Phosphorylated Sugars. Part XX.[1] Synthesis of 3-Deoxy-D-*manno*-Octulosonic Acid 8-(Dihydrogen Phosphate)

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The title compound was obtained by base-catalysed condensation of 2-*O*-benzyl-D-arabinose 5-phosphate with oxalacetate, followed by hydrogenolytic removal of the benzyl group. It was separated from the simultaneously formed D-*gluco*-isomer by ion-exchange chromatography.

3-DEOXY-D-*manno*-OCTULOSONIC ACID is a well known component of the cell envelope of gram negative bacteria. In enterobacteria it provides the link between the polysaccharide and lipid systems of the endotoxin.[2] Biologically it first appears as its 8-phosphate, which is formed from D-arabinose 5-phosphate and phosphoenolpyruvate;[3] this is then dephosphorylated[4] before being

[1] Part XIX, F. Trigale and L. Szabó, *J.C.S. Perkin I*, 1975, 604.

[2] O. Lüderitz, O. Westphal, A.-M. Staub, and H. Nikaido, in 'Microbial Toxins,' eds. E. Weinbaum, S. Kadis, and S. J. Ajl, Academic Press, New York, 1971, vol. IV, pp. 145—224.

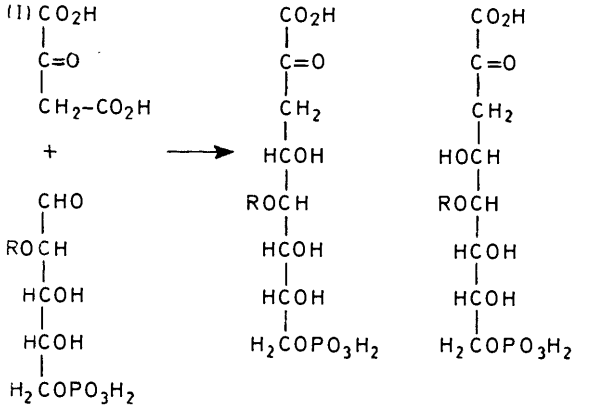
[3] D. H. Levin and E. Racker, *J. Biol. Chem.*, 1959, **234**, 2532.

[4] M. A. Ghalambor, E. M. Levine, and E. C. Heath, *J. Biol. Chem.*, 1966, **241**, 3207.

transformed into the coenzyme cytidine-5′ 3-deoxy-D-*manno*-octulosonic acid-2 phosphate,[5] the metabolically active form of this sugar acid.

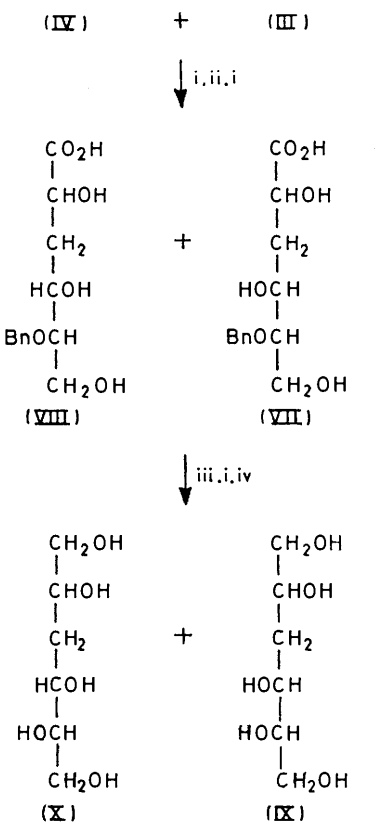
As 3-deoxy-D-*manno*-octulosonic acid can be prepared[6] by base-catalysed condensation of D-arabinose with oxalacetic acid (I), it was expected that the 5-phosphate of D-arabinose[7] would yield the 8-phosphate of the octulosonic acid under the same conditions. However, in view of the fact that D-arabinose 5-phosphate undergoes spontaneous isomerisation to ribulose 5-phosphate under alkaline and even under neutral conditions, it was considered expedient to use the stable 2-benzyl ether[7] (II) as starting material for the synthesis of the phosphorylated octulosonic acid. The condensation led to a mixture of 5-benzyl ethers of 3-deoxyoctulosonic acid 8-phosphates [(III) and (IV)] which could be separated from unchanged 2-*O*-benzyl-D-arabinose 5-phosphate by ion-exchange chromatography and isolated as their lithium salts. Although paper chromatography showed that the material was a mixture of two components, one being far more abundant than the other, conditions were not found for their separation on a preparative scale. The mixed compounds were, therefore, debenzylated by hydrogenolysis, and the isomeric, phosphorylated 3-deoxyaldulosonic acids (V) and (VI) separated by ion-exchange chromatography in a monochloroacetate system (previously used[8] for the separation of isomeric, phosphorylated aldulosonic acids) and isolated as their lithium salts.

