Synthesis and Behaviour under Acidic Conditions of 2-Deoxy-D-*arabino*-hexopyranose and 3-Deoxy-2-ketoaldonic Acids Bearing *O*-Phosphono or *O*-Glucosyl Substituents at Position β to the Carbonyl Function

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Methyl 4-*O*-phosphono- and 4-*O*-(β-D-glucopyranosyl)-3-deoxy-β-D-*erythro*-2-hexulopyranosidonic acids, and 3-*O*-phosphono-, and 3-*O*-(β-D-glucopyranosyl)-2-deoxy-α-D-*arabino*-hexopyranoses have been synthesised and their behaviour in acidic media examined. At 100 °C, release of inorganic phosphate from the 4-*O*-phosphono-hexulopyranosidonic acid is faster at pH 3—6 than at pH 0—1 and occurs through an elimination initiated by proton transfer. Phosphate release from the 3-*O*-phosphono-2-deoxy-hexopyranose occurs by a similar mechanism, but is much slower. Treatment at 100 °C with acetic acid of pH 3.4 causes release of glucose from the 4-*O*-glucosylated-3-deoxy-hexulopyranosidonic acid but not from the 3-*O*-glucosylated-2-deoxy-hexopyranose.

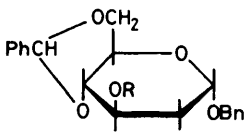
STUDIES in this laboratory have shown that the *Bordetella pertussis* endotoxin is made up of two lipopolysaccharides, LPS1 and LPS2,[1] in which lipid and polysaccharide regions are linked through the glycosidic bond of a 3-deoxy-D-*manno*-2-octulopyranosonic acid (KDO). While LPS1 was rapidly hydrolysed by 0.01M acetic acid (pH 3.4) at 100 °C—conditions used for the cleavage of the *Salmonellae* endotoxins into polysaccharides and lipid A[2]—LPS2 required treatment with 0.25M mineral acid to cleave the glycosidic bond of KDO which was shown to be phosphorylated at position 5.[3] The fact that KDO 5-phosphate was isolated after acidic hydrolysis of LPS2 does not prove unequivocally that the phosphate group originally occupied position 5 in the intact macromolecule, as it is well known that, under acidic conditions, phosphate groups can migrate. As it has been demonstrated [3] that, in the intact endotoxin, positions 7 and 8 are free, the phosphate group must necessarily have been in position 4 or 5. It appeared necessary, therefore, to investigate 4- and 5-*O*-phosphorylated 3-deoxy-2-ketoaldonic acids with respect to their behaviour in acidic medium.

In the present work the behaviour in acidic medium of 3-deoxy-D-*manno*-2-octulopyranosonic acid 5-phosphate (KDO 5-phosphate) [4] (19), 3-deoxy-D-*arabino*-2-heptulopyranosonic acid 4-phosphate (4), methyl 3-deoxy-β-D-*erythro*-2-hexulopyranosidonic acid 4-phosphate (17) and its methyl ester (18), and the barium salt of 2-deoxy-α-D-*arabino*-hexopyranose 3-phosphate (2) were examined; the last-named compound was included in the study with the aim of comparing the behaviour of a phosphate group in position α to a CH_2CHO group with that of a phosphate group in position α to a $CH_2CO{\cdot}CO_2H$ group.

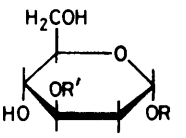
For reasons that will be discussed later, the rates of glucose release from 3-deoxy-5-*O*-β-D-glucopyranosyl-2-octulopyranosonic acid (a mixture of the D-*gluco*- and D-*manno*-isomers [5]) (20), from methyl 3-deoxy-4-*O*-β-D-glucopyranosyl-β-D-*erythro*-2-hexulopyranosidonic acid ammonium salt (15), and from 2-deoxy-3-*O*-β-D-glucopyranosyl-α-D-*arabino*-hexopyranose (8) were also determined.

The compounds required for these studies were prepared as follows. Benzyl 4,6-*O*-benzylidene-2-deoxy-α-D-*arabino*-hexopyranoside (1) was the starting material for the syntheses of the 3-phosphate (2), the disaccharide (8), and the heptulopyranosonic acid 4-phosphate (4). Phosphorylation of compound (1) with pyridinium 2-cyanoethyl phosphate,[6] followed by acidic hydrolysis, gave the free phosphate (2). That no phosphate migration had occurred during the acidic hydrolysis step was ascertained by periodate oxidation of the corresponding 2-deoxy-hexitol phosphate which reduced 2 mol equiv. of periodate, 1 mol equiv. of formaldehyde being formed. Cyanohydrin formation from compound (2), followed by selective oxidation of the hydroxy-group at position 2 of the resulting C_7-acid by Sprinson's method [7] yielded, after purification by ion-exchange chromatography, the 3-deoxy-D-*arabino*-2-heptulopyranosonic acid 4-phosphate (4), isolated as its trilithium salt. The presence of an α-keto-acid grouping was demonstrated both by the *o*-phenylenediamine [8] and by the semicarbazide [9] reactions. In the latter test, the compound gave the typical molar absorption coefficient of ε *ca.* 10 000, although this value was reached only after an incubation period of 105 min at 37 °C; it had been observed previously [10] that long incubation periods may be required in this test in order to attain the maximum ε-value. Again, the position of the phosphate group was ascertained by periodate oxidation: 2 mol equiv. of periodate were reduced and 1 mol equiv. of formaldehyde was formed. The crystalline disaccharide (8) was synthesised by condensation of compound (1) with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide to give the fully protected disaccharide (5), characterised by its ^{13}C n.m.r. spectrum, from which, successively, the acetate groups were removed by saponification, the benzylidene group by acidic hydrolysis, and the benzyl group by hydrogenolysis.

