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Enzymatic Preparation of Heparin Oligosaccharides Containing Antithrombin III Binding Sites*

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Two new oligosaccharides were prepared from heparin by its partial depolymerization using heparin lyase I (EC 4.2.2.7) in an attempt to prepare oligosaccharides having intact antithrombin III binding sites. The oligosaccharides were purified by chromatography on the basis of both size and charge and demonstrated a high level of purity by capillary electrophoresis. One- and two-dimensional ^{1}H NMR spectroscopy at 500 MHz revealed the structure of each oligosaccharide. The octasaccharide and decasaccharide are ΔUAp2S(1→4)-α-D-GlcNpS6S(1→4)-α-L-IdoAp(1→4)-α-D-GlcNpAc6S(1→4)-β-D-GlcAp(1→4)-α-D-GlcNpS3S6S(1→4)-α-L-IdoAp2S(1→4)-α-D-GlcNpS6S (where ΔUAp is 4-deoxy-α-L-*threo*-hexenopyranosyluronic acid, GlcNp is 2-amino-2-deoxy-glucopyranose, GlcAp is glucopyranosyluronic acid, S is sulfate and Ac is acetate) and ΔUAp2S(1→4)-α-D-GlcNpS6S(1→4)-α-L-IdoAp(1→4)-α-D-GlcNpAc6S (1→4)-β-D--GlcAp(1→4)-α-D-GlcNpS3S6S(1→4)-α-L-IdoAp2S(1→4)-α-D-GlcNpS6S(1→4)-α-L-IdoAp2S(1→4)-α-D-GlcNpS6S, respectively. A hexasaccharide containing a similar structural motif to that found in the antithrombin III binding site and having greatly reduced anticoagulant activity was also isolated. The structure of the hexasaccharide is ΔUAp2S(1→4)-α-D-GlcNpAc6S(1→4)-β-D-GlcAp(1→4)-α-D-GlcNpS3S6S(1→4)-α-L-IdoAp(1→4)-α-D-GlcNpS6S. The octasaccharide and decasaccharide correspond to the predominant structural motif found in porcine intestinal mucosal heparin. Sufficient quantities of the decasaccharide were obtained to examine its interaction with antithrombin III using microtitration calorimetry. This decasaccharide bound to antithrombin III with similar avidity as heparin and showed comparable anticoagulant activity, as determined using an antithrombin III dependent anti-factor Xa assay. Interestingly, while both decasaccharide and heparin bound to antithrombin with nanomolar affinity, very little heat of binding was observed.

Heparin is a glycosaminoglycan composed of *O*-sulfated and *N*-acetylated or -sulfated 1→4-linked glucosamine and uronic acid residues (1). While heparin exhibits a wide variety of biological activities (2–4), its most important is its anticoagulant activity (5). Despite its widespread use as a clinical anticoagulant for 60 years, the exact chemical structure and the precise nature of its anticoagulant and antithrombotic activities remain unclear.

The anticoagulant action of heparin is the most thoroughly studied of its activities (2–4). Anticoagulation occurs when heparin binds to antithrombin III (ATIII),[1] a serine protease inhibitor. ATIII undergoes a conformational change, becomes activated, and inhibits thrombin and other serine proteases in the coagulation cascade (5). A major breakthrough in the study of heparin-catalyzed anticoagulation resulted from the separation of distinct heparin fractions differing markedly in affinity for ATIII (6). These results led to the use of chemical and enzymatic depolymerization techniques to examine high affinity heparin for the ATIII binding site not present in low affinity heparin. Rosenberg and Lam (7) and Lindahl *et al.*, (8) studied the structure of the ATIII binding site by performing a partial chemical depolymerization of heparin and then purified the products using affinity chromatography on immobilized ATIII. The isolation of 3-*O*-sulfatase from human urine, capable of desulfating 3-*O*-sulfated glucosaminides (9), provided the crucial clue to the structure of the ATIII binding site (see Fig. 1). The presence of an unusual 3-*O*-sulfate group was demonstrated by the release of sulfation on the incubation of a pentasaccharide with 3-*O*-sulfatase (10). NMR studies of oligosaccharide mixtures having high ATIII affinity proved the presence of the 3-*O*-sulfate group (11). Chemical synthesis of a pentasaccharide containing this unique 3,6-di-*O*-sulfated sequence residue substantiated these findings (12). A number of oligosaccharide structures having sequence motifs similar to the ATIII binding sequence have been reported by our group (13) and others (14, 15). Detailed structure-activity relationship studies have also been performed using degradative methods (16, 17) and chemical synthesis (18) and have elucidated the features of the pentasaccharide that are critical for ATIII binding.

Despite an intensive effort, the pentasaccharide binding site has not yet been co-crystallized with ATIII (18). While a substantial amount of ATIII-binding site pentasaccharide has been synthesized, it is not commercially available. The lack of sufficient quantities of homogeneous oligosaccharides containing an ATIII-binding site has hindered the investigation of the ATIII-heparin interaction (19). We recently reported the x-ray co-crystal structures of basic fibroblast growth factor with a highly purified heparin-derived tetrasaccharide and hexasac-

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[1] The abbreviations and trivial names used are: ATIII, antithrombin III; CE, capillary electrophoresis; ΔUAp, 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid; GlcNp, 2-amino-2-deoxyglucopyranose; GlcNpAc, 2-acetamido-2-deoxyglucopyranose; SAX, strong anion exchange; HPLC, high performance liquid chromatography; GPC, gel permeation chromatography; PAGE, polyacrylamide gel electrophoresis; 1D, one-dimensional; 2D, two-dimensional; NOESY, nuclear Overhauser effect spectroscopy; COSY, correlation spectroscopy; TEMED, *N,N,N′,N′*-tetramethylethylenediamine.

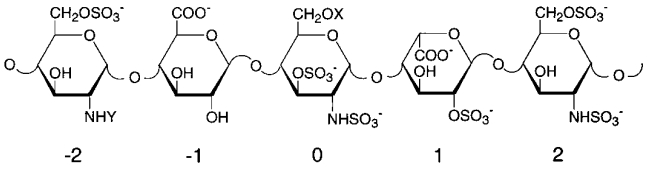

FIG. 1. **Structure of antithrombin III pentasaccharide binding site.** The central residue is numbered 0, while the non-reducing and reducing terminal residues are numbered −2 and +2, respectively. The commonly observed structural variation is indicated; X = H or SO_3^-, Y = Ac or SO_3^-.

charide (20) and examined their interaction with basic fibroblast growth factor using microtitration calorimetry. These studies required multimilligram quantities of highly purified oligosaccharides, which were obtained from heparin in a single enzymatic step (21). The current paper describes the similar enzymatic preparation of multimilligram quantities of three highly purified oligosaccharides containing intact ATIII binding sites.

