# Supporting Information

for

# Regioselective Chemoenzymatic Synthesis of Ganglioside Di-sialyl Tetrasaccharide Epitopes

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## Table of Contents


General Methods.....S2

Experimental procedures.....S3-S17

$^{1}H$ and $^{13}C$ NMR spectra for compound **3**.....S18-S19

$^{1}H$ and $^{13}C$ NMR spectra for compound **4**..... S20-S21

$^{1}H$ and $^{13}C$ NMR spectra for compound **5**.....S22-S23

$^{1}H$ and $^{13}C$ NMR spectra for compound **6**..... S24-S25

$^{1}H$ and $^{13}C$ NMR spectra for regioisomer mixture of **6** and **7**.....S26-S27

$^{1}H$ and $^{13}C$ NMR spectra for compound **9**..... S28-S29

$^{1}H$ and $^{13}C$ NMR spectra for compound **10**..... S30-S31

COSY spectra for compound **10**……..... S32

HSQC spectra for compound **10**……..... S33

HMBC spectra for compound **10**……..…..... S34

$^{1}H$ and $^{13}C$ NMR spectra for compound **11**..... S35-S36

$^{1}H$ and $^{13}C$ NMR spectra for compound **12**..... S37-S38

$^{1}H$ and $^{13}C$ NMR spectra for compound **13**..... S39-S40

$^{1}H$ and $^{13}C$ NMR spectra for compound **14**..... S41-S42

$^{1}H$ and $^{13}C$ NMR spectra for compound **15**..... S43-S44

$^{1}H$ NMR spectra for compound **16**..... S45

$^{1}H$ and $^{13}C$ NMR spectra for compound **17**..... S46-S47

$^{1}H$ and $^{13}C$ NMR spectra for compound **19**..... S48-S49

$^{1}H$ and $^{13}C$ NMR spectra for compound **20**..... S50-S51

$^{1}H$ and $^{13}C$ NMR spectra for compound **21**..... S52-S53

$^{1}H$ and $^{13}C$ NMR spectra for compound **22**..... S54-S55

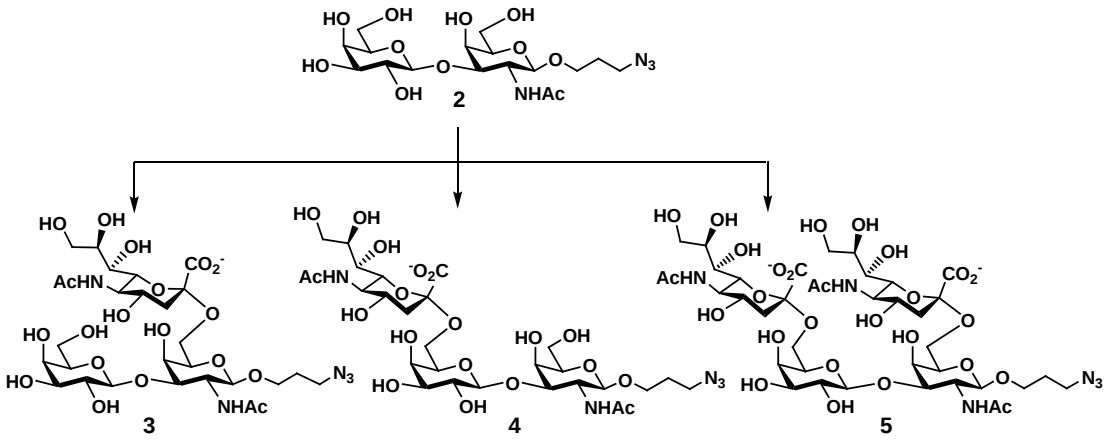
$^{1}H$ and $^{13}C$ NMR spectra for compound **24**..... S56-S57

## General Methods

Chemicals were purchased and used without further purification. $^{1}H$ and $^{13}C$ NMR spectra were recorded on Bruker AVANCE-600 spectrometer, or Bruker-AV300 spectrometer at 25 °C. High resolution electrospray ionization (ESI) mass spectra were obtained at the Mass Spectrometry Facility in the National Glycoengineering Research Center, Shandong University. Silica gel 60 (300 - 400 mesh, Haiyang, Qingdao) was used for flash column chromatography. Thin-layer chromatography (TLC) was performed on silica gel 60 $F_{254}$ plates (Merck, Billerica MA) using *p*-anisaldehyde sugar stain or 5% sulfuric acid in ethanol for detection. Gel filtration chromatography was performed using a column (100 cm × 2.5 cm) packed with BioGel P-2 Fine resins (Bio-Rad, Hercules, CA). Recombinant *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS)[1], *Pasteurella multocida* multifunctional α2–3-sialyltransferase 1 (PmST1)[1], *Photobacterium damselae* α2–6-sialyltransferase (Pd2,6ST)[2], *Escherichia coli* K-12 galactokinase (GalK)[3], and D-galactosyl-β1–3-*N*-acetyl-D-hexosamine phosphorylase cloned from *Bifidobacterium infantis* (BiGalHexNAcP)[3] were expressed and purified as described previously.

## Experimental Procedures

### Small-scale (50 μL) one-pot two-enzyme α2–6-sialylation of disaccharide 2




The small scale one-pot two-enzyme sialylation reactions were carried out in a reaction mixture of 50 μL containing disaccharide **2**[3] (10 mmol, 1.0 equiv.), Neu5Ac (varied at 0.5 equiv., 1.0 equiv., or 2.5 equiv.), CTP (varied according to Neu5Ac at 0.5 equiv., 1.0 equiv., or 2.5 equiv.), Tris-HCl buffer (100 mmol, pH 8.5), $MgCl_2$ (20 mmol), and appropriate amounts of NmCSS (0.2 μg) and Pd2,6ST (1.2 μg). Reaction mixtures were incubated in an isotherm incubator at 37 °C with agitation at 140 rpm. Reactions were monitored by thin-layer chromatography (TLC) analysis

(EtOAc: MeOH: $H_2O$: AcOH = 4: 2: 1: 0.2, v/v, was used as the developing solvent) as showed in **Figure S1**.

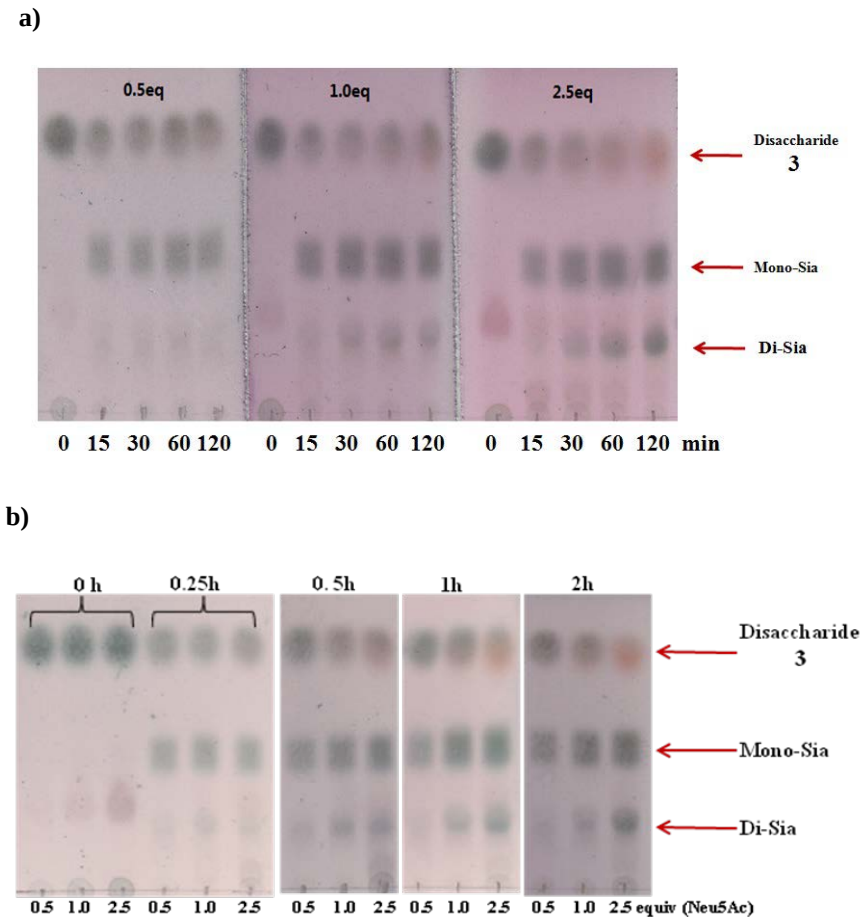
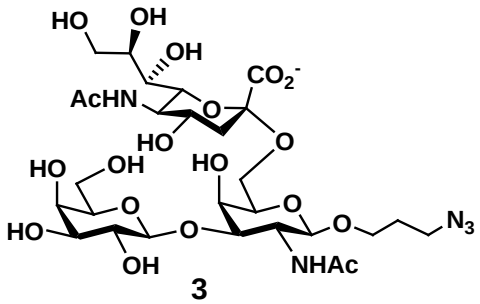



**Figure S1.** Thin-layer chromatography (TLC) analysis of small-scale one-pot two-enzyme α2–6-sialylation of disaccharide **2** using various amounts of Neu5Ac and CTP (0.5, 1.0, or 2.5 equiv.).

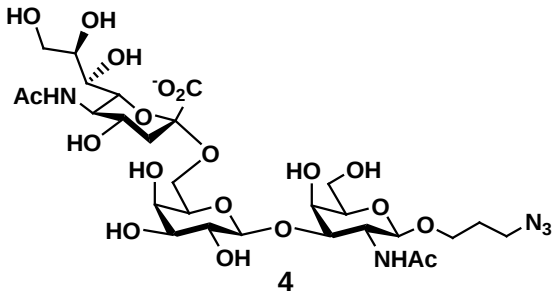
**Preparative-scale one-pot two-enzyme sialylation of disaccharide 2**

Disaccharide **2** (200 mg, 0.43 mmol ), Neu5Ac (130 mg, 1.0 equiv.), and CTP (230 mg, 1.0 equiv.) were dissolved in water in a 50 mL centrifuge tube containing Tris-HCl buffer (100 mmol, pH 8.5) and $MgCl_2$ (20 mmol). After the addition of appropriate amount of NmCSS (1.2 mg) and Pd2,6ST (1.2 mg), water was added to bring the volume of the reaction mixture to 30 mL. The reaction was carried out by incubating the solution in an isotherm incubator for 2 h at 37 °C with agitation at 140 rpm. The reaction was monitored by TLC (EtOAc: MeOH: $H_2O$: AcOH = 4: 2: 1: 0.2, v/v). The reaction was quenched by adding an equal volume of 95% EtOH. The mixture was centrifuged to remove precipitates. The supernatant was concentrated and purified by silica gel chromatography (EtOAc: MeOH: $H_2O$ = 100: 30: 15, v/v) to provide the mono-sialyl trisaccharides **3** and **4** and the di-sialyl tetrasaccharide **5**.



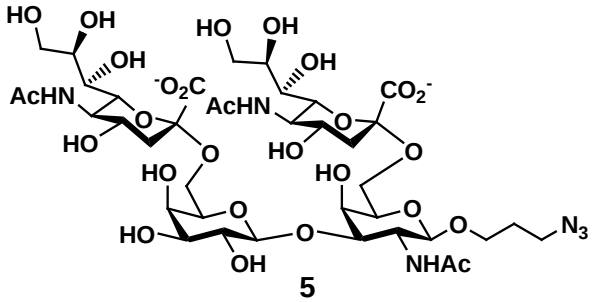

**3**

**Galβ1–3(Neu5Acα2–6)GalNAcβProN$_3$ (3).** 0.33 g, a white solid, yield 34%. $^1$H NMR (300 MHz, $D_2O$) δ 4.51 (d, *J* = 8.4 Hz, 1H), 4.45 (d, *J* = 7.6 Hz, 1H), 4.22 (d, *J* = 3.0 Hz, 1H), 4.05–3.51 (m, 20H), 3.40 (t, *J* = 6.7 Hz, 2H), 2.75 (dd, *J* = 4.6, 12.6 Hz, 1H), 2.05 (s, 6H), 1.87 (m, 2H), 1.70 (t, *J* = 12.1 Hz, 1H). $^{13}$C NMR (75 MHz, $D_2O$) δ 175.07, 174.71, 173.43, 104.88, 101.49, 100.45, 79.90, 74.98, 73.15, 72.66, 72.55, 72.07, 71.75, 70.59, 68.63, 68.25, 68.21, 67.89, 67.27, 62.67, 60.99, 51.88, 51.22, 47.82, 40.22, 28.17, 22.26, 22.05. HRMS (ESI) m/z calcd for $C_{28}H_{46}N_5O_{19}$, $[M-H]^-$, 756.2792; found, 756.2732.




**4**

**Neu5Acα2–6Galβ1–3GalNAcβProN$_3$ (4).** 0.31 g, a white solid, yield 32%. $^1$H NMR (300 MHz, $D_2O$) δ 4.51 (d, *J* = 8.4 Hz, 1H), 4.44 (d, *J* = 7.6 Hz, 1H), 4.20 (d, *J* = 3.0 Hz, 1H), 4.02–3.55 (m, 20H), 3.40 (t, *J* = 6.6 Hz, 2H), 2.75 (dd, *J* = 4.5, 12.3 Hz, 1H), 2.05 (s, 6H), 1.87 (m, 2H), 1.67 (t, *J* = 12.1 Hz, 1H). $^{13}$C NMR (75 MHz, $D_2O$) δ 175.09, 174.73, 173.44, 104.72, 101.45, 100.46, 79.97, 74.89, 73.19, 72.62, 72.40, 72.07, 71.78, 70.51, 68.51, 68.00, 67.04, 63.42, 62.66, 62.50, 61.18, 51.90, 51.26, 47.83, 40.19, 28.13, 22.26, 22.04. HRMS (ESI) m/z calcd for $C_{28}H_{46}N_5O_{19}$, $[M-H]^-$, 756.2792; found, 756.2726.

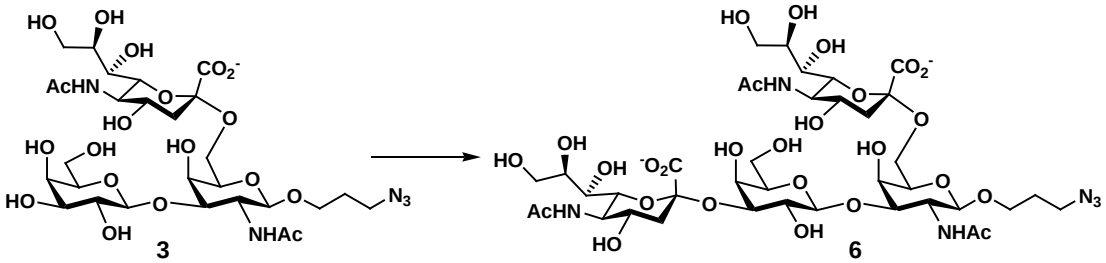



**5**

**Neu5Acα2–6Galβ1–3(Neu5Acα2–6)GalNAcβProN$_3$ (5).** 0.18 g, a white solid, yield 13%. $^1$H NMR (600 MHz, $D_2O$) δ 4.41 (d, *J* = 8.6 Hz, 1H), 4.38 (d, *J* = 7.7 Hz, 1H),

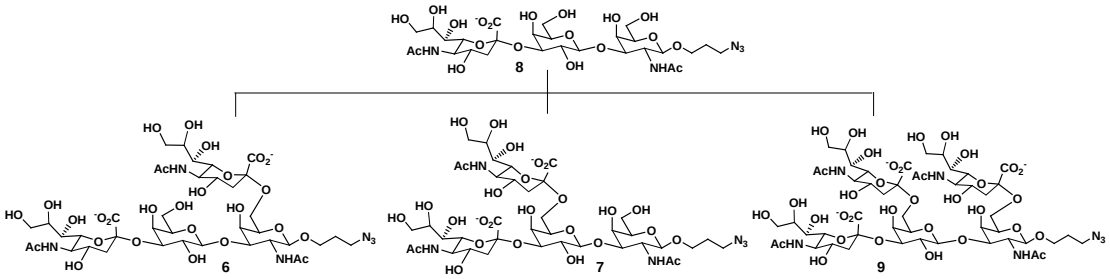
4.09 (d, *J* = 3.2 Hz, 1H,), 3.98 - 3.91 (m, 3H), 3.88 (d, *J* = 3.6 Hz, 1H ), 3.87–3.50 (m, 22H), 3.44 (dd, *J* = 7.8, 9.8 Hz, 1H), 3.33 (m, 2H), 2.68 (dd, *J* = 4.7, 12.5 Hz, 2H), 1.99 (s, 3H), 1.98 (s, 6H), 1.80 (m, 2H), 1.66 (t, *J* = 12.2 Hz, 1H), 1.64 (t, *J* = 12.2 Hz, 1H). $^{13}$C NMR (150 MHz, $D_2O$) δ 174.97, 174.92, 174.62, 174.43, 173.41, 104.45, 101.42, 100.47, 100.30, 79.02, 73.25, 73.02, 72.59, 72.52, 72.23, 71.66, 71.50, 70.37, 68.30, 68.17, 68.15, 68.08. 68.06, 67.96, 67.22, 63.85, 63.11, 62.55, 62.52, 59.20, 51.87, 51.77, 51.19, 47.68, 40.16, 40.09, 28.08, 22.17, 21.94. HRMS (ESI) m/z calcd for $C_{39}H_{65}N_6O_{27}$, $[M+H]^+$, 1049.3898; found, 1049.3781.

**One-pot two-enzyme synthesis of Neu5Acα2–3Galβ1–3(Neu5Acα2–6)GalNAcβPro$N_3$ (6) from trisaccharide 3**



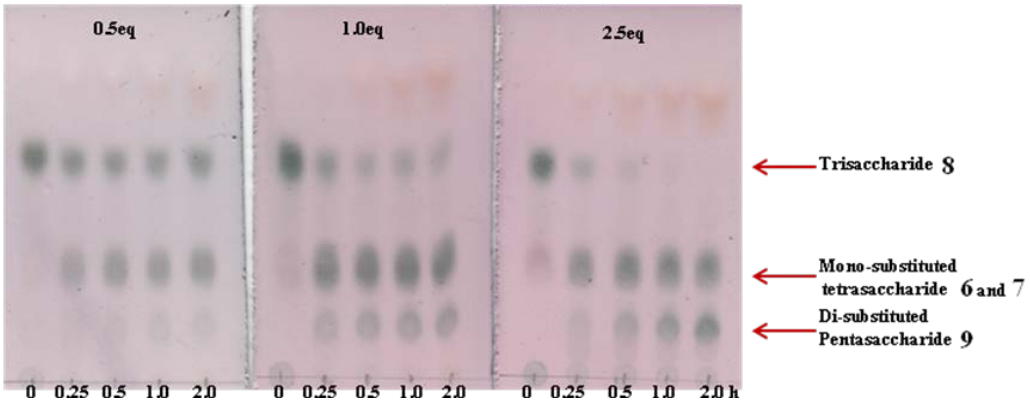

Compound **3** (100 mg, 0.13 mmol), Neu5Ac (80 mg, 2.0 equiv.), and CTP (140 mg, 2.0 equiv.) were dissolved in water in a 50 mL centrifuge tube containing Tris-HCl buffer (100 mmol, pH 8.5) and $MgCl_2$ (20 mmol). After the addition of appropriate amount of NmCSS (1.5 mg) and PmST1 (0.18 mg), water was added to bring the volume of the reaction mixture to 10 mL. The reaction was carried out by incubating the solution in an isotherm incubator for 1 h at 37 °C with agitation at 140 rpm. The product formation of compound **6** ($R_f$ 0.35 ) was monitored by TLC (EtOAc: MeOH: $H_2O$: EtOH = 4: 2: 1: 0.2, v/v) and stained with *p*-anisaldehyde sugar stain. The reaction was quenched by adding the same volume of ice-cold 95% EtOH and incubating at 4 °C for 30 min. The mixture was then centrifuged to remove precipitates. The supernatant was concentrated and passed through a BioGel P-2 gel filtration column to give the di-sialyl tetrasaccharide **6** in 95% yield (130 mg) as a white solid. $^1$H NMR (300 MHz, $D_2O$) δ 4.52 (d, *J* = 7.8 Hz, 1H), 4.51 (d, *J* = 8.4 Hz, 1H), 4.21 (d, *J* = 2.9 Hz, 1H), 4.08 (dd, *J* = 3.1, 9.8 Hz, 1H), 4.05–3.53 (m, 24H), 3.40 (t, *J* = 6.7 Hz, 2H), 2.77 (dd, *J* = 4.7, 9.3 Hz, 1H), 2.73 (dd, *J* = 3.4, 8.0 Hz, 1H), 2.05 (s, 6H), 1.93 (s, 3H), 1.86 (m, 2H), 1.81 (t, *J* = 12.0 Hz, 1H), 1.70 (t, *J* = 12.1 Hz, 1H). $^{13}$C NMR (75 MHz, $D_2O$) δ 175.06, 175.00, 174.71, 173.95, 173.42, 104.66, 101.51, 100.46, 99.73, 80.07, 75.61, 74.77, 73.17, 72.80, 72.65, 71.81, 71.73, 69.01, 68.42, 68.24, 68.09, 67.79, 67.43, 67.26, 63.44, 62.67, 62.51, 60.98, 51.87, 51.69, 51.10, 47.82, 40.23, 39.74, 28.17, 23.79, 22.32, 22.04. HRMS (ESI) m/z calcd for $C_{39}H_{65}N_6O_{27}$, $[M+H]^+$, 1049.3898; found, 1049.3815

**Small-scale one-pot two-enzyme sialylation of trisaccharide 8**



Small-scale one-pot two-enzyme sialylation of trisaccharide **8**[4] was carried out by following the similar procedures described above for sialylation of disaccharide **2**. The reaction was monitored by TLC (EtOAc: MeOH: $H_2O$ = 8: 5: 1, v/v) as showed in **Figure S2** .

**a)**




**b)**

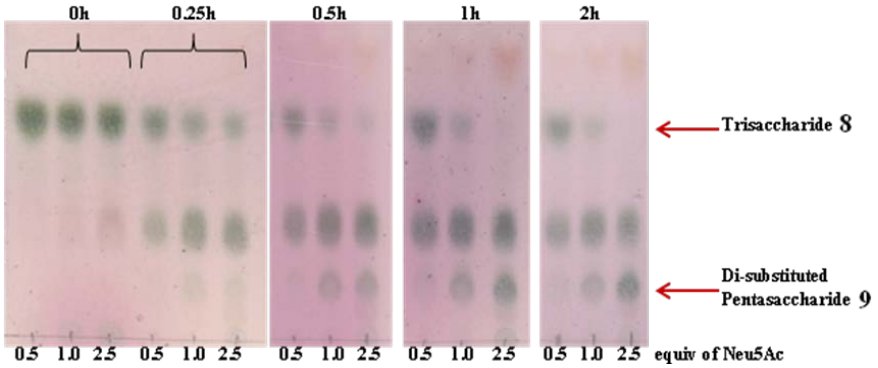


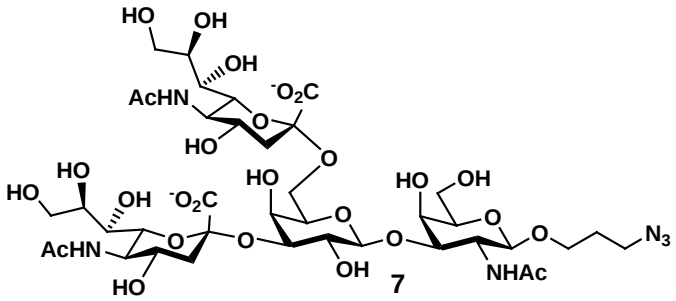

**Figure S2.** Thin-layer chromatography (TLC) analysis of small-scale one-pot two-enzyme sialylation of trisaccharide **8** using various amount of Neu5Ac and CTP (varied at 0.5, 1.0, or 2.5 equiv.).

**Preparative-scale one-pot two-enzyme sialylation of trisaccharide 8**

Compound **8** (200 mg, 0.26 mmol), Neu5Ac (80 mg, 1.0 equiv.), and CTP (170 mg, 1.2 equiv.) were dissolved in water in a 50 mL centrifuge tube containing Tris-HCl

buffer (100 mmol, pH 8.5) and $MgCl_2$ (20 mmol). After the addition of appropriate amounts of NmCSS (1.8 mg) and Pd2,6ST (0.9 mg), water was added to bring the volume of the reaction mixture to 30 mL. The reaction was carried out by incubating the solution in an isotherm incubator for 2 h at 37 °C with agitation at 140 rpm. The product formation of mono-substituted products **6** and **7** ($R_f$ 0.35) and di-substituted product **9** ($R_f$ 0.15) was monitored by TLC (EtOAc: MeOH: $H_2O$: HOAc = 4: 2: 1: 0.2, v/v) and stained with *p*-anisaldehyde sugar stain. The reaction was quenched by adding the same volume of ice-cold 95% EtOH and incubating at 4 °C for 30 min. The mixture was then centrifuged to remove precipitates. The supernatant was concentrated and purified by silica gel chromatography (EtOAc: MeOH: $H_2O$ = 80: 50: 10 to 20: 50: 10, v/v) to give a mixture of two mono-substituted disialyl tetrasaccharides in 63% yield as a white solid (**6** and **7**, 180 mg, **6**: **7** = 21: 79, based on $^1$H NMR), and the di-substituted pentasaccharide **9** in 9% yield as a white solid (40 mg).

