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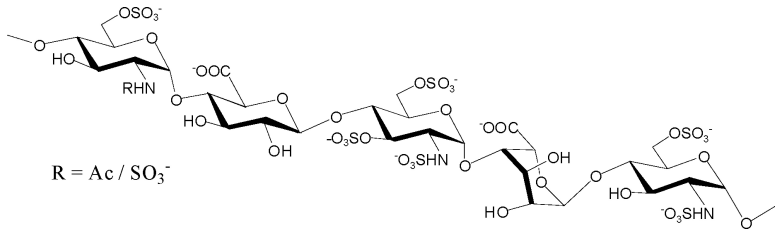

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Rapid Two-Step Synthesis of Mitrin from Heparosan: A Replacement for Heparin

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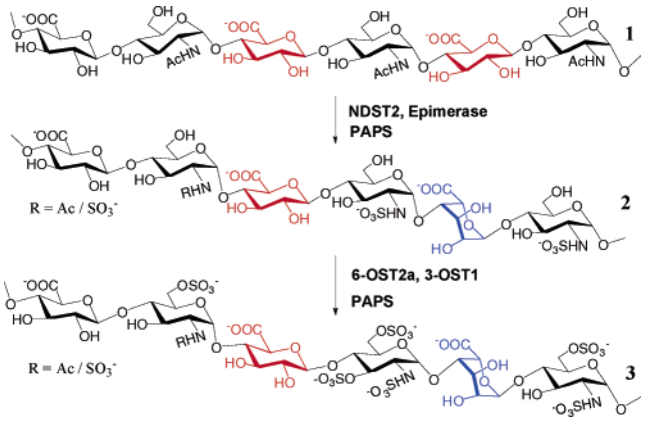
Heparin, a strongly acidic, linear sulfated polysaccharide, is used in the prevention and treatment of thrombosis. Heparin was first isolated from the liver from which it derives its name.[1] Heparin-like polysaccharides are shown to interact with numerous proteins and orchestrate many different biological functions.[2] A unique pentasaccharide domain present within heparin was found to bind to Antithrombin III (ATIII) in a highly specific manner to induce a conformational change that is sufficient to promote rapid inhibition of blood coagulation.[3,4] Sinay and co-workers pioneered the original chemical synthesis of the ATIII binding pentasaccharide and analogues.[5,6]

Heparin-induced thrombocytopenia (HIT) is an immunological disorder associated with heparin treatment.[7] HIT paradoxically increases thrombosis, which occurs in about 30% of the recognized HIT cases, and is a major cause of morbidity and mortality in patients treated with heparin. It has been shown that HIT is induced by antibodies against PF4-heparin complex. The complex formation requires a 2-O sulfated iduronic acid residue.[8] Engineering new heparin that is unable to form heparin-PF4 complexes would be a major advance in anticoagulation therapy. There is also an increased concern for the potential spread of diseases of animal origin to humans, such as bovine encephalopathy, due to the use of animal-derived heparin. The above-mentioned potential side effects of animal-derived heparin prompted the chemical synthesis of heparin-based anticoagulants. Despite many advances made in chemical synthesis, this approach is cumbersome and time-consuming. The limitations of chemical approaches prompted us to undertake a rapid approach to synthesize a heparin-like anticoagulant with improved therapeutic characteristics using cloned enzymes in a relatively simple manner.

We termed this engineered anticoagulant polysaccharide as "Mitrin" because of its origin at MIT in analogy to heparin whose origin is hepatic.[1] A nonsulfated *N*-acetyl heparosan polysaccharide **1** was isolated with an average molecular weight of 7000 Da from *E. coli* strain K5.[9] Polysaccharide **1** resembles the unmodified nascent heparan sulfate (HS) chain. It is used as a starting material in the enzymatic synthesis of Mitrin. The first step is the synthesis of N-sulfated polysaccharide **2** enriched with iduronic acid catalyzed by *N*-deacetylase-*N*-sulfotransferase (NDST) and C-5 epimerase [Scheme 1]. These two initial modifications are the essential gateway for subsequent enzymatic modifications.[10] A single protein catalyzes both N-deacetylation and N-sulfation. These two reactions are tightly coupled in vivo, because free glucosamine residues are rarely found in HS and heparin, even though each activity can be studied separately in vitro. This enzyme exists as four isoforms in humans.[11] We utilized the NDST2 isoform to selectively N-deacetylate and N-sulfate glucosamine units.[12] This step was carried out in conjunction with HS C-5 epimerase[13,14] to generate the

