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NMR investigation of chitosan derivatives formed by the reaction of chitosan with levulinic acid

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Abstract

Chitosan derivatives are obtained by reaction of chitosan with a low degree of acetylation and levulinic acid under different experimental conditions. The chemical structure of the different derivatives obtained is determined using ^{1}H and ^{13}C NMR spectroscopies. The intrinsic viscosity is used to follow the molecular weight evolution. Finally, conditions are described in which water-soluble *N*-carboxybutylchitosan is obtained. In particular, the time of the reduction step and the ratio between reagents are investigated. Under mild conditions and short times of reduction there is a very low degree of substitution and only the monocarboxybutylchitosan is formed. The dicarboxylated form is never observed. The cyclic derivative (5-methylpyrrolidinone chitosan) is obtained when the reducing agent is added slowly to the reactants.

Keywords: Chitosan; Levulinic acid; *N*-carboxybutylchitosan; 5-methylpyrrolidinone chitosan

1. Introduction

N-carboxybutylchitosan is, along with carboxymethylchitosan, one of the few chitosan derivatives water-soluble in neutral conditions previously described in the literature (Le Dung, Milas, Rinaudo & Desbrières, 1994; Muzzarelli, Wecks, Filippini & Lough, 1989). Owing to its valuable properties mainly described by Muzzarelli (1977, 1991), it has been proposed for application in many fields such as cosmetics (Muzzarelli et al., 1989), tissue expanders (Biagini et al., 1991), wound dressing (Muzzarelli, Tarsi, Filippini, Giovanetti, Biagini & Varaldo, 1990; Muzzarelli et al., 1990) and formation of organized cutaneous tissues (Biagini et al., 1991). It is claimed to be a good film-forming polymer and a good moisturizing agent and to have a good bacteriostatic capacity (Muzzarelli et al., 1989). It is obtained by the reaction of levulinic acid on chitosan. Depending on the experimental conditions, different types of molecules can be obtained and their properties may vary widely.

The reaction of chitosan in the presence of levulinic acid and a reducing agent gives three main compounds as shown in Fig. 1. According to Muzzarelli et al. (1989) depending on the chemical conditions the reaction orientates to *N*-carboxybutylchitosan (mono- or disubstituted forms) or to 5-methylpyrrolidinone chitosan (called MPC). The precise dependence of the products on the experimental condition has still to be discussed. The properties of these different derivatives will be very different, so it is important to be able to control the reaction to produce one or the other of these polymers.

Berscht, Nies, Liebendorfer and Kreuter (1994, 1995) indicated that MPC is the most biocompatible chitosan among several tested derivatives. This paper deals with the synthesis and the characterization of the polymers obtained from chitosan with a low degree of acetylation under different experimental conditions.

2. Experimental

Two samples of chitosan were used: one from Aber (France) with a degree of acetylation DA = 0.02 and the other from Protan (Norway) with a degree of acetylation DA = 0.12. The degree of acetylation was determined using ^{1}H NMR as previously discussed (Rinaudo, Le Dung, Gey & Milas, 1992). They were purified as previously described and were perfectly soluble in the presence of acid (Rinaudo, Milas & Desbrières, 1997). The viscometric average molecular weights were 100,000

