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Note

Chemical synthesis of P^1-2-acetamido-2-deoxy-α-D-glucopyranosyl P^2-dolichyl pyrophosphate*,†

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Considerable evidence for the participation of "lipid intermediates" in the transfer of the 2-acetamido-2-deoxy-D-glucosyl group from UDP-2-acetamido-2-deoxy-α-D-glucose to endogenous glycoproteins by liver microsomal fractions has been presented[2–4]. The chemical nature of the lipid carrier was not ascertained, but, on the basis of chromatography and hydrolysis studies, it was considered to be a poly(isoprenol), thus raising the possibility that the lipid intermediates are structurally similar to the compound suggested to be active in the biosynthesis of teichoic acid by *Staphylococcus lactis*[5], namely, P^1-2-acetamido-2-deoxy-D-glucosyl P^2-poly-(isoprenyl) pyrophosphate. In previous work[6], P^1-2-acetamido-2-deoxy-α-D-gluco-pyranosyl P^2-ficaprenyl pyrophosphate (**6**) was chemically synthesized in order to compare its properties with those of the biosynthesized compounds. At the time that this work was initiated, the only poly(isoprenyl) phosphates known to act as glycosyl acceptors were C_{55} compounds[7,8]. However, it has now become apparent that poly(isoprenyl) phosphates of the dolichyl phosphate type (C_{80}–C_{105}) are glycosyl acceptors in many of the biosynthetic systems derived from mammals and birds[9–15]. Several reports of dolichol-type lipids acting as carriers for 2-acetamido-2-deoxy-D-glucosyl groups have appeared[10,16,17]. In the present work, P^1-2-acetamido-2-deoxy-α-D-glucopyranosyl P^2-dolichyl pyrophosphate (**5**) has been chemically synthesized and characterized.

*Dedicated to the memory of Professor W. Z. Hassid.

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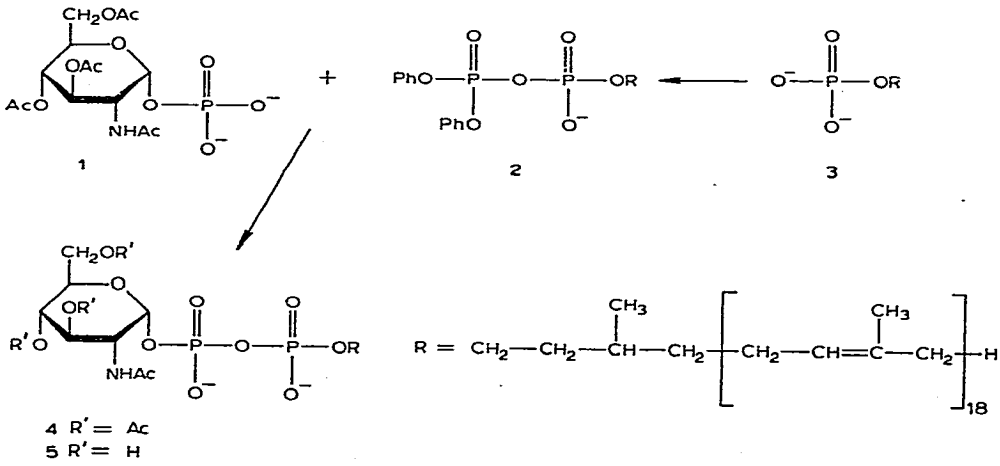
RESULTS AND DISCUSSION

Dolichyl phosphate (**3**), synthesized by a method recently developed in this laboratory[17], was converted into P^1-diphenyl P^2-dolichyl pyrophosphate (**2**) by treatment with diphenylphosphorochloridate. Condensation of **2** with 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl phosphate[6] gave a fully acetylated pyrophosphoric diester **4**, which was purified by preparative t.l.c. Compound **4** was shown to be pure by t.l.c. in two solvent systems with three spray reagents, and it was characterized by i.r. spectroscopy and polarimetry. *O*-Deacetylation with sodium methoxide in chloroform–methanol gave P^1-2-acetamido-2-deoxy-α-D-glucopyranosyl P^2-dolichyl pyrophosphate (**5**), which was characterized by t.l.c. in a variety of solvent systems, by i.r. spectroscopy, by elemental analysis, and by hydrolysis with alkali and with acid. Liberation of 2-acetamido-2-deoxy-D-glucopyranosyl phosphate after alkaline hydrolysis indicated the presence of a pyrophosphate bond.



