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Synthesis and heparanase inhibitory activity of sulfated mannooligosaccharides related to the antiangiogenic agent PI-88

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Abstract—A stepwise synthetic route to the mannooligosaccharides from the neutral fraction of *Pichia holstii* phosphomannan hydrolysate, including a tetrasaccharylamine component, was developed using only two or three readily available D-mannose building blocks. These compounds were sulfonated to give the corresponding sulfated oligosaccharides which are closely related to the constituents of the anticancer agent PI-88. The synthetic approach is well suited to the preparation of analogues as demonstrated by the synthesis of a series of (1 → 3)-linked mannooligosaccharides. The inhibitory activity of the sulfated oligosaccharides against heparanase was determined using a Microcon ultrafiltration assay. The tetra- and pentasaccharides were potent competitive inhibitors of heparanase (K_i = 200–280 nM) whilst the shorter di- and trisaccharides were partial competitive inhibitors and did not completely inhibit the enzyme even at very high concentrations.

1. Introduction

The heparan sulfate (HS) mimetic PI-88 (**1**) is a recognised inhibitor of tumour growth and metastasis currently undergoing Phase II clinical trials in patients with advanced malignancies.[1–3] It is expected to commence Phase III clinical evaluation in 2007 as an adjuvant therapy for post-resection hepatocellular carcinoma. PI-88 inhibits angiogenesis[4–6] by blocking the interactions of angiogenic growth factors such as FGF-2 and VEGF and their receptors with HS. In addition, PI-88 is a potent inhibitor of heparanase,[4] an *endo*-β-glucuronidase that cleaves the HS side chains of proteoglycans that are a major component of the extracellular matrix and basement membranes.[7] Heparanase plays a key role in both metastasis and angiogenesis and is thus an attractive target for drug development.[8–11] The biological effects of PI-88 are not restricted to anticancer activity. For example, it also displays anticoagulant,[12–14] antiproliferative,[15] antiviral[16,17] and antimalarial activity.[18]

PI-88 is prepared[19] by exhaustive sulfonation of the oligosaccharide phosphate fraction obtained following mild acid-catalysed hydrolysis of the extracellular phosphomannan produced by the yeast *Pichia holstii* NRRL Y-2448.[20] This mixture is primarily composed of the phosphorylated α(1 → 3)/α(1 → 2)-linked penta- (**5**) and tetrasaccharide **4**, which together account for approximately 90% of the total oligosaccharide content, with the remaining 10% comprised of phosphorylated di- (**2**), tri- (**3**) and hexasaccharide **6**.[21] The minor, neutral fraction from the phosphomannan hydrolysate is made up of the corresponding non-phosphorylated oligosaccharides **7**–**11** which are also present following dephosphorylation of the oligosaccharide phosphate fraction with alkaline phosphatase.[21,22] An inability to separate the oligosaccharide phosphate fraction by size exclusion chromatography (SEC) into its individual components, attributable to the presence of the phosphate group,[22] has meant that structure–activity relationship studies of PI-88 have been restricted to using partially purified fractions obtained by SEC of PI-88 itself[19] or by using the sulfated oligosaccharides (**12**–**15**)[23,16] obtained by sulfonation of the neutral oligosaccharides **7**–**10** (see Chart 1).

Like PI-88, compounds **12**–**15** display strong affinities for FGF-1, FGF-2 and VEGF,[23] and are able to inhibit herpes simplex virus (HSV) infection of cells and the cell-to-cell spread of HSV-1 and HSV-2,[16] with activity increasing with increased size. Small amounts of tetrasaccharylamine **18** have also been detected in the neutral hydrolysate fraction[21] but because of its low abundance and the difficulty in its isolation, the corresponding

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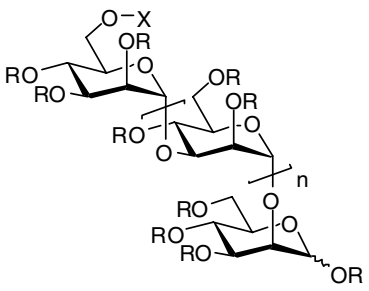
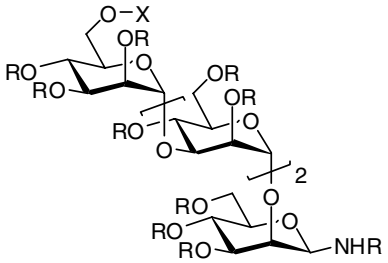




1 X = PO_3Na_2, R = SO_3Na or H (n = 0-4)
2 X = PO_3Na_2, R = H (n = 0)
3 X = PO_3Na_2, R = H (n = 1)
4 X = PO_3Na_2, R = H (n = 2)
5 X = PO_3Na_2, R = H (n = 3)
6 X = PO_3Na_2, R = H (n = 4)
7 X = R = H (n = 0)
8 X = R = H (n = 1)
9 X = R = H (n = 2)
10 X = R = H (n = 3)
11 X = R = H (n = 4)
12 X = R = SO_3Na or H (n = 0)
13 X = R = SO_3Na or H (n = 1)
14 X = R = SO_3Na or H (n = 2)
15 X = R = SO_3Na or H (n = 3)

16 X = PO_3Na_2, R = SO_3Na or H
17 X = PO_3Na_2, R = H
18 X = R = H
19 X = R = SO_3Na or H
20 X = R = Ac

Chart 1.

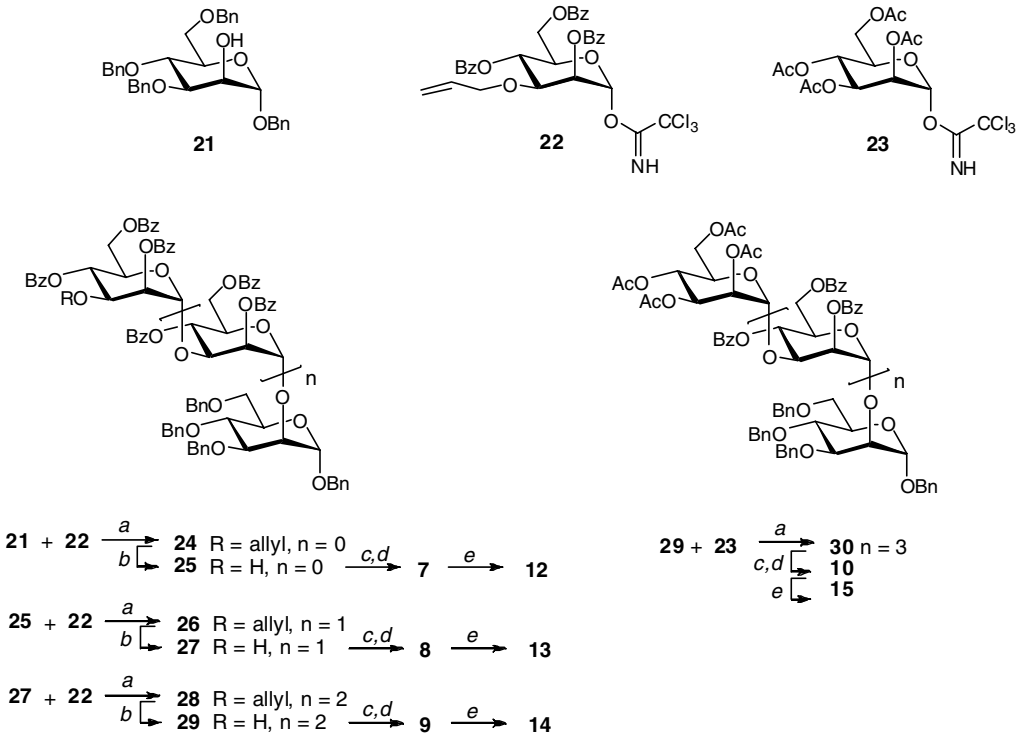
sulfated oligosaccharylamine **19** was not synthesised and therefore not assessed in the previous studies. The availability of compounds **12**–**15** and **19** for further biological evaluation is also limited by the need to access large scale *Pichia* fermentations and phosphomannan hydrolysates. To address the need for sufficient quantities of **12**–**15** and **19** for further testing, the total synthesis of these compounds and several analogues containing exclusively α(1 → 3) linkages was undertaken. The products were tested for their ability to inhibit human platelet heparanase using an improved assay.

