

Supporting Information

Synthesis and biological evaluation of a chitobiose based peptide *N*-glycanase inhibitor library

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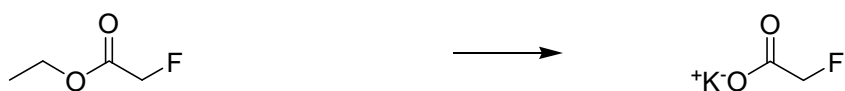
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General Procedures:

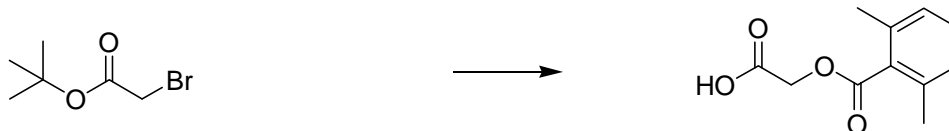
All reagents were commercial grade and were used as received unless stated otherwise. TTBP was synthesized as described. Diethyl ether (Et₂O), ethyl acetate (EtOAc), light petroleum ether (PE) and toluene (Tol) were purchased from Riedel-de Haën. Acetonitrile (MeCN), dichloroethane, dichloromethane (CH₂Cl₂), *N,N*-dimethylformamide (DMF), methanol (MeOH), pyridine (pyr), tetrahydrofuran (THF) were obtained from Biosolve. THF was distilled over LiAlH₄ prior to use. Before use CH₂Cl₂ was refluxed over CaH₂ for 2h and distilled. Trace of water were removed by refluxing *n*-Butanol over sodium for 2h followed by distilling and storing over 3Å MS. Trifluoromethanesulfonic anhydride was distilled from P₂O₅. Molecular sieves 3Å were flame dried before use. All reactions were performed under an inert atmosphere of Argon unless stated otherwise. Solvents used for flash chromatography were of pro analysi quality. Flash chromatography was performed on Screening Devices silica gel 60 (0.04 – 0.063 mm). TLC-analysis was conducted on DC-alufolien (Merck, Kieselgel60, F254) with detection by UV-absorption (254 nm) were applicable and by spraying with 20% sulfuric acid in ethanol followed by charring at ~150°C or by spraying with a solution of (NH₄)₆Mo₇O₂₄·H₂O (25 g/l) and (NH₄)₄Ce(SO₄)₄·2H₂O (10g/l) in 10% sulfuric acid in water followed by charring at ~150°C. ¹H and ¹³C NMR spectra were recorded on a 400 400/100 MHz, 500/125 MHz and a 600/150 MHz spectrometer. Chemical shifts (δ) are given in ppm relative to the chloroform residual solvent peak or tetramethylsilane as internal standard. Coupling constants are given in Hz. All given ¹³C spectra are proton decoupled. High resolution mass spectra were recorded by direct injection (2 µL of a 2 µM solution in water/acetonitrile; 50/50; v/v and 0.1% formic acid) on a mass

spectrometer equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution $R = 60000$ at m/z 400 (mass range $m/z = 150$ -2000) and dioctylphthalate ($m/z = 391.28428$) as a “lock mass”. The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). LC-MS analysis was performed on a HPLC system with a standard C_{18} (4.6 mmD \times 250 mmL, 5 μ particle size) column (detection at 200-600 nm) in combination with buffers A: H_2O , B: MeCN and C: 0.5% aq. TFA and coupled to a mass spectrometer with ESI. For RP-HPLC purifications an automated HPLC system equipped with a C_{18} (5 μ m 250 \times 10 mm) column was used. The applied buffers were A: 0.1% TFA in H_2O , B: MeCN.



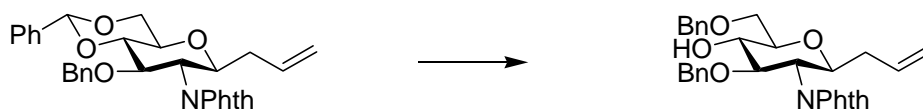
Potassium fluoroacetate (**5**)

Ethyl fluoroacetate, was synthesized as described in literature.¹ Ethyl fluoroacetate (0.107 g, 1 mmol) was dissolved in absolute EtOH, cooled to 0°C and potassium hydroxide (56.1 mg, 1 mmol) was added. The reaction was stirred overnight after which petroleum ether was added. The solution was cooled to -20°C and filtered giving title compound **5** (66%, 77 mg, 0.66 mmol) as a white solid. ¹H-NMR (400 MHz, D₂O) δ 4.75 (d, $J = 48.3$, 2H). ¹³C-NMR (101 MHz, D₂O) δ ppm 79.5 (d, $J = 176$). FT-IR: ν_{max} (neat)/cm⁻¹ 3381, 1606, 1423, 1398, 1337, 1009. Mp: 189°C (start of decomposition).



2-(2,6-dimethylbenzoyloxy)acetic acid (7)

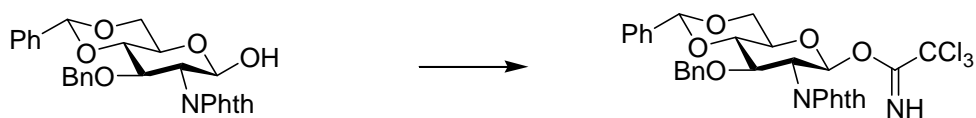
Tert-butyl bromoacetate (0.437 ml, 3 mmol), dimethylbenzoic acid (0.496 g, 3.3 mmol) and potassium fluoride (0.261 g, 4.5 mmol) were dissolved in DMF. After overnight stirring, Et₂O was added after which the solution was extracted with NaHCO₃ (sat. aq.). The organic layer was dried, concentrated *in vacuo* and subsequently dissolved in TFA/H₂O (95/5). The solution was concentrated after 1h, coevaporated with toluene to remove residual traces of TFA and applied to silica gel chromatography (0→10% EA/Tol) giving title compound **7** (81%, 0.506 g, 2.43 mmol) as a white solid. ¹H-NMR (400 MHz, CDCl₃) δ ppm 7.22 (dd, *J* = 15.2 Hz, 7.6 Hz, 1H), 7.05 (d, *J* = 7.6 Hz), 4.89 (s, 2H), 2.37 (s, 6H). ¹³C-NMR (101 MHz, CDCl₃) δ ppm 173.7, 169.1, 135.7, 132.1, 129.9, 127.7, 60.4, 19.8. FT-IR: ν_{max} (neat)/cm⁻¹ 2955.7, 1720.7, 1597.4, 1462.9, 1424.3, 1306.1, 1281.6, 1240.1, 1165.5, 1113.7, 1082.7, 1029.1, 1003.3. Mp: 128.7-130.4°C.



3-C-(3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl) propene (26)

Known 3-*C*-(3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-1-propene² (0.512 g, 1 mmol) was coevaporated with toluene (3×) before being dissolved in freshly distilled CH₂Cl₂. Activated molsieves (4 Å) and triethylsilane (0.533 mL, 3.3 mmol) were added. The mixture was stirred at room temperature for 15 min, cooled to -78°C followed by the addition of

trifluoromethanesulfonic acid (0.265 mL, 3 mmol). TLC-analysis showed complete conversion after 45 min after which the reaction was quenched with MeOH/Et₃N, extracted with NaHCO₃ and concentrated. Silica gel purification (0→30% EA/PE) afforded acceptor **26** (70%, 0.359 g, 0.70 mmol) as an oil. ¹H-NMR (400 MHz, CDCl₃) δ ppm 7.80-7.75 (m, 1H), 7.70-7.63 (m, 3H), 7.38-7.23 (m, 5H), 7.05-6.98 (m, 2H), 6.95-6.90 (m, 3H), 5.72 (dt, *J* = 16.9, 16.8, 7.0 Hz, 1H), 4.89 (d, *J* = 17.2 Hz, 1H), 4.83 (d, *J* = 10.2 Hz, 1H), 4.76 (d, *J* = 12.2 Hz, 1H), 4.64 (d, *J* = 12.1 Hz, 1H), 4.58 (d, *J* = 12.1 Hz, 1H), 4.52 (d, *J* = 12.2 Hz, 1H), 4.30-4.18 (m, 2H), 4.08 (t, *J* = 10.0, 10.0 Hz, 1H), 3.82-3.71 (m, 3H), 3.61 (td, *J* = 9.1, 4.4, 4.4 Hz, 1H), 3.04 (s, 1H), 2.27-2.13 (m, 2H). ¹³C-NMR (101 MHz, CDCl₃) δ ppm 168.1, 167.8, 138.2, 137.7, 133.8, 133.7, 133.3, 131.5, 131.4, 128.4, 128.0, 127.70, 127.65, 127.2, 123.2, 123.1, 116.9, 79.6, 77.6, 74.4, 74.2, 73.6, 70.6, 55.3, 36.9. FT-IR: ν_{max} (neat)/cm⁻¹ 3473.9, 2869.8, 1774.6, 1709.7, 1383.9, 1201.4, 1076.3, 913.3. ESI/MS: *m/z* = 514.13 (M+H)⁺. HRMS: (M + Na⁺) calc. for C₃₁H₃₁NO₆ 536.20436, found 536.20397.

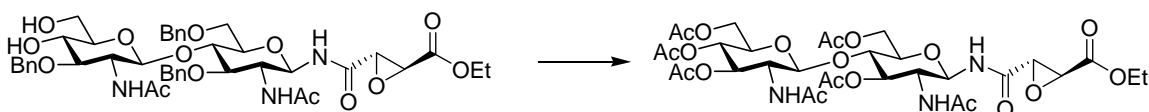


Imidate (**39**)

3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranose³ (2.88 g, 5.9 mmol) was dissolved in anhydrous CH₂Cl₂. K₂CO₃ (3.017 g, 21.83 mmol) and trichloroacetonitrile (2.97 mL, 29.5 mmol) were added. After overnight stirring, the mixture was filtered, concentrated in vacuo and purified by silicagel column chromatography. ¹H-NMR (400 MHz, CDCl₃) δ ppm 8.58 (s, 1H), 7.78-7.66 (m, 4H),

7.55-7.51 (m, 2H), 7.44-7.37 (m, 3H), 7.03-6.99 (m, 2H), 6.95-6.86 (m, 3H), 6.50 (d, $J = 8.3$ Hz, 1H), 5.64 (s, 1H), 4.82 (d, $J = 12.4$ Hz, 1H), 4.60-4.45 (m, 4H), 3.94-3.80 (m, 3H). ^{13}C -NMR (101 MHz, CDCl_3) δ ppm 167.4, 160.8, 137.7, 137.1, 134.0, 131.4, 129.1, 128.3, 128.0, 127.4, 126.1, 123.4, 101.4, 94.3, 82.6, 74.4, 74.2, 68.5, 66.9, 54.7. FT-IR: ν_{max} (neat)/ cm^{-1} 1776.4, 1714.1, 1677.8, 1453.7, 1383.8, 1294.2, 1112.9, 1053.4, 1023.1.

The imidate was hydrolyzed during mass-spectrometry. HRMS: (M-imidate + Na^+) calc. for $\text{C}_{31}\text{H}_{31}\text{NO}_6$ 510.15232, found 510.15212.



(2*S*,3*S*)-3-*N*-(*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy- β -D-glucopyranosyl)-(1 \rightarrow 4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy- β -D-glucopyranosylcarbamoyl) oxirane-2-carboxylic acid ethyl ester (49**)**

Known epoxide² (166 mg, 0.199 mmol) was dissolved in EtOH/Dioxane (2/1 v/v, 5 mL). $\text{Pd}(\text{OH})_2$ on charcoal (25 mg) was added followed by stirring under H_2 atm for 1h. The solution was filtered, concentrated, redissolved in pyridine (5 mL) and cooled to 0°C before being treated with Ac_2O (3 mL). The mixture was stirred overnight, concentrated, redissolved in CH_2Cl_2 , washed with H_2O , dried (Na_2SO_4) and concentrated in vacuo. RP-HPLC (linear gradient 22 \rightarrow 32% B in 10.00 min) gave title compound **49** (26%, 39.69 mg, 51.2 μmol) as a white solid. ^1H -NMR (400 MHz, $\text{CDCl}_3/\text{MeOD}$) δ ppm 5.27 (dd, $J = 10.2, 9.5$ Hz, 1H), 5.09 (dd, $J = 10.5, 8.4$ Hz, 1H), 5.07-4.98 (m, 2H), 4.67 (d, $J = 8.3$ Hz, 1H), 4.43 (dd, $J = 12.1, 1.4$ Hz, 1H), 4.42 (dd, $J = 12.4, 4.4$ Hz, 1H), 4.28 (m, 2H), 4.07 (dd, $J = 11.9, 4.7$ Hz, 1H), 4.05-3.98 (m, 2H), 3.78-3.69 (m, 4H), 3.62 (d, $J = 1.7$

Hz, 1H), 3.48 (d, $J = 1.7$ Hz, 1H), 2.14 (s, 3H), 2.09 (s, 3H), 2.07 (s, 3H), 2.02 (s, 6H), 1.94 (s, 3H), 1.91 (s, 3H), 1.32 (t, $J = 7.1, 7.1$ Hz, 3H). ^{13}C -NMR (101 MHz, $\text{CDCl}_3/\text{MeOD}$) δ ppm 172.3, 171.6, 171.2, 170.7, 170.6, 170.5, 169.6, 167.0, 166.6, 100.2, 78.2, 75.2, 74.2, 72.6, 71.9, 71.2, 68.1, 62.14, 62.09, 61.5, 54.4, 52.9, 52.4, 51.8, 22.11, 22.08, 20.3, 20.2, 20.1, 20.04, 19.97, 13.5. FT-IR: ν_{max} (neat)/ cm^{-1} 3288.1, 1738.9, 1665.5, 1541.9, 1435.1, 1372.0, 1228.2, 1046.1, 902.1, 600.8. LC/MS: R_t 4.700 min; linear gradient 10 \rightarrow 90% B in 13.5 min; ESI/MS: $m/z = 776.5$ ($\text{M}+\text{H}$) $^+$. HRMS: ($\text{M} + \text{H}^+$) calc. for $\text{C}_{32}\text{H}_{45}\text{N}_3\text{O}_{19}$ 776.27200, found 776.27207.

Competition assay

To 9 μL of a solution of yeast PNGase (11.1 ng/ μL) in PBS (pH = 7.2, 20 mM sodium phosphate, 150 mM NaCl, BSA (1 $\mu\text{g}/\mu\text{L}$) was added inhibitor (1 μL) and Bodipy-VAD-Fmk (1 μL , 0.5 μM final concentration). The resulting solution was incubated at 37°C for 1h, after which it was quenched by the addition of 4 \times SDS-PAGE sample buffer (4 μL) and boiling for 5 min. The proteins were separated on a 10% SDS-PAGE gel and subsequently the fluorescence was measured in the wet gel slabs by using the CY3/Tamra settings (λ_{ex} 532 λ_{em} 560) on a Typhoon Variable Mode Imager (Amersham Biosciences). The remaining fluorescence was quantified with ImageQuant followed by analysis with Graphpad Prism.

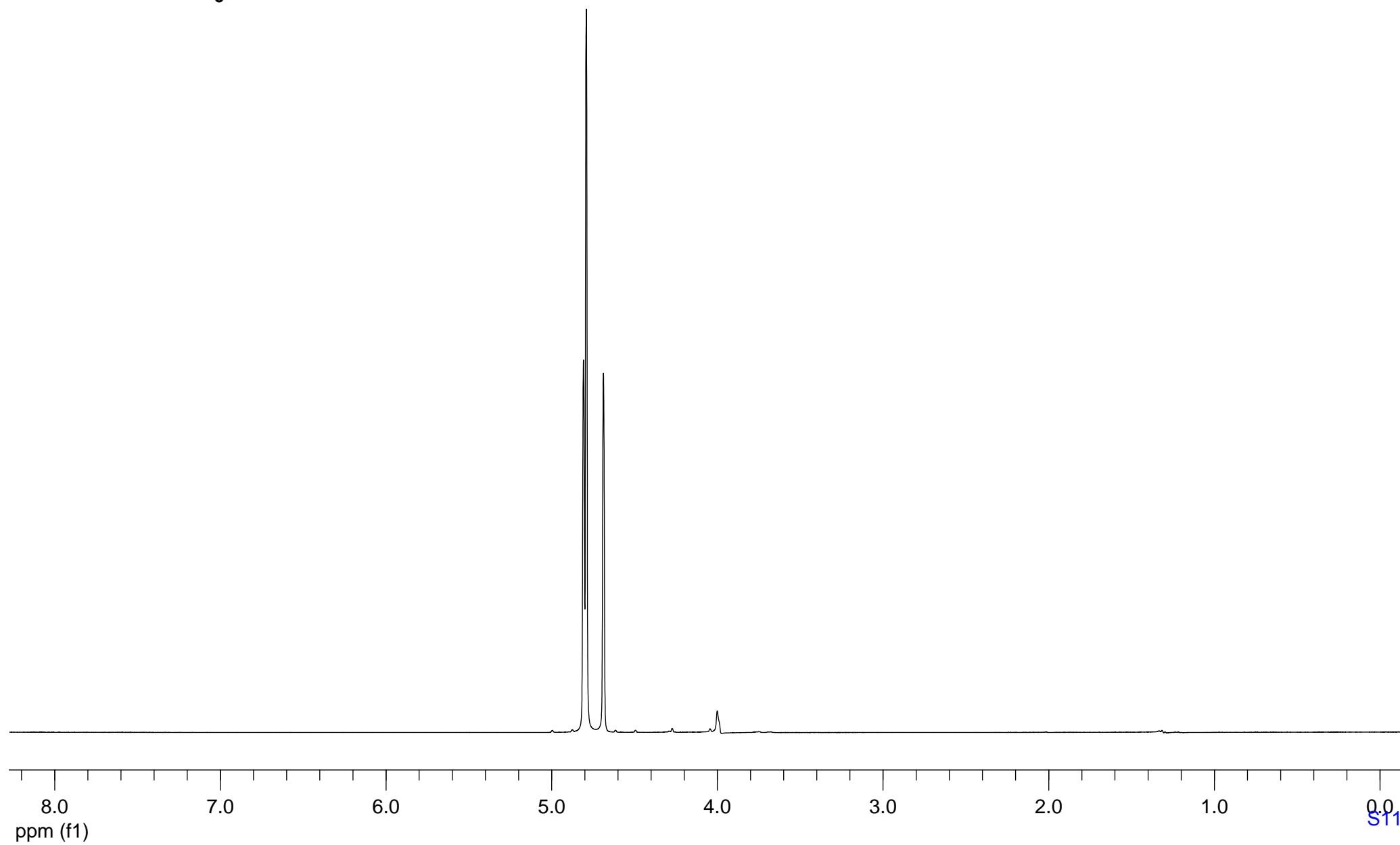
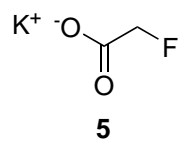
In vivo inhibition of human PNGase

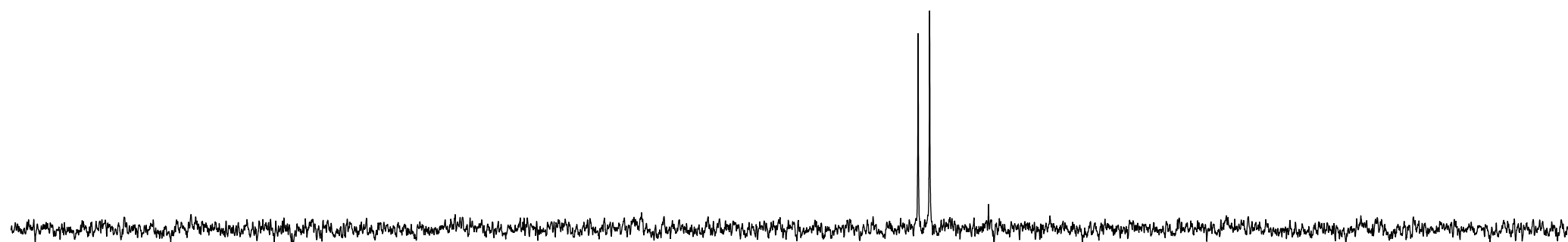
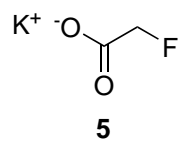
Control and US11-expressing U373 cells (1.5×10^6 cells per lane) were collected, washed with PBS and resuspended in Met $^-$ /Cys $^-$ medium (2 mL) containing 10% FCS and the

