The Synthesis and Chemical Properties of Polyisoprenyl β-D-Mannopyranosyl Phosphates*

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2,3,4,6-Tetra-O-acetyl-β-D-mannopyranosyl phosphate, free of the α anomer, was coupled with citronellol and dolichol in the presence of trisopropylbenzenesulfonyl chloride to give, after chromatographic purification and deacetylation, the respective polyisoprenyl β-D-mannopyranosyl phosphates. These compounds were compared with the previously synthesized α anomers by means of their chromatographic properties, spectra, optical rotations, and hydrolysis reactions when treated with acid and alkali. To characterize the compounds resulting from these treatments, and to determine the mechanism of the alkaline hydrolysis, β-D-mannopyranosyl phosphate was converted into β-D-mannopyranosyl 1,2-phosphate, and hence into D-mannose 2-phosphate, obtained as a mixture of α and β anomers, characterized by infrared and nuclear magnetic resonance spectra and elemental analysis. β-D-Mannopyranosyl phosphate was readily separated by thin layer chromatography from the corresponding α anomer.

Polyisoprenyl α-[14C]mannosyl phosphates are formed from GDP-α-[14C]mannose by a wide variety of biosynthetic systems and, in some cases, have been shown to act as intermediates in glycoprotein biosynthesis. This activity has been demonstrated in yeast (3, 4), mammalian (5–7), and avian systems (8). As part of a program aimed at the chemical synthesis of isoprenoid "lipid intermediates," dolichyl α-D-mannopyranosyl phosphate was synthesized (9). At the time the synthesis of this compound was undertaken, the anomeric configuration of the mannose phosphate residue in the biosynthesized compounds was unknown, and the reason for selecting the α anomer was the greater degree of accessibility by chemical methods of derivatives of α-D-mannopyranosyl phosphate. In addition, naturally occurring compounds containing a β-D-mannopyranosyl phosphate residue had not, to our knowledge, been identified. Recently, the results of experiments described in the accompanying paper (10) have suggested that the polyisoprenyl mannosyl phosphate formed by calf pancreas (11) contains a β-D-mannopyranosyl linkage. Since the configuration of the glycosyl linkage could have a bearing on the biosynthetic role of the compounds, a synthesis of dolichyl β-D-mannopyranosyl phosphate was undertaken for comparison purposes. The conditions for the synthesis were established by use of citronellol (III) as a short chain model lipid.

RESULTS AND DISCUSSION

For the chemical synthesis of both citronellyl β-D-mannopyranosyl phosphate (VIII) and dolichyl β-D-mannopyranosyl phosphate (X), it was necessary to prepare a fully protected derivative of β-D-mannopyranosyl phosphate and to couple this derivative (II) with the appropriate polyisoprenol. The peracetyl compound was a suitable starting material, as 2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl phosphate had previously been found to perform satisfactorily in the synthesis of the corresponding α-D anomers (9) and in the synthesis of ficaprenyl α-D-mannopyranosyl phosphate (12). 2,3,4,6-Tetra-O-acetyl-β-D-mannopyranosyl phosphate (II) was also the precursor in the synthesis of β-D-mannopyranosyl phosphate (I), β-D-mannopyranosyl 1,2-phosphate (V), and D-mannose 2-phosphate (VI). These three compounds were required as chromatographic standards for the identification of the product of degradative treatments of the synthetic polyisoprenyl β-D-mannopyranosyl phosphates VIII and X, and of the labeled...
mannolipid phosphate formed by calf pancreas (10).

![Chemical Structure](image)

**Scheme 1.**

**Synthesis of 2,3,4,6-Tetra O-acetyl-β-D-mannopyranosyl Phosphate (II)—** The method of preparation of compound II was based on that described by Prihar and Behrman (13) for the synthesis of β-D-mannopyranosyl phosphate. However, in order to obtain a purified tetraacetyl derivative virtually free of the α anomere, according to TLC,¹ the published procedure was modified significantly. When 2,3,4,6-tetra-O-acetyl-β-D-mannopyranose was prepared from 2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl chloride by a modification of the method of Bonner (14) and phosphorylated according to Prihar and Behrman (13), the product was found by TLC to contain some α anomere. When a recrystallized preparation of 2,3,4,6-tetra-O-acetyl-β-D-mannopyranose was used, the proportion of a α anomere in the product rose rather than fell, indicating that some anomerization was taking place during the recrystallization process, despite precautions (14). However, when 2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl chloride was converted into 2,3,4,6-tetra-O-acetyl-β-D-mannopyranose by a modified treatment with silver carbonate in aqueous diethyl ether, a suitable product could be obtained for phosphorylation purposes. It was directly treated, without recrystallization, with o-phenylene phosphorochloridate and collidine in anhydrous tetrahydrofuran, as described by Prihar and Behrman (13), except that the degree of completion of the phosphorylation was monitored by TLC rather than by paper electrophoresis. The resulting o-hydroxyphenyl phosphate was oxidized with bromine in aqueous triethylammonium buffer, as previously described (13), except that the reaction was followed by TLC.

