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THE SYNTHESIS OF P^1-(2-ACETAMIDO-2-DEOXY-α-D-GLUCOPYRANOSYL) P^2-FICAPRENYL PYROPHOSPHATE*†

CHRISTOPHER D. WARREN, YUKIKO KONAMI§, AND ROGER W. JEANLOZ‡

Laboratory for Carbohydrate Research, Departments of Biological Chemistry and Medicine, Harvard Medical School and Massachusetts General Hospital, Boston, Massachusetts 02114 (U. S. A.)

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ABSTRACT

2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α,β-D-glucopyranosylammonium phosphate was prepared by the action of crystalline phosphoric acid on 2-acetamido-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose. The α-D anomer (3) was the main product, and was isolated pure by preparative thin-layer chromatography or by removal of the β-D anomer (6) by selective acid hydrolysis. Ficaprenyl phosphate was prepared from ficaprenol, obtained as an isomeric mixture (mainly C_{55}) from an extract of *Ficus elastica*. Compound 3 was converted into the free acid and then into the tributylammonium salt, which was treated with P^1-diphenyl P^2-ficaprenyl pyrophosphate to give the acetylated pyrophosphate diester 8, characterized by analytical, spectral, and hydrogenolytic studies. Deacetylation of 8 gave the synthetic "lipid intermediate", P^1-(2-acetamido-2-deoxy-D-glucopyranosyl) P^2-ficaprenyl pyrophosphate (9), the properties of which were compared with those of natural substances considered to be active in the biosynthesis of teichoic acids.

INTRODUCTION

There is now strong evidence for the involvement of "lipid intermediates" in the biosynthesis of a wide variety of complex polysaccharides[2,3]. These substances, in which a polyisoprenoid alcohol is linked to a carbohydrate residue through a mono- or pyrophosphate bridge, are difficult to isolate owing to their high reactivity and their occurrence in small amounts. We have previously synthesized a derivative of D-

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†Dedicated to Professor O. Westphal, in honor of his 60th birthday.

§Present address: Division of Chemical Toxicology and Immunochemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan.

‡To whom communications should be addressed.

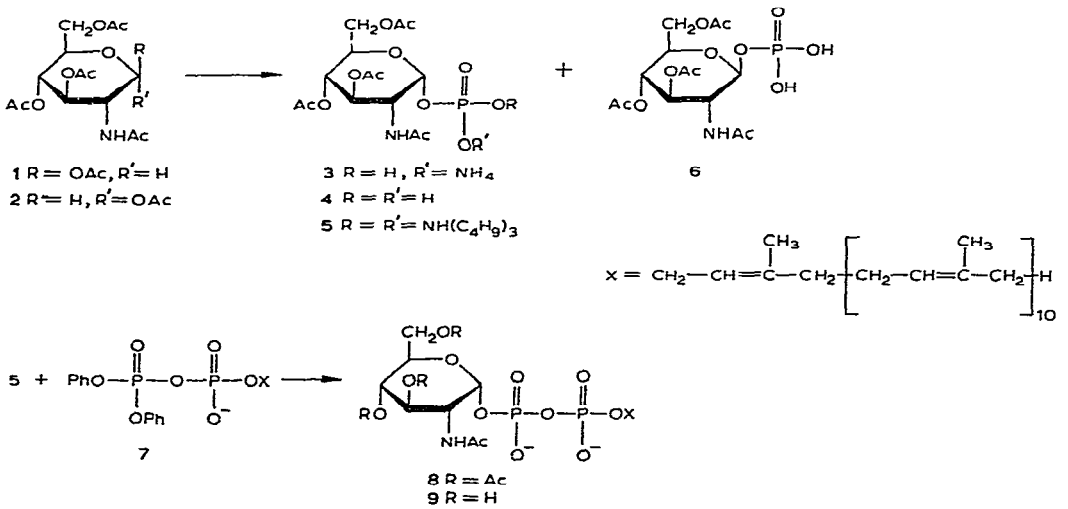
galactose[4,5] that was active in a system for the biosynthesis of Salmonella "O antigen"[6] and have discussed in detail the reasons for the importance of a chemical synthesis of these substances[4,5].

In the present paper, we describe a method for the preparation of fully acetylated 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate, and its utilization as starting material for the synthesis of a "lipid intermediate" containing a 2-acetamido-2-deoxy-α-D-glucopyranosyl residue and ficaprenol as the isoprenoid lipid.

RESULTS AND DISCUSSION

Several groups of workers have reported the use of the MacDonald procedure[7,8] for the preparation of 2-acetamido-2-deoxy-D-glycopyranosyl phosphates. In all reported examples, intermediate acetylated phosphates were not isolated but were saponified directly to yield either the α-D-glycosyl phosphate[9–11] or a mixture of anomeric glycosyl phosphates, with the β-D anomer predominating, regardless of the anomeric configuration of the starting acetate or of the duration of the reaction[12,13]. In this instance, lengthy and cumbersome chromatography on ion-exchange resins was necessary to obtain the pure α-D anomer. In the preparation of 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate, we were unable to repeat the separation according to O'Brien[12].

In the synthesis of P^1-α-D-galactopyranosyl P^2-ficaprenyl pyrophosphate[5], we prepared a crystalline, fully acetylated α-D-galactopyranosyl phosphate by a modification of the MacDonald procedure. When this procedure was repeated with 2-acetamido-2-deoxy-D-glucose, and the intermediate acetates **4** and **6** (ammonium salts) were isolated from the reaction of 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-α- or -β-D-glucopyranose (**2** or **1**, respectively) with crystalline phosphoric acid, the α-D anomer (**3**) was the main product, as shown by t.l.c. (by which the anomers can be



separated) and by optical rotation. The proportion of α to β anomer was independent of the anomeric configuration of the starting acetate and of the duration of the reaction, but varied somewhat from one preparation to another. Also the yield was much greater (by five times) when the β-D-pentaacetate (**1**) was used as the starting material instead of the α-D-pentaacetate (**2**). This result is in contrast to that reported by O'Brien[12] whose yield of phosphorylated product was independent of the anomeric configuration of the starting acetate.

According to MacDonald[8], (*a*) the yield of the acid-labile β-D-glycosyl phosphate should be greater with a short reaction-time and (*b*), the effect of acidic conditions during the isolation or during the fractionation procedures may cause a loss of the β-D anomer. Consequently, the α-D anomer is often quoted as being the main or only reaction product. Surprisingly, exposure to acid conditions during our isolation procedure did not affect our product in any way and, as a good yield (43–53%) of phosphorylated material was obtained, it seems unlikely that extensive hydrolysis had occurred during the neutralization of the reaction mixture. In addition, the total yield was also the highest in those preparations having the highest optical rotation, which is the opposite result to that which would be expected from a selective hydrolysis of the β-D anomer during the isolation.

By crystallization from ether–methanol, a small quantity of a mixture of mono- and di-ammonium salts of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl phosphate (**6**) could be obtained from some preparations, and this compound as well as pure 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl ammonium hydrogen phosphate (**3**) could be obtained by preparative t.l.c. of the crude reaction-product. However, a more convenient way of obtaining the pure α-D anomer (**3**) for subsequent synthetic purposes was selective, acid-catalyzed hydrolysis of the equatorial β-D derivative **6** at room temperature, followed by acetylation with acetic anhydride and pyridine, and removal of 2-acetamido-2-deoxy-D-glucose tetraacetate by extraction with chloroform from an aqueous solution. Previous workers[13] encountered difficulty in the application of this method. Confirmation of the structure of **3** was obtained by conversion into the known 2-acetamido-2-deoxy-α-D-glucopyranosyl dipotassium phosphate. The n.m.r. spectra of the α-D (**3**) and β-D (**6**) anomers as disodium salt in deuterium oxide solution did not differentiate them, owing to interference from the signals of other protons, notably H-3, and from HDO. Both spectra showed a group of 4 strong signals from acetyl-methyl protons, including a signal from the *N*-acetyl methyl group, which also appeared in the spectrum of the pyrophosphate diester **9**. The position of this peak agrees with the results of Onodera and Hirano[14] for an equatorial *N*-acetyl group [$^4C_1$(D) conformation]. Both spectra also showed a symmetrical triplet of peaks resulting most probably from H-3 of the pyranose ring. In the spectrum of the α-D anomer **3**, the central peak of the triplet was much larger than the others and showed signs of further splitting. It was apparently superimposed on another resonance, probably that of H-1 (part of the twin doublet arising from the coupling of H-1 with H-2 and the phosphorus atom). From the work of Raschke and Ballou[15], who observed the α-D and β-D anomeric

signals for the 2-acetamido-2-deoxy-D-glucopyranosyl residues of chitotetraose, it appears likely that the signal from H-1 in compound **6** was obscured by the HDO peak. The 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl phosphate, as the free acid **4**, was suitable for synthetic operations without purification by ion-exchange chromatography.

