SUPPLEMENTARY INFORMATION

An atypical interaction explains the high-affinity of a non-hydrolyzable S-linked 1,6-α-mannanase inhibitor

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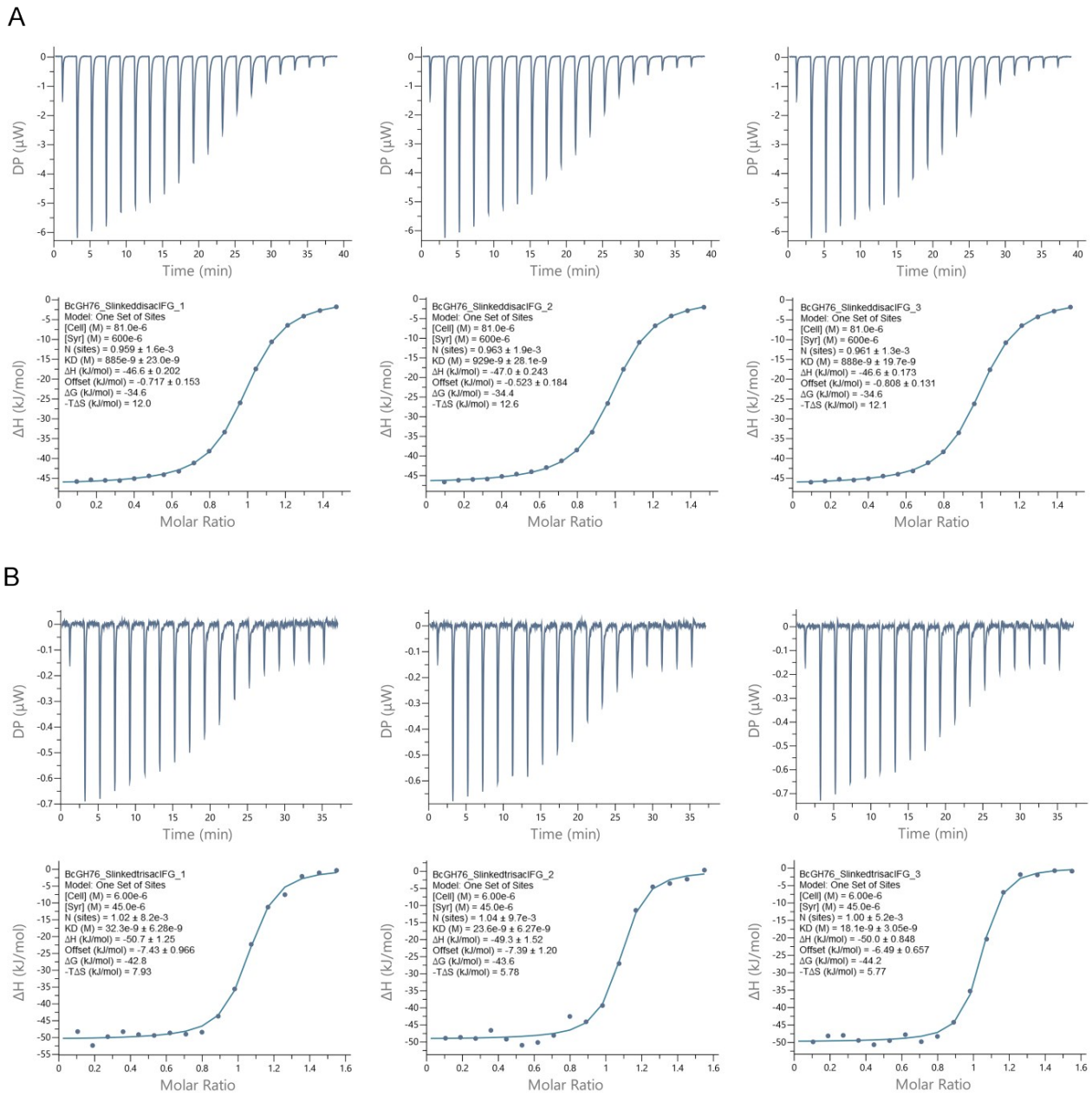


Figure S1. ITC calorimograms of (A) ManSIFG (2) and (B) $(ManS)_2$IFG (3) binding to *Bc*GH76.

Titrations were performed at 25 ºC in triplicate. ManSIFG (**2**) binds with K_D = 0.90 ± 0.03 µM, n = 0.961 ± 0.002 and ΔH = –46.7 ± 0.3 kJ mol^{-1}. $(ManS)_2$IFG (**3**) binds with K_D = 24.7 ± 7.9 nM, n = 1.02 ± 0.02 and ΔH = –50.0 ± 1.4 kJ mol^{-1}.

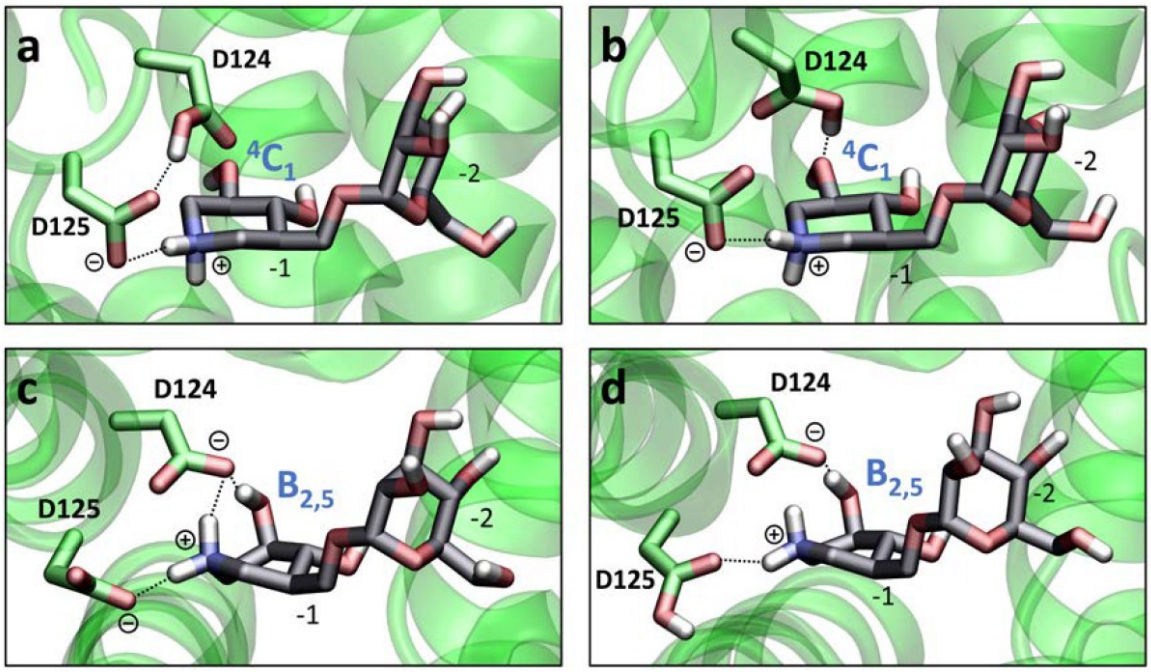


Figure S2. Active site structure resulting from the MD simulation, considering different protonation states of the two catalytic residues in *Bc*GH76-ManIFG complexes.

a) Asp124-H, Asp125; b) Asp124-H (protonation at the other carboxylic oxygen), Asp125; c) Asp124, Asp125; d) Asp124, Asp125-H. Water molecules are omitted for clarity. Only *c* and *d* are consistent with the experimentally determined crystal structure.

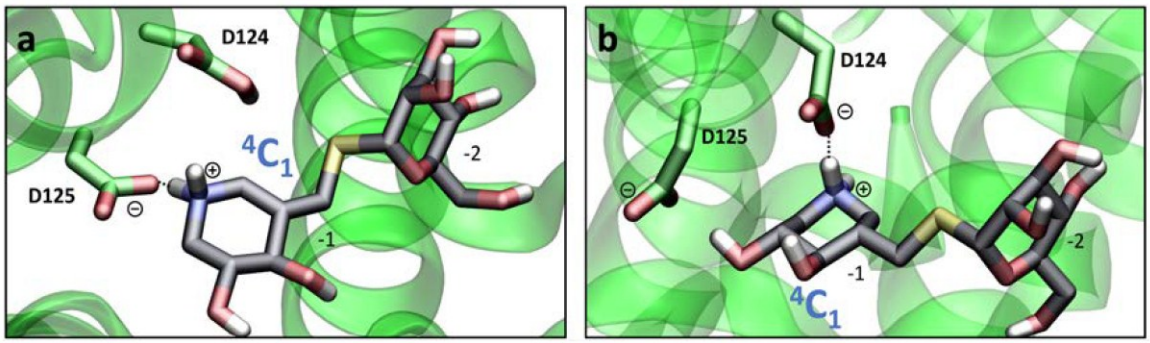


Figure S3. Active site structure resulting from the MD simulation, considering different protonation states of the two catalytic residues in *Bc*GH76-ManSIFG complexes.

a) Asp124-H, Asp125 (the result is independent of which proton of Asp124 is initially protonated); b) Asp124, Asp125. Protonation of Asp125 was not considered as this residue is directly interacting with the ammonium group of IFG. Water molecules are omitted for clarity. Only *a* is consistent with the experimentally determined crystal structure.

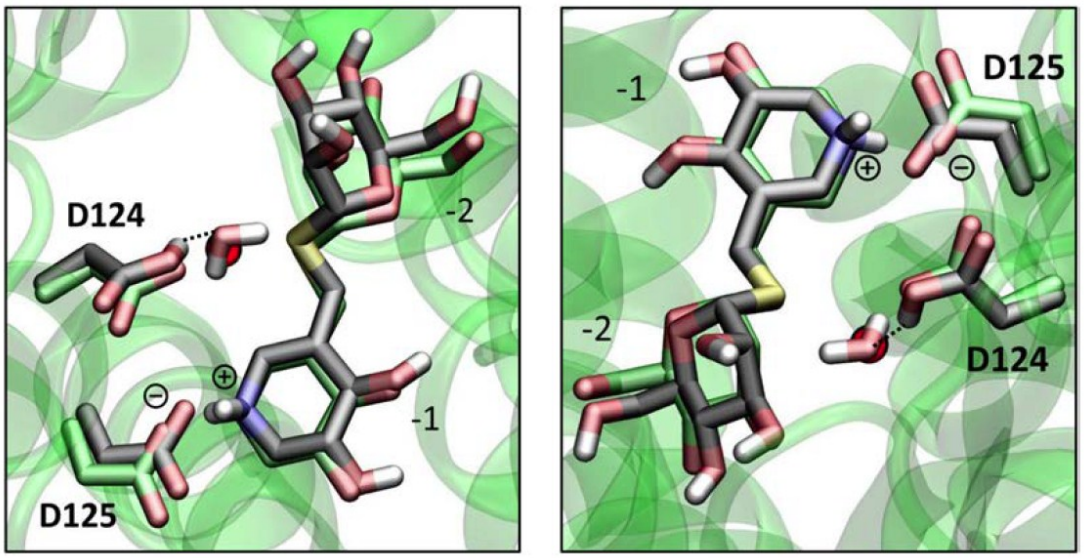


Figure S4. Superposition of the *Bc*GH76-ManSIFG structures of complexes determined by X-ray crystallography and MD simulations.