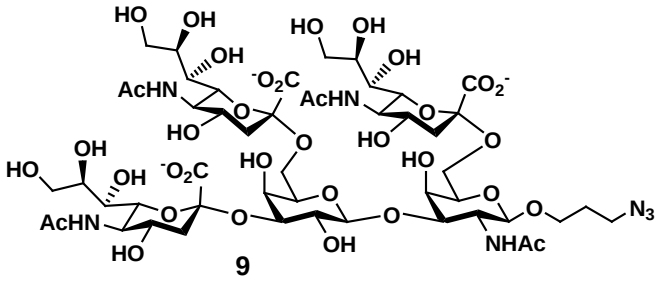
**Neu5Acα2–3(Neu5Acα2–6)Galβ1–3GalNAcβPro$N_3$ (7).**




7

$^1$H NMR (600 MHz, $D_2O$) (according to the major isomer of mixture of **6** and **7**) δ 4.35 (d, *J* = 7.6 Hz, 1H), 4.34 (d, *J* = 6.9 Hz, 1H), 4.04 (d, *J* = 2.9 Hz, 1H), 3.93 (dd, *J* = 2.8, 9.8 Hz, 1H), 3.88 - 3.66 (m, 13H), 3.59–3.37 (m, 13H), 3.24 (t, *J* = 6.7 Hz, 2H), 2.60 (dd, *J* = 4.3, 12.8 Hz, 1H), 2.58 (dd, *J* = 4.4, 12.7 Hz, 1H), 1.89 (s, 9H), 1.70 (m, 2H), 1.64 (t, *J* = 12.1 Hz, 1H), 1.51 (t, *J* = 12.1 Hz, 1H). $^{13}$C NMR (150 MHz, $D_2O$) δ 174.88, 174.84, 174.66, 173.77, 173.35, 104.37, 101.38, 100.32, 99.72, 80.09, 75.36, 74.79, 72.88, 72.68, 72.48, 71.70, 71.63, 68.81, 68.37, 68.25, 68.17, 67.95, 67.77, 67.35, 67.15, 66.91, 63.31, 62.54, 62.39, 61.10, 60.81, 51.75, 51.52, 51.01, 47.70, 40.04, 39.50, 28.03, 22.21, 21.94. HRMS (ESI) m/z calcd for $C_{39}H_{65}N_6O_{27}$ (mixture of **6** and **7**), $[M+H]^+$, 1049.3898; found, 1049.3795.

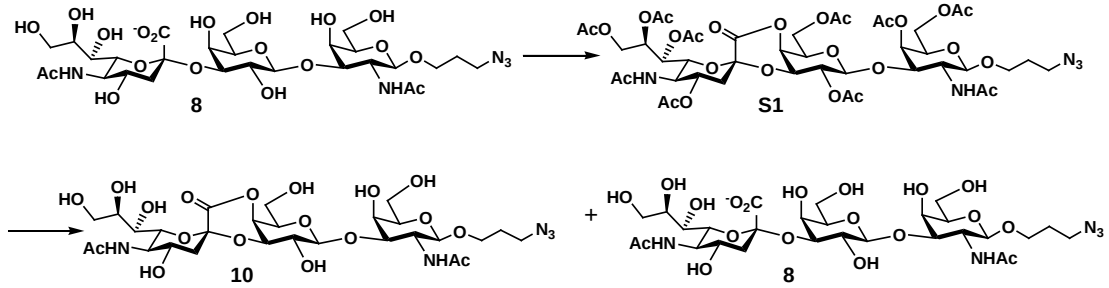
**Neu5Acα2–3(Neu5Acα2–6)Galβ1–3(Neu5Acα2–6)GalNAcβPro$N_3$ (9).**




9

$^1$H NMR (300 MHz, $D_2O$) δ 4.52 (d, *J* = 7.8 Hz, 1H), 4.48 (d, *J* = 8.4 Hz, 1H), 4.15 (d, *J* = 2.8 Hz, 1H), 4.08 (dd, *J* = 3.0, 10.0 Hz, 1H), 4.06–3.51 (m, 33H), 3.40 (t, *J* = 6.7 Hz, 2H), 2.79–2.72 (m, 3H), 2.05 (s, 9H), 2.04 (s, 3H), 1.93–1.66 (m, 5H). $^{13}$C NMR (75 MHz, $D_2O$) δ 175.03, 175.00, 174.96, 174.66, 173.79, 173.43, 173.42, 104.17, 101.54, 100.60, 100.46, 99.85, 79.19, 75.49, 73.39, 73.02, 72.79, 72.67, 72.64, 71.79, 71.75, 71.58, 71.58, 68.91, 68.47, 68.34, 68.28, 68.24, 68.12, 67.93, 67.39, 67.32, 63.97, 63.23, 62.72, 62.53, 52.00, 51.89, 51.66, 51.17, 47.83, 40.27, 40.19, 39.66, 28.18, 22.39, 22.07. HRMS (ESI) m/z calcd for $C_{50}H_{82}N_7O_{35}$, $[M+H]^+$, 1340.4852; found 1340.4685.

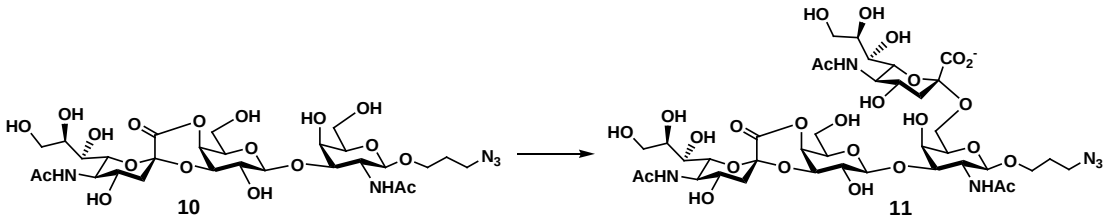
**Neu5Acα2–3Galβ1–3GalNAcβPro$N_3$ 1,4-lactone (10).**



To a solution of compound **8** (130 mg) in pyridine (2 mL) was added acetic anhydride (1 mL), and the reaction mixture was stirred at 0 °C for 12 h under argon. The mixture was concentrated, and the residue was co-evaporated with toluene (3×2 mL). The residue was dissolved in $CH_2Cl_2$ and washed with saturated aqueous $NaHCO_3$, brine, and water successively, dried over $Na_2SO_4$, and concentrated. The residue was purified by silica gel chromatography (PE: EtOAc = 1: 50, v/v) to give peracetylated trisaccharide lactone **S1** in 97% yield as a white solid (179 mg). A solution of **S1** (110 mg, 0.10 mmol) in methanol (5 mL) was treated with NaOMe to maintain the pH at 7.0. When reaction was completed as indicated by TLC (EA: MeOH: $H_2O$: HOAc = 4: 2: 1: 0.2, v/v), the reaction mixture was concentrated and purified by silica gel flash chromatography (PE: EtOAc: $H_2O$ = 2: 1: 0.04) to yield trisaccharide 1,4-lactone **10** in 61% yield as a white solid (46 mg, 0.062 mmol) and trisaccharide **8** in 31% yield as a white solid (24 mg, 0.032 mmol).

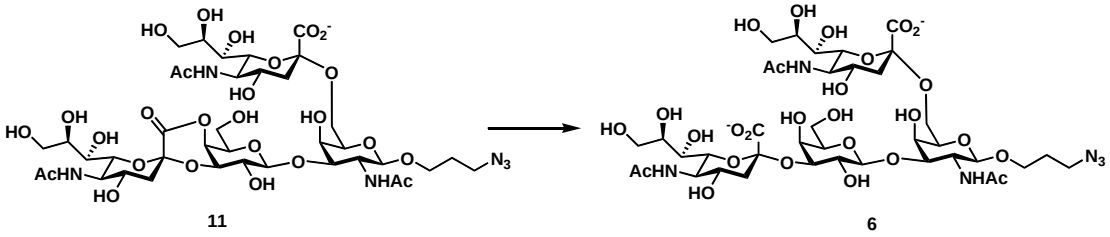
**Neu5Acα2–3Galβ1–3GalNAcβPro$N_3$ lactone (10):** $^1$H NMR (600 MHz, $D_2O$) δ 5.26 (d, *J* = 2.6 Hz, 1H), 4.53 (d, *J* = 7.9 Hz, 1H), 4.46 (d, *J* = 8.5 Hz, 1H), 4.28–4.24 (m, 2H), 4.09 (s, 1H), 3.98–3.92 (m, 3H), 3.88–3.49 (m, 14H), 3.33 (t, *J* = 6.4 Hz, 2H), 2.55 (dd, *J* = 5.3, 13.4 Hz, 1H), 1.99 (s, 3H), 1.98 (s, 3H), 1.80 (m, 2H), 1.76 (t, *J* = 12.6 Hz, 1H). $^{13}$C NMR (150 MHz, $D_2O$) δ 175.00, 174.71, 165.99, 103.74, 101.25, 95.55, 80.13, 74.67, 73.37, 72.58, 72.20, 72.05, 70.55, 70.17, 67.81, 67.56, 67.20, 66.98, 63.18, 60.81, 58.92, 51.52, 51.13, 48.78, 39.26, 28.03, 22.14, 21.96. HRMS (ESI) m/z calcd for $C_{28}H_{46}N_5O_{18}$, $[M+H]^+$, 740.2838; found, 740.2847.

**Neu5Acα2–3Galβ1–3(Neu5Acα2–6)GalNAcβPro$N_3$ 1,4-lactone (11).**



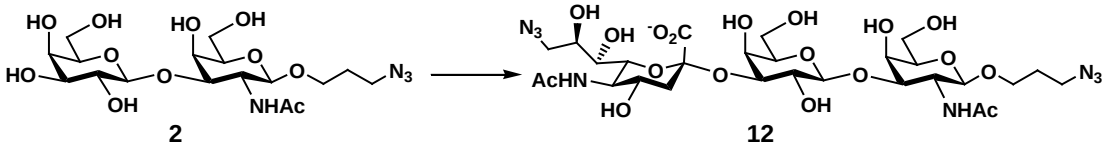
Compound **10** (40 mg, 0.054 mmol) and CMP-Neu5Ac (69 mg, 0.11 mmol) were dissolved in water in a 50 mL centrifuge tube containing Tris-HCl buffer (100 mmol, pH 7.0). After the addition of an appropriate amount of Pd2,6ST (0.75 mg), water was added to bring the volume of the reaction mixture to 10 mL. The reaction was carried out by incubating the solution in an isotherm incubator for 2 h at 37 °C with agitation at 140 rpm. The product formation of mono-substituted product **11** ($R_f$ 0.55) was monitored by TLC (EtOAc: MeOH: $H_2O$: HOAc = 4: 2: 1: 0.2, v/v) and stained with *p*-anisaldehyde sugar stain. The reaction was quenched by adding the same volume of ice-cold 95% EtOH and incubating at 4 °C for 30 min. The mixture was then centrifuged to remove precipitates. The supernatant was concentrated and purified by silica gel chromatography (EtOAc: MeOH: $H_2O$ = 80: 50: 10 to 20: 50: 10, v/v) to give tetrasaccharide 1,4-lactone **11** in 86% yield as a white solid (48 mg, 0.046 mmol). $^1$H NMR (600 MHz, $D_2O$) δ 5.24 (d, *J* = 3.8 Hz, 1H), 4.50 (d, *J* = 8.0 Hz, 1H), 4.43 (d, *J* = 8.5 Hz, 1H), 4.27–4.22 (m, 2H), 4.11 (d, *J* = 2.6 Hz, 1H), 3.96–3.48 (m, 24H), 3.33–3.29 (m, 2H), 2.67 (dd, *J* = 4.4, 12.4 Hz, 1H), 2.54 (dd, *J* = 5.4, 13.4 Hz, 1H), 1.98 (s, 3H), 1.97 (s, 3H), 1.96 (s, 3H), 1.78 (m, 2H), 1.75 (t, *J* = 11.8 Hz, 1H), 1.63 (t, *J* = 12.2 Hz, 1H). $^{13}$C NMR (150 MHz, $D_2O$) δ 181.56, 174.98, 174.66, 173.40, 165.98, 103.75, 101.31, 100.33, 95.53, 80.12, 73.39, 73.03, 72.57, 72.55, 72.22, 72.09, 71.65, 70.52, 70.13, 68.10, 67.68, 67.57, 67.19, 63.25, 63.16, 62.51, 62.36, 59.38, 58.95, 51.76, 51.50, 51.04, 47.67, 40.08, 39.27, 28.06, 23.16, 22.06, 21.95. HRMS (ESI) m/z calcd for $C_{39}H_{62}N_6O_{26}$, $[M-2H]^{2-}$, 514.1779; found, 514.1765.

**Synthesis of Neu5Acα2–3Galβ1–3(Neu5Acα2–6)GalNAcβProN$_3$ (6) from tetrasaccharide 1,4-lactone 11.**



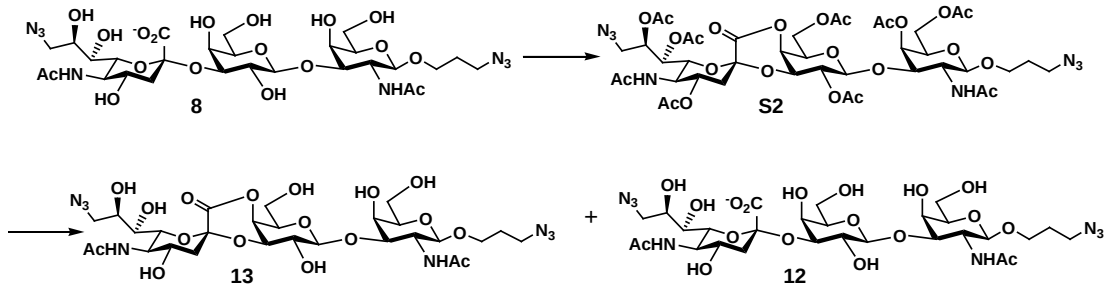
A solution of compound **11** (35 mg, 0.034 mmol) in 5 mL of 1 M NaOH was stirred for 4 h at room temperature and was subsequently neutralized by adding of acid ion-exchange resin (Amberlyst 15). After filtration, solvent was removed in vacuo, and the resulting solid was dissolved in water and purified by passing through a BioGel P-2 (2.5×100 cm) gel filtration column to afford **6** in 98% yield as a white solid (35 mg, 0.033 mmol). The NMR spectra were identical with those of compound **6** prepared from trisaccharide **3** as described above.

**Neu5Ac9N$_3$α2–3Galβ1–3GalNAcβProN$_3$ (12).**



Trisaccharide **12** was prepared by following a similar procedure as described for the trisaccharide **8**[1]. Compound **2** (47 mg, 0.1 mmol), 9N$_3$-Neu5Ac (50 mg, 0.15 mmol), and CTP (95 mg, 0.15 mmol) were dissolved in water in a 50 mL centrifuge tube containing Tris-HCl buffer (100 mmol, pH 8.5) and $MgCl_2$ (20 mmol). After the addition of appropriate amounts of NmCSS (1.1 mg) and PmST1 (0.21 mg), water was added to bring the volume of the reaction mixture to 10 mL. The reaction was carried out by incubating the solution in an isotherm incubator for 1 h at 37 °C with agitation at 140 rpm. The product formation of **12** ($R_f$ 0.65) was monitored by TLC (EtOAc: MeOH: $H_2O$: EtOH = 4: 2: 1: 0.2, v/v) and stained with *p*-anisaldehyde sugar stain. The reaction was quenched by adding the same volume of ice-cold 95% EtOH and was incubated at 4 °C for 30 min. It was then centrifuged to remove precipitates, and the supernatant was concentrated and purified by silica gel flash chromatography to give the trisaccharide **12** in 96% yield as a white solid (75 mg, 0.096 mmol). $^1$H NMR (600 MHz, $D_2O$) δ 4.46 (d, *J* = 8.1 Hz, 2H), 4.13 (s, 1H), 4.01 (d, *J* = 9.9 Hz, 1H), 3.98–3.92 (m, 3H), 3.86 (s, 1H), 3.82–3.54 (m, 13H), 3.49 (t, *J* = 8.8 Hz, 1H), 3.45 (dd, *J* = 6.4, 13.2 Hz, 1H), 3.34 (t, *J* = 6.3 Hz, 2H), 2.70 (dd, *J* = 4.0, 12.4 Hz, 1H), 1.99 (s, 3H), 1.98 (s, 3H), 1.80 (m, 2H), 1.73 (t, *J* = 12.2 Hz, 1H). $^{13}$C NMR (151 MHz, $D_2O$) δ 174.82, 174.63, 173.84, 104.49, 101.29, 99.57, 79.90, 75.48, 74.66, 72.48, 70.44, 68.93, 68.63, 68.32, 67.78, 67.73, 66.91, 60.89, 60.86, 60.28, 52.92, 51.54, 51.08, 47.69, 39.68, 28.03, 22.20, 21.96. HRMS (ESI) m/z calcd for $C_{28}H_{46}N_8O_{18}$, $[M+H]^+$, 783.3008, found 783.3036.

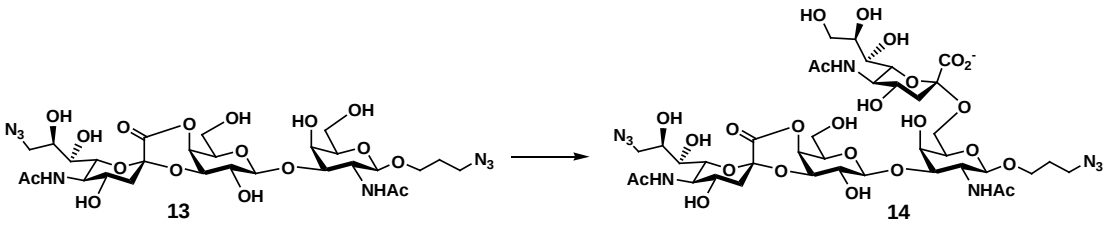
**Neu5Ac9N$_3$α2–3Galβ1–3GalNAcβProN$_3$ 1,4-lactone (13).**



Trisaccharide lactone **13** was prepared by following a similar procedure as described for trisaccharide lactone **10**. Briefly, to a solution of **12** (168 mg, 0.21 mmol) in pyridine (5.0 mL) was added acetic anhydride (2.5 mL), the reaction mixture was stirred at 0 °C for 12 h. The mixture was concentrated, and the residue was co-evaporated with toluene (3×2 mL) and dried under high vacuum. The residue was purified by silica gel flash chromatography (PE: EtOAc = 1: 50, v/v) to afford peracetylated trisaccharide 1,4-lactone **S2** in 93% as a white solid (203 mg, 0.20

mmol). To a solution of **S2** (107 mg, 0.105 mmol) in MeOH (5 mL), NaOMe was added to maintains the pH at 7.0, and the reaction mixture was stirred at room temperature until the TLC (EA: MeOH = 2:1, v/v) showed complete consumption of **S2** and the formation of **13** ($R_f$ 0.67) and hydrolysis product **12**. The reaction mixture was concentrated and the residue was purified by silica gel flash chromatography (PE: EtOAc: $H_2O$ = 3: 1: 0.1, v/v) to yield trisaccharide lactone **13** in 59% as a white solid (47.5 mg, 0.062 mmol), and the hydrolysis product **12** in 30% as a white solid (24.7 mg, 0.032 mmol). $^1$H NMR (600 MHz, $D_2O$) δ 5.23 (d, *J* = 4.0 Hz, 1H), 4.52 (d, *J* = 7.9 Hz, 1H), 4.46 (d, *J* = 8.5 Hz, 1H), 4.28–4.22 (m, 2H), 4.09 (d, *J* = 2.8 Hz, 1H), 4.09–3.48 (m, 16H), 3.39 (dd, *J* = 5.8, 13.2 Hz, 1H), 3.35–3.31 (m, 2H), 2.55 (dd, *J* = 5.4, 13.5 Hz, 1H), 2.00 (s, 3H), 1.98 (s, 3H), 1.80 (m, 2H), 1.76 (t, *J* = 11.5 Hz, 1H). $^{13}$C NMR (151 MHz, $CDCl_3$) δ 174.95, 174.69, 165.88, 103.76, 101.23, 95.56, 80.15, 74.66, 73.35, 72.42, 72.19, 71.99, 71.96, 70.11, 69.46, 67.80, 67.76, 67.52, 66.94, 62.37, 60.79, 60.23, 58.88, 53.97, 51.52, 51.13, 47.69, 39.26, 28.02, 22.13, 21.94. HRMS (ESI) m/z calcd for $C_{28}H_{45}N_8O_{17}$, $[M+H]^+$, 765.2903, found 765.2922.

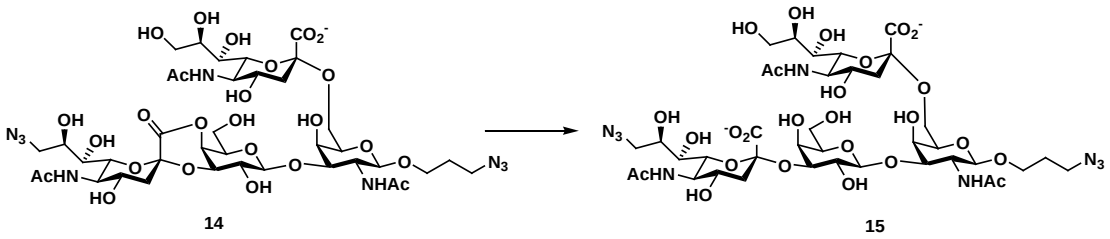
**Neu5Ac9N$_3$α2–3Galβ1–3(Neu5Acα2–6)GalNAcβProN$_3$ 1,4-lactone (14).**




Tetrasaccharide lactone **14** was prepared by following a similar procedure as described for tetrasaccharide lactone **11**. Briefly, trisaccharide lactone **13** (40 mg, 0.052 mmol) and CMP-Neu5Ac (50 mg, 0.078 mmol) were dissolved in water in a 50 mL centrifuge tube containing Tris-HCl buffer (100 mmol, pH 7.0). After the addition of an appropriate amount of Pd2,6ST (0.7 mg), water was added to bring the volume of the reaction mixture to 10 mL. The reaction was carried out by incubating the solution in an isotherm incubator for 2 h at 37 °C with agitation at 140 rpm. The product formation of tetrasaccharide lactone **14** ($R_f$ 0.55) was monitored by TLC (EtOAc: MeOH: $H_2O$: HOAc = 4: 2: 1: 0.2, v/v) and stained with *p*-anisaldehyde sugar stain. The reaction was quenched by adding the same volume of ice-cold 95% EtOH and incubating at 4 °C for 30 min. The mixture was then centrifuged to remove precipitates. The supernatant was concentrated and purified by silica gel chromatography (EtOAc: MeOH: $H_2O$ = 80: 50: 10, v/v) to give tetrasaccharide lactone **14** in 89% yield as a white solid (49.2 mg, 0.046 mmol). $^1$H NMR (600 MHz, $D_2O$) δ 5.22 (d, *J* = 4.0 Hz, 1H), 4.51 (d, *J* = 8.0 Hz, 1H), 4.44 (d, *J* = 8.5 Hz, 1H), 4.23 (dd, *J* = 3.9, 9.7 Hz, 1H), 4.11 (d, *J* = 2.9 Hz, 1H), 3.96–3.50 (m, 24H), 3.40 (dd, *J* = 5.6, 13.3 Hz, 1H), 3.35–3.30 (m, 2H), 2.68 (dd, *J* = 4.6, 12.4 Hz, 1H), 2.55 (dd, *J* = 5.4, 13.5 Hz, 1H), 2.00 (s, 3H), 1.98 (s, 3H), 1.97 (s, 3H), 1.80 (m, 2H), 1.75 (t, *J* = 11.5 Hz, 1H), 1.64 (t, *J* = 12.0 Hz, 1H). $^{13}$C NMR (151 MHz, $CDCl_3$) δ 174.95, 174.65, 173.39, 165.87, 103.77, 101.31, 100.33, 95.54, 80.14, 73.37, 73.04, 72.56, 72.41, 72.23, 72.03, 71.96, 71.64, 70.08, 69.41, 68.09, 67.68, 67.53, 67.17, 63.26,

62.52, 62.37, 58.91, 53.94, 51.75, 51.49, 51.05, 47.67, 40.09, 39.27, 28.06, 22.12, 21.93. HRMS (ESI) m/z calcd for $C_{39}H_{62}N_9O_{25}$, $[M+H]^+$, 1056.3857, found 1056.3885.

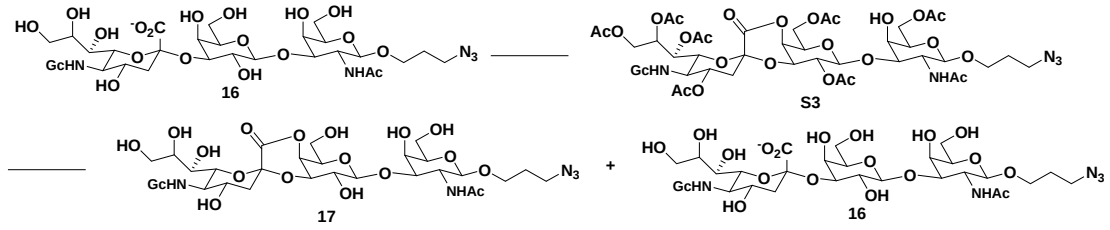
**Synthesis of Neu5Ac9N$_3$α2–3Galβ1–3(Neu5Acα2–6)GalNAcβProN$_3$ (15) from**




**tetrasaccharide 1,4-lactone 14.**

A solution of compound **14** (42 mg, 0.04 mmol) in 5 mL of 1 M NaOH was stirred at room temperature for 4 h. When TLC (EA: MeOH: $H_2O$: HOAc = 4: 2: 1: 0.2, v/v) showed complete consumption of the compound **14** and the formation of the **15** ($R_f$ 0.25), the reaction mixture was neutralized by adding acidic ion-exchange resin (Amberlyst 15). After filtration, the solvent was removed in vacuo, and the resulting solid was dissolved in water and purified by passing through a BioGel P-2 (2.5×100 cm) gel filtration column to afford **15** in 96% yield as a white solid (41 mg, 0.038 mmol). $^1$H NMR (600 MHz, $D_2O$) δ 4.35 (d, *J* = 8.2 Hz, 2H), 4.05 (d, *J* = 2.8 Hz, 1H), 3.92 (dd, *J* = 3.0, 9.8 Hz, 1H), 3.87–3.81 (m, 3H), 3.78 (d, *J* = 2.9 Hz, 1H), 3.76–3.34 (m, 22H), 3.24 (m, 2H), 2.60 (dd, *J* = 4.6, 12.5 Hz, 1H), 2.57 (dd, *J* = 4.7, 12.7 Hz, 1H), 1.89 (s, 9H), 1.70 (m, 2H), 1.66 (t, *J* = 12.1 Hz, 1H), 1.57 (t, *J* = 12.2 Hz, 1H). $^{13}$C NMR (151 MHz, $CDCl_3$) δ 174.93, 174.82, 174.62, 173.34, 172.81, 104.52, 101.37, 99.98, 99.36, 79.95, 75.47, 74.60, 72.98, 72.60, 72.53, 72.00, 71.40, 70.24, 68.91, 68.60, 68.13, 67.93, 67.67, 67.26, 67.15, 63.31, 62.61, 62.36, 60.83, 53.05, 51.71, 51.53, 50.99, 47.68, 39.87, 39.48, 28.06, 22.18, 21.96, 21.94. HRMS (ESI) m/z calcd for $C_{39}H_{64}N_9O_{26}$, $[M+H]^+$, 1074.3962, found 1074.4009.

