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Synthesis of disaccharides derived from heparin and evaluation of effects on endothelial cell growth and on binding of heparin to FGF-2

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Abstract—The disaccharide β-D-GlcA-(1→4)-α-D-GlcNAc-1→OMe and other small nonsulfated oligosaccharides related to heparin/heparan sulfate have been shown to bind to FGF and activated the fibroblast growth factor (FGF) signalling pathway in (F32) cells expressing the FGF receptor. Synthetic routes to β-D-GlcA-(1→4)-α-D-GlcNAc-1→OMe and a glucose analogue β-D-Glc-(1→4)-α-D-GlcNAc-1→OMe are described. The effects of these disaccharides on endothelial cell growth, which is relevant to angiogenesis, were evaluated and it was found they did not mimic the inhibitory effects that were observed for heparin albumin (HA) and that have also been observed by monosaccharide conjugates. They did not alter bovine aortic endothelial cell (BAEC) proliferation, in the presence of FGF-2 in serum free medium or in absence of FGF-2 in serum free and complete medium. Disaccharides (10 μg/mL) reduced by 25–31% the inhibition caused by HA (10 μg/mL) on BAEC growth in serum-free medium but had no effect in complete medium. There was no evidence obtained for the binding of these oligosaccharides to FGF-2 in competition with HA by ELISA.

Keywords: Signal transduction; Angiogenesis; Oligosaccharides; Endothelial cell growth; Heparin; Fibroblast growth factor

1. Introduction

Angiogenesis[1] provides new blood vessels to growing tissue and it relies on the up-regulation of endothelial cell proliferation and survival pathways. In adults this is tightly regulated and occurs for the most part only during pregnancy or in wound healing. However, up-regulated angiogenesis is characteristic in rheumatoid arthritis, diabetic retinopathy as well as during tumour growth and metastasis.[2] There is considerable current effort directed towards the development of angiogenesis inhibitors and there are also applications for pro-angiogenic compounds.[3] Strategies[4] being considered for the development of anti-angiogenic agents include synthesis of inhibitors of the recognition of extracellular matrix proteins to integrin receptors,[5] matrix metalloprotease inhibitors[6] natural products,[7] COX inhibitors,[8] kinase inhibitors[9] and inhibitors of growth factors such as FGF-2, VEGF and PDGF.[10] The polypeptide FGF-2 is an angiogenic factor and is also involved in pathological processes. It is considered that the development of novel inhibitors or promoters of FGF-2 could lead to new therapeutics as well as having potential in regenerative medicine.[11] The cellular receptors for FGFs are tyrosine kinases (FGFR) and these are activated by ligand-induced dimerisation, requiring heparin or heparan sulfate proteoglycans (HSPG's) as co-receptors (**1**, **2**). The general consensus is that highly sulfated oligosaccharides containing at least six saccharide units comprised of iduronic acid are required to promote

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signalling. Interestingly a paper by Ornitz et al. showed that nonsulfated disaccharides and trisaccharides (e.g., **3**, **5**), with structural features found in heparin, were active in a number of FGF dependant assays and could activate the FGF signalling pathway.[12] These oligosaccharides mimicked properties that are exhibited by heparin or heparan sulfate: they displayed binding to FGF in competition with ^{125}I-heparin, enhanced binding of FGF to FGFR and, in the presence of FGF, promoted the mitogenesis of an F-32 cell line, which expressed an FGF receptor. Evidence was provided that dimerisation or higher order clustering of FGF can occur in presence of oligosaccharides suggesting that the resulting aggregates may be the biologically active species. Alternatively, crystallographic work by Mohammadi and coworkers suggested a unifying mechanism by which both heparin and the smaller oligosaccharides could activate cellular responses dependant on FGF and FGFR.[13] Subsequently we reported the design and synthesis of a diverse series of monosaccharide conjugates as potential mimetics of **5** and heparin whose biological evaluation led to the identification of **6** (thioglucamic acid) and **7** (thioglucamide) and related conjugates, which displayed effects similar to heparin as they inhibited bovine aortic endothelial cell (BAEC) growth and inhibited, at high concentrations, binding of FGF-2 to heparin.[14] Herein we describe the synthesis of disaccharides **4** and **5**; the results from their biological evaluation has revealed that disaccharides, unlike **6** and **7** and heparin do not inhibit BAEC growth or survival (Chart 1).

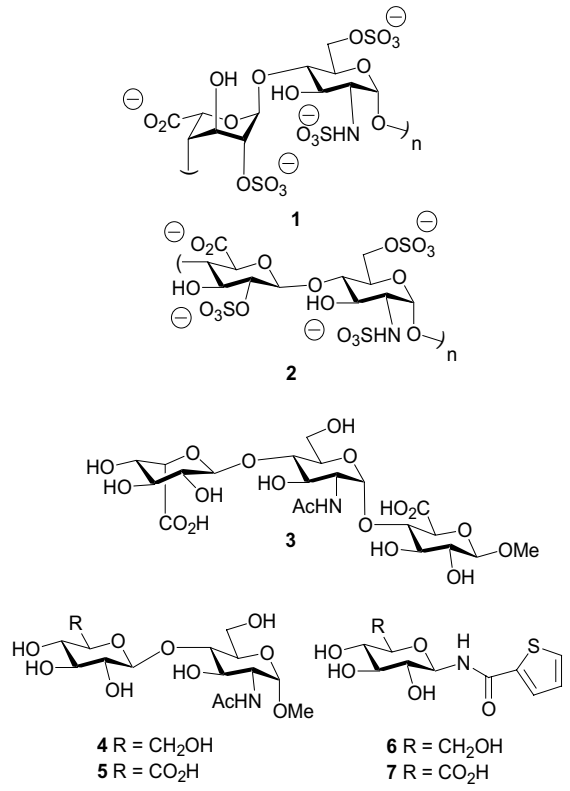
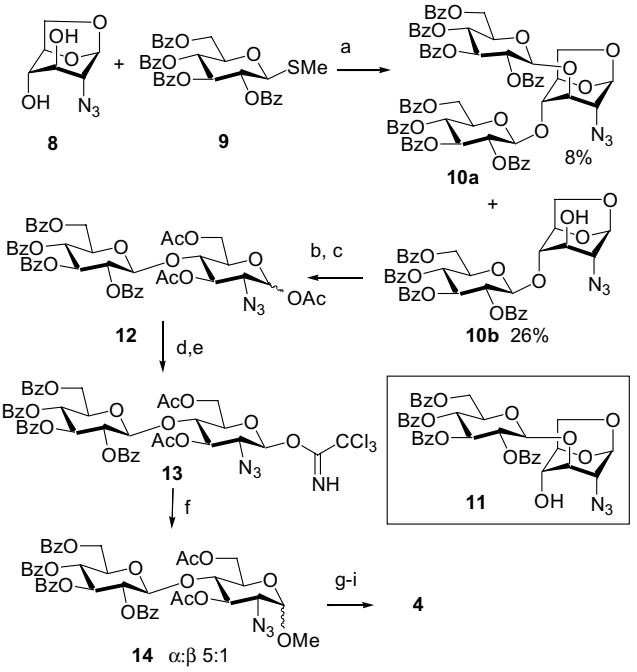


Chart 1.

2. Results and discussion

2.1. Synthesis of the disaccharides

A synthesis of **4** was first established (Scheme 1). The regioselectivity of the glycosidation reaction of the acceptor **8**[15,16] with thioglycoside donor **9**[17] was investigated; reaction of equimolar amounts of **8** and **9** gave a mixture of regioisomeric disaccharides in low yields: β-glycosidation occurred preferentially at O-4 to give desired disaccharide **10b** in 17% yield and the 3-*O*-linked disaccharide **11** was also obtained in 8% yield. The major product was **10b** (26%) when the reaction was repeated with an excess of the donor **9** (2.5 equiv); the trisaccharide **10a** was also isolated (8%). Acetylation of **10b** was followed by acetolysis to give **12**. The trichloroacetimidate donor **13** was next prepared: reaction of **12** with benzylamine gave a hemiacetal intermediate, which when treated with potassium carbonate and trichloroacetonitrile gave **13**.[18] Glycosidation of this donor with MeOH using catalytic $BF_3{\cdot}OEt_2$ gave a mixture of glycosides, the α-anomer **14** being the major product. This was converted to **4** after exchanging all benzoate esters for acetates, azide reduction in the presence of acetic



Scheme 1. Synthesis of **4**. Reagents and conditions: (a) AgOTf, NIS, CH_2Cl_2, 26%; (b) Ac_2O, Py; (c) TFA, Ac_2O; (d) $BnNH_2$, THF, 95% for three steps; (e) K_2CO_3, Cl_3CCN, CH_2Cl_2, 12 h, 28% (75% based on recovered starting material); (f) MeOH, BF_3Et_2O, CH_2Cl_2, 25% α-anomer; (g) NaOMe, MeOH then Ac_2O, Py, 86%; (h) Pd–C, H_2, Ac_2O, 59%; (i) NaOMe, MeOH, 84%.