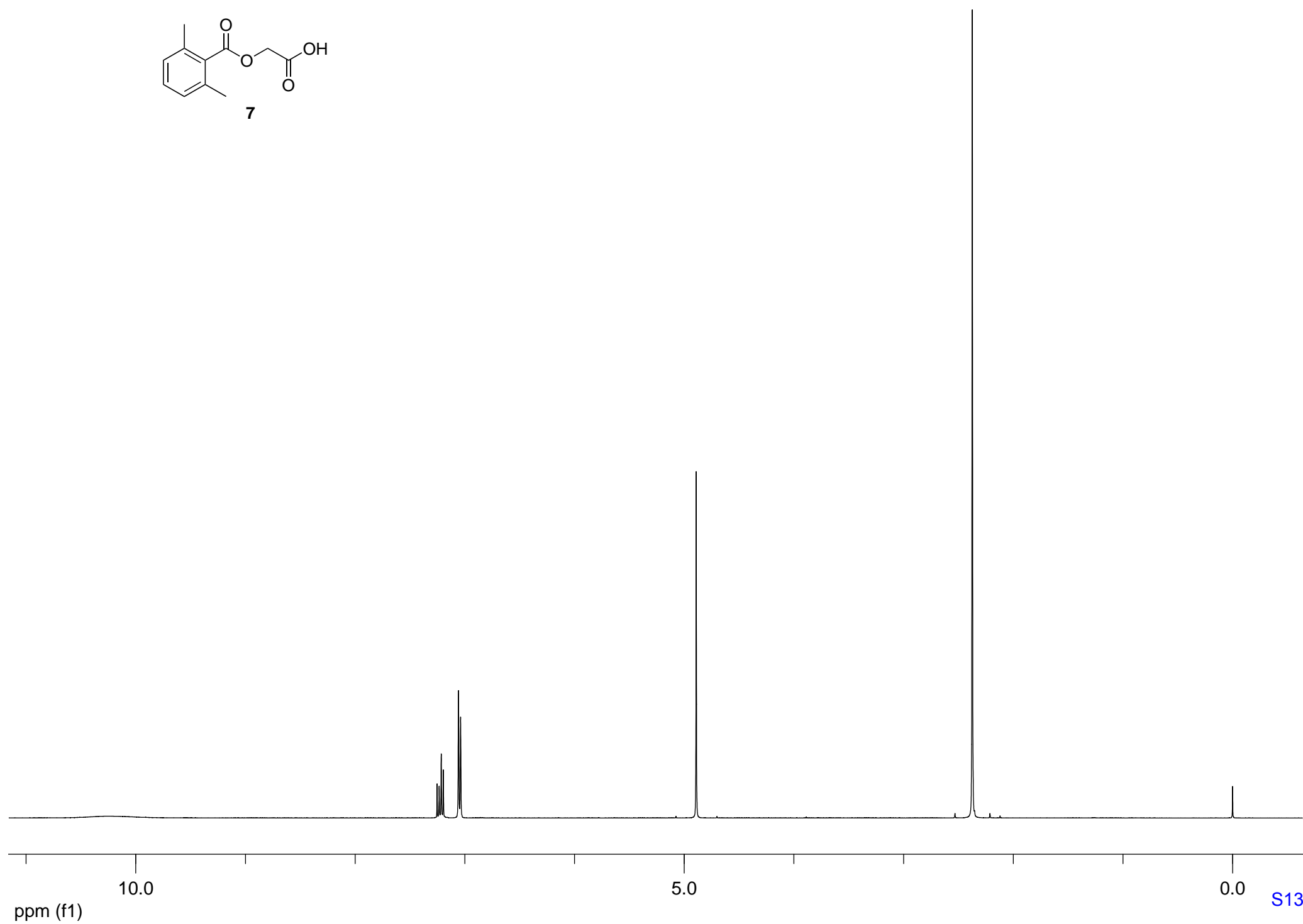
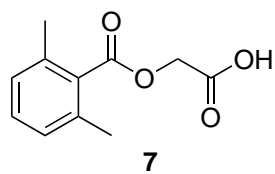
inhibitors ZL₃VS (1.25 μ M), ZVAD-Fmk, **2**, **49** (30 μ M and 100 μ M respectively) and NGT (50 μ M) as indicated. The cells were incubated for 45 min at 37°C, pelleted by centrifugation and resuspended in 250 μ L Met⁻/Cys⁻ medium, again in the presence of the inhibitors. [³⁵S]-labeled Cys/Met (5 μ L, 200 μ Ci/mL) was added and the cells were incubated for 30 minutes at 37°C. The cells were washed with PBS and subsequently lysed in 1 ml cold NP-40 buffer (0.5% NP-40, 50 mM Tris HCl pH 7.5, 5 mM MgCl₂, 10 μ M leupeptin, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride) for 45 min at 4°C. The lysed cells were centrifuged (16.000 g, 15 min) at 4°C in order to obtain post-nuclear lysates. These lysates were precleared twice by the addition of Protein G- and Protein A-sepharose beads (7.5 μ L, GE Healthcare), normal rabbit serum (1.5 μ L) and normal mouse serum (1.5 μ L), followed by incubation for 60 min at 4°C. Next, the class I heavy chains were immunoprecipitated. Specific antibodies (mAb HC10), Protein G- and Protein A-sepharose beads were added to the lysates and samples were incubated o/n at 4°C. Isolated immune complexes were washed thrice with NET buffer containing 0.1% SDS and denatured in reducing sample buffer (50 μ L). The samples were separated by SDS-PAGE and visualized by phosphoimaging.

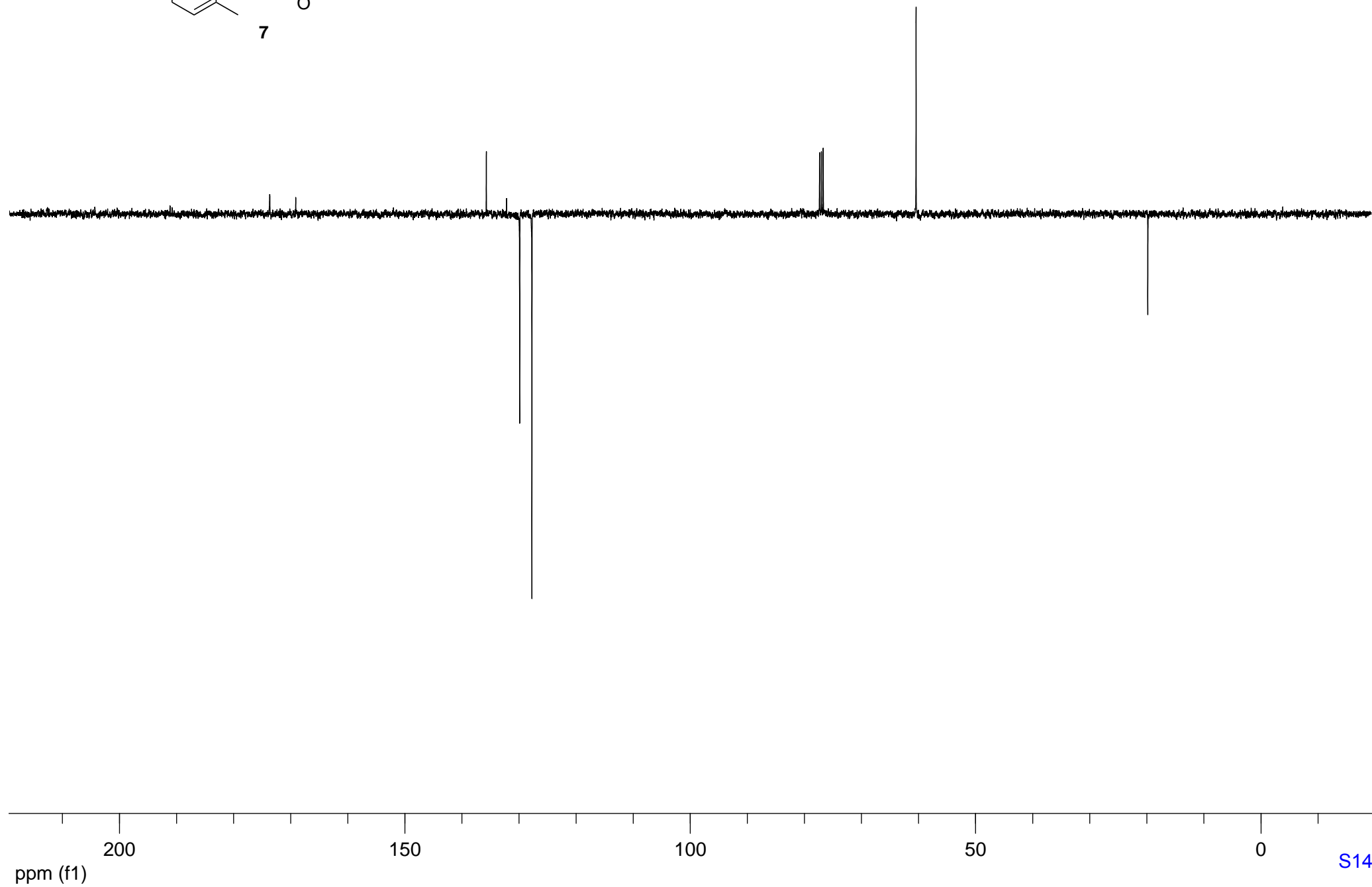
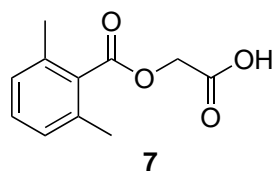
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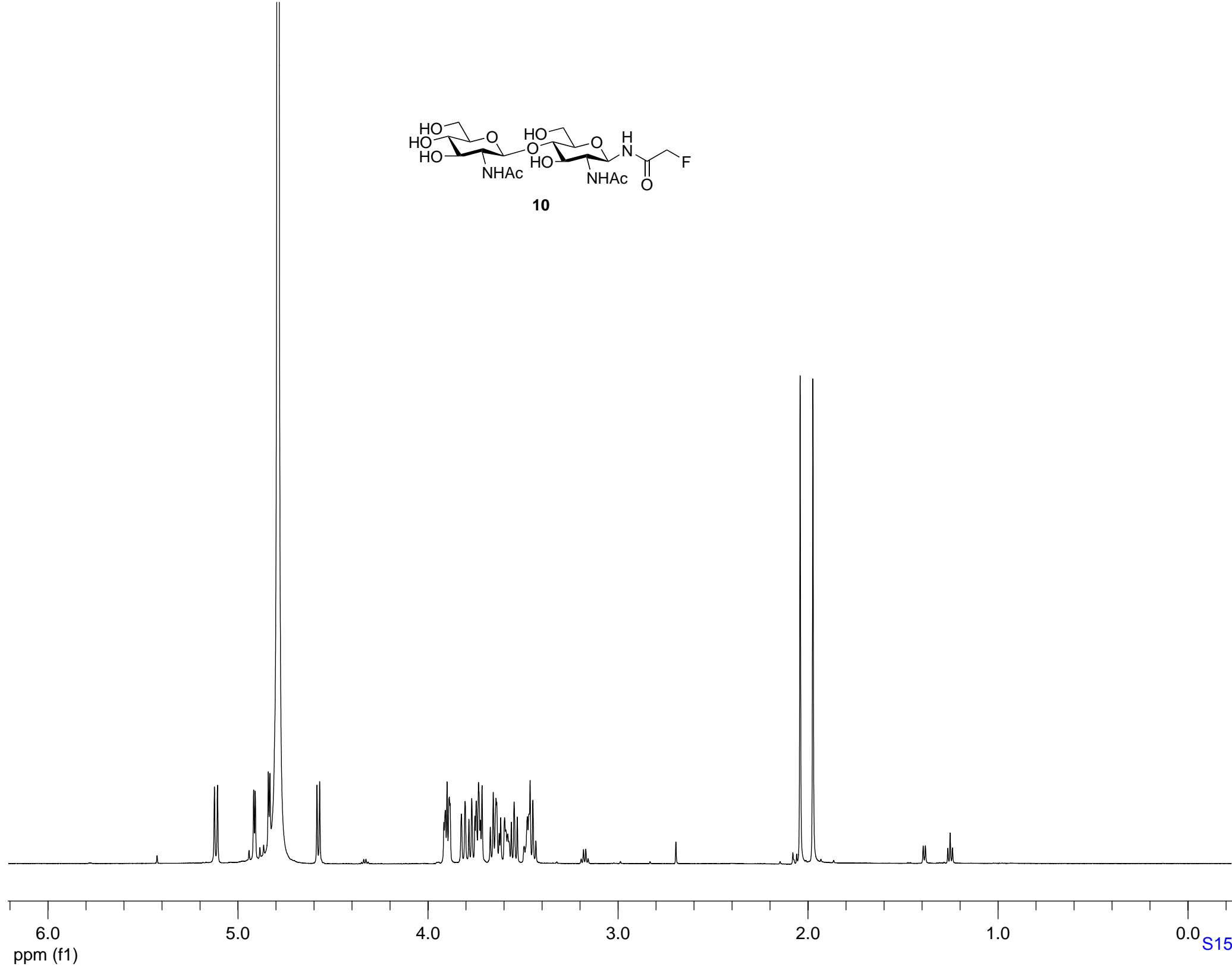
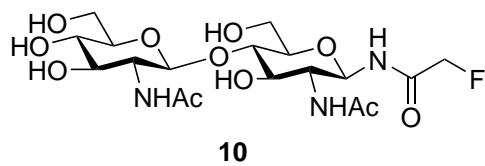
- (1) Pelter, A.; Kvicala, J. *Tetrahedron Lett.* **1997**, *38*, 4877-4880.
- (2) Witte, M. D.; Descals, C. V.; De Lavoie, S. V. P.; Florea, B. I.; Van der Marel, G. A.; Overkleeft, H. S. *Org. Biomol. Chem.* **2007**, *5*, 3690-3697.
- (3) Chiara, J. L.; García, Á.; Cristóbal-Lumbroso, G. *J. Org. Chem.* **2005**, *70*, 4142-4150

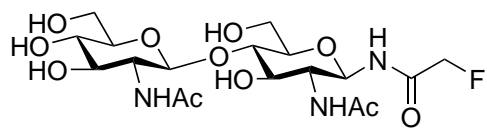








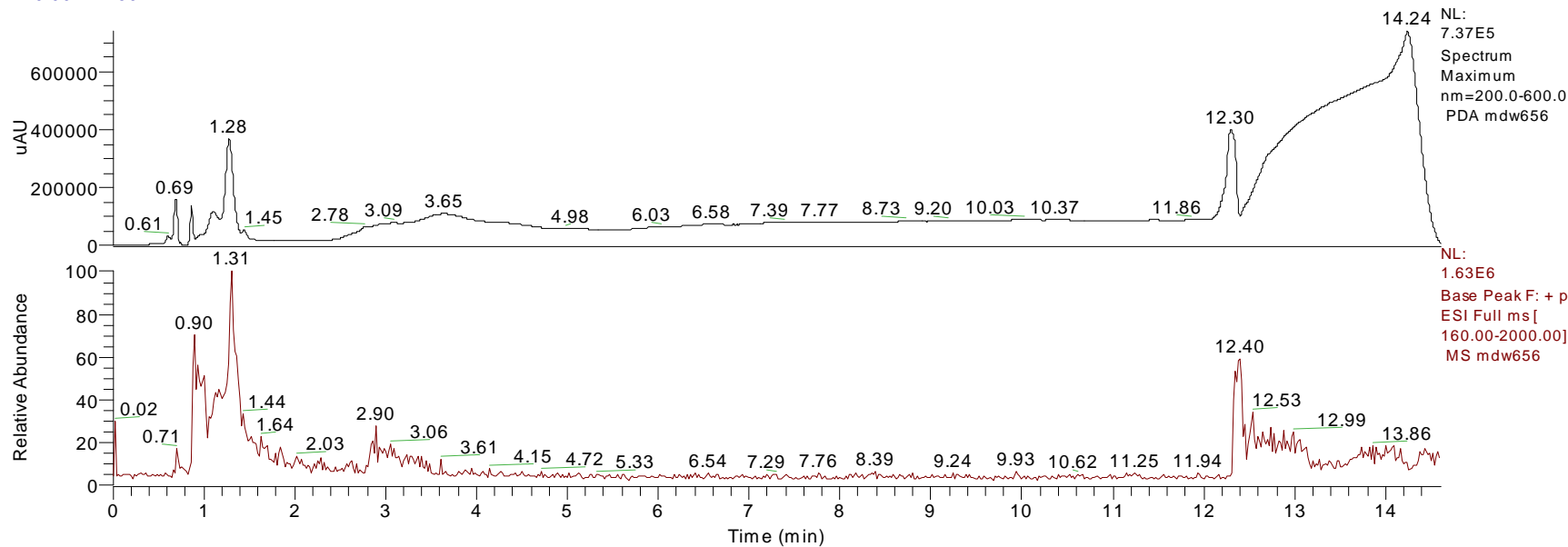




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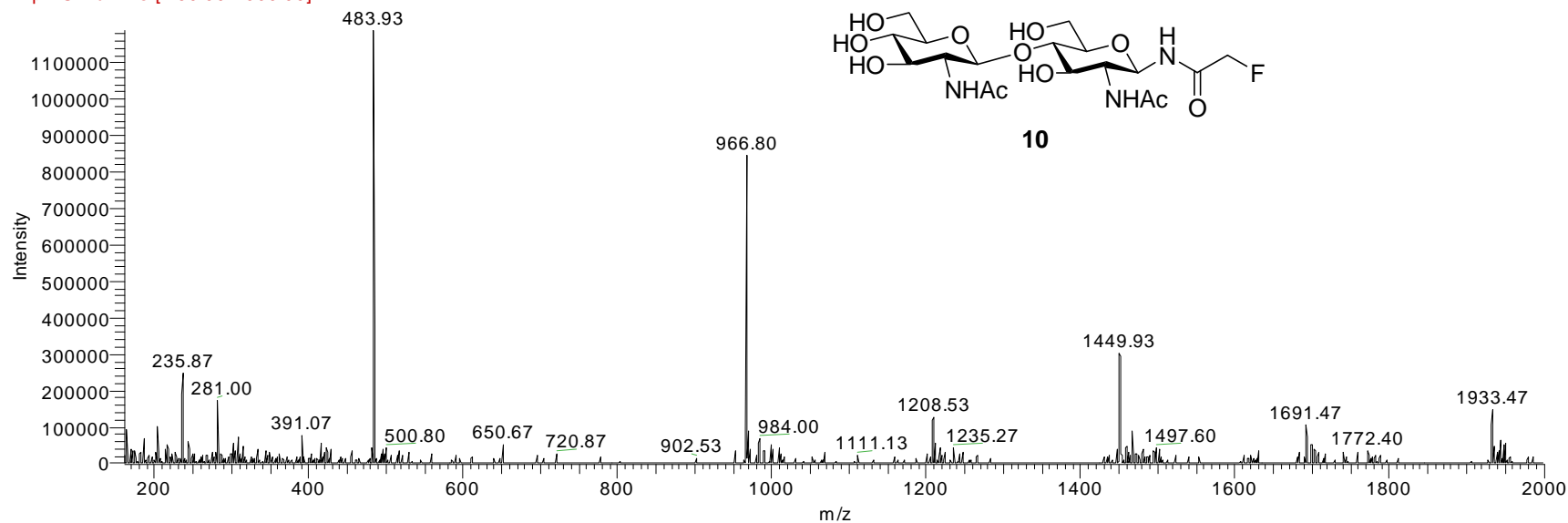


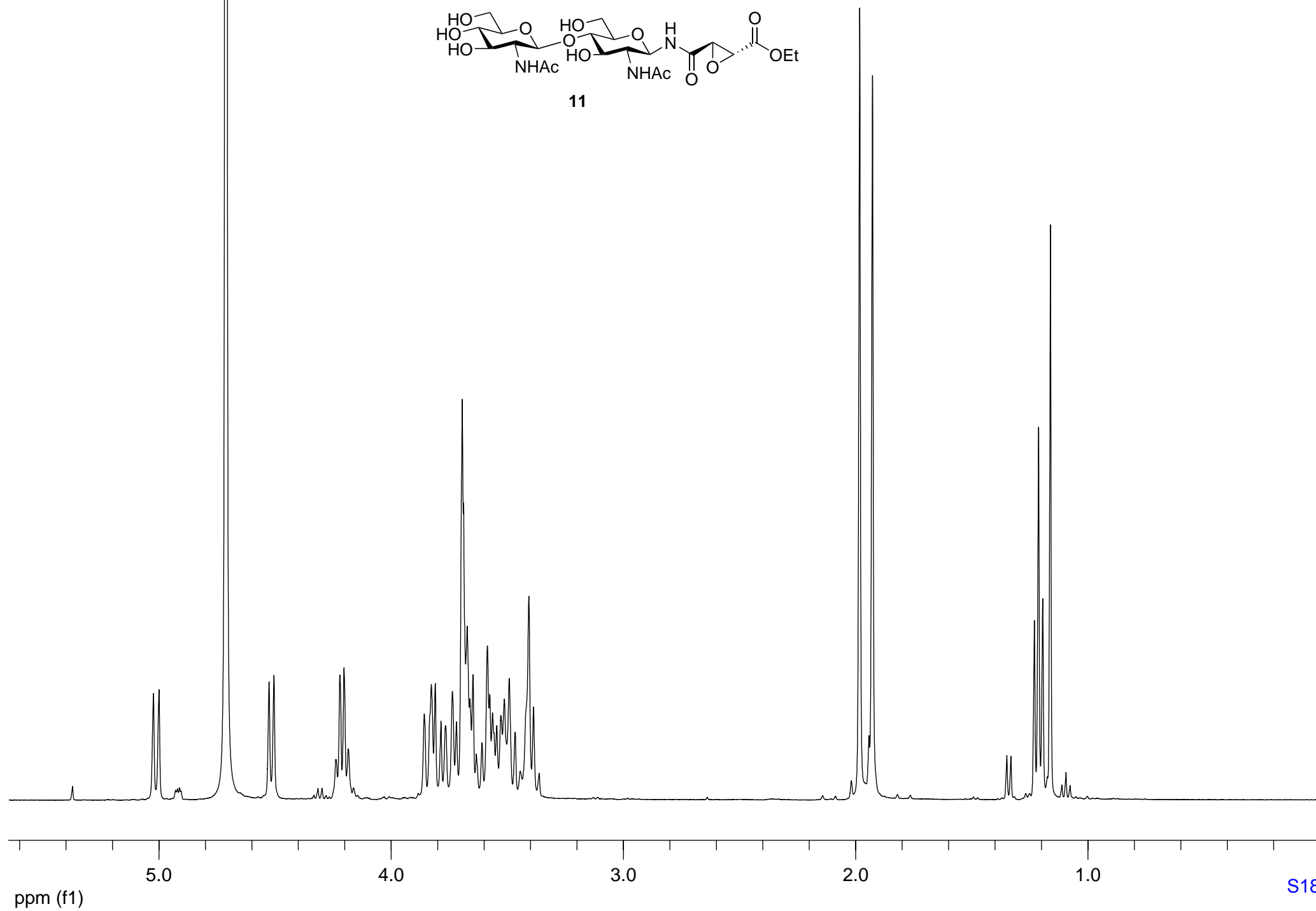
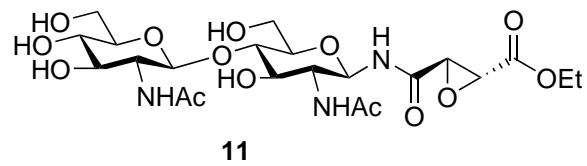
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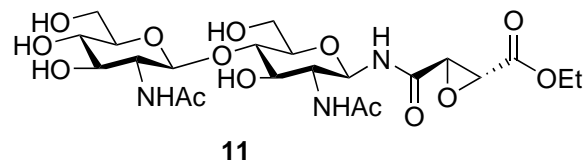


