145.13 (m, 2Car), 146.70–144.00 (m, 2Car), 146.30 (d, $J = 7.1$ Hz, Car), 134.07 (s, 2CHar), 129.25 (s, 2CHar), 123.25 (s, Car), 118.74 (s, CHar), 117.61–117.11 (m, Car), 114.88 (s, 2CHar), 114.14–113.69 (m, Car), 113.36 (s, 2CHar), 63.69 (d, $J = 7.0$ Hz, OCH₂), 62.43 (d, $J = 7.4$ Hz, OCH₂), 55.44 (s, OCH₃), 50.47 (d, $J = 158.1$ Hz, CH), 25.00 (s, ArCH₂), 16.54, 16.49, 16.42, 16.36 (2CH₃). HRMS (ESI⁺) calcd for C₂₅H₂₇F₄NO₄PS (M + H)⁺: 544.1335, found: 544.1339.

4.1.2.5 Diethyl (1-(phenylamino)-2-(2,3,5,6-tetrafluoro-4-((3-methoxyphenyl)thio)phenyl)ethyl)phosphonate (2e). Yellow oil (61 mg, 95%). ¹H NMR (400 MHz, acetone-*d*₆) $\delta = 7.23$ (t, $J = 8.0$ Hz, 1H, CHar), 7.06–7.02 (m, 2H, 2CHar), 6.85–6.80 (m, 2H, 2CHar), 6.71 (d, $J = 7.9$ Hz, 1H, 2CHar), 6.66 (d, $J = 7.9$ Hz, 1H, CHar), 6.59 (t, $J = 7.3$ Hz, 1H, CHar), 4.88–4.86 (m, NH), 4.15–4.04 (m, 5H, 2OCH₂, CH), 3.75 (s, 3H, OCH₃), 3.36–3.30 (m, 1H, ArCH₂), 3.24–3.14 (m, 1H, ArCH₂), 1.25 (t, $J = 7.1$ Hz, 3H, CH₃), 1.19 (t, $J = 7.0$ Hz, 3H, CH₃). ¹⁹F NMR (377 MHz, CDCl₃): $\delta = -133.45$ to -133.55 (m, 2F), -142.22 (dd, $J = 24.4$, 12.4 Hz, 2F). ³¹P NMR (162 MHz, acetone-*d*₆): $\delta = 23.04$ (s). ¹³C NMR (101 MHz, CDCl₃): $\delta = 159.99$ (s, Car), 148.10–145.44 (m, 2Car), 147.90–144.05 (m, 2Car), 146.28 (d, $J = 6.9$ Hz, Car), 134.52 (s, Car), 130.15 (s, CHar), 129.28–129.24 (s, 2CHar), 121.84 (s, CHar), 118.81 (s, CHar), 118.40–118.04 (m, Car), 115.25 (s, CHar), 113.34 (s, 2CHar), 113.12 (s, CHar), 111.88–111.47 (m, Car), 63.68 (d, $J = 7.0$ Hz, OCH₂), 62.45 (d, $J = 7.4$ Hz, OCH₂), 55.34 (s, OCH₃), 50.48 (d, $J = 157.9$ Hz, CH), 25.12 (d, $J = 7.3$ Hz, ArCH₂), 16.53, 16.47, 16.42, and 16.36 (2CH₃). HRMS (ESI⁺) calcd for C₂₅H₂₇F₄NO₄PS (M + H)⁺: 544.1335, found: 544.1328.

4.1.2.6 Diethyl (2-(4-((3-aminophenyl)thio)-2,3,5,6-tetrafluorophenyl)-1-(phenylamino)ethyl)phosphonate (2f). Pale

yellow oil (47 mg, 76%). ¹H NMR (400 MHz, acetone-*d*₆) $\delta = 7.06$ – 6.96 (m, 3H, 3CHar), 6.71 (d, $J = 8.3$ Hz, 2H, 2CHar), 6.57 (m, 3H, 3CHar), 6.35 (d, $J = 8.2$ Hz, 1H, CHar), 4.86–4.83 (m, 1H, NH₂), 4.76–4.74 (m, 1H, NH₂), 4.13–4.06 (m, 6H, CH, NH₂OCH₂), 3.35–3.29 (m, 1H, ArCH₂), 3.23–3.14 (m, 1H, ArCH₂), 1.24 (t, $J = 7.1$ Hz, 3H, CH₃), 1.19 (t, $J = 7.1$ Hz, 3H, CH₃). ¹⁹F NMR (377 MHz, CDCl₃) $\delta = -133.46$ (dd, $J = 23.5$, 11.4 Hz, 2F), -142.32 to -142.42 (m, 2F). ³¹P NMR (162 MHz, acetone-*d*₆) $\delta = 23.04$ (s). ¹³C NMR (101 MHz, CDCl₃) $\delta = 148.19$ – 145.53 (m, 2Car), 147.21 (s, Car), 146.65–144.08 (m, 2Car), 146.35 (d, $J = 6.6$ Hz, Car), 134.31 (s, Car), 130.12 (s, CHar), 129.35 (s, 2CHar), 119.61 (s, CHar), 118.80 (s, CHar), 118.37–117.88 (m, Car), 115.64 (s, CHar), 114.24 (s, CHar), 113.41 (s, 2CHar), 112.18–111.78 (m, Car), 63.68 (d, $J = 7.0$ Hz, OCH₂), 62.51 (d, $J = 7.4$ Hz, OCH₂), 50.56 (d, $J = 157.9$ Hz, CH), 25.09 (s, ArCH₂), 16.58, 16.52, 16.47, and 16.42 (2CH₃). HRMS (ESI⁺) calcd for C₂₄H₂₆F₄N₂O₃PS (M + H)⁺: 529.1338, found: 529.1335.

4.1.2.7 Diethyl (2-(4-((4-bromophenyl)thio)-2,3,5,6-tetrafluorophenyl)-1-(phenylamino)ethyl)phosphonate (2g). Pale yellow solid (60 mg, 86%), mp = 77–79 °C. ¹H NMR (400 MHz, acetone-*d*₆) $\delta = 7.52$ – 7.49 (m, 2H, 2CHar), 7.14–7.04 (m, 2H, 2CHar), 7.05–7.04 (m, 2H, 2CHar), 6.70 (d, $J = 8.6$ Hz, 2H, 2CHar), 6.61 (t, $J = 7.4$ Hz, 1H, CHar), 4.87–4.83 (m, 1H, NH), 4.16–4.05 (m, 5H, CH, 2OCH₂), 3.36–3.29 (m, 1H, ArCH₂), 3.24–3.18 (m, 1H, ArCH₂), 1.26 (t, $J = 7.1$ Hz, 3H, CH₃), 1.20 (t, $J = 7.0$ Hz, 3H, CH₃). ¹⁹F NMR (377 MHz, acetone-*d*₆) $\delta = -135.90$ to -136.00 (m, 2F), -142.59 to -142.69 (m, 2F). ³¹P NMR (162 MHz, acetone-*d*₆) $\delta = 22.89$ (s). ¹³C NMR (101 MHz, CDCl₃) $\delta = 148.05$ – 145.38 (m, 2Car), 146.87–144.21 (m, 2Car) 146.33 (d, $J = 7.2$ Hz, Car), 132.59 (s, Car), 132.46 (s, 2CHar), 131.50 (s, 2CHar), 129.34 (s, 2CHar), 121.74 (s, Car), 118.79 (s, CHar), 118.49 (s, Car), 113.27 (s, 2CHar), 111.45 (s, Car), 63.78 (d, $J = 7.0$ Hz, OCH₂), 62.54 (d, $J = 7.4$ Hz, OCH₂), 50.43 (d, $J = 158.6$ Hz, CH), 25.23 (d, $J = 7.5$ Hz, ArCH₂), 16.61, 16.56, 16.51, and 16.45 (2CH₃). HRMS (ESI⁺) calcd for C₂₄H₂₄BrF₄NO₃PS (M + H)⁺: 592.0334, found: 592.0336.

4.1.2.8 Diethyl (2-(4-((benzylthio)-2,3,5,6-tetrafluorophenyl)-1-(phenylamino)ethyl)phosphonate (2h). Yellow oil (40 mg, 65%). ¹H NMR (400 MHz, acetone-*d*₆) $\delta = 7.33$ – 7.20 (m, 1H, CHar), 7.17 (s, 4H, 4CHar), 7.08–7.04 (m, 2H, 2CHar), 6.70 (d, $J = 7.9$ Hz, 2H, 2CHar), 6.59 (t, $J = 7.3$ Hz, 1H, CHar), 4.82–4.79 (m, 1H, NH), 4.13–4.02 (m, 7H, SCH₂, CH, 2OCH₂), 3.30–3.24 (m, 1H, ArCH₂), 3.17–3.08 (m, 1H, ArCH₂), 1.24 (t, $J = 7.1$ Hz, 3H, CH₃), 1.17 (t, $J = 7.1$ Hz, 3H, CH₃). ¹⁹F NMR (377 MHz, acetone-*d*₆) $\delta = -136.30$ to -136.40 (m, 2F), -143.81 to -143.90 (m, 2F). ³¹P NMR (162 MHz, acetone-*d*₆) $\delta = 23.13$ (s). ¹³C NMR (101 MHz, CDCl₃) $\delta = 148.18$ – 145.53 (m, 2Car), 146.48–143.90 (m, 2Car) 146.45 (d, $J = 5.7$ Hz, Car), 136.55 (s, Car), 129.32 (s, 2CHar), 128.85 (s, CHar), 128.66 (s, 2CHar), 127.75 (s, 2CHar), 118.81 (s, CHar), 117.27–116.91 (m, Car), 113.51 (s, 2CHar), 112.49–112.08 (m, Car), 63.59 (d, $J = 7.0$ Hz, OCH₂), 62.45 (d, $J = 7.4$ Hz, OCH₂), 50.62 (d, $J = 157.1$ Hz, CH), 39.14 (s, ArCH₂S), 24.93 (d, $J = 6.9$ Hz, ArCH₂), 16.56, 16.51, 16.48, and 16.42 (2CH₃). HRMS (ESI⁺) calcd for C₂₅H₂₇F₄NO₃PS (M + H)⁺: 528.1385, found: 528.1387.

4.1.2.9 Diethyl (2-(4-(decylthio)-2,3,5,6-tetrafluorophenyl)-1-(phenylamino)ethyl)phosphonate (2i). Yellow oil (25 mg, 36%).



^1H NMR (400 MHz, CDCl_3) δ = 7.07–7.03 (m, 2H, 2CHar), 6.64 (t, J = 7.3 Hz, 1H, CHar), 6.53 (d, J = 7.9 Hz, 2H, 2CHar), 4.18–4.01 (m, 5H, CHP, 2OCH₂), 3.82–3.79 (m, 1H, NH), 3.28–3.24 (m, 1H, ArCH₂), 3.17–3.14 (m, 1H, ArCH₂), 2.80 (t, J = 7.3 Hz, 2H, CH₂S), 1.48–1.19 (m, 22H, 2CH₃, CH₃(CH₂)₈), 0.88 (t, J = 6.9 Hz, 3H, CH₃(CH₂)₉). ^{19}F NMR (377 MHz, CDCl_3) δ = –134.89 (dd, J = 24.3, 12.0 Hz, 2F), –143.26 (dd, J = 24.3, 12.1 Hz, 2F). ^{31}P NMR (162 MHz, CDCl_3) δ = 23.52 (s). ^{13}C NMR (101 MHz, CDCl_3) δ = 148.15–145.53 (m, 2Car), 146.68–143.97 (m, 2Car), 146.41 (d, J = 6.9 Hz, Car), 129.27 (s, 2CHar), 118.76 (s, CHar), 116.81–116.44 (m, Car), 113.44 (s, 2CHar), 113.15 (s, Car), 63.67 (d, J = 7.0 Hz, OCH₂), 62.46 (d, J = 7.4 Hz, OCH₂), 50.61 (d, J = 157.7 Hz, CH), 34.84 (s, SCH₂), 32.01 (s, CH₃(CH₂)₈), 29.83 (s, CH₃(CH₂)₈), 29.63 (s, CH₃(CH₂)₈), 29.59 (s, CH₃(CH₂)₈), 29.41 (s, CH₃(CH₂)₈), 29.16 (s, CH₃(CH₂)₈), 28.48 (s, CH₃(CH₂)₈), 25.03 (d, J = 7.3 Hz, ArCH₂), 22.80 (s, CH₃(CH₂)₈), 16.59, 16.53, 16.49, and 16.43 (2CH₃), 14.23 (s, CH₃(CH₂)₉) ppm. HRMS (ESI^+) calcd for $\text{C}_{28}\text{H}_{41}\text{F}_4\text{N}_3\text{O}_3\text{P}$ ($\text{M} + \text{H}$)⁺: 578.2481, found: 578.2477.

4.1.2.10 Methyl 2-acetamido-3-((4-(2-(diethoxyphosphoryl)-2-(phenylamino)ethyl)-2,3,5,6-tetrafluorophenyl)thio)propanoate (2j). Yellow oil (49 mg, 71%). ^1H NMR (400 MHz, CDCl_3) δ = 7.06 (t, J = 7.7 Hz, 2H, 2CHar), 6.65 (t, J = 7.2 Hz, 1H, CHar), 6.54–6.51 (m, 2H, 2CHar), 6.38–6.34 (m, 1H, NHCO), 4.76–4.72 (m, 1H, CHN), 4.14–4.08 (m, 5H, CHP, 2OCH₂), 3.86, 3.85 (2d, J = 3.6, 3.6 Hz, 1H, ArNH), 3.44, 3.41 (2s, 3H, CH₃O), 3.35–3.14 (m, 4H, ArCH₂, SCH₂), 1.92 (s, 3H, CH₃CO), 1.29 (t, J = 7.1 Hz, 3H, CH₃), 1.17 (td, J = 7.1, 3.4 Hz, 3H, CH₃). ^{19}F NMR (377 MHz, CDCl_3) δ = –133.64 to –133.74 (m, 2F), –142.36 to –142.49 (m, 2F) ppm. ^{31}P NMR (162 MHz, CDCl_3) δ = 23.45 and 23.41 (2s). ^{13}C NMR (101 MHz, CDCl_3) δ = 170.28 (s, CO), 169.92, 169.90 (2s, CO), 148.16–145.56 (m, 2Car), 146.31, 146.29 (2d, J = 5.0, 4.8 Hz, Car), 145.71–144.24 (m, 2Car), 129.34 (s, 2CHar), 118.86, 118.83 (2s, CHar), 118.10–117.59 (m, Car), 113.51, 113.44 (2s, 2CHar), 111.81–111.40 (m, Car), 63.6 (d, J = 7.0 Hz, POCH₂), 62.56, 62.48 (2d, J = 7.5, 7.4 Hz, POCH₂), 52.56, 52.55 (2s, CH₃O), 52.08, 52.02 (2s, CHCH₂S), 50.5, 50.4 (2d, J = 156.1, 156.2 PCH), 36.31, 36.24 (2t, J = 2.2, 2.1 Hz, SCH₂), 24.89, 24.82 (2d, J = 5.1, 6.2 Hz, ArCH₂), 22.95 (s, COCH₃), 16.5, 16.4 (2d, J = 5.3, 5.7 Hz, CH₃). HRMS (ESI^+) calcd for $\text{C}_{24}\text{H}_{30}\text{F}_4\text{N}_2\text{O}_6\text{PS}$ ($\text{M} + \text{H}$)⁺: 581.1498, found: 581.1503.

4.1.3 General procedure for the nucleophilic aromatic substitution of 1a with amines. To the mixture of **1a** (0.05 g, 0.12 mmol) in dry DMSO (1 mL), amine (3.6 mmol) was added under N₂ atmosphere in a Radley's tube. The mixture was stirred at 80 °C for 3 h. After cooling to the room temperature, the reaction mixture was poured into water (10 mL) and Et₂O (10 mL). The aqueous phase was extracted with Et₂O (3 × 10 mL), then combined organic layers were washed with water (3 × 20 mL) and dried over MgSO₄. After filtration, solvent was removed under reduced pressure and the crude was purified by column chromatography (cyclohexane/AcOEt 1 : 1, v/v).

4.1.3.1 Diethyl (1-(phenylamino)-2-(2,3,5,6-tetrafluoro-4-(methylamino)phenyl)ethyl)phosphonate (2k). Pale yellow solid (31 mg, 60%), mp = 74–76 °C. ^1H NMR (400 MHz, CDCl_3) δ = 7.10–7.06 (m, 2H, 2CHar), 6.65 (t, J = 7.4 Hz, 1H, CHar), 6.55 (d, J = 7.9 Hz, 2H, 2CHar), 4.17–3.97 (m, 5H, CH, 2OCH₂), 3.77 (dd, J = 10.6, 3.4 Hz, 1H, NH), 3.66 (bs, 1H, NH), 3.19–3.13 (m, 1H,

ArCH₂), 3.06–2.99 (m, 4H, NCH₃, ArCH₂), 1.30 (t, J = 7.0 Hz, 3H, CH₃), 1.20 (t, J = 7.1 Hz, 3H, CH₃). ^{19}F NMR (377 MHz, CDCl_3) δ = –146.46 to –146.55 (m, 2F), –161.22 to –161.30 (m, 2F). ^{31}P NMR (162 MHz, CDCl_3) δ = 24.13 (s). ^{13}C NMR (101 MHz, CDCl_3) δ = 147.18–144.46 (m, 2Car), 146.75 (d, J = 5.9 Hz, Car), 138.76–136.11 (m, 2Car), 129.22 (s, 2CHar), 124.77–124.68 (m, Car), 118.42 (s, CHar), 113.48 (s, 2CHar), 103.17–102.67 (m, Car), 63.44 (d, J = 7.0 Hz, OCH₂), 62.34 (d, J = 7.5 Hz, OCH₂), 51.06 (d, J = 156.3 Hz, CH), 33.18 (s, NCH₃), 24.03 (d, J = 6.6 Hz, ArCH₂), 16.60, 16.55, 16.52, 16.46 (2CH₃). HRMS (ESI^+) calcd for $\text{C}_{19}\text{H}_{24}\text{F}_4\text{N}_2\text{O}_3\text{P}$ ($\text{M} + \text{H}$)⁺: 435.1461, found: 435.1465.

4.1.3.2 Diethyl (1-(phenylamino)-2-(2,3,5,6-tetrafluoro-4-(propylamino)phenyl)ethyl)phosphonate (2l). Pale yellow solid (40 mg, 74%), mp = 78–80 °C. ^1H NMR (400 MHz, CDCl_3) δ = 7.08–7.04 (m, 1H, 2CHar), 6.64 (t, J = 7.3 Hz, 1H, CHar), 6.54 (d, J = 7.9 Hz, 2H, 2CHar), 4.17–3.98 (m, 5H, CH, 2OCH₂), 3.80–3.65 (m, 1H, NH), 3.65 (s, 1H, CH₂NH), 3.27–3.22 (m, 2H, CH₃CH₂CH₂N), 3.18–3.13 (m, 1H, ArCH₂), 3.06–3.00 (m, 1H, ArCH₂), 1.52 (dd, J = 14.4, 7.2 Hz, 2H, CH₃CH₂CH₂N), 1.30 (t, J = 7.1 Hz, 3H, CH₃), 1.20 (t, J = 7.1 Hz, 3H, CH₃), 0.92 (t, J = 7.4 Hz, 3H, CH₃CH₂CH₂N). ^{19}F NMR (377 MHz, CDCl_3) δ = –146.46 to –146.53 (m, 2F), –160.68 to –160.72 (m, 2F). ^{31}P NMR (162 MHz, CDCl_3) δ = 24.13 (s). ^{13}C NMR (101 MHz, CDCl_3) δ = 147.16–144.51 (m, 2Car), 146.72 (d, J = 6.3 Hz, Car), 138.79–136.16 (m, 2Car), 129.17 (s, 2CHar), 127.17–126.90 (m, Car), 118.37 (s, CHar), 113.42 (s, 2CHar), 103.16–102.64 (m, Car), 63.45 (d, J = 7.0 Hz, OCH₂), 62.31 (d, J = 7.5 Hz, OCH₂), 51.03 (d, J = 156.8 Hz, CH), 47.82 (t, J = 4.0 Hz, CH₃CH₂CH₂N), 24.04 (d, J = 7.0 Hz, ArCH₂), 23.93 (s, CH₃CH₂CH₂N), 16.57, 16.52, 16.49, and 16.43 (2CH₃), 11.17 (s, CH₃CH₂CH₂N). HRMS (ESI^+) calcd for $\text{C}_{21}\text{H}_{28}\text{F}_4\text{N}_2\text{O}_3\text{P}$ ($\text{M} + \text{H}$)⁺: 463.1774, found: 463.1773.

4.1.3.3 Diethyl (2-(4-(butylamino)-2,3,5,6-tetrafluorophenyl)-1-(phenylamino)ethyl)phosphonate (2m). Yellow oil (40 mg, 71%). ^1H NMR (400 MHz, CDCl_3) δ = 7.06 (t, J = 7.9 Hz, 2H, 2CHar), 6.64 (t, J = 7.3 Hz, 1H, CHar), 6.54 (d, J = 7.9 Hz, 2H, 2CHar), 4.17–3.97 (m, 5H, CH, 2OCH₂), 3.78 (dd, J = 10.8, 3.1 Hz, 1H, NH), 3.60 (bs, 1H, NH), 3.31–3.25 (m, 2H, NCH₂), 3.18–3.13 (m, 1H, ArCH₂), 3.06–3.00 (m, 1H, ArCH₂), 1.49–1.28 (m, 7H, CH₃, CH₂CH₂), 1.20 (t, J = 7.1 Hz, 3H, CH₃), 0.92 (t, J = 7.3 Hz, 3H, CH₃(CH₂)₃). ^{19}F NMR (377 MHz, CDCl_3) δ = –146.45 to –146.52 (m, 2F), –160.68 to –160.72 (m, 2F). ^{31}P NMR (162 MHz, CDCl_3) δ = 24.13 (s) ppm. ^{13}C NMR (101 MHz, CDCl_3) δ = 147.16–146.70 (m, 2Car), 146.73 (d, J = 6.2 Hz, Car), 138.77–136.15 (m, 2Car), 129.18 (s, 2CHar), 127.20–126.97 (m, Car), 118.38 (s, CHar), 113.43 (s, 2CHar), 103.13–102.61 (m, Car), 63.45 (d, J = 7.0 Hz, OCH₂), 62.31 (d, J = 7.5 Hz, OCH₂), 51.05 (d, J = 156.8 Hz, CH), 45.82 (t, J = 4.0 Hz, NCH₂), 32.88 (s, CH₂CH₂CH₂), 24.04 (d, J = 6.7 Hz, ArCH₂), 19.88 (s, CH₃CH₂CH₂), 16.58, 16.52, 16.49, and 16.43 (2CH₃), 13.87 (s, CH₃(CH₂)₃). HRMS (ESI^+) calcd for $\text{C}_{22}\text{H}_{30}\text{F}_4\text{N}_2\text{O}_3\text{P}$ ($\text{M} + \text{H}$)⁺: 477.1930, found: 477.1932.

4.1.3.4 Diethyl (2-(4-(allylamino)-2,3,5,6-tetrafluorophenyl)-1-(phenylamino)ethyl)phosphonate (2n). Pale yellow oil (35 mg, 65%). ^1H NMR (400 MHz, CDCl_3) δ = 7.09–7.05 (m, 2H, 2CHar), 6.64 (t, J = 7.3 Hz, 1H, CHar), 6.54 (d, J = 7.9 Hz, 2H, 2CHar), 5.88–5.81 (m, 1H, CH₂=CH), 5.15 (ddd, J = 13.7, 11.5, 1.3 Hz, 2H, CH₂=CH), 4.17–4.02 (m, 5H, CHP, 2OCH₂), 3.89–3.88 (m,



2H, NCH₂), 3.77–3.76 (m, 2H, 2 NH), 3.20–3.14 (m, 1H, ArCH₂), 3.07–2.97 (m, 1H, ArCH₂), 1.30 (t, *J* = 7.1 Hz, 3H, CH₃), 1.20 (t, *J* = 7.1 Hz, 3H, CH₃). ¹⁹F NMR (377 MHz, CDCl₃) δ = −146.13 to −146.22 (m, 2F), −159.83 to −159.92 (m, 2F). ³¹P NMR (162 MHz, CDCl₃) δ = 24.08 (s). ¹³C NMR (101 MHz, CDCl₃) δ = 147.14–146.66 (m, 2Car), 146.70 (d, *J* = 6.3 Hz, Car), 138.93–136.29 (m, 2Car), 135.27 (s, CH₂=CH), 129.20 (s, 2CHar), 126.66–126.39 (m, Car), 118.42 (s, CHar), 116.86 (s, CH₂=CH), 113.43 (s, 2CHar), 103.89–103.38 (m, Car), 63.50 (t, *J* = 6.8 Hz, OCH₂), 62.37 (t, *J* = 8.9 Hz, OCH₂), 51.01 (d, *J* = 156.8 Hz, CHP), 48.35 (t, *J* = 4.3 Hz, NCH₂), 24.09 (d, *J* = 7.0 Hz, ArCH₂), 16.58, 16.52, 16.49, 16.43 (2CH₃). HRMS (ESI⁺) calcd for C₂₁H₂₆F₄N₂O₃P (M + H)⁺: 461.1617, found: 461.1616.

4.1.3.5 Diethyl (1-(phenylamino)-2-(2,3,5,6-tetrafluoro-4-(methylamino)phenyl)ethyl)phosphonate (2o). Pale yellow oil (38 mg, 63%). ¹H NMR (400 MHz, acetone-*d*₆) δ = 7.33–7.27 (m, 5H, 5 CHar), 7.04–7.00 (m, 2H, 2CHar), 6.67–6.65 (m, 2H, 2CHar), 6.57 (t, *J* = 7.3 Hz, 1H, CHar), 5.60 (bs, 1H, NHCH₂), 4.71–4.67 (m, 1H, NH), 4.52 (d, *J* = 7.0 Hz, 2H, NCH₂), 4.08–4.01 (m, 5H, CH, 2OCH₂), 3.16–3.10 (m, 1H, ArCH₂), 3.00–2.90 (m, 1H, ArCH₂), 1.21 (t, *J* = 7.1 Hz, 3H, CH₃), 1.15 (t, *J* = 7.0 Hz, 3H, CH₃). ¹⁹F NMR (377 MHz, acetone-*d*₆) δ = −147.27 to −147.39 (m, 2F), −161.21 to −161.32 (m, 2F). ³¹P NMR (162 MHz, acetone-*d*₆) δ = 23.69 (s). ¹³C NMR (101 MHz, CDCl₃) δ = 146.91–144.51 (m, 2Car), 146.69 (d, *J* = 6.0 Hz, Car), 139.10 (s, Car), 138.95–136.33 (m, 2Car), 129.23 (s, 2CHar), 128.89 (s, 2CHar), 127.81 (s, CHar), 127.67 (s, 2CHar), 126.72–126.57 (m, Car), 118.45 (s, CHar), 113.47 (s, 2CHar), 104.00–103.63 (m, Car), 63.47 (d, *J* = 7.0 Hz, OCH₂), 62.33 (d, *J* = 7.5 Hz, OCH₂), 51.01 (d, *J* = 156.0 Hz, CH), 50.18 (s, ArCH₂N), 24.09 (d, *J* = 7.1 Hz, ArCH₂), 16.58, 16.53, 16.49, 16.43 (2 × CH₃). HRMS (ESI⁺) calcd for C₂₅H₂₈F₄N₂O₃P (M + H)⁺: 511.1774, found: 511.1774.

4.1.4 General procedure for the nucleophilic aromatic substitution of 1a with phenols. To the mixture of phenol (0.18 mmol) and K₂CO₃ (0.025 g, 0.18 mmol) in dry DMF (1 mL), a solution of **1a** (0.05 g, 0.12 mmol) in dry DMF (1 mL) was added under N₂ atmosphere in a Radley's tube. The mixture was stirred at 80 °C for 24 h. After cooling to the room temperature, the reaction mixture was poured into water (10 mL) and Et₂O (10 mL). The aqueous phase was extracted with Et₂O (3 × 10 mL), then combined organic layers were washed with water (3 × 20 mL) and dried over MgSO₄. After filtration, solvent was removed under reduced pressure and the crude was purified by column chromatography (cyclohexane/AcOEt 1 : 1, v/v).

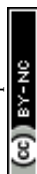
4.1.4.1 Diethyl (1-(phenylamino)-2-(2,3,5,6-tetrafluoro-4-phenoxyphenyl)ethyl)phosphonate (2p). Yellow oil (40 mg, 68%). ¹H NMR (400 MHz, CD₃C(O)CD₃) δ = 7.39–7.35 (m, 2H, 2CHar), 7.17–7.10 (m, 1H, CHar), 7.07–7.05 (m, 2H, 2CHar), 6.81 (d, *J* = 8.2 Hz, 2H, 2CHar), 6.72 (d, *J* = 7.9 Hz, 2H, 2CHar), 6.65 (t, *J* = 7.3 Hz, 1H, CHar), 4.84–4.82 (m, 1H, NH), 4.19–4.09 (m, 5H, CH, 2OCH₂), 3.33–3.30 (m, 1H, ArCH₂), 3.21–3.12 (m, 1H, ArCH₂), 1.29 (t, *J* = 7.1 Hz, 3H, CH₃), 1.22 (t, *J* = 7.2 Hz, 3H, CH₃). ¹⁹F NMR (377 MHz, CDCl₃) δ = −143.74 to −143.83 (m, 2F), −154.95 to −155.03 (m, 2F). ³¹P NMR (162 MHz, CD₃C(O)CD₃) δ = 23.02 (s). ¹³C NMR (101 MHz, CDCl₃) δ = 157.27 (s, Car), 147.14–144.45 (m, 2Car), 146.50 (d, *J* = 8.0 Hz, Car), 142.77–

140.05 (m, 2Car) 132.48–132.17 (m, Car), 129.83 (s, 2CHar), 129.37 (s, 2CHar), 123.61 (s, CHar), 118.66 (s, CHar), 115.38 (s, 2CHar), 113.17 (s, 2CHar), 112.74–112.23 (m, Car), 63.82 (d, *J* = 7.0 Hz, OCH₂), 62.55 (d, *J* = 7.4 Hz, OCH₂), 50.53 (d, *J* = 159.1 Hz, CH), 24.75 (d, *J* = 7.8 Hz, ArCH₂), 16.63, 16.57, 16.55 and 16.49 (2CH₃). HRMS (ESI⁺) calcd for C₂₄H₂₅F₄NO₄P (M + H)⁺: 498.1457, found: 498.1459.

4.1.4.2 Diethyl (1-(phenylamino)-2-(2,3,5,6-tetrafluoro-4-(4-methoxyphenoxy)phenyl)ethyl)phosphonate (2q). Yellow solid (30 mg, 48%), mp = 97–99 °C. ¹H NMR (400 MHz, CD₃C(O)CD₃) δ = 7.08–7.01 (m, 2H, 2CHar), 6.92–6.88 (m, 2H, 2CHar), 6.78–6.76 (m, 2H, 2CHar), 6.72–6.69 (m, 2H, 2CHar), 6.65 (t, *J* = 7.4 Hz, 1H, CHar), 4.83–4.80 (m, 1H, NH), 4.16–4.09 (m, 5H, CH, 2OCH₂), 3.78 (s, 3H, OCH₃), 3.33–3.27 (m, 1H, ArCH₂), 3.19–3.10 (m, 1H, ArCH₂), 1.30–1.26 (m, 3H, CH₃), 1.24–1.15 (m, 3H, CH₃). ¹⁹F NMR (377 MHz, CDCl₃) δ = −143.91 to −144.00 (m, 2F), −155.33 to −155.42 (m, 2F). ³¹P NMR (162 MHz, CDCl₃) δ = 23.42 (s). ¹³C NMR (101 MHz, CDCl₃) δ = 155.85 (s, Car), 151.42 (s, Car), 147.13–144.49 (m, 2Car) 146.51 (d, *J* = 7.9 Hz, Car), 142.78–140.10 (m, 2Car), 133.40–133.04 (m, Car) 129.36 (s, 2CHar), 118.64 (s, CHar), 116.70 (s, 2CHar), 114.79 (s, 2CHar), 113.21 (s, 2CHar), 112.34–111.83 (m, Car), 63.80 (d, *J* = 7.0 Hz, OCH₂), 62.55 (d, *J* = 7.4 Hz, OCH₂), 55.82 (s, OCH₃), 50.57 (d, *J* = 158.7 Hz, CH), 24.70 (d, *J* = 7.6 Hz, ArCH₂), 16.63, 16.58, 16.55, and 16.50 (2CH₃). HRMS (ESI⁺) calcd for C₂₅H₂₇F₄NO₅P (M + H)⁺: 528.1563, found: 528.1567.

4.1.4.3 Diethyl (2-(4-(4-chlorophenoxy)-2,3,5,6-tetrafluorophenyl)-1-(phenylamino)ethyl)phosphonate (2r). Yellow solid (35 mg, 56%), mp = 94–99 °C. ¹H NMR (400 MHz, CD₃C(O)CD₃) δ = 7.40–7.38 (m, 2H, 2CHar), 7.08–7.02 (m, 2H, 2CHar), 6.85–6.83 (m, 2H, 2CHar), 6.72 (d, *J* = 7.9 Hz, 2H, 2CHar), 6.66 (t, *J* = 7.3 Hz, 1H, CHar), 4.87–4.83 (m, 1H, NH), 4.17–4.10 (m, 5H, CH, 2OCH₂), 3.34–3.28 (m, 1H, ArCH₂), 3.21–3.14 (m, 1H, ArCH₂), 1.29 (t, *J* = 7.1 Hz, 3H, CH₃), 1.22 (t, *J* = 7.1 Hz, 3H, CH₃). ¹⁹F NMR (377 MHz, CDCl₃) δ = −143.37 to −143.45 (m, 2F), −154.94 to −155.02 (m, 2F). ³¹P NMR (162 MHz, CDCl₃) δ = 23.24 (s). ¹³C NMR (101 MHz, CDCl₃) δ = 155.79 (s, Car), 147.12–142.57 (m, 2Car), 146.47 (d, *J* = 8.1 Hz, Car), 142.57–139.85 (m, 2Car) 132.10–131.84 (m, Car), 129.77 (s, 2CHar), 129.36 (s, 2CHar), 128.73 (s, Car), 118.59 (s, CHar), 116.75 (s, 2CHar), 113.07 (s, 2CHar), 112.95–112.80 (m, Car), 63.87 (d, *J* = 7.0 Hz, OCH₂), 62.59 (d, *J* = 7.5 Hz, OCH₂), 50.38 (d, *J* = 159.4 Hz, CH), 24.74 (d, *J* = 7.9 Hz, ArCH₂), 16.62, 16.57, 16.55, and 16.49 (2CH₃) ppm. HRMS (ESI⁺) calcd for C₂₄H₂₄ClF₄NO₄P (M + H)⁺: 532.1068, found: 532.1066.

4.1.4.4 Diethyl (1-(phenylamino)-2-(2,3,5,6-tetrafluoro-4-(3-nitrophenoxy)phenyl)ethyl)phosphonate (2s). Yellow solid (31 mg, 48%), mp = 77–79 °C. ¹H NMR (400 MHz, CD₃C(O)CD₃) δ = 8.05 (ddd, *J* = 8.2, 2.1, 0.8 Hz, 1H, CHar), 7.84 (t, *J* = 2.3 Hz, 1H, CHar), 7.71 (t, *J* = 8.3 Hz, 1H, CHar), 7.24 (dd, *J* = 8.3, 2.6 Hz, 1H, CHar), 7.10–7.06 (m, 2H, 2CHar), 6.74 (d, *J* = 7.9 Hz, 2H, 2CHar), 6.63 (t, *J* = 7.3 Hz, 1H, CHar), 4.86 (d, *J* = 13.7 Hz, 1H, NH), 4.17–4.10 (m, 5H, CH, 2OCH₂), 3.35–3.30 (m, 1H, ArCH₂), 3.23–3.14 (m, 1H, ArCH₂), 1.28 (t, *J* = 7.1 Hz, 3H, CH₃), 1.22 (t, *J* = 7.1 Hz, 3H, CH₃). ¹⁹F NMR (377 MHz, CDCl₃) δ = −142.44 (dd, *J* = 22.6, 9.4 Hz, 2F), −154.52 to −154.61 (dd, *J* = 22.0, 9.3 Hz, 2F). ³¹P NMR (162 MHz, CDCl₃) δ = 23.25 (s). ¹³C NMR (101 MHz, CDCl₃) δ = 157.42 (s, Car), 149.36 (s, Car),



147.24–144.60 (m, 2Car), 146.42 (d, $J = 7.7$ Hz, Car), 142.42–139.69 (m, 2Car), 131.44–130.97 (m, Car), 130.67 (s, CHar), 129.41 (s, 2CHar), 121.39 (s, CHar), 118.79 (s, CHar), 118.74 (s, CHar), 113.97–113.75 (m, Car), 113.28 (s, 2CHar), 111.18 (s, CHar), 63.83 (d, $J = 7.0$ Hz, OCH_2), 62.60 (d, $J = 7.4$ Hz, OCH_2), 50.47 (d, $J = 158.6$ Hz, CH), 24.85 (d, $J = 7.8$ Hz, ArCH_2), 16.63, 16.58, 16.55, and 16.50 (2CH_3). HRMS (ESI^+) calcd for $\text{C}_{24}\text{H}_{24}\text{F}_4\text{N}_2\text{O}_6\text{P}$ ($\text{M} + \text{H}$) $^+$: 543.1308, found: 543.1312.

4.2 X-ray and DFT studies

4.2.1 Crystal structure determination. Reflection intensities for crystals investigated were measured on Xcalibur diffractometer (Eos detector) equipped with a graphite monochromator and MoK α radiation ($\lambda = 0.71073$ Å). Data reduction and analysis were carried out with the CrysAlisPro software. In single-crystal experiments, the temperature of the crystals was controlled with an Oxford Instruments Cryosystem cold nitrogen-gas blower and was 100 K. The structures were solved by direct methods using SHELXS⁵³ and refined by the full-matrix least-squares techniques with SHELXL.⁵³ All heavy atoms were refined anisotropically. The hydrogen atoms bound to carbon atoms were placed at calculated positions and refined using a riding model. The positions of the amine H atoms were located reliably on difference Fourier maps and their position and displacement parameters were refined. Graphical images were produced in Mercury⁵⁴ programs.

4.2.1.1 Crystals of 2k. $\text{C}_{19}\text{H}_{23}\text{F}_4\text{N}_2\text{O}_3\text{P}$, $M_r = 434.36$, $0.35 \times 0.21 \times 0.18$ mm³, triclinic, space group $P\bar{1}$ (no. 2), $a = 11.7691(9)$ Å, $b = 12.2935(5)$ Å, $c = 16.8735(9)$ Å, $\alpha = 101.120(4)^\circ$, $\beta = 99.040(5)^\circ$, $\gamma = 118.147(6)^\circ$, $V = 2023.5(2)$ Å³, $Z = 4$, $D_x = 1.426$ g cm⁻³, $F(000) = 904$, MoK α radiation, $\lambda = 0.71073$ Å, $T = 100(2)$ K, $2\theta_{\text{max}} = 57.01^\circ$, 15 277 reflections collected, 8589 unique ($R_{\text{int}} = 0.027$), Final GooF = 1.028, $R_1 = 0.0527$, $wR_2 = 0.1144$, R indices based on 6237 with $I > 2\sigma(I)$ (refinement on F^2), 545 parameters, $\mu = 0.195$ mm⁻¹.

4.2.1.2 Crystals of 2l. $\text{C}_{21}\text{H}_{27}\text{F}_4\text{N}_2\text{O}_3\text{P}$, $M_r = 462.41$, $0.56 \times 0.46 \times 0.18$ mm³, triclinic, space group $P\bar{1}$ (no. 2), $a = 8.8604(4)$ Å, $b = 9.8978(8)$ Å, $c = 13.1171(7)$ Å, $\alpha = 91.889(5)^\circ$, $\beta = 91.350(4)^\circ$, $\gamma = 109.119(6)^\circ$, $V = 1085.59(12)$ Å³, $Z = 2$, $D_x = 1.415$ g cm⁻³, $F(000) = 484$, MoK α radiation, $\lambda = 0.71073$ Å, $T = 100(2)$ K, $2\theta_{\text{max}} = 56.88^\circ$, 8189 reflections collected, 4658 unique ($R_{\text{int}} = 0.0249$), Final GooF = 1.040, $R_1 = 0.0447$, $wR_2 = 0.0951$, R indices based on 3666 with $I > 2\sigma(I)$ (refinement on F^2), 291 parameters, $\mu = 0.186$ mm⁻¹, CCDC 1912530 and 1912531 contain the supplementary crystallographic data for this paper.

