Phosphorylated Sugars. Part 21.[1] Synthesis of 3-Deoxy-D-*manno*- and 3-Deoxy-D-*gluco*-oct-2-ulosonic Acid 5-(Dihydrogen Phosphates)

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The title compounds were obtained by base-catalysed condensation of 2-*O*-phosphoryl-D-arabinose with oxalacetate and separation of the isomers by ion-exchange chromatography.

A 3-DEOXYOCT-2-ULOSONIC ACID presumed to have the D-*manno*-configuration and phosphorylated in position 5 has been isolated from the endotoxin of *Bordetella pertussis*.[2] For the identification of this compound authentic 3-deoxy-D-*manno*-oct-2-ulosonic acid 5-phosphate (4) was required. As it has been shown previously [1,3,4] that condensation of 2-*O*-substituted D-arabinose derivatives with oxalacetic acid in alkaline medium [5] leads to a mixture of 5-*O*-substituted 3-deoxy-D-*gluco*-, and -D-*manno*-oct-2-ulosonic acids in which the D-*manno*-isomer is preponderant, and also that a mixture of 3-deoxy-D-*manno*- and D-*gluco*-oct-2-ulosonic acid 8-phosphates could be separated by ion-exchange chromatography, the synthesis of the title compounds was attempted by this route.

Although 2-*O*-methyl-D-arabinose was easily obtained when 3-*O*-methyl-D-glucose was treated with 1 mol. equiv. of periodate,[3] the analogous degradation of glucose 3-phosphate to arabinose 2-phosphate gave a mixture of products and was thus unsatisfactory. The 2-*O*-phosphoryl-D-arabinose (2) required as starting material was obtained in moderate yield by phosphorylating benzyl 3,4-*O*-isopropylidene-β-D-arabinoside [6] with

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[2] R. Chaby and L. Szabó, *European J. Biochem.*, 1975, **59**, 277.
[3] D. Charon and L. Szabó, *European J. Biochem.*, 1972, **29**, 184.
[4] R. S. Sarfati, Thesis, 1976, Université de Paris-Sud, Centre d'Orsay.
[5] J. W. Cornforth, M. E. Firth, and A. Gottschalk, *Biochem. J.*, 1958, **68**, 57.
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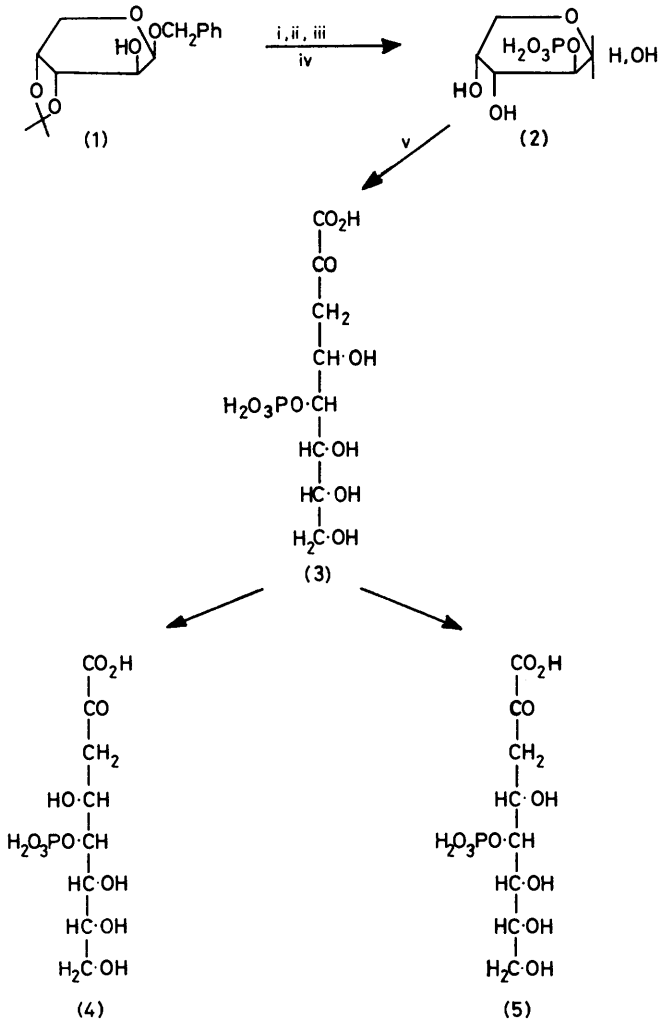
2-cyanoethyl phosphate and dicyclohexylcarbodi-imide [7] and removing the protecting groups (Scheme). The



