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Absolute Configuration of the Carboxyethyl (Lactyl) Side Chain of Muramic Acid [2-Amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose]*

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ABSTRACT: The absolute configuration of the lactyl side chain of muramic acid [2-amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose] has been confirmed as D by two procedures.

In the first, alkaline degradation of the *N*-acetyl derivative gave a lactic acid which had an optical rotation identical with that of authentic D-(−)-lactic acid. A definite proof of the configuration was obtained by degradation of 2-amino-2-deoxy-3-*O*-[(*R*)-1-(hydroxymethyl)ethyl]-α-D-glucopyranose (obtained by reduction of the side chain of muramic acid) with ninhydrin, followed by sodium borohydride reduction, periodate oxidation, and sodium borohydride reduction to (*R*)-1-(hydroxymethyl)ethyl 2-glyceryl ether, characterized by a crystalline tri-*p*-nitrobenzoate. (*S*)-1-(Hydroxymethyl)-ethyl 2-glyceryl ether, characterized by a crystalline tri-*p*-nitrobenzoate, was obtained from L-rhamnose by addition of nitromethane to give 1,7-dideoxy-1-nitro-L-*glycero*-L-*galacto*-hepitol. This compound was cyclized to give 2,6-anhydro-1,7-dideoxy-1-nitro-L-*glycero*-L-*galacto*-heptitol, which was catalytically reduced to the 1-amino-1-deoxy derivative. Treatment with sodium nitrite gave 2,6-anhydro-7-deoxy-L-*glycero*-L-*galacto*-heptitol which was oxidized with periodic acid. The resulting product was reduced with sodium borohydride to the *S* ether. Comparison of the *R* and *S* ethers and of their crystalline tri-*p*-nitrobenzoates established the D configuration of the lactyl side chain.

Muramic acid [2-amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose] is one of the two carbohydrate components of the insoluble peptidoglycan backbone found in all bacterial cell walls (Salton, 1964). Degradation of the bacterial cell wall with egg-white lysozyme involves the hydrolysis of the glycoside linkage of the 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-β-D-glucopyranosyl residue to the O-4 of the 2-acetamido-2-deoxy-β-D-glucopyranosyl residue (Salton, 1964). Similarly, egg-white lysozyme is able to split off the β-D-1→4 linkage of the 2-acetamido-2-deoxy-β-D-glucopyranosyl units of chitin, but the rate of the latter reaction is lower than that of the hydrolysis of the peptidoglycan backbone (Chipman and Sharon, 1969). Since the spatial structure of egg-white lysozyme has been elucidated by Phillips and coworkers (Blake *et al.*, 1965, 1967a,b; Johnson and Phillips, 1965; Phillips, 1966, 1967) and the site of enzyme–substrate interaction has been determined (Chipman and Sharon, 1969), exact knowledge of the configuration of the natural bacterial substrate would provide an interesting contribution to an understanding of enzyme activity.

Whereas the configuration of the 2-amino-2-deoxy-D-glucose moiety of muramic acid is well established on the basis of degradation and synthesis (Blix and Jeanloz, 1969), the exact configuration of the lactyl side chain is less firmly ascertained. Strange and Kent (1959) obtained two isomers by condensation of a racemic 2-halogenopropionic acid with a protected 2-acetamido-2-deoxy-D-glucose compound and assumed that the isomer corresponding to the natural product and having the highest optical rotation had a carboxyethyl (lactyl) side chain with the D configuration, since ethers of D-(−)-lactic acid were known to have an optical rotation higher than that of the corresponding ethers of L-(+)-lactic acid. The additivity of the optical rotations of two compounds linked through an ether bond has, however,

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never been established, and introduction of substituents in the sugar molecule may result in profound and unexpected changes of the optical rotation (Jeanloz, 1954). In addition, it was shown in the *galacto*-muramic acid series that the relationship between the optical rotations of the D- and L-1-carboxyethyl analogs could be reversed by changing the solvent in which the optical rotation was determined (Sinaÿ and Jeanloz, 1969).

A second evidence of the D configuration of the carboxyethyl side chain of natural muramic acid was based on the synthesis which starts from L-chloropropionic acid and assumes a Walden inversion (Matsushima and Park, 1962). It has been shown in the past, however, that retention of configuration (through double Walden inversion) or change of configuration (through single Walden inversion) was dependent on the concentration of optically active halogenopropionic acid and on the nature of the solvent (Cowdrey *et al.*, 1937; Hughes, 1938). Since the preparation of muramic acid derivatives is performed in a two-phase medium, evaluation of the concentration and of the solvent effect might be misleading.

A more refined interpretation of the optical rotation values has recently been presented by Listowsky *et al.* (1970) who have shown that the circular dichroism curves of muramic acid and *N*-acetylmuramic acid corresponded to the curves obtained by addition of D-lactic acid to D-glucosamine and to *N*-acetyl-D-glucosamine, respectively.

The strongest evidence of the D configuration of the lactyl side chain of muramic acid was provided by Tipper (1968) who obtained D-lactic acid by alkaline degradation of *N*-acetylmuramic acid (Perkins, 1967; Ghuysen *et al.*, 1967) and treated it with D-lactate dehydrogenase. This determination raises some questions because it is not certain that no racemization occurred during the splitting of the ether bond located in *N*-acetylmuramic acid between C-3 of the 2-acetamido-2-deoxy-D-glucose moiety and C-2 of the lactyl residue, earlier work having shown that lactic acid may be easily racemized under alkaline conditions (Jungfleisch, 1904). A second objection concerns the precision of the enzymic method of determination of D-(−)-lactic acid when performed on a submicroscale in the presence of a possible racemic mixture.

