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Synthesis of Muramic Acid 6-Phosphate [2-Amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose 6-(Dihydrogen Phosphate)]*

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ABSTRACT: Muramic acid 6-phosphate [2-amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose 6-(dihydrogen phosphate)] was synthesized starting from benzyl 2-acetamido-3-*O*-[D-1-(methoxycarbonyl)ethyl]-2-deoxy-β-D-glucopyranoside. Tritylation at C-6, acetylation at C-4, detritylation, dibenzyl phosphorylation, followed by catalytic hydrogenolysis, acid hydrolysis, and column chromatography gave crystalline muramic acid 6-phosphate. The same series of reactions was also performed in the α series.

The synthetic muramic acid 6-phosphate was characterized by infrared spectroscopy, X-ray powder diffraction, Elson–Morgan reaction, and by paper and thin-layer chromatography, and it was found to be identical with the natural muramic acid phosphates isolated from *Micrococcus lysodeikticus* cell wall and also from *Streptococcus pyogenes* cell wall.

The first evidence of the presence of an acid-stable muramic acid phosphate in bacteria was presented by Ågren and de Verdier (1958) who isolated the crystalline phosphate from the acid hydrolysate of *Lactobacillus casei*. Subsequently, an identical muramic acid phosphate was isolated from the cell walls of various Gram-positive bacteria (Hall and Knox, 1965; Heymann *et al.*, 1967; Liu and Gotschlich, 1967; Montague and Moulds, 1967; Grant and Wicken, 1968; Knox and Holmwood, 1968; Hungerer *et al.*, 1969). On the basis of the acid stability of the phosphate and of the results of the periodate oxidation, Ågren and de Verdier proposed the structure of a 6-phosphate, but the results of the periodate oxidation have in the past led to erroneous interpretations of structures containing muramic acid (Salton and Ghuysen, 1959, 1960; Ghuysen and Strominger, 1963). Since muramic acid 6-phosphate may serve as a link between the peptidoglycan structure and the cell wall polysaccharides or teichoic acids, it was of interest to obtain synthetically 2-amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose 6-*O*-(dihydrogen phosphate) and compare it to the muramic acid phosphate isolated from natural products.

Results and Discussion

Since complete removal of the benzyl aglycon group can be effected by mild catalytic hydrogenolysis, the benzyl glycoside derivatives of *N*-acetylmuramic acid [2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose] were selected as starting materials for the synthesis of muramic acid 6-phosphate (**7**) (see Scheme I). Benzyl 2-acetamido-2-deoxy-3-*O*-[D-1-(methoxycarbonyl)ethyl]-β-D-glucopyranoside (**1**) (Jeanloz *et al.*, 1968) was tritylated at C-6 and then acetylated at C-4 to give benzyl 2-acetamido-4-*O*-acetyl-2-deoxy-3-*O*-[D-1-(methoxycarbonyl)ethyl]-6-*O*-triphenylmethyl-β-D glucopyranoside (**2**). Detritylation of compound **2** with hot 60% acetic acid gave benzyl 2-acetamido-4-*O*-acetyl-2-deoxy-3-*O*-[D-1-(methoxycarbonyl)ethyl]-β-D-glucopyranoside (**3**) which was subsequently phosphorylated with dibenzyl phosphorochloridate (Smith, 1961) to give benzyl 2-acetamido-4-*O*-acetyl-2-deoxy-3-*O*-[D-1-(methoxycarbonyl)ethyl]-β-D-glucopyranoside 6-(dibenzyl phosphate) (**4**). Phosphorylation of compound **5** (the α anomer of compound **3**; Flowers and Jeanloz, 1963) with dibenzyl phosphorochloridate in the same fashion gave compound **6**. Hydrogenolysis of the benzyl glycosides of muramic acid is a very sluggish process (Flowers and Jeanloz, 1963; Osawa and Jeanloz, 1965; Osawa *et al.*, 1969), and the complete removal of the benzyl aglycon group of compound **4** or **6** required catalytic hydrogenolysis for 1 week in the presence of 10% Pd/C. In order to find out the optimal conditions of acid hydrolysis of the acetyl groups at C-2 and C-4, and of the methyl ester group at the lactyl moiety, the hydrogenolysates of compound **4** and **6** were treated with various concentrations of hydrochloric acid at 100°. The resulting hydrolysates were analyzed by paper electrophoresis and paper chromatography, and the results are reported in Table I. The hydrolysate with 1 M hydrochloric acid for 6 hr showed two spots; by treatment with acid phosphatase, as shown in Table I, one of the spots was identified