In order to conserve the acetyl substituents, which were required in the final product, the red-brown reaction solution resulting from the bromine oxidation was repeatedly extracted with toluene to remove the excess reagent and then treated with an excess of pyridine and evaporated. TLC analysis of the product at this stage showed that it contained an unphosphorylated contaminant and a trace of 2,3,4,6 tetra-O-acetyl-α-D-mannopyranosyl phosphate, as well as the required compound. The product also contained a large amount of a non-carbohydrate compound. After full purification and conversion into the pyridinium form, 2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl phosphate (II) was suitable for conversion into compounds I and VIII. Deacetylation of II in one step gave β-D-mannopyranosyl phosphate (I). After further purification by preparative TLC, II was converted into the crystalline mono-

¹The abbreviations used are: TLC, thin layer chromatography; IR, infrared; NMR, nuclear magnetic resonance.
$^1$H-NMR spectra of 2,3,4,6-tetra-O-acetyl-α-D-mannopyranose, 2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl phosphate (II), β-D-mannopyranosyl phosphate (I), D-mannose 2-phosphate (VI), and citronellyl β-D-mannopyranosyl phosphate (VIII).

Spectra (δ in parts per million) were recorded in D$_2$O and had a peak for the β anomer. Compounds I, II, and VI were potassium salts, and compound VIII was a pyridinium salt. The signal from the anomeric proton (δ 5.57 ppm) of the α anomer of II in D$_2$O (12) was shifted upfield from the other signals.

The signal from the acetyl-α- and β-D-mannopyranose ((AC)$_n$-Man), which were recorded in CDCl$_3$, the spectra from both anomers showed the same peaks: the peak δ 5.34 for the α anomer was larger than the corresponding peak for the β anomer. Compounds I, II, and VI were potassium salts, and compound VIII was a pyridinium salt. The signal from the anomeric proton (δ 5.57 ppm) of the α anomer of II in D$_2$O (12) was shifted upfield from the other signals.

<table>
<thead>
<tr>
<th>(Ac)$_n$-Man</th>
<th>I</th>
<th>II</th>
<th>VI</th>
<th>VIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.93</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.98</td>
<td></td>
<td>2.1</td>
<td></td>
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<td>2.03</td>
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<td></td>
</tr>
<tr>
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<td></td>
<td>2.1</td>
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<td>3.50</td>
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<td>CH$_2$-O-δ6 pyranose ring H</td>
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<tr>
<td>3.67$^a$</td>
<td>3.03</td>
<td></td>
<td>3.77 δ3 pyranose ring H</td>
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<td>5.28$^b$ 4-pyranose ring H</td>
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<td>5.32$^c$ β-anomeric H</td>
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<td></td>
<td>5.32$^c$ α-anomeric H</td>
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<td>6.06</td>
<td>5.17</td>
<td>5.0</td>
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</tr>
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</table>

$^a$ A doublet. $^b$ An incompletely resolved multiplet. $^c$ Showing signs of further resolution.
TABLE II

Effects of acid and alkali on \(\beta-D\)-mannopyranose 1,2-phosphate (V)

<table>
<thead>
<tr>
<th>Experiment No. and conditions</th>
<th>Temperature</th>
<th>Time</th>
<th>Extent of reaction</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Water*</td>
<td>20–23</td>
<td>24 hr</td>
<td>10</td>
<td>Man-2-P</td>
</tr>
<tr>
<td>2. Water, with cation resin*</td>
<td>20–23</td>
<td>15 min</td>
<td>80</td>
<td>Man-2-P</td>
</tr>
<tr>
<td>3. 0.1 M Acetic acid*</td>
<td>20–23</td>
<td>24 hr</td>
<td>10</td>
<td>Man-2-P</td>
</tr>
<tr>
<td>4. 0.01 M HCl*</td>
<td>20–23</td>
<td>24 hr</td>
<td>50</td>
<td>Man-2-P</td>
</tr>
<tr>
<td>5. 0.1 M HCl*</td>
<td>100</td>
<td>5 min</td>
<td>100</td>
<td>Man-2-P</td>
</tr>
<tr>
<td>6. Propanol/15 M ammonium hydroxide (1/1)</td>
<td>20–23</td>
<td>24 hr</td>
<td>40</td>
<td>Man-2-P, (\beta) Man-P (3/1)</td>
</tr>
<tr>
<td>7. Propanol/1 M NaOH (10/1)</td>
<td>100</td>
<td>10 min</td>
<td>90</td>
<td>Man-2-P, Man-2-P*</td>
</tr>
</tbody>
</table>

* Compound V, pyridinium form.