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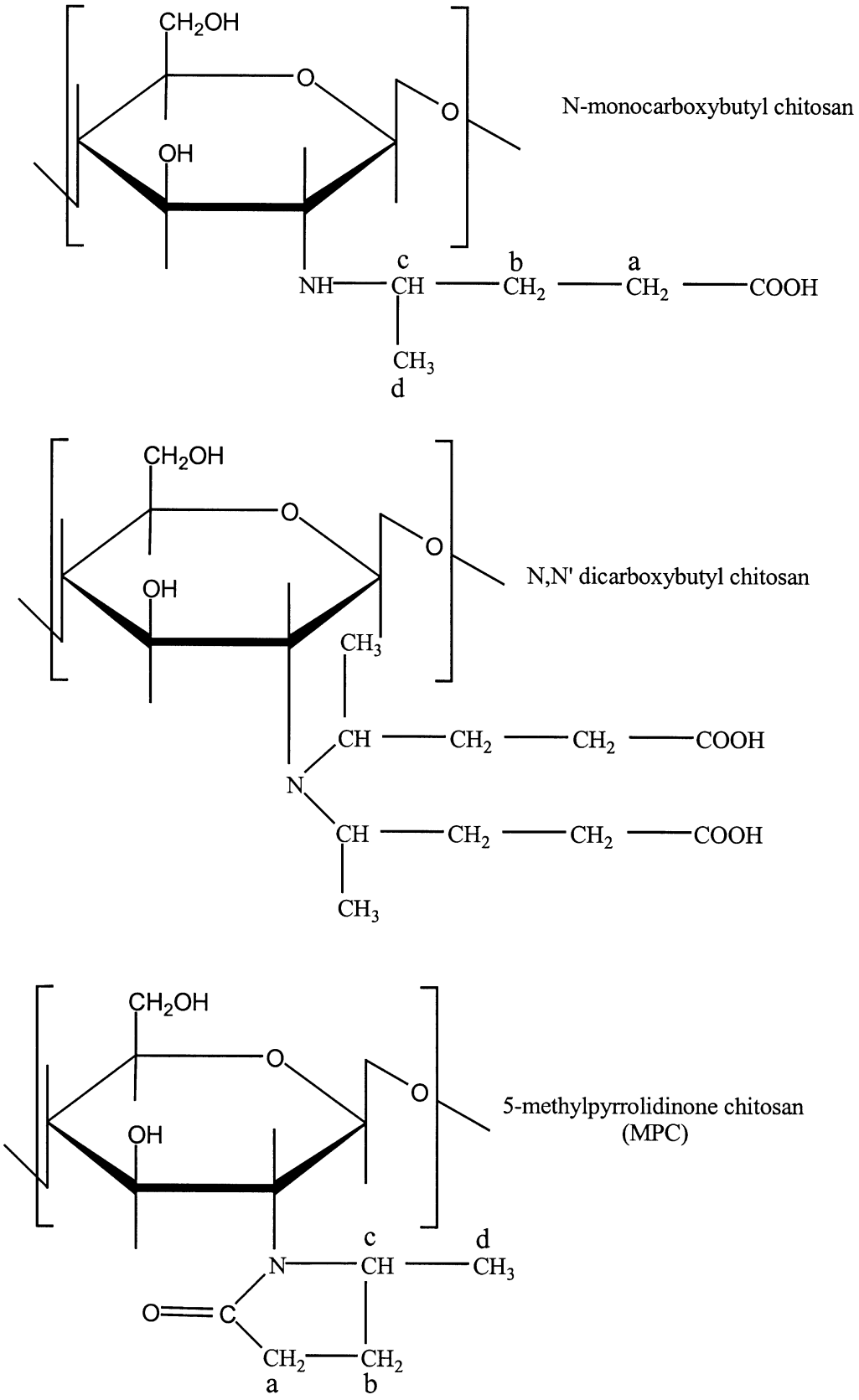

Fig. 1. Scheme of the different possible structures for chitosan–levulinic acid derivatives.

Table 1
Experimental conditions for chitosan–levulinic acid reactions (the reactions are performed on 1.6 g of chitosan in 160 ml of solution)

Sample	Molar ratio levulinic acid/chitosan/reducing agent	Nature of the reducing agent	Conditions for dissolution	Final pH	Reducing time (h)	Initial chitosan	Structure formed[a]
1	1.5/1/3.5	$NaBH_4$	1 h/25°C + 12h/25°C	6	2 h 30/25°C	Protan	(a) + (b)
2	1.5/1/3.5	$NaBH_4$	12 h/25°C + 2h/80°C	5	0 h 30/25°C + 1 h/80°C	Protan	(a)
3	10/1/10	$NaCNBH_3$	12 h/25°C	4.9	8 h 30/25°C	Aber	(b)
4	4/1/3.5	$NaCNBH_3$	12 h/25°C	4.97	8 h 30/25°C	Aber	(b)
5	4/1/3.5	$NaCNBH_3$	12 h/25°C	4.47	8 h 30/25°C	Aber	(b)
6	1/1/3	$NaCNBH_3$	12 h/25°C	6.12	8 h 30/25°C	Aber	(b)

[a] (a) represents the *N*-monocarboxybutyl chitosan and (b) the 5-methylpyrrolidinone chitosan.

and 190,000 g/mol, respectively (Rinaudo, Milas & Le Dung, 1993).

The levulinic acid used was from Aldrich (ref L 200-9); a 50% (w/v) solution was prepared in water and added to a chitosan suspension under stirring in different stoichiometric ratios. All the reactants used were the pure commercial forms.

The chitosan was dissolved in water in the presence of levulinic acid which allowed the protonation of the amino groups. After complete dissolution, the reducing agent was added at a controlled rate and left for a controlled period of time under stirring. The temperature was also controlled. The different reaction conditions are given in Table 1. The derivative being formed was isolated by precipitation with ethanol at a controlled fixed pH, washed and dried. In this process all low molecular weight materials were eliminated from the polymer. For samples 3–6 (Table 1), the pH of the final solution was first adjusted to 8 before precipitation with ethanol. For samples 1 and 2 (Table 1), the polymers were directly precipitated with ethanol in slightly acidic conditions. The exact parameters adopted for the reaction will be discussed in the next part of the paper.

The characterization of modified chitosan was performed using an Ubbelohde viscometer (inner diameter 0.58 mm) at 25°C in the dilute regime. The common solvent for chitosan and the derivatives was 0.1 M CH_3COOH/0.1 M CH_3COONa (Rinaudo et al., 1993). ^1H and ^{13}C NMR spectroscopies were realized on AC 300 or on Avance 400 spectrometers from Brüker at a temperature given in the figures, usually 353 K. The samples were dissolved directly in D_2O at a concentration of 10 mg/ml for ^1H NMR (in this case the solution was freeze dried twice in order to exchange the exchangeable protons) or 50–70 mg/ml for ^{13}C NMR and DEPT experiments. The integrated spectra were fully relaxed owing to the fact that the delay time we used (3 s) in this procedure was larger than the relaxation times of the different nuclei. All the chemical shifts (δ in ppm) are expressed with reference to TMS.

