Supplementary Information

Direct radiolabelling of proteins at cysteine using [^{18}F]-fluorosugars

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1. General Considerations

Melting points (m.p.) were recorded on a Leica Galen III hot stage microscope equipped with a Testo 720 thermocouple probe and are uncorrected. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker AVII500 (500 MHz) spectrometer, as indicated. Carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a Bruker AVII500 (125 MHz) spectrometer, as indicated. Fluorine nuclear magnetic resonance (^{19}F NMR) spectra were recorded on a Bruker AVII500 (470.4 MHz) or on a Bruker DQX400 (376.5 MHz) spectrometer, as indicated. NMR Spectra were fully assigned using COSY, HSQC, HMBC, and NOESY. All chemical shifts are quoted on the δ scale in ppm using residual solvent as the internal standard (^{1}H NMR: $CDCl_3$ = 7.26, CD_3OD = 4.87; DMSO-d_6 = 2.50 and ^{13}C NMR: $CDCl_3$ = 77.0; CD_3OD = 49.0; DMSO-d_6 = 39.5) and $CFCl_3$ as external standard for ^{19}F NMR. Coupling constants (J) are reported in Hz with the following splitting abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, and app = apparent.

Infrared (IR) spectra were recorded on a Bruker Tensor 27 Fourier Transform spectrophotometer using thin films on NaCl plates for liquids and oils and KBr discs for solids and crystals. Absorption maxima (υ_{max}) are reported in wavenumbers (cm^{-1}).

Low resolution mass spectra (LRMS) were recorded on a Waters Micromass LCT Premier TOF spectrometer using electrospray ionization (ESI) and high resolution mass spectra (HRMS) were recorded on a Bruker MicroTOF ESI mass spectrometer. Nominal and exact *m/z* values are reported in Daltons. Other methods of ionization (EI, FI, and FAB) are used where indicated and were recorded by the University of Oxford Mass Spectrometry Service in the Department of Chemistry.

Optical rotations were measured on a Perkin–Elmer 241 polarimeter with a path length of 1.0 dm and are reported with implied units of 10^{-1} deg cm^2 g^{-1}. Concentrations (c) are given in g/100 ml.

Thin layer chromatography (TLC) was carried out using Merck aluminium backed sheets coated with 60F254 silica gel. Visualization of the silica plates was achieved using a UV lamp (λ_{max} = 254 nm), and/or ammonium molybdate (5% in 2 M H_2SO_4), and/or potassium permanganate (5% $KMnO_4$ in 1 M NaOH with 5% potassium carbonate). Flash column chromatography was carried out using BDH 40–63 μm silica gel (VWR). Mobile phases are reported in relative composition (*e.g.* 1:2:4 H_2O/*i*PrOH/EtOAc v/v/v).

SDS-PAGE electrophoresis was carried out using Invitrogen system (XCell-Cell, NuPAGE Novex Bis-Tris gel, NuPAGE MES running buffer). Protein molecular weights were approximated by comparison to a protein marker (Perfect Protein Markers 15–150 kDa from Novagen). Gels were visualised by coomassie staining (Instant Blue from Expedeon).

Protein purification was performed on an AKTA Prime FPLC system (GE Healthcare). Protein concentrations were calculated using standard BCA assay or with a Labtek ND-1000 Nanodrop.

Anhydrous solvents were purchased from Fluka or Acros. Triethylamine was stored over NaOH pellets. All other solvents were used as supplied (Analytical or HPLC grade), without prior purification. Distilled water was used for chemical reactions and Milli–QR purified water for protein manipulations. Reagents were purchased from Aldrich and used as supplied, unless otherwise indicated. 'Petrol' refers to the fraction of light petroleum ether boiling in the range 40–

60 ºC. All reactions using anhydrous conditions were performed using flame-dried apparatus under an atmosphere of argon or nitrogen. Brine refers to a saturated solution of sodium chloride. Anhydrous magnesium sulfate ($MgSO_4$) was used as drying agents after reaction workup, as indicated.

DOWEX 50WX8 (H^+ form) was conditioned as follows: 100 g of the commercial resin was placed in a 500 mL sintered filter funnel and allowed to swell with 200 mL of acetone for 5 minutes. The solvent was removed by suction and the resin was washed successively with 800 mL of acetone, 500 mL methanol, 500 mL 5 M HCl, and then 1 L of water or until the pH of filtrate was ~ 7, as indicated by pH paper. The resin was partially dried on the filter and then stored and used as needed.

In addition to those specified above, the following abbreviations, designations, and formulas are used throughout the Supporting Information:

MeOH = methanol	H_2O = water	Et_2O = diethyl ether
EtOAc = ethyl acetate	CH_2Cl_2 = dichloromethane	DMF = dimethylformamide
*i*PrOH = isopropanol	PBu_3 = tributylphosphine	Et_3N = triethylamine
Boc = *tert*-butoxycarbonyl	K_2CO_3 = potassium carbonate	Cs_2CO_3 = caesium carbonate
$NaHCO_3$ = sodium bicarbonate	NaOH = sodium hydroxide	NH_4Cl = ammonium chloride
NH_4OH = ammonium hydroxide	aq. = aqueous	sat. = saturated
TFA = trifluoroacetic acid	TIS = triisopropyl silane	EDT = 1,2-ethanedithiol

Protein Mass Spectrometry

Liquid chromatography-mass spectrometry (LC–MS) was performed on a Micromass LCT (ESITOF–MS) coupled to a Waters Alliance 2790 RP-HPLC using a Phenomenex Jupiter C4 column (250 × 4.6 mm × 5μm). Water (solvent A) and acetonitrile (solvent B), each containing 0.1% formic acid, were used as the mobile phase at a flow rate of 1.0 mL min^{-1}. The gradient was programmed as follows: 95% A (5 min isocratic) to 100% B after 15 min then isocratic for 5 min. The electrospray source was operated with a capillary voltage of 3.2 kV and a cone voltage of 25 V. Nitrogen was used as the nebulizer and desolvation gas at a total flow of 600 L hr^{-1}. Spectra were calibrated using a calibration curve constructed from a minimum of 17 matched peaks from the multiply charged ion series of equine myoglobin obtained at a cone voltage of 25 V. Total

mass spectra were reconstructed from the ion series using the MaxEnt algorithm preinstalled on MassLynx software (v. 4.0 from Waters) according to the manufacturer's instructions.

Tryptic samples were analyzed with a Thermo LTQ Orbitrap mass spectrometer coupled to an ABI 4800 MALDI TOF TOF mass spectrometer in the Oxford University Central Proteomics Service (Dunn School of Pathology). Predicted peptide fragments containing the desired modification were determined by MassLynx software (v. 4.0 from Waters) according to the manufacturer's instructions.

Materials

Precursors 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-1-thio-β-D-gluco, galacto, and mannopyranose were prepared following a minor modification of the methodology described by Kawai and co-workers.[1]

Radiochemistry

[^{18}F]Fluoride and [^{18}F]FDG were produced by PETNet Solutions at Mount Vernon Hospital (UK). [^{18}F]Fluoride was produced in a tantalum target *via* the ^{18}O(p,n) ^{18}F reaction and used as [^{18}F]fluoride solution in [^{18}O]water (300–350 GBq) in a GE Healthcare TRACERlab for [^{18}F]FDG synthesis. Typical solutions of [^{18}F]FDG obtained were 130–160 GBq in 8 mL [^{18}O]water, diluted down to 3GBq/mL in saline for delivery. Radio-reversed-phase-high-performance liquid chromatography (RP-HPLC) analyses were performed using a Gilson 322 or a Dionex UltiMate 3000 HPLC system equipped with a NaI/photomultiplier tube (PMT)-radiodetector and an ultraviolet (UV) detector. Radio-thin layer chromatography (TLC) was performed on Macherey-Nagel Polygram Silica Plates and analysed using a plastic scintillator/PMT detector. Radiochemical conversions are reported non-decay corrected. Water (solvent A) and acetonitrile (solvent B), each containing 0.1% formic acid, were used as the mobile phase at a flow rate of 1.0 mL min^{-1}. The gradient was programmed as follows: 95% A (5 min isocratic) to 100% B after 15 min then isocratic for 5 min.

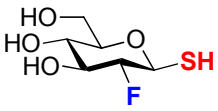
2. Experimental Section

2.1. Preparation of 2-deoxy-2-fluoro-1-thio-glycopyranoses 3–5

General procedure for the synthesis of 2-deoxy-2-fluoro-1-thio-glycopyranoses

The corresponding 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-1-thio-β-D-glycopyranose (1 mmol) was treated with 0.1 M NaOMe in MeOH (7 mL/mmol) at room temperature. The reaction mixture was stirred at the same temperature for 15 minutes and neutralized with Dowex (H^+ 50WX8-200). The ion exchanger was filtered off and washed with MeOH. The resulting solution was concentrated under reduced pressure and the residue used in the next step without further purification.

2-Deoxy-2-fluoro-1-thio-β-D-glucopyranose (3)[2,3]



The title compound was prepared following the general procedure above, starting from 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-1-thio-β-D-glucopyranose (42 mg, 0.115 mmol) and 0.1 M NaOMe in degassed MeOH (805 μL). After concentration under reduced pressure product **3** (22.8 mg, 100%) was obtained as a white hygroscopic solid. R_f (7:2:1 EtOAc/MeOH/H_2O): 0.40; $[\alpha]_D^{20}$: +12.4 (0.037, H_2O); ^{1}H NMR (D_2O, 500 MHz) δ in ppm: 4.75 (1H, overlapped, H-1), 4.10 (dt, $J_{2,F}$ = 49.5 Hz, $J_{1,2}$ = $J_{2,3}$ = 9.1 Hz, 1H, H-2), 3.85 (dd, $J_{6a,b}$ = 12.6 Hz, $J_{5,6a}$ = 1.6 Hz, 1H, H-6a), 3.73 (ddd, $J_{3,F}$ = 14.9 Hz, $J_{2,3}$ = 9.1 Hz, $J_{3,4}$ = 8.8 Hz, 1H, H-3), 3.67 (dd, $J_{6a,b}$ = 12.6 Hz, $J_{5,6b}$ = 5.4 Hz, 1H, H-6b), 3.50–3.43 (m, 2H, H-4,5); ^{13}C NMR (D_2O, 125.8 MHz) δ in ppm: 93.9 (d, $J_{2,F}$ = 184.1 Hz, C-2), 80.2 (C-5), 76.6 (d, $J_{1,F}$ = 24.8 Hz, C-1), 75.1 (d, $J_{3,F}$ = 17.2 Hz, C-3), 69.0 (d, $J_{4,F}$ = 8.6 Hz, C-4), 60.5 (C-6); ^{19}F NMR (D_2O, 376.5 MHz) δ in ppm: –188.9 (dd, $J_{2,F}$ = 49.5 Hz, $J_{3,F}$ = 14.9 Hz, F-2); FT–IR (KBr) ν in cm^{-1}: 3329, 2928, 2884, 1636, 1413, 1062; HRMS (TOF ES+) for $(M+Na)^+$ $C_6H_{10}FNaO_4S^+$ (*m/z*): calc. 221.0254; found 221.0256.

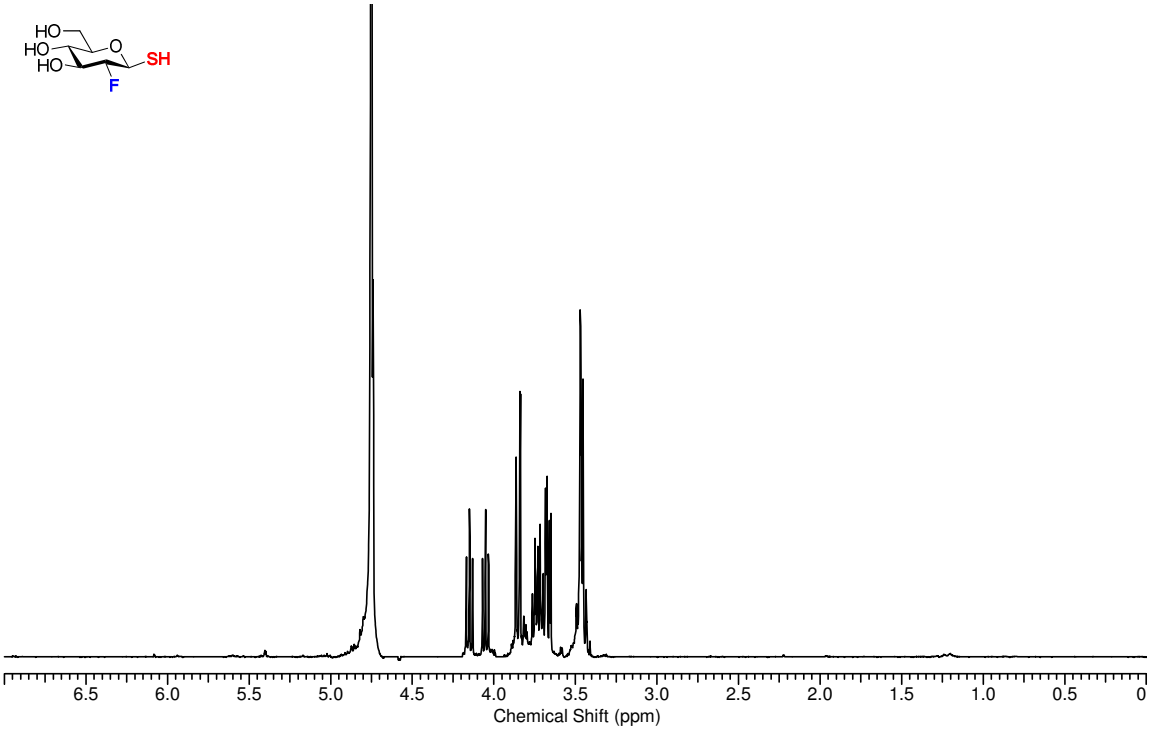


Figure ESI 1. ^{1}H NMR (500 MHz) of 2FGlc **3**.

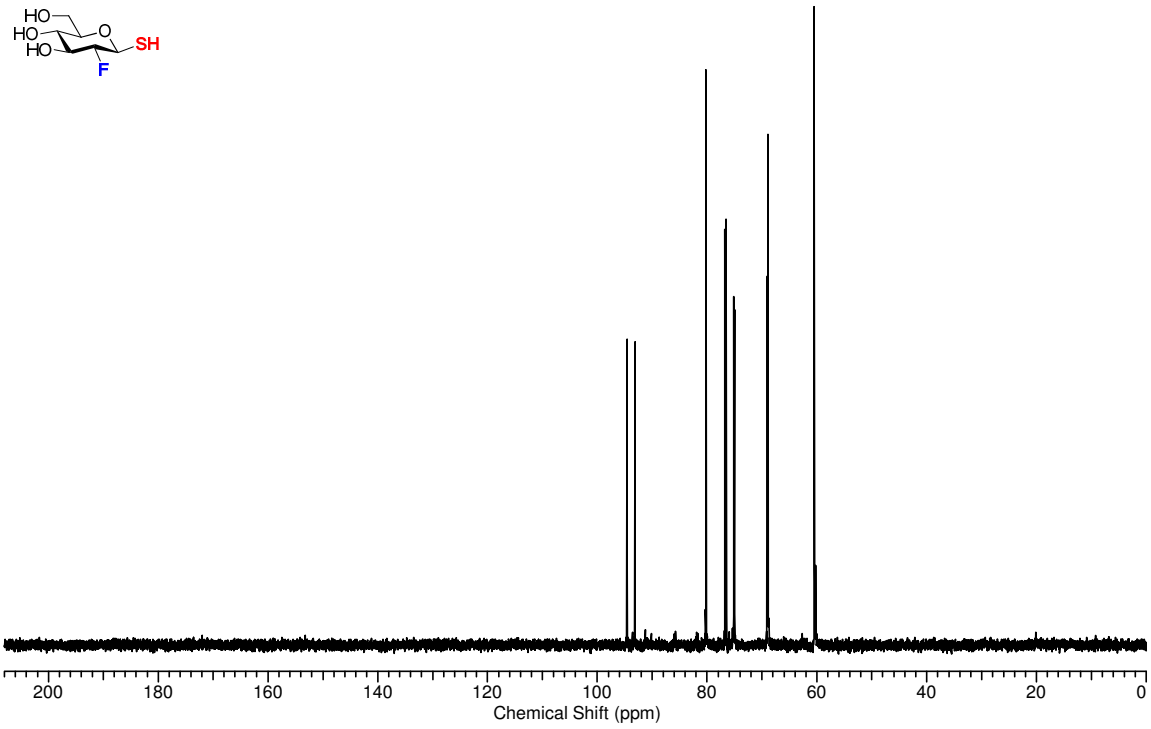


Figure ESI 2. ^{13}C NMR (125.8 MHz) of 2FGlc **3**; $^{1}J_{C1-H1}$ = 158.6 Hz.

