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Note

Evidence for a heparin derivative containing an *N*-sulfated aziridine ring that retains high anti-factor Xa activity [1]

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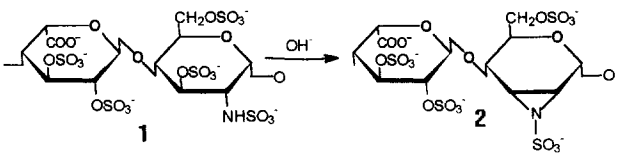
Abstract

A derivative of heparin **2** containing a high proportion of *N*-sulfated aziridine groups within the aminosugar units has been prepared from a chemically modified heparin **1** which contained the predominant repeat, → 4)-2-deoxy-2-sulfamino-α-D-glucose-3,6-bis sulfate-(1 → 4)-α-L-iduronic acid-2,3-bis sulfate-(1 →, and has been structurally characterized by ^{1}H and ^{13}C NMR employing COSY, ^{1}H coupled and decoupled HMQC, and FTIR spectroscopies. Compound **2** (16 kDa) exhibited high anti-factor Xa activity comparable to intact heparin as measured by end-point analysis.



Keywords: Heparin; Aziridine; *N*-sulfated aziridine; Anti-factor Xa activity

1. Introduction

Heparin, which has found widespread use as a blood anticoagulant in therapy and also possesses other potentially exploitable therapeutic properties, largely consists of regular sequences containing 2-deoxy-2-amino-D-glucose (D-glucosamine) and L-iduronic acid or D-glucuronic acid with a relatively constant pattern of sulfate substituents, attached to positions 2 and 6 of the glucosamine units and to position 2 of the iduronate residues. However, these chain segments are variously interrupted by sequences containing under-sulfated uronic acid and over- or under-sulfated amino sugar residues some of which are *N*-acetylated. There are a small number of

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[1] Abbreviations used: In the following sections, the *N*th proton or carbon atoms of the aminosugar residue are referred to as A-*N* and those of the iduronic acid residue as I-*N*.

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Table 1
Chemical shift assignments in ppm for **1** (*N*-acetylated derivative) and **2**, including selected coupling constants in Hz for **2**

Compound	**1** (*N*-acetylated)		**2**			
Assignment	^{1}H	^{13}C	^{1}H	^{13}C	$^{1}J_{CH}$	$^{3}J_{HH}$
A-1	5.14	98.2	5.39	91.6	186	
A-2	4.26	55.4	3.28	43.7	202	A2A3 ~ 4
A-3	4.61	79.6	3.02	41.5	198	A3A4 ~ 6
A-4	4.04	77.2	4.18	72.1	161	
A-5	4.06	72.4	3.83	68.2		
A-6	4.35	69.3	4.21	68.2		
	4.40		4.40			
I-1	5.38	102.0	5.31	102.4		
I-2	4.56	74.4	4.55	74.0		
I-3	4.79	72.7	4.90	69.7		
I-4	4.35	73.2	4.43	71.1		
I-5	5.14	70.6	5.08	70.5		
I-6	–	176.2	–	176.1		

glucosamine units which are additionally sulfated in position 3 and this residue is considered to be a marker for the pentasaccharide sequence which is essential for binding to antithrombin III [1]. Recently, much attention has been paid to investigations of synthetic or semi-synthetic oligosaccharides and mimetics and to the possibility of modulating or improving some of the activities of heparin itself or modifying other naturally occurring polysaccharides to provide alternative active compounds [2–4]. This article reports the structural elucidation and characterization of an unusual derivative **2**, which retains high anti-factor Xa activity that can be produced from an over sulfated heparin **1** containing the following predominant disaccharide repeat: → 4)-2-deoxy-2-sulfamino-α-D-glucose-3,6-bis sulfate-(1 → 4)-α-L-iduronic acid-2,3-bis sulfate-(1 → , in a one-step reaction. In contrast to other ring closure reactions that have been observed in heparin, most notably the formation of epoxides in the iduronate residues [5,6], this derivative differs in a modification within the glucosamine units.

