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PAPER

Synthesis of simple heparanase substrates

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Heparanase degrades heparan sulfate (HS) chains on proteoglycans; elevated levels of heparanase expression correlate with tumour cell metastatic potential and vascularity, and reduced post-operative survival of cancer patients. Consequently, heparanase expression is considered a biomarker for cancer detection. Although several heparanase assays have been developed, most require the preparation of heterogeneous, (radio)labelled HS substrates and rely on the separation of enzymatically-degraded products on the basis of molecular size. In studies directed towards the development of a more direct heparanase assay, a series of glucuronides and glycosyl glucuronides were synthesised as putative heparanase substrates. These compounds were designed with various aryl aglycones that could be measured spectrophotometrically upon hydrolysis of the glycosidic linkage by heparanase. It was found that the *N*-sulfated 4-nitrophenyl glycosyl glucuronide **24** and the *N*-sulfated methylumbelliferyl glycosyl glucuronide **26** were hydrolysed by recombinant human heparanase. These compounds represent the simplest substrates of heparanase reported to date.

Introduction

Heparan sulfate (HS) proteoglycans are ubiquitous macromolecules located in the extracellular matrices and basement membranes; they present a physical barrier to the movement of cells (*e.g.*, tumour cells and leukocytes) into tissues, and play an important role in a variety of biological processes including inflammation, metastasis and angiogenesis.[1] HS proteoglycans consist of a protein core, to which are attached several linear HS chains that confer most of the biological properties. These HS chains are sulfated linear polysaccharides of up to 400 sugar residues, composed of D-glucuronic acid (GlcA) 1→4 linked to D-glucosamine (GlcN), with various structural modifications, as shown in Fig. 1.[2]

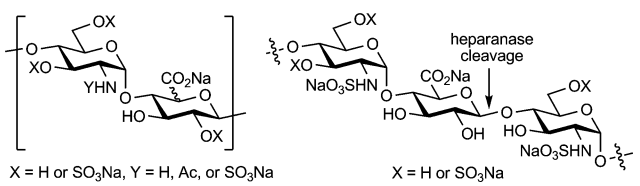

Fig. 1 Schematic of heparan sulfate repeating disaccharide unit (left) showing the heparanase cleavage site (right).

Cleavage of HS chains is critical for the modulation of the biological function of HS-binding proteins, and profoundly affects cell and tissue function involving migration and response to changes in the extracellular matrix. It is also essential in the degradation of the extracellular matrix by invading cells, particularly metastatic tumour cells and leukocytes entering inflammatory sites.[3]

Heparanase is an *endo*-β-D-glucuronidase that degrades HS chains. Elevated levels of heparanase expression correlate with metastatic potential, tumour vascularity, and reduced post-operative survival of cancer patients.[1b,3–4] Heparanase inhibitors reduce the incidence of tumour metastases and therefore heparanase is a potential target for anti-cancer drug development.[3,4b,5] Heparanase hydrolyses the glucuronide linkage in HS, but only at a few sites (*e.g.*, as indicated in Fig. 1), yielding HS fragments of 10–20 disaccharide units. This suggests that heparanase recognises a particular HS structure.[3]

There have been several attempts to define the substrate recognition properties of heparanases from various sources.[6] The approaches taken have included structural comparison between polysaccharides susceptible or resistant to an enzyme, sequence analysis of fragments generated by enzymatic cleavage, studies of inhibitory effects of heparin derivatives, a series of structurally-defined oligosaccharides isolated from heparin/HS, and synthetic polysaccharides prepared using HS biosynthetic enzymes. The majority of these studies allude to the importance of sulfate groups, but have otherwise failed to provide a unified picture. Of particular note are the studies of Okada *et al.*[6b] using defined oligosaccharides and the more recent investigations by Peterson and Liu[6c] using synthetic polysaccharides.

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The lack of a convenient, functional assay hampered early progress into the investigation of heparanase, although several assays for heparanase activity have now been published[7] or are commercially available.[8] Most assays rely on either labelled substrates or separation of enzymatically-degraded substrates on the basis of molecular size. HS fragments produced in these assays may inhibit heparanase, complicating the assays. Additionally, it is difficult to compare data from different assays. The use of a homogeneous, low molecular weight substrate with a single enzymatic cleavage point greatly simplifies measurement of heparanase activity, as recently demonstrated with the synthetic pentasaccharide fondaparinux.[9] However, whilst this assay represents a significant improvement over previous assays, fondaparinux is not an ideal substrate[10] and the assay is not suitable for use in biological matrices.

The primary objective of this investigation was to develop a simple colourimetric or fluorometric assay for heparanase activity. The general concept is shown in Fig. 2, wherein the reducing end of the HS chain is replaced with a chromogenic or fluorogenic tag; heparanase cleavage of glycosyl glucuronides like **1** would result in a measurable response. The development of such an assay would be extremely beneficial in studies on heparanase, including the kinetic evaluation of potential inhibitors and the correlation of heparanase activity with tumour progression.

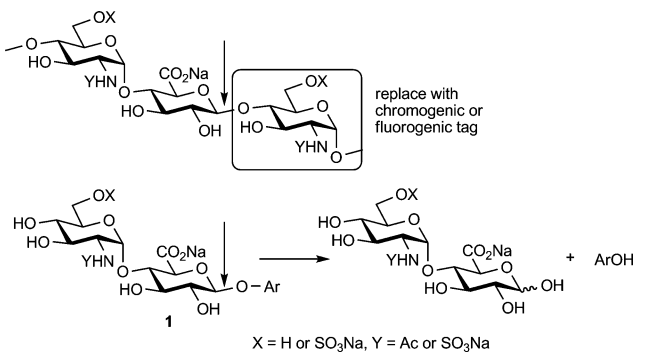


Fig. 2 Heparanase cleavage of the chromogenic or fluorogenic tag from **1** generates a measurable response.

In our approach towards the synthesis of compounds like **1**, we gave due consideration to selection of an appropriate protecting group strategy. Typically in HS/heparin oligosaccharide synthesis, hydroxyl groups that are to remain as free hydroxyl groups are protected as benzyl ethers and *O*-sulfation is performed globally with an excess of sulfating reagent. The benzyl ethers are then removed and nitrogens, masked until that stage as azides, are reduced to amines whereby they can be *N*-acetylated or *N*-sulfated.[11] In our approach towards compounds like **1**, it was necessary to remove the benzyl ethers prior to the introduction of the aromatic aglycones, as the stability of aromatic aglycones such as *para*-nitrophenyl under hydrogenolysis conditions was questionable.

An advanced synthetic intermediate was preferred that could furnish the desired target compounds with minimal synthetic steps from the branch point of the synthesis. Thus a route was devised for the synthesis of the desired glycosyl glucuronides that involved the use of the advanced synthetic intermediate **2** (Fig. 3). The

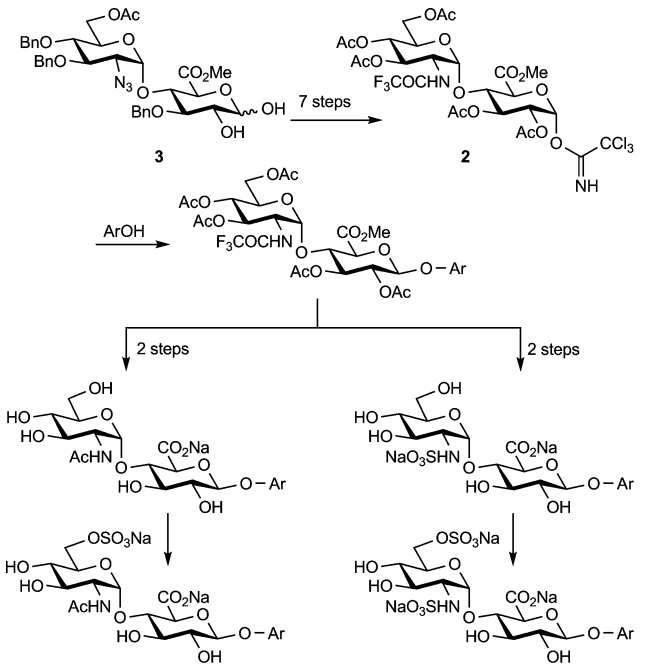


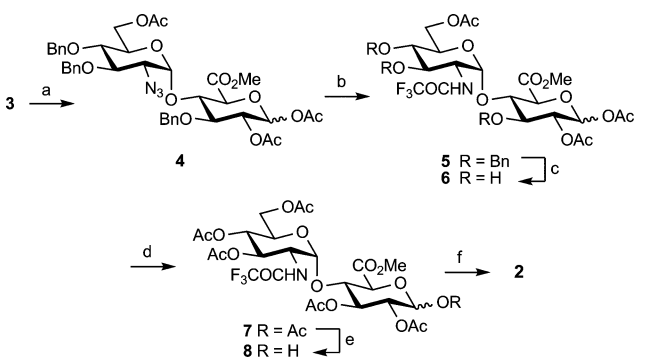
Fig. 3 Synthesis of the glycosyl glucuronide series of putative heparanase substrates.

trichloroacetimidate donor **2** has the hydroxyl groups protected as acetates and the GlcN nitrogen protected as a trifluoroacetamide.

Results and discussion

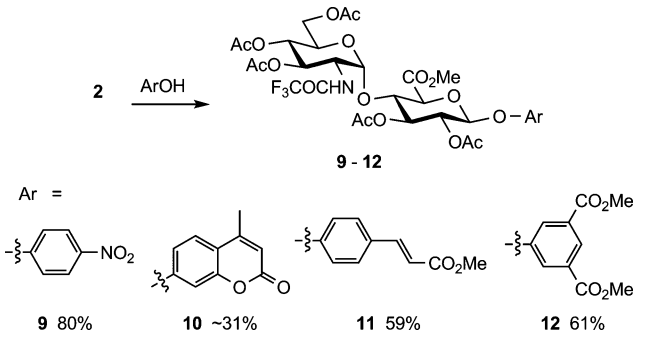
Synthesis

Our approach towards the key trichloroacetimidate donor **2** started with the known diol **3**.[12] Compound **3** was readily acetylated to furnish **4** and subsequent azide reduction and trifluoroacetylation provided the corresponding trifluoroacetamide derivative **5** (Scheme 1). Cleavage of the benzyl ethers from **5** afforded **6**, followed by acetylation to give **7**. The anomeric acetate in **7** was removed by treatment with benzylamine in THF to afford the hemiacetal **8**, which upon exposure to trichloroacetonitrile and DBU in CH_2Cl_2 gave the desired key trichloroacetimidate donor **2**.



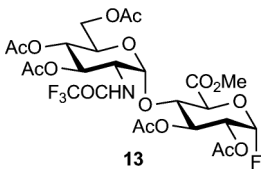
Scheme 1 Reagents and conditions: a) Ac_2O, DMAP, pyridine, quantitative; b) 1. PPh_3, THF, H_2O; 2. $(CF_3CO)_2O$, pyridine, 81% over two steps; c) H_2, Pd/C, MeOH, AcOH, quantitative; d) Ac_2O, DMAP, pyridine, quantitative; e) $BnNH_2$, THF, 87%; f) $NCCCl_3$, DBU, CH_2Cl_2, 88%.

Having developed an efficient method for the synthesis of the trichloroacetimidate donor **2**, it was possible to synthesise the protected glycosyl glucuronides **9–12** (Scheme 2).



Scheme 2 Reagents and conditions: $BF_3{\cdot}OEt_2$, CH_2Cl_2, 4 Å molecular sieves, –15 °C to room temperature.

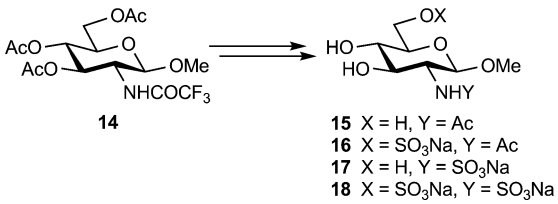
These four glycosyl glucuronides were synthesised in moderate to good yield. The four compounds prepared were chosen because the aglycones display a range of charge and spectroscopic properties. Interestingly, formation of the glycosyl glucuronosyl fluoride **13** was detected in the glycosidations of trichloroacetimidate **2** with 4-nitrophenol (25% yield of **13**) and the cinnamyl ester (16% yield of **13**). It is believed that the formation of **13** occurred *via* a similar mechanism to that previously reported by our group.[13]



Deprotection, *N*-acetylation, *N*- and *O*-sulfation

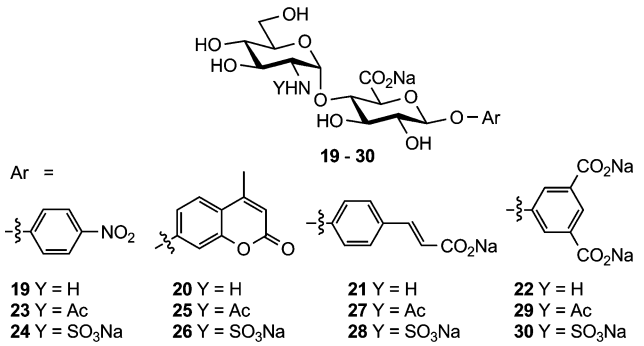
Upon completion of the synthesis of glycosyl glucuronides **9–12**, their deprotection, *N*-acetylation/*N*-sulfation and *O*-sulfation was investigated. Whilst there is literature precedence for the chemoselective sulfation of a *N*-acetylglucosamine primary hydroxyl group (using either $SO_3{\cdot}Py$ or $SO_3{\cdot}NMe_3$) in the presence of one or more secondary hydroxyl groups,[14] selective primary hydroxyl group mono-sulfation is difficult in the presence of secondary hydroxyl groups, with both di- and trisulfation products observed.[15] Simultaneous *N*- and *O*-sulfation of oligosaccharides has been reported; however, both primary and secondary hydroxyl groups were *O*-sulfated.[16] Successful *O*-sulfation in the presence of one or more *N*-sulfates has been achieved by enzyme-catalysed reactions.[6c,17]

To determine the optimal deprotection, *N*-acetylation/*N*-sulfation and *O*-sulfation conditions on the glycosyl glucuronides, the GlcN trifluoroacetamide derivative **14**[18] was utilised. The readily accessible **14** was thought to be a good model compound for this chemistry as it should behave in a similar manner to compounds such as glycosyl glucuronides **9–12** under the reaction conditions.



