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Chemoenzymatic Synthesis of Homogeneous Ultralow Molecular Weight Heparins

Yongmei Xu, Sayaka Masuko, Majde Takieddin, Haoming Xu, Renpeng Liu, Juliana Jing, Shaker A. Mousa, Robert J. Linhardt, Jian Liu

Ultralow molecular weight (ULMW) heparins are sulfated glycans that are clinically used to treat thrombotic disorders. ULMW heparins range from 1500 to 3000 daltons, corresponding from 5 to 10 saccharide units. The commercial drug Arixtra (fondaparinux sodium) is a structurally homogeneous ULMW heparin pentasaccharide constructed through a lengthy chemical process. Here, we report 10- and 12-step chemoenzymatic syntheses of two structurally homogeneous ULMW heparins (MW = 1778.5 and 1816.5) in 45 and 37% overall yield, respectively, starting from a simple disaccharide. These ULMW heparins display excellent in vitro anticoagulant activity and comparable pharmacokinetic properties to Arixtra, as demonstrated in vivo. The commercial drug Arixtra (fondaparinux sodium) is a structurally simple disaccharide-repeating unit of either iduronic acid (IdoA) or glucuronic acid (GlcA) and glucosamine (GlcN) residues, each capable of carrying sulfo groups. The locations of the sulfo groups and IdoA residues are crucial for heparin’s anticoagulant activity. The chemical synthesis of Arixtra, the most successful example to date for preparing a synthetic heparin (10), entails ~50 steps with an overall yield of ~0.1%; as such, Arixtra is the most expensive drug among heparins (12). Efforts to improve the synthesis of Arixtra with a purely chemical approach have achieved only limited success (13).

A chemoenzymatic approach, relying on a series of heparan sulfate (HS) biosynthetic enzymes, mimics the biosynthesis of heparin and HS (fig. S1) (14). Heparin and HS have similar disaccharide-repeating units of either iduronic acid (IdoA) or glucuronic acid (GlcA) and glucosamine (GlcN) residues, each capable of carrying sulfo groups. The locations of the sulfo groups and IdoA residues are crucial for heparin’s anticoagulant activity. The chemical synthesis of Arixtra, the most successful example to date for preparing a synthetic heparin (10), entails ~50 steps with an overall yield of ~0.1%; as such, Arixtra is the most expensive drug among heparins (12). Efforts to improve the synthesis of Arixtra with a purely chemical approach have achieved only limited success (13).

Heparin has been used as an anticoagulant drug for more than 50 years (1). It is currently marketed in three forms: unfractionated (UF) heparin (average molecular weight [MWavg] ~14000); low molecular weight (LMW) heparin (MWavg ~6000); and the synthetic ultralow molecular weight (ULMW) heparin pentasaccharide Arixtra (GlaxoSmithKline) (MW 1508.3). UF heparin is used in surgery and kidney dialysis due to its relatively short half-life and its safety for renal-impaired patients (2). LMW heparins and Arixtra, introduced over a decade ago, have played an increasingly important role in preventing venous thrombosis among high-risk patients (3, 4) because of their more predictable anticoagulant activity. The chemical synthesis of Arixtra, a second-generation LMW heparin pentasaccharide, the most successful example to date for preparing a synthetic heparin (10), entails ~50 steps with an overall yield of ~0.1%; as such, Arixtra is the most expensive drug among heparins (12). Efforts to improve the synthesis of Arixtra with a purely chemical approach have achieved only limited success (13).

Fig. 1. Chemoenzymatic synthetic schemes of ULMW heparin construct 1 and 2. The synthesis started from disaccharide 3, and it was then elongated to tetrasaccharide 4. Eight additional steps transformed 4 to construct 1 (left column). Steps d through h were combined in sequential one-pot reaction format. Ten additional steps transformed 4 to construct 2 (right column). The recovery yield at each purification step was determined by parallel synthesis of the corresponding radioactively labeled oligosaccharide. KfIA, N-acetyl glucosaminyl transferase of E. coli K5 strain; pmHS2, heparan synthase-2 of Pasteurella multocida; NST, N-sulfotransferase; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; C5-epi, C5-epimerase; 2-OST, 2-O-sulfotransferase; 6-OST, 6-O-sulfotransferase; 3-OST-1, 3-O-sulfotransferase isofrom 1. More synthesis details are given in table S3.
charide repeating units; however, heparin carries more sulfo groups and a higher level of IdoA residues and possesses the strongest anticoagulant activity among this class of polysaccharide isolated from natural sources (15). HS polymerases synthesize the backbone with a disaccharide repeating unit of GlcA and N-acetylated glucosamine (GlcNAc). Subsequent modification relies on sulfotransferases and an epimerase, including N-deacetylase/N-sulfotransferase [containing separate N-deacetylase and N-sulfotransferase (NST) domains], C5-epimerase (C5-epi), 2-O-sulfotransferase (2-OST), 6-O-sulfotransferase (6-OST), and 3-O-sulfotransferase (3-OST). Using these enzymes, we and others have succeeded in the preparation of heparin polysaccharides (16–19) as structurally well-defined HS oligosaccharides (20, 21). In particular, we developed a protocol to control the size of the oligosaccharides, positions of the N-sulfo glucosamine, 2-O-sulfo IdoA or 6-O,N-disulfo glucosamine residues (20). However, the utility of this approach for the targeted synthesis of medicinally significant heparin oligosaccharides requiring all four controlled sulfation steps, N-sulfation, 6-O-, 2-O- and 3-O-sulfation, has not been demonstrated. Furthermore, until now, low recovery yields in each purification step have cast doubt on the scalability of such chemoenzymatic synthesis.