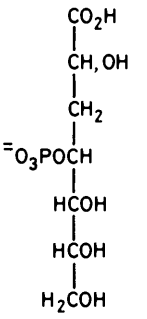
Methyl (methyl 5-*O*-benzyl-3-deoxy-β-D-*erythro*-2-hexulopyranosid)onate (13), the starting material for the synthesis of the disaccharide (15) and of the phosphate (17), was prepared in five steps from 1,2-*O*-isopropylidene-3-*O*-methyl-D-glucofuranose [11] which was tritylated



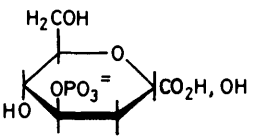
(1) R = H
(5) R = AcGlc
(6) R = Glc



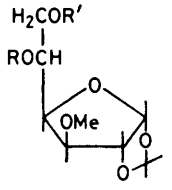
(2) R = H; R′ = $PO_3^=$
(7) R = Bn; R′ = Glc
(8) R = H; R′ = Glc



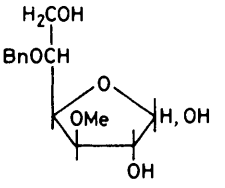
(3)



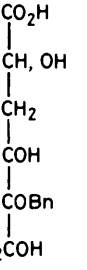
(4)



(9) R = H; R′ = Tr
(10) R = Bn; R′ = Tr



(11)



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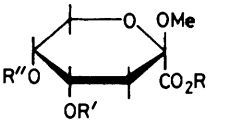
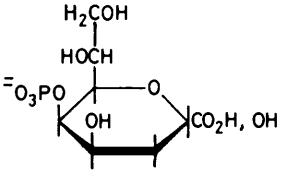


(13) R = Me; R′ = H; R″ = Bn
(14) R = Me; R′ = AcGlc; R″ = Bn
(15) R = NH_4; R′ = Glc; R″ = H
(16) R = H; R′ = $PO_3^=$; R″ = Bn
(17) R = H; R′ = $PO_3^=$; R″ = H
(18) R = Me; R′ = $PO_3^=$; R″ = H



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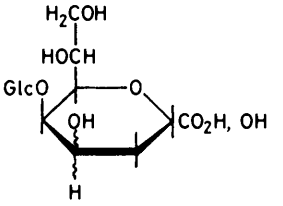

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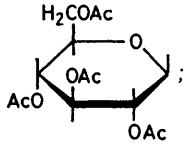
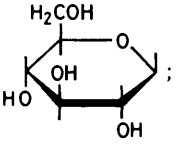
AcGlc =  ;

Glc =  ;

Tr = $(Ph)_3C$; Bn = $PhCH_2$;

at position 6 and benzylated in position 5. Acidic hydrolysis removed the trityl and isopropylidene groups to give 5-*O*-benzyl-3-*O*-methyl-D-glucofuranose (11) together with some of the corresponding 1,6-anhydro-compound. The formation of a similar anhydride was shown to occur upon acidic hydrolysis of 1,2-*O*-isopropylidene-3,5-di-*O*-methyl-6-*O*-trityl-D-glucofuranose with mineral acid,[12] but it could in that instance be avoided by performing the hydrolysis with aqueous benzoic acid. The insolubility of 5-*O*-benzyl-1,2-*O*-isopropylidene-3-*O*-methyl-D-glucofuranose did not allow us to obtain better yields of the free sugar using benzoic acid. Alkaline degradation of compound (11) afforded 5-*O*-benzyl-3-deoxy-D-*erythro*-hexonic acid (12), isolated as its cyclohexylammonium salt, which was oxidised by Sprinson's method [7] to give the corresponding keto-acid, isolated as its methyl ester methyl glycoside (13). This was first condensed with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide to give the fully protected β-disaccharide (14), obtained pure by h.p.l.c., and characterised by ^1H- and ^{13}C-n.m.r. spectroscopy. Hydrogenolysis of the benzyl group followed by saponification of the acetate and methyl ester groups gave the free disaccharide isolated as its crystalline ammonium salt (15) which liberated 1 mol equiv. of glucose upon acidic hydrolysis. Phosphorylation of compound (13) with

pyridinium 2-cyanoethyl phosphate [6] gave the 4-phosphate (16), isolated as its barium salt. This compound was used for acidic hydrolysis after catalytic removal of the benzyl group to give compound (17) [tris(cyclohexylammonium) salt].

In 1M hydrochloric acid at 100 °C the rate of release of inorganic phosphate from the heptulopyranosonic acid 4-phosphate (4) was twice as rapid as that from 3-deoxy-D-*manno*-2-octulopyranosonic acid 5-phosphate (19) (Figure 1). After 1 h the compound (4) had released 50% of its phosphate and compound (19) 25%. Under the same conditions, 3-deoxy-D-*erythro*-2-hexulofuranosonic acid 6-phosphate [13] also released 25% of phosphate whereas only 2—3% was liberated from D-glucose 6-phosphate.[14]

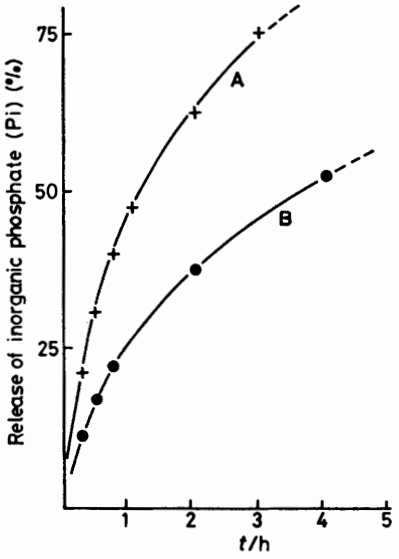


FIGURE 1 Rates of release of inorganic phosphate (P_I) from A 3-deoxy-D-*arabino*-2-heptulopyranosonic acid 4-phosphate (4) and B 3-deoxy-D-*manno*-2-octulopyranosonic acid 5-phosphate (19) in 1M hydrochloric acid at 100 °C

It is known that, in acidic medium, 3-deoxy-2-ketoaldonic acids are more easily degraded than are free sugars.[15] It is thus probable that the greater acid lability of ketoaldonate phosphates compared with that of aldose phosphates is due more to the hydrolysis of the phosphate esters of their degradation products than to the hydrolysis of the starting materials. That the heptulopyranosonate 4-phosphate is more labile than the other two ketoaldonate phosphates is probably because in acidic medium, as for all of the 3-deoxy-2-ketoaldonic acids, it is in equilibrium with its enol form in which the phosphate group is allylic: it is known that allyl phosphates are very rapidly hydrolysed in acidic medium.[16] A similar case is that of D-ribose 3-phosphate which is hydrolysed to the extent of 90% in 30 min in 0.5M sulphuric acid at 100 °C.[17]

As has already been mentioned, the glycosidic bond of 3-deoxy-D-*manno*-2-octulopyranosonic acid, which links the polysaccharide moiety of lipopolysaccharides to the lipid region, is usually cleaved in 1—2 h with 0.01M acetic acid (pH 3.4) at 100 °C. We therefore examined the release of phosphate from the ketoaldonates under these conditions. With a decrease in acidity the rate of phosphate release from the octulopyranosonate 5-phosphate (19) is considerably diminished whereas that from the heptulopyranosonate 4-phosphate (4) is increased; the rate of release from the hexulopyranosidonate 4-phosphate (17) is even more rapid (Figure 2).

