[8] Chemical Synthesis of Dolichyl Phosphate and Dolichyl Glycosyl Phosphates and Pyrophosphates or "Dolichol Intermediates"

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Chemical synthesis of "lipid intermediates" has the advantage that it yields relatively large quantities of pure compounds, with totally defined structures. The synthetic "intermediates" can then be employed (a) for comparison with biosynthesized materials, i.e., as "standards" for their identification; (b) to firmly establish physical and chemical properties, e.g., to determine pathways of hydrolytic breakdown, and (c) to explore their ability to participate in biosynthetic reactions. An extension of (c) is the use of the synthetic compounds as exogenous acceptors for purposes of enzyme activity assay and of determination of substrate specificity.

General Considerations

Phosphate Diesters

Principle. The synthesis of monophosphate "dolichol intermediates" is achieved by the coupling of a glycosyl phosphate with dolichol in the presence of a "condensing reagent," the purpose of which is the "activation" of the glycosyl phosphate. The most successful reagent is 2,4,6-triisopropylbenzenesulfonyl chloride.[1] The glycosyl phosphate must have the correct anomeric configuration and be fully protected by groups that can be readily removed after phosphate diester formation, without affecting the diester linkage. We have found that *O*-acetyl groups are suitable for this purpose, with the additional advantage that fully acetylated sugar phosphates have a greatly enhanced solubility in the preferred condensing medium, anhydrous pyridine.[1]

The chemical synthesis of a dolichyl glycosyl phosphate therefore consists of two main stages: (1) synthesis of a per-*O*-acetylglycosyl phosphate with the required anomeric configuration, and (2) coupling with dolichol followed by *O*-deacetylation.

Pyrophosphate Diesters

Principle. As it is not possible to synthesize pyrophosphate diesters by a direct coupling reaction between two phosphate monoesters, a two-stage process was devised in which one of the monoesters is first

[1] R. Lohrmann and H. G. Khorana, *J. Am. Chem. Soc.* **88**, 829 (1966).

converted into an intermediate morpholidate[2] or diphenylpyrophosphate ester.[3] For the synthesis of dolichyl glycosyl pyrophosphates, we have found that the second method[3] gives the best result. Thus, the following stages are involved: (a) Synthesis of a peracetylglycosyl phosphate having the required anomeric configuration, (b) synthesis of dolichyl phosphate, (c) conversion of dolichyl phosphate into P^1-dolichyl P^2-diphenyl pyrophosphate, (d) treatment of P^1-dolichyl P^2-diphenyl pyrophosphate with the peracetylglycosyl phosphate and isolation of a peracetyl pyrophosphate diester, and (e) *O*-deacetylation of the product.

In section **Procedures,** a description of the detailed synthetic procedure for each compound will be followed by a short discussion of properties. In each case, the original procedure has been revised, where possible, to increase yields and reproducibility or to simplify the method. The compounds to be discussed are: (1) dolichyl β-D-mannopyranosyl phosphate; (2) dolichyl β-D-glucopyranosyl phosphate; (3) dolichyl phosphate; (4) P^1-2-acetamido-2-deoxy-α-D-glucopyranosyl P^2-dolichyl pyrophosphate; (5) P^1-2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-α-D-glucopyranosyl P^2-dolichyl pyrophosphate.

Materials

Dolichol (isolated from pig liver) is obtained from Serdary Biochemicals, London, Ontario (Canada). The solution as delivered is evaporated in a weighed tube under high vacuum to obtain a dry weight, and then dolichol is redissolved in hexane to give a solution of known concentration, from which aliquots are withdrawn. 2,4,6-Triisopropylbenzenesulfonylchloride (TPS), *o*-phenylenephosphorochloridate, diphenylphosphorochloridate, tetraethylammonium chloride, and dibenzyl phosphate, are obtained from the Aldrich Chemical Co., Milwaukee, WI. Crystalline phosphoric acid and 10% palladium-on-charcoal hydrogenation catalyst are obtained from Fluka A. G., Buchs S. G., Switzerland. The cation-exchange resin used is AG 50W-X8 from Bio-Rad Laboratories, Richmond, CA. Acetyl chloride, calcium hydride (lumps), tetrahydrofuran (THF), toluene, *p*-dioxane, pyridine, 2,6-dimethylpyridine, and 2,4,6-trimethylpyridine are purchased from the Fisher Chemical Co., Fair Lawn, New Jersey.

General Methods

Thin-layer chromatography (TLC) is performed on precoated plates (0.25 mm) of Silica Gel G (E. Merck A. G., Darmstadt, Germany). The

[2] J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.* **83,** 649 (1961).
[3] A. M. Michelson, *Biochim. Biophys. Acta* **91,** 1 (1964).

plates are cut to a length of 6 cm when used for analytical purposes; otherwise they are not pretreated. Preparative-layer chromatography is performed on thick-layer plates (0.5 mm or 2 mm) from the same source. The spray reagent generally used is anisaldehyde–sulfuric acid–ethanol 1 : 1 : 18 (anisaldehyde reagent), and the plates are heated to 125°[4]; the phosphate-specific spray reagent is the one described by Dittmer and Lester,[5] and unsaturation is detected by spraying the plates with a 1% aqueous solution of potassium permanganate in 2% aqueous sodium carbonate (permanganate reagent). Solvent systems for chromatography and extraction purposes are: A, B, and C, chloroform–methanol–water (60 : 25 : 4), (60 : 35 : 6), and (10 : 10 : 3), respectively; D, 2,6-dimethyl-4-heptanone–acetic acid–water (20 : 15 : 2); E, 2-propanol–15 *M* ammonium hydroxide–water (6 : 3 : 1); and F, chloroform–methanol–15 *M* ammonium hydroxide–water (65 : 35 : 4 : 4). All proportions of solvents are v/v. The R_f values are calculated from measurement of the distance from the origin to the point of maximum intensity of the spot.