Thus compound **14** was de-esterified, *N*-acetylated to afford **15**, and selectively 6-*O*-sulfated (DMF, 2 equivalents of $SO_3{\cdot}Py$ at room temperature for 16 h) to furnish **16**. Deprotection of **14**, followed by *N*-sulfation afforded **17**. Unfortunately, selective 6-*O*-sulfation of **17** to afford **18** was not successful.

With appropriate conditions in hand for the deprotection, *N*-acetylation, and *N*-sulfation of the model compound **14**, attention was turned towards the protected glycosyl glucuronides. Compounds **9–12** were deprotected to furnish amines **19–22**, then either *N*-acetylated or *N*-sulfated to afford compounds **23–30**.



Heparanase cloning and expression

The cloning strategy to produce recombinant human heparanase followed the work of McKenzie *et al.*[19] in which an insect cell-baculovirus system was used to express the 8 and 50 kDa heparanase subunits each as a fusion protein with a signal peptide targeting for the excretion from the insect cell. In the work of McKenzie *et al.*, heparanase cDNA was amplified from a mammary gland cDNA library using undisclosed primers. In this work, repeated attempts to PCR amplify heparanase cDNA from the same library proved unsuccessful. Therefore, a plasmid incorporating the full length heparanase cDNA was used. Using this plasmid, heparanase was expressed and purified according to the literature.[19]

Heparanase assays

The synthesised compounds were evaluated for their ability to act as heparanase substrates. Significant heparanase specific activity was observed with **26** (48 nmol h^{-1} mg^{-1}) and **24** (17 nmol h^{-1} mg^{-1}). The hydrolysis of the remaining glycosyl glucuronides was at the lower limit of detection, indicating negligible hydrolysis by heparanase. The *N*-sulfated glycosyl glucuronides **24** and **26** are the smallest molecules reported to be hydrolysed by heparanase—the smallest molecules previously reported to be heparanase substrates were tetrasaccharides based on the structure shown in Fig. 4.[6b,20]

In order to ensure that **26** was indeed being hydrolysed by heparanase and not simply decomposing under the assay

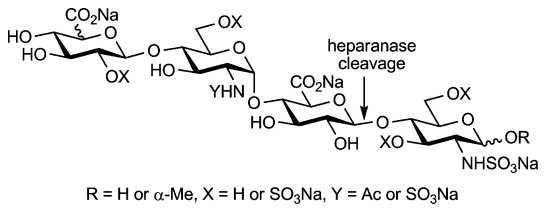
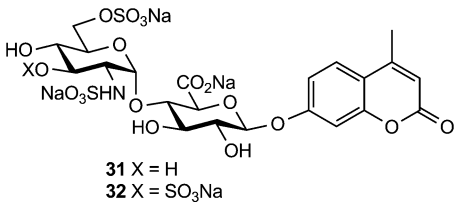


Fig. 4 HS tetrasaccharides previously reported to be the smallest substrates hydrolysed by heparanase.[6b,20]

conditions, a solution of **26**, HS, and BSA in sodium acetate buffer at pH 5.0 was incubated for 3 days. Low resolution mass spectral analysis of the assay mixture gave a peak at 592.0, consistent with the parent ion of **26**, (*m*/*z*): 592.0 $[M - 2Na + H]^-$. There were no peaks to indicate that the aglycone had been hydrolysed, or that *O*-sulfation of **26** had occurred. This would indicate that the compound was relatively stable at pH 5.0 for at least 3 days and that no cross-sulfation from HS had occurred.

The recombinant human heparanase used in this study was determined *via* an ultrafiltration assay[21] to digest 170 nmol HS h^{-1} mg^{-1} at 37 °C. This compares favourably with the heparanase activity observed with the *N*-sulfated 4-methylumbelliferyl glycosyl glucuronide **26**, which was 48 nmol h^{-1} mg^{-1}. Previously, heparanase purified from human platelets was shown to have a specific activity towards HS of 7800 nmol h^{-1} mg^{-1} using the cHRG-Sepharose beads separation method.[22] Care should be taken in comparing specific activities using different assays. Not only are the HS preparations in the two assays from different sources, but the manner by which heparanase activity is measured is different. In using a substrate such as **26** to determine heparanase activity, every heparanase catalytic event is measured. It is expected that the products resulting from the hydrolysis of **26** would not be able to bind the heparanase active site. This is in contrast to assays using HS chains, as the heparanase substrate may be hydrolysed several times, making it difficult to calculate the number of catalytic events. For example, the heparanase ultrafiltration assay uses 30 kDa HS as the substrate; degradation products are typically 7–10 kDa.[7d] This would suggest that each substrate HS chain is hydrolysed 3–4 times. Additionally, the resulting shorter HS chains may bind the heparanase active site, resulting in feedback inhibition.

The advantages of the assay used in this work include the lack of radiolabelling, the ability to process multiple samples, ease of handling, the assay measures one catalytic event per molecule, and that hydrolysed products should have no affinity for the active site of heparanase. The results presented herein indicate that 4-methylumbelliferone is an adequate leaving group for the measurement of heparanase activity. As *O*-sulfate groups on the GlcN moiety have been shown to increase hydrolysis by heparanase roughly two-fold,[6b,6c] it would be of great interest to evaluate the hydrolysis of the glycosyl glucuronides **31** and **32**. It is expected that the *O*-sulfates should increase the affinity of the substrate for the heparanase active site, leading to greater turnover by heparanase. If this is the case, it would be possible to measure heparanase activity with shorter incubation times, allowing a rapid assay for heparanase activity. Strategies towards the synthesis of compounds such as **31** and **32** are currently being pursued.



Conclusions

A series of glucuronides and glycosyl glucuronides were synthesised and evaluated as heparanase substrates. Hydrolysis of the *N*-sulfated 4-nitrophenyl glycosyl glucuronide **24** and the *N*-sulfated 4-methylumbelliferyl glycosyl glucuronide **26** by heparanase was detected. These are the smallest molecules reported to be hydrolysed by heparanase. Glycosyl glucuronide **26** should serve as a useful template in the development of a rapid diagnostic test for heparanase activity.

Experimental

General

Reactions were monitored by TLC on aluminium plates coated with Silica Gel 60 F_{254} (E. Merck) and visualised with H_2SO_4 (5% in ethanol) with heating to 200 °C or ultraviolet light where applicable. Compounds were purified by flash chromatography using either E. Merck Silica Gel 60 (0.040–0.063 mm) or Sephadex® LH-20. ^1H and ^{13}C NMR spectra were recorded using a Brüker 300 MHz spectrometer. Data acquisition and processing were performed using XWINNMR software (version 3.1) running on a Silicon Graphics O2 workstation. Data editing was performed using XWINPLOT software. Chemical shifts are given in parts per million (ppm) relative to the solvent used ($CDCl_3$: 7.26 for ^1H, 77.2 for ^{13}C; D_2O: 4.79 for ^1H). Two-dimensional COSY and HSQC experiments were run to support ^1H and ^{13}C assignments. Multiplicities of resonances are denoted as s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), m (multiplet); (b) indicates a broad signal and (obs) indicates the signal is obscured. Low resolution mass spectra (LRMS) utilising electrospray ionisation (ESI) were obtained using a Brüker Esquire 3000. High-resolution mass spectra (HRMS) were carried out at either the University of Queensland's mass spectrometry service within the School of Chemistry and Molecular Biosciences, or at the Eskitis Institute, Griffith University, Nathan campus. All HRMS were recorded using an electrospray method. Elemental microanalyses were carried out at the University of Queensland's Microanalysis Facility. All H_2O used was either distilled H_2O or Milli-Q H_2O. All commercial solvents (acetone, chloroform, dichloromethane, ethyl acetate, hexanes, methanol and toluene) were bulk distilled prior to use. Dried solvents were distilled under N_2.

General procedure for acetylation (A)

To a solution of alcohol (1.0 equiv) in pyridine (1 mL per mmol of alcohol) and 4-dimethylaminopyridine (0.3–0.5 equiv) at 0 °C under N_2 was added acetic anhydride (25–40 equiv). The mixture was allowed to regain ambient temperature, was stirred for 20 h, then concentrated. The residue was dissolved in ethyl acetate, washed with 1 N HCl, H_2O, sat. aq. $NaHCO_3$, and brine. The

organic phase was dried over Na_2SO_4, filtered, and the solvent was removed *in vacuo*.

General procedure for glycosidation (B)

To a solution of trichloroacetimidate **2** (1.0 equiv) in CH_2Cl_2 (10 mL per mmol of **2**) was added alcohol (2.0–5.0 equiv) and 4 Å sieves. The mixture was stirred for 60 min at rt, then cooled to −15 °C (4-methylumbelliferone reactions were performed with exclusion of light). $BF_3{\cdot}OEt_2$ (0.3–0.5 equiv) in CH_2Cl_2 was added. The reaction mixture was allowed to warm to rt and was stirred for 16 h. The mixture was diluted with CH_2Cl_2, filtered through Celite®, washed with aqueous Na_2CO_3, dried over Na_2SO_4, and the solvent was removed *in vacuo*.

General procedure for *N*-sulfation (C)

To a solution of methyl 2-amino-2-deoxy-β-D-glucopyranoside,[23] **19**, **20**, **21**, or **22** (1.0 equiv) in H_2O (50 mL per mmol of amine) was added $SO_3{\cdot}Py$ complex (10 equiv) portionwise. The pH was maintained at 9.5 by the addition of 1 N NaOH. After 3 h, the reaction mixture was concentrated and purified on Sephadex® LH-20, eluting with 4 : 1 methanol–H_2O.

General procedure for de-esterification (D)

To a solution of either a ~1 : 1 mixture of **8** and **10** or pure compounds **9**, **11**, or **12** (1.0 equiv) in methanol (25 mL per mmol of substrate) at 0 °C was added H_2O (5 mL per mmol of substrate) and 0.1 N LiOH (10 equiv). The solution was allowed to regain ambient temperature and was stirred for 20 h. The solution was acidified to pH 6 with dilute acetic acid, and then adjusted to pH 7.5 with dilute NaOH. The solution was concentrated and purified on Sephadex® LH-20, eluting with 4 : 1 methanol–H_2O.

General procedure for *N*-acetylation (E)

To a solution of amine **19**, **20**, **21**, or **22** (1.0 equiv) in methanol (30 mL per mmol of amine) at 0 °C under N_2 was added triethylamine (2.0 equiv) and acetic anhydride (15 equiv). The mixture was stirred for 90 min, H_2O was added, and the solution was concentrated. The product was purified by column chromatography on Sephadex® LH-20, eluting with 4 : 1 methanol–H_2O.

3,4,6-Tri-*O*-acetyl-2-deoxy-2-trifluoroacetamido-α-D-glucopyranosyl-(1→4)-methyl 2,3-di-*O*-acetyl-α-D-glucopyranuronosyl trichloroacetimidate (2). To a solution of **8** (532 mg, 788 μmol) in CH_2Cl_2 (5 mL) and trichloroacetonitrile (790 μL, 7.88 mmol) at 0 °C under N_2 was added 1,8-diazabicyclo[5.4.0]undec-7-ene (12 μL, 79 μmol). The mixture was stirred for 2 h and then concentrated. Flash chromatography on silica gel (3 : 2 hexanes–ethyl acetate, R_f 0.2) afforded **2** (571 mg, 88%); δ_H(300 MHz; $CDCl_3$) 8.74 (s, 1 H, NHC(O)CCl_3); 6.56 (d, 1 H, $J_{1,2}$ 3.6 Hz, GlcA H-1); 6.54 (bd, 1 H, NHC(O)CF_3); 5.62 (dd, 1 H, $J_{3,2}$ 10.0, $J_{3,4}$ 9.3 Hz, GlcA H-3); 5.21 (d, 1 H, $J_{1,2}$ 3.7 Hz, GlcN H-1); 5.19–5.08 (m, 2 H, GlcN H-3, GlcN H-4); 5.06 (dd, 1 H, $J_{2,3}$ 10.0, $J_{2,1}$ 3.6 Hz, GlcA H-2); 4.50 (d, 1 H, $J_{5,4}$ 9.8 Hz, GlcA H-5); 4.35 (dd, 1 H, $J_{4,5}$ 9.8, $J_{4,3}$ 9.3 Hz, GlcA H-4); 4.32–4.07 (m, 3 H, GlcN H-2, GlcN H-6a, GlcN H-6b); 3.78 (s, 3 H, CO_2Me); 3.72–3.66 (m, 1 H, GlcN H-5); 2.12, 2.03, 2.01, 2.01, 1.98 (5 × s, 15 H, 5 × OAc); δ_C(75 MHz; $CDCl_3$) 171.4, 170.7, 169.8, 169.7, 169.1 (5 × O*C*(O)CH_3); 168.0 (GlcA C-6); 160.7 (O*C*(NH)CCl_3); 157.6 (q, 38 Hz, *C*(O)CF_3); 115.3 (q, 286 Hz, C(O)*C*F_3); 96.6 (GlcN C-1); 92.5 (GlcA C-1); 90.4 (CCl_3); 73.5 (GlcA C-4); 71.7 (GlcA C-5); 71.0 (GlcA C-3); 69.8 (GlcN C-3); 69.4 (GlcA C-2); 68.9 (GlcN C-5); 67.1 (GlcN C-4); 61.0 (GlcN C-6); 53.1 (CO_2CH_3); 52.3 (GlcN C-2); 20.7, 20.6, 20.5, 20.4, 20.4 (5 × OC(O)*C*H_3); *m*/*z* (ESI) 841.059998 ([M + Na]$^+$. $C_{27}H_{32}Cl_3F_3N_2NaO_{17}$ requires 841.061086).

