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SYNTHESIS OF A 6-O-PHOSPHORYLATED ANALOGUE OF THE ANTITHROMBIN III BINDING SEQUENCE OF HEPARIN: REPLACEMENT OF ONE ESSENTIAL SULPHATE GROUP BY A PHOSPHATE GROUP NULLIFIES THE BIOLOGICAL ACTIVITY.

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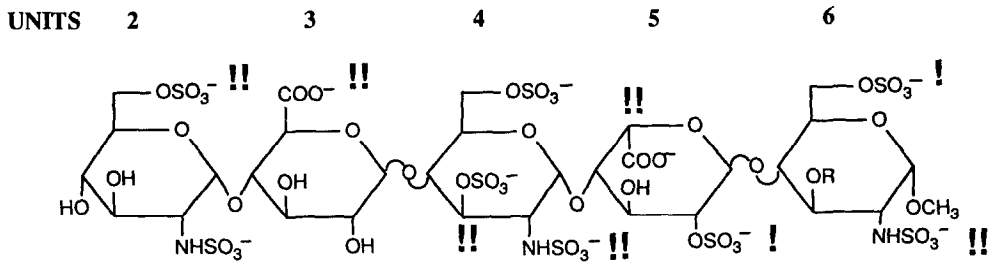
Abstract. *The synthesis of an analogue (i.e. compound* **7***) of the AT-III binding sequence of heparin is described in which an essential 6-O-SO_3 group at the non-reducing glucosamine is replaced by a phosphate group. This compound appears to be biologically inactive.*

The anticoagulant drug heparin is composed of sulphated glycosaminoglycans, part of which contains a well-defined pentasaccharide domain that activates the protease-inhibitor antithrombin III (AT-III)[1]. Through organic synthesis of this pentasaccharide (i.e. compound **Ia**) as well as analogues, and by pharmacological evaluation of these compounds it has been established which charged groups of the pentasaccharide molecule are essential to activate AT-III[2].

Some of the charged groups are strictly required for the activation of AT-III (!! in compound **Ia**, figure 1), whereas other groups contribute significantly (! in compound **Ia**) in the activation process.

On the basis of these structure-activity relationships we proposed a binding model of the pentasaccharide with AT-III that inspired us to synthesize more potent analogues containing an extra 3-O-sulphate group at the reducing glucosamine (unit 6)[3]. In this series compound **Ib** (figure 1) is the most potent analogue found thus far. Introduction of extra sulphate groups at unit 2 leads to analogues with similar or slightly reduced biological activity, whereas introduction of an extra 3-O-SO_3 group at glucuronic acid (unit 3) is accompanied by a serious decrease in biological activity[4]. It is inferred that the negative charges on the heparin fragment should be precisely positioned in space in order to interact optimally with complementary residues on AT-III[5].



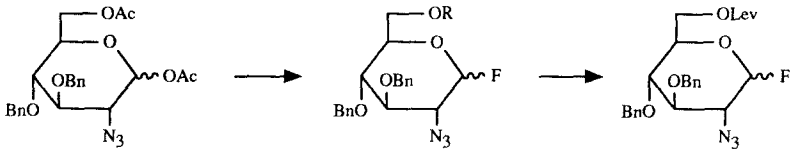
I a : R=H
I b : R=SO_3^-

Fig 1

Next, we were anxious to find out how the replacement of one of the essential sulphate groups by a more highly charged phosphate group would effect the biological activity of the heparin fragment. To this end we synthesized analogue **7** containing a 6-O-phosphate group at unit 2 instead of the essential 6-O-sulphate group.

The synthesis of analogue **7** is schematically illustrated in Scheme II. The protective group strategy is similar to that applied for the synthesis of the pentasaccharides **Ia,b**[2]. Thus, we selected acetyl-protecting groups for the hydroxyl functions to be sulphated, benzyl groups for the free hydroxyl groups and azido groups for masking the amino functions. In addition, we put one temporary levulinoyl ester[6] at 6-OH of monosaccharide **3**, which at the level of the fully protected pentasaccharide **5a** could be selectively deblocked to allow the required 6-O-phosporylation of unit 2.