The major compound was shown to have the D-*manno*-configuration in the following way (Scheme). The mixed 5-benzyl ethers [(III) + (IV)] were first treated with borohydride to reduce the carbonyl group, and then with periodate: this caused cleavage of the



(II) R = CH_2Ph (IV) R = CH_2Ph (III) R = CH_2Ph
(VI) R = H (V) R = H

C(6)–C(7) bond. The newly formed aldehyde groups were reduced with borohydride: a mixture of 5-*O*-benzyl-L-gluco- (VII) and L-galacto-metasaccharinic acids (VIII) was obtained. After esterification of the carboxy-function these were reduced with borohydride,



Reagents: i, $NaBH_4$; ii, $NaIO_4$; iii, CH_2N_2; iv, H_2–Pd Bn = benzyl

the benzyl groups were removed by hydrogenolysis, and the mixture of alditols [(IX) + (X)] was analysed as acetates by g.l.c. under conditions in which neither the pair of epimeric alditol acetates (IX) formed from glucometasaccharinic acid nor that (X) arising from galactometasaccharinic acid is resolved, but in which the respective pairs [(IX) and (X)] are well separated. The minor component present in the alditol acetate mixture resulting from the degradation of the mixed 5-*O*-benzyl-3-deoxyoctulosonic acid 8-phosphates had a retention time identical with that of the pair of alditol acetates (X) derived from galactometasaccharinic acid (VIII); the major component coincided with the alditol acetates derived from glucometasaccharinic acid (VII). This demonstrated that the condensation reaction led predominantly to the compound having the D-*manno*-configuration (III), and hence that the major phosphorylated aldulosonic acid isolated was identical with the naturally occurring 3-deoxy-D-*manno*-oct-2-ulosonic acid 8-phosphate.

In the semicarbazide test[9] both phosphorylated

[5] M. A. Ghalambor and E. C. Heath, *J. Biol. Chem.*, 1966, **241**, 3216.
[6] C. Hershberger, M. Davis, and S. B. Binkley, *J. Biol. Chem.*, 1968, **243**, 1585.
[7] J. Stverteczky, P. Szabó, and L. Szabó, *J.C.S. Perkin I*, 1973, 872.
[8] L. Szabo, Amer. Chem. Soc. Advances in Chemistry Series, 1968, No. 74, p. 86.
[9] J. MacGee and M. Doudoroff, *J. Biol. Chem.*, 1954, **210**, 617.

octulosonic acids had the expected molar absorption coefficient of 10 000. In the thiobarbiturate test [10] the molar absorption coefficient of the D-*manno*-isomer was 92 000; however the periodate cleavage had to be carried out at room temperature and thus overoxidation occurred, for it is known [11] that phosphorylated sugars give anomalous results when oxidised under the conditions of the ' cold acid ' method.

It was deemed necessary to confirm the postulated position of the phosphate group, as in these phosphorylated octulosonic acids the phosphate group is in a situation analogous to that found in glycerol phosphate, a compound that is subject to easy phosphate migration.[12] For this purpose the 3-deoxy-D-*manno*-octulosonic acid phosphate was first reduced to a mixture of phosphorylated, epimeric, 3-deoxyaldonic acids, and then treated, sequentially, with periodate and borohydride. The phosphate esters formed were then analysed [13] by paper electrophoresis: glycol phosphate alone was detected which proved that no (or negligible) phosphate migration had occurred.

EXPERIMENTAL

All evaporations were carried out under reduced pressure at 35 °C. The salts of phosphate esters were dried *in vacuo* and then exposed to ambient humidity before analysis. After borohydride reductions, solutions were decationised with Amberlite IR 120 (H^+) resin and evaporated to dryness. Boric acid was removed by repeated addition and evaporation of methanol. Periodate was determined by the method of Avigad.[14]

5-O-*Benzyl*-3-*deoxyoct*-2-*ulosonic Acid* 8-*Phosphates* [*Mixture of* D-manno- *and* D-gluco-*isomers* (III) *and* (IV)].—The pH of an ice-cold solution of oxalacetic acid (800 mg) in water (6 ml) was brought to 11 by cautious addition of 10N-sodium hydroxide. 2-*O*-Benzyl-D-arabinose 5-(dilithium phosphate) [7] (4.7 g) was added and the mixture was allowed to warm to room temperature with constant