4.2.2 Computational methods. The calculations were performed *in vacuo*, as well as using the polarisable continuum model (PCM) to take into account the effect of chloroform solution as NMR spectra for 2k were recorded for the compound sample dissolved in CDCl_3 . The structures were optimized at the WB97XD⁵⁵ level and basis set with diffuse and polarization functions namely 6-31+G(d)^{56–58} being a difficult compromise between the desired accuracy and computational time needed. The vibrational analyses were performed at the same level of theory. All calculations were carried out with the Gaussian 09

program suite⁵⁹ Boltzmann formalism,⁶⁰ and the percent of the structure Pop was computed according to the equation

$$\% \text{ Pop} = \frac{\exp(-\Delta G_x/RT)}{\sum_i \exp(-\Delta G_i/RT)} \times 100\% \quad (1)$$

The temperature for interconversion between possible stereoisomers was calculated on the basis of NMR studies using Earing's equation modified by Shanan-Atidi and Bar-Eli⁶¹

$$\Delta G = 4.57T_c \{10.62 + \log[X/2\pi(1 - \Delta P)] + \log(T_c/\Delta\nu)\} \quad (2)$$

where $\Delta P = 0.04$ and $X = 1.636$ as suggested for similar system.⁶²

The relative energies of stereoisomers calculated at WB97XD/6-31+G(d) level of theory are summarized in Table 2.

4.3 Biological evaluation

4.3.1 Cell lines and culture conditions. Glioblastoma multiforme cell lines (T98G and U-118 MG), and non-cancerous cell line (HaCaT) were purchased from ATCC and CLS (Manassas, USA; Eppelheim, Germany). All cell lines are from human origin. U-118 MG were cultured in DMEM medium. T98G and HaCaT were cultured in EMEM medium. Each medium was supplemented with 10% fetal bovine serum (FBS) and 10 mg mL⁻¹ antibiotics (penicillin and streptomycin). Cells were cultured at 37 °C with 5% CO₂ in humidified air. Cell media and other chemicals were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and ATCC. Cell concentrations in culture were adjusted to allow for exponential growth.

4.3.2 Cell viability/proliferation assay. Cell viability/proliferation was evaluated with a dye staining method using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT). The protocol is adapted from literature methods.⁶³ The monolayer cell culture was trypsinized and counted. To each well of the 96-well plate, 100 μL of the diluted cell suspension (1×10^4 cells) was added. After 24 hours, when a partial monolayer was formed, 100 μL of fresh medium with different compound concentrations (7.81, 15.625, 31.25, 62.5, 125, 250, 500 and 1000 $\mu\text{g mL}^{-1}$) were added to the cells. After 48 hours, the supernatant was washed out and 100 μL of MTT solution in medium (final concentration 0.5 mg mL⁻¹) were added to each well for 2 h. After the incubation time was complete, unreacted dye was removed by aspiration. The formazan crystals were dissolved in 100 μL per well DMSO and measured spectrophotometrically in a multi-well Synergy 2 plate reader (BioTek Instruments, USA) at a test wavelength of 492 nm and a reference wavelength of 690 nm. The results were calculated as an IC₅₀ (inhibitory concentration 50) – the IC₅₀ corresponds to the concentration of tested compound that inhibits cell viability/proliferation by 50%. Results are presented as mean of at least three independent experiments.

4.3.3 Colony formation assay. Colony formation assay is an *in vitro* cell survival assay base on the ability of single cell to grow a colony. The colony is defined to consist of at least 50 cells. The assay essentially tests every cell in the population for its ability to undergo unlimited division. Clonogenic assay is the



method of choice to determine cell reproductive death after treatment with ionizing radiation, but can also be used to determine effectiveness of other cytotoxic agents. Only a fraction of seeded cell retains the capacity to product colonies. The protocol was adapted from literature methods.⁶⁴ T98G and HaCaT cells were seeded in 6-well tissue culture plates at a density of 500 cells per well according to the morphology and growth patterns of each cell line, and were allowed adhere for 24 hours before treatment. The cells were treated with different compound concentrations (2 μ M, 10 μ M, 20 μ M, 50 μ M, 100 μ M) for 7 days. Triplicate samples were used for each treatment. Medium containing the treatment was replaced with a compound-free medium after 24 h. Plates were rinsed in milliQ water and colonies were methanol-fixed and stained with 1% gentian violet and clones were counted under a light microscope.

4.3.4 In silico pharmacokinetic prediction. Calculations of pharmacokinetic profile descriptors of synthesized compounds were performed by various software solutions accessible online. The transformation of the stoichiometric formulas of the compounds into a SMILES code (Simplified Molecular Input Line Entry System) was carried out by ChemBioDraw Ultra version 12.0 program (Cambridge Software). The SMILES code was applied to calculate log *P* values (octanol/water partition coefficient), PSA (topological polar surface area) and aPSA (apolar surface area). The log *P* values were calculated by ALOGPS 2.1 software (<http://www.vcclab.org/lab/alogps>).⁶⁵ PSA and aPSA descriptors were calculated using the VEGA ZZ program (<http://www.vegazz.net>).⁶⁶ The pharmacokinetic profiles were also evaluated according to Lipinski's "rule of five"⁶⁷ by using Molinspiration application (<http://www.molinspiration.com>), which analyses molecular weight (MW), number of hydrogen-bond acceptors (HBA), and number of hydrogen-bond donors (HBD). The Caco-2 prediction model based on descriptors generated by preADMET (<http://preadmet.bmdrc.org>) was used to compute Caco-2 apparent permeability (*t*_{Papp}), for the tested compounds. In this model a number of hydrogen bond donors and three molecular surface area properties determine membrane permeability of compounds.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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MATERIAŁY UZUPEŁNIAJĄCE DO PUBLIKACJI

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Synthesis, structural studies and biological properties of some phosphono-perfluorophenylalanine derivatives formed by S_NAr reactions

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Supplementary Information

NMR spectra and HRMS data of compounds 2a-2s

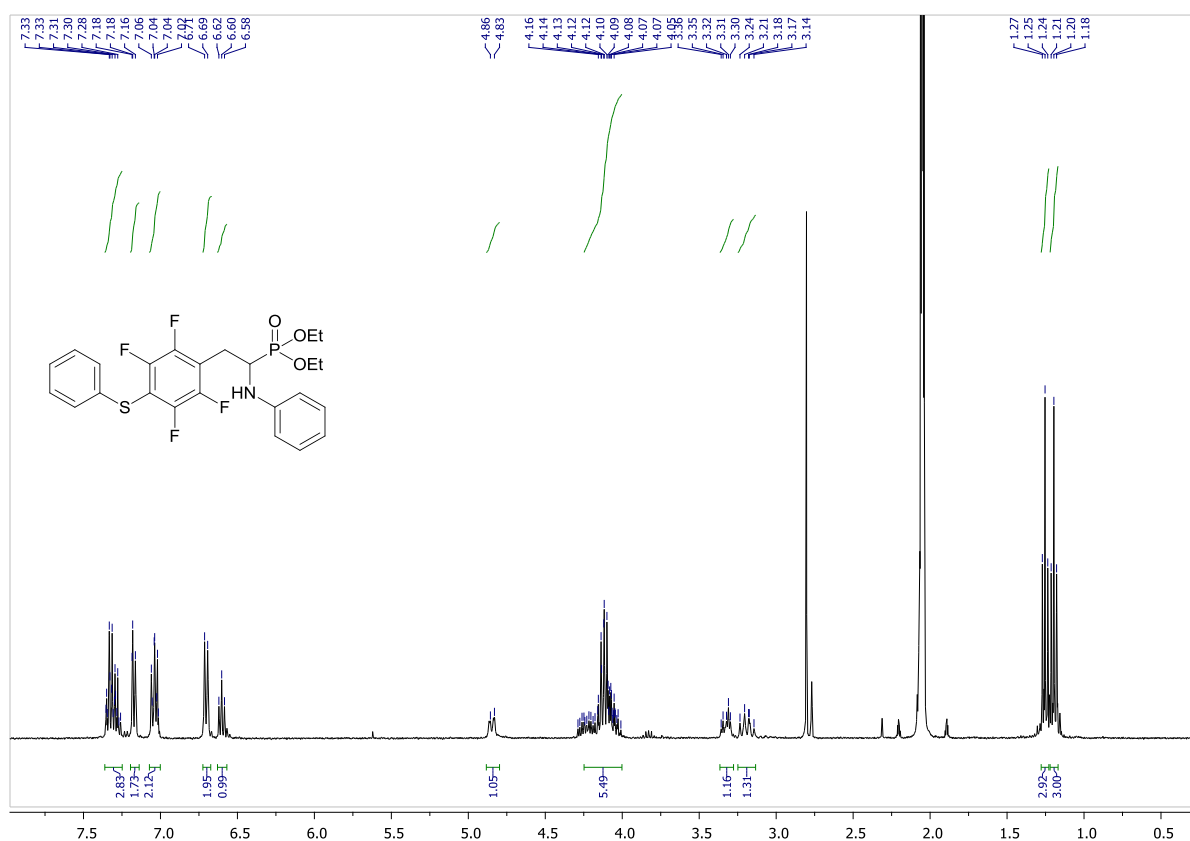


Fig. S1. ¹H NMR spectrum of 2a (Acetone-d₆)

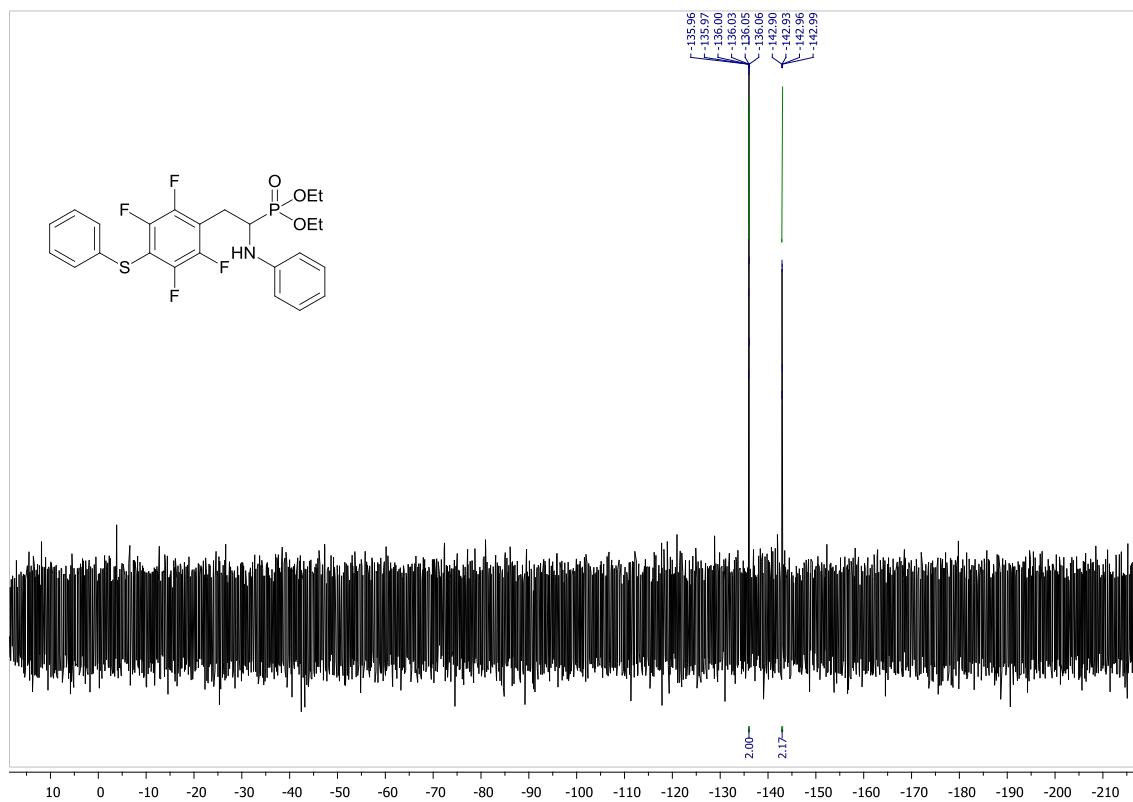


Fig. S2. ¹⁹F NMR spectrum of 2a (Acetone-d₆)

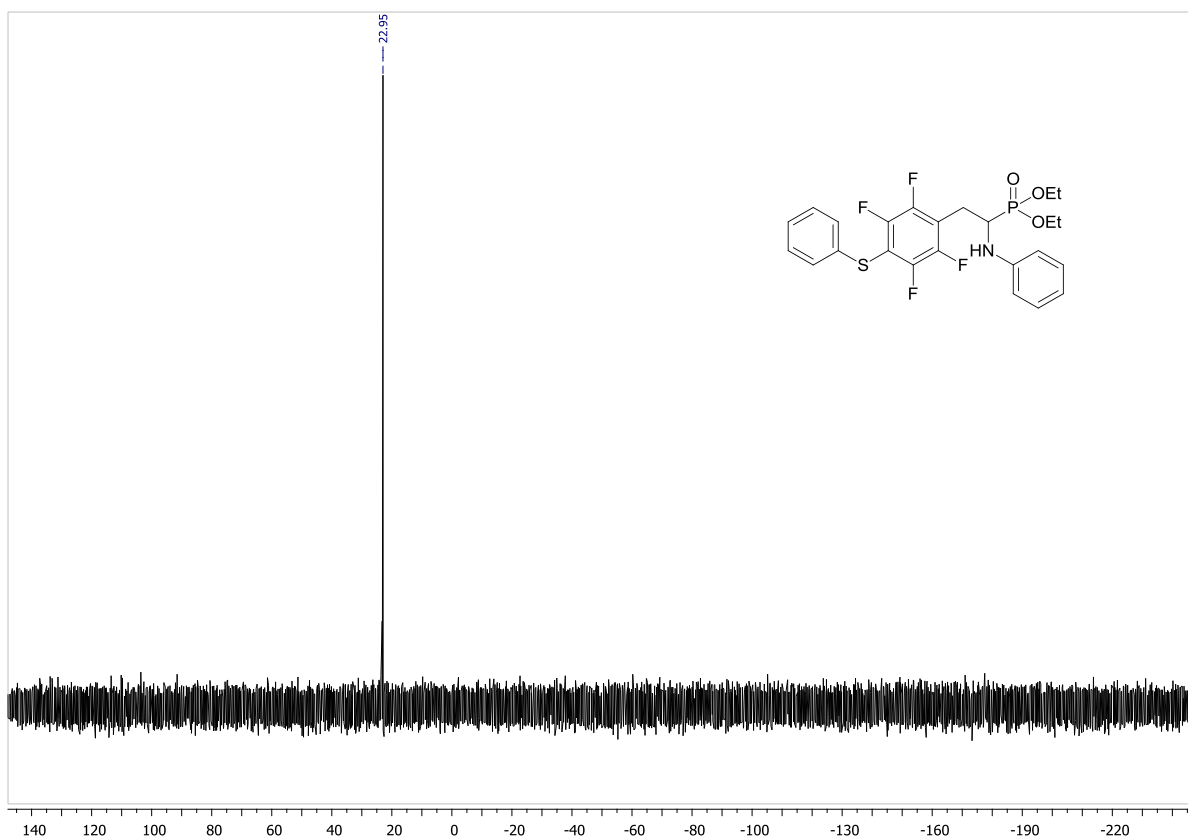


Fig. S3. ³¹P NMR spectrum of 2a (Acetone-d₆)

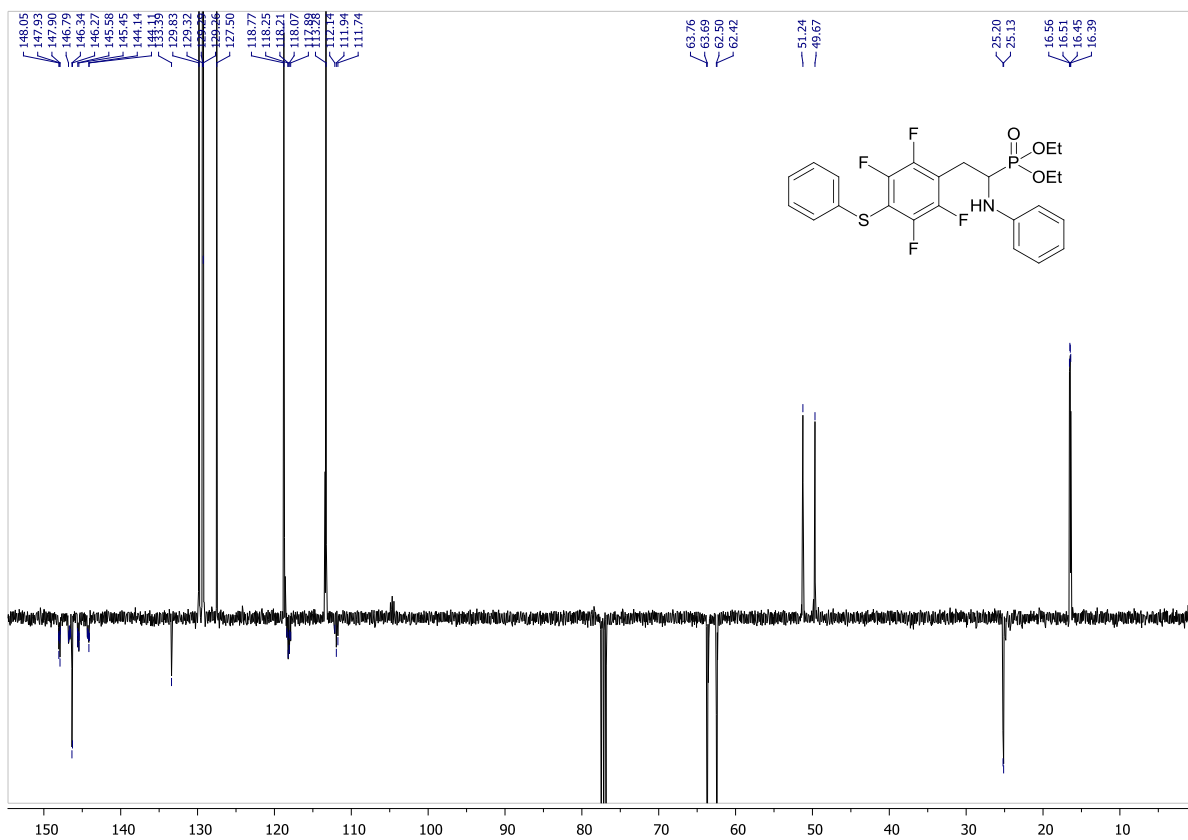


Fig. S4. ¹³C NMR spectrum of 2a (CDCl₃)

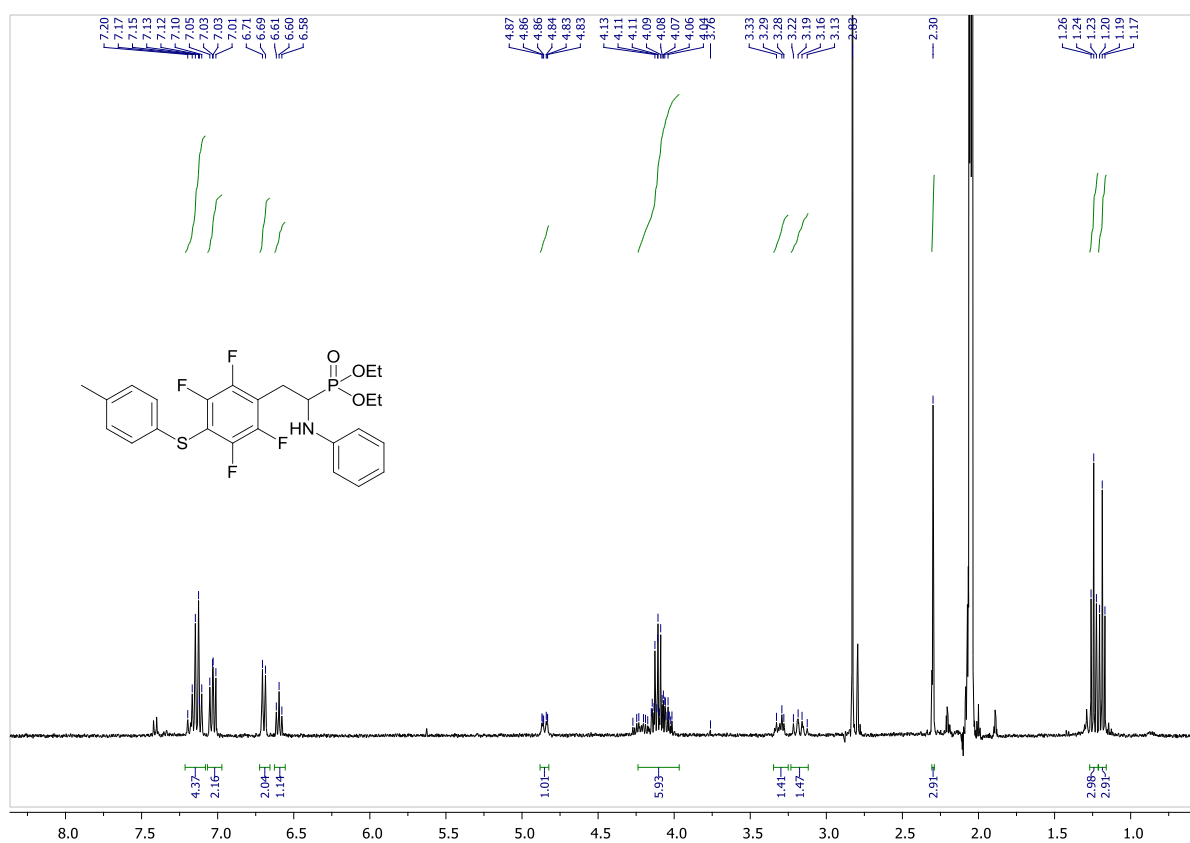


Fig. S5. ¹H NMR spectrum of 2b (Acetone-d₆)

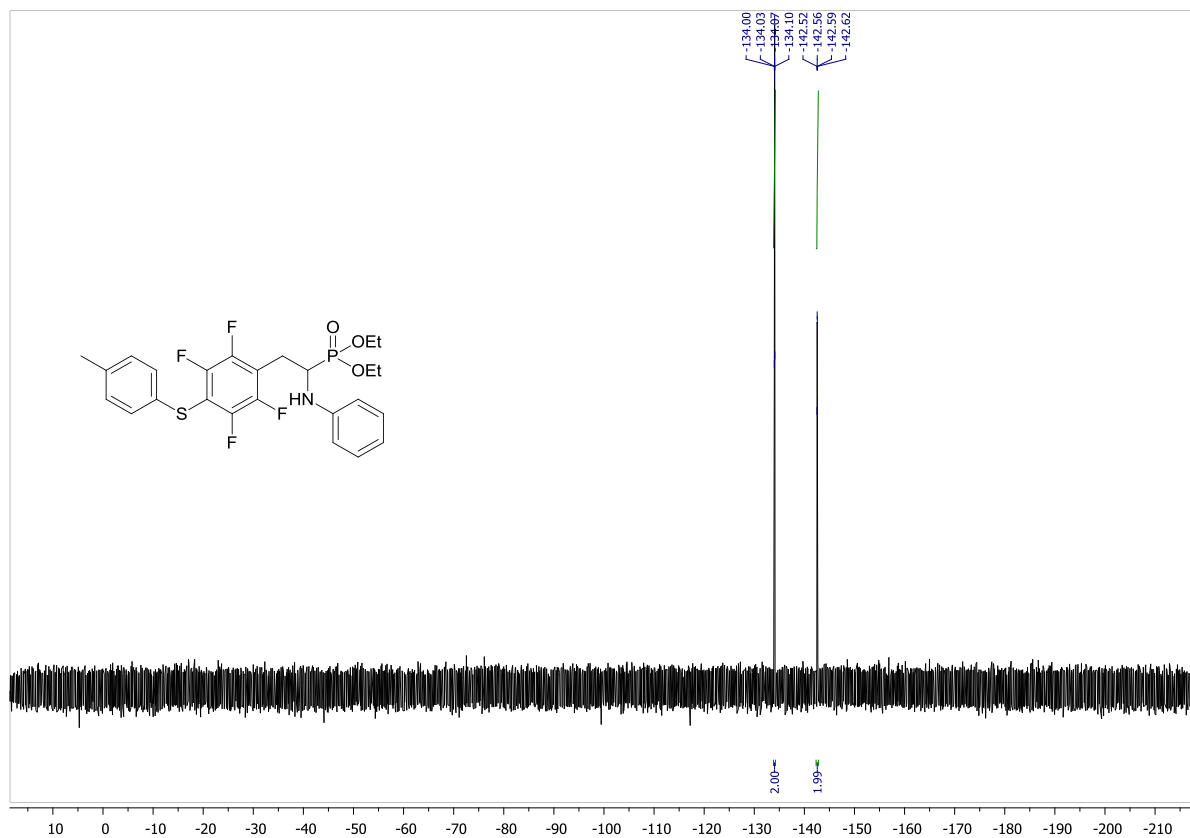


Fig. S6. ¹⁹F NMR spectrum of 2b (CDCl₃)

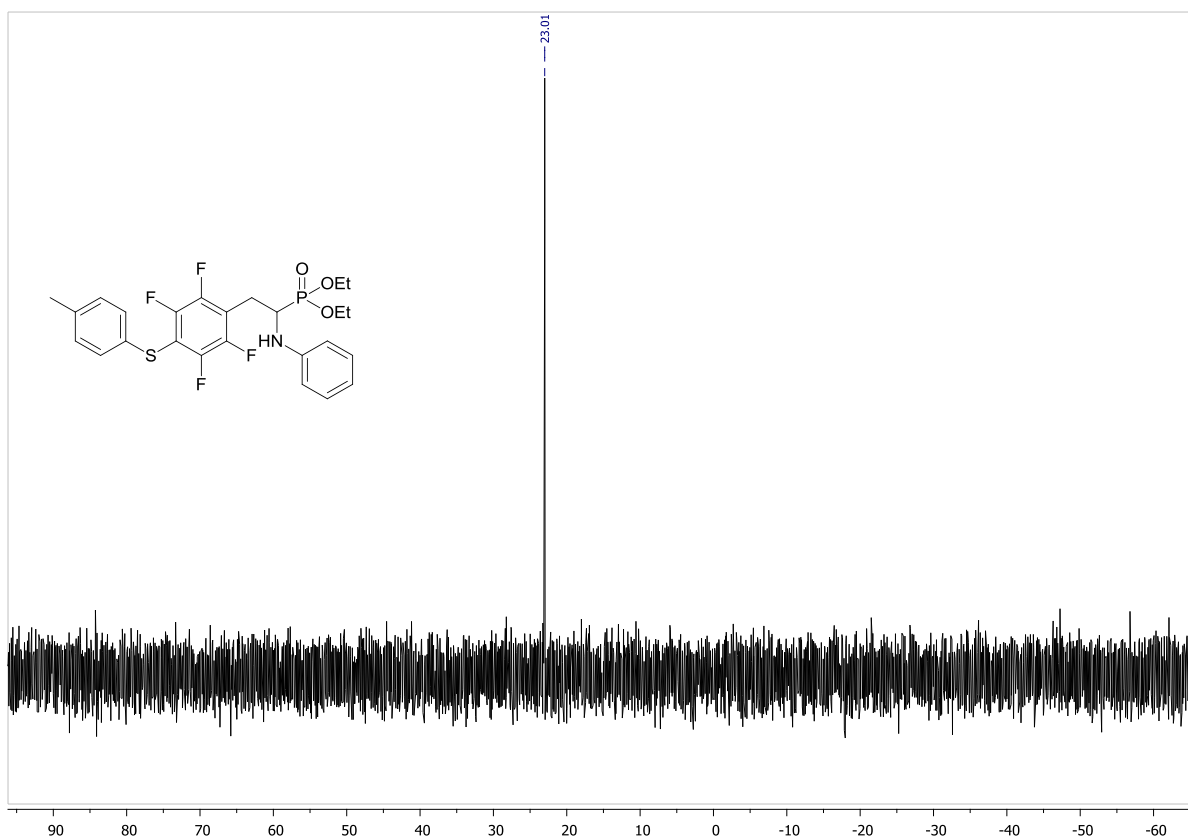


Fig. S7. ³¹P NMR spectrum of 2b (Acetone-d₆)

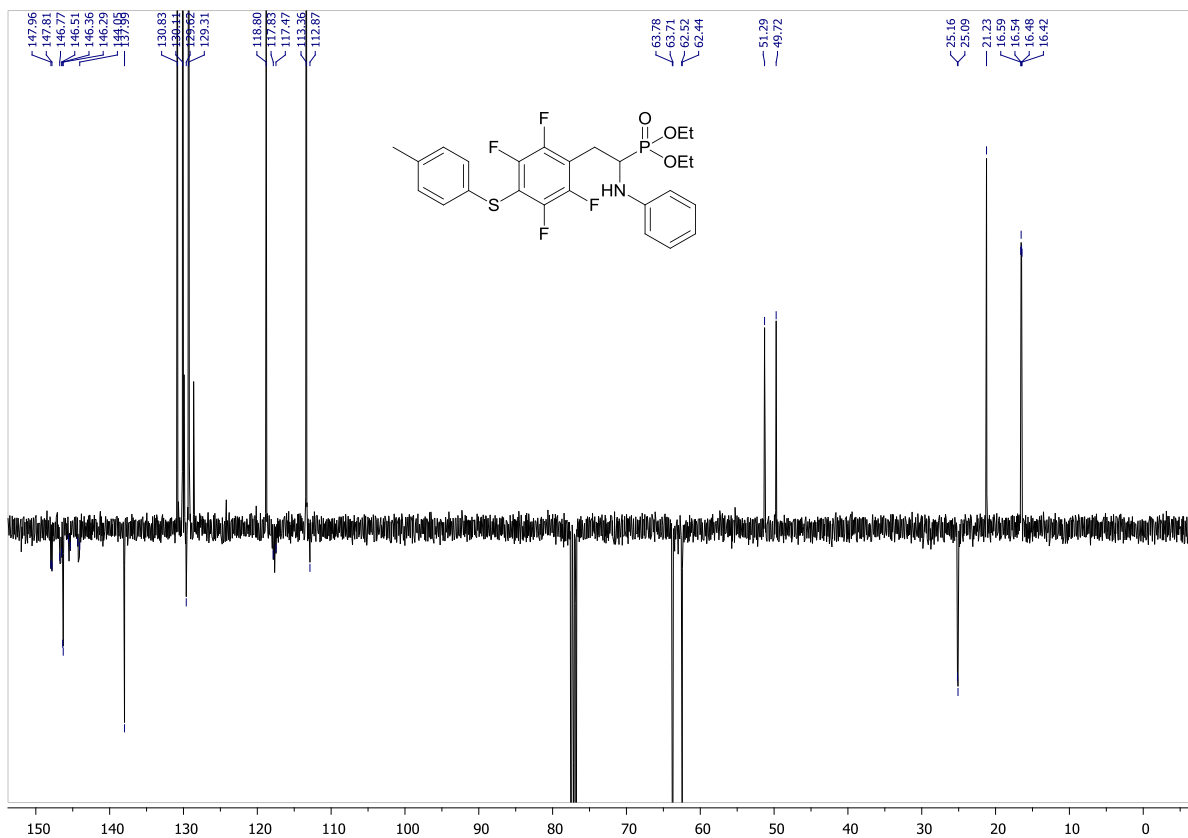


Fig. S8. ¹³C NMR spectrum of 2b (CDCl₃)

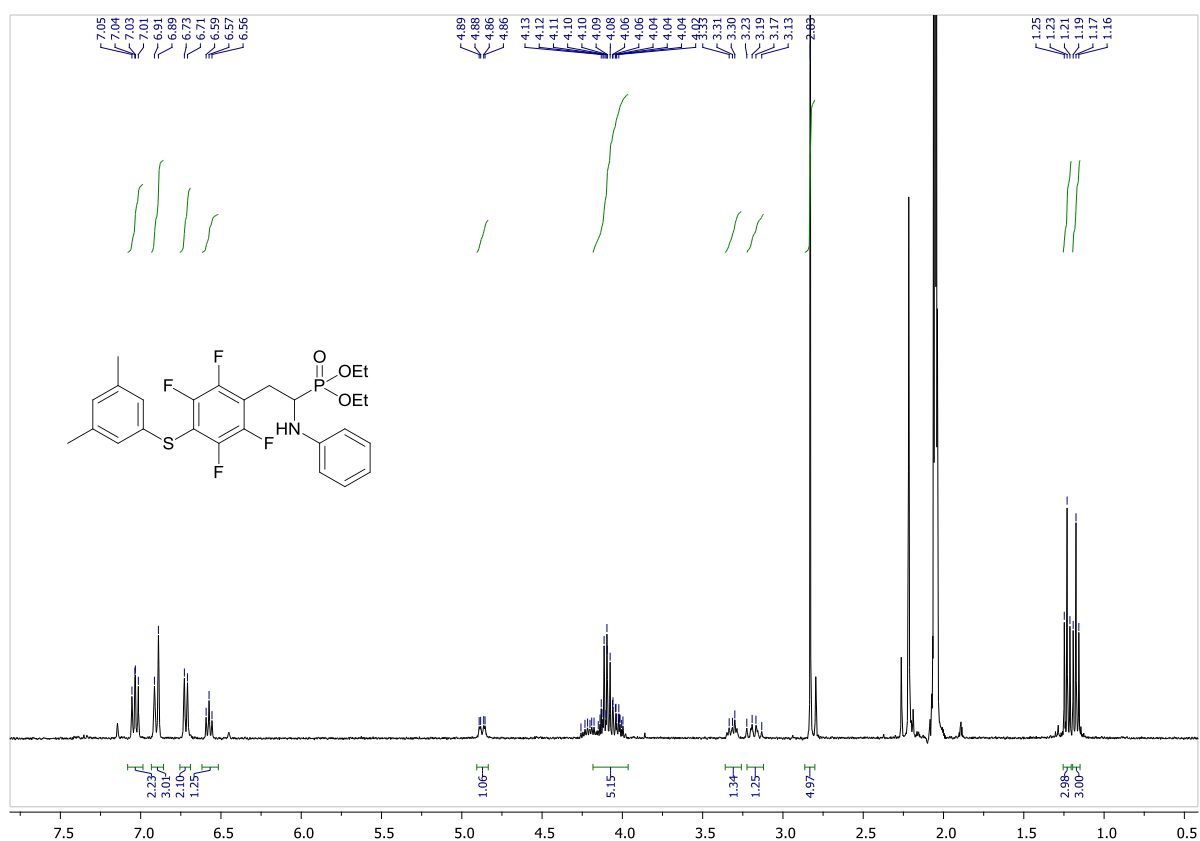


Fig. S9. ¹H NMR spectrum of 2c (Acetone-d₆)

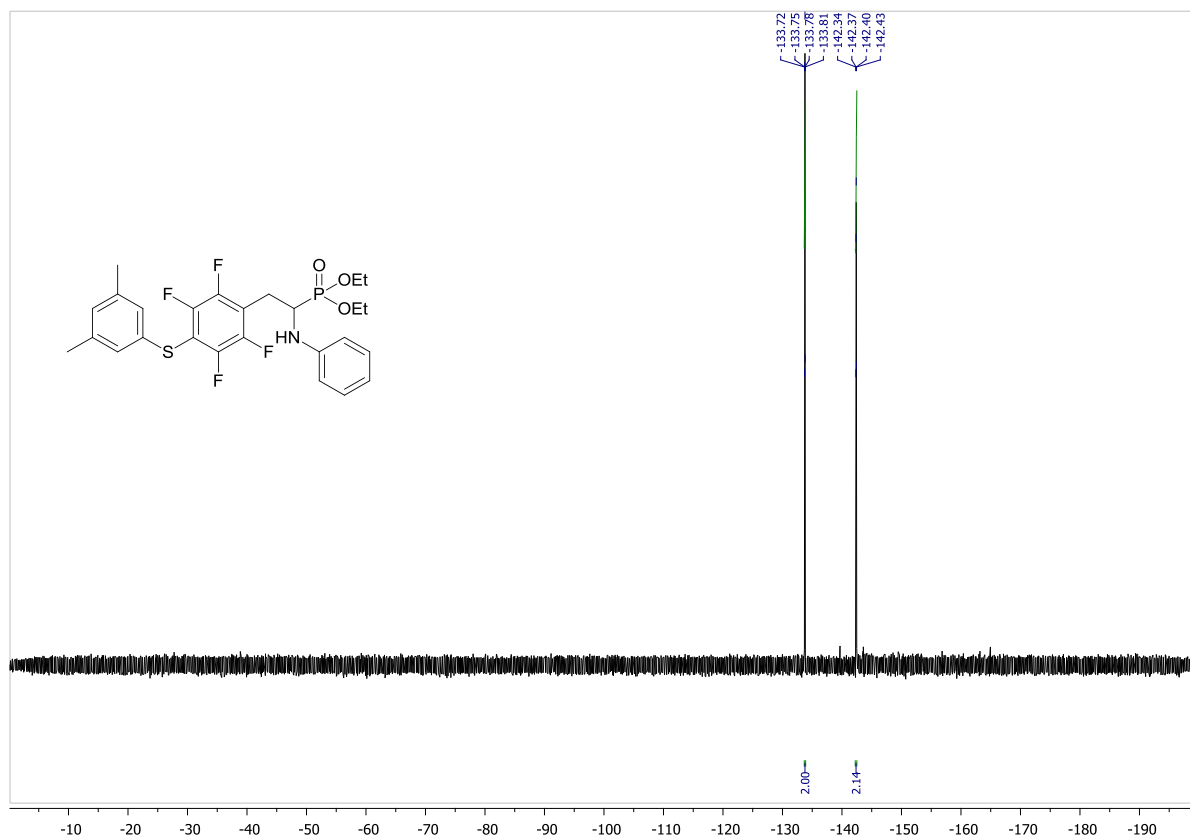


Fig. S10. ¹⁹F NMR spectrum of 2c (CDCl₃)

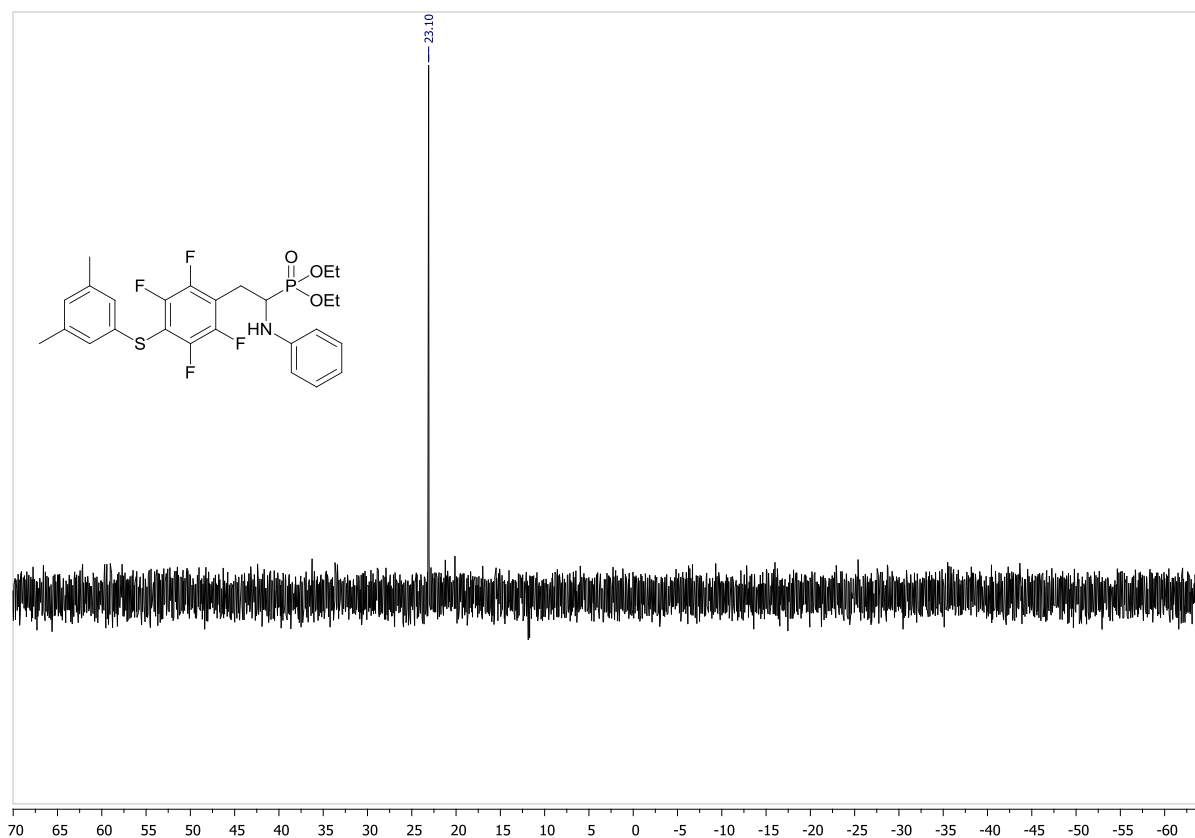


Fig. S11. ³¹P NMR spectrum of 2c (Acetone-d₆)