SCHEME Reagents: i, $NC{\cdot}CH_2{\cdot}CH_2{\cdot}O{\cdot}PO_3H_2$–pyridine–dicyclohexylcarbodi-imide; ii, M-NaOH; iii, H^+; iv, 10% Pd–C; v, oxalacetate (pH 11)

position of the phosphate group was proved by reduction to the corresponding alditol and treatment of this with periodate: 2 mol. equiv. of periodate were reduced and 1 mol. equiv. of formaldehyde was released. This proved that in the phosphorylated alditol the phosphate group occupied position 2 or 4. The quantitative migration of the phosphate group from position 2 to position 4 being improbable, it was concluded that no migration of the phosphate group had taken place during the acid-catalysed removal of the isopropylidene group. The arabinose 2-phosphate was treated at 0 °C with oxalacetic acid at pH 11 and the reaction arbitrarily stopped 2 h later by addition of an acidic ion-exchange resin. Indeed, as 5-*O*-substituted oct-2-ulosonic acids give only a very weak response [3] in the thiobarbiturate test,[8] and as 2-*O*-substituted arabinose and presumably other 2-*O*-substituted aldoses strongly interfere in this same test,[9] it was not feasible to follow the progress of the condensation by this test (as had been done [10] when unsubstituted arabinose was condensed with oxalacetic acid).

Unchanged phosphorylated arabinose was separated from the phosphorylated condensation product (3) by ion-exchange chromatography on Dowex 1 × 8 resin in a chloride system, and the phosphate esters were recovered as their ethanol-insoluble lithium salts (Figure 1). The yield of the mixed phosphorylated 3-deoxyoct-2-ulosonic acids was low: *ca.* 12—13%, or *ca.* 25% if the recovered arabinose 2-phosphate was taken into account.

The position of the phosphate group was confirmed by oxidation with periodate: 2 mol. equiv. were reduced and 1 mol. equiv. of formaldehyde was formed. As the 6-phosphate of a 3-deoxyoct-2-ulosonic acid would behave similarly, the periodate-treated material was reduced with borohydride and the phosphate esters present were analysed by paper electrophoresis under conditions in which phosphorylated 3-deoxy-hexonic- and -pentonic acids are separated: only 3-deoxy-hexonic acid phosphate was detected, which confirmed that only 5-*O*-phosphoryl 3-deoxyoct-2-ulosonic acids were present in the original material.

In the thiobarbiturate test [8] the molar absorption coefficient of the mixed isomers was 10 000, *i.e.* about one tenth of that of the unsubstituted octulosonic acid. Maximum absorbance was reached when 2 mol. equiv. of

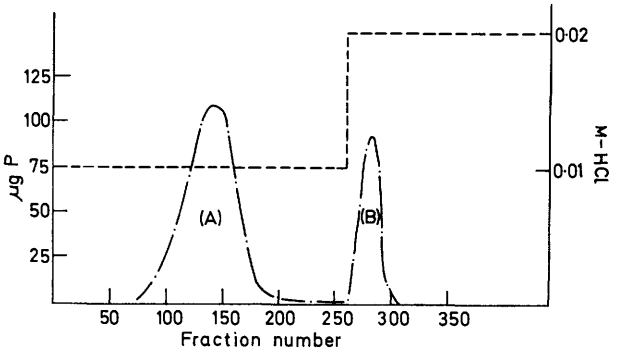


FIGURE 1 Separation of D-arabinose 2-phosphate (A) from the mixed isomeric 3-deoxyoct-2-ulosonic acid 5-phosphates (B) on Dowex 1 × 8 (Cl^-) resin

periodate were reduced and the value remained constant. In the diphenylamine test [11] the molar absorbance at 425 nm was *ca.* 45% of that of the unsubstituted 3-deoxyoct-2-ulosonic acid.

