Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 1161–1166

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

SYNTHETIC OLIGOSACCHARIDES HAVING VARIOUS FUNCTIONAL DOMAINS: POTENT AND POTENTIALLY SAFE HEPARIN MIMETICS

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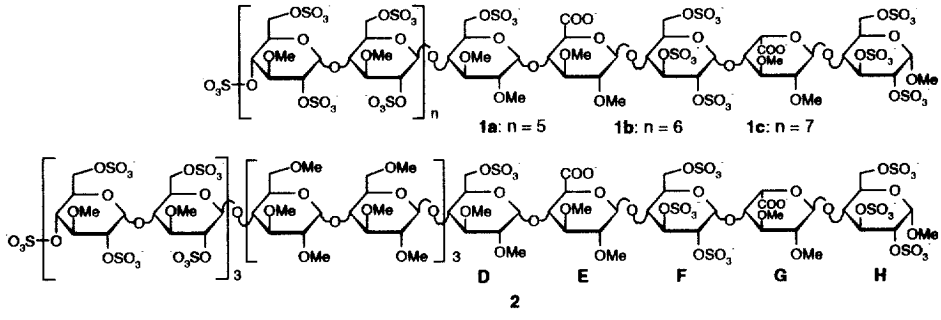
Received 25 August 1998; accepted 15 March 1999

Abstract: A synthetic heptadecasaccharide, comprising an antithrombin III binding domain, a thrombin binding domain, and a neutral methylated hexasaccharide sequence, was obtained through a convergent synthesis. This compound displayed in vitro anticoagulant properties similar to that of standard heparin but, in contrast with heparin, escaped neutralization by platelet factor 4, a protein released by activated platelets.

Heparin,[1] a complex anionic polysaccharide of animal origin, binds to antithrombin III (AT III), the main physiological inhibitor of blood coagulation, through a unique pentasaccharide sequence.[2-4] Upon binding, a conformational change occurs in AT III[5] (allosteric activation), allowing inhibition of coagulation factor Xa. In the polysaccharide chains, the pentasaccharide is prolonged, both at the reducing and the non-reducing ends, by so-called regular domains,[6] essentially made up by repeated tetrasulfated disaccharides, that may interact with the anion binding exosite II of thrombin.[7] This interaction allows efficient thrombin inhibition, by heparin-bound AT III, according to a template mechanism.[8] Like thrombin, several other proteins interact with heparin in a non-specific way. In this respect, the interaction with platelet factor 4 (PF4),[9] a basic protein released by



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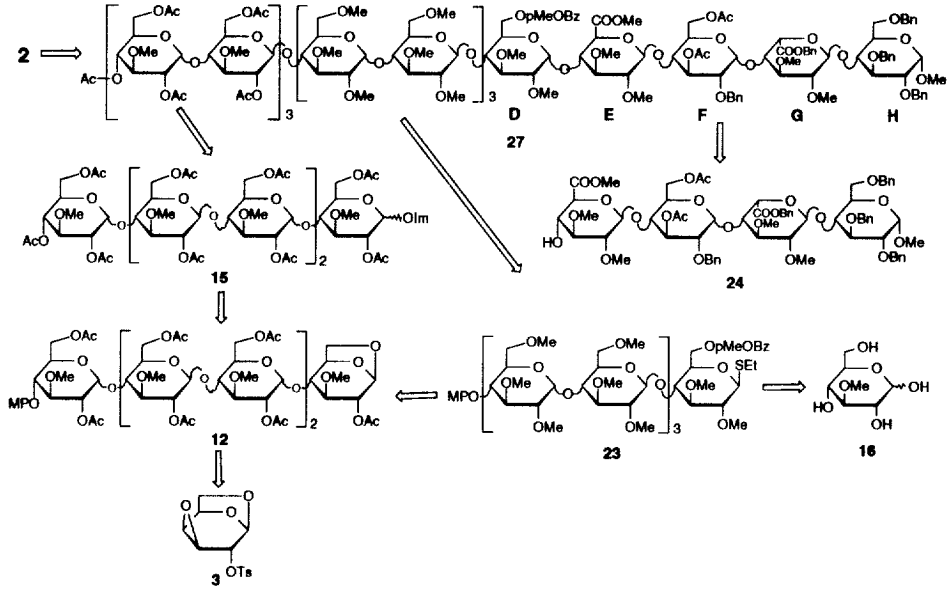
activated platelets, is at the origin of the most harmful side effect of current heparinotherapy: heparin induced thrombocytopenia which occurs in 3% of patients treated with standard heparin;[10] In contrast with the anticoagulant activity which depends on the presence of unique structural domains of the molecule, most of

