Carbohydrate Research, 169 (1987) 221–233
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

SYNTHESIS OF THE TETRASACCHARIDE LIPID INTERMEDIATE P^1-DOLICHYL P^2-[*O*-α-D-MANNOPYRANOSYL-(1→6)-*O*-β-D-MANNOPYRANOSYL-(1→4)-*O*-(2-ACETAMIDO-2-DEOXY-β-D-GLUCOPYRANOSYL)-(1→4)-2-ACETAMIDO-2-DEOXY-α-D-GLUCOPYRANOSYL] DIPHOSPHATE*

CHRISTOPHER D. WARREN, SATORU NAKABAYASHI, AND ROGER W. JEANLOZ†

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

(Received May 29th, 1987; accepted for publication, July 29th, 1987)

ABSTRACT

O-α-D-Mannopyranosyl-(1→6)-*O*-β-D-mannopyranosyl-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-D-glucopyranose was isolated from bovine or ovine mannosidosis urine. After peracetylation, treatment with trimethylsilyl trifluoromethanesulfonate gave a high yield of a peracetyl oxazoline, which was phosphorylated with dibenzyl phosphate to give a dibenzyl glycosyl phosphate that was converted into a peracetyl tetrasaccharide phosphate by catalytic hydrogenolysis. A coupling reaction with P^1-dolichyl P^2-diphenyl diphosphate, prepared in two stages from pig-liver dolichol, yielded a peracetyl diphosphoric diester, which on *O*-deacetylation gave P^1-dolichyl P^2-[*O*-α-D-mannopyranosyl-(1→6)-*O*-β-D-mannopyranosyl-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-α-D-glucopyranosyl] diphosphate. This tetrasaccharide lipid intermediate was active as an acceptor of D-mannose residues from GDP-D-mannose in the presence of calf pancreas microsomes.

INTRODUCTION

Biosynthesis of the *N*-glycoproteins involves the formation of an oligosaccharide lipid intermediate with the probable structure[1] **1**. Assembly of the oligomannosyl moiety in **1** occurs by the sequential transfer of D-mannose residues from GDP-D-mannose (for the first five residues) or from dolichyl mannosyl phosphate (for the last four residues). This paper concerns the first stage and in

*Dedicated to Dr. R. Stuart Tipson. This is publication No. 1031 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities, Harvard Medical School, and Massachusetts General Hospital, Boston, Massachusetts. This investigation was supported by research grants AM-03564 (to R. W. Jeanloz) from the National Institute of Arthritis, Diabetes, Digestive & Kidney Diseases, and DMB 84-12590 and 85-45771 (to C. D. Warren) from the National Science Foundation.

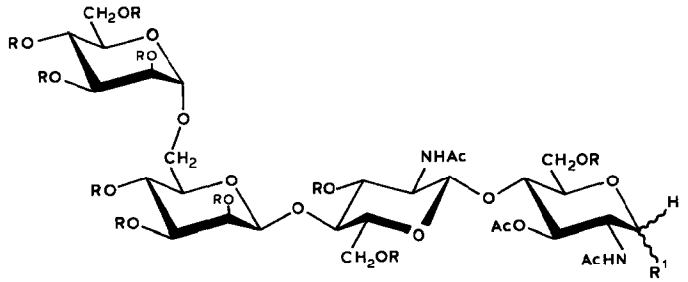
†To whom correspondence should be addressed.

0008-6215/87/$ 03.50

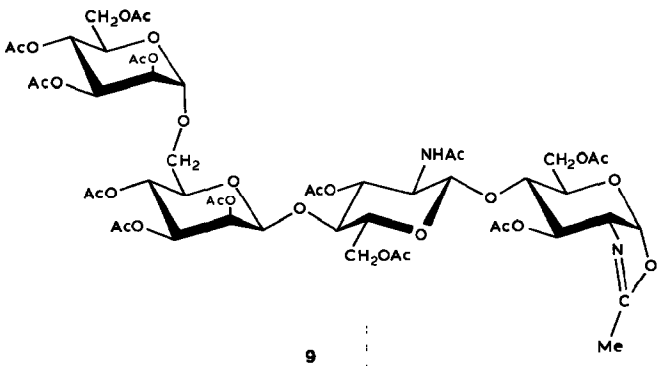
α-Glc-(1→2)-α-Glc-(1→3)-α-Glc-(1→3)-α-Man-(1→2)-α-Man-(1→2)-α-Man-(1→3)-β-Man-(1→4)-β-GlcNAc-(1→4)-α-GlcNAc-PP-O-Dolichol
6
1
α-Man-(1→2)-α-Man-(1→3)-α-Man
6
1
α-Man-(1→2)-α-Man
1
α-Man-(1→3)-β-Man-(1→4)-β-GlcNAc-(1→4)-GlcNAc
6
1
α-Man
2
β-Man-(1→4)-β-GlcNAc-(1→4)-α-GlcNAc-PP-O-Dolichol
3
PP ≡ —O—P(=O)(OH)—O—P(=O)(OH)—
α-Man-(1→3)-β-Man-(1→4)-β-GlcNAc-(1→4)-α-GlcNAc-PP-O-Dolichol
4
α-Glc ≡ α-D-Glcp
α- and β-Man ≡ α- and β-D-Manp
α-Man-(1→3)-β-Man-(1→4)-β-GlcNAc-(1→4)-α-GlcNAc-PP-O-Dolichol
6
1
α-Man
5
α- and β-GlcNAc ≡ α- and β-D-GlcpNAc