For these reasons, and in view of the importance of a clear understanding of the action of egg-white lysozyme on the bacterial peptidoglycan, additional evidence was obtained to confirm the conclusions of Tipper (1968) by isolating lactic acid after alkaline degradation of *N*-acetylmuramic acid and determining its optical rotation, and by degrading muramic acid to a known derivative without involving the ether linkage.

Results and Discussions

Degradation of *N*-acetylmuramic acid with sodium hydroxide was performed under the conditions described by Ghuysen *et al.* (1967) at the pH used by Tipper (1968). The absence of a phosphate buffer and the higher concentration of the reagents facilitated the isolation of the lactic acid. After removal of the sodium ions, lactic acid was separated on a column of Dowex 1 formate (Busch *et al.*, 1952) and isolated in a 53% yield. The optical rotation was determined in the presence of ammonium molybdate (Krebs, 1961) and found to be identical with that of an authentic sample of D-(−)-lactic acid. No racemization could be detected after treatment of an authentic sample of D-(−)-lactic acid for 2 hr at 37° at pH 12.4–12.5. This observation contradicts the earlier observation of racemization of optically active lactic acid at alkaline pH (Jungfleisch, 1904) and confirms the validity of the procedure of determination of *N*-acetylmuramic acid by alkaline degradation and D-lactate dehydrogenase described by Tipper (1968).

In order to determine independently the absolute configuration of the lactyl side chain of muramic acid, this compound was degraded through a series of intermediates without involving the two carbon atoms forming the ether link. The contribution of the four asymmetric carbons of the 2-amino-2-deoxy-D-glucose moiety to the total optical activity of muramic acid was eliminated by degradation of the 2-amino-2-deoxy-D-glucose moiety linked at C-3 to a glyceryl residue linked at C-2; the only asymmetric carbon left was that of C-2 of the lactyl side chain. This degradation was performed on the compound obtained by reduction of the carboxyl group to a hydroxymethyl group because of the possibility of formation of internal esters between the carboxy group of the lactyl residue and the two new hydroxymethyl groups created by the periodate oxidation–sodium borohydride reduction. The *p*-nitrobenzoyl esters of similar tri(hydroxymethyl) derivatives have been used for the determination of absolute configurations in the carbohydrate field (see Gorin, 1960; Rosenthal and Abson, 1964, 1965). Muramic acid having a reduced side chain **3** was prepared according to the method previously described (Jeanloz and Walker, 1967), except that the reduction of the side chain with lithium borohydride was performed at the stage of the benzylidene derivative of the methyl ester methyl α-D-glycoside **1** (see Scheme I) (Flowers and Jeanloz, 1963). Acid hydrolysis of the resulting **2** gave the hydrochloride of 2-amino-2-deoxy-3-*O*-[(*R*)-1-(hydroxymethyl)ethyl]-α-D-glucopyranose[1] (**3**) (Jeanloz and Walker, 1967). Periodate degradation of the 2-amino-2-deoxy-D-glucose moiety of **3** was not considered at this stage, because this compound exists probably in the pyranose form, stabilized by the ether linkage at C-3 (see discussion in Jeanloz *et al.*, 1963, and Sharon *et al.*, 1966), and very little oxidation would take place between C-4, C-5, and C-6. Since preliminary reduction of the aldehyde group at C-1 might degrade the ether linkage under the alkaline conditions of the reduction (Kuhn *et al.*, 1954), **3** was first degraded with ninhydrin (Gardell *et al.*, 1950; Stoffyn and Jeanloz, 1954) to the D-arabinose derivative **4** which was obtained in crystalline form. Reduction with sodium borohydride of **4** gave the syrupy arabinitol derivative **5**, characterized by a crystalline pentaacetate **6**. Periodic acid oxidation followed by sodium borohydride reduction of **5** gave in 90% yield the syrupy (*R*)-1-(hydroxymethyl)-ethyl 2-glyceryl ether (**7**) which was characterized as the crystalline tri-*p*-nitrobenzoate **8**.

