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PII: S0144-8617(96)00174-9

Carbohydrate Polymers 33 (1997) 145–151

0144-8617/97/$17.00 + .00

NMR characterization of *N*-benzyl sulfonated derivatives of chitosan

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(Received 6 June 1996; revised version received 14 October 1996; accepted 14 October 1996)

Chitosan was reacted with 2-formylbenzene sodium sulfonate and 4-formylbenzene sodium disulfonate in the presence of sodium cyanoborohydride to yield *N*-benzyl derivatives. One-dimensional and two-dimensional NMR spectroscopy were used for the characterization of both products. This study allowed us to assign all proton and carbon signals and confirmed the structure. ^{1}H NMR spectra showed that the parent polymer is 22% acetylated and that the overall degree of substitution is 83% and 47% for the monosulfonic and disulfonic derivatives, respectively. © 1997 Published by Elsevier Science Ltd

INTRODUCTION

Chitosan is a linear polysaccharide which is made from deacetylation of chitin (extracted from crustacea shells), and contains 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNac) and 2-amino-2-deoxy-β-D-glucopyranose (GlcN) residues. Commercial chitosans are water soluble at acidic pH and have become the subject of considerable interest in several aspects, including chemistry, pharmacy and biotechnology (Winterowd and Sandford, 1995; Terbojevich *et al.*, 1992; Focher *et al.*, 1990). For example, this natural amino polymer and its derivatives are able to form complexes with many of the transition metals. The chemical modification of chitosan by grafting benzyl sulfonic groups and the applications of these polymers, in the removal of metal ions when the polymer is in the form of insoluble powder or deposited on a nonwoven fabric as a filter, were reported (Weltrowski *et al.*, 1996; Martel *et al.*, 1996). The nuclear magnetic resonance (NMR) techniques are particularly useful for studying the structure and conformation of macromolecules such as polysaccharides (Varum *et al.*, 1991b; Hricovini *et al.*, 1995). So, in this paper, one-dimensional and two-dimensional NMR spectroscopy have been used to characterize our chitosan sulfonic powders.

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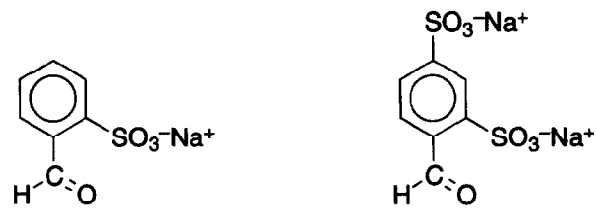
EXPERIMENTAL

Chemicals

Chitosan was supplied by Protan Inc.; the degree of acetylation as specified by the producer and confirmed by ^{1}H NMR is 22%. Sodium cyanoborohydride, 2-formylbenzenesulfonic acid, sodium salt dihydrate (**1a**), 4-formyl-1,3-benzenedisulfonic acid, disodium salt hydrate (**1b**) were Aldrich chemicals (Scheme 1).



1a **1b**

Scheme 1. Formyl benzene sulfonate derivates used for the synthesis of chitosan derivatives

NMR spectroscopy

Proton NMR spectra were measured at 500 MHz on a Bruker AMX 500 spectrometer equipped with a Bruker BGU gradient unit z system. Carbon NMR spectra were measured at 100.6 MHz on a Bruker AMX 400 spectrometer and at 75.47 MHz on a Bruker AC 300. The sample of underivatized chitosan was dissolved in 5% (w/v) 0.1 M CD_3COOD solutions in D_2O in a 5 mm tube. Chitosan sulfonate derivatives were dissolved in 0.1 M NaOD solutions (9 and 80 mg/ml for proton and carbon spectra respectively). All measurements were performed at 338 K. The chemical shift values were referenced to external sodium 3-(trimethylsilyl) propionate-d4 (TSP from Merck).