* Compound V (0.5 mg) was dissolved in a mixture of propanol/1 M NaOH (10/1, 3.5 ml). The solution was concentrated to 0.2 ml (N\(_2\) gas) and either sufficient resin (pyridinium form), or acetic acid, was added to neutralize the solution.

* Only a trace of this compound was obtained.

From these observations it can be concluded that compound VI is D-mannose 2-phosphate, the structure of which is consistent with its reducing properties, separation from \(\alpha\)- and \(\beta\)-D-mannopyranosyl phosphate by TLC (see Fig. 1) and by paper chromatography (10), and its resistance to hydrolysis by hot, dilute acid under conditions that rapidly convert both anomers of D-mannopyranosyl phosphate into D-mannose.

D-Mannose 2-phosphate (VI) was obtained from D-mannopyranosyl disodium phosphate (I), without prior purification of the latter, by dicyclohexylcarbodiimide treatment followed by acid hydrolysis of the resulting mixture. This converted the 1,2-phosphate V into the 2-phosphate VI, and I (along with traces of \(\alpha\)-D-mannopyranosyl phosphate) into D-mannose. Compound VI was separated from D-mannose by preparative TLC and converted into the solid potassium salt, which was characterized by IR and NMR spectra, optical rotation, and elemental analysis. The \(\H\) NMR spectrum of VI (Table I) was readily distinguished from that of \(\beta\)-D-mannopyranosyl phosphate (I) since it showed a large, sharp peak at \(\delta 3.77\) ppm, resulting presumably from the coincidence of signals from the pyranose ring protons with part of the multiplet arising from the \(H-2/P, H-2/H-1,\) and \(H-2/H-3\) couplings. The signals from the anomeric protons appeared as two peaks (\(\delta 5.16\) and \(\delta 5.32\) ppm) centered in the same positions as the corresponding peaks in the spectrum of 2,3,4,6-tetra-O-acetyl-\(\alpha\)- and \(\beta\)-D-mannopyranosyl. However, in the spectrum of VI, the other ring protons did not interfere, and it was apparent from the integrated peaks that the \(\alpha\) anomer was predominant in \(D_2O\) solution, the \(\alpha: \beta\) ratio being approximately 7:2. The further resolution of these peaks into doublets (H-1/H-2 coupling) was not achieved (such splitting for either anomer of a D-mannose derivative would be expected to be small). Examination of VI by TLC in solvent D showed evidence of an incompletely resolved, double spot, characteristic of a mixture of \(\alpha\) and \(\beta\) anomers.

Synthesis of Citronellyl \(\beta-D\)-Mannopyranosyl Phosphate (VIII)—The reasons for selecting citronellol (III) as a model polyenol for synthesis have been discussed previously (9, 16). For the synthesis of citronellyl \(\beta-D\)-mannopyranosyl phosphate (VIII), 2,3,4,6-tetra-O-acetyl-\(\beta\)-D-mannopyranosyl pyridinium phosphate (II) was used without extensive purification because (a) in a model synthesis the presence of a small proportion of the \(\alpha\) anomer in the product is not critical, and (b) it was necessary to ascertain whether the crude product, which contained non-carbohydrate matter, would participate satisfactorily in the coupling reaction with the polyenol. This latter point is important for a synthesis starting from \(\beta\) \([\text{\textsuperscript{13}C}]\)mannose since purification procedures involving preparative TLC and crystallization would be too wasteful to employ.