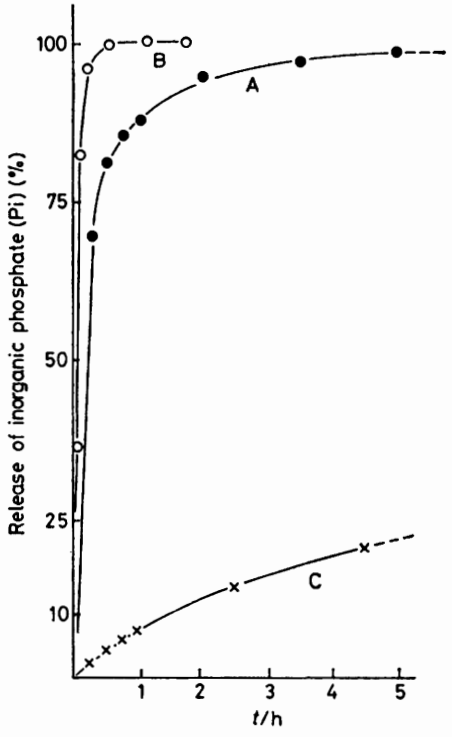


FIGURE 2 Rates of release of inorganic phosphate (P_I) from A 3-deoxy-D-*arabino*-2-heptulopyranosonic acid 4-phosphate (4), B methyl 3-deoxy-D-*erythro*-2-hexulopyranosidonic acid 4-phosphate (17), and C 3-deoxy-D-*manno*-2-octulopyranosonic acid 5-phosphate (19) in 0.01M acetic acid (pH 3.4) at 100 °C

In view of these results, compound (4) was incubated at different pH values for 30 min at 100 °C and the rate of release of phosphate was examined. The results obtained (Figure 3) suggested that release of inorganic phosphate was not entirely due to hydrolysis but to a β-elimination. There are at least three possible mechanisms (Scheme 1) which could explain the elimination of inorganic phosphate, each of which gives rise to the same compound: a 3,4-dideoxy-D-*erythro*-2-hept-3-enulosonic acid (21). At pH 2.6 the concentration of phosphate mono-anion is probably at its highest as are the rates of elimination (mechanisms I and II) at this concentration (Figure 3). With increasing pH the concentration of this mono-anion diminishes as does the liberation of inorganic phosphate. At pH *ca.* 4 the equilibrium enol–enolate (mechanism III) starts to play a role, creating a negative charge which induces elimination of inorganic phosphate. Circumstantial evidence in favour of the above hypothesis is as follows. The hypothesis, based on proton transfer, requires that the rate of

elimination should be pH-dependent. As is apparent from Figure 2, this is indeed the case: release of inorganic phosphate is slower at lower pH values where the phosphate mono-anion concentration is lower. As a corollary, a substituent that is not a good proton acceptor or

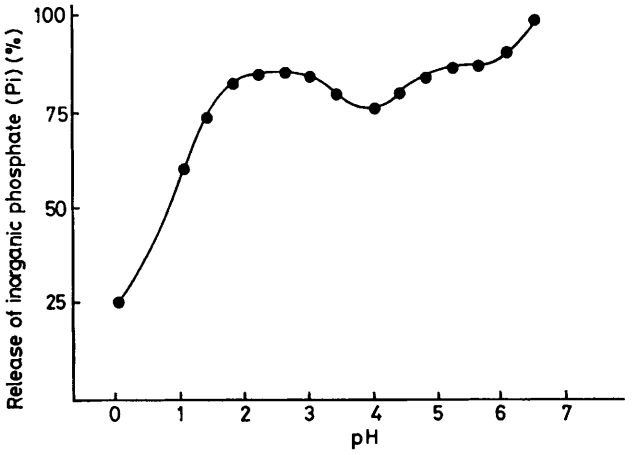
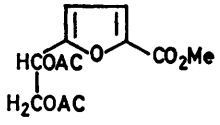


FIGURE 3 Percentage of inorganic phosphate (P_i) formed from 3-deoxy-D-*arabino*-2-heptulopyranosonic acid 4-phosphate (4) at different pH values after 30 min at 100 °C

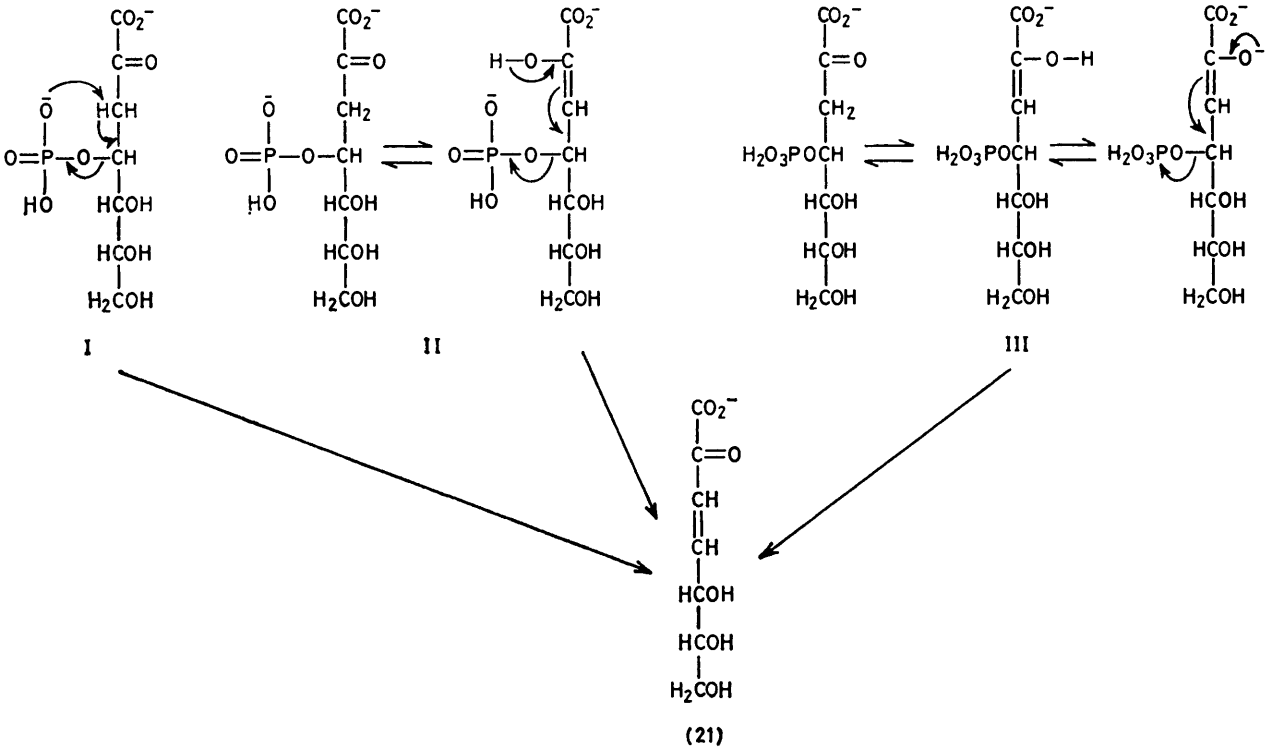
donor would be eliminated at a vastly reduced rate and perhaps by an unrelated mechanism. Thus the half-life of methyl 3-deoxy-4-*O*-(β-D-glucopyranosyl)-β-D-*erythro*-2-hexulopyranosidonic acid (15; R = Me) in 0.01M acetic acid (pH 3.4) at 100 °C [under these conditions the glycosidic bond of the hexulosidonic acid (15; R = Me) is completely hydrolysed within 10 min] is *ca.* 3 h, whereas that of the corresponding 4-phosphate (17) is 2—3 min.