particular the construction of the pentasaccharide "core" **2**. It is evident that the branch point in the "core" can be formed in two different ways, according to the order in which the α-D-Man*p*-(1→3)- and α-D-Man*p*-(1→6)-linkages are formed. The structures of oligosaccharide lipid intermediates formed in CHO cells[2] suggested the initial formation of the (1→3) linkage (route A), but observations on rabbit liver microsomes[3] suggested the alternative route B, in which the (1→6)-linkage is formed first.

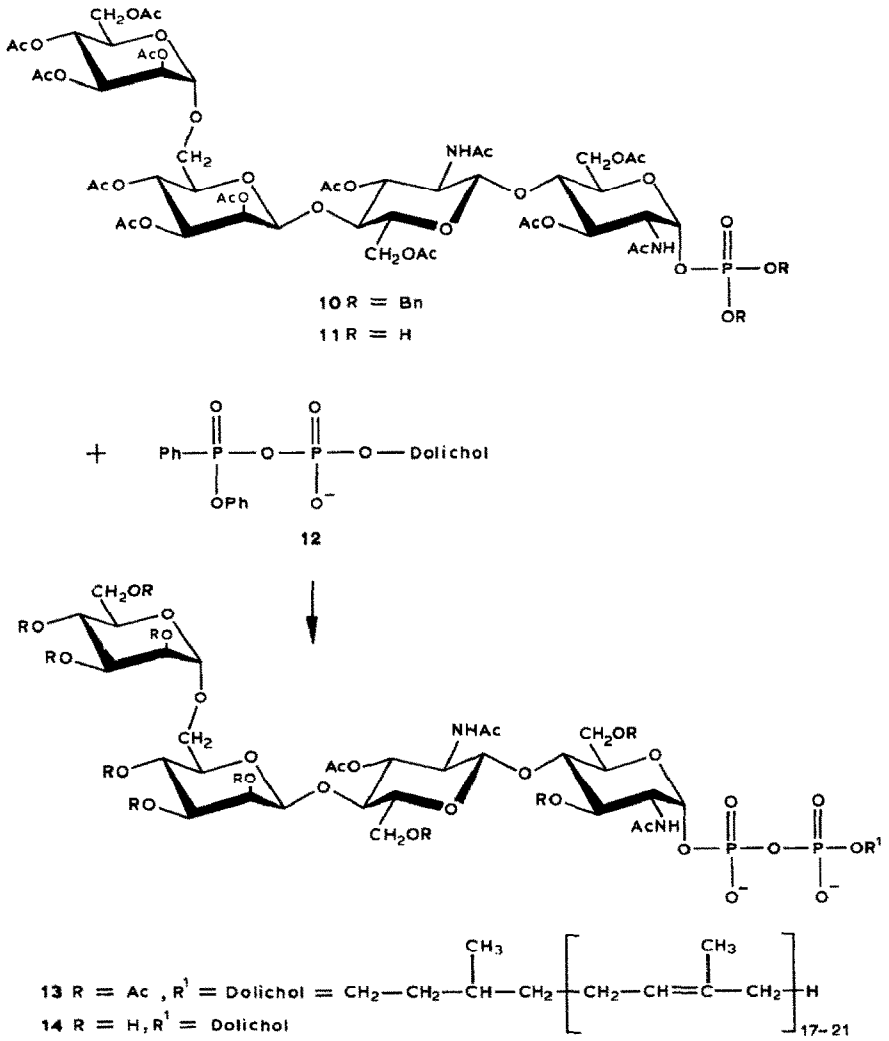
During previous work in this laboratory, the trisaccharide lipid intermediate **3** was chemically synthesized[4]. When incubated with pancreas microsomes and GDP-D-mannose, it was converted[5] into the tetrasaccharide derivative **4**. Next, the intermediate **4** was chemically synthesized[4] and, when similarly incubated, it was converted[6] into the pentasaccharide derivative **5**. These findings were in agreement with route A. In order to explore the possibility that route B may be an alternative mechanism for assembly of the "core", it was necessary to chemically synthesize the tetrasaccharide lipid intermediate **14**, and to explore its properties as a D-mannosyl acceptor. The synthesis of **14**, and a preliminary account of the chemical synthesis of **5**, are reported herein.



