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Note

Chondroitin-4-*O*-sulfatase from *Bacteroides thetaiotaomicron*: exploration of the substrate specificity

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ABSTRACT

Bacterial sulfatases can be good tools to increase the molecular diversity of glycosaminoglycan synthetic fragments. A chondroitin 4-*O*-sulfatase from the human commensal bacterium *Bacteroides thetaiotaomicron* has recently been identified and expressed. In order to use this enzyme for synthetic purposes, the minimal structure required for its activity has been determined. For that, four 4-O-sulfated monosaccharides and one 4-O-sulfated disaccharide have been synthesized and used as substrates with the sulfatase. The minimum structure was shown to be a disaccharide but in contrast to the natural substrate, which must have a 4,5-insaturation, the enzyme accepts as substrate, a disaccharide with a saturated glucuronic acid at the non-reducing end and even a glucopyranosyl moiety without the carboxylic acid functionality.

Chondroitin sulfate (CS) is a glycosaminoglycan composed of β-D-GlcA-(1–3)-β-D-GalNAc-(1–4) repeating units and found both in invertebrates and vertebrates. The position 4 or 6 of the GalNAc units is commonly sulfated, while position 2 or 3 of the GlcA units is sulfated to a minor extent. Several bacterial strains, such as *Proteus vulgaris* or *Bacteroides thetaiotaomicron* can use CS as their sole source of carbohydrate.[1,2] In this process, the polymer is first cleaved into 4′-5′-unsaturated and sulfated disaccharides by a lyase, then a series of sulfatases act sequentially to remove sulfate esters on these disaccharides. Nevertheless, if genomic analysis has revealed that these bacteria possess a large number of sulfatase coding genes,[3] their substrate specificity and selectivity are not currently understood. Interestingly, it has been demonstrated that such bacterial sulfatases may be used as tools for the structural analysis of glycosaminoglycan oligosaccharides.[1,4] Furthermore, in view of increasing the molecular diversity of synthetic fragments, chemoenzymatic diversification could benefit from the regioselectivity that some bacterial sulfatases may exhibit. Thus, the identification of new glycosaminoglycan sulfatases appears as a new opportunity to reinforce the potential of these two approaches.

In this regard, using two chondroitin disaccharide libraries as substrates[5] and a panel of cloned sulfatases, notably identified from the human commensal bacterium *Bacteroides thetaiotaomicron*,[3] we have recently described a sulfatase able to selectively remove sulfate at the 4 position of a CS *N*-acetylgalactosaminyl residue.[6] In order to better characterize the substrate specificity of this enzyme, as a prerequisite to its use in chemoenzymatic syntheses, we decided to determine the minimal structure required for its activity. We describe here the synthesis of four 4-O-sulfated monosaccharides (**1**, **7**, **13,** and **19**) and one 4-O-sulfated disaccharide (**25**) and their use to determine the minimal structure that can be accepted as substrate by the chondroitin-4-*O*-sulfatase from *Bacteroides thetaiotaomicron*.

Our first monosaccharide substrate, benzyl 4-*O*-sulfonato-β-D-galactose sodium salt **1**, was obtained following a known procedure starting from benzyl-β-D-galactopyranoside **2** (Scheme 1).[7–9]

Compound **7**, benzyl 4-*O*-sulfonato-α-D-glucose sodium salt, was prepared starting from benzyl 2,3-di-*O*-benzoyl-α-D-glucopyranoside **5** (Scheme 2).[7] Regioselective acylation of the primary hydroxyl was achieved, in 83% yield, using *Candida antartica* lipase and isopropenyl acetate.[10] Structural confirmation was achieved by ^{1}H NMR, which showed characteristic downfield shifts of the H-6, H-6′, and H-5′ protons. Sulfation using sulfurtrioxide–pyridine complex followed by transesterification with methanolic sodium methoxide gave benzyl 4-*O*-sulfonato-α-D-glucopyranoside, sodium salt **7** in 77% yield.

