

Original Article

Chemical synthesis of sulfated- $\alpha(1\rightarrow6)$ -D-mannopyranan

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Abstract

Sulfated oligosaccharides are a group of compounds with important biological properties as drug molecules. Phosphomannopentaose sulfate (PI-88) is a sulfated oligosaccharide with antitumor properties. PI-88 has been thoroughly investigated as a drug candidate to treat cancers, and it showed promising results in Phase 1 and Phase 2 clinical trials. The glycosidic linkages for the sulfated mannan in PI-88 are mainly of $\alpha(1\rightarrow3)$ and $\alpha(1\rightarrow2)$ types. It is important to determine whether the specific glycosidic linkages connecting the sulfated mannan are crucial for the biological activities of PI-88. Therefore, this study explores a method to rapidly synthesize sulfated mannan with $\alpha(1\rightarrow6)$ linkages. We report here the rapid synthesis of sulfated- α -D-mannopyranan, a PI-88 analogue, by using a regio- and stereocontrolled polymerization of a mannosyl tricyclic orthoester. After the global protecting group removal, the sulfation was successfully carried out by treatment of the unmasked mannans with sulfur trioxide-pyridine complex in dimethyl formamide.

Keywords: sulfated oligosaccharides, PI-88 analogue, sulfation, sulfur trioxide-pyridine complex, rapid synthesis

1. Introduction

Sulfated oligosaccharides are found in many sources, such as heparin in humans (Fairweather, Hammond,

Johnstone, & Ferro, 2008) and kakelokelose in tunicates (Riccio, Kinnel, Bifulco, & Scheuer, 1996). This group of compounds has many biological activities, such as anti-angiogenesis (Fairweather, Hammond, Johnstone, & Ferro, 2008) and anticoagulant (Gandhi & Mancera, 2010) as reported in the case of heparin, and anti-HIV (Riccio, Kinnel, Bifulco, & Scheuer, 1996) as reported for kakelokelose. In the past, heparin was used as an anticoagulant agent. However, as new studies were done, other non-coagulant properties of heparin have been revealed. It was shown that heparin-sulfate

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also plays an important part in tumor growth, invasion, and metastasis. Moreover, heparanase, which is the enzyme that processes heparin-sulfate, is also found to be crucial for tumor progression as well, as it degrades the heparin sulfate chain from the heparin sulfate proteoglycan and releases binding growth factors (Raman & Kuberan, 2010; Zhu, Cao, Zhang, Man, & Wu, 2013). Therefore, heparanase has become another important target for heparin mimetic compounds.

Phosphomannopentaose sulfate (PI-88) is a semi-synthetic sulfated oligosaccharide prepared from extracellular phosphomannan of yeast (Fairweather, Hammond, Johnstone, & Ferro, 2008). PI-88 resembles parts of heparin-sulfate (Raman & Kuberan, 2010). PI-88 analogues have been shown to inhibit heparanase and bind to Fibroblast Growth Factor 1 (FGF-1), Fibroblast Growth Factor 2 (FGF-2) and Vascular Endothelial Growth Factor (VEGF), which are proteins involved in angiogenesis (Karoli *et al.*, 2005). Moreover, the PI-88 analogues also show the ability to inhibit infection by Herpes Simplex Virus 1 (HSV-1) and to prevent the cell-to-cell spread of this virus (Karoli, *et al.*, 2005). In a previous Phase 1 study, PI-88 was administered to 42 cancer patients, and the results showed that PI-88 was well tolerated with appropriate efficacy (Basche *et al.*, 2006). In another Phase 1 study, PI-88 was co-administered with docetaxel to 16 cancer patients. This combination was shown to be well tolerated with appropriate efficacy (Chow *et al.*, 2008). In a previous clinical study, the adjuvant properties of PI-88 were revealed when administered with treatments of hepatocellular carcinoma (HCC) recurrences (Liu *et al.*, 2014). In addition, in a previous Phase 3 study, PI-88 was administered as an adjuvant therapy for hepatitis virus related hepatocellular carcinoma (HV-HCC) after its surgical resection. PI-88 increased the disease-free survival in 40% of the patients, which was the microvascular-invasion subgroup (Chen *et al.*, 2017). Results from these clinical trials have shown the safety and favorable clinical efficacy of PI-88. Moreover, fucoidans, which are sulfated polysaccharides derived from algae, have been shown to inhibit tumor growth by a mechanism that is independent of angiogenesis (Zhu, Cao, Zhang, Man, & Wu, 2013). This evidence highlights the need to study other biological properties and to understand the biochemical mechanisms of sulfated polysaccharides.

The PI-88 structure consists of 2 to 6 mannose units with an $\alpha(1\rightarrow2)$ glycosidic linkage as the first linkage from the reducing-end of the molecule. The rest of the mannan chain has $\alpha(1\rightarrow3)$ glycosidic linkages as shown in Figure 1 (Raman & Kuberan, 2010). There is still insufficient information on the structure-activity relationship (SAR) of PI-88. Therefore, acquiring more information on the biological activities of new PI-88 analogues would benefit the development of the essential features of this class of compounds. This information may facilitate the discovery of more potent and/or more selective drug candidates.