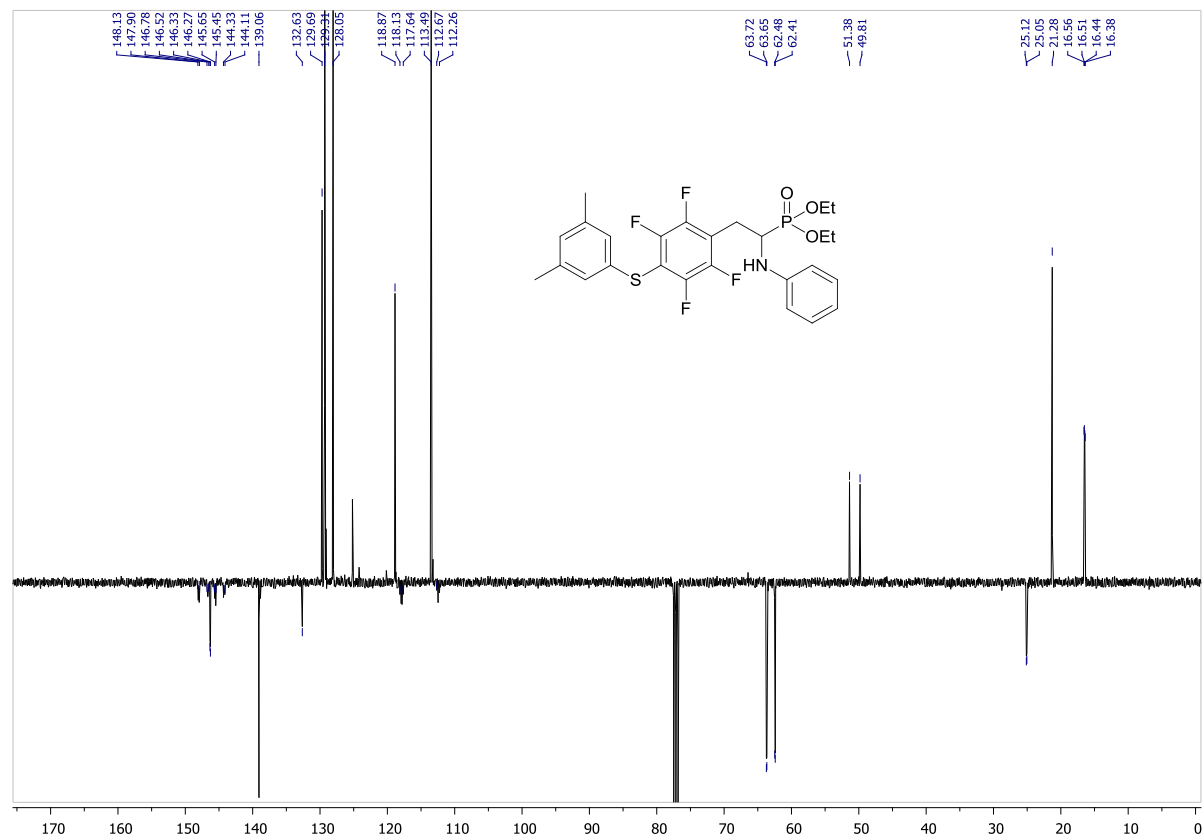


Fig. S12. ¹³C NMR spectrum of 2c (CDCl₃)

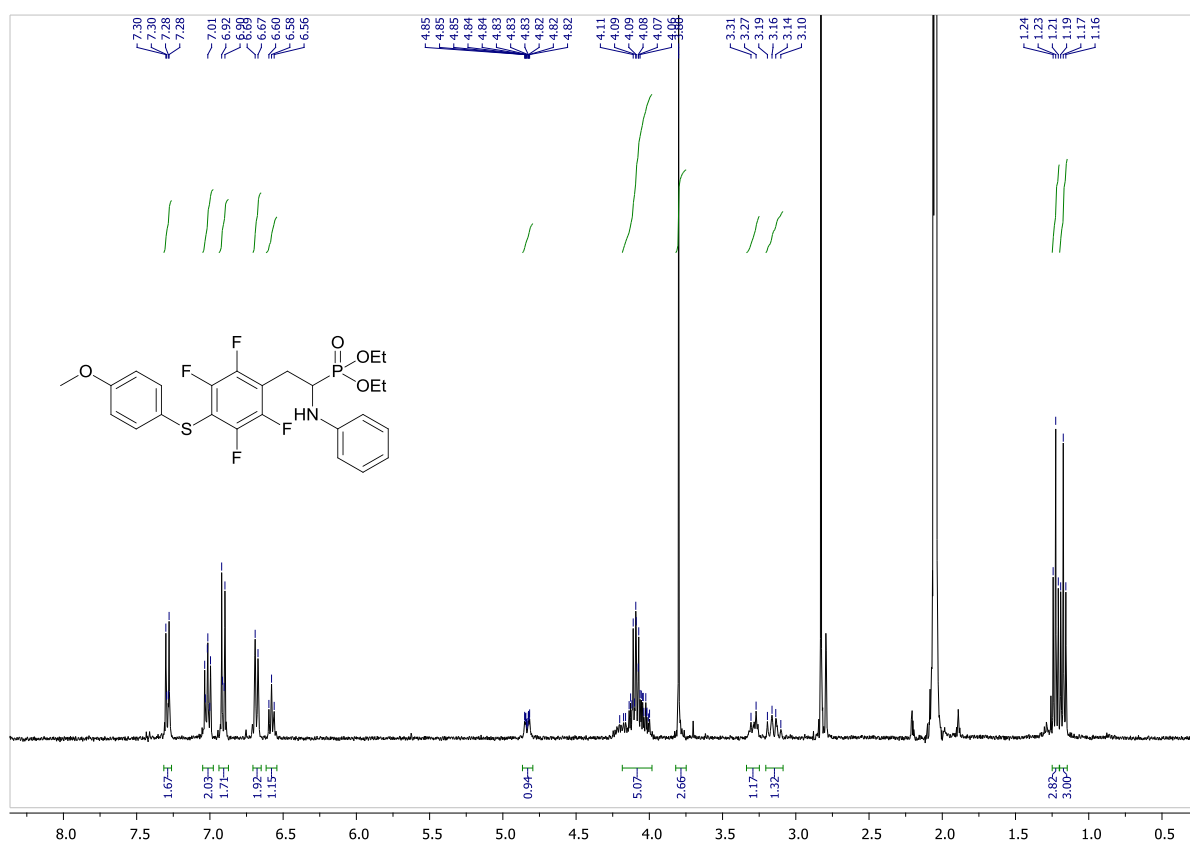


Fig. S13. ¹H NMR spectrum of 2d (Acetone-d₆)

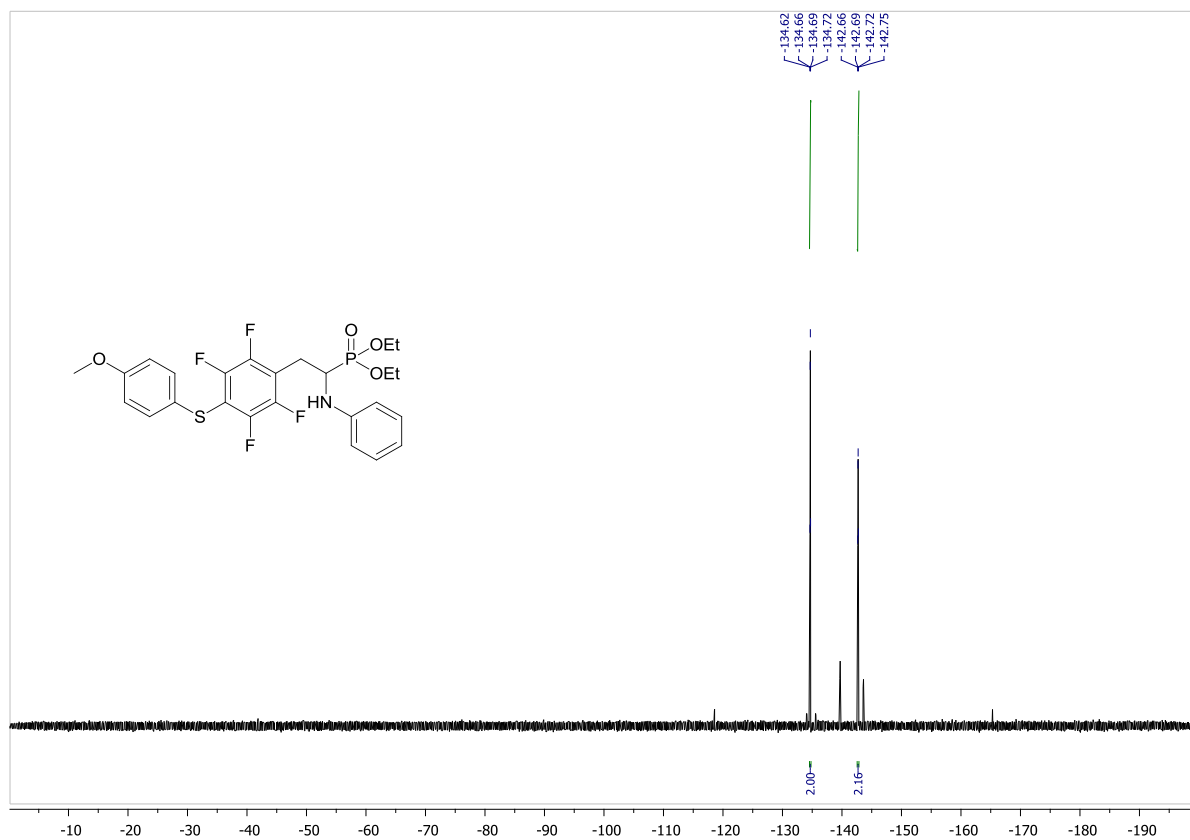


Fig. S14. ¹⁹F NMR spectrum of 2d (CDCl₃)

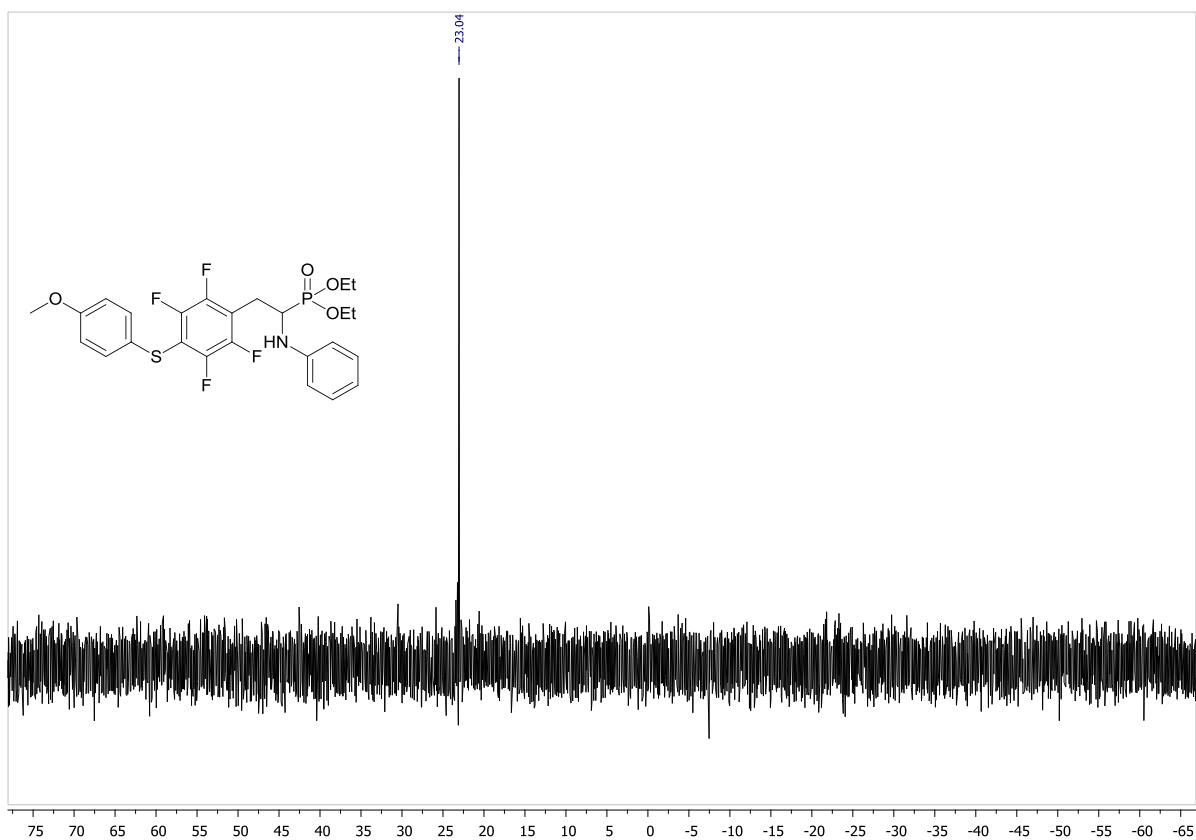


Fig. S15. ³¹P NMR spectrum of 2d (Acetone-d₆)

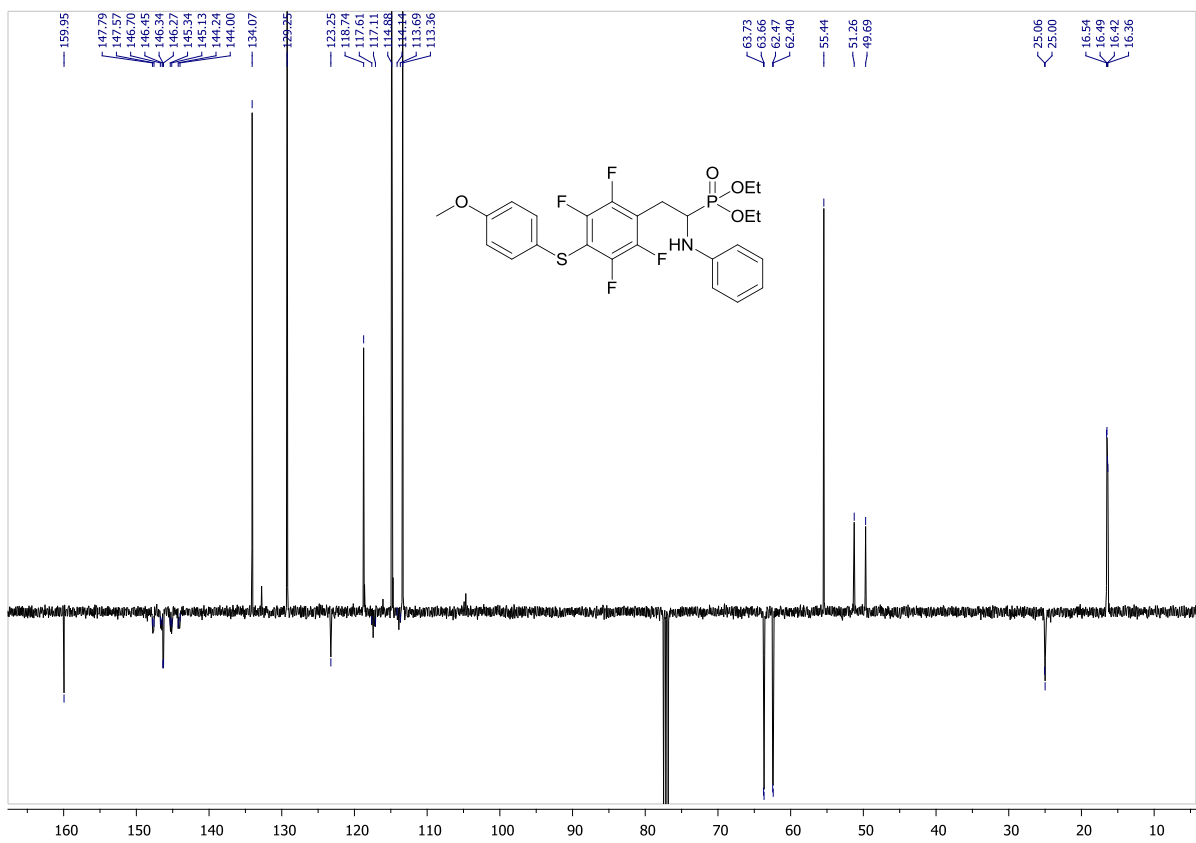


Fig. S16. ¹³C NMR spectrum of 2d (CDCl₃)

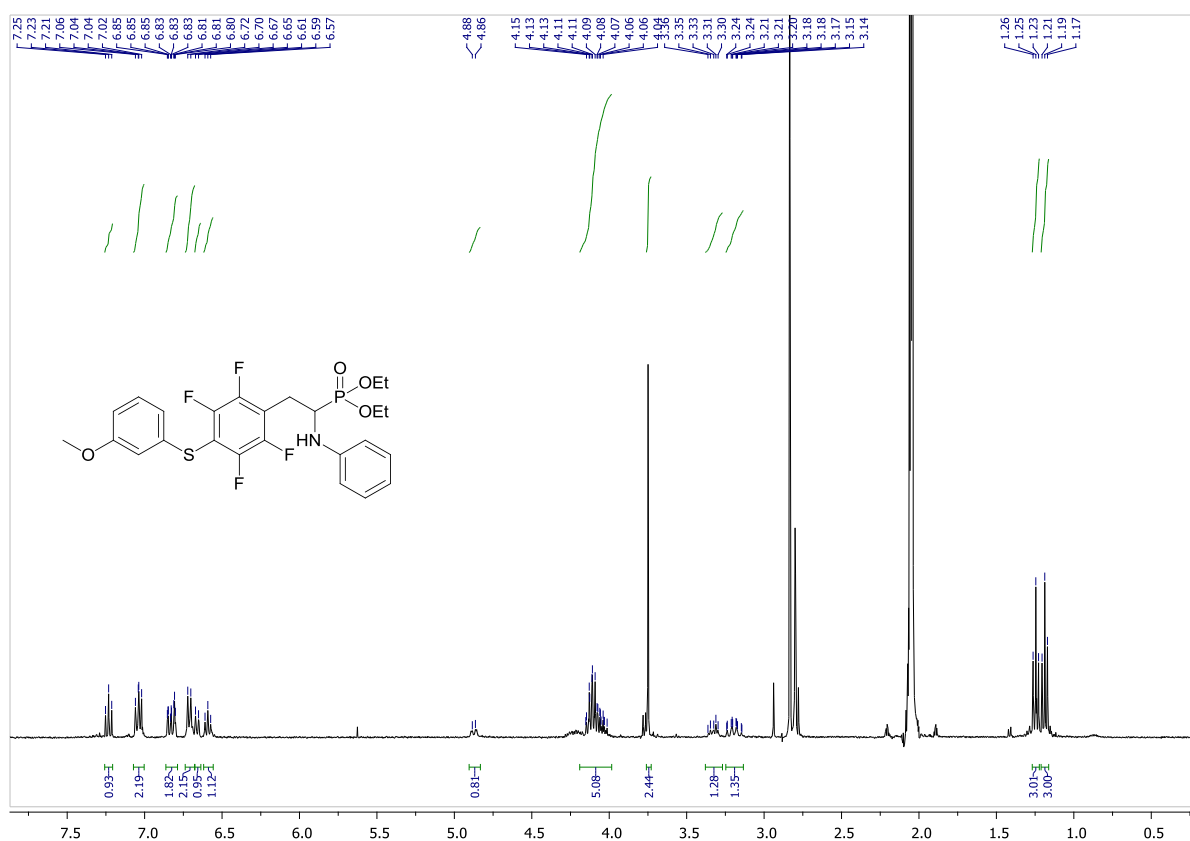


Fig. S17. ¹H NMR spectrum of 2e (Acetone-d₆)

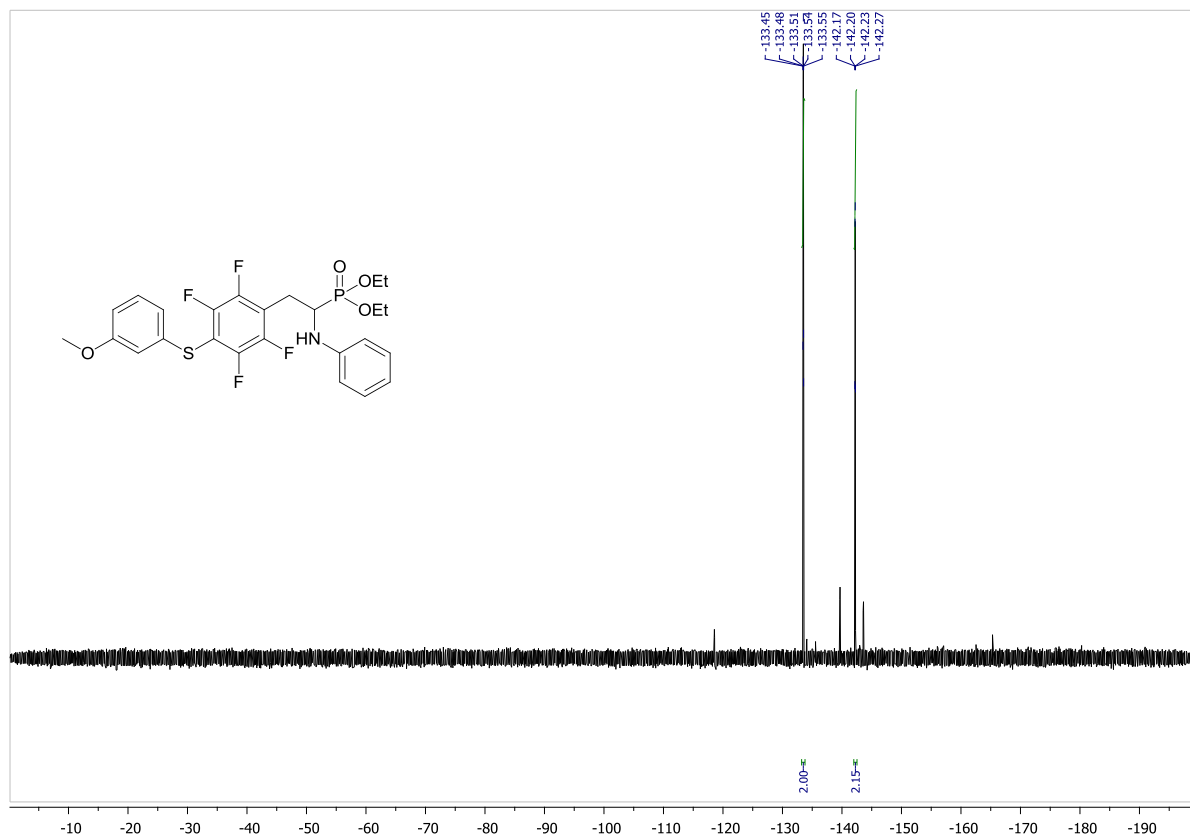


Fig. S18. ¹⁹F NMR spectrum of 2e (CDCl₃)

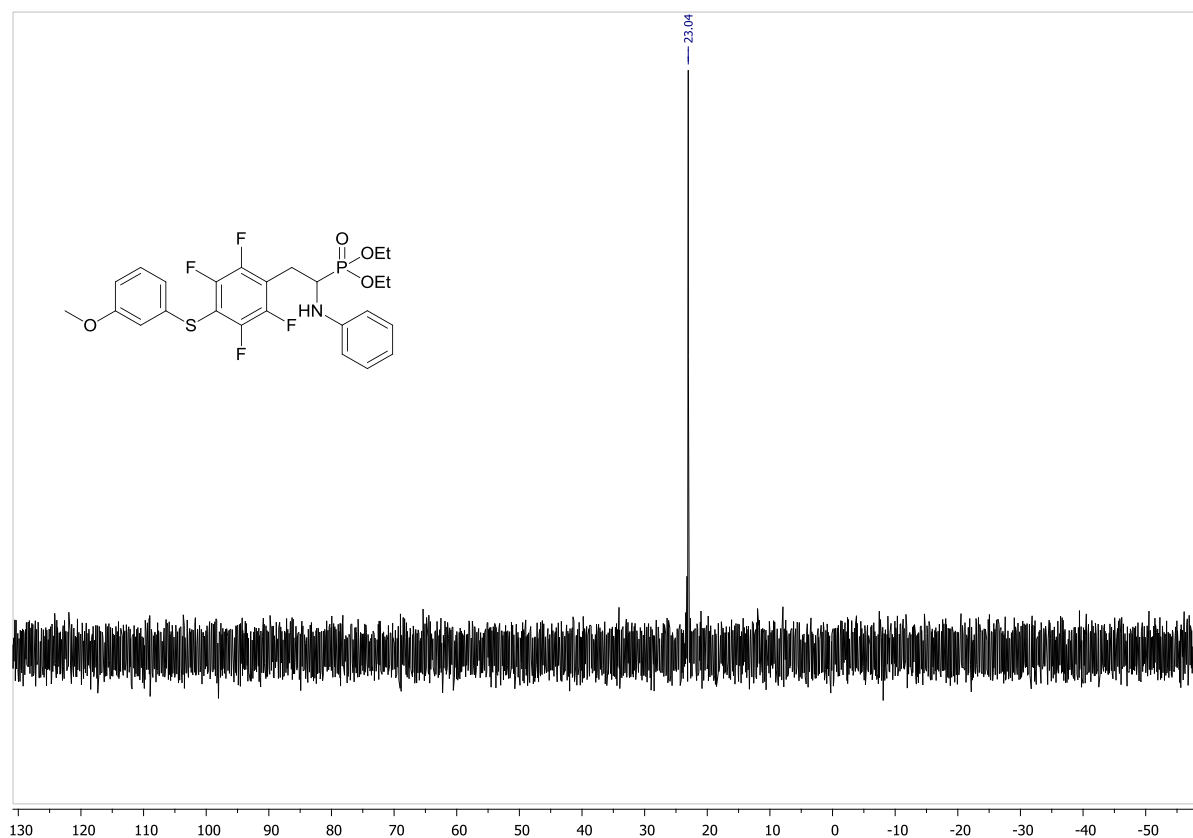


Fig. S19. ³¹P NMR spectrum of 2e (Acetone-d₆)

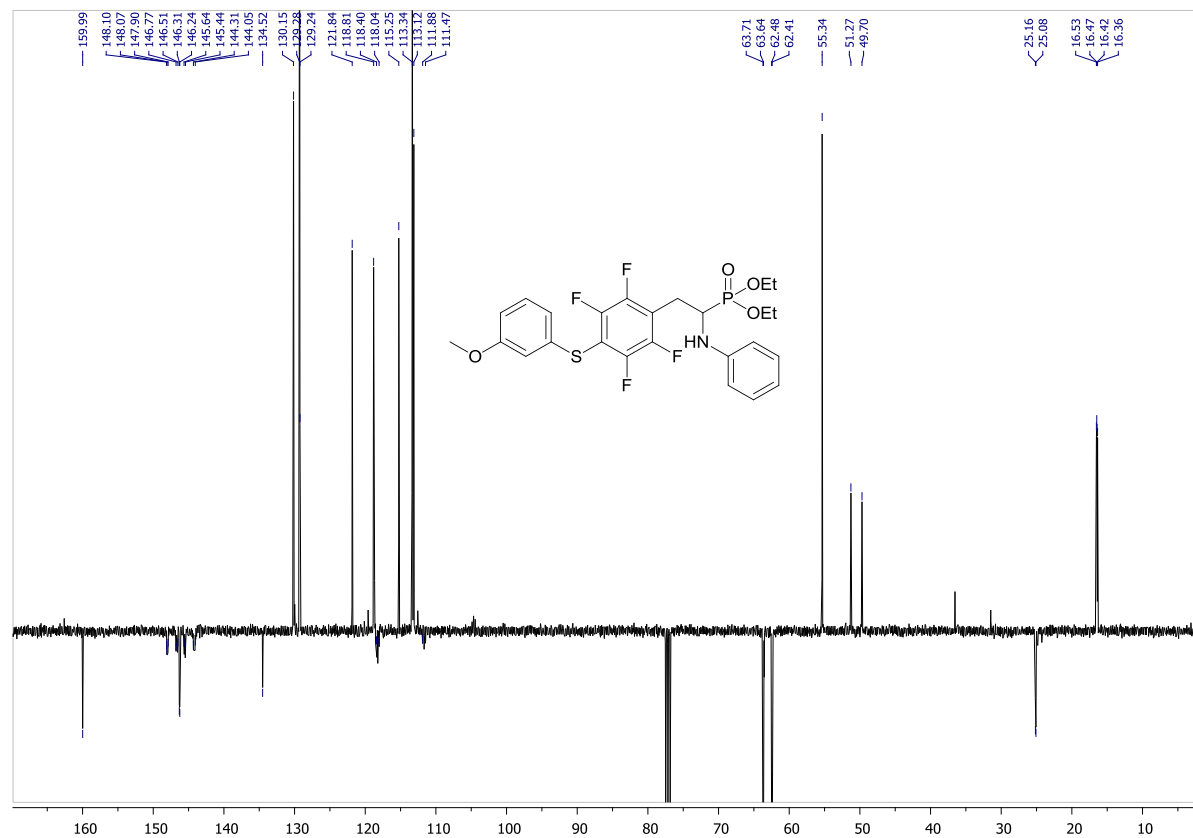


Fig. S20. ¹³C NMR spectrum of 2e (CDCl₃)

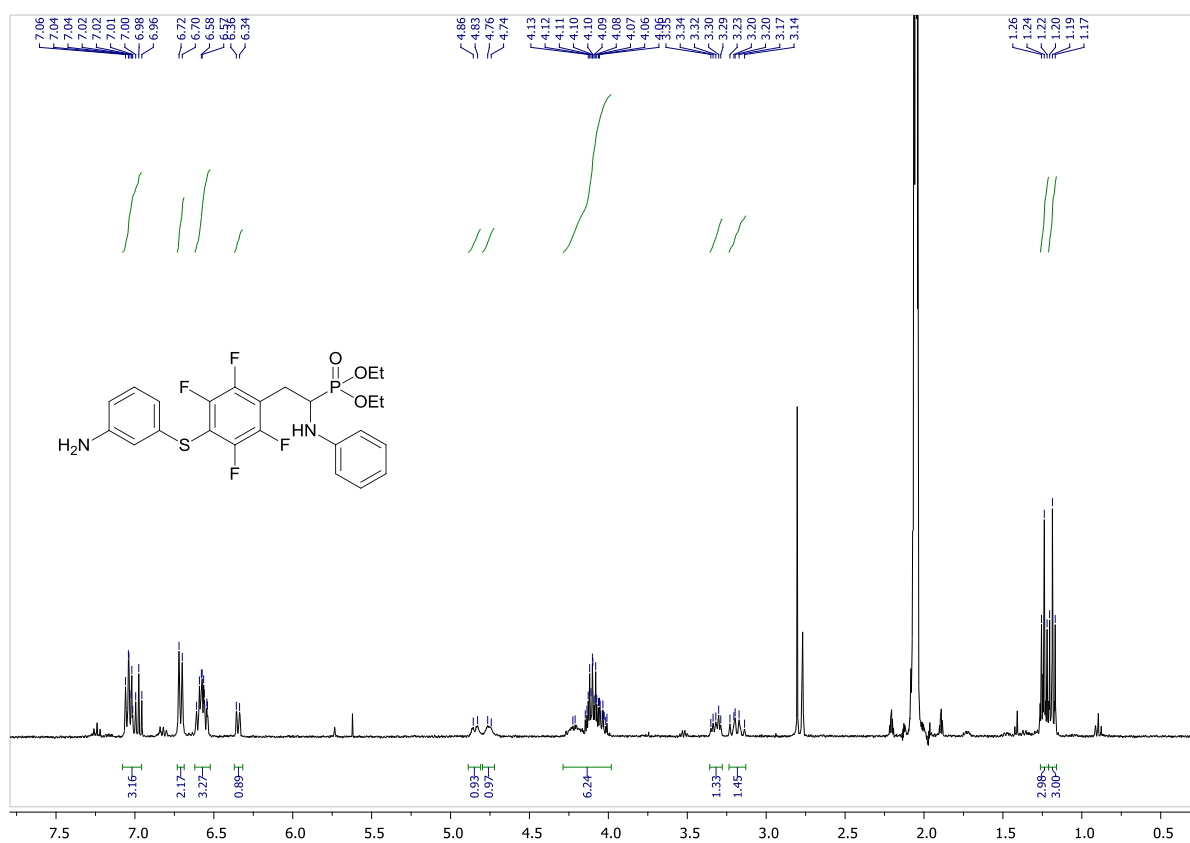


Fig. S21. ¹H NMR spectrum of 2f (Acetone-d₆)

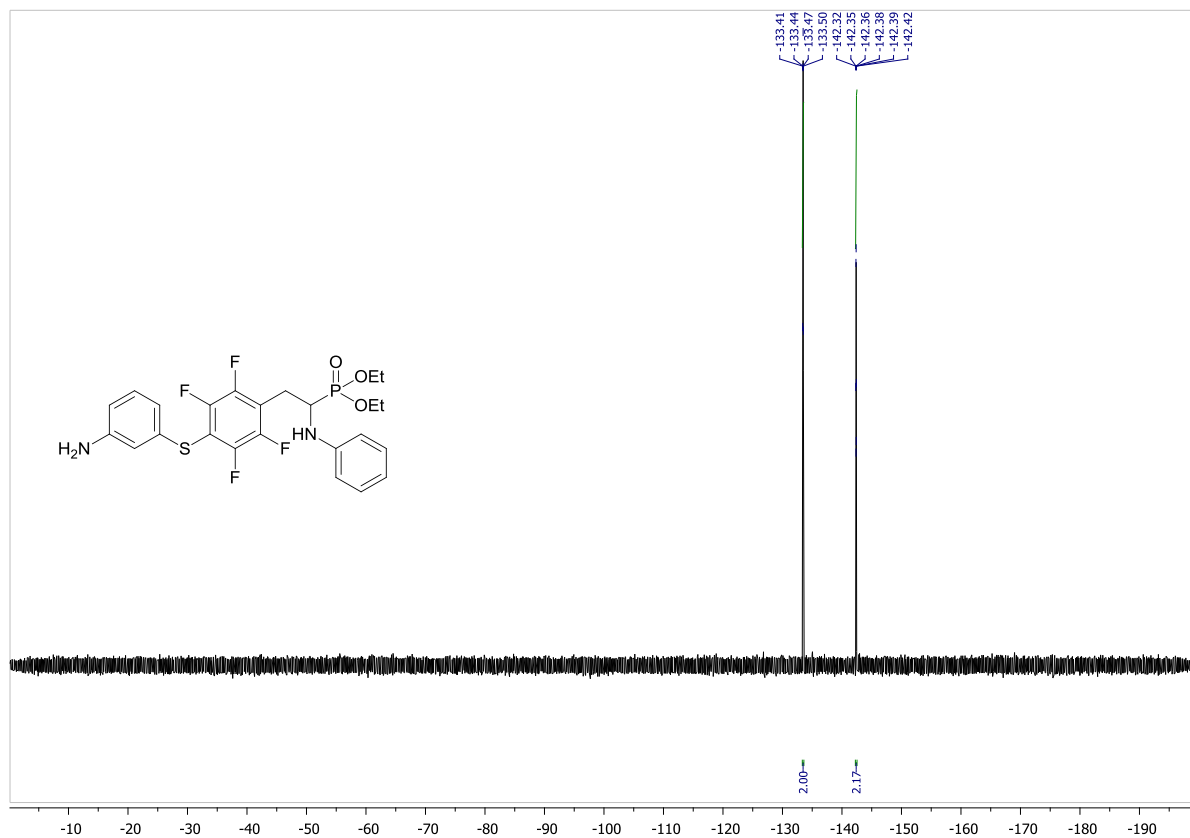


Fig. S22. ¹⁹F NMR spectrum of 2f (CDCl₃)

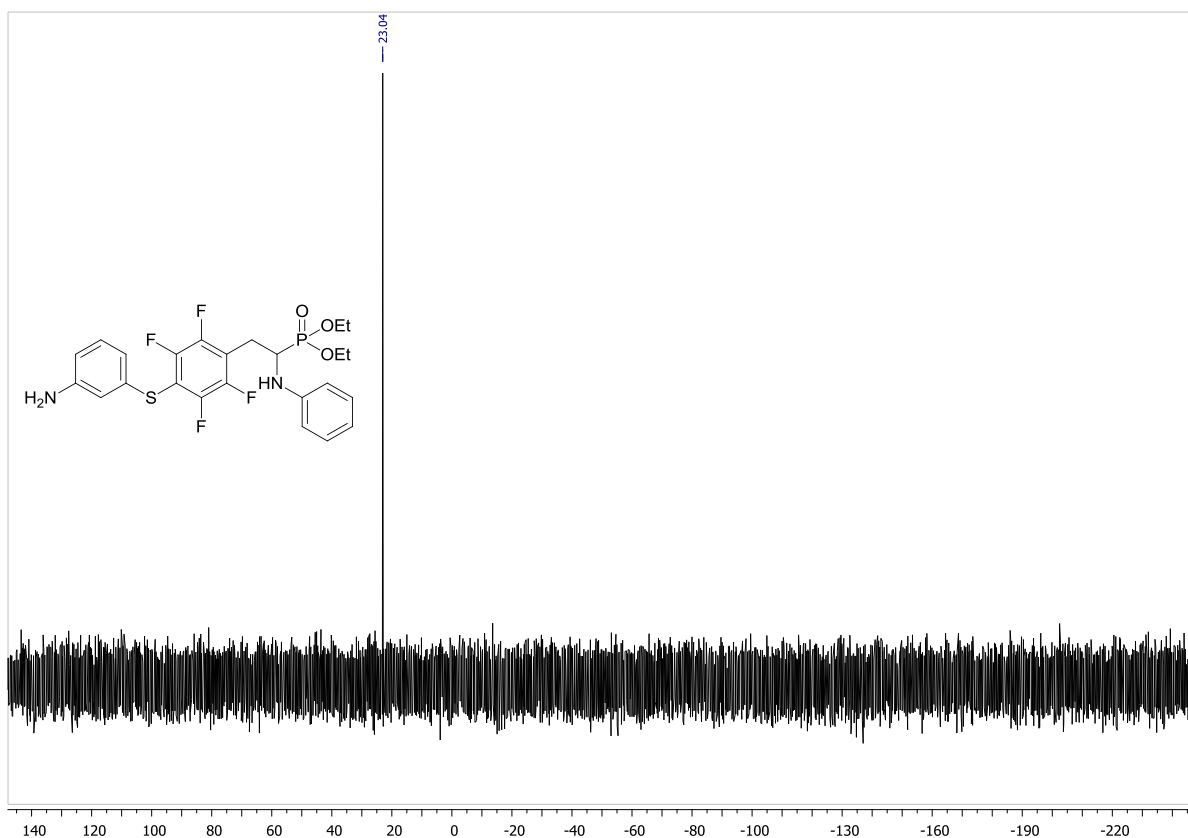


Fig. S23. ³¹P NMR spectrum of 2f (Acetone-d₆)

