Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 7, No. 22, pp. 2843-2846, 1997

0960-894X/97 $17.00 + 0.00

PII: S0960-894X(97)10082-8

SYNTHESIS AND ANTI-THROMBIN ACTIVITY OF A HEXASACCHARIDE CORRESPONDING TO THE BINDING SITE OF DERMATAN SULFATE TO HEPARIN COFACTOR II

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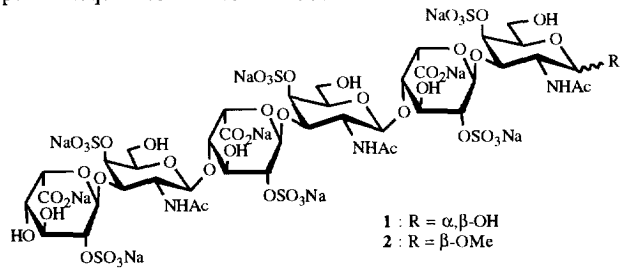
Abstract. A new synthetic route is described toward the hexasaccharide representing the heparin cofactor II binding region of dermatan sulfate. The anti-thrombin activity of this synthetic hexasaccharide is reported here for the first time. This compound is about two hundred times less active than dermatan sulfate itself for its ability to inhibit thrombin via heparin cofactor II. © 1997 Published by Elsevier Science Ltd.

The glycosaminoglycan dermatan sulfate binds to the serine protease inhibitor heparin cofactor II[1] (HC II), and considerably reinforces its thrombin inhibitory potency. The interaction is believed to occur through a specific oligosaccharide sequence. Thus, while the stucture of dermatan sulfate is mainly accounted for by the repetition of (1→4)-linked *O*-(α-L-iduronic acid)-(1→3)-4-*O*-sulfonato-β-D-*N*-acetyl galactosamine units[2], it was found that the HC II binding sequence contains several (1→4)-linked *O*-(2-*O*-sulfonato-α-L-iduronic acid)-(1→3)-4-*O*-sulfonato-β-D-*N*-acetyl galactosamine units. The hexasaccharide **1**, containing this sequence three times, has been proposed to be the HC II binding site[3]. More recently a nonasaccharide fragment of dermatan sulfate containing four such repeated sequences has been isolated[4].



1 : R = α,β-OH
2 : R = β-OMe

A chemical synthesis of **1** has been reported[5], but the corresponding biological activity was not quantitatively assessed. Two analogues of **1** have also been synthesised and shown to activate HC II[6,7]. In the present study we describe a new preparation of **1**, and compare quantitatively its activity, in an HC II dependent assay, to that of dermatan sulfate and the two analogues already mentioned.
We have chosen **2**, the methyl glycoside of **1**, as a synthesis target. Blocking the reducing end with a methyl group prevents the generation of the hemiacetal during the final deprotection steps. In this way side reactions involving this hemiacetal and the amino groups can be avoided[8].The selected route to **2** is depicted on the retrosynthetic scheme 1. The strategy was dictated by the encountered difficulty to obtain a β interglycosidic bond between a galactosamine derivative and the hydroxyl at position 4 of L-iduronic acid esters[7,9,10]. It was thus decided to use idose intermediates that were subsequentely oxidized into the corresponding L-iduronic acid and esterified. Similar strategies have previously been applied[5,7].