Ficaprenol was prepared as described previously[5]. In order to determine whether additional purification by preparative t.l.c. was desirable for synthetic purposes, a sample was purified in this way and then hydrogenated over a palladium-on-charcoal catalyst. It was compared with unpurified ficaprenol (which was also hydrogenated) by g.l.c. (Fig. 1) and mass spectrometry .The two samples were almost identical, both containing two contaminants as well as the expected mixture of reduced polyprenols, and consequently, the preparative t.l.c. step was not considered to be advantageous at this stage.

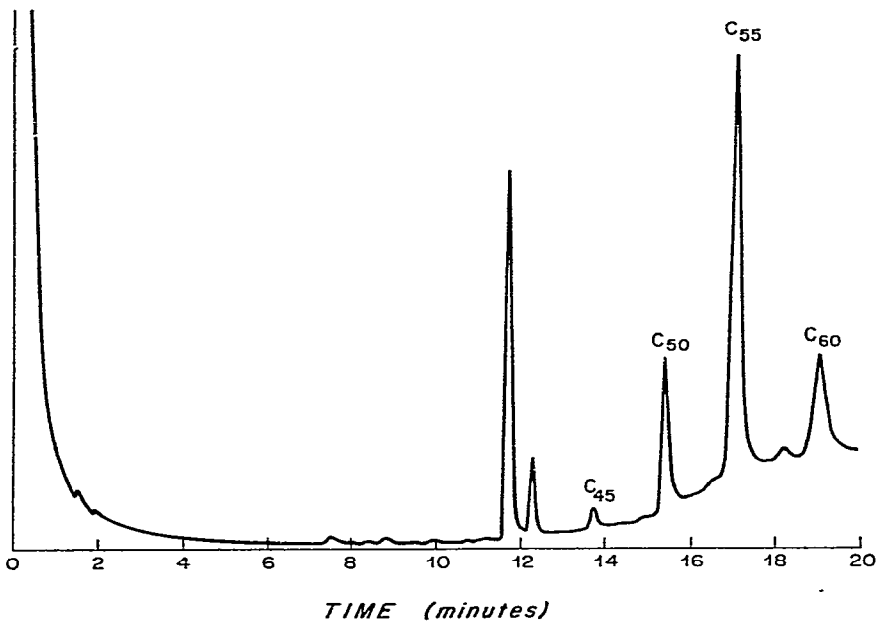


Fig. 1. G.l.c. of a preparation of ficaprenol after catalytic hydrogenation and trimethylsilylation; column temperature-programmed to increase at 12° per min from 125° to 350°.

Ficaprenol (crude) was converted into ficaprenyl phosphate. A small sample, purified by preparative t.l.c.[5], was hydrogenolyzed in the presence of palladium and platinum catalyst. G.l.c. and mass spectrometry showed that scission of the allylic phosphate bond was complete in both cases to give a mixture of isomeric hydrocarbon products, with a preponderance of the C_{55} compounds. This result also confirmed that the contaminants present in the crude ficaprenol preparation were eliminated during the phosphorylation. Specially prepared catalysts did not appear to be necessary for the hydrogenolysis reaction, although such have been used by other workers[16–18].

It is important to note that examination of the mass spectrum of the saturated hydrocarbon released by hydrogenolysis readily identified the product as a reduced polyprenol (Fig. 2); the loss of unsaturation might have been expected to yield a random fragmentation and an uninterpretable spectrum. Therefore, catalytic hydrogenation of allylic phosphate derivatives, such as those isoprenoid lipid intermediates that contain a terminal, unsaturated residue in the lipid, can produce readily identifiable lipid moieties. The procedure is thus a useful diagnostic tool, but no information on the nature or position of the double bonds can be obtained. The only other method for releasing the lipid from such a compound is by mild, acid hydrolysis, which leads to extensive decomposition and rearrangement[19,20].

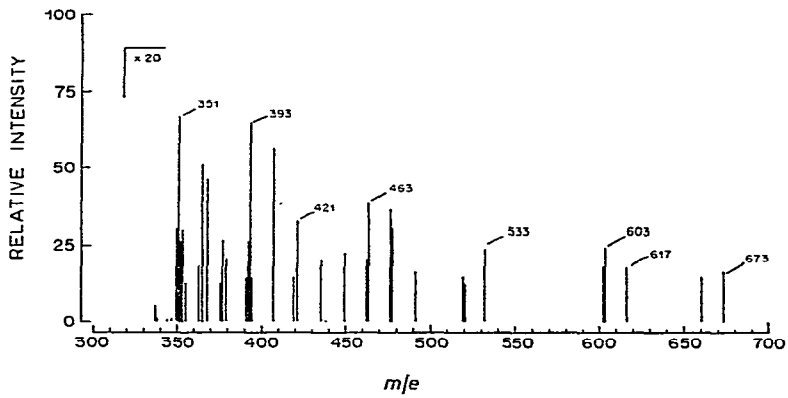


Fig. 2. Mass spectrum of the hexane-soluble products of hydrogenolysis of ficaprenyl phosphate.

Conversion of **4** into the bis(tributylammonium) form (**5**) was followed by reaction with P^1-ficaprenyl P^2-diphenyl pyrophosphate (**7**), prepared, with minor modifications, by the method previously described. The resulting pyrophosphate diester **8** was purified by column and thin-layer chromatography and deacetylated with sodium methoxide in methanol to yield P^1-(2-acetamido-2-deoxy-α-D-glucopyranosyl) P^2-ficaprenyl pyrophosphate (**9**). As expected, this compound was stable in the presence of dilute base, in contrast to the D-galactose derivative[5], and so, an excess of reagent could be used. When examined by t.l.c. on silica gel, the compound exhibited a distinct mobility in four different solvent-systems, and appeared in each case as a single spot when sprayed with reagents specific for (*a*) isoprenoid lipids, (*b*) phosphate groups, and (*c*) unsaturated hydrocarbons. However, the compound was very unstable and decomposed rapidly on exposure to air at room temperature. The decomposition products were examined by t.l.c. on silica gel and cellulose, and included substances having the mobilities of 2-acetamido-2-deoxy-D-glucose and inorganic pyrophosphate, together with highly hydrophobic products derived from the lipid moiety. This instability made determination of the physical constants of the synthetic "lipid intermediate" very difficult, and full characterization was performed with the relatively stable, acetylated compound **8**. I.r. and n.m.r. spectra were in

agreement with the expected pyrophosphate diester structure, and values for phosphate and 2-amino-2-deoxyglucose analysis were also satisfactory, showing a molar ratio of 2:1. Catalytic hydrogenolysis was performed under conditions expected to cause scission of the allylic phosphate linkage[16,19], except that commercially available catalysts were used. In related work[21], P^1-D-galactopyranosyl P^2-ficaprenyl pyrophosphate[4] has been hydrogenolyzed with these catalysts, and g.l.c. and mass spectrometry have shown that scission of the allylic phosphate bond was readily achieved under these conditions. Hydrogenolysis gave a phosphorylated 2-amino-2-deoxy-D-glucose derivative that differed from 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl phosphate (**4**). After deacetylation, this compound was examined by t.l.c. on cellulose and by paper chromatography, and although it gave a color reaction typical of a pyrophosphate, the solvent system used by previous workers[22] did not, in our hands, separate it from 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate. The product was therefore compared with the latter compound by paper electrophoresis, and by this technique the two compounds were readily separated. From these results and from the method of formation, it was concluded that the hydrogenolysis product was 2-acetamido-2-deoxy-α-D-glucopyranosyl pyrophosphate.

"Lipid intermediates" containing a 2-acetamido-2-deoxy-D-glucopyranosyl residue linked by a pyrophosphate bridge to an isoprenoid lipid have been identified in systems for the biosynthesis of teichoic acids in *Staphylococcus lactis*[22–24]. Although the amounts of material isolated from these systems were too small for complete characterization of the lipid, it was probably the same as that involved in peptidoglycan biosynthesis[25,26], as the teichoic acid- and peptidoglycan-synthesizing systems were found to compete for the same compound[27]. In this instance, the lipid would be a C_{55} alcohol that differs from ficaprenol only by having one extra double bond, in the *cis* configuration. T.l.c. of the glycolipid isolated by Hussey and Baddiley[24] showed that it had a mobility similar to that of the synthetic product **9**. Tetas *et al.*[28] and Molnar *et al.*[29] have shown that 2-acetamido-2-deoxy-D-glucose is probably transferred by microsomal fractions from rat and rabbit liver, from uridine 5′-(2-acetamido-2-deoxy-D-glucosyl pyrophosphate) into some glycoproteins *via* a "lipid intermediate" that contains a pyrophosphate bridge. Some of the experiments used to establish the nature of the intermediate were similar to those performed by Brooks and Baddiley[22]. Although the lipid was not identified, polyisoprenoid alcohols have recently been implicated as carbohydrate acceptors in a variety of biosynthetic systems in animal tissues[30–36].

