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Synthesis of *Neo*glycoproteins Containing the 3,6-di-*O*-Methyl-β-D-Glucopyranosyl Epitope and their Use in Serodiagnosis of Leprosy

DELPHI CHATTERJEE[1], JAMES T DOUGLAS[2], SANG-NAE CHO[1], THOMAS H REA[3], ROBERT H GELBER[4], GERALD O ASPINALL[5] and PATRICK J BRENNAN[1]*

[1]*Department of Microbiology, Colorado State University, Fort Collins, CO 80523, USA*
[2]*Department of Microbiology, University of Hawaii, Honolulu, HI 96822, USA*
[3]*Department of Health Services, Los Angeles County-University of Southern California Medical Center, Los Angeles, CA 90033, USA*
[4]*Hansen's Disease Program of the United States Public Health Service, Seton Medical Center, Daly City, CA 94105, USA*
[5]*Department of Chemistry, York University, Downsview, Ontario, Canada M3J 1P3*

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A stratagem for the synthesis of *neo*glycoproteins suitable for the selective serodiagnosis of leprosy is described in which synthetic 3,6-di-*O*-methyl-β-D-glucopyranose, the epitope of phenolic glycolipid I from *Mycobacterium leprae*, was used. Condensation of 8-methoxycarbonyloctanol with the acetobromo derivative of 3,6-di-*O*-methyl-glucose gave 8-methoxycarbonyloctyl 2,4-di-*O*-acetyl-3,6-di-*O*-methyl-β-D-glucopyranoside in 65% yield, and with absolute stereospecificity for the β anomer. The deacylated product was converted to the crystalline hydrazide and coupled to bovine gamma globulin, bovine serum albumin and poly-D-lysine *via* intermediate acyl azide formation to produce the 8-carbonyloctyl 3,6-di-*O*-methyl-β-D-glucopyranosyl polypeptides. The *neo*glycoproteins were highly sensitive in ELISA and emulated the specificity of the native glycolipid in analysis of sera from patients throughout the spectrum of leprosy and from different geographical regions. The 8-carbonyloctyl 3,6-di-*O*-methyl-α-D-glucopyranoside-bovine serum albumin was also synthesized and shown to have about one-half the activity of the β-linked *neo*glycoprotein. A different synthetic approach produced the 8-carbonyloctyl 4-*O*-(3′,6′-di-*O*-methyl-β-D-glucopyranosyl)-α-L-rhamnopyranoside-bovine serum albumin which was also highly sensitive and spe-

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Nomenclature: BGG, bovine gamma globulin; PGL-I, phenolic glycolipid I; PDL, poly-D-lysine; PBS, phosphate-buffered saline; 3,6-Me_2-Glc-Link-BSA, 8-carbonyloctyl 3,6-di-*O*-methyl-glucopyranoside-bovine serum albumin; 3,6-Me_2-Glc-Rha-Link-BSA, 8-carbonyloctyl 4-*O*-(3′,6′-di-*O*-methyl-β-D-glucopyranosyl)-α-L-rhamnopyranoside-BSA.

*Author for correspondence.

cific for the serodiagnosis of leprosy. The presence of the second sugar unit, similar to that in the native glycolipid but for the absence of *O*-methyl groups, seemed to provide a probe with greater felicity for the serological detection of tuberculoid leprosy.

Thus, the results indicate that highly sensitive and specific antigen probes for the serodiagnosis of leprosy can be constructed based only on the terminal one or two sugars of phenolic glycolipid I, and the synthetic approach leads to the formation of haptens with absolute stereospecificity.

The trisaccharide, 3,6-di-*O*-Me-β-D-glucopyranosyl-(1-4)-2,3-di-*O*-Me-α-L-rhamnopyranosyl-(1-2)-3-*O*-Me-α-L-rhamnopyranose [1] is the *Mycobacterium leprae*-specific segment of a phenolic phthiocerol-containing glycolipid (phenolic glycolipid I, PGL-I) which has proved highly selective for the serodiagnosis of leprosy [2, 3, 4]. With the primary intent of confirming structure, especially enantiomeric configurations, we had synthesized the trisaccharide and its inherent terminal disaccharide [5]. Both proved to be effective in inhibiting binding between phenolic glycolipid I and anti-glycolipid immunoglobulin M in human lepromatous leprosy sera, whereas analogous oligosaccharides in which the terminal sugar was 6-*O*-Me-β-D-glucopyranose or β-D-glucopyranose were much less active or entirely inactive, respectively. In addition, conjugation of the 3,6-di-*O*-Me-β-D-glucopyranosyl-(1-4)-2,3-di-*O*-Me-α-L-rhamnopyranose to protein *via* reductive amination yielded a *neo*glycoprotein which was highly reactive and specific for the serodiagnosis of leprosy. Glycoconjugates containing 6-*O*-Me-β-glycopyranosyl-(1-4)-2,3-di-*O*-Me-rhamnitol, β-glucopyranosyl-(1-4)-2,3-di-*O*-Me-rhamnitol, or the simple 3,6-di-*O*-Me-glucitol, were inactive [6]. The inability of partially deglycosylated PGL-I (i.e., the product devoid of the terminal 3,6-di-*O*-Me-glucopyranose) to bind to anti-glycolipid antibodies from infected humans [5], or to murine monoclonal antibodies [7-9] also helped confirm that the terminal sugar was the predominant epitope.

Accordingly, in the present work we have synthesized the sugar, but as the 8-methoxycarbonyloctyl 3,6-di-*O*-Me-β-D-glucopyranoside in order to preserve the natural β-glycosidic linkage and to provide a linker arm, which, by using the strategy of Lemieux *et al.* [10], could be used to attach the sugar determinant to the ε-amino group of lysine on polypeptides. Variations on this basic structure were introduced, such as replacement of the β- with the α-anomer and introduction of a second, penultimate rhamnosyl substituent. The efficacy and specificity of the resulting products in the serodiagnosis of leprosy were tested using sera from leprosy patients manifesting different clinical forms of the spectral disease and from different geographical regions. A preliminary account of a portion of this work has been published [11].

Materials and Methods

Synthesis of 8-Methoxycarbonyloctyl 3,6-di-O-Me-β-D-Glucopyranoside

3-*O*-Me-D-glucose (Sigma Chemical Co., St. Louis, MO, USA) (15 g) was acetylated and

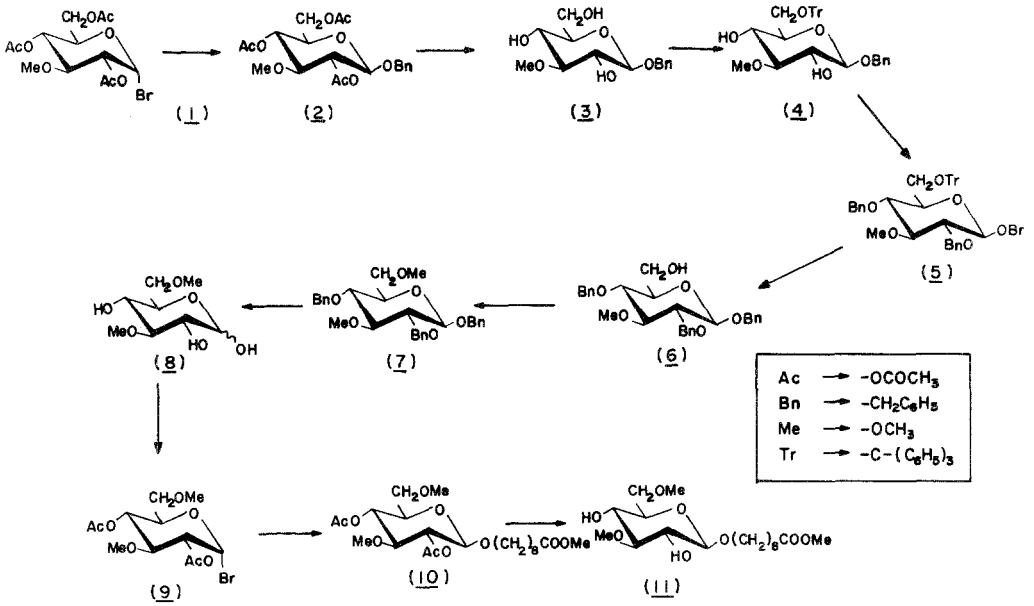

Figure 1. Pathway for the synthesis of 8-methoxycarbonyloctyl 3,6-di-*O*-Me-β-D-glucopyranoside.

