Supplementary Information

Oligomannose glycopeptide conjugates elicit antibodies targeting the glycan core rather than its extremities

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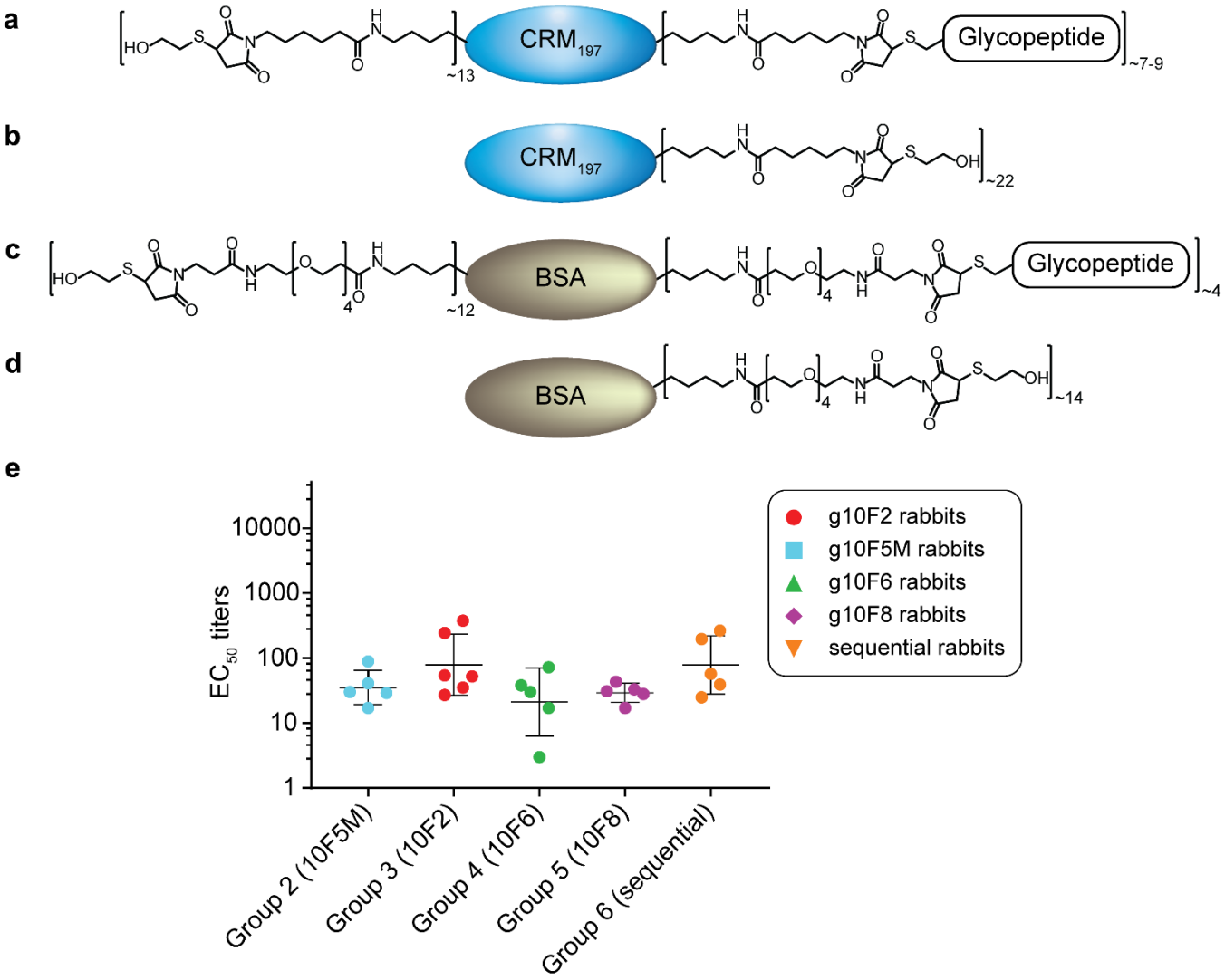


Fig. S1 Structure of linkers used in conjugation. **a** CRM_{197}-glycopeptides used in rabbit immunization. **b** CRM_{197} + linkers used in rabbit control group in multi-immunogen study. **c** BSA-glycopeptide used for coating plates in ELISA. **d** BSA+linkers used in ELISA assay. **e** Groups 2-6 post-dose 4 serum binding to BSA+linkers depicted in **d**.

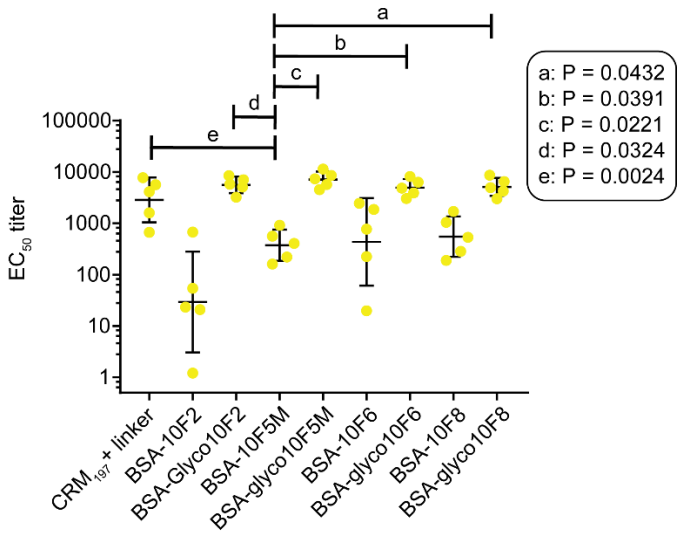


Fig. S2 Group 6 (sequential) serum selectivity for binding peptides, glycopeptides, and CRM_{197} +linker. Data are presented with geometric mean and geometric standard deviation. Statistical significance was determined by one-way ANOVA followed by multiple comparisons with Tukey's post-hoc test.

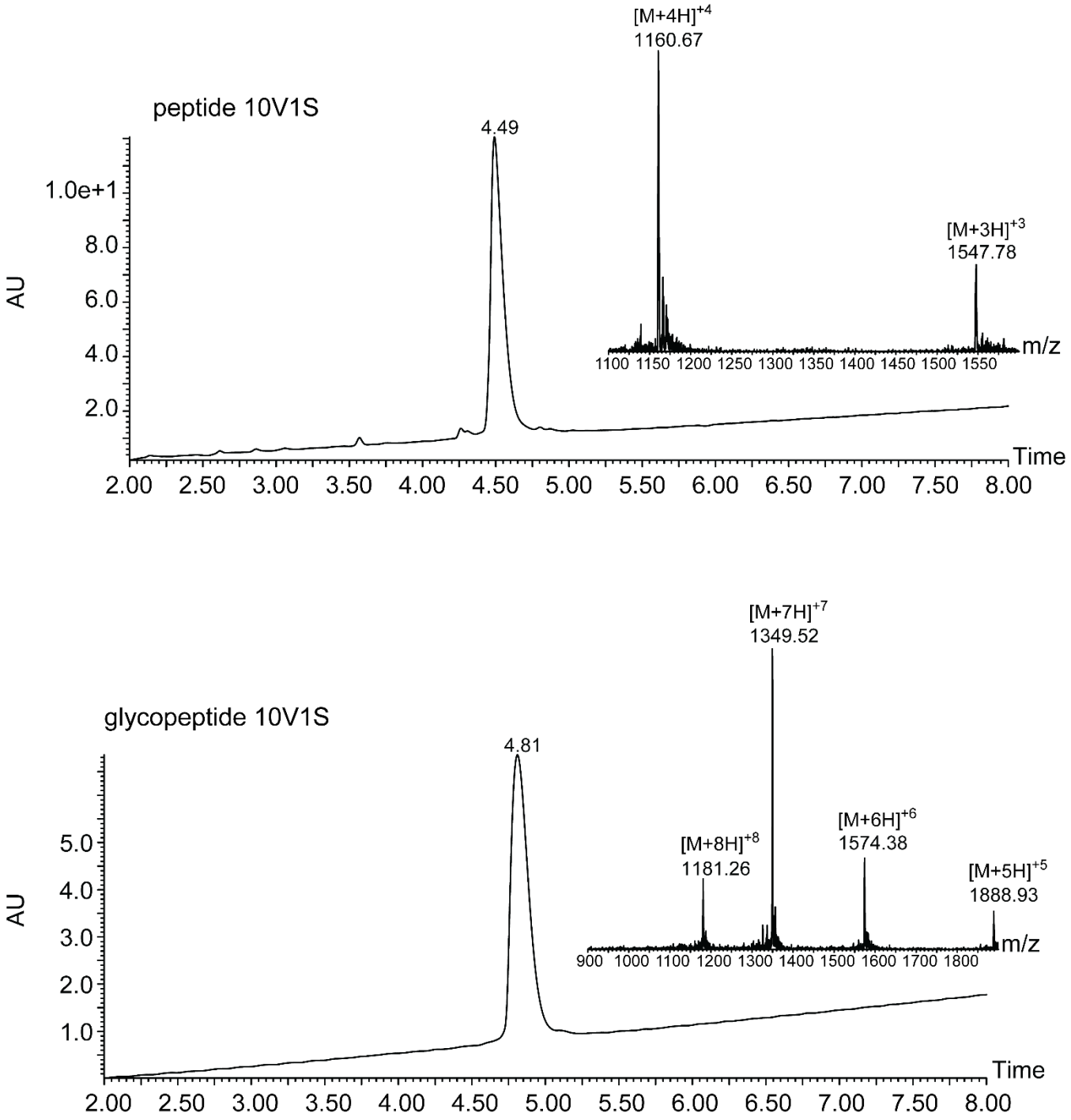


Fig. S3 LC-MS of purified peptide 10V1S and glycopeptide 10V1S.

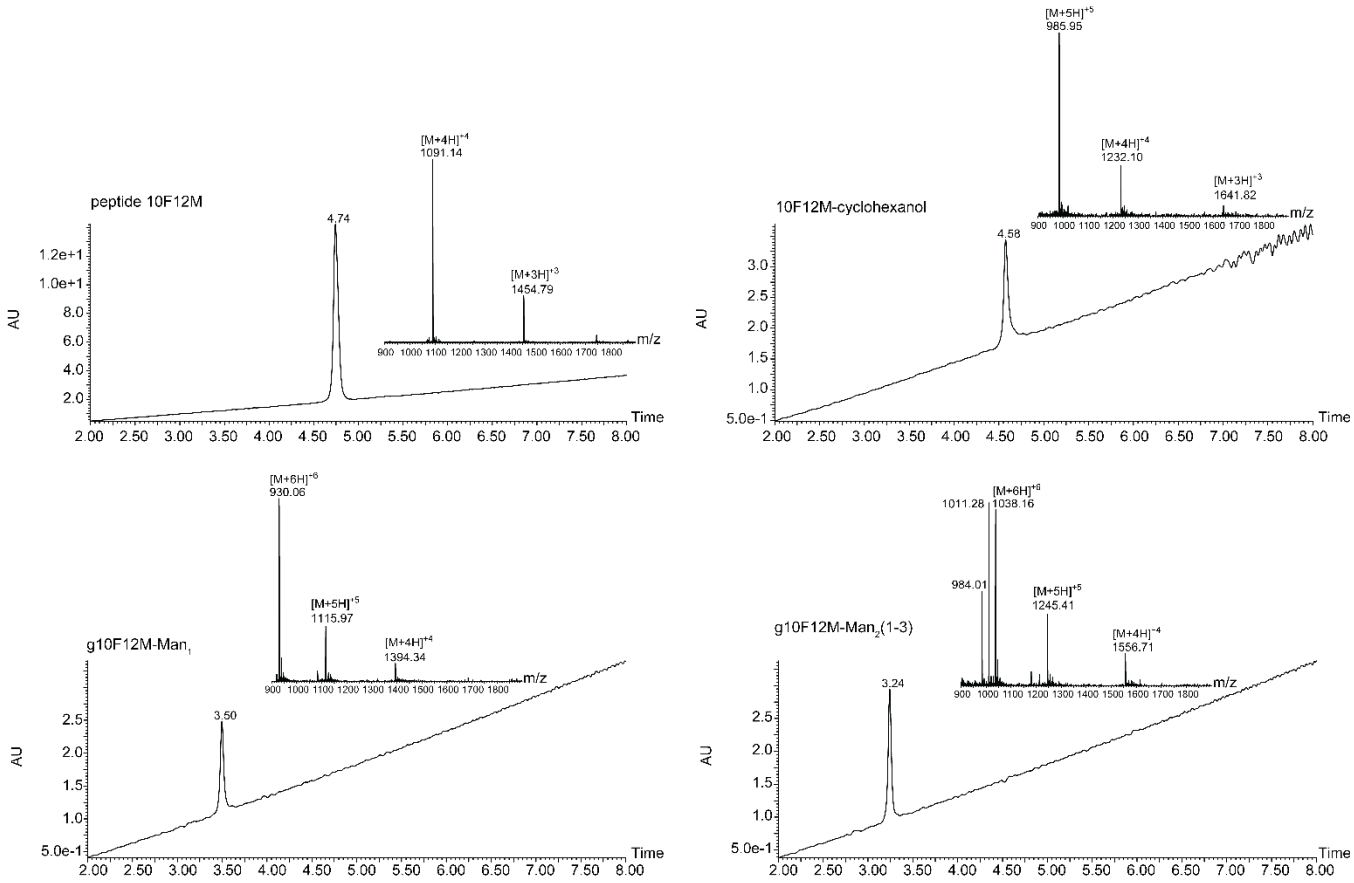


Fig. S4 LC-MS of purified peptide 10F12M, 10F12M-cyclohexanol, glyco10F12M-Man_1, and glyco10F12M-Man_2(1-3).

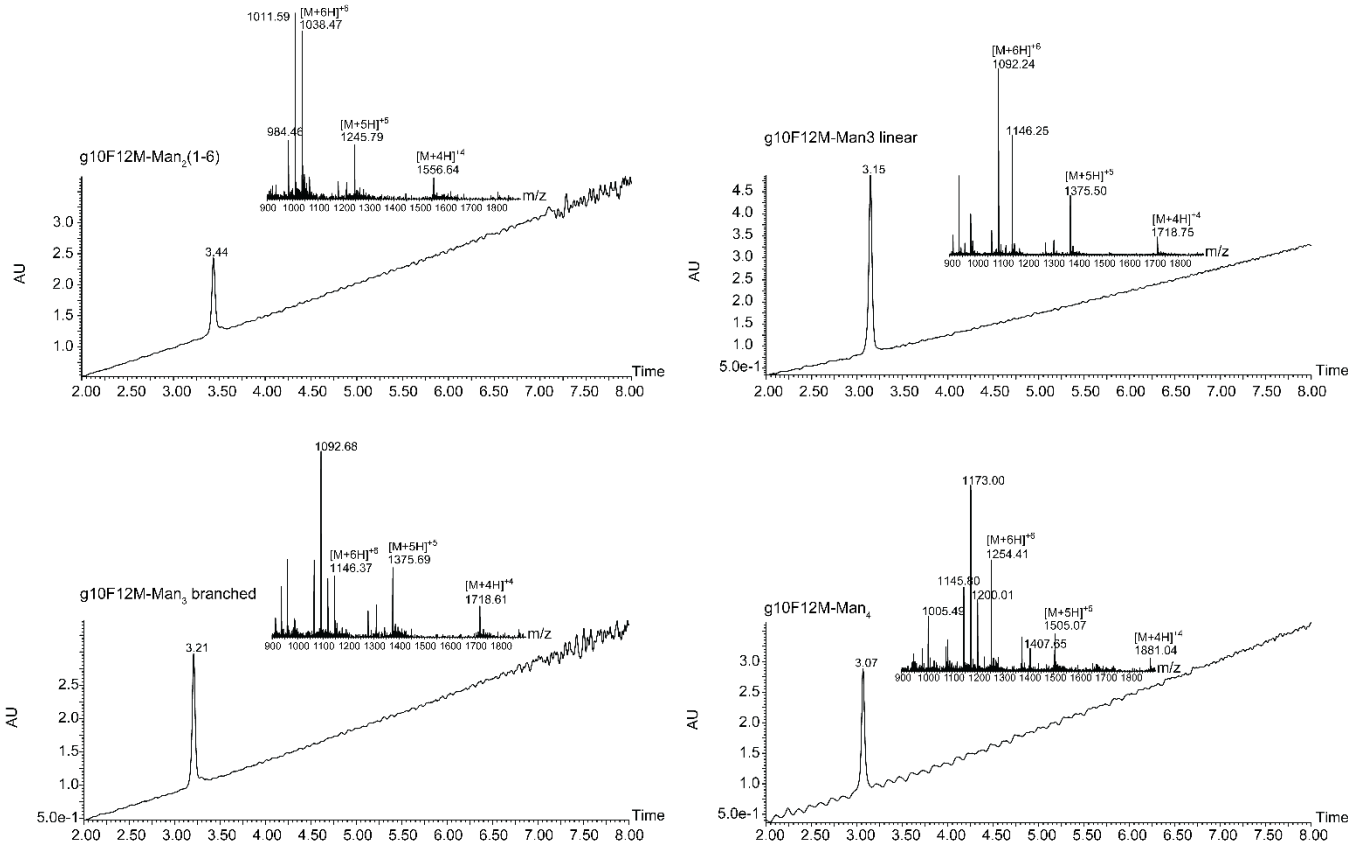


Fig. S5 LC-MS of purified glyco10F12M-Man_2(1-6), glyco10F12M Man_3 branched, glyco10F12M Man_3 linear, glyco10F12M Man_4 linear.

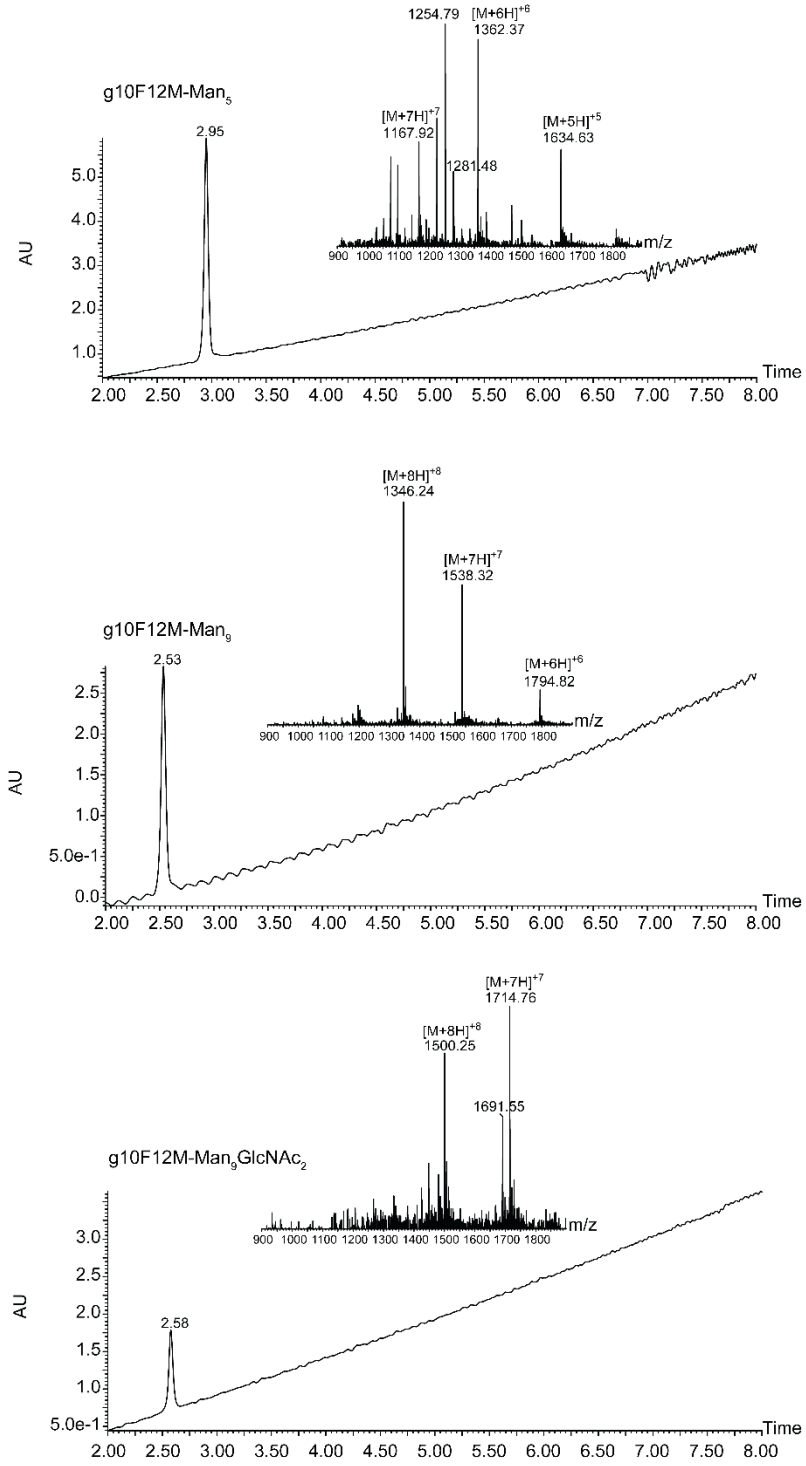


Fig. S6 LC-MS of purified glyco10F12M-Man$_5$, glyco10F12M-Man$_9$, glyco10F12M-Man$_9$GlcNAc$_2$.

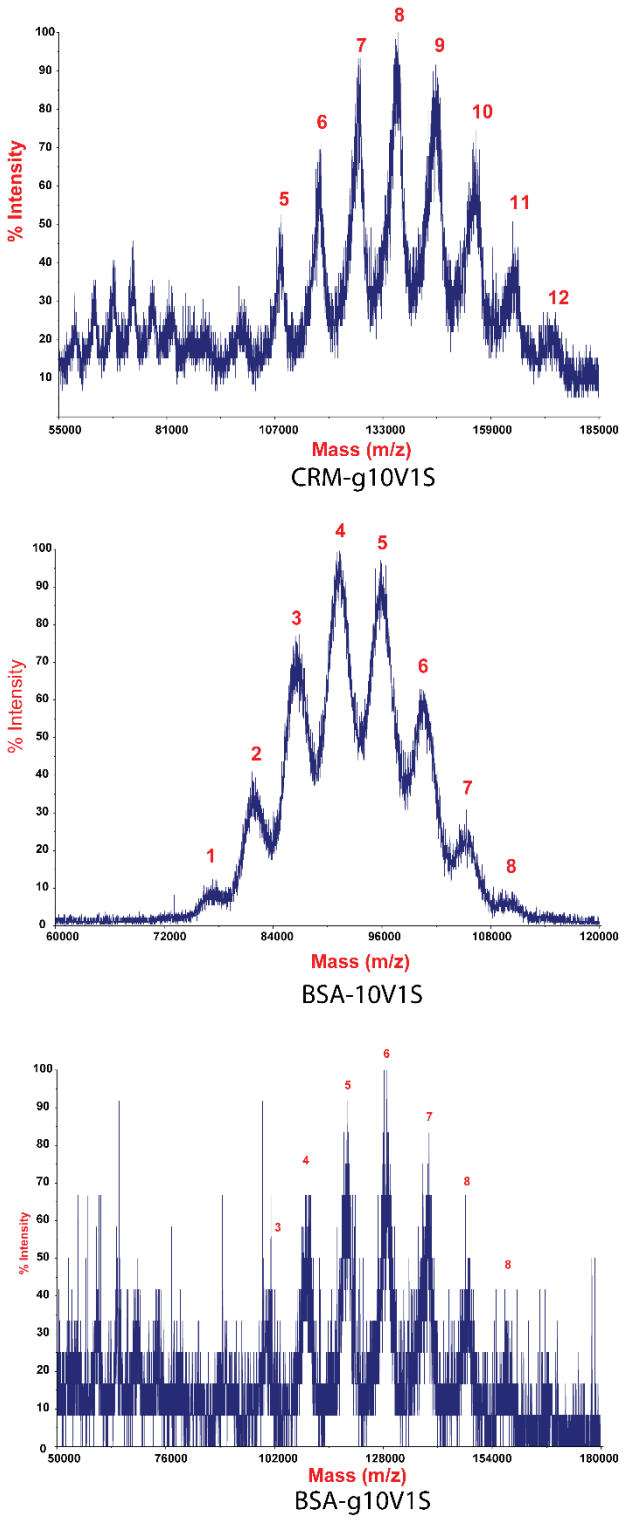


Fig. S7 MALDI-TOF MS analysis of CRM-glyco10V1S, BSA-10V1S, and BSA-glyco10V1S conjugates. The red numbers indicate the number of glycopeptides per CRM protein molecule. More closely clustered peaks at lower m/z are doubly charged ions.

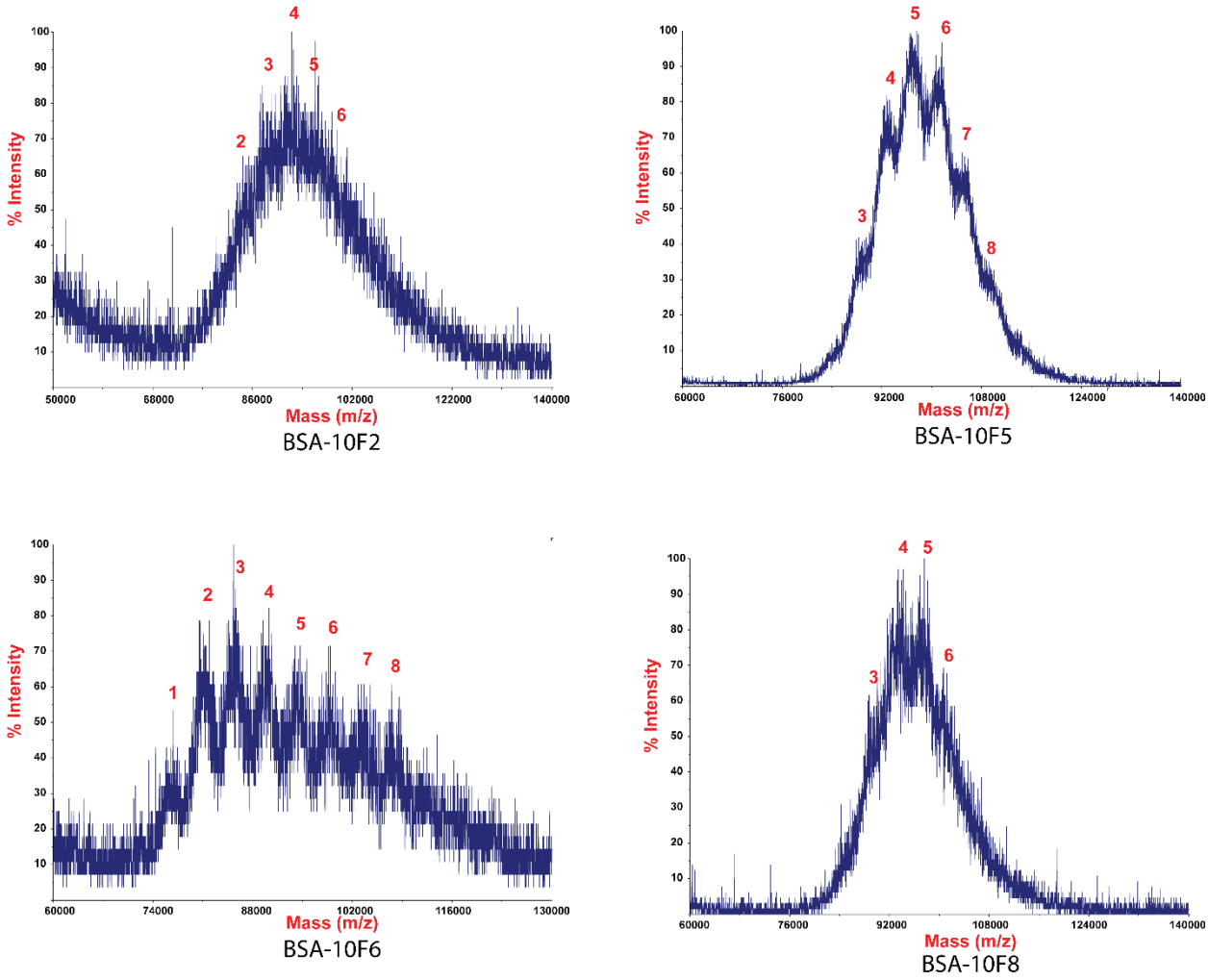


Fig. S8 MALDI-TOF MS analysis of BSA-peptide 10F2,10F5M,10F6,10F8 conjugates. The red numbers indicate the number of glycopeptides per CRM protein molecule.

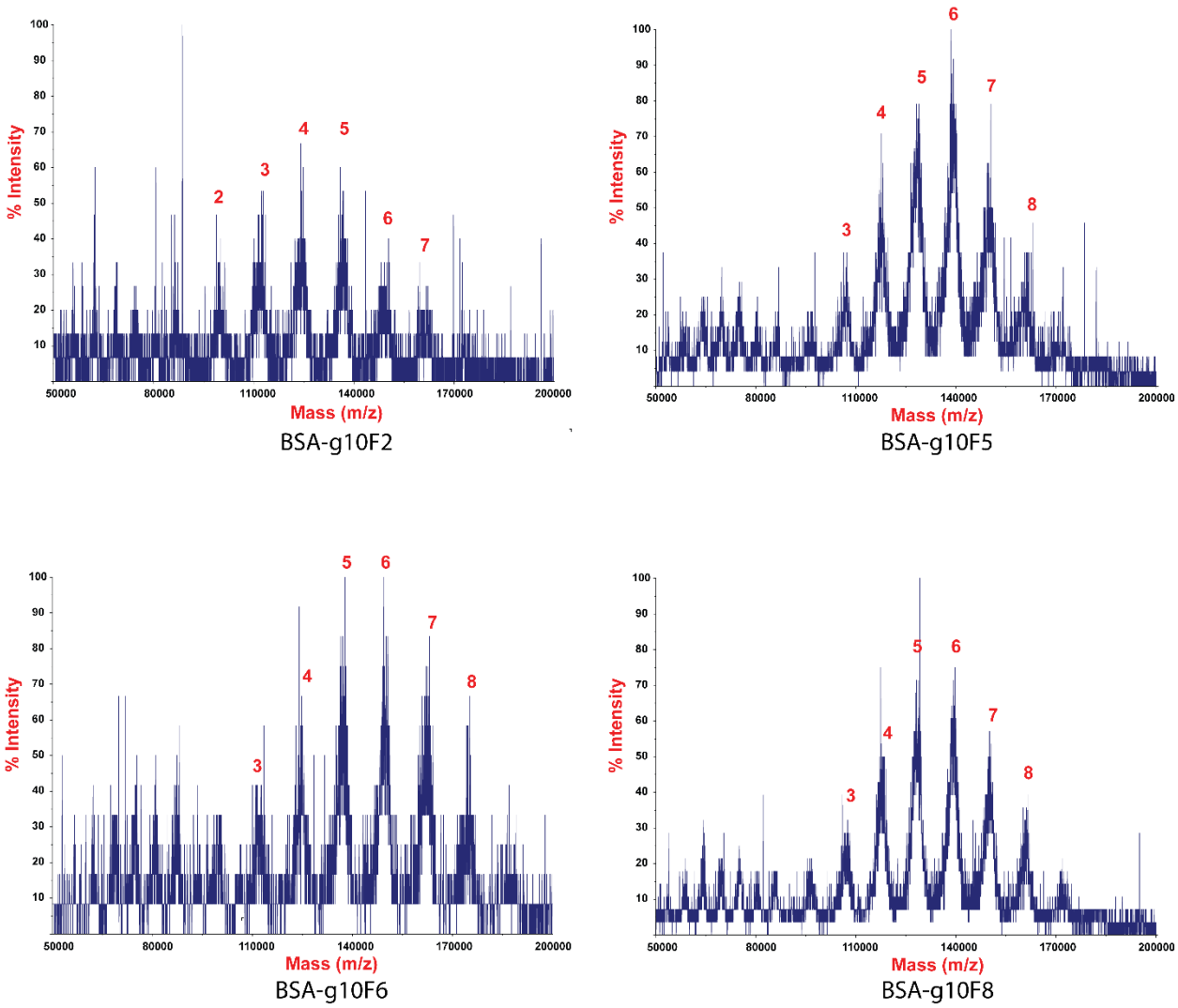


Fig. S9 MALDI-TOF MS analysis of BSA-glyco10F2, glyco10F5M, glyco10F6, glyco10F8 conjugates. The red numbers indicate the number of glycopeptides per CRM protein molecule. More closely clustered peaks at lower m/z are doubly charged ions.

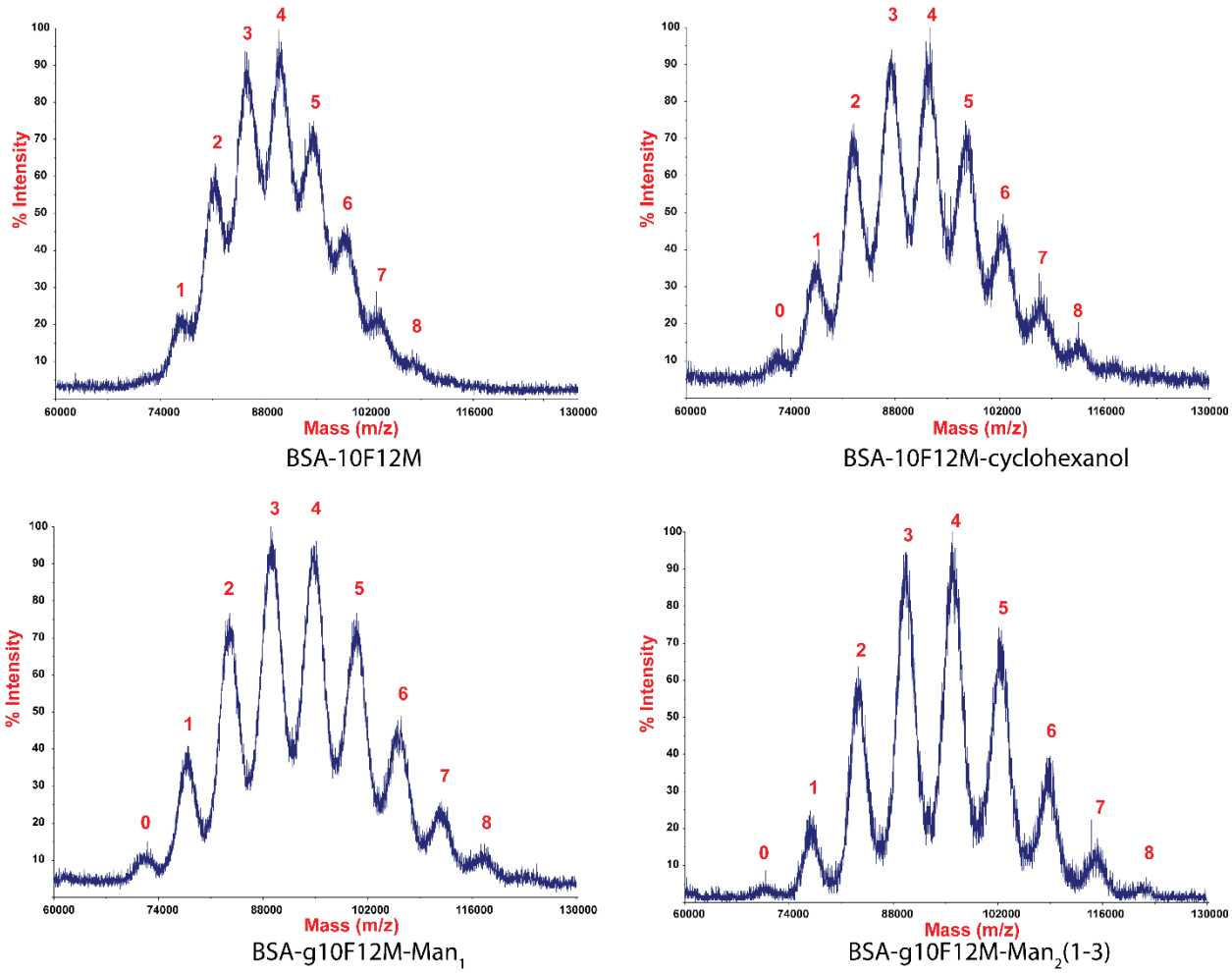


Fig. S10 MALDI-TOF MS analysis of BSA-10F12M, BSA-10F12Mcyclohexanol, BSA-10F12M Man_1, and BSA-10F12M Man_2 (1-3) conjugates. The red numbers indicate the number of glycopeptides per CRM protein molecule.

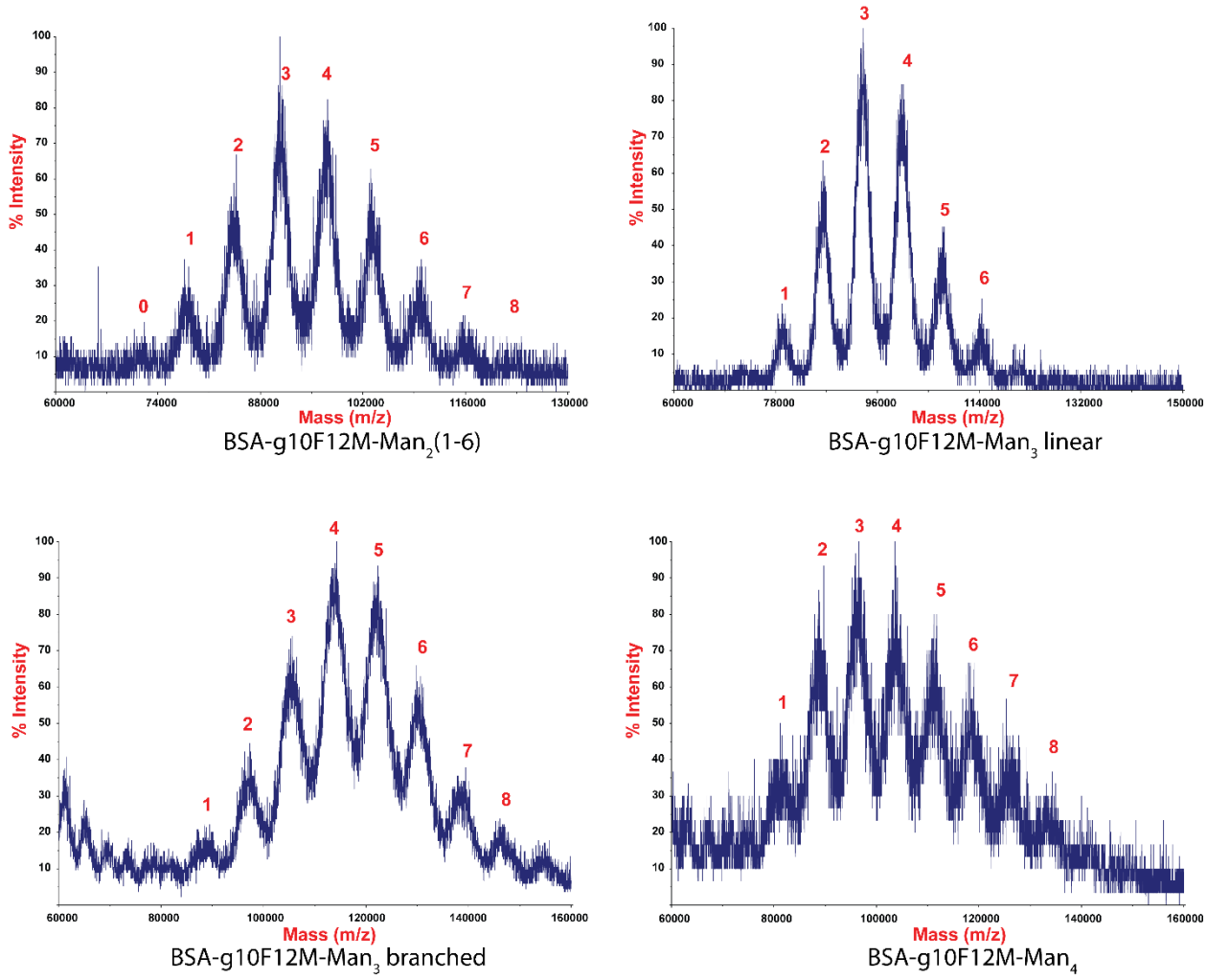


Fig. S11 MALDI-TOF MS analysis of BSA-10F12M Man_2 (1-6), BSA-10F12M Man_3 branched, BSA-10F12M Man_3 linear, and BSA-10F12M Man_4 linear conjugates. The red numbers indicate the number of glycopeptides per CRM protein molecule. More closely clustered peaks at lower m/z are doubly charged ions.

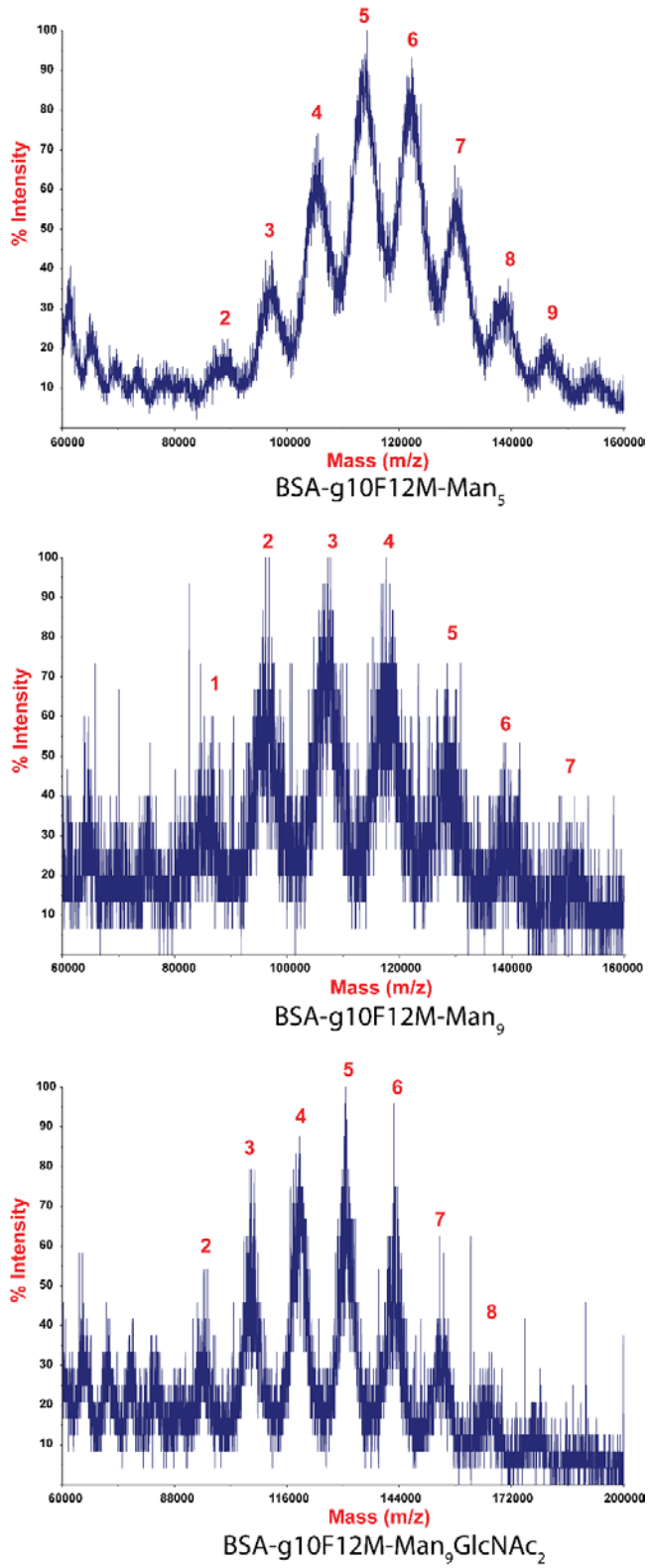


Fig. S12 MALDI-TOF MS analysis of BSA-10F12M-Man_5, BSA-10F12M-Man_9, and BSA-10F12M $Man_9GlcNAc_2$ conjugates. The red numbers indicate the number of glycopeptides per CRM protein molecule. More closely clustered peaks at lower m/z are doubly charged ions.

Crystal structure determination for 2G12-glycopeptide complexes

IgG 2G12 was expressed in 293 Freestyle cells and purified by protein A chromatography. IgG was cleaved to Fab with 2% papain for 3 hours before inactivation with 200 mM iodoacetamide. The cleavage mixture was applied to a protein A column and the unbound Fab was further purified on a S200 16/60 column (GE).

Fab was mixed with peptide at a 1:3 (Fab:peptide) molar ratio and the complex was purified by size exclusion chromatography with a Superdex 200 16/60 column. Each Fab-glycopeptide complex was concentrated to 10mg/ml. The 2G12-10V1S peptide complex was crystallized in 24-well sitting drop trays (Hampton Research) in condition 6B of Footprint 1 screen [1], which corresponded to 1.0 M sodium citrate tribasic dihydrate, 10mM sodium borate, pH 8.5. Crystals were cryoprotected with well solution augmented with glycerol to a final concentration of 30%. 2G12-10F5M peptide complex was crystallized in 24-well sitting drop trays with a well solution of 0.2M $LiSO_4$, 17.5% PEG400, 0.1M Tris, pH 8.5. Crystals were cryoprotected with the well solution plus PEG400 at a final concentration of 27.5%. All crystals were cryocooled by rapid plunging into liquid nitrogen and data were collected at SSRL beamline 12-2 using a Dectris Pilatus 6M detector. Data were processed and scaled with HKL-2000 [2] and molecular replacement was carried out with Phaser [3] using 2G12 Fab coordinates from PDB 4RBP [4] as a model. Refinement was carried out with Phenix.refine [5] and final statistics for data collection and refinement are outlined in Table S7. For both complexes, the Fab chain identifiers are L and K (light chains) and H and M (heavy chains). The 10V1S glycopeptide has chain identifiers A and C. Glycans in 10F5M are labeled A and B. In Fab1 (LH), the V_L domain is paired with the V_H' domain, while in Fab2 (KM), the V_L' domain is paired with the V_H domain (see Fig. 3).

The 2G12-10V1S complex crystals have one domain-swapped Fab dimer and two glycopeptides in the asymmetric unit, with one peptide bound to each Fab within the dimer (Figs. 3 and S13). Glycopeptide A is better ordered and has lower B values than C (38 $Å^2$ for A versus 57 $Å^2$ for C), likely due to crystal contacts from a symmetry-related Fab. Thus, glycopeptide A is used for most of the analysis and discussion. Out of the 40-residue peptide, residues 18-33 and 19-33 are visible in peptides A and C. The peptide forms a hairpin, with a type VIII non-hydrogen bonded reverse turn around residues 23-26 (IPWY). Pro24 adopts a cis conformation. Residue 20 is homopropargylglycine (HPG) to which a Man_9 glycan is attached. All 9 mannose moieties are visible in the electron density and adopt a gg-gg (3-4′ and 4′-B both adopt a gauche rotamer) rotameric arrangement. The primary glycan binding site in each Fab binds to the terminal Manα1-2 Man moieties from the D3 arm (mannose B and D3) (Fig. 3a). The Fab-glycopeptide interface is extensive with 854$Å^2$ and 884$Å^2$ buried on the glycopeptide and Fab, respectively (Tables S2 and S3). About 60% of the glycopeptide contribution is from peptide. The Fab contacts glycopeptide with CDR's L3, H1', H2', and H3', with some contributions from heavy-chain framework residues (Table S2); the largest contribution comes from CDR H2'. There are 10 hydrogen bonds from the glycopeptide to the Fab, with 6 from the peptide component and 4 from the carbohydrate (Table S4).

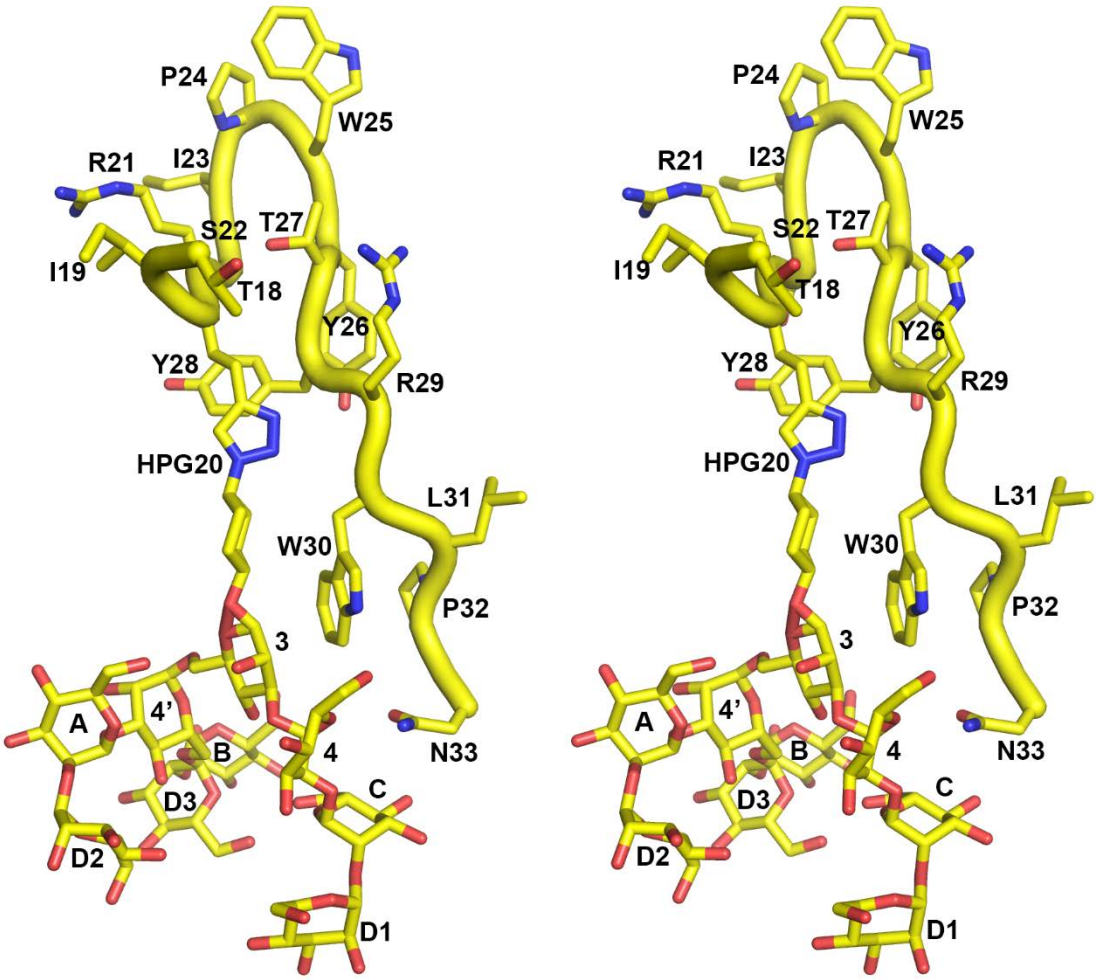


Fig. S13 Wall-eyed stereo view of glycopeptide 10V1S. Residues 18-33 of the 40-residue peptide are visible in the crystal structure.