[7] G. M. Tener, *J. Amer. Chem. Soc.*, 1961, **83**, 159.
[8] A. Weissbach and J. Hurwitz, *J. Biol. Chem.*, 1959, **234**, 705.
[9] D. Charon, R. S. Sarfati, and L. Szabó, unpublished results.
[10] C. Hershberger, M. Davis, and S. B. Binkley, *J. Biol. Chem.*, 1968, **243**, 1585.
[11] R. Chaby, R. S. Sarfati, and L. Szabó, *Analyt. Biochem.*, 1974, **58**, 123.

In the semicarbazide test [12] the compound's molar absorbance was 10 200, like that of the simple α-oxo-acids, but this value was reached only after 90 min of incubation at 37 °C. It has been observed previously [13] that long incubation periods may be required in this test to attain the maximal value.

The presence of two isomers and their approximate ratio in the 3-deoxyoct-2-ulosonate 5-phosphate preparation were demonstrated by a reaction sequence analogous to that used in the case of the isomeric 5-*O*-benzyl-3-deoxyoct-2-ulosonic acid 8-phosphates,[1] the dephosphorylation being accomplished enzymically. The ratio of the peak areas in g.l.c. of the final 3-deoxy-alditol acetate mixture (3-deoxy-L-glucitol + 3-deoxy-L-mannitol peracetates from the D-*manno*-isomer, and 3-deoxy-L-galactitol + 3-deoxy-L-talitol peracetates from the D-*gluco*-isomer) established that the D-*manno*

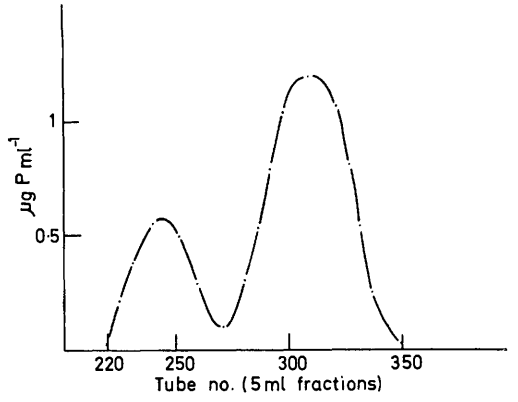


FIGURE 2 Separation of 3-deoxy-5-*O*-phosphoryl-D-*gluco*- (1) and D-manno-oct-2-ulosonic acids; the mixed lithium salts (8.5 mg) were adsorbed at pH 8.5 on a column (340 × 12 mm) of Dowex 2 × 10 resin (200—400 mesh; monochloroacetate form), and the column washed with water (250 ml) and eluted with a monochloroacetate buffer (see Experimental section)

isomer was preponderant by a factor of 3 : 1. About the same ratio of isomers was obtained when oxalacetate was condensed with 2-*O*-methyl-,[3] 3-*O*-benzyl-,[1] or 2-*O*-(β-D-glucopyranosyl)-D-arabinose.[4]

The isomers were separated (Figure 2) by ion-exchange chromatography on Dowex 2 × 10 resin in a monochloroacetate system, 0.186M with respect to monochloroacetic acid, of pH 4.7. The following conditions were unsatisfactory: 0.125M, pH 3.4 and 4.3; 0.17M, pH 3.95; 0.2M, pH 4.0; 0.25M, pH 3.6, 4.0, 4.5, and 4.8; 0.375M, pH 4.3. A second ion-exchange procedure was necessary to eliminate the monochloroacetic acid remaining after extraction of the bulk of the eluate with ether. The purity of the D-*manno*-isomer was checked by the same reaction sequence as used to establish the proportion of the isomers as described previously: [1] only one peak which had the retention time of the mixed 3-deoxy-glucitol and -mannitol peracetates was detected.

[12] J. MacGee and M. Doudoroff, *J. Biol. Chem.*, 1954, **210**, 617.
[13] D. H. Levin and E. Racker, *J. Biol. Chem.*, 1959, **234**, 2532.
[14] G. Avigad, *Carbohydrate Res.*, 1969, **11**, 119.