Two-dimensional (2D) double-quantum-filtered homonuclear correlation (DQ COSY) and 2D total correlation spectroscopy (TOCSY) were carried out in phase sensitive mode for quadrature detection in the F1 dimension using time proportional phase incrementation. The data matrix was 512×2 K (F1×F2) and the spectral width 2500 Hz. The spectra were recorded with presaturation of the residual HDO signal. The relaxation delay was 2 s followed by 2 s of presaturation, and 16 and 32 transients respectively for COSY and TOCSY experiment were accumulated per fid. The sensitivity enhanced gradient ^1H ^{13}C heteronuclear single quantum correlation (HSQC) experiment was used for the assignment of the carbon signals (Kay *et al.*, 1992). The delay τ is set to $1/(4J_{CH}) = 1.65$ ms. Four gradients pulses were applied along the z axis with strengths respectively of 20, 5, 20 and 5 G/cm, and a duration of 1 ms. The data matrix was 512×1 K with spectral widths 4000 Hz (F2) and 12 kHz (F1), respectively. A shifted ($\pi/3$) sine-bell-squared function was applied in both dimensions prior to Fourier transformation.

The pure-absorption heteronuclear multiple bond correlation (HMBC) experiment was performed with gradient pulse. The data matrix was 256×1 K with spectral widths 4000 Hz (F2) and 12 kHz (F1), using 64 scans for each point of F1. A delay of 45 ms was used for the evolution of long-range couplings. Three rectangular gradient pulses with strengths respectively of 15, 15 and 5 G/cm were used.

Synthesis of the sulfonate derivatives A1 and B1

The method was adapted from Hall and Yalpani, 1980: 5 g of chitosan were dissolved in 500 ml of 0.5% aqueous solution of acetic acid. 450 ml of methanol were added to the chitosan solution. 10 g of sodium cyanoborohydride were dissolved under vigourous stirring and 3 min later aldehyde **1a**, dissolved in 150 ml of methanol, was added. In the next 3 min the viscosity of the solution decreased and a white precipitate appeared. Stirring was maintained during the desired time at ambient temperature. The mixture was then filtered and the precipitate was washed successively with 3 liters of distilled water and 1 liter of acetone. The product **A1** was then dried overnight at 40°C. A light brown powder was obtained after crushing.

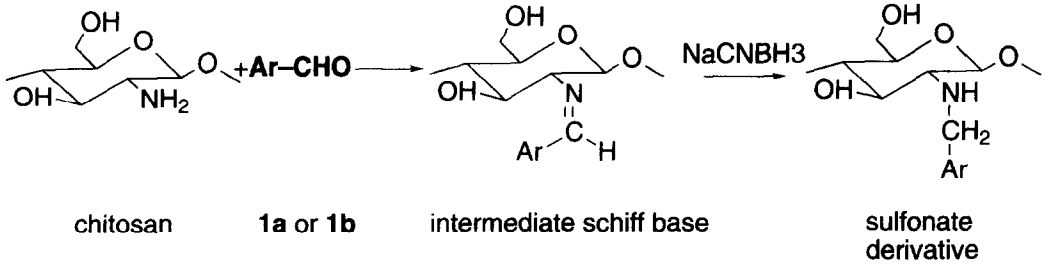
B2 compound was synthesized by a method similar to that applied to the monosulfonate derivative except that aldehyde **1b** was dissolved in 150 ml of distilled water. The amount of methanol in the initial solution was 650 ml. The modified polymer was washed with water and acetone by the centrifugation method. (See Scheme 2.)

RESULTS AND DISCUSSION

The degree of N-acetylation (DA) is an important characteristic of chitosan (Varum *et al.*, 1991b). Several methods such as elemental analysis, chromatography, titration, NMR, IR and UV spectroscopy have been used to determine the DA (Muzzarelli and Rochetti, 1986; Neugebauer *et al.*, 1989; Focher *et al.*, 1990; Varum *et al.*, 1991a).