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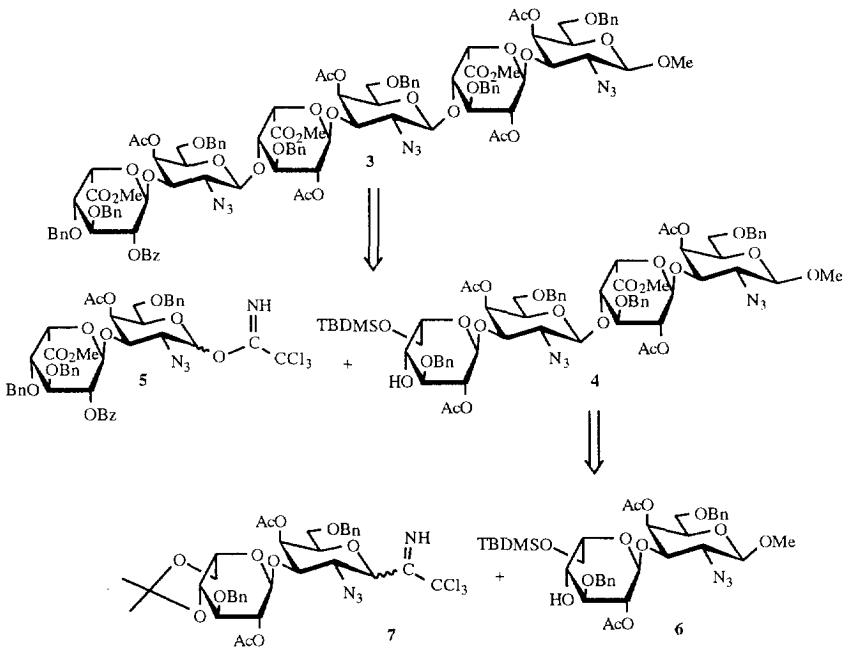
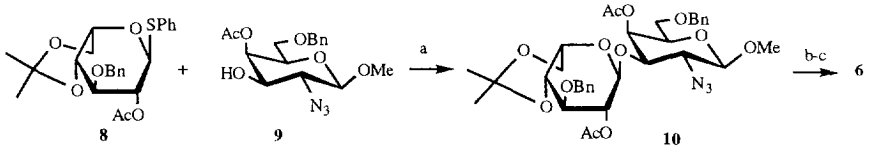


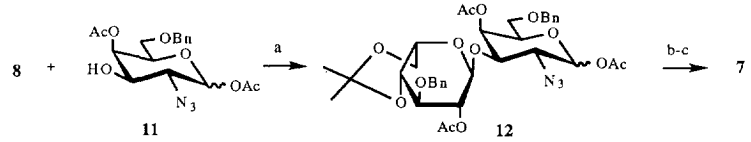
Scheme 1. Bn = benzyl; TBDMS = *tert*-butyldimethylsilyl

The reducing end disaccharide block **6** was prepared[11] as shown on scheme 2 from the known[12] thioidoside **8** which was condensed onto the known[14] alcohol **9** to give the protected disaccharide **10** in virtually quantitative yield. It was uneventfully transformed into the expected block **6** after acid hydrolysis, and selective O-silylation.



Scheme 2. a) NIS/TfOH, CH_2Cl_2, 15 min (99%); b) 80% AcOH, 80 °C, 2h (100%); c) TBDMSCl, DMAP, Et_3N, CH_2Cl_2 (96%).

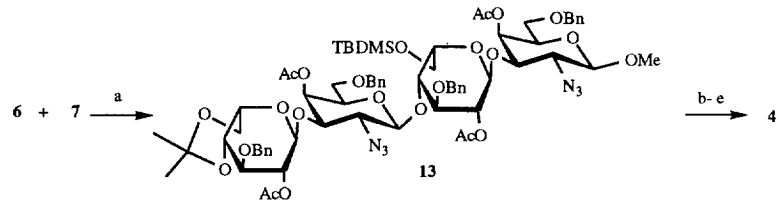
We then prepared **7** (scheme 3), the precursor of the central disaccharide unit, by high-yielding condensation of **8** onto the known[12] alcohol **11** (equimolar mixture of α and β anomers). Classical deacetylation and trichloroacetimidation[13] afforded **7**.



Scheme 3. a) NIS/TfOH, CH_2Cl_2, 15 min (90%); b) $PhCH_2NH_2$, Et_2O, 0 °C, 2 h (77%); c) CCl_3CN, DBU, CH_2Cl_2, 0 °C, 1 h (70%).