6 R = H, R^1 = OH
7 R = Ac, R^1 = OAc
8 R = Ac, R^1 = Cl



9



RESULTS AND DISCUSSION

Synthesis of tetrasaccharide lipid intermediate **14**. — The first step in the synthesis was the preparation of the glycosyl residue. The tetrasaccharide *O*-α-D-mannopyranosyl-(1→6)-*O*-β-D-mannopyranosyl-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-D-glucopyranose (**6**) was initially synthesized on a small scale from D-mannose and di-*N*-acetylchitobiose employing 2-*O*-acetyl-3-*O*-allyl-4-*O*-benzyl-6-*O*-(*tert*-butyldiphenylsilyl)-D-glucopyranosyl chloride as a specifically protected glycosyl donor[7]. Before the synthetic approach had been scaled up and optimized for overall yield, it became evident that compound **6** could be more conveniently isolated from the urine of calves with α-mannosidosis, or sheep with swainsonine-induced α-mannosidosis[8]. The required

tetrasaccharide, together with analogous compounds containing three, four, and five D-mannose residues, was isolated from urine by charcoal adsorption, gel-filtration, and de-ionization, after which the oligosaccharides were separated by preparative-layer chromatography[9]. Unlike the Man_3 and Man_4 compounds, compound **6** does not occur as an isomeric mixture, therefore further purification was not necessary.

The route for the conversion of **6** into the dolichyl diphosphate derivative **14** involved the initial formation of a peracetyl glycosyl phosphate having the α-D configuration, followed by a coupling reaction with an "activated" derivative of dolichyl phosphate, and *O*-deacetylation of the product after chromatographic purification. This was the same approach as that employed for the synthesis[4] of lipid intermediates **3** and **4**. From previous experience, it appeared likely that the best route for formation of the required tetrasaccharide phosphate would be *via* the peracetyl oxazoline[4,10]. Because of the presence of a (1→6) linkage in **6**, the formation of the peracetyl oxazoline **9** presented a synthetic challenge. Previously, we have prepared peracetyl oxazolines from peracetyl glycosyl chlorides by "chloroacetolysis"[4,10], *i.e.*, treatment with hydrogen chloride in acetyl chloride. Obviously, such a treatment was not ideal for **6** because of the probability of cleaving the (1→6) linkage. Furthermore, for a general approach to the conversion of oligosaccharides into glycosyl donors, it was necessary to develop a procedure that would completely avoid scission or modification of any inter-residue glycosidic linkages or any hydrolysis of acetamido groups. After a search for methods of forming either glycosyl halides or oxazolines from peracetyl oligosaccharides, treatment with trimethylsilyl trifluoromethanesulfonate[11] (Me_3Si triflate) was found to fulfill the stated criterion, with the further advantage that the α or β anomers of the peracetyl derivatives were equally good starting materials. Thus, peracetylation of **6** gave **7**, preponderantly the α anomer, which on treament with Me_3Si triflate gave the required peracetyl oxazoline **9** in 78% yield. For comparison, conversion of **7** into the glycosyl chloride **8** by treatment with hydrogen chloride in 1,2-dichloroethane–diethyl ether, followed by "chloride-ion catalysis"[4,10] gave **9** in 38% yield, and the product was accompanied by several other compounds arising primarily from cleavage of the α-D-Man*p*-(1→6)-D-Man linkage, or of the β-D-Glc*p*NAc-(1→4)-D-Glc*p*NAc linkage, or from other unidentified side-reactions, so that careful chromatographic purification was necessary. In contrast, the Me_3Si triflate reaction mixture showed on t.l.c. only one product, corresponding to **9**.

For phosphorylation, **9** was treated with an excess of dibenzyl phosphate under absolutely anhydrous conditions, as employed previously for the conversion of oligosaccharide oxazolines into glycosyl phosphates[4,10]. Surprisingly, even after several days at room temperature, the phosphorylation of **9** remained incomplete. [This problem became more acute with the pentasaccharide **2**, see below.] The direct preparative t.l.c. of the reaction mixture gave the dibenzyl glycosyl phosphate **10** and unreacted **9** which was hydrolyzed during the chromatography in chloroform–methanol, and could be recovered from the plates as the compound

having OH-1 free. Immediate catalytic hydrogenolysis of **10** gave the required tetrasaccharide phosphate **11** in 31% yield.

For the conversion of **11** into the dolichyl diphosphoric diester **13**, it was necessary to first prepare the diphenyl diphosphate **12**. Dolichol isolated from pig-liver (a mixture of isoprenologs C_{80}–C_{105}, with the C_{90} and C_{95} compounds preponderant) was converted into dolichyl phosphate by a modification of the phosphorus oxychloride treatment described by Danilov and Chojnacki[12]. The "activation" of dolichyl phosphate by conversion into the diphenyl diphosphate **12** was performed by treatment with diphenyl phosphorochloridate as described previously[13], and coupling of **12** with the tributylammonium form of the tetrasaccharide phosphate **11** was achieved in anhydrous conditions in the presence of a trace of pyridine in 1,2-dichloroethane[4]. For an efficient coupling reaction, and the avoidance of side reactions, it was essential that the peracetylglycosyl phosphate **11** be in excess. When the proportions of **11** and **12** were reversed, *i.e.*, **12** in excess, the required product was not formed, and only dolichyl phosphate and P^1,P^2-didolichyl diphosphate were identified as products. Even under the best conditions, some of the symmetrical diester was formed, according to t.l.c. The peracetyl diphosphate diester **13** was purified by preparative layer chromatography, and was homogeneous according to t.l.c. in a variety of solvent systems.

