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Syntheses of novel azasugar-containing mimics of heparan sulfate fragments as potential heparanase inhibitors

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ABSTRACT

A series of eight novel, highly modified mono-, di- and trisaccharide derivatives, each containing an iminoalditol moiety, has been synthesized in the quest for potential heparanase inhibitors.

1. Introduction

Mammalian heparanase (heparanase 1 or Hpa1) is an *endo*-β-D-glucuronidase that hydrolyzes heparan sulfate (HS) at specific sites, and is thus involved in the degradation of the extracellular matrix.[1] The enzyme is over-expressed in many human tumours, and this expression correlates with more aggressive and invasive cancers and with poor patient prognoses. Heparanase degradation of HS results in the release of factors that promote angiogenesis and tumour vascularization and the enzyme is also involved in metastasis and in tissue repair. Consequently, for several years heparanase inhibitors have been targeted as potential anti-cancer drugs.[2–8] Until recently, the link between metastasis and heparanase has been based mostly on circumstantial evidence because validated heparanase inhibitors have also possessed other biological activities. Recently, however, the link between heparanase and metastasis of cancer in mice has been clearly demonstrated by the use of a viral vector carrying antisense–heparanase DNA.[9]

HS is a linear polymer of repeating disaccharide units consisting of α-1,4-linked glucosamine and 1,4-linked β-D-gluc-(or α-L-id-) uronic acid. The polymer is variously N- and O-sulfated and has a block-like domain structure with regions of high sulfation, low sulfation and no sulfation.[10] Heparanase hydrolyzes HS at particular glucuronic acid residues within regions of the molecule that have relatively high degrees of sulfation.[11] The smallest heparanase substrate known to date is the tetrasaccharide ΔHexA-β-(1→4)-GlcN(NS,6S)-α-(1→4)-GlcA-β-(1→4)-GlcN(NS,6S) although the minimum size required for recognition is a trisaccharide.[11] Unfortunately, no kinetic data are available for the enzymatic cleavage of this compound due to extreme difficulty in obtaining inhibition constants for heparanase. The finding that this tetrasaccharide is hydrolyzed by the enzyme contradicts earlier studies which concluded that a 2-O-sulfated uronic acid is required in heparanase substrates. In particular, it was thought that a sulfated uronic acid moiety is required two residues towards the reducing end of the glucuronosyl moiety that is hydrolyzed by the enzyme.[12,13]

Heparanase is thought to act with a retaining hydrolytic mechanism and belongs to the clan A hydrolase family.[14,15] The two glutamic acid residues in the active site of the enzyme that are presumed to act as the proton donor and nucleophile in the hydrolysis step have been identified by homology and confirmed by site-directed mutagenesis.[14] However, there is no detailed information available on the nature of the transition state(s) of the enzymic hydrolysis of HS.

It is expected that potent inhibitors of heparanase might be attained by synthesizing stable mimics of the transition state. Enzymes can catalyze reactions by lowering the energy of activation of the reaction,[16,17] often by factors of 10^{10}–10^{15}. Stable compounds that mimic the transition state will convert a proportion of this energy of activation into thermodynamic binding energy giving rise to unusually stable enzyme inhibitor complexes.[18] The transition states of a number of *N*-ribosyltransferases have been determined using kinetic isotope effects,[19] and some exceedingly potent transition state analogue inhibitors have been designed and synthesized.[20–23] These compounds have employed a ribooxacarbenium

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ion mimic as this is an important feature of the transition states of these enzymes.

Many glycosidase inhibitors are known that are also oxacarbenium ion mimics and this feature is thought to be important in their inhibitory activity.[24–26] We have postulated that it is likely heparanase achieves catalysis via oxacarbenium ion transition state(s) and so have designed potential inhibitors that will have oxacarbenium ion character. This has led us to the choice of azasugar analogues as potential inhibitors.

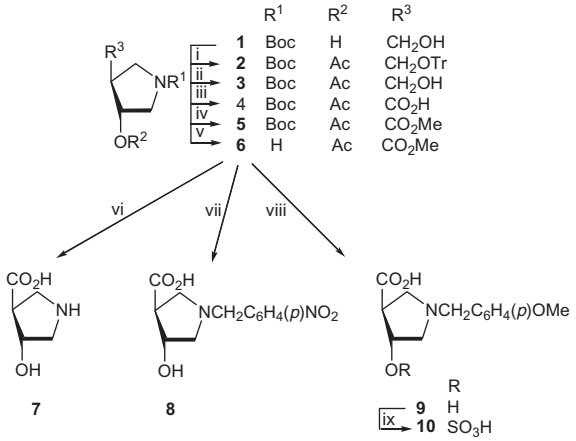
Together with the minimal structural requirements for heparanase recognition, the heparan sulfate trisaccharide mimic **46** was settled on as an inhibitor target. For comparative purposes and ease of preparation, the closely related compounds **32** and **33** were also chosen as targets. It is of interest to note that the synthesis of a GlcA-S-linked version of this trisaccharide has been described recently; but no heparanase inhibition data were reported.[27]

2. Results and discussion

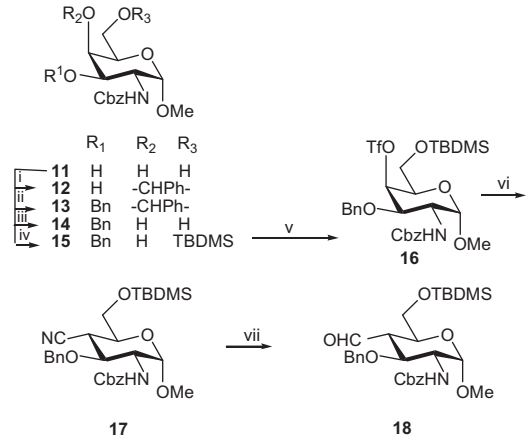
This report describes the preparation of compounds **7–10, 24, 25, 32, 33** and **46** (Schemes 1, 3, 4 and 6) which are analogues of mono-, di- and trisaccharides each containing functionalities considered likely to give rise to inhibition of heparanase.

Initially, compounds **7–10** were chosen as relatively simple and readily accessible targets. Since sugar-like pyrrolidines have good glycosidase-inhibiting properties, it was considered possible that these carboxy- and hydroxy-substituted pyrrolidine derivatives may have useful bioactivities and that sulfate **10**, in particular, might prove to be a heparanase inhibitor. Standard manipulation of the known chiral pyrrolidine **1**[28] gave the primary alcohol **3**. Oxidation with pyridinium dichromate followed by methylation generated the methyl ester **5** which on acid treatment provided the amine **6**. Saponification of **6** afforded the β-aminoacid **7** whereas reductive amination of *p*-nitrobenzaldehyde and *p*-methoxybenzaldehyde separately with **6** followed by saponification gave **8** and **9**. The latter was treated with $SO_3{\cdot}NMe_3$ affording sulfate **10**.

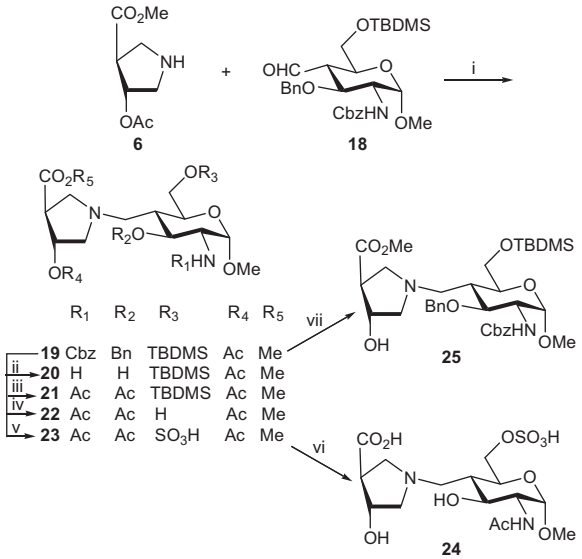
In order to construct the eventual targets **32**, **33** and **46**, the azasugar oxacarbenium ion mimics needed to be attached to the 4-position of a glucosamine residue. A stable methylene linkage was chosen which led to the *C*-formyl pyranoside **18** (Scheme 2) as an important intermediate. Reductive amination of this aldehyde with the corresponding azasugars was expected to provide the desired linkages.



Scheme 1. Reagents and conditions: (i) (a) TrCl, py, rt 48 h; (b) Ac_2O, rt 4 h (88%); ii) $Pd(OH)_2$/C (20%), H_2, 2 h, rt (97%); (iii) $Py{\cdot}H_2Cr_2O_7$, DMF, rt 20 h (86%); (iv) MeI, $KHCO_3$, DMF, rt, 20 h (90%); (v) HCl, dioxane, CH_2Cl_2, rt, 2 h (99%); (vi) NaOH, MeOH, H_2O, rt, 20 h, (93%); (vii) (a) $O_2N{\cdot}C_6H_4{\cdot}CHO(p)$, $ClCH_2CH_2Cl$; (b) $NaBH(OAc)_3$, Ar, rt, 1.5 h; (c) NaOH, H_2O, 5 h (73%); (viii) (a) $MeO{\cdot}C_6H_4{\cdot}CHO(p)$, $ClCH_2CH_2Cl$; (b) $NaBH(OAc)_3$, Ar, rt, 2 h; (c) NaOH, MeOH, H_2O, rt 4 h (77%); (ix) (a) $SO_3{\cdot}NMe_3$, DMF, 45 °C, 20 h; (b) MeOH, rt, 15 min (87%).

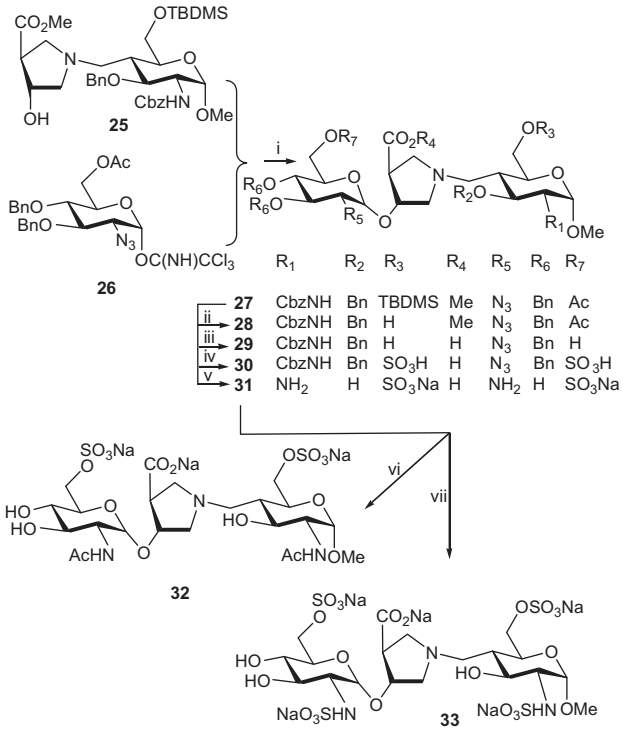


Scheme 2. Reagents and conditions: (i) (a) $PhCH(OMe)_2$, TsOH, DMF, 33 °C, 1.5 h (70%); (b) Et_3N, evaporation, 60 °C (70%); (ii) BnBr, BaO, $Ba(OH)_2{\cdot}8H_2O$, DMF, 25 °C, 22 h (64%); (iii) MeOH, H^+ resin, 65 °C, 1 h (84%); (iv) TBDMSCl, imidazole, $ClCH_2CH_2Cl$, 50 °C, 1.4 h (98%); (v) Tf_2O, CH_2Cl_2, py , 20 °C, 1 h (94%); (vi) Bu_4NCN, CH_2Cl_2, rt, 2d, 54% (from **16**); (vii) DIBAL-H, CH_2Cl_2, −78 °C, 2.5 h (59%).

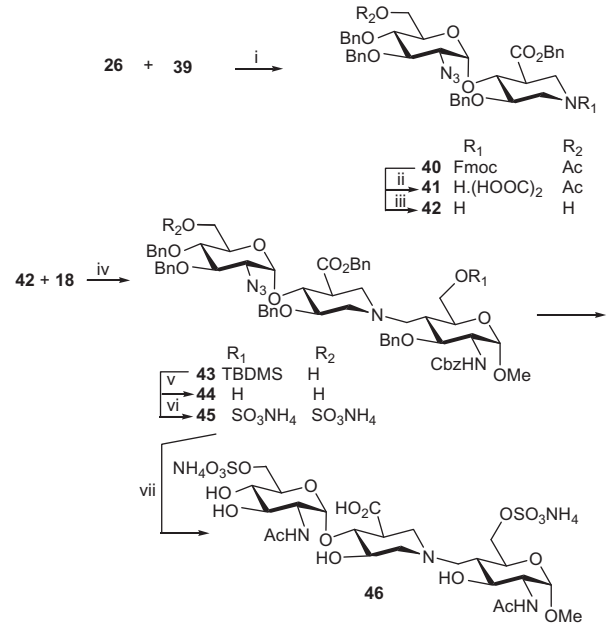


Scheme 3. Reagents and conditions: (i) $NaBH(OAc)_3$, $ClCH_2CH_2Cl$, rt, 1.5 h (81%); (ii) EtOH, NH_4OH, $Pd(OH)_2$/C (20%), H_2, rt, 3 h (98%); (iii) py, 0 °C, Ac_2O, rt, 3 h, (90%); (iv) AcOH, H_2O, 60 °C, 3 h, (94%); (v) (a) $SO_3{\cdot}NMe_3$, DMF, 45 °C, 3 h; (b) MeOH, 15 min, rt (91%); (vi) NaOH, MeOH, H_2O rt, 20 h (76%); (vii) NaOMe, MeOH, rt, 24 h (79%).