Scheme 1. Enzymatic Synthesis of Mitrin



iduronic acid-enriched polysaccharide **2**. The stereochemical nature at the C-5 carbon of uronic acid is reversed in this transformation (blue residues, Scheme 1). Epimerization proceeds only when these residues are immediately adjacent to the reducing side of the glycosidic bond of the N-sulfated glucosamine residues, but it will not react with uronic acids that are O-sulfated or that are adjacent to O-sulfated glucosamine residues or adjacent to the reducing side of the glycosidic bond of the *N*-acetylglucosamine units.[10,14] This suggests that epimerization occurs immediately after N-deacetylation and N-sulfation but before O-sulfation. For this reason, our synthetic strategy was devised to couple NDST2 and C5 epimerase to prepare in a single step predominately N-sulfated polysaccharide **2** containing a small percentage of unmodified GlcNAc residues in addition to both unmodified glucuronic and newly generated iduronic acid residues without 2-O sulfation (see Supporting Information).

The second and final step in the synthesis of anticoagulant Mitrin polysaccharide **3** was catalyzed by 6-O sulfotransferase (6-OST) and 3-O sulfotransferase (3-OST) [Scheme 1]. There are three heparan sulfate 6-O sulfotransferase isoforms: 6-OST1, 6-OST2 (6-OST2a and 6-OST2b are two splice variants), and 6-OST3.[15] It is known that all three isoforms sulfate CDSNS-heparin equally well.[15] However, *N*-sulfo-heparosan was shown to be preferentially sulfated by these isoforms in the following order: 6-OST2 > 6-OST3 ≫ 6-OST1. Thus, in essence, we have utilized the 6-OST2a isoform to catalyze the 6-O sulfation of glucosamine units. The 6-O sulfation was coupled with 3-O sulfation, which is catalyzed by 3-OST1 sulfotransferase to generate anticoagulant Mitrin.[16] There are as many as five isoforms of heparan sulfate 3-O sulfotransferases, 3-OST1, 3-OST2, 3-OST3, 3-OST4, and 3-OST5.[17,18] It was demonstrated earlier that 3-OST1 is primarily involved in generating anticoagulant heparan.[19] It was also shown

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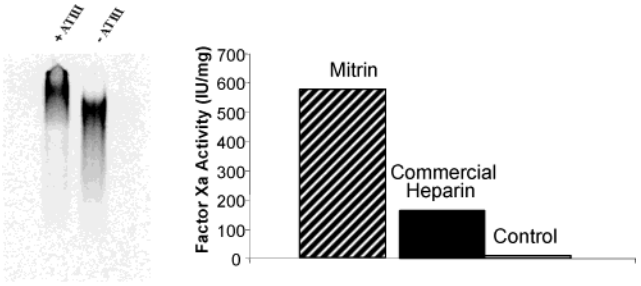

Figure 1. (Left) Gel shift analysis of polysaccharide **3**. PAP^{35}S-radiolabeled Mitrin (10 000 counts) was reacted with 5 μg of ATIII. Complex formation was analyzed by nondenaturing gel electrophoresis (4% polyacrylamide). The mobility of radiolabeled Mitrin was compared with and without ATIII. (Right) Biological activity of Mitrin. Human factor Xa was incubated with antithrombin III in the presence of Mitrin (polysaccharide **3**) or commercial heparin or polysaccharide **2** as a negative control. The percentage of inhibition of thrombin activity was calculated from three experiments performed in triplicate.

