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Chemical Synthesis and Serology of Disaccharides and Trisaccharides of Phenolic Glycolipid Antigens from the Leprosy Bacillus and Preparation of a Disaccharide Protein Conjugate for Serodiagnosis of Leprosy

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We examined the structural requirements within the species-specific 3,6-di-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3-di-*O*-methyl-α-L-rhamnopyranosyl-(1→2)-3-*O*-methyl-α-L-rhamnopyranose unit of the phenolic glycolipid I antigen of *Mycobacterium leprae* for binding to anti-glycolipid immunoglobulin M from human leprosy sera. We used chemically defined, partially deglycosylated fragments of phenolic glycolipid I, two other minor *M. leprae*-specific phenolic glycolipids (those containing 6-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3-di-*O*-methyl-α-L-rhamnopyranosyl-(1→2)-3-*O*-methyl-α-L-rhamnopyranose and 3,6-di-*O*-methyl-β-D-glucopyranosyl-(1→4)-3-*O*-methyl-α-L-rhamnopyranosyl-(1→2)-3-*O*-methyl-α-L-rhamnopyranose units), and phenolic glycolipids from other mycobacteria. Additionally, the trisaccharide of phenolic glycolipid I, the 3,6-di-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3-di-*O*-methyl-α-L-rhamnopyranose, the 6-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3-di-*O*-methyl-α-L-rhamnopyranose, and the β-D-glucopyranosyl-(1→4)-2,3-di-*O*-methyl-α-L-rhamnopyranose disaccharides were synthesized and characterized, and their activities were examined. Only the phenolic glycolipids containing 3,6-di-*O*-methyl-β-D-glucopyranosyl at the nonreducing terminus were efficient in binding the anti-glycolipid immunoglobulin M, and the 3,6-di-*O*-methyl-β-D-glucopyranosyl-containing di- and trisaccharides were the most effective in inhibiting this binding. Thus, the 3,6-di-*O*-methyl-β-D-glucopyranosyl substituent was recognized as the primary antigen determinant in phenolic glycolipid I. With this information, bovine serum albumin containing reductively aminated 3,6-di-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3-di-*O*-methyl-L-rhamnose was prepared and shown to be highly active in the serodiagnosis of leprosy.

We have recently reported on the presence in *Mycobacterium leprae* of three specific glycolipids, termed phenolic glycolipids (PhenGL)-I, -II, and -III (17, 18). PhenGL-I is secreted in large quantities (up to 2% of tissue mass) into *M. leprae*-infected armadillo tissue (16), and thus complete structural elucidation was possible. PhenGL-I consists of 3,6-di-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3-di-*O*-methyl-α-L-rhamnopyranosyl-(1→2)-3-*O*-methyl-α-L-rhamnopyranose-*para*-29-phenol-9,11-dimycocerosyl-3-methoxyl-4-methyl-nonacosane (18). PhenGL-III differs from PhenGL-I in that the nonreducing sugar terminus is 6-*O*-methyl-β-D-glucopyranosyl; thus, its characteristic trisaccharide is 6-*O*-methyl-β-D-glucopyranosyl-(1→4)-2, 3-di-*O*-methyl-α-L-rhamnopyranosyl-(1→2)-3-*O*-methyl-α-L-rhamnopyranose (17). Evidence for the structure of PhenGL-II is presented below.

The serological activity of PhenGL-I, its dissected parts, and related glycolipids from other mycobacteria have been examined by direct enzyme-linked immunosorbent assay (ELISA) against hyperimmune anti-*M. leprae* rabbit antiserum and sera from patients with leprosy and other mycobacterial diseases (3a, 4). High anti-PhenGL-I immunoglobulin M (IgM) antibodies were found in 96% of untreated or short-term-treated lepromatous leprosy patients. Sera from patients with tuberculosis or atypical mycobacterial infections were devoid of anti-PhenGL-I antibodies and the structurally related phenolic glycolipids from *Mycobacterium kansasii*, *Mycobacterium bovis* and the aglycon segments of the *M. leprae* product were devoid of significant serological activity. Thus, the trisaccharide is specific in its structure, serological activity, and, to some extent, the antibody class it evokes. In the present work, we used PhenGL-II and PhenGL-III, partially deglycosylated PhenGL-I, and synthetic di- and trisaccharides to define which sugar residues serve as antigen determinants on PhenGL-I. These results in turn led to the synthesis of a glycoprotein conjugate containing the determinant which proved to be highly active against leprosy sera. Thus, there is now the distinct promise of a synthetic hapten capable of specifically diagnosing leprosy in its humoral manifestations.

