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# Studies on Amino-hexoses XIV. Preparation of Partially *O*-Carboxymethylated Chitin and Its Component 3-*O*- and 6-*O*-Carboxymethyl-D-glucosamine, and the Corresponding Glucosaminitols

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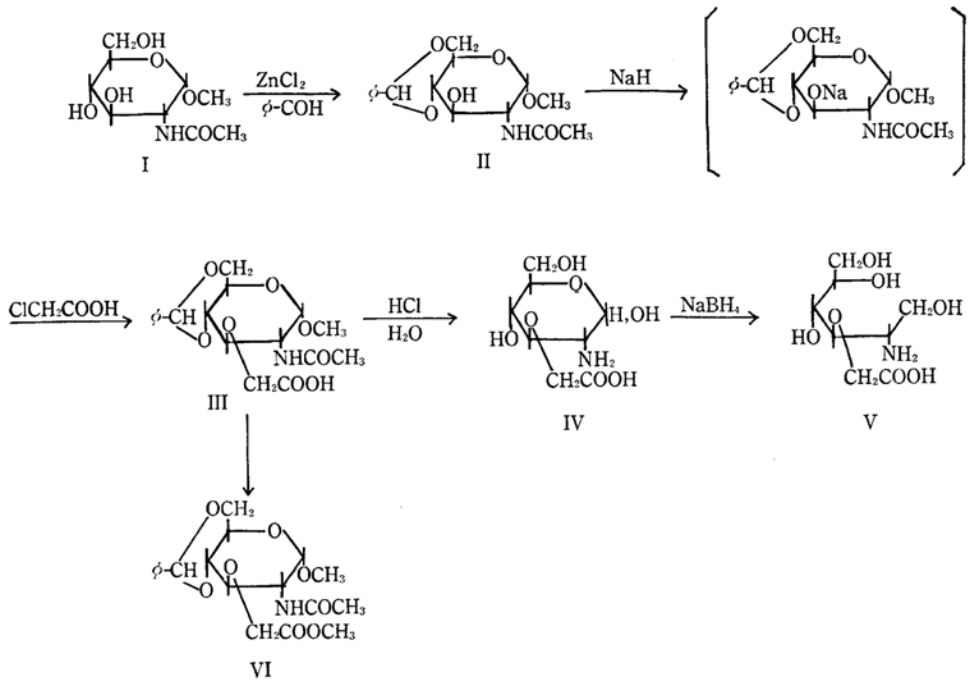
3-*O*-carboxymethyl-D-glucosamine, 6-*O*-carboxymethyl-D-glucosamine, and the corresponding glucosaminitols are prepared as reference compounds in a study of a lysozyme action on a partially-*O*-carboxymethylated chitin. The preparation of partially-*O*-carboxymethylated chitin, a substrate of hen-egg white lysozyme, is also described.

In our study of a mode of action on a partially-*O*-carboxymethylated chitin of hen-egg white lysozyme, the monomeric *O*-carboxymethylated glucosamines and the corresponding sugar alcohols were essential for characterizing the substrate and the enzymatic reaction products. In order to obtain these reference compounds, 3-*O*-carboxymethyl-D-glucosamine and the corresponding sugar alcohol were prepared *via* the synthetic route shown in Scheme 1. Because the methods we adopted in order to synthesize 6-*O*-carboxymethyl-D-glucosamine were not successful, the acid hydrolysate of the partially-*O*-carboxymethylated chitin was resolved into components by ion exchanger chromatography; a crystalline component was thus obtained which was proved analytically to be 6-*O*-carboxymethyl-D-glucosamine. The corresponding glucosaminitol was obtained by a borohydride reduction as a syrup.

Partially-*O*-carboxymethylated chitin, a substrate of the enzyme,[1] was prepared through partially-*O*-carboxymethylated chitosan,[2] different batches




Scheme 1

1) Y. Matsushima, S. Hara, T. Miyazaki and Y. Umemura, The 38 th Annual Meetings of the Japanese Biochemical Society, October, 1965.

2) T. Okimasu, *J. Agr. Chem. Soc. Japan*, **32** 303 (1958).