EXPERIMENTAL PROCEDURES

Materials—Porcine mucosal heparin, sodium salt (157 units/mg) was from Hepar Industries, Franklin, OH. Heparin lyase I used in the large scale depolymerization was from Sigma. Heparin lyase I, II, and III used in structural studies were purified in our laboratory to homogeneity from *Flavobacterium heparinum* (22). ATIII was purified to apparent homogeneity (a single band on SDS-PAGE) from bovine plasma (23). Sterile, Millex GS syringe filters (0.22 μm and 0.45 μm) were from Millipore, Bedford, MA. Bovine serum albumin (biological grade) was from Sigma. A 200 ml pressure filtration apparatus of 4.3 cm in diameter, using diaflow ultrafiltration or a membrane of 10,000 molecular weight cut-off was from Amicon, Beverly, MA. Size exclusion chromatography was performed on Sephadex G-50 (superfine) from Pharmacia Biotech Inc., Piscataway, NJ, on a glass column of dimensions 4.6 cm × 1 m from Kontes, Scientific Glassware, Morton Grove, IL. Bio-Gel P-2 (fine) from Bio-Rad, Richmond, CA, was used in a desalting column of dimensions 5 cm × 0.5 m. Calorimetric data were collected using a Model 4209 Hart Scientific microtitration calorimeter (Pleasant Grove, UT). The voltage to the instrument was regulated with a Citadel power conditioner, Model LC630, from Best Power Technology, Inc. (Necedah, WI), and temperature was controlled using an external water bath (Model 9109, Polyscience, Niles, IL). Factor Xa amidolytic kit including S-2222 (benzoyl-Ile-Glu(-OR)-Gly-Arg-*p*NA-HC1) was obtained from Pharmacia-Hepar, Inc., Franklin, OH. SAX-HPLC was performed on 5 μm Spherisorb columns from Phase Separation, Norwalk, CT, of dimensions 0.64 × 25 cm (analytical) and 2.5 × 25 cm (semi-preparative) using dual face programmable LC-7A titanium-based pumps from Shimadzu, Kyoto, Japan. This system was equipped with a Rheodyne (Catati, CA) titanium injector and a Pharmacia LKB variable wavelength UV detector from Piscataway, NJ, and with a Shimadzu Chromatopac C-R2A integrating recorder.

Gradient PAGE was performed on a 22-cm vertical slab gel unit Protean II, equipped with Model 1420B power source from Bio-Rad. Acrylamide (ultrapure), Tris, Alcian Blue, Bromophenol Blue, and ammonium persulfate were obtained from Boehringer Mannheim. EDTA, sucrose, TEMED, and sodium borate (decahydrate, 99%) were from Fisher Chemicals Co., Fairlawn, NJ. Sodium dodecylsulfate (99%) was from BDH Chemicals, Poole, United Kingdom. CE was performed using a capillary electrophoresis system with advanced computer interface, Model I, equipped with high voltage power supply capable of constant or gradient voltage control using a fused silica capillary, from Dionex Corporation, Sunnyvale, CA. UV spectroscopy was performed on a Shimadzu model UV-2101 PC spectrophotometer equipped with a thermostated cell. All other reagents used were analytical grade. A Varian 500 MHz NMR spectrometer equipped with a SUN SPARC station 2 workstation was used for all 1D and 2D NMR experiments. The NMR experiments were performed in ^2H_2O (99.996 atom %) using 3-(trimethylsilyl)propionic-*2,2,3,3-d*$_4$ acid, sodium salt (99+ atom %) as the internal reference from Aldrich WI.

Depolymerization of Heparin—The large scale, partial depolymerization of heparin was carried out on 10 g of heparin. The large scale depolymerization was carried out to only 10% reaction completion to increase the percentage of oligosaccharides containing intact ATIII binding sites. This partial digestion of heparin provided a maximum concentration of oligosaccharides of intermediate molecular weight (1500–2000) and contained substantial anti-factor Xa activity (24).

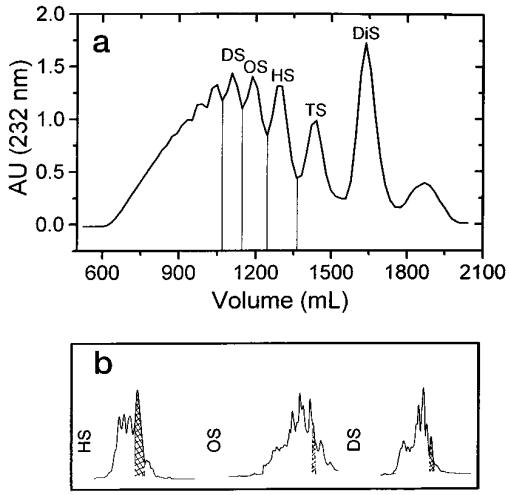

FIG. 2. **Preparation of oligosaccharides containing intact ATIII binding sites.** *a*, fractionation of oligosaccharide mixture by GPC. The fractions combined to prepare the hexasaccharide (HS), octasaccharide (OS), and decasaccharide (DS) pool for purification by SAX-HPLC are indicated. *b*, SAX-HPLC of HS, OS, and DS. The peaks corresponding to **1**, **2**, and **3** are indicated by *hatch marks*.

In a sterile hood, 10 g of heparin was prepared in 200 ml of 250 mM calcium acetate, 2.5 mM sodium acetate, 1 mM Tris, pH 7.10, and filter sterilized using a 0.22-μm syringe into a sterile polyethylene container. The reaction was carried out at 30 °C, and heparin lyase I (60 units) was added at three times (0, 19, and 48 h). A portion of this aliquot was used to monitor the reaction by diluting it in 1 ml of 0.03 M HCl and measuring the absorbance at 232 nm. When the reaction reached 10% digestion (96 h), the mixture was removed from the water bath and heated to 100 °C for 2 min.

Removal of Residual Heparin and Very High Molecular Weight Oligosaccharides—The depolymerized heparin sample contains oligosaccharides of various sizes as well as buffer salts. The sample, containing 10 g of depolymerized heparin, was pressure filtered in 2–100 ml volumes. A controlled pore 10,000 molecular weight cut-off membrane using a 200 ml stirred pressure filtration cell was used with an N_2 gas pressure of 35 p.s.i. Pressure filtration concentrated the retentate to a 20-ml volume. This volume was readjusted to 100 ml with deionized distilled water, and pressure filtration was repeated 3 times. Both the retentate (40 ml) and the filtrate (640 ml) were collected and freeze-dried. The freeze-dried retentate (oligosaccharides >10,000) was salt free and contained approximately two-thirds the oligosaccharide mass. The filtrate (oligosaccharides <10,000) contained buffer salts, and it was assumed to contain the remaining one-third of the oligosaccharide mass. Gradient PAGE confirmed that these two fractions were mixtures of oligosaccharides with the molecular weight dispersions expected (21).