The commercial chitosan used was characterized by ^1H NMR spectroscopy. The spectrum is shown in Fig. 1. The degree of acetylation was determined from the ratio between the integral of the two anomeric protons of the glucosamine (GlcN, 4.88 ppm) and N-acetylated glucosamine (GlcNac, 4.62 ppm). The spectrum was obtained at 338 K to move the water signal to a higher field (4.33 ppm). The calculated value was 22% of substitution, in agreement with the value obtained using the ratio between the integrals of the acetyl CH_3 and the anomeric signals.

The characterization of the two compounds was



Scheme 2. Synthesis of chitosan-N-benzyl sulfonate derivates by grafting aldehydes **1a** and **1b**.

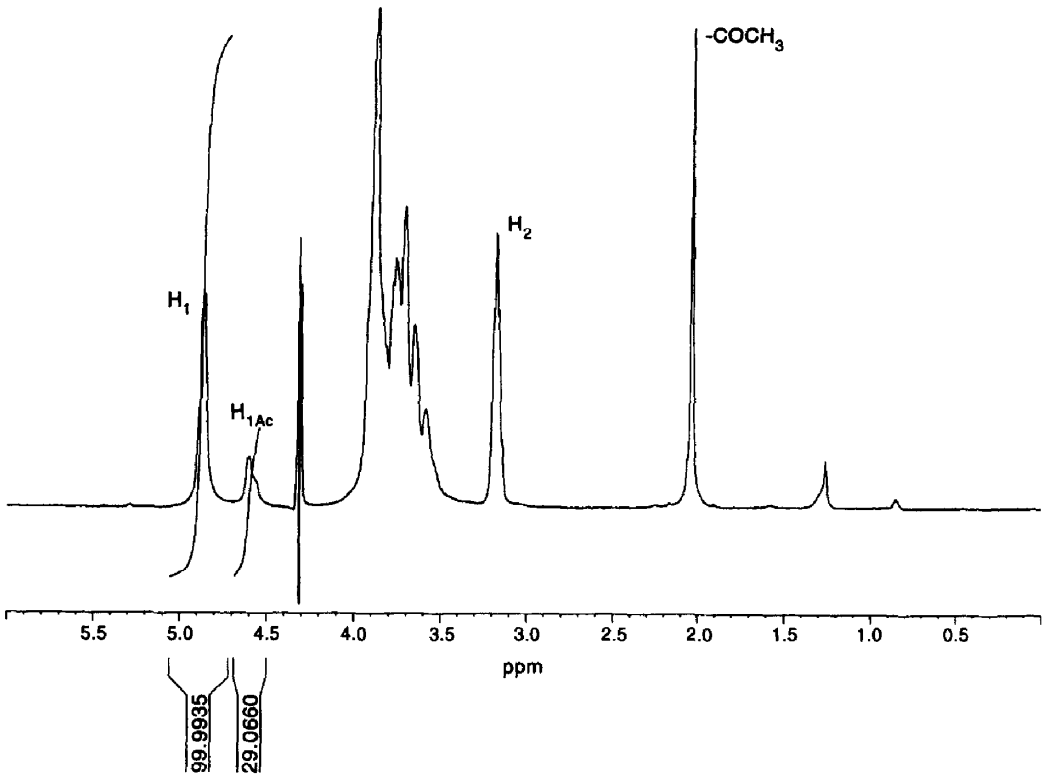


Fig. 1. ^{1}H spectrum of chitosan (CD_3COOD in D_2O, 338 K).