Building block **3** was synthesized as outlined in Scheme I. Thus, the known building block **1** was treated with 70% hydrogen fluoride pyridine in dichloromethane at 0°C to give after chromatography the fluoridated derivative **2a** in 62% yield. The presence of the fluoride at the anomeric centre of compound **2a** is of special advantage since on one hand the fluoride donor may be coupled in the presence of Lewis acids while on the other hand a deacylation - reacylation procedure can be performed. Saponification of the 6-acetate of **2a** with a catalytic amount of sodium methoxide in methanol afforded **2b** in a quantitative yield. Levulinoylation[6] of **2b** afforded the required building block **3** in 93% yield (^{1}H-NMR ($CDCl_3$), δ 5.63 ppm (dd, J = 53Hz, J = 3Hz; H1)).



1 2a R = Ac 2b R = H 3

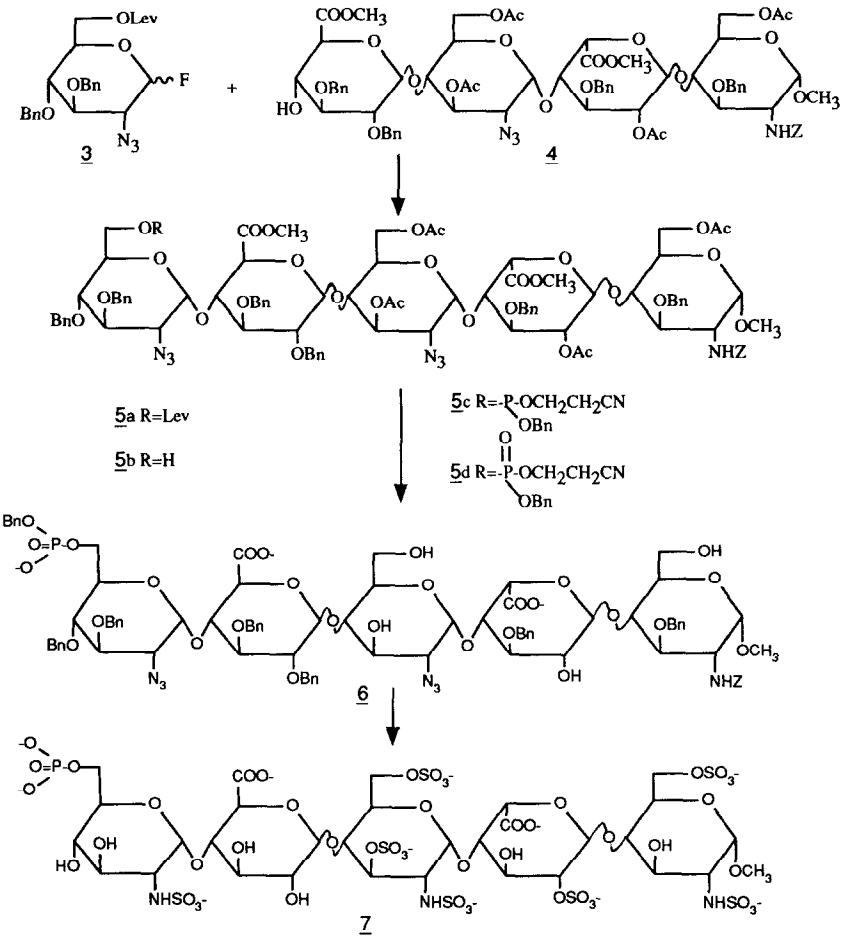
Scheme I

Compound **3** was then coupled with known tetrasaccharide **4** in the presence of borontrifluoride etherate to give after chromatography 60% of the required α-coupled pentasaccharide **5a** along with 11% of the undesired β-analogue. Controlled hydrazinolysis[6] of compound **5a** gave the key intermediate **5b** (60% yield), which can be phosphorylated at the 6-hydroxyl group of the non-reducing glucosamine unit.

