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Note

Structure elucidation of a novel acidic tetrasaccharide and hexasaccharide derived from a chemically modified heparin *

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In the course of development of potentially more bioactive forms of heparin[1] for use as an anticoagulant, we had occasion to prepare a modified heparin first reported by Jaseja et al.[2]. Treatment of unfractionated bovine lung heparin with an alkaline solution at high temperature (105–110°C) for 24 h, according to method developed by Rej and Perlin[3], results in a modified heparin. To structurally characterize the sample of modified heparin, it was depolymerized with heparinase (heparin lyase I, EC 4.2.2.7). This resulted in the isolation of the hitherto unreported tetrasaccharide and hexasaccharide. This note describes the isolation and structure elucidation of two novel oligosaccharides. The solution conformation of these oligosaccharides may aid in developing a better understanding of the conformational preferences of the uronic acid residues.

Treatment of heparin with a sodium carbonate solution according to the method developed by Perlin and co-workers[2,3] results in a modified heparin. However, because heparin is a polydisperse microheterogeneous mixture, the modified heparin is also a mixture, and thus its precise structure was ill-defined. The ^{1}H NMR spectrum of the intact modified polymer indicated the presence of

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* Abbreviations include the following: SAX-HPLC, strong anion-exchange high performance liquid chromatography; FABMS, fast atom bombardment mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; PAGE, polyacrylamide gel electrophoresis; TSP, sodium trimethylsilylpentanoate-2,2,3,3,4,4-d_6; AUFS, absorbance units full scale, NOE, nuclear Overhauser effect; 2D, two dimensional; COSY, correlated spectroscopy; HETCOR, heteronuclear correlated spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; ROESY, rotating frame nuclear Overhauser enhancement spectroscopy; FID, free-induction decay.

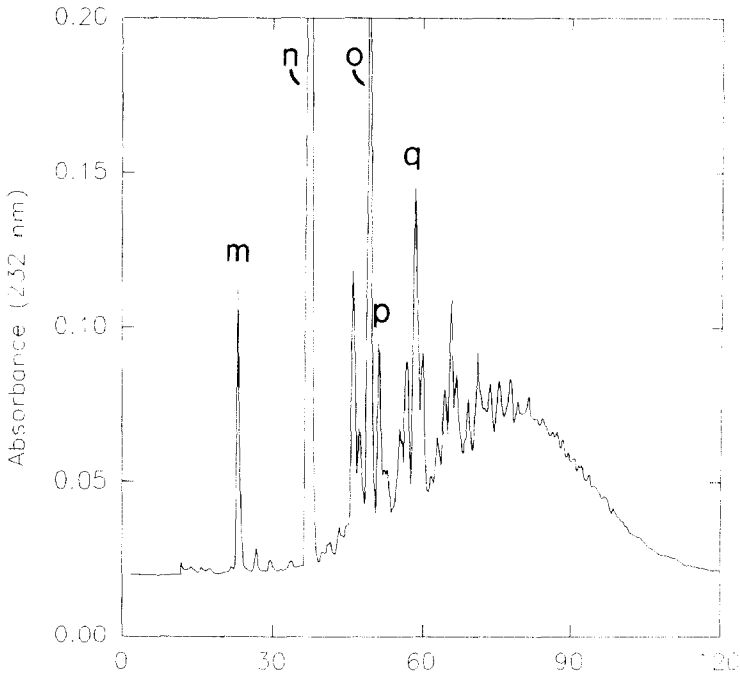

Fig. 1. SAX-HPLC chromatogram of the heparin lyase I depolymerized, modified, heparin sample.

modified uronic acid residues, but it also showed the presence of at least 25% of intact α-L-idopyranosyluronic acid residues. To better define the structure of the modified polymer, it was treated with heparin lyase I, and enzymic depolymerization was monitored by UV spectroscopy. When the reaction had reached ~ 21% completion no further depolymerization was observed. This result is consistent with the reduced sensitivity of this modified heparin towards heparinase reported by Rej and Perlin[2]. Gradient PAGE analysis of the heparinase-depolymerized, modified heparin indicated the presence of significant quantities of higher oligosaccharides in addition to the expected di-, tetra-, and hexa-saccharides. Five oligosaccharides (peaks m, n, o, p, and q in Fig. 1) could be isolated by SAX-HPLC representing 1.2% of the total mass of the modified polymer. Analysis of the oligosaccharides, corresponding to peaks m, n, o, p, and q, using analytical SAX-HPLC showed each to have a purity > 95%.

