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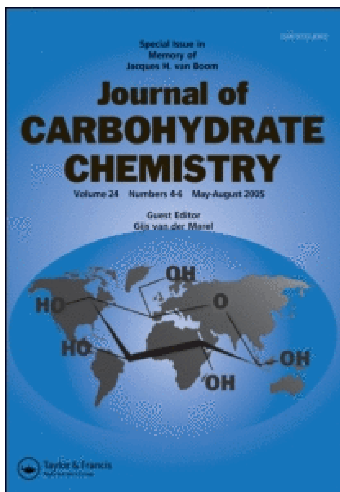

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F. Santini; G. Crini; C. Cosentino; L. Sturiale; E. A. Yates

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COMMUNICATION

INTRODUCTION OF A NITROGEN HETEROCYCLE INTO SULPHATED CHITOSAN OLIGOMERS

F.Santini, G.Crini, C.Cosentino, L.Sturiale and E.A.Yates.*

Istituto di Chimica e Biochimica "G.Ronzoni",
Via G.Colombo, 81 Milan 20133 Italy.

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Chitin is a linear polysaccharide consisting of [-4) 2-acetamido-2-deoxy-β-D-glucopyranose (1- and -4) 2-amino-2-deoxy β-D-glucopyranose $(1\text{-}]_n$ which occurs widely in nature and which after further de-*N*-acetylation yields chitosan. Chitin, chitosan and many of their derivatives have found applications in industry, medicine, pharmacology and food technology.[1] Many recent studies have reported modifications of chitosan in attempts to bestow upon it a variety of properties.[2-6]

This article reports the preparation of an unusual derivative of chitosan oligomers containing a nitrogen heterocycle which was obtained by depolymerisation of chitosan in sulphuric acid followed by a number of sulphation steps to obtain persulphated chitosan fragments **1-3**, treatment of the latter under strong basic conditions resulted in an unusual ring closure reaction to give an *N*-sulphonatoaziridine substituted product **4** (Scheme).

The ring closure reaction involves internal nucleophilic attack at C-3 of the glucosamine ring by the nitrogen atom attached at C-2 to cause inversion of configuration at C-3 giving a D-*allo* configuration product with formation of an *N*-sulphonatoaziridine ring. The nitrogen at C-2 requires an *N*-sulphoamino group for the reaction to proceed under these conditions.[7] The aziridine group in **4** is characterised by signals at high field for A-2 and A-3 (Table) in both ^{1}H and ^{13}C spectra and large $^{1}J_{CH}$ coupling constants at these positions.

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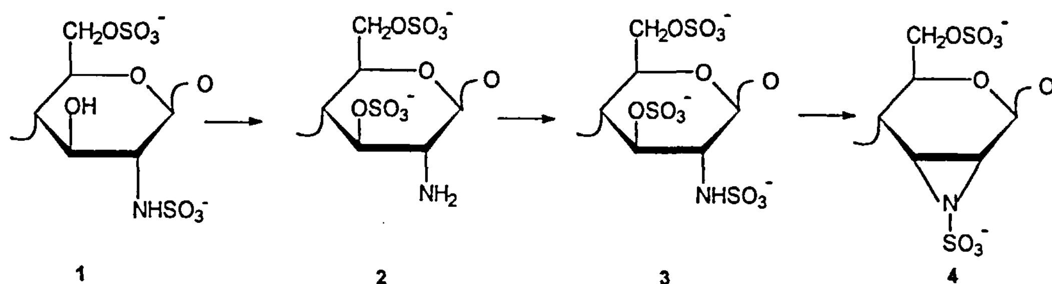

Scheme The predominant component monosaccharides of **1** to **4**.

Table ^{1}H and ^{13}C NMR chemical shifts for the predominant constituents of **2** to **4** and selected $^1J_{CH}$ coupling constant data for **4**.