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MATERIALS AND METHODS

Preparation of PhenGL-I and PhenGL-III. Details of the isolation and structure of PhenGL-I (3,6Me_2Glc→2, 3Me_2Rha→3MeRha→PhenGL) and PhenGL-III, (6MeGlc→2,3Me_2Rha→3MeRha→PhenGL) have been described previously (17, 18). The two partially deglycosylated derivatives, 2,3-di-*O*-methyl-α-L-rhamnopyranosyl-(1→2)-3-*O*-methyl-α-L-rhamnopyranosyl-phenolic dimycocerosylphthiocerol (2,3Me_2Rha→3MeRha→ PhenGL) and 3-*O*-methyl-α-L-rhamnopyranosyl-phenolic dimycocerosylphthiocerol (3MeRha→PhenGL), were obtained by partial acid hydrolysis of PhenGL-I and purified by preparative thin-layer chromatography (TLC) (18). The preparation and partial characterization of (2,4-di-*O*-methyl-rham-

nose→2-*O*-methyl-fucose→2-*O*-methyl-rhamnose) phenolic diacylphthiocerol (2,4Me_2Rha→2MeFuc→2MeRha→PhenGL) (mycoside A) from *M. kansasii* has been described previously (16). The mycoside B preparation from *M. bovis* (5) consists of a mixture of 27- and 29-[*para*-(2-*O*-methyl-α-L-rhamnopyranosyl-oxy)]-phenol-9,11-dimycocerosyl-3-methoxy-4-methyl-hepta- and nonacosane (2MeRha→PhenGL) (C. V. Knisley and P. J. Brennan, unpublished data).

Structure of PhenGL-II. The isolation of PhenGL-II has been described, but its structure has not been previously established (17). Hydrolysis and gas liquid chromatography-electron impact mass spectrometry (GLC/EI-MS) of the alditol acetates showed the presence of 3,6-di-*O*-methylglucose and 3-*O*-methyl-rhamnose in a ca. 1:2 ratio. The sequence of these sugars, established by graded acid hydrolysis (18), was shown to be 3,6-di-*O*-methylglucose→3-*O*-methylrhamnose→3-*O*-methylrhamnose. Pertrideuteriomethylation and GLC/EI-MS of the alditol acetates (17) showed 1,2,5-tri-*O*-acetyl-3-*O*-CH_3-4-*O*-CD_3-rhamnitol; 1,4,5-tri-*O*-acetyl-3-*O*-CH_3-2-*O*-CD_3-rhamnitol; and 1,5-di-*O*-acetyl-3,6-di-*O*-CH_3-2,4-di-*O*-CD_3-glucitol. Proton nuclear magnetic resonance (NMR) of PhenGL-II at 360 MHz in $CDCl_3$ allowed assignment of anomeric proton signals as before (16). Thereby, 3,6-di-*O*-methyl-β-D-glucopyranosyl-(1→4)-3-*O*-methyl-α-L-rhamnopyranosyl-(1→2)-3-*O*-methyl-α-L-rhamnopyranoside (3,6Me_2Glc→3MeRha→3MeRha) is tentatively proposed for the structure of the trisaccharide appendage of PhenGL-II.

Synthesis of 3,6-di-*O*-methyl-β-D-Glc*p*-(1→4)-2,3-di-*O*-methyl-α-L-Rha*p* and other disaccharides. The synthesis of 3,6-di-*O*-methyl-β-D-Glc*p*-(1→4)-2,3-di-*O*-methyl-α-L-Rha*p* and other disaccharides is presented in Fig. 1. Except where indicated, standard synthetic protocols were followed (7–9). All products were checked for chemical homogeneity by silica gel TLC, and structural assignments were supported by infrared and 60-MHz NMR spectroscopy and where indicated by 360-MHz (for ^1H) or 90-MHz (for ^{13}C) NMR spectroscopy. Other routine procedures and sources of materials have been described previously (17, 18).