Peaks m, n, and p were subjected to analyses by ^{1}H NMR spectroscopy, and the spectra for these compounds were found to be superimposable with the spectra of compounds **1**, **2**, and **3** (Fig. 2) already reported by our group[4]. Compounds **1**, **2**, and **3** represent residues in the heparin chain which have escaped modification and account for ~ 50% of the isolable mass. Peaks o and q had distinctly different retention times on the SAX-HPLC column as compared with the retention times of known standards prepared in our laboratory[5,6]. Their elution profiles suggested

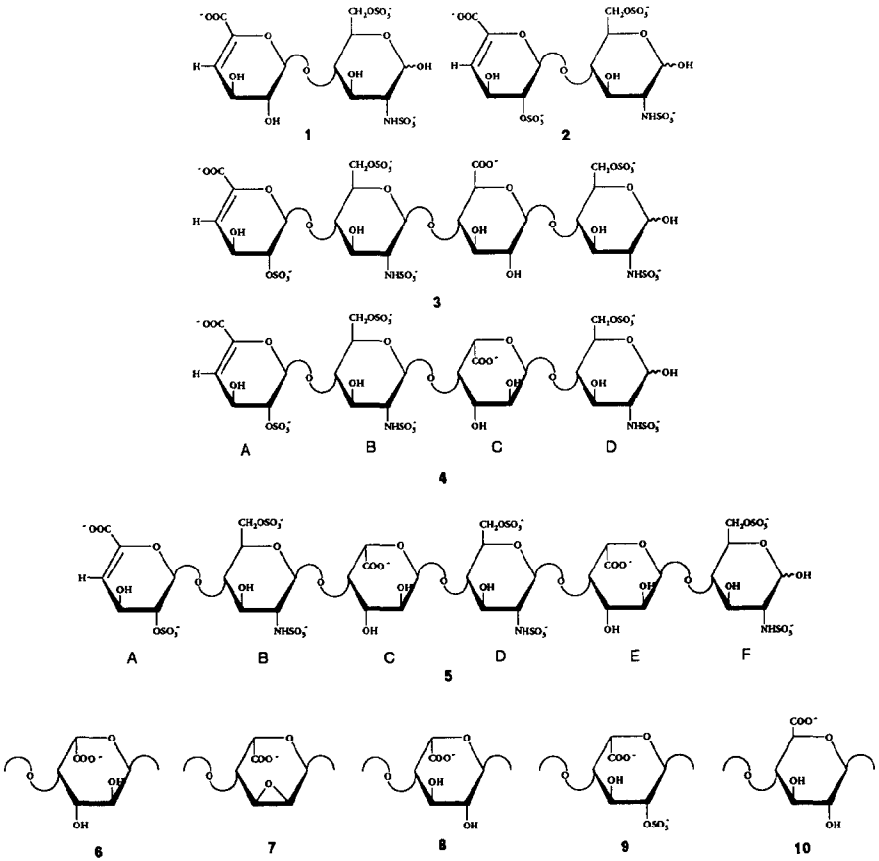

Fig. 2. Oligosaccharides (**1–5**) derived by the action of heparin lyase I (EC 4.2.2.7) on modified heparin substrate. Structures **6–10** have been reported in modified heparin[2,3].

that they were a tetrasaccharide and hexasaccharide with five and seven sulfate groups, respectively. The ^{1}H NMR spectrum of the peak o (Fig. 3a) clearly indicated the presence of four anomeric protons in addition to the proton signal at C-4 of the unsaturated uronic acid residue at the nonreducing terminus. Absence of the acetate CH_3 signal indicated that the compound was an *N*-sulfated tetrasaccharide. A homonuclear COSY spectrum provided the necessary connectivities for the assignment of all protons. Starting from the most deshielded proton of the unsaturated uronic acid at the nonreducing end (Fig. 4), the complete spin system for the A-ring could be identified. Similarly, the spin systems associated with the glucosamine and the internal uronic acid residues could also be identified. Sequence information was obtained through a transient NOE experiment (Fig. 5) in which the interresidue protons at the 1- and 4-positions could be identified. H-1 of the C-ring at 5.22 ppm showed a through-space coupling with a proton at 3.70 ppm corresponding to H-4 of the D-ring. Similarly, H-1 of the B-ring was found to