X-ray structure (PDB code: 5N0F, this work, green carbon atoms); structure from MD simulations (from Figure 3; grey carbon atoms). The distances between the O_{wat} atom and the O_{Asp124} are 2.87 and 2.86 Å, respectively.

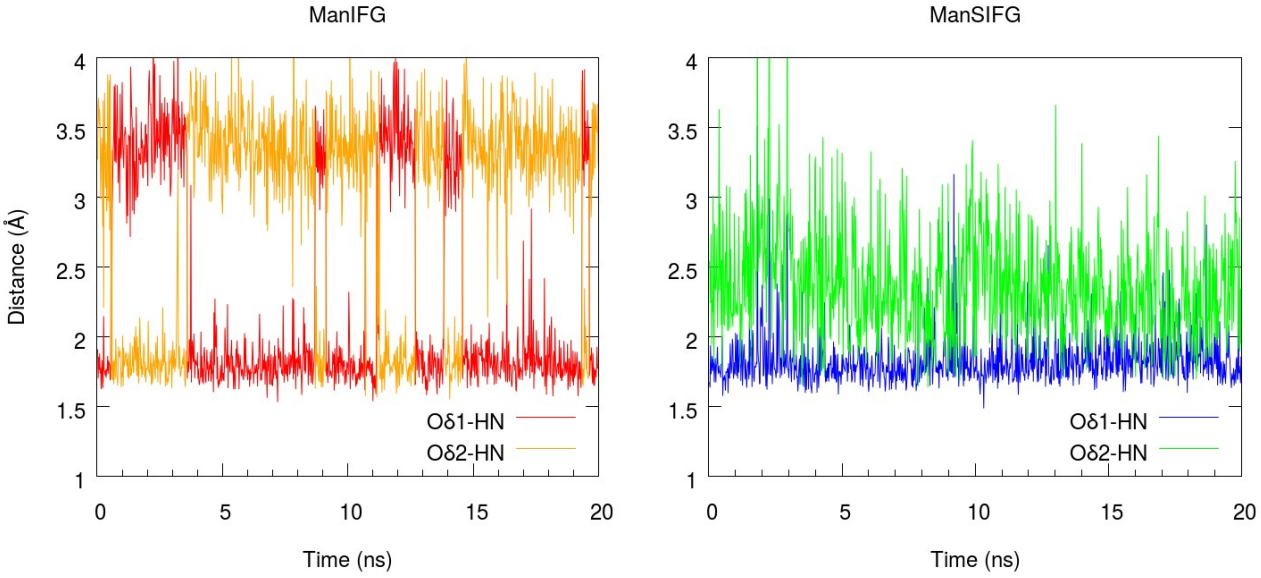


Figure S5. Ionic interaction between the ammonium group of IFG and the acid/base residue (Asp125) in both ManIFG and ManSIFG complexes of *Bc*GH76. Evolution of the H···O distance obtained from the MD simulations. Other strong interactions involving the IFG are NH_2^+···Asp124 (only in ManIFG) and OH···Asp294 (only in ManSIFG).

Table S1. Data collection and refinement statistics.

	*Bc*GH76–ManSIFG (2)	*Bc*GH76–$(ManS)_2$IFG (3)
PDB code	5N0F	5M77
Data collection		
Space group	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions		
a, *b*, *c* (Å)	85.1, 86.4, 103.7	84.1, 85.2, 101.9
α, β, γ (°)	90.0, 90.0, 90.0	90.0, 90.0, 90.0
Resolution (Å)	52.32 (1.69) *	50.93 (1.46)
R_{sym} or R_{merge}	0.084 (0.789)	0.05 (1.084)
$I/\sigma I$	13.4 (2.4)	16.2 (1.4)
Completeness (%)	98.6 (99.9)	99.9 (99.6)
Redundancy	7.6 (8.3)	6.5 (5.8)
Refinement		
Resolution (Å)	1.69	1.46
No. reflections	84733	127007
R_{work}/ R_{free}	0.152/0.218	0.121/0.165
No. atoms		
Protein	10197	10231
Ligand/ion	78	114
Water	560	455
B-factors		
Protein	26.2	26.5
Ligand/ion	21.4	21.0
Water	37.7	38.1
R.m.s deviations		
Bond lengths (Å)	0.0155	0.0194
Bond angles (°)	1.47	1.74

*Highest resolution shell is shown in parenthesis.

Table S2. Tabulation of observed N1-nucleophilic oxygen distances for isofagomine-type inhibitors bound to glycoside hydrolases.

Comparison of N1-nucleophilic oxygen (Nuc…O) distances for solved structures of glycoside hydrolases in complex with isofagomine or isofagomine derivatives. With the exception of *Bx*GH99, which has been proposed to operate through a neighboring group participation mechanism via a 1,2-anhydro sugar intermédiate, all retaining enzymes show the catalytic nucleophile (or nucleophilic water) to be within hydrogen-bonding distance to the pseudo-anomeric nitrogen of the piperidine. For several enzymes several closely-related structures are available; only one is included in the table.

PDB code	GH family	Enzyme	Inhibitor	N1-Nuc(O) distance (Å)	Mechanism	Comment	Ref.
1OIF	1	*Thermotoga maritima* β-glucosidase	isofagomine	2.6	retaining		1
4CU7	2	*Streptococcus pneumoniae* β-galactosidase, BgaA	galacto-isofagomine	2.7	retaining		2
4RE4	2	*Oryza sativa* Os7BGlu26 β-glucosidase/mannosidase	isofagomine	2.6	retaining		3
4ZOA	3	*Listeria innocua* Lin1840r β-glucosidase	isofagomine	2.6	retaining		4
2OYK	5	*Rhodococcus* sp. *endo*-glycoceramidase II	cellobiose-like isofagomine	2.7	retaining		5
1OCQ	5	*Bacillus agaradhearans* Cel5A	cellobiose-like isofagomine	2.6	retaining		6
1OCN	6	*Humicola insolens* Cel6A	cellobiose-like isofagomine	2.6	inverting	N1 to 'nucleophilic water'	7
1UP2	6	*Mycobacterium tuberculosis* Cel6	cellobiose-like isofagomine	2.8	inverting	N1 to 'nucleophilic water'	8
3RX8	9	*Alicyclobacillus acidocaldarius* Cel9A	cellobiose-like isofagomine	3.1	inverting	N1 to base (Asp146); no water molecule located nearby.	9
3CUF	10	*Cellulomonas fimi* xylanase	cellobiose-like isofagomine	2.6	retaining		see PDB

1VOL	10	*Streptomyces lividans* Xyn10A, pH 7.5	xylobiose-like isofagomine	2.7	retaining	N1-H distance = 1.26	10
1V0N	10	*Streptomyces lividans* Xyn10A, pH 7.5	xylobiose-like isofagomine	2.7	retaining	N1-H distance = 1.29	10
1FH8	10	*Cellulomonas fimi* xylanase	xylobiose-like isofagomine	2.6	retaining		11
1JAK	20	*Streptomyces plicatus* Hex	2-NHAc isofagomine	2.7	retaining	CO-N1 distance	12
1NOW	20	*Homo sapiens* HexB	2-NHAc isofagomine	2.8	retaining	CO-N1 distance	13
4CD4	26	*Cellvibrio japonicas Cj*Man26C	β-mannobiose-like isofagomine	2.8	retaining		14
3GXF	30	human lysosomal acid β-glucosidase	isofagomine	2.7	retaining		15
4UFH	59	*Mus musculus* GALC	galacto-isofagomine	2.7	retaining		16
4D4D	76	*Bacillus circulans*	α-mannosyl-1,6-isofagomine	2.8	retaining		17
3QFY	94	*Cellvibrio gilvus* cellobiose phosphorylase	isofagomine	2.9	inverting	N1 to sulfate	18
4AD2	99	*Bacteroides xylanisolvens* GH99	α-glucosyl-1,3-isofagomine	3.5	retaining	Proposed to operate through a neighboring group participation mechanism	19
4CD6	113	*Alicyclobacillus acidocaldarius* AaManA	β-mannobiose-like isofagomine	2.7	retaining		14

Biochemistry/crystallography experimental

X-ray data collection, processing and structure solution

Wildtype *Bc*GH76 (surface mutant R341Q) was expressed and purified and crystals were grown as described.[17] Complex formation with **2** and **3** was achieved by soaking native protein crystals in approximately 20 mM ligand over a period of 30 min. All crystals were cryoprotected by addition of polyethylene glycol monomethyl ether 550 to a final concentration of 20% v/v in the drop before flash-cooling with liquid nitrogen. Diffraction data were collected at 100 K at beamlines I02 and I04 of the Diamond Light Source, Didcot, UK, with all images integrated with XDS[20] and scaled with Aimless[21] in xia2. The structures were solved by molecular replacement using Molrep[22] in CCP4i2,[23] using PDB 4D4A as a search model. Model was refined numerous cycles with maximum likelihood refinement using REFMAC[24] and manually corrected using COOT.[25]

Isothermal titration calorimetry

The affinities of compounds **2** and **3** for *Bc*GH76 were measured by isothermal titration calorimetry (ITC). Titrations were performed with a MicroCal Auto-iTC200 system (GE Healthcare). Assays were conducted in triplicate at 25 ºC. Compound **2** (600 μM) and compound **3** (45 μM), respectively, were titrated into the ITC cell containing 81 μM and 6 μM, respectively, of purified *Bc*GH76. Dissociation constants (K_D) for each titration were calculated and averaged using MicroCal PEAQ-ITC analysis software. Compound **2** binds with K_D of 0.90 μM, and **3** binds with K_D of 24.7 nM.

Computational details

The initial structures of *Bc*GH76 enzyme in complex with ManSIFG, $(ManS)_2$IFG (PDB codes 5N0F and 5M77, this work) and ManIFG (PDB code 4D4, Ref.[17]) were taken from the respective crystal structures. As in previous studies, the IFG moiety was assumed to be protonated based on its high basicity.[6] Histidine residues were protonated according to its chemical environment. All Asp and Glu residues were considered deprotonated, except for the catalytic Asp residues, for which several protonation states were tested (Figures S1 and S2).