Compound II was coupled with citronellol (III) in the presence of trisopropylbenzenesulfonyl chloride (17) and anhydrous pyridine. The unreacted II, and the expected by-product di-P',P'-\(\beta\)-D-mannopyranosyl pyrophosphate, were removed, and amorphous 2,3,4,6-tetra-O-acetyl-\(\beta\)-D-mannopyranosyl citronellyl phosphate (VII) was obtained by preparative TLC. The pyridinium form was converted into the sodium and potassium forms, but both derivatives were also amorphous, in contrast to the sodium salt of the corresponding \(\alpha\) anomer (9). Compound VII was characterized by IR spectrum, optical rotation, and elemental analysis. It could be separated from the \(\alpha\) anomer on TLC in solvent systems A, G, and E. Brief treatment of VII with sodium methoxide in methanol gave solid citronellyl \(\beta\)-D-mannopyranosyl sodium phosphate (VIII), which was pure according to TLC in various solvent systems. However, most of these systems did not separate VIII from the corresponding \(\alpha\) anomer (9) and, when separations were obtained, they were very small. Compound VIII showed the expected IR and NMR spectra, optical rotation, and elemental analysis. The \(\H\) NMR spectrum (Table I) showed signals clearly derived from the protons of the \(\beta\)-D-mannopyranosyl phosphate and citronellyl residues. The main signals from the latter residue appeared as a sharp doublet (CH\(_3\) protons adjacent to a double bond) and two other doublets (CH\(_3\) and CH\(_2\) protons adjacent to a saturated C–C bond). The signals from CH\(_3\) protons adjacent to a double bond were much less defined. Most of the signals arising from the protons of the \(\beta\)-D-mannopyranosyl phosphate residue appeared as a broad complex of peaks, while the doublet arising from the anomeric proton (H-1/P coupling) was clearly shown. In the spectrum of the \(\alpha\) anomer (9), the signal from the anomeric proton was not distinguishable.
Treatment of citronellyl α-D-mannopyranosyl phosphate with hot, dilute acid (9) gave mainly D-mannose and citronellyl phosphate. Similar treatment of the β anomer gave an identical result. The compounds obtained in the alkaline treatment of the α and β anomers of citronellyl β-D-mannopyranosyl phosphate (Table III) in organic and aqueous media indicate that, while cleavage of the α-D-mannosyl phosphate bond was a dominant reaction, which confirms previous findings (16), the β-D-anomer VIII had a strong tendency to undergo hydrolytic scission between the citronellyl and β-D-mannosyl phosphate residues. The mechanism of this reaction is likely to be similar to that of the alkaline hydrolysis of the dolichol derivative (see later). A surprising feature of the hydrolysis of the β anomer was the slowness of the reaction in aqueous solution, as compared to the organic solution, whereas the α-anomer was hydrolyzed at similar rates in both solvents. The formation of 1,2-anhydro-D-mannose from the α anomer in propanolic alkali indicates that at least part of the cleavage of the α-D-mannopyranosyl phosphate bond occurred via an attack by the C-2 hydroxyl group on C-1 with concomitant scission of the C-1—O bond, rather than by the alternative mechanism in which a hydroxyl ion attacks at the P atom with scission of the P—O bond (16). Presumably the 1,2-anhydro compound was too unstable to survive the action of the hot aqueous sodium hydroxide solution. This conclusion is supported by the very hydrolysis at 80°C of p-nitrophenyl α-D-mannopyranoside, whereas a good yield was obtained by a similar treatment in alkaline treatment of p-nitrophenyl α-D-mannopyranoside, propanolic alkali.

Table III
Alkaline hydrolysis of citronellyl α- and β-β-D-mannopyranosyl phosphates

<table>
<thead>
<tr>
<th>Anomer</th>
<th>Solvent and condition</th>
<th>Hydrol. ysis</th>
<th>Products (Rₖ, solvent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>H₂O (A)</td>
<td>95</td>
<td>Cit-P (0.48, B), α-Man-P⁴⁺ (0.13, F; 0.33, D; 0.31, H)</td>
</tr>
<tr>
<td>α</td>
<td>Propanol/H₂O (10/1)</td>
<td>80</td>
<td>Cit-P, 1,2-anhydro-Man⁴⁺ (0.7, B)</td>
</tr>
<tr>
<td>β</td>
<td>H₂O (A)</td>
<td>15</td>
<td>Cit-P, Cit-OH (0.96, B), β-Man-P (0.13, F; 0.21, D; 0.29, H)</td>
</tr>
<tr>
<td>β</td>
<td>Propanol/H₂O (10/1)</td>
<td>75</td>
<td>Cit-P, Cit-OH, β-Man-P, Man-2-P⁴⁺</td>
</tr>
</tbody>
</table>

a Only a trace of this compound was obtained.
b Identified by comparison with the product obtained by treating a solution of β-nitrophenyl α-D-mannoside in aqueous propanol with 0.1 m NaOH for 1 hour at 80°C.
c Reaction time was 3 hours.

Synthesis of Dolichyl β-β-D-Mannopyranosyl Phosphate (X)—For this synthesis, a highly purified sample of 2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl pyrophosphate (II, pyridinium form), obtained from the crystalline dipotassium salt, was coupled with pig liver dolichol in the presence of triisopropylenzene sulfonyle chloride (17) under conditions based on those used for the synthesis of the citronellyl derivative. The excess II and di-P-D-P-β-D-mannopyranosyl pyrophosphate was removed by water extraction, and preparative TLC gave pure 2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl dolichyl phosphate (IX), showing the expected IR absorption maxima for an acetylated phosphate diester of D-mannose and a long chain polyisoprenol. Deacetylation of IX with sodium methoxide in methanol yielded an amorphous dolichyl β-D-mannopyranosyl sodium phosphate (X) containing small proportions of several other compounds, including dolichol. After purification of a portion of this product by TLC, a single spot was observed on TLC in solvent systems A, G, and E with spray reagents specific for lipid and carbohydrates, unsaturated compounds, and phosphate esters. Treatment of X with hot, dilute acid rapidly converted it into a mixture of dolichyl phosphate, D-mannose, and methyl D-mannoside, together with traces of dolichol. The α anomer, when similarly treated (9), was hydrolyzed into the same products, at the same rate, except that no dolichol was formed. When X was heated in aqueous chloroform/methanol without the addition of acid, it slowly gave a mixture of dolichol, dolichyl phosphate, β-D-mannose, and methyl D-mannoside.
ever, the possibility remains that ring-opening of the cyclic phosphate V may give different proportions of 1- and 2-phosphates at various temperatures. It is apparent from these results that dolichyl α- and β-D-mannopyranosyl phosphates are hydrolyzed under alkaline conditions by completely different mechanisms, and the nature of the compounds formed is diagnostic of the anomeric configuration of the mannopyranosyl residue in the original compound.