Tetrahydrofuran, toluene, pyridine, 2,6-dimethylpyridine, and 2,4,6-trimethylpyridine are distilled when necessary, and dried over calcium hydride before use. *p*-Dioxane is heated under reflux over metallic sodium, then distilled and stored over calcium hydride. Acetone, dichloromethane, and 1,2-dichloroethane are dried over molecular sieve before use.

Evaporations are conducted *in vacuo,* with bath temperatures kept below 30°, and when evaporations are for drying purposes, an oil pump with CO_2–acetone trap is employed. The melting points quoted in the procedures were determined with a Mettler FP-2 apparatus; optical rotations were determined in 1-dm semimicro tubes with a Perkin–Elmer Model 141 Polarimeter; infrared spectra were recorded with a Perkin–Elmer Model 237 spectrophotometer; and nuclear magnetic resonance (NMR) spectra were recorded at 60 MHz with a Varian T-60 spectrometer.

Procedures

Dolichyl β-D-mannopyranosyl Phosphate[6]

Preparation of 2,3,4,6-Tetra-O-acetyl-β-D-mannopyranosyl Phosphate. This procedure consists of the stereospecific conversion of

[4] P. J. Dunphy, J. D. Kerr, J. F. Pennock, K. J. Whittle, and J. Feeney, *Biochim. Biophys. Acta* **136,** 136 (1967).

[5] J. C. Dittmer and R. L. Lester, *J. Lipid Res.* **5,** 126 (1964).

[6] C. D. Warren, I. Y. Liu, A. Herscovics, and R. W. Jeanloz, *J. Biol. Chem.* **250,** 8069 (1975).

2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl chloride into 2,3,4,6-tetra-*O*-acetyl-β-D-mannopyranose, followed by phosphorylation with *o*-phenylenephosphorochloridate.[7] This reagent is chosen because the phosphorylation is very rapid, so that anomerization is avoided.

A mixture of 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl chloride[8] (1.46 g), anhydrous ether (6 ml), and silver carbonate (0.975 g) is vigorously stirred and carefully treated, over a 20-min period, with a suspension of water (0.06 ml) in ether (6 ml). The mixture is then stirred for a further 30 min; during this time solid material that collects on the sides of the reaction vessel is removed by washing with ca. 1-ml portions of ether. The reaction mixture is filtered (Celite). In order to extract some 2,3,4,6-tetra-*O*-acetyl-β-D-mannopyranose that remains associated with the solid material, the latter is scraped from the Celite pad and quickly extracted with dry acetone (4.5 ml). After refiltration (using original Celite pad), the filtrates are combined and evaporated, and the syrupy residue is treated with anhydrous ether (5 ml). At this point, crystallization of 2,3,4,6-tetra-*O*-acetyl-β-D-mannopyranose[8] is almost immediate. If no crystals have appeared after 30 min, anomerization has occurred and the first part of the procedure must be repeated with extra care. After 1 hr the crystals are filtered off and dried in a vacuum desiccator over phosphorus pentaoxide. The yield is 0.95 g; m.p. 105°–110°, $[\alpha]_D^{21}$ −12° (*c* 10.0, chloroform). If the melting point is lower than this or the optical rotation value is significantly higher, the product probably contains a large proportion of α anomer, and the first part of the procedure must be repeated, because any attempts at recrystallization may cause more anomerization.

Phosphorylation is achieved by dissolving 2,3,4,6-tetra-*O*-acetyl-β-D-mannopyranose (0.14 g) in dry THF (1.2 ml) containing 2,4,6-trimethylpyridine (84 μl), cooling to 0° with stirring, and quickly adding a solution of *o*-phenylenephosphorochloridate (0.12 g) in dry THF (0.75 ml). The reaction mixture is allowed to attain room temperature, and the stirring is continued for 25 min, after which more THF (1–2 ml) is added, and the precipitate of 2,4,6-trimethylpyridinium hydrochloride is filtered off and washed with a small amount of THF. The combined filtrates are treated with THF (1.2 ml) containing 2,4,6-trimethylpyridine (84 μl) and water (20 μl), and the mixture is kept at room temperature for 30 min before evaporation of the solvents. The residual gum is dissolved in aqueous triethylammonium hydrogencarbonate buffer, pH 7.5 (15 ml, prepared by passing CO_2 gas into 0.2 *M* aqueous triethylamine); the resulting solution is poured into a separatory funnel and treated with bromine (0.25 ml). The

[7] H. S. Prihar and E. J. Behrman, *Carbohydr. Res.* **23**, 456 (1972).
[8] W. A. Bonner, *J. Am. Chem. Soc.* **80**, 3372 (1958).

