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Supporting Information

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Multivalent Glycocluster Design through Directed Evolution**

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Supporting Information

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Materials and general synthetic methods for Man_4 preparation:

The original oligonucleotide library, PCR primers and the library regeneration primer were purchased from Integrated DNA Technologies. A complete list of primers is in Table 1. Vent polymerase, Vent(exo) polymerase, Bst polymerase, T4 polynucleotide kinase, Exonuclease I, Taq polymerase and streptavidin magnetic beads were purchased from New England Biolabs. Centrisep desalting columns were purchased from Princeton Separations. Sephadex G-50 superfine resin was purchased from GE Healthcare. Antibody 2G12 was purchased from Immune Technology Corp. Protein A Dynabeads and a TOPO-TA cloning kit were purchased from Invitrogen. ATP (γ-^{32}P) was purchased from Perkin Elmer. Synthetic oligos were purchased from Integrated DNA Technologies or ELLA Biotech.

All synthesis reagents were purchased from Sigma-Aldrich, Acros Organics, Fluka, Alfa Aesar, or Strem, and used without further purification unless otherwise noted. Toluene, THF, and DCM were dried by passage through activated alumina columns and stored under argon gas. Acetonitrile was distilled over calcium hydride. Glassware was flame-dried or dried in a 150° C oven. Silicycle Siliaflash® P60 silica was used for column chromatography. All ^{1}H and ^{13}C NMR spectra were obtained on a Varian iNova 400 instrument in $CDCl_3$, internally referenced to TMS, or D_2O, internally or externally referenced to sodium 3-(trimethylsilyl)propanesulfonate. Chemical shifts are reported in parts per million (ppm), and coupling constants are reported in Hz. Coupling is referred to with the following abbreviations (d = doublet, t = triplet, q = quartet, m = multiplet, app = apparent, and br = broad). For NMR spectra in which large numbers of resonances are unresolved, only the clearly-resolved "selected signals" are listed in text-format listing of data. LC/MS analysis was performed on a Waters Acquity UPLC chromatograph with a reverse phase C_{18} or C_8 column, and a Waters Micromass Z/Q mass detector. Optical rotation was measured using a Jasco digital polarimeter. Infrared spectra were obtained using a Varian 640-IR spectrometer with a ZnSe ATR.

Selection method

Table 1: DNA oligonucleotides used

Oligonucleotide	Sequence
Hairpin library	5'CTGTTGTTCCGCAGTCACCTTNNNNNNNNNNNNNNNNNNNNNNNNNCCCGTACCCGTATTTGGTGGCAAGGATGACAAGGATTTTATATTTTATATTTTTATTTTATTATCGGGTACGGG Blue=aptamerrev binding region Red=stem region Green=loop region underlined=aptamerfor binding region N_{25}=Randomized region
Regeneration primer	5'biotin/CCCGTACCCGATAATAAAATAAAAATATAAAATA

	TAAAATCCTTGTCATCCTTGCCACCA
Aptamerfor	5′CCTTGTCATCCTTGCCACCA
Aptamerfor-biotin	5′biotin/CCTTGTCATCCTTGCCACCA
Aptamerrev	5′CTGTTGTTCCGCAGTCACCTT
Aptamerrev-biotin	5′biotin/CTGTTGTTCCGCAGTCACCTT
Hairpin primer	5′CACCAAATACGGGTACGGG

Incorporation of alkyne-containing thymidine analogues

The original oligonucleotide library consists of a stem-loop region connected to a typical aptamer library- a randomized portion flanked by primer regions for aptamerfor and aptamerrev (Table 1). In a PCR tube, 40 pmol of library, 2.5 µl 10X Thermopol buffer (New England Biolabs), and 17 µl autoclaved H_2O were combined, after which the temperature was raised to 95° C for 15 seconds and allowed to cool to room temperature. Then, a 0.5 µl of a solution containing 10 mM deoxyadenosine triphosphate, 10 mM deoxycytosine triphosphate, 10 mM deoxyguanosine triphosphate, and 10 mM alkyne-containing thymidine triphosphate analogue 5-ethynyl-deoxyuridine (EdU) triphosphate was added to afford a final concentration of 200 µM each. 8 U of Bst polymerase (large fragment) was added to the reaction, yielding a final reaction volume of 25 µl. The reaction was mixed and incubated at 60° C for 2 minutes.

Click reaction

The reaction was diluted to 50 µl with H_2O and transferred to a cap-less 0.5 ml microcentrifuge tube. 5 µl of 10 mM tris(3-hydroxypropyl-4-triazolylmethyl)amine (THPTA), 2 µl of 25 mM $CuSO_4$, and 5 µl of 35 mM mannose sugar-azide were added and the solution was mixed by pipetting. Then, 2 µl of freshly dissolved 250 mM sodium ascorbate was added followed by immediate mixing by pipetting. The microcentrifuge tube was quickly placed in a 5 ml round bottom flask and a rubber septum used to seal the tube, and argon was flushed into the flask for 5 minutes. The reaction was allowed to proceed for 2 hours. The modified DNA was then desalted twice through Centrisep desalting columns containing Sephadex G-50 superfine resin.

Note: Following the addition of sodium ascorbate, it is important to flush argon as quickly as possible to minimize damage to the DNA.

Strand Displacement

To the desalted reaction product, Thermopol buffer (1X final concentration), Aptamerfor primer (50 pmol), dNTPs (200 µM each final concentration), 8 U of Bst polymerase (large fragment) and H_2O was added to a final volume of 50 µl. The reaction was incubated at 65° C for 5 minutes followed immediately by buffer exchanging through a Centrisep column loaded with Sephadex G-50/binding buffer (20 mM Tris pH 7.5, 100 mM NaCl, 2 mM MgSO4). Then, binding buffer plus 0.02% Tween-20 was added to a final volume of 50 µl and the solution was heated to 75° C for 3 minutes and allowed to cool to room temperature.

Note: It is important to keep the mixture on ice prior to incubation at 65° C to avoid unwanted side reactions. After strand displacement, it is important to quickly buffer exchange the reaction to remove dNTPs thus minimizing unwanted side reactions. At

each desalting/buffer exchange step, the overall volume decreases. This is exacerbated by the inclusion of detergent (Triton X-100) in the polymerase buffer.

Selection for 2G12 antibody affinity
2G12 antibody was added to a final concentration of 50 nM and the solution was incubated at room temperature for 1 hr. Then, the mixture was added to 1.5 mg protein A Dynabeads and incubated for 45 minutes with rotation. The mixture was applied to a magnetic separator and the supernatant was removed by pipetting. Then, the mixture was washed with 100 µl, 150 µl, and 200 µl of binding buffer/0.02 % Tween-20. Following washing, the beads were then resuspended in 30 µl elution buffer (20 mM Tris pH 8, 100 mM NaCl, 0.02% Tween-20) and placed in a boiling water bath for 2 minutes The beads were immediately applied to a magnetic separator and the supernatant placed in a PCR tube.

Amplification of selected mannose-DNA
Thermopol buffer (1X final conc.), 60 pmol aptamerfor-biotin and 60 pmol aptamerrev, dNTPs (200 µM final conc.), 4 U Vent(exo) polymerase and H_2O were added to a final volume of 200 µl. The reaction was separated into 3 tubes and cycled at
1) 95° C for 1.5 minutes,
2) 95° C for 15 seconds,
3) 64° C for 20 seconds,
4) 72° C for 10 seconds,
5) Cycles 2 through 4 repeated for 12 cycles.
Note: Cycle number was empirically determined by removing the PCR tubes at varying cycle numbers (8-12) and running a portion (5 µl) of the reaction product on an agarose gel. Subsequently, all tubes are brought up to the optimal cycle number. It is important to avoid excessive cycling as this can lead to unwanted side reactions.

Regeneration of the library
30 U Exonuclease I was added followed by incubation at 37° C for 30 minutes and inactivation at 80° C for 20 minutes to remove excess primer from the previous PCR reaction. 4 M NaCl was added to a final concentration of 500 mM and EDTA was added to a final concentration of 5 mM. The PCR product was then incubated with streptavidin magnetic beads for 30 minutes with intermittent mixing. The beads were washed twice with wash buffer (20 mM Tris pH 8.0, 500 mM NaCl) followed by the addition of 40 µl 100 mM NaOH for 4 minutes to elute the unbiotinylated strand. The supernatant was immediately mixed with 4 µl of 1 M HCl and the solution was desalted through a Centrisep column loaded with Sephadex G-50.

Thermopol buffer (1X final concentration), library regeneration primer (40 pmol), dNTPs (200 µM each final concentration), 2 U of Vent polymerase and H_2O was added to a final volume of 100 µl. The reaction was heated at 64° C for 30 seconds followed by 2 minutes at 72° C. 30 U of Exonuclease I was added and the reaction was incubated at 37° C for 30 minutes followed by 20 minutes at 80° C. 4 M NaCl was added to a final concentration of 500 mM and EDTA was added to a final concentration of 5 mM. The product was then incubated with streptavidin magnetic beads for 30 minutes with

intermittent mixing. The beads were washed twice with wash buffer (20 mM Tris pH 8.0, 500 mM NaCl) followed by the addition of 40 µl 100 mM NaOH for 4 minutes to elute the unbiotinylated strand. The supernatant was immediately mixed with 4 µl of 1 M HCl followed by 1 µl of 1 M Tris pH 8.

Subsequent rounds of library generation/selection
10 µl of the 45 µl recovered from the library regeneration step were used in each subsequent round of library generation/selection. 4 U of Bst polymerase were added instead of 8 U in both steps using this enzyme. 10 pmol aptamerfor was used for the strand displacement reaction. 50 nM antibody 2G12 were used in rounds 1 and 2, 10 nM antibody in rounds 3 and 4, and 5 nM antibody in rounds 5, 6, and 7. In rounds 2, 4, and 6, the library was counterselected against protein A magnetic beads by incubation with 0.75 mg beads for 30 minutes and using the supernatant to select for antibody 2G12 binding.

Cloning of selected library

After 7 rounds of library generation/selection and amplification of the selected mannose-DNA from round 7, 2 µl of the amplification PCR product was used in a 100 µl amplification reaction using Vent(exo) polymerase according to the same parameters as used previously, except primer aptamerfor was used instead of primer aptamerfor-biotin. 5 U Taq polymerase was added to the PCR product and the reaction was incubated for 30 minutes at 72° C to ensure optimal incorporation of overhanging adenosine nucleotides at the 3' ends of both strands. A TOPO TA cloning kit was then used to clone the library according to manufacturer's instructions, using blue-white colony screening to identify positive clones. 20 white colonies were picked into LB broth and the plasmid isolated and sequenced:

Sequences of clones, (+) strand, 5'->3':

1
CCTTGTCATCCTTGCCACCAAATACGGGTAAGGATGTTATAAGATCAACGAATCATTATAAGGTGACTGCGGAACAACAG
3
CCTTGTCATCCTTGCCACCAAATACGGCCACGGGCGCACGTCTCACCGCACTCTTAAGTAAGGTGACTGCGGAACAACAG
4/5
CCTTGTCATCCTTGCCACCAAATACGCGTACGGGGACGCCTGTCATCCTGGTCATTACTGAGGTGACTGCGGAACAACAG
6
CCTTGTCATCCTTGCCACCAAATACGGGTGCGGGCGCGCTTTGTTTCAGCTCATGATATAAGGTGACTGCGGAACAACAG
8
CCTTGTCATCCTTGCCACCAAATACGGATACGGGTCGGTCATGATCATCAGTATGTCATAAGGTGACTGCGGAACAACAG
9
CCTTGTCATCCTTGCCACCAAATACAGGTACGGGTCCATTATCGCGTGTCGTGTGCCGAAAGGTGACTGCGGAACAACAG
11
CCTTGTCATCCTTGCCACCAA-TACGGGTACGGGAGGCCTTTCTCCATTGGGACGTCTCAAGGTGACTGCGGAACAACAG
13
CCTTGTCATCCTTGCCACCAAATACGGGTATGGGTTCGTTCATTCTCCTTACCATTGTCAAGGTGACTGCGGAACAACAG
14
CCTTGTCATCCTTGCCACCAAATACGTACACGGGGCAATTCAGAGCTCCATTGCGCTCTAAGGTGACTGCGGAACAACAG
15
CCTTGTCATCCTTGCCACCAA-TACGGGCACGGGGCGTTTGTCTCATTACGTGCTAATCAAGGTGACTGCGGAACAACAG
16/23
CCTTGTCATCCTTGCCACCAATTACGGGTACGGGCCCGGCTGTTTCAGATGCTGTAAGTAAGGTGACTGCGGAACAACAG
18
CCTTGTCATCCTTGCCACCAAATACGGGTACGGGCCGCGGTGTCTCATCCGCATTTATAAGGTGACTGCGGAACAACAG
19
CCTTGTCATCCTTGCCACCAAATACGGGTACGGGCGCTTTGTCGCTATGGTCGTTGACTAAGGTGACTGCGGAACAACAG
21

CCTTGTCATCCTTGCCACCAAATACGGGTACGGGTCAGCTCGTCTCACCTGCTGTGTGTAAGGTGACTGCGGAACAACAG
22
CCTTGTCATCCTTGCCACCAAATAAGGGTACGGGCCATTGACCGCCATTGCCGATTCCAAAGGTGACTGCGGAACAACAG

Preparation of selected clones for filter binding assay

Clones were amplified using Vent(exo) polymerase in 100 µl reactions and 20 pmol each of primers hairpinfor and aptamerrev-biotin and the conditions/thermal cycling used previously for library amplification. In these reactions, deoxythymidine triphosphate was replaced by 5'ethynyl-deoxyuridine triphosphate. The non-biotinylated strand was isolated using streptavidin magnetic beads as described, and 1 µl of 1M Tris pH 8 was added to the isolated strand. 10 µl isolated single-stranded DNA was used in a 25 µl reaction containing 1X Thermopol buffer, 200 µM dNTPs, 15 pmol aptamerrev-biotin, and 0.5 U Vent polymerase. The reaction was incubated at 64° C for 30 seconds followed by 72° C for 2 minutes. Then, 10 U exonuclease I was added and the reaction was incubated at 37° C for 30 minutes followed by inactivation at 80° C for 20 minutes. The reaction was transferred to a cap-less 0.5 ml microcentrifuge tube. Added to the reaction was 2.5 µl 10 mM THPTA ligand, 1 µl 25 mM $CuSO_4$, 2.5 µl of 35 mM mannose sugar-azide, and the reaction was mixed by pipetting. Then, 1 µl of fresh 250 mM sodium ascorbate was added followed by immediate mixing by pipetting. The microcentrifuge tube was quickly placed in a 5 ml round bottom flask and a rubber septum used to seal the tube, and argon was flushed into the flask for 5 minutes. The reaction was allowed to proceed for 2 hours. Then, 25 µl H_2O was added and the reaction was immediately desalted twice through Centrisep desalting columns containing Sephadex G-50 superfine resin. The desalted modified DNA was then radioactively phosphorylated using polynucleotide kinase and ATP (γ-^{32}P) according to manufacturer's instructions. The non-biotinylated, radiolabeled strand was then isolated using streptavidin magnetic beads as described, however four washes were performed to extensively remove unincorporated ^{32}P. 1 µl of 1 M Tris pH 8 was added and the resulting modified, labeled DNA was stored on ice or at 4° C.

Preparation of mutant clones from (-)-strand synthetic oligos (sequences in manuscript Figure 3)

All synthetic mutant clones were truncated to remove the loop portion of the sequence (24 green residues in above clone seqences).