The galactosamine derivative **11**[29] was converted to the 4,6-*O*-benzylidene compound **12** and O-benzylated under mild conditions to give **13**. Removal of the benzylidene and silylation of the primary hydroxy group then afforded the D-*galacto*-alcohol **15**. After conversion into the triflate **16** this material was treated with tetrabutylammonium cyanide under argon in dichloromethane at room temperature for two days to give the D-*gluco*-nitrile **17**. That configurational inversion at C-4 occurred in this reaction was established from the relevant vicinal ^1H–^1H NMR coupling constants of compounds **16** and **17**. The $J_{3,4}$ and $J_{4,5}$ values for the former are 2.5 and <1 Hz, respectively, indicating that H-4 is equatorially oriented as is required for D-galactopyranoid compounds in the $^4C_1$ conformation. The $J_{2,3}$ value of 10.5 Hz for



Scheme 4. Reagents and conditions: (i) TBDMSOTf, CH_2Cl_2, −20 °C, 25 min (65%); (ii) HCO_2H, THF, H_2O, 60 °C, 5 h (78%); (iii) NaOH, H_2O, MeOH, rt, 5 h (81%); (iv) SO_3·NMe_3, DMF, 50 °C, 24 h (77%); (v) $Pd(OH)_2$/C (20%), EtOH, NH_4OH, H_2, 17 h, rt (76%); (vi) Ac_2O, MeOH, H_2O, rt, 3 h (78%); (vii) SO_3·NMe_3, Na_2CO_3, H_2O, rt, 3 d (66%).



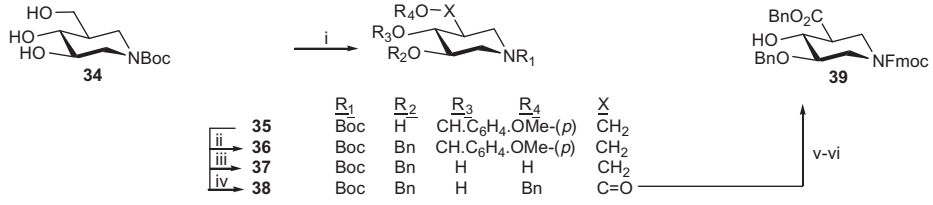
Scheme 6. Reagents and conditions: (i) TBDMSOTf, CH_2Cl_2, sieves, −20 °C, 25 min (64%); (ii) (a) morpholine, 18 °C, 35 min; (b) $(CO_2H)_2$·$2H_2O$ (57% from compound **38**); (iii) LiOH, H_2O, THF, 4 °C, 17 h (84%); (iv) $NaBH(OAc)_3$, $ClCH_2CH_2Cl$, rt, 18 h (82%); (v) (a) HCO_2H, THF, H_2O, 60 °C, 3 h; (b) NH_4OH, MeOH, 20 °C, 30 min (91%); (vi) SO_3·NMe_3 DMF, 45 °C, 16 h (26%); (vii) (a) $Pd(OH)_2$/C (20%), EtOH, H_2O, NH_4OH, 20 °C, 24 h (64% from **43**); (b) Ac_2O, H_2O, MeOH, $NaHCO_3$, rt, 18 h. (30%).

compound **16** confirms this conclusion. Contrastingly, the $J_{3,4}$ and $J_{4,5}$ values for cyano compound **17** are both 9.5 Hz indicating that H-4 is axially oriented and that the compound has the D-*gluco* configuration and exists in the $^4C_1$ conformation. Selective reduction of the nitrile to the corresponding imine was effected using DIBAH at −78 °C and after hydrolytic workup the desired aldehyde **18** was obtained.

Reductive amination of **18** with pyrrolidine **6** afforded the disaccharide mimic **19** (Scheme 3) in good yield. Zemplen deacetylation then gave the disaccharide glycosyl acceptor **25**. In addition, compound **19** was converted into the sulfated disaccharide mimic **24** on the basis that it might show heparanase inhibitor properties. Hydrogenolysis of **19** followed by acetylation produced the triacetate **21** which after desilylation, sulfation and then saponification afforded **24**.

Condensation of the disaccharide acceptor **19** with the known trichloroacetimidate donor **26**[30] gave the trisaccharide analogue **27** (Scheme 4). The α anomeric configuration of the new glycosidic bond was determined from the small $J_{1,2}$ coupling constant (3.5 Hz) while the large $J_{2,3}$ and $J_{3,4}$ values (9.0 and 9.2 Hz, respectively) showed that the pyranoside remained in the $^4C_1$ conformation. Desilylation of **27** followed by basic hydrolysis then gave carboxylic acid diol **29**. This was converted into the disulfate **30** which was deprotected by hydrogenolysis to give the trisaccharide analogue **31**. The two amine groups were then separately acetylated and sulfated to give the trisaccharide analogue targets **32** and **33**.

The other sulfated trisaccharide analogue target **46** has an isofagomine derivative as the oxacarbenium ion mimic. This required the synthesis of the oxidized isofagomine derivative **39** (Scheme 5) to be used as a glycosyl acceptor. The *N*-Boc-protected isofagomine derivative **34** (itself derived from isofagomine[31]) was converted into the 4,6-*O*-*p*-methoxybenzylidene derivative **35** followed by benzylation of the remaining hydroxy group and hydrolysis of the benzylidene acetal to give **37**. Selective oxidation of the primary hydroxy group was followed by benzyl ester formation to give **38**. Attempted glycosylation of this *N*-Boc-protected acceptor **38** with the trichloroacetimidate donor **26** used previously (Scheme 4) was unsuccessful due to loss of the Boc group. Replacement of the Boc by the Fmoc-protecting group afforded the glycosyl acceptor **39** and glycosylation of this acceptor with the trichloroacetimidate donor **26** was successful affording disaccharide analogue **40** which was characterized after removal of the Fmoc group as the oxalic acid salt **41**. The α configuration of the



Scheme 5. Reagents and conditions: (i) $(MeO)_2HC$·C_6H_4·OMe-(*p*), TsOH, DMF, evaporation 33 °C, 1.5 h (83%); (ii) (a) BnBr, NaH, THF, (b) NaI, H_2O, 65 °C, 2 h, (65%); (iii) H^+ resin, MeOH, 20 °C, 2 h, (95%); (iv) (a) TEMPO, Bu_4NBr, CH_2Cl_2, $Ca(OCl)_2$, $NaHCO_3$, H_2O, 5 °C, 1.5 h; (b) BnBr, DMF, $KHCO_3$, MeOH, 20 °C, 17 h (42%); (v) TFA, CH_2Cl_2, rt, 1 h; (vi) FmocCl, CH_2Cl_2, H_2O, Na_2CO_3, $NaHCO_3$, rt, 16 h.

new glycosidic bond in **41** was again established by the small $J_{1,2}$ coupling constant (3.5 Hz) while the large $J_{2,3}$ and $J_{3,4}$ values (10 and 9 Hz, respectively) showed that the pyranoside remained in the $^4C_1$ conformation.

The desired trisaccharide structure was generated by the reductive coupling of free amine **42** with the branched aldehyde **18** afforded trisaccharide analogue **43**. Desilylation was effected with formic acid followed by mild base treatment to remove traces of formate esters affording diol **44**. Then sulfation of the hydroxy groups gave compound **45** which was globally deprotected by hydrogenolysis and then N-acetylated to give the target **46**. We found that the corresponding *N*-sulfate of **46** proved too unstable for characterization.

3. Biochemical results

Human heparanase was cloned and recombinant active enzyme produced in cell culture and purified. The enzyme activity was measured with and without the potential inhibitors using HPLC analysis of a fluorescent substrate. Compounds **7–10, 24, 25, 32, 33** and **46** were tested but none demonstrated any observable inhibition at a concentration of 500 μM. The known inhibitor suramin was used as a control. Details are provided in Section 5.

4. Conclusions

We have synthesized mono- di- and trisaccharide azasugar derivatives that mimic a putative oxacarbenium ion transition state of the heparanase-catalyzed hydrolysis of HS. None showed any inhibitory activity up to a concentration of 500 μM suggesting that either the transition state of the reaction does not involve oxacarbenium ion character or the trisaccharide mimic is too small to induce recognition by the enzyme.

5. Experimental

5.1. General methods

Melting points were measured on a Reichert hot stage microscope and are uncorrected. Optical rotations were determined with a Perkin–Elmer 214 polarimeter and are in units of 10^{-1} deg cm^2 g^{-1}. ^{1}H NMR spectra were obtained at either 300 or 500 MHz and are referenced to TMS. Coupling constants (*J*) are reported to the nearest 0.5 Hz. ^{13}C NMR spectra were recorded at 75 or 125 MHz. Electro-spray ionization (ESI) mass spectra were recorded on a PerSeptive Biosystems Mariner time of flight mass spectrometer run in either positive- or negative-ion mode (as indicated by the charge on the charged species). Elemental analyses were carried out at the Campbell Microanalytical Laboratory, University of Otago, Dunedin, New Zealand. TLC was performed on aluminium-backed Silica Gel 60 F254 (E. Merck) with detection by UV absorption and/or by heating after dipping in $(NH_4)_6Mo_7O_{24}{\cdot}6H_2O$ (5 g) and $Ce(SO_4)_2$ (100 mg) in aqueous H_2SO_4 (100 mL, 5%). Flash column chromatography was performed on Scharlau silica gel (40–60 μm). Organic solutions were dried over $MgSO_4$.

5.1.1. (3*R*,4*R*)-3-Acetoxy-1-*tert*-butoxycarbonyl-4-(trityloxymethyl)pyrrolidine (2)

Boc-protected amine (**1**)[28] (1.5 g, 6.9 mmol) in dry pyridine (30 mL) was treated with recrystallized trityl chloride (3.85 g, 13.81 mmol). The reaction mixture was stirred at rt for 48 h, Ac_2O (0.846 g, 8.28 mmol) was added and after stirring for 4 h at rt the solution was taken to dryness. EtOAc was added and the mixture was washed with water (×2), dried and concentrated. Chromatography (EtOAc/hexanes 1:4) gave trityl derivative **2** (3.05 g, 88%) as a white amorphous solid. ^{1}H NMR ($CDCl_3$) δ 7.39–7.43 (m, 5H), 7.24–7.31 (m, 10H), 5.1–5.14 (m, 1H), 4.08–4.15 (m, 2H), 3.23–3.64 (m, 2H), 3.05–3.13 (m, 2H), 2.48–2.52 (m, 1H), 2.02 (s, 3H), 1.44 (s, 9H); ^{13}C NMR δ 170.8, 154.7, 144.1, 129.0, 128.2, 127.5, 127.5, 87.2, 79.9, 75.6, 75.0, 63.1, 60.7, 51.3, 50.9, 47.4, 47.0, 44.9, 43.9, 28.9, 21.4. HRMS (ESI) calcd for $C_{31}H_{35}NO_5Na$ (M+Na)$^+$ *m/z* 524.2413, found 524.2394.

5.1.2. (3*R*,4*R*)-3-Acetoxy-1-*tert*-butoxycarbonyl-4-(hydroxymethyl)pyrrolidine (3)

Trityl derivative (**2**) (3 g, 5.98 mmol) was dissolved in EtOH (50 mL) and treated with palladium hydroxide on carbon (200 mg, 20% Pd, moisture ca. 60%). The mixture was stirred for 2 h under hydrogen at rt and atmospheric pressure. The catalyst was filtered off and washed with abs. EtOH (15 mL), and the filtrate and washings were taken to dryness. Chromatography of the residue (EtOAc/hexanes 1:2) furnished alcohol **3** (1.5 g, 97%) as a clear syrup. ^{1}H NMR ($CDCl_3$) δ 5.13–5.17 (m, 1H), 4.08–4.15 (m, 2H), 3.65–3.71 (m, 2H), 3.3–3.6 (m, 2H), 2.4–2.48 (m, 1H), 2.07 (s, 3H), 1.45 (s, 9H); ^{13}C NMR δ 171.2, 154.9, 80.1, 75.2, 74.3, 62.1, 60.8, 51.2, 50.6, 46.8, 46.6, 45.9, 28.8, 21.4. HRMS (ESI) calcd for $C_{12}H_{21}NO_5Na$ (M+Na)$^+$ *m/z* 282.1317, found 282.1306.

5.1.3. (3*S*,4*R*)-4-Acetoxy-1-(*tert*-butoxycarbonyl)pyrrolidine-3-carboxylic acid (4)

Alcohol (**3**) (0.7 g, 2.7 mmol) in dry DMF (15 mL) was treated with pyridinium dichromate (5 g, 13.2 mmol). Molecular sieves (1 g) were added, the reaction mixture was stirred at rt for 20 h, diluted with EtOAc and filtered through a pad of silica gel. Concentration afforded acid **4**, 0.634 g, (86%) as a syrup which was used in the next reaction without further purification. ^{1}H NMR ($CDCl_3$) δ 8.35–8.43 (m, 1H), 5.2–5.4 (m, 1H), 4.09–4.2 (m, 2H), 3.05–3.1 (m, 1H), 2.01 (s, 3H), 1.39 (s, 9H); ^{13}C NMR δ 173.8, 169.3, 153.4, 79.4, 73.5, 72.8, 59.5, 50.1, 49.6, 47.6, 46.7, 45.6, 27.4, 19.9. HRMS (ESI) calcd for $C_{12}H_{19}NO_6Na$ (M+Na)$^+$ *m/z* 296.1110, found 296.1101.