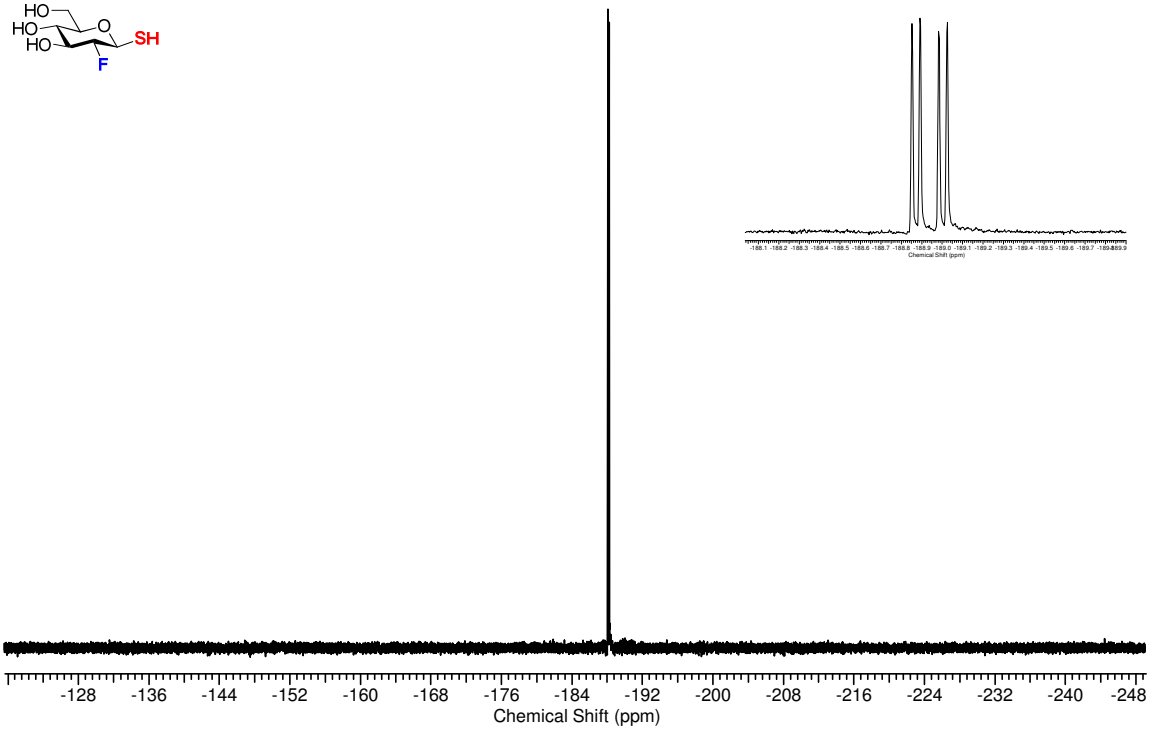
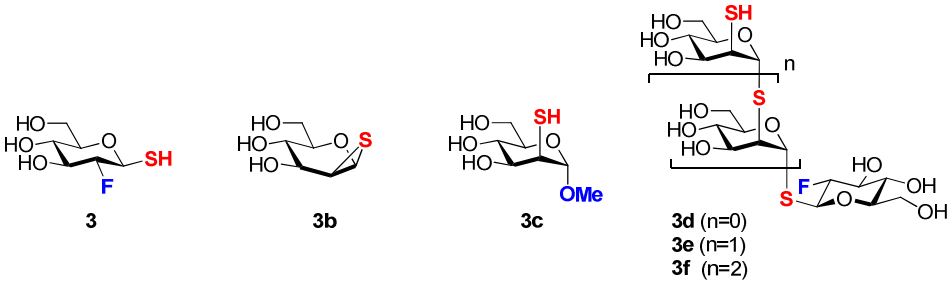
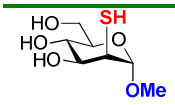
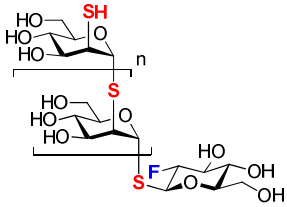


Figure ESI 3. ^{19}F NMR (376.5 MHz) of 2FGlc **3**.

Table ESI 1. MS (TOF ES) analysis of the possible products obtained from 2FGlc **3** upon standing in aqueous solution at room temperature for >12 h.



Compound[a]	[M–H]$^-$		[M+Na]$^+$	
	Calc. mass	Obs. mass	Calc. mass	Obs. mass
3	197.0289	-	221.0254	221.0256
3b	177.0227	177.0227	201.0192	-

 **3c**	209.0489	-	233.0454	-
 **3d** (n=0)	375.0589	375.08[b]	399.0554	399.0553
3e (n=1)	553.0889	-	577.0854	-
3f (n=2)	731.1189	-	755.1154	-

(a) High resolution analysis unless otherwise indicated. (b) Low resolution analysis.

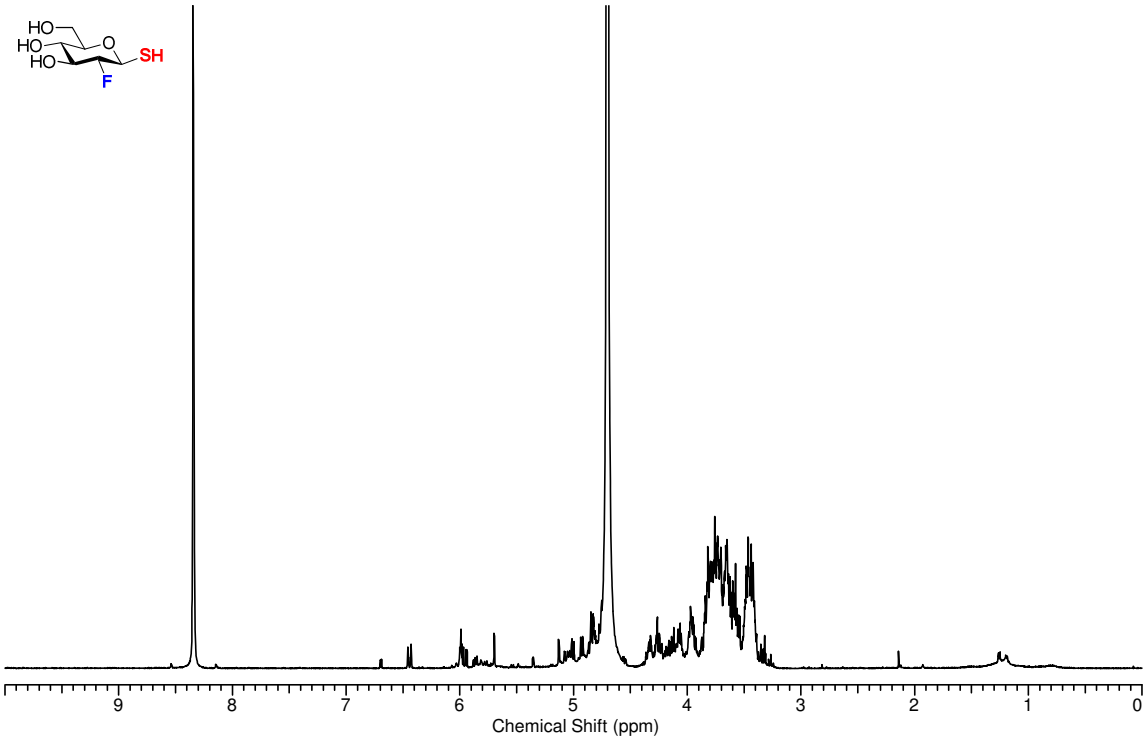


Figure ESI 4. ^{1}H NMR (500 MHz) of 2FGlc **3** upon standing in D_2O at room temperature for >12 h.

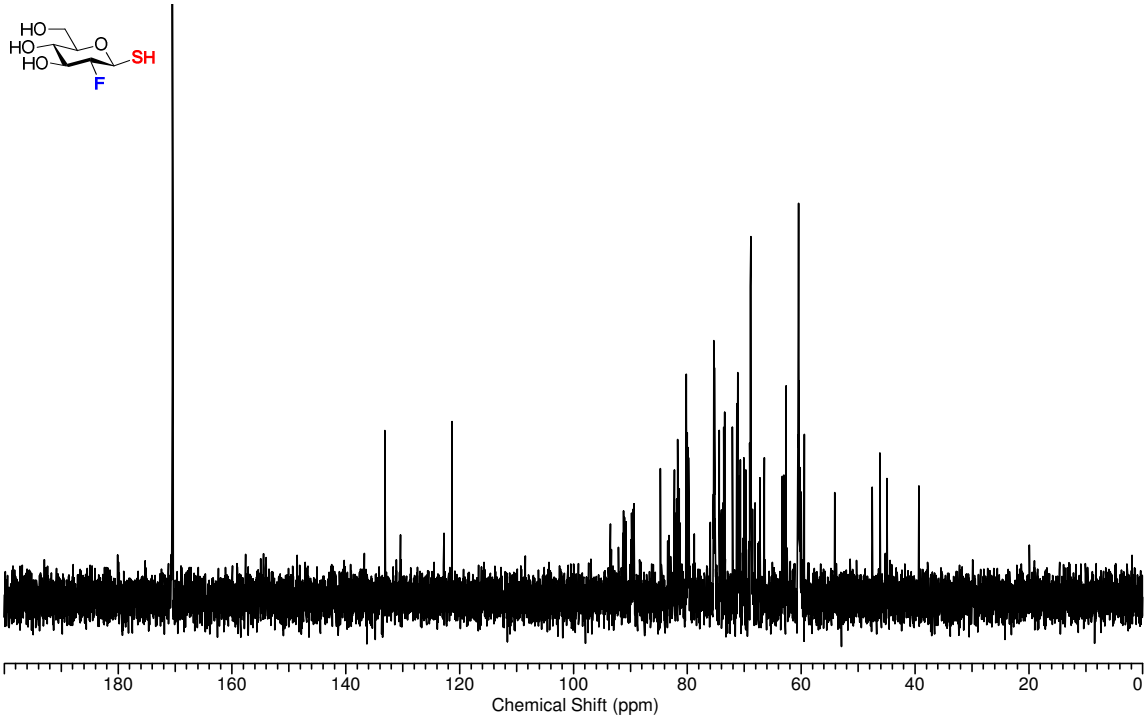


Figure ESI 5. ^{13}C NMR (125.8 MHz) of 2FGlc **3** upon standing in D_2O at room temperature for >12 h.

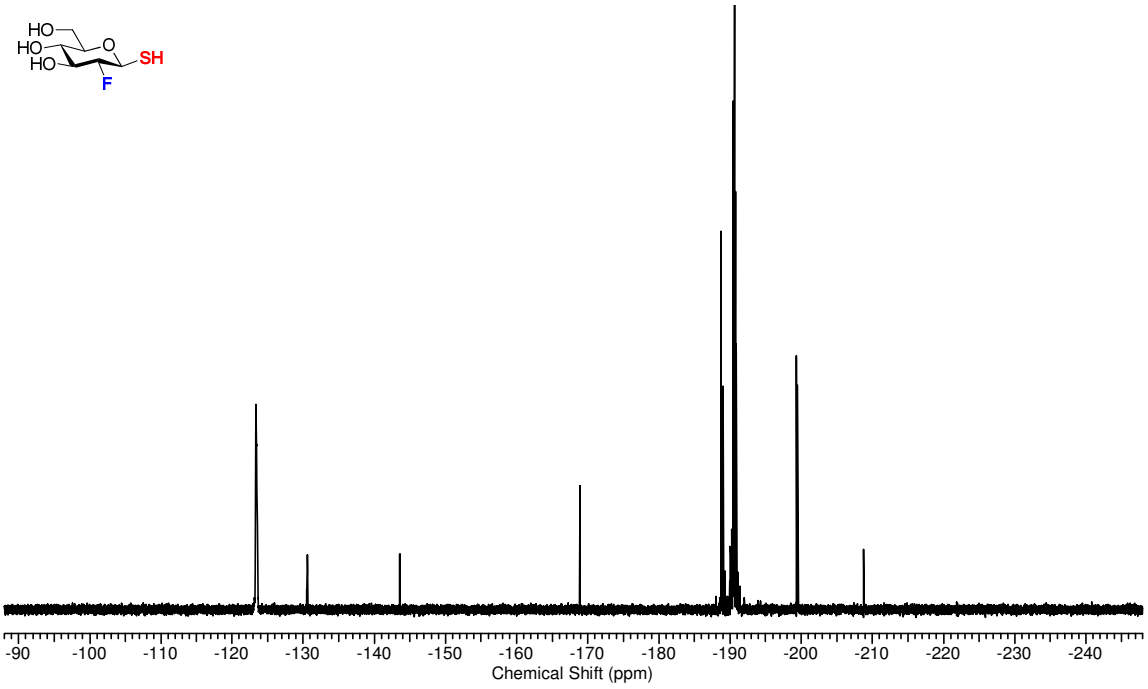
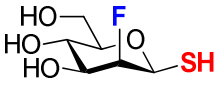


Figure ESI 6. ^{19}F NMR (470.4 MHz) of 2FGlc **3** upon standing in D_2O at room temperature for >12 h.

2-Deoxy-2-fluoro-1-thio-β-D-mannopyranose (4)[3]



The title compound was prepared following the general procedure above, starting from 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-1-thio-β-D-mannopyranose (32 mg, 0.087 mmol) and 0.1 M NaOMe in degassed MeOH (610 μL). After concentration under reduced pressure product **4** (17.2 mg, 100%) was obtained as a white hygroscopic solid. R_f (7:2:1 EtOAc/MeOH/H_2O): 0.36; $[\alpha]_D^{20}$: +16.4 (0.043, H_2O); ^{1}H NMR (D_2O, 500 MHz) δ in ppm: 4.99 (d, $J_{1,F}$ = 26.5 Hz, 1H, H-1), 4.83 (dd, $J_{2,F}$ = 49.2 Hz, $J_{2,3}$ = 2.5 Hz, 1H, H-2), 3.87 (dd, $J_{6a,b}$ = 12.3 Hz, $J_{5,6a}$ = 2.0 Hz, 1H, H-6a), 3.77 (ddd, $J_{3,F}$ = 30.3 Hz, $J_{3,4}$ = 9.8 Hz, $J_{2,3}$ = 2.5 Hz, 1H, H-3), 3.68 (dd, $J_{6a,b}$ = 12.3 Hz, $J_{5,6b}$ = 6.0 Hz, 1H, H-6b), 3.60 (appt, $J_{3,4}$ = $J_{4,5}$ = 9.8 Hz, 1H, H-4), 3.45 (ddd, $J_{3,4}$ = 9.8 Hz, $J_{5,6b}$ = 6.0 Hz, $J_{5,6a}$ = 2.0 Hz, 1H, H-5); ^{13}C NMR (D_2O, 125.8 MHz) δ in ppm: 93.1 (d, $J_{2,F}$ = 180.2 Hz, C-2), 80.5 (C-5), 75.8 (d, $J_{1,F}$ = 19.1 Hz, C-1), 72.6 (d, $J_{3,F}$ = 19.1 Hz, C-3), 66.3 (C-4), 60.7 (C-6); ^{19}F NMR (D_2O, 376.5 MHz) δ in ppm: –214.5 (ddd, $J_{2,F}$ = 49.2 Hz, $J_{3,F}$ = 30.3 Hz, $J_{1,F}$ = 26.5 Hz, F-2); FT–IR (KBr) ν in cm^{-1}: 3326, 2930, 1636, 1411, 1358, 1061; HRMS (TOF ES+) for $(M+Na)^+$ $C_6H_{10}FNaO_4S^+$ (*m/z*): calc. 221.0254; found 221.0264.

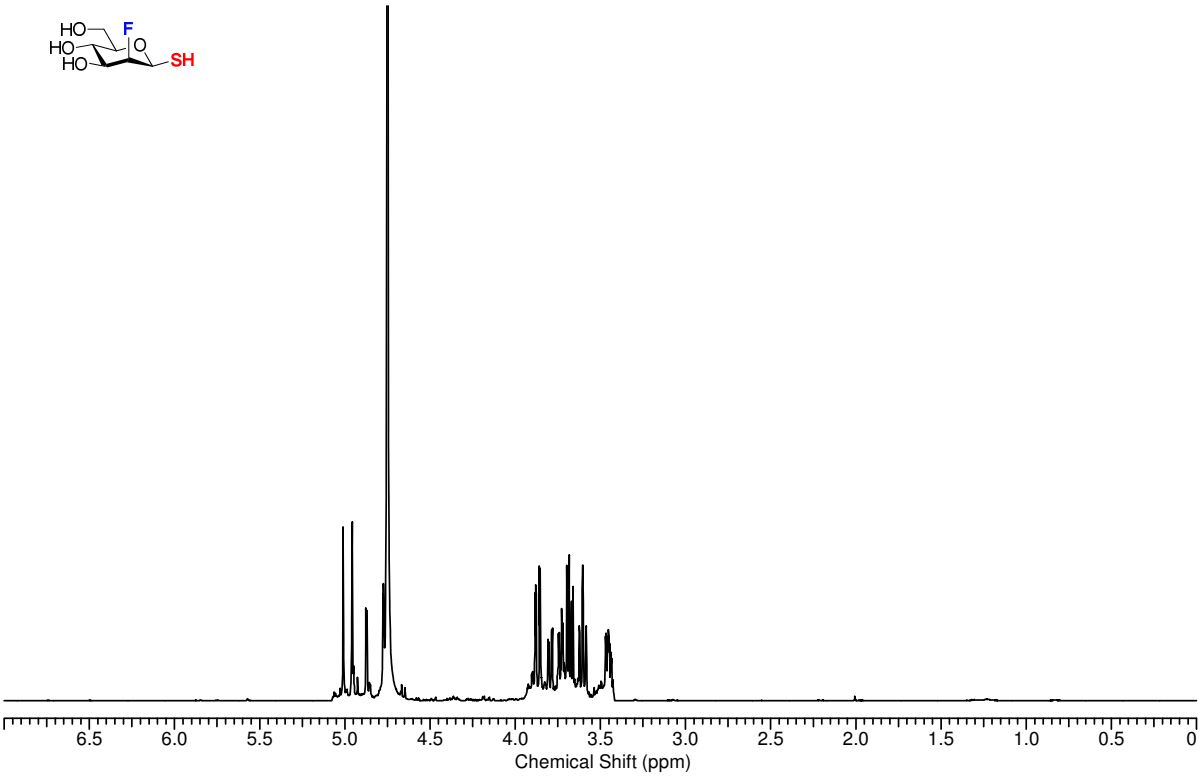


Figure ESI 7. ^{1}H NMR (500 MHz) of 2FMan **4**.