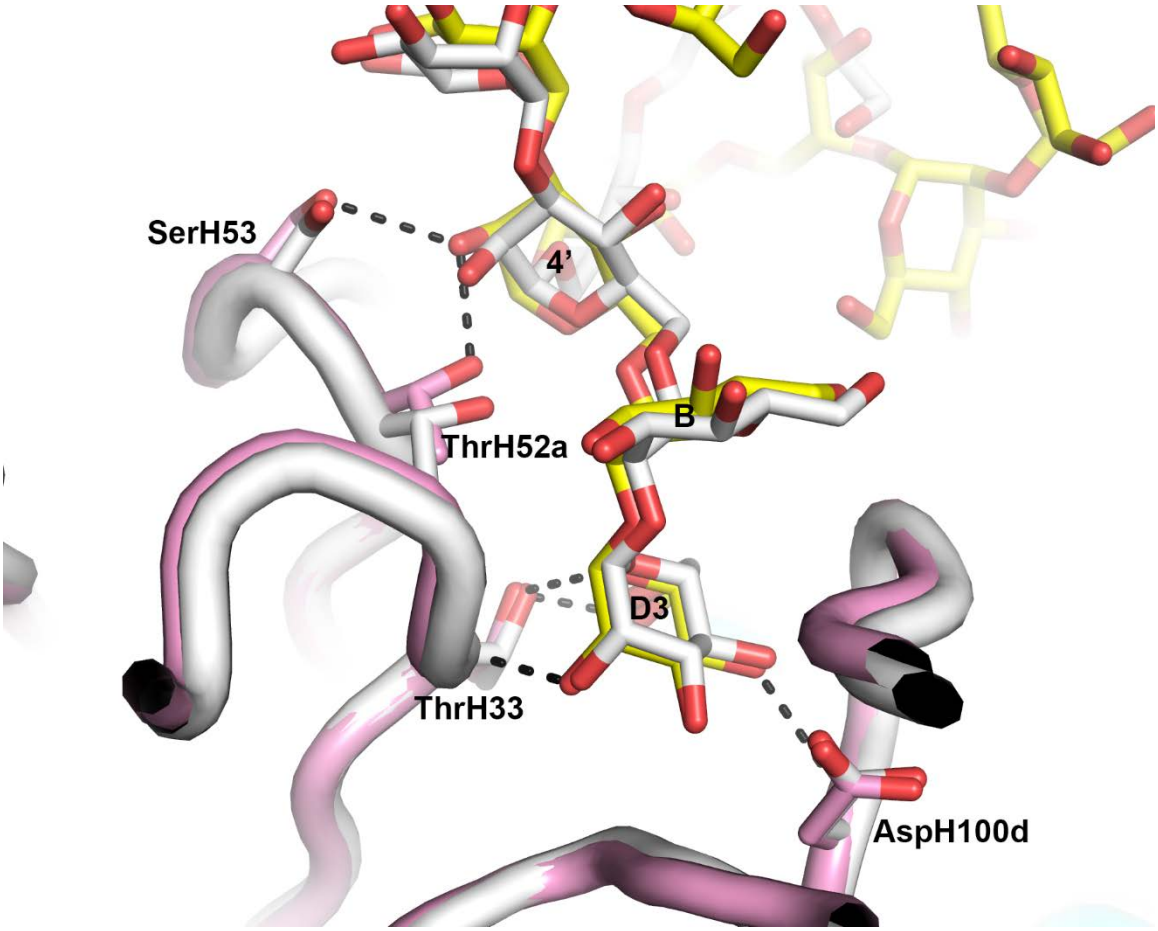


Fig. S14. Superposition of 10V1S with 2G12+Man_8 (6MNF). The 10V1S heavy chain and carbohydrate are colored pink and yellow, while the 2G12/Man_8 heavy chain and carbohydrate are both colored white. In both the 10V1S structure and 6MNF, there are 6 hydrogen bonds involving ThrH33, ThrH52a, SerH53 and AspH100d to the D3 or 4' mannose moieties.

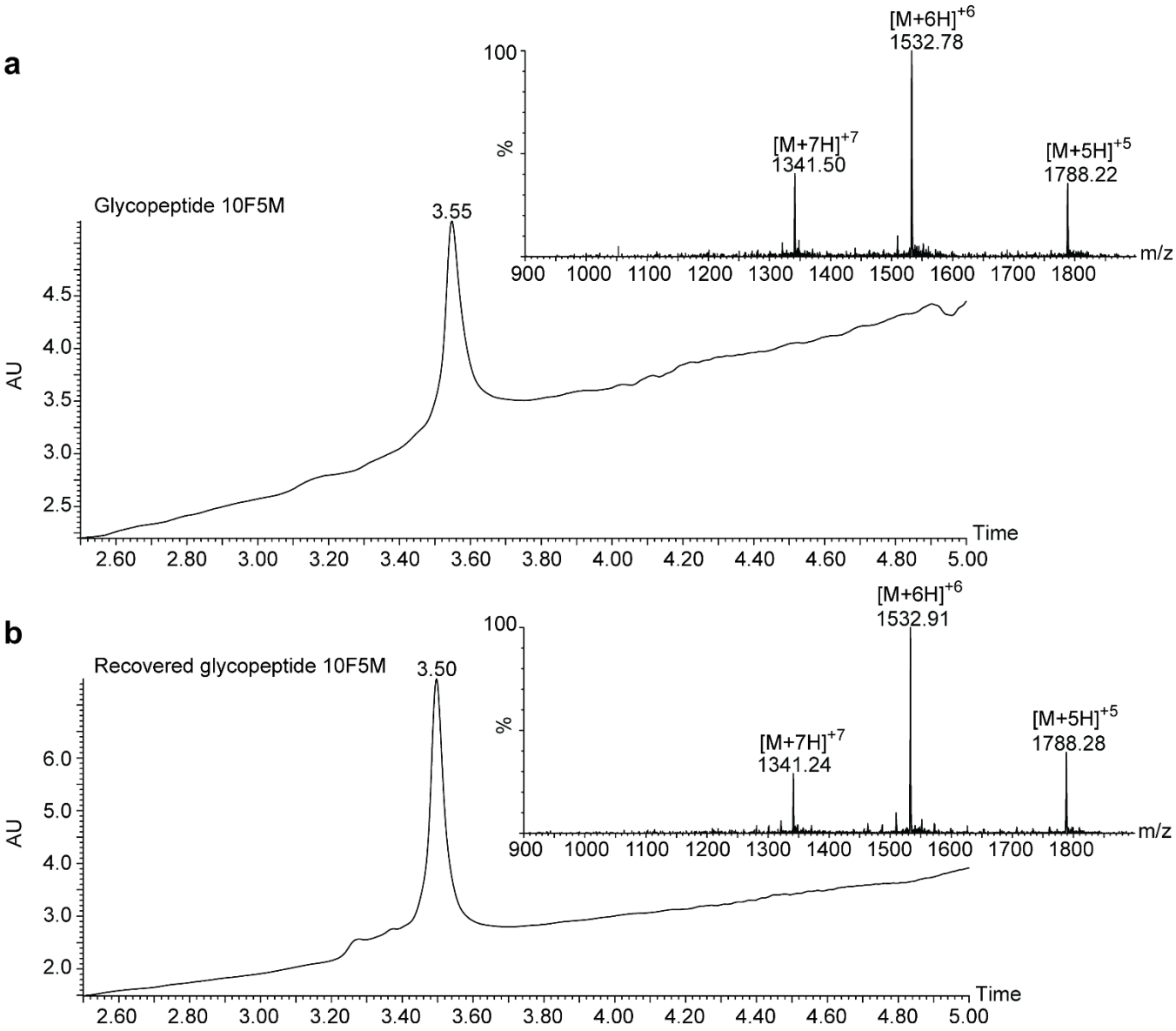


Fig. S15 Stability of glycopeptide 10F5M in crystallization buffer. UPLC chromatograms of glycopeptide 10F5M are shown **a)** prior to and **b)** after incubation in crystal growth medium. The conditions for growth of 10F5M-2G12 cocrystals were room temperature for 2 days in 0.2M Li_2SO_4, 17.5% PEG400, 0.1M Tris, pH 8.5. To test stability, glycopeptide 10F5M was dissolved in the same mixture for a week at room temperature, then exchanged back to water by Amicon filtration and analyzed by UPLC/ESI/MS.

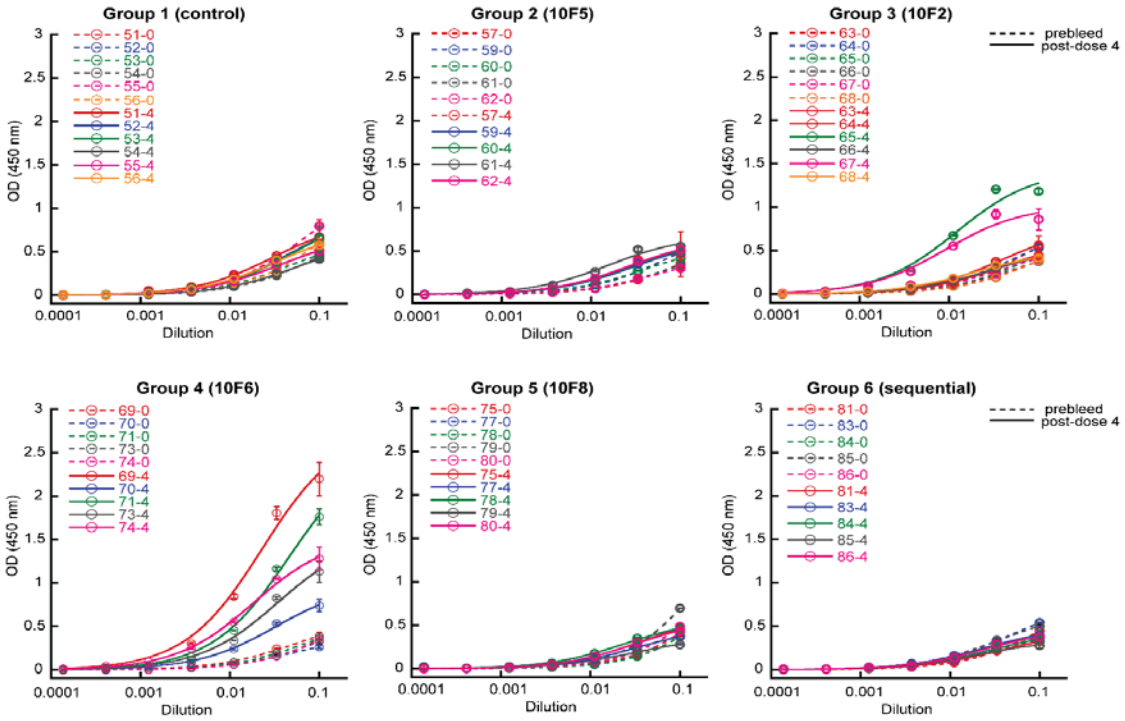


Fig. S16 ELISA of all groups binding to 293F SOSIP trimer at 200 ng/well.

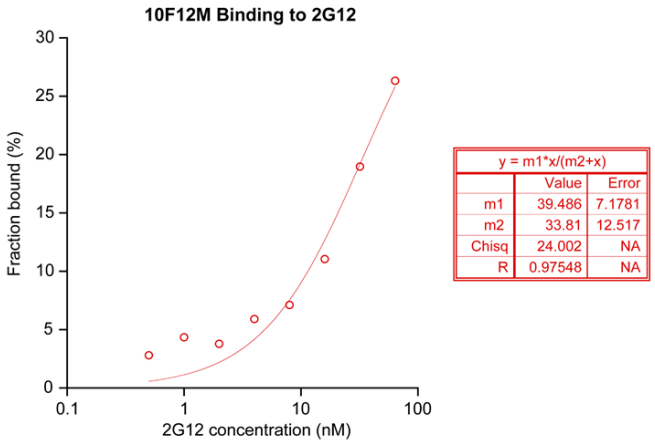


Fig. S17 Binding of 2G12 to glycopeptide 10F12M. Binding was measured by a bead-based radioactive binding assay [6]. Glycopeptide 10F12M was produced by *in vitro* translation of the peptide followed by click glycan attachment, and contained a C-terminal LGHis$_6$-FLAG sequence instead of the shorter GCA sequence of synthetic 10F12M shown in Fig. 8. The sequence of 10F12M, including tags for this assay, is XS**S**YVTVIPAXNXPEARLGIV**S**HXPGIRRGKALYGSGSLGHHHHHHRDYKDDDDK (X = homopropargylglycine).

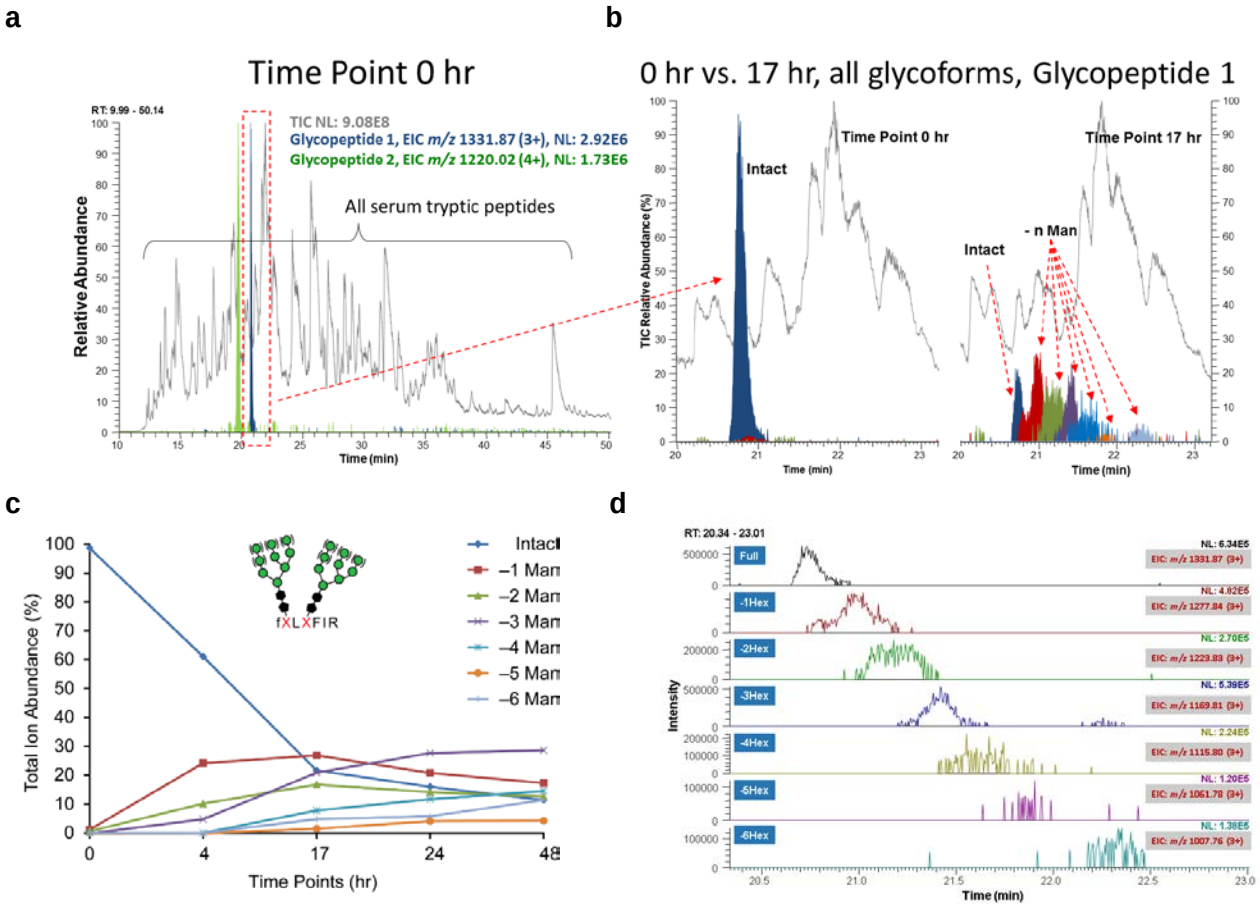


Fig. S18. Glycan analysis showing high mannose glycoform distribution for alkyne-CRM$_{197}$-Ac-glycopeptide 10F6 collected at different time points. **a** nanoUPLC/MS Total Ion Chromatogram of tryptic digest of conjugate, with colored superimposed Extracted Ion Chromatograms for two 10F6 tryptic glycopeptides. **b** Closeup of EICs for all glycoforms of N-terminal glycopeptide (f**X**L**X**FIR) at t = 0 and 17hr exposure to serum. **c** graph of glycoform relative abundance vs. time **d** Representative data (time point 17h) showing Extracted Ion Chromatograms corresponding to distinct glycoforms of the glycopeptide in b and c.

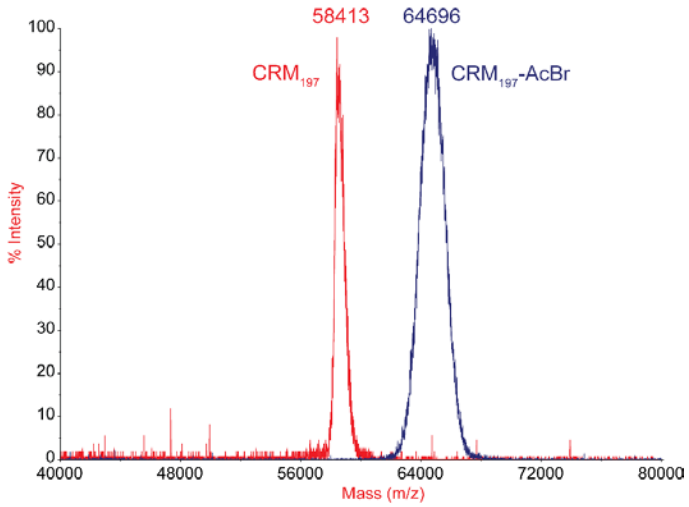


Figure S19. MALDI-TOF MS analysis of CRM$_{197}$-AcBr.

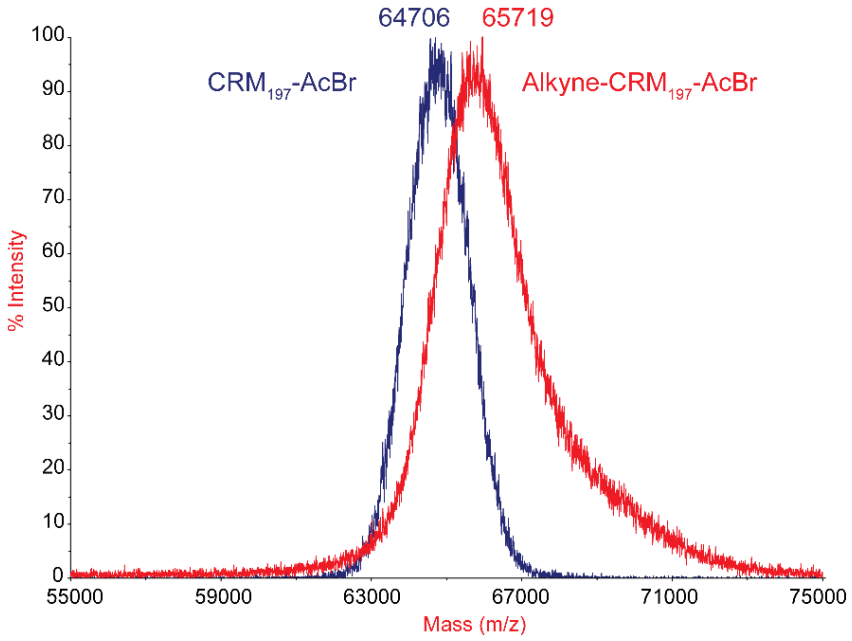


Figure S20. MALDI-TOF MS analysis of alkyne-CRM$_{197}$-AcBr.

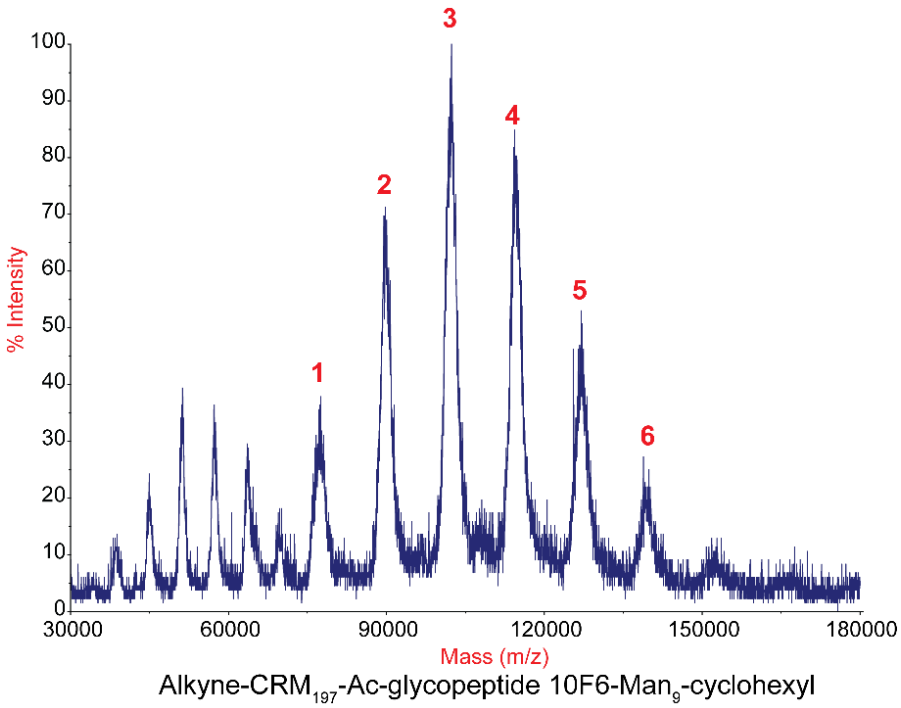


Figure S21. MALDI-TOF MS analysis of alkyne-CRM$_{197}$-Ac-glycopeptide 10F6. The red numbers indicate the number of glycopeptides per CRM protein molecule. More closely clustered peaks at lower m/z are doubly charged ions.

a

	Sequences	K_D (nM)
10V1	XATKTNCKREKTXDNHVTIXRSIPWYTYRWLPN	1.90 ± 0.17
10V1S	XATKTNSKREKTXDNHVTIXRSIPWYTYRWLPN	2.06 ± 0.04

b

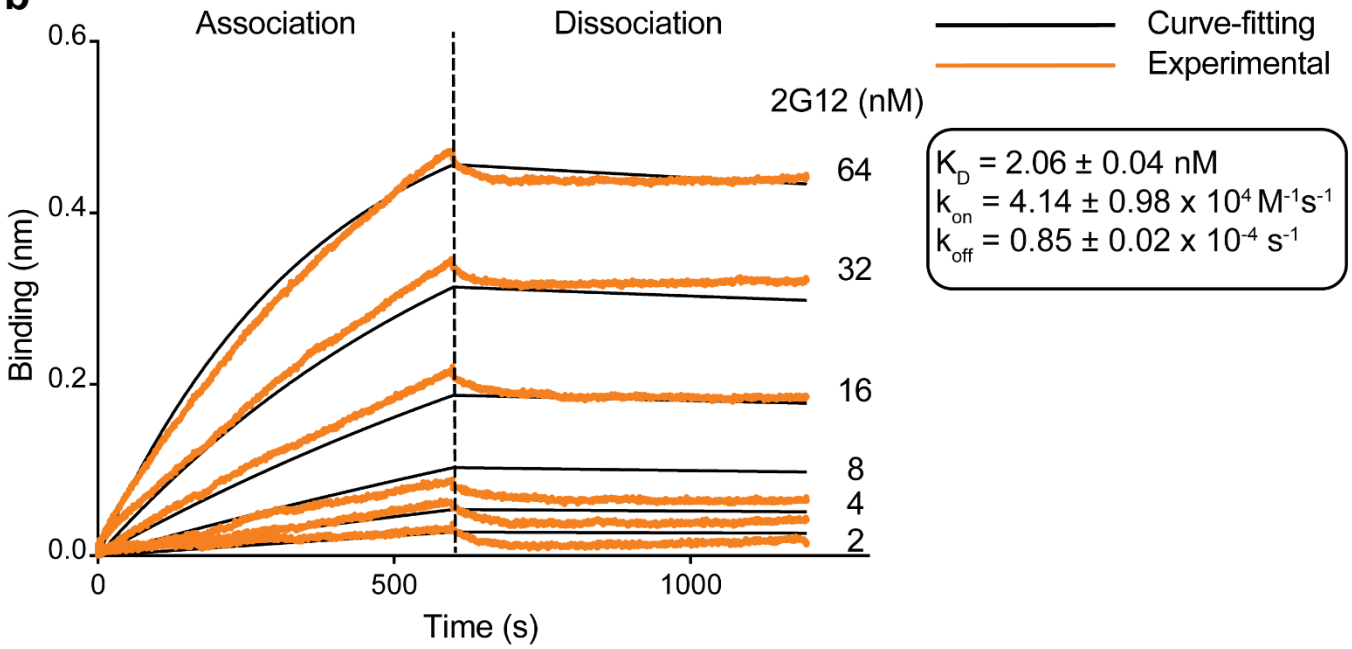


Table S1. a 10V1 vs 10V1S sequences and K_Ds. X = Man_9-Cy- "click" glycosylated homopropargylglycine residue, as depicted in Fig. 1. **b** BioLayer Interferometry (BLI) measurement of 2G12 interacting with surface-immobilized synthetic 10V1S glycopeptide. The K_D for 10V1 was published previously [6]. Uncertainties reported are standard errors of measurement.

Table S2. Surface area (Å^2) buried on Fab by glycopeptides 10V1S or 10F5M. Contacts made to the neighboring Fab within the domain -swapped dimer are indicated .

	10V1S		10F5M	
CDR	Glycopeptide	Glycopeptide	Glycan A	Glycan
L1	0	0	14	2
L2	0	0	0	0
L3	93	84	74	50
H1	45	44	51	48
H2	288	303	76	109
H3	141	140	191	130
H framework	138	147	0	0
H1 (from neighboring Fab)	38	41	0	0
H framework (from neighboring Fab)	111	120	0	0
Total	854	879	405	339

Table S3. Surface area (Å^2) buried on glycopeptide 10V1S or 10F5M by Fab.

	10V1S		10F5M	
	Fab1	Fab2	Fab1	Fab2
Peptide	528	530	N/A	N/A
Man3	28	38	14	1
Man4	0	0	3	53
ManC	27	0	72	71
ManD1	0	0	133	131
Man4´	58	49	21	18
ManA	28	26	8	1
ManD2	0	0	49	15
ManB	72	73	9	5
ManD3	143	143	0	0
Total carbohydrate	356	329	359	317
Total peptide+carbohydrate	884	859	N/A	N/A

Table S4. Hydrogen bonds from Fab to glycopeptide 10V1S.				
Peptide residue	atom	Fab residue	atom	Distance (Å)
TyrA26	OH	ArgH57	O	2.46
SerA22	OG	TyrH59	OH	2.78
SerA22	OG	GluH´(M)75	OE2	2.52
TyrA28	OH	GluH´(M)75	OE2	2.70
ArgA21	NH1	AspH´(M)76	OD2	3.10
ArgA21	NH2	AspH´(M)76	OD2	3.15
Carbohydrate	atom	Fab residue	atom	Distance (Å)
ManA D3	O2	ThrH33	N	2.74
ManA D3	O5	ThrH33	OG1	2.92
ManA D3	O6	ThrH33	OG1	2.92
ManA 4’	O2	ThrH52a	OG1	2.64
ManA 4’	O2	SerH53	OG	3.02
ManA D3	O4	AspH100d	N	3.01

Table S5a. TZMbl Neutralization assay results of post-dose 4 sera from multi-immunogen study.

		ID_{50} in TZM-bl Cells[1]			
		SVA-MLV	**MW965.26**	**BG505/T332N**	**JR-FL**
		Neg Ctrl	**Clade C**	**Clade A**	**Clade B**
			Tier 1A	**Tier 2**	**Tier 2**
Animal ID	**Groups**	**ID#8075**	**ID#7847**	**ID#9212**	**ID#**
19251	1	<20	<20	<20	<20
19252	1	<20	<20	<20	<20
19253	1	<20	<20	<20	<20
19254	1	<20	<20	<20	<20
19255	1	<20	<20	<20	<20
19251	1	<20	<20	<20	<20
19263	3	<20	<20	<20	<20
19264	3	<20	<20	<20	<20
19265	3	<20	<20	<20	<20
19266	3	<20	**23**	<20	<20
19267	3	<20	<20	<20	<20
19268	3	<20	<20	<20	<20
19269	4	<20	<20	<20	<20
19270	4	<20	**24**	<20	<20
19271	4	<20	<20	<20	<20
19272	4	<20	<20	<20	<20
19273	4	<20	<20	<20	<20
CH01-31		>25	3.00	<0.01	<0.01

[1]Values are the serum dilution at which relative luminescence units (RLUs) were reduced 50% compared to virus control wells (no test sample).

Note: Values in bold are considered positive for neutralizing antibody activity in the post-immune sample based on the criterion of >3X the observed background against the SVA-MLV negative control pseudovirus.

Table S5b. TZMbl Neutralization assay results of post-dose 6 sera from multi-immunogen study.

		ID_{50} in TZM-bl Cells[1]					
		SVA-MLV	**MW965.26**	**BG505ΔCT/T332N**	**JR-FL**	**SVA-MLV/GnTI-**	**JR-FL/GnTI-**
		Neg Ctrl	**Clade C**	**Clade A**	**Clade B**	**neg ctrl**	**Clade B**
			Tier 1A	**Tier 2**	**Tier 2**		**Tier 2**
Animal ID	**Group**	**ID#6565**	**ID#7847**	**ID#7012**	**ID#730**	**ID#7190**	**ID#7204**
19251	1	<20	**994**	<20	<20	<20	**69**
19252	1	<20	**151**	<20	<20	<20	**40**
19253	1	<20	**1172**	**30**	<20	<20	**152**
19254	1	<20	**1166**	<20	<20	<20	**768**
19255	1	<20	**1475**	<20	<20	<20	**528**
19256	1	<20	**45**	<20	<20	<20	**239**
19257	2	<20	**524**	**200**	<20	<20	**367**
19259	2	<20	**870**	**203**	**25**	<20	**206**
19260	2	<20	**255**	**34**	<20	<20	**349**
19261	2	<20	**976**	<20	<20	<20	**905**
19262	2	<20	**1089**	<20	<20	<20	**662**
19263	3	<20	**2004**	<20	<20	<20	**48**
19264	3	<20	**640**	<20	<20	<20	**356**
19265	3	<20	**959**	<20	<20	<20	**406**
19266	3	<20	**774**	<20	<20	<20	**424**
19268	3	<20	**619**	<20	<20	<20	**265**
19269	4	<20	**1239**	**49**	<20	<20	**162**
19270	4	<20	**394**	<20	<20	<20	**131**
19271	4	<20	**304**	<20	<20	<20	**386**
19273	4	<20	**599**	<20	<20	<20	**351**
19274	4	<20	**306**	<20	<20	<20	**60**
19275	5	<20	**581**	<20	<20	<20	**290**
19277	5	<20	**1105**	<20	<20	<20	**280**
19278	5	<20	<20	<20	<20	<20	**437**
19279	5	<20	**244**	<20	<20	<20	**591**
19280	5	<20	**53**	<20	<20	<20	**22**
19281	6	<20	**689**	<20	<20	<20	**155**
19283	6	<20	**84**	<20	<20	<20	**257**
19284	6	<20	**292**	<20	<20	<20	**509**
19285	6	<20	**44**	<20	<20	<20	**700**
19286	6	<20	**1097**	<20	<20	<20	**286**
CH01-31			2.14	0.03	0.02	>25	<0.01

[1]Values are the serum dilution at which relative luminescence units (RLUs) were reduced 50% compared to virus control wells (no test sample).

Note: Values in bold are considered positive for neutralizing antibody activity in the post-immune sample based on the criterion of >3X the observed background against the SVA-MLV negative control pseudovirus.

Glycans compared	Mean diff. (log IgG titer)	Adjusted p-value
Groups 2-5 (aggregate)		
Cy10F12M vs. Man_1Cy10F12M	-.32	.011
Cy10F12M vs. Man_2Cy10F12M	-1.07	< .0001
Man_1Cy10F12M vs. Man_2Cy10F12M	-.75	< .0001
Group 2 (10F5M)		
Cy10F12M vs. Man_1Cy10F12M	.054	.973
Cy10F12M vs. Man_2Cy10F12M	-0.79	.141
Man Cy10F12M_1 vs. Man_2Cy10F12M	-0.85	.0184
Group 3 (10F2)		
Cy10F12M vs. Man_1Cy10F12M	-.26	.293
Cy10F12M vs. Man_2Cy10F12M	-.96	.0014
Man_1 Cy10F12M vs. Man_2Cy10F12M	-.70	.0145
Group 4 (10F6)		
Cy10F12M vs. Man_1Cy10F12M	-.80	.141
Cy10F12M vs. Man_2Cy10F12M	-2.1	.0072
Man_1 Cy10F12M vs. Man_2Cy10F12M	-1.3	.0564
Group 5 (10F8)		
Cy10F12M vs. Man_1Cy10F12M	-.26	.0926
Cy10F12M vs. Man_2Cy10F12M	-.57	.0322
Man_1 Cy10F12M vs. Man_2Cy10F12M	-.31	.234
Group 6 (sequential)		
Cy10F12M vs. Man_1Cy10F12M	-.33	.214
Cy10F12M vs. Man_2Cy10F12M	-.94	.235
Man_1Cy10F12M vs. Man_2Cy10F12M	-.61	.288

Table S6. P-values for comparison of serum binding to BSA-10F12M antigens bearing cyclohexyl (Cy), $CyMan_1$ and $CyMan_2$(1-3 linked) when groups are considered in aggregate or separately. P-values are calculated by matched one-way ANOVA, followed by Tukey's post-hoc test. Overall p values for the ANOVA indicated significance for all groups except group 6 (aggregated groups: $p < .0001$, Group 2: $p = .0356$; Group 3: $p = .0002$; Group 4: $p = .0011$; Group 5: $p = .0162$; Group 6: $p = .124$). ANOVA for this table was performed to compare only these three antigens, whereas ANOVA for **Fig. 8b, c** was performed to compare all antigens.

Table S7. Crystal structure data collection and refinement statistics.

	2G12+10V1S	**2G12+10F5M**
Beamline	SSRL 12-2	SSRL 12-2
Wavelength (Å)	0.97950	0.97950
Resolution range (Å)[a]	42.4 - 2.3 (2.34- 2.30)	46.8-3.6 (3.68-3.60)
Space group	$P2_12_12_1$	I222
Unit cell	45.22, 131.18, 169.54	137.64, 146.31, 148.21
Total reflections	247,212 (11,195)	150,342 (10,062)
Unique reflections	45,804 (2195)	17,760 (1170)
Multiplicity	5.4 (5.1)	8.5 (8.6)
Completeness (%)	99.5 (97.6)	99.8 (99.9)
Mean I/sigma(I)	8.8 (1.9)	5.8 (1.5)
Wilson B-value ($Å^2$)	27	82
R_{merge} (%)[b]	17.4 (>100)	29.6 (>100)
R_{meas} (%)[c]	19.3 (>100)	31.6 (>100)
R_{pim} (%)[d]	9.5 (50.6)	14.2 (48.0)
$CC_{1/2}$[e]	99.0 (51.0)	92.0 (72.0)
Reflections [R_{work} (R_{free})]	43,502 (2230)	16,789 (927)
R_{work} (%)	19.7 (28.4)	21.9 (33.4)
R_{free} (%)	23.7 (31.3)	24.8 (37.9)
No. non-hydrogen atoms		
Fab	6586	6573
Ligands	477	198
Solvent	441	0
Protein residues	898	869
RMS (bonds)	0.009	0.005
RMS (angles)	1.35	1.06
Ramachandran favored (%)	96.4	96.4
Ramachandran allowed (%)	3.6	3.6
Ramachandran outliers (%)	0.0	0.0
Clashscore[f]	2.8	6.7
Average B-value ($Å^2$)	33	90
Fab	32	90

Glycopeptide	47	93
Solvent	33	NA

[a]Numbers in parentheses are for highest resolution shell

[b]$R_{merge} = \Sigma_{hkl} \Sigma_{i=1,n} |I_i(hkl) - \langle I(hkl) \rangle| / \Sigma_{hkl} \Sigma_{i=1,n} I_i(hkl)$

[c]$R_{meas} = \Sigma_{hkl} \sqrt{(n/n-1)} \Sigma_{i=1,n} |I_i(hkl) - \langle I(hkl) \rangle| / \Sigma_{hkl} \Sigma_{i=1,n} I_i(hkl)$

[d]$R_{pim} = \Sigma_{hkl} \sqrt{(1/n-1)} \Sigma_{i=1,n} |I_i(hkl) - \langle I(hkl) \rangle| / \Sigma_{hkl} \Sigma_{i=1,n} I_i(hkl)$

[e]$CC_{1/2}$ = Pearson Correlation Coefficient between two random half datasets

[f] Number of unfavorable all-atom steric overlaps ≥ 0.4Å per 1000 atoms

Table S8. P-values for serum binding to autologous vs. heterologous glycopeptides

Group 2 (10F5M vaccinated)		
comparison	average Δ log EC_{50} titer	adjusted p-value
10F5M vs. 10F2 response	.254	.0025
10F5M vs. 10F6 response	.285	.0013
10F5M vs. 10F8 response	.291	.0016
Group 3 (10F2 vaccinated)		
comparison	average Δ log EC_{50} titer	adjusted p-value
10F2 vs. 10F5M response	.153	.24
10F2 vs. 10F6 response	.277	.10
10F2 vs. 10F8 response	.276	.029
Group 4 (10F6 vaccinated)		
comparison	average Δ log EC_{50} titer	adjusted p-value
10F6 vs. 10F5M response	.428	.034
10F6 vs. 10F2 response	.445	.033
10F6 vs. 10F8 response	.364	.033
Group 5 (10F8 vaccinated)		
comparison	average Δ log EC_{50} titer	adjusted p-value
10F8 vs. 10F5M response	.527	.022
10F8 vs. 10F2 response	.482	.020
10F8 vs. 10F6 response	.233	.035

For each rabbit, post dose 2-4 titers (Fig. 5) were log transformed and averaged over the three doses. Matched one-way ANOVA was performed, comparing each rabbit's serum selectivity for its own (autologous) glycopeptide immunogen vs. the other glycopeptides. Adjusted p-values were calculated with Dunnett's multiple comparison test.

Table S9. Effect of mannose on Dose 6 serum binding to BG505SOSIP.664T332N

	rabbit ID	A: EC_{50} titer (0 mM mannose)	B: EC_{50} titer (554 mM mannose)	Mannose effect (log (A/B))	Average
Group 1	**51-6**	1645	1082	**0.18**	0.11
	52-6	544	418	**0.11**	
	53-6	2570	1913	**0.13**	
	54-6	4595	4021	**0.06**	
	55-6	3295	2401	**0.14**	
	56-6	1568	1511	**0.02**	
Group 2	**57-6**	2272	1515	**0.18**	0.14
	59-6	9553	8066	**0.07**	
	60-6	2164	1472	**0.17**	
	61-6	1954	1536	**0.10**	
	62-6	1823	1195	**0.18**	
Group 3	**63-6**	3333	2444	**0.13**	0.08
	64-6	2327	2180	**0.03**	
	65-6	2588	1936	**0.13**	
	66-6	3324	3504	**-0.02**	
	68-6	4318	3175	**0.13**	
Group 4	**69-6**	6111	5112	**0.08**	0.10
	70-6	2905	1931	**0.18**	
	71-6	1312	943	**0.14**	
	73-6	2315	1648	**0.15**	
	74-6	1922	1563	**0.09**	
Group 5	**75-6**	1402	1066	**0.12**	0.10
	77-6	3924	3605	**0.04**	
	78-6	1995	1697	**0.07**	
	79-6	714	556	**0.11**	
	80-6	1458	1014	**0.16**	
Group 6	**81-6**	1954	1462	**0.13**	0.06
	83-6	2316	1826	**0.10**	
	84-6	3607	3517	**0.01**	
	85-6	5248	4751	**0.04**	
	86-6	4662	4396	**0.03**	

bnAb controls					
	EC_{50} (nM) 0 mM mannose	**EC_{50} (nM) 554 mM mannose**	**EC_{50} (nM) 554 mM glycerol**	**Mannose effect**	**Glycerol effect**
2G12	2.8	129.58	2.24	**1.67**	-0.10
b12	4.21	18.34	9.43	**0.64**	0.35
VRC01	8.53	14.17	10.08	**0.22**	0.07

Table S10. Relative quantification of serum mannosidase trimming of g10F6 conjugate								
Tryptic glycopeptide 1 (formyl**X**LX**F**IR)*								
	Full	-1 Man	-2 Man	-3 Man	-4 Man	-5 Man	-6 Man	
0	98.62	1.13	0.61	-0.20	0.00	-0.16	0.00	
4	61.06	24.16	10.16	4.71	0.00	-0.10	0.00	
17	21.59	26.84	16.75	20.87	7.73	1.46	4.76	
24	15.99	20.78	14.11	27.65	11.66	4.05	5.76	
48	11.38	17.28	12.61	28.65	14.47	4.24	11.37	
Tryptic glycopeptide 2 (**X**QYVYHAPLLT**X**VR)*								
	Full	-1 Man	-2 Man	-3 Man	-4 Man	-5 Man	-6 Man	-7 Man
0	90.78	2.47	6.81	-0.05	0.00	0.00	0.00	0.00
4	52.17	27.37	14.94	3.64	1.56	0.32	0.00	0.00
17	17.39	20.21	14.86	8.94	18.66	7.72	11.78	0.44
24	9.11	12.67	14.46	21.67	16.88	7.71	16.64	0.88
48	6.35	10.46	5.88	22.64	20.46	8.82	23.99	1.41
*__X__ refers to click-glycosylated homopropargylglycine residue. Data are graphed in Figure 9d and SI Figure S18. See Methods for LC and mass spectrometric quantification procedures.								

Synthesis of peptide 10F12M

10F12M (X**S**YVTVIPAXNXPEARLGIV**S**HXPGIRRGKALYGSGSGC(StBu)A, X = homopropargylglycine) was synthesized according to previously reported methods [7] and was prepared with two C→S mutations (bold **S**'s) from the original evolved sequence, 10F12 [6]. These mutations were deleterious to 2G12 binding (K_D > 20 nM, Fig. S16, vs. 0.77 nM for 10F12). Briefly, starting with 120 mg of trityl ChemMatrix® resin loaded with 0.28 mequiv/g serine (33.5 µmol scale), 41.8 mg of crude peptide 10F12M was obtained. HPLC purification (Waters Symmetry 300 C4, 5 µm, 10×250 mm, 4 mL/min, 10-45% MeCN in H_2O with 0.1% formic acid, over 60 min, retention time: 28.4 min) of 22 mg of crude peptide yielded 3.5 mg of pure 10F12M, corresponding to 16 % overall yield if all crude peptide had been purified. LR ESI-MS: observed average m/z of multiply charged ions 1091.14 $[M+4H]^{4+}$, 1454.47 $[M+3H]^{3+}$, corresponding to 4360.49 observed average mass, calculated mass for $C_{195}H_{311}N_{59}O_{51}S_2$: 4362.05.

Synthesis of glycopeptides 10F12M – Man_1, Man_2, Man_3, Man_4, Man_5, Man_9, $Man_9GlcNAc_2$

Glycans were attached by Copper-Assisted Alkyne Azide Cycloaddition chemistry as reported previously [7]. Briefly, starting with 0.42 mg (97 nmol, 1 equiv) of peptide 10F12M and 0.68 mg (427 nmol, 4.4 equiv) of Man_9cyclohexyl azide. HPLC purification (Waters Symmetry 300 C4, 5 µm, 10×250 mm, 4 mL/min, 10-45% MeCN in H_2O with 0.1% formic acid, over 60 min, retention time 15.4 min) to afford 0.28 mg of pure glycopeptide 10F12M-Man_9 (quantified by BCA assay), corresponding to 22% yield. LR ESI-MS: observed average m/z of multiply charged ions 1345.70 $[M+8H]^{8+}$, 1537.80 $[M+7H]^{7+}$, corresponding to 10759.76 observed average mass, calculated average mass for $C_{435}H_{715}N_{71}O_{235}S_2$: 10763.80.

Synthesis of BSA-maleimide

BSA (Fisher BioReagents, BP9706-100) (0.7 mg, 10.5 nmol) was dissolved in 0.63 mL of PBS buffer (pH 7.5). SM-PEG4-NHS (0.7 mg, 1.36 µmol, 130 equiv) was added to the solution to have the final concentration of 1 mg/mL. The reaction stood at room temperature for 30 min. Excess SM-PEG_4-NHS was removed by buffer exchanged through an Amicon centrifugal filter (30 kDa cutoff, Ultra-0.5, 4 rounds of dilution with PBS pH 6.5). The molecular weight of the activated BSA-maleimide was determine by MALDI-TOF MS analysis. A 6991.6-dalton average mass increase indicated an average of 17.5 linkers. A yield of 0.72 mg of activated BSA-maleimide was obtained based on BCA assay.

General conjugation procedure: CRM-g10V1S, BSA- peptide10V1S, g10V1S, g10F2, g10F5M, g10F6, g10F8, 10F12M-Man_3 branched, 10F12M-Man_3 linear, 10F12M-Man_4, 10F12M-Man_5, 10F12M-Man_9, 10F12M-$Man_9GlcNAc_2$

Conjugations to CRM197 were performed according to previously reported methods [7]. For 10V1S, CRM197 from Reagent Proteins (expressed in *Pseudomonas fluorescens*) was used. For other glycopeptides, ecoCRM197 from Fina Biosciences (expressed in *E. coli*) was used; this was identical to the Reagent Proteins CRM197 by MALDI-TOF mass spectrometry and SDS PAGE, and provided similar glycopeptide-specific titers. Briefly, g10V1S (2.9 mg, 310 nmol) in 150 µL of water was treated with 12.31 µL of 500 mM TCEP·HCl/ 1M Tris-HCl buffer (pH 7.8, 20 equiv). Complete deprotection of the cysteine was confirmed by UPLC-ESI-MS after the reaction stood overnight at room temperature under N_2. Excess TCEP was removed by buffer-exchanged through an Amicon centrifugal filter (3-kDa cutoff, Ultra-0.5, 20 min in the first round of filtration, and 30 min for the second round) with PBS buffer (pH 6.5). The deprotected glycopeptide was added to the freshly made CRM_{197}-maleimide [7] (1.7 mg quantified by BCA, 27.5 nmol, ~22 average linkers/CRM) in PBS buffer (pH 6.5). The solution stood overnight under N_2 at room temperature. The CRM_{197}-glycopeptide 10V1S conjugate was purified by using an Amicon centrifugal filter (30 kDa cutoff, Ultra-0.5, 5 min for at least 4 rounds of filtration) to remove salts and unreacted glycopeptide. MALDI-TOF MS analysis indicated the distribution of conjugates with an average loading of 8. The CRM_{197}-glycopeptide 10V1S conjugates were capped with β-mercaptoethanol (.29µL neat, .32 mg, 4.1 µmol, 100 equiv, 1h) in 1 mL PBS (pH 6.5), purified and buffer-exchanged to water by Amicon centrifugal filter (30 kDa cutoff, Ultra-0.5, 5 min for at least 4 rounds of filtration). BCA quantification assay (corrected to add carbohydrate

content)[7] indicated 4 mg of CRM_{197}-glycopeptide 10V1S conjugate (containing 2.2 mg of glycopeptide antigen). BSA conjugates of g10V1S, g10F2, g10F5M, g10F6, g10F8,10F12M-Man_3 branched, 10F12M-Man_3 linear, 10F12M-Man_4, 10F12M-Man_5, 10F12M-Man_9, and 10F12M-$Man_9GlcNAc_2$ were prepared in an analogous manner with the lower-density BSA-maleimide reported above, yielding 72.6 µg BSA-g10V1S, 305 µg BSA-g10F2, 265 µg BSA-g10F5M, 282 µg BSA-g10F6, 263 µg BSA-g10F8, 54 µg BSA-10F12M-Man_3 branched, 48 µg BSA-10F12M-Man_3 linear, 62 µg BSA-10F12M-Man_4, 69 µg BSA-10F12M-Man_5 , 64 µg BSA-10F12M-Man_9 , and 49 µg BSA-10F12M-$Man_9GlcNAc_2$, all with average loadings of ~4.

Conjugation procedure (6M guanidine PBS): BSA-peptide 10F2, 10F5M, 10F8

Peptides 10F2, 10F5M, 10F8 were each dissolved in 0.5% acetic acid for quantification by BCA assay, and appropriate aliquots of appropriate size for the reaction below were lyophilized and redissolved in 6M guanidine PBS buffer for the conjugation. Generally, capping was performed with slightly substoichiometric BME to avoid driving the reversible loss of glycopeptides from the conjugate.