2. Results

Compounds **1** and **2** were prepared as described in the Experimental section and the structure of **2** was elucidated by a combination of ^{1}H and ^{13}C NMR spectroscopy, FTIR spectroscopy, and chemical evidence as described below. The anti-Xa activity (U mg^{-1}), as determined by end-point analysis, of **1** is 59, of a de-*N*-sulfated version of **1** is 58, of intact heparin is 193 and of **2** is 189 (16 kDa samples in each case). Table 1 shows the NMR chemical shift data for the *N*-acetylated derivative of **1** and chemical shift and coupling constant data for **2**. A detail of the ^{1}H COSY spectrum of **2** demonstrating the connectivity from A-1 to A-4 is shown in Fig. 1. The ^{1}H and ^{13}C spectra of **2** are shown in Fig. 2a and b, respectively.

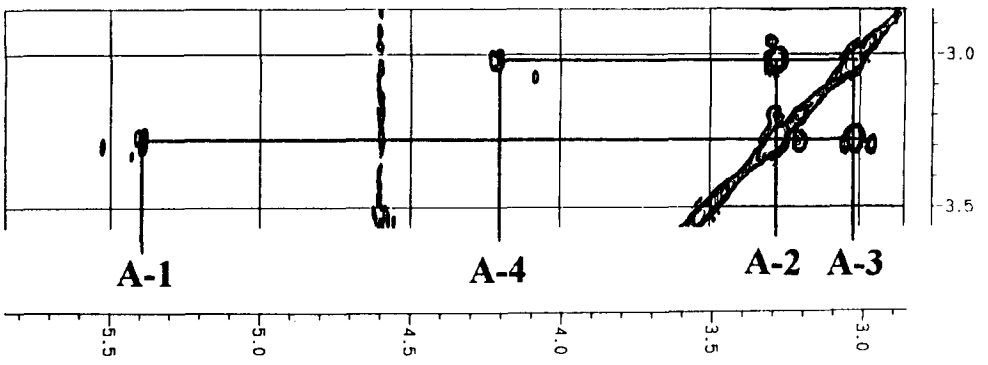

Fig. 1. Detail of the ^{1}H COSY NMR spectrum of **2** showing connectivities of A-1 to A-4 signals.

The ^{1}H COSY NMR spectrum of **2** (Fig. 1) exhibits a large up-field shift of the signals due to protons 2 and 3 (A-2 and A-3, respectively) of the glucosamine residues (at ~3.3 and ~3.0 ppm, respectively). These high field resonances are typical for small strained structures such as aziridines. For example, a small model aziridine compound (3-ethyl-2-methyl-*N*-phenylsulfonylaziridine) exhibited ring proton resonances at 2.72 and 2.90 ppm [7], while an unsubstituted 3,4-aziridine containing monosaccharide exhibited proton shifts for the two protons (C–H) of the three membered ring at 2.0 and 2.2 ppm and