Compound **5**, lacking the allylic phosphate group (because dolichol contains a saturated, α-isoprene residue), was much more stable than the previously synthesized[6] ficaprenyl derivative (**6**). The dolichyl and ficaprenyl derivatives were well separated by t.l.c. in systems consisting of mixtures of chloroform, methanol, and water; otherwise, good separations were not achieved (see Table I). In the presence of

TABLE I

COMPARISON OF MOBILITY, IN T.L.C. ON SILICA GEL, OF P^1-2-ACETAMIDO-2-DEOXY-α-D-GLUCOPYRANOSYL P^2-DOLICHYL PYROPHOSPHATE (**5**) AND P^1-2-ACETAMIDO-2-DEOXY-α-D-GLUCOPYRANOSYL P^2-FICAPRENYL PYROPHOSPHATE (**6**)

Solvent system[a]	R_F *value*[b]	
	5	**6**
Chloroform–methanol–water (60:25:4) (A)	0.21	0.10
Chloroform–methanol–water (60:35:6) (B)	0.64	0.37
2,6-Dimethyl-4-heptanone–acetic acid–water (20:15:2) (D)	0.53	0.4–0.5[c]
2-Propanol–15M ammonium hydroxide–water (6:3:1) (E)	0.80[c]	0.80
Chloroform–methanol–15M ammonium hydroxide–water (65:35:4:4) (F)	0.25	0.23

[a]All proportions are volume to volume. [b]The R_F value was calculated from the distance from the origin of the chromatogram to the point of maximum intensity of the spot (anisaldehyde spray). [c]With streaking.

concentrated ammonium hydroxide, the ficaprenyl derivative **6** proved to be more stable than the dolichyl compound (**5**). When compound **5** was treated with hot, dilute, propanolic sodium hydroxide solution, rapid hydrolysis occurred, to give mainly dolichyl phosphate and 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate. This result contrasts markedly with that of the alkaline hydrolysis of P^1-α-D-mannopyranosyl P^2-dolichyl pyrophosphate[18], where the glycosyl phosphate bond was predominantly cleaved.

The hydrolysis of P^1-2-acetamido-2-deoxy-α-D-glucopyranosyl P^2-dolichyl pyrophosphate (**5**) in dilute acid followed a course distinct from that[6] of the ficaprenyl derivative **6**. Brief treatment of **5** with dilute hydrochloric acid in aqueous methanol–chloroform at 80–100° caused either (*a*) scission of the pyrophosphate bond, or (*b*) cleavage of the glycosyl phosphate bond; of the two competing mechanisms, the latter occurred somewhat more rapidly than the former. Prolongation of the acid treatment caused hydrolysis of the dolichyl pyrophosphate formed in reaction (*b*), giving dolichyl phosphate and inorganic phosphate. As expected, the lipid–phosphate bond in this compound is very stable, whereas, in the ficaprenyl derivative, this bond is extremely labile to acid[6]. Hydrolysis of the ficaprenyl derivative cleaved both the ficaprenyl phosphate and the glycosyl phosphate bonds, and degraded and rearranged the ficaprenyl residue. It is evident from the stability of the dolichol–phosphate bond in **5** that the formation of a glycosyl phosphate under the hydrolytic conditions described is proof of the presence of a pyrophosphate bridge in this compound.