The synthetic compound **9** was treated under conditions similar to those used to determine the structures of these "lipid intermediates", which involve dilute acid hydrolysis in aqueous methanol ("methanolysis"). However, it was necessary to add some chloroform to the reaction mixture to overcome the insolubility of **9** in aqueous methanol (a property expected for long-chain isoprenoid derivatives). The hydrolysis products, mainly identified by comparison with standards on silica gel and cellulose t.l.c., were 2-acetamido-2-deoxy-D-glucose, inorganic pyrophosphate, and a com-

pound having the mobility of methyl 2-acetamido-2-deoxy-β-D-glucopyranoside; this compound, and 2-acetamido-2-deoxy-D-glucose, were also the products of a parallel hydrolysis of 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate. A fourth product of the "methanolysis" of **2** had the same properties as the product of hydrogenolysis of **9**, presumably 2-acetamido-2-deoxy-D-glucopyranosyl pyrophosphate; however, Brooks and Baddiley[22] reported a different mobility for the latter compound. A product having this lower mobility was detected on our chromatograms, but it was not carbohydrate-containing, and a spot in the same position could readily be obtained by acid treatment of sodium pyrophosphate. The hexane extract of the hydrolysis products contained the expected rearrangement- and decomposition-products derived from the lipid moiety, together with a small proportion of ficaprenol.

Column chromatography on DEAE-cellulose has been used by many workers, including Brooks and Baddiley[22] and Molnar *et al.*[29] for the purification and identification of "lipid intermediates". As a model for this procedure, we have examined the mobility of the synthetic compound **9** on thin layers of DEAE-cellulose (AcO^-), with increasing concentrations of ammonium acetate in methanol as the eluting solvent. The expected, linear relationship between R_F and salt concentration was confirmed (Fig. 3), and the optimal separation from impurities occurred at a buffer strength of 0.25–0.3M. The previous investigators[22,29] reported a concentration of 0.21–0.25M ammonium acetate in methanol for column elution of the "lipid intermediate".

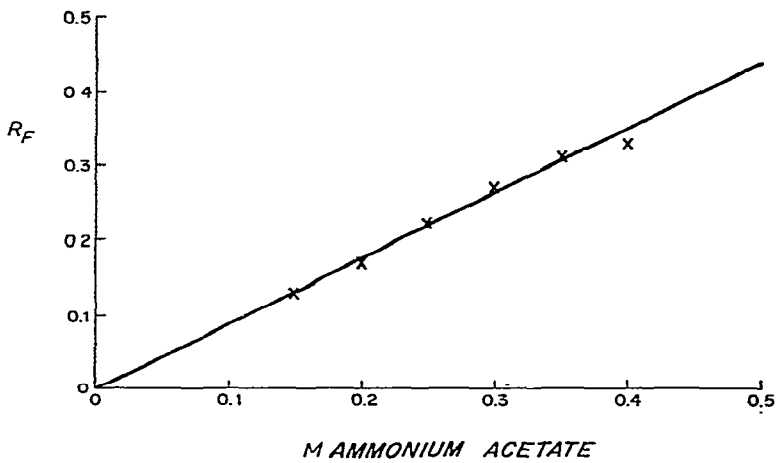


Fig. 3. Relationship of the mobility of compound **9** on thin layers of DEAE-cellulose acetate to the concentration of ammonium acetate in the eluent.

The synthetic product **9** is being tested for its ability to act as an acceptor for D-mannose from guanosine 5'-(α-D-mannopyranosyl pyrophosphate), and for D-glucose from uridine 5'-(α-D-glucopyranosyl pyrophosphate), in a system employing a microsomal fraction obtained from liver[37]. It is hoped also to test **9** for its ability to function as a lipid intermediate in the biosynthesis of a teichoic acid in *Staphylococcus lactis*.

EXPERIMENTAL

General methods. — Melting points were determined on a Mettler FP2 hot-stage equipped with a microscope, and correspond to "corrected melting points". Optical rotations were determined in 1-dm semimicro tubes with a Perkin–Elmer No. 141 polarimeter. I.r. spectra were recorded with a Perkin–Elmer spectrophotometer, Model 237. Absorbancy at 820 nm was measured with a Zeiss Spectrophotometer, Model PMQ 11. N.m.r. spectra were recorded at 100 MHz with a Perkin–Elmer R-20 spectrometer, with deuterium oxide as solvent and sodium 4,4-dimethyl-4-silapentane-1-sulfonate as internal standard, or with chloroform-*d* as solvent, containing 1% of tetramethylsilane as internal standard (Silanor-C, MSD Isotopic Products, Montreal, Canada). Mass spectra were recorded with a single-focusing, magnetic-scanning, mass spectrometer (Hitachi–Perkin–Elmer RMU-6) on line with an IBM-1800 computer. The cation-exchange resin used was AG-50W X8 (200–400 mesh, Bio-Rad Laboratories, Richmond, Calif.); in all instances, the amount of resin used was in at least a twofold excess over the quantity necessary to effect complete ion-exchange. Evaporations were conducted *in vacuo* with a bath temperature kept below 30°. The microanalyses were performed by Dr. M. Manser, Zürich, Switzerland.

Chromatographic methods. — Silica gel column chromatography was performed on silica gel (0.05–0.2 mm, 70–325 mesh, E. Merck A.-G., Darmstadt, Germany; the material was used without pretreatment). Paper chromatography (ascending) was performed on Whatman No. 3MM paper. T.l.c. was performed on precoated plates of Silica Gel G (Merck) or Cellulose F (Merck); the plates supplied (20 × 20 cm) were cut to a length of 6 cm before use. Preparative t.l.c. was performed on precoated p.l.c. plates, Silica Gel F 254 (Merck). The spray reagent used, unless otherwise stated, was 1:1:18 anisaldehyde–sulfuric acid–ethanol[38], and the plates were heated to 125°. The spray reagent used to detect unsaturation was a 1% solution of potassium permanganate in 2% sodium carbonate[39]. The spray reagent used to detect phosphate groups was described by Dittmer and Lester[40]. The solvents for t.l.c. were (*A*) 60:25:4, (*B*) 60:35:6, and (*C*) 10:10:3 chloroform–methanol–water.

Gas–liquid chromatographic determination of 2-acetamido-2-deoxy-D-glucose was performed on a Perkin–Elmer Model 900 instrument, equipped with a flame-ionization detector, on a column of stainless steel (150 × 0.3 cm) packed with 0.1% OV-17 on Corning glass beads GLC-110 (120–140 mesh, Supelco, Inc., Bellefonte, Pa.), with the per(trimethylsilyl) ether of *myo*-inositol as internal standard. G.l.c. interfaced with the mass spectrometer was performed on the same model of gas chromatograph, on a column of stainless steel (150 × 0.3 cm) packed with 0.05% OV-17 on glass beads. The temperature of the column was programmed to increase from 125° to 350° at a rate of 12° per min. The samples were per(trimethylsilylated) with Tri-Sil (Pierce Chemical Co., Rockford, Ill.) in culture tubes (1.3 cm diameter × 10 cm) equipped with teflon-lined screw tops.

*2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α,β-*D*-glucopyranosyl ammonium hydrogen phosphate* (**3** *and* **6**). — (a) *From 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-β-*

D-*glucopyranose* (**1**). Compound **1** [1.0 g, prepared from 2-acetamido-2-deoxy-D-glucose (Pfanstiehl Laboratories, Inc., Waukegan, Ill.) according to Bergmann and Zervas[41]] was placed in the upper part of a Thunberg oxidation tube and, in the bottom part, there was placed crystalline phosphoric acid (1.2 g, Fluka A.-G. Buchs, S.G., Switzerland, dried overnight at room temperature *in vacuo* over magnesium perchlorate before use). The tube was evacuated (oil pump) and the phosphoric acid fused at 60°. Compound **1** was mixed into the melt, whereupon a vigorous evolution of acetic acid vapor took place. After 3 h at 65°, most of the gas evolution had ceased, and the brown, fused residue was dissolved in anhydrous tetrahydrofuran. The solution was cooled to −10°, and conc. (58%) ammonium hydroxide (*ca.* 1.5 ml) was added rapidly with vigorous stirring until the pH of the solution reached 5–6. The precipitate of ammonium phosphate was filtered off and washed with tetrahydrofuran at room temperature. The combined filtrate and washings were evaporated to give a syrupy residue. Examination by t.l.c. (silica gel) in solvent *B* showed two main products, 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl ammonium hydrogen phosphate (**3**) (R_F 0.24) and the β-D anomer **6** (R_F 0.13), and a smaller amount of contaminants including the starting material (R_F 0.9); the main product was the α-D anomer **3**. The two spots were not always well resolved, but on heating the t.l.c. plate, the less-mobile material always appeared well before the other product. Two similar spots were observed with a spray specific for phosphate ester groups.