Molecular dynamics (MD) simulations were performed using AMBER14 software,[26] together with FF99SB[27] (protein residues), GLYCAM06[28] (mannose) and TIP3P[29] (water) force fields. The isofagomine inhibitor was parametrized using the antechamber module; considering the structural parameters of GLYCAM06 and gaff[30] force fields, together with the atomic charges (ESP) obtained from first principles calculations using Gaussian09[31] at the HF/6-31G* level of theory. The MD protocol for each enzyme complex consisted in the following. First, all water molecules were relaxed by energy minimization, holding the protein and the substrate fixed. Afterwards, the whole system was relaxed. To gradually reach the temperature of 300 K, the system was heated to 100 K, 200 K, 250 K and 300 K; in intervals of 50 ps (except for the last two heating steps, which took 100 ps each). In the first heating step, spatial constraints were initially added to the protein and ligands, while water molecules and sodium ions were able to move freely. Then, the constraints were removed while the whole system reached 100 K. Afterwards, the density was converged up to water density at 300 K in the constant-pressure, constant-temperature (NPT) ensemble. The simulations were further extended until equilibrium was reached, according to the convergence of the RMSD (20 ns approximately). Analysis of the trajectories was carried out using standard tools of AMBER and VMD.[32]

Protonation state of the catalytic residues

The protonation state of Asp124 (nucleophile residue) and Asp125 (acid/base residue) assumed in the previously reported *Bc*GH76-ManIFG complex (both residues deprotonated) did not reproduce the binding mode observed in the S-linked complexes (*Bc*GH76-ManSIFG and *Bc*GH76-$(ManS)_2$IFG). Therefore, other protonation states were examined for the O- and S-linked disaccharide complexes (Figures S2 and S3).

In silico O- and S-linkage replacement

For each experimentally observed binding mode, the oxygen/sulfur atom of the glyosidic linkage was replaced with the other element (i.e., oxygen to sulfur for ManIFG complex, Figures S2a and

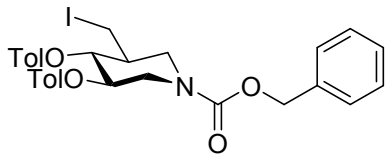
S2b; and *vice versa* for the ManSIFG complex, Figures S2c and S2d) and the corresponding structures were submitted to MD simulation, following the protocol described above. The protonation state of the catalytic residues (Asp124 and Asp125) was tested in each case. After the *in* silico O- or S-linkage replacement, neither the binding mode nor the ring conformation reverted to the experimentally determined poses. For instance, starting from the *Bg*GH76-ManIFG complex, when the O-linkage is replaced by sulfur, the resulting complex did not show the binding mode and the ring conformation of the *Bc*GH76-ManSIFG complex observed in the X-ray experiments. In the same way, when the S-linkage of the *Bc*GH76-ManSIFG complex was replaced by O, the binding mode and the ring conformation of the equilibrated structure do not correspond to the ones obtained experimentally for the *Bc*GH76-ManIFG complex. These results suggest that the energy barrier for the transition between the two binding modes sufficiently high so that interconversion between them cannot occur once the inhibitor is bound to the active site. The binding mode and the conformation of the IFG ring were maintained when oxygen was replaced by sulfur on ManIFG complex (Figures S2a and S2b). Nevertheless, in the case in which Asp125 was protonated (Figure S2b), the interaction between the IFG 'ammonium' group and the carboxylic group of the acid/base residue was lost. On the other hand, replacement of S by O on the ManSIFG complex disrupts the binding mode independently of the protonation state of the catalytic residues (Figures S2c and Figure S2d).

Synthetic chemistry

General

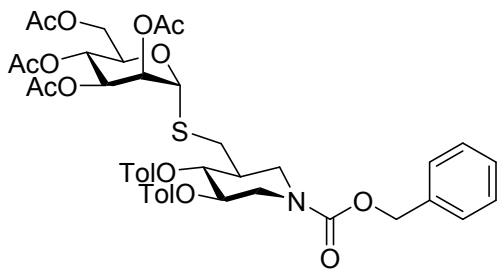
Proton nuclear magnetic resonance spectra (^{1}H NMR, 400, 500 or 700 MHz) and proton decoupled carbon nuclear magnetic resonance spectra (^{13}C NMR, 100 or 125 MHz) were obtained in deuterochloroform, dimethylsulfoxide-d_6, with residual protonated solvent or solvent carbon signals as internal standards, or for D_2O solutions to an external D_2O solutions of the sodium salt of 3-(trimethylsilyl)propanesulfonic acid. Abbreviations for multiplicity are s, singlet; d, doublet; t, triplet; q, quartet. For oligosaccharides, rings are labelled A,B,C… from the reducing end. Optical rotations were obtained using a JASCO DIP-1000 polarimeter. $[\alpha]_D$ values are given in 10^{-1} cm^2 g^{-1}. Flash chromatography was carried out on silica gel 60 according to the procedure of Still *et al*.[33] Analytical thin layer chromatography (t.l.c.) was conducted on aluminium-backed 2 mm thick silica gel 60 F_{254} and chromatograms were visualized with ceric ammonium molybdate (Hanessian's stain), potassium permanganate or 5% H_2SO_4/MeOH, with charring as necessary. Melting points were obtained using a hot-stage or capillary apparatus and are corrected. High resolution mass spectra (HRMS) were obtained using an ESI-TOF-MS; all samples were run using 0.1% formic acid. Dry CH_2Cl_2, THF, and Et_2O were obtained from a dry solvent apparatus (Glass Contour of SG Water, Nashua, U.S.A.) as per the procedure of Pangborn *et al*.[34] Dry DMF was dried over 4 Å molecular sieves. Pet. spirits refers to petroleum ether, boiling range 40–60 °C.

3,4-Di-O-(4-methylbenzoyl)-6-deoxy-6-iodo-N-benzyloxycarbonyl-isofagomine (5)



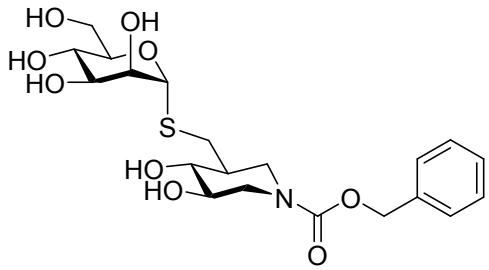
A solution of alcohol (**4**)[17] (41.3 mg, 0.080 mmol) in toluene (2.5 ml) was treated sequentially with triphenylphosphine (31.5 mg, 0.120 mmol), imidazole (16.3 mg, 0.239 mmol) and iodine (20.3 mg, 0.160 mmol) at 70 C with stirring for 2 h. The mixture was evaporated to dryness under reduced pressure. The residue was purified by flash column chromatography (0.5 % Et_3N in 10:90 EtOAc/pet. spirts) to give iodide (**5**) (38.8 mg, 78%), $[\alpha]_D^{23}$ −50.6 (c 0.25 in $CHCl_3$); δ_H (400 MHz, d_6-DMSO) 7.84–7.27 (13 H, m, Ph,C_6H_4), 5.43 (1 H, t, $J_{3,4}$ 7.5, $J_{4,5}$ 7.5 Hz, H4), 5.13–5.06 (7 H, m, H2,3,6,6',7,C**H**$_2$Ph), 5.03 (1 H, m, H3), 4.27 (1 H, m, H2), 4.19 (1 H, m, H7), 2.34 (6 H, s, 2 × CH_3, Ar), 2.16 (1 H, m, H5); δ_C (125 MHz, d_6-DMSO) 165.6, 165.3 (OC=O), 154.9 (NC=O), 144.6, 144.5, 137.1, 129.9–126.6 (18 C, Ph,Ar), 74.4 (C4), 70.9 (C3), 67.2 (**C**H_2Ph), 47.3 (C2), 45.2 (C6), 40.5 (C5), 21.6 (2 C, 2 × CH_3), 5.6 (C7); HRMS (ESI^+) calcd for $C_{30}H_{30}INO_8Na$ $(M + Na)^+$ 682.2608. Found 682.2597.

(2,3,4,6-Tetra-O-acetyl-α-D-mannopyranosyl)-(1→6)-6-deoxy-3,4-di-O-(4-methylbenzoyl)-6-thio-N-benzyloxycarbonyl-isofagomine (7)



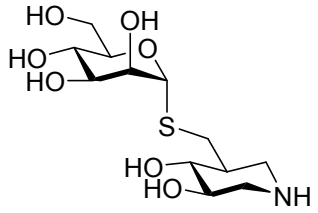
A solution of iodide (**5**) (50.0 mg, 0.0797 mmol) and tetra-*O*-acetyl-α-D-mannopyranosyl isothiouronium bromide (**6**)[35] (53.3 mg, 0.120 mmol) in dry and deoxygenated DMF (1 ml) was treated with triethylamine (0.129 ml, 0.925 mmol) at r.t with stirring for 1 h. The solvent was evaporated to dryness under reduced pressure and the residue was purified by flash column chromatography (3:7 EtOAc/pet. spirits) to give disaccharide (**7**) (78.9 mg, 80%); δ_H (700 MHz, d_6-DMSO) 7.82–7.26 (13 H, m, Ar,Ph), 5.47 (1 H, s, $H1^B$), 5.39 (1 H, t, $J_{3,4}$ 8.6, $J_{4,5}$ 8.6 Hz, $H4^A$), 5.18 (1 H, dd, $J_{2,3}$ 3.5, $J_{1,2}$ 1.3 Hz, $H2^B$), 5.11–5.00 (7 H, m, $H3^A$,(3,4,5,6,6')^B,C**H**$_2$Ph), 4.26-4.17 (1 H, m, $H2^A$), 4.17 (1 H, m, $H7^A$), 3.77 (1 H, m, $H7^A$), 3.43 (1 H, m, $H6^A$), 3.33 (1 H, m, $H6'^A$), 3.21 (1 H, m, $H2^A$), 2.84 (1 H, m, $H2^A$), 2.61 (1 H, m, $H5^A$), 2.32 (2 × 3 H, 2s, 2 × C**H**$_3$Ar), 2.10, 2.01, 1.99, 1.93 (4 × 3 H, 4s, 4 × C**H**$_3$CO), 1.87 (1 H, m, $H5^A$); δ_C (125 MHz, d_6-DMSO) 169.8, 169.6, 169.5, 169.4, 165.3, 164.8 (OC=O), 154.6 (NC=O), 144.1, 144.0, 136.6, 129.4–126.1 (18 C, Ar,Ph), 80.8 ($C1^B$), 73.4 ($C4^A$), 70.5, 69.7, 69.0, 68.4, 66.7, 65.3, 61.8, 59.7 (**C**H$_2$Ph, C(3,6)^A,(2,3,4,5)^B), 45.0 ($C2^A$), 44.7 ($C7^A$), 40.0 ($C5^A$), 29.0 ($C6^B$), 21.1 (2C, 2 ×Ar**C**H$_3$), 20.6, 20.4, 20.3, 20.2 (CO**C**H$_3$); HRMS (ESI$^+$) calcd for $C_{12}H_{23}NO_7SNa$ $(M + Na)^+$ 886.2715. Found 886.2707.