	Assignment[a]					
compound	A-1	A-2	A-3	A-4	A-5	A-6
2	5.04	3.49	4.66	4.30	4.06	4.36-4.40
	98.7	57.5	78.4	75.4	75.1	69.1
3	4.65	3.41	4.42	3.90	3.85	4.31-4.39
	104.4	60.4	80.0	75.5	75.3	69.0
4	5.24	3.09	3.32	4.28	3.81	4.31-4.37
	99.5	42.2	44.4	74.8	71.0	69.0

$^1J_{C\text{-}2,H\text{-}2} = 189$ Hz
$^1J_{C\text{-}3,H\text{-}3} = 183$ Hz

a. A-*N* refers to the *N*th hydrogen or carbon atom of the aminosugar residue. Some signals are multiplets, in this case, the signals given refer to the centre of the peaks. Signals for A-6 are given as intervals.

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RESULTS AND DISCUSSION

A sample of chitosan was subjected to a series of *O*- and *N*-sulphation reactions to give the predominant repeating unit [-4) 2-deoxy-2-sulphoamino-3,6-di-*O*-sulphonato-β-D-glucopyranose $(1\text{-}]_n$ **3**. After treatment under basic conditions, the product **4**, consisting predominantly of [-4) 2,3-aziridino-2,3-dideoxy-*N*-sulpho-6-*O*-sulphonato-β-D-allopyranose $(1\text{-}]_n$ was formed. The ^{1}H and ^{13}C NMR data of **2** to **4** are given in the Table.

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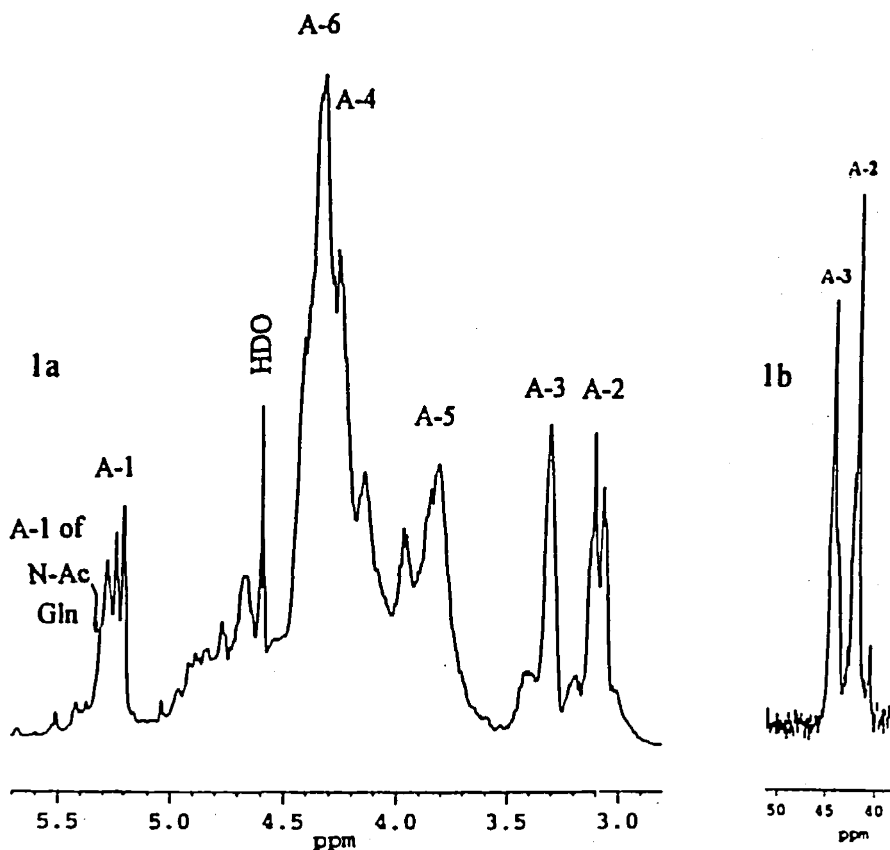

Figure 1(a) ^1H NMR spectrum of 4 with assignments of major signals. **(b)** Relevant section of the ^{13}C NMR spectrum of 4 showing characteristic high field positions of A-2 and A-3 signals.

