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The synthesis of a novel thio-linked disaccharide of chondroitin as a potential inhibitor of polysaccharide lyases

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Abstract—A thio-linked disaccharide based on the structure of the glycosaminoglycan chondroitin was synthesized as a potential inhibitor of chondroitin AC lyase from *Flavobacterium heparinum* for structural analysis of the active site. Instead it was found to be a slow substrate, thereby demonstrating that lyases, in contrast to glycosidases, can cleave thioglycoside links between sugars.
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Keywords: Glycosaminoglycan; Polysaccharide lyase; *Flavobacterium heparinum*; Chondroitin; Chondroitin AC

1. Introduction

The highly heterogeneous and acidic polysaccharide chains of glycosaminoglycans (GAGs) contribute to a variety of important physiological roles such as simple mechanical support, the lubrication and cushioning of joints, the modulation of cell signals, as well as the control of cellular adhesion and motility.[1,2] GAGs are composed of a repeating sequence of disaccharide units consisting of hexosamine and uronic acid residues. The chondroitin sulfates are the most common type of GAG chain and consist of an *N*-acetyl-D-galactosamine residue (usually O-sulfated at C-4 or C-6) attached through a β-(1 → 4) linkage to D-glucuronic acid, or sometimes L-iduronic acid (dermatan sulfate), that is in turn attached via a β-(1 → 3) linkage to the next hexosamine residue. Polysaccharide lyases are one class of enzymes responsible for the degradation of GAG chains. They do so via an elimination mechanism, resulting in disaccharides or oligosaccharides with Δ4,5-unsaturated uronic acid residues at their nonreducing ends (Fig. 1).[3,4]

Compared with other carbohydrate-degrading enzymes, such as the well studied and characterized glycosidases, there is a paucity of information regarding the polysaccharide lyases. This is especially true regarding their mechanism of action and the enzymatic residues responsible for their function. The structures of several lyases have been determined,[5–11] but few structures of complexes of native enzymes with substrates, or analogues thereof bound across the active site are available.† Part of the problem here is that no known, specific, tight-binding inhibitors of polysaccharide lyases

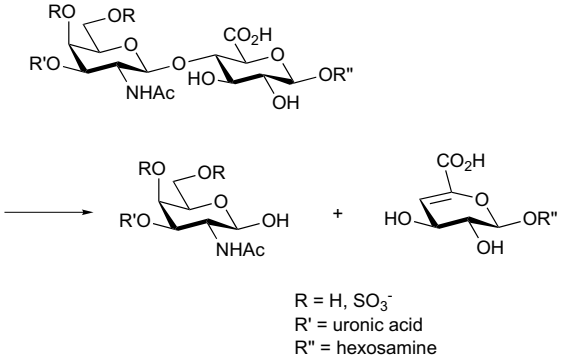

Figure 1. Elimination reaction of polysaccharide lyases.

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† For a more complete listing of the available 3D structures of the polysaccharide lyases, please go to the Web at http://afmb.cnrs-mrs.fr/CAZY.

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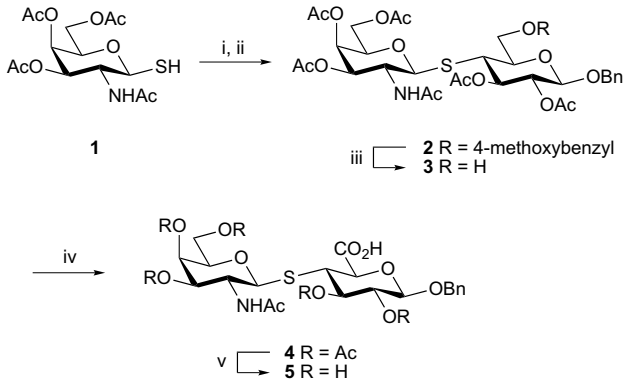
that might be useful in such complexes have been developed. Not only might such inhibitors prove useful in the identification of the key residues involved in binding and catalysis, but they also may provide important therapeutic agents, since lyases have not been found in mammals but are common in some bacteria. In addition, inhibitors may be used to gain detailed information regarding the nature of the transition state of the enzyme-catalyzed reaction.