α-D-Mannopyranosyl-(1→6)-6-deoxy-6-thio-N-benzyloxycarbonyl-isofagomine (8)



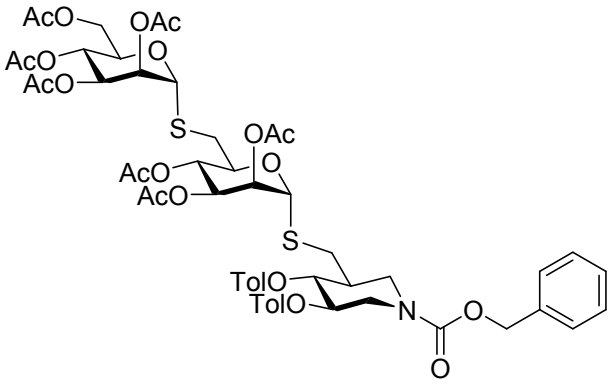
A solution of disaccharide (**7**) (60.7 mg, 0.0702 mmol) was treated with 1 M methanolic NaOMe (24 μL) in methanol (2 ml) at rt with stirring for 3 h. The solution was adjusted to pH 5–6 with Amberlite IR-120 resin (H^+ form), filtered, and the filtrate evaporated to dryness under reduced pressure. Water (5 ml) was added and the solution was washed with ether (2 × 5 ml). The aqueous layer was evaporated and the residue was purified by flash chromatography (7:2:1→5:4:1 EtOAc/MeOH/H_2O) to give disaccharide polyol (**8**) (23.4 mg, 73%), $[\alpha]_D^{24}$ + 148.1 (c 0.25 in MeOH); δ_H (700 MHz, D_2O) 7.40–7.35 (5 H, m, Ph), 5.18 (1 H, s, $H1^B$), 5.10 (2 H, m, C**H**$_2$Ph), 4.24–3.71 (8 H, m, H(3,6)^A,(2,3,4,5,6,6')^B), 3.61 (1 H, t, $H6'^A$), 3.38 (1 H, m, $H4^A$), 3.25 (1 H, t, $H3^A$), 3.03 (1 H, dd, $H7^A$), 2.69 (2 H, m, H(2,7')^A), 2.44 (1 H, m, $H2'^A$), 1.69 (1 H, m, $H5^A$); δ_C (125 MHz, D_2O) 156.5 (NC=O), 136.3, 128.8–127.7 (6 C, Ph), 84.3 ($C1^B$), 75.7, 73.2, 71.7, 71.0, 70.6, 67.8, 67.0, 60.8 (C(3,4,7)^A,(2,3,4)^B,Ph**C**H$_2$), 47.8, 46.3 ($C2^A$,6^B), 41.6 ($C5^B$), 41.1 ($C5^A$), 29.4 ($C6^A$); HRMS (ESI$^+$) calcd for $C_{20}H_{29}NO_9S$ $(M + H)^+$ 460.1630. Found 460.1636.

α-D-Mannopyranosyl)-(1→6)-6-deoxy-6-thio-isofagomine (ManSIFG; 2)



A mixture of disaccharide polyol (**8**) (23.4 mg, 0.0509 mmol) in thioanisole (0.3 ml) was treated with TFA (1.91 ml, 25.7 mmol) at rt with stirring for 2 h. The TFA was evaporated under a stream of nitrogen for 15 min and the mixture was extracted into water (5 ml). The aqueous layer was washed with ether (2 × 5 ml) and evaporated to dryness under reduced pressure. The residue was purified by ion-exchange chromatography (Dowex 1X-8, OH^- form, eluted with H_2O; Amberlite CG50 Type 1 H^+ form, washed with H_2O, then eluted with 3 M aqueous NH_3) followed by C18-reverse phase chromatography (1 → 19:1 → 9:1 H_2O/MeCN, 200 μL fractions) to give ManSIFG (**2**) (11.5 mg, 70%), $[\alpha]_D^{24}$ + 95.1 (c 0.05 in MeOH); δ_H (700 MHz, D_2O) 5.19 (1 H, s, $H1^B$), 3.98 (1 H, d, $J_{1,2}$ 2.0 Hz, $H2^B$), 3.93–3.91 (1 H, m, $H5^B$), 3.83 (1 H, dd, $J_{5,6}$ = $J_{6,6}$ 2.0 Hz, $H6^B$), 3.74–3.70 (2 H, m, $H(3,6')^B$), 3.61 (1 H, t, $J_{3,4}$ 9.9 Hz, $H4^B$), 3.44–3.40 (1 H, m, $H6^A$), 3.22–3.15 (2 H, m, $H(6',7)^A$), 3.09 (1 H, dd, $J_{6,7}$ 5.3, $J_{7,7}$ 13.6 Hz, $H7'^A$), 3.00 (1 H, m, $H4^A$), 2.48 (1 H, dd, $J_{3,4}$ 9.2 Hz, $H3^A$), 2.38-2.30 (2 H, m, $H(2,2')^A$), 1.75-1.69 (1 H, m, $H5^A$); δ_C (125 MHz, D_2O) 84.5 ($C1^B$), 75.9, 73.2, 71.8, 71.7, 71.0, 67.1, 60.8 ($C(3,4,7)^A$,$(2,3,4,6)^B$,Ph**C**H_2), 49.5, 47.8 ($C(5,6)^A$), 42.24 ($C5^B$), 29.9 ($C2^A$); HRMS (ESI^+) calcd for $C_{12}H_{23}NO_7SNa$ $(M + Na)^+$ 348.1087. Found 348.1108.

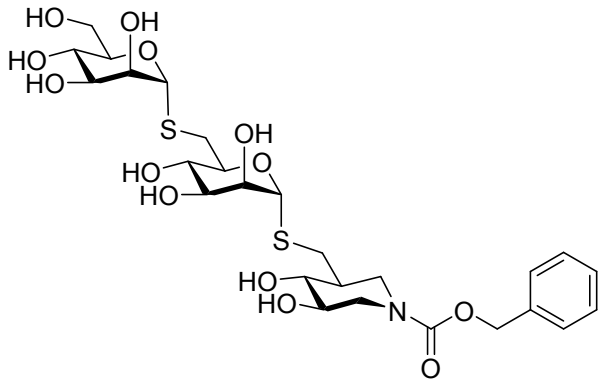
(2,3,4,6-Tetra-O-acetyl-α-D-mannopyranosyl)-(1→6)-(2,3,4-tri-O-acetyl-6-deoxy-1,6-dithio-α-D-mannopyranosyl)-(1→6)-6-deoxy-3,4-O-di-(4-methylbenzoyl)-6-thio-N-benzyloxycarbonyl-isofagomine (10)



A solution of iodide (**5**) (37.8 mg, 0.0602 mmol) and *S*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→6)-(2,3,4-tri-*O*-acetyl-1-*S*-acetyl-1,6-dithio-α-D-mannopyranose) (**9**)[36] (64.2 mg, 0.0904 mmol) in dry and deoxygenated DMF (1 ml) was treated with diethylamine (18.7 μL, 0.181 mmol) at r.t with stirring for 1 h. The solvent was evaporated to dryness under reduced pressure and the residue was purified by flash chromatography (3:7 → 1:1 EtOAc/pet. spirits) to give trisaccharide (**10**) (87.6 mg, 81%), $[\alpha]_D^{23}$ +21.0 (c 0.25 in $CHCl_3$); δ_H (700 MHz, d_6-DMSO) 7.83–7.26 (13 H, m, Ar,Ph), 5.41–5.39 (2 H, m, $H4^A$,1^C), 5.32 (1 H, s, $H1^B$), 5.20–5.00 (13 H, m, $H3^A$,$(2,3,4,6,6')^B$,$(2,3,4,6,6')^C$,C**H**$_2$Ph), 4.26–3.98 (5 H, m, $H(2,6,6',7,7')^A$), 2.91 (1 H, m, $H2^A$), 2.70–2.57 (3 H, m, $H5^A$,5^B,5^C), 2.33, 2.33 (6 H, 2s, 2 × Ar**C**H_3), 2.07, 2.07 (6 H, 2s, Ac), 2.03 (6 H, s, Ac), 2.00 (3 H, s, Ac), 1.95, 1.95 (6 H, 2s, Ac); δ_c (125 MHz, d_6-DMSO) 170.4, 170.1, 170.05, 170.00, 169.9, 165.7, 165.3 (OC=O), 154.9 (NC=O), 144.6, 144.4, 137.1, 129.9-126.6 (13 C,

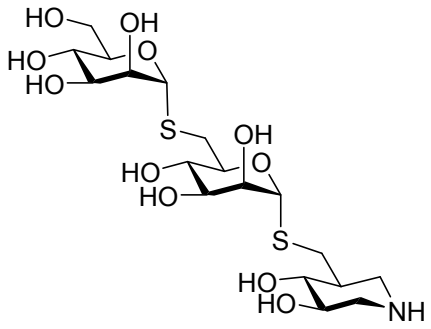
Ar,Ph), 82.0, 81.4 ($C1^{B}$,1^{C}), 73.8, 71.0, 70.6, 70.3, 70.0, 69.4, 69.22, 69.15, 68.0, 67.1, 66.1, 62.3, 60.2 ($C(3,4)^{A}$,$(2,3,4,5)^{B,C}$,6^{C},**C**H_2Ph), 45.6 ($C2^{A}$), 45.2 ($C7^{A}$), 40.5 ($C5^{A}$), 31.6 ($C6^{B}$), 29.5 ($C6^{A}$), 21.6–20.8 (9 C, 9 × CH_3); HRMS (ESI^+) calcd for $C_{18}H_{34}NO_{11}S_2$ $(M + H)^+$ 1168.3512 Found 1168.3564.

α-D-Mannopyranosyl)-(1→6)-(6-deoxy-1,6-dithio-α-D-mannopyranosyl)-(1→6)-6-deoxy-6-thio-N-benzyloxycarbonyl-isofagomine (11)



A solution of trisaccharide (**10**) (62.0 mg, 0.053 mmol) was treated with 1 M NaOMe (50 μL) in methanol (2 ml) at rt with stirring for 3 h. The solution was adjusted to pH 5–6 with Amberlite IR-120 resin (H^+ form), filtered, and the filtrate evaporated to dryness under reduced pressure. Water (5 ml) was added and the solution was washed with ether (2 × 5 ml). The aqueous layer was evaporated and the residue was purified by flash chromatography (7:2:1→5:4: EtOAc/MeOH/H_2O) to give trisaccharide polyol (**11**) (26.0 mg, 90%), $[\alpha]_D^{24}$ +175.1 (c 0.16 in MeOH); δ_H (700 MHz, D_2O) 7.39–7.35 (5 H, m, Ph), 5.24–5.10 (4 H, $H1^{B}$,1^{C},C**H**$_2$Ph), 4.26–3.59 (13 H, m, $H(3,7,7')^{A}$,$(2,3,4,5)^{B}$,$(2,3,4,5,6,6')^{C}$), 3.39 (1 H, m, $H4^{A}$), 3.26 (1 H, m, $H6^{B}$), 3.08 (2 H, m, $H6^{A}$,$6'^{B}$), 2.72 (2 H, m, $H(2,6')^{A}$), 2.45 (1 H, m, $H2'^{A}$), 1.70 (1 H, m, $H5^{A}$); δ_C (125 MHz, D_2O) 156.5 (NC=O), 136.3, 128.8–127.8 (6C, Ph), 84.6, 84.2 ($C1^{B}$,1^{C}), 76.0, 73.2, 71.6, 71.5, 71.1, 70.9, 70.6, 69.8, 67.9, 67.0, 60.8 ($C(3,4,5,7)^{A}$,$(2,3,4)^{B}$,$(2,3,4,6)^{C}$,Ph**C**H_2), 47.8, 46.4 $(C2,6)^{A}$, 41.6, 41.0 ($C5^{B}$,5^{C}), 31.2, 29.6 ($C6^{A}$,6^{B}); HRMS (ESI^+) calcd for $C_{26}H_{39}NO_{13}S_2$ $(M + H)^+$ 638.1934. Found 638.1946.