EXPERIMENTAL

General methods. — Optical rotations were determined in 1-dm, semimicro tubes with a Perkin–Elmer polarimeter, Model 141. I.r. spectra were recorded with a Perkin–Elmer spectrophotometer, Model 237. The cation-exchange resin used was AG 50W X-8 (Bio-Rad Lab., Richmond, California 94804), and, in all cases, the

amount of resin used was in an at least two-fold excess over the quantity necessary to obtain complete ion-exchange. T.l.c. was performed on precoated plates of Silica Gel G (Merck, Darmstadt, Germany) or Cellulose F (Merck); the plates supplied (20 × 20 cm) were cut to a length of 6 cm, and used without pretreatment. However, in experiments in which one (or more) of the samples was applied in aqueous solution to the plate, all the other samples were treated with water (1–2 μl) before the plate was dried in a current of air or nitrogen, prior to development. T.l.c. refers to thin-layer chromatography on Silica Gel, unless otherwise stated. Preparative t.l.c. was conducted on precoated, PLC plates, Silica Gel F 254 (Merck). The spray reagent used, unless otherwise stated, was 1:1:18 anisaldehyde–sulfuric acid–ethanol[19], and the plates were heated to 125°. The spray reagent used to detect unsaturation was 1% aqueous potassium permanganate in 2% aqueous sodium carbonate. The spray reagent of Dittmer and Lester[20] was used to detect phosphate groups. Solvent systems used for t.l.c. were: A, 60:25:4 chloroform–methanol–water; B, 60:35:6 chloroform–methanol–water; C, 10:10:3 chloroform–methanol–water; D, 20:15:2 2,6-dimethyl-4-heptanone–acetic acid–water; E, 6:3:1 2-propanol–15M ammonium hydroxide–water; F, 65:35:4:4 chloroform–methanol–15M ammonium hydroxide–water; and G, 49:1 toluene–methanol. All proportions are volume to volume. Evaporations were conducted under diminished pressure with the bath temperature below 30°. Toluene and pyridine were dried over calcium hydride before use.

The microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, Tennessee 37921. The proportion of 2-acetamido-2-deoxy-D-glucose in compound **4** (80 μg) was determined by methanolysis followed by g.l.c. The proportion of acid-labile phosphate was determined after digestion of the sample (48 μg) with conc. hydrochloric acid for 4 h at 100°, followed by removal of the hydrochloric acid under vacuum. The details of both determinations were as previously described for the ficaprenyl derivative[6].

P^1-*Dolichyl* P^2-*diphenyl pyrophosphate* (**2**). — Dolichyl phosphate[17] (dipotassium salt, from 28 mg of dolichol), used without chromatographic purification, was converted into the pyridinium form (**3**) by dissolving in chloroform (2 ml), adding methanol to the point of turbidity, and stirring overnight with a cation-exchange resin (pyridinium$^+$). After addition of methanol (3 ml), the resin was filtered off, and washed with 2:1 chloroform–methanol. The filtrates were combined, treated with tributylamine (8 mg), and evaporated; three additions and evaporations of toluene (2 ml) gave tributylammonium dolichyl phosphate, which was dissolved in 1,2-dichloroethane (2 ml) and treated with tributylamine (6 mg). The mixture was cooled to −10°, stirred, treated with a solution of diphenylphosphorochloridate (6 mg) in 1,2-dichloroethane (0.6 ml), with exclusion of moisture, and kept at room temperature for 2 h, when analysis by t.l.c. showed the formation of a single product, R_F 0.73 (5:1 chloroform–methanol), with traces of material near the origin. Methanol (1 ml) was added, and the mixture was kept for a further 30 min at room temperature. Evaporation, followed by three additions and evaporations of toluene, gave the desired dolichyl diphenyl pyrophosphate (tributylammonium form, **2**).