have a dipolar coupling with proton H-4 of the C-ring. Although this NOE experiment resulted in sequence information, it was not possible to determine the precise structure of the internal uronic acid residue. In their studies on modified heparin, Rej and Perlin[3] report the formation of either an epoxy uronic acid derivative (structure **7**, Fig. 2), or inversion of stereochemistry at the 2- and 3-positions (structure **6**), or a uronic acid residue having retention of the stereochemistry with the loss of a 2-sulfate group (structure **8**). The chemical shift of the H-1 of the C-ring (5.22 ppm) clearly showed that neither a nonsulfated idopyranosyluronic (**8**) nor a glucopyranosyluronic (**10**) acid residue is present in **4**. Since 2-sulfation causes the deshielding of the anomeric proton in uronic acids by ~ 0.2 ppm, a 2-sulfated α-L-idopyranosyluronic acid residue (structure **9**) could be ruled out. An inverse heteronuclear $^{1}H-^{13}C$ correlation (HETCOR) experiment (Fig. 6) showed that C-2 and C-3 of the internal uronic acid residue resonated at 72.5 and 73.7 ppm, respectively (Table I). The low chemical shift dispersion in the ^{1}H and ^{13}C signals at the 2-position and the 3-position of the internal uronic acid, together with the chemical shift of H-1 (5.22 ppm), suggested that the internal uronic acid was α-L-galactopyranosyluronic acid (**6**) and the structure of peak o (Fig. 1) was probably **4**. The presence of this residue was confirmed by a NOESY experiment (Fig. 5), which shows a weak crosspeak associated with protons at the 5-position and the 3-position, in addition to the expected dipolar couplings between H-4 and H-5, H-1 and H-2, and H-4 and H-3 of the C-ring. The absence of a dipolar coupling between H-2 and H-3 of the C-ring also suggests an *antiperiplanar* arrangement for the two protons. The ^{3}J coupling constant for H-1 of the C-ring is approximately 2.2 Hz implying a *synclinal* orientation of protons at the 1- and 2-positions. Since the stereochemical configurations of C-1 and C-2 of the C-ring are expected to remain unchanged in the chemical transformation, and the NOESY experiment establishes the relative stereochemistry of protons at the 2-, 3-, 4-, and 5-positions, the configuration of the internal uronic acid residue was established as α-L-galactopyranosyluronic acid. The negative ion FABMS spectrum of the compound showed the $[M - Na]^+$ ion peak at m/z 1205 ruling out the possibility of the epoxy derivative (structure **7**) which would have shown peaks at either m/z 1209 $[M]^+$ or 1187 $[M - Na]^+$. The ^{1}H NMR spectrum (Fig. 3b) of peak q in the SAX-HPLC, showed the presence of six signals that could be ascribed to anomeric protons, indicating it to be a hexasaccharide. Concerted application of 2D NMR techniques including COSY, inverse heteronuclear correlation, NOESY, and ROESY, in a manner described for tetrasaccharide **4**, led to the structured **5**.

The conformational analysis of α-L-idopyranosyluronic acid (**8** and **9**) has been the subject of study[7,8] in order to elucidate special features in the pentasaccharide binding sequence. The conformation of uronic acid residues is known to be significantly affected by the presence of sulfate groups within the residue as well as on adjacent residues. α-L-Idopyranosyluronic acid (**8**, **9**) exhibits conformational flexibility while β-D-glucopyranosyluronic acid (**10**) appears to be rather rigid[7,8].