α-D-Mannopyranosyl-(1→6)-(6-deoxy-1,6-dithio-α-D-mannopyranosyl)-(1→6)-6-deoxy-6-thio-isofagomine (ManS)$_2$IFG; 3)



A mixture of trisaccharide polyol (**11**) (21.0 mg, 0.0329 mmol) in thioanisole (0.2 ml) was treated with TFA (1.2 ml, 16.6 mmol) at rt with stirring for 2 h. The TFA was evaporated under a stream of nitrogen for 15 min and the mixture was extracted into water (5 ml). The aqueous layer was washed with ether (2 × 5 ml) and evaporated to dryness under reduced pressure. The residue was purified by ion-exchange chromatography (Dowex 1X-8, OH^- form, eluted with H_2O; Amberlite CG50 Type 1 H^+ form, washed with H_2O, then eluted with aqueous 3 M NH_3) followed by reverse phase chromatography (1 → 19:1 → 9:1 H_2O/MeCN, 200 μL fractions) to give (ManS)$_2$IFG (**3**) (8.4 mg, 51%), $[\alpha]_D^{24}$ +211.7 (c 0.04 in MeOH); δ_H (700 MHz, D_2O) 5.29 (1 H, d, $J_{1,2}$ 0.7 Hz, H1^C), 5.18 (1 H, d, $J_{1,2}$ 0.6 Hz, H1^B), 4.06–4.03 (1 H, m, H4^B), 4.01-3.99 (3 H, m, H2^B,(2,5)^C), 3.94 (1 H, m, H5^B), 3.82 (1 H, dd, $J_{5,6}$ 2.2, $J_{6,6}$ 12.3 Hz, H6^C), 3.76–3.56 (7 H, m, H(3,6)^A,(3,6)^B,(3,4,6')^C), 3.45–3.36 (1 H, m, H6'^{A},6^B), 3.12 (2 H, m, H(7,7')^A), 2.84–2.76 (3 H, m, H(2,2',4)^A), 2.59 (1 H, m, H3^A), 2.02–1.97 (1 H, m, H5^A); δ_C (125 MHz, D_2O) 84.3, 84.1 (C1^B,1^C), 73.8, 73.2, 71.6, 71.5, 71.0, 70.9, 69.7, 68.8, 67.0, 60.8 (C(3,4,6,7)^A,(2,3,4,6)^B,(2,3,4,6)^C,Ph**C**H$_2$), 47.0, 46.1 (C5^A,5^B), 39.0 (C5^C), 31.3, 29.0 (C5^A,6^A), 23.2 (C2^A); HRMS (ESI$^+$) calcd for $C_{18}H_{33}NO_{11}S_2$ $(M + H)^+$ 504.15678. Found 504.15720.

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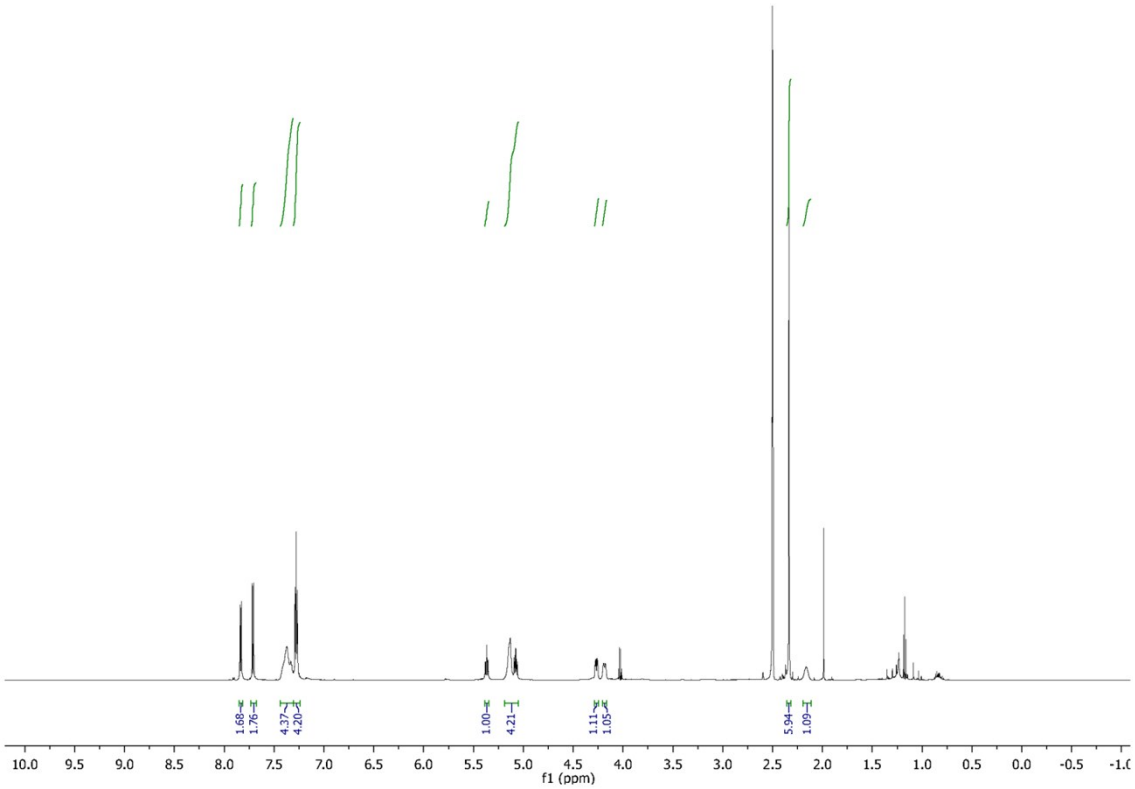
NMR Spectra

3,4-Di-O-(4-methylbenzoyl)-6-deoxy-6-iodo-N-benzyloxycarbonyl-isofagomine (5)

^{1}H NMR



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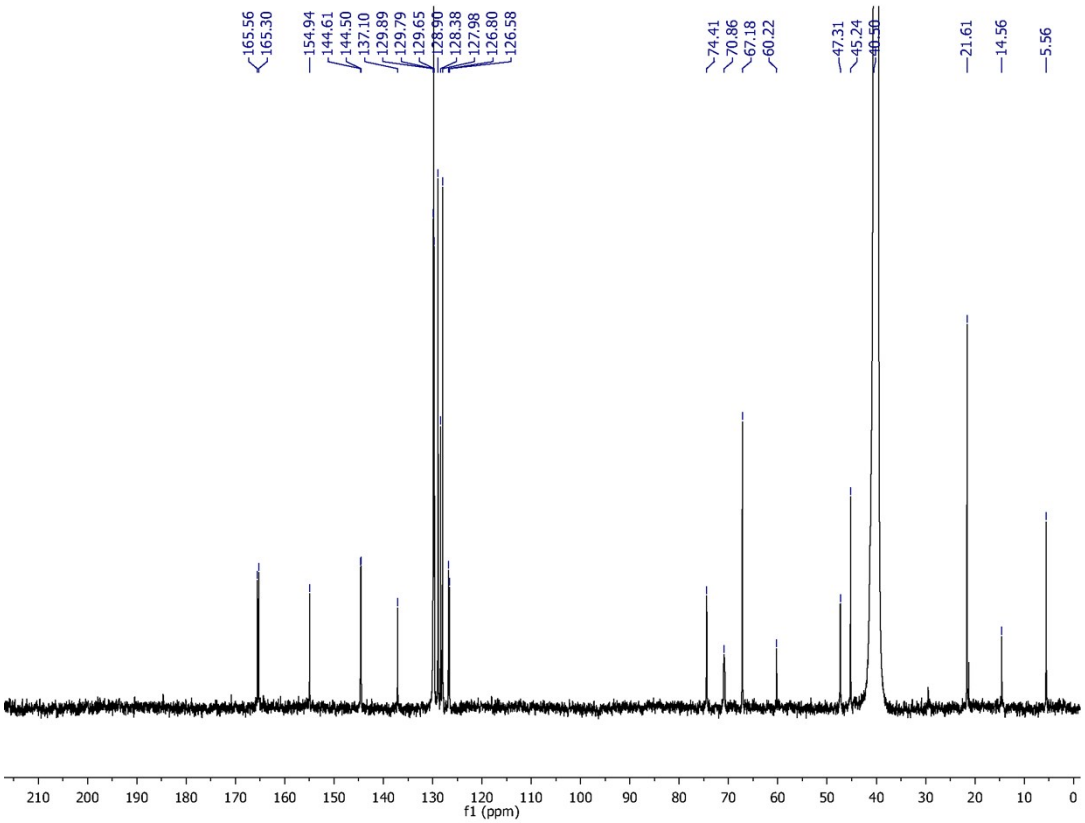
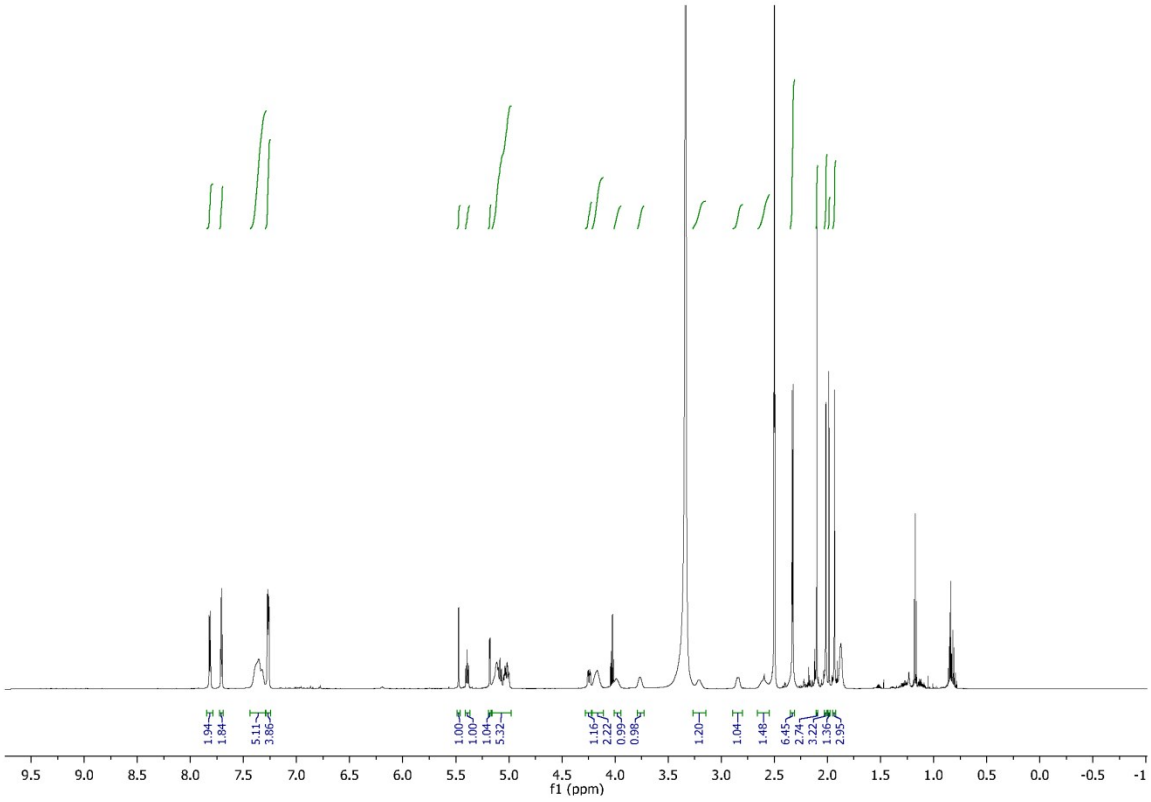


