Electronic Supplementary Material (ESI) for Chemical Science.

Cellular delivery of dinucleotides by conjugation with small molecules: targeting translation initiation for anticancer applications

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Abstract: Targeting cap-dependent translation initiation is one of experimental approaches that could lead to the development of novel anti-cancer therapies. Synthetic cap analogs in the form of dinucleoside 5',5'-triphosphates are potent antagonists of eukaryotic translation initiation factor 4E (eIF4E) in vitro and consequently could counteract elevated levels of eIF4E in cancer cells. However, transformation of these compounds into therapeutic agents remains challenging because they do not penetrate into cells and are susceptible to enzymatic cleavage by endogenous enzymes. Here, we tested several small molecule ligands – folic acid, biotin, glucose and cholesterol – as potential cell-penetrating vehicles for both hydrolysable and cleavage-resistant cap analogs. We performed a broad structure-activity relationship (SAR) study for model fluorescent probes and cap-ligand conjugates to determine that cholesterol greatly facilitates cap analog uptake without disturbing interaction with eIF4E. The most potent cholesterol conjugate identified based on SAR studies showed apoptosis-mediated cytotoxicity towards cancer cells.

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General Information

Starting materials and chemical reagents

Nucleoside and nucleotide components, folic acid cholesteryl hemisuccinate, cholesteryl-TEG-azide, acetobromo-*α*-D-glucose and phosphorylating agents were purchased from Sigma Aldrich (Merck). Fluorescent label 6-carboxyfluorescein-*N*-hydroxysuccinimide (FAM-NHS) and biotin(+)-amido caproate-*N*-hydroxysuccinimide were purchased from ChemGenes. Bis(3-aminopropyl)amine were aquired form TCI. PEGylated linkers were aquired from BroadPharm. Solvents and other chemical reagents were purchased form commercial vendors and used without further purification. Non modified mono- and diphosphates were obtained as triethylammonium salts (m^7GMP, m^7GDP, their imidazolides and AMP with linker attached in *N*-6 position) as described[1]. Thiophosphonates (m^7GMPαS and m^7GDPαS) were synthesized using procedure described previously[2]. GMP-L_{6N} was synthesized as presented in the paper[3]. Folic acid derivatives were synthesized according to the published procedure[4]. Cholesteryl hemisuccinate-NHS was synthesized according to the procedure[5]. Compounds for biological evaluation were dissolved in water at millimolar concentrations and stored at -20 °C if necessary.

Chromatography

Flash chromatography

The isolation of synthesized compounds was performed using Büchi Reveleris® Preparative Flash Chromatograph with UV and ELSD detection. The crudes were dissolved in DCM or chloroform and injected onto the volume suitable Reveleris cartriges. As eluents were used DCM or chloroform and methanol in appropriate linear gradient. The fractions containing desired product were collected, combined and solvents were evaporated under reduced pressure.

Ion-exchange chromatography

The synthesized nucleoside derivatives were isolated using ion-exchange chromatography on DEAE Sephadex A-25 (HCO_3^- form) column. The loading was washed thoroughly with MQ-water, after that the products were eluted using buffer TEAB in gradient: TEAB:MQ-water in 0-1.0 M for nucleoside triphosphates. Collected fractions were analyzed spectrophotometrically (at 260 nm) and by analytical RP HPLC. The fractions containing desired product were combined and evaporated, the buffer was co-evaporated with ethanol twice and with acetonitrile. The residues were dissolved in MQ-water and freeze-dried repeated thrice. The products isolated using ion-exchange chromatography were obtained as triethylamine salts. Calculation of the yields was done on the basis of optical density miliunits (mOD = volume in mL × absorbance of the solution) of the isolated products and corresponding starting materials (nucleotides or nucleotide *P*-imidazolide derivatives). Optical unit quantifications were performed in a 0.1 M phosphate buffer at 260 nm (pH 7.0).

Analytical and semi-preparative reverse phase (RP) HPLC

Analytical and semi-preparative RP HPLC was performed on Agilent Tech. Series 1260 Infinity. Analytical RP HPLC was performed using Supelcosil LC-18-T HPLC column (4.6 x 250 mm, 5µm, flow rate 1.3 mL/min) for nucleosides, nucleotides and dinucleotide triphosphates derivatives. Different conditions were applied for linear gradient of ammonium acetate buffer (pH 5.9, 50 mM) named as buffer A and buffer B (methanol and buffer A in 1:1; v/v), such as: Method A: 0-50% in 15 min; Method B: 0-100% in 15 min.
Analytical RP HPLC for folic acid derivatives was performed using Phenomenex Gemini NX-C18 column (4.6 x 250 mm, 5µm, flow rate 1mL/min) as a gradient of ammonium acetate buffer (pH 8.4, 200 mM) as a buffer C and acetonitrile in 5-30% in 20 min (Method C).
RP HPLC separation technique for cholesteryl containing compounds was established using Thermo Scientific Hypersil GOLD™ C4 HPLC column (4.6 x 150 mm, 5µm, flow rate 1.25 ml/min) and mobile phase gradient was optimized as Method D: Buffer A : Buffer B 0-100% in 15 min, 5 min back to buffer A, 10 min from 0-100% of acetonitrile. Profiles of purified compounds containing cholesteryl was recorded using Method E: Buffer A : acetonitrile 0-100% in 20 min (Method E).
Semi-preparative RP HPLC was performed using Grace Vision HT C18 HL chromatography column (22 x 250 mm, 10 µm, flow rate 5.0 mL/min) with a linear gradient of buffer A and acetonitrile in different ratios.
All nucleoside analytes were detected with UV-detection at 254 nm and fluorescence detection were fixed at Ex: 260 nm and Em: 370 nm and for FAM containing products the UV-detection was fixed at 490 nm and for fluorescence Ex: 495 nm and Em: 513 nm.

NMR and MS analysis

NMR analysis

The structures of resulted compounds were confirmed by NMR spectra, which were recorded at 25°C on Bruker Avance III HD spectrometer at 500.24 MHz (^{1}H NMR), 125.80 MHz (^{13}C NMR) and 202.49 MHz (^{31}P NMR), probe: 5 mm PABBO BB/ 19F-1H/ D Z-GRD. ^{1}H NMR chemical shifts were calibrated to sodium 3-trimethylsilyl-[2,2,3,3-D4]propionate (TSP) in D_2O and for ^{31}P NMR to H_3PO_4 (20%) in D_2O as an external standard. Signal assignments was based on COSY spectra analysis. The raw NMR spectroscopic data were processed by use of MestReNova v12.0.2-20910 Software.

MS analysis

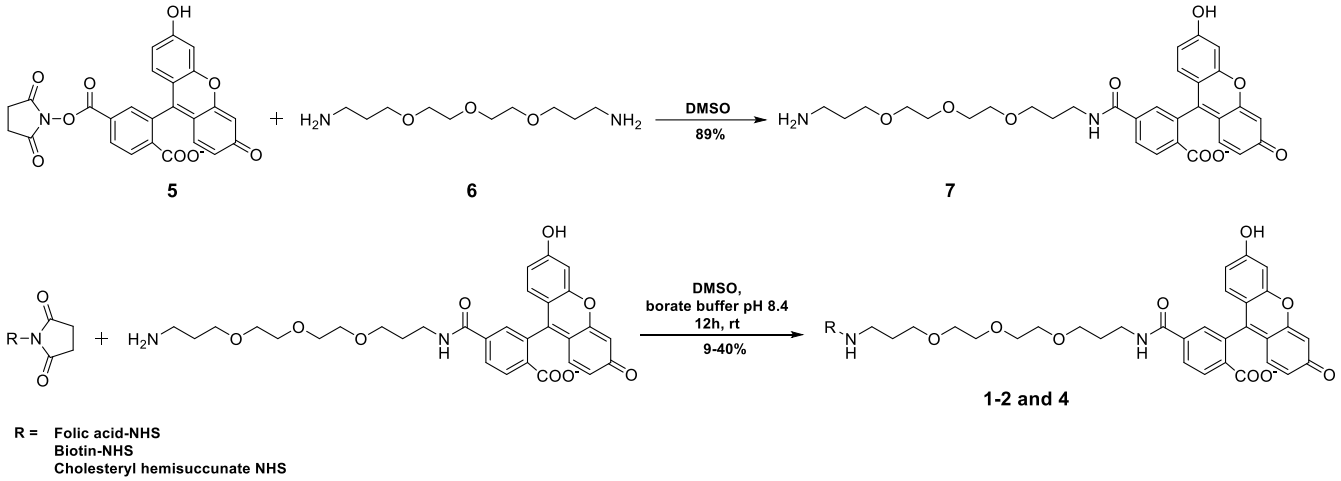
The confirmation of resulted compounds was performed with high resolution mass spectrometry using negative or positive electrospray ionization (HRMS-ESI). Mass spectra were recorded with LTQ Orbitrap Velos (Thermo Scientific) spectrometer.

Quantification

Absorption spectra in the range of 700-220 nm were recorded on Shimadzu UV-1800 with a 1.0 nm slit, 0.1 nm sampling interval, and medium scan speed. Emission spectra were recorded on Cary Eclipse (Agilent) spectrofluorimeter equipped with xenon lamp set at excitation wavelengths of 495 nm for FAM-labeled compound with a 5 nm bandwidths. For FAM containing products the extinction coefficient was ε = 65000 $M^{-1}cm^{-1}$ at pH 7.0. For triphosphate dinucleotide derivatives, the extinction coefficient was calculated as an additive effect of absorbances of m^7GDP (ε = 11.400 $M^{-1}cm^{-1}$) and AMP-HDA (ε = 15.000 $M^{-1}cm^{-1}$) or GMP-L_{6N} (13.711 $M^{-1}cm^{-1}$) performed in a 0.1 M phosphate buffer at 260 nm (pH 7.0) using an equation $\varepsilon_{dinucleotide} = 0.9 \times (\varepsilon_1 + \varepsilon_2)$, where ε_1 and ε_2 represent extinction coefficients of both nucleotides subunits. For folic acid dinucleotide conjugates, the extinction coefficient of folic acid was estimated using 3 samples dissolved in 1 mL of DMSO. The absorbance was measured tree times for each sample at 260 nm in a 0.1 M phosphate buffer at 260 nm (pH 7.0) and ε = 15782.7 $M^{-1}cm^{-1}$. The obtained results were taken to the calculation using an equation for A_{260} as $\varepsilon_{folic\_acid\_conjugate} = 0.9 \times (\varepsilon_{dinucleotide} + \varepsilon_{folic\_acid})$ and the extinction coefficient was estimated ε = 35588.43 $M^{-1}cm^{-1}$ at pH = 7.0 for m^7GpppA-folic acid derivatives and ε = 34544.43 $M^{-1}cm^{-1}$ for m^7GpppG-folic acid derivatives.

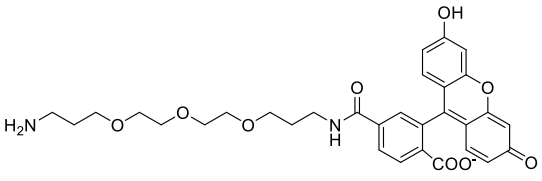
Synthetic procedures

Cell-permeability fluorescent probes (1-2 and 4)



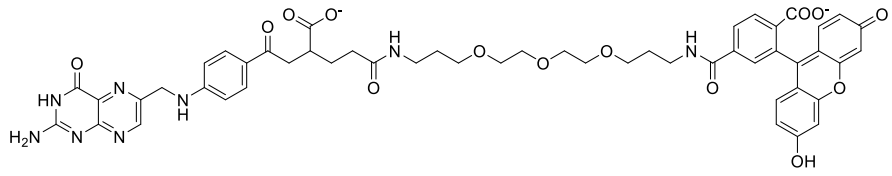
Scheme S1. Synthesis of cell-permeability fluorescent probes (**1-2** and **4**).

Compound: FAM-PEG (7)



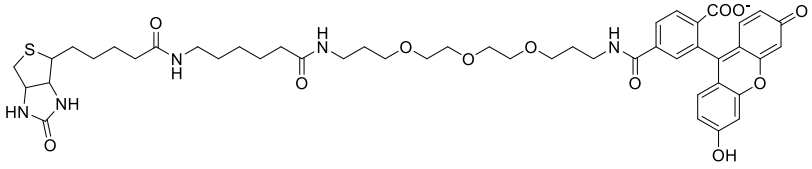
To a solution of FAM-NHS (**5**, 10.75 mg, 0.022 mmol, 1 eq.) in DMSO (200 µL) was added a solution of 4,7,10-trioxa-1,13-diaminotridecane (**6**, 0.045 mmol, 2 eq.). The mixture was stirred for 2 hours at room temperature and the reaction progress was monitored using HPLC chromatography, then quenched with 1% of acetic acid. After conversion, the crude product was purified by semi-preparative HPLC column (ammonium acetate pH 5.9: acetonitrile gradient) to give desired product **7** (11.87 mg, 89% yield) as orange powder. **^{1}H NMR** (500 MHz, D_2O) δ 1.87 – 2.02 (m, 6H), 3.08 (t, *J* = 7.1 Hz, 2H), 3.48 – 3.55 (m, 2H), 3.58 – 3.69 (m, 13H), 6.63 (s, 2H), 6.66 (d, *J* = 9.6 Hz, 2H), 7.08 (d, *J* = 9.2 Hz, 2H), 7.26 (d, *J* = 7.9 Hz, 1H), 7.91 (d, *J* = 7.9 Hz, 1H), 8.24 (s, 1H). **HRMS-ESI** (*m/z*): $[M-H]^-$ calcd for $C_{31}H_{33}N_2O_9^-$, 577.21915; found, 577.21976.

Compound: FAM-PEG-FA (1)



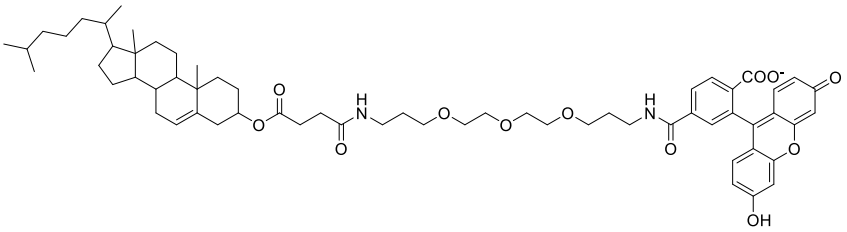
FAM-PEG (**7**, 1 mg, 1.75 µmol, 1 eq.) was dissolved in 180 µL of a borate buffer (pH 8.4). Folic acid-NHS (R-NHS, 1.39 mg, 2.59 µmol, 1.5 eq.) substrate was dissolved in 20 µL of DMSO and this mixture was added to FAM-PEG solution. The reaction mixture was stirred for 12 h at room temperature. The progress of the reaction was monitored using HPLC chromatography (Gemini NX-C-18 column in ammonium acetate buffer pH 8.4:acetonitrile gradient 5-30% in 20 min. The purification of the resulted product was performed using HPLC column chromatography with described above elution conditions to give 0.173 µmol, yield 10% of product **1**.
HRMS-ESI (*m/z*): $[M-H]^-$ calcd for $C_{50}H_{50}N_9O_{14}^-$, 1000.34827; found, 1000.35001.

Compound: FAM-PEG-Biotin (2)



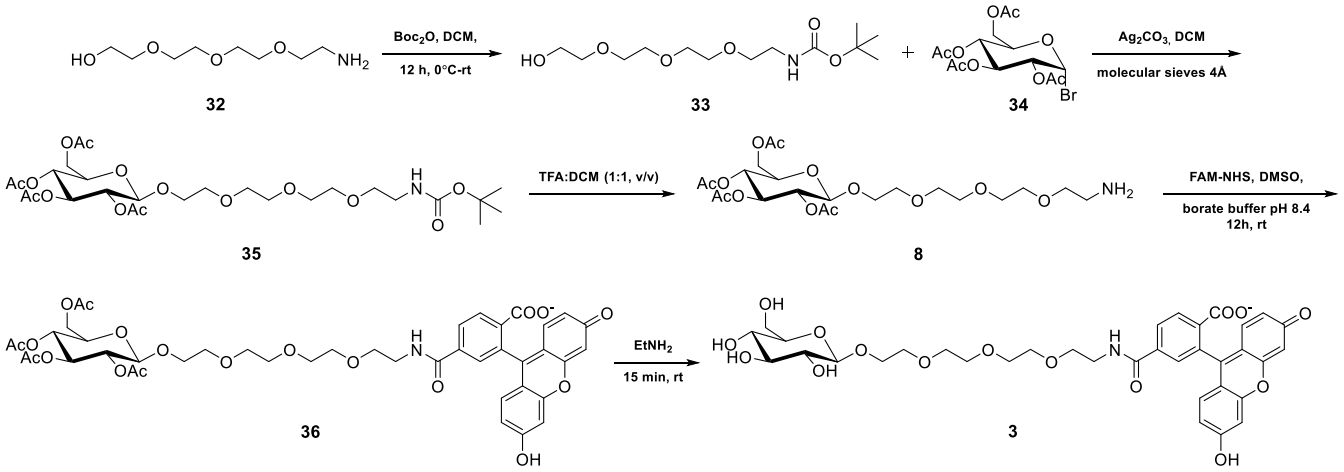
FAM-PEG (**7**, 1 mg, 1.73 µmol, 1 eq.) was dissolved in 180 µL of a borate buffer (pH 8.4). Biotin(+)-amido caproate-*N*-hydroxysuccinimide (R-NHS, 1.18 mg, 2.6 µmol, 1.5 eq.) was dissolved in 20 µL of DMSO and added into FAM-PEG solution. The mixture was stirred for 12 h at room temperature. The progress of the reaction was monitored using HPLC chromatography (Supelco C8 column in ammonium acetate buffer pH 5.9:acetonitrile in a gradient 0-100% in 20 min). The crude product was then purified using HPLC chromatography using the same separation conditions to give 0.74 µmol, yield 43% of compound **2**. **HRMS-ESI** (*m/z*): $[M-H]^-$ calcd for $C_{47}H_{58}N_5O_{12}S^-$, 916.38082; found, 916.38245.

Compound: FAM-PEG-CHOL (4)



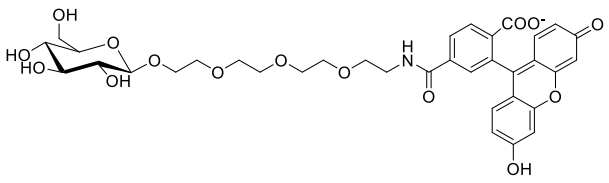
FAM-PEG (**7**, 9.9 mg, 0.017 mmol, 1 eq.) was dissolved in 0.2 mL of DMSO. Cholesteryl hemisuccinate *N*-hydroxysuccinimide (10 mg, 0.026 mmol, 1.5 eq.) was dissolved in 0.69 mL of DMSO and added into FAM-PEG solution following the addition of triethylamine (10 µL). The mixture was stirred for 12 h at room temperature. The reaction progress was monitored using TLC chromatography using 0.5% MeOH in DCM as a mobile phase. The purification of the crude product was performed using preparative layer chromatography PLC using 1% of MeOH in DCM as elution phase. The band related to the desired compound was then removed from the plate and extracted tree times with the 5% solution of MeOH in DCM. The organic phase was filtered and evaporated under reduced pressure giving 7.2 mg, 6.88 µmol, yield 40% as an orange powder of compound **4**. **^{1}H HPLC** (500 MHz, CD_3OD) δ 0.69 (s, 3H), 0.87 (dd, *J* = 6.6, 2.0 Hz, 8H), 0.93 (d, *J* = 6.5 Hz, 5H), 1.01 (s, 4H), 1.05 – 1.18 (m, 7H), 1.26 – 1.31 (m, 4H), 1.34 – 1.40 (m, 4H), 1.46 – 1.55 (m, 5H), 1.55 – 1.61 (m, 2H), 1.74 (p, *J* = 6.4 Hz, 2H), 1.91 – 1.97 (m, 3H), 1.98 – 2.06 (m, 1H), 2.26 – 2.31 (m, 2H), 2.45 (t, *J* = 6.8 Hz, 2H), 2.56 (t, *J* = 6.8 Hz, 2H), 3.24 (t, *J* = 6.8 Hz, 2H), 3.47 – 3.59 (m, 7H), 3.61 – 3.67 (m, 8H), 3.65 – 3.69 (m, 4H), 4.47 – 4.53 (m, 3H), 5.32 – 5.37 (m, 1H), 6.52 (d, *J* = 2.0 Hz, 3H), 6.54 (d, *J* = 2.2 Hz, 2H), 7.03 (d, *J* = 8.9 Hz, 2H), 7.31 (d, *J* = 7.9 Hz, 1H), 7.98 (dd, *J* = 7.9, 1.9 Hz, 1H), 8.43 (d, *J* = 1.9 Hz, 1H). **^{13}C NMR** (126 MHz, CD_3OD) δ ^{13}C NMR (126 MHz, CD_3OD) δ 12.02, 18.95, 19.48, 21.83, 22.66, 22.91, 24.67, 25.00, 28.49, 28.86, 29.03, 30.09, 30.20, 30.51, 31.26, 32.70, 32.88, 36.83, 37.07, 37.55, 37.91, 38.45, 38.85, 40.13, 40.39, 40.79, 48.66, 48.83, 49.00, 49.17, 49.34, 51.28, 57.24, 57.78, 69.60, 69.91, 70.91, 71.03, 71.25, 75.31, 104.12, 123.35, 123.52, 128.49, 128.94, 130.91, 131.95. **HRMS-ESI** (*m/z*): $[M-H]^-$ calcd for $C_{62}H_{81}N_2O_{12}^-$, 1045.57950; found, 1045.58073.

Cell-permeability fluorescent probe (3)



Scheme S2. Synthesis of cell-permeability fluorescent probe (**3**).

Compound: FAM-PEG-β-Glc (3)



11-amino-3,6,9-trioxaundecanol (**32**, PEG4) (1 g, 4.52 mmol) was dissolved in 5 mL of DCM and stirred in a round bottom flask. Di-*tert*-butyl dicarbonate (0.723 g, 3.32 mmol, 1.1 eq.) dissolved in 3 mL of DCM was poured into a dropping funnel and installed to the reaction flask. Then, solution of di-*tert*-butyl dicarbonate was added drop by drop into PEG4 solution during 30 min at room temperature. The reaction mixture was stirred for 12 h. After, Et_2O was added and extracted with H_2O trice. The organic phase was evaporated and taken to the next step without further purification (compound **33**, 860 mg, yield 57%). Then, to a mixture of acetobromo-α-D-glucose (**34**, 200 mg, 0.49 mmol), compound **33** (143 mg, 0.49 mmol) and molecular sieves 4 Å stirred in DCM (2 mL) at 0 °C under N_2 was added Ag_2CO_3 (134.5 mg, 0.49 mmol). The reaction mixture was warmed to room temperature and stirred for 3 h. The reaction was monitored using TLC chromatography with 1% of MeOH in DCM staining the spots with 20% H_2SO_4 and heating the plate. The reaction mixture was filtrated through celite and the solvent was evaporated under reduced pressure. The crude product **35** was purified by silica gel Flash chromatography eluted in gradient of MeOH in DCM 0-30% to obtain the product as β-isomer (128 mg, yield 42%). Dried Boc-protected aceto-PEG4-β-glucose (**35**, 100 mg, 0.16 mmol) was dissolved in 200 µL of DCM and 200 µL of TFA was added. After deprotection completion the mixture was thoroughly co-evaporated with DCM and obtained quantitatively. The crude **8** (1 mg, 2.9 µmol) was then dissolved in 80 µL of a borate buffer (pH 8.4) solution and added to a solution of FAM-NHS (**5**, 0.9 mg, 1.9 µmol) in 20 µL of DMSO. The reaction mixture was stirred for 12 h at room temperature. The reaction progress was monitored by HPLC (Supelco C18 column, ammonium acetate buffer pH 5.9:acetonitrile 0-100% in 15 min.) to obtain product **36**. The cleavage of acetate protection groups was done by dissolving the product in $EtNH_2$ 40% in water (100 µL) and

stirred for 15 min, then desired product **3** was purified using HPLC chromatography (C18 column in gradient of ammonium acetate pH 5.9:acetonitrile 0-100% in 15 min to give 0.21 µmol, yield 11%.

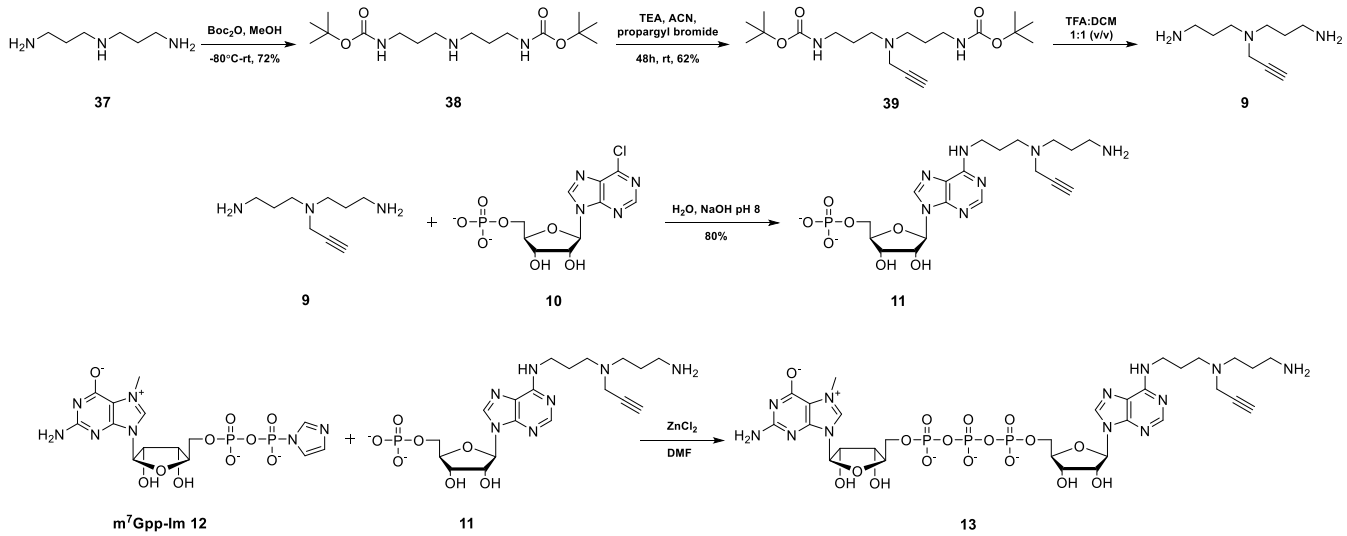
Compound **33**
^{1}H NMR (500 MHz, $CDCl_3$) δ 1.24 (s, 9H), 3.10 (d, J = 5.5 Hz, 2H), 3.33 (t, J = 5.2 Hz, 2H), 3.39 – 3.58 (m, 13H), 5.54 (s, 1H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 28.12, 40.01, 61.08, 69.73, 69.86, 69.98, 70.05, 70.19, 72.36, 76.90, 77.16, 77.42, 78.58, 155.92. **HRMS-ESI of compound 33** (*m/z*): $[M-H]^-$ calcd for $C_{13}H_{26}NO_6^-$, 292.17656; found 292.17686.

Compound **35**
^{1}H NMR (400 MHz, $CDCl_3$) δ 1.32 – 1.42 (m, 10H), 1.64 – 1.66 (m, 2H), 1.88 – 2.14 (m, 12H), 3.21 – 3.26 (m, 2H), 3.36 – 3.41 (m, 1H), 3.45 – 3.49 (m, 2H), 3.51 – 3.61 (m, 12H), 4.09 – 4.16 (m, 2H), 4.26 – 4.34 (m, 1H), 4.79 – 4.87 (m, 1H), 5.09 – 5.13 (m, 1H), 5.62 – 5.72 (m, 1H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 20.62, 20.74, 20.76, 20.78, 20.80, 28.42, 40.35, 50.53, 51.02, 62.90, 63.09, 66.89, 68.24, 70.04, 70.07, 70.19, 70.21, 70.25, 70.48, 70.51, 70.54, 72.91, 96.90, 121.20, 156.04, 156.21, 169.15, 169.68, 170.71.
HRMS-ESI of compound 35 (*m/z*): $[M+H]^+$ calcd for $C_{27}H_{46}NO_{15}^+$, 624.28620; found 624.28560 .

Compound **36**
^{1}H NMR (500 MHz, $CDCl_3$) δ 1.94 – 2.07 (m, 16H), 3.52 – 3.77 (m, 22H), 3.88 (s, 1H), 4.12 (d, J = 12.0 Hz, 1H), 4.19 – 4.28 (m, 1H), 4.55 (d, J = 7.9 Hz, 1H), 4.96 (t, J = 9.0 Hz, 1H), 5.06 (t, J = 9.7 Hz, 1H), 5.19 (t, J = 9.5 Hz, 1H), 6.44 – 6.74 (m, 6H), 8.10 (s, 1H), 8.44 (s, 1H). **HRMS-ESI of compound 36** (*m/z*): $[M-H]^-$ calcd for $C_{43}H_{46}NO_{19}^-$, 880.26695; found 880.26841.

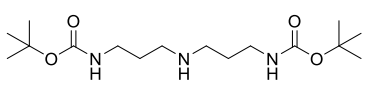
Compound **3**
^{1}H NMR (500 MHz, D_2O) δ 3.19 – 3.27 (m, 1H), 3.30 – 3.36 (m, 2H), 3.43 (t, J = 9.0 Hz, 1H), 3.61 – 3.85 (m, 25H), 3.94 – 4.02 (m, 1H), 4.36 (d, J = 7.9 Hz, 1H), 6.63 – 6.70 (m, 4H), 7.20 (d, J = 9.2 Hz, 2H), 7.50 (d, J = 7.9 Hz, 1H), 8.00 (dd, J = 7.9, 1.9 Hz, 1H), 8.22 (d, J = 1.9 Hz, 1H). **HRMS-ESI of compound 4** (*m/z*): $[M-H]^-$ calcd for $C_{35}H_{38}NO_{15}^-$, 712.22469; found 712.22599.

Double-functionalized cap-ligand fluorescent probes (m^{7}GpppA-FAM-Ligand) (15-17)



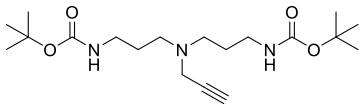
Scheme S3. Synthesis of double-functionalized cap-analogue (**13**).

Bis(3-Boc-aminopropyl)amine (38)



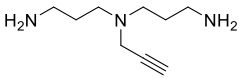
Bis(3-aminopropyl)amine (**37**, 5 g, 5.33 mL, 38 mmol) was dissolved in MeOH (20 mL) at -80°C, and the solution of di-*tert*-butyl dicarbonate (12.5 g, 57.2 mmol, 1.5 eq.) in 30 mL of MeOH was added to the mixture. The reaction was stirred for 2 h at -80°C and the was allowed to come to room temperature. After, MeOH was evaporated under reduced pressure and crude was purified using a flash chromatography on silica gel with $CHCl_3$:MeOH (8:92) as a mobile phase to give 9.09 g, yield 72%. **^{1}H NMR** (500 MHz, $CDCl_3$) δ 1.35 (s, 18H, $(CH_3)_3$ x2 Boc), 1.56 – 1.61 (m, 4H, C-CH_2-C x2), 2.11 (s, 1H, C-NH-C), 2.56 – 2.60 (m, 4H, N-CH_2-C x2,), 3.12 (s, 4H, C-CH_2-NBoc x2), 5.31 (s, 2H, C-NH-Boc x2). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 28.42, 29.58, 38.72, 47.21, 78.92, 156.22. **HRMS-ESI** (*m/z*): $[M+H]^+$ calcd for $C_{16}H_{34}N_3O_4^+$, 332.25493; found, 332.25420.

Bis(3-Boc-aminopropyl)propargylamine (39)



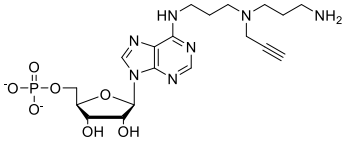
To the solution of bis(3-Boc-aminopropyl)amine (**38**, 3.6 g, 10.87 mmol), TEA (4.5 mL, 32.63 mmol) in ACN (20 mL), propargyl bromide (80% v/v in toluene) was added (3.7 mL, 32.62 mmol). The mixture was stirred for 48 h and solvent was evaporated under reduced pressure. The residue was dissolved in DCM (30 mL) and was washed with water (3 x 30 mL), dried with $MgSO_4$ and concentrated. The resulting residue was purified by a flash chromatography on silica gel with DCM:MeOH (80:20) as a mobile phase. The product was obtained as a colorless oil 2.5 g, yield 62%. **^{1}H NMR** (500 MHz, DMSO-d_6) δ 1.37 (s, 18H, $(CH_3)_3$ x2 Boc), 1.46 (p, J = 7.1 Hz, 4H, C-CH_2-C), 2.36 (t, J = 6.9 Hz, 4H, N-CH_2-C x2), 2.91 (q, J = 6.6 Hz, 4H, C-CH_2-NBoc x2), 3.04 (t, J = 2.3 Hz, 1H, $CH_{propargyl}$), 3.32 (s, 2H CH_2-$C_{propargyl}$), 6.75 (t, J = 5.7 Hz, 2H, C-NH-Boc x2). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 27.22, 28.54, 39.13, 41.40, 51.36, 73.18, 79.05, 156.17. **HRMS–ESI** (*m/z*): $[M+H]^+$ calcd for $C_{19}H_{36}N_3O_4^+$, 370.27058; found, 370.26970.

Bis(3-aminopropyl)propargylamine (9)



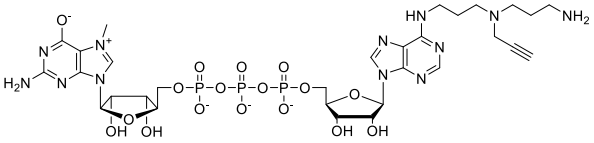
Bis(3-Boc-aminopropyl)propargylamine (**39**, 2.23 mL, 5.68 mmol) was dissolved in DCM (3 mL) and TFA was added (3 mL). The mixture was stirred for 6 h at room temperature and evaporated under reduced pressure. The residue was co-evaporated with DCM 3 times. The product was obtained quantitively and was used without further purification 1.09 g, yield 95%. **^{1}H NMR** (500 MHz, DMSO-d_6) δ 1.67 (p, J = 6.9 Hz, 4H, C-CH_2-C), 2.46 (t, J = 6.7 Hz, 4H), 2.85 – 2.75 (m, 4H), 3.16 – 3.11 (m, 1H), 3.37 (d, J = 2.4 Hz, 5H, overlapped with a solvent), 7.92 (s, 5H). **^{13}C NMR** (126 MHz, DMSO-d_6) δ 24.61, 37.22, 40.35, 49.66, 76.07, 78.26. **HRMS–ESI** (*m/z*): $[M+H]^+$ calcd for $C_9H_{20}N_3^+$, 170.16572; found, 170.16515.

Adenosine-Bis(3-aminopropyl)propargylamine 5'-monophosphate / NH_4^+ (11)



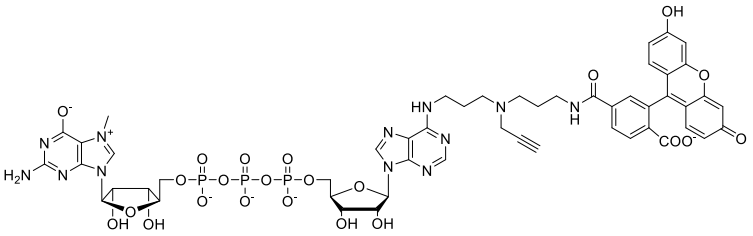
6-Chlor-AMP/TEA$^+$ (**10**, 200 mg, 0.32 mmol, 6245 mOD) was dissolved in H_2O and Bis(3-aminopropyl)propargylamine (**9**, 5.29 mmol) was added and pH was adjusted to 8. The mixture was stirred for 4-5 days at 65°C. The progress of the reaction was monitored using HPLC. The desired product was then purified by ion-exchange chromatography on DEAE Sephadex A-25 and evaporated to dryness. The product was additionally purified by semi-preparative HPLC to afford 4586 mOD, yield 73%. **^{1}H NMR** (500 MHz, D_2O) δ 2.03 – 2.10 (m, 4H, CH_2 x2), 2.92 – 2.93 (m, 1H, $CH_{propargyl}$), 3.03 – 3.04 (m, 2H, CH_2), 3.23 – 3.30 (m, 4H, CH_2 x2), 3.60 (s, 2H, NH_2), 3.99 (s, 2H, CH_2-propargyl), 4.02 – 4.10 (m, 2H, CH_2-5', 5"), 4.31 – 4.33 (m, 1H, CH-4'), 4.42 – 4.44 (m, 1H, CH-3'), 4.68 (t, J = 5.4 Hz, 1H, CH-2', overlapped with D_2O), 6.04 (d, J = 5.6 Hz, 1H, CH-1'), 8.16 (s, 1H, CH-8), 8.39 (s, 1H, CH-2). **^{31}P NMR** (202 MHz, D_2O) δ 1.70 (t, J = 4.9 Hz). **HRMS–ESI** (*m/z*): $[M-H]^-$ calcd for $C_{19}H_{29}N_7O_7P^-$, 498.18716; found, 498.18749.

m^{7}GpppA-Bis(3-aminopropyl)propargylamine / NH_4^+ (13)



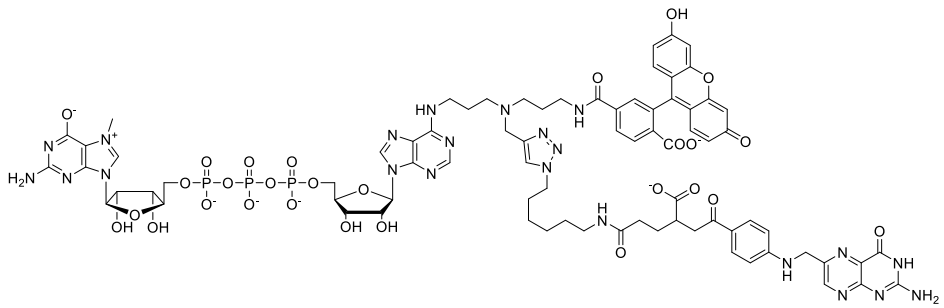
The TEA salt of 7-methylguanosine 5'-diphosphate imidazolide (**12**, 100 mg, 0.18 mmol) and adenosine-Bis(3-aminopropyl)propargyl amine 5'-monophosphate (**11**, 130 mg, 0.18 mmol) were dissolved in anhydrous DMF (5 mL) and then $ZnCl_2$ (200 mg, 1.46 mmol) was added. The mixture was stirred at room temperature for 12 h. Next, a solution of EDTA (1.46 mmol) in 40 mL of H_2O was poured into the reaction mixture and pH was adjusted to 7 with 1M $NaHCO_3$. The product was isolated using DEAE Sephadex on a linear gradient of TEAB (0–0.8 M) to obtain the product as the TEA salt. The final product was purified additionally on a semi-preparative liquid chromatography column resulting 190.75 mg, 0.203 mmol, 3720 mOD, yield 78%.
^{1}H NMR (500 MHz, D_2O) δ 2.07 – 2.10 (m, 4H), 3.06 (t, J = 7.7 Hz, 2H), 3.19 – 3.24 (m, 4H), 3.69 (s, 2H), 3.95 (s, 2H), 4.03 (s, 3H), 4.24 – 4.27 (m, 3H), 4.34 – 4.39 (m, 3H), 4.44 (t, J = 5.2 Hz, 1H), 4.52 (dd, J = 5.0, 3.5 Hz, 1H), 4.57 (dd, J = 4.9, 3.5 Hz, 1H), 4.72 (t, J = 5.4 Hz, 1H), 5.92 (d, J = 3.5 Hz, 1H), 6.05 (d, J = 5.8 Hz, 1H), 8.20 (s, 1H), 8.44 (s, 1H). **^{31}P NMR** (202.49 MHz, D_2O) δ -10.62, -22.13 (t, J = 19.2 Hz). **HRMS–ESI** (*m/z*): $[M-H]^-$ calcd for $C_{30}H_{44}N_{12}O_{17}P_3^-$, 937.21657; found, 937.21588.

m^{7}GpppA-propargyl-FAM / NH_4^+(14)



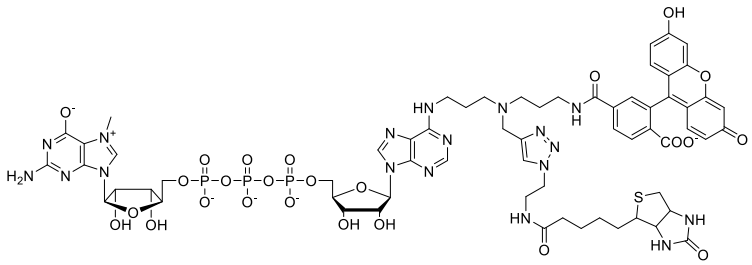
To m^{7}GpppA-Bis(3-aminopropyl)-propargyl amine (**13**, 5 mg, 5.3 µmol, 1 eq.) dissolved in 180 µL of a borate buffer (pH 8.4) was added 6-isomer of carboxyfluorescein-*N*-hydroxy-succinimide (FAM-NHS, 8.7 mg, 18.4 µmol, 4 eq.) dissolved in 20 µL of DMSO. The reaction mixture was stirred in room temperature for 12 h. The progress of the reaction was monitored using HPLC chromatography (C18 column in ammonium acetate buffer pH 5.9:acetonitrile in a gradient 0-100% in 20 min). The crude product was then purified using HPLC chromatography using the same separation conditions to give 3.46 µmol, yield 47%. **HRMS–ESI** (*m/z*): $[M-H]^-$ calcd for $C_{51}H_{53}N_{12}O_{23}P_3^-$, 1295.26431; found, 1295.26547.

m^{7}GpppA-FAM-FA / NH$_4^+$ (15)



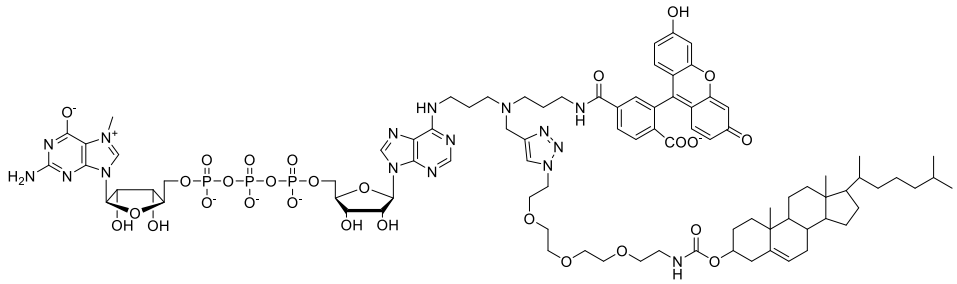
Folic acid-hexamine-azide (0.43 mg, 0.75 µmol, 1 eq.) was stirred with DMSO (150 µL) in 50°C resulting clear orange solution of FA-N_3. To this solution dinucleotide analogue (**14**, 1 mg, 0.75 µmol, 1 eq.) dissolved in 100 µL of H_2O was added. To the mixture was added in the following order sodium ascorbate (0.3 µmol, 0.4 eq.) and copper(II) sulfate pentahydrate (0.3 µmol, 0.4 eq.) both dissolved in 25 µL of H_2O. The reaction mixture was stirred at room temperature for 4 h and then EDTA solution was added to quench the reaction. The purification of the resulting product was performed using RP-HPLC on Phenomenex Gemini NX-C18 chromatography column in the gradient of ammonium acetate pH 8.4: acetonitrile 5-30% in 15 min to give 0.24 µmol, 22%. **HRMS–ESI** (*m/z*): $[M-2H]^{2-}$ calcd for $C_{76}H_{85}N_{23}O_{28}P_3^{2-}$, 929.75400; found, 929.75578.

m^{7}GpppA-FAM-Biotin / NH$_4^+$ (16)



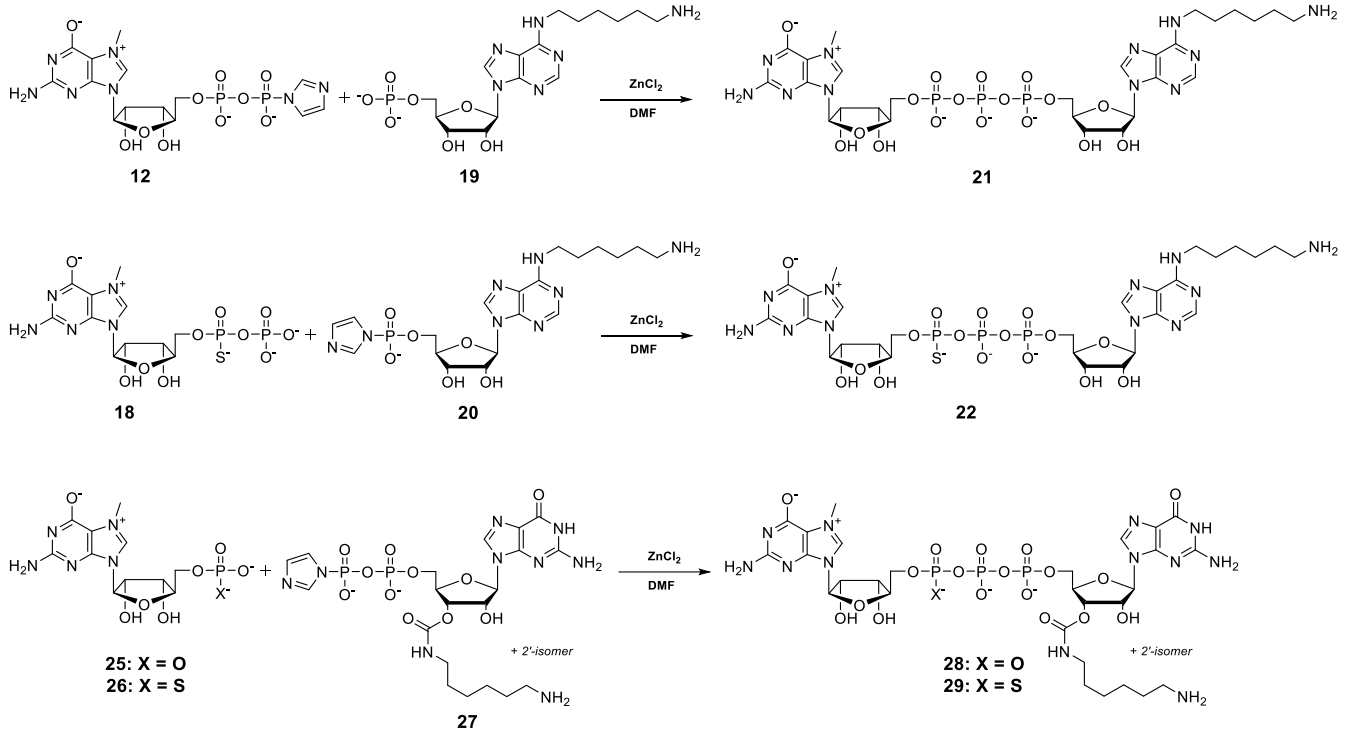
Biotin azide (0.24 mg, 0.75 µmol, 1 eq.) and dinucleotide analogue (**14**, 1 mg, 0.75 µmol, 1 eq.) were dissolved in 100 µL of H_2O. To the mixture was added in the following order sodium ascorbate (0.6 µmol, 0.4 eq.) and copper(II) sulfate pentahydrate (0.48 µmol, 0.4 eq.) both dissolved in 25 µL of H_2O. The reaction mixture was stirred at room temperature for 4 h and then EDTA solution was added to quench the reaction. The purification of the resulting product was performed using RP-HPLC on Supelco C8 column in the gradient of ammonium acetate pH 5.9: acetonitrile 0-100% in 15 min to give 0.45 µmol, 48%. **HRMS–ESI** (*m/z*): $[M-H]^-$ calcd for $C_{63}H_{74}N_{18}O_{25}P_3S^-$, 1607.40115; found, 1607.40325.

m^{7}GpppA-FAM-CHOL / NH$_4^+$ (17)



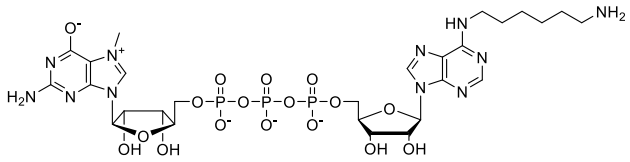
FAM-labeled dinucleotide (**14**, 3.8 mM, 0.76 µmol, 21.58 mOD) was dissolved in 2M triethylammonium acetate buffer pH 7.0 (123.5 µL) and 172 µL of DMSO. Then, 7.6 µL of 10 mM solution of cholesteryl-TEG-azide dissolved in DMSO was added and vortexed. To this solution, 38 µL of sodium ascorbate (5 mM in H_2O) was added and mixed shortly. The mixture was degassed under inert atmosphere for 30 seconds. Then, 19 µL of Cu(II)-TBTA (10 mM, 55% in DMSO) was added to the reaction mixture and stirred for 6h in room temperature. After reaction completion, EDTA solution was added to quench the reaction. The crude was purified using RP-HPLC Thermo Scientific Hypersil GOLD™ C4 column in the gradient of BufferA:BufferB 0-100% in 15 min, then 5 min BufferA:BufferB 100-0%, then 10 min BufferA:acetonitrile 0-100%. Analytical profile was recorded using the gradient BufferA:acetonitrile 0-100% in 20 min to give 0.31 µmol, yield 27%. **HRMS–ESI** (*m/z*): $[M-2H]^{2-}$ calcd for $C_{87}H_{115}N_{16}O_{28}P_3^{2-}$, 962.364525; $[M+2H]^{2+}$ calcd for $C_{87}H_{119}N_{16}O_{28}P_3^{2+}$, 964.37908.

Cap-Linker analogues (21-22, 28-29)



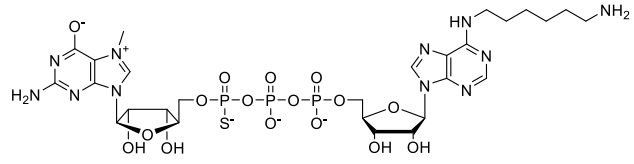
Scheme S4. Synthesis of cap-linker conjugates strategies (**21-22**, **28-29**).

m^{7}GpppA-HDA / NH$_4^+$ (21)



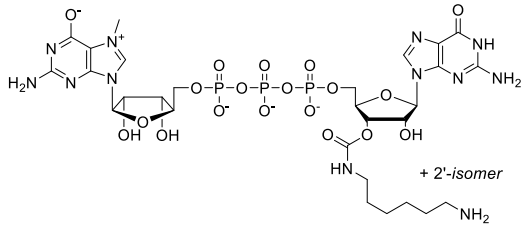
The TEA salt of 7-methylguanosine 5'-diphosphate imidazolide (**12**, 100 mg, 0.18 mmol, 2150 mOD) and adenosine-6-hexamine 5'-monophosphate (**19**, 200 mg, 0.37 mmol, 4860 mOD) were dissolved in anhydrous DMF (5 mL) and then $ZnCl_2$ (400 mg, 2.94 mmol) was added. The mixture was stirred at room temperature for 12 h. Next, a solution of EDTA (2.94 mmol) in 40 mL of H_2O was poured into the reaction mixture and pH was adjusted to 7 with 1M $NaHCO_3$. The product was isolated using DEAE Sephadex on a linear gradient of TEAB (0–0.8 M to obtain the product as the TEA salt. The final product was purified additionally on a semi-preparative liquid chromatography column resulting the 0.14 µmol, 133.6 mg, 2341 mOD, yield 84%. **^{1}H NMR** (500 MHz, D_2O) *δ* 1.40 (m, 5H), 1.65 (m, 5H), 2.92 – 3.00 (m, 2H), 3.47 (s, 1H), 3.99 (s, 3H), 4.25 – 4.28 (m, 2H), 4.34 – 4.36 (m, 1H), 4.39 (m, 2H), 4.51 (m, 2H), 4.69 (t, *J* = 5.6 Hz, 1H), 5.86 (d, *J* = 3.4 Hz, 1H), 6.01 (d, *J* = 6.1 Hz, 1H), 8.12 (s, 1H), 8.39 (s, 1H). **^{31}P NMR** (202 MHz, D_2O) *δ* -22.19 (t, *J* = 19.3 Hz, 1P), -10.65 (m, 2P). **HRMS–ESI** (*m/z*): $[M-H]^-$ calcd for $C_{27}H_{43}N_{11}O_{17}P_3^-$, 884.19112; found, 884.19180.

m^{7}Gp$_S$ppA-HDA-D1/D2 / NH$_4^+$ as a mixture of diastereoisomers D1/D2 (22)



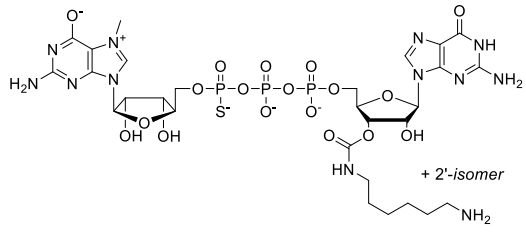
The TEA salt of 7-methylguanosine-5′-dithiophosphate D1/D2 (**18**, 5 mg, 7.42 µmol, 1 eq.) and adenosine-6-hexamine 5'-monophosphate imidazolide (**20**, 4.4 mg, 7.42 µmol, 1 eq.) were dissolved in anhydrous DMF (1.5 mL) and then $ZnCl_2$ (8.0 mg, 0.06 mmol, 8 eq.) was added. The mixture was stirred at room temperature for 12 h. Next, a solution of EDTA (0.06 mmol) in 12 mL of H_2O was poured into the reaction mixture and pH was adjusted to 7 with 1M $NaHCO_3$. The product was isolated using a semi-preparative liquid chromatography column resulting the yield 4.8 mg, 81 mOD, 38%. **^{1}H NMR D1/D2 isomer** ^{1}H NMR (500 MHz, D_2O) δ 1.37 – 1.50 (m, 6H), 1.59 – 1.76 (m, 6H), 2.97 (t, *J* = 7.8, 2.9 Hz, 3H), 3.56 (s, 2H), 4.02 (s, 3H), 4.26 – 4.31 (m, 2H), 4.34 – 4.41 (m, 3H), 4.46 – 4.49 (m, 1H), 4.52 – 4.54 (m, 1H), 4.55 – 4.60 (m, 1H), 4.70 (t, *J* = 5.5 Hz, 1H), 5.89 (d, *J* = 3.6 Hz, 1H), 6.02 (d, *J* = 5.9 Hz, 1H), 8.17 (s, 1H), 8.41 (s, 1H). **HRMS–ESI** (*m/z*): $[M-H]^-$ calcd for $C_{27}H_{41}N_{11}O_{16}P_3S^-$, 900.16718; found, 900.16877.

m^7GpppG-L_{6N} / NH_4^+ as a mixture of 2'-O/3'-O-isomers (28)



The TEA salt of 7-methylguanosine-5′-monophosphate (**25**, 1 eq.) and 2'-*O*/3'-*O*-carbamoyl-GDP-L_{6N} imidazolide (**27**, 1 eq.) were dissolved in a mixture of anhydrous DMF and DMSO (2:1 v/v) and then $ZnCl_2$ (16 eq.) was added. The mixture was stirred at room temperature for 2 days. Next, a solution of EDTA (8 eq.) in H_2O was poured into the reaction mixture and pH was adjusted to 7 with 1M $NaHCO_3$. The product was isolated using DEAE Sephadex on a linear gradient of TEAB (0–0.9 M) to obtain the product as the TEA salt. As described in the literature.[3] **^{1}H NMR** (400 MHz, D_2O) δ 1.15 – 1.32 (m, 3H), 1.33 – 1.44 (m, 6H), 1.47 – 1.71 (m, 6H), 2.89 – 3.06 (m, 5H), 3.06 – 3.25 (m, 3H), 4.01 – 4.04 (m, 3H), 4.21 – 4.31 (m, 5H), 4.31 – 4.49 (m, 9H), 4.51 – 4.59 (m, 2H), 4.62 – 4.67 (m, 1H), 4.84 – 4.91 (m, 1H), 5.23 – 5.30 (m, 1H), 5.40 (t, *J* = 5.3 Hz, 1H), 5.81 (d, *J* = 6.9 Hz, 1H), 5.88 – 5.94 (m, 2H), 8.01 (s, 1H), 8.05 (s, 1H), 9.00 – 9.03 (m, 1H), 9.06 (s, 1H). **^{31}P NMR** (162 MHz, D_2O) δ -22.48 – -21.99 (m, 1P), -10.70 (dt, *J* = 19.6, 5.6 Hz, 2P). **HRMS–ESI** (*m/z*): $[M-H]^-$ calcd for $C_{28}H_{42}N_{12}O_{19}P_3^-$, 943.19075; found 943.19182.

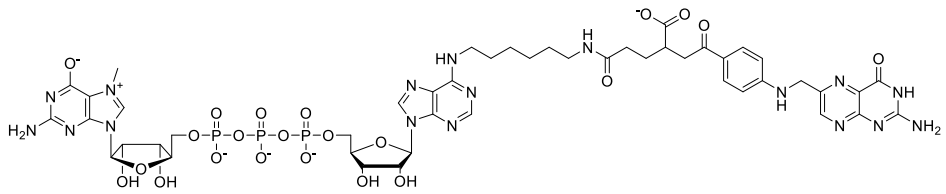
m^7Gp_SppG-L_{6N} D1/D2 / NH_4^+ as a mixture of D1/D2 diastereoisomers and 2'-O/3'-O isomers (29)



The TEA salt of 7-methylguanosine-5′-monothiophosphate (**26**, 1 eq.) and 2'-*O*/3'-*O*-carbamoyl-GMP-L_{6N} imidazolide (**27**, 1 eq.) were dissolved in a mixture of anhydrous DMF and DMSO (1:1 v/v) and then $ZnCl_2$ (8 eq.) was added. The mixture was stirred at room temperature for 4 days. Next, a solution of EDTA (8 eq.) in H_2O was poured into the reaction mixture and pH was adjusted to 7 with 1M $NaHCO_3$. The product was isolated using DEAE Sephadex on a linear gradient of TEAB (0–0.9 M) to obtain the product as the TEA salt. As described in the literature. [3] **^{1}H NMR** (400 MHz, D_2O) δ 1.14 – 1.33 (m, 3H), 1.34 – 1.37 (m, 3H), 1.37 – 1.42 (m, 2H), 1.46 – 1.61 (m, 3H), 1.61 – 1.70 (m, 2H), 2.89 – 3.06 (m, 5H), 3.08 – 3.23 (m, 2H), 4.02 – 4.11 (m, 5H), 4.24 – 4.62 (m, 16H), 5.27 (dd, *J* = 5.4, 3.1 Hz, 1H), 5.37 – 5.46 (m, 1H), 5.81 (dd, *J* = 6.9, 2.5 Hz, 1H), 5.86 – 5.96 (m, 2H), 8.00 – 8.08 (m, 2H), 9.08 – 9.17 (m, 2H). **^{31}P NMR** (162 MHz, D_2O) δ -23.53 – -22.95 (m, 2P), -11.07 – -10.55 (m, 2P), 43.79 (d, *J* = 26.1 Hz, 1P), 44.22 (d, *J* = 27.3 Hz, 1P). **HRMS–ESI** (*m/z*): $[M-H]^-$ calcd for $C_{28}H_{42}N_{12}O_{18}P_3S^-$, 959,16791; found, 959.16939, 959.16902, 959.16910, 959.16890.