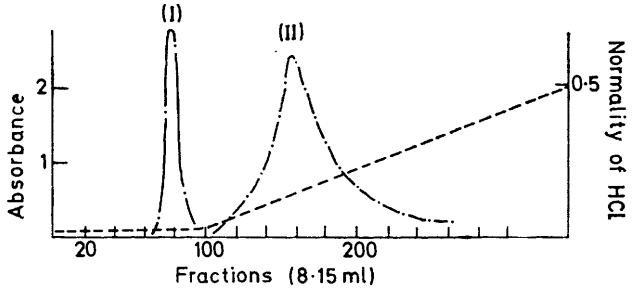


FIGURE 1 Separation of phosphate esters (II) ($\lambda_{max.}$ 485 nm) from unphosphorylated material (I) ($\lambda_{max.}$ 475 nm) following condensation of D-arabinose 5-phosphate with oxalacetic acid

stirring over 2 h. The precipitate (phosphorus content 427 mg) formed upon addition of ethanol (500 ml) was centrifuged off, washed with ethanol (3 × 50 ml) and stored undried at −20 °C. A solution of part of this material (P content 316 mg) in water (100 ml), adjusted to pH 7 with N-hydrochloric acid, was percolated through a column of Dowex 1 × 8 resin (19 × 2.4 cm; Cl^- form) in 0.03N-hydrochloric acid. Elution (5 ml min^{-1}) was carried out (Figure 1) with the same acid. Fractions (8.15 ml) were collected and every fifth was analysed: [15] to the

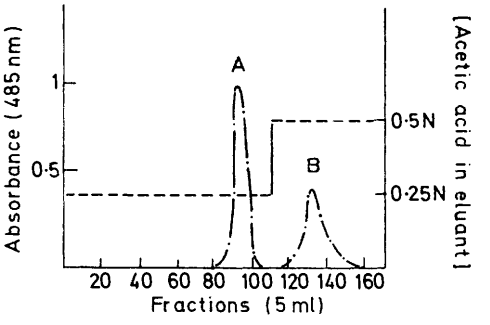


FIGURE 2 Separation of 2-*O*-benzyl-D-arabinose 5-phosphate (A) from the mixture of 5-*O*-benzyl-3-deoxyoctulosonic acid 8-phosphates (B) by anion-exchange chromatography on DEAE-cellulose (AcO^-)

sample (150 μl) a solution of phenol (5% w/v; 500 μl) was added, followed by concentrated sulphuric acid (2.5 ml). An unidentified, unphosphorylated substance, having $\lambda_{max.}$ 475 nm in the test, was eluted (fractions 60—100). Thereafter a linear gradient of hydrochloric acid solution (0.02—0.05N) was applied. Fractions 120—250, containing the phosphate esters, were pooled, neutralised with lithium hydroxide solution, and concentrated (50 ml). The lithium salts of the phosphate esters (2.624 g) were precipitated with ethanol (500 ml), centrifuged off, washed with ethanol until free of chloride, and dried (P_2O_5). The material obtained was a mixture of 2-*O*-benzyl-D-arabinose 5-phosphate and the 5-*O*-benzyl-3-deoxyoctulosonic acid 8-phosphates. A portion (315 mg) was dissolved in a buffer prepared by adjusting the pH of a 0.25M-solution of acetic acid to 5, with pyridine, and the solution was passed through a column (4.5 × 30 cm) containing a mixture of DEAE-cellulose (Schleicher and Schüll; 0.5 mequiv. g^{-1}; 4 parts) and cellulose powder (Whatman CF 1, coarse fibre; 1 part) equilibrated with the buffer. Elution (Figure 2) with the same buffer (25 ml h^{-1}; 5 ml fractions) gave 2-*O*-benzyl-D-arabinose 5-phosphate (A) (fractions 80—105); the 5-*O*-benzyl-3-deoxyoctulosonic acid 8-phosphates (B) were then eluted (fractions 115—150) with 0.5M-pyridinium acetate buffer at the same pH. Corresponding fractions of three such preparations (total 1 034 mg of mixed phosphate esters) were pooled and concentrated, and the residue was dried overnight (KOH pellets). The pH of an aqueous solution of this residue was adjusted to 7 with lithium hydroxide solution. The lithium salts (240 mg, 55%; based on oxalacetic acid) of the isomeric mixture of 8-phosphates, precipitated with ethanol and dried, had $[\alpha]_D^{22}$ +33° (*c* 1.2 in H_2O) (Found: C, 37.4; H, 4.8; Li, 4.45; P, 6.5. Calc. for $C_{15}H_{18}Li_3O_{11}P,3H_2O$: C, 37.5; H,

