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PII: S0144-8617(96)00088-4

Carbohydrate Polymers **31** (1996) 29–33
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0144-8617/96/$15.00 + .00

Muraminan (polymuramic acid) and related compounds derived from chitosan*

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(Received 29 April 1996; revised version received 2 July 1996; accepted 2 July 1996)

Chitosan was modified to give *N*-1-naphthylmethylenechitosan (d.s. 0.92, **1**), which was tritylated to give *N*-(1-naphthylmethylene)-6-*O*-tritylchitosan (d.s. 0.97 for trityl, **2**). Compound **2** was acylated to give a series of 3-*O*-acyl derivatives (d.s. 0.24–1.0, **4–14**), and was treated with 2-D, L-chloropropionic acid to give 3-*O*-(1-D, L-carboxyethyl) derivative (d.s. 0.37, **15**). Compound **15** was treated in aqueous 1 N HCl to give 3-*O*-(1-D, L-carboxyethyl) chitosan (d.s. 0.37, **16**, muraminan). Compound **2** was treated with 2-D, L-halopropionic acids in sodium methylsulfinylcarbanion to give 3-*O*-(1-D, L-carboxyethyl)-*N*, *N*-(1-D, L-dicarboxyethyl)-*O*-tritylchitosan (d.s. 2.9 for carboxyethyl, **17**). Compound **17** was treated with HCl in methanol to afford 3-*O*-(1-carboxyethyl)-*N*, *N*-(1-dicarboxyethyl) chitosan (d.s. 2.4 for carboxyethyl, **18**). Compounds **16** and **18** were soluble in water. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Bacterial cell wall murein, a peptide glycan, has the structural backbone of chitosan, (1 → 4)-2-amino-2-deoxy-β-D-glucan (Jap. Soc. Chitin/Chitosan, 1985). Therefore, chitosan can be used as a starting material for the preparation of murein. In murein, 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-3-*O*-(1-D-carboxyethyl)-2-deoxy-β-D-glucopyranose (muramic acid) residues are linked alternatively by 1,4-linkage, and a peptide is linked to the carboxyl group of the muramic acid residue (Schleifer & Kandler, 1972; Sharon, 1975). Only the murein is used as a substrate for lysozyme reaction (Osserman *et al.*, 1974). 2-Amino-3-*O*-(1-D-carboxyethyl)-2-deoxy-D-glucose (Matsushima & Park, 1962), 2-acetamido-3-*O*-(1-D-carboxyethyl-L-alanyl-D-isoglutamine)-2-deoxy-D-glucose (Shiba *et al.*, 1978; Hasegawa *et al.*, 1978), and *N*, *O*-(1-D, L-carboxyethyl) chitosan (d.s. 0.5–2.0) (Shigemasa *et al.*, 1995) have been prepared as a minimum unit of adjuvant active compounds. However, no report has specifically dealt with one 3-O-substituted derivative, 2-amino-3-*O*-(1-carboxyethyl)-2-deoxy-D-glucan. Now we report the preparation of (1 → 4)-2-amino-3-*O*-(1-D, L-carboxyethyl)-2-deoxy-D-glucan (muraminan), and some 3-*O*-acyl, 3-*O*-alkyl and *N*, *N*-dicarboxyethyl derivatives of chitosan as muraminan-like compounds.

*Abbreviations used: Ac, acetyl; d.s., degree of substitution; DSS, dimethylsilapentanesufonate (Na); DMSO, dimethylsulfoxide; DMAC, *N*, *N*-dimethylacetamide

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EXPERIMENTAL

Materials and methods

Crab shell chitosan (d.s. 0.01 for NAc/GlcN), a product of Katakurachikkarin Co., Ltd, Tokyo, was used in the present study. ^{13}C FTNMR spectra (D_2O) were recorded on a Jeol JNM-GX 270 FTNMR spectrometer (Jeol Co., Ltd, Tokyo), ^{13}C CPMAS NMR spectra on a Chemagnetics CMX 360 NMR spectrometer (Chemagnetics Co., Ltd, Fort Collins), FTIR spectra (KBr) on a Jasco FTIR 5300 spectrometer (Jasco Co., Ltd, Tokyo), and specific rotations on a Horiba SEPA-200 autopolarimeter (Horiba Co., Ltd, Kyoto). Elemental analyses were performed at the Micro-analytical Center of Kyoto University, Kyoto, and d.s. values for substituents were calculated from the elemental analysis data.

