*ca.* 3.5 mmoles) was protected from moisture and added to a green solution of 0.116 g. (0.5 mmole) of 1-phenylazoazulene[5] in 10 ml. of carbon tetrachloride; the mixture became red immediately. After standing at room temperature for 10 min., the reaction mixture was washed with 10% aqueous sodium carbonate, whereupon the original green color returned to the solution. The organic solution was then washed three times with water and dried over anhydrous sodium sulfate. The solvent was then removed and the residue was chromatographed over basic alumina. Ether eluted a green band which yielded 0.115 g. of yellow-green needles which were identified by m.p. (119–120°) and m.m.p. (no depression) as 1-phenylazoazulene.[5]

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# Stereospecific Synthesis of 2-Amino-3-*O*-(D-1′-carboxyethyl)-2-deoxy-D-glucose (Muramic Acid) and Related Compounds[1,2]

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Muramic acid, 2-amino-3-*O*-(D-1′-carboxyethyl)-2-deoxy-D-glucose, an important component of the structural mucopeptide of bacterial cell walls, has been synthesized by a stereospecific method. Data are presented which indicate that the lactic acid moiety of muramic acid belongs to the D-series. The following derivatives of muramic acid and isomuramic acid have been synthesized and described: methyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-(D-1′-carboxyethyl)-2-deoxy-α-D-glucopyranoside, methyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-(D-1′-carboxyethyl)-2-deoxy-β-D-glucopyranoside, ethyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-(D-1′-carboxyethyl)-2-deoxy-α-D-glucopyranoside, ethyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-(methyl D-ethyl-1′-carboxylate)-2-deoxy-α-D-glucopyranoside, methyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-(L-1′-carboxyethyl)-2-deoxy-α-D-glucopyranoside, methyl 2-acetamido-3-*O*-(L-1′-carboxyethyl)-2-deoxy-α-D-glucopyranoside, and the potassium salt of methyl 2-acetamido-3-*O*-(D-1′-carboxyethyl)-2-deoxy-α-glucopyranoside.

A new amino sugar was discovered[5] bound to uridine pyrophosphate peptide complexes in *Staphylococcus aureus* and this amino sugar was found subsequently in bacterial spores,[6] cell walls of gram positive bacteria,[7] and cell walls of *Escherichia coli*.[8] This amino sugar, named muramic acid, has been crystallized[9] and its provisional structure[10] confirmed by Kent[11] who synthesized 2-amino-3-*O*-(1′-carboxyethyl)-2-deoxy-D-glucose. Lambert and Zilliken[12] and Gigg and Carroll[13] have also synthesized muramic acid. All of the methods employed have required the resolution of a stereoisomeric mixture to obtain the desired product.

We synthesized muramic acid following basically the method of Kent but using optically active α-chloropropionic acid as the condensing reagent. Use of the optically active compound permitted us to establish more conclusively the stereostructure of the lactic acid moiety of muramic acid and incidentally to avoid tedious chromatographic operations in the preparative work. The reaction course of our synthesis is shown in the diagram below. The preparation of optically active α-chloropropionic acid was carried out as described in the literature,[14] that is, by deamination of active alanine with a mixture of nitrous and hydrochloric acids. We obtained a yield of about 50% of the corresponding optically active α-chloropropionic acid from either L-or-D-alanine. Isolation of methyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-(1′-carboxyethyl)-2-deoxy-α-D-glucopyranosides proved easy since they behaved as water-insoluble, alkali-soluble organic acids. As shown in the Experimental, this initial condensation product was obtained in good yield (usually more than 70% as pure recrystallized product). After removal of the benzylidene group by heating with 66% acetic acid, methyl 2-acetamido-3-*O*-(1′-carboxyethyl)-2-deoxy-α-D-glucopyranoside was obtained. Hydrolysis with dilute hydrochloric acid gave 2-amino-3-*O*-(1′-carboxyethyl)-2-deoxy-D-glucopyranoside. It was found that L-α-chloro-

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(5) J. T. Park, *J. Biol. Chem.*, **194**, 885 (1952).

(6) R. E. Strange and J. F. Powell, *Biochem. J.*, **58**, 80 (1954); J. T. Park and J. L. Strominger, *Science*, **125**, 99 (1957); J. L. Strominger, J. T. Park, and R. E. Thompson, *J. Biol. Chem.*, **234**, 3263 (1959).