(2,3,4,6-Tetra-O-acetyl-α-D-mannopyranosyl)-(1→6)-6-deoxy-3,4-di-O-(4-methylbenzoyl)-6-thio-N-benzyloxycarbonyl-isofagomine (7)

^{1}H NMR



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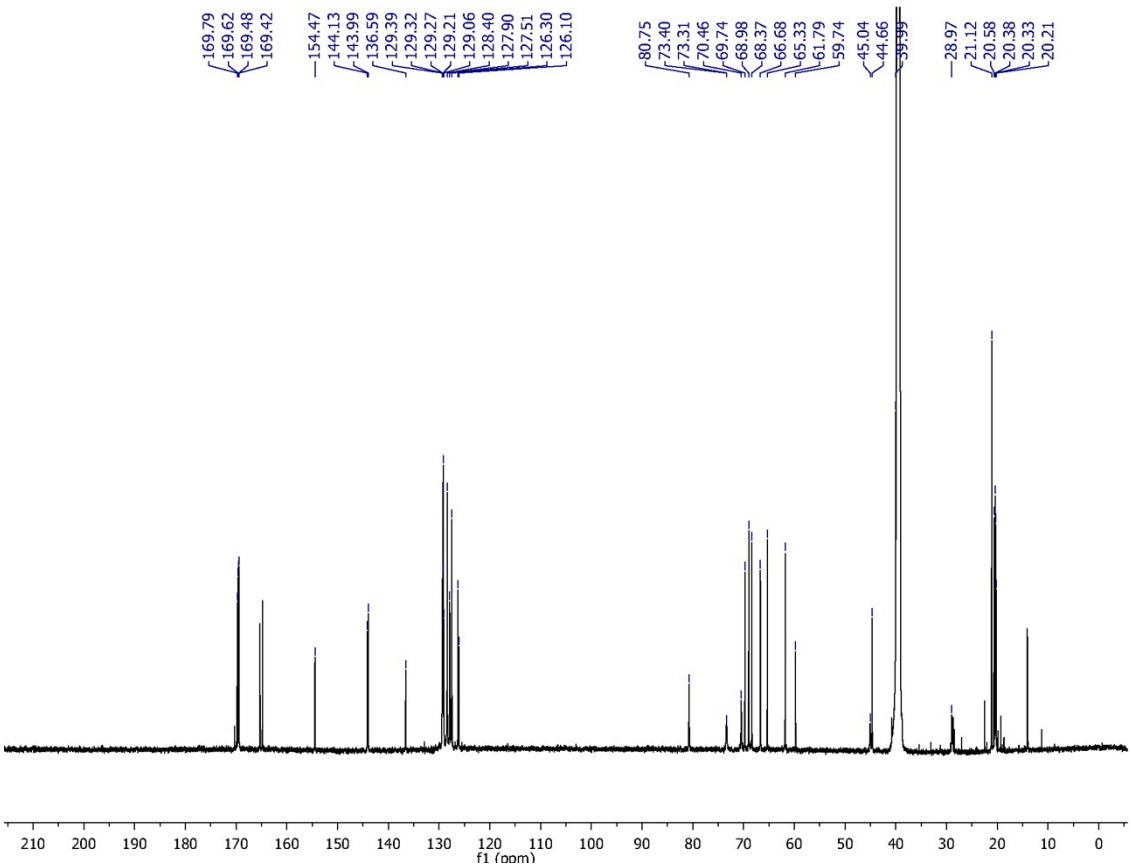

α-D-Mannopyranosyl-(1→6)-6-deoxy-6-thio-N-benzyloxycarbonyl-isofagomine (8)

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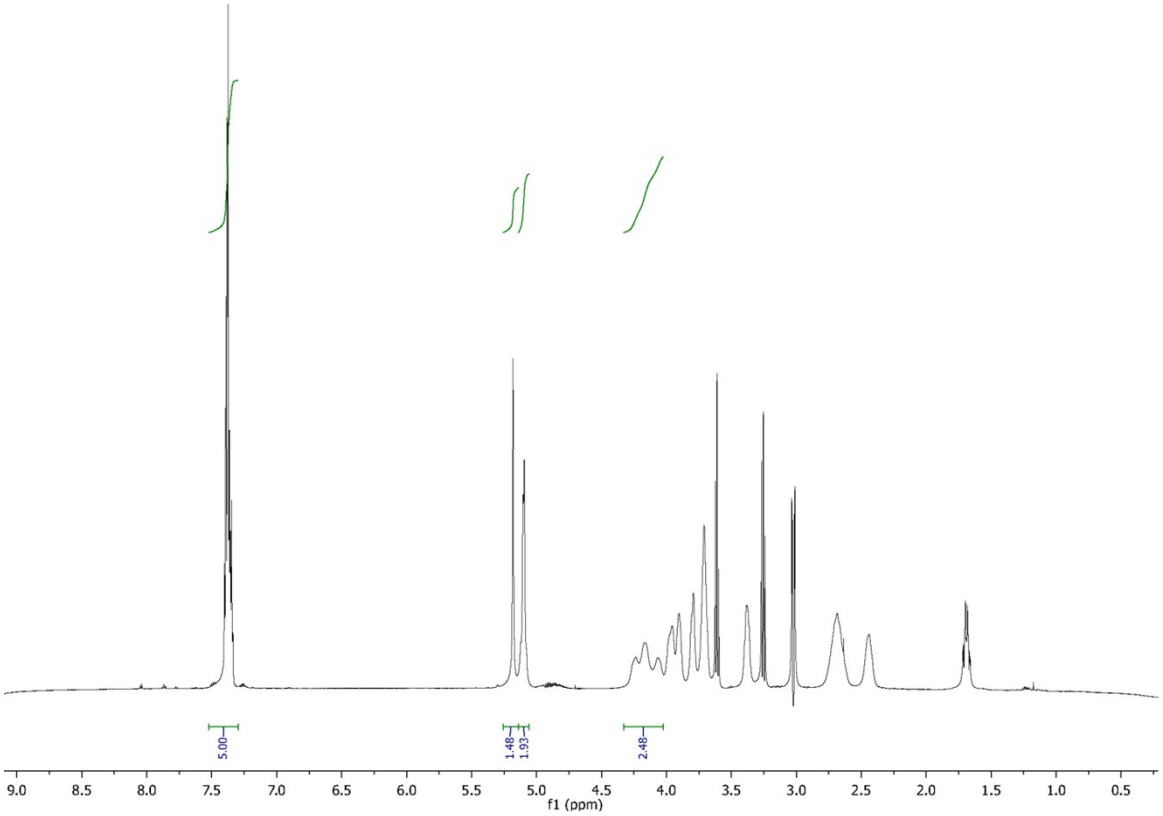

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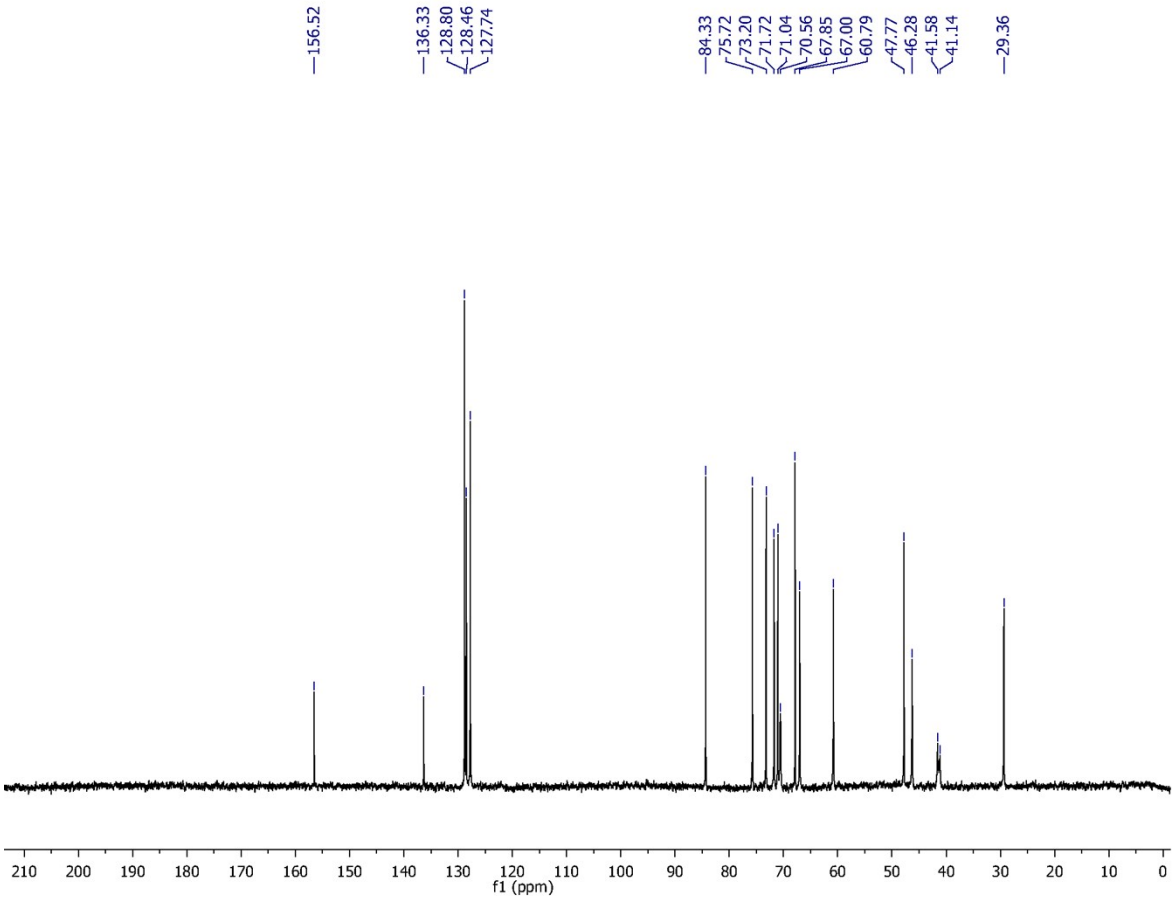