In a PCR tube, 40 pmol of (-)-strand synthetic oligo complementary to a sequence in Figure 3, 2.5 µl 10X Thermopol buffer (New England Biolabs), 15 µl autoclaved H_2O, and 2 µl 25 µM primer were combined. To this was added 0.5 µl of a solution containing 10 mM deoxyadenosine triphosphate, 10 mM deoxycytosine triphosphate, 10 mM deoxyguanosine triphosphate, and 10 mM alkyne-containing thymidine triphosphate analogue 5-ethynyl-deoxyuridine (EdU) triphosphate to afford a final concentration of 200 µM each. 8 U of Bst polymerase (large fragment) was added and the mixture was

incubated at 60° C for 2 minutes to complete synthesis of the duplex. The reaction was diluted with 25 µl of autoclaved H_2O to a final volume of 50 µl.

For entry 21 of Figure 3, a synthetic (+)-sense strand containing the desired sequence was simply annealed to the (-)-sense strand to produce a similar duplex structure.

The reaction was transferred into a 0.5 mL microcentrifuge tube containing 5.0 µl 10 mM THPTA ligand and 2.0 µl 25 mM $CuSO_4$. 5.0 µl of 35 mM mannose sugar-azide was added and the reaction was mixed by pipetting. Then, 2 µl of fresh 250 mM sodium ascorbate was added followed by immediate mixing by pipetting. The microcentrifuge tube was quickly placed in a 5 ml round bottom flask and a rubber septum used to seal the tube, and argon was flushed into the flask for 5 minutes. The reaction was allowed to proceed for 2 hours under argon. The modified DNA was then desalted twice through Centrisep desalting columns containing Sephadex G-50 superfine resin.

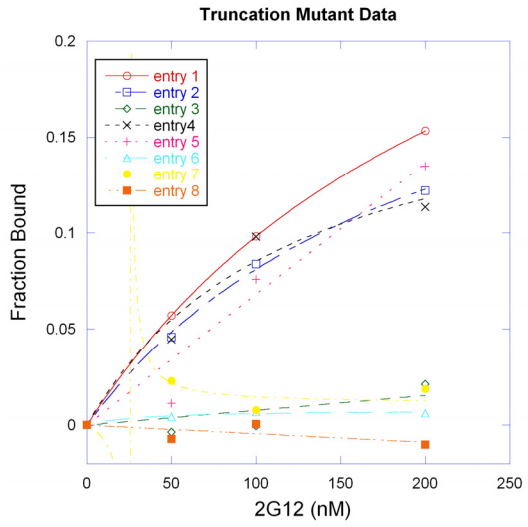
24.5 µl of the desalted modified DNA was added to a PCR tube containing 1ul 100 mM freshly prepared dithiothreitol (DTT) and 3.0 µl T4 Polynucleotide Kinase Reaction Buffer (10X). To the reaction was added 0.5-1.0 µl ATP (γ-^{32}P-Perkin Elmer), followed by 10 U T4 Polynucleotide Kinase (New England Biolabs). The reaction was incubated at 37 ^{o}C for 2 hours and then the labeled product was incubated with streptavidin magnetic beads for 30 minutes at RT with rotation. The beads were washed four times with 150 µl wash buffer (20 mM Tris pH 8.0, 500 mM NaCl) followed by the addition of 40 µl 100 mM NaOH for 4 minutes to elute the unbiotinylated strand. The supernatant was immediately mixed with 4 µl of 1 M HCl followed by 1 µl of 1 M Tris pH 8.

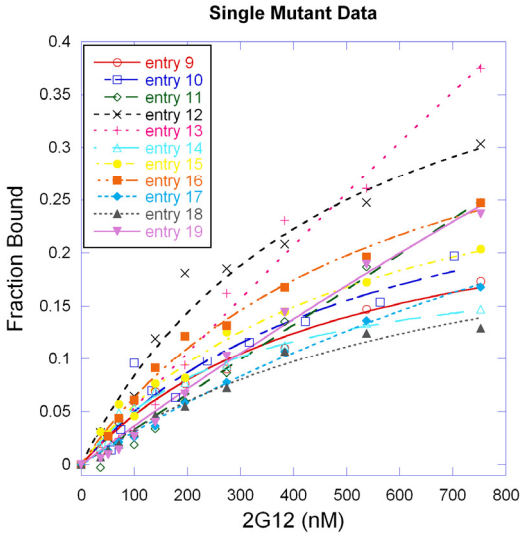
These labeled ssDNA were then directly used in the labeling procedures as described in the filter binding section

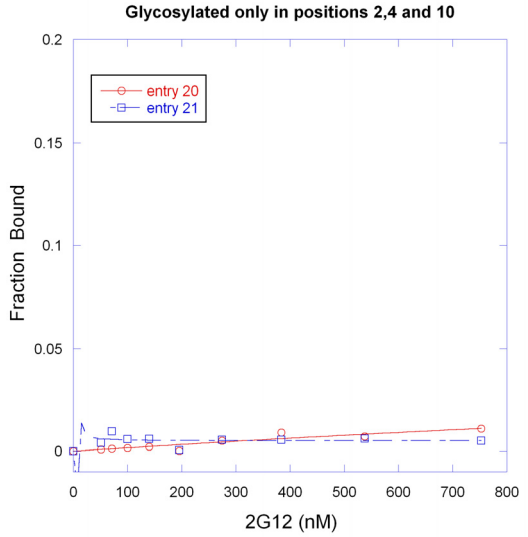
Filter binding

2.5 µl of modified, radiolabeled DNA was added to 50 µl binding buffer/0.02 % Tween-20. The solution was heated to 75° C for 3 minutes and allowed to cool to room temperature. Then, the desired amount of antibody 2G12 was added to the solution and binding allowed for 3 hours at room temperature. The solution was then filtered through a nitrocellulose/PVDF sandwich and the radioactivity in each membrane quantified by exposure to a phosphor screen followed by phosphor imaging.
Note: Nitrocellulose was exposed to 0.4 M NaOH for 10 minutes, washed extensively with H_2O, and then soaked in binding buffer prior to the filter binding assay. PVDF was soaked in methanol prior to extensive washing with H_2O and soaking in binding buffer prior to the filter binding assay.

Binding Curves for Mutants of Clone 16/23 (see manuscript Figure 3c):





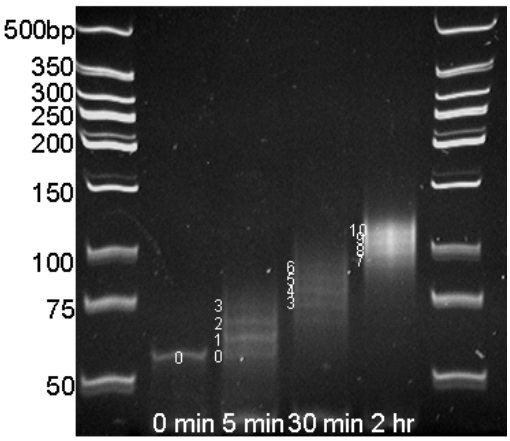


Measurement of Click Glycosylation Efficiency

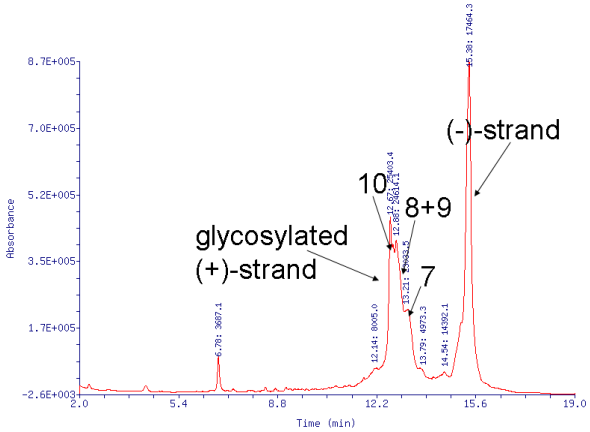
The PAGE gel below (20% acrylamide, 29:1 acrylamide:bis-acrylamide, EtBr staining) shows the progress of the click glycosylation of clone 16/23 at several timepoints over two hours. Roughly 10-11 bands can be seen in the gel (corresponding to starting oligo and one- through ten-fold-glycosylated species). The identity of the 3 major bands after 2 hrs was confirmed to be the 8, 9, and 10-clicked species by RP-HPLC/ESI-MS analysis (analysis by Novatia, Inc., 2x50 mm ACE C18 300 column, 60 °C, 0.4 mL/min, 5-20% B over 19 min). In a control experiment, the fully-glycosylated material was separated from underglycosylated material by preparative PAGE. Pure material bound with a somewhat higher F_{max}, but not a better K_d, than the mixture.

Supplementary Figure 1: Click Glycosylation Efficiency

PAGE of Click Reaction Aliquots:



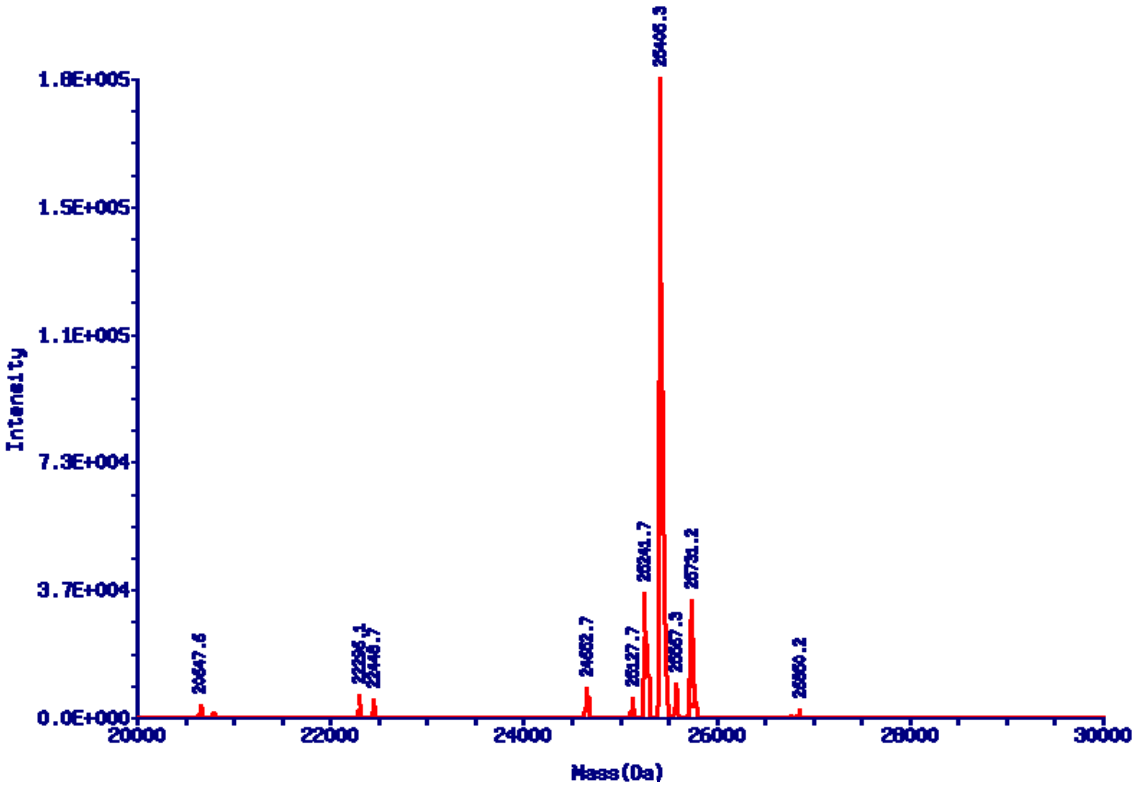
RP-HPLC/ESI-MS Chromatogram after 2 hr:



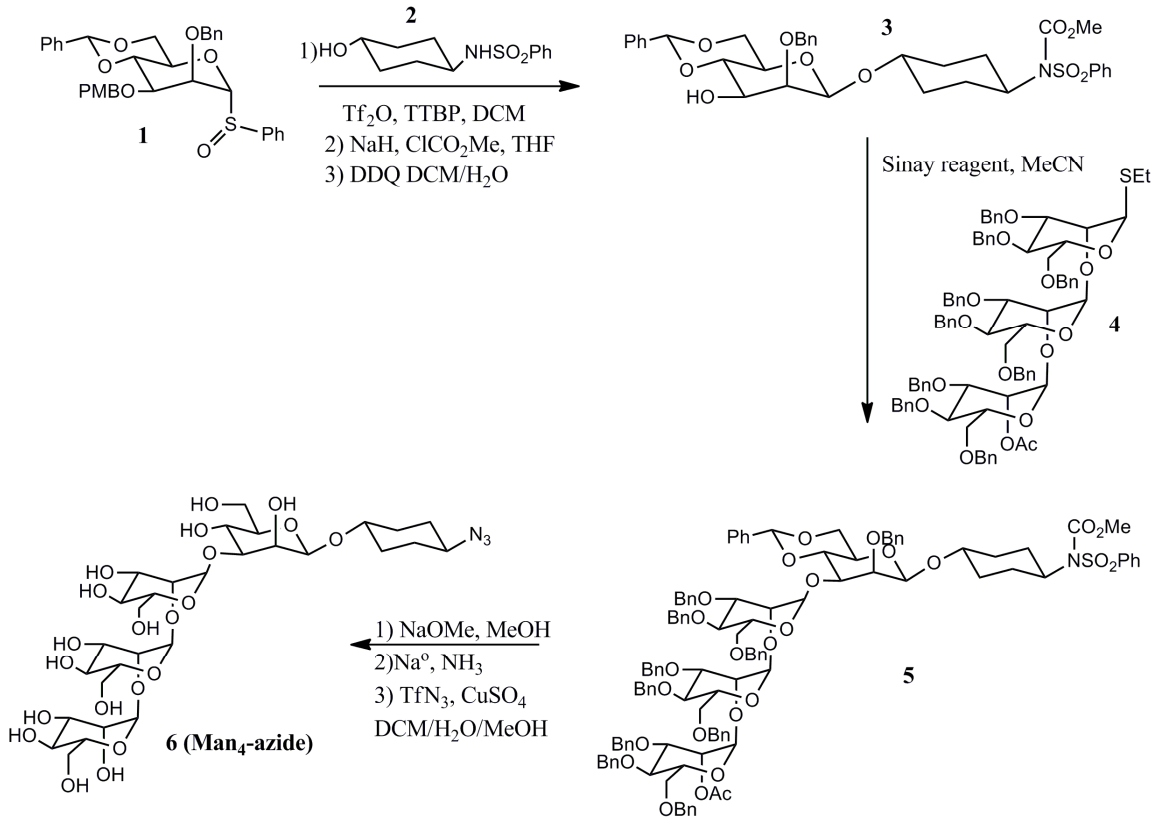
Masses and Abundance of Observed Species in Chromatogram:

RT (min)	Target Mass (Da)	Observed Mass (Da)	Mass Error	LC/MS Area Percent	Identity
12.66	25407.5	25403.4	-4.1 Da (-0.016 %)	19.15	(click 10x)
12.87	24617.8	24614.1	-3.7 Da (-0.015 %)	22.60	(click 9x)
12.87	23828.0	23824.6	-3.4 Da (-0.014 %)	9.16	(click 8x)
13.20	23038.3	23033.5	-4.8 Da (-0.021 %)	0.36	(click 7x)
15.37	17465.5	17464.3	-1.2 Da (-0.007 %)	46.25	(-)-strand

Supplementary Figure 2: Deconvoluted ESI mass spectrum of 10-fold glycosylated species (RT = 12.66)



Synthetic Scheme:

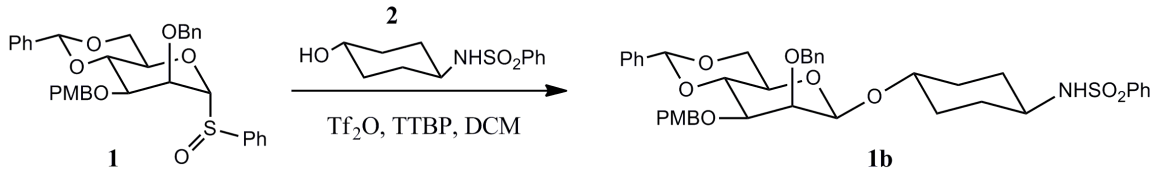


For synthesis of starting materials **1** and **3** see references:

(1) **1:** Crich, D.; Li, W.; Li, H. *J. Am. Chem. Soc.* **2004**, *126*, 15081–15086.