(7) C. S. Cummins and H. Harris, *Biochem. J.*, **57**, XXXII (1954); C. S. Cummins and H. Harris, *J. Gen. Microbiol.*, **14**, 583 (1956).

(8) W. Weidel and J. Primosigh, *Z. Naturforsch.*, **12**, 441 (1957).

(9) R. E. Strange and F. A. Dark, *Nature*, **177**, 186 (1956).

(10) R. E. Strange, *Biochem. J.*, **64**, 23 (1956).

(11) L. H. Kent, *ibid.*, **67**, 5 (1957); R. E. Strange and L. H. Kent, *ibid.*, **71**, 333 (1959).

(12) R. Lambert and F. Zilliken, *Chem. Ber.*, **93**, 2915 (1960).

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

(14) Shou-Cheng J. Fu, S. M. Birnbaum, and J. P. Greenstein, *J. Am. Chem. Soc.*, **76**, 6054 (1954).

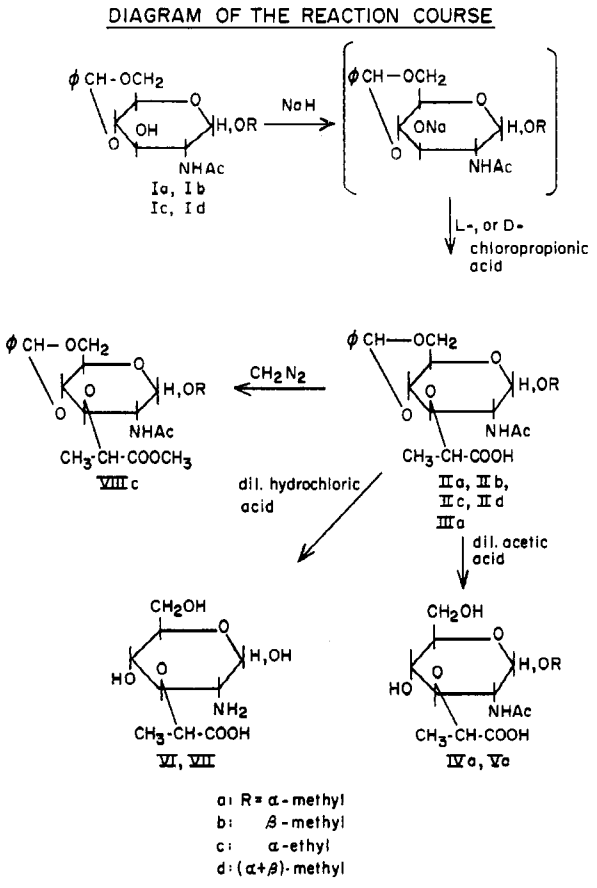


Figure 1

propionic acid gave muramic acid and the D-acid gave the stereoisomer which we shall call isomuramic acid for the sake of convenience. Muramic and isomuramic acids were easily separated by paper chromatography using either 75% phenol or 2-butanol, formic acid, water (7:1:2) as solvent.

As shown by Cowdrey, Hughes, and Ingold,[15] α-halogenopropionic acids undergo Walden inversion when condensed with an alcoholate through the so-called SN2 reaction mechanism though a partial retention of the configuration occurs when a large amount of the corresponding alcohol coexists. Inversion must have been essentially quantitative in our experiment as would be expected since the solvent used was anhydrous dioxane rather than an alcohol. This is born out by the fact that the yield of the condensation was usually more than 90% referred to the product before recrystallization and stereospecificity of the condensation was perfect as shown by paper chromatography of the product after hydrolysis. Therefore, the configuration of the lactic acid moiety of muramic acid belongs most probably to the D-series. This conclusion is supported by comparison of the optical rotation values of several derivatives of muramic acid and isomuramic acid. The literature shows (references below Table I) that the alkoxyl derivatives of D-lactic acid have considerably higher positive rotation values than those of the corresponding L-lactic acid derivatives. If we roughly apply the additivity rule of optical rotation, muramic acid must have D-lactic acid configuration because, as shown in Table I, muramic acid and its derivatives have higher positive values than those of the corresponding isomuramic acid derivatives.