N-(1-naphthylmethylene) chitosan (1)

To a solution of chitosan (1.61 g) in aqueous 2% acetic acid (60 ml)-methanol (100 ml), 1-naphthaldehyde (10

ml, 7 mol/GlcN) dissolved in methanol (5 ml) was added under stirring, and the mixture was kept at room temperature for 12 h to give a slightly yellow gel (Nud'ga *et al.*, 1973; Hirano *et al.*, 1979). The gel was crushed into small pieces by a homogenizer, collected by filtration, washed with methanol, ethanol and ether successively, and dried to give **1** in 2.8 g yield (92%). ν_{max}^{KBr} cm^{-1}: 2880, 1640 (—N = CH—), 1500, 810 and 760 (naphthyl). ^{13}C CPMAS NMR data: δ (ppm) 192.0 and 187.0 (—N = CH—), 131.3 and 127.5 (naphthyl), 104.9 (C1), 82.9 (C4), 75.6 (C3 and 5), 60.7 (C2 and C6). Anal. calc. for $[C_6H_9O_4N\ (C_{11}H_8)_{0.92}(H_2)_{0.04}\ 0.33H_2O]_n$: C, 65.84; H, 5.82; N, 4.76. Found: C, 65.69; H, 5.80; N, 4.72.

N-(1-naphthylmethylene)-6-*O*-tritylchitosan (2)

Compound **1** (1.0 g) was swollen in dry pyridine (50 ml) by stirring at 80°C for 10 h, and tritylchloride (4.0 g, 3 mol eq./GlcN) was added. The mixture was stirred at 100°C for 15 h under anhydrous conditions to give a viscous brown solution, and was poured into methanol (250 ml) to give a precipitate, which was collected by centrifugation, washed with methanol and ether, and dried to give **2** in 1.7 g yield (96%). ν_{max}^{KBr} cm^{-1}: 1640 (—N = CH—), 2888, 1500, 800 and 760 (naphthyl), 3050, 1450, 740 and 700 (phenyl). ^{13}C CPMAS NMR data: δ (ppm) 186.9 (—N = CH— and —C^* =). 127.0 (phenyl and naphthyl), 87.5 (C1), 74.0 (C4, 3 and 5), 66.9 (C2 and 6). Anal. calc. for $[C_6H_8O_4N(C_{11}H_8)_{0.92}(H_2)_{0.04}(C_{19}H_{15})_{0.97}(H)_{0.03}\ 0.47H_2O]_n$: C, 78.07; H, 5.82; N, 2.64. Found: C, 77.84; H, 5.78; N, 2.68.

O-acetyl-*N*-(1-naphthylmethylene) chitosan (3)

Compound **1** (0.10 g) was swollen in pyridine (10 ml) by stirring at 80°C for 12 h. Acetic anhydride (0.1 ml, 3 mol eq./GlcN) was added. The mixture was stirred at 100°C for 12 h, and was poured into ethanol (50 ml). The produced precipitate was collected by filtration, washed with ethanol and ether, and dried to give **3** in 0.10 g yield (90%). ν_{max}^{KBr} cm^{-1}: 1780 (C = O of OAc), 1640 (—N = CH—), 2880, 1500, 810 and 760 (naphthyl). Anal. calc. for $[C_6H_7O_4N(C_{11}H_8)_{0.50}(C_2H_3O)_{1.99}(H)_{1.01}]_n$: C, 59.24; H, 5.73; N, 4.46. Found: C, 59.44; H, 5.46; N. 4.52.