In the previous studies, the rapid synthesis of $\alpha(1\rightarrow6)$ mannopyranan was done in a single polymerization reaction (Wattanasiri, Paha, Ponpuak, Ruchirawat, & Boonyarattanakalin, 2017; Yongyat, Ruchirawat, & Boonyarattanakalin, 2010). Mannosyl tricyclic orthoester **2**, as a monomer, has high strain in its tricyclic structure. The Lewis acid, trimethylsilyl trifluoromethanesulfonate (TMSOTf) can trigger a ring-opening polymerization of the monomer. This monomer has a unique spatial geometry, which leads to regio-

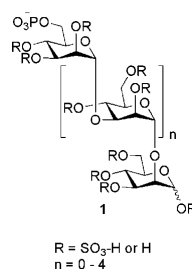


Figure 1. Structure of Phosphomannopentaose sulfate (PI-88) **1**.

stereoselective polymerization. During the reaction, TMSOTf coordinates at the least hindered oxygen on the monomer, C-6 oxygen moiety, which leads to the regioselective transformation. The tricyclic structure opens up and turns into a tricyclic cation intermediate, which contains a bicyclic ring with a free hydroxyl group. The presence of the benzyl groups helps to stabilize the orthoester intermediate by electron donation. The hydroxyl group at C-6 can then attack the bottom face of another intermediate at C-1 position and create a dimer. The spatial conditions in this process ensure stereoselectivity throughout the propagation of polymerization. This provides access to chemically characterized regio- and stereocontrolled mannopyranan molecules that can be further sulfonated.

We propose a method to install the sulfate groups onto the mannan backbone and create a larger analogue of the PI-88. This compound would contain an $\alpha(1\rightarrow6)$ glycosidic linkage, whereas the PI-88 has $\alpha(1\rightarrow2)$ and $\alpha(1\rightarrow3)$ glycosidic linkages. Although the linkages are not identical, we speculate that this stereo-similarity may provide a compound with similar properties. In addition, the larger number of mannose units in our target sulfated mannan may also strengthen the binding with a target protein, possible through multivalent bindings. For example, a larger $\alpha(1\rightarrow6)$ mannan has been experimentally demonstrated to have a higher binding affinity with Concanavalin A than that of a smaller $\alpha(1\rightarrow6)$ mannan (Leelayuwapan *et al.*, 2017; Wattanasiri, Paha, Ponpuak, Ruchirawat, & Boonyarattanakalin, 2017; Yongyat, Ruchirawat, & Boonyarattanakalin, 2010).

2. Materials and Methods

2.1 Chemicals

All chemicals were of reagent grade and they were used as supplied except where noted. All reactions were performed with oven-dried glassware under an inert atmosphere unless otherwise noted. Dried dichloromethane (CH₂Cl₂) was obtained from a Pure-Solv solvent purification system. Lutidine was treated with potassium hydroxide (KOH), and allyl alcohol was treated with potassium carbonate (K₂CO₃) prior to use. The monomers used for polymerizations were dried using a Kugelrohr apparatus (Buchi GKR-51). Analytical thin layer chromatography (TLC) was performed on Merck silica gel 60 F254 plates (0.25 mm). Compounds were visualized by staining with cerium sulfate-ammonium molybdate (CAM) solution or with phosphomolybdic acid (PMA) solution. Flash column chromatography was carried out using forced flow of the indicated solvent on Fluka Kieselgel 60 (230-400 mesh).

2.2 Equipment

All new compounds were characterized by NMR spectroscopy (NMR), high resolution mass spectrometry (HRMS), alpha rotation, and melting point (for a solid). NMR spectra were recorded on a Varian Gemini 2000 (200 MHz), Bruker Avance III (300 MHz), Avance 400 (400 MHz), and Bruker Avance 600 (600 MHz). NMR chemical shifts (δ) are reported in ppm with chemical shift (δ) reference to internal standards (CDCl_3 , δ 7.26 ppm for ^1H and δ 77.0 ppm for ^{13}C ; D_2O , δ 4.79 ppm for ^1H). Splitting patterns in ^1H NMR data are indicated as s for singlet; br s for broad singlet; d for doublet; dd for doublet of doublet; and m for multiplet. H-H COSY NMR spectra of compounds **3**, **4**, and **5** are provided in appendix (Figures S1-S3). High resolution mass spectral (HRMS) analyses were performed by the MS-service at CRI. Peaks are reported as m/z . MALDI-TOF was measured using Microflex Bruker Daltonics. The data was interpreted by Flex Analysis 2.4. Optical rotations were measured using a JASCO P-1020 polarimeter. Melting points were measured in capillaries on a BÜCHI apparatus (Model BÜCHI 535).

2.3 Synthesis of $\alpha(1\rightarrow6)$ mannopyranan **4**

(Detailed experiments are described in Appendix.)