Benzyl 2-acetamido-2-deoxy-4-*O*-sulfonato-β-D-galactopyranoside sodium salt **13** and 2-acetamido-2-deoxy-3-*O*-methyl-4-*O*-sulfonato-β-D-galactopyranose sodium salt **19** were obtained from their *gluco* counterparts using, as key steps, the known and elegant stereospecific inversion of configuration at C-4 of D-glucosamine (Schemes 3 and 4).[11] To this aim, benzyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside **8** was first obtained by O-anomeric alkylation of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-D-glucose using NaH and allylbromide in CH_2Cl_2.[12] Zemplèn deacet-

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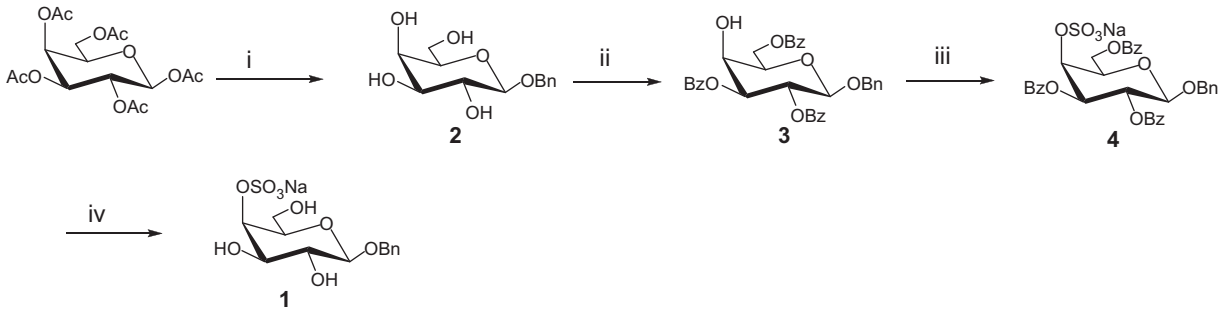
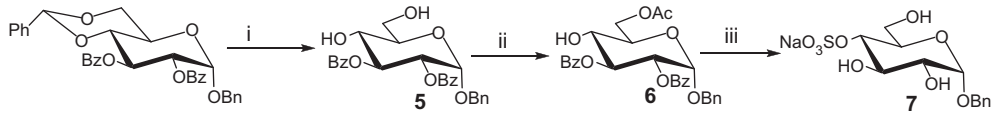
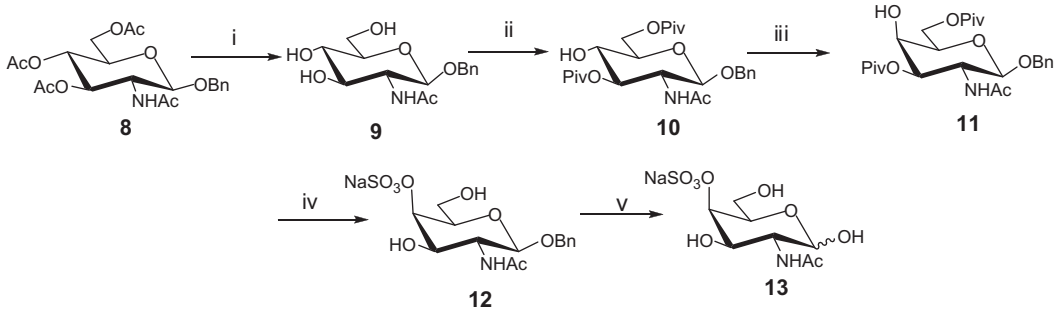


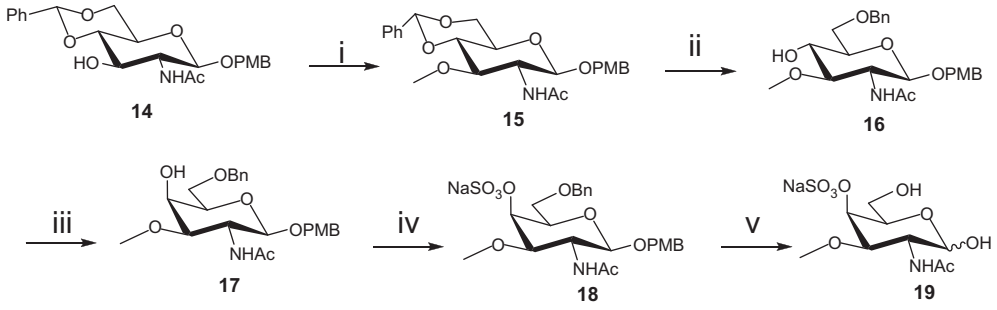
Scheme 1. Reagents and conditions: (i) BnOH (1.8 equiv) $BF_3{\cdot}Et_2O$ (1.2 equiv), CH_2Cl_2, t.a., 15 h; NaOMe (0.4 M), MeOH, t.a., 2 h, 56.5%; (ii) BzCl (4.2 equiv), pyridine, −40 °C, 2 h, then 15 h at 4 °C , 27%; (iii) $SO_3{\cdot}$pyr (7.6 equiv), pyridine, 65 °C, 30 min, 57%; (iv) MeONa (0.2 M), MeOH, 15 h, rt, 97%.