mixture is shaken vigorously, care being taken to release evolved CO_2; after 2–3 min the excess bromine is removed by 4 or 5 extractions with toluene (10 ml each). The aqueous phase, containing 2,3,4,6-tetra-*O*-acetyl-β-D-mannopyranosyl phosphate, is treated with pyridine (2 ml), and evaporated, and the residue is dried by 2 or 3 additions and evaporations of toluene (2 ml each). This residue contains, together with required glycosyl phosphate, a large proportion of noncarbohydrate material, much of which is removed at this stage by trituration with 1,2-dichloroethane (15–20 ml), and filtration through Celite. The filtrate is evaporated, and the residue (0.6 g) is dissolved in water (20 ml). The resulting solution is extracted three or four times with chloroform (10–15 ml each) to remove unphosphorylated carbohydrate contaminants. The aqueous solution is then passed slowly through a column (12 × 1 cm) of cation-exchange resin (pyridinium form), the column is washed with water, and the combined eluates are treated with pyridine (5 ml) and evaporated. Residual pyridine is removed by three additions and evaporations of toluene (2 ml each), and the residue is triturated with 1,2-dichloroethane. The clear supernatant is decanted from the semisolid residue to give 2,3,4,6-tetra-*O*-acetyl-β-D-mannopyranosyl phosphate (0.25 g). This product shows a single major spot on TLC (R_f 0.39; solvent B) with the anisaldehyde and phosphate-specific reagents, but contains a trace of the α anomer, with slightly lower R_f. Purification is performed by preparative-layer chromatography on two 20 × 20 cm plates (thickness 2 mm). The plates are eluted with solvent B, and the band containing the product is located with the phosphate-specific reagent. After removal from the plate, the silica gel is stirred overnight with solvent C, and the suspension is filtered (Celite) and evaporated. The residue is extracted by trituration with chloroform–methanol (1 : 1), and filtration and evaporation gives pure 2,3,4,6-tetra-*O*-acetyl-β-D-mannopyranosyl phosphate (70 mg, pyridinium form), $[\alpha]_D^{21}$ −13° (*c* 1.5, methanol).

Coupling Reaction with Dolichol. A mixture of 2,3,4,6-tetra-*O*-acetyl-β-D-mannopyranosyl phosphate (9 mg, pyridinium form) and dolichol (12 mg) is dried by five additions and evaporations of toluene (1 ml each), and TPS (10 mg) is added. After several more additions and evaporations of toluene for drying purposes, the mixture of reactants is quickly treated with dry pyridine (0.2 ml), and the resulting solution is kept at room temperature for 5 days with total exclusion of moisture. After treatment with methanol (0.2 ml), the reaction mixture is kept overnight at room temperature, and then the solvents are evaporated. The residue is dissolved in chloroform (10 ml), and the resulting solution is washed three times with water (5 ml each) to remove per-*O*-acetyl-β-D-mannopyranosyl phosphate and pyrophosphate (a by-product). The chloroform solution is

dried over magnesium sulfate, and evaporation gives a crude product that is purified by preparative-layer chromatography on a 20 × 5 cm plate (2 mm thick), eluted with chloroform–methanol (5 : 1). The band containing the required per-*O*-acetyl phosphate diester is located with the anisaldehyde and phosphate-specific spray reagents; after removal from the plate, the silica gel is stirred overnight with solvent C. Filtration (Celite) and evaporation give a residue that is triturated with chloroform–methanol (5 : 1). Filtration and evaporation yields 2,3,4,6-tetra-*O*-acetyl β-D-mannopyranosyl dolichyl phosphate (11 mg, pyridinium form), TLC R_f 0.45 (chloroform–methanol 5 : 1), 0.80 (solvent A), a single spot according to the three spray reagents (see General Methods). The compound is deacetylated by dissolution in chloroform–methanol (5 : 1) and treatment at room temperature with an excess of 1% sodium methoxide in dry methanol (pH paper). After 30 min, the excess base is neutralized with cation-exchange resin (pyridinium), and then the resin is filtered off and washed (chloroform–methanol, 5 : 1). Evaporation of the resulting solution gives dolichyl β-D-mannopyranosyl phosphate. TLC shows that this product contains several minor compounds, which can be eliminated by preparative TLC on a 20 × 20-cm plate with solvent A. The location of the compound on the plate (R_f 0.57) and extraction from the silica gel are performed as described for the per-*O*-acetyl compound, to give pure dolichyl β-D-mannopyranosyl phosphate (8 mg).

The synthetic compound is amorphous, with no measurable optical rotation, and an infrared spectrum (film) shows maxima at 3300, 3040, 2940, 2740, 1730, 1655, 1450, 1378, 1220, 1070, 1020, 923, and 835 cm^{-1}. The compound shows a single spot on TLC in solvents A (R_f 0.57), D (R_f 0.60), and F (R_f 0.54) (using the three spray reagents described in General Methods). In solvent A it migrates slightly ahead of dolichyl α-D-mannopyranosyl phosphate,[9] and in solvents D and F, slightly behind the α-linked compound.

Dolichyl β-D-mannopyranosyl phosphate is stable when stored at −15° in chloroform–methanol solution in the dark in a sealed tube. When the compound is dissolved in solvent C and kept at 90° for 2 hr, TLC shows a ca. 75% decomposition into D-mannose, methyl D-mannopyranoside, β-D-mannopyranose 1,2-phosphate,[6] dolichol, and dolichyl phosphate. When the compound is dissolved in chloroform–methanol–0.08 *M* HCl (10 : 10 : 3) and the mixture is kept at 80° for 5 min, TLC shows that more than 95% is converted into dolichyl phosphate, D-mannose, and methyl D-mannopyranoside. This behavior is similar to that of the α anomer.[9]

When dolichyl β-D-mannopyranosyl phosphate is treated with hot, dilute alkali (propanol–1 *M* sodium hydroxide, 10 : 1, 100°), TLC shows

[9] C. D. Warren and R. W. Jeanloz, *Biochemistry* **12**, 5038 (1973).