3-*O*-Acyl derivatives (4-14) of *N*-(1-naphthylmethylene)-6-*O*-tritylchitosan

Compound **2** (0.1 g) was swollen in dry pyridine (10 ml) by stirring at 80°C for 12 h. Each (3 mol eq./GlcN) of a fatty acid anhydride (acetyl to isobutyryl) and a fatty acylchloride (pentanoyl to palmitoyl) was added. The mixture was stirred at 100°C for 12 h to give a homogenous brown solution, and was poured into methanol (50 ml). The produced precipitate was collected by filtration, washed with methanol and ether, and dried. Table 1 shows analytical data for these derivatives. ν_{max}^{KBr} cm^{-1}: 3060, 2880, 1750 (C = O of *O*-Acyl), 1640 (—N = CH—), 1650 and 1550 (C = O and NH of NAc), 1500, 810, 780, 760 (naphthyl), 1450, 740 and 700 (trityl). No absorptions at 1650 and 1550 (C = O and NH of *N*-Acyl) cm^{-1}.

3-*O*-(1-D, L-carboxyethyl)-*N*-(1-naphthylmethylene)-6-*O*-tritylchitosan (15)

Compound **2** (0.10 g) was swollen in DMAC (10 ml) by stirring at 80°C for 12 h, and 2-D, L-chloropropionic acid (0.1 g, 5 mol eq./GlcN) was added. The mixture was stirred at 100°C for 12 h to give a homogenous solution, and was added dropwise to ice-water to give a precipitate. The produced precipitate was collected by filtration washed with water, and dried to give a 1-D, L-carboxyethyl derivative (**15**) in 0.07 g (74%). The product was soluble in pyridine, DMSO and DMAC, but insoluble in water. $[\alpha]_D^{20}$ −32° (*c* 0.1, DMSO). ν_{max}^{KBr} cm^{-1}: 1750 (COOH), 1640 (—N = CH—), 3060, 1450,

Table 1. Data for some 3-*O*-fatty acyl derivatives of *N*-(1-naphthylmethylene)-6-*O*-tritylchitosan

Acyl	Yield (%)	Formula[a]	Calc.			Found		
			C	H	N	C	H	N
Acetyl (**4**)	83	$R(C_{11}H_8)_{0.53}(C_2H_3O)_{1.00}(H)_{0.94}0.72H_2O$	73.90	5.95	2.67	73.90	5.59	2.66
Propionyl (**5**)	88	$R(C_{11}H_8)_{0.35}(C_3H_5O)_{1.00}(H)_{1.33}1.63H_2O$	70.90	6.41	2.64	71.25	6.00	2.65
Butyryl (**6**)	98	$R(C_{11}H_8)_{0.40}(C_4H_7O)_{0.98}(H)_{1.25}0.92H_2O$	72.84	6.42	2.58	72.86	6.14	2.58
Isobutyryl (**7**)	73	$R(C_{11}H_8)_{0.50}(C_4H_7O)_{0.24}(H)_{1.79}0.70H_2O$	75.12	6.02	2.84	75.02	5.74	2.79
Pentanoyl (**8**)	91	$R(C_{11}H_8)_{0.16}(C_5H_9O)_{0.25}(H)_{2.46}1.67H_2O$	70.34	6.58	2.97	70.37	6.33	2.97
Hexanoyl (**9**)	98	$R(C_{11}H_8)_{1.00}(C_6H_{11}O)_{1.00}(H)_{0.05}0.88H_2O$	76.28	6.57	2.15	76.28	6.98	2.15
Octanoyl (**10**)	92	$R(C_{11}H_8)_{0.63}(C_8H_{15}O)_{1.00}(H)_{0.74}0.85H_2O$	75.69	7.06	2.24	75.43	7.70	2.26
Decanoyl (**11**)	94	$R(C_{11}H_8)_{1.00}(C_{10}H_{19}O)_{1.00}(H)_{0.03}1.17H_2O$	76.91	7.18	1.98	77.10	7.91	2.00
Lauroyl (**12**)	83	$R(C_{11}H_8)_{0.60}(C_{12}H_{23}O)_{1.00}(H)_{0.80}2.86H_2O$	72.52	7.86	1.97	72.25	8.20	1.97
Myristoyl (**13**)	66	$R(C_{11}H_8)_{0.12}(C_{14}H_{27}O)_{0.72}(H)_{2.07}1.14H_2O$	73.79	7.93	2.39	73.69	7.89	2.39
Palmitoyl (**14**)	95	$R(C_{11}H_8)_{0.84}(C_{16}H_{31}O)_{1.00}(H)_{0.67}0.35H_2O$	77.83	8.02	1.83	77.56	8.55	1.85