O-Deacetylation of **13** to yield the tetrasaccharide lipid intermediate **14** was performed overnight at room temperature by treatment with sodium methoxide in chloroform–methanol, and **14** was pure according to t.l.c. in four solvent systems with three spray reagents[4]. The identities of the dolichyl phosphate and tetrasaccharide residues in **14** were confirmed by a treatment with hot, dilute acid, followed by t.l.c. and l.c. analysis of the hydrolysis products, which showed that **14** was converted into dolichyl phosphate, dolichyl diphosphate, and **6**. The tetrasaccharide phosphate was not identified by this treatment owing to the lability of the glycosyl phosphate bond. The formation of small proportions of a trisaccharide having a single terminal GlcNAc residue suggested that some hydrolysis of the β-D-Glc*p*NAc-(1→4)-D-Glc*p*NAc linkage also occurred, but the possibility remains that a trace of *O*-α-D-mannopyranosyl-(1→6)-*O*-β-D-mannopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucose may have been present in the starting material.

When the tetrasaccharide lipid intermediate **14** was incubated with GDP-D-[^{14}C]mannose and calf pancreas microsomes, the labeled mannose was incorporated into three compounds having the t.l.c. properties of oligosaccharide lipid intermediates and more than two residues of D-mannose[14]. After hydrolysis with hot, dilute acid, the ^{14}C-labeled oligosaccharides were analyzed by a variety of chromatographic, chemical, and enzymic procedures[14]. These experiments showed that the synthetic lipid intermediate was an efficient acceptor of D-mannose residues in the presence of appropriate detergents and manganese chloride[14], forming products with structures that would have been expected on the basis of previous work in this[6] and other laboratories[2,3]. These findings suggest that the "core" pentasaccharide **5** can be formed[14] by both of the alternative routes discussed in the "Introduction".

Synthesis of pentasaccharide lipid intermediate **5**. — The pentasaccharide *O*-α-D-mannopyranosyl-(1→3)-*O*-[α-D-mannopyranosyl-(1→6)]-*O*-β-D-mannopyranosyl-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-D-glucopyranose (**2**) was isolated from bovine α-mannosidosis urine[8]. The compound was identified by l.c. comparison with a synthetic reference[15] compound, pending full characterization by spectroscopic procedures, and purified by preparative-layer chromatography[9]. A portion of the product was further purified by semi-preparative l.c., and the structure confirmed by ^{1}H-n.m.r. spectroscopy and combinations of permethylation and mass-spectrometry[8], but it was not possible to perform the l.c. purification on a scale large enough for synthetic purposes, owing to the inferior resolution obtained with "aminopropyl" modified silica columns when samples are not reduced to the corresponding alditols[9].

The crude pentasaccharide was converted into a peracetyl oxazoline in 78% yield by the Me_3Si triflate procedure[11] starting from the peracetyl derivative, and phosphorylation was performed as described for **9**, but the reaction was very incomplete, resulting in only a 16% yield. Coupling of the peracetyl pentasaccharide phosphate with P^1-dolichyl P^2-diphenyl diphosphate[13] gave a peracetyl diphosphoric diester, after which *O*-deacetylation as described for **13** gave a pentasaccharide lipid intermediate. Examination of the product by t.l.c. in various mixtures of chloroform–methanol–water and chloroform–methanol–15M ammonium hydroxide–water indicated that it was not homogeneous, so a portion was treated with 0.02M hydrochloric acid for 30 min at 100°. The released glycosyl residue was reduced to the alditol with sodium borohydride and analyzed by l.c., which showed a 2:1 mixture of **2** and *O*-α-D-mannopyranosyl-(1→3)-*O*-α-D-mannopyranosyl-(1→6)-*O*-β-D-mannopyranosyl-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-D-glucopyranose. This analysis showed that the synthetic pentasaccharide lipid intermediate was a mixture of two compounds, presumably because the preparative-layer chromatography of starting pentasaccharide **2** had failed to separate it from the isomeric component present in the urine. Further attempts to synthesize pure **5** will therefore depend on the development of alternative chromatographic procedures[16] for the purification of **2**.

Despite the heterogeneity of the synthetic pentasaccharide lipid intermediate **5**, it was useful for some preliminary biosynthetic studies. In t.l.c., the compound co-chromatographed with the pentasaccharide product resulting from the incubation of the tetrasaccharide lipid intermediate **14** with GDP-D-mannose and calf pancreas microsomes[14].