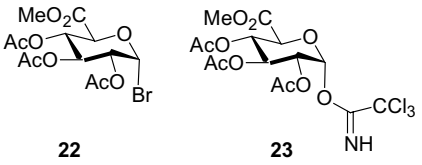
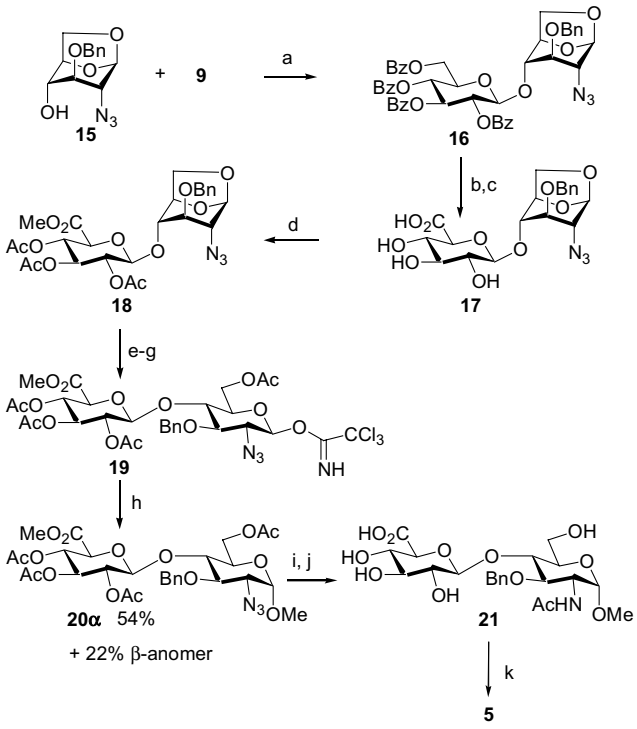


Chart 2.

anhydride and finally *O*-deacetylation using sodium methoxide and MeOH. Synthetic routes to oligosaccharides containing glucuronic acid are less straightforward than to the corresponding glucosides because of the lower reactivity of the glucuronide donors.[19] The attempted synthesis of the desired disaccharide **5** via the coupling of acceptors **8** or **15**[20] with donors **22** or **23** (Chart 2) was not successful. The glucuronic acid was instead obtained by oxidation of the corresponding glucoside. Thus glycosidation of **15** with **9** promoted by NIS and AgOTf gave **16** in 74% yield. The benzoyl groups were then removed and selective oxidation of the primary alcohol of the resulting intermediate using a TEMPO-sodium hypochlorite procedure[21] gave the carboxylic acid **17**. This acid was methylated using methyl iodide in DMF and subsequent acetylation gave **18**. Acetolysis of **18** followed by conversion of the product in two steps to the β-trichloroacetimidate **19** was achieved as for **13**. Glycosidation of **19** with MeOH using catalytic $BF_3{\cdot}Et_2O$ gave the desired α-anomer **20α** in 54% yield as well as the β-anomer (22%). A number of standard methods (e.g., catalytic hydrogenation) were investigated for the reduction of the azide group of **20α** but these proved unsatisfactory. The best result was achieved using sodium borohydride in presence of nickel(II) chloride hexahydrate and boric acid in EtOH.[22] Subsequently the amine that was generated was acetylated to give acetamide **21**. Finally the removal of the benzyl group from **21** by catalytic hydrogenation gave **5**, which had analytical data that agreed well with that reported previously (Scheme 2).[23]



Scheme 2. Synthesis of **5**. Reagents and conditions: (a) AgOTf, NIS, CH_2Cl_2, 74%; (b) NaOMe, MeOH; (c) TEMPO, NaOCl, KBr, THF, aq $NaHCO_3$; (d) MeI, DMF then Ac_2O, Py, 67% for four steps; (e) TFA, Ac_2O; (f) $BnNH_2$, Et_2O, 65% for two steps; (g) K_2CO_3, Cl_3CCN, CH_2Cl_2, 12 h, 81%; (h) MeOH, $BF_3{\cdot}OEt_2$, CH_2Cl_2, 54%, α-anomer; (i) $NaBH_4$, $NiCl_2[H_2O]_6$, $B(OH)_3$, EtOH, then Ac_2O, Py, 89%; (j) LiOH, MeOH/water/THF, 81%; (k) $Pd(OH)_2$, H_2, EtOH, water, 99%.

2.2. Biological evaluation of the disaccharides

2.2.1. Binding assay. Binding of disaccharides **4**, **5** and **21** to FGF-2 (100 ng/mL) was investigated in competition with heparin albumin (HA) by ELISA (Fig. 1).[13] Heparin albumin was found to have an IC_{50} of 46.2 ng/mL and the maximum inhibition observed was 80%; this contrasted with the disaccharides, which did not have any effect on binding.

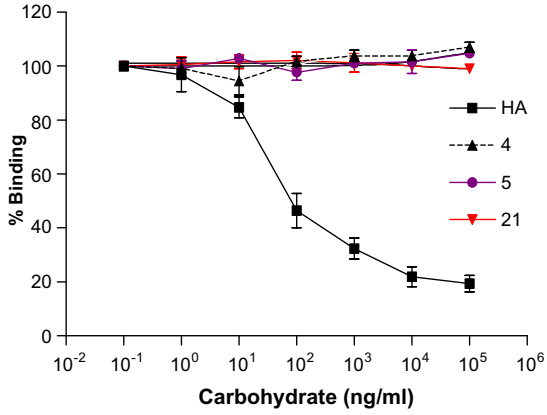


Figure 1. The binding of **4**, **5** and **21** to FGF-2 (100 ng/mL) in competition with HA (bound) by ELISA. Data points represent the mean of triplicate experiments, where each experiment was conducted in quadruplicate. HA was included as the standard inhibitor.

2.2.2. BAEC assays. The effects of the disaccharides on BAEC growth were also determined. The BAECs express both FGFR and heparan sulfate proteoglycans and release FGF-2 that drives proliferation (important in angiogenesis) and potently suppresses apoptotic cell death.[24] The effect of the disaccharides on BAEC proliferation in complete medium (containing serum) where the cells grow optimally was first investigated. The thiophene conjugates **6** and **7** and other monosaccharide conjugates inhibited BAEC growth under these conditions (~25% @ 10 μg/mL).[14b] However, the disaccharides did not inhibit growth over 72 h whereas HA did,

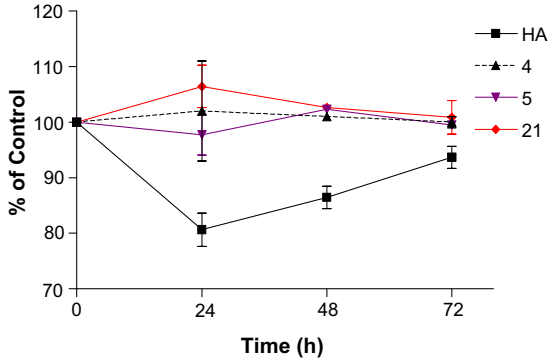


Figure 2. The effect of disaccharides on BAEC proliferation in complete medium. Data are the means of quadruplicate values. Heparin albumin (HA) was included as a standard inhibitor.

the maximum inhibition (19.6%) by HA occurring after 24 h (Fig. 2) with growth again increasing, albeit at a lower rate, between 24 and 72 h. A small increase in BAEC growth was observed for only disaccharide **21** after 24 h. In contrast to the inhibitory effect of HA, 24 h exposure to FGF-2 increased BAEC proliferation by 7% in complete medium ($p < 0.05$ vs control, *t*-test).

The effect of disaccharides on HA-mediated inhibition of BAEC growth in complete medium was also studied. The disaccharides did not alter the effect of HA (Fig. 3).

Conditions where the cells grow optimally (i.e., in complete medium) may not be suitable for the determination of the mitogenic activity of the disaccharides. It has been reported for bovine lens epithelial cells (BLEC) that the addition of heparin to serum-free medium containing FGF-2 increased cell proliferation, whilst the addition of heparin to serum containing medium caused inhibition.[25] The former conditions were thus investigated over 72 h to establish if disaccharides are mitogenic towards BAECs and the results are summarised in Figure 4. The addition of FGF-2 to cells grown in serum free medium caused a small but statistically significant increase in growth ($p < 0.001$, ANOVA followed by a post ANOVA Bonferroni test). Heparin albumin caused 33% inhibition of growth compared to the control ($p < 0.001$) but none of the disaccharides altered cell proliferation in serum free medium (Fig. 4).

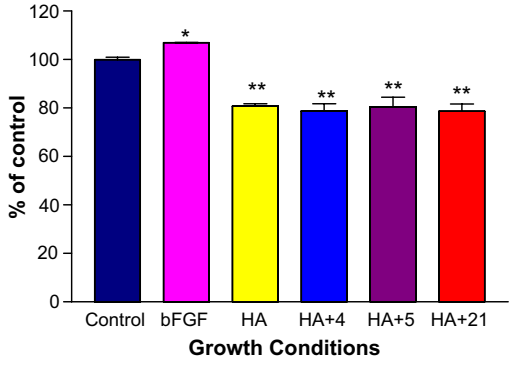


Figure 3. The effect of bFGF (10 ng/mL), HA (10 μg/mL) and HA (10 μg/mL) in combination with disaccharides (10 μg/mL) on BAEC proliferation in complete medium after 24 h. Data are the means of quadruplicate values. $^{**}p < 0.01$ versus control, ANOVA followed by a post-ANOVA Bonferroni test.