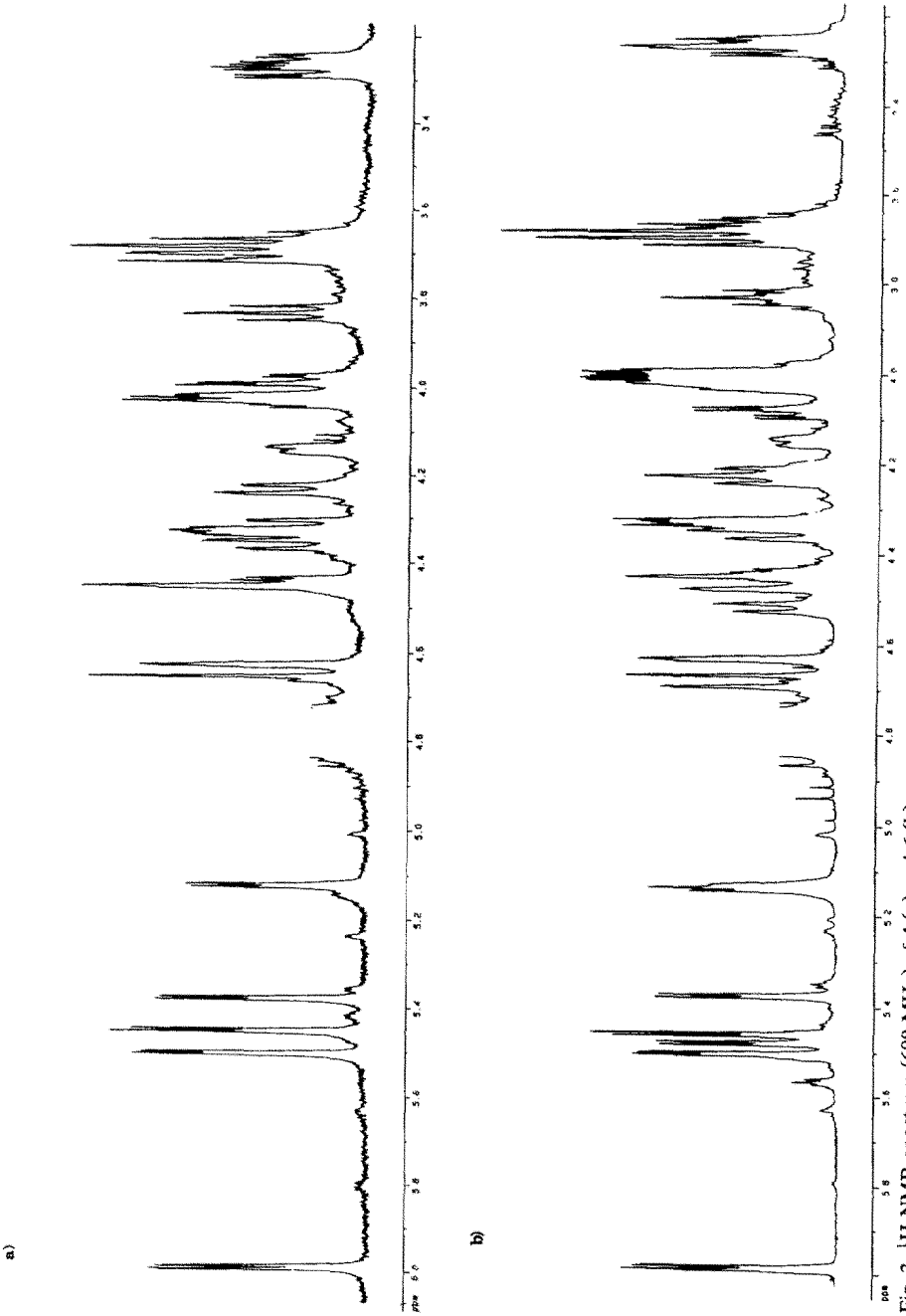

Fig. 3. ^{1}H NMR spectrum (600 MHz) of **4** (a) and **6** (b).