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 reductions with borohydride, solutions were decationised with Amberlite IR 120(H^+) resin and evaporated to dryness; boric acid was removed by repeated addition of methanol containing a few drops of acetic acid and evaporation to dryness. Periodate was estimated according to the method of Avigad.[14] 2-Cyanoethyl phosphate pyridinium salt was prepared by treating an aqueous solution (30 ml) of the barium-salt (16.1 g, 50 mmol) with an excess Amberlite IR 120(H^+) resin; the mixture was filtered and the solid was washed with water; pyridine (20 ml) was added to the filtrate, from which all solvents were then removed. The residual syrup was dissolved in anhydrous pyridine and the volume adjusted to 50 ml. The solution appeared to be stable for at least 1 month in a refrigerator.

Benzyl 2-O-*Phosphoryl*-β-D-*arabinopyranoside*.—To benzyl 3,4-*O*-isopropylidene-β-D-arabinopyranoside [6] (560 mg, 2 mmol) (1), anhydrous pyridine (20 ml) was added, followed by 4 ml of the pyridinium 2-cyanoethyl phosphate solution (4 mmol). Solvents were removed and anhydrous pyridine (40 ml) was evaporated from the residue, which was then kept *in vacuo* over phosphoric oxide overnight at 20 °C. A solution of *NN'*-dicyclohexylcarbodi-imide (2.4 g, 24 mmol) in anhydrous pyridine (20 ml) was added, and the mixture stirred at 20 °C for 4 days. Water (6 ml) was added, and after 30 min the solvents were removed and more water (20 ml) was added. The precipitate was filtered off and washed with water (20 ml), and to the filtrate M-sodium hydroxide (40 ml) was added. The mixture was kept on a boiling water-bath for 40 min, cooled, and treated with an excess of Amberlite IR 120(H^+) resin for 30—45 min with stirring. The removal of the isopropylidene group can be easily followed by paper electrophoresis at pH 5—6. After removal and washing of the resin, the pH of the solution was brought to 10 with barium hydroxide solution, and the barium phosphate was removed by centrifugation. The pH of the supernatant was lowered to 7 [IR 120(H^+) resin] and the volume of the filtered solution brought to about 0.5 ml. The barium salt (560 mg, 62%) of the title compound was precipitated with acetone, recovered by centrifugation, and dried *in vacuo* over phosphoric oxide. The calcium salt was obtained when the pH of the decationised solution of the barium salt (500 mg) was adjusted to 7.3 with calcium hydroxide solution in a nitrogen atmosphere. After concentration of the solution (to *ca.* 1 ml) and addition of ethanol, the precipitated *calcium salt* (390 mg) had $[\alpha]_D^{20}$ −132° (*c* 0.43 in 0.1M-HCl) (Found: C, 36.3; H, 4.8; P, 7.8. $C_{12}H_{15}CaO_8P,2H_2O$ requires C, 36.5; H, 4.8; P, 7.9%).

The biscyclohexylammonium salt was formed when an aqueous solution of the calcium salt (500 mg) was treated with IR 120(H^+) resin, and, after removal of the solid, the pH of the solution was adjusted to 7 with cyclohexylamine. The solvent was removed and the residue was taken up in a small amount of ethanol; dropwise addition of ether induced crystallisation. Recrystallisation from ethanol gave the pure *product*, m.p. 177—178 °C, $[\alpha]_D^{20}$ −76° (*c* 0.4

in water) (Found: C, 55.4; H, 8.5; N, 5.3; P, 5.9. $C_{24}H_{43}N_2O_8P$ requires C, 55.6; H, 8.3; N, 5.4; P, 6.0%).

2-O-*Phosphoryl*-D-*arabinose* (2).—A solution of the above-mentioned calcium salt (250 mg) was decationised [Amberlite IR 120(H^+)] and its volume reduced to 15 ml. Palladium–carbon (10%; 15 mg) was added and the mixture was stirred overnight in hydrogen. The pH of the filtered solution was brought to 7 with saturated calcium hydroxide solution; the solution was filtered if necessary, and concentrated (to *ca.* 0.5 ml). Upon addition of acetone the *calcium salt* of the title compound (185 mg) precipitated, and was collected by centrifugation; $[\alpha]_D^{20}$ −44.6° (*c* 0.5 in 0.1M-HCl) (Found: C, 22.4; H, 3.35; P, 11.6. $C_5H_8CaO_9P$ requires C, 22.4; H, 3.5; P, 11.7%). The barium salt was obtained by using barium hydroxide solution.