3. Results and discussion

When chitosan is in the presence of an excess of levulinic acid, it dissolves. As proposed by Muzzarelli in 1985, a ketimine is formed, which is then reduced to form the *N*-carboxylated derivative or a 5-methylpyrrolidinone; the formation of cyclic substituent was described by Kitano, Tanimoto and Ohabayashi (1975) and Leonard (1956). This reaction gives an amphoteric polymer in the case of carboxybutylchitosan, which is soluble in acidic, neutral and basic conditions when chitosan itself is only soluble in acidic conditions. The range of solubility is restricted with the cyclic derivative and limited to acidic conditions.

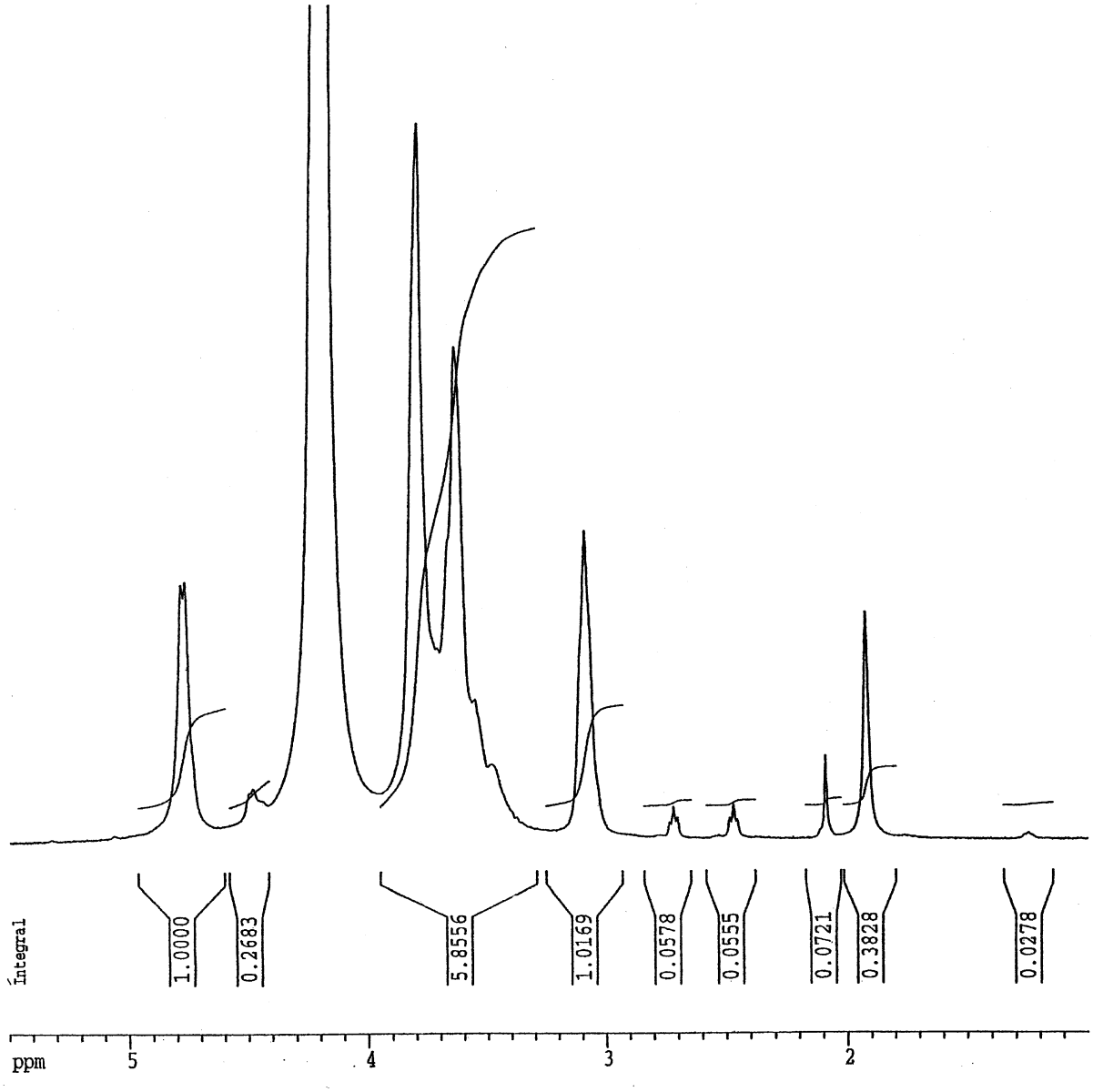

Fig. 2. ^{1}H NMR spectrum for linear monocarboxybutylchitosan (sample 2). Concentration: 10 mg/ml in D_2O at 70°C.