6-*O*-Acetyl-2-azido-3,4-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl-(1→4)-methyl 3-*O*-benzyl-D-glucopyranosyluronate (3). Prepared as previously reported.[12] α anomer δ_H(300 MHz; $CDCl_3$) 7.39–7.26 (m, 15 H, 3 × Ph); 5.43 (d, 1 H, $J_{1,2}$ 2.4 Hz, GlcA H-1); 5.25 (d, 1 H, $J_{1,2}$ 3.7 Hz, GlcN H-1); 4.89 (s, 2 H, PhCH_2); 4.86, 4.59 (AB q, 2 H, J_{AB} 11.2 Hz, PhCH_2); 4.74, 4.68 (AB q, 2 H, J_{AB} 11.2 Hz, PhCH_2); 4.62–4.56 (obs, 1 H, GlcA H-5); 4.31 (dd, 1 H, $J_{6a,6b}$ 12.1, $J_{6a,5}$ 2.0 Hz, GlcN H-6a); 4.27–4.24 (m, 1 H, GlcA H-4); 4.20 (dd, 1 H, $J_{6b,6a}$ 12.1, $J_{6b,5}$ 4.6 Hz, GlcN H-6b); 3.99–3.90 (m, 2 H, GlcA H-3, GlcN H-3); 3.81–3.74 (m, 2 H, GlcA H-2, GlcN H-5); 3.63 (s, 3 H, CO_2Me); 3.57–3.49 (m, 2 H, GlcN H-2, GlcN H-4); 2.05 (s, 3 H, OAc); δ_C(75 MHz; $CDCl_3$) 170.9 (O*C*(O)CH_3); 169.4 (C-6); 137.4, 137.3, 137.3 (3 × $_{ipso}$Ph); 128.6, 128.6, 128.5, 128.2, 128.2, 128.1, 128.0, 128.0, 127.6 (Ph); 96.9 (GlcN C-1); 90.5 (GlcA C-1); 80.6 (GlcN C-3); 77.5 (GlcN C-4); 76.3 (GlcA C-3); 75.8, 75.1, 73.5 (3 × Ph*C*H_2); 73.2 (GlcA C-5); 72.7 (GlcA C-4); 70.2 (GlcN C-5); 69.9 (GlcA C-2); 63.5 (GlcN C-2); 62.5 (GlcN C-6); 52.5 (CO_2CH_3); 20.8 (OC(O)*C*H_3); β anomer δ_C(75 MHz; $CDCl_3$) 170.9 (O*C*(O)CH_3); 169.2 (C-6); 138.2, 137.6, 137.5 (3 × $_{ipso}$Ph); 128.6–127.6 (Ph); 97.5, 97.0 (C-1, GlcN C-1); 82.9, 80.2, 77.4, 74.7, 74.4, 69.7, 64.4, 63.3 (GlcA C-2, GlcA C-3, GlcA C-4, GlcA C-5, GlcN C-2, GlcN C-3, GlcN C-4, GlcN C-5); 77.6, 77.1, 76.1 (3 × Ph*C*H_2); 62.3 (GlcN C-6); 52.9 (CO_2CH_3); 20.9 (OC(O)*C*H_3); *m*/*z* (ESI) 729.9 ([M + Na]$^+$. $C_{36}H_{41}N_3NaO_{12}$ requires 730.3).

6-*O*-Acetyl-2-azido-3,4-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl-(1→4)-methyl 1,2-di-*O*-acetyl-3-*O*-benzyl-D-glucopyranosyluronate (4). Prepared according to general procedure A from **3**. Flash chromatography on silica gel (3 : 1 hexanes–ethyl acetate, R_f 0.3) afforded **4** (100%) as a 2.5 : 1 α : β mixture (Found: C, 60.64; H, 5.79; N, 5.17. $C_{40}H_{45}N_3O_{14}$ requires C, 60.68; H, 5.73; N, 5.31%); α anomer δ_H(300 MHz; $CDCl_3$) 7.41–7.25 (m, 15 H, 3 × Ph); 6.33 (d, 1 H, $J_{1,2}$ 3.6 Hz, GlcA H-1); 5.51 (d, 1 H, $J_{1,2}$ 3.9 Hz, GlcN H-1); 5.09 (dd, 1 H, $J_{2,3}$ 9.6, $J_{2,1}$ 3.6 Hz, GlcA H-2); 4.94–4.78 (m, 4 H, 2 × PhCH_2); 4.86, 4.57 (AB q, 2 H, J_{AB} 11.1 Hz, PhCH_2); 4.36 (d, 1 H, $J_{5,4}$ 9.3 Hz, GlcA H-5); 4.30–4.16 (m, 3 H, GlcN H-6a, GlcN H-6b, GlcA H-4); 4.09 (dd, 1 H, $J_{3,4}$ 9.6, $J_{3,2}$ 9.6 Hz, GlcA H-3); 3.91 (dd, 1 H, $J_{3,2}$ 10.2, $J_{3,4}$ 8.7 Hz, GlcN H-3); 3.77 (s, 3 H, CO_2Me); 3.65–3.58 (m, 1 H, GlcN H-5); 3.52 (dd, 1 H, $J_{4,5}$ 10.2, $J_{4,3}$ 8.4 Hz, GlcN H-4); 3.36 (dd, 1 H, $J_{2,3}$ 10.2, $J_{2,1}$ 3.6 Hz, GlcN H-2); 2.20, 2.04, 1.95 (3 × s, 9 H, 3 × OAc); δ_C(75 MHz; $CDCl_3$) 170.7, 169.7, 168.8 (3 × O*C*(O)CH_3); 168.4 (GlcA C-6); 137.8, 137.5, 137.4 (3 × $_{ipso}$Ph); 128.8, 128.6, 128.5, 128.1, 128.1, 127.9, 127.8, 127.5, 127.2 (3 × Ph); 97.9 (GlcN C-1); 89.2 (GlcA C-1); 80.2 (GlcN C-3); 79.3 (GlcA C-3); 77.4 (GlcN C-4); 75.6, 75.2, 75.0 (3 × Ph*C*H_2); 74.8 (GlcA C-4); 72.3 (GlcA C-5); 71.4 (GlcA C-2); 69.8 (GlcN C-5); 63.3 (GlcN C-2); 62.2 (GlcN C-6); 53.0 (CO_2CH_3); 20.9, 20.9, 20.6 (3 × OC(O)*C*H_3); β anomer δ_H(300 MHz; $CDCl_3$) 7.40–7.24 (m, 15 H, 3 × Ph); 5.73 (d,

1 H, $J_{1,2}$ 6.9 Hz, GlcA H-1); 5.43 (d, 1 H, $J_{1,2}$ 3.6 Hz, GlcN H-1); 5.16 (dd, 1 H, $J_{2,3}$ 8.1, $J_{2,1}$ 6.9 Hz, GlcA H-2); 4.89–4.81 (m, 2 H, PhCH_2); 4.85, 4.72 (AB q, 2 H, J_{AB} 11.1 Hz, PhCH_2); 4.85, 4.56 (AB q, 2 H, J_{AB} 10.8 Hz, PhCH_2); 4.31–4.13 (m, 4 H, GlcN H-6a, GlcN H-6b, GlcA H-4, GlcA H-5); 3.93–3.84 (m, 2 H, GlcN H-3, GlcA H-3); 3.74 (s, 3 H, CO_2Me); 3.66–3.57 (m, 1 H, GlcN H-5); 3.52 (dd, 1 H, $J_{4,5}$ 10.2, $J_{4,3}$ 8.7 Hz, GlcN H-4); 3.32 (dd, 1 H, $J_{2,3}$ 10.2, $J_{2,1}$ 3.6 Hz, GlcN H-2); 2.09, 2.05, 1.99 (3 × s, 9 H, 3 × OAc); δ_C(75 MHz; $CDCl_3$) 170.7, 170.5, 169.3 (3 × O*C*(O)CH_3); 168.2 (GlcA C-6); 137.5, 137.4, 137.4 (3 × $_{ipso}$Ph); 128.8–127.2 (3 × Ph); 97.8 (GlcN C-1); 91.7 (GlcA C-1); 81.5 (GlcN C-3); 80.0 (GlcA C-3); 77.1 (GlcN C-4); 75.6, 75.0, 74.7 (3 × Ph*C*H_2); 74.6, 74.4 (GlcA C-4, GlcA C-5); 71.7 (GlcA C-2); 69.9 (GlcN C-5); 63.2 (GlcN C-2); 62.2 (GlcN C-6); 52.9 (CO_2CH$_3$); 20.9, 20.8, 20.86 (3 × OC(O)*C*H_3); *m*/*z* (ESI) 814.0 ([M + Na]$^+$. $C_{40}H_{45}N_3NaO_{14}$ requires 814.3).

6-*O*-Acetyl-3,4-di-*O*-benzyl-2-deoxy-2-trifluoroacetamido-α-D-glucopyranosyl-(1→4)-methyl 1,2-di-*O*-acetyl-3-*O*-benzyl-D-glucopyranosyluronate (5). To a solution of **4** (1.16 g, 1.46 mmol) in THF (10 mL) at 0 °C under N_2 was added PPh_3 (421 mg, 1.61 mmol). After 10 min, H_2O (263 μL, 14.6 mmol) was added, the solution was allowed to regain ambient temperature and was stirred for 20 h. The solvents were removed *in vacuo*, the residue was dissolved in pyridine (25 mL) at 0 °C under N_2, to which was added trifluoroacetic anhydride (2.03 mL, 14.6 mmol). The solution was allowed to regain ambient temperature and was stirred for 20 h. The mixture was then concentrated, the residue was dissolved in ethyl acetate, washed with 1 N HCl, and H_2O. The organic phase was dried over Na_2SO_4, filtered, and the solvent was removed *in vacuo*. Flash chromatography on silica gel (2 : 1 hexanes–ethyl acetate, R_f 0.3) afforded **5** (1.02 g, 81%) as a 3 : 1 α : β mixture; α anomer δ_H(300 MHz; $CDCl_3$) δ 7.38–7.17 (m, 15 H, 3 × Ph); 6.74 (d, 1 H, $J_{NH,2}$ 9.5 Hz, NH); 6.29 (d, 1 H, $J_{1,2}$ 3.6 Hz, GlcA H-1); 5.23 (d, 1 H, $J_{1,2}$ 3.4 Hz, GlcN H-1); 5.05 (dd, 1 H, $J_{2,3}$ 9.7, $J_{2,1}$ 3.6 Hz, GlcA H-2); 4.84–4.76 (m, 2 H, PhCH_2); 4.62 (s, 2 H, PhCH_2); 4.60–4.54 (m, 2 H, PhCH_2); 4.35–4.15 (m, 4 H, GlcN H-2, GlcN H-6a, GlcN H-6b, GlcA H-5); 4.14–4.08 (m, 1 H, GlcA H-4); 3.92 (dd, 1 H, $J_{3,2}$ 9.7, $J_{3,4}$ 9.2 Hz, GlcA H-3); 3.77 (s, 3 H, CO_2Me); 3.74–3.57 (m, 3 H, GlcN H-3, GlcN H-4, GlcN H-5); 2.18, 2.06, 1.77 (3 × s, 9 H, 3 × OAc); δ_C(75 MHz; $CDCl_3$) 170.7, 169.5, 168.6 (3 × O*C*(O)CH_3); 168.0 (GlcA C-6); 157.1 (q, 37 Hz, N*C*(O)CF_3); 137.3, 137.3, 137.3 (3 × $_{ipso}$Ph); 128.7–127.0 (3 × Ph); 115.7 (q, 286 Hz, NC(O)*C*F_3); 97.7 (GlcN C-1); 89.2 (GlcA C-1); 78.9 (GlcA C-3); 78.8 (GlcN C-3); 77.1 (GlcN C-4); 75.6 (GlcA C-4); 75.1, 74.9, 74.9 (3 × Ph*C*H_2); 72.6 (GlcA C-5); 71.3 (GlcA C-2); 70.9 (GlcN C-5); 61.9 (GlcN C-6); 53.1 (CO_2CH$_3$); 52.9 (GlcN C-2); 20.9, 20.8, 20.3 (3 × OC(O)*C*H_3); β anomer δ_H(300 MHz; $CDCl_3$) 7.38–7.17 (m, 15 H, 3 × Ph); 6.65 (d, 1 H, $J_{NH,2}$ 9.6 Hz, NH); 5.69 (d, 1 H, $J_{1,2}$ 6.7 Hz, GlcA H-1); 5.23 (d, 1 H, $J_{1,2}$ 3.4 Hz, GlcN H-1); 5.12 (dd, 1 H, $J_{2,3}$ 7.9, $J_{2,1}$ 6.7 Hz, GlcA H-2); 4.84–4.76 (m, 2 H, PhCH_2); 4.62 (s, 2 H, PhCH_2); 4.60–4.54 (m, 2 H, PhCH_2); 4.35–4.08 (m, 6 H, GlcN H-2, GlcN H-6a, GlcN H-6b, GlcA H-3, GlcA H-4, GlcA H-5); 3.75 (s, 3 H, CO_2Me); 3.74–3.57 (m, 3 H, GlcN H-3, GlcN H-4, GlcN H-5); 2.08, 2.06, 1.86 (3 × s, 9 H, 3 × OAc); δ_C(75 MHz; $CDCl_3$) 170.7, 169.1, 169.0 (3 × O*C*(O)CH_3); 168.1 (GlcA C-6); 157.1 (q, 37 Hz, N*C*(O)CF_3); 137.3, 137.2, 136.8 (3 × $_{ipso}$Ph); 128.7–127.0 (3 × Ph); 115.7 (q, 286 Hz, NC(O)*C*F_3); 97.2 (GlcN C-1); 91.8 (GlcA C-1); 81.0, 78.6, 77.2, 74.6, 72.6, 71.9, 70.9 (GlcN C-3, GlcN C-4, GlcN C-5, GlcA C-2, GlcA C-3, GlcA C-4, GlcA C-5); 75.1, 74.9, 74.2 (3 × Ph*C*H_2); 62.0 (GlcN C-6); 53.0 (CO_2CH$_3$); 52.8 (GlcN C-2); 20.8, 20.8, 20.6 (3 × OC(O)*C*H_3); *m*/*z* (ESI): 884.272582 ([M + Na]$^+$. $C_{42}H_{46}F_3NNaO_{15}$ requires 884.271116).