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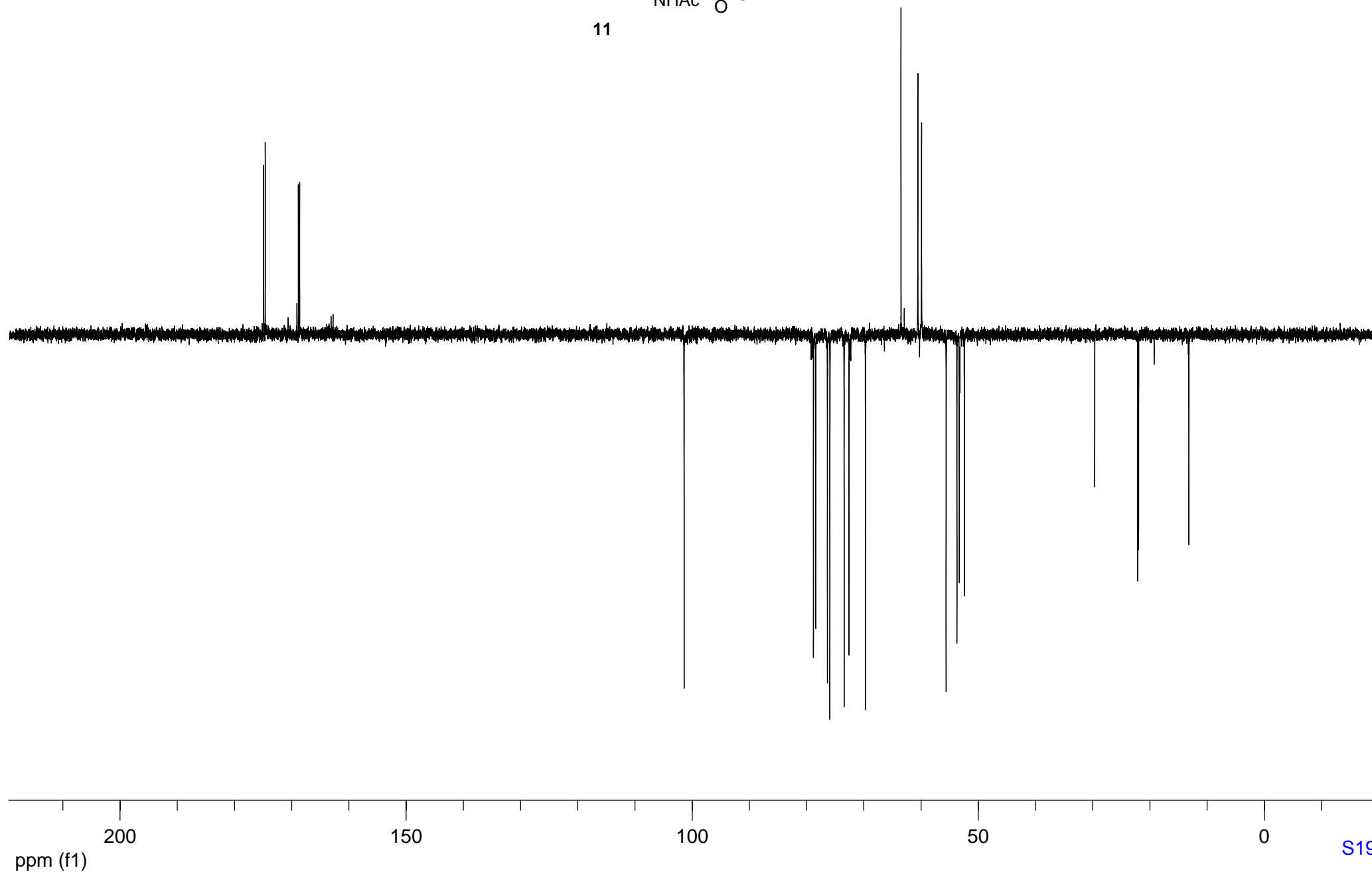
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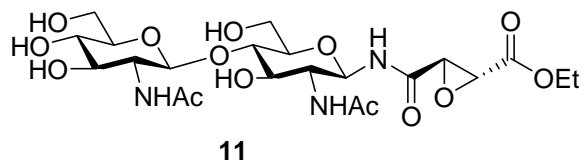
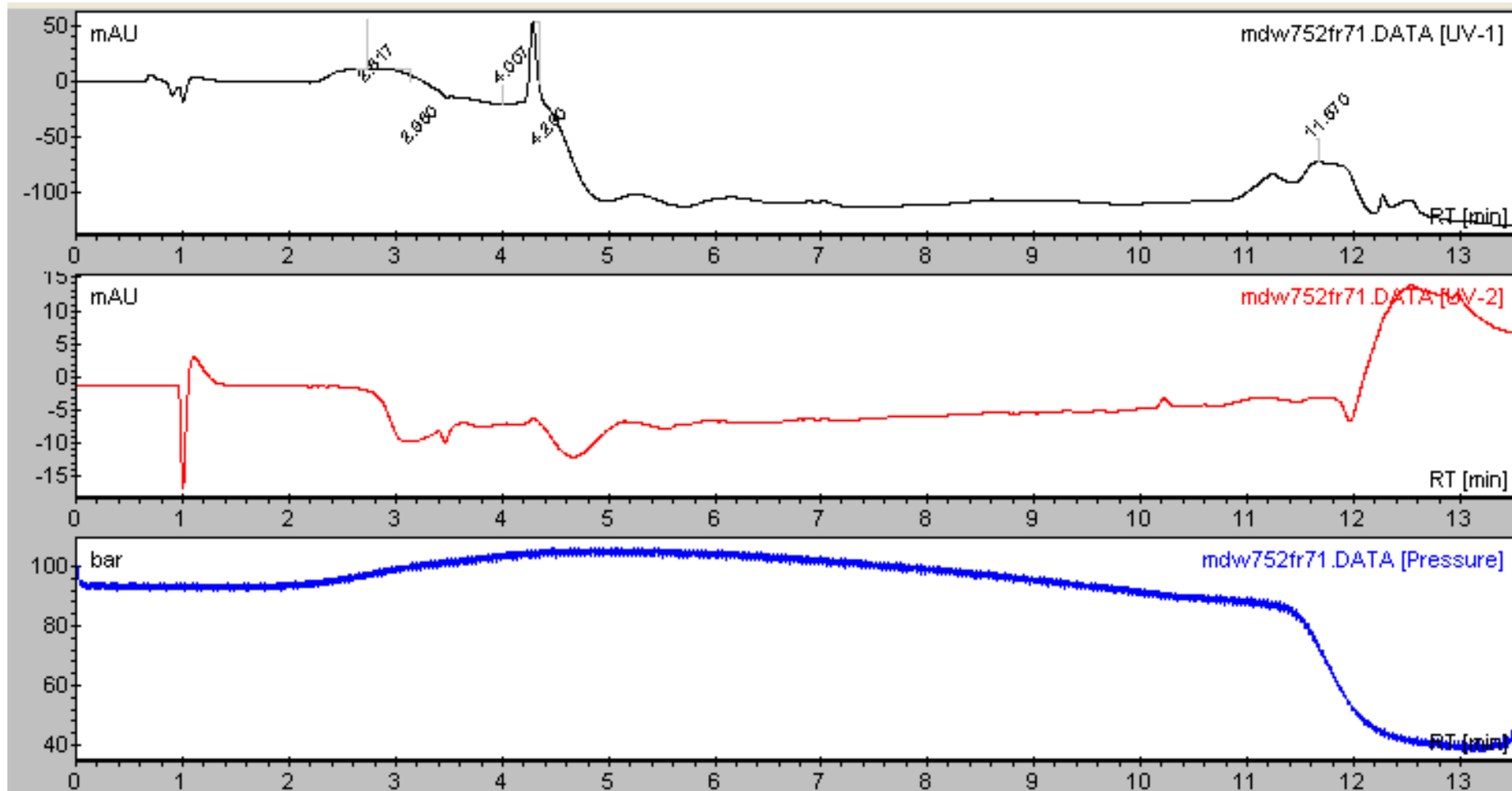


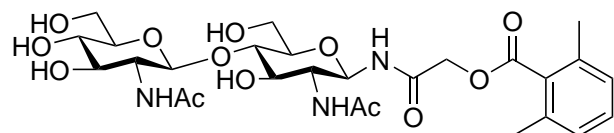




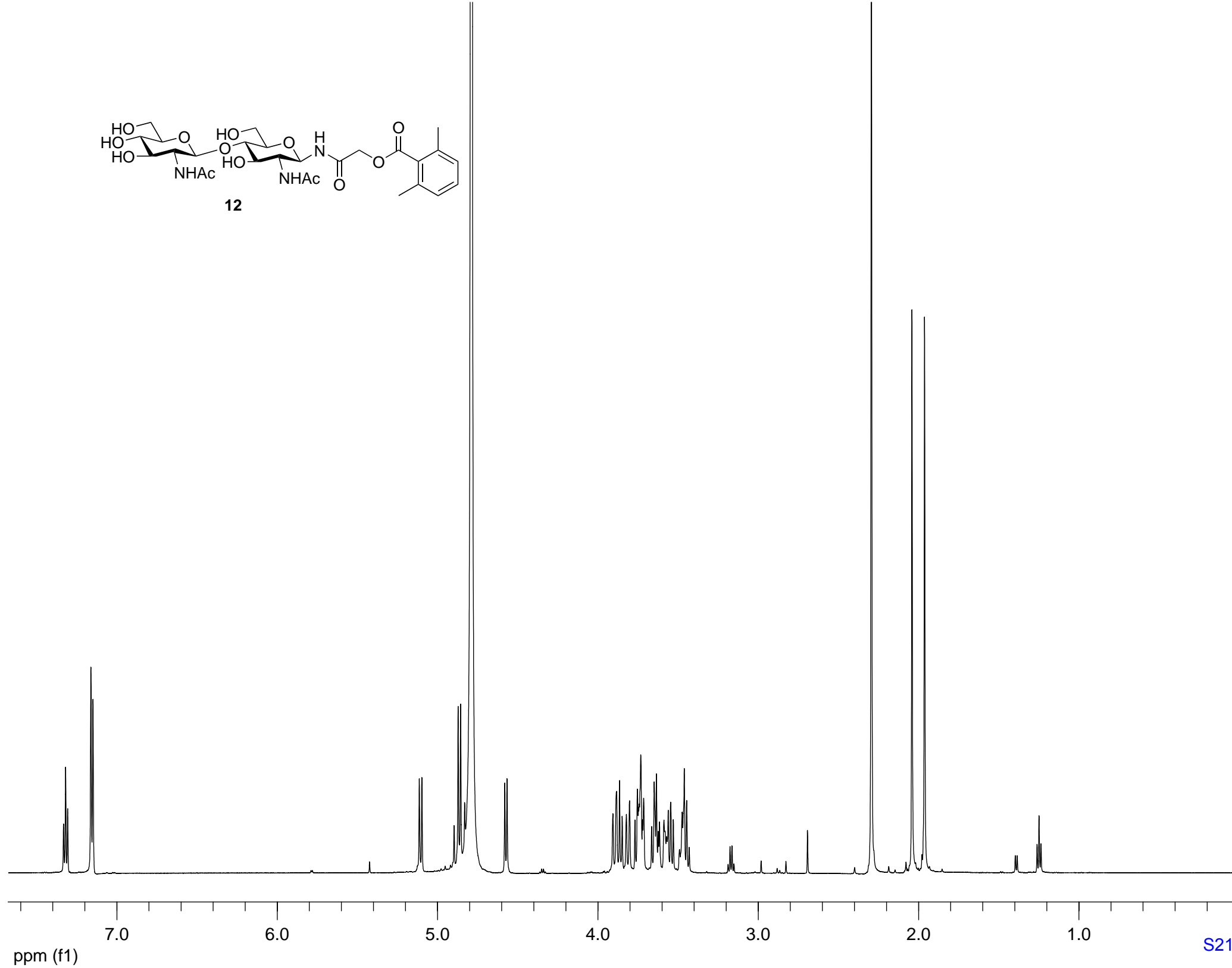
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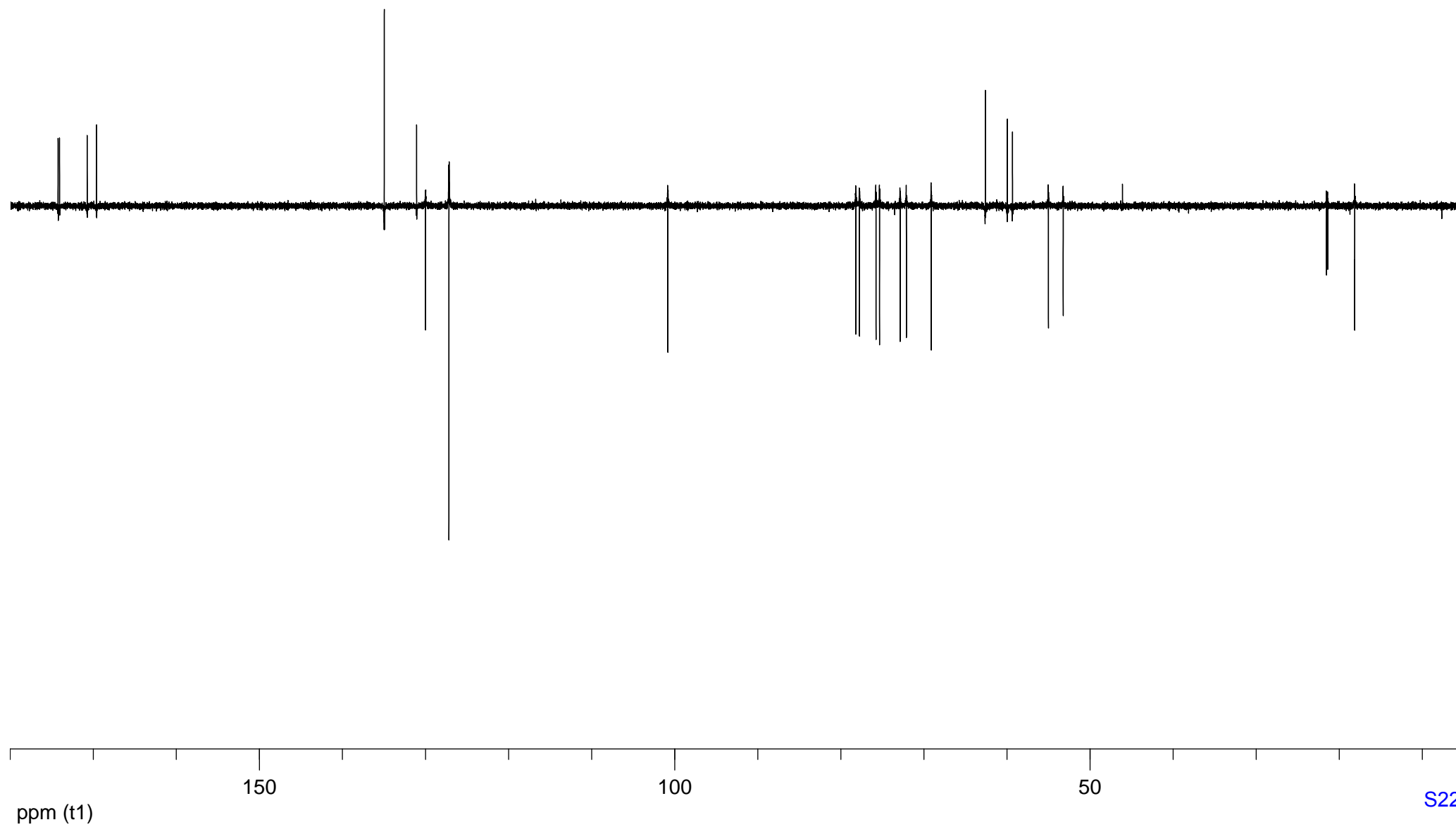
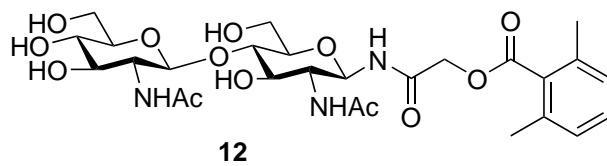


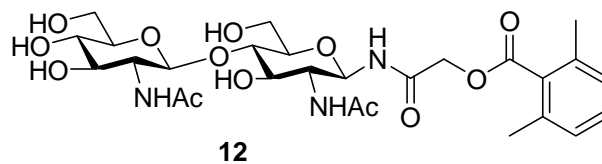
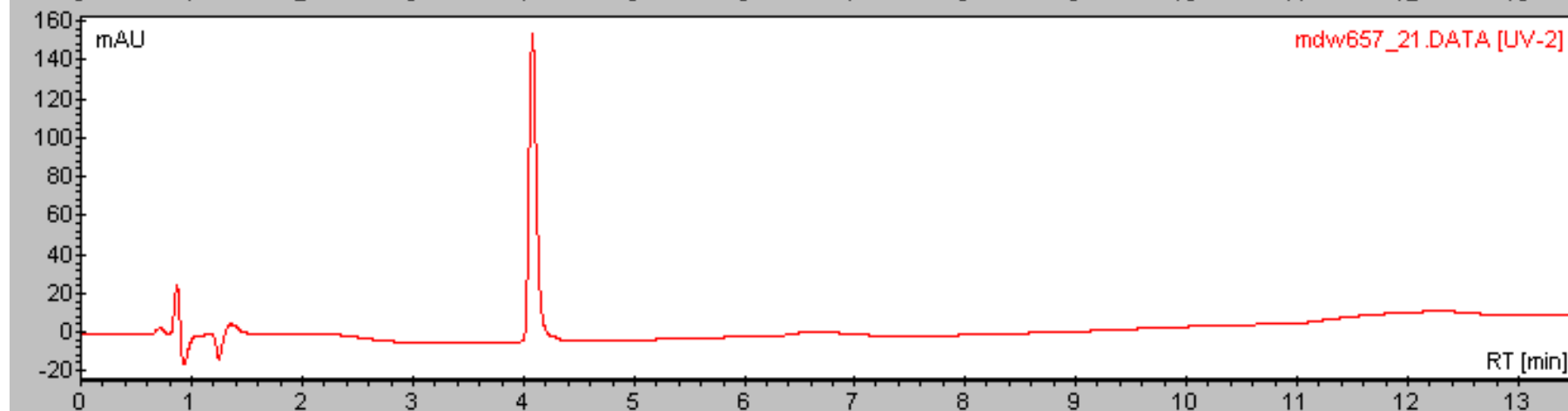
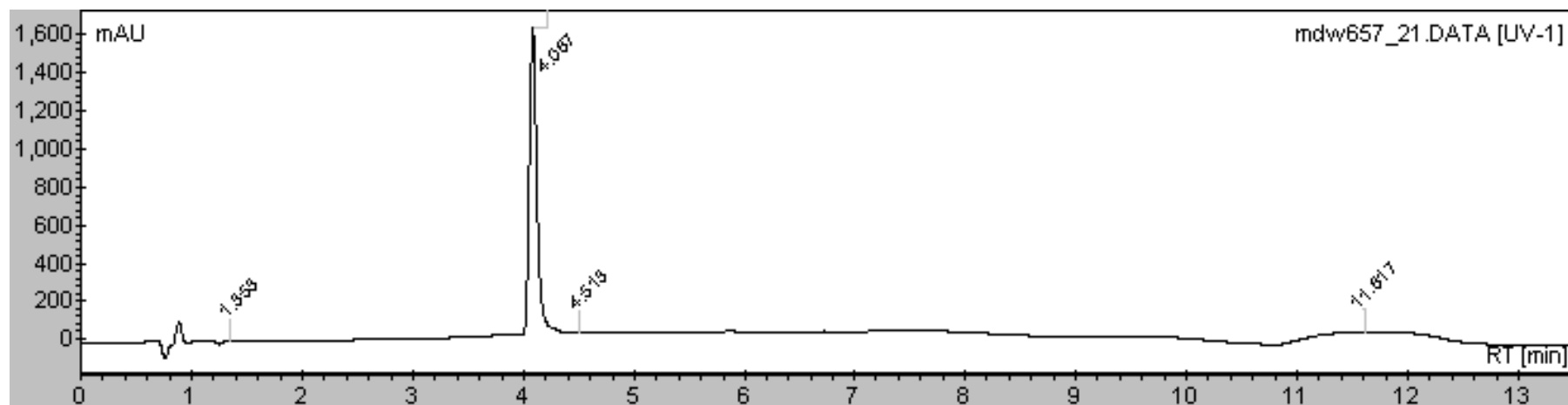