that 3-OST1 generally acts on glucosamine units flanked by the reducing side of GlcA and the nonreducing side of IdoA2S to generate ATIII binding structures containing GlcA-GlcNS3S and GlcA-GlcNS3S6S.[19−21] Because 6-O sulfation and 3-O sulfation are not coupled to generate anticoagulant structures in vivo, it was anticipated that coupling both modifications in vitro would dramatically shorten the time required for total synthesis of Mitrin. Mitrin was successfully prepared from polysaccharide **2** using 3-OST1 and 6-OST2a sulfotransferases. The final step was also carried out in the presence of radioactive PAP^{35}S to prepare the radiolabeled Mitrin to test its ability to bind to ATIII by gel mobility shift assay.[22] The synthesized Mitrin polysaccharide **3** was found to bind to ATIII. In the presence of ATIII, Mitrin binds specifically to ATIII and hence its mobility is retarded, whereas in the absence of ATIII, Mitrin migrates more rapidly [Figure 1, left]. A greater percentage of Mitrin binds to ATIII as compared to in vitro modified commercial heparin. This result is confirmed by a heparin-dependent factor Xa inhibition assay [Figure 1, right]. The specific activity of Mitrin is approximately 4−5 times that of commercial heparin. Finally, Mitrin was cleaved by heparitinases I, II, and III for structural analysis by capillary liquid chromatography coupled to electrospray mass spectrometry (LC/MS).[23] The LC/MS analysis showed one major trisulfated disaccharide containing a 3-O sulfated glucosamine unit, ΔU-GlcNS3S6S, corresponding to molecular ion 576.0 $[M - 1H]^{-1}$ and two other minor disaccharides, ΔU-GlcNS and ΔU-GlcNS6S, corresponding to molecular ion 416.1 and 496.1 $[M - 1H]^{-1}$, respectively. It was shown earlier by us that the absence of 2-O sulfation could increase the number of 3-O sulfation sites within the polymer which may account for the presence of 3-O sulfate containing trisulfated disaccharide as a major component.[21] The LC/MS analysis also confirmed the presence of many tetrasaccharides, which are resistant to any further cleavage by heparitinases, due to the presence of 3-O sulfate groups. These 3-O sulfated tetrasaccharides are ΔU-GlcNAc6S-GlcA-GlcNS3S6S with molecular ion 517.0 $[M - 2H]^{-2}$; ΔU-GlcNAc6S-GlcA-GlcNS3S with molecular ion 477.1 $[M - 2H]^{-2}$; and ΔU-GlcNS6S-GlcA-GlcNS3S6S with molecular ion 536.0 $[M - 2H]^{-2}$. This result demonstrates that Mitrin consists of multiple ATIII binding sites within the polymer and indicates why Mitrin has a greater ability to inhibit factor Xa. Because Mitrin is free of 2-O sulfated iduronic acid residues, we expect that it will have a reduced ability to bind to PF4 which should decrease its ability to cause HIT and at the same time increase its anticoagulant activity against the platelet-rich thrombi present on the arterial side of the circulation. It seems likely that Mitrin will be relatively more resistant to be cleaved by endogenous heparanases due to its lack of 2-O sulfated iduronic acid and hence it could exhibit a longer in vivo half-life.[24] Furthermore, this engineered polysaccharide would be free of animal-derived pathogens. The molecular weight of Mitrin can be tailored at any stage during this two-step synthesis by standard chemical or enzymatic cleavage techniques which have been utilized in similar fashion to produce low molecular weight heparin. Engineered Mitrin with the desired pharmacological properties is predicted to emerge as a replacement drug for the animal-derived low molecular weight heparins that are currently in use. We acknowledge that use of the regenerating PAPS system would substantially reduce the cost of producing Mitrin. This enzymatic approach would also permit the generation of virtually any structure of this class of molecules to be used as a probe in uncovering its functions in many biological systems.

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Supporting Information Available: Experimental details, mass spectrometric analysis, cloning and expression of enzymes (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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