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THE SYNTHESIS OF A TRISACCHARIDE AND A TETRASACCHARIDE LIPID INTERMEDIATE. P^1-DOLICHYL P^2-[*O*-β-D-MANNOPYRANOSYL-(1→4)-*O*-(2-ACETAMIDO-2-DEOXY-β-D-GLUCOPYRANOSYL)-(1→4)-2-ACETAMIDO-2-DEOXY-α-D-GLUCOPYRANOSYL] DIPHOSPHATE AND P^1-DOLICHYL P^2-[*O*-α-D-MANNOPYRANOSYL-(1→3)-*O*-β-D-MANNOPYRANOSYL-(1→4)-*O*-(2-ACETAMIDO-2-DEOXY-β-D-GLUCOPYRANOSYL)-(1→4)-2-ACETAMIDO-2-DEOXY-α-D-GLUCOPYRANOSYL] DIPHOSPHATE

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ABSTRACT

Synthetic benzyl *O*-(2,3,4,6,-tetra-*O*-acetyl-β-D-mannopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranoside was converted, by catalytic hydrogenolysis of the benzyl groups, followed by treatment with acetyl chloride–hydrogen chloride and then "chloride-ion catalysis", into *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-mannopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-methyl-(3,6-di-*O*-acetyl-1,2-dideoxy-α-D-glucopyrano)-[2,1-*d*]-2-oxazoline. This compound was phosphorylated by treatment with dibenzyl phosphate under strictly anhydrous conditions to give after catalytic hydrogenolysis, a peracetyl trisaccharide phosphate. This was coupled with P^1-dolichyl P^2-diphenyl diphosphate to give, after *O*-deacetylation, P^1-dolichyl P^2-[*O*-β-D-mannopyranosyl-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-α-D-glucopyranosyl] diphosphate, a trisaccharide "lipid intermediate". *O*-(2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→3)-*O*-(2,4,6-tri-*O*-

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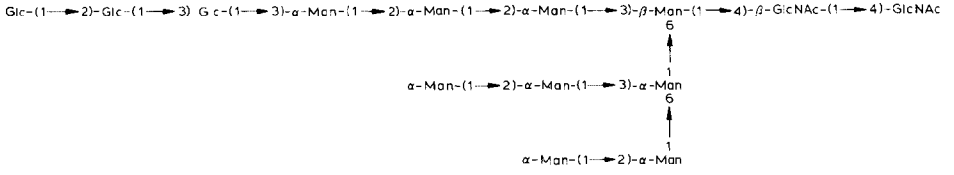
acetyl-β-D-mannopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-α-D-glucopyranosyl) phosphate, synthesized from *O*-α-D-mannopyranosyl-(1→3)-*O*-β-D-mannopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranose that had been isolated from mannosidosis urine was coupled with P^1-dolichyl P^2-diphenyl diphosphate to give, after *O*-deacetylation, P^1-dolichyl P^2-[*O*-α-D-mannopyranosyl-(1→3)-*O*-β-D-mannopyranosyl-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-α-D-glucopyranosyl] diphosphate, a tetrasaccharide "lipid intermediate".

INTRODUCTION

Biosynthesis of the *N*-glycoproteins takes place by the co-translational transfer of a pre-formed "high-mannose" oligosaccharide chain from a "lipid intermediate" to the nascent protein[1]. The "lipid" consists of dolichyl diphosphate, so that the "lipid intermediate" is a dolichyl glycosyl diphosphate, and evidence[1] from several laboratories suggests that the sugar residue consists of the tetradecasaccharide **1**. The biosynthesis of **1** occurs by the sequential transfer of nine D-mannose residues to P^1-di-*N*-acetylchitobiosyl P^2-dolichyl diphosphate[2] (**2**), the D-mannosyl donor being GDP-D-mannose for the first five residues, and dolichyl mannosyl phosphate for the last four residues transferred. The transfer is believed to occur by a discrete series of reactions[3] in which the first α-D-mannosyl residue transferred is (1→3)-linked, but evidence from one system[4] indicates that isomers can be formed for several of the stages, suggesting that alternative pathways may exist. This possibility is strengthened by the finding that, in another system[5], the second α-D-mannosyl residue transferred is apparently (1→3)- rather than (1→6)-linked, suggesting that a (1→6)-linked residue is already present in the D-mannosyl acceptor. Also, in a mutant cell-line[6], lipid intermediates having glycosyl moieties with a structure different from **1** have been identified.



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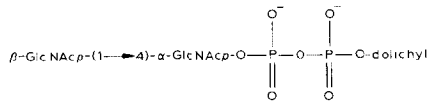
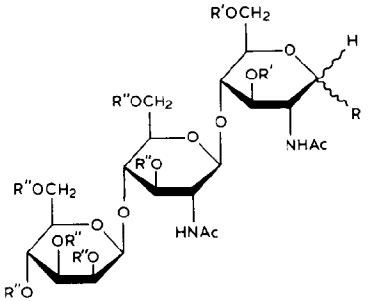


2

In order to perform experiments that will decide the exact order of addition of the D-mannose residues and characterize the mannosyl transferases involved, it is necessary to employ pure, exogenous acceptors of known structure, and available in amounts large enough to facilitate kinetic measurements. This paper reports the synthesis of two such acceptors or synthetic "lipid intermediates", P^1-dolichyl P^2-[*O*-β-D-mannopyranosyl-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-α-D-glucopyranosyl] diphosphate (**16**), and P^1-dolichyl P^2-[*O*-α-D-mannopyranosyl-(1→3)-*O*-β-D-mannopyranosyl-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-α-D-glucopyranosyl] diphosphate (**28**).

RESULTS AND DISCUSSION

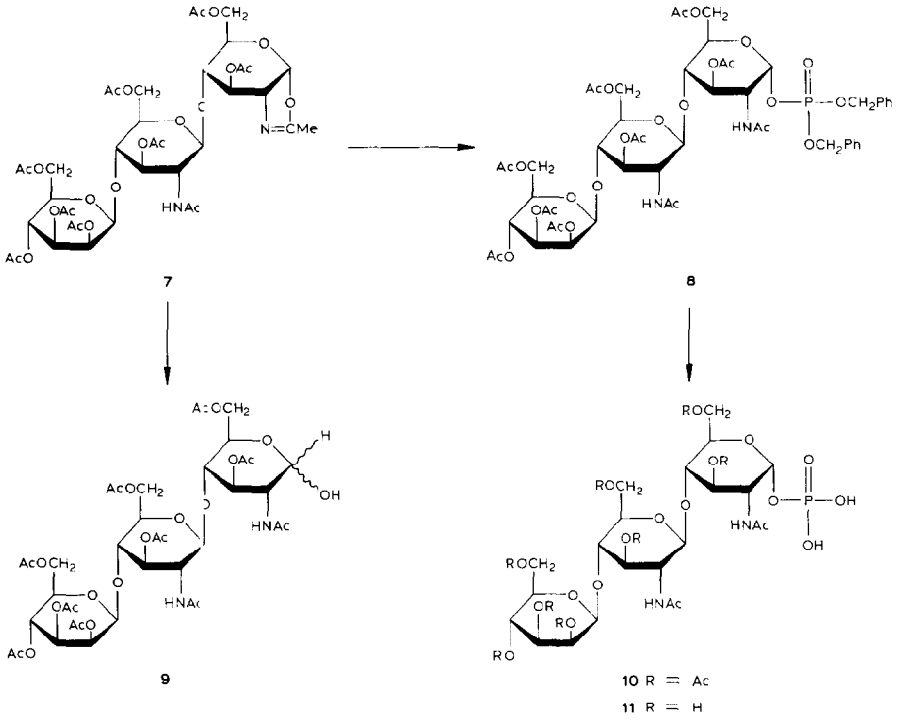
Synthesis of trisaccharide "lipid intermediate" (**16**). — The first stage in the synthesis of each "lipid intermediate" was the preparation of the glycosyl residue. The trisaccharide *O*-β-D-mannopyranosyl-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-D-glucopyranose (**3**) has been synthesized by two routes[7,8], both of which were suitable for this work. However, the