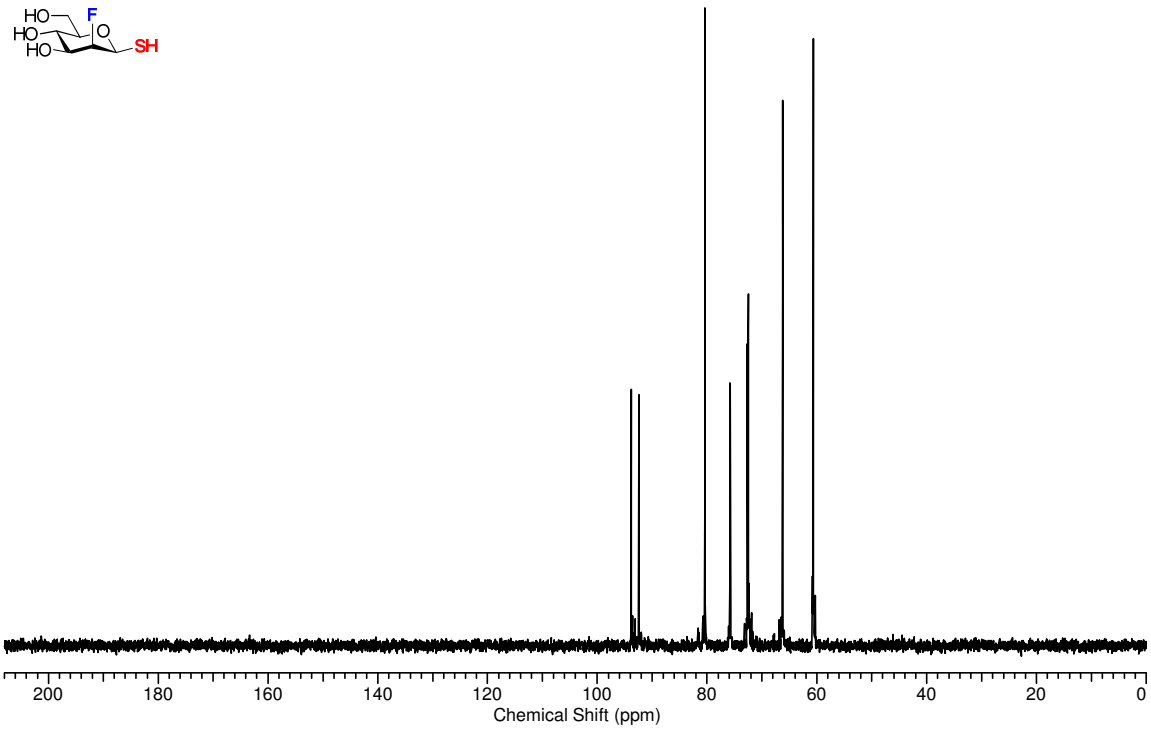


Figure ESI 8. ^{13}C NMR (125.8 MHz) of 2FMan **4**; $^{1}J_{C1-H1}$ = 155.2 Hz.

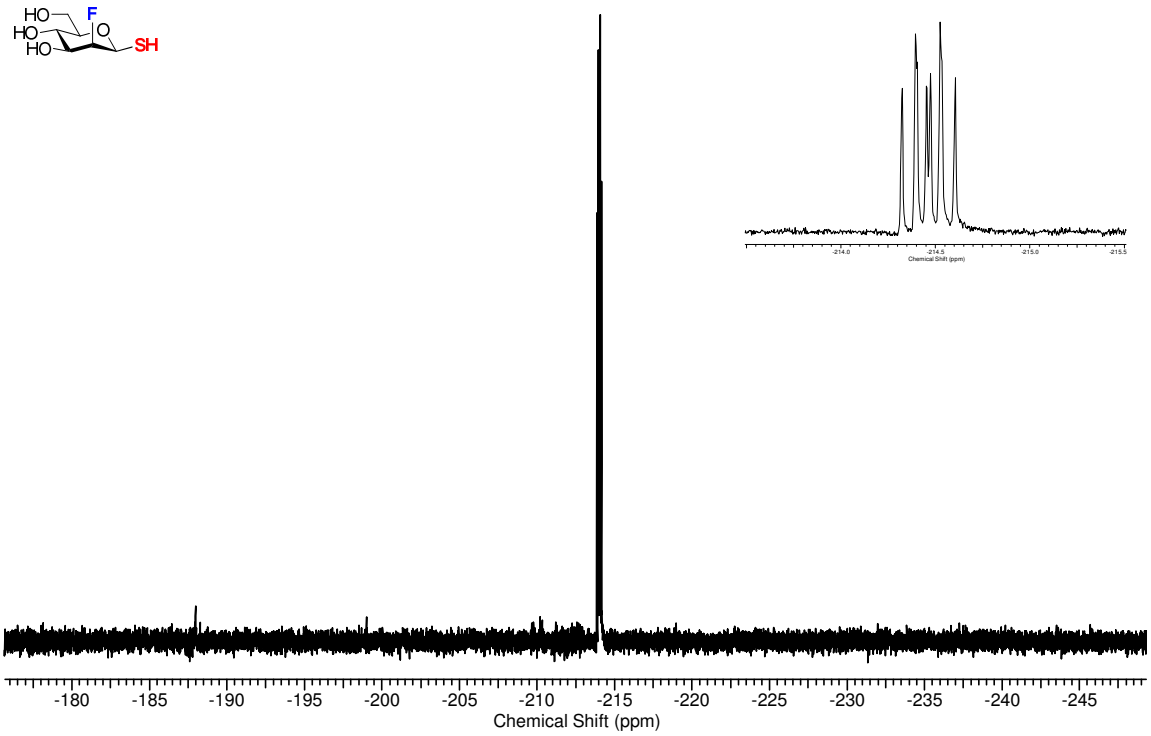
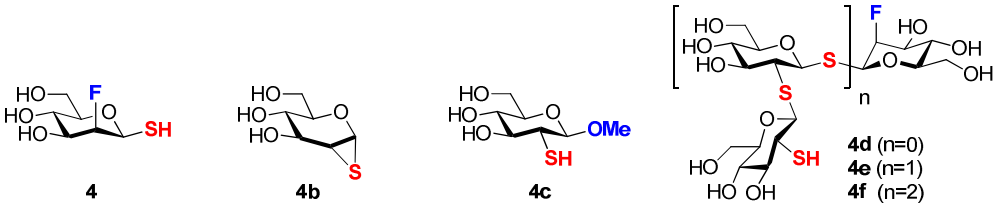
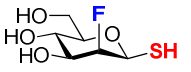
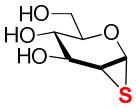
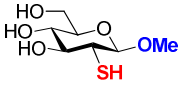
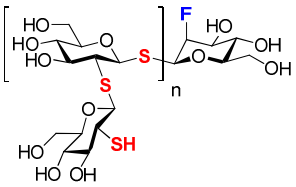


Figure ESI 9. ^{19}F NMR (376.5 MHz) of 2FMan **4**.

Table ESI 2. MS (TOF ES) analysis of the possible products obtained from 2FMan **4** upon standing in aqueous solution at room temperature for >12 h.



Compound[a]	[M–H]$^-$		[M+Na]$^+$	
	Calc. mass	Obs. mass	Calc. mass	Obs. mass
 **4**	197.0289	197.04[b]	221.0254	221.0264
 **4b**	177.0227	177.0228	201.0192	-
 **4c**	209.0489	-	233.0454	-
 **4d** (n=0)	375.0589	-	399.0554	-
4e (n=1)	553.0889	-	577.0854	-
4f (n=2)	731.1189	-	755.1154	-

(a) High resolution analysis unless otherwise indicated. (b) Low resolution analysis.

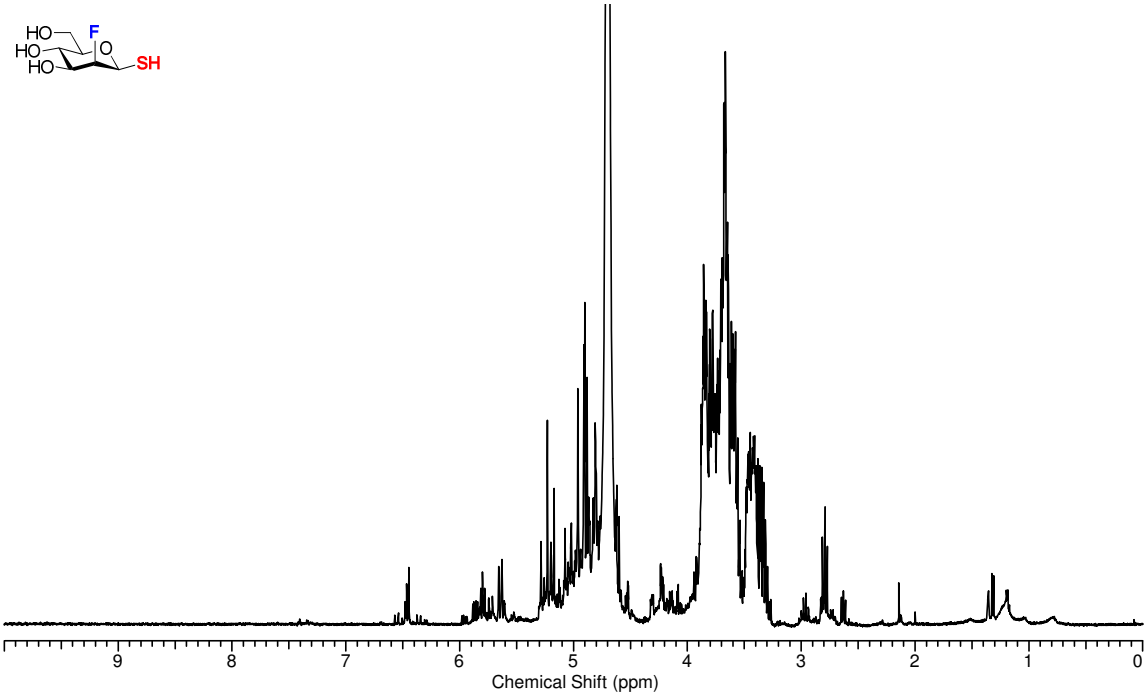


Figure ESI 10. ^{1}H NMR (500 MHz) of 2FMan **4** upon standing in D_2O at room temperature for >12 h.

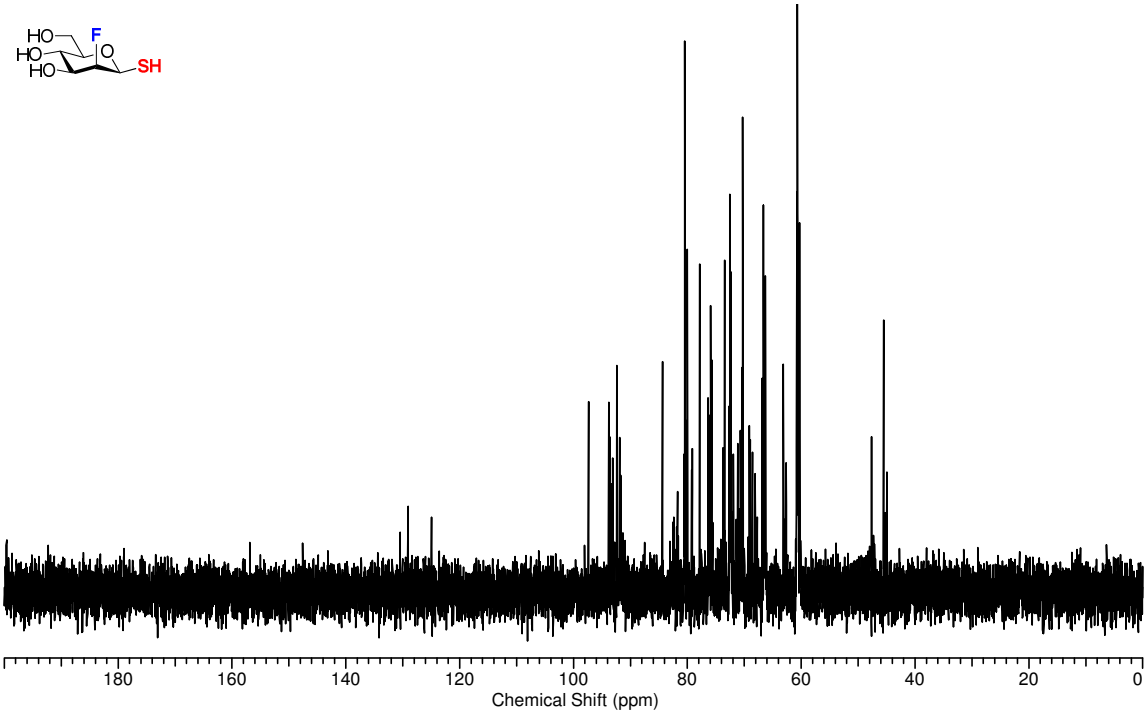


Figure ESI 11. ^{13}C NMR (125.8 MHz) of 2FMan **4** upon standing in D_2O at room temperature for >12 h.

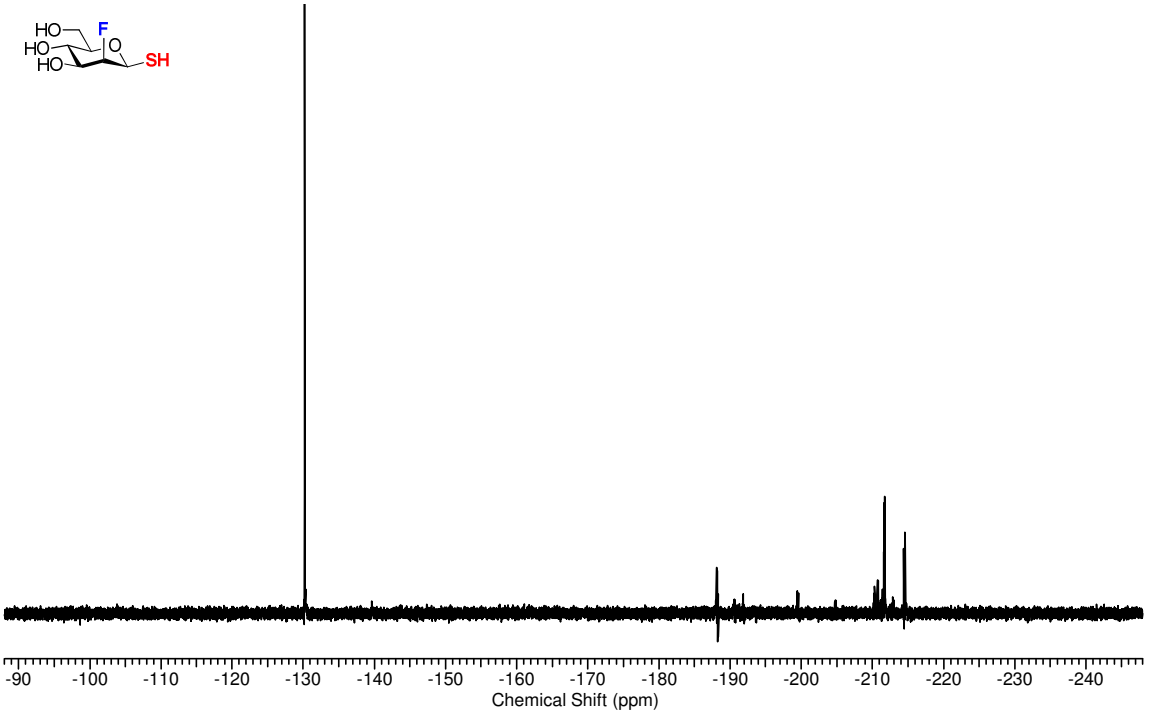
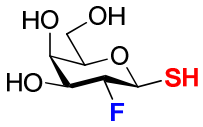


Figure ESI 12. ^{19}F NMR (470.4 MHz) of 2FMan **4** upon standing in D_2O at room temperature for >12 h.

2-Deoxy-2-fluoro-1-thio-β-D-galactopyranose (5)



The title compound was prepared following the general procedure above, starting from 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-1-thio-β-D-galactopyranose (27.7 mg, 0.076 mmol) and 0.1 M NaOMe in degassed MeOH (530 μL). After concentration under reduced pressure product **5** (15.1 mg, 100%) was obtained as a white solid. R_f (7:2:1 EtOAc/MeOH/H_2O): 0.25; mp: 155–159 ºC; $[\alpha]_D^{20}$: –44.1 (0.017, H_2O); ^{1}H NMR (D_2O, 500 MHz) δ in ppm: 4.70 (dd, $J_{1,2}$ = 9.5 Hz, $J_{1,F}$ = 2.5 Hz, 1H, H-1), 4.33 (dt, $J_{2,F}$ = 49.7 Hz, $J_{2,3}$ = $J_{1,2}$ = 9.5 Hz, 1H, H-2), 4.01 (m, 1H, H-4), 3.93–3.86 (m, 1H, H-3), 3.75–3.62 (m, 3H, H-5,6a,b); ^{13}C NMR (D_2O, 125.8 MHz) δ in ppm: 93.5 (d, $J_{2,F}$ = 181.2 Hz, C-2), 79.5 (C-5), 77.0 (d, $J_{1,F}$ = 24.8 Hz, C-1), 72.0 (d, $J_{3,F}$ = 18.1 Hz, C-3), 69.4 (d, $J_{4,F}$ = 9.5 Hz, C-4), 60.8 (C-6); ^{19}F NMR (D_2O, 376.5 MHz) δ in ppm: –197.2 (ddd, $J_{2,F}$ = 50.5 Hz, $J_{3,F}$ = 14.9 Hz, $J_{1,F}$ = 2.5 Hz, F-2); FT–IR (KBr) ν in cm^{-1}: 3357, 3342, 3300, 2936, 2883, 1636, 1413, 1056; HRMS (TOF ES–) for $(M–H)^-$ $C_6H_9FO_4S^-$ (*m/z*): calc. 197.0289; found 197.0286.