When compound (4) was heated at 100 °C in a pH 6.5 buffer and the reaction was stopped after 15 min, practically all of the phosphate had been eliminated. Paper electrophoresis showed the presence of a non-phosphorylated, acidic substance which could be visualised with the periodate–benzidine spray (test for vicinal diols).[18] The acid was treated with diazomethane and then acetylated, and the mass spectrum of the product suggested it had structure (22). The mass spectrum showed peaks of



(22)

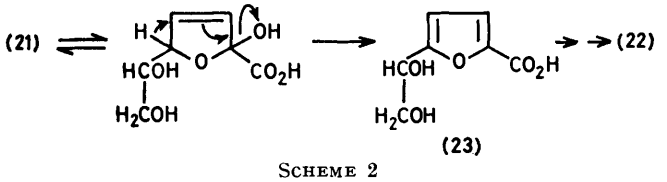
high intensity (*m/e* 123 and 87) derived by a well known fragmentation pattern of furanoid rings.[19] This furoic acid derivative is postulated to be formed from the olefinic α-keto-acid (21) *via* the elimination reaction shown in Scheme 2. This mechanism is similar to that proposed by Charon [15] for the acidic degradation of the unsubstituted 3-deoxy-D-*arabino*-2-heptulosonic acid and in both cases the same 5-substituted 2-furoic acid is formed. In the case of the acyclic form of compound (4) the formation of the acid (23) is much faster than with the unsubstituted heptulosonate. In the latter case, the enolic 1,4-lactone is the first intermediate: the rate-limiting step for the degradation of the unsubstituted heptulosonate appears



SCHEME 1

to be the formation of compound (21) from this lactone.

As expected, the same type of elimination is observed with 2-deoxyaldose 3-phosphates. In 0.01M acetic acid (pH 3.4) at 100 °C, 2-deoxy-α-D-*arabino*-hexopyranose 3-phosphate (2) has a half-life of 10—15 min, whereas under the same conditions there is no release of glucose from 2-deoxy-3-*O*-β-D-glucopyranosyl-α-D-*arabino*-hexopyranose (8). It appears from these experiments



SCHEME 2

that the carboxy-group present in the 3-deoxy ketoaldonic acid derivatives is not an absolute requirement for the elimination reaction to take place but it considerably enhances its rate. However, the presence of a free carbonyl group in the molecule *is* necessary for the elimination to occur. When the methyl ester (18) was treated with acetic acid under the usual conditions, less than 25% of inorganic phosphate was formed after 150 min, whereas with the free acid (17) the reaction was complete within 15 min. In the case of the methyl ester the rate of phosphate release reflects the rate of hydrolysis of the glycosidic bond. Charon [20] has shown that esterification of the carboxy-group stabilises the glycosidic bond of methyl 3-deoxy-D-*manno*-2-octulopyranosidonic acid.

The aforementioned release of glucose from compound (15; R = Me) under conditions in which glucosides are not readily hydrolysed could have some importance in the analysis of bacterial endotoxins. Although 3-deoxy-D-*manno*-2-octulopyranosonic acid is known to be a characteristic constituent of these lipopolysaccharides and is required for the accomplishment of their biosynthesis, endotoxins have been described which apparently do not contain KDO and in which the terminal reducing sugar of the polysaccharide chain is a heptose. In view of the ready release of an aldose from position 4 of a 3-deoxy-2-ketoaldonic acid occurring with concomitant destruction of the ketoaldonic acid it is possible that in these endotoxins position 4 of the terminal KDO molecule is substituted by heptose, the latter being usually the penultimate unit of the sugar chain.

EXPERIMENTAL

Evaporations were carried out under reduced pressure at 40 °C. Products were dried *in vacuo* (P_2O_5) and phosphates were then equilibrated in air. M.p.s were determined on a Kofler hot-plate and are uncorrected. Optical rotations were determined with a Perkin-Elmer model 141 polarimeter. ^{1}H N.m.r. spectra at 400 MHz were obtained on a prototype I.E.F. 400 instrument,[21] using Me_4Si as internal standard. ^{13}C N.m.r. spectra were recorded on a Varian CFT 20 spectrometer operating at 20 MHz in the Fourier-transform mode. T.l.c. was performed on silica gel (F 1 500 LS_{254}, Schleicher and Schüll) and compounds were located by spraying with sulphuric acid (10%) in ethanol and heating with an infrared lamp. Preparative t.l.c. was performed on glass plates coated with a 1.5 mm layer of silica gel (Merck 60 PF_{254}) and h.p.l.c. on a stainless steel column (20 in × 1 in) packed with the same silica gel at a pressure of 40 bar.* Paper electrophoresis was carried out on Whatman 3MM paper with a flat-plate electrophoresis apparatus in pyridine–acetic acid–water buffer (25 : 2.5 : 972.5 v/v/v; pH 6.2) at 70 V cm^{-1} for 45 min. Phosphate esters were detected according to the method of Hanes and Isherwood.[22] Migrations (*R*) are given with respect to that of picric acid. Periodate oxidations were performed by the method of Avigad,[23] and formaldehyde was determined with chromotropic acid.[24] The kinetics of hydrolysis of the disaccharides were followed using the 'Gluco-Quant' system of Boehringer for the estimation of free glucose and those of the phosphate by using the Boehringer colorimetric method of estimation of inorganic phosphate (ammonium molybdate and ammonium vanadate in acidic solution). Total phosphorus was estimated by the method of Macheboeuf and Delsal.[25] Light petroleum refers to that fraction boiling in the range 40—65 °C.