Figure 1 shows the ^1H NMR spectrum of 4 with assignments and a relevant section of the ^{13}C spectrum.

The commercial chitosan starting material was characterised by ^1H NMR spectroscopy. The degree of acetylation was determined (at 338K to move the water signal to higher field) from the ratio between the integral of two anomeric protons of the glucosamine (4.88 ppm) and *N*-acetyl-D-glucosamine (4.62 ppm). This calculation indicated that 22% of the glucosamine residues of the starting material were *N*-acetylated and agreed with the value calculated from one-third of the integral for the acetyl signal at 2.1 ppm compared to the total of the integral for the anomeric signals.

6-*O*-sulphation was performed according to a literature procedure[8] and as expected resulted in concomitant depolymerisation to give **1** as a mixture of oligo- and polysaccharides. After neutralisation and dialysis the remaining product fraction was subjected to *O*- and *N*-sulphation reactions which produced no significant further depolymerisation, as determined by HPLC against a set of standards of known molecular weight.

In the final product **4**, 10% *N*-acetylation was indicated by ^{1}H NMR. This de-*N*-acetylation was presumably the result of the treatment of **3** with strong basic conditions[9] to form **4**, but products **3** and **4** both gave identical molecular weights (2.4 KDa, polydispersity 1.5). The reaction of **3** under basic conditions to form **4** does not therefore cause significant depolymerisation.

The FT IR spectrum of **4** showed significant peaks in the range 1000 to 1200 cm^{-1} at 1004, 1052, 1152 and 1237 cm^{-1}. The first two and the last of these are also present in the spectrum of a sample of 6-*O*-sulphonato chitosan[10] but the third can be assigned to the *N-S* stretch.[11-13]

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Notable features of the NMR spectra of **4** are the high field positions for carbons A-2 and A-3 (at 42.2 and 44.4 ppm) and protons A-2 and A-3 (at 3.09 and 3.32 ppm). In addition, the $^{1}J_{CH}$ coupling constants for these positions are large (189 Hz and 183 Hz, respectively) which is typical for small strained ring structures such as aziridines. The relative chemical shift positions of A-2 and A-3 are reversed compared with the same group in heparin[7] in which the glucosamine is α and not β linked as it is in chitosan. The $^{1}J_{CH}$ coupling constants in **4** are also slightly lower (189 and 183 Hz) than those found in the heparin derivative (198 and 202 Hz). These observations presumably reflect slightly different conformations in the two cases.

The *N*-sulpho function can be removed by treatment under standard solvolytic de-*N*-sulphation conditions[14] to give an unprotected aziridine group which is characterised by higher chemical shift values for carbons 2 and 3 at 37-39 ppm (results not shown). Upon re-*N*-sulphation under standard conditions,[15] the spectrum is identical with **4**, further demonstrating the substituted nature of the aziridine ring in **4**.

It is well known that the substituent attached to an aziridine nitrogen atom largely determines its susceptibility to nucleophilic attack.[16] In general, two groups of substituents have been defined: the first, which includes -COR, $-CO_2R$ and $-SO_2R$ tends to activate the aziridine group by stabilising the negative charge which develops on the nitrogen atom during nucleophilic attack; the second group, which includes such substituents as -H, alkyl and aryl, has the effect of deactivating the aziridine group. The negative charge on the *N*-sulphonato group of **4** may be expected to hinder the formation of a negative charge on the nitrogen during attack at C-2 or C-3 by a nucleophile

resulting in low reactivity. Deprotection of 4 followed by derivitisation with an activating derivative should open-up the possibility of a large number of derivatives.[16] Possible reactions may include acid catalysed ring opening or nucleophilic attack which could be used to generate structures with altered stereochemistry, labelled oligomers or to introduce a wide variety of functional groups or side chains.