0960-894X/99/$ - see front matter
PII: S0960-894X(99)00156-0

these nonspecific interactions are directly correlated with the charge density and the size of the heparin chains that may contain up to a hundred saccharide units.[6]

To obtain better tolerated drugs, we have synthesized oligosaccharide heparin mimetics displaying the AT III mediated anticoagulant activity of heparin, but the size and the charge of which have been adapted to exclusively obtain optimized anticoagulant/antithrombotic effects.[11-15] We found that the minimum number of saccharide units to be included in the chain to observe thrombin inhibition was fifteen[14,15] (**1a**) while comparison of the activity of **1a**, **1b**, and **1c** showed that this inhibition increased with the length of the chain.[15] However the anticoagulant activities of **1a-c** could be neutralized by PF4, indicating that reducing the size down to the minimum still allowing thrombin inhibition was not sufficient to abolish undesired interactions.

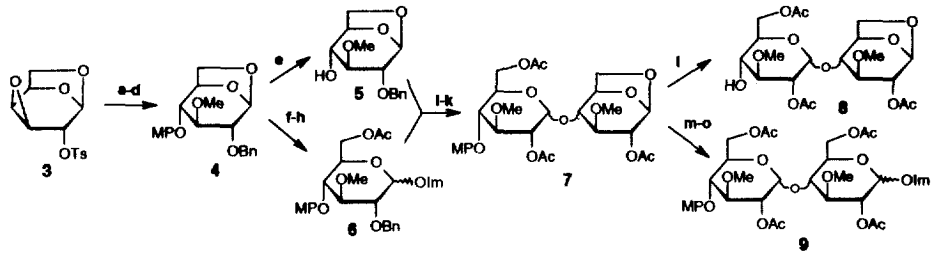
We therefore reduced the charge of the molecule, the second parameter governing nonspecific interactions of heparin. The design of our target molecules was based on the following observations: (i) a pentasaccharide sequence DEFGH, the AT III binding domain (ABD) is required to bind and activate AT III toward factor Xa inhibition; (ii) the thrombin binding domain (TBD) must be, according to the literature,[16] two to three disaccharides in length; and (iii) the required number of saccharide units being fifteen, the six central saccharide units in **1a-c** are probably not critically involved in the interaction neither with AT III, nor with thrombin, and the charges on these units can therefore be decreased or suppressed without affecting the anticoagulant activity. This analysis, which was supported by modelling studies of the ternary AT III/thrombin/heparin complex[12], led us to synthesize **2** (Scheme 1), which comprises an ABD having a very high affinity for AT III.[11,13]



Scheme 1. Retrosynthetic analysis of **2**.

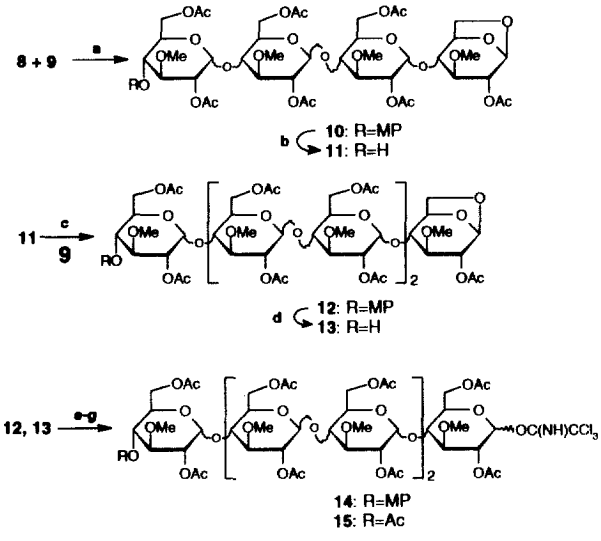
The fully protected **27** is a synthetic equivalent of **2**. It can be obtained from the tetrasaccharide **24**, available from previous work,[17] which is first coupled to the heptasaccharide **23**, followed by addition of **15**. To minimize the number of steps **15** and **23** derive from the same hexasaccharide precursor **12**, the key synthon of this synthesis. A *p*-methoxybenzoyl ester was chosen to protect the position 6 of the D unit in **23** because we observed that such an ester strongly oriented a glycosylation reaction toward the formation of the α-anomer, when a benzyl ether was present at position 2 of glucose.[18] The choice of a *p*-methoxyphenyl (MP) ether[19] to protect the position 4 of the non-reducing end unit of **12** was dictated by the many different types of reaction

conditions the temporary protective group at this position had to withstand (alkylation, acetolysis, base treatment, catalytic hydrogenation, conditions for activation of imidates and thiol functions, fluoride ions treatment). Moreover this group must be removed under conditions that do not affect acetates, benzyl ethers, and benzyl esters.