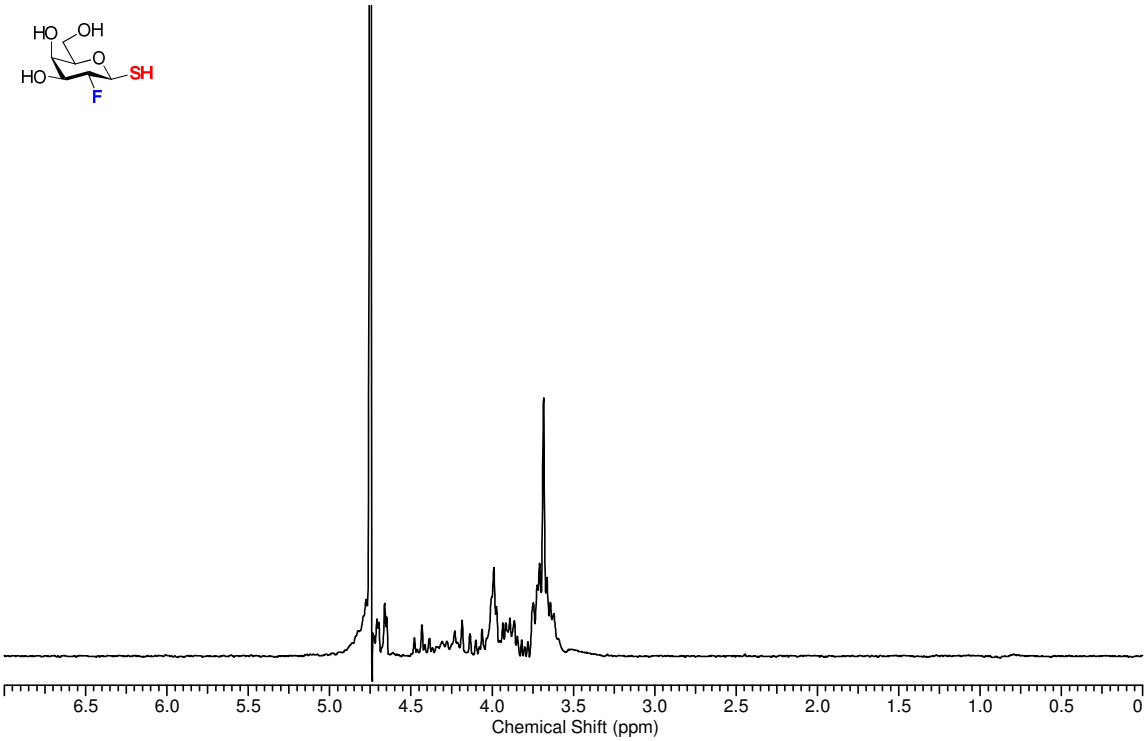


Figure ESI 13. ^{1}H NMR (500 MHz) of 2FGal **5**.

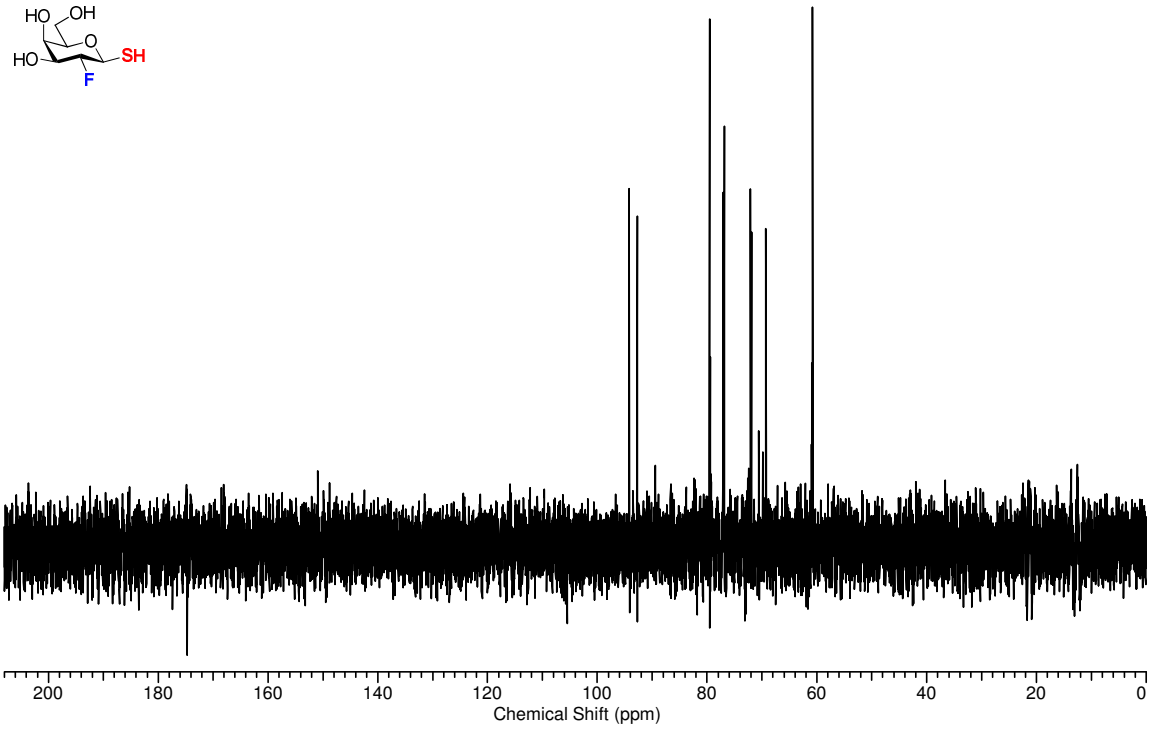


Figure ESI 14. ^{13}C NMR (125.8 MHz) of 2FGal **5**; $^{1}J_{C1-H1}$ = 157.5 Hz.

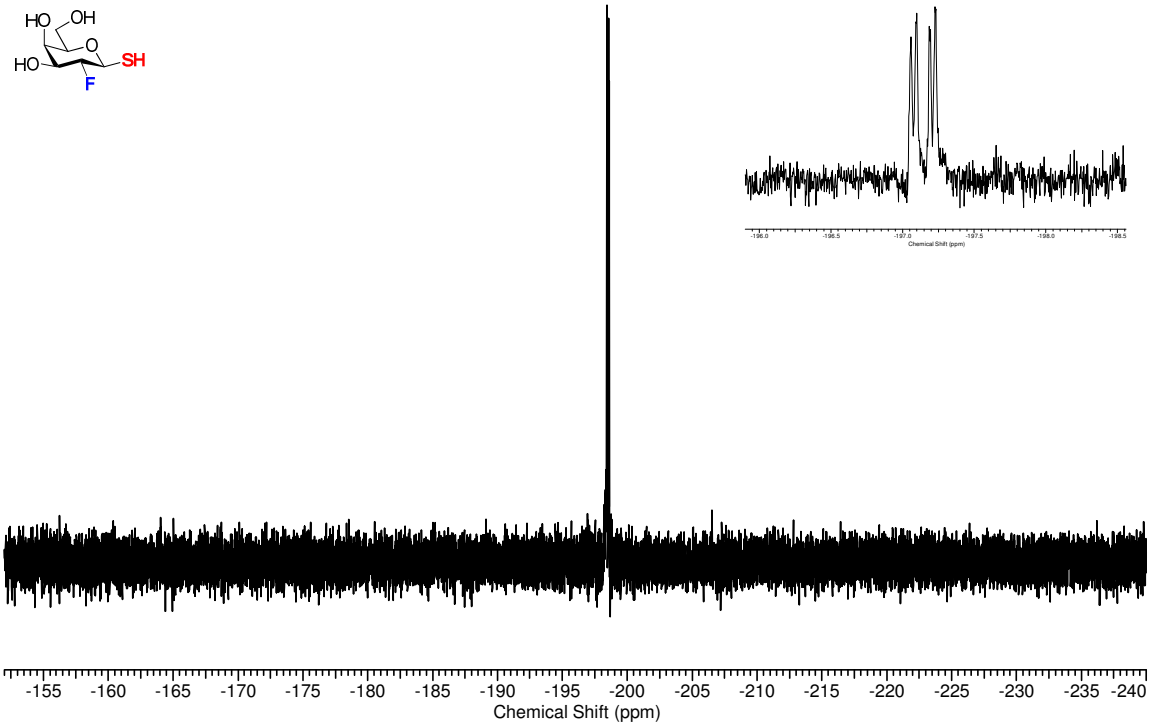
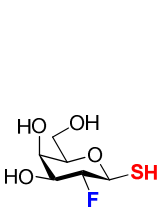
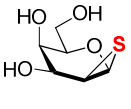


Figure ESI 15. ^{19}F NMR (376.5 MHz) of 2FGal **5**.

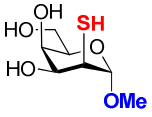
Table ESI 3. MS (TOF ES) analysis of the possible products obtained from 2FGal **5** upon standing in aqueous solution at room temperature for >3 h.



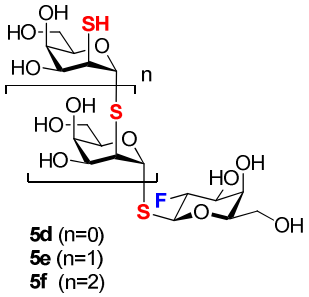
5



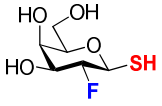
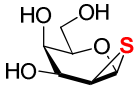
5b

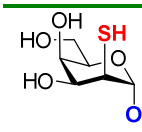
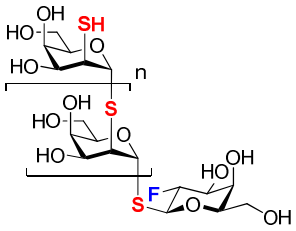


5c



5d (n=0)
5e (n=1)
5f (n=2)

Compound[a]	[M–H]$^-$		[M+Na]$^+$	
	Calc. mass	Obs. mass	Calc. mass	Obs. mass
 **5**	197.0289	197.0286	221.0254	-
 **5b**	177.0227	177.04[b]	201.0192	-

 **5c**	209.0489	-	233.0454	233.0457
 **5d** (n=0)	375.0589	375.07[b]	399.0554	399.0557
5e (n=1)	553.0889	553.10	577.0854	577.0850
5f (n=2)	731.1189	731.13[b]	755.1154	755.10[b]

(a) High resolution analysis unless otherwise indicated. (b) Low resolution analysis.

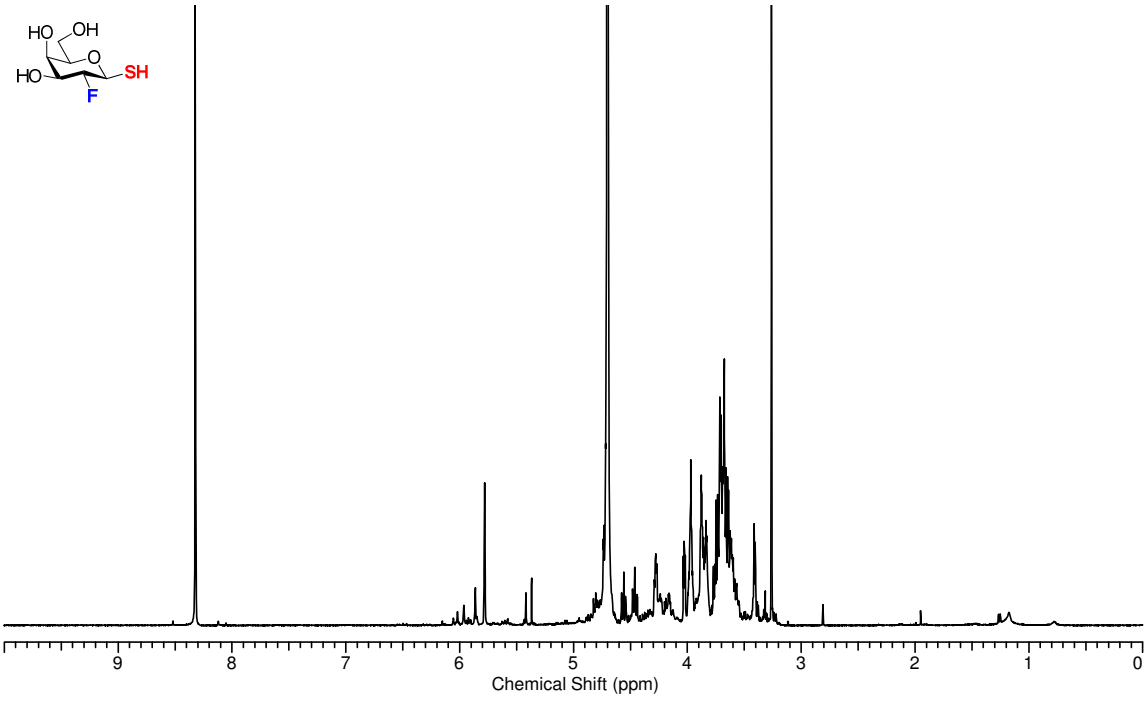


Figure ESI 16. ^{1}H NMR (500 MHz) of 2FGal **5** upon standing in D_2O at room temperature for >3 h.

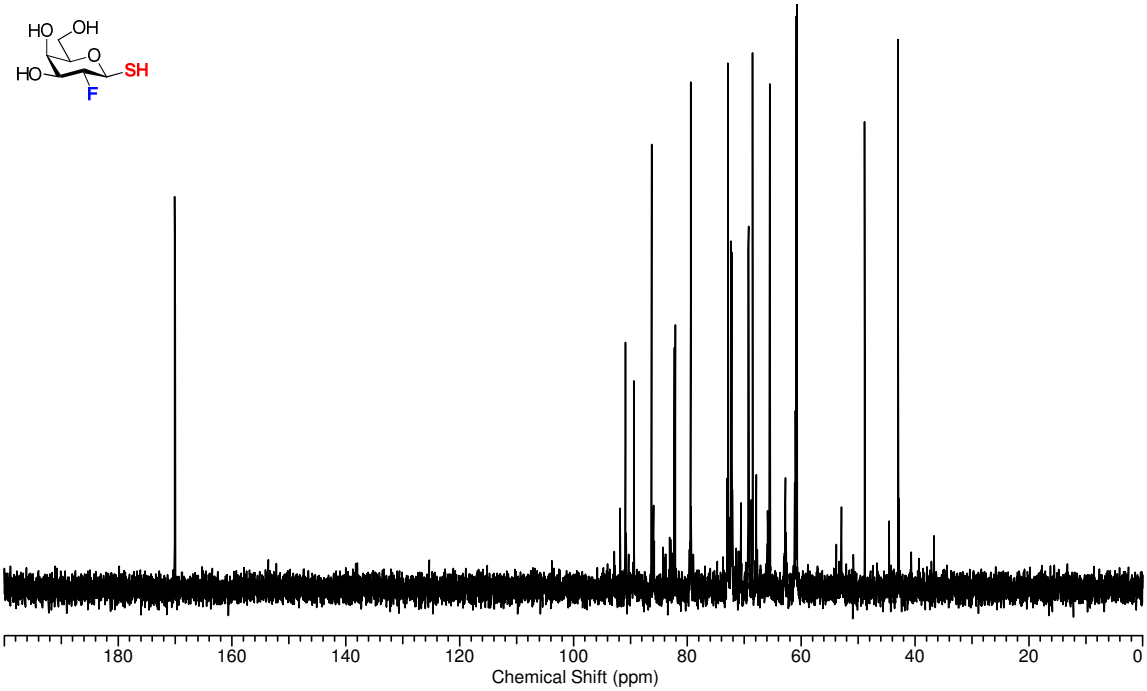


Figure ESI 17. ^{13}C NMR (125.8 MHz) of 2FGal **5** upon standing in D_2O at room temperature for >3 h.