(2) **3:** Geng, X.; Dudkin, V.Y.; Mandal, M.; Danishefsky. S.J. *Angew. Chem. Int. Ed.* **2004**, *116*, 2616–2619.

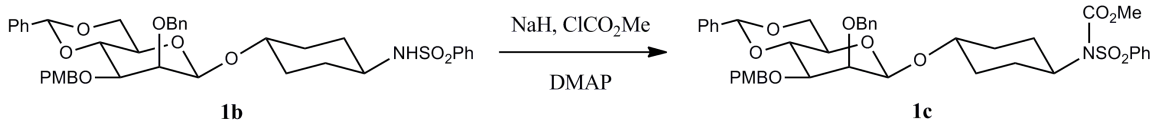
Monosaccharide sulfonamide (1b)



To a 50 mL flask was added 717 mg (1.22 mmol) of starting material **1**. Toluene was added, the solution was cooled to -78 °C, vacuum was applied and the mixture was allowed to warm to R.T. and stir until the toluene was removed. This procedure was repeated twice. Then, 8 mL of dry dichloromethane, along with 606 mg (2.44 mmol) of tri tert-butylpyrimidine and freshly flame-dried powdered 4Å molecular sieves were added. This was cooled to -78° C and allowed to stir for 30 minutes. After this time, 0.16 ml (0.978 mmol) of distilled triflic anhydride was added dropwise. This was allowed to stir for 30 minutes, and 630 mg (2.44 mmol) of trans-(4-hydroxycyclohexyl)-benzenesulfonamide (**2**) in 8.5 ml of dichloromethane was added dropwise. After 1 hour, the reaction was allowed to slowly warm to -20° C over ~1.5 hours, quenched with saturated aqueous $NaHCO_3$ solution, and filtered through celite. The solution was washed with 50 mL of saturated aqueous $NaHCO_3$ solution, and the aqueous phase was extracted with 3 × 50 mL ethyl acetate. The combined organic layers were dried with $MgSO_4$, filtered and concentrated. Crude mass was 2.00 g. Purification by flash column chromatography with 1:2:1 ----> 1:1.5:1 ethyl acetate/hexanes/dichloromethane gave 638 mg (.891 mmol, 91% based on Tf_2O) of **1b** as a white foam. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.89 (d, 2H, J = 7.3 Hz), 7.60-7.17 (m, 15H), 6.82 (d, 2H, J = 8.6 Hz), 5.59 (s, 1H), 4.93 (d, 1H, J = 12.2 Hz), 4.83 (d, 1H, J = 12.2 Hz), 4.55 (m, 4H), 4.26 (dd, 1H, J = 4.9, 10.4 Hz), 4.17 (app t, 1H), 3.90 (app t, 1H), 3.79 (s, 3H), 3,79 (m, 1H), 3.59 (m, 1H), 3.53 (dd, 1H, J = 9.8 Hz, 3.1 Hz), 3.27 (m, 1H), 3.18 (m, 1H), 1.97 (m, 1H), 1.85 (m,

3H), 1.40 (m, 1H), 1.24 (m, 3H). ^{13}C-NMR (100 MHz, CDCl3, selected signals): δ 29.5, 31.0, 51.7, 55.4, 67.7, 68.7, 72.1, 74.7, 75.6, 76.1, 77.7, 78.6, 100.2, 101.5, 113.8, 126.2, 127.0, 127.7, 128.2, 128.3, 128.8, 129.0, 129.3, 130.4, 132.8, 137.7, 138.5, 141.1, 159.2. IR (cm^{-1}): 3267 (br), 2936, 2863, 1610 (s), 1512, 1448, 1325, 1246, 1159, 1076. HRMS (ESI+): calcd. for $C_{40}H_{46}NO_9S^+$ [M + H$^+$] 716.2893, found 716.2892.

Monosaccharide(phenylsulfonyl)carbamate (1c)



1b **1c**

To a 25 mL 0 °C suspension of 213 mg (5.32 mmol) of 60% wt NaH powder in 5 mL THF was added a solution of 760 mg (1.06 mmol) of **1b** in 8.5 mL of THF, dropwise. This was allowed to stir for 30 minutes, and 0.269 mL (3.19 mmol) of methyl chloroformate was added, follwed by 130 mg (1.06 mmol) of recrystallized 4-(dimethylamino)pyridine. The cooling bath was removed, and the reaction stirred at room temperature for 17 hours. The flask was then cooled to 0° C and quenched with saturated aqueous NH_4Cl solution. The organic phase was washed with 40 mL of NH_4Cl solution, and the aqueous phase was extracted with 3 × 40 mL of DCM. The combined organic layers were dried with $MgSO_4$, filtered, and concentrated. Purification by flash chromatography in 1:2 ethyl acetate/hexanes afforded 724 mg of product **1c** as a white foam (0.936 mmol, 88%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.92 (d, 2H, *J* = 8.6 Hz), 7.62 (app t, 1H), 7.57-7.44 (m, 6H), 7.40-7.27 (m, 6H), 7.20 (d, 2H, *J* = 8.5 Hz), 6.83 (d, 2H, *J* = 8.6 Hz), 5.61 (s, 1H), 4.98 (d, 1H, *J* = 12.8 Hz), 4.88 (d, 1H, *J* = 12.2 Hz), 4.61 (d, 1H, *J* = 12.2 Hz), 4.55 (s, 1H), 4.53 (d, 1H, *J* = ~12 Hz), 4.44 (m, 1H), 4.31 (dd, 1H, *J* = 10.4

Hz, 4.9 Hz), 4.20 (app t, 1H), 3.94 (app t, 1H), 3.83 (d, 1H, J = 2.7 Hz), 3.80 (s, 3H), 3.66 (s, 3H), 3.66 (m, 1H), 3.56 (dd, 1H, J = 9.8 Hz, 2.4 Hz), 3.31 (m, 1H), 2.27 (m, 3H), 2.05 (m, 1H), 1.87 (m, 2H), 1.58 (m, 1H), 1.41 (m, 1H). ^{13}C-NMR (100 MHz, CDCl3, selected signals): δ 28.6, 31.7, 33.3, 53.6, 55.4, 58.6, 67.7, 68.7, 72.1, 74.7, 76.1, 76.3, 77.7, 78.7, 100.3, 101.5, 113.8, 126.2, 127.6, 128.0, 128.2, 128.3, 128.9, 129.3, 130.5, 133.5, 137.7, 138.5, 140.3, 152.7, 159.2. IR (cm^{-1}): 2939, 2866, 1731, 1512, 1449, 1356, 1269, 1247, 1169, 1085, 1045, 733, 697. HRMS (ESI+): calcd. for $C_{42}H_{48}NO_{11}S^+$ [M + H^+] 774.2948, found 774.2961.

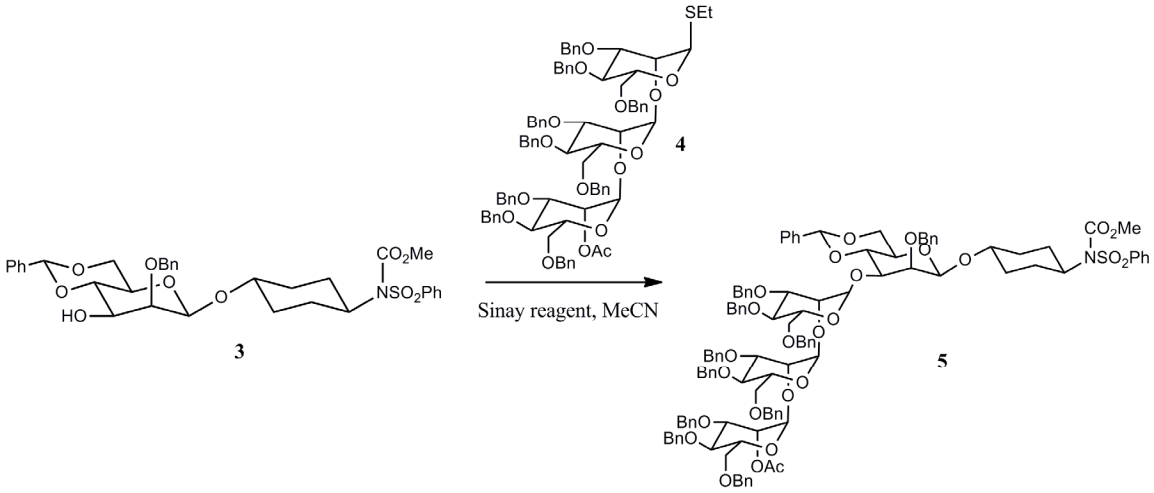
PMB deprotected monosaccharide(phenylsulfonyl)carbamate (3)

1c **3**

To a flask containing 2.04 g (2.64 mmol) of **1c** was added 28 mL of DCM and 1.55 mL of 1M pH 7 phosphate buffer. This was cooled to 0° C, and 1.44 g (6.34 mmol) of 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) was added. This was allowed to stir for 1 hour, and the reaction was quenched with aqueous $NaHCO_3$ solution. The mixture was diluted with DCM, and the organic phase was washed with 375 mL of water. The aqueous phase was extracted with 3 × 300 mL DCM, and the combined organic layers were dried with $MgSO_4$, filtered, and concentrated. Purification by flash chromatography (1:2 ethyl acetate/hexanes) afforded 1.53 g (2.34 mmol, 87%) of **3** as an off-white foam. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.90 (d, 2H, J = 7.3 Hz), 7.60 (app t, 1H) 7.57-7.27 (m, 12H), 5.52 (s, 1H), 5.05 (d, 1H, J = 11.6 Hz), 4.67 (s, 1H), 4.65 (d, 1H, J = 12.2), 4.43 (m, 1H), 4.29 (dd, 1H, J = 10.4 Hz, 4.9 Hz), 3.92-3.63 (m, 5H), 3.64 (s, 3H), 3.31 (m,

1H), 2.36-2.11 (m, 5H), 1.86 (m, 2H), 1.61-1.40 (m, 2H). ^{13}C-NMR (100 MHz, CDCl3, selected signals): δ 28.5, 31.7, 33.4, 53.6, 58.5, 67.2, 68.7, 70.9, 75.8, 76.4, 78.9, 79.4, 100.3, 102.1, 126.4, 128.0, 128.1, 128.4, 128.5, 128.6, 128.9, 129.2, 133.5, 137.3, 138.2, 140.3, 152.6. IR (cm^{-1}): 3528 (br), 2949, 2872, 1733, 1449, 1358, 1272, 1171, 1090, 751, 700. HRMS (ESI+): calcd. for $C_{34}H_{40}NO_{10}S^+$ [M + H$^+$] 654.2373, found 654.2366.

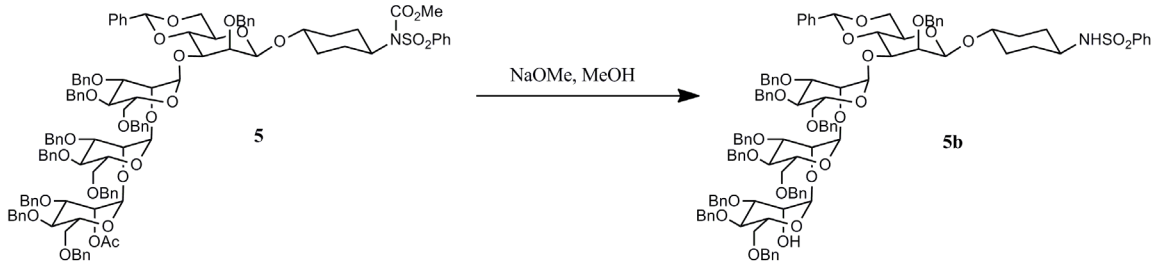
Fully Protected Tetrasaccharide (5)



210 mg (0.333 mmol) of **3** and 700 mg (0.500 mmol) of **4** in a 25 mL flask were dissolved in toluene and cooled to -78 °C. Vacuum was applied and the cooling bath was removed and allowed to warm to room temperature as the toluene evaporated. This procedure was repeated twice. The residue was redissolved in 12 mL of acetonitrile, freshly flame-dried 4Å molecular sieves were added, and this was allowed to stir for 1 hour. The flask was then wrapped in foil, cooled to 0° C, and 525 mg (0.799 mmol) Sinaÿ reagent, (p-BrC_6H_4)$_3$N$^+$ $SbCl_6^-$, was added. This was allowed to react at 0° C for 30 minutes, then at room temperature for 30 minutes. After this time, 1 mL triethylamine was added, and the reaction was filtered through celite and concentrated in vacuo. The

crude residue was purified by flash chromatography with 1:3.5:1 ethyl acetate / hexanes / DCM to give 440 mg (.230 mmol, 69%) **5** as a white foam. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.92 (d, 2H, J = 6.4 Hz), 7.62 (t, 1H), 7.53 (app t, 2H), 7.43 (d, 2H, J=7.3 Hz), 7.39 (d, 2H, J= 7.0 Hz), 7.37-6.96 (m, 50H + residual $CHCl_3$), 6.93 (app t, 1H), 5.53 (s, 1H), 5.40 (s, 1H), 5.34 (s, 1H), 5.24 (s, 1H), 4.98 (s, 1H), 4.9-3.6 (complex region), 3.66 (s, 3H), 3.60-3.40 (m, 4H), 3.33 (br d, 1H, J=11.0), 3.13 (m, 1H), 2.24 (m, 2H), 2.17 (m, 1H), 2.12 (s, 3H), 1.84 (m, 3H), 1.51 (m, 1H), 1.26 (m, 1H + grease). ^{13}C-NMR (100 MHz, $CDCl_3$, selected signals): δ 21.3, 28.5, 31.6, 33.3, 53.6, 58.6, 67.4, 68.9, 69.2, 78.2, 78.4, 100.0, 100.1, 101.3, 126.0, 133.5, 137.4, 138.2, 138.3, 138.7, 138.7, 139.1, 140.5, 152.7, 170.2. IR (cm^{-1}): 3029, 2863, 1735, 1452, 1360, 1085, 1055, 736, 697. HRMS (ESI+): calcd. for $C_{117}H_{126}NO_{26}S^+$ [M + H$^+$] 1992.8289, found 1992.8224.