3-*O*-Methyl-D-glucose (Sigma Chemical Co., St. Louis, Mo.) was acetylated and brominated with HBr to give 2,4,6-tri-*O*-acetyl-3-*O*-methyl-α-D-glucopyranosyl bromide (compound I). L-rhamnose was stirred in benzyl alcohol saturated with HCl and acetylated to give crystalline benzyl 2,3,4-tri-*O*-acetyl-L-rhamnopyranoside in ca. 80% yield. The latter was deacetylated and acetonated in a mixture of Amberlite IR 120 (H^+) and 2,2-dimethoxypropane in dry acetone to give benzyl 2,3-di-*O*-isopropylidene-α-L-rhamnopyranoside (compound II), a known compound (12). Compounds I (21.5 g) and II (12.5 g) were stirred in acetonitrile with mercury cyanide (9), and benzyl 2,3-di-*O*-isopropylidene-4-*O*-(3-*O*-methyl-2,4,6-tri-*O*-acetyl-β-D-glucopyranosyl)-α-L-rhamnopyranoside [21.8 g; mp, 136.5-137°C; specific optical rotation ($[\alpha]_D^{24}$), −51.94; concentration in grams per 100 ml (*c*), 0.93] (compound III) was purified on Florisil in benzene. Compound III was deacetylated with sodium methoxide, tritylated with trityl chloride in pyridine, and acetylated. The product was applied to Florisil in benzene-hexane (1:1) and the 6-*O*-tritylated derivative (compound IV) was eluted with benzene (11.5 g; $[\alpha]_D^{24}$, −6.18; *c*, 1.44). Benzylation of compound IV (8.0 g) was achieved by heating at 130 to 140°C in benzyl chloride-potassium hydroxide. Purification by Florisil column chromatography yielded 3.17 g of benzyl 2,3-di-*O*-isopropylidene-4-*O*-(3-*O*-methyl-2,4-di-*O*-benzyl-6-*O*-trityl-β-D-glucopyranosyl)-α-L-rhamnopyranoside (compound V) ($[\alpha]_D^{24}$, −16.64; *c*, 1.46). Compound V (3.8 g) was hydrolyzed in 60% acetic acid-ethanol (1:1), and the products were applied to a column of silica gel in benzene and eluted with benzene-ethyl acetate (1:1). The detritylated disaccharide (120 mg; $[\alpha]_D^{24}$, −73.57; *c*, 0.14) and the detritylated deisopropylidenated disaccharide (compound VI) (1.57 g; $[\alpha]_D^{24}$, −75.10; *c*, 7.54) were well separated. The latter (950 mg) was methylated (13), and the products were purified on silica gel in benzene-ethyl acetate (8:1) to give the benzyl 2,4-di-*O*-benzyl-3,6-di-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3-di-*O*-methyl-α-L-rhamnopyranoside (compound VII) in 90% yield. Hydrogenolysis of compound VII (820 mg) with 5% palladium–charcoal (8) gave the disaccharide, 2,3-di-*O*-methyl-4-*O*-(3,6-di-*O*-methyl-β-D-glucopyranosyl)-α-L-rhamnopyranose (3,6Me_2Glc→2,3Me_2Rha) (compound VIII) (390 mg; $[\alpha]_D^{24}$, −27.08; *c*, 7.8).

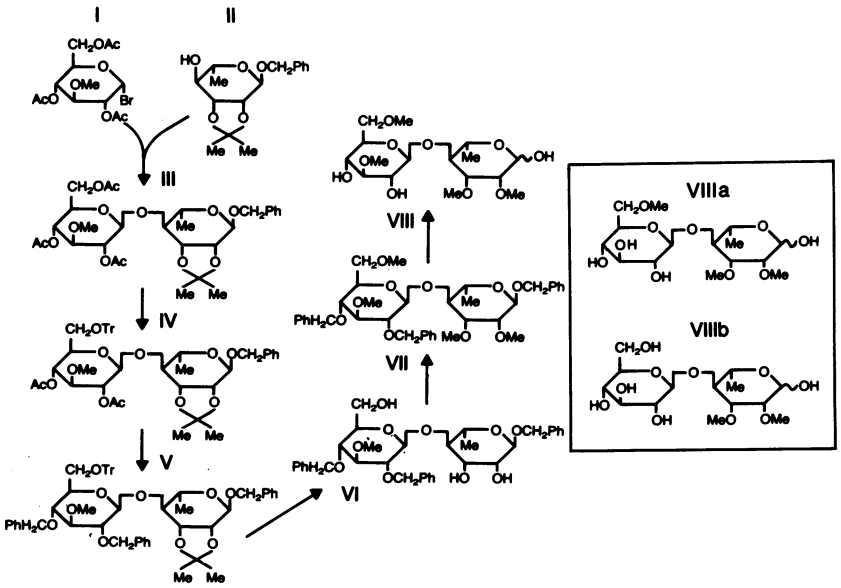

FIG. 1. Pathway for the synthesis of the three disaccharides, compounds VIII, VIIIa, and VIIIb.