[a] R:$C_6H_7O_4N(C_{19}H_{15})_{0.97}$.

Table 2. Reaction conditions for *O*- and *N,N*-carboxyethylation[a]

2 M Sodium methylsulfinyl carbanion (ml)	2-Halopropionic acid (ml)	Reaction		Yield (g)
		Temperature (°C)	Time (h)	
5.0	D,L-bromo (0.5)	70	20	0.15
5.0	D,L-chloro (0.5)	70	20	0.14
2.5	D,L-chloro (0.5)	70	20	0.15
3.0	D,L-chloro (1.5)	100	18	0.14

[a]Compound **2** (0.13 g) was used.

760, 740 and 700 (trityl), 2880, 1640, 1500, 810 and 780 (naphthyl). Anal. calc. for $[C_6H_7O_4N(C_{19}H_{15})_{0.97}(C_{11}H_8)_{0.58}(C_3H_5O_2)_{0.37}(H)_{1.54}]_n$: C, 76.13; H, 5.89; N, 2.80. Found: C, 76.09; H, 5.87; N, 2.80.

3-*O*-(1-D, L-carboxyethyl) chitosan (muraminan, 16)

A suspension of **15** (0.20 g) in 1 N hydrochloric acid (20 ml) was stirred at room temperature for 3 h to give a homogenous solution. The reaction mixture was poured into methanol (100 ml), and the produced precipitate was collected by filtration, washed with methanol and ether, and dried to give **16** in 0.04 g (51%); $[\alpha]_D^{20}$ −22° (*c* 0.1, water). The product was soluble in water, but insoluble in common organic solvents. ν_{max}^{KBr} cm^{-1}: 1730 (COOH), 1620 (NH_2), and no phenyl absorptions at 1500, 1450, 760, 740 and 700. ^{13}C-NMR data (D_2O): δ 171.0 (COOH), 100.2 (C1), 79.1 (C4), 77.4 (C5), 72.7 and 70.5 (C3), 62.7 (C2), 44.4, 24.9 (CH_3). ^{1}H NMR data: δ (ppm) 1.30 (CH_3). Anal. calc. for $[C_6H_{10}O_4N(C_3H_5O_2)_{0.37}(H)_{0.63}0.40H_2O]_n$: C, 43.79; H, 6.82; N, 7.19. Found: C, 43.59; H, 6.84; N, 7.17.

3-*O*-(1-carboxyethyl)-*N*, N-(1-dicarboxyethyl)-6-*O*-tritylchitosan (17)