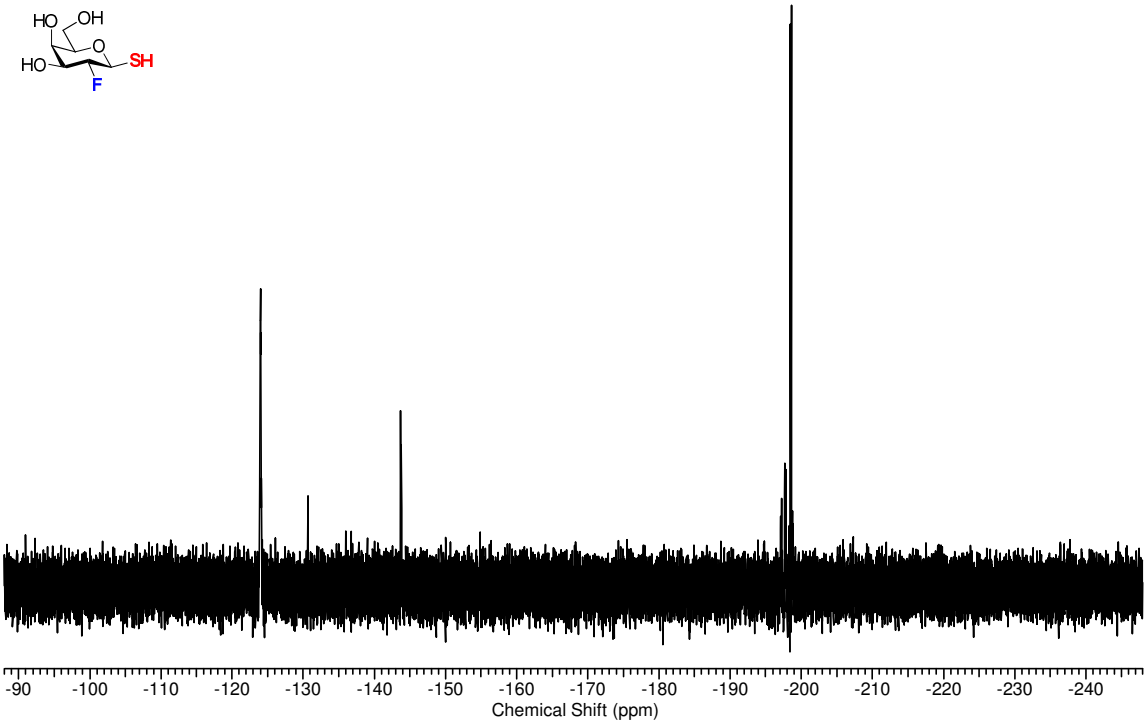
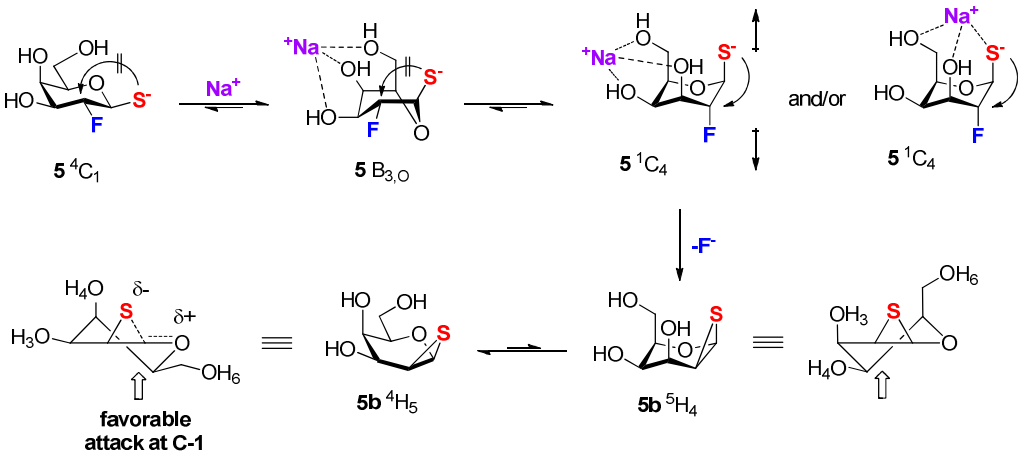


Figure ESI 18. ^{19}F NMR (376.5 MHz) of 2FGal **5** upon standing in D_2O at room temperature for >3 h.

Supplementary Information

In order to gain insight into the likely mechanism from which oligosaccharide units are formed under certain conditions, we studied in detail the formation of starting 2-deoxy-2-fluoro-1-thio-β-D-glycopyranoses **3**–**5**. Analysis of starting 2-deoxy-2-fluoro-1-thio-β-D-glycopyranoses **3**–**5** by TLC and LRMS showed reasonable pure material although traces of impurities were detected. However, the complexity of the mixture increased upon standing in aqueous and/or methanolic solution at room temperature for several hours as indicated by TLC, HRMS, ^{1}H, ^{13}C, and ^{19}F NMR (Tables ESI 1–3 and Figures ESI 1–18). Since deprotection of peracetylated 2-deoxy-2-fluoro-1-thio-glycopyranoses proceeds smoothly when mild reaction conditions and short reaction times are employed, it is crucial to control this step to obtain pure starting materials and avoid contamination with undesired by-products. We rationalized the formation of these by-products from 2FGalSH **5** as illustrated in Scheme ESI 1.



Scheme ESI 1. Proposed mechanism for the formation of episulfide **5b** from 2FGal **5** which may lead to subsequent oligomerization reactions (accordingly applicable to Glc and Man configurations although to a lesser extent).

The formation and structural assignment of oligosaccharides is tentatively explained by oligomerization[4] of sugar 1,2-episulfide **5b** (detected by HRMS) which is in turn obtained after a 1→2 intramolecular thiol migration.[5] The $^{1}C_4$ conformation in **5**, which is stabilized by favourable dipole interactions[6] and complexation[7] with Na^+ ions, leads to corresponding episulfide **5b** $^{5}H_4$ after elimination of fluorine at C-2. Regioselective ring opening of episulfides/epoxides with soft nucleophiles[8] (*e.g.* FGalSH) occurs at C-1 through the lower-energy chair-like transition state.[9] Chelation of Na^+ ions may also assist during this process.[10] Accordingly, we assume a 1,2-trans diaxial relative stereochemistry in disaccharide **5d** (added to

SBL C156-Dha **2**) although the presence of anomeric mixtures due to the oxocarbenium character of sugar 1,2-episulfides cannot be ruled out.[11] In addition, negative Ellman's test reinforces our structural hypothesis that suggests disaccharide **5d** is connected to the protein by the thiol at C-2. Mutarotation and further oligomerization can also account for the increasing complexity of the reaction mixture occurring at different pH in aqueous media as recently revealed by Ramström.[12] However, this is only a tentative model which attempts to rationalize experimental data and the origin of this abnormal reactivity is still unclear.

2.2. Preparation of 2-fluoroglycoproteins 6–9

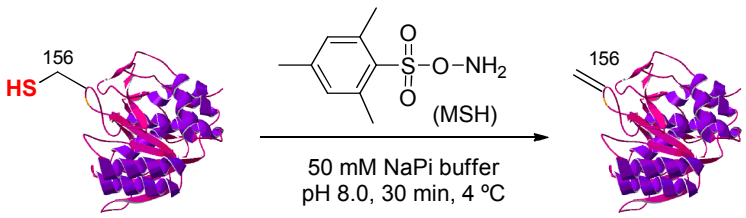
Sequence of SBL-S156C (1)

(BPN' numbering, PDB code for wild type SBL = 1GCI)

AQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPSTQDGN
GHGTHVAGTIAALNNSIGVLGVAPSAELYAVKVLGASGSGSVSSIAQGLEWAGNNGMHV
ANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNCGAGSISYPARYANAMAVGATDQNN
NRASFSQYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPSWSN
VQIRNHLKNTATSLGSTNLYGSGLVNAEAATR

Calculated average isotopic mass = 26714.5

SBL-C156-Dha (2)[13]



All manipulations were carried out in a cold room maintained at 4 ºC. D/L-Dithiothreitol (DTT) (5.77 mg, 37.43 µmol) was added as a solid to a solution of SBL-S156C **1** (5 mL, 2 mg/mL, 374.0 nmol) in 50 mM sodium phosphate buffer at pH 8.0 and the resulting mixture shaken for 30 minutes. Small molecules were removed from the reaction mixture by loading the sample onto a PD10 desalting column (GE Healthcare) previously equilibrated with 10 column volumes of pH 8.0 sodium phosphate buffer (50 mM) and eluting with 7 mL of the same buffer.

To a solution of SBL-S156C **1** (2.5 mL, 1.4 mg/mL, 131.0 nmol) in 50 mM sodium phosphate buffer at pH 8.0, a freshly prepared solution of *O*-mesitylsulfonylhydroxylamine (MSH) (20 µL of

7 mg/mL, 655.0 nmol) in DMF was added and the resulting mixture vortexed for 30 seconds. After 30 minutes of additional shaking, a 20 µL aliquot was analyzed directly by LC–MS and complete conversion to SBL-C156-Dha **2** was observed (calculated mass, 26681; observed mass, 26681). Small molecules were removed from the reaction mixture by loading the sample onto a PD10 desalting column (GE Healthcare) previously equilibrated with 10 column volumes of pH 8.0 sodium phosphate buffer (50 mM) and eluting with 3.5 mL of the same buffer. The protein sample was flash frozen with liquid nitrogen and stored at –80 ºC.

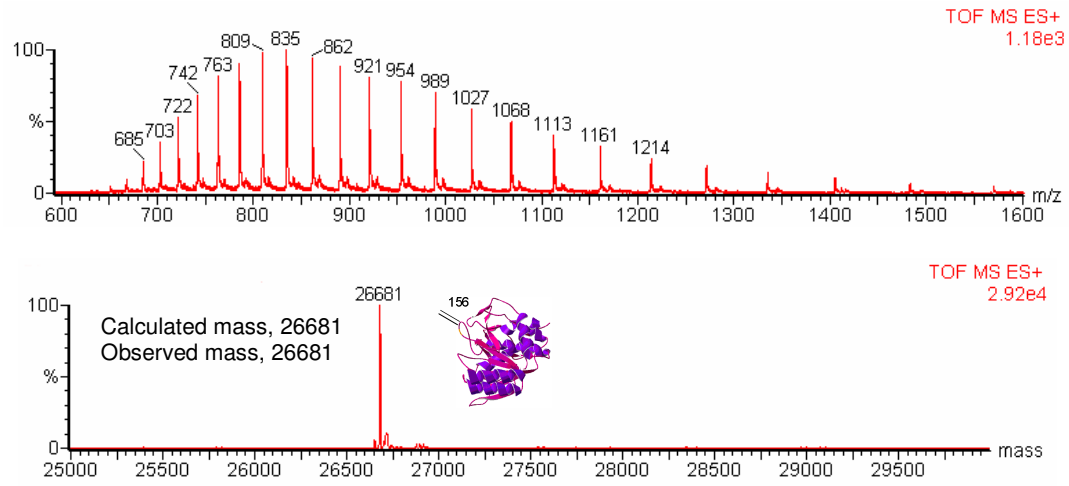
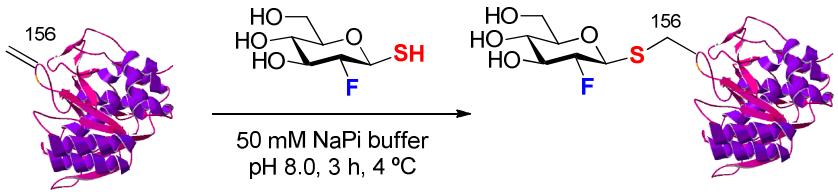


Figure ESI 19. ESI–MS spectrum after 30 min at 4 ºC.

SBL-C156-*S*Glc2F (6)[3]



All manipulations were carried out in a cold room maintained at 4 ºC. A 300 µL aliquot of SBL-C156-Dha **2** (0.4 mg/mL, 4.49 nmol) previously prepared in 50 mM sodium phosphate buffer at pH 8.0 was thawed. To the protein solution was added 2-deoxy-2-fluoro-1-thio-β-D-glucopyranose **3** (3.2 mg, 16.1 µmol) as a solid and the resulting mixture vortexed for 30 seconds. After 3 h of shaking, the reaction was analyzed directly by LC–MS and complete conversion to SBL-C156-*S*Glc2F **6** was observed (calculated mass, 26879; observed mass, 26884). The protein solution was dialyzed against 50 mM sodium phosphate buffer (pH 7.0) and stored at –80 ºC.

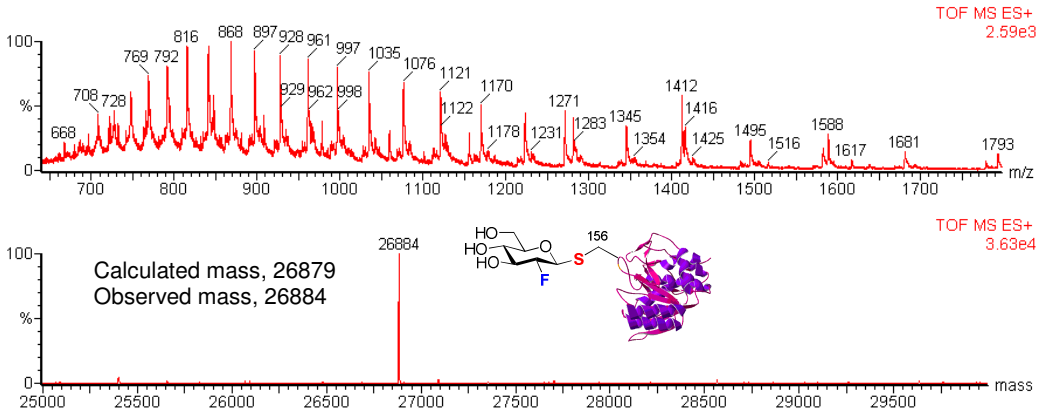
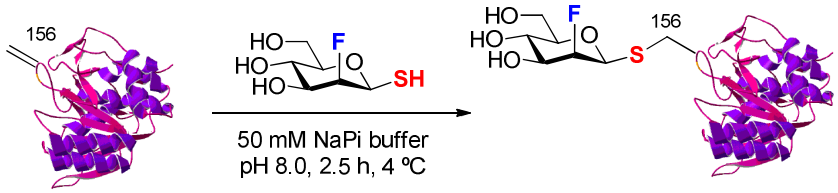


Figure ESI 20. ESI–MS spectrum after 3 h at 4 ºC.

SBL-C156-*S*Man2F (7)[3]



All manipulations were carried out in a cold room maintained at 4 ºC. A 300 µL aliquot of SBL-C156-Dha **2** (0.4 mg/mL, 4.49 nmol) previously prepared in 50 mM sodium phosphate buffer at pH 8.0 was thawed. To the protein solution was added 2-deoxy-2-fluoro-1-thio-β-D-mannnopyranose **4** (3.7 mg, 18.7 µmol) as a solid and the resulting mixture vortexed for 30 seconds. After 2.5 h of shaking, the reaction was analyzed directly by LC–MS and complete conversion to SBL-C156-*S*Man2F **7** was observed (calculated mass, 26879; observed mass, 26884). The protein solution was dialyzed against 50 mM sodium phosphate buffer (pH 7.0) and stored at –80 ºC.

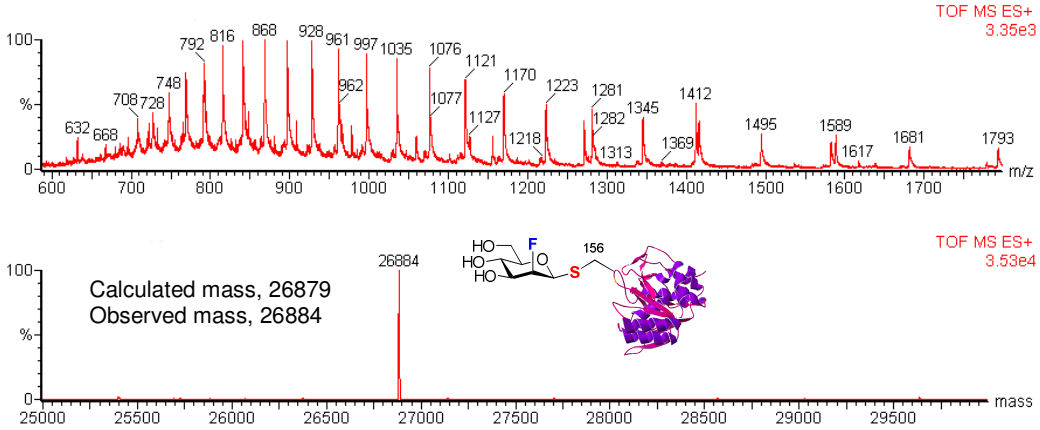
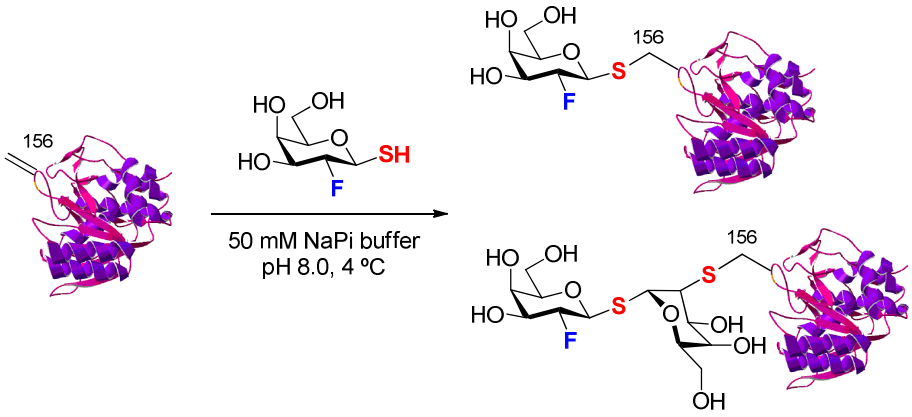


Figure ESI 21. ESI–MS spectrum after 2.5 h at 4 ºC.

SBL-C156-*S*Gal2F (8) and SBL-C156-*S*TalGal2F (9)



All manipulations were carried out in a cold room maintained at 4 ºC. A 250 µL aliquot of SBL-C156-Dha **2** (0.4 mg/mL, 3.70 nmol) previously prepared in 50 mM sodium phosphate buffer at pH 8.0 was thawed. To the protein solution was added 2-deoxy-2-fluoro-1-thio-β-D-galactopyranose **5** (1.9 mg, 9.6 µmol) as a solid and the resulting mixture vortexed for 30 seconds. After 10 h of shaking, the reaction was analyzed directly by LC–MS and 65% conversion to SBL-156-*S*Gal2F **8** (calculated mass, 26879; observed mass, 26878) and 35% conversion to SBL-156-*S*TalGal2F **9** (calculated mass, 27057; observed mass, 27057) was observed. The protein sample was flash frozen with liquid nitrogen and stored at –80 ºC.