Scheme 2. Reagents and conditions: (i) AcOH 60%, 70 °C, 4 h, 68%; (ii) isopropenyl acetate (7 equiv), *Candida antartica* lipase, THF, 24 h, 40 °C, 83%; (iii) $SO_3{\cdot}$pyr (7 equiv), pyridine, 65 °C, 15 min; then MeONa, (0.2 M), MeOH, 15 h, rt, 77%.



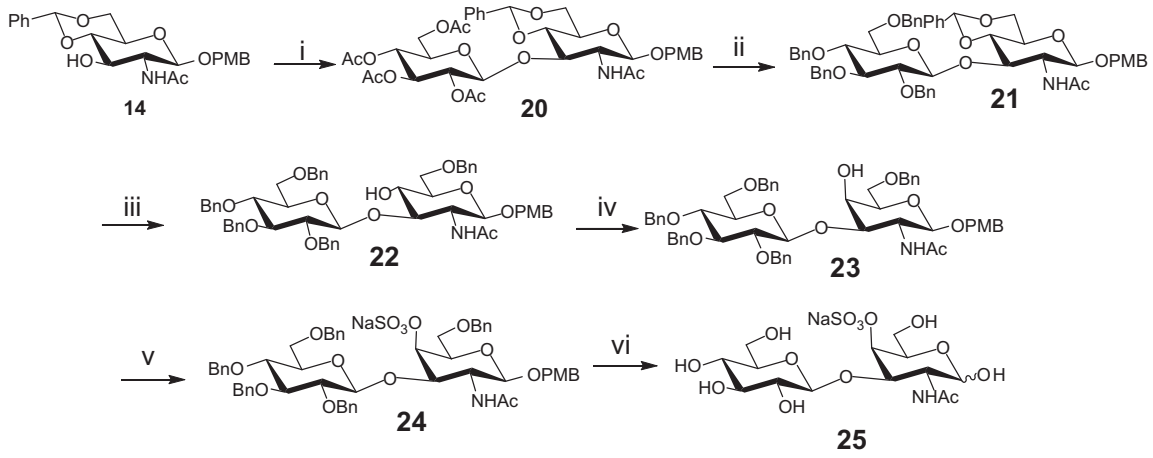
Scheme 3. Reagents and conditions: (i) MeONa (0.3 M), MeOH, 90 min, rt, 95%; (ii) PivCl (2.8 equiv), pyridine, 0 °C, 2 h, 69%; (iii) Tf_2O (1.3 equiv), pyridine/1,2 dichloroethane (1:9), −15 0°C to rt, 15 h; then NBu_4NO_2 (4 equiv), DMF, 3 h, 57%; (iv) $SO_3{\cdot}$pyridine (2.6 equiv), pyridine, 65 °C; then MeONa (0.4 M), MeOH, 16 h, rt, 76%; (v) H_2, Pd/C, EtOH–H_2O (9:1), 15 h, 95%.



Scheme 4. Reagents and conditions: (i) CH_3I (3 equiv), BaO (12 equiv), $Ba(OH)_2{\cdot}8H_20$ (1.8 equiv), DMF, 16 h, rt (64%); (ii) CH_3COOH 60%, 5 h, 50 °C; Bu_2SnO (1.1 equiv), toluene, 5 h, Dean-start; then $C_6H_5CH_2Br$ (2 equiv), Bu_4NBr (1 equiv), 36 h, 70 °C, 82%; (iii) Tf_20 (1.3 equiv), pyridine (6 equiv), 1,2 dichloroethane, −15 °C, 2 h; then NBu_4NO_2 (4 equiv), DMF, 3 h, rt (51%); (iv) $SO_3{\cdot}$pyridine (2.6 equiv), pyridine, 65 °C, 1 h, 85%; (v) H_2, Pd/C, EtOH–H_2O (4:1), 15 h, 61%.

ylation of **8** further gave triol **9**, which was then treated with pivaloyl chloride and pyridine in dichloromethane at 0 °C to give **10** in 69% yield.[11a] Inversion of the configuration at C-4 of **10** was achieved by first treatment with triflic anhydride and pyridine in dichloromethane at −15 °C, followed by nucleophilic displacement of the intermediate 4-*O*-triflate with tetrabutylammonium nitrite in DMF affording, after aqueous workup, the D-*galacto* derivative **11** in 57%[14] yield. Treatment of alcohol **11** with the sulfurtrioxide–pyridine complex in pyridine gave the 4-*O*-sulfonato derivative, which after transesterification with methanolic sodium methoxide, gave monosaccharide **12** in 76% yield.