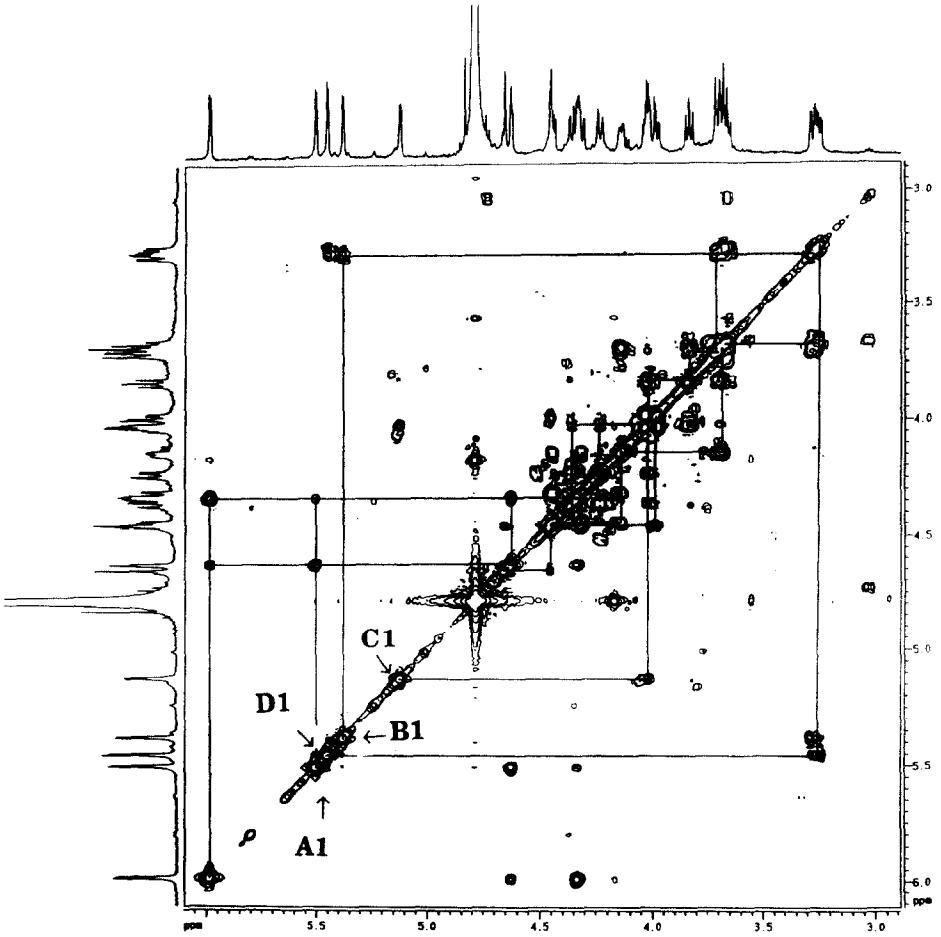

Fig. 4. The homonuclear COSY spectrum (600 MHz) for **4**. The appropriate connectivities can be followed by the lines drawn from the appropriate H-1 signal of the different rings. (A1 = H-1 of the A-ring, etc.). The connectivity pattern for the H-2 and H-3 of the C-ring is clear even though the signal dispersion is very low. The digital resolution was 1.6 Hz/pt.

Hence, it was important to analyze the conformational preference of the α-L-galactopyranosyluronic acid residue (**6**) and compare the differences. On performing a 2D *J*-resolved NMR experiment, coupling constants for most of the protons in compound **4** were obtained (Table II). The 2D *J*-resolved spectrum for compound **5** could not be analyzed because of severe overlap due to nearly equivalent chemical shifts. The coupling constants for the H-2 and H-3 (~ 10 Hz) of the α-L-galactopyranosyluronic acid residue of tetrasaccharide **4** indicate that the protons are *antiperiplanar*. The ^{3}J coupling constants of H-1 (2.2 Hz) and H-4 (2.5 Hz) suggest that the substituents at these positions are axially oriented. Hence, the internal α-L-galactopyranosyluronic acid ring is conformationally biased towards