P^1-2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-glucopyranosyl P^2-dolichyl pyrophosphate (**4**). — 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl dihydrogen phosphate[6] (20 mg) was converted into the bis(tributylammonium) salt **1** by dissolution in methanol (2 ml) and treatment with tributylamine (20 mg). After addition of water (0.5 ml), the excess of tributylamine was removed by three extractions with hexane. The aqueous methanol solution was evaporated, and three additions and evaporations of toluene gave the desired compound as a syrup. A mixture of **1** and **2** was dried by two additions and evaporations of toluene, and then dissolved in a solution of pyridine (4 mg) in 1,2-dichloroethane (0.4 ml). The mixture was kept for 48 h at room temperature, when t.l.c. (performed on an aliquot treated with a cation-exchange resin, pyridinium$^+$ form) showed the formation of a major product, R_F 0.53 (solvent A), together with traces of byproducts moving faster than the main spot, and a considerable proportion of material having the same R_F (0.8) as **2**. In this solvent system, t.l.c. did not clearly separate the main product from dolichyl phosphate, but a good separation was achieved in solvent D, which showed that dolichyl phosphate (R_F 0.71) was also a byproduct in the formation of **4** (R_F 0.6).

The reaction mixture was diluted with chloroform (20 ml), and extracted three times with water (5 ml) to remove unreacted **1**. The chloroform solution was dried (magnesium sulfate), and evaporated to yield crude **4** (36 mg). A solution of the product in 5:1 chloroform–methanol (2 ml) was applied to a preparative t.l.c. plate (20 × 10 cm), which was developed in solvent A. The plate was dried, and the band (2–2.5 cm) containing the expected product was located by spraying a narrow zone near the center with (*a*) the potassium permanganate spray and (*b*) the phosphate-specific spray. It was necessary to distinguish between the desired band and another band (containing diphenyl phosphate) that had migrated farther on the plate and that gave an intense reaction with the phosphate-specific spray. After the sprayed area (blue) had been carefully discarded, the silica gel was removed from the plate and extracted with solvent C by stirring overnight. The solution was filtered through Celite, and the residue washed well with solvent C. Evaporation of the combined filtrates gave a residue that was extracted with 5:1 chloroform–methanol. The extract was filtered and evaporated, to give the fully acetylated, pyrophosphoric diester **4** (8 mg, tributylammonium form). Extraction of an additional band (~1 cm wide), from the area of the chromatogram adjoining the main band on the side of the origin, gave an additional yield of **4** (4 mg), slightly less pure according to t.l.c. (solvent A); **4** was a syrup, $[\alpha]_D^{20}$ +2.0° (*c* 0.4, 5:1 chloroform–methanol); ν_{max}^{film} 2965 (CH_3), 2930 and 2860 (CH_2), 1745 (C=O, acetyl), 1660 (C=C), 1450 (–CH_2, –CH_3), 1375 (CCH_3), 1230 [P=O, O–C–(C=O)], 1140 (CH_3CCH_3), and 930 cm^{-1} (P–O–P); pure according to t.l.c. in solvents A and D with the anisaldehyde, potassium permanganate, and phosphate-specific spray reagents, except for the presence of a trace of dolichyl phosphate.

Anal. Ratio of 2-acetamido-2-deoxy-D-glucosyl residue to phosphate group; Calc.: 1:2. Found: 1:2.14 (average of 2 determinations).

P^1-2-Acetamido-2-deoxy-α-D-glucopyranosyl P^2-dolichyl pyrophosphate (**5**). —

A solution of the fully acetylated pyrophosphoric diester (**4**, 8 mg; pure material obtained by preparative t.l.c.) in 2:1 chloroform–methanol (2 ml) was treated with 1% sodium methoxide in methanol, to give a strongly basic solution. The mixture was kept for 30 min at room temperature, when t.l.c. (solvent A) showed that **4** (R_F 0.53) had been converted into a new product (R_F 0.21). A small excess of cation-exchange resin (pyridinium$^+$ form, prewashed with 2:1 chloroform–methanol) was added, and the mixture was stirred overnight at room temperature. The resin was removed by filtration through sintered glass, and washed with 2:1 chloroform–methanol, and the filtrates were combined and evaporated; three additions and evaporations of toluene gave **5** (7 mg). Compound **5** showed a single major spot on t.l.c. in solvents A, B, D, and F, with the anisaldehyde, potassium permanganate, and phosphate-specific spray reagents; it contained a trace of dolichyl phosphate, moving ahead of the main spot in solvents A, B, and D, and behind it in solvent F. Compound **5** was amorphous; ν_{max}^{film} 3350 (OH, NH), 2965 (CH_3), 2930 and 2860 (CH_2), 1660 (broad, C=C, and C=O, amide), 1450 (–CH_2, –CH_3), 1375 (CCH_3), 1230 [P=O, O–C–(C=O)], and 925 cm^{-1} (P–O–P).