Representative procedure for 10F5M: Peptide 10F5M (0.143 mg, 33 nmol) was dissolved in 230 µl 6M guanidine PBS buffer (pH 6.5). TCEP (0.6 µL of 500 mM aqueous solution, 9 equiv) was added and the mixture stood under N_2 overnight, after which time UPLC-ESI-MS indicated complete deprotection of the cysteine. Excess TCEP was removed by Amicon centrifugal filter (3-kDa cutoff, 2 rounds of filtration: 20 min and then 30 min) with using 6M guanidine PBS buffer (pH 6.5). The deprotected peptide (50 µL volume) was added to activated BSA-maleimide (0.1 mg, 1.31 nmol, ~25 maleimide linkers, in 85 µL of the same buffer) and the solution stood overnight at room temperature under N_2. The BSA-peptide 10F5M conjugate was purified by Amicon centrifugal filter (30-kDa, 4 rounds, 5 min each round) with water to remove salts and unreacted peptide. MALDI-TOF MS analysis indicated an average loading of 5. The BSA-peptide 10F5M was then diluted to 100 µL PBS/Guanidine and capped with mercaptoethanol (2.6 µL of 10mM solution in PBS pH 6.5, 20 equiv., 1h). The conjugate was then purified and buffer-exchanged with water using 30K Amicon filter (4 rounds: 5 min). BCA quantification assay indicated a yield of 72.5 µg of the BSA-peptide 10F5M conjugate. BSA conjugates of 10F2, 10F5M, and g10F8 were prepared in an analogous manner with the lower-density BSA-maleimide reported above, yielding, 154 µg 10F2-BSA, 72.5 µg 10F5M-BSA, and 34.4 µg 10F8-BSA, all with average loadings of ~4.

Conjugation procedure (0.5% acetic acid PBS): BSA-10F12M, 10F12M-cyclohexanol, 10F12M-Man_1, 10F12M-Man_2

HPLC-purified lyophilized 10F12M peptide was dissolved in 10% acetic acid and diluted to 0.5% acetic acid solution before quantification by BCA assay (1.26 mg/mL result). This peptide 10F12M solution (260µL, 0.129 mg, 29.6 nmol) was transferred to a 0.5 mL low protein binding Eppendorf tube which was placed in a two-neck flask flushed with nitrogen. TCEP (0.6 µL of 500 mM aqueous solution, 10 equiv) was added and the solution was incubated under N_2 atmosphere in a water bath at either 45˚C overnight or 60˚C for 7 hours, after which time UPLC-ESI-MS showed complete deprotection of the cysteine. Sometimes, another 10 equiv of TCEP was needed for the deprotection to go to completion. Excess TCEP was removed by Amicon centrifugal filter (3-kDa cutoff, 2 rounds of filtration: 20 min and then 30 min) with 0.5% acetic acid in PBS buffer (pH 5). The deprotected peptide (50 µL volume) was added to the activated BSA-maleimide (0.125 mg, 1.7 nmol, 17.4 maleimide linkers, in 101 µL of the same buffer) and the solution stood under N_2 atmosphere overnight at room temperature. The BSA-peptide conjugates were then purified by Amicon centrifugal filter (30-kDa, 4 rounds, 5 min each round) with 0.5% AcOH/H_2O to remove salts and unreacted peptide. The conjugates were capped with mercaptoethanol (1.4 µL of 10 mM solution, 8 equiv, 1h), then purified using 3K Amicon centrifugal filter with 0.5% AcOH/ H_2O (4 rounds: 5 min each round). BCA assay indicated 0.123 mg of BSA-peptide 10F12M conjugate. BSA conjugates of 10F12M-cyclohexanol, 10F12M-Man_1, and 10F12M-Man_2(1-3), and 10F12M-Man_2(1-6) were prepared in an analogous manner, yielding 84 µg BSA-10F12M-cyclohexanol, 52 µg BSA-10F12M-Man_1, 50 µg BSA-10F12M-Man_2(1-3), 17µg BSA-10F12M-Man_2(1-6), all with average loadings of ~4.

Conjugation procedure (6M guanidine and 1% acetic acid): BSA-peptide 10F6

HPLC-purified lyophilized 10F6 (2.2 mg) was dissolved in 10% acetic acid and diluted to 0.5% acetic acid solution (0.5 ml) before quantification by BCA assay (1.797 mg/mL result). Solution containing 27 µg of peptide 10F6 (15 µL) was transferred to a 0.5 mL low protein binding Eppendorf tube which was lyophilized. The lyophilized peptide 10F6 was then dissolved in 50 µL 6M guanidine PBS buffer (pH 6.5) and placed in a two-neck flask flushed with nitrogen. TCEP (0.6 µL of 100 mM aqueous solution, 10 equiv) was added and the solution stood overnight at room temperature after which time UPLC-ESI-MS showed complete deprotection of the cysteine. Excess TCEP was removed by Amicon centrifugal filter (3-kDa cutoff, 2 rounds of filtration: 20 min and then 30 min) with 6M guanidine in PBS buffer (pH 6.5). The deprotected peptide was added to the activated BSA-maleimide (0.05 mg, 0.68 nmol, 17.7 maleimide linkers, in 42.4 µL of the same buffer) and the solution stood overnight at room temperature under N_2. The peptide-BSA conjugates were purified by Amicon centrifugal filter (30-kDa, 4 rounds, 5 min each round) with 1% AcOH/ H_2O to remove salts and unreacted peptide. The conjugate was then capped with mercaptoethanol (0.68 µL of 10 mM solution, 10 equiv, 1h), and purified using 30K Amicon filter with 1% AcOH/ H_2O (4 rounds: 5 min each round). BCA quantification assay indicated a yield of 34.4 µg of the BSA-peptide 10F6 conjugate.

Synthesis of cyclohexanol-azide

Cyclohexanol-azide was synthesized by previously reported method [8]. Briefly, imidazole-sulfonyl-azide·HCl (0.52 mmol, 109 mg) was added to a solution of 4-aminocylohexanol (0.434 mmol, 50 mg), $CuSO_4$ (4.35 µmol, 1.1 mg), and K_2CO_3 (0.478 mmol, 66 mg) in 2 mL MeOH. The mixture was stirred overnight, and then concentrated in vacuo. Water (5 mL) and concentrated HCl (0.25 mL) were then added to the residue and the mixture was extracted with 10 mL of EtOAc for 3 times. The organic layer was washed 3 times with brine (10 mL), dried over $MgSO_4$, and concentrated. The crude was purified by flash column chromatography (1:1 hexane/ EtOAc). Product-containing fractions were combined and dried under vacuum for 1.5 h, affording 34 mg of pure product, corresponding to 55% yield.

Pilot rabbit study

Three groups of three female New Zealand White rabbits were used to test the dose response to CRM_{197}-glycopeptide 10V1S conjugates. Each group was immunized subcutaneously with CRM-g10V1S conjugate containing either 10µg, 50 µg or 100 µg antigen formulated in 50µL Adjuplex adjuvant, 4 times at 4-week intervals and blood was collected 2 weeks after each immunization. A prebleed was collected just before the first immunization.

Multi-immunogen rabbit study

Six groups of six female New Zealand White rabbits were used for the multi-immunogen study. Group 1 rabbits as a control group received subcutaneous immunizations of 50 µg CRM_{197}-maleimide (with BME cap) and 50 µL Adjuplex. Group 2,3,4,5 were immunized with CRM_{197}-glycopeptide 10F5M, CRM_{197}-glycopeptide 10F2, CRM_{197}-glycopeptide 10F6, CRM_{197}-glycopeptide 10F8, respectively, each containing 50 µg of respective glycopeptides per dose. Group 6 received sequential immunizations of 10F5M, 10F2, 10F6 and 10F8 glycopeptide conjugates with adjuvant as above. Four immunizations were performed at 4-week intervals and blood was collected 2 weeks after each immunization, with a pre-bleed just before the first immunization. All rabbits received 2 booster injections of 50 µg BG505.SOSIP.664 (T332N) and 50 µL Adjuplex. Throughout the study, 5 animals (roughly one per group) died of unknown causes with no obvious relation to the immunizations.

ELISA analysis

High-protein-binding flat-bottomed Maxisorp ELISA plates (Nunc-Immuno) were coated with 120 ng/mL antigen in coating buffer (50 mM carbonate/bicarbonate buffer, pH 9.6, 100 µL/well) and incubated at 4 °C overnight. The

wells were washed twice with PBS-0.05% Tween 20 (PBS-T) and then blocked for 1 h at room temperature with 5% fat-free milk PBS-T (200 µL/well). After washing again twice with PBS-T, the wells were then incubated with either 3-fold or 4-fold serial dilutions of rabbit serum (starting at different concentrations: either 1:10 or 1:100) in 1% fat-free milk in PBS-T for 2 h at room temperature. The wells were washed 3 times before incubating with 100 µL of a horseradish peroxidase (HRP) conjugated sheep anti-rabbit antibody (Novex, part number A16172) at 1: 10,000 dilutions for 1 h at room temperature. After 3 washes, the wells were developed by adding 100 µL of 3,3′,5,5′-tetramethylbenzidine (TMB solution, Abcam Ab171522) for 3 min. The reaction was stopped by adding 100 µL of 1 M sulfuric acid and absorbance was measured at 450 nm wavelength. All measurements were performed in triplicate.

Synthesis of CRM_{197}-AcBr

CRM_{197} (1.0 mg, 17 nmol) was dissolved in 0.9 mL of PBS buffer (pH 7.5). Bromoacetamido-PEG_4-NHS ester (BroadPharm, BP-20569, $BrCH_2(CO)NH(CH_2CH_2O)_4$-$(CH_2)_2$-CO-NHS) (1.2 mg, 2.5 µmol, 147 equiv) was added to the solution to have the final concentration of 1 mg/mL. The reaction stood at room temperature for 1.5 hours. Excess bromoacetamido-PEG_4-NHS ester was removed by buffer exchange through an Amicon centrifugal filter (30 kDa cutoff, Ultra-0.5, 4 rounds of dilution with PBS pH 8.5). The molecular weight of the activated BSA-bromoacetamido (BSA-AcBr) was determined by MALDI-TOF MS analysis. A 6283.8-dalton average mass increase indicated an average of 17 linkers. A yield of 1.1 mg of activated BSA-AcBr was obtained based on BCA assay.

Synthesis of Alkyne-CRM_{197}-AcBr

Alkyne-PEG_5-NHS (Sigma-Aldrich, catalog # 764191, $HCC-CH_2-(OCH_2CH_2)_5$-CO-NHS) (0.4 mg, 0.93 µmol, 200 equiv) was added to the solution of CRM_{197}-AcBr (0.3 mg, 4.6 nmol) in 167 µL of PBS buffer (pH 7.5) to a final concentration of 1 mg/mL. The reaction stood at room temperature for 1.5 h. Excess alkyne-PEG_5-NHS was removed by buffer exchange through an Amicon centrifugal filter (30 kDa cutoff, Ultra-0.5, 4 rounds of dilution with PBS pH 8.5). The molecular weight of alkyne-CRM_{197}-AcBr was determined by MALDI-TOF MS analysis. A 1012.7-dalton average mass increase indicated an average of 3.5 alkyne linkers. A yield of 0.25 mg of alkyne-CRM_{197}-AcBr was obtained based on BCA assay.

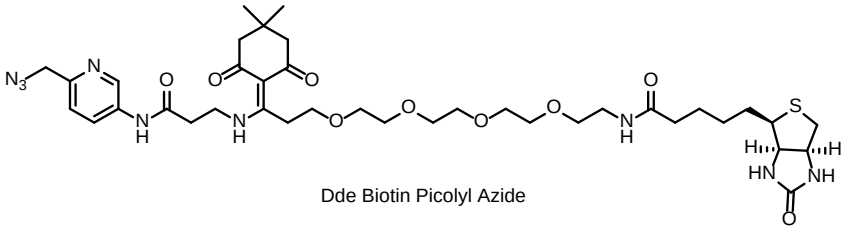
Synthesis of alkyne-CRM_{197}-Ac-glycopeptide 10F6-Man_9-cyclohexyl

Glycopeptide 10F6 (0.202 mg, 16.2 nmol) in 96 µL of water was treated with 1.62 µL of 100 mM TCEP·HCl/ 1M Tris-HCl buffer (pH 7.8, 10 equiv). Complete deprotection of the cysteine was confirmed by UPLC-ESI-MS after the reaction stood overnight at room temperature under N_2. Excess TCEP was removed by buffer-exchange through an Amicon centrifugal filter (3-kDa cutoff, Ultra-0.5, 20 min in the first round of filtration, and 45 min for the second round) with PBS buffer (pH 8.5). The deprotected glycopeptide was added to the freshly-made alkyne-CRM_{197}-AcBr (0.125 mg quantified by BCA, 1.9 nmol, ~17 average AcBr linkers/CRM_{197}) in PBS buffer (pH 8.5). The solution stood overnight in the dark under N_2 at room temperature. The alkyne-CRM_{197}-glycopeptide 10F6 conjugate was purified using an Amicon centrifugal filter (30 kDa cutoff, Ultra-0.5, 5 min for at least 4 rounds of filtration) to remove salts and unreacted glycopeptide. MALDI-TOF MS analysis indicated the distribution of conjugates with an average loading of 3. The alkyne-CRM_{197}-glycopeptide 10F6 conjugates were capped with β-mercaptoethanol (2.28 µL from 10 mM stock, 22.8 nmol, 12 equiv, 1h) in 125 µL PBS (pH 8.5), purified and buffer-exchanged to water by Amicon centrifugal filter (30 kDa cutoff, Ultra-0.5, 5 min for at least 4 rounds of filtration). BCA quantification assay (corrected to add carbohydrate content) indicated 0.162 mg of alkyne-CRM_{197}-glycopeptide 10F6 conjugate.

***In vitro* trimming of CRM_{197}-glycopeptide 10F6 conjugates using rabbit serum**

Alkyne-CRM-Ac-g10F6 conjugate (30 µg) was added to five 1.5 mL Eppendorf tubes, each containing 0.3 mL of rabbit serum. The serum mixtures were incubated at 37˚C and the reaction was stopped at different time points (4, 17, 24, 48 h) by adding two mannosidase inhibitors (kifunensine and swainsonine, Santa Cruz Biotechnology, catalog # sc-201364 and sc-201362, respectively) directly to the serum mixture to obtain a 5 µg/mL concentration.

A 0 hr timepoint was generated by adding conjugate to serum already containing inhibitors. The quenched reactions were immediately frozen and stored at -80˚C until the last time point sample was obtained.



Dde Biotin Picolyl Azide

Next, Dde-biotin-picolyl-azide (Click Chemistry Tools, catalog # 1186-5) (25 µL from 10 mM stock in DMSO) and THPTA (50 µL from 100 mM stock in PBS buffer pH 7.5) were added to the serum mixtures. The mixtures were then vortexed briefly to mix. Next, $CuSO_4$ $5H_2O$ (50 µL from 20 mM stock in PBS buffer) was added and mixed briefly. Lastly, sodium ascorbate (50 µL from 300 mM stock in PBS buffer) was added and also vortexed to mix. The click reactions were covered with aluminum foil to protect from light and they were stood for 2h at room temperature. Excess Dde-biotin-picolyl-azide linker was removed by buffer exchange through a 30K Amicon filter (6 rounds of filter, 10 min each round). The clicked CRM-Ac-g10F6 conjugates were incubated with 100 µL of NeutrAvidin resin (ThermoFisher, catalog # 29200) in 0.8 mL centrifuge columns for 1.5 h at room temperature. The NeutrAvidin resin was then washed 4 times with 200 µL water. Then, the washed resin was incubated in elution buffer (2% v/v hydrazine solution) for 1h at room temperature and washed 4 times with 200 µL water. The eluant and all the washes were combined and buffer exchanged by 30K Amicon filter with water. The recovered CRM-Ac-g10F6 conjugates were concentrated and lyophilized.

Identification and quantification of intact and trimmed glycoforms of the tryptic glycopeptides from g10F6 by mass spectrometry (MS) analysis:

To determine the presence of G10F6 trimmed glycoforms resulting from serum mannosidase activity on the G10F6 glycopeptide, a pure G10F6-CRM (standard) sample and the five time-point serum-incubated samples (0, 4, 17, 24, and 48 hr) described above were analyzed the same way, as described below. Prior to mass spectrometry analysis, the samples were reduced, alkylated, trypsin digested, SP-C18 desalted and concentrated. Tryptic digestion would be expected to generate two glycopeptides (formyl**X**L**X**FIR and **X**QYVYHAPLLT**X**VR), each containing two homopropargylglycine residues with triazole cyclohexyl linker and glycan attached (**X**).

Three types of MS experiments were performed on each sample: a glycopeptide identification focus nanoUPLC-MS/MS [HCD (Higher Energy Collisional Dissociation (HCD) NCE (Normalized Collision Energy) experiment at 45 v, a glycan composition focus nanoUPLC-MS/MS (HCD NCE) experiment at 15 v, and a relative quantification focus nanoUPLC-MS-only experiment. Each dried peptide/glycopeptide mixture was resuspended in 40 µL of Mobile Phase A (1% ACN/0.1% FA/Water). A one-µL aliquot was analyzed in each MS experiment. The aliquot was injected into a nanoAcquity UPLC M-Class (Waters) equipped with reversed phase columns: nanoEase™ M/Z Symmetry C18, 100 Å, 5 µm, 1/PK 180 µm x 20 mm, trap column and nanoEase™ M/Z HSS C18 T3 Col 100 Å, 1.8 µm, 1/PK 75 µm x 100 mm, analytical column (Waters). The nano-UPLC was connected online to an Orbitrap Fusion Lumos Tribid Mass Spectrometer (Thermo Scientific) equipped with a Triversa NanoMate (Advion) electrospray ionization (ESI) source operated at 1.7 kV, in order to generate a constant nanoESI plume. The sample was loaded onto the precolumn, washed for 4 min at a flow of 4 µL/min with 100% Mobile Phase A/% (1% ACN/0.1% FA/Water). After the trapping event, the peptides were eluted to the analytical column and resolved by a gradient of 3-40% mobile phase B (1% Water/0.1% FA/ACN) delivered over 40 min at a flow rate of 500 nL/min.

The data for all MS acquisition experiments were acquired as follows: the mass spectrometer was operated in positive ion mode and the Orbitrap analyzer was used as the detector for all scan events; all data were acquired in Profile mode. The sample ions were introduced into the mass spectrometer (MS) through an Ion Transfer Tube operated at 300 °C. To minimize in-source fragmentation, the source RF Lens was operated at 5%. Data from the MS tandem experiments were acquired with the following scan event parameters: The MS^1 scan was set at a resolution of 120,000 @ *m/z* 200, over the full scan range *m/z* 350-1500, 1 µscan/spectrum, maximum injection time (ion accumulation time) of 50 ms with a target automatic gain control (AGC) of 4×10^5 ion population. The following filters were applied to the data dependent acquisition scan events: Monoisotopic Peak Determination was set to Peptide; Charge States included 2-7, the Dynamic Exclusion was set to exclude after 1 time, for a duration of 10 s with a ± 10 ppm window, Excluding Isotopes was set to True; the Intensity Threshold was set to 4×10^4; the Data Dependent Mode was set to Cylce Time (Top Speed Methodology) with a Master Scan every 3 s. The MS^2 scan event used the Quadrupole for Isolation Mode, with an Isolation Window of *m/z* 1.6; the activation used was HCD at 45 % for the glycopeptide identification focus experiment or 15% for the glycan composition focus experiment. The MS^2 scan range was set to Auto with *m/z* range set to High; the first mass was fixed to *m/z* 100; the AGC target was set to 5.5×10^5 ion population and maximum injection time of 150 ms; 2 µscan/spectrum.

The relative quantification focus MS-only experiment MS^1 scan followed the same parameters of the tandem MS^1 scan events in the tandem methods, the only difference being that the MS scan range was set as *m/z* 350-2000. The glycopeptides were identified by manually assigning peptide backbone and glycan loss peaks in the HCD 45% tandem data; the glycan compositions of such glycopeptides were confirmed in the HCD 15% tandem data. To quantify the relative abundances of the g10F6 glycopeptides' glycoform distributions (the full [Man_9] and trimmed glycoform versions) for the standard sample (pure glycopeptide-CRM conjugate) and all time point samples (serum-treated samples) the area under the chromatographic peak corresponding to each glycopeptide precursor ion was calculated by the Thermo Scientific Xcalibur's Qual Browser Software, using the extracted ion chromatogram for each observed charge state. The extracted areas were normalized by charge ($Total\ Area = \sum z\ (Area/z)$). The percentage for each chromatographically resolved Man_n glycoform in the standard sample were calculated and then subtracted from the total signal observed for the corresponding Man_n glycoform at each time point. The values were also corrected to take into account a small amount of in-source fragmentation (~4-5%) observed for the standard. This corrected amount was then calculated as a percentage distribution and plotted with Excel. The results are shown in Table S10.

LC/MS characterization data of peptides and glycopeptides:

Peptide 10V1S

Peptide 10V1S was synthesized according to previously reported methods [7]. Briefly, starting with 85 mg of trityl ChemMatrix® resin loaded with 0.3 mequiv/g alanine (26 µmol scale), 50 mg of crude peptide 10V1S was obtained. HPLC purification (Waters Symmetry 300 C4, 5 µm, 10×250 mm, 4 mL/min, 10-45% MeCN in H_2O with 0.1% formic acid, over 60 min, retention time, t_R = 30.8 min) of 14 mg of crude peptide yielded 2 mg of pure 10V1S, corresponding to 6 % overall yield if all crude peptide had been purified. LR ESI-MS: observed average m/z of multiply charged ions 1160.67 $[M+4H]^{4+}$, 1547.78 $[M+3H]^{3+}$, corresponding to 4639.51 observed average mass, calculated average mass for $C_{206}H_{313}N_{59}O_{60}S_2$: 4640.18.

Glycans were "clicked" to peptides according to the procedure in Ref. 7.

Glycopeptide 10V1S

Starting with 4.7 mg (1.1 µmol, 1 equiv) of peptide 10V1S and 6.2 mg (3.9 µmol, 3.6 equiv) of Man_9cyclohexyl azide. LR ESI-MS: observed average m/z of multiply charged ions 1181.26 $[M+8H]^{8+}$, 1349.52 $[M+7H]^{7+}$, 1574.38 $[M+6H]^{6+}$, 1888.93 $[M+5H]^{5+}$, corresponding to 9440.41 observed average mass, calculated average mass for $C_{386}H_{616}N_{680}O_{198}S_2$: 9441.49. RP-HPLC purification (Waters Symmetry 300 C4, 5 µm, 10×250 mm, 4 mL/min, 2-42% MeCN in H_2O with 0.1% formic acid, 60 min, retention time, t_R = 30.7 min) afforded 3.9 mg of pure glycopeptide 10V1S, corresponding to 41 % yield.

Peptide 10F12M-cyclohexanol

Starting with 0.5 mg (115 nmol, 1 equiv) of peptide 10F12M and 0.89 mg (6.3 µmol, 55 equiv) of cyclohexanol azide. LR ESI-MS: observed average m/z of multiply charged ions 985.95 $[M+5H]^{5+}$, 1232.1 $[M+4H]^{4+}$, 1642.82 $[M+3H]^{3+}$, corresponding to 4924.87 observed average mass, calculated average mass for $C_{219}H_{355}N_{71}O_{55}S_2$: 4926.74. RP-HPLC purification (Waters Symmetry 300 C4, 5 µm, 10×250 mm, 4 mL/min, 10-45% MeCN in H_2O with 0.1% formic acid, 60 min, retention time, t_R = 26.6 min) afforded 0.13 mg of pure 10F12M-cyclohexanol, corresponding to 23 % yield.

Most glycopeptides 10F12M were quantified by BCA assay without correction factor accounting for the glycan weight, which resulted in lower yields than anticipated:

Glycopeptide 10F12M-Man_1

Starting with 0.5 mg (115 nmol, 1 equiv) of peptide 10F12M and 0.15 mg (504 nmol, 4.4 equiv) of Man_1cyclohexyl azide. LR ESI-MS: observed average m/z of multiply charged ions 930.06 $[M+6H]^{6+}$, 1115.97 $[M+5H]^{5+}$, 1394.34 $[M+4H]^{4+}$, corresponding to 5574.19 observed average mass, calculated average mass for $C_{243}H_{395}N_{71}O_{75}S_2$: 5575.30. RP-HPLC purification (Waters Symmetry 300 C4, 5 µm, 10×250 mm, 4 mL/min, 10-45% MeCN in H_2O with 0.1% formic acid, 60 min, retention time, t_R = 21.6 min) afforded 0.2 mg of pure glycopeptide10F12M-Man_1, corresponding to 31 % yield. Most 10F12M-derived glycopeptides were quantified by BCA assay without a correction factor to account for the glycan weight, which resulted in lower yields than anticipated.

Glycopeptide 10F12M-Man_2 (1-3)

Starting with 0.22 mg (51 nmol, 1 equiv) of peptide 10F12M and 0.10 mg (224 nmol, 4.4 equiv) of Man_2(1-3) cyclohexyl azide. LR ESI-MS: observed average m/z of multiply charged ions 1038.16 $[M+6H]^{6+}$, 1245.41 $[M+5H]^{5+}$, 1556.71 $[M+4H]^{4+}$, corresponding to 6222.62 observed average mass, calculated average mass for $C_{267}H_{435}N_{71}O_{95}S_2$: 6223.86. RP-HPLC purification (Waters Symmetry 300 C4, 5 µm, 10×250 mm, 4 mL/min, 10-45% MeCN in H_2O with 0.1% formic acid, 60 min, retention time, t_R = 20.4 min afforded 0.16 mg of pure glycopeptide 10F12M-Man_2(1-3), corresponding to 52 % yield.

Glycopeptide 10F12M-Man_2 (1-6)

Starting with 0.5 mg (115 nmol, 1 equiv) of peptide 10F12M and 0.23 mg (504 nmol, 4.4 equiv) of Man_2(1-6) cyclohexyl azide. LR ESI-MS: observed average m/z of multiply charged ions 1038.47 $[M+6H]^{6+}$, 1245.79 $[M+5H]^{5+}$, 1556.64 $[M+4H]^{4+}$, corresponding to 6223.78 observed average mass, calculated average mass for $C_{267}H_{435}N_{71}O_{95}S_2$: 6223.86. RP-HPLC purification (Waters Symmetry 300 C4, 5 µm, 10×250 mm, 4 mL/min, 10-45% MeCN in H_2O with 0.1% formic acid, 60 min, retention time, t_R = 20.3 min) afforded 0.13 mg of pure glycopeptide 10F12M-Man_2(1-6), corresponding to 18 % yield.

Glycopeptide 10F12M-Man_3 (linear)

Starting with 0.61 mg (140 nmol, 1 equiv) of peptide 10F12M and 0.39 mg (616 nmol, 4.4 equiv) of Man_3cyclohexyl azide. LR ESI-MS: observed average m/z of multiply charged ions 1146.25 $[M+6H]^{6+}$, 1375.50 $[M+5H]^{5+}$, 1718.75 $[M+4H]^{4+}$, corresponding to 6871.67 observed average mass, calculated average mass for $C_{291}H_{475}N_{71}O_{115}S_2$:

6872.43. RP-HPLC purification (Waters Symmetry 300 C4, 5 µm, 10×250 mm, 4 mL/min, 10-45% MeCN in H_2O with 0.1% formic acid, 60 min, retention time, t_R = 18.9 min) afforded 0.8 mg of pure glycopeptide 10F12M-Man_3 (linear), corresponding to 83 % yield.

Glycopeptide 10F12M-Man_3 (branched)

Starting with 0.5 mg (115 nmol, 1 equiv) of peptide 10F12M and 0.32 mg (504 nmol, 4.4 equiv) of Man_3cyclohexyl azide. LR ESI-MS: observed average m/z of multiply charged ions 1146.37 $[M+6H]^{6+}$, 1375.69 $[M+5H]^{5+}$, 1718.61 $[M+4H]^{4+}$, corresponding to 6872.04 observed average mass, calculated average mass for $C_{291}H_{475}N_{71}O_{115}S_2$: 6872.43. RP-HPLC purification (Waters Symmetry 300 C4, 5 µm, 10×250 mm, 4 mL/min, 10-45% MeCN in H_2O with 0.1% formic acid, 60 min, retention time, t_R = 18.6 min) afforded 0.12 mg of pure glycopeptide 10F12M-Man_3(branched), corresponding to 15 % yield.

Glycopeptide 10F12M-Man_4

Starting with 0.5 mg (115 nmol, 1 equiv) of peptide 10F12M and 0.45 mg (573 nmol, 5 equiv) of Man_4cyclohexyl azide. LR ESI-MS: observed average m/z of multiply charged ions 1254.41 $[M+6H]^{6+}$, 1505.07 $[M+5H]^{5+}$, 1881.04 $[M+4H]^{4+}$, corresponding to 7520.32 observed average mass, calculated average mass for $C_{315}H_{515}N_{71}O_{135}S_2$: 7520.99. RP-HPLC purification (Waters Symmetry 300 C4, 5 µm, 10×250 mm, 4 mL/min, 10-45% MeCN in H_2O with 0.1% formic acid, 60 min, retention time, t_R = 18.4 min) afforded 0.32 mg of pure glycopeptide 10F12M-Man_4, corresponding to 37 % yield.

Glycopeptide 10F12M-Man_5

Starting with 0.5 mg (115 nmol, 1 equiv) of peptide 10F12M and 0.48 mg (504 nmol, 4.4 equiv) of Man_5cyclohexyl azide. LR ESI-MS: observed average m/z of multiply charged ions 1167.92 $[M+7H]^{7+}$, 1362.37 $[M+6H]^{6+}$, 1634.63 $[M+5H]^{5+}$, corresponding to 8168.27 observed average mass, calculated average mass for $C_{339}H_{555}N_{71}O_{155}S_2$: 8169.55. RP-HPLC purification (Waters Symmetry 300 C4, 5 µm, 10×250 mm, 4 mL/min, 10-45% MeCN in H_2O with 0.1% formic acid, 60 min, retention time, t_R = 16.7 min) afforded 0.35 mg of pure glycopeptide 10F12M-Man_5, corresponding to 37 % yield.

Glycopeptide 10F12M-$Man_9GlcNAc_2$

Starting with 0.5 mg (115 nmol, 1 equiv) of peptide 10F12M and 0.96 mg (504 nmol, 4.4 equiv) of Man_9cyclohexyl azide. LR ESI-MS: observed average m/z of multiply charged ions 1500.25 $[M+8H]^{8+}$, 1714.76 $[M+7H]^{7+}$, corresponding to 11995.16 observed average mass, calculated average mass for $C_{479}H_{787}N_{79}O_{267}S_2$: 11996.77. RP-HPLC purification (Waters Symmetry 300 C4, 5 µm, 10×250 mm, 4 mL/min, 10-45% MeCN in H_2O with 0.1% formic acid, 60 min, retention time, t_R = 14.7 min) afforded 0.2 mg of pure glycopeptide 10F12M- $Man_9GlcNAc_2$, corresponding to 14 % yield.

Glycan synthesis methods

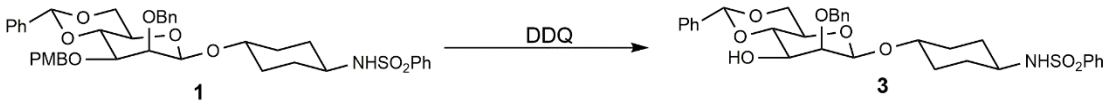
General synthetic methods for $Man_{1–5}$–Cy–N_3 preparation

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, DCM, Ethyl Ether and Pentane were deoxygenated by argon purging and dried by passage through activated alumina columns, then stored under argon gas only briefly before use. DriSolv Acetonitrile, DMSO and Methanol were purchased from EMD. Amines (Et_3N, iPr_2NEt, Pyridine, 2,6-Lutidine and 2,6-di-t-butylpyridine) were refluxed over CaH_2 and freshly distilled before use. Glassware was flame dried or dried in a 150 °C oven. For glycosylations, carbohydrate donors and acceptors were azeotropically dried by the following procedure: the intermediate was dissolved in dry toluene, the solution was

cooled to –78 °C, vacuum was applied, and the cooling bath was removed to allow the toluene to evaporate while the mixture warmed to room temperature. The flask was then backfilled with dry nitrogen and this procedure was repeated a total of three times. SiliCycle Siliaflash P60 silica was used for flash column chromatography. Analytical thin layer chromatography (TLC) was performed using SiliCycle glass backed plates (Cat# TLG-R10011B323). TLC plates were analyzed by short wave UV illumination, or by staining with dipping in cerium-ammonium-molybdate (CAM) stain (40 g of ammonium pentamolybdate, 1.6 g of cerium (IV) sulfate, 800 mL of diluted sulfuric acid (1:9, with water, v/v)) and heating on a hot plate. 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 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 (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublets, app d = apparent doublet, app t = apparent triplet). 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 equipped with photodiode array and Waters Micromass ZQ4000 mass detector (Column: Waters ACQUITY UPLC BEH® C18, 1.7 µm, 130Å, 2.1 x 50 mm. Waters ACQUITY UPLC BEH® HILIC, 1.7 µm, 130Å, 2.1 x 150 mm.). Optical rotation was measured using a Jasco digital polarimeter. Infrared spectra were obtained using a Nicolet IR200 spectrometer with a diamond ATR. DCM stands for dichloromethane, DDQ stands for 2,3–dichloro–5,6–dicyanobenzoquinone, DTBP stands for 2,6–di–tert–butylpyridine, EA stands for ethyl acetate, TES-H stands for triethylsilane, THF stands for tetrahydrofuran, NIS stands for N-Iodosuccinimide.

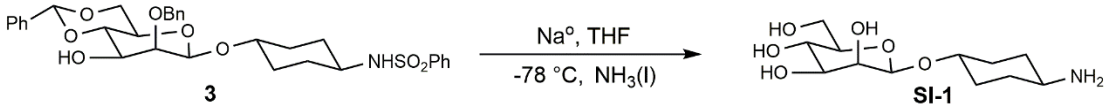
Synthesis of Man_1–Cy–N_3 (**5**)

PMB deprotected monosaccharide (**3**)



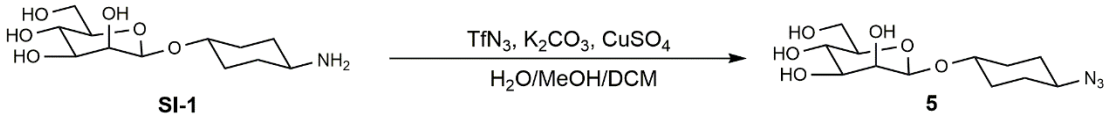
To a flask containing 120 mg (0.168 mmol, 1 equiv) of **1** [9-12] was added 1.8 mL of DCM and 0.1 mL of 1M phosphate buffer pH 7, then cooled to 0 °C, and 91 mg (0.40 mmol, 2.4 equiv) DDQ was added. This mixture was allowed to stir for 6 hours, and the reaction was quenched with aqueous $NaHCO_3$ solution. The mixture was diluted with DCM, and the organic phase was washed with water. The aqueous phase was extracted with DCM three times, and the combined organic layers were dried over $MgSO_4$, filtered, and concentrated. Purification by flash chromatography (1:1 ethyl acetate/hexanes) afforded 83 mg (0.14 mmol, 83%) of **3** as an off–white foam. ^{1}H NMR (400MHz, $CDCl_3$): δ 7.89 (app d, J = 7.2 Hz, 2H), 7.59 (app t, J = 7.6 Hz, 1H), 7.53 (app t, J = 8.0, 2H), 7.49 – 7.43 (m, 2H), 7.41 – 7.29 (m, 8H), 5.52 (s, 1H), 5.03 (app d, J = 11.8 Hz, 1H), 4.63 (app d, J = 11.8 Hz, 1H), 4.74 (s, 1H), 4.32 – 4.24 (m, 2H), 3.90 – 3.81 (m, 2H), 3.81 – 3.70 (m, 2H), 3.70 – 3.60 (m, 1H), 3.35 – 3.26 (m, 1H), 3.25 – 3.15 (m, 1H), 2.33 (app d, J = 8.6 Hz, 1H), 1.81 – 2.08 (m, 4H), 1.52 – 1.16 (m, 4H). MS (ESI+): calcd. for $C_{32}H_{38}NO_8S^+$ [M + H^+] 596.23, found 596.17.

Fully deprotected cyclohexyl linked monosaccharide amine (**SI-1**)



Along with a stream of N_2, ammonia gas was condensed against a -78 °C cold finger into a -78 °C-cooled 50 mL 3-necked flask equipped with glass-coated stir bar until 25 mL had accumulated. 70 mg (2.7 mmol, 40 equiv) Na metal was then added, and the resulting blue solution was left to stir for 1 hour. 40 mg (0.067 mmol, 1 equiv) of **3** in 4 mL of THF was then added. The reaction progress was monitored by LC–MS. After 6 hours, 165 mg (3.09 mmol, 46 equiv) NH_4Cl was added to quench the reaction. At this time, the cooling bath was removed and the ammonia was blown off under a stream of N_2. The resulting white solids were dissolved in water and desalted by passage through a Biorad P-2 Biogel (Fine) size exclusion column to give 16 mg of partially purified **SI-1**. ^{1}H NMR (400MHz, D_2O): δ 4.78 (s, 1H), 3.98 – 3.86 (m, 2H), 3.86 – 3.76 (m, 1H), 3.71 (dd, J = 12.5, 6.4 Hz, 1H), 3.63 (app d, J = 9.7 Hz, 1H), 3.56 (app t, J = 9.6 Hz, 1H), 3.35 (app t, J = 6.8 Hz, 1H), 3.24 – 3.10 (m, 1H), 2.29 – 1.99 (m, 4H), 1.63 – 1.26 (m, 4H). ^{13}C NMR (100 MHz, D_2O): δ 100.78, 79.06, 78.77, 75.88, 73.86, 69.62, 63.84, 51.94, 33.16, 31.86, 31.05, 30.90. IR (cm^{-1}): 3178, 2943, 1645, 1062. MS (ESI+): calcd. for $C_{12}H_{24}NO_6^+$ [M + H^+] 278.16, found 277.88. $[\alpha]_D$ (c 1.0, H_2O, –8.5).

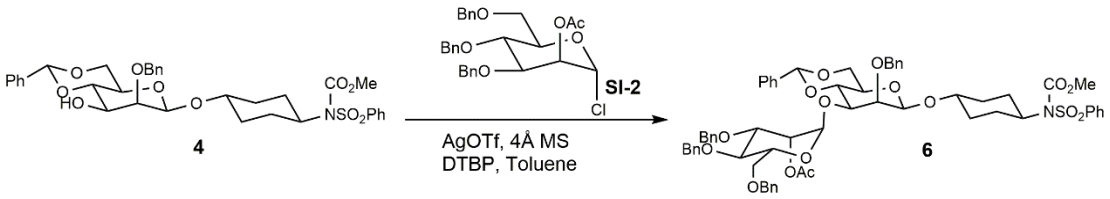
Cyclohexyl linked monosaccharide azide (**5**, Man_1–Cy–N_3)



Into a 20 mL vial containing 10 mg (0.036 mmol, 1.0 equiv) of crude amine **SI-1** was added 0.16 mL water, 150 µL (0.030 mmol, 0.8 equiv) of 0.2M aqueous $CuSO_4$ and 15 mg (0.11 mmol, 3.0 equiv) of K_2CO_3. 1 mL of MeOH was added, followed by the addition of 1.4 mL (0.36 mmol, 10.0 equiv) of freshly prepared 0.25M TfN_3[13] in DCM. The resulting homogeneous reaction was left to stir at room temperature for 3 hours until complete conversion was observed by LC–MS. The reaction was quenched with 30 mg (0.36 mmol, 10.0 equiv) solid $NaHCO_3$ and concentrated in vacuo. The residue was desalted on a Biorad P-2 Biogel (Fine) size exclusion column. Reversed phase HPLC purification (Column: Waters Xbridge Prep, C_{18}, 5µm, 130Å, 10x250mm. Method: 4mL/min flow rate. A= H2O/0.1%FA, B= ACN/0.1%FA. 98.2% A for 1min, then 98.2% A to 60% A over 24 minutes, then 60% to 5% A over 5 minutes. Desired product eluted at 17.9 minutes.) providing 3.5 mg (0.012 mmol, 18%, 2 steps) of Man_1–Cy–N_3 (**5**) as a glassy solid. ^{1}H NMR (400MHz, D_2O): δ 4.80 (s, 1H), 3.98 – 3.89 (m, 2H), 3.87 – 3.78 (m, 1H), 3.73 (dd, J = 12.3, 6.2 Hz, 1H), 3.64 (dd, J = 9.6, 3.1 Hz, 1H), 3.57 (app t, J = 10.4 Hz, 1H), 3.54 – 3.44 (m, 1H), 3.40 – 3.32 (m, 1H), 2.13 – 1.95 (m, 4H), 1.56 – 1.31 (m, 4H). ^{13}C NMR (100 MHz, D_2O): δ 100.71, 79.03, 78.93, 75.90, 73.90, 69.63, 63.84, 61.72, 32.99, 31.58, 31.31, 31.15. IR (cm^{-1}): 3338, 2935, 2863, 2091, 973. MS (ESI+): calcd. for $C_{12}H_{21}N_3NaO_6^+$ [M + Na^+] 326.13, found 325.87. $[\alpha]_D$ (c 0.1, H_2O, –21.0).

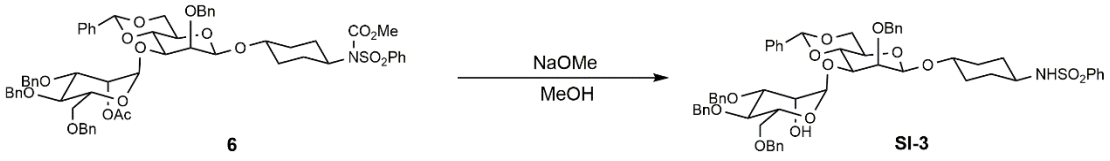
Synthesis of Man_2–Cy–N_3 (**7**)

Fully protected cyclohexyl linked disaccharide (**6**)



To a 25 mL flask containing 54 mg (0.083 mmol, 1 equiv) of **4** [9-12] and a 10 mL flask containing 64 mg (0.12 mmol, 1.5 equiv) of **SI-2** [9-12] was added toluene and freeze pumped for three times. 37 μL (0.16 mmol, 2 equiv) of DTBP and 100 mg freshly dried 4Å molecular sieves were added to the 25 mL flask. **4** was redissolved in 1 mL toluene and cooled in a –20 °C salt ice bath. **SI-2** was dissolved in 1 mL toluene and transferred *via* cannula to the 25 mL flask. The 25 mL flask was then wrapped in foil and 38 mg (0.15 mmol, 1.8 equiv) AgOTf was added. This was allowed to react for 3 hours, and the reaction was quenched with aqueous $NaHCO_3$ solution. The mixture was extracted with EA three times. The combined organic layers were dried over $MgSO_4$, filtered, and concentrated. Purification by flash chromatography (1:3 ethyl acetate/hexanes) afforded 55 mg (0.049 mmol, 59%) of **6** as an off–white foam. ^{1}H NMR (400MHz, $CDCl_3$): δ 7.92 (app d, J = 7.4 Hz, 2H), 7.61 (app t, J = 7.4 Hz, 1H), 7.52 (app t, J = 7.5 Hz, 2H), 7.48 – 7.41 (m, 2H), 7.41 – 7.35 (m, 2H), 7.36 – 7.09 (m, 21H + $CHCl_3$), 5.67 – 5.57 (m, 2H), 4.95 – 4.84 (m, 2H), 4.79 (app d, J = 12.3 Hz, 1H), 4.70 (app d, J = 11.2 Hz, 1H), 4.63 – 4.37 (m, 6H), 4.32 (dd, J = 10.6, 5.0 Hz, 1H), 4.19 (app t, J = 9.7 Hz, 1H), 4.00 – 3.86 (m, 3H), 3.85 – 3.55 (m, 10H), 3.37 – 3.25 (m, 1H), 2.36 – 2.15 (m, 3H), 2.01 (s, 3H), 2.00 – 1.91 (m, 1H), 1.90 – 1.78 (m, 2H), 1.64 – 1.46 (m + H_2O, 1H), 1.42 – 1.28 (m, 1H). ^{13}C NMR (100MHz, $CDCl_3$, selected signals): δ 169.95, 152.50, 140.28, 138.57, 138.27, 138.09, 137.82, 137.23, 133.34, 128.76, 128.72, 128.36, 128.30, 128.29, 128.21, 128.18, 128.11, 128.07, 127.87, 127.72, 127.64, 127.63, 127.56, 127.50, 125.91, 101.08, 100.05, 98.75, 78.63, 77.87, 77.67, 76.34, 75.14, 74.95, 74.88, 74.24, 73.32, 71.99, 71.59, 69.10, 68.47, 68.09, 67.31, 58.42, 53.42, 33.18, 31.48, 28.39, 20.98. IR (cm^{-1}): 2941, 2866, 1734, 1044, 1026, 735, 696. MS (ESI+): calcd. for $C_{63}H_{73}N_2O_{16}S^+$ [M + NH_4^+] 1145.47, found 1145.22. $[\alpha]_D$ (c 0.4, DCM, –24.2)

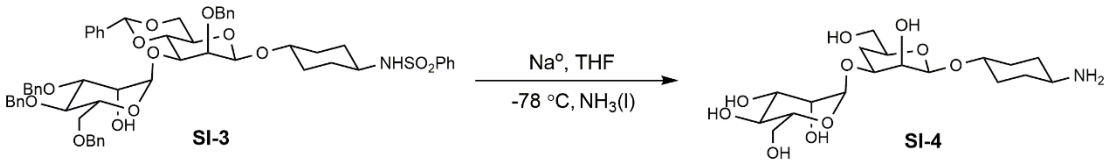
Partially deprotected cyclohexyl linked disaccharide (**SI-3**).



100 mg (0.0887 mmol, 1 equiv) of **6** was dissolved in 27 mL MeOH in a 50 mL flask. 1.25 mL (3.55 mmol, 40 equiv) of a 25% NaOMe solution in MeOH was added. The reaction was left stirring at room temperature under N_2 for 13 hours until reaction was complete based on LC–MS and TLC monitoring. Upon completion, the volume of the mixture was reduced to a half by rotary evaporation, then saturated NH_4Cl solution was used to quench the

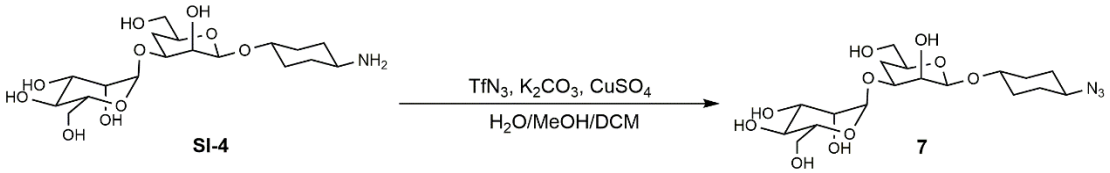
reaction. The mixture was extracted with DCM three times. The combined organic layers were dried over $MgSO_4$, filtered and concentrated. Purification by flash chromatography (1:1 ethyl acetate/hexanes) afforded 70 mg (0.068 mmol, 77%) of **SI-3** as an off–white foam. ^{1}H NMR (400MHz, $CDCl_3$): δ 7.91 (app d, J = 7.4 Hz, 2H), 7.58 (app t, J = 7.2 Hz, 1H), 7.52 (app t, J = 8.0 Hz, 2H), 7.45 (app d, J = 8.0 Hz, 2H), 7.42 – 7.12 (m, 23H + $CHCl_3$), 5.55 (s, 1H), 5.33 (s, 1H), 5.02 (app d, J = 7.4 Hz, 1H), 4.87 (app t, J = 11.5 Hz, 2H), 4.76 (app d, J = 12.3 Hz, 1H), 4.67 (app d, J = 11.6 Hz , 1H), 4.60 (app d, J = 11.6 Hz, 1H), 4.56 – 4.43 (m, 4H), 4.32 – 4.23 (m, 1H), 4.21 – 4.16 (m, 1H), 4.13 (app t, J = 9.6 Hz, 1H), 3.98 (dd, J = 10.1, 3.0 Hz, 1H), 3.94 – 3.61 (m, 7H), 3.56 – 3.45 (m, 1H), 3.36 – 3.25 (m, 1H), 3.21 – 3.08 (m, 1H), 2.48 (s, 1H), 2.01 – 1.89 (m, 1H), 1.87 – 1.67 (m, 3H), 1.47 – 1.33 (m, 1H), 1.29 – 1.10 (m, 3H). ^{13}C NMR (100MHz, $CDCl_3$, selected signals): δ 141.12, 138.46, 138.30, 138.26, 137.75, 137.32, 132.59, 129.13, 128.95, 128.51, 128.30, 128.29, 128.27, 128.21, 128.16, 127.92, 127.86, 127.70, 127.68, 127.58, 127.53, 126.87, 125.98, 101.46, 100.34, 100.01, 79.82, 78.63, 78.12, 75.66, 75.29, 74.89, 74.31, 73.35, 71.83, 69.29, 68.58, 67.96, 67.31, 51.58, 31.05, 30.77, 30.70, 29.27. IR (cm^{-1}): 3400, 3268, 2862, 1452, 1066, 734, 694. MS (ESI+): calcd. for $C_{59}H_{69}N_2O_{13}S^+$ [M + NH_4^+] 1045.45, found 1045.34. $[\alpha]_D$ (c 0.4, DCM, –22.0).