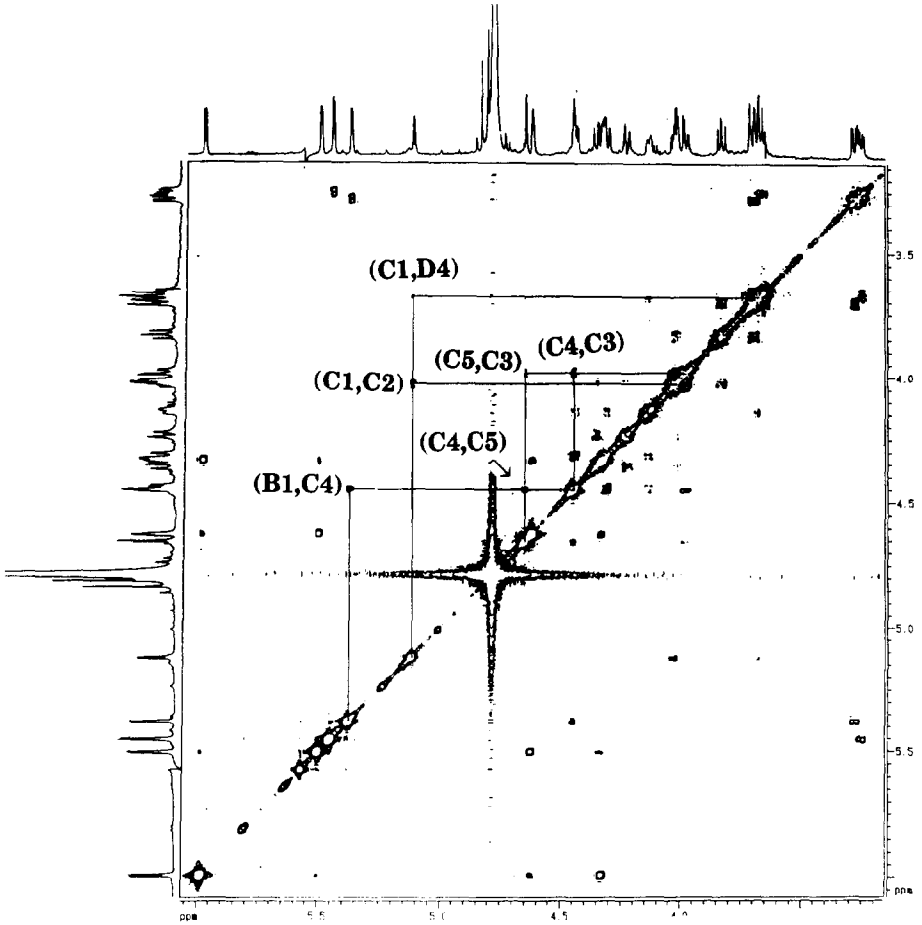

Fig. 5. The 2D transient NOE experiment for **4**. A presaturation of the HO^2H signal was avoided so as to not obscure the connectivities of the C-ring protons. (Note B1 = H-1 of the B-ring, etc.). Note the clear difference in intensity between the (C5, C3) and the (C4, C3) crosspeaks. Also the crosspeak for C1, C2 clearly shows the *synclinal* nature of the two protons.

the $^1C_4$ chair form (Fig. 7). The discrepancy observed in the ^3J coupling constants of H-1 and H-2 of the C-ring (Table II), probably arises due to the second ordered nature of these couplings, or due to the presence of a distorted $^1C_4$ chair form of an α-L-galactopyranosyluronic acid ring in relatively reduced proportion. The presence of the $^4C_1$ chair conformation, however, can be ruled out, by the ^3J coupling constant analysis. This conformational preference of the unsulfated α-L-galactopyranosyluronic acid residue for the $^1C_4$ chair form suggests that the presence of a sulfate group at the 2-position on the α-L-idopyranosyluronic acid residue brings about the conformational change to a distorted $^1C_4$ chair form rather than the expected $^4C_1$ chair form.

EXPERIMENTAL

Materials and methods.—Heparin (sodium salt) from bovine lung (167 U/mg), was obtained from Sigma Chemical Co., St. Louis, MO. Heparin lyase I (EC 4.2.2.7) was purified from *Flavobacterium heparinum* (5 mIU/mg)[9] or purchased (11 mIU/mg) from Sigma Chemical Co., St. Louis, MO. Sulfopropyl (SP) Sephadex C-50 was purchased from Sigma Chemical Co., St. Louis, MO, while Bio-Gel P-2 was from Bio-Rad Laboratories, Richmond, CA. SAX-HPLC was performed using a Shimadzu LC-7A dual pump system, and the detection system consisted of an