Compound **2** (0.13 g) was dissolved in DMSO (10 ml) by stirring at 80°C for 12–20 h. 2 M Sodium methylsulfinylcarbanion (2.5–5.0 ml) (Hakomori, 1964; Sandford & Conrad, 1966; Conrad, 1972) was added under cooling in an ice bath in a stream of nitrogen gas, and the mixture was stirred at room temperature for about 1 h. 2-D, L-chloro- or bromo-propionic acid (0.5–1.5 ml) was added dropwise with stirring under cooling in an ice bath. After cooling to room temperature, the mixture was stirred at 70–100°C for 18–20 h. The reaction mixture was poured into ice-water with stirring. The produced precipitate was collected by filtration, washed with water, and dried to give **17** in 0.14–0.15 g yields (93–98%) (Table 2). ν_{max}^{KBr} cm^{-1}: 1750 (COOH), 1640 (—CH = N—), 1600 (NH_2), 3060, 1450, 740, 700 (trityl), no absorption at 2880, 1500, 810 and 780 (naphthyl). Anal. calc. for $[C_6H_7O_4N(C_{19}H_{15})_{0.97}(C_3H_5O_2)_{2.93}(H)_{0.10}\ 0.61H_2O]_n$: C, 64.53; H, 6.08; N, 2.27. Found: C, 64.61; H, 6.08; N, 2.26.

3-*O*-(1-carboxyethyl)-*N*, *N*-(1-dicarboxyethyl) chitosan (18)

A suspension mixture of **17** (0.15 g) in methanol (5 ml)-conc. HCl (0.5 ml) was stirred at room temperature for 20 h to give a homogenous solution. The reaction mixture was poured into ether (50 ml), and the produced precipitate was collected by centrifugation, washed with ether, and dried to give **18** in 0.07 g yield (88%). $[\alpha]_D^{14}$ +94° (*c* 0.37, water). ν_{max}^{KBr} cm^{-1}: 1740 (COOH), 1640, 1380, 1240. Anal. calc. for $[C_6H_8O_4N(C_3H_5O_2)_{2.44}(H)_{0.56}]_n$: C, 47.90; H, 6.22; N, 4.20. Found: C, 47.96; H, 5,82; N, 4.14.

RESULTS AND DISCUSSION

As shown in Fig. 1, the amino group at C2 of 2-amino-2-deoxy-D-glucose residue in chitosan was protected with 1-naphthylmethylene (Schiff's base)

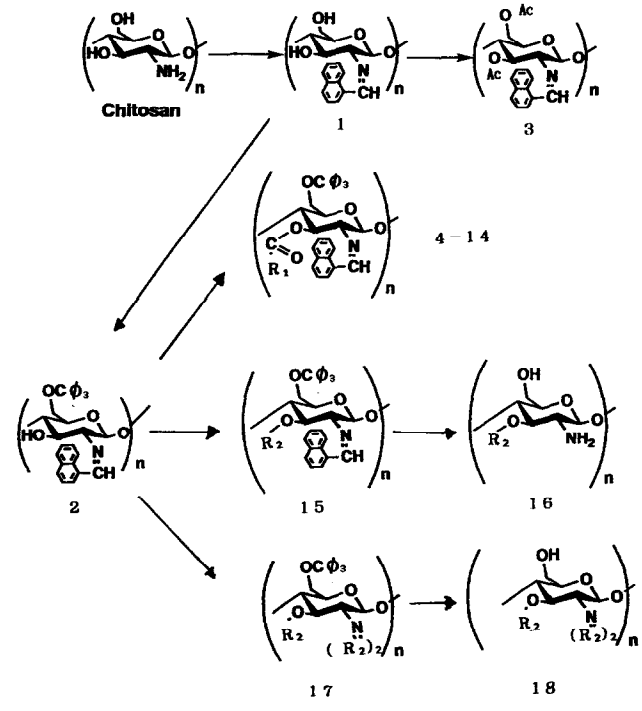

Fig. 1. Reaction scheme. R_1, alkyl; R_2, 1-D, L-carboxyethyl ($CH_3CHCOOH$).