The crude product was dissolved in water, and the solution was extracted with chloroform (six times) to remove the nonphosphorylated contaminants. After addition of a small amount of pyridine, the solution was evaporated. Toluene was added to the residue and evaporated three times from it to give a syrupy mixture of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α- and -β-D-glucopyranosyl ammonium hydrogen phosphates (**3** and **6**, respectively). The yield varied from 0.50 g (43%) to 0.61 g (53%), presumably according to the quality of the phosphoric acid, which had a marked tendency to deteriorate. Optical rotatory measurements indicated that the proportion of α-D and β-D anomers also varied, and those preparations having the highest rotation also gave the highest yields (Table I).

TABLE I

YIELD, OPTICAL ROTATION, AND RELATIVE PROPORTIONS OF **3** AND **6**

Preparation no.	*Yield* (%)	*Ratio of* **3** *to* **6**[a]	$[\alpha]_D^{22}$ (*degrees*)[b]
1	43	3:1	+30 (2)
2	48	4:1	+43 (1.6)
3	53	4.5:1	+47 (1)

[a]Estimated from silica gel t.l.c. in Solvent *B*. [b]In 1:1 (v/v) methanol–water; *c* in parentheses.

(b) *From 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-α-D-glucopyranose* (**2**). Treatment of **2** (1.0 g), prepared from 2-acetamido-2-deoxy-D-glucose according to

O'Brien[12], with phosphoric acid (1.2 g) was performed under conditions similar to those just described for the preparation from **1**. Examination of the product by t.l.c. in solvent *B* again showed two spots (R_F 0.24 and R_F 0.13). The α-D anomer (**3**) was the main product, and the proportions of α-D (**3**) to β-D (**6**) anomer appeared to the be same as in (a). The yield, however, was very low (0.11 g, 8.6%).

Effect of acidic conditions on the yield of compounds **3** *and* **6**. — In order to ascertain the effect of acidic conditions before neutralization, the tetrahydrofuran solution described in the preparation of **3** and **6** was divided into five parts (10 ml), and each part was treated with conc. (58%) ammonium hydroxide (0.3 ml) at 10-min intervals. After filtering off the precipitate of ammonium phosphate and washing with tetrahydrofuran, the combined filtrate and washings were evaporated. The residue was dissolved in water, and the solution extracted with chloroform (six times), and the aqueous solution was evaporated in the presence of pyridine. Toluene was added and evaporated off three times to give a syrup. In each example t.l.c. was performed with solvent *B* and the optical rotation was determined. The appearance of the t.l.c. plate, and the value of the optical rotation, were approximately the same for each preparation. Therefore, the results indicate that the proportions of **3** and **6** did not vary, and that the β-D anomer (**6**) was not being preferentially hydrolyzed by the acidic conditions of the isolation.

Separation of compounds **3** *and* **6** *by preparative t.l.c.* — (a) *As monoammonium salts.* The mixture of **3** and **6** (85 mg) was applied in conc. methanolic solution to a preparative t.l.c. plate (20 × 20 cm), which was developed in solvent *B*. The band containing the required material was revealed by spraying the edges of the plate with a spray specific for phosphate ester groups, and it was divided into two parts by analogy with the results of t.l.c. Each part was extracted by stirring with solvent *C* overnight at room temperature. After filtration through Celite, the residue was washed with the same solvent, and the combined filtrate and washings were evaporated to dryness. The residue was extracted with methanol, and the solution filtered and evaporated. From the upper part (75% of the total) a syrup was obtained (45 mg), t.l.c. (Solvent *B*): R_F 0.24, $[\alpha]_D^{20}$ +55° (*c* 0.9, 1:1 water–methanol). From the lower part of the band a syrup [3 mg; t.l.c. (Solvent *B*): R_F 0.13] was obtained, but in an amount insufficient for optical rotatory measurement.

(b) *As sodium salts.* The mixture of **3** and **6** was converted into the sodium salts by passage of an aqueous solution through a cation-exchange resin (Na^+ form). Evaporation gave a syrup (0.1 g), which was chromatographed in the same way as described in the preceding paragraph. In the first experiment, the upper half of the total band was removed from the plate to give a syrup (42 mg; t.l.c. (Solvent *B*): R_F 0.24, $[\alpha]_D^{20}$ +49° (*c* 1, 1:1 water–methanol); n.m.r. data (after drying throughly *in vacuo* over phosphorus pentaoxide and 3 additions and evaporations of deuterium oxide): δ 1.96, 2.07, and 2.10 (Me, OAc), 2.02 (Me, NAc), 4.25 (CH_2, H-6 of pyranose), 4.60 (HDO), 4.87 (pyranose-ring proton), 5.02, 5.14, and 5.27 (H-3 of pyranose, δ 5.14 peak consists of more than one signal), and 5.43 (pyranose-ring proton). When only the top one-fourth of the total band was removed, the syrup

obtained (20 mg) had the same chromatographic mobility; $[\alpha]_D^{20}$ +55° (*c* 1.1, 1:1 water–methanol).

Anal. Calc. for $C_{14}H_{21}NNaO_{12}P \cdot 0.5H_2O$: C, 36.69; H, 4.84; N, 3.06. Found: C, 36.87; H, 5.10; N, 3.09.

The lower section (20%) of the band gave a syrup (19 mg), $[\alpha]_D^{20}$ −7° (*c* 0.95, 1:1 water–methanol); t.l.c.: (Solvent *B*) R_F 0.13; n.m.r. spectrum (after preparation of sample as described for the α-D anomer): δ 1.98, 2.07, and 2.10 (Me, OAc), 2.03 (Me, NAc), 4.26 (CH_2, C-6 of pyranose), 4.60 (HDO), 5.03, 5.14, and 5.28 (H-3 of pyranose), 5.10 (pyranose-ring proton), and 5.45 (pyranose-ring proton).

*2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-*D*-glucopyranosyl ammonium hydrogen (and diammonium) phosphate* (**6**). — Compound **1** was treated with phosphoric acid (1.2 g) under conditions similar to those just described for the preparation of **3** and **6**, except that the duration of the reaction was 2 instead of 3 h. The methanolic solution of **3** and **6** was treated with ether (to the point of cloudiness) and kept at room temperature. After 15 h, the crystalline precipitate was filtered off (13 mg, 1.1%). The compound was recrystallized from ether–methanol as needles, m.p. 126–126.8°, $[\alpha]_D^{20}$ −8.8° (*c* 0.65, 1:1 water–methanol); t.l.c. (Solvent *B*): R_F 0.13; i.r. spectrum: ν_{max}^{KBr} 1750 (Ac), 1660, 1550 (NHAc), and 1250 cm^{-1} (P=O and C=O, stretching).

Anal. Calc. for $C_{14}H_{21}N_1O_{12}PNH_4$: C, 37.84; H, 5.67; N, 6.31. Calc. for $C_{14}H_{20}N_1O_{12}P(NH_4)_2$: C, 36.44; H, 6.12; N, 9.11. Calc. for a 1:1 mixture of mono- and di-ammonium salts: C, 37.13; H, 5.90; N, 7.73. Found: C, 37.26; H, 5.91; N, 7.92.

*2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-*D*-glucopyranosyl dihydrogen phosphate* (**4**) *by preferential acid hydrolysis of* **6**. — The mixture of **3** and **6** (monoammonium, 0.24 g) was dissolved in 0.2M sulfuric acid (6 ml) and the solution was kept for 5 h at room temperature. After neutralization with barium carbonate and filtration through Celite, evaporation gave a product that was treated with 1:1 acetic anhydride–pyridine (4 ml). The mixture was stirred until a clear solution was obtained, kept for 15 h at room temperature, treated with pyridine (2 ml) and water (2 ml), and then kept for 2 h at room temperature. After evaporation, the residual pyridine was removed by repeated addition and evaporation of toluene. The residue was dissolved in water and extracted with chloroform (six times) to remove **1** and **2**, and evaporation gave a syrupy product (0.22 g) that was purified as follows: a sample (41 mg) was applied to a preparative t.l.c. plate (20 × 20 cm) in conc. methanol solution. After development in solvent *B*, the required band was located and the product extracted, as described before, to give **4** (20 mg), as an amorphous powder. Examination of this material by t.l.c. on silica gel with solvent *B* showed that it was very pure (R_F 0.24), $[\alpha]_D^{20}$ +50° (*c* 1, 1:1 water–methanol).