TABLE 1. COMPONENT OF PARTIALLY-*O*-CARBOXYMETHYLATED CHITIN

| Component | Lot No. 1 | 2 | 3 |
|---|---|---|---|
| Unknown-$X_1$ | 1.3% | 3.6% | 3.0% |
| Unknown-$X_2$ | 0.9% | 2.5% | 5.5% |
| 3-*O*-CM-Glucosamine | 2.3% | 2.5% | 5.5% |
| 6-*O*-CM-Glucosamine | 22.6% | 33.1% | 43.1% |
| Glucosamine | 72.9% | 56.9% | 43.1% |

For the estimation of the unknown components, color yield of the ninhydrin reaction was assumed to be the same with that of 3-*O*-CM-glucosamine.

of which had naturally varying compositions. The compositions of the specimens which we obtained are summarized in Table 1. The specimens are composed mostly of unsubstituted glucosamine, 3-*O*-, and 6-*O*-carboxymethyl-glucosamines. The enzymatic action will be described elsewhere.

## Experimental

**Methyl 2-Acetamido-2-deoxy-3-*O*-carboxymethyl-4,6-*O*-benzylidene-α-D-glucopyranoside (III).** To a solution of 6.5 g of methyl 2-acetamido-2-deoxy-4,6-*O*-benzylidene-α-D-glucopyranoside (II,[3] mp 248—250°C) in 400 m*l* of dry dioxane, was added, at 90°C, a suspension of 2.5 g of sodium hydride in 15 m*l* of dioxane under mechanical stirring. After 30 min, 10 g of chloroacetic acid in 30 m*l* of dioxane was added, and then the stirring was continued for 3.5 hr at the same temperature. After an additional 10 g portion of sodium hydride in 50 m*l* of dioxane had been added to the reaction mixture, the heater was removed and the stirring was continued overnight. The excess sodium hydride was decomposed by the careful addition of 30 m*l* of water under ice-water cooling, after which the mixture was evaporated *in vacuo*. The residue was dissolved in 250 m*l* of warm water, and the insoluble matter was removed by filtration. The filtrate gave a colorless precipitate when the pH of the solution was adjusted to 2 by adding 6 N hydrochloric acid under ice-water cooling. The precipitate was washed thoroughly with water and recrystallized in methanol, 3.6 g of colorless crystals which melted at 215—217°C. giving $[\alpha]_D^{20}$ +69.1 (*c* 0.79, methanol).

Found: C, 56.54; H, 6.44; N, 3.54%. Calcd for $C_{18}H_{23}O_8N$: C, 56.68; H, 6.08; N, 3.67%.

An aglycon homologue of III, ethyl 2-acetamido-2-deoxy-3-*O*-carboxymethyl-4,6-*O*-benzylidene-α-D-glucopyranoside, was likewise prepared; it melted at 203—205°C. $[\alpha]_D^{20}$ +92 (*c* 0.90, methanol).

Found: C, 57.64; H, 6.35; N, 3.52%. Calcd for $C_{19}H_{25}O_8N$: C, 57.71; H, 6.37; N, 3.54%.

**2-Amino-2-deoxy-3-*O*-carboxymethyl-D-glucose (IV).** Three grams of III were hydrolyzed in 35 m*l* of 2.5 N hydrochloric acid at 100°C for 4 hr. The hydrolysate was then washed with ether in order to remove the benzaldehyde that had been released. After the addition of 50 m*l* of water, the pH of the solution was adjusted to 6.1 by the addition of Dowex-1 ($OH^-$ form) resin. The filtrate from the resin was decolorized with a small amount of charcoal, and the solution was concentrated *in vacuo*. The concentrated solution (20 m*l*) gave, after standing in a refrigerator, colorless crystals which melted at 160—162°C (decomp.) after having been once recrystallized in a small amount of water. $[\alpha]_D^{20}$ +108 (*c* 1.36, water, equil.).

Found: C, 40.23; H, 6.63; N, 5.82%. Calcd for $C_8H_{15}O_7N$: C, 40.50; H, 6.37; N, 5.91%.

The physical constants agreed well with those given by Gigg and Carroll,[4] who prepared the compound *via* a different route.

**2-Amino-2-deoxy-3-*O*-carboxymethyl-D-glucitol (V).** To a solution of 250 mg of IV in 15 m*l* of water, was added a solution of 100 mg of sodium borohydride in 5 m*l* of water, after which the mixture was stirred overnight at room temperature. Then the pH of the mixture was adjusted to 4.5 by the addition of Amberlite IR-120 ($H^+$ form) resin. The filtrate from the resin was concentrated *in vacuo*. A small amount of methanol was then added to the residue, and the mixture was evaporated. This procedure was repeated six times in order to remove any boric acid. The residue was dissolved in 0.7 m*l* of water, and the subsequent addition of 7 m*l* of methanol gave 150 mg of colorless crystals which melted at 197—198°C. $[\alpha]_D^{23}$ +38 (*c* 2.0, methanol).