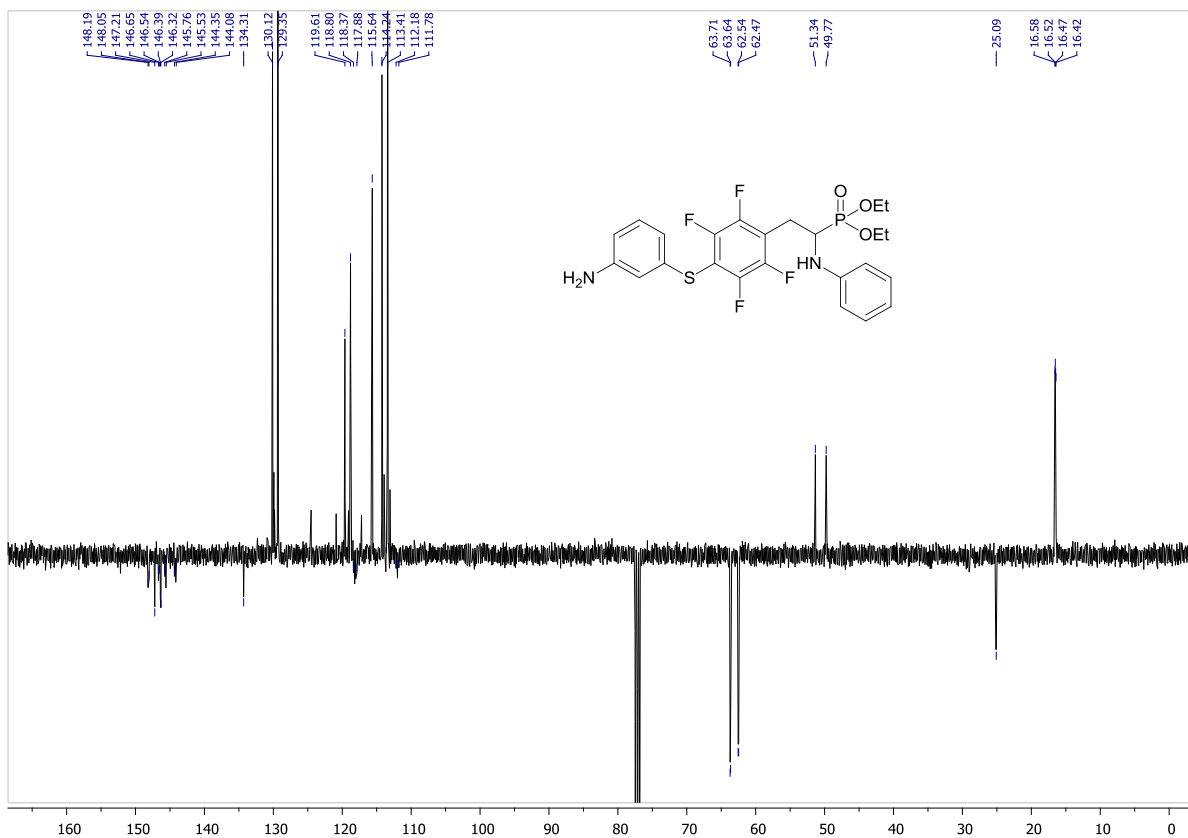


Fig. S24. ¹³C NMR spectrum of 2f (CDCl₃)

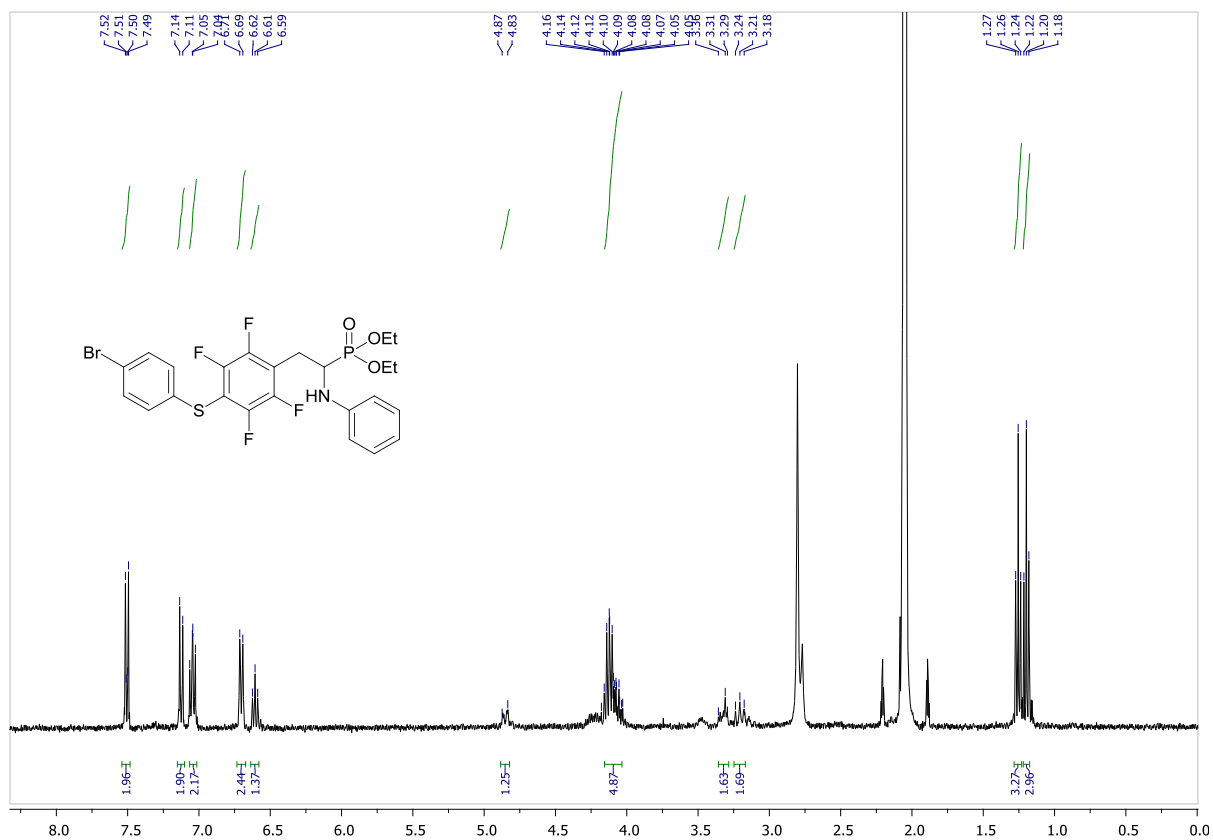


Fig. S25. ¹H NMR spectrum of 2g (Acetone-d₆)

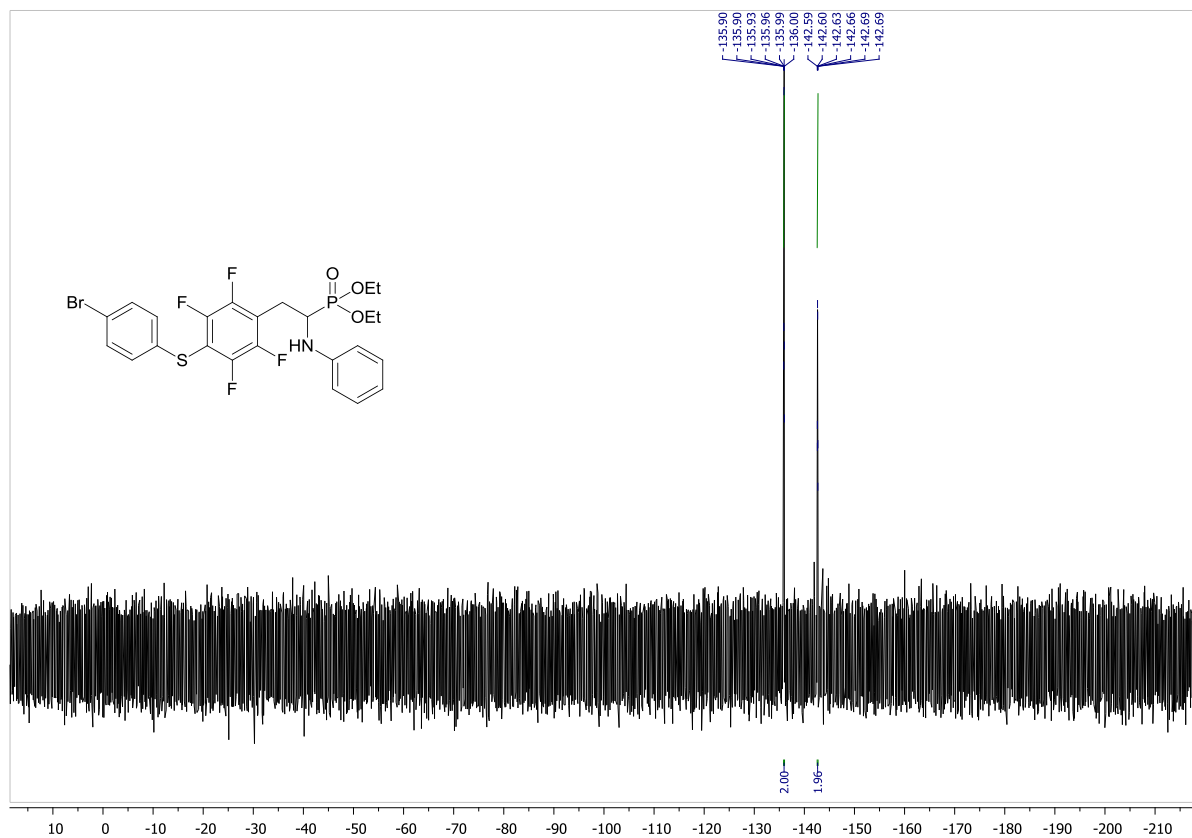


Fig. S26. ¹⁹F NMR spectrum of 2g (Acetone-d₆)

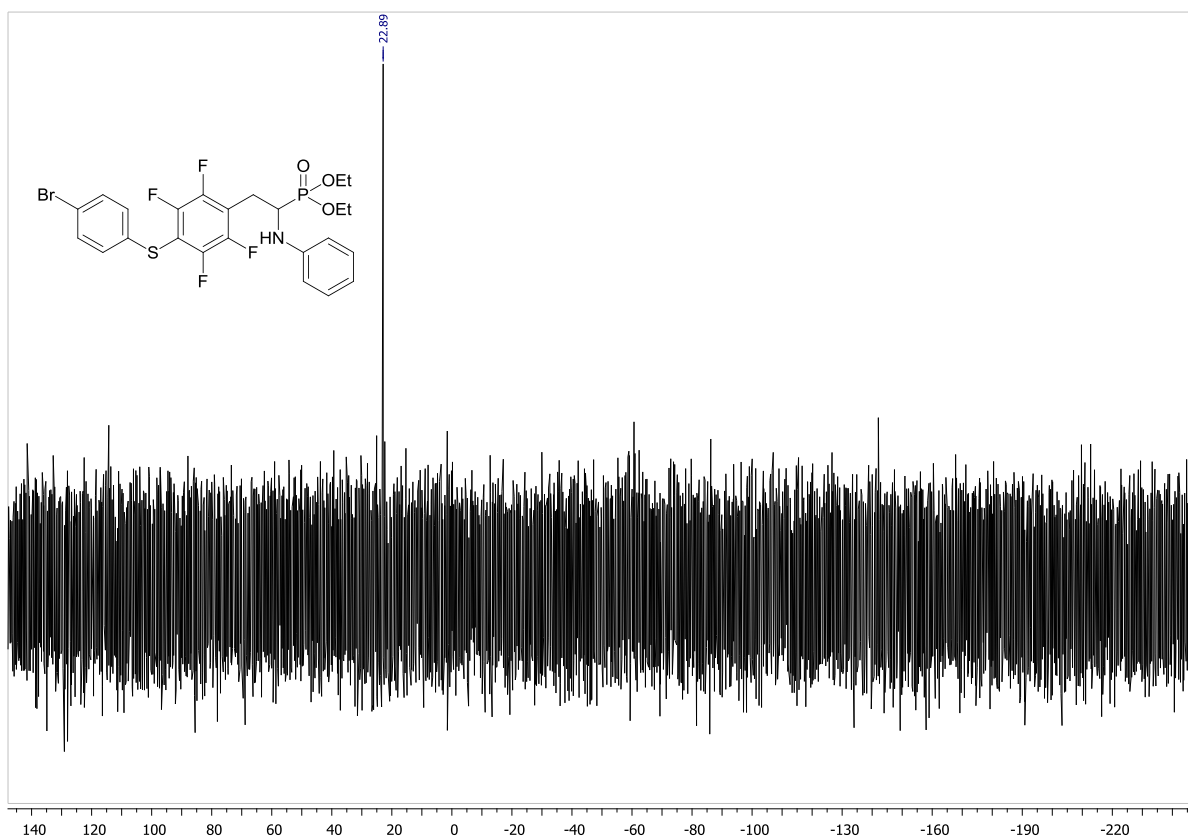


Fig. S27. ³¹P NMR spectrum of 2g (Acetone-d₆)

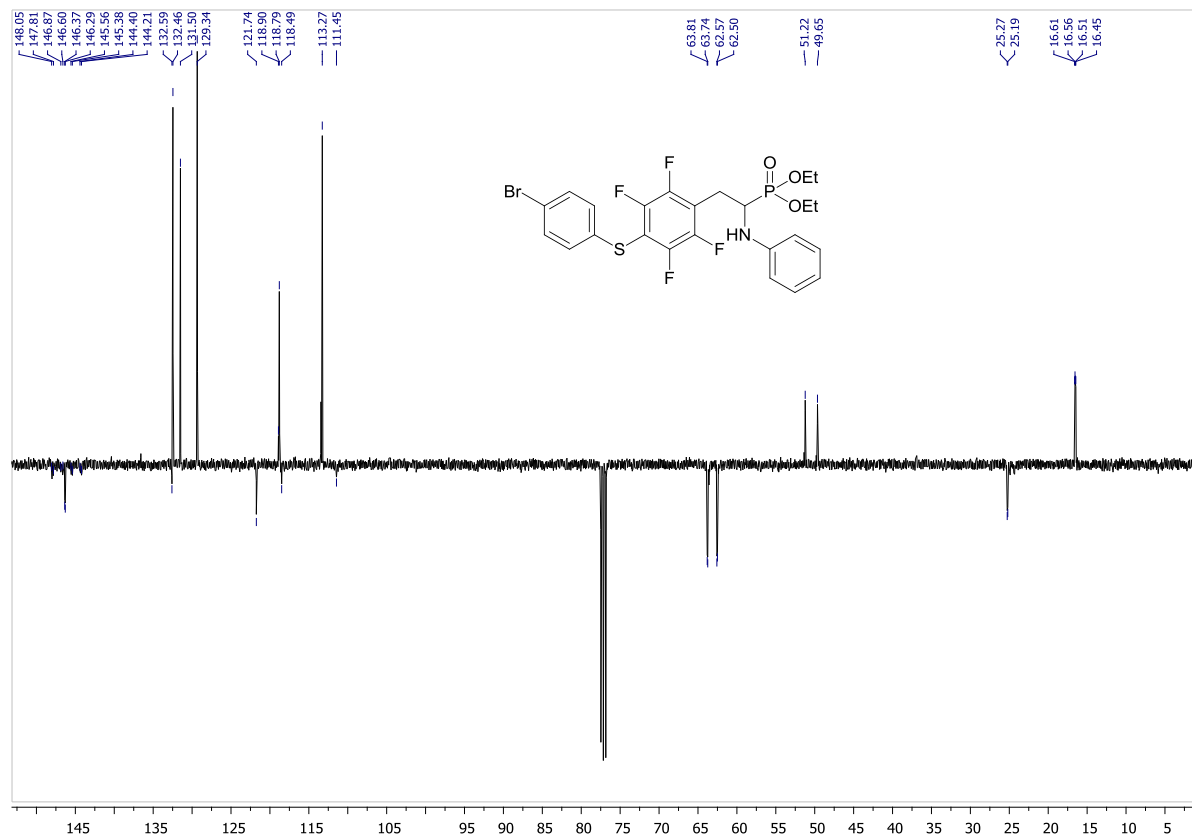


Fig. S28. ¹³C NMR spectrum of 2g (CDCl₃)

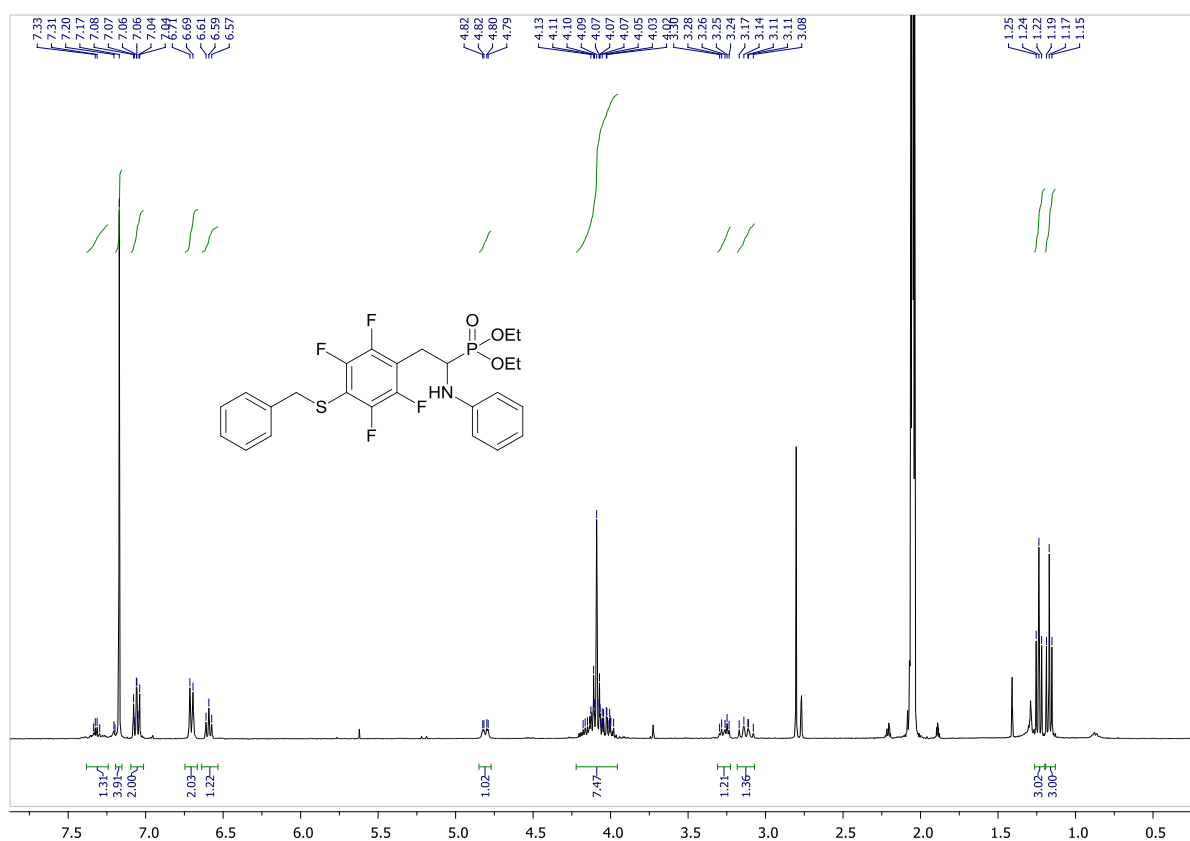


Fig. S29. ¹H NMR spectrum of 2h (Acetone-d₆)

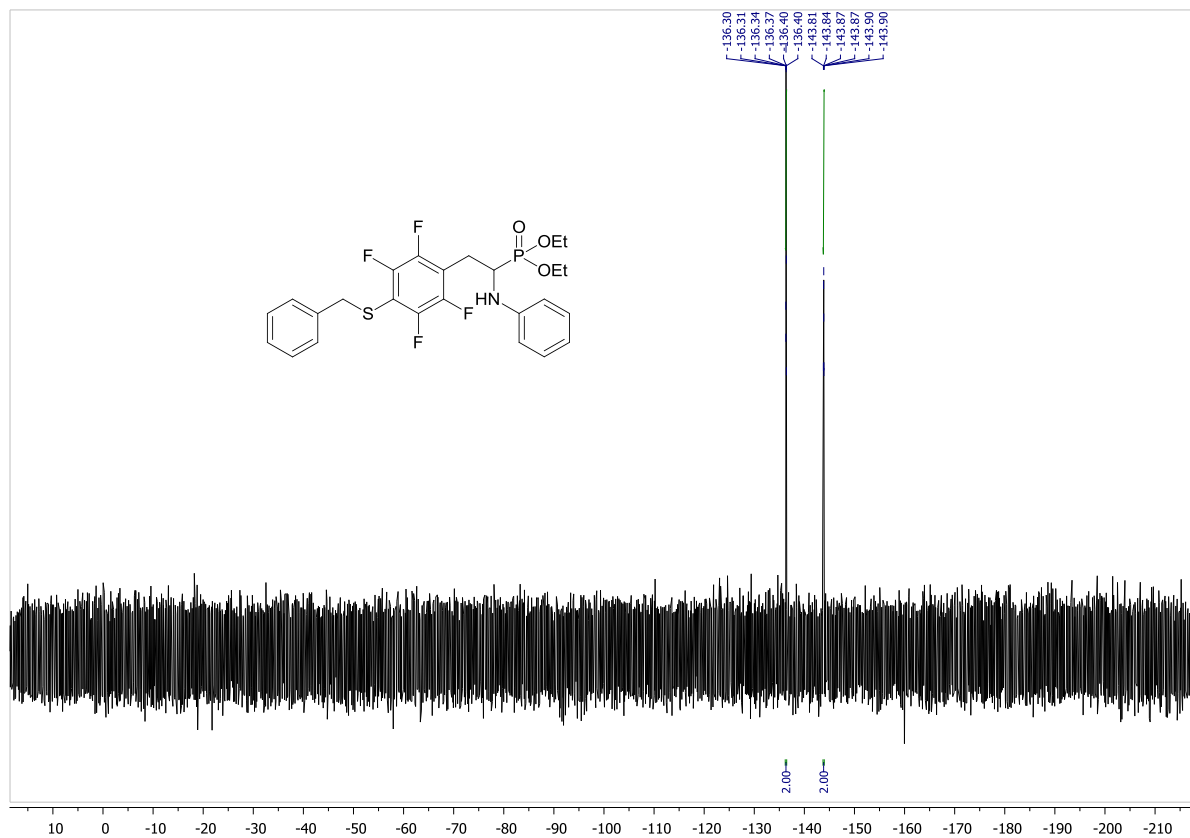


Fig. S30. ¹⁹F NMR spectrum of 2h (Acetone-d₆)

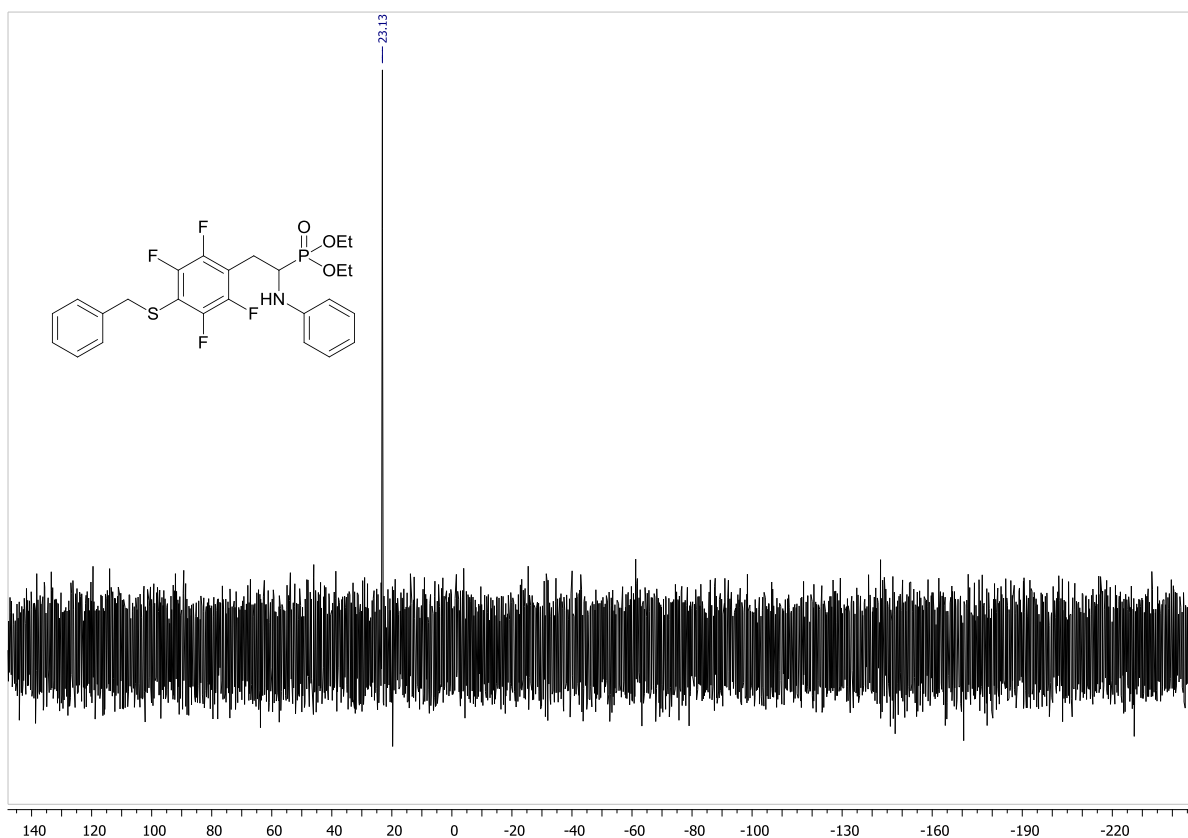


Fig. S31. ³¹P NMR spectrum of 2h (Acetone-d₆)

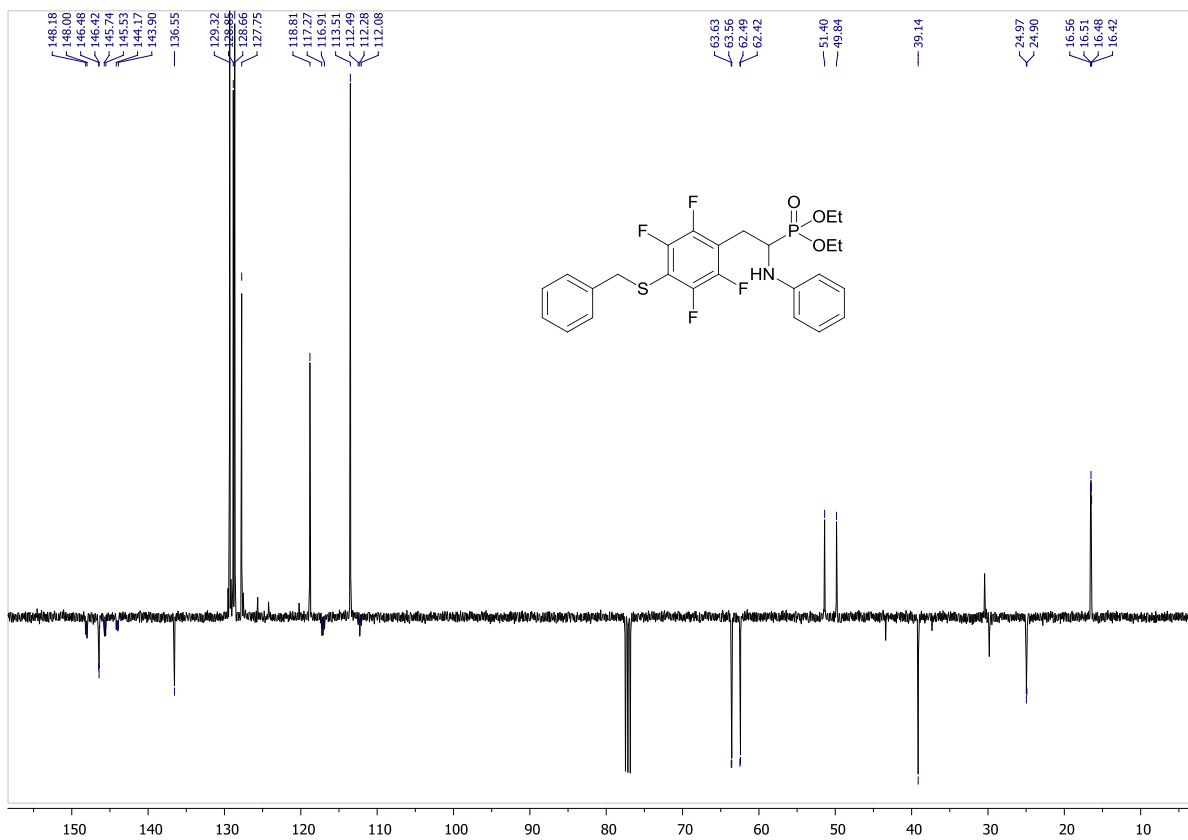


Fig. S32. ¹³C NMR spectrum of 2h (CDCl₃)

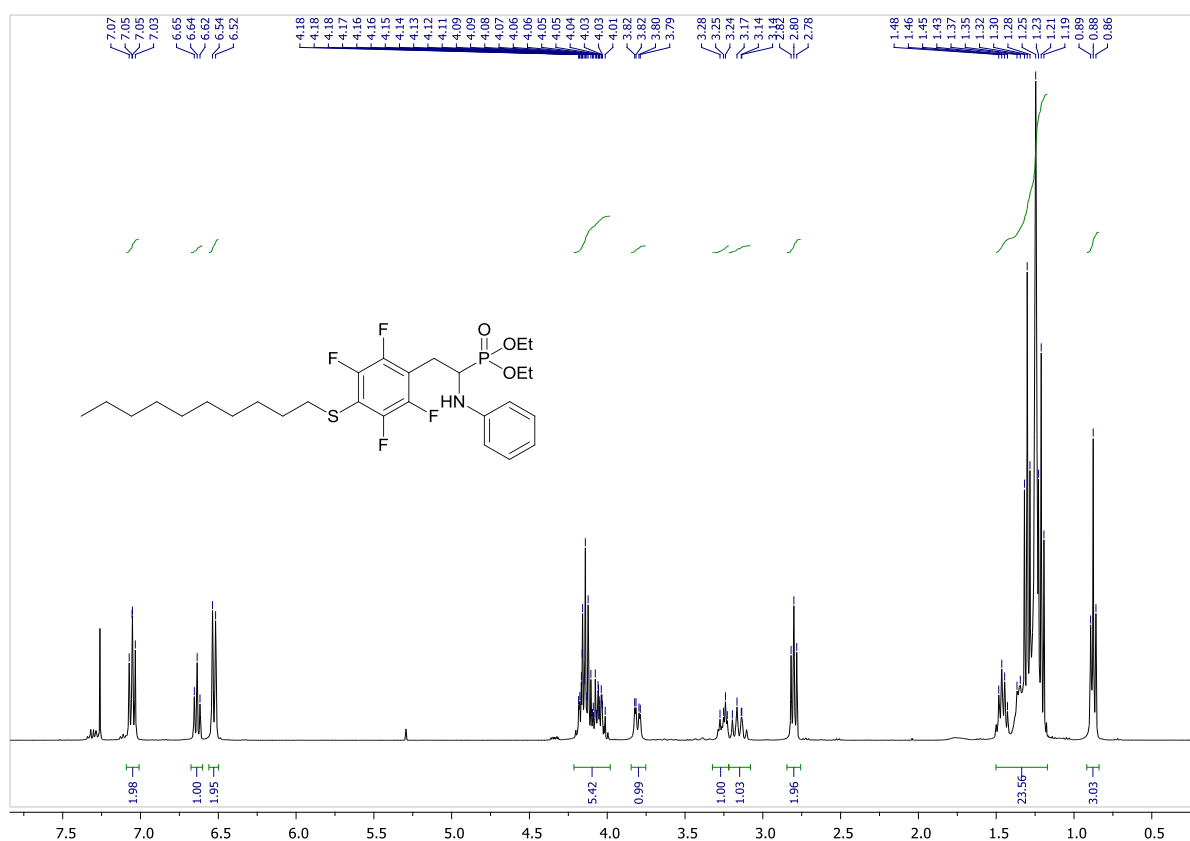


Fig. S33. ¹H NMR spectrum of 2i (CDCl₃)

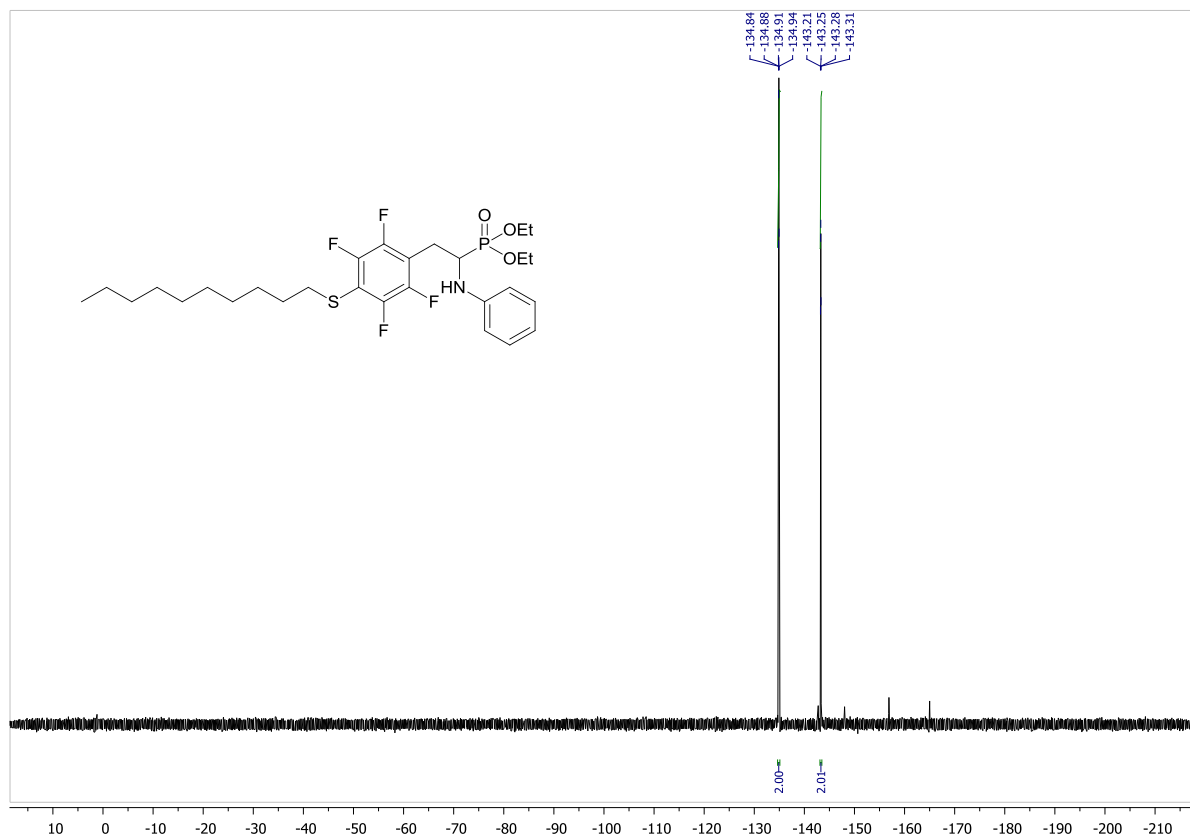


Fig. S34. ¹⁹F NMR spectrum of 2i (CDCl₃)

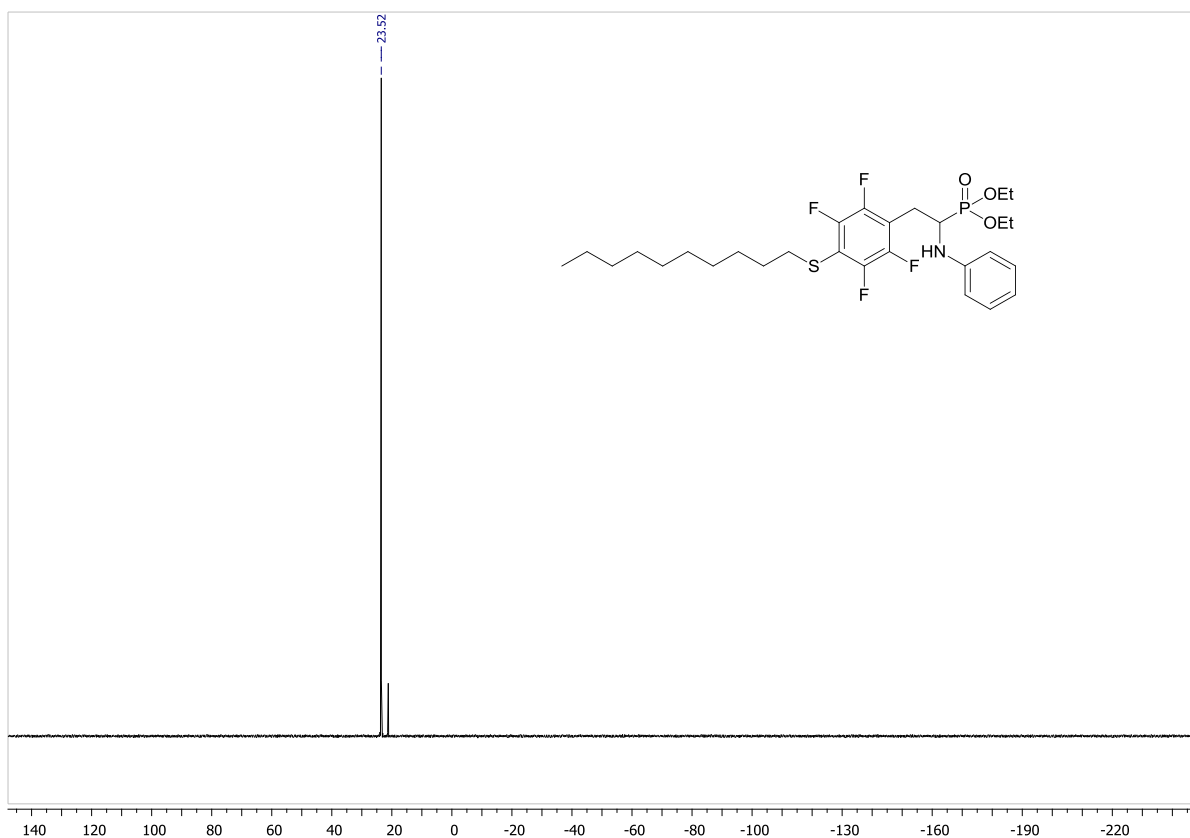


Fig. S35. ³¹P NMR spectrum of 2i (CDCl₃)

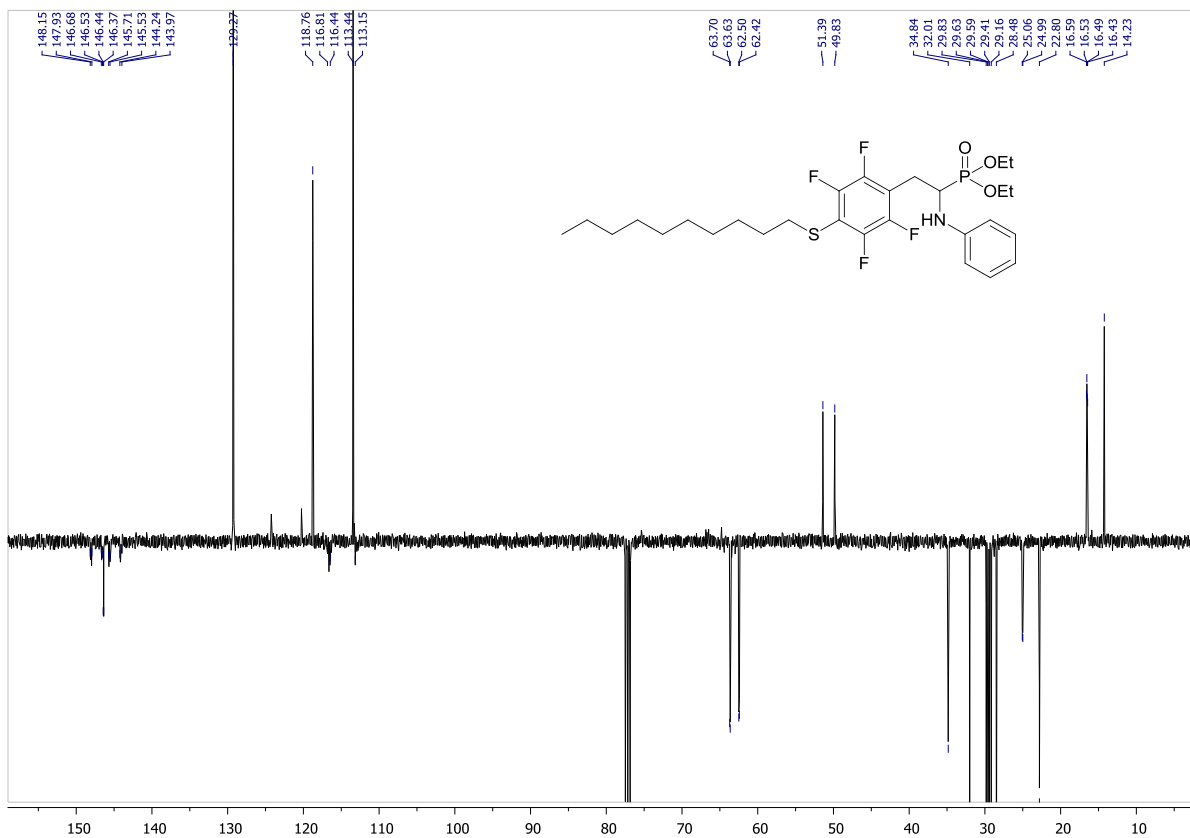


Fig. S36. ¹³C NMR spectrum of 2i (CDCl₃)