Furthermore, when crude **5** was itself incubated under similar conditions, it acted as an acceptor of D-mannose to form two products that were co-eluted in t.l.c. with the hexa- and hepta-saccharide lipid intermediates formed[14] by the transfer of D-mannose to compound **14**, suggesting that **5** and **14** are converted into the same compounds.

EXPERIMENTAL

General methods. — These were as described previously[4], except that ^{1}H-n.m.r. spectra were recorded either at 60 MHz, with a Varian T-60 spectrometer, or at 500 MHz, with a Bruker WM-500 spectrometer at the Northeast Regional NSF-NMR Facility, Yale University.

Chromatographic methods. — These were described previously[4], except that preparative t.l.c. was performed on 20 cm × 20 cm pre-coated plates of Silica gel F254, layer thickness 0.5 mm (E. Merck, Darmstadt, W. Germany). Liquid chromatography at 7 MPa was performed with a Model 5020 instrument (Varian Associates, Palo Alto, CA) equipped with a u.v. detector, Model ERC 7210 (Erma Optical Co., Japan), and a printer-plotter integrator, Model 3380A (Hewlett Packard, Avondale, PA). Two types of column were used: (*a*) a 5-μm Hi-chrom reversible Amino-Spherisorb (Regis Chemical Co., Morton Grove, IL) eluted with 7:3 acetonitrile–15mM KH_2PO_4 at a flow rate of 2 mL/min with detection at 195 nm; and (*b*) a Microsorb Short One-3μm C-8 reversed phase (Rainin Instruments, Woburn, MA) eluted with a gradient of 80–100% acetonitrile–water over a period of 20 min ending with pure acetonitrile at a flow rate of 1 mL/min, and detection at 230 nm. For all types of chromatography, solvent proportions are v/v.

Peracetylation of the starting material. — Compound **6** (200 mg), prepared from the urine of swainsonine-intoxicated sheep or calves with α-mannosidosis[8] without purification beyond the stage of preparative-layer chromatography, was acetylated as described previously for *O*-α-D-mannopyranosyl-(1→3)-*O*-β-D-mannopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranose[4] to give O-*(2,3,4,6-tetra*-O-*acetyl*-α-D-*mannopyranosyl)-(1→6)*-O-*(2,3,4-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-1,3,6-tri*-O-*acetyl-2-deoxy*-D-*glucopyranose* (**7**; 240 mg, 79%); $[\alpha]_D^{20}$ +2.3° (*c* 0.13, chloroform); t.l.c. (20:1 chloroform–methanol) R_F 0.27 (α anomer), 0.25 (β anomer): ^{1}H-n.m.r. (500 MHz, $CDCl_3$): δ 6.12 (d, $J_{1,2}$ 9.8 Hz, H-1β), 6.08 (d, $J_{1,2}$ 3.6 Hz, H-1α). This material was suitable for the next step without purification.

O-*(2,3,4,6-Tetra*-O-*acetyl*-α-D-*mannopyranosyl)-(1→6)*-O-*(2,3,4-tri*-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* (**9**). — (*a*) A solution of **7** (a mixture of anomers, ratio α to β ~3:2, calculated from height of peaks in ^{1}H-n.m.r. spectrum) (215 mg, 0.172 mmol) in 1,2-dichloroethane (5 mL) was treated with trimethylsilyl triflate (78 mg, 0.35 mmol; Aldrich Chemical Co., Milwaukee, WI) and the mixture was stirred for 40 h at room temperature, when t.l.c. (20:1 chloroform–methanol) showed complete conversion of **7** into **9**, R_F 0.31. After the addition of triethylamine (0.02 mL), the mixture was applied directly to a column of silica gel (30 g) which was eluted with 20:1:0.1 chloroform–methanol–triethylamine. Fractions containing **9** were combined and evaporated to yield 160 mg (78% based on crude **7**), $[\alpha]_D^{20}$ +0.8° (*c* 0.12,

chloroform); ^{1}H-n.m.r. (500 MHz, $CDCl_3$): δ 5.99 (d, 1 H, J 9.4 Hz, N*H*Ac) and 5.89 (d, 1 H, $J_{1,2}$ 7.3 Hz, H-1).

Anal. Calc. for $C_{50}H_{68}N_2O_{31}$: C, 50.34; H, 5.75; N, 2.35. Found: C, 50.14; H, 5.90; N, 2.17.