[10] A. Weissbach and J. Hurwitz, *J. Biol. Chem.*, 1959, **234**, 705.
[11] P. Szabó and L. Szabó, *Carbohydrate Res.*, 1967, **4**, 206.
[12] M. C. Bailly, *Compt. rend.*, 1938, **206**, 1902; P. E. Verkade, J. C. Stoppelenburg, and W. D. Cohen, *Rec. Trav. chim.*, 1940, **59**, 886.
[13] F. Trigalo, P. Szabó, and L. Szabó, *J. Chem. Soc.* (*C*), 1968, 901.
[14] G. Avigad, *Carbohydrate Res.*, 1969, **11**, 119.
[15] M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Analyt. Chem.*, 1956, **28**, 350

5.0; Li, 4.4; P, 6.5%); $\lambda_{max.}$ 255 nm (ε 240); $\lambda_{max.}$ of semicarbazones 250 (ε 10 000) and 217 nm (6 600) [$\lambda_{min.}$ 223 nm (ε 5 300)]; electrophoretic mobility at pH 3.5 (0.3M pyridinium acetate buffer) $R_{picric\ acid}$ 0.93. On an ascending paper chromatogram (acetone–formic acid–water, 75 : 5 : 20 v/v) two phosphorylated α-oxo-acids were revealed with the phosphate [16] and *o*-phenylenediamine [17] sprays.

Determination of the Structures of the 5-O-*Benzyl*-3-*deoxyoctulosonic Acid* 8-*Phosphates*.—The mixture of phosphorylated benzyl ethers (20 mg) was dissolved in water (2 ml) and treated with sodium borohydride (20 mg) in water (2 ml) for 2 h. The syrup obtained after work-up was dissolved in water (2 ml) and the pH of the solution was brought to 8 with lithium hydroxide solution. The solvents were removed, the residue was triturated with ethanol (10 ml), and the precipitated lithium salts of the 5-*O*-benzyl-3-deoxyoctonic acid phosphates (15 mg) were collected by centrifugation and dried. This material was treated with 0.04M-sodium periodate (2.5 ml): the oxidation was complete within 30 min, 1 mol. equiv. of periodate being reduced; no further reaction was observed within the next 4 h. The reaction mixture was treated with sodium borohydride (45 mg) for 2 h and then passed through a column (10 ml) of Amberlite IR 120 (H^+) resin. The residue obtained after work-up was dissolved in water and the pH of the solution was brought to 9 with barium hydroxide solution. The solvents were removed, the residue was triturated with ethanol (20 ml), and the precipitate was recovered by centrifugation and thoroughly dried (P_2O_5). It was then suspended in methanol and dissolved by addition of Amberlite IR 120 (H^+) resin moistened with absolute methanol. The filtered solution was treated with a slight excess of ethereal diazomethane. The solvents were removed and the residue was treated with sodium borohydride (15 mg) in water (2 ml) and left overnight. After removal of boric acid an aqueous solution of the material was passed through a column (10 ml) of Dowex 1 × 8 resin (carbonate form). The effluent was evaporated to 5 ml, palladium–carbon (10%; 5 mg) and acetic acid (2 drops) were added, and the mixture was stirred in hydrogen. When debenzylation (followed by the periodate–thiobarbiturate test) was complete, the catalyst was filtered off, the solvents were removed, and the dry residue was acetylated with acetic anhydride and pyridine (1 ml each) at 40 °C overnight. The mixture, directly analysed by g.l.c. [5% XE 60 on Varaport 30 (100—200 mesh), 1/8 in × 10 ft, isothermal at 235 °C, carrier gas nitrogen at 25 ml min^{-1}], was separated into two components of retention times identical with those of the alditol acetates derived from glucometasaccharinic acid (single peak) and from galactometasaccharinic acid (single peak) and present in the ratio 7 : 1 in favour of the glucometasaccharinic acid derivatives.