brominated to give 2,4,6-tri-*O*-Ac-3-*O*-Me-α-D-glucopyranosyl bromide (27 g) (**1** in Fig. 1). Condensation of **1** with equimolar quantities of benzyl alcohol in the presence of mercuric cyanide (16 g) and powdered, predried, molecular sieves (4 Å) [12] gave benzyl 2,4,6-tri-*O*-Ac-3-*O*-Me-β-D-glycopyranoside **2** (20 g, 73.5%, m.p. 88-91°C). Deacetylation of **2** gave **3** as a crystalline compound (m.p. 106°C; $[\alpha]_D$ -55.26°, c 2.87 in $CHCl_3$; 13 g, 94% yield). Compound **3** was tritylated using triphenylmethylchloride in the presence of dimethylaminopyridine [13] in dry dichloromethane to give benzyl-3-*O*-Me-6-*O*-trityl-β-D-glucopyranoside **4** which was benzylated [14] using sodium hydride and benzyl bromide in dry tetrahydrofuran to give benzyl 2,4-di-*O*-benzyl-3-*O*-Me-6-*O*-trityl-β-D-glucopyranoside **5**, which was detritylated by heating under reflux with 1 N HCl for 3 h. Column chromatography in chloroform/ether, 6/1 by vol, gave the pure detritylated compound **6**, which was methylated [14] to give benzyl 2,4-di-*O*-benzyl-3,6-di-*O*-Me-β-D-glucopyranoside **7** (m.p. 82°C; $[\alpha]_D$ -22.37°, c 4.13 in $CHCl_3$; 8 g, 91% yield). Hydrogenolysis of compound **7** in ethanol, containing 10% palladium on charcoal, gave 3,6-di-*O*-Me-D-glucose **8**, a known compound [11, 15]. Acetylation and bromination gave 2,4-di-*O*-Ac-3,6-di-*O*-Me-α-D-glucopyranosyl bromide **9** which was not isolated due to its thermal instability but condensed directly with 8-methoxycarbonyloctanol, prepared as described [16], in benzene and nitromethane in the presence of mercuric cyanide with stirring for 16 h at room temperature. 8-Methoxycarbonyloctyl 2,4-di-*O*-Ac-3,6-di-*O*-Me-β-D-glucopyranoside **10** was obtained after column purification in benzene/ethyl acetate, 3/1 by vol, as a syrup. Deacylation of **10** gave **11** in 70% overall yield ($[\alpha]_D$ -30°, c 1.0 in CH_3OH).

2,4-Di-*O*-benzyl-3,6-di-*O*-Me-D-glucose **12** (m.p. 75-77°C) was prepared as described previously [11]. It was acetylated, and compound **13** was dissolved in anhydrous dichloromethane, cooled to -10°C, and brominated with trimethylsilylbromide [17]. Evaporation of the solvents after 1.5 h gave a syrupy product. Due to the instability of the compound, no attempts were made to isolate **14**, but it was added directly to a well-stirred mixture of 8-methoxycarbonyloctanol and tetrabutylammonium bromide [18] in dry dichloromethane in the presence of powdered molecular sieves. The reaction mixture was stirred for 3 days at room temperature, quenched with methanol and filtered through a bed of Celite. Extraction with water and evaporation of the solvent followed by purification by preparative TLC using petroleum ether/ether, 1.5/1 by vol, gave pure **15**. Compound **15** was dissolved in ethanol and hydrogenolysed using 10% palladium on charcoal. Pure compound **16** ($[\alpha]_D$ +32°, c 1.0 in CH_3OH) was obtained as a syrup in 55% overall yield starting from 0.080 g of compound **15**.

Synthesis of 8-Methoxycarbonyloctyl 4-0-(3'6'di-O-Me-β-D-Glucopyranosyl)-α-L-Rhamnopyranoside

L-Rhamnose (25 g) was dissolved in pyridine (100 ml) and cooled to 0°C. Acetic anhydride (100 ml) was added and the mixture maintained at room temperature overnight. 1,2,3,4-Tetra-*O*-Ac-L-rhamnopyranose was obtained as a syrup. Bromination using HBr in acetic acid gave the acetobromorhamnose **17** in 50% yield. The acetobromorhamnose (5 g) was dissolved in dichloromethane and added dropwise to a well-stirred mixture of 8-methoxycarbonyloctanol, mercuric cyanide, and powdered molecular sieves in dichloromethane. Compound **18** was obtained in 70% yield after column chromatography using chloroform/ether, 4/1 by vol. Deacylation of compound **18** gave **19**, in quantitative yield. Treatment of **19** with 2,2-dimethoxypropane, acetone, and a catalytic quantity of *p*-toluenesulfonic acid resulted in 8-methoxycarbonyloctyl 2,3-*O*-isopropylidene-α-L-rhamnopyranoside **20**, a syrup ($[\alpha]_D$ -19,5°, in $CHCl_3$) [19]. Compound **20** (0.090 g, 0.2406 mmol) was condensed with 2,4-di-*O*-Ac-3,6-di-*O*-Me-α-D-glucopyranosyl bromide (**9** in Fig. 1) (0.250 g, 0.563 mmol) in anhydrous dichloromethane containing mercuric cyanide:mercuric bromide (5:1) and powdered molecular sieves and with stirring overnight under dry argon [20]. The fully protected disaccharide, 8-methoxycarbonyloctyl 2,3-*O*-isopropylidene-4-*O*-(2',4'di-*O*-Ac-3',6'-di-*O*-Me-β-D-glucopyranosyl)-α-L-rhamnopyranoside **21** was obtained as a syrup ($[\alpha]_D$ -40°, c 1.0 in $CHCl_3$; 0.1 g, 72% yield) after column chromatography in benzene/acetone, 4/1 by vol. Compound **21** was deacylated with a catalytic amount of sodium methoxide in anhydrous methanol at 0°C and the resulting compound **22** was hydrolysed with 99% trifluoroacetic acid at 0°C for 15 min. 8-Methoxycarbonyloctyl 4-*O*-(3',6'-di-*O*-Me-β-D-glucopyranosyl) -α- L-rhamnopyranoside **23** was obtained as a syrup ($[\alpha]_D$ -39.07°, c 4.01 in CH_3OH) after preparative TLC using chloroform/methanol, 9/1 by vol, as solvent.

Preparation of the Hydrazides, Azides and Neoglycoproteins

The 8-methoxycarbonyloctyl glycosides **11**, **16**, **23**, approximately 50 mg, were converted to their hydrazides (see Fig. 7) by the addition of hydrazine hydrate (85%, 0.5 g) in ethanol (3 ml) and stirring for 24-48 h at room temperature [21]. Dilution with water and evaporation rendered the products hydrazine-free and gave white solids which crystallized from aqueous ethanol.

The hydrazides, in general, were dissolved in anhydrous dimethylformamide and the solution cooled to -30°C. A solution of freshly prepared 3.5-4 N HCl in dioxane was added, followed by 10% *tert*-butyl nitrite in dimethylformamide. The mixture was stirred at -30°C for 30 min. Sulfamic acid in dimethylformamide was added and stirring continued for an additional 15 min. The acyl azides were not isolated, but the cold solution was used directly in the preparation of the *neo*glycoproteins. Bovine gamma globulin (BGG; fraction II, electrophoretic purity grade; Sigma), poly-D-lysine (PDL; 15-30 000 mol wt range; Sigma) or bovine serum albumin (BSA; fraction IV; Sigma) were used as carrier proteins.

Some examples of the preparation of the various hydrazides, acyl azides and *neo*glycoproteins are as follows. The α- and β- monosaccharide hydrazides **24** and **27** (2.5 mg, 6.2 μmol) were each dissolved in dimethylformamide (100 μl) and 3.6 N HCl in dioxane (15 μl) was added followed by *tert*-butyl nitrite (30 μl). The reaction mixtures were neutralized after 30 min with sulfamic acid (30 μl). The cold (-50°C) solution of acyl azide was added dropwise, by means of a micropipette (precooled to -70°C), to a solution of BSA (4 mg, 0.05 μmol) in 0.4 ml of 0.08 M $Na_2B_4O_7$ and 0.3 M $KHCO_3$, pH 9.2, at 0°C, stirred overnight at 0°C and dialysed against five changes of deionized water in an ultrafiltration cell (Amicon Corp., Danvers, MA, USA). Most preparations of monosaccharide-containing products were further fractionated on columns of Sephadex G-75 (Pharmacia, Uppsala, Sweden), and material containing the highest ratio of carbohydrate to protein was used.

In the case of the disaccharide-containing *neo*glycoprotein, the hydrazine-free hydrazide **27** (6 mg, 11 μmol). was dissolved in 200 μl dimethylformamide, a 3.6 N HCl/dioxane solution (15 μl) was added, and the reaction mixture cooled to -20°C. *tert*-Butyl nitrite (30 μl) was added followed by sulfamic acid (0.5 M, 30 μl) to neutralize the excess nitrous acid. The acyl azide formed at -70°C was added dropwise to a solution of BSA (10 mg, 0.14 μmol, in 1 ml buffer, as described above). The reaction mixture was stirred at 0°C overnight and dialyzed against five changes of distilled water before applying to a column (1 × 100 cm) of Sephadex G-75. The product in the void volume was used.