Fully deprotected cyclohexyl linked disaccharide amine (**SI-4**)



Along with a stream of N_2, ammonia gas was condensed against a -78 °C cold finger into a -78 °C-cooled 50 mL 3-necked flask equipped with a glass stir bar, until 25 mL had accumulated. 94 mg (4.1 mmol, 60 equiv) Na metal was then added, and the resulting blue solution was left to stir for 1 hour. 70 mg (0.068 mmol, 1 equiv) of **SI-3** in 4 mL of THF was then added. The reaction progress was monitored by LC–MS. After 5 hours, 251 mg (4.70 mmol, 69 equiv) NH_4Cl was added to quench the reaction. At this time, the cooling bath was removed and the ammonia was blown off under a stream of N_2. The crude white solids were dissolved in water and desalted by passage through a Biorad P-2 Biogel (Fine) size exclusion column to give 28 mg of partially purified **SI-4**. ^{1}H NMR (400MHz, D_2O): δ 5.11 (s, 1H), 4.81 (s, 1H), 4.13 – 4.02 (m, 2H), 3.96 – 3.84 (m, 4H), 3.84 – 3.71 (m, 5H), 3,71 – 3.59 (m, 2H), 3.44 – 3.35 (m, 1H), 3.10 (s, 1H), 2.29 – 1.81 (m, 4H), 1.58 – 1.04 (m, 4H). ^{13}C NMR (100 MHz, D_2O): δ 105.14, 100.63, 83.34, 79.07, 78.87, 76.14, 73.63, 73.19, 72.87, 69.63, 68.99, 63.80, 51.82, 33.28, 32.05, 31.77, 31.66. IR (cm^{-1}): 3228, 2935, 2092, 1632, 1052. MS (ESI+): calcd. for $C_{18}H_{34}NO_{11}^+$ [M + H^+] 440.21, found 440.16. $[\alpha]_D$ (c 1.0, H_2O, 6.1).

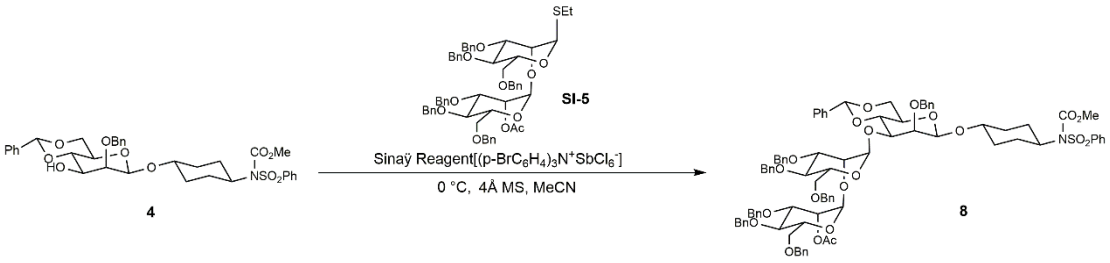
Cyclohexyl linked disaccharide azide (**7**, Man_2–Cy–N_3)



To a 20 mL vial containing 24 mg (0.054 mmol, 1.0 equiv) of crude amine **SI-4** was added 0.27 mL water, 1.4 mg (0.0054 mmol, 0.1 equiv) of $CuSO_4.5H_2O$ and 22 mg (0.16 mmol, 3.0 equiv) of K_2CO_3. 1 mL of MeOH was added, followed by the addition of 2.2 mL (0.54 mmol, 10.0 equiv) of freshly prepared 0.25M TfN_3 in DCM. The resulting homogeneous reaction was left to stir at room temperature for 4 hours until complete conversion was observed by LC–MS. The reaction was quenched with 45 mg (0.54 mmol, 10.0 equiv) solid $NaHCO_3$ and concentrated in vacuo. The residue was desalted on a Biorad P-2 Biogel (Fine) size exclusion column. Reversed phase HPLC purification (Column: Waters Xbridge Prep, C18, 5µm, 130Å, 10x250mm. Method: 4mL/min flow rate. A= H2O/0.1%FA, B= ACN/0.1%FA. 98.2% A for 1min, then 98.2% A to 60% A over 24 minutes, then 60% to 5% A over 5 minutes. Desired product eluted at 16.4 minutes.) providing 12 mg (0.026 mmol, 38%, 2 steps) of Man_2–Cy–N_3 (**7**) as a glassy solid. ^{1}H NMR (400MHz, D_2O): δ 5.11 (s, 1H), 4.81 (s, 1H), 4.13 – 4.04 (m, 2H), 3.95 – 3.86 (m, 3H), 3.87 – 3.79 (m, 2H), 3.79 – 3.72 (m, 4H), 3.71 – 3.61 (m, 2H), 3.58 – 3.46 (m, 1H), 3.45 – 3.33 (m, 1H), 2.20 – 1.80 (m, 4H), 1.54 – 1.32 (m, 4H).^{13}C NMR (100 MHz, D_2O): δ 105.15, 100.54, 83.39, 78.83, 76.12, 73.64, 73.17, 72.86, 69.62, 68.97, 63.81, 61.68, 32.82, 31.46, 31.13, 30.98. IR (cm^{-1}): 3340, 2937, 2094, 1057. MS (ESI+): calcd. for $C_{18}H_{31}KN_3O_{10}^+$ [M + K$^+$] 488.16, found 488.12. $[\alpha]_D$ (c 0.5, H_2O, 3.0).

Synthesis of Man_3–Cy–N_3 (**9**)

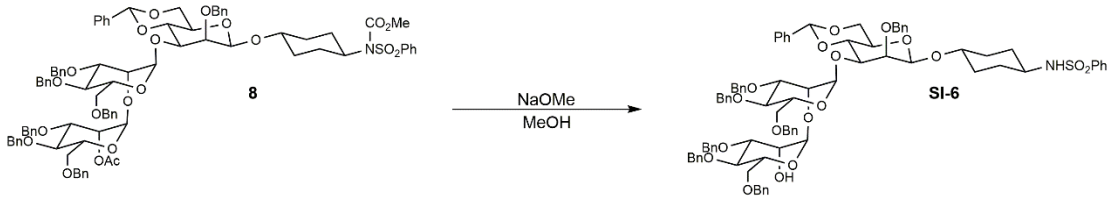
Fully protected cyclohexyl linked trisaccharide (**8**)



50 mg (0.077 mmol, 1 equiv) of **4**[9-12] and 111 mg (0.115 mmol, 1.5 equiv) of **SI-5** [9-12] were dissolved in toluene in a 25 mL flask and freeze pumped for three times. The dry residue was dissolved in 3 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 150 mg (0.184 mmol, 2.4 equiv) Sinaÿ reagent, [(p-$BrC_6H_4)_3N^+$ $SbCl_6^-$,] was added. This was allowed to react at 0 °C for 30 minutes and then at room temperature for 30 minutes. After this time, 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 60 mg (0.038 mmol, 50%) **8**, as an off-white foam. ^{1}H NMR (400MHz, $CDCl_3$): δ 7.92 (app d, J = 7.6 Hz, 2H), 7.60 (app t, J = 7.4 Hz, 1H), 7.52 (app t, J = 7.7 Hz, 2H), 7.46 – 7. 36 (m, 4H), 7.36 – 7.09 (m, 35H + $CHCl_3$), 7.06 (app t, J = 7.4 Hz, 1H), 5.47 (s, 1H), 5.43 (s, 1H), 5.10 (s, 1H), 4.92 – 4.75 (m, 5H), 4.75 – 4.16 (m, 15H), 4.07 (app t, J = 9.5 Hz, 1H), 4.01 – 3.60 (m, 15H), 3.59 – 3.46 (m, 1H),3.40 (app d, J = 10.9 Hz, 1H), 3.28 – 3.13 (m, 2H), 2.33 – 2.20 (m, 2H), 2.20 – 2.11 (m, 1H + Acetone), 2.08 (s, 3H), 1.59 – 1.41 (m, 1H), 1.35 – 1.13 (m, 2H + Ethyl acetate). ^{13}C NMR (100MHz, $CDCl_3$, selected signals): δ 170.15, 152.51, 140.30, 138.66, 138.60, 138.44, 138.37, 138.29, 138.11, 138.08, 137.45,

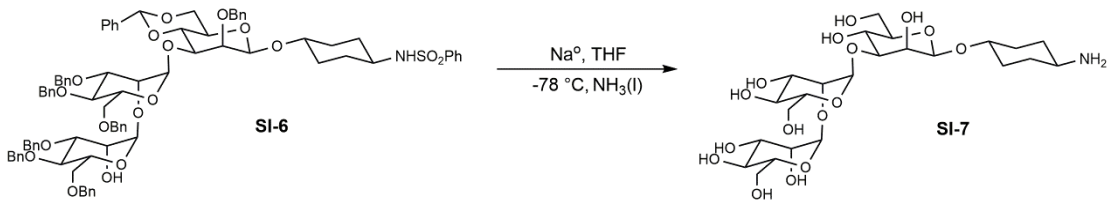
133.35, 128.77, 128.30, 128.27, 128.22, 128.15, 128.10, 127.96, 127.88, 127.86, 127.58, 127.53, 127.50, 127.46, 127.44, 127.32, 125.98, 101.32, 100.00, 99.67, 98.60, 78.48, 78.15, 76.36, 75.14, 75.02, 74.73, 74.14, 73.24, 73.04, 72.36, 71.93, 71.79, 69.70, 68.67, 68.56, 68.48, 67.32, 58.44, 53.44, 33.15, 31.44, 28.41, 21.13. IR (cm^{-1}): 2867, 1735, 1052, 1026, 734, 696. MS (ESI+): calcd. for $C_{90}H_{101}N_2O_{21}S^+$ [M + NH_4^+] 1577.66, found 1577.86. $[\alpha]_D$ (c 1.0, DCM, –13.0).

Partially deprotected cyclohexyl linked trisaccharide (**SI-6**)



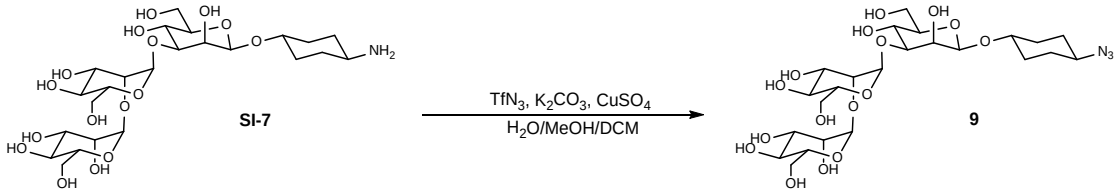
55 mg (0.035 mmol, 1 equiv) of **8** was dissolved in 15 mL MeOH in a 25 mL flask. 0.4 mL (1.4 mmol, 40 equiv) of a 25% NaOMe solution in MeOH was added. The reaction was left stirring at room temperature under N_2 for 13 hours until reaction was completed based on LC–MS and TLC monitoring. Upon completion, the volume of the mixture was reduced to a half by rotary evaporation, and saturated NH_4Cl solution was added to quench the reaction. The mixture was extracted with DCM three times. The combined organic layers were dried over $MgSO_4$, filtered and concentrated. Purification by flash chromatography (2:3 ethyl acetate/hexanes) afforded 40 mg of **SI-6** as an off–white foam. Analysis by LC–MS shows tiny amount of impurity. Reversed phase HPLC purification (Column: Waters Xbridge Prep, C18, 5µm, 130Å, 10x250mm. Method: 4mL/min flow rate. A= H2O/0.1%FA, B= ACN/0.1%FA. 21% A to 1% A over 25 minutes, then keep for 15 minutes. Desired product eluted at 20.9 minutes.) providing 37 mg (0.025 mmol, 71%) of **SI-6** as an off–white foam. ^{1}H NMR (400MHz, $CDCl_3$): δ 7.94 – 7.84 (m, 2H), 7.58 (app t, J = 7.3 Hz, 1H), 7.52 (app t, J = 7.5 Hz, 2H), 7.37 (app t, J = 8.0 Hz, 4H), 7.34 – 7.14 (m, 35H + $CHCl_3$), 7.08 (app t, J = 7.3Hz, 1H), 5.40 (s, 1H), 5.31 (s, 1H), 5.17 (s, 1H), 4.87 – 4.68 (m, 4H), 4.66 – 4.57 (m, 1H), 4.56 – 4.32 (m, 9H), 4.23 (dd, J = 12 Hz, 2.4 Hz, 2H), 4.19 – 4.12 (m, 1H), 4.07 – 3.98 (m, 2H), 3.95 – 3.82 (m, 2H), 3.83 – 3.68 (m, 5H), 3.65 (s, 2H), 3.52 – 3.41 (m, 1H), 3.41 – 3.33 (m, 1H), 3.27 (app d, J = 10.8 Hz, 1H), 3.21 – 3.07 (m, 2H), 1.96 – 1.87 (m, 1H), 1.86 – 1.75 (m, 2H), 1.73 – 1.63 (m, 1H), 1,44 – 1.29 (m, 1H), 1.28 – 1.04 (m, 3H). ^{13}C NMR (100MHz, $CDCl_3$, selected signals): δ 141.07, 138.57, 138.45, 138.38, 138.21, 138.06, 138.01, 137.37, 132.58, 129.11, 128.84, 128.42, 128.38, 128.33, 128.25, 128.23, 128.07, 127.97, 127.78, 127.75, 127.65, 127.60, 127.46, 127.42, 127.38, 127.30, 126.85, 125.94, 101.28, 99.82, 79.94, 78.37, 78.11, 75.60, 75.07, 74.93, 74.53, 74.12, 73.35, 73.19, 73.00, 72.42, 72.05, 71.94, 71.46, 69.69, 68.52, 68.45, 67.31, 51.59, 40.68, 31.02, 30.80, 29.26. IR (cm^{-1}): 3502, 3301, 3029, 2860, 1452, 1046, 734, 694. MS (ESI+): calcd. for $C_{86}H_{97}N_2O_{18}S^+$ [M + NH_4^+] 1477.65, found 1477.73. $[\alpha]_D$ (c 1.0, DCM, –5.5).

Fully deprotected cyclohexyl linked trisaccharide amine (**SI-7**)



Along with a stream of N_2, ammonia gas was condensed against a -78 °C cold finger into a -78 °C-cooled 50 mL 3-necked flask, equipped with glass-coated stir bar, until 25 mL had accumulated. 52 mg (2.3 mmol, 100 equiv) Na metal was then added, and the resulting blue solution was left to stir for 1 hour. 33 mg (0.023 mmol, 1 equiv) of **SI-6** in 4 mL of THF was then added. The reaction progress was monitored by LC–MS. After 3 hours, 139 mg (2.60 mmol, 115 equiv) NH_4Cl was added to quench the reaction. At this time, the cooling bath was removed and the ammonia was blown off under a stream of N_2. The crude white solids were dissolved in water and desalted by passage through a Biorad P-2 Biogel (Fine) size exclusion column to give 50 mg crude. The mixture was desalted again and 18 mg of partially purified **SI-7** was obtained. This material was used in the next step without further purification. ^{1}H NMR (400MHz, D_2O): δ 5.36 (s, 1H), 5.05 (s, 1H), 4.80 (s, 1H), 4.13 – 4.09 (m, 1H), 4.09 – 4.05 (m, 2H), 4.00 (dd, J = 9.5, 3.3 Hz, 1H), 3.96 – 3.82 (m, 4H), 3.82 – 3.61 (m, 10H), 3.39 (app t, J = 8.6 Hz, 1H), 3.04 – 2.77 (m, 1H), 2,17 – 2.02 (m, 2H), 2.01 – 1.87 (m, 2H), 1.56 – 1.09 (m, 4H).^{13}C NMR (100 MHz, D_2O): δ 112.94, 105.12, 103.49, 100.61, 83.43, 81.27, 79.62, 78.88, 76.18, 76.08, 73.65, 73.17, 72.93, 72.81, 69.82, 69.57, 69.05, 63.82, 51.72, 33.58, 33.24, 33.08, 32.34. IR (cm^{-1}): 3223, 2934, 1686, 1025. MS (ESI+): calcd. for $C_{24}H_{44}NO_{16}^+$ [M + H^+] 602.27, found 602.15. $[\alpha]_D$ (c 0.5, H_2O, 8.5).

Cyclohexyl linked trisaccharide azide (**9**, Man_3–Cy–N_3)

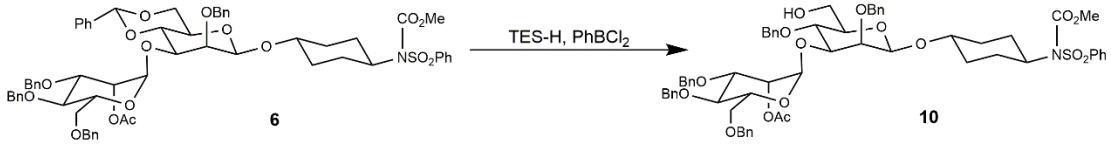


Into a 20 mL vial containing 13.5 mg (0.0230 mmol, 1.0 equiv) of crude amine **SI-7** was added 113 µL water, 9.2 µL (0.0023 mmol, 0.1 equiv) of 0.25 M aqueous $CuSO_4$ and 9.4 mg (0.068 mmol, 3.0 equiv) of K_2CO_3. 0.5 mL of MeOH was added, followed by the addition of 0.9 mL (0.226 mmol, 10.0 equiv) of freshly prepared 0.25M TfN_3 in DCM. The resulting homogeneous reaction was left to stir at room temperature for 3 hours until complete conversion was observed by LC–MS. The reaction was quenched with 19 mg (0.23 mmol, 10.0 equiv) solid $NaHCO_3$ and concentrated in vacuo. The residue was desalted on a Biorad P-2 Biogel (Fine) size exclusion column. Reversed phase HPLC purification (Column: Waters Xbridge Prep, C18, 5µm, 130Å, 10x250mm. Method: 4mL/min flow rate. A= H2O/0.1%FA, B= ACN/0.1%FA. 98.2% A for 1min, then 98.2% A to 60% A over 24 minutes, then 60% to 5% A over 5 minutes. Desired product eluted at 15.7 minutes.) providing 5 mg (0.008 mmol, 35%, 2 steps) of Man_3–Cy–N_3 (**9**) as a glassy solid. ^{1}H NMR (400MHz, D_2O): δ 5.34 (s, 1H), 5.03 (s, 1H), 4.77 (s, 1H), 4.11 – 4.07 (m, 1H), 4.07 – 4.03 (m, 2H), 3.98 (dd, J = 9.5, 3.3 Hz, 1H), 3.93 – 3.78 (m, 5H), 3.78 – 3.56 (m, 9H), 3.53 – 3.43 (m, 1H), 3.37 (app t, J = 7.2 Hz, 1H), 2.06 – 1.92 (m, 4H), 1.56 – 1.32 (m, 4H). ^{13}C NMR (100

MHz, D_2O, selected signals): δ 112.96, 105.15, 103.55, 100.61, 83.51, 81.30, 78.99, 78.90, 76.21, 76.10, 73.69, 73.20, 72.96, 72.84, 69.86, 69.61, 69.08, 63.85, 61.76, 32.92, 31.56, 31.22, 31.07. IR (cm^{-1}): 3365, 2933, 2095, 1059. MS (ESI+): calcd. for $C_{24}H_{42}N_3O_{16}^+$ [M + H^+] 628.26, found 628.08. $[\alpha]_D$ (c 0.18, H_2O, 11.8).

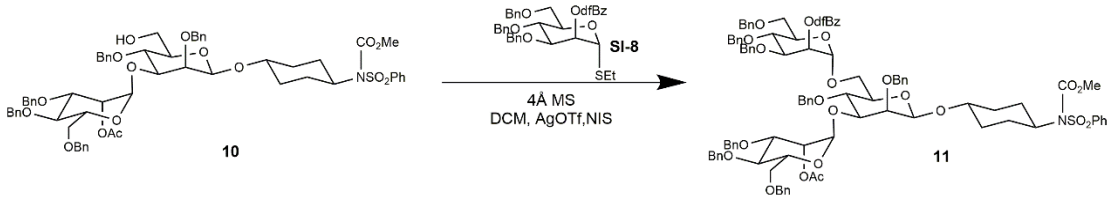
Synthesis of Man_3–Cy–N_3 (**12**)

Reduction of fully protected cyclohexyl linked disaccharide (**10**)



To a 10 mL flask containing 102 mg (0.0904 mmol, 1 equiv) of **6** toluene was added and freeze pumped three times. 2 mL of DCM and 300 mg freshly dried 4Å molecular sieves were added to the flask. The flask was cooled to –78 °C, and 43 µL (0.27 mmol, 3 equiv) of TES–H was added to the flask, followed by addition of 38 µL (0.31 mmol, 3.4 equiv) $PhBCl_2$. This mixture was allowed to react for 1 hour, and the reaction was quenched with triethylamine and methanol. The mixture was filtered through celite, washed with sat. $NaHCO_3$, dried over $MgSO_4$, filtered, and concentrated. Purification by flash chromatography (2:3 ethyl acetate/hexanes to 1:1 ethyl acetate/hexanes) afforded 71 mg (0.062 mmol, 68%) of **10** as an off–white foam. ^{1}H NMR (400MHz, $CDCl_3$): δ 7.91 (app d, J = 7.6 Hz, 2H), 7.59 (app t, J = 7.4 Hz, 1H), 7.50 (app t, J = 7.7 Hz, 2H), 7.43 – 7.09 (m, 25H + $CHCl_3$), 5.50 (s, 1H), 5.21 (s, 1H), 4.90 (dd, J = 26.9, 11.8 Hz, 2H), 4.78 (app t, J = 10.8 Hz, 2H), 4.65 (dd, J = 26.0, 11.1 Hz, 2H), 4.56 – 4.35 (m, 6H), 4.01 – 3.91 (m, 2H), 3.90 – 3.60 (m, 10H), 3.60 – 3.42 (m, 1H), 3.34 – 3.21 (m, 1H), 2.34 – 2.11 (m, 4H), 2.08 (s, 3H), 1.98 – 1.74 (m, 4H), 1.61 – 1.45 (m, 1H), 1.41 – 1.28 (m, 1H). ^{13}C NMR (100MHz, $CDCl_3$, selected signals): δ 171.20, 153.70, 141.45, 139.73, 139.64, 139.38, 138.97, 134.55, 129.97, 129.63, 129.58, 129.50, 129.39, 129.38, 129.33, 129.32, 129.07, 128.98, 128.83, 128.79, 128.68, 128.60, 100.76, 81.02, 79.08, 79.06, 77.52, 77.07, 76.39, 76.34, 76.03, 75.53, 75.45, 74.55, 73.28, 73.06, 70.43, 69.90, 63.28, 61.56, 59.60, 54.63, 34.45, 32.70, 29.62, 22.17, 15.40. IR (cm^{-1}): 3025, 2931, 2870, 1733, 1043, 734, 696. MS (ESI+): calcd. for $C_{63}H_{75}N_2O_{16}S^+$ [M + NH_4^+] 1147.48, found 1147.62. $[\alpha]_D$ (c 1.0, DCM, –14.8).

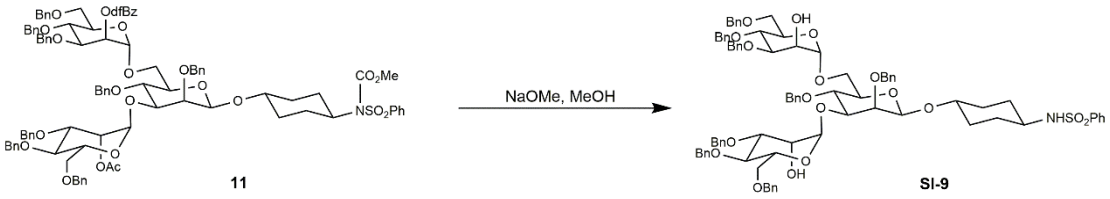
Fully protected cyclohexyl linked trisaccharide (**11**)



To a 25 mL flask containing 70 mg (0.062 mmol, 1 equiv) of **10** and 79 mg (0.12 mmol, 2 equiv) of donor **SI-8** [14] was added toluene and freeze pumped for three times. 200 mg freshly dried 4Å molecular sieves were added and the mixture was redissolved in 2 mL DCM and cooled in a –20 °C salt ice bath for 30 minutes. The 25 mL flask

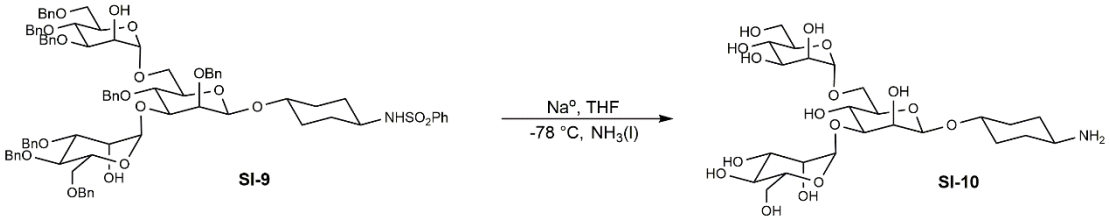
was then wrapped in foil and 8 mg (0.03 mmol, 0.5 equiv) AgOTf was added, followed by 29 mg (0.13 mmol, 2.1 equiv) of NIS. This mixture was allowed to react for 2.5 hours, and the reaction was quenched with triethylamine. The mixture was filtered through celite and extracted with EA three times. The combined organic layers were dried over $MgSO_4$, filtered, and concentrated. Purification by flash chromatography (1:3:1 ethyl acetate/hexanes/DCM) afforded 70 mg (0.041 mmol, 66%) of **11** as an off–white foam. ^{1}H NMR (400MHz, $CDCl_3$): δ 7.87 (app d, J = 7.8 Hz, 2H), 7.74 – 7.61 (m, 1H), 7.56 (app t, J = 7.5 Hz, 1H), 7.50 – 7.40 (m, 4H), 7.37 – 7.01 (m, 40H + $CHCl_3$), 5.71 (s, 1H), 5.52 (s, 1H), 5.20 (s, 1H), 5.11 – 4.98 (m, 2H), 4.86 (dd, J = 15.6, 11.0 Hz, 2H), 4.82 – 4.61 (m, 5H), 4.59 – 4.30 (m, 10H), 4.09 – 3.94 (m, 3H), 3.95 – 3.68 (m, 8H), 3.70 – 3.61 (m, 3H), 3.60 – 3.48 (m, 4H), 3.46 – 3.31 (m, 1H), 2.32 – 2.14 (m, 3H), 2.07 (s, 3H), 2.02 – 1.91 (m, 1H), 1.87 – 1.78 (m, 1H), 1.75 – 1.58 (m, 2H), 1.56 – 1.43 (m, 1H), 1.40 – 1.25 (m, 1H). ^{13}C NMR (100MHz, $CDCl_3$, selected signals): δ 169.95, 152.36, 140.25, 138.70, 138.51, 138.42, 138.23, 138.09, 137.78, 137.74, 137.64, 133.15, 128.63, 128.29, 128.23, 128.19, 128.11, 128.07, 128.02, 127.90, 127.79, 127.67, 127.61, 127.60, 127.55, 127.50, 127.38, 127.33, 127.21, 118.44, 118.18, 99.91, 99.63, 97.90, 80.30, 77.99, 77.68, 77.59, 76.39, 75.19, 74.96, 74.77, 74.49, 74.25, 74.10, 73.94, 73.31, 73.14, 71.97, 71.86, 71.49, 71.36, 69.46, 69.07, 68.67, 66.79, 58.43, 53.22, 33.26, 31.57, 28.51, 28.37, 20.90. IR (cm^{-1}): 2890, 2800, 1734, 1046, 733, 695. MS (ESI+): calcd. for $C_{97}H_{105}F_2N_2O_{22}S^+$ [M + NH_4^+] 1720.69, found 1720.78. $[\alpha]_D$ (c 1.0, DCM, –3.4).

Partially deprotected cyclohexyl linked trisaccharide (**SI-9**)



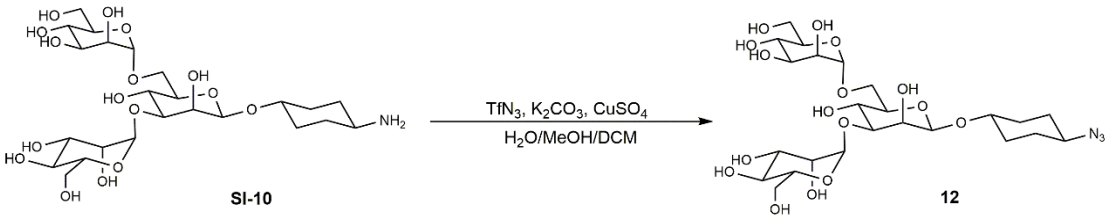
60 mg (0.035 mmol, 1 equiv) of **11** was dissolved in 15 mL MeOH in a 25 mL flask. 0.4 mL (1.4 mmol, 40 equiv) of a 25% NaOMe solution in MeOH was added. The reaction was left stirring at room temperature under N_2 for 13 hours until reaction was completed based on LC–MS and TLC monitoring. Upon completion, the volume of the mixture was reduced to a half by rotary evaporation, and saturated NH_4Cl solution was added to quench the reaction. The mixture was extracted with DCM three times. The combined organic layers were dried over $MgSO_4$, filtered and concentrated. Purification by flash chromatography (1:1 ethyl acetate/hexanes to 3:2 ethyl acetate/hexanes) afforded 38 mg (0.026 mmol, 74%) of **SI-9** as an off–white foam. ^{1}H NMR (400MHz, $CDCl_3$): δ 7.82 (app d, J = 7.4 Hz, 2H), 7.54 (app t, J = 7.3 Hz, 1H), 7.47 (app t, J = 8.4 Hz, 2H), 7.41 – 7.02 (m, 40H + $CHCl_3$), 5.21 (s, 1H), 4.99 (s, 1H), 4.91 (app d, J = 12.2 Hz, 1H), 4.80 (app t, J = 10.3 Hz, 2H), 4.73 – 4.55 (m, 6H), 4.56 – 4.37 (m, 8H), 4.20 – 4.02 (m, 2H), 4.02 – 3.94 (m, 1H), 3.90 – 3.55 (m, 15H), 3.52 – 3.37 (m, 1H), 3.36 – 3.24 (m, 1H), 3.14 – 2.91 (m, 1H), 2.36 (s, 2H), 1.89 – 1.66 (m, 4H), 1.37 – 1.18 (m, 1H), 1.16 – 0.90 (m, 3H). ^{13}C NMR (100MHz, $CDCl_3$, selected signals): δ 141.11, 138.96, 138.55, 138.43, 138.16, 137.85, 137.77, 132.48, 129.03, 128.52, 128.49, 128.34, 128.29, 128.26, 128.22, 128.08, 127.96, 127.94, 127.92, 127.88, 127.85, 127.78, 127.71, 127.69, 127.60, 127.55, 127.51, 127.26, 126.81, 101.55, 99.59, 99.16, 80.94, 79.94, 79.73, 78.13, 75.22, 75.17, 75.12, 75.07, 74.78, 74.33, 74.24, 74.10, 73.39, 73.34, 72.14, 71.76, 71.38, 70.92, 69.23, 68.79, 68.67, 67.82, 66.22, 51.62, 31.28, 30.89, 30.74, 29.15. IR (cm^{-1}): 3029, 2920, 1067, 1043. MS (ESI+): calcd. for $C_{86}H_{99}N_2O_{18}S^+$ [M + NH_4^+] 1479.66, found 1479.75. $[\alpha]_D$ (c 1.0, DCM, 10.0).

Fully deprotected cyclohexyl linked trisaccharide amine (**SI-10**)



Along with a stream of N_2, ammonia gas was condensed against a -78 °C cold finger into a -78 °C-cooled 50 mL 3-necked flask equipped with a glass-coated stir bar, until 25 mL had accumulated. 43 mg (1.8 mmol, 90 equiv) Na metal was then added, and the resulting blue solution was left to stir for 1 hour. 30 mg (0.021 mmol, 1 equiv) of **SI-9** in 2 mL of THF was then added. The reaction progress was monitored by LC–MS. After 4 hours, 113 mg (2.11 mmol, 103 equiv) NH_4Cl was added to quench the reaction. At this time, the cooling bath was removed and the ammonia was blown off under a stream of N_2. The crude white solids were dissolved in water and desalted by passage through a Biorad P-2 Biogel (Fine) size exclusion column to give 70 mg crude. The mixture was desalted again and 13 mg of partially purified **SI-10** was obtained. This material was used in the next step without further purification. ^{1}H NMR (400MHz, D_2O): δ 5.10 (s, 1H), 4.91 (s, 1H), 4.81 (s, 1H), 4.17 – 4.04 (m, 2H), 4.01 – 3.85 (m, 5H), 3.84 – 3.62 (m, 11H), 3.59 – 3.50 (m, 1H), 3.07 – 2.92 (m, 1H), 2.22 – 1.80 (m, 4H), 1.54 – 1.12 (m, 4H).^{13}C NMR (100 MHz, D_2O): δ 164.06, 102.38, 99.37, 98.07, 80.75, 76.85, 74.06, 73.31, 72.69, 70.78, 70.65, 70.37, 70.06, 69.94, 66.78, 66.71, 65.99, 65.63, 60.97, 48.92, 30.73, 29.73, 29.61, 29.41. IR (cm^{-1}): 3275, 2946, 1436, 1027. MS (ESI+): calcd. for $C_{24}H_{44}NO_{16}^+$ [M + H$^+$] 602.27, found 602.15. $[\alpha]_D$ (c 0.26, H_2O, 3.0).

Cyclohexyl linked trisaccharide azide (**12**, Man_3–Cy–N_3)

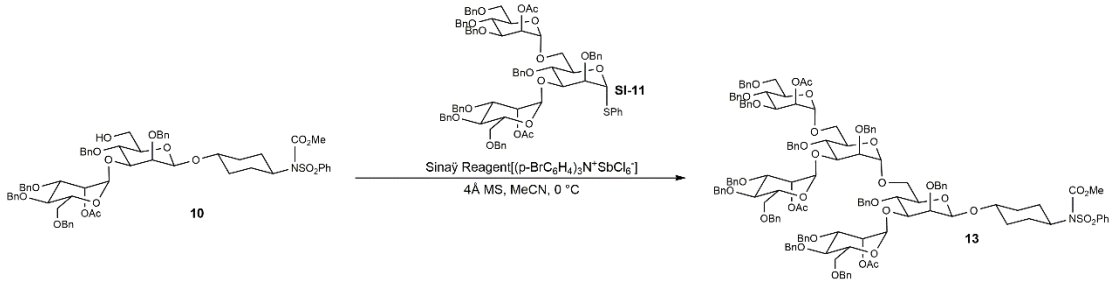


Into a 20 mL vial containing 10 mg (0.017 mmol, 1.0 equiv) of crude amine **SI-10** was added 83 µL water, 6.8 µL (0.0017 mmol, 0.1 equiv) of 0.25M aqueous $CuSO_4$ and 7 mg (0.05 mmol, 3.0 equiv) of K_2CO_3. 166 µL of MeOH was added, followed by the addition of 0.7 mL (0.17 mmol, 10.0 equiv) of freshly prepared 0.25M TfN_3 in DCM. The resulting homogeneous reaction was left to stir at room temperature for 3 hours until complete conversion was observed by LC–MS. The reaction was quenched with 18 mg (0.22 mmol, 13.0 equiv) solid $NaHCO_3$ and concentrated in vacuo. The residue was desalted on a Biorad P-2 Biogel (Fine) size exclusion column. Reversed phase HPLC purification (Column: Waters Xbridge Prep, C18, 5µm, 130Å, 10x250mm. Method: 4mL/min flow

rate. A= H2O/0.1%FA, B= ACN/0.1%FA. 98.2% A for 1min, then 98.2% A to 60% A over 24 minutes, then 60% to 5% A over 5 minutes. Desired product eluted at 15.1 minutes.) providing 6 mg (0.01 mmol, 48%, 2 steps) of Man_3–Cy–N_3 (**12**) as a glassy solid. ^{1}H NMR (400MHz, D_2O): δ 5.09 (s, 1H), 4.90 (s, 1H), 4.79 (s, 1H), 4.07 (app d, J = 16 Hz, 2H), 4.01 – 3.84 (m, 4H), 3.83 – 3.61 (m, 12H), 3.59 – 3.38 (m, 2H), 2.13 – 1.92 (m, 4H), 1.63 – 1.29 (m, 4H). ^{13}C NMR (100 MHz, D_2O, selected signals): δ 105.23, 102.20, 100.87, 83.64, 79.24, 76.89, 76.14, 75.51, 73.65, 73.46, 73.20, 72.90, 72.77, 69.63, 69.55, 68.78, 68.47, 63.82, 61.71, 32.99, 31.59, 31.20, 31.08. IR (cm^{-1}): 3301, 2940, 2095, 1044. MS (ESI+): calcd. for $C_{24}H_{42}N_3O_{16}^+$ [M + H$^+$] 628.26, found 628.23. $[\alpha]_D$ (c 0.21, H_2O, – 7.0).

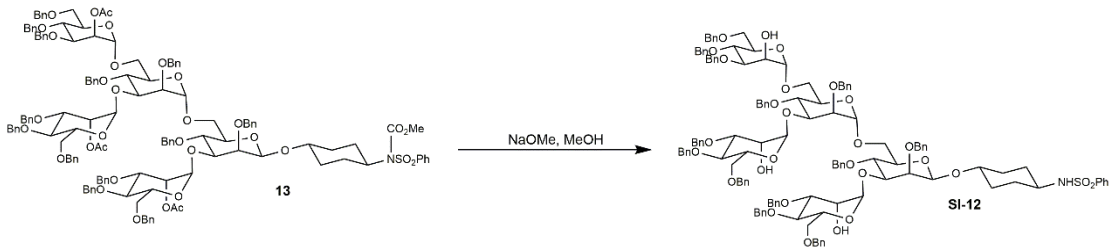
Synthesis of Man_5–Cy–N_3 (**14**)

Fully protected cyclohexyl linked pentasaccharide (**13**)



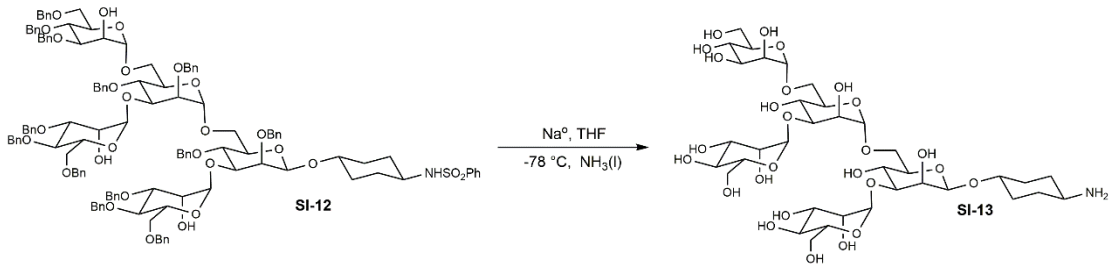
101 mg (0.0894 mmol, 1 equiv) of **10** and 200 mg (0.143 mmol, 1.6 equiv) of **SI-11**[9-12] in a 25 mL flask were dissolved in toluene and freeze pumped for three times. The dry residue was dissolved in 4 mL of acetonitrile, freshly flame-dried 4Å molecular sieves were added, and was allowed to stir for 1 hour. The flask was then wrapped in foil, cooled to 0 °C, and 175 mg (0.214 mmol, 2.4 equiv) Sinaÿ reagent, [(p-$BrC_6H_4)_3N^+$ $SbCl_6^-$] was added. This mixture was allowed to react at 0 °C for 30 minutes and react at room temperature for 30 minutes. After this time, triethylamine was added, and the reaction was filtered through celite and concentrated in vacuo. The crude residue was purified by flash chromatography with 1:2.5:1 ethyl acetate / hexanes / DCM to give 138 mg (0.0570 mmol, 63.8%,) **13**, as an off-white foam. ^{1}H NMR (400MHz, $CDCl_3$): δ 7.89 (app d, J = 7.7 Hz, 2H), 7.56 (app t, J = 7.3 Hz, 1H), 7.48 (app t, J = 7.6 Hz, 2H), 7.40 – 6.95 (m, 65H + $CHCl_3$), 5.58 (s, 1H), 5.55 – 5.41 (m, 2H), 5.23 (app d, J = 7.7 Hz, 2H), 5.12 (s, 1H), 4.98 (s, 1H), 4.94 – 4.79 (m, 6H), 4.76 – 4.30 (m, 24H), 4.21 – 3.41 (m, 34H), 3.30 – 3.10 (m, 1H), 2.28 – 1.63 (m, 9H + ethyl acetate), 1.52 – 1.17 (m, 2H + ethyl acetate). ^{13}C NMR (100MHz, $CDCl_3$, selected signals): δ 170.29, 170.14, 170.03, 152.65, 140.48, 138.80, 138.78, 138.72, 138.60, 138.41, 138.35, 138.33, 138.27, 138.06, 137.99, 137.96, 133.39, 128.88, 128.50, 128.44, 128.37, 128.33, 128.27, 128.25, 128.17, 128.10, 128.05, 127.89, 127.77, 127.74, 127.71, 127.63, 127.58, 127.49, 127.37, 100.18, 99.82, 98.32, 97.83, 79.80, 78.43, 78.08, 76.65, 75.66, 75.33, 75.29, 75.16, 75.01, 74.90, 74.47, 74.23, 73.81, 73.61, 73.46, 72.43, 72.27, 72.12, 72.08, 71.93, 71.70, 71.30, 71.20, 69.31, 69.03, 68.92, 68.76, 68.47, 66.46, 66.19, 58.59, 53.49, 33.46, 31.87, 28.71, 28.54, 21.27, 21.10, 14.36. IR (cm^{-1}): 3030, 2940, 1738, 1047, 733, 695. MS (ESI+): calcd. for $C_{141}H_{157}N_2O_{33}S^+$ [M + NH_4^+] 2439.04, found 2439.09. $[\alpha]_D$ (c 1.0, DCM, 21.0).

Partially protected cyclohexyl linked pentasaccharide (**SI-12**)



130 mg (0.0537 mmol, 1 equiv) of **13** was dissolved in 1.5 mL MeOH and 3 mL THF in a 25 mL flask. 77 µL (2.15 mmol, 5 equiv) of a 25% NaOMe solution in MeOH was added. The reaction was left stirring at room temperature under N_2 for 13 hours until reaction was completed based on LC–MS and TLC monitoring. Upon completion, the volume of the mixture was reduced to a half by rotary evaporation, and saturated NH_4Cl solution was added to quench the reaction. The mixture was extracted with DCM three times. The combined organic layers were dried over $MgSO_4$, filtered and concentrated. Purification by flash chromatography (1:1 ethyl acetate/hexanes to 3:2 ethyl acetate/hexanes) afforded 73 mg (0.033 mmol,61%) of **SI-12** as an off–white foam. ^{1}H NMR (400MHz, $CDCl_3$): δ 7.84 (app d, J = 7.5 Hz, 2H), 7.48 (app t, J = 7.3 Hz, 1H), 7.41 (app t, J = 7.5 Hz, 1H), 7.37 – 7.07 (m, 66H + CHCl3), 5.43 – 5.27 (m, 1H), 5.22 (s, 1H), 5.18 (s, 1H), 5.14 (s, 1H), 4.97 – 4.77 (m, 5H), 4.77 – 4.33 (m, 22H), 4.22 (s, 1H), 4.19 – 3.54 (m, 27H), 3.53 – 3.35 (m, 2H), 3.21 – 2.95 (m, 2H), 2.88 (s, 1H), 2.44 (s, 1H), 2.35 (s, 1H), 2.00 – 1.76 (m, 2H), 1.75 – 1.63 (m, 2H), 1.60 – 1.45 (m, 1H), 1.40 – 0.60 (m, 4H). ^{13}C NMR (100MHz, $CDCl_3$, selected signals): δ 142.09, 138.75, 138.69, 138.61, 138.59, 138.49, 138.43, 138.33, 138.23, 138.15, 137.96, 137.89, 137.79, 132.28, 129.05, 128.74, 128.67, 128.61, 128.56, 128.50, 128.46, 128.44, 128.38, 128.25, 128.06, 128.02, 127.91, 127.87, 127.81, 127.76, 127.67, 127.45, 126.98, 101.91, 101.62, 99.93, 98.49, 96.79, 80.56, 80.29, 80.05, 80.02, 79.86, 76.63, 75.49, 75.25, 75.16, 74.98, 74.94, 74.79, 74.70, 74.66, 74.46, 74.04, 73.68, 73.56, 73.53, 72.38, 72.32, 72.22, 72.05, 71.89, 71.75, 71.61, 71.48, 69.80, 69.34, 69.06, 68.91, 68.78, 68.14, 66.07, 65.76, 51.67, 31.94, 31.85, 31.29, 29.46. IR (cm^{-1}): 3020, 2927, 1068, 1026. MS (ESI+): calcd. for $C_{133}H_{149}N_2O_{28}S^+$ [M + NH_4^+] 2255.00, found 2255.05. $[\alpha]_D$ (c 1.0, DCM, 22.5).

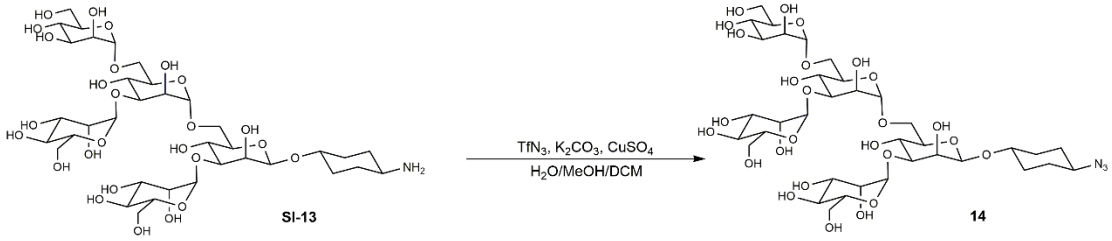
Fully deprotected cyclohexyl linked pentasaccharide amine (**SI-13**)



Along with a stream of N_2, ammonia gas was condensed against a -78 °C cold finger into a -78 °C-cooled 50 mL 3-necked flask equipped with glass-covered stir bar, until 25 mL had accumulated. 101 mg (4.38 mmol, 140 equiv) Na metal was then added, and the resulting blue solution was left to stir for 1 hour. 70 mg (0.031 mmol, 1 equiv) of **SI-12** in 4 mL of THF was then added. The reaction progress was monitored by LC–MS. After 3 hours, 270 mg (5.04 mmol, 161 equiv) NH_4Cl was added to quench the reaction. At this time, the cooling bath was removed and the ammonia was blown off under a stream of N_2. The crude white solids were dissolved in water and desalted by passage through a Biorad P-2 Biogel (Fine) size exclusion column to give 70 mg crude. The mixture was desalted again and 29 mg of partially purified **SI-13** was obtained. This material was used in the next step without further purification. ^{1}H NMR (400MHz, D_2O): δ5.15 (s, 1H), 5.11 (s, 1H), 4.92 (s, 1H), 4.89 (s, 1H), 4.82 (s, 1H), 4.22 – 4.14 (m, 1H), 4.13 – 4.07 (m, 3H), 4.04 – 3.63 (m, 26H), 3.62 – 3.50 (m, 1H), 3.20 – 3.10 (m, 1H), 2.18 – 2.03 (m,

4H), 1.57 – 1.34 (m, 4H). ^{13}C NMR (100 MHz, D_2O, selected signals): δ 105.21, 105.16, 102.38, 102.17, 101.12, 83.60, 81.43, 79.51, 76.93, 76.16, 75.58, 73.76, 73.63, 73.44, 73.24, 72.96, 72.92, 72.83, 72.36, 69.65, 69.61, 69.57, 68.78, 68.58, 68.14, 63.84, 63.76, 51.94, 33.39, 32.14, 31.59, 31.46. IR (cm^{-1}): 3273, 2969, 1024. MS (ESI+): calcd. for $C_{36}H_{64}NO_{26}^+$ [M + H^+] 926.37, found 926.15. $[\alpha]_D$ (c 1.0, H_2O, 42.7).

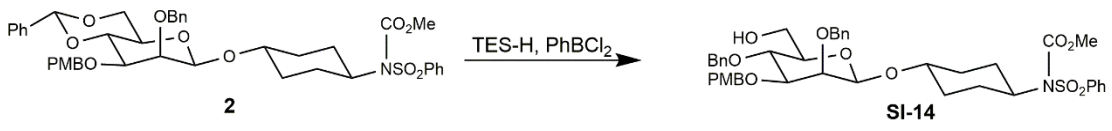
Cyclohexyl linked pentasaccharide azide (**14**, Man_5–Cy–N_3)



Into a 20 mL vial containing 26 mg (0.028 mmol, 1.0 equiv) of crude amine **SI-13** was added 140 µL water, 11 µL (0.0028 mmol, 0.1 equiv) of 0.25M aqueous $CuSO_4$ and 11.6 mg (0.0840 mmol, 3.0 equiv) of K_2CO_3. 280 µL of MeOH was added, followed by the addition of 1.1 mL (0.167 mmol, 10.0 equiv) of freshly prepared 0.25M TfN_3 in DCM. The resulting homogeneous reaction was left to stir at room temperature for 4 hours until complete conversion was observed by LC–MS. The reaction was quenched with 30.6 mg (0.365 mmol, 13.0 equiv) solid $NaHCO_3$ and concentrated in vacuo. The residue was desalted on a Biorad P-2 Biogel (Fine) size exclusion column. Reversed phase HPLC purification (Column: Waters Xbridge Prep, C18, 5µm, 130Å, 10x250mm. Method: 4mL/min flow rate. A= H_2O/0.1% FA, B= ACN/0.1% FA. 98.2% A for 1min, then 98.2% A to 60% A over 24 minutes, then 60% to 5% A over 5 minutes. Desired product eluted at 13.6 minutes.) providing 11 mg (0.012 mmol, 39%, 2 steps) of Man_5–Cy–N_3 (**14**) as a glassy solid. ^{1}H NMR (400MHz, D_2O): δ 5.12 (s, 1H), 5.09 (s, 1H), 4.90 (s, 1H), 4.87 (s, 1H), 4.79 (s, 1H), 4.19 – 4.12 (m, 1H), 4.11 – 4.04 (m, 3H), 4.03 – 3.94 (m, 2H), 3.94 – 3.61 (m, 24H), 3.59 – 3.41 (m, 2H), 2.10 – 1.94 (m, 4H), 1.54 – 1.24 (m, 4H). ^{13}C NMR (100 MHz, D_2O, selected signals): δ 105.21, 102.27, 102.14, 100.83, 83.64, 81.56, 79.20, 76.91, 76.16, 75.55, 73.73, 73.63, 73.42, 73.23, 73.20, 72.92, 72.89, 72.80, 72.33, 69.63, 69.58, 68.78, 68.56, 68.47, 68.10, 63.81, 61.67, 33.01, 31.58, 31.21, 31.09. IR (cm^{-1}): 3340, 2931, 2096, 1027. MS (ESI+): calcd. for $C_{36}H_{62}N_3O_{26}^+$ [M + H^+] 952.36, found 952.55. $[\alpha]_D$ (c 0.5, H_2O, 41.4).