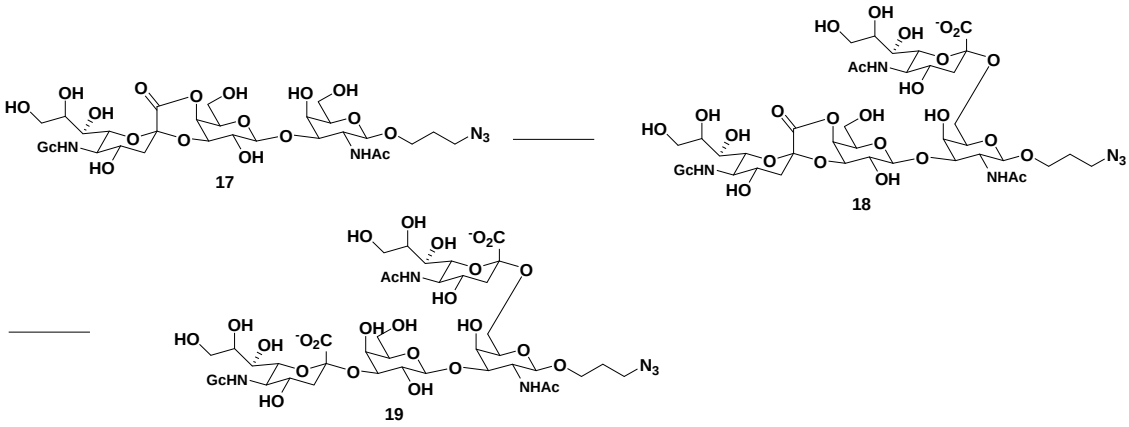
**Neu5Gcα2–3Galβ1–3GalNAcβProN$_3$1,4-lactone (17).**




Trisaccharide lactone **17** was prepared by following a similar procedure as described for trisaccharide lactone **10**. Briefly, to a solution of **16**[4] (65 mg, 0.084 mmol) in pyridine (5.0 mL) was added acetic anhydride (2.5 mL), the reaction mixture was stirred at 0 °C for 12 h. The mixture was concentrated, and the residue was co-evaporated with toluene (3×1.5 mL) and dried under high vacuum. The residue was purified by silica gel flash chromatography (PE: EtOAc = 1: 50, v/v) to afford peracetylated trisaccharide 1,4-lactone **S3** in 96% as a white solid (82 mg, 0.075

mmol). To a solution of **S3** (82 mg, 0.075 mmol) in MeOH (4 mL), NaOMe was added to maintains the pH at 7.5, and the reaction mixture was stirred at room temperature until the TLC (EA: MeOH: $H_2O$ = 2:1:0.2, v/v) showed complete consumption of **S3** and the formation of **17** ($R_f$ 0.30) and hydrolysis product **16**. The reaction mixture was concentrated and the residue was purified by silica gel flash chromatography (EtOAc: MeOH: $H_2O$ = 2: 1: 0.1, v/v) to yield trisaccharide lactone **17** in 47% as a white solid (26.4 mg, 0.048 mmol). $^1$H NMR (600 MHz, $D_2O$) δ 5.31 (d, *J* = 3.8 Hz, 1H), 4.57 (d, *J* = 8.0 Hz, 1H), 4.49 (d, *J* = 8.5 Hz, 1H), 4.40 (m, 1H), 4.29 (dd, *J* = 9.6, 4.0 Hz, 1H), 4.12(s, 2H), 4.04–3.48 (m, 15H), 3.37 (t, *J* = 6.5 Hz, 2H), 2.61 (dd, *J* = 5.5, 13.5 Hz, 1H), 2.02 (s, 3H), 1.84 (m, 2H), 1.82 (t, *J* = 10.0 Hz, 1H). $^{13}$C NMR (150 MHz, $D_2O$) δ 175.73, 174.67, 165.99, 103.69, 101.22, 95.57, 80.11, 74.64, 73.38, 72.26, 72.00, 70.59, 70.17, 67.81, 67.31, 67.14, 66.98, 63.13, 62.38, 60.89, 60.80, 58.94, 51.17, 48.78, 47.71, 39.29, 28.02, 22.15. HRMS (ESI) m/z calcd for $C_{28}H_{45}N_5NaO_{19}$, $[M+Na]^+$, 778.2606, found 778.2596.

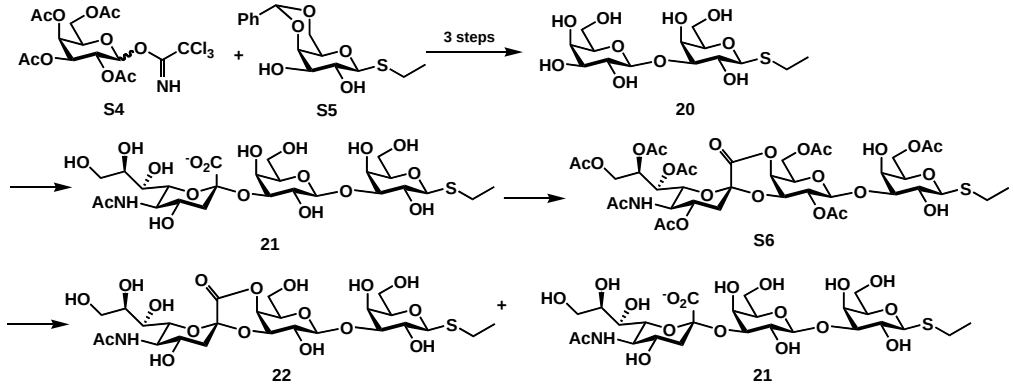
**Synthesis of Neu5Gcα2–3Galβ1–3(Neu5Acα2–6)GalNAcβPro$N_3$ (19) from trisaccharide 1,4-lactone 17.**




Compound **17** (17 mg, 0.023 mmol) and CMP-Neu5Ac (37 mg, 0.056 mmol) were dissolved in water in a 50 mL centrifuge tube containing Tris-HCl buffer (100 mmol, pH 7.0). After the addition of an appropriate amount of Pd2,6ST (0.075 mg), water was added to bring the volume of the reaction mixture to 3 mL. The reaction was carried out by incubating the solution in an isotherm incubator for 2 h at 37 °C with agitation at 140 rpm. The product formation of mono-substituted product **18** ($R_f$ 0.55) was monitored by TLC (EtOAc: MeOH: $H_2O$: HOAc = 4: 2: 1: 0.2, v/v) and stained with *p*-anisaldehyde sugar stain. The reaction was quenched by adding the same volume of ice-cold 95% EtOH and incubating at 4 °C for 30 min. The mixture was then centrifuged to remove precipitates. The supernatant was concentrated and purified by silica gel chromatography (EtOAc: MeOH: $H_2O$ = 80: 50: 5 to 30: 50: 5, v/v) to give tetrasaccharide 1,4-lactone **18** in 93% yield as a white solid (22 mg, 0.021 mmol). A solution of compound **18** (22 mg, 0.021 mmol) in 2 mL of 1 M NaOH was stirred for 2 h at room temperature and was subsequently neutralized by adding of acid ion-exchange resin (Amberlyst 15). After filtration, solvent was removed in vacuo, and the resulting solid was dissolved in water and purified by passing through

a BioGel P-2 (2.5×100 cm) gel filtration column to afford **19** in 98% yield as a white solid (21.9 mg, 0.021 mmol). $^{1}$H NMR (600 MHz, $D_2O$) δ 4.49 (d, *J* = 8.0 Hz, 1H), 4.47 (d, *J* = 8.7 Hz, 1H), 4.18 (d, *J* = 2.5 Hz, 1H), 4.10 (s, 2H), 4.06 (dd, *J* = 9.8, 2.8 Hz, 1H), 4.00–3.51 (m, 32 H), 3.36 (m, 2H), 2.75 (dd, *J* = 12.4, 4.5 Hz, 1H), 2.70 (dd, *J* = 12.4, 4.4 Hz, 1H), 2.02 (s, 6H), 1.82 (m, 2H), 1.79 (t, *J* = 12.2 Hz, 1H), 1.67 (t, *J* = 12.2 Hz, 1H). $^{13}$C NMR (150 MHz, $D_2O$) δ 175.61, 174.92, 174.58, 173.87, 173.31, 104.56, 101.38, 100.31, 99.59, 79.96, 75.47, 74.65, 73.03, 72.52, 72.39, 71.94, 71.61, 68.88, 68.11, 68.04, 67.87, 67.66, 67.28, 67.13, 63.32, 62.52, 62.36, 61.33, 60.86, 59.24 , 51.74, 51.26, 50.98, 47.68, 40.11, 39.67, 28.05, 22.19, 21.92. HRMS (ESI) m/z calcd for $C_{39}H_{64}N_6O_{28}$, $[M+Na]^+$, 1087.3666, found 1087.3671.

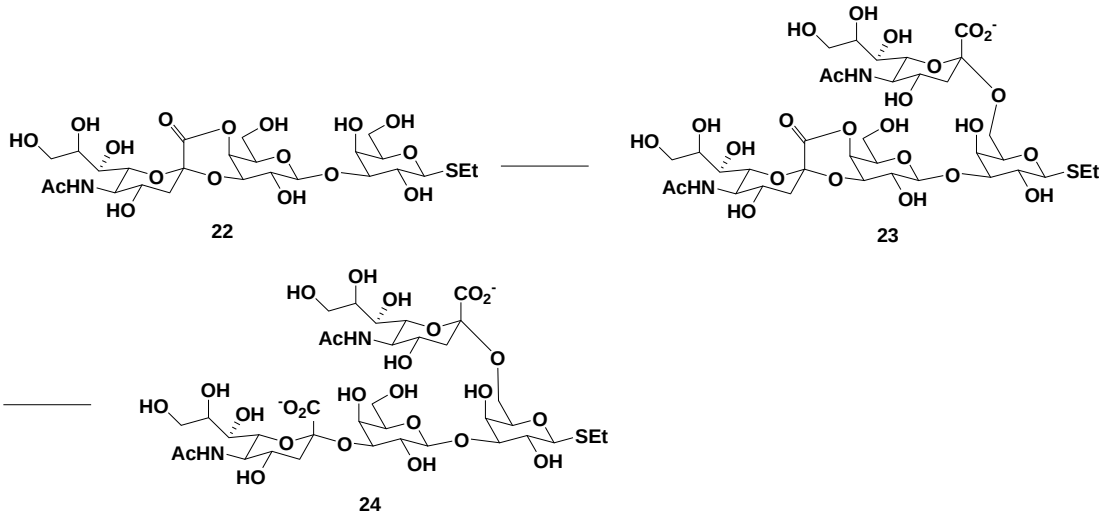
**Neu5Acα2–3Galβ1–3GalβSEt1,4-lactone (22).**




Trisaccharide lactone **22** was synthesized starting from disaccharide **20**, which was prepared following standard chemical glycosylation protocol from monosaccharide donor **S4** and monosaccharide acceptor **S5** in 3 steps. The disaccharide was then transformed to sialylated trisacchaaride **21** by following a similar procedure as described for synthesis of tetrasaccharide **6** from trisaccharide **3**. To a solution of **20** (248 mg, 0.366 mmol) in pyridine (10.0 mL) was added acetic anhydride (5.0 mL), the reaction mixture was stirred at 0 °C for 12 h. The mixture was concentrated, and the residue was co-evaporated with toluene (3×2 mL) and dried under high vacuum. The residue was purified by silica gel flash chromatography (EtOAc: MeOH = 100:1, v/v) to afford peracetylated trisaccharide 1,4-lactone **S7** in 90% as a white solid (328 mg, 0.33 mmol). To a solution of **S6** (328 mg, 0.33 mmol) in MeOH (8 mL), NaOMe was added to maintains the pH at 7.5, and the reaction mixture was stirred at room temperature until the TLC (EA: MeOH: $H_2O$ = 2:1:0.5, v/v) showed complete consumption of **S7** and the formation of **22** ($R_f$ 0.70) and hydrolysis product **22**. The reaction mixture was concentrated and the residue was purified by silica gel flash chromatography (EtOAc: MeOH: $H_2O$ = 2: 1: 0.1, v/v) to yield trisaccharide lactone **20** in 50% as a white solid (103 mg, 0.156 mmol). $^{1}$H NMR (600 MHz, $D_2O$) δ 5.32 (d, *J* = 3.7 Hz, 1H), 4.85 (d, *J* = 8.0 Hz, 1H), 4.55 (d, *J* = 8.8 Hz, 1H), 4.33–4.29 (m, 2H), 4.07–3.53 (m, 16H), 2.75 (m, 2H), 2.61 (dd, *J* = 5.4, 13.4 Hz, 1H), 2.04 (s, 3H), 1.81 (t, *J* = 12.6 Hz, 1H), 1.25 (t, *J* = 7.4 Hz, 3H). $^{13}$C NMR (150 MHz, $D_2O$) δ 174.97, 166.09, 102.24, 95.54, 82.83, 78.80, 77.10, 74.15, 73.68, 72.59, 72.15, 72.06, 71.95, 71.07, 70.50, 68.94, 67.57, 67.22, 63.20, 60.86, 58.70, 51.52, 48.77, 39.24,

23.45, 21.94, 13.79. HRMS (ESI) m/z calcd for $C_{25}H_{41}NO_{17}S$, $[M+NH_4]^+$, 677.2439, found 677.2436.

**Synthesis of Neu5Acα2–3Galβ1–3(Neu5Acα2–6)GalβSEt (24) from trisaccharide 1,4-lactone 22.**




Compound **22** (50 mg, 0.076 mmol) and CMP-Neu5Ac (150 mg, 0.228 mmol) were dissolved in water in a 50 mL centrifuge tube containing Tris-HCl buffer (100 mmol, pH 7.0). After the addition of an appropriate amount of Pd2,6ST (0.56 mg), water was added to bring the volume of the reaction mixture to 7 mL. The reaction was carried out by incubating the solution in an isotherm incubator for 2 h at 37 °C with agitation at 140 rpm. The product formation of mono-substituted product **23** ($R_f$ 0.6) was monitored by TLC (EtOAc: MeOH: $H_2O$: HOAc = 4: 2: 1: 0.2, v/v) and stained with *p*-anisaldehyde sugar stain. The reaction was quenched by adding the same volume of ice-cold 95% EtOH and incubating at 4 °C for 30 min. The mixture was then centrifuged to remove precipitates. The supernatant was concentrated and purified by silica gel chromatography (EtOAc: MeOH: $H_2O$ = 100: 50: 5, v/v) to give tetrasaccharide 1,4-lactone **23** in 92% yield as a white solid (66.3 mg, 0.07 mmol). A solution of compound **23** (35 mg, 0.037 mmol) in 2 mL of 1 M NaOH was stirred for 2 h at room temperature and was subsequently neutralized by adding of acid ion-exchange resin (Amberlyst 15). After filtration, solvent was removed in vacuo, and the resulting solid was dissolved in water and purified by passing through a BioGel P-2 (2.5×100 cm) gel filtration column to afford **24** in 98% yield as a white solid (35.7 mg, 0.037 mmol). $^1$H NMR (600 MHz, $D_2O$) δ 4.54 (t, *J* = 4.3 Hz, 1H), 4.09 (dd, *J* = 2.9, 9.6 Hz, 1H), 3.98–3.53 (m, 25 H), 2.78–2.70 (m, 4H), 2.02 (s, 6H), 1.80 (t, *J* = 12.2 Hz, 1H), 1.67 (t, *J* = 12.2 Hz, 1H), 1.25 (t, *J* = 7.3 Hz, 3H). $^{13}$C NMR (150 MHz, $D_2O$) $^{13}$C NMR (125 MHz, $D_2O$) δ 174.92, 174.87, 173.75, 173.33, 102.84, 100.31, 99.72, 83.23, 77.08, 76.32, 75.63, 74.76, 74.09, 72.74, 72.53, 71.95, 71.67, 69.79, 68.88, 68.19, 68.01, 67.39, 63.25, 62.49, 62.37, 61.24, 60.69, 59.29, 51.75, 51.58, 48.76, 40.08, 39.56, 23.73, 21.93, 13.85. HRMS (ESI) m/z calcd for $C_{36}H_{60}N_2O_{26}S$, $[M+Na]^+$, 991.3053, found 991.3055.

## References

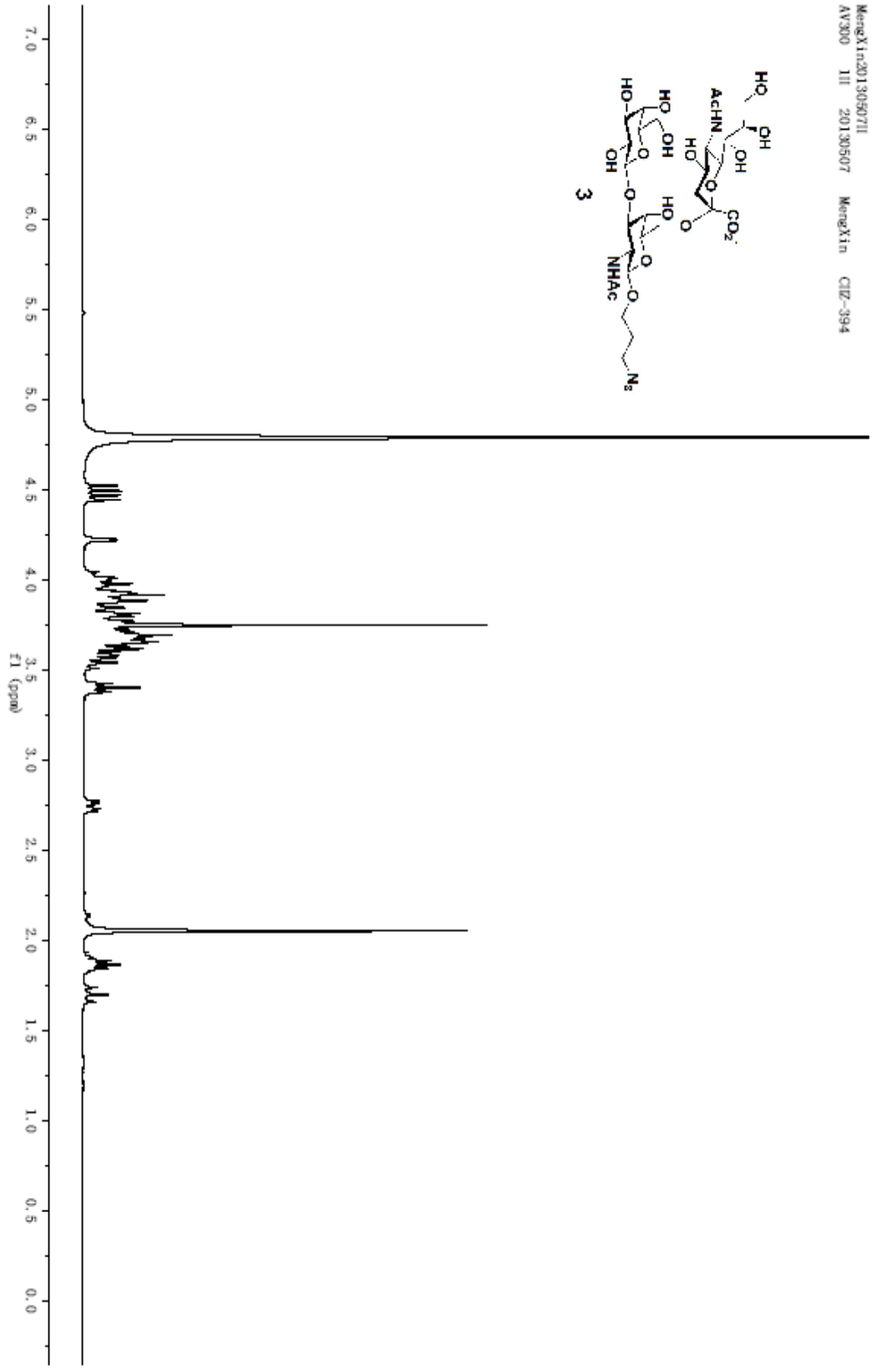

1. Yu, H., Chokhawala, H., Karpel, R., Yu, H., Wu, B.; Zhang, J.; Zhang, Y.; Jia, Q.; Chen, X. *J. Am. Chem. Soc.* **2005**, *127*, 17618-17619.

2. Yu, H.; Huang, S.; Chokhawala, H.; Sun, M.; Zheng, H.; Chen, X. *Angew. Chem., Int. Ed.* **2006,** *45*, 3938-3944.

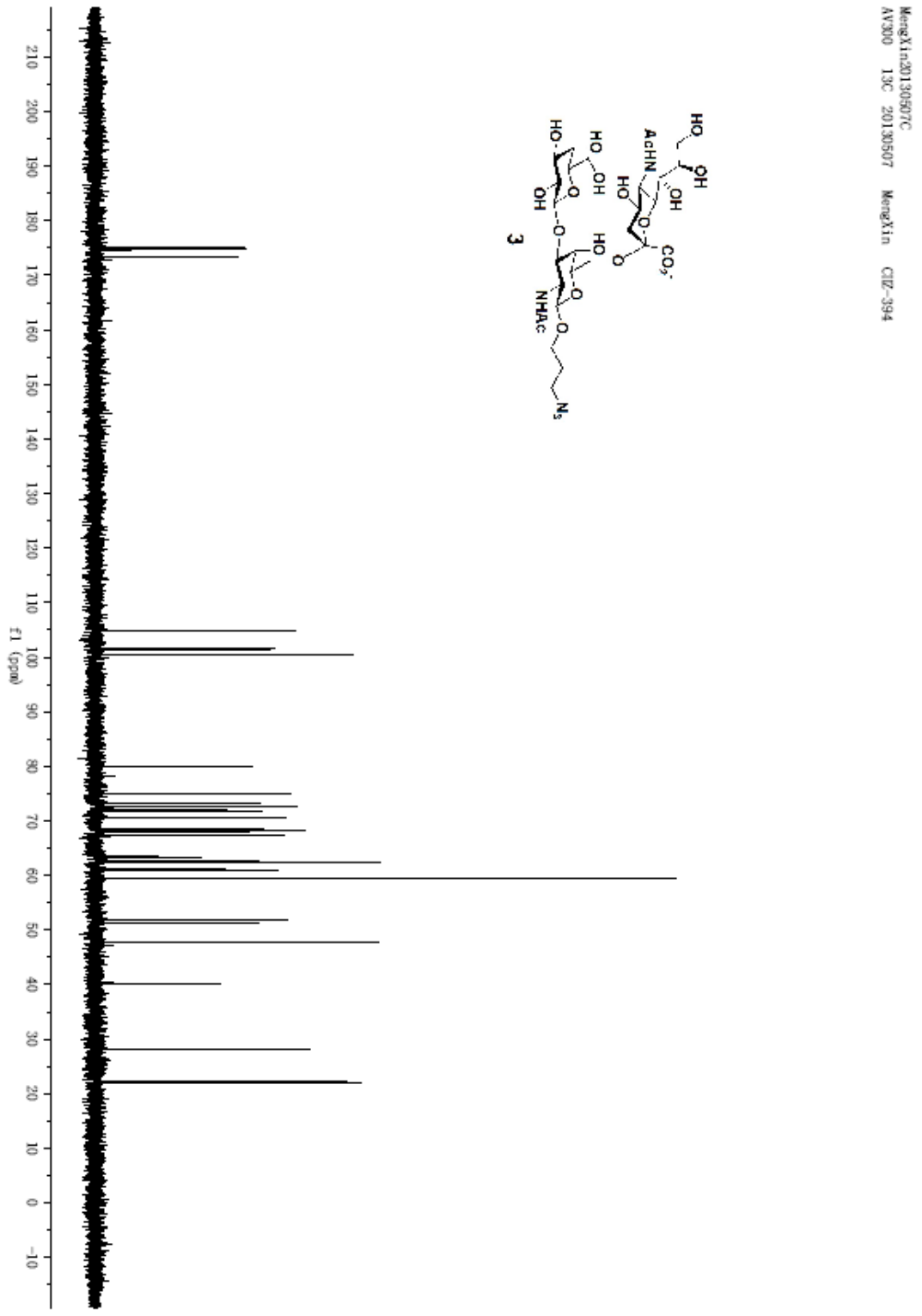
3. Yu, H.; Thon, V.; Lau, K.; Cai, L.; Chen, Y.; Mu, S.; Li, Y.; Wang, P. G.; Chen, X.; *Chem. Commun.*, **2010,** *46*, 7507–7509.