Phosphorylation of **5b** was performed with the phosphorylating reagent, benzyl-(2-cyanoethyl)-N,N-diethyl phosphoramidite[8] in the presence of 1H tetrazole in dichloromethane to give after 15 minutes the intermediate phosphite (**5c**). Then in situ oxidation of **5c** with t-butylhydroperoxide afforded the phosphotriester derivative **5d** in 77% yield.

The fully protected pentasaccharide was then treated with sodium hydroxide (4M) to remove simultaneously the acetyl esters, the carboxylic acid methyl ester as well as the cyanoethyl group. ^{1}H-NMR and ^{31}P-NMR analysis revealed that no concomitant cleavage of the phosphate group had occurred. Then, compound **6** was O-sulphated, hydrogenolyzed and selectively N-sulphated under conditions described earlier[2,3,7].

Finally the required analogue was purified by Sephadex -DEAE ion - exchange chromatography and desalted on Sephadex G25 to give compound **7** in 27% overall yield from **5b**.



Scheme II

Analogue **7** thus obtained was then submitted for testing. To our great surprise compound **7** did not display significant AT-III mediated αXa activity in an amidolytic assay. In contrast, we expected that replacement of an essential sulphate group by a more negatively charged phosphate group would enhance the interaction with the positively charged heparin binding domain of AT-III[5].

At first we suspected that the compound obtained might be structurally different from compound **7**. However, thorough analysis[9] of compound **7**, including ^{31}P-NMR and ^{1}H-NMR spectroscopy, HPLC-analysis on ion-exchange columns (Mono Q)[10], and FAB-mass spectrometry unambiguously confirmed the structure and identity of compound **7**. Therefore it can be concluded that the biological activity of the heparin pentasaccharide **Ia** disappears when the essential 6-O-sulphate group at unit 2 is replaced by a phosphate group.

There is no obvious explanation for this clear difference in molecular recognition between phosphate and sulphate, although several examples are described in literature demonstrating that particular proteins may distinguish between phosphate and sulphate. The specificity of these proteins towards either phosphate or sulphate is conferred through differences in the networks of hydrogen bonds and salt bridges which are formed between the substrate and the protein[11].

In addition, it may be argued that phosphorylated analogues may bind less tightly with sulphate binding proteins, because the required dehydration energy of phosphate outweighs the increase in electrostatic interaction(s) between the protein and the charged carbohydrate. Apparently, phosphate and sulphate substituents on biomolecules are deliberately selected by nature for their distinct, specific properties. As far as we know, no biologically active molecules have been described in which functional sulphate groups may be interchanged by phosphate groups or vice versa. In this respect it is noteworthy that we recently found that the tris-sulphated analogue of the well-known second messenger D-myo-inositol-1,4,5-trisphosphate (IP_3) is also biologically inactive[12a,b].

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9. Compound 7: ^1H NMR (360 MHz, D_2O): H1(2):(5.61 ppm, J = 3.8 Hz); H1(4):(5.53 ppm; J = 3.4 Hz); H1(5):(5.18 ppm J = 3.8 Hz); H1(6):(5.02 ppm, J = 3.6 Hz); H1(3):(4.62 ppm, J = 8.0 Hz).
   13C NMR (90.56 MHz): C1(3):(101.4 ppm); C1(5):(99.9 ppm); C1(6):(98.5 ppm); C1(2):(97.9 ppm); C1(4);(96.2 ppm).
   31P NMR (145 MHz): 5.0 ppm.
   FAB MS, Both Fab (+) and Fab(-) (thioglycerol as the matrix) showed a molecular mass peak of 1705, which corresponds to the proposed structure with 9Na and 2H.
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