Studies on Amino-hexoses. XVIII. A Simplified Method of Preparing 2-Amino-3-*O*-(D-1′-carboxyethyl)-2-deoxy-D-glucose 6-(Dihydrogen Phosphate) (Muramic Acid 6-Phosphate)

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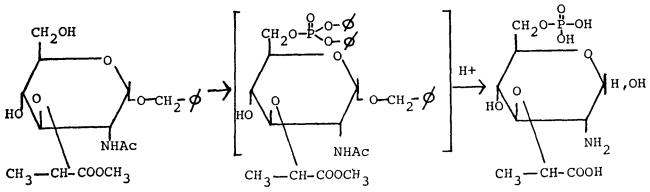
Muramic acid 6-phosphate was prepared from benzyl 2-acetamido-2-deoxy-3-*O*-[D-1′(methoxycarbonyl)-ethyl]-α-D-glucopyranoside *via* two reaction stages: diphenylphosphorylation under restricted conditions, followed by hydrolysis. When the reaction mixture was chromatographed on a Dowex 1×8 (formate form) column, the product was obtained as fine needles. Among the periodate oxidation products of the compound, glycolaldehyde phosphate was detected, while formaldehyde was not. Equimolar amounts of muramic acid and inorganic phosphate were produced by an alkaline phosphatase. The physical data are consistent with those of authentic muramic acid 6-phosphate.

The first synthetic muramic acid 6-phosphate was reported by Konami, Osawa, and Jeanloz[1] to be identical with the muramic acid phosphate obtained from bacterial cell walls, thus establishing the structure of the natural compound. We tried more or less to simplify the preparation of the compound and the estimation of a phosphate group at the 6 position of muramic acid. The route of the reactions is shown in Scheme 1. We simplified the synthesis of muramic acid 6-phosphate in a better yield (25%) to two reaction stages; selective phosphorylation and acid hydrolysis, whereas the previously reported method used 6 reaction stages. The physical constants of the synthesized compound (mp, $[\alpha]_D$, infrared spectrum, and R_{GlcNAc}) are consistent with those reported.[1] It has been reported that[2] a primary hydroxyl could be phosphorylated preferentially with a limiting amount of the reagent. For example, D-glucosamine 6-phosphate[3] was synthesized by selective diphenylphosphorylation under restricted conditions. In addition, the introduction of the bulky diphenylphosphate group at the C-4 hydroxyl of the muramic acid derivative seemed to be interfered by the lactyl group on C-3. The resulting compound was hydrolyzed with strong acid to remove the blocking groups. It seemed that phosphate migration did not occur, because a phosphate group on C-6 was more stable than that on C-4, as it was reported that D-glucosamine 4-phosphate was converted to D-glucosamine 6-phosphate during acid hydrolysis.[4] The hydrolysate of the diphenylphosphate of the muramic acid derivative showed, besides muramic acid and its 6-phosphate (main product), two minor spots on tlc. The main peak was isolated by Dowex 1×4 (formate form) column chromatography. The physical data were identical with those of the authentic compound, so the phosphate group might be at C-6. In the course of the reactions, hydrolysis by strong acid was used; therfore, the possibility of the migration of the phosphate group could not be excluded. The position of the phosphate group at muramic acid was thus estimated in the following way. Periodate oxidation study was performed by the previously reported method.[5] The periodate oxidation products of muramic acid 6-phosphate or muramicitol 6-phosphate gave a peak of glycolaldehyde phosphate which was positive for organic phosphate and diphenylamine reactions (Fig. 1). This fact indicates the 6-phosphate of muramic acid. The absorbtion spectrum of the fraction developed by diphenylamine was the same as that obtained from the reference compounds (Fig. 2). Formaldehyde was not detected in the periodate oxidation products; this is further evidence of the presence of the phosphate group at C-6 (Table 3). The infrared spectrum of the muramic acid-6-phosphate synthesized agreed well with the reported data.



Scheme 1.

Experimental

Abbreviations used: Glc, glucose; Mur, muramic acid; Mur 6-P, muramic acid 6-(dihydrogen phosphate); GlcN, glucosamine; GlcNAc, N-acetyl-glucosamine.