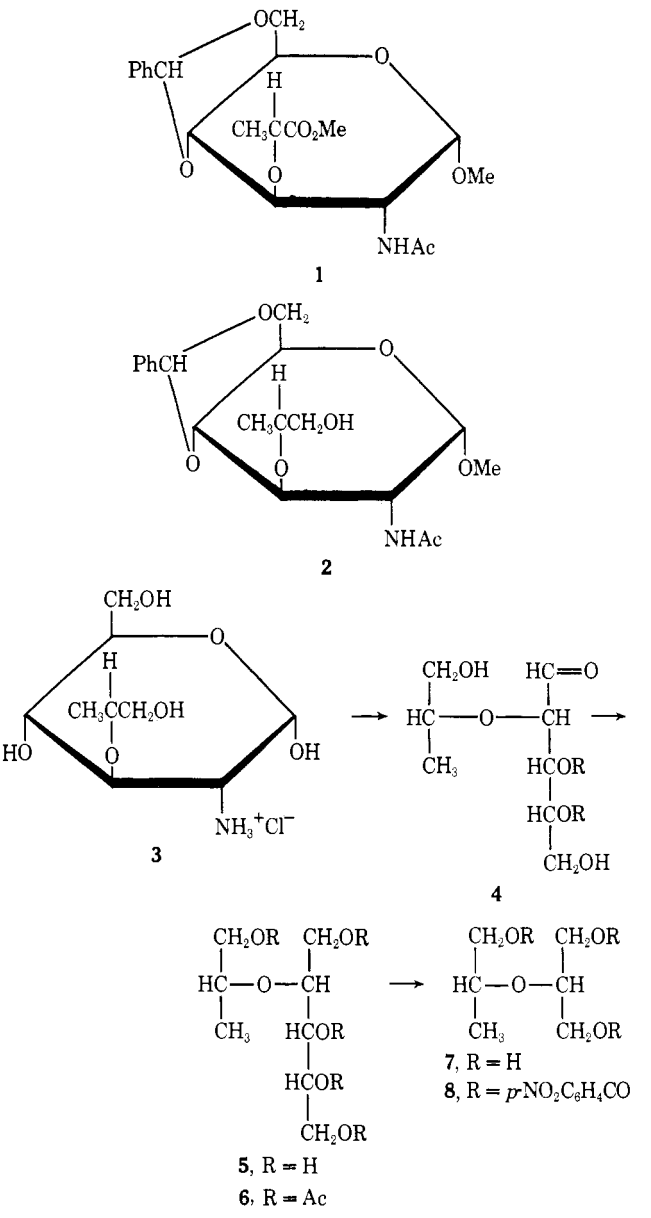
Because of the lengthy work involved in the preparation of D-rhamnose, as starting material for an independent synthesis of **7**, such a synthesis was not considered, but

[1] Erroneously referred to as 2-amino-2-deoxy-3-*O*-[(*S*)-1-(hydroxymethyl)ethyl]-α-D-glucopyranose in Jeanloz and Walker (1967) and Blix and Jeanloz (1969).

instead that of its *S* enantiomorph **21**, starting from the readily available L-rhamnose (**9**) (Scheme II). Condensation of **9** with nitromethane according to Sowden (1951) in the presence of barium hydroxide gave the two epimeric nitro alcohols **10** and **11** already described by Yoshimura and Ando (1964). These authors (see also Yoshimura *et al.*, 1966) had attributed the structure of 1,7-dideoxy-L-*glycero*-L-*talo*-heptitol (**11**) to the compound having mp 177–178° and $[\alpha]_D$ +8.9° (in water) which was identical with the compound (mp 171–172°, $[\alpha]_D^{20}$ +6.6° (water)) that we obtained in 30% yield. We were able to show, however, that this compound is in fact 1,7-dideoxy-1-nitro-L-*glycero*-L-*galacto*-heptitol (**10**) by treatment with concentrated sulfuric acid (Nef, 1894) to give the known 7-deoxy-L-*glycero*-L-*galacto*-heptose (**12**) (Jackson and Hudson, 1953). Heating of an aqueous solution of **10** (Sowden and Oftedahl, 1961) gave crystalline 2,6-anhydro-1,7-dideoxy-1-nitro-L-*glycero*-L-*galacto*-heptitol (**13**) in addition to a small proportion of L-rhamnose (**9**). Both L-*glycero*-L-*galacto* and L-*glycero*-L-*talo* configurations can be formed during the cyclization; the former configuration was selected on the basis of the earlier work of Sowden, Hough, and associates (see Sowden *et al.*, 1962). Compound **13** was hydrogenated (Sowden and Oftedahl, 1961) into 1-amino-2,6-anhydro-1,7-dideoxy-L-*glycero*-L-*galacto*-heptitol (**14**), not previously described, and isolated as the crystalline tetraacetyl derivative **15**. Treatment of **14** with sodium nitrite (Sowden and Oftedahl, 1961) afforded 2,6-anhydro-7-deoxy-L-*glycero*-L-*galacto*-heptitol (**16**) and 1,7-dideoxy-L-*manno*-heptulose (**18**), which could be separated by silica gel chromatography of the crystalline per-*O*-acetyl derivatives 1,3,4,5-tetra-*O*-acetyl-2,6-anhydro-7-deoxy-L-*glycero*-L-*galacto*-heptitol (**17**) and 3,4,5-tri-*O*-acetyl-1,7-dideoxy-L-*manno*-heptulose (**19**), respectively. Assignment of structures to **17** and **19** was based on elementary analysis and nuclear magnetic resonance data. Separation of the 2,6-anhydroheptitol **16** and the heptulose **18** could also be achieved by formation of the crystalline 2,5-dichlorophenylhydrazone **20** of the heptulose **18**; the yields of **16** and **18** were in the approximate proportion of 1.6 to 1. After removal of the hydrazone **20**, the 2,6-anhydroheptitol **16** was obtained from the mother liquors in crystalline form, while the crystalline heptulose **18** was obtained by treatment of the hydrazone **20** with benzaldehyde.

Periodic acid oxidation of the 2,6-anhydroheptitol **16** followed by sodium borohydride reduction gave, in 95% yield, the syrupy (*S*)-1-(hydroxymethyl)ethyl 2-glyceryl ether (**21**) which was characterized by a crystalline tri-*p*-nitrobenzoate **22**. Both ethers **7** (obtained from muramic acid) and **21** (obtained from L-rhamnose) and their respective tri-*p*-nitrobenzoates **8** and **22** were identical on the basis of melting point, thin-layer chromatography, nuclear magnetic resonance, and infrared spectra, except that the optical rotations of the ethers and of the tri-*p*-nitrobenzoates were, respectively, of the same magnitude, but of opposite signs. During the degradation of the muramic acid molecule the C-2 atom of the lactyl chain and the C-3 atom of the 2-amino-2-deoxy-D-glucose moiety did not partake in the reaction. Similarly, during the degradation of the 2,6-anhydroheptitol **16** the C-1, C-6, and C-7 atoms were not involved. Consequently, no isomerization could take place during these two degradations, and, the absolute configuration of L-rhamnose having been previously established (Fischer and Zach, 1912), it is possible to conclude that the lactyl side chain of muramic acid possess the D configuration at C-2.