Gel Permeation Chromatography (GPC) of Oligosaccharides <10,000—The low molecular weight heparin oligosaccharides (M_r <10,000) obtained from pressure filtration were fractionated on a Sephadex G-50 (superfine) column (4.8 cm × 1 m) eluted with 200 mM sodium chloride at a flow rate of 2 ml/min. Oligosaccharide mixture (750 mg) was dissolved in 10 ml of deionized, distilled water and applied to this column, and 200 fractions were collected (10 ml/tube) and absorbance was measured at 232 nm. The fraction number was plotted *versus* absorbance affording a chromatograph that showed a partial separation of disaccharide through tetradecasaccharides. This separation was repeated 4 times giving a reproducible profile that permitted the combination of like fractions. Three fractions, consisting of hexasaccharides, octasaccharides, and decasaccharides were collected, and each fraction was evaporated to dryness.

Desalting the Sized Oligosaccharide Fractions by GPC and Dialysis—The sized oligosaccharide mixtures were desalted by GPC on Bio-Gel P-2 column (5 cm × 0.5 m) eluted with water at 6 ml/min. The eluent was collected, and the fractions containing oligosaccharides having absorbance at 232 nm were combined. The volume was reduced using a rotary-evaporator, and the samples were freeze-dried. The desalted, sized oligosaccharide mixtures were light yellow colored powders and were stored at −60 °C. Gradient PAGE confirmed the oligosaccharide size in each mixture (21).

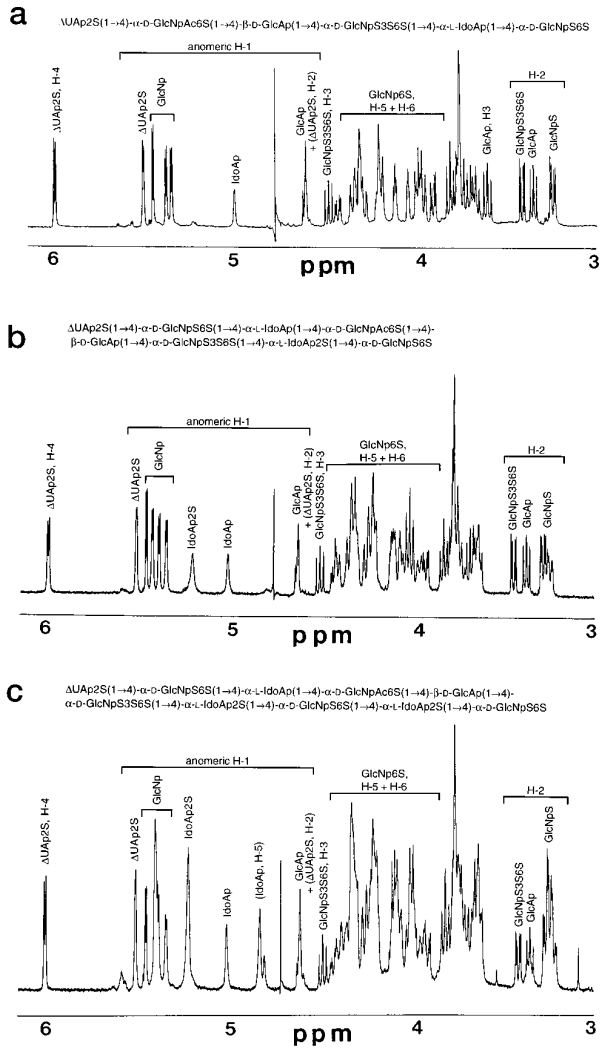

FIG. 3. **One-dimensional 500 MHz ^{1}H NMR spectra for oligosaccharides containing intact ATIII binding sites.** *a*, hexasaccharide **1**; *b*, octasaccharide **2**; and *c*, decasaccharide **3**. The methyl of the *N*-acetyl group at 2 ppm is not shown.

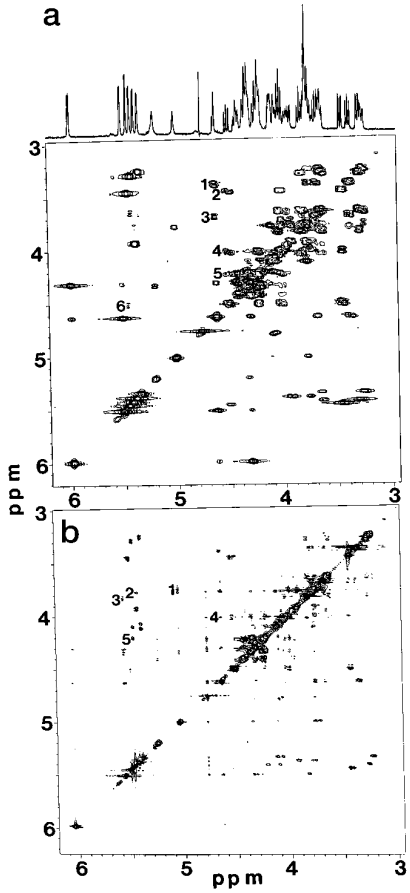

FIG. 4. **Two-dimensional ^{1}H NMR spectra for the octasaccharide containing ATIII binding site 2.** *a*, 2D-relayed COSY spectrum. The major COSY cross-peaks are: 1, GlcAp (H-1/H-2); 2, GlcNpS3S6S (H-2/H-3); 3, GlcAp (H-1/H-3); 4, GlcNpS3S6S (H-3/H-4); 5, GlcNpS3S6S (H-3/H-5); and 6, GlcNpS3S6S (H-1/H-3). The numbers are placed immediately to the left of the cross-peaks. *b*, 2D-NOESY spectrum. The NOE cross-peaks are: 1, IdoAp (H-1) to GlcNpAc6S (H-4); 2, GlcNpAc (H-1) to GlcAp (H-4); 3, ΔUAp2S (H-1) to GlcNpS6S (H-4); 4, GlcAp (H-1) to GlcNpS3S6S (H4); and 5, GlcNpS6S (H-1) to IdoAp (H-4). The GlcNpS3S6S (H-1) to IdoAp2S (H-4) is observed at 5.5 ppm, and 4.1 ppm in a spectrum obtained with a lower threshold value.

Purification of Sized Oligosaccharides into Homogeneous Oligosaccharides Using Semi-preparative SAX-HPLC—Charge separation of sized oligosaccharide fractions was carried out by semi-preparative SAX-HPLC using a linear gradient of sodium chloride at pH 3.5. The column was pre-equilibrated with 0.2 M sodium chloride, pH 3.5. The desalted oligosaccharide mixture (50–100 mg) was applied and eluted using a 180-min linear gradient from 0.2 to 1.9 M sodium chloride, pH 3.5, at a flow rate of 4 ml/min. The elution profile was monitored by absorbance at 232 nm at 0.5–1.0 absorbance units full scale. After each separation, the column was washed with 2.0 M sodium chloride, followed by a water wash and reequilibrated with 0.2 M sodium chloride solution of pH 3.5. Each sized oligosaccharide mixture was applied multiple times to the same column, and each resulted in nearly identical elution profiles. The major peaks were pooled, freeze-dried and desalted on a Bio-Gel P-2 column. Each peak, corresponding primarily to a single oligosaccharide (as demonstrated by analytical SAX-HPLC performed at this stage in the purification) was again applied to the semi-preparative SAX-HPLC column (using a newly optimized gradient), and its fractionation and desalting was repeated. From 10 gm of heparin, 9.7, 4.0, and 5.4 mg of oligosaccharides **1**, **2**, and **3** were obtained. Each purified oligosaccharide was freeze-dried and stored at −60 °C.