2. Results and discussion

2.1. Synthesis

Du et al. have reported a convergent '3 + 2' block strategy for the synthesis of PI-88 pentasaccharide analogues which was successfully applied to the preparation of a 6^V-monosulfated octyl mannopentasaccharide.[24] A more general approach amenable to the synthesis of all PI-88 oligosaccharide components (di- to hexasaccharides) and adaptable to the preparation of analogues is a reiterative '1 + 1' strategy. A similar 1 + 1 strategy has been reported by Ikegami for the synthesis of a protected 1-*C*-methyl-substituted mannosyl pentasaccharide analogue of PI-88.[25] Using this 1 + 1 strategy, the target oligosaccharides can all be synthesised with the use of only two simple and readily available mannose building blocks (Scheme 1): a starting block (**21**)[26] and a differentially protected, elongating block (**22**).[27,28] The elongating block may be partially deprotected thus providing a site for further extension or in conjunction with the molecule as a whole, completely liberated of protecting groups. Alternatively, a non-differentiable block (**23**) may be used at the non-reducing end thus 'capping' the oligosaccharide at a fixed length. Part of this strategy has been easily modified to allow for the preparation of phosphorylated PI-88-related oligosaccharides by using a phosphorylated or differentially 6-O-protected capping block.[29]

The alcohol **25** was prepared in good yield by the sequential processes of glycosylation (of the alcohol **21** with the imidate **22**, catalysed by TMSOTf) and de-O-allylation of the resultant disaccharide **24** (Scheme 1). The alcohol **25** served as an acceptor for further elongation and following a similar glycosylation/de-O-allylation strategy, the imidate **22** was incorporated into the growing chain yielding first the trisaccharides **26** and **27** and then the tetrasaccharides **28** and **29** in good overall yields. The alcohol **29** was ultimately glycosylated with the peracetylated imidate **23** in 87% yield to afford the pentasaccharide **30**. Compounds **25**, **27**, **29** and **30** were deprotected through the sequential processes of transesterification and hydrogenolysis to yield the oligomannans **7**–**10**, respectively. The products displayed ^1H and ^{13}C NMR spectroscopic properties in accord with those reported in the literature.[22,30] The oligomannans were sulfonated as previously described[23] to give the sulfated oligomannans **12**–**15**. Compounds **12**–**15** displayed identical ^1H NMR spectroscopic and capillary electrophoretic (CE) properties to authentic samples previously prepared.[23] The sulfonation of PI-88-related oligosaccharides larger than disaccharides with reagents such as sulfur trioxide pyridine or trimethylamine complex does not go to completion. Following precedent,[23,31] the target compounds were all obtained as reproducible mixtures of highly but incompletely sulfated forms. The

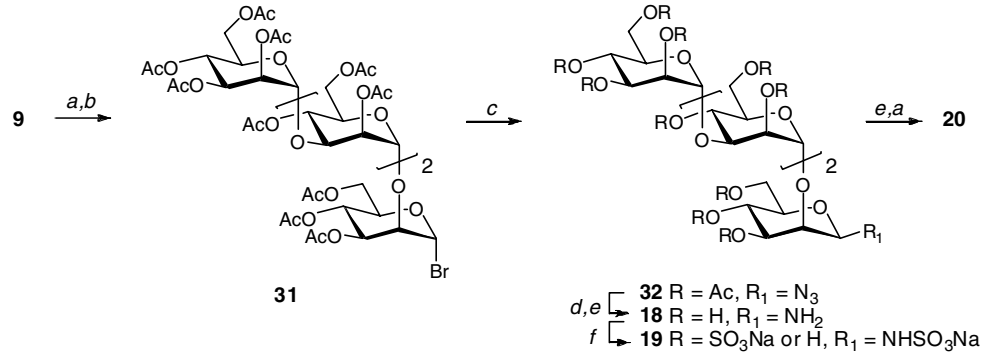


Scheme 1. Synthesis of mannooligosaccharides **12**–**15**. Reagents and conditions: (a) TMSOTf, 1,2-DCE, 0 °C, 10 min, 84–94%; (b) $PdCl_2$, MeOH/1,2-DCE, 70 °C, 40 min, 64–91%; (c) NaOMe, MeOH/THF, rt, o/n; (d) $Pd(OH)_2/C$, H_2 (100 psi), THF/H_2O/AcOH, 3 h, 56–67%, two steps; (e) i—SO_3·Py, DMF, 60 °C, 6 h; ii—1 M NaOH, 40–50%.

purity of the compounds was therefore determined by a combination of CE and ^{1}H NMR spectroscopy (see Section 4).

The sulfated tetrasaccharylamine derivative **19** was synthesised as outlined in Scheme 2. The tetrasaccharide **9** was first acetylated under standard conditions and the resulting peracetate treated with HBr in acetic acid to form the α-bromide **31** which was subsequently displaced with azide ion to give the β-azide **32** in moderate yield (41% from the peracetate). Azide **32** was deacetylated under Zemplén conditions and then hydrogenated with Adam's catalyst to give, presumably, tetrasaccharylamine **18**, which was not isolated but instead immediately sulfonated to give the target compound **19**, characterised by ^{1}H NMR spectroscopy and CE. The presence of small quantities of **18** in the neutral fraction of *Pichia* phosphomannan hydrolysates was previously inferred by the isolation and NMR structure elucidation of slightly impure peracetate **20**.[21] In the present study, whilst the isolation of **18** was not required, peracetate **20** was readily obtained by hydrogenation of **32** followed by acetylation. Flash chromatographic purification afforded a sample of **20** of greater purity than the previously isolated sample but which was otherwise identical by ^{1}H and ^{13}C NMR spectroscopy, thus confirming the earlier structural assignment.

The stepwise synthetic approach utilized for the synthesis of **12**–**15** and **19** is well suited to the preparation of



Scheme 2. Synthesis of tetrasaccharylamine derivatives **19** and **20**. Reagents and conditions: (a) Ac_2O, Py, DMAP, rt, o/n; (b) HBr/HOAc/1,2-DCE, rt, o/n; (c) NaN_3, DMF, 110 °C, o/n, 41% two steps; (d) NaOMe, MeOH, rt, 4 h, 72%; (e) PtO_2, H_2 (100 psi), MeOH/EtOAc/AcOH, 3 h; (f) i—SO_3·Py, DMF, 60 °C, 6 h; ii—1 M NaOH, 55% two steps.

PI-88 analogues. A simple change to the nature of the starting, elongating or capping blocks allows access to a variety of different analogues. This is illustrated in Scheme 3 whereby changing the starting block to the alcohol **33**[27] gives rise to all (1 → 3)-linked analogues such as **41** and **43**, which are useful for examining the nature of the carbohydrate linkages on biological activity.

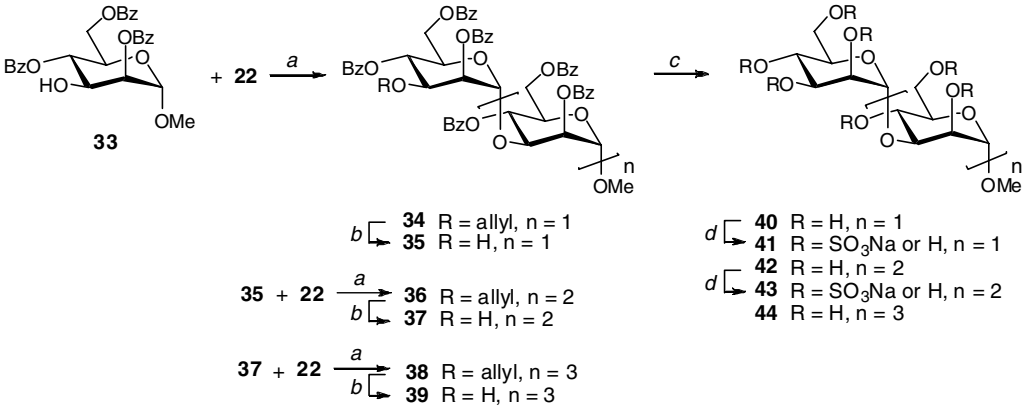
2.2. Heparanase inhibition studies

Heparanase for use in the inhibition studies was purified from human platelets by a modification of the procedure of Freeman and Parish[32] via the sequential fractionation of a crude platelet extract on Concanavalin A Sepharose, Sulfopropyl Sepharose and Sephacryl S-200 HR chromatography columns. This modification results in a more efficient purification (one less chromatographic step is required) with an improved overall yield of protein.