SCHEME I

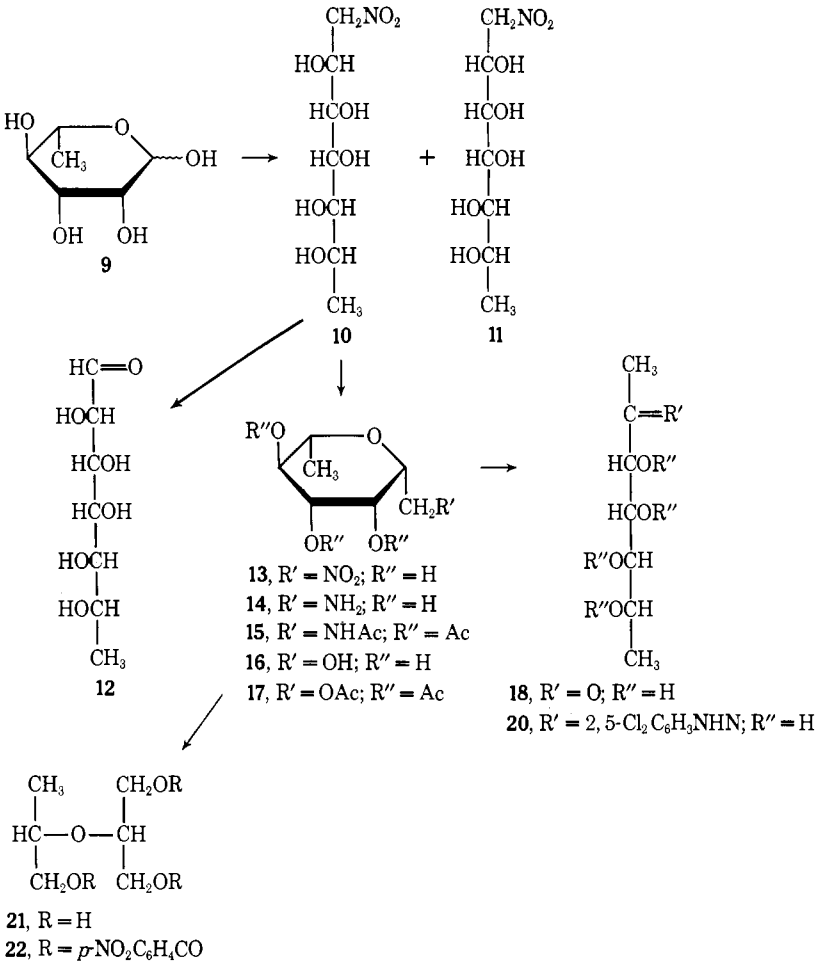


Experimental Section

General. Melting points were determined with a Mettler FP-2 apparatus and correspond to "corrected melting points."

Optical rotations were determined in 1-dm semimicro tubes with a Perkin-Elmer polarimeter no. 141. The chloroform used was analytical reagent grade and contained approximately 0.75% ethanol. Infrared spectra were recorded with a Perkin-Elmer Model 237 spectrophotometer, in chloroform solutions or as Nujol mulls. Nuclear magnetic resonance spectra were recorded with a Varian A-60 spectrometer, in chloroform-*d* as solvent, and with tetramethylsilane as internal standard. Evaporations were performed *in vacuo*, with an outside bath temperature kept below 45°. The

SCHEME II



microanalyses were performed by Dr. W. Manser, Zurich, Switzerland.

Chromatographies. Thin-layer chromatographies (ascending) were performed on Merck silica gel G in the solvent systems specified. The components were detected by spraying with anisaldehyde–sulfuric acid, and heating at 110°. Column chromatographies were performed on Merck silica gel no. 7734 (0.05–0.20 mm), Darmstadt. The paper chromatographies were performed on Whatman paper No. 1, and the spots were detected with the silver nitrate reagent.

Alkali-Catalyzed Elimination of D-(−)-*Lactic Acid from N-Acetylmuramic Acid. N*-Acetylmuramic acid (Pfanstiehl, Waukegan, Ill.; 98 mg, 0.33 mmole) was treated with 68 ml (3.4 mmoles) of 0.05 M sodium hydroxide for 2 hr at 37°, the pH of the solution remaining fairly constant (12.5–12.4). Amberlite 1R-120 (H^+, 5 ml) was added to the reaction mixture to remove the sodium ions, and the pH decreased to 3. After filtration, the solution was examined by cellulose (microcrystalline, Merck) thin-layer chromatography in ether–formic acid–water (13:3:1, v/v), lactic acid being detected by spraying with bromocresol green (R_F 0.8).

The solution was passed through a column of Dowex 1-X8 (200–400 mesh, formate form, 40 × 2 cm), and the column was thoroughly washed with water to remove the nonacidic compounds. Lactic acid was eluted with 0.3 M formic acid, 5-ml fractions being collected, and it was detected in fractions 60–90 by the Eegriwe (1933) reaction. The fractions containing lactic acid were evaporated *in vacuo* at 32°, traces of formic acid being eliminated by addition of toluene, followed by distillation. The residue was dried *in vacuo* for 2 days in the presence of potassium hydroxide pellets.