The same synthetic pathway with D-glucose as the starting material led to the synthesis of 2,3-di-*O*-methyl-4-*O*-(6-*O*-methyl-β-D-glucopyranosyl)-α-L-rhamnopyranose (6MeGlc→2,3Me_2Rha) (compound VIIIa) ($[\alpha]_D^{24}$, −32.57; *c*, 1.10). Likewise, when D-glucose was used again and the tritylation reactions were omitted, the product was 2,3-di-*O*-methyl-4-*O*-(β-D-glucopyranosyl)-α-L-rhamnopyranose (Glc→2,3Me_2Rha) (compound VIIIb) ($[\alpha]_D$, −23.90; *c*, 1.05).

Synthesis of benzyl 3-*O*-methyl-4-*O*-benzyl-2-*O*-[2,3-di-*O*-methyl-4-*O*-(2,4-di-*O*-acetyl-3,6-di-*O*-methyl-β-D-glucopyranosyl)-α,β-L-rhamnopyranosyl]-α-L-rhamnopyranosides. Compound II (Fig. 1) was benzylated with benzyl chloride-potassium hydroxide to give crystalline compound XI (Fig. 2) in good yield. Compound XI was hydrolyzed with 0.01 N hydrochloride–ethanol to give benzyl 4-*O*-benzyl-α-L-rhamnopyranoside (compound XII), a known compound (12). Specific methylation of the 3-OH was achieved through phase-transfer catalysis via the 2-*O*-allylated compound (10). Compound XII (8.53 g) was shaken in a mixture of tetrabutyl-ammonium bromide, allyl bromide, dichloromethane, and 10% aqueous sodium hydroxide for 2 days. The 2-*O*-allylated (4.85 g; 70% of mixture) (compound XIII) was readily separated from the 3-*O*-allylated (15%) derivative, the 2,3-di-*O*-allylated (5%) derivative, and unreacted benzyl 4-*O*-benzyl-α-L-rhamnopyranoside by silica gel column chromatography with 1% *t*-butanol in benzene as the elutrient. Compound XIII (4.0 g) was methylated with methyl iodide and silver oxide (15) to give benzyl 2-*O*-allyl-4-*O*-benzyl-3-*O*-methyl-α-L-rhamnopyranoside (compound XIV; 3.5 g) which was treated with potassium *t*-butoxide and dilute acid to yield benzyl 3-*O*-methyl-4-*O*-benzyl-α-L-rhamnopyranoside (compound XV).

Compound VIII (Fig. 1) was acetylated with pyridine-acetic anhydride, and 500 mg of the peracetate (compound IX, Fig. 2) was treated with titanium (IV) bromide (24) in 7.7 ml of dichloromethane-ethyl acetate (10:1) to give the 1-brominated acetylated disaccharide (compound X; 502 mg). Without further purification it was stirred with compound XV in acetonitrile in the presence of mercury cyanide (9). The reaction products were applied to a column of silica gel which was eluted with benzene-ethyl acetate. The derivatized trisaccharides (compounds XVI and XVII; 177 mg) emerged with benzene-ethyl acetate (2:1), whereas the hydrolyzed products were removed with benzene-ethyl acetate (1:1).

Coupling of disaccharide to BSA. The reductive amination procedure described by Gray (11) was used. Bovine serum albumin (BSA) (13.6 mg, 200 nmol), 3,6-di-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3-di-*O*-methyl-α-L-rhamnopyranose (20 mg, 52.3 μmol), and sodium cyanoborohydride (20 mg, 318 nmol) were dissolved in 1 ml of 0.2 M potassium phosphate (pH 8.0), and the reaction was incubated at 37°C for 200 h. The mixture was applied to a Biogel P-2 column (2.5 by 47 cm) in 0.1 M potassium phosphate (pH 7.0) containing 0.2% sodium azide. Fractions (2 ml) were assayed for protein by absorbance at 280 nm and for total carbohydrate (18). The conjugate eluted at the void volume (ca. 70 ml), and the unreacted disaccharide eluted at ca. 150 ml.