6-*O*-Acetyl-2-deoxy-2-trifluoroacetamido-α-D-glucopyranosyl-(1→4)-methyl 1,2-di-*O*-acetyl-D-glucopyranosyluronate (6). A solution of **5** (700 mg, 812 μmol) in methanol (20 mL) and acetic acid (232 μL, 4.06 mmol) was stirred under an atmosphere of H_2 in the presence of Pd/C (10%) for 20 h at rt. After filtration through a pad of Celite®, the solvent was removed *in vacuo* to afford **6** (480 mg, 100%) as a 2.3 : 1 α : β mixture; α anomer δ_H(300 MHz; $CDCl_3$) 8.28–8.20 (m, 1 H, GlcN NH); 6.30 (d, 1 H, $J_{1,2}$ 3.6 Hz, GlcA H-1); 5.07 (d, 1 H, $J_{1,2}$ 3.0 Hz, GlcN H-1); 4.90 (dd, 1 H, $J_{2,3}$ 9.6, $J_{2,1}$ 3.6 Hz, GlcA H-2); 4.45–3.93 (m, 8 H, GlcN H-2, GlcN H-3, GlcN H-4, GlcN H-6a, GlcN H-6b, GlcA H-3, GlcA H-4, GlcA H-5); 3.78 (s, 3 H, CO_2Me); 3.54–3.44 (m, 1 H, GlcN H-5); 2.17, 2.11, 2.07 (3 × s, 9 H, 3 × OAc); δ_C(75 MHz; $CDCl_3$) 172.0, 170.7, 169.8 (3 × O*C*(O)CH_3); 167.7 (GlcA C-6); 158.7 (q, 37 Hz, *C*(O)CF_3); 115.8 (q, 285 Hz, C(O)*C*F_3); 99.9 (GlcN C-1); 89.1 (GlcA C-1); 80.4, 72.7, 72.0, 71.5, 71.5, 70.0, 69.9 (GlcN C-3, GlcN C-4, GlcN C-5, GlcA C-2, GlcA C-3, GlcA C-4, GlcA C-5); 62.5 (GlcN C-6); 55.0 (GlcN C-2); 53.1 (CO_2CH$_3$); 20.8, 20.7, 20.5 (3 × OC(O)*C*H_3); β anomer δ_H(300 MHz; $CDCl_3$) 8.28–8.20 (m, 1 H, GlcN NH); 5.67 (d, 1 H, $J_{1,2}$ 8.0 Hz, GlcA H-1); 5.04 (d, 1 H, $J_{1,2}$ 3.0 Hz, GlcN H-1); 4.90 (obs, 1 H, GlcA H-2); 4.45–3.93 (m, 8 H, GlcN H-2, GlcN H-3, GlcN H-4, GlcN H-6a, GlcN H-6b, GlcA H-3, GlcA H-4, GlcA H-5); 3.82 (s, 3 H, CO_2Me); 3.54–3.44 (m, 1 H, GlcN H-5); 2.12, 2.09, 2.04 (3 × s, 9 H, 3 × OAc); δ_C(75 MHz; $CDCl_3$) 172.3, 171.2, 168.9 (3 × O*C*(O)CH_3); 167.3 (GlcA C-6); 158.7 (q, 37 Hz, *C*(O)CF_3); 115.8 (q, 285 Hz, C(O)*C*F_3); 99.9 (GlcN C-1); 91.5 (GlcA C-1); 80.7, 75.3, 73.7, 73.1, 71.5, 71.2, 69.9 (GlcN C-3, GlcN C-4, GlcN C-5, GlcA C-2, GlcA C-3, GlcA C-4, GlcA C-5); 62.6 (GlcN C-6); 54.9 (GlcN C-2); 53.1 (CO_2CH$_3$); 20.7, 20.6, 20.5 (3 × OC(O)*C*H_3); *m*/*z* (ESI) 614.130081 ([M + Na]$^+$. $C_{21}H_{28}F_3NNaO_{15}$ requires 614.130325).

3,4,6-Tri-*O*-acetyl-2-deoxy-2-trifluoroacetamido-α-D-glucopyranosyl-(1→4)-methyl 1,2,3-tri-*O*-acetyl-D-glucopyranosyluronate (7). Prepared according to general procedure A from **6**. Flash chromatography on silica gel (3 : 2 hexanes–ethyl acetate, R_f 0.3) afforded **7** (100%) as a 2.3 : 1 α : β mixture (Found: C, 45.13; H, 4.86; N, 1.91. Microanalysis calculated for $C_{27}H_{34}F_3NO_{18}$: C, 45.19; H, 4.78; N, 1.95%); α anomer δ_H(300 MHz; $CDCl_3$) 6.57 (d, 1 H, $J_{NH,2}$ 9.3 Hz, NH); 6.30 (d, 1 H, $J_{1,2}$ 3.6 Hz, GlcA H-1); 5.49 (dd, 1 H, 10.1, 9.0 Hz, GlcA H-3); 5.21–4.98 (m, 4 H, GlcN H-1, GlcN H-3, GlcN H-4, GlcA H-2); 4.42–4.06 (m, 5 H, GlcN H-2, GlcN H-6a, GlcN H-6b, GlcA H-4, GlcA H-5); 3.77 (s, 3 H, CO_2Me); 3.73–3.63 (m, 1 H, GlcN H-5); 2.23, 2.10, 2.02, 2.00, 1.98, 1.97 (6 × s, 18 H, 6 × OAc); δ_C(75 MHz; $CDCl_3$) 171.5, 170.7, 169.9, 169.6, 169.1, 168.7 (6 × O*C*(O)CH_3); 168.1 (GlcA C-6); 157.6 (q, 38 Hz, *C*(O)CF_3); 115.4 (q, 286 Hz, C(O)*C*F_3); 96.6 (GlcN C-1); 88.7 (GlcA C-1); 73.4 (GlcA C-4); 71.6 (GlcA C-5); 71.2 (GlcA C-3); 69.7 (GlcN C-3); 68.9, 68.9 (GlcN C-5, GlcA C-2); 67.0 (GlcN C-4); 61.0 (GlcN C-6); 53.0 (CO_2CH$_3$); 52.4 (GlcN C-2); 20.9, 20.7, 20.5, 20.5, 20.4, 20.3 (6 × OC(O)*C*H_3); β anomer δ_H(300 MHz; $CDCl_3$) 6.71–6.62 (bd, 1 H, NH); 5.77 (d, 1 H, $J_{1,2}$ 7.3 Hz, GlcA H-1); 5.30 (dd, 1 H, $J_{3,2}$ 8.8, $J_{3,4}$ 8.8 Hz, GlcA H-3); 5.21–4.98 (m, 4 H, GlcN H-1, GlcN H-3, GlcN H-4, GlcA H-2); 4.42–4.06 (m, 5

H, GlcN H-2, GlcN H-6a, GlcN H-6b, GlcA H-4, GlcA H-5); 3.77 (s, 3 H, CO_2Me); 3.73–3.63 (m, 1 H, GlcN H-5); 2.10, 2.07, 2.01, 2.00, 1.99, 1.98 (6 × s, 18 H, 6 × OAc); δ_C(75 MHz; $CDCl_3$) 171.4, 170.7, 169.8, 169.3, 169.1, 168.7 (6 × O*C*(O)CH_3); 167.8 (GlcA C-6); 157.6 (q, 38 Hz, *C*(O)CF_3); 115.4 (q, 286 Hz, C(O)*C*F_3); 96.5 (GlcN C-1); 91.4 (GlcA C-1); 74.1, 73.7, 73.0 (GlcA C-3, GlcA C-4, GlcA C-5); 70.2, 69.7, 68.9 (GlcN C-3, GlcN C-5, GlcA C-2); 67.0 (GlcN C-4); 61.1 (GlcN C-6); 53.0 (CO_2CH_3); 52.4 (GlcN C-2); 20.9–20.3 (6 × OC(O)*C*H_3); *m/z* (ESI) 740.1 ([M + Na]$^+$. $C_{27}H_{34}F_3NNaO_{18}$ requires 740.2).

3,4,6-Tri-*O*-acetyl-2-deoxy-2-trifluoroacetamido-α-D-glucopyranosyl-(1→4)-methyl 2,3-di-*O*-acetyl-D-glucopyranosyluronate (8). To a solution of **7** (2.69 g, 3.75 mmol) in THF (20 mL) at 0 °C under N_2 was added benzylamine (820 μL, 7.50 mmol). The mixture was allowed to regain ambient temperature and was stirred for 16 h. The mixture was diluted with $CHCl_3$ and washed with H_2O. The aqueous layer was back-extracted with $CHCl_3$ and the combined organic phase was washed with 1 N HCl, sat. aq. $NaHCO_3$, brine, H_2O, dried over Na_2SO_4, and the solvent was removed *in vacuo*. Flash chromatography on silica gel (1 : 1 hexanes–ethyl acetate, R_f 0.3) afforded **8** (2.19 g, 87%) as a 5 : 1 α : β mixture; α anomer δ_H(300 MHz; $CDCl_3$) 6.75 (d, 1 H, $J_{NH,2}$ 9.3 Hz, NH); 5.58 (dd, 1 H, $J_{3,2}$ 9.3, $J_{3,4}$ 9.3 Hz, GlcA H-3); 5.47 (d, 1 H, $J_{1,2}$ 3.4 Hz, GlcA H-1); 5.20 (d, 1 H, $J_{1,2}$ 3.7 Hz, GlcN H-1); 5.18–5.07 (m, 2 H, GlcN H-3, GlcN H-4); 4.83 (dd, 1 H, $J_{2,3}$ 9.3, $J_{2,1}$ 3.4 Hz, GlcA H-2); 4.58 (d, 1 H, $J_{5,4}$ 9.5 Hz, GlcA H-5); 4.36–4.08 (m, 4 H, GlcN H-2, GlcN H-6a, GlcN H-6b, GlcA H-4); 3.80 (s, 3 H, CO_2Me); 3.78–3.72 (m, 1 H, GlcN H-5); 2.12, 2.05, 2.03, 2.00, 2.00 (5 × s, 15 H, 5 × OAc); δ_C(75 MHz; $CDCl_3$) 171.2, 171.0, 170.4, 169.9, 169.4 (5 × O*C*(O)CH_3); 169.4 (GlcA C-6); 157.8 (q, 38 Hz, *C*(O)CF_3); 115.4 (q, 286 Hz, C(O)*C*F_3); 96.4 (GlcN C-1); 90.2 (GlcA C-1); 73.8 (GlcA C-4); 71.3 (GlcA C-3); 70.8 (GlcA C-2); 69.8 (GlcN C-3); 69.6 (GlcA C-5); 68.6 (GlcN C-5); 67.4 (GlcN C-4); 61.2 (GlcN C-6); 53.0 (CO_2CH_3); 52.2 (GlcN C-2); 20.7, 20.5, 20.5, 20.5, 20.3 (5 × OC(O)*C*H_3); β anomer δ_H(300 MHz; $CDCl_3$) 6.61 (d, 1 H, $J_{NH,2}$ 9.3 Hz, NH); 5.32 (dd, 1 H, $J_{3,2}$ 9.3, $J_{3,4}$ 9.3 Hz, GlcA H-3); 5.22–5.07 (m, 3 H, GlcN H-1, GlcN H-3, GlcN H-4); 4.88–4.80 (m, 2 H, GlcA H-1, GlcA H-2); 4.36–4.08 (m, 5 H, GlcN H-2, GlcN H-6a, GlcN H-6b, GlcA H-4, GlcA H-5); 3.81 (s, 3 H, CO_2Me); 3.78–3.72 (m, 1 H, GlcN H-5); 2.12–2.00 (5 × s, 15 H, 5 × OAc); δ_C(75 MHz; $CDCl_3$) 171.4, 171.0, 170.8, 170.1, 169.3 (5 × O*C*(O)CH_3); 168.8 (GlcA C-6); 157.8 (q, 38 Hz, *C*(O)CF_3); 115.4 (q, 286 Hz, C(O)*C*F_3); 96.3 (GlcN C-1); 95.4 (GlcA C-1); 73.8, 73.7, 73.5, 72.7, 69.8, 68.7, 67.5 (GlcN C-3, GlcN C-4, GlcN C-5, GlcA C-2, GlcA C-3, GlcA C-4, GlcA C-5); 61.1 (GlcN C-6); 53.1 (CO_2CH_3); 52.3 (GlcN C-2); 20.7–20.3 (5 × OC(O)*C*H_3); *m/z* (ESI) 698.150885 ([M + Na]$^+$. $C_{25}H_{32}F_3NNaO_{17}$ requires 698.151454.

4′-Nitrophenyl (3,4,6-tri-*O*-acetyl-2-deoxy-2-trifluoroacetamido-α-D-glucopyranosyl)-(1→4)-methyl 2,3-di-*O*-acetyl-β-D-glucopyranosiduronate (9). Prepared according to general procedure B from **2** and 4-nitrophenol. Flash chromatography on silica gel (2 : 1 hexanes–ethyl acetate, R_f 0.2) afforded **9** (80%); (Found: C, 47.06; H, 4.56; N, 3.25. $C_{31}H_{35}F_3N_2O_{19}$ requires C, 46.74; H, 4.43; N, 3.52%); δ_H(300 MHz; $CDCl_3$) 8.26–8.19 (AB m, 2 H, H-3′, H-5′); 7.10–7.04 (AB m, 2 H, H-2′, H-6′); 6.57 (d, 1 H, $J_{NH,2}$ 9.1 Hz, NH); 5.36 (d, 1 H, $J_{1,2}$ 6.0 Hz, GlcA H-1); 5.36 (dd, 1 H, $J_{3,2}$ 8.7, $J_{3,4}$ 8.7 Hz, GlcA H-3); 5.27 (d, 1 H, $J_{1,2}$ 3.8 Hz, GlcN H-1); 5.19 (dd, 1 H, $J_{2,3}$ 8.7, $J_{2,1}$ 6.0 Hz, GlcA H-2); 5.21–5.09 (m, 2 H, GlcN H-3, GlcN H-4); 4.54 (dd, 1 H, $J_{4,3}$ 8.7, $J_{4,5}$ 8.4 Hz, GlcA H-4); 4.33 (d, 1 H, $J_{5,4}$ 8.4 Hz, GlcA H-5); 4.32–4.11 (m, 3 H, GlcN H-2, GlcN H-6a, GlcN H-6b); 3.81–3.76 (m, 1 H, GlcN H-5); 3.65 (s, 3 H, CO_2Me); 2.10, 2.05, 2.04, 2.03, 2.01 (5 × s, 15 H, 5 × OAc); δ_C(75 MHz; $CDCl_3$) 171.5, 170.7, 169.9, 169.3, 169.1 (5 × O*C*(O)CH_3); 167.6 (GlcA C-6); 160.7 (C-1′); 157.5 (q, 38 Hz, *C*(O)CF_3); 143.3 (C-4′); 125.8 (C-3′, C-5′); 116.5 (C-2′, C-6′); 115.4 (q, 286 Hz, C(O)*C*F_3); 98.0 (GlcA C-1); 96.7 (GlcN C-1); 74.0 (GlcA C-5); 73.1 (GlcA C-3); 72.8 (GlcA C-4); 71.3 (GlcA C-2); 69.8 (GlcN C-3); 69.0 (GlcN C-5); 67.1 (GlcN C-4); 61.2 (GlcN C-6); 53.0 (CO_2CH_3); 52.6 (GlcN C-2); 20.7, 20.5, 20.5, 20.5, 20.4 (5 × OC(O)*C*H_3); *m/z* (ESI) 819.1 ([M + Na]$^+$. $C_{31}H_{35}F_3N_2O_{19}$ requires 819.2).