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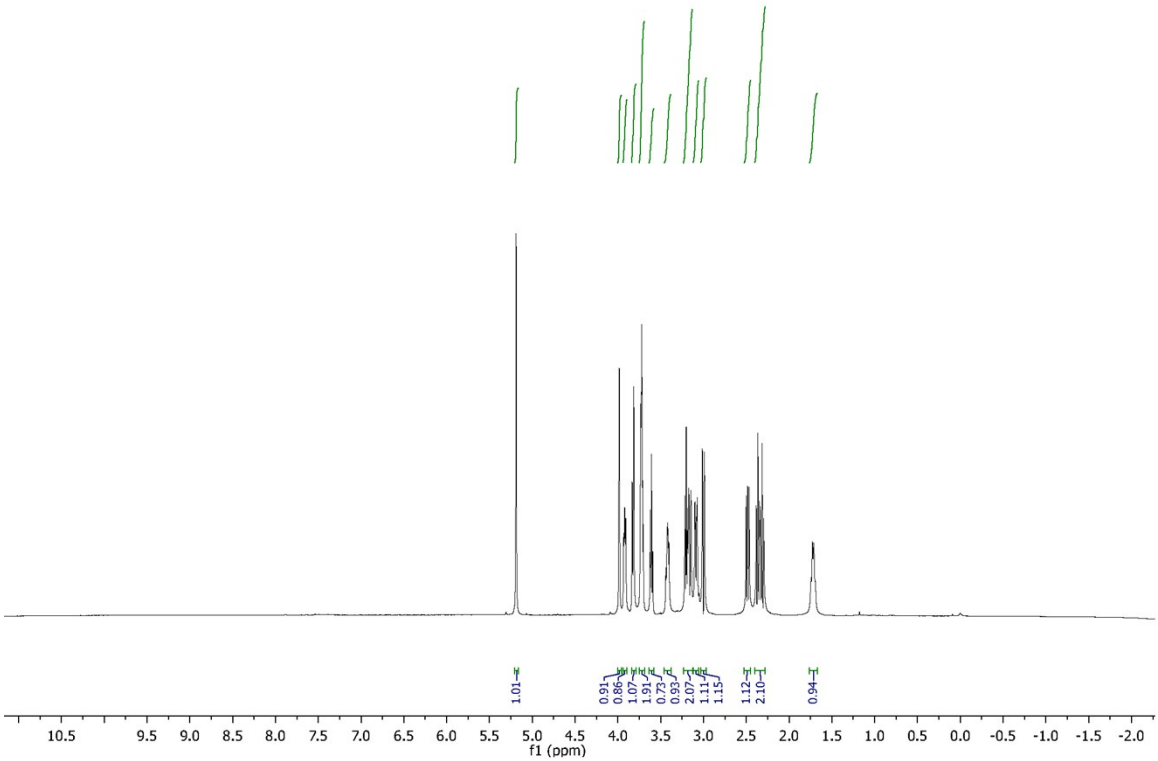

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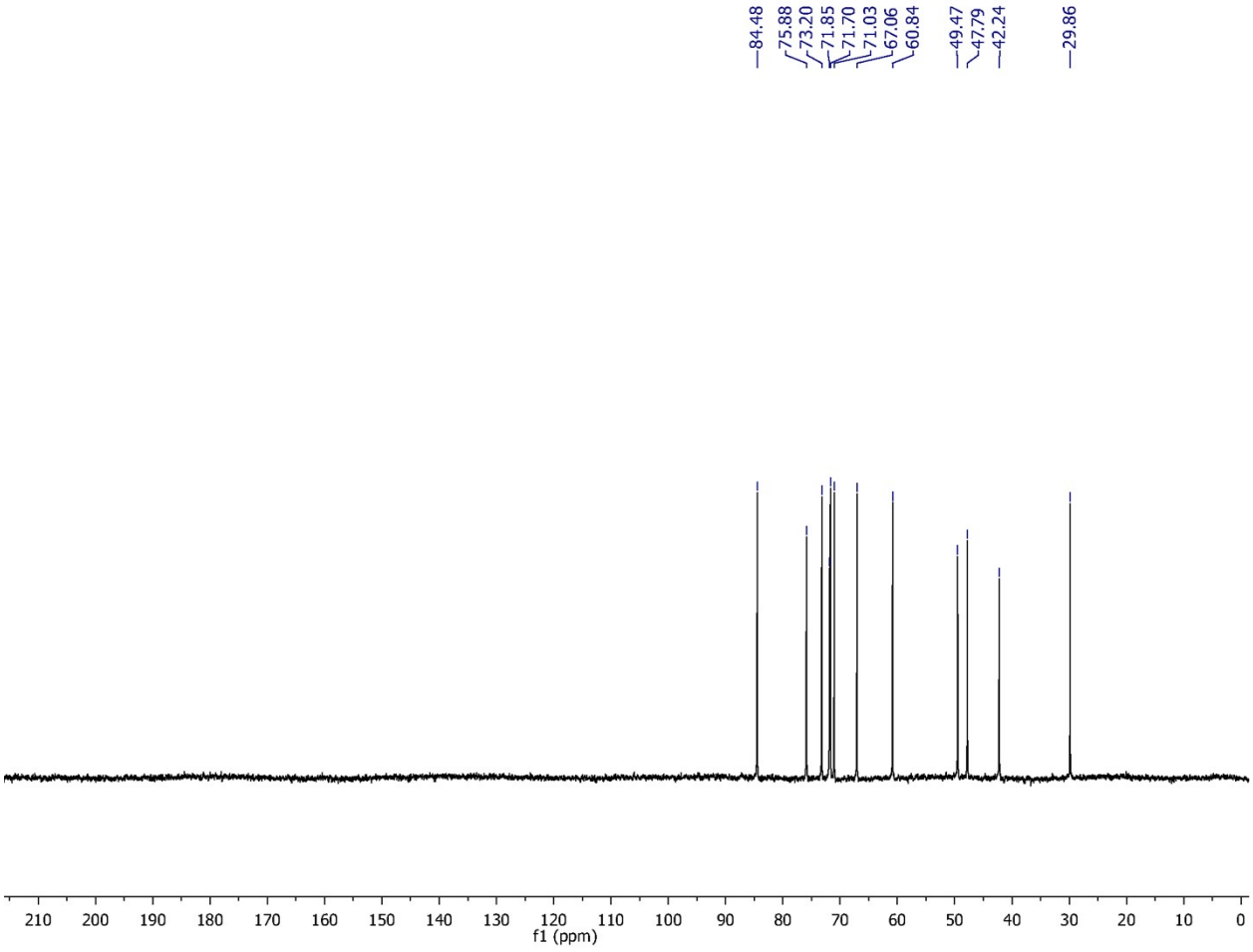
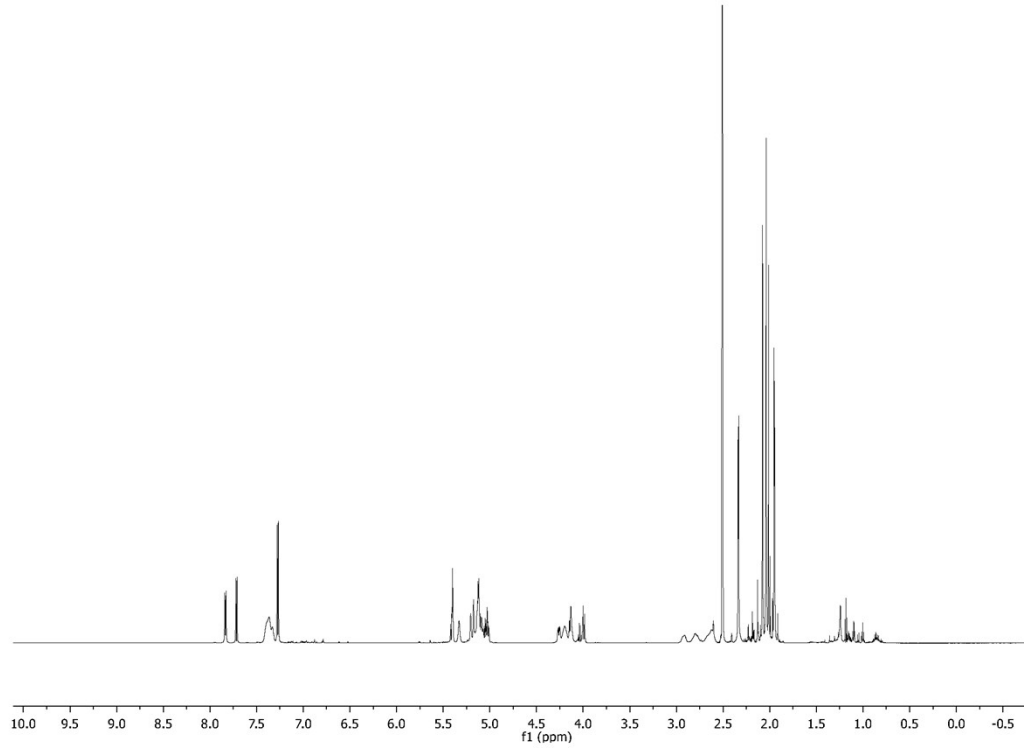
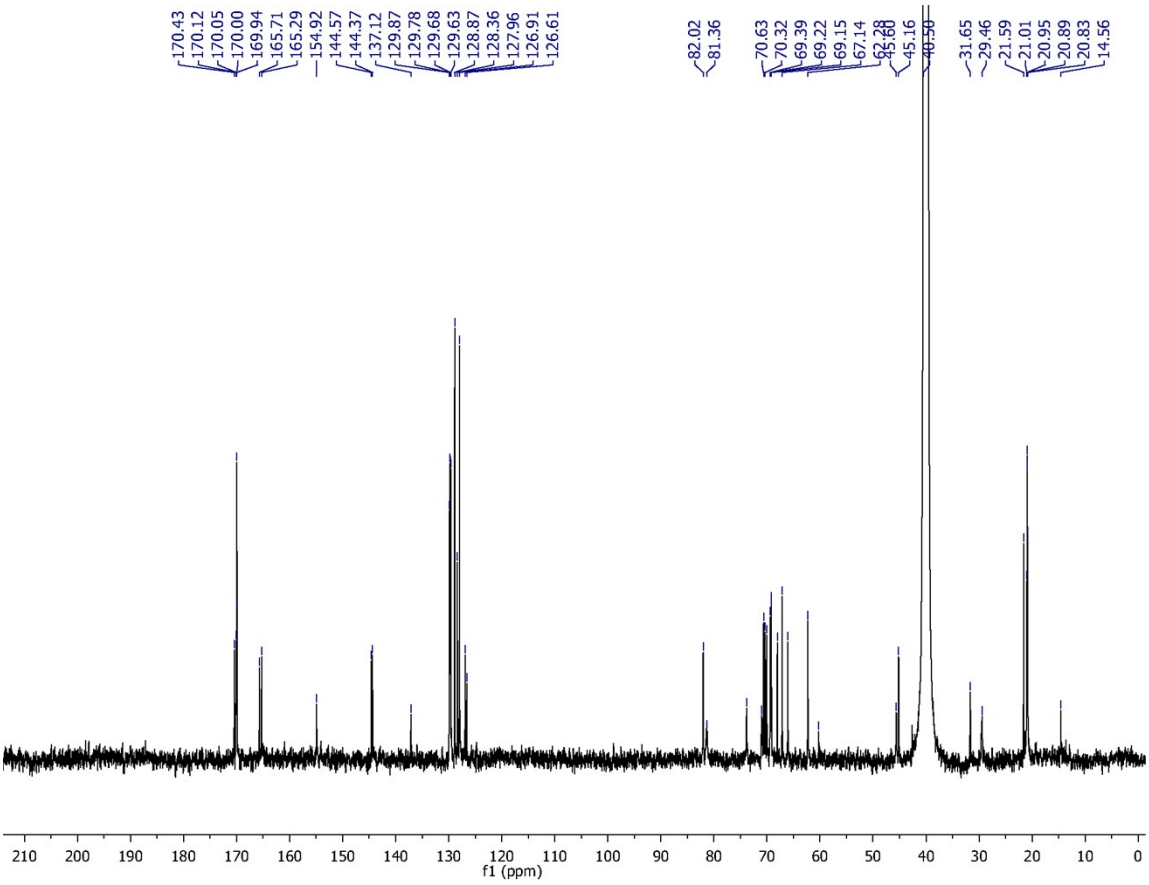