TABLE I

OPTICAL ROTATION VALUES OF MURAMIC, ISOMURAMIC, AND LACTIC ACID DERIVATIVES

| | |
|---|---|
| Methyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-(D-1′-carboxyethyl)-2-deoxy-α-D-glucopyranoside | +107 |
| Methyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-(L-1′-carboxyethyl)-2-deoxy-α-D-glucopyranoside | + 39.1 |
| Methyl 2-acetamido-3-*O*-(D-1′-carboxyethyl)-2-deoxy-α-D-glucopyranoside-(K-salt) | +111 |
| Methyl 2-acetamido-3-*O*-(L-1′-carboxyethyl)-2-deoxy-α-D-glucopyranoside-(K-salt) | + 13.7 |
| Muramic acid (2-amino-3-*O*-(D-1′-carboxyethyl)-2-deoxy-D-glucose) | +103 |
| Isomuramic acid (2-amino-3-*O*-(L-1′-carboxyethyl)[a]-2-deoxy-D-glucose) | +24.0 |
| D-α-*O*-Methyl lactic acid[b] | + 72 |
| L-α-*O*-Methyl lactic acid[c] | − 75.47 |
| D-α-*O*-Methyl lactic acid methyl ester[d] | + 97.16 |
| L-α-*O*-Methyl lactic acid methyl ester[e] | − 93.42 |

[a] *ca.* 54% purity. [b] K. Freudenberg and L. Markert, *Ber.*, **60**, 2447 (1927). [c] T. Purdie and J. C. Irvine, *J. Chem. Soc.*, **75**, 483 (1899). [d] T. S. Patterson and W. C. Forsyth, *ibid.*, **103**, 2263 (1913). [e] T. S. Patterson and A. Lawson, *ibid.*, 2042 (1929).

An interesting fact that we observed during this work was that hydrolysis with dilute hydrochloric acid to obtain muramic acid followed by removal of the hydrogen chloride under reduced pressure gave rise to several ninhydrin-positive substances which had papergram mobilities slower than that of muramic acid. These substances seemed to be ester derivatives of muramic acid because incubation of the hydrolysis mixture at pH 8 for two hours at room temperature caused the disappearance of all the ninhydrin-positive spots except that of muramic acid which increased in intensity. Thus treatment of the hydrolysis mixture at slightly alkaline pH is important for isolation of either synthetic or natural muramic acid in pure form.

## Experimental

**Methyl 2-Acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside (Ia).**—Methyl 2-acetamido-2-deoxy-α-D-glucopyranoside, which is separated from the β-anomer by charcoal column chromatography[16] was treated with benzaldehyde and dry zinc chloride.[17] The resulting 4,6-benzylidene compound melted at 245–247° when recrystallized once; $[\alpha]^{25}D$ +67.1 (ethanol, *c* 0.245).

(15) W. A. Cowdrey, E. D. Hughes, and C. K. Ingold, *J. Chem. Soc.*, 1208 (1937); E. D. Hughes, *Trans. Faraday Soc.*, **34**, 185 (1938).

(16) F. Zilliken, C. S. Rose, G. A. Braun, and P. Gyorgy, *Arch. Biochem. Biophys.*, **54**, 392 (1955).

(17) A. Neuberger, *J. Chem. Soc.*, 50 (1941).

*Anal.* Calcd. for $C_{16}H_{21}O_6N$: C, 59.44; H, 6.55; N, 4.33. Found: C, 59.17; H, 6.84; N, 4.22.

**Methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside** (Ib), m.p. 265–270° dec., $[\alpha]^{25}D$ −93.1 (ethanol, *c* 0.233), ethyl 2-acetamido-4,6-O-benzylidene-2-deoxy-α-D-glucopyranoside (Ic), m.p. 230°, $[\alpha]^{25}D$ +76.8 (ethanol, *c* 0.214) and methyl 2-acetamido-4,6-O-benzylidene-2-deoxy-(α + β)-D-glucopyranoside (Id), m.p. 215–230°, were all prepared likewise.