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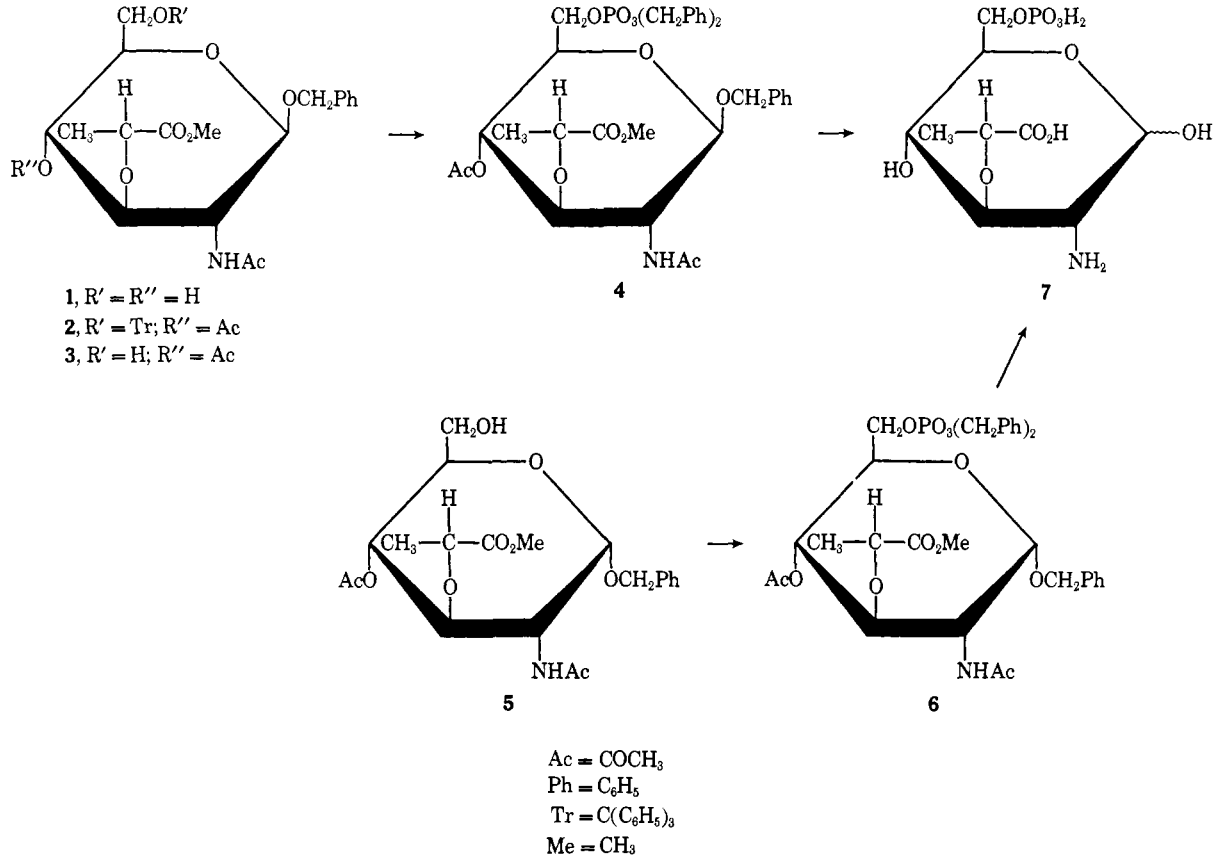
SCHEME I



as *N*-acetylmuramic acid 6-phosphate [2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose 6-(dihydrogen phosphate)] and the second spot as compound **7**. Hydrolysis with 3 M hydrochloric acid for 12 hr gave an appreciable amount of muramic acid [2-amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose] in addition to compound **7**. Acid hydrolysis with 3 M hydrochloric acid for 6 hr at 100° was, therefore, selected. After hydrolysis of the catalytic hydrogenolysates of compound **4** and **6** under this condition, small amounts of muramic acid and *N*-acetylmuramic acid 6-phosphate contaminating the hydrolysate were removed by successive passage through columns of Dowex-50 (H^+) and Dowex-1 (HCO_2^-) to yield pure, crystalline compound **7**.