Most of the heparanase assays developed to date are suitable for qualitative assessment of heparanase inhibitors but not so for detailed kinetic analysis.[33–36] To address this, we developed a sensitive heparanase assay which uses [^{3}H]-labelled HS as substrate and allows for the variation of substrate concentration. This assay is similar to that developed independently by Tsuchida et al.[37] in that it uses ultrafiltration devices, in this case a Microcon YM-10, to separate the longer native HS from the smaller, cleaved products of heparanase catalysis which are then quantified by scintillation counting.

The sulfated oligosaccharides were evaluated for their heparanase inhibitory activity using the assay described above (Table 1 and Fig. 1). The data indicate that the di- and trisaccharide members of this series (**12**, **13**, **41**, **43**) deviate from the expected result for putative competitive inhibitors. Competitive inhibitors should approach 100% inhibition of their target enzyme at saturating concentrations. Under these conditions, nearly all enzyme active sites are occupied by inhibitor molecules preventing catalysis. This near 100% inactivation of the enzyme clearly occurs with the longer compounds of the series (**14**, **15** and **19**) but does not for the di- and trisaccharides which reach between approximately 30% and 90% inhibition at inhibitor concentrations 10-fold higher than their respective IC_{50} values. At these saturating concentrations, the disaccharides (**12** and **41**) inhibit heparanase less than the trisaccharides (**13** and **43**). The IC_{50} values determined for the compounds were used, along with the determined K_m of heparanase for HS (1.64 μM, data not shown), to calculate K_{ic} values.[38] The affinity of these compounds for the active site of heparanase is very similar to that of PI-88 as indicated by their similar K_{ic}.

The di- and trisaccharides did not conform to simple competitive inhibition so more experiments were performed using example compounds (**12** and **15**) to deter-



Scheme 3. Synthesis of all α(1 → 3)-linked mannooligosaccharides. Reagents and conditions: (a) TMSOTf, 1,2-DCE, 0 °C → rt, 30 min; (b) $PdCl_2$, MeOH/1,2-DCE, 70 °C, 40 min, 54–70%, two steps; (c) NaOMe, MeOH/THF, rt, o/n, 85–98%; (d) i—SO_3·Py, DMF, 60 °C, 6 h; ii—1 M NaOH, 36–48%.

Table 1. Inhibition of human platelet heparanase by sulfated oligosaccharides **12**–**15**, **19**, **41** and **43** as determined by Microcon ultrafiltration assay

Compound	IC_{50} (μM)	K_{ic} (μM)	K_{iu} (μM)	Maximum inhibition (%)
1	0.98 ± 0.11	0.24 ± 0.03	NA	100
12	1.57 ± 0.31	2.23 ± 0.20	68.3 ± 22.4	58.6
13	2.69 ± 0.46	ND	ND	84.3
14	0.80 ± 0.06	0.20 ± 0.02	NA	100
15	1.14 ± 0.18	0.28 ± 0.04	NA	100
19	0.99 ± 0.11	0.24 ± 0.03	NA	100
41	0.25 ± 0.12	ND	ND	34.2
43	4.15 ± 0.36	ND	ND	91.3

Inhibition data for PI-88 (**1**) are given for comparison. NA, not applicable; ND, not determined.

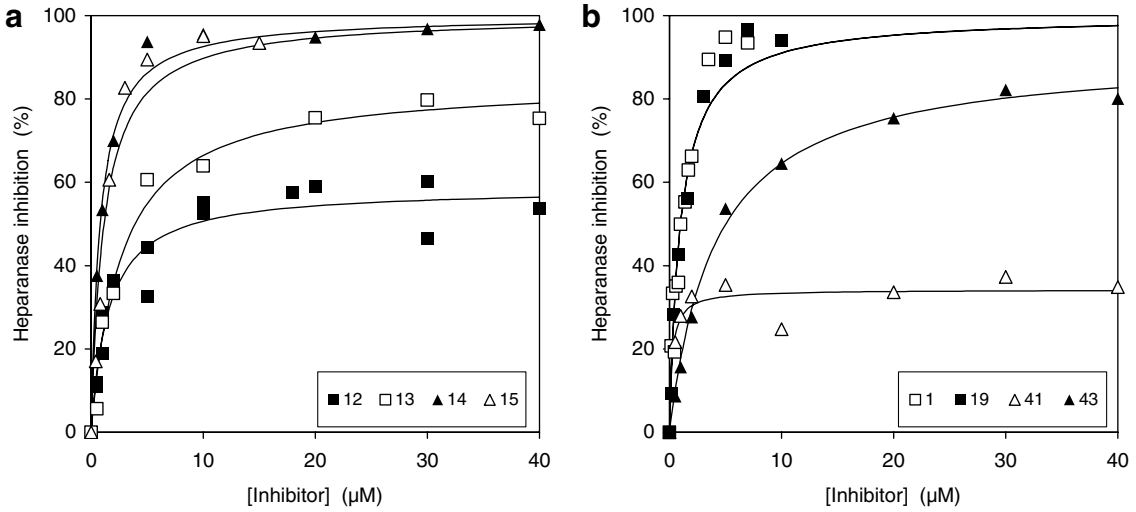


Figure 1. Inhibition of heparanase by the sulfated oligosaccharides **12**–**15** (a) and **1**, **19**, **41** and **43** (b). Heparanase assays were conducted at 0.9 μg/mL heparanase, 5 μM [^{3}H]HS, 40 mM sodium acetate, pH 5.0, and 0.1 mg/mL BSA at 37 °C for 4 h.

mine their mode of inhibition and also to confirm that the longer oligosaccharides were acting as competitive inhibitors. Double reciprocal plot analysis confirmed that pentasaccharide **15** behaves as a simple competitive inhibitor of heparanase with respect to substrate HS (Fig. 2). The linear plots intercept at the *y*-axis indicating that **15** increases the apparent K_m but has no effect upon V_m, traits characteristic of competitive inhibitors. The double reciprocal plot for disaccharide **12** is similar (Fig. 3) but this compound is not a simple competitive inhibitor due to its inability to completely inhibit heparanase at saturating concentrations. Dixon plots of the data are nonlinear, which, in combination with the other observations, indicate that **12** is a partial competitive inhibitor. The rate equation for partial competitive inhibition was used to determine K_{ic} and K_{iu} for compound **12** by nonlinear regression.[39]

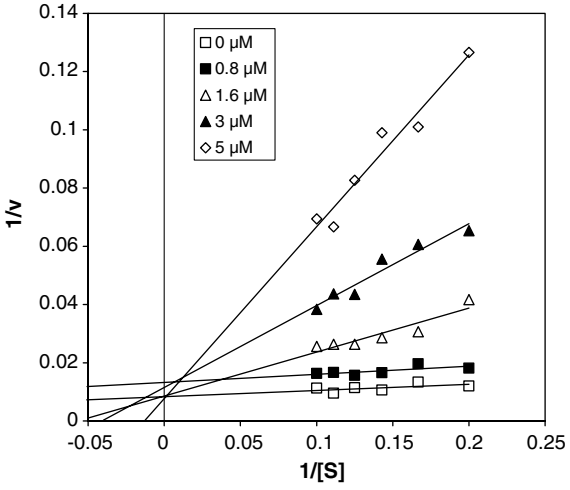


Figure 2. Double reciprocal plot of heparanase inhibition by sulfated pentasaccharide **15**. Heparanase assays were conducted at 0.9 μg/mL heparanase, various concentrations of ^{3}[H]HS, 40 mM sodium acetate, pH 5.0, and 0.1 mg/mL BSA at 37 °C for 4 h.

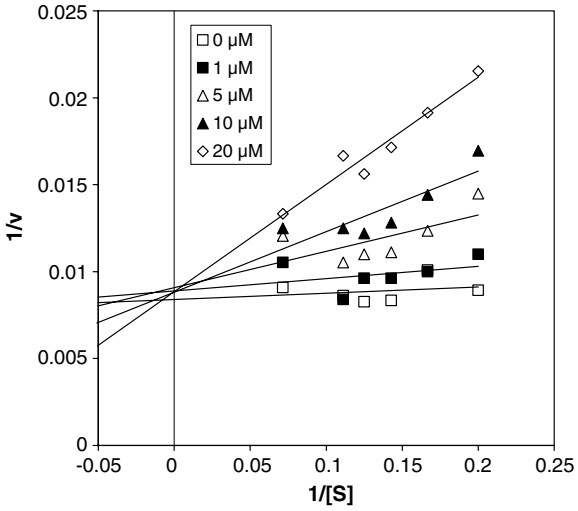


Figure 3. Double reciprocal plot of heparanase inhibition by sulfated disaccharide **12**. Heparanase assays were conducted at 0.9 μg/mL heparanase, various concentrations of ^{3}[H]HS, 40 mM sodium acetate, pH 5.0, and 0.1 mg/mL BSA at 37 °C for 4 h.