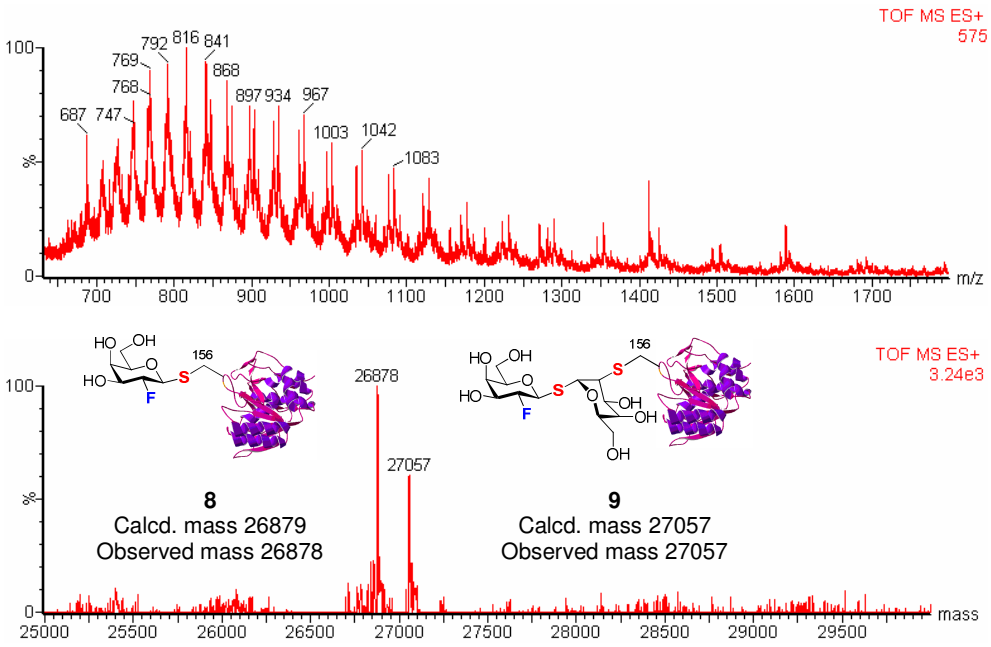
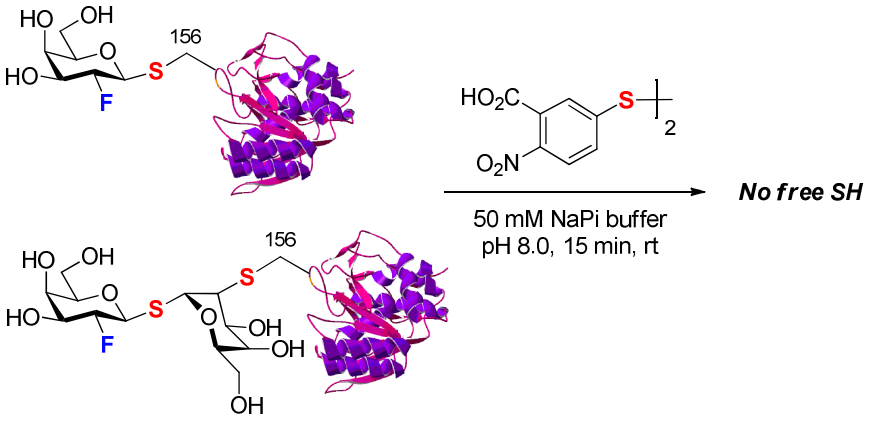


Figure ESI 22. ESI–MS spectrum after 10 h at 4 ºC.

Control: Ellman's reagent addition



A 25 µL aliquot of SBL-156-Dha **2** (*ca.* 0.375 nmol) reaction with 2-deoxy-2-fluoro-1-thio-β-D-galactopyranose **5** after 10 h at 4 ºC was transferred to a 0.5 mL eppendorf tube. Ellman's reagent (1.1 mg, 2.7 µmol) in 25 µL sodium phosphate buffer (50 mM, pH 8.0) was added at room temperature and the resulting mixture vortexed for 30 seconds. After 15 minutes of additional shaking, a 30 µL aliquot was analyzed directly by LC–MS and no free SH was detected.

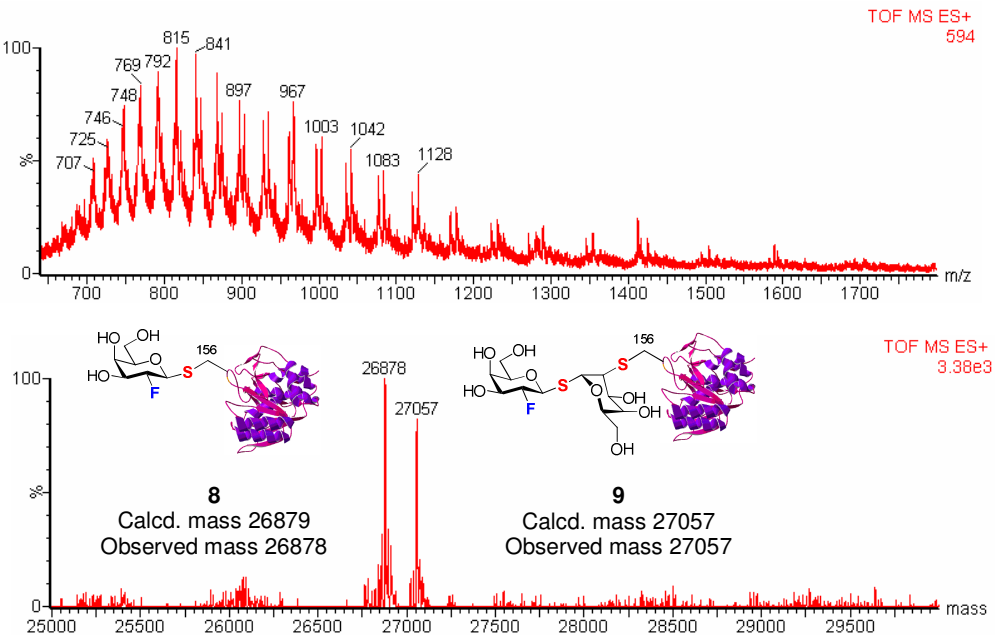
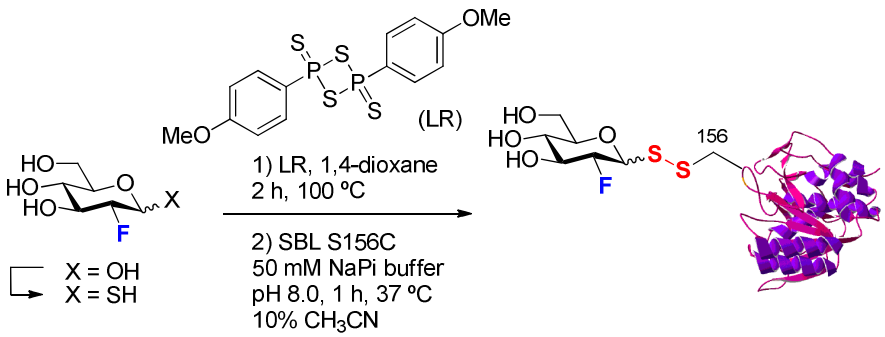


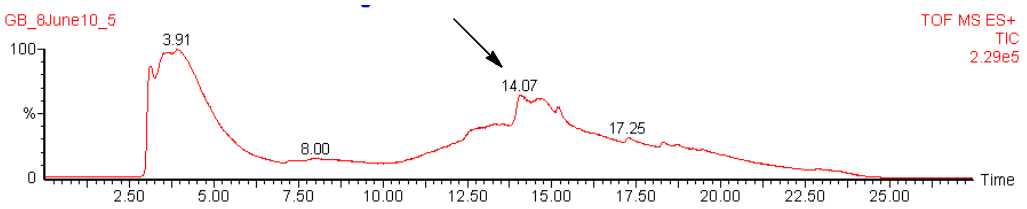
Figure ESI 23. ESI–MS spectrum after 15 min at room temperature.

SBL-C156-*SS*Glc2F (10)



Conditions were chosen in order to be the "most" suitable to apply using [^{18}F]FDG. Lawesson's reagent (LR) (27 mg, 0.066 mmol) was partially dissolved in anhydrous 1,4-dioxane (4 mL) in a flame dried round-bottomed flask. The mixture was heated to reflux and stirred for 1 h under argon atmosphere. 2-Deoxy-2-fluoro-D-glucose (FDG) (10 mg, 0.055 mmol) was then added as a solid and the mixture stirred for an extra 2 h period. After this time, an aliquot was taken for LRMS analysis, and showed formation of FDGSH **3** (*m/z* (ES^-) 197.0 $[M–H]^-$). Although the reaction did not seem to be complete (*ca.* 15% by LRMS), the reaction mixture was cooled to room temperature, filtered through Celite® and concentrated *in vacuo*. The crude reaction was used without further purification.

Crude reaction of FDG and LR (2 mg, 0.01 mmol) was dissolved in 25 µL of acetonitrile and added to 250 µL of SBL-S156C **1** (0.71 mg/mL, 6.64 µmol) in 50 mM sodium phosphate buffer at pH 8.0. The mixture was vortexed and shaked for 60 minutes at 37 ºC. The reaction mixture was passed through a PD minitrap G-25 desalting column (GE Healthcare) to remove excess small molecules. The protein was eluted with 50 mM sodium phosphate buffer at pH 8.0 to a final concentration of *ca.* 0.36 mg/mL. An aliquot of the protein sample was analyzed by LC–MS and complete conversion to SBL-C156-*SS*Glc2F **10** was observed (calculated mass, 26912; observed mass, 26910). The protein solution was stored at –20 ºC.



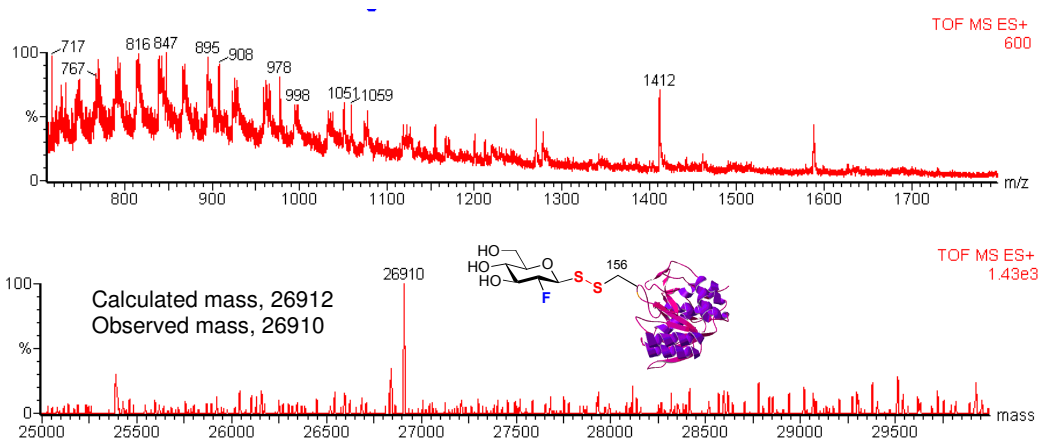
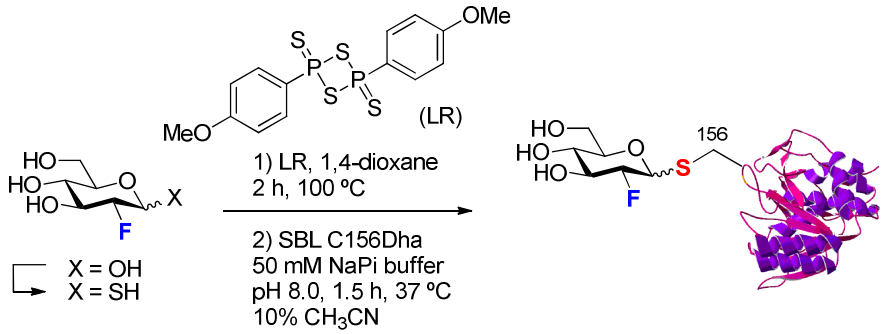
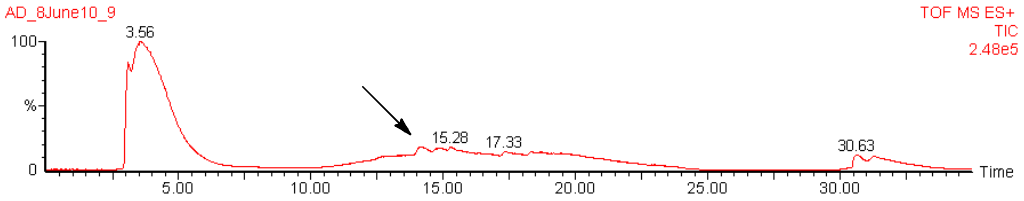


Figure ESI 24. ESI–MS spectrum after 1 h at 37 ºC; arrow indicates region containing all protein

SBL-C156-*S*Glc2F (6)



Crude reaction of FDG and LR (1 mg, 5 µmol) was dissolved in 10 µL of acetonitrile and added to 100 µL of SBL-C156-Dha **2** (0.25 mg/mL, 0.93 µmol) in 50 mM sodium phosphate buffer at pH 8.0. The mixture was vortexed and shaked for 90 minutes at 37 ºC. A second aliquot of the crude reaction (1 mg, 5 µmol) was added and the mixture vortexed and shaked for 30 minutes at 37 ºC. After 2 h of total reaction time, the reaction mixture was passed through a PD minitrap G-25 desalting column (GE Healthcare). The protein was eluted with 50 mM sodium phosphate buffer at pH 8.0 to a final concentration of *ca.* 0.13 mg/mL. An aliquot of the protein sample was analyzed by LC–MS and complete conversion to SBL-C156-*S*Glc2F **6** was observed (calculated mass, 26879; observed mass, 26872). The protein solution was stored at –20 ºC.



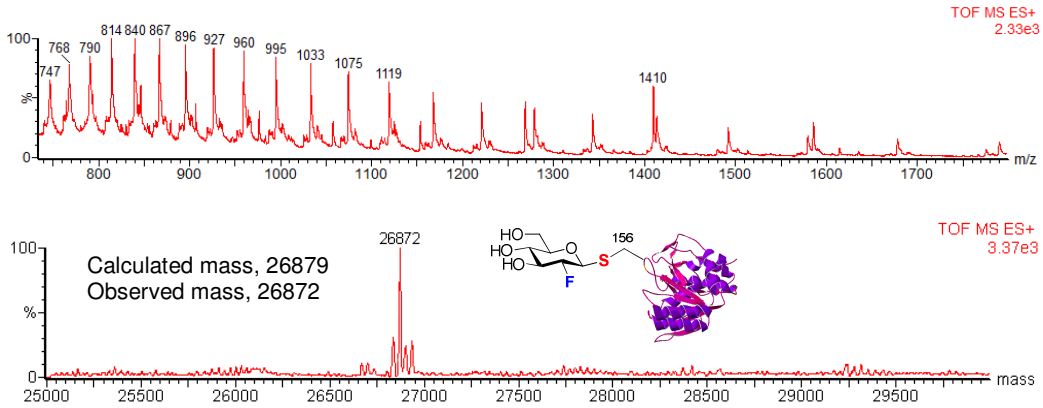


Figure ESI 25. ESI–MS spectrum after 2 h at 37 ºC; arrow indicates region containing all protein

2.3. SDS-PAGE analysis of 2-fluoroglycoproteins 6–9

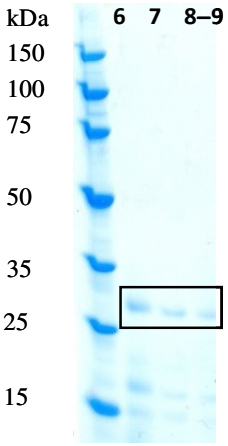


Figure ESI 26. SDS-PAGE analysis of *S*-linked fluoroglycoproteins **6–9** (MES running buffer and coomassie blue stain).

2.4. Tryptic digest of 2-fluoroglycoproteins 8 and 9

In Gel tryptic digest protocol

An SDS PAGE gel (NuPAGE 4–12 % Bis-Tris, Invitrogen) of protein sample was run (see above). A clean razor blade was used to excise the gel band corresponding to the protein. The gel slice was placed into a microcentrifuge tube and was cut into ~ 1 mm pieces. The gel slices were washed sequentially as follows and rotated on a lab rotisserie: a) 100 µL solution B for 30 min then discard supernatant; b) repeat wash with 100 µL solution B for 30 min then discard supernatant (repeat until bands are no longer a strong blue colour); c) wash in 100 µL acetonitrile for 10 min and

remove (gel pieces should dehydrate and go white); d) dry in SpeedVac for 10 min (all acetonitrile should have evaporated); e) add 100 µL (10 mM) DTT for 30 min at 37 ºC and discard; f) wash gel pieces with 25 mM ammonium bicarbonate solution and discard; g) wash in acetonitrile until gel pieces are white and discard; h) add 100 µL (55 mM) iodoacetamide for 60 min (in dark) and discard; i) wash bands with 100 µL solution B for 10 min and discard; j) wash in 100 µL acetontrile until gel pieces are white, then discard; k) dry in SpeedVac for 10 min and add 20 uL trypsin to each tube; l) digest overnight at 37 ºC; m) add 1 µL of formic acid to stop digest; n) remove supernatant containing peptides to clean new tube, add 50 µL of extraction buffer to gel piece and incubate for 30 min; o) remove supernatant containing peptides and pool with existing supernatant. Tryptic samples were analyzed with a Thermo LTQ Orbitrap mass spectrometer coupled to an ABI 4800 MALDI TOF TOF mass spectrometer in the Oxford University Central Proteomics Service (Dunn School of Pathology).