4. Lau, K.; Yu, H.; Thon, V.; Khedri, Z.; Leon, M. E.; Tran, B. K.; Chen, X. *Org. Biomol. Chem.* **2011**, 9, 2784.

$^{1}$H NMR spectrum for compound **3**



$^{13}$C NMR spectrum for compound **3**



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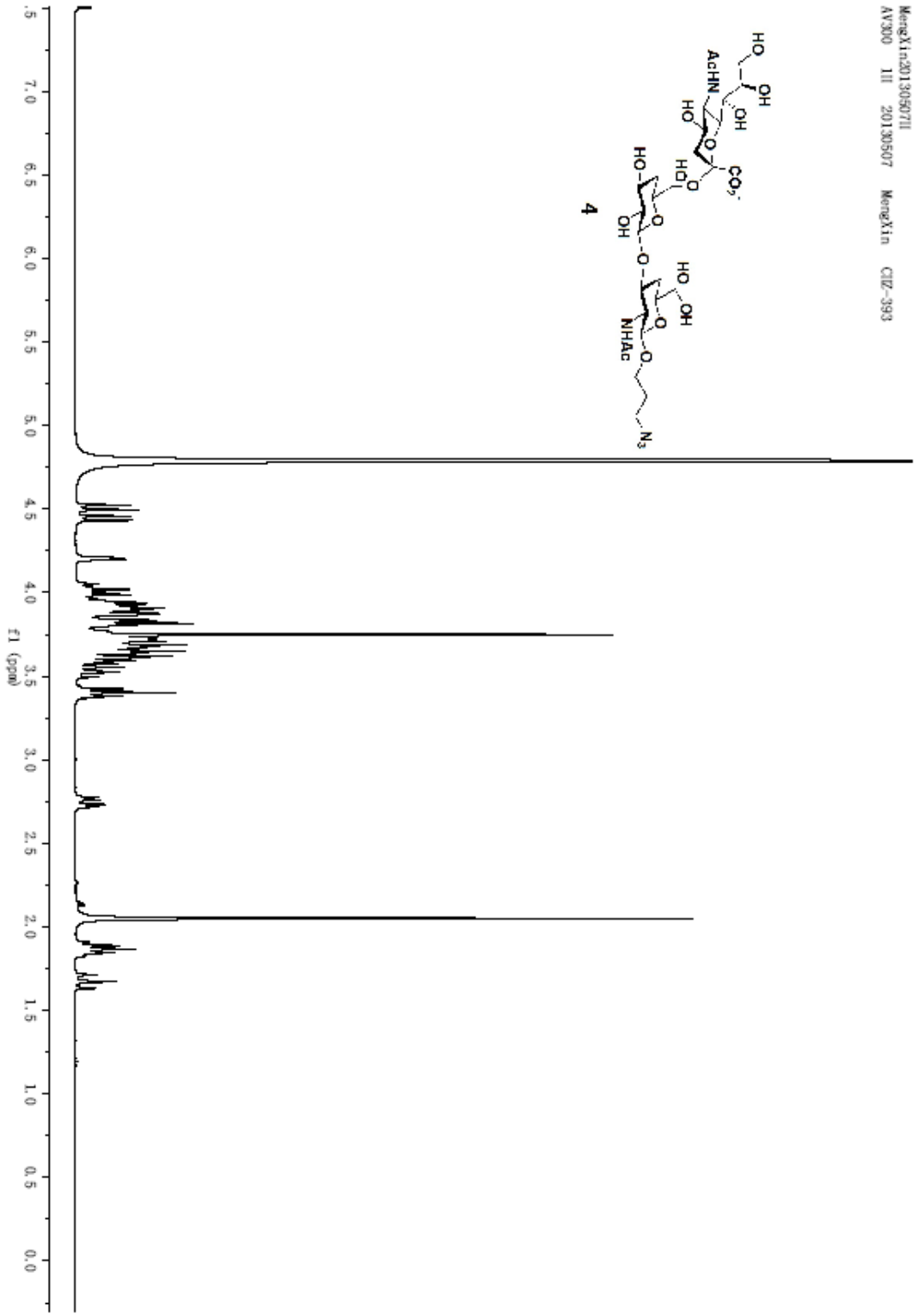

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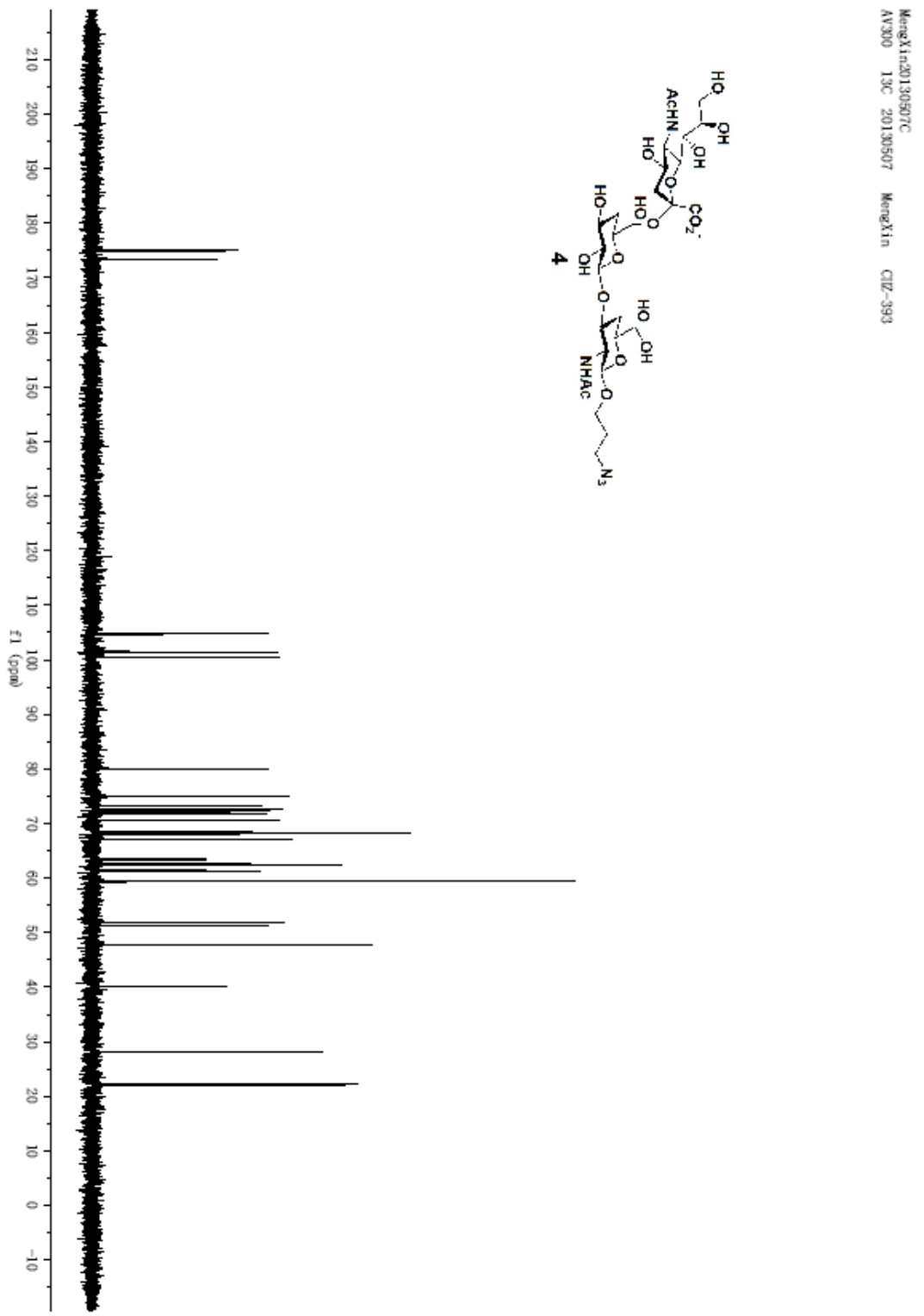

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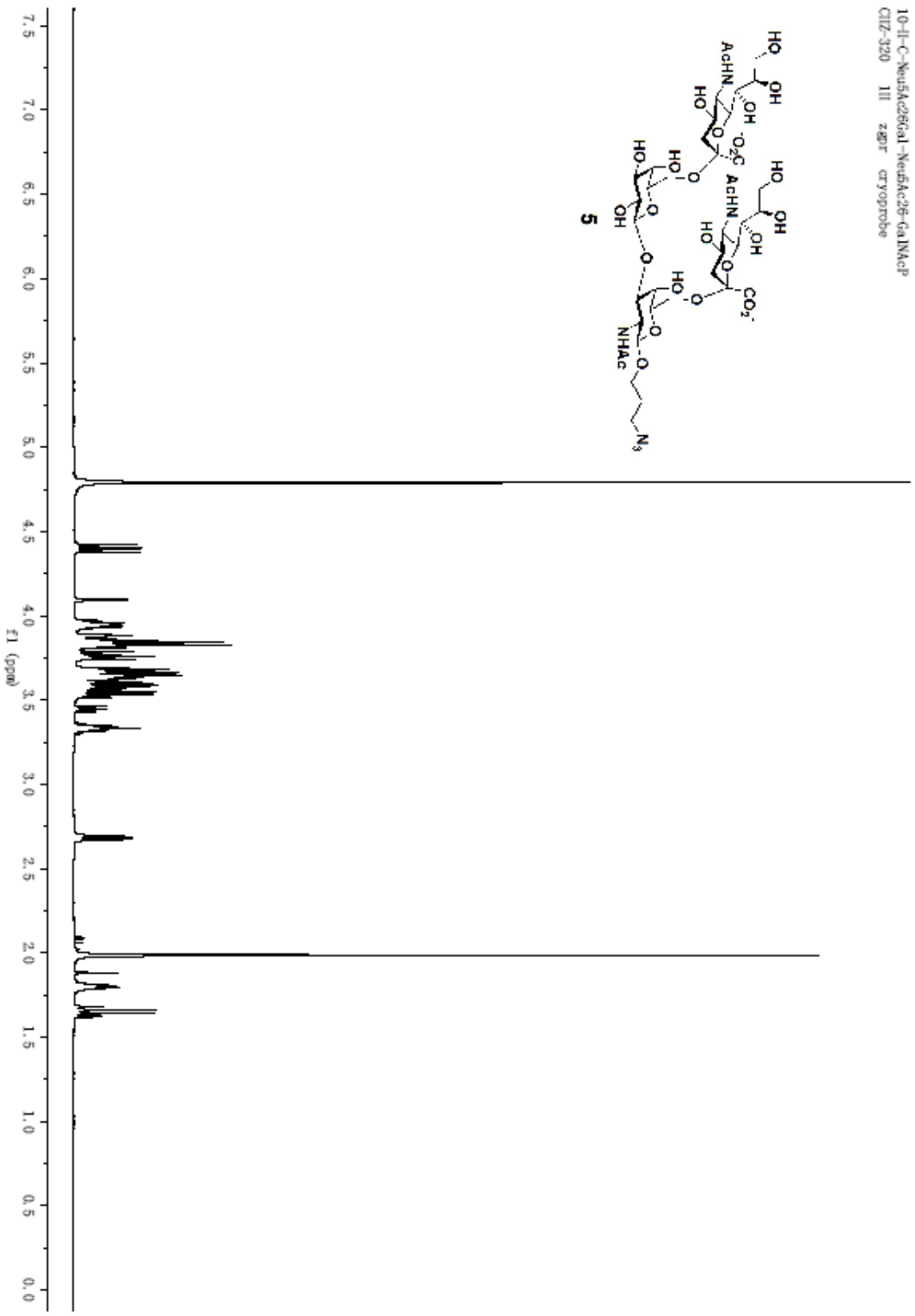

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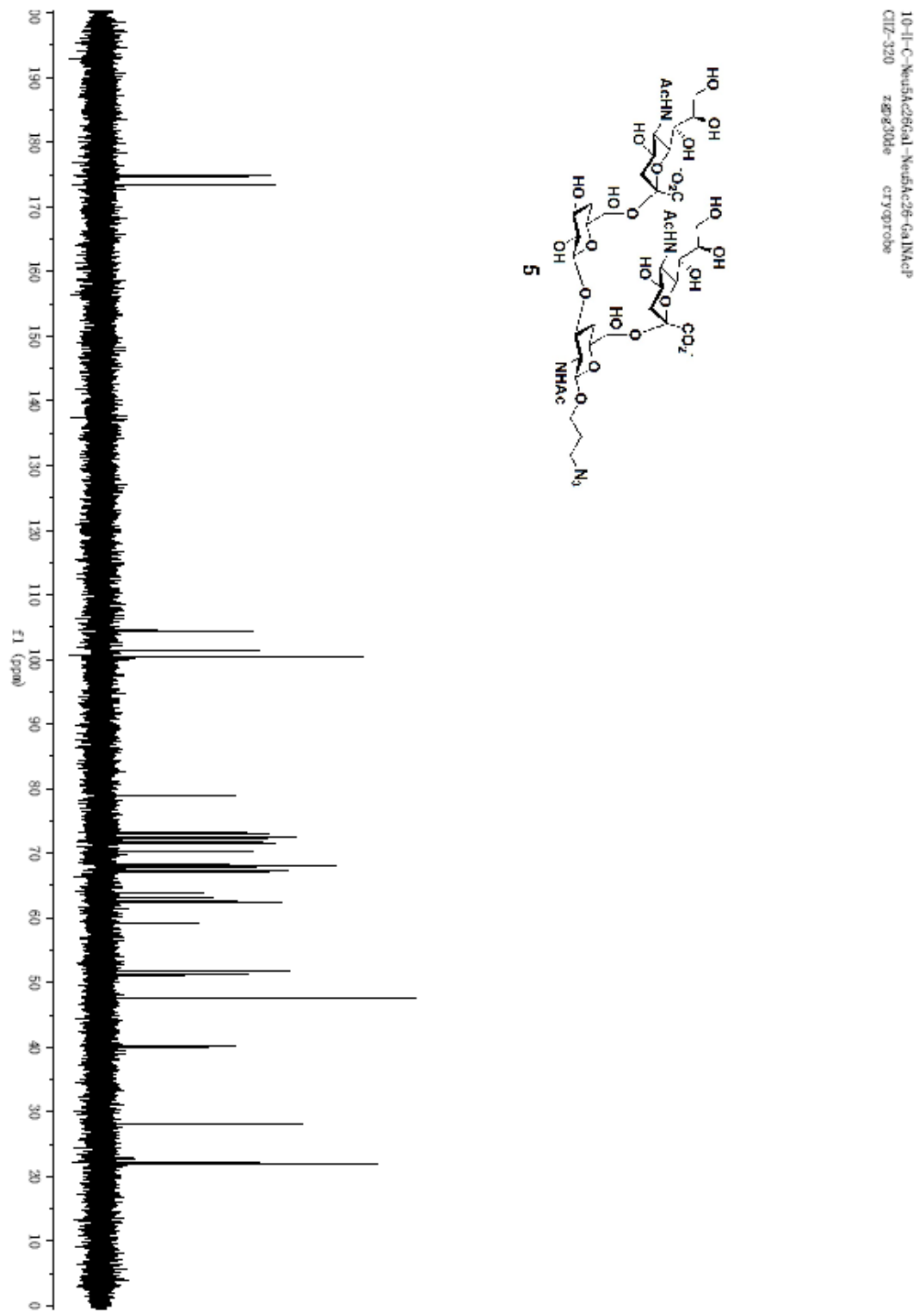
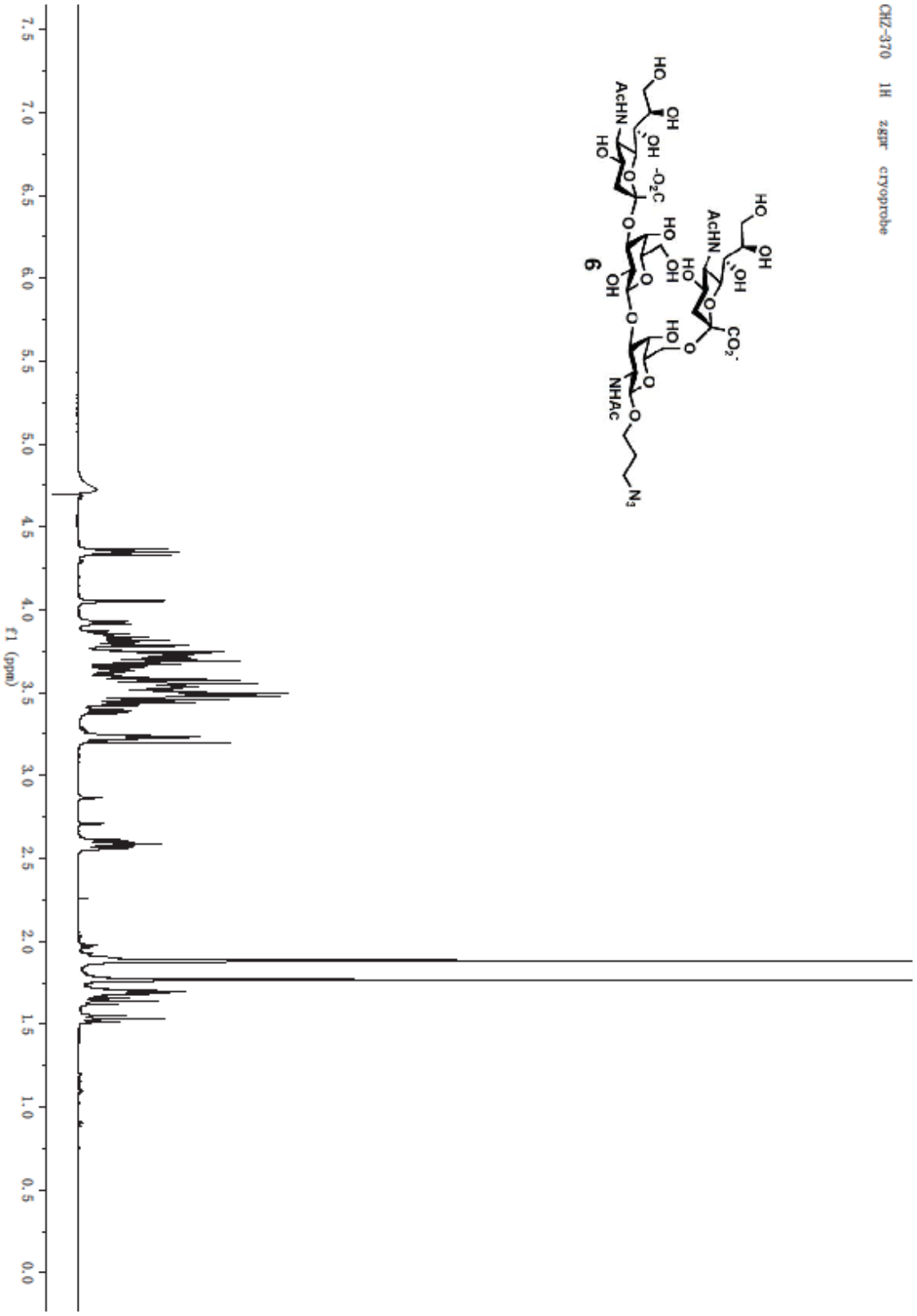
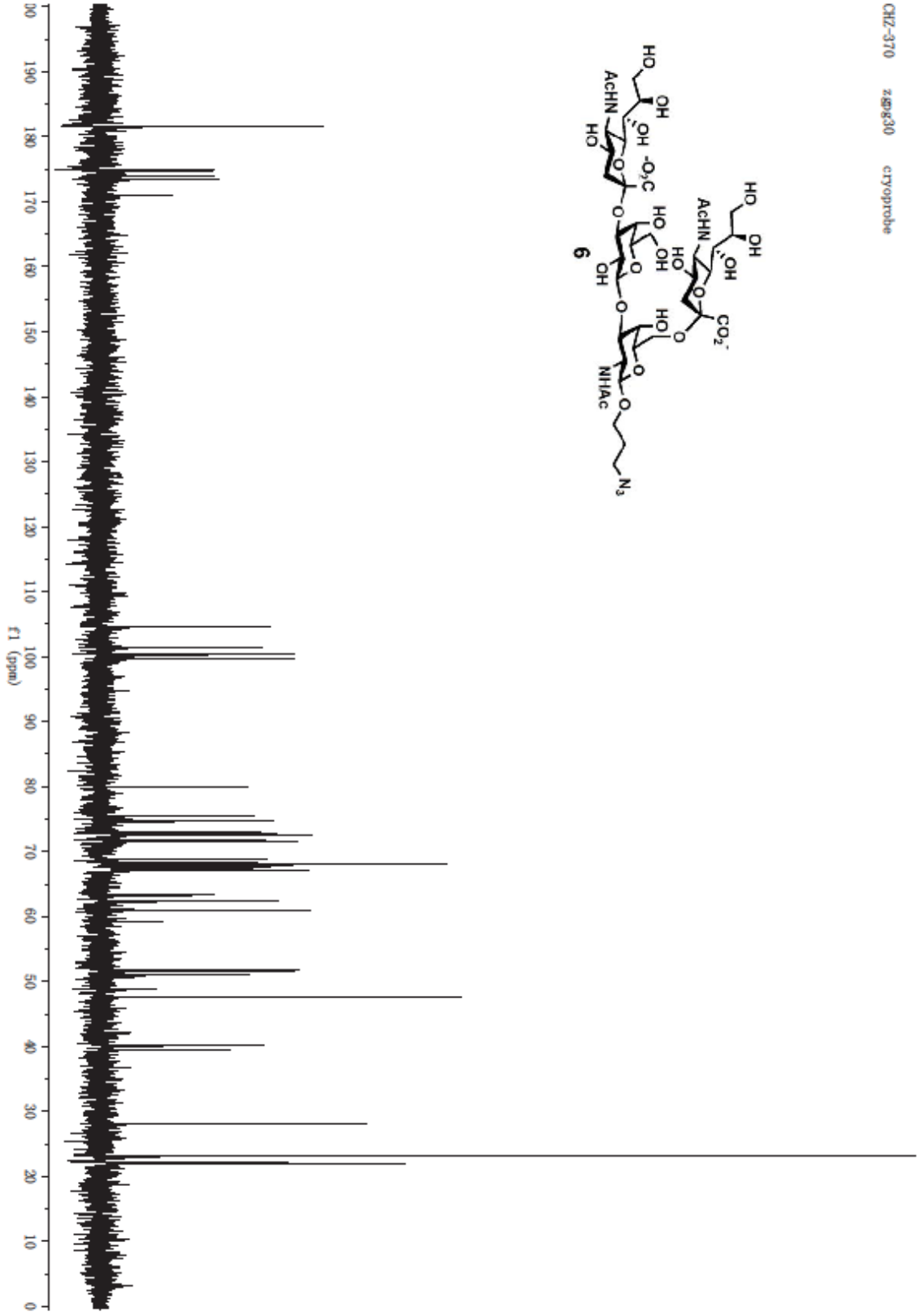


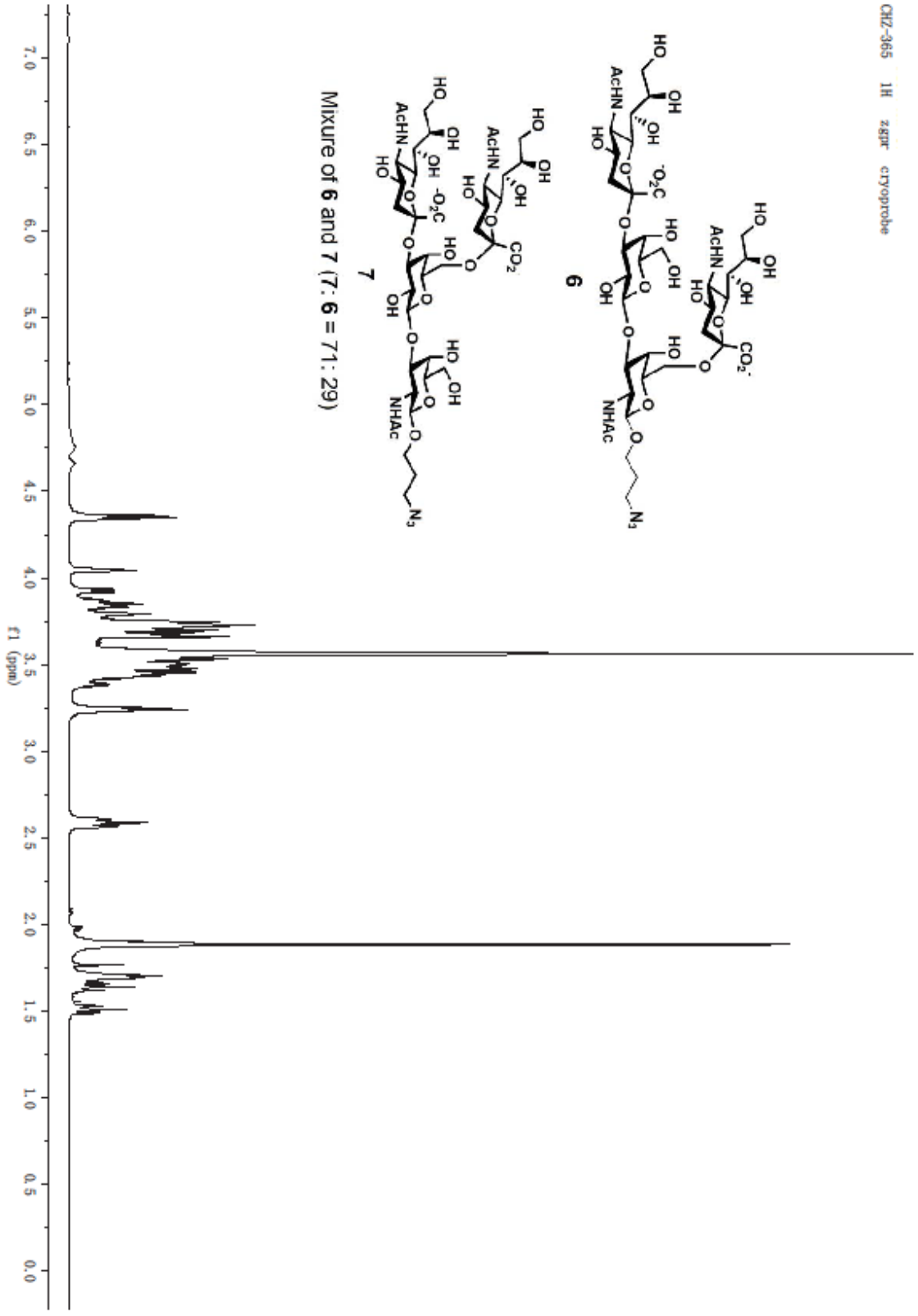
$^{1}$H NMR spectrum for compound **6**



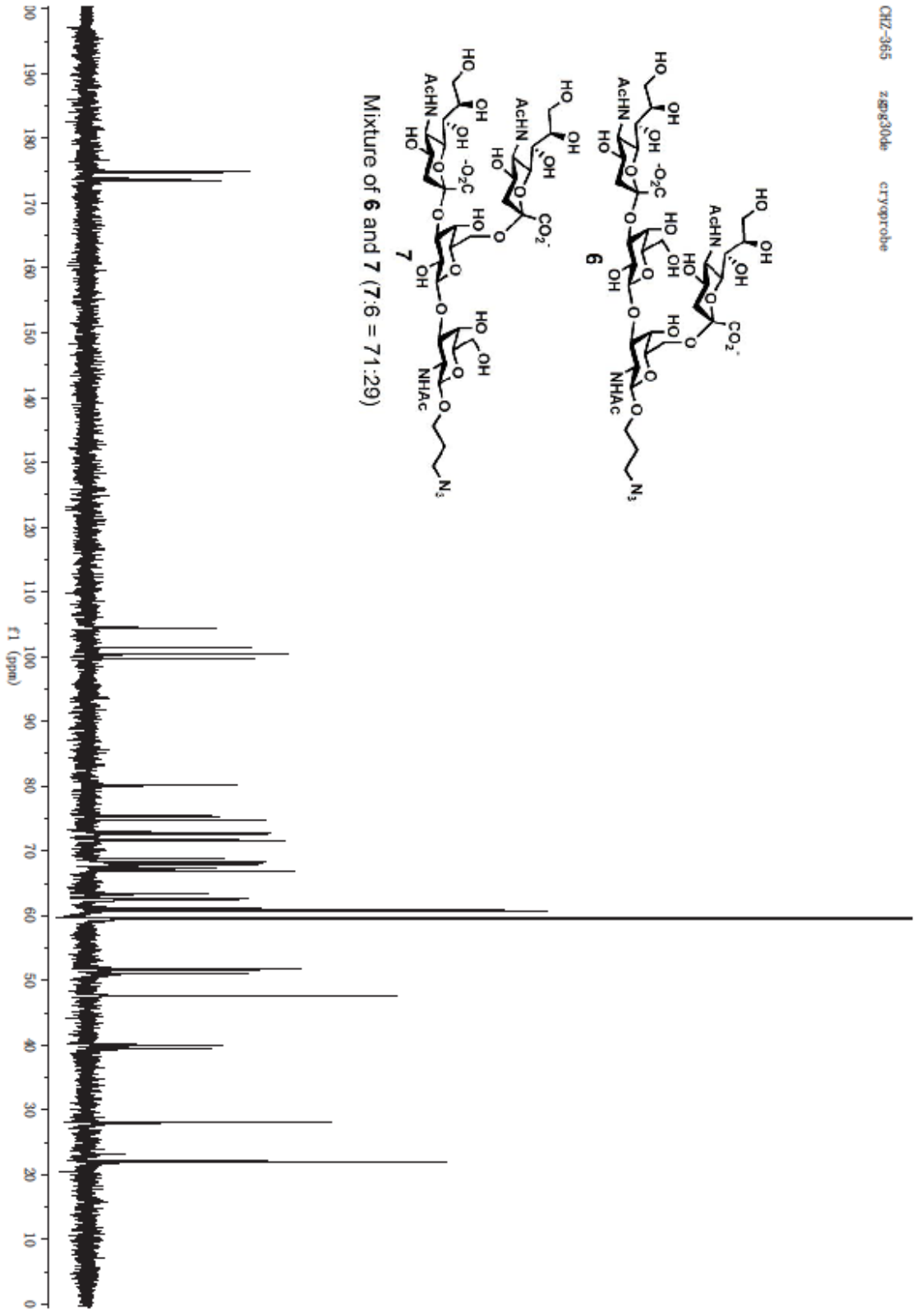
$^{13}$C NMR spectrum for compound **6**