Analysis of Oligosaccharides by Analytical SAX-HPLC—Purified oligosaccharides were analyzed by analytical SAX-HPLC to confirm their purity. The SAX-HPLC column was equilibrated with 0.2 M sodium chloride at pH 3.5. Each oligosaccharide sample (10–100 μg) was analyzed using a 120-min linear gradient of 0.2–2 M sodium chloride at pH 3.5 at a flow rate of 1.0 ml/min. The elution profile was monitored by absorbance at 232 nm at 0.02 absorbance units full scale. Sample purity was confirmed by the presence of a single symmetrical peak.

Analysis of Oligosaccharides by Capillary Electrophoresis—The purity of each oligosaccharide was confirmed by the presence of a single symmetrical peak on analysis using CE. The sample was separated and analyzed using a freshly prepared fused silica capillary (75 μm i.d., 375 μm o.d., 65 cm long). Reverse polarity analyses were carried out for 25 min at 18 kV using 20 mM phosphoric acid buffer adjusted to pH 3.48 with a saturated solution of dibasic sodium phosphate (25).

NMR Spectral Analysis of Oligosaccharides—The pure oligosaccharide samples were dissolved in ^2H_2O (99.9%) filtered through a 0.45 μm syringe filter and freeze dried to remove exchangeable protons. After exchanging the sample three times, the sample was dissolved in ^2H_2O (99.996 atom %) containing 0.03% 3-(trimethylsilyl)-1-propanoic acid sodium salt as internal reference. 1D ^{1}H NMR experiments were performed on 5-mm broad band probe with standard Varian software at 298 K on 700 μl samples at 1–6 mM. Spectral width/block size were 6 K and 48 K, respectively. Chemical shifts were determined relative to the internal reference. Proton assignments for all of the heparin oligosaccharides were made from 2D COSY and multiple relayed COSY experiments carried out using standard Varian software at 298 K. The number of the data points in F1 and F2 dimensions were 512 and 2048, respectively, and were obtained with 32 scans. The free induction decays were Fourier transformed onto a data matrix of 2 × 1 K with a phase-shifted sine-bell window function (26). NOESY experiments used a $\pi/2$-t_1 -$\pi/2$-τ_m - $\pi/2$-FID pulse program with 512 F1 increments and with 2048 data points for F2 acquired with 32 scans. The free induction decays were Fourier transformed onto a data matrix of 2 × 1 K with a phase-shifted sine-bell window function. The mixing times were 150–500 ms with the strongest nuclear Overhauser effects observed at a

mixing time of 300 ms.

Determination of ATIII Affinity of Heparin Oligosaccharide—Calorimetric data were collected at 25 °C, and the instrument was calibrated as described previously (27). For all titrations, ten 10-μl injections of decasaccharide (0.5 mM) were pipetted automatically into the reaction cell containing 1 ml of the 20 μM ATIII in buffer containing 50 mM sodium phosphate, 100 mM sodium chloride, 1 mM dithiothreitol, pH 7.4, at 250 s intervals from a 100-μl syringe while stirring at 75 rpm. In all experiments, the thermal reference cell contained 1 ml of water. Integration of the thermogram peaks was carried out using the software supplied with the calorimeter (Hart Scientific). The total corrected heats were obtained after subtraction of the control heats of dilution for the ligand at each injection. In all control experiments, the ligand was simply diluted into buffer with ATIII omitted. The corrected heats were fitted using a non-linear least squares algorithm that minimized the sum of squared residuals while varying ΔH, n, and K_a as described previously (28–30).

Characterization of Oligosaccharides by Enzymatic Methods—Oligosaccharide structures were examined using enzymes of known specificity to confirm the structures deduced by NMR spectroscopy. Oligosaccharides were further treated with heparin lyases I, II, and III, and the products formed were analyzed by CE. The identity of the oligosaccharide products were identified by co-migration with oligosaccharides that had already been characterized in our laboratory (21, 31). Each oligosaccharide sample (three vials each containing 50 μg in 40 μl of 50 mM sodium phosphate buffer, pH 7.0, containing 2 mg/ml bovine serum albumin) was treated overnight with 50 m-units of heparin lyase I, heparin lyase II or heparin lyase III at 30 °C. After reaction completion, each sample was heated at 100 °C for 1 min to inactivate the enzymes. The oligosaccharides were converted into their disaccharide, tetrasaccharide, or hexasaccharide components depending on their structure and the specificity (31–33) of the heparin lyase used.

RESULTS AND DISCUSSION

Porcine intestinal mucosal heparin was treated with heparin lyase I, and the reaction was terminated prior to its completion. The oligosaccharide products formed when 10% of the heparin lyase I susceptible linkages had been cut was rich in hexasaccharide, octasaccharide, and decasaccharide components and contained approximately 95% of the heparin anti-factor Xa activity but <30% of the heparin anti-factor IIa activity (24). Thus, while most ATIII binding sites were intact (Fig. 1), they were primarily in oligosaccharides smaller than the hexadecasaccharides required for anti-factor IIa activity (5). Fractionation of this oligosaccharide mixture by gel permeation chromatography (Fig. 2*a*) affords sized oligosaccharide fractions. The hexasaccharide, octasaccharide, and decasaccharide fractions, expected to contain intact pentasaccharide ATIII-binding sites, were collected, desalted, and fractionated by SAX-HPLC. The chromatographs obtained (Fig. 2*b*) contained multiple peaks. Each was collected, desalted, and freeze-dried. SAX-HPLC, using a shallow salt gradient, was used to purify each fraction to homogeneity. Oligosaccharide purity was assessed at 90% or greater using analytical SAX-HPLC and reversed polarity CE.