The large size of the active site of heparanase may be the reason why similar molecules have such different interactions with this enzyme. During catalysis, heparanase interacts with at least three saccharides within substrate HS.[40] These interactions are largely between negatively charged groups on HS (sulfates and carboxylates) and positively charged residues (lysines and arginines) flanking both sides of the catalytic glutamate residues.[41] The sulfated oligosaccharides described here are likely to interact with these positively charged residues but to have little affinity for the negatively charged glutamate residues. It is conceivable that the smaller oligosaccharides such as **12** bind to some of the positive residues but due to their small size leave access to the catalytic glutamate residues allowing substrate to bind and catalysis to occur. This hypothesis explains how these smaller oligosaccharides, apparently interacting with heparanase as competitive inhibitors, do not approach complete

enzyme inactivation even at very high concentrations. The higher affinity of **12** for free enzyme than enzyme with substrate bound ($K_{ic} < K_{iu}$) is consistent with this hypothesis because substrate bound to heparanase will restrict access to the positive residues. The interaction between heparanase and **12** is contrasted with that of the longer compounds such as **14** and **15** which, due to their length, may block substrate access to the catalytic glutamate residues and completely inhibit catalysis.

3. Conclusions

In conclusion, a simple synthetic route to the oligosaccharides from the neutral fraction of *P. holstii* phosphomannan hydrolysate, including the tetrasaccharylamine component, was developed. These compounds were sulfonated to give the corresponding sulfated oligosaccharides which are closely related to the constituents of the anticancer agent PI-88. The synthetic approach is well suited to the preparation of analogues as demonstrated by the synthesis of a series of all-(1 → 3)-linked derivatives. The inhibitory activity of the sulfated oligosaccharides against human platelet heparanase was determined using a Microcon ultrafiltration assay. The tetra- and pentasaccharides were potent competitive inhibitors of heparanase whilst the shorter di- and trisaccharides were partial competitive inhibitors and did not completely inhibit the enzyme at saturating concentrations. This study now gives a clearer picture of the biological activity of the PI-88 components and of the size requirements of sulfated oligosaccharides for efficient inhibition of heparanase. This information may be useful in the design of new heparanase inhibitors.

4. Experimental

4.1. General

General experimental procedures have been given previously.[29] Capillary electrophoresis (CE) was performed in reverse polarity mode on a Beckman P/ACE 5000 System equipped with a P/ACE UV absorbance detector, using 10 mM 5-sulfosalicylic acid (pH 3) as the background electrolyte, as previously described.[19] Radioactivity (^{3}H) was counted on a Wallac 1450 MicroBeta Trilux liquid scintillation counter using Optiphase HiSafe 2 (Perkin-Elmer) as the scintillation fluid.

4.2. Benzyl 2-*O*-(3-*O*-allyl-2,4,6-tri-*O*-benzoyl-α-D-mannopyranosyl)-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside (24)

A mixture of the imidate **22** (902 mg, 1.21 mmol)[28,27] and the alcohol **21** (723 mg, 1.34 mmol)[26] in 1,2-DCE (10 mL) was stirred in the presence of mol. sieves (1.0 g of 3 Å powder) under an atmosphere of argon (30 min). The mixture was cooled (0 °C) with continued stirring (10 min) prior to the addition of TMSOTf (219 μL, 1.21 mmol). After 10 min, Et_3N (100 μL) was introduced and the mixture was filtered. The solvent was evaporated and the residue subjected to FC (10–50% EtOAc/hexane) to yield the allyl ether **24** as a colourless oil (1.14 g, 84%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.67–3.81, 3.88–3.95, 4.06–4.15, 4.30–4.35 (4 m, 12H, H-2^I, -3^I, -4^I, -5^I, -6a^I, -6b^I, -3^{II}, -5^{II}, -6a^{II}, -6b^{II}, OCH_2), 4.94–4.70 (m, 7H, CH_2Ph), 4.84 (d, 1H, $J_{A,B}$ = 10.8 Hz; A of AB q, CH_2Ph), 4.93–4.96, 5.04–5.09 (2 m, 2H, =CH_2), 5.02 (d, 1H, $J_{1,2}$ = 1.9 Hz, H-1^I), 5.24 (d, 1H, $J_{1,2}$ = 1.9 Hz, H-1^{II}), 5.59–5.69 (m, 1H, =CH), 5.72 (dd, 1H, $J_{2,3}$ = 3.1 Hz, H-2^{II}), 5.75 (dd, 1H, $J_{3,4}$ = 9.8, $J_{4,5}$ = 9.9 Hz, H-4^{II}), 7.09–7.58, 7.97–8.06 (2 m, 35H, Ph); ^{13}C NMR (100 MHz, $CDCl_3$) δ 61.5, 63.5 (C-6^I, -6^{II}), 68.6, 69.2, 69.3, 69.5, 69.6, 71.1, 72.0, 72.6, 73.6, 74.7, 75.3, 75.4 (13 C; C-3^I, -4^I, -5^I, -2^{II}, -3^{II}, -4^{II}, -5^{II}, OCH_2, *C*H_2Ph), 80.0 (C-2^I), 98.5, 99.6 (C-1^I, -1^{II}), 117.7 (=CH_2), 127.7–138.4 (43 C; =CH, Ph), 165.6, 165.7, 166.4 (C=O); ESMS: *m/z* 1072.2 $[M+NH_4]^+$.

4.3. Benzyl 2-*O*-(2,4,6-tri-*O*-benzoyl-α-D-mannopyranosyl)-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside (25)

$PdCl_2$ (40 mg) was added to a solution of the allyl ether **24** (1.09 g, 0.97 mmol) in MeOH (10 mL) and 1,2-DCE (10 mL) and the combined mixture was heated (70 °C, 40 min). The solvents were evaporated and the residue subjected to FC (20–30% EtOAc/hexanes) to yield the alcohol **25** as a colourless oil (0.96 g, 91%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.68–3.81, 3.97–4.06, 4.32–4.71 (3 m, 18H, H-2^I, -3^I, -4^I, -5^I, -6a^I, -6b^I, -3^{II}, -5^{II}, -6a^{II}, -6b^{II}, CH_2Ph), 4.84 (d, 1H, $J_{A,B}$ = 12 Hz A of AB q, CH_2Ph), 5.05 (d, 1H, $J_{1,2}$ = 1.9 Hz, H-1^I), 5.26 (d, 1H, $J_{1,2}$ = 1.9 Hz, H-1^{II}), 5.61 (dd, 1H, $J_{2,3}$ = 3.3 Hz, H-2^{II}), 5.67 (dd, 1H, $J_{3,4}$ = 9.8, $J_{4,5}$ = 9.9 Hz, H-4^{II}), 7.13–7.40, 7.48–7.59, 7.98–8.06 (3 m, 35H, Ph); ^{13}C NMR (100 MHz, $CDCl_3$) δ 60.6, 63.3 (C-6^I, -6^{II}), 69.06, 69.12, 69.2, 69.4, 70.4, 72.1, 72.6, 72.8, 73.5, 74.8, 75.47, 75.48, 76.2 (C-3^I, -4^I, -5^I, -2^{II}, -3^{II}, -4^{II}, -5^{II}, OCH_2, *C*H_2Ph), 79.7 (C-2^I), 98.3, 99.4 (C-1^I, -1^{II}), 127.7–138.5 (42 C, Ph), 166.0, 166.4, 167.0 (C=O); ESMS: *m/z* 1015.2 $[M+H]^+$, 1037.2 $[M+Na]^+$.