Solutions

a) Solution B: 0.04 g ammonium bicarbonate in 10 mL Milli-Q grade water and 10 mL HPLC grade acetonitrile.

b) 25 mM ammonium bicarbonate: 0.04 g in 20 mL Milli-Q grade water.

c) 10 mM DTT: 0.031 g in 20 mL (25 mM) ammonium bicarbonate solution.

d) 55 mM iodoacetamide: 0.2 g in 20 mL (25 mM) ammonium bicarbonate solution.

e) Promega sequencing grade modified trypsin (catalogue number: V5111): add 100 µL Promega resuspension buffer and store in 5 µL aliquots at –20 ºC until use. Prior to use add 95 µL (25 mM) ammonium bicarbonate – the tryspin in now active. Any trypsin remaining after use should be disposed off.

f) Extraction buffer: 10 mL Milli-Q grade water + 10 mL acetonitrile + µL formic acid.

MALDI-TOF analysis of 8 and 9

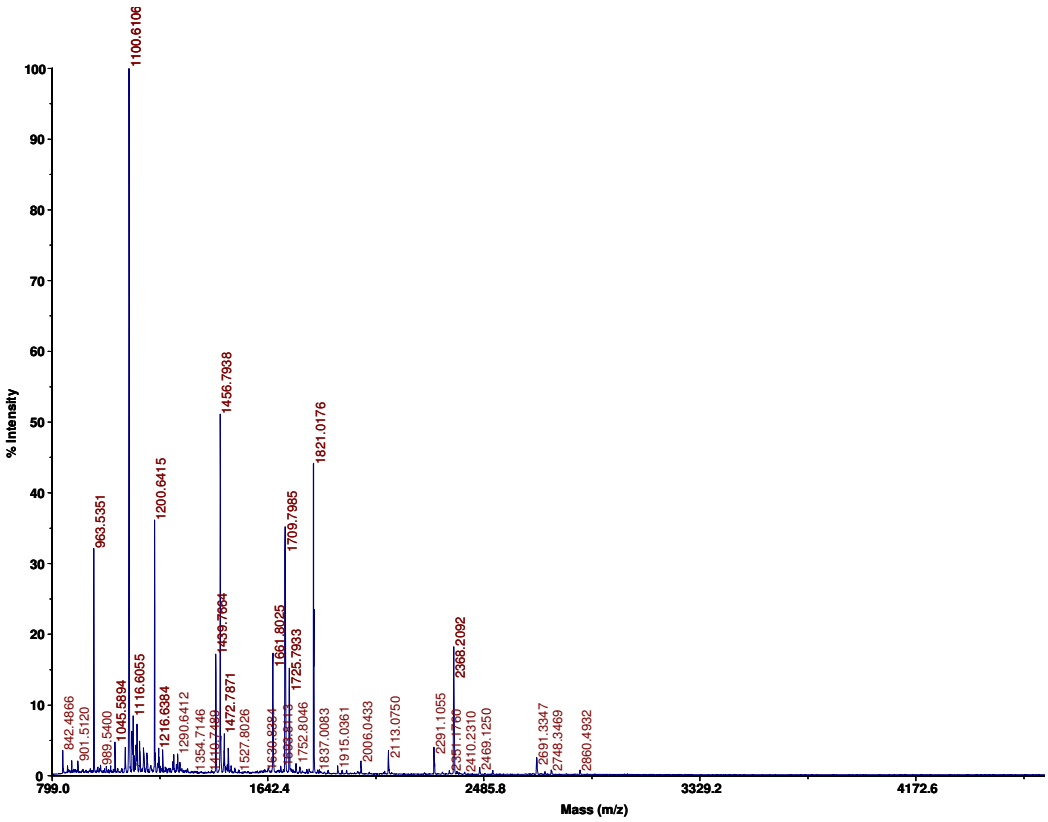
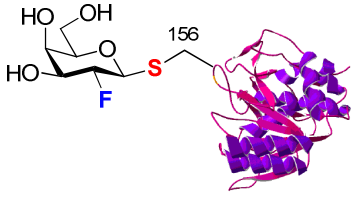


Figure ESI 27. MALDI-TOF spectrum of **8** and **9** after tryptic digest.

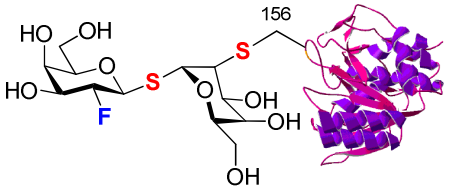
Table ESI 4. Selected results for SBL-C156-*S*Gal2F **8**.



Frag #	Res #	Sequence[a]	Calc. mass	Obs. mass	Charge[b]	Error (Δ)
T7	144–164	(R)GVLVVAASGN[***S*Gal2F**]GAGSISYPAR(Y)	2113.04	2113.08	1+	0.04
T7–8	144–180	(R)GVLVVAASGN[***S*Gal2F**] GAGSISYPARYANAMAVGATDQNNNR(A)	2002.52	2006.04	2+	3.52
T6–7	93–164	(K)VLGASGSGSVSSIAQGLEWAGNNGMHVANLSL GSPSPSATLEQAVNSATSRGVLVVAASGN[***S*Gal2F**] GAGSISYPAR(Y)	2473.55	2469.13	3+	4.42

(a) Predicted peptide fragments containing 156*S*Gal2F moiety were determined by MassLynx software (v. 4.0 from Waters) according to the manufacturer's instructions. (b) Charge corresponding to $[M+nH]^{n+}$

Table ESI 5. Selected results for SBL-C156-*S*TalGal2F **9**.

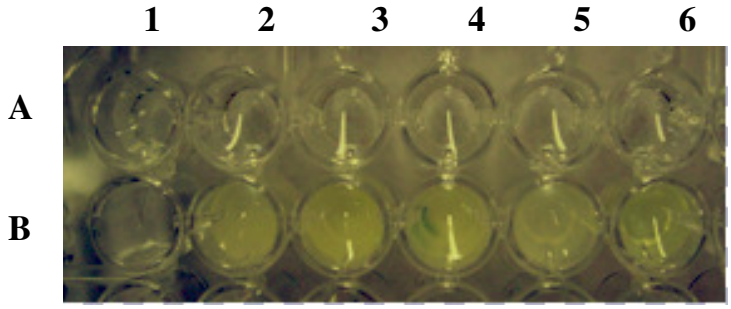


Frag #	Res #	Sequence[a]	Calc. mass	Obs. mass	Charge[b]	Error (Δ)
T7	144–164	(R)GVLVVAASGN**[*S*Gal2F]**GAGSISYPAR(Y)	2291.07	2291.11	1+	0.04
T7–8	144–180	(R)GVLVVAASGN**[*S*TalGal2F]** GAGSISYPARYANAMAVGATDQNNNR(A)	1579.39	-	3+	-
T6–7	93–164	(K)VLGASGSGSVSSIAQGLEWAGNNGMHVANLSL GSPSPSATLEQAVNSATSRGVLVVAASGN**[*S*TalGal2F]** GAGSISYPAR(Y)	2651.58	-	3+	-

(a) Predicted peptide fragments containing 156*S*TalGal2F moiety were determined by MassLynx software (v. 4.0 from Waters) according to the manufacturer's instructions. (b) Charge corresponding to $[M+nH]^{n+}$

2.5. Peptidase activity of 2-fluoroglycoproteins 1, 2 and 6–10

SBL-S156C **1** (unmodified), SBL-C156-Dha **2** and SBL-C156-*S*Glyco2F (modified) **6–10** were prepared at a concentration of 0.01 mg/mL in pH 8.0 sodium phosphate (50 mM). 200 µL aliquots of each protein sample were added to a 96-well plate. A 2 µL aliquot of SucAAPF*p*NA (0.20 M in DMSO, Bachem) was added to each of the protein samples. All SBL samples turned yellow immediately upon addition of the peptide substrate. The yellow solution indicates liberation of *p*-nitroaniline (*p*NA), confirming peptidase activity of all SBL samples. All protein solutions and the peptide solution alone at the same concentration are colourless (See below).



Well A1: SucAAPF*p*NA

Well A2: SBL-S156C **1**

Well A3: SBL-C156-Dha **2**

Well A4: SBL-C156-*S*Glc2F **6**

Well A5: SBL-C156-*S*Man2F **7**

Well A6: SBL-C156-*S*Gal2F (+2FGalTal) **8** and **9**

Well B1: (empty)

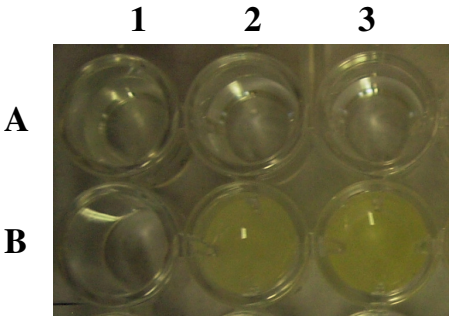
Well B2: SBL-S156C **1** + SucAAPF*p*NA

Well B3: SBL-C156-Dha **2** + SucAAPF*p*NA

Well B4: SBL-C156-*S*Glc2F **6** + SucAAPF*p*NA

Well B5: SBL-C156-*S*Man2F **7** + SucAAPF*p*NA

Well B6: SBL-C156-*S*Gal2F (+2FGalTal) **8** and **9** + SucAAPF*p*NA



Well A1: SucAAPF*p*NA

Well A2: SBL-C156-*S*Glc2F **6** (from LR reaction)

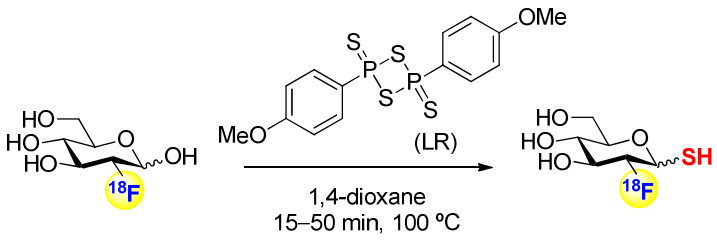
Well A3: SBL-C156-*SS*Glc2F **10** (from LR reaction)

Well B1: (empty)

Well B2: SBL-C156-*S*Glc2F **6** (from LR reaction) + SucAAPF*p*NA

Well B3: SBL-C156-*SS*Glc2F **10** (from LR reaction) + SucAAPF*p*NA

2.6. Preparation of 2-deoxy-2-[^{18}F]fluoro-1-thio-glucopyranose 11



[^{18}F]FDG (72.3–23.7 MBq) solution was diluted with dry 1,4-dioxane (0.5 mL) at room temperature. The solution was evaporated using a stream of nitrogen at 100 °C and co-evaporated

to dryness with dry 1,4-dioxane (3 x 0.3 mL). Lawesson's reagent (20 mg, 0.048 mmol) was dissolved in dry 1,4-dioxane (0.5 mL) and heated at 100 °C for 60 minutes. This solution was added to dry [^{18}F]FDG (31.5–18.4 MBq) and the resulting mixture heated at 100 °C for 15–60 min. The Reaction was analyzed by radio-TLC and radio-RP-HPLC (Activity = 8.9–17.4 MBq, >98% conv. in 45–100 min, n=3). Used in the next step without further purification.

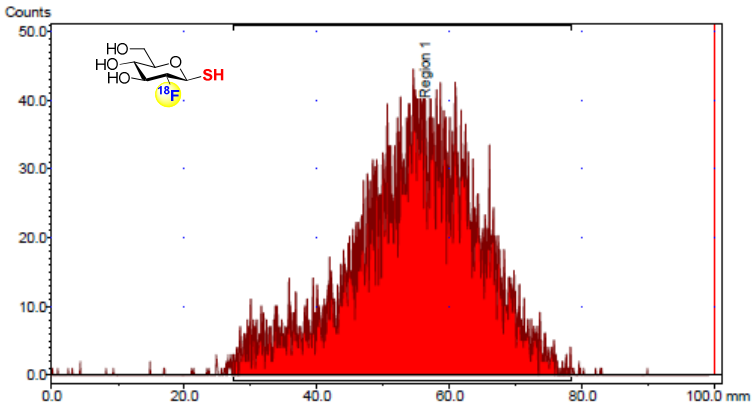


Figure ESI 28. Radio-TLC showing reaction mixture after 15 min at 100 °C; Solvent system = water/isopropanol/ethyl acetate (WIPE 1:4:4 v/v/v).

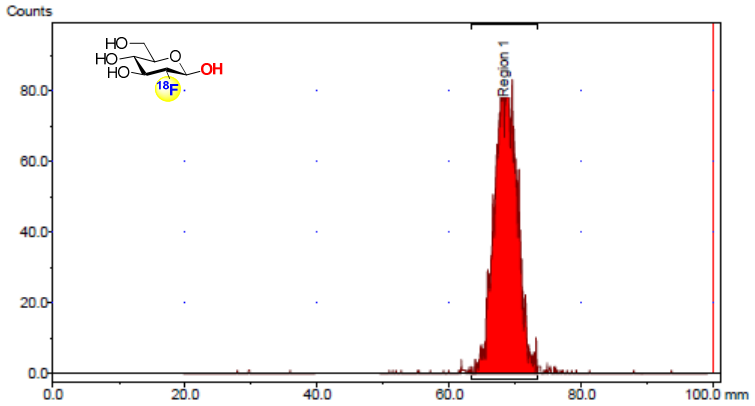


Figure ESI 29. Radio-TLC control ([^{18}F]FDG); Solvent system = water/isopropanol/ethyl acetate (WIPE 1:4:4 v/v/v).

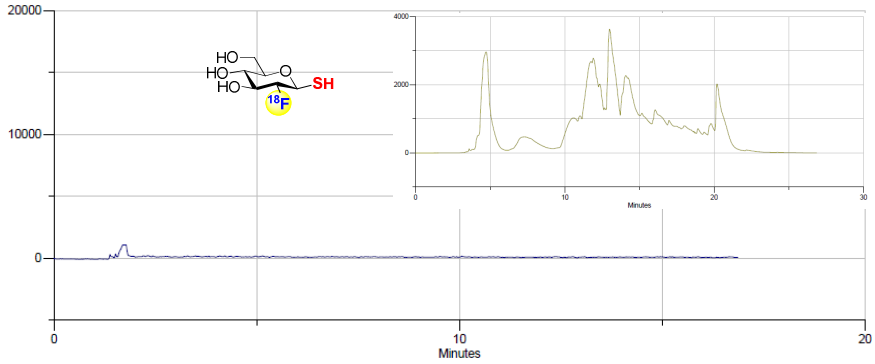


Figure ESI 30. Full radio-RP-HPLC trace showing reaction mixture after 15 min at 100 °C; inset shows UV monitoring at 280 nm [Phenomenex Jupiter C4 (250 × 4.6 mm× 5µm) column; flow rate was 1.0 mL/min; t_R: 1.7 min].

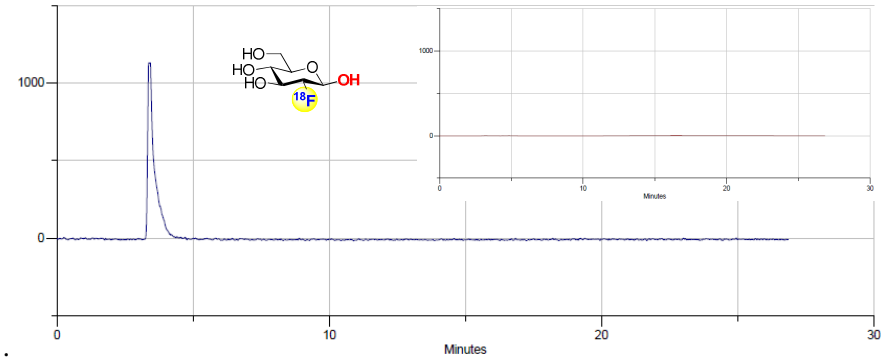
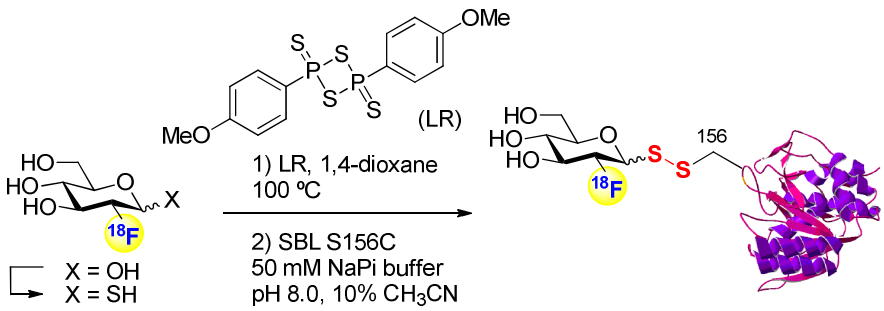


Figure ESI 31. Full radio-RP-HPLC trace showing control ([^{18}F]FDG); inset shows UV monitoring at 280 nm [Phenomenex Jupiter C4 (250 × 4.6 mm× 5µm) column; flow rate was 1.0 mL/min; t_R: 3.3 min].

2.7. Preparation of [^{18}F]-labelled glycoproteins 12 and 13

SBL-C156-*SS*Glc2F (12)



Crude [^{18}F]FDGSH **11** solution was evaporated using a stream of nitrogen at 100 °C. Dry [^{18}F]FDGSH **11** (8.9–17.4 MBq) was re-dissolved in 50 mM sodium phosphate buffer at pH 8.0 (100 µL) and CH_3CN (10 µL). To this solution was added SBL-S156C **1** in 50 mM sodium phosphate buffer at pH 8.0 (10 µL, 1 mg/mL, 0.374 nmol) and the resulting mixture stirred at room temperature or 37 °C for 15–90 min. The reaction was analyzed by radio-RP-HPLC (Conv. and time over two steps = 43–60% in 90–210 min, n=3); Protein elute between 13 and 16 min.