3-*Deoxy*-D-manno- *and* 3-*Deoxy*-D-gluco-*octulosonic Acid* 8-*Phosphates*, (V) *and* (VI).—The mixture of lithium salts of 5-*O*-benzyl-3-deoxyoctulosonic acid 8-phosphates [(III) and (IV)] (150 mg) was dissolved in water (10 ml), the pH of the solution was brought to 3 with a solution of chloroacetic acid, and the benzyl groups were removed by hydrogenolysis over 5% palladium–carbon. When cleavage (followed by paper electrophoresis in pyridinium acetate buffer at pH 5) was complete, the catalyst was filtered off and the lithium salts of the mixture of 3-deoxyoctulosonic acid 8-phosphates were isolated by precipitation (ethanol) from the concentrated (2 ml) solution. The material was dissolved in a sodium chloroacetate buffer (pH 4; 0.2M with respect to chloroacetate ion) and adsorbed on a column (120 × 1.2 cm) of Dowex 2 × 10 resin (chloroacetate form) equilibrated with the same buffer. Elution (Figure 3) with this buffer (10 ml h^{-1}; 3 ml fractions) afforded two incompletely separated peaks, detected by the periodate–thiobarbiturate test, the first (fractions 140—172) about seven times more abundant than the other, representing the D-*manno*-isomer, and the second (fractions 172—195) the D-*gluco*-isomer. Fractions containing the pure isomers were pooled, decationised with Amberlite IR 120 (H^+) resin, and continuously extracted with ether until no further acid was extracted. The aqueous phases were neutralised with lithium hydroxide solution and concentrated to *ca.* 0.2 ml, and the lithium salts of the phosphorylated acids were precipitated with ethanol and collected by centrifugation. The products obtained from two identical separations were combined, and the lithium salts were reprecipitated twice (to remove traces of chloroacetic acid) and dried (P_2O_5). The D-manno-*isomer* (86 mg)

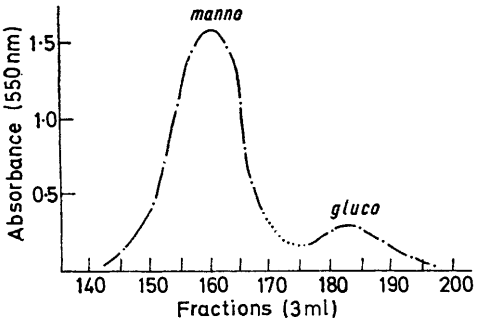


FIGURE 3 Separation of 3-deoxy-D-*manno*-octulosonic acid 8-phosphate from the D-*gluco*-isomer by anion-exchange chromatography on Dowex 2 × 10 ($ClCH_2{\cdot}CO_2^-$)

had $[\alpha]_D^{20}$ +28° (*c* 1 in H_2O) (Found: C, 26.4; H, 4.4; P, 8.4. $C_8H_{12}Li_3O_{11}P,1.5H_2O$ requires C, 26.4; H, 4.1; P, 8.5%). In the semicarbazide test it had $\lambda_{max.}$ 250 nm (ε 10 000). The acid (2 mmol), when treated in 0.1N-sulphuric acid at room temperature with sodium periodate (10 mmol) was overoxidised, but had set free 1 mol. equiv. of 2,4-dioxobutyrate (molar absorption 92 000 in the thiobarbituric acid test) after 1 h exposure to the oxidant and had liberated no formaldehyde after 4 h. The D-gluco-*isomer* had $[\alpha]_D^{20}$ +21° (*c* 0.3 in H_2O) (Found: C, 25.8; H, 4.2; P, 8.1. $C_8H_{12}Li_3O_{11}P,2H_2O$ requires C, 25.8; H, 4.3; P, 8.3%). In the semicarbazide test it had $\lambda_{max.}$ 250 nm (ε 10 000).

Proof of the Position of the Phosphate Group.—3-Deoxy-D-*manno*-octulosonic acid 8-phosphate (lithium salt) (18 mg) was treated with sodium borohydride (20 mg) in water (0.5 ml) for 45 min. The residue obtained after the usual work-up was dissolved in water (0.5 ml), the solution was neutralised with lithium hydroxide solution, and the lithium salts of the mixed epimeric, phosphorylated 3-deoxyoctonic acids (12 mg) were precipitated with ethanol and collected by centrifugation (Found: C, 26.7; H, 5.2; P, 8.35. Calc. for $C_8H_{14}Li_3O_{11}P,H_2O$: C, 27.0; H, 4.5; P, 8.7%). This material (4.25 mg) was treated with sodium periodate (480 mg) and the reduction of periodate

[16] C. S. Hanes and F. A. Isherwood, *Nature*, 1949, **164**, 1107.
[17] J. De Ley, *Enzymologia*, 1954, **17**, 55.

was followed: 2.8 mol. equiv. of oxidant were reduced. The mixture was treated for 45 min with sodium borohydride and the lithium salts of the phosphate esters present were precipitated as described above. The precipitate was subjected to paper electrophoresis in pyridinium acetate buffer (pH 5). Only one phosphate ester was detected: its mobility corresponded to that of authentic glycol phosphate ($R_{\text{picric acid}}$ 1.43; glycerol 2-phosphate had $R_{\text{picric acid}}$ 1.29).

[5/2430 *Received, 15th December,* 1975]