NMR spectroscopy was next used to determine the structure

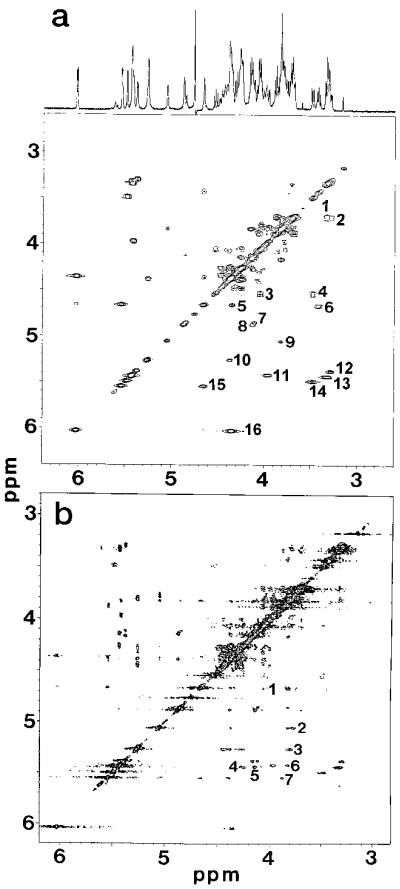

FIG. 5. **Two-dimensional ^{1}H NMR spectra for the decasaccharide 3.** *a*, 2D-COSY spectrum. The major COSY cross-peaks are: 1, GlcNpS6S (H-2/H-3) (2H); 2, GlcNpS6Sα (H-2/H-3); 3, GlcNpS3S6S (H-3/H-4); 4, GlcNpS3S6S (H-2/H-3); 5, ΔUAp2S (H-2/H-3); 6, GlcAp (H-1/H-2); 7, IdoAp (H-4/H-5); 8, IdoAp2S (H-4/H-5) (2H); 9, IdoAp (H-1/H-2); 10, IdoAp2S (H-1/H-2) (2H); 11, GlcNpAc6S (H-1/H-2); 12, GlcNpS6Sα (H-1/H-2); 13, GlcNpS6S (H-1/H-2) (2H); 14, GlcNpS3S6S (H-1/H-2); 15, ΔUAp2S (H-1/H-2); and 16, ΔUAp2S (H-3/H-4). *b*, 2D-NOESY spectrum. The major NOESY cross-peaks are: 1, GlcAp (H-1) to GlcNpS3S6S (H-4); 2, IdoAp (H-1) to GlcNpS6S (H-4) (2H); 3, IdoAp2S (H-1) to GlcNpS6S (H-4) (2H); 4, GlcNpS6S (H-1) to IdoAp (H-4); 5, GlcNpS6S (H-1) to IdoAp2S (H-4); 6, GlcNpAc6S (H-1) to GlcAp (H-4); and 7, ΔUAp2S (H-1) to GlcNpS6S (H-4). The GlcNpS3S6S (H-1) to IdoAp2S (H-4) is observed at 5.5 ppm, 4.3 ppm in a spectrum obtained with a lower threshold value.

TABLE I
*Chemical shifts (ppm) and coupling constants (Hz) of ATIII binding site hexasaccharide (**1**)*

	ΔUAp2S	GlcNpAc6S	GlcAp	GlcNpS3S6S	IdoAp	GlcNpS6S
H-1	5.472	5.386	4.605	5.411	4.974	5.322
$J_{1,2}$	1.5	3.9	8.0	2.9	<1	3.4
H-2	4.588	3.931	3.342	3.408	3.75	3.233
$J_{2,3}$	<1	10.4	9.4	10.1	3.2	9.8
H-3	4.302	3.787	3.602	4.472	4.101	3.664
$J_{3,4}$	3.9	ND[b]	8.9	9.1	4.1	9.6
H-4	5.922	3.784	3.723	3.942	4.21	3.72
$J_{4,5}$		9.2	8.4	9.2	ND	10.4
H-5	–[a]	4.119	3.724	4.31	4.756	4.002
$J_{5,6a}$		4.5		4.3		4.3
H-6a	–	4.328	–	4.402	–	4.321
$J_{6a,6b}$		ND		−10.9		−11.3
H-6b	–	4.22	–	4.28	–	4.28
$J_{5,6b}$		8.1		8.1		ND
N-acetyl methyl	–	2.004	–	–	–	–

[a] –, not present.
[b] ND, not determined because of overlap with other peaks.

TABLE II
*Chemical shifts (ppm) and coupling constants (Hz) of ATIII binding site octasaccharide (**2**)*

	ΔUAp2S	GlcNpS6S	IdoAp	GlcNpAc6S	GlcAp	GlcNpS3S6S	IdoAp2S	GlcNpS6S
H-1	5.508	5.422	5.009	5.386	4.634	5.455	5.202	5.347
$J_{1,2}$	2.0	3.4	<1	3.9	8.0	3.3	<1	3.7
H-2	4.626	3.293	3.784	3.931	3.384	3.454	4.329	3.254
$J_{2,3}$	<1	10.7	3.7	10.4	9.4	10.3	7.0	10.6
H-3	4.317	3.643	4.111	3.787	3.688	4.508	4.214	3.650
$J_{3,4}$	4.3	9.9	4.3	ND[b]	9.2	9.3	ND	9.5
H-4	5.989	3.833	4.222	3.784	3.784	4.021	4.074	3.757
$J_{4,5}$		9.0	ND	9.2	8.6	9.3	2.9	10.4
H-5	–[a]	4.065	4.756	4.119	3.757	4.321	4.756	4.110
$J_{5,6a}$		4.5		4.5		4.3		4.3
H-6a	–	4.343	–	4.328	–	4.432	–	4.411
$J_{6a,6b}$		ND[b]		ND		−11.5		−11.5
H-6b	–	4.22	–	4.22	–	4.32	–	4.32
$J_{5,6b}$		8.9		8.1		8.5		9.1
N-acetyl methyl	–	–	–	2.048	–	–	–	–

[a] –, not present.
[b] ND, not determined because of overlap with other peaks.

TABLE III
*Chemical shifts (ppm) and coupling constants (Hz) of ATIII binding site decasaccharide (**3**)*

	ΔUAp2S	GlcNpS6S	IdoAp	GlcNpAc6S	GlcAp	GlcNpS3S6S	IdoAp2S	GlcNpS6S	IdoAp2S	GlcNpS6S
H-1	5.508	5.446	5.054	5.424	4.674	5.498	5.272	5.446	5.272	5.382
$J_{1,2}$	2.0	3.4	<1	3.7	8.2	3.1	<1	3.6	<1	3.5
H-2	4.626	3.336	3.806	3.981	3.422	3.489	4.38	3.324	4.38	3.302
$J_{2,3}$	<1	10.6	ND[b]	10.4	8.9	10.6	ND	10.4	ND	10.1
H-3	4.317	3.702	4.126	3.868	3.722	4.522	4.26	3.702	4.26	3.710
$J_{3,4}$	4.3	ND[b]	ND	9.4	9.1	9.4	ND	ND	ND	9.4
H-4	5.989	3.82	4.282	3.803	3.802	4.088	4.15	3.82	4.15	3.781
$J_{4,5}$		ND	ND	9.2	ND	9.4	<1	ND	<1	10.1
H-5	–[a]	4.280	4.852	4.283	3.788	4.382	4.881	4.280	4.881	4.130
$J_{5,6a}$		ND		ND		ND		ND		4.1
H-6a	–	4.38	–	4.38	–	4.42	–	4.38	–	4.44
$J_{6a,6b}$		ND		ND		ND		ND		ND
H-6b	–	4.32	–	4.26	–	4.38	–	4.32	–	4.38
$J_{5,6b}$		ND		ND		ND		ND		ND
N-acetyl methyl	–	–	–	2.097	–	–	–	–	–	–

[a] –, not present.
[b] ND, not determined because of overlap with other peaks.

of oligosaccharides **1**, **2**, and **3**. 1D ^{1}H NMR spectroscopy of each purified oligosaccharide was used as a preliminary means to locate the potential ATIII-binding site containing oligosaccharides through the presence of a triplet at ~4.5 ppm, characteristic of the signal for the H-3 of the critical GlcNpS3S6S residue (Fig. 3). Three such oligosaccharides, corresponding to peaks **1**, **2** and **3** (see Fig. 2 legend), were obtained.