Materials and Analytical Methods

All solvents and reagents were purified according to standard procedures [22]. Deacylations were effected using anhydrous sodium methoxide in dry methanol. Analytical TLC was performed on precoated silica gel 60-F_{254} (E. Merck, Darmstadt, W. Germany) and products were visualized by charring after spraying with 5% sulfuric acid in ethanol. For column chromatography, silica gel (type 60-200 mesh) and distilled solvents were used.

Preparative TLC was conducted on the Chromatotron (Model 7924, Harrison Research,

Palo Alto, CA, USA) centrifugal TLC device using a 1 or 2 mm rotor. Structural assignments were supported by 270 or 360 MHz NMR spectroscopy recorded on the appropriate Bruker spectrophotometers. ^{1}H- and ^{13}C-NMR chemical shifts are given in parts per million (ppm) relative to internal 1% tetramethylsilane (TMS). Melting points are reported uncorrected.

Enzyme Linked Immunosorbent Assay

The IgM ELISA protocol described previously [3] was used with the alterations described in the Results section for the studies involving sera from the Southern and Northern California clinics. In these assays, only IgM was assayed for reasons described previously [3]. Analysis of sera from Hawaii, Ponape (Federated States of Micronesia) and the Philippines was conducted in the Honolulu laboratory and conditions were as follows. The PGL-I antigen (100 μg/ml) was sonicated in 0.01 M ammonium acetate-ammonium carbonate buffer (pH 8.2) for 3 × 30 s periods with a 3 mm probe. This suspension was then diluted to a concentration of 4 μg/ml in the same buffer and routinely used at this concentration. Whole *M. leprae* [1] were suspended in the same volatile coating buffer, at a concentration of 0.02 absorbance units at 420 nm. The 8-carbonyloctyl 3,6-di-*O*-Me-β-glucopyranosyl-BGG (320 μg/ml of glucose equivalent) was diluted 1:10 000 to give 32 ng glucose/ml and 50 μl was applied to each well. The 8-carbonyloctyl 3,6-di-*O*-Me-β-glucopyranosyl-BSA (300 μg/ml) was diluted 1:200 to give 1.5 μg/ml of which 50 μl were applied to each well. The 8-carbonyloctyl 3,6-di-*O*-Me-α-glucopyranosyl-poly-D-lysine (1130 μg/ml) was diluted 1:5 000 (226 ng/ml) and 50 μl applied to each well. The 8-hydrazinocarbonyloctyl 4-*O*-(3',6'-di-*O*-Me-β-D-glucopyranosyl)-α-L-rhamnopyranoside-BSA (300 μg/ml) was diluted 1:200 and 50 μl applied to each well. Immulon 2, "U" microtitre plates (Dynatech Labs., Alexandria, VA, USA) were used and antigen was coated by incubating to dryness at 37°C overnight. Antigen-coated wells were blocked to prevent non-specific binding by adding 0.075 ml of 5% goat serum (Gibco, Chagrin Falls, OH, USA) in phosphate buffered saline (PBS; Na_2HPO_4, 12.8 g; NaH_2PO_4, 2.62 g; NaCl, 0.58 g; H_2O, 1 l; pH 7.4) and incubating overnight at 4°C. Other details of this ELISA protocol have been described [23]. A multiclass conjugate (IgG + IgA + IgM) of goat antihuman immunoglobulins conjugated to horseradish peroxidase (Cappel Labs., Cochranville, PA, USA) diluted 1:2000, was used.

Sera

Sera were obtained from individual leprosy patients attending the Hansen's Disease Clinics at Seton Medical Center, Daly City, CA, USA, or Los Angeles County-University of Southern California, Los Angeles, CA, USA. A pool of serum was obtained by mixing equal volumes of sera from several patients (of R.H. Gelber) with lepromatous leprosy. Patients were classified clinically and pathologically according to the Ridley and Jopling scale [24, 25]. Also, three reactional states (reversal reactions, erythema nodosum leprosum (ENL) and Lucio reaction) were diagnosed according to published criteria [26]. Sera were also obtained from patients with mycobacterial infections other than *M. leprae* attending the National Jewish Hospital and Research Center, Denver, CO, USA. In all cases, clinical symptoms of disease were evident, acid fast bacilli were detected in sputa or other body fluids or organs, and mycobacteria had been cultured.

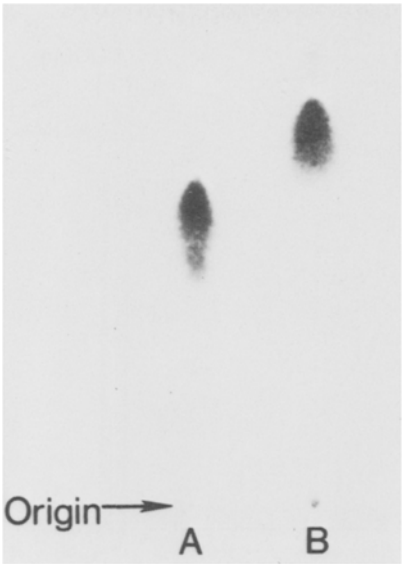

Figure 2. TLC of the synthetic 3,6-di-*O*-Me-D-glucopyranose mixture, A; the 8-methoxycarbonyloctyl 3,6-di-*O*-Me-β-D-glucopyranoside preparation, B. Solvent: $CHCl_3/CH_3OH$, 5/1 by vol. The plate was slightly charred by spraying with 10% H_2SO_4 and heating at 110°C for 10 min.

The Hawaiian study involved separate sera. In Ponape, blood was collected *via* finger prick into a standard heparinized microhematocrit tube marked to 75 μl [23]. Sera from leprosy and tuberculosis patients and normal volunteers in Hawaii were collected by venipuncture, as were sera from leprosy patients at the Leonard Wood Memorial Leprosy Research Center, Cebu, Philippines. Such sera were usually diluted 1:700 in PBS containing 1% goat serum.

Results

Synthesis of 8-Methoxycarbonyloctyl 3,6-di-O-Me-α- and β-D-Glucopyranosides

Synthesis of 3,6-di-*O*-Me-glucose and the 8-methoxycarbonyloctyl β-glucoside is summarized in Fig. 1. 3-*O*-Me-D-glucose (15 g) gave 10 g of the 3,6-di-*O*-Me-D-glucose **8** which in turn produced methoxycarbonyloctyl 3,6-di-*O*-Me-β-D-glucopyranoside **11** in 70% yield. TLC of the monosaccharide preparation showed a mixture, possibly of the α- and β-anomers (Fig. 2, A). GLC of the acetate of 3,6-di-*O*-Me-glucitol on SP-2340 at 190°C showed a single peak with a retention time of 14.73 which corresponded to the 3,6-di-*O*-Me-glucitol acetate prepared from PGL-I [1]. The mass spectrum showed major peaks (m/z 87, 113, 129, 173, 189 and 233) of a 1,2,4,5-tetra-*O*-Ac-3,6-di-*O*-Me-hexitol [1]. TLC of the 8-methoxycarbonyloctyl glucoside showed slight traces of an unknown impurity (Fig. 2, B). The ^{1}H-NMR spectrum of the acetylated 8-methoxycarbonyloctyl glucoside (**10** in