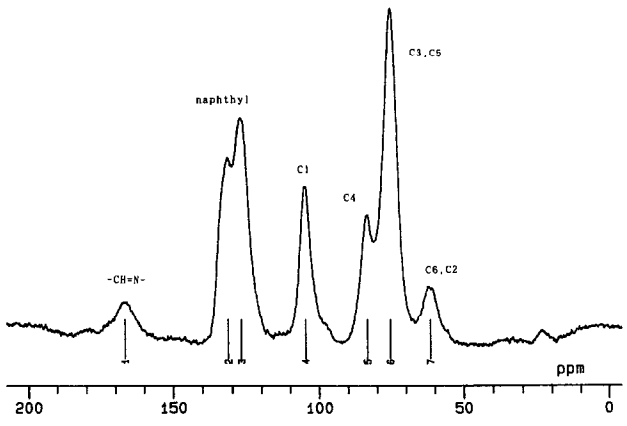

Fig. 2. ^{13}C CPMAS NMR spectrum of *N*-(1-naphthylmethylene)-chitosan (**1**).

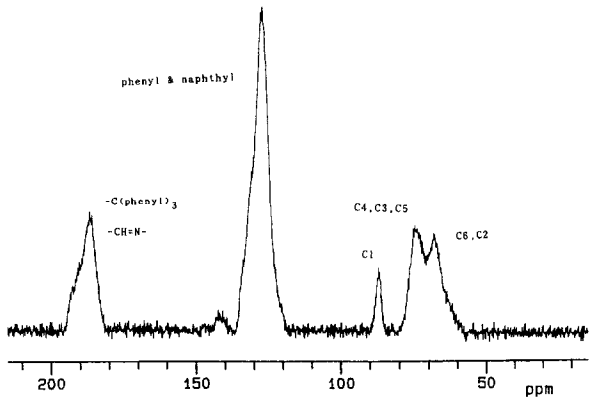

Fig. 3. ^{13}C CPMAS NMR spectrum of *N*-(1-naphthylmethylene)-3-*O*-(1-carboxyethyl)-6-*O*-tritylchitosan (**2**).

group (Nud'ga *et al.*, 1973; Hirano *et al.*, 1979) to give **1** (d.s. 0.92), and the hydroxyl group at C6 was protected with trityl group to give **2** (d.s. 0.97). Then the hydroxyl group at C3 was acylated with fatty acylchlorides or carboxylic anhydrides to give **4**–**14**, and was alkylated with 2-D, L-halopropionic acids to give **15**. Compound **1** was acetylated to give **3**. Compounds **1**–**15**, **17** and **18** were isolated in over 74% yield, and **16** was isolated in 51% yield. Compounds **1**–**15** and **17** were insoluble in water, **2** was soluble in DMSO after stirring at 80°C for 12–20 h, **15** was soluble in pyridine, DMSO and DMAC, and **16** and **18** in water.

In the ^{13}C CPMAS spectrum of **1** (Fig. 2), —N=C*H— of naphthyl appeared at 167.6, naphthyl at 131.2 and 127.5, C1 at 104.9, C4 at 82.9, C3 and C5 at 75.6, and C2 and C6 at 60.7 ppm. In the ^{13}C CPMAS spectrum of 2 (Fig. 3), —N=C*H— of naphthyl was overlapped with –C*(phenyl)$_3$ and appeared at 186.9, naphthyl and trityl were overlapped and appeared at 127.3, C1 appeared at 104.9, and C4 at 82.9, C3 and C5 at 75.6, and C2 and C6 at 60.7 ppm. In the ^{13}C NMR spectrum (D_2O) of muraminan (**16**, Fig. 4), COOH appeared at 171.0, C1 at 100.2, C4 at 79.1, C5 at 77.5,