5.1.4. Methyl (3*S*,4*R*)-4-acetoxy-(1-*tert*-butoxycarbonyl)pyrrolidine-3-carboxylate (5)

Acid **4** (0.634 g, 2.32 mmol) in dry DMF (25 mL) was treated with MeI (0.433 mL, 6.96 mmol) and $KHCO_3$ (0.697 g, 6.96 mmol). The reaction mixture was stirred at rt for 20 h and concentrated to dryness. The residue was dissolved in EtOAc, filtered through a microfibre filter and the filtrate was concentrated to dryness. Chromatography (EtOAc/hexanes 1:4) gave methyl ester **5** (0.6 g, 90%) as a clear syrup. ^{1}H NMR ($CDCl_3$) δ 5.3–5.6 (m, 1H), 4.35–4.22 (m, 2H), 3.66 (s, 3H), 3.28–3.4 (m, 2H), 3.03–3.08 (m, 1H), 2.01 (s, 3H), 1.39 (s, 9H); ^{13}C NMR δ 170.4, 169.2, 153.1, 135.9, 78.9, 73.6, 72.8, 51.5, 50.8, 49.9, 49.4, 47.7, 46.7, 45.7, 45.5, 27.4, 19.9. HRMS (ESI) calcd for $C_{13}H_{21}NO_6Na$ (M+Na)$^+$ *m/z* 310.1291, found 310.1263.

5.1.5. Methyl (3*S*,4*R*)-4-acetoxypyrrolidine-3-carboxylate (6)

Methyl ester **5** (0.386 g, 1.344 mmol) in dry CH_2Cl_2 (5 mL) was treated with HCl in dioxane (4 M, 3 mL). The reaction mixture was stirred at rt for 2 h and concentrated to dryness. Chromatography (EtOAc/MeOH 4:1) gave the amine **6**, 0.249 g (99%) as a clear syrup. ^{1}H NMR (MeOH-d_4) δ 5.46–5.48 (m, 1H), 4.68 (s, 3H), 3.6–3.66 (m, 2H), 3.25–3.49 (m, 3H), 2.02 (s, 3H); ^{13}C NMR δ 172.1, 171.8, 75.9, 53.9, 51.6, 48.9, 47.8, 21.2. HRMS (ESI) calcd for $C_8H_{14}NO_4$ (M+H)$^+$ *m/z* 188.0923, found 188.0914.

5.1.6. (3*S*,4*R*)-4-Hydroxypyrrolidine-3-carboxylic acid (7)

Amine **6** (46 mg, 246 μmol) in MeOH (2 mL) and water (1 mL) was cooled to 0 °C and treated with NaOH solution (aq, 2 M,

300 μL). The reaction mixture was stirred at rt for 20 h and concentrated to dryness. Chromatography (CH_2Cl_2/MeOH/NH_4OH 5:4:1) furnished acid **7** (33 mg, 93%) as a clear syrup. ^1H NMR (D_2O) δ 3.56–3.59 (m, 3H), 3.37–3.43 (m, 2H), 2.99–3.04 (m, 1H); ^{13}C NMR δ 177.7, 73.5, 54.0, 52.1, 47.2. HRMS (ESI) calcd for $C_5H_{10}NO_3$ $(M+H)^+$ *m/z* 132.0666, found 132.0652.

5.1.7. (3*S*,4*R*)-4-Hydroxy-1-(4-nitrobenzyl)pyrrolidine-3-carboxylic acid (8)

Amine (**6**) (23 mg, 123 μmol) in dry 1,2-dichloroethane (2 mL) was treated with *p*-nitrobenzaldehyde (22 mg, 147 μmol) followed by $NaBH(OAc)_3$ (33 mg, 142 μmol), and the reaction mixture was stirred at rt under argon for 1.5 h. The reaction solution was diluted with CH_2Cl_2 (13 mL), washed with water and satd $NaHCO_3$ solution, dried and taken to dryness. The residue, dissolved in MeOH (2 mL) and water (1 mL), was cooled to 0 °C and treated with NaOH solution (2 M, 200 μL), stirred at rt for 5 h and concentrated to dryness. Chromatography [CH_2Cl_2/MeOH/NH_4OH (26%) 7:2:0.5] furnished nitrobenzyl derivative **8**, (24 mg. 73%) as a clear syrup. ^1H NMR (DMSO-d_6) δ 8.22–8.26 (m, 2H), 7.62–7.66 (m, 2H), 4.4–4.45 (m, 1H), 2.89–2.95 (m, 2H), 2.63–2.79 (m, 3H), 2.48–2.57 (m, 2H); ^{13}C NMR δ 175.2, 147.5, 146.8, 129.7, 123.7, 73.1, 62.4, 58.6, 55.9, 52.9. HRMS (ESI) calcd for $C_{12}H_{15}N_2O_5$ $(M+H)^+$ *m/z* 267.0986, found 267.0931.

5.1.8. (3*S*,4*R*)-4-Hydroxy-1-(4-methoxybenzyl)pyrrolidine-3-carboxylic acid (9)

Amine **6** (28 mg, 150 μmol) in dry 1,2-dichloroethane (2 mL) was treated with *p*-anisaldehyde (20 mg, 179 μmol). $NaBH(OAc)_3$ (40 mg, 190 μmol) was added and the reaction mixture was stirred at rt under argon for 2 h. The reaction solution was diluted with CH_2Cl_2 (15 mL), washed with water (5 mL) and $NaHCO_3$ solution (5 mL), dried and taken to dryness. The residue was dissolved in MeOH (2 mL) and water (1 mL) and the solution was cooled to 0 °C, treated with NaOH solution (2 M, 300 μL) and stirred at rt for 4 h and concentrated to dryness. Chromatography (CH_2Cl_2/MeOH/NH_4OH 7:2:0.5) furnished methoxybenzyl derivative **9** (29 mg, 77%) as a clear syrup. ^1H NMR (MeOH-d_4) δ 7.32–7.35 (m, 2H), 6.86–6.89 (m, 2H), 5.51–5.53 (m, 1H), 4.15–4.30 (m, 2H), 3.7 (s, 3H), 3.45–3.50 (m, 2H), 3.3–3.36 (m, 2H), 2.88–3.09 (m, 1H); ^{13}C NMR δ 177.8, 162.6, 133.5, 124.8, 115.9, 74.9, 61.5, 60.5, 56.7, 56.2, 48.8. HRMS (ESI) calcd for $C_{13}H_{18}NO_4$ $(M+H)^+$ *m/z* 252.1239, found 252.1240.

5.1.9. (3*S*,4*R*)-1-(4-Methoxybenzyl)-4-(sulfoxy)pyrrolidine-3-carboxylic acid (10)

Methoxybenzyl derivative (**9**) (20 mg, 80 μmol) in dry DMF (1.5 mL) was treated with $SO_3{\cdot}NMe_3$ (55 mg, 0.4 mmol). The reaction mixture was heated at 45 °C for 20 h, MeOH (0.5 mL) was added and the mixture was stirred for 15 min and concentrated to dryness. Chromatography [CH_2Cl_2/MeOH/NH_4OH (26%) 7:2:1] afforded sulfate **10** (23 mg, 87%) as a clear syrup. ^1H NMR (D_2O) δ 7.43–7.46 (d, *J* = 8.6 Hz, 2H), 7.03–7.06 (d, *J* = 8.6 Hz, 2H), 5.22–5.23 (m, 1H), 4.34–4.43 (m, 2H), 3.83 (s, 3H), 3.59–3.66 (m, 2H), 3.34–3.42 (m, 2H), 3.23–3.32 (m, 1H); ^{13}C NMR δ 175.9, 160.6, 132.6, 122.7, 115.1, 79.7, 59.1, 58.1, 55.9, 55.0, 52.1. HRMS (ESI) calcd for $C_{13}H_{18}NO_4$ $(M-SO_3+H)^+$ *m/z* 252.1236, found 252.1240; calcd for $C_{13}H_{16}NO_7S$ $(M-H)^-$ *m/z* 330.0642, found 330.0656.

5.1.10. Methyl 4,6-*O*-benzylidene-2-*N*-(benzyloxycarbonyl)-amino-2-deoxy-α-D-galactopyranoside (12)

To methyl 2-*N*-(benzyloxycarbonyl)amino-2-deoxy-α-D-galactopyranoside[29] (**11**, 16.4 g, 50 mmol), DMF (174 mL), benzaldehyde dimethylacetal (32 mL, 213 mmol) and TsOH (0.56 g) were added. The solution was subjected to rotary evaporation at 33 °C for 90 min. Et_3N (2 mL) was added and the solvent was evaporated under high vacuum at 60 °C. The product was dissolved in CH_2Cl_2 (750 mL) and washed with HCl (0.5 M, 750 mL) followed by $NaHCO_3$ solution (satd, aq). The solution was dried and concentrated and the product was crystallized from CH_2Cl_2/hexanes to give title compound **12** (14.62 g, 70%) as a white solid: mp 194.5–195.0 °C; $[\alpha]_D$ +118 (*c* 0.9, EtOAc). ^1H NMR ($CDCl_3$) δ 7.30–7.55 (m, 10H), 5.69 (s, 1H), 5.10 (m, 3H), 4.85 (d, *J* = 3.5 Hz, 1H), 4.28 (dd, *J* = 12.5, 1.5 Hz, 1H), 4.22 (d, *J* = 3 Hz, 1H), 4.16 (br dd, *J* = 3, 10 Hz, 1H), 4.07 (dd, *J* = 12.5, 1.5 Hz, 1H), 3.81 (dt, *J* = 11, 11, 3.5 Hz, 1H), 3.66 (m, *J* = 1.5,1.5 Hz, 1H), 3.38 (s, 3H), 2.58 (d, *J* = 11 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 157.3, 137.9, 136.7, 126.7–129.6, 101.7, 100.1, 75.9, 69.7, 69.5, 67.5, 63.2, 55.9, 52.6. HRMS (ESI) calcd for $C_{22}H_{26}NO_7$ $[M+H]^+$ *m/z* 416.1709, found 416.1714. Anal. Calcd for $C_{22}H_{25}NO_7{\cdot}0.66H_2O$: C, 61.82; H, 6.21; N, 3.28. Found: C, 61.59; H, 6.26; N, 3.22.

5.1.11. Methyl 3-*O*-benzyl-4,6-*O*-benzylidene-2-*N*-(benzyloxycarbonyl)amino-2-deoxy-α-D-galactopyranoside (13)

Galactoside **12** (10 g, 20 mmol), BaO (20 g, 130 mmol), $Ba(OH)_2{\cdot}8H_2O$ (6.1 g, 20 mmol), benzyl bromide (4.8 mL, 40 mmol) and dry DMF (240 mL) were combined and the mixture was stirred under argon at 25 °C for 22 h. MeOH (50 mL) was added and the mixture was stirred for 1 h after which CH_2Cl_2 (1 L) and H_2O (1 L) were added and the mixture was shaken and filtered. The organic phase was washed twice with water and then dried. After removal of the solvent, the product was crystallized from CH_2Cl_2/hexanes to give the title compound **13** as a white solid (7.8 g, 64%): mp 214–215 °C; $[\alpha]_D$ +158 (*c* 0.1, EtOAc). ^1H NMR ($CDCl_3$) δ 7.2–7.6 (m, 15H), 5.49 (s, 1H), 5.15 (d, *J* = 12.5 Hz, 1H), 5.06 (d, *J* = 12.5 Hz, 1H), 4.90 (br s, 1H), 4.80 (br d, *J* = 9.5 Hz, 1H), 4.68 (d, *J* = 12.5 Hz, 1H), 4.59 (d, *J* = 12.5 Hz, 1H), 4.44 (ddd, *J* = 11, 9.5, 3.5 Hz, 1H), 4.25 (dd, *J* = 12.5, 1.5 Hz, 1H), 4.20 (d, *J* = 3 Hz, 1H), 4.01 (dd, *J* = 12.5, 1.5 Hz, 1H), 3.65 (dd, *J* = 3.5, 11 Hz, 1H), 3.54 (br s, 1H), 3.34 (s, 3H); ^{13}C NMR ($CDCl_3$), δ 156.5, 138.7, 138.2, 137.0, 126.7–138.7, 101.3, 100.2, 75.0, 73.4, 71.1, 69.9, 67.2, 63.2, 55.9, 50.6; HRMS (ESI) calcd for $C_{29}H_{32}NO_7$ $[M+H]^+$ *m/z* 506.2179, found 506.2169. Anal. Calcd for $C_{29}H_{31}NO_7$: C, 68.88; H, 6.18; N, 2.77. Found: C, 68.88; H, 6.33; N, 2.77.