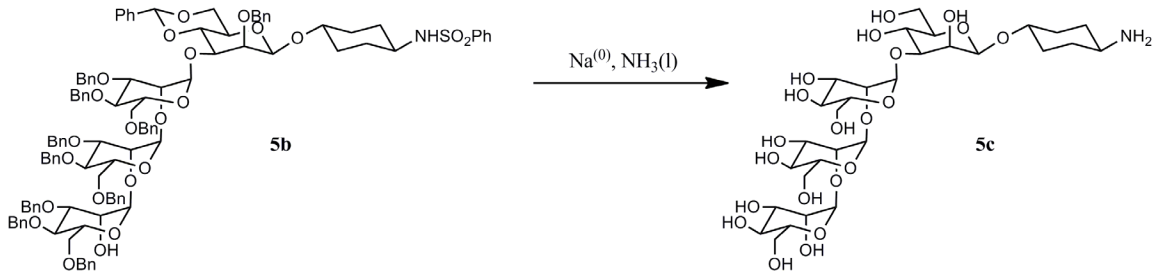
Partially deprotected Tetrasaccharide (5b)



100 mg (0.050 mmol) **5** was dissolved in 12 mL anhydrous methanol and 0.500 mL (2.00 mmol) of 25% wt NaOMe solution in methanol was added. After 3 hours, Amberlite IR-120 H$^+$ ion exchange resin was added until the solution was neutral (NOTE: avoid acidifying beyond pH 4). The mixture was filtered through celite and concentrated to give 97 mg crude material. Purification by flash chromatography in 40% ethyl acetate / hexanes gave 87.6 mg (0.0463 mmol, 93%) product **5b** as a white foam. ^{1}H NMR (400

MHz, $CDCl_3$): δ 7.89 (d, 2H, *J* = 8 Hz), 7.60 (app t, 1H), 7.55 (m, 2H), 7.43 (d, 2H, *J* = 7.9 Hz) 7.40-7.07 (m, 52H + residual $CHCl_3$), 6.92 (app t, 1H) 5.41 (s, 1H), 5.33 (s, 1H), 5.26 (s, 1H), 5.05 (s, 1H), 4.82-3.20 (complex region), 3.13 (m, 2H), 2.34 (s, 1H), 1.91 (m, 1H), 1.82 (m, 2H), 1.67 (m, 1H), 1.40 (m, 1H), 1.2-1.1 (m, 3H). ^{13}C-NMR (100 MHz, CDCl3, selected signals): δ 24.8, 31.1, 36.8, 51.8, 67.4, 68.6, 69.3, 69.4, 69.8, 71.5, 78.2, 79.5, 80.3, 99.9, 100.1, 101.3, 126.0, 132.8, 137.3, 138.2, 138.3, 138.6, 138.7, 138.8, 139.2, 141.2. IR (cm^{-1}): 3460 (br) 3261 (br) 3063, 3027, 2920, 2862, 1453, 1362, 1073, 1055, 737, 697. HRMS (ESI+): calcd. for $C_{113}H_{121}NO_{23}S^+$ [M + H^+] 1892.8128, found 1892.8042.

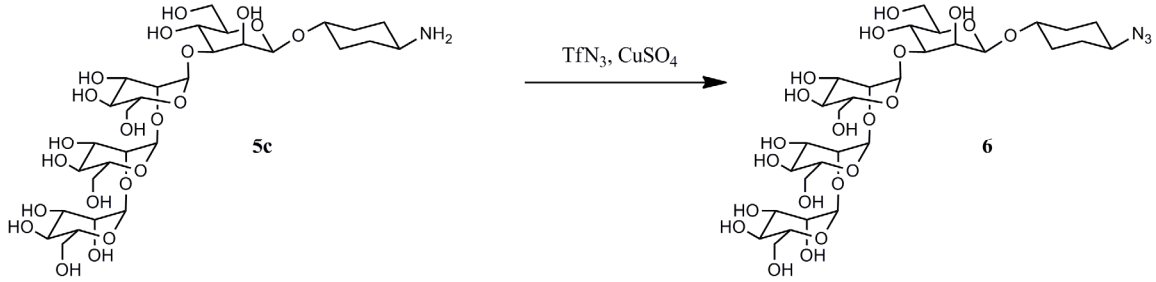
Fully deprotected Tetrasaccharide Amine (5c)



Along with a stream of N_2, ammonia gas was was condensed against a -78 °C cold finger into a -78 °C-cooled 500 mL 3-necked flask until ~200 mL had accumulated. 320 mg (13.8 mmol) Na^0 was then added, and the resulting blue solution was monitored for 1 hour to ensure that color did not disappear. 131 mg (0.0691 mmol) **5b** in 3 mL THF was then added, and this was allowed to react at -78° C for 2 hours. 1.11 g (20.7 mmol) of solid NH_4Cl was added portionwise, the cooling bath was removed, and the ammonia was blown off under a stream of nitrogen. The crude product was dissolved in minimal water and desalted by passage through a Biogel P-2 size exclusion gel column to give

51.8 mg (0.0678 mmol, 98%) compound **5c** as a brittle colorless glass. ^{1}H NMR (400 MHz, D_2O): δ 5.35 (s, 1H), 5.30 (s, 1H), 5.04 (s, 1H), 4.81 (s, 1H), 4.15-3.60 (m, 24H), 3.39 (m, 1H), 3.19 (m, 1H), 2.20-2.03 (m, 4H), 1.5-1.3 (m, 4H). ^{13}C-NMR (100 MHz, CDCl3, selected signals): δ 30.9, 31.0, 31.9, 33.2, 52.0, 63.8, 69.0, 69.7, 69.8, 72.8, 73.2, 73.6, 76.1, 76.2, 78.8, 81.4, 83.5, 100.7, 103.5, 105.1. IR (cm^{-1}): 3300 (v br), 2925, 1739, 1629, 1448, 1363, 1030. HRMS (ESI+): calcd. for $C_{30}H_{54}NO_{21}^{+}$ [M + H^{+}] 764.3188, found 764.3184.

Fully deprotected Tetrasaccharide Man$_4$-azide (6)



15.2 mg (0.234 mmol) sodium azide was suspended in a vial in 50 μL each of DCM and water. This was cooled to 0° C, and 20 μL (0.117 mmol) of triflic anhydride was added. After 2 hours, this was quenched with aqueous $NaHCO_3$ solution, and the aqueous layer was extracted twice with DCM. The combined organic layers containing triflyl azide were reduced to ~0.1 mL under vacuum.

Into a 5 mL flask containing 9 mg (0.0117 mmol) of **5c** was added 125 μL water and 57 μL of .02 M aqueous $CuSO_4$ solution (0.0011 mmol). The triflyl azide solution (prepared above) was then added, followed by 0.5 mL of methanol. After 2.5 hours, the reaction was quenched with 10 mg (10 eq) solid $NaHCO_3$ and concentrated *in vacuo*. The crude material was desalted on a Biogel P-2 size exclusion gel column, and then purified by HPLC (gradient shown in the table below). Product was detected by UV at 220 nm and

eluted at ~18 minutes. Concentration of fractions afforded 7.0 mg (0.00889 mmol, 76%) of **Man_4-azide** (**6**), a colorless glass. ^{1}H NMR (400 MHz, D_2O): δ 5.36 (s, 1H), 5.31 (s, 1H), 5.05 (s, 1H), 4.80 (s, 1H), 4.10-3.62 (m, 25H), 3.53-3.47 (m, 1H), 3.42-3.38 (m, 1H), 3.34(s residual MeOH), 2.1-1.95 (m, 4H), 1.5-1.3 (m, 4H). ^{13}C-NMR (100 MHz, D_2O, selected signals): δ 31.1, 31.2, 31.5, 32.9, 61.7, 63.8, 63.9, 69.0, 69.7, 69.8, 72.8, 73.2, 73.7, 76.1, 76.2, 78.9, 79.0, 81.4, 81.6, 83.5, 100.6, 103.5, 105.1. IR (cm^{-1}): 3344, 2933, 2096, 1629, 1367, 1124, 1055. HRMS (ESI+): calcd. for $C_{40}H_{52}N_3O_{21}^+$ [M + H^+] 790.3093, found 790.3087.

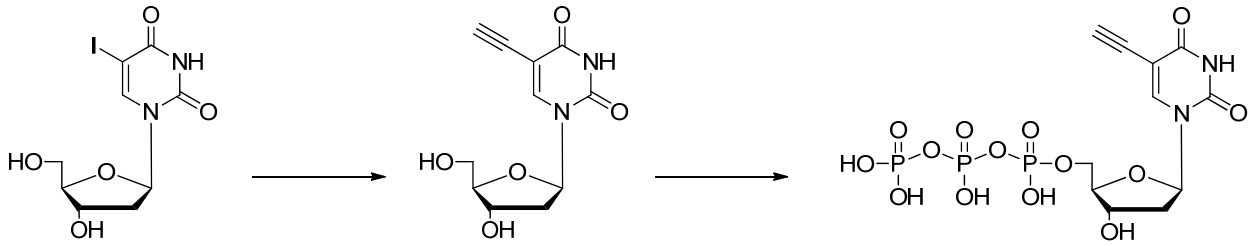
HPLC gradient used for the purification of 5:

Time (min.)	MeOH (%)	H_2O (%)	Flow Rate (mL/min.)
0	1	99	4
6	1	99	4
6.5	1	99	3
16	60	40	3
60	60	40	3

Synthesis of 5-ethynyl-2'deoxyuridine-5'-triphosphate(EdUTP).

General Methods (EdUTP synthesis). All commercial reagents (Sigma-Aldrich, Alfa Aesar) were used as provided unless otherwise indicated. An anhydrous solvent dispensing system (J. C. Meyer) using 2 packed columns of neutral alumina was used for drying THF, Et_2O, and CH_2Cl_2, while 2 packed columns of molecular sieves were used to dry DMF. Solvents were dispensed under argon. Analytical HPLC was performed on a Varian Microsorb column (C18, 5 μ, 4.6×250 mm) with a flow rate of 0.5 mL/min while a Varian Dynamax column (C18, 8 μ, 41.4×250 mm) with a flow rate of 40 mL/min was used for preparative HPLC. An isocratic or linear gradient of 0.1 M triethylammonium bicarbonate (TEAB) and aqueous MeCN (70%) were used. Teledyne ISCO CombiFlash Rf equipped with Teledyne ISCO RediSep Rf flash column silica cartridges (www.isco.com/combiflash) were used for flash chromatography with the indicated solvent system. Nuclear magnetic resonance spectra were recorded on a Varian 600 MHz with Me_4Si, DDS or signals from residual solvent as the internal standard for ^1H and external H_3PO_4 for ^{31}P. Chemical shifts are reported in ppm , and signals are described as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), brs (broad singlet), and dd (double doublet). Values given for coupling constants are first order. High resolution mass spectra were recorded on an Agilent TOF II TOF/MS instrument equipped with either an ESI or APCI interface. All reactions were performed under an inert atmosphere of dry Ar in oven dried (150 °C) glassware.

Synthesis of 5-ethynyl-2'-deoxyuridine 5'-triphosphate (EdUTP)



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5-Ethynyl-2'-deoxyuridine. 5-Iodo-2'-deoxyuridine (5, 1.0 g, 2.82 mmol) was dissolved in MeCN/Et_3N (66 mL of 1:1, v/v) under argon atmosphere. Trimethylsilylacetylene (1.6 mL, 11.3 mmol), bis-(triphenylphosphine)-palladium(II) chloride (42.2 mg, 0.60 mmol), and CuI (28 mg, 0.15 mmol) were added, and the mixture was heated for 3.5 h in the flask immersed into a preheated oil bath (50 °C). The solvents were removed in vacuo to give a residue that was purified by silica gel flash column chromatography. Elution with $CHCl_3$/MeOH (9:1, v/v) afforded trimethylsilyl intermediate as a solid (0.75 g, 82%). To the solution of this intermediate (0.7 g, 2.16 mmol) in anhydrous MeOH (16 mL) under argon atmosphere, a solution of NaOMe in MeOH (145 mL of 0.05 N) was added, and the reaction was stirred at 25 °C for 2 h. The pH of the solution was adjusted to 5-6 using Dowex 50 WX8-200 (H^+), the mixture was filtered, and concentrated in vacuo to give a residue that was purified by silica gel column flash chromatography using $CHCl_3$/MeOH (8:2, v/v) as eluent to yield 5-ethynyl-2'-deoxyuridine (EdU) as a white solid (395 mg, 73%); ^{1}H NMR (DMSO-d_6) δ 11.62 (s, 1H, NH), 8.29 (s, 1H, H-6), 6.10 (dd, J = 6.56, 6.56 Hz, 1H, H-1'), 5.24 (d, J = 4.31 Hz, 1H, C-3' OH), 5.12 (t, J = 4.91 Hz, 1H, C-5' OH), 4.23 (m, 1H, H-3'), 4.10 (s, 1H, C≡CH), 3.79 (q, J = 3.25, 3.25, 3.26 Hz, 1H, H-4'),

3.59 (m, 2H, H-5', H-5"), 2.16 (m, 2H, H-2', H-2"). HRMS calcd for $C_{11}H_{11}N_2O_{14}$ 251.0673 $(M-H)^-$, found 251.0683.

5-Ethynyl-2'-deoxyuridine-5'-triphosphate. EdU was dried by coevaporation with dry pyridine, and left over P_2O_5 under vacuo overnight. The compound (75 mg, 0.3 mmol) was dissolved in solution of trimethylphosphate (2 mL), cooled in ice-bath, and a powdered Proton Sponge (96.4 mg, 0.45 mmol) was added followed by $POCl_3$ (30 μL, 0.33 mmol). After 2 h of stirring, a solution of tributylammonium pyrophosphate in DMF (3 mL, 1.5 mmol) containing tributylamine (300 μL, 1.26 mmol) was quickly added to the reaction mixture. After 2 min of stirring mixture was poured into 30 mL of 0.2 M TEAB, stirred and evaporated to dryness. Proton-Sponge was removed on small column with Dowex 50 WX8-200 (Na^+). The crude product was then purified by preparative HPLC with 70% MeCN/0.1M TEAB (2 - 10 linear gradient) to give a residue which was dissolved in water, and passed through a small column of Dowex 50 WX8-200 (Na^+). Fractions containing product were combined and lyophilized to give the product as a white powder (46.5 mg, 28%). ^{1}H NMR (D_2O) δ 8.02 (s, 1H, H-6), 6.76 (t, J = 6.59 Hz, 1H, H-1'), 4.44 (m, 1H, H-3'), 4.00 (m, 3H, H-4', H-5', H-5'), 3.39 (s, 1H, C≡CH), 2.12 (m, 2H, H-2', H-2"). ^{31}P NMR (243 MHz, D_2O) δ ppm -8.94 (d, J = 20.38 Hz), -10.49 (d, J = 20.19 Hz), -22.16 (t, J = 20.17 Hz). HRMS calcd for $C_{11}H_{14}N_2O_{14}P_3$ 490.9663 $(M-H)^-$, found 490.9673.

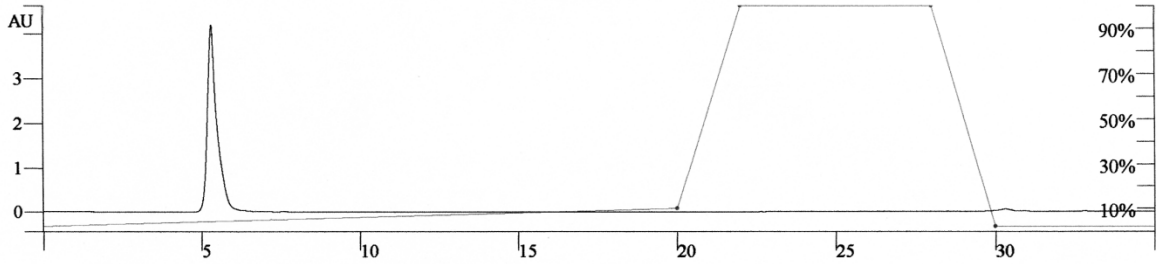
For other synthetic reports on EdU and EdUTP see references:

(3) Kovacs, T.; Otvos, L. *Tetrahedron Lett.* **1988**, *29*, 4525-4528.

(4) Borsonberger, V.; Kukwikila, M.; Howorka, S. *Org. Biomol. Chem.,* **2009**, *7*, 3826-3835.