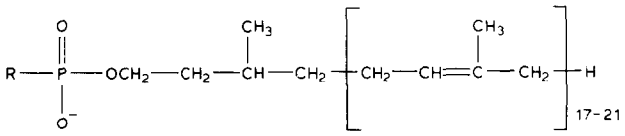
3 R = OH, R' = R" = H
4 R = OBn, R' = Bn, R" = Ac
5 R = OH, R' = H, R" = Ac
6 R = Cl, R' = Ac, R" = Ac

route[7] that involves initial formation of the disaccharide β-D-Man*p*-(1→4)-D-GlcNAc*p*, which is converted into a peracetyl oxazoline and employed as the glycosyl donor for formation of the trisaccharide **3**, was easier to scale up than the other, in which the chitobiosyl residue is formed first[8]. This was because the latter involves the glycosylation of OH-4 of a D-glucopyranose derivative by a benzylated oxazoline[9], a procedure that, in our hands, gave a very low yield[10]. Thus, benzyl *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-mannopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranoside (**4**) was converted by catalytic hydrogenolysis into the triol **5**,

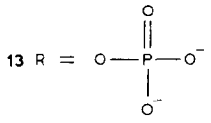
which was the precursor, *via* the peracetyl glycosyl chloride **6**, of the peracetyl oxazoline **7**. The "chloroacetolysis" conditions employed to generate the chloride **6** also caused some cleavage of the bond between the two 2-acetamido-2-deoxy-D-glucose residues, so it was necessary to chromatograph the oxazoline **7** to separate it from mono- and di-saccharide derivatives; the same purification was also necessary for the synthesis of a tetrasaccharide oxazoline[11].



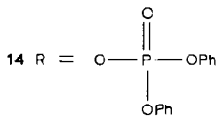
Conversion of the trisaccharide oxazoline **7** into the dibenzylglycosyl phosphate **8** was extremely sensitive to the presence of traces of water or methanol, and, unless these were eliminated by careful drying of starting materials, reagents, solvent, and apparatus, the major product was the OH-1 compound **9**. Thus, it was imperative to perform the procedure in a dry-box in order to obtain a reasonably good yield of **8**. The separation of the desired compound **8** from excess phosphorylating reagent was achieved by p.l.c., after which the benzyl groups were removed from the compound by a brief catalytic hydrogenolysis to give the peracetylglycosyl phosphate **10**. On the basis of previous experience with the formation of glycosyl phosphates from oxazolines[2,12], the anomeric configuration of **10** was expected to be the desired α-D, and this was confirmed by the ^{1}H-n.m.r. spectrum of **11**, obtained by *O*-deacetylation of **10**.



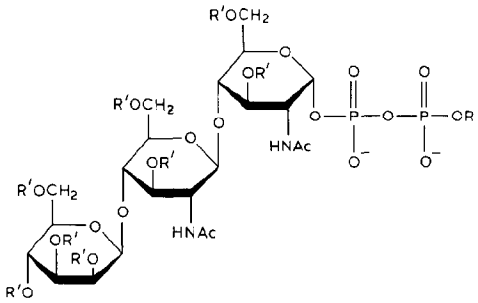
12 R = O^-

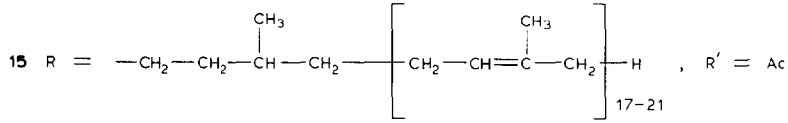


13 R =

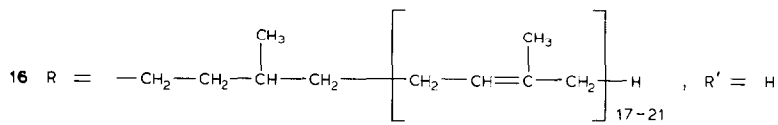


14 R =





15 R = , R′ = Ac



16 R = , R′ = H

Dolichyl phosphate (**12**) was synthesized from pig-liver dolichol as described previously[13], but in order to achieve particularly favorable conditions for the coupling reaction between the peracetyl trisaccharide phosphate **10** and P^1-dolichyl P^2-diphenyl diphosphate **14**, an additional preparative t.l.c. step was performed, which gave **12** in a state of high purity. The conversion of pure **12** into the "activated" derivative **14** was performed as described previously[14]. The condensation of **14** with the tributylammonium form of **10** was performed in dry 1,2-dichloroethane

with a trace of dry pyridine as catalyst, and examination of the mixture by t.l.c. revealed the formation of the required diphosphate diester **15**, with almost no side-reactions. This t.l.c. readily separated **15** from dolichyl phosphate (**12**). The separation of **15** from diphenyl phosphate and unreacted glycosyl phosphate **10** was readily achieved by p.l.c., and compound **15** was obtained pure according to t.l.c. in a variety of solvent systems; this t.l.c. also clearly distinguished it from the dolichyl diphosphate derivatives of peracetylated 2-acetamido-2-deoxy-D-glucose[14], and di-*N*-acetylchitobiose[2].

O-Deacetylation of **15** to give the trisaccharide "lipid intermediate" **16** was achieved by overnight treatment with sodium methoxide in chloroform–methanol, and **16** was obtained pure according to t.l.c. in five solvent systems with three spray reagents (see Table I). When **16** was treated with hot, dilute acid the observed products were dolichyl phosphate **12** and trisaccharide **3**, indicating a simultaneous scission of the diphosphate and glycosyl phosphate bonds. A similar treatment of **16** with hot, dilute alkali produced dolichyl phosphate **12** by the expected base-catalyzed hydrolysis of the diphosphate bond. However, this reaction did not occur during the *O*-deacetylation of **15**. When **16** was kept at room temperature for 3 days, t.l.c. showed that it had almost completely decomposed, to yield a mixture of dolichyl phosphate **12**, trisaccharide **3**, dolichyl diphosphate[14] (**13**), and trisaccharide phosphate **11**. Therefore, the synthetic lipid intermediate was routinely stored in the peracetyl form **15**, which was more stable.

When synthetic P^1-di-*N*-acetylchitobiosyl P^2-dolichyl diphosphate[2] (**2**), was incubated with calf pancreas microsomes and GDP-D-[^{14}C]mannose, a labeled compound was formed[15] that was chromatographically indistinguishable from the synthetic trisaccharide "lipid intermediate" **16**. When **16** was employed as the glycosyl acceptor in a similar incubation, it was converted into several ^{14}C-labeled compounds, one of which had the properties of a tetrasaccharide "lipid intermediate"[16] (see synthesis of compound **28**).