that in 5 min at least 50% is converted into dolichol, β-D-mannopyranosyl phosphate, and a trace of D-mannose 2-phosphate.[6] At lower temperatures (e.g., 37°) and longer reaction times (4 hr), the main carbohydrate product is D-mannose 2-phosphate, and TLC also indicates traces of β-D-mannopyranose 1,2-phosphate and β-D-mannopyranosyl phosphate.[6]

When dolichyl α-D-mannopyranosyl phosphate[9] was treated with hot, dilute propanolic alkali, it behaved quite differently, the only identifiable product being dolichyl phosphate.[10]

Dolichyl β-D-Glucopyranosyl Phosphate

Preparation of 2,3,4,6-Tetra-O-acetyl-β-D-Glucopyranosyl Phosphate. This procedure is a modification of the MacDonald[11] fusion method.

A mixture of 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose[12] (1 g) and crystalline phosphoric acid (1.2 g, predried under reduced pressure over magnesium perchlorate for 48 hr) is fused at 65° under high vacuum. The thick, viscous mixture is stirred for 1 min (a strong magnetic stirrer is required), then THF (ca. 5 ml) is added to quickly dissolve the syrupy product. The resulting solution is immediately cooled to −10° and treated with 15 *M* ammonium hydroxide (ca. 1.5 ml) to give pH 6 (pH paper). This neutralization procedure must be performed rapidly; if successful, TLC of the solution (solvent B) will show a major compound with R_f 0.35, and minor compounds with slightly higher and lower R_f values. However, if TLC shows a significant amount of a compound migrating just ahead of the main spot, partial anomerization has occurred, and the phosphorylation should be repeated. (A minor degree of anomerization cannot be avoided, but content of α anomer at this stage should be less than 5%). The THF solution is decanted from a semisolid precipitate and evaporated, after which toluene (2 ml) is added and evaporated twice. The resulting residue is dissolved in chloroform–methanol (1 : 1), inorganic material is filtered off, and the product is purified by preparative-layer chromatography on several 2 mm thick plates (20 × 20 cm), one plate being used for each 100 mg of crude product. Elution is performed with solvent B, after which the plate is dried in air for at least 30 min, and reeluted. The band containing the required compound is located with the phosphate-specific spray reagent, and the silica gel is removed and stirred with solvent C to extract the compound [however, the upper and lower extremities of the band (ca. 10% of the total) are discarded, as they contain impurities]. The suspension is filtered (Celite), and the filtrate is

[10] C. D. Warren and R. W. Jeanloz, *Biochemistry* **14,** 412 (1975).
[11] D. L. MacDonald, *Carbohydr. Res.* **3,** 117 (1966).
[12] M. L. Wolfrom and A. Thompson, *Methods Carbohydr. Chem.* **II,** 212 (1963).

passed through a column of cation-exchange resin (pyridinium form). The column is washed with two volumes of solvent C, and the combined eluates are evaporated to dryness, giving an amorphous residue that is triturated with dichloromethane. The resulting solution is filtered and evaporated, to give 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl phosphate (0.58 g, pyridinium form). TLC of the product (solvent E) indicates the presence of only a trace of the α anomer, R_f values 0.20 (α) and 0.14 (β); $[\alpha]_D^{20} + 3°$ (*c* 2.9, dichloromethane).

Coupling Reaction with Dolichol. A mixture of 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl phosphate (20 mg, pyridinium form) and dolichol (20 mg) is dried in a vacuum desiccator over phosphorus pentoxide for 24 hr and then quickly treated with a solution of TPS (20 mg) in dry pyridine (0.4 ml). The reactants are thoroughly mixed with a Vortex mixer, and the resulting solution is kept at room temperature for 48 hr with total exclusion of moisture. After treatment with methanol (0.5 ml), the mixture is kept overnight at room temperature, and the solvents are evaporated, after which toluene (1 ml) is added and evaporated twice. The residue is dissolved in a small volume of chloroform–methanol (5 : 1) and applied to a preparative-layer plate (20 × 20 cm, 0.5 mm thick) which is eluted with solvent A. The broad band migrating with approximate R_f 0.8 that contains the required per-*O*-acetyl phosphate diester is detected with the potassium permanganate spray reagent, and the product is extracted from the silica gel as described for dolichyl β-D-mannopyranosyl phosphate, to give a crude product (14 mg).

O-Deacetylation is performed by treatment with an excess (pH paper) of 3% sodium methoxide in methanol, plus an equal volume of dry dichloromethane to give a clear solution. After 30 min, TLC shows a major product (R_f 0.56, solvent A); after removal of excess base with cation-exchange resin, the product is purified by preparative TLC as described for dolichyl β-D-mannopyranosyl phosphate, to give dolichyl β-D-glucopyranosyl phosphate (7 mg).

As prepared by this route, the compound is pure according to TLC in solvents A and D, and the R_f values are very close to those of the D-mannosyl derivative.[6] As expected, there is good separation from ficaprenyl β-D-glucopyranosyl phosphate in both solvent systems. Chromatography cannot be performed in solvent F because of extreme lability to alkali. When dolichyl β-D-glucopyranosyl phosphate is treated with 0.1 *M* sodium hydroxide in aqueous propanol and the mixture is kept at 65° for 10 min, TLC shows at least 90% conversion into dolichyl phosphate and a carbohydrate product having the appearance on TLC of an anhydro derivative, but migrating differently from 1,6-anhydro-D-glucose (solvents A and F). In contrast, dolichyl α-D-glucopyranosyl

phosphate is stable for at least 2 hr at 65° when treated similarly. When treated with dilute acid (10 m*M* HCl) in chloroform–methanol–water, hydrolysis of dolichyl β-D-glucopyranosyl phosphate occurs to give D-glucose, methyl D-glucopyranoside, and dolichyl phosphate.