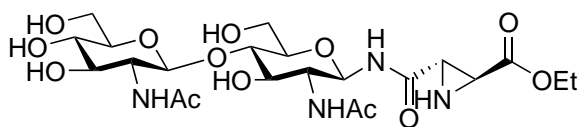


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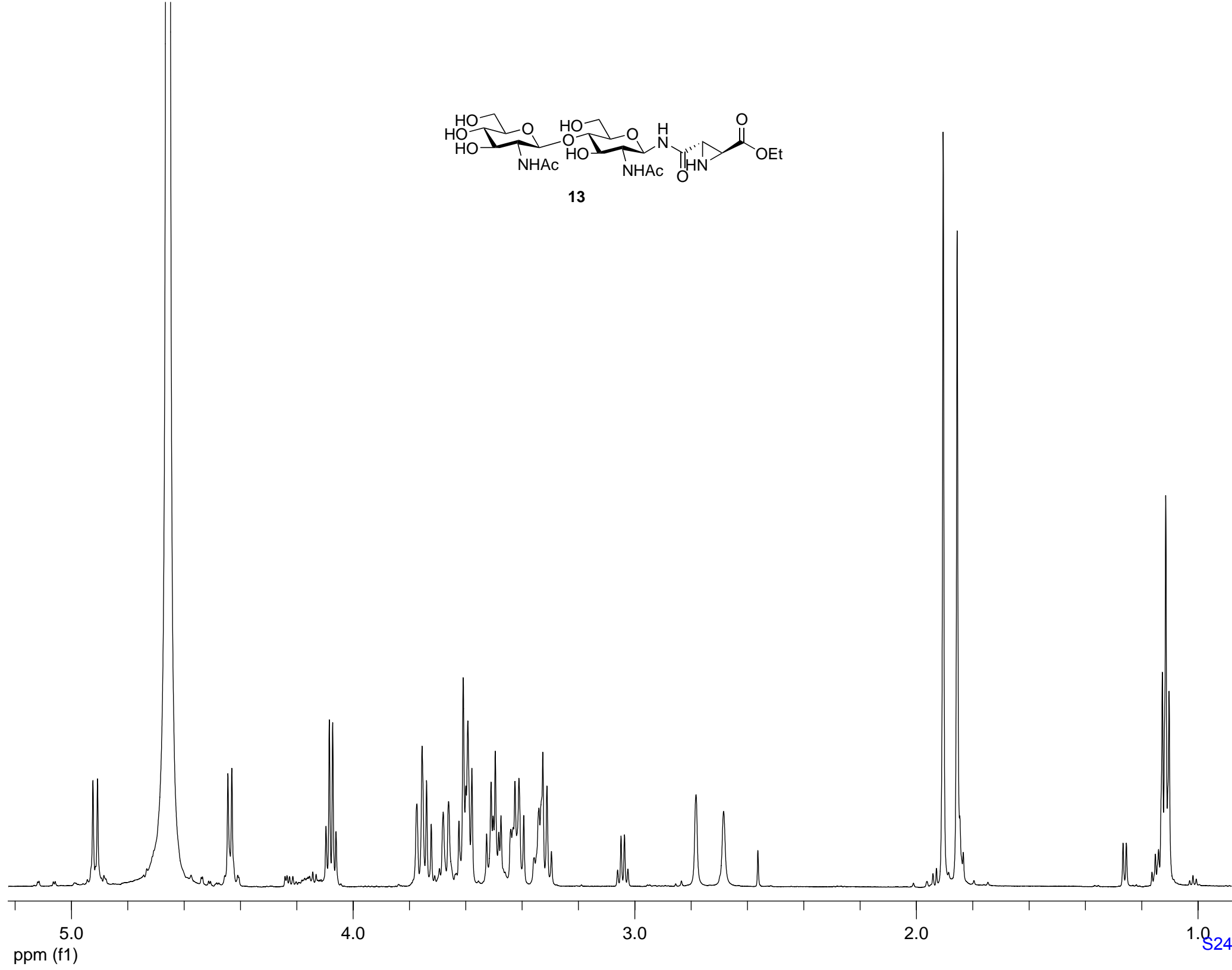


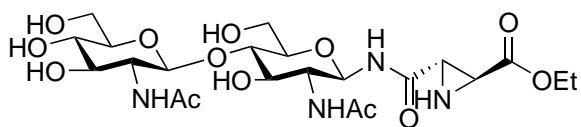




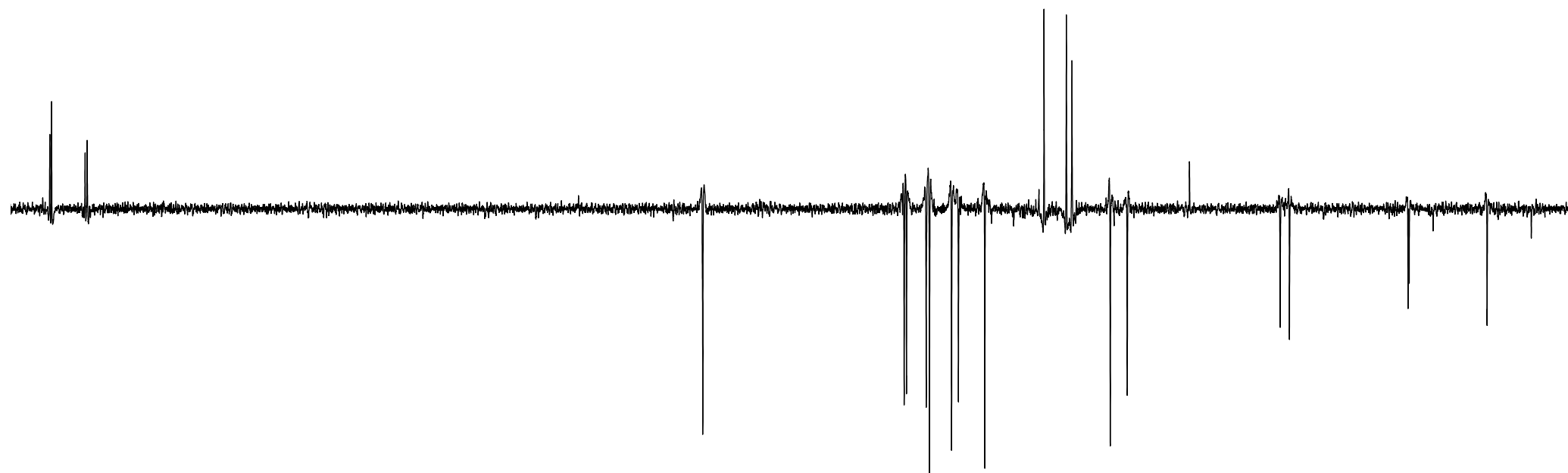


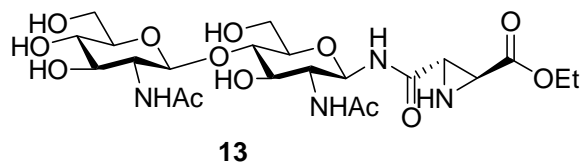
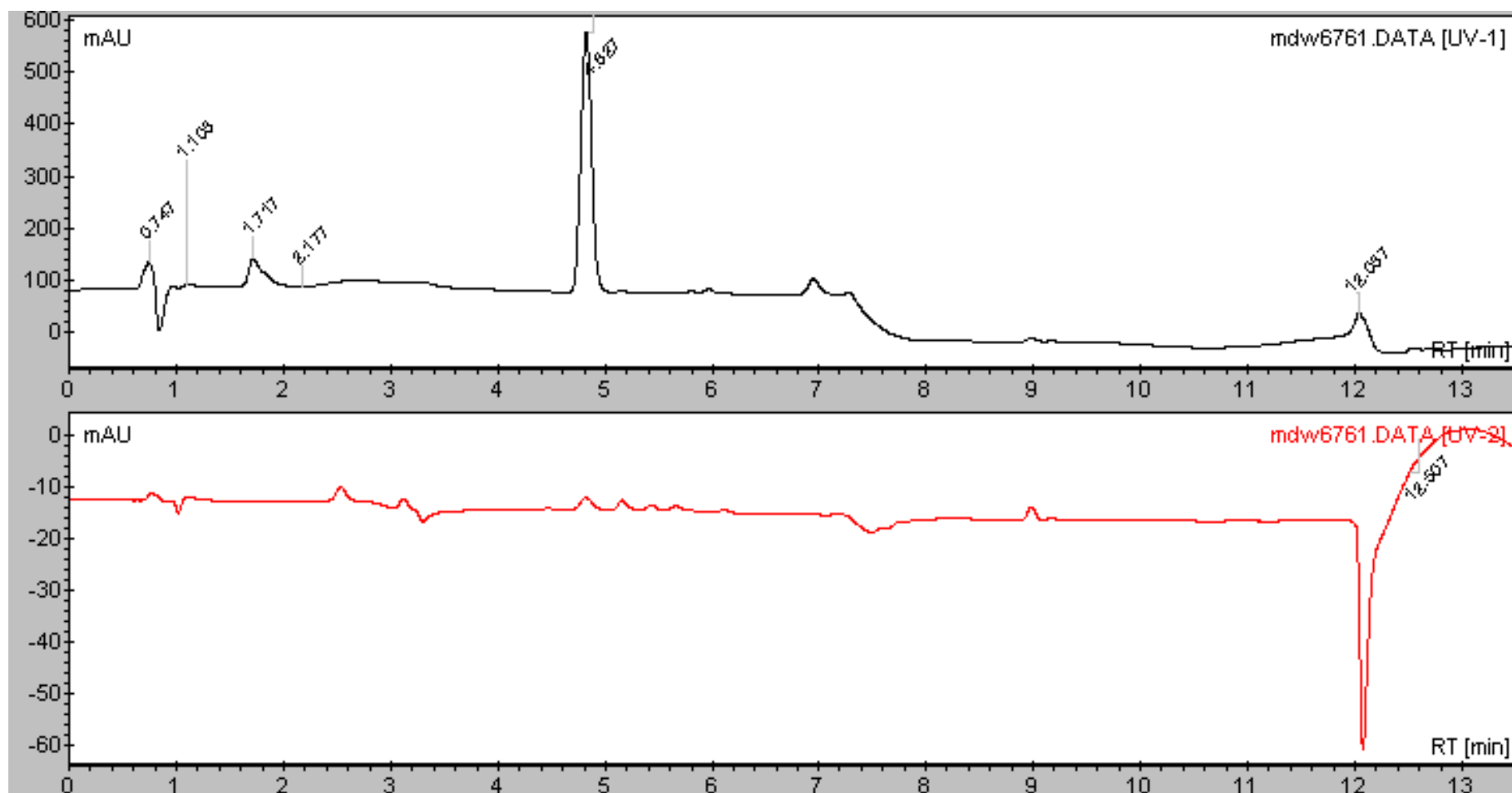
13

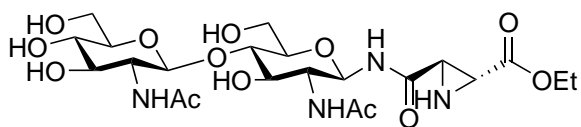




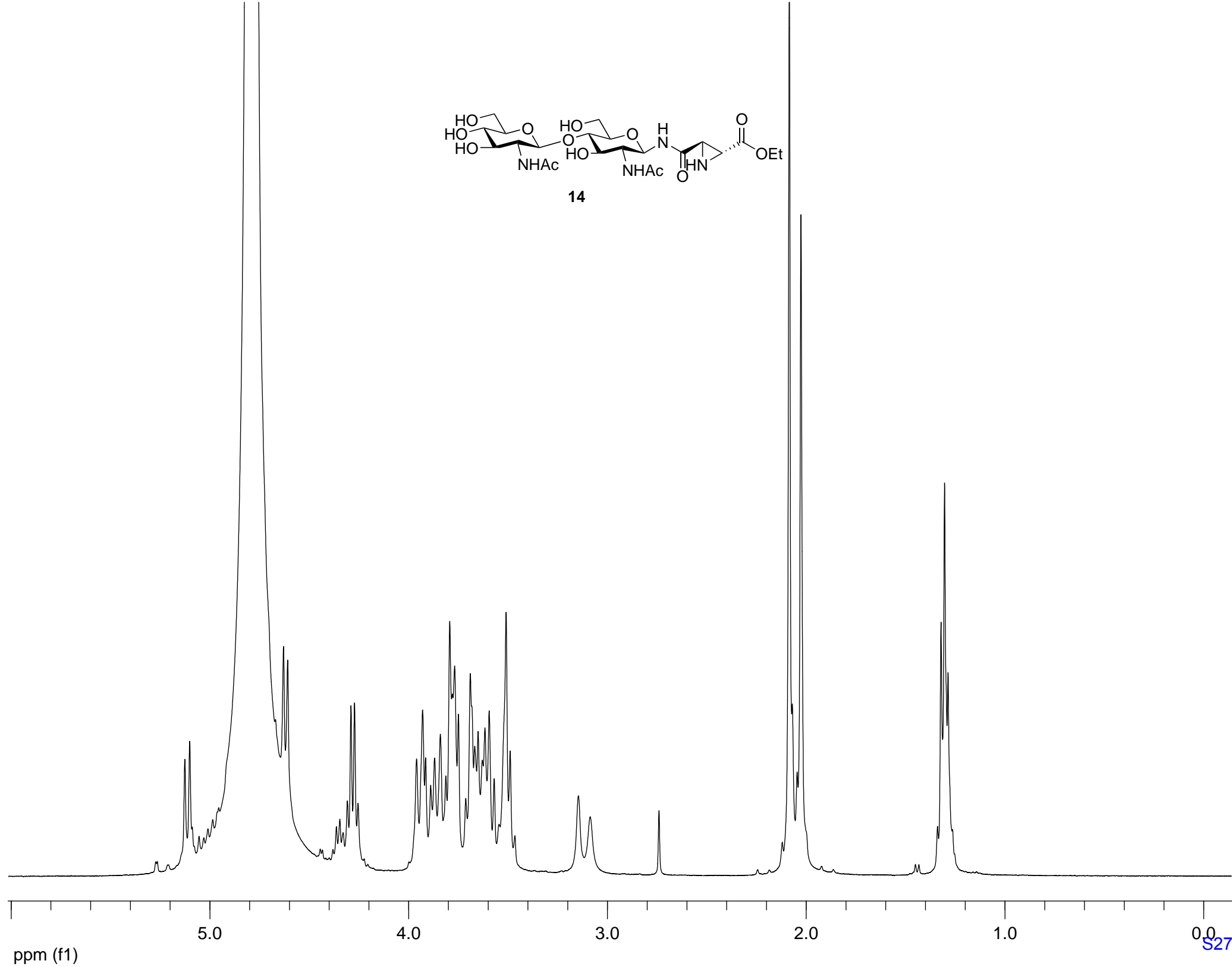
13

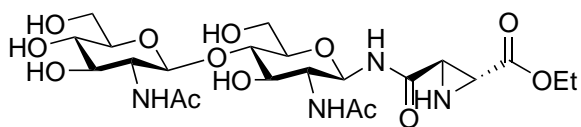




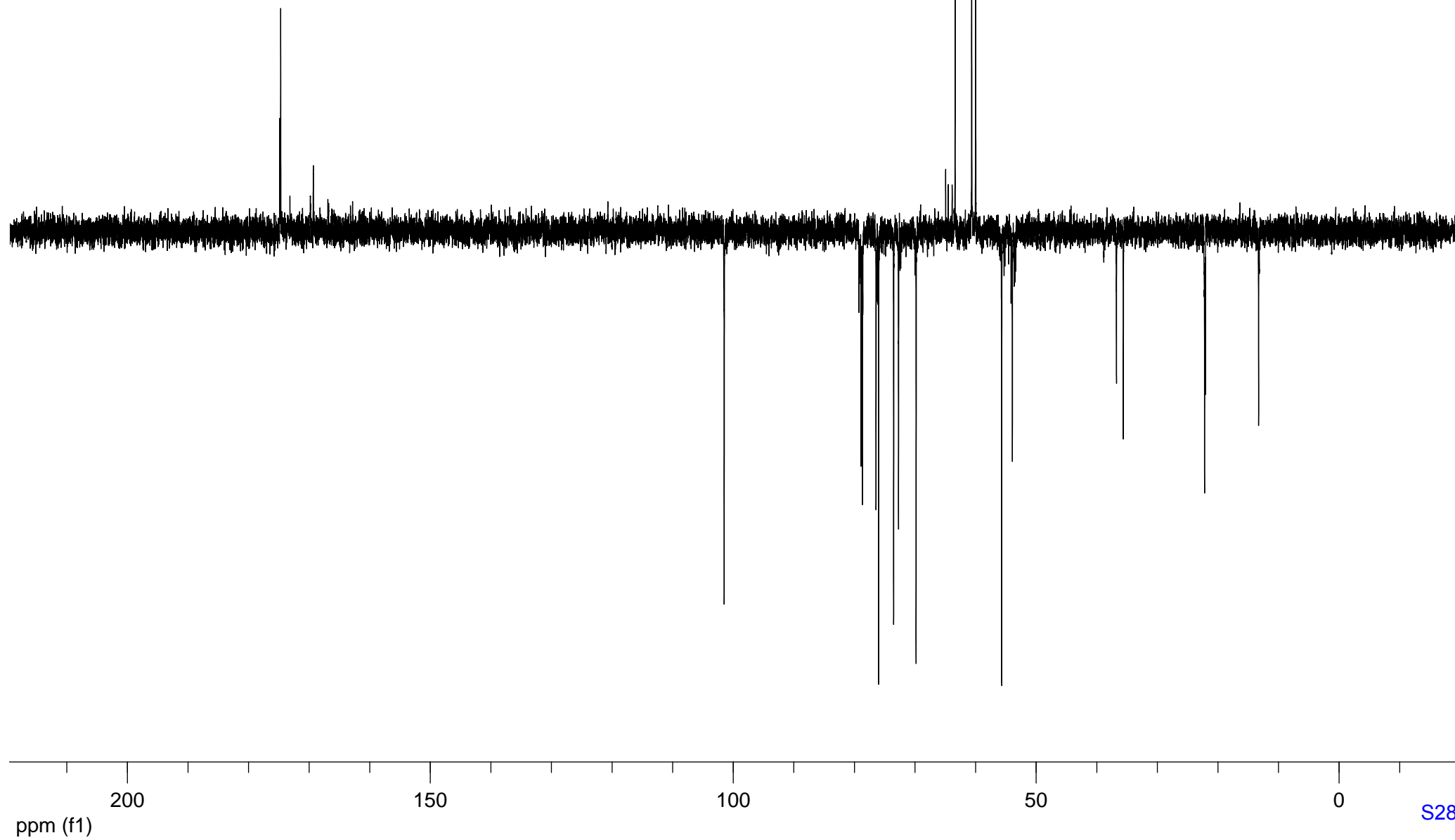


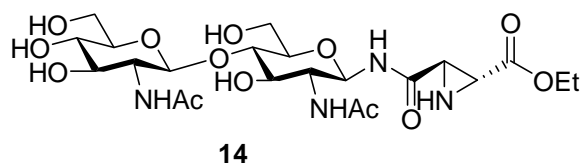
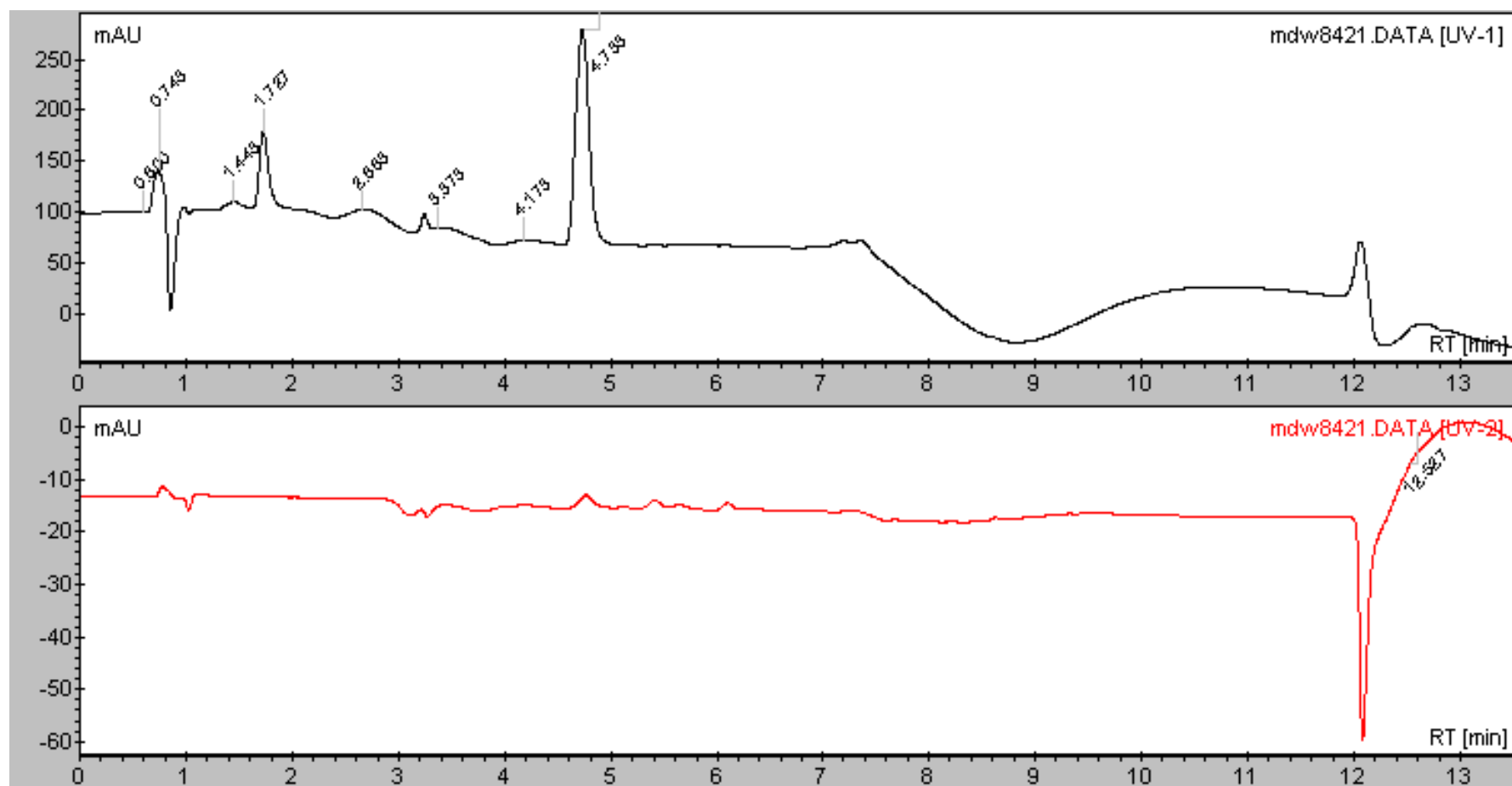
14