Compounds **19** and **25** (Schemes 4 and 5) were obtained starting from the known benzylidene **14**.[13] On one hand, chemoselective 3-O-methylation of **14**, with methyl iodide and barium hydroxide in



Scheme 5. Reagents and conditions: (i) Bromo peracetate glucose (1.33 equiv), $Hg(CN)_2$ (1.33 equiv), toluenenitromethane (2:1), 16 h, 55 °C, 76%; (ii) MeOH–NEt_3–H_20 (8:1:1), 4 h, 40 °C then $PhCH_2Br$ (8 equiv), NaH (4 equiv), DMF, 3 h, 0 °C, 69%; (iii) Et_3SiH (3 equiv), TfOH (2 equiv), MS 4 Å, CH_2Cl_2, 30 min, −78 °C, 55%; (iv) (a) $(COCl_2)$ (5 equiv), DMSO (25 equiv), NEt_3 (25 equiv), CH_2Cl_2, −60 to −30 °C, (b) K Selectride (1.2 equiv), −78 to −30 °C, 70%; (v) SO_3·pyr (3 equiv), pyridine, 65 °C, 1 h 30 min, 84%; (vi) H_2, Pd/C, MeOH–phosphate buffer (2:1), 25%.

DMF, gave **15** in 64% yield. Hydrolysis of the 4,6-benzylidene acetal, with 60% acetic acid at 60 °C, followed by regioselective stannylene-promoted alkylation, allowed obtaining compound **16** in 82% yield. Inversion of the configuration at C-4 of **16** was achieved by SN_2 displacement of C-4 triflate, as described above for compound **10**, giving the D-*galacto* derivative **17** in 51% yield. Further treatment with the sulfurtrioxide–pyridine complex in DMF gave the 4-*O*-sulfonato derivative **18** in 85% yield.

Alternatively, monosaccharide **14** was glycosylated with acetobromoglucose to give **20** in 76% yield, following standard procedure.[13] Zemplèn deacetylation, followed by benzylation using NaH (1 equiv/OH) and benzyl bromide (2 equiv/OH) in DMF at 0 °C gave **21** in 69% yield, without noticeable side reaction on the *N*-acetamido moiety. Further treatment with triethylsilane and trifluoromethanesulfonic acid allowed regioselective opening of the benzylidene acetal to provide alcohol **22** in 55% yield. Swern oxidation followed by stereoselective K Selectride reduction, gave the D-*galacto* derivative **23** in 70% yield.[5] As shown previously on other disaccharides,[5] this oxidation/reduction method gave a better yield than SN_2 displacement of a *C*-4 triflate. Then, sulfation of the alcohol with the sulfurtrioxide–pyridine complex in pyridine gave the 4-*O*-sulfonato derivative **24** in 85% yield. The ^1H NMR spectrum of **12**, **18,** and **24** showed signals at relatively high field (4.6–5.1) and display small coupling constant ($J_{3,4}$ = 3.0 Hz), as awaited for 4-*O*-sulfonato-D-*galacto* derivatives.

Hydrogenolysis of benzyl group from compounds **12**, **18,** and **24** using Pd/C as catalyst, afforded monosaccharide **13**, its 3-O-methylated counterpart **19,** and disaccharide **25**, all isolated as their sodium salts.

Recently, we used capillary electrophoresis and a synthetic library of the eight sulfo forms of the CS basic disaccharide[5] to screen a library of potential new sulfatases, cloned notably from *Bacteroides thetaiotaomicron*. Interestingly, only in the case of one enzyme, we were able to detect sulfatase activity.[6] We further demonstrated that this enzyme hydrolyzes regioselectively sulfate at the 4 position of the different libraries members, establishing it as an authentic and specific chondroitin 4-*O*-sulfatase. In addition, we observed that the 4,6-disulfated CS disaccharides were desulfated five times slower than their non-sulfated counterpart in position 6. Considering that 4,6-disulfated moieties are rare in CS polymers, such result was not unexpected. Nevertheless, the sensitivity of this new sulfatase to the overall pattern of sulfation of CS disaccharides appeared to be a decisive advantage for its use in analytical or synthetic applications. In order to better characterize the substrate structural requirements of this enzyme, compounds **1**, **7**, **12**, **13**, **19,** and **25** were tested as potential substrate for this 4-*O*-sulfatase using capillary electrophoresis to follow the desulfation reaction. Interestingly, none of the benzylglycoside monosaccharides were found to be a substrate: benzyl galactopyranoside **1**, benzyl glucopyranoside **7,** and benzyl *N*-acetylgalactosaminopyranoside **12** remained fully intact upon incubation with the sulfatase. The benzyl aglycon moiety was not at the origin of the absence of activity, since compound **13**, obtained by hydrogenolysis of **12** was not a substrate either. Not surprisingly, a methyl at the position 3 of *N*-acetylgalactosamine was not sufficient to mimic a pyranosyl ring and no reaction was observed when compound **19** was incubated with the sulfatase. In contrast, the disaccharide **25**, that contains a β-D-glucopyranosyl moiety in position 3 of the galactosaminyl ring, was accepted as substrate. As a result of the sulfatase action, compound **25** was converted into the non-sulfated corresponding disaccharide as demonstrated by the capillary electropherograms (Fig. 1). Although, in this case, the reaction was slower than with original CS disaccharides (factor 15 at 2 mM), these results clearly indicated that the presence of a glucuronic acid residue at the non-reducing end is not necessary for the enzyme activity.