Thioglycosides, in which the glycosidic oxygen atom has been replaced by a sulfur atom, have been shown to be stable glycoside analogues, acting as competitive inhibitors of many glycosidases.[12–15] We were therefore interested in exploring whether or not the polysaccharide lyase enzymes could cleave a thiol linkage. If they are stable towards elimination, then thioglycosides might prove to be useful inhibitors of these enzymes, and help expand our understanding of these enzymes, which are still in their infancy of mechanistic investigation. The thio-linked disaccharide **5** was therefore synthesized with the hope that it would be an inhibitor of chondroitin AC lyase from *Flavobacterium heparinum*[8,16] that might be useful for structural analyses.

2. Results and discussion

The protected and activated monosaccharide moieties used to create the target disaccharide compound were prepared as described in the literature.[4,17,18] Briefly, compound **1** was synthesized starting from 2-acetamido-2-deoxy-D-galactopyranose, which was reacted with neat acetyl chloride to give the fully acetylated α-D-galactopyranosyl chloride. This crude material was then treated with thiourea in acetone at 80 °C, yielding the thiopseudourea hydrochloride salt, which was subsequently treated with $K_2S_2O_5$ to yield the free thiol **1**.[18] To produce the other coupling partner, benzyl 2,3-di-*O*-acetyl-6-*O*-(4-methoxybenzyl)-4-*O*-trifluoromethanesulfonyl-β-D-galactopyranoside,[4] benzyl β-D-galactopyranoside was treated with *p*-anisaldehyde dimethyl acetal and *p*-TsOH in DMF to install a benzylidene functionality across hydroxyls 4 and 6. The remaining hydroxyls were acetylated, and the benzylidene ring was selectively opened with trifluoroacetic acid (TFA) and sodium cyanoborohydride, leaving the 4-hydroxyl free, onto which the triflate leaving group was installed via reaction with trifluoromethanesulfonic anhydride and pyridine.

The coupling of the two monosaccharides was accomplished by deprotonating the thiol **1** with an equivalent of sodium hydride, followed by the addition of benzyl 2,3-di-*O*-acetyl-6-*O*-(4-methoxybenzyl)-4-*O*-trifluoromethanesulfonyl-β-D-galactopyranoside[4] (Scheme 1). Prior experiments attempting to carry out this S_N2 displacement of a triflate in a compound where C-6 had previously been oxidized to the methyl ester, resulted in only the elimination product being isolated, with no substitution product evident. Thus the oxidation of the C-6 hydroxyl was left until the end of the synthesis. Following the coupling reaction, the 4-methoxybenzyl ether protecting group was selectively removed by a brief exposure to a solution of 10% TFA in CH_2Cl_2, giving compound **3** as a white solid (86%). Formation of a β-glycosidic linkage was confirmed by the 10.4 and 10.7 Hz coupling constants of the anomeric proton of the GlcNAc moiety with H-2 in **2** and **3**. The selective oxidation of the newly exposed primary hydroxyl group over that of the sulfur moiety was accomplished using a sonicated Jones oxidation[19] with CrO_3 and H_2SO_4, in acetone/water at 35 °C, to give **4** as a white solid (73%). The global deprotection of the remaining O-linked acetates was accomplished using sodium methoxide in methanol, to give the desired target **5** as a white solid (46% isolated). High resolution mass spectrometric analysis of **5** confirmed its structure, and particularly that oxidation had not taken place at the sulfur atom.



Scheme 1. Reagents and conditions: (i) NaH, THF, 0 °C → rt; (ii) benzyl 2,3-di-*O*-acetyl-6-*O*-(4-methoxybenzyl)-4-*O*-trifluoromethanesulfonyl-β-D-galactopyranoside,[4] DMF; (iii) 10% TFA in CH_2Cl_2; (iv) CrO_3, H_2SO_4, acetone, water, sonication, 35 °C; (v) NaOMe, MeOH.