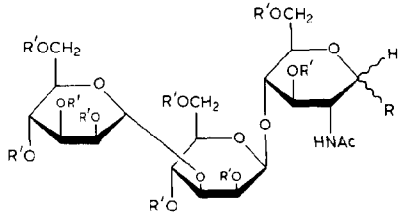
TABLE I

COMPARISON OF MOBILITY IN T.L.C. OF DI-, TRI-, AND TETRA-SACCHARIDE "LIPID INTERMEDIATES", COMPOUNDS **2**[a], **16**, AND **28**, RESPECTIVELY

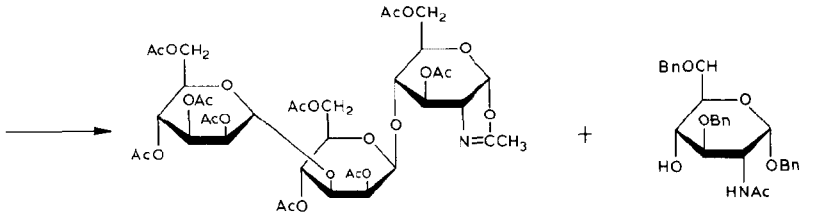
	R_F *value*[c]		
Solvent system[b]	**2**	**16**	**28**
60:25:4 Chloroform–methanol–water (*A*)	0.12	0.09	
60:35:6 Chloroform–methanol–water (*B*)	0.35	0.25	0.31
10:10:3 Chloroform–methanol–water (*C*)		0.80	0.74
70:45:9 Chloroform–methanol–water (*D*)	0.54	0.49	0.37
75:44:5:5 Chloroform–methanol–15M NH_4OH–water (*E*)	0.36	0.26	0.21
65:35:4:4 Chloroform–methanol–15M NH_4OH–water (*F*)	0.20	0.15	

[a]Ref. 2. [b]All proportions are solvents v/v. [c]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).

Synthesis of tetrasaccharide "lipid intermediate" (**28**). — The previously reported synthesis[11] of the peracetyl tetrasaccharide phosphate **25**, employed as the starting material the trisaccharide **17**, isolated from mannosidosis urine by charcoal–Celite adsorption and preparative paper-chromatography[17]. For the synthesis reported here, a new source of **17** was required, and therefore **17** was isolated from human mannosidosis urine by rapid liquid-chromatographic methods[18]. In order to

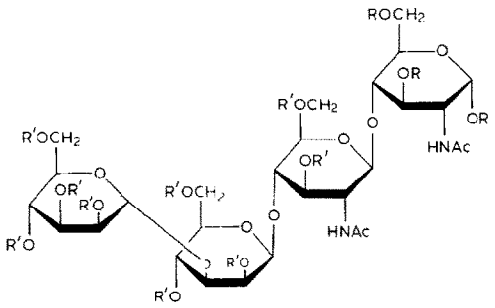


17 R = OH, R′ = H
18 R = OAc, R′ = Ac
19 R = Cl, R′ = Ac

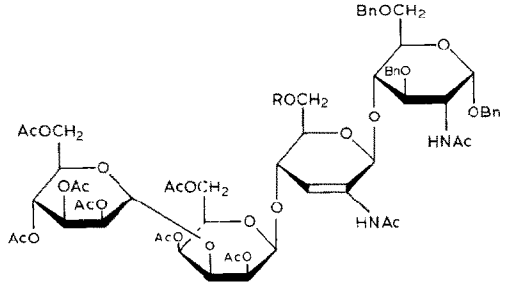


20 **21**

be certain that the trisaccharide isolated was **17**, a purified sample was compared by t.l.c. and l.c. with various solvent systems, and by high-field ^{1}H-n.m.r., and field-desorption mass-spectrometric analysis, to the material prepared by Strecker *et al.*[17]. Nevertheless, in an attempt to convert **17** into the peracetylglycosyl chloride **19** *en route* to the oxazoline **20**, the sample prepared by l.c. was treated with acetyl chloride–hydrogen chloride, but did not undergo a satisfactory conversion into **19**, presumably owing to the presence of (noncarbohydrate) contaminants. To overcome this problem, it was more convenient to modify the synthetic route than to submit the trisaccharide **17** to further purification steps. Thus **17** was peracetylated to give **18**, which was readily converted into the glycosyl chloride **19**, and hence into the peracetyl oxazoline **20**. At this point, it was convenient to separate **20** from unwanted materials by p.l.c., after which the coupling with benzyl 2-acetamido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranoside (**21**) was performed in a way similar to that described previously[11]. However, in a re-examination of the coupling reaction, shorter reaction times (~2 h), combined with carefully controlled coupling-conditions, were found to give improved yields of the tetrasaccharide



22 R = Bn, R′ = Ac
23 R = R′ = H

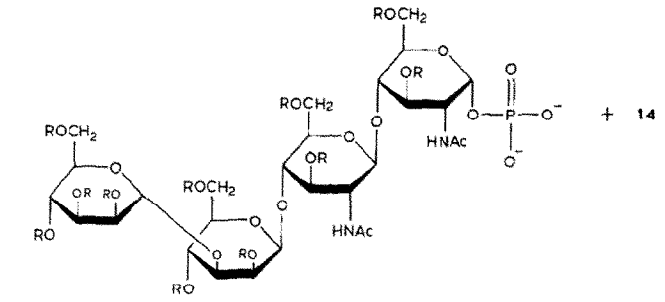


24

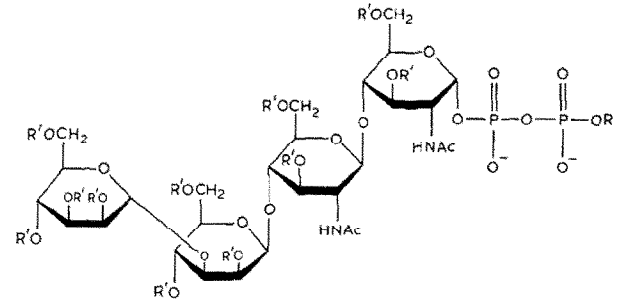
derivative **22**. A second coupling product **24** was also isolated, which, on the basis of its properties and previous experience with glycosidations involving oligosaccharide oxazolines[7], was concluded to be a 2′,3′-unsaturated analog of **22**. The transformation of **22** into the peracetyl tetrasaccharide phosphate **25** was performed as described previously[11], except that the yield in the phosphorylation step was almost doubled (to 50%) by application of the same precautions as those employed in the phosphorylation of **7**. To obtain a sample of the tetrasaccharide *O*-α-D-mannopyranosyl-(1→3)-*O*-β-D-mannopyranosyl-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-D-glucopyranose (**23**), needed for chromatographic purposes, 2 mg of **22** was subjected to *O*-deacetylation and catalytic hydrogenolysis. For the same reason, a sample of **25** was *O*-deacetylated to give tetrasaccharide phosphate **26**.

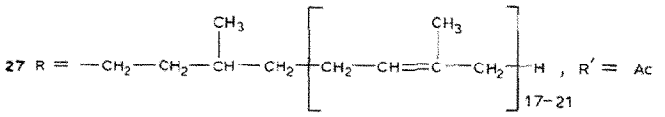
The coupling of **25** (tributylammonium form) with P^1-dolichyl P^2-diphenyl diphosphate (**14**) was performed by the same procedure as that for the trisaccharide phosphate **10**, and *O*-deacetylation gave the tetrasaccharide "lipid intermediate" **28**.