Previous studies have shown that mannosyl tricyclic orthoester **2** can be used as a building block to create $\alpha(1\rightarrow6)$ mannopyranan by polymerization (Leelayuwapan, *et al.*, 2017; Wattanasiri, Paha, Ponpuak, Ruchirawat, & Boonya rattanakalin, 2017; Yongyat, Ruchirawat, & Boonyarattana kalin, 2010). Monomer **2** was dried under *vacuum* at 80 °C for 4 hours prior to the polymerization reaction. The reactant was diluted in dichloromethane (CH_2Cl_2) and stirred at -40 °C. Trimethylsilyl trifluoromethanesulfonate (TMSOTf) was added to trigger the polymerization. The reaction mixture was stirred for 4 hours while the temperature was kept at -40 °C. The reaction was quenched with cold aqueous saturated NaHCO_3 solution. The crude reaction product was purified by flash silica gel column chromatography to yield the protected $\alpha(1\rightarrow6)$ mannopyranan **3** as a desired product. The protected glycan **3** was subjected to a global protecting group removal reaction by Birch reduction. Protected glycan **3** was dissolved in a minimal amount of tetrahydrofuran (THF) and stirred at -78 °C. After that, liquid ammonia (NH_3) was added by condensation of NH_3 gas. Then, 300-500 mg of solid sodium chips were gradually added until a dark blue solution was generated. The dark blue solution was maintained for 4 hours. Afterwards, the reaction mix was neutralized with formic acid and extracted with water. The aqueous solution was then purified by dialysis in large volumes of deionized water. After 3 cycles of dialysis, the water in the solution was evaporated to yield the unmasked $\alpha(1\rightarrow6)$ mannopyranan **4** (Scheme 1). The molecular weight of compound **4** was confirmed by MALDI-TOF analysis. The MALDI-TOF spectra show a series of peaks separated by an interval of 162 m/z . The mass of $\alpha(1\rightarrow6)$ mannopyranan **4**, calculated for $\text{C}_96\text{H}_{164}\text{O}_{81}^{2+}$, is 1306.435 and the observed mass was 1307.243. The mass analysis indicates 16 repeating units of mannose in the mannan chain.

$\alpha(1\rightarrow6)$ Mannopyranan **3**: 55 mg, 61% by mass, mp = 100.1-104.3 °C; $[\alpha]_D = +16.19$ ($c = 0.1$, 1 mg/mL, CH_2Cl_2); ^1H NMR (600 MHz, CDCl_3) δ 3.34-3.45 (m, 1H, H-6a), 3.52-

3.62 (m, 1H, H-5), 3.69-3.77 (m, 1H, H-6b), 3.88-4.09 (m, 2H, H-3,4), 4.10-4.78 (m, 4H, 2H, $-\text{CH}_2\text{Ph}$), 4.88 (s, 1H, H-1) 5.68 (s, 1H, H-2), 6.87-7.15 (m, 10 Arom-H), 7.20-7.56 (m, 3 Arom-H), 7.88-8.05 (m, 2H, ortho-H of benzoyl group); ^{13}C NMR (150 MHz, CDCl_3) δ 65.86, 68.53, 71.06, 71.30, 71.39, 73.86, 75.09, 75.24, 78.27, 98.65 (C-1), 127.17, 127.22, 127.40, 128.44, 128.47, 128.52, 129.81, 130.02, 133.22, 137.53, 138.45, 165.50.

$\alpha(1\rightarrow6)$ Mannopyranan **4**: 5 mg, 27%, MALDI-TOF (m/z): $[\text{M}+2\text{H}^+]$ calculated for $\text{C}_96\text{H}_{164}\text{O}_{81}^{2+}$, 1306.435; found 1307.243. mp was undetermined due to quick decomposition at high temperature; $[\alpha]_D = +61.20$ ($c = 0.1$, 1 mg/mL, H_2O); ^1H NMR (600 MHz, D_2O) δ 3.64-3.66 (s, 1H, H-4), 3.69-3.71 (s, 1H, H-6), 3.72-3.74 (s, 1H, H-3), 3.75-3.77 (s, 1H, H-5), 3.81-3.83 (s, 1H, H-6), 3.88-3.90 (s, 1H, H-2), 4.80-4.82 (s, 1H, H-1); ^{13}C NMR (150 MHz, D_2O) δ 65.45, 66.51, 69.91, 70.57, 70.82, 99.26.

2.4 Synthesis of sulfated- $\alpha(1\rightarrow6)$ mannopyranan **5**

(Detailed experiments are described in Appendix.)