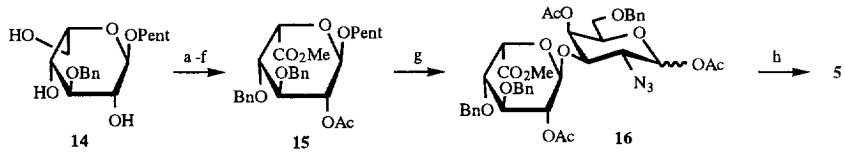
The reducing end tetrasaccharide **4** was then prepared as shown in the self explanatory scheme 4. The use of acetonitrile as a solvent led[15] to the stereoselective formation of the β-linked tetrasaccharide (51% yield), in spite of the presence of a non-participating azido group at position 2. The formation of the β anomer **13** was

confirmed by ^{1}H NMR analysis (δ 3.96 ppm, $J_{1,2}$ = 8.5 Hz). No α-linked tetrasaccharide could be detected.



Scheme 4. a) TBDMSOTf, 2:1 CH_3CN/CH_2Cl_2, -50 °C, 10 min (51%); b) Bu_4NF, THF, 0 °C, 2 h (83%); c) ClCOCOCl, DMSO, CH_2Cl_2, -78 °C, 1 h, *i*Pr_2EtN; then $NaClO_2/NaH_2PO_4$, 2-methyl-2-butene/*t*-BuOH, r.t., 16 h; then CH_2N_2, MeOH-EtOH (83%); d) 80% AcOH, 80 °C, 2h (100%); e) TBDMSCl, DMAP, Et_3N, CH_2Cl_2 (96%).

The third required disaccharide building block **5** was now prepared according to scheme 5. The pentenyl glycosyl donor **15** was first obtained from the known compound **14**[12] then condensed onto **11** to provide **16** in 76% yield. **16** was finally converted into imidates **5**.



Scheme 5. a) PhCHO, CF_3COOH, r.t., 1 h (81%); b) Ac_2O, pyridine, r.t., 2 h (96%); c) $TiCl_4/Et_3SiH$, CH_2Cl_2, - 78 °C, then MeONa (67%); d) TBDMSCl, DMAP, Et_3N, CH_2Cl_2 (87%); e) Ac_2O, pyridine, r.t., 2 h (97%); f) CrO_3/H_2SO_4, then CH_2N_2, Et_2O (67%); g) **11**, NIS/TfOH, CH_2Cl_2, 15 min (76%); h) $PhCH_2NH_2$, Et_2O, 0 °C, then CCl_3CN, DBU, CH_2Cl_2, 0 °C, 1 h (69%). Pent= $-(CH_2)_3-CH=CH_2$

Finally **5** was condensed onto **4** under the conditions used for the preparation of the tetrasaccharide **13**. The expected β-linked hexasaccharide **3** was obtained in 42% yield. After cleavage of the silyl group (nBu_4NF, 76%), oxidation of the resulting primary alcohol was performed in two steps using first Dess-Martin reagent[16] then sodium hypochlorite. Diazomethane treatment of the resulting acid provided **3** (77%).

The target hexasaccharide **2** was obtained after saponification ($LiOH/H_2O_2$; 82%), sulfation (SO_3/Et_3N complex in DMF; 82%), hydrogenation, and selective *N*-acetylation by acetic anhydride in a methanol-water mixture (75%). Careful ^{1}H NMR analysis and mass spectrometry confirmed the structure of **2**[17].