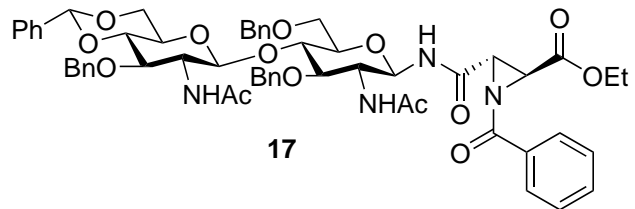




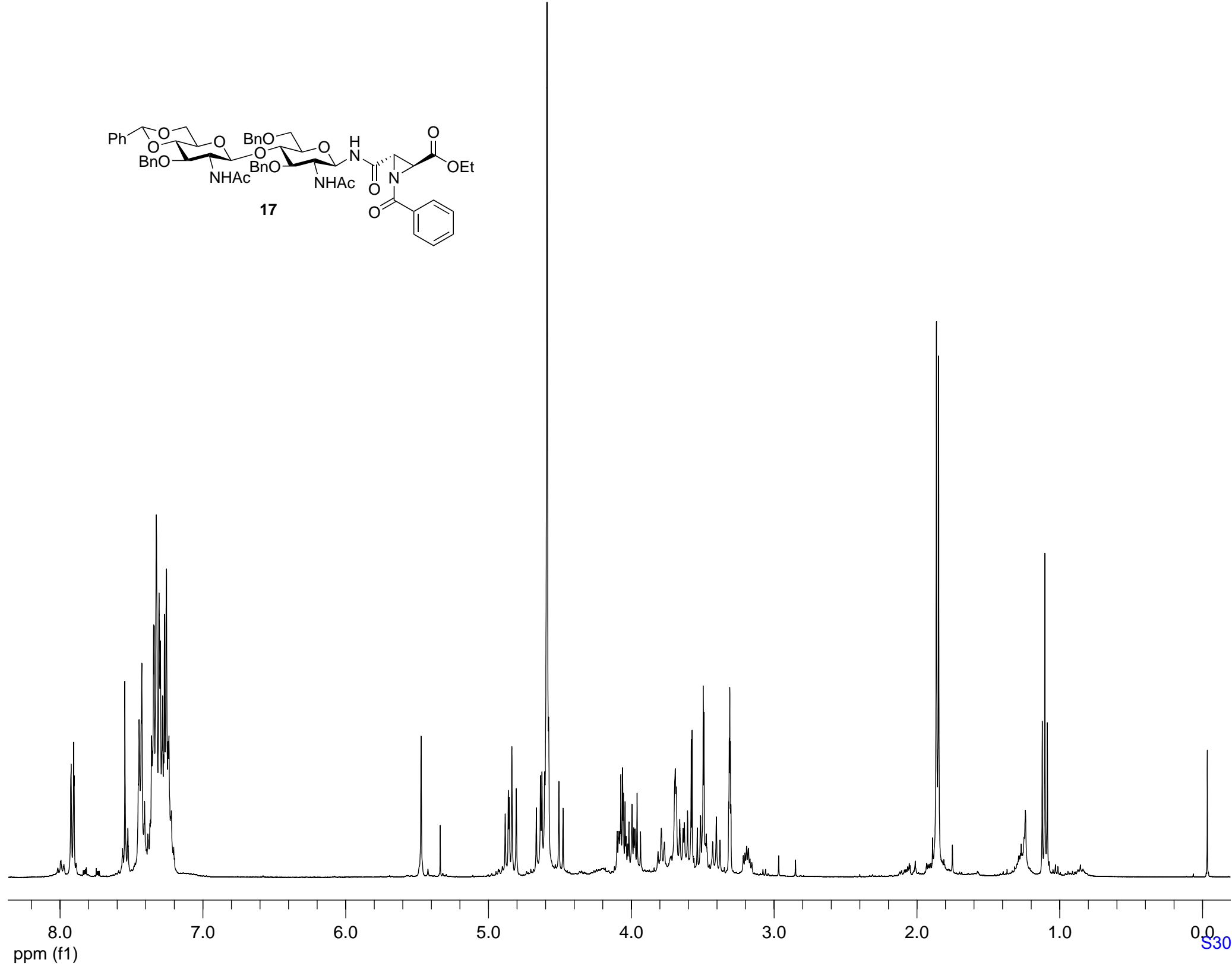
14

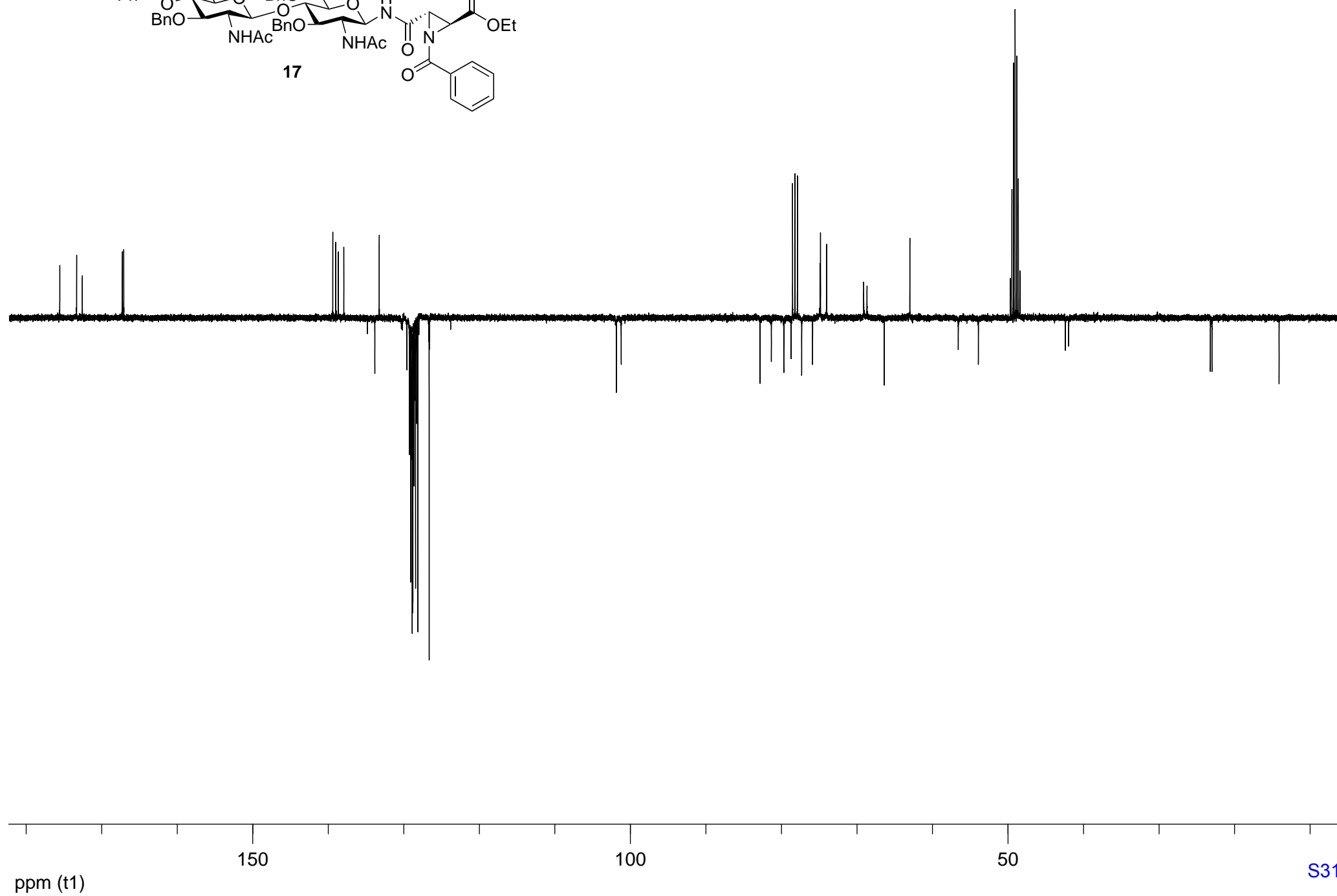
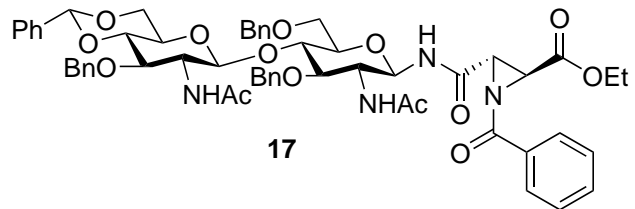


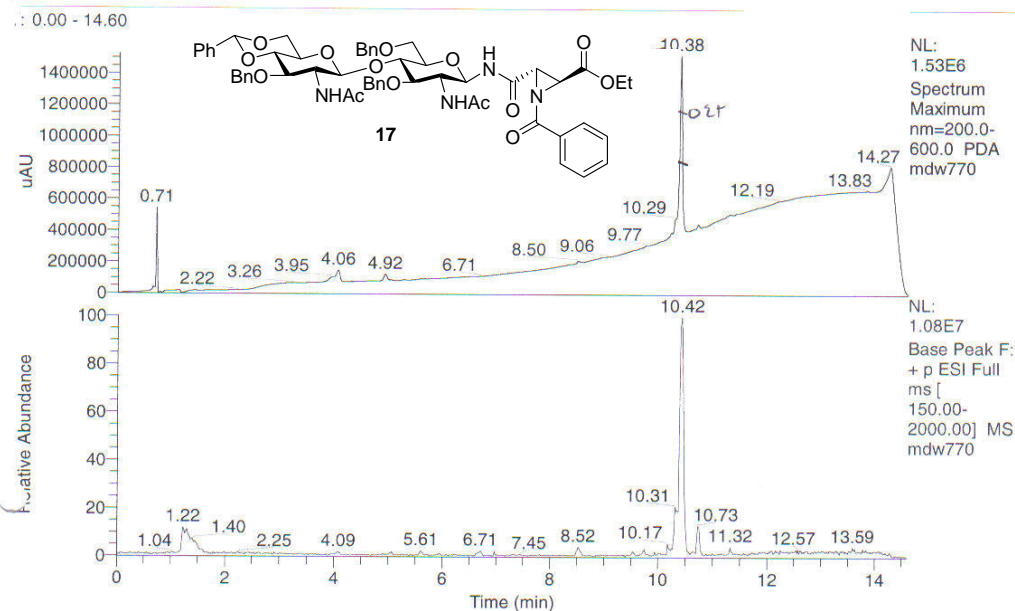




17

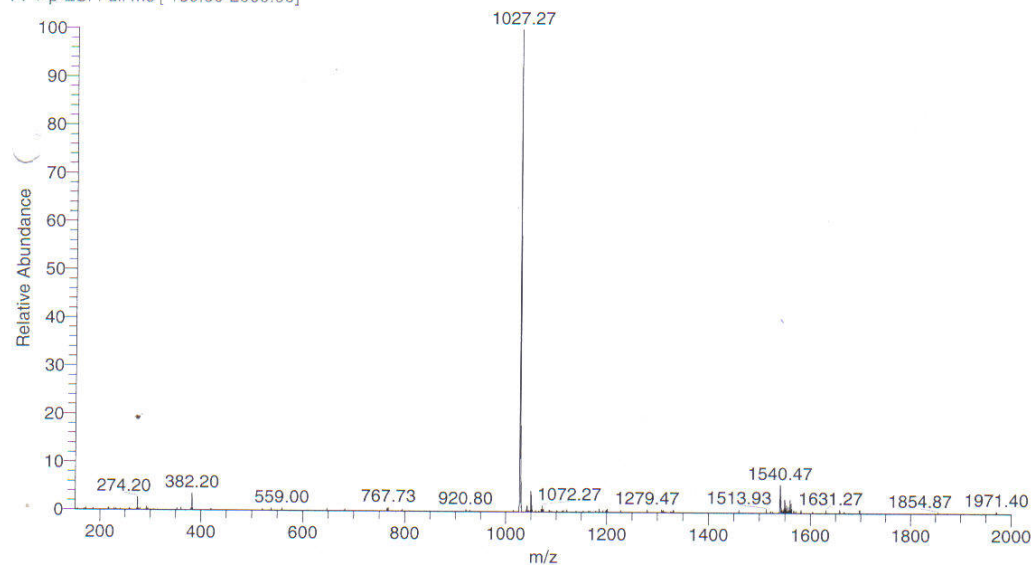


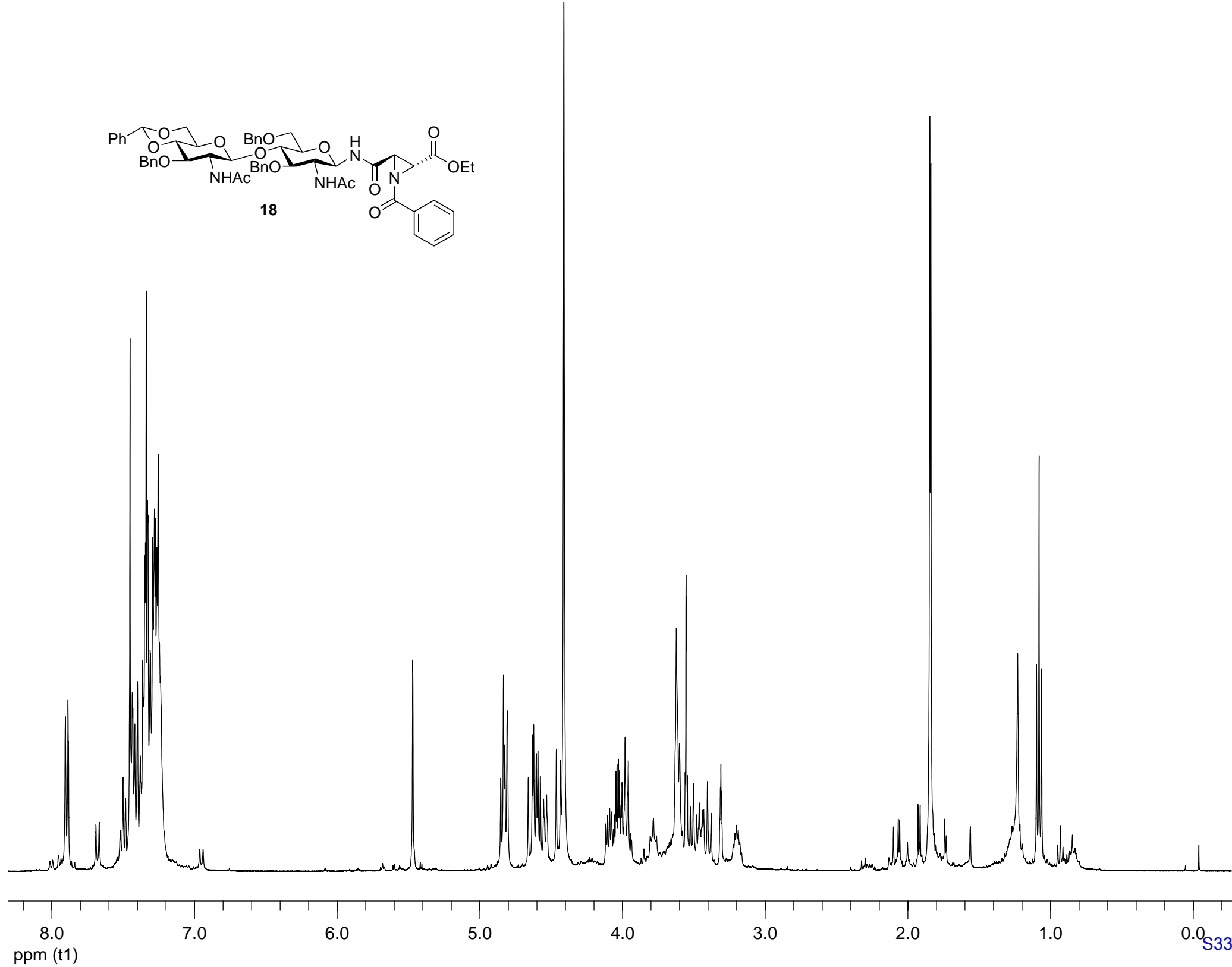
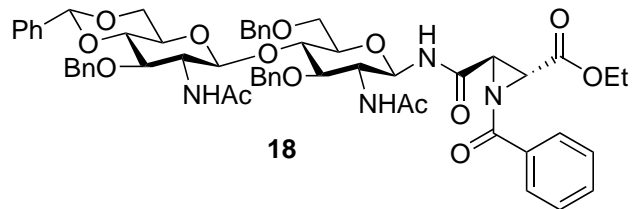


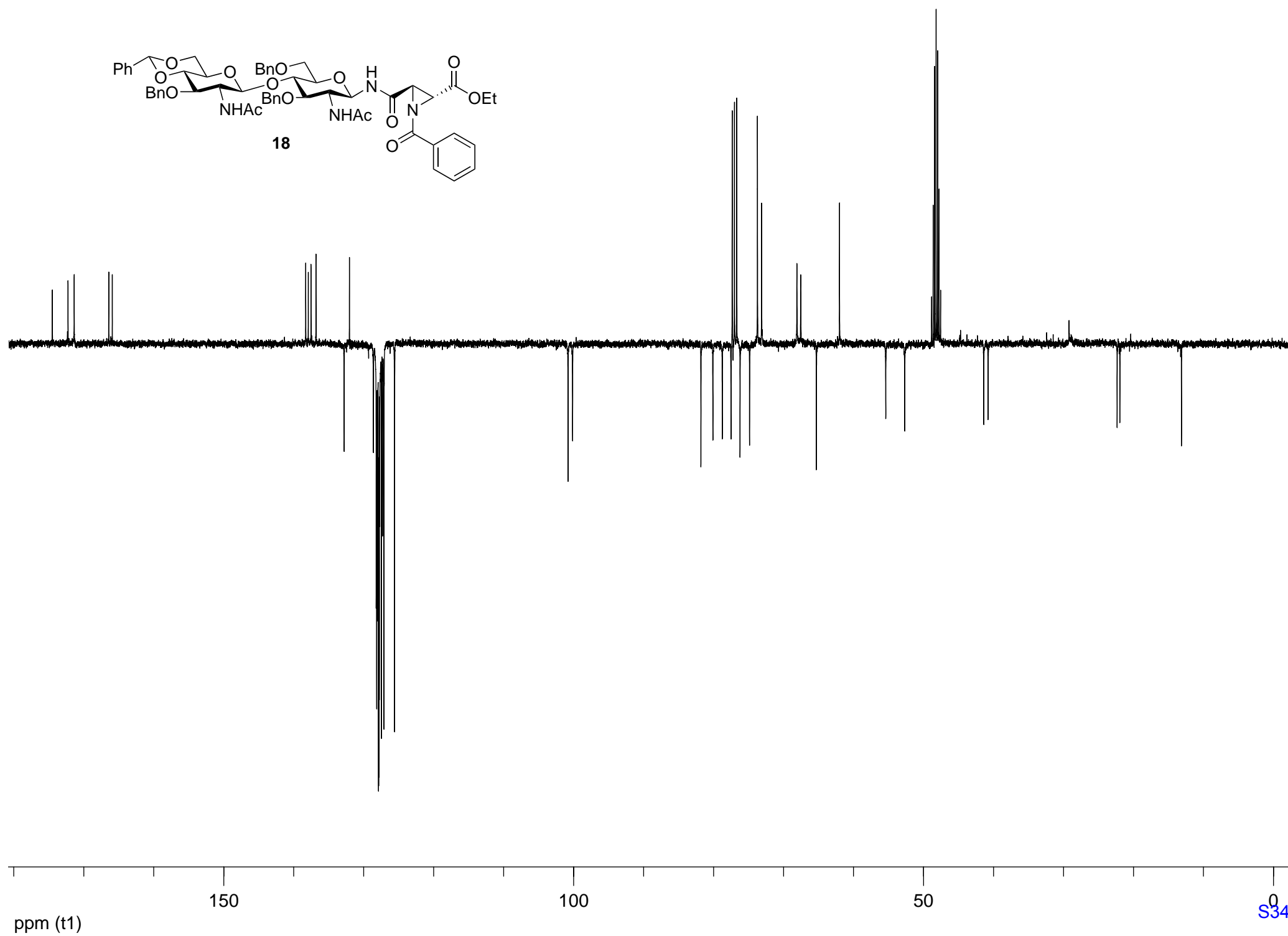
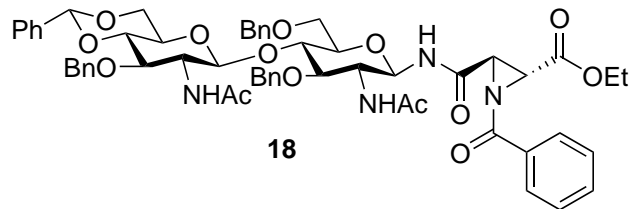