We targeted two ULMW heparins, constructs 1 and 2, because of their apparent compatibility with our chemoenzymatic approach (Fig. 1). These new constructs contain the antithrombin (AT)-binding domains of porcine and bovine heparin, respectively, which constitute the pharmacophores of anticoagulant heparin (22). Construct 1 also resembles the AT-binding site of human heparin (23). Construct 2 has the same structure as Arixtra except for replacement of a methyl aglycone with disaccharide 3. This structural similarity facilitates comparison of the synthetic efficiency and the in vitro and in vivo biological activities of these two homogeneous ULMW heparins to Arixtra.

Our synthesis of ULMW heparins includes backbone elongation and saccharide modification (Fig. 1). Disaccharide 3 was chosen as the starting material because it can be elongated by glycosyl transferases and can be prepared in multigram quantities from heparosan, readily obtained by fermentation (24). Elongation of disaccharide 3 to tetrasaccharide 4 was completed using two bacterial glycosyl transferases, N-acetyl glucosaminyl transferase of Escherichia coli K5 (KfA) (25) and heparosan synthase-2 (pmHS2) from Pasteurella multocida (26). Tetrasaccharide 4 was designed with an unnatural monosaccharide, GlcNTFA (N-trifluoroacetyl glucosamine), because the N-TFA group can be readily converted to an N-sulfo group (20) in a later step. In preparation of ULMW heparin construct 1, tetrasaccharide 4 was elongated to heptasaccharide 5 in three steps, with an overall yield of 80% (Fig. 1, steps a, b, and c). Heptasaccharide 5 was converted to the final product by a series of chemoenzymatic reactions, including conversion of the GlcNTFA

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**Fig. 2.** Structural characterization of ULMW heparin construct 1. (A) The DEAE-HPLC profile of a 35S-labeled product. (B) The ESI-MS spectrum of construct 1. Peaks 1 to 3 represent the desulfated signals of quadruply charged ions. Peaks 4 to 7 represent the desulfated signals of triply charged ions. Peaks 8 to 12 represent the desulfated signals of doubly charged ions. (C) The 1D 1H NMR spectrum of construct 1. Peaks assigned to the anomeric protons of each hexose ring (A to F) are labeled. (D) The 2D correlation spectroscopy spectrum of construct 1 and the corresponding peak assignments of the anomeric protons that resonate as doublets at δ5.48 (d, J = 3.23 Hz, 2H), 5.35 (d, J = 2.94 Hz, 1H), 5.09 (broad doublet, 1H), 4.53 (d, J = 8.11 Hz, 1H), and 4.46 (d, J = 8.07 Hz, 1H) ppm. The small coupling constants (~3 Hz) of the anomeric protons indicate an α linkage, and the large coupling constants (~8 Hz) indicate a β linkage.
residue to GlcNS (Fig. 1, left column, steps d and e), epimerization and 2-O-sulfation (Fig. 1, left column, step f; here, epimerization of GlcA to IdoA is accompanied by 2-O-sulfation using 2-OST to form an IdoA2S at residue D), 6-O-sulfation (Fig. 1, left column, step g), and 3-O-sulfation (Fig. 1, left column, step h; the 3-O-sulfation occurred at residue C). After these 10 steps, we obtained 3.5 mg of construct 1, corresponding to 45% overall yield as assessed by nuclear magnetic resonance (NMR) spectral integration (fig. S7). Selective epimerization/2-O-sulfation of residue D but not residue B (step f) takes advantage of known enzyme specificity, as residue D is flanked by two GlcNS residues (27). Similarly, in the 3-O-sulfation step (step h), 3-OST-1 selectively adds a 3-O-sulfate group to residue C but not to residue E, because residue C is flanked by a GlcA residue at its nonreducing end (28). In the conversion of heptasaccharide 5 to ULMW heparin construct 1, it was critical to ensure that each modification was completed. For this purpose, small-scale reactions were carried out in parallel using [35S]3′-phosphoadenosine 5′-phosphosulfate ([35S]PAPS) to form the 35S-labeled intermediates; monitoring by diethylaminoethyl high-performance liquid chromatography (DEAE-HPLC) enabled optimization of the reagent concentrations and reaction times required in the synthesis (fig. S2).