Found: C, 39.89; H, 7.25; N, 5.77%. Calcd for $C_8H_{17}O_7N$: C, 40.16; H, 7.16; N, 5.86%.

**Methyl 2-Acetamido-2-deoxy-3-*O*-methylcarboxylate-4,6-*O*-benzylidene-α-D-glucopyranoside (VI).** An ethereal solution of diazomethane was added to a solution of 300 mg of III in 100 m*l* of methanol. The addition of the reagent was stopped when a persistent yellow color remained. The mixture was then evaporated *in vacuo*, and the residue was dissolved in a small amount of methanol. After an amorphous, insoluble material had been removed, colorless crystals were obtained when the solution was cooled in a refrigerator. The specimen thus obtained (180 mg) melted at 225—226°C. $[\alpha]_D^{20}$ +77 (*c* 0.56, methanol).

Found: C, 57.84; H, 6.44; N, 3.53%. Calcd for $C_{19}H_{25}O_8N$: C, 57.71; H, 6.37; N, 3.54%.

An aglycon homologue (ethyl 2-acetamido-2-deoxy-3-*O*-methylcarboxylate-4,6-*O*-benzylidene-α-D-glucopyranoside) was likewise obtained; it melted at 174—175°C. $[\alpha]_D^{20}$ +79.4 (*c* 0.68, methanol).

Found: C, 58.54; H, 6.51; N, 3.47%. Calcd for $C_{20}H_{27}O_8N$: C, 58.67; H, 6.65; N, 3.42%.

**2-Amino-2-deoxy-6-*O*-carboxymethyl-D-glucose.** Two grams of *O*-carboxymethylated chitin (Lot 3 in Table 1) were hydrolyzed in a nitrogen atmosphere at 100°C for 5 hr with 200 m*l* of 4 N hydrochloric acid. The hydrolysate was then dried up *in vacuo*, and the residue was dissolved in 50 m*l* of a 0.2 M citrate buffer solution with a pH of 3.11. The solution was placed on an Amberlite IR-120 column ($Na^+$ form, 3×65 cm) which had been bufferized with the same buffer solution. The column was then also eluted with the same buffer solution. The elution speed was 50 m*l* per hour, and each 8.1 m*l* portion of ninhydrin-positive fraction was collected. At Fraction No. 120, the

3) Y. Matsushima and J. T. Park, *J. Org. Chem.*, **27**, 3581 (1962).

4) R. Gigg and P. Carroll, *Nature*, **191**, 495 (1961).

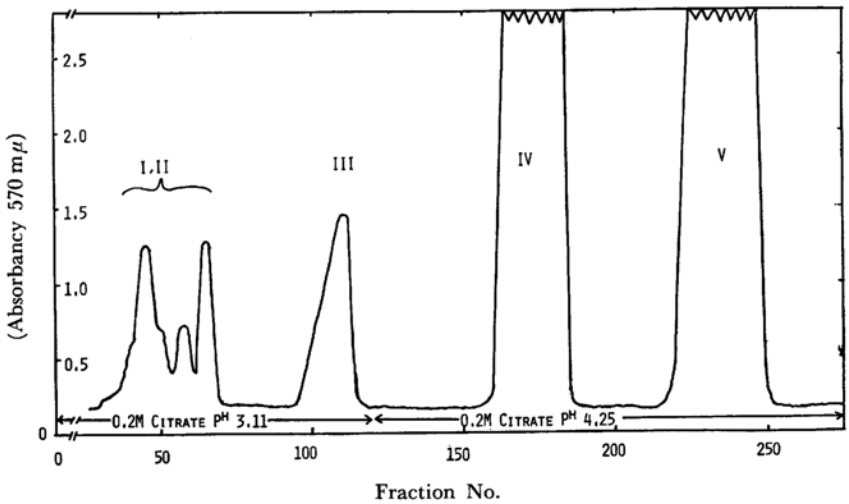



Fig. 1. Preparative chromatography of the acid hydrolyzate of partially carboxymethylated chitin (Lot 3 in Table 1). The ninhydrin-positive peaks represent unknown substances (I, II), 3-*O*-carboxymethyl-glucosamine contaminated with an unknown substance (III), 6-*O*-carboxymethyl-glucosamine (IV) and glucosamine (V).