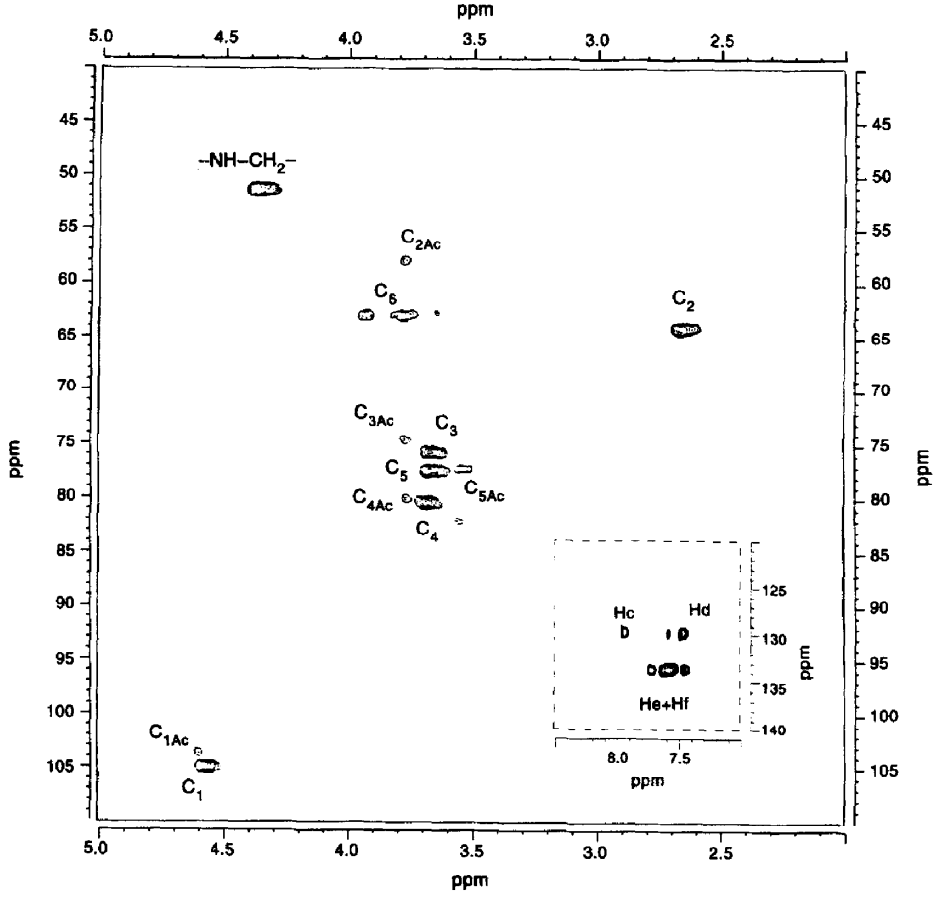


Fig. 2. ^{1}H–^{13}C heteronuclear correlation NMR spectrum of chitosan **A1** (NaOD, 338 K).