Anal. Calc. for $C_{14}H_{22}NO_{12}P \cdot H_2O$: C, 37.76; H, 5.43; N, 3.11. Found: C, 37.71; H, 5.08; N, 2.93.

As t.l.c. before purification showed that no β-D anomer was present, preparative t.l.c. was not used for synthetic purposes.

Conversion of **4** *into 2-acetamido-2-deoxy-α-*D*-glucopyranosyl dipotassium*

phosphate. — In order to prove the assignment of the α-D configuration to the major phosphorylation product, compound **4** (100 mg) (obtained as described in the preceding paragraph without t.l.c. purification) was dissolved in dry methanol and treated with potassium methoxide (0.1%) in small portions until the solution was strongly basic and t.l.c. (solvent *B*) showed complete deacetylation. After addition of a large excess of abs. ethanol, the solution was kept for 15 h at 0°. The solid product was filtered off and washed with ethanol and ether (73 mg). After recrystallization from aqueous ethanol, a solid was obtained (50 mg), $[\alpha]_D^{20}$ +57° (*c* 1, 1:1 water–methanol).

In order to eliminate any of the barium ions that may have been present, a solution of this compound in water was passed through a column of cation-exchange resin (pyridinium$^+$), and then very slowly through a column of cation-exchange resin (K^+), and the resin was washed with water. The combined solutions were evaporated in presence of pyridine, and toluene was added and evaporated off three times to give the 2-acetamido-2-deoxy-α-D-glucopyranosyl dipotassium phosphate (38 mg) as a hygroscopic solid, $[\alpha]_D^{20}$ +66° (*c* 0.8, 1:1 water–methanol) or $[\alpha]_D^{20}$ +69° for anhydrous material, as the analysis of acid-labile phosphate groups with $NaH_2PO_4 \cdot H_2O$ as standard (see method used for the analysis of **6**) indicated a water content of 4.2%. O'Brien[12] reported $[\alpha]_D^{20}$ +76° (*c* 3.44, water) for a hygroscopic product that had been dried *in vacuo* overnight over phosphorus pentaoxide.

The deacetylated product was also compared by t.l.c. (silica gel, Solvent *C*) with an authentic sample of 2-acetamido-2-deoxy-α,β-D-glucopyranosyl dilithium phosphate prepared in this laboratory[42]. A separation of the two anomers was achieved: α-D, R_F 0.36 and β-D, R_F 0.27. The behavior of both compounds during detection on the t.l.c. plate was similar to that of the acetylated compound, that is the β-D anomer always appeared first on heating. After cooling of the plate, the α-D and β-D anomers appeared as green and brown spots, respectively. This t.l.c. indicated that the product obtained from **4** was the pure α-D anomer.

Homogeneity of ficaprenol. — A sample of crude ficaprenol[5] (10 mg) was dissolved in hexane and applied to a precoated preparative t.l.c. plate (5 × 20 cm), which was developed with 49:1 benzene–methanol. The plate was dried, and the band containing ficaprenol was located by spraying the edges with 1% potassium permanganate in 2% sodium carbonate solution[39]. The silica gel was removed and extracted with 5:1 chloroform–methanol by stirring for 15 h at room temperature. Filtration and evaporation gave a purified sample of ficaprenol (6 mg). Small samples of crude and purified ficaprenol (2 mg) in 2:1 chloroform–methanol (2 ml) were hydrogenated at 1 atm for 15 h over (*a*) 5% palladium-on-charcoal (Fluka A.-G., Buchs, Switzerland) and (*b*) platinum dioxide (Engelhard Industries, Newark, New Jersey 07105). In each instance, the catalyst was filtered off and washed with 2:1 chloroform–methanol, and the filtrate and washings were evaporated to dryness. The products were converted into trimethylsilyl ethers and analyzed by g.l.c. This indicated that the reduced polyprenol samples (Fig. 1) were in an almost identical state of purity, that is, no significant purification had been achieved by preparative t.l.c., and

both catalysts were equally effective in the reduction. At a column temperature of 267°, a major and a minor contaminant, R_t 11.8 min and R_t 12.3 min (retention times are measured from the point of injection of sample) were respectively eluted, and when the column temperature reached 311°, the elution of the ficaprenols began. The retention times were in agreement with the expected values for C_{45} (R_t 13.8 min), C_{50} (R_t 15.5 min), C_{55} (R_t 17.2 min), and C_{60} (R_t 19.2 min) components. A good, straight line was obtained when log R_t was plotted against C_n (see also Ref. 43).

The proportions of the different isomers were similar to those reported[43] for the acetates of the unreduced materials, the C_{55} compound being the major component of the mixture, whereas the C_{45} compound was present in only very small proportion. In order to confirm that these products were in fact reduced polyprenols, the mass spectra were recorded. Where possible, this was done directly on the fractions obtained by g.l.c., by using a fritted-glass g.l.c.–m.s. interface. However, these long-chain lipids would pass through this interface only with difficulty, and in order to obtain a satisfactory mass spectrum of the C_{55} and C_{60} isomers, it was necessary to inject a small sample of the mixture of trimethylsilylated, reduced polyprenols directly into the mass spectrometer. The two methods gave very similar spectra. The main fragment-ions were found at intervals of 70 (C_5H_{10}) and 14 (CH_2) mass units, giving a pattern obviously typical of a saturated polyisoprenoid. Most of the ions for the expected fragmentation could be observed (*m/e* values are given in Table II). In contrast to the fragmentation pattern observed with unreduced polyprenols, where the bond primarily spiit is the one between the two methylene groups[45], fragmentation occurred mainly at the positions adjacent to the methyl branch-points.

Ficaprenyl phosphate. — The procedure previously described[5] was improved. Ficaprenol (77 mg) in 1,4-dioxane (1.3 ml) containing 2,6-dimethylpyridine (25 mg) was stirred at 0° and treated with *o*-phenylene phosphochloridate[46] (45 mg) in 1,4-dioxane (0.45 ml). The temperature was allowed to rise to 20° and, after 10 min, the 2,6-dimethylpyridinium hydrochloride was filtered off and washed with 1,4-dioxane (1 ml). The filtrate was treated with 2,6-dimethylpyridine (25 mg) and water (45 µl) and, after 5 min, the 1,4-dioxane was evaporated off. Toluene was then added to, and evaporated from, the residue (twice). Small amounts of 2,6-dimethylpyridinium chloride were removed by filtration, after dissolution of the residue in chloroform, and the solution was dried over magnesium sulfate. After filtration, evaporation gave the *o*-hydroxyphenyl phosphate as a syrup (R_F 0.7, silica gel, Solvent *A*). The product was dissolved in 1,4-dioxane (5 ml) and treated with lead tetraacetate (0.1 g containing 10% of acetic acid, Ventron, Beverly, Mass. 01915). The dark-brown mixture was stirred for 30 min, and then treated with M potassium hydroxide in methanol until the pH reached ~12. After 30 min, a slight excess of glacial acetic acid was added. Evaporation of the solvents, followed by three additions and evaporations of toluene, gave a solid, brown residue, which was dissolved in 5:1 chloroform–methanol and added with vigorous stirring to methanol (75 ml). The light-brown precipitate was collected on a Celite pad, washed with methanol, and redissolved in 5:1 chloroform–methanol. Filtration and evaporation gave ficaprenyl dipotassium phosphate (75 mg),

a syrup in a good state of purity according to t.l.c. (R_F 0.5, silica gel, Solvent *A*). A small quantity was purified by preparative t.l.c. as previously described[5], and two small samples (2 mg) in 2:1 chloroform–methanol were hydrogenated at 16 atm for 15 h. In one experiment, the catalyst was 5% palladium-on-charcoal (Fluka A.-G., Buchs, Switzerland) and, for comparison, the other hydrogenation was performed with platinum dioxide (Engelhard Industries, Newark, New Jersey 07105) as catalyst. The products from both hydrogenations were processed by the following procedure: the catalyst was filtered off and washed with the same solvent mixture, and the combined filtrate and washings evaporated to dryness. The residue was extracted with hexane, and the hexane-insoluble material was dissolved in a mixture of 10:10:3 chloroform–methanol–conc. hydrochloric acid and heated for 4 h at 100° to hydrolyze any reduced prenyl phosphate that may have been present. The solvents and hydrochloric acid were removed by evaporation overnight *in vacuo* over potassium hydroxide, and, after per(trimethylsilyl)ation, this product was compared with the hexane-extracted material by g.l.c. The hexane extract showed three major peaks having the same retention times as the reduced C_{50}, C_{55}, and C_{60} ficaprenols obtained earlier from the hydrogenation of ficaprenol, and a small peak from the C_{45} ficaprenol. The only other peaks were minor, and occurred in a closely spaced, regular pattern that would be expected for pyrolysis products. The material obtained by acid hydrolysis and trimethylsilylation showed no significant peaks, indicating that scission of the allylic phosphate linkage had been complete during hydrogenation with each catalyst. These experiments also showed that the retention times for ficaprenols that had been trimethylsilylated after reduction were the same as for the