4′-Methylumbelliferyl (3,4,6-tri-*O*-acetyl-2-deoxy-2-trifluoroacetamido-α-D-glucopyranosyl)-(1→4)-methyl 2,3-di-*O*-acetyl-β-D-glucopyranosiduronate (10). Prepared according to general procedure B from **2** and 4-methylumbelliferone. Flash chromatography on silica gel (3 : 2 hexanes–ethyl acetate, R_f 0.3) afforded **10** (~31%); δ_H(300 MHz; $CDCl_3$) 7.52 (d, 1 H, $J_{5',6'}$ 9.5 Hz, H-5′); 6.94–6.91 (m, 2 H, H-6′, H-8′); 6.75 (d, 1 H, $J_{NH,2}$ 9.3 Hz, NH); 6.19 (q, 1 H, $J_{3',Me'}$ 1.2 Hz, H-3′); 5.39–5.25 (m, 2 H, GlcA H-1, GlcA H-3); 5.22–5.05 (m, 4 H, GlcN H-1, GlcN H-3, GlcN H-4, GlcA H-2); 4.49 (dd, 1 H, $J_{4,3}$ 8.7, $J_{4,5}$ 8.4 Hz, GlcA H-4); 4.33–4.07 (m, 5 H, GlcN H-2, GlcN H-5, GlcN H-6a, GlcN H-6b, GlcA H-5); 3.70 (s, 3 H, CO_2Me); 2.40 (d, $J_{Me',3'}$ 1.2 Hz, Me′); 2.11–1.98 (5 × s, 15 H, 5 × OAc); δ_C(75 MHz; $CDCl_3$) 171.5–169.1 (5 × O*C*(O)Me); 167.7 (GlcA C-6); 160.9 (C-2′); 158.8 (C-7′); 157.8 (q, 38 Hz, *C*(O)CF_3); 154.7, 152.3 (C-4′, C-8a′); 125.8 (C-5′); 115.6 (C-4a′); 115.4 (q, 286 Hz, C(O)*C*F_3); 113.8 (C-6′); 113.2 (C-3′); 103.9 (C-8′); 98.2 (GlcA C-1);96.5 (GlcN C-1); 74.0–72.9 (GlcA C-3, GlcA C-4, GlcA C-5); 71.3 (GlcA C-2); 69.9 (GlcN C-3); 69.0 (GlcN C-5); 67.2 (GlcN C-4); 61.1 (GlcN C-6); 52.9 (CO_2CH_3); 52.5 (GlcN C-2); 20.7–20.4 (5 × OC(O)*C*H_3); 18.7 (C-4′ *C*H_3); *m/z* (ESI) (*m/z*) 856.187146 ([M + Na]$^+$. $C_{35}H_{38}F_3NNaO_{19}$ requires 856.188241) and **6** (~31%), which were not separated.

4′-[3″-Methyl prop-2″(*E*)-enoate]phenyl (3,4,6-tri-*O*-acetyl-2-deoxy-2-trifluoroacetamido-α-D-glucopyranosyl)-(1→4)-methyl 2,3-di-*O*-acetyl-β-D-glucopyranosiduronate (11). Prepared according to general procedure B from **2** and methyl 4-hydroxycinnamate.[24] Flash chromatography on silica gel (3 : 2 hexanes–ethyl acetate) afforded **11** (59%, R_f 0.3); δ_H(300 MHz; $CDCl_3$) 7.64 (d, 1 H, $J_{3'',2''}$ 16.0 Hz, H-3″); 7.48 (d, 2 H, $J_{3',2'}$ 8.7 Hz, H-3′); 6.97 (d, 2 H, $J_{2',3'}$ 8.7 Hz, H-2′); 6.53 (d, 1 H, $J_{NH,2}$ 9.0 Hz, NH); 6.34 (d, 1 H, $J_{2'',3''}$ 16.0 Hz, H-2″); 5.36 (dd, 1 H, $J_{3,2}$ 8.8, $J_{3,4}$ 8.8 Hz, GlcA H-3); 5.29–5.26 (m, 2 H, GlcN H-1, GlcA H-1); 5.20–5.12 (m, 3 H, GlcN H-3, GlcN H-4, GlcA H-2); 4.53 (dd, 1 H, $J_{4,3}$ 8.8 Hz, $J_{4,5}$ 8.8 Hz, GlcA H-4); 4.32–4.11 (m, 4 H, GlcN H-2, GlcN H-6a, GlcN H-6b, GlcA H-5); 3.82–3.77 (m, 4 H, GlcN H-5, CO_2Me); 3.64 (s, 3 H, CO_2Me); 2.11, 2.05, 2.04, 2.03, 2.02 (5 × s, 15 H, 5 × OAc); δ_C(75 MHz; $CDCl_3$) 171.4, 170.7, 170.0, 169.4, 169.1 (5 × O*C*(O)CH_3); 167.8, 167.5 (GlcA C-6, C-1″); 157.7 (C-1′); 157.5 (q, 38 Hz, *C*(O)CF_3); 143.8 (C-3″); 129.6 (C-4′); 129.6 (C-3′, C-5′); 116.8 (C-2′, C-6′); 116.8 (C-2″); 115.4 (q, 286 Hz, C(O)*C*F_3); 98.4 (GlcA C-1); 96.6 (GlcN C-1); 73.9 (GlcA C-5); 73.5 (GlcA C-3); 72.9 (GlcA C-4); 71.6 (GlcA C-2); 69.8 (GlcN C-3); 68.9 (GlcN C-5); 67.2 (GlcN C-4); 61.1 (GlcN

C-6); 52.9, 51.7 (2 × CO_2CH_3); 52.5 (GlcN C-2); 20.7, 20.5, 20.5, 20.5, 20.4 (5 × OC(O)CH_3); *m/z* (ESI) 858.206055 ([M + Na]$^+$. $C_{35}H_{40}F_3NNaO_{19}$ requires 858.20443) and 3,4,6-tri-*O*-acetyl-2-deoxy-2-trifluoroacetamido-α-D-glucopyranosyl-(1→4)-methyl 2,3-di-*O*-acetyl-α-D-glucopyranosyluronate fluoride (**13**) (16%, R_f 0.4); δ_H(300 MHz; $CDCl_3$) 6.67 (d, 1 H, $J_{NH,2}$ 9.3 Hz, NH); 5.73 (dd, 1 H, $J_{1,F}$ 52.8 Hz, $J_{1,2}$ 2.7 Hz, GlcA H-1); 5.56 (dd, 1 H, $J_{3,2}$ 9.9 Hz, $J_{3,4}$ 9.3 Hz, GlcA H-3); 5.20 (d, 1 H, $J_{1,2}$ 3.9 Hz, GlcN H-1); 5.18–5.10 (m, 2 H, GlcN H-3, GlcN H-4); 4.89 (ddd, 1 H, $J_{2,F}$ 24.0, $J_{2,3}$ 9.9, $J_{2,1}$ 2.7 Hz, GlcA H-2); 4.47 (d, 1 H, $J_{5,4}$ 9.6 Hz, GlcA H-5); 4.32 (dd, 1 H, $J_{4,5}$ 9.6, $J_{4,3}$ 9.3 Hz, GlcA H-4); 4.30–4.09 (m, 3 H, GlcN H-2, GlcN H-6a, GlcN H-6b); 3.82 (s, 3 H, CO_2Me); 3.72–3.66 (m, 1 H, GlcN H-5); 2.12, 2.06, 2.03, 2.01, 2.01 (5 × OAc); δ_C(75 MHz; $CDCl_3$) 171.5, 170.8, 169.9, 169.7, 169.2 (5 × O*C*(O)CH_3); 167.9 (GlcA C-6); 157.6 (q, 38 Hz, *C*(O)CF_3); 115.4 (q, 286 Hz, C(O)*C*F_3); 103.6 (d, $J_{1,F}$ 230 Hz, GlcA C-1); 96.5 (GlcN C-1); 73.0 (GlcA C-4); 71.3 (d, $J_{5,F}$ 4 Hz, GlcA C-5); 70.0 (GlcA C-3); 69.9 (d, $J_{2,F}$ 24 Hz, GlcA C-2); 69.7 (GlcN C-3); 69.0 (GlcN C-5); 67.0 (GlcN C-4); 61.0 (GlcN C-6); 53.2 (CO_2CH_3); 52.4 (GlcN C-2); 20.7, 20.5, 20.4, 20.4, 20.4 (5 × OC(O)CH_3); *m/z* (ESI) 700.14607 ([M + Na]$^+$. $C_{25}H_{31}F_4NNaO_{16}$ requires 700.147111).

3′,5′-Dimethoxycarbonyl (3,4,6-tri-*O*-acetyl-2-deoxy-2-trifluoroacetamido-α-D-glucopyranosyl)-(1→4)-methyl 2,3-di-*O*-acetyl-β-D-glucopyranosiduronate (12). Prepared according to general procedure B from **2** and dimethyl 5-hydroxyisophthalate.[25] Flash chromatography on silica gel (3 : 2 hexanes–ethyl acetate, R_f 0.3) afforded **12** (61%); δ_H(300 MHz; $CDCl_3$) 8.40 (t, 1 H, $J_{4',2'} = J_{4',6'}$ 1.4 Hz, H-4′); 7.83 (d, 2 H, $J_{2',4'} = J_{6',4'}$ 1.4 Hz, H-2′, H-6′); 6.52 (d, 1 H, $J_{NH,2}$ 9.1 Hz, NH); 5.40–5.30 (m, 2 H, GlcA H-1, GlcA H-3); 5.27 (d, 1 H, $J_{1,2}$ 3.8 Hz, GlcN H-1); 5.22–5.09 (m, 3 H, GlcN H-3, GlcN H-4, GlcA H-2); 4.54 (dd, 1 H, $J_{4,3}$ 8.6, $J_{4,5}$ 8.4 Hz, GlcA H-4); 4.34 (d, 1 H, $J_{5,4}$ 8.4 Hz, GlcA H-5); 4.32–4.08 (m, 3 H, GlcN H-2, GlcN H-6a, GlcN H-6b); 3.95 (s, 6 H, 2 × $PhCO_2Me$); 3.84–3.77 (m, 1 H, GlcN H-5); 3.65 (s, 3 H, CO_2Me); 2.11, 2.07, 2.05, 2.04, 2.02 (5 × s, 15 H, 5 × OAc); δ_C(75 MHz; $CDCl_3$) 171.4, 170.7, 169.9, 169.4, 169.1 (5 × O*C*(O)CH_3); 167.8 (GlcA C-6); 165.5 (2 × Ph*C*O_2Me); 157.5 (q, 38 Hz, *C*(O)CF_3); 156.3 (C-1′); 132.1 (C-3′, C-5′); 125.5 (C-4′); 121.9 (C-2′, C-6′); 115.4 (q, 286 Hz, C(O)*C*F_3); 98.5 (GlcA C-1); 96.7 (GlcN C-1); 73.8 (GlcA C-5); 73.3 (GlcA C-3); 72.9 (GlcA C-4); 71.4 (GlcA C-2); 69.8 (GlcN C-3); 69.0 (GlcN C-5); 67.1 (GlcN C-4); 61.2 (GlcN C-6); 52.8 (CO_2CH_3); 52.6 (2 × CO_2CH_3); 52.5 (GlcN C-2); 20.7, 20.5, 20.5, 20.5, 20.4 (5 × OC(O)CH_3); *m/z* (ESI) 890.194956 ([M + Na]$^+$. $C_{35}H_{40}F_3NNaO_{21}$ requires 890.19426).

Methyl 2-acetamido-2-deoxy-6-*O*-sulfo-β-D-glucopyranoside, sodium salt (16). To a solution of **15**[26] (89 mg, 378 μmol) in DMF (2 mL) was added SO_3·Py complex (120 mg, 757 μmol). The mixture was stirred at rt for 16 h, and then cooled to 0 °C, methanol (1 mL) was added and the mixture was concentrated. The product was purified on Sephadex® LH-20, eluting with 4 : 1 methanol–H_2O afforded **16** (100 mg, 78%); δ_H(300 MHz; D_2O) 4.40 (d, 1 H, $J_{1,2}$ 8.4 Hz, H-1); 4.29 (dd, 1 H, $J_{6a,6b}$ 11.2, $J_{6a,5}$ 2.1 Hz, H-6a); 4.17 (dd, 1 H, $J_{6b,6a}$ 11.2, $J_{6b,5}$ 5.1 Hz,H-6b); 3.68–3.58 (m, 2 H, H-2, H-5); 3.54–3.42 (m, 5 H, H-3, H-4, OMe); 1.98 (s, 3 H, NAc); δ_C(75 MHz; D_2O) 174.6 (NH*C*(O)CH_3); 102.0 (C-1); 73.7, 73.6, 71.7 (C-3, C-4, C-5); 66.9 (C-6); 57.1 (OCH_3); 55.3 (C-2); 22.1 (NHC(O)*C*H_3); *m/z* (ESI) 360.032980 ([M + Na]$^+$. $C_9H_{16}NNa_2O_9S$ requires 360.033568).

Methyl 2-deoxy-2-sulfonamido-β-D-glucopyranoside sodium salt (17). Prepared according to general procedure C from methyl 2-amino-2-deoxy-β-D-glucopyranoside[23] to afford **17** (86%); δ_H(300 MHz; D_2O) 4.46 (d, 1 H, $J_{1,2}$ 8.4 Hz, H-1); 3.93 (dd, 1 H, $J_{6a,6b}$ 12.3, $J_{6a,5}$ 1.9 Hz, H-6a); 3.67 (AX m, 1 H, H-6b); 3.64 (AX m, 1 H, H-3); 3.55 (s, 3 H, OMe); 3.44–3.40 (m, 2 H, H-4, H-5); 2.99 (dd, 1 H, $J_{2,3}$ 10.0, $J_{2,1}$ 8.4 Hz, H-2); δ_C(75 MHz; D_2O) 102.5 (C-1); 75.6 (C-4/5); 74.7 (C-3); 70.0 (C-4/5); 60.7 (C-6); 59.9, 57.2 (C-2, O*C*H_3); *m/z* (ESI) 318.023732 ([M + Na]$^+$. $C_7H_{14}NNa_2O_8S$ requires 318.02355).