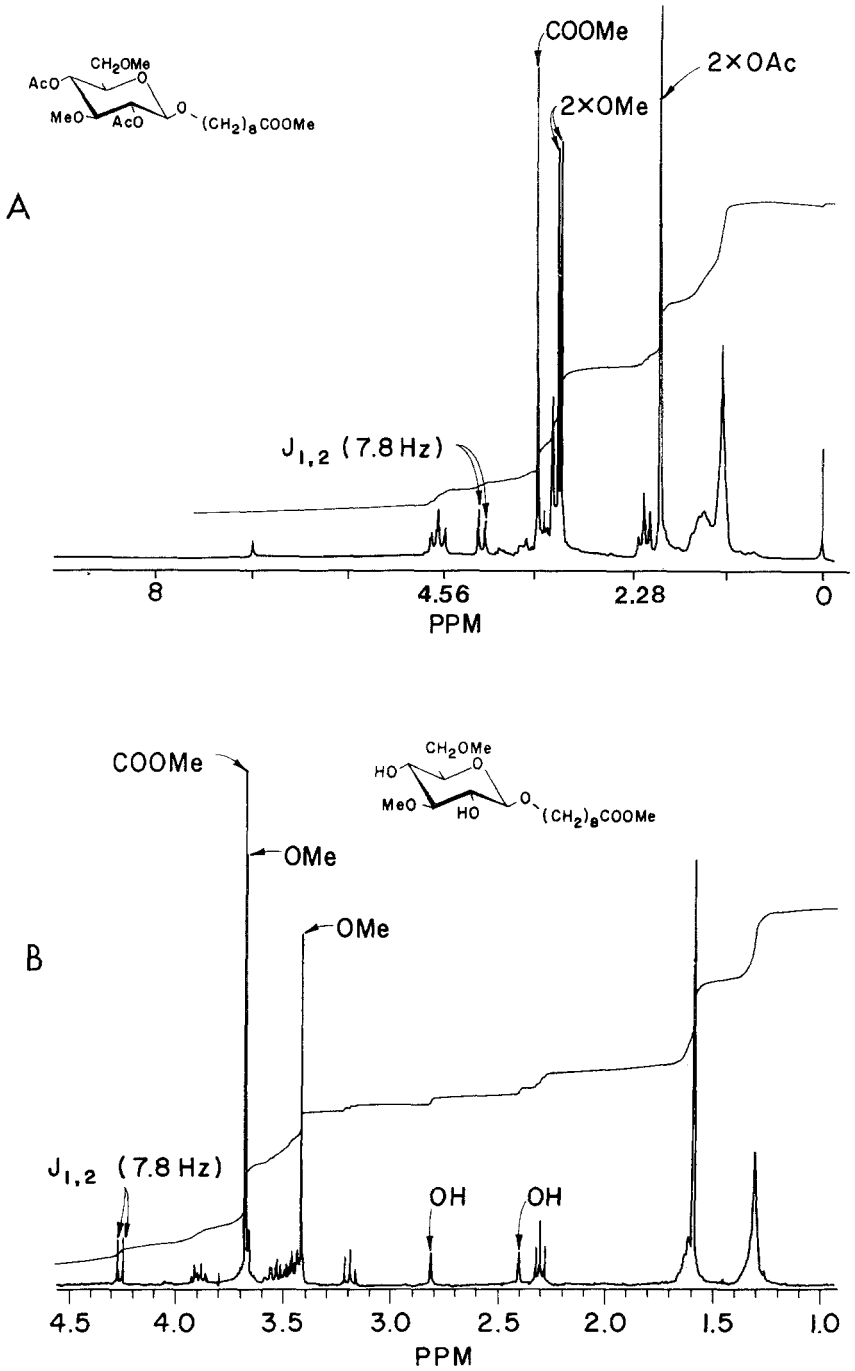

Figure 3. ^{1}H-NMR at 360 MHz of the 3,6-di-*O*-Me-glucopyranosyl derivatives. A, 8-methoxycarbonyloctyl 2,4-di-*O*-Ac-3,6-di-*O*-Me-β-D-glucopyranoside; B, 8-methoxycarbonyloctyl 3,6-di-*O*-Me-β-D-glucopyranoside.

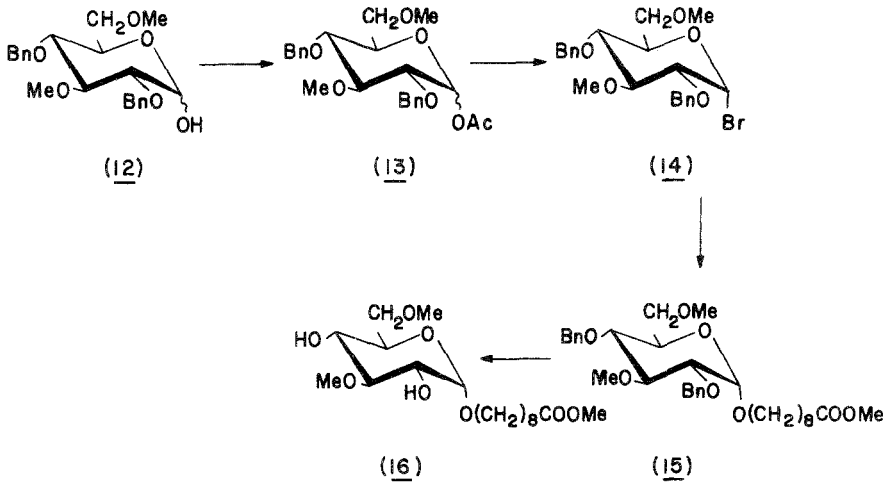

Figure 4. Pathway for the synthesis of 8-methoxycarbonyloctyl 3,6-di-*O*-Me-α-D-glucopyranoside.

Fig. 1) showed a multiplet at 4.6 ppm, due to H-2 and H-4, and a doublet at 4.4 ppm ($J_{1,2}$ 7.8 Hz) due to the β-anomeric proton (Fig. 3A). The singlet at 3.66 ppm arises from the ester methyl (-$COOCH_3$) and the two singlets at 3.39 ppm and 3.35 ppm are due to the two methyl esters. The ^{13}C-NMR spectrum of the acetylated 8-methoxycarbonyloctyl glycoside showed the presence of three C=0 groups between 173.7 and 168.6 ppm and the β-anomeric carbon at 100.8 ppm (results not shown). ^{1}H-NMR of the deacetylated glycoside (**11** in Fig. 1) showed a doublet at 4.25 ppm ($J_{1,2}$ 7.79 Hz) and two singlets at 3.66 Hz due to ester methyl and methyl ether (Fig. 3B). The ^{13}C-NMR spectrum showed a resonance at 182.5 ppm due to the C=0 function and at 102 ppm due to the anomeric carbon, and infra-red spectroscopy showed strong absorption at 1740 cm^{-1} due to the terminal methyl ester function (results not shown). Thus, all of the analytical data were in accord with the named compounds.

8-Methoxycarbonyloctyl 3,6-di-*O*-Me-α-D-glucopyranoside was synthesized as described in Fig. 4. When 2,4-di-*O*-Ac-3,6-di-*O*-Me-glucopyranosyl halide was allowed to react with 8-methoxycarbonyloctanol in the presence of silver triflate and silver carbonate, we obtained only the β-anomer, even when the reaction was conducted at low temperature (-30°C). An alternative approach as described in the Materials and Methods section using Lemieux's "common ion" catalyst [18] gave only the α-anomer **16** in moderate yield (56%).

The relative mobility on thin layer plates of the α-anomer was identical to that of the β-anomer, and the ^{1}H-NMR spectrum of compound **16** was also similar except that the anomeric proton resonated at 4.5 ppm and had a coupling constant of 3.2 Hz. Three singlets at 3.69, 3.66, and 3.33 were due to ester methyl and the two ether methyls. The optical rotation was positive (+32° in CH_3OH) as compared to its β-anomer. Although this compound was not obtained in a crystalline state, the analytical and spectral data were in accord with the proposed structure.

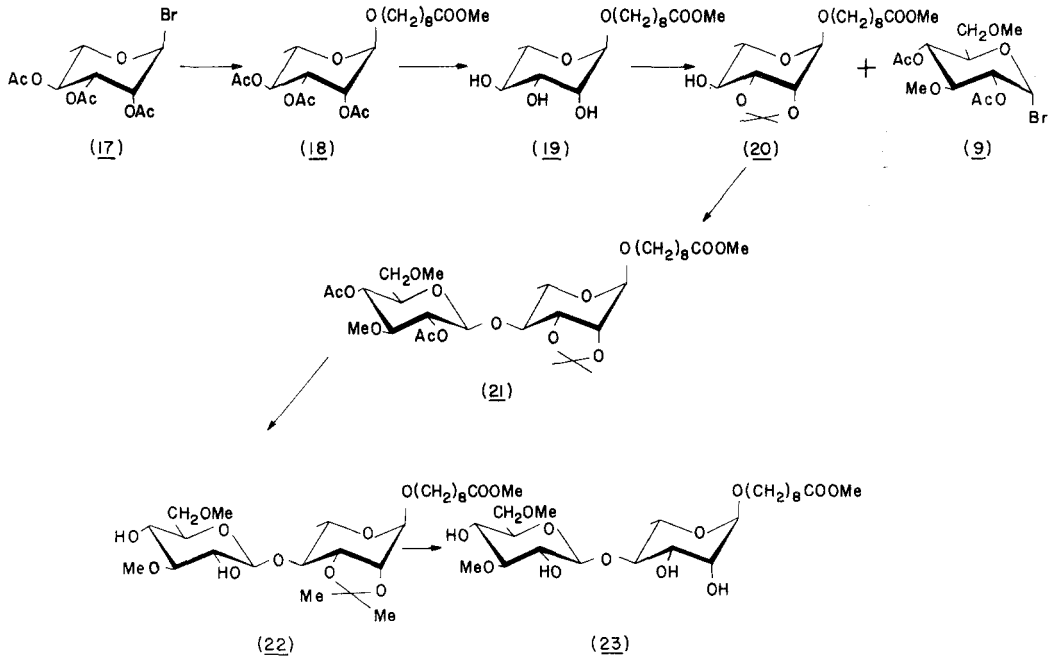

Figure 5. Pathway for the synthesis of 8-methoxycarbonyloctyl 4-*O*-(3′,6′-di-*O*-Me-β-glucopyranosyl)-α-L-rhamnopyranoside.