(2,3,4,6-Tetra-O-acetyl-α-D-mannopyranosyl)-(1→6)-(2,3,4-tri-O-acetyl-6-deoxy-1,6-dithio-α-D-mannopyranosyl)-(1→6)-6-deoxy-3,4-O-di-(4-methylbenzoyl)-6-thio-N-benzyloxycarbonyl-isofagomine (10)

^{1}H NMR

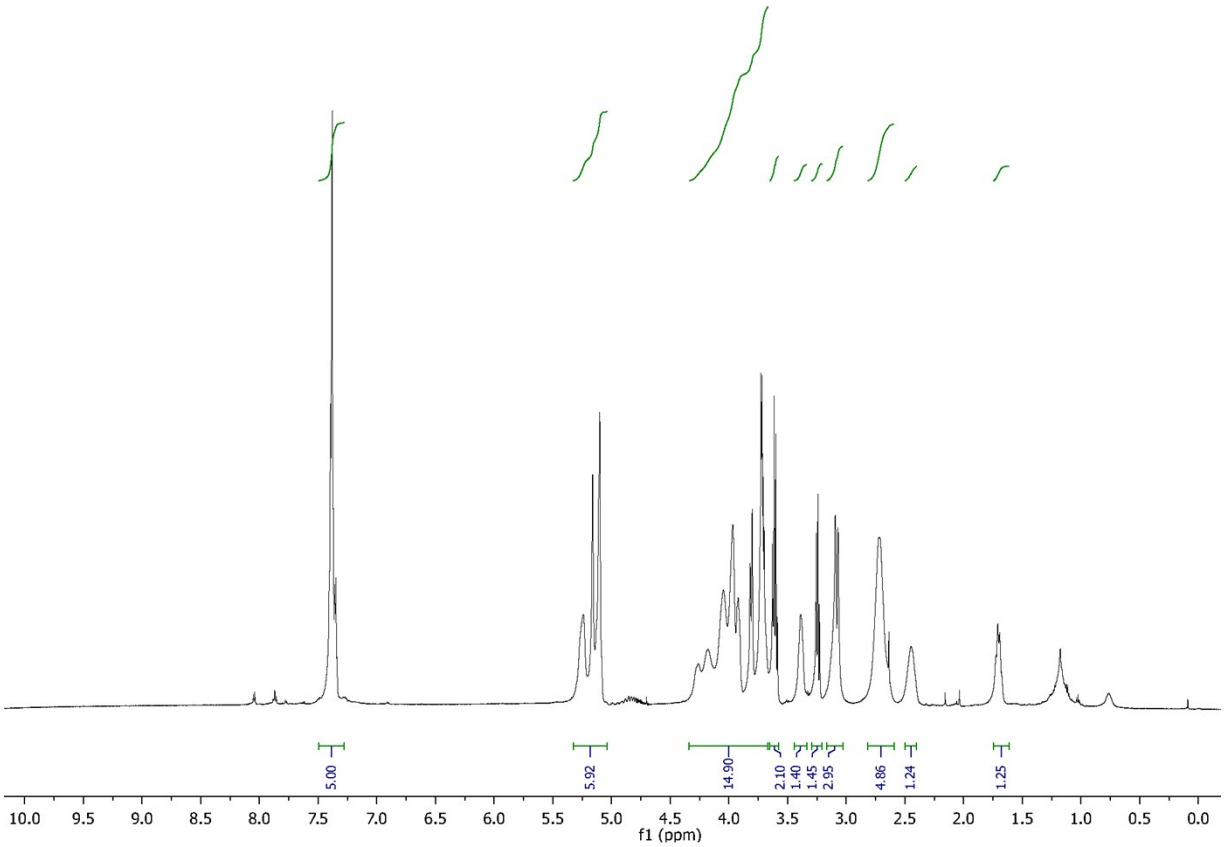


^{13}C NMR

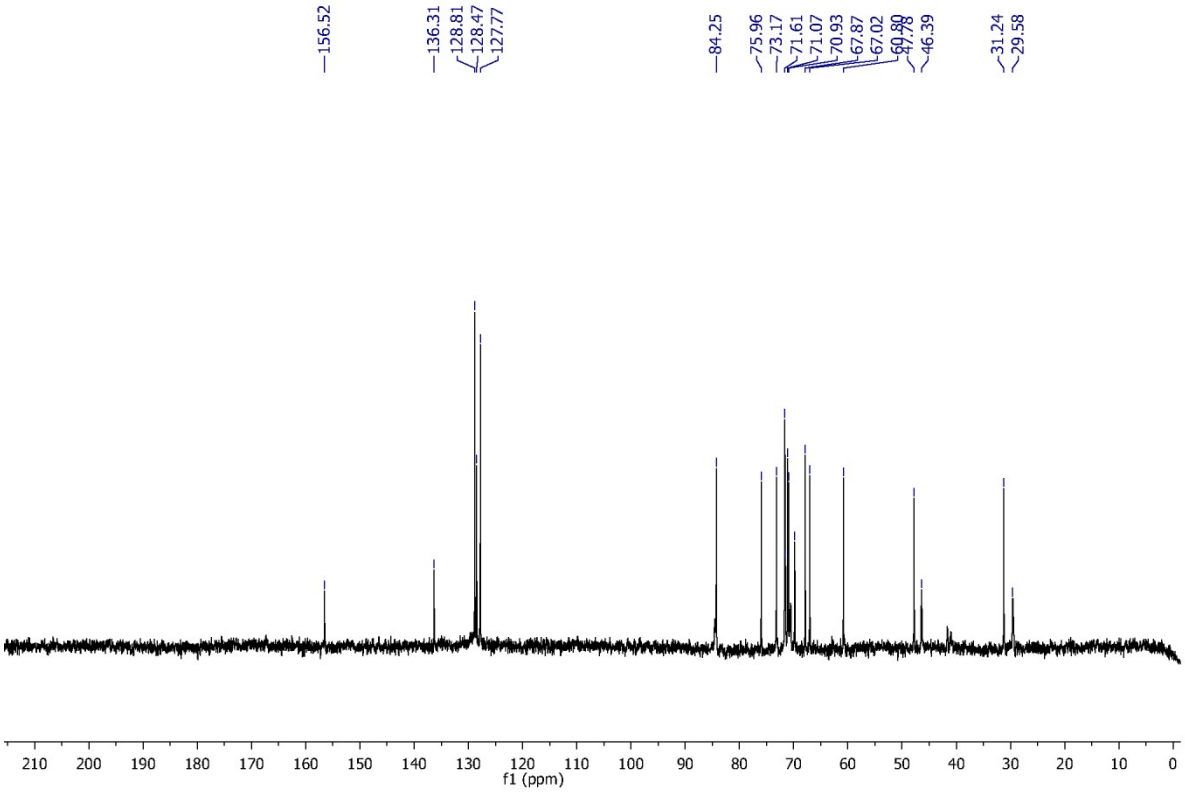


α-D-Mannopyranosyl)-(1→6)-(6-deoxy-1,6-dithio-α-D-mannopyranosyl)-(1→6)-6-deoxy-6-thio-N-benzyloxycarbonyl-isofagomine (11)

^{1}H NMR

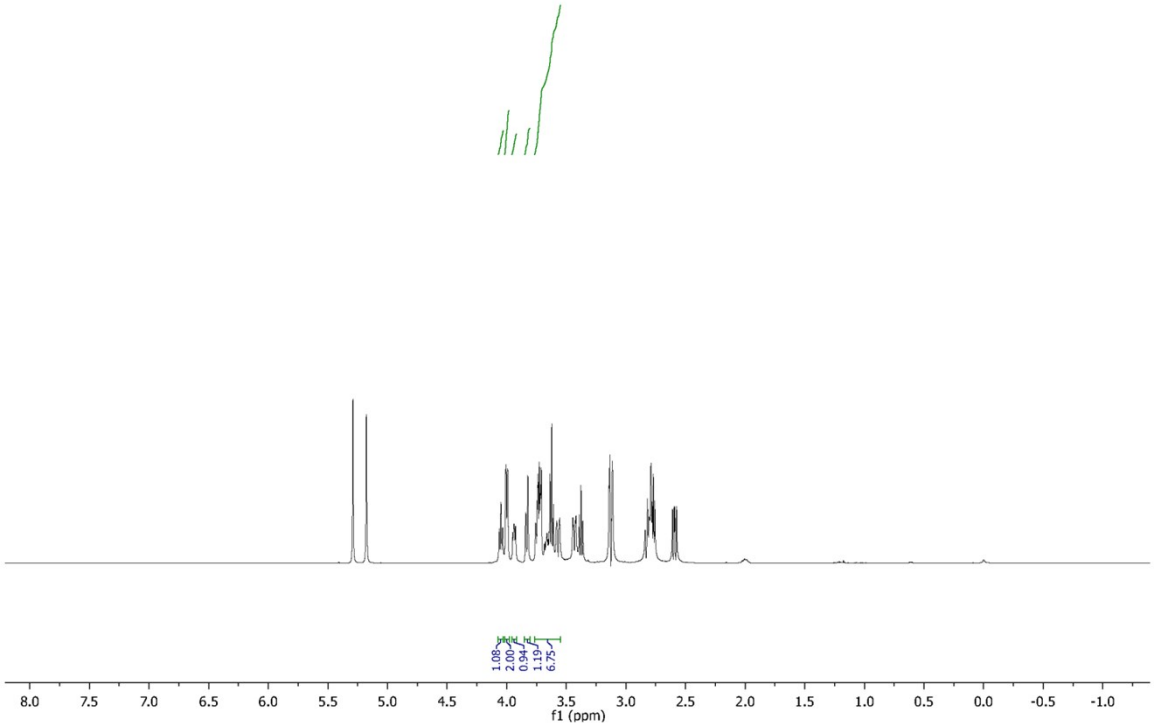


^{13}C NMR



α-D-Mannopyranosyl-(1→6)-(6-deoxy-1,6-dithio-α-D-mannopyranosyl)-(1→6)-6-deoxy-6-thio-isofagomine (3)

^{1}H NMR



^{13}C NMR