3.1. Chemical structure identification

For the reduction, two reducing agents were used: sodium borohydride $NaBH_4$ and sodium cyanoborohydride $NaCNBH_3$, which was previously demonstrated to be very efficient for the reaction with glyoxilic acid (Le Dung et al., 1994). $NaCNBH_3$ is more reactive and selective than more usual reducing agents and another advantage is its stability in acidic media (Lane, 1975). Its hydrolysis rate at pH 3 is smaller than other common reducing agents and at pH 7 it is only 0.5 mol% after 24 h. Moreover, the reduction of the imminium ion by the BH_3CN^- anion is rapid at pH values in the range of 6–7 and the reduction of aldehydes or ketones is negligible in this pH range (it becomes fast at pH values smaller than 3.5). However, $NaBH_4$ is obviously suitable for this preparation as demonstrated, but is less efficient in the pH reaction range considered. The reaction is usually performed at room temperature; the initial and final pH are measured (in the range 4–6). The conditions for the syntheses are given in Table 1.

The nature of the substitution was identified by ^{1}H and ^{13}C NMR spectroscopies. From spectra analysis, two different characteristic structures were identified. This analysis allows both the determination of the DS and also the identification of the type of derivative obtained as a function of the experimental conditions adopted for the modification (see Fig. 1). *N,N′*-dicarboxybutylchitosan was never observed in our conditions.

The ^{1}H NMR spectrum given in Fig. 2 was obtained for sample 2. This indicates a low degree of substitution

Table 2
Assignments of the signals of NMR spectra

^{1}H NMR spectra				^{13}C NMR spectra			
N-monocarboxybutyl chitosan (Fig. 2)		5-methylpyrrolidinone chitosan (Fig. 4)		*N*-monocarboxybutyl chitosan (Fig. 3)		5-methylpyrrolidinone chitosan (Fig. 5)	
H-1	4.80 ppm	H-1	4.36 ppm	$-CH_3$ (acetamido)	23.1 ppm	$-CH_3$ (d)	20.3 and 21.1 ppm
H-2	3.09 ppm	H-2	2.58 ppm	$-CH_3$ (butyl)	28.8 ppm	$-CH_2$ (a and b)	33.8–36.4 ppm
$-CH_3$ (butyl)	2.10 ppm	–CH (cycle)	2.96 ppm	$-CH_2$	35.4 and 38.7 ppm	–CH (c)	54.3 and 58.8 ppm
$-CH_3$ (acetamido)	1.96 ppm	$-CH_2$ (b)	1.43 and 1.71 ppm	C-2	56.9 ppm	C-2	61.6 ppm
$-CH_2$ (a and b)	2.75 and 2.51 ppm	$-CH_2$ (a)	2.08 ppm	C-6	61.3 ppm	C-6	62.6 ppm
$-CH_3$ (MPC)	1.25 ppm	$-CH_3$ (MPC)	0.97 ppm	C-3	71 ppm	C-3	76.4 ppm
				C-5	75.7 ppm	C-5	77 ppm
				C-4	78 ppm	C-4	80.7 ppm
				C-1	98.3 ppm	C-1	104.7 ppm
				Carboxyl	175.3 ppm	Carboxyl	184.7 ppm

suggesting a monocarboxylated derivative. It was obtained under mild conditions but with a rapid addition of the reducing agent. This result confirms the results obtained previously by Muzzarelli (Muzzarelli et al., 1989). The small signal at 1.2 ppm demonstrates the presence of traces of MPC as discussed later.

From the integral of the signal corresponding to $-CH_3$ of the acetamido group at 1.9 ppm (Fig. 2) and that of the H-1 proton we determined the degree of acetylation of the chitosan DA = 0.12 and from the integral of the signal at 2.1 ppm (CH_3 of the butyl substituent), the degree of substitution DS = 0.03. These values are confirmed from the ^{13}C spectrum in the region of 20–40 ppm (Table 2). There is no modification of the acetylation degree during the reaction.

From the ^{13}C NMR spectrum (Fig. 3), we proposed the attribution of the different signals (Table 2). In Fig. 3a, the ^{13}C NMR spectrum confirms the chemical modification with a larger signal at 175 ppm compared with initial chitosan and three signals at 28.8, 35.4 and 38.7 ppm attributed to the $-CH_3$ group and the two $-CH_2$ of the substituent, respectively (see Fig. 3b). From these data, it seems clear that in mild conditions and short times of reduction, we get a very low degree of substitution and only the monocarboxybutylchitosan.

In Fig. 4, the ^{1}H NMR spectrum obtained for sample 4 in different experimental conditions on the Aber sample is given. It must be pointed out that some HCl was needed to solubilize the derivative for NMR study; in addition, the spectrum is completely different from that of Fig. 2 but the signal located at 0.97 ppm is large. From literature, it is attributed to the $-CH_3$ of the methylpyrrolidinone (see Fig. 1). The correlation ^{1}H–^{1}H (not shown) indicates a correlation between $-CH_3$ of the ring and the proton at 3.0 ppm, this position was attributed to the –CH of the ring. The three other signals (at 2.08, 1.71 and 1.43 ppm) corresponding to four protons compared to the $-CH_3$ are identified as the $-CH_2$ groups. Considering the integral of the signal at 0.97 ppm and that of the H-1 proton (at 4.36 ppm), one gets a DS = 0.35 for this sample. The ratio of the different integrals attributed to the substitution are 3/1/1/2/1 for signals at 0.97, 1.43, 1.71, 2.08 and 2.96 ppm, respectively. This seems to indicate that only one type of derivative is formed in these conditions with a relatively high degree of substitution. As mentioned previously, this derivative is soluble only in acidic conditions. The small signal at 1.96 ppm reflects the low degree of acetylation of the initial Aber sample.