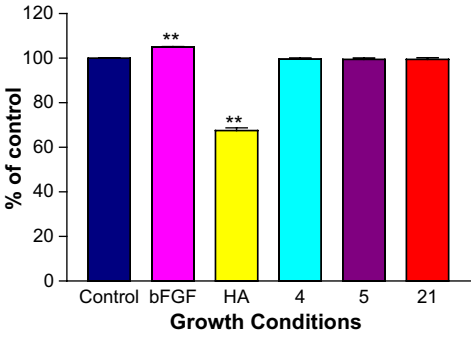


Figure 4. The effect of bFGF (10 ng/mL), HA (10 μg/mL), and disaccharides (10 μg/mL), on BAEC proliferation in serum-free medium after 24 h. Data are the means of quadruplicate values. Heparin albumin was included as a standard inhibitor. $^{**}p < 0.001$, ANOVA followed by post-ANOVA Bonferroni test.

In separate experiments, the effects of the disaccharides on the FGF-2-induced cell proliferation was examined at different time points and results are summarised in Figure 5. The addition of **5** or **22** did not alter the proliferative effect of FGF-2 whereas **4** weakly inhibited FGF-2-induced proliferation at the 24 h time point only. Heparin albumin displayed no effect on FGF-2-induced proliferation after 24 h but after 48 h it had reduced the number of viable cells to the level of the control.

Finally the disaccharides were investigated for their effect on heparin-mediated inhibition of BAEC proliferation in serum-free medium. In this case the maximum inhibition of cell growth exhibited by HA (33%) was decreased by all three disaccharides by 25–31% (Fig. 6).

Previous work has shown that the disaccharide **5** and other heparin derived di- and trisaccharides were mimics of heparin/heparan sulfate and were mitogenic towards

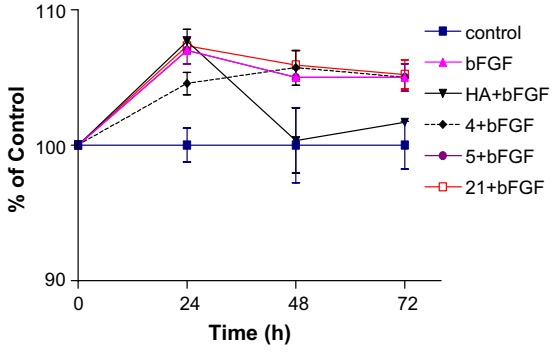


Figure 5. The effect of disaccharides and HA on BAEC proliferation induced by bFGF (10 ng/mL). Data are the means of triplicate values.

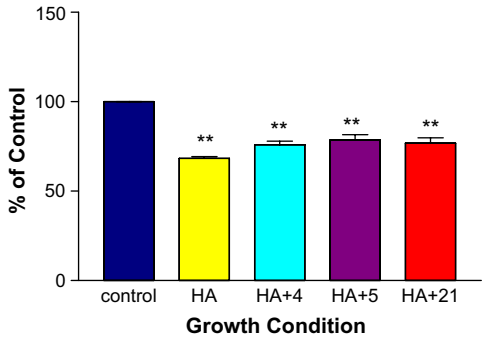


Figure 6. The effect of disaccharides (10 μg/mL) on HA (10 μg/mL), induced inhibition of BAEC proliferation in serum-free medium after 24 h. Data are the means of quadruplicate values. $^{**}p < 0.01$ versus control, ANOVA followed by post-ANOVA Bonferroni test.

F-32 cells in presence of FGF-2.[12] It is not possible to draw similar conclusions regarding the effects of heparin disaccharides on endothelial cell growth or survival based on the experiments described herein. The reasons why **5** has shown differing behaviour in the binding assay carried out by Ornitz et al. and in that described herein is unclear; this may be due to the different conditions employed in both studies. The different effects observed in growth assays may be explained to be due to different cell types being used in the experiments. It has been shown that binding of heparin to FGF and the FGF receptor can lead to internalisation of heparin by cells and this can lead to apoptosis.[26,27] It is clear, based on the experiments described herein, that the disaccharides are unable to mimic the effects of heparin as they do not bind to FGF and neither do they inhibit endothelial cell growth under a variety of conditions whereas heparin (HA) is an inhibitor in all cases.

3. Summary and conclusions

A route to disaccharide **5** has been reported, that is, suitable for synthesis of analogues with a view to investigation of their biological properties. Biological evaluation of the disaccharides has shown that, under a variety of conditions, they are not heparin mimics. It would seem that synthetic fragments larger than disaccharides will be required if agents are to be developed from heparin that will have potential application in angiogenesis therapy.

4. Experimental section

4.1. General experimental conditions

Optical rotations were determined with a Perkin–Elmer 241 or 341 model polarimeter at the sodium D line at 23 °C. NMR spectra were recorded with JEOL JNM-GX270 and Varian Inova 300 and 500 spectrometers. Chemical shifts are reported relative to internal Me_4Si in $CDCl_3$ (δ 0.0) or HOD for D_2O (δ 4.63) or CD_2HOD (δ 3.36) for ^1H and $CDCl_3$ (δ 77.0) or CD_3OD (δ 47.7) for ^{13}C. ^1H NMR signals were assigned with the aid of coupling constants are in hertz; data are reported in Tables 1 and 2. ^{13}C NMR signals were assigned with the aid of DEPT. IR spectra were recorded with a Mattson Galaxy Series FTIR 3000 using either thin film between NaCl plates or KBr discs, as specified. Melting points were measured on a Gallenkamp melting point apparatus. Elemental analysis was performed on an Exeter Analytical CE440 elemental analyser. Low- and high-resolution mass spectra were measured at the University of York, UK or on a Micromass LCT KC420 or on Micromass Quattro. TLC was performed on aluminium sheets precoated with Silica Gel 60 (HF254, E. Merck) and spots visualised by UV and charring with 1:20 H_2SO_4–EtOH. Flash column chromatography was carried out with Silica Gel 60 (0.040–0.630 mm, E. Merck) and employed a stepwise solvent polarity gradient correlated with the TLC mobility. Chromatography solvents used were EtOAc, MeOH, acetone and dichloromethane (Riedel-deHaen), petroleum ether (bp 40–60 °C, BDH laboratory supplies) and toluene (Aldrich). Reaction solvents were dried and distilled where stated.

4.2. 1,6-Anhydro-2-azido-2-deoxy-4-*O*-(2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl)-β-D-glucopyranose (10b)

Thioglycoside **9**[15,16] (12.5 g, 20.0 mmol, 2.5 equiv) and **8**[17] (1.5 g, 8.0 mmol, 1.0 equiv) were stirred in CH_2Cl_2 (30 mL) containing 4 Å molecular sieves under an inert atmosphere. After 20 min *N*-iodosuccinimide (7.2 g, 32.0 mmol) was added. Silver triflate (40 mg, 0.16 mmol) was added after a further 20 min. The reaction was allowed to stir overnight and was then quenched with Et_3N (1.5 mL). The soln was filtered, concentrated and purified by chromatography to provide the title compound **10b** as a white foam (1.73 g) and the trisaccharide **10a** (0.83 g). Analytical data for **10b**: $[\alpha]_D$ +11.6 (*c* 0.4, $CHCl_3$); R_f 0.47 (1:1 EtOAc–petroleum ether); IR (KBr) cm^{-1} 3463, 2921, 2109, 1718, 1452, 1378, 1252, 1191, 911, 706; ^{13}C NMR (75 MHz, $CDCl_3$): δ 166.6, 166.1, 165.5, 165.0 (each s), 134.0, 133.7, 133.6, 130.2, 130.0 (each d), 129.5, 129.3, 129.0, 128.9 (each s), 128.8, 128.7, 128.6, 101.7, 101.5, 83.8, 76.2, 72.9, 72.1, 72.0, 69.6, 67.1 (each d), 64.5, 63.1 (each t); FABMS *m/z* $[M+Na]^+$ calcd 788.2068, found 788.2072. Analytical data for **10a**: $[\alpha]_D$ +5.1 (*c* 1.2, $CHCl_3$); R_f 0.07 (1:3 EtOAc–petroleum ether); IR (KBr) cm^{-1} 3434, 2976, 2104, 1734, 1451, 1267, 1109, 713; ^{13}C NMR (75 MHz, $CDCl_3$): δ 166.4, 166.3,