The natural muramic acid phosphate isolated from *Micrococcus lysodeikticus* cell wall (M. Tomoda and R. W. Jeanloz, unpublished data) and that from *Streptococcus pyogenes* cell wall (Heymann *et al.*, 1967) were compared with the synthetic crystalline sample reported here, and both compounds were found to be identical with the synthetic material on the basis of paper and thin-layer chromatography, each in two solvent systems, and of the Elson–Morgan reaction (Table I). Furthermore, the infrared spectrum (Figure 1), the optical rotation (Table II), and the X-ray powder diffraction data (Table II) of the synthetic crystalline sample were in good agreement with those of the natural crystalline muramic acid phosphate isolated from *Lactobacillus casei* (Ågren and de Verdier, 1958).

Experimental Section

General Methods. Melting points were taken on a hot stage, equipped with a microscope. Specific rotations were determined in semimicropolarimeter tubes having lengths of 1 dm or 2 dm, with a Zeiss polarimeter having a scale reading to 0.01° or with a Yanagimoto automatic polarimeter, Model OR-50. Infrared spectra were determined on a Nihon-Bunko (JASCO) spectrophotometer, Model DS-402G, on potassium bromide disks. Evaporations were performed *in vacuo*, with an outside bath temperature kept below 45°. Small amounts of volatile solvents (less than 20 ml) were evaporated under a stream of dry nitrogen. Microanalyses were performed by the members of the Central Analysis Room of the Faculty of Pharmaceutical Sciences, University of Tokyo, and by Dr. W. Manser, Zurich, Switzerland.

Chromatographies. The silicic acid used for column chromatography was "Silica Gel Davison" (grade 950, 60–200 mesh) or "Wakogel C-100" (100 mesh, Wako Chemical Co.), used without pretreatment. The eluents were used in the

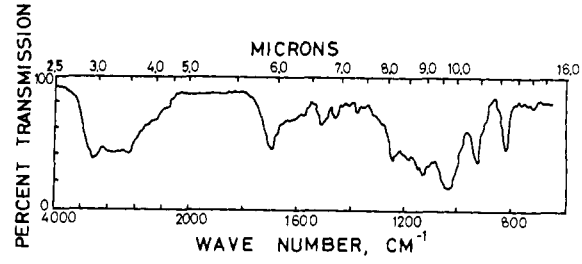


FIGURE 1: Infrared spectrum of synthetic muramic acid 6-phosphate [2-amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose 6-(dihydrogen phosphate)] determined with the Nihon-Bunko (JASCO) spectrophotometer, Model DS-402G, on KBr disk.

TABLE I: Paper and Thin-Layer Chromatography and Elson–Morgan Reaction of Muramic Acid 6-Phosphate and of the Products Derived by Acid and Enzymic Hydrolysis.