4.4. Benzyl 2-*O*-[(3-*O*-allyl-2,4,6-tri-*O*-benzoyl-α-D-mannopyranosyl)-(1 → 3)-(2,4,6-tri-*O*-benzoyl-α-D-mannopyranosyl)]-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside (26)

A mixture of the imidate **22** (742 mg, 1.01 mmol) and the alcohol **25** (908 mg, 0.84 mmol) was treated with TMSOTf as described for **24** to yield the allyl ether **26** as a colourless oil (1.26 g, 90%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.51–3.56, 3.66–4.06, 4.23–4.27, 4.30–42, 4.47–4.72, 4.78–4.86 (6 m, 26H, H-2^I, -3^I, -4^I, -5^I, -6a^I, -6 b^I, -3^{II}, -5^{II}, -6a^{II}, -6b^{II}, -3^{III}, -5^{III}, -6a^{III}, -6b^{III}, OCH_2, =CH_2, CH_2Ph), 5.04 (d, 1H, $J_{1,2}$ = 1.7 Hz, H-1^I), 5.15 (dd, 1H, $J_{1,2}$ = 1.8, $J_{2,3}$ = 2.7 Hz, H-2^{II}), 5.26 (d, 1H, H-1^{II}), 5.28 (d, 1H, $J_{1,2}$ = 1.7 Hz, H-1^{III}), 5.33–5.43 (m, 1H, =CH), 5.77–5.82 (m, 2H, H-4^{II}, -2^{III}), 5.92 (dd, 1H, $J_{3,4}$ = 9.5, $J_{4,5}$ = 9.8 Hz, H-4^{III}), 7.00–7.61, 7.80–8.19 (2 m, 50H, Ph); ^{13}C NMR (100 MHz, $CDCl_3$) δ 62.0, 62.9, 67.3, 68.2, 68.6, 68.9, 69.0, 69.1, 69.6, 70.2, 71.3, 71.7, 72.2, 73.1, 73.7, 74.5, 75.0, 75.8, 76.1, 77.2, 79.1, 97.8, 99.0, 99.4, 117.1, 127.3, 127.4, 127.5, 127.6, 127.8, 127.9, 128.1, 128.2, 128.3, 128.4, 128.5, 128.7, 129.2, 129.4, 129.5, 129.7, 129.8, 129.9,

132.5, 132.9, 133.4, 133.6, 136.8, 138.0, 164.7, 165.1, 165.5, 165.7, 165.9; ESMS: *m/z* 1552.5 $[M+Na]^+$.

4.5. Benzyl 2-*O*-[(2,4,6-tri-*O*-benzoyl-α-D-mannopyranosyl)-(1 → 3)-(2,4,6-tri-*O*-benzoyl-α-D-mannopyranosyl)]-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside (27)

The allyl ether **26** (394 mg, 241 μmol) was treated with $PdCl_2$ as described for **25** to yield the alcohol **27** as a colourless oil (317 mg, 84%). ^1H NMR (400 MHz, $CDCl_3$) δ 3.67–3.82, 3.91–3.99, 4.01–4.21, 4.29–4.71 (4 m, 21H, H-2^I, -3^I, -4^I, -5^I, -6a^I, -6b^I, -3^{II}, -5^{II}, -6a^{II}, -6b^{II}, -3^{III}, -5^{III}, -6a^{III}, -6b^{III}, CH_2Ph), 4.83 (d, 1H, $J_{A,B}$ = 10.9 Hz, A of AB q, CH_2Ph), 5.03–5.05 (m, 2H, H-1^I, -2^{II}), 5.25–5.28 (m, 2H, H-1^{II}, -1^{III}), 5.63 (dd, 1H, $J_{3,4} = J_{4,5}$ = 9.9 Hz, H-4^{II}), 5.77 (dd, 1H, $J_{1,2}$ = 2.0, $J_{2,3}$ = 3.1 Hz, H-2^{III}), 5.92 (dd, 1H, $J_{3,4}$ = 9.7, $J_{4,5}$ = 9.9 Hz, H-4^{III}), 6.99–7.62, 7.80–8.16 (2 m, 50H, Ph); ESMS: *m/z* 1511.4 $[M+Na]^+$.

4.6. Benzyl 2-*O*-[(3-*O*-allyl-2,4,6-tri-*O*-benzoyl-α-D-mannopyranosyl)-(1 → 3)-(2,4,6-tri-*O*-benzoyl-α-D-mannopyranosyl)-(1 → 3)-(2,4,6-tri-*O*-benzoyl-α-D-mannopyranosyl)]-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside (28)

A mixture of the imidate **22** (102 mg, 138 μmol) and the alcohol **27** (135 mg, 86.5 μmol) was treated with TMSOTf as described for **24** to yield the allyl ether **28** as a colourless oil (173 mg, 94%). ^1H NMR (400 MHz, $CDCl_3$) δ 3.44–3.49, 3.60–3.99, 4.05–4.16, 4.42–4.44, 4.48–4.68, 4.73–4.77 (6 m, 30H, H-2^I, -3^I, -4^I, -5^I, -6a^I, -6b^I, -3^{II}, -5^{II}, -6a^{II}, -6b^{II}, -3^{III}, -5^{III}, -6a^{III}, -6b^{III}, -3^{IV}, -5^{IV}, -6a^{IV}, -6b^{IV}, OCH_2, =CH_2, CH_2Ph), 4.83 (d, 1H, $J_{A,B}$ = 10.9 Hz; A of AB q, CH_2Ph), 5.01–5.04 (m, 2H, H-1^I, -2^{III}), 5.19–5.23 (m, 1H, H-2^{II}), 5.27–5.40 (m, 4H, H-1^I, -1^{II}, -1^{III}, =CH_2), 5.61 (dd, 1H, $J_{3,4}$ = $_{4,5}$ = 9.9 Hz, H-4^{IV}), 5.77 (dd, 1H, $J_{1,2}$ = 2.0, $J_{2,3}$ = 3.1 Hz, H-2^{IV}), 5.90–5.96 (m, 2H, H-4^{II}, -4^{III}), 7.01–7.56, 7.70–8.16 (2 m, 65H, Ph); ^{13}C NMR (100 MHz, $CDCl_3$) δ 61.9, 62.3, 62.9, 67.3, 67.3, 68.0, 68.6, 68.9, 69.0, 69.1, 69.2, 69.5, 70.2, 71.2, 71.3, 71.8, 72.3, 73.2, 73.8, 74.6, 75.1, 75.9, 76.0, 79.1, 97.8, 98.9, 99.0, 99.2, 117.1, 127.3, 127.4, 127.4, 127.7, 127.8, 127.9, 128.0, 128.2, 128.3, 128.5, 128.6, 128.7, 128.9, 129.0, 129.1, 129.5, 129.6, 129.7, 129.7, 129.8, 130.0, 132.6, 132.6, 132.8, 132.8, 133.0, 133.3, 133.5, 133.7, 136.9, 138.5, 164.7, 164.7, 165.1, 165.3, 165.6, 165.7, 166.0.

4.7. Benzyl 2-*O*-[(2,4,6-tri-*O*-benzoyl-α-D-mannopyranosyl)-(1 → 3)-(2,4,6-tri-*O*-benzoyl-α-D-mannopyranosyl)-(1 → 3)-(2,4,6-tri-*O*-benzoyl-α-D-mannopyranosyl)]-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside (29)

The allyl ether **28** (155 mg, 70.4 μmol) was treated with $PdCl_2$ as described for **25** to yield the alcohol **29** as a colourless oil (97 mg, 64%). ^1H NMR (400 MHz, $CDCl_3$) δ 3.67–3.82, 3.90–4.10, 4.24–4.68 (3 m, 26H, H-2^I, -3^I, -4^I, -5^I, -6a^I, -6b^I, -3^{II}, -5^{II}, -6a^{II}, -6b^{II}, -3^{III}, -5^{III}, -6a^{III}, -6b^{III}, -3^{IV}, -5^{IV}, -6a^{IV}, -6b^{IV}, CH_2Ph), 4.84 (d, 1H, $J_{A,B}$ = 11.2 Hz, A of AB q, CH_2Ph), 4.86 (d, $J_{1,2}$ = 1.8 Hz, H-1^I), 4.90 (dd, 1H, $J_{1,2}$ 1.8, $J_{2,3}$ = 3.1 Hz, H-2^{III}), 5.03 (d, 1H, $J_{1,2}$ = 1.5 Hz, H-1^{IV}), 5.22 (dd, 1H, $J_{1,2}$ = 2.1, $J_{2,3}$ 2.6 Hz, H-2^{II}), 5.27–5.29 (m, 2H, H-1^{III}, -1^{IV}), 5.46 (dd, 1H, $J_{3,4}$ = 9.7, $J_{4,5}$ = 9.9 Hz, H-4^{IV}), 5.79 (dd, 1H, $J_{2,3}$ = 2.9 Hz, H-2^{IV}), 5.90–5.96 (m, 2H, H-4^{II}, -4^{III}), 7.01–7.56, 7.68–8.16 (2 m, 65H, Ph).