After transfer to a 10-ml graduated flask, lactic acid was titrated with 0.1 M sodium hydroxide in the presence of phenolphthalein, and the titration was completed at the boiling point of the solution in order to hydrolyze any lactide present. *Anal.* Found: 1.76 ml, equivalent to 15.85 mg of lactic acid (53% yield from *N*-acetylmuramic acid). Glacial acetic acid (15 μl) and 29% ammonium molybdate (0.15 ml) were added to the cooled solution, and the mixture was diluted to 10 ml with water; $[\alpha]_D^{20}$ +57 ± 1° (*c* 0.1585).

D-(−)-Lactic acid (Mann Research Laboratory, 16.4 mg) was titrated as described above with 0.1 M sodium hydroxide. *Anal.* Found: 1.80 ml, equivalent to 16.21 mg of lactic acid. Glacial acetic acid (15 μl) and 29% ammonium molybdate (0.15 ml) were added, and the mixture was diluted to 10 ml; $[\alpha]_D^{20}$ +56 ± 1° (*c* 0.162).

Alkaline Treatment of D-(−)-*Lactic Acid.* D-(−)-Lactic acid (Mann Research Laboratory, 105 mg) was treated with 0.05 M sodium hydroxide (233 ml) for 2 hr at 37° and pH 12.3–12.4. Amberlite 1R-120 (H^+, 12 ml) was added to the reaction mixture. After filtration and concentration

to a few milliliters *in vacuo* at 32°, the lactic acid was titrated with 0.1 M sodium hydroxide. *Anal.* Found: 10.0 ml, equivalent to 90.08 mg of lactic acid. Glacial acetic acid (0.1 ml) and 29% ammonium molybdate (1 ml) were added to the cooled solution, and the mixture was diluted to 25 ml; $[\alpha]_D^{20}$ +54 ± 1° (*c* 0.360).

An equivalent amount of D-(−)-lactic acid (98.6 mg determined by titration) showed: $[\alpha]_D^{20}$ +52 ± 1° (*c* 0.394).

Methyl 2-Acetamido-4,6-O-benzylidene-2-deoxy-3-O-[(R)-1-(hydroxymethyl)ethyl]-α-D-glucopyranoside (**2**). Methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-[D-1-(methoxycarbonyl)ethyl]-α-D-glucopyranoside (**1**, 1 g, 2.4 mmoles) (Flowers and Jeanloz, 1963) dissolved in dry tetrahydrofuran (20 ml) was heated for 2 hr under reflux with lithium borohydride (0.1 g, 4.6 mmoles). After dropwise addition of 0.5 M hydrochloric acid (9.2 ml) to the cooled mixture, the tetrahydrofuran was evaporated at a bath temperature below 30°. The remaining mixture was diluted with water (50 ml) and extracted with chloroform (three 20-ml portions); the extracts were washed with water and dried with sodium sulfate. After evaporation, the residue was crystallized from methanol–water (0.7 g, 75%): mp 285°, $[\alpha]_D^{20}$ +38° (*c* 0.54, methanol). *Anal.* Calcd for $C_{19}H_{27}NO_7$: C, 59.83; H, 7.14; N, 3.67; O, 29.36. Found: C, 59.94; H, 7.08; N, 3.61; O, 29.57.

2-O-[(R)-1-(Hydroxymethyl)ethyl]-D-arabinose (**4**). 2-Amino-2-deoxy-3-*O*-[(*R*)-1-(hydroxymethyl)ethyl]-α-D-glucopyranose hydrochloride (**3**, 1 g, 3.7 mmoles) (Jeanloz and Walker, 1967), prepared by direct acid hydrolysis of **2**, was treated for 1 hr at 100° with ninhydrin (1.19 g, 6.7 mmoles) in 4% aqueous pyridine (20 ml). The cooled reaction mixture was diluted with water (100 ml) and extracted with chloroform (four 50-ml portions). The yellow, aqueous layer was evaporated, the residue dissolved in ethanol, and the solution treated with charcoal. After the filtration and evaporation, the residual syrup was chromatographed on silica gel in chloroform–95% ethanol (2:1, v/v). The main fraction (R_F 0.40) gave a crystalline compound (0.47 g, 62%), which was recrystallized from ethanol–pentane: mp 132°, $[\alpha]_D^{23}$ −66 (after 7 min) to −71° (after 30 min, *c* 1.24, water). *Anal.* Calcd for $C_8H_{16}O_6$: C, 46.15; H, 7.75; O, 46.11. Found: C, 46.22; H, 7.82; O, 46.14.

2-O-[(R)-1-(Hydroxymethyl)ethyl]-D-arabinitol Pentaacetate (**6**). To a solution of **4** (0.7 g, 3.3 mmoles) in water (15 ml) was added a solution of sodium borohydride (0.25 g, 6.6 mmoles) in water (10 ml). The mixture was kept for 24 hr at room temperature, and then stirred with Amberlite 1R-120 (H^+, 10 ml). After filtration, the solution was evaporated to a colorless syrup (**5**, 0.70 g, 100%); thin-layer chromatography in chloroform–95% ethanol (2:1, v/v) showed only one spot (R_F 0.31).