Comparison of X with the polypropenyl α-mannosyl phosphates performed by Galbraith and D for TLC were chloroform/methanol/water at ratios of 60/25/4, and a carbon dioxide/acetone trap. Filtrations were performed with a 2-fold excess over the necessary quantity to obtain complete ion exchange, and after use in a column the resin was washed with 3 bed volumes of anhydrous ether. However, in 2,3,4,6-tetra-O-acetyl-β-D-mannopyranose, its 1H NMR spectrum (Table I) showed an extra absorption at 6.37 ppm of the proton of the β anomer when compared with the spectrum of the α anomer (this peak was composed, however, of signals from two other protons as well as from H-1). As prepared by Bonnor (14), this compound had m.p. 93-94°, [α]D = +22.8° (c 2.2, in chloroform); this product was, unusually, crude 2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl phosphates (VII) were readily identified on thin layer chromatography (Table I). An absorption at 3.37 ppm of the proton of the α anomer was missing, and no absorption was found at 3.97 ppm of the proton of the β anomer (this peak was composed, however, of signals from two other protons as well as from H-1). As prepared by Bonnor (14), this compound had m.p. 93-94°, [α]D = +22.8°.

When the crude crystalline product (m.p. 105-110°, [α]D = +12°) was converted into β-D-mannopyranosyl phosphate (I) by the method of Prihar and Behrmann (13), analysis by TLC (solvent G) showed that the product was a small proportion of a major product (Rf 0.8). This material was used, this proportion was increased distinctly, showing that the mutarotation of the starting material had occurred. However, by use of a modified phosphorylation method (described later), traces of the α anomer were readily removed from the peracetylated β-D-mannopyranosyl phosphate (III), so that further steps to purify 2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl Phosphate (II) were unnecessary.

Chromatographic Methods

Thin layer chromatography was performed on precoated plates of Silica Gel G (Merck). The plates supplied were 20 × 20 cm; they were cut to a length of 6 cm and used without pretreatment. However, in experiments where one or more of the samples were applied to the plate in aqueous solution, all the other spots were treated with water (1 to 2 μl), and the plate was dried in a current of air prior to elution. All preparative TLC was performed on precoated PLC plates, Silica Gel F 254 (Merck). The spray reagent, unless otherwise stated, was anisaldehyde/sulfuric acid/ethanol (1/1/18) (20), and the plates were heated to 125°. Unidentification was detected with a 1% aqueous solution of potassium permanganate in 2% aqueous sodium carbonate. Phosphate groups were detected with the spray reagent described by Dittmer and Lester (21). Solvents A, B, C, and D for TLC were chloroform/methanol/water at ratios of 80/25/4, 60/35/6, 10/10/3, and 14/15/4, respectively. Solvent E was 2,6-di-tert-butyl-4-heptanone/acetic acid/water (20/15/2), solvent F was 2-propanol/15 m ammonium hydroxide/water (6/3/1), solvent G was chloroform/methanol/15 m ammonium hydroxide/water (65/35/4/4), and solvent H was propanol/15 m ammonium hydroxide (1/1). The Rf was calculated from a measurement of the distance from the origin of the chromatogram to the point of maximum intensity of the spot after development.
the intermediate 3-hydroxypropyl phosphate diester (R<sub>H</sub> 0.00) had been converted into II (R<sub>H</sub> 0.29, with streaking); when conversion was incomplete, an additional quantity of bromine (0.10 ml) was added to complete the reaction. The aqueous reaction solution was extracted repeatedly with toluene, treated with pyridine (2 ml), and evaporated. The residue (0.8 g) was dried by two additions and evaporations of toluene (2 ml); it contained II, unphosphorylated contaminants (R<sub>H</sub> 0.70 and 0.90), and a large quantity of noncarbohydrate material. Therefore, it was extracted with 1,2-dichloroethane (15 to 70 ml), and the resulting solution filtered through Celite and evaporated. A solution of the residue (0.6 g) in water (20 ml) was extracted four times with chloroform (10 to 16 ml) to which each removed the compounds having R<sub>H</sub> 0.70 and 0.90 (TLC, solvent A). The aqueous solution was passed slowly through a column (12 x 1 cm) of a cation exchange resin (pyridinium form), treated with pyridine (2 ml), and evaporated. After three more additions and evaporations of toluene (2 ml), the residue (0.5 g) was triturated with 1,2-dichloroethane (20 ml), and the clear liquid was decanted from a semisolide residue and evaporated to give compound II (0.25 g), showing a single major spot on TLC (R<sub>H</sub> 0.39, solvent B, anisaldehyde and phosphate-specific sprays) but containing a trace of the α anomer (running just ahead of the main spot), as well as noncarbohydrate material. This product, without further purification, was suitable for deacylation to yield β-d-mannopyranosyl phosphate (I), and for the synthesis of citronellyl β-d-mannopyranosyl phosphate (VIII).