Benzyl 4,6-O-*Benzylidene*-2-*deoxy*-α-D-arabino-*hexopyranoside* (1).—A mixture of benzyl 2-deoxy-α-D-*arabino*-hexopyranoside [26] (1.95 g), benzaldehyde (5 ml), and zinc chloride (1.5 g) was vigorously shaken. After 1 h, further benzaldehyde (5 ml) was added and the mixture was stirred at room temperature for 48 h. The resulting solution was poured into a mixture of water (10 ml) and light petroleum (20 ml) and the mixture was stirred for 15 min. The precipitated *title compound* (2 g, 77%) was filtered off, washed with a mixture of water (10 ml) and light petroleum (20 ml), dried, and recrystallised from methanol; m.p. 138—139 °C; $[\alpha]_D^{20}$ +71° (*c* 0.8, acetone); δ_C ($CDCl_3$) 137.31 and 137.24 (quaternary aromatics), 129.07; 128.82, 128.20, 127.74, and 126.15 (*m,p,o*-aromatic), 101.89 [PhHC(-O)O], 97.14 (C-1), 83.79 (C-4), 69.03 and 68.89 (CH_2Ph and C-6), 65.69 and 62.77 (C-3 and C-5), and 37.27 p.p.m. (C-2) (Found: C, 69.9; H, 6.5. $C_{20}H_{22}O_5$ requires C, 70.2; H, 6.4%).

2-*Deoxy*-α-D-arabino-*hexopyranose* 3-(*Barium Phosphate*) (2; barium salt).—A solution of pyridinium 2-cyanoethyl phosphate [6] (4 ml, 4 mmole) in pyridine was added to a solution of compound (1) (0.68 g, 2 mmol) in pyridine (20 ml) and the solvents were removed. Anhydrous pyridine (2 × (20 ml) was added to the product and the mixture was evaporated; the residue was dried overnight. A solution of *N,N'*-dicyclohexylcarbodi-imide (2.4 g, 12 mmol) in anhydrous pyridine (20 ml) was added to the residue and the mixture was stirred at room temperature for 4 d. Water (6 ml) was added and after 30 min the solvent was removed. More water (20 ml) was added and the precipitate was filtered off and washed with water (20 ml). To the filtrate and washings was added 1M sodium hydroxide (40 ml) and the mixture was heated for 40 min in a boiling-water bath, cooled, and treated with an excess of Amberlite IR 120 (H^+-form) resin; removal of the benzylidene- and benzyl-groups was monitored by paper electrophoresis. When hydrolysis was complete, the pH of the mixture was adjusted to 10 with saturated aqueous barium hydroxide and the precipitated inorganic phosphate was removed by centrifugation. The pH of the supernatant layer was adjusted to 7 [Amberlite IR 120 (H^+-form) resin] and the solution was concentrated

* 1 bar = 10^5 Pa.

to *ca.* 10 ml. The *barium salt* (460 mg, 55%) was precipitated with ethanol and recovered by centrifugation; $[\alpha]_D^{20}$ +14° (*c* 0.63, 0.1M HCl) (Found: C, 17.35; H, 3.3; P, 7.45. $C_6H_{11}BaO_8P\cdot 2H_2O$ requires C, 17.34; H, 3.61; P, 7.46%).

3-*Deoxy*-D-arabino-*heptonic Acid* 4-*Phosphate, Barium Salt* (3; barium salt).—Potassium cyanide (0.97 g) was added to a cold solution of the barium phosphate (2) (1.2 g) in water (5 ml) and the mixture was kept at 4 °C for 18 h, then diluted with cold water (50 ml), and passed through a column (150 ml) of Amberlite IR 120 (H^+-form) resin. The pH of the eluant and washings was brought to 8.5 with aqueous barium hydroxide and the mixture was heated on a boiling-water bath for 15 min, clarified with charcoal, and concentrated to *ca.* 10 ml. The *barium salt* of the title compound, precipitated by addition of ethanol and recovered by centrifugation, had $[\alpha]_D^{20}$ +6° (*c* 0.4, 0.1M HCl) (Found: C, 15.85; H, 3.05; P, 5.8. $C_7H_{12}Ba_{1.5}O_{10}P\cdot 2H_2O$ requires C, 15.90; H, 3.03; P, 5.87%).

3-*Deoxy*-D-arabino-2-*heptulopyranosonic Acid* 4-*Phosphate, Trilithium Salt* (4; Lithium Salt).—A suspension of the barium salt (3) (1.05 g) in water (10 ml) was decationised with Amberlite IR 120 (H^+-form) resin and the pH of the resulting solution was adjusted to 9 with cyclohexylamine. To the concentrated (*ca.* 10 ml) solution, sodium chlorate (0.1 g) and freshly prepared Sprinson oxidation catalyst [7] (3 ml) were added and the pH of the solution was adjusted to 4.7 with either pyridine or dilute hydrochloric acid as necessary. The solution was stirred in a closed tube for 24 h, then passed through a column of Amberlite IR 120 (H^+-form) resin (50 ml). The pH of the eluant and washings was adjusted to 6.9 with aqueous lithium hydroxide, and the lithium salts were precipitated by addition of ethanol (120 ml) to the concentrated (*ca.* 5 ml) solution, then centrifuged off, washed with ethanol until free of lithium chloride, dried, and dissolved in water (150 ml). The solution was passed through a column of Dowex 1−X8 resin (100—200 mesh, Cl^--form, 10 ml) which was washed with water (100 ml), and then eluted with 0.01M hydrochloric acid until the fractions (8 ml) were free of phosphorus [25] and then with 0.02M hydrochloric acid. The fractions were tested for the presence of phosphorus and α-keto acids; [9] those containing the phosphorylated heptulosonic acid were pooled and the pH of the solution was brought to 6.9 with aqueous lithium hydroxide. The solution was concentrated to *ca.* 3 ml and the pH was adjusted with aqueous lithium hydroxide to 7.2. The *trilithium salt* (115 mg, 18%) of the title compound was precipitated with ethanol, recovered by centrifugation, washed free of lithium chloride with ethanol, and dried; $[\alpha]_D^{25}$ +31° (*c* 0.52, H_2O) (Found: C, 22.95; H, 4.7; P, 8.5. $C_7H_{10}Li_3O_{10}P\cdot 3.5H_2O$ requires C, 22.76; H, 4.46; P, 8.40%).