5.1.12. Methyl 3-*O*-benzyl-2-*N*-(benzyloxycarbonyl)amino-2-deoxy-α-D-galactopyranoside (14)

Galactoside **13** (6.9 g) was dissolved in MeOH (400 mL) and Amberjet 1200 acid resin (Rohm and Haas, H^+, 50 mL) was added. The mixture was heated under reflux with stirring for 1 h, and the resin was removed by filtration and washed with MeOH (150 mL). The MeOH solutions were combined, Et_3N (1 mL) was added and the solution was concentrated to dryness. The product was crystallized from CH_2Cl_2/hexanes to give the title compound **14** as a white solid (4.8 g, 84%): mp 157–158 °C; $[\alpha]_D$ +107 (*c* 1, EtOAc). ^1H NMR ($CDCl_3$) δ 7.23–7.41 (m, 10H), 5.19 (d, *J* = 12 Hz, 1H), 5.07 (d, *J* = 12 Hz, 1H), 4.81 (br d, *J* = 9.5 Hz, 1H), 4.76 (d, *J* = 3.5 Hz, 1H), 4.66 (d, *J* = 12 Hz, 1H), 4.52 (d, *J* = 12 Hz, 1H), 4.24 (br m, 1H), 4.07 (d, *J* = 2.5 Hz, 1H), 3.96 (dd, *J* = 6, 11.5 Hz, 1H), 3.81 (dd, *J* = 4.5, 11 Hz, 1H), 3.72 (dd, *J* = 4.5, 6 Hz, 1H), 3.51 (dd, *J* = 3, 10.5 Hz, 1H), 3.33 (s, 3H), 2.67 (br s, 1H), 2.28 (br s, 1H); ^{13}C NMR ($CDCl_3$) δ 156.5, 137.8, 136.8, 128.1–128.9, 99.6, 76.7, 71.8, 69.9, 67.5, 67.3, 63.4, 55.7, 50.5. HRMS (ESI) calcd for $C_{22}H_{28}NO_7$ $[M+H]^+$ *m/z* 418.1866, found: 418.1884. Anal. Calcd for $C_{22}H_{27}NO_7$: C, 63.30; H, 6.52; N, 3.36. Found: C, 63.45; H, 6.62; N, 3.33.

5.1.13. Methyl 3-*O*-benzyl-2-*N*-(benzyloxycarbonyl)amino-6-*O*-*tert*-butyldimethylsily-4-*C*-cyano-2,4-dideoxy-α-D-glucopyranoside (17)

Galactoside derivative **14** (4.60 g, 11.0 mmol) and imidazole (3.68 g, 54 mmol) were dried together by the addition of dry MeCN

followed by its evaporation. The mixture was dissolved under argon in CH_2ClCH_2Cl (150 mL) and TBDMSCl (3.27 g, 21.7 mmol) was added. The mixture was heated at 50 °C with stirring for 85 min (reaction complete in 70 min, tlc), cooled and concentrated to a syrup. The product was taken up in CH_2Cl_2 (500 mL), washed with brine (×2), then water, dried ($MgSO_4$) and purified by column chromatography (EtOAc/hexanes, 2:3) to give methyl 3-*O*-benzyl-2-*N*-(benzyloxycarbonyl)amino-6-*O*-*tert*-butyldimethylsilyl-2-deoxy-α-D-galactopyranoside (**15**) as a slightly impure syrup (5.73 g, 98%). ^{1}H NMR ($CDCl_3$) δ 7.2–7.4 (m, 10H), 5.17 (d, *J* = 12 Hz, 1H), 5.05 (d, *J* = 12 Hz, 1H), 4.86 (d, *J* = 9.7 Hz, 1H), 4.70 (d, *J* = 3 Hz, 1H), 4.65 (d, *J* = 12 Hz, 1H), 4.52 (d, *J* = 12 Hz, 1H), 4.15–4.30 (dt, *J* = 3, 10, 10 Hz, 1H), 4.04 (d, *J* = 2.5 Hz, 1H), 3.78 (dd, *J* = 6, 10 Hz, 1H), 3.66 (t, *J* = 6 Hz, 1H), 3.48 (dd, *J* = 10.5, 3.5 Hz, 1H), 3.30 (s, 3H), 2.62 (br s, 1H), 0.89 (s, 9H), 0.07 (s, 6 H); ^{13}C NMR ($CDCl_3$) δ 156.6, 138.2, 137.0, 128–129, 99.5, 77.2, 71.7, 70.7, 67.2, 66.1, 62.9, 55.4, 50.7, 26.3, −5.0, −5.0. The slightly impure galactoside **15** (4 g, 9.6 mmol) was dissolved in CH_2Cl_2 (67 mL) and pyridine (40 mL), the solution was cooled and stirred in an ice/water/methanol bath at −10 °C. Triflic anhydride (4.84 mL, 29 mmol) was added dropwise with stirring over 10 min, the stirring was continued for 10 min at −10 °C then for 1 h at 20 °C. The mixture was concentrated at 40 °C and residual reagents were evaporated by azeotropic distillation with toluene. CH_2Cl_2 (500 mL) was added to the residue and the solution was washed with cold HCl (M, 500 mL), then with cold water followed by cold $NaHCO_3$ (cold, satd, 500 mL). The solution was dried and concentrated. Crystallization of the residue from CH_2Cl_2/hexanes yielded methyl 3-*O*-benzyl-2-*N*-(benzyloxycarbonyl)amino-6-*O*-*tert*-butyldimethylsilyl-2-deoxy-4-*O*-trifluoromethanesulfonyl-α-D-galactopyranoside (**16**) as a white solid (4.69 g, 94%). ^{1}H NMR ($CDCl_3$) δ 7.23–7.39 (m, 10H), 5.37 (d, *J* = 2.5 Hz, 1H), 5.11 (br s, 2H), 4.82 (d, *J* = 12 Hz, 1H), 4.74 (br s, 1H), 4.65 (d, *J* = 8 Hz, 1H), 4.44 (d, *J* = 12 Hz, 1H), 4.12–4.25 (br m, 1H), 3.83 (t, *J* = 7 Hz, 1H), 3.66–3.76 (m, 2H), 3.57 (dd, *J* = 2.5, 10.5 Hz, 1H), 3.30 (s, 3H), 0.90 (s, 9H), 0.08 (s, 6H). Dry CH_2Cl_2 (43 mL) was added to a mixture of the crude triflate (**16**) (4.32 g) and dry tetrabutylammonium cyanide (4.44 g, 16.5 mmol) under Ar. The resulting solution was stirred for 2 d whereupon it was diluted with CH_2Cl_2 (400 mL) and washed with water (×3). After drying ($MgSO_4$) and evaporation of the solvent, the product was purified by flash column chromatography (EtOAc/hexanes 1:3 then 2:3) and crystallized from EtOAc/hexanes to give title cyano-D-*gluco*-compound **17** (1.9 g, 3.58 mmol, 54% from **16**) as a white solid: mp 98 °C; $[\alpha]_D$ +69 (*c* 0.7, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 7.25–7.35 (m, 10H), 5.14 (d, *J* = 12 Hz, 1H), 5.07 (d, *J* = 12 Hz, 1H), 4.87 (d, *J* = 11 Hz, 1H), 4.81 (br d, *J* = 8.5 Hz, 1H), 4.67–4.71 (m, 2H), 3.77–3.94 (m, 5H), 3.35 (s, 3H), 2.99 (dd, *J* = 9.5, 9.5 Hz, 1H), 0.91 (s, 9H), 0.10 (s, 3H), 0.09 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 156.2, 137.5, 136.6, 128.4–129.0, 118.4, 99.7, 76.3, 69.8, 67.6, 63.5, 55.8, 54.8, 35.8, −5.1, −5.0. HRMS (ESI) calcd for $C_{29}H_{41}N_2O_6Si$ $[M+H]^+$ *m/z* 541.2734, found 541.2731. Anal. Calcd for $C_{29}H_{40}N_2O_6Si$: C, 64.41; H, 7.46; N, 5.18. Found: C, 64.38; H, 7.58; N, 5.05.

5.1.14. Methyl 3-*O*-benzyl-2-*N*-(benzyloxycarbonyl)amino-6-*O*-*tert*-butyldimethylsilyl-2,4-dideoxy-4-*C*-formyl-α-D-glucopyranoside (18)

The *C*-cyano compound **17** (0.89 g, 1.65 mmol) was dried azeotropically with toluene and dissolved in dry CH_2Cl_2 (30 mL). After the solution was cooled to −78 °C, DIBAL-H (20% solution in toluene, 3.43 mL, 4.1 mmol) was added over 10 min. The solution was then stirred for 150 min after which potassium hydrogen tartrate buffer (M, pH 6.25, 60 mL) was added, and the mixture was stirred at 20 °C for 15 min. CH_2Cl_2 (300 mL) was added and the organic phase was washed with $NaHCO_3$ solution (satd, aq ×2) and dried. Removal of the solvent and purification of the residue by flash column chromatography (EtOAc/hexanes, 20:80 and 40:60) gave title compound **18** as a white solid (0.531 g, 0.98 mmol, 59%). The product was recrystallized from a small volume of hexanes: mp 73–75 °C; $[\alpha]_D$ +43.1 (*c* 1, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.79 (d, *J* = 2.5 Hz, 1H), 7.16–7.36 (m, 10H), 5.14 (d, *J* = 12 Hz, 1H), 5.06 (d, *J* = 12 Hz, 1H), 4.95 (br d, *J* = 7 Hz, 1H), 4.68 (br s, 1H), 4.55 (d, *J* = 11 Hz, 1H), 4.48 (d, *J* = 11 Hz, 1H), 3.97–4.05 (m, 2H), 3.91 (ddd, *J* = 4.5, 5.5, 10.5 Hz, 1H), 3.74 (dd, *J* = 10.5, 4.5 Hz, 1H), 3.66 (dd, *J* = 10.5, 5.5 Hz, 1H), 3.35 (s, 3H), 3.01 (br m, 1H), 0.88 (s, 9H), 0.05 (s, 3H), 0.04 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 200.8, 156.3, 138.0, 136.7, 128.2–128.9, 99.7, 75.9, 74.4, 70.2, 67.4, 64.7, 57.7, 55.6, 55.2, 26.2, −5.2, −5.1. Anal. Calcd for $C_{29}H_{41}NO_7Si$: C, 64.06; H, 7.60; N, 2.58. Found: C, 63.79; H, 7.61; N, 2.59.

5.1.15. Methyl 4-*C*-[(*3S,4R*)-4-acetoxy-3-(methoxycarbonyl)-pyrrolidin-1-yl]methyl-3-*O*-benzyl-2-(benzyloxycarbonyl)-amino-6-*O*-*tert*-butyldimethylsilyl-2,4-dideoxy-α-D-glucopyranoside (19)

Amine (**6**) (166 mg, 887 μmol) in dry 1,2-dichloroethane (10 mL) was treated with aldehyde **18** (530 mg, 975 μmol) followed by $NaBH(OAc)_3$ (207 mg, 975 μmol), and the reaction mixture was stirred at rt under argon for 1.5 h. The solution was diluted with CH_2Cl_2, washed with water and satd $NaHCO_3$ solution, dried and concentrated. Chromatography (EtOAc/hexanes 1:4) furnished the N-Cbz-protected disaccharide analogue **19** (564 mg, 81%) as a clear syrup. ^{1}H NMR ($CDCl_3$) δ 7.15–7.22 (m, 10H), 5.26–5.28 (m, 1H), 4.95–5.03 (m, 2H), 4.44–4.53 (m, 3H), 3.98–4.05 (m, 2H), 3.74 (s, 3H), 3.69–3.71 (m, 1H), 3.61–3.65 (m, 1H), 3.27 (s, 3H), 3.06–3.08 (m, 1H), 2.90–2.91 (m, 1H), 2.7–2.72 (m, 1H), 2.46–2.6 (m, 2H), 2.31–2.41 (m, 1H), 2.14–2.2 (m, 2H), 1.96 (s, 3H), 1.81–1.93 (m, 1H), 0.83 (s, 9H), 0.0 (s, 6H); ^{13}C NMR δ 171.6, 169.5, 154.9, 137.2, 135.5, 127.5, 127.4, 127.3, 127.2, 127.1, 126.9, 126.7, 126.6, 98.2, 75.7, 74.8, 72.2, 69.4, 65.8, 63.7, 59.8, 58.0, 55.5, 53.9, 53.8, 52.1, 51.1, 49.1, 40.5, 24.9, 21.6, 0.0. HRMS (ESI) calcd for $C_{37}H_{55}N_2O_{10}Si$ $(M+H)^+$ *m/z* 715.3631, found 715.3645.

5.1.16. Methyl 4-*C*-[(*3S,4R*)-4-acetoxy-3-(methoxycarbonyl)-pyrrolidin-1-yl]methyl-2-amino-6-*O*-*tert*-butyldimethylsilyl-2,4-dideoxy-α-D-glucopyranoside (20)

N-Cbz-protected disaccharide analogue 19 (170 mg, 238 μmol) in EtOH (9 mL), containing NH_4OH (26%, 1.5 mL), was treated with $Pd(OH)_2$ on carbon (20 mg, 20%). The reaction mixture was stirred for 3 h under hydrogen at ambient temperature and pressure, the catalyst was filtered off and washed with EtOH (2 mL), the filtrate and washings were taken to dryness and chromatography of the residue (EtOAc) gave amino-disaccharide analogue **20** (114 mg, 98%) as a clear syrup. ^{1}H NMR ($CDCl_3$) δ 5.28–5.31 (m, 1H), 4.62 (d, *J* = 3.5 Hz, 1H), 3.66 (s, 3H), 3.6–3.65 (m, 2H), 3.31–3.34 (m, 1H), 3.29 (s, 3H), 3.0–3.06 (m, 2H), 2.93–2.98 (m, 1H), 2.62–2.92 (m, 4H), 2.52–2.61 (m, 2H), 1.99 (s, 3H), 1.85–1.91 (m, 1H), 0.84 (s, 9H), 0.01 (s, 6H); ^{13}C NMR δ 172.7, 170.7, 100.9, 76.3, 76.1, 70.8, 64.2, 60.6, 60.2, 57.5, 57.1, 56.7, 55.4, 52.7, 50.0, 40.9, 26.2, 21.2, 0.0. HRMS (ESI) calcd for $C_{22}H_{43}N_2O_8Si$ $(M+H)^+$ *m/z* 491.2789, found 491.2745.