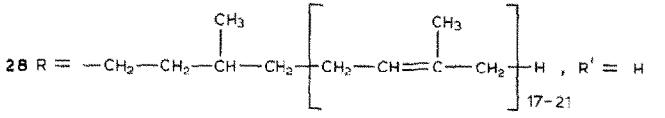
When the peracetyl tri- and tetra-saccharide "lipid intermediates" were compared in t.l.c., tetrasaccharide **27** unexpectedly had an R_F value higher than that of



25 R = Ac
26 R = H







trisaccharide **15** (see Table II). However, after *O*-deacetylation, the situation was reversed, and the tetrasaccharide derivative **28** had the expected lower R_F value (see Table I). This t.l.c. also showed that **28** was obtained free of dolichyl phosphate (**12**) or any other compound that might be active as a glycosyl acceptor in biosynthetic experiments (see later). The identity of **28** was confirmed by treatment with hot, dilute acid, which resulted in the formation of tetrasaccharide **23**, and dolichyl phosphate **12**, together with traces of tetrasaccharide phosphate **26** and dolichyl diphosphate **13**. Under hot, dilute alkaline conditions, **28** was hydrolyzed

TABLE II

COMPARISON OF MOBILITY IN T L C OF P^1-DOLICHYL P^2-PERACETYLGLYCOSYL DIPHOSPHATES **15** AND **27**

	R_F *value*[a]	
Solvent system[a]	**15**	**27**
60:25:4 Chloroform–methanol–water (*A*)	0.58	0.61
65:35:4:4 Chloroform–methanol–15M NH_4OH–water (*F*)	0.63	0.67
46:21:1:3 Chloroform–methanol–15M NH_4OH–water (*G*)	0.56	0.61

[a]See footnotes to Table I.

to yield tetrasaccharide phosphate **26** and dolichyl phosphate **12**, together with a small proportion of tetrasaccharide **23**.

The tetrasaccharide "lipid intermediate" **28** was shown to act as an efficient exogenous mannosyl acceptor in a system employing GDP-D-[^{14}C]mannose and calf pancreas microsomes[19].

EXPERIMENTAL

General methods. — Melting points were determined with a Mettler FP2 hot-stage equipped with a microscope, and correspond to "corrected melting points". Optical rotations were determined for solutions in 1-dm, semimicro tubes with a Perkin–Elmer No. 141 polarimeter. I.r. spectra were recorded with a Perkin–Elmer spectrophotometer, Model 237. ^{1}H-N.m.r. spectra were recorded, at 60 MHz, with a Varian T-60 spectrometer; at 200 MHz, with a Varian XL-200 spectrometer at the National Cancer Institute, N.I.H., Bethesda, MD 20014; at 360 MHz, with a Nicolet NT-360 spectrometer in the Department of Biological Chemistry, University of California School of Medicine, Davis, CA 95616; at 500 MHz, with an instrument made at the Francis Bitter National Magnet Laboratory, Massachusetts Institute of Technology, Cambridge, MA 02139; or, at 500 MHz, with a Bruker WM-500 spectrometer at the Northeast Regional NSF-NMR Facility, Yale University, New Haven, CT 06520. The cation-exchange resin used was AG-50W X-8 (200–400 mesh; Bio-Rad Laboratories, Richmond, CA 94804). Evaporations were conducted *in vacuo*, with the bath temperature kept below 30°. Dichloromethane, acetonitrile, and 1,2-dichloroethane were dried by distillation from phosphorus pentaoxide, and addition of 3A molecular sieve (No. M-9882, Sigma Chemical Co., St. Louis, MO 63178). Dimethyl sulfoxide was dried by distillation *in vacuo*, and addition of 4A molecular sieve (No. M-0133, Sigma). Other solvents were dried (where stated) by treatment with molecular sieve followed by addition of calcium hydride (in lump form; Fisher Scientific Co., Pittsburgh, PA 15219). The microanalyses were performed by Dr. W. Manser, CH-8704 Herrliberg, Zurich, Switzerland, and by Galbraith Laboratories, Inc., Knoxville, TN 37821.

Chromatographic methods. — T.l.c. and preparative t.l.c. were performed on precoated plates of silica gel G, 0.25-mm thick (E. Merck AG, Darmstadt, Germany); for t.l.c., the plates supplied were cut to a length of 6-cm before use, but otherwise were used without pretreatment. All proportions of solvents are v/v. Preparative-layer chromatography (p.l.c.) was performed on precoated silica gel F254 PLC plates, 2-mm thick (Merck), or on precoated plates of silica gel F254, 0.5-mm thick (Merck). The spray reagent, unless otherwise stated, was 1:1:18 anisaldehyde–sulfuric acid–ethanol[20], and the plates were heated to 125°. Unsaturation was detected by spraying with a solution of 1% potassium permanganate in 2% aqueous sodium hydrogencarbonate. Phosphate groups were detected with the spray reagent described by Dittmer and Lester[21]. When plates were eluted more than once, they were dried in air between each elution. Solvents *A, B, C,* and *D* were 60:25:4, 60:35:6, 10:10:3, and 70:45:9 chloroform–methanol–water, respectively. Solvents *E, F,* and *G* were 75:44:5:5, 65:35:4:4, and 46:21:1:3 chloroform–methanol–15M ammonium hydroxide–water, respectively. Column chromatography (l.c.) was performed on silica gel (0.05–0.2 mm, 70–325 mesh, Merck). Gas–liquid chromatography (g.l.c.) was performed with a Perkin–Elmer Model 900 instrument, equipped with a flame-ionization detector; sugars were analyzed as per(trimethylsilyl) ethers, in a column (300 × 0.3 cm) of Gas Chrom Q coated with 3% of OV 17. The column temperature was programmed to rise from 120 to 300° at 8°/min.

O-(2,3,4,6-Tetra-O-acetyl-β-D-mannopyranosyl)-(1→4)-O-(2-acetamido-3,6-di-O-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-methyl-(3,6-di-O-acetyl-1,2-dideoxy-α-D-glucopyrano-[2,1-d]-2-oxazoline (**7**). — A solution of benzyl *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-mannopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranoside[7] (**4**, 80 mg) in glacial acetic acid (3 mL), was treated with 10% palladium-on-charcoal (40 mg, Fluka Chemical Corp., Hauppauge, NY 11788) and hydrogenated overnight at 0.2 MPa pressure. The catalyst was filtered off (Celite), and examination of the filtrate by t.l.c. (5:1 chloroform–methanol) showed complete conversion of **4** (R_F 0.8) into **5** (R_F 0.37). Evaporation gave **5** (50 mg), which was dissolved in acetyl chloride (5 mL), and the resulting solution was stirred vigorously and treated with conc. hydrochloric acid (100 μL). After sealing the reaction tube, the stirring was continued at room temperature for 24 h. Evaporation (N_2 gas), followed by six additions and evaporations of toluene (0.2 mL), gave a residue consisting of **6**. Before conversion into the oxazoline **7**, the drying of **6** was completed *in vacuo* over phosphorus pentaoxide for 2 h. A stirred solution of **6** in acetonitrile (2 mL) was treated with tetraethylammonium chloride (50 mg) and sodium hydrogencarbonate (50 mg), and the stirring continued for 3 h at room temperature, when t.l.c. (10:1 chloroform–methanol) showed the formation of **7** and several by-products (see Results and Discussion). Therefore, the reaction mixture was diluted with dichloromethane (30 mL) and filtered. The filtrate was concentrated to 1 mL and applied to four t.l.c. plates, which were developed twice in 15:1

chloroform–methanol. To locate the band containing **9**, the plates were viewed under u.v. light (a strongly absorbing band migrated just ahead of **7**), and then a 0.5-cm strip was cut from each plate and sprayed with the anisaldehyde reagent. The product was extracted from the silica gel by stirring for 1 h with 2:1 chloroform–methanol. After filtration (Celite) and washing, the combined filtrates were evaporated to yield **7** (28 mg, 42.9%), amorphous, m.p. 102–106°, $[\alpha]_D^{20}$ −20° (*c* 0.87, dichloromethane); ν_{max}^{KBr} 1745 ($OCOCH_3$), 1670 (C=N and amide I), and 1545 cm^{-1} (amide II); ^{1}H-n.m.r. ($CDCl_3$, 60 MHz): δ 5.91 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1), and 2.18–1.98 (30 H, 8 $OCOCH_3$, $NHCOCH_3$, and CH_3 of oxazoline).