Dolichyl Phosphate[13]

This procedure consists of treatment of dolichol with *o*-phenylenephosphorochloridate and 2,6-dimethylpyridine, hydrolysis of the intermediate triester with aqueous base, and oxidation of the resulting *o*-hydroxyphenyl phosphate with lead tetraacetate. The oxidation step is performed very quickly to avoid unwanted side reactions. A recently introduced,[14] alternative procedure utilizes 2-chloromethyl-4-nitrophenylphosphorodichloridate instead of *o*-phenylenephosphorochloridate.

Dolichol (22 mg) is dried under reduced pressure over phosphorus pentoxide for 24 hr, dissolved in a mixture of 2,6-dimethylpyridine (20 mg) and *p*-dioxane (2 ml); the resulting solution is stirred (magnetic bar). The reaction vessel is fitted with a drying tube and then cooled briefly in an ice–water bath (until the *p*-dioxane is on the point of freezing), and a solution of *o*-phenylenephosphorochloridate (18 mg) in *p*-dioxane (1.8 ml) is added dropwise. For this phosphorylation to be successful, it is essential that all solvents and reagents, and the reaction tube or flask, be perfectly dry. Immediately after addition of the phosphorylating agent, a precipitate of 2,6-dimethylpyridinium hydrochloride will form, and the reaction mixture is then allowed to attain room temperature. After 15 min, the reaction is checked by TLC in (a) chloroform–methanol (5 : 1), which will show the required product R_f ca. 0.5, and (b) in chloroform, showing some material at the solvent front, the product at the origin, and two minor compounds that have the same R_f as dolichol and are not phosphorylated (these contaminants are present in commercial and noncommercial preparations of dolichol). However, it is imperative that an intense spot corresponding to the required product be observed at this stage; otherwise the phosphorylation is a failure owing to the presence of moisture. If the TLC is satisfactory, the reaction mixture is filtered, the solid material is quickly washed with 1–2 ml of *p*-dioxane, and the combined filtrates are treated with 2,6-dimethylpyridine (20 mg) and water (0.4 ml). The mixture is kept for at least 5 min at room temperature, and the solvents are evaporated, after which toluene (2 ml) is added and evaporated 4 times, for total removal of water. The residue is triturated with *p*-dioxane (4 ml), and the resulting solution is decanted or

[13] J. F. Wedgwood, C. D. Warren, and J. L. Strominger, *J. Biol. Chem.* **249,** 6316 (1974).
[14] C. A. Rupar and K. K. Carroll, *Chem. Phys. Lipids* **17,** 193 (1976).

filtered into a dry Kimax tube fitted with a magnetic stirrer (the undissolved material is not the required product). The *p*-dioxane solution of dolichyl *o*-hydroxyphenyl phosphate is now treated with lead tetraacetate (100 mg), and the brown mixture is stirred for 5 min at room temperature before adding 1 *M* KOH in methanol (4 ml) to give pH 13–14, this addition being performed quickly and with vigorous stirring (i.e., not in small portions). After 20 min, TLC (solvent A) will show an intense spot with the mobility of dolichyl phosphate (R_f ca. 0.6), and the solvents are evaporated (N_2 gas) to ca. 2 ml. The brown suspension is directly applied to two 20 × 20 cm (2 mm thick) preparative-layer plates, which are eluted with solvent A, dried in air for 1 hr, and reeluted with the same solvent. The band containing dolichyl phosphate is located with the permanganate reagent (the lower band, less than halfway up the plate), and the product is extracted from the silica gel by stirring overnight with solvent C, followed by filtration (Celite) and evaporation. The residue is triturated with chloroform–methanol (2 : 1), and the resulting solution is treated with cation-exchange resin [200 mg, pyridinium form, prewashed with chloroform–methanol (2 : 1)], and stirred for 2–3 hr. The resin is filtered off and washed with chloroform–methanol (2 : 1), and the combined filtrates are evaporated to yield dolichyl phosphate (12 mg, pyridinium form).

The compound is a syrup, pure according to TLC in solvents A, F, D, and E (3 spray reagents) with infrared maxima at 3400, 2965, 2930, 2860, 1730, 1660, 1450, 1376, 1070, and 835 cm^{-1}.

P^1-2-Acetamido-2-deoxy-α-D-glucopyranosyl P^2-dolichyl Pyrophosphate[15]

Preparation of 2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-glucopyranosyl Phosphate. This compound is best prepared by a modification of the "oxazoline procedure." This method has been shown to give 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate derivatives having the α anomeric configuration.[16,17]

A mixture of 2-methyl-(3,4,6-tri-*O*-acetyl-1,2-dideoxy-α-D-glucopyrano)[2,1-*d*]-2-oxazoline[18] (80 mg) and dibenzyl phosphate (100 mg) is dissolved in 1,2-dichloroethane (1 ml), and the solution is kept at room temperature for 24 hr with total exclusion of moisture, after which TLC (chloroform–methanol 5 : 1) shows a single, major phosphate product, R_f 0.7. The reaction mixture is directly chromatographed on two 20 × 20-cm

[15] C. D. Warren and R. W. Jeanloz, *Carbohydr. Res.* **37**, 252 (1974).
[16] A. Y. Khorlin, S. E. Zurabyan, and T. S. Antonenko, *Tetrahedron Lett.* 4803 (1970).
[17] C. D. Warren, A. Herscovics, and R. W. Jeanloz, *Carbohydr. Res.* **61** (1978) (in press).
[18] R. U. Lemieux and H. Driguez, *J. Am. Chem. Soc.* **97**, 4063 (1975).