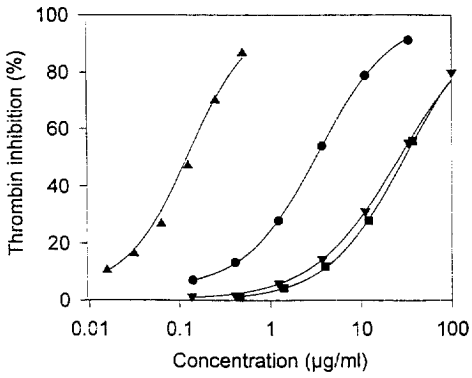


Figure 1. Determination of the IC_{50} for thrombin inhibition by HCII in the presence of dermatan sulfate (triangles pointing up; IC_{50}=0.14 µg/mL), hexasaccharide **2** (squares; IC_{50}=25 µg/mL), an analogue of **2**[7] (triangles pointing down; IC_{50}= 25.1 µg/mL), and an oversulfated analogue of **2**[6] (circles; IC_{50} = 3 µg/mL)

The biological activity of **2** was determined[18] and compared to that of dermatan sulfate, and of the corresponding compound in the non-glycosaminoglycan series[7]. Another hexasaccharide where *N*-acetyl groups have been replaced by *O*-sulfonato groups[6] was also included. The results (figure 1) indicate that **2** is about 180 times less active than dermatan sulfate. This observation is somewhat disappointing if we refer to the activation of heparin by antithrombin III where the pentasaccharide that binds to AT III triggers the same anti-factor Xa activity as the full heparin molecule[19]. This further confirms that the mechanisms involved in AT III activation by heparin and HC II activation by dermatan sulfate are very different from each other. The present data support the claim that the hexasaccharide sequence in dermatan sulfate is able to induce thrombin inhibition as proposed by Tollefsen[20].

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17. Analytical data for **2** : ^{1}H-NMR (500 MHz, D_2O, δ, ppm) : the following resonances were identified for the six units from the non-reducing end *unit 1* to the reducing end *unit 6*. ***Unit 1***: 5.16 (H-1), 4.16 (H-2), 3.96 (H-3), 3.98 (H-4), 4.79 (H-5); ***unit 2***: 4.68 (H-1, $J_{1,2}$ 8 Hz), 3.99 (H-2), 4.0 (H-3); 4.63 (H-4); 3.8 (H-5); 3.7-3.8 (H-6, H-6'); ***unit 3***: 5.12 (H-1), 4.15 (H-2), 4.15 (H-3), 4.04 (H-4), 4.74 (H-5); ***unit 4***: 4.68 (H-1, $J_{1,2}$ 8 Hz), 3.99 (H-2), 4.0-4.1 (H-3); 4.63 (H-4); 3.8 (H-5); 3.7-3.8 (H-6, H-6'); ***unit 5***: 5.12 (H-1), 4.14 (H-2), 4.17 (H-3), 4.03 (H-4), 4.79 (H-5); ***unit 6***: 4.51 (H-1, $J_{1,2}$ 8.3 Hz), 4.03 (H-2), 4.06 (H-3); 4.63 (H-4); 3.78 (H-5); 3.7-3.8 (H-6, H-6'); 2.048, 2.092, 2.096 (3s, 3NHAc).
    MS (Electrospray ionisation, negative mode) : M = 1847.96 ± 0.31 (calculated 1848.22).
18. Anti-thrombin activity in the presence of HCII : Human alpha thrombin (6 NIH/ml) was incubated for 1 min with human HCII (0.26 u/ml) at 37 °C in the presence of the compound to be tested. To measure the residual thrombin activity, a chromogenic substrate (CBS 61.50; Stago, Asnières, France: EtM-SPro-Arg-pNA, AcOH) was added (0.1 mL of a solution containing 8 µmoles of chromogenic substrate, prepared by adding 4 mL of distilled water to the content of the vial, according to the manufacturer). After 1 min, the reaction was stopped by addition of 50% acetic acid (0.1 mL) and the absorbance at 405 nm was measured. The percentage of inhibition was then calculated [inhibition % = 100 x (OD blank - OD sample)/OD blank] and plotted vs concentration. IC_{50} were determined using the 4-parameter logistic model with a confidence interval of 95%. The adjustment was obtained by non-linear regression using the Levenberg-Marquard algorithm in RS/1 software (BBN, Cambridge, USA).
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(*Received in Belgium* 8 *July* 1997; *accepted* 3 *October* 1997)