Anal. Calc for $C_{38}H_{52}N_2O_{23}$: C, 50.44; H, 5.79; N, 3.10; O, 40.67. Found: C, 50.21; H, 5.97; N, 2.96; O, 40.86.

O-(2,3,4,6-Tetra-O-acetyl-β-D-mannopyranosyl)-(1→4)-O-(2-acetamido-3,6-di-O-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-3,6-di-O-acetyl-2-deoxy-α-D-glucopyranosyl phosphate (**10**). — Compound **7** (22 mg, 24 μmol) was exhaustively dried, by six additions and evaporations of dry toluene (0.5 mL), followed by storage *in vacuo* over phosphorus pentaoxide for 4 days. With the protection of a dry box, dibenzyl phosphate (~100 mg) was dissolved in dry 1,2-dichloroethane to give a 50 mg/mL solution, and 0.4 mL (20 mg, 72 μmol) (using a predried syringe and pipette) was added to the reaction tube containing **7**. The tube was flushed with dry nitrogen and then sealed, and kept in the dry box at room temperature for 48 h, when t.l.c. (10:1 chloroform–methanol) showed the formation of a major product that migrated just ahead of **7**, but giving a positive reaction with the phosphate-specific spray reagent. This t.l.c. also showed that some oxazoline **7** remained, but the formation of the OH-1 compound **9** with lower R_F value had been minimized. (During the preparative t.l.c. of **8**, any oxazoline **7** was converted into **9**.) The mixture was directly applied to two 0.5-mm plates, which were developed with 10:1 chloroform–methanol. The band containing **8** was located by viewing under u.v. light, and by spraying a narrow strip of the plate with the phosphate-specific reagent. Extraction of **8** from the silica gel was achieved by stirring with 2:1 chloroform–methanol for 30 min, allowing the gel to settle, decanting off the clear extract, and repeating the process twice. The combined supernatant solutions were evaporated to ~0.5 mL, treated with methanol (3 mL) and with 10% palladium-on-charcoal (20 mg, Fluka), and hydrogenated at 0.2 MPa pressure for 1 h, when t.l.c. showed that **8** had been completely converted into a single compound **10**, R_F 0.15 (solvent *A*), 0.25 (solvent *B*), and 0.8 (solvent *C*) (anisaldehyde and phosphate spray-reagents). The catalyst was filtered off (sintered glass) and evaporation gave **10** (12 mg, 49%); amorphous, $[\alpha]_D^{20}$ +8.5° (*c* 0.47, methanol); ν_{max}^{KBr} 1750 ($OCOCH_3$), 1675 (amide I), and 1545 cm^{-1} (amide II).

For analytical purposes, **10** (1 mg) was divided into portions of 100 μg and 200 μg for phosphate determination, and 700 μg, for determination of D-mannose and 2-acetamido-2-deoxy-D-glucose content. For phosphate determination, **10** was treated with conc. sulfuric acid (70 μL) for 30 min at 150°. The cooled solution was treated with 30% aqueous hydrogen peroxide (50 μL), and again kept at 150° for

30 min. The excess of hydrogen peroxide was removed by the addition of 5% aqueous urea, and heating to 150° for 30 min. After being cooled, the solution was treated with water (0.65 mL) and 5% ammonium molybdate solution (0.2 mL), and, after mixing, with a solution of reducing agent[22] (50 μL). The mixture was boiled for 7 min to develop a blue color, and the absorbance was recorded at 830 nm. A calibration curve was drawn using standards of uridine monophosphate.

For determination of D-mannose and 2-acetamido-2-deoxy-D-glucose, **10** (700 μg) was treated with M hydrochloric acid (0.2 mL), and the mixture kept in a sealed tube for 4 h at 100°. After evaporation (N_2 gas), water (0.2 mL) was added and evaporated (twice), and then toluene (0.2 mL) was added and evaporated three times. Drying of the residue was completed in the presence of potassium hydroxide pellets *in vacuo* (24 h), after which the sugars were acetylated, subjected to methanolysis, and converted into per(trimethylsilyl) ethers of methyl glycosides, which were analyzed by g.l.c. (for details, see analysis of **16**).

Anal. Ratios of methyl D-mannosides to methyl 2-acetamido-2-deoxy-D-glucosides to phosphate. Calc.: 1.0:2.0:1.0. Found: 1.0:2.25:1.05.

To record the ^{1}H-n.m.r. spectrum, a sample of **10** was *O*-deacetylated. Compound **10** (9 mg) was dissolved in dry methanol (1 mL) and treated with 0.5% sodium methoxide in methanol until an excess of base was present (pH paper). The mixture was kept for 15 h at room temperature, when t.l.c. (solvent *C*) showed that **10** (R_F 0.8) had been completely converted into **11** (R_F 0.3). The solution was made neutral with cation-exchange resin, and filtration and evaporation gave a residue that was chromatographed preparatively on a t.l.c. plate (solvent *C*). The band containing **11** was located by cutting a strip from the plate and spraying with the anisaldehyde reagent, and **11** was extracted from the silica gel by stirring overnight with the same solvent mixture as for the elution. Filtration (glass sinter) and evaporation gave **11** (7 mg, containing traces of silica gel); ^{1}H-n.m.r. (360 MHz, D_2O): δ 5.375 (dd, 1 H, $J_{1,P}$ 7.0, $J_{1,2}$ 2.5–3 Hz, H-1), [assignment confirmed by selective decoupling: irradiation of H-2 caused collapse of 2.5–3 Hz coupling, but did not affect 7.0 Hz coupling], 4.85 (d, 1 H, $J_{1'',2''}$ <1 Hz, H-1''), 4.63 (d, 1 H, $J_{1',2'}$ 7.5 Hz, H-1'), 4.06 (dd, 1 H, $J_{2,3}$ 5.0 Hz, H-2), 3.93 (m, H-6a,-6a'-6a''), 3.90 (s), 3.87 (d, 1 H, *J* 3.0 Hz), 3.84, 3.82 (s, d at 78°), 3.77 (s), 3.76 (s), 3.74 (d, *J* 7.5 Hz), 3.68 (m), 3.63 (d, *J* 5 Hz), 3.57 (t, *J* 10 Hz) (δ 3.90–3.57, 28 H), 3.42 (m, 2 H), 2.07 (s, 3 H, $NHCOCH_3$), and 2.05 (s, 3 H, $NHCOCH_3$).