(2 mm thick) plates, being eluted with chloroform–methanol (5 : 1). The band containing 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl dibenzyl phosphate is located with the phosphate-specific spray reagent, and the compound is extracted from the silica gel by stirring overnight with solvent C. After filtration (Celite) and evaporation, the residue is triturated with chloroform–methanol (2 : 1), and the resulting solution is filtered and evaporated to give the required dibenzyl glycosyl phosphate (60 mg). The compound is dissolved in methanol (2 ml) and hydrogenated, in the presence of 10% palladium-on-charcoal, in a Parr apparatus at ca. 2 atm pressure. After 2.5 hr the reaction mixture is analyzed by TLC (chloroform–methanol, 5 : 1) to verify that no starting compound remains (phosphate-specific reagent). If the hydrogenolysis is complete, pyridine (0.5 ml) is added, the solution is filtered and evaporated, and toluene (2 ml) is added and evaporated twice to give the required compound (58 mg, pyridinium form). (If hydrogenolysis is not complete, the catalyst is filtered off and replaced by a fresh batch, and hydrogenation is continued for another 2 hr.) 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl phosphate as prepared by this route gives a single major spot on TLC (R_f 0.24, solvent B) and is suitable for synthetic purposes without chromatographic purification. However, it must be converted into the tributylammonium form, by dissolution of the product (20 mg) in methanol (2 ml), and treatment with tributylamine (20 mg). After the addition of water (0.5 ml), excess tributylamine is removed by three extractions with hexane (1 ml). The aqueous methanol solution is evaporated, and the tributylammonium salt of the required glycosyl phosphate is dried by three additions and evaporations of toluene (2 ml each).

Preparation of Dolichyl Phosphate. See above in section on dolichyl phosphate.

Conversion of Dolichyl Phosphate into P^1-Dolichyl P^2-Diphenyl Pyrophosphate. Dolichyl phosphate (pyridinium, 12 mg) is converted into the tributylammonium form by dissolution in chloroform–methanol (2 : 1, 5 ml) and treatment with tributylamine (8 mg). The solvents are evaporated, and toluene (2 ml) is added and evaporated three times. The residue is dissolved in 1,2-dichloroethane (2 ml) and treated with more tributylamine (6 mg); then the reaction vessel is fitted with a drying tube and magnetic stirrer, and the solution is cooled to −10°. The cold solution is stirred and treated with a solution of diphenylphosphorochloridate (6 mg) in 1,2-dichloroethane (0.6 ml), with total exclusion of moisture. When the addition is complete, the reaction vessel is tightly stoppered, and the mixture is kept for 2 hr at room temperature, when TLC (solvent A) shows that the dolichyl phosphate, R_f ca. 0.6, has been converted into

P^1-dolichyl P^2-diphenyl pyrophosphate, R_f ca. 0.85, and a minor by-product migrating near the origin. After the addition of methanol (1 ml), the reaction mixture is kept for a further 1 hr at room temperature, then the solvents are evaporated, and the residue is dried by three additions and evaporations of toluene (2 ml each).

Preparation of P^1-2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-glucopyranosyl P^2-Dolichyl Pyrophosphate. A mixture of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl tributylammonium phosphate (from 20 mg of the pyridinium form) and P^1-dolichyl P^2-diphenyl pyrophosphate (from 12 mg of dolichyl phosphate) is dried by three additions and evaporations of toluene (2 ml each) and dissolved in a solution of pyridine (4 mg) in 1,2-dichloroethane (0.4 ml). The reaction mixture is kept for 48 hr at room temperature, when TLC (solvent A) shows the formation of the required compound, R_f ca. 0.6, together with several by-products and a considerable amount of residual material, R_f ca. 0.85. Processing can be performed in two ways; if necessary, the excess of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl phosphate can be recovered by diluting the reaction mixture with chloroform (20 ml), extracting three times with water (5 ml each), and evaporating the combined aqueous extracts. The chloroform solution containing the per-*O*-acetyl pyrophosphate diester is dried over magnesium sulfate and evaporated; the residue is dissolved in chloroform–methanol (5 : 1, 2 ml) and chromatographed on a preparative-layer plate (2 mm thick, 20 × 10 cm). If recovery of unreacted sugar phosphate is not required, the solvents are evaporated from the reaction mixture, and the residue is dissolved in chloroform–methanol (5 : 1) and chromatographed directly. In either case, elution is performed with solvent A, and the band containing the required compound is detected with the permanganate and phosphate-specific spray reagents. The band migrates about halfway up the plate, and it is important not to confuse it with diphenyl phosphate, which has a higher R_f and gives a very intense blue color with the phosphate reagent. The silica gel is removed from the plate and stirred overnight with solvent C to extract the product. Filtration (Celite) and evaporation gives a residue that is triturated with chloroform–methanol (5 : 1). The resulting solution is filtered and evaporated to yield the required per-*O*-acetyl pyrophosphate diester (8 mg, tributylammonium form). The compound is pure according to TLC in solvents A (R_f 0.53) and D (R_f 0.60) with three spray reagents (see General Methods), and has infrared maxima at 2965, 2930, 2860, 1745, 1660, 1450, 1375, 1230, 1140, and 930 cm^{-1}.