TABLE II

CHARACTERISTIC MASS-SPECTRAL FRAGMENT-IONS OF HYDROCARBONS DERIVED FROM CATALYTIC REDUCTON OF FICAPRENYL PHOSPHATE (7) AND OF THE TRIMETHYLSILYL ETHERS OF REDUCED FICAPRENOL, SHOWING ASSOCIATED FRAGMENTS LOST FROM THE MOLECULAR ION OF THE TRIMETHYLSILYL ETHER OF REDUCED C_{55} FICAPRENOL[a]

Ficaprenyl phosphate (m/e)	*Ficaprenols* (m/e)	*Fragments*[b]
729	729	$CH_3CH(CH_3)$
715	716	$CH_2CH_2CH(CH_3)$
687	687	$CH_3CH(CH_3)(CH_2)_3$
673	674	$CH_2CH_2CH(CH_3)(CH_2)_3$
617	617	$CH_3CH(CH_3)(CH_2)_3CH(CH_3)(CH_2)_3$
603	604	$CH_2CH_2CH(CH_3)(CH_2)_3CH(CH_3)(CH_2)_3$
589	589	$CH_3CH(CH_3)(CH_2)_3CH(CH_3)(CH_2)_3CH(CH_3)$
575	576	$CH_2CH_2CH(CH_3)(CH_2)_3CH(CH_3)(CH_2)_3CH(CH_3)$
547	547	$CH_3CH(CH_3)(CH_2)_3CH(CH_3)(CH_2)_3CH(CH_3)(CH_2)_3$

[a]For detailed information on the mass spectra of unreduced polyprenols, see Ref. 43 and 44.
[b]Fragment-ions derived from ficaprenyl phosphate (7) will differ by one mass unit from those derived from ficaprenol when the fragmentation is from the end of the molecule adjacent to the phosphate linkage, because the CH_2–O–P group has been replaced by CH_3 during the reduction.

hydrocarbon products obtained by hydrogenolysis of ficaprenyl phosphate. The mass spectrum of the hexane-extracted, hydrocarbon products obtained by direct introduction of the sample, without g.l.c., showed a very clear fragmentation pattern derived from a saturated polyisoprenoid (Fig. 2) and the fragments observed were essentially the same as those from ficaprenol (Table II).

P^1-*Diphenyl* P^2-*ficaprenyl pyrophosphate* (**7**). — Ficaprenyl phosphate (prepared from 77 mg of ficaprenol) was converted into the tributylammonium form as described previously[5]. The product was dissolved in 1,2-dichloroethane (2 ml) and treated with tributylamine (20 mg). The mixture was cooled to −10° and, while stirring, a solution of diphenyl phosphochloridate (20 mg) in 1,2-dichloroethane (2 ml) was slowly added. The temperature was allowed to rise to ~20° and, after 2 h, t.l.c. (silica gel) in 5:1 chloroform–methanol showed the formation of the diphenyl pyrophosphate (R_F 0.8), with only small quantities of decomposition products (R_F 1.0). The reaction mixture was treated with methanol (0.5 ml) and kept for 30 min at room temperature. Evaporation, followed by three additions and evaporations of toluene, gave **7** as a syrup.

P^1-(*2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-*D*-glucopyranosyl*) P^2-*ficaprenyl pyrophosphate* (**8**). — 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl dihydrogen phosphate (**4**, 40 mg) was converted into the bis(tributylammonium) form (**5**) by passage of an aqueous solution through a cation-exchange resin (pyridinium$^+$) to remove traces of barium ions, followed by concentration of the solution to ~5 ml and addition of methanol (5 ml). Tributylamine (40 mg) was added, and a homogeneous solution was obtained. The excess tributylamine was removed by three extractions with hexane. Evaporation of the solution *in vacuo*, followed by three additions and evaporations of toluene, gave the required syrupy product **5**. This was dissolved in 1,2-dichloroethane and added to P^1-diphenyl P^2-ficaprenyl pyrophosphate (**7**, obtained from 77 mg of ficaprenol), and, after the solvent had been evaporated, the residue was dissolved in 1,2-dichloroethane (0.4 ml) containing pyridine (1.2 mg). Formation of the pyrophosphate **8** was monitored by removal of a small sample from the reaction mixture, dilution with chloroform, extraction three times with water to remove unreacted **5**, and t.l.c. (silica gel, Solvent *A*), which showed one new, major product having R_F 0.5. After 20 h, when the starting materials (R_F 0.93 and 0.2) had almost disappeared, the reaction mixture was diluted with chloroform (25 ml) and extracted three times with water. The chloroform solution was dried with magnesium sulfate and evaporated to yield a residue (0.1 g), which was chromatographed on silica gel (8.4 g). Elution with 40:1 and subsequently 20:1 chloroform–methanol gave the components having on t.l.c. (silica gel, Solvent *A*) R_F 1.0 and R_F 0.9, respectively. When t.l.c. showed that no further material was eluted, the eluent was changed to 10:1 chloroform–methanol and 5-ml fractions were collected. T.l.c. showed that early fractions contained a by-product (R_F 0.8), as well as the desired compound **8** (R_F 0.5). Later fractions contained the bulk of this material (R_F 0.5) with only traces of contaminant, and elution of **8** was completed with 5:1 chloroform–methanol. The main fractions were combined and evaporated to give a syrup (30 mg), almost pure on t.l.c. (silica gel, Solvent *A*), but still containing traces

of other components (R_F 0.7 and R_F 0.9). Complete purification of **8** could be achieved by preparative t.l.c., but with great loss of yield. A concentrated solution of the product in chloroform was applied to a plate (25 × 10 cm). After development with Solvent *A*, the position of the required band was located by spraying the edges of the plate with 1% potassium permanganate in 2% aq. sodium carbonate[39]. After removal from the plate, the product was extracted from the silica gel by stirring with Solvent *C* for 15 h at room temperature. The silica gel was filtered off and washed with the same solvent mixture. After evaporation to dryness, the residue was extracted with chloroform. The suspension was filtered, and evaporation of the filtrate gave pure **8** as a syrup (11 mg).

An alternative procedure used preparative t.l.c. without prior column chromatography, but this could only be done when the reaction mixture showed a discrete, single product on t.l.c., without the presence of minor by-products having closely similar chromatographic mobility (the proportion of these by-products varied considerably with each experiment). In this instance, a chloroform solution of the total reaction-products, after removal of excess **5**, was applied to a plate (25 × 25 cm), which was developed with Solvent *A*. Location of the required band and extraction of the product were performed as just described, to give pure **8** (22 mg) in the bis(tributylammonium) form, a syrup, $[\alpha]_D^{20}$ +29° (*c* 1.55, chloroform); i.r. data: ν_{max}^{film} 925 (P–O–P), 1250 (P=O and C=O, stretching), 1585 and 1660 ($NHCOCH_3$), 1750 (C=O, Ac), and 3350 cm^{-1} (NH); t.l.c. (silica gel, detection with anisaldehyde[38] or the reagent specific for phosphate groups[40]): R_F 0.4 (Solvent *A*), 0.54 (Solvent *B*), 0.7 (Solvent *C*), and 0.42 (20:15:2 2,6-dimethyl-4-heptanone–acetic acid–water). For determination of the n.m.r. spectrum, **8** (10 mg) was dissolved in chloroform (2 ml), and methanol was added until the point of turbidity was reached. A few drops of chloroform were added to give a clear solution, which was stirred with a cation-exchange resin (pyridinium$^+$) and, over a period of 1 h, a further 10 ml of methanol was added in portions. The resin was filtered off and washed with methanol, and the combined filtrate and washings were passed slowly through a column of cation-exchange resin (Na^+). The eluate and washings (methanol) from the resin were combined and evaporated to dryness, followed by three additions and evaporations of toluene, and three additions and evaporations of chloroform-*d*. The residue (**8**, 8 mg, sodium salt) was dissolved in chloroform-*d* (0.5 ml) containing 1% of tetramethylsilane; n.m.r. data: δ 2–2.15 [unresolved group of peaks due to *N*–Ac(Me), *O*–Ac (Me_3), and $(CH_2)_{20}$], 1.67 (Me_7, adjacent to *cis* double bond), 1.58 (Me_5, adjacent to *trans* double bond), and 1.26. This last peak, also observed by others[26,37], is not present in pure ficaprenol. It most probably results from autoacid-catalyzed decomposition and cyclization of the ficaprenol residue, due to formation of a trace of 2-acetamido-2-deoxy-D-glucosyl pyrophosphate, which causes a progressive lowering of the pH in the medium. T.l.c. (silica gel) of the sample after the spectrum had been taken showed partial decomposition into highly mobile substances (R_F 1.0, Solvent *A*). A signal at δ 1.26, which could not be assigned, was also observed in the n.m.r. spectrum of a long-chain polyprenol from *Mycobacterium tuberculosis*[47].