Anal. Calc. for $C_{103}H_{172}NO_{12}P_2$: C, 73.65; H, 10.34. Calc. for $C_{103}H_{172}NO_{12}P_2 \cdot 3.5\ CH_3OH$: C, 71.41; H, 10.50. Found: C, 71.59; H, 11.35.

Because of the hygroscopic properties and instability of **5**, and the small quantities available (owing to the scarcity of dolichol), a correct value for the percentage of H was not obtained.

Similar treatment of the less pure fraction of **4** (4 mg), obtained from the preparative t.l.c., with 1% sodium methoxide in chloroform–methanol gave a product which contained, as shown by t.l.c. (solvent A), in addition to the main spot (R_F 0.21), several minor components ahead of and behind it, and some material at the solvent front. Therefore, the product was purified by preparative t.l.c. on a plate (20 × 2.5 cm) in solvent B, without treatment with cation-exchange resin. The detection of the location, and the extraction, of the band containing the product were performed as in the purification of compound **4**. After evaporation of the extract (solvent C), the residue was dissolved in 2:1 chloroform–methanol, and the solution was filtered through a sintered glass before evaporation to yield compound **5** (2 mg, disodium salt); t.l.c. (solvent B) showed the presence of a trace of a component having R_F 0.25, in addition to the main spot (R_F 0.64).

Properties of compound **5**. — P^1-2-Acetamido-2-deoxy-α-D-glucopyranosyl P^2-dolichyl pyrophosphate (**5**) is insoluble in methanol, slightly soluble in chloroform, and very soluble in mixtures of chloroform and methanol (20:1–2:1). It is much more stable than the corresponding ficaprenyl derivative[6] **6**, and neither evaporation nor storage overnight at room temperature causes noticeable decomposition. T.l.c. showed no appreciable decomposition after a solution of the compound in chloroform–methanol had been stored for several weeks at −15°.

The synthetic dolichyl and ficaprenyl pyrophosphoric diesters were poorly separated by solvents D and F, but well separated by solvents A and B (see Table I). Although solvent E gave no separation in terms of R_F value, the two compounds could

be distinguished by differences in their stability in this solvent. Compound **5** exhibited serious streaking, and the appearance of some material at the origin of the chromatogram indicated that decomposition had occurred in the alkaline medium. In contrast, P^1-2-acetamido-2-deoxy-α-D-glucopyranosyl P^2-ficaprenyl pyrophosphate (**6**) gave a discrete spot, indicating relative stability to the alkaline conditions.

Acid hydrolysis of compound **5**. — (*a*). A solution of compound **5** (1 mg) in 10:10:3 chloroform–methanol–0.08M hydrochloric acid (0.2 ml) was kept for 5 min in a sealed tube at 80°. The tube was cooled and opened, and a 50-μl aliquot was neutralized with a solution of 1% sodium methoxide in methanol. T.l.c. (solvent A) showed that ~50% of the starting material had been hydrolyzed to a mixture of dolichyl phosphate (R_F 0.63) and dolichyl pyrophosphate[18] (R_F 0.14). In solvent E, dolichyl phosphate and **5** were not separated (R_F 0.8, with streaking), but t.l.c. revealed the formation of dolichyl pyrophosphate and 2-acetamido-2-deoxyglucose as two close-running spots, R_F 0.55–0.58. T.l.c. (solvent C) showed the formation of 2-acetamido-2-deoxyglucose (R_F 0.62) [*cf.*, compound **5** (R_F 0.87)] and a trace of 2-acetamido-2-deoxyglucopyranosyl phosphate (R_F 0.18–0.25), the latter compound being detected by the phosphate-specific spray. Owing to the streaking of the spot, the anomeric configuration could not be distinguished.