Table 1. ^{1}H NMR chemical shifts for disaccharides

No	Solvent	Glucose or glucuronic acid residue							Glucosamine residue							Other protons
		H-1	H-2	H-3	H-4	H-5	H_a-6	H_b-6	H-1	H-2	H-3	H-4	H-5	H_a-6	H_b-6	
10b	$CDCl_3$	5.1	5.57	5.94	5.67	nd	4.8	nd	5.29	3.23	3.88	3.63	4.25	3.77	3.53	7.95–7.25 (Ar–H), 3.82 (OH)
11	$CDCl_3$	4.95	5.53	5.93	5.66	4.22	4.77	4.47–4.39	5.28	3.2	3.59	3.73	4.47–4.39	3.92	3.59	8.12–7.25 (Ar–H)
12α	$CDCl_3$	4.76	nd	nd	nd	nd	nd	nd	6.1	3.26	nd	3.81–3.67	3.81–3.67	4.20–4.05	4.20–4.05	7.98–6.88 (Ar–H), 2.03, 1.99, 1.97 (OAc)
12β	$CDCl_3$	nd	nd	nd	nd	4.20–4.05	nd	nd	nd	3.38	5.17	nd	3.47–3.43	4.20–4.05	4.20–4.05	7.98–6.88 (Ar–H), 2.03, 1.99, 1.97 (OAc)
13	$CDCl_3$	4.85	5.5	5.86	5.67–5.57	4.20–4.12	4.67–4.52	4.3	5.67–5.57	3.15	5.12	3.85	3.6	4.67–4.52	4.20–4.12	8.70 (NH), 2.06, 2.05 (OAc)
14α	$CDCl_3$	4.86	5.55–5.48	5.87	5.66	4.12–4.04	4.6	4.52	4.24	3.12	5.55–5.48	3.76–3.63	3.76–3.63	4.2	4.12–4.04	8.05–7.23 (Ar–H), 3.33 (OMe), 2.03, 2.02 (OAc)
14β	$CDCl_3$	4.76	5.42	5.78	5.56	4.08–4.04	4.53	4.44	4.08–4.04	3.2	4.91	3.67	3.39–3.35	4.24	4.08–4.04	8.05–7.23 (Ar–H), 3.20 (OMe), 1.96, 1.94 (OAc)
4	CD_3OD	4.44	3.21	nd	nd	nd	nd	nd	4.68	3.96	3.77	nd	nd	nd	nd	3.38 (OMe), 1.99 (NHAc)
16	$CDCl_3$	5.13	5.6	5.94	5.72	4.15	4.66–4.57	4.51	5.41	3.22	3.87–3.82	3.87–3.82	3.65	4.66–4.57	4.01	8.06–7.28 (Ar–H), 4.66–4.57 (CH_2Ph)
17	$CDCl_3$	4.52	3.29	3.37	3.56	3.65	—	—	5.58	3.61	4.01	3.95	3.74	4.8	4.22	7.40–7.20 (Ar–H), 4.73 (CH_2Ph)
18	$CDCl_3$	4.82	5.03	5.27	5.27	4.04	—	—	5.47	3.21	3.78	3.78	4.61	4.09	3.85	7.37–7.28 (Ar–H), 4.65 (CH_2Ph), 3.74 (OMe), 2.05, 2.04, 2.03 (OAc)
19	$CDCl_3$	4.72	5.06–4.98	5.21–5.16	5.21–5.16	3.90–3.84	—	—	5.58	3.58	3.65–3.62	3.90–3.84	4.16–4.10	4.46	3.65–3.62	8.73 (NH), 7.37–7.27 (Ar–H), 4.81 (C(H)HPh), 5.06–4.98 (C(H)HPh), 3.58–3.51 (OMe), 2.10, 2.05, 2.00, 2.00 (OAc)
20α	$CDCl_3$	4.83–4.78	5.09	5.31–5.23	5.31–5.23	4.01–3.90	—	—	4.83–4.78	3.4	4.01–3.90	3.89–3.80	3.89–3.80	4.53	4.21	7.32–7.17 (Ar–H), 5.17–5.13 (C(H)HPh), 4.83–4.78 (C(H)HPh), 3.56 (OMe), 3.48 (OMe), 2.18, 2.14, 2.08, 2.07 (OAc)
20β	$CDCl_3$	4.66	4.95–4.88	5.12–5.08	5.12–5.08	3.76–3.65	—	—	4.08	3.39–3.25	3.39–3.25	3.39–3.25	3.76–3.65	4.39	4.02	7.32–7.18 (Ar–H), 4.95–4.88 (CH_2Ph), 3.46 (OMe), 3.45 (OMe), 2.03, 1.98, 1.93, 1.92 (OAc)
21	D_2O	nd	3.87–3.70	3.53–3.44	3.53–3.44	3.6	—	—	4.61	3.3	3.87–3.70	3.87–3.70	3.87–3.70	3.93	3.87–3.70	7.35–7.24 (Ar–H), 4.86 (CH_2Ph), 3.30 (OMe), 1.81 (NHAc)
5	D_2O	4.54	3.36	3.53	3.81–3.84	3.53	—	—	4.78	3.93	3.84	3.7	3.81–3.84	3.94	3.88	3.38 (OMe), 2.03 (NHAc)

Table 2. ^{1}H,^{1}H coupling constants

No	Glucose or glucuronic acid residue							Glucosamine residue						
	$^3J(1,2)$	$^3J(2,3)$	$^3J(3,4)$	$^3J(4,5)$	$^3J(5,6_a)$	$^3J(5,6_b)$	$^2J_a(6_a,6_b)$	$^3J(1,2)$	$^3J(2,3)$	$^3J(3,4)$	$^3J(4,5)$	$^3J(5,6_a)$	$^3J(5,6_b)$	$^2J(6_a,6_b)$
10b	8	9.6	9.6	9.6	2.5	nd	12	nd	5	5	nd	nd	3.5	7.5
11	8	10	10	10	2.6	nd	12	nd	5	1	5	nd	nd	7
12α	8	nd	nd	nd	nd	nd	nd	4	10	nd	nd	nd	nd	nd
12β	nd	nd	nd	nd	nd	nd	nd	8	10	nd	nd	nd	nd	nd
13	8	9.5	9.5	nd	nd	nd	11	8.5	9.5	9.5	9.5	2	nd	nd
14α	8	9.5	9.5	9.5	3	5	12	4	10	nd	nd	2	nd	12
14β	8	9.5	9.5	9.5	3	5	12	8	10	10	10	2	nd	12
4	8	8	nd	nd	nd	nd	nd	4	10	8.5	nd	nd	nd	nd
16	7.9	9.6	9.6	9.6	5.2	nd	12	nd	nd	nd	nd	7.5	5	12
17	7.6	9	2	9	—	—	—	nd	nd	nd	nd	nd	6	8
18	7.7	nd	nd	9.5	—	—	—	nd	nd	nd	nd	7.5	nd	nd
19	8	nd	nd	nd	—	—	—	8	nd	nd	nd	nd	nd	12
20α	8	8	nd	9.6	—	—	—	3.5	10	nd	8.2	nd	4	11.4
20β	8	nd	nd	nd	—	—	—	8	nd	nd	nd	1.8	5.2	12
21	8	nd	nd	10	—	—	—	4	nd	nd	nd	4	nd	10
5	7.8	nd	nd	nd	—	—	—	4	nd	9	9	nd	nd	nd

166.0, 165.9, 165.6, 165.5, 165.3, 165.1 (each s), 133.7, 133.7, 133.5, 133.4, 130.2, 130.2, 130.1, 130.0, 129.9 (each d), 129.9, 129.7, 129.5, 129.3, 129.2, 129.1, 129.1 (each s), 129.0, 128.9, 128.7, 128.7, 128.6, 128.6, 128.5, 101.4, 101.2, 100.2, 77.4, 76.5, 74.5, 73.2, 72.8, 72.6, 72.2, 71.9, 70.0, 69.6, (each d), 64.7, 63.0, 62.1 (each t); ESMS *m/z* $[M+Na]^+$ calcd 1366.3623, found 1366.3644.

4.3. 1,6-Anhydro-2-azido-3-*O*-acetyl-2-deoxy-4-*O*-(2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl)-β-D-glucopyranose

Azide **10b** (40 mg, 0.052 mmol) was suspended in Ac_2O (1 mL) and pyridine (1 mL). The soln was stirred for 12 h at rt, concentrated to dryness under diminished pressure and the resulting residue purified by flash chromatography to give the title compound (quantitative yield); $[\alpha]_D$ +17.8 (*c* 1.2, $CHCl_3$); R_f 0.54 (1:1 EtOAc–petroleum ether); IR (KBr) cm^{-1} 3465, 2901, 2105, 1728, 1515, 1253, 1070, 1027, 707; ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.8, 166.2, 166.1, 165.5, 165.2 (each s), 133.7, 133.4, 133.3, 130.1, 130.1 (each s), 130.0, 130.0, 129.9 (each d), 129.5, 129.1 (each s), 128.7, 128.6, 128.5, 101.3, 100.3, 76.6, 74.4, 73.3, 72.9, 72.1, 70.6, 69.9 (each d), 65.2, 63.3 (each t), 59.0 (d), 21.0 (q); FABMS *m/z* $[M+Na]^+$ calcd 830.2173, found 830.2176.