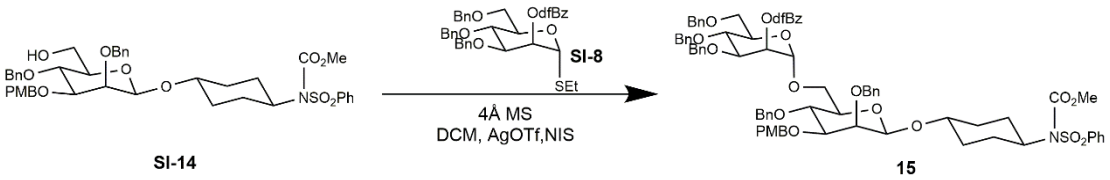
Synthesis of Man_2–Cy–N_3 (**16**)

Partially deprotected cyclohexyl linked monosaccharide (**SI-14**)



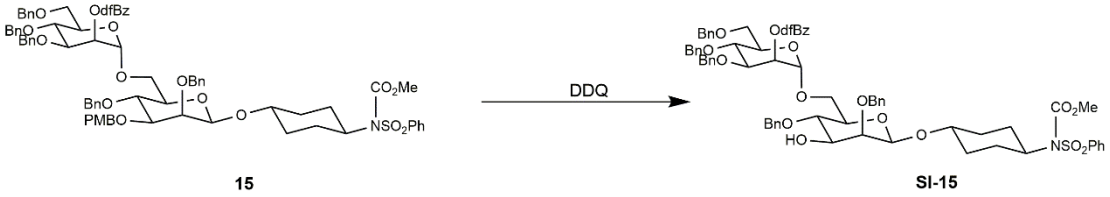
To a 10 mL flask containing 50 mg (0.065 mmol, 1 equiv) of **2** toluene was added and freeze pumped three times. 1 mL of DCM and 100 mg freshly dried 4Å molecular sieves were added to the flask. The flask was cooled to –78 °C, and 31 µL (0.19 mmol, 3 equiv) of TES–H was added to the flask, followed by addition of 28 µL (0.22 mmol, 3.4 equiv) $PhBCl_2$. This mixture was allowed to react for 1 hour, and the reaction was quenched with triethylamine and methanol. The mixture was filtered through celite, washed with sat. $NaHCO_3$, dried over $MgSO_4$, filtered, and concentrated. Purification by flash chromatography (2:3 ethyl acetate/hexanes) afforded 12mg (0.016 mmol, 25%) of **SI-14** as an off–white foam. ^{1}H NMR (400MHz, $CDCl_3$): δ 7.92 (app d, J = 7.6 Hz, 2H), 7.61 (app t, J = 7.6 Hz, 1H), 7.52 (app t, J = 7.8 Hz, 2H), 7.46 (app d, J = 7.8 Hz, 2H), 7.40 – 7.25 (m, 8H + $CHCl_3$), 7.21 (app d, J = 7.6 Hz, 2H), 6.84 (app d, J = 7.6 Hz, 2H), 5.05 – 4.82 (m, 3H), 4.73 – 4.58 (m, 1H), 4.55 – 4.37 (m, 3H), 3.97 – 3.85 (m, 2H), 3.85 – 3.73 (m, 4H), 3.72 – 3.56 (m, 4H), 3.56 – 3.40 (m, 1H), 3.40 – 3.23 (m, 1H), 2.42 – 1.21 (m, 11H). ^{13}C NMR (100MHz, $CDCl_3$, selected signals): δ 159.52, 152.87, 140.58, 138.88, 138.60, 133.70, 130.51, 129.51, 129.12, 128.78, 128.75, 128.46, 128.45, 128.23, 128.12, 127.85, 114.11, 100.04, 82.43, 76.63, 76.15, 75.55, 75.13, 74.72, 74.34, 71.63, 62.90, 58.77, 55.61, 53.80, 33.67, 32.00, 28.85, 28.78. IR (cm^{-1}): 3441, 2933, 2094, 1731, 1026. MS (ESI+): calcd. for $C_{42}H_{53}N_2O_{11}S^+$ [M + NH_4^+] 793.34, found 793.25. [α]$_D$ (c 1.0, DCM, –44.1).

Fully protected cyclohexyl linked disaccharide (**15**)



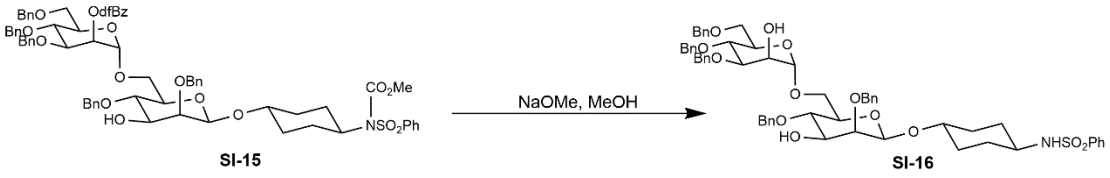
To a 25 mL flask containing 22 mg (0.028 mmol, 1 equiv) of **SI-14** and 36 mg (0.057 mmol, 2 equiv) of donor **SI-8** [14] toluene was added and freeze pumped three times. 100 mg freshly dried 4Å molecular sieves were added to the 25 mL flask. **SI-14** was redissolved in 2 mL DCM and cooled in a –20 °C salt ice bath for 30 minutes. The 25 mL flask was then wrapped in foil and 3.6 mg (0.014 mmol, 0.5 equiv) AgOTf was added, followed by 14 mg (0.060 mmol, 2.1 equiv) of NIS. This mixture was allowed to react for 2.5 hours, and the reaction was quenched with triethylamine. The mixture was filtered through celite and extracted with EA three times. The combined organic layers were dried over $MgSO_4$, filtered, and concentrated. Purification by flash chromatography (1:3:1 ethyl acetate/hexanes/DCM) afforded 18 mg (0.013 mmol, 46%) of **15** as an off–white foam. ^{1}H NMR (400MHz, $CDCl_3$): δ 7.88 (app d, 2H), 7.72 – 7.62 (m, 1H), 7.58 (app t, 1H), 7.52 – 7.44 (m, 4H), 7.36 – 7.02 (m, 27H + $CHCl_3$), 6.83 (app d, 2H), 5.71 (s, 1H), 5.09 – 4.98 (m, 2H), 4.97 – 4.86 (m, 2H), 4.86 – 4.76 (m, 1H), 4.76 – 4.59 (m, 2H), 4.53 – 4.34 (m, 8H), 4.09 – 3.89 (m, 3H), 3.88 – 3.79 (m, 6H), 3.77 – 3.69 (m, 2H), 3.64 – 3.57 (m, 2H), 3.57 – 3.46 (m, 4H), 3.43 – 3.26 (m, 1H), 2.36 – 2.17 (m, 3H), 2.13 – 2.00 (m, 1H), 1.96 – 1.79 (m, 1H), 1.78 – 1.68 (m, 1H), 1.64 – 1.38 (m, 2H). ^{13}C NMR (100MHz, $CDCl_3$, selected signals): δ 160.66, 153.93, 141.85, 140.38, 140.09, 139.87, 139.86, 139.38, 134.72, 131.72, 130.72, 130.20, 129.98, 129.81, 129.77, 129.69, 129.65, 129.55, 129.37, 129.27, 129.20, 129.12, 129.05, 129.02, 128.87, 120.01, 119.76, 115.24, 101.72, 99.37, 83.76, 79.44, 78.05, 76.48, 76.21, 76.02, 75.72, 75.43, 75.24, 74.70, 73.01, 72.91, 72.52, 71.10, 70.33, 68.71, 60.03, 56.77, 54.78, 34.95, 33.34, 30.16, 29.96. IR (cm^{-1}): 3020, 2820, 1733, 1084. MS (ESI+): calcd. for $C_{76}H_{83}F_2N_2O_{17}S^+$ [M + NH_4^+] 1365.54, found 1365.51. [α]$_D$ (c 0.5, DCM, –37.8).

PMB deprotected disaccharide (**SI-15**)



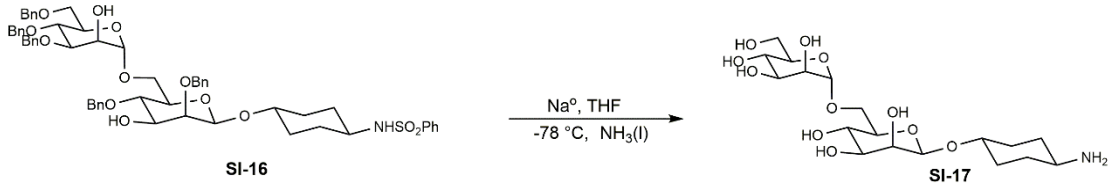
To a flask containing 15 mg (0.013 mmol, 1 equiv) of **15** was added 1 mL of DCM and 0.06 mL of 1M pH 7 phosphate buffer. This mixture was cooled to 0 °C, and 6 mg (0.03 mmol, 2.4 equiv) DDQ was added, allowed to stir for 6 hours, and the reaction was quenched with aqueous $NaHCO_3$ solution. The mixture was diluted with DCM, and the organic phase was washed with water. The aqueous phase was extracted with DCM three times, and the combined organic layers were dried over $MgSO_4$, filtered, and concentrated. Purification by flash chromatography (1:2 ethyl acetate/hexanes) afforded 7 mg (0.006 mmol, 46%) of **SI-15** as an off–white foam. ^{1}H NMR (400MHz, $CDCl_3$): δ 7.88 (app d, J = 8.0 Hz, 2H), 7.74 – 7.04 (m, 31H + $CHCl_3$), 5.70 (s, 1H), 5.23 – 5.02 (m, 2H), 4.80 – 4.97 (m, 2H), 4.80 – 4.29 (m, 9H), 4.14 – 3.32 (m, 16H), 2.49 (app d, J = 10.1 Hz, 1H), 2.39 – 2.18 (m, 2H), 2.18 – 2.06 (m, 1H), 1.97 – 1.81 (m, 1H), 1.81 – 1.66 (m, 1H), 1.52 – 1.39 (m, 2H). MS (ESI+): calcd. for $C_{68}H_{75}F_2N_2O_{16}S^+$ [M + NH_4^+] 1245.48, found 1245.48.

Partially deprotected cyclohexyl linked disaccharide (**SI-16**)



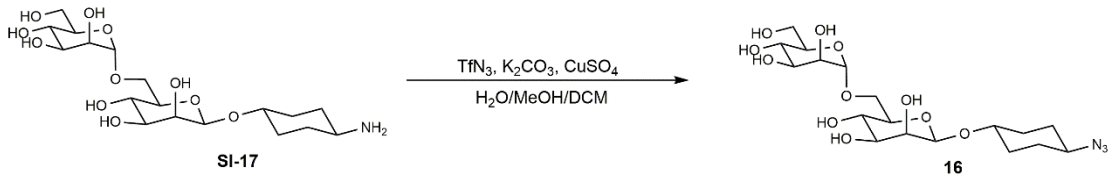
12 mg (0.011 mmol, 1 equiv) of **SI-15** was dissolved in 5 mL MeOH and 2 mL THF in a 25 mL flask. 111 µL (0.40 mmol, 40 equiv) of a 25% NaOMe solution in MeOH was added. The reaction was left stirring at room temperature under N_2 for 13 hours until reaction was completed based on LC–MS and TLC monitoring. Upon completion, the volume of the mixture was reduced to a half by rotary evaporation, and saturated NH_4Cl solution was added to quench the reaction. The mixture was extracted with DCM three times. The combined organic layers were dried over $MgSO_4$, filtered and concentrated. Purification by flash chromatography (1:2 ethyl acetate/hexanes) afforded 6 mg (0.006 mmol, 55%) of **SI-16** as an off–white foam. ^{1}H NMR (400MHz, $CDCl_3$): δ 7.81 (app d, J = 7.2 Hz, 2H), 7.55 (app t, J = 7.6 Hz, 1H), 7.48 (app t, J = 8 Hz, 2H), 7.36 – 7.23 (m, 23H + $CHCl_3$), 7.21 – 7.10 (m, 2H), 5.03 – 4.95 (m, 2H), 4.92 – 4.76 (m, 2H), 4.72 – 4.61 (m, 2H), 4.60 – 4.53 (m, 2H), 4.53 – 4.43 (m, 4H), 4.11 – 4.02 (m, 1H), 3.96 (app d, J = 7.4 Hz, 1H), 3.91 – 3.52 (m, 11H), 3.40 (app t, J = 9.1 Hz, 1H), 3.35 – 3.20 (m, 1H), 3.12 – 2.97 (m, 1H), 2.47 – 2.35 (m, 2H), 1.97 – 1.74 (m, 3H), 1.40 – 1.08 (m, 4H), 1.08 – 0.92 (m, 1H).

Fully deprotected cyclohexyl linked disaccharide amine (**SI-17**)

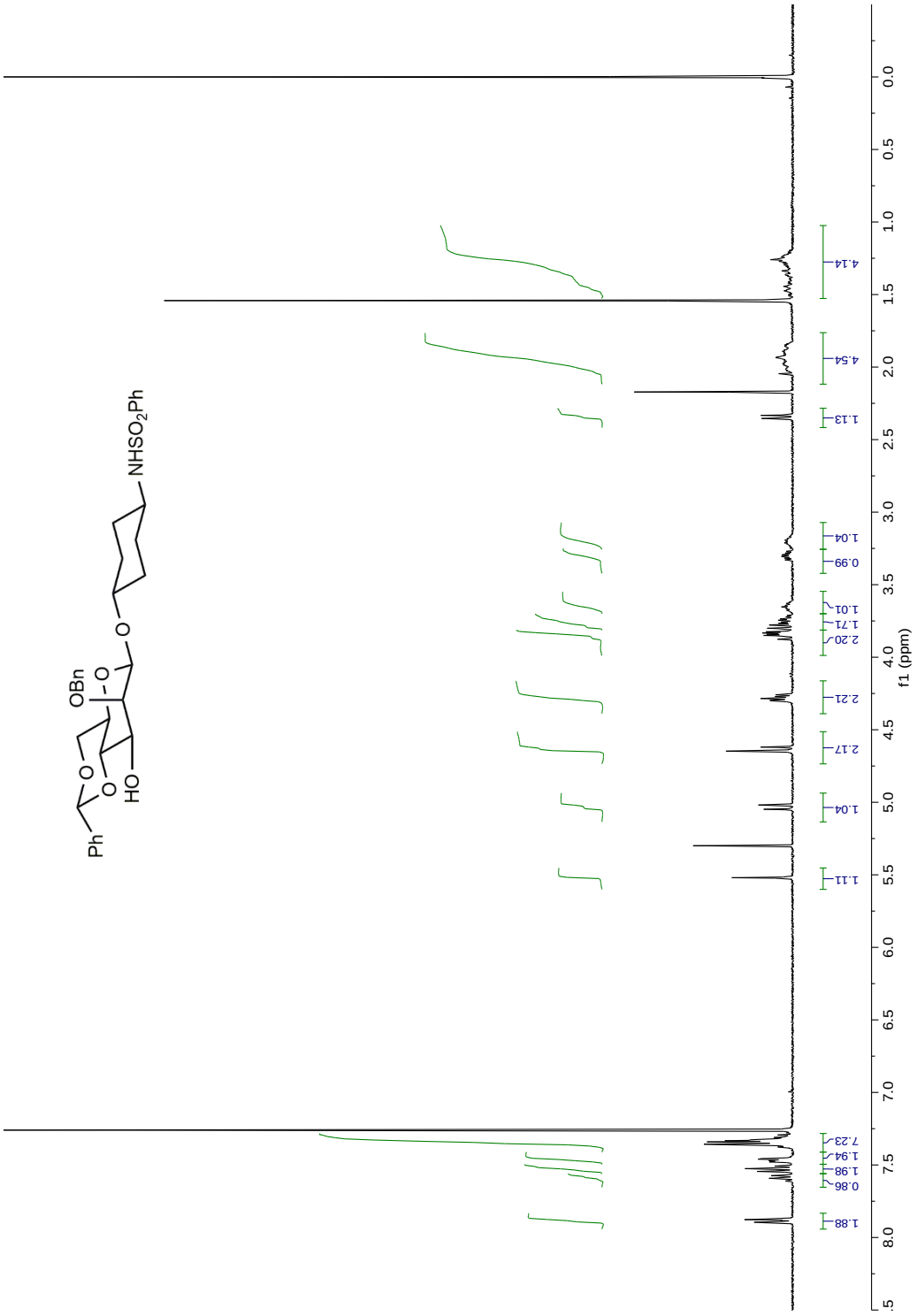


Along with a stream of N_2, ammonia gas was condensed against a -78 °C cold finger into a -78 °C-cooled 50 mL 3-necked flask equipped with a glass-coated stir bar, until 25 mL had accumulated. 8 mg (0.4 mmol, 60 equiv) Na metal was then added, and the resulting blue solution was left to stir for 1 hour. 6 mg (0.006 mmol, 1 equiv) of **SI-16** in 2 mL of THF was then added. The reaction progress was monitored by LC–MS. After 3 hours, 21 mg (0.40 mmol, 69 equiv) NH_4Cl was added to quench the reaction. At this time, the cooling bath was removed and the ammonia was blown off under a stream of N_2. The crude white solids were dissolved in water and desalted by passage through a Biorad P-2 Biogel (Fine) size exclusion column to give 4 mg of partially purified **SI-17**. This material was used in the next step without further purification. ^{1}H NMR (400MHz, D_2O): δ 4.95 (s, 1H), 4.84 (s, 1H), 4.04 – 3.89 (m, 4H), 3.88 – 3.62 (m, 8H), 3.60 – 3.47 (m, 1H), 3.30 – 3.17 (m, 1H), 2.25 – 2.06 (m, 4H), 1.60 – 1.35 (m, 4H). MS (ESI+): calcd. for $C_{18}H_{34}NO_{11}^+$ [M + H^+] 440.21, found 440.35.

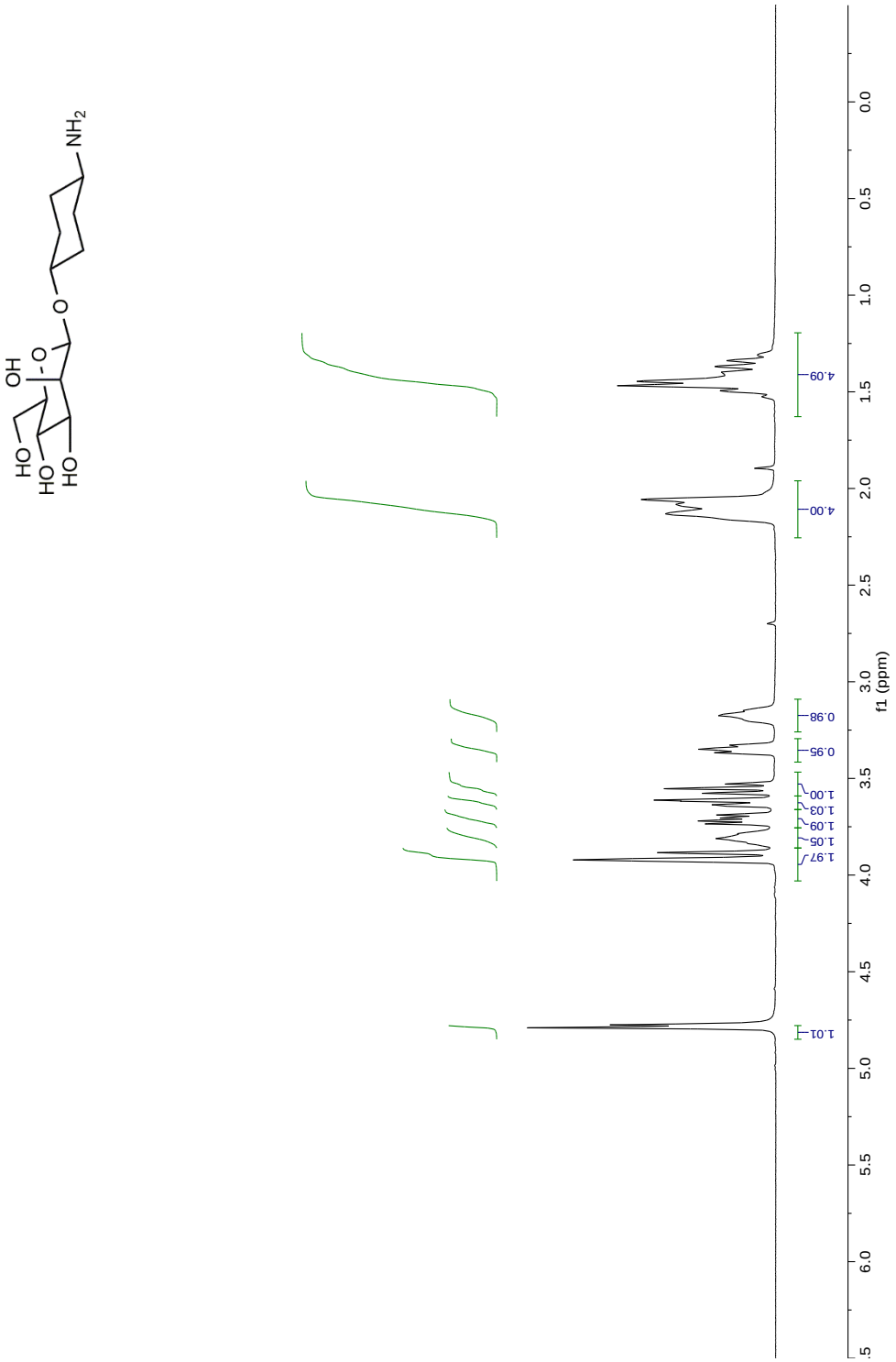
Cyclohexyl linked disaccharide azide (**16**, Man_2–Cy–N_3)



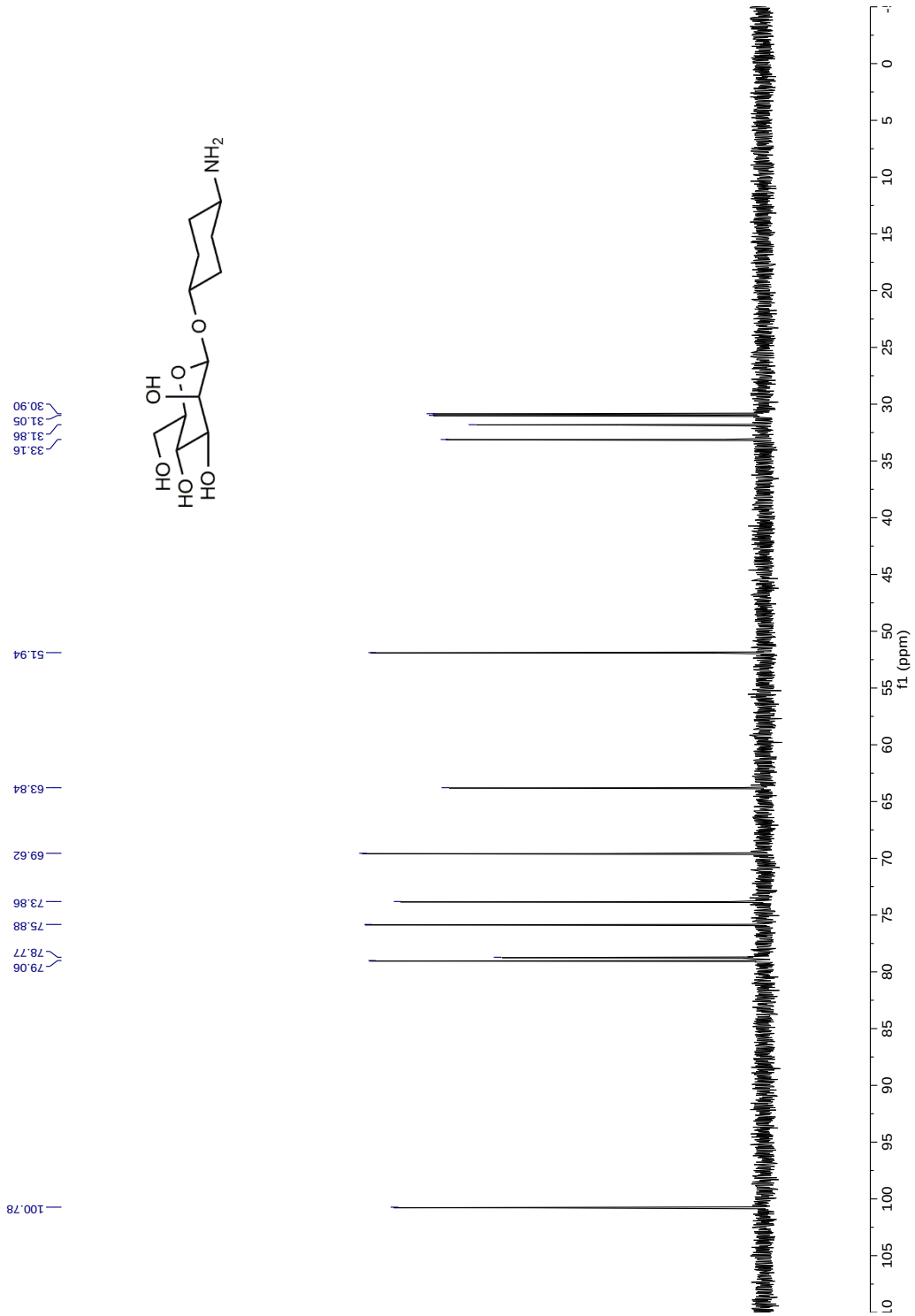
Into a 20 mL vial containing 2.6 mg (0.0060 mmol, 1.0 equiv) of crude amine **SI-17** was added 29 µL water, 2.3 µL (0.0006 mmol, 0.1 equiv) of 0.25M aqueous $CuSO_4$ and 2.4 mg (0.017 mmol, 3.0 equiv) of K_2CO_3. 60 µL of MeOH was added, followed by the addition of 0.23 mL (0.060 mmol, 10.0 equiv) of freshly prepared 0.25M TfN_3 in DCM. The resulting homogeneous reaction was left to stir at room temperature for 4 hours until complete conversion was observed by LC–MS. The reaction was quenched with 6.3 mg (0.075 mmol, 13.0 equiv) solid $NaHCO_3$ and concentrated in vacuo. The residue was desalted on a Biorad P-2 Biogel (Fine) size exclusion column. Reversed phase HPLC purification (Column: Waters Xbridge Prep, C18, 5µm, 130Å, 10x250mm. Method: 4mL/min flow rate. A= H_2O/0.1% FA, B= ACN/0.1% FA. 98.2% A for 1min, then 98.2% A to 60% A over 24 minutes, then 60% to 5% A over 5 minutes. Desired product eluted at 16.2 minutes.) providing 1.4 mg (0.0030 mmol, 50%, 2 steps) of Man_2–Cy–N_3 (**16**) as a glassy solid. ^{1}H NMR (400MHz, D_2O): δ 4.91 (s, 1H), 4.78 (s, 1H), 4.00 – 3.95 (m, 1H), 3.95 – 3.86 (m, 3H), 3.86 – 3.58 (m, 8H), 3.56 – 3.40 (m, 2H), 2.15 – 1.93 (m, 4H), 1.53 – 1.34 (m, 4H).^{13}C NMR (100 MHz, D_2O): δ 102.23, 101.05, 79.33, 77.06, 76.08, 75.53, 73.89, 73.46, 72.79, 69.70, 69.56, 68.80, 63.82, 61.76, 33.19, 31.72, 31.37, 31.25. IR (cm^{-1}): 3338, 2933, 2094, 1046, 974. MS (ESI+): calcd. for $C_{18}H_{35}N_4O_{11}^+$ [M + NH_4^+] 483.23, found 483.40. $[\alpha]_D$ (c 0.06, H_2O, 32.1). ^{1}H NMR Spectrum of **3** (400MHz, $CDCl_3$)


NHSO2Ph
OBn
HO
Ph
f1 (ppm)
4.14
4.54
1.13
1.04
0.99
1.01
1.71
2.20
2.21
2.17
1.04
1.11
7.23
1.94
1.98
0.86
1.88

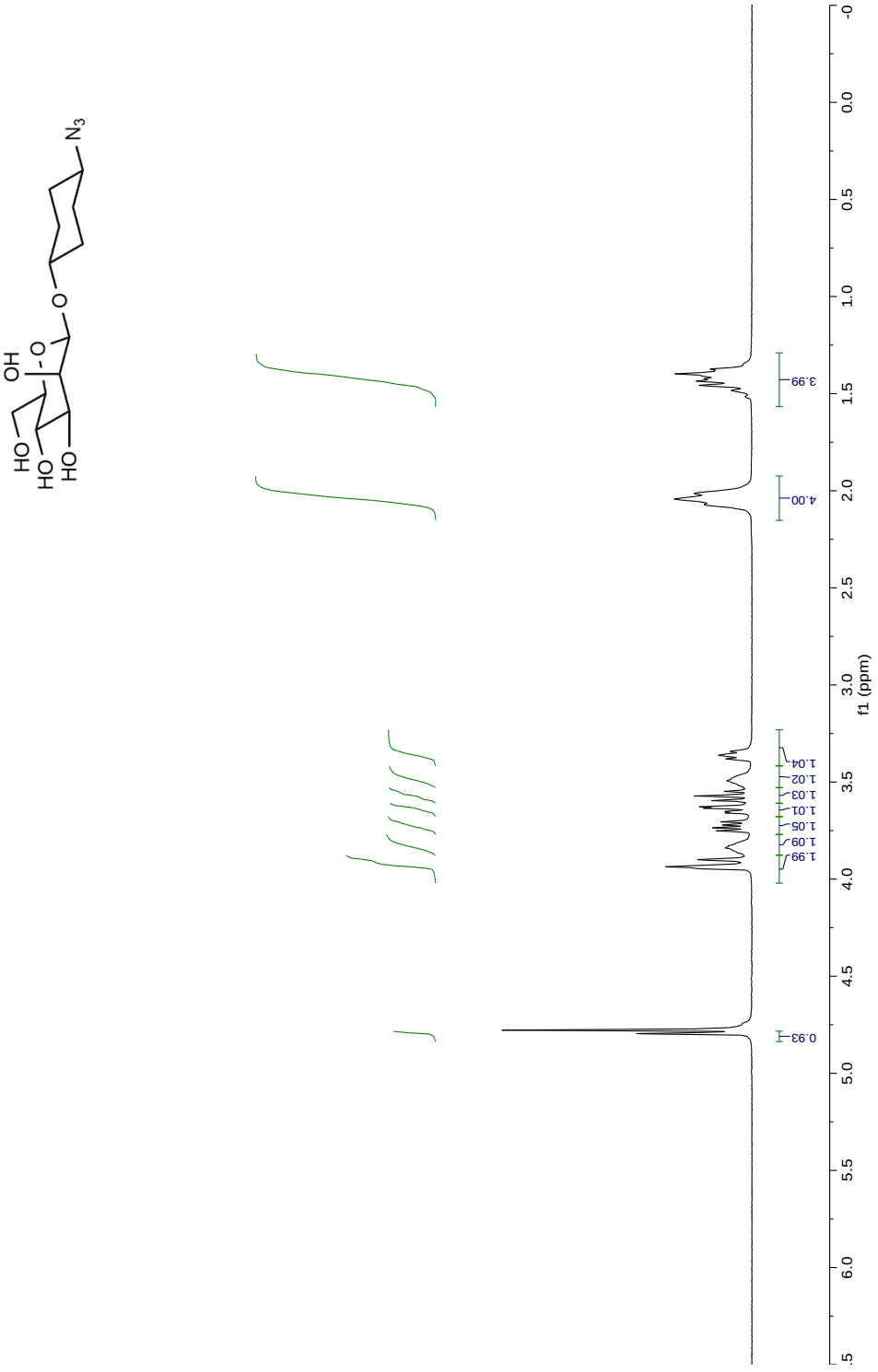
^{1}H NMR Spectrum of **SI-1** (400MHz, D_2O)



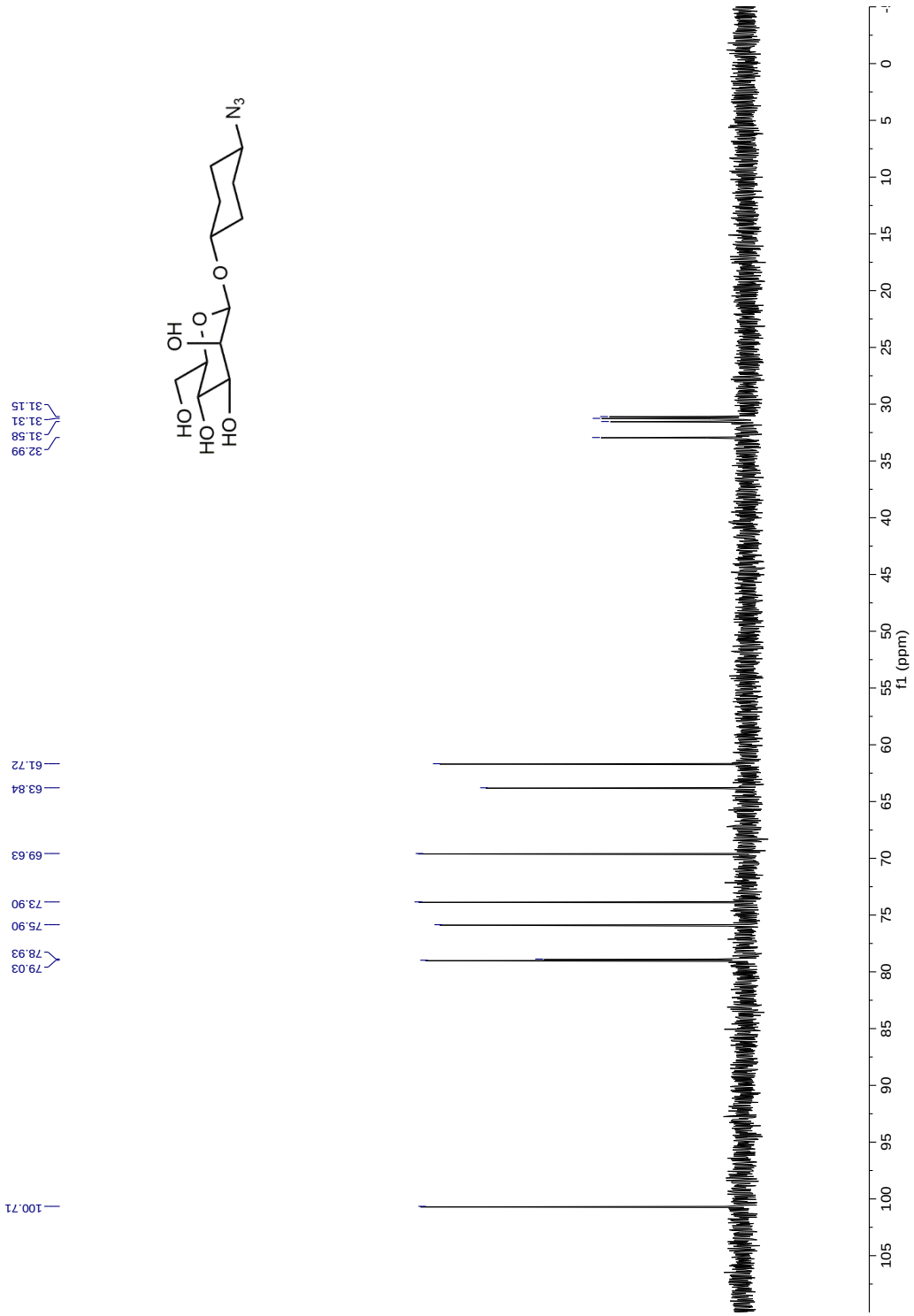
^{13}C NMR Spectrum of **SI-1** (100MHz, D_2O)



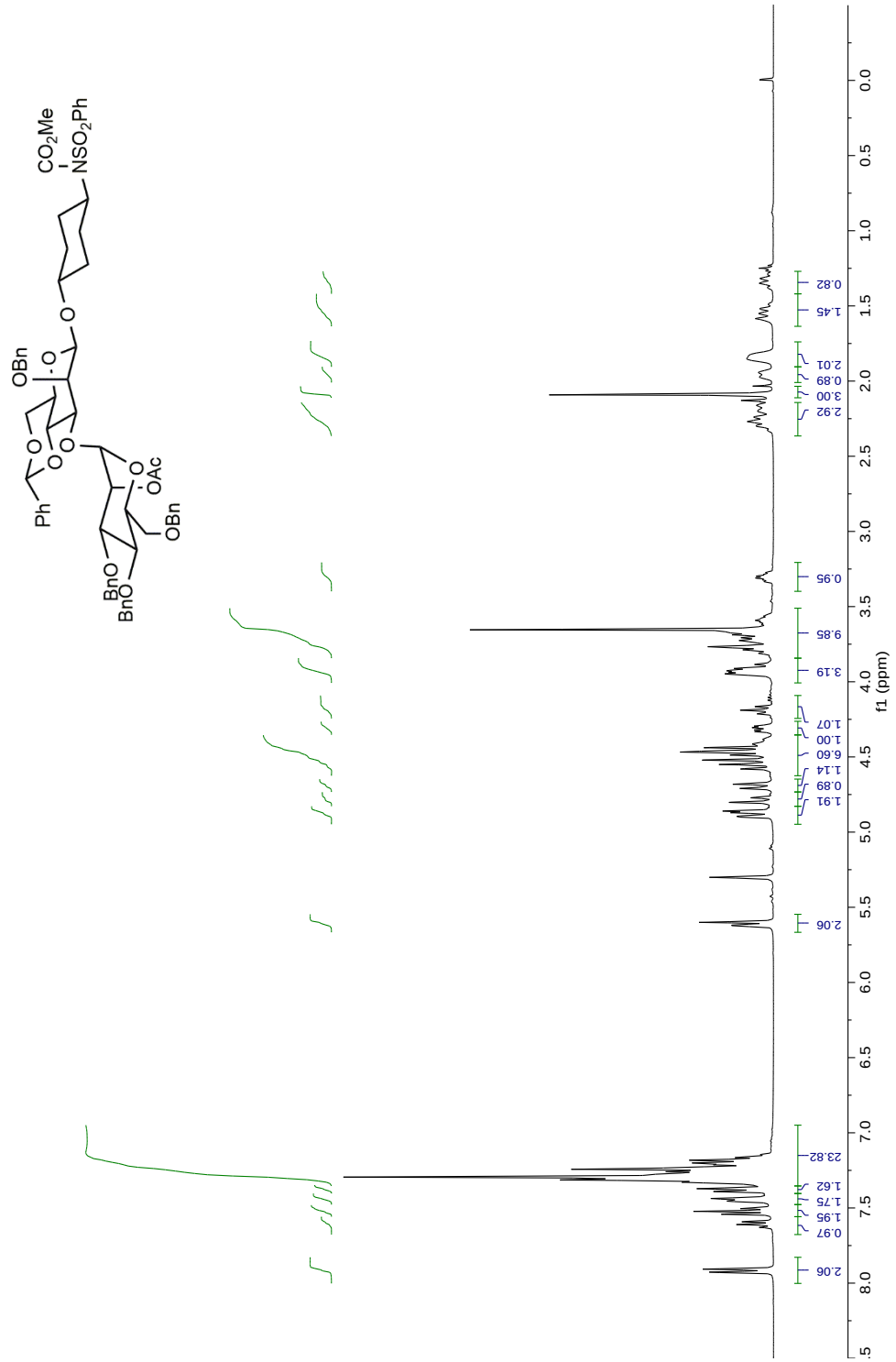
^{1}H NMR Spectrum of **5** (400MHz, D_2O)



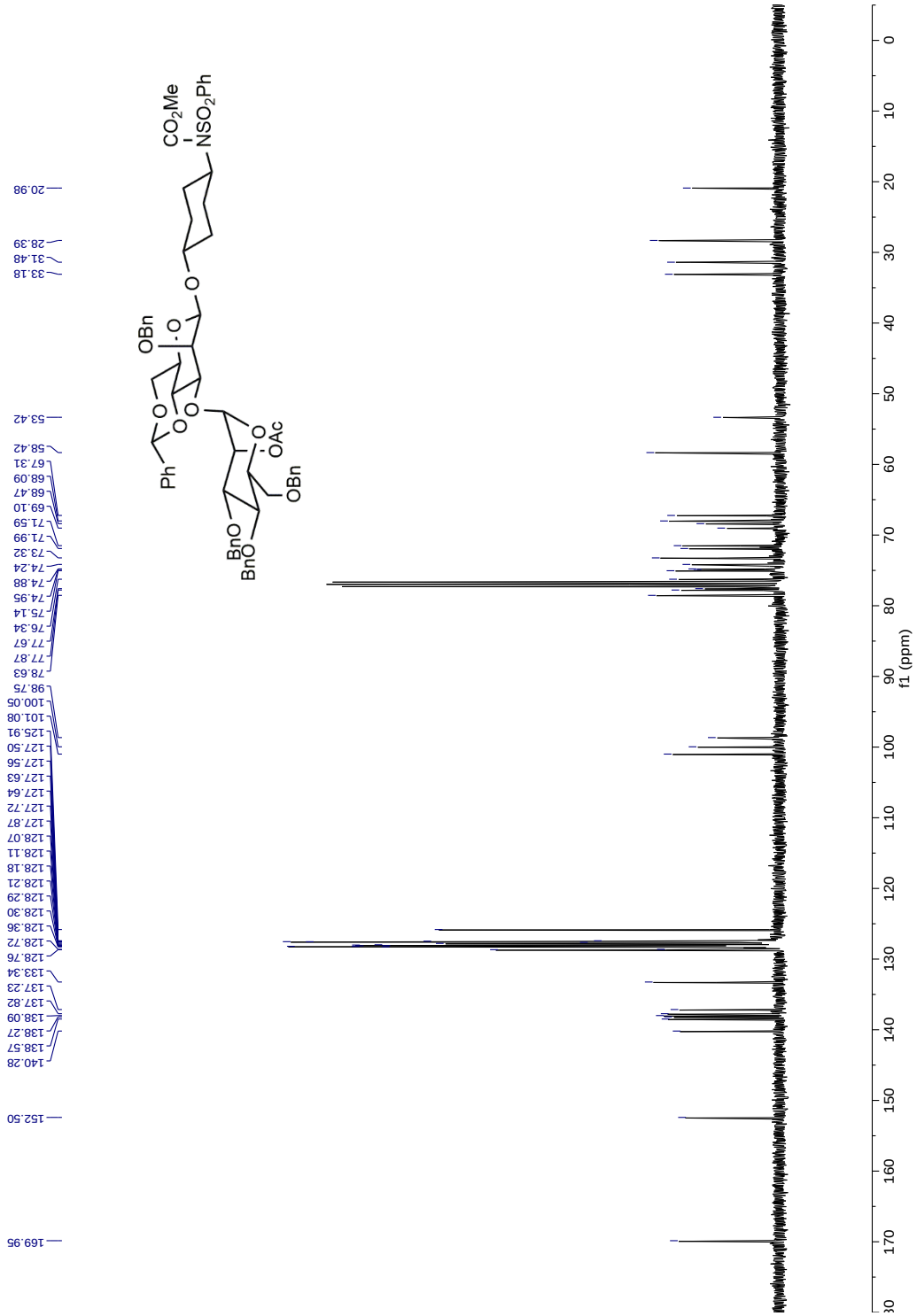
^{13}C NMR Spectrum of **5** (100MHz, D_2O)



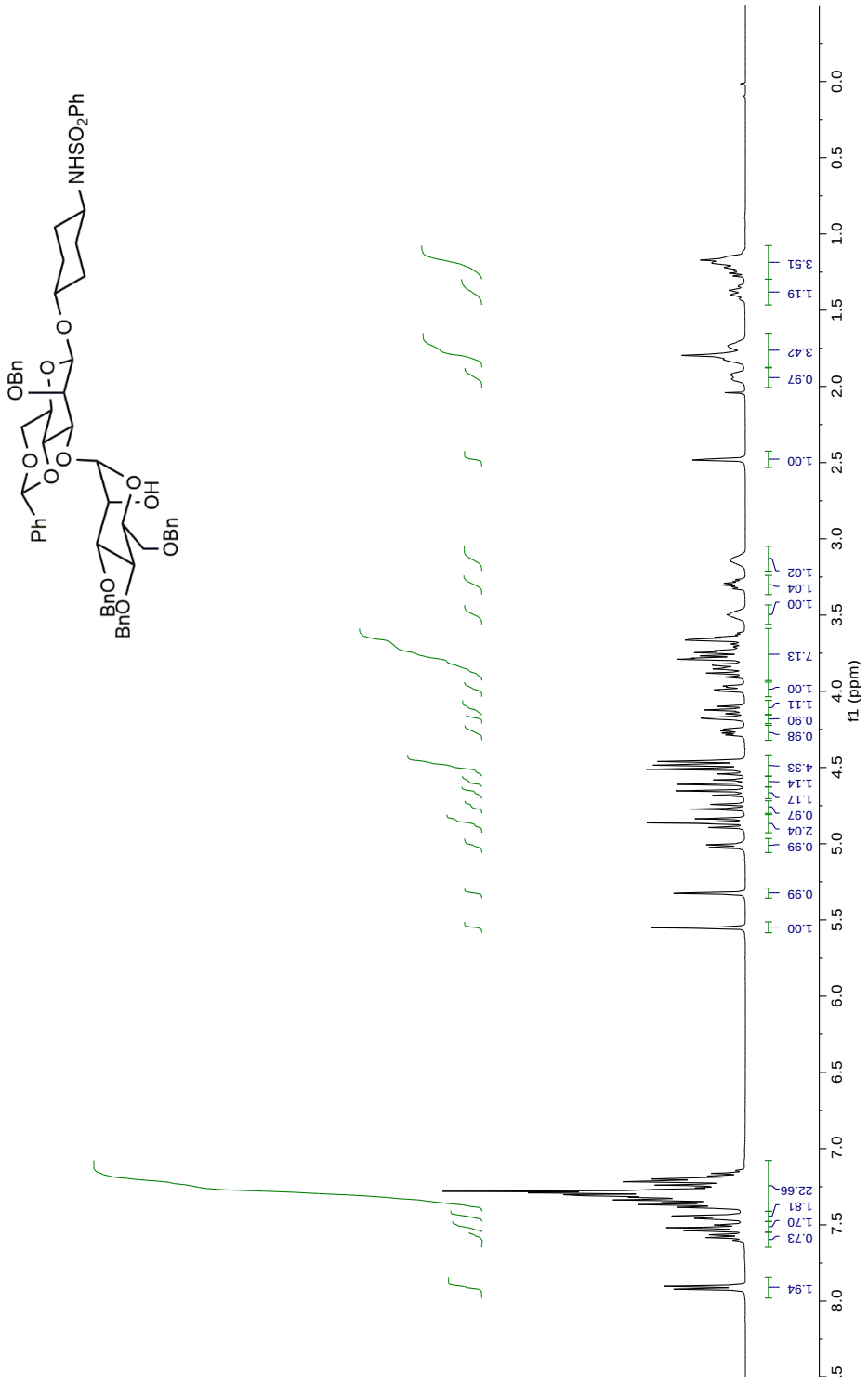
^{1}H NMR Spectrum of **7** (400MHz, $CDCl_3$)



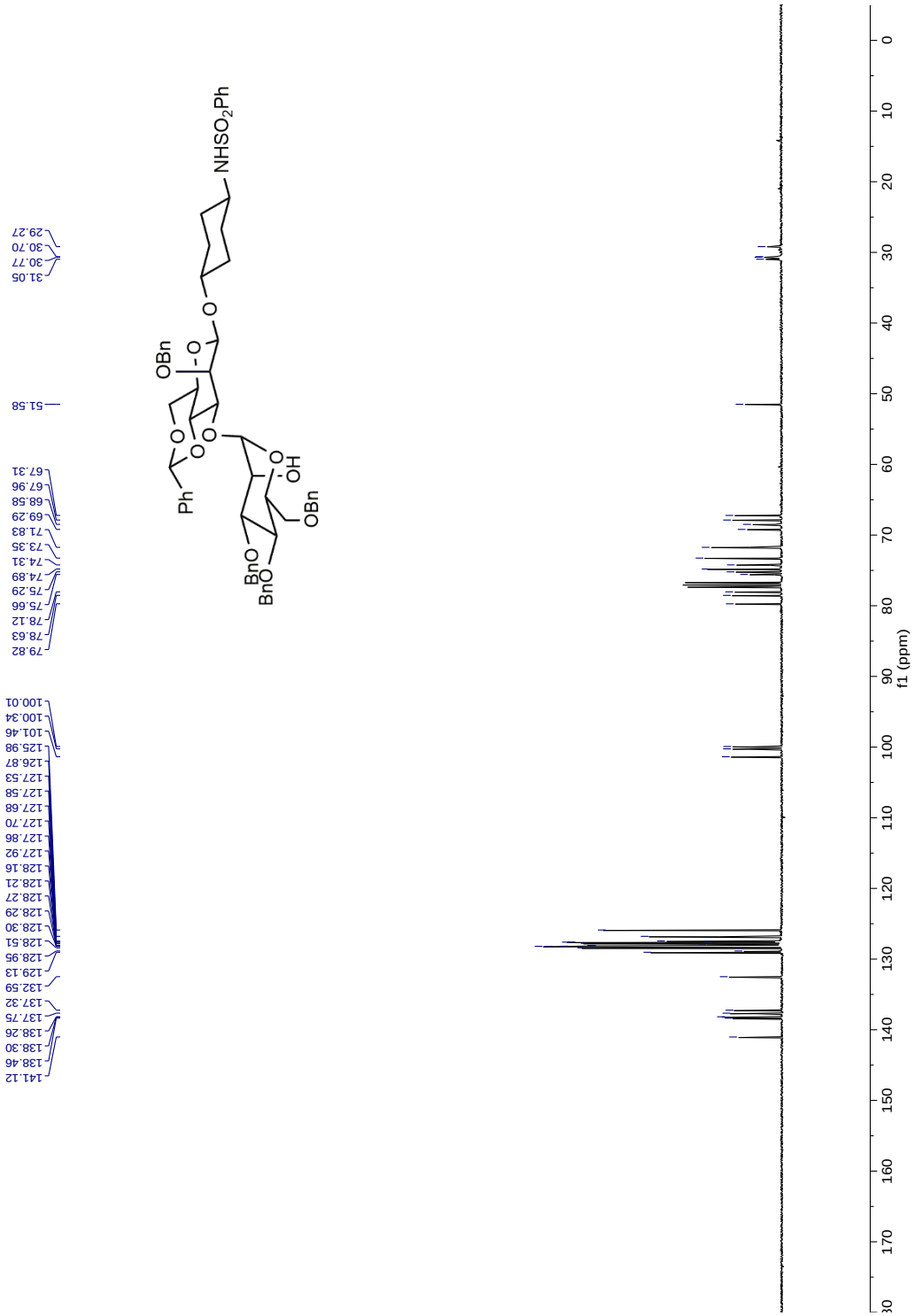
^{13}C NMR Spectrum of **7** (100MHz, $CDCl_3$)