The 1D spectra of **1**, **2**, and **3** (Fig. 3) showed that each oligosaccharide contained a single IdoAp (H-1, 5.0 ppm), GlcAp (H-1, 4.7 ppm), and ΔUAp2S (H-1, 5.5 ppm) residue. Octasaccharide **2** and decasaccharide **3** showed an anomeric signal at 5.2–5.3 ppm with an integration of 1H and 2H, demonstrating the presence of one and two IdoAp2S residues, respectively. The integrals of the H-1 signals, corresponding to the GlcNp residues (5.3–5.5 ppm) were 3H, 4H, and 5H for oligosaccharides **1**, **2**, and **3**, respectively. Thus, the 1D 500 MHz spectra (Fig. 3) show 6, 8, and 10 anomeric (H-1) signals, indicating that **1**, **2**, and **3** are a hexasaccharide, octasaccharide, and decasaccharide, respectively. Failure to observe additional, extraneous, anomeric (H-1) signals confirmed the high level of purity of these three oligosaccharides.

2D ^{1}H NMR spectroscopy was next used to assign each 1D spectra and establish the structure of these three purified oligosaccharides. Since the cross-peaks observed in 2D ^{1}H multiple relayed COSY are due to indirect scaler (through-bond) coupling, the spin system present in each saccharide residue can be assigned. The position of the saccharide residues (sequence) were established by NOESY. Here the cross-peaks result from dipolar (through-space) coupling of spatially adjacent nuclei. COSY and NOESY spectra for octasaccharide **2** and decasaccharide **3** are presented in Figs. 4 and 5. 2D-relayed COSY, taken using a 32-msec mixing time, was used to determine positions of the sulfate groups of the critical GlcNpS3S6S residue in octasaccharide **2** and decasaccharide **3**. The structure of this saccharide was unambiguously established by H-1/H-3, H-2/H-3, H-3/H-4, and H-3/H-5 cross-peaks in the relayed COSY spectra (Figs. 4*a* and 5*a*).

The NOESY spectrum in Fig. 4*b* was used to determine the sequence of octasaccharide **2**. Although the intensity of each cross-peak obtained from the NOE spectrum are different, NOEs can be observed between all of the H-1 anomeric protons and the H-4 protons. The ^{1}H NMR assignments for each structure are presented in Tables I-III. The signals for hexasaccharide **1** and octasaccharide **2** could be easily identified from the 1D and 2D spectra because each signal resonated at a different position.

The structure of decasaccharide **3** was next determined by 2D COSY and NOESY (Fig. 5, *a* and *b*). The ring protons of each saccharide unit were first assigned by COSY. The assignment of the H-4 protons, critical for sequence determination, was possible; however, overlap of the signals corresponding to the H-5 protons and the two H-6 protons of GlcNp6S complicated the definitive assignment of these signals. The sequence of decasaccharide **3** was definitively established by NOESY (Fig. 5*b*). Cross-peaks between the H-1/H-4 signals of all of the glycosidic linkages are observed in the NOESY spectrum (Fig. 5b) confirming the sequence of decasaccharide **3**.

Next, the oligosaccharide structures were reconfirmed by CE

TABLE IV
Alignment of oligosaccharides containing portions of the sequence found in the antithrombin III binding sites of heparin[a]

Oligosaccharide	Source preparation[b]	RESIDUE NUMBER										Reference
		-5	-4	-3	-2	-1	0	1	2	3	4	
Penta	Syn[c]				GlcNpS**6S**	GlcAp	GlcNpS3S6S	IdoAp2S	GlcNpS6S			Van Boeckel & Petitou (18)
Tetra	BL or PIMcH1			ΔUAp2S	GlcNpS6S	GlcAp	GlcNpS3S6S					Loganathan et al. (13)
Tetra	PIMcH1, 2, 3			ΔUAp	GlcNpAc6S	GlcAp	GlcNpS3S					Yamada et al. (14)
Tetra	PIMcH1, 2, 3			ΔUAp	GlcNpAc6S	GlcAp	GlcNpS3S6S					Yamada et al. (14)
Tetra	PIMcH1, 2, 3			ΔUAp	GlcNpS6S	GlcAp	GlcNpS3S6S					Yamada et al. (14)
Hexa	PIMcH1	ΔUAp2S	GlcNpS6S	IdoAp	GlcNpAc6S	GlcAp	GlcNpS3S6S					Linhardt et al. (36)
Hexa	PIMcH1	ΔUAp2S	GlcNpS6S	IdoAp	GlcNpAc6S	GlcAp	GlcNpS3S					Tsuda et al. (15)
Hexa	PIMcH1	ΔUAp2S	GlcNpS6S	IdoAp	GlcNpAc6S	GlcAp	GlcNpS6S[d]					Linhardt et al. (26)
Hexa **1**	PIMpH1			ΔUAp2S	GlcNAc6S	GlcAp	GlcNpS3S6S	IdoAp	GlcNpS6S			
Octa	PIMpH[e], AT			ΔUAp	GlcNpAc6S	GlcAp	GlcNpS3S6S	IdoAp2S	GlcNpS6S	IdoAp2S	GlcNpS6S[f]	Ototani & Yosizawa (39)
Octa	WHpH1, AT	ΔUAp2S	GlcNpS	IdoAp	GlcNpAc6S	GlcAp	GlcNpS3S	IdoAp2S	GlcNpS			Ototani et al. (40)
Octa3	PIMpNA, AT			IdoAp	GlcNpAc6S	GlcAp	GlcNpS3S6S	IdoAp2S	GlcNpS6S	IdoAp2S	AM6S	Atha et al. (17) Thunberg et al. (37)
Octa	PIMpNA, AT	IdoAp2S	GlcNpS6S	IdoAp	GlcNpAc6S	GlcAp	GlcNpS3S6S	IdoAp2S	AM6S			Atha et al. (17)
Octa	PIMpNA, AT			IdoAp	GlcNpS6S	GlcAp	GlcNpS3S6S	IdoAp2S	GlcNpS6S	IdoAp2S	AM[f]	Casu et al. (11)
Octa	PIMpH1, AT			IdoAp	GlcNpS6S	GlcAp	GlcNpS3S6S	IdoAp2S	GlcNpS6S[f]			Casu et al. (11)
Octa **2**	PIMpH1	ΔUAp2S	GlcNpS6S	IdoAp	GlcNpAc6S	GlcAp	GlcNpS3S6S	IdoAp2S	GlcNpS6S			
Deca **3**	PIMpH1	ΔUAp2S	GlcNpS6S	IdoAp	GlcNpAc6S	GlcAp	GlcNpS3S6S	IdoAp2S	GlcNpS6S	IdoAp2S	GlcNpS6S	
Deca	PIMFrAT	IdoAp2S	GlcNpS6S	IdoAp	GlcNpS6S	GlcAp	GlcNpS3S6S	IdoAp2S	GlcNpS6S	IdoAp2S	GlcNpS6S[f]	Casu et al. (11)
Overall[g]	Major	IdoAp2S	GlcNpS6S	IdoAp	GlcNpAc6S	GlcAp	GlcNpS3S6S	IdoAp2S	GlcNpS6S	IdoAp2S	GlcNpS6S	
	Minor		GlcNpS	IdoAp2S	GlcNpS6S		GlcNpS3S GlcNpS6S	IdoAp	GlcNpS			