(*b*). The remaining solution (0.15 ml) from (*a*) was evaporated under a stream of nitrogen, and the residue dissolved in 10:10:3 chloroform–methanol–0.8M hydrochloric acid (0.15 ml) in a tube. The tube was sealed, kept for 3 min at 80°, cooled, and a 50-μl aliquot neutralized with a 1% solution of sodium methoxide in methanol. T.l.c. in the same solvents as in (*a*) showed that at least 90% of the starting material had been hydrolyzed to give the same products as in (*a*). As 2-acetamido-2-deoxy-D-glucose gives a faint spot with the anisaldehyde spray, it was possible to confirm its identity as a product of hydrolysis of **5** by cellulose t.l.c. in 7:1:2 1-propanol–ethyl acetate–water (v/v). Spots were detected by the chlorine–starch–potassium iodide method[6,21], which revealed the presence of a methyl glycoside (R_F 0.45), as well as 2-acetamido-2-deoxyglucose (R_F 0.33). Under identical conditions of acid hydrolysis, the ficaprenyl derivative[6] gave 2-acetamido-2-deoxy-D-glucose, the same methyl glycoside, 2-acetamido-2-deoxy-α-D-glucopyranosyl pyrophosphate, inorganic pyrophosphate, and a trace of ficaprenol, together with a mixture of hydrophobic, degradation products.

(*c*). The rest of the solution from (*b*) was kept in a sealed tube for 1 h at 80°. T.l.c. (solvents C and E) was performed without neutralization, and the plates were examined by the use of the phosphate-specific spray. The presence of 2-acetamido-2-deoxy-α-glucopyranosyl phosphate (R_F 0.18) was clearly observed in both chromatograms. The proportion of this compound in the hydrolyzate was much greater than in that in (*a*). The t.l.c. also indicated the formation of inorganic phosphate. The complete hydrolysis of dolichyl pyrophosphate to dolichyl phosphate was shown by t.l.c. in solvents A and E, and the absence of free dolichol by t.l.c. in solvent G.

Alkaline hydrolysis of compound **5**. — Compound **5** (0.5 mg) was treated with a solution (0.1 ml) of M sodium hydroxide in 1-propanol (1:10). The mixture was heated

to 80° in a sealed tube, whereupon the synthetic glycolipid **5** immediately dissolved in the alkaline medium. The solution was kept at 80°, and examined by t.l.c. after elapse of various time intervals. (a) After 5 min, t.l.c. (solvent A) showed that ~25% of the starting material (R_F 0.21) had been hydrolyzed to afford dolichyl phosphate (R_F 0.63); no dolichyl pyrophosphate was detectable. (b) After 10 min, t.l.c. in solvent A indicated that ~60% of **5** had been hydrolyzed to give dolichyl phosphate; t.l.c. in solvent C showed no 2-acetamido-2-deoxyglucose, but indicated the presence of a compound giving a positive reaction with the anisaldehyde and phosphate-specific spray reagents, and remaining near the origin of the chromatogram. (c) After 20 min, t.l.c. in solvent A showed that at least 80% of **5** had been hydrolyzed to give dolichyl phosphate, but t.l.c. in solvent G showed that no free dolichol had been liberated. The 1-propanol was evaporated under a stream of nitrogen, and water (10 μl) was added to the residue. T.l.c. (solvent C) of this solution showed two faint spots corresponding to a standard of 2-acetamido-2-deoxy-D-glucose (R_F 0.56, 0.62), and an intense spot near the origin. In order to ascertain whether this major product was 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate (the retarded mobility on t.l.c. being due to the presence of sodium hydroxide), t.l.c. was performed in solvent E, which gave an intense spot (R_F 0.18) corresponding to that for the standard 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate.

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