The crude arabinitol (0.21 g) was treated overnight at room temperature with acetic anhydride (5 ml) and pyridine (5 ml). The solution was evaporated, and the residue dried by several additions of toluene, followed by distillation. Thin-layer chromatography in chloroform–95% ethanol (19:1, v/v) showed only one spot (R_F 0.63). After distillation at 250° and 3 mm, the compound crystallized from ether–hexane (0.38 g, 90%): mp 82°, $[\alpha]_D^{22}$ +0.5° (*c* 1.36, chloroform). *Anal.* Calcd for $C_{18}H_{28}O_{11}$: C, 51.42; H, 6.71; O, 41.86. Found: C, 51.35; H, 6.72; O, 41.98.

(R)-1-(Hydroxymethyl)ethyl 2-Glyceryl Ether (**7**). Crude 2-*O*-[(*R*)-1(hydroxymethyl)ethyl-D-arabinitol (**5**, 0.5 g, 2.4 mmoles) was treated for 24 hr at +5° in the dark with 0.1 M paraperiodic acid (172 ml). The mixture was neutralized with barium carbonate and filtered. Thin-layer chromatography in chloroform–95% ethanol (4:1, v/v) showed a main spot at R_F 0.49 and an impurity at R_F 0.75, but no starting material.

Sodium borohydride (0.24 g, 6.3 mmoles) was added to the solution, and the reaction mixture was kept overnight at room temperature. Glacial acetic acid (0.5 ml) was added, and the solution was stirred with Amberlite 1R-120 (H^+, 20 ml). After filtration, the solution was evaporated to a syrup which was distilled at 200° and 3 mm (0.32 g, 90%), $[\alpha]_{20}^{D}$ −29° (*c* 1.0, water). *Anal.* Calcd for $C_6H_{14}O_4$: C, 47.98; H, 9.40; O, 42.62. Found: C, 47.96; H, 9.50; O, 42.58.

(R)-1-(Hydroxymethyl)ethyl 2-Glyceryl Ether Tri-p-nitrobenzoate (**8**). Compound **7** was treated with *p*-nitrobenzoyl chloride as described for **21**. After recrystallization from ethyl acetate, the *p*-nitrobenzoate **8** had mp 120–122°, $[\alpha]_D^{20}$ −15° (*c* 1.21, chloroform), and nuclear magnetic resonance and infrared spectra identical with those of **22**. *Anal.* Calcd for $C_{27}H_{23}N_3O_{13}$: C, 54.27; H, 3.88; N, 7.03; O, 34.81. Found: C, 54.16; H, 3.90; N, 6.90; O, 34.90.

1,7-Dideoxy-1-nitro-L-glycero-L-galacto-heptitol (**10**). To a stirred mixture of L-rhamnose monohydrate (**9**, 20 g, 0.11 mole), water (10 ml), and nitromethane (22 ml), was added barium hydroxide octahydrate (26.9 g, 0.085 mole), resulting in a homogeneous brown solution after 10 min. The temperature of the reaction mixture rose quickly, and it was kept for 15 min at 45° by cooling from time to time with an ice bath. Absolute ethanol (40 ml) was added, and the mixture was cooled to 0° to precipitate the barium acid salts of the nitroheptitols. The precipitate was filtered off, washed with cold absolute ethanol, and dissolved in 2 M acetic acid (200 ml). The solution was immediately stirred for 30 min with Amberlite 1R-120 (H^+, 100 ml). After filtration, the solution was evaporated to dryness. The residue was dried by addition of toluene followed by distillation, and then crystallized from 95% ethanol. The crystalline material (9 g) was filtered off, and recrystallized first from 95% ethanol, and then from water (7.5 g, 30%): mp 171–172°, $[\alpha]_D^{20}$ +6.6° (*c* 2.35, water). Yoshimura and Ando (1964) reported mp 177–178°, $[\alpha]_D$ +8.9° (*c* 2.1, water). *Anal.* Calcd for $C_7H_{15}NO_7$: C, 37.33; H, 6.71; N, 6.22; O, 49.74. Found: C, 37.22; H, 6.76; N, 6.18; O, 49.62.

Evaporation of the mother liquors gave a red syrup (8.6 g) containing the other epimeric nitroheptitol **11** which could not be crystallized.

7-Deoxy-L-glycero-L-galacto-heptose (**12**). A solution of **10** (2.25 g, 0.01 mole) in 2 M sodium hydroxide (6 ml) was added dropwise, at room temperature, to a stirred solution of concentrated sulfuric acid (3 ml) in water (3.5 ml). After addition, stirring was continued for 5 min, and then the mixture was diluted with water (100 ml). The pH was raised to 5 by addition of barium hydroxide octahydrate (about 15 g), and then to pH 7 by addition of a barium acetate solution. After filtration through Celite, the solution was passed through a column of Amberlite 1R-120 (H^+, 50 ml). The effluents and washings were concentrated to a syrup which crystallized after dilution with ethanol (1 g, 52%). Recrystallization from methanol–water gave small prisms: mp 182°, $[\alpha]_D^{22}$ −63° (at equilibrium, *c* 1.98, water); Fischer

and Piloty (1890) reported mp 180–181°, $[\alpha]_D^{20}$ −61.4° (at equilibrium, *c* 10, water); Jackson and Hudson (1953) mp 186.5–187.5°, $[\alpha]_D^{20-21}$ −62.5° (*c* 2.48, water).