(*b*) A solution of **8** (110 mg, 88 μmol) in a mixture of 1,2-dichloroethane (13 mL) and diethyl ether (10 mL) was saturated with dry HCl gas for 15 min at −10°. The reaction vessel was sealed and kept for 64 h at room temperature, and then the reagent and solvents were removed by passage of dry N_2 gas. To the residual syrup was added dry acetonitrile (0.5 mL), followed by $NaHCO_3$ (100 mg) and tetraethylammonium chloride (100 mg). The mixture was stirred for 4 h at room temperature with exclusion of moisture, then passed through a short column of silica gel (30 g) and concentrated to 0.5 mL. The concentrated solution was applied to two preparative t.l.c. plates which were dried in a stream of air and irrigated with 15:1 chloroform–methanol. After the first irrigation, the plates were dried in a stream of air and redeveloped in the same solvent. The band containing **9** was located by viewing under u.v. light, and extraction of the silica gel with 2:1 chloroform–methanol gave **9** (40 mg, 38%), with the same properties as the compound prepared by method (*a*).

To confirm the identity of **9** and make sure that no modification of the saccharide residue had occurred during procedures (*a*) and (*b*), a sample (3 mg) was dissolved in a mixture of 500:1 acetonitrile–M *p*-toluenesulfonic acid (0.1 mL) and the solution kept for 3 h at room temperature. After neutralization with 0.1M NaOH and evaporation to dryness, the residue was treated with an excess of 0.5% sodium methoxide in dry methanol, and the mixture kept overnight at room temperature. The solution was then treated with water (0.25 mL) and with $NaBH_4$ (2 mg). The mixture was kept overnight at room temperature and treated with M acetic acid until no more H_2 was evolved, and then passed through a small column (Pasteur pipette) of AG 50 (H^+) cation exchange resin. The column was rinsed with water (2 × 1 mL) and the eluent evaporated (N_2 gas). After 4 additions and evaporations of methanol (0.25 mL each), the residue was analyzed by l.c. (7:3 acetonitrile–15mM KH_2PO_4). For comparison, authentic samples of the alditols derived from *O*-β-D-mannopyranosyl-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-D-glucopyranose[17] (T_R 12.05 min) and compound **6** (T_R 15.5 min) were co-chromatographed. The sample derived from **9** by acid hydrolysis, *O*-deacetylation, and borohydride reduction showed a single peak corresponding to the alditol of compound **6**.

O-(2,3,4,6-Tetra-O-acetyl-α-D-mannopyranosyl)-(1→6)-O-(2,3,4-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**). — In a 7-mL septum vial, compound **9** (140 mg, 0.12 mmol) was exhaustively dried by repeated additions and evaporations of dry toluene (1 mL each), followed by storage in *vacuo* in the presence of P_2O_5 for 16 h. In a dry box (predried with several changes of P_2O_5 and flushed with dry N_2 gas), a solution of dibenzyl

phosphate (Aldrich; 83.4 mg, 0.3 mmol) in dry 1,2-dichloroethane (2 mL) was quickly added to the vial containing **9**, the vial capped, and the contents mixed. After being kept for 3 days in the dry box at room temperature, t.l.c. (10:1 chloroform–methanol) showed the incomplete conversion of **9** (R_F 0.57) into **10** (R_F 0.61), giving a blue color with the phosphate specific reagent. The mixture was directly applied to five preparative t.l.c. plates, solvent was quickly evaporated in a stream of N_2 gas, and the plates were developed with 10:1 chloroform–methanol. The band corresponding to **10** was located by spraying three narrow zones with the phosphate-specific reagent, and **10** was extracted from the silica gel by repeated stirring, filtering, and washing with 2:1 chloroform–methanol. The combined extracts were evaporated to dryness, the residue dissolved in methanol (2 mL) and hydrogenated in the presence of 10% Pd–C (Fluka Chemical Corp., Ronkonkoma, NY) for 2.5 h at 0.2 MPa. After the addition of pyridine (0.04 mL), the catalyst was filtered off and washed with methanol and the combined filtrates were evaporated to yield **11** (48 mg, 31%), $[\alpha]_D^{23}$ +21.5° (*c* 0.13, methanol), R_F (10:10:3 chloroform–methanol–water) 0.85, and (65:35:4:4 chloroform–methanol–15M ammonium hydroxide–water) 0.31; ^{1}H-n.m.r. (500 MHz, CD_3OD): δ 5.40 (dd, 1 H, $J_{1,P}$ 5.0, $J_{1,2}$ 1.5 Hz, H-1), and 2.09–1.91 (39 H, 11 OCOCH_3, 2 NHCOCH_3).

Anal. Calc. for $C_{50}H_{71}N_2O_{35}P \cdot 0.5\ H_2O$: C, 46.19; H, 5.58. Found: C, 46.19; H, 5.64.