**Methyl 2-Acetamido-4,6-*O*-benzylidene-3-*O*-(D-1′-carboxyethyl)-2-deoxy-α-D-glucopyranoside (IIa).**—Ia (1.2 g.) was dissolved in hot dry dioxane (100 ml.) and after the addition of sodium hydride (0.4 g., fine powder dispersed in mineral oil at 53% concentration) the mixture while being stirred mechanically was once brought to just below the boiling point. L-α-Chloropropionic acid (2.0 g., b.p. 73–75° (6 mm.), $[\alpha]^{25}D$ −19.1 (ethanol, *c* 3.95) was added when the temperature had cooled to 60° and no heat was applied thereafter. The mixture became a heavy paste and so the flask was shaken by hand for about 30 min. Again sodium hydride (1.6 g.) was added. The mixture became thinner and mechanical stirring was resumed overnight. Then water (20 ml.) was added cautiously and the mixture was concentrated *in vacuo* to a thick sirup which was dissolved in water (30 ml.) and washed three times with 25-ml. portions of chloroform. The water layer was filtered from a trace of insoluble matter and the clear, slightly yellow solution thus obtained was made acid (pH 2.5–3.0) by adding dropwise 6 *N* hydrochloric acid under ice water cooling. The mixture made a crystalline cake which was placed in a refrigerator overnight for improving crystallization. The crystals were filtered and washed thoroughly with water. The crystals thus obtained (1.37 g.) were recrystallized from methanol (30 ml.); yield 1.06 g., m.p. 263–266°, $[\alpha]^{25}D$ +107 (ethanol, *c* 0.172).

*Anal.* Calcd. for $C_{19}H_{25}O_8N$: C, 57.71; H, 6.37; N, 3.54. Found: C, 58.08; H, 6.55; N, 3.41.

The following compounds were prepared likewise:

**Methyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-(D-1′-carboxyethyl)-2-deoxy-β-D-glucopyranoside (IIb)**, m.p. 277–280° dec., $[\alpha]^{25}D$ −79.0 (ethanol, *c* 0.166).

*Anal.* Calcd. for $C_{19}H_{25}O_8N$: C, 57.71; H, 6.37; N, 3.54. Found: C, 57.74; H, 6.42; N, 3.28.

**Ethyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-(D-1′-carboxyethyl)-2-deoxy-α-D-glucopyranoside (IIc)**, m.p. 208–210°, $[\alpha]^{25}D$ +98.3 (ethanol, *c* 0.166).

*Anal.* Calcd. for $C_{20}H_{27}O_8N$: C, 58.67; H, 6.65; N, 3.42. Found: C, 58.16; H, 6.85; N, 3.28.

**Methyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-(D-1′-carboxyethyl)-2-deoxy-(α + β)-D-glucopyranoside (IId)**, m.p. 262–263°.

**Methyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-(L-1′-carboxyethyl)-2-deoxy-α-D-glucopyranoside (IIIa)**, m.p. 280–283°, $[\alpha]^{25}D$ +39.1 (ethanol, *c* 0.202).

*Anal.* Calcd. for $C_{19}H_{25}O_8N$: C, 57.71; H, 6.37; N, 3.54. Found: C, 57.10; H, 6.58; N, 3.35.

**Potassium Salt of Methyl 2-acetamido-3-*O*-(D-1′-carboxyethyl)-2-deoxy-α-D-glucopyranoside (IVa).**—A mixture of methyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-(D-1′-carboxyethyl)-2-deoxy-α-D-glucopyranoside (IIa, 0.185 g.), water (10 ml.) and acetic acid (20 ml.) was boiled for exactly 10 min. After drying *in vacuo* in a rotary evaporator, water (10 ml.) was added and the solution was again taken to dryness *in vacuo*. This was repeated once again. The glassy residue now devoid of benzaldehyde and acetic acid odor was dissolved in water (10 ml.) and was neutralized with N potassium hydroxide accurately to cresol red. The solution was concentrated *in vacuo* and the heavy sirup remaining was triturated with ethanol. Crystallization occurred soon; yield 0.112 g., m.p. 305° dec., $[\alpha]^{25}D$ +111 (water, *c* 0.268).

*Anal.* Calcd. for $C_{12}H_{20}O_8NK$: N, 4.06. Found: N, 3.79.