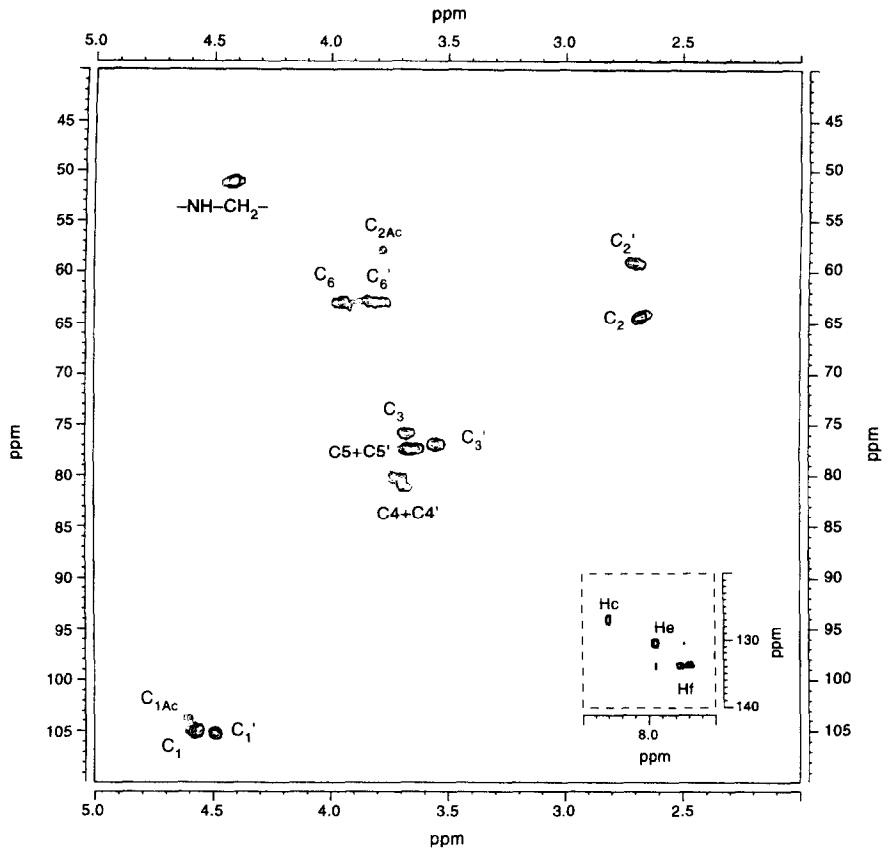


Fig. 3. ^{1}H–^{13}C heteronuclear correlation NMR spectrum of chitosan **B1** (NaOD, 338 K).

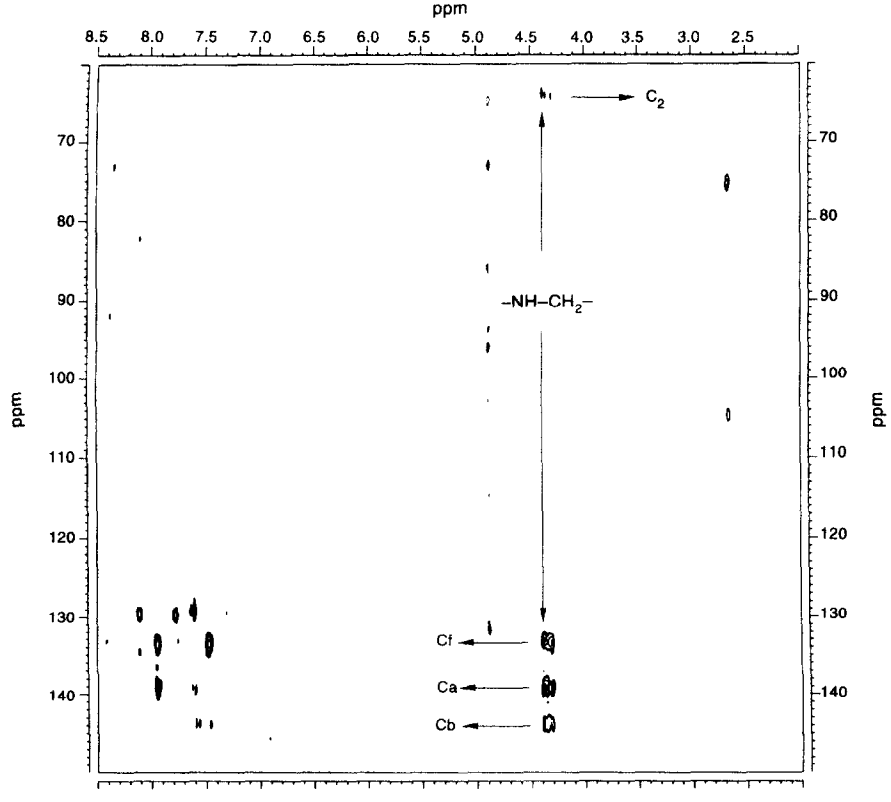


Fig. 4. HSQC spectrum of chitosan **A1** (NaOD, 338 K).