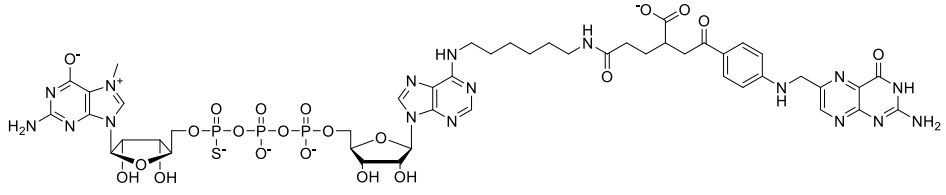
Cap-Ligand analogues (23-24 a-b and 30-31 a-b)

m^7GpppA-HDA-FA / NH_4^+ (23a)



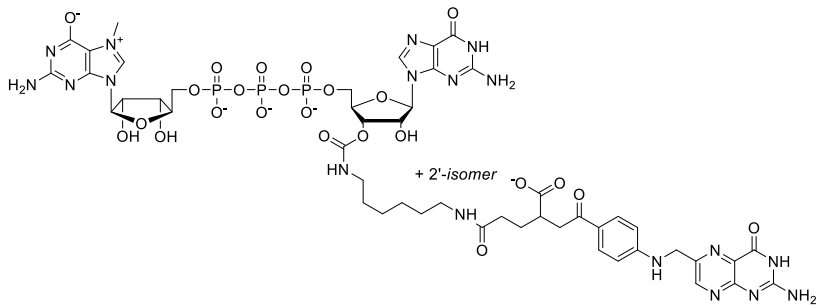
To the solution of m^7GpppA-HDA (**21**, 1 mg, 1.38 µmol, 1.5 eq.) dissolved in 80 µL of a borate buffer (pH 8.4) was added folic acid-*N*-hydroxy-succinimide (FA-NHS, 0.5 mg, 0.92 µmol, 1 eq.) dissolved in 20 µL of DMSO. The reaction mixture was stirred in room temperature for 12 h. The progress of the reaction was monitored using HPLC chromatography (Phenomenex Gemini NX-C18 column in ammonium acetate buffer pH 8.4:acetonitrile in a gradient 5-30% in 20 min). The crude product was then purified using HPLC chromatography using the same separation conditions to give 0.53 µmol, yield 31%. **^{1}H NMR** (500 MHz, D_2O) δ 0.96 – 1.02 (m, 4H), 1.23 – 1.33 (m, 3H), 2.26 – 2.38 (m, 2H), 2.41 – 2.48 (m, 1H), 2.87 – 2.94 (m, 2H), 3.00 (s, 1H), 3.99 (s, 4H), 4.24 – 4.33 (m, 4H), 4.34 – 4.45 (m, 6H), 4.50 – 4.54 (m, 2H), 4.57 (s, 2H), 4.64 – 4.71 (m, 1H), 5.84 – 5.86 (m, 1H), 5.99 (d, *J* = 5.8 Hz, 1H), 6.85 (d, *J* = 8.4 Hz, 2H), 7.68 (d, *J* = 8.4 Hz, 2H), 8.03 (d, *J* = 5.1 Hz, 1H), 8.34 (s, 1H), 8.58 (d, *J* = 3.6 Hz, 1H), 9.00 (s, 1H). **HRMS–ESI** (*m/z*): $[M-H]^-$ calcd for $C_{46}H_{58}N_{18}O_{22}P_3^-$, 1307.31914; found, 1307.31904.

m^7Gp_SppA-HDA-FA D1/D2 / NH_4^+ as a mixture of D1/D2 diastereoisomers (24a)



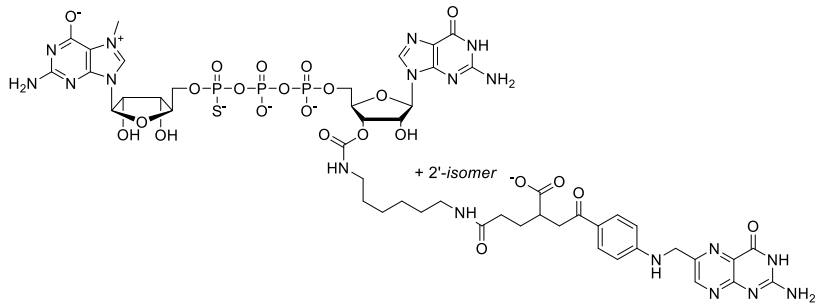
To the solution of m^7Gp_SppA-HDA D1/D2 (**22**, 1 mg, 1.36 µmol, 1.5 eq.) dissolved in 80 µL of a borate buffer (pH 8.4) was added folic acid-*N*-hydroxysuccinimide (FA-NHS, 0.5 mg, 0.90 µmol, 1 eq.) dissolved in 20 µL of DMSO. The reaction mixture was stirred in room temperature for 12 h. The progress of the reaction was monitored using HPLC chromatography (Phenomenex Gemini NX-C18 column in ammonium acetate buffer pH 8.4:acetonitrile in a gradient 5-30% in 20 min). The crude product was then purified using HPLC chromatography using the same separation conditions to give 0.21 µmol, yield 12%. **HRMS–ESI D1** (*m/z*): $[M-H]^-$ calcd for $C_{46}H_{58}N_{18}O_{21}P_3S^-$, 1323.29629; found, 1323.29742. **HRMS–ESI D2** (*m/z*): $[M-2H]^{2-}$ calcd for $C_{46}H_{57}N_{18}O_{21}P_3S^{2-}$, 661.14451; found, 661.14527.

m^{7}GpppG-L$_{6N}$-FA / NH$_4^+$ as a mixture of 2'-O/3'-O-isomers (30a)



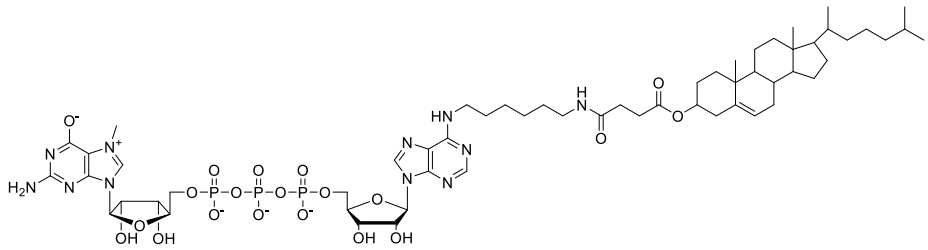
To the solution of m^{7}GpppG-L$_{6N}$ (**28**, 0.5 mg, 0.66 µmol, 1.5 eq.) dissolved in 50 µL of a borate buffer (pH 8.4) was added Folic acid-*N*-hydroxysuccinimide (FA-NHS, 0.23 mg, 0.44 µmol, 1 eq.) dissolved in 10 µL of DMSO. The reaction mixture was stirred in room temperature for 12 h. The progress of the reaction was monitored using HPLC chromatography (Phenomenex Gemini NX-C18 column in ammonium acetate buffer pH 8.4:acetonitrile in a gradient 5-30% in 20 min). The crude product was then purified using HPLC chromatography using the same separation conditions to give 0.17 µmol, yield 20%. **HRMS–ESI** (*m/z*): [M-H]$^-$ calcd for $C_{47}H_{59}N_{19}O_{24}P_3^-$, 1366.31987; found, 1366.32061.

m^{7}Gp$_S$pppG-L$_{6N}$-FA D1/D2 / NH$_4^+$ as a mixture of D1/D2 diastereoisomers and 2'-O/3'-O isomers (31a)



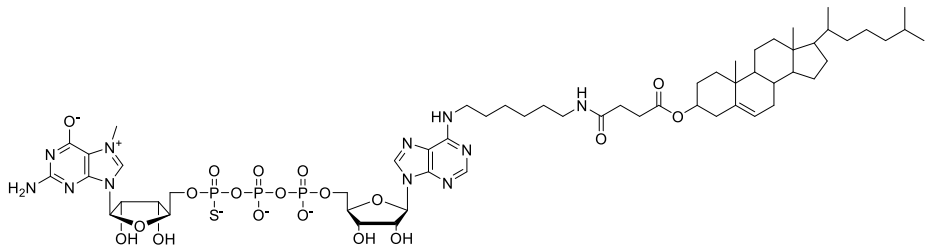
To the solution of m^{7}Gp$_S$ppG-L$_{6N}$ (**29**, 1.0 mg, 1.3 µmol, 1.5 eq.) dissolved in 80 µL of a borate buffer (pH 8.4) was added Folic acid-*N*-hydroxysuccinimide (FA-NHS, 0.46 mg, 0.86 µmol, 1 eq.) dissolved in 20 µL of DMSO. The reaction mixture was stirred in room temperature for 12 h. The progress of the reaction was monitored using HPLC chromatography (Phenomenex Gemini NX-C18 column in ammonium acetate buffer pH 8.4:acetonitrile in a gradient 5-30% in 20 min). The crude product was then purified using HPLC chromatography using the same separation conditions to give 0.45 µmol, yield 28%. **HRMS–ESI** (*m/z*): [M-H]$^-$ calcd for $C_{47}H_{59}N_{19}O_{23}P_3S^-$, 1382.29702; found, 1382.29795.

m^{7}GpppA-HDA-CHOL / NH$_4^+$ (23b)



To the solution of m^{7}GpppA-HDA (**21**, 0.25 mg, 0.35 µmol, 1.5 eq.) dissolved in 10 µL of a borate buffer (pH 8.4) was added cholesteryl hemisuccinate-NHS (CHOL-NHS, 0.13 mg, 0.23 µmol, 1 eq.) dissolved in 10 µL of DMSO. The reaction mixture was stirred in room temperature for 12 h. The progress of the reaction was monitored using HPLC chromatography. The crude product was then purified using HPLC chromatography (C4 column) to give 0.18 µmol, yield 39%. **HRMS–ESI** (*m/z*): [M-H]$^-$ calcd for $C_{58}H_{89}N_{11}O_{20}P_3^-$, 1352.55037; found, 1352.55164.

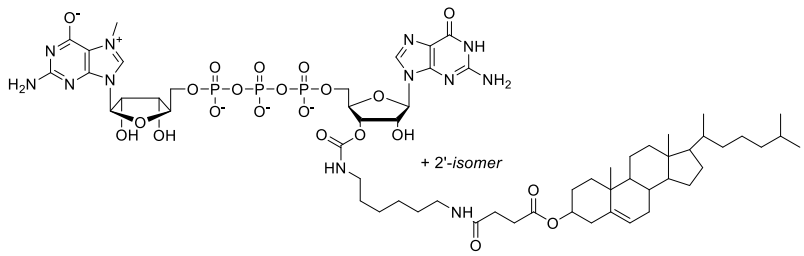
m^{7}Gp$_S$ppA-HDA-CHOL D1/D2 / NH$_4^+$ as a mixture of D1/ D2 diastereoisomers (24b)



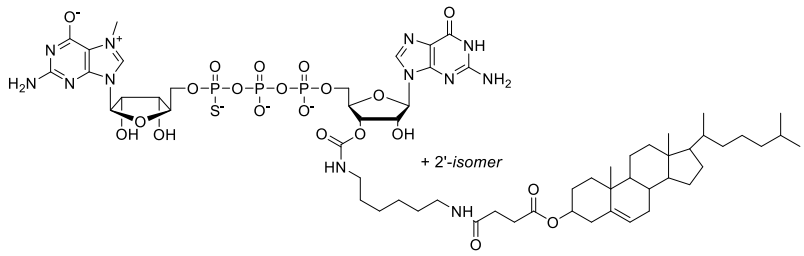
To the solution of m^{7}Gp$_S$ppA-HDA D1/D2 (**22**, 0.5 mg, 0.69 µmol, 1.5 eq.) dissolved in 25 µL of a borate buffer (pH 8.4) was added cholesteryl hemisuccinate-NHS (CHOL-NHS, 0.25 mg, 0.5 µmol, 1 eq.) dissolved in 25 µL of DMSO. The reaction mixture was stirred in room temperature for 12 h. The progress of the reaction was monitored using HPLC chromatography. The crude product was then purified using HPLC chromatography (C4 column) to give 0.16 µmol, yield 18%. **HRMS–ESI** (*m/z*): [M-2H]$^{2-}$ calcd for $C_{58}H_{88}N_{11}O_{19}P_3S^{2-}$, 683.76013; found, 684.77740.

m^7GpppG-L_{6N}-CHOL / NH_4^+ as a mixture of 2'-O/3'-O-isomers (30b)

To the solution of m^7GpppG-L_{6N} (**28**, 0.5 mg, 0.65 µmol, 1.5 eq.) dissolved in 25 µL of a borate buffer (pH 8.4) was added cholesteryl hemisuccinate-NHS (CHOL-NHS, 0.24 mg, 0.44 µmol, 1 eq.) dissolved in 25 µL of DMSO. The reaction mixture was stirred in room temperature for 12 h. The progress of the reaction was monitored using HPLC chromatography. The crude product was then purified using HPLC chromatography (C4 column) to give 0.2 µmol, yield 20%. **HRMS–ESI** (*m/z*): $[M-H]^-$ calcd for $C_{59}H_{90}N_{12}O_{22}P_3^-$, 1411.55110; found, 1411.55191.



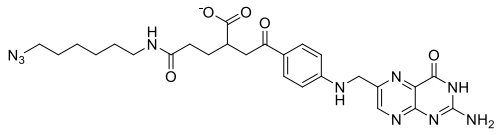
m^7Gp_SppG-L_{6N}-CHOL D1/D2 / NH_4^+ as a mixture of D1/D2 diastereoisomers and 2'-O/3'-O isomers (31b)



To the solution of m^7Gp_SppG-L_{6N} D1/D2 (**29**, 0.5 mg, 0.65 µmol, 1.5 eq.) dissolved in 25 µL of a borate buffer (pH 8.4) was added cholesteryl hemisuccinate-NHS (CHOL-NHS, 0.23 mg, 0.44 µmol, 1 eq.) dissolved in 25 µL of DMSO. The reaction mixture was stirred in room temperature for 12 h. The progress of the reaction was monitored using HPLC chromatography. The crude product was then purified using HPLC chromatography (C4 column) to give 0.63 µmol, yield 47%. **HRMS–ESI** (*m/z*): $[M-H]^-$ calcd for $C_{59}H_{90}N_{12}O_{21}P_3S^-$, 1427.52825; found 1427.52933.

Miscellaneous

Folate-HDA-N_3 (40)



To the solution of folic acid N-hydroxysuccinimide (FA-NHS, 200 mg, 0.37 mmol, 1.5 eq.) dissolved in 10 mL of DMSO was added 6-azidohexan-1-amine (21.3 mg, 0.25 mmol, 1 eq.) and 50 µL of TEA. The reaction mixture was stirred in room temperature for 12 h. The progress of the reaction was monitored using HPLC chromatography. The crude product was then purified using HPLC chromatography to give 142 mg, yield 75%. **^{1}H NMR** (500 MHz, DMSO-d_6) δ 1.17 – 1.31 (m, 5H), 1.31 – 1.39 (m, 2H), 1.44 – 1.53 (m, 2H), 1.98 – 2.09 (m, 1H), 2.09 – 2.19 (m, 2H), 2.95 – 3.04 (m, 2H), 3.28 (t, *J* = 6.9 Hz, 4H), 3.36 (s, 3H), 4.15 – 4.23 (m, 1H), 4.47 (d, *J* = 5.9 Hz, 2H), 6.64 (d, *J* = 8.4 Hz, 2H), 6.91 (t, *J* = 6.1 Hz, 1H), 7.31 (s, 1H), 7.62 (d, *J* = 8.3 Hz, 2H), 7.81 (t, *J* = 5.7 Hz, 1H), 7.88 – 7.95 (m, 1H), 8.63 (s, 1H). **HRMS–ESI** (*m/z*): $[M-H]^-$ calcd for $C_{25}H_{30}N_{11}O_5^-$, 564.24369; found, 564.24396.

Biological procedures

Flow cytometry

FR positive (FR+) MDA-MB-231 (human mammary gland carcinoma, ATCC HTB-26) cells and FR negative (FR-) A549 (human lung carcinoma, ATCC CCL-185) cells were grown in RPMI 1640 medium without folic acid (Gibco) supplemented with 10% FBS (Sigma), GlutaMAX (Gibco) and 1% penicillin/streptomycin (Gibco) at 5% CO_2 and 37 °C. SK-RB-3 (human mammary gland carcinoma, ATCC HTB-30) cells were grown in in DMEM (Gibco) supplemented with 10% FBS, GlutaMAX and 1% penicillin/streptomycin at 5% CO_2 and 37 °C. In a typical experiment, 24 h before treatment $5 \cdot 10^5$ cells were seeded in 3 ml medium per well of 6-well plate. In the day of experiment, cells were washed with PBS and selected fluorescent compound was added in medium without FBS. After 2 hours incubation medium was removed, cells were washed with PBS and subjected to trypsinization. Detached cells were incubated with Zombie Violet dye (BioLegend) prior flow cytometry analysis using LSR Fortessa flow cytometer (BD Biosciences). Data was analysed using FlowJo software v10 (Tree Star).

For assessing cell apoptosis, 10^5 K562 cells were seeded in 200 ul Opti-MEM medium containing tested compound per well of 48-well plate. After 20 hours incubation cells were washed with PBS and FACS Buffer (1 x PBS, 2% BSA) and incubated with Annexin V-FITC (BioLegend) and propodium iodide (BioLegend) for 15 min at RT. Samples were analysed using LSR Fortessa flow cytometer and data was analysed using FlowJo software

Cell viability assay

MDA-MB-231 (FR+) cells were grown as mentioned before. In a typical experiment, 24 h before transfection $4 \cdot 10^3$ cells were seeded in 100 µl medium per well of 96-well plate. In the day of experiment, cells were washed with PBS and selected compound was added in medium without FBS. Moreover, K562 (human bone marrow carcinoma, ATCC CCL-243) cells were grown in RPMI 1640 (Gibco) supplemented with 10% FBS, sodium pyruvate (Gibco), 1% penicillin/streptomycin at 5% CO_2 and 37 °C. In the day of experiment, 10^4 K562 cells were seeded in 100 µl Opti-MEM (Gibco) medium containing selected compound per well of 96-well plate. After 72 hours incubation, cells were subjected to Rezasurin viability assay. Briefly, 10 µl of 440 mM Rezasurin (Sigma) was added to each well and after 4 hours incubation fluorescence (exc. 550 nm, em. 580 nm) was measured on Synergy H1 (BioTek) microplate reader. Before normalization of fluorescence values to mock treated cells background fluorescence of medium was extracted.

Microscopy

MDA-MB-231 (FR+) and A549 (FR-) cells were grown as mentioned before. In a typical experiment, 24 h before treatment 2·104 cells were seeded in 500 µl medium per well supplemented with glass coverslips of 24-well plate. In the day of experiment, cells were washed with PBS and selected compound in medium without FBS was added. After 2 hours incubation, cells were washed with PBS and fixed using 4% paraformaldehyde in PBS for 10 min at RT. Then, cells nuclei were stained using Hoechst (Sigma-Aldrich). Cells samples were imaged using Carl Zeiss LSM 700 with Axio Imager Z2 confocal laser scanning microscope, using a 40x/1.3 oil objective. The Hoechst emission and FAM emission were detected at emission spectra of 435-483 nm and 505-600 nm, respectively, after excitation at 405 nm for Hoechst and 488 nm for FAM.

For cap immunofluorescence, cells after fixation were stained with mouse anti-cap antibody (H20, Merck Millipore) followed by staining with secondary goat anti-mouse Alexa 594 conjugated antibody. Then, cells nuclei were stained using Hoechst. Cells samples were imaged also using Carl Zeiss LSM 700 with Axio Imager Z2 confocal laser scanning microscope, using a 40x/1.3 oil objective. The Hoechst emission and Alexa 594 emission were detected at emission spectra of 435-483 nm and 585-800 nm, respectively, after excitation at 405 nm for Hoechst and 555 nm for Alexa 594.

Translation inhibition in rabbit reticulocyte lysate

Inhibition of cap dependent translation in rabbit reticulocyte lysate (RRL) system (Promega) by cap analogues were performed as described previously[6]. In vitro translation reaction (9 µl) contained a tested compound was incubated at 30 °C for 1 hour and then 1 µl of 10 µg/ml ARCA-capped mRNA encoding Gaussia luciferase was added to start translation. Reactions were further incubated at 30 °C for 1 hour and stopped afterwards by freezing in liquid nitrogen. To detect luminescence from Gaussia luciferase, 50 µl of 10 ng/ml h-coelenterazine (NanoLight) in PBS was added to 10 µl of RRL and the luminescence was measured on Synergy H1 (BioTek) microplate reader. Obtained luminescence values were normalized to control reaction without any inhibitor.

Determination of dissociation constants with murine eIF4E protein (pyrene fluorescence intensity binding method, PyFLINT-B)

To determine dissociation constants of the ligands binding to the eIF4E we used previously described pyrene fluorescence intensity binding method[7]. Briefly, the competitive binding experiments were performed using plate reader (Synergy H1 Microplate Reader Biotek) in 96-well, black, non-binding assay plates with point fluorescence measurements (λ_{exc} = 345 nm; λ_{em} = 378 nm). Each well contained a buffer (50 mM Hepes/KOH pH = 7.2 containing 100 mM KCl and 0.5 mM EDTA), 10 nM of pyrene-labeled 7-methylguanosine pentaphosphate probe, non-fluorescent ligand (half-log dilutions of $C_{lig.}$ from 100 μM to 0.003 μM or from 31.6 μM to 0.001 μM), and 75 nM of eIF4E protein. The reagents and protein were pre-incubated for 15 min at 20°C and stirred at 300 rpm. Measurements were performed at three different temperatures: 20, 30, and 37°C in three or two repetitions. To the registered dependences of fluorescence intensity on ligand concentration the following equation was fitted:

$$F = F_0 + \frac{F_{\max} - F_0}{1 + \left(\frac{C_{\text{lig.}}}{K_{\text{D,app.}}}\right)^p}$$

where $K_{D,app.}$ is the inhibitor concentration required to replace 50% of the fluorescent probe from the protein binding site, $C_{lig.}$ is the concentration of non-fluorescent ligand and p is the hill slope. To fit the curve we used Origin 2017 (OriginLab Corp., Northampton, MA, USA) software.

Fluorescence Correlation Spectroscopy (FCS) and Fluorescence Lifetime Imaging Microscopy (FLIM)

FCS and FLIM measurements were performed using Nikon Eclipse TE2000U confocal microscope (Nikon, Japan) combined with the FCS PicoHarp 300 set (PicoQuant, Germany). The measurements were carried out in an Okolab climate chamber (Okolab, Italy) at 36° C. Fluorescence was induced by 485 nm or 561 nm pulse laser (depending on the label dye used, FITC or TRITC, respectively). Observations were carried out using a 60x lens (N.A. 1.2) with water immersion. Fluorescent signal was collected by Single Photon Avalanche Diodes (MPD and PerkinElmer). Each measurement was preceded by calibration in a suitable reference medium[8].

FCS in living cells was preceded with positioning of the focal volume in cytoplasmic area of the cell using confocal imaging module. Next, fluctuations of fluorescence intensity were recorded at a single spot. Autocorrelation curves were calculated using SymphoTime software and then fitted with appropriate diffusion model using QuickFit 3.0 software.

FLIM imaging and analysis was performed using SymphoTime software.

Probes

Calcein-AM (Sigma-Aldrich) and TRITC dextran conjugates: TRITC-dex 4.4 kDa and TRITC-dex 155 kDa (Sigma-Aldrich) were used to sample the nanoscale viscosity of MDA-MB-231 cells. These probes are well characterized for diffusion in the cytoplasm[8].

Results and Discussion

Supporting tables

Table S1. Diffusion coefficients predicted for the probes at temperature 36°C.

Probe	FAM-PEG	FAM-PEG-FA (4)	FAM-PEG-Chol (6)	m^7GpppA-FAM-FA (15)	m^7GpppA-FAM-Chol (17)
r_p [nm]	0.81	0.85	0.79	1.4	2.0
D_{aq} [$\mu m^2/s$]*	395	376	405	235	159
D_{cyto} [$\mu m^2/s$]	198	187	205	96.6	58.5

r_p – hydrodynamic radius, D_{aq} – diffusion coefficient in water, D_{cyto} – diffusion coefficient in cytoplasm, * based on Stokes'a-Sutherland's-Einstein'a.

Table S2. Results of the measurement of the probes in water at 36°C.

Probe	FAM-PEG	FAM-PEG-FA (4)	FAM-PEG-Chol (6)	m^7GpppA-FAM-FA (15)	m^7GpppA-FAM-Chol (17)
D_{aq} [$\mu m^2/s$]	409 ± 70	386 ± 63	423 ± 81	235 ± 15	159 ± 36
r_p [nm]	0.81	0.85	0.79	1.4	2.0

D_{aq} – diffusion coefficient in water, r_p – hydrodynamic radius,

Table S3. Results of nanoviscosity probing in MDA-MB-231 cells (36°C)

Probe	Calcein-AM	TRITC-dex 4.4 kDa	TRITC-dex 155 kDa
r_p [nm][a]	0.65	1.3	8.6
η_{cyto}/η_{H2O}	2.2	3.2	5.2

r_p – hydrodynamic radius, η_{cyto} – diffusion coefficient in cytoplasm, η_{H2O} – diffusion coefficient in water, [a] – Kalwarczyk et al 2017

Supplementary figures

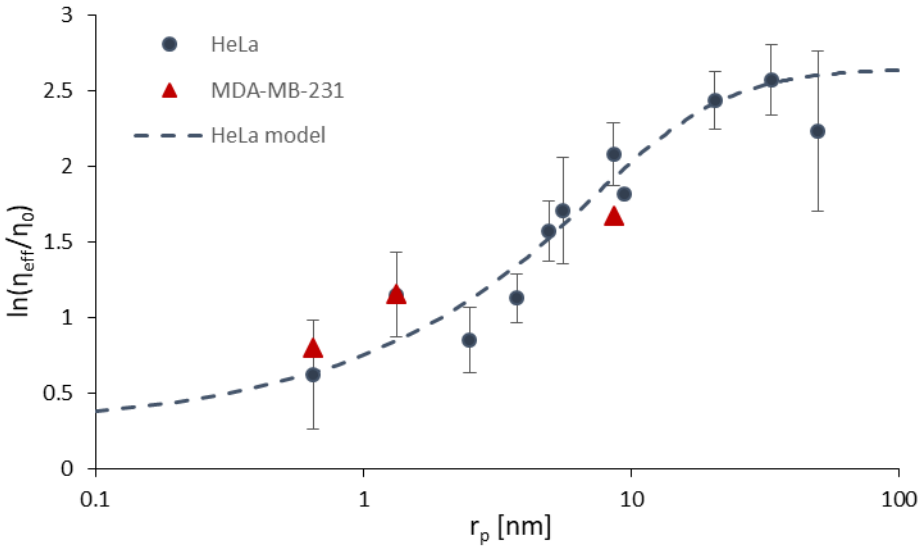


Figure S1. Comparison of nanoviscosity of the cytoplasm of MDA-MB-231 cells and HeLa cells.

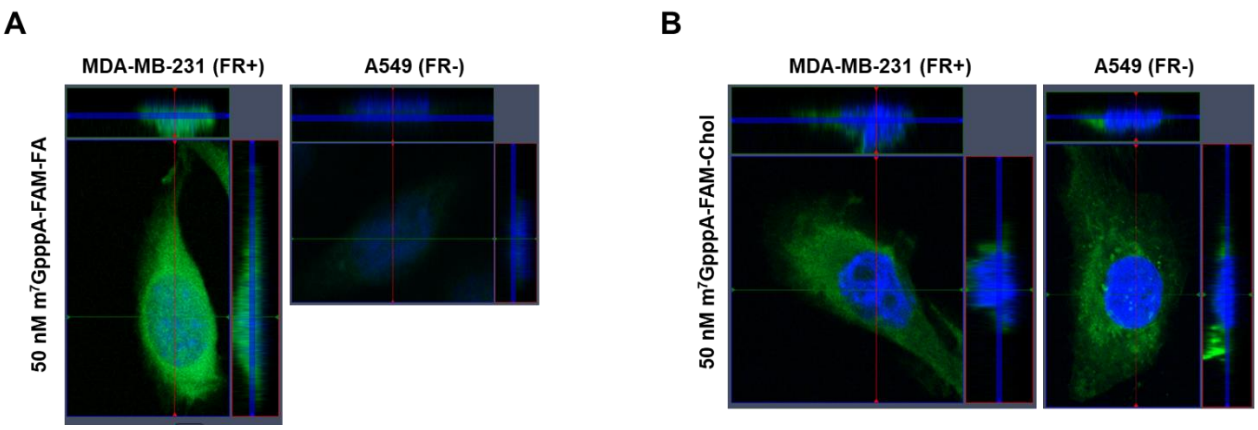


Figure S2. Cellular uptake of FAM labeled cap analog probes by (A) MDA-MB-231 and (B) A549 cells assessed by confocal microscopy with respective orthogonal axis view to verify cellular uptake.

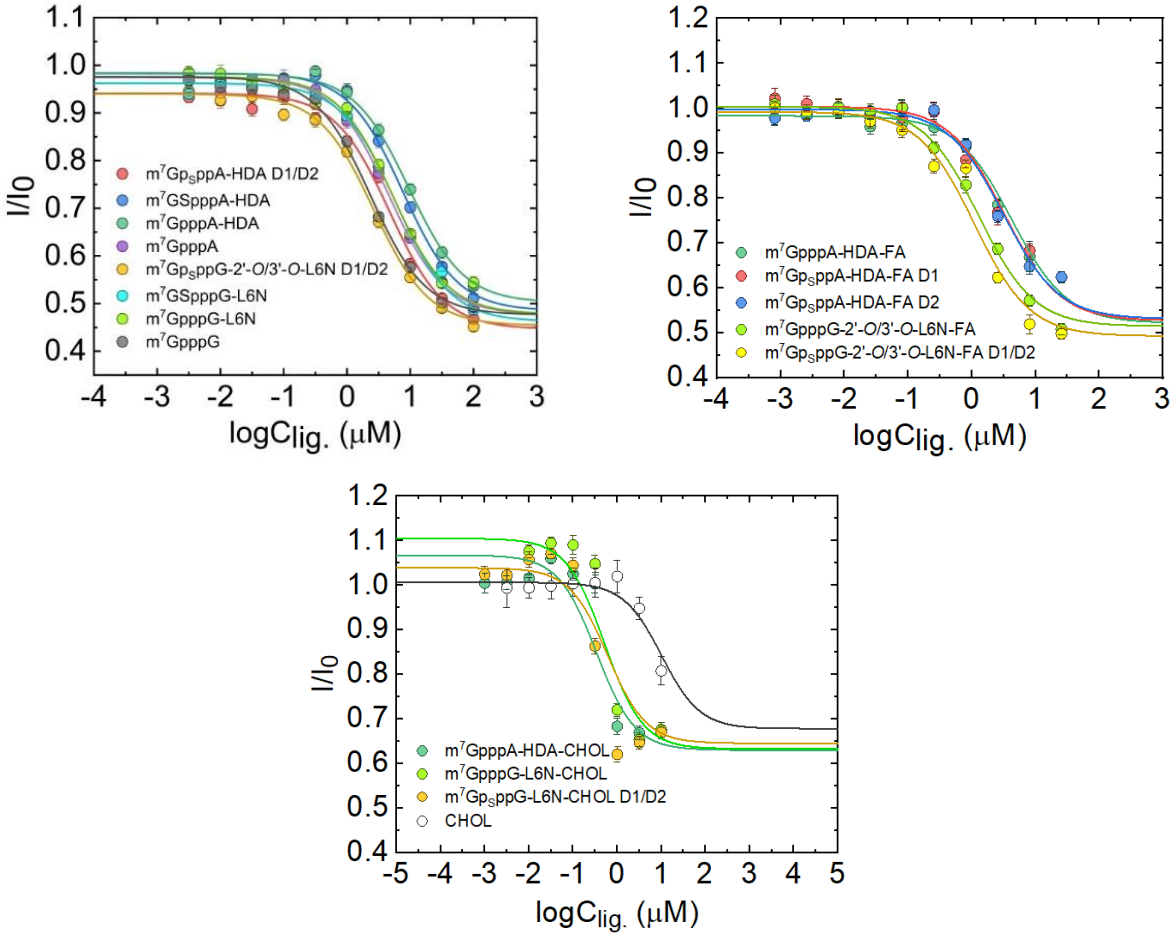


Figure S3. Competitive binding curves of synthesized cap analogues. Competitive binding of cap analogues to eIF4E protein. The experiment was carried out on plate reader with fluorescence intensity detection (λ_{exc} = 345 nm, λ_{em} = 378 nm) in 50 mM Tris/HCl pH 7.6 buffer containing 200 mM KCl and 0.5 mM EDTA at 20°C. Data shown are mean values ± SD of 3 separate experiments

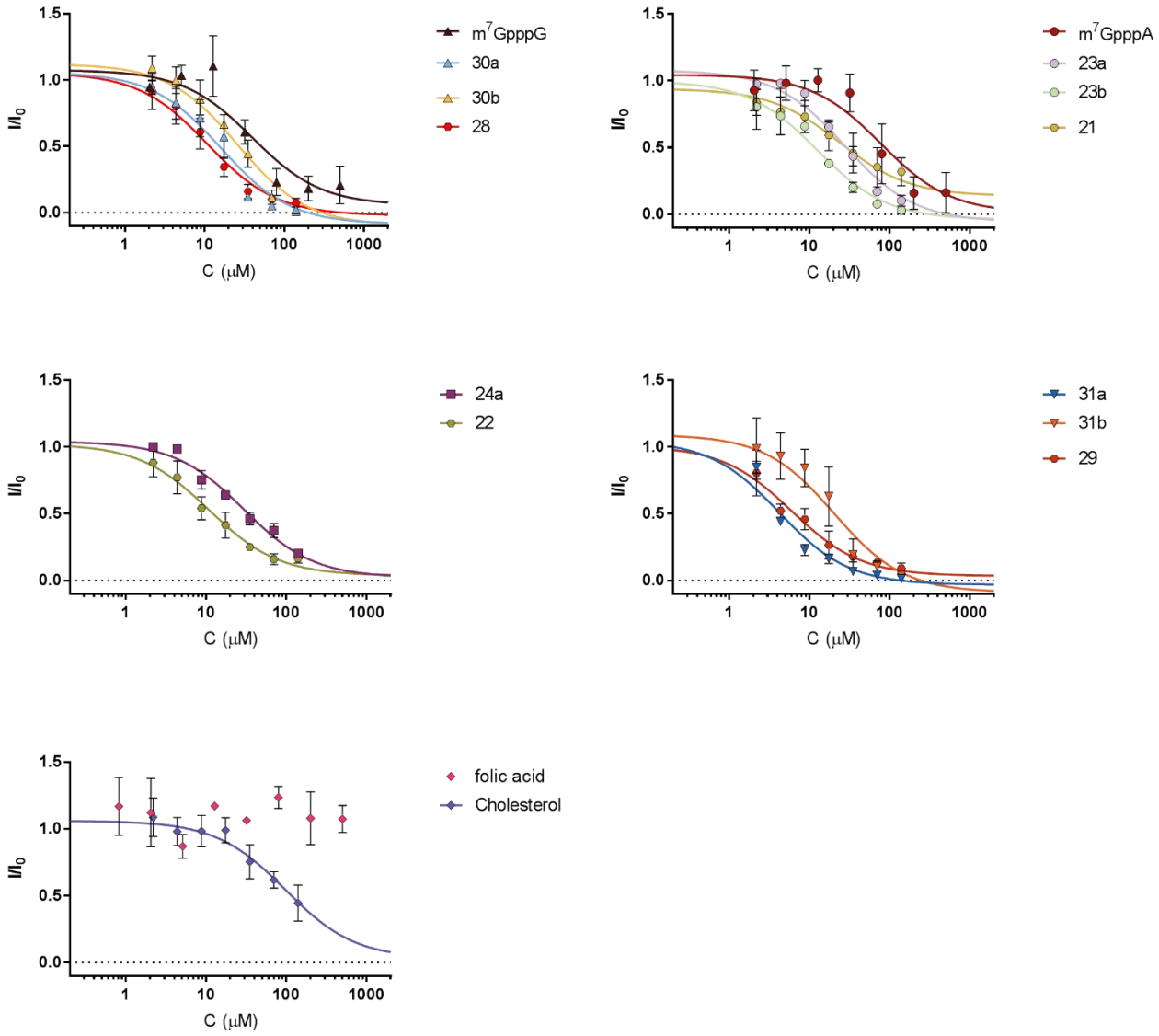


Figure S4. Inhibition of translation in RRL system. *In vitro* translation reaction (9 µl) contained a tested compound was incubated at 30 °C for 1 hour and then 1 µl of 10 µg/ml ARCA-capped mRNA encoding *Gaussia* luciferase was added to start translation. Reactions were further incubated at 30 °C for 1 hour and stopped afterwards by freezing in liquid nitrogen. Data shown represent mean value ± SD from triplicate.

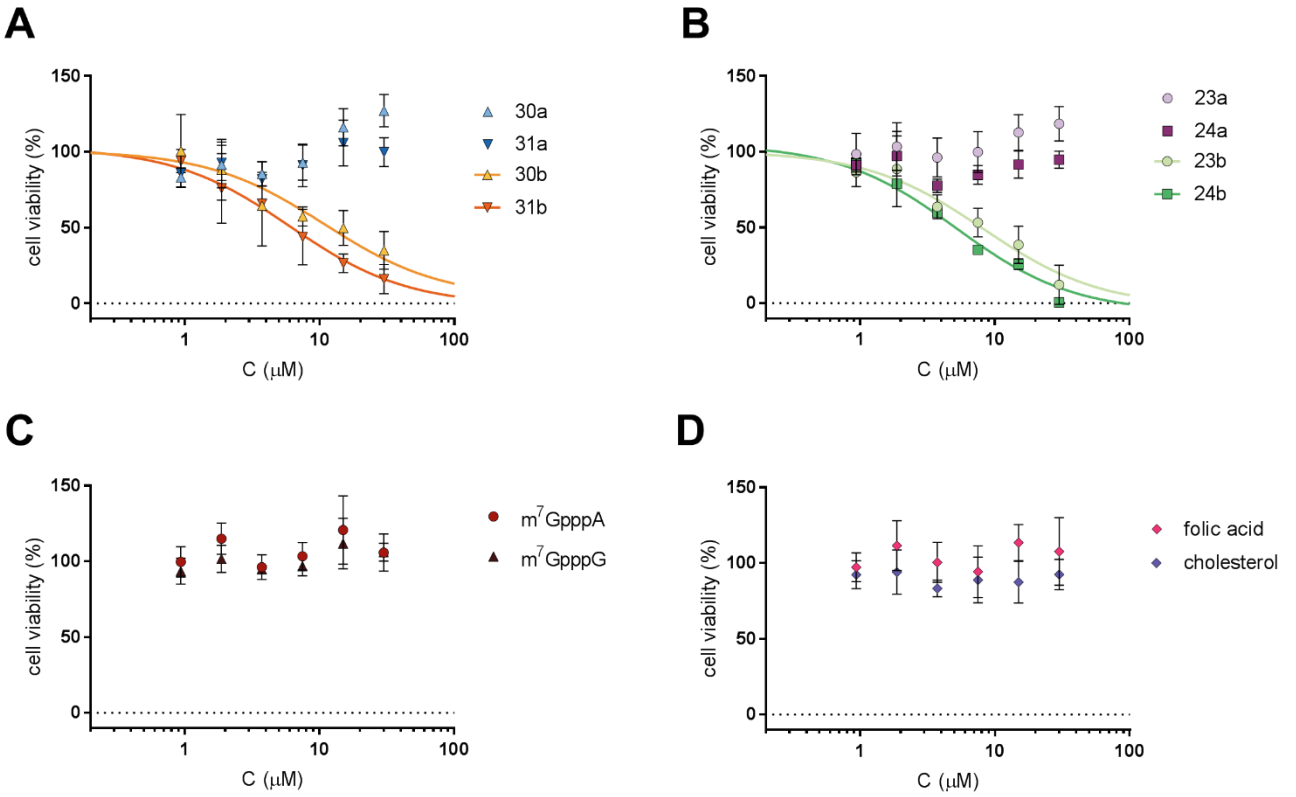


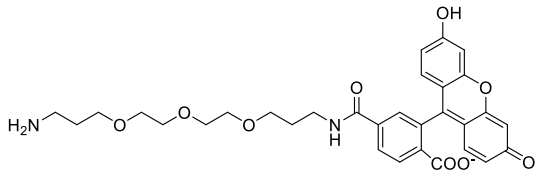
Figure S5. Cell viability of MDA-MB-231 cells treated with different concentration of selected compounds. After 72 hours of incubation, cells viability was assessed with resazurin assay. Data points were normalized to mock treated cells for each time point and represent mean value ± SD from at least 2 independent biological replications with 2 technical replicates per treatment.

NMR, HPLC profiles and HRMS

Cell-permeability fluorescent probes (1-4)

FAM-PEG (5)

Chemical structure



HPLC profile

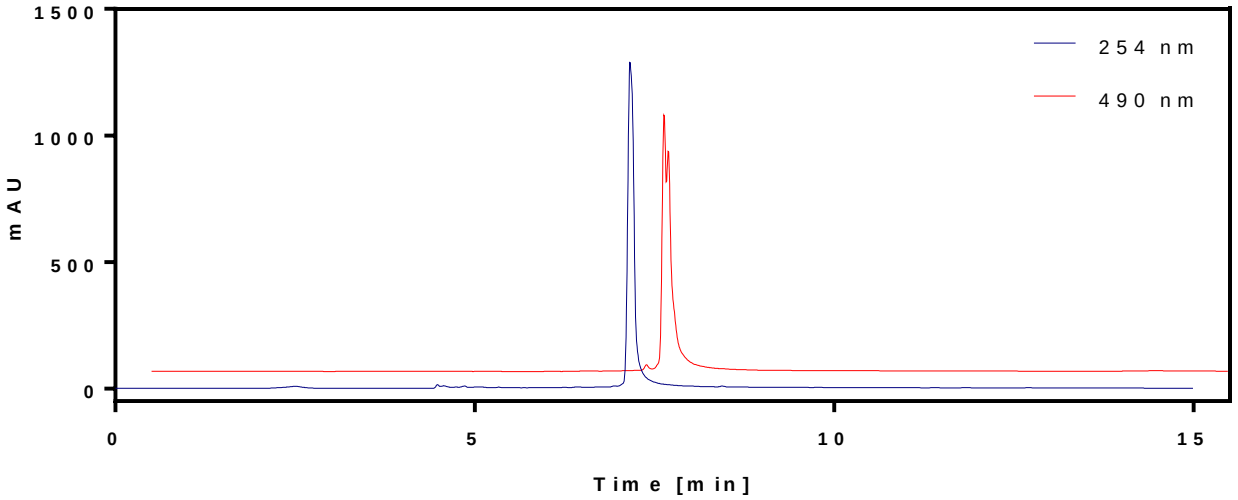
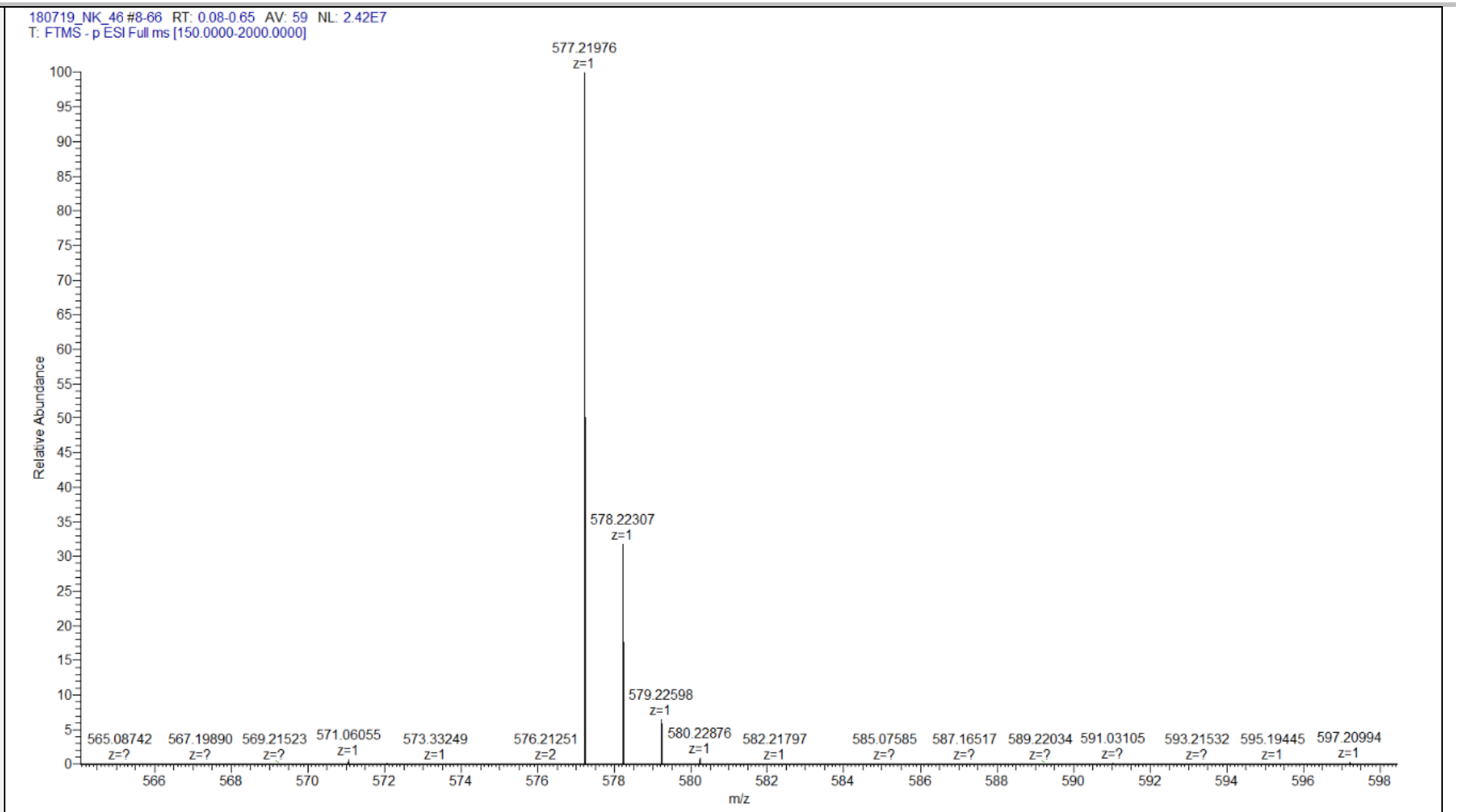
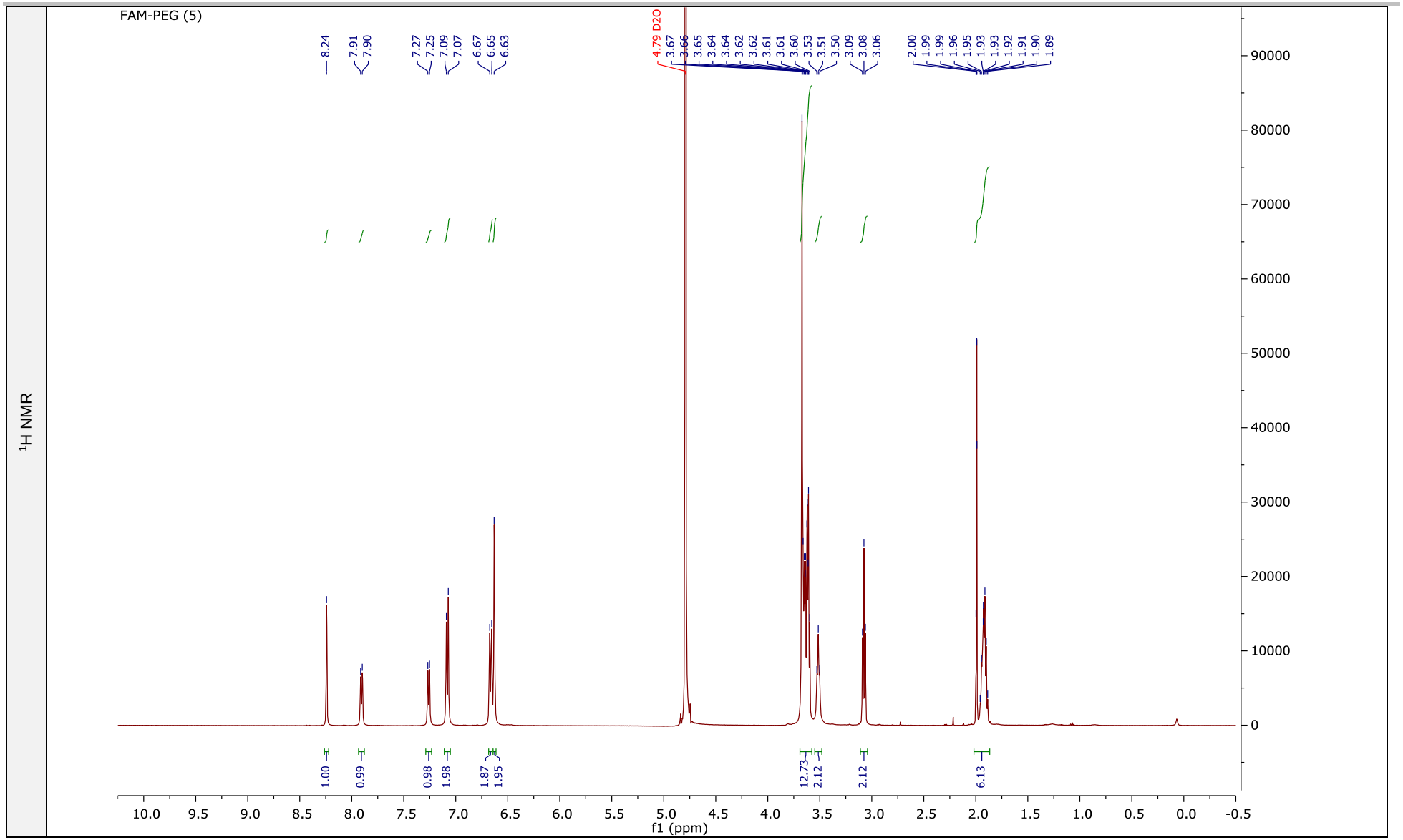


Figure S5. Analytical HPLC profile of compound **5**: column C18, Buffer A:ACN 0-100% in 15 min.

HRMS–ESI (*m/z*): [M-H]$^-$ calcd for $C_{31}H_{33}N_2O_9^-$, 577.21915.




FAM-PEG (5)
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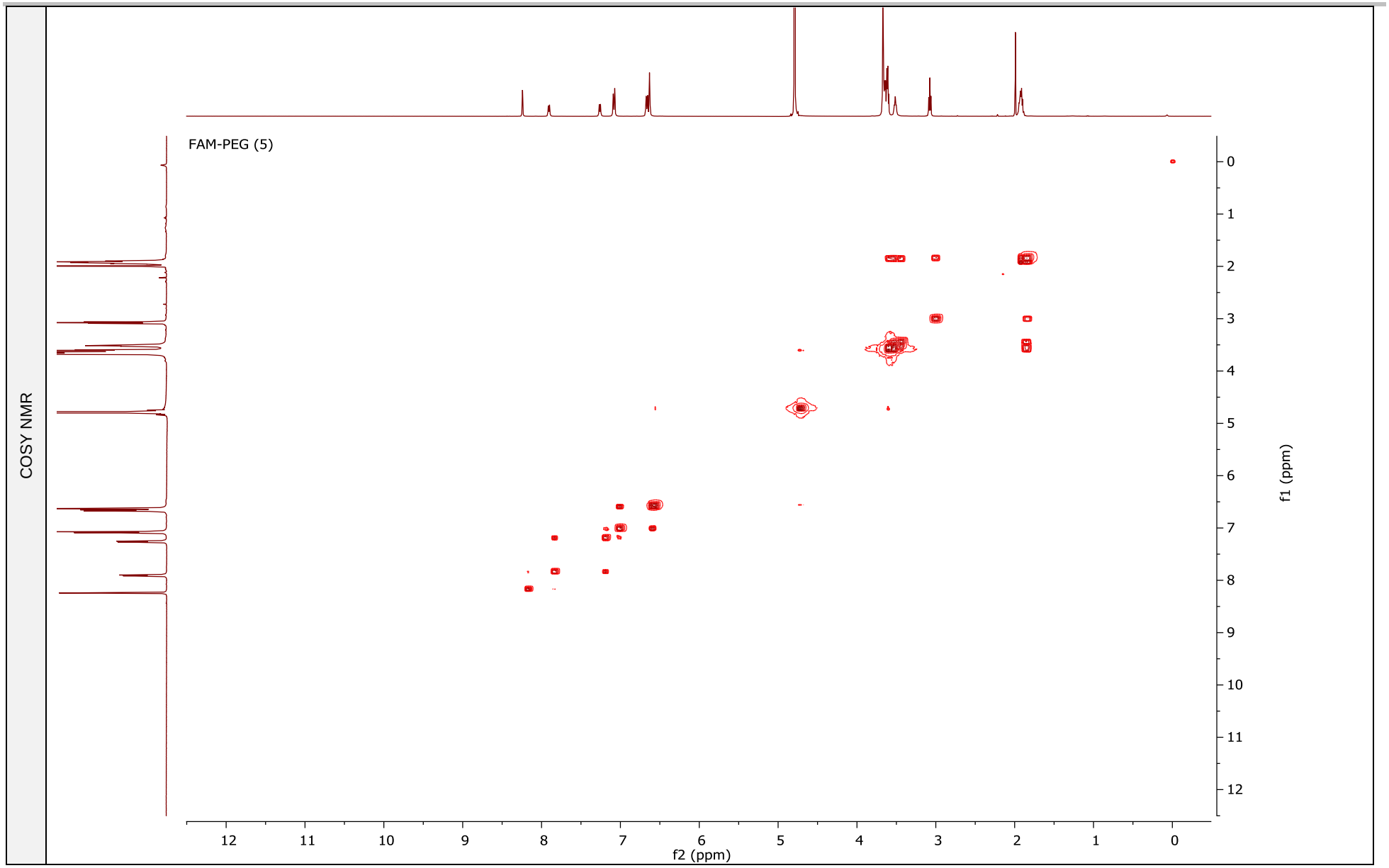
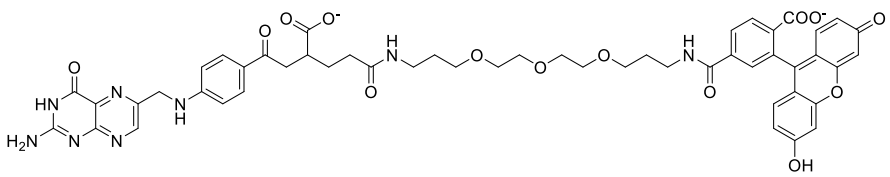
8.24
7.91
7.90
7.27
7.25
7.09
7.07
6.67
6.65
6.63
4.79 D2O
3.67
3.66
3.65
3.64
3.64
3.62
3.62
3.61
3.61
3.60
3.53
3.51
3.50
3.09
3.08
3.06
2.00
1.99
1.99
1.96
1.95
1.93
1.93
1.92
1.91
1.90
1.89
1.00
0.99
0.98
1.98
1.87
1.95
12.73
2.12
2.12
6.13
f1 (ppm)


COSY NMR
FAM-PEG (5)
f2 (ppm)
f1 (ppm)
12
11
10
9
8
7
6
5
4
3
2
1
0

FAM-PEG-FA (1)

Chemical structure



HPLC profile

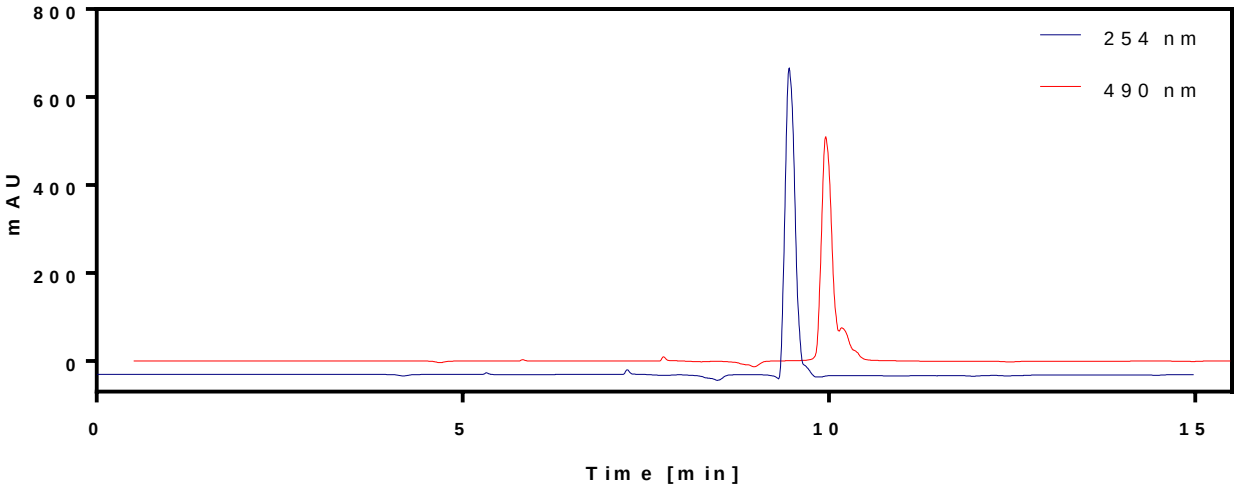
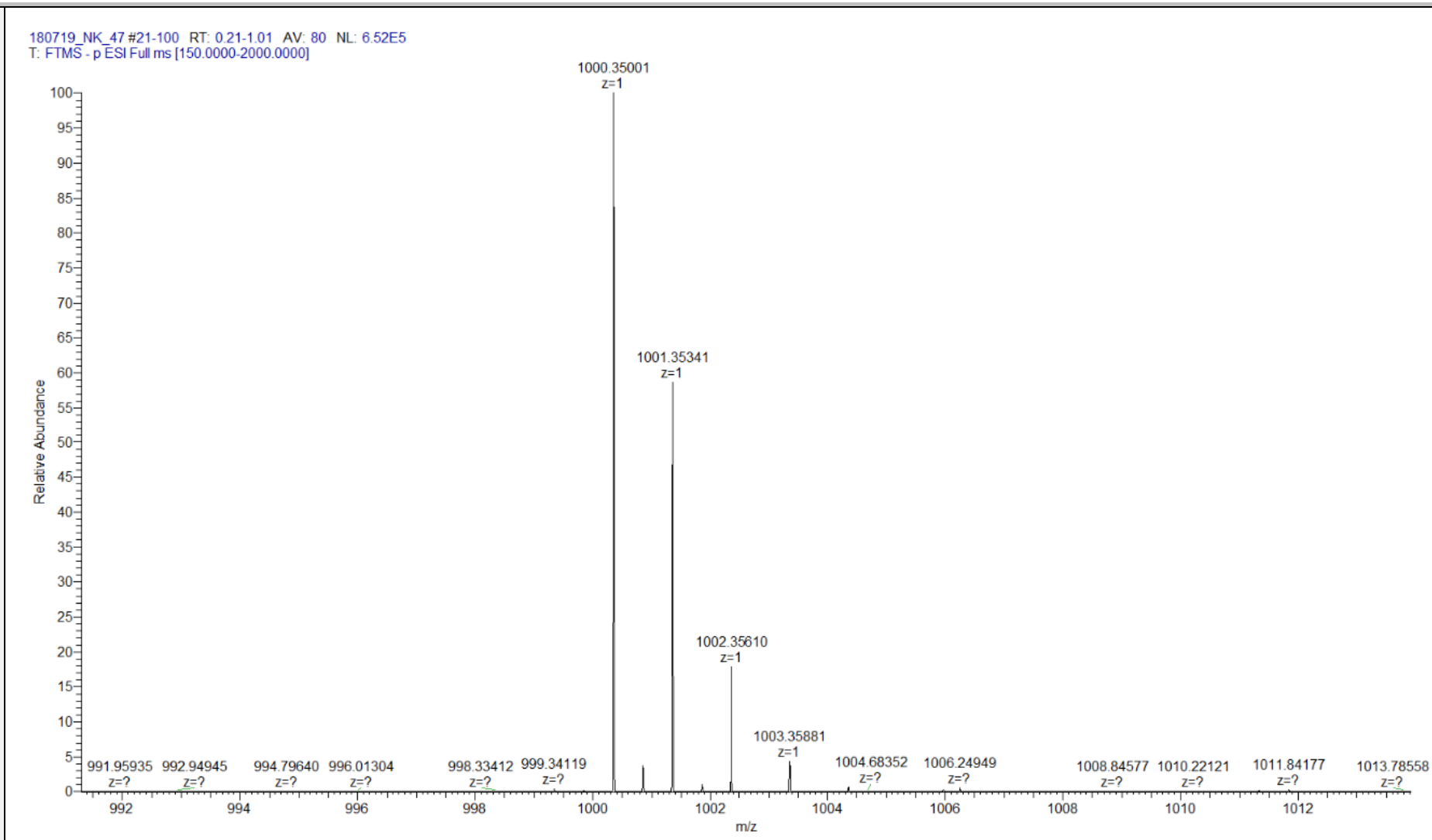


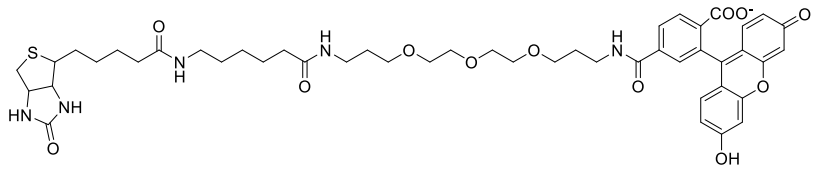
Figure S6. Analytical HPLC profile of compound **1**: column Gemini NX-C18, ammonium acetate pH 8.4:ACN 5-30% in 15 min.