HPLC purity profile of 5-Ethynyl-2'-deoxyuridine 5'-triphosphate:

Data File:	c:\star\data\krzysztof\5_ethynyl_dutp\pr	Operator (Calc):	
Channel:	1 = 254 nm RESULTS	Calc Date:	04/28/2009 05:28:28
Sample ID:	analytical_2_10	Times Calculated:	1
Operator (Inj):	Chen	Calculation Method:	c:\star\methods\ana methods\2-10_
Injection Date:	04/28/2009 04:53:25	Instrument (Calc):	Varian Star #1
Injection Method:	c:\star\methods\ana methods\2-10_	Run Mode:	Analysis
Run Time (min):	35.000	Peak Measurement:	Peak Area
Workstation:		Calculation Type:	Percent
Instrument (Inj):	Varian Star #1	Calibration Level:	N/A
		Verification Tolerance:	N/A



Peak No	Peak Name	Result ()	Ret Time (min)	Time Offset (min)	Peak Area (counts)	Rel Ret Time	Sep. Code	Width 1/2 (sec)	Status Codes	Group
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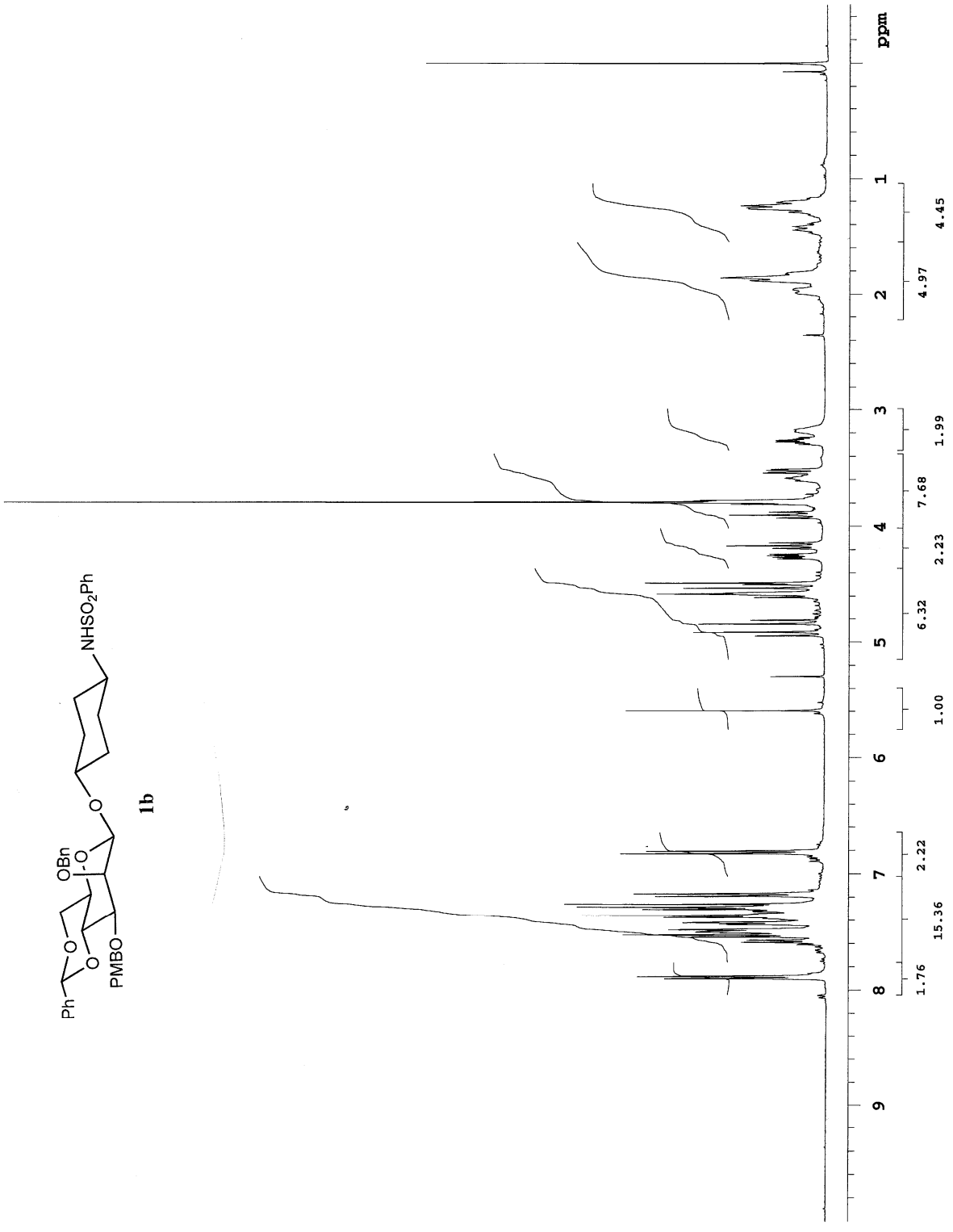
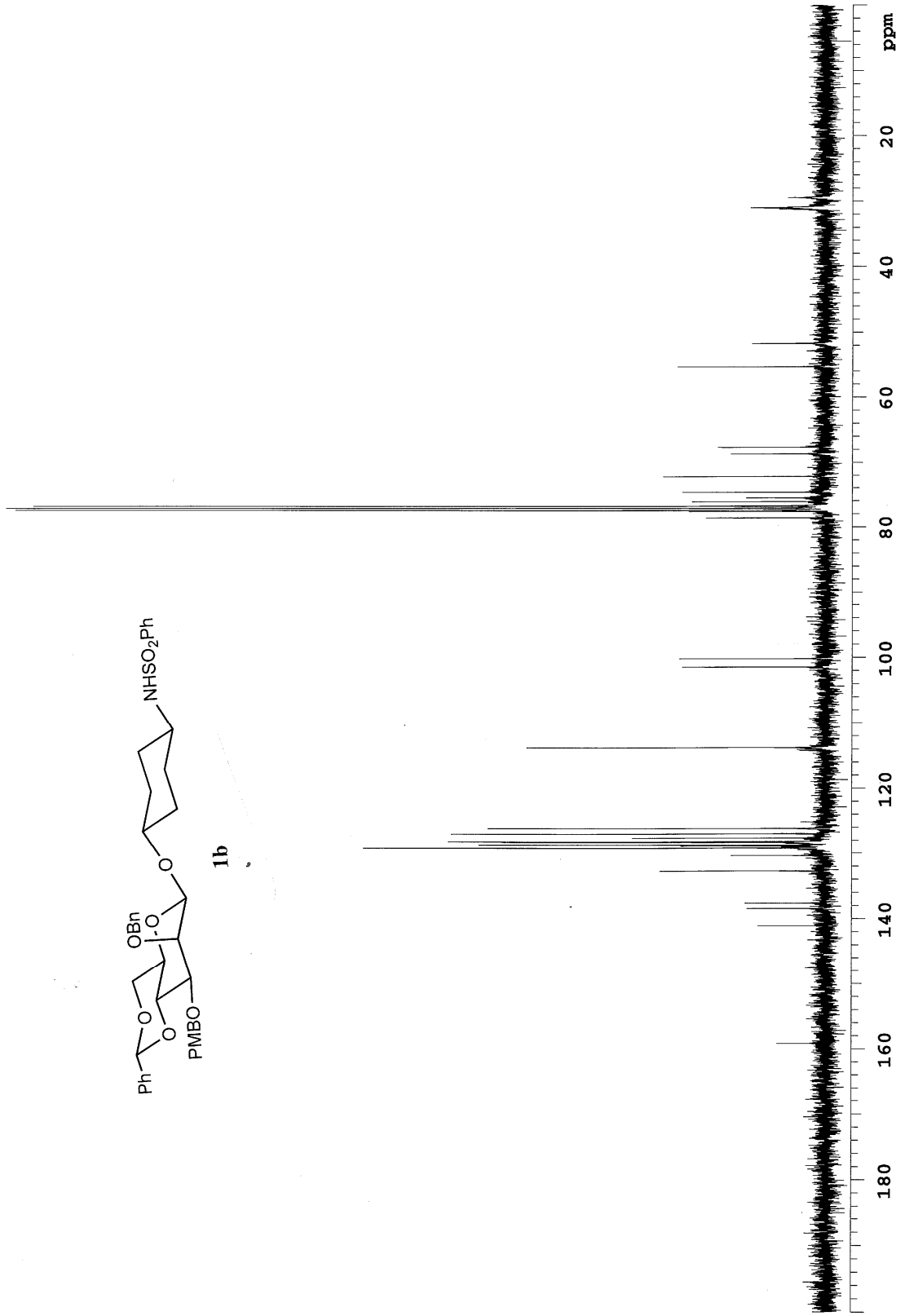
2		0.0127	6.322	0.000	115337	0.00	TS	0.0		0
3		0.1314	7.568	0.000	1197488	0.00	BB	12.8		0
4		1.7113	30.303	0.000	15590579	0.00	BV	26.0		0
5		0.2071	30.851	0.000	1886981	0.00	VB	20.1		0
6		0.1969	32.766	0.000	1794188	0.00	BB	0.0		0
	Totals	**99.9999**		**0.000**	**911029248**					

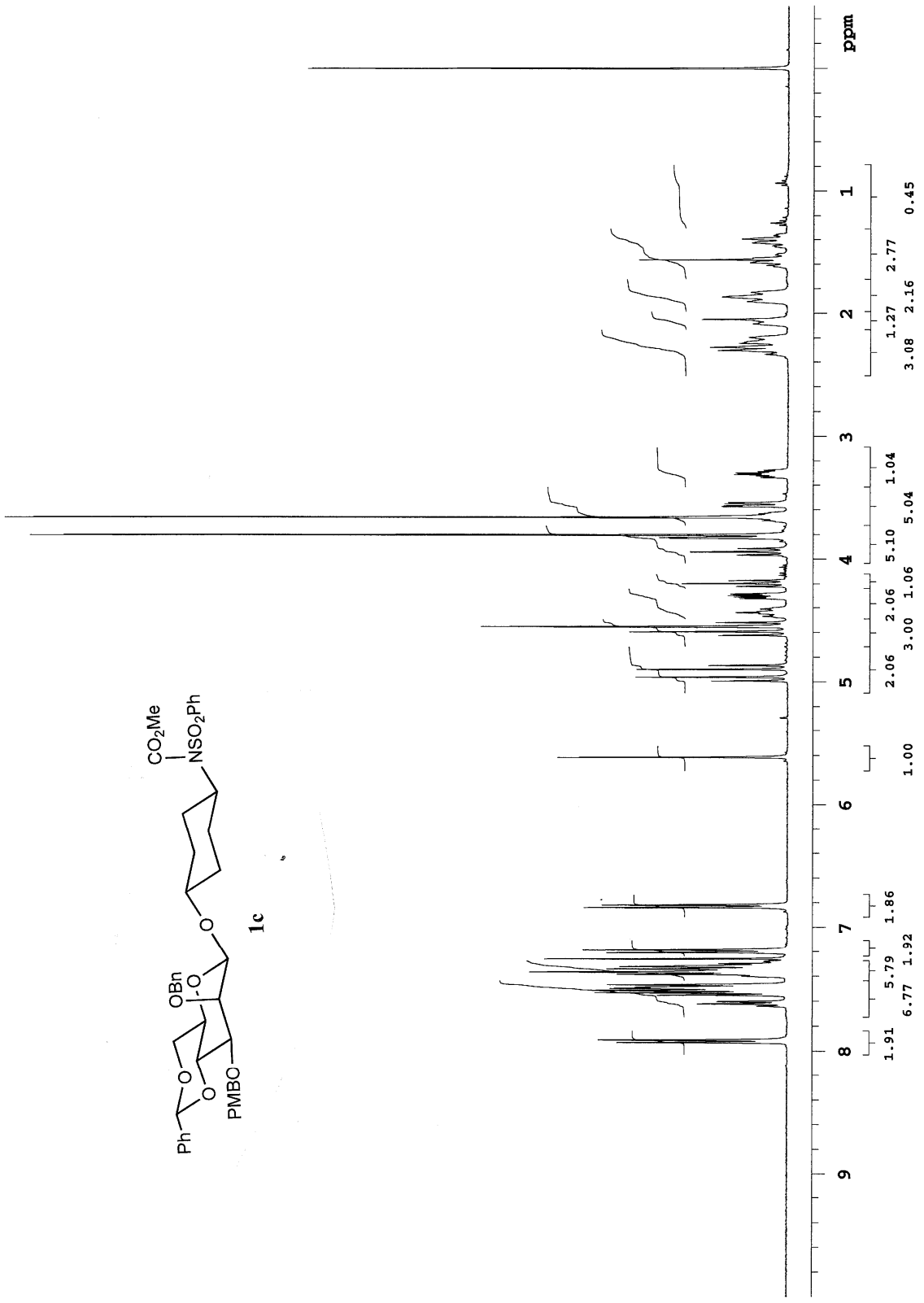
^{1}H NMR spectrum of **1b** (400 MHz, $CDCl_3$)



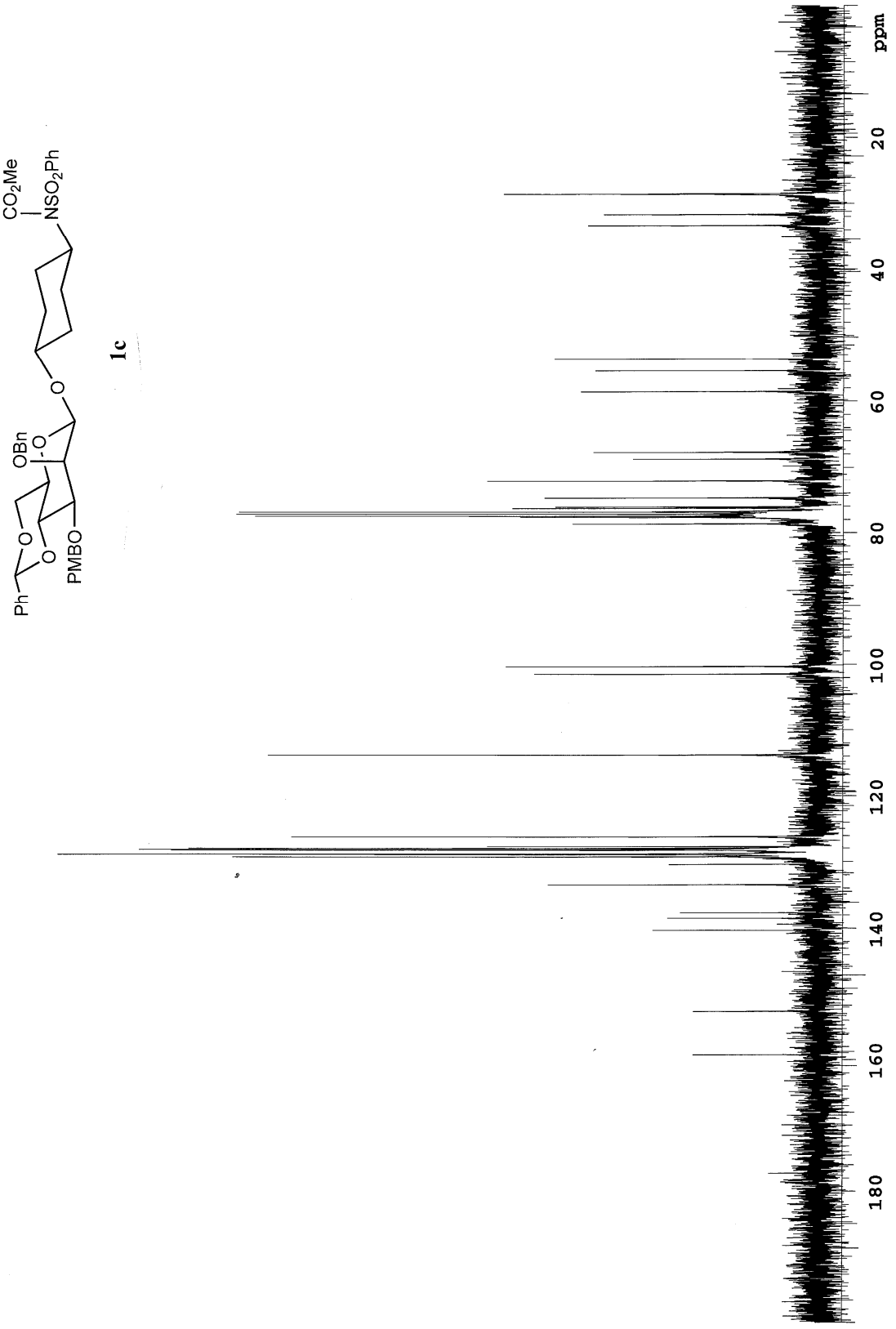
^{13}C NMR spectrum of **1b** (100 MHz, $CDCl_3$)