performed by 2D homonuclear and heteronuclear spectroscopy. COSY and TOCSY spectra (data not shown) permit only a few signals to be assigned because there is a large degree of signal overlap. In particular in both compounds the correlation H_1/H_2 is evident (4.60/3.78 ppm for compound **A1** and 4.59/3.77 ppm for compound **B1**), corresponding to GlcNAc units present in the chitosan. Compound **A1** presents another anomeric signal (H_1 4.57 ppm) correlated to H_2 at 2.65 ppm, while compound **B1** has two other anomeric signals at 4.56 and 4.48 ppm correlated to their H_2 signals at 2.67 and 2.69 ppm. These H_2 resonances are separated by very small frequency intervals and are very close to those of H_2 of the GlcN unit.

^1H–^{13}C correlation experiments permitted a resolution of overlap problem. The results are shown in Figs 2 and 3 for compounds **A1** and **B1** respectively. The spectrum resolution was improved because the signals were spread out into the 2D domain.

In compound **B1** two different C_2 signals at 59.1 and 64.2 ppm, corresponding respectively to unsubstituted and substituted glucosamine units, are evident. An important contribution to the characterization was achieved by using an HMBC experiment. In Fig. 4 the partial portion of the 2D HMBC spectrum of compound **A1** is shown. The real substitution was demonstrated by the correlations between the CH_2 protons of the benzylsulfonate group (4.34 ppm) with the C_2 of glucosamine and aromatic carbons.

The ^1H NMR spectra of **A1** and **B1** derivatives are shown in Figs 5 and 6 respectively. The compound **A1** exhibits the GlucN units completely substituted by *N*-benzyl sulfonate groups while compound **B1** is partially substituted.

For compound **A1** the degree of substitution of the non-acetylated amine group was calculated from the ratio between the area of proton H_d of the aromatic signal and of proton H_2 of the GlucN unit. The value was 1:1; this confirms the total substitution of GlucN units. By comparing the integral of H_d to the total area of H_2 (non-acetylated) plus one-third of the area of the acetyl CH_3 signal we have found the overall degree of substitution to be 83% (80% from the elemental analysis). Therefore, 17% of the glucosamine units carry acetyl groups.

For compound **B1** the degree of substitution with acetyl groups was determined as for **A1** and was found to be 18%. The non-acetylated H_2 protons of glucosamine exhibit two very similar chemical shifts (H_2 and H_2'). The degree of substitution at this position relative to the total non-acetylated glucosamine was calculated from the ratio between the area of H_f' and ($H_2 + H_2'$) and was found to be 57%. The degree of substitution overall was calculated from the ratio between the areas of the signals of H_f' and ($H_2 + H_2' + 1/3$ of the area of the acetyl CH_3 signal) and was found to be 47% (50% from the elemental analysis).

The discrepancy between the degree of substitution with acetyl groups given by the manufacturer (22%) and that found by our calculation (18%) is most likely to be caused by *N*-deacetylation during the reaction.

Tables 1 and 2 show the complete assignment of the resonances of the ^1H and ^{13}C NMR spectra obtained for the compounds **A1** and **B1** respectively.