P^1-[O-*(2,3,4,6-tetra*-O-*acetyl-β*-D-*mannopyranosyl)-(1→4)*-O-*(2-acetamido-3,6-di*-O-*acetyl-2-deoxy-β*-D-*glucopyranosyl)-(1→4)-2-acetamido-3,6-di*-O-*acetyl-2-deoxy-α*-D-*glucopyranosyl*] P^2-*dolichyl diphosphate* (**15**). — A solution of **10** (15 mg, 0.015 mmol) in methanol (2 mL) was treated with tributylamine (15 mg). After addition of water (0.5 mL), the excess of tributylamine was removed by three extractions (1 mL each) with hexane. The aqueous methanol solution was evaporated, and three additions and evaporations of toluene (1 mL each) gave the bis(tributylammonium)salt of **10**.

Dolichyl phosphate (**12**, pyridinium$^+$) was prepared from pig-liver dolichol

(14 mg, 0.011 mmol, Sigma Chemical Co.), shown by l.c. analysis under elevated pressure to consist mainly of almost equal proportions of C_{90} and C_{95} isoprenologs) employing a method similar to that previously described[23]. The product was subjected to an additional purification step consisting of preparative t.l.c. on two 0.25 mm-thick plates with 60:25:4 chloroform–methanol–water as developing solvent. Detection of **12** was performed with the potassium permanganate and phosphate-specific spray reagents, and extraction from the silica gel[13] gave pure **12** (8 mg). It was converted into the tributylammonium form by dissolution of the pyridinium salt in 2:1 chloroform–methanol, treatment with tributylamine (4 mg), and evaporation, followed by three additions and evaporations of toluene (0.5 mL each) to give the tributylammonium salt of **12**. The preparation of P^1-dolichyl P^2-diphenyl diphosphate (**14**) from **12** was carried out as described previously[14].

A mixture of **14** (from 8 mg of **12**, 5.4 μmol) and **10** (15 mg, 15 μmol, tributylammonium$^+$) was exhaustively dried by repeated additions and evaporations of toluene (1 mL each, rotary evaporator). The residue was treated with a freshly prepared 1% solution of dry pyridine in 1,2-dichloroethane (0.2 mL). The contents of the vessel were thoroughly mixed, and kept under a dry nitrogen atmosphere for 72 h at room temperature. Examination of the mixture by t.l.c. (solvent *A*) showed that **14** (R_F 0.8) had been completely transformed into **15**, (R_F 0.6) and diphenyl phosphate having the same R_F value as **14**, but detectable only by the phosphate-specific spray. T.l.c. (solvents *A* and *F*) also showed that dolichyl phosphate (**12**) had not been formed. The reaction mixture was directly applied to two 0.5 mm-thick p.l.c. plates, and eluted with solvent *A*. The bands containing **15** and unchanged **10** were located by spraying a narrow zone with (a) the potassium permanganate, and (b) the phosphate-specific spray reagents, and the compounds were extracted from the silica gel by stirring overnight with solvent *C*. After filtration (Celite) and evaporation, the residue was extracted with 1:1 chloroform–methanol, and the resulting solution was filtered (sintered glass) and evaporated, to give **15** (7 mg, 26% based on **10** used up in the reaction), $[\alpha]_D^{20}$ +2° (*c* 0.4, 2:1 chloroform–methanol); t.l.c. R_F 0.58 (solvent *A*), 0.63 (solvent *F*), (see Table II). Similarly, extraction of the lower band from the p.l.c. plate gave unreacted **10** (5 mg).

P^1-*Dolichyl* P^2-[O-β-D-*mannopyranosyl-(1→4)*-O-*(2-acetamido-2-deoxy-β*-D-*glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-α*-D-*glucopyranosyl*] *diphosphate* (**16**). — A solution of **15** (400 μg) in 1:1 chloroform–methanol (0.2 mL) was treated with a 0.05% solution of sodium methoxide in methanol (50 μL), and the mixture kept for 2 h at room temperature, then overnight at 4°, with exclusion of moisture. The mixture was treated with cation-exchange resin to give a neutral pH, and the resin was filtered off and washed with 1:1 chloroform–methanol (2 × 1 mL). The combined filtrates were evaporated to give **16** (318 μg, 100%); ^{1}H-n.m.r. [500 MHz, ($^2H_6$)Me_2SO]: δ 5.08 (s, C=CH-), 3.8–3.0 (ring protons and HDO), 1.98 [m, CH_2-C(CH_3)=CH-CH_2], 1.83, 1.85 (s, 2NHCOCH_3), 1.62 (s, C*H*-C=, *cis*), 1.54 (s, CH_3-C=, *trans*), 1.47–1.23 [m, CH_2-C*H*(CH_3)-CH_2], and 0.853 (s, CH_3-CH, sat.): detailed analysis and integration was not possible because of large

solvent peaks at δ 3.28 (HDO) and 2.50 (Me_2SO); ^{1}H-n.m.r. (200 MHz, 2:1 $CDCl_3$:CD_3OD): δ 5.28 (s, C=C*H*-), 4.3–3.15 (ring protons and CH_3OD), 2.05 [m, CH_2-C(CH_3)=CH-CH_2], 1.83, 1.86 (s, 2NHCOCH_3), 1.65 (s, CH_3-C=, *cis*), 1.59 (s, CH_3-C=, *trans*), 1.26 (s, CH_2-C*H*(CH_3)-CH_2, and 0.91 (m, CH_3-CH, sat.); integration was not possible because of large solvent peaks at δ 4.53 (HDO) and 3.35 (CH_3OD); t.l.c. (see Table I). Additional analyses of **16** were as follows.

(*a*) Compound **16** (160 μg) was treated with 0.1M hydrochloric acid (100 μL) for 5 min, at 95°, and then the mixture was cooled, diluted with water (0.5 mL), and extracted with chloroform (3 × 1 mL). The aqueous and organic layers were separated and evaporated to ~100 μL, and examined by t.l.c., which showed that **16** had been hydrolyzed to ~70% to give dolichyl phosphate **12** (R_F 0.29, solvent *F*) and trisaccharide **3** (R_F 0.4, solvent *B*). The t.l.c. showed that no dolichyl diphosphate[24] (R_F 0.18, solvent *F*) or trisaccharide phosphate **11** (R_F 0.22, solvent *B*) had been formed.

(*b*) Compound **16** (320 μg) was treated with M hydrochloric acid (200 μL) for 4 h at 100°. After evaporation (N_2 gas), water (200 μL) was added and evaporated 4 times, after which the drying was completed *in vacuo* overnight in the presence of potassium hydroxide. For comparison, samples (150 μg) of trisaccharide **3**, and a 1:2 mixture of D-mannose and 2-acetamido-2-deoxy-D-glucose were treated in exactly the same way. T.l.c. of the mixture (solvent *D*) showed the formation of D-mannose and 2-amino-2-deoxy-D-glucose hydrochloride. The dried hydrolyzate was acetylated with 1:1 acetic anhydride–pyridine (0.2 mL), and the resulting acetylated sugars were subjected to methanolysis with M hydrogen chloride in methanol (1 mL) for 20 h at 80°, followed by evaporation, *N*-reacetylation with acetic anhydride–pyridine for 2 min at room temperature, evaporation, and per(trimethylsilyl)ation. The per(trimethylsilyl) derivatives were analyzed by g.l.c. in a column (300 × 0.3 cm) packed with Gas-chrom Q (80–100 mesh) coated with 3% of OV-17. The column temperature was programmed to rise from 120° to 290° at 8°/min. The ratio of the peak-area of methyl α- and -β-D-mannosides to that of methyl 2-acetamido-2-deoxy-α- and -β-D-glucosides, after correction for a 20% loss of D-mannose (calculated from the expected and found values for the hydrolyzate of the mixture of D-mannose and 2-acetamido-2-deoxy-D-glucose) was 1:2.50 for compound **16** and trisaccharide **3** (expected: 1:2.0).

