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Research Note

N-Alanyl and some *N*-(*N'*-aryl)glycyl derivatives of chitosan

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Chemical modification on solid *N*-α-chloropropionyl and *N*-chloroacetyl derivatives of chitosan was performed. The *N*-alanyl derivative was prepared by treating the *N*-α-chloropropionyl derivative with aqueous 25% ammonia at room temperature for 18 h. *N*-(*N'*-Phenyl)glycyl, *N*-(*N'*-*o*-tolyl)glycyl and *N*-(*N'*-*p*-methoxyphenyl)glycyl derivatives were prepared by treating the *N*-chloroacetyl derivative with each of aniline, *o*-toluidine and *p*-anisidine at 100° to 150°C for 2 days.

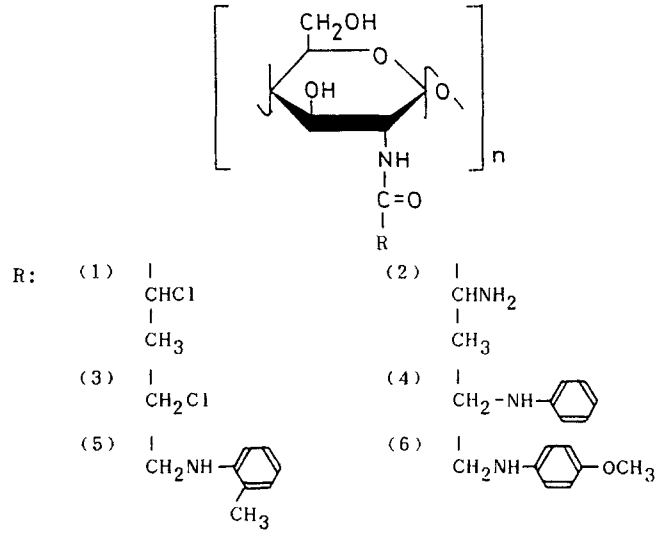
INTRODUCTION

Chemical reactions on solid phases are well known in multistepped organic syntheses (Letsinger & Kornet, 1963; Merrifield, 1963; Leznoff, 1978). Chitosan, (1 → 4)-2-amino-2-deoxy-β-D-glucan, is insoluble and stable in aqueous alkaline solutions, and it has reactive amino groups in the molecule (Muzzarelli, 1977). It is also known that glycine is produced by treatment of chloroacetic acid with aqueous ammonia at room temperature (Orten & Hill, 1948). These facts enabled us to modify chitosan in the solid phase in aqueous alkaline solution. We wish to report some novel *N*-acyl (NAc) derivatives prepared by treating solid *N*-(α-chloropropionyl) and *N*-chloroacetyl derivatives of chitosan (d.s. 0·20 for NAc) with aqueous ammonia and with some arylamines (Scheme 1).

EXPERIMENTAL

Methods

Infrared (IR) spectra (KBr) were recorded with a Hitachi 215 grating spectrometer. Carbon-13 CP/MAS NMR spectra were recorded with a Chemagnetics CMX 360 NMR spectrometer at the Research Institute of Ohtsuka Pharmaceuticals Inc., Tokushima. Paper chromatography was performed by the descending method on Whatman No. 1 filter paper with 6:4:3 (v/v) 1-butanol–pyridine–water, and the spots were made



Scheme 1. Chemical structures of the main repeating unit of each of the *N*-α-chloropropionyl (**1**), *N*-alanyl (**2**), *N*-chloroacetyl (**3**), *N*-(*N'*-phenyl)glycyl (**4**), *N*-(*N'*-*o*-tolyl)glycyl (**5**), and *N*-(*N'*-*p*-methoxyphenyl)glycyl (**6**) derivatives of chitosan.

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visible by spraying with 0·1% alcoholic ninhydrin. Chlorine was quantitatively detected by Beilstein's test (Shriner *et al.*, 1974). Elemental analysis was performed at the Analytical Centre of Kyoto University. The d.s. for the *N*-acyl group was calculated from the elemental analysis data.

Materials

Crab shell chitosan (d.s. 0·20 for NAc; $[\alpha]_D^{26}$ − 15° (*c* 1·0, aqueous 10% acetic acid)) was a product of Katakura Chikkarin Inc., Tokyo, and *N*-acetylchitosan (d.s. 1·0 for Nac) was prepared by *N*-acetylation of chitosan (Hirano *et al.*, 1976). Chitinase from *Streptomyces* was a product from Wakunaga Kono Inc., Osaka.