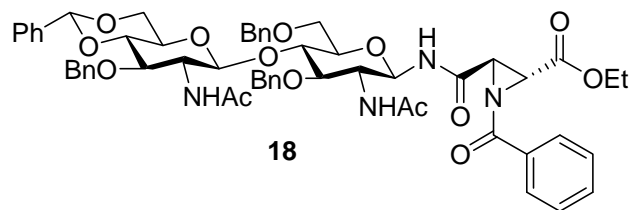
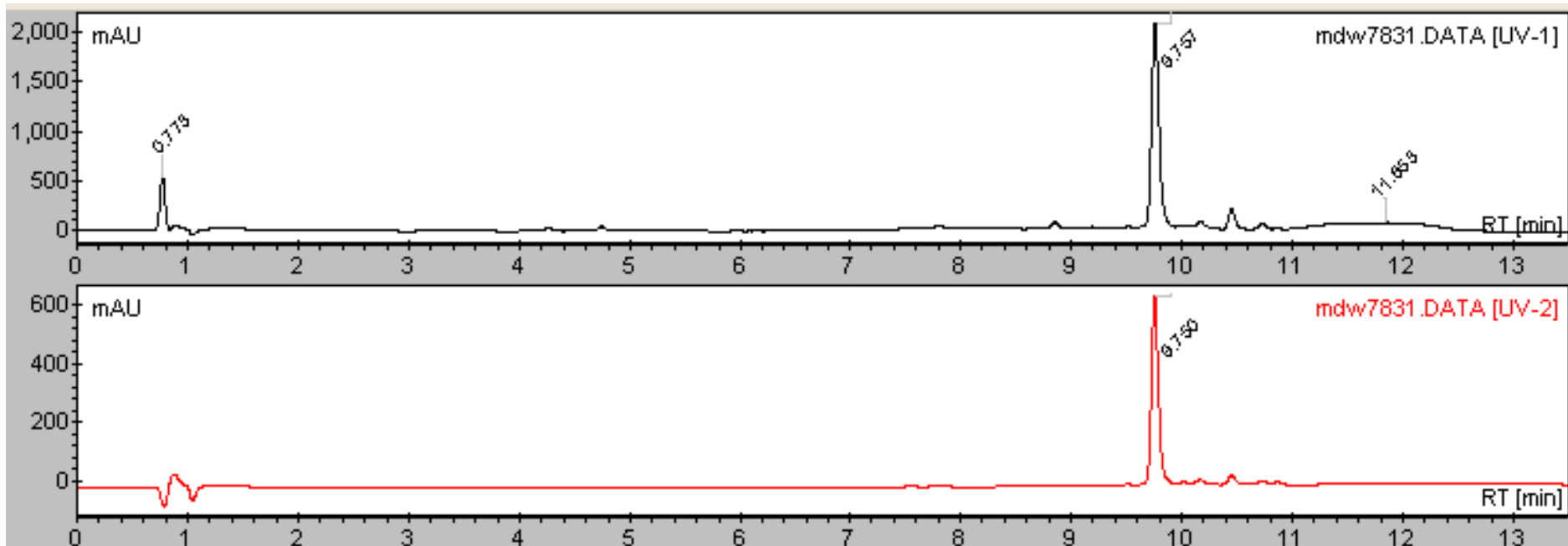
mdw770
Type: Unknown ID: 1 Row: 1
Sample Name:
Study:
Client:
Laboratory:

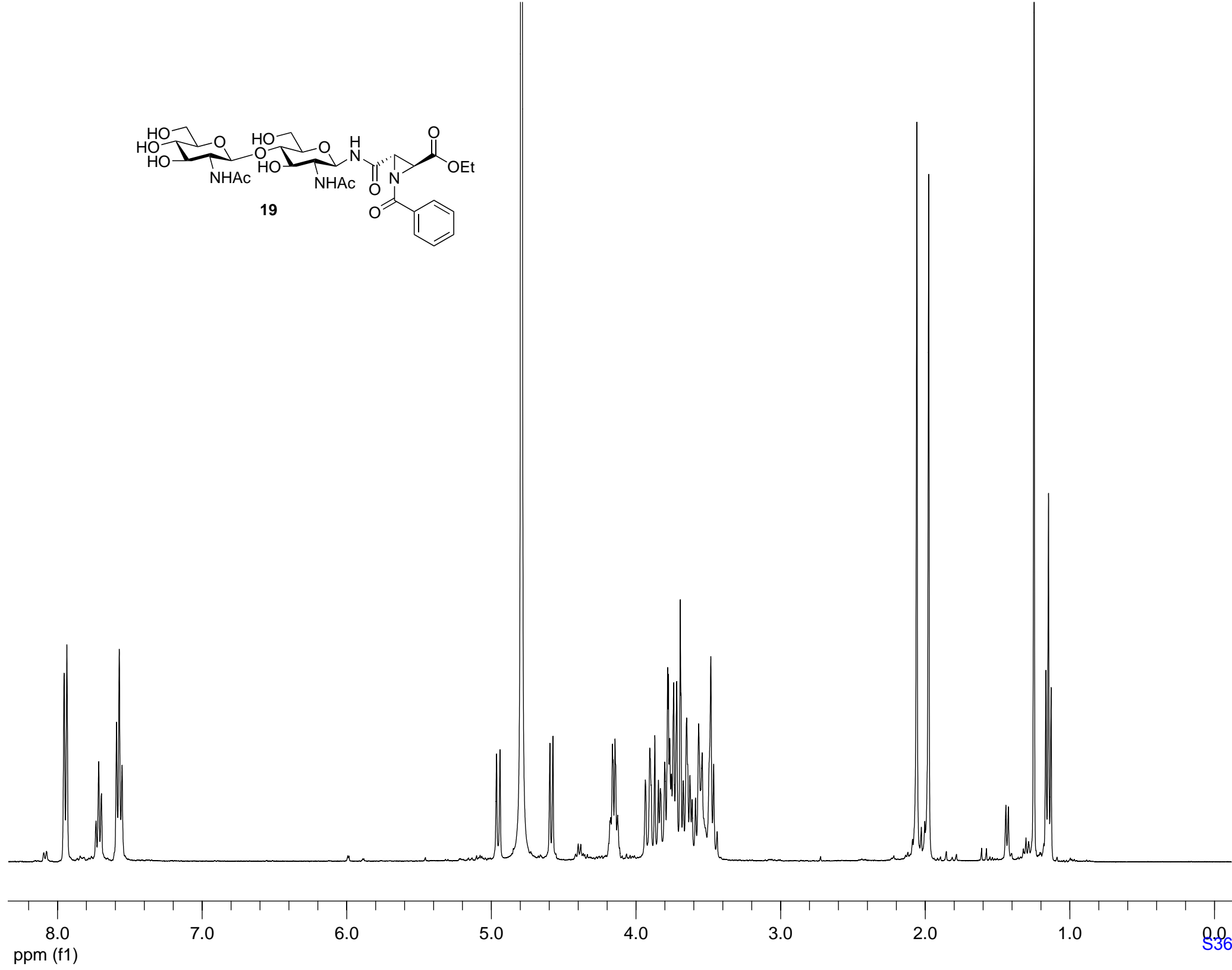
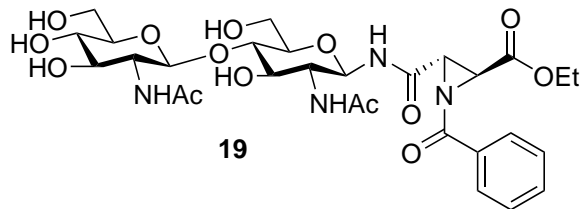
mdw770 #524 RT: 10.40 AV: 1 NL: 1.04E7
F: + p ESI Full ms [150.00-2000.00]

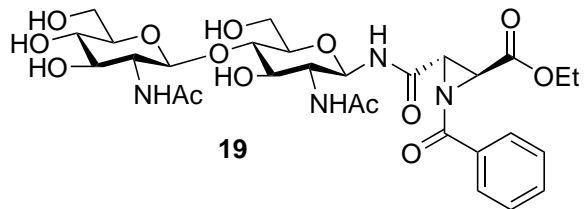




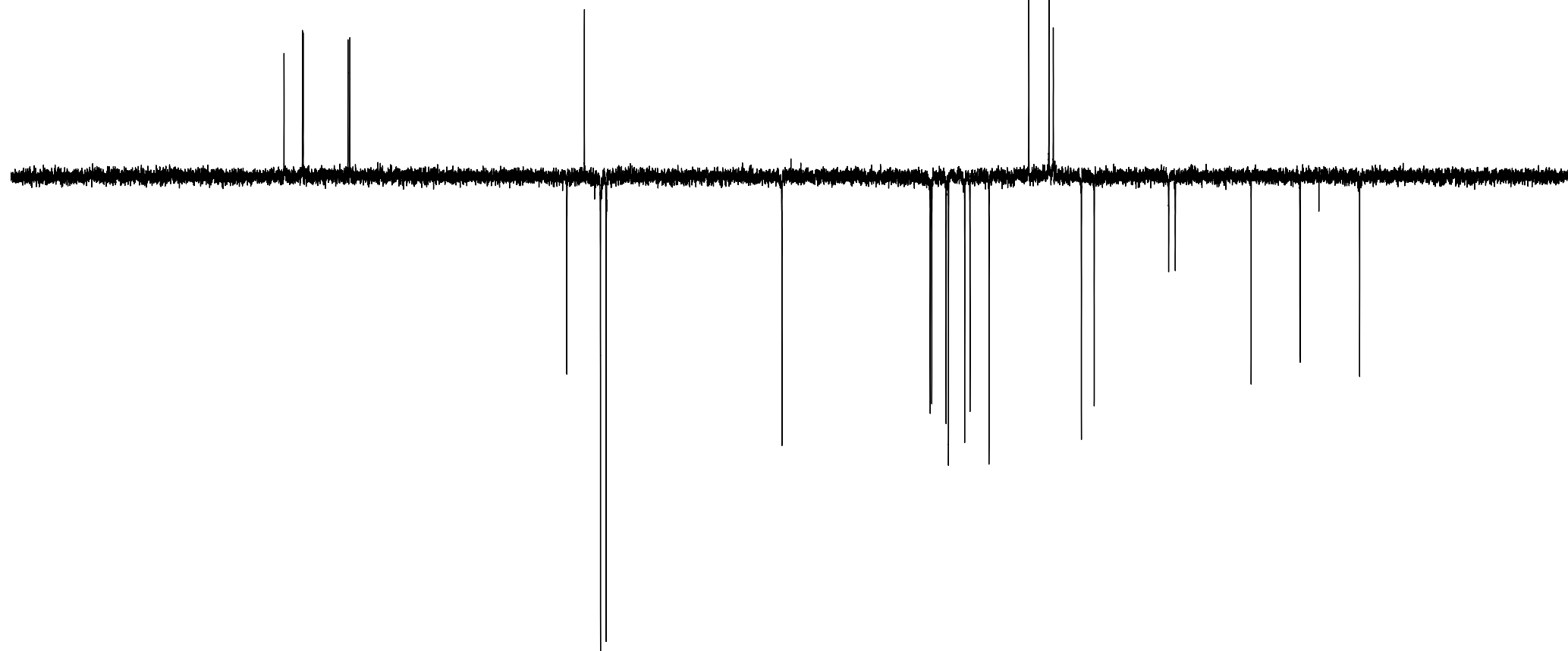








19



200

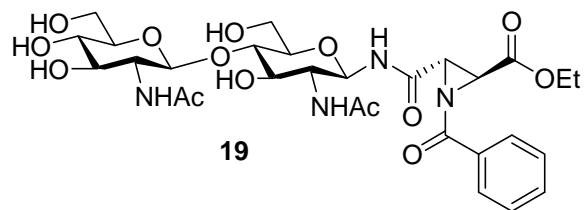
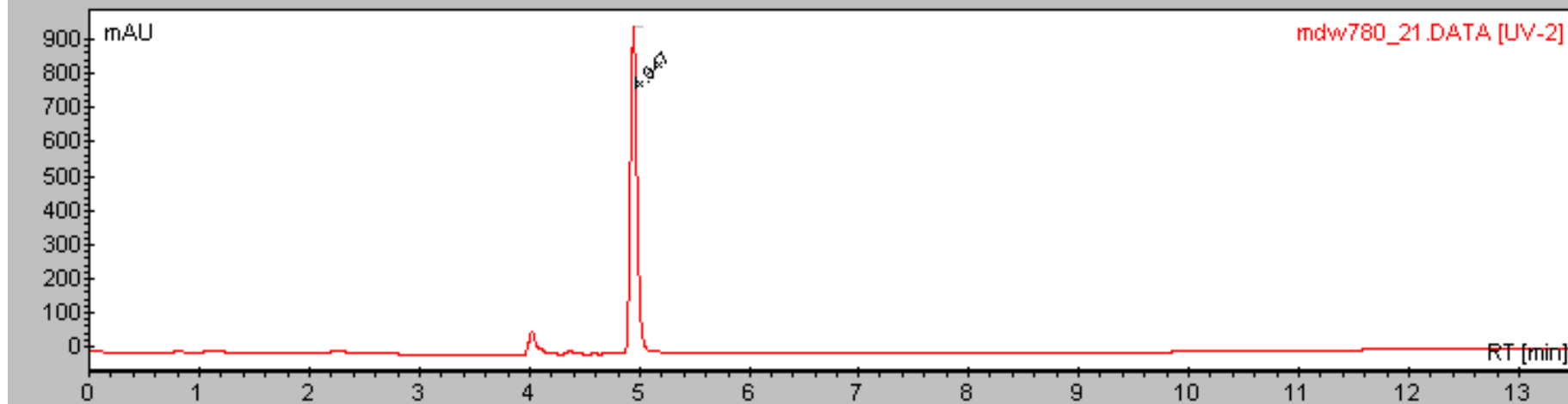
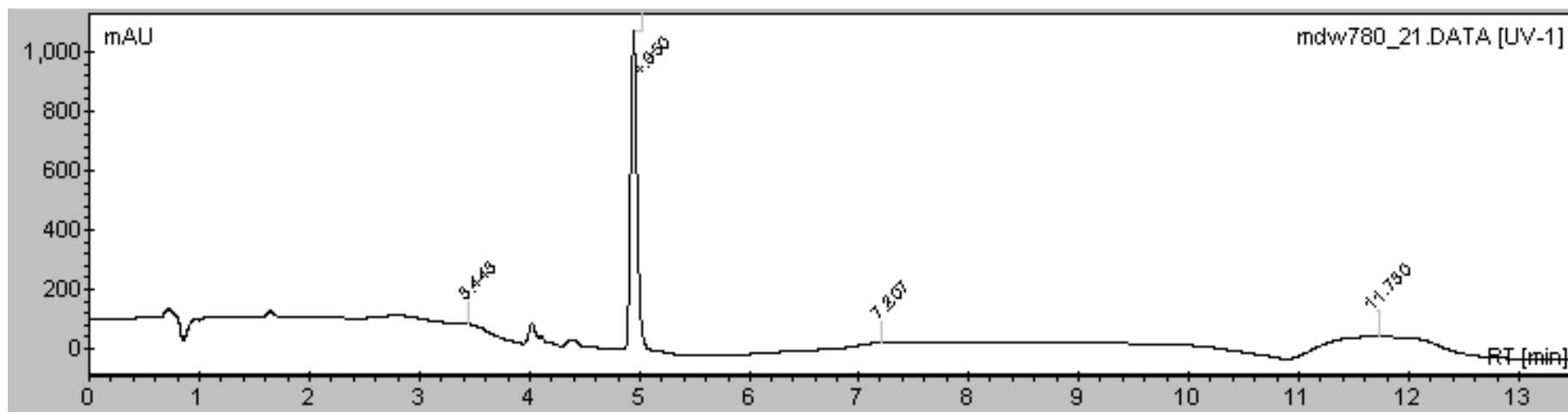
150

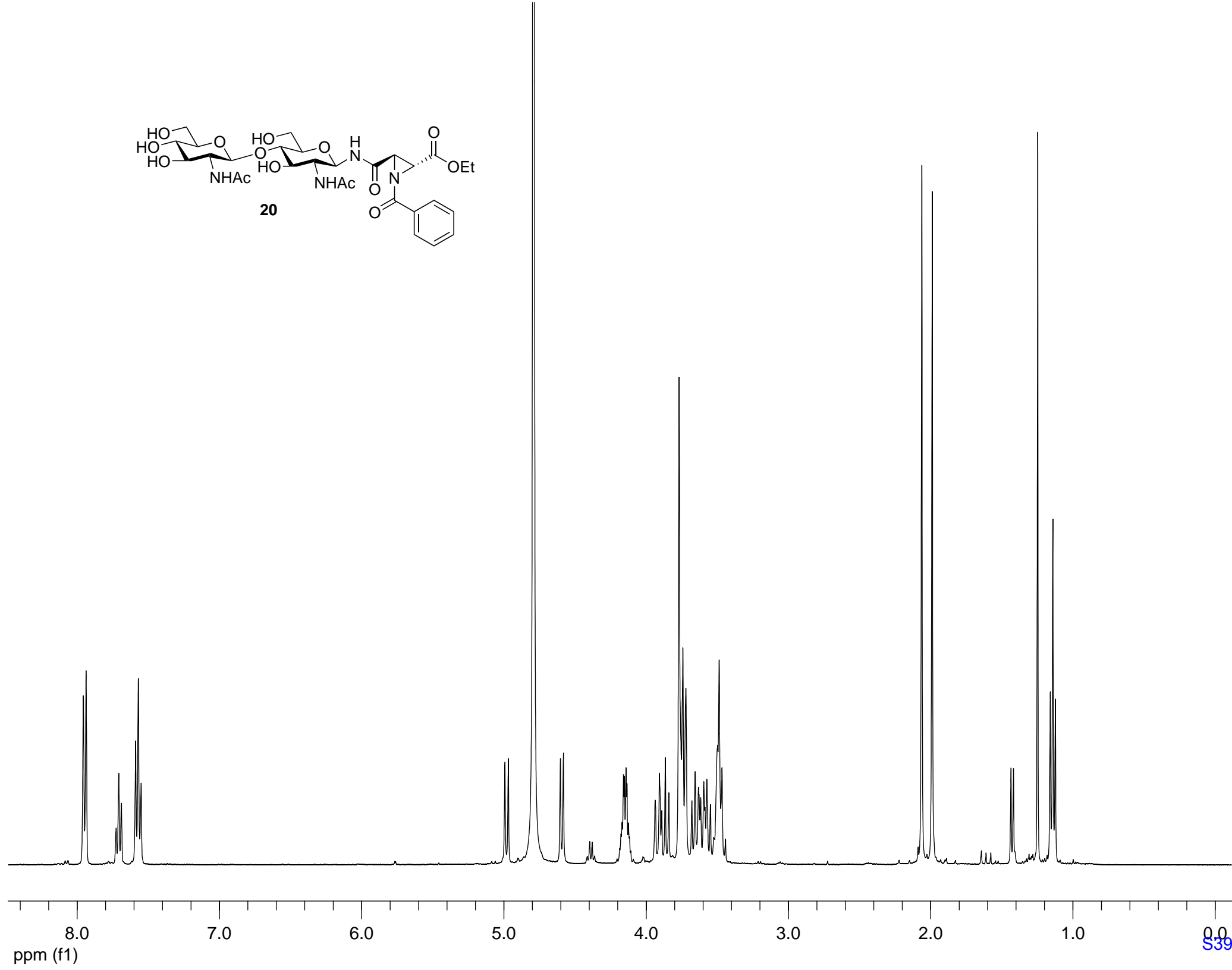
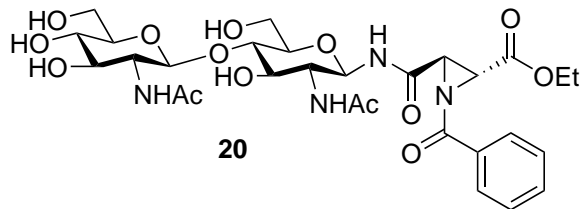
100