HRMS–ESI (*m/z*): [M-H]$^-$ calcd for $C_{50}H_{50}N_9O_{14}^-$, 1000.34827.



FAM-PEG-Biotin (2)

Chemical structure



HPLC profile

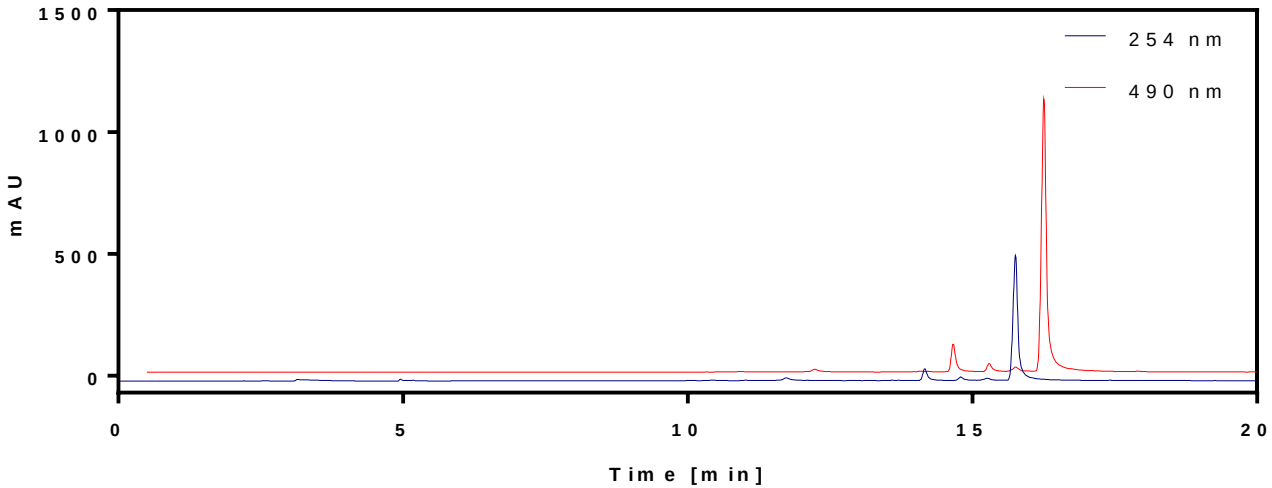
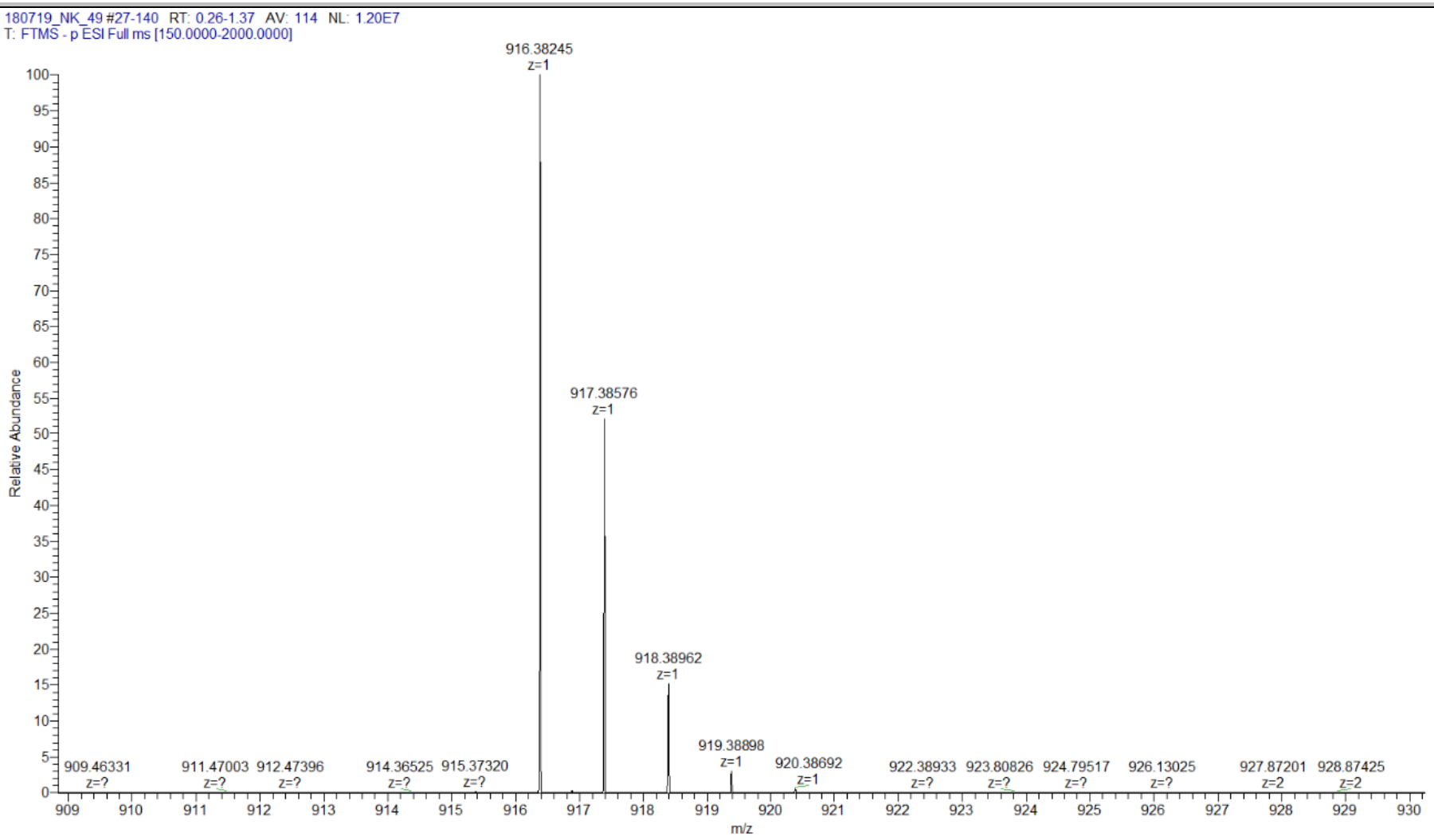


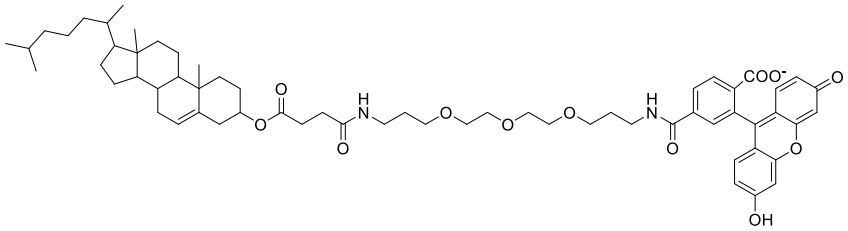
Figure S7. Analytical HPLC profile of compound **2**: column Supelco C18, ammonium acetate pH 5.9:ACN 5-30% in 15 min.

HRMS–ESI (*m/z*): $[M-H]^-$ calcd for $C_{47}H_{58}N_5O_{12}S^-$, 916.38082.



FAM-PEG-CHOL (4)

Chemical structure



HPLC profile

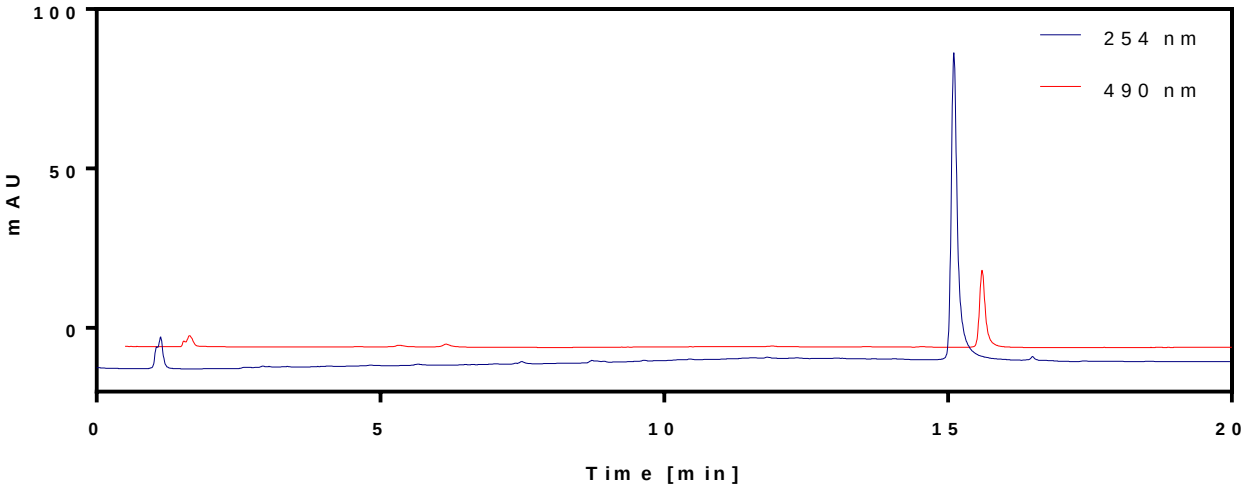
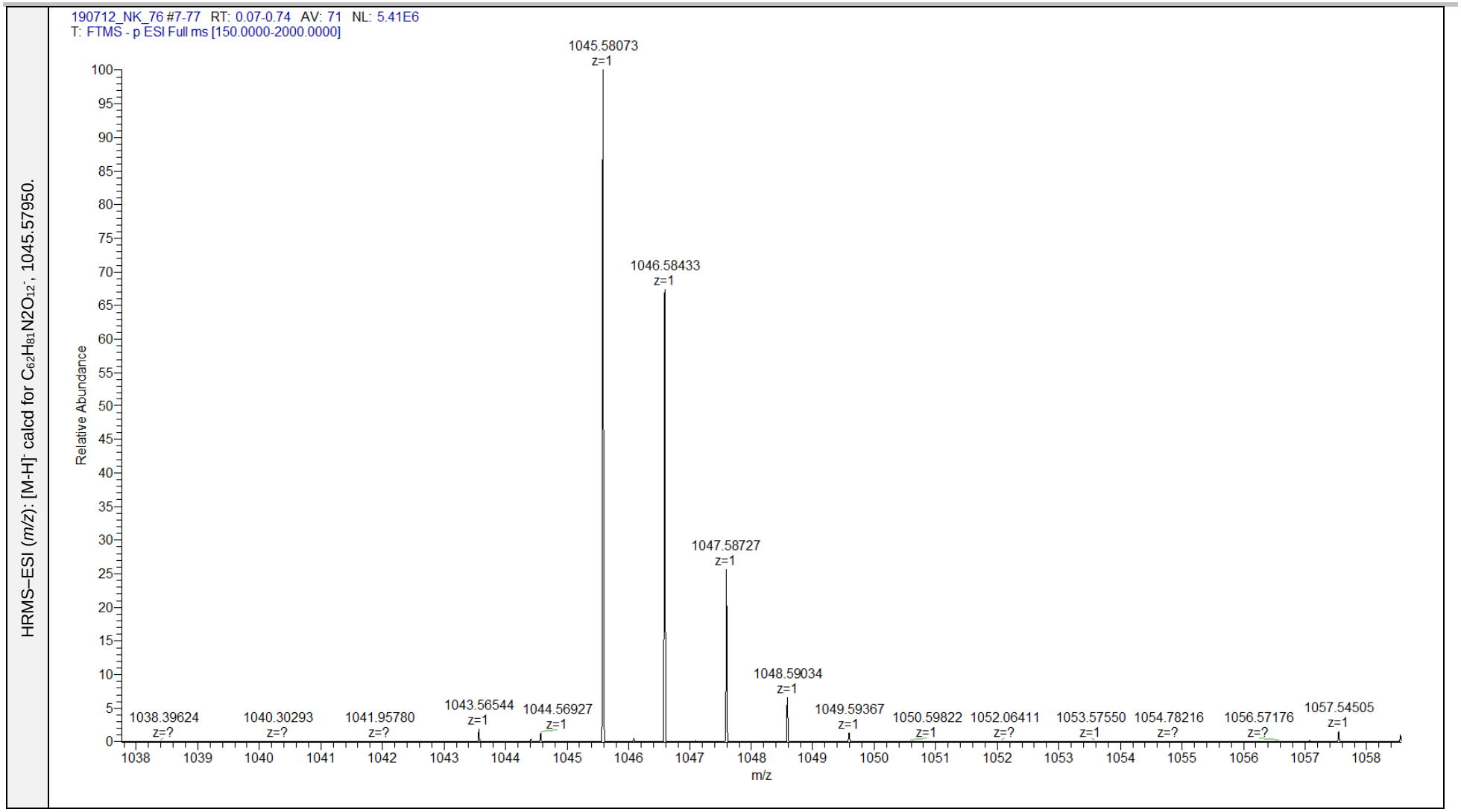
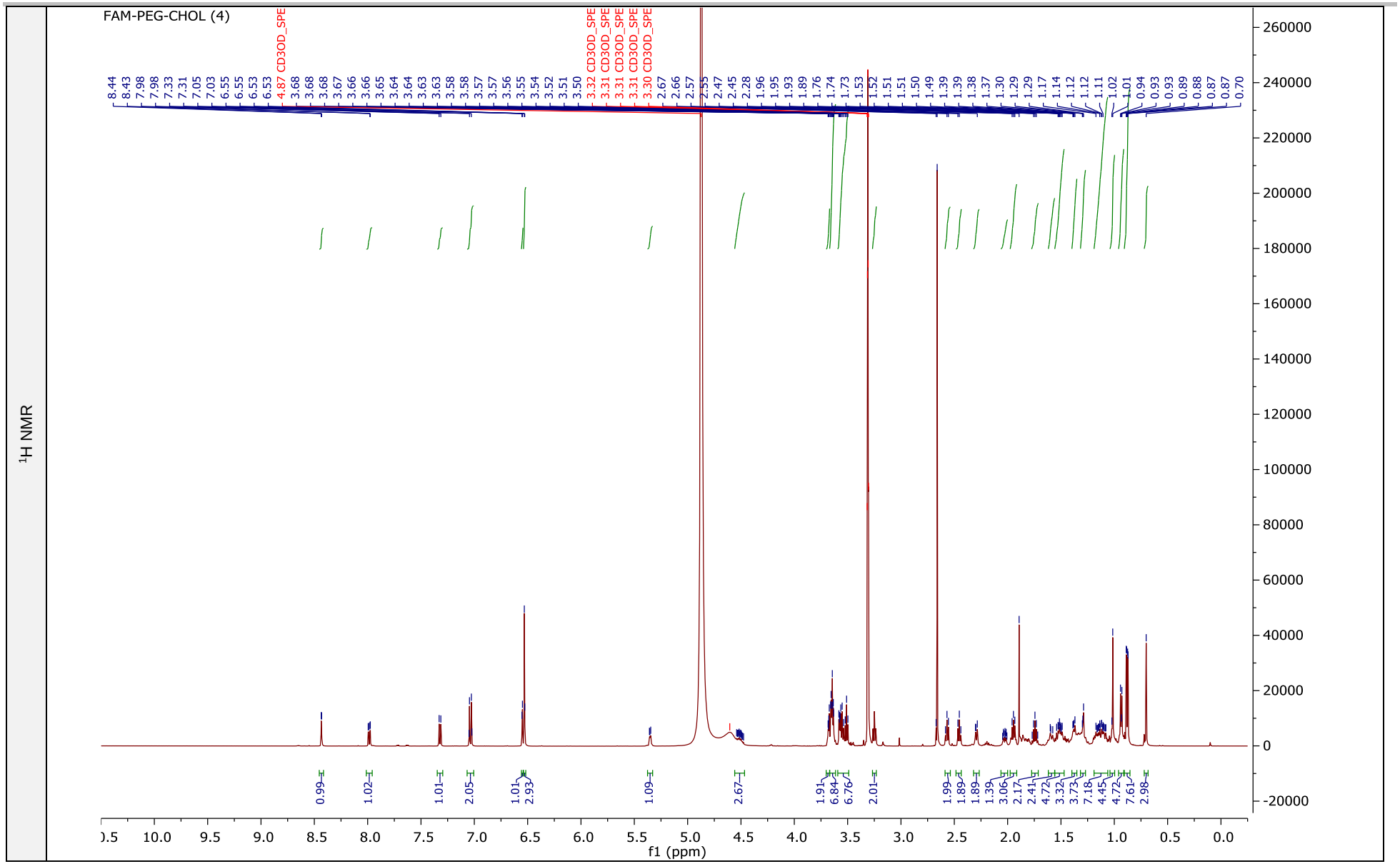
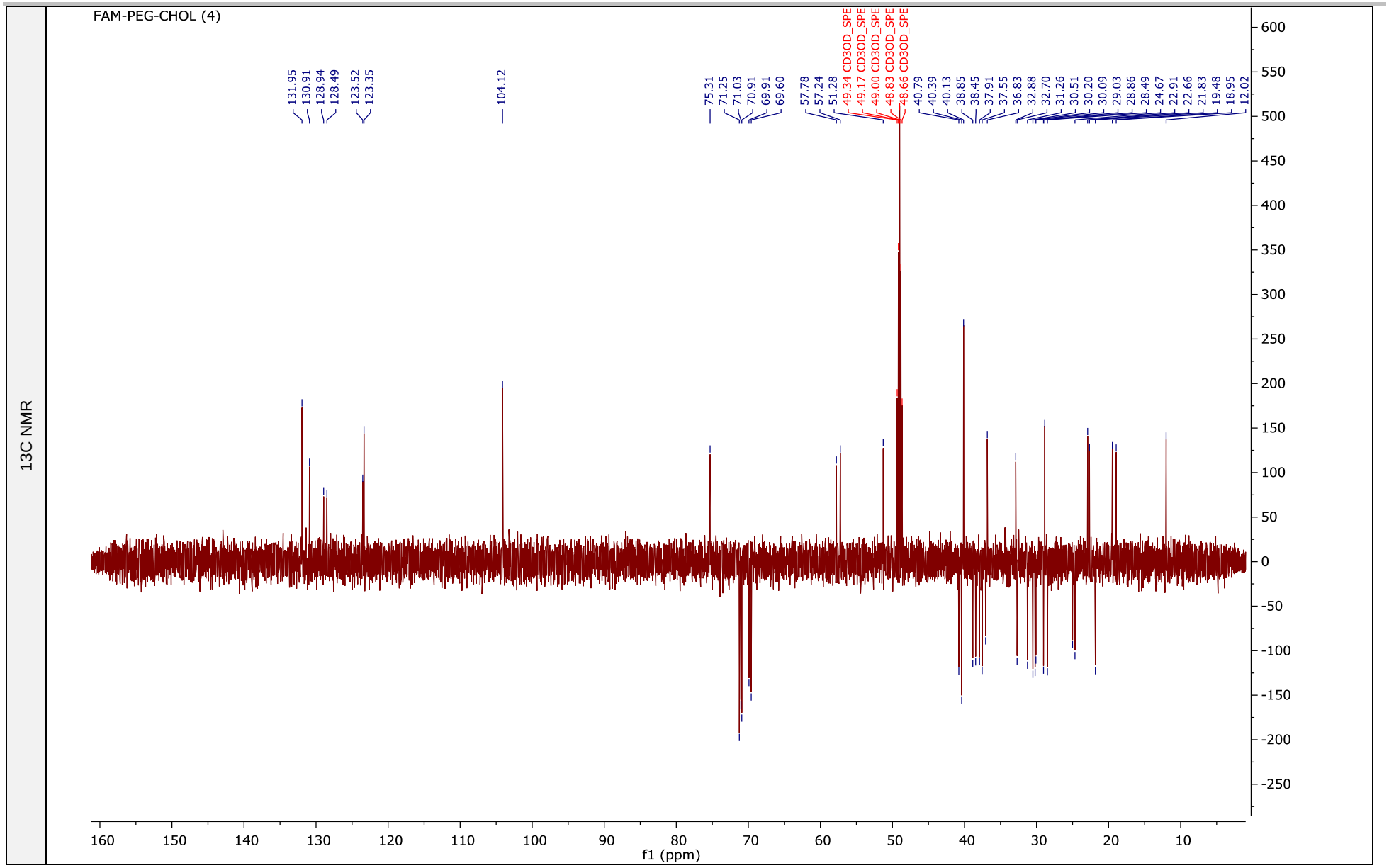
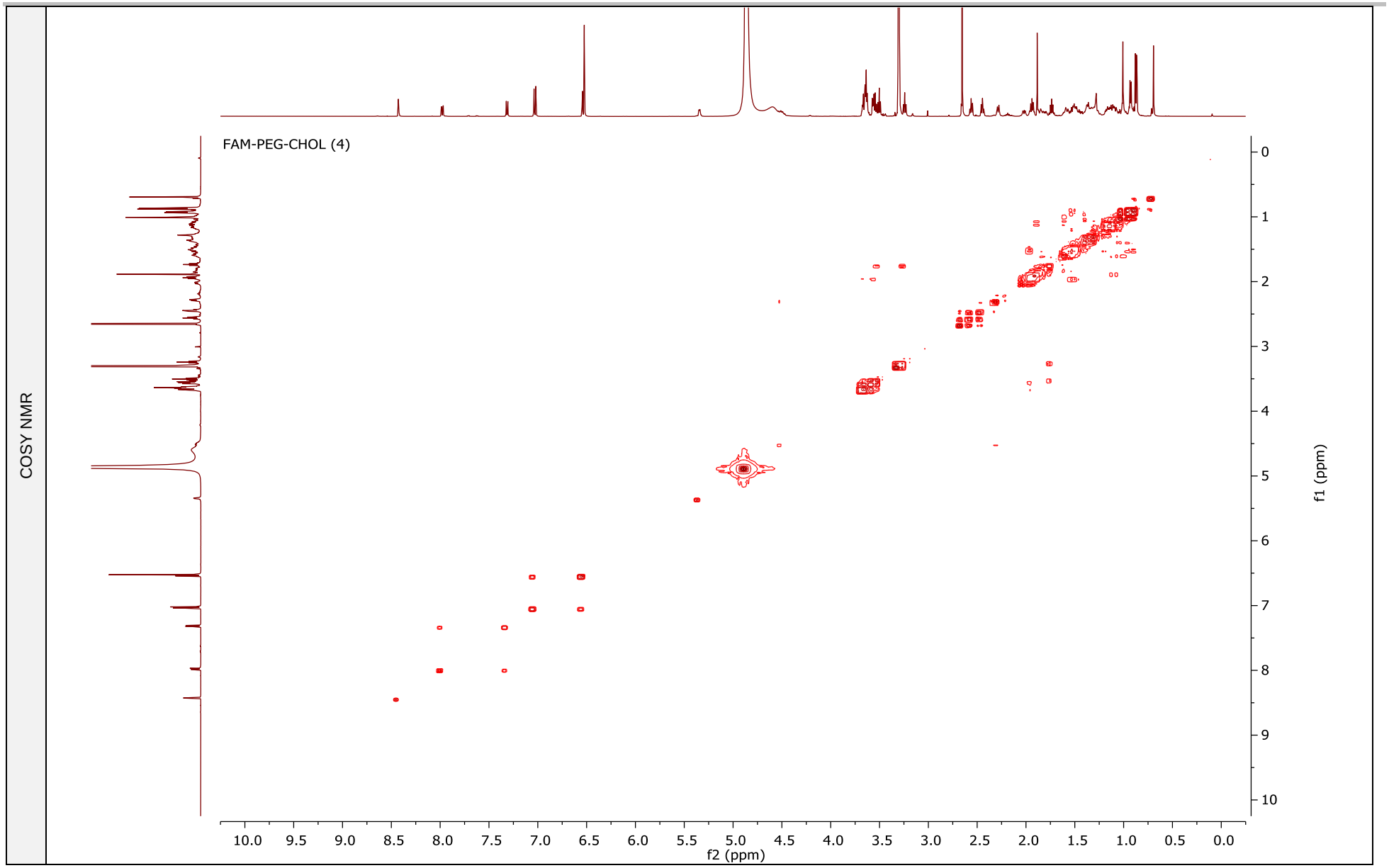


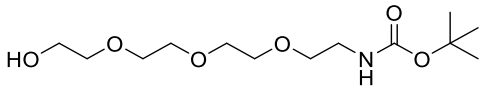
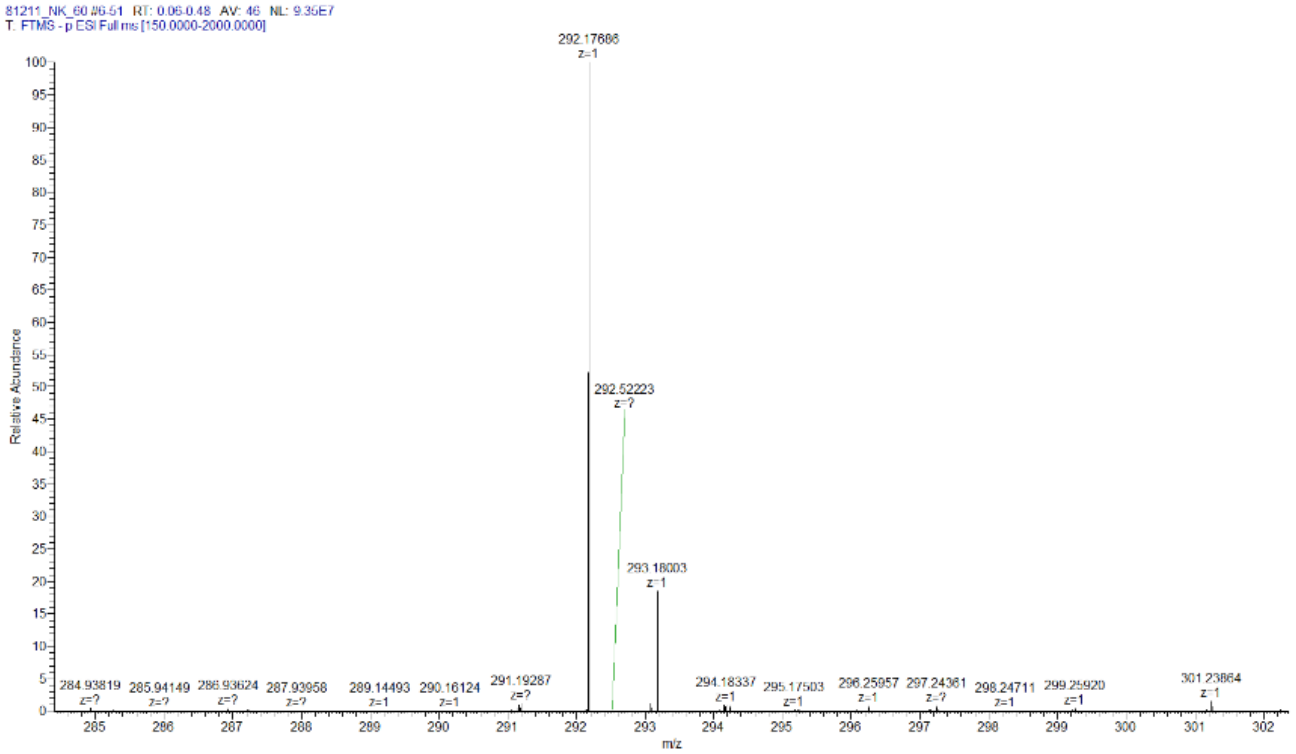
Figure S8. Analytical HPLC profile of compound **4**: column C4, Buffer A:ACN 0-100% in 15 min.

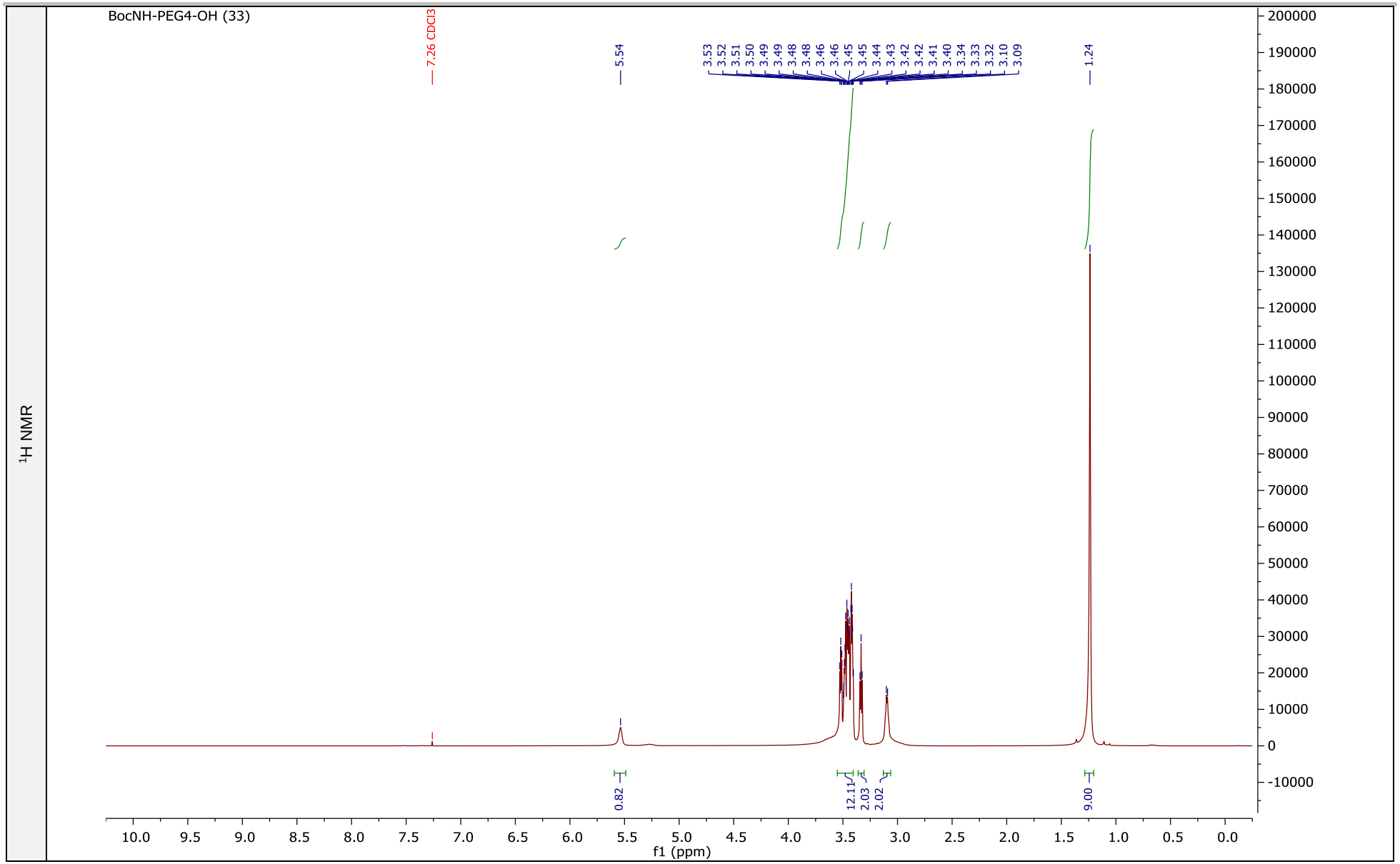

HRMS–ESI (m/z): [M-H]- calcd for C62H81N2O12-, 1045.57950.
190712_NK_76 #7-77 RT: 0.07-0.74 AV: 71 NL: 5.41E6
T: FTMS - p ESI Full ms [150.0000-2000.0000]
Relative Abundance
m/z
1045.58073 z=1
1046.58433 z=1
1047.58727 z=1
1048.59034 z=1
1038.39624 z=?
1040.30293 z=?
1041.95780 z=?
1043.56544 z=1
1044.56927 z=1
1049.59367 z=1
1050.59822 z=1
1052.06411 z=?
1053.57550 z=1
1054.78216 z=?
1056.57176 z=?
1057.54505 z=1

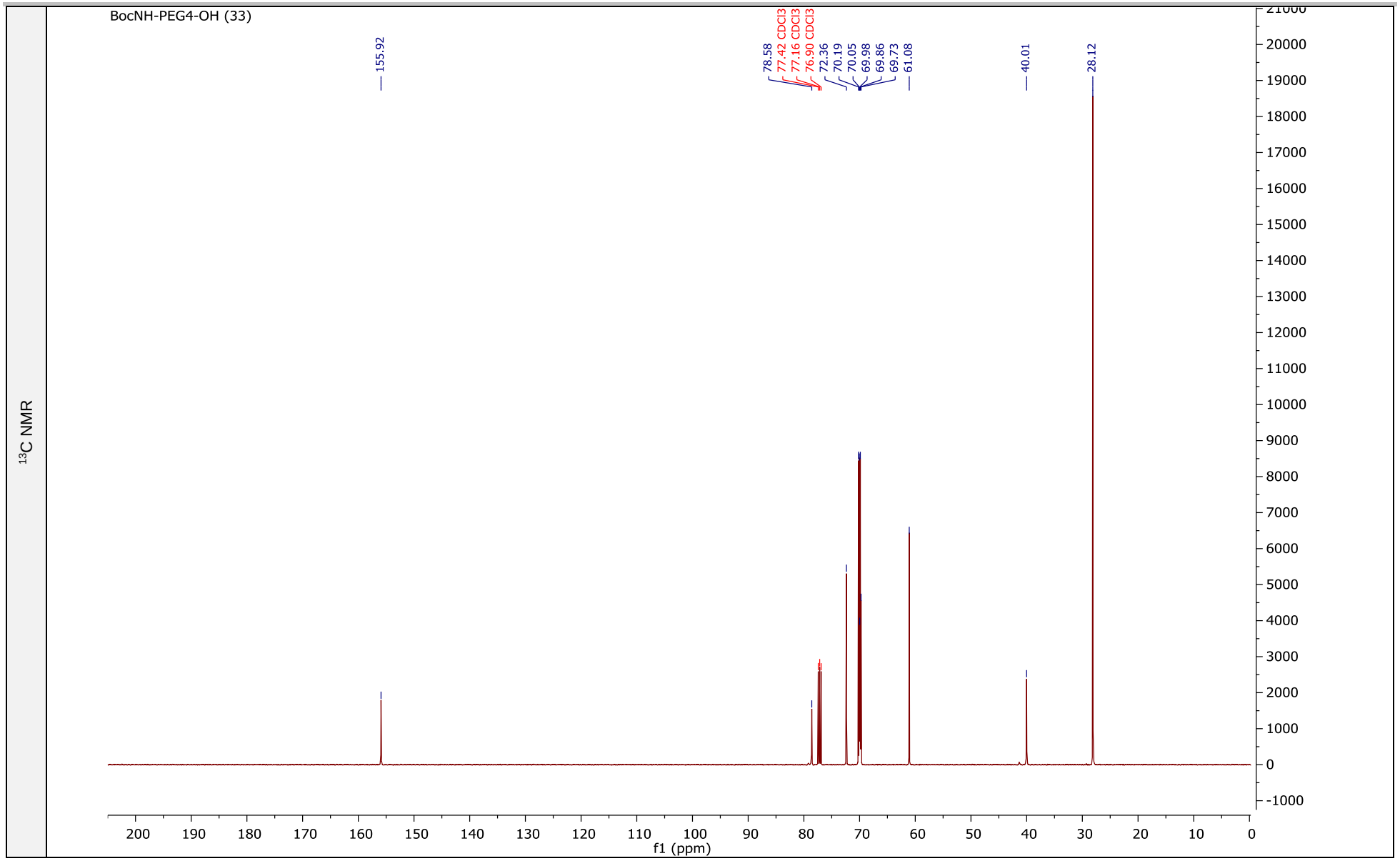

FAM-PEG-CHOL (4)
^{1}H NMR
f1 (ppm)

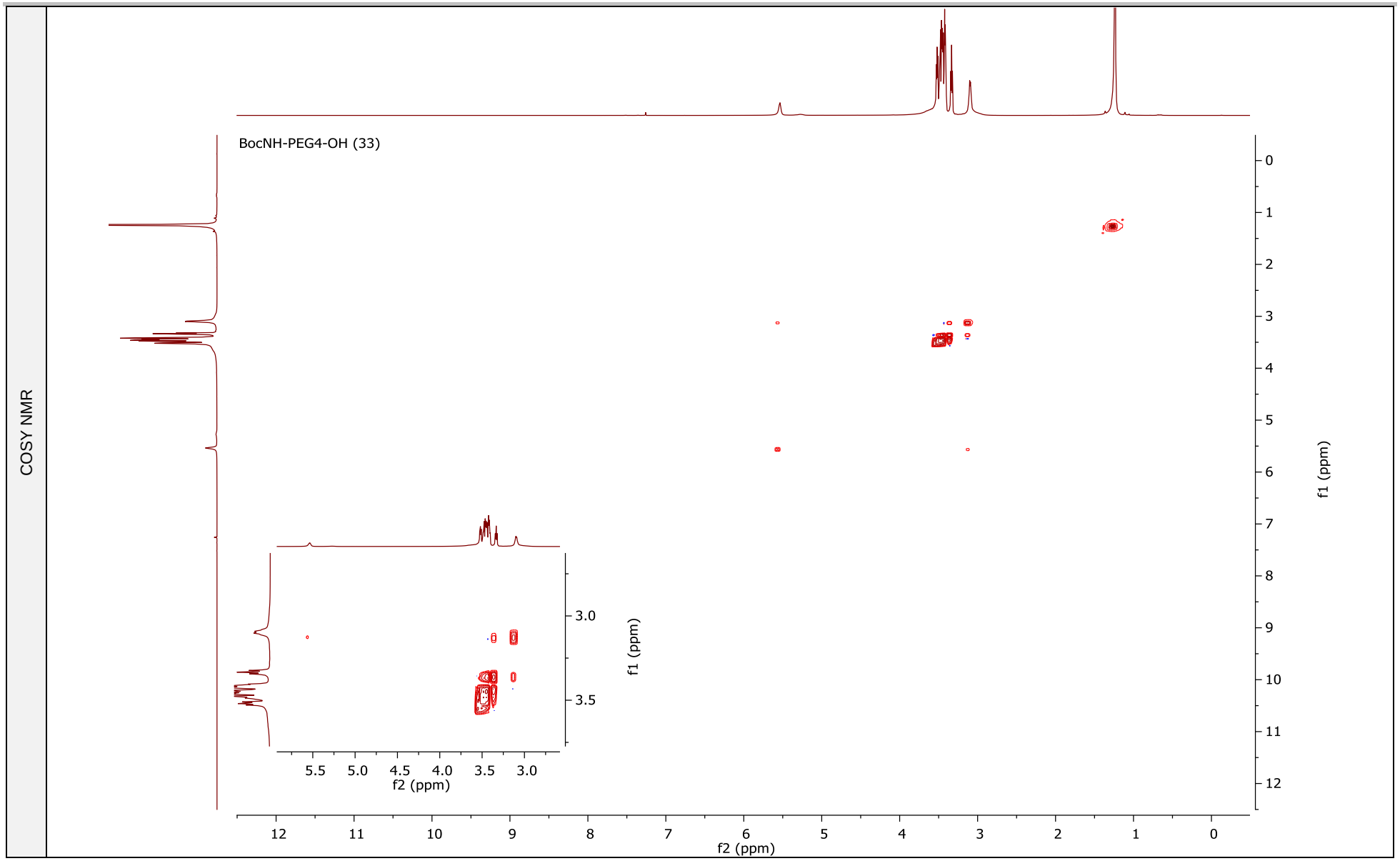

FAM-PEG-CHOL (4)
13C NMR
131.95
130.91
128.94
128.49
123.52
123.35
104.12
75.31
71.25
71.03
70.91
69.91
69.60
57.78
57.24
51.28
49.34 CD3OD_SPE
49.17 CD3OD_SPE
49.00 CD3OD_SPE
48.83 CD3OD_SPE
48.66 CD3OD_SPE
40.79
40.39
40.13
38.85
38.45
37.91
37.55
36.83
32.88
32.70
31.26
30.51
30.20
30.09
29.03
28.86
28.49
24.67
22.91
22.66
21.83
19.48
18.95
12.02
600
550
500
450
400
350
300
250
200
150
100
50
0
-50
-100
-150
-200
-250
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

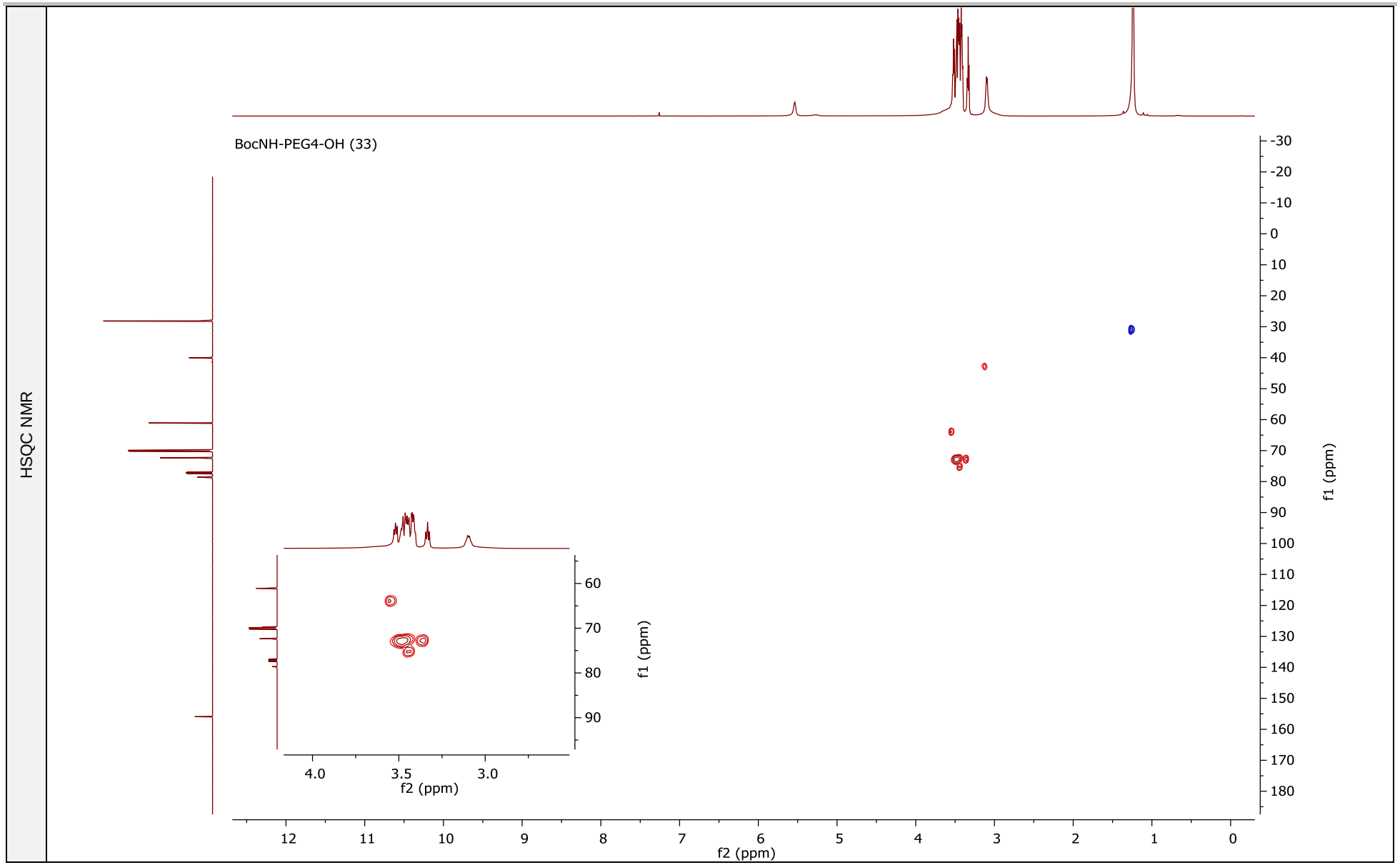

COSY NMR
FAM-PEG-CHOL (4)
f2 (ppm)
f1 (ppm)
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
5
6
7
8
9
10

BocNH-PEG4-OH (33)	
Chemical structure	
HRMS–ESI (*m/z*): [M-H]$^-$ calcd for $C_{13}H_{26}NO_6^-$, 292.17656.	


BocNH-PEG4-OH (33)
^{1}H NMR
7.26 CDCl3
5.54
3.53
3.52
3.51
3.50
3.49
3.49
3.48
3.48
3.46
3.46
3.45
3.45
3.44
3.43
3.42
3.42
3.41
3.40
3.34
3.33
3.32
3.10
3.09
1.24
0.82
12.11
2.03
2.02
9.00
f1 (ppm)

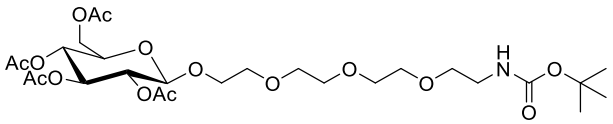

BocNH-PEG4-OH (33)
155.92
78.58
77.42 CDCl3
77.16 CDCl3
76.90 CDCl3
72.36
70.19
70.05
69.98
69.86
69.73
61.08
40.01
28.12
13C NMR
f1 (ppm)


COSY NMR
BocNH-PEG4-OH (33)
f1 (ppm)
f2 (ppm)
12
11
10
9
8
7
6
5
4
3
2
1
0
5.5
5.0
4.5
4.0
3.5
3.0

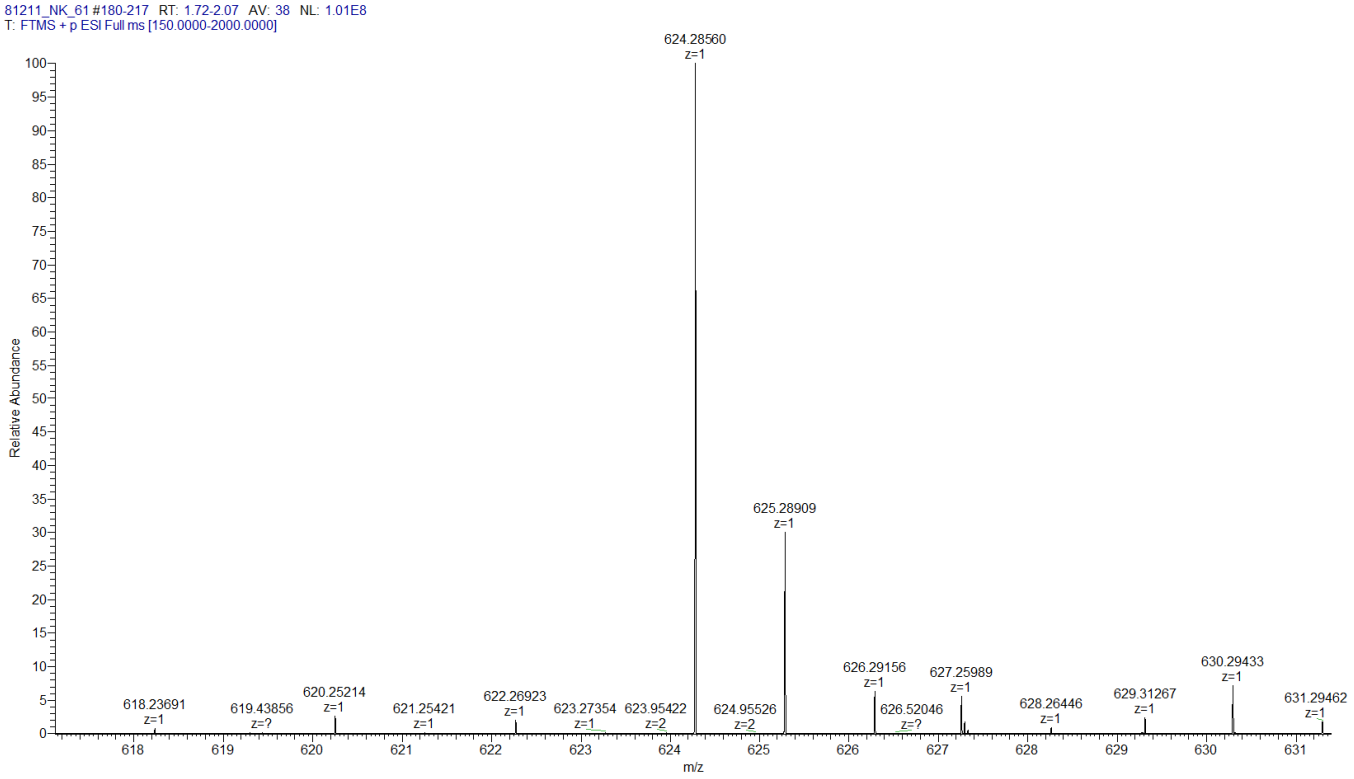

HSQC NMR
BocNH-PEG4-OH (33)
f2 (ppm)
f1 (ppm)
4.0
3.5
3.0
60
70
80
90
12
11
10
9
8
7
6
5
4
3
2
1
0
-30
-20
-10
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180

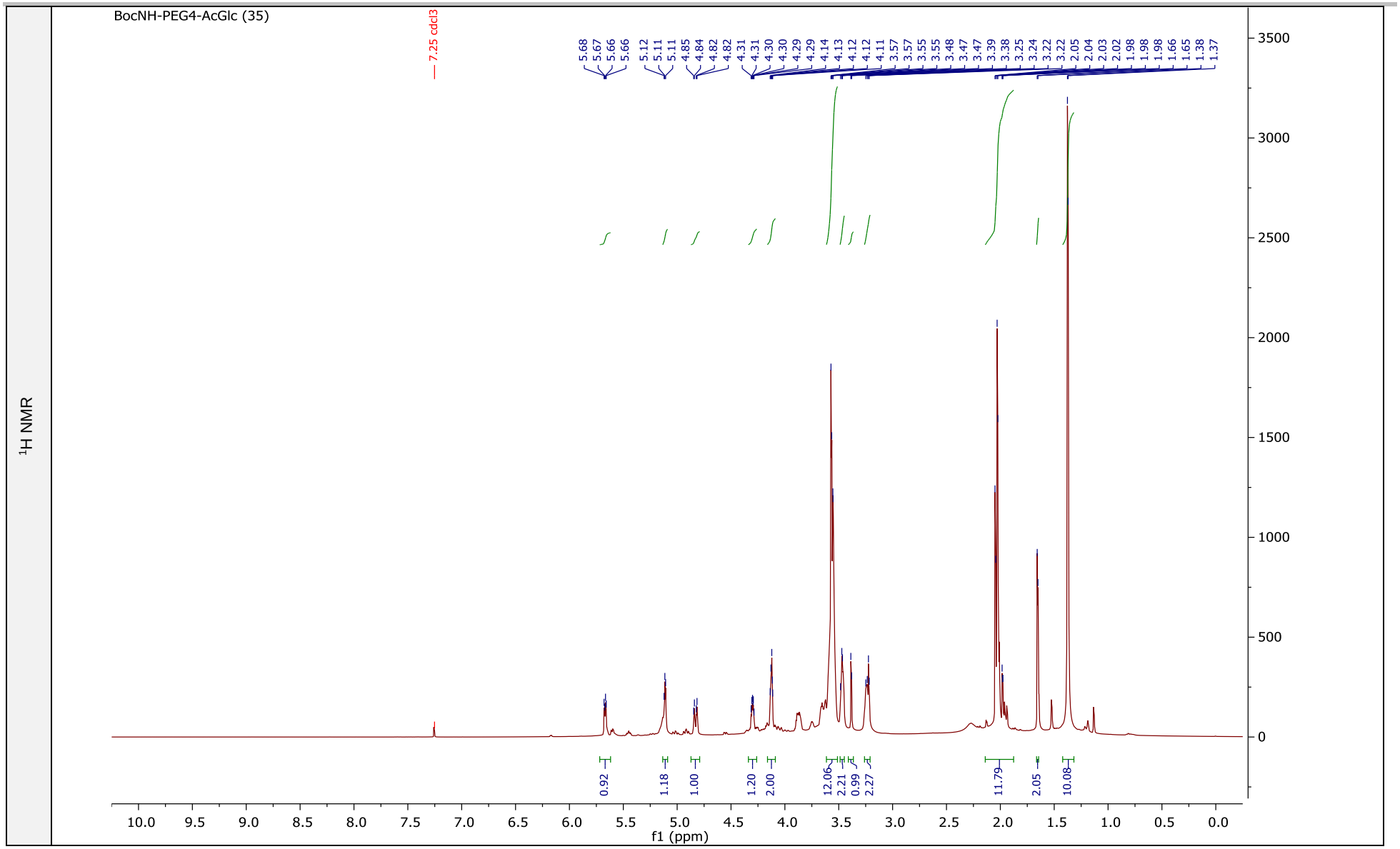
BocNH-PEG4-AcGlc (35)

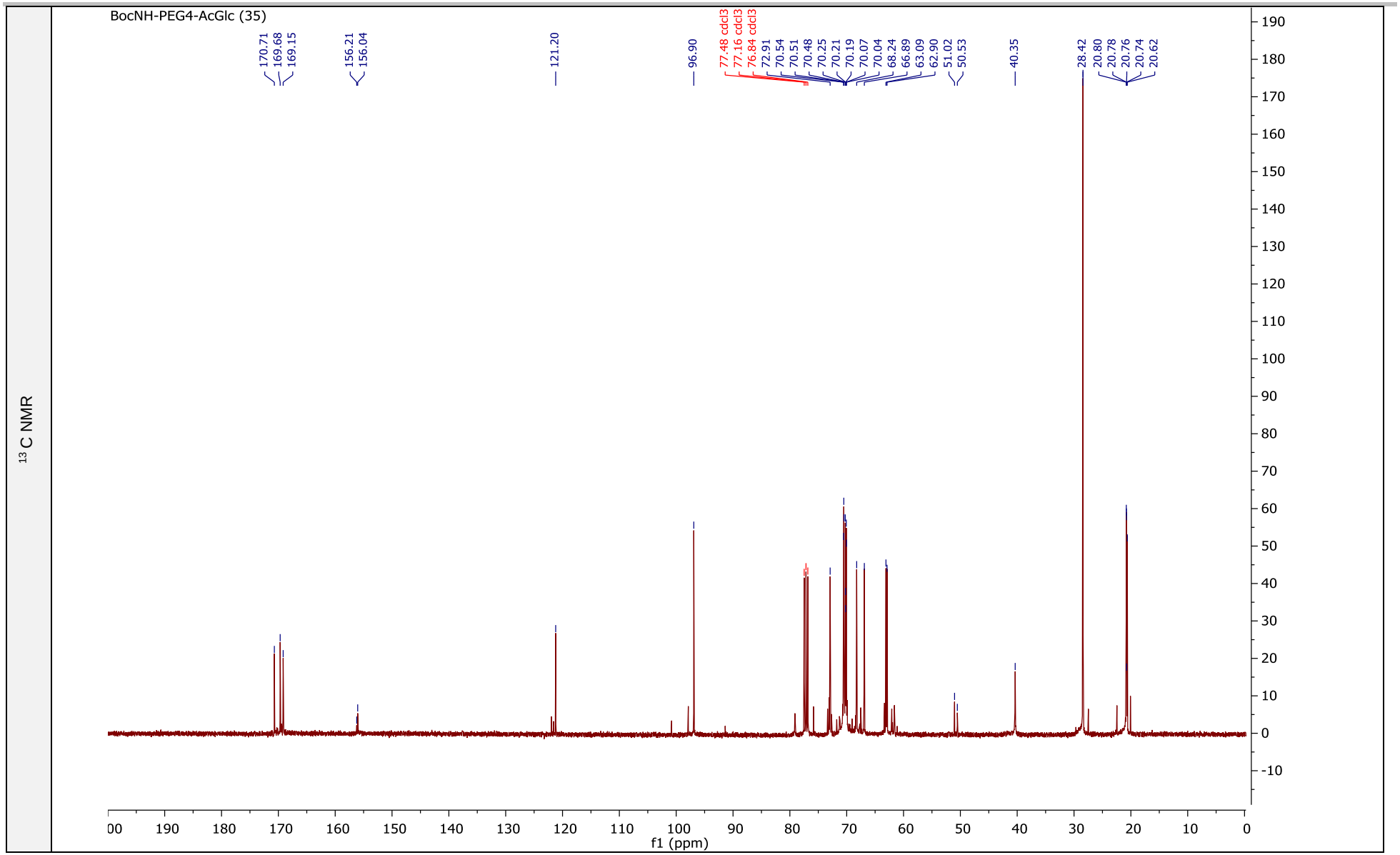
Chemical structure

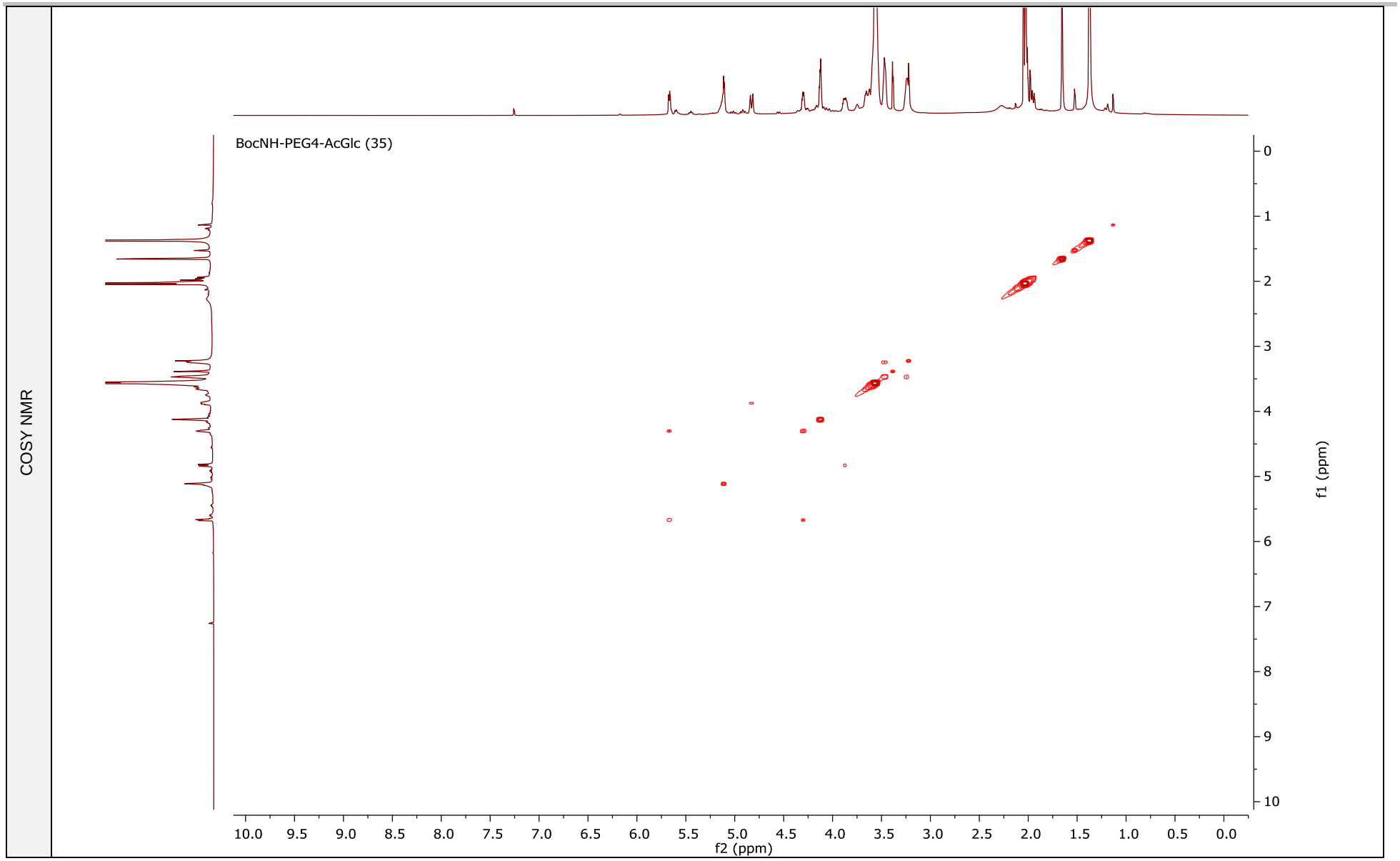


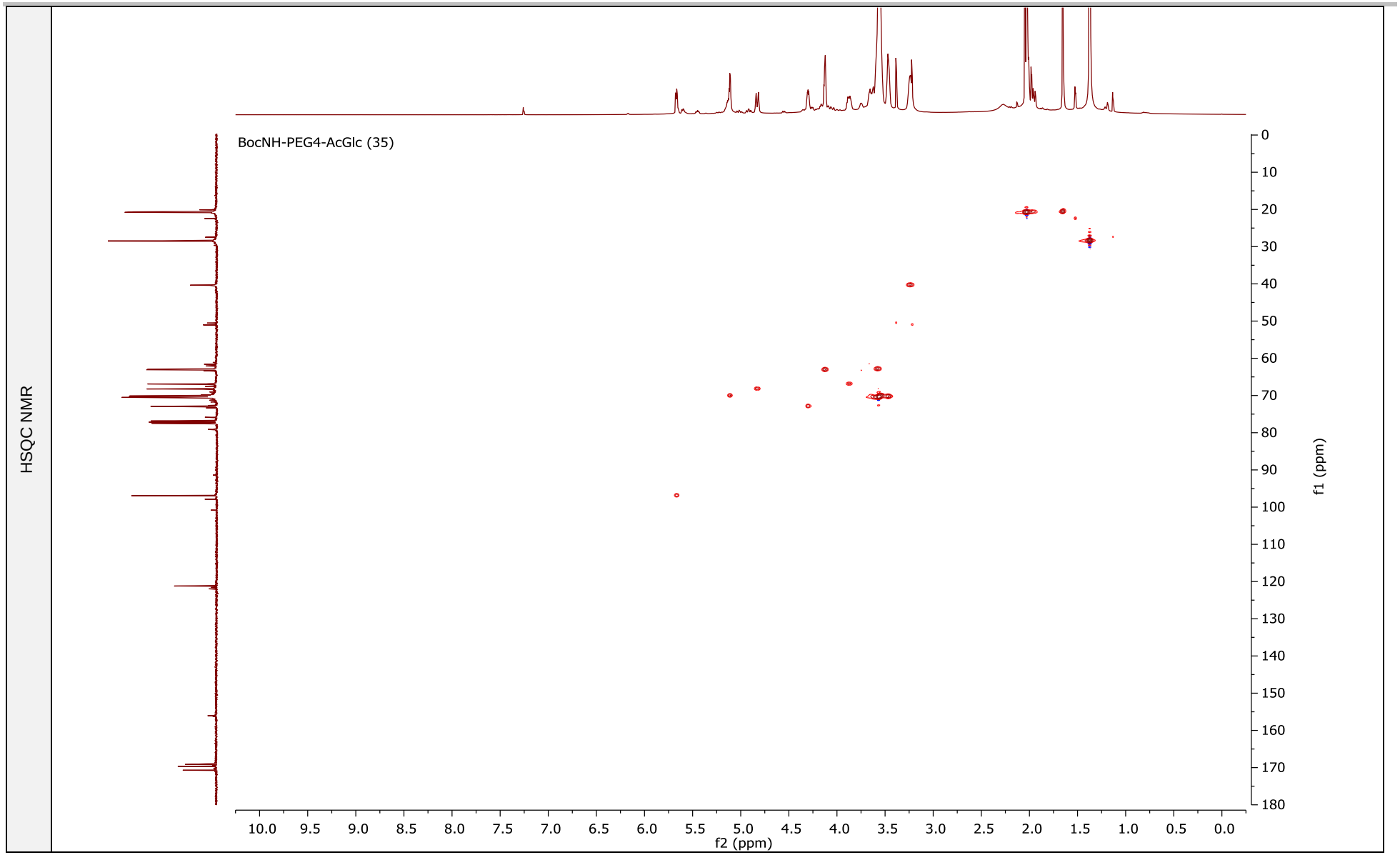
HRMS–ESI (*m/z*): $[M+H]^+$ calcd for $C_{27}H_{46}NO_{15}^+$, 624.28620.