Synthesis of 8-Methoxycarbonyloctyl 4-O-(3′,6′-di-O-Me-β-D-Glucopyranosyl)α-L-Rhamnopyranoside.

The 8-methoxycarbonyloctyl 2,3-*O*-isopropylidene-4-*O*-(3′,6′-di-*O*-Me-β-D-glucopyranosyl)α-L-rhamnopyranoside was synthesized according to the scheme in Fig. 5. Condensation of compounds **20** and **9** as described in Materials and Methods gave exclusively the desired β(1-4) linkage. The optimum ratio of mercuric cyanide and mercuric bromide was achieved after experimenting with a wide variety of catalysts and different solvents. This observation supports Paulsen's [20] observation that the course of oligosaccharide synthesis is influenced by the reactivity of the halide, catalyst, and alcohol. The ^{1}H-NMR spectrum of compound **21** showed a doublet with 7.52 Hz spacing at 4.37 ppm for the glucosyl unit (results not shown). The H-1′ of the glucosyl unit was somewhat obscure due to the deshielding of H-2′ and H-4′ at 4.9 ppm due to acetyl substitutents. However, compound **23** obtained after deprotection of **21** showed a singlet at 4.74 ppm attributable to H-1 and a doublet at 4.5 ppm for H-1′ ($J_{1',2'}$ 7.7 Hz) (Fig. 6). Two singlets at 3.67 and 3.66 ppm for an ester methyl and an ether methyl, and a singlet at 3.41 ppm for a second ether methyl, were evident. ^{13}C-NMR spectroscopy showed a resonance for carbonyl at 174.57 ppm (results not shown). The lines at 105.1 and 99.3 were due to C-1′ and C-1, and three resonances at 60.3, 59.6, and 51.50 were due to two methoxyl and one ester methyl. The methylene carbons of the spacer arm resonated between 29.3 and 24.9 ppm. All these data supported the structure of the 8-methoxycarbonyl-disaccharide.

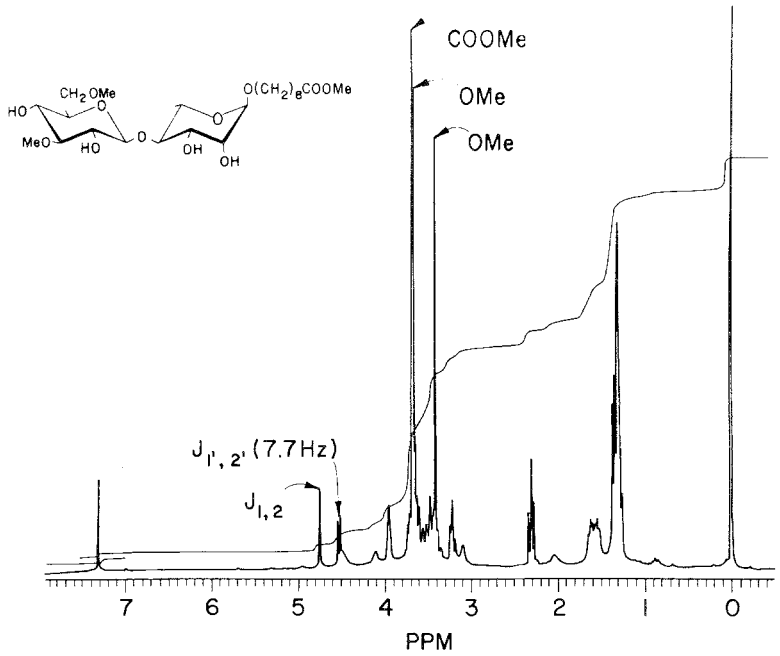

Figure 6. ^{1}H-NMR at 360 MHz of 8-methoxycarbonyloctyl 4-*O*-(3′,6′-di-*O*-Me-β-D-glucopyranosyl)-α-L-rhamnopyranoside.

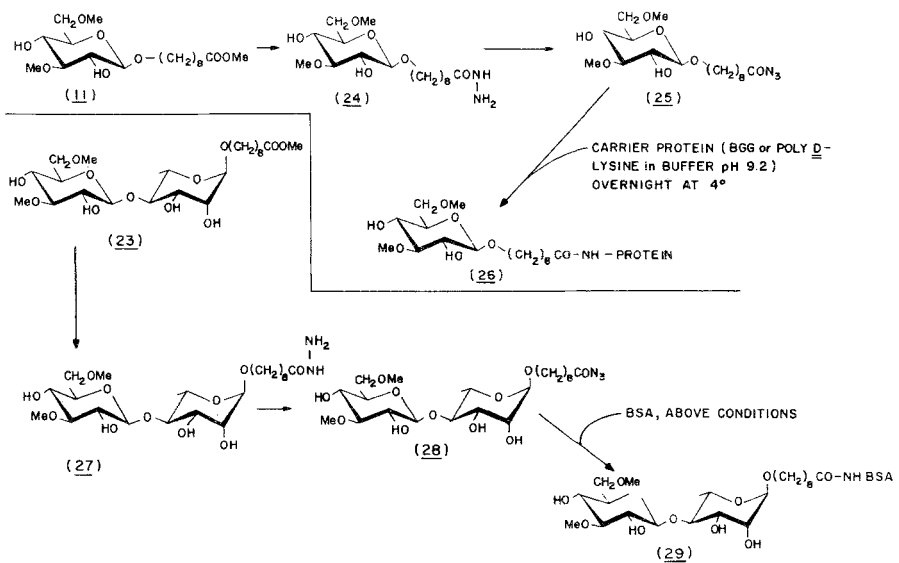

Figure 7. Pathway for the preparation of the *neo*glycoproteins.

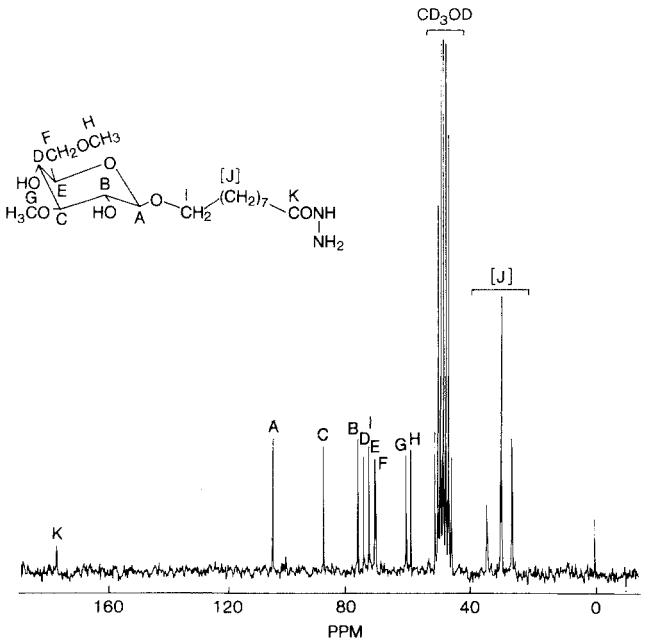

Figure 8. ^{13}C-NMR (360 MHz) of the 8-hydrazinocarbonyloctyl 3,6-di-*O*-Me-β-D-glucopyranoside.

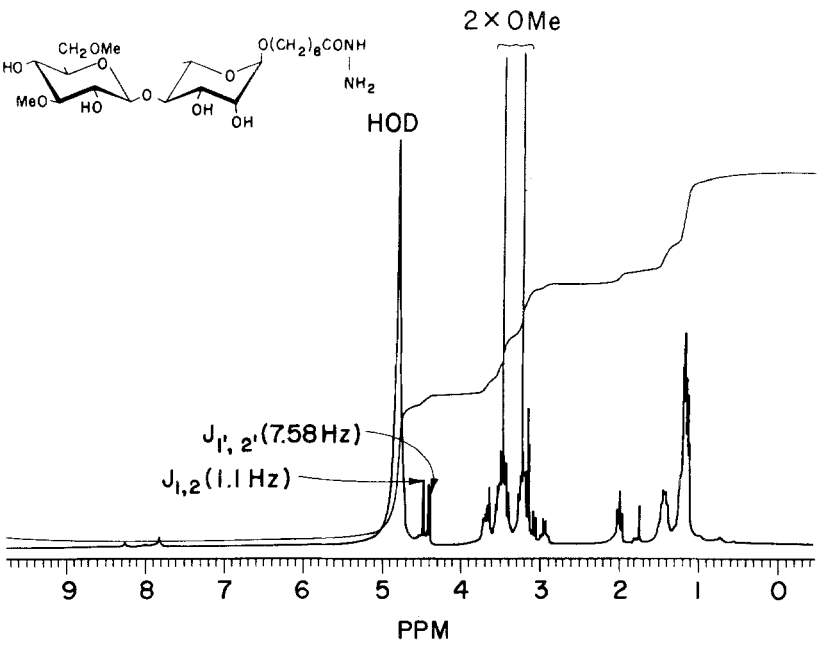

Figure 9. ^{1}H-NMR (360 MHz) of the 8-hydrazinocarbonyloctyl 4-*O*-(3',6'-di-*O*-Me-β-D-glucopyranosyl)-α-L-rhamnopyranoside.