Dolichyl phosphate. — Dolichol, isolated from pig liver (50 mg, 38 μmol; Sigma Chemical Co., St. Louis, MO) was dissolved in hexane (3 mL) and the solution added dropwise over 10 min to a stirred mixture of $POCl_3$ (340 μmol, Aldrich), hexane (1.7 mL), and triethylamine (500 μmol, previously dried in the presence of CaH_2). The mixture was stirred for 15 min at room temperature in an atmosphere of Ar, then poured into 44:5:1 acetone–water–triethylamine (10 mL), and kept for 18 h at room temperature. After the addition and evaporation of propanol (2 × 15 mL), and then toluene (15 mL), the residue was dissolved in toluene (15 mL) and the suspension filtered to remove precipitated triethylammonium hydrochloride. The filtrate was evaporated to dryness, and the residue (80 mg) dissolved in 2:1 chloroform–methanol and applied to two preparative t.l.c. plates which were developed in 60:25:4 chloroform–methanol–water. The band corresponding to dolichyl phosphate was located with the $KMnO_4$ reagent, and the product extracted from the silica gel by stirring with the chloroform–methanol–water mixture used for the development. The silica gel was filtered off and washed with the same solvent, and the combined filtrates were evaporated to yield dolichyl phosphate (40 mg, 66%), R_F (60:25:4 chloroform–methanol–water) 0.46 (triethylammonium hydrochloride, 0.36) and (46:21:1:3 chloroform–methanol–15M NH_4OH–water) 0.19 (triethylammonium hydrochloride, 0.68). It was important to ascertain that all the triethylammonium salt was removed prior to the next stage.

P^1-*Dolichyl* P^2-[O-*(2,3,4,6-tetra*-O-*acetyl-α*-D-*mannopyranosyl)-(1→6)*-O-*(2,3,4-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-*gluco-*

pyranosyl] *diphosphate* (**13**). — Compound **11** (9 mg, 7 μmol) was converted into the bis(tributylammonium) salt by treatment with tributylamine as previously described for *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[4]. P^1-Dolichyl P^2-diphenyl diphosphate (**12**) was prepared from dolichyl phosphate (8 mg, 4.8 μmol, triethylammonium salt) as described previously[13]. A mixture of **11** (tributylammonium form) and **12** (tributylammonium form) was dried in a 7-mL septum vial by repeated additions and evaporations of dry toluene (1 mL). The residue was treated with 0.1 mL of a freshly prepared 1% solution of dry pyridine in 1,2-dichloroethane, and then the contents of the vial were thoroughly mixed and kept under an atmosphere of dry Ar for 72 h at room temperature, when t.l.c. (46:21:1:3 chloroform–methanol–15M NH_4OH–water) showed the conversion of **11** (R_F 0.13) into **13** (R_F 0.63) and diphenyl phosphate (detected only by the phosphate-specific spray reagent). The mixture was directly applied to a preparative t.l.c. plate and, after removal of solvents in a stream of N_2 gas, the plate was developed in 46:21:1:3 chloroform–methanol–15M NH_4OH–water. The band corresponding to **13** was detected by the $KMnO_4$ and phosphate-specific spray reagents, and **13** extracted from the silica gel by stirring for 2 h with 10:10:3 chloroform–methanol–water. After filtration and evaporation, the crude product was rechromatographed on a similar t.l.c. plate, which was developed with 60:25:4 chloroform–methanol–water. Detection and extraction as before gave **13** (9 mg, 47%), $[\alpha]_D^{20}$ +9.2° (*c* 0.25, chloroform), pure according to t.l.c. in three solvent systems and detection using three spray reagents, R_F (60:25:4 chloroform–methanol–water) 0.62, (46:21:1:3 chloroform–methanol–15M NH_4OH–water) 0.63, and (65:35:4:4 chloroform–methanol–15M NH_4OH–water) 0.69; ^{1}H-n.m.r. (500 MHz, $CDCl_3$): δ 2.05 (38 H, 12 $OCOCH_3$,CH_2–C(CH_3)=CH–CH_2), 1.99, and 1.98 (2s, 6 H, 2 $NHCOCH_3$), 1.69 (s, CH_3–C=, *cis*), and 1.62 (s, CH_3–C=, *trans*) (it was not possible to integrate the last two signals).

P^1-*Dolichyl* P^2-[O-α-D-*mannopyranosyl-(1→6)*-O-β-D-*mannopyranosyl-(1→4)*-O-*(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-α-D-glucopyranosyl*] *diphosphate* (**14**). — A solution of **13** (120 μg) in 1:1 chloroform–methanol (0.12 mL) was treated with dry methanol (0.12 mL) and a 0.25% solution of sodium methoxide in dry methanol (40 μL), and the mixture kept for 17 h at room temperature with exclusion of moisture. The solution was made neutral (pH paper) with AG 50W-X8 (Et_3N^+) cation-exchange resin, the resin washed with 1:1 chloroform–methanol (2 mL), and the combined eluents were evaporated to give **14** (100 μg, 100%), R_F (60:35:6 chloroform–methanol–water) 0.29, (70:4:9 chloroform–methanol–water) 0.37, (10:10:3 chloroform–methanol–water) 0.60, and (75:44:5:5 chloroform–methanol–15M NH_4OH–water) 0.17.