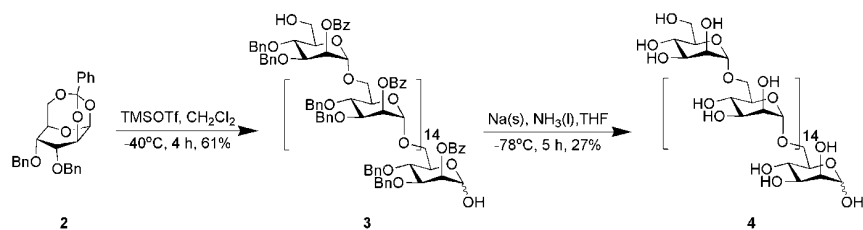
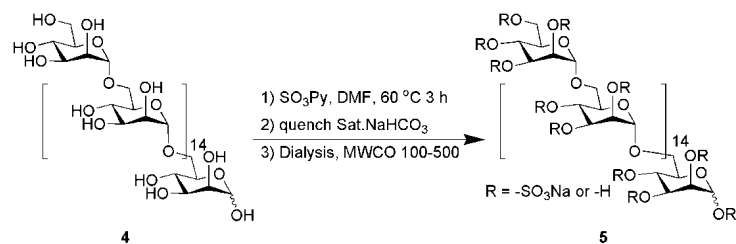
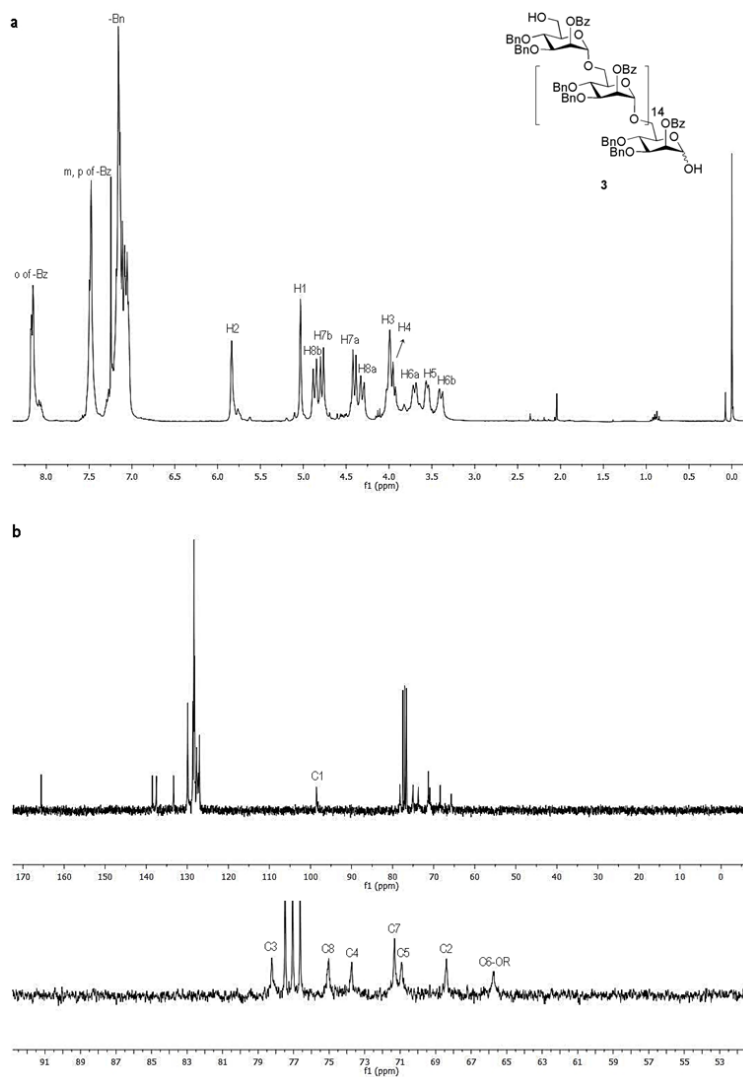
The unmasked $\alpha(1\rightarrow6)$ mannopyranan **4** (5 mg) was dissolved in dimethyl formamide (2 mL) and stirred at 60 °C. After that, sulfur trioxide-pyridine complex was added to the solution, and the mixture was stirred at 60 °C for 3 hours. Then the reaction was terminated by adding cold aqueous saturated NaHCO_3 solution to neutralize the reaction mixture. The neutralized solution was filtered and then the filtrate was subjected to dialysis in large volumes of deionized water. After 3 cycles of dialysis, the water in the solution was evaporated to yield the sulfated mannopyranan **5** as a brown transparent solid (Scheme 2).

Sulfated- $\alpha(1\rightarrow6)$ mannopyranan **5**: mp was undetermined due to quick decomposition at high temperature; ^1H NMR (400 MHz, D_2O) δ 3.41-4.41 (m, 5H, H3, H4, H5, H6), 4.41-4.66 (m, 1H, H-2), 5.06-5.35 (m, 1H, H-1); ^{13}C NMR (100 MHz, D_2O) δ 64.14, 65.29, 67.40, 68.32, 70.71, 75.00, 76.73, 79.10, 97.28, 100.00, 116.46, 129.53, 145.20.

3. Results and Discussion

3.1 Chemical synthesis

Polymerization can generate multiple chemical bonds and produce large molecules in a short period of time. This is an advantage over stepwise synthesis that requires multiple steps and takes a long time to execute. Moreover, a stepwise synthesis also often has a low product yield. In the production of PI-88, yeast cells are cultivated in large quantities, and the PI-88 precursor polysaccharides are then extracted as mixture (Fairweather, Hammond, Johnstone, & Ferro, 2008). These processes also require a large amount of culture media and months of nurturing in order to produce sufficient amounts of the PI-88 precursor. The protected $\alpha(1\rightarrow6)$ mannopyranan **3** can be prepared on a gram scale in 2 steps and can be obtained within a few weeks. The ^1H and ^{13}C NMR spectra of protected $\alpha(1\rightarrow6)$ mannopyranan **3** and peak assignments are shown in Figure 2. The major peaks of anomeric protons and anomeric carbons are at 5.03 ppm and 98.6 ppm, respectively. Both peaks indicate high regio- and stereoregularity of synthetic glycan **3**. The C-2 protons are at 5.82 ppm. The integrated area of the C-2 protons is equal to

Scheme 1. Synthesis of $\alpha(1\rightarrow6)$ mannopyranan **4**.Scheme 2. Synthesis of sulfated- $\alpha(1\rightarrow6)$ mannopyranan **5**.Figure 2. NMR spectra of protected $\alpha(1\rightarrow6)$ mannopyranan **3** (CDCl_3 as solvent), (a) 600 MHz ^1H NMR spectrum; (b) 150 MHz ^{13}C NMR spectrum.

the area of the anomeric protons, implying the regioselectivity of the $\alpha(1\rightarrow6)$ glycosidic bonds in the polysaccharide chain.