^{1}H NMR analysis was used to determine whether or not compound **5** was a substrate for chondroitin AC lyase. Upon addition of enzyme to compound **5** in D_2O buffer, the ^{1}H NMR spectrum revealed the gradual appearance of peaks characteristic of the unsaturated uronic acid product of the enzymatic reaction.[4] Clearly visible were doublets at 5.95 ppm (*J* 4.0 Hz) and 5.26 ppm (*J* 5.4 Hz) corresponding to the C-4 alkene and anomeric protons, respectively. The H-3 proton of the product was also evident at 4.26 ppm (dd, *J* 4.0, 0.8 Hz), however the signal of the H-2 proton was obscured under peaks arising from the starting compound (**5**). The enzymatic cleavage of the disaccharide **5** was very slow (~9% product after 24 h) and was not observed to go to completion, even after a few days under the conditions used (12.5 mg/mL **5**, 0.4 mg/mL enzyme, pH 7). This corresponds to a k_{cat} value of less than 0.3 min^{-1}.

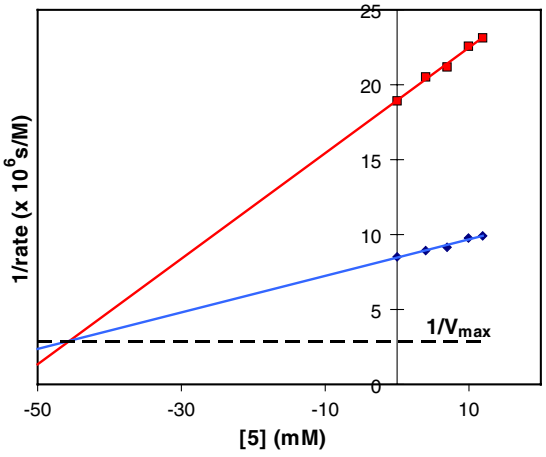


Figure 2. Dixon plot showing the inhibition by compound **5**. Kinetic analysis was carried out as described in Ref. 4 using substrate concentrations of $0.25K_m$ and $0.75K_m$. A K_m value of 45 mM was determined.

A control experiment consisting of the same concentration of compound **5** in D_2O buffer in the absence of enzyme showed no degradation whatsoever as observed by ^1H NMR after a few days.

The thio-disaccharide **5** displayed surprisingly weak binding to the enzyme, acting as a competitive inhibitor with a K_i value of 45 mM, measured using phenyl 4-*O*-(2′,4′-dinitrophenyl)-β-D-glucopyranosiduronic acid[4,20,‡] as substrate (Fig. 2). This low binding was quite unexpected, given that the monosaccharide substrate has a K_m value of 7 mM. The presence of the second sugar moiety in the disaccharide should provide additional binding interactions that improve affinity. It is possible that differences in bond length and bond angle associated with the thioglycosidic bond versus the O-linked parent might be responsible. However it has been pointed out[13] that the greater C–S bond length (1.8 Å vs 1.4 Å for C–O) is somewhat countered by the smaller C–S–C bond angle (105° vs 110° for C–O–C)[21] resulting in almost equivalent relative positioning of the two sugar moieties. Indeed thioglycosides typically bind to glycosidases with similar affinities to those of their oxygen-linked counterparts.[12,13]

In summary, a thio-linked disaccharide, based on the structure of chondroitin, was synthesized and observed to be slowly cleaved by chondroitin AC lyase from *F. heparinum*. This, combined with the fact that the enzyme binds this disaccharide with a lower affinity than the monosaccharide substrate, suggests that the strategy of replacing the glycosidic oxygen with sulfur is not the correct avenue to follow in the quest for a useful inhibitor of the polysaccharide lyase enzymes.

‡ Synthesized as for the appropriate benzyl glycoside found in Ref. 4.