5.1.17. Methyl 2-acetamido-4-*C*-[(*3S,4R*)-4-acetoxy-3-(methoxycarbonyl)pyrrolidin-1-yl]methyl-3-*O*-acetyl-6-*O*-*tert*-butyldimethylsilyl-2,4-dideoxy-α-D-glucopyranoside (21)

A solution of amino-disaccharide analogue **20** (113 mg, 230 μmol) in dry pyridine (2.5 mL) was cooled to 0 °C, Ac_2O (1 mL) was added and the reaction mixture was stirred at rt for 3 h. The resulting solution was concentrated in vacuo and co-evaporated with toluene to afford a syrup. Chromatography (EtOAc/hexanes 1:1) gave tri-*N,O,O*-acetyl compound (**21**) (119 mg, 90%) as a clear syrup. ^{1}H NMR ($CDCl_3$) δ 5.60–5.63 (m, 1H), 5.25–5.27 (m, 1H), 4.92–4.99 (m, 1H), 4.62 (d, *J* = 3.5 Hz, 1H), 4.04–4.11

(m, 2H), 3.88–3.93 (m, 2H), 3.69–3.74 (m, 1H), 3.64 (s, 3H), 3.46–3.47 (m, 1H), 3.29 (s, 3H), 3.04–3.1 (m, 1H), 2.9–2.91 (m, 1H), 2.64–2.66 (m, 2H), 2.29–2.35 (m, 2H), 1.97 (s, 6H), 1.87 (s, 3H), 0.83 (s, 9H), 0.0 (s, 6H); ^{13}C NMR δ 172.9, 171.9, 170.8, 170.3, 98.8, 76.2, 72.8, 72.0, 64.2, 60.8, 57.0, 55.2, 54.4, 52.9, 52.5, 50.4, 40.2, 26.3, 23.6, 21.3, 21.2, 18.7, 14.5, −4.8. HRMS (ESI) calcd for $C_{26}H_{47}N_2O_{10}Si$ $(M+H)^+$ *m/z* 575.2995, found 575.2982.

5.1.18. Methyl 2-acetamido-4-*C*-[(3*S*,4*R*)-4-acetoxy-3-(methoxycarbonyl)pyrrolidin-1-yl]methyl-3-*O*-acetyl-2,4-dideoxy-α-D-glucopyranoside (22)

Triacetyl derivative (**21**) (93 mg, 162 μmol) in AcOH (3 mL) and water (0.7 mL) was heated at 60 °C for 3 h, the solution was diluted with water and concentrated in vacuo. Chromatography (EtOAc/MeOH 4:1) afforded hydroxy-derivative **22** (70 mg, 94%) as a clear syrup. ^{1}H NMR (MeOH-d_4) δ 5.2–5.22 (m, 1H), 4.92–4.99 (m, 1H), 4.56–4.57 (d, J = 3.5 Hz, 1H), 4.0–4.05 (m, 1H), 3.63–3.69 (m, 2H), 3.61 (s, 3H), 3.53–3.59 (m, 1H), 3.3 (s, 3H), 3.08–3.11 (m, 1H), 2.95–2.95 (m, 1H), 2.66–2.73 (m, 2H), 2.39–2.44 (m, 2H), 1.93 (s, 3H), 1.91 (s, 3H), 1.82 (s, 3H); ^{13}C NMR δ 174.6, 173.9, 173.1, 172.7, 100.5, 77.5, 73.9, 72.8, 64.8, 61.9, 61.5, 57.7, 56.0, 55.4, 54.4, 53.1, 51.5, 50.3, 43.3, 22.9, 21.4, 21.2. HRMS calcd for $C_{20}H_{33}N_2O_{10}$ $(M+H)^+$ *m/z* 461.2140, found 461.2128.

5.1.19. Methyl 2-acetamido-4-*C*-[(3*S*,4*R*)-4-acetoxy-3-(methoxycarbonyl)pyrrolidin-1-yl]methyl-3-*O*-acetyl-2,4-dideoxy-6-*O*-sulfo-α-D-glucopyranoside (23)

Hydroxy-derivative **22** (64 mg, 139 μmol) was treated with $SO_3{\cdot}NMe_3$ complex (96 mg, 0.7 mmol) in dry DMF (3 mL). The reaction mixture was heated at 45 °C for 3 h, MeOH (1 mL) was added and the mixture was stirred for 15 min and concentrated in vacuo. Chromatography (EtOAc/MeOH 4:1) afforded sulfate **23** (68 mg, 91%) as a clear syrup. ^{1}H NMR (MeOH-d_4) δ 5.29–5.31 (m, 1H), 5.1–5.17 (m, 1H), 4.65–4.66 (d, J = 3.5 Hz, 1H), 4.33–4.37 (m, 1H), 4.13–4.19 (m, 2H), 4.09–4.11 (m, 1H), 3.72 (s, 3H), 3.4 (s, 3H), 3.31–3.35 (m, 2H), 3.3–3.31 (m, 1H), 3.1–3.3 (m, 1H), 2.8–2.81 (m, 2H), 2.51–2.53 (m, 2H), 2.04 (s, 3H), 2.0 (s, 3H), 1.92 (s, 3H); ^{13}C NMR δ 174.8, 173.9, 173.2, 172.9, 100.4, 77.7, 73.1, 71.3, 69.6, 61.9, 61.6, 58.1, 56.1, 55.5, 55.0, 54.3, 53.1, 51.6, 42.3, 22.9, 21.5, 21.3. HRMS (ESI) calcd for $C_{20}H_{31}N_2O_{13}S$ $(M-H)^-$ *m/z* 539.1542, found 539.1549.

5.1.20. Methyl 2-*O*-acetamido-2,4-dideoxy-4-*C*-[(3*S*,4*R*)-4-hydroxy-3-(carboxy)pyrrolidin-1-yl]methyl-6-*O*-sulfo-α-D-glucopyranoside (24)

Sulfate **23** (40 mg, 74 μmol) in MeOH (2 mL) and water (1 mL) was cooled to 0 °C and treated with NaOH solution (2 M aq, 150 μL). The reaction mixture was stirred at rt for 20 h and concentrated to dryness. Chromatography [CH_2Cl_2/MeOH/NH_4OH (26%) 6:3:0.5] furnished acid **24** (25 mg, 76%) as a clear syrup. ^{1}H NMR (D_2O) δ 4.88–4.89 (d, J = 3.5 Hz, 1H), 4.81–4.82 (m, 1H), 4.42–4.45 (m, 2H), 4.26–4.3 (m, 2H), 4.04–4.11 (m, 2H), 3.93–3.99 (m, 3H), 3.8–3.87 (m, 4H), 3.7–3.75 (m, 4H), 3.64–3.68 (m, 1H), 3.6–3.63 (m, 2H), 3.38 (s, 3H), 3.15–3.25 (m, 1H), 2.45–2.59 (m, 2H), 2.02 (s, 3H); ^{13}C NMR δ 176.5, 175.1, 101.0, 73.9, 72.4, 70.2, 68.2, 67.9, 67.1, 65.7, 56.0, 55.1, 54.3, 47.9, 22.3. HRMS (ESI) calcd for $C_{15}H_{26}N_2O_{11}S$ $(M-H)^-$ *m/z* 442.1252, found: 442.1248.

5.1.21. Methyl 4-*C*-[(3*S*,4*R*)-3-*O*-benzyl-2-*O*-(benzyloxycarbonyl)* amino-6-*O*-*tert*-butyldimethylsilyl-2,4-dideoxy-4 *C*-[4-hydroxy-3-(methoxycarbonyl)pyrrolidin-1-yl]methyl-α-D-glucopyranoside (25)

N-Cbz-protected disaccharide analogue (**19**) (0.5 g, 0.699 mmol) in dry MeOH (7 mL) was stirred with NaOMe solution (1%, 150 μL) at rt for 4 h. The solution was neutralized with Amberlyst-15 ion-exchange resin and concentrated to dryness. Chromatography (EtOAc/petroleum ether 1:4) afforded alcohol **25** (0.372 g, 79%) as a clear syrup. ^{1}H NMR ($CDCl_3$) δ 7.15–7.23 (m, 10H), 4.95–5.03 (m, 2H), 4.63–4.64 (d, J = 3.5 Hz, 1H), 4.38–4.53 (m, 2H), 4.36–4.37 (m, 1H), 3.91–3.95 (m, 2H), 3.66–3.7 (m, 1H), 3.61 (s, 3H), 3.49–3.51 (m, 1H), 3.4–3.48 (m, 2H), 3.27 (s, 3H), 3.02–3.05 (m, 1H), 2.79–2.81 (m, 1H), 2.65–2.67 (m, 1H), 2.4–2.54 (m, 3H), 1.88–1.97 (m, 1H), 0.82 (s, 9H), 0.0 (s, 6H); ^{13}C NMR δ 172.5, 154.9, 137.2, 135.4, 127.5, 127.3, 127.2, 127.1, 126.9, 126.6, 98.2, 73.0, 72.3, 72.1, 65.9, 63.2, 61.8, 54.8, 54.1, 53.9, 52.2, 51.8, 50.9, 40.4, 24.9, 0.0. HRMS (ESI) calcd for $C_{35}H_{53}N_2O_9Si$ $(M+H)^+$ *m/z* 673.3525, found 673.3487.

5.1.22. Methyl 4-*C*-[(3*S*,4*R*)-4-{6-*O*-acetyl-2-azido-3,4-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl}oxy-3-(methoxycarbonyl)pyrrolidin-1-yl]methyl-3-*O*-benzyl-2-*O*-(benzyloxycarbonyl)amino-6-*O*-*tert*-butyldimethylsilyl-2,4-dideoxy-α-D-glucopyranoside (27)

The acceptor **25** (370 mg, 550 μmol) and the glycosyl donor **26** (503 mg, 880 μmol)[30] were dried together in vacuo. Powdered molecular sieves (4 Å, 300 mg) and CH_2Cl_2 (10 mL) were added and the mixture was stirred for 45 min at rt. After the mixture had been cooled to −20 °C TBDMS triflate (52 μL) was added and stirring was carried out at this temperature for 25 min. $NaHCO_3$ (70 mg) and water (20 μL) were added and the mixture was allowed to warm to 20 °C. CH_2Cl_2 (20 mL) was added and the mixture was filtered through a pad of Celite and, after evaporation of the solvent, the residue was applied directly to a column of silica for flash chromatography. Elution with EtOAc/hexanes/$CHCl_3$ (1:3:3) gave the TBDMS-protected trisaccharide analogue **27** (387 mg, 65%) as a syrup. ^{1}H NMR ($CDCl_3$) δ 7.17–7.32 (m, 20H), 4.96–5.04 (m, 2H), 4.83–4.84 (m, 1H), 4.76–4.8 (m, 3H), 4.63–4.64 (d, J = 3.5 Hz, 1H), 4.49–4.54 (m, 3H), 4.2–4.24 (m, 3H), 3.88–3.94 (m, 3H), 3.6 (s, 3H), 3.41–3.51 (m, 3H), 3.28 (s, 3H), 3.2–3.24 (m, 4H), 2.95–2.96 (m, 2H), 2.43–2.56 (m, 4H), 1.97 (s, 3H), 1.86–1.95 (m, 1H), 0.83 (s, 9H), 0.0 (s, 6H); ^{13}C NMR δ 174.5, 172.3, 157.7, 139.9, 139.5, 139.2, 138.2, 133.8, 133.6, 133.3, 132.9, 130.2, 130.1, 129.8, 129.3, 100.9, 98.8, 85.3, 82.7, 82.3, 81.8, 80.4, 77.1, 76.9, 75.0, 74.8, 71.3, 69.4, 68.6, 68.3, 67.9, 65.9, 64.8, 64.3, 61.8, 60.1, 58.2, 57.3, 56.9, 56.6, 55.3, 54.8, 53.8, 51.8, 43.1, 27.7, 22.5, 0.0. HRMS (ESI) calcd for $C_{57}H_{76}N_5O_{14}Si$ $(M+H)^+$ *m/z* 1082.5157, found 1082.5190.

5.1.23. Methyl 4-*C*-[(3*S*,4*R*)-4-{6-*O*-acetyl-2-azido-3,4-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl}oxy-3-(methoxycarbonyl)pyrrolidin-1-yl]methyl-3-*O*-benzyl-2-*O*-(benzyloxycarbonyl)amino-2,4-dideoxy-α-D-glucopyranoside (28)

TBDMS-protected trisaccharide analogue **27** (190 mg, 176 μmol) in THF (6 mL) was dissolved in formic acid (3 mL) and water (1 mL) and the solution was heated at 60 °C for 5 h, diluted with water and concentrated in vacuo. Chromatography (EtOAc/petroleum ether 1:1) afforded hydroxy-derivative **28** (133 mg, 78%) as a syrup. ^{1}H NMR ($CDCl_3$) δ 7.17–7.32 (m, 20H), 4.95–5.08 (m, 2H), 4.82–4.84 (d, 1H, J = 3.7 Hz), 4.75–4.8 (m, 3H), 4.56–4.61 (m, 2H), 4.51–4.53 (d, J = 3.5 Hz, 1H), 4.38–4.47 (m, 1H), 4.16–4.21 (m, 3H), 3.86–3.95 (m, 3H), 3.61 (s, 3H), 3.41–3.49 (m, 3H), 3.26 (s, 3H), 3.2–3.25 (m, 4H), 3.23–3.24 (m, 1H), 3.02–3.04 (m, 2H), 2.48–2.59 (m, 3H), 1.96 (s, 3H), 1.87–1.95 (m, 1H); ^{13}C NMR δ 171.3, 169.5, 154.8, 136.7, 136.5, 135.3, 127.5, 127.4, 127.17, 127.0, 126.9, 126.7, 98.3, 96.3, 78.9, 77.7, 76.9, 75.6, 74.4, 72.8, 72.2, 68.7, 65.9, 64.0, 62.0, 61.6, 59.3, 58.5, 54.5, 54.2, 54.0, 52.7, 51.3, 48.7, 43.5, 28.6. HRMS (ESI) calcd for $C_{51}H_{62}N_5O_{14}$ $(M+H)^+$ *m/z* 968.4298, found 968.4242.