BocNH-PEG4-AcGlc (35)
^{1}H NMR
7.25 cdcl3
5.68 5.67 5.66 5.66 5.12 5.11 5.11 4.85 4.84 4.82 4.82 4.31 4.31 4.30 4.30 4.29 4.29 4.14 4.13 4.12 4.12 4.11 3.57 3.57 3.55 3.55 3.48 3.47 3.47 3.39 3.38 3.25 3.24 3.22 3.22 2.05 2.04 2.03 2.02 1.98 1.98 1.98 1.66 1.65 1.38 1.37
0.92 1.18 1.00 1.20 2.00 12.06 2.21 0.99 2.27 11.79 2.05 10.08
f1 (ppm)

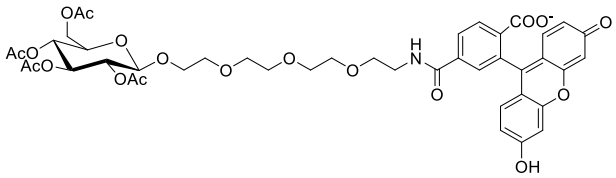

BocNH-PEG4-AcGlc (35)
13 C NMR
170.71
169.68
169.15
156.21
156.04
121.20
96.90
77.48 cdcl3
77.16 cdcl3
76.84 cdcl3
72.91
70.54
70.51
70.48
70.25
70.21
70.19
70.07
70.04
68.24
66.89
63.09
62.90
51.02
50.53
40.35
28.42
20.80
20.78
20.76
20.74
20.62
f1 (ppm)


COSY NMR
BocNH-PEG4-AcGlc (35)
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f2 (ppm)
0 1 2 3 4 5 6 7 8 9 10
f1 (ppm)

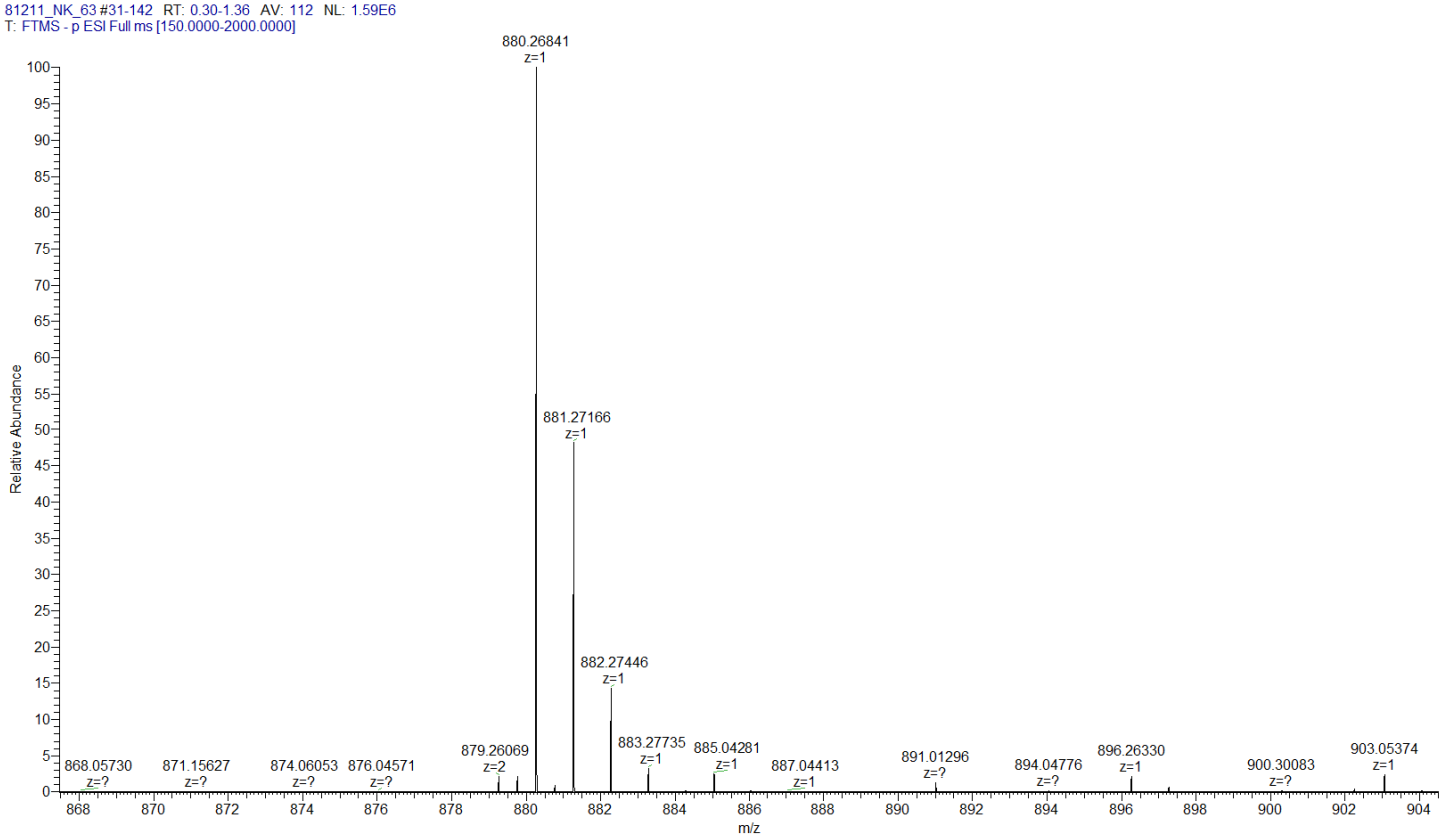

HSQC NMR
BocNH-PEG4-AcGlc (35)
f2 (ppm)
f1 (ppm)

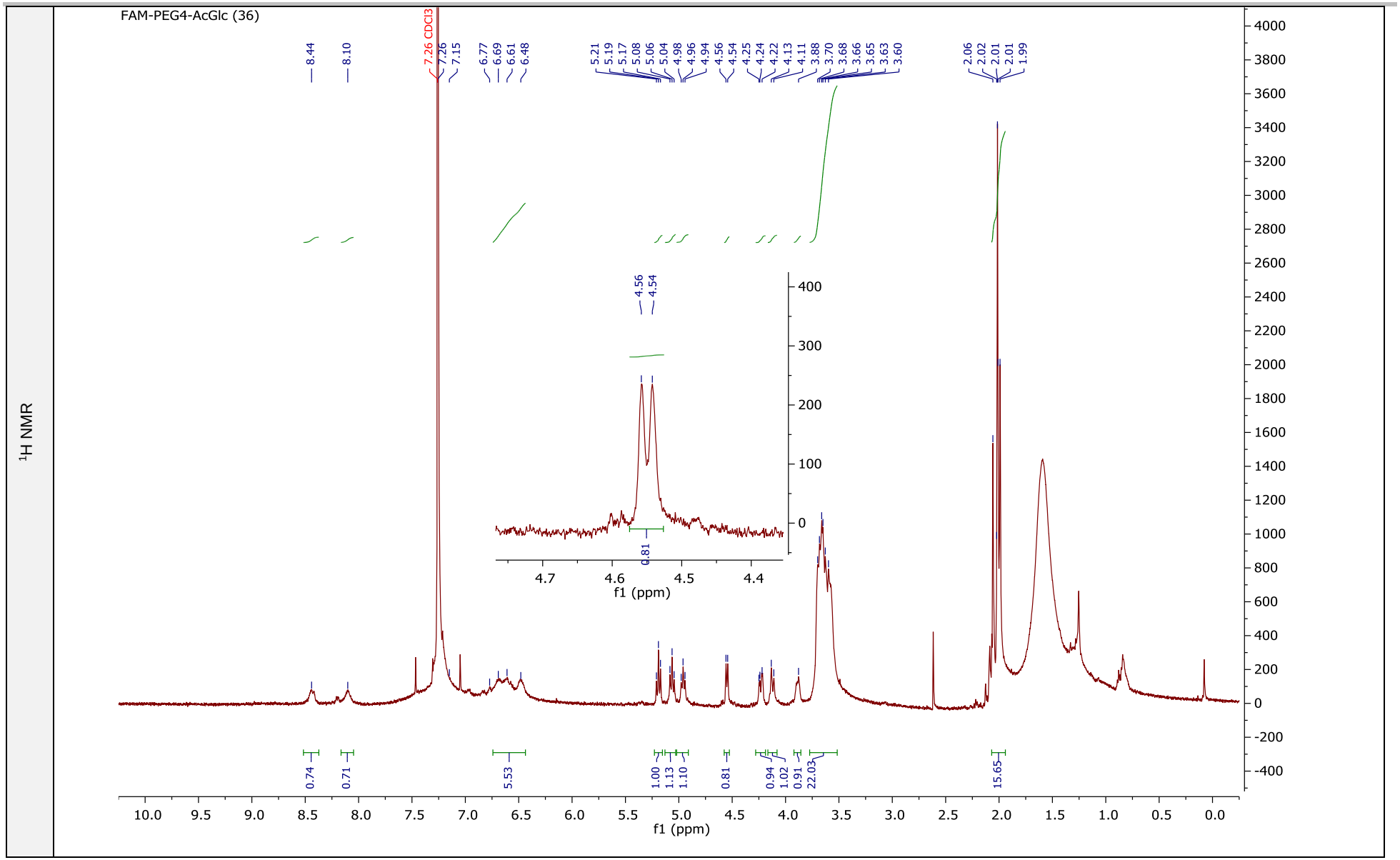
FAM-PEG4-AcGlc (36)

Chemical structure



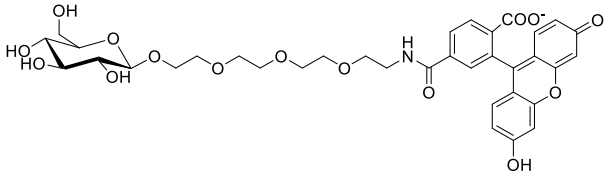
HRMS–ESI (*m/z*): $[M-H]^-$ calcd for $C_{43}H_{46}NO_{19}^-$, 880.26695.




FAM-PEG4-AcGlc (36)
1H NMR
f1 (ppm)

FAM-PEG4-β-Glc (3)

Chemical structure



HPLC profile

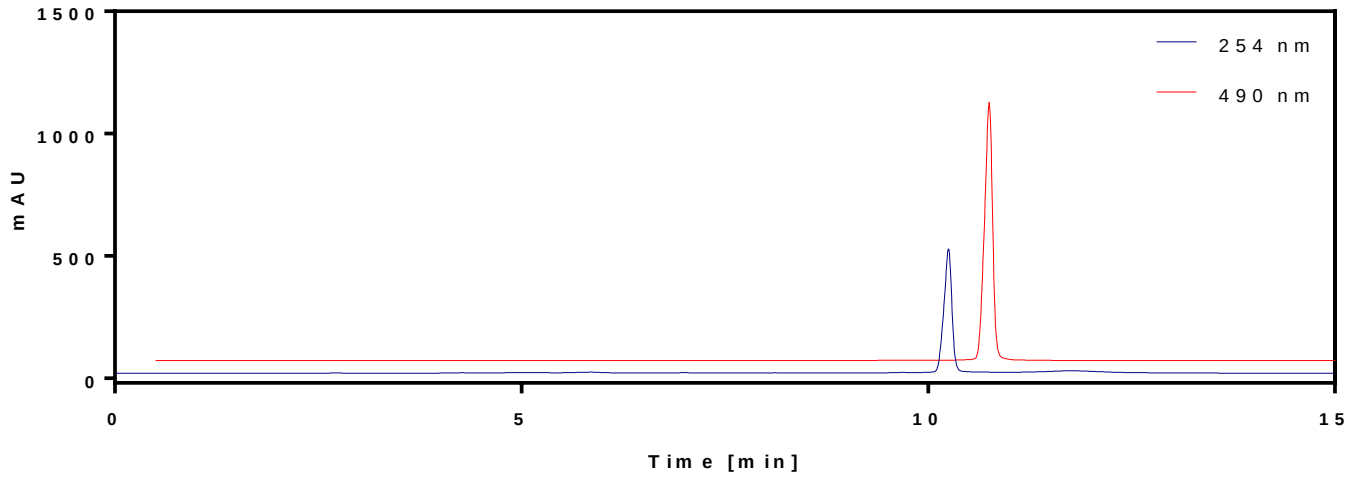
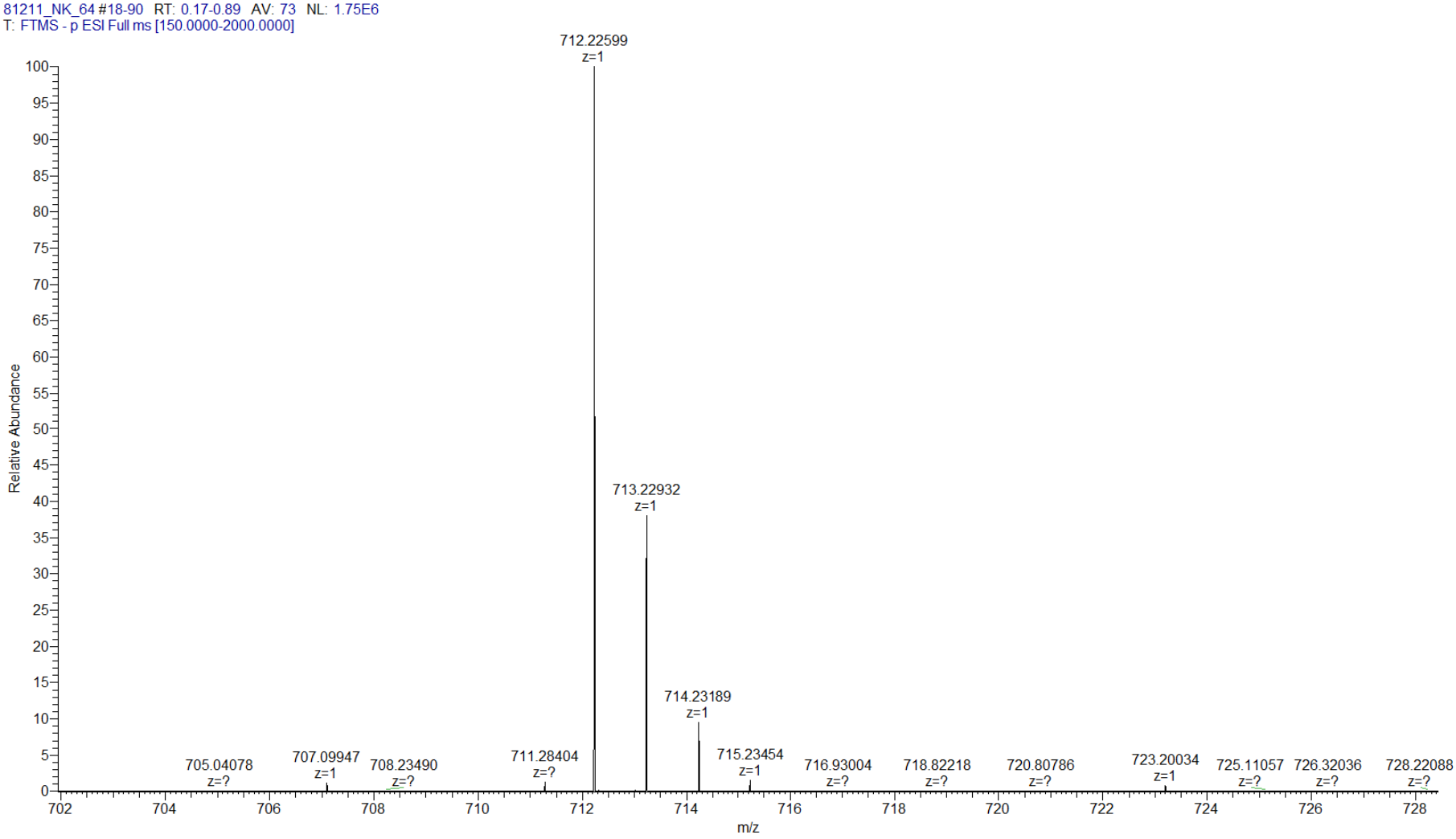
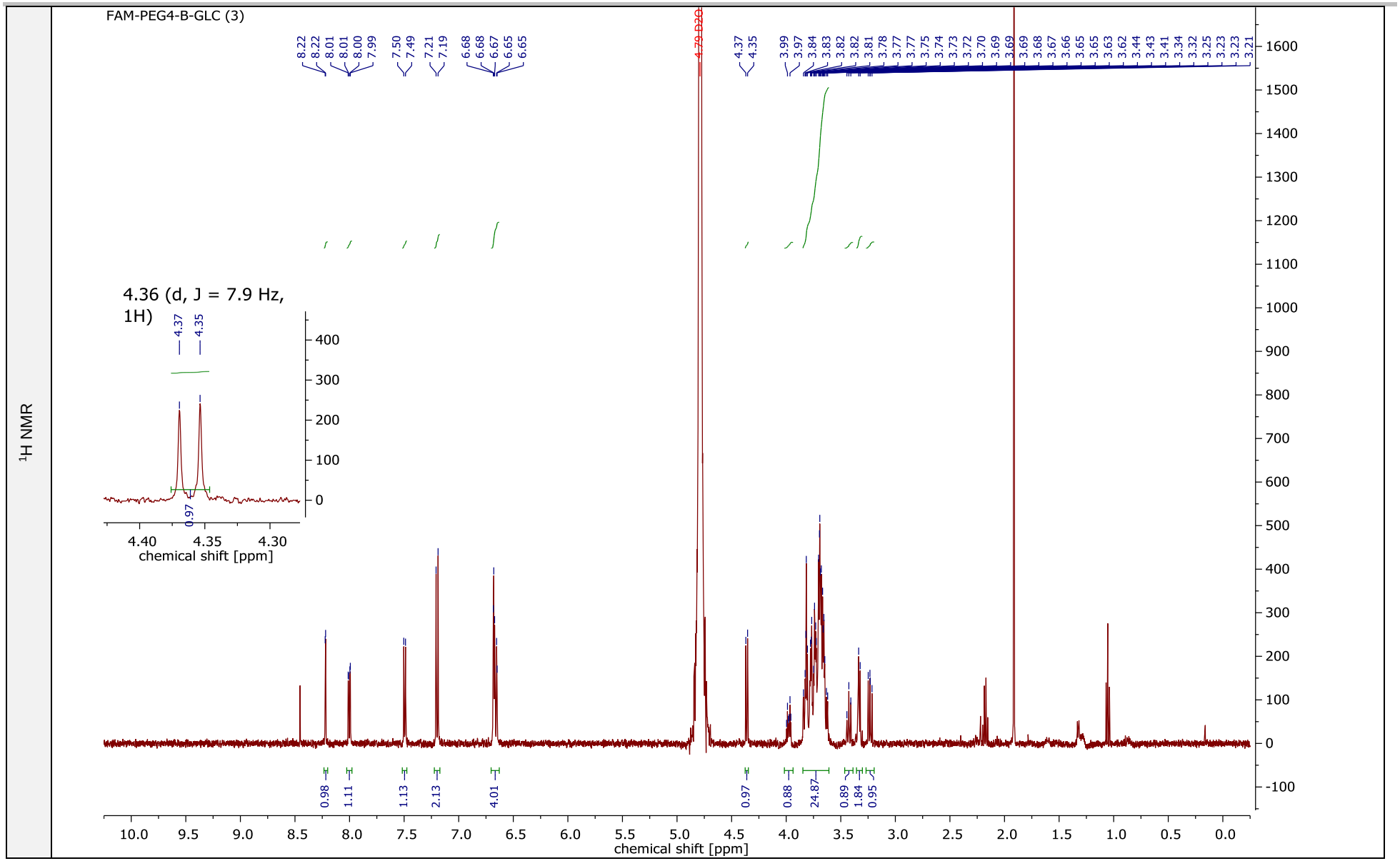


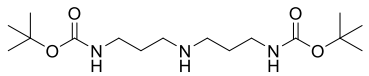
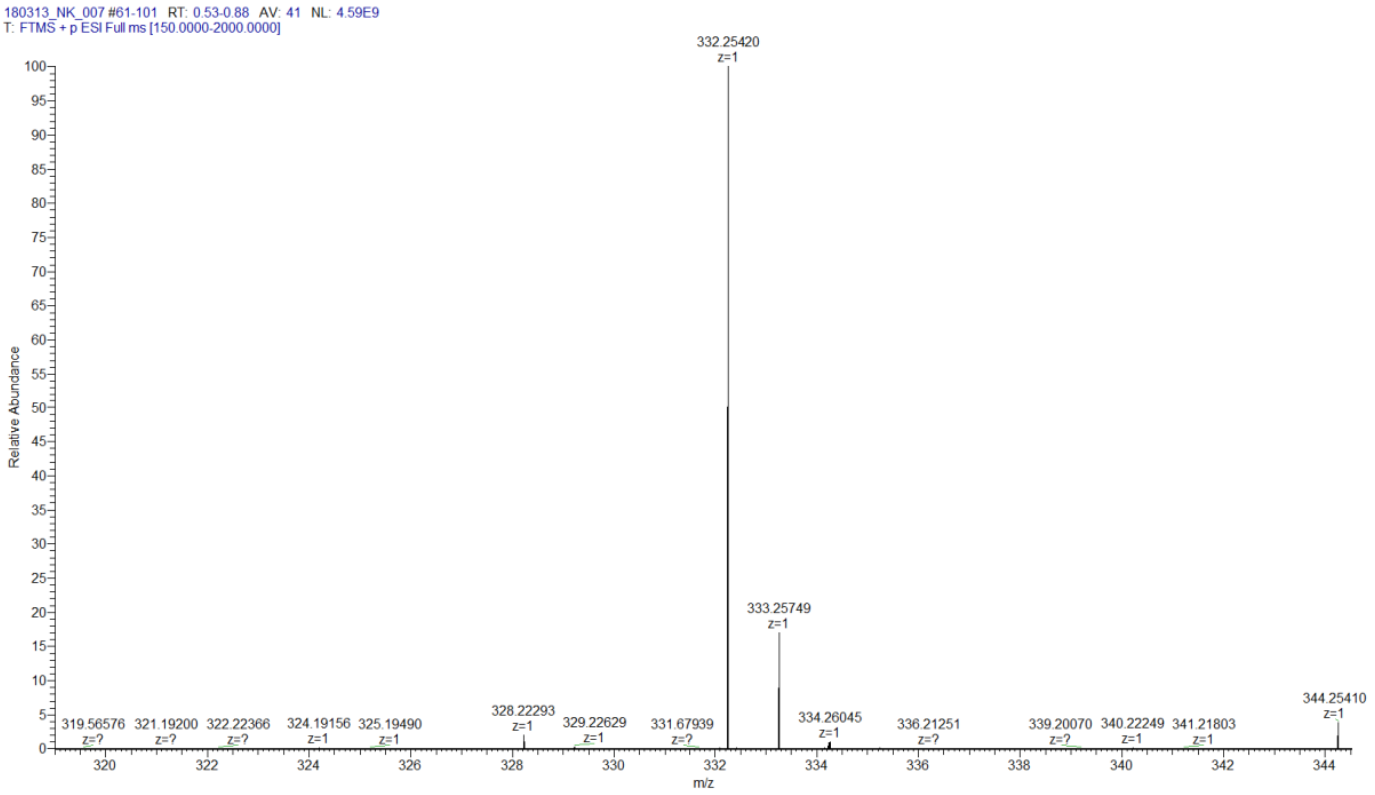
Figure S9. Analytical HPLC profile of compound **3**: column C18, Buffer A:ACN 0-100% in 15 min.

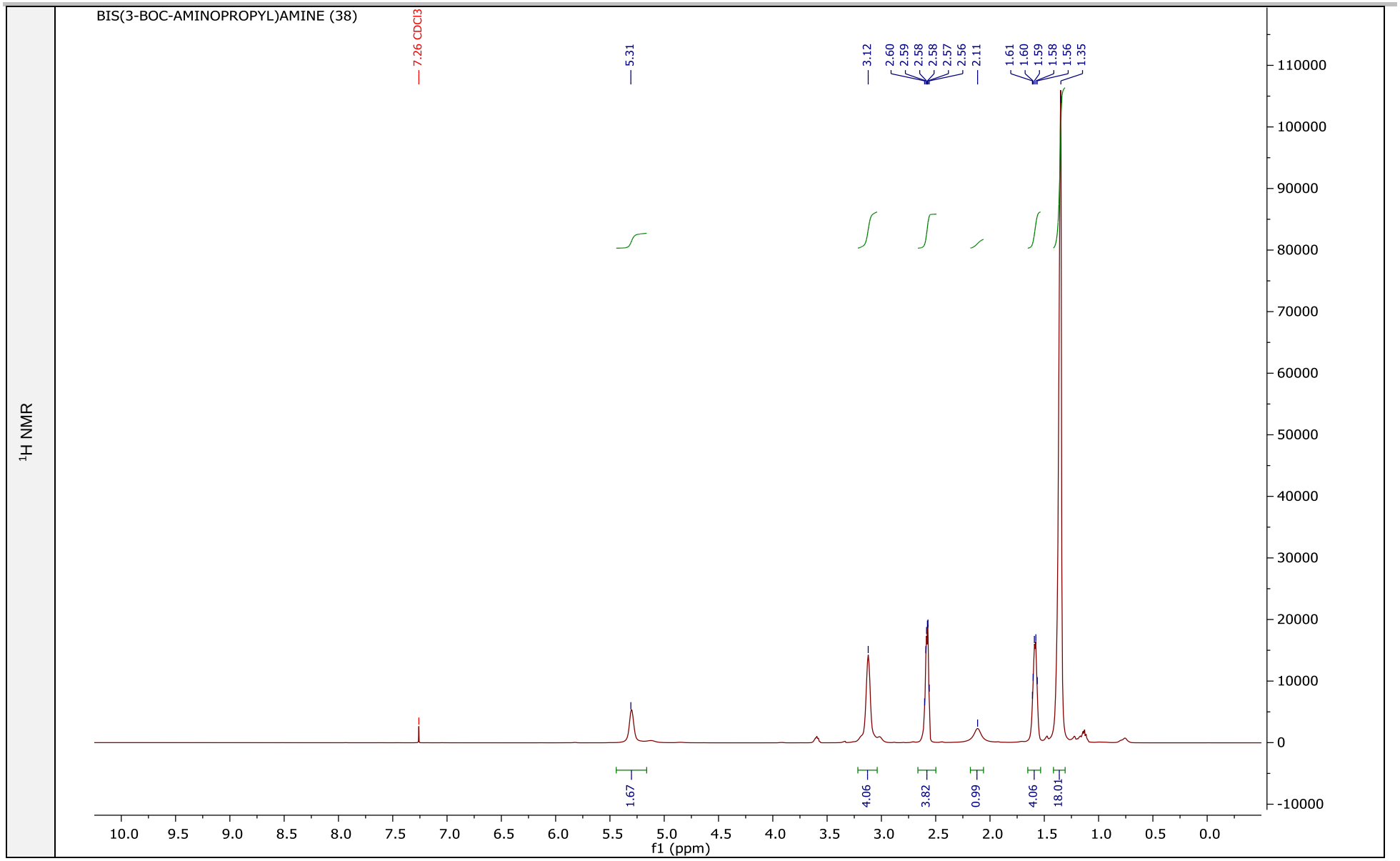
HRMS–ESI (m/z): [M-H]$^-$ calcd for $C_{35}H_{38}NO_{15}^-$, 712.22469.

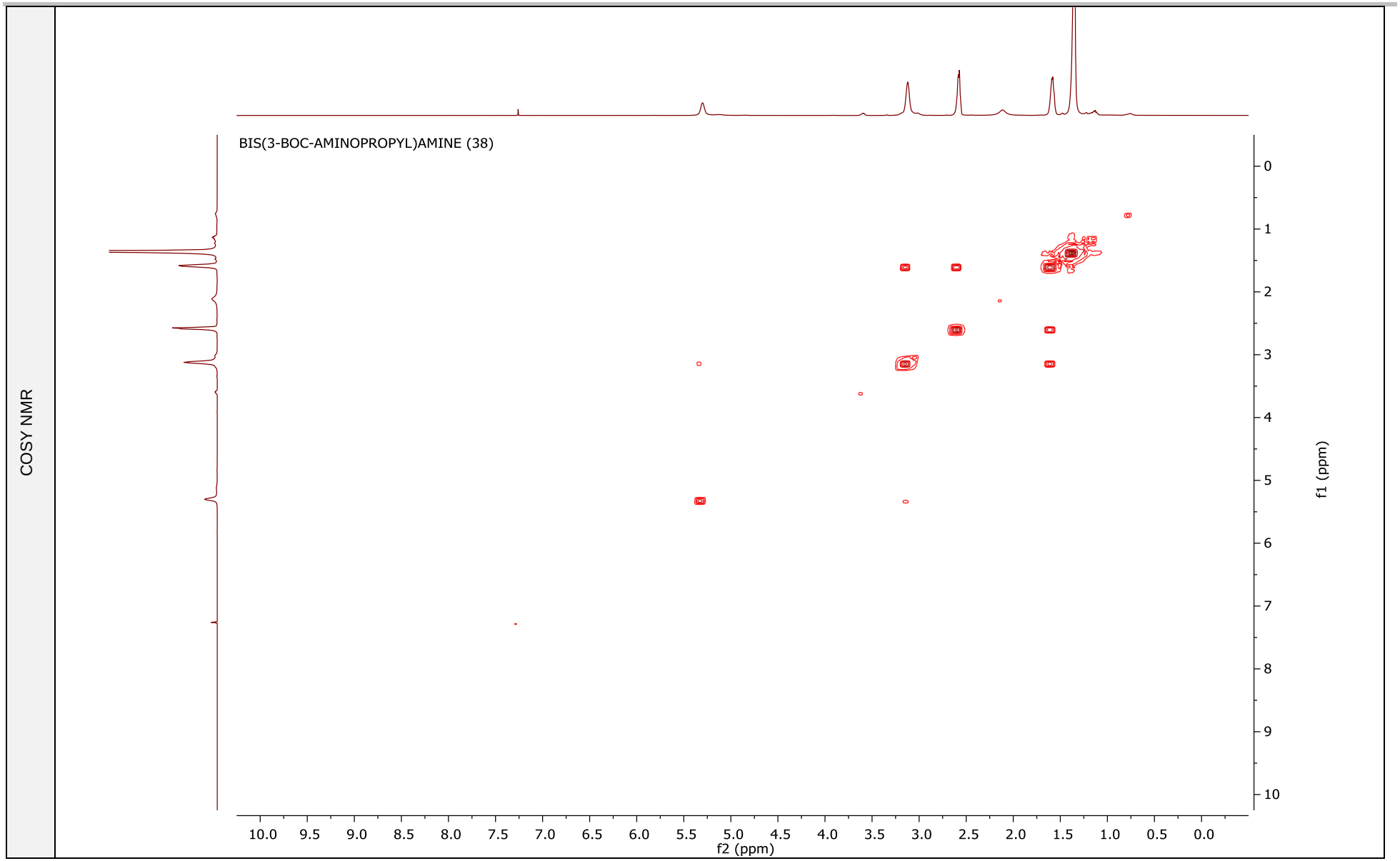


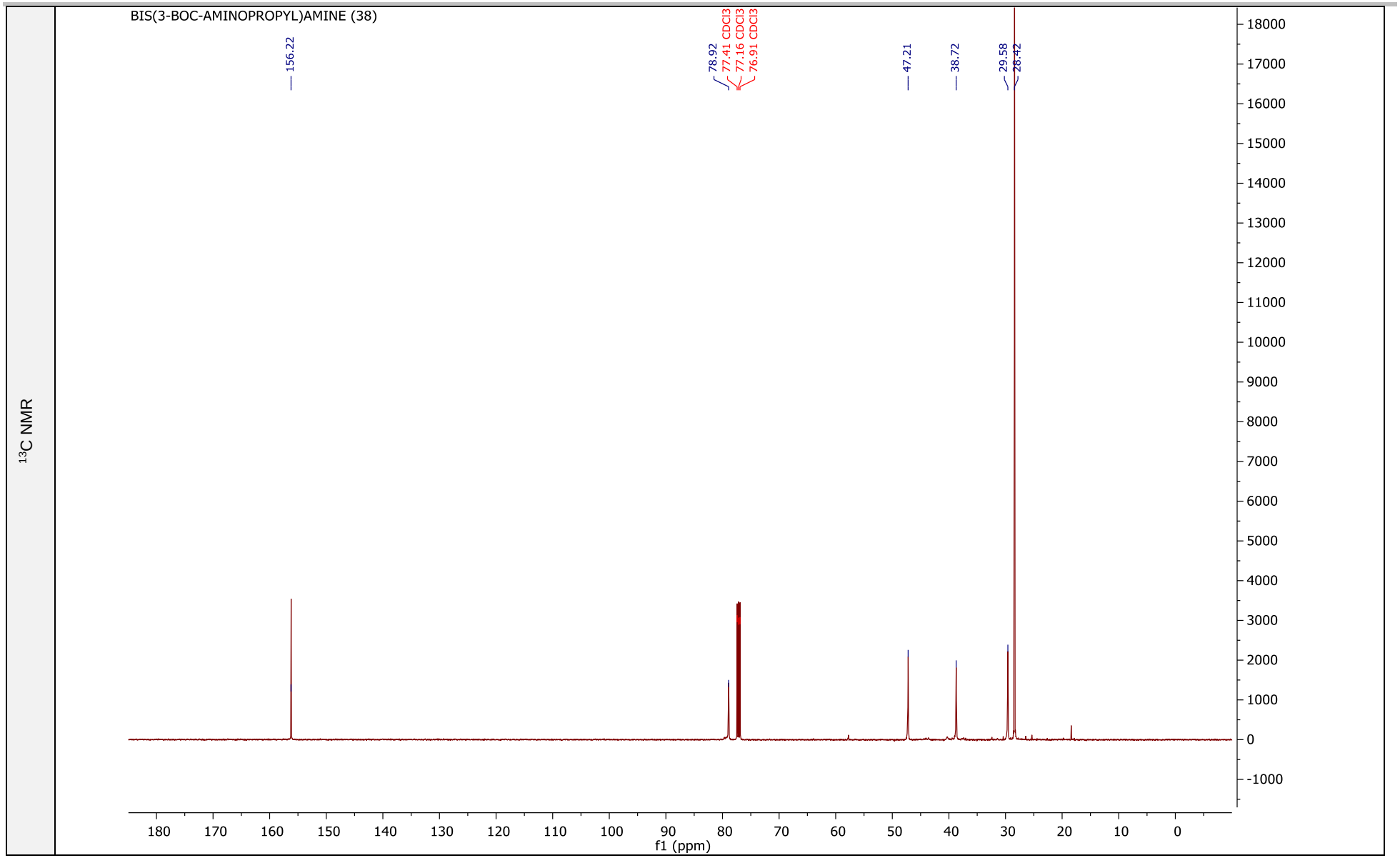

FAM-PEG4-B-GLC (3)
^{1}H NMR
4.36 (d, J = 7.9 Hz, 1H)
chemical shift [ppm]
chemical shift [ppm]

Double-functionalized cap-ligand fluorescent probes (15-17)

Bis(3-Boc-aminopropyl)amine (38)	
Chemical structure	
HRMS–ESI (*m/z*): $[M+H]^+$ calcd for $C_{16}H_{34}N_3O_4^+$, 332.25493.	

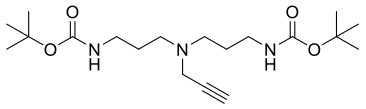

BIS(3-BOC-AMINOPROPYL)AMINE (38)
1H NMR
7.26 CDCl3
5.31
3.12
2.60
2.59
2.58
2.58
2.57
2.56
2.11
1.61
1.60
1.59
1.58
1.56
1.35
1.67
4.06
3.82
0.99
4.06
18.01
f1 (ppm)


COSY NMR
BIS(3-BOC-AMINOPROPYL)AMINE (38)
f2 (ppm)
f1 (ppm)

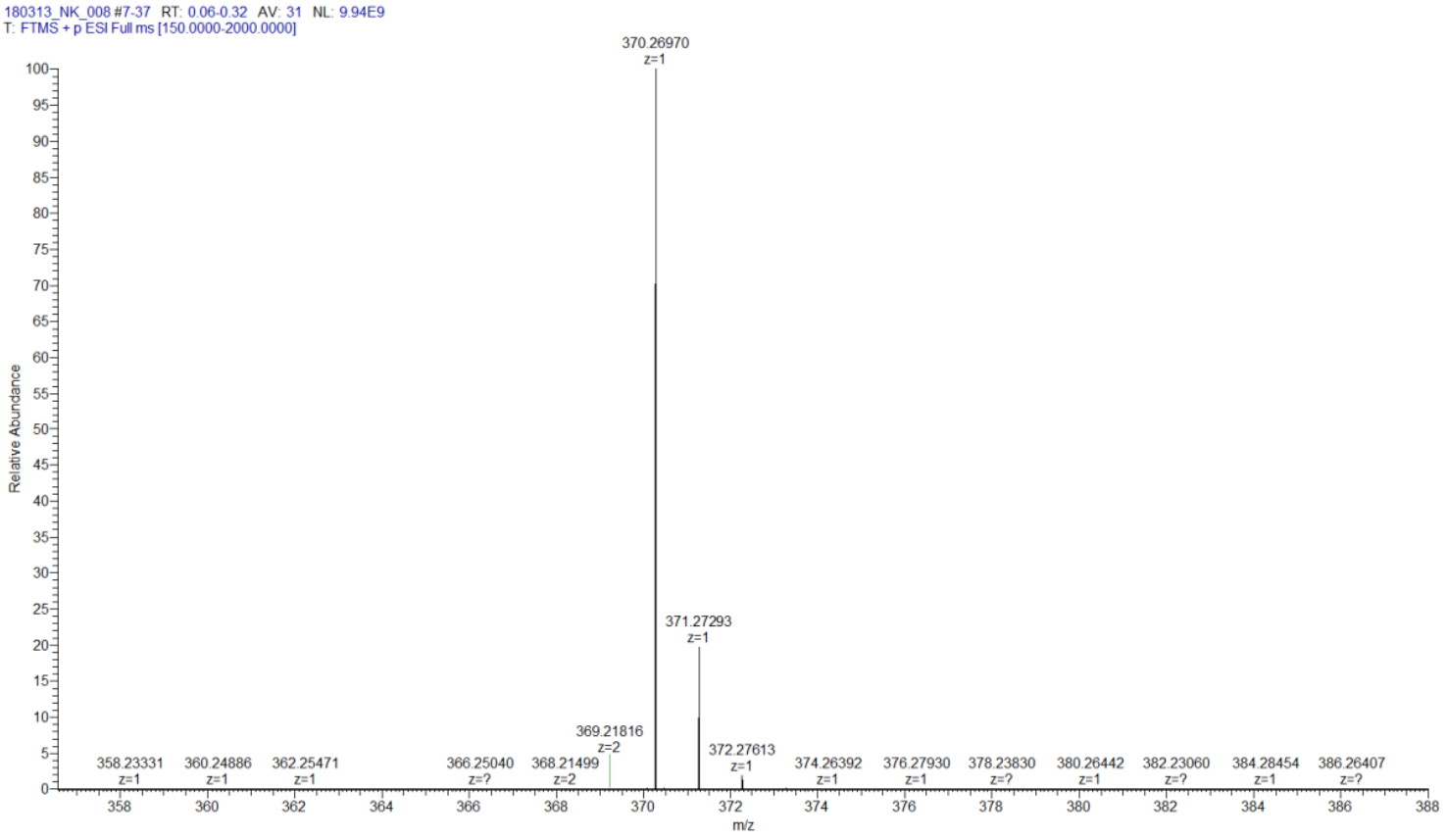

BIS(3-BOC-AMINOPROPYL)AMINE (38)
13C NMR
156.22
78.92
77.41 CDCl3
77.16 CDCl3
76.91 CDCl3
47.21
38.72
29.58
28.42
f1 (ppm)

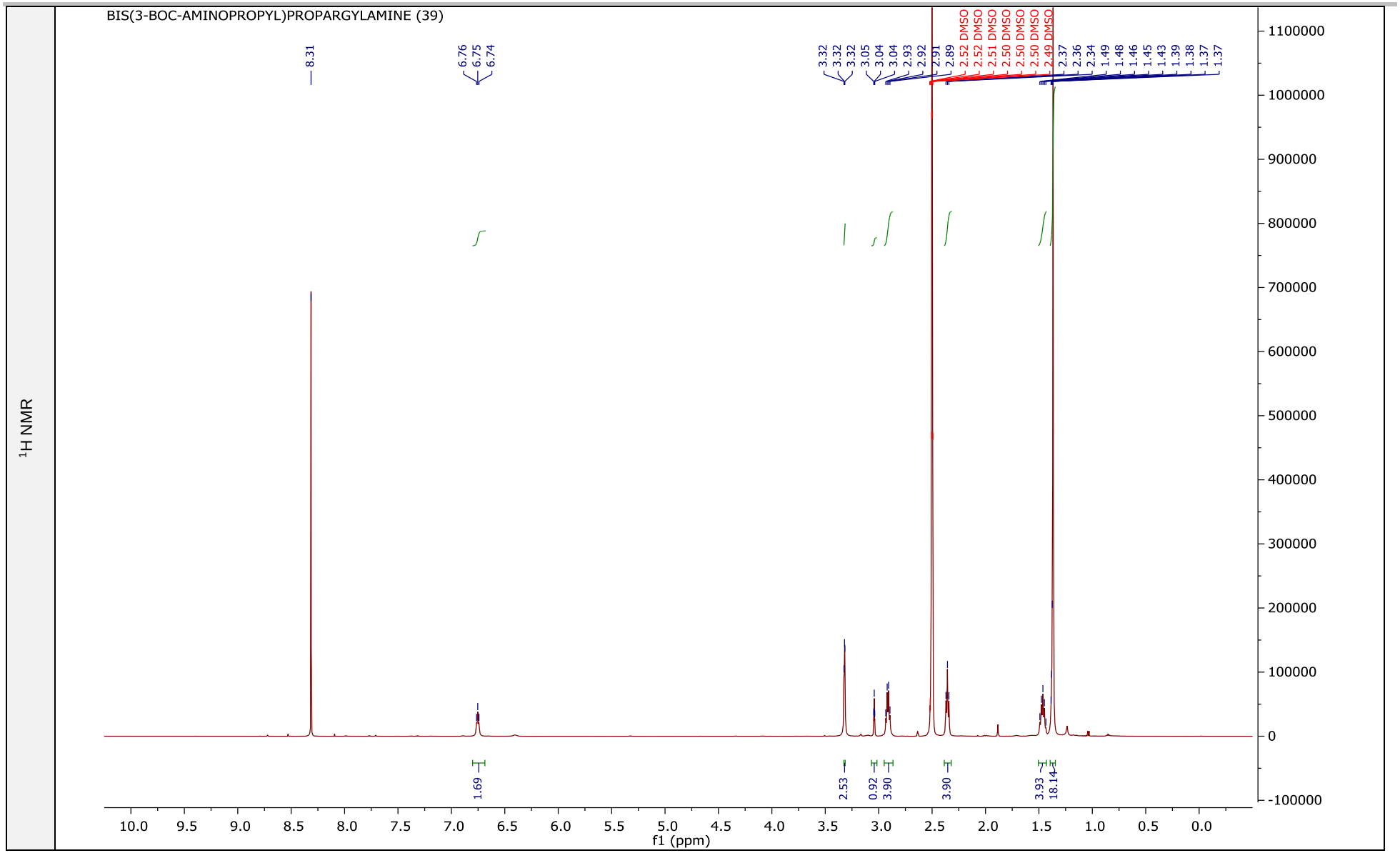
Bis(3-Boc-aminopropyl)propargylamine (39)

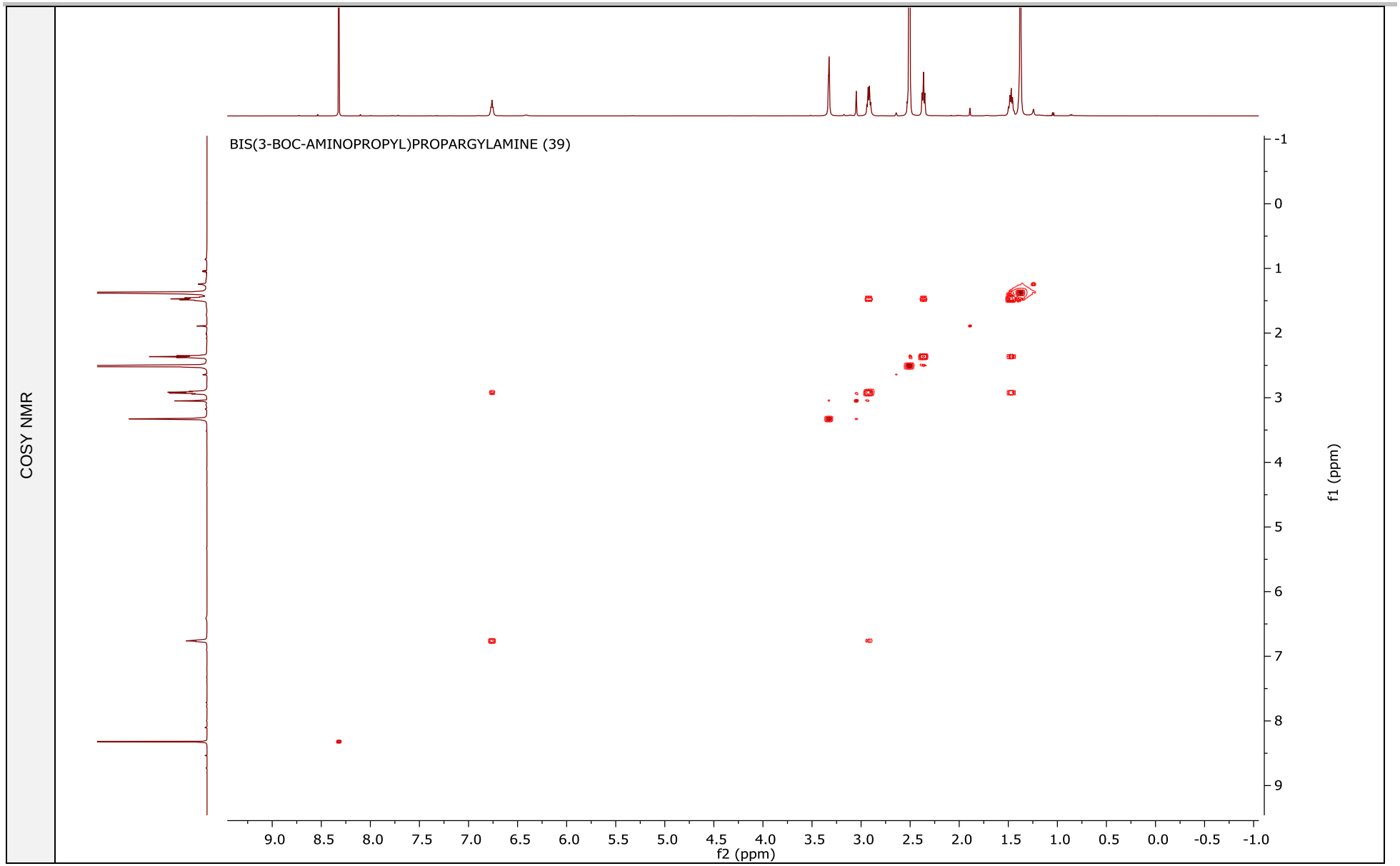
Chemical structure

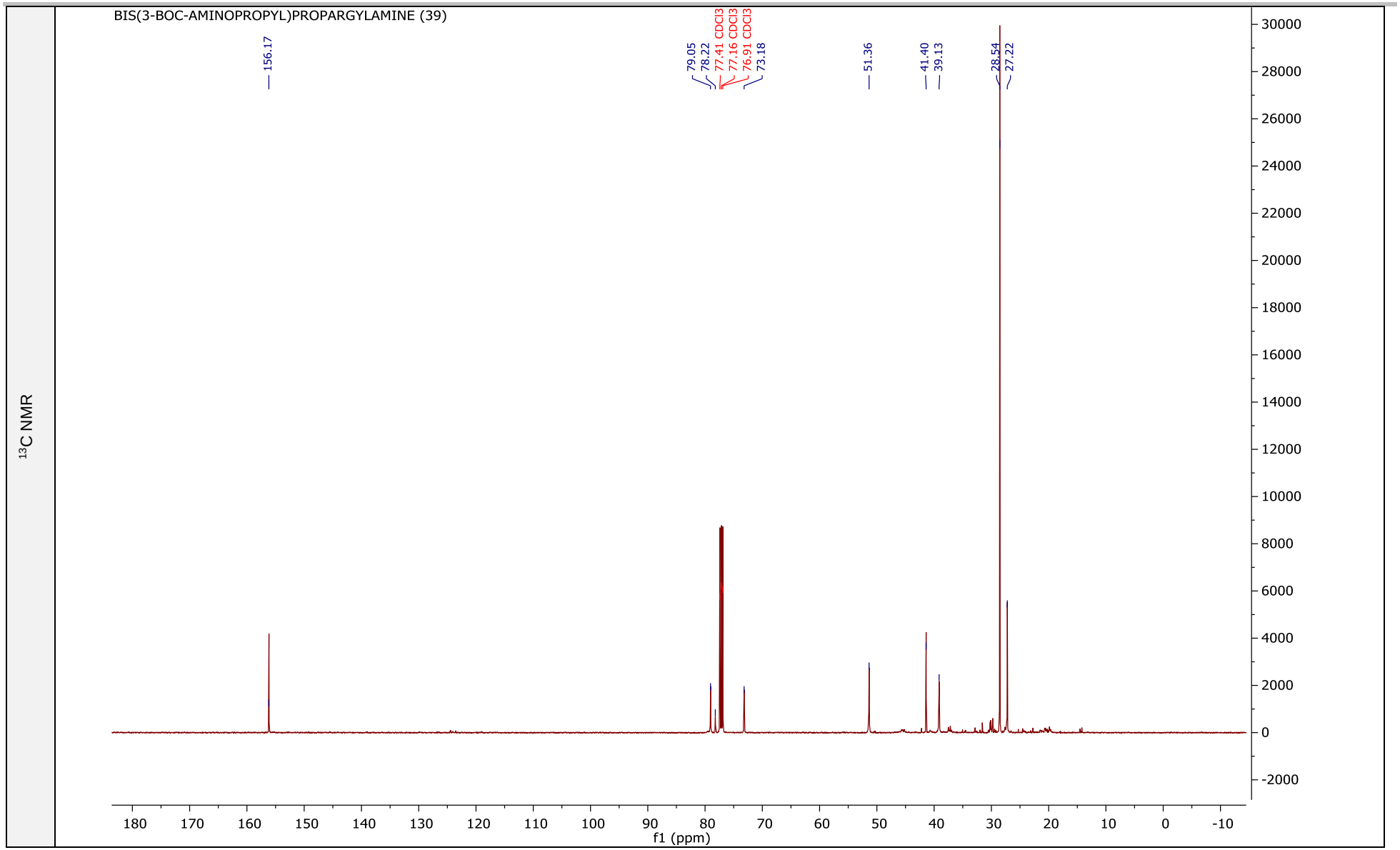


HRMS–ESI (*m/z*): $[M+H]^+$ calcd for $C_{19}H_{36}N_3O_4^+$, 370.27058.