The ^{13}C spectrum (Fig. 5) and a DEPT experiment (Fig. 6) show the presence of two isomers as mentioned by Muzzarelli, Ilari & Tomasetti (1993). As the DA of the initial chitosan is very low, the two signals at 20.3 and 21.8 ppm are due only to $-CH_3$ of the ring. The four signals between 33 and 36 ppm found using DEPT correspond to the two $-CH_2$ groups, the two signals at 54 and 58.2 ppm are attributed to –CH of the two isomers. The ^{13}C spectrum allows an approximate equilibrium ratio between the two isomers to be determined. For samples 3 and 4 one gets 50/50 and 60/40, respectively.

For the first time, the NMR spectra of these two types of chitosan derivatives are reported. For all the experimental conditions tested, there was no evidence of the formation of a dicarboxybutylchitosan. With *N*-carboxymethylchitosan, the situation was different and we rapidly obtained the dicarboxylic form (Le Dung et al., 1994). This may be due to the steric hindrance of the substituted group. The formation of the five-membered ring is certainly favoured by room temperature and long reaction time in the presence of the reducing agent but then the absence of carboxylic groups decreases the range of solubility compared with the linear form of the substituent. Considering this NMR analysis, it is possible now to identify the chitosan derivatives obtained and to relate their structure to the preparation conditions.

3.2. Relation between structure and experimental conditions

First of all, from Table 1, it appears that the amount of levulinic acid must be larger than that of amino groups to solubilize the polymer in water. This observation is consistent with the fact that only one third of the levulinic acid is available for substitution (Muzzarelli et al., 1993). The time