Synthetic glycan **3** was subjected to Birch reduction to provide globally unmasked glycan **4**. The ^1H and ^{13}C NMR spectra of glycan **4** and peak assignments are shown in Figure 3. The major peaks of anomeric protons and anomeric carbons are at 4.81 ppm and 99.3 ppm, respectively. Both peaks indicate the high regio- and stereoregularity of synthetic glycan **4**. The C-2 protons are at 3.89 ppm. The integrated area of the C-2 protons is equal to the area of the anomeric protons, indicating the regioselectivity of the $\alpha(1\rightarrow6)$ glycosidic bonds.

Synthetic glycan **4** was further modified into sulfated- $\alpha(1\rightarrow6)$ mannopyranan **5** through sulfation with sulfur trioxide-pyridine complex in a single step (Fairweather, Hammond, Johnstone, & Ferro, 2008). The ^1H and ^{13}C NMR spectra of glycan **5** and the peak assignments are shown in Figure 4. The major peaks of the anomeric protons and anomeric carbons are at 5.18 ppm and 99.3 ppm, respectively.

Most of the ^1H and ^{13}C NMR spectra of sulfated- $\alpha(1\rightarrow6)$ mannopyranan **5** are shifted upfield when compared to the $\alpha(1\rightarrow6)$ mannopyranan **4** due to the electron-withdrawing effects of the installed sulfate groups. This up-field shift due to the installed sulfate groups is also reported in previous PI-88 analogue studies (Tony Mong, Shiau, Lin, Cheng, & Lin, 2015).

4. Conclusions

A polymerization reaction has enabled the rapid synthesis of sulfated- $\alpha(1\rightarrow6)$ mannopyranan **5** with a relatively high yield and high regio- and stereoregularity. The synthetic sulfated- $\alpha(1\rightarrow6)$ mannopyranan **5** can be prepared in only 3 steps from the monomer, mannosyl tricyclic orthoester **2**. The chemical characteristics of the sulfated- $\alpha(1\rightarrow6)$ mannopyranan **5** correspond to the installed sulfate moieties, as shown by an up-field shift of all ^1H NMR peaks, except for the anomeric protons and carbons, when compared to the ^1H

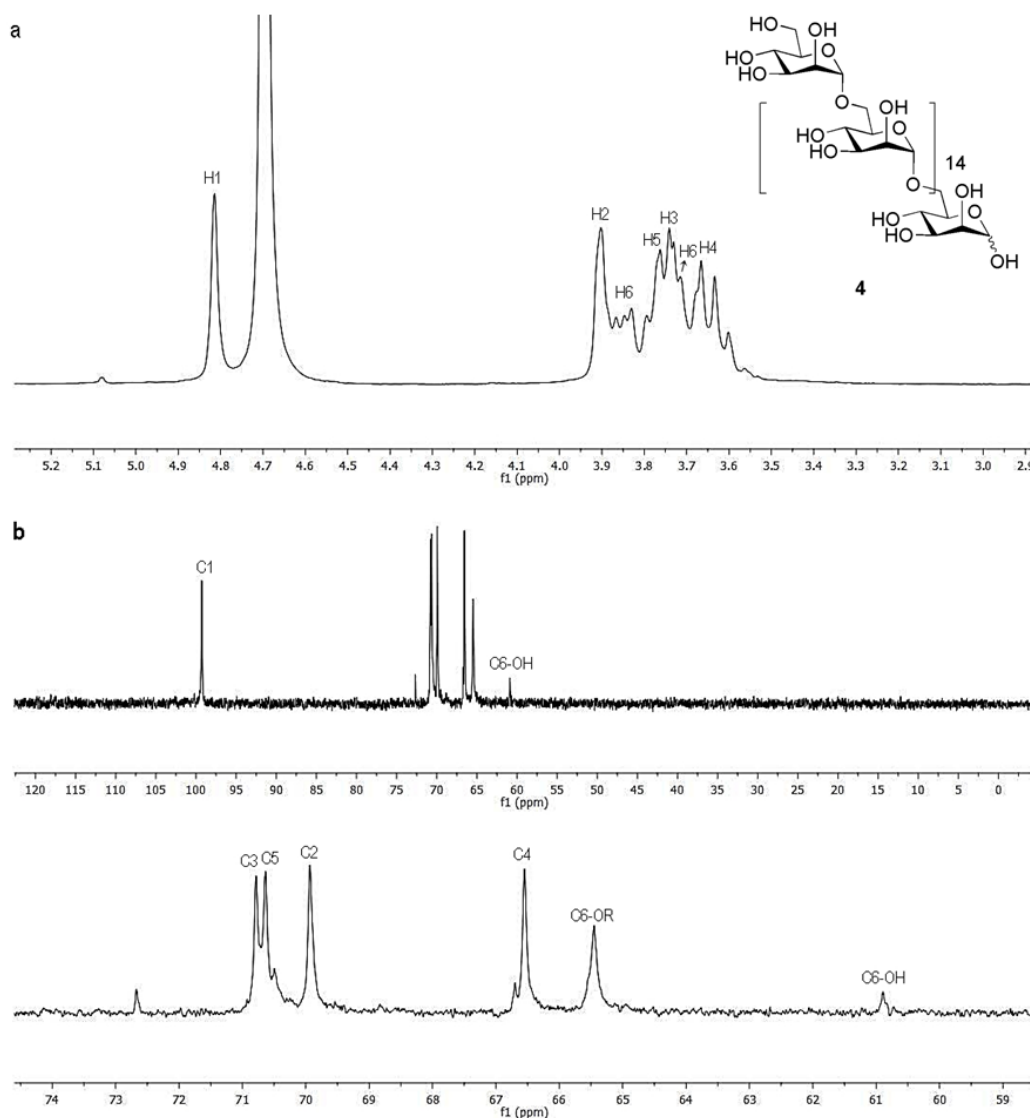


Figure 3. NMR spectra of $\alpha(1\rightarrow6)$ mannopyranan **4** (D_2O as solvent), (a) 600 MHz ^1H NMR spectrum; (b) 150 MHz ^{13}C NMR spectrum.