5.1.24. Methyl 4-*C*-[(3*S*,4*R*)-4-{2-azido-3,4-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl}oxy-3-(carboxy)pyrrolidin-1-yl]methyl-2-*O*-(benzyloxycarbonyl)amino-3-*O*-benzyl-2,4-dideoxy-α-D-glucopyranoside (29)

Hydroxy derivative **28** (110 mg, 114 μmol) was dissolved in MeOH (2 mL) and water (0.5 mL), and the solution was cooled to 0 °C and treated with NaOH solution (2 M, 120 μmol) with stirring at rt for 5 h and concentrated to dryness. Chromatography (EtOAc) furnished dihydroxy acid **29** (84 mg, 81%) as a clear syrup. ^{1}H NMR ($CDCl_3$) δ 7.16–7.47 (m, 20H), 5.06–5.26 (m, 2H), 4.86–5.02 (m, 3H), 4.75–4.76 (d, 1H, J = 3.5 Hz), 4.53–4.71 (m, 3H), 4.51–4.52 (d, J = 3.5 Hz, 1H), 4.37–4.52 (m, 4H), 4.05–4.03 (m, 2H), 3.88–3.96 (m, 1 H), 3.82–3.85 (m, 4H), 3.65–3.77 (m, 2H), 3.50 (t, J = 10 Hz, 1H), 3.23 (s, 3H), 3.15–3.23 (m, 2H), 2.67–2.88 (m, 1H), 2.54–2.58 (m, 2H), 2.39–2.45 (m, 3H), 1.87–1.95 (m, 1H); ^{13}C NMR δ 156.2, 138.3, 138.2, 138.0, 136.7, 128.9, 128.8, 128.7, 128.6, 128.5, 128.4, 128.3, 99.6, 98.3, 80.33, 79.2, 75.7, 75.5, 74.3, 73.1, 72.5, 67.4, 64.8, 63.5, 61.96, 58.9, 57.3, 55.6, 54.6, 51.1, 45.0. HRMS (ESI) calcd for $C_{48}H_{56}N_5O_{13}$ $(M-H)^-$ m/z 910.3870, found 910.3870.

5.1.25. Methyl 4-*C*-[(3*S*,4*R*)-4-{2-amino-2-deoxy-6-*O*-sulfo-α-D-glucopyranosyl}oxy-3-(carboxy)pyrrolidin-1-yl]methyl-2-amino-2,4-dideoxy-6-*O*-sulfo-α-D-glucopyranoside trisodium salt (31)

Acid (**29**) (150 mg, 164 μmol) in dry DMF (3 mL) was treated with $SO_3{\cdot}NMe_3$ (137 mg, 1 mmol) and the mixture was heated at 50 °C under argon for 24 h. MeOH (1 mL) was added and the mixture was stirred for 15 min and concentrated in vacuo. Chromatography (CH_2Cl_2/MeOH/NH_4OH (26%), 7:2:0.5) furnished disulfate **30** (135 mg, 77%) as a syrup which was not formally characterized. This disulfate **30** (60 mg, 56 μmol) in EtOH (5 mL) and NH_4OH (26%, 1.5 mL) was treated with palladium hydroxide on carbon (20%, 30 mg). The reaction mixture was stirred for 17 h under hydrogen at rt and ambient pressure, the catalyst was filtered off and washed with EtOH (1 mL), and the filtrate and washings were concentrated to dryness. Chromatography of the residue [CH_2Cl_2/MeOH/NH_4OH (26%), 5:4:0.5] gave di-amino-trisaccharide disulfate analogue **31** (34 mg, 76%) as a syrup. ^{1}H NMR (D_2O) δ 5.32–5.33 (d, J = 3.5 Hz, 1H), 5.14–5.15 (d, J = 3.5 Hz, 1H), 4.62 (dd, J = 5, 12 Hz, 1H), 4.24–4.29 (m, 4H), 4.18–4.19 (m, 3H), 4.14–4.15 (m, 3H), 4.07–4.11 (br d, J = 11 Hz, 1H), 3.98–4.04 (m, 3H), 3.93–3.95 (m, 2H), 3.87–3.89 (m, 3H), 3.81–3.83 (br d, J = 11 Hz, 1H), 3.74–3.78 (m, 2H), 3.69–3.71 (m, 2H), 3.59 (t, J = 10 Hz, 1H), 3.28 (s, 3H), ^{13}C NMR δ 175.3, 99.2, 96.1, 79.1, 71.6, 71.3, 70.8, 70.0, 69.2, 68.7, 67.0, 66.8, 66.3, 65.5, 56.2, 55.3, 53.9, 52.6, 47.1. HRMS (ESI) calcd for $C_{19}H_{34}N_3O_{17}S_2$ $(M-H)^-$ m/z 641.1403, found 641.1429.

5.1.26. Methyl 4-*C*-{(3*S*,4*R*)-4-[2-acetamido-2-deoxy-6-*O*-sulfo-α-D-glucopyranosyl}oxy-3-(carboxy)pyrrolidin-1-yl]methyl}-2-acetamido-2,4-dideoxy-6-*O*-sulfo-α-D-glucopyranoside trisodium salt (32)

Di-amino-trisaccharide analogue (**31**) (30 mg, 43.8 μmol) was dissolved in MeOH (2 mL) and water (200 μL) and cooled to 0 °C. Acetic anhydride (400 μL) was added and the reaction mixture was stirred at rt for 3 h. The resulting solution was concentrated in vacuo and co-evaporated with toluene to afford a syrup. Chromatography [CH_2Cl_2/MeOH/NH_4OH (26%), 5:4:0.5] gave di-*N*-acetate (**32**), 27 mg (78%) as a clear syrup. NMR (MeOH-d_4) δ 4.94–4.95 (d, J = 3.5 Hz, 1H), 4.69–4.7 (d, J = 3.5 Hz, 1H), 4.64 (br s, 1H), 4.3–4.34 (m, 2H), 4.25–4.29 (m, 3H), 4.2–4.24 (m, 2H), 4.14–4.18 (m, 2H), 4.0 (dd, J = 2, 12 Hz, 1H), 3.98–3.99 (m, 2H), 3.95–3.96 (m, 2H), 3.94 (t, J = 10 Hz, 1H), 3.87–3.88 (m, 1H), 3.74–3.87 (m, 2H), 3.69 (t, J = 9 Hz, 1H), 3.55–3.59 (m, 2H), 3.4–3.47 (m, 2H), 3.3 (s, 3H), 2.2–2.3 (m, 1H), 2.05 (s, 3H), 1.99 (s, 3H); ^{13}C NMR δ 176.45, 174.4, 100.61, 98.73, 80.59, 73.13, 72.77, 72.48, 72.3, 69.26, 68.87, 68.54, 61.72, 58.15, 57.97, 57.34, 56.42, 56.21, 55.4, 53.37, 42.18, 23.33, 23.12. HRMS (ESI) calcd for $C_{23}H_{38}N_3O_{19}S_2$ $(M-H^+)^-$ m/z 724.1546, found 724.1549.

5.1.27. Methyl 4-*C*-{(3*S*,4*R*)-4-[2-deoxy-6-*O*-sulfo-2-sulfoamino-α-D-glucopyranosyl}oxy-3-(carboxy)pyrrolidin-1-yl]methyl}-2,4-dideoxy-6-*O*-sulfo-2-sulfoamino-α-D-glucopyranoside pentasodium salt (33)

Di-amino-trisaccharide (**31**) (40 mg, 62.3 μmol) was dissolved in water (2.5 mL) at rt, treated with $SO_3{\cdot}NMe_3$ (100 mg) and Na_2CO_3 (100 mg), and the reaction mixture was stirred at rt for 3 d. The resulting solution was applied on a column of silica gel and eluted with CH_2Cl_2/MeOH/NH_4OH (5:4:0.5) to afford di-*N*-sulfate (**33**), (33 mg, 66%) as a syrup. NMR (D_2O) δ 5.26–5.27 (d, 1H, J = 3.5 Hz), 5.03–5.04 (d, 1H, J = 3.5 Hz), 4.37–4.44 (m, 3H), 4.32–4.4.35 (m, 4H), 4.19–4.29 (m, 4H), 4.01–4.15 (m, 2H), 3.86–3.93 (m, 4H), 3.77–3.83 (m, 3H), 3.62–3.73 (m, 3H), 3.43–3.59 (m, 3H), 3.33 (s, 3H), 2.41–2.59 (m, 1H); ^{13}C NMR δ 175.36, 101.5, 99.36, 71.25, 70.77, 70.31, 69.92, 69.73, 68.72, 68.33, 67.09, 65.77, 59.42, 58.58, 57.81, 52.27, 49.37, 47.38, 39.03. HRMS (ESI) calcd for $C_{19}H_{31}N_3O_{23}S_4$ $(M-4H)^{-4}$, Z = −4, m/z 199.2556, found 199.2556; for $C_{19}H_{32}N_3O_{23}S_4$ $(M-3H)^{-3}$, Z = −3, m/z 266.0103, found 266.0119; for $C_{19}H_{33}N_3O_{23}S_4$ $(M-2H)^{-2}$, Z = −2, 399.5194, found 399.5204.

5.1.28. (3*R*,4*R*,5*R*)-*N*-*tert*-Butoxycarbonyl-3,4-dihydroxy-5-hydroxymethyl-piperidine (34)

The dihydroxy-hydroxymethylpiperidine isofagomine[31] (2.08 g, 14.2 mmol) was dissolved in H_2O (100 mL) and MeOH (50 mL). Et_3N (3 mL) and di-*tert*-butyl dicarbonate (6.17 g, 28.3 mmol) were added and the solution was stirred for 16 h at 20 °C. Concentration followed by dry flash column chromatography (MeOH/CH_2Cl_2, 1:15, 2:15) and recrystallization of the residue from Me_2CO, Et_2O and hexanes gave title compound **34** as a white solid (1.85 g, 7.5 mmol, 53%): mp 99–101 °C; $[\alpha]_D$ +13.2 (c 2, MeOH); ^{1}H NMR ($CDCl_3$) δ 4.71 (br s, 1H), 4.18 (d, J = 12 Hz, 1H), 4.02 (br s, 1H), 3.78 (dd, J = 10.5, 4.5 Hz, 1H), 3.70 (dd, J = 10.5, 4 Hz, 1H), 3.42–3.51 (m, 2H), 2.50–2.68 (m, 2H), 1.69 (br m, 1H), 1.45 (s, 9H). ^{13}C NMR ($CDCl_3$) δ 155.4, 80.9, 76.4, 71.9, 62.5, 48.3, 45.1, 43.8, 28.8. Anal. Calcd for $C_{11}H_{21}NO_5$: C, 53.43; H, 8.56; N, 5.66. Found: C, 53.12; H, 8.48; N, 5.69.

5.1.29. (3*R*,4*R*,5*R*)-*N*-*tert*-Butoxycarbonyl-3,4-dihydroxy-5-hydroxymethyl-4,5'-*O*-*p*-methoxybenzylidene-piperidine (35)

The piperidine amide **34** (14.14 g, 57 mmol) was dissolved in dry DMF (160 mL) and *p*-anisaldehyde dimethyl acetal (11.8 mL, 69 mmol), methyl orange (25 mg) and TsOH (533 mg) were added. The solution was subjected to rotary evaporation at 33 °C for 90 min, then $NaHCO_3$ solution (satd, aq, 40 mL), Na_2CO_3 solution (aq, M, 20 mL) and H_2O (100 mL) were added. The mixture was concentrated to a small volume under high vacuum, the residue was extracted with Et_2O (1 L) and the solution was washed with brine (700 mL). The aqueous layer was back-extracted with diethyl ether (×2). The ether solutions were combined, dried and concentrated and the residue was purified by flash column chromatography (EtOAc/hexanes 4:6) to give impure title compound **35** which was recrystallized from diethyl ether/hexanes to give a white solid (17.3 g, 47 mmol, 83%): mp 112.5–113 °C; $[\alpha]_D$ +3.6 (c 2, EtOAc); ^{1}H NMR δ 7.41 (d, J = 9 Hz, 1H), 6.89 (d, J = 9 Hz, 1H), 5.52 (s, 1H), 4.37 (br s, 1H), 4.15 (dd, J = 4.5, 11 Hz, 1H), 3.99 (br s, 1H), 3.80 (s, 3H), 3.66–3.75 (m, 1H), 3.57 (t, J = 11 Hz, 1H), 3.43 (t, J = 10 Hz, 1H), 2.68 (d, J = 2.5 Hz, 1H), 2.64 (dd, J = 11.5, 11.5 Hz, 1H), 2.38 (br t, J = 12 Hz, 1H), 1.87–2.01 (m, 1H), 1.46 (s, 9H). ^{13}C NMR ($CDCl_3$) δ 160.6, 154.9, 130.7, 128.0, 114.1, 102.3, 85.5, 80.8, 68.8, 68.7, 55.7, 48.4, 43.5, 37.4, 28.8. Anal. Calcd for $C_{19}H_{27}NO_6$: C, 62.45; H, 7.45; N, 3.83. Found: C, 62.57; H, 7.42; N, 3.78.