4.4. 1,3,6-Tri-*O*-acetyl-2-azido-2-deoxy-4-*O*-(2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl)-β-D-glucopyranose (12)

A soln of 1,6-anhydro-2-azido-3-*O*-acetyl-2-deoxy-4-*O*-(2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl)-β-D-glucopyranose (0.60 g, 0.79 mmol) in Ac_2O (4.5 mL) and trifluoroacetic acid (0.5 mL) was stirred for 12 h at rt, the solvent was removed under diminished pressure and the resulting residue purified by flash chromatography to yield the title compound **12** as a white solid (0.67 g, quantitative); mp 79–82 °C; $[\alpha]_D$ +37.4 (*c* 0.48, $CHCl_3$); R_f 0.66 (1:1 EtOAc–petroleum ether); IR (KBr) cm^{-1} 3463, 2930, 2112, 1736, 1584, 1436, 1370, 1266, 1095, 916, 697; ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.4, 169.9, 168.9, 166.3, 165.9, 165.4, 164.9 (each s), 133.8, 133.7, 133.5, 139.2, 130.1, 130.0, 130.0 (each d), 129.7, 128.9 (each s), 128.8, 128.7, 128.5, 101.4, 90.1, 75.9, 73.2, 72.6, 72.2, 70.9, 70.0, 69.8 (each d), 63.4, 61.6 (each t), 21.2, 21.0, 20.9 (each q); FABMS *m/z* $[M+Na]^+$ calcd 932.2497, found 932.2499. Anal. Calcd for $C_{46}H_{43}N_3O_{17}$: C, 60.72; H, 4.76; N, 4.62. Found: C, 60.60; H, 4.85; N, 4.29.

4.5. 3,6-Di-*O*-acetyl-2-azido-2-deoxy-4-*O*-(2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl)-β-D-glucopyranose

Benzylamine (0.12 mL, 1.1 mmol, 1.5 equiv) was added to a stirred soln of **12** (0.67 g, 0.79 mmol) in THF. After 12 h, the mixture was concentrated, and CH_2Cl_2 (150 mL) was added. The reaction was washed with 1 M HCl, dried over anhyd $MgSO_4$ and the solvent evaporated to give the title compound as a white solid (quantitative yield); mp 84–88 °C; $[\alpha]_D$ +24.3 (*c* 1.3, $CHCl_3$); R_f 0.43 (1:1 EtOAc–petroleum ether); IR (KBr) cm^{-1} 2359, 2113, 1739, 1647, 1268, 1178, 1069, 918; ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.8, 170.7, 170.0, 169.9, 166.3, 166.0, 166.0, 165.4, 164.9 (each s), 133.8, 133.6, 133.5, 130.1, 130.0 (each d), 129.6, 129.6, 128.9, 128.9 (each s), 128.8, 128.7, 128.5, 101.1, 101.0 (each d), 96.1, 92.2, 76.6, 76.2, 73.2, 73.2, 73.1, 72.6, 72.2, 71.8, 69.8, 69.6, 68.7 (each d), 65.4, 62.1 (each t), 21.0, 20.9 (each q); FABMS *m/z* $[M+Na]^+$ calcd 890.2385, found 890.2390.

4.6. 3,6-Di-*O*-acetyl-2-azido-2-deoxy-4-*O*-(2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl)-β-D-glucopyranosyl trichloroacetimidate (13)

A soln of 3,6-di-*O*-acetyl-2-azido-2-deoxy-4-*O*-(2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl)-β-D-glucopyranose (0.62 g, 0.72 mmol) in dry CH_2Cl_2 (4 mL) was stirred at rt for 5 min in the presence of 4 Å molecular sieves. Dry potassium carbonate (0.2 g, 0.14 mmol) and trichloroacetonitrile (0.65 mL, 6.4 mmol) were added and the reaction was allowed to stir for 12 h. The solvent was removed and the residue purified by chromatography to give **13** as an off-white solid (0.23 g, 28%, 75% based on recovered starting material); $[\alpha]_D$ +4.4 (*c* 0.23, $CHCl_3$); R_f 0.41 (1:2 EtOAc–petroleum ether); IR (KBr) cm^{-1} 3363, 2948, 2867, 2190, 1758, 1693, 1614, 1309, 1079, 692; ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.3, 169.6, 166.2, 165.8, 165.3, 164.7 (each s), 160.6 (s), 133.6, 133.4 (each d), 130.0, 129.9 (each s), 129.5, 128.7, 128.7, 128.6, 128.5, 101.1, 96.4, 75.5, 73.6, 73.1, 72.6, 72.1, 71.9, 69.7, 63.7 (each d), 63.3, 61.6 (each t), 20.9, 20.8 (each q, each OAc); ESMS *m*/*z* $[M+Na]^+$ calcd 1033.15, found 1033.0.

4.7. Methyl 3,6-di-*O*-acetyl-2-azido-2-deoxy-4-*O*-(2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl)-α-D-glucopyranoside (14α)

A soln of MeOH (0.1 mL, 5.0 mmol, 10.0 equiv) and boron trifluoride-diethyl-ether (0.01 mL, 0.05 mmol, 0.1 equiv) in anhyd CH_2Cl_2 (4.0 mL) over 4 Å molecular sieves was stirred under a nitrogen atmosphere for 10 min at 0 °C. A 0.6 M soln of **13** (489 mg, 0.5 mmol) in anhyd CH_2Cl_2 was added dropwise to the reaction over 10 min. The mixture was allowed to stir at rt for 12 h, filtered through Celite, diluted with CH_2Cl_2 (50 mL) and washed with satd $NaHCO_3$. The organic layer was dried over anhyd $MgSO_4$, the solvent removed and the resulting residue purified by flash chromatography to give the title compound **14α** as an off-white solid (0.10 g, 25%) as well as its β-anomer **14β** (0.02 g, 5%); $[\alpha]_D$ +55.8 (*c* 0.22, $CHCl_3$); R_f 0.70 (EtOAc–petroleum ether, 1:1); IR (KBr) cm^{-1} 3437, 2935, 2394, 2110, 1735, 1454, 1371, 1095, 594; ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.5, 169.9, 166.3, 165.9, 165.3, 164.8 (each s), 133.6, 133.5, 130.1, 130.0, 129.9, 129.8 (each d), 129.6, 128.9 (each s), 128.7, 128.6, 128.5, 101.1, 98.8, 76.5, 73.2, 72.5, 72.1, 69.5, 68.5 (each d), 63.2, 62.0 (each t), 55.5, 20.9 (each q); FABMS *m*/*z* $[M+Na]^+$ calcd 904.2541, found 904.2542. Analytical data for **14β**: R_f 0.58 (1:1 EtOAc–petroleum ether); $[\alpha]_D$ −1.9 (*c* 0.95, $CHCl_3$); IR (KBr) cm^{-1} 3441, 2925, 2394, 2113, 1735, 1624, 1451, 1267, 1095, 790; ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.3, 169.1, 166.1, 165.7, 165.2, 164.5 (each s), 133.5, 133.5, 133.3, 129.9, 129.9, 129.8, 129.7 (each d), 129.4, 128.7, 128.7 (each s), 128.6, 128.5, 128.5, 128.3, 102.8, 101.0, 76.3, 73.1, 73.0, 72.6, 72.2, 71.8, 64.4 (each d), 63.3, 62.1 (each t), 57.5 (q), 20.8, 20.7 (each q); FABMS *m*/*z* $[M+Na]^+$ calcd 904.2541, found 904.2535.

4.8. Methyl 3,6-*O*-acetyl-2-azido-2-deoxy-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-α-D-glucopyranoside

To a stirred soln of compound **14α** (40.0 mg, 0.05 mmol) in MeOH (5.0 mL) was added NaOMe (0.1 mL, 0.25 M). After 12 h the soln was diluted with MeOH (20 mL), and the pH adjusted to 5.0 with Amberlite IR120 (H^+). The Amberlite was removed by filtration and solvent evaporated under diminished pressure. The resulting residue was stirred in pyridine (1.0 mL) and Ac_2O for 3 h. On completion the solvent was removed under diminished pressure and the resulting residue purified by flash chromatography to give the title compound as an off-white solid (27.0 mg, 86%); R_f 0.20 (1:2 EtOAc–petroleum ether); ^1H NMR (300 MHz, $CDCl_3$): δ 5.44 (t, 1H, *J* 10.0), 5.15–5.08 (overlapping signals, 2H), 4.93 (t, 1H, *J* 8.0), 4.82 (d, 1H, *J* 8.0), 4.52 (d, 1H, *J* 8.0), 4.46 (dd, 1H, *J* 2.0, 12.0), 4.39 (dd, 1H, *J* 4.0, 12.0), 4.15 (dd, 1H, *J* 5.0, 12.0), 4.05 (dd, 1H, *J* 2.0, 12.0), 3.96–3.88 (m, 1H), 3.73–3.63 (m, 2H), 3.44 (s, 3H), 3.20 (dd, 1H, *J* 10.0, 4.0), 2.12, 2.09 (2s), 2.03, 2.01, 1.98 (each s, each 3H).