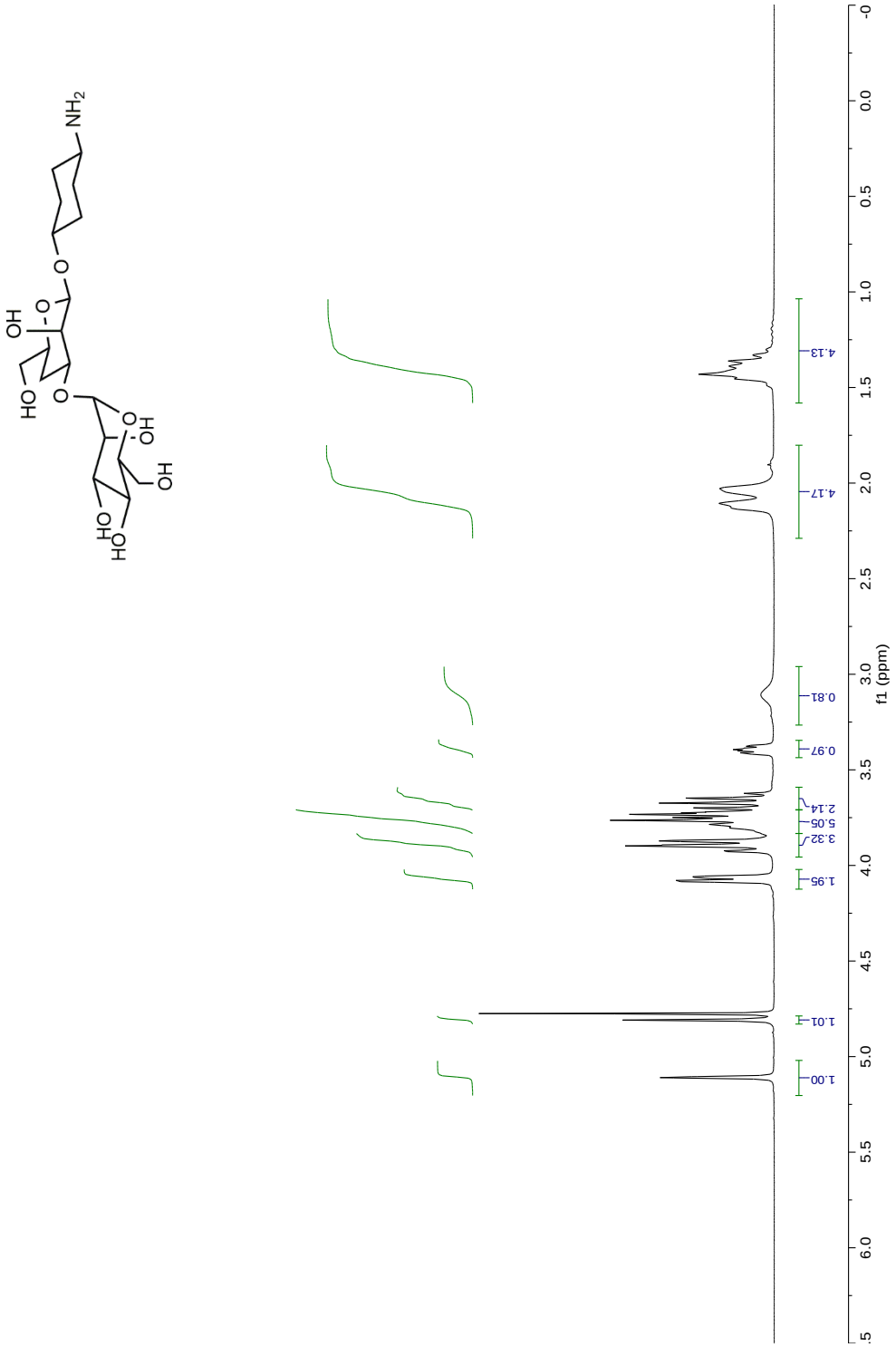
^{1}H NMR Spectrum of **SI-3** (400MHz, $CDCl_3$)



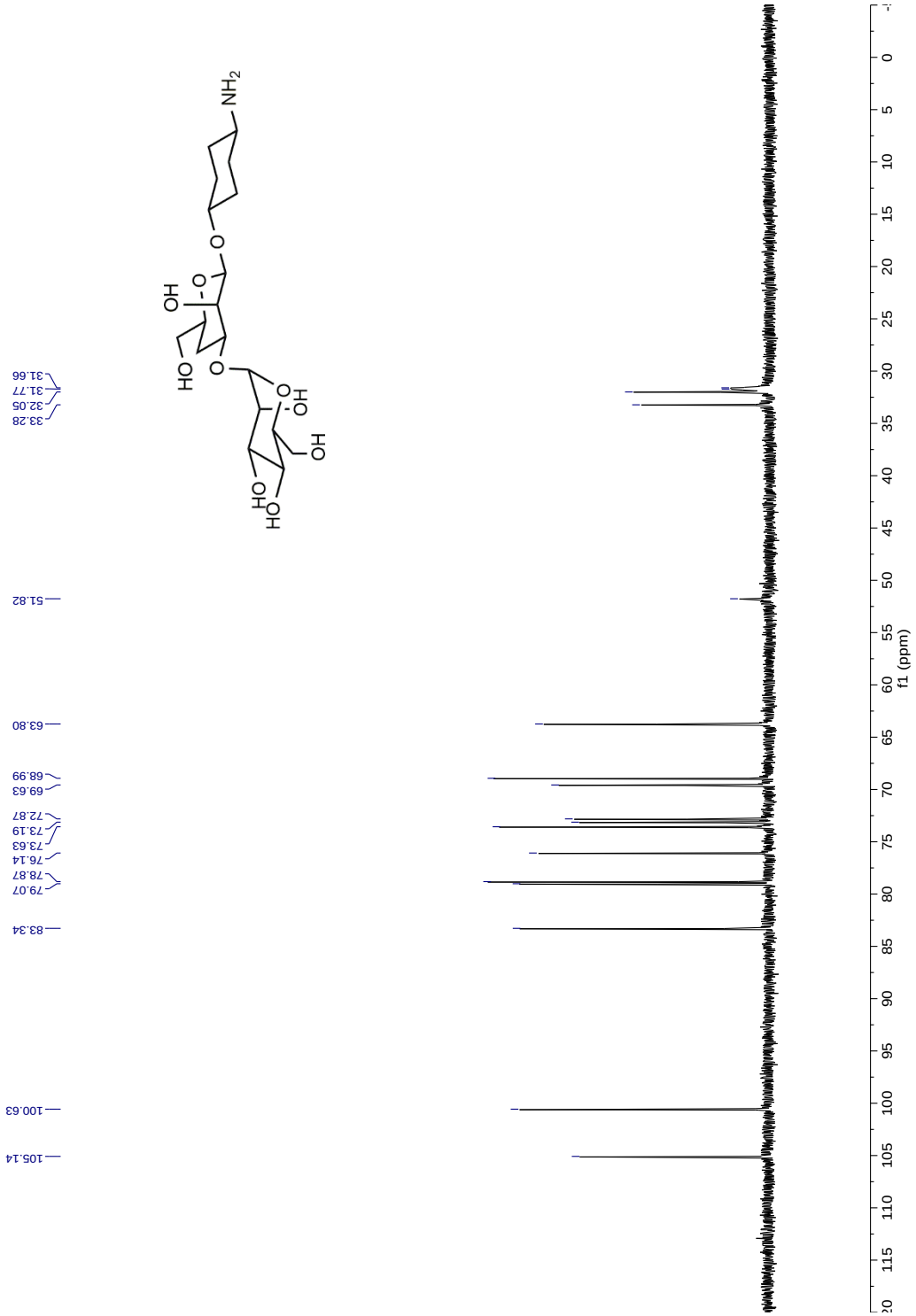
^{13}C NMR Spectrum of **SI-3** (100MHz, $CDCl_3$)



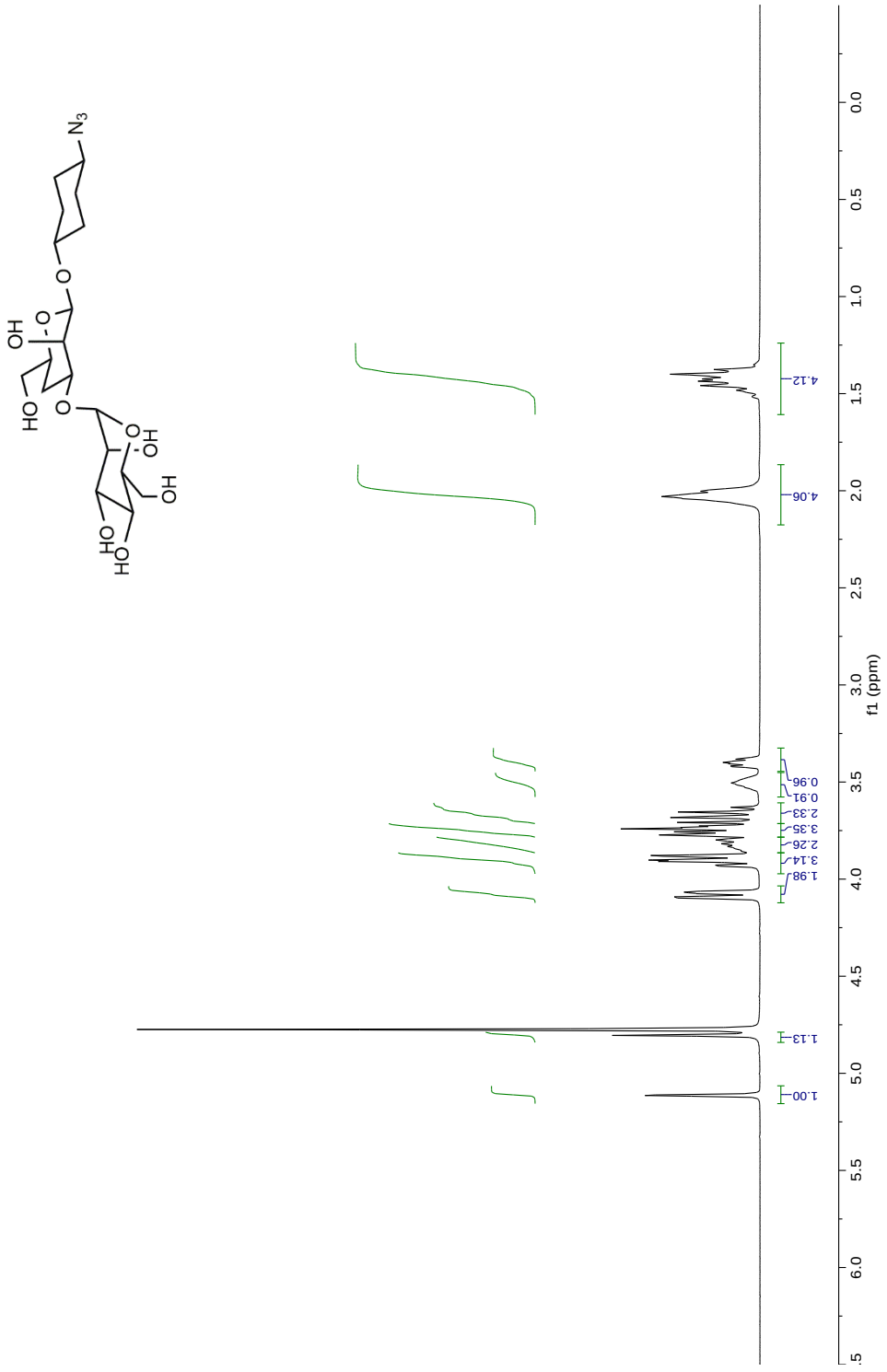
^{1}H NMR Spectrum of **SI-4** (400MHz, D_2O)



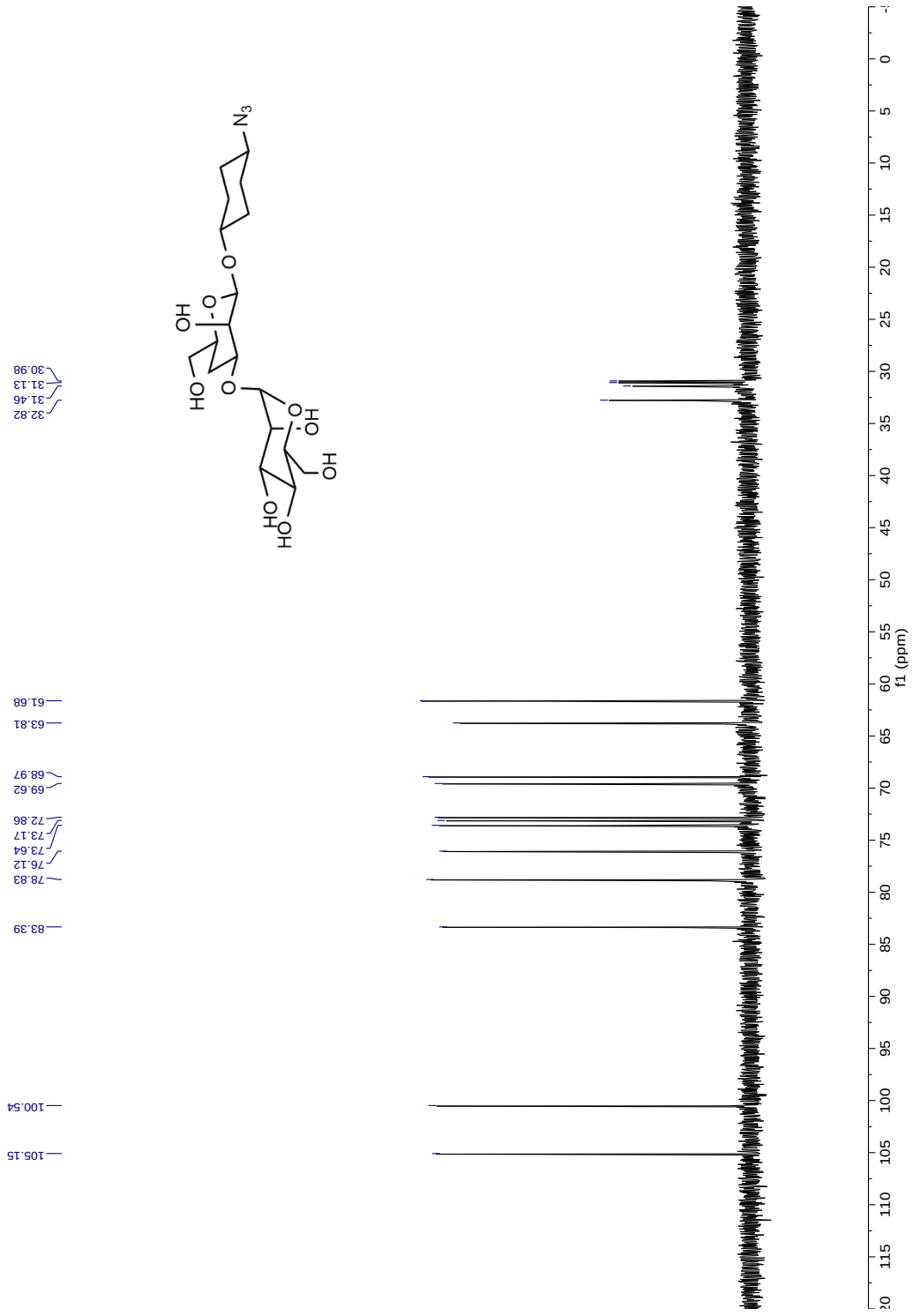
^{13}C NMR Spectrum of **SI-4** (100MHz, D_2O)



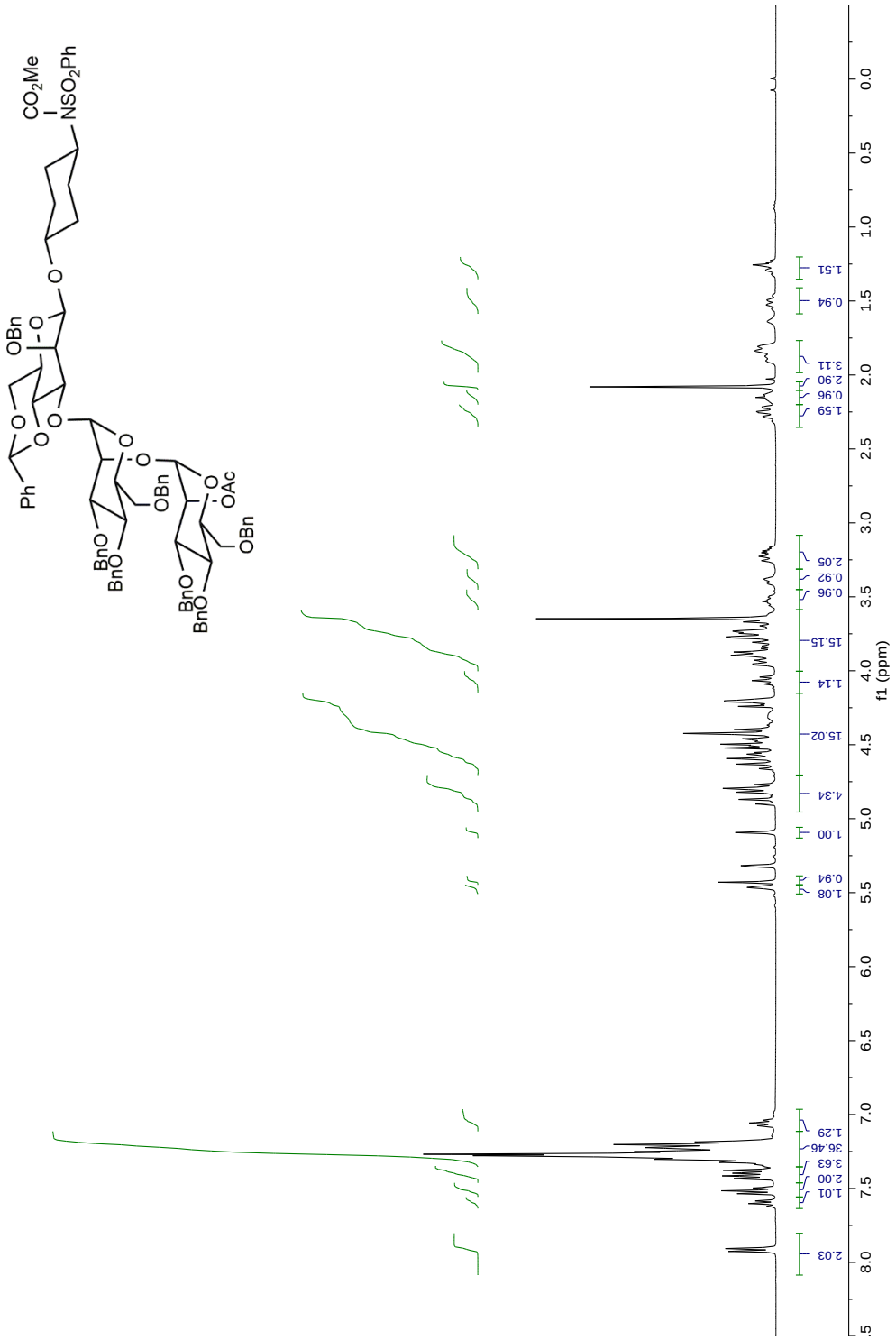
^{1}H NMR Spectrum of **7** (400MHz, D_2O)



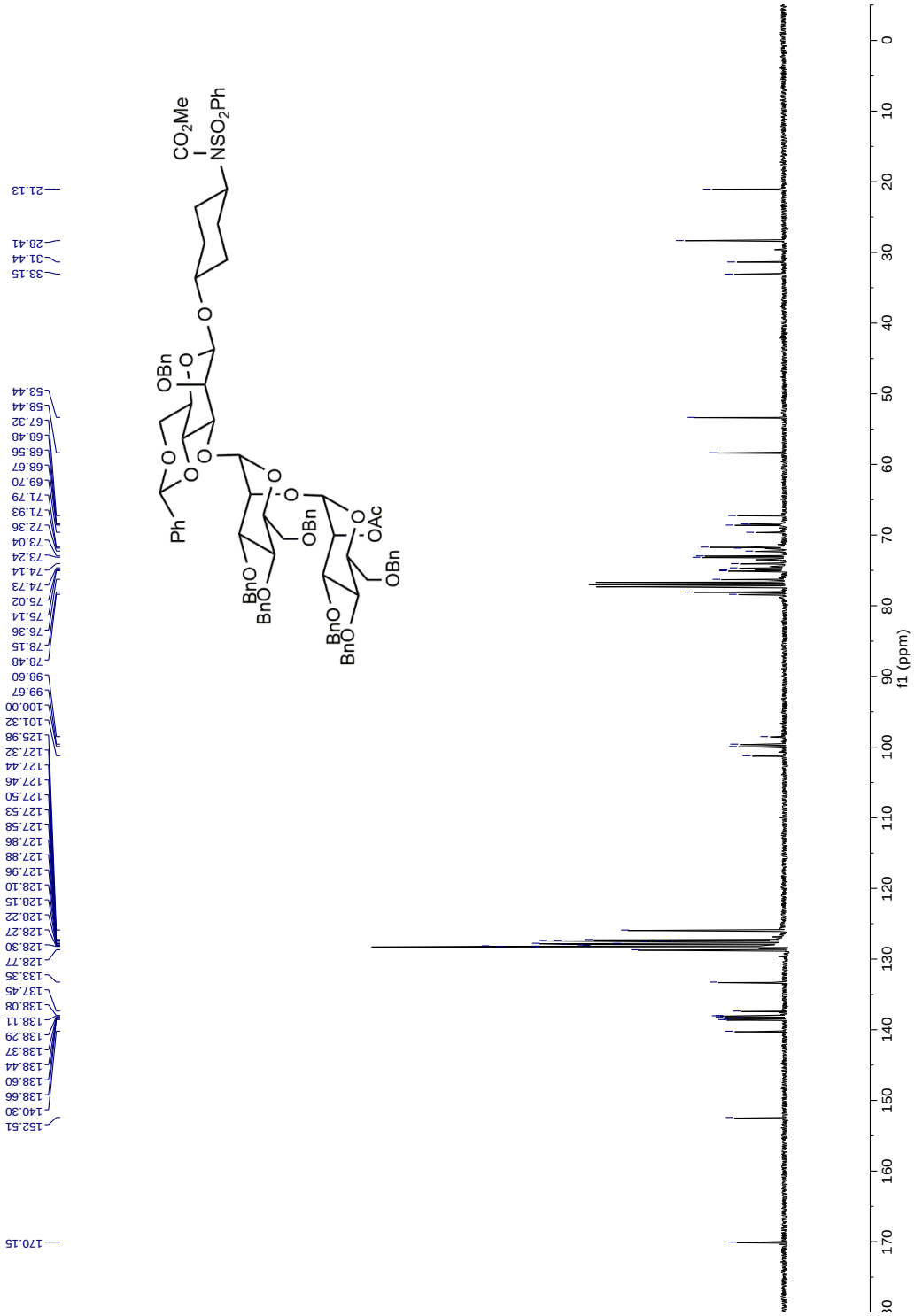
^{13}C NMR Spectrum of **7** (100MHz, D_2O)



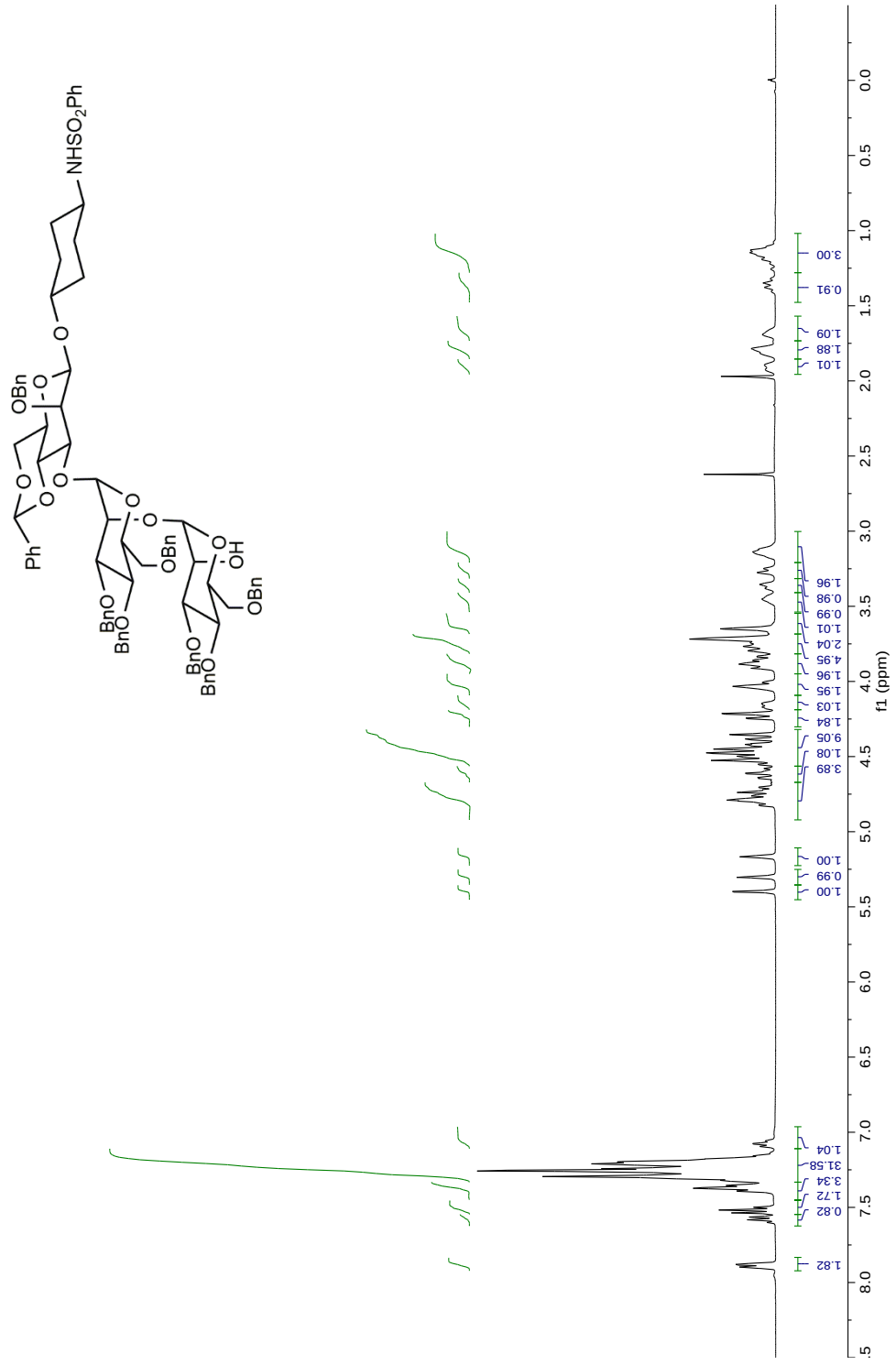
^{1}H NMR Spectrum of **8** (400MHz, $CDCl_3$)



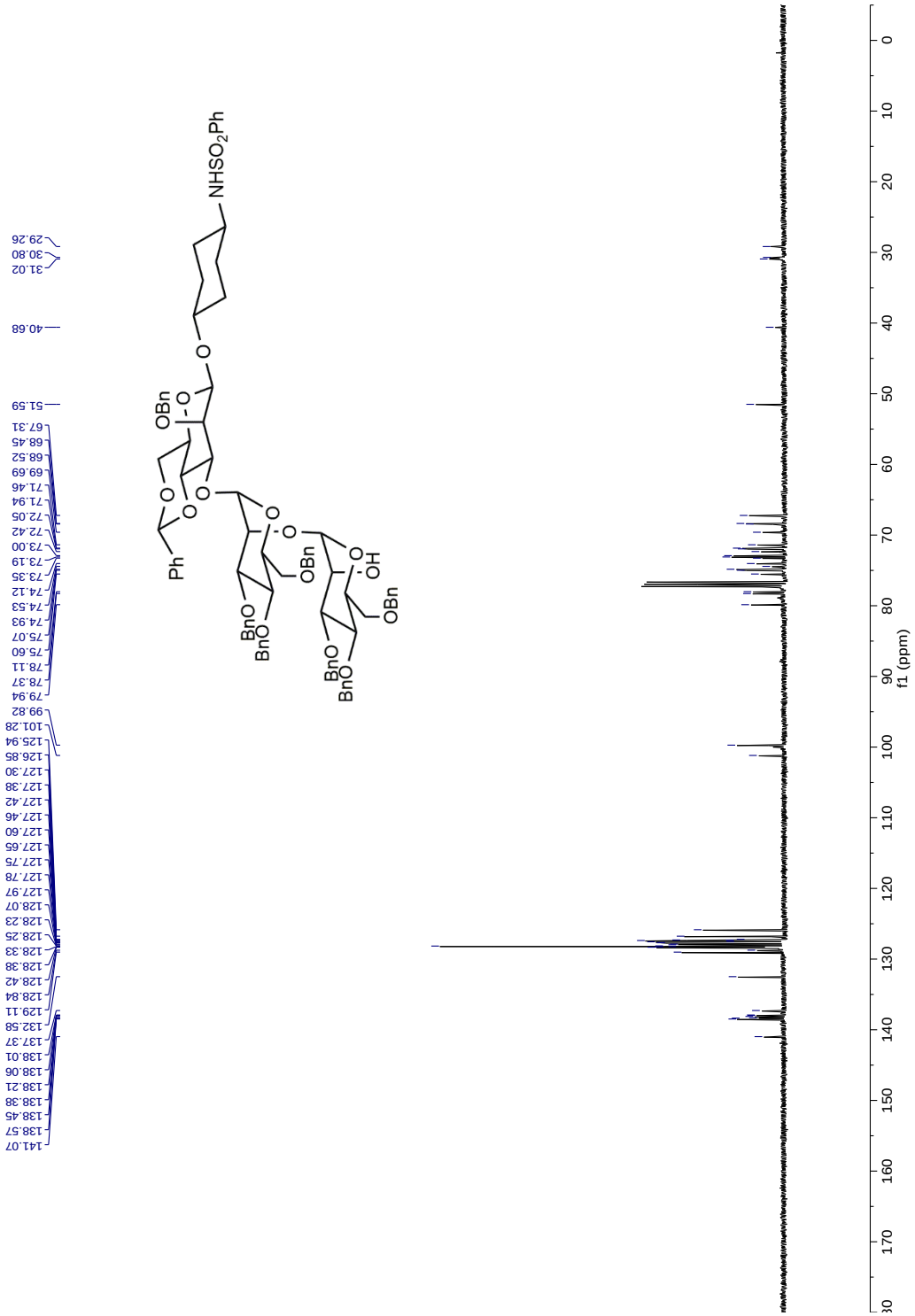
^{13}C NMR Spectrum of **8** (100MHz, $CDCl_3$)



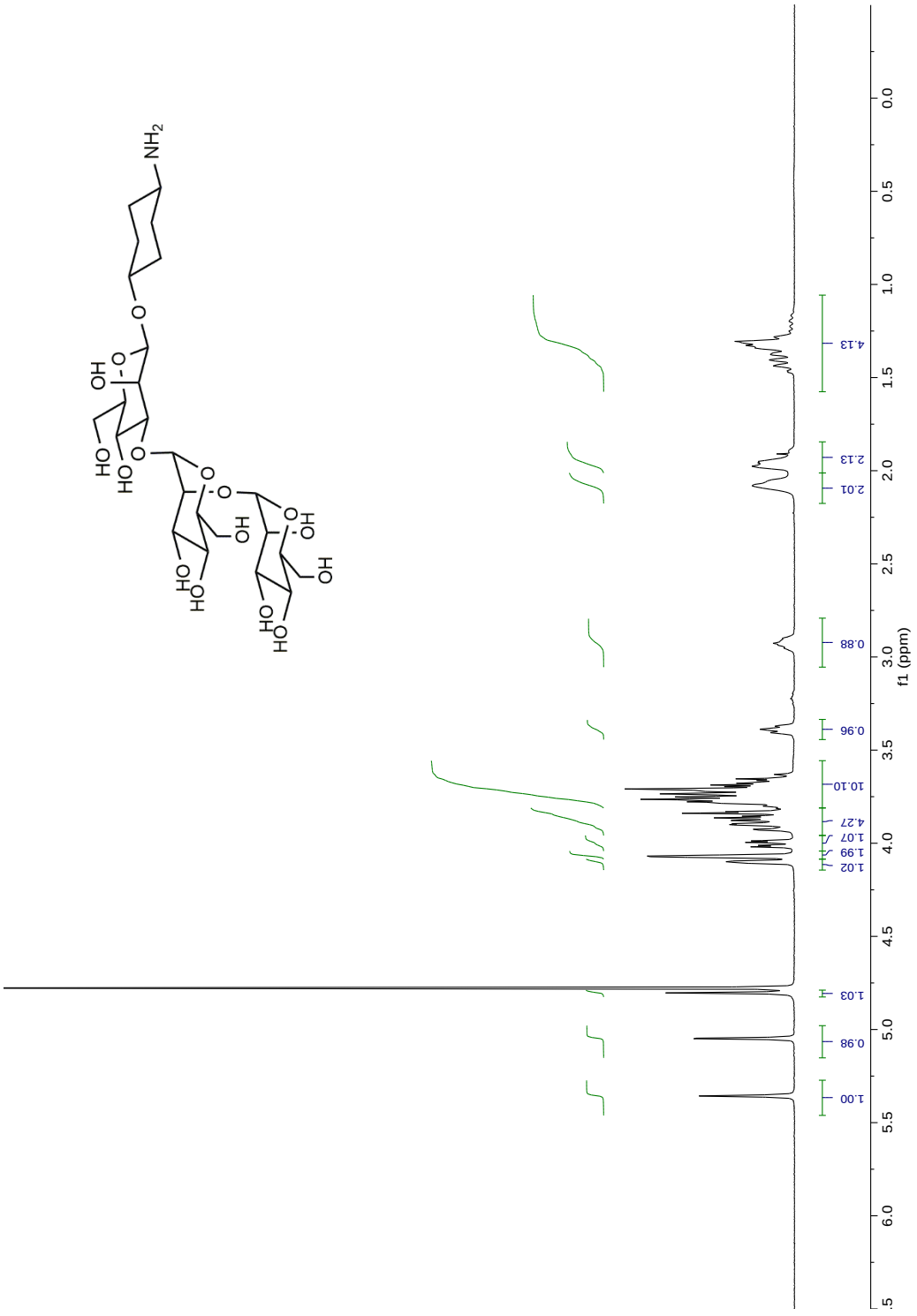
^{1}H NMR Spectrum of **SI-6** (400MHz, $CDCl_3$)



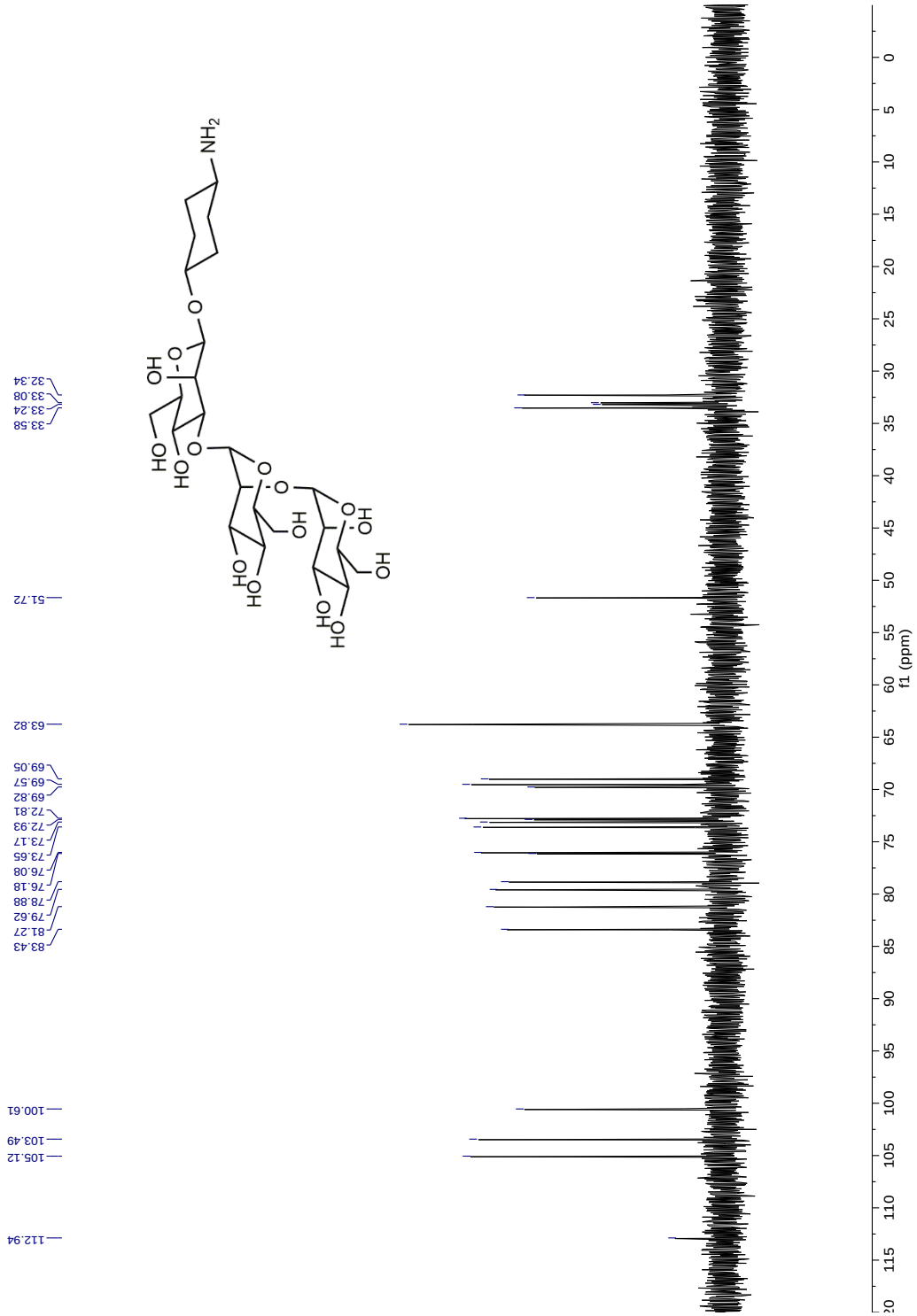
^{13}C NMR Spectrum of **SI-6** (100MHz, $CDCl_3$)



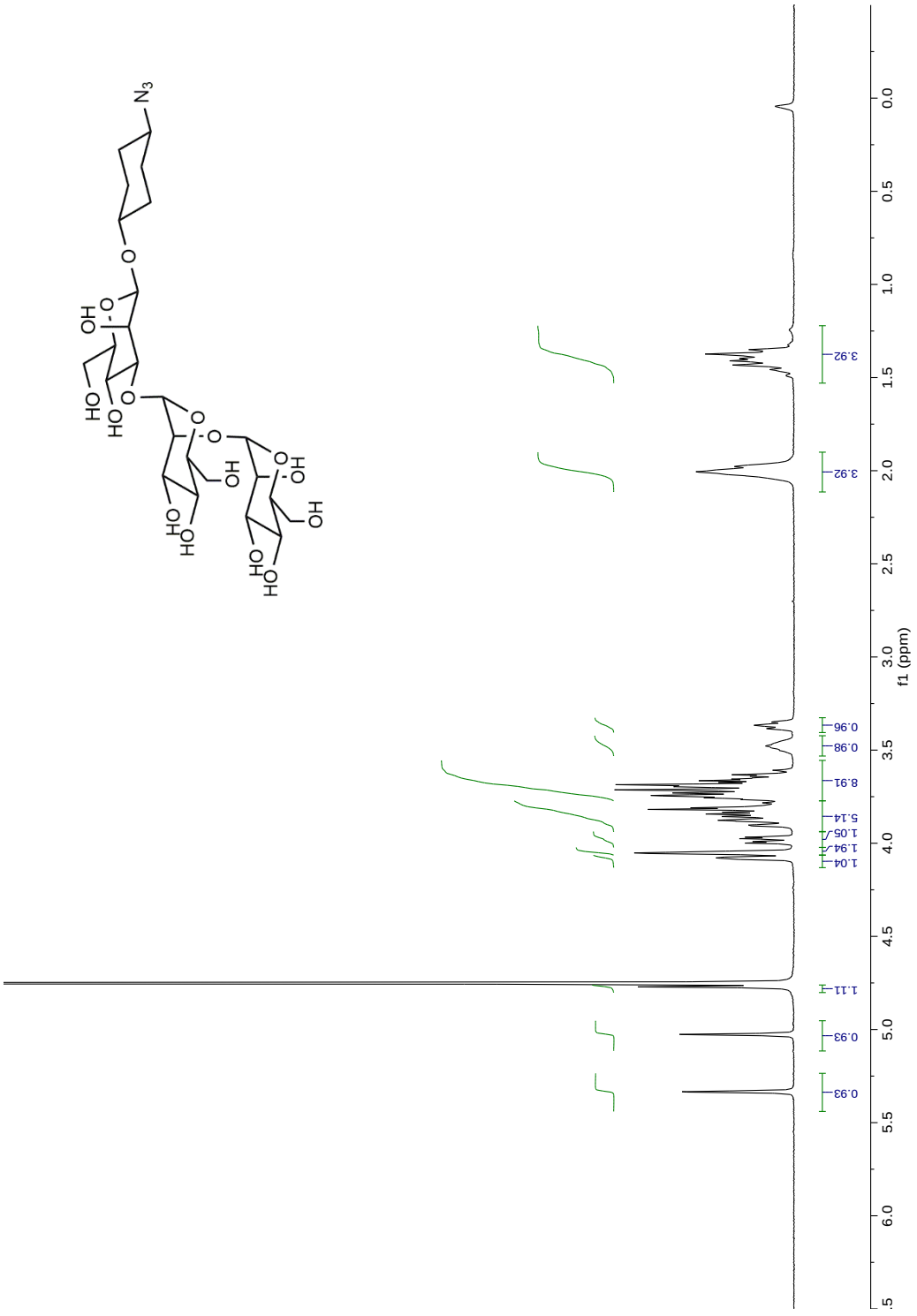
^{1}H NMR Spectrum of **SI-7** (400MHz, D_2O)



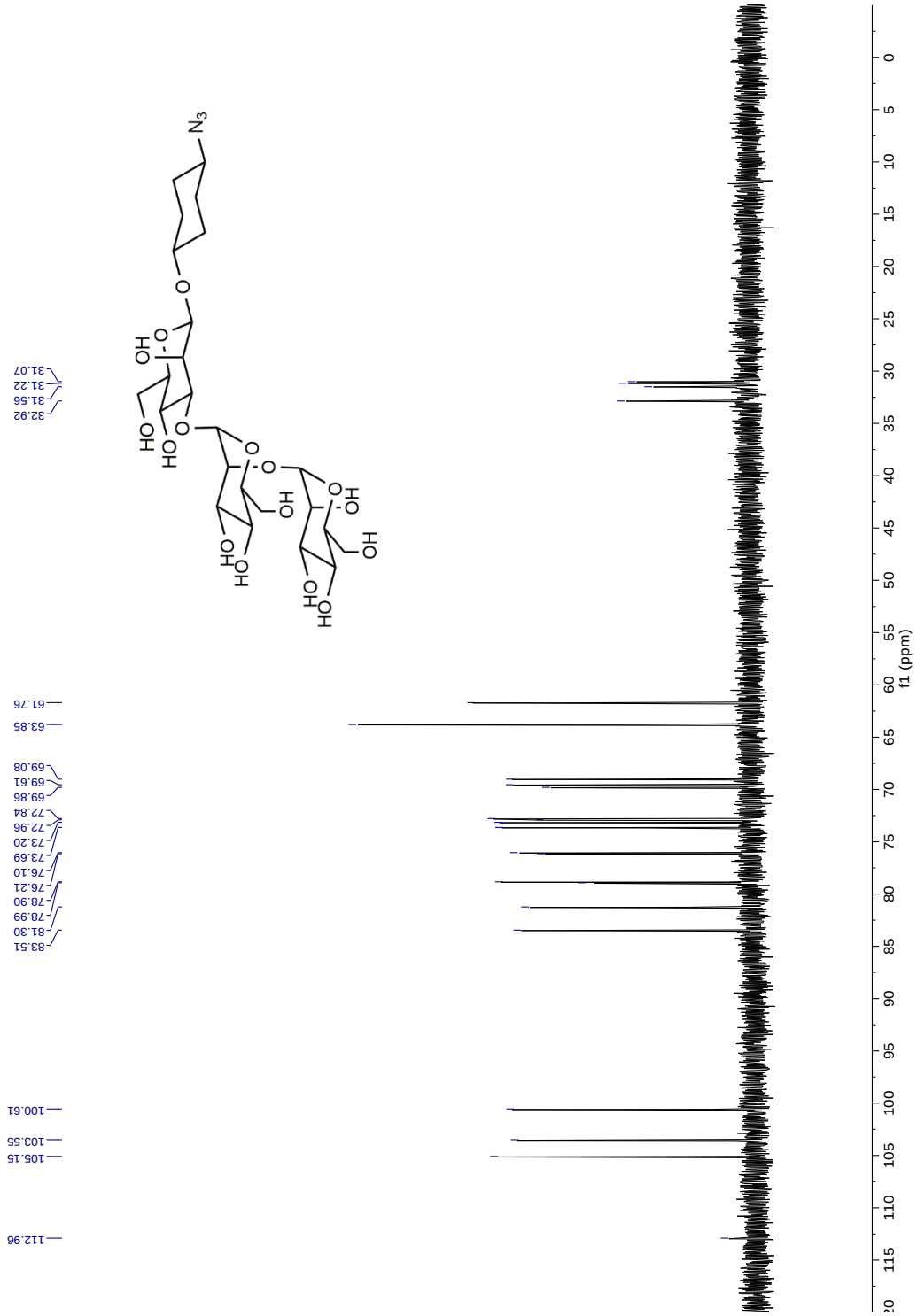
^{13}C NMR Spectrum of **SI-7** (100MHz, D_2O)



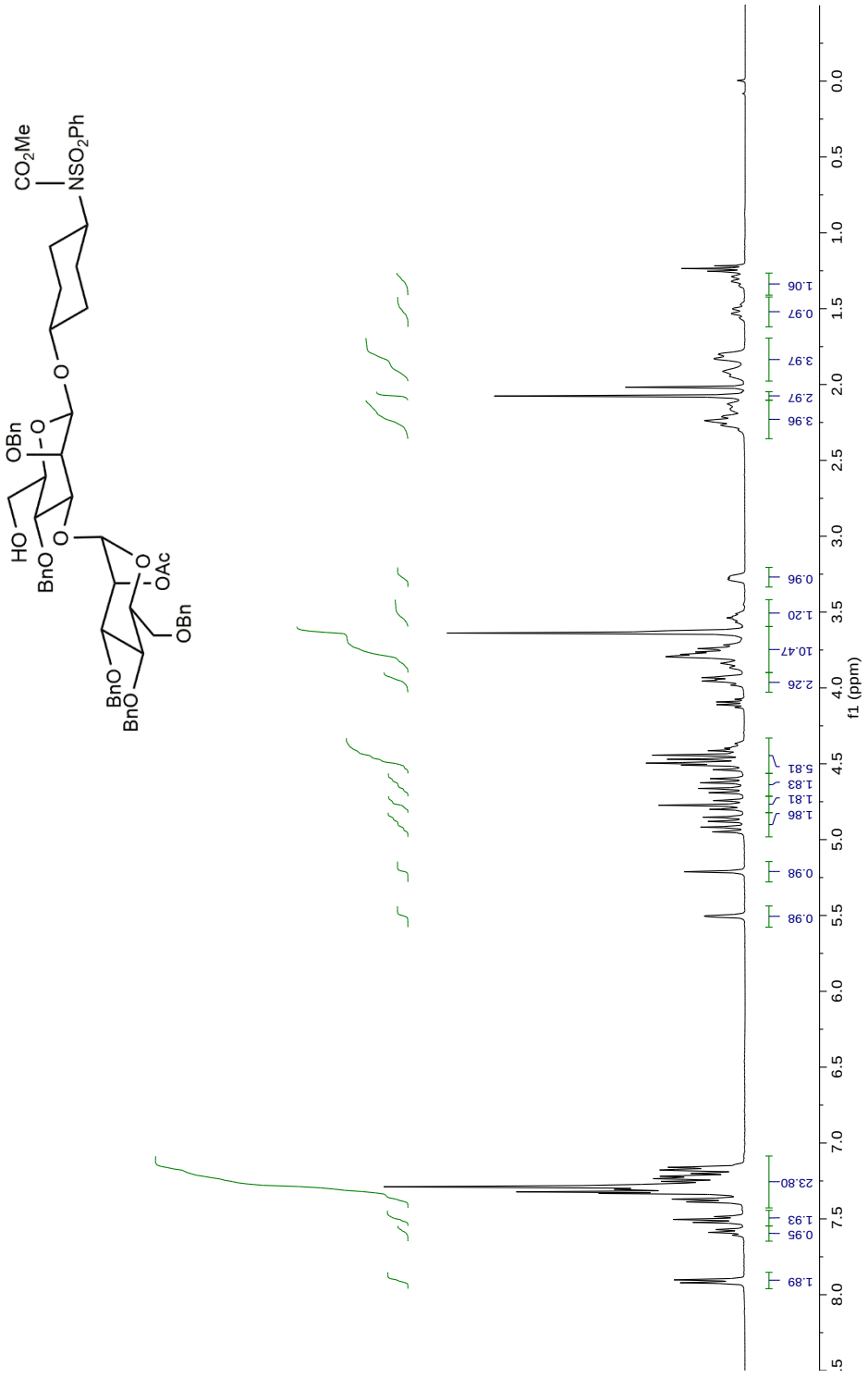
^{1}H NMR Spectrum of **9** (400MHz, D_2O)