**Methyl 2-Acetamido-3-*O*-(L-1′-carboxyethyl)-2-deoxy-α-D-glucopyranoside.**—Methyl 2-acetamido-4,6-*O*-benzylidene 3-*O*-(L-1′-carboxyethyl)-2-deoxy-α,D-glucopyranoside (IIIa, 0.138 g.) was treated likewise but in this case the free acid crystallized in fine needles during removal of the solvent; yield was 0.048 g. The crystals had double melting points. They melted at 198°, solidified again and finally melted at 245–250°; $[\alpha]^{25}D$ +60.4 (water, *c* 0.211).

*Anal.* Calcd. for $C_{12}H_{21}O_8N$: C, 47.13; H, 6.89; N, 4.56. Found: C, 47.33; H, 6.98; N, 4.29.

The specimen (0.032 g.) was dissolved in water (8 ml.) and neutralized to pH 8 by adding 0.1 *N* potassium hydroxide and diluted to exactly 10 ml. The specific rotation of the potassium salt thus obtained was $[\alpha]^{25}D$ +13.7 (water, *c* 0.363).

**2-Amino-3-*O*-(D-1′-carboxyethyl)-2-deoxy-D-glucose (Muramic Acid) (VI).**—Methyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-(D-1′-carboxyethyl)-2-deoxy-(α + β)-D-glucopyranoside (IId, 4.0 g.) was heated in a boiling water bath with 2.5 *N* hydrochloric acid (60 ml.) for 4 hr. The reaction mixture was shaken three times with 20-ml. portions of ether in order to remove benzaldehyde and then treated with a small amount of decolorizing charcoal. The colorless solution thus obtained was concentrated *in vacuo* to completely dried material (3.0 g.) and a part of this material (2.63 g.) was dissolved in water (20 ml.). The pH of the solution was made 8.0 g. by the dropwise addition of sodium hydroxide and the solution was left overnight at room temperature. Amberlite XE-64 was added to the solution until the pH was 5.5. The resin was filtered off, and the solution was decolorized with a small amount of charcoal and lyophillized; yield was 2.76 g. A portion of the light yellow substance (2.00 g.) thus obtained was dissolved in absolute methanol (50 ml.) and the insoluble substance was filtered off. The solution was concentrated *in vacuo* to about 10 ml. and placed in a refrigerator overnight. Colorless crystals formed which were filtered and washed with methanol; yield was 0.49 g. In order to obtain an ash-free specimen the raw crystals (0.35 g.) were recrystallized from an equal weight of water. The colorless crystals thus obtained (0.084 g.) melted at 152–154° dec., $[\alpha]^{25}D$ +103 (water, *c* 0.260).

*Anal.* Calcd. for $C_9H_{17}O_7N$: C, 43.03; H, 6.82; N, 5.58. Found: C, 42.26; H, 6.97; N, 5.54.

**2-Amino-3-*O*-(L-1′-carboxyethyl)-2-deoxy-D-glucose (Isomuramic Acid) (VII).**—Ethyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-(L-1′-carboxyethyl)-2-deoxy-α-D-glucopyranoside was treated likewise. However, the compound did not crystallize from methanol solution. The sirup was made crystalline by triturating with ether–methanol (9:1). The crystals which presumably had a high salt content, melted at 80–92° dec., $[\alpha]^{25}D$ +24 (water, *c* 0.927). The purity of the specimen was about 54% on the basis of its reaction with ninhydrin and the assumption that muramic and isomuramic acid had the same molar extinction.

**Ethyl 2-Acetamido-4,6-*O*-benzylidene-3-*O*-(methyl D-1′-ethylcarboxylate)-2-deoxy-α-D-glucopyranoside (VIIIc).**—Ethyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-(D-1′-carboxyethyl)-2-deoxy-α-D-glucopyranoside (IIc, 3.00 g.) was dissolved in methanol (50 ml.). An ether solution of diazomethane was added dropwise at room temperature until the yellow color remained consistently. The reaction mixture was filtered from a trace of insoluble material and then dried *in vacuo*. The crystalline colorless residue was recrystallized from methanol–water (20:10). Colorless needles (2.83 g.) thus obtained melted at 185–186°, $[\alpha]^{25}D$ +104 (ethanol, *c* 0.750).

*Anal.* Calcd. for $C_{21}H_{29}O_8N$: C, 59.57; H, 6.90; N, 3.31. Found: C, 59.53; H, 6.92; N, 3.21.