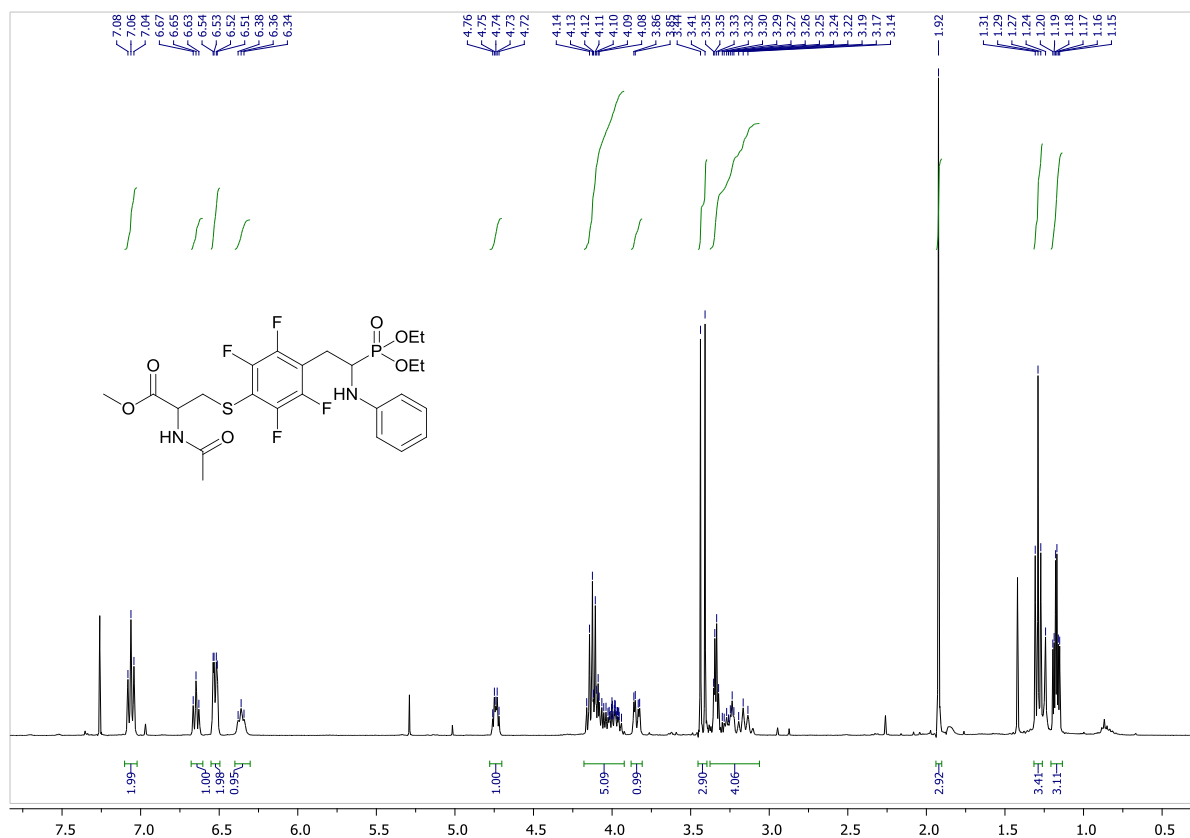


Fig. S37. ¹H NMR spectrum of 2j (CDCl₃)

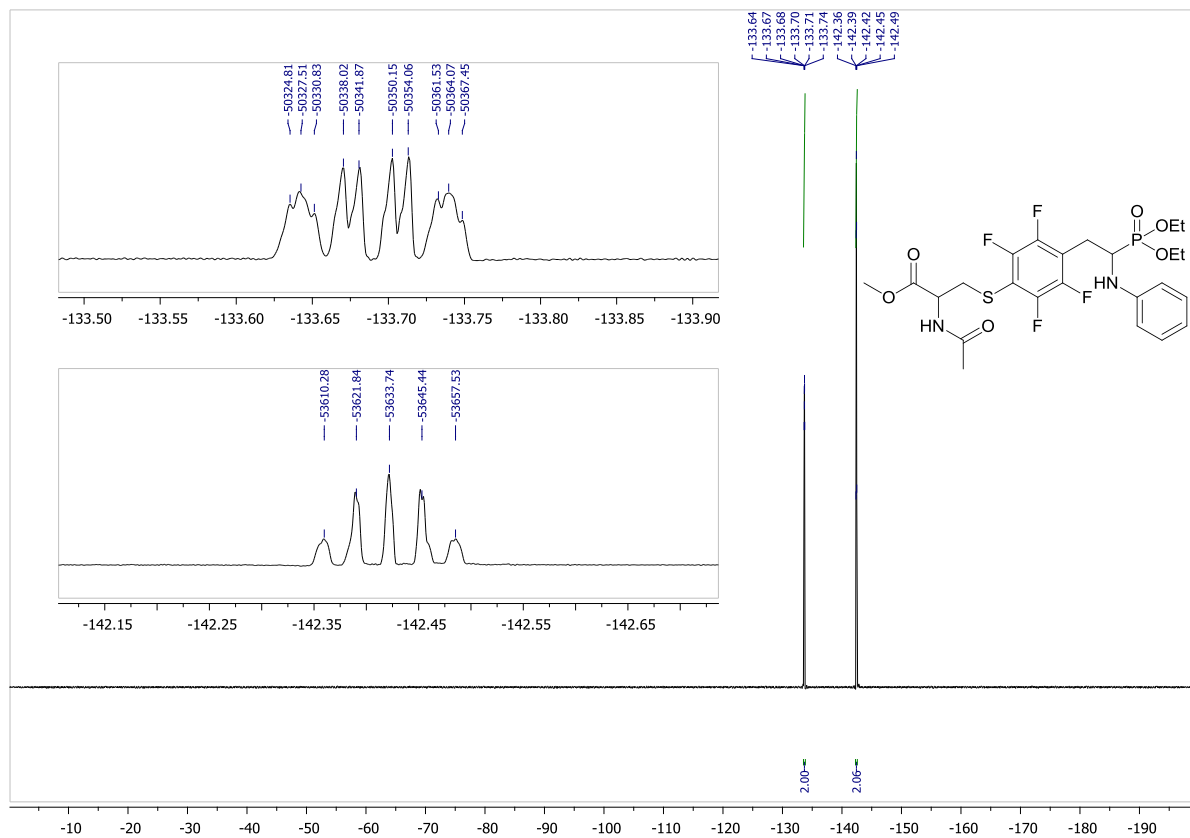


Fig. S38. ¹⁹F NMR spectrum of 2j (CDCl₃)

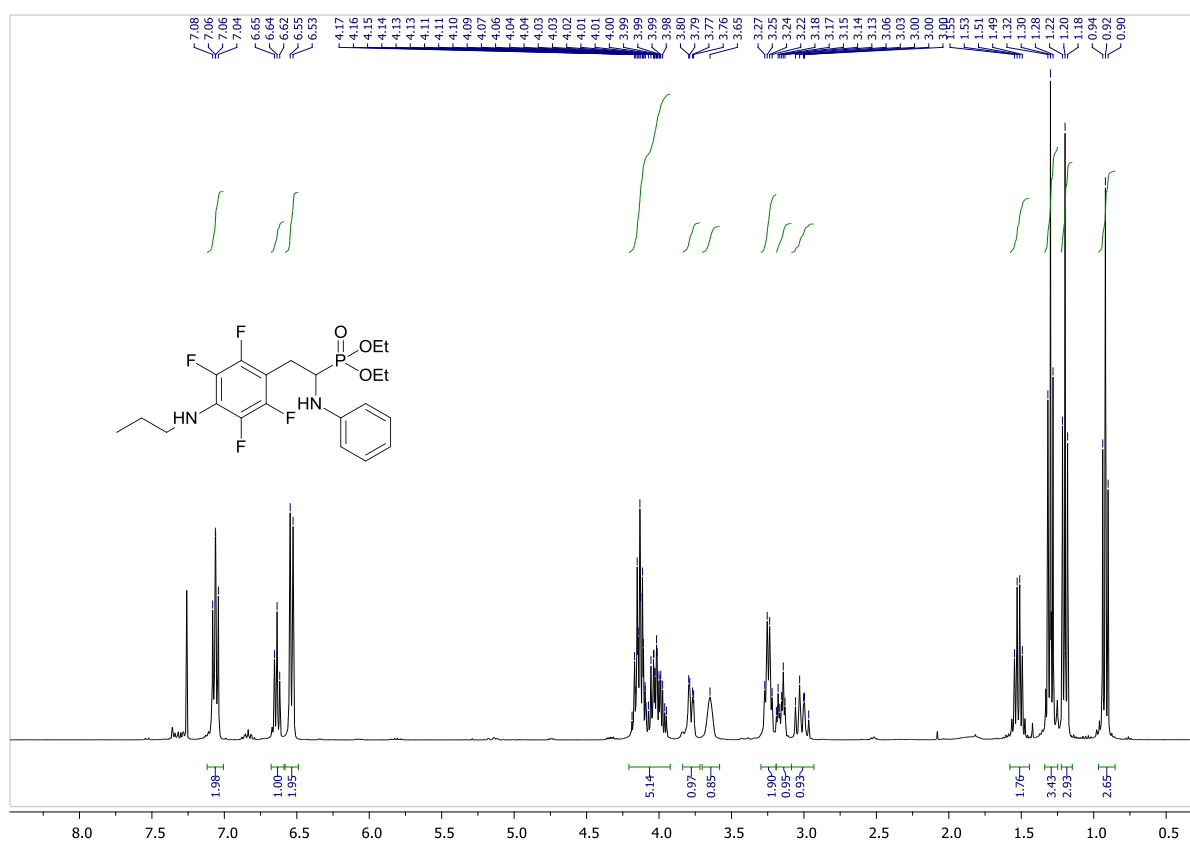


Fig. S41. ¹H NMR spectrum of 2l (CDCl₃)

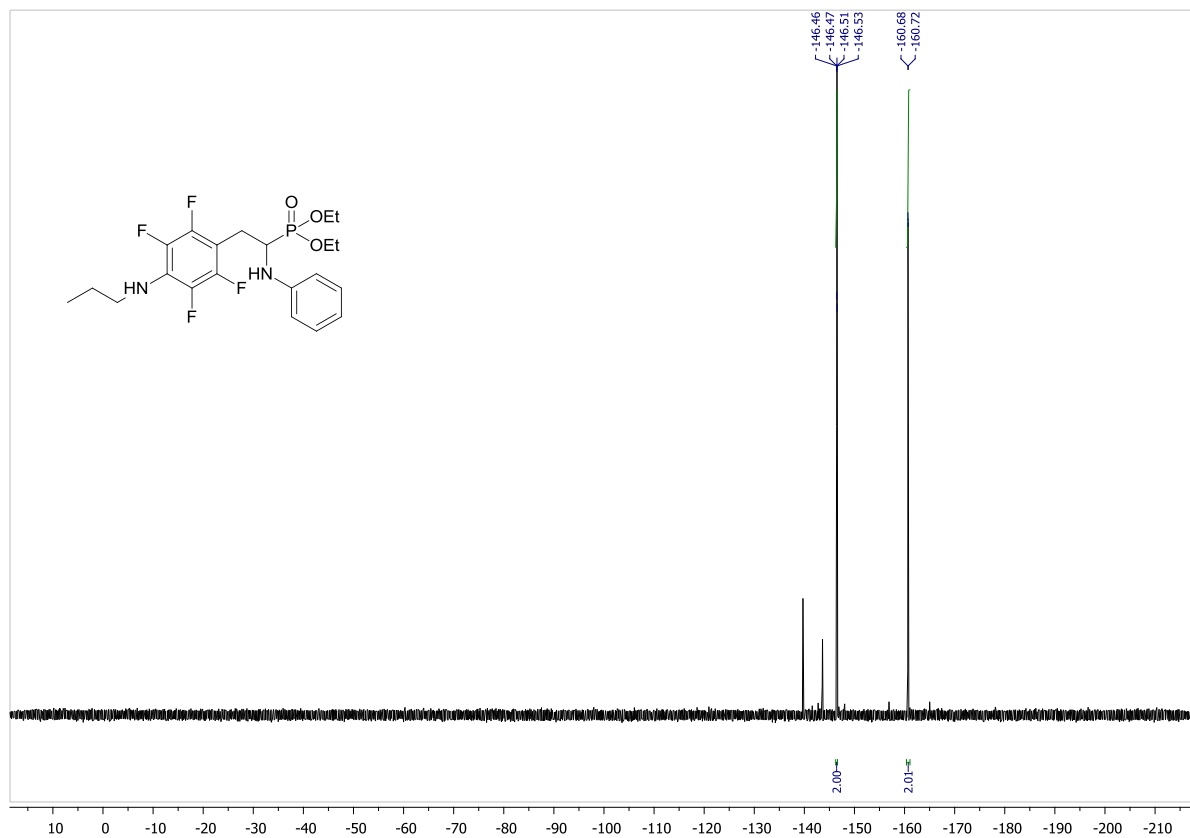


Fig. S42. ¹⁹F NMR spectrum of 2l (CDCl₃)

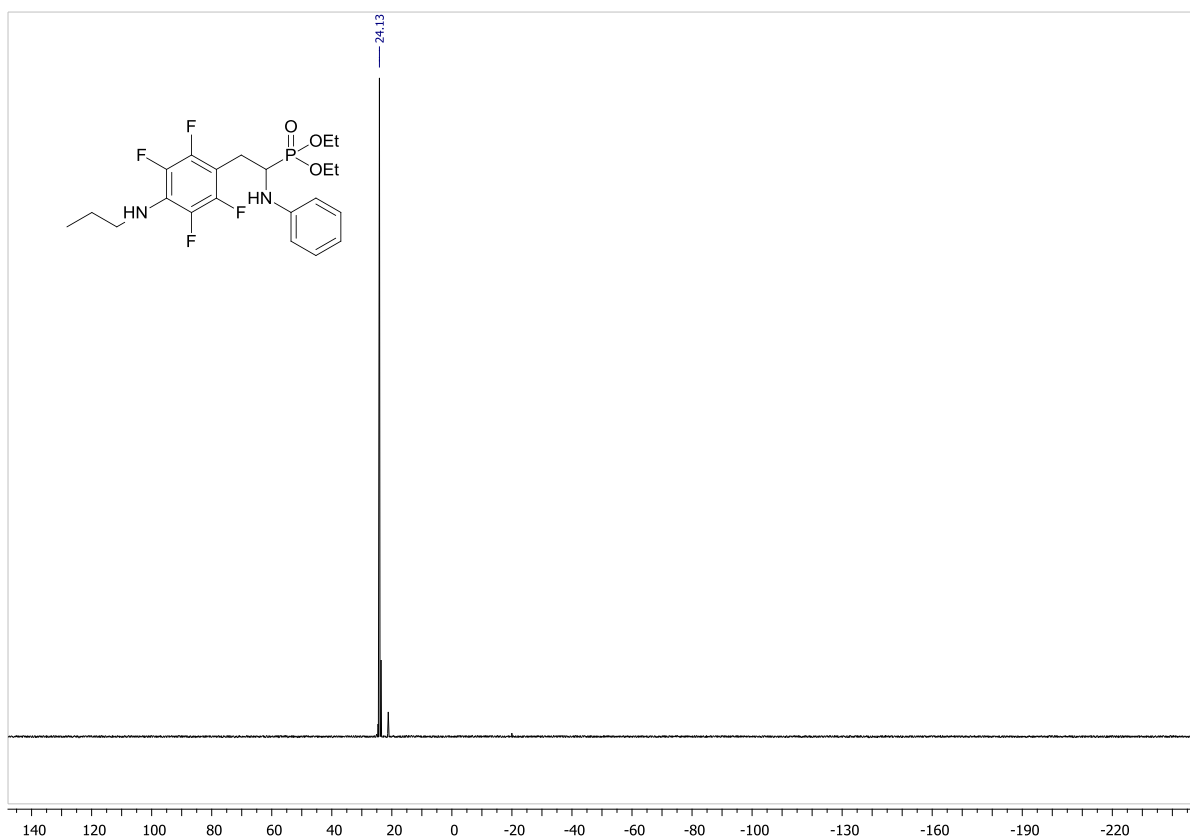


Fig. S43. ³¹P NMR spectrum of 2l (CDCl₃)

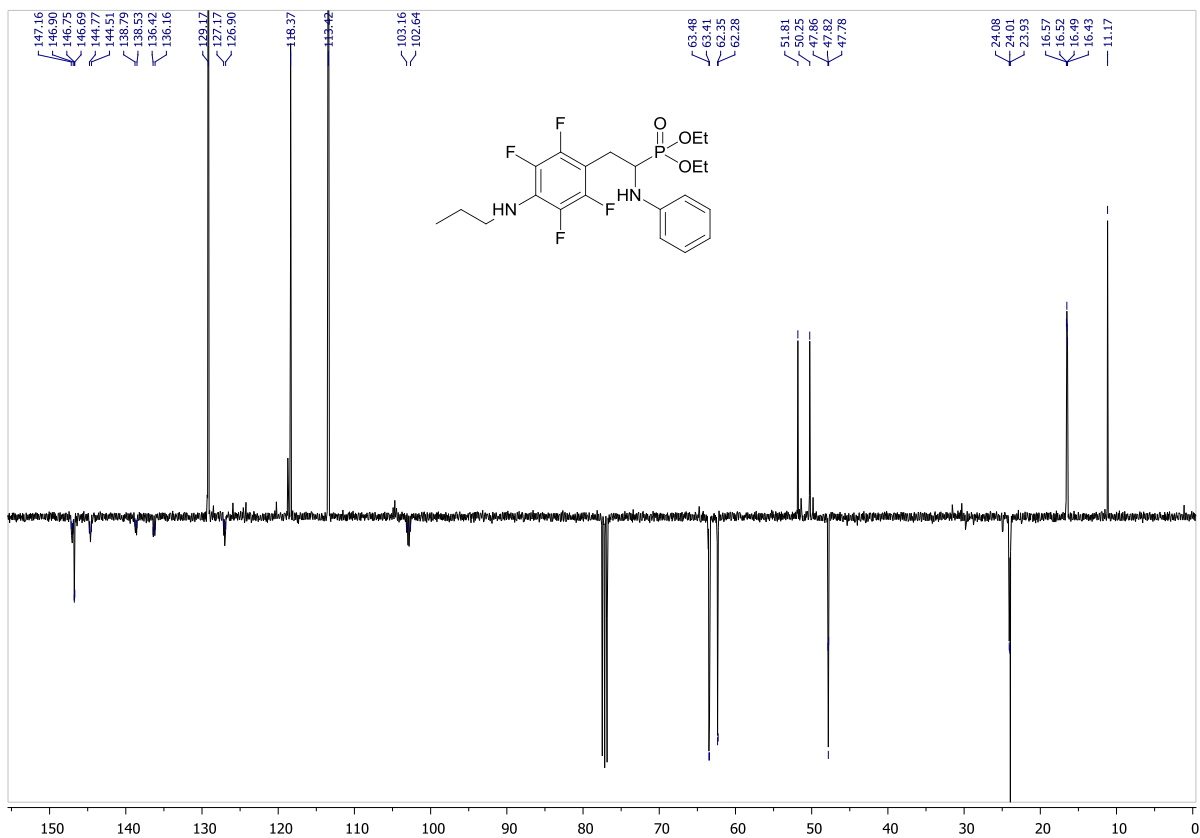


Fig. S44. ¹³C NMR spectrum of 2l (CDCl₃)

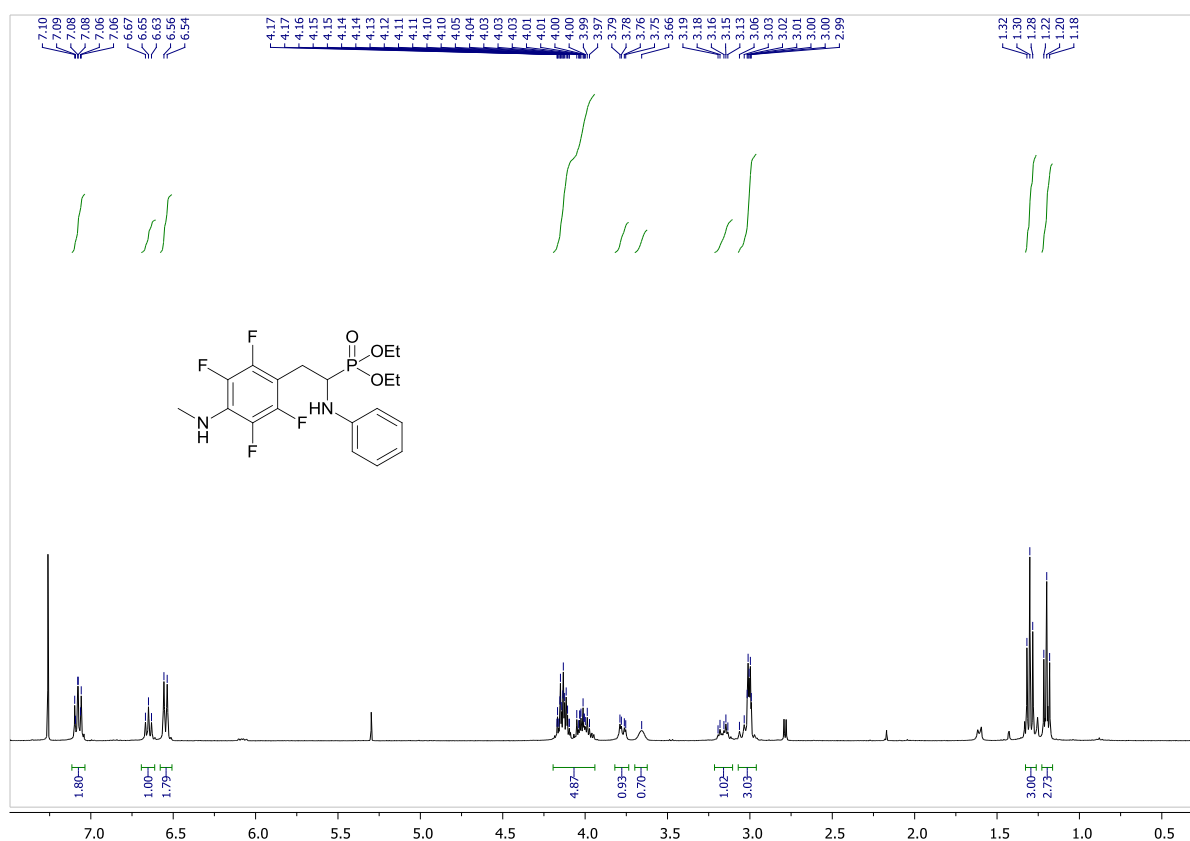


Fig. S45. ¹H NMR spectrum of 2k (CDCl₃)

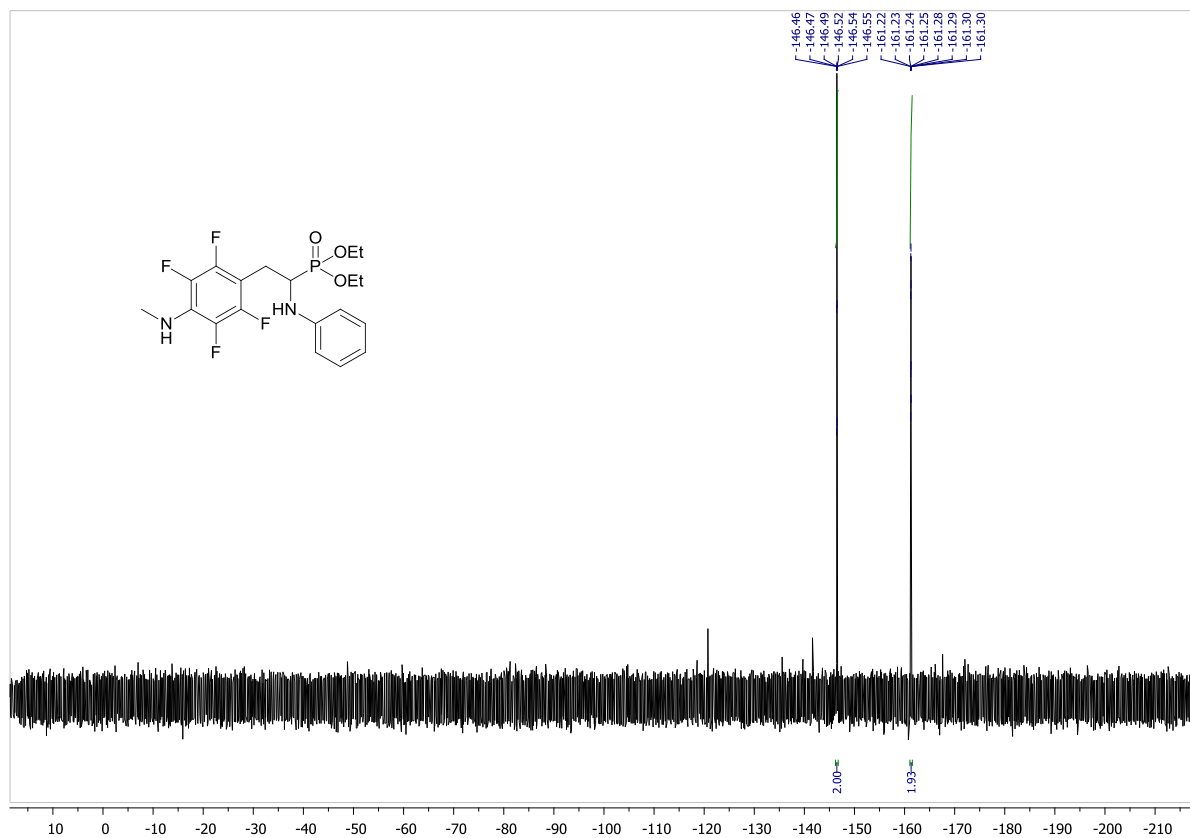


Fig. S46. ¹⁹F NMR spectrum of 2k (CDCl₃)

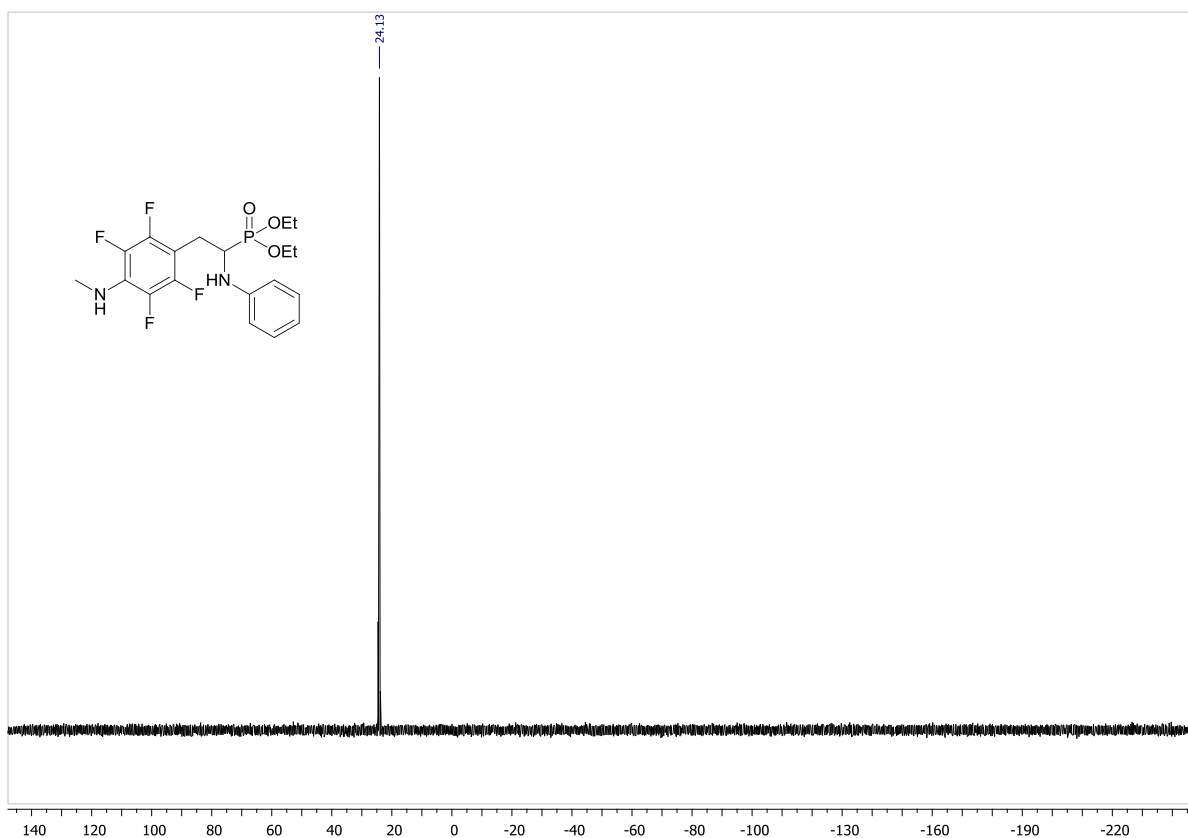


Fig. S47. ³¹P NMR spectrum of 2k (CDCl₃)

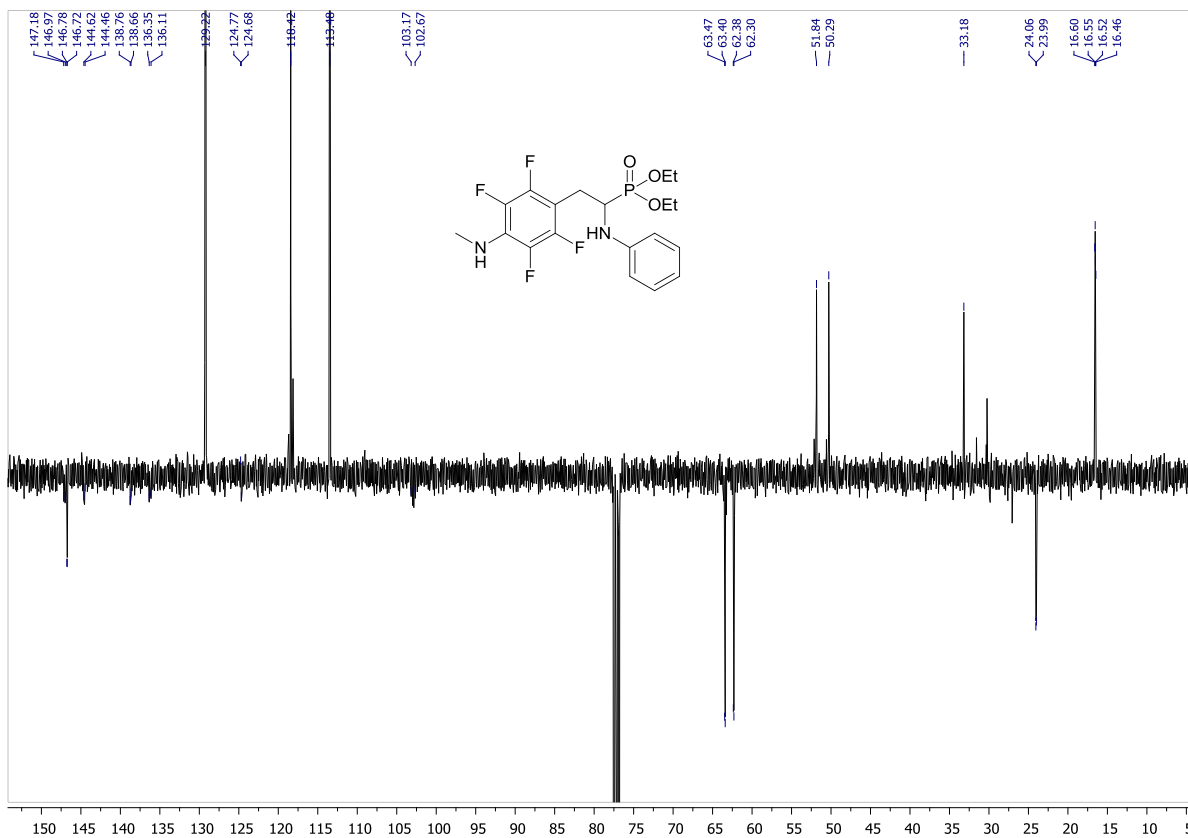


Fig. S48. ¹³C NMR spectrum of 2k (CDCl₃)

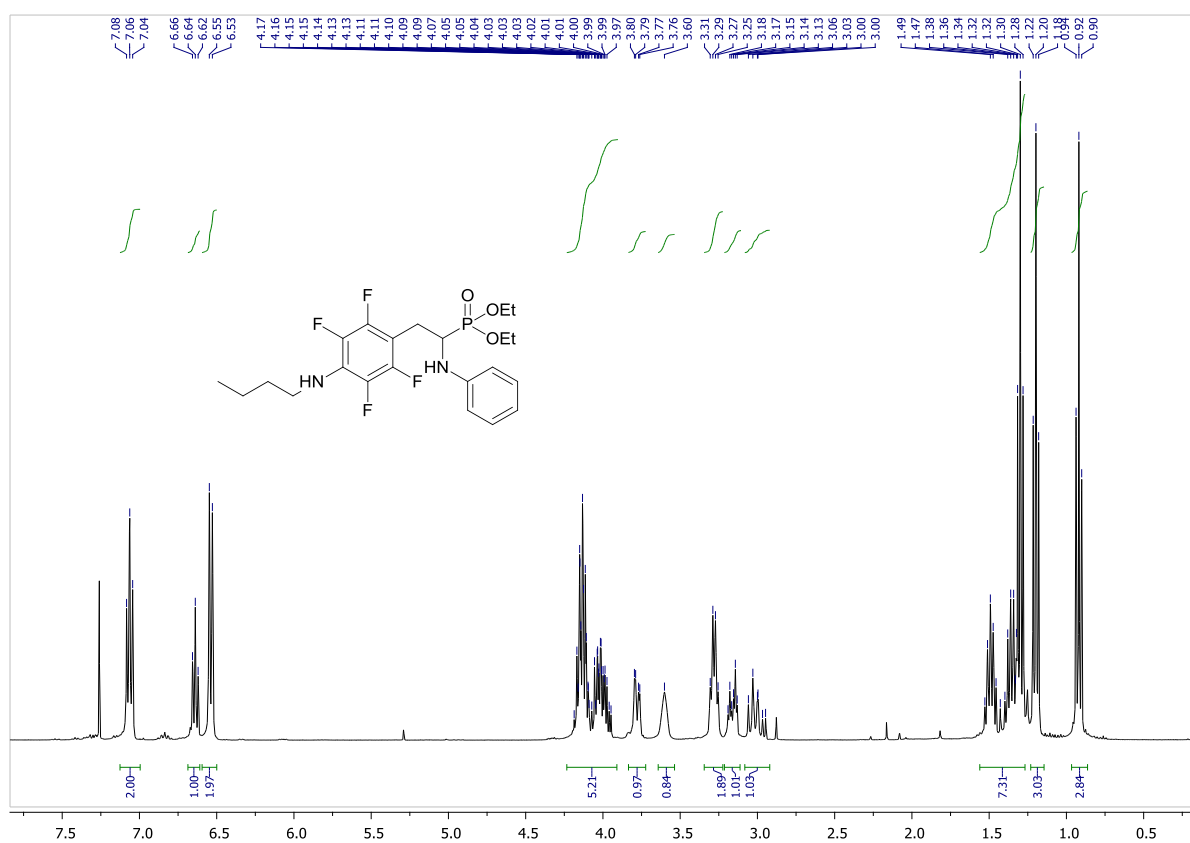


Fig. S49. ¹H NMR spectrum of 2m (CDCl₃)

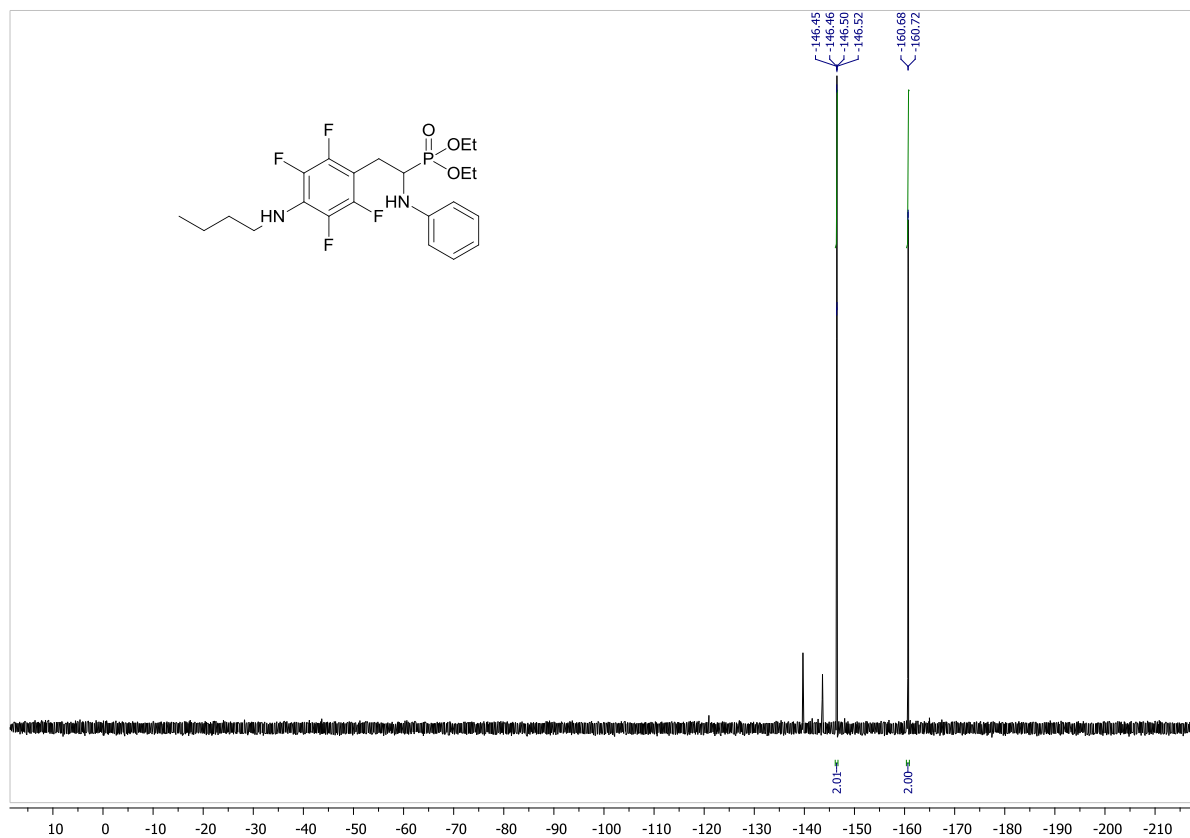


Fig. S50. ¹⁹F NMR spectrum of 2m (CDCl₃)

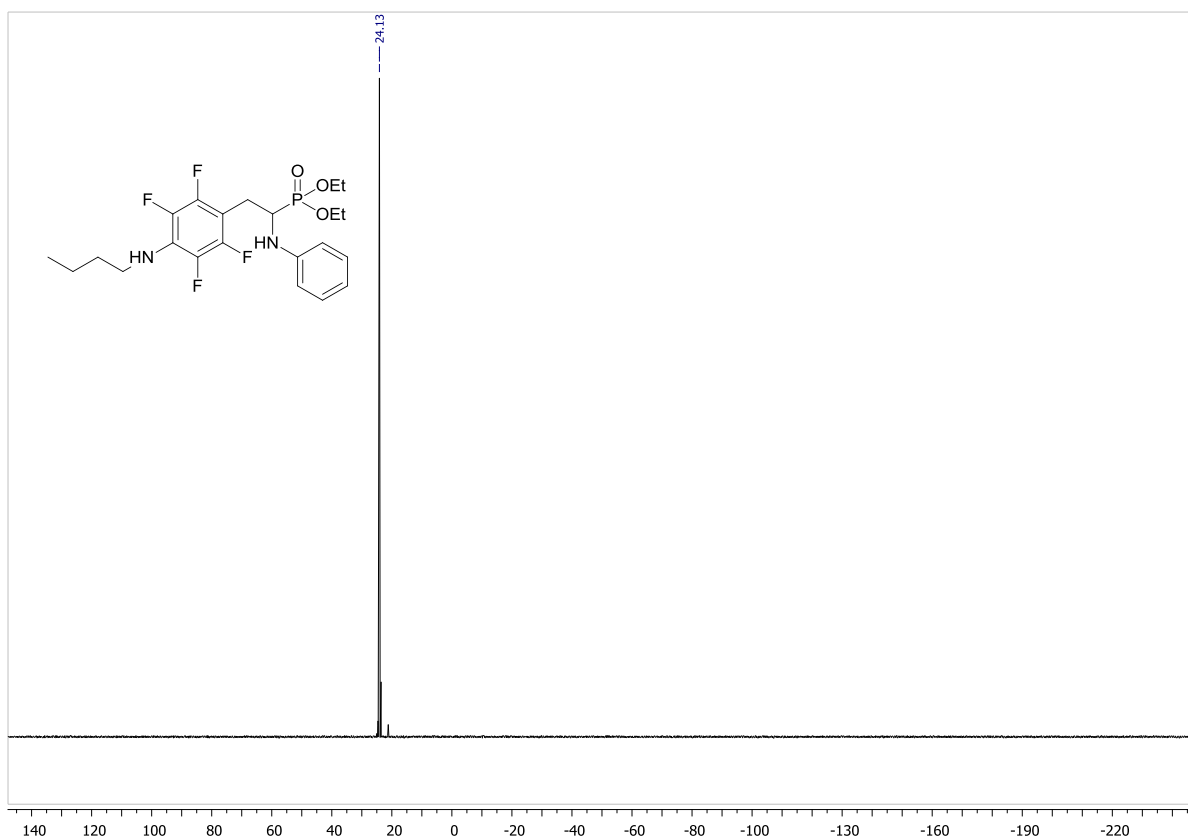


Fig. S51. ³¹P NMR spectrum of 2m (CDCl₃)