5.1.30. (3*R*,4*R*,5*R*)-3-Benzyloxy-*N*-*tert*-butoxycarbonyl-4-hydroxy-5-hydroxymethyl-4,5'-*p*-methoxybenzylidene-piperidine (36)

Cyclic acetal **35** (2.17 g, 5.94 mmol) was dissolved in dry THF (100 mL) containing BnBr (1.38 mL, 11.9 mmol). NaH (60% in oil, 3.6 g, 89 mmol) was slowly added followed by H_2O (dropwise, 0.32 mL, 18 mmol) and NaI (50 mg). The mixture was heated under reflux for 2 h, after which it was cooled to 40 °C and MeOH (40 mL) was added. Stirring was applied for 30 min, H_2O (500 mL) and CH_2Cl_2 (500 mL) were added and the mixture was shaken. The resulting organic layer was washed again with water and dried ($MgSO_4$). Removal of the solvent and flash column chromatography (EtOAc/hexanes 3:7) gave crude product which was recrystallized from Et_2O/hexanes to give title compound **36** (1.77 g, 3.89 mmol, 65.4%) as a white solid**:** mp 91.5–92 °C; $[\alpha]_D$ +26.3 (*c* 1, EtOAc); ^{1}H NMR ($CDCl_3$) δ 7.43 (d, *J* = 9 Hz, 2H), 7.23–7.37 (m, 5H), 6.90 (d, *J* = 9 Hz, 2H), 5.58 (s, 1H), 4.84 (t, *J* = 12 Hz, 1H), 4.70 (t, *J* = 12 Hz, 1H), 4.20–4.55 (br s, 1H), 4.15 (dd, *J* = 11, 4.5 Hz 1H), 3.85–4.00 (br s, 1H), 3.80 (s, 3H), 3.53–3.70 (m, 3H), 2.66 (dd, *J* = 11, 12.5 Hz, 1H), 2.37 (br m, 1H), 1.89–2.02 (m, 1H), 1.44 (s, 9H). ^{13}C NMR δ 160.4, 154.9, 138.9, 131.1, 127.1–128.8, 114.0, 101.8, 85.3, 80.7, 75.6, 73.5, 68.7, 55.7, 47.3, 43.4, 38.1, 28.7. Anal. Calcd for $C_{26}H_{33}NO_6$: C, 68.55; H, 7.30; N, 3.07. Found: C, 68.55; H, 7.25; N, 3.07.

5.1.31. (3*R*,4*R*,5*R*)-3-Benzyloxy-*N*-*tert*-butoxycarbonyl-4-hydroxy-5-hydroxymethyl-piperidine (37)

The methoxybenzylidene piperidine derivative **36** (3.70 g, 8.12 mmol) and Amberjet 1200 (H^+) (15 g) were added to MeOH (250 mL). The mixture was stirred at 20 °C for 2 h when the resin was removed by filtration and washed with methanol. The filtrate and washings were combined and taken to pH 7 with Na_2CO_3 (aq, M, ca. 0.5 mL). The resulting solution was concentrated and purified by flash column chromatography (EtOAc/hexanes 7:3) to yield a white solid (2.61 g, 7.73 mmol, 95%) that was recrystallized from Et_2O/hexanes to give title compound **37**: mp 90.5–91 °C; $[\alpha]_D$ +12.7 (*c* 1.6, EtOAc); ^{1}H NMR ($CDCl_3$) δ 7.26–7.39 (m, 5H), 4.71 (d, *J* = 11.5 Hz, 1H), 4.57 (d, *J* = 11.5 Hz, 1H), 4.1–4.7 (br s, 1H), 4.00 (d, *J* = 12 Hz, 1H), 3.68 (t, *J* = 5 Hz, 2H), 3.53 (dd, *J* = 9, 9.5 Hz, 1H), 3.24–3.32 (m, 1H), 3.14 (s, 1H), 2.78 (br m, 1H), 2.52 (t, *J* = 12 Hz, 2H), 1.70–1.83 (m, 1H), 1.45 (s, 9H). ^{13}C NMR ($CDCl_3$) δ 155.0, 138.4, 128.2–129.0, 80.6, 79.4, 76.3, 72.5, 63.6, 45.6, 44.8, 43.6, 28.7. Anal. Calcd for $C_{18}H_{27}NO_5$: C, 64.07; H, 8.07; N, 4.15. Found: C, 64.19; H, 8.06; N, 4.09.

5.1.32. Benzyl (3*R*,4*R*,5*S*)-3-benzyloxy-*N*-*tert*-butoxycarbonyl-4-hydroxy-piperidine-5-carboxylate (38)

The BOC-protected piperidine **37** (500 mg, 1.48 mmol), TEMPO (7.5 mg) and Bu_4NBr (50 mg) were dissolved in CH_2Cl_2 (15 mL). While the solution was stirred vigorously in an ice/water bath, a suspension of $Ca(OCl)_2$ (463 mg, 3.23 mmol) in $NaHCO_3$ solution (aq, 0.28 M, 15 mL) was added over 15 min such that the reaction temperature remained below 5 °C. The biphasic mixture was stirred on ice for a further 70 min when EtOH (10 mL) was added, and the mixture was dried by rotary evaporation at 40 °C to give a solid residue which was extracted by stirring with acetone (50 mL). The remaining solids were removed by filtration and washed with additional Me_2CO (50 mL). The acetone solutions were combined and the solvent was removed to give a crude residue (600 mg). This was added to DMF (25 mL), and $KHCO_3$ (350 mg), MeOH (0.60 mL) and BnBr (0.54 mL, 4.5 mmol) were added, and the suspension was then stirred for 17 h at 20 °C. The resulting mixture was concentrated to a syrup under high vacuum, taken up in CH_2Cl_2 (100 mL) and washed with H_2O (100 mL). The organic layer was dried and concentrated and the residue was purified by flash column chromatography (EtOAc/hexanes 25:75) to give a white solid that was recrystallized from ether/hexanes to give the title ester **38** (274 mg, 0.62 mmol, 42%): mp 114–115 °C; $[\alpha]_D$ −1.5 (*c* 1, EtOAc); ^{1}H NMR ($CDCl_3$) δ 7.24–7.40 (m, 10H) 5.18 (t, *J* = 13 Hz, 2H), 5.13 (d, *J* = 13 Hz, 1H), 4.70 (d, *J* = 11.5 Hz, 1H), 4.63 (d, *J* = 11.5 Hz, 1H), 4.26 (br m, 1H), 3.89 (dt *J* = 2, 9.5 Hz, 1H), 3.24–3.31 (m, 1H), 2.99 (s, 1H), 2.80 (br s, 1H), 2.51–2.63 (m, 2H), 1.44 (s, 9H). ^{13}C NMR ($CDCl_3$) δ 171.5, 154.6, 138.4, 135.9, 128.2–129.0, 80.9, 78.5, 74.4, 72.8, 67.2, 48.6, 45.7, 44.7, 28.7. Anal. Calcd for $C_{25}H_{31}NO_6$: C, 68.01; H, 7.08; N, 3.17. Found C, 67.88; H, 7.04; N, 3.07.

5.1.33. Benzyl (3*R*,4*R*,5*S*)-3-benzyloxy-*N*-[(9-fluorenylmethyloxy)-carbonyl]-4-hydroxy-piperidine-5-carboxylate (39)

The benzyl ester **38** (1.47 g, 3.33 mmol) was dissolved in CH_2Cl_2 (125 mL) and TFA (12.5 mL) was added. After 1 h at 20 °C, toluene (80 mL) was added and the solution was concentrated. Toluene addition and evaporation were repeated twice more. The syrupy product was then dissolved in CH_2Cl_2 (90 mL) and 9-fluorenylmethyl chloroformate (1.97 g, 7.61 mmol), aqueous Na_2CO_3 (1 M, 45 mL) and aqueous $NaHCO_3$ (satd, 45 mL) were added while the mixture was stirred in an ice/water bath. Stirring of the two-phase mixture was continued for 30 min at 0 °C and then at 20 °C for 16 h. After this time, additional CH_2Cl_2 (100 mL) was added and the organic layer was washed with water (200 mL) and dried. Flash column chromatography (EtOAc/hexanes 1:3) gave the Fmoc-protected **39** as a colourless syrup (1.82 g, 3.23 mmol, 97%); $[\alpha]_D$ −18.4 (*c* 1, EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.71 (d, *J* = 7 Hz, 2H), 7.53 (d, 2H), 7.17–7.45 (m, 14H), 5.16 (s, 2H), 4.24–4.75 (br m, 5H), 4.19 (t, *J* = 6 Hz, 1H), 3.94–4.15 (br m, 1H), 3.83 (br m, 1H), 2.95–3.30 (br m, 1H), 2.70–2.95 (m, 2H), 2.50 (s, br, 2H). ^{13}C NMR ($CDCl_3$) δ 171.3, 155.1, 144.2, 141.8, 138.3, 135.9, 127.5–129.0, 125.2, 120.4, 78.5, 78.3, 74.2, 72.8, 67.9, 67.8, 67.3, 48.3, 47.8, 45.9, 44.6. Anal. Calcd for $C_{35}H_{33}NO_6$: C, 74.58; H, 5.90; N, 2.49. Found C, 74.49; H, 5.93; N, 2.48.

5.1.34. Benzyl (3*R*,4*R*,5*S*)-4-(6-*O*-acetyl-2-azido-3,4-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl)oxy-3-benzyloxy-piperidine-5-carboxylate. Mono-oxalic acid salt (41)

The piperidine alcohol **39** (2.00 g, 3.55 mmol) and 6-*O*-acetyl-2-azido-3,4-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl trichloroacetimidate (3.78 g, 6.61 mmol) were dried together by benzene azeotroping. Powdered molecular sieves (4 Å, 1.6 g) and CH_2Cl_2 (20 mL) were added and the mixture was stirred for 45 min at 20 °C and cooled to −20 °C. TBDMSOTf (122 μL, 0.53 mmol) was added, and the mixture was stirred at this temperature for 25 min. $NaHCO_3$ (700 mg) and H_2O (100 μL) were added, and the mixture was allowed to warm to 20 °C. CH_2Cl_2 (100 mL) was added and the mixture was filtered through a pad of Celite and, after evaporation of the solvent, the residue was applied directly to a column of silica gel for flash chromatography. Elution with EtOAc/hexanes/$CHCl_3$ (25:65:10) produced partly purified product which was re-chromatographed in the same way to give compound **40** as an impure syrup which was dissolved in morpholine (80 mL) and stirred at 18 °C for 35 min whereupon the resulting solution was poured onto ice cold water (800 mL) and extracted (×3) with Et_2O (400 mL, 80 mL and 80 mL). The ether extracts were combined, benzene (120 mL) was added and the solution was concentrated to a syrup. Flash column chromatography (EtOAc/hexanes/$CHCl_3$/Et_3N, 7:2:1:0.05) gave a syrup (1.70 g, 2.26 mmol, 64%) from which mono-oxalate **41** (1.71 g, 2.03 mmol, 57% from **39**) was obtained as a white solid, after the addition of oxalic acid dihydrate (0.29 g, 2.30 mmol), followed by crystallization from EtOH (40 mL), by addition of *i*PrOH, Et_2O and hexanes; mp 131–132° ; $[\alpha]_D$ +31.4 (*c* 1.5, EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.13–7.39 (m, 20H), 5.09 (d, *J* = 3.5 Hz, 1H), 5.01 (d, *J* = 12 Hz, 1H), 4.80–4.92 (m, 4H), 4.60 (d, *J* = 11.5 Hz, 1H), 4.57

(d, J = 11 Hz, 1H), 4.44 (d, J = 11.5 Hz, 1H), 4.33 (t, J = 4.5 Hz, 1H), 4.24 (dd, J = 2, 12 Hz, 1H), 4.13 (dd, J = 5, 12 Hz, 1H), 3.93 (dd, J = 9, 10 Hz, 1H), 3.83–3.89 (m, 1H), 3.78–3.83 (m, 1H), 3.35–3.65 (m, 4H), 3.49 (dd, J = 9, 9.5 Hz, 1H), 3.39 (dd, J = 3.5, 10 Hz, 1H), 3.05 (br d, J = 4 Hz, 1H), 1.92 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.5, 169.4, 163.3, 137.5, 137.2, 136.3, 134.6, 128.7–128.0, 98.3. 80.1, 77.8, 75.7, 75.2, 72.6, 72.2, 71.3, 70.6, 68.0, 63.4, 62.6, 43.0, 41.8. 40.8, 20.6. Anal. Calcd for $C_{44}H_{48}N_4O_{13}$: C, 62.85; H, 5.75; N, 6.66. Found C, 62.85; H, 5.79; N, 6.62. HRMS (ESI) calcd for $C_{42}H_{47}N_4O_9$ $[M-COOHCOO^-]^+$ m/z 751.3338; found 751.3344.