O-Deacetylation. A solution of P^1-2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl P^2-dolichyl pyrophosphate (8 mg) in chloro-

form–methanol (2 : 1) is treated with an excess of 3% sodium methoxide in methanol (pH paper). The mixture is kept for 30 min at room temperature, and treated with a small excess of cation-exchange resin (pyridinium form, prewashed with chloroform–methanol). The resin is filtered off and washed with chloroform–methanol; the combined filtrates are evaporated to give P^1-2-acetamido-2-deoxy-α-D-glucopyranosyl P^2-dolichyl pyrophosphate (7 mg).

The compound is amorphous, but TLC with three spray reagents (see General Methods) shows that it is pure, R_f values being 0.23 (solvent A), 0.42 (solvent B), 0.27 (solvent F), and 0.77 (solvent E). The infrared maxima are 3350, 2965, 2930, 2860, 1660, 1450, 1375, 1230, and 925 cm^{-1}. It can be stored in chloroform–methanol solution at −15° for at least a week, or at room temperature for several hours, after which decomposition starts to occur. When a solution in chloroform–methanol is briefly subjected to treatment with hot dilute hydrochloric acid (0.1 *M* at 80° for 3 min), TLC indicates at least a 90% conversion into dolichyl phosphate, dolichyl pyrophosphate,[10] 2-acetamido-2-deoxy-D-glucose, a methyl glycoside, and some 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate.[15] After a longer period (1 hr), dolichyl pyrophosphate is not observed as a product, as it is converted into dolichyl phosphate and inorganic phosphate. When treated with hot, dilute alkali (0.1 *M* sodium hydroxide in aqueous propanol at 80°), hydrolysis occurs to give a mixture of dolichyl phosphate and 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate. The reaction is ca. 60% complete after 10 min, and at least 80% complete after 20 min.

P^1-2-Acetamido-4-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-α-D-glucopyranosyl P^2-dolichyl Pyrophosphate[17]

Preparation of Per-O-acetyl-di-N-acetyl-α-chitobiosyl Phosphate. This method also employs the "oxazoline procedure" (see above). The required oxazoline is best prepared by an adaptation of the method of Lemieux and Driguez.[18]

Octaacetylchitobiose (impure, obtained by acetolysis of chitin,[19] 200 mg) is treated with an excess of 3% sodium methoxide in methanol (pH paper). The mixture is stirred to give a clear solution, kept for 2 hr at room temperature, and treated with a small excess of cation-exchange resin (pyridinium form). The resin is filtered off and washed with methanol; the combined filtrates are evaporated to dryness. After three additions and evaporations of toluene (2 ml each), the residue is treated

[19] M. Shaban and R. W. Jeanloz, *Carbohydr. Res.* **19**, 311 (1971).

with acetyl chloride (3 ml) and the mixture is stirred for 24 hr at room temperature. The reagent is evaporated, and residual traces are removed by five additions and evaporations of toluene (2 ml each). The residue is dissolved in dry acetonitrile (2 ml) and stirred at room temperature with tetraethylammonium chloride (80 mg) and sodium hydrogencarbonate (80 mg). After 1 hr the solution is diluted with dichloromethane (150 ml), washed twice with water (25 ml) and once with saturated aqueous potassium chloride, and dried over sodium hydrogencarbonate. The dried solution is evaporated, the residue is dissolved in methanol (2.5 ml), and the resulting solution is diluted with ether (50 ml). The precipitate, which is not the required product, is filtered off and washed with ether–methanol (30: 1). The combined filtrates are evaporated to yield an impure oxazoline, showing on TLC (chloroform–methanol, 10: 1) a major spot (R_f 0.48) corresponding to the required compound, plus a number of minor spots mostly derived from impurities in the starting material. These are removed by preparative-layer chromatography on a 2 mm thick plate (20 × 20 cm) which is eluted with chloroform–methanol (10: 1). For detection, a strip is cut from the center of the plate and sprayed (anisaldehyde), and the product is extracted from the silica gel by stirring overnight with chloroform–methanol (2: 1). Filtration and evaporation gives 2-methyl-[4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-*O*-acetyl-1,2-dideoxy-α-D-glucopyranol]-[2,1-*d*]-2-oxazoline (55 mg), $[\alpha]_D^{20}$ −3° (*c* 1.1, dichloromethane); infrared maxima 3280, 3090, 2950, 1745, 1670 (double peak), 1555, 1430, 1375, 1320, 1230, 1170, 1130, 1035, and 945 cm^{-1}; NMR (in chloroform-d) δ 1.27 (1 H), 1.95 (18 H), 3.55 and 4.21 (8 H), and 5.15 (2 H).

A mixture of the oxazoline and dibenzyl phosphate (95 mg) is dissolved in 1,2-dichloroethane (2 ml). The reaction mixture is processed as for the preparation of the dibenzyl phosphate of 2-acetamido-2-deoxy-D-glucose (see above in section on P^1-2-acetamido-2-deoxy-α-D-glucopyranosyl P^2-dolichyl pyrophosphate) to give the required compound (30 mg). Hydrogenation is performed as described in the subsection cited above to give a crude per-*O*-acetyl-di-*N*-acetylchitobiosyl phosphate, which must be purified by preparative-layer chromatography. This is performed on a 2 mm-thick plate (20 × 8 cm) by eluting with solvent C and detecting the required compound with the phosphate-specific spray reagent. The silica gel is extracted overnight by stirring with solvent C and filtered off (Celite). Evaporation of the filtrate gives a residue that is triturated with methanol. After filtration the solution is evaporated to yield 2-acetamido-4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-*O*-acetyl-2-deoxy-α-D-glucopyranosyl phosphate

(13 mg, pyridinium form), an amorphous solid, m.p. 228°–229°, $[\alpha]_D^{20}$ +22° (*c* 0.65, methanol); infrared maxima 3350, 2950, 1745, 1655, 1550, 1375, 1240, 1120, 1045, 950, 905, 845, and 720 cm^{-1}; TLC (solvent B) R_f 0.22. Conversion into the tributylammonium form is by the same method as described in the subsection cited above.