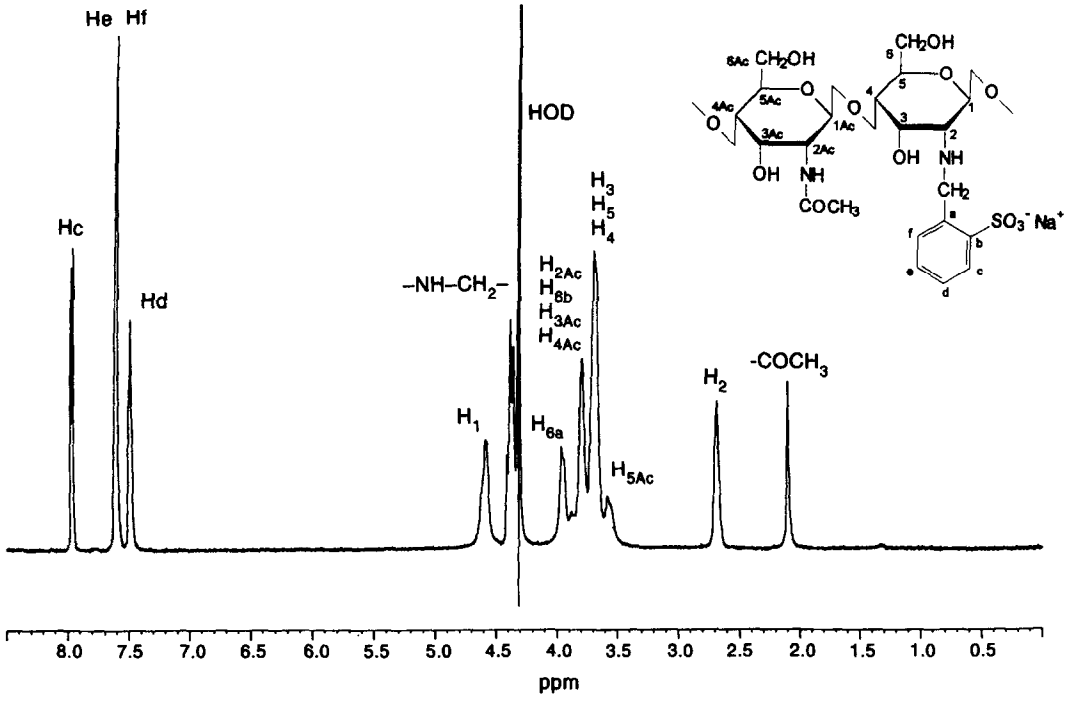


Fig. 5. ^1H spectrum and structure of chitosan **A1** (NaOD, 338 K).

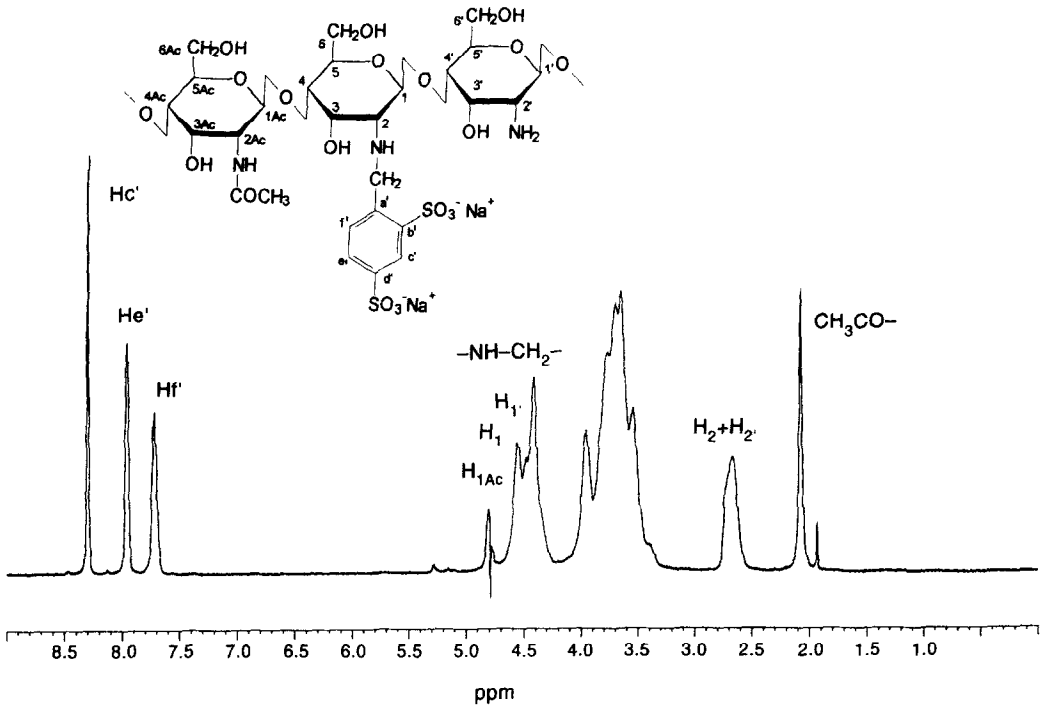


Fig. 6. ^{1}H spectrum and structure of chitosan **B1** (NaOD, 303 K).