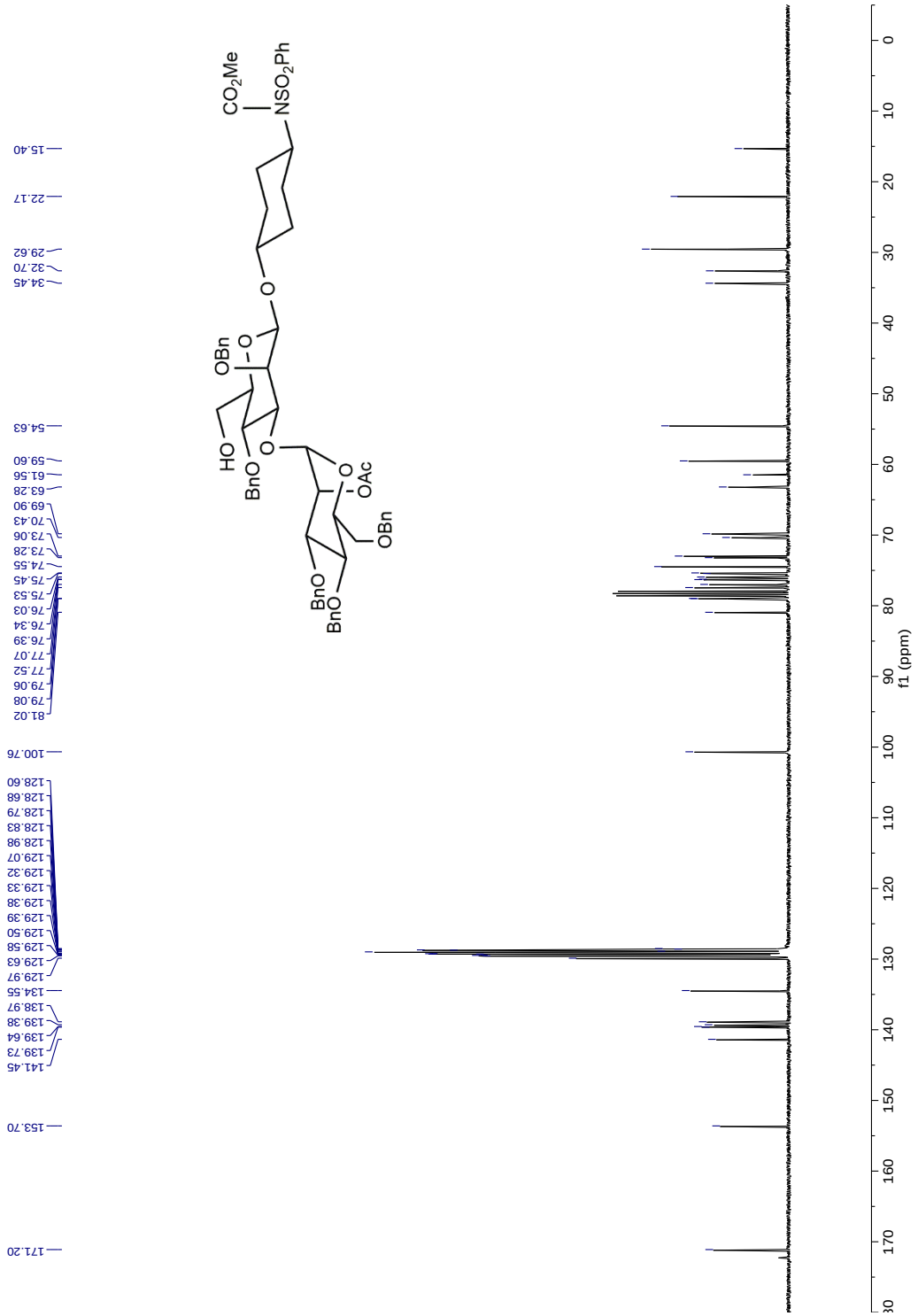
^{13}C NMR Spectrum of **9** (100MHz, D_2O)



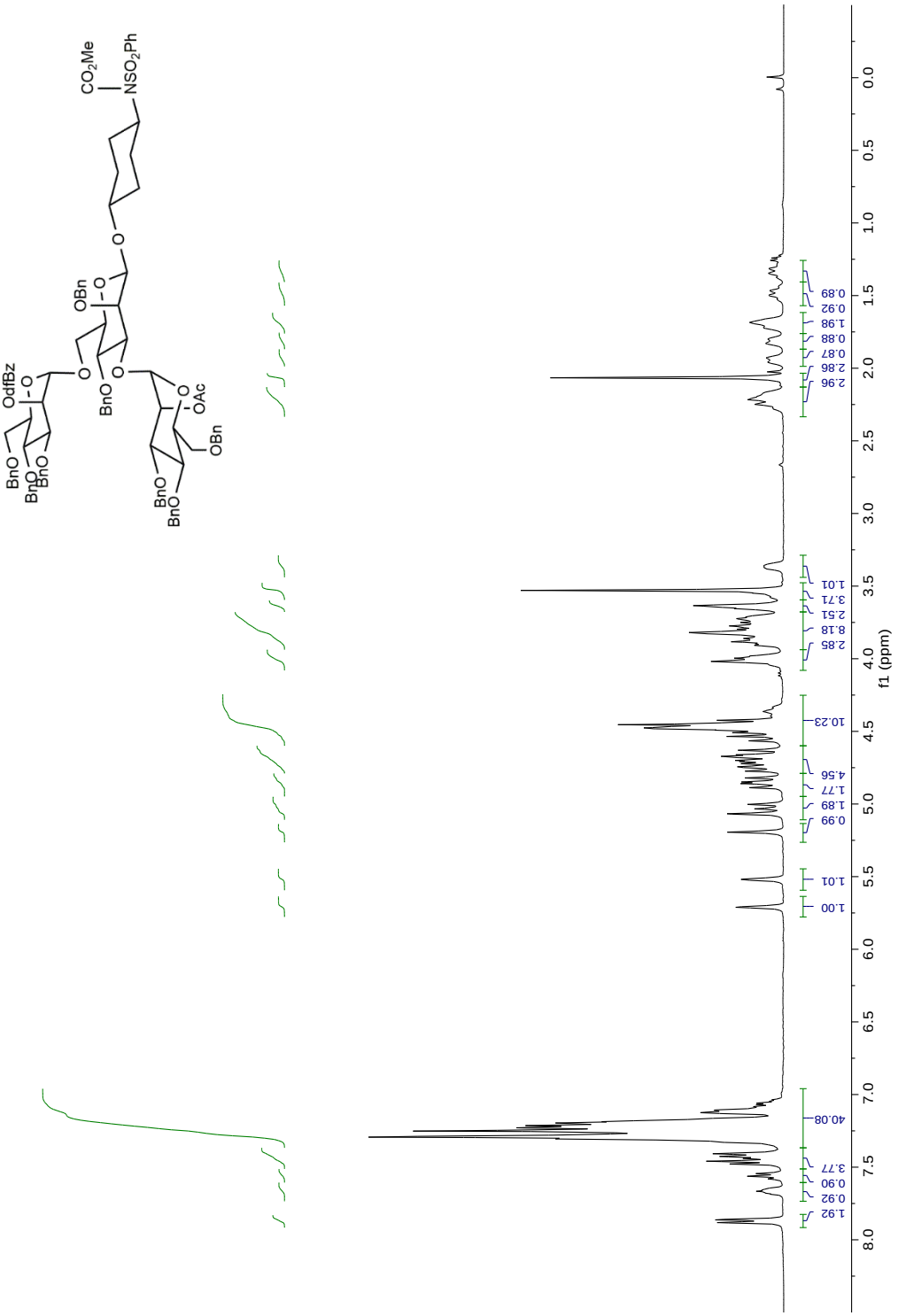
^{1}H NMR Spectrum of **10** (400MHz, $CDCl_3$)



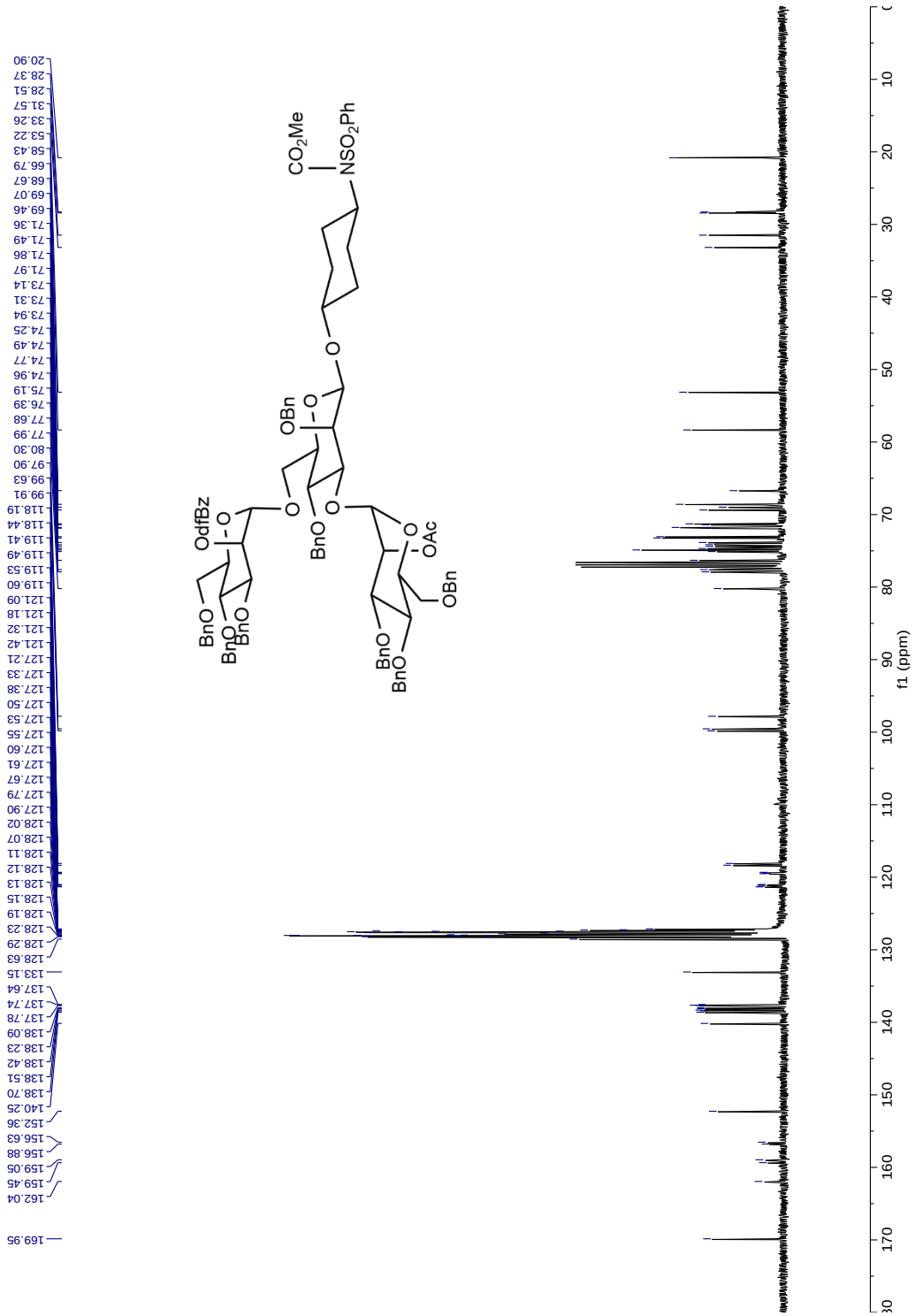
^{13}C NMR Spectrum of **10** (100MHz, $CDCl_3$)



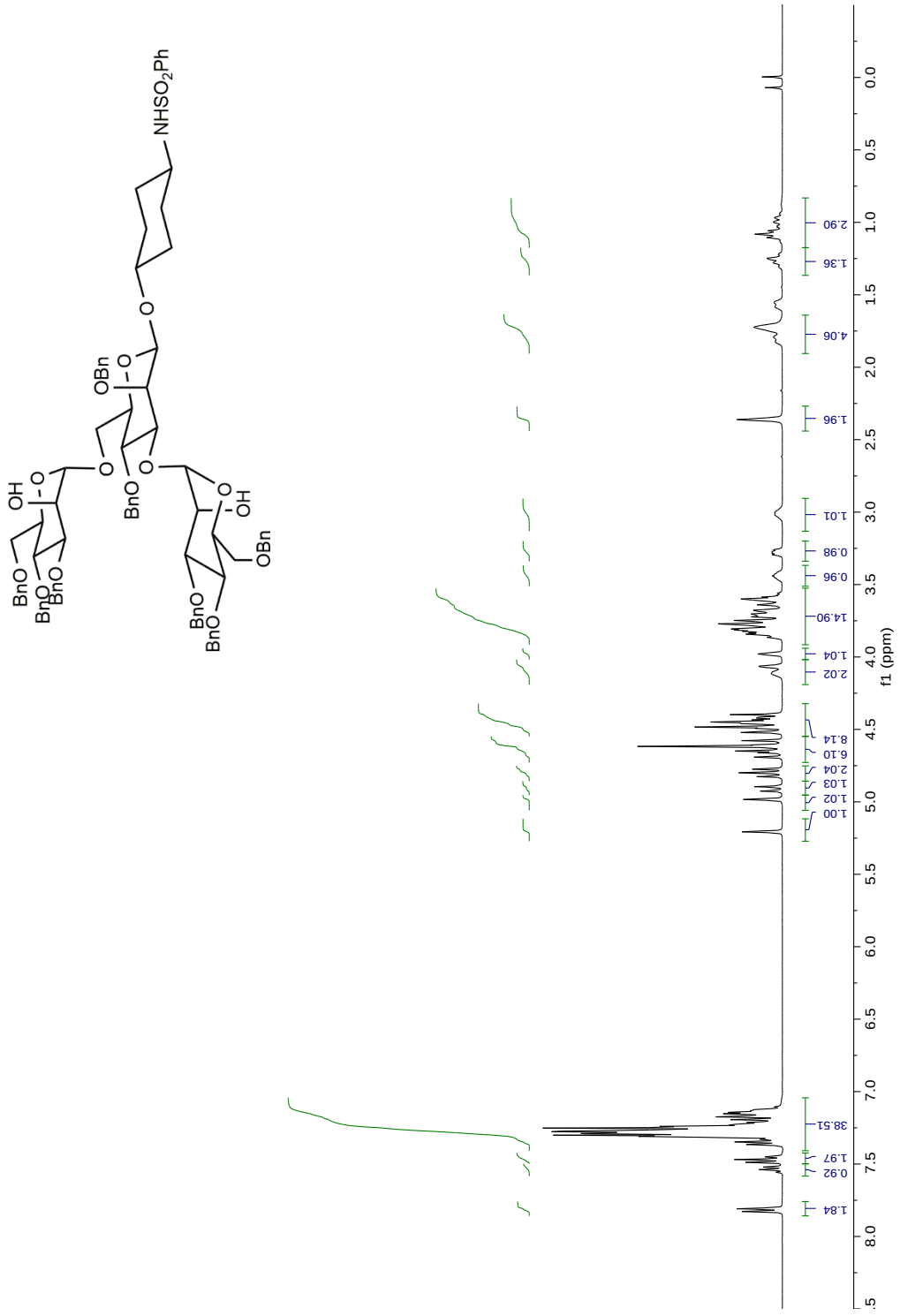
^{1}H NMR Spectrum of **11** (400MHz, $CDCl_3$)



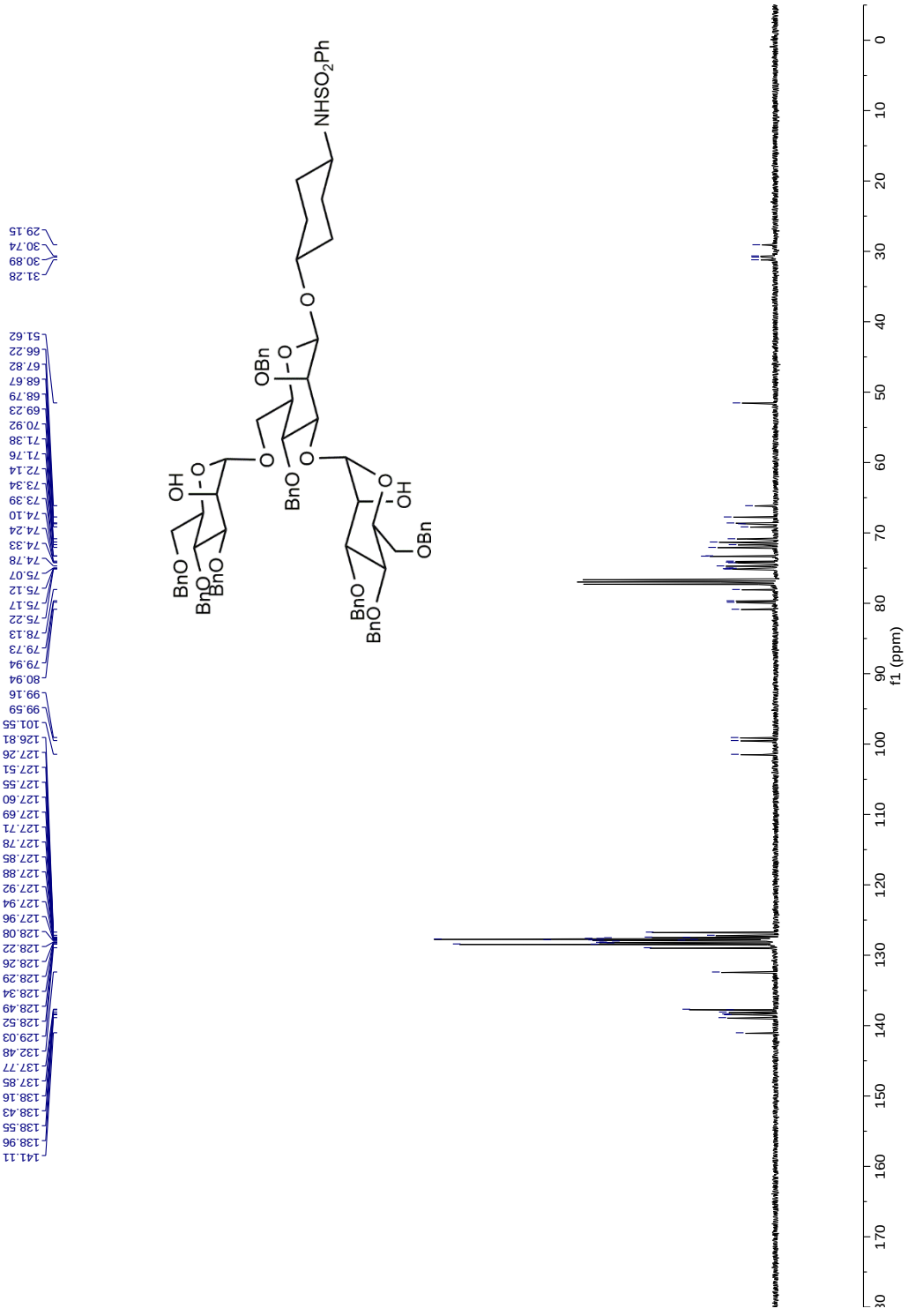
^{13}C NMR Spectrum of **11** (100MHz, $CDCl_3$)



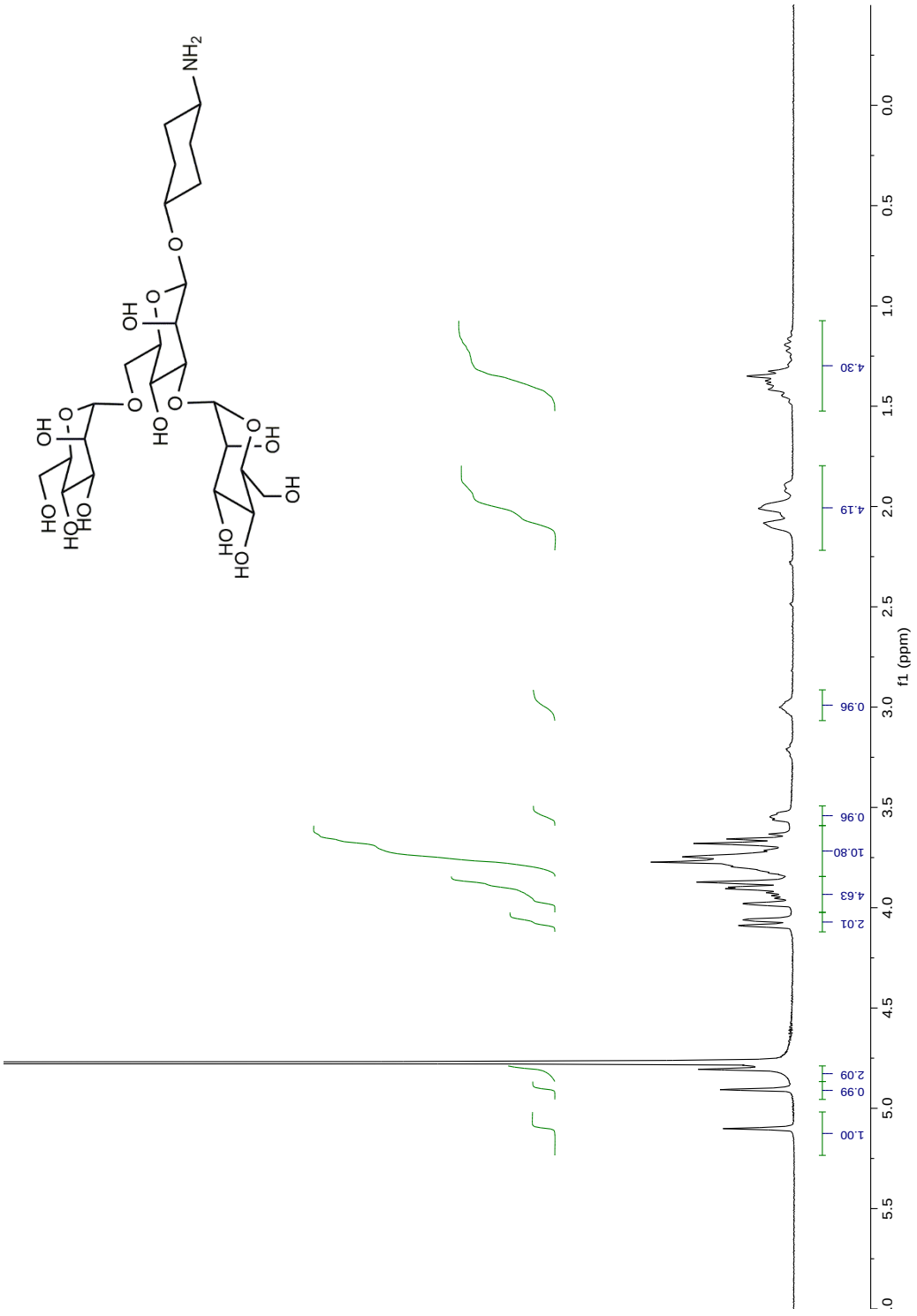
^{1}H NMR Spectrum of **SI-9** (400MHz, $CDCl_3$)



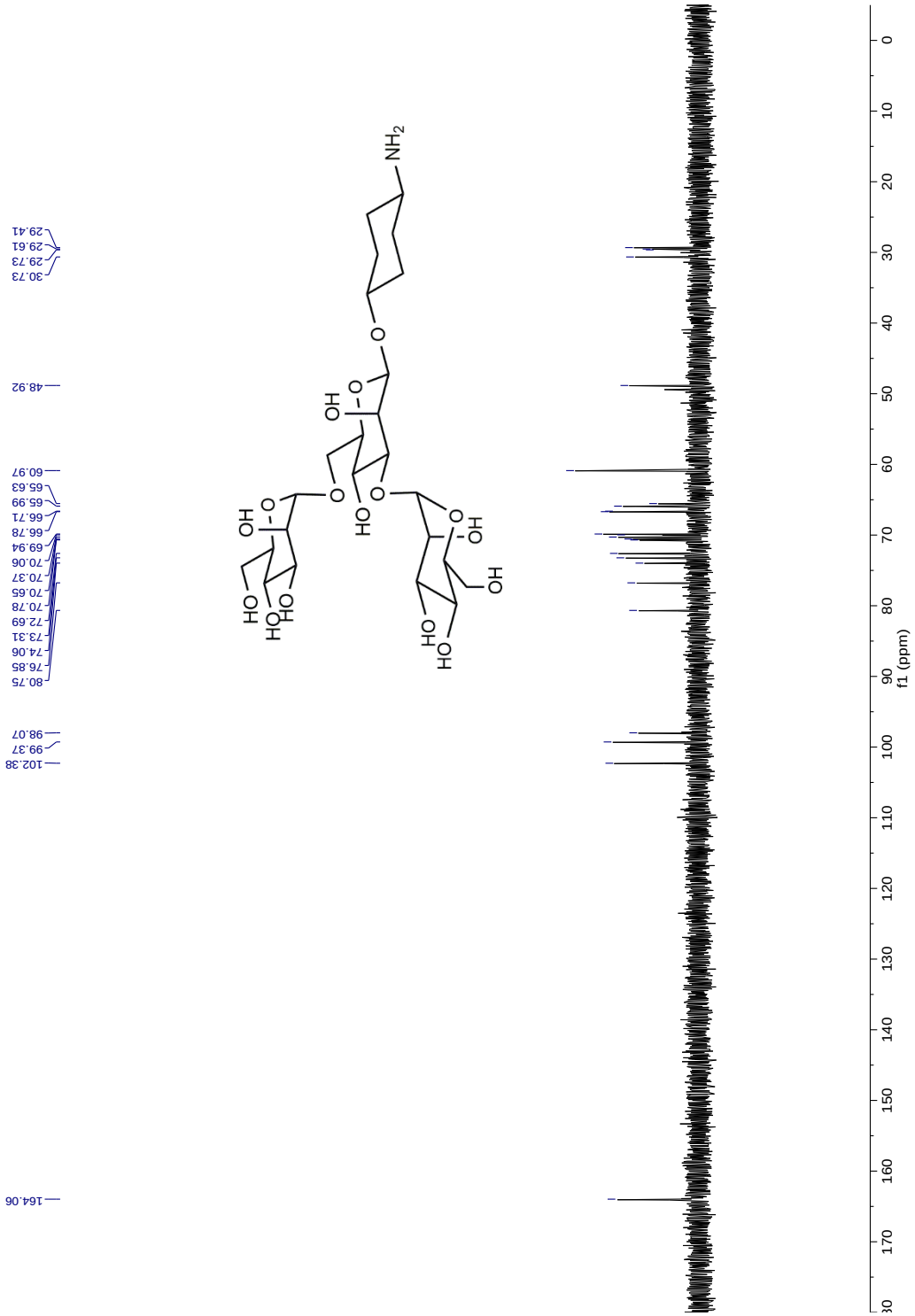
^{13}C NMR Spectrum of **SI-9** (100MHz, $CDCl_3$)



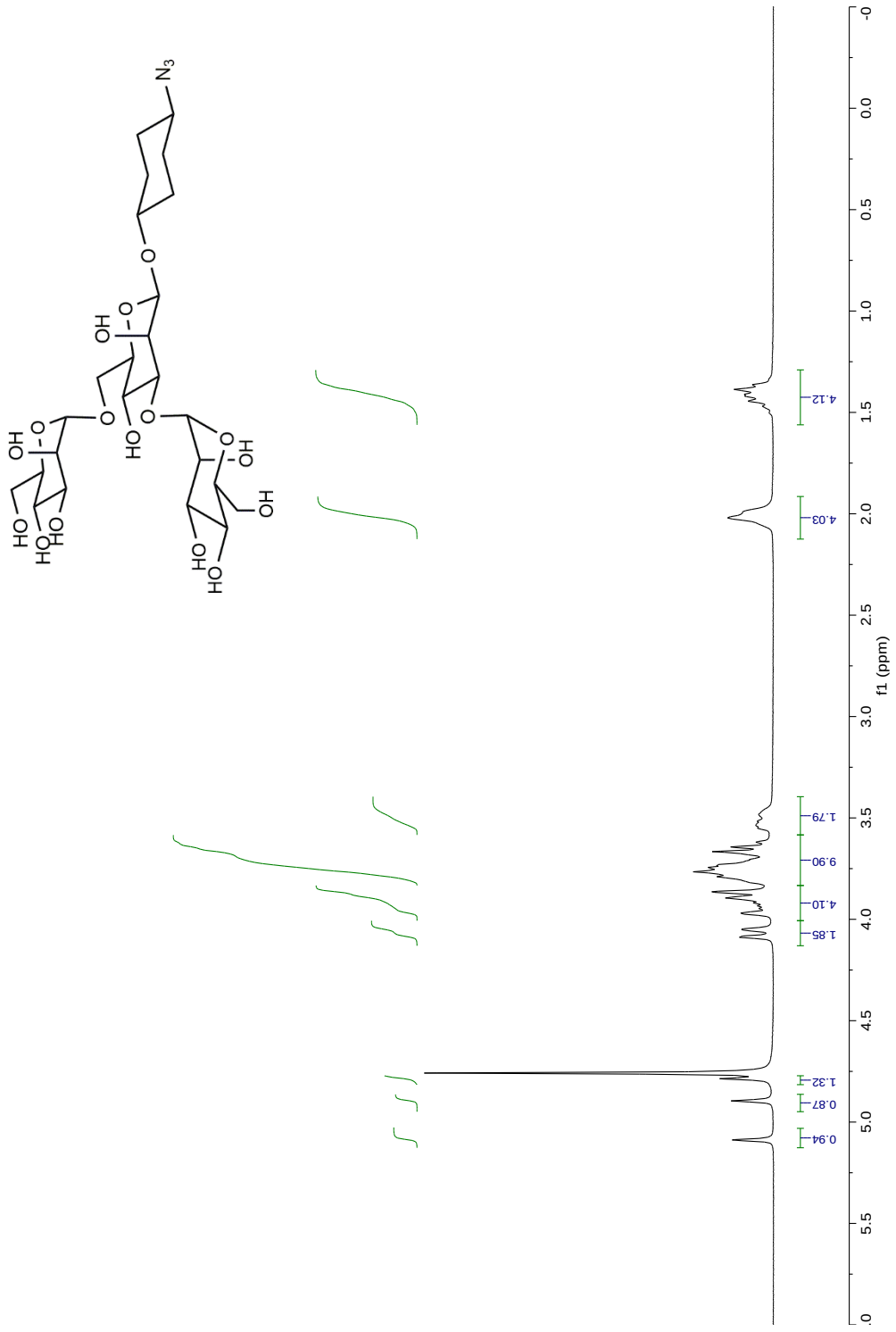
^{1}H NMR Spectrum of **SI-10** (400MHz, D_2O)



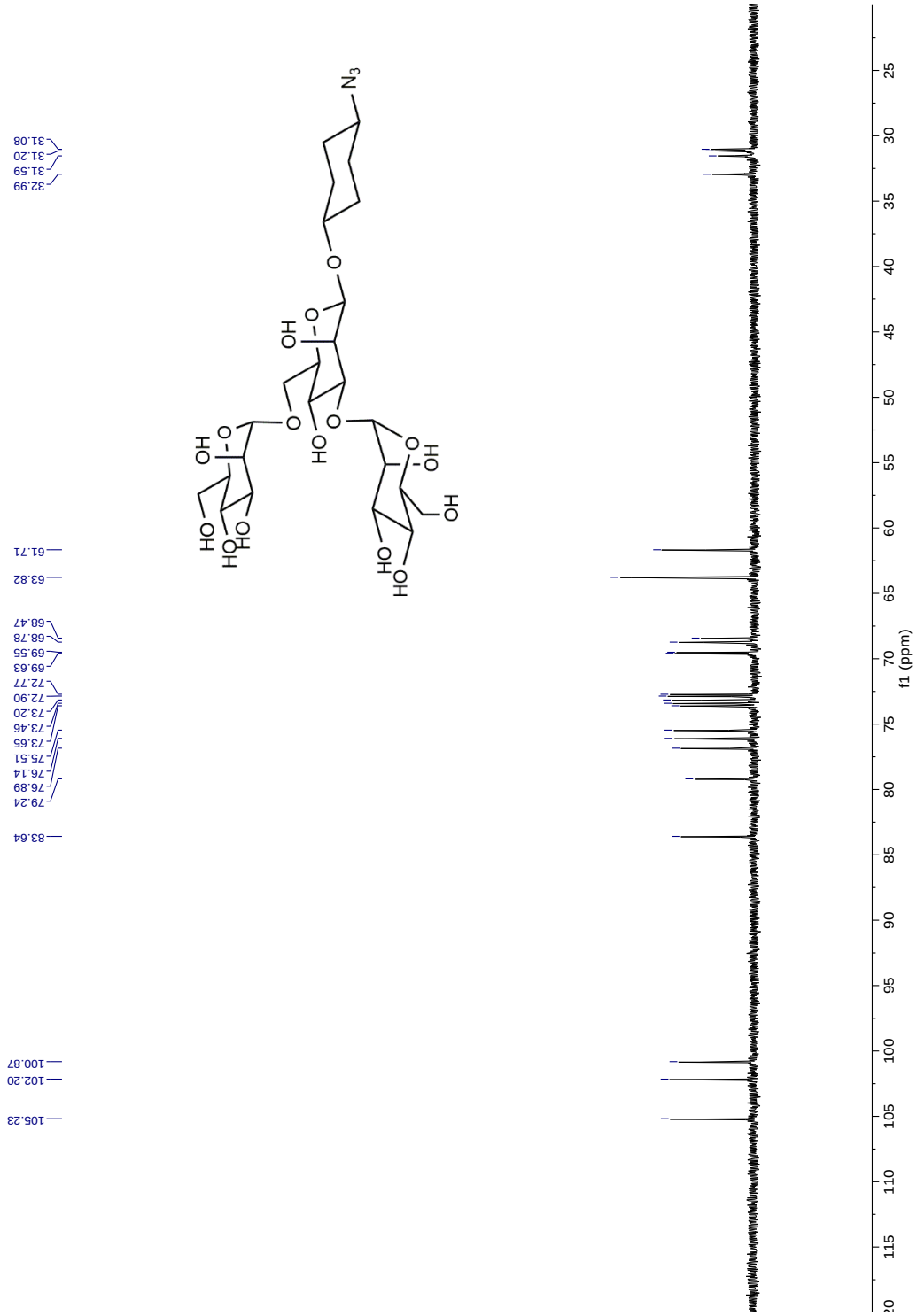
^{13}C NMR Spectrum of **SI-10** (100MHz, D_2O)



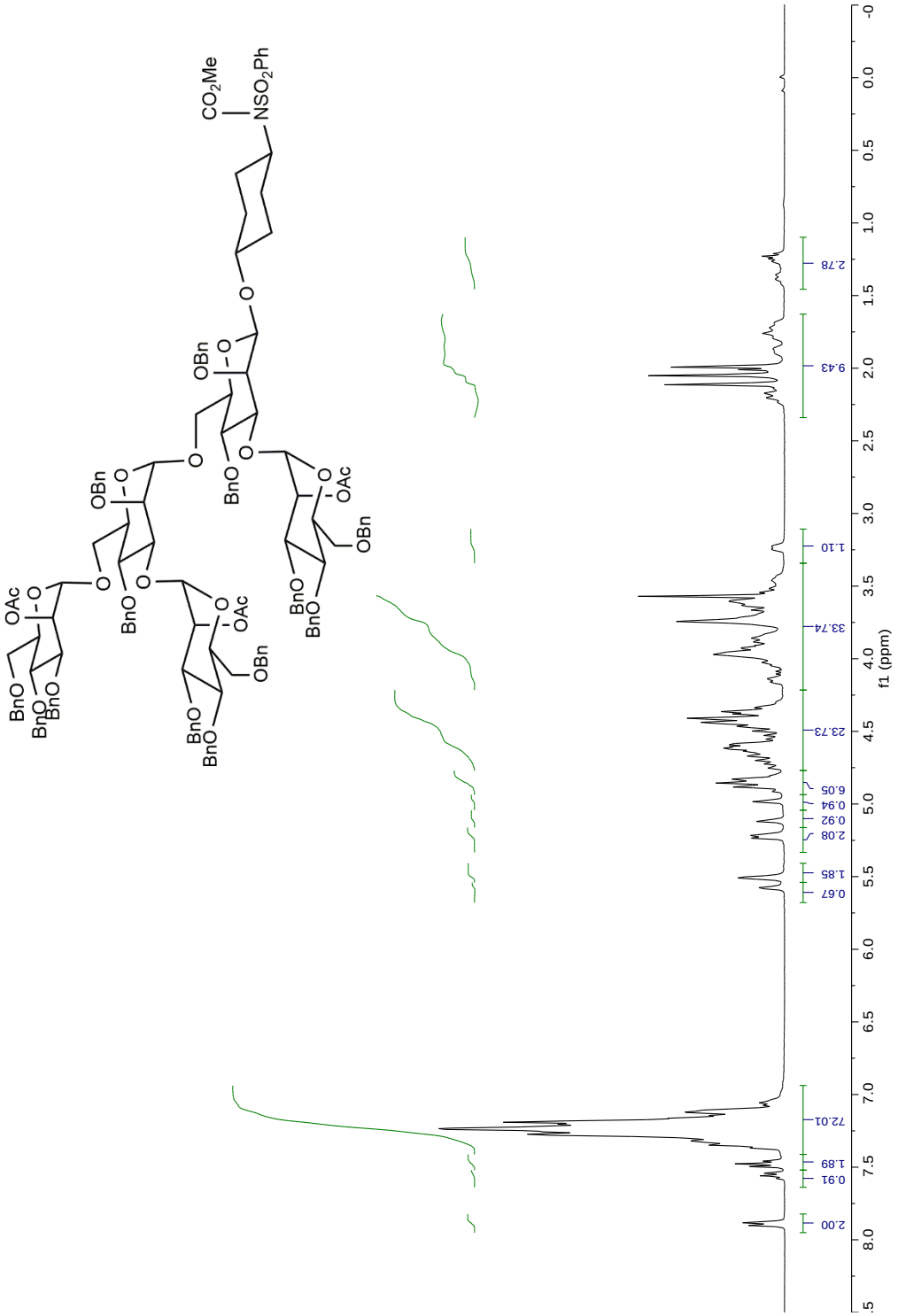
^{1}H NMR Spectrum of **12** (400MHz, D_2O)



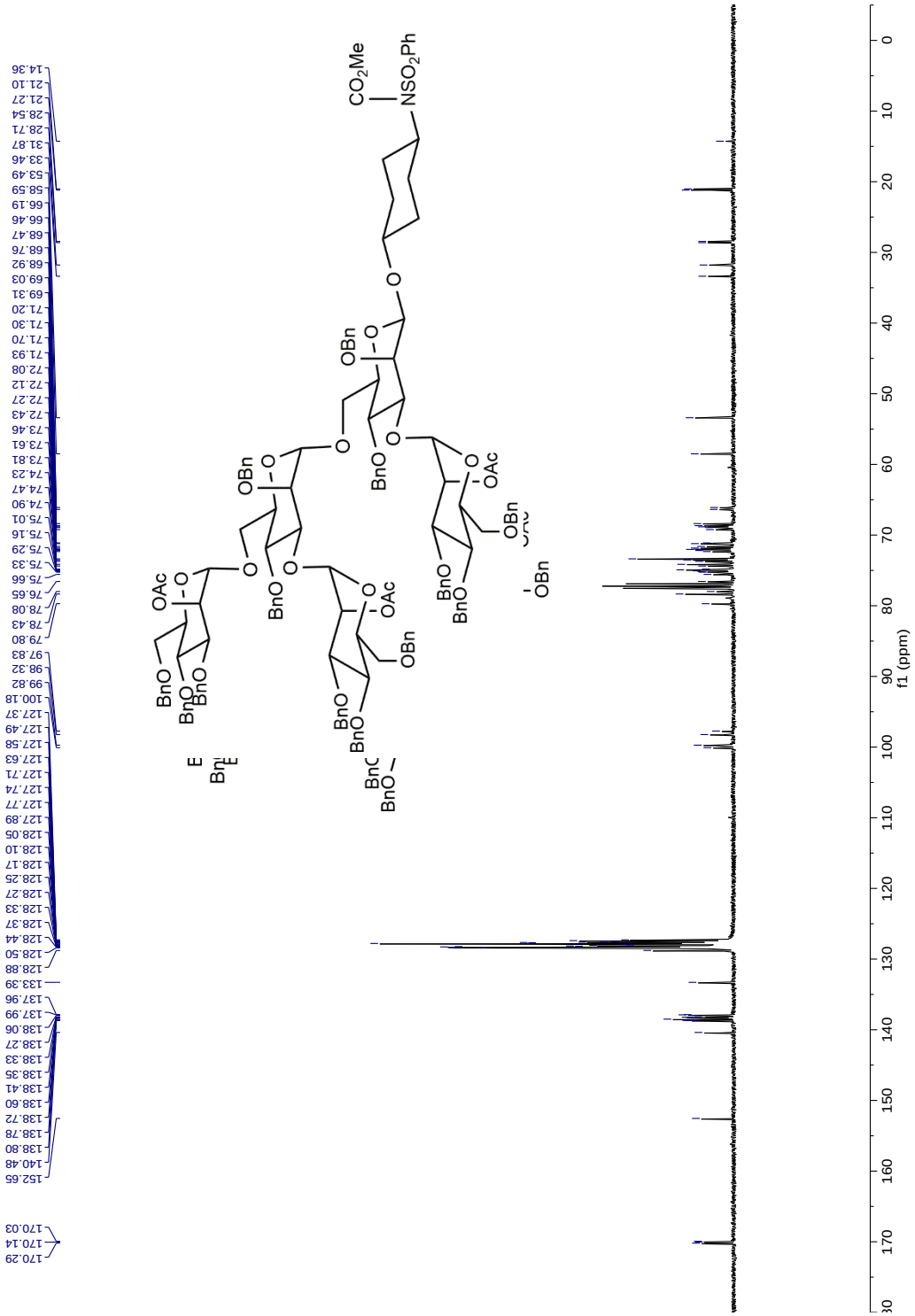
^{13}C NMR Spectrum of **12** (100MHz, D_2O)



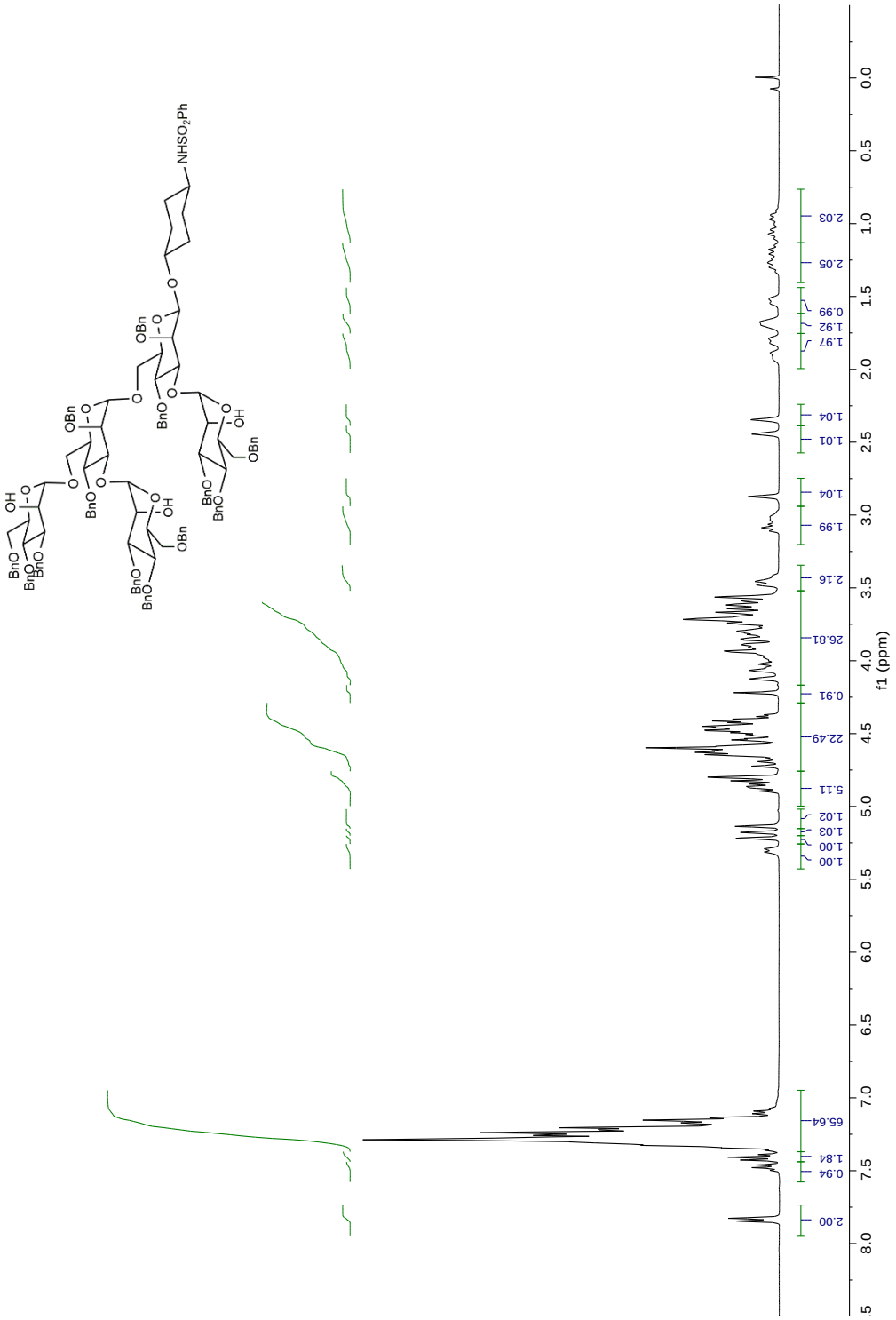
^{1}H NMR Spectrum of **13** (400MHz, $CDCl_3$)



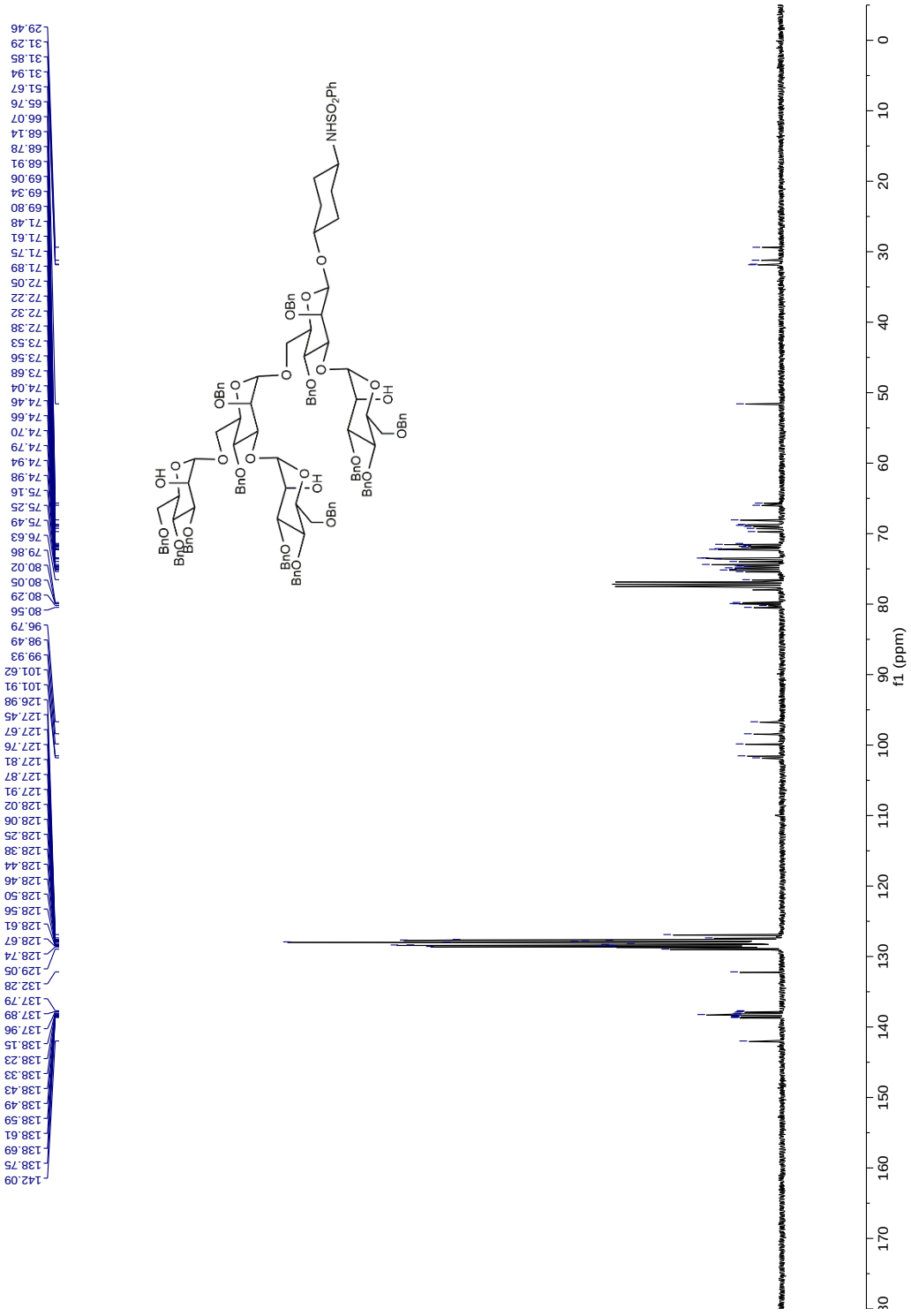
^{13}C NMR Spectrum of **13** (100MHz, $CDCl_3$)