4.9. Methyl 2-acetamido-3,6-di-*O*-acetyl-2-deoxy-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-α-D-glucopyranoside

Palladium on carbon (14.0 mg) was added to a stirred soln of methyl 3,6-di-*O*-acetyl-2-azido-2-deoxy-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-α-D-glucopyranoside (27.0 mg, 0.05 mmol) in Ac_2O (4.0 mL) under an atmosphere of hydrogen. After 12 h the reaction was passed through a pad of Celite, which was subsequently washed with hot MeOH. The solvent was removed under diminished pressure and the resulting residue purified by flash chromatography to give the title compound as a yellow syrup (16.6 mg, 59%); $[\alpha]_D$ +12.0 (*c* 0.83, $CHCl_3$); R_f 0.42 (toluene–EtOH, 6:1); IR (KBr) cm^{-1} 3456, 2924, 2853, 2360, 1743, 1657, 1362, 1238, 1059, 796, 668; ^1H NMR (300 MHz, $CDCl_3$): δ 5.71 (d, 1H, *J* 9.5), 5.21–5.02 (overlapping signals, 2H), 4.92 (t, 1H, *J* 8.0), 4.65 (d, 1H, *J* 4.0), 4.53 (d, 1H, *J* 8.0), 4.48 (dd, 1H, *J* 12.0, 2.0), 4.36 (dd, 1H, *J* 12.0, 4.0), 4.20 (m, 1H), 4.12 (dd, 1H, *J* 12.0, 4.0), 4.03 (dd, 1H, *J* 12.0, 2.0), 3.81–3.75 (overlapping signals, 2H), 3.68–3.62 (overlapping signals, 2H), 3.37 (s, 3H), 2.12, 2.08, 2.04, 2.03, 2.00, 1.98, 1.94 (each s, each 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.3, 170.7, 170.6, 170.5, 170.3, 169.6, 169.4 (each s), 101.1, 98.4, 73.2, 72.2, 72.0, 71.4, 68.7,

68.1 (each d), 62.1, 61.9 (each t), 55.6 (q), 23.4, 22.9, 21.1, 20.9, 20.8, 20.8 (each q); ESMS *m/z* $[M+Na]^+$ calcd 672.6, found 672.0.

4.10. Methyl 2-acetamido-2-deoxy-4-*O*-(β-D-glucopyranosyl)-α-D-glucopyranoside (4)

Sodium methoxide (0.1 mL, 0.25 M) was added to a stirred soln of methyl 2-acetamido-3,6-di-*O*-acetyl-2-deoxy-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-α-D-glucopyranoside (21.5 mg, 0.04 mmol) in MeOH (3.0 mL). After 12 h the soln was diluted with MeOH (20.0 mL), and the pH adjusted to 5.0 with Amberlite IR120 (H^+). The Amberlite was removed by filtration and solvent evaporated. The resulting residue was purified by flash chromatography to give **4** as a heavy syrup (10.4 mg, 81%); $[\alpha]_D$ +16.8 (*c* 0.57, $CHCl_3$); ^{13}C NMR (125 MHz, CD_3OD): δ 176.3 (s), 107.5, 102.4, 84.2, 80.9, 80.9, 80.7, 77.8, 74.9, 74.2, 74.0 (each d), 65.2, 64.6 (each t), 58.4 (d), 57.8 (q), 20.9 (q); ESMS *m/z* $[M+Na]^+$ calcd 420.1482, found 420.1487.

4.11. 1,6-Anhydro-2-azido-3-*O*-benzyl-2-deoxy-4-*O*-(2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl)-β-D-glucopyranose (16)

Thioglycoside **9** (13.8 g, 22.0 mmol, 2 equiv) and **15** (3.0 g, 11.0 mmol, 1.0 equiv) were stirred in CH_2Cl_2 (40 mL) containing 4 Å molecular sieves under an inert atmosphere. After 20 min *N*-iodosuccinimide (6.4 g, 28.0 mmol) was added, after a further 20 min AgOTf (113 mg, 0.44 mmol) was added to the reaction mixture. The reaction was allowed to stir overnight and was then quenched with Et_3N (1.5 mL). The soln was filtered, concentrated and purified by chromatography to provide the title compound as a white solid (7.0 g, 74%); mp 66–71 °C; $[\alpha]_D$ +24.7 (*c* 0.3, $CHCl_3$); IR (KBr) cm^{-1} 3426, 2973, 2102, 1730, 1452, 1265, 1098, 713, 622; ^{13}C NMR (75 MHz, $CDCl_3$): δ 166.3, 166.1, 165.4, 165.2 (each s), 137.6 (d), 133.8 (s), 133.5, 133.5, 130.1, 130.1, 130.0, 130.0 (each d), 129.7, 129.5, 129.0 (each s), 128.8, 128.7, 128.6, 128.6, 128.2, 128.0 (each d), 100.8, 100.2, 77.4, 76.6, 74.2, 73.1, 72.8 (each d), 72.8 (t), 72.1, 69.7 (each d), 65.4, 63.1 (each t), 59.8 (d). Anal. Calcd for $C_{47}H_{41}N_3O_{13}$: C, 65.96; H, 4.83; N, 4.91. Found: C, 65.72; H, 4.85; N, 4.51.

4.12. 1,6-Anhydro-2-azido-3-*O*-benzyl-2-deoxy-4-*O*-(β-D-glucopyranosyl)-β-D-glucopyranose

Disaccharide **16** (4.0 g, 4.6 mmol) was suspended in a soln of MeOH (80 mL) and THF (20 mL). Sodium methoxide (0.5 mL of a 0.25 M soln) was added and the reaction mixture was allowed to stir at rt. TLC analysis (1:4 MeOH–EtOAc) showed that the reaction was complete after 2 days. Amberlite (H^+) was added and after 5 min the reaction mixture was filtered and the solvent removed to give the title compound as a heavy syrup (2.0 g, quantitative); $[\alpha]_D$ +13.9 (*c* 0.36, CH_3CN); IR (KBr) cm^{-1} 3410, 2921, 2110, 1739, 1647, 1647, 1259, 1082, 802, 617; ^{13}C NMR (75 MHz, CD_3OD): δ 138.3 (s), 128.3, 127.9, 127.7, 103.3, 100.6, 77.3, 76.9, 76.7, 76.2, 75.0, 73.8 (each d), 72.3 (t), 70.4 (d), 65.1, 61.7 (each t), 60.5; ESMS *m/z* $[M+NH_4]^+$ calcd 457.1935, found 457.1939.

4.13. 1,6-Anhydro-2-azido-3-*O*-benzyl-2-deoxy-4-*O*-(β-D-glucopyranosyluronic acid)-β-D-glucopyranose (17)

A soln of 1,6-anhydro-2-azido-3-*O*-benzyl-2-deoxy-4-*O*-(β-D-glucopyranosyl)-β-D-glucopyranose (2.0 g, 4.6 mmol), KBr (49.0 mg, 0.41 mmol) and 2,2,6,6-tetramethylpiperdine-1-oxide (TEMPO, 45.0 mg, 0.3 mmol), in satd aq 100:15 $NaHCO_3$–THF (100:15, 115 mL) was stirred at 0 °C while a soln of sodium hypochlorite [30 mL, 39.0 mmol, 8.5 equiv, (1.3 M, Aldrich)] was added dropwise. The reaction was the allowed to attain rt, and monitored by TLC (R_f 0.4, 10:9:2 *i*-PrOH–$MeNO_2$–water). After 30 min the THF was removed under diminished pressure at 30 °C, and the remaining soln lyophilised to give **17** as a white solid; $[\alpha]_D$ −6.4 (*c* 0.7, water); IR (KBr) cm^{-1} 3435, 2922, 2391, 2144, 1652, 1261, 1072; ^{13}C NMR (75 MHz, D_2O): δ 175.6, 137.1 (each s), 129.1, 128.9, 128.7, 102.9, 99.6, 77.2, 76.1 (each d), 75.5 (t), 75.2, 74.7, 72.7, 72.1, 65.1 (each d), 60.0 (t); FABMS *m/z* $[M-H+2Na]^+$ calcd 498.1101, found 498.1096.

4.14. 1,6-Anhydro-2-azido-3-*O*-benzyl-2-deoxy-4-*O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate)-β-D-glucopyranose (18)

Acid **17** was suspended in anhyd DMF (30.0 mL) and stirred with methyl iodide (0.37 mL, 5.9 mmol, 1.3 equiv) for 12 h. After completion of the methylation [the ester had R_f 0.6 (2:1 EtOAc–MeOH)] Ac_2O (5.0 mL) and DMAP (40 mg) were added and stirring continued for a further 12 h at rt. The reaction was diluted with water (100 mL) and extracted with EtOAc (3 × 100 mL). The combined extracts were dried over anhyd $MgSO_4$ and concentrated under diminished pressure, the resulting residue was purified by flash chromatography to give the title compound **18** as white solid (1.68 g, 67%); mp 87–90 °C; $[\alpha]_D$ −12 (*c* 0.23, CH_3CN); R_f 0.48 (4:1 toluene–acetone); IR (KBr) cm^{-1} 3457, 2918, 2107, 1758, 1437, 1377, 1255, 1046, 631; ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.4, 169.6, 169.4, 167.2, 137.6 (each s), 128.8, 128.7, 128.2, 128.0, 101.0, 99.2, 76.9, 75.9, 73.7 (each d), 72.8 (t), 72.3, 71.4, 69.4 (each d), 65.3 (t), 59.8, 53.2, 20.8, 20.7 (each q). Anal. Calcd for $C_{26}H_{31}N_3O_{13}$: C, 52.61; H, 5.26; N, 7.08. Found: C, 52.75; H, 5.30; N, 6.58.