Benzyl 4,6-O-*Benzylidene*-2-*deoxy*-3-O-(2,3,4,6-*tetra*-O-*acetyl*-β-D-*glucopyranosyl*)-α-D-arabino-*hexapyranoside* (5).—A solution of compound (1) (4 g) in freshly distilled (P_2O_5) chloroform (150 ml) was stirred with mercury(II) cyanide (4 g) and molecular sieve 3A (3 g) under reflux for 1 h, and 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (8 g) was added. Refluxing and stirring were continued for 24 h, the condensation being monitored by t.l.c. (ethyl acetate–hexane, 2 : 3 v/v). The mixture was cooled and filtered and the filtrate was washed successively with ice-cold water, cold saturated aqueous sodium hydrogen carbonate, and ice–water, and dried (Na_2SO_4). Chloroform was removed under reduced pressure and the residue (7 g) was crystallised from ethyl acetate–light petroleum to afford the disaccharide (5), m.p. 130 °C; $[\alpha]_D^{20}$ +42° (*c* 1, $CHCl_3$); δ_C ($CDCl_3$) 170.89, 170.02, and 169.04 (C=O), 137.26 and 137.00 (quaternary aromatics) 128.85, 128.31, 127.97, and 125.85 (*o*, *m*, *p* aromatic), 101.22 and 100.35 [Ph*C*H(-O)O and C-1′], 96.78 (C-1), 81.73 (C-4), 74.48, 72.70, and 71.67 (C-2′, C-3′, C-5′, and C-3), 69.09 and 68.75 (PhCH_2 and C-6), 67.87 (C-4′), 63.03 (C-5), 61.45 (C-6′), 36.01 (C-2), and 20.34 p.p.m. (CH_3) (Found: C, 60.45; H, 6.05. $C_{34}H_{39}O_{14}$ requires C, 60.80; H, 5.81%).

Benzyl 4,6-O-*Benzylidene*-2-*deoxy*-3-O-(β-D-*glucopyranosyl*)-α-D-arabino-*hexopyranoside* (6).—The tetra-acetate (5) (6 g) was dissolved in anhydrous methanol (250 ml) containing sodium (80 mg). The solution was cooled in ice–water and the course of deacetylation was followed by t.l.c. (ethyl acetate–hexane, 2 : 3 v/v). During the reaction part of the title compound crystallised out of solution. At the end of the reaction the product was filtered off and the filtrate was decationised with Amberlite IR 120 (H^+-form) resin and concentrated until the rest of the product started to crystallise; recrystallisation from butan-2-one gave the *disaccharide* (6), m.p. 177 °C; $[\alpha]_D^{20}$ +55° (*c* 1, butan-2-one) (Found: C, 61.95; H, 6.3. $C_{26}H_{32}O_{10}$ requires C, 61.90; H, 6.34%).

Benzyl 2-*Deoxy*-3-O-(β-D-*glucopyranosyl*)-α-D-arabino-*hexopyranoside* (7).—Compound (6) (0.4 g) was dissolved in a mixture of water (5 ml), methanol (5 ml), and glacial acetic acid (1 ml) and the mixture was heated at 60 °C. The hydrolysis was monitored by t.l.c. (butan-1-ol–propan-2-ol-water, 5 : 3 : 1 v/v/v). When the reaction had finished, the solution was neutralised with Amberlite IR 45 (HO^--form) resin and evaporated to dryness to afford the *title compound* (7) which, after crystallisation from methanol–ethyl acetate, and recrystallisation from methanol–butan-2-one, had m.p. 98—100 °C; $[\alpha]_D^{20}$ +57° (*c* 1, MeOH) (Found: C, 52.3; H, 6.75. $C_{19}H_{28}O_{10}\cdot H_2O$ requires C, 52.53; H, 6.90%).

2-*Deoxy*-3-O-(β-D-*glucopyranosyl*)-α-D-arabino-*hexopyranose* (8).—Compound (7) (150 mg) in ethanol (10 ml) was hydrogenated over 10% palladium–charcoal for 48 h, the reaction being monitored by t.l.c. (butan-1-ol–propan-2-ol–water, 5 : 3 : 1 v/v/v). The catalyst was filtered off and the filtrate was evaporated to dryness to afford the *disaccharide* (8), m.p. 134 °C (methanol–ethanol–acetone); $[\alpha]_D^{20}$ −6° (equilibrium, 35 min) (*c* 1, H_2O) (Found: C, 43.65; H, 6.95. $C_{12}H_{22}O_{10}\cdot 0.25\ H_2O$ requires C, 43.57; H, 6.80%).

1,2-O-*Isopropylidene*-3-O-*methyl*-6-O-*triphenylmethyl*-D-*glucofuranose* (9).—A solution of triphenylmethyl chloride (86 g) in anhydrous pyridine (250 ml) was added dropwise with stirring to a solution of 1,2-*O*-isopropylidene-3-*O*-methyl-D-glucofuranose [11] and the mixture was left at room temperature for 3 d. After the usual work-up, the syrupy residue was crystallised, contaminated with triphenylmethanol, from ethanol. A sample, purified by p.l.c. (benzene), gave the *furanose* (9) as a foam having $[\alpha]_D^{20}$ −26.5° (*c* 1, $CHCl_3$) (Found: C, 72.9; H, 6.65. $C_{29}H_{32}O_6$ requires C, 73.11; H, 6.72%).