Determination of 2-acetamido-2-deoxy-D-glucose (as 2-amino-2-deoxy-D-glucose hydrochloride) and acid-labile phosphate was performed in the following way: the synthetic product (~460 μg) was dissolved in a mixture of 10:10:3 chloroform–methanol–M hydrochloric acid (1 ml), and the solution was heated for 1 h under reflux. The resulting decomposition products, after evaporation of the solvents *in vacuo*, were water soluble, except for the products derived from the lipid moiety. The residue was therefore treated with 3M hydrochloric acid (1 ml) for 6 h at 100° and the solution evaporated to dryness *in vacuo* over potassium hydroxide. The Elson–Morgan determination of 2-amino-2-deoxy-D-glucose hydrochloride was performed according to the Blix[48] and Gardell[49] modifications, and the determination of acid-labile phosphate groups was performed by the method of Chen *et al.*[50]. The molar ratio of acid-labile phosphate groups to 2-acetamido-2-deoxy-D-glucose residues was found to be 2:0.94 (average of 2 determinations).

The proportion of 2-acetamido-2-deoxy-D-glucose was also determined by methanolysis of **6** (136 μg) with 0.5M hydrogen chloride in methanol overnight at reflux temperature. This was followed by evaporation *in vacuo*, acetylation with 1:1 acetic anhydride–pyridine for 1 min at room temperature, evaporation *in vacuo*, *O*-deacetylation with 0.5M hydrogen chloride in methanol at reflux temperature, and g.l.c. after per(trimethylsilyl)ation: The molar ratio of acid-labile phosphate groups to 2-acetamido-2-deoxy-D-glucose residues was found to be 2:0.91 (average of 2 determinations).

Hydrogenolysis of **8**. — (*a*) *Without added base.* A solution of **6** (8 mg) in 2:1 chloroform–methanol was hydrogenated at 1 atm over a 5% platinum-on-charcoal catalyst (Matheson, Coleman, and Bell, Norwood, Ohio 45212) for 15 h at room temperature. The catalyst was filtered off and washed with methanol, and filtrate and washings combined and evaporated. T.l.c. (silica gel) in Solvent *B* showed the presence of an acetylated 2-amino-2-deoxy-D-glucose derivative (R_F 0.54), which gave rise, after spraying the plate with anisaldehyde reagent, to a green spot on the hot plate, and to a pink spot after the plate had cooled; this behavior appears to be typical for nonphosphorylated hexosamine derivatives. In addition, a phosphate derivative of 2-acetamido-2-deoxy-D-glucose (R_F 0.1) was observed, but there was no indication of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl phosphate (R_F 0.24). In Solvent *C*, this latter compound has R_F 0.7 and the new product R_F 0.5. The phosphate-specific spray showed an intense spot, presumably inorganic pyrophosphate, at the origin of the plate. Measurement of the pH of a methanolic solution of the hydrogenolysis products showed that it was strongly acidic, presumably from the generation of the fully acetylated 2-acetamido-2-deoxy-D-glucopyranosyl pyrophosphate; this would explain the presence of inorganic pyrophosphate and an acetylated 2-amino-2-deoxy-D-glucose derivative. The hydrogenolysis product was deacetylated by treatment of a methanol solution with sodium methoxide. After 30 min, the sodium ions were removed with a cation-exchange resin (pyridinium$^+$) and the products formed examined by t.l.c. (cellulose) and paper chromatography with standards of 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-α-D-gluco-

pyranosyl phosphate, and inorganic phosphate and pyrophosphate. Mono- and pyrophosphates, both inorganic and derivatives of 2-amino-2-deoxy-D-glucose, showed a distinctive behavior on t.l.c. (cellulose) plates and paper chromatographs when sprayed with the reagent specific for phosphate groups[40]. Monophosphates, immediately after being sprayed, always gave a pale-blue spot on a white background, which after a few min, changed to a white spot on a blue background. Pyrophosphates always gave an intense-violet spot which appeared quickly and remained. Generally, t.l.c. (cellulose) gave reproducible, compact spots, whereas on paper a varying degree of streakiness of the spots was observed. Chromatography in 7:1:2 propanol–ethyl acetate–water and detection with chlorine–starch–potassium iodide[51] confirmed the presence of 2-acetamido-2-deoxy-D-glucose as a major product of hydrogenolysis followed by autoacid hydrolysis; in 5:2 M ammonium acetate–ethanol the products appeared to be inorganic pyrophosphate (R_F 0.7) and 2-acetamido-2-deoxy-D-glucopyranosyl pyrophosphate (R_F 0.83), each having the same mobility as inorganic monophosphate and 2-acetamido-2-deoxy-D-glucopyranosyl phosphate, respectively.

(*b*) *With added base.* The hydrogenation was performed as described under (*a*), except that tributylamine (10 mg) and a slight excess of glacial acetic acid were added to the reaction mixture. After filtration from the catalyst and evaporation, the resultant residue was dissolved in chloroform and extracted three times with water. After evaporation in the presence of a small amount of pyridine, t.l.c. (silica gel) of the material with Solvent *B* showed only the new product (R_F 0.1). As expected, 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl phosphate (R_F 0.7, Solvent *C*) was not produced, t.l.c. (silica gel, Solvent *C*) showing a single spot (R_F 0.5). This gave a positive reaction with the phosphate group-specific spray[40] and was, therefore, not 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-D-glucose, which might conceivably have arisen from mild acid hydrolysis due to the acetic acid in the hydrogenation solvent. Deacetylation was performed as described under (*a*) and, in order to show that *O*-deacetylation was complete, the product was chromatographed on silica gel in Solvent *C*. This gave a spot (R_F 0.1) having the characteristic violet coloration given by the pyrophosphate derivatives with the phosphate group-specific spray[40] and no starting material (R_F 0.5) was observed; in contrast, 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate gave a blue spot (R_F 0.36). Thus, t.l.c. (cellulose) and paper chromatography indicated the formation of a single product having the properties of 2-acetamido-2-deoxy-α-D-glucopyranosyl pyrophosphate, R_F 0.83 in M ammonium acetate–ethanol (5:2).

Finally, the mono- and pyrophosphates of 2-acetamido-2-deoxy-D-glucose were compared by paper electrophoresis, in order to confirm the identity of the pyrophosphate. This was performed in a buffer solution, pH 3.9, consisting of pyridine (24 ml), glacial acetic acid (80 ml), and water (4 l), at 35 V/cm, on Whatman filter paper No. 1. After 1.25 h, the paper was dried in a current of air for 30 min at 75° and cut into two parts. On one part, the spots were visualized by dipping the paper in a solution of periodic acid (114 mg) in acetone (95 ml) containing water (5 ml), drying for 5 min at room temperature, and spraying with 1% potassium permanganate in

2% aq. sodium carbonate to give yellow spots on a mauve background; alternatively, the dried paper was immersed in chlorine gas and then sprayed with potassium iodide–starch solution[51] (as described in the acid hydrolysis of compound **7**): the monophosphate moved 9.5 cm from the origin, the pyrophosphate moved 15 cm. The second part of the paper was sprayed with the reagent specific for phosphate ester-groups[40]. The pyrophosphate gave an intense, violet spot at 14 cm on a white background which gradually turned blue. A trace of a second component, at 16 cm, was also observed. The monophosphate gave a faint blue spot on a white background at 9 cm.