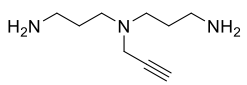

BIS(3-BOC-AMINOPROPYL)PROPARGYLAMINE (39)
^{1}H NMR
8.31
6.76
6.75
6.74
3.32
3.32
3.32
3.05
3.04
3.04
2.93
2.92
2.91
2.89
2.52 DMSO
2.52 DMSO
2.51 DMSO
2.50 DMSO
2.50 DMSO
2.50 DMSO
2.49 DMSO
2.37
2.36
2.34
1.49
1.48
1.46
1.45
1.43
1.39
1.38
1.37
1.37
1.69
2.53
0.92
3.90
3.90
3.93
18.14
f1 (ppm)


COSY NMR
BIS(3-BOC-AMINOPROPYL)PROPARGYLAMINE (39)
f2 (ppm)
f1 (ppm)

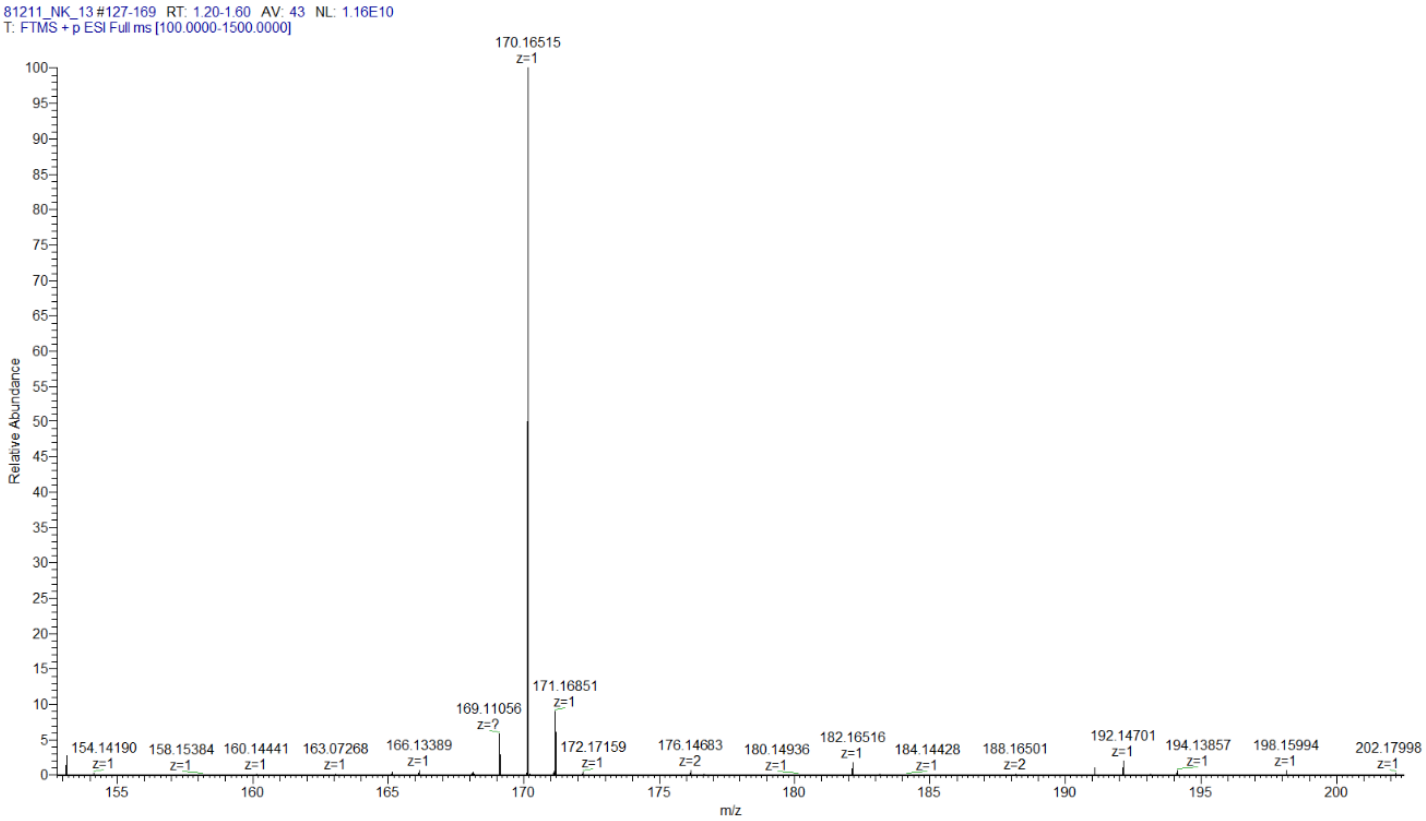

BIS(3-BOC-AMINOPROPYL)PROPARGYLAMINE (39)
^{13}C NMR
156.17
79.05
78.22
77.41 CDCl3
77.16 CDCl3
76.91 CDCl3
73.18
51.36
41.40
39.13
28.54
27.22
f1 (ppm)

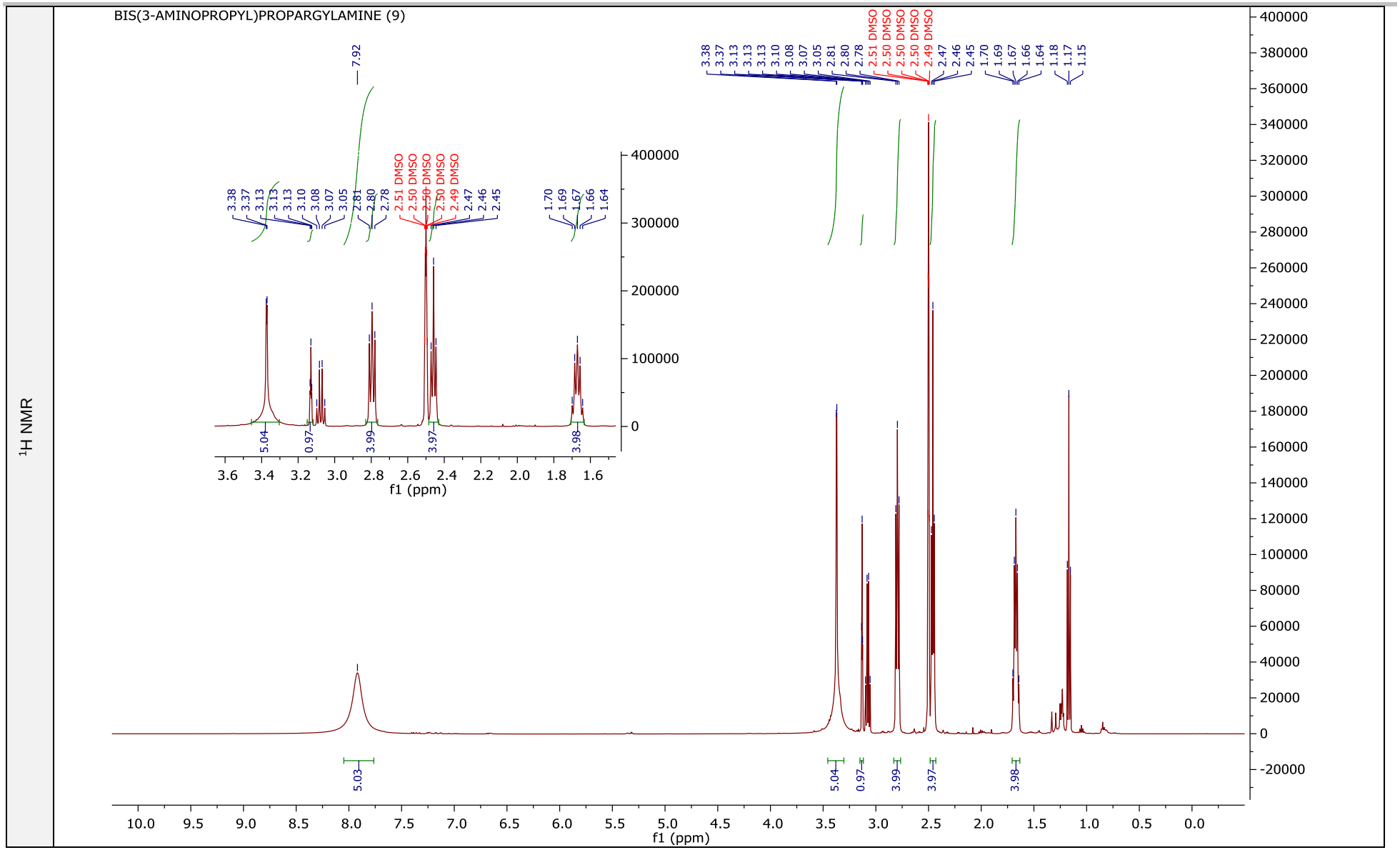
Bis(3-aminopropyl)propargylamine (9)

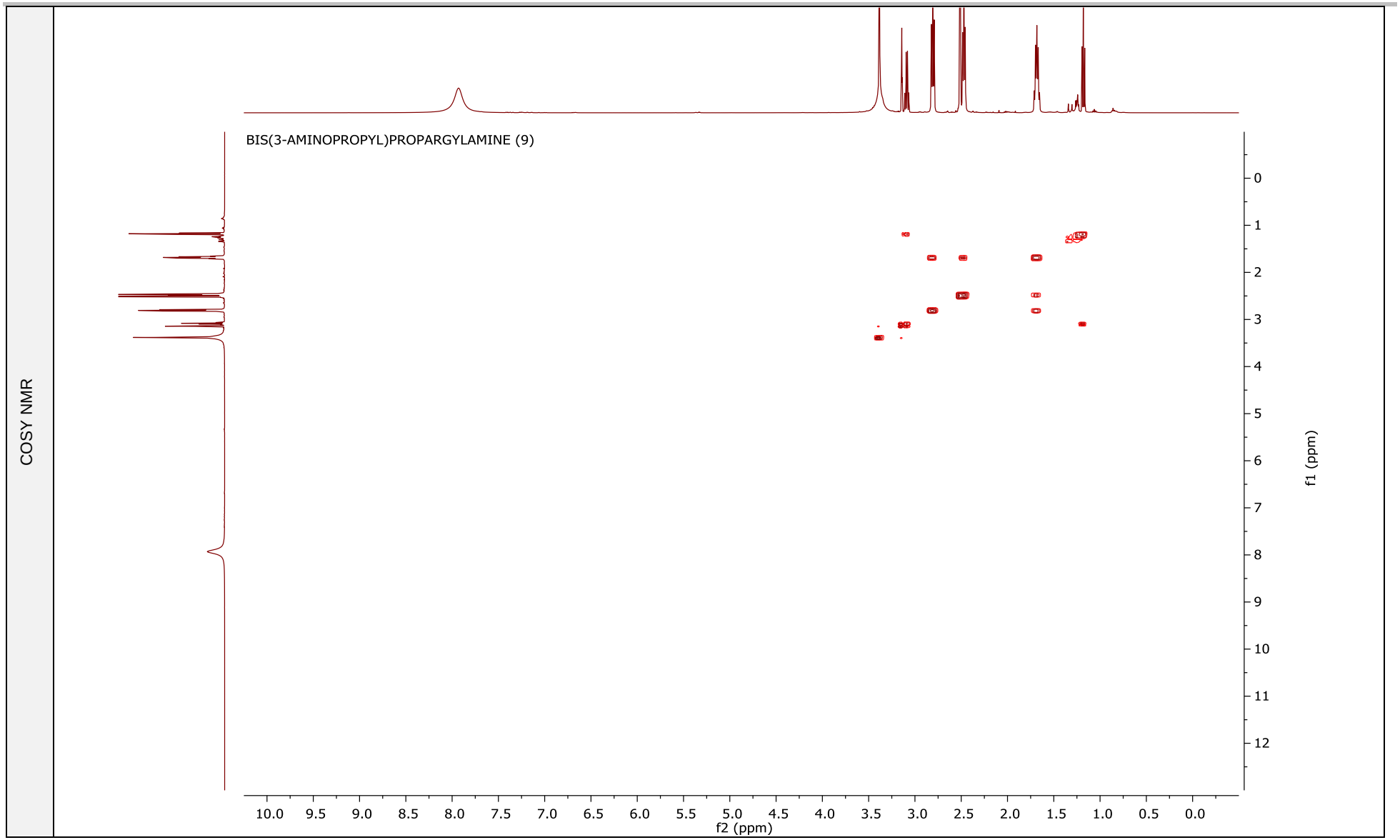
Chemical structure

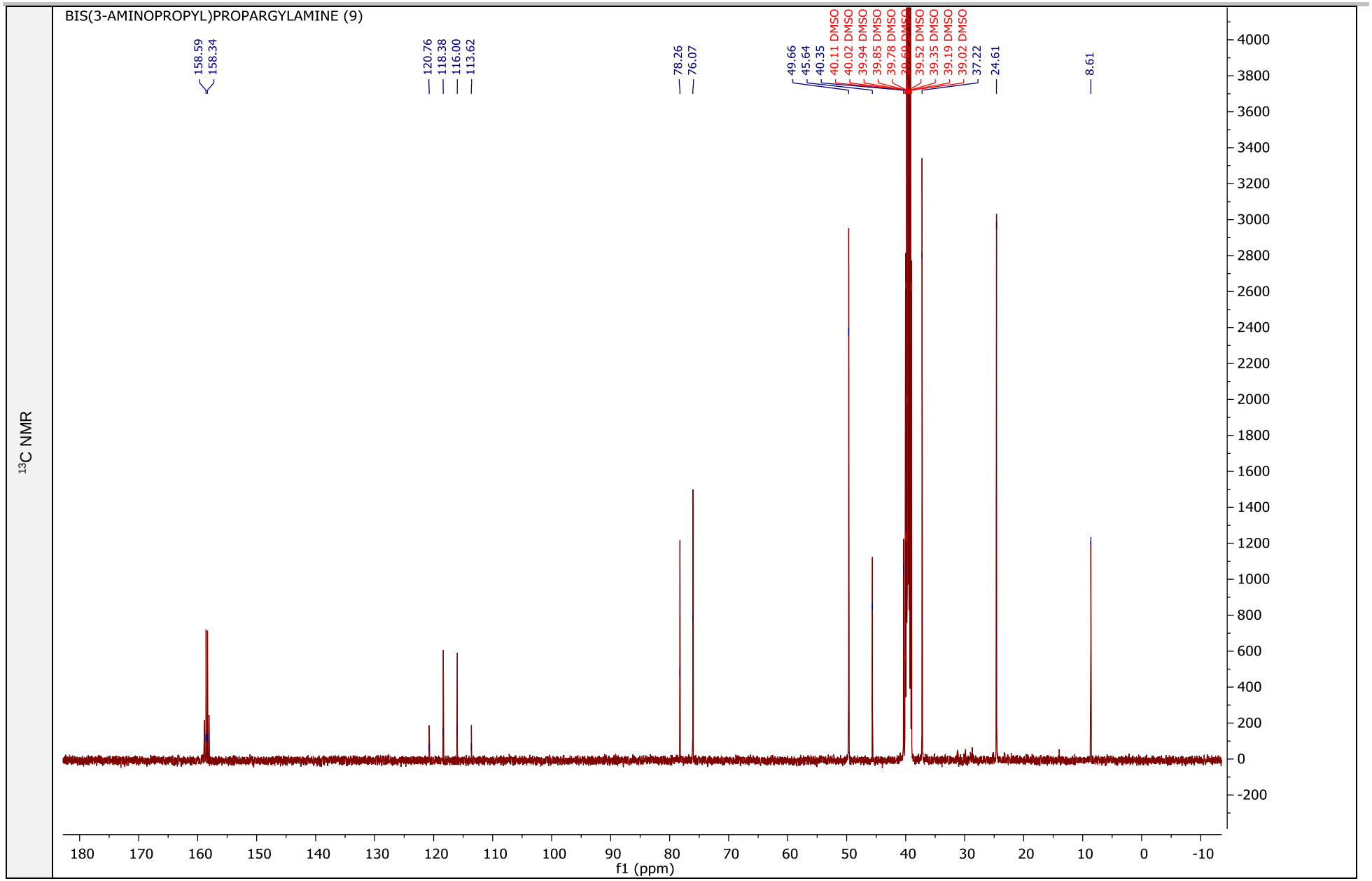


HRMS–ESI (*m/z*): $[M+H]^+$ calcd for $C_9H_{20}N_3^+$, 170.16572.



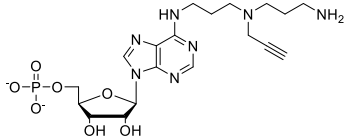

BIS(3-AMINOPROPYL)PROPARGYLAMINE (9)
1H NMR
f1 (ppm)


COSY NMR
BIS(3-AMINOPROPYL)PROPARGYLAMINE (9)
f2 (ppm)
f1 (ppm)


BIS(3-AMINOPROPYL)PROPARGYLAMINE (9)
13C NMR
158.59
158.34
120.76
118.38
116.00
113.62
78.26
76.07
49.66
45.64
40.35
40.11 DMSO
40.02 DMSO
39.94 DMSO
39.85 DMSO
39.78 DMSO
39.60 DMSO
39.52 DMSO
39.35 DMSO
39.19 DMSO
39.02 DMSO
37.22
24.61
8.61
f1 (ppm)

AMP-Bis(3-aminopropyl)propargylamine / NH_4^+ (11)

Chemical structure



HPLC profile

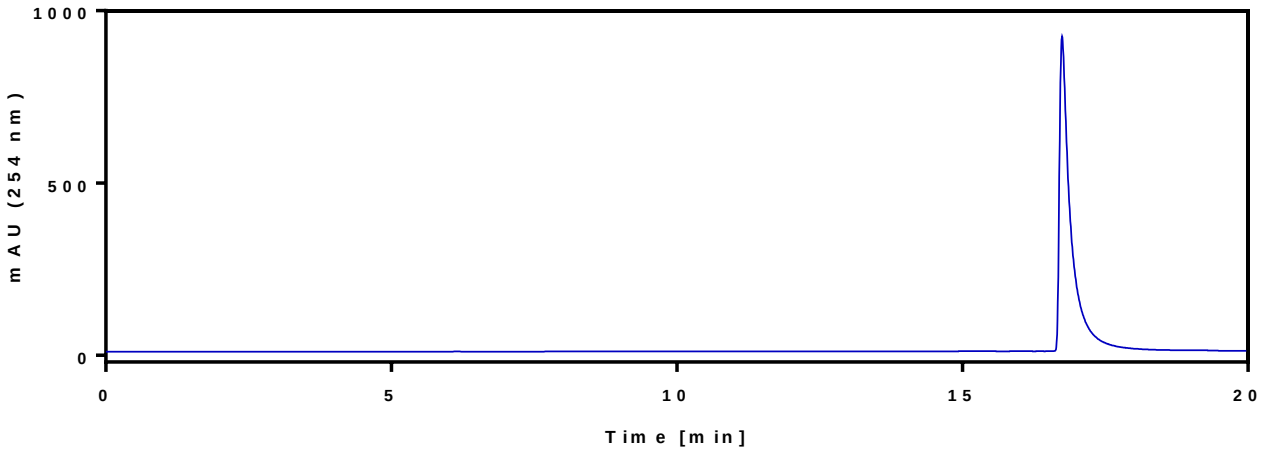
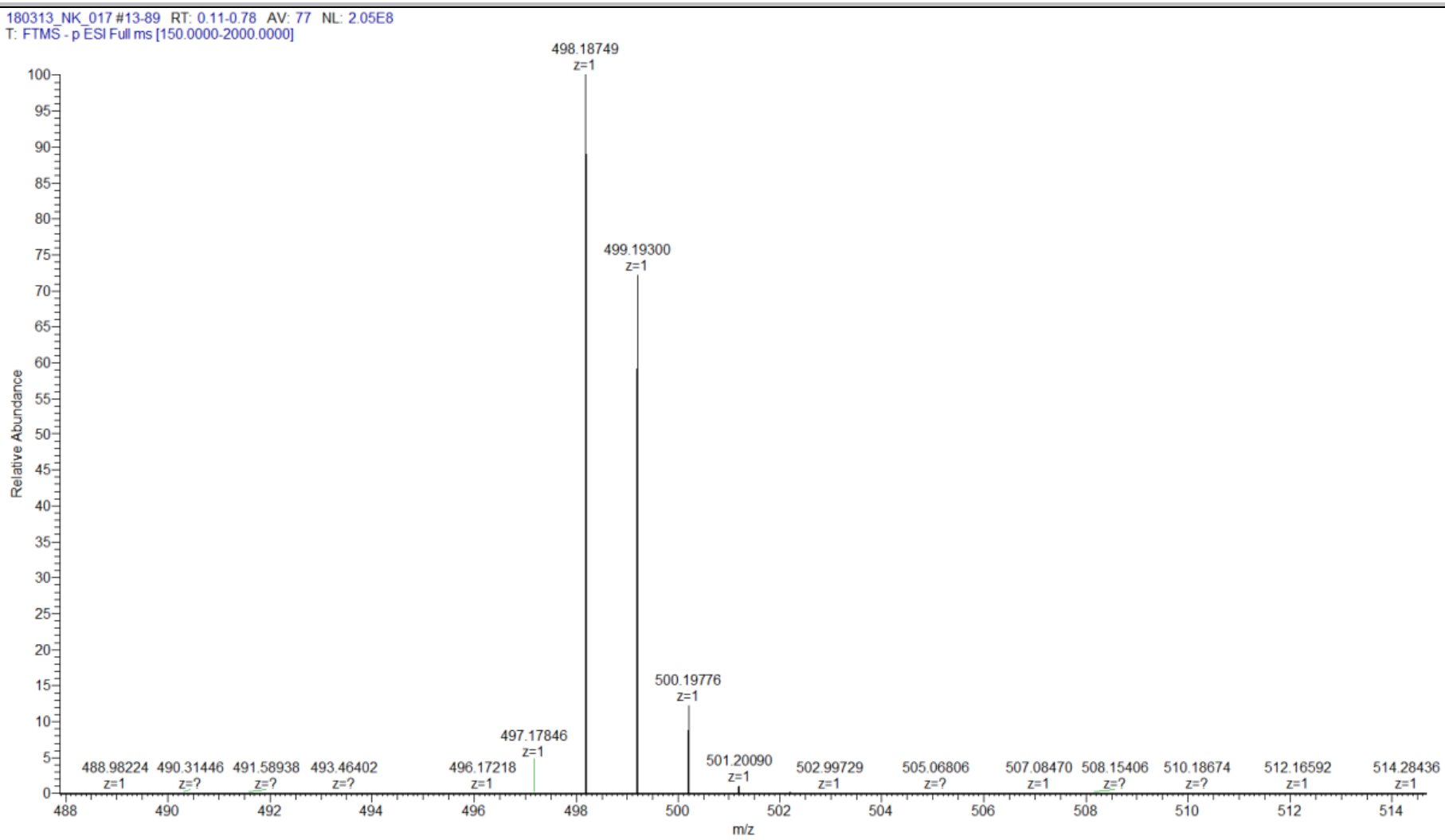
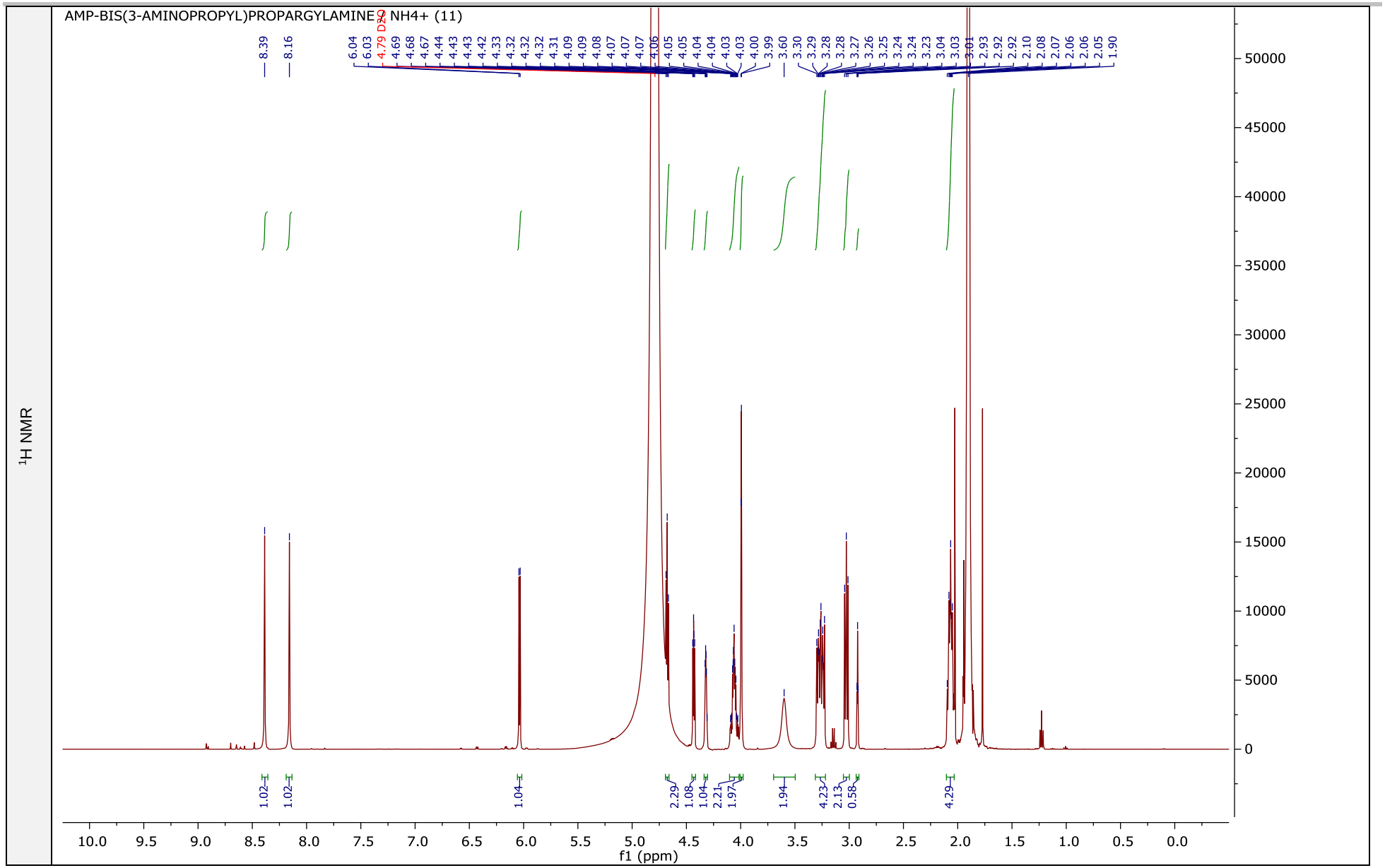
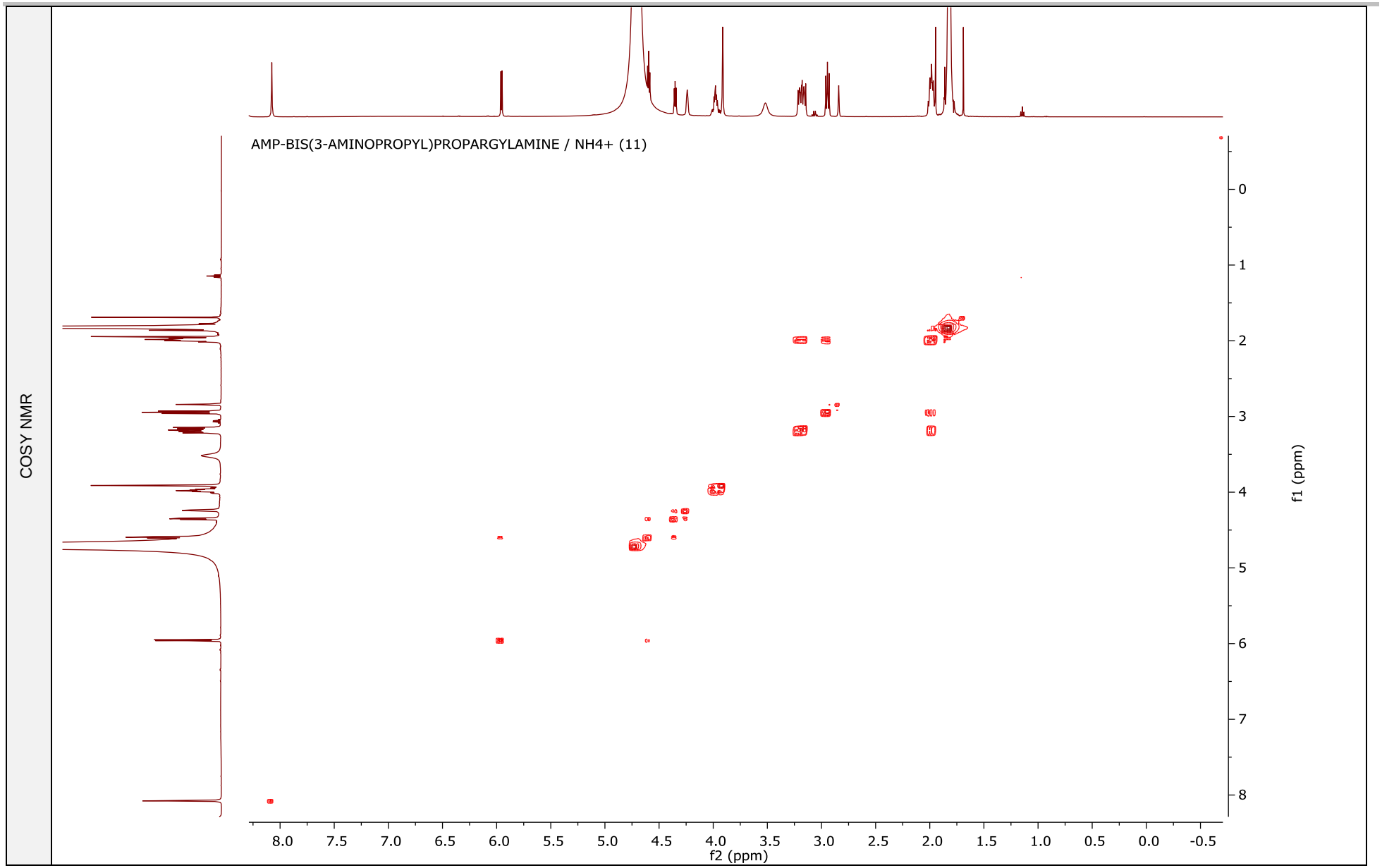


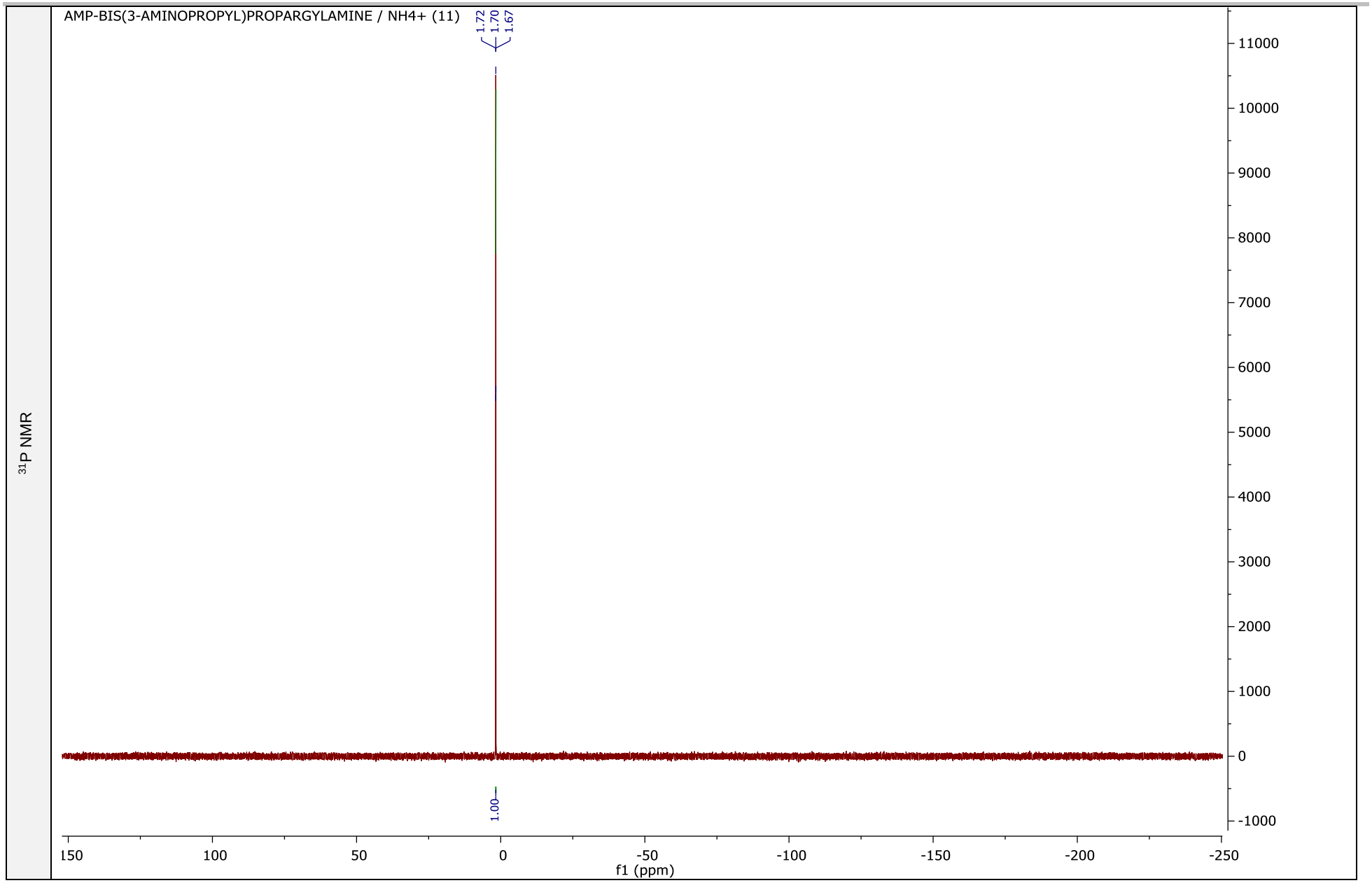
Figure S10. Analytical HPLC profile of compound **11**: column C18, Buffer A:Buffer B 0-100% in 15 min.

HRMS–ESI (*m/z*): $[M-H]^-$ calcd for $C_{19}H_{29}N_7O_7P^-$, 498.18716.




AMP-BIS(3-AMINOPROPYL)PROPARGYLAMINE + NH4+ (11)
1H NMR
f1 (ppm)


COSY NMR
AMP-BIS(3-AMINOPROPYL)PROPARGYLAMINE / NH4+ (11)
f2 (ppm)
f1 (ppm)
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
0
1
2
3
4
5
6
7
8


AMP-BIS(3-AMINOPROPYL)PROPARGYLAMINE / NH4+ (11)
1.72
1.70
1.67
31P NMR
1.00
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-1000
150
100
50
0
-50
-100
-150
-200
-250
f1 (ppm)

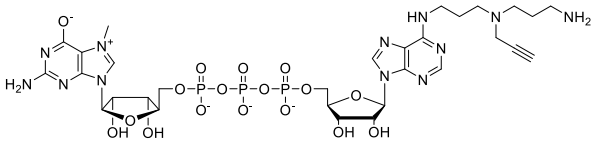
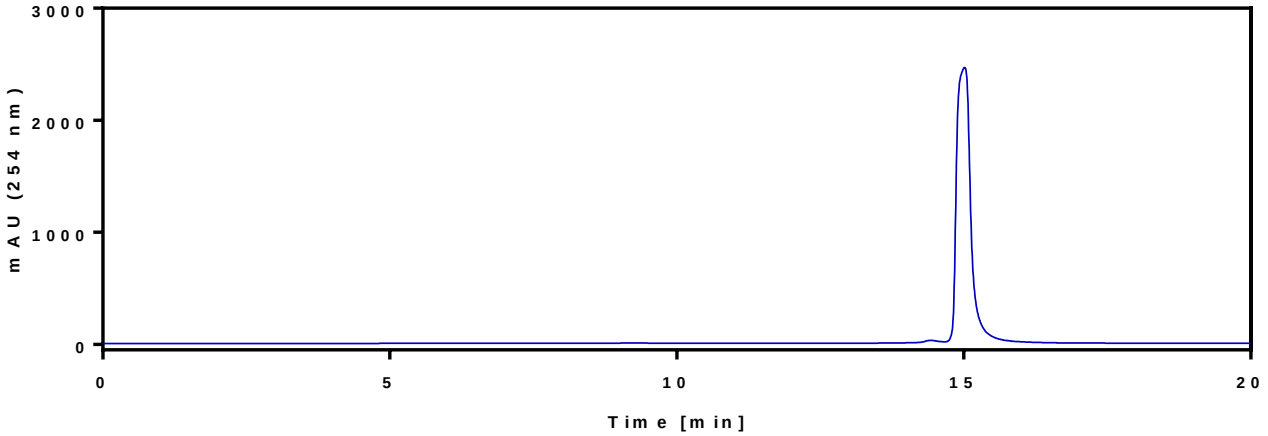
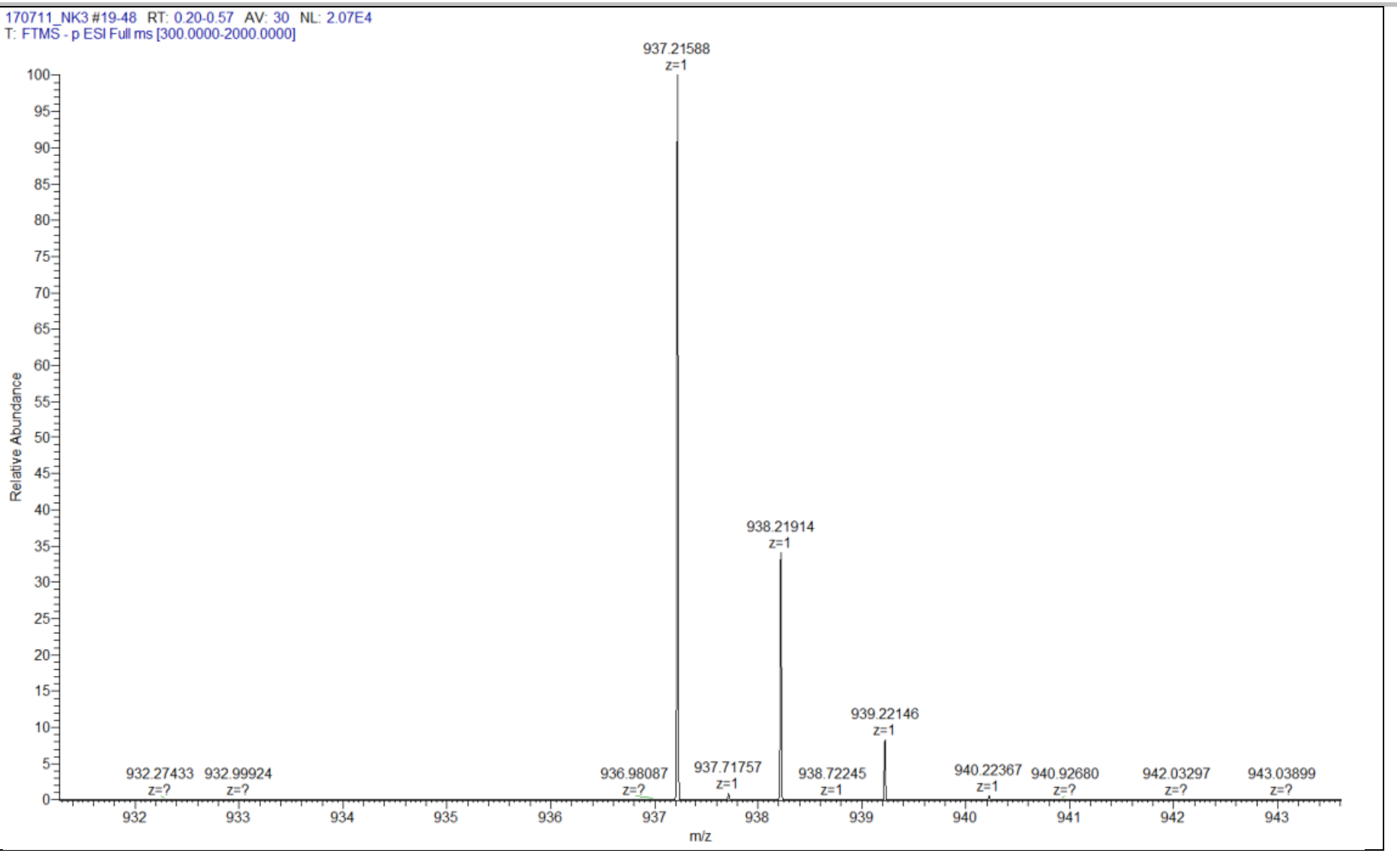
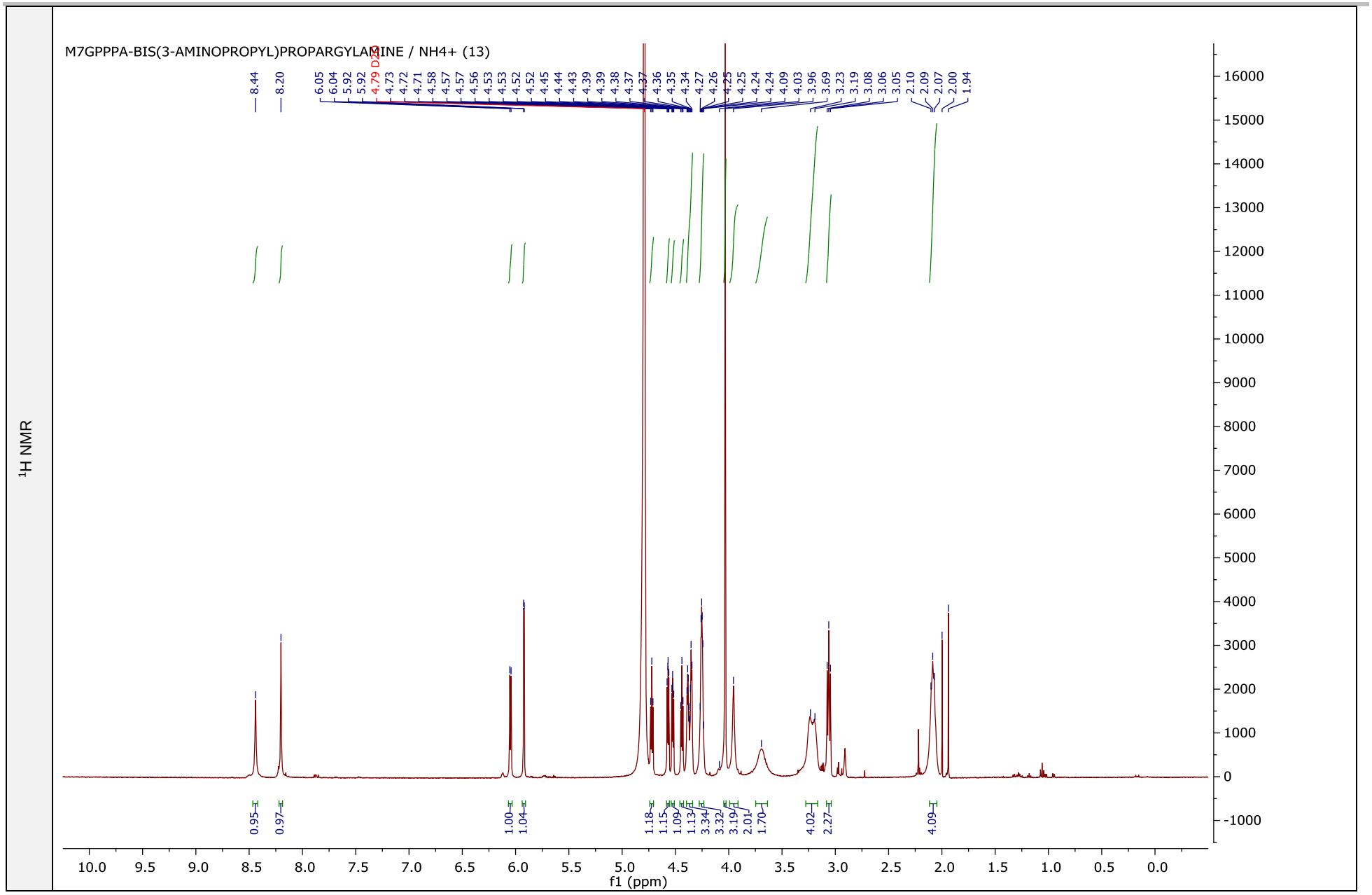
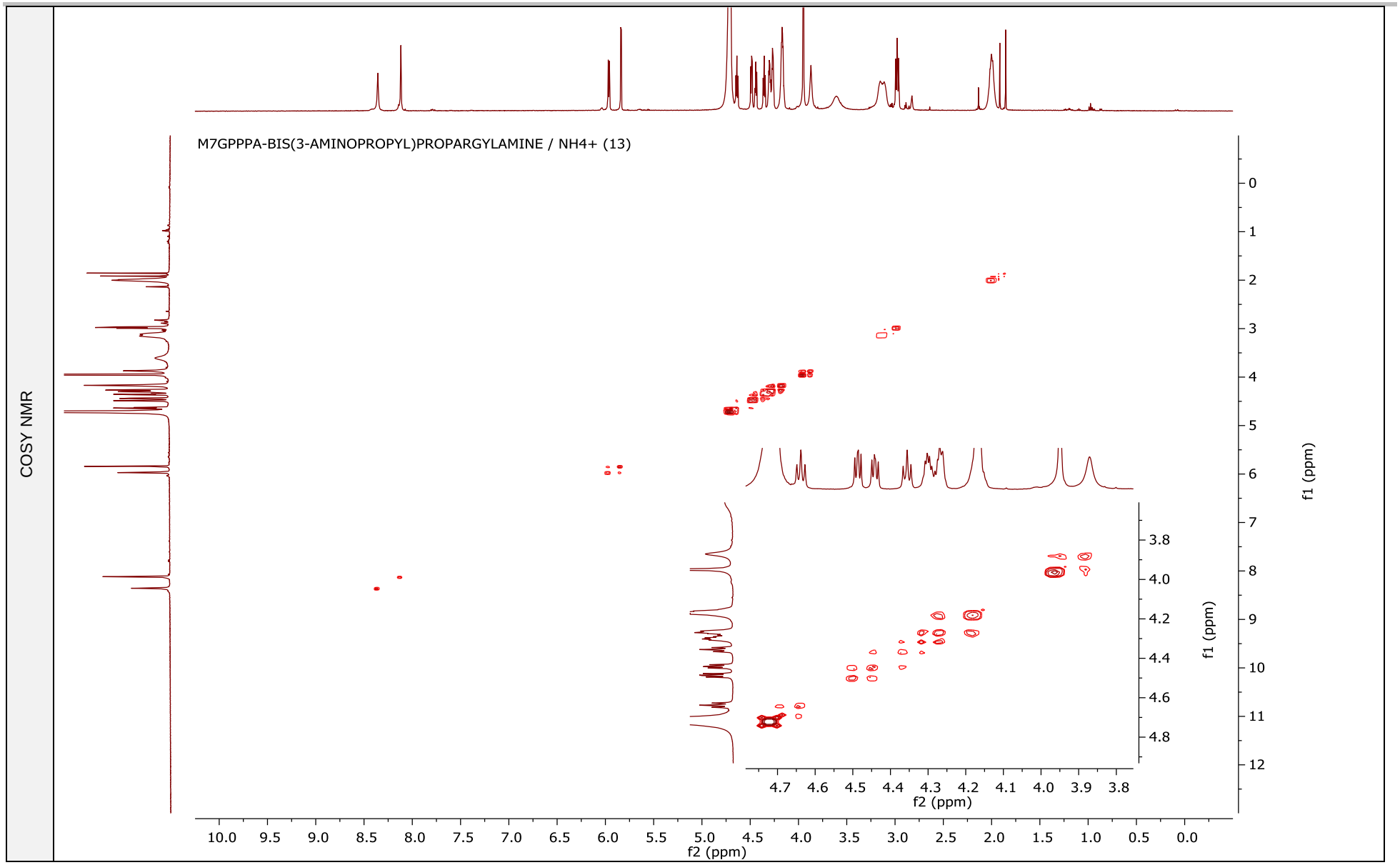
m^7GpppA-Bis(3-aminopropyl)propargylamine / NH_4^+ (13)	
Chemical structure	
HPLC profile	

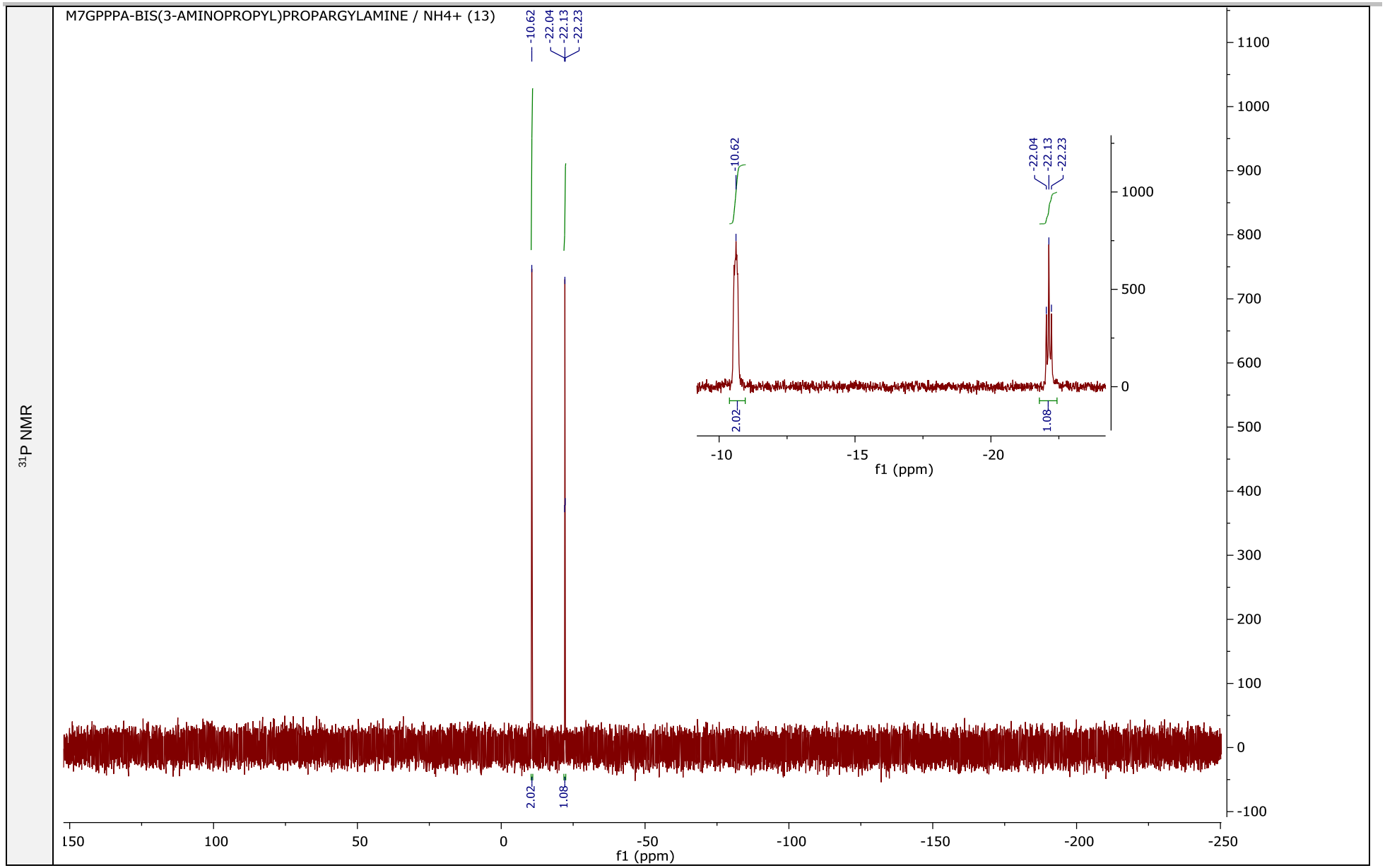
Figure S11. Analytical HPLC profile of compound **13**: column C18, Buffer A:Buffer B 0-100% in 15 min.

HRMS–ESI (*m/z*): [M-H]$^-$ calcd for $C_{30}H_{44}N_{12}O_{17}P_3^-$; 937.21657.




M7GPPPA-BIS(3-AMINOPROPYL)PROPARGYLAMINE / NH4+ (13)
1H NMR
f1 (ppm)


COSY NMR
M7GPPPA-BIS(3-AMINOPROPYL)PROPARGYLAMINE / NH4+ (13)
f2 (ppm)
f1 (ppm)


M7GPPPA-BIS(3-AMINOPROPYL)PROPARGYLAMINE / NH4+ (13)
31P NMR
-10.62
-22.04
-22.13
-22.23
2.02
1.08
f1 (ppm)

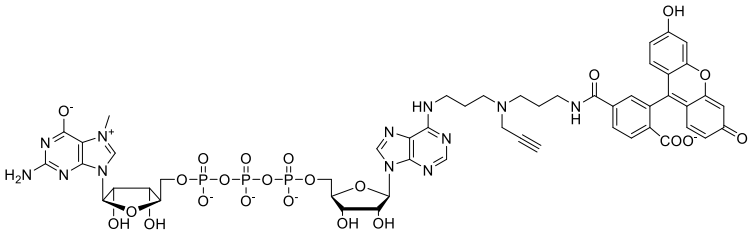
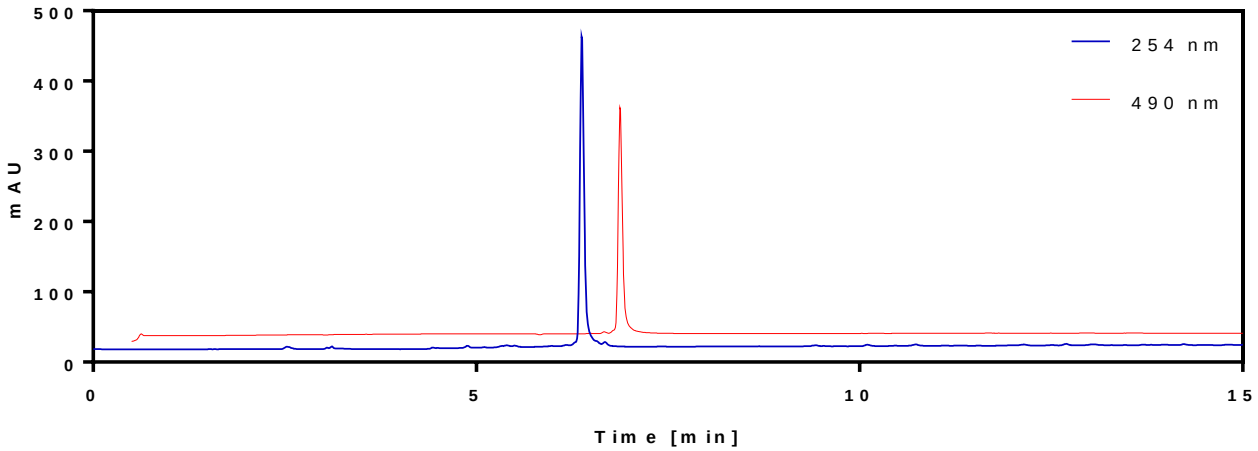
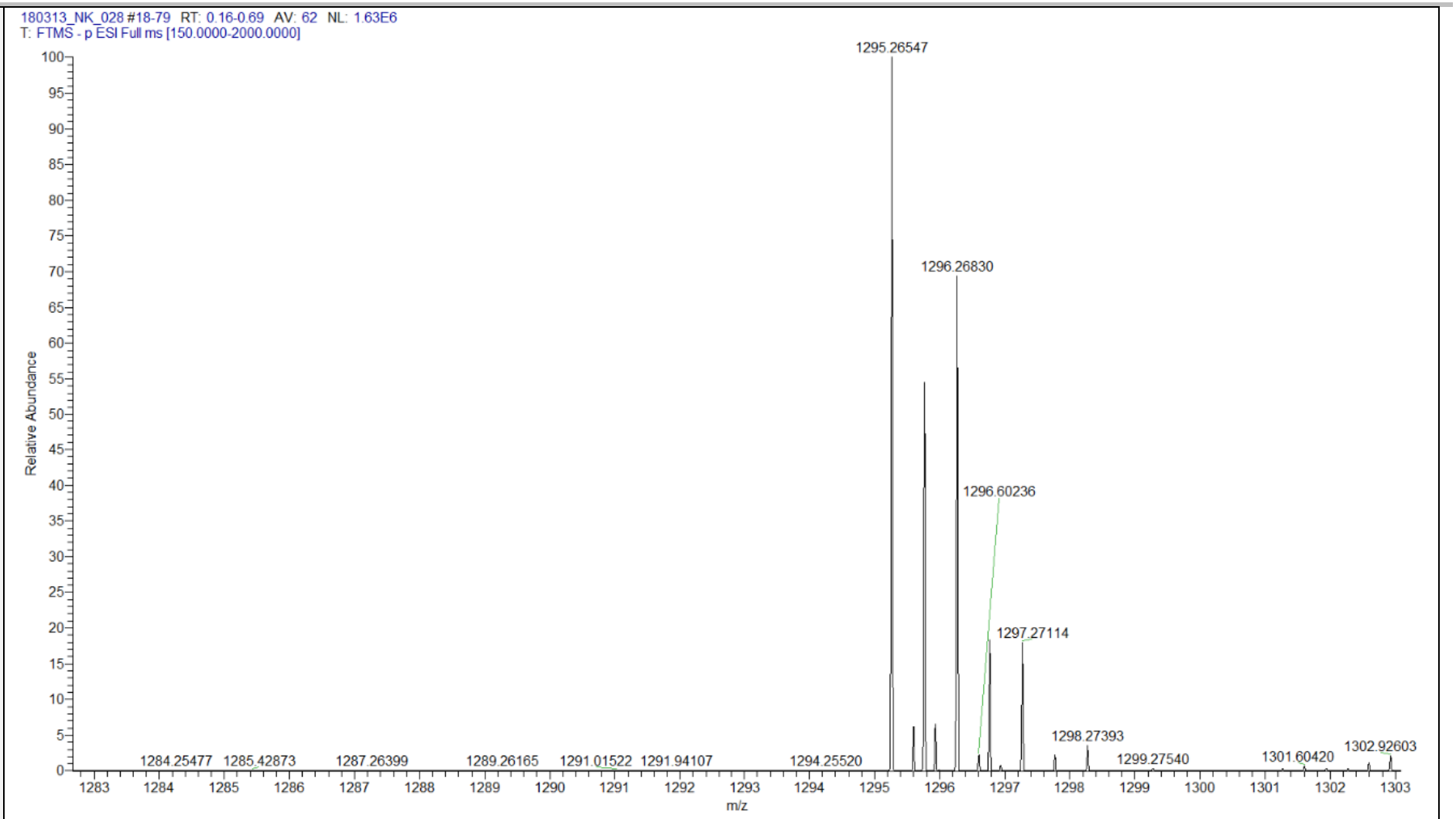
m^{7}GpppA-propargyl-FAM / NH$_4^+$ (14)	
Chemical structure	
HPLC profile	

Figure S12. Analytical HPLC profile of compound **14**: column C18, Buffer A:ACN 0-100% in 15 min.