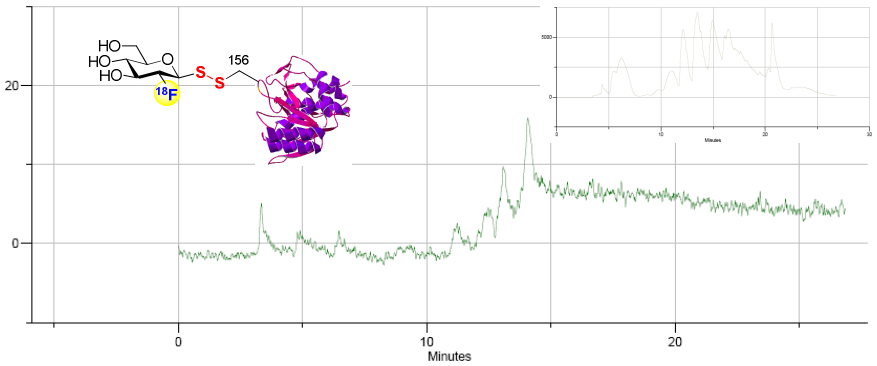


Figure ESI 32. Expanded radio-RP-HPLC trace showing reaction mixture after **15 min at 37 °C**; inset shows UV monitoring at 280 nm [Phenomenex Jupiter C4 (250 × 4.6 mm× 5µm) column; flow rate was 1.0 mL/min].

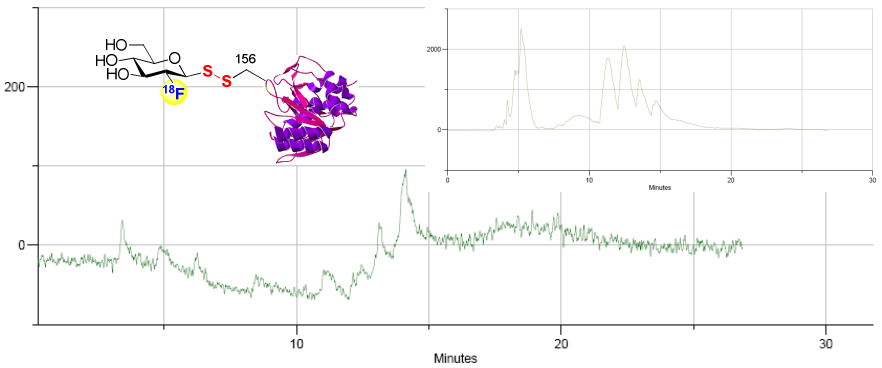


Figure ESI 33. Expanded radio-RP-HPLC trace showing reaction mixture after **90 min at 37 °C**; inset shows UV monitoring at 280 nm [Phenomenex Jupiter C4 (250 × 4.6 mm× 5µm) column; flow rate was 1.0 mL/min].

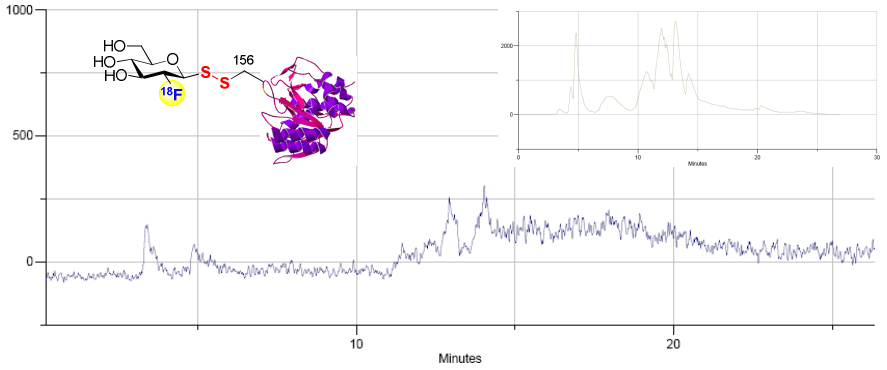


Figure ESI 34. Expanded radio-RP-HPLC trace showing reaction mixture after **15 min at room temperature**; inset shows UV monitoring at 280 nm [Phenomenex Jupiter C4 (250 × 4.6 mm×5µm) column; flow rate was 1.0 mL/min].

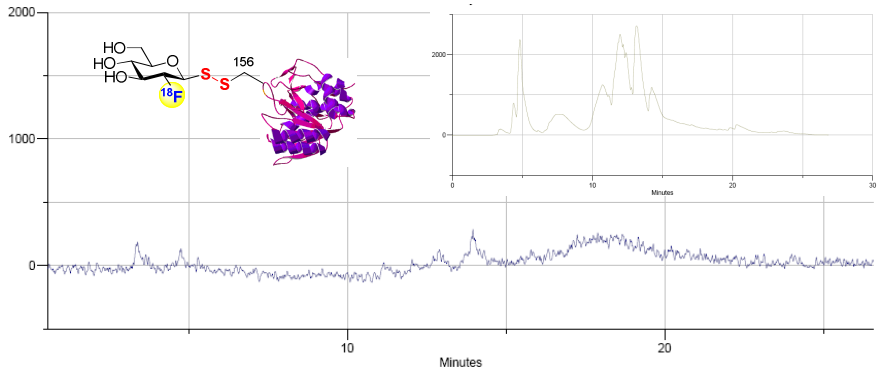


Figure ESI 35. Expanded radio-RP-HPLC trace showing reaction mixture after **90 min at room temperature**; inset shows UV monitoring at 280 nm [Phenomenex Jupiter C4 (250 × 4.6 mm×5µm) column; flow rate was 1.0 mL/min].

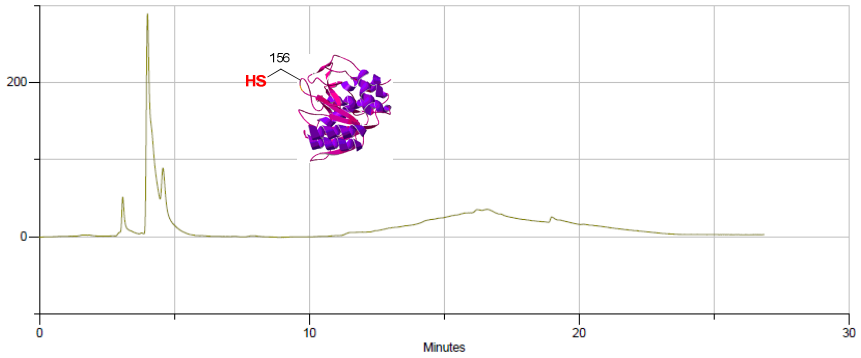


Figure ESI 36. Full RP-HPLC trace showing control (SBL S156C); UV monitoring at 280 nm [Phenomenex Jupiter C4 (250 × 4.6 mm× 5µm) column; flow rate was 1.0 mL/min].

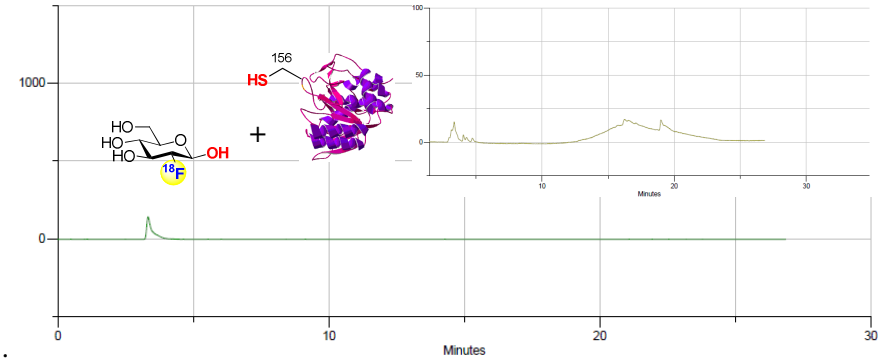
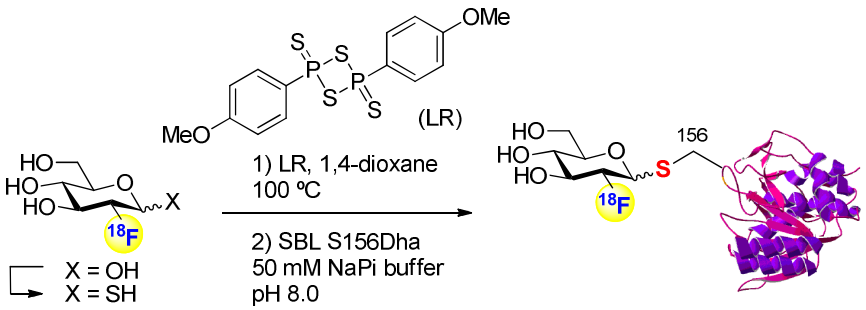


Figure ESI 37. Full radio-RP-HPLC trace showing control (SBL S156C + [^{18}F]FDG after incubation at 37 °C for 15 min); inset shows UV monitoring at 280 nm [Phenomenex Jupiter C4 (250 × 4.6 mm× 5µm) column; flow rate was 1.0 mL/min].

SBL-C156-*S*Glc2F (13)



Crude [^{18}F]FDGSH **11** solution was evaporated using a stream of nitrogen at 100 °C. Dry [^{18}F]FDGSH **11** (9.2–10.0 MBq) was re-dissolved in 50 mM sodium phosphate buffer at pH 8.0 (100 µL). To this solution was added SBL-C156Dha **2** in 50 mM sodium phosphate buffer at pH 8.0 (10 µL, 0.25 mg/mL, 0.094 nmol) and the resulting mixture stirred at room temperature or 37 °C for 15–105 min. The reaction was analyzed by radio-RP-HPLC (Conv. and time over two steps = 50–60% in 90–180 min, n=2); Protein elute between 13 and 16 min.

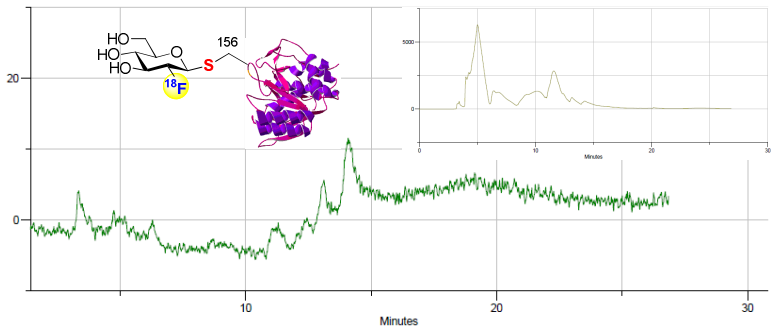


Figure ESI 38. Expanded radio-RP-HPLC trace showing reaction mixture after **20 min at 37 °C**; inset shows UV monitoring at 280 nm [Phenomenex Jupiter C4 (250 × 4.6 mm× 5µm) column; flow rate was 1.0 mL/min].

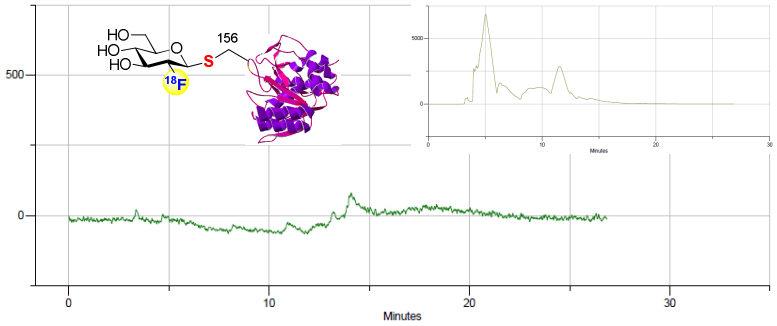


Figure ESI 39. Expanded radio-RP-HPLC trace showing reaction mixture after **105 min at 37 °C**; inset shows UV monitoring at 280 nm [Phenomenex Jupiter C4 (250 × 4.6 mm× 5µm) column; flow rate was 1.0 mL/min].

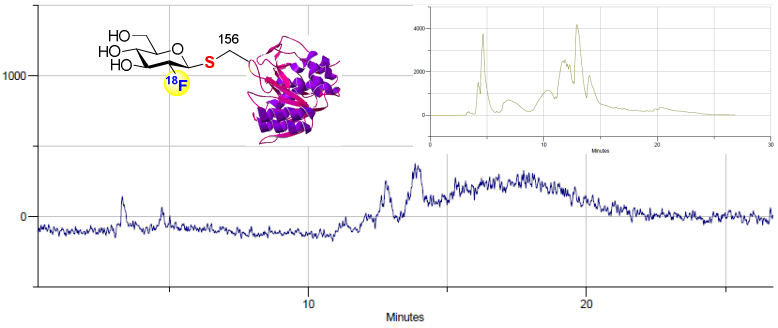


Figure ESI 40. Expanded radio-RP-HPLC trace showing reaction mixture after **15 min at room temperature**; inset shows UV monitoring at 280 nm [Phenomenex Jupiter C4 (250 × 4.6 mm× 5µm) column; flow rate was 1.0 mL/min].

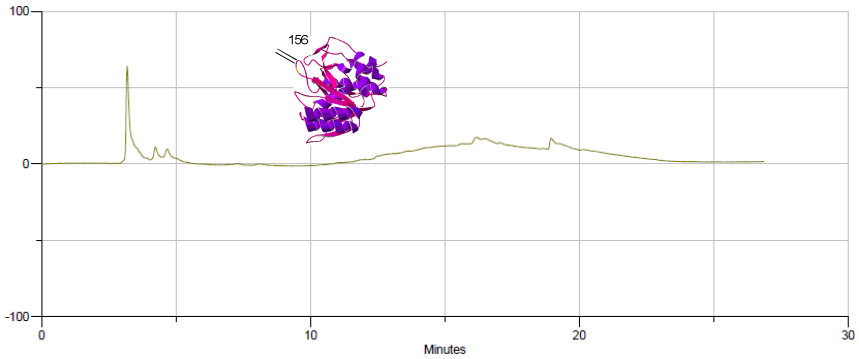


Figure ESI 41. Full RP-HPLC trace showing control (SBL-C156Dha); UV monitoring at 280 nm [Phenomenex Jupiter C4 (250 × 4.6 mm× 5µm) column; flow rate was 1.0 mL/min].

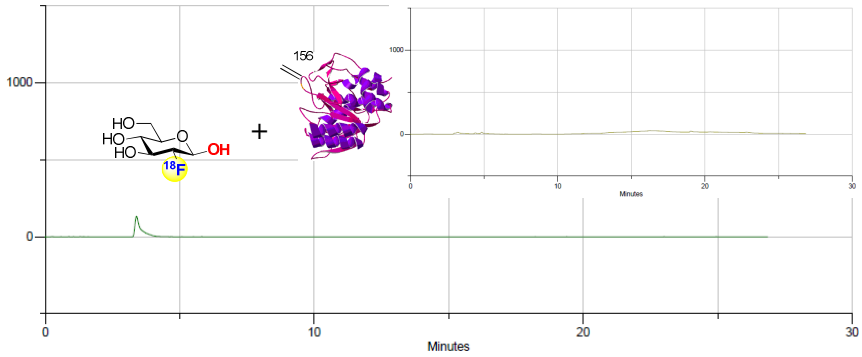


Figure ESI 42. Full radio-RP-HPLC trace showing control (SBL-C156Dha + [^{18}F]FDG alter incubation at 37 °C for 15 min); inset shows UV monitoring at 280 nm [Phenomenex Jupiter C4 (250 × 4.6 mm× 5µm) column; flow rate was 1.0 mL/min].

Double peaks in RP-HPLC analysis

RP-HPLC of a chemically pure protein leads in most cases to a single peak. However, proteins are sometimes eluted from HPLC columns as multiple or/and irregular-shaped peaks. This could lead to chromatograms with poor resolution or to wrong analysis. Proteins are stabilized by a combination of hydrogen bonding, electrostatic interactions, and hydrophobic interactions. The same forces are also involved in the chromatographic processes, thus conformational changes may occur induced by mobile or stationary phase or both, leading to loss of bioactivity and denaturation. The native and denaturated forms can be resolved if the kinetic process of conformational change is slow relative to the time scale of migration through the column, otherwise, a single broad peak will be obtained, which is the weighted average of the forms in equilibrium.

Such a chromatographic behaviour can be due to chemical transformations, conformational changes (denaturation, renaturation), or other structural changes (aggregation, chain disruption, *etc*.) undergone during protein elution. These changes in the analyte can be promoted by mobile phase and/or stationary phase mediated by temperature. A conformational change of the solute that takes place during its migration through the column can result in broadened peaks or multiple peaks if the kinetics of this change is on the time scale of the chromatographic experiment and the products have a retention time different from that of the original solutes. In other cases, multiple peaks are not caused by the chromatographic system, but rather due to chemical transformations, conformational changes or other structural changes taking place in the sample during the isolation, purification or other processing steps prior to its elution through the column.[14]
In particular, peak shapes for globular proteins above 20 KDa are very responsive to mobile phase mediated phenomena which affect the diffusion coefficients of these solutes as well as the kinetic resistance to migration of the solutes onto and off the alkylsilica support.[15]

Importantly, the following references on multiple peaks found in HPLC analysis of radioactive peptides[16] show that multiple molecular configurations of identical molecular weight but differing in shape, charge, isomerism or lipophilicity such that they are resolved under the conditions of certain analyses. **One conclusion from this investigation is that the appearance of a single peak by any HPLC analysis offers no assurance that multiple peaks would not appear on alternative HPLC analyses. It will always be difficult by HPLC to prove that a single radiochemical species is present. Evidence that each species is due to radiolabelled active peptide and not to radiocontaminants is therefore potentially more important than evidence of a single peak.**

3. References

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