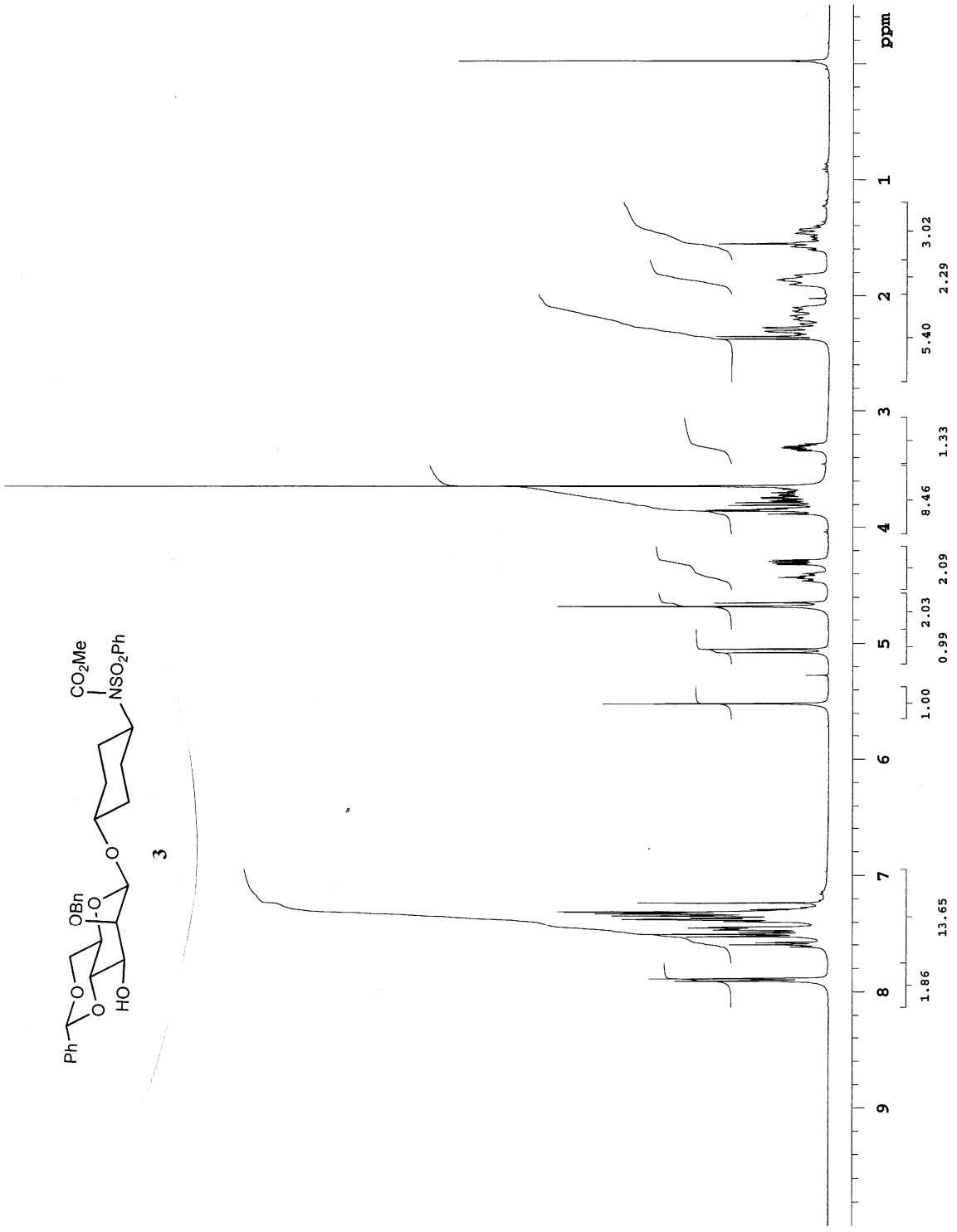
^{1}H NMR spectrum of **1c** (400 MHz, $CDCl_3$)



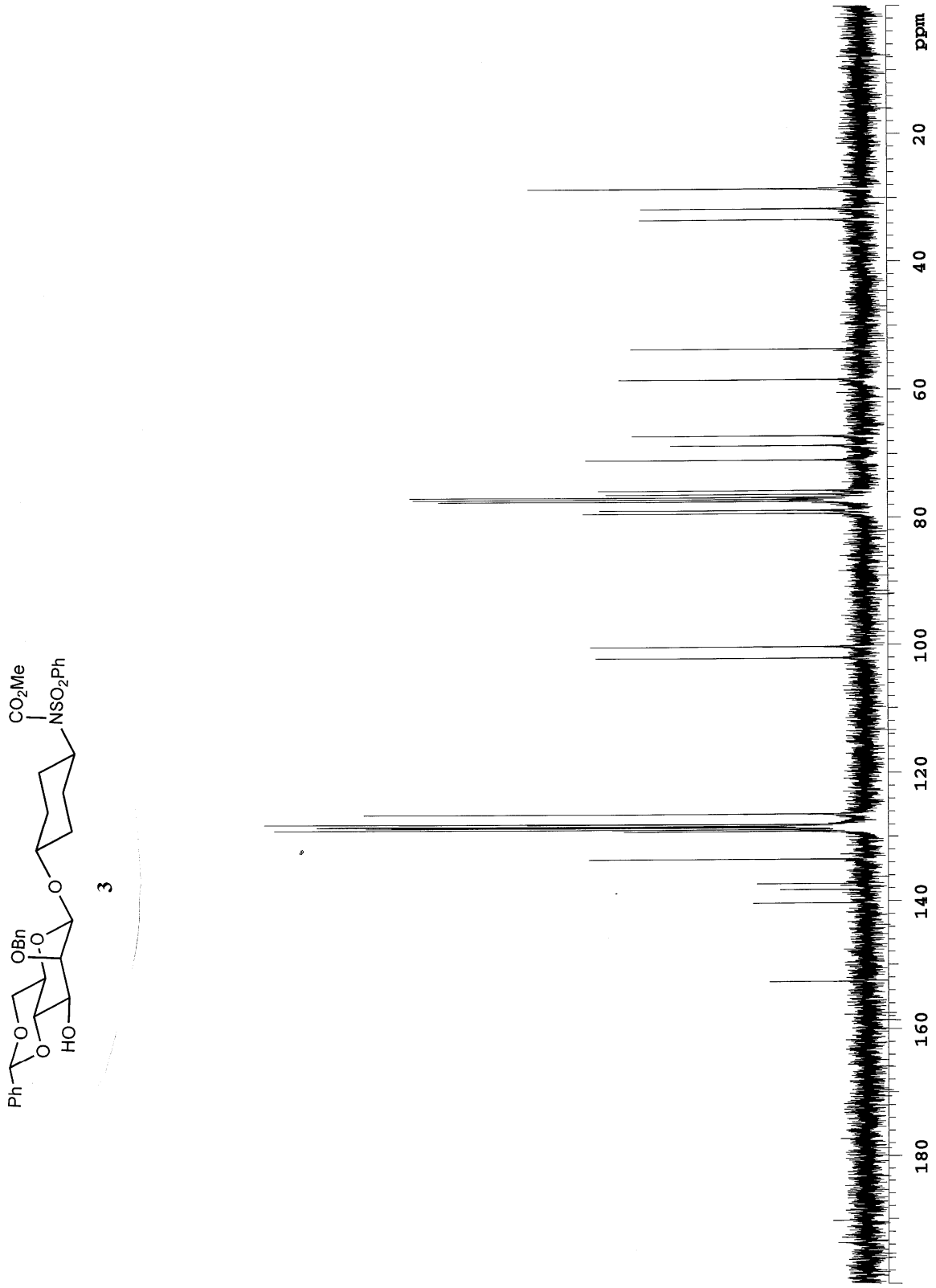
^{13}C NMR spectrum of **1c** (100 MHz, $CDCl_3$)



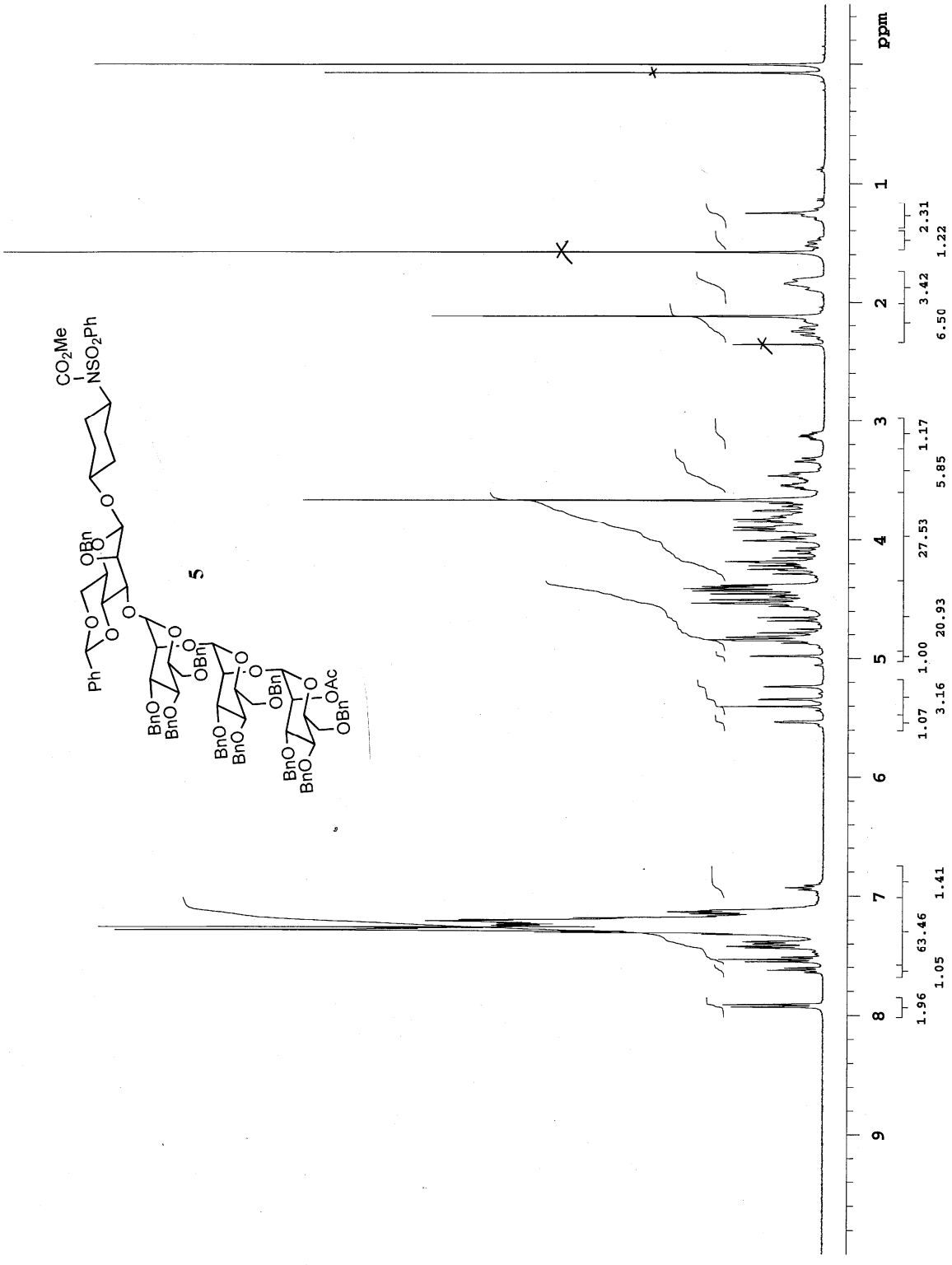
^{1}H NMR spectrum of **3** (400 MHz, $CDCl_3$)



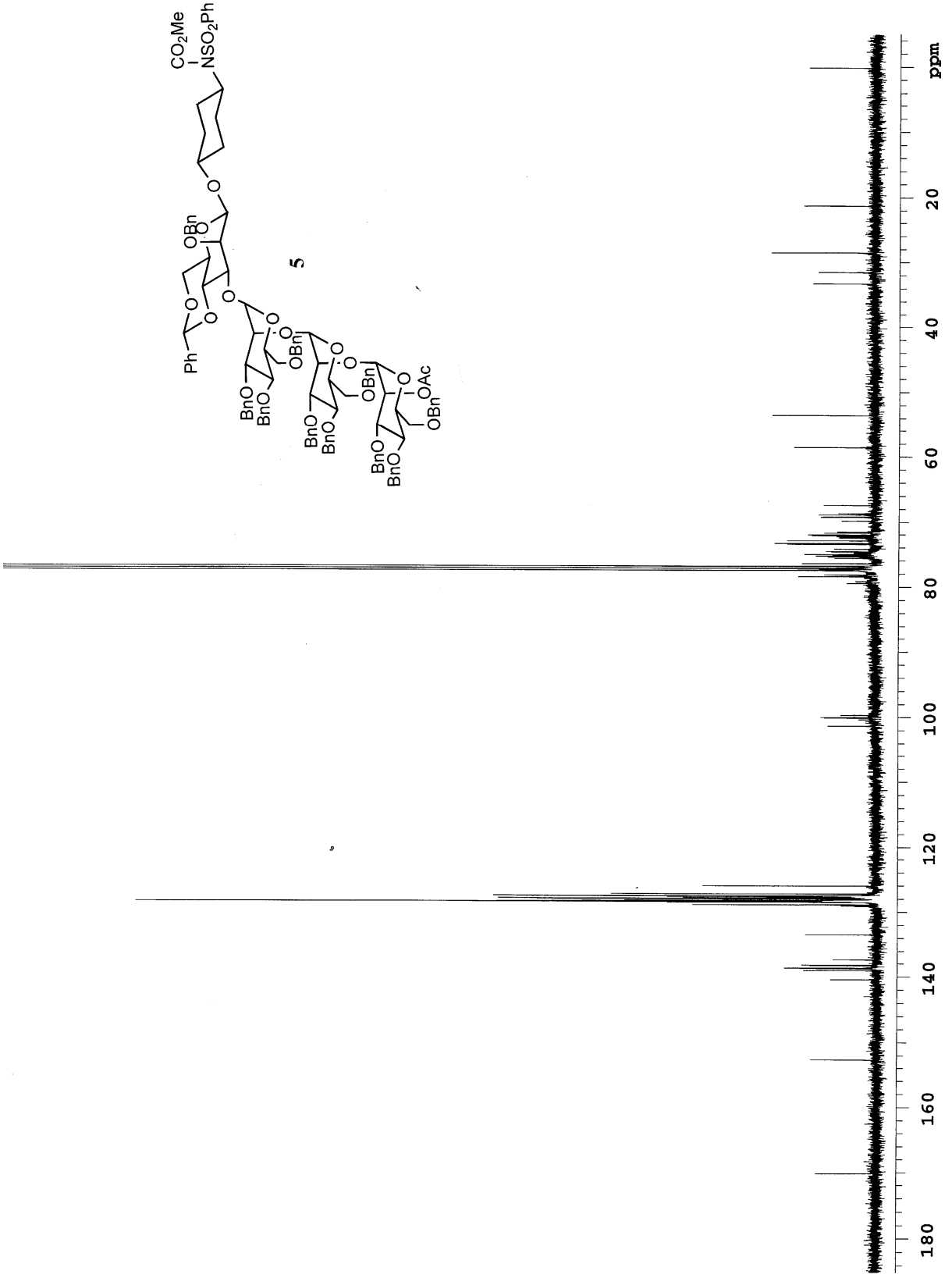
^{13}C NMR spectrum of **3** (100 MHz, $CDCl_3$)