HRMS–ESI (*m/z*): $[M-H]^-$ calcd for $C_{51}H_{53}N_{12}O_{23}P_3^-$, 1295.26431.



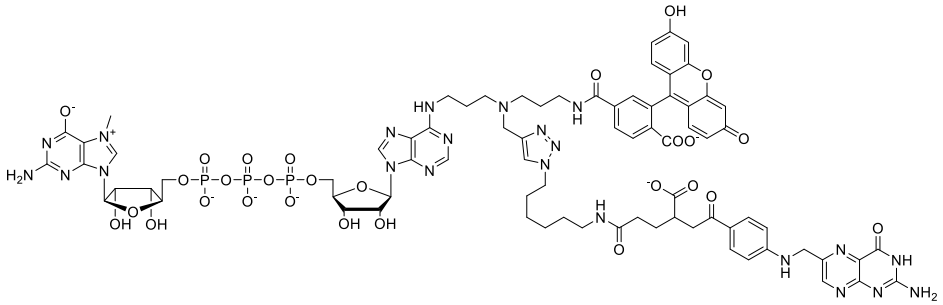
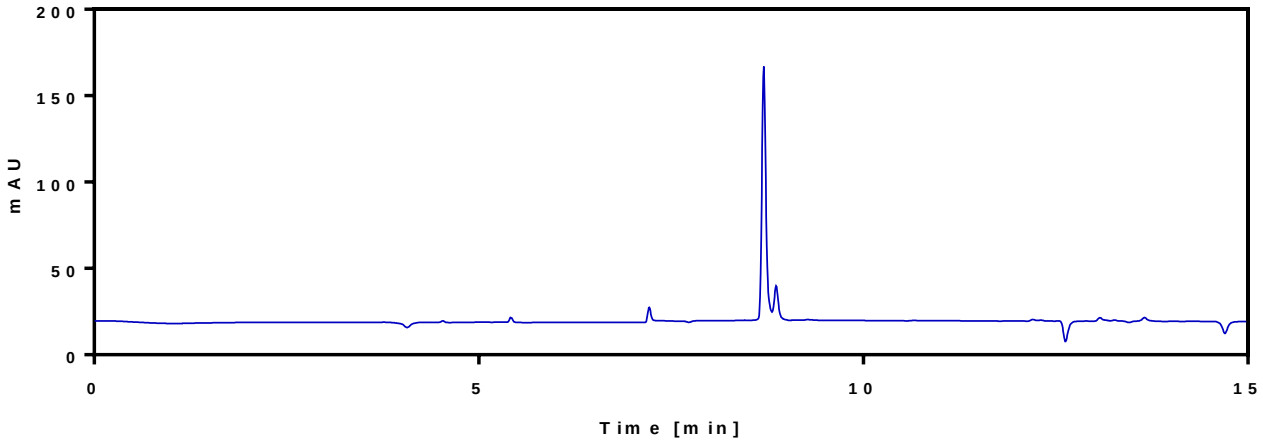
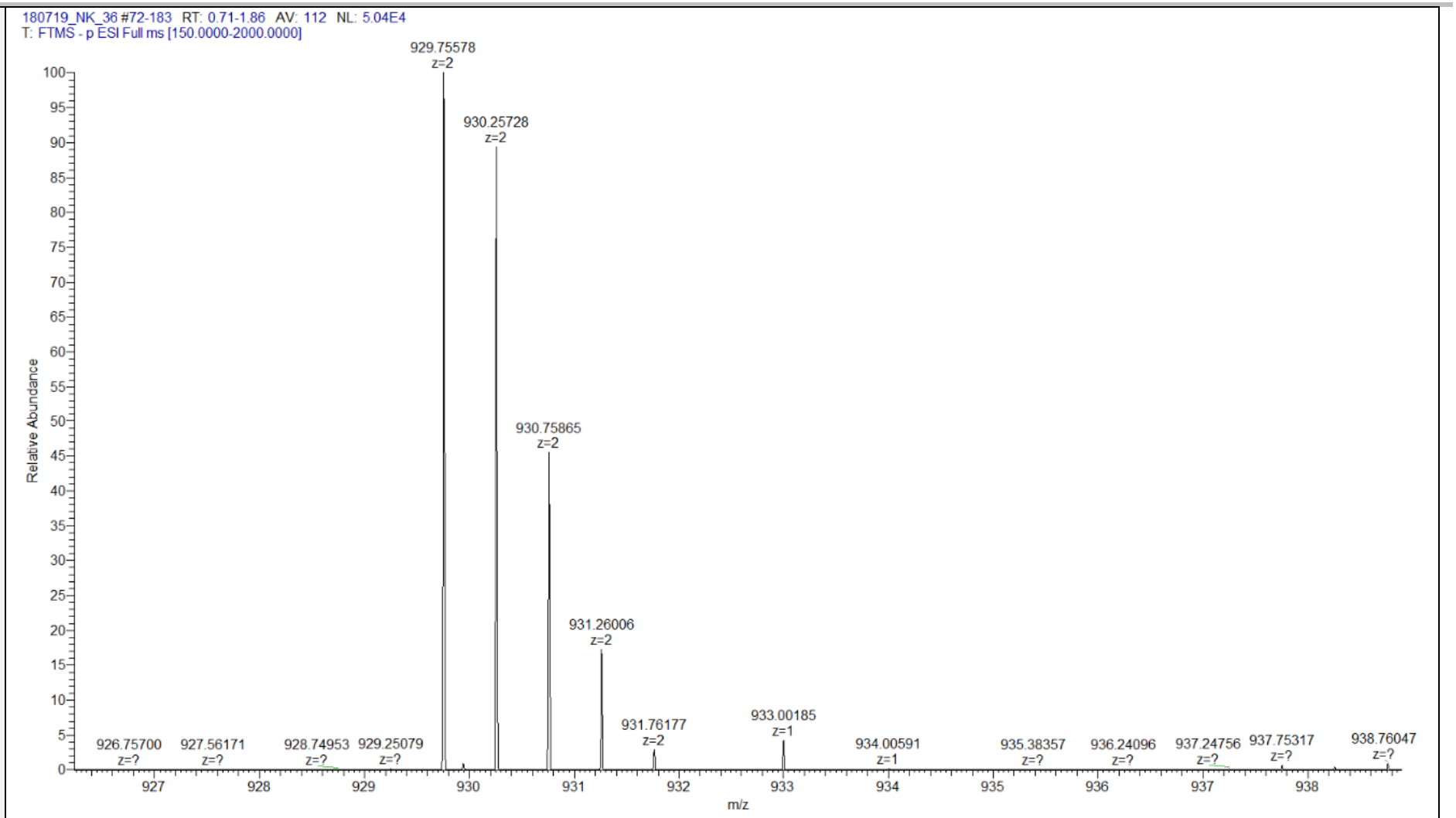
m^7GpppA-FAM-FA / NH_4^+ (15)	
Chemical structure	
HPLC profile	

Figure S13. Analytical HPLC profile of compound **15**: column Gemini NX C18, ammonium acetate pH 8.4:ACN 5-30% in 15 min.

HRMS–ESI (*m/z*): $[M-2H]^{2-}$ calcd for $C_{76}H_{85}N_{23}O_{28}P_3^{2-}$, 929.75400.



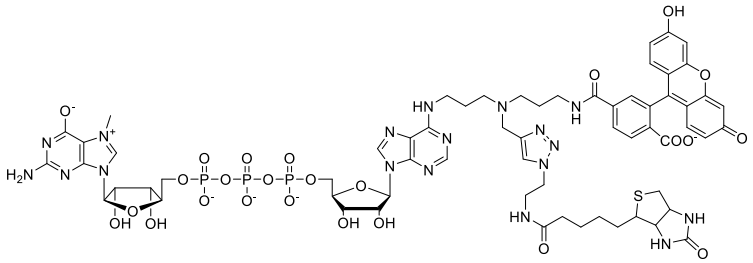
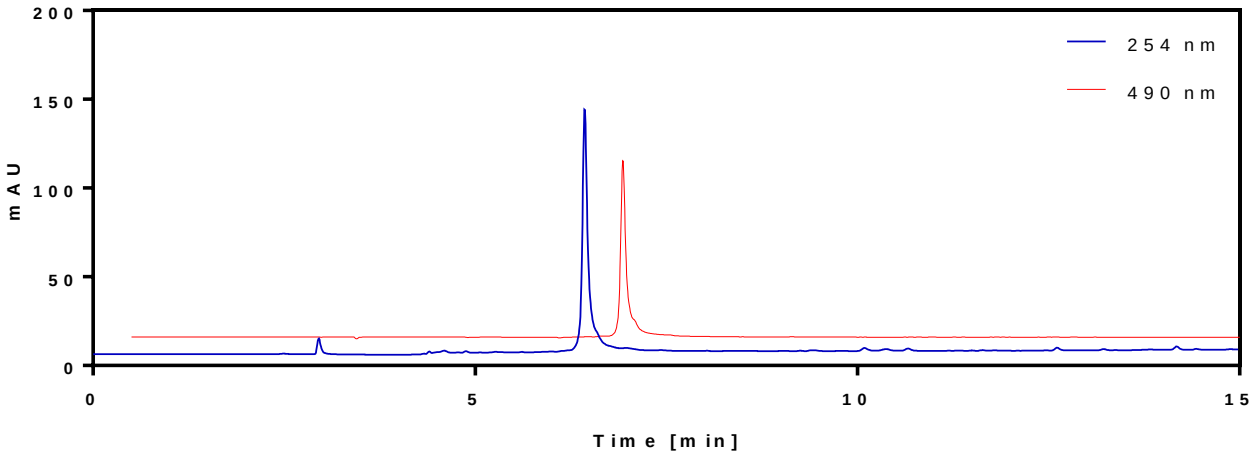
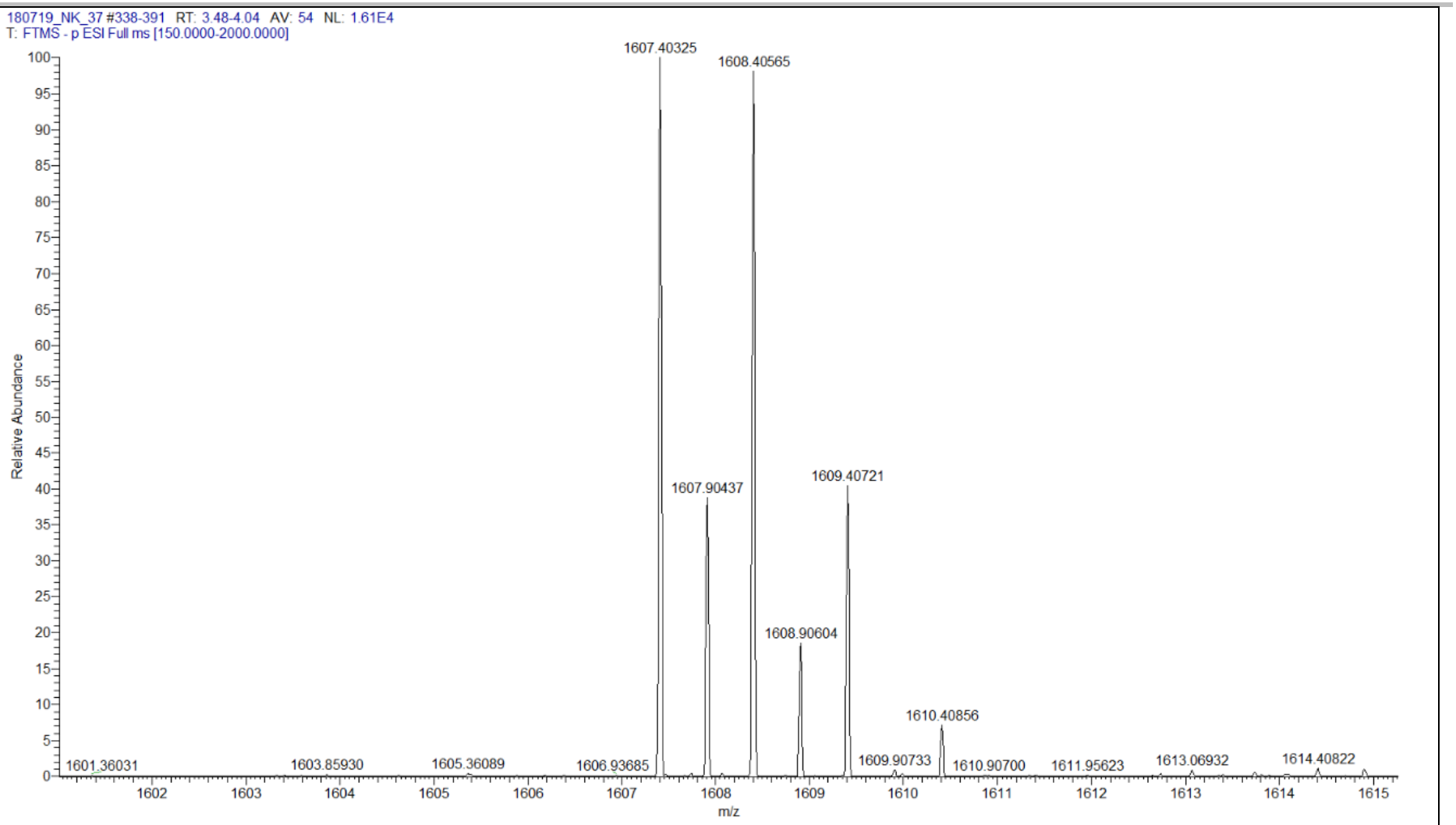
m^7GpppA-FAM-Biotin / NH_4^+ (16)	
Chemical structure	
HPLC profile	

Figure S14. Analytical HPLC profile of compound **16**: column C8, Buffer A:ACN 0-100% in 15 min.

HRMS–ESI (*m/z*): [M-H]$^-$ calcd for $C_{63}H_{74}N_{18}O_{25}P_3S^-$, 1607.40115.



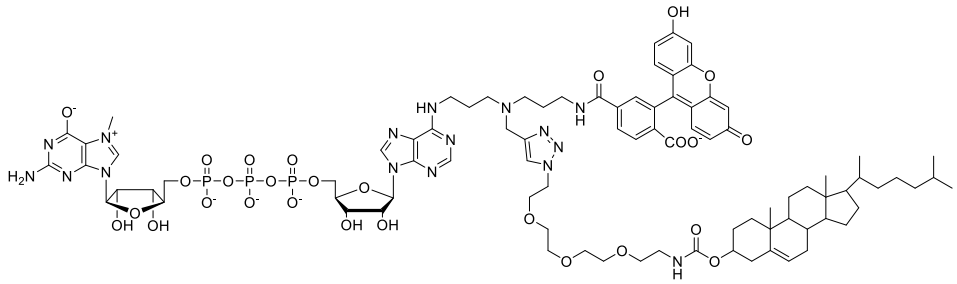
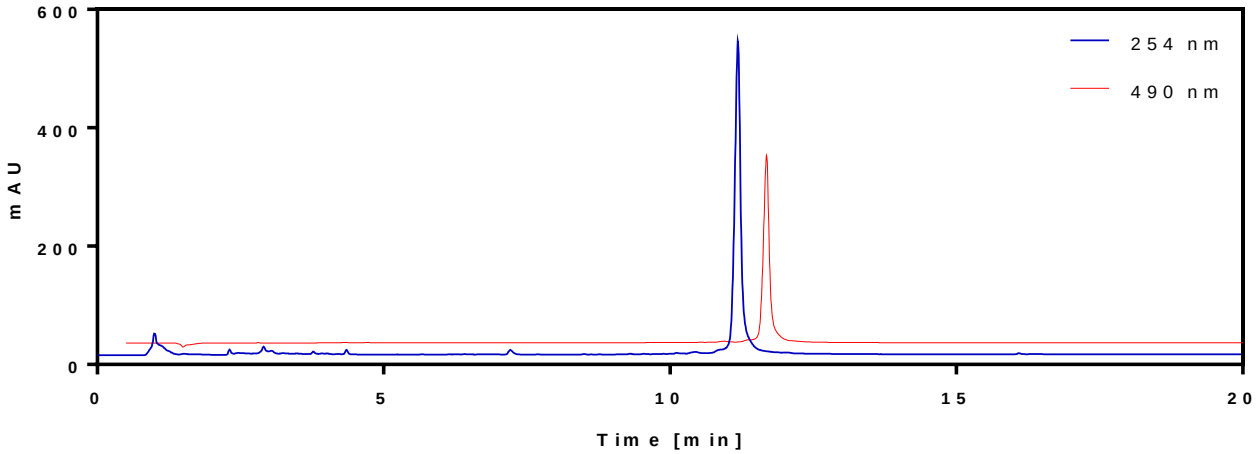
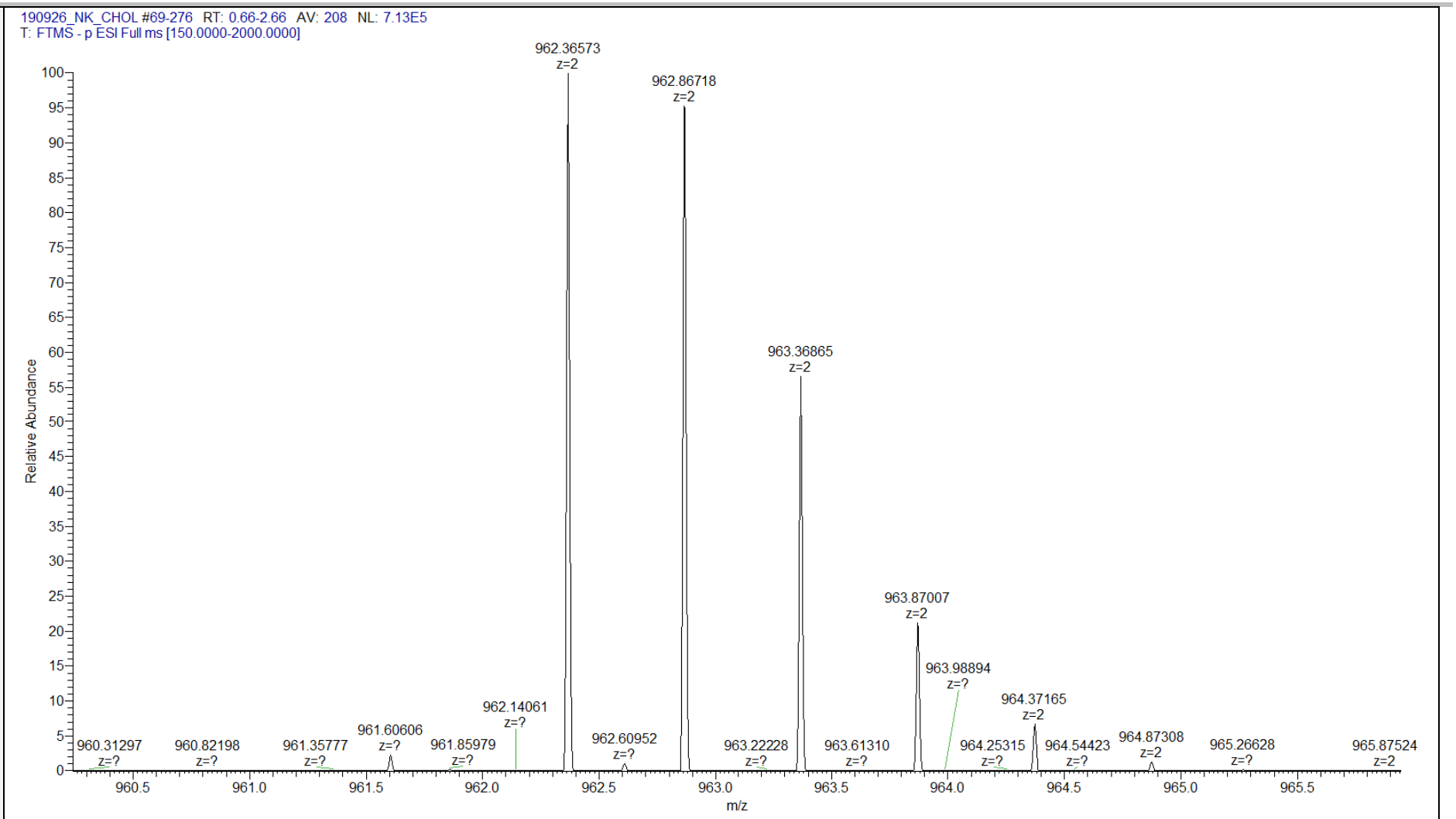
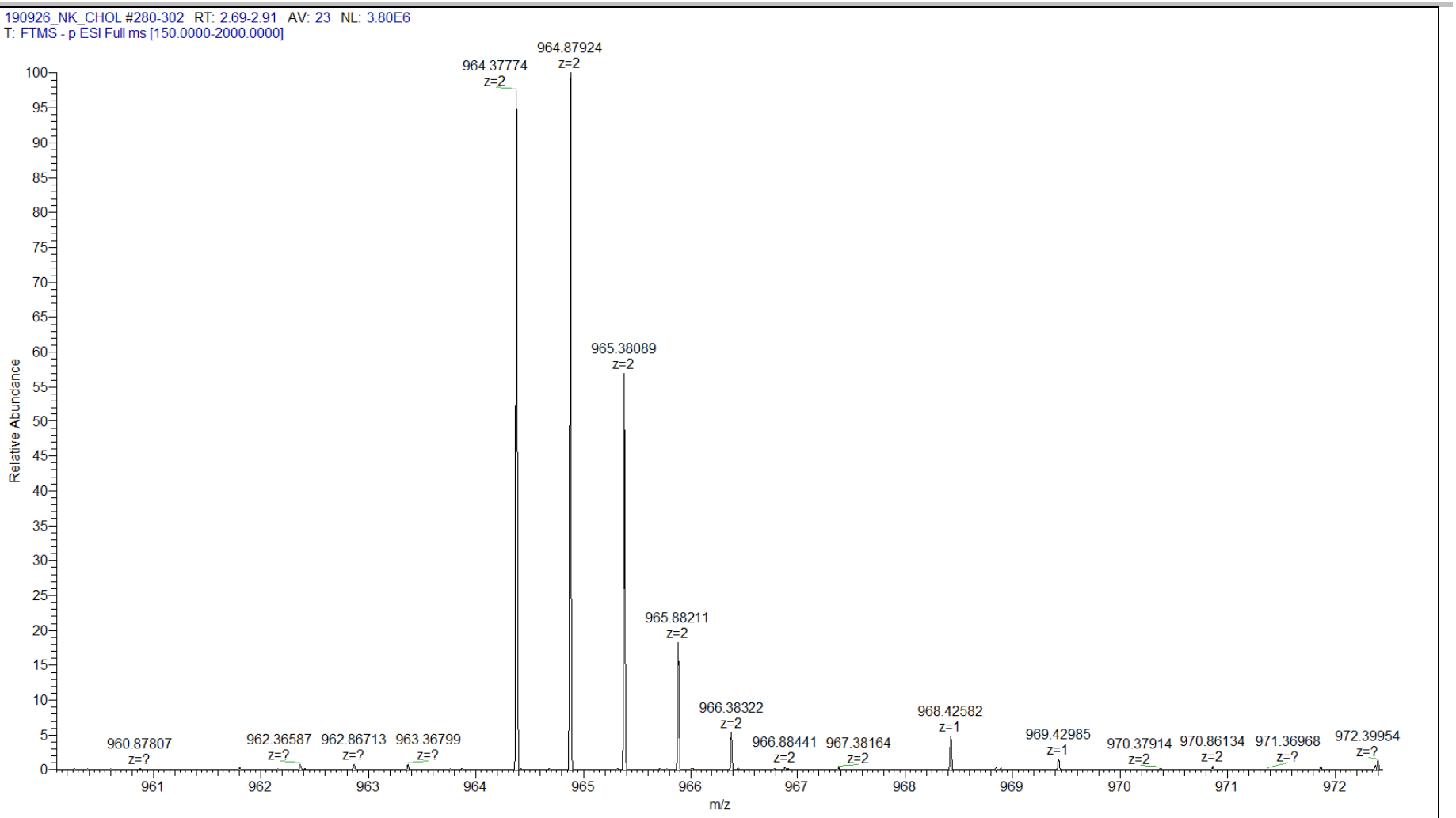
m^{7}GpppA-FAM-CHOL/ NH$_4^+$ (17)	
Chemical structure	
HPLC profile	

Figure S15. Analytical HPLC profile of compound **17**: column C4, Buffer A:ACN 0-100% in 20 min

HRMS–ESI (*m/z*): $[M-2H]^{2-}$ calcd for $C_{87}H_{115}N_{16}O_{28}P_3^{2-}$, 962.364525.



HRMS–ESI (*m/z*): $[M+2H]^{2+}$ calcd for $C_{87}H_{119}N_{16}O_{28}P_3^{2+}$, 964.37908.



Cap-Linker analogues (21-22, 28-29)

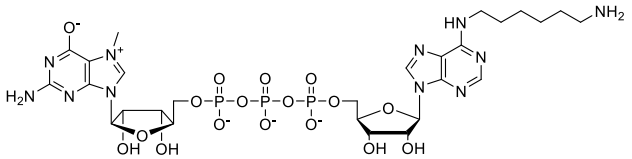
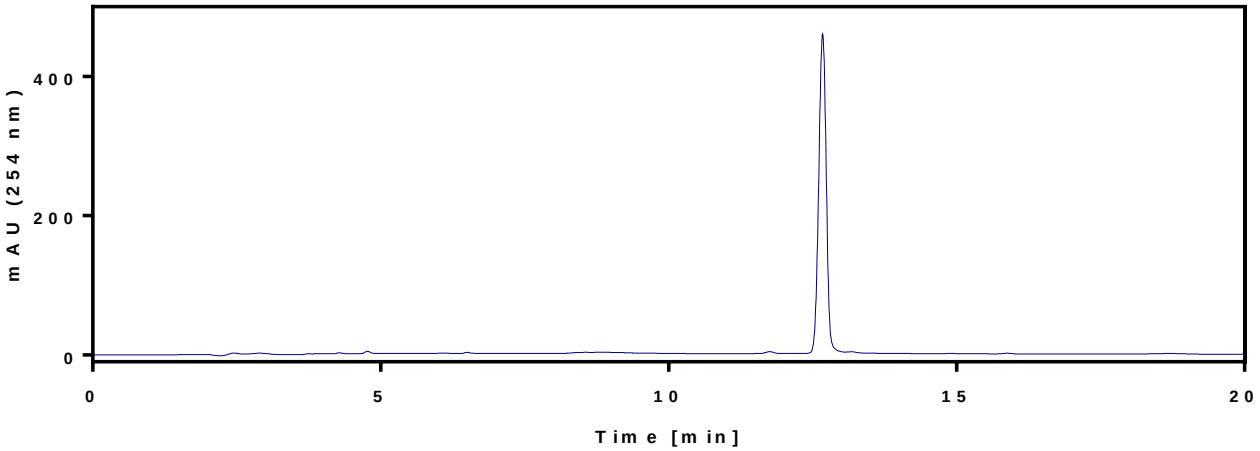
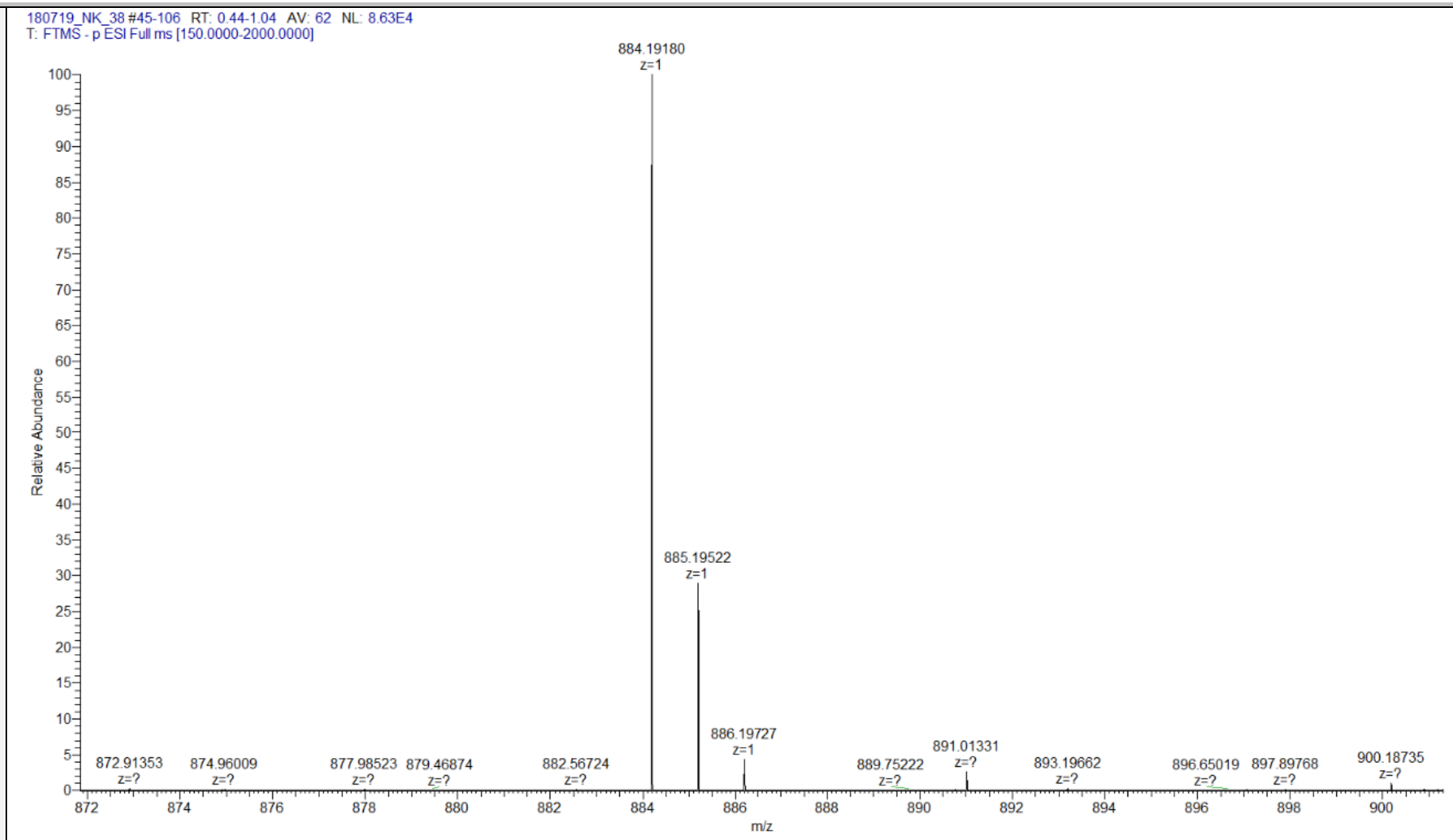
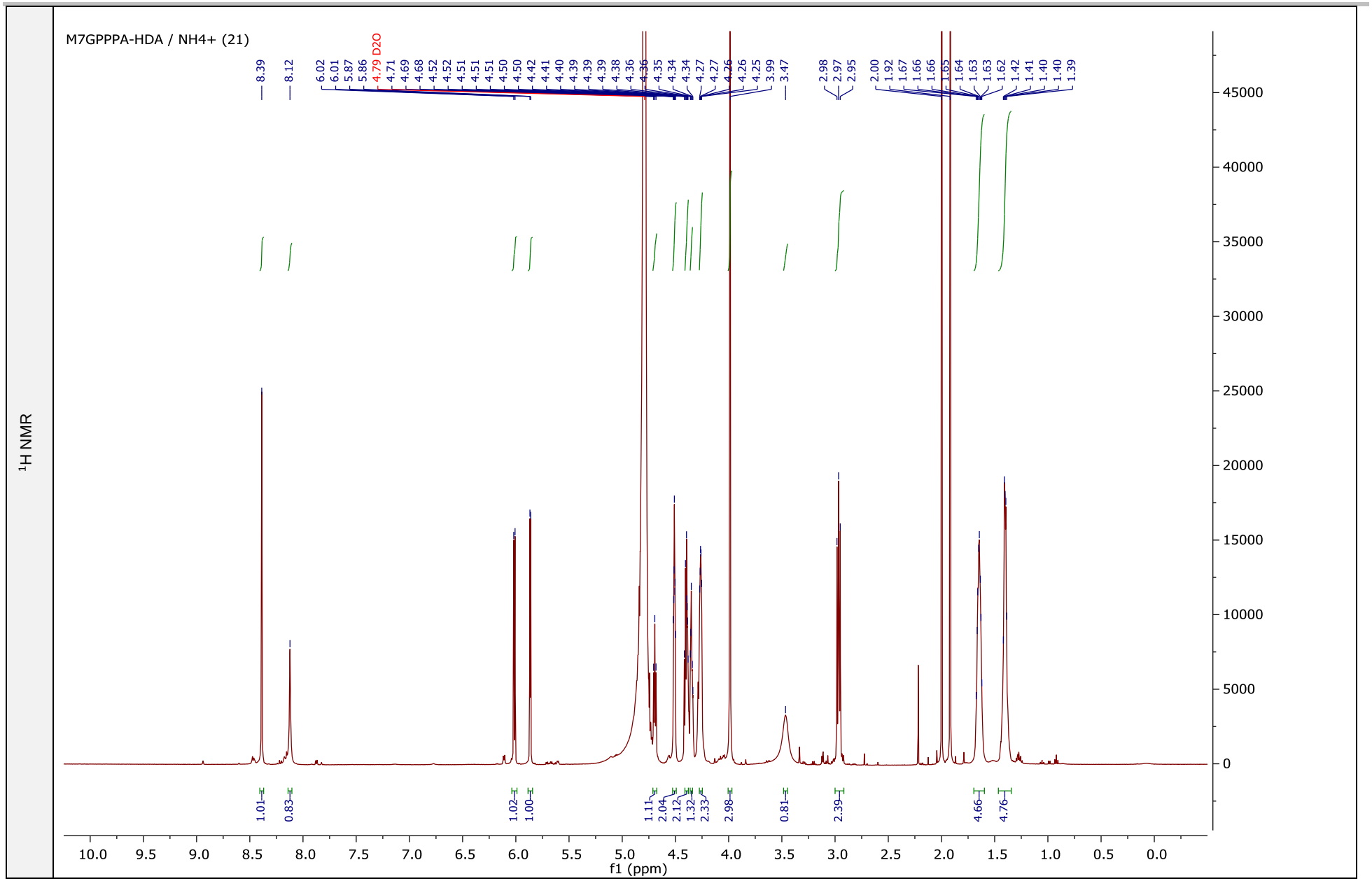
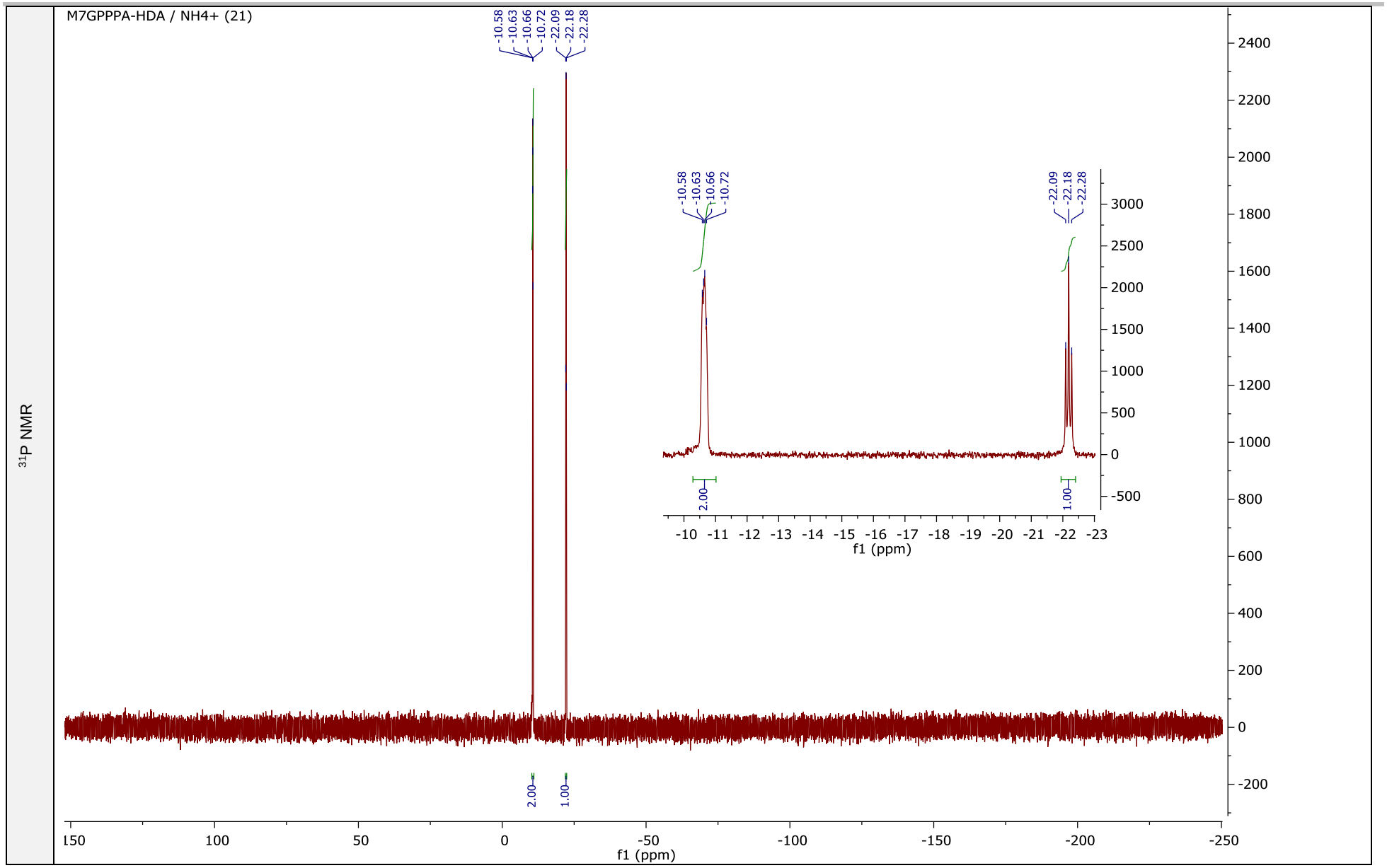
m^7GpppA-HDA / NH_4^+ (21)	
Chemical structure	
HPLC profile	

Figure S16. Analytical HPLC profile of compound **21**: column C18, Buffer A:Buffer B 0-50% in 15 min.

HRMS–ESI (*m/z*): $[M-H]^-$ calcd for $C_{27}H_{43}N_{11}O_{17}P_3^-$, 884.19112.




M7GPPPA-HDA / NH4+ (21)
1H NMR
8.39
8.12
6.02
6.01
5.87
5.86
4.79 D2O
4.71
4.69
4.68
4.52
4.52
4.51
4.51
4.51
4.50
4.50
4.42
4.41
4.40
4.39
4.39
4.39
4.38
4.36
4.36
4.35
4.34
4.34
4.27
4.27
4.26
4.26
4.25
3.99
3.47
2.98
2.97
2.95
2.00
1.92
1.67
1.66
1.66
1.65
1.64
1.63
1.63
1.62
1.42
1.41
1.40
1.40
1.39
1.01
0.83
1.02
1.00
1.11
2.04
2.12
1.32
2.33
2.98
0.81
2.39
4.66
4.76
f1 (ppm)


M7GPPPA-HDA / NH4+ (21)
31P NMR
-10.58
-10.63
-10.66
-10.72
-22.09
-22.18
-22.28
2.00
1.00
f1 (ppm)

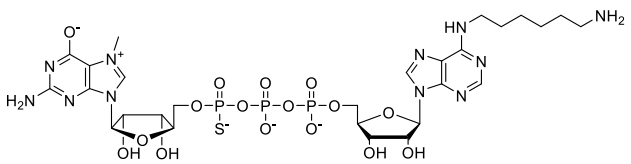
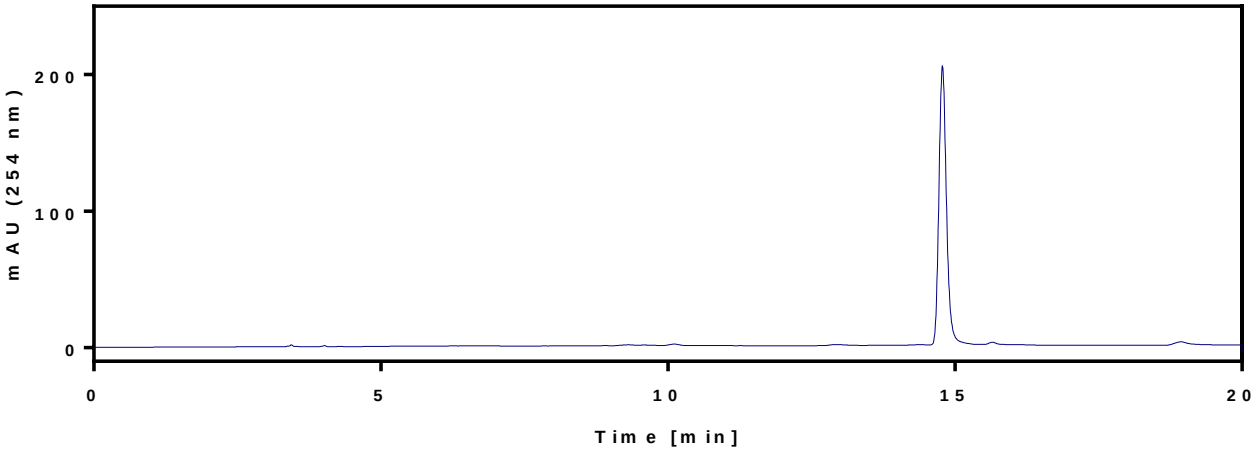
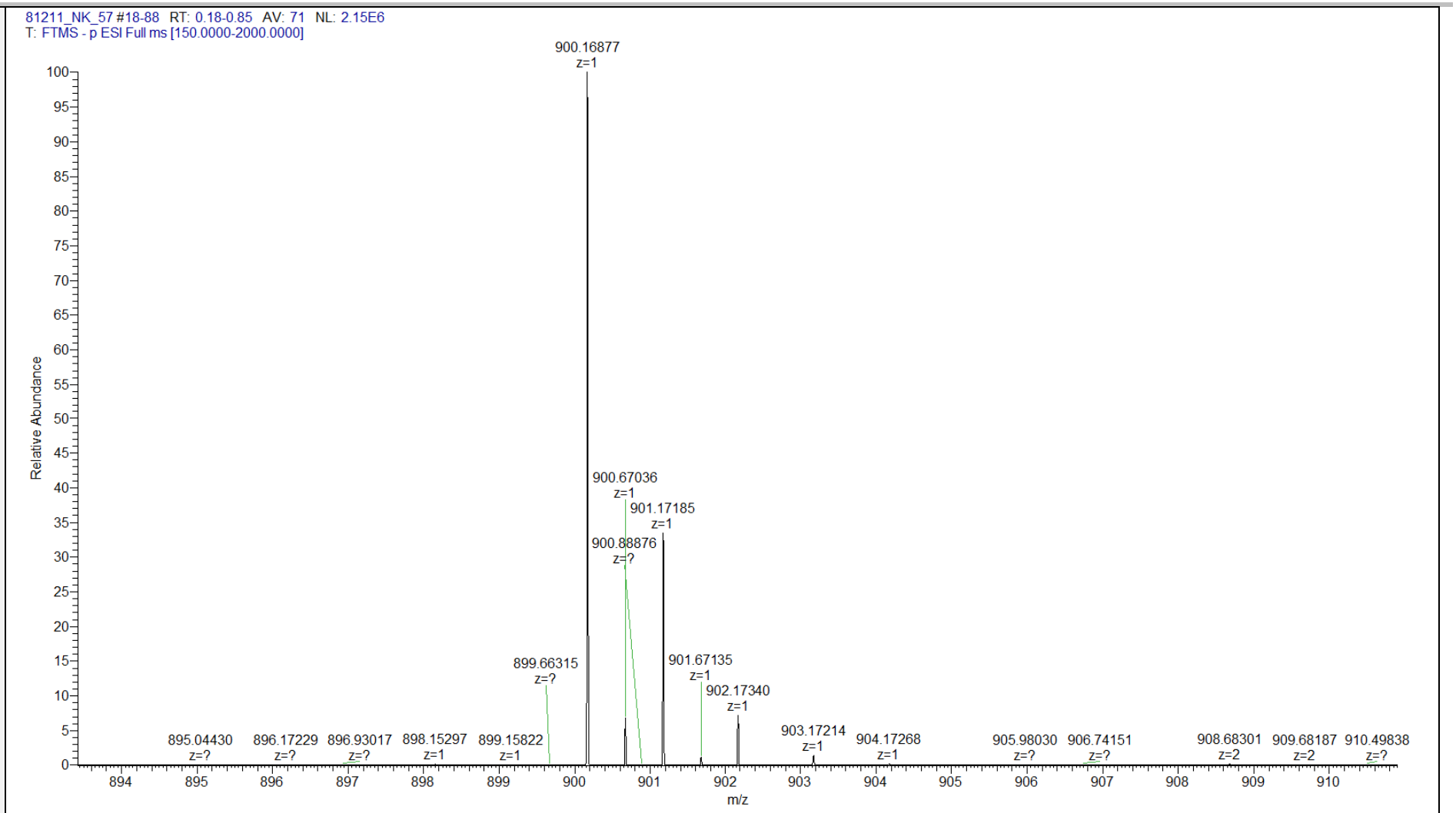
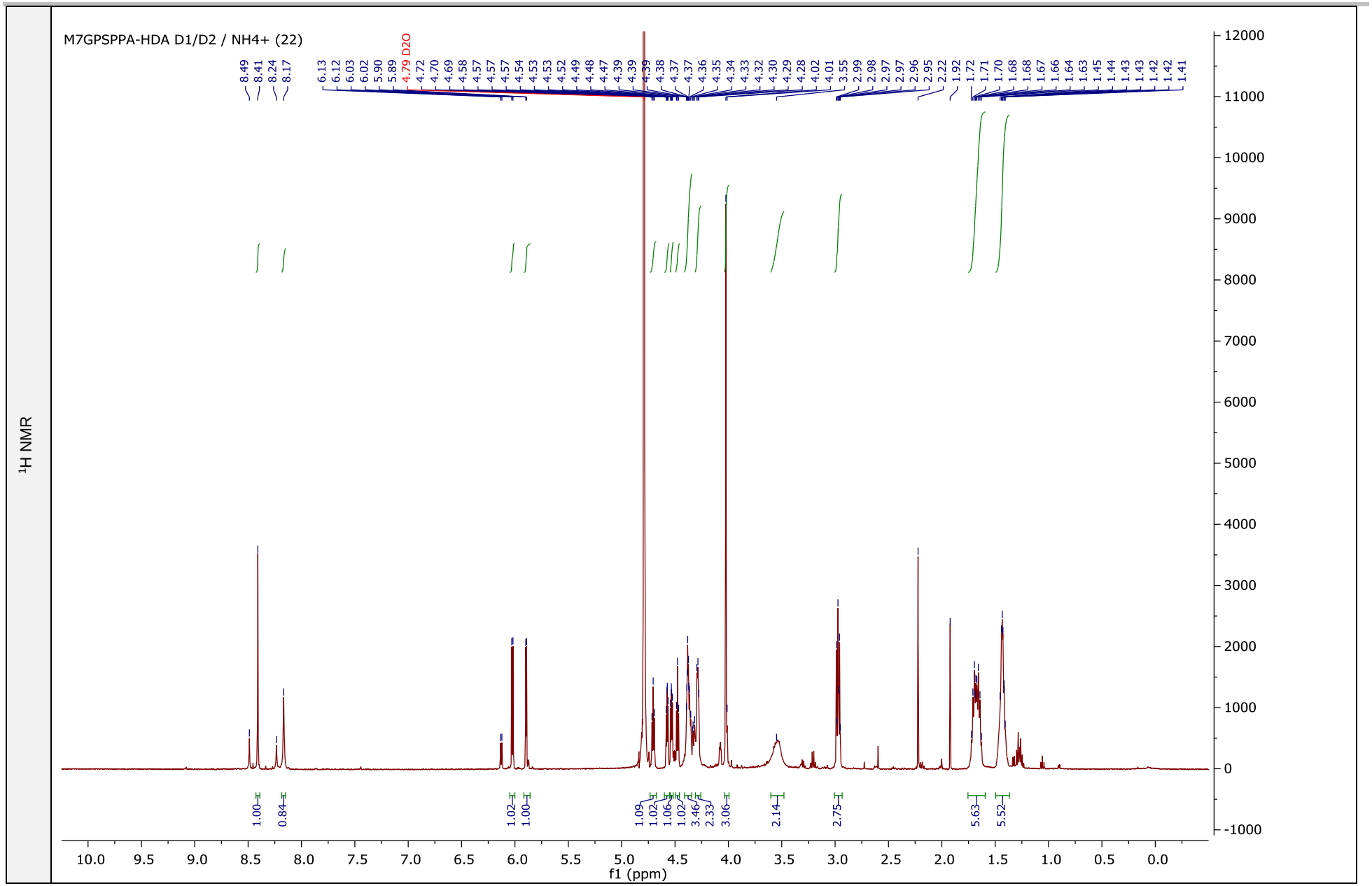
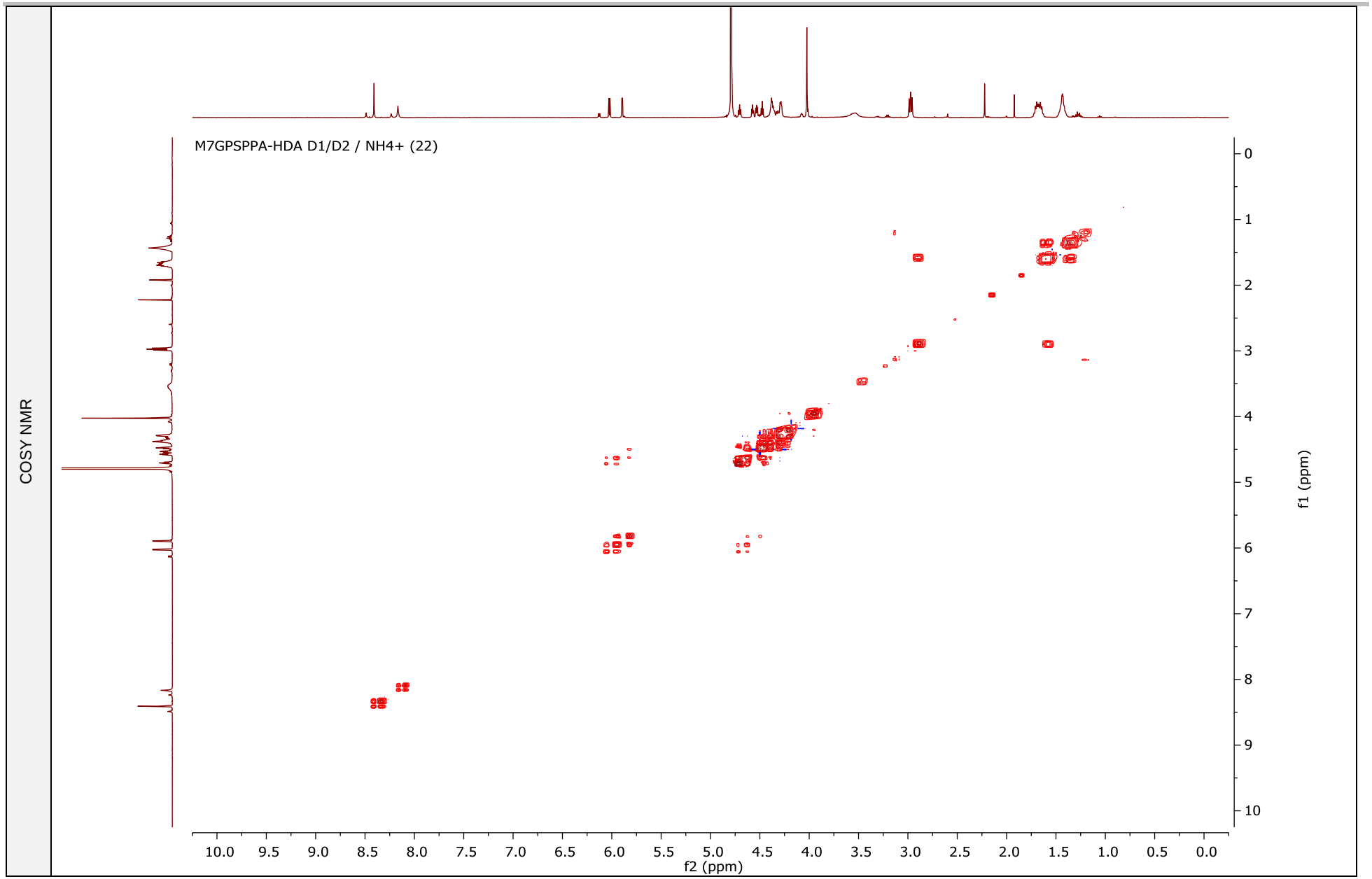
$m^7GpsppA$-HDA-D1/D2 / NH_4^+ as a mixture of diastereoisomers D1/D2 (22)	
Chemical structure	
HPLC profile	

Figure S17. Analytical HPLC profile of compound **22**: column C18, Buffer A:Buffer B 0-50% in 15 min.

HRMS–ESI (*m/z*): $[M-H]^-$ calcd for $C_{27}H_{41}N_{11}O_{16}P_3S^-$, 900.16718.