Table 1. C/H correlation and assignments of A1 chitosan derivatives

Carbons	(ppm)	Hydrogens	(ppm)
C_1Ac	103.22	H_1Ac	4.60
C_2Ac	57.80	H_2Ac	3.78
C_3Ac	74.32	H_3Ac	3.77
C_4Ac	79.76	H_4Ac	3.78
C_5Ac	77.32	H_5Ac	3.51
C_6Ac	62.79	H_6Ac	3.94–3.92
			3.79–3.77
CH_3	24.39	CH_3	2.06
C=O	175.89		
C_1	104.91	H_1	4.57
C_2	64.00	H_2	2.65
C_3	75.48	H_3	3.66
C_4	14.14	H_4	3.68
C_5	77.32	H_5	3.66
C_6	62.79	$H_{6a,b}$	3.94–3.92
			3.79–3.77
C_a	141.70		
C_b	144.20		
C_c	129.13	H_c	7.95
C_d	129.68	H_d	7.47
C_e	133.44	H_e	7.59
C_f	134.22	H_f	7.59
CH_2	51.23	CH_2	4.34

CONCLUSION

This study allowed us to identify, characterize and confirm the structure of chitosan *N*-benzyl sulfonate derivatives. The complete assignments of the ^{1}H and ^{13}C resonances were obtained with the assistance of 2D NMR experiments. Preliminary nOe experiments have demonstrated the presence of an interaction between H_f' of the benzylsulfonate group (compound **B1**) and protons belonging to the sugar ring. There is also the possibility of interactions between the amino group of the polymer and the sulfonate group in the ortho position of the grafted moiety (Weltrowski *et al.*, 1996). Both of these aspects are being investigated. The

Table 2. C/H correlation and assignments of B1 chitosan derivatives

Carbons	(ppm)	Hydrogens	(ppm)
C_1Ac	103.70	H_1Ac	4.59
C_2Ac	57.80	H_2Ac	3.77
C_3Ac	74.40	H_3Ac	3.75
C_4Ac	80.20	H_4Ac	3.71
C_5Ac	77.40	H_5Ac	3.66–3.63
C_6Ac	62.80	$H_6Ac_{a,b}$	3.95–3.92
			3.82–3.78
CH_3	24.52	CH_3	2.02
C=O	176.78		
C_1'	105.10	H_1'	4.48
C_2'	59.10	H_2'	2.69
C_3'	76.80	H_3'	3.54
C_4'	80.20	H_4'	3.71
C_5'	77.40	H_5'	3.66–3.63
C_6'	62.80	$H_{6'a,b}$	3.95–3.92
			3.82–3.78
C_1	104.90	H_1	4.56
C_2	64.20	H_2	2.67
C_3	75.80	H_3	3.67
C_4	81.00	H_4	3.68
C_5	77.40	H_5	3.66–3.63
C_6	62.80	$H_{6a,b}$	3.95–3.92
			3.82–3.78
C_a'	141.93		
C_b'	144.40		
C_c'	126.90	H_c'	8.30
C_d'	144.30		
C_e'	130.40	H_e'	7.94
C_f'	134.23	H_f'	7.69
CH_2	51.06	CH_2	4.41

conformation of these polymers is also being determined by means of NOESY and relaxation experiments.

ACKNOWLEDGEMENTS

The authors thank Dr Edwin Yates for useful discussions about the manuscript and Mr Cesare Cosentino (Istituto Ronzoni, Milan) for his technical assistance during this study.

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