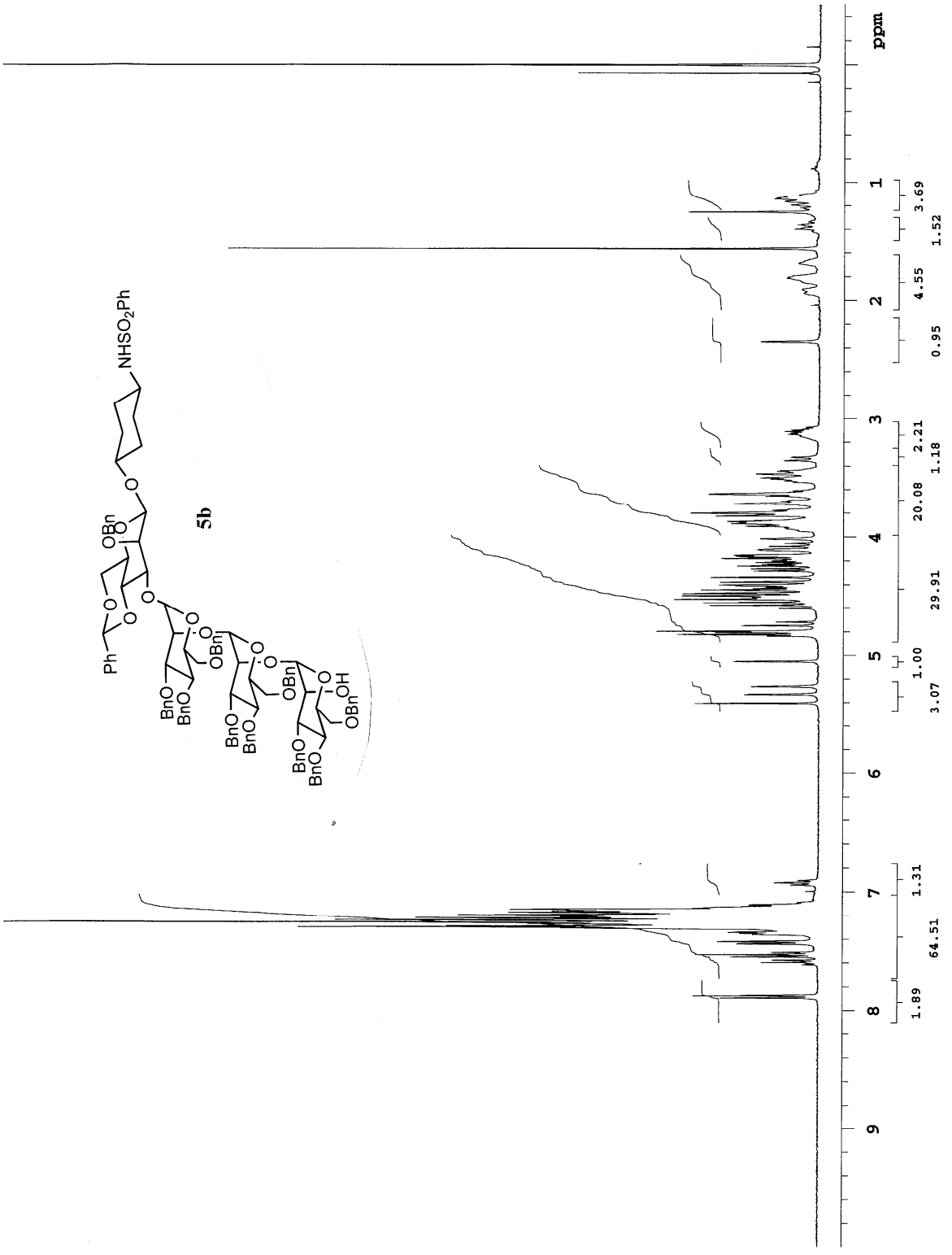
^{1}H NMR spectrum of **5** (400 MHz, $CDCl_3$)



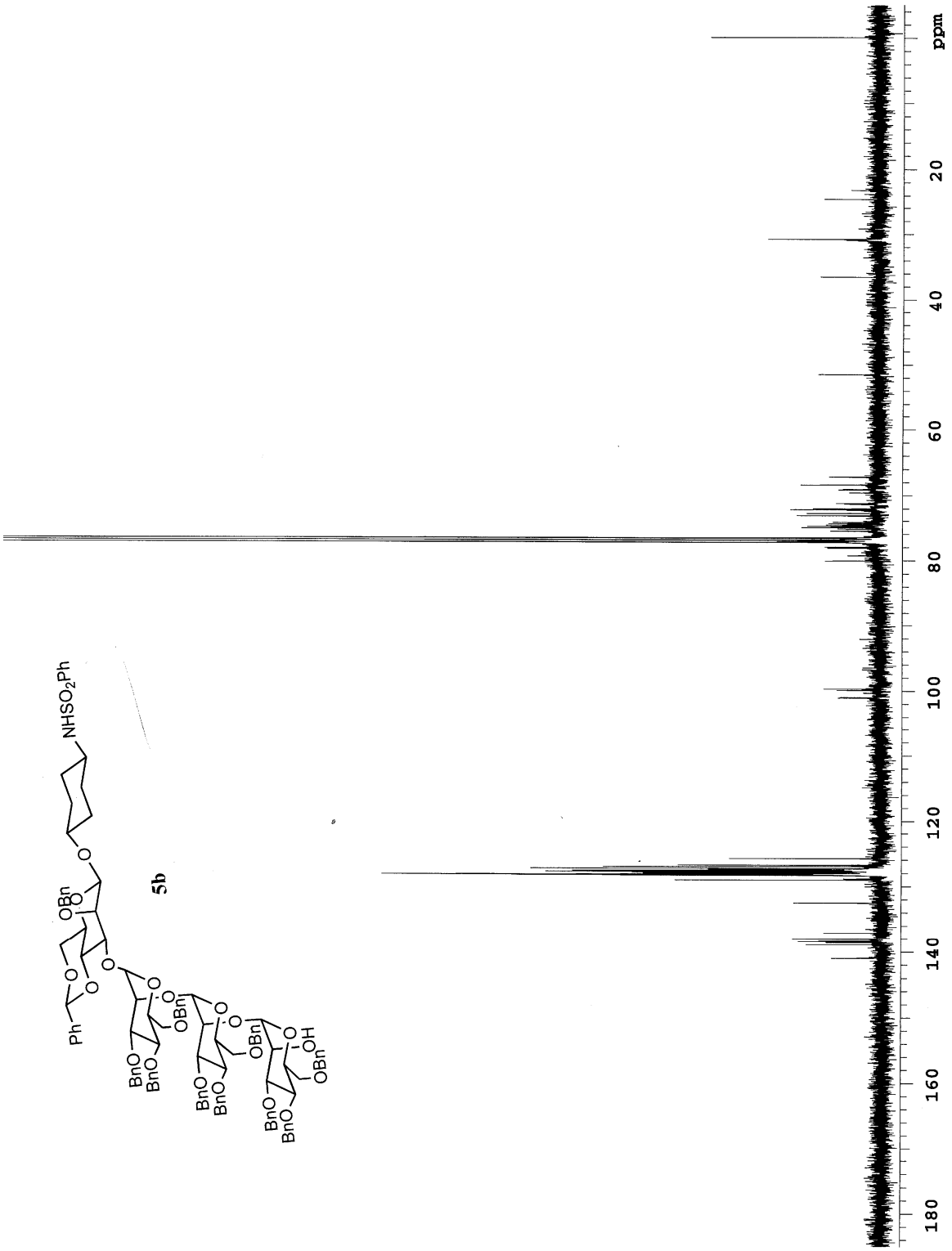
^{13}C NMR spectrum of **5** (100 MHz, $CDCl_3$)



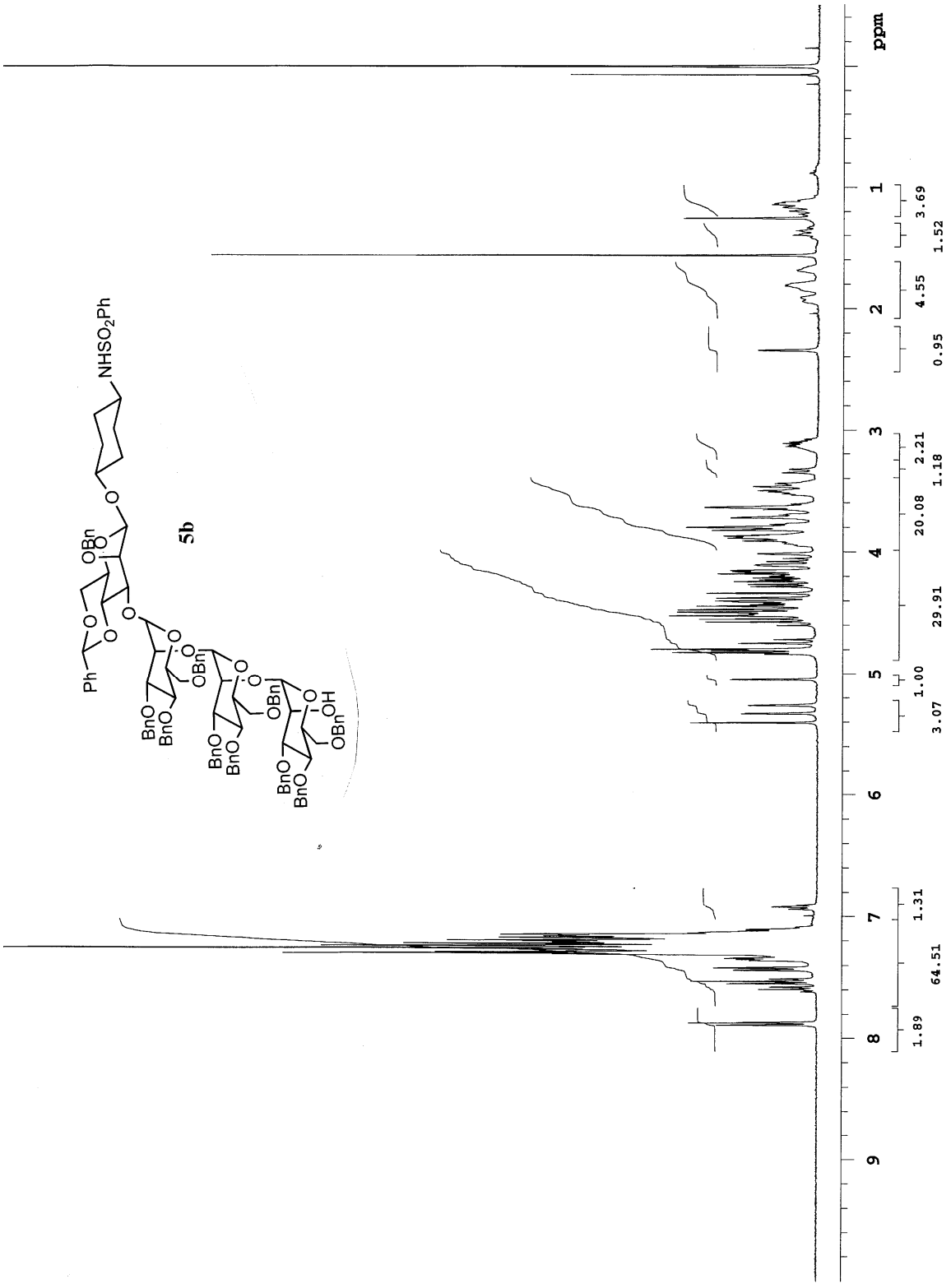
^{1}H NMR spectrum of deprotected sulfonamide **5b** (400 MHz, $CDCl_3$)



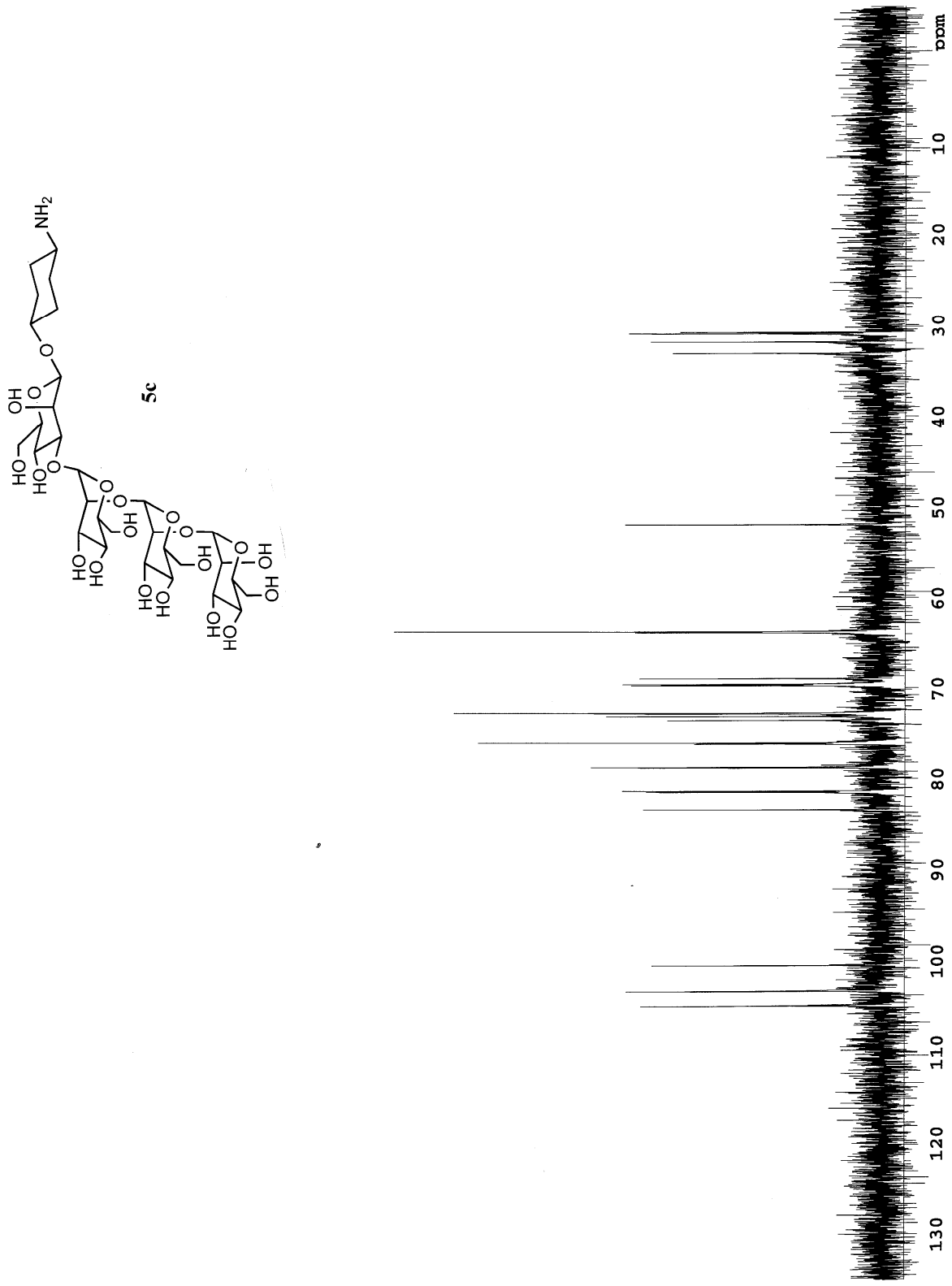
^{13}C NMR spectrum of **5b** (100 MHz, $CDCl_3$)