4′-Nitrophenyl (2-amino-2-deoxy-α-D-glucopyranosyl)-(1→4)-sodium β-D-glucopyranosiduronate (19). Prepared according to general procedure D from **9** to afford **19** (65%); δ_H(300 MHz; D_2O) 8.21 (AB m, 2 H, H-3′, H-5′); 7.18 (AB m, 2 H, H-2′, H-6′); 5.41 (d, 1 H, $J_{1,2}$ 4.0 Hz, GlcN H-1); 5.22 (d, 1 H, $J_{1,2}$ 7.8 Hz, GlcA H-1); 3.98 (AX m, 1 H, GlcA H-5); 3.87–3.81 (m, 2 H, GlcA H-3, GlcA H-4); 3.77–3.72 (m, 2 H, GlcN H-6a, GlcN H-6b); 3.70–3.60 (m, 2 H, GlcN H-5, GlcA H-2); 3.51 (dd, 1 H, $J_{3,2}$ 10.1, $J_{3,4}$ 9.6 Hz, GlcN H-3); 3.35 (dd, 1 H, $J_{4,3}$ 9.6, $J_{4,5}$ 9.6 Hz, GlcN H-3); 2.67 (dd, 1 H, $J_{2,3}$ 10.1, $J_{2,1}$ 4.0 Hz, GlcN H-2); δ_C(75 MHz; D_2O) 174.5 (GlcA C-6); 161.6 (C-1′); 142.5 (C-4′); 126.0 (C-3′, C-5′); 116.4 (C-2′, C-6′); 99.2 (GlcA C-1); 99.0 (GlcN C-1); 76.7 (GlcA C-5); 76.0, 75.9, 73.6, 72.6, 72.0 (GlcN C-3, GlcN C-5, GlcA C-2, GlcA C-3, GlcA C-4); 69.4 (GlcN C-4); 60.0 (GlcN C-6); 55.1 (GlcN C-2); *m/z* (ESI) 499.116688 ([M + Na]$^+$. $C_{18}H_{24}N_2NaO_{13}$ requires 499.117061).

4′-Methylumbelliferyl (2-amino-2-deoxy-α-D-glucopyranosyl)-(1→4)-sodium β-D-glucopyranosiduronate acetate (20). Prepared according to general procedure D from a ~1 : 1 mixture of **8** and **10** to afford **20** (100% from **10**); δ_H(300 MHz; D_2O) 7.34 (d, 1 H, $J_{5',6'}$ 8.9 Hz, H-5′); 6.87 (dd, 1 H, $J_{6',5'}$ 8.9, $J_{6',8'}$ 2.3 Hz, H-6′); 6.73 (d, 1 H, $J_{8',6'}$ 2.3 Hz, H-8′); 5.95 (s, 1 H, H-3′); 5.70 (d, 1 H, $J_{1,2}$ 3.7 Hz, GlcN H-1); 5.12 (d, 1 H, $J_{1,2}$ 7.8 Hz, GlcA H-1); 4.07–4.00 (AX m, 1 H, GlcA H-5); 3.98–3.88 (m, 2 H, GlcA H-3, GlcA H-4); 3.88–3.80 (m, 3 H, GlcN H-3, GlcN H-6a, GlcN H-6b); 3.80–3.73 (m, 1 H, GlcN H-5); 3.72–3.65 (AX m, 1 H, GlcA H-2); 3.49 (dd, 1 H, $J_{4,3}$ 9.6, $J_{4,5}$ 9.6 Hz, GlcN H-4); 3.24 (dd, 1 H, $J_{2,3}$ 10.6 Hz, $J_{2,1}$ 3.7 Hz, GlcN H-2); 2.18 (s, 3 H, CH_3′); δ_C(75 MHz; D_2O) 176.2 (GlcA C-6); 166.0 (C-2′); 161.2, 157.9, 155.2 (C-4′, C-7′, C-8a′); 128.4 (C-5′); 116.6 (C-4a′); 115.8 (C-6′); 112.9 (C-3′); 105.1 (C-8′); 101.2 (GlcA C-1); 97.8 (GlcN C-1); 78.4 (GlcA C-5); 77.9, 77.7 (GlcA C-3, GlcA C-4); 74.8 (GlcA C-2); 74.2 (GlcN C-5); 72.0 (GlcN C-3); 71.1 (GlcN C-4); 61.7 (GlcN C-6); 56.3 (GlcN C-2); 19.8 (CH_3′); *m/z* (ESI) 512.143037 ([M – Na]$^-$. $C_{22}H_{26}NO_{13}$ requires 512.140962) and a mixture (900 mg) of sodium acetate and 2-amino-2-deoxy-α-D-glucopyranosyl-(1→4)-sodium β-D-glucopyranosyluronate acetate.[27]

4′-[3″-(Sodium prop-2″(*E*)-enoate)]phenyl (2-amino-2-deoxy-α-D-glucopyranosyl)-(1→4)-sodium β-D-glucopyranosiduronate acetate (21). Prepared according to general procedure D from **11** to afford **21** (77%); δ_H(300 MHz; D_2O) 7.50 (d, 2 H, $J_{3',2'}$ 8.8 Hz, H-3′); 7.28 (d, 1 H, $J_{3'',2''}$ 16.0 Hz, H-3″); 7.04 (d, 2 H, $J_{2',3'}$ 8.8 Hz, H-2′); 6.35 (d, 1 H, $J_{2'',3''}$ 16.0 Hz, H-2″); 5.53 (d, 1 H, $J_{1,2}$ 3.7 Hz, GlcN H-1); 5.07 (d, 1 H, $J_{1,2}$ 7.8 Hz, GlcA H-1); 3.95–3.90 (m, 1 H, GlcA H-5); 3.84–3.80 (m, 1 H, GlcA H-4); 3.77–3.54 (m, 6 H,

GlcN H-3, GlcN H-5, GlcN H-6a, GlcN H-6b, GlcA H-2, GlcA H-3); 3.38 (dd, 1 H, $J_{4,3}$ 9.5 Hz, $J_{4,5}$ 9.5 Hz, GlcN H-4); 2.95 (dd, 1 H, $J_{2,3}$ 10.3 Hz, $J_{2,1}$ 3.7 Hz, GlcN H-2); δ_C(75 MHz; D_2O) 181.5 (O*C*(O)CH_3); 175.9, 174.5 (GlcA C-6, C-1″); 157.4 (C-1′); 140.2 (C-3″); 130.6 (C-4′); 129.3 (C-3′, C-5′); 122.7 (C-2″); 116.6 (C-2′, C-6′); 99.8 (GlcA C-1); 97.1 (GlcN C-1); 76.4 (GlcA C-5); 76.0, 75.8 (GlcA C-3, GlcA C-4); 72.8, 72.1, 71.5 (GlcN C-3, GlcN C-5, GlcA C-2); 69.2 (GlcN C-4); 59.8 (GlcN C-6); 54.5 (GlcN C-2); 23.2 (OC(O)*C*H_3).

3′,5′-Disodium carbonyl (2-amino-2-deoxy-α-D-glucopyranosyl)-(1→4)-sodium β-D-glucopyranosiduronate acetate (22). Prepared according to general procedure D from **12** to afford **22** (97%); δ_H(300 MHz; $CDCl_3$) 7.93 (bt, 1 H, H-4′); 7.58 (bd, 2 H, H-2′, H-6′); 5.62 (d, 1 H, $J_{1,2}$ 3.7 Hz, GlcN H-1); 5.10 (d, 1 H, $J_{1,2}$ 7.8 Hz, GlcA H-1); 4.03–3.97 (AX m, 1 H, GlcA H-5); 3.89–3.79 (m, 2 H, GlcA H-3, GlcA H-4); 3.79–3.71 (m, 3 H, GlcN H-3, GlcN H-6a, GlcN H-6b); 3.71–3.64 (m, 1 H, GlcN H-5); 3.61 (dd, 1 H, $J_{2,3}$ 8.2, $J_{2,1}$ 7.8 Hz, GlcA H-2); 3.42 (dd, 1 H, $J_{3,2}$ 9.5, $J_{3,4}$ 9.5 Hz, GlcN H-4); 3.21 (dd, 1 H, $J_{2,3}$ 9.5, $J_{2,1}$ 3.7 Hz, GlcN H-2); 1.84 (s, 3 H, OAc); δ_C(75 MHz; $CDCl_3$) 183.3 (O*C*(O)CH_3); 176.4, 176.1 (GlcA C-6, 2 × Ph*C*O_2Na); 158.2 (C-1′); 139.9 (C-3′, C-5′); 125.7 (C-4′); 121.2 (C-2′, C-6′); 102.1 (GlcA C-1); 97.4 (GlcN C-1); 78.1, 77.7, 77.6 (GlcA C-3, GlcA C-4, GlcA C-5); 74.8 (GlcA C-2); 74.1 (GlcN C-5); 71.6 (GlcN C-3); 70.9 (GlcN C-4); 61.6 (GlcN C-6); 56.0 (GlcN C-2); 25.2 (OC(O)*C*H_3).

4′-Nitrophenyl (2-acetamido-2-deoxy-α-D-glucopyranosyl)-(1→4)-sodium β-D-glucopyranosiduronate (23). Prepared according to general procedure E from **19** to afford **23** (48%); δ_H(300 MHz; D_2O) 8.29–8.24 (AB m, 2 H, H-3′, H-5′); 7.27–7.22 (m, 2 H, H-2′, H-6′); 5.44 (d, 1 H, $J_{1,2}$ 4.0 Hz, GlcN H-1); 5.33 (d, 1 H, $J_{1,2}$ 7.8 Hz, GlcA H-1); 4.22 (d, 1 H, $J_{5,4}$ 9.3 Hz, GlcA H-5); 3.96–3.63 (m, 8 H, GlcN H-2, GlcN H-3, GlcN H-5, GlcN H-6a, GlcN H-6b, GlcA H-2, GlcA H-3, GlcA H-4); 3.54 (dd, 1 H, 10.1, 8.8 Hz, GlcN H-4); 2.06 (s, 3 H, NAc); δ_C(75 MHz; D_2O) 174.4 (GlcA C-6); 172.0 (NH*C*(O)CH_3); 161.4 (C-1′); 142.7 (C-4′); 126.0 (C-3′, C-5′); 116.5 (C-2′, C-6′); 99.2 (GlcA C-1); 97.5 (GlcN C-1); 75.8, 75.4 (GlcA C-3, GlcA C-4); 74.4 (GlcA C-5); 72.8, 72.3, 70.5 (GlcN C-3, GlcN C-5, GlcA C-2); 69.3 (GlcN C-4); 59.8 (GlcN C-6); 53.5 (GlcN C-2); 21.9 (NHC(O)*C*H_3); *m*/*z* (ESI) 517.131970 ([M − Na]$^-$. $C_{20}H_{25}N_2O_{14}$ requires 517.131129).

4′-Nitrophenyl (2-deoxy-2-sulfonamido-α-D-glucopyranosyl)-(1→4)-sodium β-D-glucopyranosiduronate, sodium salt (24). Prepared according to general procedure C from **19** to afford **24** (32%); δ_H(300 MHz; D_2O) 8.26–8.23 (AB m, 2 H, H-3′, H-5′); 7.24–7.20 (m, 2 H, H-2′, H-6′); 5.68 (d, 1 H, $J_{1,2}$ 3.6 Hz, GlcN H-1); 5.30 (d, 1 H, $J_{1,2}$ 8.1 Hz, GlcA H-1); 4.05–3.90 (m, 3 H, GlcA H-3, GlcA H-4, GlcA H-5); 3.80 (m, 2 H, GlcN H-6a, GlcN H-6b); 3.75–3.66 (m, 2 H, GlcN H-5, GlcA H-2); 3.63 (dd, 1 H, $J_{3,2}$ 10.2, $J_{3,4}$ 9.6 Hz, GlcN H-3); 3.50 (dd, 1 H, $J_{4,3}$ 9.6, $J_{4,5}$ 9.6 Hz, GlcN H-4); 3.26 (dd, 1 H, $J_{2,3}$ 10.2, $J_{2,1}$ 3.6 Hz, GlcN H-2); δ_C(75 MHz; D_2O) 174.4 (GlcA C-6); 162.0 (C-1′); 142.9 (C-4′); 126.5 (C-3′, C-5′); 116.8 (C-2′, C-6′); 99.6 (GlcA C-1); 97.7 (GlcN C-1); 77.1, 76.4, 76.3 (GlcA C-3, GlcA C-4, GlcA C-5); 72.8, 72.0, 71.5 (GlcN C-3, GlcN C-5, GlcA C-2); 69.9 (GlcN C-4); 60.4 (GlcN C-6); 58.4 (GlcN C-2); *m*/*z* (ESI) 577.059334 ([M − Na]$^-$. $C_{18}H_{22}N_2NaO_{16}S$ requires 577.059315).