Analysis of **14**. — (*a*) Compound **14** (150 μg) was treated with 0.1M HCl (0.2 mL) and the mixture kept for 5 min at 90°, and then cooled, diluted with water (0.5 mL), and extracted with chloroform (3 × 0.5 mL). The chloroform and aqueous

layers were concentrated to 0.1 mL and examined by t.l.c. (46:21:1:3 chloroform–methanol–15M NH_4OH–water), which showed that the products of acid hydrolysis were dolichyl phosphate (R_F 0.31), dolichyl diphosphate (R_F 0.15), and *O*-α-D-mannopyranosyl-(1→6)-*O*-β-D-mannopyranosyl-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-D-glucopyranose (**6**). The tetrasaccharide phosphate corresponding to **6** was not detected.

(*b*) Compound **14** (50 μg) was treated with 0.1M HCl (0.1 mL) for 30 min at 90°. After evaporation (N_2 gas), followed by the addition and evaporation of water (2 × 0.2 mL), the residue was dissolved in water (0.2 mL) and treated with $NaBH_4$ (2 mg). The mixture was kept overnight at room temperature, and then processed and analyzed by l.c. as described earlier for the acid hydrolyzate of **9**. This showed a single major peak corresponding to the alditol derived from tetrasaccharide **6**. To confirm this result, a portion of the reduced hydrolyzate was perbenzoylated and examined by reversed phase l.c.[18]. This showed a major peak corresponding to the alditol derived from **6** together with a small proportion of *O*-α-D-mannopyranosyl-(1→6)-*O*-β-D-mannopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucitol, probably derived from the acid hydrolysis of the di-*N*-acetylchitobiose residue in **6**.

ACKNOWLEDGMENTS

The authors thank Prof. H. Paulsen for the gift of synthetic pentasaccharide **2** and Ms. Birgitte Bugge for performing the l.c. analyses. The authors acknowledge the support of the Northeast Regional NSF-NMR Facility at Yale University, New Haven, CT 06520. The Facility is funded by Grant No. CDP-791620 from the Chemistry Division of the National Science Foundation.

REFERENCES

1 R. KORNFELD AND S. KORNFELD, *Annu. Rev. Biochem.*, 54 (1985) 631–664.
2 A. CHAPMAN, E. LI, AND S. KORNFELD, *J. Biol. Chem.*, 254 (1979) 10,243–10,249.
3 J. W. JENSEN, J. D. SPRINGFIELD, AND J. S. SCHUTZBACH, *J. Biol. Chem.*, 255 (1980) 11,268–11,272.
4 C. D. WARREN, M.-L. MILAT, C. AUGÉ, AND R. W. JEANLOZ, *Carbohydr. Res.*, 126 (1984) 61–80.
5 A. HERSCOVICS, C. D. WARREN, AND R. W. JEANLOZ, *FEBS Lett.*, 156 (1983) 298–302.
6 W. SASAK, C. LEVRAT, C. D. WARREN, AND R. W. JEANLOZ, *J. Biol. Chem.*, 259 (1984) 332–337.
7 C.-M. LIU, C. D. WARREN, AND R. W. JEANLOZ, *Carbohydr. Res.*, 136 (1985) 275–284.
8 P. F. DANIEL, C. D. WARREN, L. F. JAMES, AND R. D. JOLLY, in A. A. SEAWRIGHT, L. F. HEGARTY, L. JAMES, AND R. F. KEELER (Eds.), *Plant Toxicology*, The Queensland Poisonous Plants Committee, Queensland, Australia, 1985, pp. 290–300.
9 C. D. WARREN, P. F. DANIEL, B. BUGGE, N. O'NEILL, AND R. W. JEANLOZ, unpublished results.
10 C. D. WARREN, R. W. JEANLOZ, AND G. STRECKER, *Carbohydr. Res.*, 92 (1981) 85–101.
11 S. NAKABAYASHI, C. D. WARREN, AND R. W. JEANLOZ, *Carbohydr. Res.*, 150 (1986) c7–c10.
12 L. L. DANILOV AND R. CHOJNACKI, *FEBS Lett.*, 131 (1981) 310–312.
13 C. D. WARREN AND R. W. JEANLOZ, *Carbohydr. Res.*, 37 (1974) 252–260.
14 Y. GOUSSAULT, S. NAKABAYASHI, C. D. WARREN, B. BUGGE, AND R. W. JEANLOZ, unpublished results.
15 H. PAULSEN AND R. LEBUHN, *Carbohydr. Res.*, 130 (1984) 85–101.
16 Y. GOUSSAULT, C. D. WARREN, B. BUGGE, AND R. W. JEANLOZ, *Glycoconjugate J.*, 3 (1986) 239–245.

17 C. D. WARREN, C. AUGÉ, M. L. LAVER, S. SUZUKI, D. POWER, AND R. W. JEANLOZ, *Carbohydr. Res.*, 82 (1980) 71–83.
18 P. F. DANIEL, D. F. DEFEUDIS, I. T. LOTT, AND R. H. MCCLUER, *Carbohydr. Res.*, 97 (1981) 161–180.