Hydrolysis by chitinase

Each 15 mg of powdered chitosan derivative (>120 mesh) was suspended in 1·0 ml of 0·05 M citric acid–0·1 M Na_2HPO_4 buffer solution (pH 5·5), and the suspension was mechanically stirred with 0·5 ml of chitinase solution (0·5 mg/ml) at 37°C for 1 h. The enzymic reaction was stopped by boiling in a water-bath for 10 min, and the mixture was centrifuged at 2800 × *g* for 10 min. An increase in the reducing sugar value of the supernatant solution was measured as *N*-acetyl-D-glucosamine by a modified Schalles' method (Imoto & Yagishita, 1971), and the rate of hydrolysis was compared with that of *N*-acetylchitosan.

***N*-α-Chloropropionyl derivative**

Chitosan (0·16 g) was dissolved in 5 g of α-chloropropionic acid by heating at 70 to 75°C. To the solution was added acetic anhydride (3 ml) with stirring. After standing at room temperature overnight, the mixture was poured on to ice-water (about 500 ml) with stirring, and the precipitate was collected by filtration, washed with water, and dried to give the *N,O*-α-chloropropionyl derivative in 0·28 g yield. ν_{max}KBr 1750 (C=O of *O*-acyl), 1660 and 1540 (C=O and NH of Nac), 710 cm^{-1} (C-Cl).

Anal. Calc. for $[C_6H_8O_4N(C_2H_3O)_{0.20}(C_3H_4OCl)_{1.86}(H)_{0.94}\ 0{\cdot}92H_2O]n$: C, 40·61; H, 5·32; N, 3·96; Cl, 18·65. Found: C, 40·27; H, 5·09; N, 3·88; Cl, 20·50.

A suspension of the *N,O*-α-chloropropionyl derivative (100 mg) was stirred in 10 ml of 0·1 M NaOH–methanol (2:1, v/v) at room temperature for 18 h. The insoluble material was collected by filtration, washed with methanol and dried to give the named derivative as a white powder in 65 mg yield. ν_{max}KBr 1670 and 1560 (C=O and NH of Nac), 710 cm^{-1} (C-Cl), no absorption at 1750 cm^{-1} for *O*-acyl.

Anal. Calc. for $[C_6H_9O_4N(C_2H_3O)_{0.20}(C_3H_4OCl)_{0.84}(H)_{0.96}\ 0{\cdot}33H_2O]n$: C, 42·58; H, 5·81; N, 5·57; Cl, 11·86. Found: C, 42·88; H, 6·23; N, 5·54; Cl, 11·62.

***N*-Alanyl derivative**

A suspension of the *N*-α-chloropropionyl derivative (50 mg) in aqueous 25% ammonia (20 ml) was stirred at room temperature for 4 days. The mixture was poured on to water (about 100 ml) to afford a precipitate, which was collected by filtration, washed with water and ethanol, and dried. Since the precipitate still exhibited a positive Beilstein's test for chlorine, the precipitate was retreated with aqueous 25% ammonia to give the named compound as a white powder in 34 mg yield. The compound exhibited a negative Beilstein's test for chlorine. ν_{max}KBr 1660 and 1560 cm^{-1} (C=O and NH of Nac). The *N*-alanyl derivative (d.s. 0·8 for *N*-alanyl and d.s. 0·20 for Nac) was insoluble in water and in the common organic solvents examined. In the hydrolysate of the compound with 6 N HCl at 100°C for 20 h, two ninhydrin-positive spots (R_f 0·12 and 0·24) were detected in almost equal quantities by paper-chromatography, and they were identified as DL-alanine and D-glucosamine, respectively, in reference to their authentic sample.

Anal. Calc. for $[C_6H_{10}O_4N(C_2H_3O)_{0.20}(C_3H_6ON)_{0.80}\ 0{\cdot}10H_2O]n$: C, 44·57; H, 7·09; N, 11·41. Found: C, 44·45; H, 7·20; N, 11·32.

***N*-Chloroacetyl derivative**

Chitosan (1·6 g) was dissolved in chloroacetic acid (66 g) by heating at 65 to 70°C. To the solution was added chloroacetic anhydride (26 g), and the mixture was treated as described in the preparation of the *N,O*-α-chloropropionyl derivative to give the *N,O*-chloroacetyl derivative (d.s. 2·68 for chloroacetyl) as a white powder in 3·39 g yield. ν_{max}KBr 1760 (C=O of *O*-acyl), 1670 and 1550 (C=O and NH of Nac), 710 cm^{-1} (C-Cl).