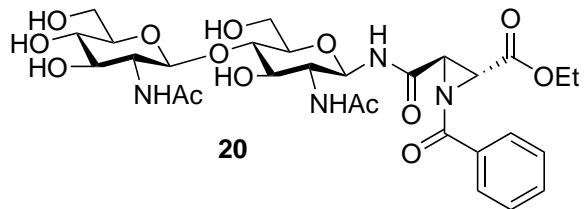
50

0

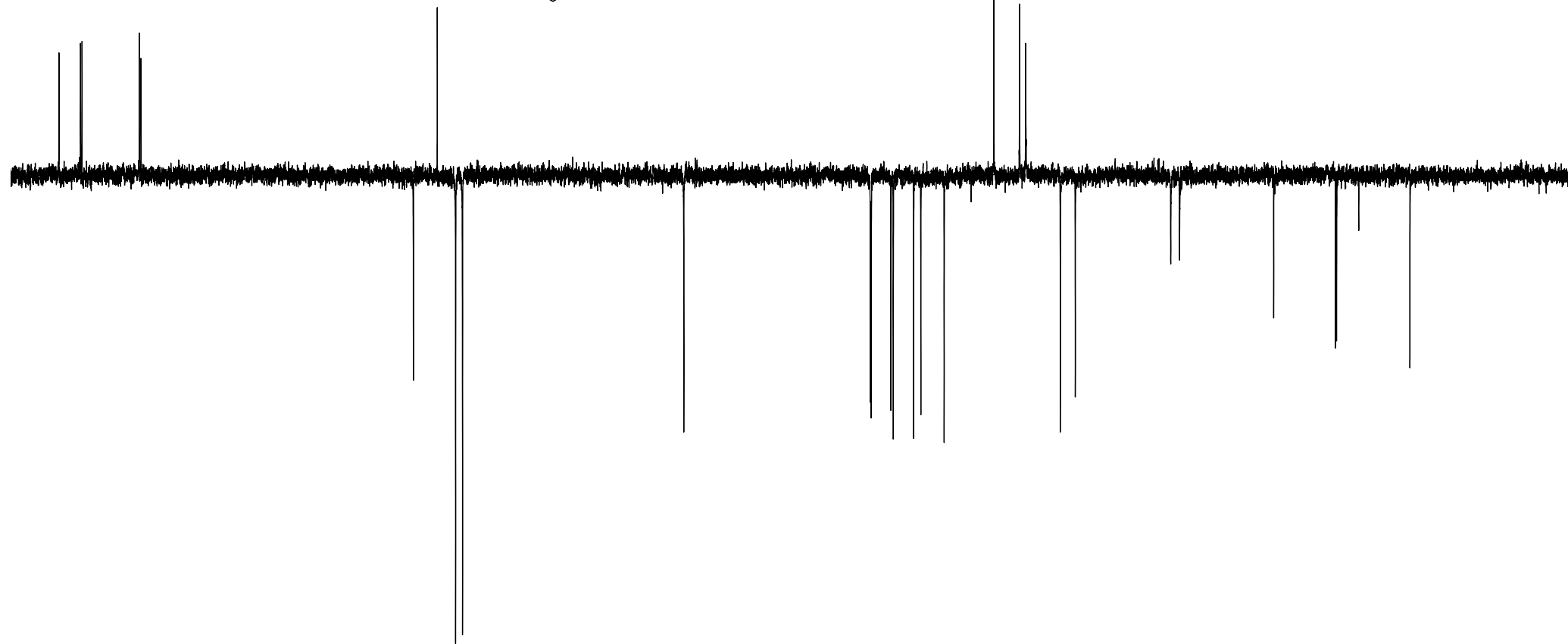
ppm (f1)

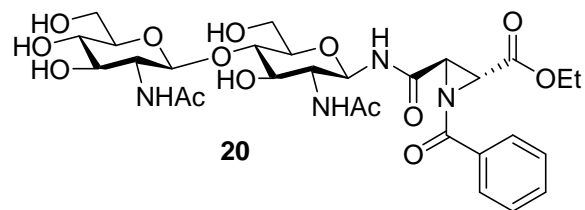
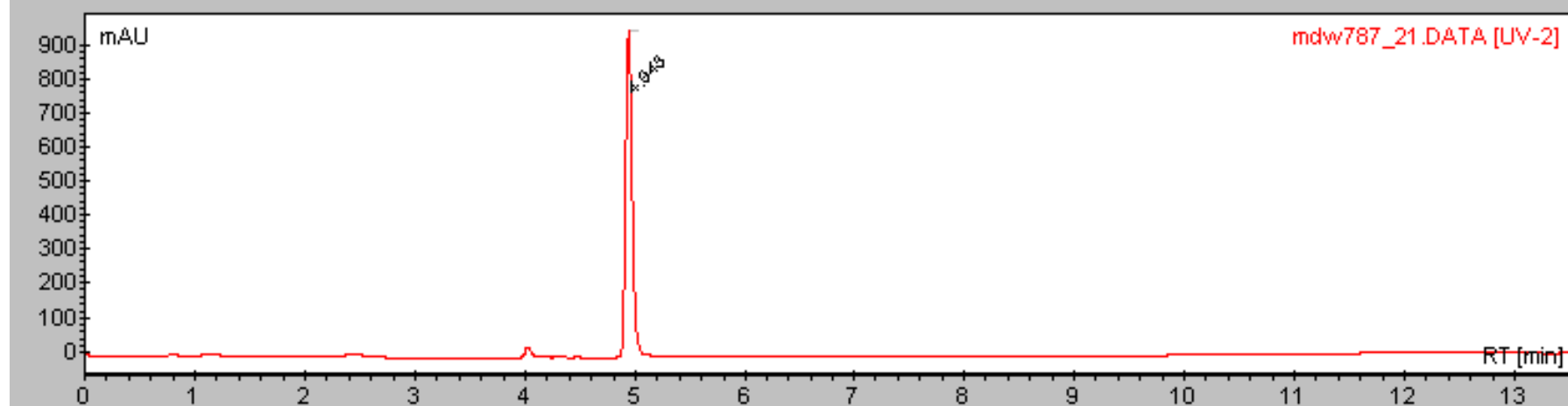
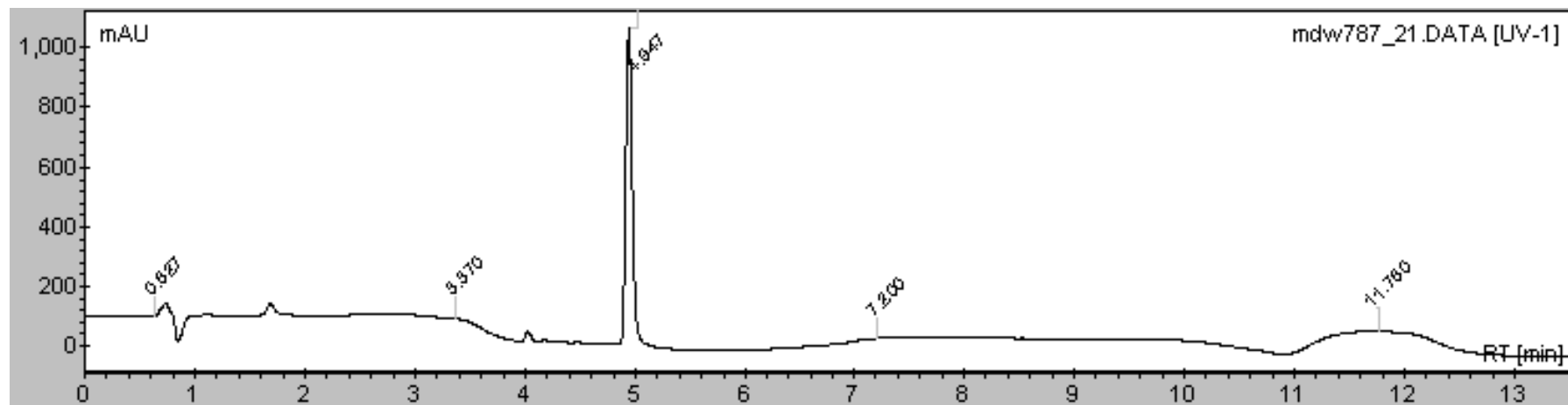


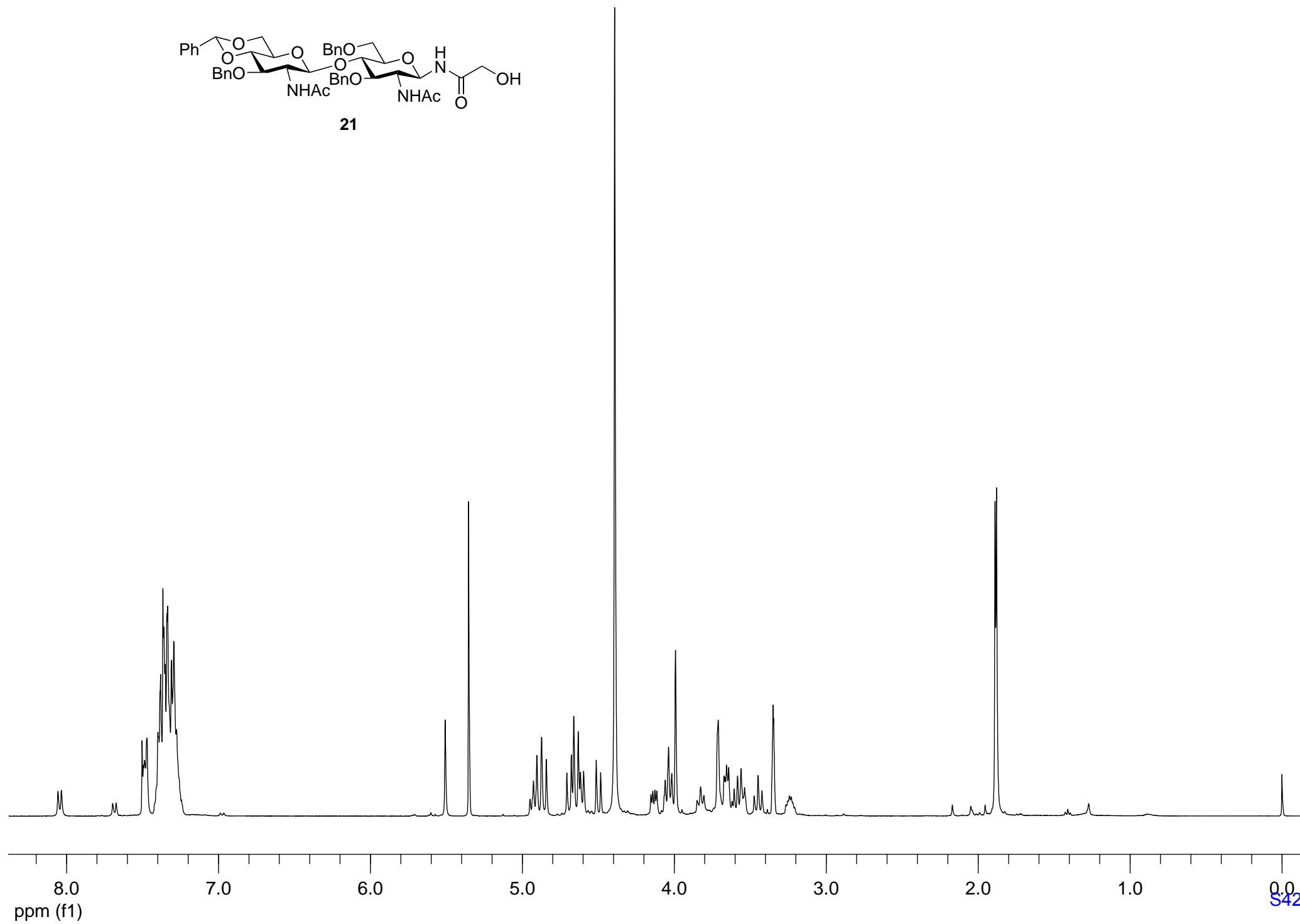
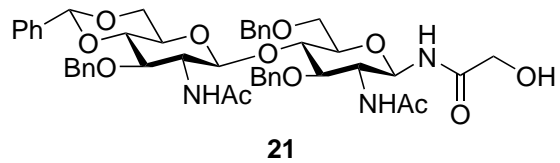


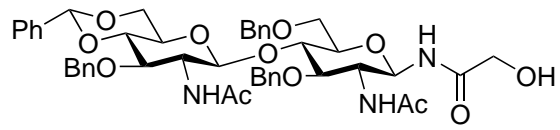


20

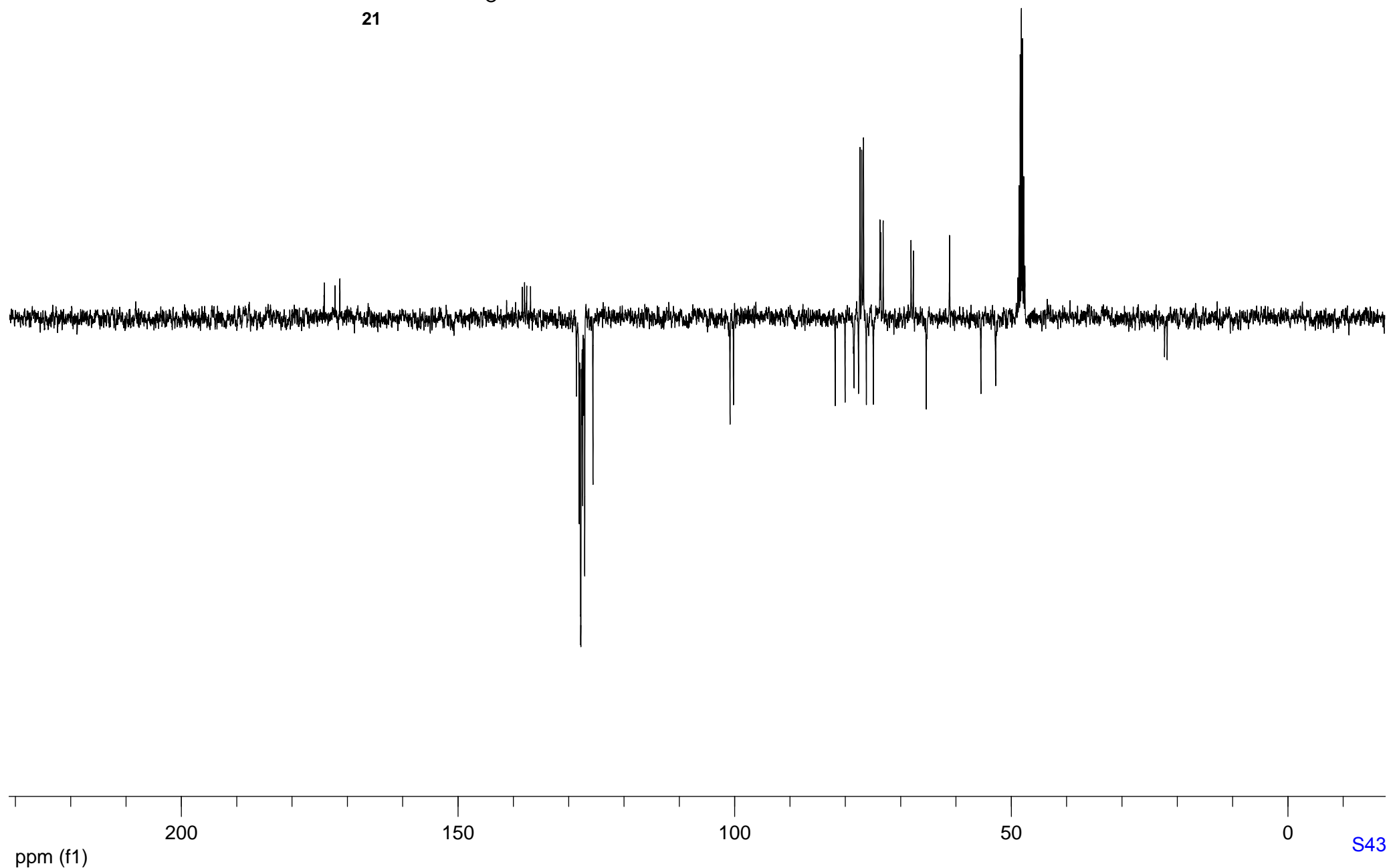


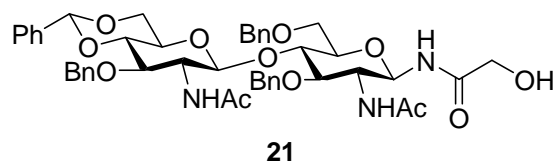
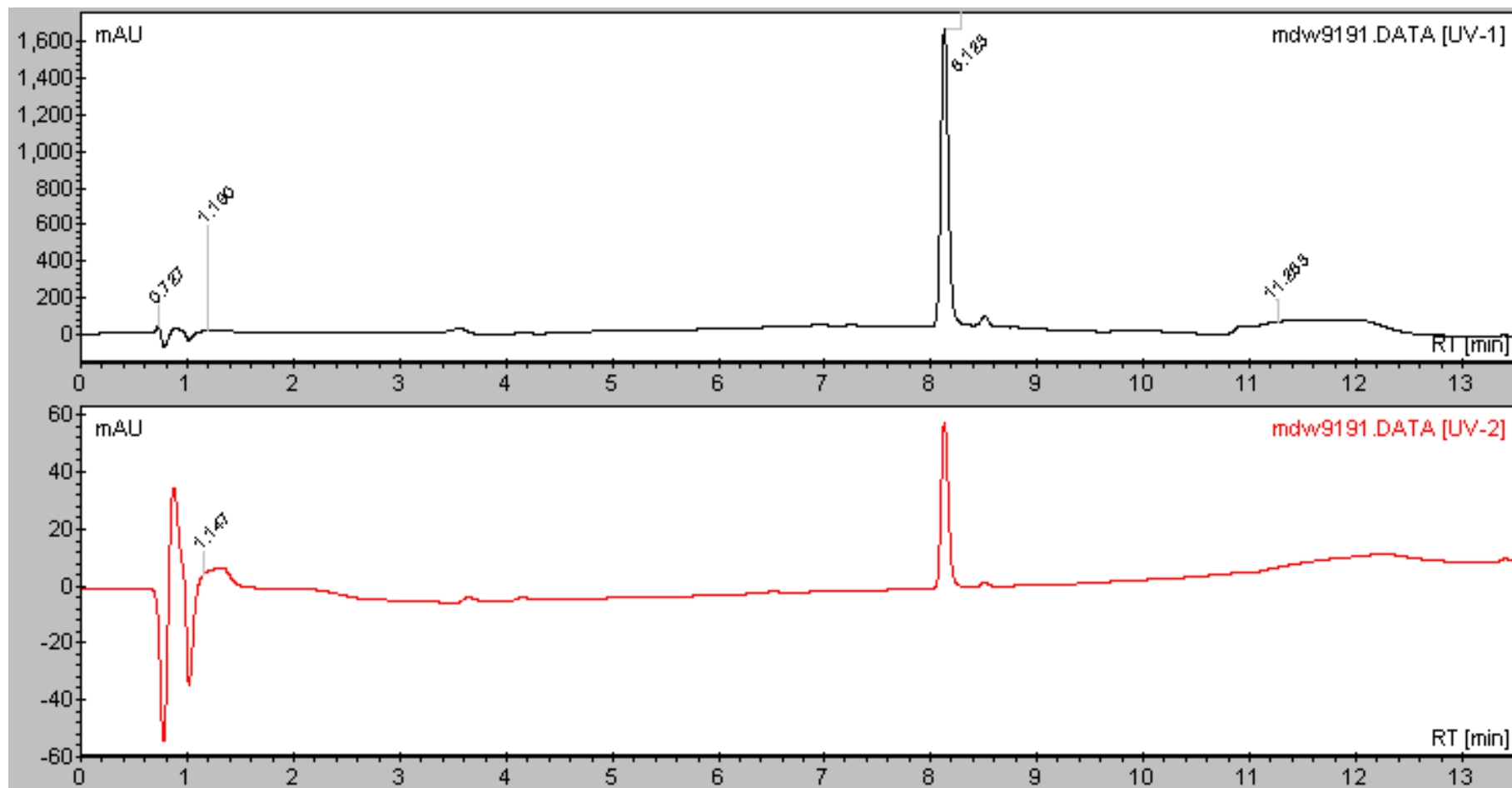