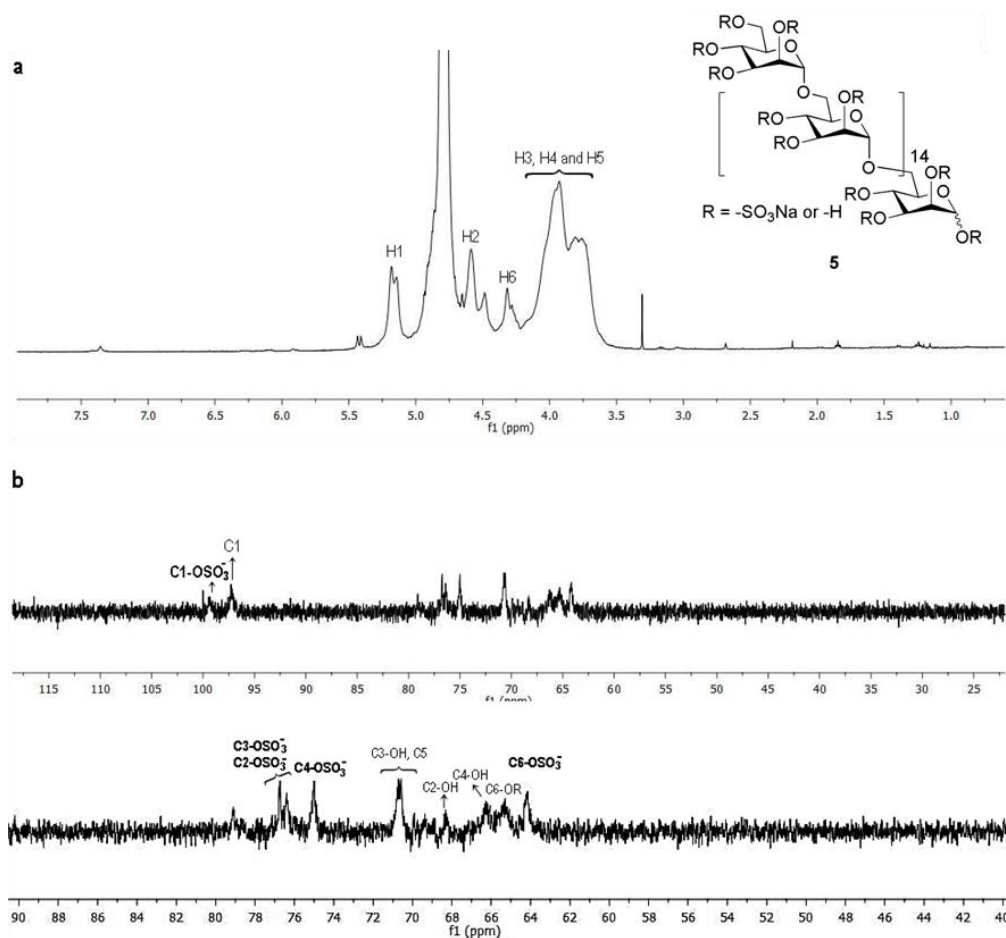


Figure 4. NMR spectra of sulfated- $\alpha(1\rightarrow6)$ mannopyranan **5** (D_2O as solvent), (a) 400 MHz ^1H NMR spectrum; (b) 100 MHz ^{13}C NMR spectrum.

NMR of mannopyranan **4**. This report provides a new rapid chemical synthesis method with reasonable yield to produce a sulfated polysaccharide that is a PI-88 analogue. Further biological investigation of the biological activity of this compound shall be conducted in the future.

Acknowledgements

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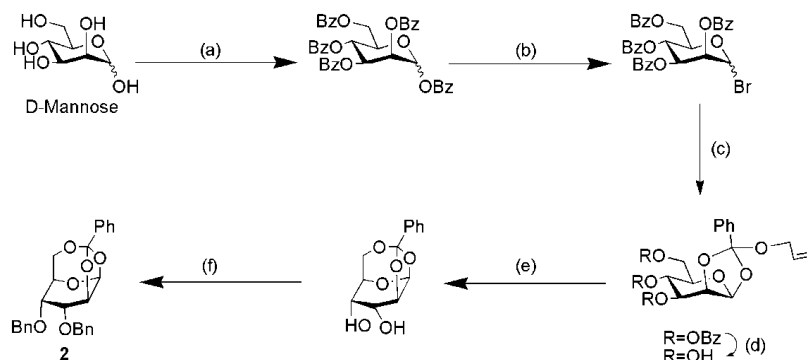
Appendix

Detailed Experiments

Synthesis

Preparation of mannosyl tricyclic orthoester building block 2

According to our previous work (Yongyat, Ruchirawat, & Boonyarattanakalin, 2010), the synthesis of the mannosyl tricyclic orthoester building block 2 started from the protection of D-mannose hydroxyl groups with benzoyl groups. The perbenzoylated mannose was modified into the bicyclic, and then the tricyclic orthoester intermediates, as shown in Scheme S1. In this process, the overall steps were 6 reactions with 2 column purifications, before yielding the mannosyl building block 2 at around 30 % over all (6 steps) for the oligosaccharide assembly.