Two extra steps were required to add a GlcNS6S residue to the nonreducing end in the synthesis of ULMW heparin construct 2. Tetrasaccharide 4 was first converted to hexasaccharide 6, and the N-TFA groups were replaced by N-sulfo groups to afford hexasaccharide 7 (Fig. 1, right column). Hexasaccharide 7 was elongated to a heptasaccharide with a nonreducing end GlcNTFA (residue A). This heptasaccharide was treated with C5-epi and 2-OST enzymes (Fig. 1, right column, step f) to place an IdoA2S at residue D forming heptasaccharide 8. The introduction of a GlcNTFA residue at the nonreducing end was a critical control point because it prevented the action of C5-epi and 2-OST on the GlcA (residue B in step f). Heptasaccharide 8 was then converted to construct 2 in a sequential one-pot reaction format (Fig. 1, right column, steps d, e, g, and h). Carrying out a small-scale reaction using [35S]PAPS ensured complete 6-O-sulfation (fig. S3). We obtained 7.2 mg of ULMW heparin construct 2 in 12 steps with an overall yield of 37%, as determined by NMR spectral integration (fig. S7). The synthesis of construct 2 was achieved by rearranging the order of the modifications and elongation steps without employing additional enzymes or reagents, thus demonstrating that both structural control and target diversification are possible in chemoenzymatic synthesis.

The structure of construct 1 was confirmed by electrospray ionization mass spectrometry (ESI-MS), as well as one-dimensional (1D) and two-dimensional (2D) NMR analysis (Fig. 2). The 3-O-[35S]sulfate labeled construct 1 showed a single symmetric peak in a high-resolution DEAE-HPLC trace (Fig. 2A), demonstrating that the purity of the product was above 95% [in the large-scale reaction, purity was confirmed by polyacrylamide gel electrophoresis (PAGE)] (fig. S4). The ESI-MS analysis revealed construct 1 to have a molecular mass of 1778.5 ± 0.8 daltons, which is identical to the expected calculated molecular mass (1778.5 daltons) (Fig. 2B). High-resolution ESI-MS exhibited a signal at a mass/charge ratio of 887.5313, consistent with [M-2H]2− (calculated mass/charge ratio, 887.5324). The 2D 1H NMR spectrum clearly demonstrates the presence of six anomic protons that resonate as doublets (Fig. 2, C and D). The small coupling constants (~3 Hz) of three anomic protons indicate α linkages between the A-B, C-D, and E-F rings; larger coupling constants (~8 Hz) indicate β linkages between the B-C, D-E and F-G rings. The presence of an internal IdoA2S is clearly indicated by a broad characteristic
9.1

The inhibitory concentration (IC50) values of constructs AT to constructs forming a 1:1 complex with AT, which subse- 
hemoglobin would exhibit anticoagulant activity by 
the substrate specificities of the enzymes. Although our chemoenzymatic approach, with 
appropriate optimization, provides a general method for preparing different heparins, includ- 
ing ULMW heparin, and in principle LMW hepa- 
rin and UF heparin, target selection is restricted by 
the substrate specificities of the enzymes. Smaller targets, such as the Arixtra pentasac- 
charide, are more difficult to prepare because 
the disaccharide, and about 90% of the hexuronic acid 
substrate size and optimizing the sequence of 
sulfo group installation. Careful design of tar- 
gent structures offers a major advance over our 
previous study (20) by avoiding by-product for- 
iation. For example, the order for installing 
different types of sulfo groups is critically im- 
portant in synthesizing construct 1. The optimized 
sequence is N-sulfation followed by epimerization/ 
2-O-sulfation, 6-O-sulfation, and 3-O-sulfation. 
Reversal of the order in which epimerization/2- 
O-sulfation and 6-O-sulfation (or 6-O-sulfation 
and 3-O-sulfation) take place results in very low 
yields of products. Target size selection is also 
critical, as heptasaccharides and larger oligosac- 
charides are highly susceptible to sulfotransfer- 
ase modification and undergo nearly quantitative 
conversion to the desired intermediate without 
side products in each modification step. Another 
crucial innovation was the improvement of pu-
rification protocols, raising the 30 to 40% yields 
from each purification step described in our pre-
vious study (20) to ~90% herein by coupling 

dumbbell, a direct thrombin inhibitor, was 
recently approved by the U.S. Food and Drug 
Administration, a culmination of intensified ef-
fors new anticoagulant drug development 
(32). However, none of the currently marketed 
anticoagulant drugs can replace heparin because 
of its unique pharmacological properties. A cost-
effective approach to prepare heparin is impor-
tant not only to secure the safety of the heparin 
supply chain but also to provide the opportunity 
to design derivatives that eliminate side effects. 
Although our chemoenzymatic approach, with 
appropriate optimization, provides a general 
method for preparing different heparins, includ-
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ing ULMW heparin, and in principle LMW hepa-

References and Notes


15. On average, heparin carries 2.6 sulfo groups per disaccharide, and about 90% of the hexuronic acid 
is IdA, whereas HS carries 0.6 sulfo groups per disaccharide, and about 20% of the hexuronic acid 
is IdA.


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ULMW heparins has been filed with the U.S. 

Supporting Online Material 
www.sciencemag.org/cgi/content/full/334/6055/498/DC1 
Materials and Methods 
Figs. S1 to S10 
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References (S3–40) 
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