4.15. 1,6-Di-*O*-acetyl-2-azido-3-*O*-benzyl-2-deoxy-4-*O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate)-D-glucopyranose

A soln of **18** (1.67 g, 2.8 mmol) in Ac_2O (4.5 mL) and trifluoroacetic acid (0.5 mL) was stirred for 12 h at rt, the solvent removed under diminished pressure and the resulting residue purified by flash chromatography to yield the title compound as a white solid (1.96 g, quantitative); mp 40–45 °C; $[\alpha]_D$ +24.0 (*c* 0.28, $CHCl_3$); R_f 0.50 (4:1 toluene–acetone); IR (KBr) cm^{-1} 3462, 2930, 2393, 2114, 1758, 1457, 1373, 1218, 1030, 775; ^1H NMR (300 MHz, $CDCl_3$, 5:2 mixture of α:β anomers): δ 7.39–7.26 (m, 5H, Ar–H), 6.18 (d, 1H, *J* 4.0), 5.45 (m, 1H), 5.22–5.20 (m, 2H), 5.12 (d, 1H, *J* 11.4), 5.04–5.00 (m, 1H), 4.80–4.71 (m, 2H), 4.42 (d, 1H, *J* 12.0), 4.12 (d, 1H, *J* 12.0), 3.89–3.84 (m, 3H), 3.55 (overlapping signals, 5H), 2.16, 2.10, 2.07, 2.01, 1.99 (each s, each 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.3, 169.0, 167.7, 165.6, 137.1 (each s), 127.3, 126.5, 126.3, 99.9, 99.7, 89.1, 77.3, 77.2 (each d), 74.2 (t), 72.4, 71.7, 71.1, 70.7, 70.6, 69.7, 68.2, 63.7, 61.4 (each d), 60.6 (t), 51.7 (q), 19.9, 19.8, 19.6, 19.5, 19.4 (each q); FABMS *m/z* $[M+Na]^+$ calcd 718.2072, found 718.2067.

4.16. 6-*O*-Acetyl-2-azido-3-*O*-benzyl-2-deoxy-4-*O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate)-D-glucopyranose

Benzylamine (11.2 mL, 2.6 mmol, 39 equiv) was added to a soln of 1,6-di-*O*-acetyl-2-azido-3-*O*-benzyl-2-deoxy-4-*O*-(methyl 2,3,4-tri-*O*-β-D-glucopyranosyluronate)-β-D-glucopyranose in anhyd diethyl ether at 0 °C. After 1 h, the solvent was removed, and CH_2Cl_2 (150 mL) was added. The soln was washed with 1 M HCl, dried over anhyd $MgSO_4$ and the solvent evaporated to give the title compound as a white foam (1.1 g, 65%); $[\alpha]_D$ +30 (*c* 0.3, $CHCl_3$); R_f 0.27 (1:1 EtOAc–petroleum ether); IR (KBr) cm^{-1} 3424, 2939, 2115, 1751, 1648, 1383, 1229, 1074, 869, 688; ^1H NMR (300 MHz, $CDCl_3$, 1.5:1 mixture of α and β anomers): δ 7.39–7.26 (m, 5H), 5.25 (d, 1H, *J* 4.0), 5.22–4.95 (overlapping signals, 5H), 4.82 (d, 1H, *J* 11.0), 4.74 (d, 1H, *J* 8.0), 4.58 (d, 1H, *J* 8.0), 4.50–4.45 (overlapping signals), 4.13–4.06 (overlapping signals), 3.96 (d, 1H, *J* 10.0), 3.87–3.71 (overlapping signals), 3.51 (s, 3H), 3.50 (s, 3H, OCH_3), 3.37–3.31 (overlapping signals), 2.10, 2.05, 2.00, 1.98 (each s, each 3H), 1.89 (s, 1H, OH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.8, 170.3, 169.6, 169.6, 169.5, 167.0, 167.0, 138.6, 138.3 (each s), 129.0, 128.6, 128.1, 127.9, 127.7, 127.4, 101.0, 96.3, 92.1, 81.2, 79.0, 78.6, 78.1 (each d), 75.3, 75.1 (each t), 72.9, 72.9, 72.8, 72.4, 72.0, 69.5, 68.7, 67.5, 63.9 (each d), 62.6, 62.3 (each t), 53.0, 21.1, 21.1, 20.8, 20.7 (each q); FABMS *m/z* $[M+Na]^+$ calcd 676.1966, found 676.1965. Anal. Calcd for $C_{28}H_{35}N_3O_{13}$: C, 51.45; H, 5.40; N, 6.43. Found: C, 51.27; H, 5.34; N, 6.22.

4.17. [6-*O*-Acetyl-2-azido-3-*O*-benzyl-2-deoxy-4-*O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate)-β-D-glucopyranosyl] trichloroacetimidate (19)

A soln of 6-*O*-acetyl-2-azido-3-*O*-benzyl-2-deoxy-4-*O*-(methyl 2,3,4-tri-*O*-β-D-glucopyranosyluronate)-β-D-glucopyranose (0.9 g, 1.37 mmol) in dry CH_2Cl_2 (4 mL) was stirred at rt for 5 min in the presence of 4 Å molecular sieves. Dry potassium carbonate (0.57 g, 4.1 mmol, 3 equiv) and trichloroacetonitrile (1.24 mL, 12.4 mmol, 9 equiv) were added and the reaction was allowed to stir for 12 h. The mixture was concentrated and the residue purified by chromatography to give **19** as an off-white solid (0.74 g, 81%); $[\alpha]_D$ +1.0 (*c* 0.21, $CHCl_3$); R_f 0.62 (1:1 EtOAc–petroleum ether); IR (KBr) cm^{-1} 3439, 2936, 2392, 2116, 1758, 1663, 1369, 1244, 1053, 616; ^{13}C NMR(75 MHz, $CDCl_3$): δ 169.3, 169.0, 168.3, 168.2, 165.6, 159.8, 137.1 (each s), 127.3, 126.6, 126.4, 99.7, 95.4, 79.9, 76.9 (each d), 74.1 (t), 72.3, 71.6, 71.1, 70.6, 68.2, 64.3 (each d), 60.8 (t), 51.8, 19.8, 19.5, 19.4 (each q); ESMS *m/z* $[M+Na]^+$ calcd 819.1, found 819.0.

4.18. Methyl 6-*O*-acetyl-2-azido-3-*O*-benzyl-2-deoxy-4-*O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate)-α-D-glucopyranoside (20α)

A soln of MeOH (0.3 mL, 0.92 mmol, 10 equiv) and boron trifluoride-diethyl ether (0.017 mL, 0.09 mmol, 0.1 equiv) in anhyd CH_2Cl_2 (4.0 mL) over 4 Å molecular sieves was stirred under a nitrogen atmosphere for 10 min at 0 °C. A soln of **19** [736 mg, 0.92 mmol, 1 equiv, 0.6 M in anhyd CH_2Cl_2] was added dropwise to the reaction over 10 min. The reaction was allowed to stir at rt for 12 h, filtered through a pad of Celite, diluted with CH_2Cl_2 (50 mL) and washed with satd $NaHCO_3$. The organic layer was dried over anhyd $MgSO_4$, concentrated and the resulting residue purified by flash chromatography to give the title compound **20α** as an off-white solid (331 mg, 54%) as well as the β-anomer **20β** (136 mg, 22%). Analytical data for **20α**: $[\alpha]_D$ +58 (*c* 0.25, $CHCl_3$); R_f 0.14 (1:2 EtOAc–petroleum ether); IR (KBr) cm^{-1} 3445, 2956, 2363, 2110, 1455, 1362, 1250, 1102, 892; ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.4, 169.1, 168.2, 165.6, 137.3 (each s), 127.3, 126.4, 99.8, 97.5, 77.8, 77.1 (each d), 74.0 (t), 71.7, 71.7, 71.2, 70.7, 68.2, 67.4, 62.2 (each d), 61.0 (t), 54.4, 51.7, 19.8, 19.6, 19.5, 19.4 (each q); FABMS *m/z* $[M+Na]^+$ calcd 690.2122, found 690.2120. Analytical data for **20β**: R_f 0.16 (1:2 EtOAc–petroleum ether); $[\alpha]_D$ −7.0 (*c* 0.26, $CHCl_3$); IR (KBr) cm^{-1} 3470, 2962, 2114, 1375, 1261, 1098, 801; ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.4, 169.0, 168.3, 168.3, 165.6 (each s), 137.4 (q), 101.8, 99.8, 80.0, 77.5 (each d), 73.8 (t), 71.5, 71.5, 71.1, 70.7, 68.2,

65.1 (each d), 61.2 (t), 56.2 (d), 51.7, 19.8, 19.5, 19.4 (each q); FABMS *m/z* $[M+Na]^+$ calcd 690.2122, found 690.2125.