4′-Methylumbelliferyl (2-acetamido-2-deoxy-α-D-glucopyranosyl)-(1→4)-sodium β-D-glucopyranosiduronate (25). Prepared according to general procedure E from **20** to afford **25** (100%); δ_H(300 MHz; D_2O) 7.41 (d, 1 H, $J_{5',6'}$ 8.9 Hz, H-5′); 6.92 (dd, 1 H, $J_{6',5'}$ 8.9, $J_{6',8'}$ 2.4 Hz, H-6′); 6.81 (d, 1 H, $J_{8',6'}$ 2.4 Hz, H-8′); 6.01 (q, 1 H, $J_{3',Me'}$ 1.1 Hz, H-3′); 5.45 (d, 1 H, $J_{1,2}$ 3.7 Hz, GlcN H-1); 5.15 (d, 1 H, $J_{1,2}$ 7.8 Hz, GlcA H-1); 4.04–3.96 (AX m, 1 H, GlcA H-5); 3.91 (dd, 1 H, $J_{2,3}$ 10.7, $J_{2,1}$ 3.7 Hz, GlcN H-2); 3.90–3.84 (m, 2 H, GlcA H-3, GlcA H-4); 3.83–3.80 (m, 2 H, GlcN H-6a, GlcN H-6b); 3.80–3.73 (m, 2 H, GlcN H-3, GlcN H-5); 3.69–3.62 (AX m, 1 H, GlcA H-2); 3.49 (dd, 1 H, $J_{4,3}$ 9.5, $J_{4,5}$ 9.5 Hz, GlcN H-4); 2.22 (d, 3 H, $J_{Me',3'}$ 1.1 Hz, CH_3′); 2.07 (s, 3 H, NAc); δ_C(75 MHz; D_2O) 176.9 (GlcA C-6); 176.9 (NH*C*(O)CH_3); 166.6 (C-2′); 161.8, 158.5, 155.9 (C-4′, C-7′, C-8a′); 129.1 (C-5′); 117.3 (C-4a′); 116.4 (C-6′); 113.5 (C-3′); 105.7 (C-8′); 101.7 (GlcA C-1); 99.6 (GlcN C-1); 79.4 (GlcA C-5); 78.7, 78.3 (GlcA C-3, GlcA C-4); 75.6 (GlcA C-2); 74.4, 73.2 (GlcN C-3, GlcN C-5); 72.2 (GlcN C-4); 62.6 (GlcN C-6); 56.2 (GlcN C-2); 24.5 (NHC(O)*C*H_3); 20.4 (CH_3′); *m*/*z* (ESI) 554.151152 ([M − Na]$^-$. $C_{24}H_{28}NO_{14}$ requires 554.151525).

4′-Methylumbelliferyl (2-deoxy-2-sulfonamido-α-D-glucopyranosyl)-(1→4)-sodium β-D-glucopyranosiduronate, sodium salt (26). Prepared according to general procedure C from **20** to afford **26** (67%); δ_H(300 MHz; D_2O) 7.52 (d, 1 H, $J_{5',6'}$ 8.9 Hz, H-5′); 7.00 (dd, 1 H, $J_{6',5'}$ 8.9, $J_{6',8'}$ 2.4 Hz, H-6′); 6.92 (d, 1 H, $J_{8',6'}$ 2.4 Hz, H-8′); 6.09 (q, 1 H, $J_{3',Me'}$ 1.1 Hz, H-3′); 5.69 (d, 1 H, $J_{1,2}$ 3.7 Hz, GlcN H-1); 5.23 (d, 1 H, $J_{1,2}$ 7.9 Hz, GlcA H-1); 4.07–3.89 (m, 3 H, GlcA H-3, GlcA H-4, GlcA H-5); 3.85–3.80 (m, 2 H, GlcN H-6a, GlcN H-6b); 3.79–3.62 (m, 3 H, GlcN H-3, GlcN H-5, GlcA H-2); 3.51 (dd, 1 H, $J_{4,3}$ 9.5, $J_{4,5}$ 9.5 Hz, GlcN H-4); 3.27 (dd, 1 H, $J_{2,3}$ 10.3 Hz, $J_{2,1}$ 3.7 Hz, GlcN H-2); 2.31 (d, 3 H, $J_{Me',3'}$ 1.1 Hz, CH_3′); δ_C(75 MHz; D_2O) 174.4 (GlcA C-6); 164.3 (C-2′); 159.3, 156.1, 153.5 (C-4′, C-7′, C-8a′); 126.6 (C-5′); 114.9 (C-4a′); 113.9 (C-6′); 111.1 (C-3′); 103.3 (C-8′); 99.2 (GlcA C-1); 97.4 (GlcN C-1); 76.8 (GlcA C-5); 76.2, 75.9 (GlcA C-3, GlcA C-4); 72.4, 71.6, 71.2 (GlcN C-3, GlcN C-5, GlcA C-2); 69.6 (GlcN C-4); 60.0 (GlcN C-6); 58.0 (GlcN C-2); 17.9 (CH_3′); *m*/*z* (ESI) 592.095721 ([M − 2 Na + H]$^-$. $C_{22}H_{26}NO_{16}S$ requires 592.097775).

4′-[3″-(Sodium prop-2″(*E*)-enoate)]phenyl (2-acetamido-2-deoxy-α-D-glucopyranosyl)-(1→4)-sodium β-D-glucopyranosiduronate (27). Prepared according to general procedure E from **21** to afford **27** (100%); δ_H(300 MHz; D_2O) 7.51 (d, 2 H, $J_{3',2'}$ 8.8 Hz, H-3′); 7.37 (d, 1 H, $J_{3'',2''}$ 16.0 Hz, H-3″); 7.04 (d, 2 H, $J_{2',3'}$ 8.8 Hz, H-2′); 6.34 (d, 1 H, $J_{2'',3''}$ 16.0 Hz, H-2″); 5.37 (d, 1 H, $J_{1,2}$ 3.7 Hz, GlcN H-1); 5.08 (d, 1 H, $J_{1,2}$ 7.8 Hz, GlcA H-1); 3.91–3.64 (m, 8 H, GlcN H-2, GlcN H-3, GlcN H-5, GlcN H-6a, GlcN H-6b, GlcA H-3, GlcA H-4, GlcA H-5); 3.54 (dd, 1 H, $J_{2,3}$ 8.7, $J_{2,1}$ 7.8 Hz, GlcA H-2); 3.42 (dd, 1 H, $J_{4,3}$ 9.5, $J_{4,5}$ 9.5 Hz, GlcN H-4); 1.99 (s, 3 H, NAc); δ_C(75 MHz; D_2O) 175.0, 174.5, 174.3 (GlcA C-6, C-1″, NH*C*(O)CH_3); 157.6 (C-1′); 140.9 (C-3″); 129.6 (C-4′); 129.4 (C-3′, C-5′); 121.8 (C-2″); 116.6 (C-2′, C-6′); 99.6 (GlcA C-1); 96.9 (GlcN C-1); 76.8 (GlcA C-5); 76.3, 75.6 (GlcA C-3, GlcA C-4); 73.1 (GlcA C-2); 71.8, 70.6 (GlcN C-3, GlcN C-5); 69.6 (GlcN C-4); 60.0 (GlcN C-6); 53.6 (GlcN C-2); 21.8 (NHC(O)*C*H_3); *m*/*z* (ESI) 542.151100 ([M − 2 Na + H]$^-$. $C_{23}H_{28}NO_{14}$ requires 542.151525).

4′-[3″-(Sodium prop-2″(*E*)-enoate)]phenyl (2-deoxy-2-sulfonamido-α-D-glucopyranosyl)-(1→4)-sodium β-D-glucopyranosiduronate, sodium salt (28). Prepared according to general procedure C from **21** to afford **28** (76%); δ_H(300 MHz; D_2O) 7.57–7.51 (AX m, 2 H, H-3′); 7.31 (d, 1 H, $J_{3'',2''}$ 16.0 Hz, H-3″); 7.10–7.06 (AX m, 2 H, H-2′); 6.38 (d, 1 H, $J_{2'',3''}$ 16.0 Hz, H-2″); 5.63 (d, 1 H, $J_{1,2}$ 3.7 Hz, GlcN H-1); 5.15 (d, 1 H, $J_{1,2}$ 8.0 Hz, GlcA H-1); 3.98–3.81 (m, 3 H, GlcA H-3, GlcA H-4, GlcA H-5); 3.78–3.53 (m, 5 H, GlcN H-3, GlcN H-5, GlcN H-6a, GlcN H-6b, GlcA H-2); 3.45 (dd, 1 H, 9.7, 9.3 Hz, GlcN H-4); 3.22 (dd, 1 H, $J_{2,3}$ 10.3, $J_{2,1}$ 3.7 Hz, GlcN H-2); δ_C(75 MHz; D_2O) 175.9, 174.6 (GlcA C-6, C-1″); 157.4 (C-1′); 140.2 (C-3″); 130.1 (C-4′); 129.3 (C-3′, C-5′); 122.8 (C-2″); 116.7 (C-2′, C-6′); 99.8 (GlcA C-1); 97.3 (GlcN C-1); 76.7, 76.1, 76.0 (GlcA C-3, GlcA C-4, GlcA C-5); 72.5, 71.6, 71.2 (GlcN C-3, GlcN C-5, GlcA C-2); 69.6 (GlcN C-4); 60.0 (GlcN C-6); 58.0 (GlcN C-2); *m/z* (ESI) 580.099737 ($[M - 3\ Na + 2\ H]^-$. $C_{21}H_{26}NO_{16}S$ requires 580.097774).

3′,5′-Disodium carbonyl (2-acetamido-2-deoxy-α-D-glucopyranosyl)-(1→4)-sodium β-D-glucopyranosiduronate (29). Prepared according to general procedure E from **22** to afford **29** (61%); δ_H(300 MHz; $CDCl_3$) 7.98 (bt, 1 H, H-4′); 7.63 (bd, 2 H, H-2′, H-6′); 5.43 (d, 1 H, $J_{1,2}$ 3.7 Hz, GlcN H-1); 5.14 (d, 1 H, $J_{1,2}$ 7.9 Hz, GlcA H-1); 4.03–3.97 (AX m, 1 H, GlcA H-5); 3.89 (dd, 1 H, $J_{2,3}$ 10.7, $J_{2,1}$ 3.7 Hz, GlcN H-2); 3.87–3.82 (m, 2 H, GlcA H-3, GlcA H-4); 3.82–3.78 (m, 2 H, GlcN H-6a, GlcN H-6b); 3.78–3.69 (m, 2 H, GlcN H-3, GlcN H-5); 3.61 (dd, 1 H, $J_{2,3}$ 8.4, $J_{2,1}$ 7.9 Hz, GlcA H-2); 3.47 (dd, 1 H, $J_{3,2}$ 9.4, $J_{3,4}$ 9.4 Hz, GlcN H-4); 2.04 (s, 3 H, NAc); δ_C(75 MHz; $CDCl_3$) 176.7, 174.4, 174.3 (GlcA C-6, NH*C*(O)CH_3, 2 × Ph*C*O_2Na); 156.4 (C-1′); 138.1 (C-3′, C-5′); 123.7 (C-4′); 119.3 (C-2′, C-6′); 100.4 (GlcA C-1); 96.9 (GlcN C-1); 76.8 (GlcA C-5); 76.2, 75.7 (GlcA C-3, GlcA C-4); 73.2 (GlcA C-2); 71.8, 70.6 (GlcN C-3, GlcN C-5); 69.6 (GlcN C-4); 60.0 (GlcN C-6); 53.6 (GlcN C-2); 21.9 (NHC(O)*C*H_3); *m/z* (ESI) 560.128451 ($[M - 3\ Na + 2\ H]^-$. $C_{22}H_{26}NO_{16}$ requires 560.125706).

3′,5′-Disodium carbonyl (2-deoxy-2-sodium sulfonamido-α-D-glucopyranosyl)-(1→4)-sodium β-D-glucopyranosiduronate, sodium salt (30). Prepared according to general procedure C from **22** to afford **30** (39 mg, 65%); δ_H(300 MHz; $CDCl_3$) 8.03 (bs, 1 H, H-4′); 7.68 (bd, 2 H, H-2′, H-6′); 5.66 (d, 1 H, $J_{1,2}$ 3.7 Hz, GlcN H-1); 5.20 (d, 1 H, $J_{1,2}$ 7.9 Hz, GlcA H-1); 4.07–3.85 (m, 3 H, GlcA H-3, GlcA H-4, GlcA H-5); 3.83–3.78 (m, 2 H, GlcN H-6a, GlcN H-6b); 3.76–3.59 (m, 3 H, GlcN H-3, GlcN H-5, GlcA H-2); 3.48 (dd, 1 H, $J_{4,3}$ 9.5, $J_{4,5}$ 9.5 Hz, GlcN H-4); 3.25 (dd, 1 H, $J_{2,3}$ 10.3, $J_{2,1}$ 3.7 Hz, GlcN H-2); δ_C(75 MHz; $CDCl_3$) 174.6, 174.3 (GlcA C-6, 2 × Ph*C*O_2Na); 156.3 (C-1′); 138.0 (C-3′, C-5′); 123.7 (C-4′); 119.2 (C-2′, C-6′); 100.2 (GlcA C-1); 97.3 (GlcN C-1); 76.6, 76.1, 75.9 (GlcA C-3, GlcA C-4, GlcA C-5); 72.5 (GlcA C-2); 71.5, 71.1 (GlcN C-3, GlcN C-5); 69.5 (GlcN C-4); 59.9 (GlcN C-6); 57.9 (GlcN C-2); *m/z* (ESI) 598.074728 ($[M - 4\ Na^+ + 3\ H]^-$. $C_{20}H_{24}NO_{18}S$ requires 598.071956).

Materials

Ampicillin was purchased from Astral Scientific (Sydney, NSW, Australia). One Shot® TOP10 Competent Cells were purchased from Invitrogen (Carlsbad, CA, USA). Restriction enzymes and buffers, DNA ladders, and Calf Intestinal Alkaline Phosphatase (CIP) were purchased from New England Biolabs (Ipswich, MA, USA). Bacto®-tryptone, Bacto®-yeast extract, and agarose were purchased from Oxoid (Basingstoke, Hampshire, UK). The QIAprep® spin miniprep kit, QIAquick® gel extraction kit, and the QIAGEN plasmid midi kit were supplied by QIAGEN Pty Ltd (Doncaster, VIC, Australia). All custom made primers were supplied by Bio-strategy Ltd (Paddington, QLD, Australia) and GeneWorks Pty Ltd (Thebarton, South Australia). All sequencing was carried out by the Australian Genome Research Facility (AGRF, QLD, Australia). pCR-Blunt and the Zero Blunt® PCR cloning kit were purchased from Invitrogen (Carlsbad, CA, USA). The pAcGP67-A baculovirus transfer vector and the pAcUW51 dual expression vector were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Microcon YM-10 ultrafiltration devices for centrifugation were obtained from Millipore (Bedford, MA, USA).

[^{3}H]-HS was prepared by Progen Pharmaceuticals Ltd, by de-*N*-acetylation of HS with hydrazine sulfate and re-*N*-acetylation with [^{3}H]-acetic anhydride (16 μmol, 8 mCi).[7a] The concentrations of the [^{3}H]-HS preparations were accurately determined using the dimethylmethylene blue assay for GAGs.[28] Unlabelled HS from the same source was used as standard.