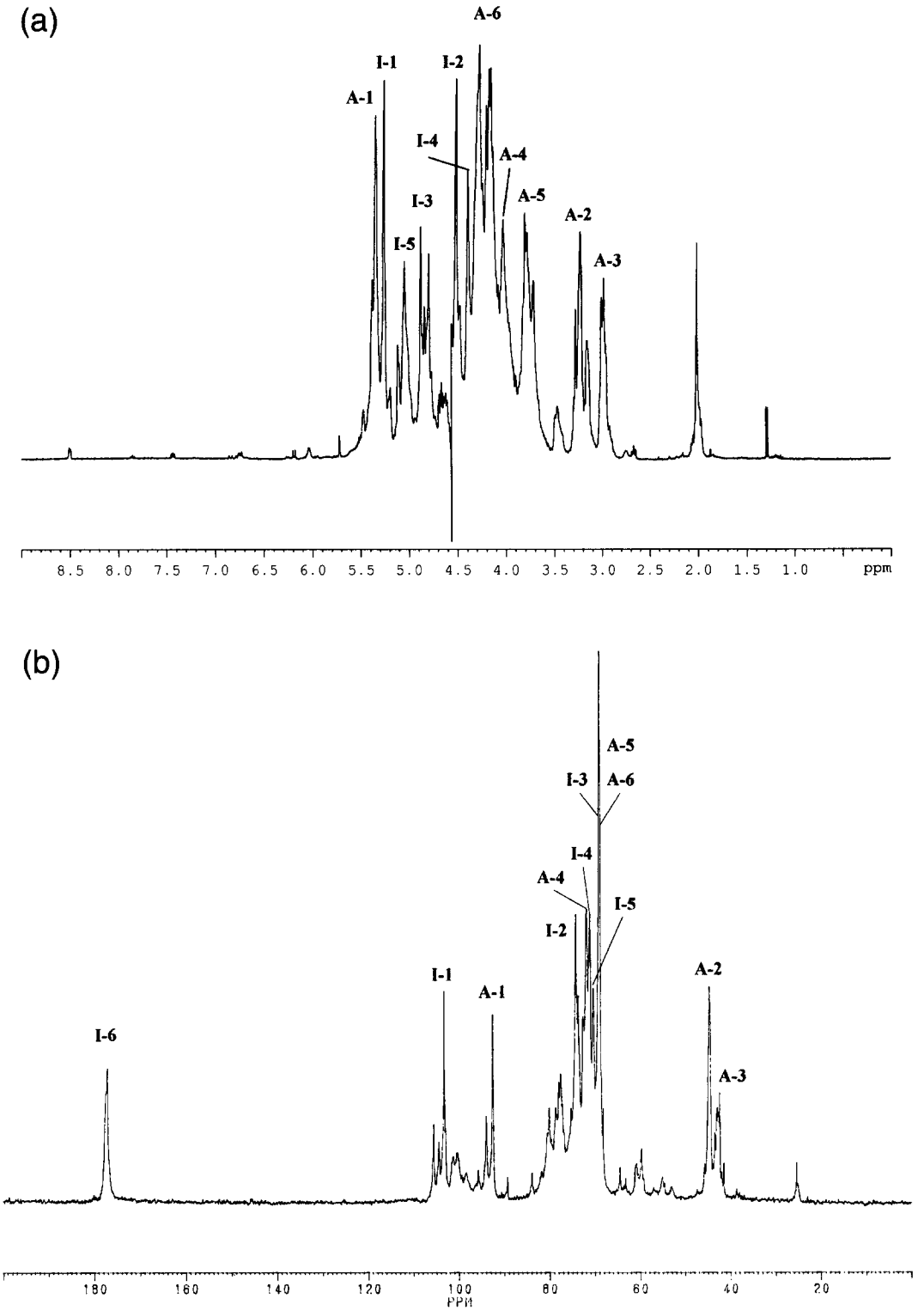

Fig. 2. (a) ^{1}H and (b) ^{13}C NMR spectra of a low molecular weight version of **2**.

for a trifluoroacetate substituted aziridine derivative the resonances were at 2.52 and 2.75 ppm [8] (personal communication Professor A. Vigevani, Pharmacia-Upjohn). $^{3}J_{H,H}$ coupling constants of A-2 and A-3 could not be measured in higher molecular weight samples of **2** (M_w, 16 kDa) but in a low molecular weight sample (M_w, 4.8 kDa) they were measured at ~4.5 and ~6 Hz which is consistent with values found in *cis*-aziridines [9] which also supports the assertion that an inversion of stereochemistry has occurred at C-3 of the aminosugar unit. The ^{13}C NMR spectrum of **2** (Fig. 2b) was assigned by the use of HMQC spectra. Notably, the resonances due to carbons 2 and 3 (A-2 and A-3) of the glucosamine residue are at high field (43.7 and 41.5 ppm, respectively), 3-ethyl-2-methyl-*N*-phenylsulfonylaziridine exhibits resonances at 40.4 and 46.6 ppm [7] and similar high field resonances have also been observed in monosaccharides containing 3,4-aziridine groups (personal communication Professor A. Vigevani) and at 53.5 and 54.5 ppm in analogous derivatives containing epoxide rings in the uronic acid residues [5,6]. The $^{1}J_{C,H}$ coupling constants for **2** were measured by coupled HMQC experiments at 202 and 198 Hz for A-2 and A-3, respectively, compared to 150 and 139 Hz in normal heparin, 149 and 140 Hz in **1** and are similar to those observed for I-2 and I-3 in the epoxide derivative of heparin which were both measured as 192 Hz in an authentic sample. These data are consistent with a small, strained ring structure.