3. Experimental

3.1. Inhibition kinetics

Assays were carried out in quartz cuvettes (1 cm path length), total solution volume 125 μL. Mixtures containing buffer (50 mM sodium phosphate, 100 mM NaCl, pH 6.8) and the desired amount of substrate (phenyl 4-*O*-(2′,4′-dinitrophenyl)-β-D-glucopyranosiduronic acid)[4] and inhibitor **5** were incubated at 30 °C for at least 15 min to thermally equilibrate them. Enzyme (9 μL of 9.0×10^{-5} M chondroitin AC lyase in buffer) was added, the solution mixed, and the change in absorption at 400 nm was monitored over 3 min. Rates were calculated using an extinction coefficient of $\varepsilon = 10{,}910\ M^{-1}\,cm^{-1}$ for 2,4-dinitrophenolate.

3.2. General methods

All reagents were obtained from commercial suppliers and were used without further purification. Column chromatography was performed with Silica Gel (230–400 mesh). TLC was performed on E. Merck pre-coated 60 F-254 silica plates and visualized using UV light and/or by applying a solution of 10% ammonium molybdate in 2 M H_2SO_4 followed by heating. CH_2Cl_2 was distilled over CaH_2. MeOH was distilled over magnesium and iodine. DMF was dried successively over 4 Å molecular sieves (3×). THF was distilled over sodium benzophenone. All ^1H NMR assignments were based on COSY experiments.

3.2.1. 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-1-thio-β-D-galactopyranose (1).[18] Prepared from 2-acetamido-2-deoxy-D-galactopyranose according to the procedure for the corresponding glucopyranose compound:[17,18,22] ^1H NMR ($CDCl_3$, 400 MHz): δ 5.54 (1H, d, *J* 9.4 Hz, NH), 5.35 (1H, dd, *J* 3.4, 1.2 Hz, H-4), 5.04 (1H, dd, *J* 11.0, 3.4 Hz, H-3), 4.58 (1H, dd, *J* 9.8, 9.1 Hz, H-1), 4.26 (1H, ddd, *J* 9.7, 9.7, 9.4 Hz, H-2), 4.14–4.05 (2H, m, H-6_a, H-6_b), 3.90 (1H, ddd, *J* 6.7, 6.7, 1.2 Hz, H-5), 2.59 (1H, 9.1 Hz, SH), 2.15, 2.03, 1.99, 1.97 (12H, 4s, 4Ac). Anal. Calcd for $C_{14}H_{21}NO_8S$: C, 46.27; H, 5.82; N, 3.85. Found: C, 46.53; H, 5.76; N, 3.83.

3.2.2. Benzyl *S*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-galactopyranosyl)-(1 → 4)-2,3-di-*O*-acetyl-4-deoxy-6-(4-methoxybenzyl)-4-thio-β-D-glucopyranoside (2). A suspension of **1** (345 mg, 0.95 mmol), in dry THF (10 mL) was added to a suspension of NaH (38 mg of a 60% dispersion in mineral oil, 0.95 mmol, 1 equiv) in dry THF (9 mL) at 0 °C. The reaction mixture was then allowed to warm to ambient temperature until the evolution of hydrogen gas ceased (~15 min), concentrated under diminished pressure and the residue dissolved in dry DMF (7 mL). To this was added a solution of benzyl