Compound	Paper Chromatography[a]		Thin-Layer Chromatography[b]		Paper Electrophoresis[c]	Elson–Morgan Reaction[d]
	Solvent A R_{GNAc}[e]	Solvent B R_{GNAc}[e]	Solvent C R_{GN}[f]	Solvent D R_{GN}[f]	M_{GNAc}[e]	
Hydrolyzate of product of hydrogenolysis of compound **4** or **6** with						
1 M HCl, 6 hr, 100°	0.50, 0.14				3.24, 3.83	
3 M HCl, 6 hr, 100°	0.50[g], 0.14, 0.62[g]				3.84,[g] 3.24, 0.78[g]	
3 M HCl, 12 hr, 100°	0.14, 0.62[g]				3.24, 0.78[g]	
Hydrolyzate with acid phosphatase of R_{GNAc} 0.50 (solvent A)[h]	1.00	1.45				
Hydrolyzate with acid phosphatase of R_{GNAc} 0.14 (solvent A)[h]	0.62	1.09			0.78	
2-Amino-2-deoxy-D-glucose hydrochloride			1.00	1.00		530
2-Amino-2-deoxy-D-glucose 6-phosphate			0.65	0.44		
2-Acetamido-2-deoxy-D-glucose	1.00	1.00			1.00	
Muramic acid	0.62	1.09	1.90	1.26	0.78	503
N-Acetylmuramic acid	1.00	1.45				
Muramic acid 6-phosphate (**7**)	0.14	0.63	1.19	0.59	3.24	517
Compound isolated from *M. lysodeikticus* cell wall	0.14	0.63	1.19	0.59		517
Compound isolated from *S. pyogenes* cell wall[i]	0.14	0.63	1.19	0.59		517

[a] Paper chromatography on Toyo Roshi No. 53 (ascending) in ethyl acetate–pyridine–acetic acid–water (5:5:1:3, v/v, solvent A) and in 1-butanol–acetic acid–water (5:3:2, v/v, solvent B). [b] Thin-layer chromatography on cellulose (Avicel) plate (ascending) in 1-butanol–acetic acid–water (2:1:1, v/v, solvent C) and in ethanol–1 M ammonium acetate (pH 3.8) (7:3, v/v, solvent D). [c] Paper electrophoresis on Whatmann No. 3 MM in 0.02 M pyridine-acetic acid buffer, pH 3.5, 20 V/cm, 3 hr. [d] The wavelength (nm) of absorption maximum in Rondle and Morgan (1955) modification. [e] GNAc, 2-acetamido-2-deoxy-D-glucose. [f] GN, 2-amino-2-deoxy-D-glucose hydrochloride. [g] Faint spot. [h] The areas corresponding to R_{GNAc} value indicated were cut out, extracted with distilled water, and evaporated. The residue was dissolved in a small amount of buffer solution, incubated with acid phosphatase, and rechromatographed. [i] Kindly provided by Dr. H. Heymann.

following sequence, individually or in binary mixtures; hexane, benzene or 1,2-dichloroethane, ether, ethyl acetate, acetone, and methanol. The proportion of weight of substance to be adsorbed to weight of adsorbent was 1 to 50–100. The proportion of weight of substance in grams to volume of fraction of eluent in milliliters was 1 to 100. The ratio of diameter to length of the column was 1 to 20.

The fractions of ion-exchange column chromatographies were analyzed with the ninhydrin reagent, as modified by Yemm and Cocking (1955), or with the Elson–Morgan reagent, as modified by Belcher *et al.* (1954).

The homogeneity of the nonpolar substances was controlled by thin-layer chromatography (ascending) on plates covered by silica gel (Wakogel B-O, Wako Chemical Co.) revealed by concentrated sulfuric acid. The polar substances were examined on thin-layer plates of microcrystalline cellulose (Avicel) and the spots were detected with the ninhydrin reagent (0.2% in water-saturated 1-butanol). Some results of the chromatographies are reported in Table I.

The paper chromatographies were performed (ascending) on Toyo Roshi paper No. 53, and the spots were detected with the silver nitrate reagent. The results are reported in Table I.

Paper Electrophoresis. Paper electrophoresis was carried out on Whatman No. 3MM paper at pH 3.5 (0.02 M pyridine–acetic acid buffer) at 20 V/cm for 3 hr. The spots were detected with the silver nitrate reagent.

Color Reaction. Synthetic and natural muramic acid 6-phosphate muramic acid, and 2-amino-2-deoxy-D-glucose hydrochloride (0.1 mg in 1 ml of water) were treated with the Elson–Morgan reagent, as modified by Rondle and Morgan (1955). The results are reported in Table I.

Acid Phosphatase Degradation. Treatment with wheat-germ acid phosphatase (Seikagaku Kogyo Co., lot No. 4301) was

TABLE II: Physical Properties of Synthetic and Natural Muramic Acid 6-Phosphate.