The FTIR spectrum of **2** was assigned by comparison with a number of other spectra which were recorded on a range of standard heparin and other derivatives including totally de-acetylated, de-*N*- and de-*O*-sulfated, re-*N*-sulfated heparin, intact heparin, the starting material **1** and glucosamine *N*-sulfate (data not shown). The most notable features of this spectrum are the presence of the band at 1149 cm^{-1}, which can be attributed to the N–S stretch [10–12] of the *N*-sulfate group, and the band at 1362 cm^{-1} (O=S=O stretch).

Compound **2** gave a negative result to ninhydrin [13] but after hydrolysis with conc HCl (100 °C, 2 h) and removal of excess acid, it gave a strong positive result thereby demonstrating the presence of a primary amine group in the hydrolysed products. This can be interpreted as resulting from the opening of the aziridine ring (preceded by de-*N*-sulfation) to expose the primary amine function. In addition, attempts to re-*N*-sulfate **2** showed no change in the ^{1}H spectrum of the products, further supporting its fully substituted nature. When the aziridine reaction was attempted on a starting material containing glucosamine-3,6-di-*O*-sulfate (i.e with free amine groups at A-2) no change in the ^{1}H spectrum of the product was observed suggesting that the *N*-sulfate group is essential for the reaction to occur under these conditions. Furthermore, when a low molecular weight de-*N*-sulfated version of **2** was prepared, the spectra of the purified products revealed ^{1}H and ^{13}C chemical shifts for the signals due to A-2 and A-3 at 2.9 and 2.8 ppm and 36.7 and 34.2 ppm, respectively, corresponding to de-*N*-sulfated aziridine signals. Upon re-*N*-sulfation under standard conditions [14], chemical shifts identical with **2** were observed for A-2 and A-3, thereby further supporting the structure elucidation.

3. Discussion

The possibility that other, more open ring forms represent the structure of **2** seems to be unlikely because they do not produce such large up-field shifts and are inconsistent, for example, with the chemical shift data, observed coupling constant values and the FTIR results. For instance, cyclic sulfates typically exhibit ^{13}C chemical shifts in the 79–81 ppm region [15]. We conclude that the reaction of **1** under the conditions described involves an essentially quantitative conversion of glucosamine units bearing sulfate groups at A-3 and sulfamino groups at A-2 to the *N*-sulfated aziridine form present in **2**.

The high anti-Xa activity exhibited by a 16 kDa sample of **2** appears to be related to the formation of *N*-sulfated aziridine groups within the glucosamine units and is not due to active portions remaining in the starting material because **1** (and its free-amino precursor) exhibited only low anti-Xa activities. The exact nature and degree of specificity of the interaction between **2**, antithrombin III, and factor Xa remains unknown.

The simplest mechanism which can be proposed for this reaction involves an internal nucleophilic attack by the nitrogen atom attached to C-2 (A-2) upon C-3 (A-3) with the displacement of the *O*-sulfate group at C-3 resulting in an inversion of stereochemistry at C-3 to give D-*allo* configuration. It appears that under the conditions used here, the nitrogen requires a sulfamino group for activation because attempts to carry out this reaction with a free amino group at C-2 and *O*-sulfate at C-3 failed to produce any indication of the formation of an aziridine group. The stereochemistry of the nitrogen atom of the

aziridine group remains unknown but there is the possibility of an equilibrium between chiral forms resulting from nitrogen inversion [16].

The formation of an aziridine group allows the preparation of a wide range of possible derivatives for example via a range of ring opening reactions [17] or by deamination [18] to introduce a double bond and subsequent modification. Such compounds may prove interesting both in the preparation of labelled derivatives and in the search for alternative active structures and, from the pharmacological point of view, for structures which could survive longer in the system of a potential host because they are not so efficiently hydrolysed by physiological enzymes. The reaction may also be found to be applicable to a number of other amino sugar containing compounds. A number of other biological tests on **2** are currently in progress.