Scheme 2. (a) *p*-MeOPhOH, $AlCl_3$, 90 °C, 20 min, 74%; (b) 1 M BnONa/BnOH, 35 min; (c) then 110 °C, 1.5 h, 94% over the two steps; (d) MeI, NaH, DMF, 0 °C→RT, 97%; (e) $(NH_4)_2Ce(NO_3)_6$, CH_3CN/H_2O, 0 °C, 10 min, 94%; (f) CF_3COOH, Ac_2O, 16 h; (g) $BnNH_2$, Et_2O, 5 h, 96% over the two steps; (h) CCl_3CN, Cs_2CO_3, CH_2Cl_2, 1 h, 98%; (i) TBDMSOTf, CH_2Cl_2, 4Å MS, -20 °C, 10 min; (j) H_2, Pd/C, *t*-BuOH/ CH_2Cl_2, 16 h; (k) Ac_2O, DMAP, Et_3N, CH_2Cl_2, 1.5 h, 66% over the three steps; (l) $(NH_4)_2Ce(NO_3)_6$, DMF/H_2O, 0 °C, 4 h, 84%; (m) CF_3COOH, Ac_2O, 1.5 h; (n) $BnNH_2$, Et_2O, 2 h, 92% over the two steps; (o) CCl_3CN, K_2CO_3, CH_2Cl_2, 16 h, 87%.

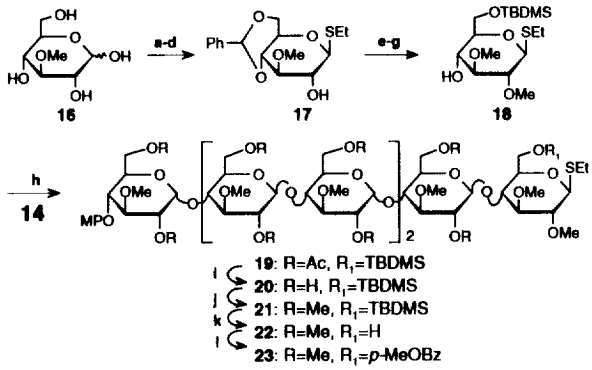
Introducing the MP group at position 4 in the *gluco* configuration was easily achieved (Scheme 2) through inversion of the configuration at position 4 of the known *galacto* epoxide **3**,[20] by treatment with *p*-methoxyphenol in the presence of aluminium chloride. Base treatment of the resulting 3-hydroxy-2-*O*-tosyl derivative, at room temperature, with sodium benzylate, gave the 1,2-*manno* epoxide. Simple heating of the reaction mixture then resulted, as expected, in *trans*-diaxial opening of the epoxide by selective attack at position 2, leading to the desired 2-*O*-benzylated derivative (94% over the two steps). Methylation then delivered **4**[21] in excellent yield. Selective deprotection of position 4, using ceric ammonium nitrate (CAN),[19,22] gave **5** while, after acetolysis of the 1,6-anhydro ring of **4**, anomeric deacetylation using benzylamine in ether[23] followed by reaction with trichloroacetonitrile in the presence of cesium carbonate[24] yielded the imidate **6**. Condensation of **5** and **6** (molar ratio 1.4/1) gave a mixture of the corresponding disaccharides (α/β 3/2), which was submitted to catalytic hydrogenation followed by acetylation, at which stage column chromatography gave pure **7** (66% from **6**). This latter was treated as described for **4** to deliver **8** and **9**, that reacted together to give the tetrasaccharide **10** (Scheme 3). After removal of the *p*-methoxyphenyl protecting group,



Scheme 3. (a) TBDMSOTf, CH_2Cl_2, 4Å MS, -20 °C, 20 min, 62%; (b) $(NH_4)_2Ce(NO_3)_6$, CH_3CN/H_2O, 0 °C, 1 h, 81%; (c) like a, 75%; (d) like b, 82%; (e) CF_3COOH, Ac_2O, 16 h; (f) $BnNH_2$, THF, 2 h, 89% over the two steps from **12**; 78% over the two steps from **13**; (g) CCl_3CN, Cs_2CO_3, CH_2Cl_2, 1 h, 82% for **14**; 88% for **15**.