5-O-*Benzyl*-1,2-O-*isopropylidene*-3-O-*methyl*-6-O-*triphenylmethyl*-D-*glucofuranose* (10).—A solution of crude furanose (9) (40 g) in dimethylformamide (200 ml) was stirred for 2 h with sodium hydride (50% suspension in oil) (20 g) previously washed with hexane, and benzyl bromide (25 ml) was then added dropwise to the mixture which was kept at room temperature overnight. After the usual work-up, the residual oil crystallised slowly from ethanol. The mother liquors were purified by column chromatography on

alumina (chloroform–hexane, 1 : 1 v/v as eluant) to afford the *title compound* (10), m.p. 100 °C; $[\alpha]_D^{21}$ −36° (*c* 1, $CHCl_3$) (Found: C, 76.0; H, 6.85. $C_{36}H_{38}O_6$ requires C, 76.3; H, 6.71%).

5-O-*Benzyl*-3-O-*methyl*-D-*glucofuranose* (11).—A solution of the fully protected furanose (10) (5 g) in a mixture of dioxan (80 ml), water (10 ml), and 0.5M H_2SO_4 (10 ml) was heated for 2.5 h in an oil-bath at 100 °C, then cooled, neutralised with dry Amberlite IR 45 (HO^--form) resin, filtered, and the filtrate was evaporated to dryness. The residue was purified by h.p.l.c. (ethyl acetate) to give the *title compound* (11) (0.85 g, 37%) as crystals from benzene, m.p. 69—71 °C; $[\alpha]_D^{20}$ −77° (*c* 1 ,EtOH) (Found: C, 58.8; H, 7.1. $C_{14}H_{20}O_6$ requires C, 59.15; H, 7.04%).

5-O-*Benzyl*-3-*deoxy*-D-erythro-*hexonic Acid* (12). A solution of the furanose (11) (2.47 g) in baryta (240 ml) was kept at room temperature under nitrogen for 3 d, then neutralised with Amberlite IR 120 (H^+-form) resin and the mixture was concentrated to small volume and passed through a column (15 × 2 cm) of Amberlite IR 120 (cyclohexylammonium-form) resin. The eluant was evaporated to dryness and the residue was crystallised from ethanol–ether to give the *cyclohexylammonium salt* of the acid (12) (1.47 g, 44%), m.p. 150—155 °C; $[\alpha]_D^{20}$ −6.2° (*c* 1, H_2O) (Found: C, 61.7; H, 8.65; N, 3.95. $C_{19}H_{31}NO_6$ requires C, 61.79; H, 8.40; N, 3.79%).

Methyl (*Methyl*-5-O-*benzyl*-3-*deoxy*-β-D-erythro-2-*hexulopyranosid*)*onate* (13).—To a solution of the hexonic acid (12) (3.16 g) in water (40 ml) containing potassium perchlorate (0.47 g) was added freshly prepared Sprinson oxidation catalyst [7] (12.5 ml). The pH of the mixture was adjusted to 4.7 with either pyridine or dilute hydrochloric acid as appropriate and the mixture was then stirred at room temperature for 24 h and passed through a column (48 × 4 cm) of Amberlite IR 120 (H^+-form) resin. The eluant was neutralised with solid calcium oxide, filtered, and the filtrate was evaporated to dryness. The residue was dissolved in anhydrous methanol (250 ml) and was stirred at 60 °C with dry Amberlite IR 120 (H^+-form) resin for 24 h. The resin was filtered off and the filtrate was evaporated to dryness. The residue was purified by h.p.l.c. (benzene–ethyl acetate, 1 : 1 v/v) to give the *title compound* (1 g, 40%), $[\alpha]_D^{20}$ −101° (*c* 1, $CHCl_3$); δ_H ($CDCl_3$) 1.94 (1 H, t, $J_{3ax,3eq}$ 10 Hz, 3_{ax}-H), 2.16 (1 H, q, $J_{3ax,3eq}$ 10 Hz, 3_{eq}-H), 3.23 (3 H, s, OMe), 3.62 (2 H, m, 5- and 6′-H), 3.8 (3 H, s, CO_2Me), 4.0 (1 H, m, 4-H), 4.16 (1 H, q, $J_{6,6'}$ 13, $J_{6,5}$ 2.5 Hz, 6-H), 4.5 and 4.8 (2 H, dd, CH_2Ph), and 7.3 (6 H, m, Ph) (Found: C, 60.95; H, 6.9. $C_{15}H_{20}O_6$ requires C, 60.81; H, 6.75%).

The derived crystalline 4-*O*-*p*-nitrobenzoate had m.p. 78—79 °C (EtOH); $[\alpha]_D^{20}$ −86.5° (*c* 0.478, $CHCl_3$) (Found: C, 59.30; H, 5.30; N, 3.30. $C_{22}H_{23}NO_9$ requires C, 59.32; H, 5.17; N, 3.15%).

Methyl [*Methyl*-5-O-*benzyl*-3-*deoxy*-4-O-(2,3,4,6-*tetra*-O-*acetyl*-β-D-*glucopyranosyl*)-β-D-erythro-*hexulopyranosid*]-*onate* (14).—2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl bromide (1.11 g) was added to a solution of the compound (13) (0.8 g) in dry dichloromethane (10 ml) which had previously been stirred with mercury(II) cyanide (0.8 g) and molecular sieve 3A (1 g) at 50 °C for 2 h. Heating and stirring were continued and the condensation was monitored by t.l.c. (ethyl acetate–hexane, 3 : 2 v/v). After 20 h, further ' acetobromoglucose ' (0.5 g) and mercury(II) cyanide (0.5 g) were added and the reaction was allowed to proceed for a further 24 h. The mixture was cooled, diluted with dichloromethane, and filtered. The filtrate was washed successively with ice–water, cold saturated aqueous sodium hydrogen carbonate, and ice–water, and dried (Na_2SO_4). The solvent was removed under reduced pressure and the residue (2.1 g) was purified by h.p.l.c. (ethyl acetate–hexane, 1 : 1 v/v) to give the β-*disaccharide* (14) (0.612 g), which crystallised from ethyl acetate–hexane, m.p. 136—137 °C; $[\alpha]_D^{20}$ −63.5° (*c* 1.015, $CHCl_3$); δ_C ($CDCl_3$) 20.4 (CH_3CO), 34.4 (C-3), 50.9 (OCH_3), 62.0 and 62.4 (C-6 and C-6′), 68.5 (C-4′), 71.4, 72.8, and 73.9 (C-2′, C-3′, C-5′, and C-4), 99.1 and 99.4 (C-2 and C-1′), 126.9, 127.5, and 128.3 (aromatic), 138.4 (quaternary aromatic), and 168.3, 169.0, 169.2, 170.1, and 170.5 p.p.m. (C=O of acetates and acid); δ_H ($CDCl_3$) 1.93, 1.992, 2.02, and 2.09 (12 H, 4 s, CH_3CO), 2.23 (1 H, q, $J_{3eq,3ax}$ 13.5, $J_{3eq,4}$ 6 Hz, 3_{eq}-H), 2.30 (1 H, t, $J_{3eq,3ax}$ 13.5, $J_{3ax,4}$ 13.5 Hz, 3_{ax}-H), 3.21 (3 H, s, 1-OCH_3), 3.62 (3 H ,m, 5-, 5′-, and 6-H), 3.79 (3 H, s, CO_2CH_3), 4.06 (2 H, m, 6′-H_a and 6′-H_b, J(6′$_a$,6′$_b$) 12, J(6′$_b$,5′) 6, J (6′$_a$,5′) 3 Hz, 6′$_b$$H_2$), 4.22 (2 H, m, 4- and 6-H), 4.63 (1 H, d, $J_{1,2'}$ 8 Hz, 1′-H), 4.66 (2 H, s, CH_2Ph), 5.00 (1 H, t, $J_{2',1'} = J_{2',3'}$ 8 Hz, 2′-H), 5.04 (1 H, t, $J_{3',4'} = J_{4',5'}$ 10 Hz, 4′-H), 5.17 (1 H, t, 3′-H), and 7.35 (5 H, m, Ph) (Found: C, 55.5; H, 6.15. $C_{29}H_{38}O_{15}$ requires C, 55.58, H, 6.07%).