*2,6-Anhydro-1,7-dideoxy-1-nitro-*L*-glycero-*L*-galacto-heptitol* (**13**). A solution of **10** (30 g, 0.133 mole) in water (300 ml) was heated at reflux for 48 hr. Paper chromatography of the reaction mixture in 1-butanol–ethanol–water (40:11:19, v/v) showed the presence of **13** (R_F 0.62) and of rhamnose (**9**) (R_F 0.41). The solution was evaporated to a syrup which crystallized from chloroform–ethanol (23.2 g, 84%). The crystals were recrystallized several times from the same mixture: mp 120–122°, $[\alpha]_D^{22}$ +50° (*c* 2.05, water). *Anal.* Calcd for $C_7H_{13}NO_6$: C, 40.58; H, 6.32; N, 6.76; O, 46.33. Found: C, 40,67; H, 6.41; N, 6.80; O, 46.18.

*1-Acetamido-3,4,5-tri-O-acetyl-2,6-anhydro-1,7-dideoxy-*L*-glycero-*L*-galacto-heptitol* (**15**). A solution of **13** (1 g, 4.8 mmoles) in water (30 ml) was hydrogenated for 1 hr at room temperature and atmospheric pressure in the presence of Adams' platinum catalyst (0.2 g). The catalyst was filtered off, and the filtrate was evaporated to give a white amorphous foam (0.89 g) which could not be crystallized and was not stable at room temperature. Paper chromatography in 1-butanol–ethanol–water (40:11:19, v/v) showed only one spot (R_F 0.18).

The crude amine **14** (0.89 g) was treated for 24 hr at room temperature with acetic anhydride (15 ml) and dry pyridine (15 ml). The reaction mixture was then poured into cold water (200 ml), and extracted a few times with chloroform. The combined extracts were washed with 2 M hydrochloric acid, aqueous 5% sodium hydrogen carbonate, and water, and then evaporated to give a syrup which crystallized spontaneously. Recrystallization from alcohol–ether gave crystals (1.1 g, 70%): mp 146–147°, $[\alpha]_D^{23}$ +13° (*c* 1.9, chloroform). *Anal.* Calcd for $C_{15}H_{23}NO_8$: C, 52.17; H, 6.71; N, 4.06; O, 37.06. Found: C, 52.16; H, 6.73; N, 3.97; O, 37.17.

*2,6-Anhydro-7-deoxy-*L*-glycero-*L*-galacto-heptitol* (**16**). A solution of **13** (10 g, 0.048 mole) in water (220 ml) and glacial acetic acid (6 ml) was hydrogenated for 4 hr at room temperature and atmospheric pressure in the presence of Adams' platinum catalyst (0.4 g). After removal of the catalyst, glacial acetic acid (10 ml) was added to the filtrate. The stirred solution was treated for 20 hr at room temperature with sodium nitrite (10 g, 0.145 mole) and then deionized by stirring with a mixture of Amberlite 1R-120 (H^+, 150 ml) and Amberlite 1R-45 (OH^-, 100 ml). After filtration, the solution was evaporated to a syrup (10 g) which was dried by the addition of toluene followed by distillation.

A portion of the crude syrup (2.8 g) was treated for 24 hr at room temperature with acetic anhydride (12 ml) and dry pyridine (18 ml). The reaction mixture was then poured into water (200 ml) and extracted with chloroform. The extracts were washed with aqueous 5% sodium hydrogen carbonate, and then with water, and dried with sodium sulfate. Evaporation gave a syrup which was dried under vacuum (4.6 g); thin-layer chromatography in chloroform–95% ethanol (19:1, v/v) showed the presence of two products (R_F 0.36 and 0.66) which were separated by column chromatography on silica gel, using chloroform–95% ethanol (19:1, v/v) as eluent. The faster moving compound was *1,3,4,5-tetra-O-acetyl-2,6-anhydro-7-deoxy-*L*-glycero-*L*-galacto-heptitol* (**17**), isolated as a syrup, which crystallized after distillation at 200° under 3 mm: mp 79–81°, $[\alpha]_D^{26}$ +15° (*c* 2.24, chloroform); nuclear magnetic resonance data showed (chloroform-*d*): τ 7.83, 7.93, and 8.02 (12 protons, OAc); 8.75 (doublet, CH_3). *Anal.* Calcd for $C_{15}H_{22}O_9$: C, 52.02; H, 6.40; O, 41.58; CH_3CO, 49.72. Found: C, 52.05; H, 6.34; O, 41.62; CH_3CO, 50.80.

The slower moving compound was *3,4,5-tri-O-acetyl-1,7-dideoxy-*L*-manno-heptulose* (**19**), isolated as a syrup, which crystallized from ether–hexane: mp 119–121°, $[\alpha]_D^{26}$ −38° (*c* 1.91, chloroform); nuclear magnetic resonance data showed (chloroform-*d*): τ 6.48 (OH) (this peak disappeared after addition of a drop of deuterium oxide); 7.82, 7.93, and 8.02 (9 protons, OAc); 8.62 (singlet, CH_3 at C-1); and 8.80 (doublet, CH_3 at C-7). *Anal.* Calcd for $C_{13}H_{20}O_8$: C, 51.31; H, 6.63; O, 42.06; CH_3CO, 42.44. Found: C, 51.51; H, 6.62; O, 41.93; CH_3CO, 44.19.