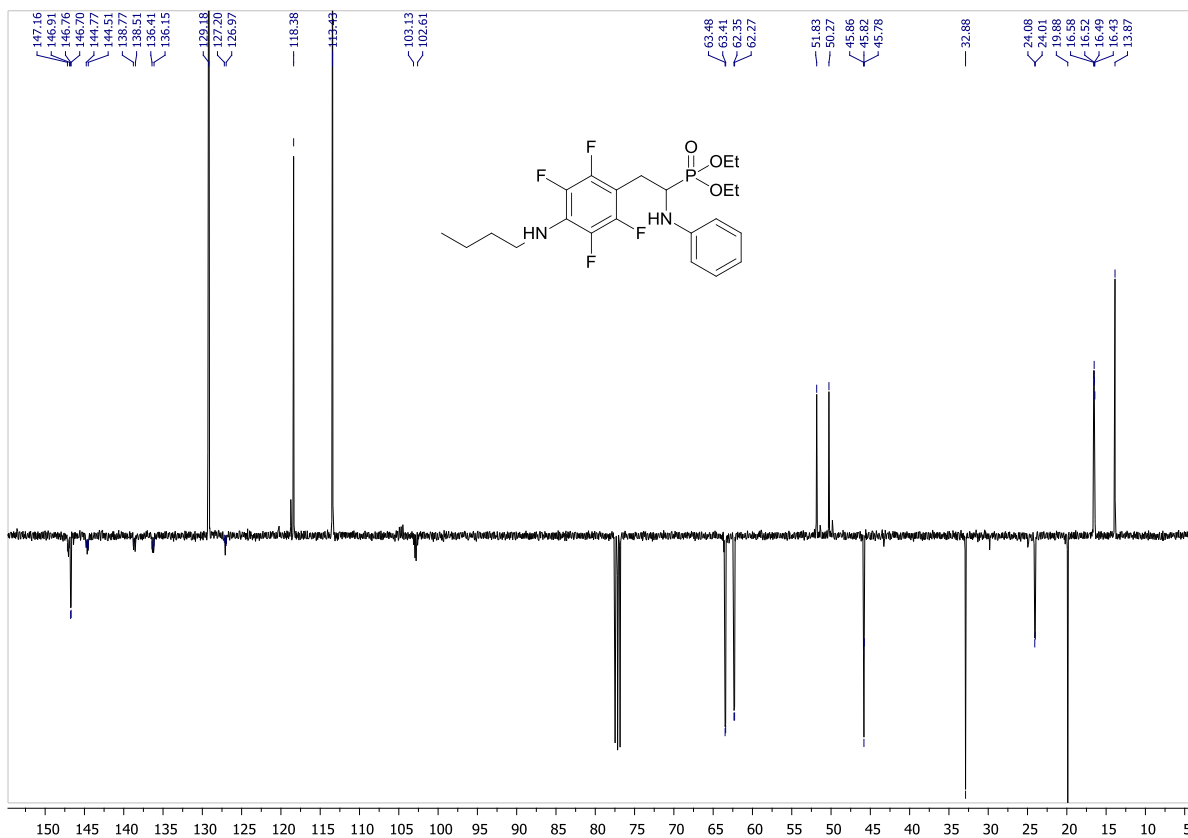


Fig. S52. ¹³C NMR spectrum of 2m (CDCl₃)

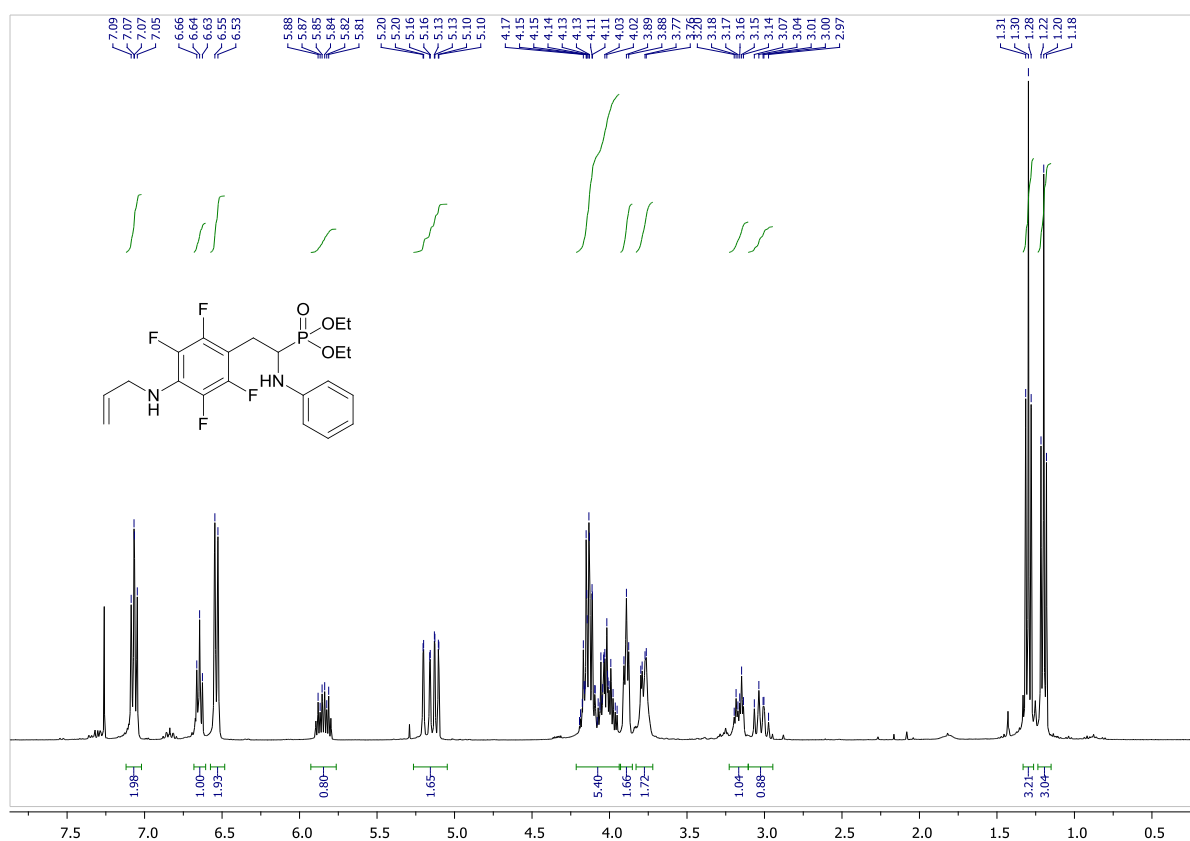


Fig. S53. ¹H NMR spectrum of 2n (CDCl₃)

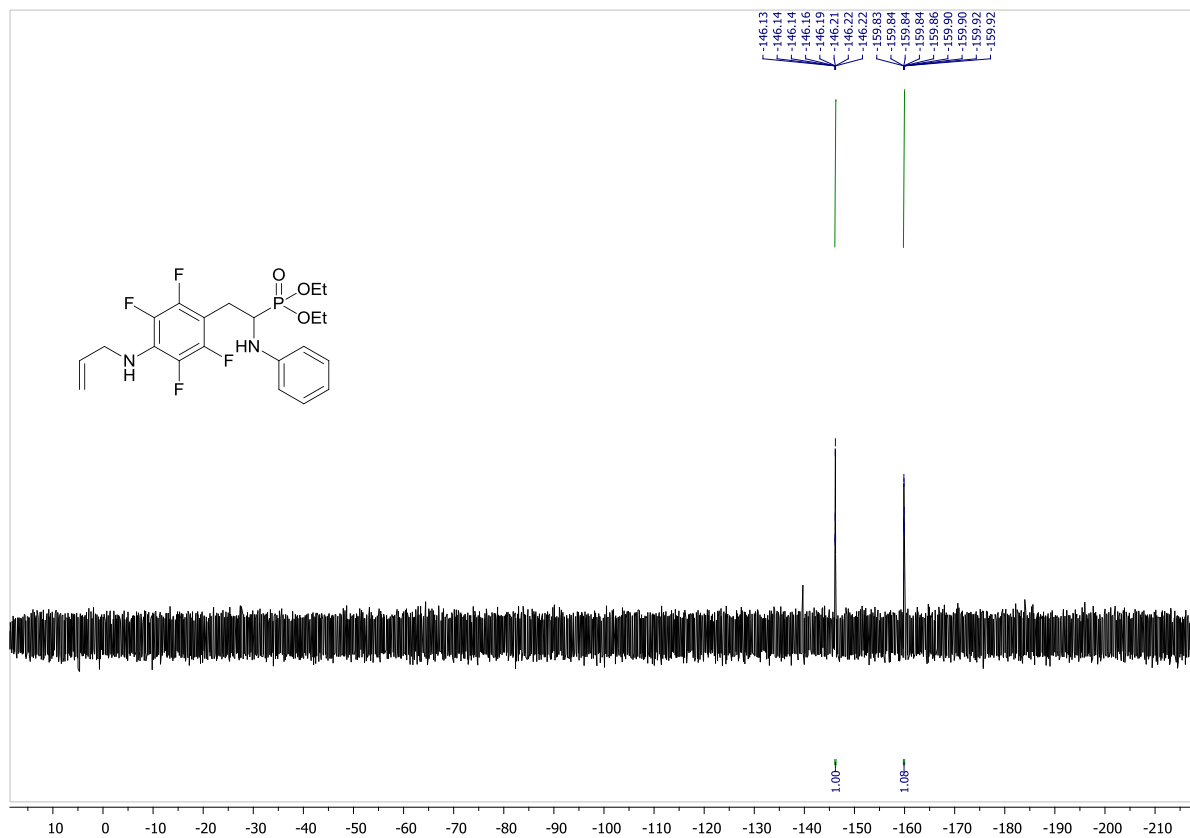


Fig. S54. ¹⁹F NMR spectrum of 2n (CDCl₃)

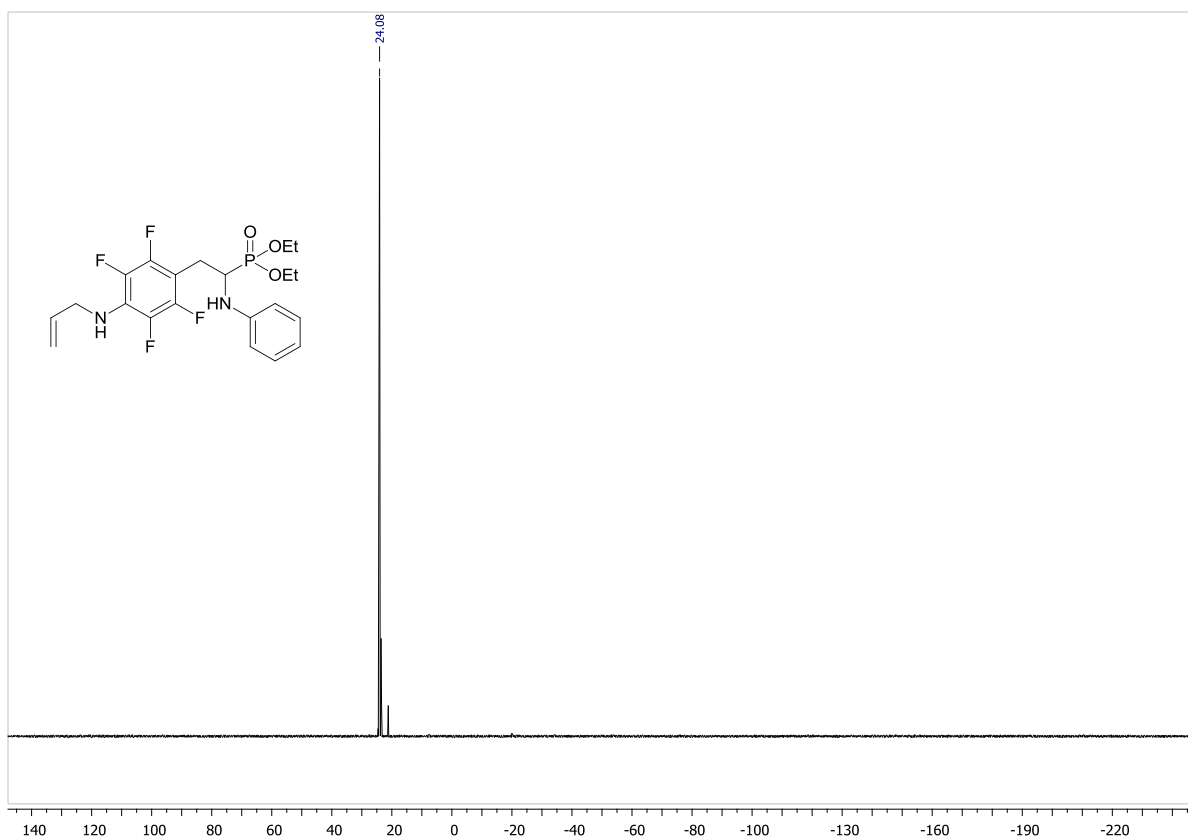


Fig. S55. ³¹P NMR spectrum of 2n (CDCl₃)

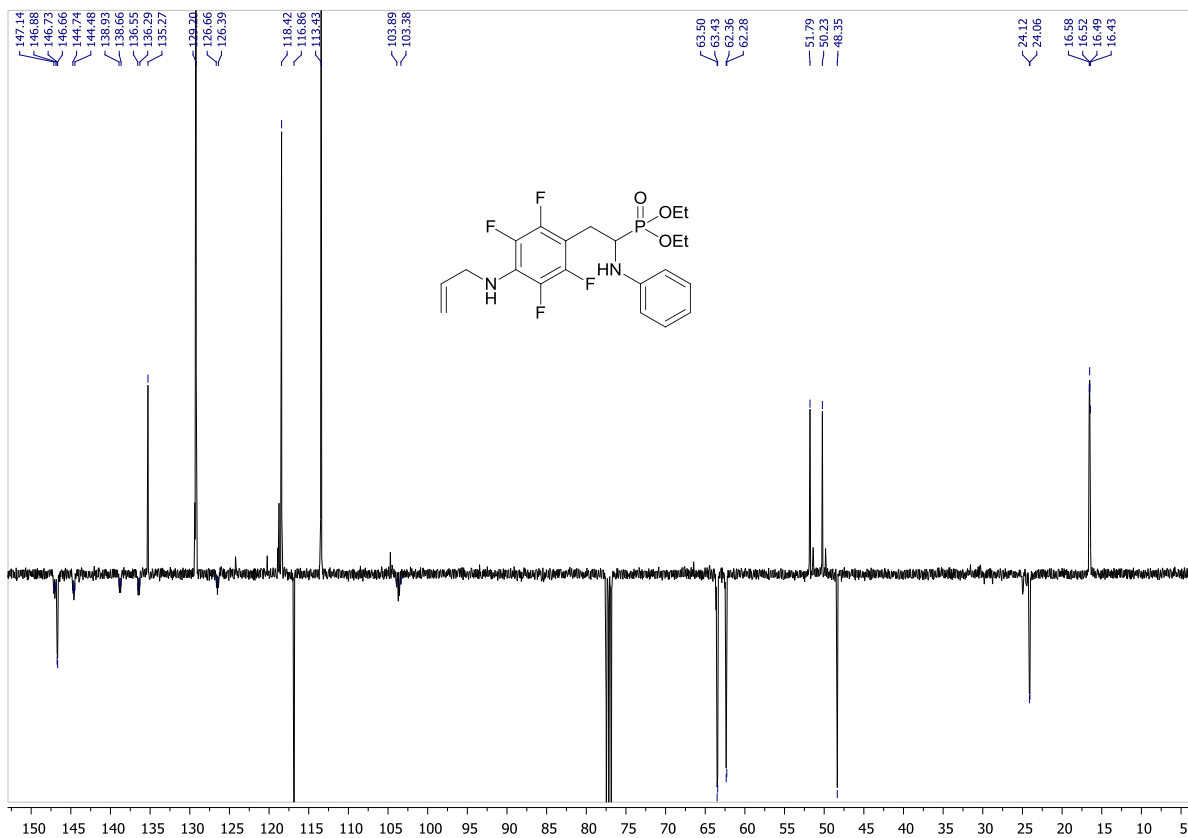


Fig. S56. ¹³C NMR spectrum of 2n (CDCl₃)

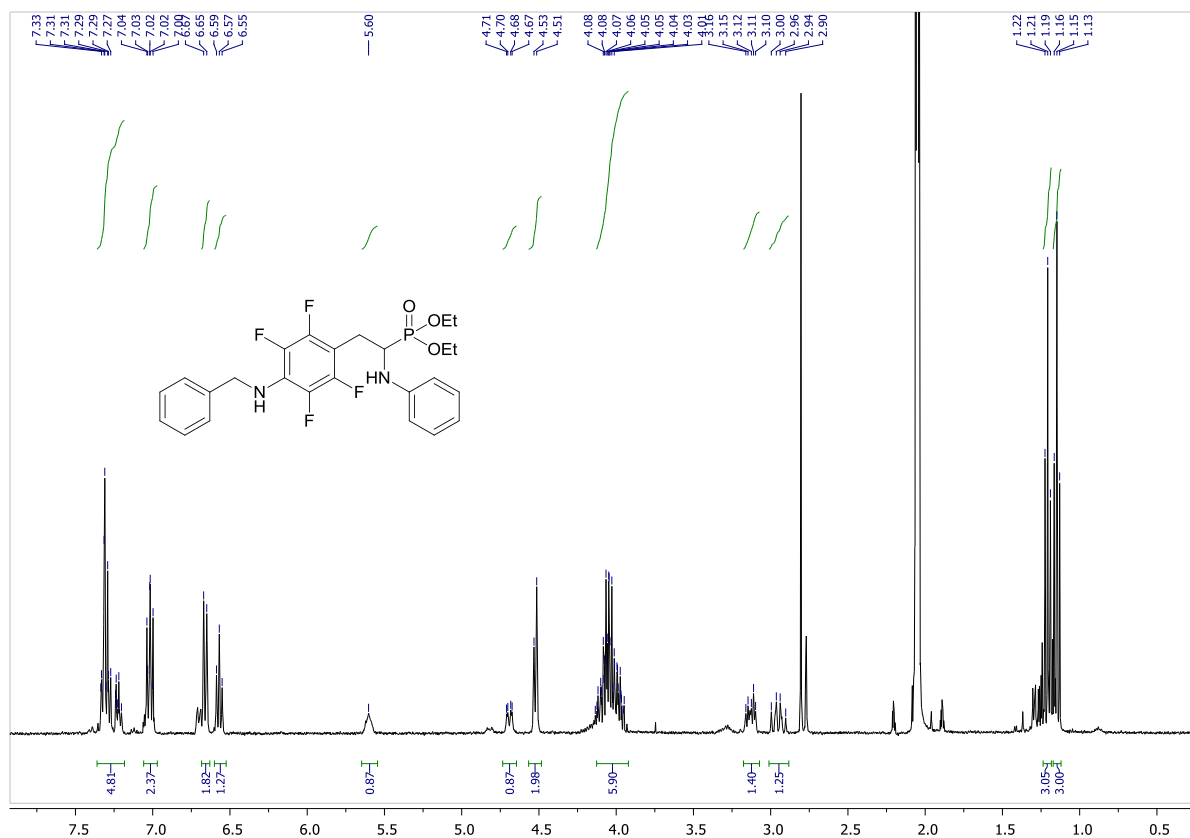


Fig. S57. ¹H NMR spectrum of 2o (Acetone-d₆)

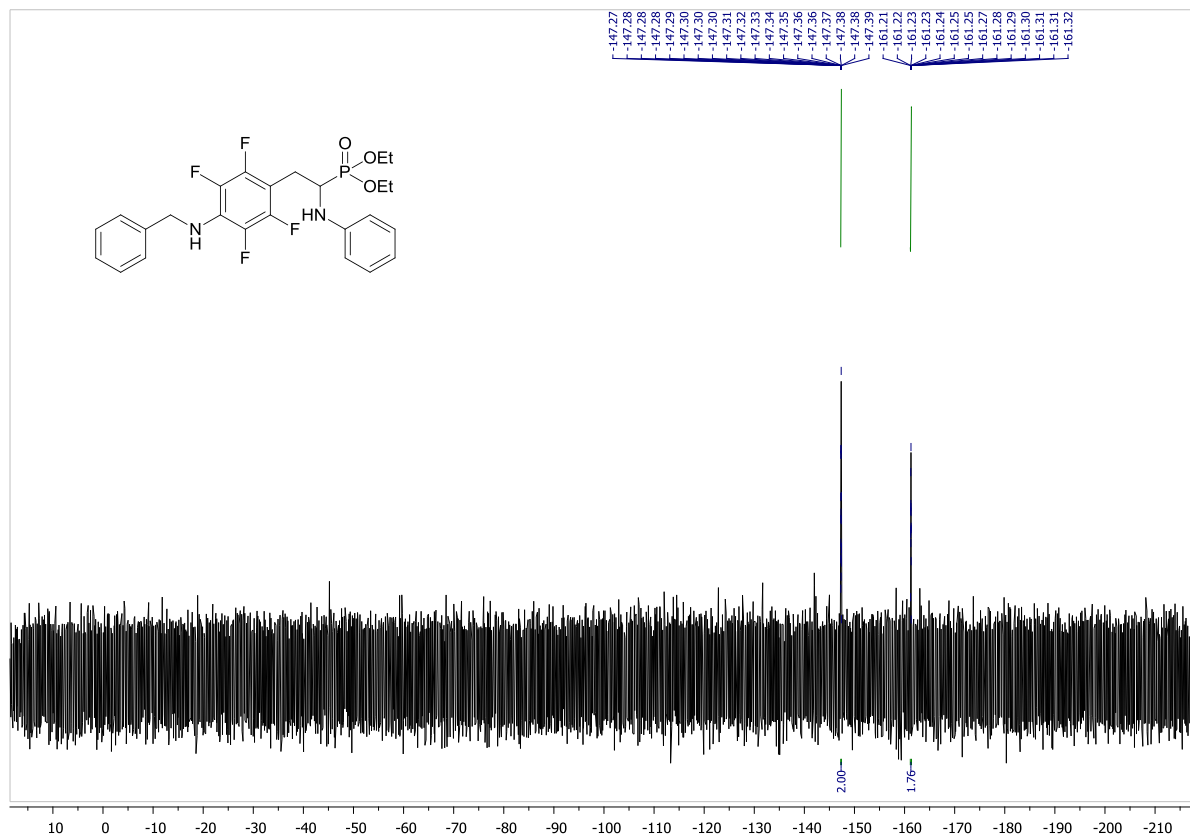


Fig. S58. ¹⁹F NMR spectrum of 2o (Acetone-d₆)

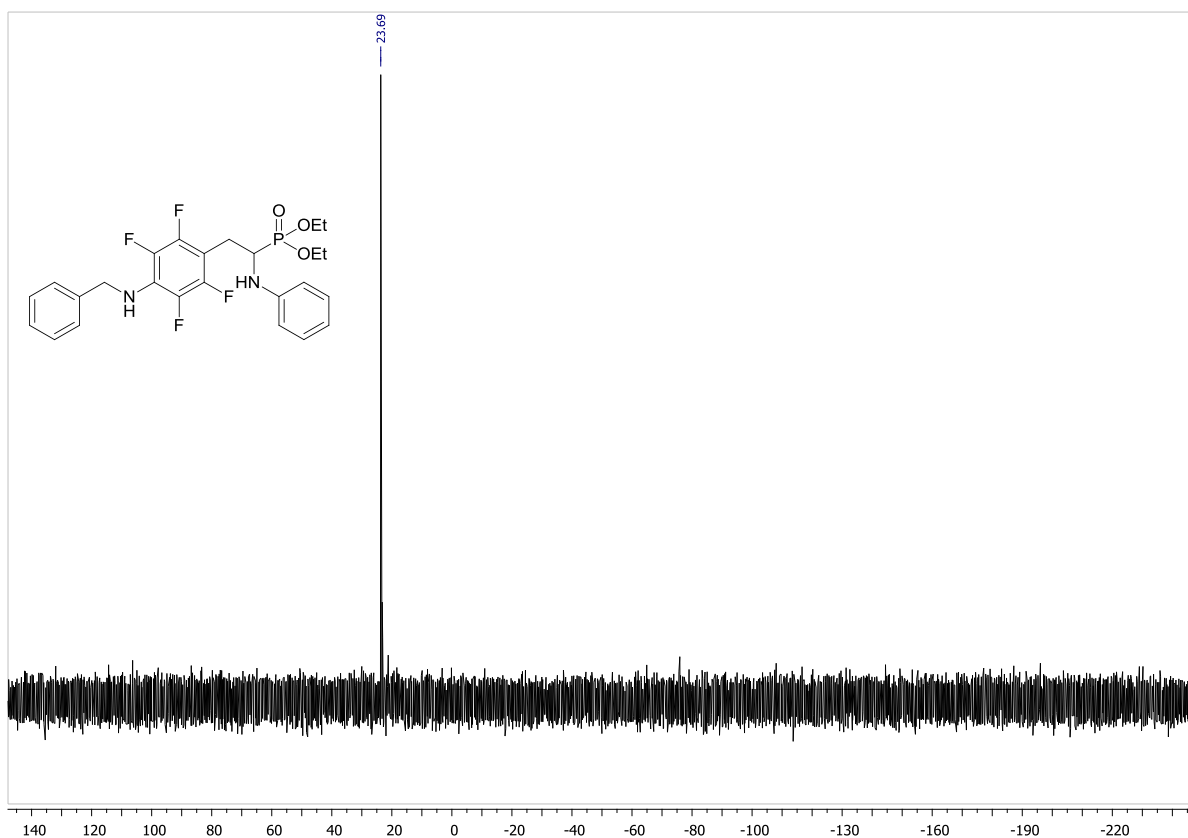


Fig. S59. ³¹P NMR spectrum of 2o (Acetone-d₆)

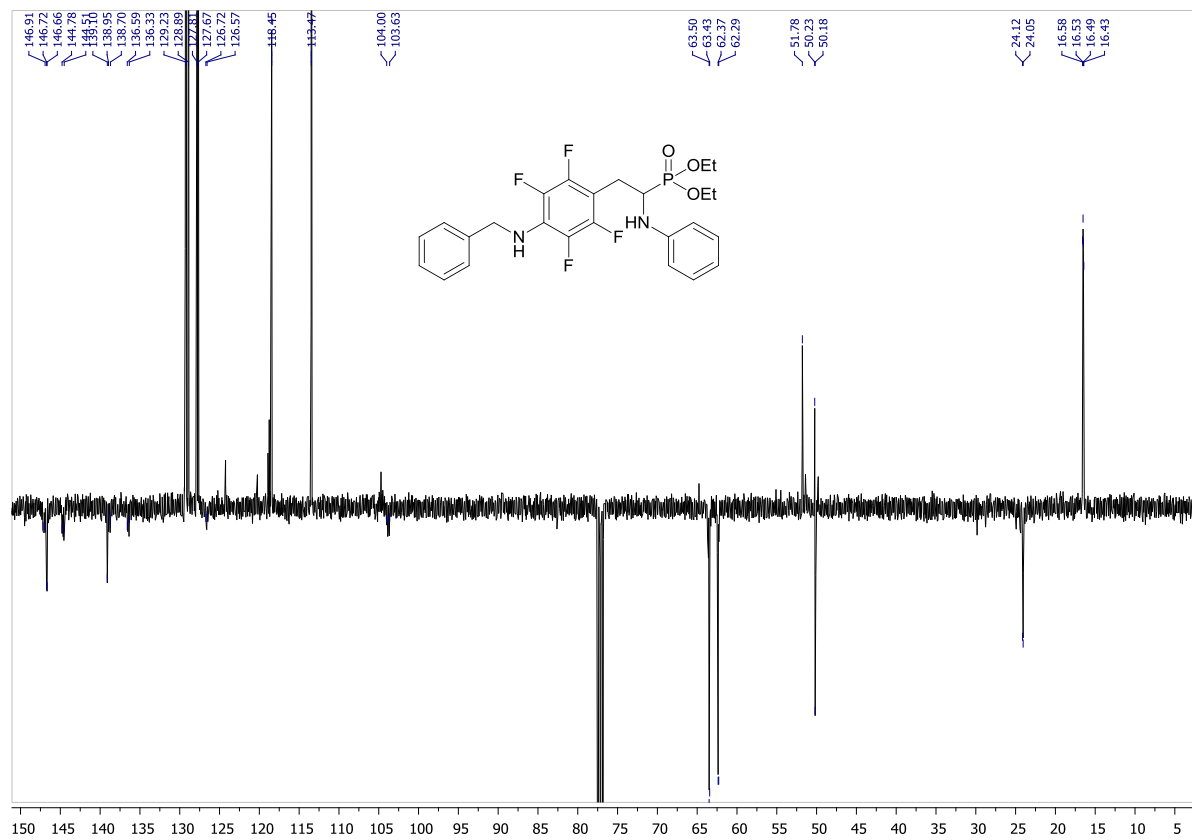


Fig. S60. ¹³C NMR spectrum of 2o (CDCl₃)

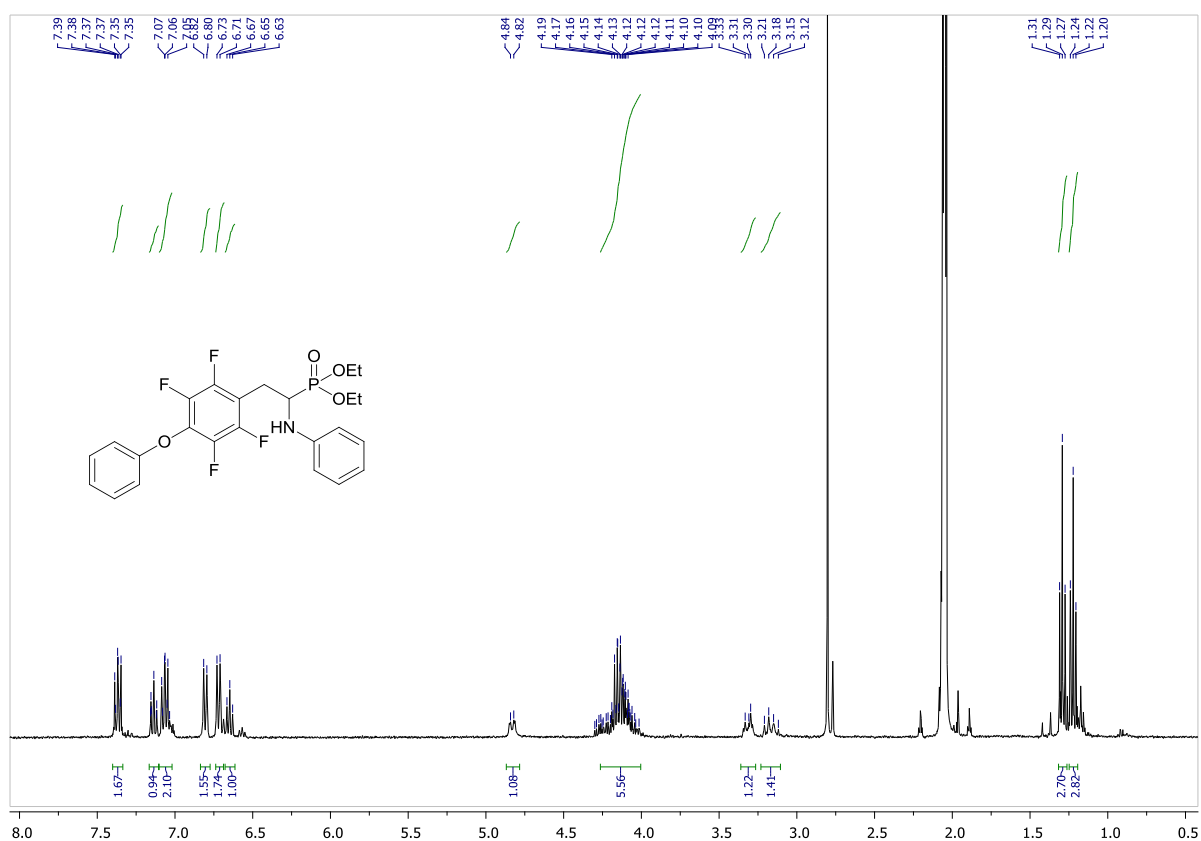


Fig. S61. ¹H NMR spectrum of 2p (Acetone-d₆)

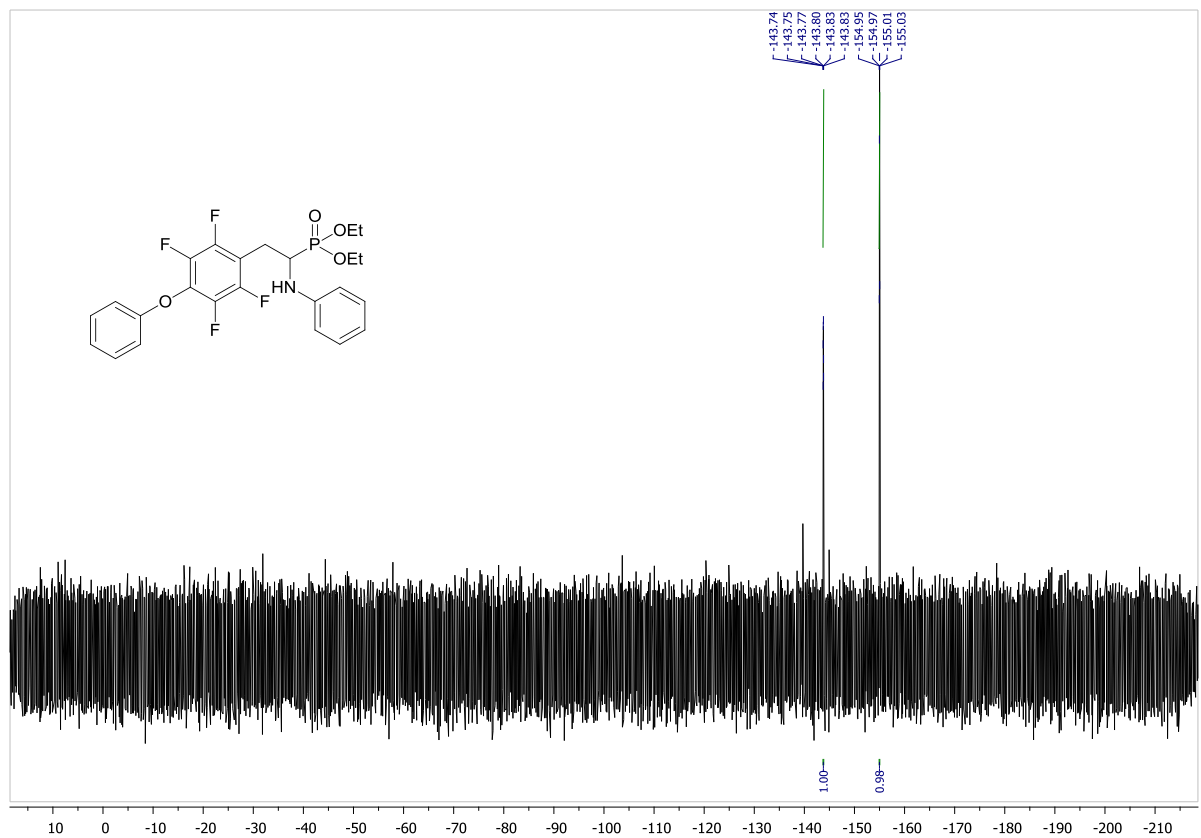


Fig. S62. ¹⁹F NMR spectrum of 2p (CDCl₃)

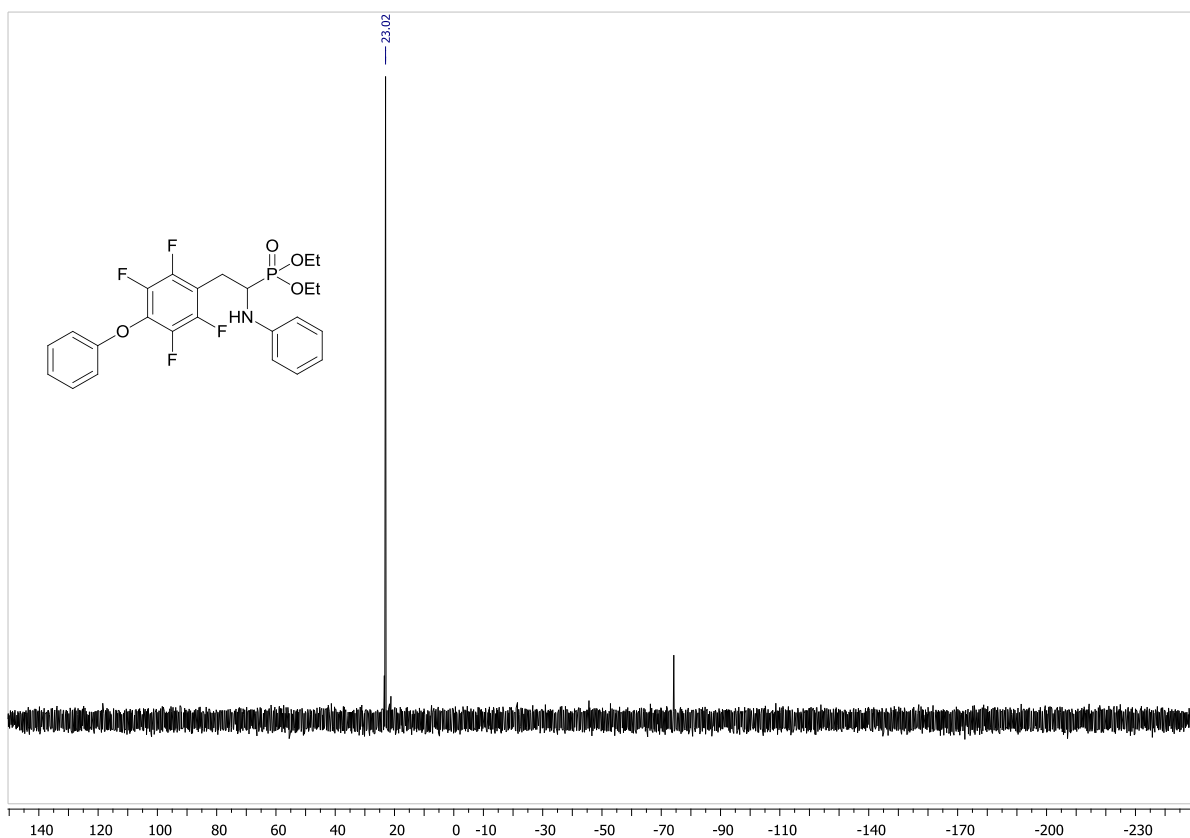


Fig. S63. ³¹P NMR spectrum of 2p (Acetone-d₆)