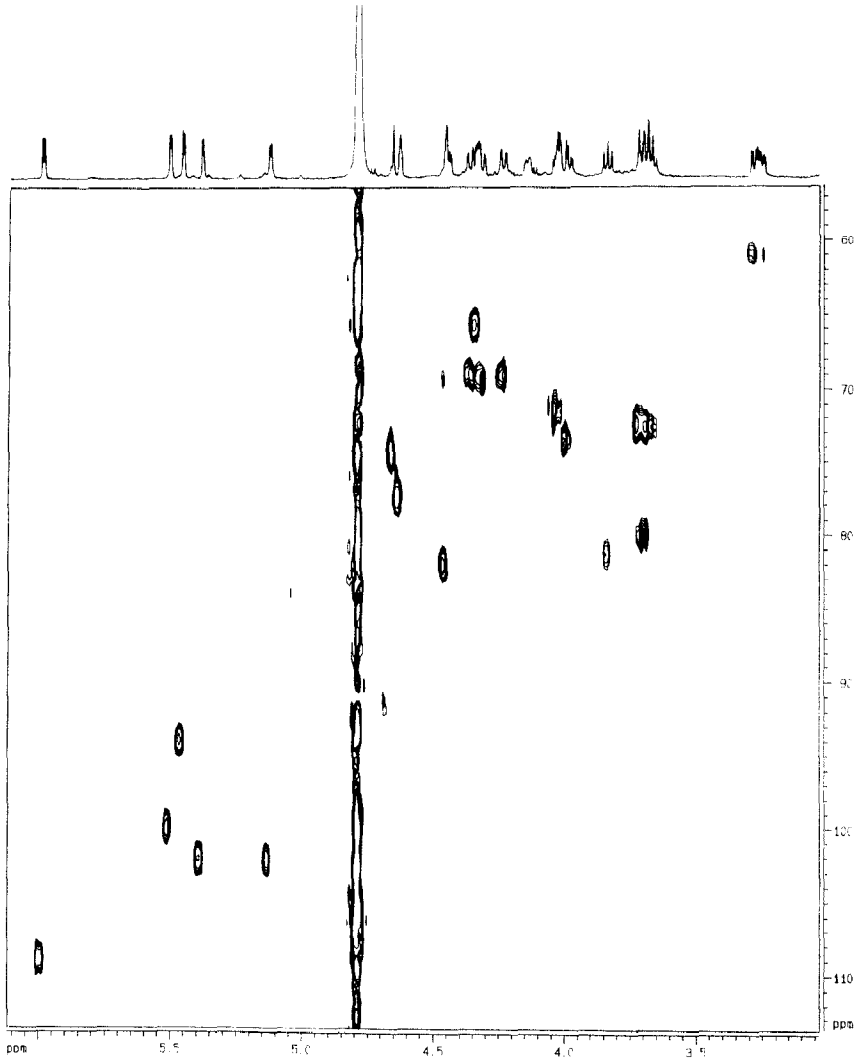

Fig. 6. The 2D inverse heterocorrelation (HETCOR) experiment for **4**.

TABLE I [a]

The ^{1}H and ^{13}C NMR chemical shifts of **4** and **5**

4					**5**						
Ring proton	A	B	C	D	Ring proton	A	B	C	D	E	F
1	5.51	5.37	5.22	5.45	1	5.50	5.48	5.14	5.38	5.12	5.46
2	4.62	3.27	4.05	3.26	2	4.63	3.25	4.09	3.30	3.98	3.27
3	4.34	3.75	4.00	3.67	3	4.33	3.67	3.99	3.64	4.00	3.70
4	5.99	3.83	4.46	3.70	4	5.99	3.69	4.47	3.70	4.44	3.69
5		4.02	4.62	4.13	5		4.14	4.67	4.02	4.69	4.02
6		4.23		4.32	6		4.32		4.23		4.21
6′		4.35		4.44	6′		4.43		4.35		4.51
Carbon					Carbon						
1	100.2	102.2	102.1	94.2	1	101.59	102.74	103.53	103.64	103.53	95.50
2	77.5	61.1	72.5	60.9	2	78.94	62.93	no	62.93	nf	62.34
3	65.8	72.5	73.7	72.9	3	67.32	73.98	nf	73.98	nf	73.98
4	109	81.5	82	79.9	4	110.27	81.08	82.05	no	83.84	no
5	nf	71.5	74.2	71.5	5	no	nf	75.83	nf	75.90	nf
6	nf	69	nf	69.5	6	no	70.94	nf	70.54	nf	70.81

[a] No, not observed; nf, not found due to signal overlap

TABLE II

The $^{3}J_{H,H}$ vicinal coupling constant (Hz) for **4** derived from the *J*-resolved NMR experiment

Ring proton	A	B	C	D
1	1.38, 3.08	3.52	2.19	3.6
2	1.38, 5.54	10.46, 3.69	4.00, 10.31	3.53, 8.31
3	4.66, 5.24, 3.08	8.31, 10.61	2.77, 10.05	9.54
4	4.66, 1.21	8.62, 10.16	2.46	10.01
5		10.31	2.37	m [a]
6		3.94, 10.62		10.62, 1.38
6′		1.69, 10.54		10.46, 1.38

[a] Multiplet not analyzable

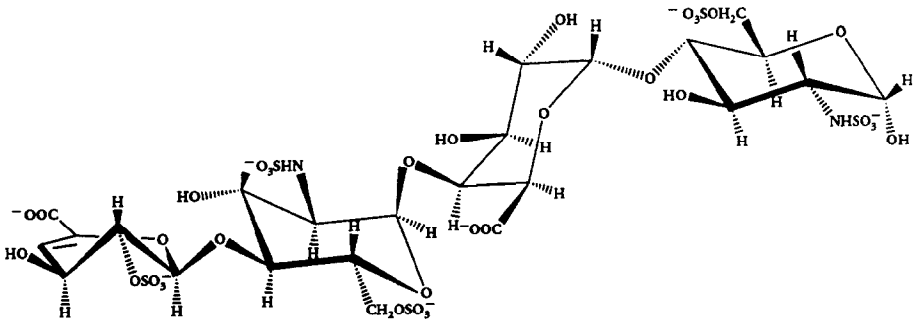

Fig. 7. The $^{1}C_4$ conformation of α-L-galactopyranosyluronic acid residue and a probable global conformation for **4** based on literature reports of the preferred conformations[7,8].