A portion of the syrupy mixture (2.2 g) of crude anhydroheptitol and heptulose was also treated with 2,5-dichlorophenylhydrazine (2.47 g) in boiling methanol (15 ml), which was evaporated almost to dryness. The brown residual syrup thus obtained was diluted with methanol (15 ml), and the process was repeated. After cooling, the solid mass was triturated six times with 40-ml portions of ether to remove the excess of 2,5-dichlorophenylhydrazine, and the supernatant solutions were discarded. The pale yellow residue was then extracted with water (five 40-ml portions) to remove the anhydroheptitol. The crystalline residue was filtered off, washed with water, and recrystallized from aqueous methanol to give *1,7-dideoxy-*L*-manno-heptulose 2,5-dichlorophenylhydrazone* (**20**) (0.8 g, 22% based on **13**): mp 187° (dec), $[\alpha]_D^{22}$ +6.2° (*c* 2.25, methanol). *Anal.* Calcd for $C_{13}H_{18}Cl_2N_2O_4$: C, 46.30; H, 5.38; Cl, 21.03; N, 8.31. Found: C, 46.21; H, 5.46; Cl, 21.30, N, 8.36.

The aqueous extract from the crude 2,5-dichlorophenylhydrazone was concentrated to a syrup which was purified by silica gel column chromatography with chloroform–95% ethanol (1:1, v/v) as eluent. The main fraction (R_F 0.35) gave a syrup (0.65 g, 35% based on **13**) which crystallized from absolute ethanol. The anhydroheptitol **16**, probably solvated by ethanol, was dried for 48 hr at 50° under vacuum: mp 105.5–106.5°, $[\alpha]_D^{22}$ +58° (*c* 2.18, water). *Anal.* Calcd for $C_7H_{14}O_5$: C, 47.18; H, 7.92; O, 44.90. Found: C, 47.25; H, 7.86; O, 44.82.

*1,7-Dideoxy-*L*-manno-heptulose* (**18**). A mixture of 2,5-dichlorophenylhydrazone (3.4 g), benzaldehyde (5 ml), and benzoic acid (0.5 g) in water (50 ml) and ethanol (10 ml) was heated for 150 min at reflux. After cooling, the alcohol was evaporated, and the solution was diluted with water (50 ml), and then extracted three times with chloroform to remove benzaldehyde 2,5-dichlorophenylhydrazone. The aqueous layer was evaporated to a yellowish syrup which was chromatographed on silica gel in chloroform–95% ethanol (1:1, v/v). The homogeneous syrup (1.7 g) crystallized after a few months from ethanol: mp 67–68°, $[\alpha]_D^{20}$ −7.4° (*c* 2.1, water); no mutarotation was observed. The product was dried for 12 hr at 60–70° in high vacuum. *Anal.* Calcd for $C_7H_{14}O_5$: C, 47.18; H, 7.92; O, 44.90. Found: C, 47.03; H, 7.86; O, 44.80.

(S)-1-(Hydroxymethyl)ethyl 2-Glyceryl Ether (**21**). The anhydroheptitol **16** (1 g, 5.6 mmoles) was treated for 24 hr at 5° in the dark with 0.1 M paraperiodic acid (169 ml). The dialdehyde was not isolated, but thin-layer chromatog-

raphy in chloroform–95% ethanol (1:1, v/v) showed only one spot (R_F 0.82). The reaction mixture was neutralized with barium carbonate (about 2 g), filtered, and sodium borohydride (0.560 g, 15 mmoles) was added to the filtrate. After the solution had been kept for 30 min at room temperature, glacial acetic acid (1 ml) was added, and then the solution was stirred with Amberlite 1R-120 (H^+, 20 ml). After filtration, the solution was evaporated, and the residual syrup was distilled at 200° under 3 mm (0.8 g, 95%), $[\alpha]_D^{22}$ +28° (*c* 1.07, water); thin-layer chromatography in chloroform–95% ethanol (2:1, v/v) showed one spot (R_F 0.47). *Anal.* Calcd for $C_6H_{14}O_4$: C, 47.98; H, 9.40; O, 42.62. Found: C, 47.77; H, 9.47; O, 42.72.

(S)-1-(Hydroxymethyl)ethyl 2-Glyceryl Ether Tri-(p-nitrobenzoate) (**22**). A solution of the triol **21** (0.13 g, 2 mmoles) and *p*-nitrobenzoyl chloride (2.2 g, 12 mmoles) in pyridine (10 ml) was heated for 1 hr at 80°. The cooled solution was poured into an aqueous solution of 5% sodium hydrogen carbonate (50 ml). The mixture was extracted several times with chloroform, and the combined extracts were washed with aqueous 5% sodium hydrogen carbonate, and then with water. Evaporation gave a syrup which was chromatographed on silica gel with chloroform containing 0.75% alcohol as eluent. After evaporation of the solvent, crystallization from ethyl acetate gave 0.96 g (80%): mp 119–120°; $[\alpha]_D^{22}$ +17° (*c* 1.23 chloroform); nuclear magnetic resonance data (chloroform-*d*); τ 1.70 and 1.78 (12 aromatic protons); 5.33–5.60 (8 protons, two overlapping multiplets); and 8.62 (doublet, CH_3); infrared data ($\nu_{max}^{CHCl_3}$): 3120 (aromatic CH), 3030 (CH_3), 1730 (Ar-CO-O), 1615 (phenyl), 1535 (NO_2), 1360 (NO_2), 1275 (Ar-CO-O), 1110 (Ar-CO-O), 1025 (ether), 875 (NO_2), and 715 (phenyl) cm^{-1}. *Anal.* Calcd for $C_{27}H_{23}N_3O_{13}$: C, 54.27; H, 3.88; N, 7.03; O, 34.81. Found: C, 54.30; H, 3.98; N, 6.49; O, 34.71.

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