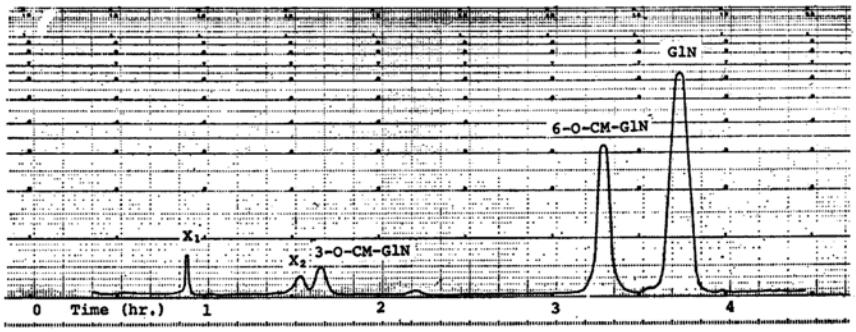



Fig. 2. Ninhydrin-positive fractions of the acid hydrolysate of partially carboxymethylated chitin. GlN: glucosamine, CM: carboxymethyl

buffer solution was changed to 0.2 M citrate with a pH of 4.25. The peak IV in Fig. 1 was evaporated *in vacuo*, and the residue was dissolved in 30 m*l* of water and placed on a column of Amberlite IR-120 ($H^+$ form, 2.8×12 cm). The column was then washed thoroughly with water and eluted with 0.1 N hydrochloric acid. A ninhydrin-positive fraction was obtained and evaporated *in vacuo*. The syrup thus obtained was dissolved in 50 m*l* of water, and the pH of the solution was adjusted to 6.1 by the addition of Dowex-1 ($OH^-$ form) resin. The filtrate from the resin was decolorized with a small amount of charcoal and evaporated *in vacuo*. The remaining syrup was crystallized in 10 m*l* of ethanol. The yield of faintly yellow crystals which melted at 178—180°C (decomp.) was 430 mg. $[\alpha]_D^{18}$ +72 (*c* 1.03, water, equil.).

Found: C, 39.84; H, 6.50; N, 5.84%. Calcd for $C_8H_{13}O_7N$: C, 40.50; H, 6.37; N, 5.91%.

The position of the carboxymethyl group was determined by the periodate oxidation method. As is shown in Table 2 the specimen did not produce formaldehyde at all after the consumption of the theoretical amount of periodate. This is strong evidence of the position-6 for the carboxymethyl group. The elemental analyses agreed with its identification as a monocarboxymethylated glucosamine; its behavior was

Table 2.

| | Consumption of periodate after 46 hr oxidation* | Formation of formaldehyde after 46 hr oxidation** |
|---|---|---|
| Glucose | 6.7 mol | 1.0 mol |
| Glucosamine | 5.4 | 1.0 |
| 3-*O*-CM-Glucosamine | 2.6 | 0.84 |
| 6-*O*-CM-Glucosamine | 4.3 | 0.00 |

* By the method of Ikenaka.[5]

** By the method of Speck.[6] The amount of formaldehyde produced by glucose was taken as the standard.

5) T. Ikenaka, *J. Biochem.*, **54**, 328 (1963).

6) J. C. Speck, Jr., "Methods in Carbohydrate Chem.," Vol. I, Academic Press (1962), p. 441.

quite different from that of synthetic 3-*O*-carboxymethylglucosamine. All these facts indicate that the specimen is 6-*O*-carboxymethylglucosamine.

**Partially-*O*-carboxymethylated Chitin.** Ten grams of finely-powdered commercial chitin were soaked in 60 g of a 42% sodium hydroxide solution and then kept overnight *in vacuo*. To this mixture crushed ice was then added, portion by portion and under vigorous stirring, until the total volume reached about one liter. A solution of 300 m*l* of 4 N sodium chloroacetate was added to the paste thus obtained, after which the stirring was continued at room temperature for 24 hr. In order to *N*-acetylate the partially-released amino groups, 100 m*l* of acetic anhydride were gradually stirred in, while the pH of the solution was prevented from going below 10 by the addition of sodium hydroxide. Stirring was continued for 24 hr. The mixture was dialyzed against running water for 3 days and against deionized water for 24 hr. The insoluble material was removed in a centrifuge, and the supernatant was lyophilized. The sodium salt of partially-carboxymethylated chitin thus obtained weighed 8.5 g; it contained 14% of water and did not reveal any van Slyke nitrogen. A sample of chromatographic pattern of a specimen (Lot No. 2 in Table 1) derived by component analysis using an automatic amino-acid analyzer is shown in Fig. 2.

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