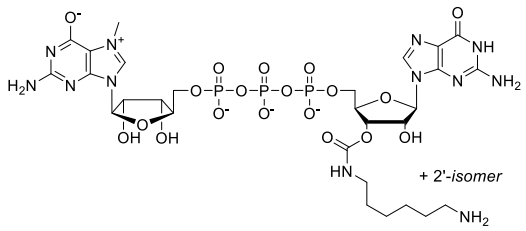



M7GPSPPA-HDA D1/D2 / NH4+ (22)
1H NMR
f1 (ppm)


COSY NMR
M7GPSPPA-HDA D1/D2 / NH4+ (22)
f2 (ppm)
f1 (ppm)

m^7GpppG-L_{6N} / NH_4^+ as a mixture of 2'-O/3'-O-isomers (28)

Chemical structure



HPLC profile

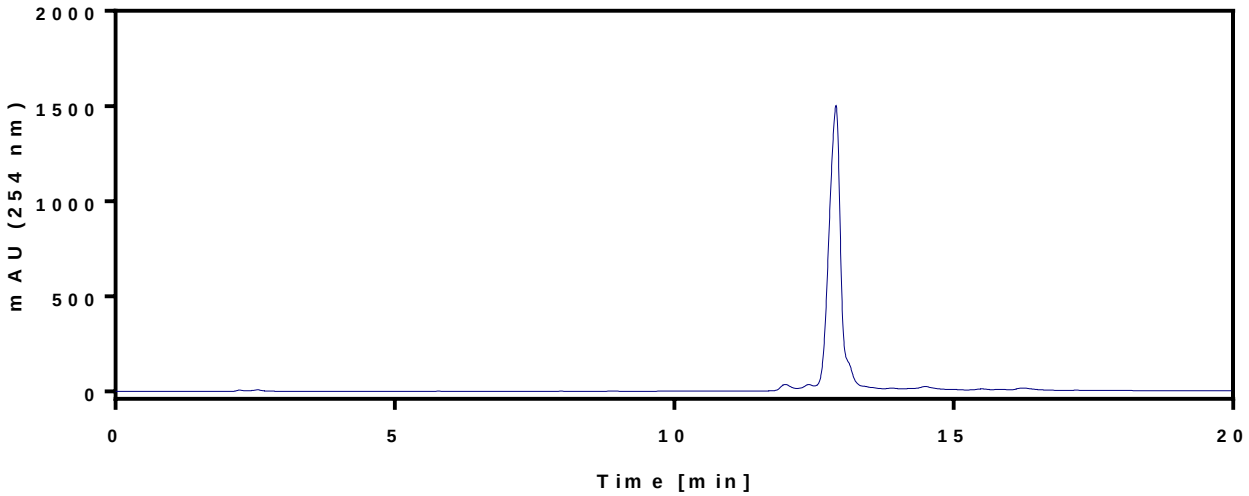
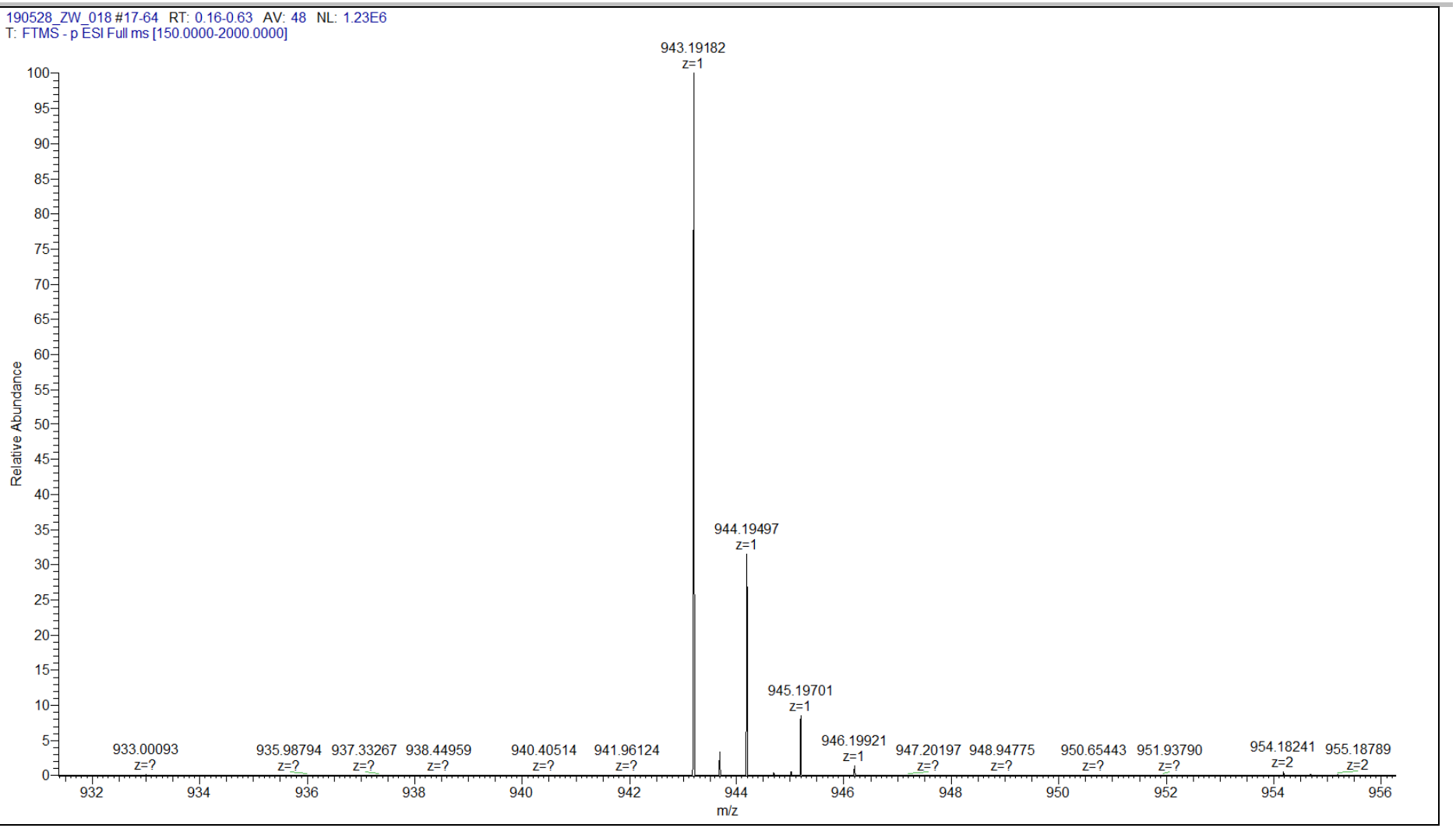
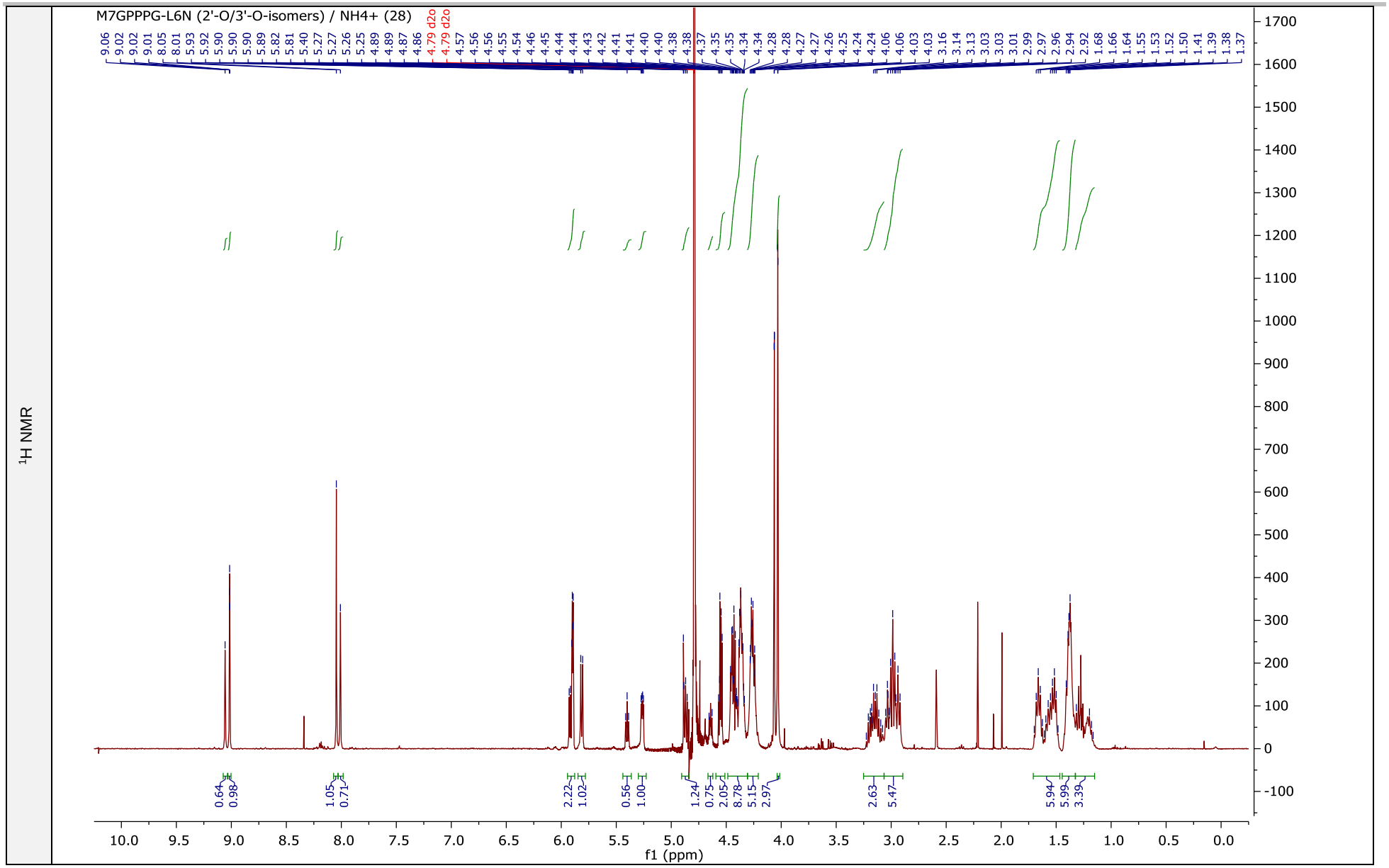
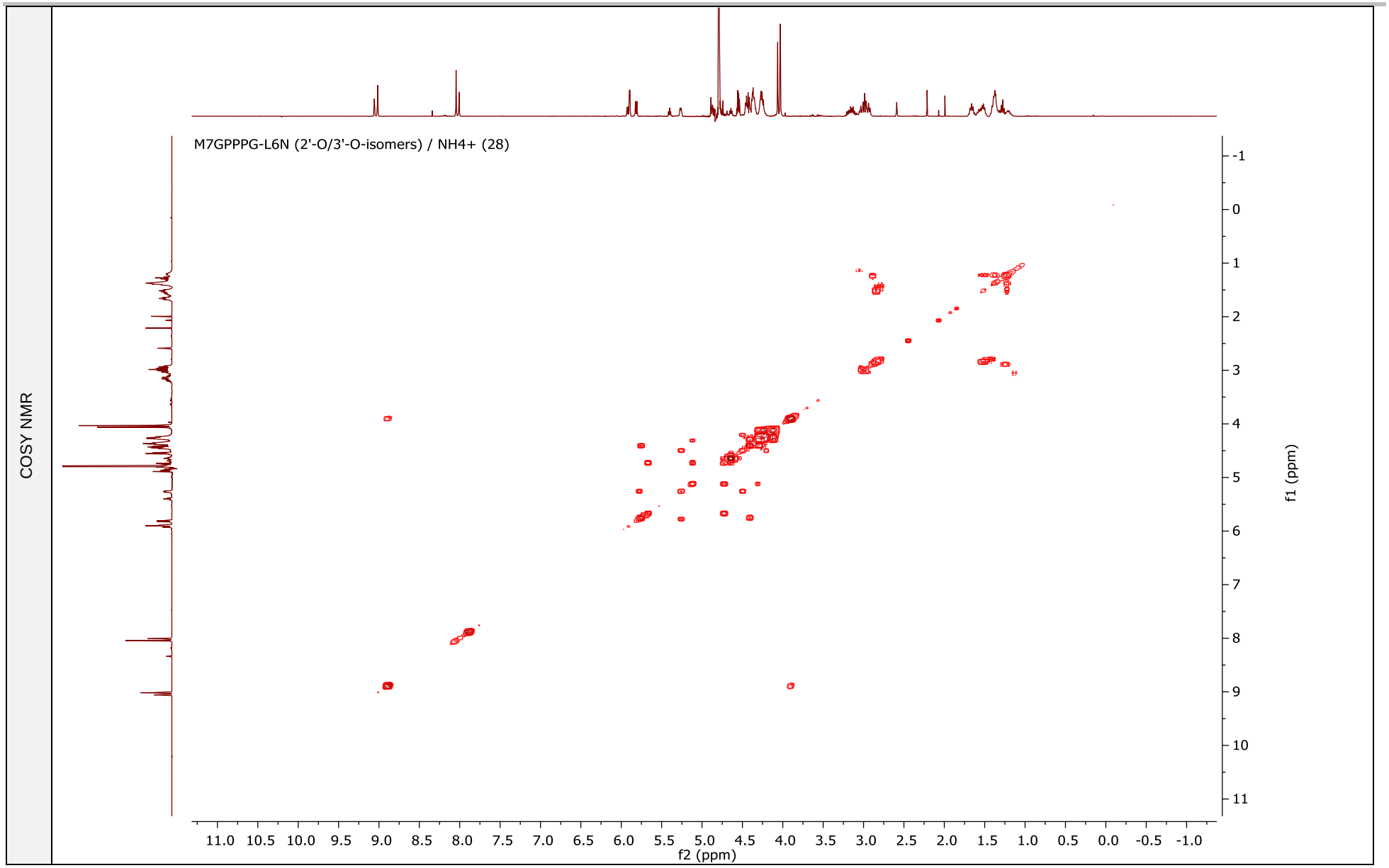


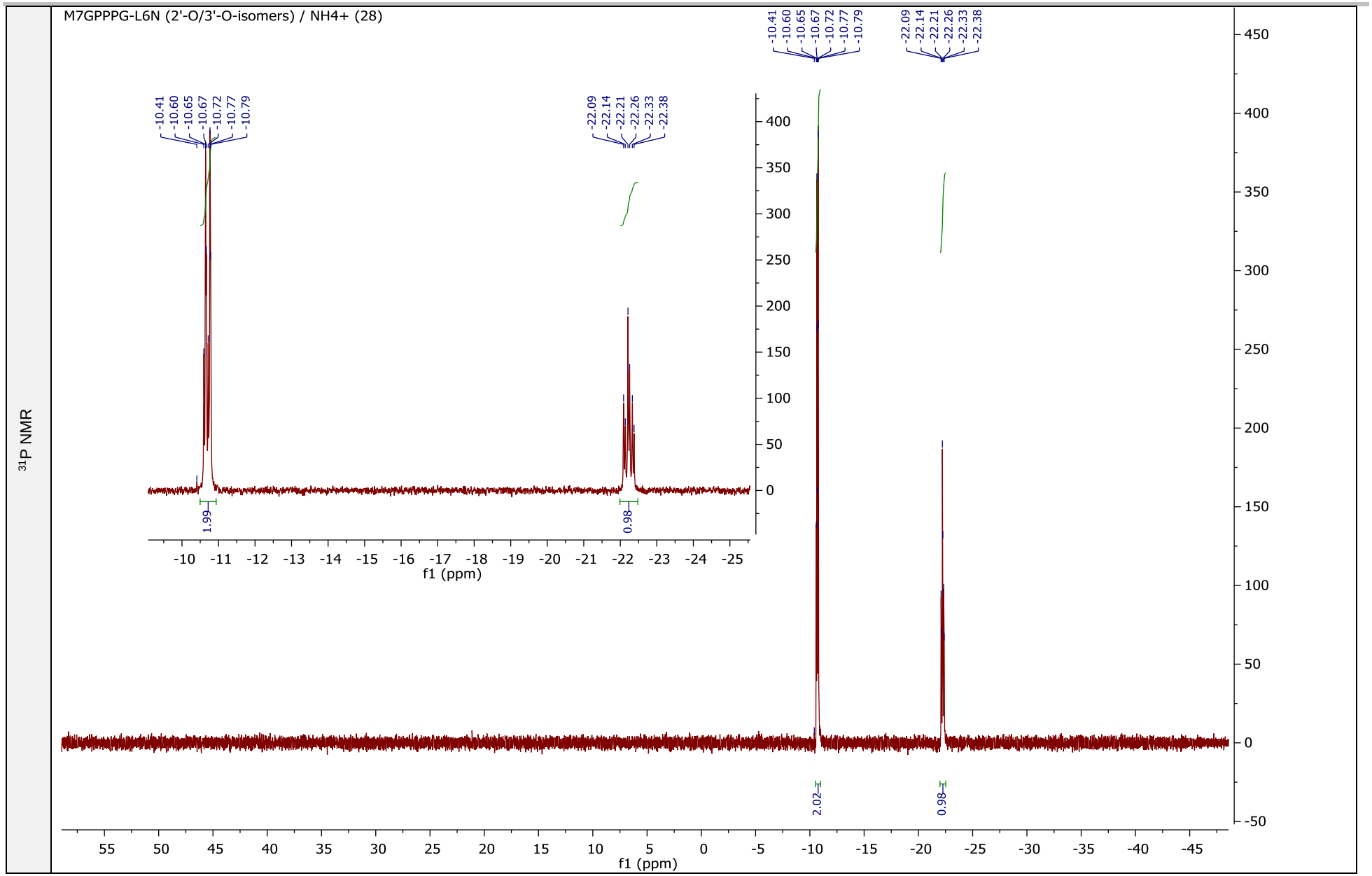
Figure S18. Analytical HPLC profile of compound **28**: column C18, Buffer A:Buffer B 0-50% in 15 min.

HRMS–ESI (*m/z*): [M-H]$^-$ calcd for $C_{28}H_{42}N_{12}O_{19}P_3^-$, 943.19075.



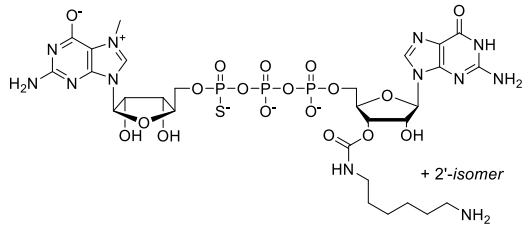

M7GPPPG-L6N (2'-O/3'-O-isomers) / NH4+ (28)
1H NMR
f1 (ppm)


COSY NMR
M7GPPPG-L6N (2'-O/3'-O-isomers) / NH4+ (28)
f2 (ppm)
f1 (ppm)


M7GPPPG-L6N (2'-O/3'-O-isomers) / NH4+ (28)
31P NMR
-10.41
-10.60
-10.65
-10.67
-10.72
-10.77
-10.79
-22.09
-22.14
-22.21
-22.26
-22.33
-22.38
1.99
0.98
2.02
0.98
f1 (ppm)

$m^7GpSppG\text{-}L_{6N}$ D1/D2 / NH_4^+ as a mixture of D1/D2 diastereoisomers and 2'-O/3'-O isomers (29)

Chemical structure



HPLC profile

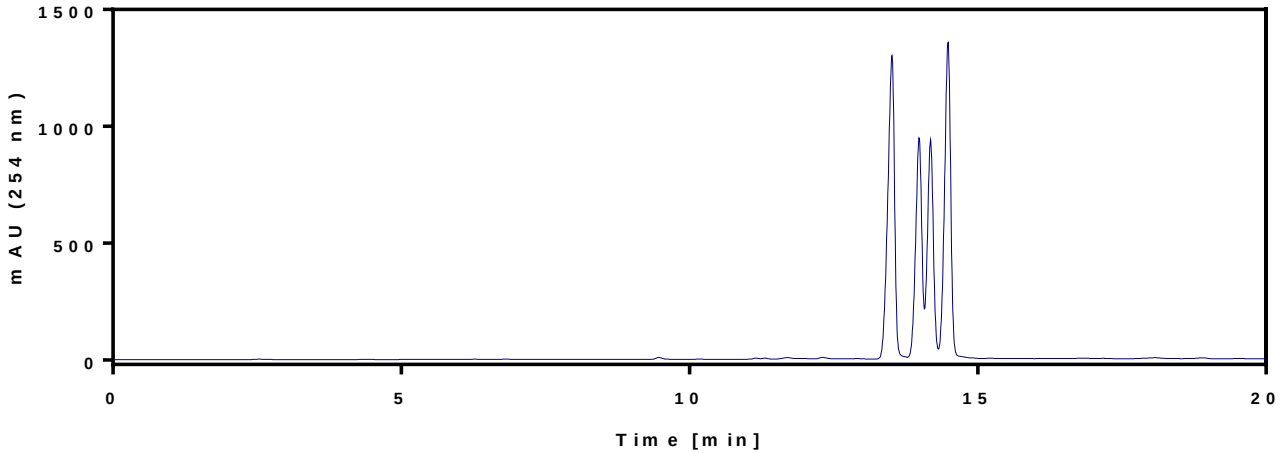
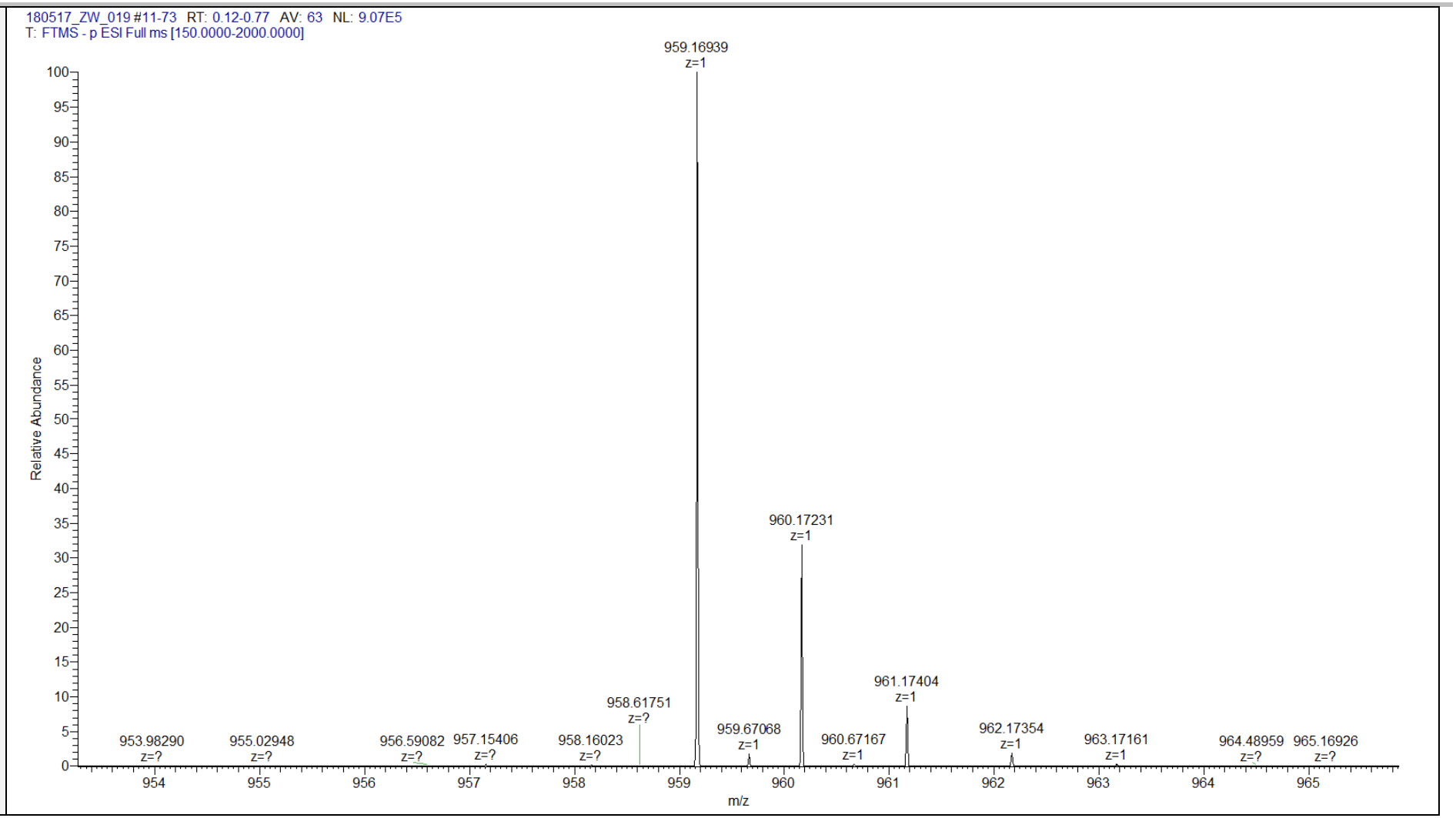
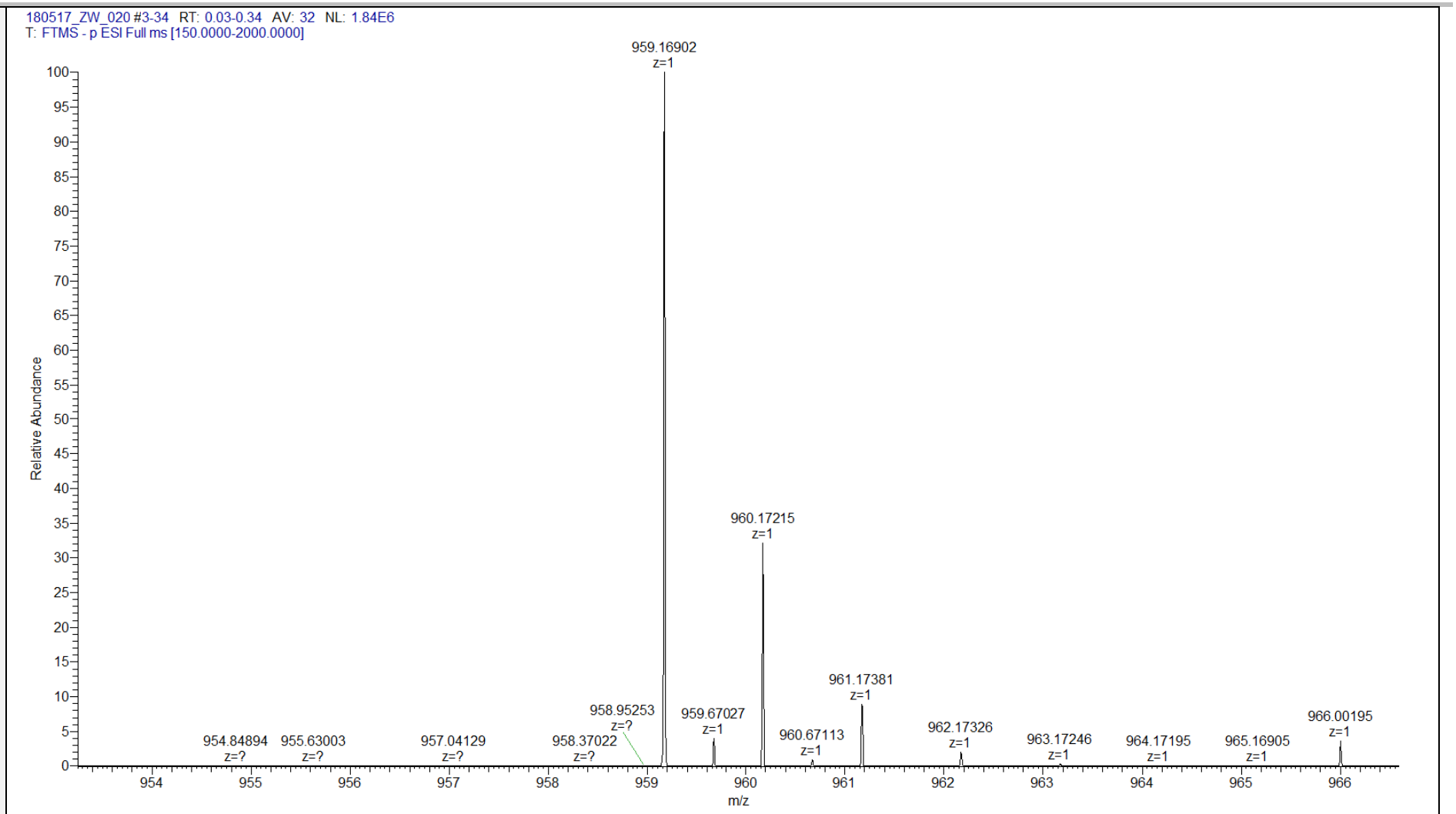


Figure S19. Analytical HPLC profile of compound **29**: column C18, Buffer A:Buffer B 0-100% in 15 min.

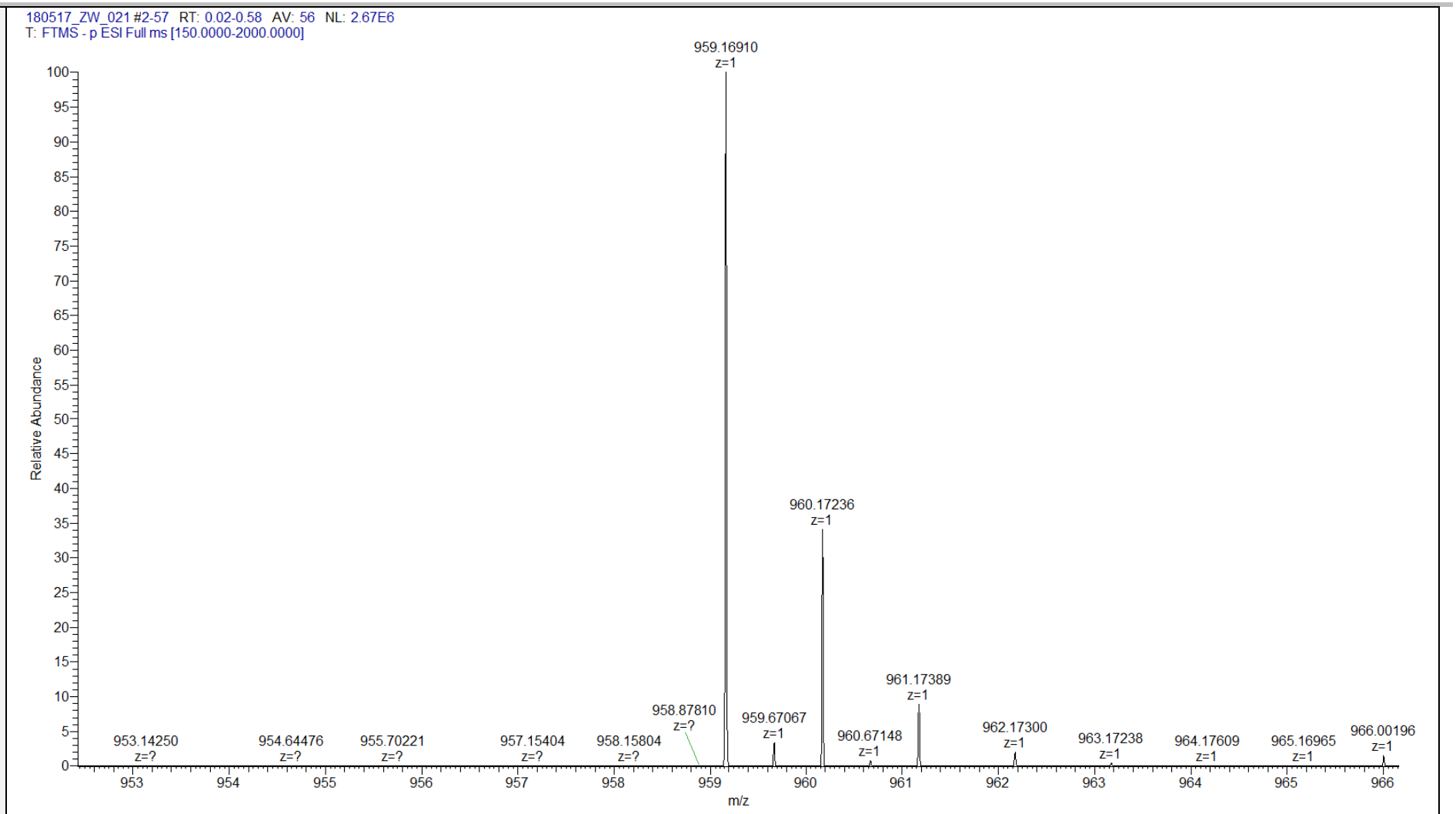
HRMS–ESI (*m/z*): [M-H]$^-$ calcd for $C_{28}H_{42}N_{12}O_{18}P_3S^-$; 959,16791.



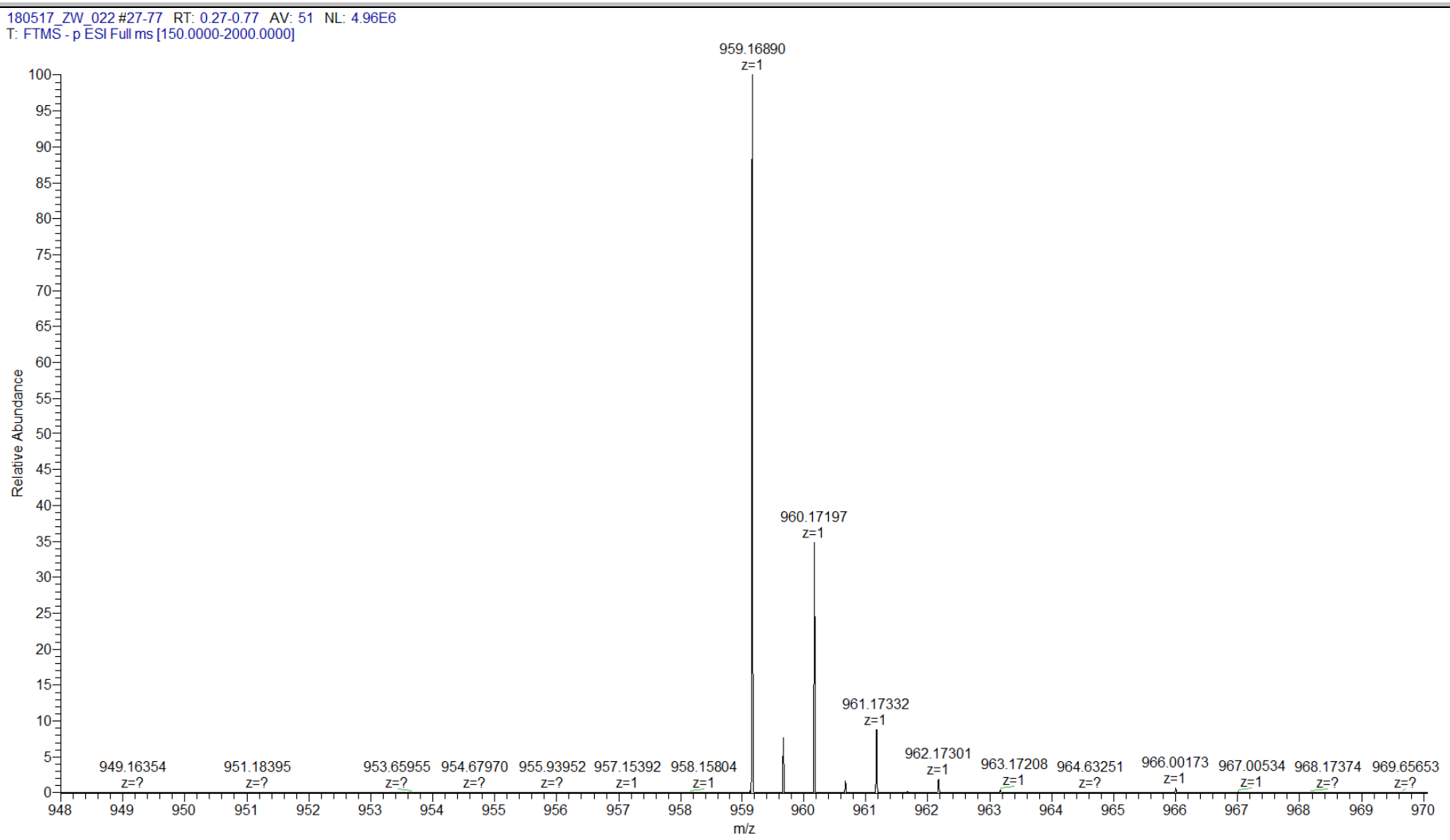
HRMS–ESI (*m/z*): $[M-H]^-$ calcd for $C_{28}H_{42}N_{12}O_{18}P_3S^-$, 959.16791.

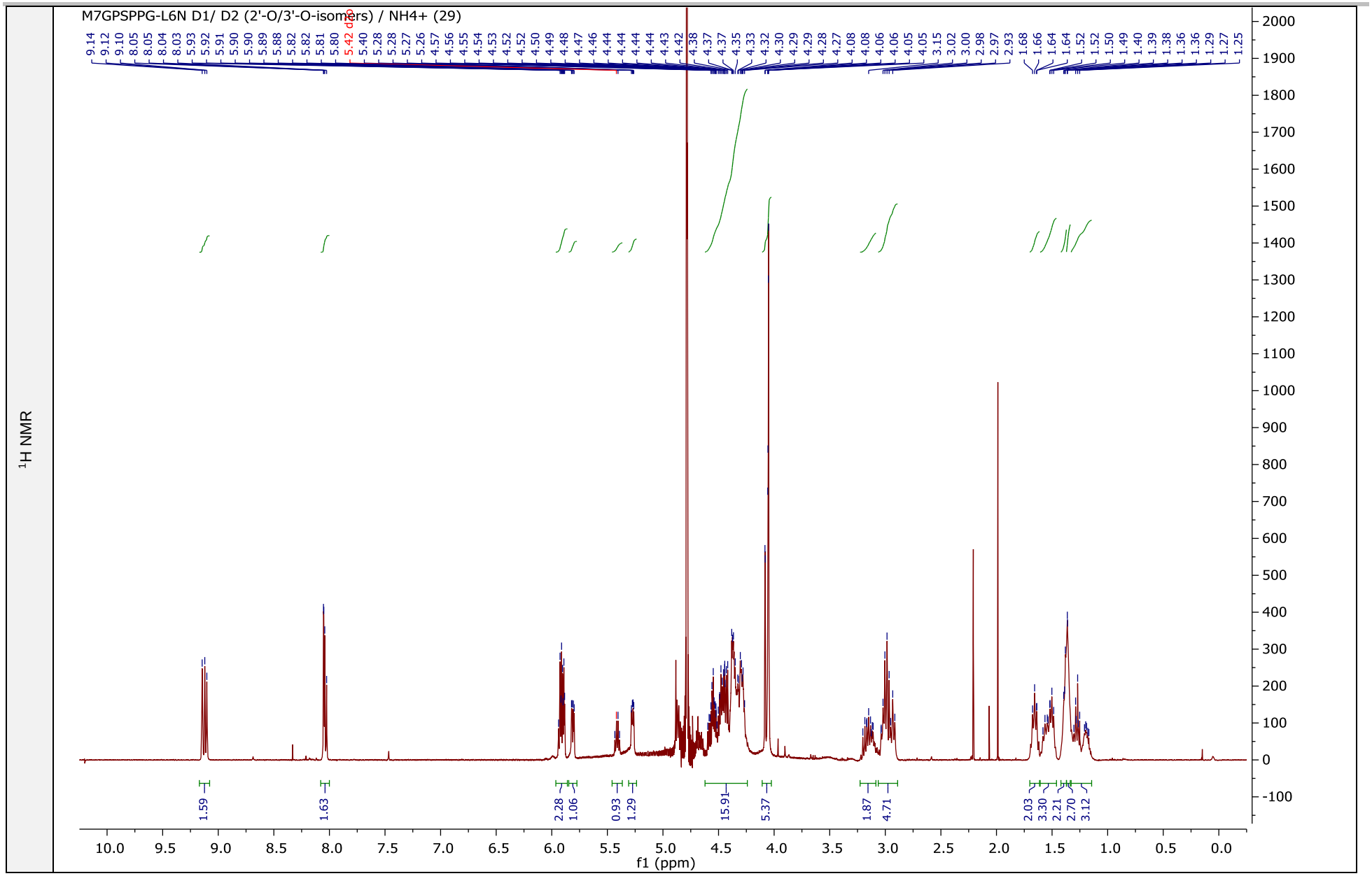


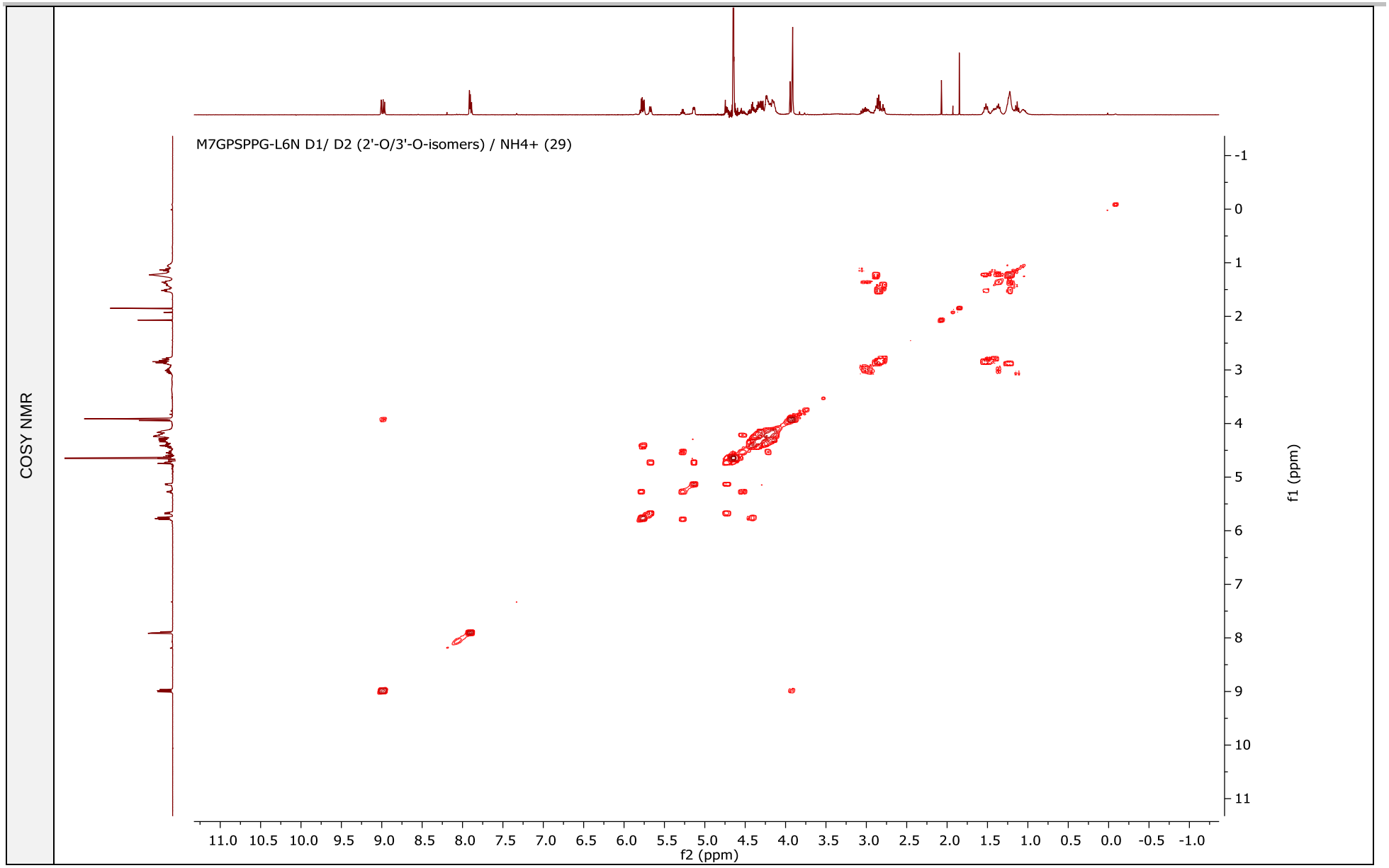
HRMS–ESI (*m/z*): $[M-H]^-$ calcd for $C_{28}H_{42}N_{12}O_{18}P_3S^-$, 959.16791.

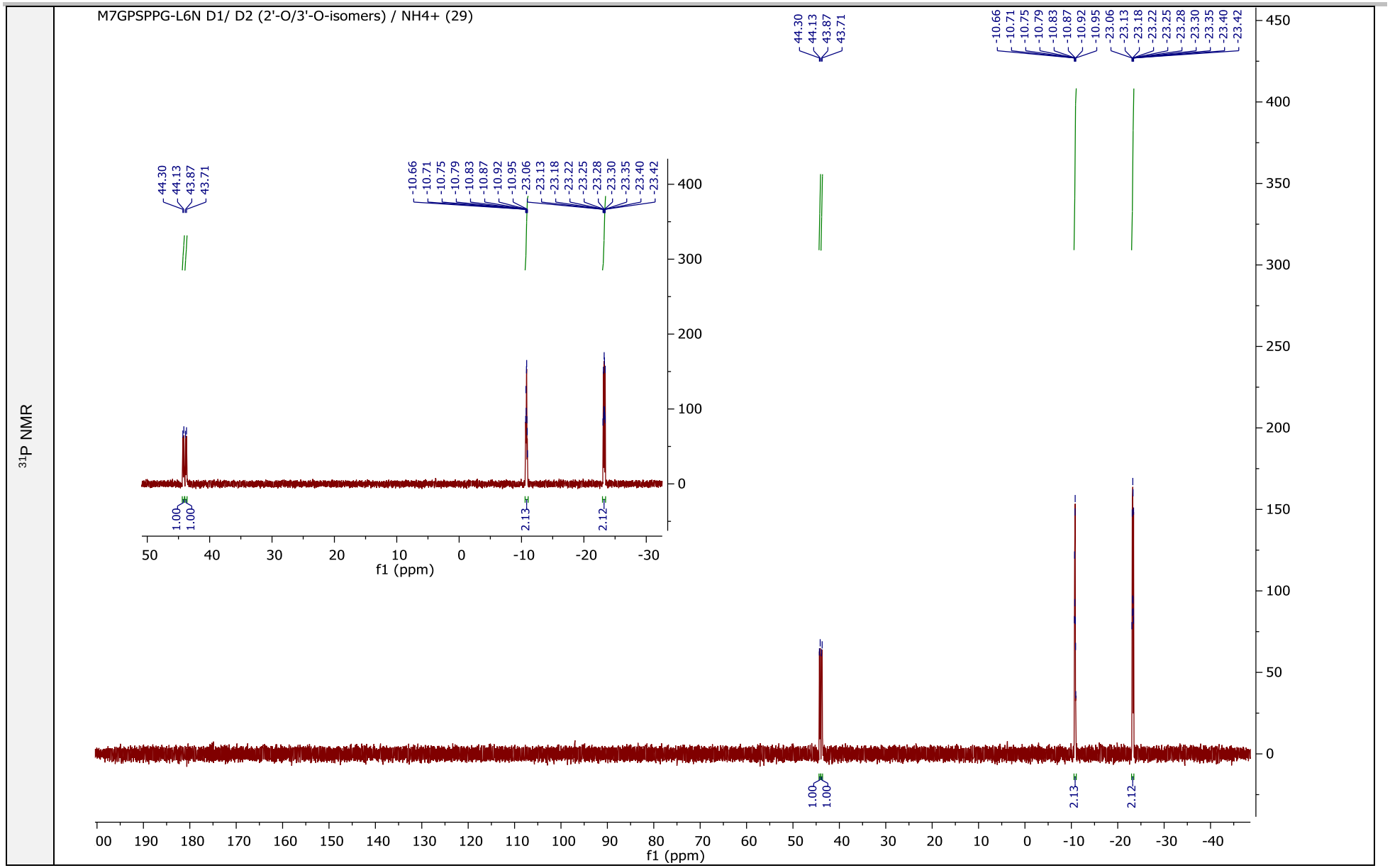


HRMS–ESI (*m/z*): [M-H]$^-$ calcd for $C_{28}H_{42}N_{12}O_{18}P_3S^-$, 959.16791.




M7GPSPPG-L6N D1/ D2 (2'-O/3'-O-isomers) / NH4+ (29)
1H NMR
f1 (ppm)

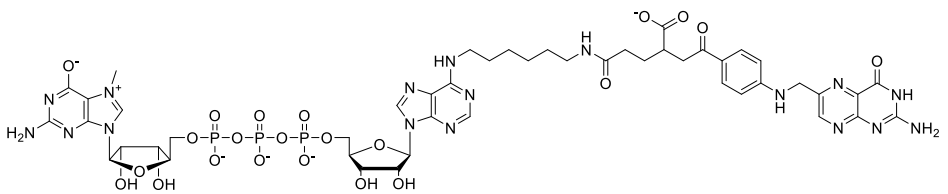

COSY NMR
M7GPSPPG-L6N D1/ D2 (2'-O/3'-O-isomers) / NH4+ (29)
f2 (ppm)
f1 (ppm)


M7GPSPPG-L6N D1/ D2 (2'-O/3'-O-isomers) / NH4+ (29)
31P NMR
44.30
44.13
43.87
43.71
-10.66
-10.71
-10.75
-10.79
-10.83
-10.87
-10.92
-10.95
-23.06
-23.13
-23.18
-23.22
-23.25
-23.28
-23.30
-23.35
-23.40
-23.42
1.00
1.00
2.13
2.12
f1 (ppm)

Cap-Ligand conjugates (23-24 a-b and 30-31 a-b)

m^7GpppA-HDA-FA / NH_4^+ (23a)

Chemical structure



HPLC profile

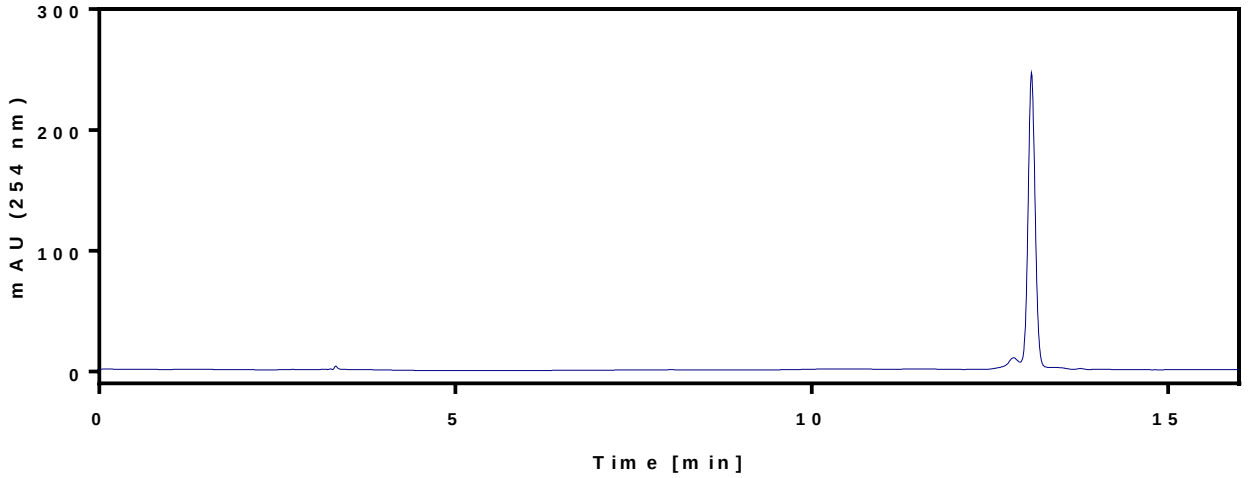
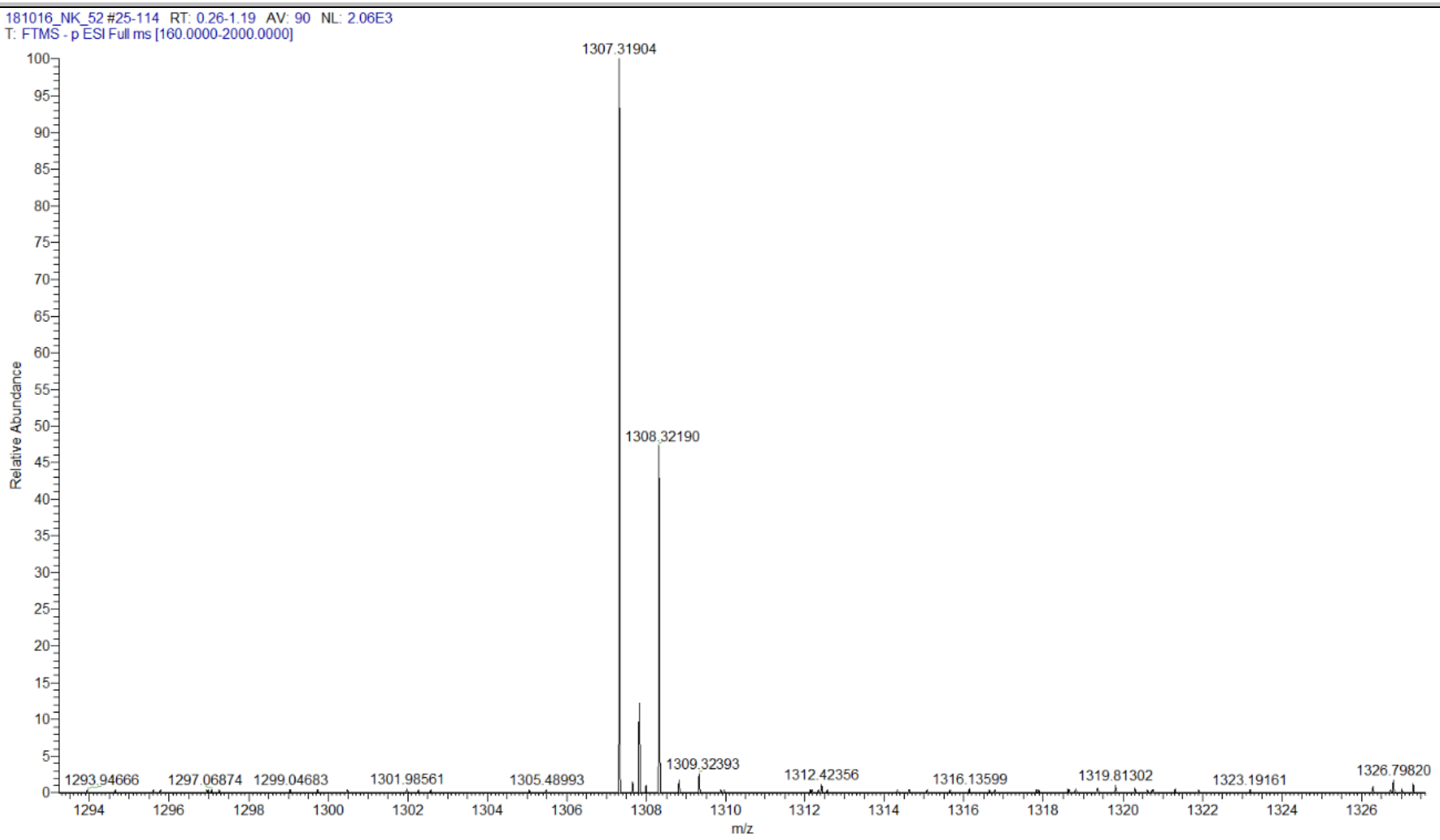
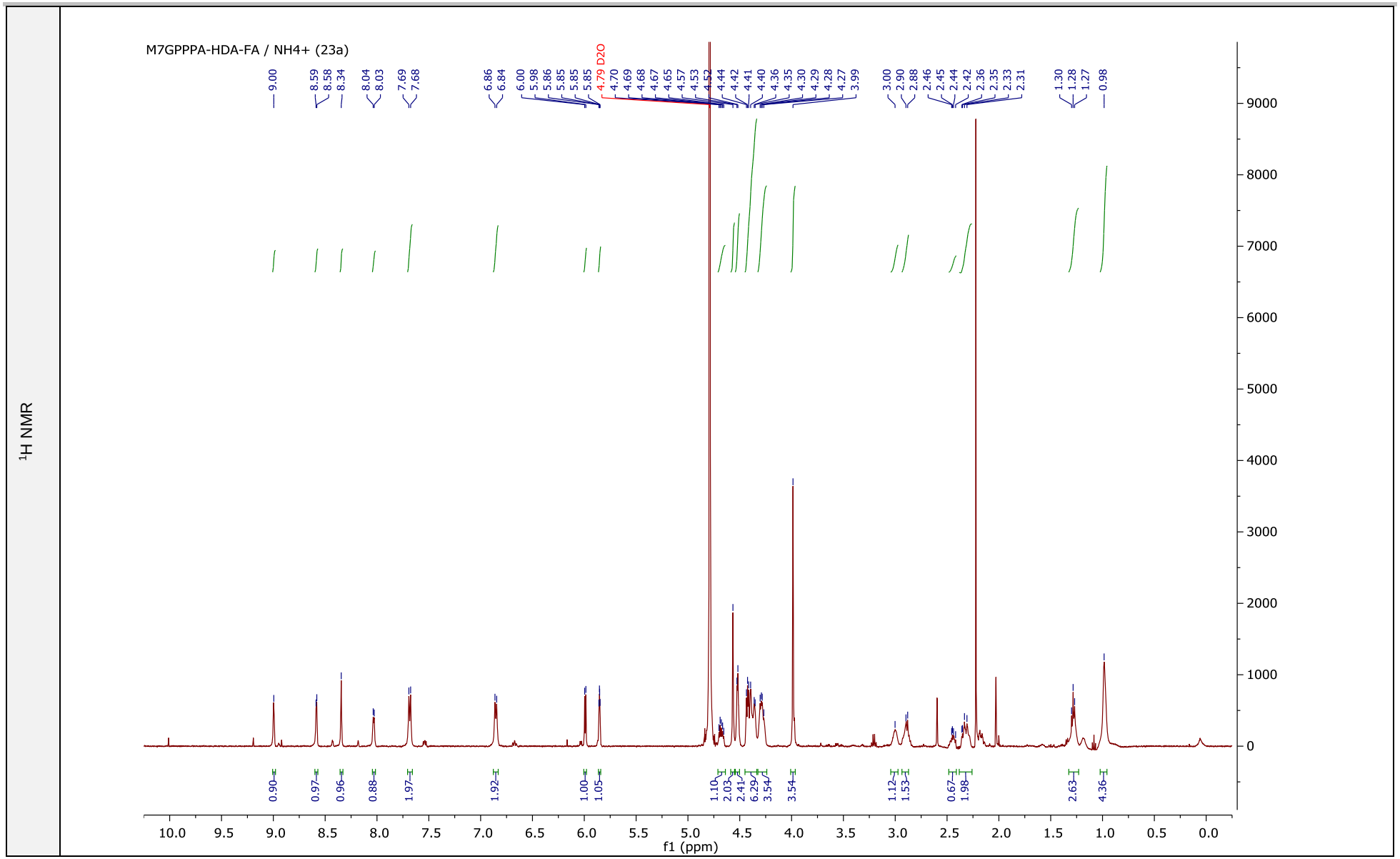


Figure S20. Analytical HPLC profile of compound **23a**: column Gemini NX C18, ammonium acetate pH 8.4:ACN 5-30% in 15 min.

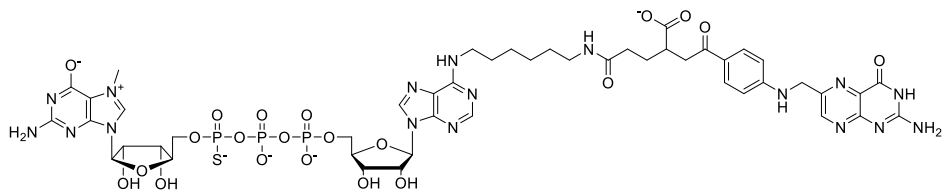
HRMS–ESI (*m/z*): $[M-H]^-$ calcd for $C_{46}H_{58}N_{18}O_{22}P_3^-$; 1307.31914.




M7GPPPA-HDA-FA / NH4+ (23a)
1H NMR
9.00
8.59
8.58
8.34
8.04
8.03
7.69
7.68
6.86
6.84
6.00
5.98
5.86
5.85
5.85
5.85
4.79 D2O
4.70
4.69
4.68
4.67
4.65
4.57
4.53
4.52
4.44
4.42
4.41
4.40
4.36
4.35
4.30
4.29
4.28
4.27
3.99
3.00
2.90
2.88
2.46
2.45
2.44
2.42
2.36
2.35
2.33
2.31
1.30
1.28
1.27
0.98
0.90
0.97
0.96
0.88
1.97
1.92
1.00
1.05
1.10
2.03
2.41
6.29
3.54
3.54
1.12
1.53
0.67
1.98
2.63
4.36
f1 (ppm)

$m^7GpsppA$-HDA-FA D1/D2 / NH_4^+ as a mixture of D1/D2 diastereoisomers (24a)

Chemical structure



HPLC profile

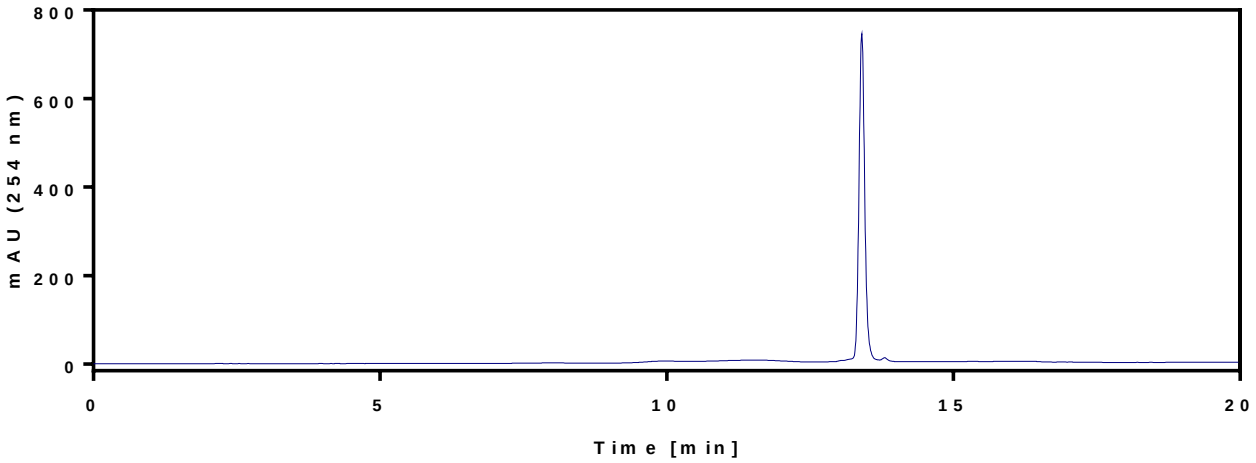
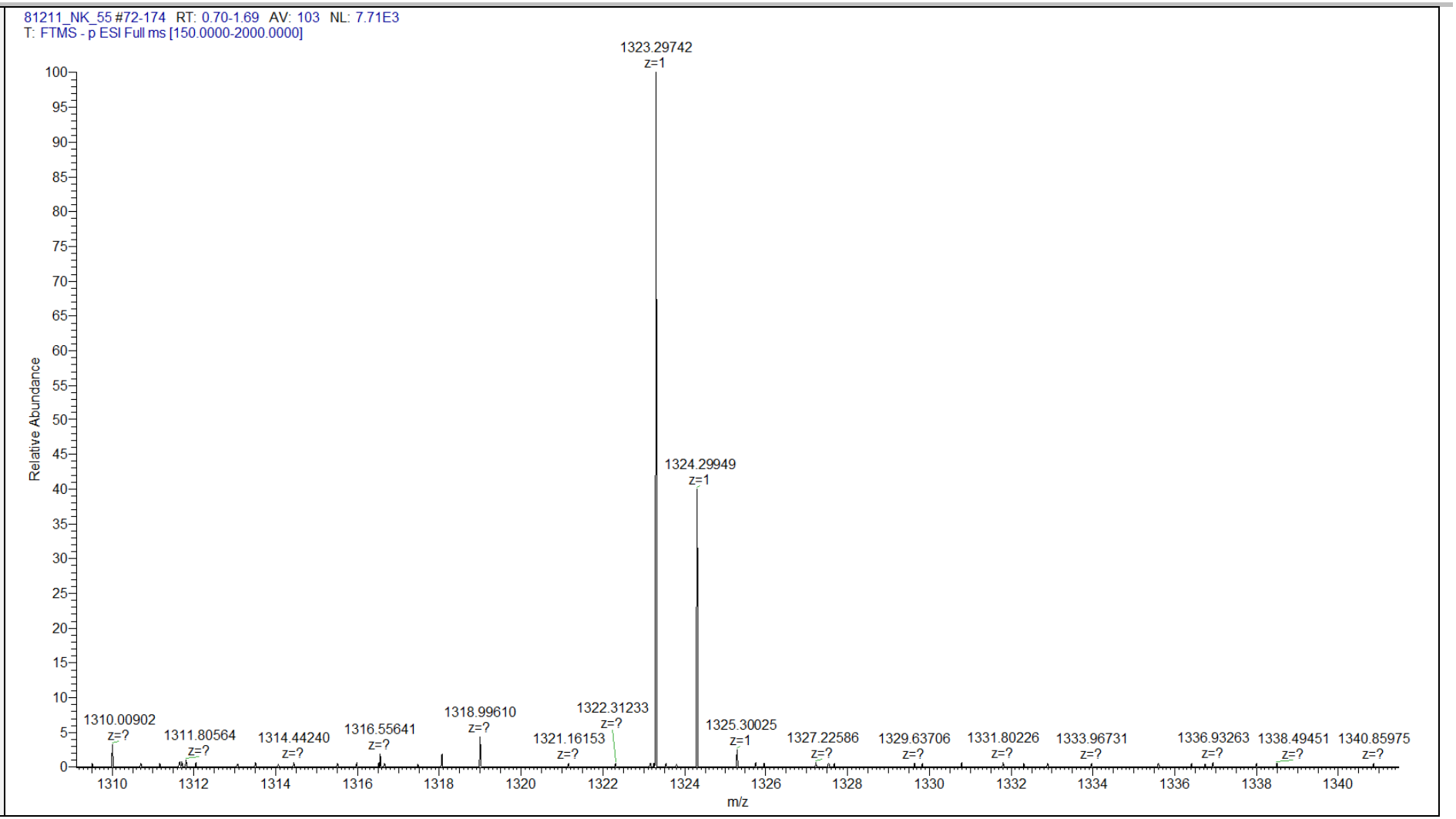


Figure S21. Analytical HPLC profile of compound **24a**: column Gemini NX C18, ammonium acetate pH 8.4:ACN 5-30% in 15 min.