4.19. Methyl 2-acetamido-6-*O*-acetyl-3-*O*-benzyl-2-deoxy-4-*O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate)-α-D-glucopyranoside

A soln of sodium borohydride (15 mL, 0.25 M (EtOH), 9.6 equiv) was added dropwise to a stirred soln of **20α** (0.25 g, 0.4 mmol, 1 equiv), nickel(II) chloride hexahydrate (0.99 g, 4.2 mmol, 10.5 equiv) and boric acid (0.49 g, 7.9 mmol, 20 equiv) in EtOH (30 mL). On completion of the addition the soln went from a light green to permanent black colour and after 30 min the reaction was judged complete by TLC analysis (R_f amine = 0.04, EtOAc–CH_2Cl_2, 2:1). The soln was filtered through a pad of Celite, and the solvent removed under diminished pressure. The residue was stirred for 12 h in Ac_2O (2 mL) and pyridine (2 mL). The reaction was concentrated and the resulting residue purified by flash chromatography (initially CH_2Cl_2, followed by a gradient 3:1 to 1:1 CH_2Cl_2–EtOAc) to give the title compound as an off-white solid (51.0 mg, 89%); $[\alpha]_D$ +41.0 (*c* 0.73, $CHCl_3$); R_f 0.38 (2:1 EtOAc–CH_2Cl_2); IR (KBr) cm^{-1} 3447, 2967, 1754, 1667, 1535, 1458, 1368, 1261, 1114, 1044; ^1H NMR (300 MHz, $CDCl_3$): δ 7.37–7.26 (m, 5H), 5.23–5.18 (m, 2H), 5.16–5.03 (m, 2H), 5.00 (d, 1H, *J* 12.6), 4.68 (d, 1H *J* 8.0), 4.62 (d, 1H, *J* 4.0), 4.55 (d, 1H, *J* 12.6), 4.46 (1H, dd, *J* 2.0, 11.2), 4.17–4.08 (m, 2H), 3.92 (d, 1H, *J* 9.0), 3.83–3.70 (m, 2H), 3.61 (dd, 1H, *J* 11.2, *J* 9.0), 3.56 (s, 3H, OCH_3), 3.30 (s, 3H, OCH_3), 2.12, 2.07, 2.01, 2.0, 1.73 (each s, each 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.5, 169.0, 168.8, 168.4, 168.4, 165.8, 137.9 (each s), 127.4, 126.9, 126.5, 100.0, 97.2, 78.4, 76.7 (each d), 73.8 (t), 71.5, 71.3, 70.7, 68.3, 65.6 (each d), 61.0 (t), 54.2 (d), 51.7, 51.2, 22.3, 19.9, 19.6, 19.5, 19.5 (each q); FABMS *m/z* $[M+Na]^+$ calcd 706.2323, found 706.2321.

4.20. Methyl 2-acetamido-3-*O*-benzyl-2-deoxy-4-*O*-(β-D-glucopyranosyluronic acid)-α-D-glucopyranoside (21)

A soln of 0.05 M LiOH in 2.5:1.0:0.5 MeOH–water–THF (20.6 mL, 6.0 equiv) was added to methyl 2-acetamido-6-*O*-acetyl-3-*O*-benzyl-2-deoxy-4-*O*-(methyl 2,3,4-tri-*O*-β-D-glucopyranosyluronate)-α-D-glucopyranoside (112 mg, 0.16 mmol, 1.0 equiv). The reaction was stirred at rt for 12 h. The soln was then diluted with water and the pH adjusted to 5.0 with sodium hydrogen phosphate buffer. The MeOH and THF were removed under diminished pressure. The resulting aq soln was freeze dried and the residue purified first by flash chromatography and then semi-preparative reverse phase HPLC (C-4 column, flow rate: 10 mL/min; 95:5 water–MeCN, retention time 9.18 min) to give, after lyophylisation, **21** as a white solid (66.0 mg, 81%); $[\alpha]_D$ +80 (*c* 0.15, water); IR (KBr) cm^{-1} 3410, 2940, 2360, 1609, 1419, 1080, 650; ^{13}C NMR (75 MHz, D_2O): δ 175.6, 171.1, 137.9 (each s), 128.7, 128.6, 128.1, 102.7, 98.0, 78.5, 77.1, 76.6, 75.5 (each d), 74.5 (t), 73.7, 71.8, 70.8 (each d), 60.0 (t), 55.1 (d), 52.4, 21.9 (each q); ESMS *m/z* $[M-H]^-$ calcd 500.5, found 500.5.

4.21. Methyl 2-acetamido-2-deoxy-4-*O*-(β-D-glucopyranosyluronic acid)-α-D-glucopyranoside (5)[23]

Benzyl ether **21** was stirred over palladium hydroxide (51.0 mg) and H_2 in a soln of EtOH (5 mL) and water (1.5 mL) for 12 h. The reaction was filtered through Celite, the EtOH removed under diminished pressure and the resulting aqueous soln was freeze dried to give the title compound (quantitative yield); $[\alpha]_D$ +35.0 (*c* 0.29, water) {lit.[23] +36.0, (*c* 0.5, water)}; R_f 0.47 (lit.[23] R_f 0.55; 4:3:2:2 EtOAc–EtOH–acetic acid–water); IR (KBr) cm^{-1} 3433, 2985, 2361, 1643, 1416, 1261, 1098, 802; ^1H NMR (300 MHz, D_2O): δ 4.78 (d, 1H, *J* 4.0), 4.54 (d, 1H, *J* 7.8), 3.94 (d, 1H, *J* 4.0), 3.93 (m, 1H), 3.88 (d, 1H, *J* 4.0), 3.84 (t, 1H, *J* 5.0), 3.81–3.84 (m, 2H), 3.70 (t, 1H, *J* 9.0), 3.53 (m, 2H), 3.38 (s, 3H), 3.36 (m, 1H), 2.03 (s, 3H); ^{13}C NMR (125 MHz, D_2O): δ 178.3, 177.4 (each s), 105.2, 100.6, 82.1, 78.6, 78.2, 75.9, 74.6, 73.2, 72.5 (each d), 62.8 (t), 58.2, 56.2 (each d), 24.8 (q); ESMS *m/z* $[M+Na]^+$ calcd 410.1, found 410.4.

4.22. FGF binding assay

A stock soln of heparin albumin (5.0 mg/mL) was made in distilled water and diluted to a final working concentration in a buffer containing 0.1 M sodium carbonate and 0.1 M $NaHCO_3$ and coated onto 96-well assay plates. Disaccharides, heparin albumin and FGF-2 (final concentration 100 ng/mL) were added to the wells in a 100 μL volume of distilled water and incubated for 4 h at 37 °C. Wells were then washed sequentially with PBS/0.05% T20 to remove any unbound protein and blot dried after each wash. Goat polyclonal IgG antibody was added (100 μL/well) and incubated overnight at 37 °C. Wells were washed as before. The amount of bound protein retained in the wells was determined by ELISA using an alkaline phosphatase-conjugated rabbit anti-goat IgG heavy and light chain antibody. The ELISA absorbance readings were read at 405 nm. Results were analysed using a nonlinear curve fitting programme (GraphPad PRISM).

4.23. Endothelial cell assays

BAEC were maintained in RPMI 1640 medium supplemented with 10% heat inactivated FCS, 25 mM glutamine, 75 U/mL penicillin and 75 μg/mL streptomycin.

Cells were grown to confluency in 75 cm^2 tissue culture flasks and maintained at 37 °C in a humidified atmosphere containing 95% O_2 and 5% CO_2. Subcultures were created by passaging using a trypsin/EDTA (0.125%/0.05%) mixture in phosphate buffered saline (PBS), harvested by centrifugation (4 min at 210*g*) and seeded at the appropriate density. The methylthiazol tetrazolium (MTT) assay, adapted from that described in the literature,[28] was used to assess cell viability.

A 24-well plate is seeded with 1 mL of cell suspension 1×10^5 cells/mL in complete or serum free medium. After 24 h, 1 mL of control or disaccharide or HA at 10 μg/mL is added. After 24 h incubation the wells are analysed. Following aspiration and washing with PBS, each well was incubated with MTT (0.45 mg/mL) in RPMI 1640 for 3 h at 37 °C. The overlying solution was then aspirated and the cells solubilised by the addition of 1 mL dimethyl sulfoxide. Absorbance was measured at 600 nm and viability expressed as percentage of control (untreated) wells Statistical significance of differences between group means was determined by ANOVA followed by a post ANOVA Dunnett's test. For experiments where cell growth was monitored over 72 h wells were seeded with 0.5 mL of 1×10^5 cell suspension. The wells were analysed for 72 h post drug addition, with the control and compound added being changed at 48 h.[29]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2004.07.018.

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