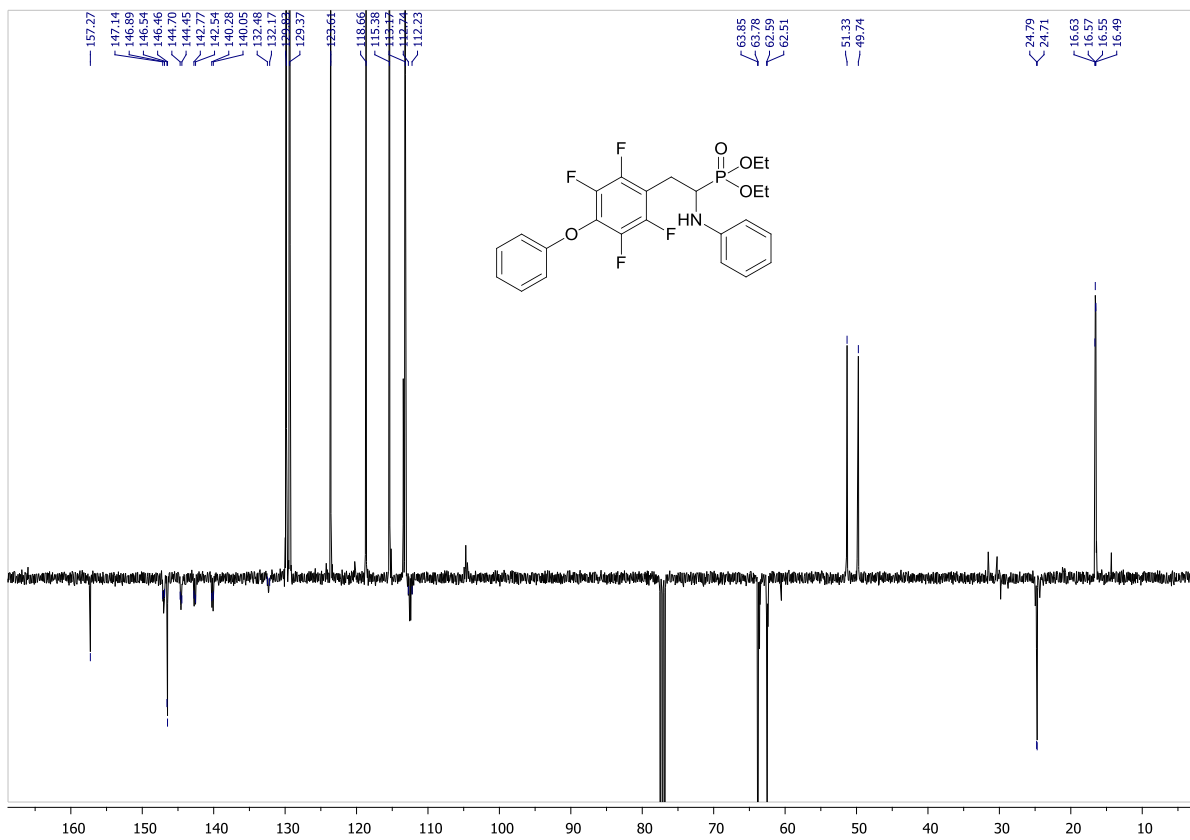


Fig. S64. ¹³C NMR spectrum of 2p (CDCl₃)

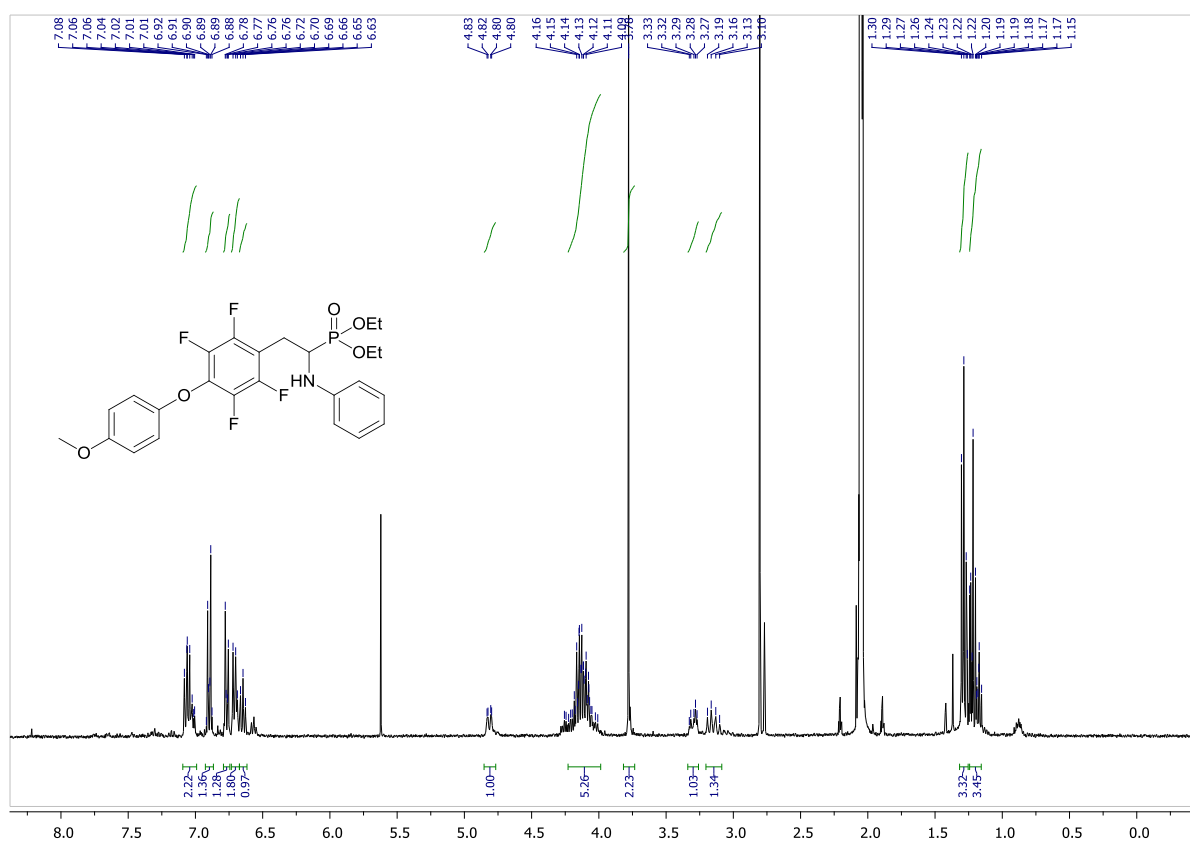


Fig. S65. ^1H NMR spectrum of 2q (Acetone- d_6)

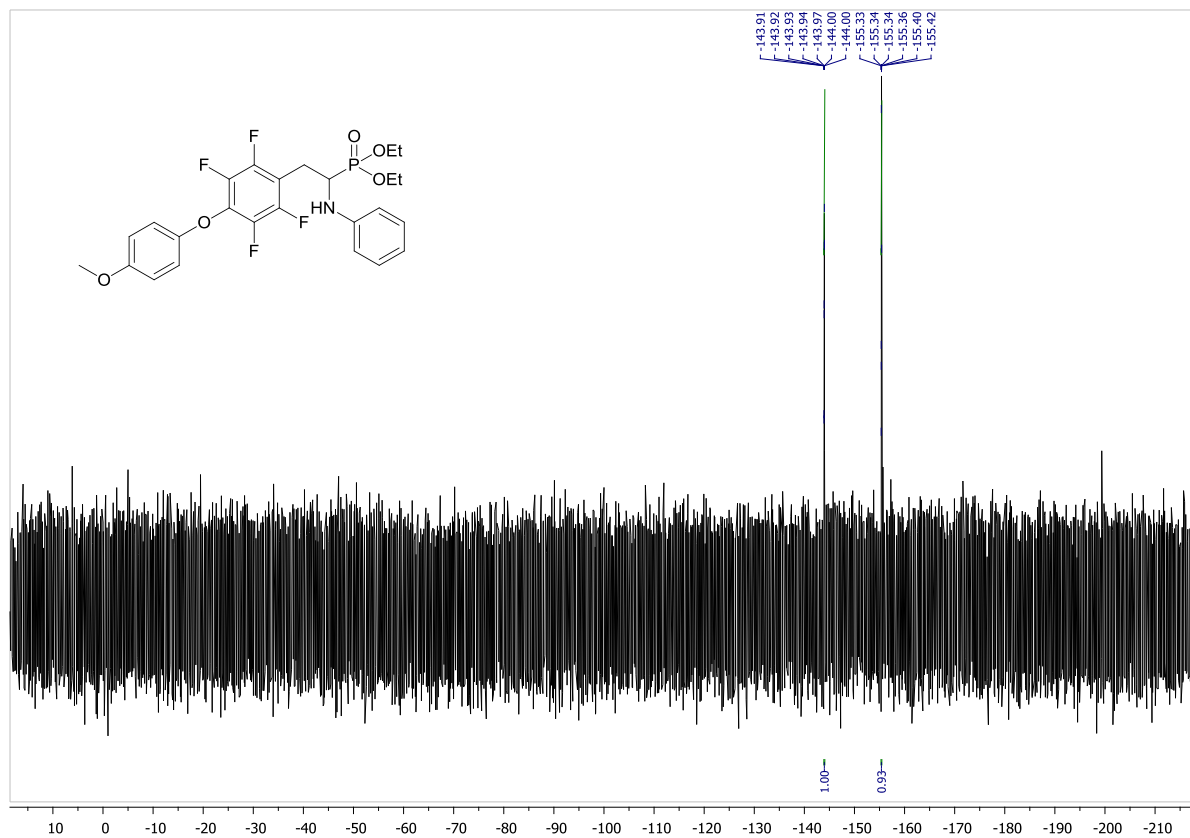


Fig. S66. ^{19}F NMR spectrum of 2q (CDCl_3)

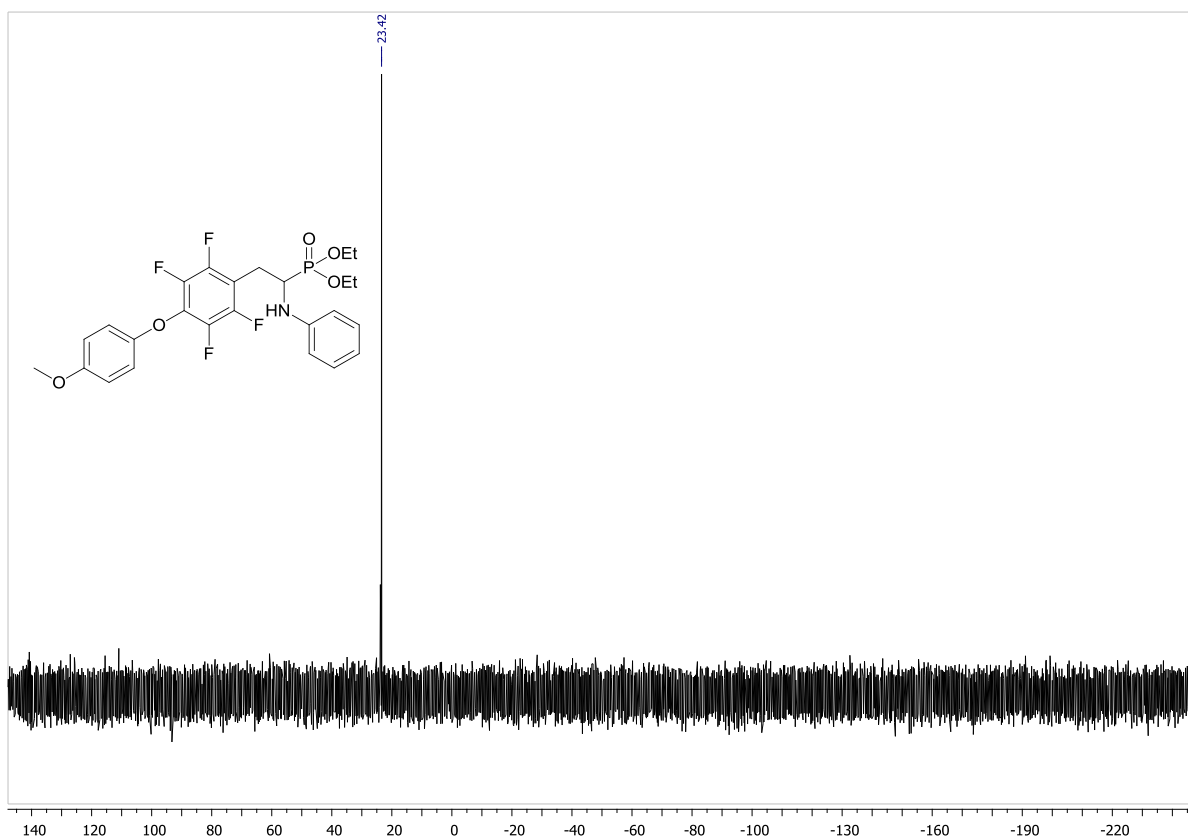


Fig. S67. ³¹P NMR spectrum of 2q (CDCl₃)

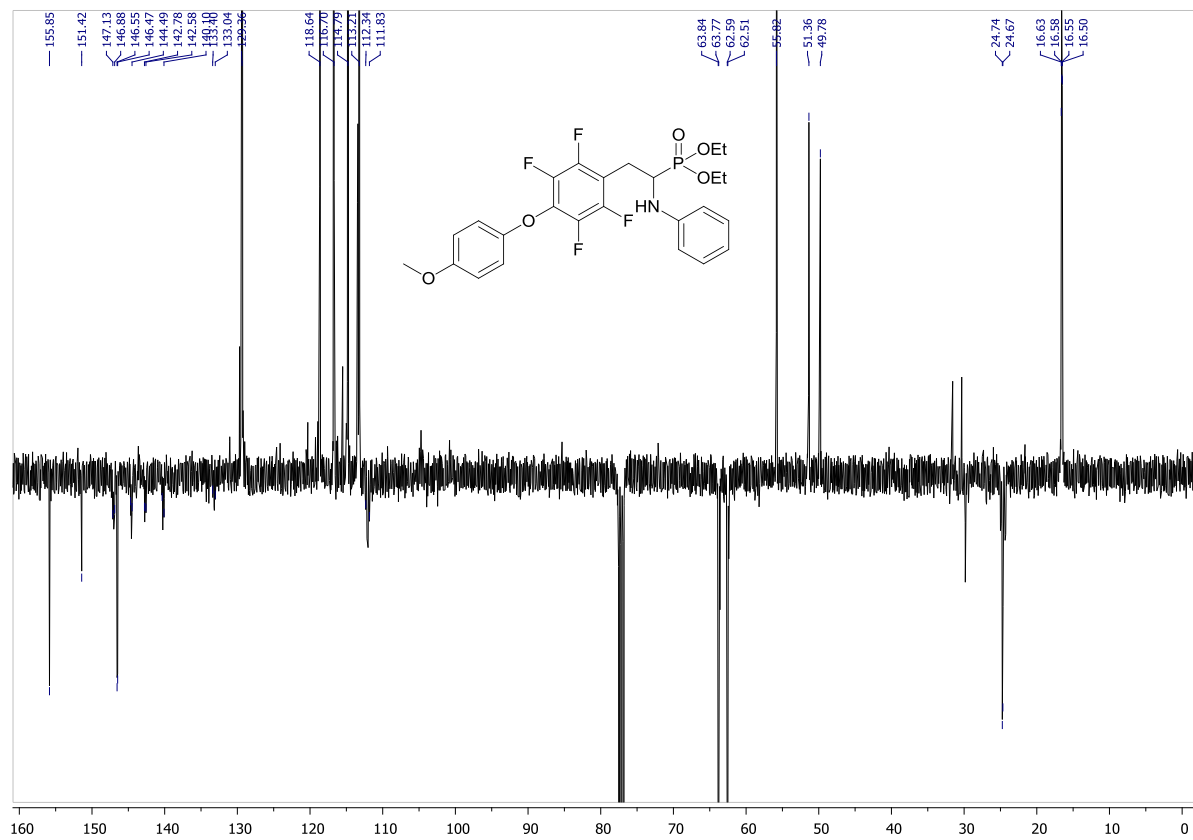


Fig. S68. ¹³C NMR spectrum of 2q (CDCl₃)

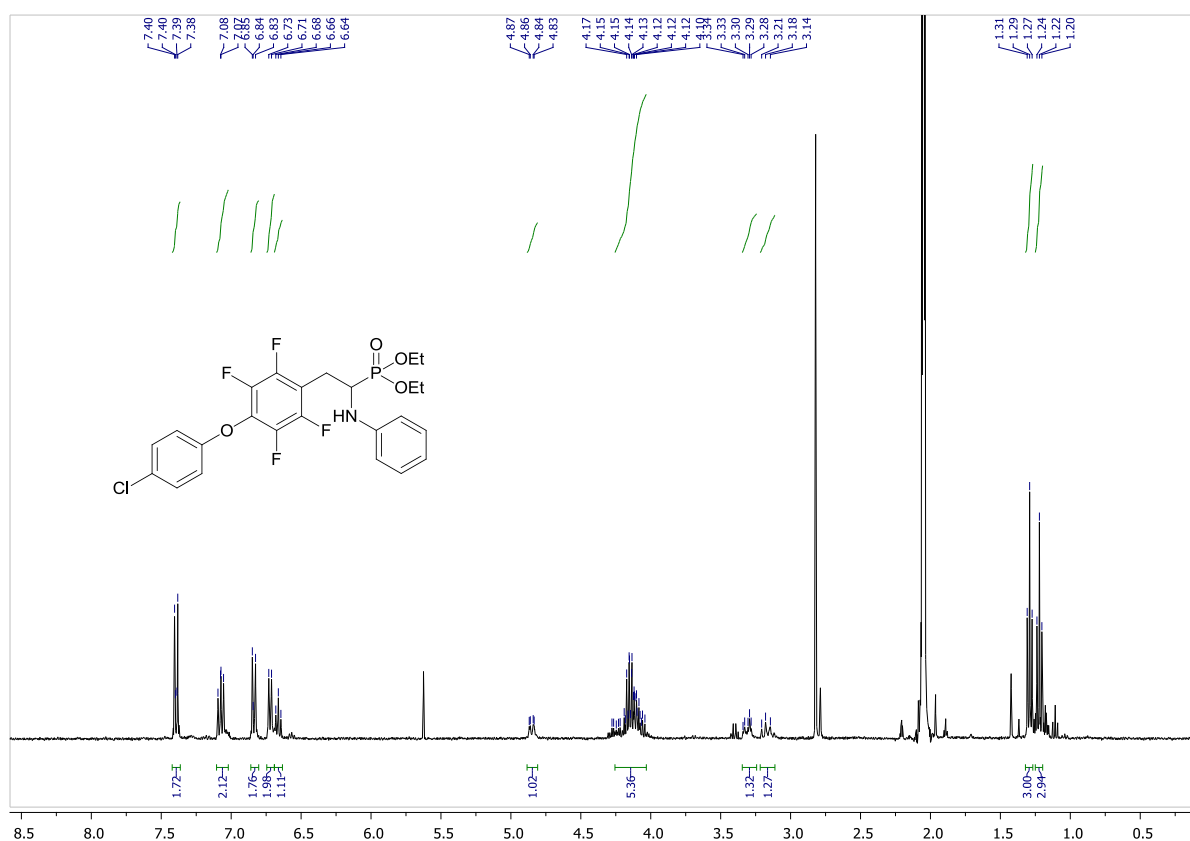


Fig. S69. ^1H NMR spectrum of 2r (Acetone- d_6)

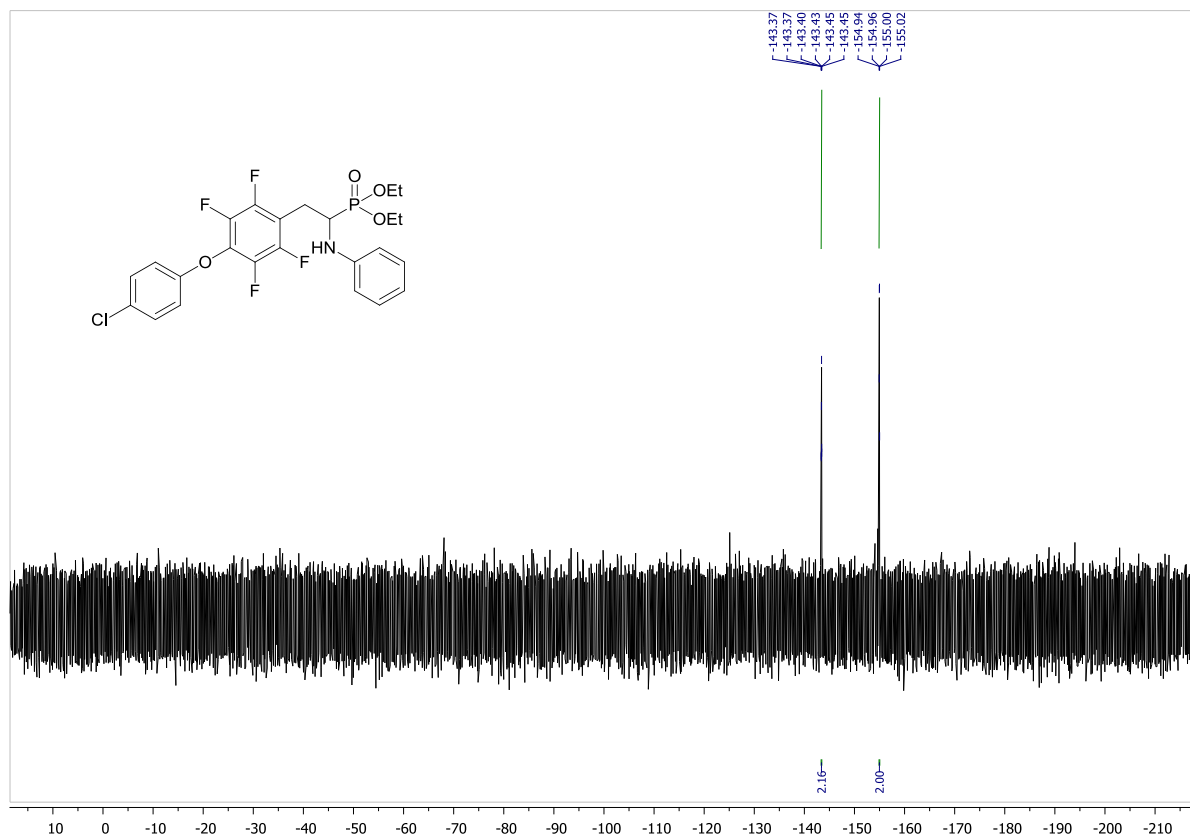


Fig. S70. ^{19}F NMR spectrum of 2r (CDCl_3)

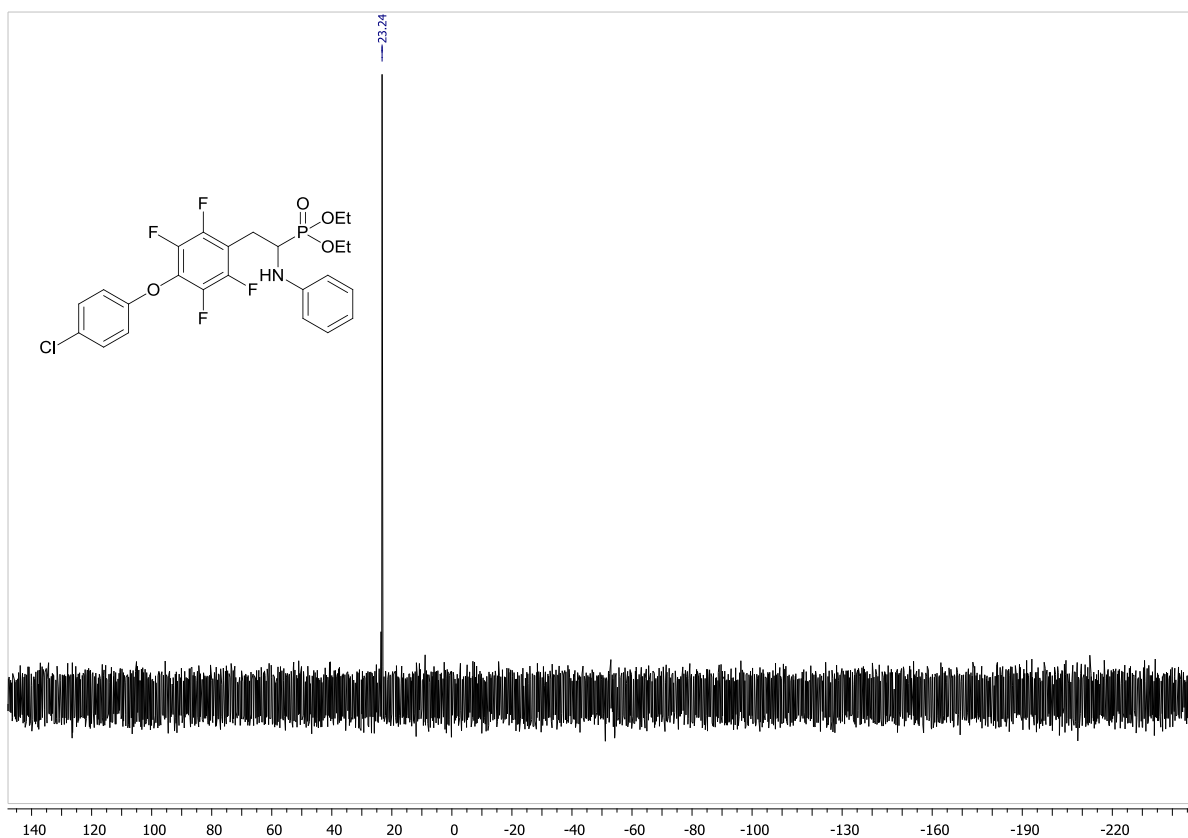


Fig. S71. ^{31}P NMR spectrum of 2r (CDCl_3)

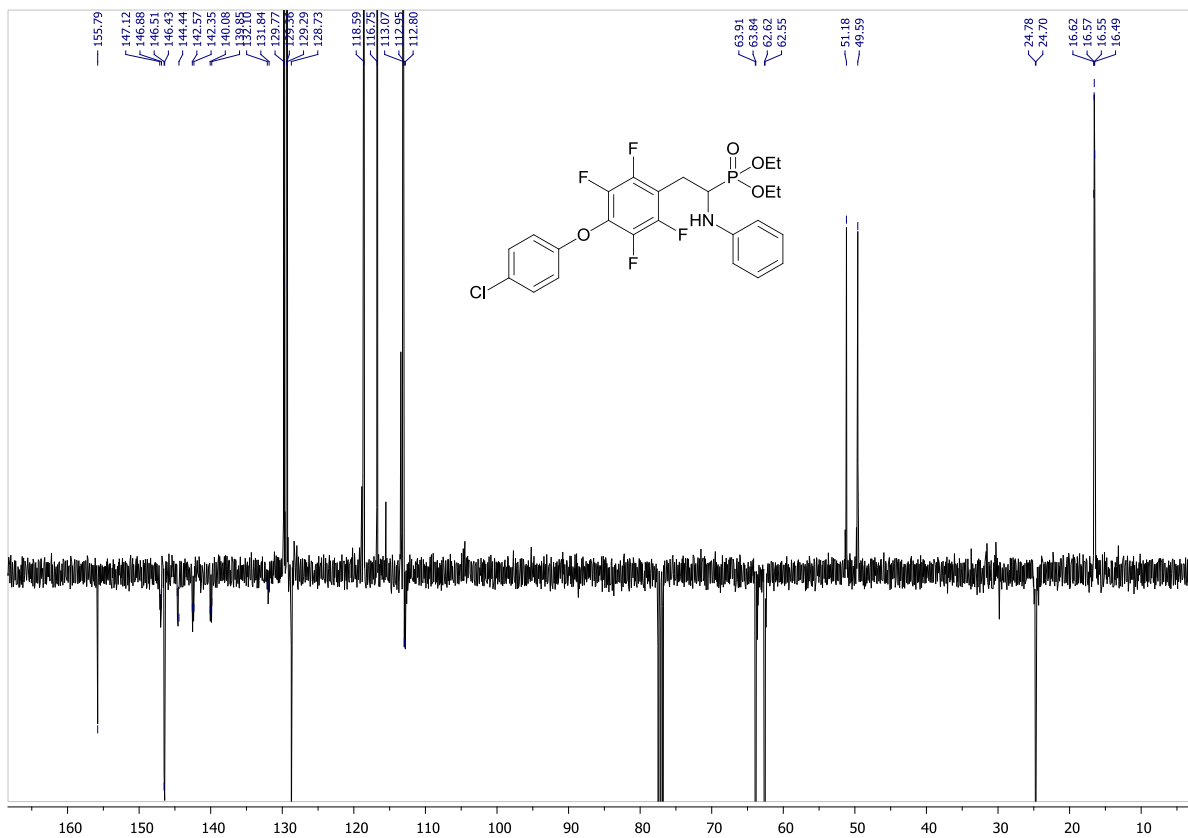


Fig. S72. ^{13}C NMR spectrum of 2r (CDCl_3)

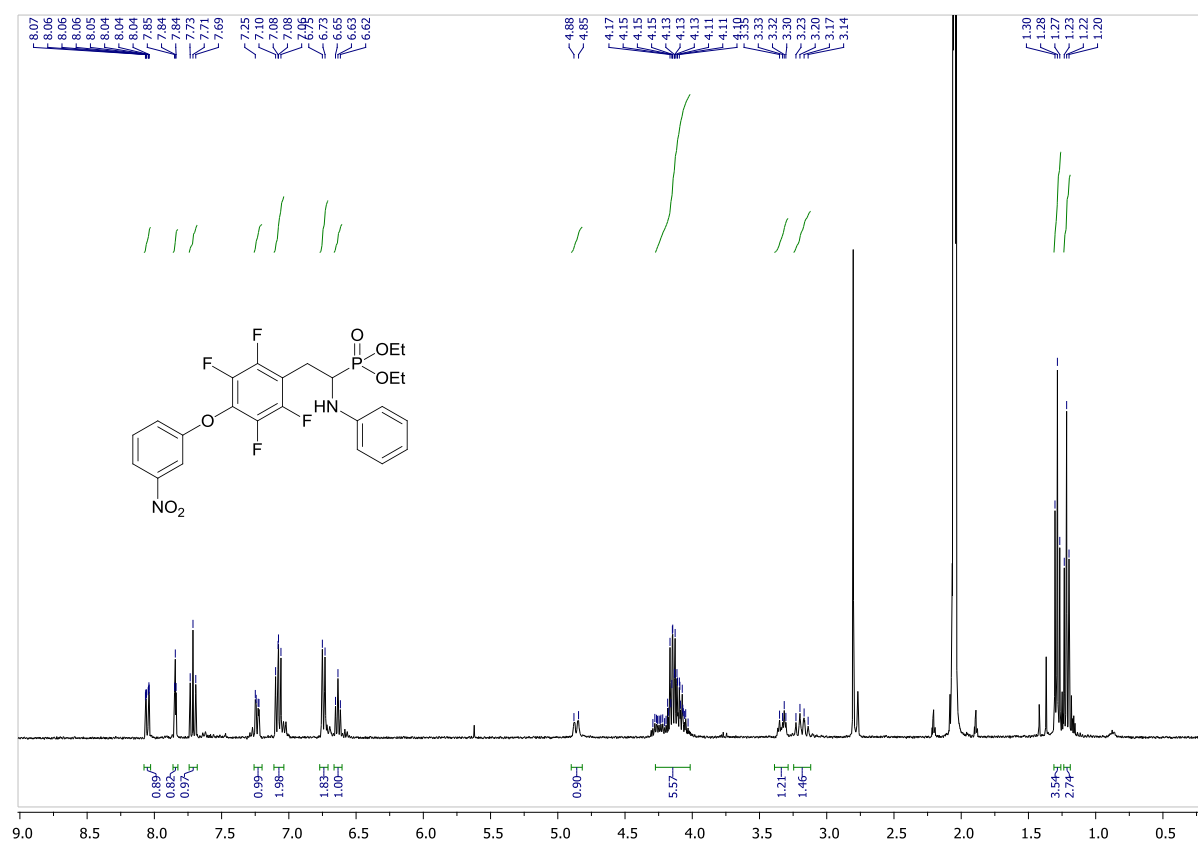


Fig. S73. ¹H NMR spectrum of 2s (Acetone-d₆)

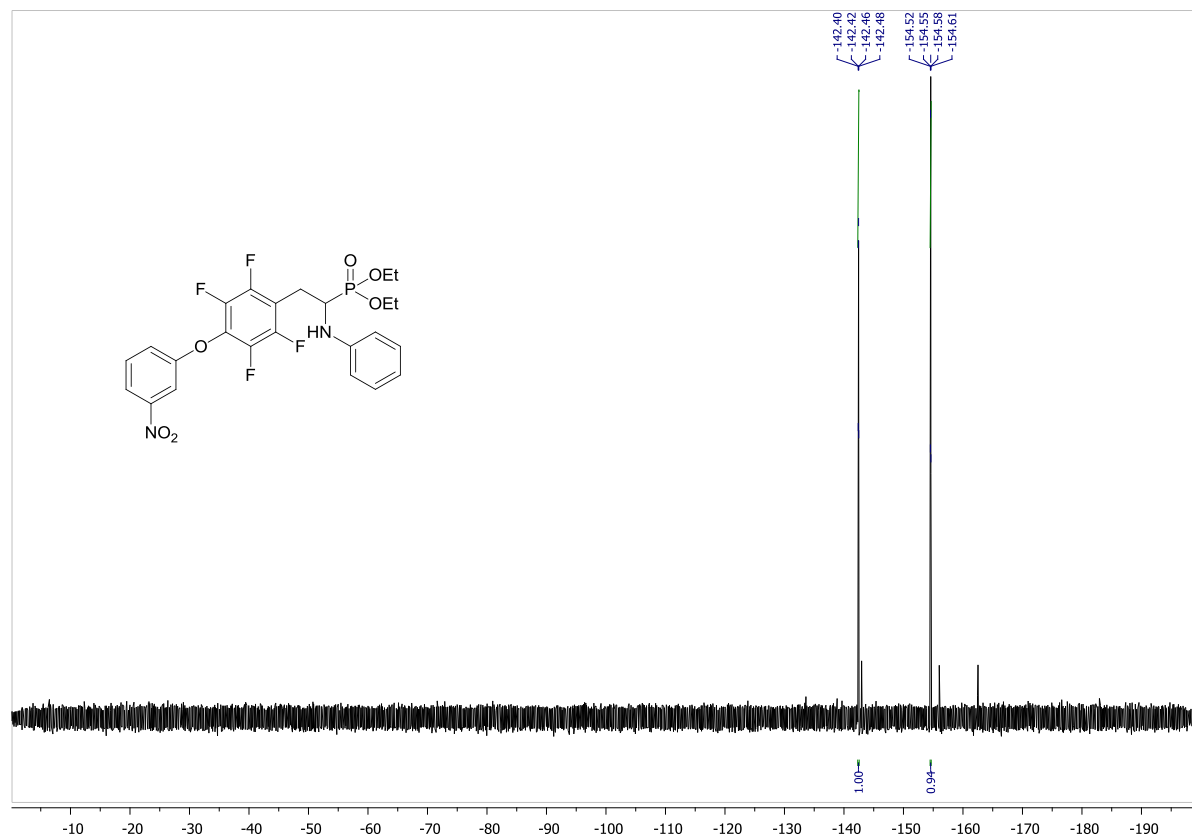


Fig. S74. ¹⁹F NMR spectrum of 2s (CDCl₃)

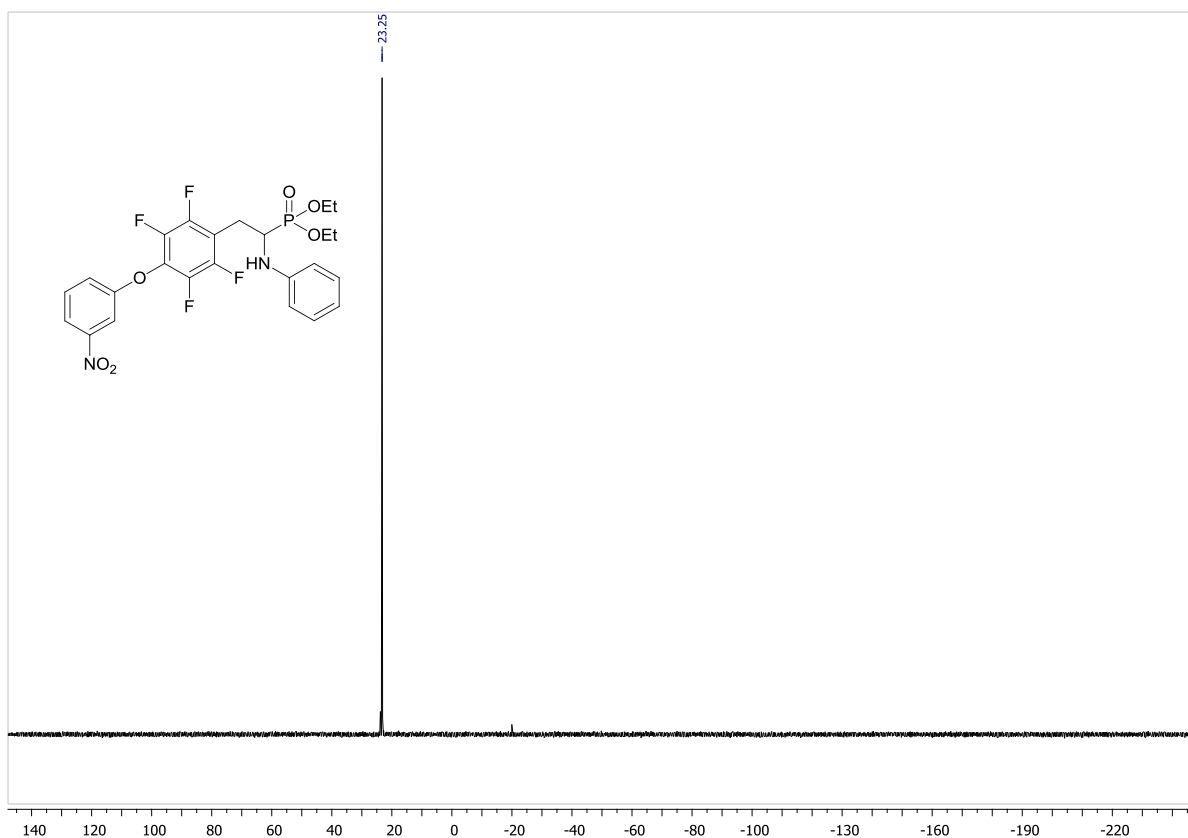


Fig. S75. ³¹P NMR spectrum of 2s (CDCl₃)

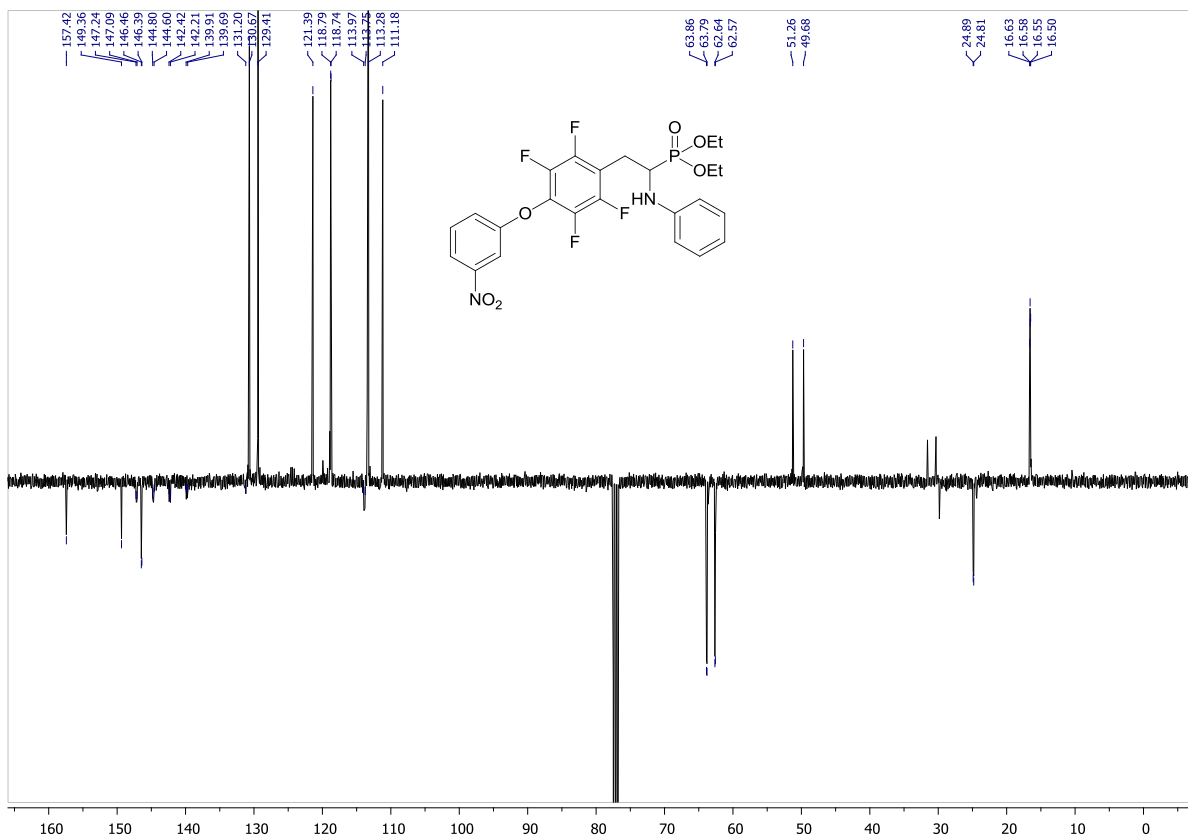


Fig. S76. ¹³C NMR spectrum of 2s (CDCl₃)