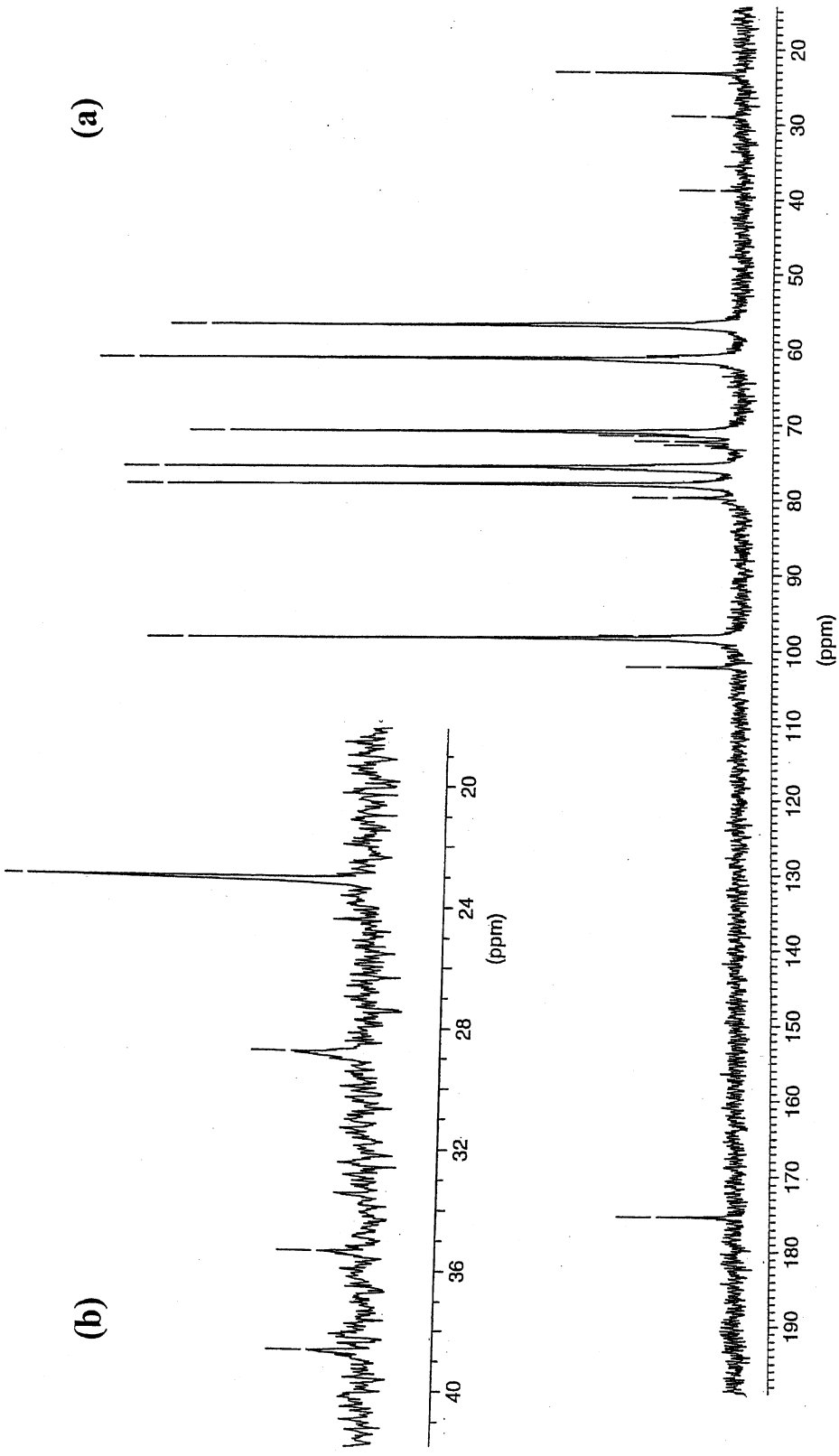

Fig. 3. ^{13}C NMR for the same derivative (sample 2) in D_2O at 80°C. Concentration: 50 mg/ml. (a) Complete spectrum. (b) Substituents region.

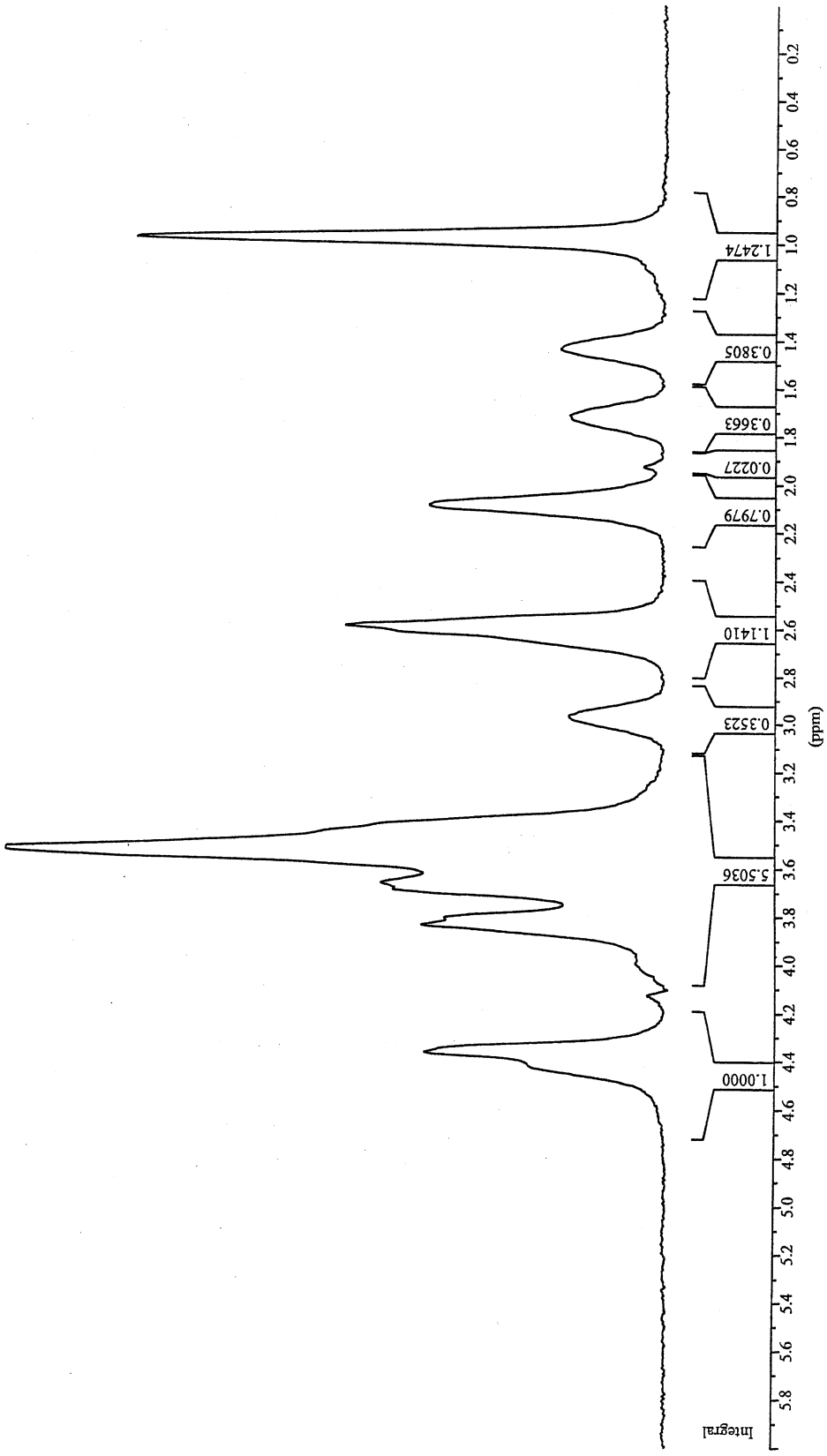

Fig. 4. ^{1}H NMR spectrum for the cyclic chitosan derivative in D_2O (sample 4). Concentration: 10 mg/l; temperature 80°C.