[a] All saccharide units are in the pyranose form and 1 → 4 linked, GlcNp is α-D-glucosamine, GlcAp is β-D-glucuronic acid and IdoAp is α-L-iduronic acid, S is sulfate, Ac is acetate and AM is anhydromannose.

[b] Sources include: syn, chemical synthesis; PIM, porcine intestinal mucosa; BL, bovine lung; WH, whale heparin; c, complete; p, partial; H1, 2, 3, heparin lyase I, II, and III, respectively; NA, nitrous acid; AT, antithrombin III affinity fractionated; Fr, fractionated by gel permeation chromatography.

[c] Acidic groups that are underlined are important and those bolded are essential for interaction with ATIII (18).

[d] Oligosaccharide containing a portion of the biosynthetic precursor of the ATIII binding site.

[e] Enzyme listed as heparinase (39) probably contained heparin lyase I and II.

[f] Tentative structure based on ^{13}C NMR assigned on a partially purified oligosaccharide mixture.

[g] The most common AT binding sequence found in PIM heparin (Major) and less commonly observed (Minor) sequences are shown.

analysis following their breakdown with heparin lyase I, II, or III. Exhaustive treatment of these oligosaccharides with heparin lyases I, II, and III afforded oligosaccharide products expected based on the reported specificity of these enzymes (31–33). Heparin lyases I and II both cleaved →4)-α-D-GlcNpS6S(1→4)-α-L-IdoAp2S(1→ linkages, and heparin lyase II also acted on →4)-α-D-GlcNpS6S(1→4)-α-L-IdoAp(1→. Heparin lyase III cleaved the →4)-α-D-GlcNpAc6S(1→4)-β-D-GlcAp(1→ linkage found in these oligosaccharides. Interestingly, the →4)-α-D-GlcNpS3S6S(1→4)-α-L-IdoAp(1→ linkage appeared to be slightly sensitive to heparin lyase II. The heparin lyase sensitivity of this linkage had not been previously reported.

Two of the three binding sites that were characterized (**2** and **3**) contain the identical pentasaccharide motif, →4)-α-D-GlcNpS6S(1→4)-β-D-GlcAp(1→4)-α-D-GlcNpS3S6S(1→4)-α-L-IdoAp2S(1→4)-α-D-GlcNpS6S(1→, associated with the major form of the heparin ATIII binding site (Table IV). Decasaccharide **3** was available in sufficient quantities to perform detailed binding studies using microtitration calorimetry. Decasaccharide **3** bound to bovine ATIII with the expected 1:1 stoichiometry (0.9 mol oligosaccharide:1 mol of ATIII). While the K_d of 290 nM, obtained for decasaccharide binding to ATIII, is within the range of reported values for heparin binding to ATIII (19, 34, 35), the heat of binding ($\Delta H = -1.7$ kCal/mol) was lower than that observed for ATIII (34) and other heparin binding proteins such as basic fibroblast growth factor (20). The ATIII mediated anti-factor Xa activity measured for decasaccharide was ~180 units/mg, comparable with that observed for heparin.

The synthetically prepared ATIII pentasaccharide binding site corresponds to a minor structural form, containing a GlcNpS6S residue in place of the GlcNpAc6S residue (found in the natural product) at its non-reducing terminus (Table IV). This pentasaccharide was chosen for commercial synthesis since its additional *N*-sulfate group contributes to ATIII binding, enhancing its affinity and presumably increasing its potency as an anticoagulant agent (18). The occurrence of *N*-sulfate or *N*-acetate at this position in heparin appears to depend on both its tissue (organ) and species source (13). These structural differences may also result in some of the pharmacological differences observed in pharmaceutical heparins prepared from beef lung and porcine intestinal mucosa (containing an *N*-sulfated and *N*-acetylated ATIII binding sites, respectively) (13).

The third oligosaccharide isolated (**1**), contains all the structural features of the most commonly found ATIII binding site except that it is lacking a 2-*O*-sulfate group in its IdoAp residue. Structure activity studies performed on synthetic ATIII binding site pentasaccharides demonstrated the importance of this 2-*O*-sulfate group in binding affinity (18). The current study confirms these previous results by the marked reduction in the ATIII-mediated anti-factor Xa activity of hexasaccharide **1**, to ~8 units/mg.

A number of oligosaccharides containing a portion of the pentasaccharide sequence required for ATIII binding have been isolated and characterized by our group (13, 36) and others (14, 15) (Table IV). These were prepared by exhaustive treatment of heparin with heparin lyases. Intact ATIII binding sites have been obtained through synthesis (18), fractionation (1), and partial depolymerization with nitrous acid (11, 17, 37). A number of attempts have been made to enzymatically prepare oligosaccharides containing intact ATIII binding sites by our group and others (11, 38–40). These efforts were complicated by both the unavailability of pure heparin lyases (22) and the ability of these lyases to cleave glycosidic linkages that are found within the ATIII binding site (14, 31–33). The heparin lyase sensitivity of glycosidic linkages, within the ATIII binding site, can be overcome, in part, by performing a controlled partial depolymerization (24, 25, 41). Ototani and Yosizawa (39) used this approach to isolate an octasaccharide with ATIII affinity and anticoagulant activity using heparinase. The structure of this octasaccharide was inferred from the similarity of its ^{13}C NMR to an octasaccharide mixture prepared by Casu *et al.* (11) using a similar method. The non-reducing terminus of this octasaccharide, however, while containing a ΔUAp (4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid) residue characteristic of the action of a polysaccharide lyase (33), was missing a 2-*O*-sulfate group suggesting that it did not result from the action of heparin lyase I (31–33). Thus, either the heparinase used by Ototani and Yosizawa (39) contained both heparin lyase I and II (able to cleave the →4)-α-D-GlcNpS or Ac (6S or 3OH) (1→4)-α-L-IdoAp (1→ linkage)), or the structure ascribed to this octasaccharide was misassigned. A whale heparin-derived octasaccharide reported by Ototani *et al.*, (38, 40) appears to be consistent with the heparin lyase I used, the known structure of heparin prepared from this source, and the difference in enzymatic susceptibility of linkages containing GlcNpS (3S or OH) compared with their more fully sulfated counterpart GlcNpS (3S or 3OH)6S. The restricted availability of whale heparin, however, makes it unusable as a source for the enzymatic preparation of oligosaccharides having intact ATIII binding sites.