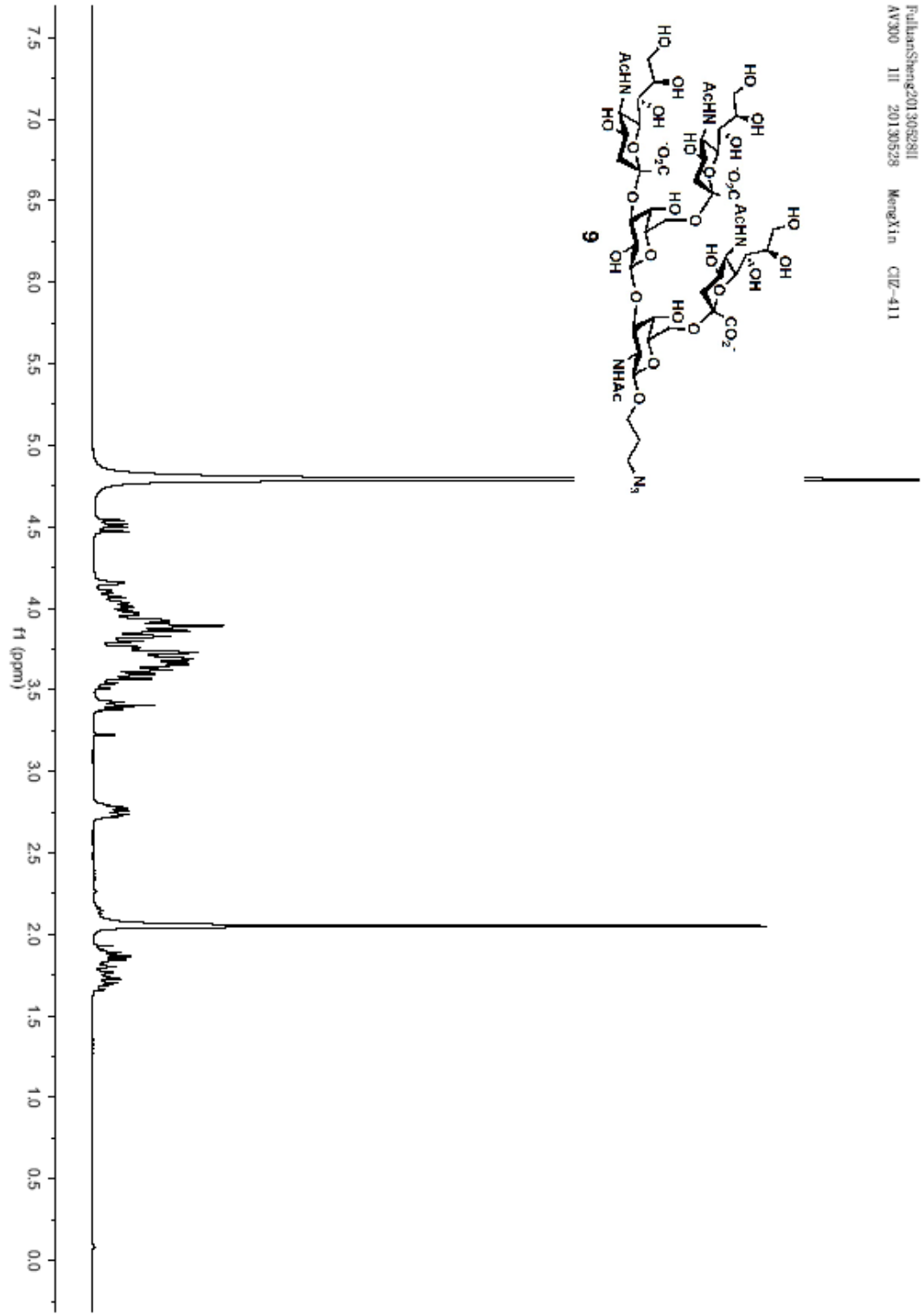
$^{1}$H NMR spectrum for compound **6** and **7** mixture



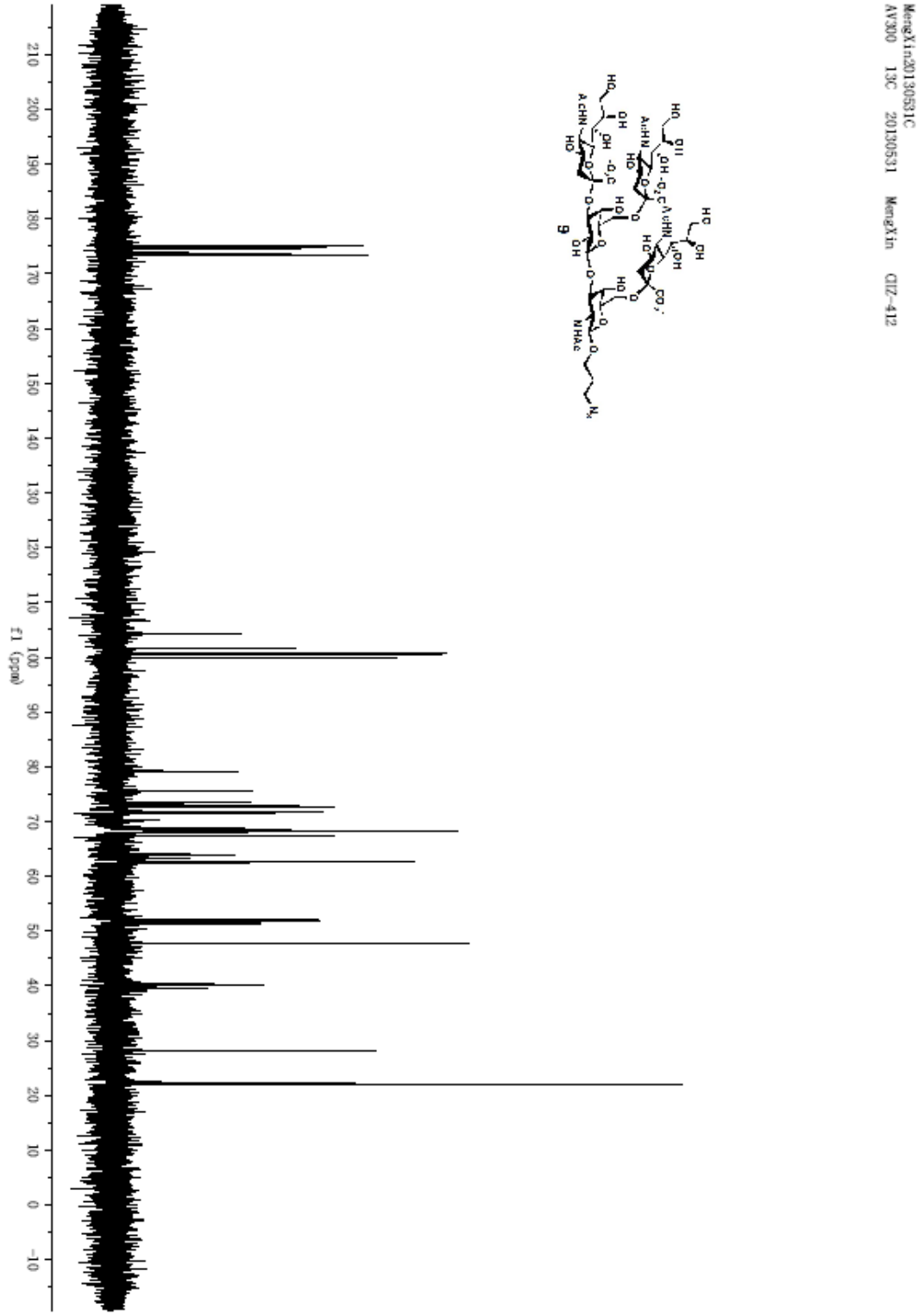
$^{13}$C NMR spectrum for compound **6** and **7** mixture



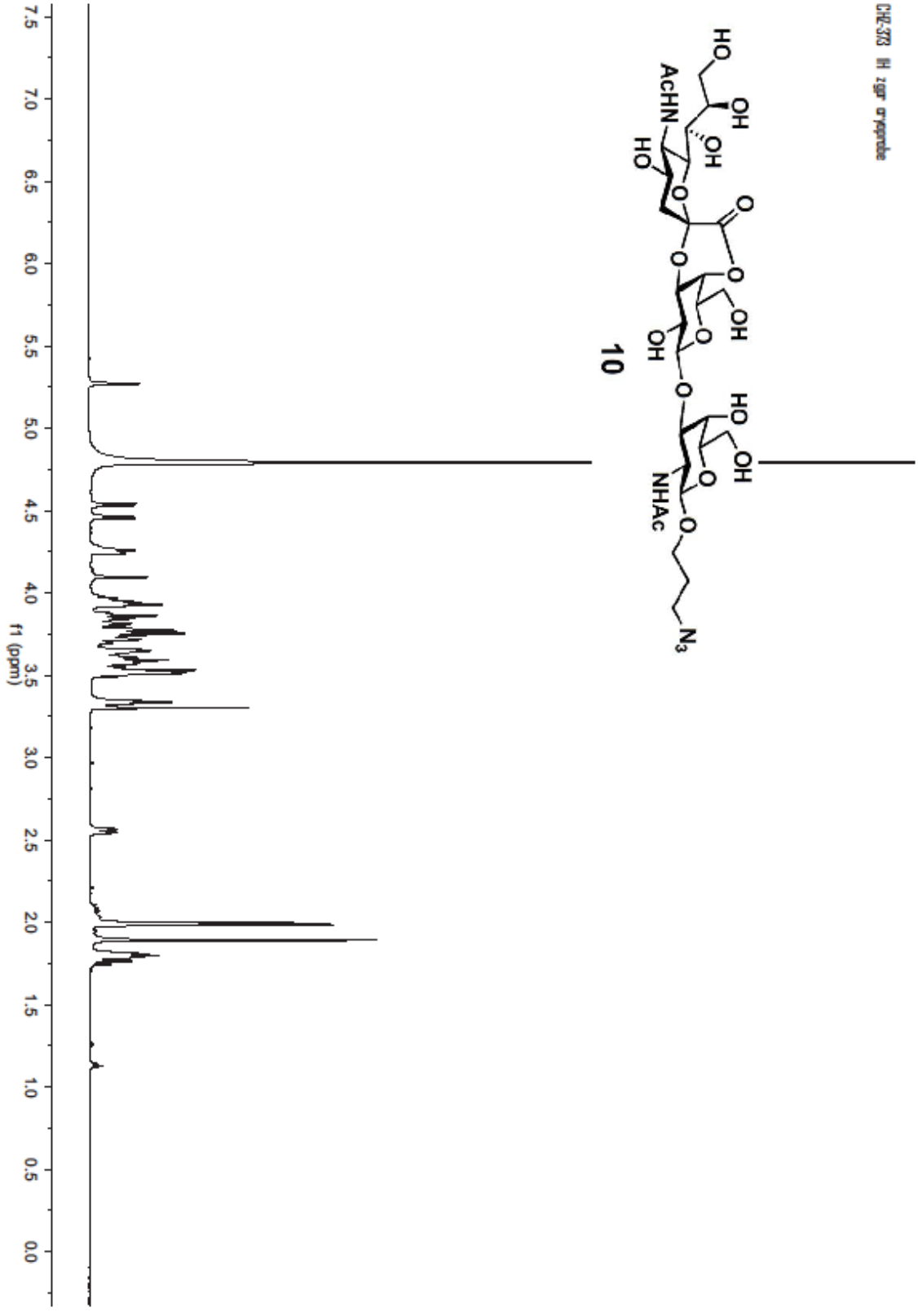
$^{1}$H NMR spectrum for compound **9**



$^{13}$C NMR spectrum for compound **9**



$^{1}$H NMR spectrum for compound **10**



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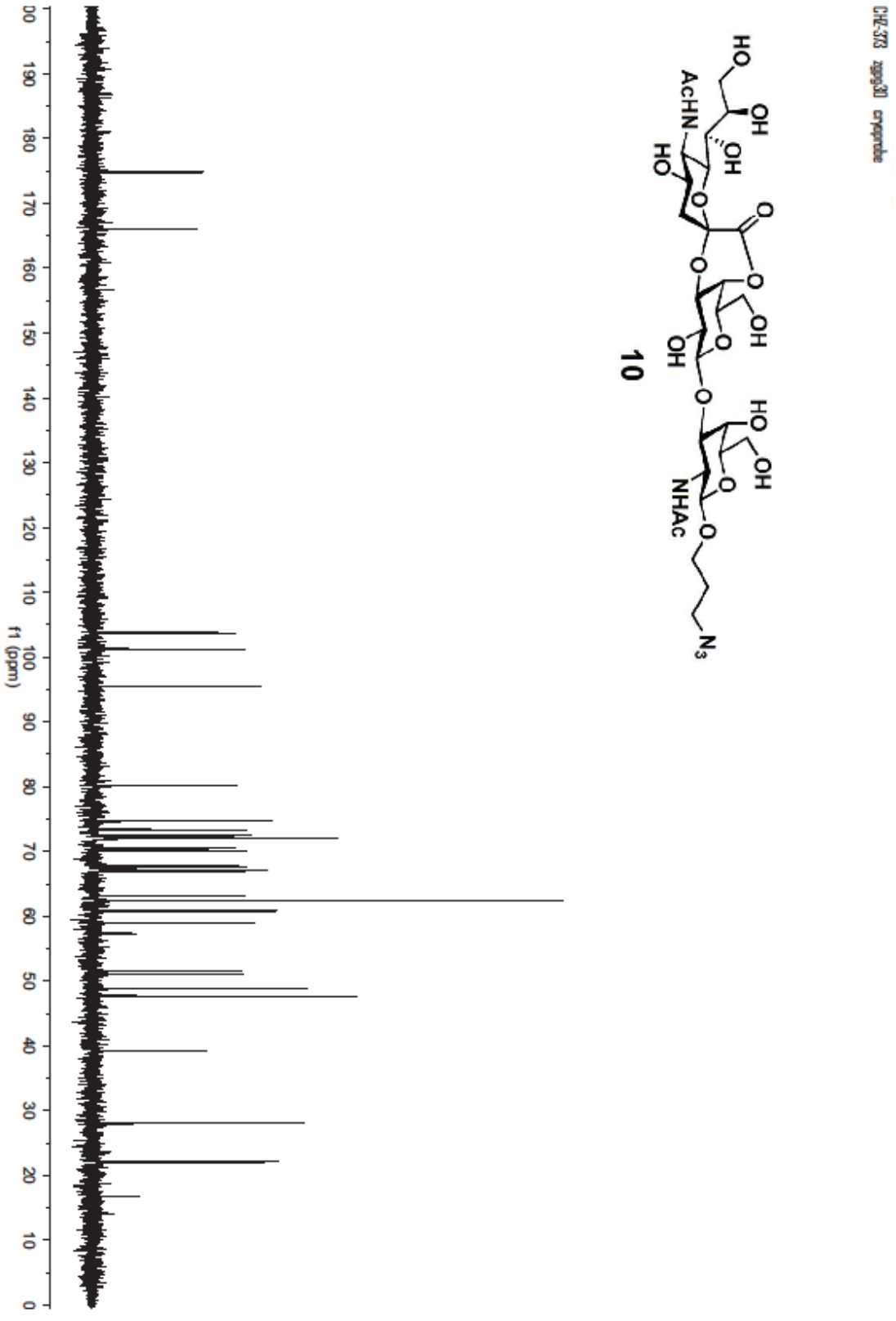
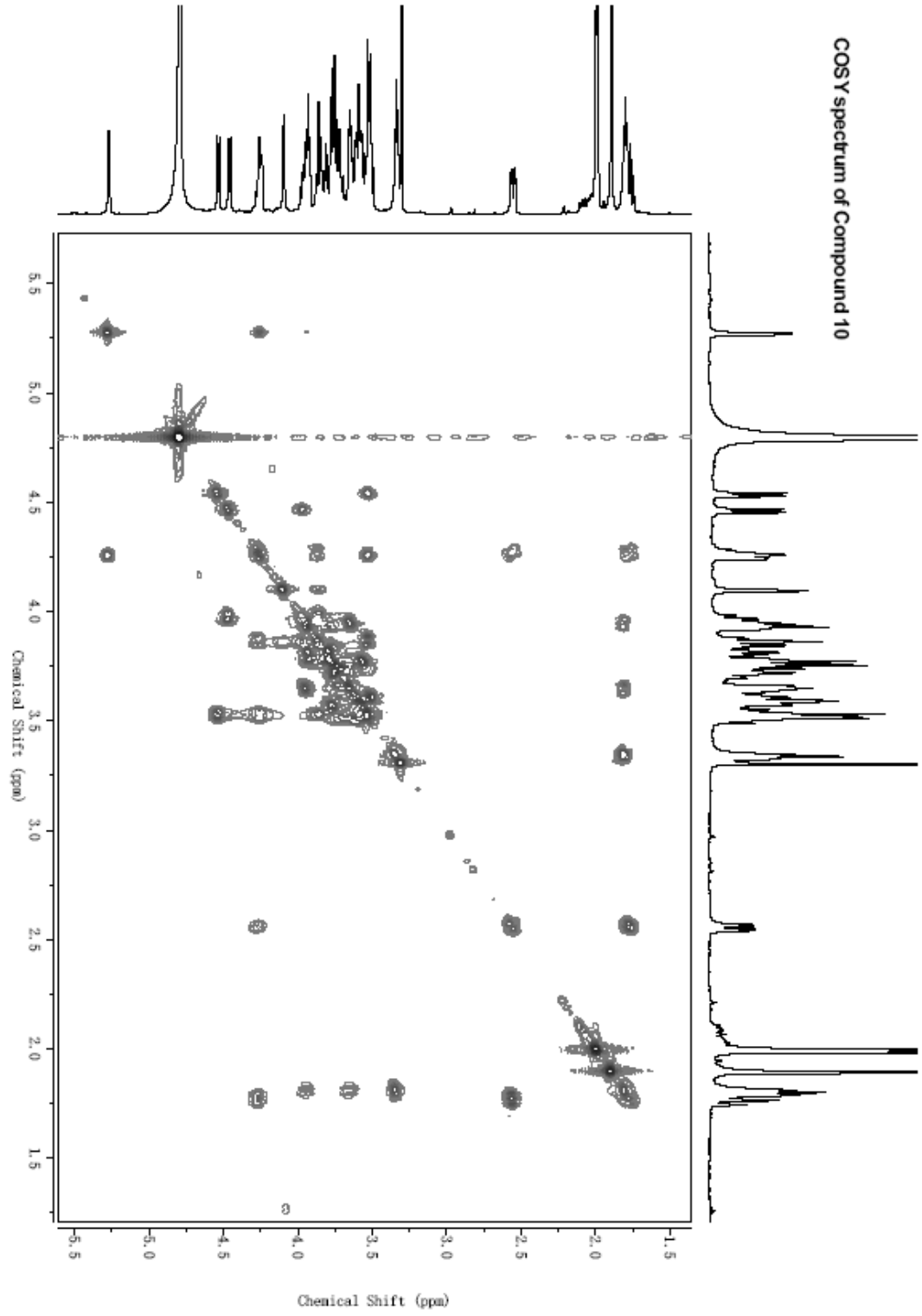
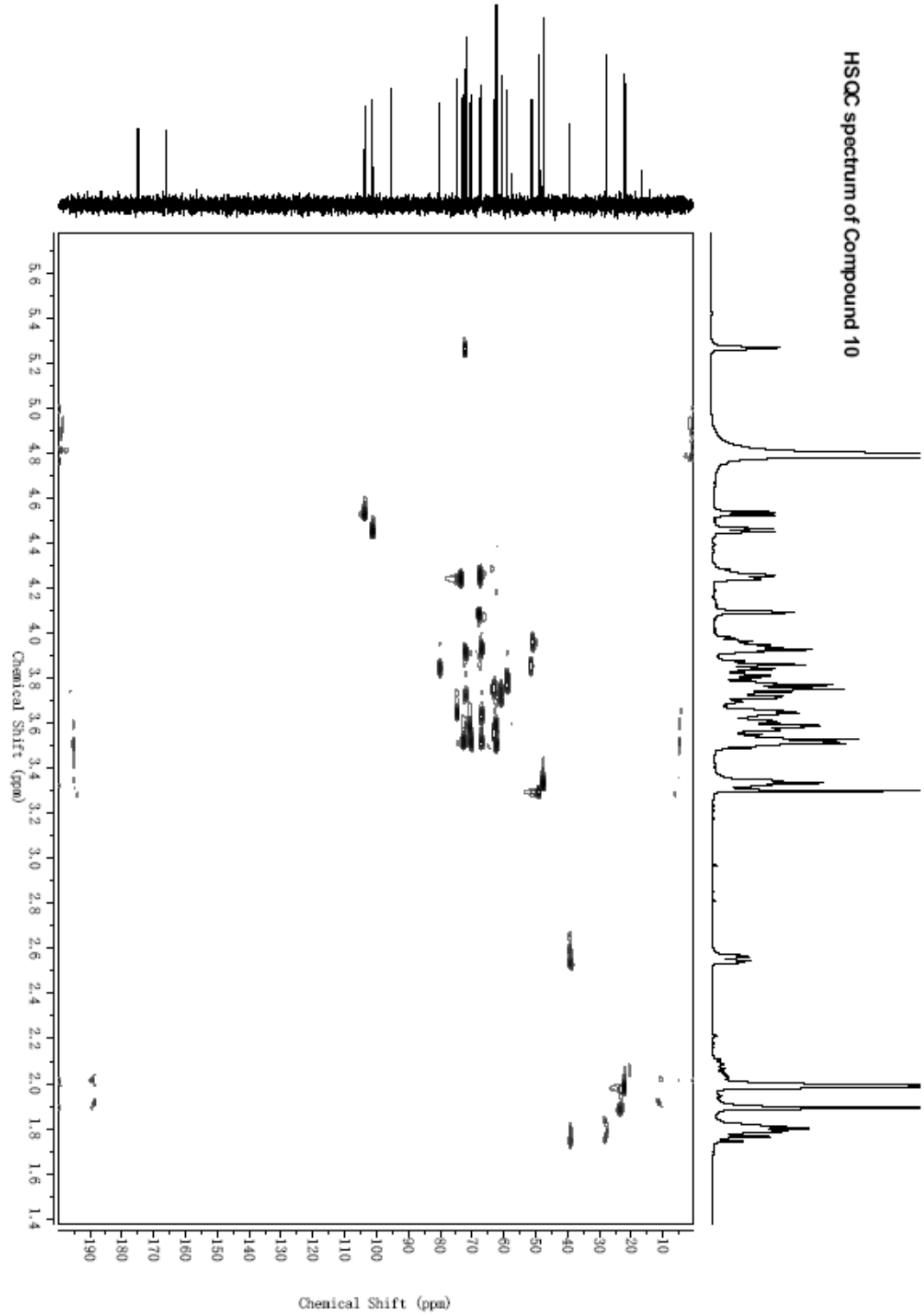


COSY spectrum for compound **10**



HSQC spectrum for compound **10**



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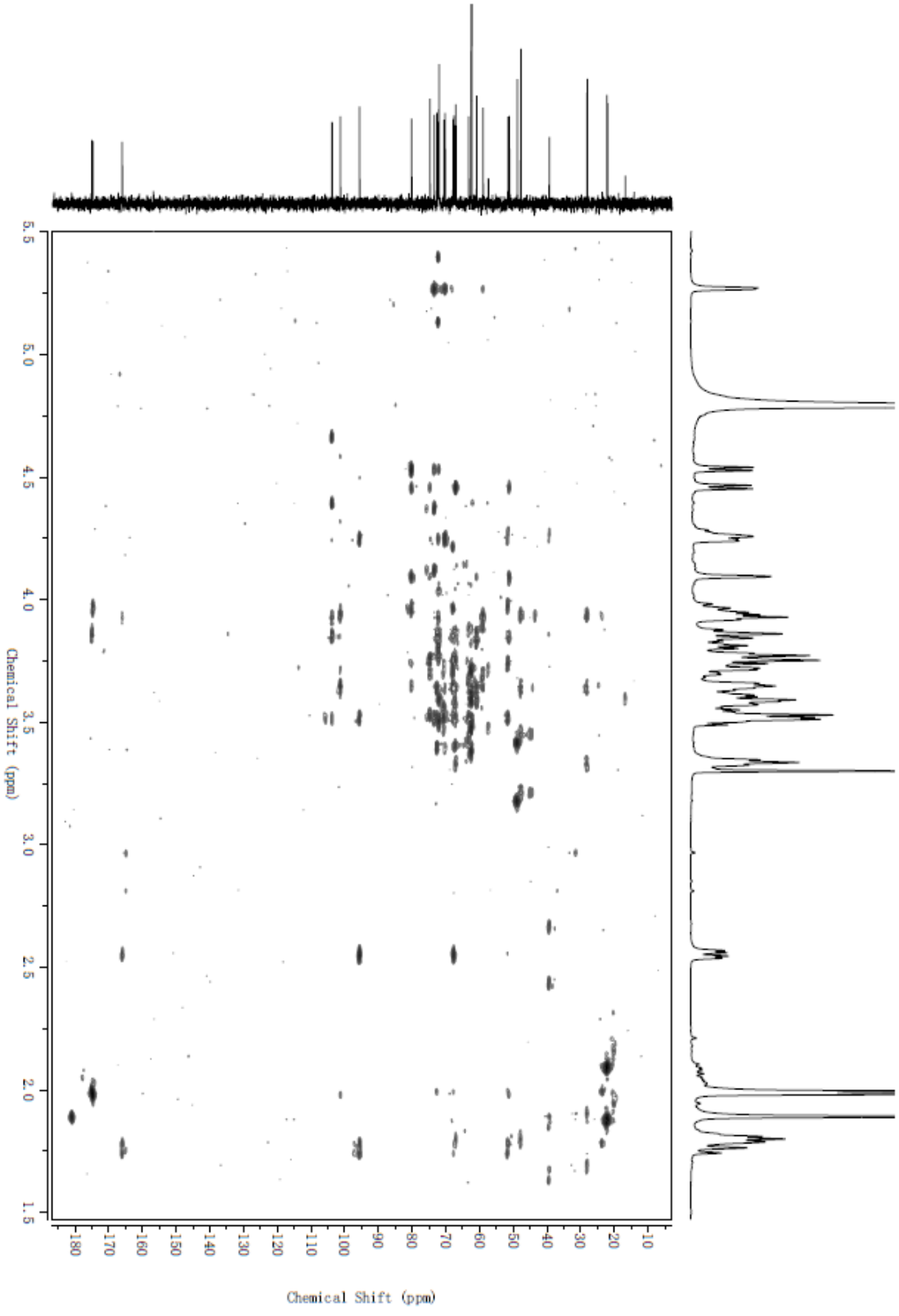