2,3-di-*O*-acetyl-6-*O*-(4-methoxybenzyl)-4-*O*-trifluoromethanesulfonyl-β-D-galactopyranoside[4] (750 mg, 1.24 mmol, 1.3 equiv) in dry DMF (10 mL), and the reaction mixture was stirred for 2 h at rt, concentrated under diminished pressure, redissolved in CH_2Cl_2, washed with water (1×), saturated $NaHCO_3$ (1×), water (1×). The combined aq phases were extracted once with CH_2Cl_2. The combined organic phases were then dried over $MgSO_4$, concentrated under diminished pressure and the resulting residue purified by column chromatography (8:1 PE–EtOAc) giving **2** as a pale yellow foam (334 mg isolated, 43%): ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.32–7.20 (7H, m, Ar), 6.86 (2H, d, *J* 8.5 Hz, Ar), 5.49 (1H, d, *J* 10.4 Hz, NH), 5.28 (1H, dd, *J* 3.4, 1.2 Hz, H-4′), 5.09 (1H, dd, *J* 11.0, 9.1 Hz, H-3), 5.02 (1H, dd, *J* 9.1, 7.9 Hz, H-2), 4.85 (1H, d, *J* 12.5 Hz, OCH_2Ar), 4.83 (1H, dd, *J* 10.4, 3.4 Hz, H-3′), 4.64 (1H, d, *J* 10.4 Hz, H-1′), 4.56 (1H, d, *J* 12.5 Hz, OCH_2Ar), 4.54 (1H, d, *J* 11.6 Hz, OCH_2Ar), 4.46 (1H, d, *J* 11.6 Hz, OCH_2Ar), 4.43 (1H, d, *J* 7.9 Hz, H-1), 4.27 (1H, ddd, *J* 10.4 Hz, H-2′), 4.00 (1H, dd, *J* 11.3, 5.8 Hz, H-6′$_a$), 3.98–3.92 (2H, m, H-6$_a$, H-6′$_b$), 3.83 (1H, dd, *J* 11.0, 1.5 Hz, H-6$_b$), 3.77 (3H, s, OMe), 3.76 (1H, m, H-5′), 3.67 (1H, ddd, *J* 10.7, 4.0, 1.5 Hz, H-5), 3.01 (1H, dd, *J* 11.0, 10.7 Hz, H-4), 2.12, 2.03, 1.99, 1.94, 1.93, 1.85 (18H, 6s, 6Ac); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 171.32, 170.47, 170.24, 170.17, 169.66, 169.17 (6C=O), 159.18, 136.98, 130.14 (3C), 129.42, 128.35, 127.80, 127.37, 113.73, 99.34, 82.85, 75.69, 74.27 (9CH), 73.27 (CH_2), 72.49, 71.99, 70.56 (3CH), 70.21, 68.69 ($2CH_2$), 66.65 (CH), 62.07 (CH_2), 55.194 (CH_3), 48.13, 46.03 (2CH), 23.01, 20.90, 20.64, 20.62, 20.55, 20.48 (6Ac).

3.2.3. Benzyl *S*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-galactopyranosyl)-(1 → 4)-2,3-di-*O*-acetyl-4-deoxy-4-thio-β-D-glucopyranoside (3). Trifluoroacetic acid (2.0 mL) was added to a soln of **2** (300 mg, 0.37 mmol) in dry CH_2Cl_2 (20 mL) and stirred at rt for 20 min. The reaction mixture was then poured into saturated $NaHCO_3$ (300 mL). The aq layer was extracted with CH_2Cl_2 (3×) and the combined organic phases were dried over $MgSO_4$, and concentrated under diminished pressure. The residue was purified by column chromatography (1:9 PE–EtOAc to 100% EtOAc) giving **3** as an off-white solid (220 mg, 86%): ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.36–7.21 (5H, m, Ar), 5.62 (1H, d, *J* 10.7 Hz, NH), 5.33 (1H, dd, *J* 3.4, 1.2 Hz, H-4′), 5.12 (1H, dd, *J* 10.7, 9.1 Hz, H-3), 5.01 (1H, dd, *J* 9.1, 7.9 Hz, H-2), 4.92 (1H, dd, *J* 10.7, 3.4 Hz, H-3′), 4.85 (1H, d, *J* 12.5 Hz, OCH_2Ar), 4.70 (1H, d, *J* 10.7 Hz, H-1′), 4.61 (1H, d, *J* 12.5 Hz, OCH_2Ar), 4.59 (1H, d, *J* 7.9 Hz, H-1), 4.30 (1H, ddd, *J* 10.7 Hz, H-2′), 4.10–4.02 (3H, m, H-6$_a$, H-6$_b$, H-6′$_a$), 3.87 (1H, ddd, *J* 7.9, 6.7, 1.2 Hz, H-5′), 3.60 (1H, ddd, *J* 10.7, 4.3, 2.4 Hz, H-5), 2.93 (1H, dd, *J* 10.7 Hz, H-4), 2.14, 2.05 (6H, 2s, 2Ac), 2.01 (6H, s, 2Ac), 1.96, 1.89 (6H, 2s, 2Ac); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 171.35, 170.57, 170.29, 170.26, 169.80, 169.23 (6C=O), 136.85 (C), 128.49, 128.04, 127.45, 99.62, 83.04, 76.03, 74.47, 72.50, 71.96 (9CH), 70.80 (CH_2), 70.46, 66.74 (2CH), 62.23, 62.20 ($2CH_2$), 48.29, 46.17 (2CH), 23.10, 20.94, 20.72, 20.66, 20.62 (5Ac).