Final synthesis of the *neo*glycoproteins is summarized in Fig. 7. TLC of the 8-hydrazinocarbonyloctyl β-glucoside **24** in $CHCl_3/CH_3OH$, 6/1 by vol, showed one component with R_F 0.28. This new compound readily crystallized from aqueous ethanol (m.p. 179°C, $[\alpha]_D$ -70°, c 0.5 in H_2O). The ^{1}H-NMR spectrum showed a doublet at 4.2 ppm ($J_{1,2}$ 7.2 Hz) and two singlets at 3.65 ppm and 3.30 ppm due to the two methyl groups. The C-1 resonated at 104.2 ppm (Fig. 8). The ^{1}H-NMR spectrum of the 8-hydrazinocarbonyloctyl α-glucoside in $C^2H_3O^2H$ showed a doublet at 4.62 for the H-1 ($J_{1,2}$ 3.56 Hz).

The disaccharide (**23**, Fig. 7) was also converted to its hydrazide **27** and made hydrazine-free by co-evaporation with water and freeze drying. The ^{1}H-NMR of compound **27** showed a singlet at 4.5 ppm for H-1 and a doublet at 4.35 ppm ($J_{1',2'}$ 7.58 Hz) for H-1' (Fig. 9). The two methoxyl peaks were at 3.5 and 3.2 ppm.

The acyl azides were not isolated but were used directly in the preparation of the *neo*glycoproteins, care being taken to conduct the condensation at 0°C. The hapten azide solution was added dropwise to a cold (0°C) solution of BGG, BSA or PDL in a buffer and stirred overnight at 0°C. The *neo*glycoproteins were finally purified on Sephadex G-75 or G-150. In initial experiments, the molar ratio of hapten to protein was varied and incorporation was determined on the basis of carbohydrate content assayed by the phenol-H_2SO_4 method.

In the case of the monosaccharide conjugate with BGG (3,6-Me_2-Glc-Link-BGG), assuming a mol wt for BGG of 150 000, a hapten (as glucose equivalents) to protein molar ratio of 100:1 provided antigens with covalently bound carbohydrate corresponding to about 46 mol/mol BGG, and in the case of the monosaccharide conjugate with poly-D-lysine (3,6-Me_2-Glc-Link-PDL), a molar ratio of 95:1 provided antigens with about 40 mol hapten/mol polypeptide. The results of other conjugations are summarized in Table 1. An

Table 1. Ratio of hapten to protein in the 3,6-di-*O*-methyl-glucopyranose-containing *neo*glycoproteins.

Experiment no.	Molar ratio of hapten to protein used in the conjugation reaction	% incorporation of sugar	Proposed structure[a]
1	100:1	66	[3,6-Me_2Glc-β-Link]$_{46}$-BGG[b]
2	94:1	57	[3,6-Me_2Glc-β-Link]$_{40}$-PDL[c]
3	100:1	41	[3,6-Me_2Glc-α-Link]$_{25}$-BSA[d]
	100:1	41	[3,6-Me_2Glc-β-Link]$_{25}$-BSA
4	78:1	70	[3,6-Me_2Glc(1-4)-β-L-Rha-α-Link]$_{46}$-BSA

[a] Link = Spacer arm, - $O(CH_2)_8CO$-
[b] BGG, bovine gamma globulin.
[c] PDL, poly-D-lysine.
[d] BSA, bovine serum albumin.

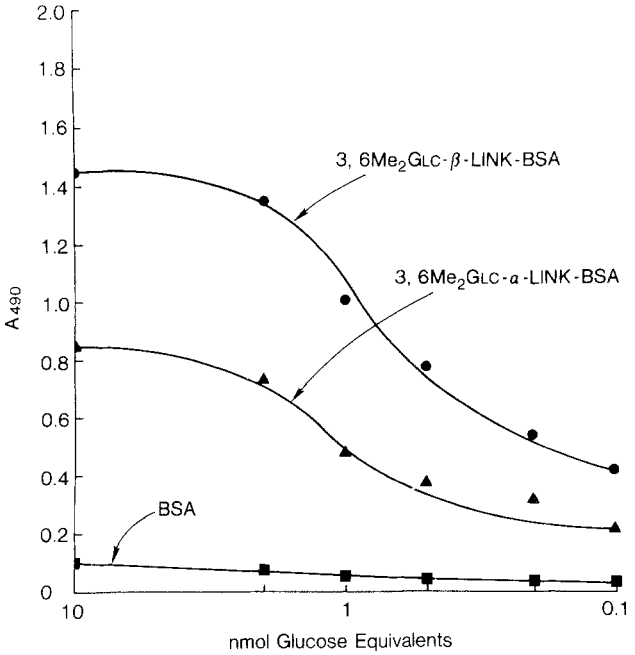

Figure 10. Comparison of the activities of the 3,6-Me_2-Glc-α- and -β-Link-BSA glycoconjugates in the standard ELISA against pooled lepromatous leprosy sera.

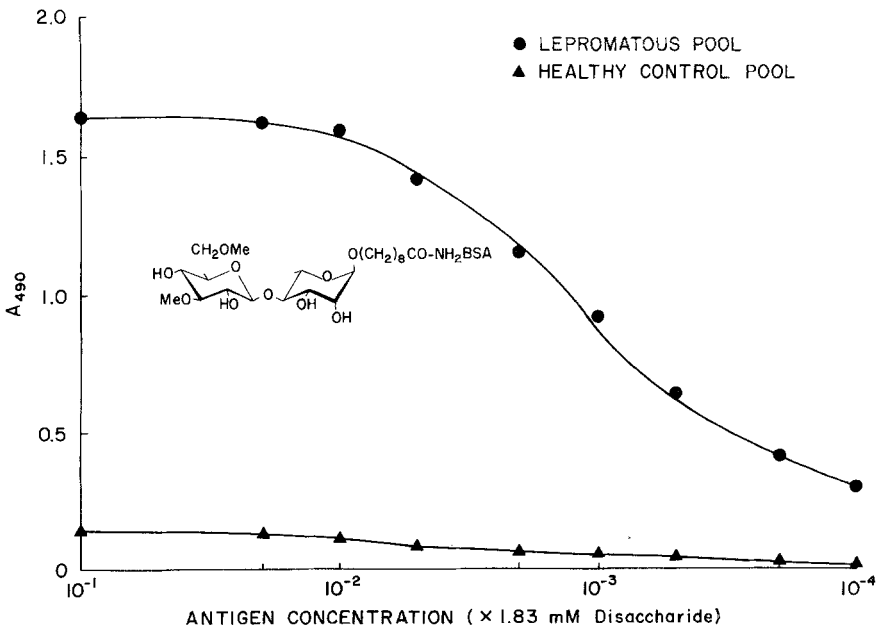

Figure 11. Titration of the activity of the 3,6-Me_2-Glc-Rha-Link-BSA.

optimum incorporation of 60-70% was achieved when the molar ratios were approximately 80-100:1. Thus, these glycoproteins contained about six times more hapten than the products of reductive amination described previously [6]. The azide-coupling procedure is one of the most efficient procedures for introducing high numbers of haptens to lysine-containing proteins [10].

To compare directly the reactivity of the α- and β-anomers, parallel incorporation experiments were conducted. The number of incorporated haptens was determined by the carbohydrate assay and were both found to be about 25:1 (Table 1). A comparison of the serological activities of the 3,6-Me_2-Glc-α- and -β-Link-BSA glycoproteins are compared in Fig. 10 over a broad antigen concentration range. In general, the unnatural α link resulted in about 50% diminution of activity. The 3,6-Me_2-Glc-Rha-Link-BSA was also highly active against pooled lepromatous leprosy serum (Fig. 11).