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
514.1230	514.1229	0.1	0.2	11.5	1195.0	0.000	100.00	C24 H25 N O3 S P F4
	514.1234	-0.4	-0.8	4.5	1208.9	13.921	0.00	C9 H21 N13 O4 S P F4
	514.1221	0.9	1.8	-0.5	1210.0	15.058	0.00	C8 H25 N9 O8 S P F4

Fig. S77. HRMS of 2a

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
528.1387	528.1385	0.2	0.4	11.5	806.4	0.000	100.00	C25 H27 N O3 S P F4
	528.1390	-0.3	-0.6	4.5	817.7	11.299	0.00	C10 H23 N13 O4 S P F4
	528.1377	1.0	1.9	-0.5	818.7	12.316	0.00	C9 H27 N9 O8 S P F4

Fig. S78. HRMS of 2b

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
542.1542	542.1542	0.0	0.0	11.5	870.5	0.000	100.00	C26 H29 N O3 S P F4
	542.1547	-0.5	-0.9	4.5	883.7	13.124	0.00	C11 H25 N13 O4 S P F4
	542.1534	0.8	1.5	-0.5	885.1	14.526	0.00	C10 H29 N9 O8 S P F4

Fig. S79. HRMS of 2c

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
544.1339	544.1335	0.4	0.7	11.5	1238.4	0.021	97.94	C25 H27 N O4 S P F4
	544.1348	-0.9	-1.7	16.5	1242.3	3.883	2.06	C26 H23 N5 S P F4
	544.1340	-0.1	-0.2	4.5	1249.4	11.046	0.00	C10 H23 N13 O5 S P F4

Fig. S80. HRMS of 2d

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
544.1328	544.1335	-0.7	-1.3	11.5	1030.0	0.000	100.00	C25 H27 N O4 S P F4
	544.1326	0.2	0.4	-0.5	1039.9	9.924	0.00	C9 H27 N9 O9 S P F4

Fig. S81. HRMS of 2e

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
529.1335	529.1338	-0.3	-0.6	11.5	1186.3	0.000	100.00	C24 H26 N2 O3 S P F4
	529.1343	-0.8	-1.5	4.5	1199.4	13.093	0.00	C9 H22 N14 O4 S P F4
	529.1330	0.5	0.9	-0.5	1200.3	14.056	0.00	C8 H26 N10 O8 S P F4

Fig. S82. HRMS of 2f

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
592.0336	592.0334	0.2	0.3	11.5	1018.3	0.000	99.99	C24 H24 N O3 S Br P F4
	592.0339	-0.3	-0.5	4.5	1028.1	9.765	0.01	C9 H20 N13 O4 S Br P F4

Fig. S83. HRMS of 2g

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
528.1387	528.1385	0.2	0.4	11.5	1242.2	0.000	100.00	C25 H27 N O3 S P F4
	528.1390	-0.3	-0.6	4.5	1253.8	11.556	0.00	C10 H23 N13 O4 S P F4
	528.1377	1.0	1.9	-0.5	1255.0	12.819	0.00	C9 H27 N9 O8 S P F4

Fig. S84. HRMS of 2h

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
578.2477	578.2481	-0.4	-0.7	7.5	892.0	0.000	100.00	C28 H41 N O3 S P F4
	578.2486	-0.9	-1.6	0.5	905.7	13.678	0.00	C13 H37 N13 O4 S P F4

Fig. S85. HRMS of 2i

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
581.1503	581.1498	0.5	0.9	9.5	1256.7	0.053	94.80	C24 H30 N2 O6 S P F4
	581.1512	-0.9	-1.5	14.5	1259.6	2.956	5.20	C25 H26 N6 O2 S P F4
	581.1503	0.0	0.0	2.5	1267.9	11.301	0.00	C9 H26 N14 O7 S P F4

Fig. S86. HRMS of 2j

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
463.1773	463.1774	-0.1	-0.2	7.5	1496.4	n/a	n/a	C21 H28 N2 O3 F4 P

Fig. S87. HRMS of 2l

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
435.1465	435.1461	0.4	0.9	7.5	1402.8	0.000	100.00	C19 H24 N2 O3 F4 P
	435.1466	-0.1	-0.2	0.5	1422.0	19.215	0.00	C4 H20 N14 O4 F4 P

Fig. S88. HRMS of 2k

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
477.1932	477.1930	0.2	0.4	7.5	1626.9	0.000	100.00	C22 H30 N2 O3 F4 P
	477.1935	-0.3	-0.6	0.5	1640.7	13.792	0.00	C7 H26 N14 O4 F4 P

Fig. S89. HRMS of 2m

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
461.1616	461.1617	-0.1	-0.2	8.5	1595.0	n/a	n/a	C21 H26 N2 O3 F4 P

Fig. S90. HRMS of 2n

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
511.1774	511.1774	0.0	0.0	11.5	1541.3	0.000	100.00	C25 H28 N2 O3 F4 P
	511.1779	-0.5	-1.0	4.5	1552.5	11.176	0.00	C10 H24 N14 O4 F4 P

Fig. S91. HRMS of 2o

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
498.1459	498.1457	0.2	0.4	11.5	1594.7	0.000	100.00	C24 H25 N O4 F4 P
	498.1462	-0.3	-0.6	4.5	1606.2	11.464	0.00	C9 H21 N13 O5 F4 P

Fig. S92. HRMS of 2p

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
528.1567	528.1563	0.4	0.8	11.5	1428.7	0.000	100.00	C25 H27 N O5 F4 P
	528.1568	-0.1	-0.2	4.5	1442.4	13.692	0.00	C10 H23 N13 O6 F4 P

Fig. S93. HRMS of 2q

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
532.1066	532.1068	-0.2	-0.4	11.5	1483.7	n/a	n/a	C24 H24 N O4 F4 P Cl

Fig. S94. HRMS of 2r

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
543.1312	543.1308	0.4	0.7	12.5	1381.2	0.000	100.00	C24 H24 N2 O6 F4 P
	543.1313	-0.1	-0.2	5.5	1395.1	13.933	0.00	C9 H20 N14 O7 F4 P

Fig. S95. HRMS of 2s

OŚWIADCZENIA WSPÓŁAUTORÓW

Poznań, 16 września 2019 r.

Dr hab. Katarzyna Rolle, prof. IChB PAN
Instytut Chemii Bioorganicznej PAN
ul. Noskowskiego 12/14
61-704 Poznań

OŚWIADCZENIA

Dotyczy rozprawy doktorskiej mgr Dariusza Wawrzyniaka

Mgr Dariusz Wawrzyniak wykonywał pracę doktorską w Instytucie Chemii Bioorganicznej PAN. Poniżej przedstawiam zakres prac wykonanych przez mgr Dariusza Wawrzyniaka, oraz mój udział w poszczególnych publikacjach:

- Rolle K*, Piwecka M*, Belter A*, Wawrzyniak D, Jeleniewicz J, Barciszewska MZ, Barciszewski J. The sequence and structure determine the function of mature human miRNAs. *PLoS One*, 2016, 11(3):e0151246

*Autorzy mieli taki sam wkład w przygotowanie publikacji

Mgr Dariusz Wawrzyniak przeprowadził eksperymenty związane z analizą sekwencji nukleotydowych oraz tych zawierających pojedyncze powtórzenia (SSR), jak również przygotował odpowiadające im ryciny i tabele.

Mój udział w niniejszej publikacji polegał na: przygotowaniu koncepcji pracy, analizie i interpretacji wyników, tworzeniu manuskryptu, wykonaniu większości rycin i tabel oraz odpowiedzi na uwagi recenzentów. Wykonane eksperymenty: badanie zależności pomiędzy sekwencją poszczególnych miRNA, a ich rolą w procesach biologicznych i szlakach komórkowych (analiza z wykorzystaniem programu PANTHER System oraz DIANA MirPath). Mój udział polegał również na pozyskaniu środków finansowych na realizację projektu w ramach grantu „Poszukiwanie i analiza niekodujących RNA specyficznych dla złośliwych guzów mózgu” (5955/B/PO1/2010/38).

- Grabowska M, Grześkowiak BF, Szutkowski K, Wawrzyniak D, Głodowicz P, Barciszewski J, Jurga S, Rolle K, Mrówczyński R. Nano-Mediated Delivery of Double-Stranded RNA for Gene Therapy of Glioblastoma Multiforme. *PLoS One*, 2019, 14(3):e0213852

Rola mgr Dariusza Wawrzyniaka w przygotowaniu tej publikacji polegała na przeprowadzeniu eksperymentów biologicznych obejmujących właściwości cytotoksyczne nanocząstek oraz kompleksów

nanocząstek z dsRNA. Przeprowadził on również analizę statystyczną oraz brał udział w przygotowaniu manuskryptu, włączając w to przygotowanie rycin i materiałów suplementarnych.

Mój udział w tej publikacji polegał na zaplanowaniu badań funkcjonalnych oraz koordynacji i nadzorowaniu eksperymentów i analiz. Moja główna rola polegała na przygotowaniu tekstu manuskryptu.

- Wawrzyniak D, Rolle K, Barciszewski J. Aktywność biologiczna N6-furfuryloadenozyny. *Postępy Biochemii*, 2019, 65(2):109-117

Rola mgr Dariusza Wawrzyniaka w przygotowaniu niniejszego artykułu polegała na zebraniu danych literaturowych dotyczących aktywności biologicznej cytokinin w kontekście leczenia chorób nowotworowych oraz napisaniu manuskryptu.

Mój udział w przygotowaniu niniejszej pracy przeglądowej polegał na koordynacji i nadzorowaniu pracy nad manuskryptem. Brałam również udział w tworzeniu ilustracji.

Proszę o kontakt w przypadku jakichkolwiek pytań odnośnie przedstawionych powyżej oświadczeń.



Poznań, 18 września 2019 r.

Prof. dr hab. Jan Barciszewski
Instytut Chemii Bioorganicznej PAN
ul. Noskowskiego 12/14
61-704 Poznań

OŚWIADCZENIA

Dotyczy rozprawy doktorskiej mgr Dariusza Wawrzyniaka

Poniżej przedstawiam mój udział w poszczególnych publikacjach:

- Szymańska-Michalak A*, Wawrzyniak D*, Framski G, Kujda M, Zgoła P, Stawiński J, Barciszewski J, Boryski J, Kraszewski A. New 3'-O-aromatic acyl-5-fluoro-2'-deoxyuridine derivatives as potential anticancer agents. *European Journal of Medicinal Chemistry*, 2016, 115:41-52
*Autorzy mieli taki sam wkład w przygotowanie publikacji
- Szymańska-Michalak A*, Wawrzyniak D*, Framski G, Stawiński J, Barciszewski J, Kraszewski A. New antiglioma zwitterionic pronucleotides with a FdUMP framework. *European Journal of Medicinal Chemistry*, 2018, 144:682-691
*Autorzy mieli taki sam wkład w przygotowanie publikacji
- Framski G, Wawrzyniak D, Jahnz-Wechmann Z, Szymańska-Michalak A, Kraszewski A, Barciszewski J, Boryski J, Stawiński J. Searching for anti-glioma activity. Ribonucleoside analogues with modifications in nucleobase and sugar moieties. *Acta Biochimica Polonica*, 2016, 63(4):765-771
- Michalska L, Wawrzyniak D, Szymańska-Michalak A, Barciszewski J, Boryski J, Baraniak D. Synthesis and biological assay of new 2'-deoxyuridine dimers containing a 1,2,3-triazole linker. Part I. *Nucleosides, Nucleotides and Nucleic Acids*, 2019, 3:218-235
- Kwiczak-Yigitbaşı J, Pirat J-C, Virieux D, Volle J-N, Janiak A, Hoffmann M, Mrzygłód J, Wawrzyniak D, Barciszewski J, Pluskota-Karwatka D. Synthesis, structural studies and biological properties of some phosphonoperfluoro-phenylalanine derivatives formed by SNAr reactions. *RSC Advances*, 2019, 9:24117-24133

Moja rola polegała na nadzorowaniu i koordynacji eksperymentów biologicznych oraz udziale w przygotowaniu manuskryptu.

- Rolle K*, Piwecka M*, Belter A*, Wawrzyniak D, Jeleniewicz J, Barciszewska MZ, Barciszewski J. The sequence and structure determine the function of mature human miRNAs. *PLoS One*, 2016, 11(3):e0151246

*Autorzy mieli taki sam wkład w przygotowanie publikacji

- Grabowska M, Grześkowiak BF, Szutkowski K, Wawrzyniak D, Głodowicz P, Barciszewski J, Jurga S, Rolle K, Mrówczyński R. Nano-Mediated Delivery of Double-Stranded RNA for Gene Therapy of Glioblastoma Multiforme. *PLoS One*, 2019, 14(3):e0213852

Mój wkład w powstanie tych prac polegał na dyskusji wyników oraz współtworzeniu manuskryptów.

- Wawrzyniak D, Rolle K, Barciszewski J. Aktywność biologiczna N⁶-furfuryloadenozyny. *Postępy Biochemii*, 2019, 65(2):109-117

Mój udział w przygotowaniu niniejszej pracy przeglądowej polegał na zaplanowaniu koncepcji pracy. Ponadto koordynowałem i nadzorowałem prace nad manuskryptem.

Proszę o kontakt w przypadku jakichkolwiek pytań odnośnie przedstawionych powyżej oświadczeń.



Poznań, 16 września 2019 r.

Prof. dr hab. Mirosława Z. Barciszewska
Instytut Chemii Bioorganicznej PAN
ul. Noskowskiego 12/14
61-704 Poznań

Oświadczenie o współautorstwie w publikacji

Rolle K*, Piwecka M*, Belter A*, Wawrzyniak D, Jeleniewicz J, Barciszewska MZ, Barciszewski J†. **The sequence and structure determine the function of mature human miRNAs.** PLoS One, 2016, 11(3)e0151246

*Autorzy mieli taki sam wkład w przygotowanie publikacji

Mój wkład w powstanie pracy

Powyższa praca jest wynikiem realizacji kierowanego przeze mnie projektu „*Rybozymy anty-miRNA jako potencjalne terapeutyki w leczeniu guzów mózgu u ludzi*”, nr umowy UDA-POIG.01.03.01-30-050/09-02 (2009-2014), projekt współfinansowany ze środków Unii Europejskiej, POIG, Działanie 1.3, Poddziałanie 1.3.1.

Oświadczam, iż mój udział w przedstawionej pracy dotyczył analizy i dyskusji wyników.



Poznań, 19 września 2019 r.

Prof. dr hab. Adam Kraszewski
Instytut Chemii Bioorganicznej PAN
ul. Noskowskiego 12/14
61-704 Poznań

OŚWIADCZENIA

Dotyczy rozprawy doktorskiej mgr Dariusza Wawrzyniaka

Poniżej przedstawiam zakres prac wykonanych przez mgr Dariusza Wawrzyniaka, oraz mój udział w poszczególnych publikacjach:

- Szymańska-Michalak A*, Wawrzyniak D*, Framski G, Kujda M, Zgoła P, Stawiński J, Barciszewski J, Boryski J, Kraszewski A. New 3'-O-aromatic acyl-5-fluoro-2'-deoxyuridine derivatives as potential anticancer agents. *European Journal of Medicinal Chemistry*, 2016, 115:41-52

*Autorzy mieli taki sam wkład w przygotowanie publikacji

- Szymańska-Michalak A*, Wawrzyniak D*, Framski G, Stawiński J, Barciszewski J, Kraszewski A. New antiglioma zwitterionic pronucleotides with a FdUMP framework. *European Journal of Medicinal Chemistry*, 2018, 144:682-691

*Autorzy mieli taki sam wkład w przygotowanie publikacji

Rola mgr Dariusza Wawrzyniaka w przygotowaniu powyższych prac polegała na zaplanowaniu koncepcji badań biologicznych, przeprowadzeniu wszystkich eksperymentów dotyczących aktywności biologicznej związków, analizie statystycznej i opracowaniu rezultatów. Wspólnie z dr Agnieszką Szymańską-Michalak przeprowadził analizę stabilności chemicznej i enzymatycznej pronukleotydów. Wyzначzył on również parametry fizykochemiczne determinujące biodostępność badanych związków.

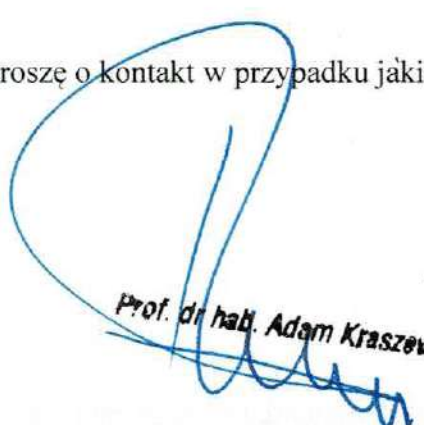
Mój udział w niniejszych publikacjach polegał na opracowaniu koncepcji badań, dobór zespołu realizującego, organizację i koordynację badań oraz przygotowanie, przy współudziale pozostałych członków zespołu w tym mgr Dariusza Wawrzyniaka, manuskryptu do publikacji.

- Framski G, Wawrzyniak D, Jahnz-Wechmann Z, Szymańska-Michalak A, Kraszewski A, Barciszewski J, Boryski J, Stawiński J. Searching for anti-glioma activity. Ribonucleoside analogues with modifications in nucleobase and sugar moieties. *Acta Biochimica Polonica*, 2016, 63(4):765-771

Mgr Dariusz Wawrzyniak przeprowadził w tej pracy badania aktywności biologicznej związków. Ponadto, doktorant wykonał analizę statystyczną oraz opracował wyniki.

Mój udział w tej publikacji polegał na uzgodnieniu koncepcji badań, dobór zespołu realizującego, organizację i koordynację badań oraz udział w przygotowaniu, manuskryptu do publikacji.

Proszę o kontakt w przypadku jakichkolwiek pytań odnośnie przedstawionych powyżej oświadczeń.



Prof. dr hab. Adam Kraszewski

Poznań, 19 września 2019 r.

Prof. dr hab. Jacek Stawiński
Instytut Chemii Bioorganicznej PAN
ul. Noskowskiego 12/14
61-704 Poznań

OŚWIADCZENIA

Dotyczy rozprawy doktorskiej mgr Dariusza Wawrzyniaka

Poniżej przedstawiam mój udział w poszczególnych publikacjach:

- Szymańska-Michalak A*, Wawrzyniak D*, Framski G, Kujda M, Zgoła P, Stawiński J, Barciszewski J, Boryski J, Kraszewski A. New 3'-O-aromatic acyl-5-fluoro-2'-deoxyuridine derivatives as potential anticancer agents. *European Journal of Medicinal Chemistry*, 2016, 115:41-52

*Autorzy mieli taki sam wkład w przygotowanie publikacji

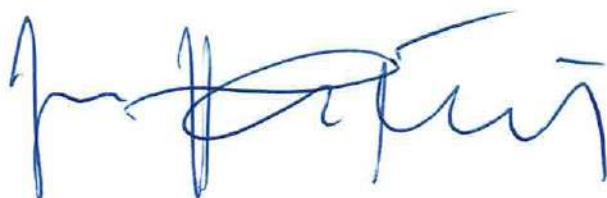
- Szymańska-Michalak A*, Wawrzyniak D*, Framski G, Stawiński J, Barciszewski J, Kraszewski A. New antiglioma zwitterionic pronucleotides with a FdUMP framework. *European Journal of Medicinal Chemistry*, 2018, 144:682-691

*Autorzy mieli taki sam wkład w przygotowanie publikacji

- Framski G, Wawrzyniak D, Jahnz-Wechmann Z, Szymańska-Michalak A, Kraszewski A, Barciszewski J, Boryski J, Stawiński J. Searching for anti-glioma activity. Ribonucleoside analogues with modifications in nucleobase and sugar moieties. *Acta Biochimica Polonica*, 2016, 63(4):765-771

Mój udział w tych publikacjach polegał na uzgodnieniu koncepcji badań i ich koordynacji oraz udziale w przygotowaniu manuskryptu do publikacji.

Proszę o kontakt w przypadku jakichkolwiek pytań odnośnie przedstawionych powyżej oświadczeń.



19. września 2019

Poznań, 16 września 2019 r.

Oświadczenie

Niniejszym oświadczam, że z p. mgr Dariuszem Wawrzyniakiem z Instytutu Chemii Bioorganicznej PAN posiadamy następujące wspólne publikacje:

1. A. Szymańska-Michalak*, D. Wawrzyniak*, G. Framski, M. Kujda, P. Zgoła, J. Stawiński, J. Boryski, A. Kraszewski. New 3'-O-aromatic acyl-5-fluoro-2'-deoxyuridine derivatives as potential anticancer agents. *Eur. J. Med. Chem.* 115, 41-52 (2016).

*Autorzy mieli taki sam wkład w przygotowanie publikacji

2. G. Framski, D. Wawrzyniak, Z. Jahnz-Wechmann, A. Szymańska-Michalak, A. Kraszewski, J. Boryski, J. Barciszewski, J. Stawiński. Searching for anti-glioma activity. Ribonucleoside analogues with modifications in nucleobase or sugar moieties. *Acta Biochimica Polonica*, 63, 765-771 (2016).
3. L. Michalska, D. Wawrzyniak, A. Szymańska-Michalak, J. Barciszewski, J. Boryski, D. Baraniak. Synthesis and biological assay of new 2'-deoxyuridine dimers containing a 1,2,3-triazole linker. Part I. *Nucleosides, Nucleotides and Nucleic Acids*, 38, 218-235 (2019).

Jednocześnie oświadczam, że w wyżej wymienionych pracach zajmowałem się wyłącznie ich częścią chemiczną, to jest projektowaniem i syntezą modyfikowanych nukleozydów. Natomiast mgr Wawrzyniak odpowiedzialny był za badanie aktywności biologicznej tych związków, analizę stabilności chemicznej i enzymatycznej oraz wyznaczenie parametrów fizykochemicznych determinujących biodostępność badanych związków.



Prof. dr. hab. Jerzy Boryski
Zakładu Chemii Komponentów
Kwasów Nukleinowych

Poznań, 15 października 2019 r.

Prof. dr hab. Stefan Jurga
Centrum NanoBioMedyczne UAM
ul. Wszechnicy Piastowskiej 3
61-614 Poznań

OŚWIADCZENIE

Dotyczy rozprawy doktorskiej mgr Dariusza Wawrzyniaka

- Grabowska M, Grześkowiak BF, Szutkowski K, Wawrzyniak D, Głodowicz P, Barciszewski J, Jurga S, Rolle K, Mrówczyński R. Nano-Mediated Delivery of Double-Stranded RNA for Gene Therapy of Glioblastoma Multiforme. *PLoS One*, 2019, 14(3):e0213852

Mój udział w tej publikacji polegał na uzgodnieniu koncepcji badań, organizacji i koordynacji badań oraz udział w przygotowaniu manuskryptu do publikacji.



Poznań, 24 września 2019 r.

Prof. zw. dr hab. Marcin Hoffmann
Wydział Chemii
Uniwersytet im. Adama Mickiewicza w Poznaniu
ul. Uniwersytetu Poznańskiego 8
61-614 Poznań

Oświadczenie o współautorstwie publikacji naukowej

Dotyczy rozprawy doktorskiej mgr Dariusza Wawrzyniaka

Mój wkład jako współautora w powstanie pracy zatytułowanej: „Synthesis, structural studies and biological properties of some phosphonoperfluoro-phenylalanine derivatives formed by S_NAr reactions”, autorstwa J. Kwiczak-Yiğitbaşı, J-L. Pirat, D. Virieux, J-N. Volle, A. Janiak, M. Hoffmanna, J. Mrzygłoda, D. Wawrzyniaka, J. Barciszewskiego, D. Pluskoty-Karwatki, (*RSC Advances*, 2019, 9, 24117-24133) polegał na opracowaniu koncepcji badań obliczeniowych, koordynacji i nadzorowaniu realizacji tych badań, interpretacji i przygotowaniu wyników badań *in silico* do publikacji.

W przypadku jakichkolwiek pytań dotyczących przedstawionego powyżej oświadczenia, proszę o kontakt.



Poznań, 24 września 2019 r.

Dr hab. Donata Pluskota-Karwatka, prof. ucz.
Wydział Chemii
Uniwersytet im. Adama Mickiewicza w Poznaniu
ul. Uniwersytetu Poznańskiego 8
61-614 Poznań

Oświadczenie o współautorstwie publikacji naukowej

Dotyczy rozprawy doktorskiej mgr Dariusza Wawrzyniaka

Niniejszym oświadczam, że mój wkład jako współautora w powstanie pracy naukowej zatytułowanej: „Synthesis, structural studies and biological properties of some phosphonoperfluoro-phenylalanine derivatives formed by S_NAr reactions”, autorstwa J. Kwiczak-Yiğitbaşı, J-L. Pirat, D. Virieux, J-N. Volle, A. Janiak, M. Hoffmanna, J. Mrzygłóda, D. Wawrzyniaka, J. Barciszewskiego, D. Pluskoty-Karwatki, (*RSC Advances*, 2019, 9, 24117-24133) polegał na opracowaniu koncepcji całości badań, doboru zespołu realizującego, organizacji i koordynacji badań, interpretacji wyników badań syntetycznych i strukturalnych oraz przygotowaniu, przy współudziale pozostałych członków zespołu w tym mgr Dariusza Wawrzyniaka, manuskryptu do publikacji.

Rola mgr Dariusza Wawrzyniaka w przygotowaniu wyżej wymienionej pracy polegała na zaplanowaniu koncepcji badań biologicznych, przeprowadzeniu wszystkich eksperymentów dotyczących aktywności biologicznej związków, analizie statystycznej i opracowaniu rezultatów. Mgr Wawrzyniak wyznaczył również parametry fizykochemiczne determinujące biodostępność badanych związków.

Ponadto, oświadczam, że rola J. Kwiczak-Yiğitbaşı polegała na przeprowadzeniu syntezy chemicznej nowych pochodnych fosfono-perfluorofenyloalaniny, jak również na wykonaniu badań spektroskopowych i spektrometrycznych otrzymanych związków. Rola J-L. Pirat, D. Virieux oraz J-N. Volle polegała na opracowaniu koncepcji prac syntetycznych w tym metod izolacji otrzymanych związków, natomiast A. Janiak i J. Mrzygłód przeprowadzili badania rentgenograficzne oraz opracowali uzyskane w zakresie tych badań wyniki.

W przypadku jakichkolwiek pytań dotyczących powyższego oświadczenia, proszę o kontakt.

Donata Pluskota

Poznań, 15 października 2019 r.

Dr hab. Kosma Szutkowski
Centrum NanoBioMedyczne UAM
ul. Wszechnicy Piastowskiej 3
61-614 Poznań

OŚWIADCZENIE

Dotyczy rozprawy doktorskiej mgr Dariusza Wawrzyniaka

- Grabowska M, Grześkowiak BF, Szutkowski K, Wawrzyniak D, Głodowicz P, Barciszewski J, Jurga S, Rolle K, Mrówczyński R. Nano-Mediated Delivery of Double-Stranded RNA for Gene Therapy of Glioblastoma Multiforme. *PLoS One*, 2019, 14(3):e0213852

Mój udział w tej publikacji polegał na wykonaniu badań obrazowania nanocząstek z wykorzystaniem magnetycznego rezonansu jądrowego.



Poznań, 28 czerwca 2019 r.

dr Agnieszka Szymańska-Michalak
Instytut Chemii Bioorganicznej PAN
ul. Noskowskiego 12/14
61-704 Poznań

Oświadczenie o współautorstwie w publikacjach

1. Szymańska-Michalak A*, Wawrzyniak D*, Framski G, Kujda M, Zgoła P, Stawiński J, Barciszewski J, Boryski J, Kraszewski A. New 3'-O-aromatic acyl-5-fluoro-2'-deoxyuridine derivatives as potential anticancer agents. *European Journal of Medicinal Chemistry*, 2016, 115:41-52

*Autorzy mieli taki sam wkład w przygotowanie publikacji

2. Szymańska-Michalak A*, Wawrzyniak D*, Framski G, Stawiński J, Barciszewski J, Kraszewski A. New antiglioma zwitterionic pronucleotides with a FdUMP framework. *European Journal of Medicinal Chemistry*, 2018, 144:682-691

*Autorzy mieli taki sam wkład w przygotowanie publikacji

3. Framski G, Wawrzyniak D, Jahnz-Wechmann Z, Szymańska-Michalak A, Kraszewski A, Barciszewski J, Boryski J, Stawiński J. Searching for anti-glioma activity. Ribonucleoside analogues with modifications in nucleobase and sugar moieties. *Acta Biochimica Polonica*, 2016, 63(4):765-771

Oświadczam, że moja rola jako współautora powyższych publikacji polegała na wykonaniu pracy eksperymentalnej, tj. syntezy chemicznej nukleozydów i nukleotydów oraz analizy NMR i HPLC.

4. Michalska L, Wawrzyniak D, Szymańska-Michalak A, Barciszewski J, Boryski J, Baraniak D. Synthesis and biological assay of new 2'-deoxyuridine dimers containing a 1,2,3-triazole linker. Part I. Nucleosides, *Nucleotides and Nucleic Acids*, 2019, 3:218-235

Oświadczam, że moja rola jako współautora powyższej publikacji polegała na wykonaniu pracy eksperymentalnej, tj. analizy HPLC.

28.06.19 Agnieszka - Michalak

Poznań, 16 września 2019 r.

dr Grzegorz Framski
Instytut Chemii Bioorganicznej PAN
ul. Noskowskiego 12/14
61-704 Poznań

Oświadczenie o współautorstwie w publikacjach

1. Szymańska-Michalak A*, Wawrzyniak D*, Framski G, Kujda M, Zgoła P, Stawiński J, Barciszewski J, Boryski J, Kraszewski A. New 3'-O-aromatic acyl-5-fluoro-2'-deoxyuridine derivatives as potential anticancer agents. *European Journal of Medicinal Chemistry*, 2016, 115:41-52

*Autorzy mieli taki sam wkład w przygotowanie publikacji

2. Szymańska-Michalak A*, Wawrzyniak D*, Framski G, Stawiński J, Barciszewski J, Kraszewski A. New antiglioma zwitterionic pronucleotides with a FdUMP framework. *European Journal of Medicinal Chemistry*, 2018, 144:682-691

*Autorzy mieli taki sam wkład w przygotowanie publikacji

3. Framski G, Wawrzyniak D, Jahnz-Wechmann Z, Szymańska-Michalak A, Kraszewski A, Barciszewski J, Boryski J, Stawiński J. Searching for anti-glioma activity. Ribonucleoside analogues with modifications in nucleobase and sugar moieties. *Acta Biochimica Polonica*, 2016, 63(4):765-771

Oświadczam, że moja rola jako współautora powyższych publikacji polegała na wykonaniu pracy eksperymentalnej, tj. syntezy chemicznej nukleozydów oraz analizy NMR.



Poznań, 16.09.2019

dr Dagmara Baraniak

Zakład Chemii Komponentów Kwasów Nukleinowych

Instytut Chemii Bioorganicznej PAN

Ul. Z. Noskowskiego 12/14

61-704 Poznań

Oświadczenie o współautorstwie w publikacji

L. Michalska, D. Wawrzyniak, A. Szymańska-Michalak, J. Barciszewski, J. Boryski, and D. Baraniak, Synthesis and biological assay of new 2'-deoxyuridine dimers containing a 1,2,3-triazole linker. Part I, *Nucleosides, Nucleotides and Nucleic Acids*, **2019**, 38(3), 218–235.

Oświadczam, że moja rola jako współautora powyższej publikacji polegała na pomocy w kierowaniu pracą eksperymentalną oraz w przygotowaniu manuskryptu.

Dagmara Baraniak

dr Dagmara Baraniak

Poznań, 16 września 2019 r.

dr Zofia Jahnz-Wechmann
Instytut Chemii Bioorganicznej PAN
ul. Noskowskiego 12/14
61-704 Poznań

Oświadczenie o współautorstwie w publikacji

Framski G, Wawrzyniak D, Jahnz-Wechmann Z, Szymańska-Michalak A, Kraszewski A, Barciszewski J, Boryski J, Stawiński J. Searching for anti-glioma activity. Ribonucleoside analogues with modifications in nucleobase and sugar moieties. *Acta Biochimica Polonica*, 2016, 63(4):765-771

Oświadczam, że moja rola jako współautora powyższej publikacji polegała na wykonaniu pracy eksperymentalnej, tj. syntezy chemicznej nukleozydów oraz analizy NMR.

Z. Jahnz-Wechmann

Poznań, 16 września 2019 r.

dr Agnieszka Belter
Instytut Chemii Bioorganicznej PAN
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Oświadczenie o współautorstwie w publikacji

Rolle K*, Piwecka M*, Belter A*, Wawrzyniak D, Jeleniewicz J, Barciszewska MZ, Barciszewski J†. **The sequence and structure determine the function of mature human miRNAs.** PLoS One, 2016, 11(3)e0151246

*Autorzy mieli taki sam wkład w przygotowanie publikacji

Mój wkład w powstanie pracy

Oświadczam, iż mój wkład w powstanie tej pracy polegał na przeprowadzeniu analiz dotyczących struktury drugorzędowej dojrzałych miRNA oraz współudziale w tworzeniu manuskryptu i dyskusji wyników.

Agnieszka Belter

Berlin, 16.09.2019

dr Monika Piwecka
Max-Delbrück-Centrum for Molecular Medicine (MDC)
Robert-Rössle-Str. 10
13092 Berlin

Oświadczenie o współautorstwie w publikacji

Rolle K*, Piwecka M*, Belter A*, Wawrzyniak D, Jeleniewicz J, Barciszewska MZ, Barciszewski J†. **The sequence and structure determine the function of mature human miRNAs**. PLoS One, 2016, 11(3)e0151246

*Autorzy mieli taki sam wkład w przygotowanie publikacji

Mój wkład w powstanie pracy

Oświadczam, iż mój wkład w powstanie tej pracy polegał na współtworzeniu jej koncepcji, przeprowadzeniu analizy bioinformatycznej z użyciem programu Diana miRPath, dyskusji wyników oraz tworzeniu manuskryptu.

Monika Piwecka

Kraków, 16.10.2019

dr Marta Kujda

Oświadczenie o współautorstwie w publikacji

Szymańska-Michalak A*, Wawrzyniak D*, Framski G, Kujda M, Zgoła P, Stawiński J, Barciszewski J, Boryski J, Kraszewski A. New 3'-*O*-aromatic acyl-5-fluoro-2'-deoxyuridine derivatives as potential anticancer agents. *European Journal of Medicinal Chemistry*, 2016, 115:41-52

*Autorzy mieli taki sam wkład w przygotowanie publikacji

Mój wkład w powstanie pracy

Oświadczam, że moja rola jako współautora powyższej publikacji polegała na wykonaniu pracy eksperymentalnej, tj. badania zdolności do tworzenia mikromicel przez 5-fluoro-3'-*O*-benzoilo-2'-deoksyurydynę metodą dynamicznego rozproszenia światła.

dr Marta Kujda

Poznań, 20 września 2019 r.

Dr Radosław Mrówczyński
Centrum NanoBioMedyczne UAM
ul. Wszechnicy Piastowskiej 3
61-614 Poznań

OŚWIADCZENIE

Dotyczy rozprawy doktorskiej mgr Dariusza Wawrzyniaka

- Grabowska M, Grześkowiak BF, Szutkowski K, Wawrzyniak D, Głodowicz P, Barciszewski J, Jurga S, Rolle K, Mrówczyński R. Nano-Mediated Delivery of Double-Stranded RNA for Gene Therapy of Glioblastoma Multiforme. *PLoS One*, 2019, 14(3):e0213852

Mój udział w tej publikacji polegał na zaplanowaniu syntez chemicznych i ich przeprowadzeniu oraz koordynacji i nadzorowaniu eksperymentów i analiz. Brałem udział w pisaniu i korekcie manuskryptu.

Radosław Mrówczyński

Poznań, 16 października 2019 r.

Dr inż. Bartosz Grześkowiak
Centrum NanoBioMedyczne UAM
ul. Wszechnicy Piastowskiej 3
61-614 Poznań

OŚWIADCZENIE

Dotyczy rozprawy doktorskiej mgr Dariusza Wawrzyniaka

- Grabowska M, Grześkowiak BF, Szutkowski K, Wawrzyniak D, Głodowicz P, Barciszewski J, Jurga S, Rolle K, Mrówczyński R. Nano-Mediated Delivery of Double-Stranded RNA for Gene Therapy of Glioblastoma Multiforme. *PLoS One*, 2019, 14(3):e0213852

Mój udział w tej publikacji polegał na wykonaniu pracy eksperymentalnej, tj. przeprowadzeniu testów WST-1 oraz Live/Dead, wnikania nanomateriałów do komórek oraz charakterystyce fizykochemicznej. Brałem, także udział w przygotowaniu rycin i tekstu manuskryptu.

Bartosz Grześkowiak

Brno, 16.09.2019

mgr Lucyna Michalska

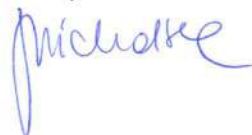
Laboratory of Organic Synthesis and Medicinal Chemistry
Department of Organic Chemistry
Faculty of Science
Masaryk University
Ul. Kamenice 753/5
625 00 Brno - Bohunice

Oświadczenie o współautorstwie w publikacji

L. Michalska, D. Wawrzyniak, A. Szymańska-Michalak, J. Barciszewski, J. Boryski, and D. Baraniak, Synthesis and biological assay of new 2'-deoxyuridine dimers containing a 1,2,3-triazole linker. Part I, *Nucleosides, Nucleotides and Nucleic Acids*, **2019**, 38(3), 218–235.

Oświadczam, że moja rola jako współautora powyższej publikacji polegała na wykonaniu pracy eksperymentalnej tj. syntezy chemicznej oraz analizy NMR i MS otrzymanych analogów dimerów nukleozydów.

mgr Lucyna Michalska



Poznań, 20 września 2019 r.

Mgr Małgorzata Grabowska
Instytut Chemii Bioorganicznej PAN
ul. Noskowskiego 12/14
61-704 Poznań

OŚWIADCZENIE

Dotyczy rozprawy doktorskiej mgr Dariusza Wawrzyniaka

- Grabowska M, Grześkowiak BF, Szutkowski K, Wawrzyniak D, Głodowicz P, Barciszewski J, Jurga S, Rolle K, Mrówczyński R. Nano-Mediated Delivery of Double-Stranded RNA for Gene Therapy of Glioblastoma Multiforme. *PLoS One*, 2019, 14(3):e0213852

Mój udział w przygotowaniu niniejszej pracy polegał na wykonaniu pracy eksperymentalnej, tj. przygotowaniu ATN-RNA, przeprowadzeniu testów funkcjonalnych oraz analiz ekspresji tenascyny-C. Brałam, także udział w przygotowaniu rycin.

Grabowska

Poznań, 20.09.2019

mgr Paulina Zgoła

Oświadczenie o współautorstwie w publikacji

Szymańska-Michalak A*, Wawrzyniak D*, Framski G, Kujda M, Zgoła P, Stawiński J, Barciszewski J, Boryski J, Kraszewski A. New 3'-O-aromatic acyl-5-fluoro-2'-deoxyuridine derivatives as potential anticancer agents. *European Journal of Medicinal Chemistry*, 2016, 115:41-52

*Autorzy mieli taki sam wkład w przygotowanie publikacji

Mój wkład w powstanie pracy

Oświadczam, że moja rola jako współautora powyższej publikacji polegała na wykonaniu pracy eksperymentalnej, tj. syntezy chemicznej blokowanych nukleozydów.

20 09 2019 Paulina Zgoła

Poznań, 20 września 2019 r.

Mgr Paweł Głodowicz
Instytut Chemii Bioorganicznej PAN
ul. Noskowskiego 12/14
61-704 Poznań

OŚWIADCZENIE

Dotyczy rozprawy doktorskiej mgr Dariusza Wawrzyniaka

- Grabowska M, Grześkowiak BF, Szutkowski K, Wawrzyniak D, Głodowicz P, Barciszewski J, Jurga S, Rolle K, Mrówczyński R. Nano-Mediated Delivery of Double-Stranded RNA for Gene Therapy of Glioblastoma Multiforme. *PLoS One*, 2019, 14(3):e0213852

Mój udział w przygotowaniu niniejszej pracy polegał na wykonaniu pracy eksperymentalnej, tj. przygotowaniu ATN-RNA oraz przeprowadzeniu analiz ekspresji genów odpowiedzi immunologicznej.

Głodowicz