For purification, a solution of crude II (0.25 g) in methanol (2 ml) was applied to two preparative TLC plates (20 x 20 cm). The reaction was neutralized with silver carbonate, filtered through Celite, and evaporated. The resulting solution was filtered and concentrated to 2 ml, was applied to two preparative thin layer plates (20 x 20 cm), which were dried thoroughly in a stream of air before development in solvent D. In order to locate the band containing VI, a 1-cm strip was cut from each plate and sprayed with the anisaldehyde reagent. After removal of the silica gel from the plate, the product was extracted by stirring overnight with solvent D.

β-d-Mannopyranosyl 1,2-phosphate (VIII) — β-d-Mannopyranosyl phosphate (I, disodium salt, 30 mg) was converted into the pyridinium form (as described in the preparation of I, dicyclohexylammonium salt) and dissolved in a mixture of water (1.2 ml) and pyridine (7.2 ml). The addition of dicyclohexylcarbodiimide (120 mg), the mixture was stirred until a clear solution was obtained, and then kept at room temperature. After 3 hours, TLC (solvent C) showed that about 50% of compound I (R<sub>H</sub> 0.10) had been converted into a new product (R<sub>H</sub> 0.50) of m.p. 137.5, 1245 (broad), 1120, 1080, 1055, 925, and 825 cm<sup>-1</sup>. For details of the NMR spectrum (potassium salt), see Table I.

A solution of III (disodium salt, 50 mg) in water (10 ml) was passed slowly through a column (8 x 1 cm) of a cation exchange resin (pyrididine form), treated with pyridine (2 ml), evaporated to dryness, and treated with cyclohexylamine (0.15 g). The resulting solution was filtered and evaporated to yield amorphous β-d-mannopyranosyl phosphate (III). After the addition of dicyclohexylcarbodiimide (120 mg), the mixture was stirred until a clear solution was obtained, and then kept at room temperature. After 3 hours, TLC (solvent C) showed that about 50% of compound I (R<sub>H</sub> 0.10) had been converted into a new product (R<sub>H</sub> 0.50) of m.p. 137.5, 1245 (broad), 1120, 1080, 1055, 925, and 825 cm<sup>-1</sup>. For details of the NMR spectrum (potassium salt), see Table I.

β-d-Mannopyranosyl 1,2-phosphate (V) — β-d-Man-
mose 2-phosphate (VI, 110 mg). A solution of this product, in water, was passed through a small column (1 x 5 cm) of a cation exchange resin (pyridinium form), and the eluate was stirred with a large excess of the same resin (potassium form) at room temperature for 48 hours, after which the resin was filtered off. The combined filtrates were evaporated to yield a glass, which was triturated with methanol to give β-mannose 2-phosphate (VI, dipotassium salt, 65 mg), a hygroscopic solid decomposing slowly when heated above 145°C, [α]D 8 - 1 ° (c 1.1 methanol/water, 1/1, unchanged after 20 hours); IR spectrum: νmax 3400, 1125, 975, and 940 cm⁻¹. For details of the NMR spectrum, see Table I.

Compound VI (30 mg) was treated with an excess of a 1% solution of sodium methoxide in methanol. After 30 min at room temperature, thin layer chromatography (solvent A) showed that the starting material ([α]D 97) had been converted into a new compound (R 0.18). After the addition of sufficient cation exchange resin (pyridinium form) to give a neutral solution, the resin was filtered off. The combined filtrates were evaporated to dryness, and the residue was extracted with chloroform/methanol (2/1). The resulting solution was filtered and evaporated, to give citronellol β-D-mannopyranosyl pyridinium phosphate (VIII, 26 mg), a solid decomposing above 250°C, [α]D 4 - 2 ° (c 1.4, methanol); IR spectrum: νmax 3250, 3110, 2960, 2930, 2860, 1650, 1620, 1450, 1375, 1360, 1220, 1170, 1090, 1075, 1025, 925, 870, 775, and 765 cm⁻¹. (The IR absorption maxima of citronellol α-D-mannopyranosyl phosphate were similar, except that the peaks at 1005, 1026, 870, and 765 cm⁻¹ were replaced by peaks at 1105, 1095, 865, 885, and 805 cm⁻¹.) For details of the NMR spectrum, see Table I.