Ammonium [*Methyl*-3-*deoxy*-4-O-(β-D-*glucopyranosyl*)-β-D-erythro-2-*hexulopyranosid*]*onate* (15).—The tetra-acetate (14) (77 mg, 0.123 mmol) in methanol (2 ml) was hydrogenated over 10% palladium–charcoal. T.l.c. (ethyl acetate–hexane, 3 : 2 v/v) showed the reaction to be complete after 2 h. The catalyst was filtered off and the filtrate was evaporated to dryness. The residue was dried (P_2O_5) and dissolved in anhydrous methanol (1 ml). Freshly prepared 0.5M methanolic sodium methoxide (0.05 ml, 0.025 mmol) was added and after 1 h the solution was evaporated to dryness, the residue was dissolved in 0.1M aqueous sodium hydroxide (1.1 ml, 0.11 mmol), and the mixture was left for 2 d at room temperature after which paper electrophoresis showed the saponification to be complete (*R* 0.86). The solution was passed through a small column of Amberlite IR 120 (NH_4^+-form) resin and the eluant and aqueous washings were concentrated to small volume. Upon addition of ethanol, the *title compound* crystallised out, m.p. 166—167 °C; $[\alpha]_D^{20}$ −77° (*c* 0.47, H_2O) (Found: C, 41.95; H, 6.65; N, 3.8. $C_{13}H_{25}NO_{11}$ requires C, 42.05; H, 6.74; N, 3.77%).

Methyl 5-O-*Benzyl*-3-*deoxy*-β-D-erythro-2-*hexulopyranosidonic Acid* 4-*Phosphate* (16).—Compound (13) (4 mmol) was phosphorylated with pyridinium cyanoethyl phosphate [6] as described for compound (1). After treatment with 1M aqueous sodium hydroxide the solution was cooled in ice and the pH was adjusted to 7 with Amberlite IR 120 (H^+-form) resin. This solution was passed through a column of Amberlite IR 120 (pyridinium-form) resin, the eluant was concentrated to *ca.* 10 ml, and saturated aqueous barium hydroxide was added until the mixture was at pH 10. The precipitated inorganic phosphate was removed by centrifugation, the supernatant liquid was concentrated to *ca.* 3 ml, and acetone was added. The resulting precipitate (1.1 g) was recovered by centrifugation, then washed with acetone, and dried. On electrophoresis it showed one major phosphate-containing spot (*R* 1.40). A sample (150 mg) was converted into the ammonium salt of the acid (16) with Amberlite IR 120 (NH_4^+-form) resin and purified by preparative descending chromatography (propan-2-ol–ammonium hydroxide–water, 7 : 1 : 2 v/v/v) on Whatman 3MM paper previously washed with the solvent and then with water. The ammonium salt (63 mg), recovered by elution of the

appropriate area of paper with water, was transformed into the *barium salt* which was precipitated from aqueous solution with ethanol, centrifuged off, washed with ethanol, and dried. It had $[\alpha]_D^{20}$ −48.5° (*c* 0.57, H_2O) (Found: C, 22.7; H, 3.3; P, 5.0. $C_{14}H_{16}Ba_{1.5}O_9P\cdot 3H_2O$ requires C, 22.63; H, 3.56; P, 5.01%).

Methyl 3-*Deoxy*-β-D-erythro-2-*hexulopyranosidonic Acid* 4-*Phosphate* (17).—A solution of compound (16) (215 mg) in water (5 ml) was passed through a column of Amberlite IR 120 (cyclohexylammonium-form) resin and the eluant and aqueous washings were evaporated to dryness; two cycles of dissolution in ethanol, filtration, and evaporation gave a residue which was dissolved in methanol (3 ml) and hydrogenated over 10% palladium–charcoal. The catalyst was filtered off and the filtrate was concentrated to small volume whence the *tris*(*cyclohexylammonium*) *salt* of the title acid crystallised out and was homogeneous by paper electrophoresis (*R* 1.76), m.p. 174—177 °C (decomp.); $[\alpha]_D^{20}$ −29° (*c* 0.47, H_2O) (Found: C, 49.35; H, 9.0; N, 6.5; P, 5.2. $C_{25}H_{52}N_3O_9P\cdot 2H_2O$ requires C, 49.58; H, 9.26; N, 6.94; P, 5.12%).

Methyl (*Methyl* 3-*deoxy*-β-D-erythro-2-*hexulopyranosid*)-*onate* 4-*Phosphate* (18).—The acid (17) (25 mg) in anhydrous methanol (5 ml) was stirred for 24 h with dry Amberlite IR 120 (H^+-form) resin. The resin was filtered off and washed (methanol), the filtrate and washings were neutralised with ammonium hydroxide, and evaporated to dryness. The residue was dissolved in water (1 drop) and the *title compound* was precipitated with ethanol. The product was homogeneous by electrophoresis (*R* 1.1) (Found: C, 30.2; H, 6.3. $C_8H_{21}N_2O_9P$ requires C, 30.00; H, 6.56%).

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