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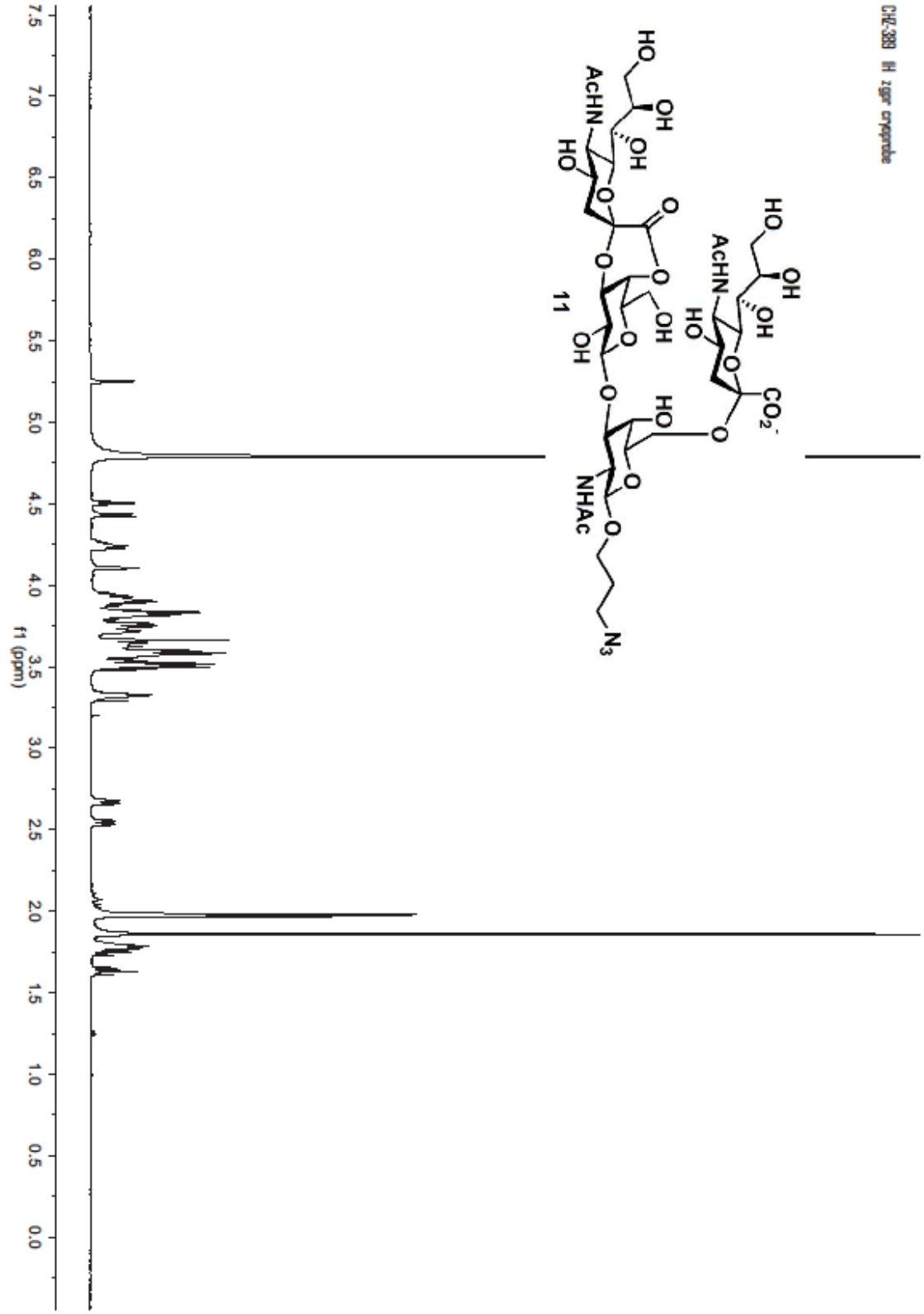

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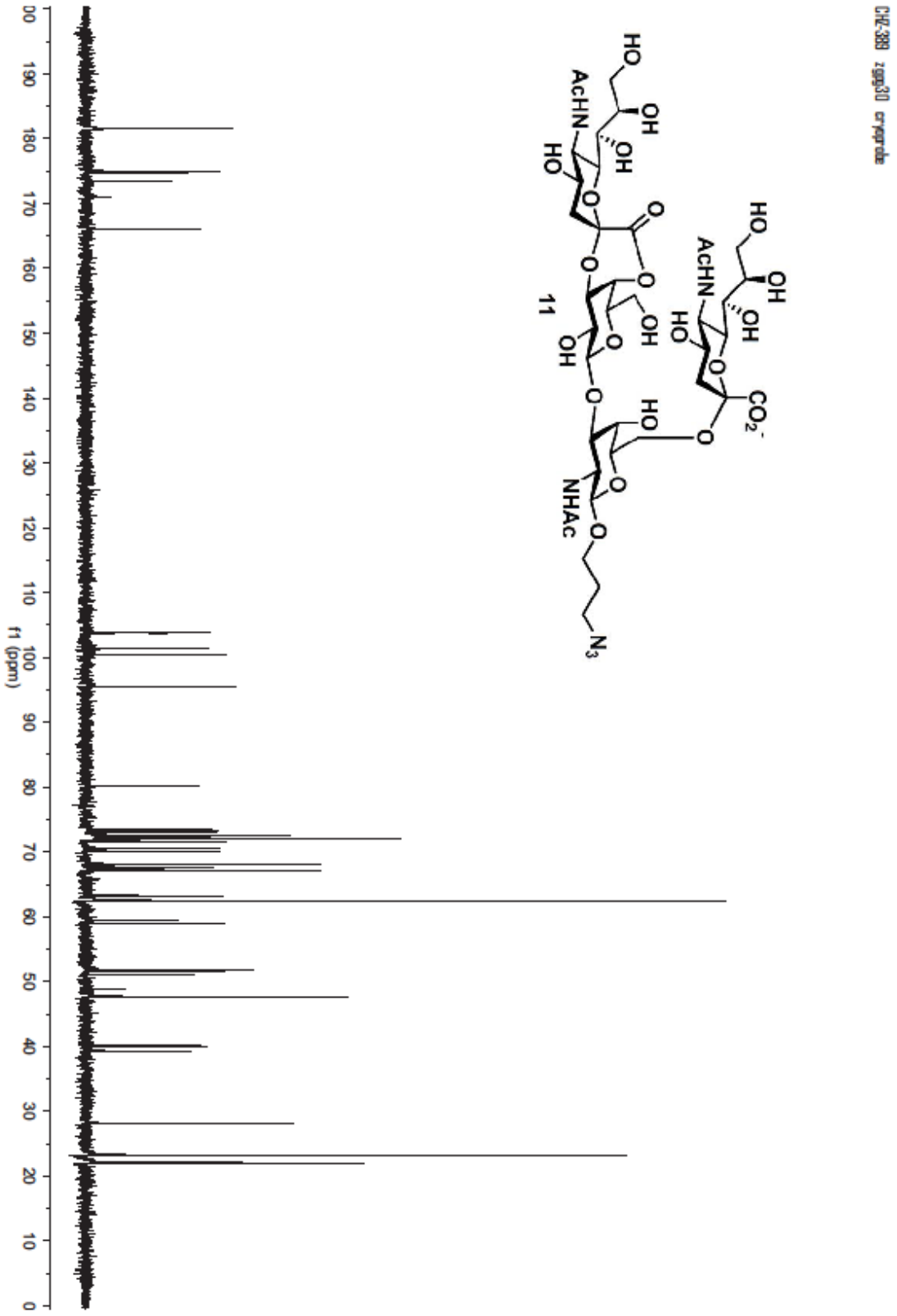

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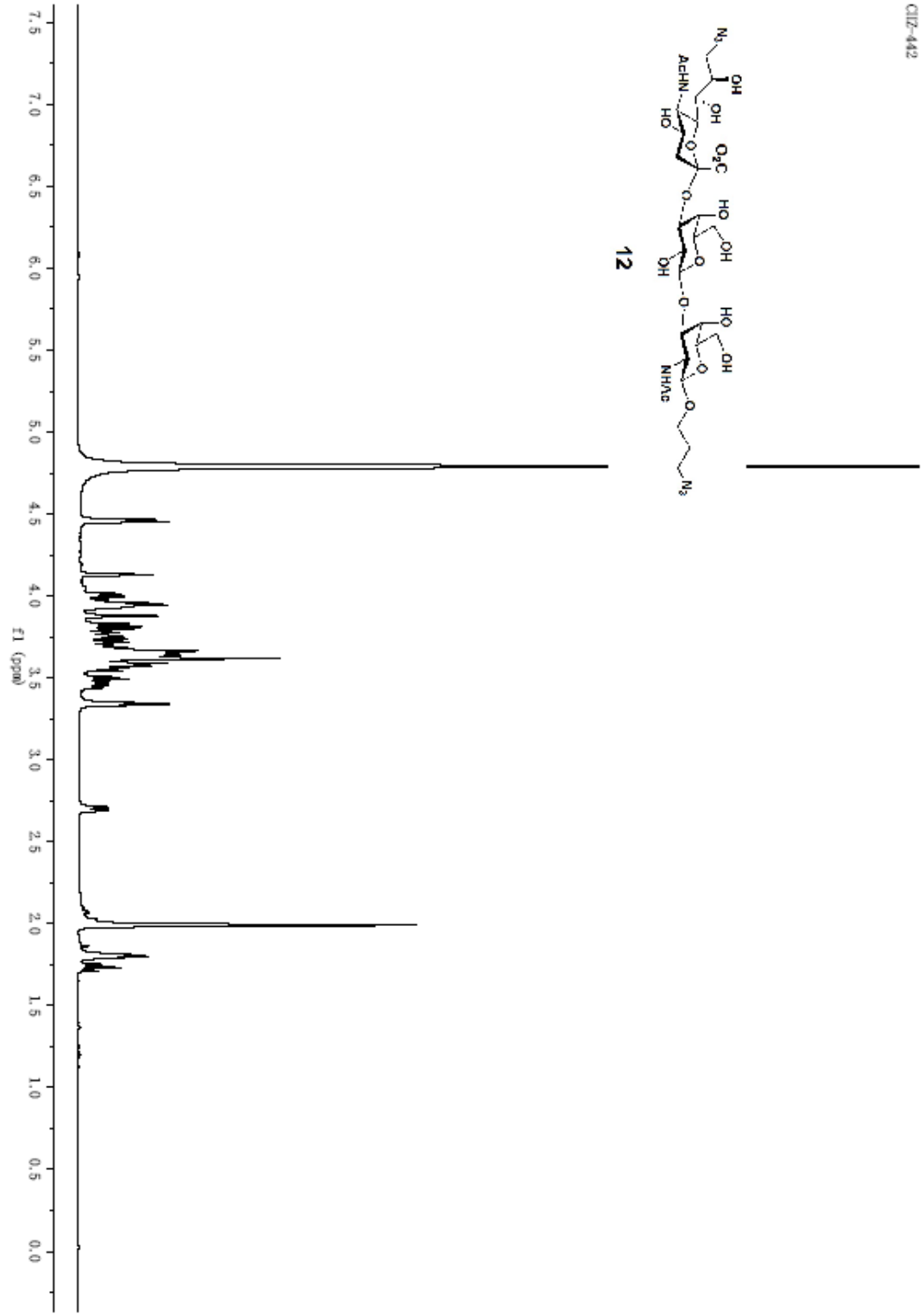

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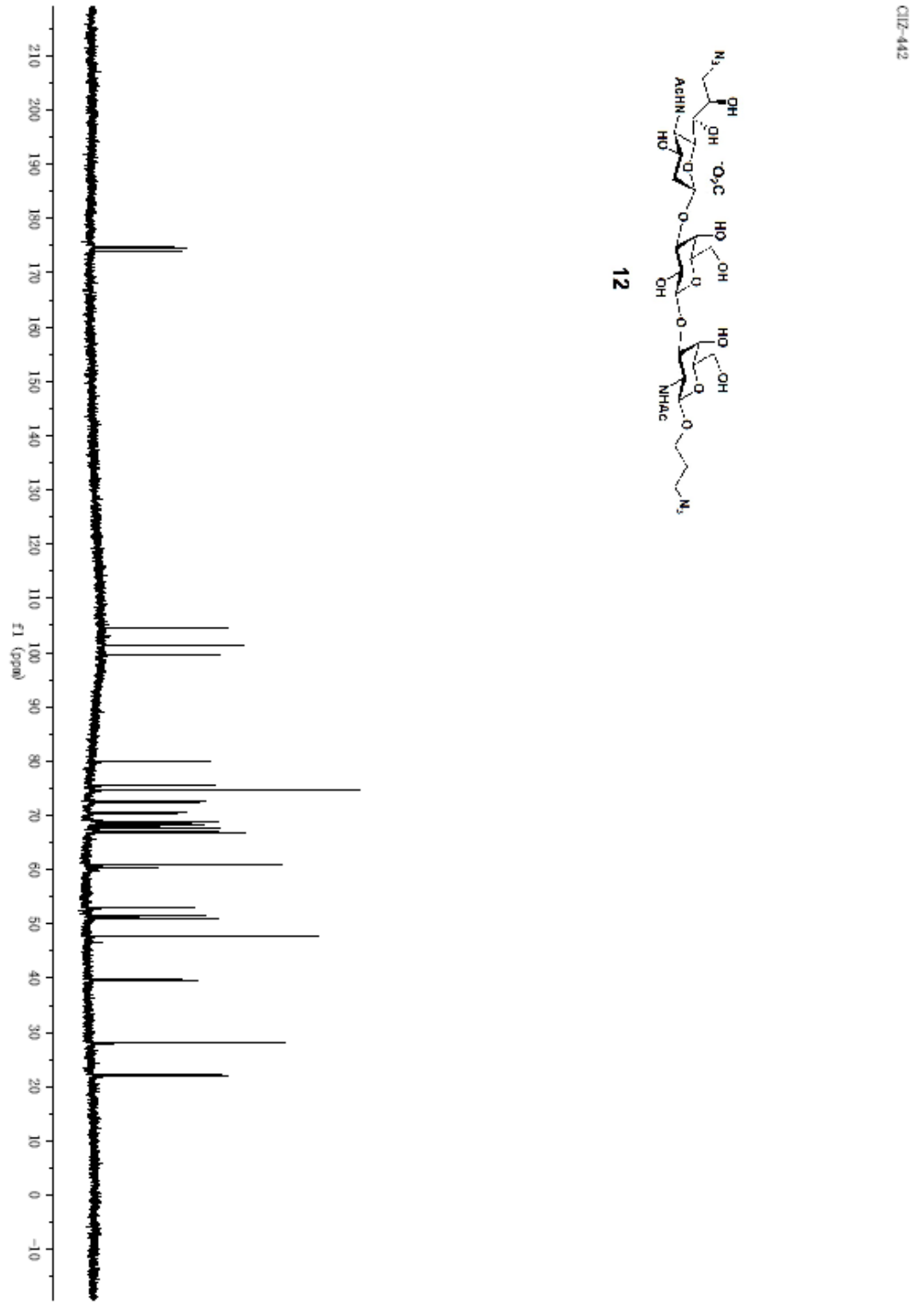

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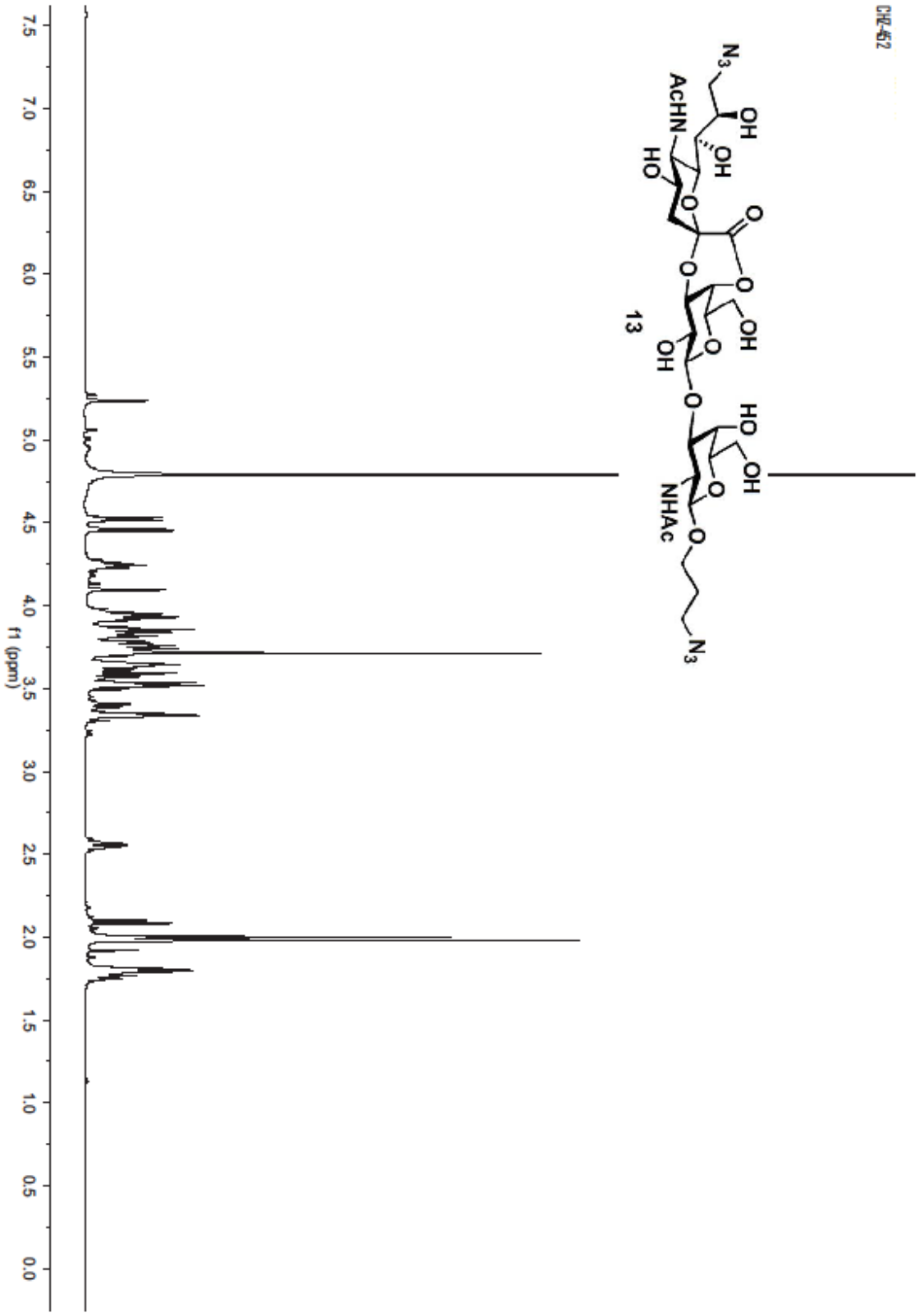

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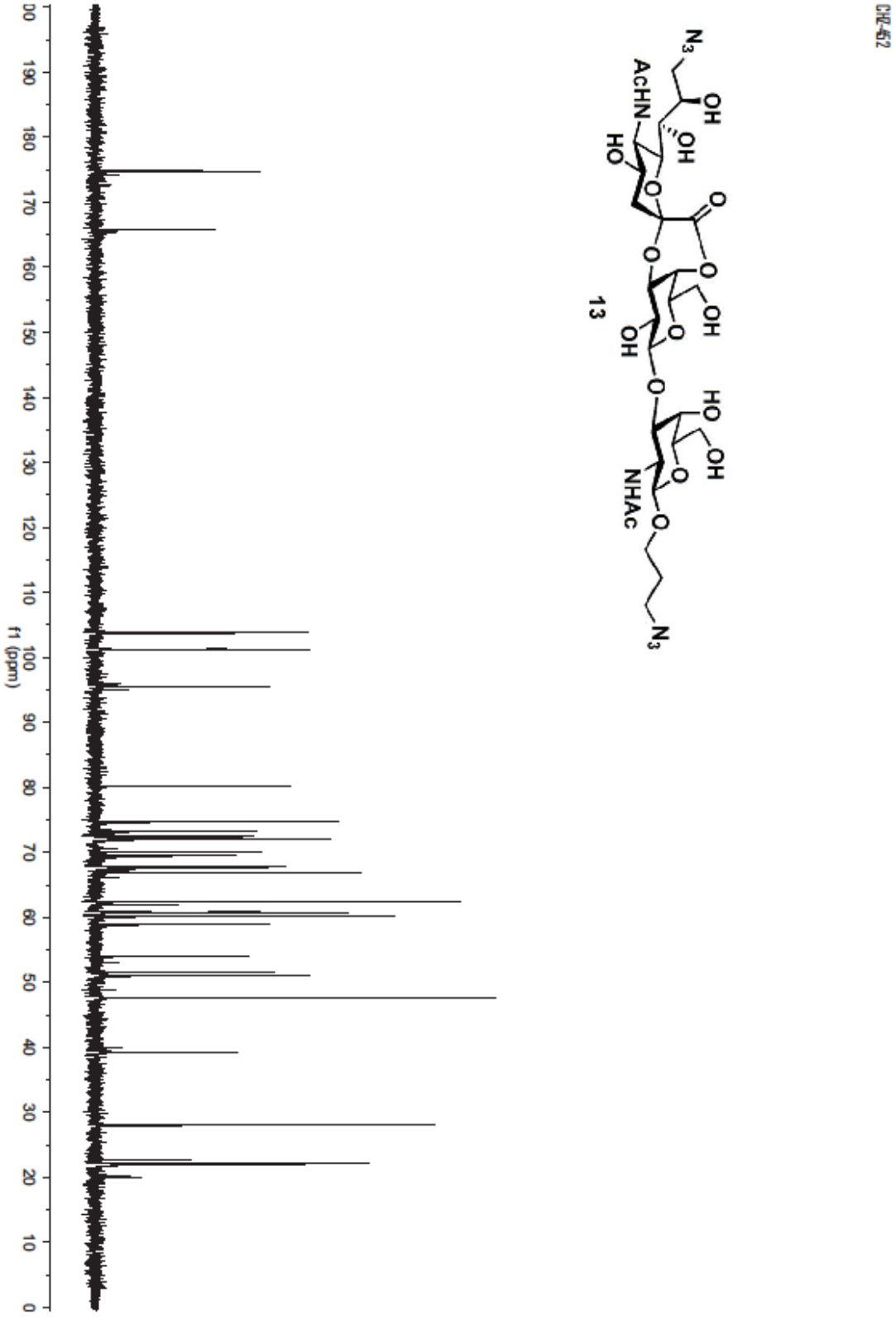

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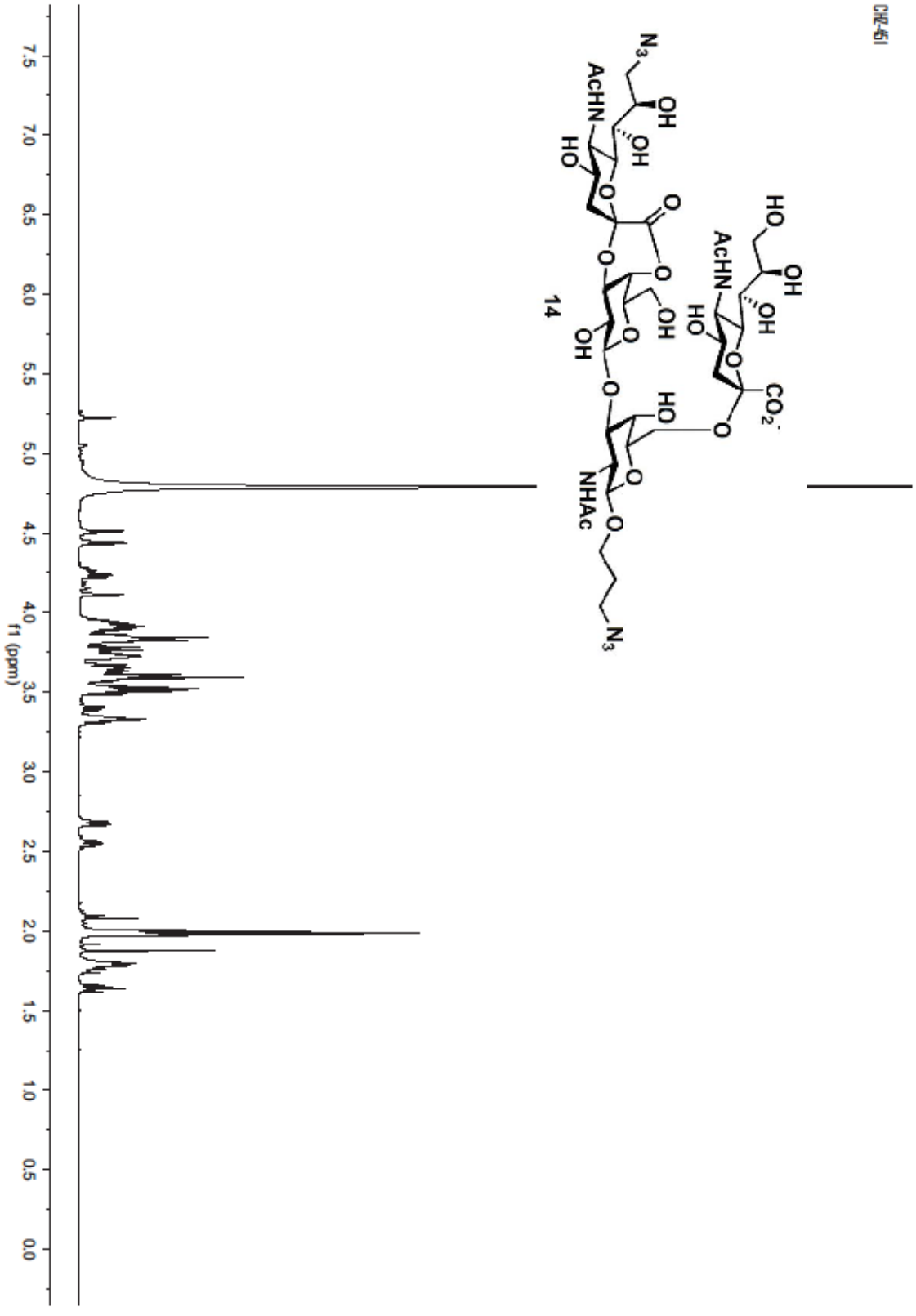