Thin layer chromatography gave no separation of the α and β anomers of citronellol β-D-mannopyranosyl phosphate in solvents A (R 0.18), B (R 0.40), C (R 0.67), G (R 0.20), or H (R 0.71). Small separations were obtained, however, in solvent E, R values for the α and β anomers being, respectively, 0.20 and 0.11, and in solvent F, R values being 0.73 and 0.71, respectively.

Dilute Acid Hydrolysis—Compound VIII (1 mg) was treated with 0.1 M HCl (0.2 ml) for 1 min at 80°C. Thin layer chromatography (solvent B) indicated about 30% hydrolysis to yield β-mannose (R 0.97) and citronellol phosphate (R 0.54). No formation of another derivative of citronellol, as by dilute acid treatment of citronellol α-D-mannopyranosyl phosphate (9), or of any β-D-mannopyranosyl phosphate (R 0.10, solvent C), was observed.

Dilute Alkaline Hydrolysis—This hydrolysis was performed in aqueous and organic media, and parallel experiments were conducted with the α anomer. The hydrolysis products were identified by thin layer chromatography, and the results are reported in Table III.

Dolichyl β-D-Mannopyranosyl Phosphate (X)

Synthesis

2,3,4,6-Tetra-O-acetyl-β-D-mannopyranosyl potassium hydrogen phosphate (XI, purified, 9 mg) was converted into the pyridinium form by passage of an aqueous solution through a small column (0.5 x 2 cm) of cation exchange resin (pyridinium form). The pyridinium salt was dissolved in dry pyridine (0.5 ml) and evaporated. After three additions and evaporations of dry toluene (1 ml), the residue (compound II, pyridinium form) was treated with dolichol (IV, 12 mg, isolated from pig liver, Sigma Chemical Co., St. Louis, Mo.) and the mixture was dried by several more additions and evaporations of toluene. Triisopropylbenzenesulfonyl chloride (48 mg, Aldrich) and the mixture was dried by several more additions and evaporations of toluene. Triisopropylbenzenesulfonyl chloride (48 mg, Aldrich) was added to the mixture (20 x 10 cm) plate. This treatment gave amorphous 2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl citronellyl phosphate (VII, 30 mg), pure according to TLC in solvents C and G and detection with the anisaldehyde, potassium permanganate, and phosphate-specific spray reagents. Thin layer chromatography gave small separations from the corresponding α anomer (9), the Rf values for the α and β anomers being, respectively, 0.68 and 0.63 (solvent A), 0.85 and 0.81 (solvent G), and 0.59 and 0.54 (solvent E). Both anomers gave an incompletely resolved double spot on thin layer chromatography, owing to the presence of more than one isomer in citronellol (see Ref. 9). Compound VII had [α]D 9 - 7 ° (c 1.5, methanol); IR spectrum: νmax 3026, 2930, 2890, 1740, 1410, 1375, 1230, 1075, and 1060 cm⁻¹.

Calcified: C 50.09, H 7.00
Found: C 50.09, H 7.35

Table I.
per-β-acetyl-D-mannopyranosyl) phosphates and purified by preparative TLC as described for compound VII, except that the plate (20 x 3 cm) was eluted with chloroform/methanol (5/1), and the second extraction was performed with chloroform/methanol 5/1, rather than 2/1. This gave amorphous 2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl dolichyl phosphate (IX, 11 mg, pyridinium form); IR spectrum: ν:~5300, 3300, 3040, 2960, 2930, 2860, 1750, 1665, 1450, 1378, 1249 (broad), 1070 (broad, double peak), 835 cm⁻¹. The product gave, on TLC in chloroform/methanol (5/1) and in solvent A (Rₜ 0.80), a single spot detected by the three spray reagents, as described under “Chromatographic Methods.”

Compound IX (11 mg) was dissolved in chloroform/methanol (5/1) and treated at room temperature with an excess of 1% sodium methoxide in methanol (pH of the solution > 12). After 30 min, TLC (Solvent A) showed that the starting material (Rₜ 0.80) had been converted into a compound having Rₜ 0.57 (main spot), together with a small proportion of another compound having Rₜ 0.75, a trace of a compound having Rₜ 0.25, and a trace of dolichol (identified by comparison with a standard on TLC in toluene/methanol, 49/1). The solution was neutralized with a cation exchange resin (pyridinium form). After filtration and evaporation, the residual solvent was removed by three additions and evaporation of toluene to give amorphous dolichyl β-D-mannopyranosyl phosphate IX, 11 mg, which contained residual toluene but was suitable for TLC and hydrolysis experiments without further purification; [α]°D 20.0° (c 0.47, chloroform/methanol, 5/1); IR spectrum: ν:~5300, 3300, 3040, 2940 (broad), 1740, 1370, 1460, 1378, 1220, 1070, 1020, 923, and 835 cm⁻¹. Dolichyl α-D-mannopyranosyl phosphate (IX) had [α]°D +5.5°, and the IR spectrum had a prominent sharp peak at 975 cm⁻¹, and no absorption at 1020 or 923 cm⁻¹. However, evaporation of toluene was not used in the preparation of this compound, and its analysis showed the presence of residual water instead of toluene.