Analysis of Sera from Hawaii, the Federated States of Micronesia, and the Philippines

Table 2 presents a comparison of the efficacy of the different 3,6-Me_2-Glc-containing antigens in detecting anti-glycolipid antibodies of immunoglobulin classes IgG, IgM and

Table 2. Comparison of PGL-I, 3,6-Me_2Glc-Link-BGG and 3,6-Me_2Glc-Rha-Link-BSA in assay of anti-glycolipid antibodies in individual sera.

Description of serum	Antigen		
	PGL-I	3,6-Me_2Glc-Link-BGG	3,6-Me_2Glc-Rha-Link-BSA
	A_{480}[a]		
No serum	0.06	0.07	0.07
138, Normal	0.06	0.09	0.08
535, Normal	0.09	0.08	0.08
537, Normal	0.05	0.12	0.09
005, tuberculin-reactive[b]	0.12	0.11	0.12
35, TT	0.29	0.18	0.17
151, TT	0.09	0.08	0.08
48, TT	0.16	0.11	0.14
137, BL	0.66	0.42	0.59
0064, LL	0.59	0.47	0.54
124, LL	1.24	1.58	1.70
271, LL	1.34	1.27	0.93
Pool, LL	0.72	0.72	0.65

[a] ELISA conditions are those attributed to the Honolulu laboratory in Materials and Methods.
[b] See [24-26] for details on patient classification. TT, Tuberculoid leprosy; BT, Borderline tuberculoid; BL, Borderline lepromatous leprosy; LL, Lepromatous leprosy.

Table 3. Comparison of activities against leprosy sera of whole *M. leprae*, PGL-I and 3,6-Me_2Glc-Link-BGG.[a]

Patient classification (No.)	No. (%) positive[b] for			Mean ± SD A_{488} for		
	M. leprae	PGL-I	3,6-Me_2Glc-Link-BGG	*M. leprae*	PGL-I	3,6-Me_2Glc-Link-BGG
LL (22)	22 (100)	22 (100)	22 (100)	1.11 ± 0.31	1.13 ± 0.35	0.99 ± 0.40
BT (30)	16 (53)	12 (40)	25 (83)	0.54 ± 0.18	0.28 ± 0.20	0.34 ± 0.21
TT (17)	7 (14)	11 (65)	14 (82)	0.53 ± 0.15	0.30 ± 0.13	0.27 ± 0.05
High responder tuberculous (16)[c]	16 (100)	ND[d]	1 (6)	1.29 ± 0.35	ND	0.14 ± 0.04
Low responder tuberculous (17)[c]	8 (47)	ND	0 (0)	0.46 ± 0.15	ND	0.11 ± 0.03
Normal (14)	0 (0)	0 (0)	0 (0)	0.38 ± 0.04	0.17 ± 0.02	0.15 ± 0.02
No antigen	—	—	—	0.22	0.17	0.11

[a] Abbrevations are described in Table 2.
[b] Criterion for positivity: $>A_{490}\,\bar{x} + 3 \times$ SD of controls. ELISA conditions are those described in Table 2.
[c] Sera from tuberculosis patients were previously selected on the basis of their reactivity to the mycobacterial common antigens.
[d] ND = not determined.

Table 4. Incidence of anti-glycolipid activity in sera from control groups after allowance for non-specific reaction.[a]

Subject (No.)	No. (%) positive/ No. negative[b]	A_{488} (mean ± SD)	
Healthy controls (142)[c]	1 (0.7)/141	0.048	0.032
Tuberculosis (54)[d]	3 (5.6)/51	0.057	0.043

[a] Tested against the 3,6-Me_2-Glc-Rha-Link-BSA as described under Materials and Methods. The IgM ELISA protocol [3] was used.
[b] Criteria for positive: Mean A_{490} + 3 × SD of healthy controls, i.e., 0.145 (n = 142).
[c] Residents of Northern Colorado.
[d] All high reactors.

IgA in individual leprosy sera. The extent of concordance between the different antigens was satisfactory. The results of a larger study are shown in Table 3. All sera within the lepromatous leprosy group were positive against whole bacilli, the natural glycolipid antigen, and the 3,6-Me_2-Glc-Link-BGG and each antigen gave comparable ELISA values. At the tuberculoid end of the spectrum, the 3,6-Me_2-Glc-Link-BGG resulted in a higher case detection (over 80%) compared to either whole bacilli or the glycolipid. This is an important consideration, since tuberculoid leprosy shows only low titre anti-*M. leprae* antibodies, and previously it was shown that PGL-I was not an efficacious antigen in detecting tuberculoid leprosy [3, 6].

Table 3 also shows the results of a limited study of the specificity of the 3,6-Me_2-Glc-Link--BGG. With whole bacillus as antigen, both high and low reactor tuberculous sera showed a corresponding response, which is to be expected in view of the presence of shared common antigens in both the leprosy and tuberculous bacilli [27]. However, even with the high reactor tuberculous sera, the 3,6-Me_2-Glc-Link-BGG showed minimal reactivity. Thus, from these preliminary results, conducted in one global leprosy-endemic area, the portents for a semisynthetic antigen for the serodiagnosis of leprosy look good. In particular, the apparent lack of cross reactivity against high reactor tuberculous sera and a high positivity rate against tuberculoid leprosy sera, are encouraging.

Analysis of Sera from Northern and Southern California

We have previously reported that sera from healthy subjects from within the non-endemic United States showed a false positive rate of 2.4% (four of 169) against PGL-I and 3.6% (six of 169) against the *neo*glycoprotein described earlier, the ε-*N*-1[1-deoxy-2,3-di-*O*-Me-4-*O*-(3′,6′-di-*O*-Me-β-D-glucopyranosyl)-rhamnitol]-lysyl-BSA [6]. Comparable figures for samples of sera of tuberculous patients were 3.0% (two of 66) and 9.0% (six of 66), respectively. In order to study this apparent spurious activity further, six of the highly cross-reactive sera were re-examined by IgM ELISA against the native glycolipid and against the new set of *neo*glycoproteins and then re-assayed after absorption with these antigens (results not shown). Analysis of the data suggested two reasons for the occasional positive activity among healthy and tuberculosis control groups. Firstly, there was evi-

Table 5. Human IgM seroreactivities to the 3,6-Me_2Glc-Link-BGG *neo*glycoprotein.

Patient classification (No.)[a]	No. (%) positive/ No. negative[b]	Mean ± SD A_{488}	Patient classification (No.)[c]	No. (%) positive/ No. negative[b]	Mean ± SD A_{488}
TT and BT (25)	8 (32)/17	0.180 ± 0.201	TT (17)	5 (29)/12	0.165 ± 0.179
			BT (8)	3 (38)/5	0.133 ± 0.087
BB (4)[d]	2 (50)/2	0.394 ± 0.398			
BL and LL			BL (6)	5 (83)/1	0.421 ± 0.317
Less than two years of dapsone			LL Active (15)	14 (93)/1	1.070 ± 0.744
therapy (43)	32 (74)/11	0.541 ± 0.523	Inactive (18)	7 (39)/11	0.221 ± 0.170
More than two years of dapsone					
therapy (54)	32 (59)/22	0.365 ± 0.382			
Total (97)	64 (66)/33	0.443 ± 0.456			
			ENL (16)[d]	12 (75)/4	0.642 ± 0.623
			Lucio reaction (7)	7 (100)/0	1.254 ± 0.780
			Reversal (10)	8 (80)/2	0.729 ± 0.685

[a] Sera from patients attending the Northern California clinic. The IgM ELISA protocol was used.
[b] Mean + 3 × SD of healthy controls (No. = 142), i.e., 0.064 + 3 × 0.037 = 0.175. Therefore, ⩾0.175 is considered positive.
[c] Sera from patients attending the Southern California clinic.
[d] BB, borderline. ENL, erythema nodosum leprosum. Other abbrevations are described in Table 2.