4.8. Benzyl 2-*O*-[(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1 → 3)-(2,4,6-tri-*O*-benzoyl-α-D-mannopyranosyl)-(1 → 3)-(2,4,6-tri-*O*-benzoyl-α-D-mannopyranosyl)-(1 → 3)-(2,4,6-tri-*O*-benzoyl-α-D-mannopyranosyl)]-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside (30)

A mixture of the trichloroacetimidate **23** (39 mg, 78 μmol) and the alcohol **29** (85 mg, 39 μmol) was treated with TMSOTf as described for **24** to yield the pentasaccharide **30** as a colourless oil (85 mg, 87%). ^1H NMR (400 MHz, $CDCl_3$) δ 1.82–2.04 (4 s, 3H each; CH_3CO), 3.67–3.95, 4.05–4.72, 4.82–5.03, 5.21–5.28, 5.69–5.50 (m, 43 H; H-$1^{I–IV}$, -$2^{I–IV}$, -$3^{I–IV}$, -$4^{I–IV}$, -$5^{I–IV}$, -6ab$^{I–IV}$, CH_2Ph), 7.01–7.56, 7.68–8.16 (2 m, 65H′ Ar).

4.9. α-D-Mannopyranosyl-(1 → 2)-D-mannopyranose (7)

(A) A small piece of sodium was added to a solution of the tetrabenzyl ether **25** (86 mg, 85 μmol) in MeOH/THF (4 mL of 1:1) and the combined mixture was stirred (rt, o/n). The mixture was neutralised with Dowex 50-X8 resin (H^+ form) and filtered. The solvent was evaporated and co-evaporated (MeOH) and used in the following reaction without further purification. (B) $Pd(OH)_2$ (10% on C, 20 mg) was added to a solution of the crude product from (A) in THF/H_2O (4 mL of 1:1) containing a little AcOH (50 μL) and the combined mixture was vigorously stirred under hydrogen (100 psi, 3 h). The mixture was filtered, the solvent evaporated and the residue was subjected to size exclusion chromatography (Bio-Gel P-2; H_2O; 60 mL/h) to yield, after lyophilisation, the disaccharide **7** as a colourless powder (17 mg, 59%, two steps). The ^1H NMR (400 MHz, D_2O) spectrum was consistent with that reported in the literature.[22] HRMS: *m/z* 343.1180 $[M+H]^+$, 365.0972 $[M+Na]^+$.

4.10. α-D-Mannopyranosyl-(1 → 3)-α-D-mannopyranosyl-(1 → 2)-D-mannopyranose (8)

The trisaccharide **27** (53 mg, 36 μmol) was deprotected as described for **7** to yield the trisaccharide **8** as a colourless powder (12 mg, 67%). The ^1H NMR (400 MHz, D_2O) spectrum was consistent with that reported in the literature.[22] HRMS: *m/z* 505.1702 $[M+H]^+$, 527.1516 $[M+Na]^+$.

4.11. α-D-Mannopyranosyl-(1 → 3)-α-D-mannopyranosyl-(1 → 3)-α-D-mannopyranosyl-(1 → 2)-D-mannopyranose (9)

The tetrasaccharide **29** (42 mg, 21 μmol) was deprotected as described for **7** to yield the tetrasaccharide **9** as a colourless powder (9 mg, 63%). The ^1H NMR (400 MHz, D_2O) spectrum was consistent with that reported in the literature.[22] HRMS: *m/z* 667.2235 $[M+H]^+$, 689.2015 $[M+Na]^+$.

4.12. α-D-Mannopyranosyl-(1 → 3)-α-D-mannopyranosyl-(1 → 3)-α-D-mannopyranosyl-(1 → 3)-α-D-mannopyranosyl-(1 → 2)-D-mannopyranose (10)

The pentasaccharide **30** (64 mg, 28 μmol) was deprotected as described for **7** to yield the pentasaccharide **10** as a colourless powder (13 mg, 56%). The ^{1}H NMR (400 MHz, D_2O) spectrum was consistent with that reported in the literature.[22,30] HRMS: *m/z* 829.2810 $[M+H]^+$.

4.13. Sulfonation of oligosaccharides (7–10)

Oligosaccharides **7**–**10** were sulfonated (SO_3·Py complex) and purified (Bio-Gel P-2) as previously described[23] to yield the sulfated oligosaccharides **12**–**15** as amorphous white powders (40–50%). Compounds **12**–**15** were identical in all respects to the previously prepared samples.[23] The purity of samples as determined by CE was >95%.

4.14. 2,3,4,6-tetra-*O*-Acetyl-α-D-mannopyranosyl-(1 → 3)-2,4,6-tri-*O*-acetyl-α-D-mannopyranosyl-(1 → 3)-2,4,6-tri-*O*-acetyl-α-D-mannopyranosyl-(1 → 2)-3,4,6-tri-*O*-acetyl-β-D-mannopyranosyl azide (32)

(A) A mixture of the tetrasaccharide **9** (126 mg, 189 μmol) and *N*,*N*-dimethylaminopyridine (10 mg) was stirred in pyridine (3 mL) and Ac_2O (2 mL) (rt, o/n). The mixture was cooled (0 °C), quenched (MeOH) and the solvents evaporated. The residue was subjected to workup (EtOAc) and FC (30–60% EtOAc/hexanes) to yield a colourless powder (193 mg, 81%). This sample was used in the next reaction without further purification or characterisation. (B) HBr (1.0 mL of 30% in HOAc, 3.7 mmol) was added to a solution of the peracetate from (A) (180 mg, 0.14 mmol) in 1,2-DCE (10 mL) and the mixture stirred (rt, o/n). The mixture was poured onto an ice-water mixture and immediately subjected to workup ($CHCl_3$) to yield, presumably, the bromide **31** as a pale yellow coloured oil. This sample was used in the next reaction without further purification. (C) The product from (B) (0.14 mmol, max) and NaN_3 (93 mg, 1.43 mmol) in DMF (5 mL) was heated (110 °C, o/n). The mixture was cooled and the solvent evaporated. The residue was dissolved (EtOAc) and subjected to workup to yield the azide **32** as a colourless oil (73 mg, 41%, two steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.97, 2.01, 2.02, 2.06, 2.07, 2.08, 2.10, 2.10(5), 2.11(2), 2.12(7), 2.13(0), 2.16, 2.17 (13 s, 13H, OAc), 3.74 (ddd, 1H, H-5), 3.87 (ddd, 1H, H-5), 3.93 (ddd, 1H, H-5), 3.99–4.31 (m, 11H), 4.40 (ddd, 1H, H-5^I), 4.91 (d, 1H, $J_{1,2}$ = 1.2 Hz, H-1), 4.95 (d, 1H, $J_{1,2}$ = 2.0 Hz, H-1,), 4.97 (d, 1H, $J_{1,2}$ = 1.6 Hz, H-1), 5.00–5.03 (m, 3H), 5.16–5.33 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.7, 20.83, 20.87, 20.91, 20.94, 20.97, 21.00, 21.2, 21.3, 62.1, 62.3, 62.6, 65.86, 65.90, 67.0, 67.2, 68.6, 69.5, 69.7, 69.9, 70.0, 71.1, 71.4, 72.7, 74.8, 74.9, 75.5, 75.9, 87.1, 98.5, 99.2, 99.7, 169.2, 169.6, 169.75, 169.85, 169.96, 170.04, 170.1, 170.2, 170.3, 170.6, 170.67, 170.69, 170.8; ESMS: *m/z* 1260.3 $[M+Na]^+$.

4.15. *N*-Acetyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl-(1 → 3)-2,4,6-tri-*O*-acetyl-α-D-mannopyranosyl-(1 → 3)-2,4,6-tri-*O*-acetyl-α-D-mannopyranosyl-(1 → 2)-3,4,6-tri-*O*-acetyl-β-D-mannopyranosylamine (20)

(A) PtO_2 (20 mg) was added to a solution of the azide **32** (30 mg, 24.3 μmol) in 1:1 MeOH/EtOAc (4 mL) containing a little AcOH (50 μL) and the combined mixture was vigorously stirred under hydrogen (100 psi, 3 h). The mixture was filtered and the solvent evaporated and co-evaporated (CH_3CN) to yield, presumably, the amine as a colourless glass. This sample was used in the next reaction without further purification. (B) The crude product from (A) was treated with pyridine (1 mL), Ac_2O (1 mL) and DMAP (20 mg) and the combined mixture stirred (rt, o/n). The mixture was treated with MeOH (1 mL) and subjected to workup and FC (40–70% EtOAc/hexane) to yield the amide **20** as a colourless powder (12 mg, 40%, two steps). The sample was of greater purity than that previously isolated[21] but otherwise displayed identical ^{1}H and ^{13}C NMR spectroscopic properties.