5.1.35. Benzyl (3*R*,4*R*,5*S*)-4-(2-azido-3,4-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl)oxy-3-benzyloxy-piperidine-5-carboxylate (42)

The piperidine salt **41** (1.51 g, 1.80 mmol) was dissolved in cold THF (200 mL) and water (46 mL). LiOH (aq, M, 10 mL) was added and the mixture was stirred for 17 h at 4 °C. It was then shaken with CH_2Cl_2 (800 mL) and brine (50%, 1L), and the brine was back-extracted twice with CH_2Cl_2 (2 × 100 mL). The organic phases were combined and washed again with dilute brine, which was again back-extracted with CH_2Cl_2 (100 mL). The organic phase was dried and flash column chromatography (EtOAc/hexanes/$CHCl_3$/Et_3N, 7:2:1:0.05) followed by (EtOAc/Et_3N, 99:1) gave alcohol **42** (1.07 g, 1.51 mmol, 84%) as a colourless syrup: $[\alpha]_D$ +12.6 (c 0.5, EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.23–7.37 (m, 20H), 5.46 (d, 4 Hz, 1H), 5.07 (d, J = 12.5 Hz, 1H), 4.99 (d, J = 12.5 Hz, 1H), 4.81–4.85 (m, 3H), 4.54–4.67 (m, 3H), 4.22 (dd, J = 7.5, 7.5 Hz, 1H), 3.92 (dd, J = 8.5, 10.5 Hz, 1H), 3.82 (dd, J = 2, 11.5 Hz, 1H), 3.46–3.70 (m, 4H), 3.19–3.28 (m, 1H), 3.23 (dd, J = 4, 10.5 Hz, 1H), 3.11 (dd, J = 4, 13 Hz, 1H), 2.87 (dd, J = 8.5, 13 Hz, 1H), 2.56–2.70 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.3, 138.4, 138.3, 135.8, 128.0–129, 98.4, 80.2, 79.2, 78.5, 77.0, 75.7, 72.4, 72.1, 67.3, 61.8, 48.5, 47.9, 46.7. Anal. Calcd for $C_{40}H_{44}N_4O_8$: C, 67.78; H, 6.26; N, 7.90. Found C: 67.95; H, 6.56; N 7.60.

5.1.36. Methyl 4-*C*-[(3*R*, 4*R*, 5*S*)-4-(2-azido-3,4-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl)-3-benzyloxy-5-benzyloxycarbonyl-piperidin-1-yl)methyl]-3-*O*-benzyl-2-(benzyloxycarbonyl)-amino-2,4-dideoxy-α-D-glucopyranoside (44)

The piperidine glycoside **42** (0.56 g, 0.79 mmol) and aldehyde **18** (0.45 g, 0.82 mmol) were mixed and dried by addition and removal of benzene. $NaBH(OAc)_3$ (0.69 g, 3.3 mmol) was added followed by $(CH_2)_2Cl_2$ (13 mL), and the mixture was stirred for 18 h. CH_2Cl_2 (200 mL) was then added and the organic solution was washed with a mixture of Na_2CO_3 (aq, M) and $NaHCO_3$ (satd, aq) 1:1 (200 mL). The aqueous phase was back-extracted twice with CH_2Cl_2 (40 mL). The organic solutions were combined and dried and flash column chromatography (EtOAc/hexanes/Et_3N 3:7:0.05) gave the adduct **43** as a slightly impure, colourless syrup (0.801 g, 0.65 mmol, 82%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.20–7.35 (m, 30H), 5.61 (d, J = 4 Hz, 1H), 4.78–5.16 (m, 5H), 4.69 (d, J = 3 Hz, 1H), 4.43–4.66 (m, 5H), 3.94–4.05 (m, 3H), 3.87 (dd, J = 8, 10 Hz, 1H), 3.82 (br d, J = 10.5 Hz, 1H), 3.47–3.68, m, 6H), 3.34 (s, 3H), 3.31 (t, J = 10 Hz, 1H), 3.19 (dd, J = 4, 10.5 Hz, 1H), 2.97–3.04 (m, 2H), 2.75 (ddd, J = 4, 10.5, 11 Hz, 1H), 2.43 (d, J = 12.5 Hz, 1H), 2.16–2.29 (m, 1H), 1.87–2.05 (m, 2H), 1.65–1.80 (m, 2H), 0.89 (s, 9H), 0.064 (s, 3H), 0.057 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.7, 156.3, 138.5, 138.4, 136.8, 135.5, 128.1–129.0, 99.5, 98.1, 80.8, 80.3, 78.6, 78.1, 75.7, 75.5, 74.6, 74.4, 72.5, 72.0, 67.7, 67.4, 64.8, 63.8, 61.9, 57.5, 56.6, 55.9, 55.2, 54.6, 48.0, 41.8, 26.4, −4.6. This silyl ether of diol **44** (0.801 g, 0.65 mmol) was dissolved in THF/HCOOH/H_2O (6:3:1, 120 mL) and heated at 60 °C for 3 h. The mixture was concentrated, dissolved in acetone, water (40 mL, 1:1) and again concentrated. This addition/concentration was repeated and finally the sample was taken up in acetone and concentrated to a syrup. The resulting syrup was dissolved in dry MeOH (96 mL) and methanolic NH_4OH (7 M, 16 mL) was added. After 30 min at 20 °C the sample was concentrated and flash column chromatography (EtOAc/hexanes/$CHCl_3$/Et_3N 5:2:1:0.05) gave the diol **44** (0.662 g, 0.59 mmol, 91%) as a colourless syrup; $[\alpha]_D$ +45.9 (c 0.5, EtOAc); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.19–7.36 (m, 30H), 5.36 (br s, 1H), 5.15 (d, J = 12 Hz, 1H), 5.09 (d, J = 12 Hz, 1H), 5.01–5.07 (m, 3H), 4.80–4.85 (m, 3H), 4.69 (d, J = 2.5 Hz, 1H), 4.64 (d, J = 11 Hz, 1H), 4.56 (d, J = 11.5 Hz, 1H), 4.50 (d, J = 11.5 Hz, 1H), 4.42 (d, J = 11 Hz, 1H), 4.13 (t, J = 6.5 Hz, 1H), 4.05 (dt, J = 2.5, 9.5 Hz, 1H), 3.86 (dd, J = 8.5, 10.5 Hz, 1H), 3.52–3.82 (m, 7H), 3.45–3.50 (m, 1H), 3.32 (s, 3H), 3.22–3.30 (br m, 1H), 3.24 (dd, J = 3.5, 10 Hz, 1H), 2.96 (br s, 1H), 2.76 (dt, J = 8.5, 4 Hz, 1H), 2.72 (br s, 1H), 2.49 (d, J = 12.5 Hz, 1H), 2.34 (br s, 2H), 2.18 (dd, J = 9.5, 12.5 Hz, 1H), 2.18 (dd, J = 9.5, 12.5 Hz, 1H), 1.82–2.00 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.6, 156.2, 138.2, 138.0, 136.7, 135.4, 127.9–128.9, 99.8, 98.0, 80.2, 78.3, 77.7, 76.1, 75.6, 75.4, 74.5, 73.9, 72.4, 72.2, 67.6, 67.3, 64.1, 63.8, 61.7, 55.9, 55.8, 55.7, 55.4, 53.1, 46.6, 42.4. Anal. Calcd for $C_{63}H_{71}N_5O_{14}$: C, 67.42; H, 6.38; N, 6.24. Found: C, 67.59; H, 6.53; N, 6.22.

5.1.37. Methyl 2-acetamido-4-*C*-[(3*R*,4*R*,5*S*)-4-(2-acetamido-2-deoxy-6-*O*-sulfo-α-D-glucopyranosyl)-5-carboxy-3-hydroxy-piperidin-1-yl)methyl]-2,4-dideoxy-6-*O*-sulfo-α-D-glucopyranoside diammonium salt (46)

The trisaccharide analogue **44** (75 mg, 0.067 mmol) was dried azeotropically with benzene then $Me_3N{\cdot}SO_3$ (185 mg, 1.33 mmol) and dry DMF (6 mL) were added, and the mixture was stirred for 16 h at 45 °C under Ar. It was concentrated under high vacuum at 40 °C and then dissolved in MeOH. Silica gel was added and the solvent was evaporated under reduced pressure. The resulting silica was transferred to the top of a flash silica gel column, and elution with CH_2Cl_2/MeOH/NH_4OH (aq, 26%) (7:2:0.5) gave disulfate diammonium salt **45** as an impure solid (95 mg, 88%). The crude material was dissolved in EtOH/H_2O (11 mL, 7.5:1) and NH_4OH (28%, 0.28 mL), $Pd(OH)_2$ on carbon (260 mg, ca. 20%) were added, and the mixture was stirred for 24 h at 20 °C under an atmosphere of hydrogen. The product was filtered through Celite and the filtrate was concentrated at 37 °C. The resulting material was stirred with water, MeOH (5.0 mL, 2:3), Ac_2O (600 μL) and $NaHCO_3$ (500 mg) for 3 h. Additional $NaHCO_3$ (300 mg) and Ac_2O (600 μL) were added and the mixture was stirred for 18 h at 20 °C and adsorbed onto flash column silica gel and applied to a chromatographic column containing pure gel. Elution with ^{i}PrOH/MeOH/NH_4OH (28%, aq), 4:4:3 gave crude **46**. This was re-columned in the same solvent system to give **46** as a glassy solid (22 mg, 64% from compound **44**). ^{1}H NMR (500 MHz, D_2O) δ 5.31 (s, br, 1H), 4.90 (d, J = 3.5 Hz, 1H), 3.90–4.50 (m, 12 H), 3.86 (t, J = 10.0 Hz, 1H), 3.67 (dd, J = 9.0, 10.0 Hz, 1H), 3.66 (s, br, 1H), 3.47 (s, 3H), 3.20–3.55 (br, m, 4H), 2.92 (br, s, 1H), 2.46 (br m, 1H), 2.12 (s, 6H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 176.8, 175.7, 175.3, 99.5, 97.8, 72.3, 71.5, 71.4, 70.4, 68.5, 68.2, 67.6, 58.9, 56.9, 56.3, 55.2, 54.6, 39.6, 23.0, 22.9. HRMS (ESI) Calcd for $C_{24}H_{47}N_5O_{20}S_2$ $[M-2NH_4]^{2-}$ m/z 376.5790. Found 376.5788.

6. FITC-labelled heparan sulfate degradation assay

6.1. Heparanase expression in COS-7 cells and purification

COS-7 (African green monkey kidney, ATCC) cells stably transfected with human heparanase cDNA were cultured and passaged at confluence into T-175 triple flasks. Cells from three clones were harvested with 1 mL 0.25% trypsin (Medica Pacifica), centrifuged and washed in PBS. Preparation of the cell lysate and isolation of heparanase were carried out as reported.[32] Cell extracts were

sonicated briefly then centrifuged at 12,000*g* for 10 min at 4 °C to remove insoluble debris. Soluble fractions were stored at −80 °C in 1 mL aliquots. The presence of Heparanase in the purified fractions was confirmed using western blotting (anti-heparanase polyclonal antibodies, Insight, Ins-AB-04002) and its activity tested by ELISA assay (Heparan Degrading Enzyme Assay Kit (Takara)) and an HPLC assay.

6.2. Substrate preparation

FITC-labelled heparan sulfate (HS) was prepared by adding FITC (fluorescein-5-isothiocyanate) to HS from bovine kidney (Sigma) dissolved in 0.1 M Na_2CO_3 (pH 9.35) in a 1:2 ratio (weight), respectively, and the labelling reaction was stirred overnight at 4 °C in the dark. The labelled material was purified using a G-25 spin column (GE Healthcare) to remove excess FITC and then fractionated on a sephacryl-300 10/60 gel filtration column (GE Healthcare). The labelled substrate was collected in 1 mL fractions, and 80 fractions were assayed in a multiwell format with a fluorescent plate reader (excitation 492, emission 524). The labelled substrate was present in two peaks over 40 fractions. To maximize peak resolution, each fraction was concentrated separately and material from the first peak (largest molecular weight) was used for the inhibitor studies. The concentration of FITC-labelled heparan sulfate (fraction 37) was determined to be 0.33 mg/mL using the Blyscan glucosaminoglycan assay. The labelled substrate was diluted 100 fold and 20 μL was used in 50 μL reactions, resulting in a final substrate concentration of 1.3 μg/mL (65 ng per reaction).

6.3. HPLC assay

An HPLC assay was developed to monitor the degradation of labelled HS by heparanase. A variety of buffers were tested and it was found that buffers containing 0.2 mg/mL BSA, pH 4.5, with 10% glycerol and 0.1% Triton X-100 added were the most suitable in terms of best enzyme activity and minimal column blockage. The introduction of a pre-column in-line filter unit and an extra column filter was necessary to prevent column blockages.

Assay experiments were prepared with and without 5 μL of semi-purified heparanase in reaction buffer (25 mM sodium dimethylglutarate, 0.1 M NaCl, 0.1% Triton X-100, 10% glycerol, pH 4.5). After incubation at 37 °C for 90 min, each assay was quenched by the addition of 5 μl of 1.5 M Tris–HCl, pH 8.8, then heated to 60 °C for 5 min, before being filtered through a 0.22-μm filter. Separation was effected by loading 25 μL of each assay experiment onto a Superdex 200 column connected to a fluorescence detector and eluting with the buffer at a flow rate of 0.3 ml/min. The substrate peak (retention time 35 min) was degraded in the presence of heparanase to about one third the original peak height, with a prominent product peak appearing with a retention time of 55 min.

In control experiments, extracts containing equivalent protein amounts from wild-type COS-7 cells not expressing heparanase did not degrade the substrate.

6.4. Inhibitor studies

Compounds **7–10**, **24**, **25**, **32**, **33** and **46** were tested for efficacy as inhibitors at 500 μM concentration using the same reaction conditions as mentioned above. The potential inhibitors were initially tested at high substrate concentration (30 μg/mL) and then with lower concentration (0.33 μg/mL). Control reactions with substrate only and substrate plus semi-purified extracts from transfected COS-7 cells without inhibitor were prepared in duplicate with each experiment, together with a suramin control reaction. No inhibition was seen with any of the compounds at 500 μM whereas the IC_{50} for suramin under these conditions was approximately 25 μM.

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