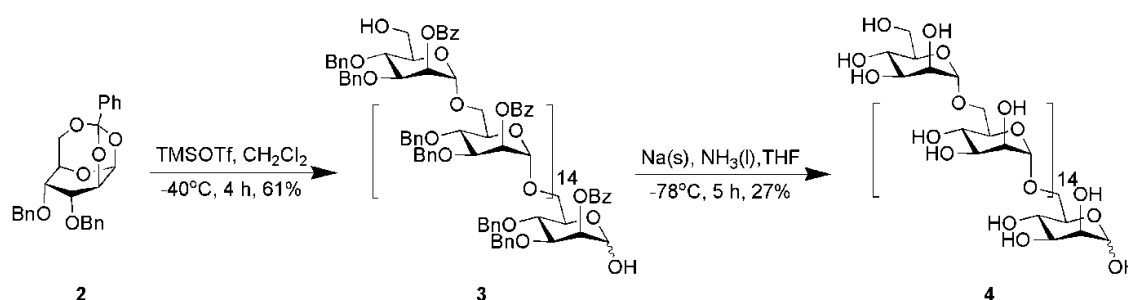
Scheme S1. Synthesis of mannosyl orthoester building block 2. Reagents and conditions: (a) BzCl, Pyridine, 0 °C to rt, 12 hours; (b) HBr/HOAc (33%) 24 hours; (c) Allyl alcohol, Lutidine, 83% (three steps); (d) KOH, H₂O, MeOH, THF, rt, 1 hour; (e) 0.05 equiv CSA, CH₂Cl₂, 30 mins; (f) BnBr, NaH, DMF, 0 °C to rt, 12 hours, 37% (three steps).

Preparation of 3,4-*O*-benzyl-2-*O*-benzoyl- α (1 \rightarrow 6)-D-mannopyranan **3**

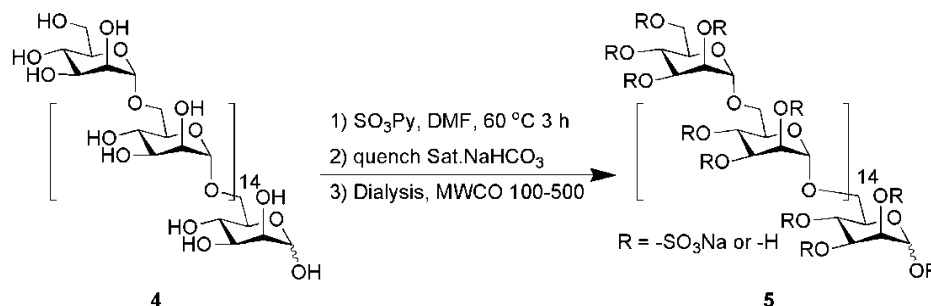
Monomer **2** (90 mg) was transferred into the reaction flask and dried under high vacuum at 80 °C for 4 hours. Dichloromethane (300 μ L) was transferred under inert atmosphere. To a solution of monomer in solvent at -40 °C (dry ice-acetonitrile bath), TMSOTf (0.010 mmol) was added and kept at the desired temperature for 4 hours. The reaction mixture was quenched by the addition of saturated aqueous NaHCO₃ and extracted with CH₂Cl₂ and saturated aqueous NaHCO₃ (3 \times 10 mL). The combined organic layer was dried over anhydrous Na₂SO₄ and concentrated in *vacuo*. The crude product was dissolved in a minimum amount of CH₂Cl₂ and precipitated with pentane. The precipitate was allowed to settle overnight. After the mother liquor was removed, the precipitated polymer was dried under high vacuum to give a white powder (55 mg, 61% by mass) (Scheme S2).

Preparation of α (1 \rightarrow 6)-D-mannopyranan **4**

Protected 3,4-*O*-benzyl-2-*O*-benzoyl- α (1 \rightarrow 6)-D-mannopyranans **3** (50 mg) were dissolved in anhydrous tetrahydrofuran (5 mL) was added to a reaction flask. Anhydrous NH₃ gas was condensed into liquid NH₃ (25 mL) at -78 °C, followed by an addition of small pieces of sodium metal, until the reaction mixture turned dark blue. The reaction was continued stirring for 5 hours to yield α (1 \rightarrow 6)-D-mannopyranan **4** followed by successive addition of *tert*-butanol. After the reaction solution turned colourless, the reaction mixture was allowed to gradually warm to room temperature under a stream of argon gas in order to remove ammonia. Formic acid was added slowly to the reaction flask to adjust the pH to about 7. Dichloromethane (5 mL) was added to the crude product. The organic layer was extracted with water (3 x 5 mL). The aqueous layers containing the desired product were combined and concentrated under high *vacuum*. Unmasked mannopyranan **4** was dialyzed with water at 4 °C and freeze-dried to give a clear solid (5 mg, 27%) (Scheme S2).