3.2.4. Benzyl *S*-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-(1 → 4)-4-deoxy-4-thio-β-D-glucopyranosiduronic acid (5). Jones reagent (0.55 mL of a soln made of 1 g CrO_3, 0.857 mL H_2SO_4 and 7.14 mL water) was added dropwise to a soln of **3** (220 mg, 0.31 mmol) in acetone (4 mL). The reaction mixture was sonicated at 35 °C for 1 h, after which time another aliquot of Jones reagent (0.2 mL) was added and the reaction mixture sonicated for a further hour. Isopropanol was added to quench, and the solution was decanted from the resulting green solid. The solvent was removed under diminished pressure and the residue was passed through a short column of silica (40:2:1 EtOAc–MeOH–water+0.4% AcOH) to yield benzyl *S*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-galactopyranosyl)-(1 → 4)-2,3-di-*O*-acetyl-4-deoxy-4-thio-β-D-glucopyranosiduronic acid (**4**) as a white solid (164 mg, 73%). This was then dissolved in dry MeOH (5 mL) to which NaOMe was added until the soln remained basic. When the reaction was judged to be complete by TLC (7:4:1 EtOAc–MeOH–water+0.4% HOAc), the reaction mixture was briefly neutralized with Amberlite IR-120(H^+) resin, filtered and concentrated under diminished pressure. Note that prolonged exposure to the acidic resin results in the formation of the methyl ester. The residue was purified by column chromatography (17:2:1 to 7:2:1 to 7:4:1 EtOAc–MeOH–water all include 0.4% acetic acid) and the resulting material dissolved in water, passed down an ion exchange column (Bio-Rad AG 50W-X2, 200–400 mesh, H^+ form), and freeze-dried to give **5** as a white solid (55 mg isolated, 48%): ^{1}H NMR (D_2O, 400 MHz): δ 7.55–7.45 (5H, m, Ar), 4.97 (1H, d, *J* 11.6 Hz, OCH_2Ar), 4.81 (1H, d, *J* 11.6 Hz, OCH_2Ar), 4.63 (1H, d, *J* 8.0 Hz, H-1), 4.62 (1H, d, *J* 10.4 Hz, H-1′), 4.14 (1H, d, *J* 11.1 Hz, H-5), 4.04 (1H, d, *J* 3.1 Hz, H-4′), 3.97 (1H, dd, *J* 10.4, 10.4 Hz, H-2′), 3.87 (1H, dd, *J* 11.9, 8.0 Hz, H-6′$_a$), 3.83–3.72 (3H, m, H-3′, H-5′, H-6′$_b$), 3.57 (1H, dd, *J* 10.6, 9.2 Hz, H-3), 3.47 (1H, dd, *J* 9.2, 8.0 Hz, H-2), 3.08 (1H, dd, *J* 11.1, 10.6 Hz, H-4), 2.11 (3H, s, Ac); ^{13}C NMR (D_2O, 100 MHz): δ 174.71, 172.02 (2C=O), 136.33 (C), 128.67, 101.40, 84.70, 78.97, 76.63, 74.07, 72.68 (7CH), 72.68 (CH_2), 71.69, 67.79 (2CH), 61.20 (CH_2), 51.19, 48.61 (2CH), 22.20 (Ac); MS calcd for $C_{21}H_{29}NO_{11}S$ $[M-H]^-$ 502.15, found *m*/*z* 502.14. Anal. Calcd for $C_{21}H_{29}NO_{11}S{\cdot}0.5H_2O$: C, 49.18; H, 5.90; N, 2.73. Found: C, 48.94; H, 5.75; N, 2.74.

Acknowledgements

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