Table 6. Comparison of activities against leprosy sera of the 3,6-Me_2Glc-Link-PDL and the 3,6-Me_2Glc-Rha-Link-BSA.[a]

Patient classification (No.)	3,6-Me_2Glc-Link-PDL		3,6-Me_2Glc-Rha-Link-BSA	
	No. (%) positive/ No. negative[b]	Mean ± SD A_{488}	No. (%) positive/ No. negative[b]	Mean ± SD A_{488}
TT and BT (13)	4 (31)/9	0.167 ± 0.079	7 (54)/6	0.158 ± 0.097
BB (2)	1 (50)/1	0.277 ± 0.260	1 (50)/1	0.241 ± 0.257
BL and LL				
Less than two years of therapy (24)	22 (92)/2	0.706 ± 0.523	22 (92)/2	0.727 ± 0.476
More than two years of therapy (9)	4 (44)/5	0.224 ± 0.096	7 (78)/2	0.231 ± 0.245
Total (33)	26 (79)/7	0.575 ± 0.503	29 (88)/4	0.592 ± 0.478

[a] Abbreviations are described in Table 2.
[b] For the negative pool, the mean A_{488} was 0.098. Therefore, $A_{488} \geqslant 0.200$ was considered positive in this study.

dence for the occurrence of non-specific binding of some sera to the ELISA plate, particularly tuberculous sera. Secondly, whereas with some normal sera, most of the activity could be absorbed by pretreatment with the synthetic antigens, with others, this was not so. Accordingly, this limited study indicates that sera positivity among healthy and tuberculosis control patients may be due to either non-specific binding of sera to the plate or to real, albeit weak, antigen-antibody binding. To counteract the first of these possibilities, "no antigen controls" were conducted on all sera, i.e., all plates were divided in half and coated with either antigen or coating buffer alone with addition of serum to both. The results of this adaptation on the standard ELISA are shown in Table 4. The percentage of positivity in the healthy control group was reduced to 0.7%. The tuberculosis control group remained high at 5.6%, although lower than the figures reported previously [6]. Since the responsible patients were from a leprosy endemic area, some of the activity may be due to true anti-glycolipid antibodies.

With these revised, controlled conditions, sets of sera previously examined in the context of the first generation of synthetic antigens [6] were re-examined with 3,6-Me_2-Glc-Link-BGG (Table 5) and both sets of results were compared. The degree of correspondence was striking: the number of positives were the same except for the inactive LL group, which against the ε-*N*-1-[1-deoxy-2,3-di-*O*-Me-4-*O*-(3′,6′-di-*O*-Me-β-D-glucopyranosyl)-rhamnitol]-lysyl-BSA showed a 60% positivity rate compared to 38.7% with the 3,6-Me_2-Glc-Link-BGG.

Some of the sera from the Northern California clinic used to test the efficacy of the 3,6-Me_2-Glc-Link-BGG were also applied to the 3,6-Me_2-Glc-Rha-Link-BSA (Table 6). In this study, the 3,6-Me_2-Glc-Link-PDL was also included for comparison. The most striking difference between either of the monosaccharide-link-glycoconjugates and the disaccharide-link-product was the comparative success of the latter in detecting anti-glycolipid IgM antibodies at the tuberculoid end of the disease spectrum, and within the long-time treated lepromatous population. Perhaps, contrary to prevailing evidence

(see Introduction), the penultimate sugar of the natural PGL-I, emulated in the disaccharide-Link-BSA by a rhamnosyl substituent, contributes to anti-glycolipid IgM binding.

Discussion

Previously, we described a successful synthesis of the trisaccharide and disaccharide substituents of phenolic glycolipid I and some related products devoid of some of the methoxyl groups [5]. Others have evolved a different synthetic strategem for the terminal disaccharide [28]. We had shown that in an ELISA inhibition assay, only those oligosaccharides with the full complement of methoxyl groups at the non-reducing end were active in binding human leprosy anti-glycolipid IgM. Subsequently, it was shown that of the first generation of *neo*glycoproteins (those derived by reductive amination of synthetic disaccharides) only ε-*N*-1-[1-deoxy-2,3-di-*O*-Me-4-*O*-(3′,6′-di-*O*-Me-β-D-glucopyranosyl)-rhamnitol]-lysyl-BSA was active, whereas those based on the 3′-mono-*O*-Me-β-D-glucopyranoside or the nonmethylated β-D-glucopyranoside were virtually inactive.

The fact that the 3,6-di-*O*-Me-β-D-glucopyranosyl-containing product of reductive amination was so highly active against lepromatous leprosy and showed excellent concordance with phenolic glycolipid I sera had itself indicated that only the terminal 3,6-di-*O*-Me-β-D-glucopyranosyl unit was necessary for recognition of anti-glycolipid IgM. In addition, it had been shown that partially deglycosylated PGL-I, the product devoid of the terminal di-*O*-Me-glucose, had lost the majority of its activity against IgM antibodies in human lepromatous leprosy and murine monoclonal IgM and IgG antibodies [5, 7-9]. Thus, there was every rationale for the synthesis of *neo*glycoproteins based on the single sugar epitope.

The strategy used for the successful synthesis of 8-hydrazinocarbonyloctyl 3,6-di-*O*-Me-β-D-glucopyranoside, the 8-hydrazinocarbonyloctyl 4-*O*-(3′,6′-di-*O*-Me-β-D-glucopyranosyl)-α-L-rhamnopyranoside, and their coupling to protein and polypeptide carriers *via* the intermediate acyl azide is based on that pioneered by Lemieux *et al.* [10]. In their classical work, Goebel and Avery [29] had shown that antibodies specific for glycosyl substituents could be generated with diazotized *p*-aminophenyl or *p*-aminobenzyl glycosides covalently linked to proteins, and Luderitz *et al.* [30] in a variation on this method, converted the amino group to isothiocyanate for attachment of epitopic O-antigen monosaccharides. Arakatsu *et al.* [31] oxidized the reducing end to aldonic acid, which was subsequently attached to protein amino groups by way of a mixed anhydride. Himmelspach *et al.* [32] converted the sugar to the 1-(*m*-nitrophenyl)-flavazole derivative and then coupled this, after reduction to form an amino function followed by diazotization. Fielder *et al.* [33] prepared the 1-amino monosaccharide derivatives and, with cyanuric trichloride, linked the sugar amino functions to the amino groups of glycine.

These methods suffer when one is dealing with minimal epitopic sugars, because they destroy the terminal reducing end of the sugar, which we already know [6] is necessary for antibody recognition. Of course, if the hapten is large, such as the entire triglycosyl or the terminal disaccharide units of PGL-I, these procedures are highly applicable, as already noted by Himmelspach *et al.* [32] in a different context. We were anxious to en-

gage in minimal synthesis and, accordingly, to preserve the epitope in its natural β-anomeric cyclic hemiacetal configuration. The use of a nine-carbon-long aliphatic chain is convenient and is thought to minimize intramolecular reaction of the activated azidocarbonyloctyl aglycon [10]. Besides a variety of synthetic antigens related to the blood group substances [34], this ploy has been used to produce the highly immunogenic artificial lactose antigen, 8-hydrazinocarbonyloctyl 4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosyl-BSA [35], and more recently, for synthesis of α-D-Gal(1-2)-[3,6-dideoxy-α-D-hexopyranosyl(1-3)]-α-D-Man-*O*-$(CH_2)_8$COOMe [36], the branched trisaccharide structure which corresponds to the O-antigenic determinant of *Salmonella* serogroup B.

In the present context, the utility of the 8-methoxycarbonyloctanol-linking arm has been demonstrated in the context of leprosy serology. It allows introduction of structural variations on the epitopic molecule and analysis of the molecular requirements for antibody recognition. Previously, we had implicated the intact pyranosyl structure in anti-glycolipid IgM binding, in that ε-*N*-1-(1-deoxy-3,6-di-*O*-Me-glucitol)-lysyl-BSA, unlike the ε-*N*-1-[1-deoxy-2,3-di-*O*-Me-4-*O*-(3′,6′-di-*O*-Me-β-D-glucopyranosyl)-rhamnitol]-lysyl-BSA was virtually without activity [6]. The present results suggest less stringency in whether the β- or α-glucopyranoside is presented. The latter shows about one-half the activity of the β-glucopyranoside throughout a broad concentration range. Likewise, the need for the two methoxyl groups at the 3′ and 6′positions of the glucopyranose was previously stressed, in that the 3′mono-methoxyl ε-*N*-1-[1-deoxy-2,3-di-*O*-Me-4-*O*-(3′-*O*-Me-β-D-glucopyranosyl)-rhamnitol]-lysyl-BSA or the ε-*N*-1-[1-deoxy-2,3-di-*O*-Me-4 -*O*-(β-D-glucopyranosyl)-rhamnitol]-lysyl-BSA were virtually devoid of activity [6]. Indeed, in results not presented above, it was shown that the product with "extra" methoxyl groups, namely, 8-hydrazinocarbonyloctyl 3,4,6-tri-*O*-Me-β-D-glucopyranosyl-lysyl-BGG, was about 75% less active than the di-*O*-methylated product over a broad concentration range (unpublished observations with A. Liav and M.B. Goren). The present results indicate that the nature of the protein carrier has little bearing on antibody recognition by the hapten group. Accordingly, in more recent large-scale serological studies, we have opted for BSA-containing glycoconjugates. It should be emphasized that all of the above observations apply only to antibody binding. The molecular requirements for immunogenicity have not been studied. Perhaps therein may be found a role for the remainder of the unique triglycosyl entity of phenolic glycolipid I, the 2,3-di-*O*-Me-α-L-rhamnopyranosyl(1-2)-3-*O*-Me-α-L-rhamnopyranoside unit.

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