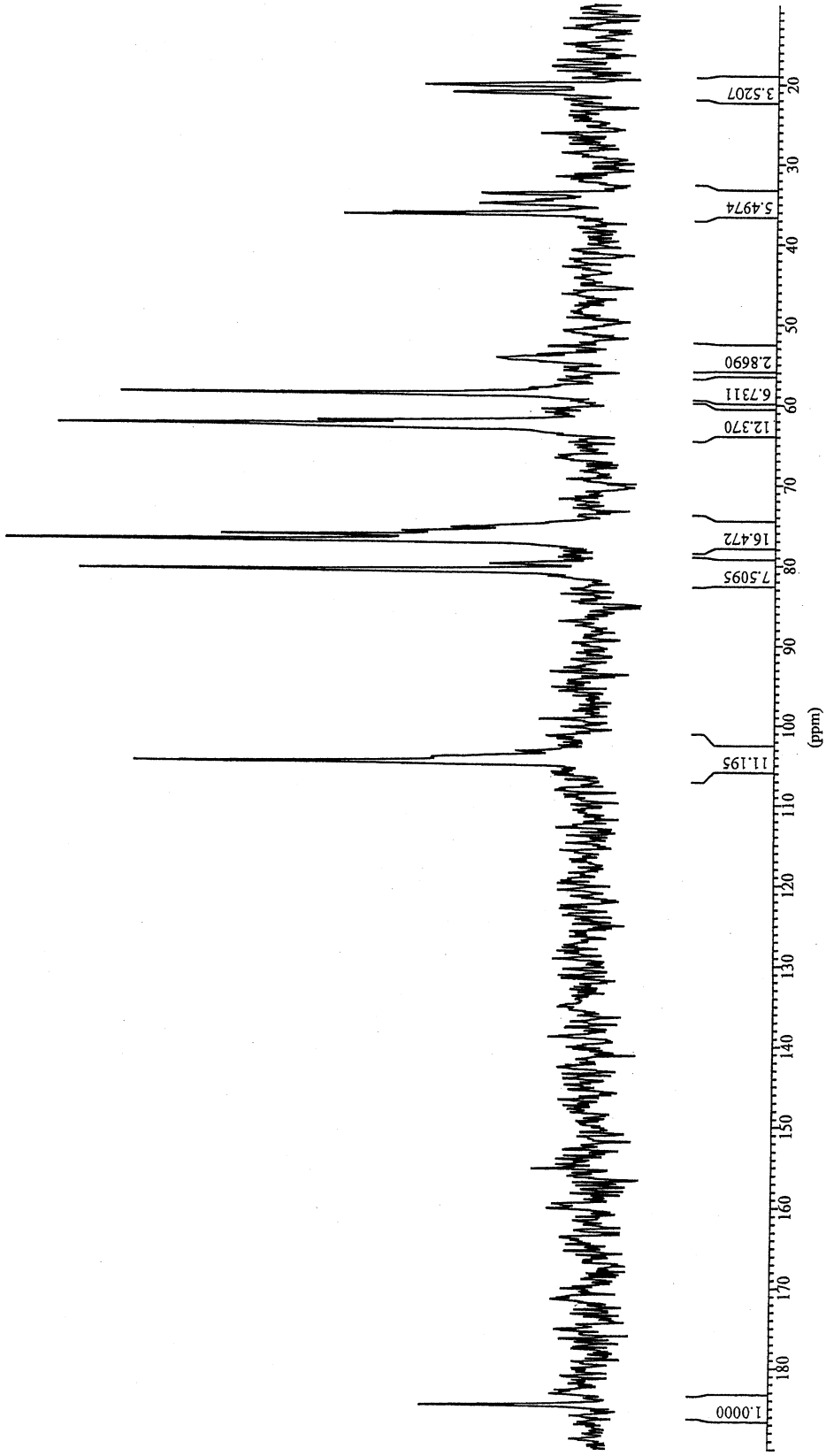

Fig. 5. ^{13}C NMR spectrum for the cyclic derivatives. Same conditions as for Fig. 4 (sample 4).