Anal. Calc. for $[C_6H_8O_4N(C_2H_3O)_{0.20}(C_2H_2OCl)_{2.68}(H)_{0.12}\ 0{\cdot}38H_2O]n$: C, 37·03; H, 3·93; N, 3·67; Cl, 24·91. Found: C, 37·33; H, 3·75; N, 3·64; Cl, 24·61.

The *N,O*-chloroacetyl derivative (2·0 g) was treated in 200 ml of 0·1 M NaOH–methanol (2:1, v/v) at room temperature for 18 h to give the named compound as a white powder in 1·26 h yield. ν_{max}KBr 1670 and 1560 (C=O and NH of Nac), 710 cm^{-1} (C-Cl), no absorption at 1760 cm^{-1} for *O*-acyl; ^{13}C CP/MAS NMR data: 174.5 (C=O for CH_3), 169·9 (C=O for CH_2Cl), 103·5 (C-1), 83·9 (C-4), 75·6 (C-5 and C-3), 61·4 (C-6), 56·8 (C-2), 43·8 (CH_2Cl), 23·8 (CH_3) ppm.

Anal. Calc. for $[C_6H_9O_4N(C_2H_3O)_{0.20}(C_2H_2OCl)_{0.89}(H)_{0.91}\ 0{\cdot}51H_2O]n$: C, 39·79; H, 5·45; N, 5·68; Cl, 12·78. Found: C, 39·83; H, 5·36; N, 5·56; Cl, 12·78.

***N*-(*N'*-Phenyl)glycyl derivative**

A suspension of the *N*-chloroacetyl derivative (0·12 g) in aniline (5 ml) was stirred under anhydrous conditions at 100 to 105°C for 2 days. The reaction mixture

was poured on to ice-water (about 300 ml) with stirring. The precipitate was collected by filtration, washed with water and ethanol and dried to give the named compound as a brown powder in 0·12 g yield. ν_{max}KBr 1670 and 1550 (C=O and NH of Nac), 760 and 700 cm^{-1} (monosubstituted phenyl).

Anal. Calc. for $[C_6H_{10}O_4N(C_2H_3O)_{0\cdot20}(C_8H_8ON)_{0\cdot80}]n$: C, 55·67; H, 6·22; N, 9·13. Found: C, 55·97; H, 6·37; N, 9·39.

N-(N'-*o*-Tolyl)glycyl derivative

A suspension of the *N*-chloroacetyl derivative (0·12 g) in *o*-toluidine (5 ml) was treated as described above to afford the named compound as a grey powder in 0·12 g yield. ν_{max}KBr 1670 and 1550 (C=O and NH of Nac), 760 cm^{-1} (*o*-substituted Ph).

Anal. Calc. for $[C_6H_{10}O_4N(C_2H_3O)_{0\cdot20}(C_9H_{10}ON)_{0\cdot80} \cdot 1\cdot31H_2O]n$: C, 52·55; H, 6·83; N, 8·11. Found: C, 52·26; H, 6·17; N, 8·03.

N-(*N'-p*-Methoxyphenyl)glycyl derivative

A suspension of the *N*-chloroacetylchitosan (0·12 g) in *p*-anisidine (5 ml) was treated as described above to afford the named compound as a brown powder in 0·13 g yield. ν_{max}KBr 1670 and 1550 (C=O and NH of Nac), 830 cm^{-1} (*p*-substituted phenyl).

Anal. Calc. for $[C_6H_{10}O_4N(C_2H_3O)_{0\cdot20}(C_9H_{210}O_2N)_{0\cdot80} 0\cdot54H_2O]n$: C, 52·71; H, 6·41; N, 8·14. Found: C, 52·72; H, 6·39; N, 8·21.

RESULTS AND DISCUSSION

The *N*-α-chloropropionyl derivative (d.s. 0·84 for chloropropionyl) (**1**) was prepared by treatment of chitosan (d.s. 0·20 for *N*-acetyl) with chloropropionic acid in acetic anhydride (Hirano & Kondo, 1982). Compound **1** was insoluble in water and in the common organic solvents examined. Its suspension in aqueous 25% ammonia was stirred at room temperature to give the *N*-alanyl derivative (**2**), which exhibited a negative Beilstein's test for chlorine and no absorption at 710 cm^{-1} (C-Cl) in the IR spectrum. Alanine was identified by paper chromatography in an acidic hydrolysate of **2**. The structure was further confirmed by elemental analyses (d.s. 0·8 for *N*-alanyl group). The *N*-glycyl derivative has been prepared by treating the *N*-chloroacetyl derivative of chitosan with aqueous ammonia (Hirano & Yagi, 1980).