LKB Bromma 2141 variable wavelength detector operating at 232 nm. The injection system was a Rheodyne 7125 injector with a Spherisorb SAX-HPLC column from Phase Separations, Norwalk, CT. UV spectroscopy was performed with a Shimadzu UV-160 spectrophotometer, and mass spectrometry was carried out with a VG Analytical ZAB-HF spectrometer using ethanolamine as the matrix.

All ^{1}H and ^{13}C NMR experiments on tetrasaccharide **4** and hexasaccharide **5** were performed on a Bruker AMX 600 MHz spectrometer equipped with an X32 computer. Each oligosaccharide (~2 mg) was dissolved in $^{2}H_2O$ (0.5 mL) exchanged thrice from 99.96% $^{2}H_2O$, and 0.03% (w/v) TSP was added as internal standard. The final solution was filtered through a 0.45-μm membrane. The 2D NMR experiments were performed using standard Bruker software at 298 K. The COSY experiment utilized $\pi/2$–t_1–$\pi/2$–FID pulse program. The number of data points in the F_1-dimension were 256, each obtained with 16 scans. The FIDs were Fourier-transformed onto a data matrix of 1K × 1K with a sine-bell window function. The NOESY experiment utilized a $\pi/2$–t_1–$\pi/2$–τ_m–$\pi/2$–FID pulse program with 512 t_1, increments acquired with 32 scans each. The FIDs were Fourier-transformed onto a data matrix of 1K × 1K with a phase shifted sine-bell window function and the two frequency domains symmetrized. A τ_m value of 800 ms was found to provide the required connectivities in the NOESY experiments. The mixing time was randomly varied by 5% to suppress the *J*-connectivities. The inverse heteronuclear correlation (HETCOR) experiment was performed using standard Bruker software with 128 t_1 increments, each acquired with 104 scans. A recovery delay of 4.25 s was employed. The FIDs were Fourier-transformed onto a data matrix of 1K × 512W using sine-bell window function.

Base-catalysed modification of heparin.—A solution of bovine lung heparin (1 g) and sodium carbonate (0.45 g) in water (50 mL) was heated for 24 h at 105–110°C in an oil bath. After cooling, the solution was exhaustively dialyzed for 72 h against deionized, distilled water and freeze-dried to yield 653 mg of the modified heparin.

Enzymatic depolymerization of modified heparin.—The modified heparin (500 mg in 25 mL) was exhaustively depolymerized for 70 h at 30°C with 2.5 IU (1 IU = 1 μmol product formed/min) heparin lyase I (EC 4.2.2.7) in a 5 mM sodium phosphate buffer containing 0.2 M NaCl at pH 7.0. The reaction was monitored by removing aliquots and measuring absorbance at 232 nm, after a 1:100 dilution in 30 mM HCl. A constant absorbance value in the presence of active enzyme indicated that the reaction was complete. The reaction was then terminated by heating for 1 min at 100°C. The mixture was adjusted to pH 2.5 with concd HCl and passed through a 15 × 0.5 cm SP Sephadex column to remove protein. After readjusting to pH 7.0, the same was desalted on a 35 × 2.5 cm Bio-Gel P-2 column, freeze-dried, and reconstituted at 100 mg/mL in deionized, distilled water for SAX-HPLC purification step.

Purification of the oligosaccharide mixture with SAX-HPLC.—A semipreparative SAX-HPLC column[4] was preequilibrated with 0.2 M NaCl at pH 3.5. The desalted, reconstituted, enzymically depolymerized sample was loaded (75–100 mg

each injection) and eluted using a linear gradient (concentration (y, in M) at any time (x in s) = 0.0015x + 0.2) of NaCl at pH 3.5 at a flow rate of 6–8 mL/min. The elution profile was monitored by absorbance at 232 nm (0.5–1.0 AUFS). Fractions having the same retention times from different runs were combined, freeze-dried, desalted on Bio-Gel P-2 column, and freeze-dried to obtain five major oligosaccharide products.

CONCLUSIONS

Treatment of heparin with sodium carbonate solution at high temperature leads to a modified polymer that could be depolymerized by heparin lyase I (EC 4.2.2.7) leading to a mixture of oligosaccharides. A novel tetrasaccharide and hexasaccharide with internal α-L-galactopyranosyluronic acid residues were isolated and characterized by 2D NMR techniques. Complete assignment of ^{1}H NMR spectra and partial assignment of ^{13}C NMR spectra of these oligosaccharides was carried out. The conformational preference of the α-L-galactopyranosyluronic acid residue was deduced to be $^{1}C_{4}$ as determined by NOE as well as coupling constants analyses.

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