HRMS–ESI (*m/z*): [M-H]$^-$ calcd for $C_{46}H_{58}N_{18}O_{21}P_3S^-$, 1323.29629.



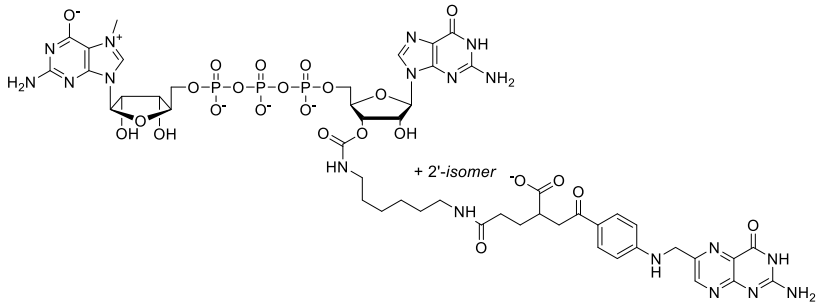
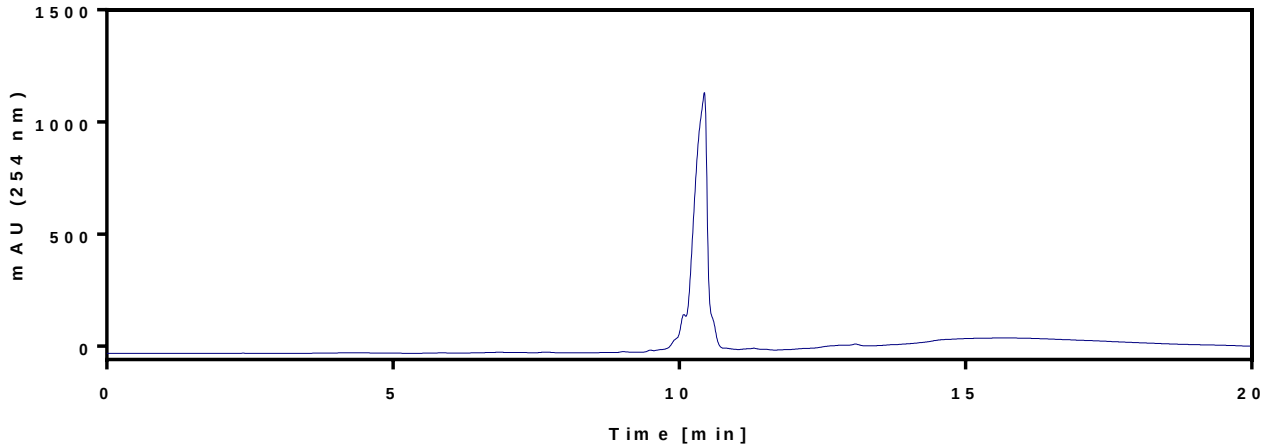
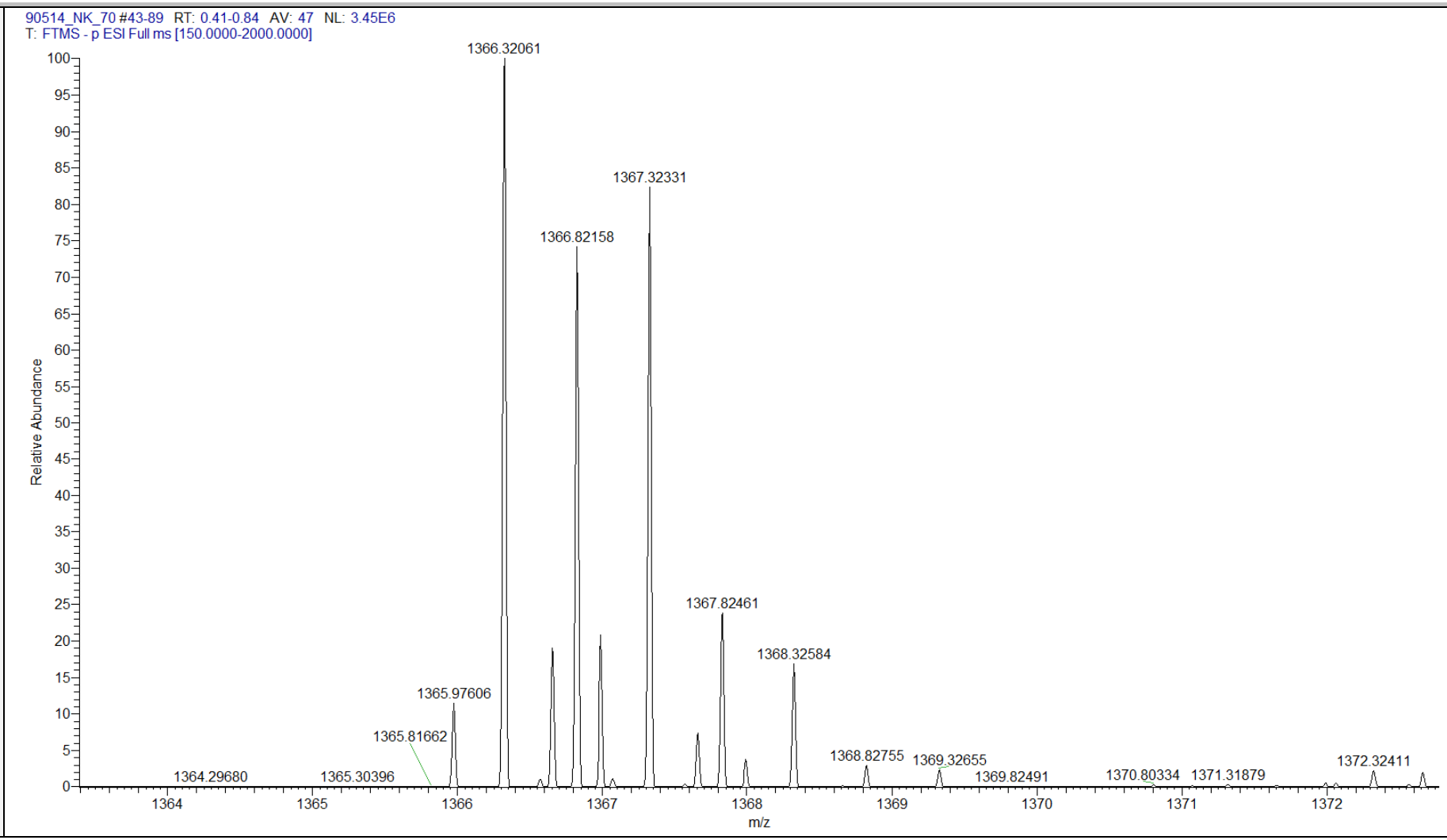
m^7GpppG-L_{6N}-FA / NH_4^+ as a mixture of 2'-O/3'-O-isomers (30a)	
Chemical structure	
HPLC profile	

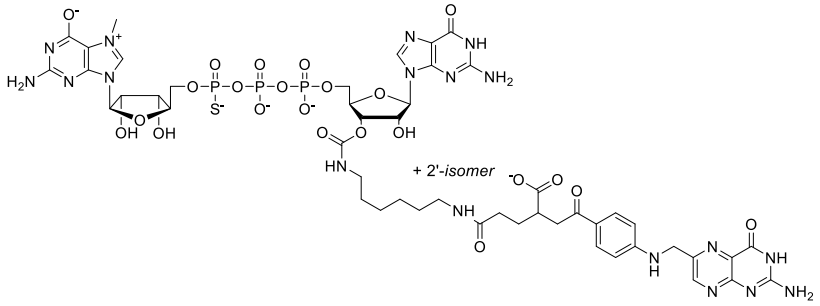
Figure S22. Analytical HPLC profile of compound **30a**: column Gemini NX C18, ammonium acetate pH 8.4:ACN 5-30% in 15 min.

HRMS–ESI (*m/z*): $[M-H]^-$ calcd for $C_{47}H_{59}N_{19}O_{24}P_3^-$, 1366.31987.



$m^7GpsppG$-L_{6N}-FA D1/D2 / NH_4^+ as a mixture of D1/D2 diastereoisomers and 2'-O/3'-O isomers (31a)

Chemical structure



HPLC profile

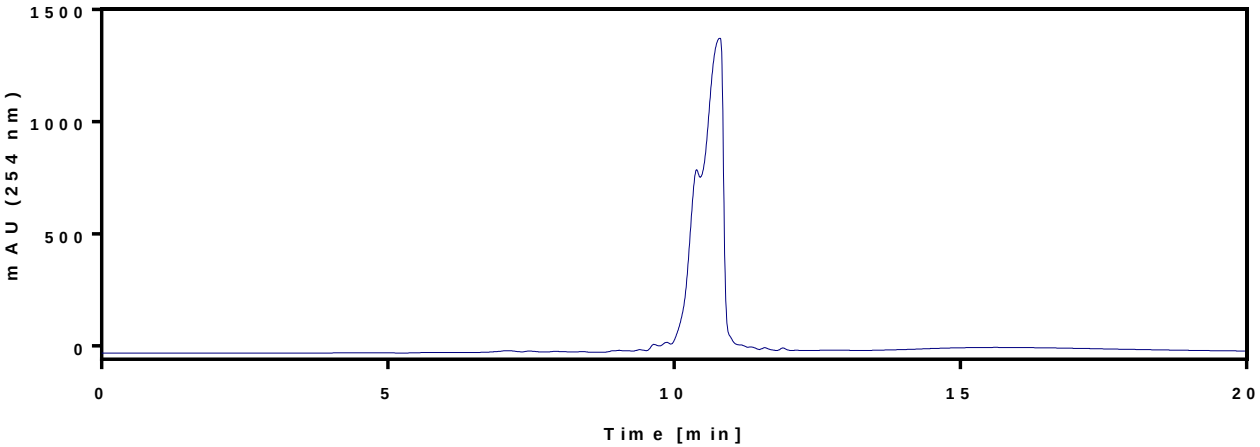
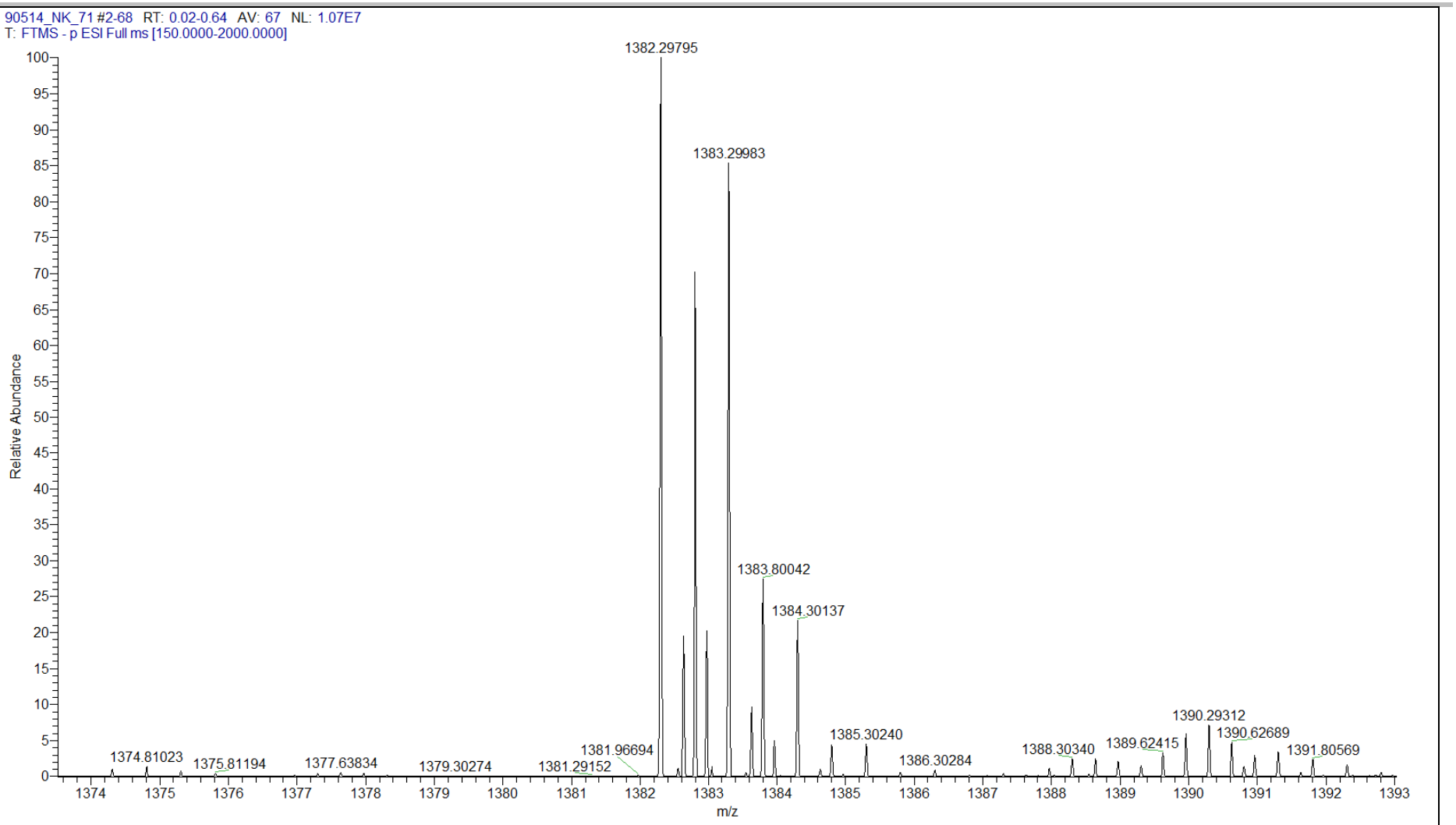


Figure S23. Analytical HPLC profile of compound **31a**: column Gemini NX C18, ammonium acetate pH 8.4:ACN 5-30% in 15 min.

HRMS–ESI (*m/z*): [M-H]$^-$ calcd for $C_{47}H_{59}N_{19}O_{23}P_3S^-$, 1382.29702.



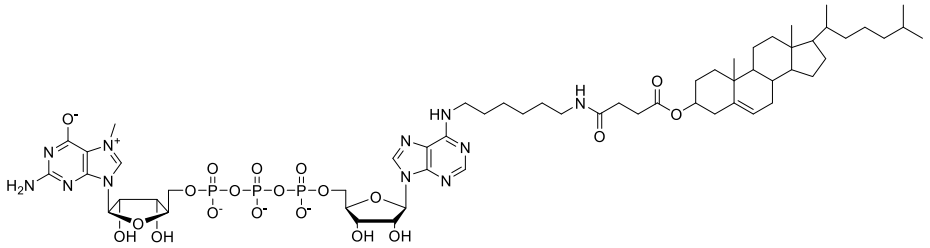
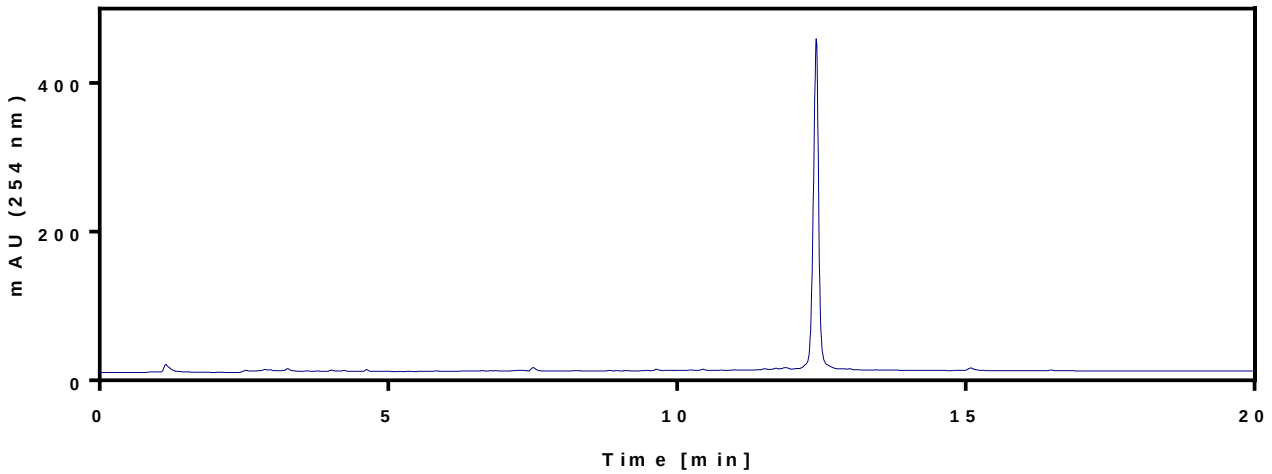
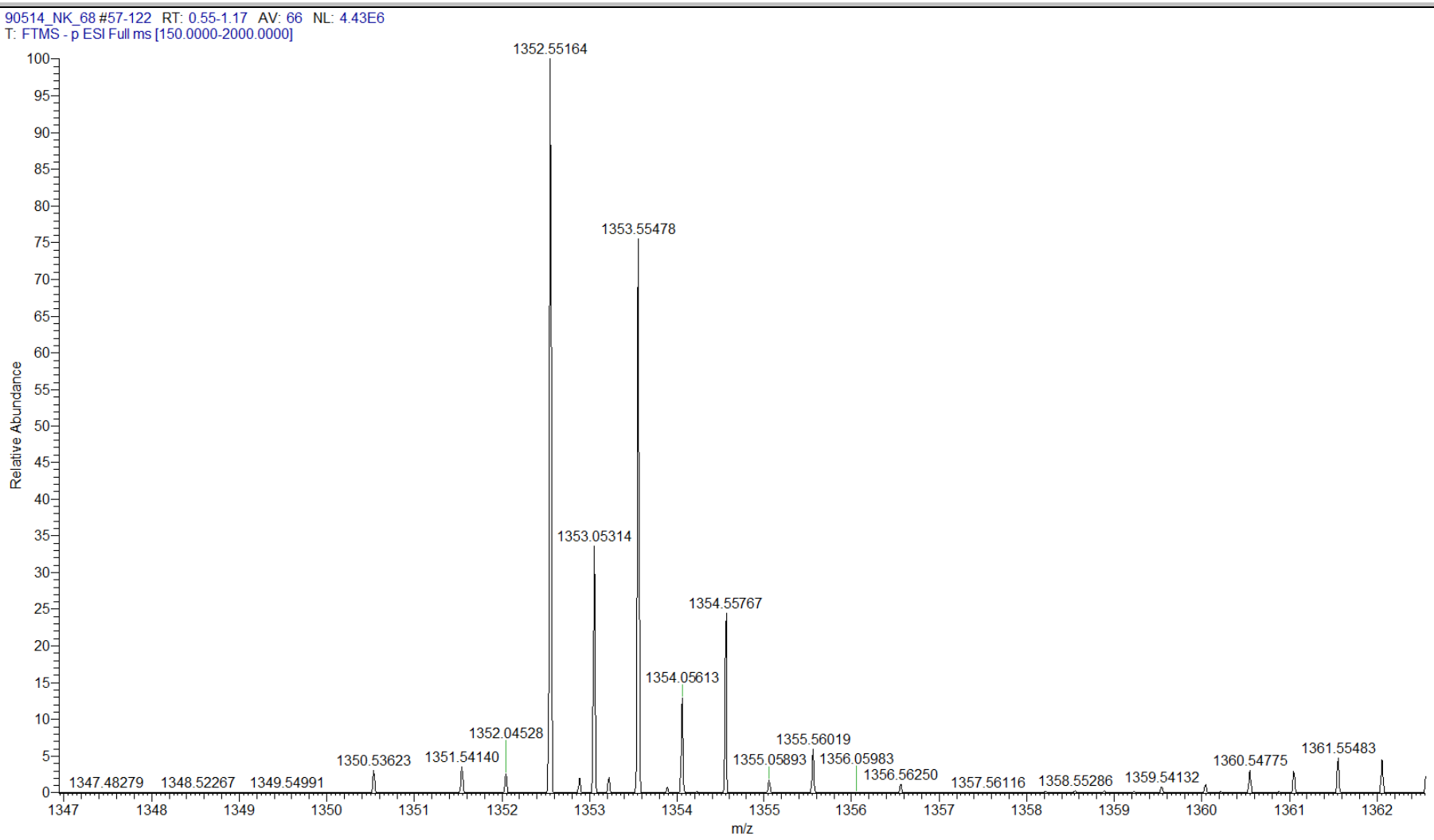
m^7GpppA-HDA-CHOL / NH_4^+ (23b)	
Chemical structure	
HPLC profile	

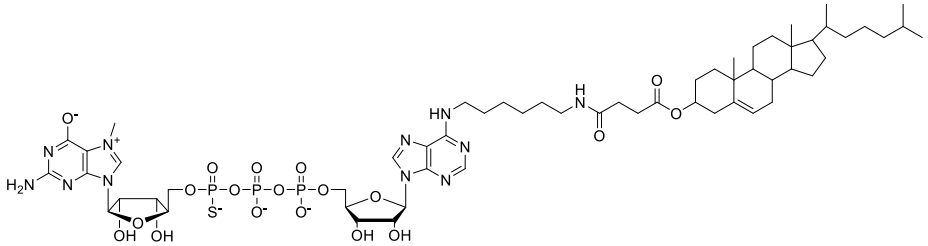
Figure S24. Analytical HPLC profile of compound **23b**: column C4, Buffer A : ACN 0-100% in 15 min.

HRMS–ESI (*m/z*): [M-H]$^-$ calcd for $C_{58}H_{89}N_{11}O_{20}P_3^-$, 1352.55037.



$m^7GpsppA$-HDA-CHOL D1/D2 / NH_4^+ as a mixture of D1/ D2 diastereoisomers (24b)

Chemical structure



HPLC profile

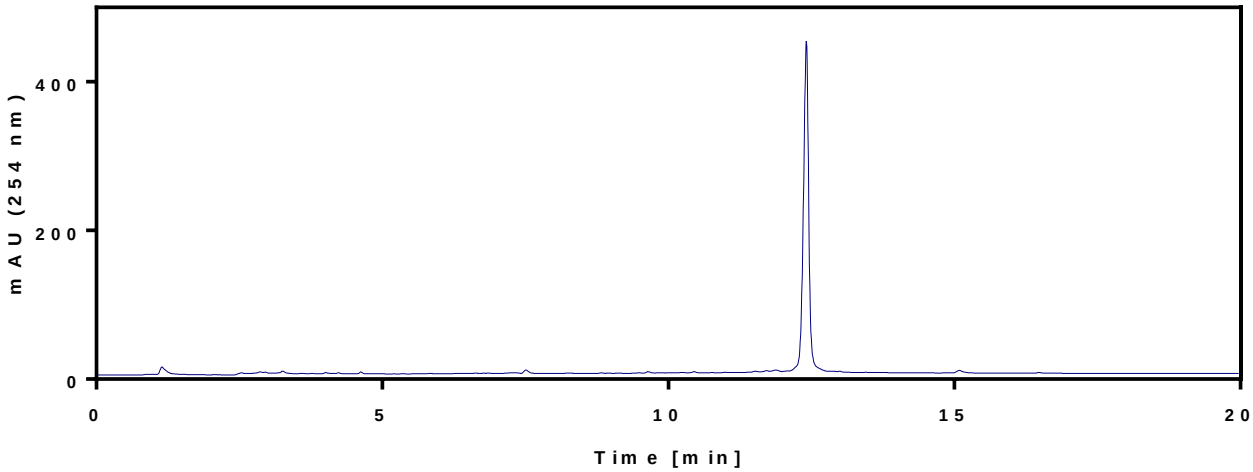
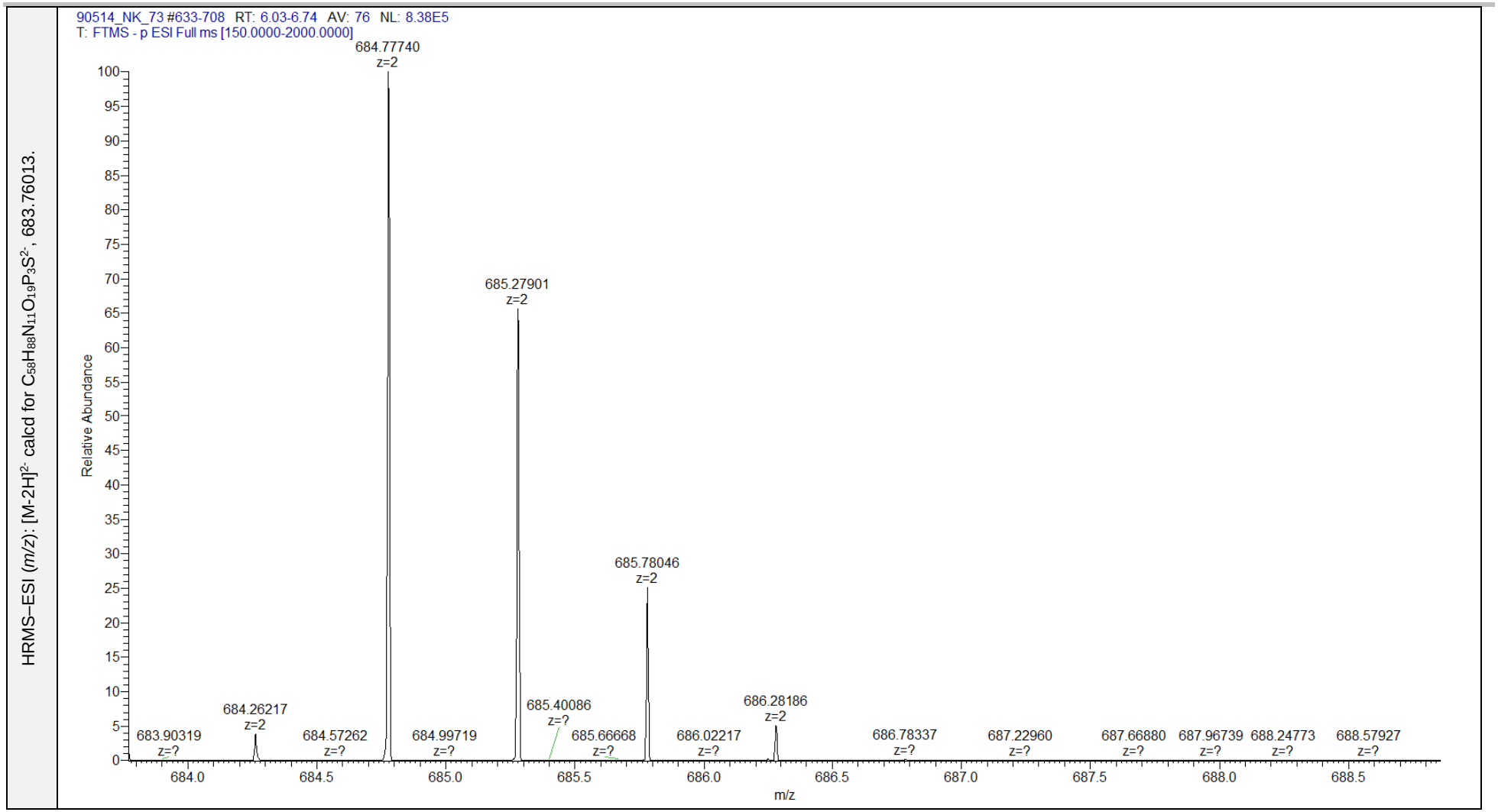
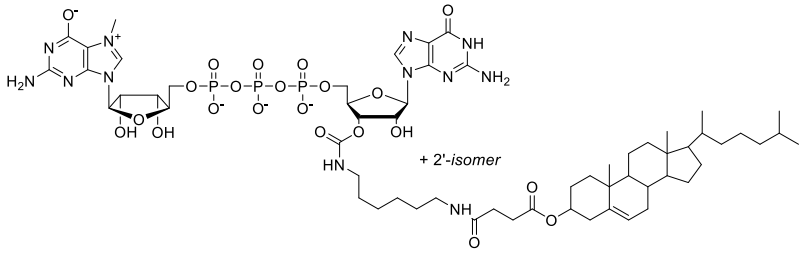


Figure S25. Analytical HPLC profile of compound **24b**: column C4, Buffer A : ACN 0-100% in 15 min.


HRMS–ESI (m/z): [M-2H]2- calcd for C58H88N11O19P3S2-, 683.76013.
90514_NK_73 #633-708 RT: 6.03-6.74 AV: 76 NL: 8.38E5
T: FTMS - p ESI Full ms [150.0000-2000.0000]
Relative Abundance
m/z
684.77740 z=2
685.27901 z=2
685.78046 z=2
684.26217 z=2
686.28186 z=2
683.90319 z=?
684.57262 z=?
684.99719 z=?
685.40086 z=?
685.66668 z=?
686.02217 z=?
686.78337 z=?
687.22960 z=?
687.66880 z=?
687.96739 z=?
688.24773 z=?
688.57927 z=?

m^{7}GpppG-L$_{6N}$-CHOL / NH$_4^+$ as a mixture of 2'-O/3'-O-isomers (30b)

Chemical structure



HPLC profile

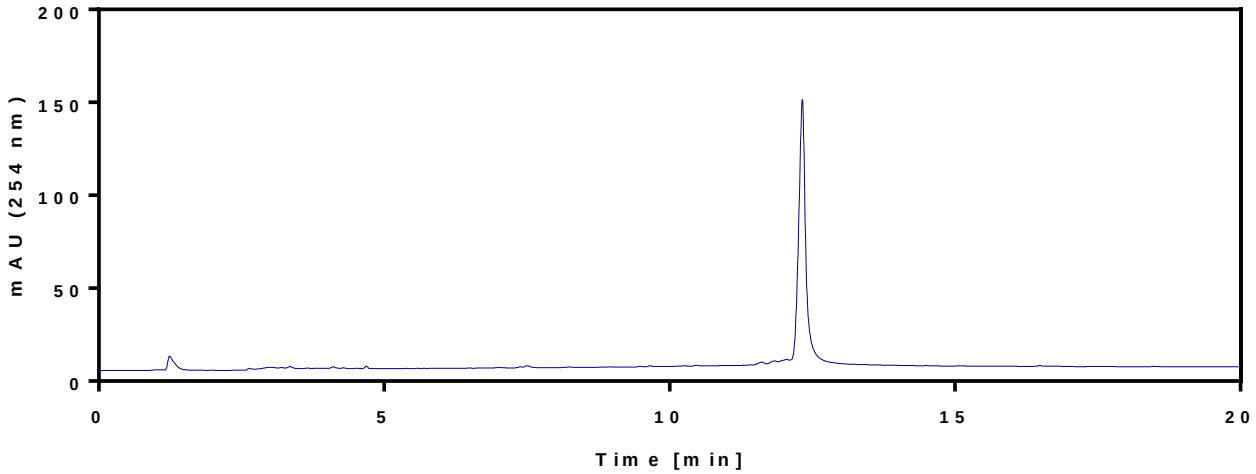
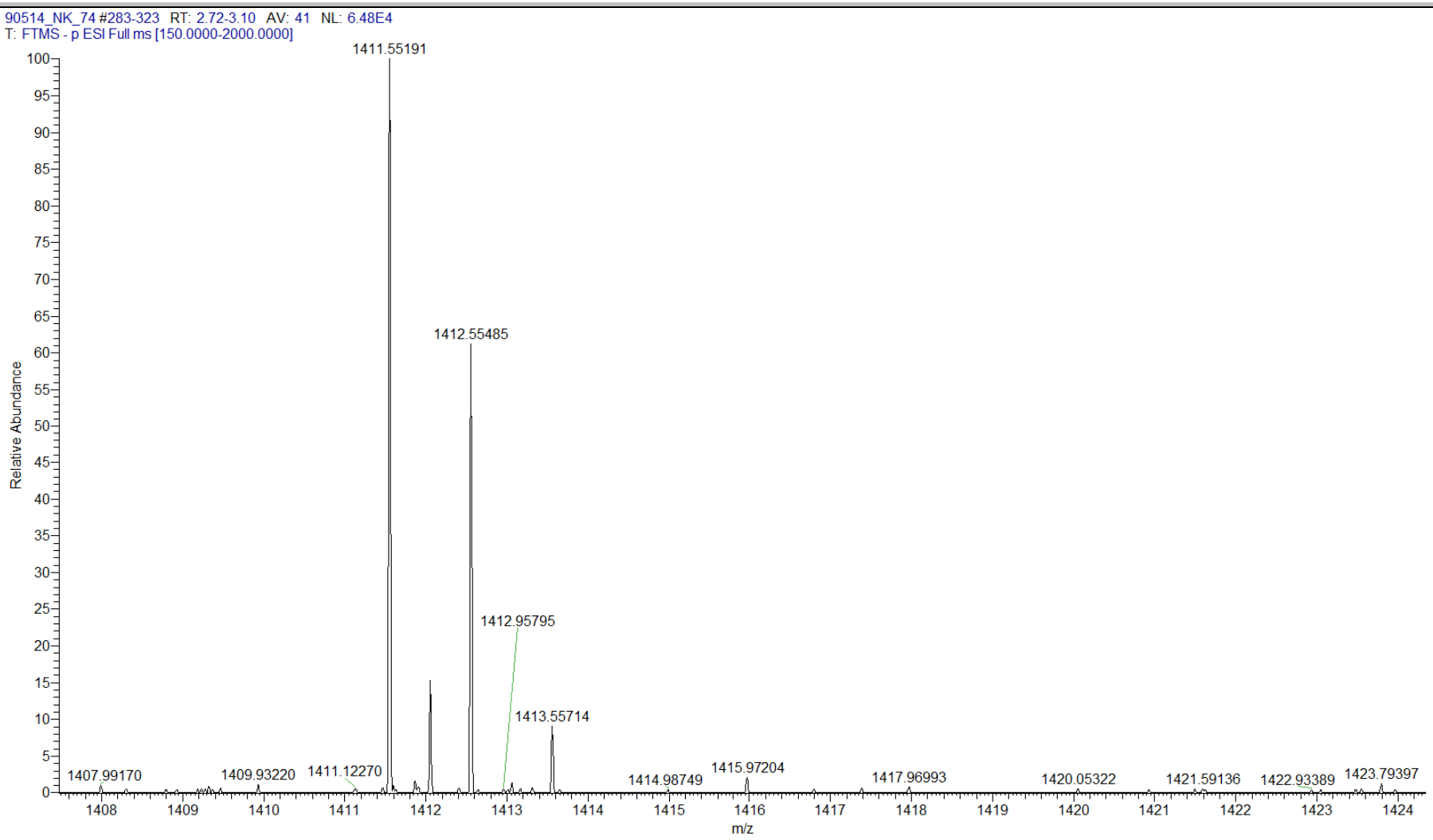


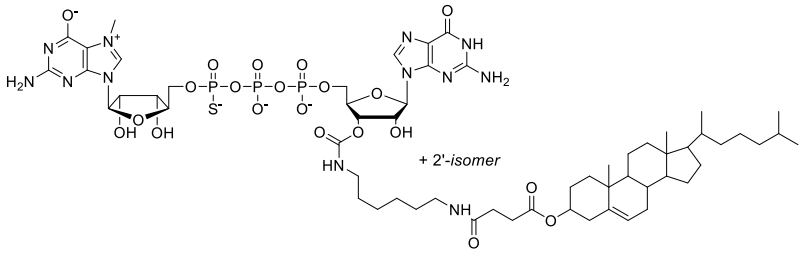
Figure S26. Analytical HPLC profile of compound **30b**: column C4, Buffer A : ACN 0-100% in 15 min.

HRMS–ESI (*m/z*): $[M-H]^-$ calcd for $C_{59}H_{90}N_{12}O_{22}P_3^-$, 1411.55110.



$m^7GpsppG\text{-}L_{6N}$-CHOL D1/D2 / NH_4^+ as a mixture of D1/D2 diastereoisomers and 2'-O/3'-O isomers (31b)

Chemical structure



HPLC profile

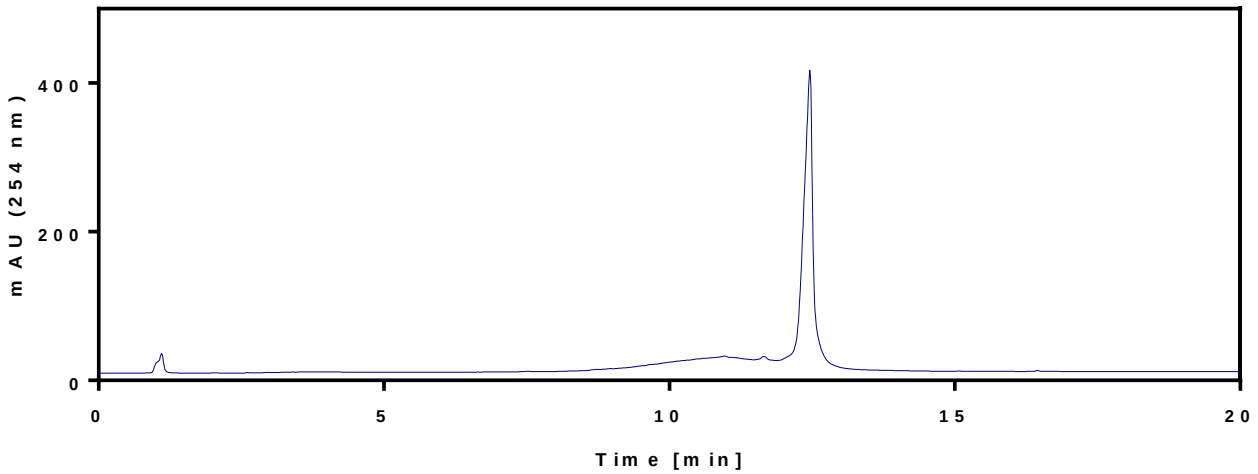
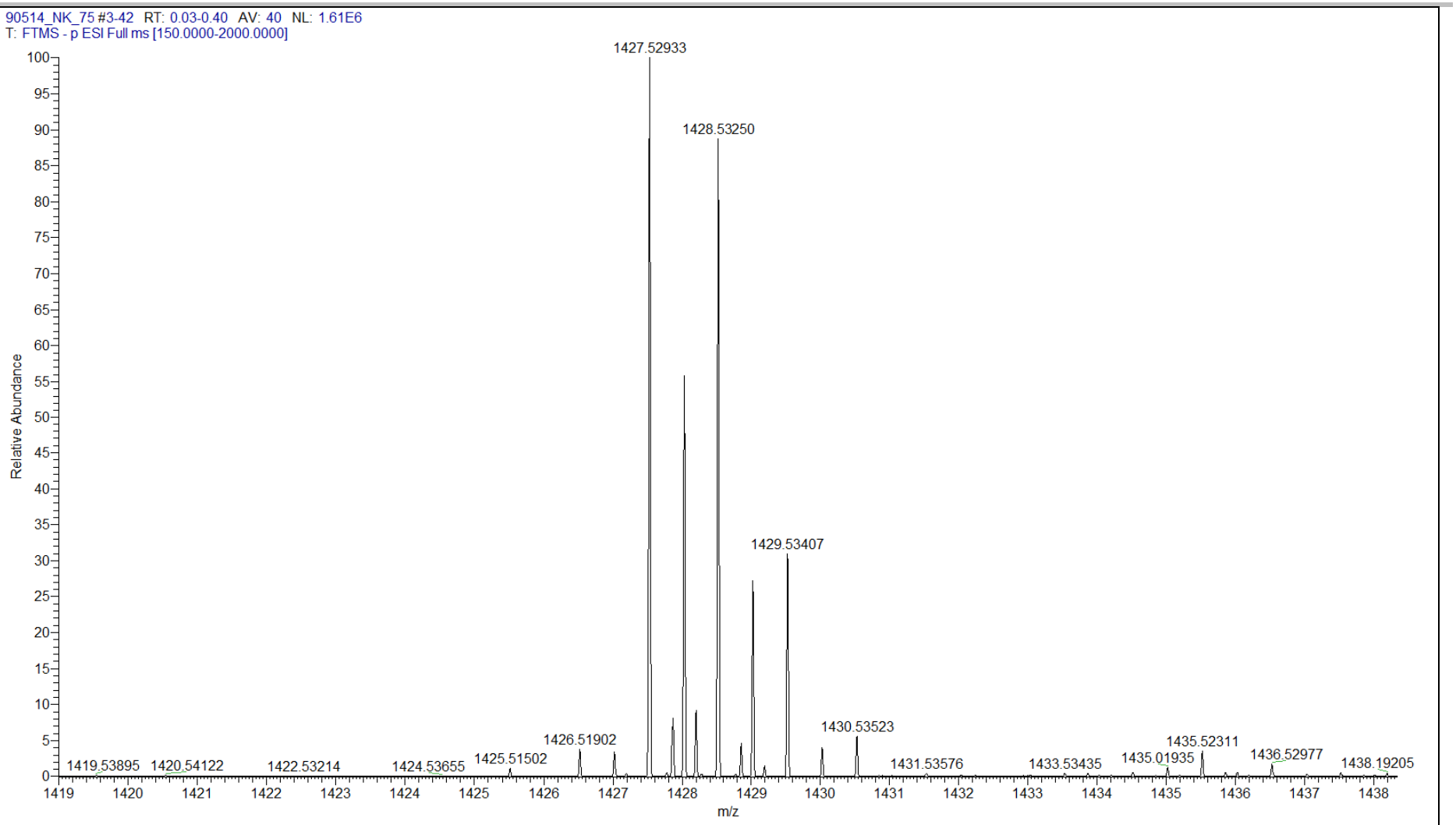


Figure S27. Analytical HPLC profile of compound **31b**: column C4, Buffer A : ACN 0-100% in 15 min.

HRMS–ESI (*m/z*): [M-H]$^-$ calcd for $C_{59}H_{90}N_{12}O_{21}P_3S^-$, 1427.52825.



Miscellaneous

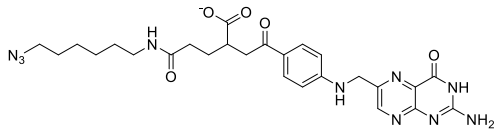
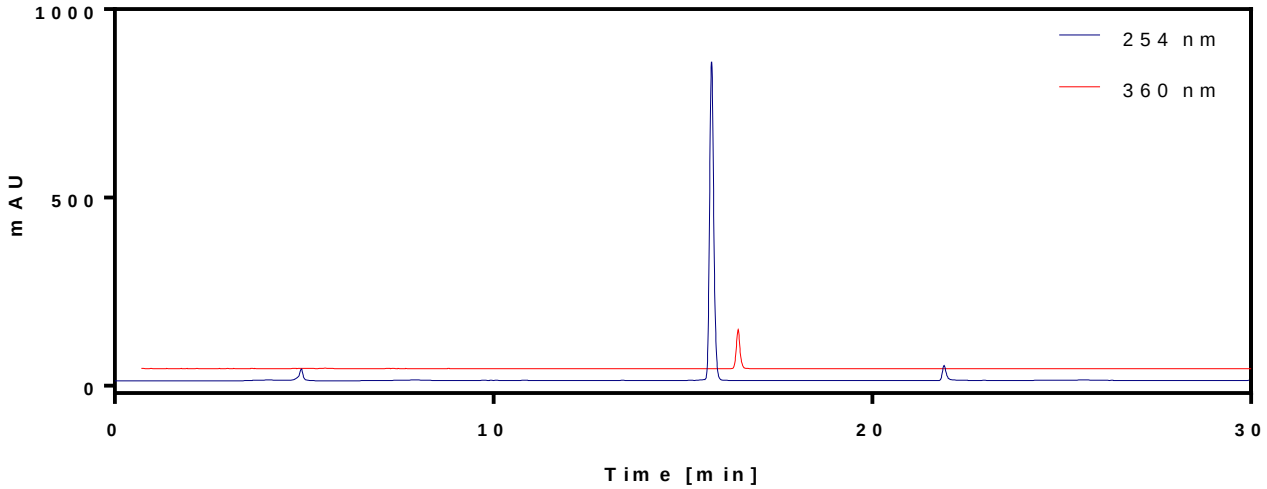
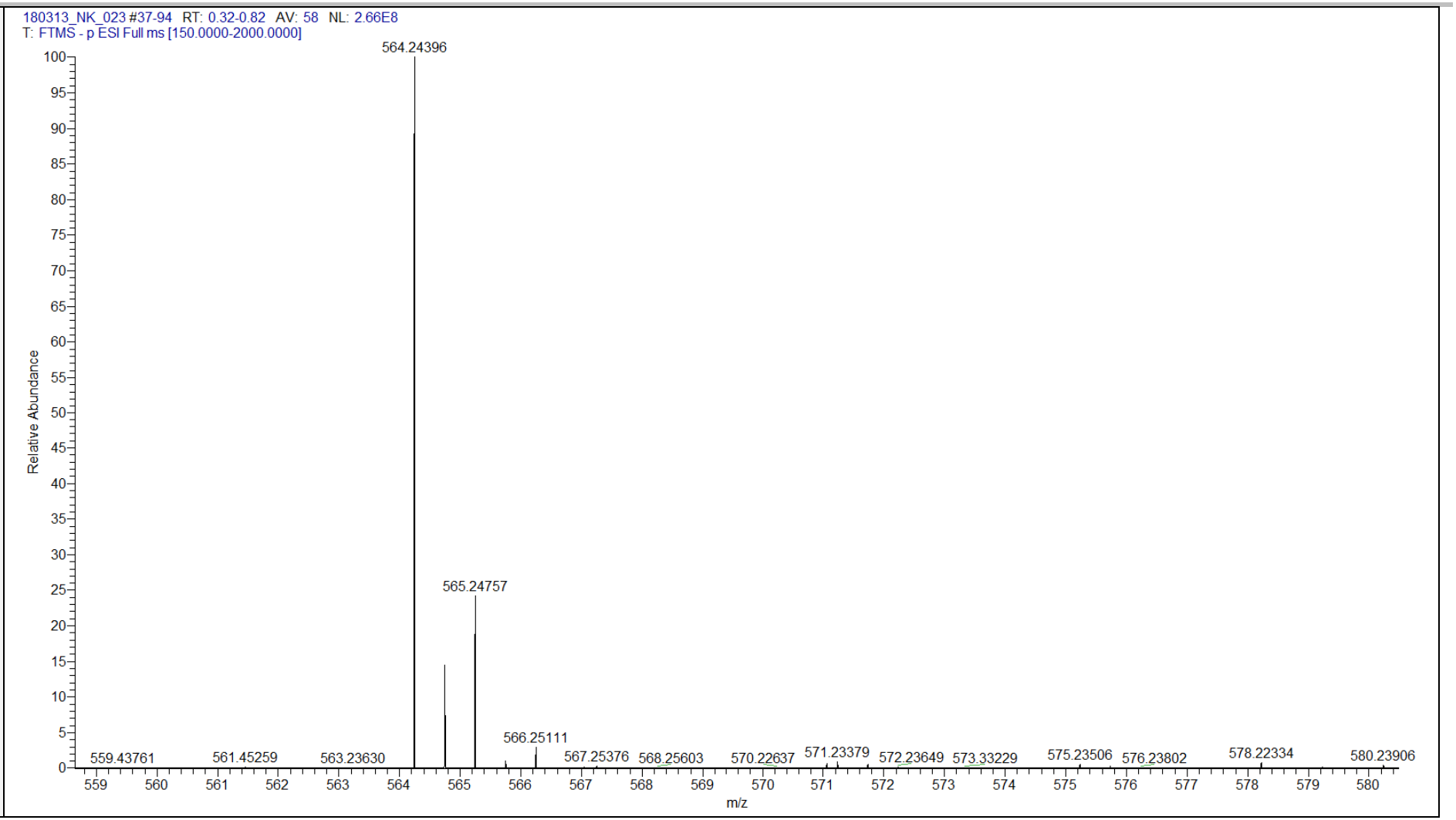
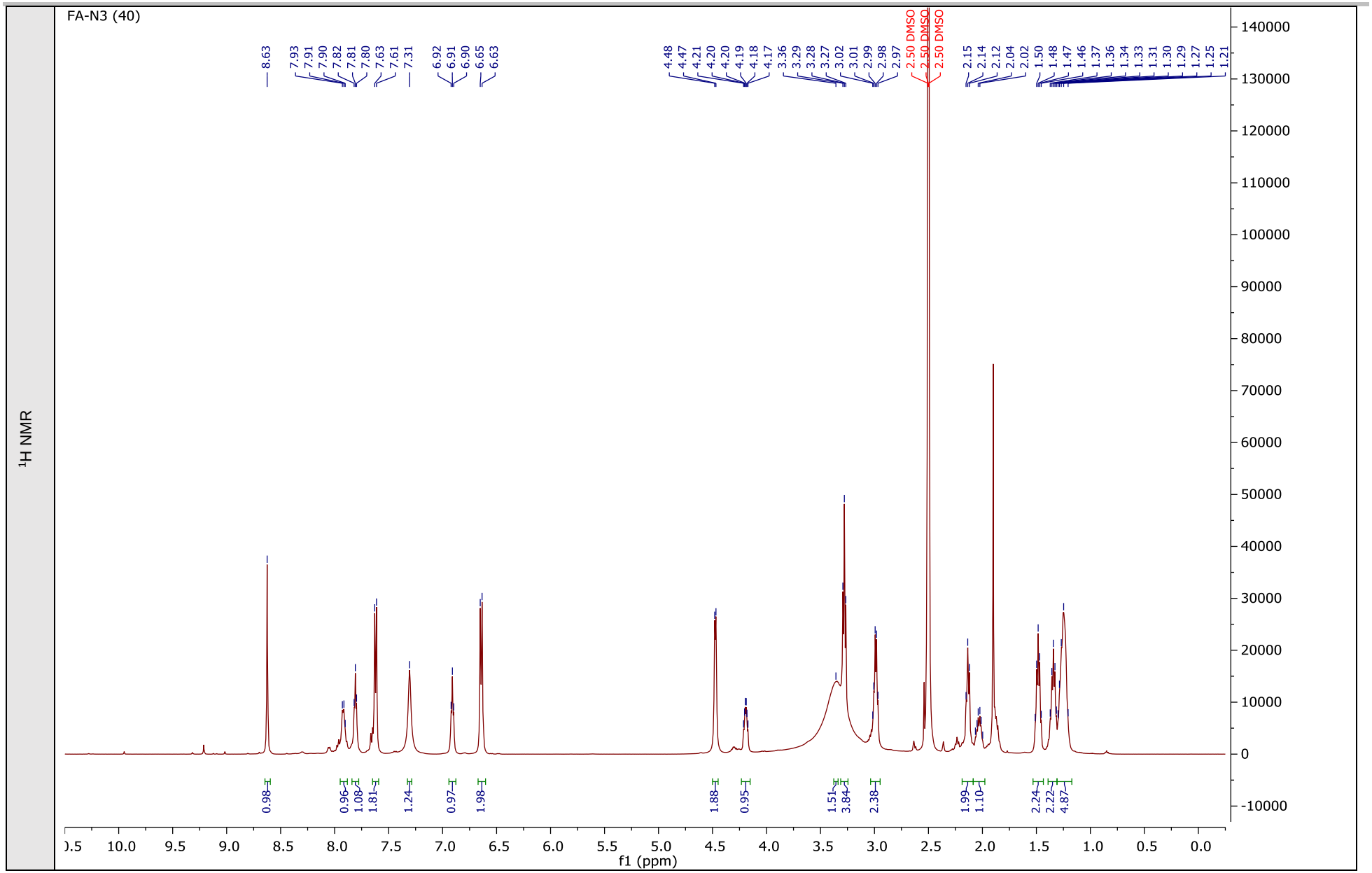
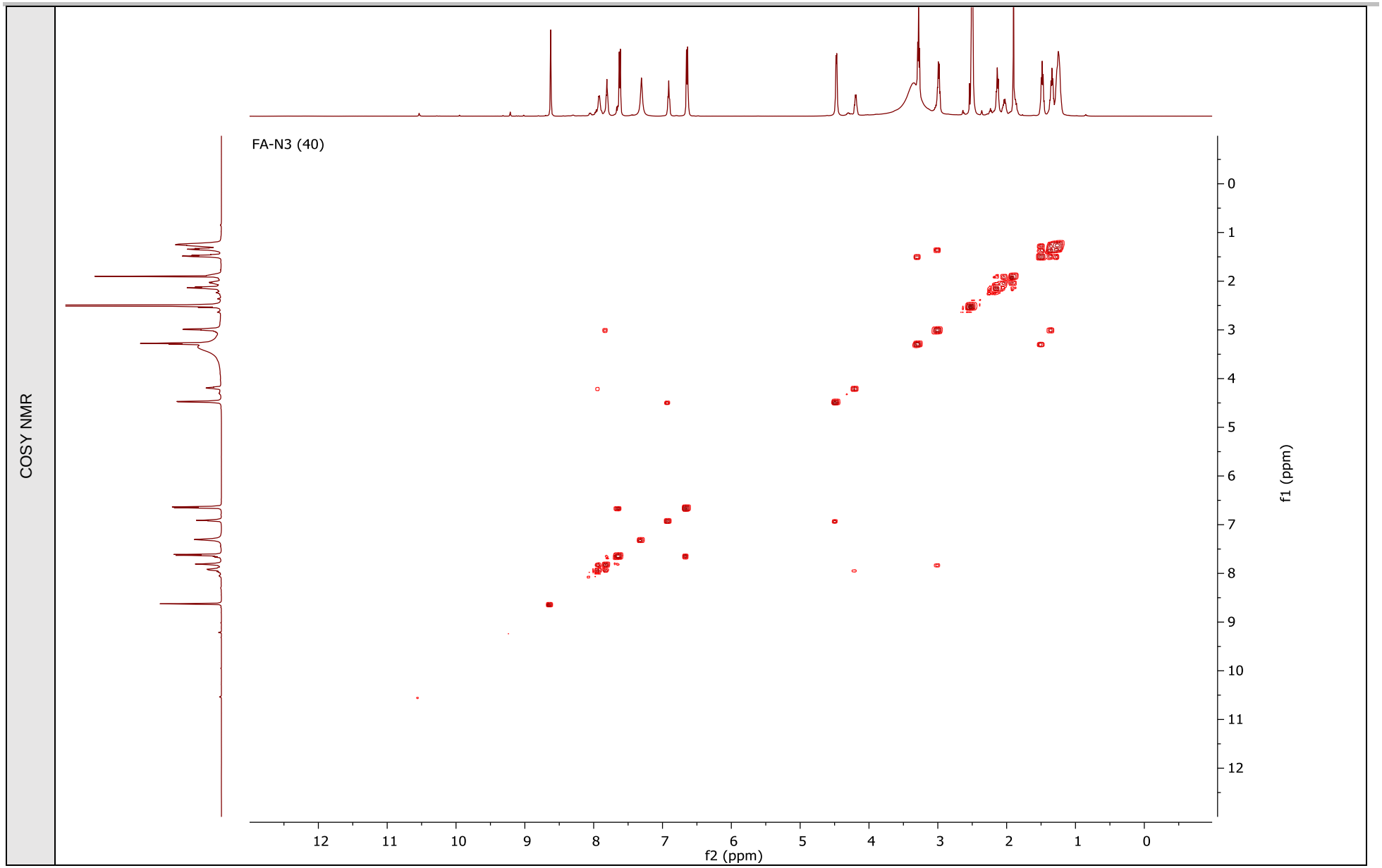
Folic acid azide (40)	
Chemical structure	
HPLC profile	

Figure S28. Analytical HPLC profile of compound **40**: column Gemini NX C18, ammonium acetate pH 8.4:ACN 5-50% in 15 min.

HRMS–ESI (*m/z*): [M-H]$^-$ calcd for $C_{25}H_{30}N_{11}O_5^-$, 564.24369.




FA-N3 (40)
1H NMR
8.63
7.93
7.91
7.90
7.82
7.81
7.80
7.63
7.61
7.31
6.92
6.91
6.90
6.65
6.63
4.48
4.47
4.21
4.20
4.19
4.18
4.17
3.36
3.29
3.28
3.27
3.02
3.01
2.99
2.98
2.97
2.50 DMSO
2.50 DMSO
2.50 DMSO
2.15
2.14
2.12
2.04
2.02
1.50
1.48
1.47
1.46
1.37
1.36
1.34
1.33
1.31
1.30
1.29
1.27
1.25
1.21
0.98
0.96
1.08
1.81
1.24
0.97
1.98
1.88
0.95
1.51
3.84
2.38
1.99
1.10
2.24
2.22
4.87
f1 (ppm)


COSY NMR
FA-N3 (40)
f2 (ppm)
f1 (ppm)
12
11
10
9
8
7
6
5
4
3
2
1
0

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