Compound	Melting point	Optical Rotation $[\alpha]_D$ (degrees)	X-Ray Powder Diffraction Data[a]
Synthetic muramic acid 6-phosphate (7)	170–172° dec	+106 (8 min) → +83 (3 hr) → +79 (equilibrium, water)	3.44 W, 3.68 W, 4.06 S, 4.62 S, 5.37 W, 5.95 M, 6.66 VW, 10.63 VW, 11.95 M, 13.00 VW
Natural muramic acid 6-phosphate from *L. casei*[b]		+82 (water)	3.20 VW, 3.38 W, 3.53 W, 4.11 S, 4.68 S, 5.43 W, 6.03 M, 6.73 W, 10.43 VW, 12.22 M

[a] Data give interplanar spacings for Cu Kα radiation with relative intensities estimated visually: S, strong; M, moderate; W, weak; VW, very weak. [b] Reported by Ågren and de Verdier (1958).

performed at 37°, for 4 hr, in 0.05 M acetate buffer (pH 5.6) with 300 μg of phosphatase per ml.

Benzyl 2-Acetamido-4-O-acetyl-2-deoxy-3-O-[D-*1-(methoxycarbonyl)ethyl*]*-6-O-triphenylmethyl-β-*D-*glucopyranoside* (**2**). To a solution of benzyl 2-acetamido-2-deoxy-3-*O*-[D-1-(methoxycarbonyl)ethyl]-β-D-glucopyranoside (**1**, 2.57 g) (Jeanloz *et al.*, 1968) in dry pyridine (10 ml) was added chlorotriphenylmethane (2.2 g). The solution was heated at 100° for 30 min, and then kept overnight at room temperature. After it was cooled with an ice bath to 4°, acetic anhydride (7 ml) was added, and the solution was kept overnight at 4°, and then poured into ice water and extracted three times with chloroform. The chloroform extract was washed successively with 10% potassium hydrogen sulfate and saturated sodium hydrogen carbonate, and then dried and evaporated. The residue was dissolved in 1,2-dichloroethane and chromatographed on silica gel. Elution with a mixture of 1,2-dichloroethane and ether (9:1, v/v) gave 2.75 g (62%) of crystalline material, which was recrystallized from a mixture of methanol and chloroform to give needles, mp 256–257°, $[\alpha]_D^{20}$ +12° (*c* 0.87, chloroform). *Anal.* Calcd for $C_{40}H_{43}NO_9$: C, 70.47; H, 6.35; N, 2.05. Found: C, 70.11; H, 6.18; N, 2.25.

Benzyl 2-Acetamido-4-O-acetyl-2-deoxy-3-O-[D-*1-(methoxycarbonyl)ethyl*]*-2-deoxy-β-*D-*glucopyranoside* (**3**). A solution of compound **2** (480 mg) in 60% acetic acid (20 ml) was heated for 30 min at 100°. The solution was evaporated, and the last trace of acetic acid was removed by addition of toluene, followed by evaporation. The residue was dissolved in benzene and chromatographed on silica gel. A mixture of ether and ethyl acetate (4:1, v/v) eluted 176 mg (56%) of crystalline fractions which were recrystallized from a mixture of acetone, ether, and petroleum ether to give needles, mp 188–190°, $[\alpha]_D^{20}$ −6° (*c* 0.54, chloroform). *Anal.* Calcd for $C_{21}H_{29}NO_9$: C, 57.30; H, 6.62; N, 3.19. Found: C, 57.74; H, 6.89; N, 3.01.