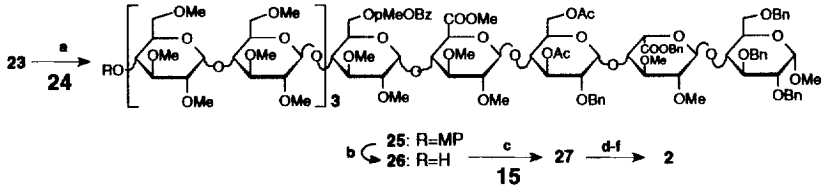
similar addition of a new disaccharide unit to **11** gave the pivotal hexasaccharide **12**.

The hexasaccharide **13** was treated like **4** to give in three steps the imidate **15**. The same sequence of reactions also converted **12** into **14**. This latter reacted with **18** (obtained as shown in Scheme 4), the precursor of the D-unit of **2**. Thank to the participating group present in **14** the reaction delivered stereospecifically **19** in excellent yield (87%). **18** was equipped with a temporary *t*-butyldimethylsilyl ether at position 6 to allow the next deacetylation and methylation steps that furnished **21**. Selective cleavage of the silyl ether by fluoride ions,[25] followed by reaction with *p*-methoxybenzoyl chloride, then provided the glycosyl donor **23**.



Scheme 4. (a) AcONa, Ac_2O, 150 °C, 1.5 h, quantitative; (b) EtSH, BF_3-Et_2O, toluene, 1 h, 74%; (c) MeONa, MeOH/CH_2Cl_2, 1 h, quantitative; (d) $PhCH(OMe)_2$, CSA, CH_3CN, 0 °C, 70 min, 59%; (e) MeI, NaH, DMF, 0 °C→RT; (f) 60% aq AcOH, 80 °C, 45 min; (g) TBDMSCl, DMAP, Et_3N, CH_2Cl_2, 5 h, 88% over the three steps; (h) TBDMSOTf, CH_2Cl_2, 4Å MS, -20 °C, 50 min, 87%; (i) 1 M aq NaOH, MeOH/CH_2Cl_2, 4 h; (j) MeI, NaH, DMF, 0 °C, 2.5 h, 86% over the two steps; (k) Bu_4N^+ F^-, THF, 65 °C, 1 h, 94%; (l) *p*-MeOBzCl, DMAP, pyridine, 60 °C, 1.5 h, 97%.

Having all the synthons in hand we then assembled **27** (Scheme 5). Reaction of the heptasaccharide **23** and **24** in the presence of *N*-iodosuccinimide and triflic acid[26,27] gave **25** in 56% yield. Cleavage of the MP protecting group gave the acceptor **26**, which reacted with the imidate **15** to provide **27**.



Scheme 5. Reagents and conditions: (a) NIS/TfOH, CH_2Cl_2/Et_2O, -45 °C, 3 h, 56%; (b) $(NH_4)_2Ce(NO_3)_6$, CH_3CN/H_2O, 0 °C, 30 min, 84%; (c) TBDMSOTf, CH_2Cl_2, 4Å MS, -20 °C, 1 h, 70%; (d) H_2, Pd/C, AcOH, 16 h; (e) 5M NaOH, MeOH, 1.5 h; (f) Et_3N:SO_3, DMF, 55 °C, 20 h, 77% over the three steps.

Finally **27** was converted into **2** using the following steps:[11,28] (i) Pd/C catalyzed hydrogenation of the benzyl groups, (ii) saponification of the esters using sodium hydroxide, and (iii) sulfation of the generated hydroxyl functions by triethylamine sulfur trioxide complex in DMF. The structure and the purity of **2** (360 mg were obtained) was confirmed by spectroscopic methods[21]. ^1H NMR analysis indicated that **2** was about 95% pure.

The in vitro biological activities of **1b**, **2**, and heparin are shown in Table 1. The three compounds displayed similar anticoagulant potencies. In vivo, in various animal models of venous and arterial thrombosis, **2** was five to ten times more potent than standard heparin (ED_{50} 15-66 μg/kg vs. 77-700 μg/kg). Noteworthy, while PF4 was able to fully neutralize thrombin inhibition by **1b** and heparin, the activity of **2** was not affected by the presence of this protein, even when added at very large concentration (100 μg/mL). These results demonstrate that synthetic substitutes for heparin, endowed with the full anticoagulant activity but potentially

devoid of harmful interactions with platelets and PF4, can be obtained by chemical synthesis of oligosaccharides having functional domains separated by neutral oligosaccharide sequences.