The *N*-chloroacetyl derivative (d.s. 0·89 for chloroacetyl) (**3**) was prepared by treating chitosan (d.s. 0·20 for NAc) with chloroacetic anhydride in chloroacetic acid (Hirano & Kondo, 1982). In its ^{13}C CP/MAS NMR spectrum (Fig. 1), C=O signals appeared at 174·5 ppm for *N*-acetyl and at 169·9 ppm for *N*-chloroacetyl, while the CH_2Cl signal appeared at 43·8 ppm and the CH_3 signal at 23·8 ppm. C-1 to C-6 signals were essentially the same as those of *N*-acetylchitosan (Hirano *et al.*, 1988). The *N*-chloroacetyl derivative of chitosan was treated in each of aniline, *o*-toluidine, and *p*-anisidine at 100 to 105°C for 2 days to give *N*-(*N'*-phenyl)glycyl (**4**), *N*-(*N'-o*-tolyl)glycyl (**5**) and *N*-(*N'-p*-methoxyphenyl) glycyl (**6**) derivatives, respectively, in 64 to 90% yields. These compounds exhibited a negative Beilstein's test for chlorine and no absorption at 710 cm^{-1} (C-Cl) in the IR spectra. No significant degradation of chitosan occurred throughout the treatments. Their structures were confirmed by absorptions at 760 and 700 cm^{-1} (monosubstituted phenyl) for **4**, at 760 cm^{-1} (*o*-substituted phenyl) for **5** and at 830 cm^{-1} (*p*-substituted phenyl) for **6** in the IR spectra. Their elemental analyses also confirmed the structures (Scheme 1). Compounds **2, 4, 5** and **6** were hydrolysed by chitinase from *Streptomyces* at 1/10 to 2/5 rates of *N*-acetylchitosan

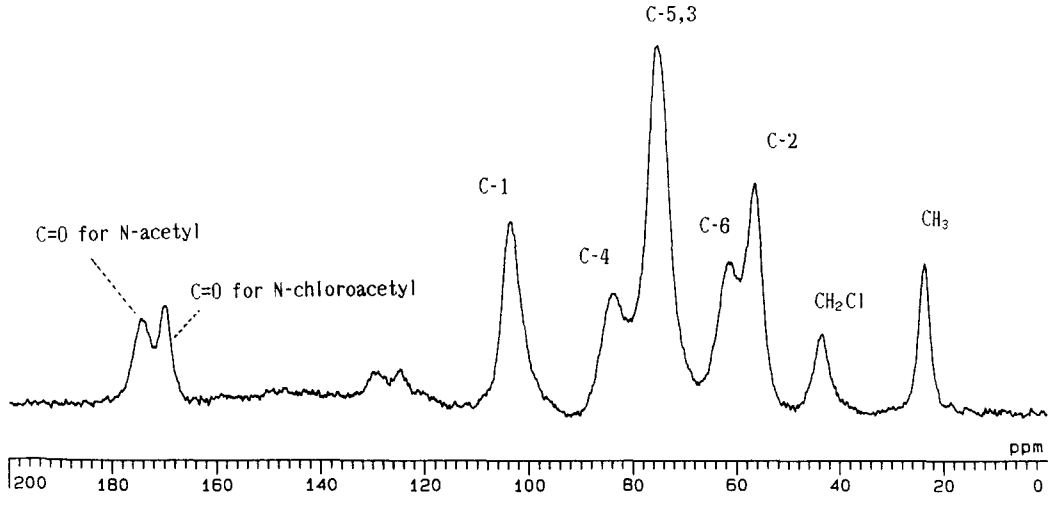

Fig. 1. ^{13}C CP/MAS NMR spectrum of *N*-chloroacetyl derivative (d.s. 0·89 for chloroacetyl and d.s. 0·20 for Nac) of chitosan.

(d.s. 1·0 for NAc). In conclusion, the present study provides a new two-stepped method for the chemical modification of chitosan on a solid phase in alkaline conditions. The method is suitable for the preparation of *N*-acyl derivatives which were difficult to prepare by direct *N*-acylation (Hirano & Nishiguchi, 1985; Kurita *et al.*, 1988).

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