The diphenylphosphorochloridate was purchased from Merck Co. and was used without purification. The muramic acid was synthesized by the reported method.[6] The glucosamine 6-phosphate sodium salt and α-DL-glycerophosphate disodium salt were commercial specimens from the Sigma Chem. Co. Avicel SF (microcrystalline cellulose) was purchased from the Funacoshi, Co. Ltd., and the Kiesel Gel F_{254} from the Merck Co. The calf mucosa alkaline phosphatase (grade II, 10 mg/ml) [EC 3.1.3.1] was purchased from Boehringer Mannheim.

*2-Amino-3-*O*-(D-1′-carboxyethyl)-2-deoxy-D-glucose 6-(Dihydrogen Phosphate).* Benzyl 2-acetamido-2-deoxy-3-*O*-[D-1′-(methoxycarbonyl)ethyl]-α-D-glucopyranoside[7] (1.0 g) was dissolved in 30 ml of pyridine, after which the solution was cooled in a dry ice-acetone bath. To this solution was added 0.65 ml of diphenylphosphorochloridate. After the reaction mixture had been maintained at −20 °C for 16 hr, 1.0 ml of water was added to this solution. The solution was then allowed to stand for 30 min and was concentrated. The residue was dissolved in chloroform. The solution was washed with water, concentrated, and placed on a silicic acid column. The conditions were as follows: a column (2.5×20 cm; 52 g

silicic acid activated overnight at 150 °C) was eluted with ethyl acetate: toluene (5:2 v/v) and 8 ml fractions were collected. The main peak appeared at the 14—21 fractions. The main fraction was concentrated to give a colorless sirup which gave a single spot on a thin-layer chromatogram (Merck Kiesel Gel F_{254}, ethyl acetate: toluene=5:2 v/v, R_f=0.37).

To the sirup we then added 20 ml of 3 M hydrochloric acid, and the solution was heated for 6 hr at 100 °C.[1] The water-soluble fraction of the hydrolysate was concentrated and placed on a Dowex 1×4 column (100—200 mesh, formate form), which was then eluted with 0.5 M formic acid.[8] Ten-ml fractions were collected, and the main peak appeared at tubes number 10—16. The main fraction was concentrated, and the residue gave fine needles in ethanol-water in a refrigerator. Yield, 230 mg (25% of theory). mp 170.5—171.5 °C (dec.); reported mp 170—172 °C (dec.).[1] Found: C, 31.29; H, 6.20; N, 4.10; P, 9.11%. Calcd for $C_9H_{18}O_{10}NP\cdot H_2O$: C, 30.95; H, 5.77; N, 4.01; P, 8.87%. Mol wt. 349.2. $[\alpha]_D^{22}$ +107.7°→+104.1° (8 min)→+90.2° (3 hr)→+90.2±7.5° (at equilibrium; *c* 0.194, water). Reported $[\alpha]_D$ +106° (8 min)→+79° (at equilibrium).[1]

Table 1. Paper and thin layer chromatography of muramic acid 6-phosphate

Relative mobilities to glucosamine or *N*-acetyl-glucosamine were shown.

Solvent	GlcN	GlcNAc	Mur	Mur 6-P
Ethylacetate: Acetic acid: Pyridine: Water (5:1:5:3) (Whatman No. 1)[a]	—	1.00	0.60	0.15
Butanol: Acetic acid: Water (5:3:2) (Whatman No. 1)[a]	—	1.00	1.13	0.67
Butanol: Acetic acid: Water (2:1:1) (Avicel SF)[b]	1.00	—	1.84	1.22
Butanol: Acetic acid: Water (2:1:1) (Kiesel Gel F_{254})[c]	1.00	—	1.12	0.45

Spots were detected. a) by silver nitrate reagent[9]. b) by ninhydrin reagent. c) by sulfuric acid.

Analyses of Muramic Acid 6-Phosphate Synthesized. The compound gave one spot on paper and thin-layer chromatograms. The results are summarized in Table 1. On a paper electrophoretogram the compound gave a single spot with $M_{\text{gluconic acid}}$=1.0 at 700 V/15 cm for 35 min in an acetic acid pyridine buffer (pH 3.5).