EXPERIMENTAL

Chitosan (containing 20% *N*-acetylated glucosamine units by ^{1}H NMR) was supplied by Protan. All chemicals used were reagent grade or better. Pyridine sulphur trioxide complex (Fluka) was washed (cold distilled H_2O), filtered and dried over P_2O_5 before use. Dimethylformamide was stored over activated molecular sieves (4Å) before use. The starting material consisted of polymeric chitosan of undetermined molecular weight. All subsequent reactions were followed by gel permeation chromatography (GPC).

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Compounds were isolated by precipitation in a solution of ethanol saturated with sodium acetate, filtered, dialysed, purified by·GPC and subjected to cation exchange to form the sodium salt. The samples were exchanged with high quality D_2O (3 times) prior to NMR analysis. NMR chemical shift values were recorded downfield from external 3-trimethylsilyl propionic acid (TSP) as standard at 40 °C.

^{1}H NMR spectra were assigned with the use of double quantum filtered COSY spectra with gradient enhancement[17] 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 1K x 512 W data points with zero filling in F1, and a squared cosine function was applied before Fourier transformation. ^{1}H-^{13}C chemical shift correlations were obtained with ^{1}H detection via gradient enhanced heteronuclear multiple-quantum coherence spectra[18] which employed a square wave with a maximum amplitude of 15, 20 and 15 $G.cm^{-1}$. The first value was optimised to obtain a good presaturation of the HDO signal in the sample. 16 scans were collected for each series in 1K x 256W data points before processing and were zero filled to 2K x 512 W by application of a squared cosine function prior to Fourier transformation. The FT IR spectrum of 4 was recorded with a Bruker IFS-25 FT IR instrument utilising the standard software.

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Chemical Synthesis

(1) Chitosan was subjected to 6-*O*-sulphation according to the method described.[8,19] Following neutralisation, the product was isolated as described above and then subjected to re-*N*-sulphation under standard conditions.[15] The product was then purified to yield **1** and its structure verified [8,10,19-21] by NMR spectroscopy.

(2) Chitosan *N*-,6-*O*-disulphonato **1** was subjected to *O*-sulphation as follows; the tetrabutylammonium salt form of **1** (500 mg) was dissolved in DMF (10 mL) and to this solution an excess of pyridine sulphur trioxide complex (200 mg) was added. The solution was stirred at a temperature of 55 °C for 16 h, after which the reaction mixture was cooled and the pH adjusted to 9 with 1.0 M NaOH. The products were isolated and purified to yield **2** (274 mg).

(3) Compound **2** (274 mg) was re-*N*-sulphated according to the reported procedure.[15] After the reaction was complete, the polysaccharide products were retrieved and purified to give **3** in essentially quantitative yield.

(4) Compound **3** (250 mg) was dissolved in 0.5 M NaOH (25 mL) to give a concentration of 10 gL^{-1}, frozen and lyophilised. The products were isolated and were then re-*N*-sulphated as described above for the preparation of **3**. The compound was again subjected to the aziridine formation reaction (as described above for the preparation of **4**). This was carried out for two reasons; first, the re-*N*-sulphation step above (preparation of **3**) is rarely complete, presumably as a result of the presence of 3-*O*-sulphate groups adjacent to the amino group) and second, the basic conditions used during the preparation of **4** may cause further de-*N*-acetylation.[9] After the first reaction, the aziridine formation was estimated as 58% from ^{1}H NMR analysis and after two steps as 81 %. Of the remainder, 10% is *N*-acetylated. Other possible structures include free amine with either hydroxyl or *O*-sulphate at position-3, but no attempt was made to investigate the sequence further. It was found previously[7] that both sulphates at A-2 and A-3 are essential for this reaction to occur. The second re-*N*-sulphation step ensures that any free amino groups adjacent to 3-*O*-sulphates are themselves *N*-sulphated allowing maximum aziridine formation in **4** to take place. After two reactions, the products were isolated and purified to yield **4** (187 mg).

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