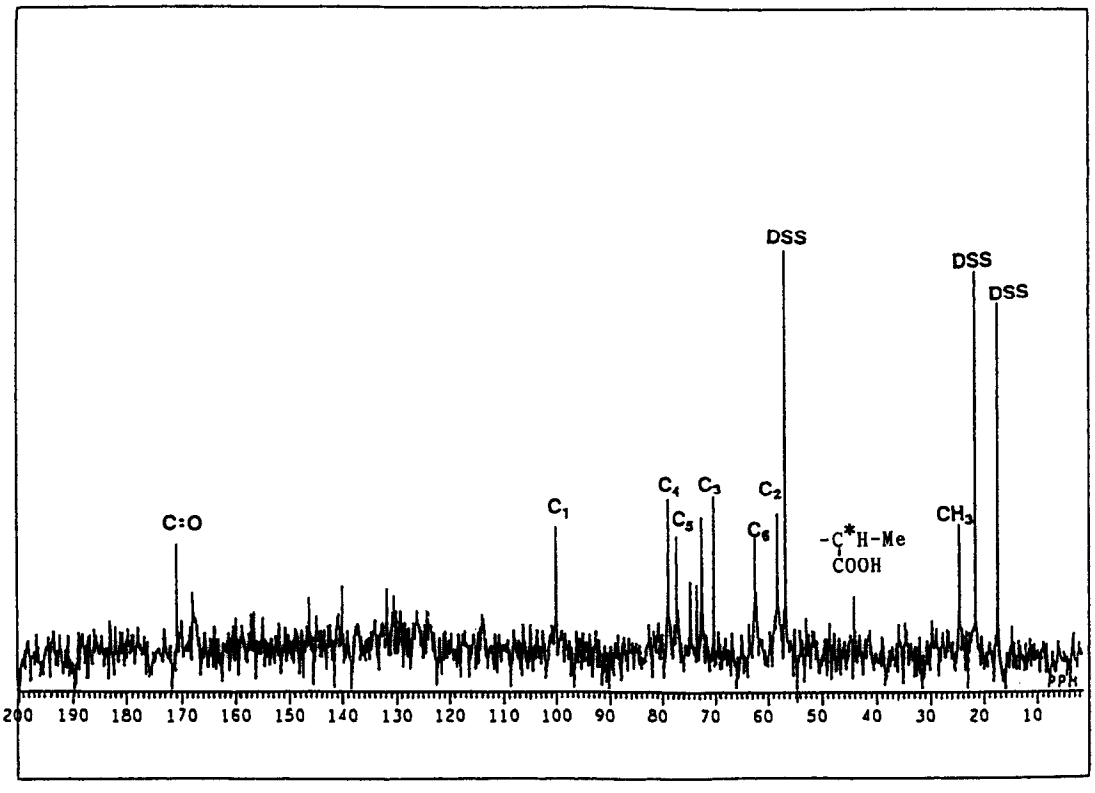

Fig. 4. ^{13}C FTNMR spectrum (D_2O) of 3-*O*-(1-D, L-carboxyethyl) chitosan (**16**).

and C3 appeared at 72.8 and 70.5 due to both the presence and absence of carboxyethyl group at C3, and C6 appeared at 62.8, C2 at 58.5, —C* (COOH)H—Me at 44.5, and CH_3 at 24.9 ppm.

The partial cleavage of *N*-naphthylmethylene group was observed with the reaction of 2-D, L-chloropropionic acid even in a pyridine solvent under the present conditions. In *O*-acyl products of **1** and **2**, no IR absorptions were detected at 1650 and 1550 cm^{-1} for *N*-acyl, indicating that free amino group is present in the products. On the other hand, the complete cleavage of the *N*-naphthylmethylene group was observed with the reaction of **2** in sodium methylsulfinylcarbanion (Hakomori, 1964; Sandford & Conrad, 1966; Conrad, 1972) to afford **18**, which have both *N*, *N*-dicarboxyethyl and 3-*O*-carboxyethyl groups (d.s. 2.44).

In the FTIR spectrum of fully acetylated **18** (d.s. 2.44 for carboxyethyl), only *O*-acetyl absorptions were detected at 1760 and 1240 cm^{-1}, but no *N*-acetyl absorptions were detected at 1650 and 1550 (C = O and NH of NAc) cm^{-1}. These data support *N*, *N*-dicarboxyethyl structure at C2, and in agreement with *N*-methylation of acetamido group (Stellner *et al.*, 1973).

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