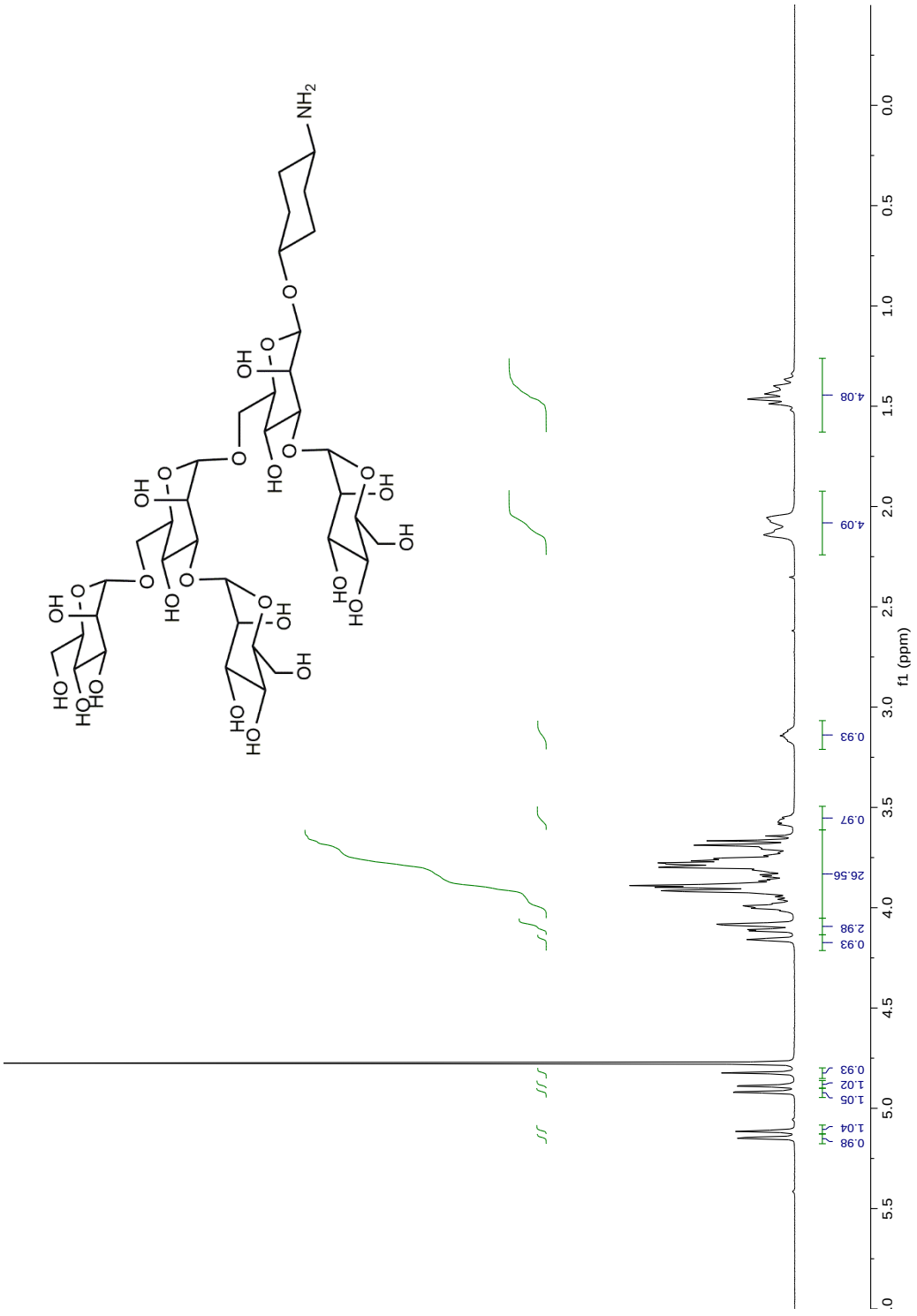
^{1}H NMR Spectrum of **SI-12** (400MHz, $CDCl_3$)



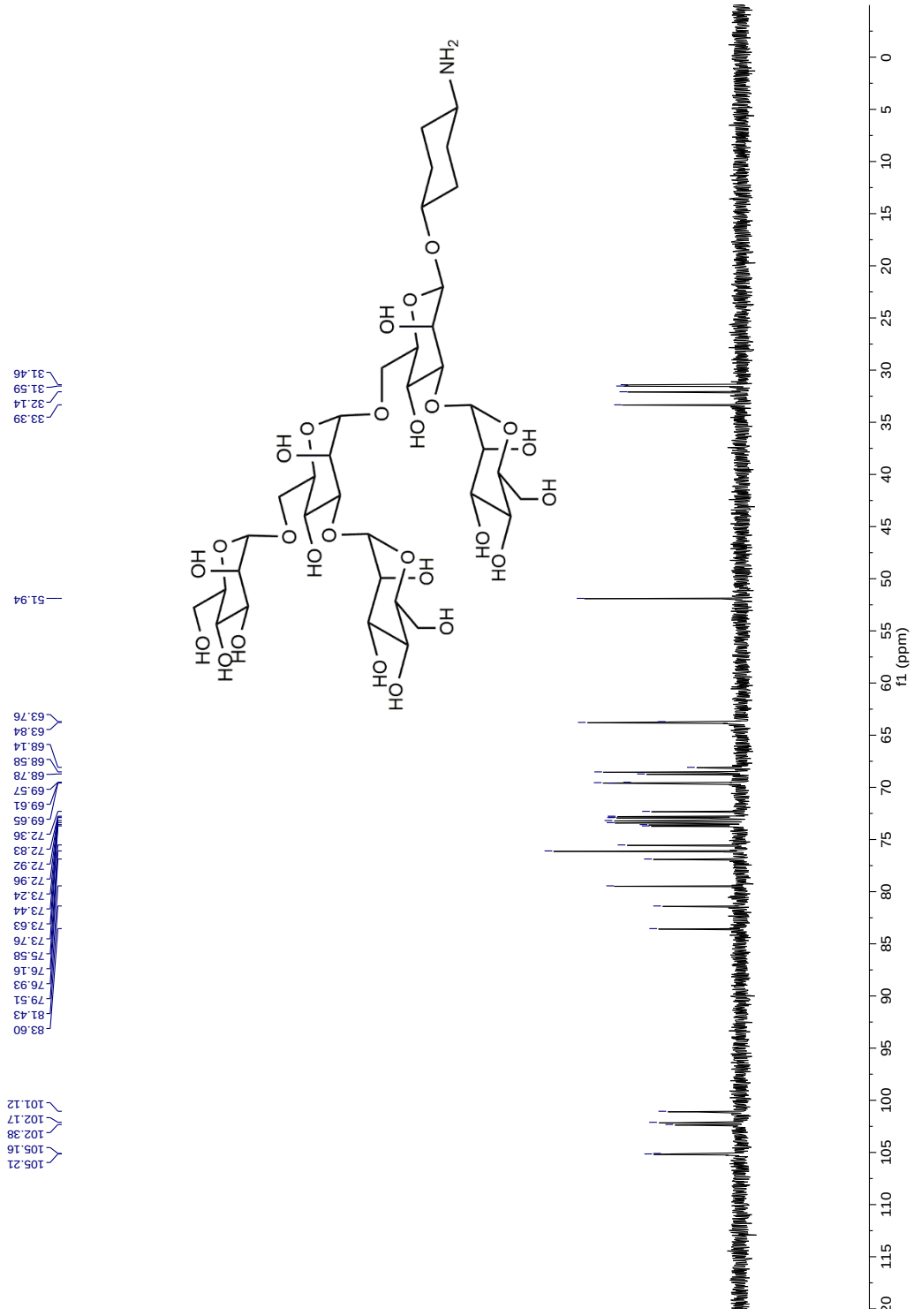
^{13}C NMR Spectrum of **SI-12** (100MHz, $CDCl_3$)



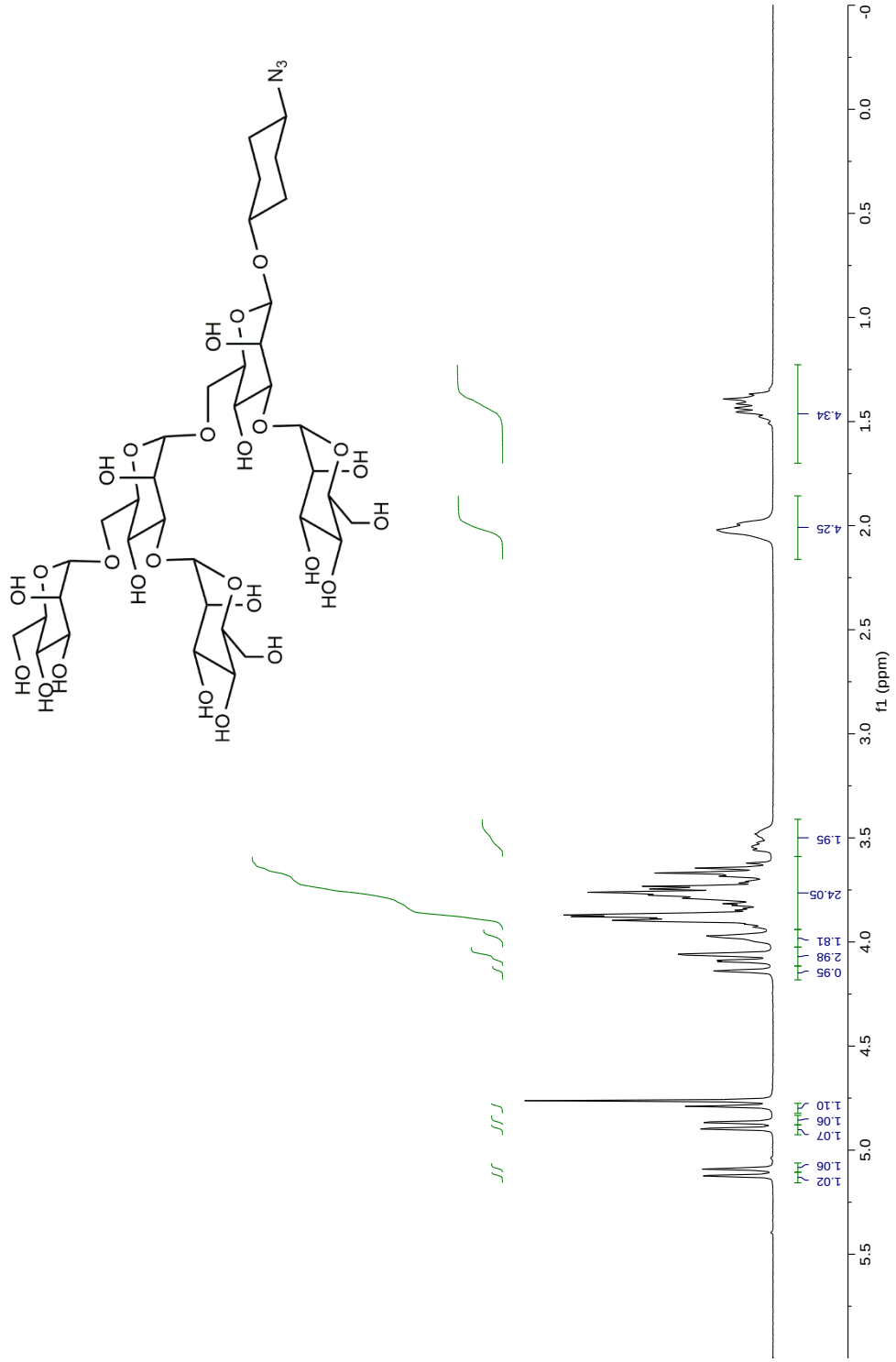
^{1}H NMR Spectrum of **SI-13** (400MHz, D_2O)



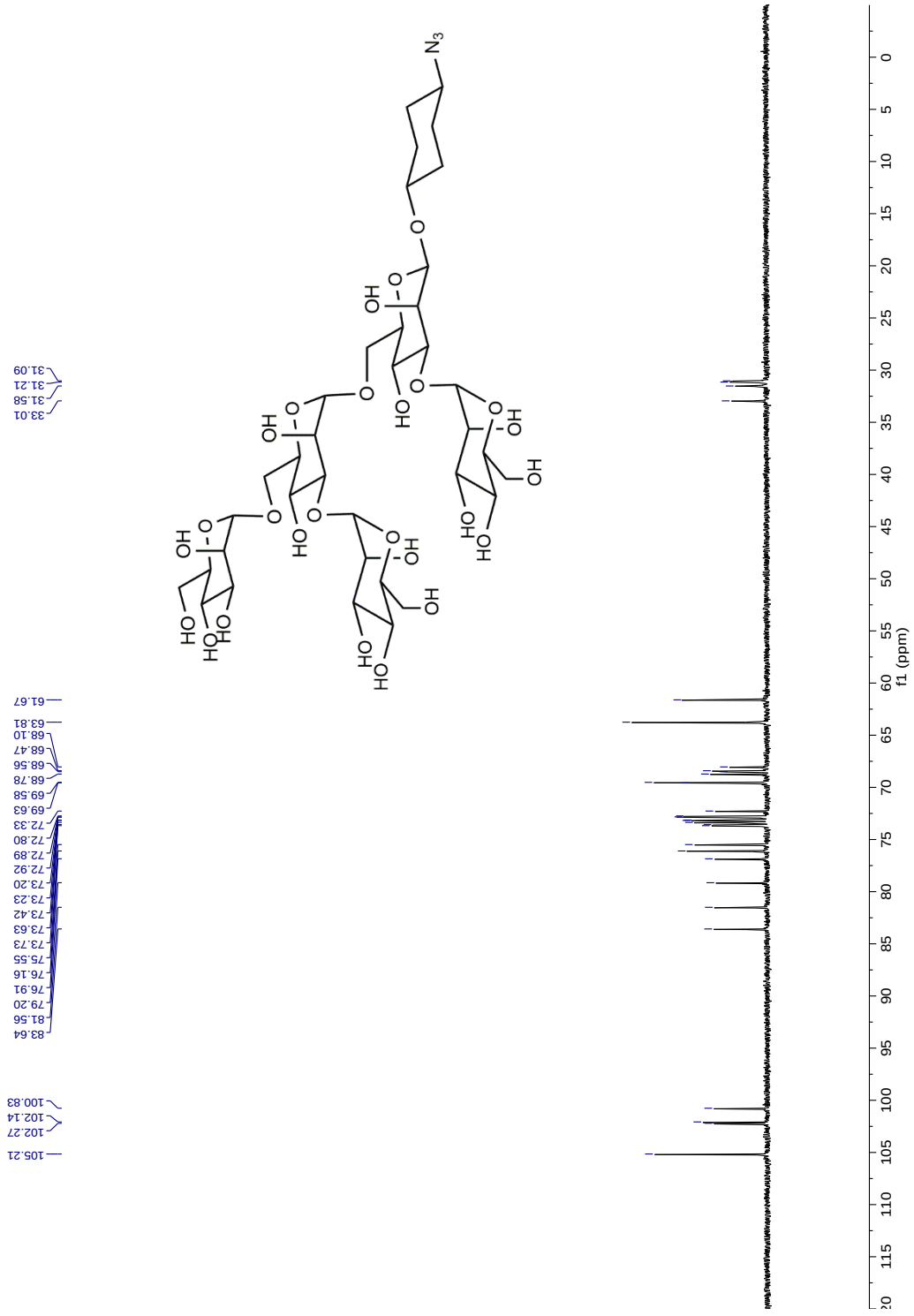
^{13}C NMR Spectrum of **SI-13** (100MHz, D_2O)



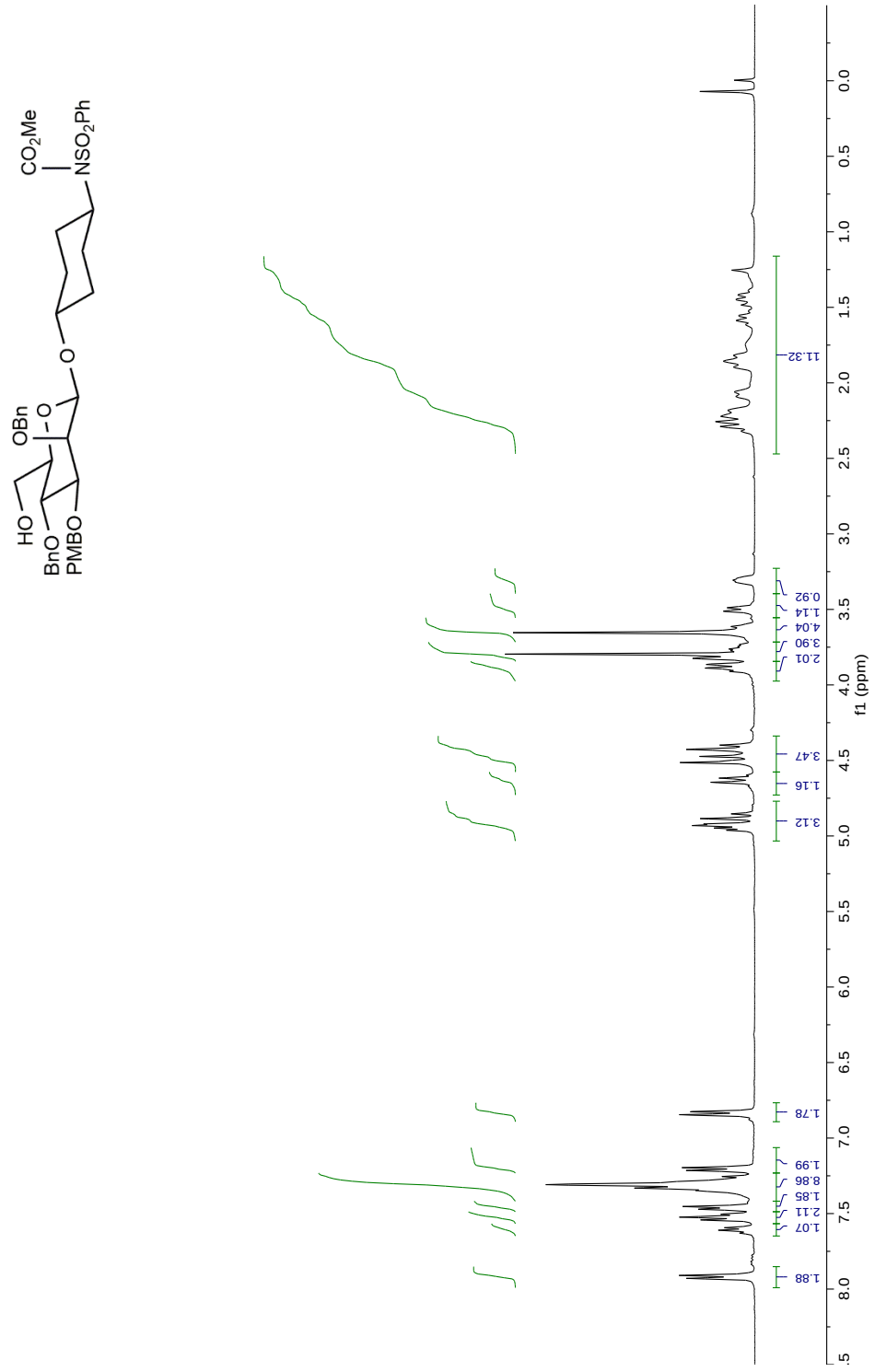
^{1}H NMR Spectrum of **14** (400MHz, D_2O)



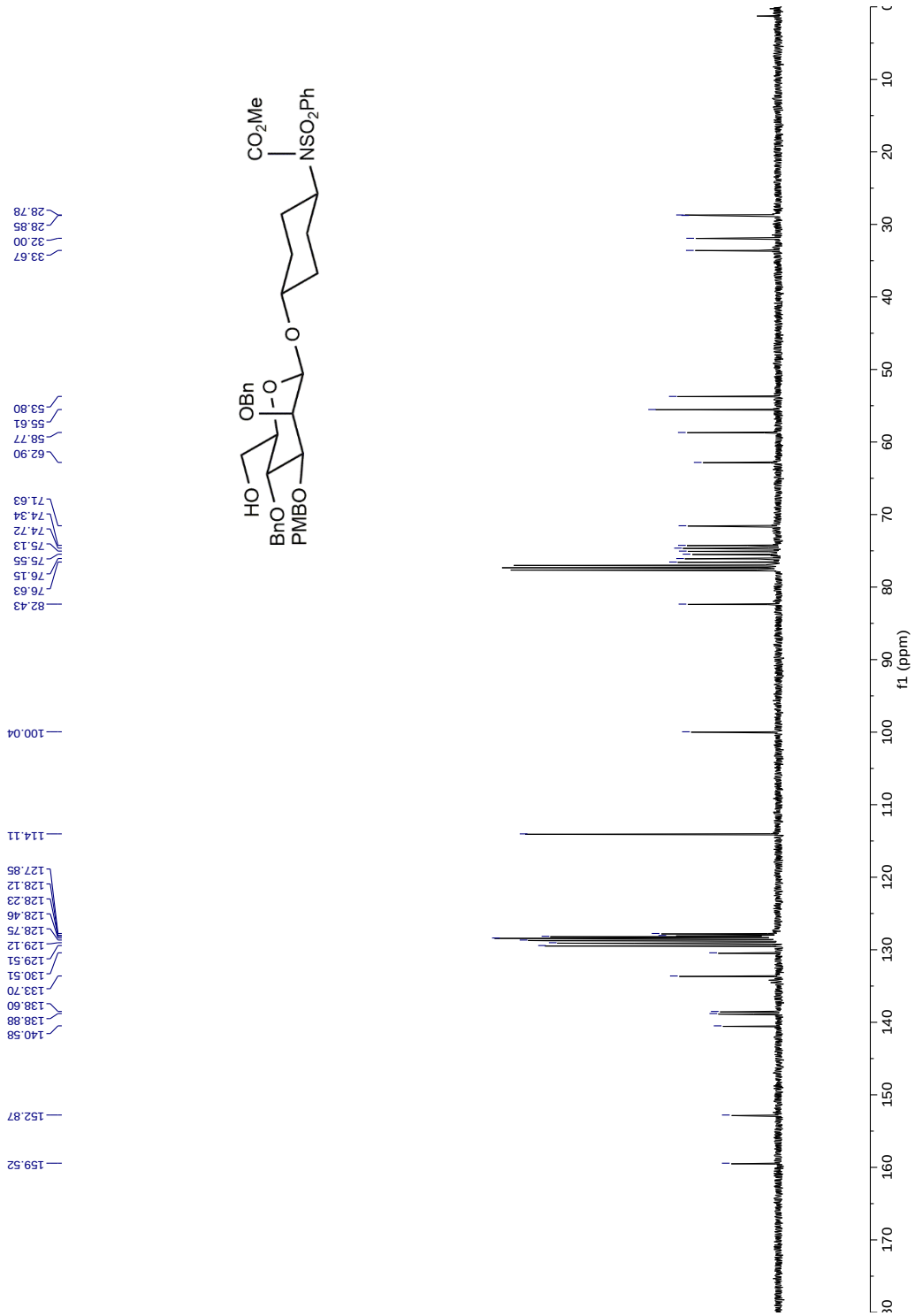
^{13}C NMR Spectrum of **14** (100MHz, D_2O)



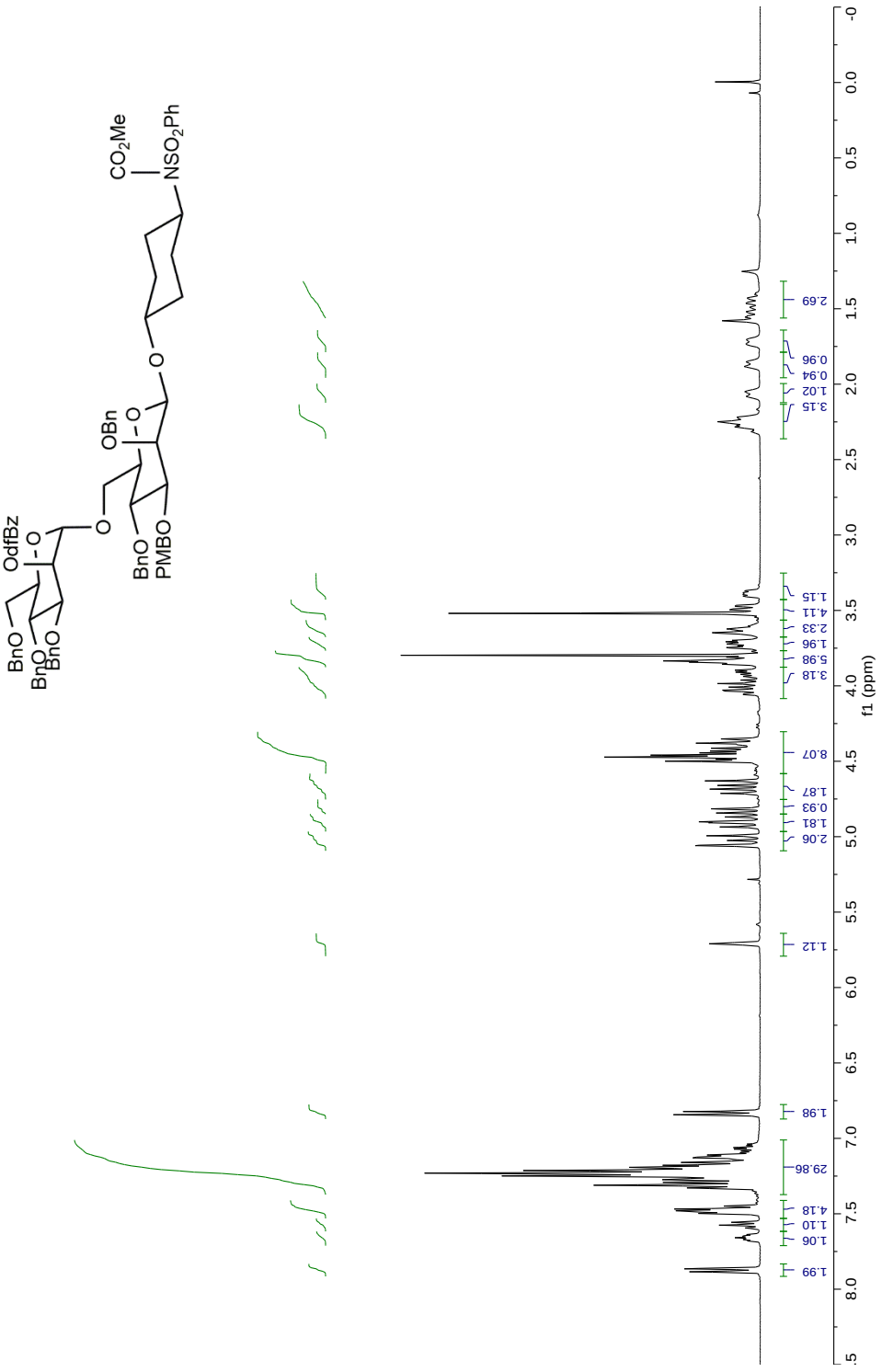
^{1}H NMR Spectrum of **SI-14** (400MHz, $CDCl_3$)



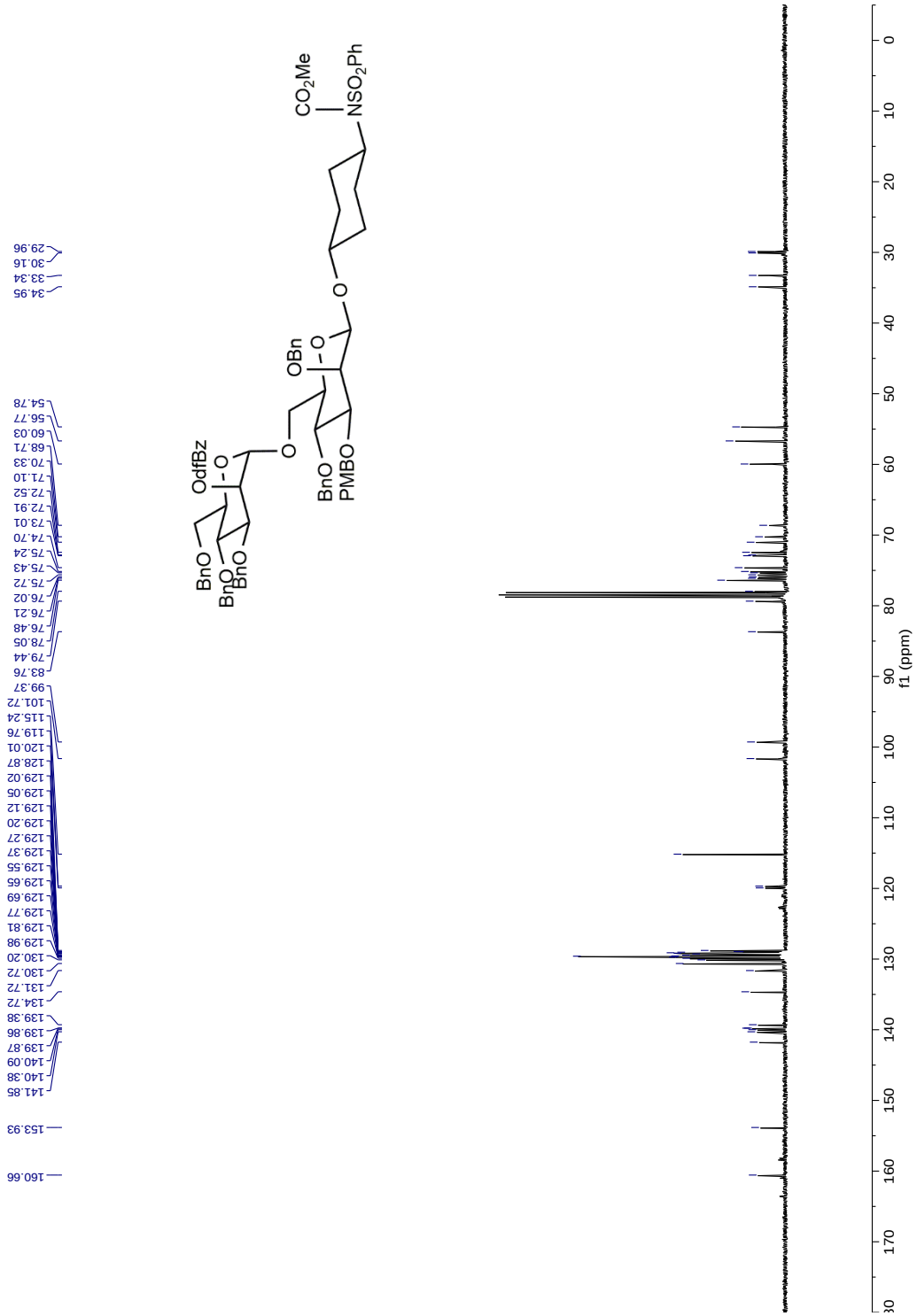
^{13}C NMR Spectrum of **SI-14** (100MHz, $CDCl_3$)



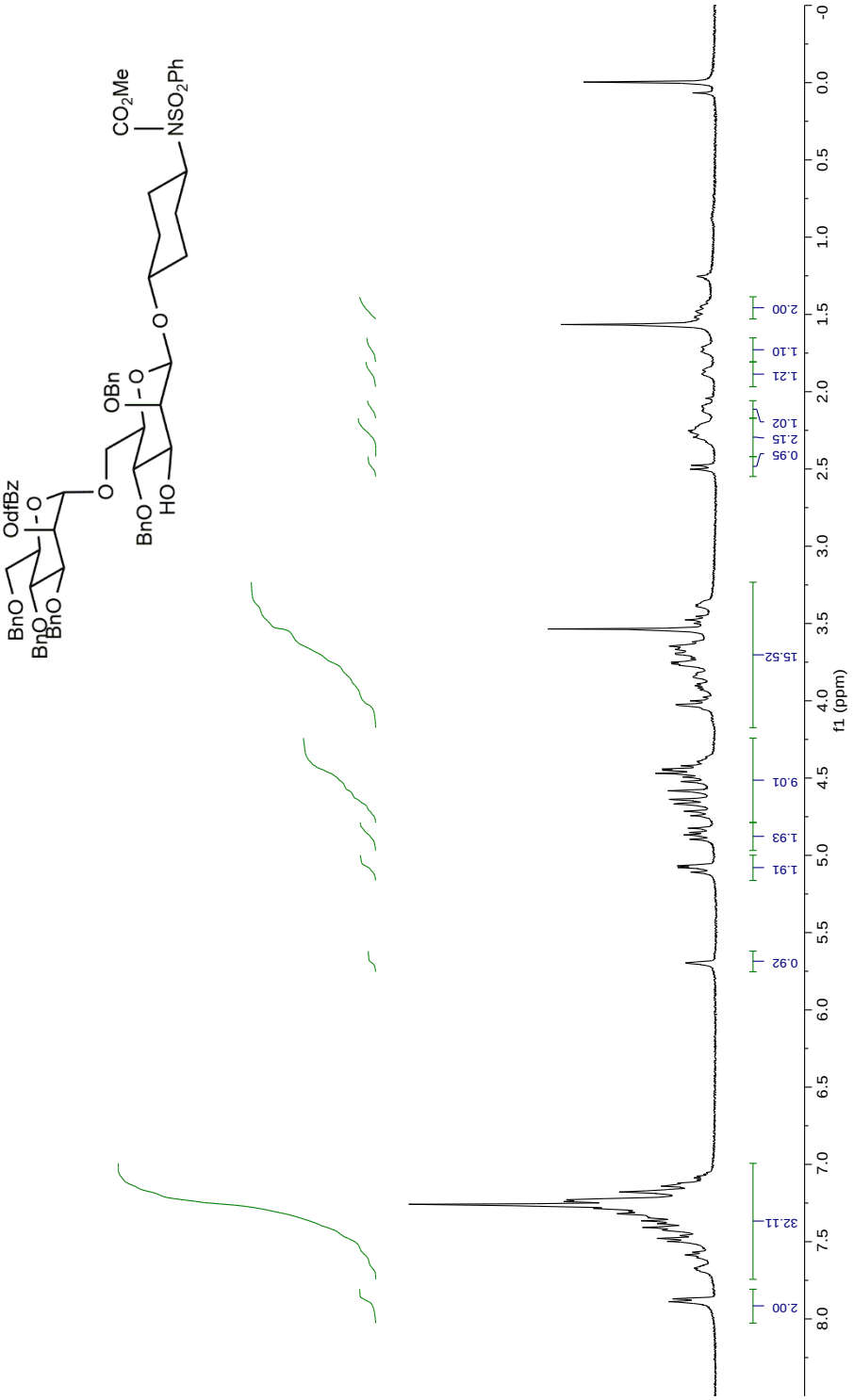
^{1}H NMR Spectrum of **15** (400MHz, $CDCl_3$)



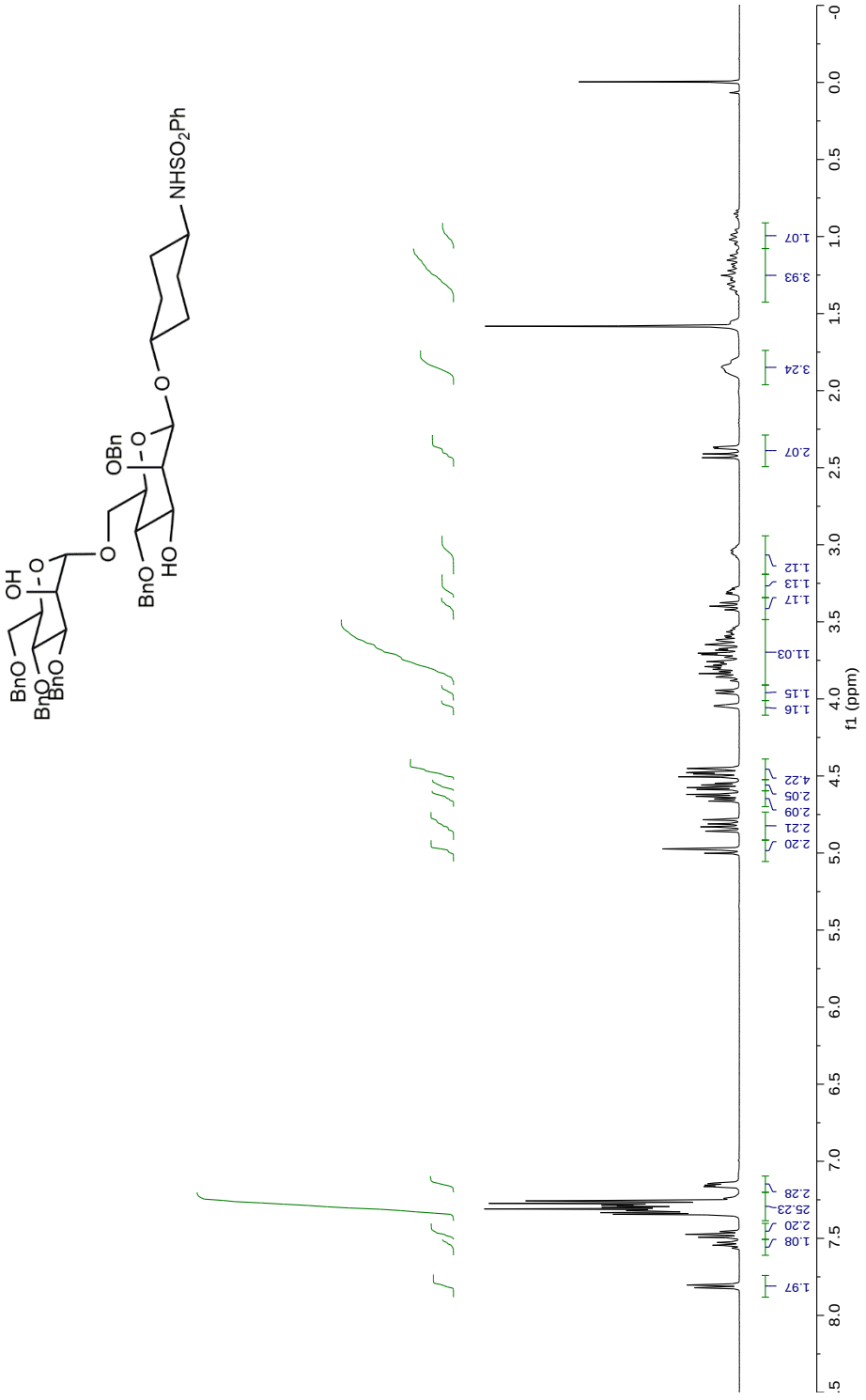
^{13}C NMR Spectrum of **15** (100MHz, $CDCl_3$)



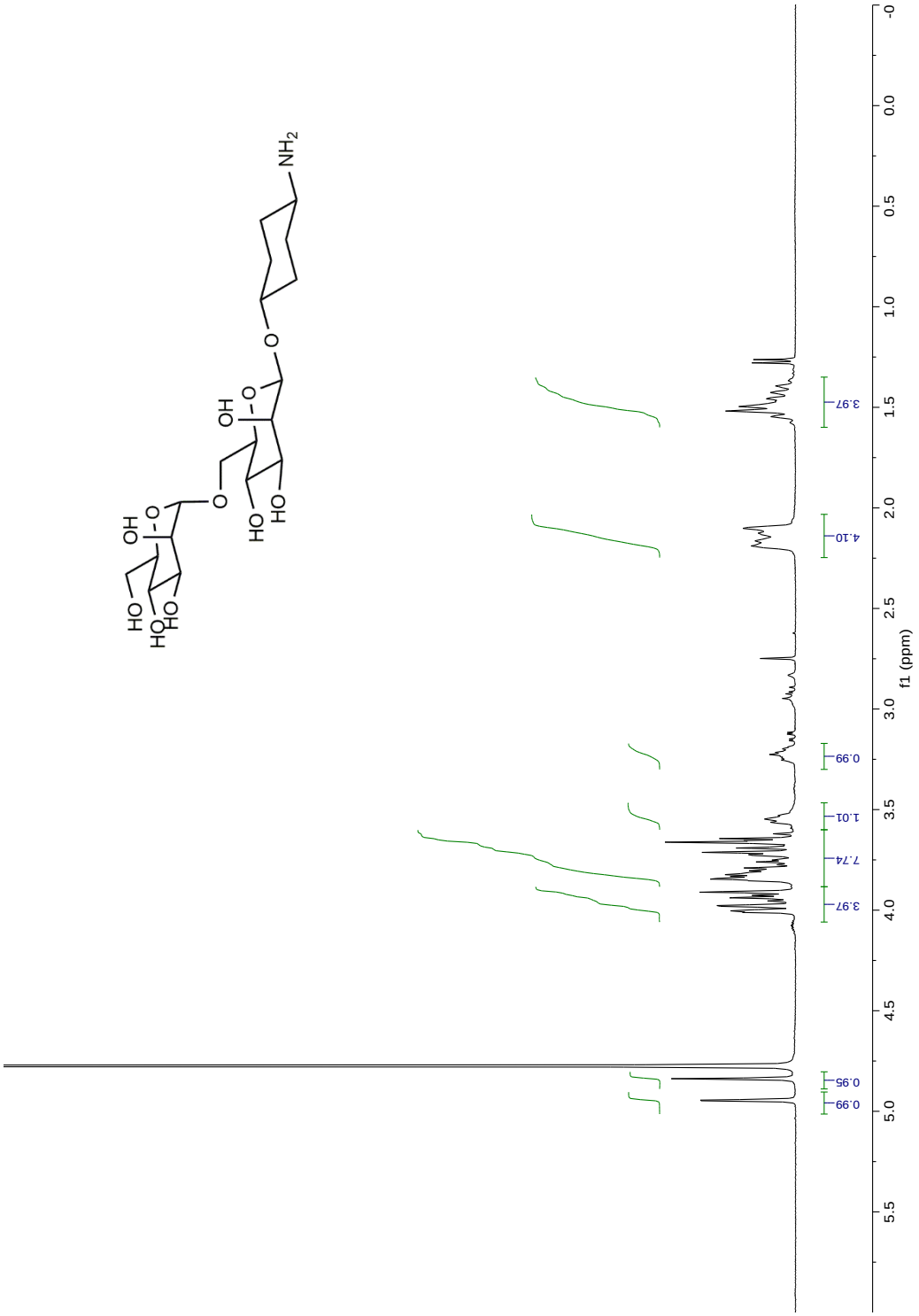
^{1}H NMR Spectrum of **SI-15** (400MHz, $CDCl_3$)



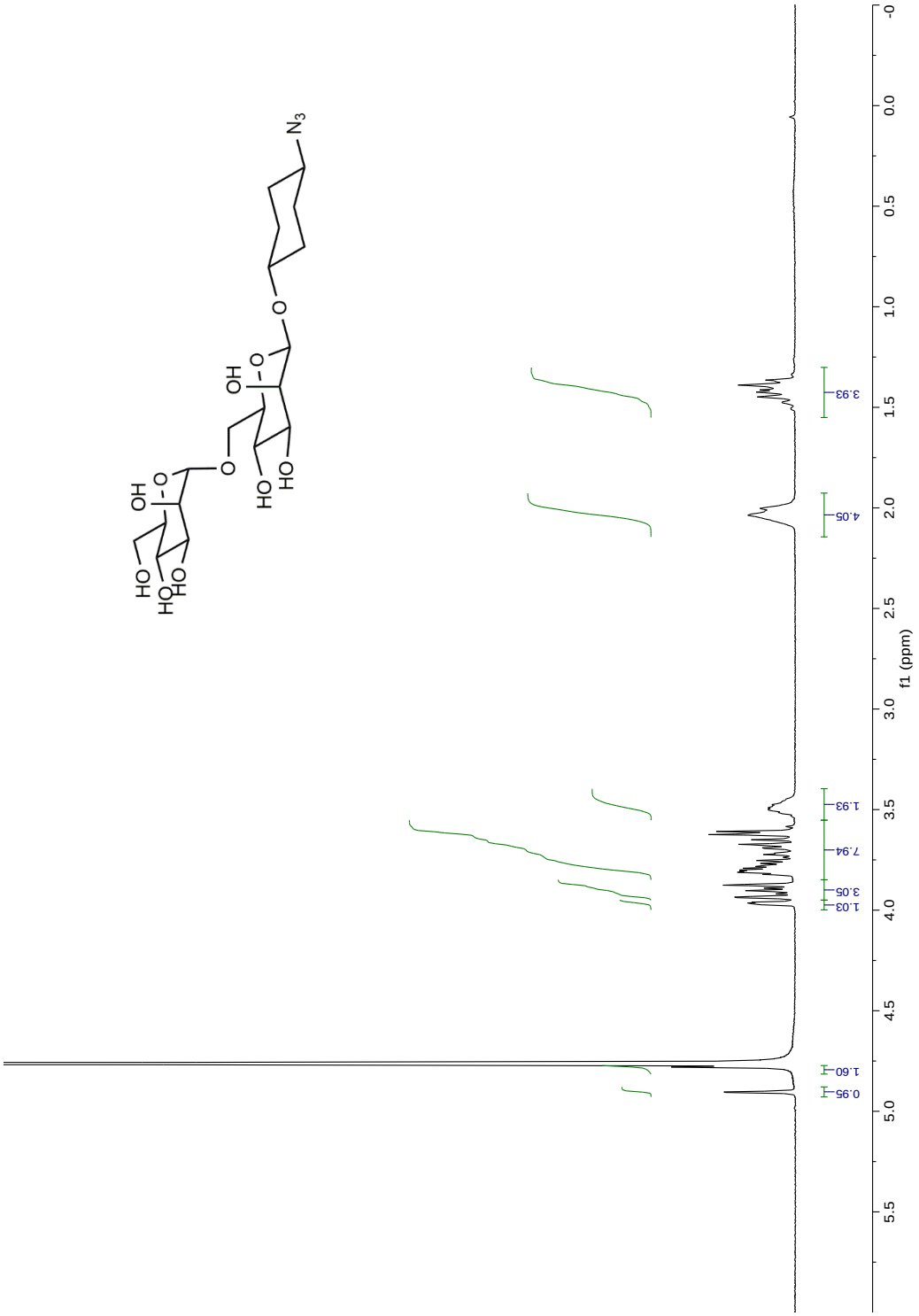
^{1}H NMR Spectrum of **SI-16** (400MHz, $CDCl_3$)



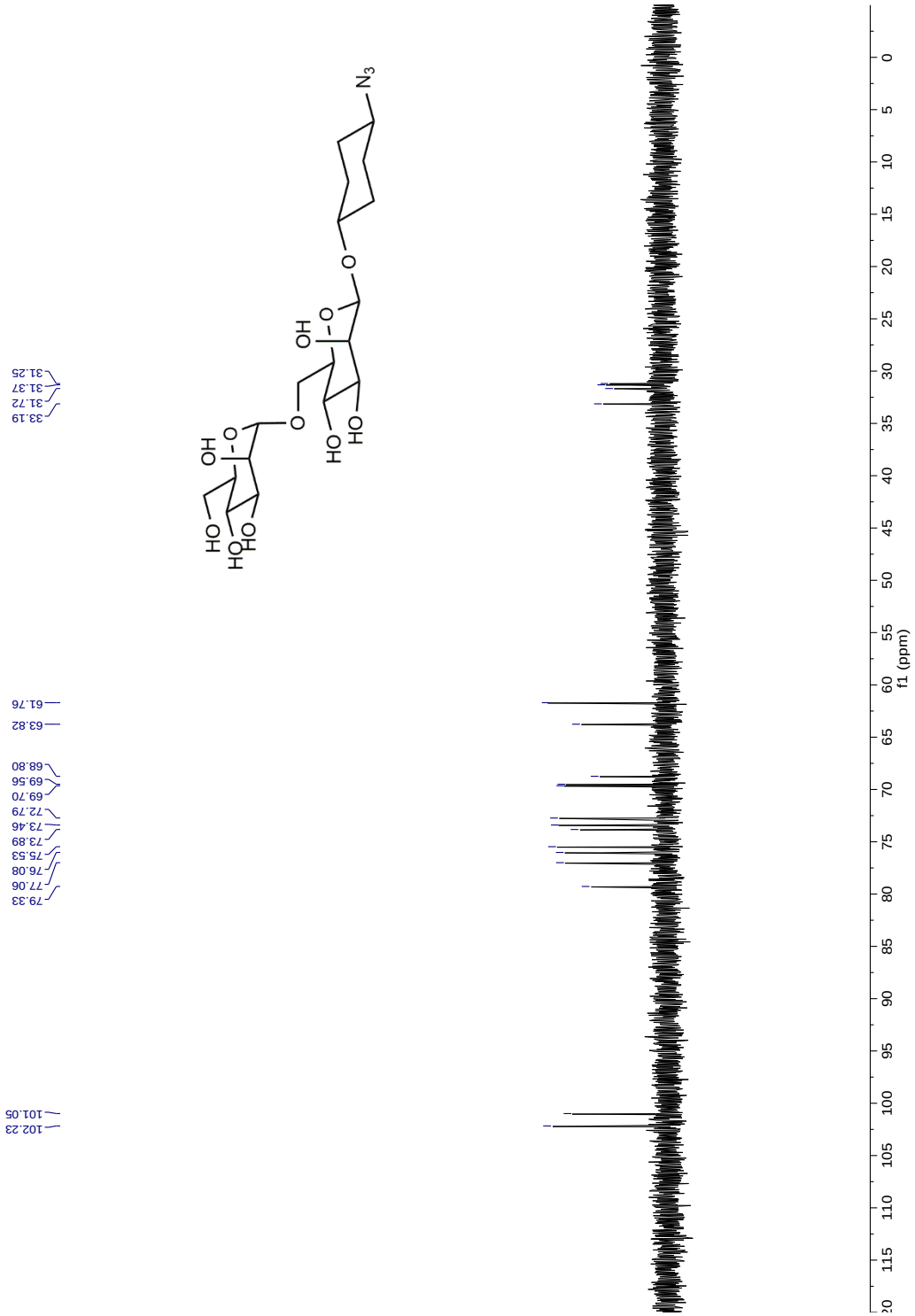
^{1}H NMR Spectrum of **SI-17** (400MHz, D_2O)



^{1}H NMR Spectrum of **16** (400MHz, D_2O)



^{13}C NMR Spectrum of **16** (100MHz, D_2O)



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