In conclusion, we have prepared four 4-O-sulfated monosaccharides and one 4-O-sulfated disaccharide that allowed us to better characterize the substrate specificity of a newly cloned chondroitin 4-*O*-sulfatase. We have shown that the enzyme needed a minimum structure, which should be a disaccharide. In contrast to the natural substrate, which is supposed to bear a 4,5-insaturation, this disaccharide can contain a saturated glucuronic acid at the non-reducing end or even a glucopyranosyl moiety. The presence of carboxylic acid is thus not essential to the recognition by the sulfatase. Interestingly, these results show that the enzyme from *Bacteroides thetaiotaomicron* had a substrate profile similar to the one found for the 4-*O*-sulfatase purified from *Proteus vulgaris*.[1]

1. Experimental

1.1. Enzymatic tests for chondroitin 4-*O*-sulfatase

For the libraries, 1 mL of enzyme was added to 68 mL Tris–HCl buffer 34 mM, pH 7.5 containing 6 mL of libraries solution (1 mg/mL). The hydrolysis was followed by capillary electrophoresis. For other compounds, sulfatase activity was assayed at 25 °C using

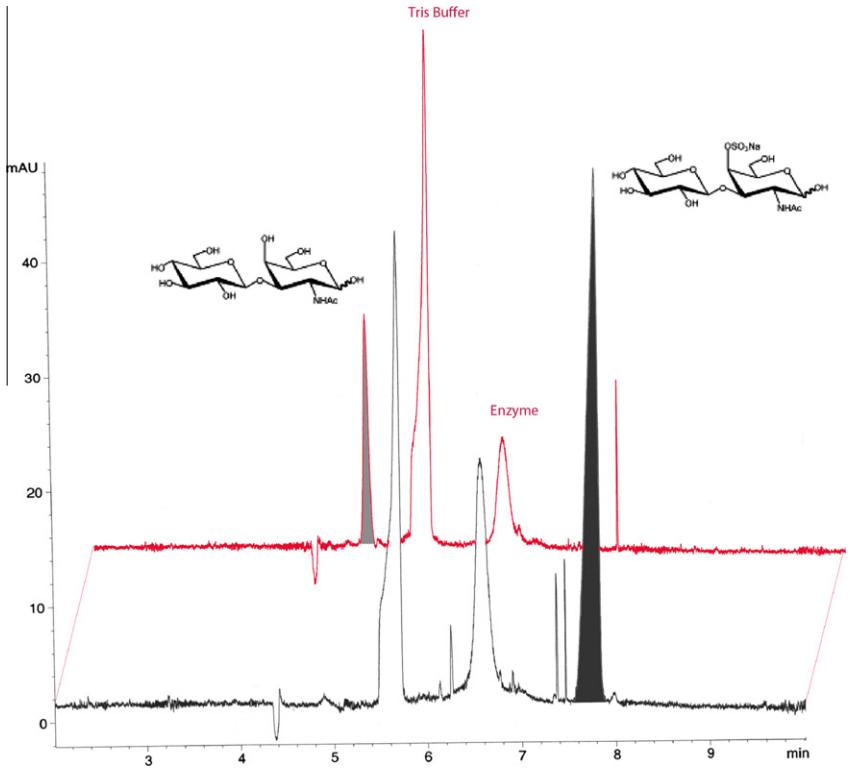


Figure 1. Capillary electropherograms of disaccharide treated with chondro-4-sulfatase. (a) Reaction at $t = 0$ after addition of B4 enzyme. (b) Reaction at $t = 24$ h after addition of B4 enzyme.

3 mM mono or disaccharide sulfate in Tris–HCl buffer 34 mM, pH 7.5.

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Supplementary data

Supplementary data (experimental details, product characterizations for compounds **1**–**25**) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carres.2012.03.033.

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