4.16. Sulfated tetrasaccharylamine (19)

(A) A small piece of sodium metal was added to a mixture of the azide **32** (30 mg, 24.3 μmol) in MeOH (3 mL) and the combined mixture stirred (rt, 4 h). The solution was neutralised by addition of Dowex AG50-X8 (H^+) resin and filtered. The filtrate was evaporated and the residue lyophilised to yield, presumably, α-D-mannopyranosyl-(1 → 3)-α-D-mannopyranosyl-(1 → 3)-α-D-mannopyranosyl-(1 → 2)-β-D-mannopyranosyl azide as a pale yellow coloured glass (12 mg, 72%). This was used in the following reactions without further purification or characterisation. (B) PtO_2 (20 mg) was added to a solution of the azide **32** from (A) (24.3 μmol, max) in H_2O (3 mL) and the combined mixture was vigorously stirred under hydrogen (100 psi, o/n). The mixture was filtered and the solvent evaporated and lyophilised (H_2O) to yield, presumably, the amine **18** as a colourless glass. This sample was used in the next reaction without further purification. (C) The amine **18** from (B) was sulfonated as described for the preparation of **12**–**15** to yield **19** (20 mg, 55%) as an amorphous white powder after lyophilisation. ^{1}H NMR (400 MHz, D_2O): δ 3.70–4.51 (26H, m), 4.67–4.73 (2H, m), 4.91 (1H, m), 5.12 (1H, m), 5.44–5.25 (4H, m), 5.68 (1H, m).

4.17. Methyl 3-*O*-(2,4,6-tri-*O*-benzoyl-α-D-mannopyranosyl)-2,4,6-tri-*O*-benzoyl-α-D-mannopyranoside (35)

(A) A mixture of the imidate **22** (410 mg, 0.57 mmol) and the alcohol **33** (300 mg, 0.51 mmol)[27] in 1,2-DCE (6 mL) in the presence of mol. sieves (700 mg of 3 Å powder) was treated with TMSOTf (30 μL, 0.17 mmol) and the combined mixture stirred (0° → rt, 30 min). Et_3N (100 μL) was introduced, the mixture was filtered and the solvent was evaporated. The residue was subjected to FC (10–50% EtOAc/hexane) to yield, presumably, disaccharide **34** as a colourless oil. (B) $PdCl_2$ (40 mg) was added to a solution of the product from (A) in MeOH (10 mL) and 1,2-DCE (10 mL) and the combined mixture was heated (70 °C, 40 min). The sol-

vents were evaporated and the residue subjected to FC (10–50% EtOAc/hexanes) to yield the alcohol **35** as a colourless oil (316 mg, 68%, two steps). The ^{1}H and ^{13}C NMR ($CDCl_3$) spectra were in accord with those reported in the literature.[27]

4.18. Methyl 3-*O*-[3-*O*-(2,4,6-tri-*O*-benzoyl-α-D-mannopyranosyl)-2,4,6-tri-*O*-benzoyl-α-D-mannopyranosyl]-2,4,6-tri-*O*-benzoyl-α-D-mannopyranoside (37)

(A) A mixture of trichloroacetimidate **22** (269 mg, 0.37 mmol) and the alcohol **35** (306 mg, 0.31 mmol) was treated with TMSOTf (20 μL, 0.11 mmol) as described for **34** to yield, presumably, the trisaccharide **36** as a colourless oil. (B) The product from (A) was deprotected as described for **35** to yield the alcohol **37** as a colourless oil (316 mg, 70%, two steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.14–7.22 (m, 45 H, Ph), 6.63 (dd, 1H, $J_{1III,2III}$ = 1.8, $J_{2III,3III}$ = 3.3 Hz, H2^{III}), 5.94 (dd, 1H, $J_{3III,4III}$ = 10.0, $J_{4III,5III}$ = 10.0 Hz, H4^{III}), 5.84 (dd, 1H, $J_{3II,4II}$ = 9.9, $J_{4II,5II}$ = 9.9 Hz, H4^{II}), 5.48 (dd, 1H, $J_{3I,4I}$ = 9.8, $J_{4I,5I}$ = 9.8 Hz, H4^{I}), 5.26 (d, 1H, $J_{1I,2I}$ = 1.9 Hz, H1^{I}), 5.22 (dd, 1H, $J_{1II,2II}$ = 2.1, $J_{2II,3II}$ = 3.0 Hz, H2^{II}), 4.91 (d, 1H, H1^{III}), 4.90 (dd, 1H, $J_{2I,3I}$ = 3.2 Hz, H2^{I}), 4.86 (dd, 1H, $J_{1II,2II}$ = 1.7 Hz, H1^{II}). 4.67–4.63 (m, 12H, H3$^{I–III}$, 5$^{I–III}$, 6a,b$^{I–III}$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.5, 166.4, 166.2, 166.1, 165.9, 165.8, 165.6, 165.19, 165.15, 133.8, 133.7, 133.61, 133.58, 133.5, 133.2, 133.1, 130.22, 130.16, 130.1, 130.05, 130.02, 129.97, 129.91, 129.88, 129.8, 129.5, 129.4, 129.3, 129.22, 129.17, 129.1, 129.0, 128.8, 128.6, 128.53, 128.50, 128.46, 99.4, 99.2, 98.7, 76.5, 76.1, 72.4, 71.8, 71.6, 69.9, 69.7, 69.0, 68.9, 68.6, 68.5, 67.8, 63.2, 62.8, 62.4, 55.7; ESMS: *m/z* 1373.4 $[M-Bz+H+Na]^+$, 1269.4 $[M-2Bz+2H+Na]^+$.

4.19. Methyl 3-*O*-{3-*O*-[3-*O*-(2,4,6-tri-*O*-benzoyl-α-D-mannopyranosyl)-2,4,6-tri-*O*-benzoyl-α-D-mannopyranosyl]-2,4,6-tri-*O*-benzoyl-α-D-mannopyranosyl}-2,4,6-tri-*O*-benzoyl-α-D-mannopyranoside (39)

(A) A mixture of trichloroacetimidate **22** (121 mg, 0.17 mmol) and the alcohol **37** (201 mg, 0.14 mmol) was treated with TMSOTf (1 μL, 0.06 mmol) as described for **34** to yield, presumably, the tetrasaccharide **38** as a colourless oil. (B) The product from (A) was deprotected as described for **35** to yield the alcohol **39** as a colourless oil (145 mg, 54%, two steps). The ^{1}H and ^{13}C NMR ($CDCl_3$) spectra were in accord with those already reported in the literature.[27]

4.20. Methyl α-D-mannopyranosyl-(1 → 3)-α-D-mannopyranoside (40)

The alcohol **35** (10 mg, 0.10 mmol) was transesterified as described for the deprotection of **25** to yield the disaccharide **40** as a colourless oil (3 mg, 85%), identical by ^{1}H NMR to that reported in the literature.[42–44]

4.21. Sulfated disaccharide (41)

The disaccharide **40** (25 mg, 70 μmol) was sulfonated as described for the preparation of **12**–**15** to yield **41** (27 mg, 36%) as an amorphous white powder after lyophilisation. ^{1}H NMR (400 MHz, D_2O) δ 5.26 (d, 1H, $J_{1,2}$ = 1.8 Hz, H1^{II}), 4.98 (dd, 1H, $J_{2,3}$ = 2.4 Hz, H2^{II}), 4.87 (d, 1H, $J_{1,2}$ = 1.9 Hz, H1^{I}), 4.60–4.55 (m, 1H, H3^{II}), 4.53 (dd, 1H, $J_{2,3}$ = 2.3 Hz, H2^{I}), 4.41–4.19 (m, 5H, H4^{I}, 4^{II}, 6a^{I}, 6a^{II}, 6b^{II}), 4.15 (dd, 1H, $J_{3,4}$ = 9.3 Hz, H3^{I}), 4.06–3.91 (m, 3H, H5^{I}, 5^{II}, 6b^{I}), 3.29 (s, 3H, OCH_3).