(*c*) Compound **16** (160 μg) was treated with propanol (100 μL) and M sodium hydroxide (10 μL) at 95°, and analyzed by t.l.c. after various time-intervals. This showed that the degree of hydrolysis of **16** was ~60 (5 min), ~80 (10 min), and >95% (20 min). The only definite product observed was dolichyl phosphate **12** [R_F 0.29 (solvent *F*) and 0.9 (solvent *C*)]. A spot having a low R_F value, similar to that of trisaccharide phosphate **11** (R_F 0.3, solvent *C*) could not be definitely assigned because of streaking.

O-α-D-*Mannopyranosyl-(1→3)*-O-β-D-*mannopyranosyl-(1→4)-2-acetamido-2-deoxy*-D-*glucopyranose* (**17**). — Oligosaccharides were isolated from mannosidosis urine and chromatographed on silica gel with a Chromatospac Prep-10

preparative, medium-pressure (0.7 MPa) liquid chromatograph; for full details, see ref. 18. The yield of crude **17** from 2.5 g urine extract was 0.66 g. Examination by t.l.c. (solvent *C*, 2 elutions) showed that **17** was free of other oligosaccharides, but contained low-molecular-weight material (probably monosaccharides and noncarbohydrate urine metabolites). Therefore crude **17** was chromatographed on a column (23 × 2.5 cm) of Bio-Gel P-2 (200–400 mesh, Bio-Rad Labs, Richmond, CA 94804) with water as solvent. Fractions (1 mL) were monitored by t.l.c. (solvent *C*, 2 elutions), and those containing pure **17** were combined to give an amorphous solid (35 mg), $[\alpha]_D^{20}$ +34→+35° (20 h, *c* 2.1, 1:1 ethanol–water); ν_{max}^{KBr} 3400 (OH, NH), 2945, 1650 (amide I), 1565 (amide II), 1380, and 1050 cm^{-1} (broad); ^{1}H-n.m.r. (500 MHz, D_2O): δ 5.19 (d, 1 H, $J_{1,2}$ 3.1 Hz, H-1α), 5.09 (s, 1 H, H-1″), 4.77 (s, 1 H, H-1′), 4.75–4.71 (m, H-1β + HDO), 4.23 (d, 1 H, $J_{2',3'}$ 3.0 Hz, H-2′), 4.06 (d, 1 H, $J_{2'',3''}$ 1.6 Hz, H-2″), 3.93–3.62 (m, 17 H, ring protons), and 2.02 (s, 3 H, NHCOCH_3) (see ref. 25); m.s. (field desorption): *m/z* 568 (M + Na), 550 (M + Na − H_2O), 527 (M − H_2O), 508 (M + Na − $COCH_3$), 263, 201, 172, and 160.

Anal. Calc. for $C_{20}H_{35}NO_{16}$: C, 44.04; H, 6.47; O, 46.93. Found: C, 43.96; H, 6.57; O, 46.62.

For further analysis, **17** (0.3 mg) was hydrolyzed with M hydrochloric acid, and the released sugars were converted into per(trimethylsilyl) ethers of methyl glycosides as described for **16**.

Anal. Calc. ratio of methyl D-mannosides to methyl 2-acetamido-2-deoxy-D-glucopyranosides, 2.0:1. Found (corrected for D-mannose decomposition as for **16**): 2.16:1.

Samples of **17** isolated from mannosidosis urine as just described, and obtained from G. Strecker[17], were compared in t.l.c. on Merck plates (solvent *C*, 3 elutions) or on Redi-plates (Fisher Scientific Co., Pittsburgh, PA 15219) (solvent systems: 3:3:2 2-propanol–ethyl acetate–water, 3:3:2 propanol–acetic acid–water, and 3:3:2 butanol–acetic acid–water). In all cases, the materials were indistinguishable. Similarly, samples of **17** were examined by l.c. under elevated pressure on a column of Micropak-NH_2 (Varian Assoc., Palo Alto, CA 94303), in the solvent system 31:19 acetonitrile–water (for full details see ref. 18). In each case, the peak for **17** showed a shoulder on the leading edge, probably due to a partial resolution of anomers, and the samples from the two sources were identical.

*O-(2,3,4,6-Tetra-*O*-acetyl-α-*D*-mannopyranosyl)-(1→3)-*O*-(2,4,6-tri-*O*-acetyl-β-*D*-mannopyranosyl)-(1→4)-2-methyl-3,6-di-*O*-acetyl-1,2-dideoxy-α-*D*-glucopyrano-[2,1-*d*]-2-oxazoline*[11] (**20**). — Compound **17** (crude, 200 mg) was treated with 1:2 acetic anhydride–pyridine (7.5 mL) and the mixture stirred for 48 h at room temperature. After treatment with water (2 mL), the solvents were evaporated, and the residue dissolved in chloroform (100 mL). The solution was washed with water (4 × 4 mL), dried ($MgSO_4$), and evaporated to yield **18**, (233 mg), identical according to t.l.c. (10:1 chloroform–methanol) with **18** as prepared from a sample of **17** provided by G. Strecker[17]. Compound **18** was converted into **19**, and thence into **20** by treatment with hydrogen chloride–acetyl chloride, fol-

lowed by chloride-ion catalysis, employing the same experimental conditions as described previously for the conversion of **17** into **19**, and **19** into **20**, respectively[11]. Examination of **20** by t.l.c. (10:1 chloroform–methanol) showed the presence of minor contaminants having R_F values higher and lower than that of **20** (R_F 0.67); therefore, crude **20** (180 mg) was purified by p.l.c. on four 0.5 mm-thick plates, as described previously[11], to give **20** (110 mg), pure according to t.l.c. and indistinguishable chromatographically from a material prepared from a sample of **17** provided by G. Strecker[17].

Benzyl O-*(2,3,4,6-tetra*-O-*acetyl-α*-D-*mannopyranosyl)-(1→3)*-O-*(2,4,6-tri*-O-*acetyl-β*-D-*mannopyranosyl)-(1→4)*-O-*(2-acetamido-3,6-di*-O-*acetyl-2-deoxy-β*-D-*glucopyranosyl)-(1→4)-2-acetamido-3,6-di*-O-*benzyl-2-deoxy-α*-D-*glucopyranoside* (**22**) *and benzyl* O-*(2,3,4,6-tetra*-O-*acetyl-α*-D-mannopyranosyl)-(1→*3)*-O-*(2,4,6-tri*-O-*acetyl-β*-D-*mannopyranosyl)-(1→4)*-O-*(2-acetamido-6*-O-*acetyl-2,3-dideoxy-β*-D-erythro-*hex-2-enopyranosyl)-(1→4)-2-acetamido-3,6-di*-O-*benzyl-2-deoxy-α*-D-*glucopyranoside* (**24**). — The coupling of **20** (110 mg, 0.12 mmol) with benzyl 2-acetamido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranoside **21** (200 mg, 0.46 mmol) was performed as described previously[11], with the following improvements. The reaction temperature was kept between 70 and 75°, and the disappearance of **20** was carefully monitored by t.l.c. (10:1 chloroform–methanol). Also the pH of the mixture was frequently checked, and small amounts of *p*-toluenesulfonic acid added when necessary to maintain the pH at 3–4 (pH paper). When checking pH or performing t.l.c., it was very important to maintain an atmosphere of dry nitrogen in the reaction tube. When ~90% of **20** had reacted (2–2.5 h), the reaction was stopped by cooling and addition of sodium methoxide[11]. The yield of **22** after purification was 32% based on **20**. When the reaction time was 7 h (ref. 11), the yield was 19%.