4. Experimental

Preparation of **1**.—Over-sulfation of heparin or heparin-like polysaccharides can be achieved by a number of methods [2,3]. In our experiments we prepared **1** by a variation of the method described [3,19] from totally de-*O*-sulfated, re-*N*-sulfated heparin (tetrabutylammonium salt form) by re-*O*-sulfation employing excess pyridine-sulfur trioxide complex (3 equivalents per free hydroxyl group) in DMF at a temperature of 55 °C for 16 h. After the reaction was complete, the reactants were cooled and the pH was adjusted with dilute aq NaOH to 9.0. The products were isolated and purified as described below to yield totally *O*-sulfated heparin which was then subjected to re-*N*-sulfation under standard conditions as described [14], purified as described below to yield **1** in essentially quantitative yield.

Preparation of **2**.—The formation of **2** occurred in essentially identical conditions to those reported for the formation of epoxide groups in iduronate residues [5]. Typically, **1** (250 mg) was dissolved in 0.1 M NaOH (25 mL) to give a concentration of 10 mg mL^{-1}, frozen, and the sample lyophilized. After lyophilization was complete, the sample was dissolved in 2 mL of distilled water and carefully neutralized with dilute HCl. The total volume was reduced by rotary evaporation and then purified as described below to yield **2**.

Purification of compounds.—The samples were subjected to dialysis (6 kDa cut-off membrane in the case of 16 kDa samples and 2 kDa in the case of 4.5 kDa samples) against distilled water (5 × 2 L). The dialysed polysaccharide material was then dried under reduced pressure and de-salted by gel filtration on a column of Sephadex G-25 eluting with an ethanol/degassed distilled water mixture (1/10, v/v). The eluted polysaccharide products and salts were detected by monitoring their absorbance at 215 nm. The polysaccharide containing fractions were pooled and their volume reduced by rotary evaporation. The product was then converted into its sodium salt form by cation exchange on a column of Dowex W-50 (H^+ form) followed by careful neutralization with sodium hydroxide, dried, and prepared for NMR analysis as described below.

NMR spectroscopy.—A sample of **2** was dissolved in high quality D_2O (containing low paramagnetic impurities, 99.99% D_2O) and exchanged three times with intermediate drying. Finally, the sample was dissolved in 0.5 mL of D_2O in a 5-mm tube (or in 2.0 mL of D_2O in a 10-mm tube in the case of one-dimensional ^{13}C experiments) and used for subsequent experiments. The ^1H (COSY and HMQC) spectra were obtained at 500 MHz with a Bruker AMX 500 spectrometer equipped with a 5-mm $^1H/X$ inverse probe and the ^{13}C spectra were obtained at 100 MHz using a Bruker AM400 instrument equipped with a 10-mm broad band probe. Chemical shift values were recorded downfield from external trimethylsilyl propionate (TSP) as standard at 40 °C. Mono-dimensional ^1H spectra were obtained with presaturation of the HDO signal with digitization of 0.1 Hz/point and mono-dimensional ^{13}C spectra were recorded using the standard Bruker sequence with powergated proton decoupling utilizing signal enhancement by the nuclear Overhauser effect. Proton spectra were assigned with the use of double quantum filtered COSY spectra with gradient enhancement [20] and were recorded employing a Bruker BGU (unit-*z*) with a maximum strength of 50 $G\,cm^{-1}$. The *z*-gradient was a square wave of 1 ms duration and with a maximum amplitude of 5, 5, and 15 $G\,cm^{-1}$. These gradient strengths were determined empirically to be the minimum required to completely eliminate the HDO signal in the sample. Data were acquired using 8 scans per series in 1 K × 512 W data points with zero filling in F1 and a squared cosine function was applied before Fourier transformation. ^1H–^{13}C chemical shift correlations were obtained with ^1H detection via gradient enhanced heteronuclear multiple-quantum coherence (HMQC) spectra [21] which employed a square wave with a maximum amplitude of 15, 20, and 15 $G\,cm^{-1}$. The first value was optimized to obtain a good

presaturation of the HDO signal in the sample. 16 scans were collected for each series in 1 K × 256 W data points before processing and were zero filled to 2 K × 512 W by application of a squared cosine function prior to Fourier transformation.

Anti-factor Xa assay.—Tests for anti-factor Xa activity were performed using a chromogenic end-point assay according to the method described [22] employing an Accucolour Heparin kit (Sigma). The data reported are the average of three tests.

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