Preparation of Dolichyl Phosphate. See in dolichyl phosphate section.

Preparation of P^1-Dolichyl P^2-Diphenyl Pyrophosphate. See above in subsection on conversion of dolichyl phosphate into P^1-dolichyl P^2-diphenyl pyrophosphate.

Preparation of P^1-2-Acetamido-4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-O-acetyl-2-deoxy-α-D-glucopyranosyl P^2-dolichyl Pyrophosphate. A mixture of per-*O*-acetyl-di-*N*-acetyl-α-chitobiosyl phosphate (tributylammonium form, derived from 20 mg of pyridinium form) and P^1-dolichyl P^2-diphenyl pyrophosphate (from 6 mg of dolichyl phosphate), is dried and then treated with pyridine (4 mg) and 1,2-dichloroethane (0.4 ml) as described for the 2-acetamido-2-deoxy-D-glucose derivative above. Processing and preparative-layer chromatography give the required compound (6 mg, tributylammonium form), pure according to TLC in solvents A (R_f 0.70), D (R_f 0.52), and F (R_f 0.70); infrared maxima are at 3340, 2905, 2930, 2860, 1745, 1655, 1545, 1450, 1375, 1230, 1135, and 930 cm^{-1}.

O-Deacetylation. The per-*O*-acetyl pyrophosphate diester (6 mg) is dissolved in dichloromethane and treated with an excess of 3% sodium methoxide in methanol (pH paper). The reaction is followed by TLC (solvent F); as soon as this indicates a single major product (1–2 hr), the reaction solution is applied directly onto a TLC plate (20 × 20 cm), which is eluted with solvent B. The band containing the required compound is located with the permanganate and phosphate-specific spray reagents, and the silica gel is stirred overnight with solvent C to extract the product. After filtration (Celite) and evaporation, the residue is triturated with chloroform–methanol (2 : 1), and the resulting solution is filtered and evaporated to give P^1-di-*N*-acetyl-α-chitobiosyl P^2-dolichyl pyrophosphate (5 mg, sodium salt). The compound is pure according to TLC in solvent A (R_f 0.11), solvent B (R_f 0.31), solvent F (R_f 0.23), and solvent E (R_f 0.67) with 3 spray reagents as described in General Methods.

The stability of this compound is similar to that of the 2-acetamido-2-deoxy-D-glucose derivative (see above). Brief treatment with hot, dilute acid (10 m*M* hydrochloric acid in chloroform–aqueous methanol, or 25 m*M* in aqueous 2-propanol, 5 min at 93°) causes more than 90% conversion into (mainly) dolichyl pyrophosphate, di-*N*-acetylchitobiose, and a methyl glycoside of the latter. However, some dolichyl phosphate is

also formed. Treatment with hot, dilute alkali (10 m*M* sodium hydroxide in aqueous propanol) also causes hydrolysis, but the products are dolichyl phosphate and di-*N*-acetyl-α-chitobiosyl phosphate. The reaction is 80% complete after 15 min at 85°, but some *N*-deacetylation of the sugar phosphate also occurs.

[9] Covalent Attachment of Glycolipids to Solid Supports and Macromolecules

By WILLIAM W. YOUNG, JR., ROGER A. LAINE, and SEN-ITIROH HAKOMORI

Glycosphingolipids covalently linked to solid supports and macromolecules are useful in the studies of: (1) interaction of glycolipid to cell surface, (2) purification of antiglycolipid antibodies, (3) possible affinity purification of glycosylhydrolases or transferases whose specificity is directed toward glycosphingolipids, and (4) glycosphingolipids as immunogens.

A procedure has been described previously for the coupling of glycolipids to solid supports.[1] That method utilized oxidative ozonolysis of the olefinic bond of the sphingosine moiety of the glycolipid to yield carboxyl-bearing products, which were then coupled to the amino groups of the solid supports. In the present, simplified method, oxidation of the olefinic bond to the carboxyl function is accomplished with potassium permanganate that has been solubilized in benzene by the crown ether dicyclohexyl-18-crown-6.[2,3] Figure 1 illustrates the formation of hematoside acid and its coupling to glass beads as described below.

Reagents

Dowex 50-W X8 200–400 mesh, hydrogen form (Bio-Rad Laboratories, Richmond, California)
Pyridine dried over barium oxide and distilled
Acetic anhydride, reagent grade
Dicyclohexyl-18-crown-6 (Aldrich Chemical Co., Inc., Milwaukee, Wisconsin).

[1] R. A. Laine, G. Yogeeswaran, and S. Hakomori, *J. Biol. Chem.* **249**, 4460 (1974).
[2] D. J. Sam and H. E. Simmons, *J. Am. Chem. Soc.* **94**, 4024 (1972).
[3] G. W. Gokel and H. D. Durst, *Aldrichimica Acta* **9**, 3 (1976).