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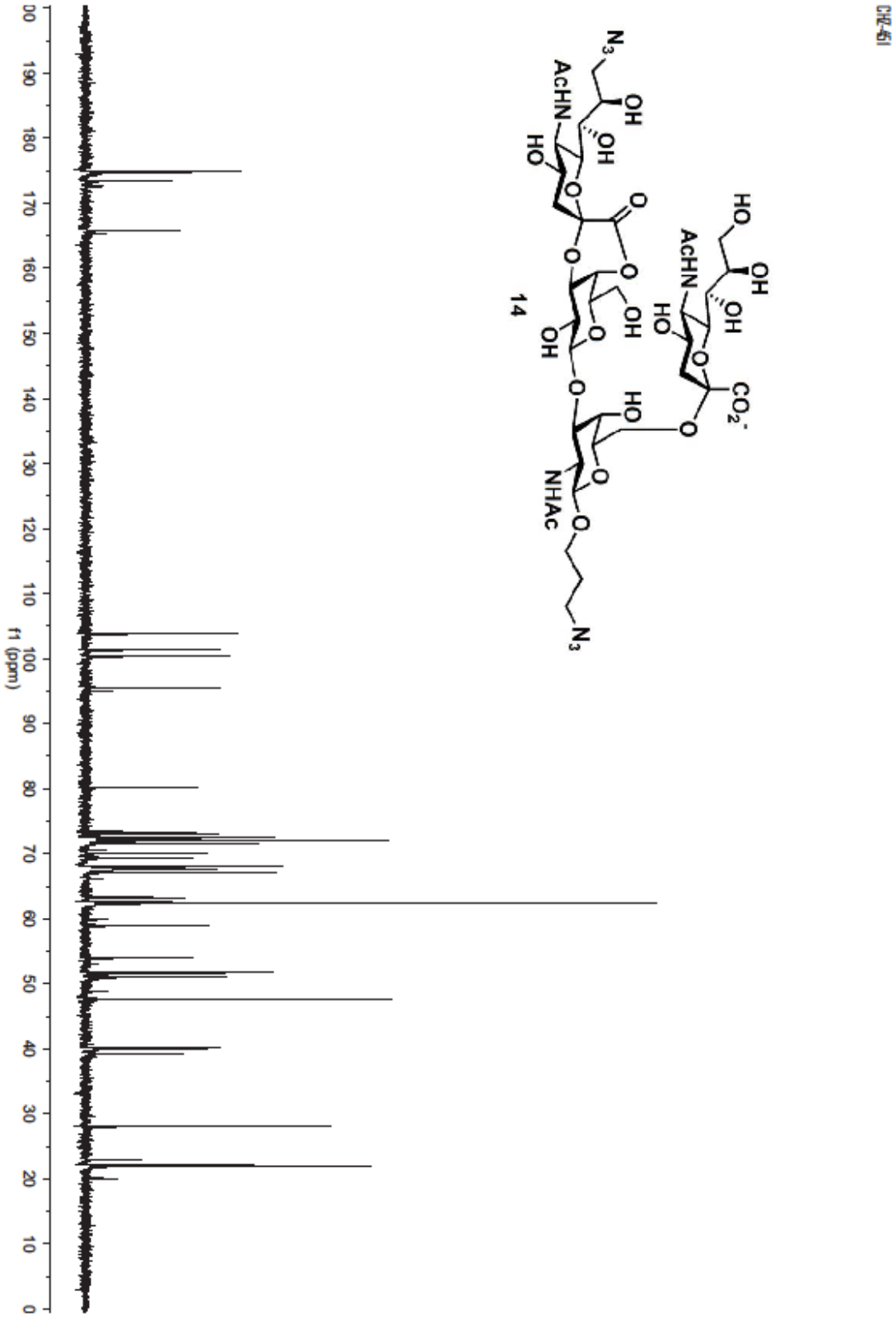

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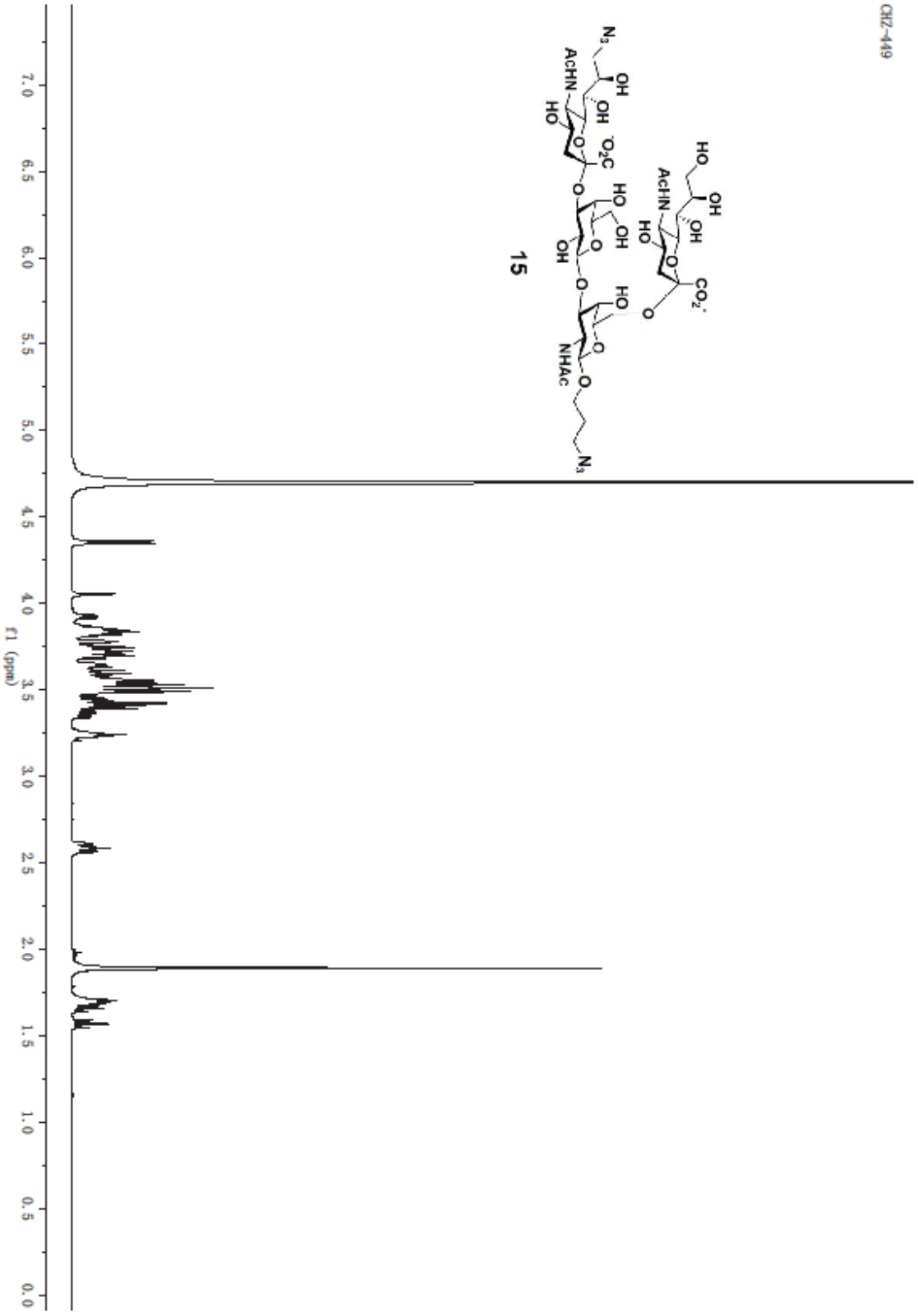

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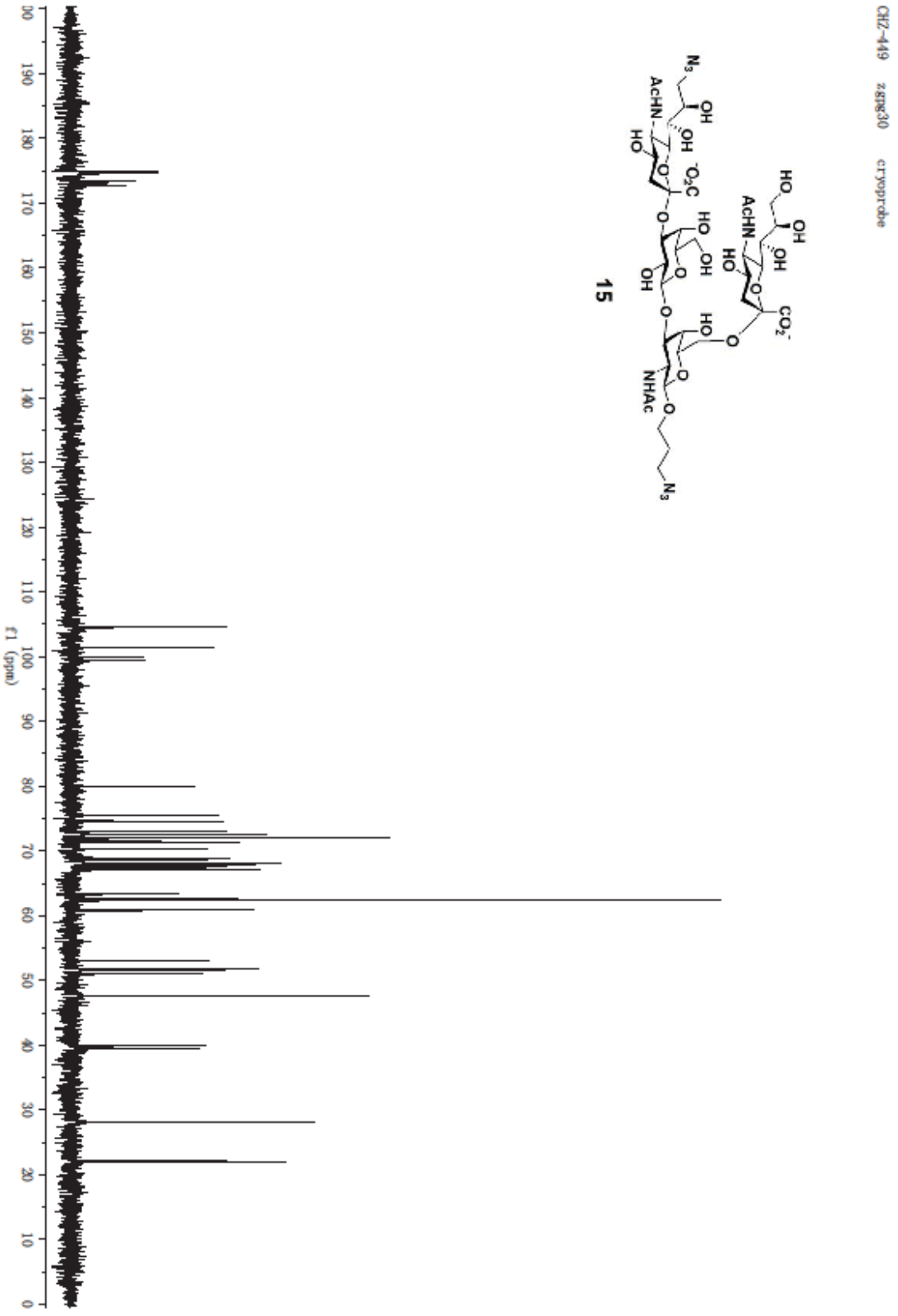

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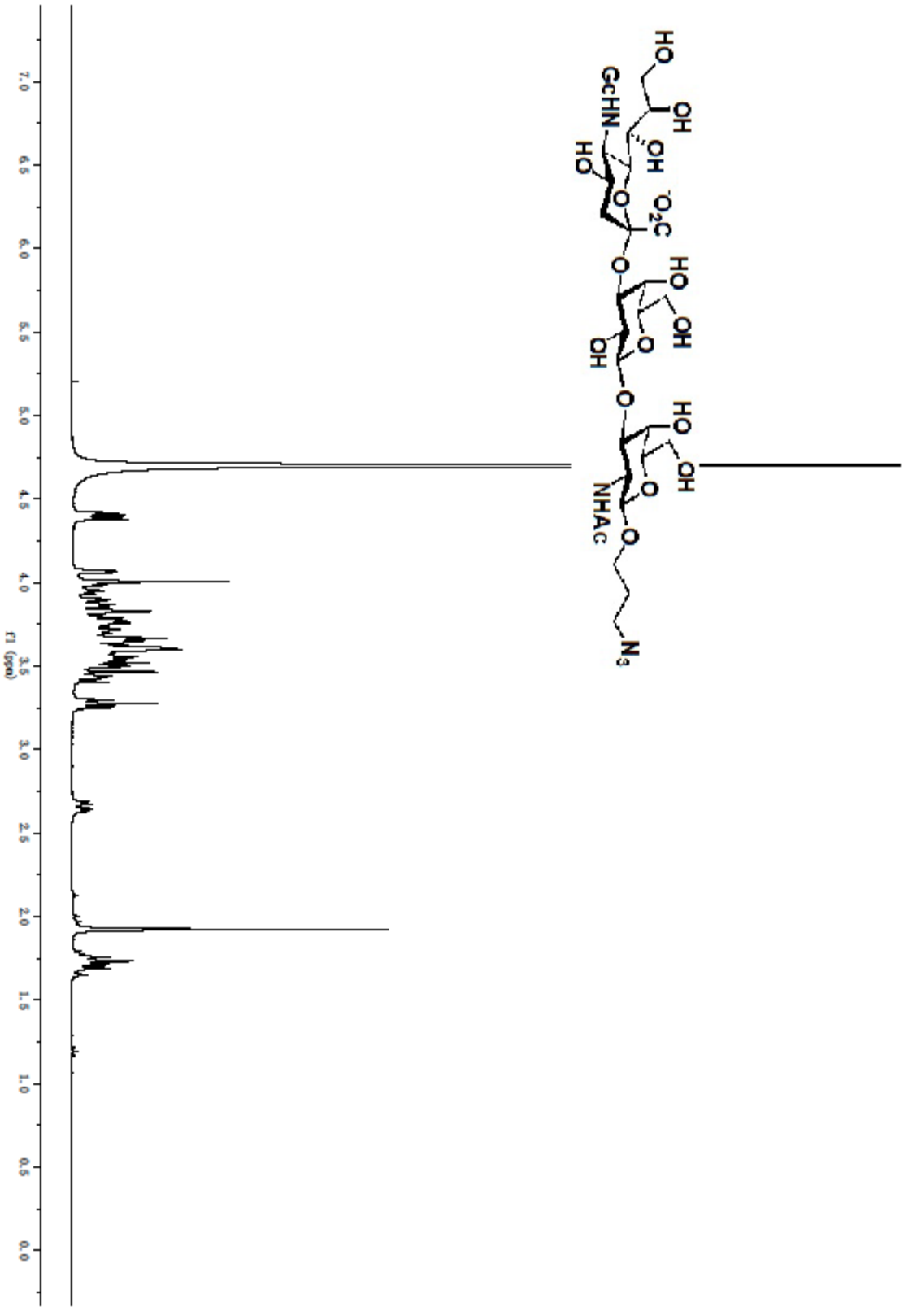

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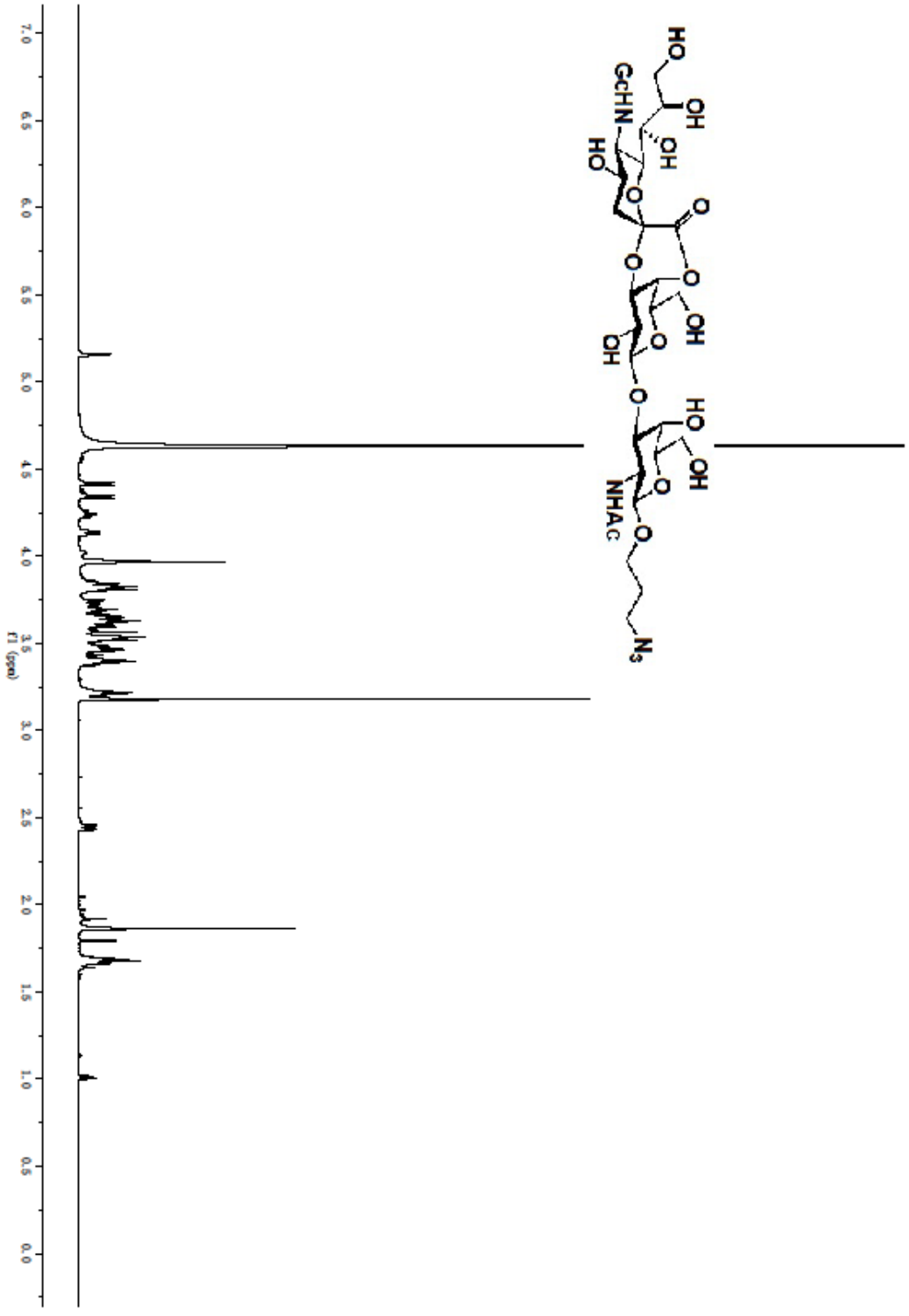

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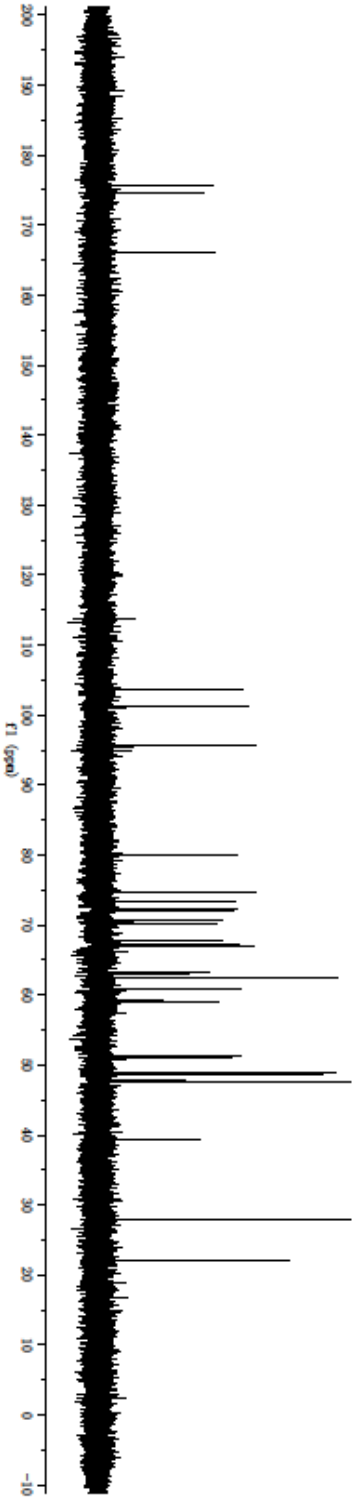


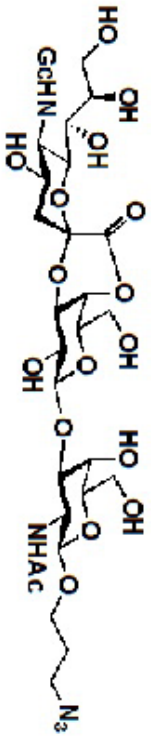

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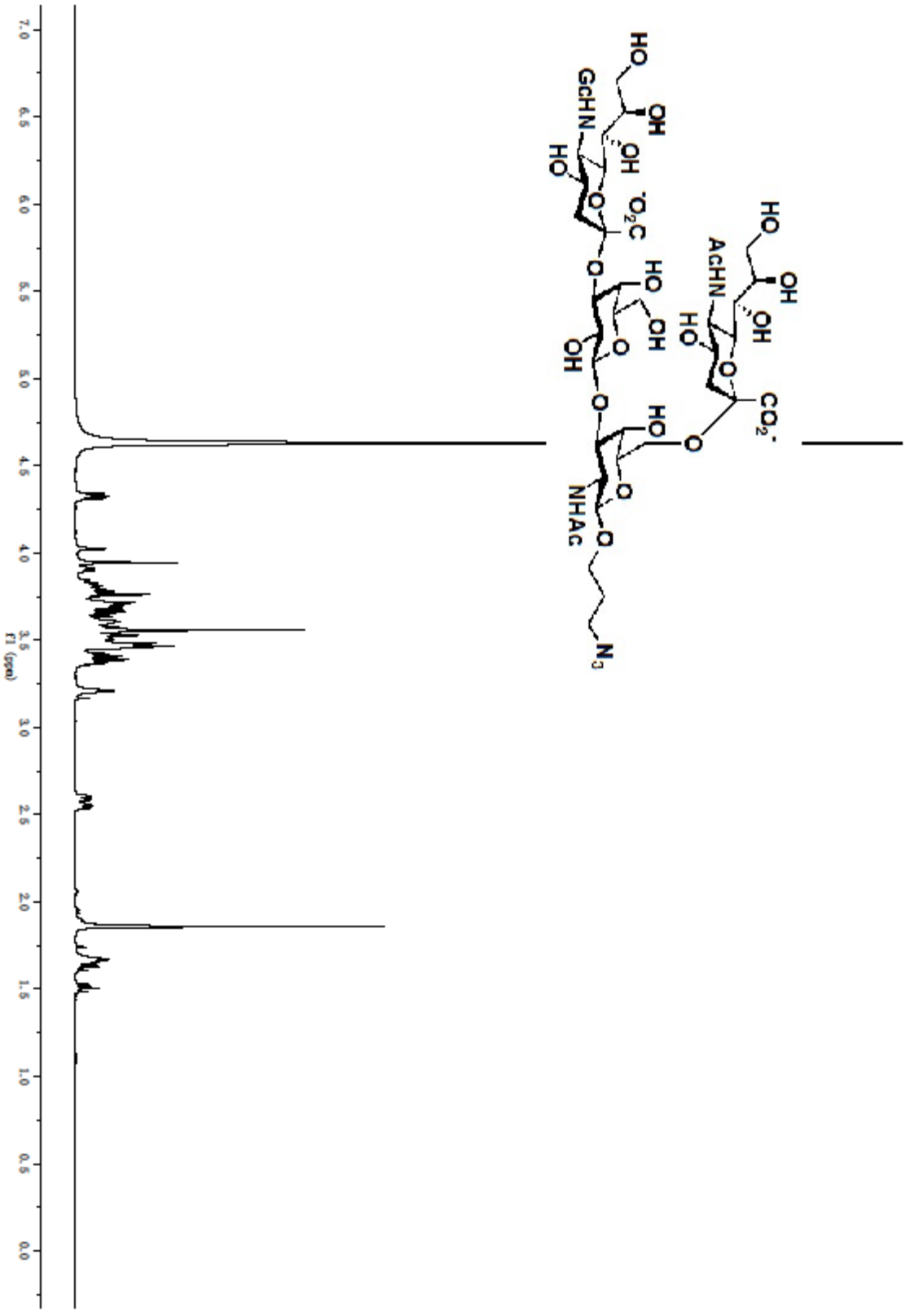