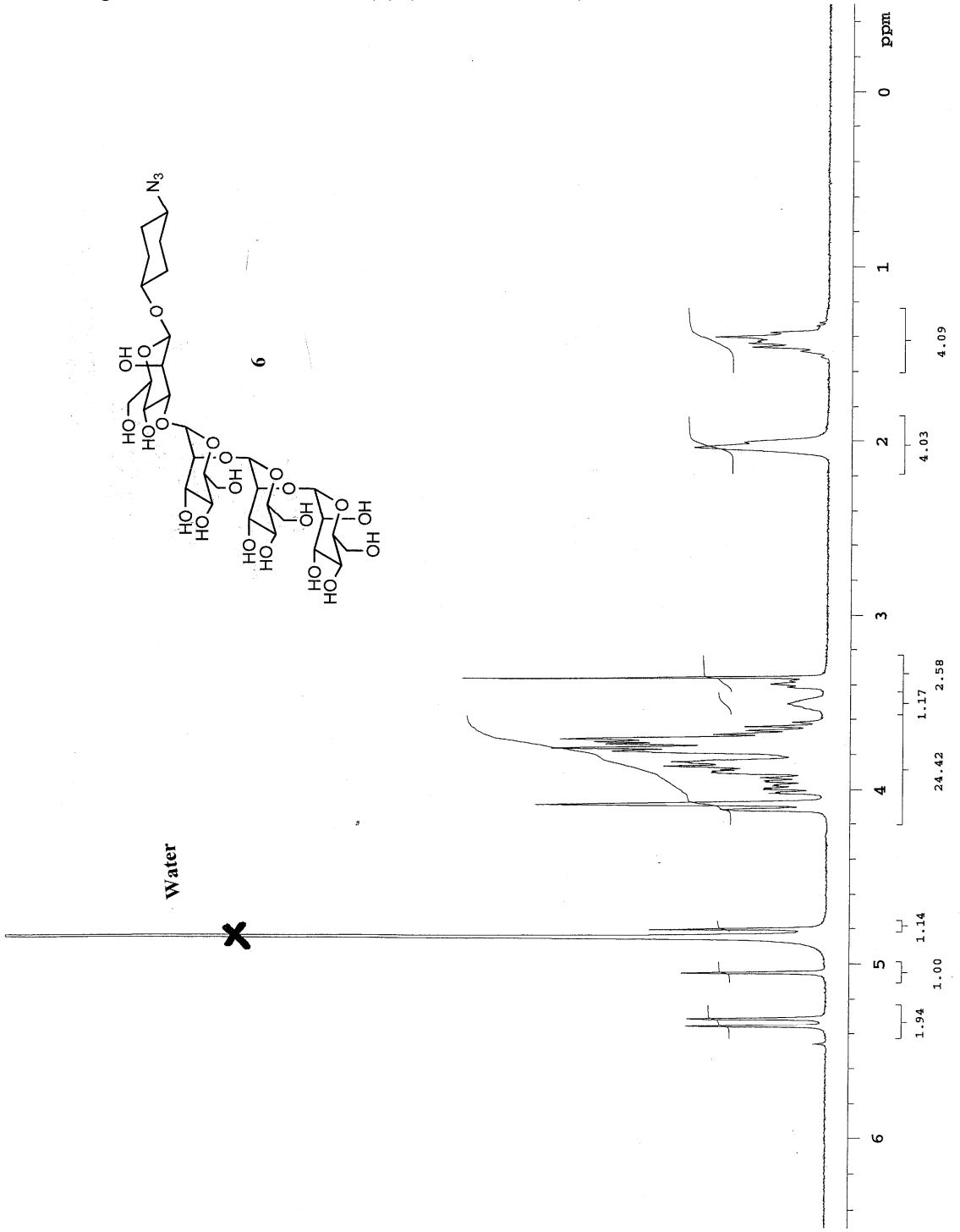
^{1}H NMR spectrum of amine **5c** (400 MHz, D_2O)



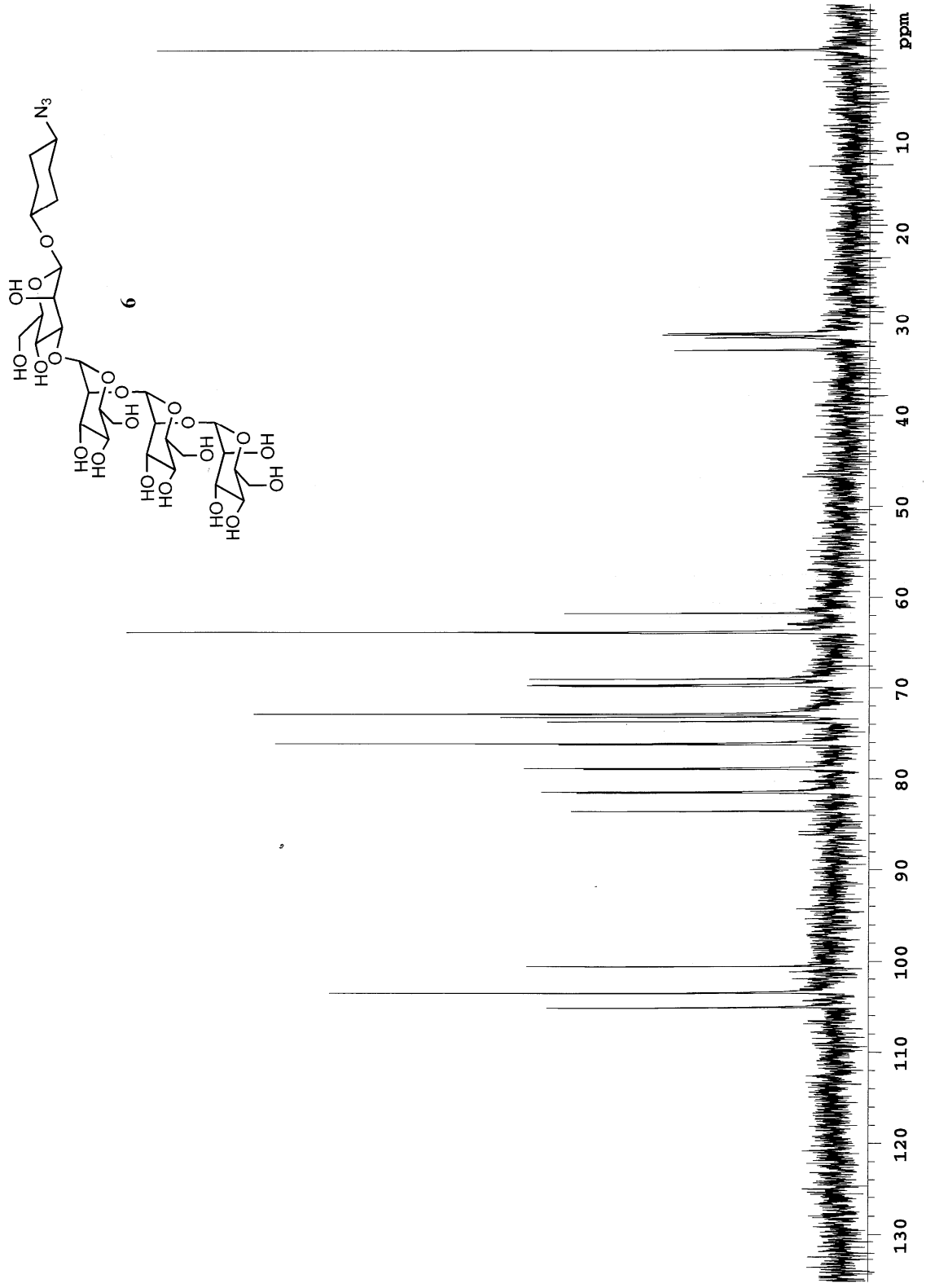
^{13}C NMR spectrum of **5c** (100 MHz, D_2O)



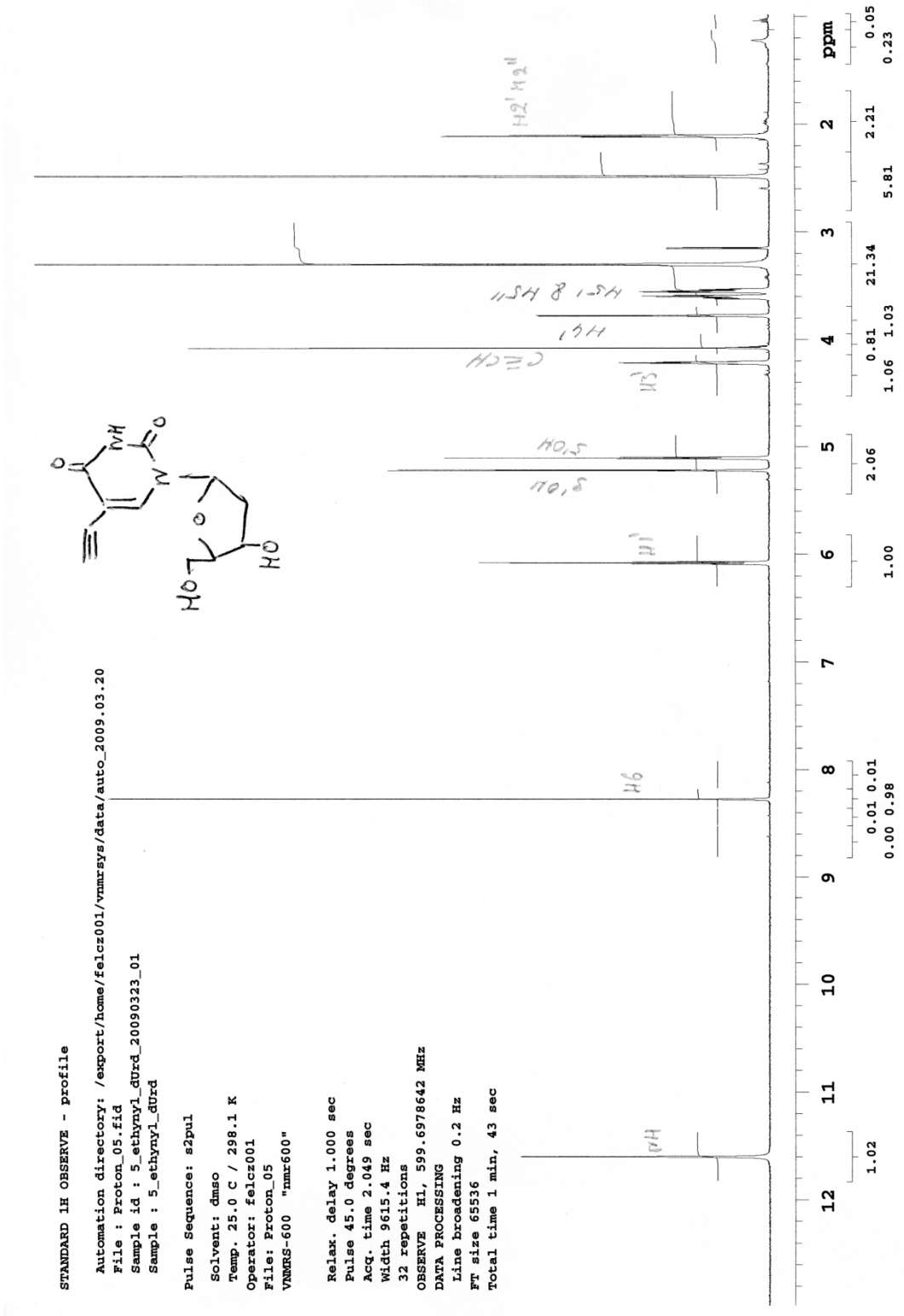
^{1}H NMR spectrum of **Man$_4$-azide** (**6**) (400 MHz, D_2O)



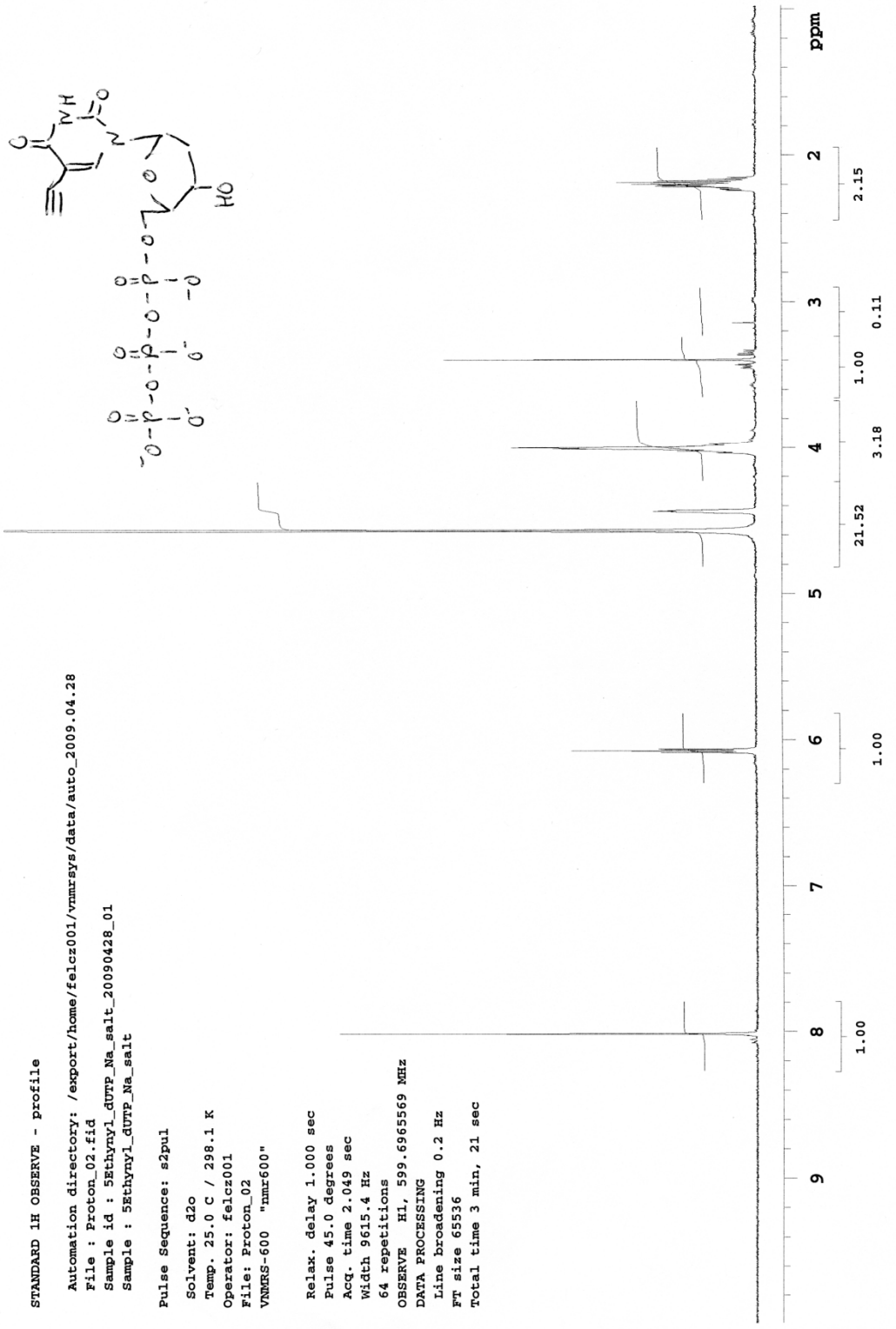
^{13}C NMR spectrum of **6** (100 MHz, D_2O)



[1]HNMR of 5-Ethynyl-2'-deoxyuridine (600 MHz, DMSO-d_6)



^{1}H NMR of 5-Ethynyl-2'-deoxyuridine-5'-triphosphate (600 MHz, D_2O)



^{31}P NMR of 5-Ethynyl-2'-deoxyuridine 5'-triphosphate (243 MHz, D_2O)