P^1-(*2-Acetamido-2-deoxy-α-*D*-glucopyranosyl*) P^2-*ficaprenyl pyrophosphate* (**9**). — Compound **8** (tributylammonium form, 22 mg) was converted into the pyridinium form as described previously for the n.m.r. sample. The chloroform–methanol solution was evaporated to dryness, and then toluene was added and evaporated off (three times). The material was dissolved in methanol (2 ml) containing a trace of chloroform, and treated with sodium methoxide (2 mg). T.l.c. (silica gel) in Solvent *B* showed that deacetylation was complete within 15 min, giving a new product (R_F 0.37; starting material, R_F 0.54) that was stable in the presence of excess base. The reaction mixture was treated with a cation-exchange resin (pyridinium$^+$) to remove sodium ions. The resin was filtered off and washed with methanol, and the highly unstable product **9** (pyridinium form) was characterized as far as possible in solution, as evaporation (below 25°, *in vacuo*) was found to cause appreciable decomposition. T.l.c. (silica gel) in six solvent systems and detection with the anisaldehyde[38], permanganate[39], and phosphate group-specific[40] spray-reagents showed a single spot in each case (Table III). Subsequent evaporation gave ~16 mg of a syrup, $[\alpha]_D^{20}$ +15° (*c* 1.2, chloroform).

Anal. Calc. for $C_{68}H_{110}N_2O_{12}P_2 \cdot 2H_2O$: C, 65.55; H, 9.24; N, 2.24. Found: C, 65.57; H, 9.88; N, 2.18.

TABLE III

RELATIONSHIP OF MOBILITY OF **9** ON T.L.C. (SILICA GEL) WITH ELUTING SOLVENT

Solvent	R_F
Chloroform–methanol–water (60:25:4)	0.10
Chloroform–methanol–water (13:6:1)	0.20[a]
Chloroform–methanol–water (60:35:6)	0.37
Chloroform–methanol–water (10:10:3)	0.84
Isopropyl alcohol–15M ammonium hydroxide–water (6:3:1)	0.80
2,6-Dimethyl-4-heptanone–acetic acid–water (20:15:2)	0.4–0.5

[a]Hussey and Baddiley[24] reported in this solvent mixture R_F 0.3 for a compound that is probably P^1-2-acetamido-2-deoxy-α-D-glucopyranosyl P^2-undecaprenyl pyrophosphate, but their silica gel contained 10% (w/w) of ammonium sulfate.

The n.m.r. spectrum was observed on the sodium salt, obtained by slow percolation of a solution in 1:10 chloroform–methanol through a cation-exchange

resin (Na^+), as previously described for **8**, special care being taken in the evaporations owing to the instability of the material; n.m.r. data: δ 2.02 (with a downfield shoulder, *N*–AcMe), 2.0 [$(CH_2)_{20}$], 1.67 (Me_7, adjacent to *cis* double bond), 1.58 (Me_5, adjacent to *trans* double bond), and 1.26 (the probable origin of this peak is as described for **8**).

Chromatography of **9** *on DEAE-cellulose.* — Cellex D (Bio Rad, Richmond, Calif. 94804) was prepared by the method of Dankert *et al.*[52], dried in air, and mixed with water to give a slurry (15 g in 100 ml). Microscope slides, freed from grease with chloroform, were dipped into the slurry, dried for 10 min at 120°, and cooled. Compound **9** (~5 μg) in conc. methanol solution was applied to each plate, and the chromatograms were developed in solutions of increasing concentration of ammonium acetate in methanol (see Table IV). After being dried, each plate was sprayed with the reagent specific for phosphate groups[40] which showed that three decomposition products were present; t.l.c. on silica gel also showed some decomposition products. The best separation of these materials from **9** occurred with salt concentrations of 0.25–0.3M. The mobilities of (*a*) the synthetic lipid intermediate **9** and (*b*) the decomposition products A, B, and C at different salt concentrations are shown in Table IV, and the relation between the R_F of **9** and the concentration of ammonium acetate in Fig. 3.

TABLE IV

MOBILITY OF **9** AND DECOMPOSITION PRODUCTS A, B, C ON THIN LAYERS OF DEAE-CELLULOSE WITH DIFFERENT CONCENTRATIONS OF AMMONIUM ACETATE IN METHANOL

Concentration of ammonium acetate (M)	R_F			
	A	*B*	*C*	**9**
0.15	0.3			0.13
0.20	0.7	0.4		0.17
0.25	0.8	0.45	0.35	0.22
0.30	0.9	0.64	0.40	0.27
0.35		0.80	0.58	0.31
0.40		0.90	0.62	0.33

Dilute acid hydrolysis of **9**. — To compound **9** (1 mg), which was not soluble in aqueous methanol, was added 0.1M hydrochloric acid in 1:1 water–methanol (0.2 ml) and then chloroform (0.1 ml), and lastly methanol to obtain a homogeneous solution. The mixture was placed in a water-bath at 100°, and, after 1 min, the volume of the solution had been reduced to ~0.2 ml. The heating was continued for a further 2 min, and, after cooling, the lipid products were extracted with hexane and examined by t.l.c. on silica gel with 49:1 benzene–methanol. This showed that a small amount of material having the mobility of ficaprenol had been formed, together with rearrangement and decomposition products derived from it (R_F 0.5, 0.6, 0.8, and 1.0), the fastest moving being the major product. T.l.c. of the aqueous methanol solution in

Solvent *C* showed that no starting material remained. T.l.c. on cellulose and paper chromatography was performed in 5:2M ammonium acetate–ethanol in the presence of standards of inorganic mono- and pyrophosphate and 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate and pyrophosphate, the latter compound being obtained by hydrogenation of **8** and deacetylation, as described earlier. The spots were detected with the phosphate-specific spray reagent[40]. The peculiarities of mono- and pyrophosphate, when detected with this reagent, were the same as in the analysis of the hydrogenolysis products of **8**. These chromatograms indicated that the hydrolysis products of **9** contained inorganic pyrophosphate (R_F 0.7), 2-acetamido-2-deoxy-α-D-glucopyranosyl pyrophosphate (R_F 0.83), and a compound (R_F 0.5) showing considerable streaking, especially on paper, but not detected with the periodate–benzidine spray-reagent[53]. When a sample of sodium pyrophosphate was treated with hydrochloric acid under similar conditions, t.l.c. of the resulting solution showed the same spot of R_F 0.5 with much streaking. Brooks and Baddiley[22] reported that 2-acetamido-2-deoxy-α-D-glucopyranosyl pyrophosphate moved approximately half as far as 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate in 5:2M ammonium acetate–ethanol; in our chromatograms on paper and cellulose, the mono- and pyrophosphate always appeared to have the same mobility in this solvent system.

In order to identify the products devoid of phosphate groups resulting from the hydrolysis, 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate was subjected to a hydrolysis similar to that described for **9**, and the products from both experiments were examined by t.l.c. (cellulose) and paper chromatography in 7:1:2 propanol–ethyl acetate–water in the presence of a standard of 2-acetamido-2-deoxy-D-glucose. The spots were detected by immersion of the dried chromatograms in chlorine gas for 10 min, and removal of the excess chlorine with a current of air (for at least 30 min). The plate was then sprayed with 1% potassium iodide in 1% starch solution to give black spots on a light-gray background[51]. The hydrolysis products of **9** also contained 2-acetamido-2-deoxy-D-glucose (R_F 0.33) and a component (R_F 0.45), presumably methyl 2-acetamido-2-deoxy-β-D-glucopyranoside, that was formed in the parallel hydrolysis of 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate[22].

Stability of **9**. — Solutions of **9** in 2:1 chloroform–methanol were stored at −15°, when the substance could be kept for several days without appreciable decomposition. At room temperature, t.l.c. indicated ~50% decomposition in 30 min, and when a sample was exposed to air at room temperature, decomposition was complete in less than 5 min. The products formed were examined by t.l.c. on silica gel with the anisaldehyde[38], permanganate[39], and phosphate group-specific[40] spray reagents, and on cellulose with the phosphate reagent and the chlorine–starch–iodide method[51] described previously. In Solvents *B* and *C*, two of these products contained phosphate groups and had a mobility higher than that of the starting material; a third substance moved slowly, and showed considerable streaking. In 6:3:1 isopropyl alcohol–15M ammonium hydroxide–water the decomposition products had R_F 0.6, 0.9, and 1.0 (**9**: R_F 0.8), but the permanganate spray gave much streaking. In 20:15:2 2,6-dimethyl-4-heptanone–acetic acid–water (**9**: streaking, R_F 0.4–0.5), the main products had

R_F 0.85 and 1.0. In order to identify the substances derived from ficaprenol, t.l.c. (silica gel) was performed in 49:1 benzene–methanol, but only decomposed hydrocarbon material migrating near the solvent front was observed. Cellulose t.l.c. in 7:1:2 propanol–ethyl acetate–water showed that the decomposition products included 2-acetamido-2-deoxy-D-glucose (R_F 0.33) and (probably) methyl 2-acetamido-2-deoxy-β-D-glucopyranoside (R_F 0.45). In 5:2M ammonium acetate–ethanol the products observed were inorganic pyrophosphate and 2-acetamido-2-deoxy-D-glucopyranosyl pyrophosphate.

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