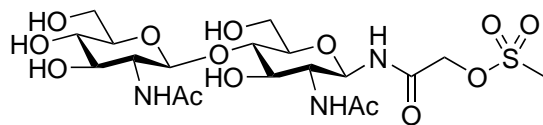




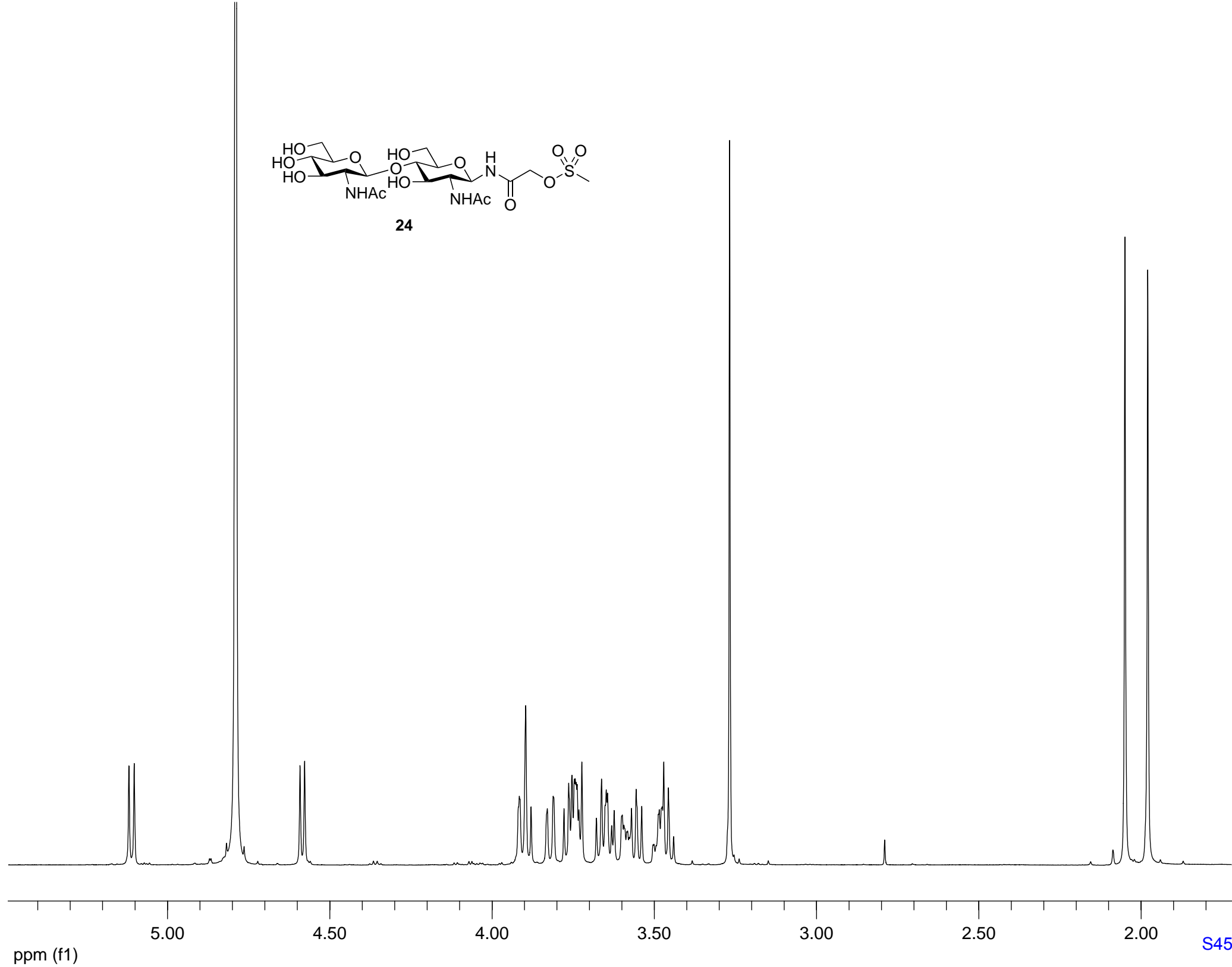
21

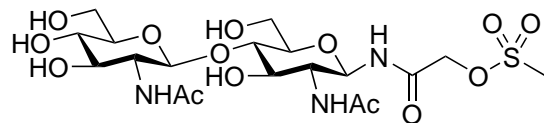




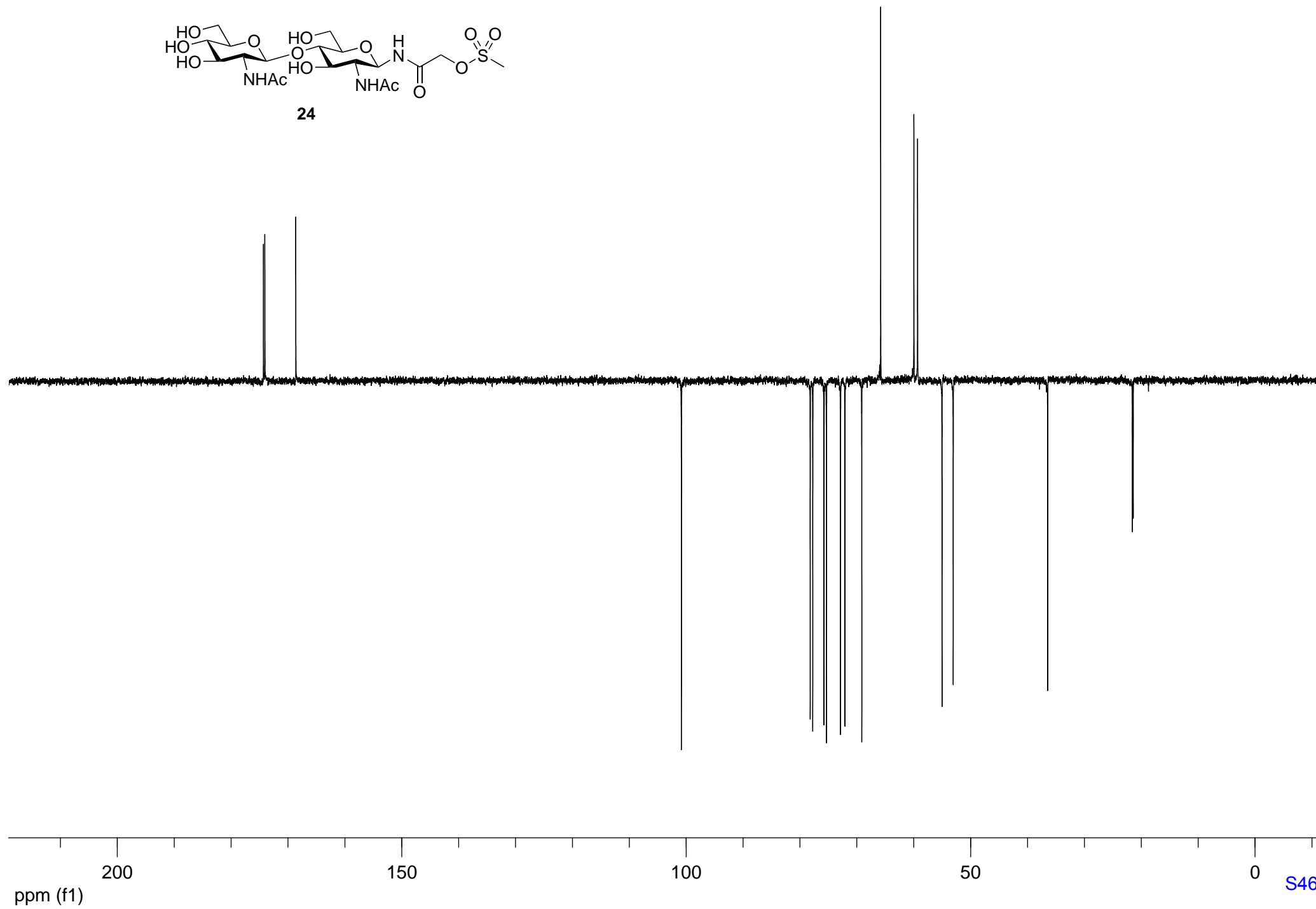


24





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The figure displays two stacked chromatograms sharing a common x-axis representing Time in minutes (0 to 14.5).

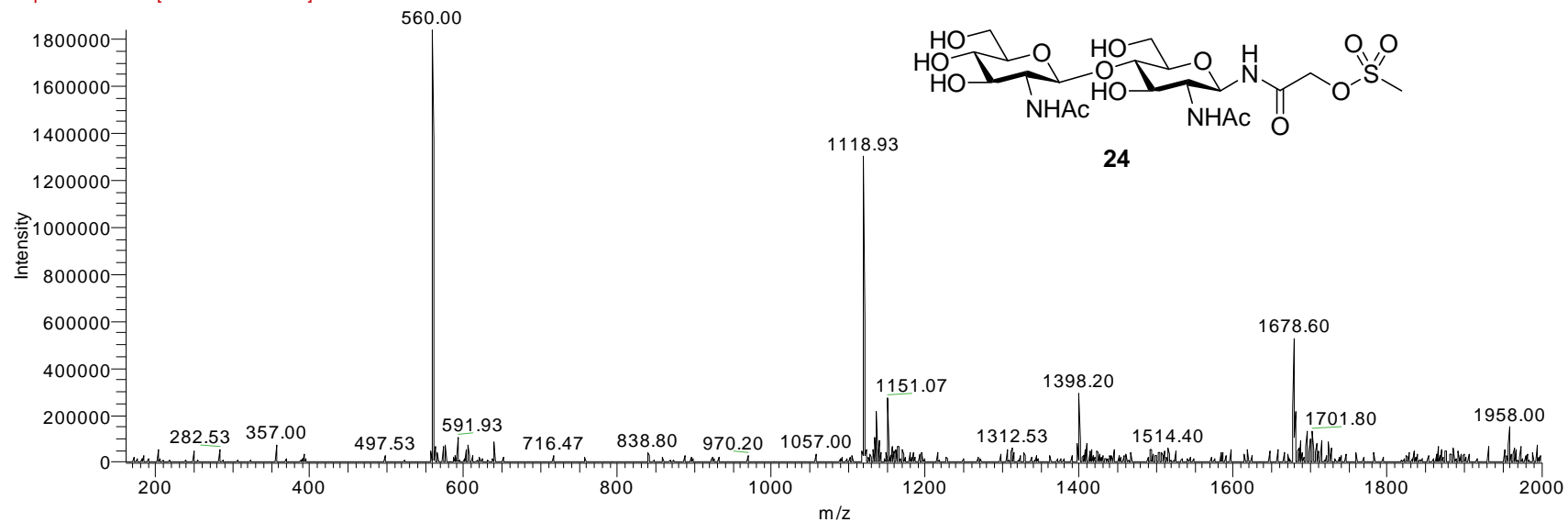
Top Chromatogram (PDA Spectrum):

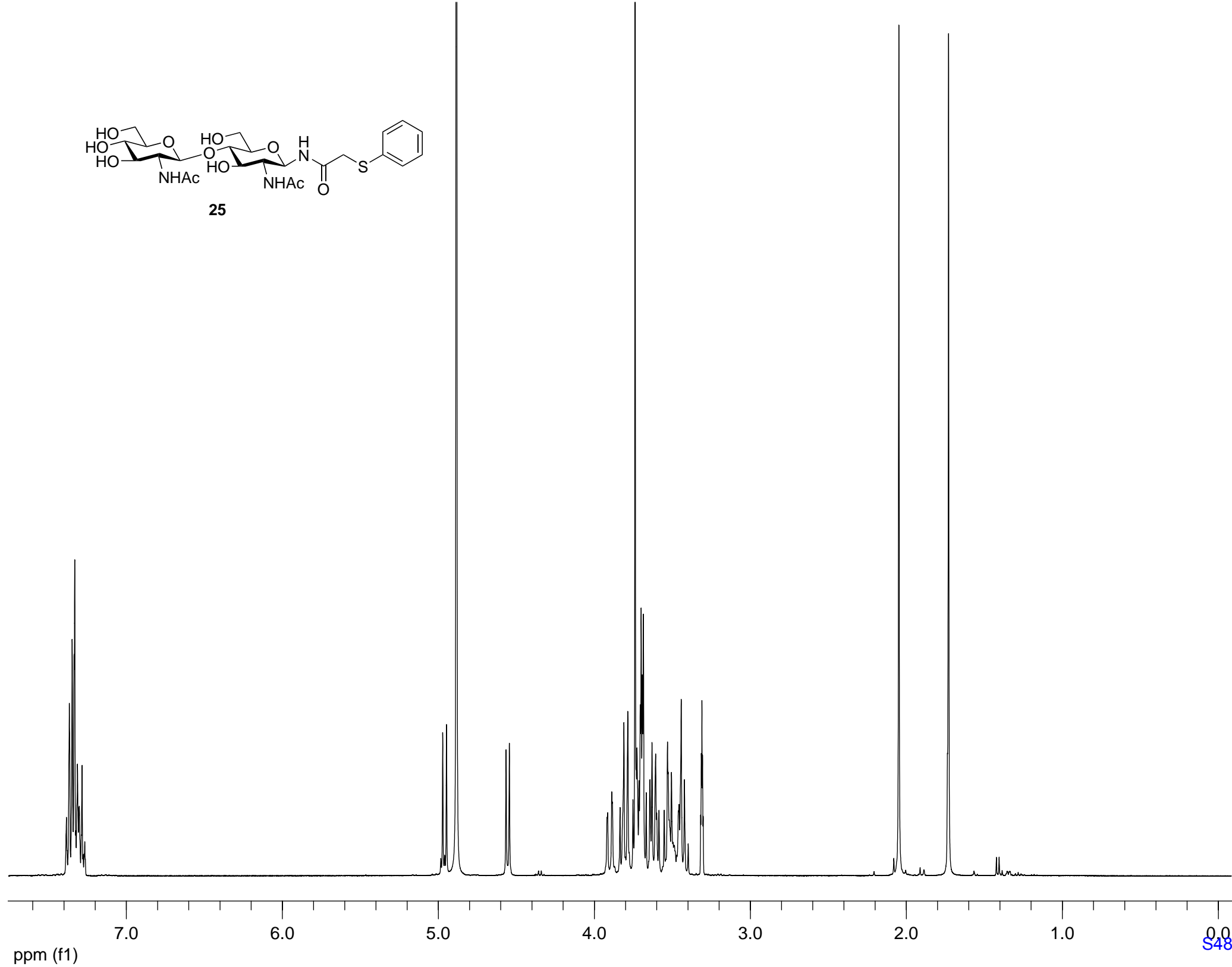
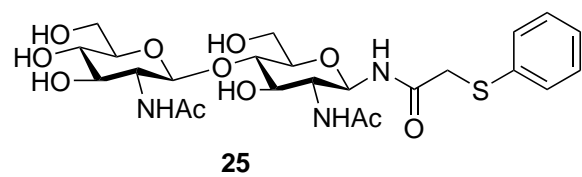
- Y-axis:** uAU (0 to 600,000)
- Major Peaks:** 12.29, 14.27
- Other Labeled Peaks:** 0.61, 0.68, 1.51, 2.33, 2.77, 3.70, 4.51, 6.61, 7.80, 8.09, 8.84, 9.35, 10.15, 11.38, 11.83
- Metadata:** NL: 7.30E5, Spectrum Maximum, nm=200.0-600.0, PDA mdw707

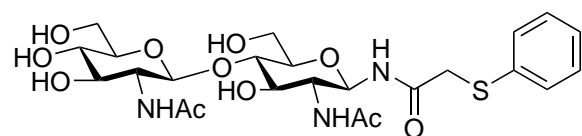
Bottom Chromatogram (ESI-MS Spectrum):

- Y-axis:** Relative Abundance (0 to 100)
- Base Peak:** 2.79
- Other Labeled Peaks:** 0.51, 0.88, 1.50, 1.93, 4.01, 4.63, 4.79, 4.96, 5.91, 6.73, 7.07, 7.87, 8.02, 8.18, 9.51, 10.73, 12.00, 12.10, 12.40, 13.78, 13.83, 14.06
- Metadata:** NL: 1.99E6, Base Peak F: + p, ESI Full ms [160.00-2000.00], MS mdw707

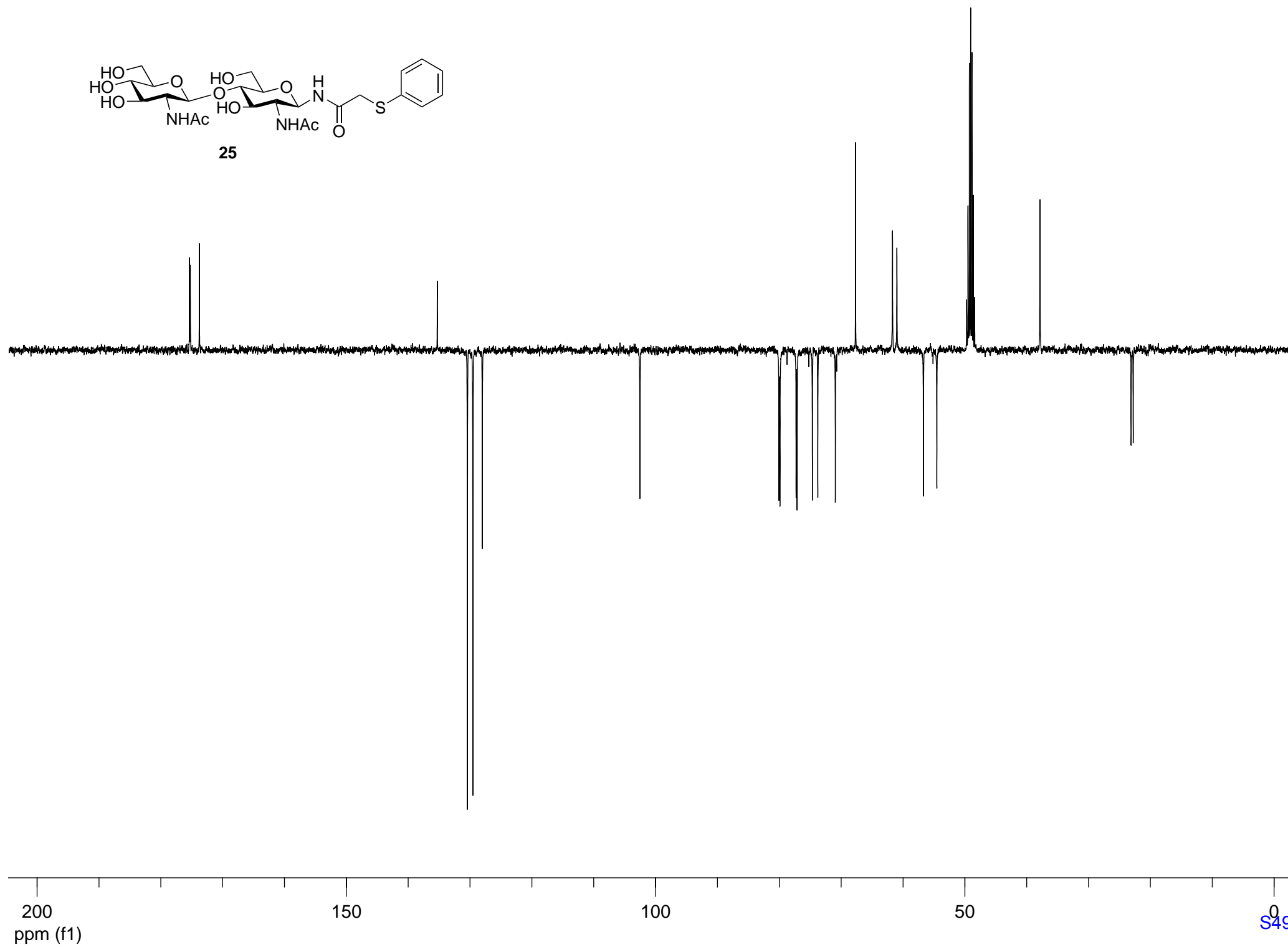
mdw707 #143 RT: 2.77 AV: 1 NL: 1.84E6
F: + p ESI Full ms [160.00-2000.00]



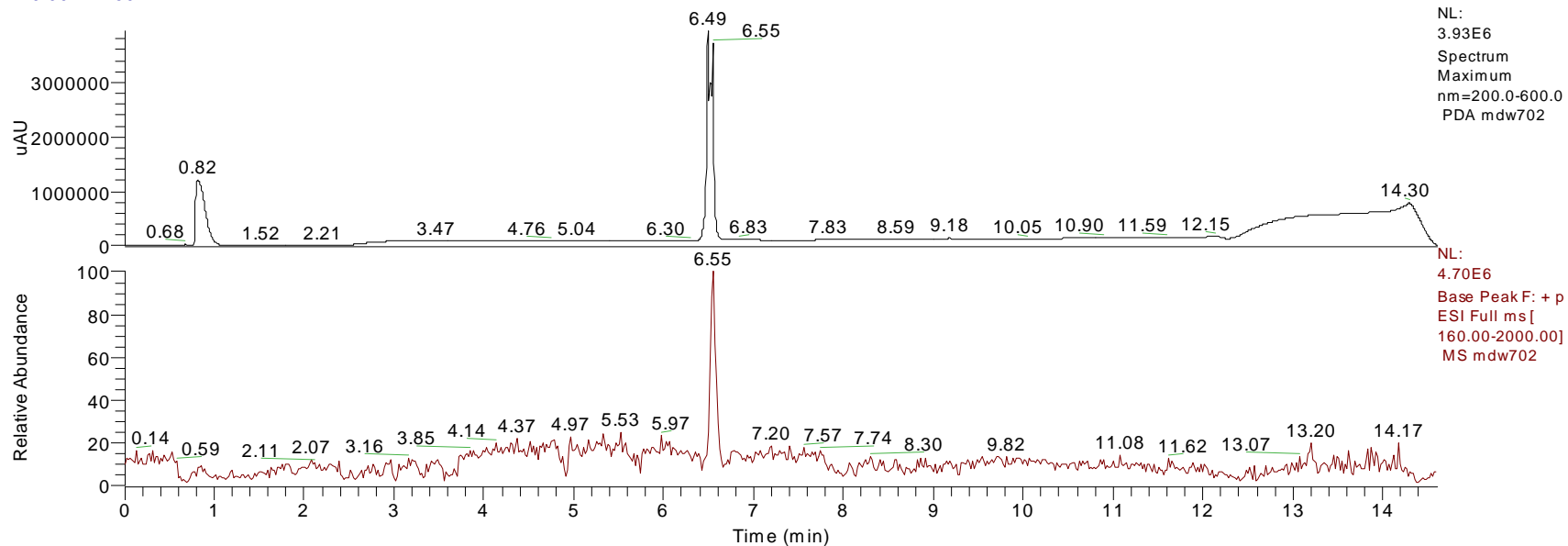




25



RT: 0.00 - 14.60



Instrument Method: C:\Xcalibur\methods\General(TFA)\0050%B00_C=TFA_20ul_+pf 15min.meth
Processing Method:
Vial: C:24
Injection Volume (µl): 20.00
Sample Weight: 0.00

mdw702 #339 RT: 6.53 AV: 1 NL: 4.15E6
F: + p ESI Full ms [160.00-2000.00]