Periodate Oxidation of Muramic Acid 6-Phosphate. As a reference, α-DL-glycerophosphate or D-glucosamine 6-phosphate (4.1 and 5.0 μmol each) in 0.5 ml of water was treated with 9.5 μmoles of sodium periodate in 0.5 ml at pH 5. The reaction mixture was allowed to stand at 25 °C for 48 hr in the dark. It was then placed on a Dowex 1×4 column (200—400 mesh, chloride form, 0.9×10 cm); the column was eluted first with 20 ml of 0.052 M hydrochloric acid and then with 0.05 M hydrochloric acid.[5] One and a half-ml fractions were collected and the aliquots were analyzed for organic phosphate[10] and diphenylamine-positive substances[11] (Fig. 1, A, B). Muramic acid 6-phosphate (4.9 μmol) was treated in the same way, and the oxidation was continued for 62 hr (Fig. 1, C). Muramic acid 6-phosphate was reduced with sodium borohydride and then treated in the same way (Fig. 1, D). The absorbtion spectra of the color developed by diphenylamine[5] with peak fractions at tubes number 24—26 are shown in Fig. 2.

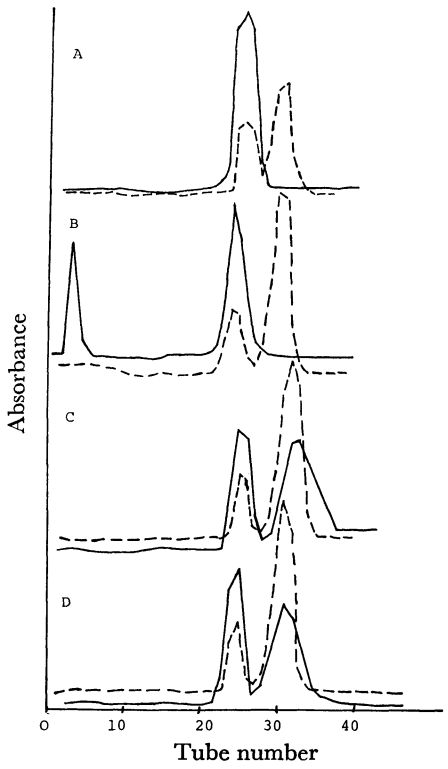


Fig. 1. Column chromatography of the periodate oxidation products of muramic acid 6-phosphate and standard samples.
A: α-glycerophosphate, B: Glucosamine 6-phosphate, C: Muramic acid 6-phosphate, D: Muramic acid 6-phosphate reduced with sodium borohydride. ——, Organic phosphate at 820 mμ. ----, Color at 660 mμ developed by diphenylamine reaction.

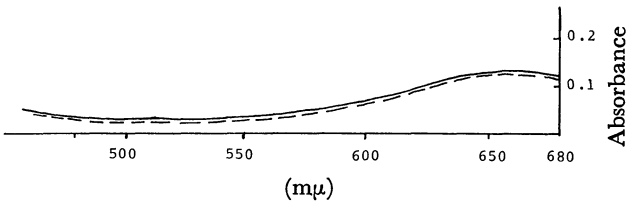


Fig. 2. Absorbtion spectra of the color developed by diphenylamine with peak fractions appeared at tube number 24—26 shown in Fig. 1.
Muramic acid 6-phosphate or reduced muramic acid 6-phosphate ----; glucosamine 6-phosphate or α-glycerophosphate ——.

Table 2. Analyses by the alkaline phosphatase of muramic acid 6-phosphate

	Recovery		
	with the enzyme	without the enzyme	incubation time (hr)
Mur 6-P	<1.0%	83%	4
Mur produced from Mur 6-P	103%	<2.5%	4
Inorganic phosphate	101%	<1%	1

Analyses by Alkaline Phosphatase. To a 0.2 ml solution of 0.40 μmole of muramic acid 6-phosphate, was added 0.4 ml of water and 0.2 ml of a 0.1 M ethanolamine–HCl buffer (pH 9.5, containing 0.005 M $MgCl_2$). To this solution 2 μl of the enzyme solution was added, after which the mixture was incubated at 37 °C.[12] Aliquots were analyzed for inorganic phosphate, and aminosugars were determined by means of an automatic amino acid analyzer (Hitachi KLA 3B). The results are shown in Table 2.

Detection of Formaldehyde in Periodate Oxidation Products. The pH of a sample solution (0.8 μmol in 0.08 ml of water) was adjusted to 5 if necessary. The oxidation was carried out with 4.3 μmol of sodium periodate in 0.02 ml at 25 °C for 48 hr. The excess periodate was decomposed with arsenite, and chromotropic acid was used for the determination of formaldehyde.[13] When glucose was used as an standard, 0.04 mol of formaldehyde was detected from one mole of muramic acid 6-phosphate, and 0.91 mol from one mol of muramic acid.

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