This current study demonstrates that oligosaccharides containing intact ATIII binding sites can be prepared by partial enzymatic depolymerization of porcine intestinal mucosal heparin with pure heparin lyase I. A two-step fractionation on the basis of size and charge affords multimilligram amounts of oligosaccharides containing intact binding sites at a high level of purity. These oligosaccharides exhibit the expected affinity toward ATIII and anticoagulant activity. Finally, such highly purified oligosaccharides should be useful in the studies of heparin-ATIII interaction (19) and in the effort to prepare protein-oligosaccharide co-crystals (20) for x-ray crystallographic analysis.

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REFERENCES

1. Casu, B. (1984) *Adv. Carbohydr. Chem. Biochem.* **43,** 51–134
2. Jacques, L. B. (1979) *Science* **206,** 528–530
3. Lane, D. A., and Lindahl U. (eds) (1989) *Heparin: Chemical and Biological Properties, Clinical Applications*, Edward Arnold, London
4. Linhardt, R. J., and Toida, T. (1997) in *Carbohydrates as Drugs* (Witczak, Z. B., and Nieforth, K. A., eds), Marcel Dekker, New York, in press
5. Bourin, M.-C., and Lindahl, U. (1993) *Biochem. J.* **289,** 313–330
6. Höök, M., Bjork, I., Hopwood, J., and Lindahl, U. (1976) *FEBS Lett.* **66,** 90–93
7. Rosenberg, R. D., and Lam, L., (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 1218–1222
8. Lindahl, U., Backstrom, G., Höök, M., Thunberg, L., Fransson, L.-A., and Linker, A. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 3198–3202
9. Leder, I. G. (1980) *Biochem. Biophys. Res. Commun.* **94,** 1183–1189
10. Lindahl, U., Backstrom, G., Thunberg, L., and Leder, I. G. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 6551–6555
11. Casu, B., Oreste, P., Torri, G., Zoppetti, G., Choay, L., Lormeau, J.-C., Petitou, M., and Sinaÿ, P. (1981) *Biochem. J.* **197,** 599–609
12. Sinaÿ, P., Jacquinet, J.-C., Petitou, M., Duchaussay, P., Lederman, I., Choay, J., and Torri, G. (1984) *Carbohydr. Res.* **132,** C5–C9
13. Loganathan, D., Wang, H. M., Mallis, L. M., and Linhardt, R. J. (1990) *Biochemistry* **29,** 4362–4368
14. Yamada, S., Yoshida, K., Sugiura, M., Sugahara, K., Khoo, K.-H., Morris, H. R., and Dell, A. (1993) *J. Biol. Chem.* **268,** 4780–4787
15. Tsuda, H., Yamada, S., Yamane, Y., Yoshida, K., Hopwood, J. J., and Sugahara, K. (1996) *J. Biol. Chem.* **271,** 10495–10502
16. Lindahl, U. Backstrom, G., and Thunberg, L. (1983) *J. Biol. Chem.* **258,** 9826–9830
17. Atha, D. H., Stephens, A. W., and Rosenberg, R. D. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 1030–1034
18. Van Boeckel, C. A. A., and Petitou, M. (1993) *Angew. Chem. Int. Ed. Engl.* **32,** 1671–1818
19. Bae, J. H., Desai, U. R., Pervin, A. P., Caldwell, E. O., Weiler, J. M., and Linhardt, R. J. (1994) *Biochem. J.* **301,** 121–129

20. Faham, S., Hileman, R. E., Fromm, J. R., Linhardt, R. J., and Rees, D. C. (1996) *Science* **271,** 1116–1120
21. Pervin, A., Gallo, C., Jandik, K., Han, X.-J., Linhardt, R. J. (1995) *Glycobiology* **5,** 83–95
22. Lohse, D. L., and Linhardt, R. J. (1992) *J. Biol. Chem.* **267,** 24347–24355
23. Kim, Y. S., Lee, K. B., and Linhardt, R. J. (1988) *Thromb. Res.* **51,** 97–104
24. Linhardt, R. J., Grant, A., Cooney, C. L., and Langer, R. (1982) *J. Biol. Chem.* **257,** 7310–7313
25. Pervin, A., Al-Hakim, A., and Linhardt, R. J. (1994) *Anal. Biochem.* **221,** 182–188
26. Linhardt, R. J., Wang, H., Loganathan, D., and Bae, J. (1992) *J. Biol. Chem.* **267,** 2380–2387
27. Fromm, J. R., Hileman, R. E., Caldwell, E. E. O., Weiler, J. M., and Linhardt, R. J. (1995) *Arch. Biochem. Biophys.* **323,** 279–287
28. Wiseman, T., Williston, S., Brandts, J. F., and Lin, L.-N. (1989) *Anal. Biochem.* **179,** 131–137
29. Connelly, P. R., Varadarajan, R., Sturtevant, J. M., and Richards, F. M., (1990) *Biochemistry* **29,** 6108–6114
30. Freire, E., Mayorga, O. L., and Straume, M. (1990) *Anal. Chem.* **62,** 950A-959A
31. Desai, U. R., Wang, H. M., and Linhardt, R. J. (1993) *Arch. Biochem. Biophys.* **306,** 461–468
32. Desai, U. R., Wang, H. M., and Linhardt, R. J. (1993) *Biochemistry* **32,** 8140–8145
33. Linhardt, R. J. (1994) in *Current Protocols in Molecular Biology, Preparation and Analysis of Glycoconjugates*, (Varki, A., ed.) Vol. 2, pp. 17.13.17–17.13.29, Wiley Interscience, New York
34. DeLauder, S., Schwarz, F. P., Williams, J. C., and Atha, D. H. (1992) *Biochem. Biophys. Acta* **1159,** 141–149
35. Edens, R. E., Fromm, J. R., Fromm, S. J., Linhardt, R. J., and Weiler, J. M. (1995) *Biochemistry* **34,** 2400–2407
36. Linhardt, R. J., Rice, K. G., Merchant, Z. M., Kim, Y. S., Lohse, D. L. (1986) *J. Biol. Chem.* **261,** 14448–14454
37. Thunberg, L., Backstrom, G., and Lindahl, U. (1982) *Carbohydr. Res.* **100,** 393–410
38. Ototani, N., Kikuchi, M., and Yosizawa, Z. (1981) *J. Biochem.* (*Tokyo*) **90,** 241–246
39. Ototani, N., and Yosizawa, Z. (1981) *J. Biochem.* (*Tokyo*) **90,** 1553–1556
40. Ototani, N. Kikuchi, M., and Yosizawa, Z. (1981) *Biochem. J.* **205,** 23–30
41. Rice, K. G., and Linhardt, R. J. (1989) *Carbohydr. Res.* **190,** 219–233