Thin Layer Chromatography of Dolichyl α- and β-D-Mannopyranosyl Phosphates

The two compounds gave small separations in solvent A (α, Rₜ 0.54; β, Rₜ 0.57), solvent B (α, Rₜ 0.67; β, Rₜ 0.60), and solvent G (α, Rₜ 0.60; β, Rₜ 0.54), and no separation in solvent B (Rₜ 0.83). When plates were developed three times in solvents A or G (with drying in air between each development) small improvement of the separation was observed, but multiple development in solvent E gave no separation. The most successful combination of multiple development and 2-dimensional thin layer chromatography tried was (α) solvent A (three developments) followed by solvent E in the second dimension, or (b) solvent G (three developments) followed by solvent E in the second dimension.

Mild Acid Hydrolysis

A solution of compound X (0.5 mg) in 0.1 ml of a mixture of chloroform/methanol/0.08 m HCl (10/10/3) was kept for 5 min at 90°, when thin layer chromatography showed that more than 95% of X had been converted into a mixture of dolichyl phosphate (Rₜ 0.63, solvent A), dolichyl α-D-mannopyranosyl phosphate (Rₜ 0.27, solvent B; Rₜ 0.32, solvent F), and methyl β-D-mannopyranoside (Rₜ 0.55, solvent B; Rₜ 0.61, solvent F) Thin layer chromatography (toluene/methanol, 49/1) showed that a trace of dolichol (IV) was also formed, but that β-D-mannopyranosyl phosphate (Rₜ 0.21, solvent D) was not a product. When compound X (0.5 mg) was dissolved in chloroform/methanol/water (10/10/3, 0.1 ml), and the solution kept at 90°, thin layer chromatography (solvent A) showed the formation of dolichyl, methyl β-D-mannopyranoside, β-D-mannopyranosyl 1,2-phosphate (V, Rₜ 0.61, solvent H), dolichol (IV), and dolichyl phosphate. The extent of decomposition was estimated to be 25% after 5 min, 60% after 45 min, and 75% after 2 hours. When dolichyl α-D-mannopyranosyl phosphate was hydrolyzed with dilute acid in aqueous chloroform/methanol, the products were dolichyl phosphate, D-mannose, and methyl β-mannosides (9).

Mild Alkaline Hydrolysis

Procedure a — A solution of compound X (0.5 mg) in propanol/1 m NaOH (10/1, 1 ml) was kept for 5 min at 100°, when thin layer chromatography (solvent A) showed 75% hydrolysis. After the addition of 1 m NaOH (0.5 ml) the solution was kept for further 5 min at 100°, when TLC indicated more than 90% hydrolysis. Neutralization, and identification of products as mainly dolichol and β-D-mannopyranosyl phosphate (I) was performed as in Procedure a.

Procedure b — A solution of compound X (0.5 mg) in propanol/1 m NaOH (10/1, 1 ml) was kept for 4 hours at 37° and, then neutralized with 0.1 m acetic acid (0.5 ml). The solvents were evaporated (N₂ gas) and the products were separated into organic and aqueous phases as in Procedure a. The thin layer chromatography of the organic phase showed that hydrolysis was 75% complete and gave the same lipid-derived products as in Procedure a, whereas thin layer chromatography of the aqueous phase showed the presence of a major product (β-D-mannose 2-phosphate, VI), a minor product (β-D-mannopyranosyl phosphate, I), and a trace of β-D-mannopyranosyl 1,2-phosphate (V, Rₜ 0.61, solvent D; Rₜ 0.51, solvent F). When compound X (0.5 mg) was dissolved in 1.25 m NaOH in water-saturated butanol (1 ml) was treated with just enough methanol to obtain a single phase (22), and kept for 2 hours at 54°, when thin layer chromatography (Solvent A) indicated 80% hydrolysis. After 3 hours, thin layer chromatography indicated more than 90% hydrolysis, and the solution was neutralized with a cation exchange resin (pyridinium form). The resin was filtered off, the filtrate was evaporated (N₂ gas) and the residue was dissolved in chloroform/methanol (5/1) and solvents A, B, and G, and detection with the anisaldehyde, potassium permanganate, and phosphate-specific spray reagents. The two compounds gave small separations in solvent A (α, Rₜ 0.54; β, Rₜ 0.57); solvent B (α, Rₜ 0.67; β, Rₜ 0.60); and solvent G (α, Rₜ 0.60; β, Rₜ 0.54), and no separation in solvent B (Rₜ 0.83). The products were identified as in (a), showing that the lipid-derived compounds were the same, and the non-mannose derived compounds were β-D-mannose 2-phosphate (VI) and β-D-mannopyranosyl phosphate (I), in a 2:1 ratio, together with a trace of β-D-mannopyranosyl 1,2-phosphate (V).

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