Under the modified coupling conditions, t.l.c. revealed the formation of a second product having a higher R_F value than **22**, and giving a positive test for unsaturation with the potassium permanganate spray reagent. This compound (**24**) was isolated by the same procedure[11] of p.l.c., acetylation with acetic anhydride–pyridine (1:2), and second p.l.c., as employed for **22**, except that the final p.l.c. was performed on one 0.5-mm plate, with 15:1 chloroform–methanol, detection by viewing under u.v. light, and spraying with the potassium permanganate reagent. Extraction of **24** from the silica gel with 2:1 chloroform–methanol gave 12 mg (7% based on **20**), amorphous, $[\alpha]_D^{21}$ +34° (*c* 1.95, 1,2-dichloroethane); ν_{max}^{KBr} 3380, 1750, 1660, 1450, 1375, 1230, 1050, 730, and 680 cm^{-1}; ^{1}H-n.m.r. (60 MHz, $CDCl_3$): δ 7.54 (s, 15 H, 3 Ph-H) and 2.23–2.08 (m, 30 H, 8 $COCH_3$, 2 $NHCOCH_3$).

Anal. Calc. for $C_{67}H_{84}N_2O_{30}$: C, 57.59; H, 6.06; N, 2.00; O, 34.35. Found: C, 57.60; H, 5.90; N, 1.93; O, 34.25.

P^1-[O-*(2,3,4,6-tetra*-O-*acetyl-α*-D-*mannopyranosyl)-(1→3)*-O-*(2,4,6-tri*-O-*acetyl-β*-D-*mannopyranosyl)-(1→4)*-O-*(2-acetamido-3,6-di*-O-*acetyl-2-deoxy-β*-D-*glucopyranosyl-(1→4)-2-acetamido-3,6-di*-O-*acetyl-2-deoxy-α*-D-*glucopyranosyl*]

P^2-*dolichyl diphosphate* (**27**). — Dolichyl phosphate (**12**, 8 mg, 5.4 μmol) was purified and converted into P^1-dolichyl P^2-diphenyl diphosphate (**14**) as described for the synthesis of **15**. The coupling reaction between **14** and *O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→3)-*O*-(2,4,6-tri-*O*-acetyl-β-D-mannopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-α-D-glucopyranosyl phosphate[11] (**25**) (15 mg, 11.3 μmol) was also performed as for the coupling of **14** and **10**, except that t.l.c. of the mixture and p.l.c. of the product **27** were performed with solvent *G*. The yield of amorphous **27** was 7 mg (31% based on the amount of **25** used in the reaction); ν_{max}^{film} 2930, 2850, 1745, 1660, 1550, 1450, 1375, 1225, and 920 cm^{-1}. In t.l.c., **27** was clearly separated from **15** (see Table II).

As in the synthesis of **15**, unreacted peracetylglycosyl phosphate **25** (5 mg) was recovered from the p.l.c. of the mixture.

P^1-*Dolichyl* P^2-[O-α-D-*mannopyranosyl-(1→3)*-O-β-D-*mannopyranosyl)-(1→4)*-O-*(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-α-D-glucopyranosyl*] *diphosphate* (**28**). — *O*-Deacetylation of **27** (400 μg) was performed as described for **15**, to give **28** (310 μg, 100%); ^{1}H-n.m.r. (500 MHz, Me_2SO): δ 5.08 (s, 17–18 H, =C*H*), 3.8–3.0 (m, ring protons), 2.00–1.98 [m, 68–74 H, CH_2-C(CH_3)=CH-CH_2], 1.81, 1.83 (s, 6 H, 2 NHCOCH_3), 1.61 (s, 42–45 H, CH_3-C=, *cis*), and 1.54 (s, 12 H, CH_3-C=, *trans*); a detailed analysis of the spectrum was not possible because of large, unavoidable solvent signals at δ 3.26–3.32 (HDO) and 2.50–2.48 (Me_2SO); the number of protons represents a ~1:1 mixture of C_{90} and C_{95} isoprenologs in the dolichol used for the preparation of **14**; t.l.c., see Table II. Further analyses of **28** were as follows.

(*a*) Compound **28** (150 μg) was treated with 0.1M hydrochloric acid (200 μL) for 5 min at 90°. The mixture was cooled and diluted with water (0.5 mL), and then extracted with chloroform (3 × 0.5 mL). The aqueous and organic layers were each evaporated to ~50 μL, and examined by t.l.c., which showed that **28** had been hydrolyzed to ~60% to give dolichyl phosphate (**12**, R_F 0.38, solvent *G*), traces of dolichyl pyrophosphate[24] (**13**) (R_F 0.24, solvent *G*), tetrasaccharide **23** (R_F 0.44, solvent *C*), and traces of tetrasaccharide phosphate **26** (R_F 0.16, solvent *C*). To provide a chromatographic "standard" of **23**, a sample of **22** (2 mg) was *O*-deacetylated by treatment with a 0.5% solution of sodium methoxide in methanol (200 μL). The mixture was kept overnight at room temperature, and then neutralized with cation-exchange resin (pyridinium$^+$). The resin was filtered off, and the filtrate evaporated to dryness, followed by two additions and evaporations of toluene (0.2 mL). The residue was dissolved in glacial acetic acid (0.25 mL), treated with 10% palladium-on-charcoal (2 mg, Fluka) and hydrogenated at 0.2 MPa overnight. The catalyst was filtered off and washed with acetic acid (0.25 mL), and the combined filtrates were evaporated, followed by two additions and evaporations of toluene, to give **23**, pure according to t.l.c. in solvent *C* (R_F 0.44) and according to t.l.c. on Redi-plates (Fisher) in 3:3:2 butanol–acetic acid–water. To provide a chromatographic "standard" of **26**, a sample of **25** (1 mg) was *O*-deacetylated by treatment

with a 0.5% solution of sodium methoxide in methanol (0.5 mL). The mixture was kept overnight at room temperature, and then made neutral with cation-exchange resin (pyridinium$^+$). The resin was filtered off and washed with 1:1 methanol–water (0.5 mL), and the combined filtrates were evaporated to give **26**, pure according to t.l.c. in solvent *C* (R_F 0.16).

(*b*) Compound **28** (150 μg) was treated with M hydrochloric acid (200 μL) for 4 h at 100°, and the mixture processed as described for the analysis of **16**. The ratio of the g.l.c. peak area for methyl D-mannosides to methyl 2-acetamido-2-deoxy-D-glucosides was 1:1.06 (calc. 1:1).

(*c*) Compound **28** (150 μg) was dissolved in propanol (100 μL), and the solution treated with M sodium hydroxide (10 μL). The mixture was shaken with a Vortex instrument and kept at 90°. After 5 min, t.l.c. (solvent *E*) showed hydrolysis of ~90% to give dolichyl phosphate (**12**) (R_F 0.38), and this had increased to 100% after 10 min. The cooled mixture was evaporated (N_2 gas), and the residue dissolved in water (50 μL) and made neutral with 10% aq. acetic acid (10 μL). Evaporation, followed by two additions and evaporations of toluene (100 μL) gave a residue that was examined by t.l.c. (solvent *C*); it showed formation of a ~10:1 mixture of tetrasaccharide phosphate **26** and tetrasaccharide **23**, R_F 0.16 and 0.44, respectively.

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