3-*Deoxy*-5-O-*phosphoryl*-*Oct*-2-*ulosonic Acids* (*Mixture of* D-manno- *and* D-gluco-*Isomers*) (3).—To a suspension of oxalacetic acid (265 mg, 2 mmol) in ice–water (4 ml), sufficient 10M-sodium hydroxide was added to bring the pH to 11. To this solution, D-arabinose 2-(barium phosphate) (1.54 g, 4 mmol) was added with constant stirring, and the pH was maintained at 11 by addition of 10M-sodium hydroxide. After 2 h at 0 °C the mixture was neutralised [IR 120(H^+) resin; pH 7] and filtered, and the filtrate and washings were passed through a column (14 × 220 mm) of Dowex 1 × 8 (100—200 mesh; Cl^-) resin. The column was washed with water and then eluted with 0.01M-hydrochloric acid (2 l); the eluate contained unchanged arabinose 2-phosphate, which was recovered. Elution was continued with 0.02M-hydrochloric acid. Fractions (8 ml) containing the phosphorylated octulosonic acids (detection in 0.1 ml samples by the thiobarbiturate test [8]) were pooled, neutralised (pH 7) with lithium hydroxide solution, and concentrated (to *ca.* 3 ml). Upon addition of ethanol the *lithium salts* of the title compounds (210 mg, 13%) were precipitated; the material was collected by centrifugation and washed with ethanol until free of chloride ions. When dry, it had $[\alpha]_D^{20}$ +40° (*c* 0.45 in water) (Found: C, 24.4; H, 4.5; P, 7.9. $C_8H_{12}Li_3O_{11}P,3H_2O$ requires C, 24.6; H, 4.6; P, 7.9%).

Separation of the D-manno- *and* D-gluco-*Isomers* [(4) *and* (5).]—The pH of an aqueous solution (5 ml) of the mixed lithium salts (400 mg) was adjusted to 8.5 with dilute aqueous ammonia; it was then percolated through a column (340 × 12 mm) of Dowex 2 × 10 resin (200—400 mesh; monochloroacetate form). The column was washed with water (250 ml) and eluted (20 ml h^{-1}) with a monochloroacetate buffer solution (11.75 g l^{-1} of monochloroacetic acid brought to pH 4.7 with sodium hydroxide solution). Fractions (8 ml) were collected and the elution was monitored by estimation of the phosphorus content of samples (1 ml).[15] Those containing the pure compounds (D-*gluco*-isomer: fractions 210—320; D-*manno*-isomer: fractions 370—550) were pooled, treated separately with Amberlite IR 120 (H^+) resin, filtered, extracted with ether (3 times), and diluted 3-fold, and the pH was adjusted (M-NaOH) to 7.5. Each solution was then slowly percolated through a small column (20 × 100 mm) of Dowex 1 × 8 resin (200—400 mesh; Cl^-). The resin was washed with water (100 ml) and then eluted first with 0.01M-hydrochloric acid until no inorganic phosphate was detected in the eluate and then with 0.03M-hydrochloric acid. Fractions (100 ml) were collected and elution continued until the thiobarbiturate reaction of a sample (0.1 ml) was negligible (400—500 ml); the phosphorylated 3-deoxy-octulosonic acids were isolated as described for the mixed isomers. 3-*Deoxy*-5-O-*phosphoryl*-D-gluco-*oct*-2-*ulosonic acid trilithium salt* (peak I; 95 mg, 23%) had $[\alpha]_D^{20}$ +54° (*c* 0.8 in water) (Found: C, 24.1; H, 4.7; P, 8.35. $C_8H_{12}Li_3O_{11}P,3.5H_2O$ requires C, 24.1; H, 4.8; P, 7.8%). The D-*manno*-isomer (peak II; 220 mg, 54%) had $[\alpha]_D^{20}$ +46° (*c* 0.8 in water) (Found: C, 24.3; H, 4.4; P, 8.0. $C_8H_{12}Li_3O_{11}P,3H_2O$ requires C, 24.6; H, 4.6; P, 7.9%).

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[15] P. S. Chen, T. Y. Toribara, and H. Warner, *Analyt. Chem.*, 1956, **28**, 1756.