Cloning and expression of recombinant human heparanase

Heparanase cDNA was provided by Dr Mark Hulett and Prof. Chris Parish, (ANU, Canberra, Australia). The strategy of McKenzie *et al.* was then followed and proceeded essentially as described.[19] Insect cell expression was carried out at the Protein Expression Facility, Institute for Molecular Biosciences, University of Queensland. The expression protocol published by McKenzie *et al.*[19] was followed. The resultant crude supernatants containing heparanase were kept at 4 °C until purification, which was carried out at Progen Pharmaceuticals Ltd, Darra, QLD. Media was clarified by centrifuging at 17 700 *g* for 30 min at 4 °C and the pH was adjusted by the addition of 10 mM sodium phosphate, pH 7.0. The material was applied to a 145 mL SP Sepharose FF (GE Healthcare) column. The column was then eluted with a 1.3 L gradient of 0–0.75 M NaCl in 10 mM sodium phosphate, pH 7.0 buffer. Heparanase fractions were detected by electrophoresis and pure heparanase was pooled and exchanged into 10 mM sodium phosphate, pH 7.0 buffer. The amount of heparanase purified from 1 L of insect cell culture media was 0.4 mg.

Heparanase activity assay

The putative heparanase substrates were assayed against recombinant human heparanase as follows; reaction mixtures (50 μL) were prepared in 96-well plates containing putative substrate (5.0 mM), BSA (0.1 mg mL^{-1}), and heparanase (1.2 μg mL^{-1}) in 60 mM sodium acetate buffer, pH 5.0. The samples were incubated at 37 °C with shaking at 400 rpm for 4 h–3 d. Reactions were stopped by the addition of 200 μL of 0.2 M glycine buffer, pH 10.7. Fluorescence was measured using a VICTOR^3 Multilabel Plate Reader at emission and excitation wavelengths of 460 and 355 nm, respectively. Absorbance was measured using a VICTOR^3 Multilabel Plate Reader at 405 nm for 4-nitrophenol, and at 355 nm for 3-nitrophenol, 4-hydroxycinnamic acid, and 5-hydroxyisophthalic acid. The assays were performed at least in duplicate and were

corrected for background fluorescence/absorbance (reactions were performed in the absence of heparanase).

Stability assay

A 50 μL reaction mixture was prepared in a 96-well plate that contained **26** (5 mM), HS (4 mM), and BSA (0.1 mg mL^{-1}) in 60 mM sodium acetate buffer, pH 5.0. The plate was incubated at 37 °C with shaking at 400 rpm for 3 d and then analysed by low resolution mass spectrometry.

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References

1 For recent reviews see: (*a*) K. Raman and B. Kuberan, Chemical Tumor Biology of Heparan Sulfate Proteoglycans, *Curr. Chem. Biol.*, 2010, **4**, 20–31; (*b*) J. P. Li and I. Vlodavsky, Heparin, heparan sulfate and heparanase in inflammatory reactions, *Thromb. Haemost.*, 2009, **102**, 823–828.

2 L. Kjellen and U. Lindahl, Proteoglycans: structures and interactions, *Annu. Rev. Biochem.*, 1991, **60**, 443–475.

3 N. Ilan, M. Elkin and I. Vlodavsky, Regulation, function and clinical significance of heparanase in cancer metastasis and angiogenesis, *Int. J. Biochem. Cell Biol.*, 2006, **38**, 2018–2039.

4 (*a*) L. Fux, N. Ilan, R. D. Sanderson and I. Vlodavsky, Heparanase: busy at the cell surface, *Trends Biochem. Sci.*, 2009, **34**, 511–519; (*b*) E. Hammond, I. Bytheway, V. Ferro, Heparanase as a target for anticancer therapeutics: New developments and future prospects In *New Developments in Therapeutic Glycomics*, M. Delehedde, H. Lortat-Jacob, ed. Research Signpost: Kerala, India, 2006 pp 251–282.

5 (*a*) I. Vlodavsky, N. Ilan, A. Naggi and B. Casu, Heparanase: structure, biological functions, and inhibition by heparin-derived mimetics of heparan sulfate, *Curr. Pharm. Des.*, 2007, **13**, 2057–2073; (*b*) E. A. McKenzie, Heparanase: a target for drug discovery in cancer and inflammation, *Br. J. Pharmacol.*, 2007, **151**, 1–14; (*c*) V. Ferro, E. Hammond and J. K. Fairweather, The development of inhibitors of heparanase, a key enzyme involved in tumour metastasis, angiogenesis and inflammation, *Mini Rev. Med. Chem.*, 2004, **4**, 693–702.

6 For example see: (*a*) D. S. Pikas, J. P. Li, I. Vlodavsky and U. Lindahl, Substrate specificity of heparanases from human hepatoma and platelets, *J. Biol. Chem.*, 1998, **273**, 18770–18777; (*b*) Y. Okada, S. Yamada, M. Toyoshima, J. Dong, M. Nakajima and K. Sugahara, Structural recognition by recombinant human heparanase that plays critical roles in tumour metastasis: hierarchical sulfate groups with differential effects and the essential target disulfated trisaccharide sequence, *J. Biol. Chem.*, 2002, **277**, 42488–42495; (*c*) S. B. Peterson and J. Liu, Unraveling the specificity of heparanase utilizing synthetic substrates, *J. Biol. Chem.*, 2010, **285**, 14504–14513.

7 (*a*) C. Freeman and C. R. Parish, A rapid quantitative assay for the detection of mammalian heparanase activity, *Biochem. J.*, 1997, **325**, 229–237; (*b*) F. Behzad and P. E. C. Brenchley, A multiwell format assay for heparanase, *Anal. Biochem.*, 2003, **320**, 207–213; (*c*) K. S. Huang, J. Holmgren, L. Reik, D. Lucas-McGady, J. Roberts, C. M. Liu and W. Levin, High-throughput methods for measuring heparanase activity and screening potential antimetastatic and anti-inflammatory agents, *Anal. Biochem.*, 2004, **333**, 389–398; (*d*) S. Tsuchida, K. A. Podyma-Inoue and M. Yanagishita, Ultrafiltration-based assay for heparanase activity, *Anal. Biochem.*, 2004, **331**, 147–152; (*e*) K. Enomoto, H. Okamoto, Y. Numata and H. Takemoto, A simple and rapid assay for heparanase activity using homogeneous time-resolved fluorescence, *J. Pharm. Biomed. Anal.*, 2006, **41**, 912–917.

8 (*a*) TaKaRa Bio, Heparan degrading enzyme assay kit. Available from: <http://takaramirusbio.com>; (*b*) C. Tokuda, L. Jacquemart, M. Préaudat, Development of a homogeneous heparanase assay using HTRF technology, Cisbio international technical document. Available from: <http://www.htrf-assays.com>.

9 E. Hammond, C. P. Li and V. Ferro, Development of a colorimetric assay for heparanase activity suitable for kinetic analysis and inhibitor screening, *Anal. Biochem.*, 2010, **396**, 112–116.

10 A. Bisio, A. Mantegazza, E. Urso, A. Naggi, G. Torri, C. Viskov and B. Casu, High-performance liquid chromatographic/mass spectrometric studies on the susceptibility of heparin species to cleavage by heparanase, *Semin. Thromb. Hemost.*, 2007, **33**, 488–495.

11 For example see: (*a*) A. Adibekian, P. Bindschädler, M. S. M. Timmer, C. Noti, N. Schützenmeister and P. H. Seeberger, *De novo* synthesis of uronic acid building blocks for assembly of heparin oligosaccharides, *Chem.–Eur. J.*, 2007, **13**, 4510–4522; (*b*) L. Poletti and L. Lay, Chemical contributions to understanding heparin activity: synthesis of related sulfated oligosaccharides, *Eur. J. Org. Chem.*, 2003, **16**, 2999–3024; (*c*) B. K. S. Yeung, P. Y. C. Chong and P. A. Petillo, Synthesis of glycosaminoglycans, *J. Carbohydr. Chem.*, 2002, **21**, 799–865.

12 H. A. Orgueira, A. Bartolozzi, P. Schell, R. Litjens, E. R. Palmacci and P. H. Seeberger, Modular synthesis of heparin oligosaccharides, *Chem.–Eur. J.*, 2003, **9**, 140–169.

13 A. G. Pearson, M. J. Kiefel, V. Ferro and M. von Itzstein, Towards the synthesis of aryl glucuronides as potential heparanase probes. An interesting outcome in the glycosidation of glucuronic acid with 4-hydroxycinnamic acid, *Carbohydr. Res.*, 2005, **340**, 2077–2085.

14 For example see: (*a*) T. Sawada, S. Fujii, H. Nakano, S. Ohtake, K. Kimata and O. Habuchi, Synthesis of sulfated phenyl 2-acetamido-2-deoxy-D-galactopyranosides. 4-*O*-Sulfated phenyl 2-acetamido-2-deoxy-β-D-galactopyranoside is a competitive acceptor that decreases sulfation of chondroitin sulfate by *N*-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase, *Carbohydr. Res.*, 2005, **340**, 1983–1996; (*b*) H. Uzawa, Y. Nishida, K. Sasaki, T. Nagatsuka, H. Hiramatsu and K. Kobayashi, Sulfatase-catalysed assembly of regio selectively *O*-sulfonated *p*-nitrophenyl α-D-gluco- and α-D-mannopyranosides, *Carbohydr. Res.*, 2004, **339**, 1597–1602; (*c*) A. K. Misra, G. Agnihotri, S. K. Madhusudan and P. Tiwari, Practical synthesis of sulfated analogs of lactosamine and sialylated lactosamine derivatives, *J. Carbohydr. Chem.*, 2004, **23**, 191–199.

15 S. A. Gerber, F. Turecek and M. H. Gelb, Design and synthesis of substrate and internal standard conjugates for profiling enzyme activity in the Sanfilippo syndrome by affinity chromatography/electrospray ionisation mass spectrometry, *Bioconjugate Chem.*, 2001, **12**, 603–615.

16 (*a*) A. Lubineau, H. Lortat-Jacob, O. Gavard, S. Sarrazin and D. Bonnaffe, Synthesis of tailor-made glycoconjugate mimetics of heparan sulfate that bind IFN-γ in the nanomolar range, *Chem.–Eur. J.*, 2004, **10**, 4265–4282; (*b*) H. Z. Cao and B. Yu, Synthesis of a *S*-linked heparan sulfate trisaccharide as the substrate mimic of heparanase, *Tetrahedron Lett.*, 2005, **46**, 4337–4340; (*c*) L. D. Lu, C. R. Shie, S. S. Kulkarni, G. R. Pan, X. A. Lu and S. C. Hung, Synthesis of 48 disaccharide building blocks for the assembly of a heparin and heparan sulfate oligosaccharide library, *Org. Lett.*, 2006, **8**, 5995–5998.

17 B. Kuberan, M. Z. Lech, D. L. Beeler, Z. L. L. Wu and R. D. Rosenberg, Enzymatic synthesis of antithrombin III-binding heparan sulfate pentasaccharide, *Nat. Biotechnol.*, 2003, **21**, 1343–1346.

18 D. J. Silva, H. M. Wang, N. M. Allanson, R. K. Jain and M. J. Sofia, Stereospecific solution- and solid-phase glycosylations. Synthesis of β-linked saccharides and construction of disaccharide libraries using phenylsulfenyl 2-deoxy-2-trifluoroacetamido glycopyranosides as glycosyl donors, *J. Org. Chem.*, 1999, **64**, 5926–5929.

19 E. McKenzie, K. Young, M. Hircock, J. Bennett, M. Bhaman, R. Felix, P. Turner, A. Stamps, D. McMillan, G. Saville, S. Ng, S. Mason, D. Snell, D. Schofield, H. P. Gong, R. Townsend, J. Gallagher, M. Page, R. Parekh and C. Stubberfield, Biochemical characterisation of the active heterodimer form of human heparanase (Hpa1) protein expressed in insect cells, *Biochem. J.*, 2003, **373**, 423–435.

20 (*a*) S. H. Yamada, K. Yoshida, M. Sugiura, K. Sugahara, K. H. Khoo, H. R. Morris and A. Dell, Structural studies on the bacterial lyase-resistant tetrasaccharides derived from the antithrombin III-binding site of porcine intestinal heparin, *J. Biol. Chem.*, 1993, **268**, 4780–4787; (*b*) J. F. Chen, Y. Zhou, C. Chen, W. C. Xu and B. Yu, Synthesis of a tetrasaccharide substrate of heparanase, *Carbohydr. Res.*, 2008, **343**, 2853–2862.

21 J. K. Fairweather, E. Hammond, K. D. Johnstone and V. Ferro, Synthesis and heparanase inhibitory activity of sulfated mannooligosaccharides related to the antiangiogenic agent PI-88, *Bioorg. Med. Chem.*, 2008, **16**, 699–709.
22 C. Freeman and C. R. Parish, Human platelet heparanase: purification, characterisation and catalytic activity, *Biochem. J.*, 1998, **330**, 1341–1350.
23 P. Stangier and O. Hindsgaul, Solid-phase transimidation for the removal of *N*-phthalimido and *N*-tetrachlorophthalimido protecting groups on carbohydrates, *Synlett*, 1996, **2**, 179–181.
24 A. M. S. Silva, I. Alkorta, J. Elguero and V. L. M. Silva, A ^{13}C NMR study of the structure of four cinnamic acids and their methyl esters, *J. Mol. Struct.*, 2001, **595**, 1–6.
25 S. R. Turner, F. Walter, B. I. Voit and T. H. Mourey, Hyperbranched aromatic polyesters with carboxylic acid terminal groups, *Macromolecules*, 1994, **27**, 1611–1616.
26 S. J. Perkins, L. N. Johnson, D. C. Phillips and R. A. Dwek, High resolution ^{1}H- and ^{13}C-NMR spectra of D-glucopyranose, 2-acetamido-2-deoxy-D-glucopyranose, and related compounds in aqueous media, *Carbohydr. Res.*, 1977, **59**, 19–34.
27 M. Kosakai and Z. Yosizawa, Isolation and characterisation of heparin disaccharides, *Carbohydr. Res.*, 1977, **58**, 153–164.
28 J. G. N. de Jong, R. A. Wevers, C. Laarakkers and B. J. H. M. Poorthuis, Dimethylmethylene blue-based spectrophotometry of glycosaminoglycans in untreated urine - a rapid screening-procedure for mucopolysaccharidoses, *Clin. Chem.*, 1989, **35**, 1472–1477.