4.22. Sulfated trisaccharide (43)

The alcohol **37** (115 mg, 0.79 mmol) was transesterified as described for the deprotection of **25** to yield methyl α-D-mannopyranosyl-(1 → 3)-α-D-mannopyranosyl-(1 → 3)-α-D-mannopyranoside (**42**)[45] as a colourless oil (35 mg, 86%), used without further purification in the next step; HRMS: *m/z* 519.1862 $[M+H]^+$, 541.1646 $[M+Na]^+$. The trisaccharide **42** (25 mg, 48 μmol) was sulfonated as described for the preparation of **12**–**15** to yield **43** (36 mg, 49%) as an amorphous white powder after lyophilisation. ^{1}H NMR (400 MHz, D_2O) δ 5.26 (d, 1H, $J_{1,2}$ = 1.9 Hz, H1^{III}), 5.22 (d, 1H, $J_{1,2}$ = 1.8 Hz, H1^{II}), 5.04 (dd, 1H, $J_{2,3}$ = 2.4 Hz, H2^{III}), 4.89 (d, 1H, $J_{1,2}$ = 1.6 Hz, H1^{I}), 4.76–4.75 (m, 1H, H2^{II}), 4.60–4.55 (m, 1H, H3^{III}), 4.55 (dd, 1H, $J_{2,3}$ = 3.1 Hz, H2^{I}), 4.50 (dd, 1H, $J_{3,4}$ = 9.6, $J_{4,5}$ = 9.7 Hz, H4^{III}), 4.41–4.12, 4.04–3.91 (2 m, 12H, H3^{II}, 4^{I}, 4^{II}, 5$^{I–III}$, 6a$^{I–III}$, 6b$^{I–III}$), 4.10 (dd, 1H, $J_{3,4}$ = 9.5 Hz, H3^{I}), 3.29 (s, 3H, OCH_3).

4.23. Methyl α-D-mannopyranosyl-(1 → 3)-α-D-mannopyranosyl-(1 → 3)-α-D-mannopyranosyl-(1 → 3)-α-D-mannopyranoside (44)

The alcohol **39** (127 mg, 0.66 mmol) was transesterified as described for the deprotection of **25** to yield the tetrasaccharide **44** as a colourless oil (45 mg, 98%). ^{1}H NMR (400 MHz, CD_3OD): δ 3.37 (1H, s, CH_3), 3.49–3.89 (18H, m), 3.93–4.00 (4H, m), 4.16–4.19 (2H, m), 4.61 (1H, d, $J_{1,2}$ = 1.2 Hz, H-1^{I}), 5.03 (1H, d, $J_{1,2}$ = 1.2 Hz, H-1), 5.07 (1H, d, $J_{1,2}$ = 1.2 Hz, H-1), 5.10 (1H, d, $J_{1,2}$ = 1.2 Hz, H-1); ^{13}C NMR (100 MHz, CD_3OD) δ 54.0, 61.6, 61.7, 62.0, 62.1, 66.3, 66.5, 66.6, 66.8, 67.8, 70.18, 70.23, 70.9, 71.3, 73.5, 73.8, 73.9, 74.1, 78.7, 79.3, 101.5, 102.5, 102.6, 102.7; HRMS calcd for $C_{25}H_{45}O_{21}$ $[M+H]^+$ 681.2453, found 681.2390.

4.24. Preparation of [^{3}H]-heparan sulfate (HS)

HS (18 mg) was dissolved in hydrazine hydrate (1 mL) containing 2% hydrazine sulfate and heated at 100 °C for 170 min to remove the N-linked acetate groups. The mixture was cooled and co-evaporated with toluene (3× 1 mL) and the residue was dissolved in 1 M NaCl (2 mL) and desalted into water using a PD-10 column. The eluate from the PD-10 column was passed through a Dowex 50 column (1 × 5 cm, Na^+ form) to remove remaining impurities. The eluate was concentrated and the residue dissolved in 500 mM $NaHCO_3$ (1.5 mL) containing 10% MeOH, chilled to 0 °C and a solution of [^{3}H]acetic anhydride (16 μmol, 8 mCi) in toluene (80 μL) added. After 3 h excess unlabelled acetic anhydride (50 μL) was added to ensure complete re-acetylation of amino groups and the reaction was adjusted to alkaline pH with 0.5 M Na_2CO_3. The mixture was

subjected to size exclusion chromatography (Sephacryl S-200, 1.6 × 58 cm) equilibrated and eluting with 10% EtOH. Fractions containing high molecular weight HS fragments were pooled, evaporated and dissolved in 10% EtOH to an approximate concentration of 5 mg/mL. The concentration of the [^{3}H]HS preparation was accurately determined (5.6 mg/mL) using the DMB assay for glycosaminoglycans.[46] Unlabelled HS from the same source was used as standard.

4.25. Isolation and purification of heparanase from human platelets

Heparanase was purified to homogeneity from human platelets using three chromatography steps: Concanavalin A Sepharose, Sulfopropyl Sepharose and Sephacryl S-200 HR. The platelets were extracted by repeatedly freezing the cells in buffer consisting of 15 mM dimethylglutarate, pH 6.0, 500 mM NaCl. After centrifugation to pellet the cell debris the extract was adjusted to 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 0.2% Triton X-100. The extract was then applied to a 100 mL Concanavalin A Sepharose column equilibrated with buffer consisting of 15 mM dimethylglutarate, pH 6.0, 500 mM NaCl and 0.2% Triton X-100. The Concanavalin A column was at 4 °C. Once the extract had been loaded, the column was washed with buffer minus Triton X-100 for one column volume and the column temperature was increased to 23 °C. Once equilibrated to 23 °C, glycosylated proteins (including heparanase) were eluted from the column by washing the column with buffer containing 20% methyl α-D-mannopyranoside. Subsequent chromatography columns in the procedure were run at room temperature. The eluate from the Concanavalin A column was diluted 1/5 into 10 mM Na phosphate, pH 7.0, and then applied to a 150 mL Sulfopropyl Sepharose column equilibrated with 20 mM Na phosphate, pH 7.0. The column was then eluted with a 1.3 L gradient of 0–750 mM NaCl in 20 mM Na phosphate, pH 7.0. Fractions containing heparanase were pooled and concentrated using centrifugal concentrators (Centriplus YM-30, Millipore) to approximately 5 mL. The concentrated sample was loaded onto Sephacryl S-200 HR columns (two 500 mL columns in series) equilibrated with 15 mM dimethylglutarate, pH 6.0, 500 mM NaCl. The same buffer was used to elute this column. Fractions were analysed by gel electrophoresis and those containing pure heparanase were pooled. The heparanase sample was then concentrated using Centriplus concentrators and desalted into the final buffer (10 mM dimethylglutarate, pH 6.0). The purified heparanase prepared this way was stored at −80 °C until use.

4.26. Heparanase inhibition assays

Reactions were set up in a volume of 100 μL containing 40 mM sodium acetate buffer (pH 5.0), BSA (0.1 mg/mL), heparanase (90 ng), 5 μM [^{3}H]HS and various concentrations of sulfated oligosaccharide inhibitors. Initially, all components except the [^{3}H]HS were allowed to equilibrate for 10 min at 22 °C. The assays were then initiated by adding the [^{3}H]HS and immediately 20 μL was taken, quenched with 80 μL of 10 mM phosphate (pH 7.0) and the 100 μL transferred to a Microcon YM-10 concentrator which was centrifuged at approximately 14,000*g* for 5 min. The solution that passed through the membrane (filtrate) was retained. This sample was considered the time = 0 sample. The assays (now 80 μL in volume) were allowed to react at 37 °C for 4 h and then the filtration step was repeated for three aliquots of 20 μL from each assay. The time = 0 filtrate and the three 4-h filtrate samples were counted for ^{3}H. The difference between the time = 0 and the averaged 4-h samples gave the amount of heparanase activity. Relative inhibition assays were run with a heparanase standard assay which was identical to the assay composition above except no inhibitor was present and the amount of heparanase inhibition in the other assays determined by comparison with this standard. In assays containing a higher concentration of HS than 5 μM the volume of assay mixture quenched was proportionally reduced. For example, a 10 μM assay would have a 10 μL aliquot quenched in 90 μL of 10 mM phosphate buffer (pH 7.0). This was done to prevent concentration effects during ultrafiltration which can occur at high solute concentrations. The results are presented in Table 1.

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