Table 1. Biological properties of **1b**, **2**, and heparin. Affinity for AT III,[29] factor Xa inhibition,[30] and thrombin inhibition[31] were determined using published procedures.

Compound N°	**1b**	**2**	**heparin**
Number of saccharide units	17	17	≈ 10-50
Molecular weight	6378	5322	≈ 15000
Affinity for AT III (Kd, nM ± SD, n = 3)	3.3 ± 0.8	7.0 ± 1.5	25 ± 0.2
Factor Xa inhibition (units/mg ± SD, n = 3)	270 ± 8	270 ± 8	180
Thrombin inhibition (IC50, ng/mL, 95% confidence interval)	5.3 (5-5.4)	9.3 (9.0-9.6)	3.3 (3-4)

Acknowledgements: This work is part of a collaborative project between N. V. Organon (The Netherlands) and Sanofi Recherche (France) on antithrombotic oligosaccharides.

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21. All new compounds were analysed by ^{1}H NMR, mass spectrometry and occasionally by HPLC. Combustion analyses were systematically performed on monosaccharides and disaccharides only. Selected analytical data: **4**: mp 81 °C (from EtOAc-cyclohexane); $[\alpha]_D$ -29 (*c* 1, CH_2Cl_2). **5**: $[\alpha]_D$ -62° (*c* 1.3, CH_2Cl_2). **6**: ^{1}H NMR δ 6.45 (d, $J_{1,2}$=3.6 Hz, H-1α), 5.85 (d, $J_{1,2}$=8.0 Hz, H-1β). **7**: mp 107 °C (from diethylether). **8**: mp 133 °C (from EtOAc-cyclohexane). **9**: ^{1}H NMR δ 6.47 (d, $J_{1,2}$=3.7 Hz, H-1α), 5.50 (d, $J_{1,2}$=4.0 Hz, H-1'α), 5.84 (d, $J_{1,2}$=6.9 Hz, H-1'β), 5.44 (d, $J_{1,2}$=4.4 Hz, H-1β). **10** : $[\alpha]_D$ +88° (*c* 1.4, CH_2Cl_2). **17**: mp 134 °C (from diethylether); $[\alpha]_D$ -60° (*c* 1.46, CH_2Cl_2). **18**: $[\alpha]_D$ -44° (*c* 1.33, CH_2Cl_2). For longer oligosaccharides ^{1}H NMR data were collected at 500 MHz in D_2O (external TSP), δ for anomeric protons and $J_{1,2}$ are reported (detailed data are available on request). Mass Spectrometry data (ESI MS) were collected using Electron Spray Ionisation in the negative mode, monoisotopic mass/average mass/experimental mass are given. Compound **2** : $[\alpha]_D$ +51 (*c* 0.83, water). ESI MS, 5318.09/5322.11/5319.14 ± 0.99 a.m.u.. ^{1}H NMR from nonreducing-end (unit 1) to reducing-end (unit 17): unit 1, 5.70 ($J_{1,2}$=3.5 Hz); unit 2, 4.79 ($J_{1,2}$=7.9 Hz); unit 3, 5.47 ($J_{1,2}$=3.5 Hz); unit 4, 4.77 ($J_{1,2}$=7.9 Hz); unit 5, 5.46 ($J_{1,2}$=3.5 Hz); unit 6, 4.55 ($J_{1,2}$=7.9 Hz); unit 7, 5.59 ($J_{1,2}$=3.3 Hz); unit 8, 4.43 ($J_{1,2}$=7.7 Hz); unit 9, 5.64 ($J_{1,2}$=3.3 Hz); unit 10, 4.43 ($J_{1,2}$=7.7 Hz); unit 11, 5.64 ($J_{1,2}$=3.3 Hz); unit 12, 4.63 ($J_{1,2}$=7.7 Hz); unit 13, 5.45 ($J_{1,2}$=3.5 Hz); unit 14, 4.67 ($J_{1,2}$=7.7 Hz); unit 15, 5.42 ($J_{1,2}$=3.5 Hz); unit 16, 5.07 ($J_{1,2}$=3.1 Hz); unit 17, 5.16 ($J_{1,2}$=3.5 Hz).
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