Benzyl 2-Acetamido-4-O-acetyl-2-deoxy-3-O-[D-*1-(methoxycarbonyl)ethyl*]*-β-*D-*glucopyranoside 6-(Dibenzyl Phosphate)* (**4**). Dibenzyl phosphorochloridate, freshly prepared from dibenzyl phosphite (1.2 g) and *N*-chlorosuccinimide (620 mg) (Smith, 1961), was added to a solution of compound **3** (564 mg) in dry pyridine (5 ml), which had previously been frozen in a solid carbon dioxide–acetone bath. The mixture was shaken until homogeneous, and then replaced in the solid carbon dioxide–acetone bath for 1 hr. Subsequently, the reaction vessel was cooled to −15° for 15 hr. Distilled water (1 ml) was added, and the solution was kept at room temperature for 15 min and then concentrated to a syrup. The remaining pyridine was removed by addition of toluene, followed by distillation. The residue was dissolved in chloroform, and the solution was chromatographed on silica gel (30 g). Elution with a mixture of benzene and ether (4:1, v/v) gave 547 mg (66%) of crystalline fractions, which were recrystallized from a mixture of acetone, ether, and petroleum ether to give needles, mp 105–108°, $[\alpha]_D^{20}$ −12° (*c* 0.69, chloroform). *Anal.* Calcd for $C_{35}H_{42}NO_{12}P$: C, 60.08; H, 6.05; N, 2.00; P, 4.43. Found: C, 59.97; H, 6.14; N, 2.26; P, 4.07.

Benzyl 2-Acetamido-4-O-acetyl-2-deoxy-3-O-[D-*1-(methoxycarbonyl)ethyl*]*-α-*D-*glucopyranoside 6-(Dibenzyl Phosphate)* (**6**). Dibenzyl phosphorochloridate, freshly prepared from dibenzyl phosphite (336 mg) and *N*-chlorosuccinimide (171 mg), was added to a solution of benzyl 2-acetamido-4-*O*-acetyl-2-deoxy-3-*O*-[D-1-(methoxycarbonyl)ethyl]-α-D-glucopyranoside (467 mg) (Flowers and Jeanloz, 1963) in dry pyridine, which had previously been frozen in a solid carbon dioxide–acetone bath. The mixture was treated as described for the preparation of compound **4**. After chromatography on silica gel (40 g), elution with 1,2-dichloroethane and ether (4:1, v/v) gave 319 mg (43%) of crystalline fractions. Recrystallization from a mixture of acetone, ether, and pentane gave needles, mp 122–124°, $[\alpha]_D^{20}$ +90° (*c* 0.87, chloroform). *Anal.* Calcd for $C_{35}H_{42}NO_{12}P$: C, 60.08; H, 6.05; N, 2.00; P, 4.43. Found: C, 60.06; H, 6.23; N, 1.96; P, 4.59.

2-Amino-3-O-(D-*1-carboxyethyl*)*-2-deoxy-*D-*glucose 6-(Dihydrogen Phosphate)* (**7**). A solution of compound **4** (500 mg) in 90% ethanol (30 ml) was hydrogenolyzed at room temperature and normal pressure in the presence of 10% Pd/C as catalyst. After 1 week, the solution was filtered, and the filtrate was evaporated. The substance was homogeneous on silica gel thin-layer chromatography in butyl alcohol–acetone–water (4:5:1, v/v); R_{GNAc} 1.51. It was heated in 3 M hydrochloric acid for 6 hr at 100°. After evaporation, the residue was applied to a column of Dowex 50-X8 (H^+, 200–400 mesh) and eluted with water at a flow rate of 10 ml/hr. Fractions of 2.0 ml were collected, and aliquots of 20 μl were taken from each fraction and analyzed with the Elson–Morgan reagent and with ninhydrin. Elson–Morgan-positive and ninhydrin-positive fractions were pooled and further purified by application to a column of Dowex 1-X10 (HCO_2^-, 200–400 mesh). The column was eluted with 0.5 M formic acid at a flow rate of 10 ml/hr. Elson–Morgan-positive fractions were pooled and lyophilized (81 mg) (29%). The amorphous

residue crystallized from a mixture of aqueous ethanol and ether, after being kept in a refrigerator for 0.5 month, to give needles. This substance was found to be pure by paper and thin-layer chromatography, as indicated in Table I, and showed mp 170–172° dec, $[\alpha]_D^{21}$ +106° (8 min) → +83° (3 hr) → +79° (at equilibrium; *c* 0.66, water). *Anal.* Calcd for $C_9H_{18}NO_{10}P \cdot 3H_2O$: C, 28.06; H, 6.28; P, 8.04. Found: C, 27.81; H, 5.78; P, 7.44.

The same treatment of compound **6** (100 mg) gave 20 mg (36%) of compound **7**.

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