Scheme S2. Synthesis of α (1 \rightarrow 6)mannopyranan **4**.Preparation of sulfated- α (1 \rightarrow 6)-D-mannopyranan **5**

Unmasked mannopyranan **4** (5 mg) was dissolved in a solution of sulfur trioxide pyridinium complex in dimethyl formamide (1 mL). The mixture was stirred at 60 °C for 3 hours. Then the reaction was quenched with cold sat. NaHCO₃ solution to neutralize the reaction mixture. The neutralized solution was filtered through 2micron RC Filter disc. Then, the solution was subjected to dialysis with RC tube 100-500 micron. Water in the final purified solution was removed via *vacuo*. The precipitated sulfated polymer **5** was dried under high vacuum to give a clear solid (4 mg) (Scheme S3).

Scheme S3. Synthesis of sulfated- α (1 \rightarrow 6)mannopyranan **5**.

H-H COSY NMR spectra

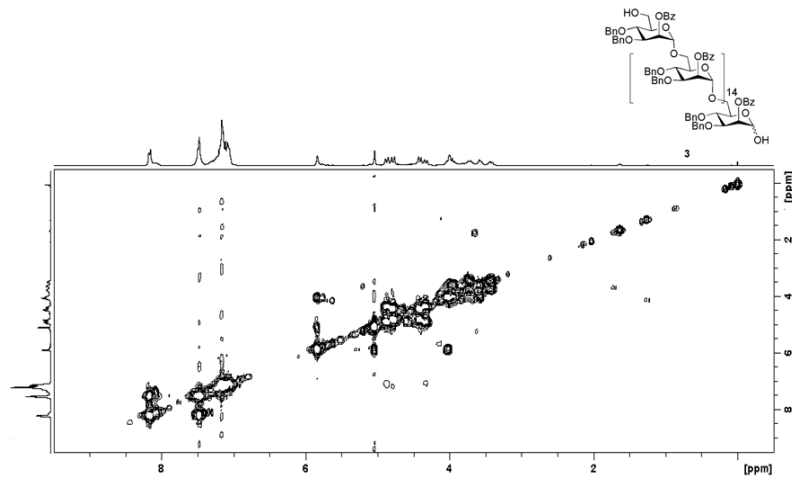


Figure S1. H-H COSY NMR spectra of protected $\alpha(1\rightarrow6)$ mannopyranan **3** (CDCl_3 as solvent).

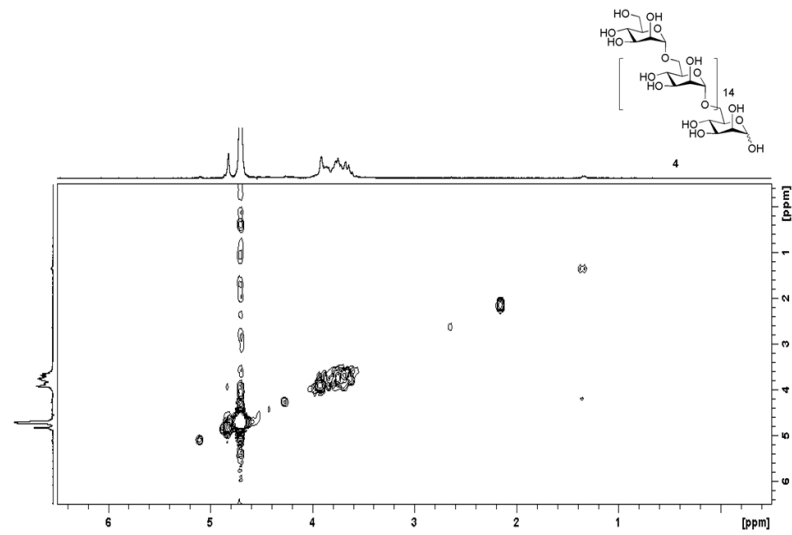


Figure S2. H-H COSY NMR spectra of $\alpha(1\rightarrow6)$ mannopyranan **4** (D_2O as solvent).

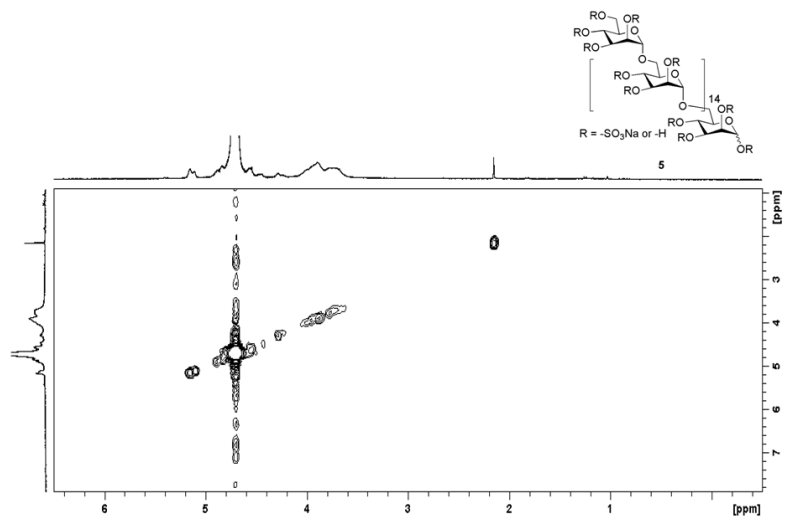


Figure S3. H-H COSY NMR spectra of sulfated- $\alpha(1\rightarrow6)$ mannopyranan **5** (D_2O as solvent).