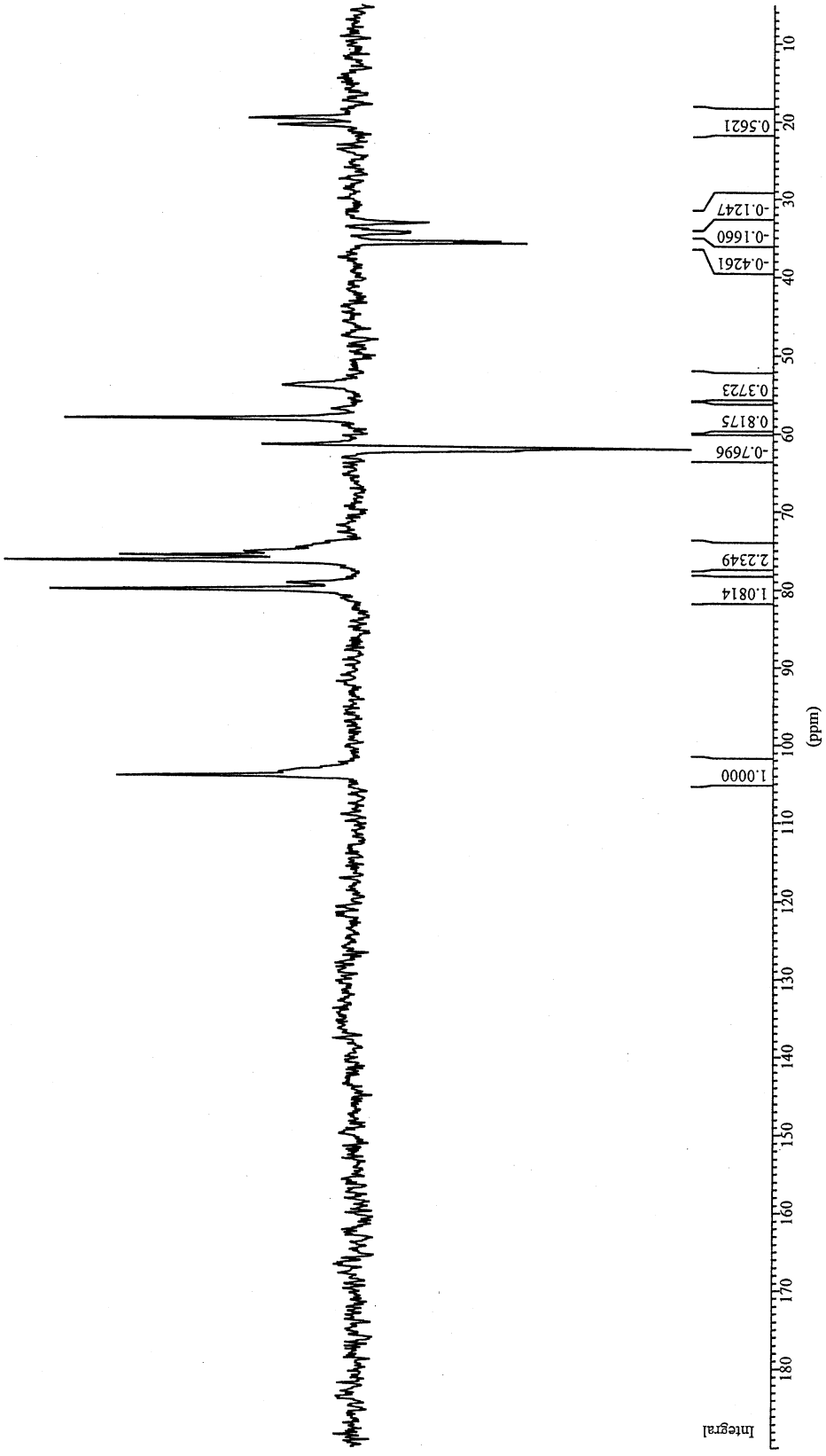

Fig. 6. DEPT experiment on the cyclic derivative to evidence the two isomers. Same conditions as for Fig. 5.

Table 3
Characteristics of some chitosan derivatives prepared

Samples	$[\eta]$ (ml/g)	k'	Degree of substitution by NMR (type of structure)
Initial chitosan	520	0.38	–
3	318	0.40	0.46 (MPC)
4	437	0.42	0.35 (MPC)

of reduction must be short to get a soluble polymer as proposed by Muzzarelli (sample 2).

The thermal treatment adopted to increase the solubilization rate or after addition of the reducing agent does not have an important role on the synthesis (compare samples 1 and 2). The use of $NaCNBH_3$ as reducing agent does not modify the result but gives a better stability under acidic conditions; the final pH remains lower than 5 (samples 3–5) but not for sample 6 in which no excess of levulinic acid was used.

The important parameters to derivatize chitosan are the content of levulinic acid, which must be in excess to allow both the dissolution of chitosan and the formation of enough ketimine form (sample 6). Excess of the reducing agent is necessary but too much may cause a larger degradation of the polymer. This is shown by the intrinsic viscosity of the polymer reported in Table 3 (samples 3 and 4). The rate of addition of the reducing agent is important as pointed out by Muzzarelli; rapid addition (sample 2) gives the linear form, whereas slow addition and longer time of reduction favours the cyclic form (samples 3 and 4). For an intermediate time of reduction (sample 1), a complex NMR spectrum is obtained corresponding to the mixture of the two forms.

4. Conclusions

In this paper, the assignment of the NMR signals was proposed based on two distinct chemical structures for the derivatization of chitosan in the presence of levulinic acid. The type of structures represented in Fig. 1 are directly connected with the experimental conditions adopted.

Water-soluble chitosan derivatives are obtained when the molar ratio of levulinic acid to the amino groups is high and for short times for the reduction reaction. Rapid addition of the reducing agent gives a linear monocarboxylated derivative while slow addition leads to the ring form. A large excess of reducing agent degrades the polymeric chain reducing its intrinsic viscosity.

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