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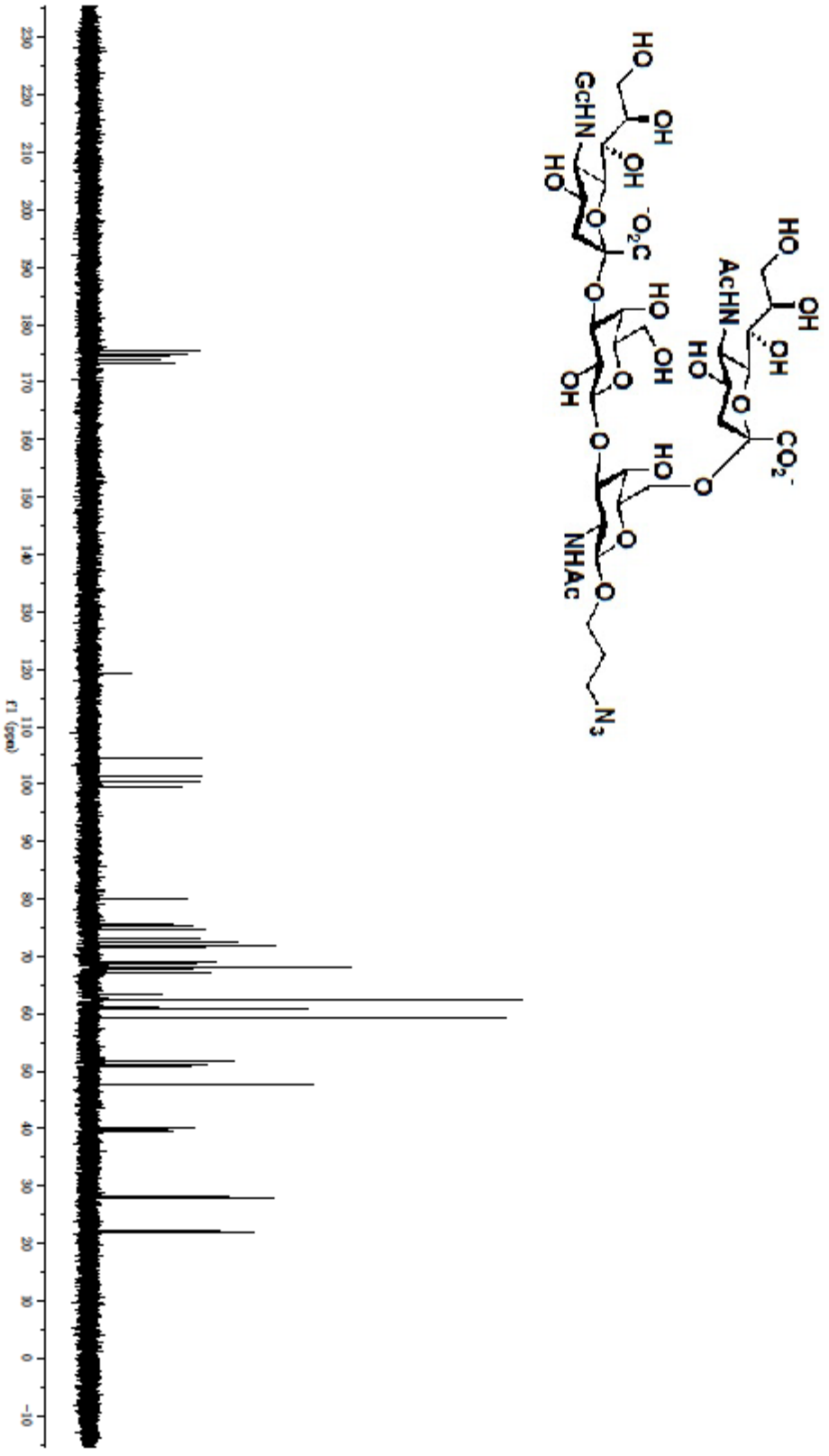

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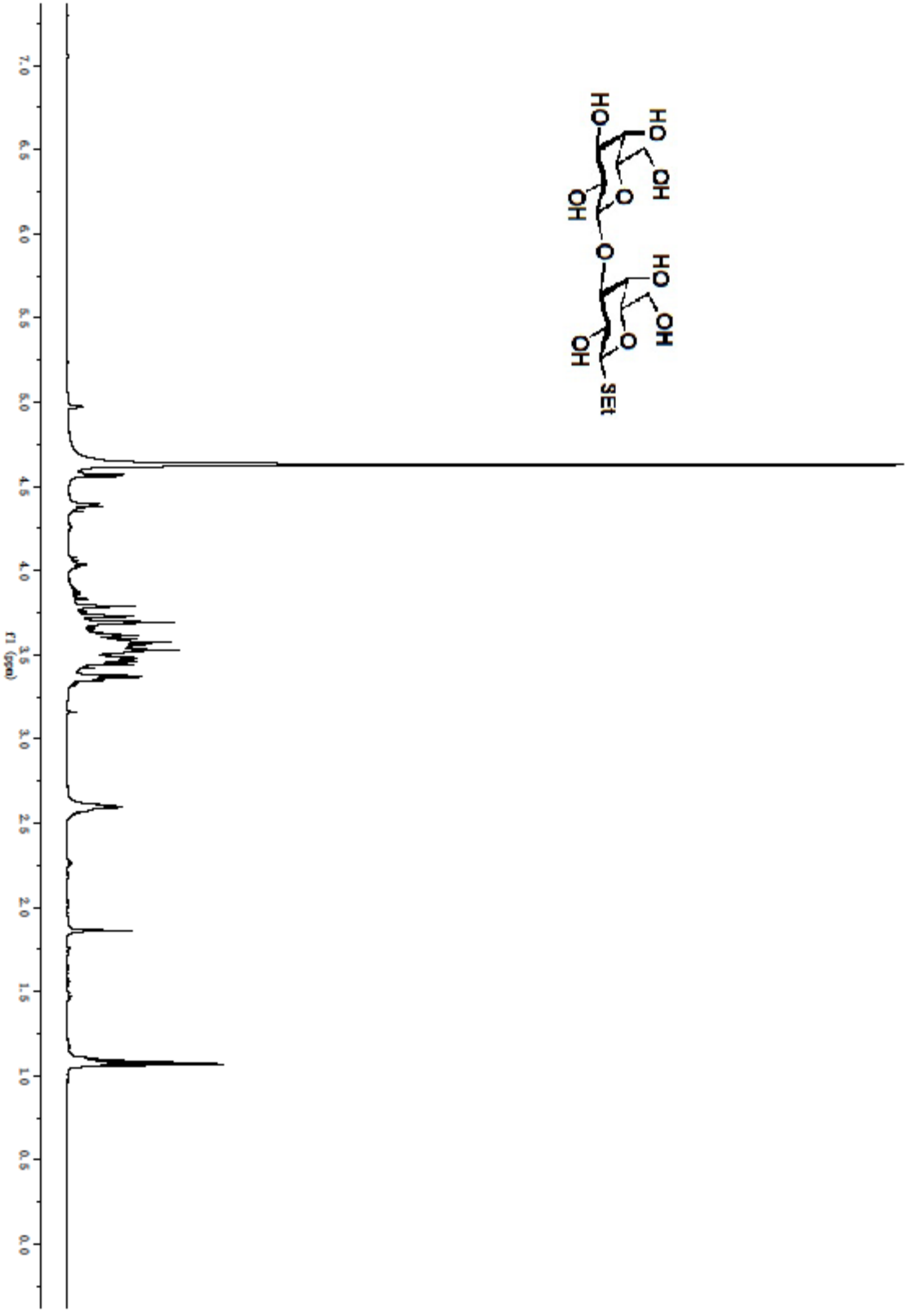

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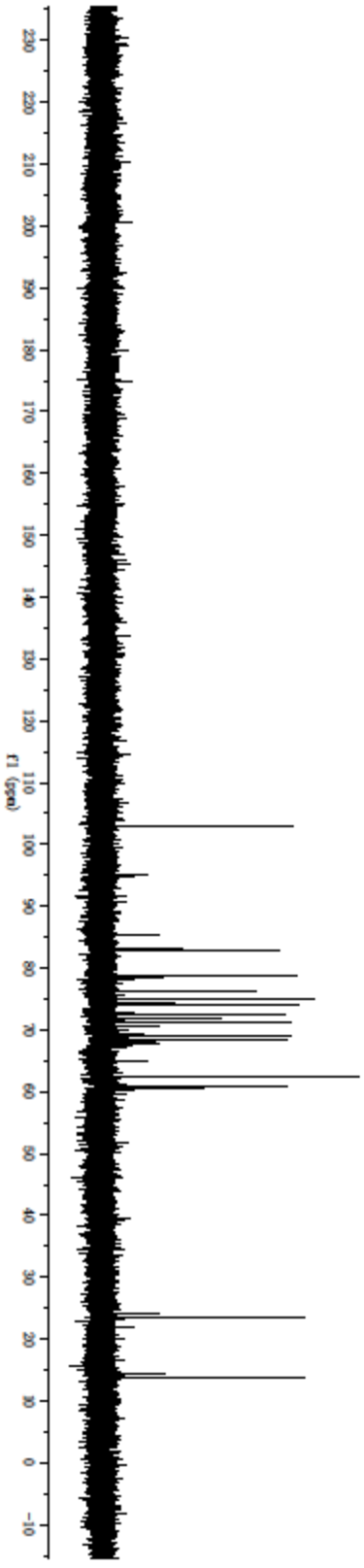


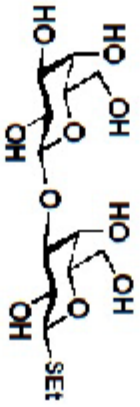

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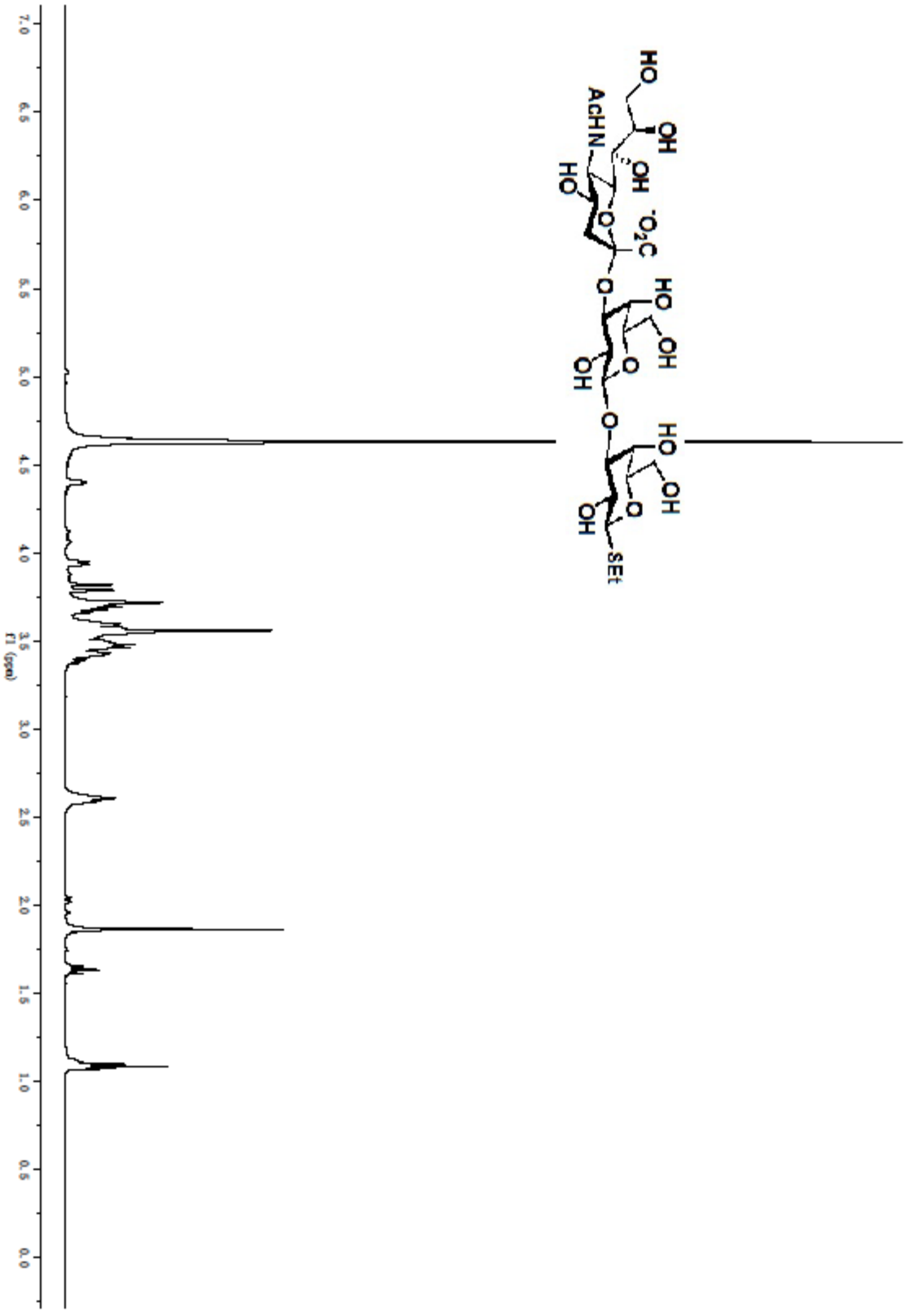

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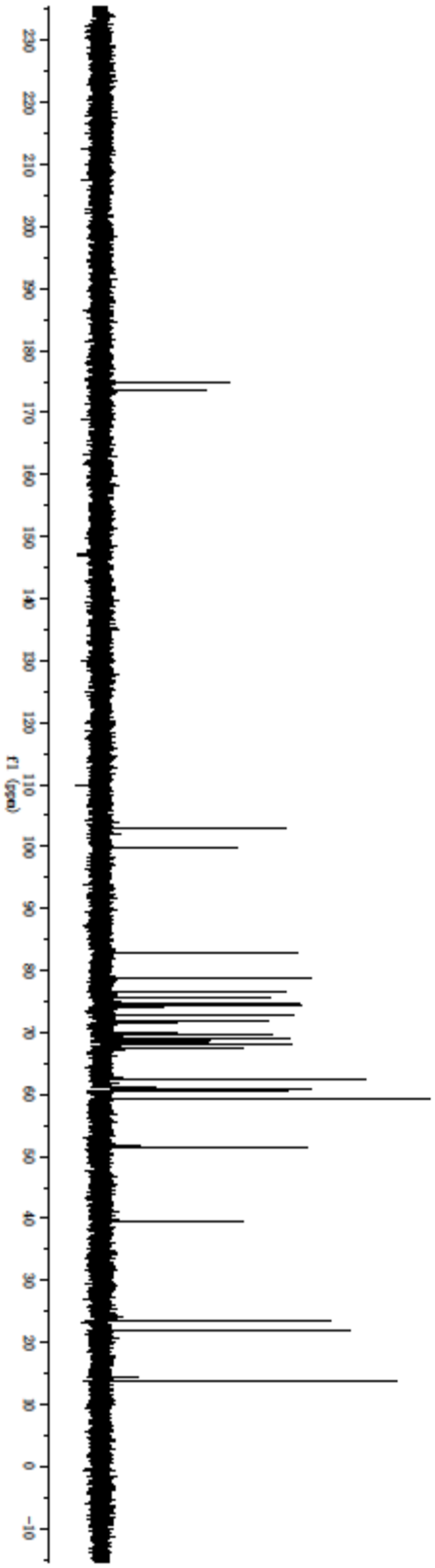


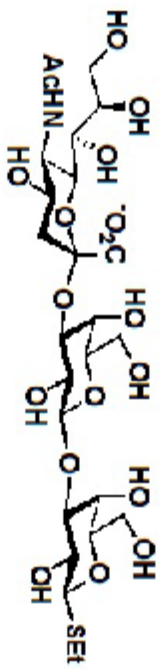

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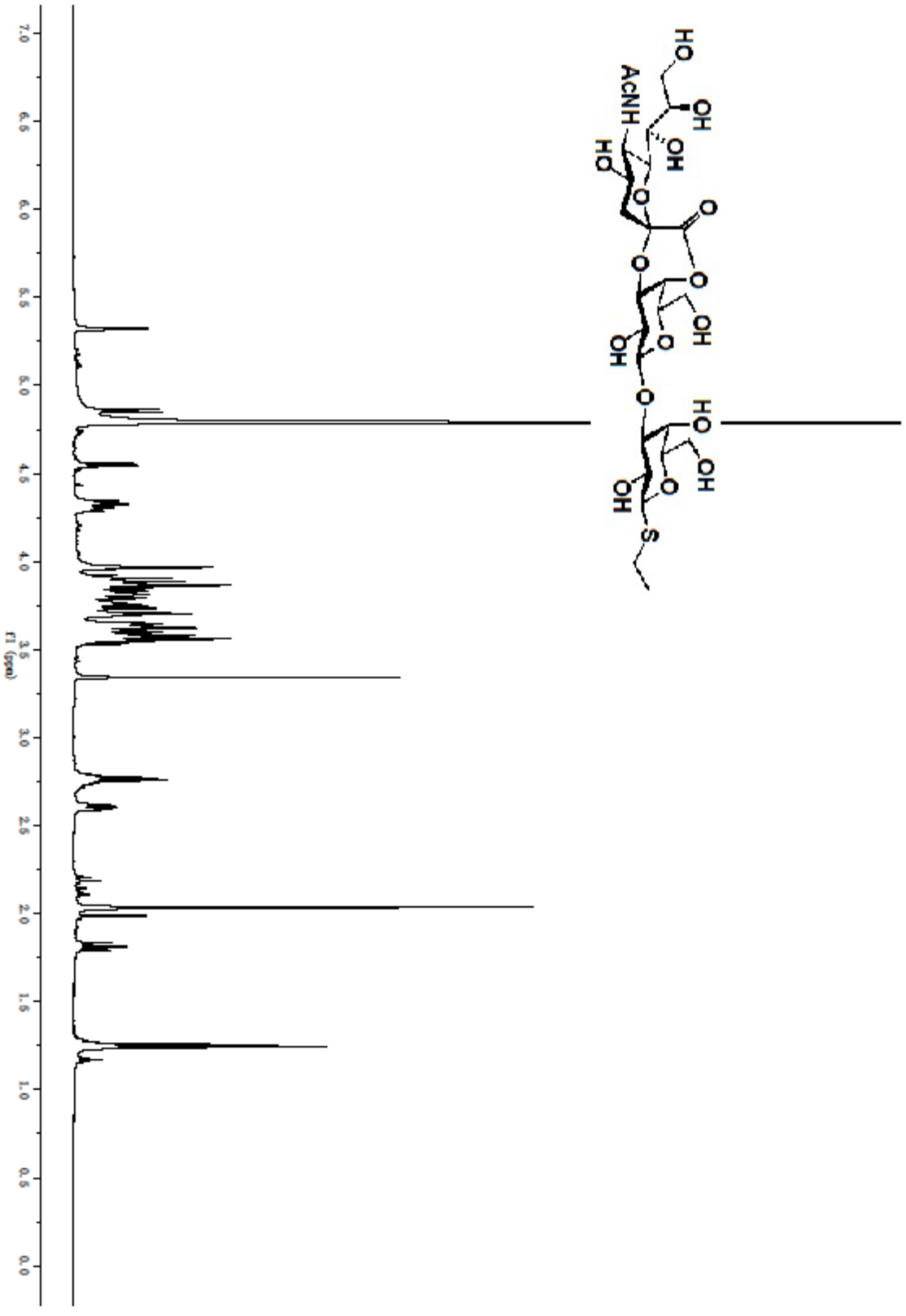

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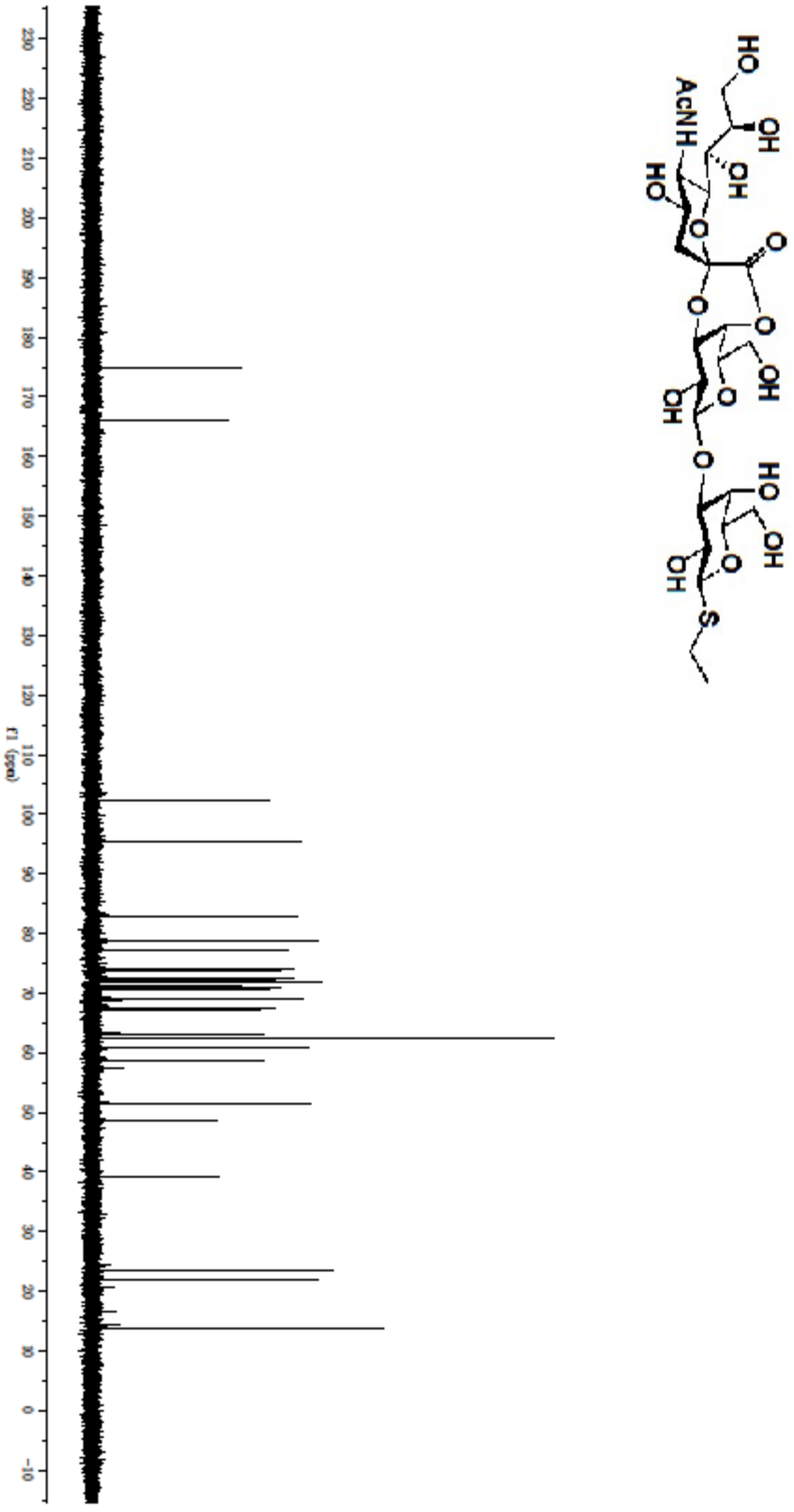

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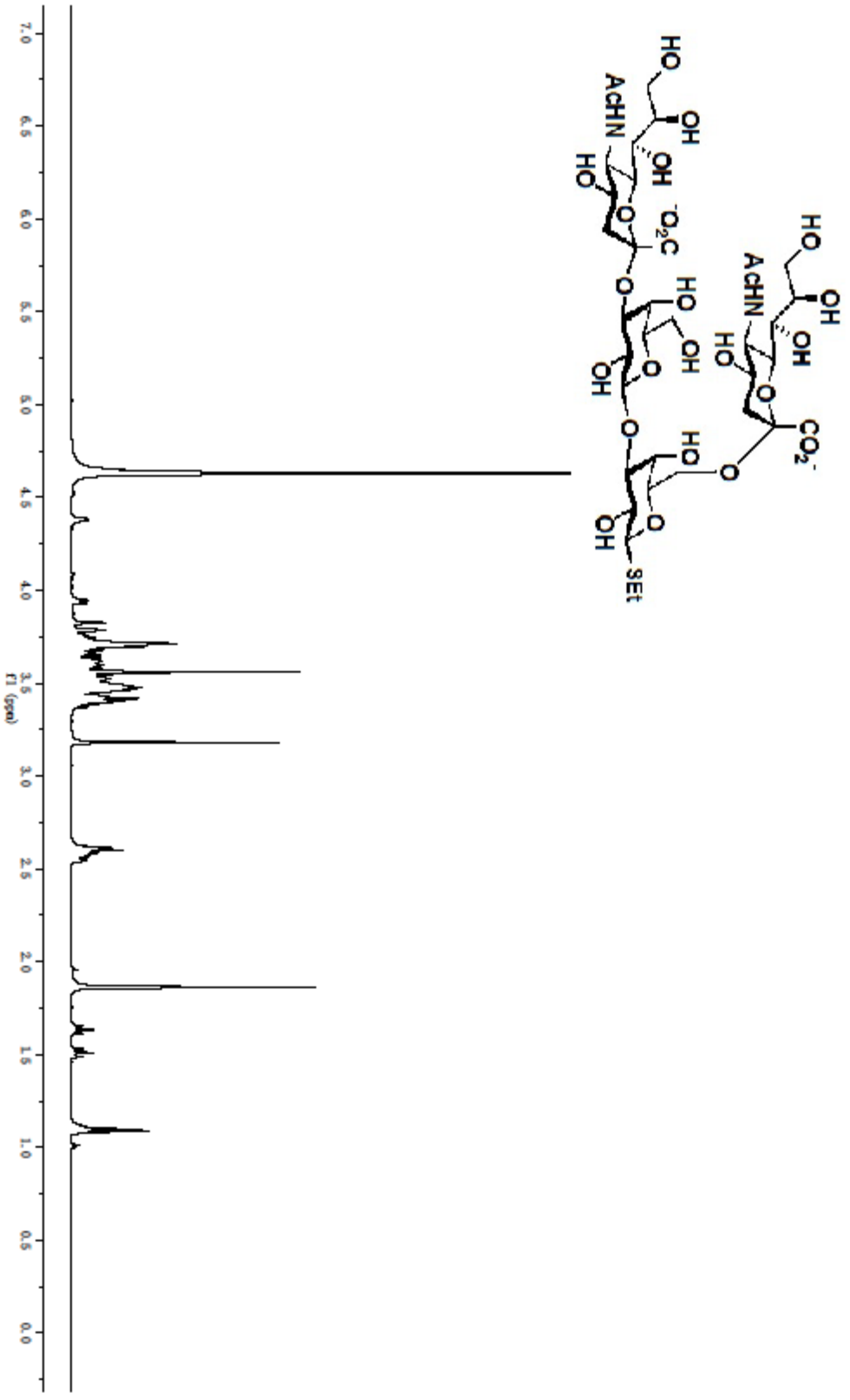

$^{13}$C NMR spectrum for compound **24**