Serological procedures. Lipids were suspended by direct sonication with a 3-mm probe in a carbonate-bicarbonate buffer (pH 9.6) (4) and absorbed to polystyrene microwells at 37°C over 18 h. Pooled human lepromatous leprosy sera diluted 1/300 with the carbonate-bicarbonate buffer was added to wells, followed by a blocking step with 5% BSA in phosphate-buffered saline. Goat anti-human IgM-peroxidase-conjugated reagent (Cappel Laboratories, Downingtown, Pa.) diluted 1/1,000 in 20% normal goal serum was added, followed by H_2O_2-*O*-phenylenediamine (4). Absorbances were read at 488 nm. Sera from lepromatous leprosy patients and people without the disease arose from a survey of anti-PhenGL-I antibodies in leprosy patients conducted with R. H. Gelber, Seton Medical Center, Daly City, Calif. (4).

An inhibition assay was also developed to compare the effects of the synthetic oligosaccharides and the various glycolipids on binding between PhenGL-I and human leprosy IgM. Oligosaccharides were dissolved in the carbonate-bicarbonate buffer, whereas lipids were suspended by sonication as described above. Preparations were added to the pooled human lepromatous leprosy sera to achieve a final dilution of 1/300

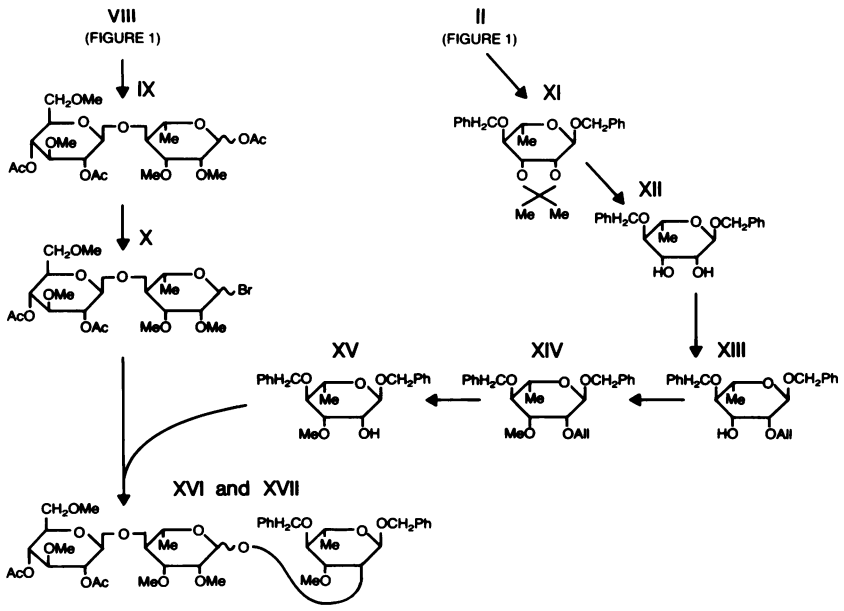

FIG. 2. Pathway for the synthesis of the two 3,6-di-*O*-methyl-D-glucopyranose-containing trisaccharides.

and were incubated at 37°C for 3 h and at 4°C for 16 h. Sera so treated were added to polystyrene wells precoated with 2 μg of sonicated PhenGL-I per ml, and the steps described above were followed.

RESULTS

Synthesis of disaccharides and trisaccharides. Synthesis of the three disaccharides is summarized in Fig. 1. Starting with 10 g of 3-*O*-methyl-D-glucose, the yield of compound VIII was about 400 mg. Benzylation of compound IV gave poor yields due to formation of an intractable emulsion. The ^{1}H-NMR spectrum of the final disaccharide (compound VIII) in D_2O (Fig. 3A) showed signals which may be assigned as follows: (i) two protons at 5.02 ppm ($J_{1,2}$ = 1.91 Hz) due to the anomeric proton of the α-2,3-Me_2-L-Rha*p* and at 4.39 ppm ($J_{1,2}$ = 7.59 Hz) due to the hydrogen-1 of a β-3,6-Me_2-D-Glc*p* residue (1); (ii) one C-CH_3 group at 1.01 ppm ($J_{5,6}$ = 4.65 Hz, somewhat lower than the expected 6 to 6.5 Hz); and (iii) four OCH_3 group at 3.12, 3.17, 3.18, and 3.34 ppm. To confirm the position of the linkage and OCH_3 groups, the disaccharide was deuteriomethylated, and the alditol acetates were analyzed by GLC/EI-MS on an OV-225 support coated open tubular (SCOT) column. Two peaks in a ca. 1:1 ratio were detected with relative retention times (1,5-di-*O*-Ac-2,3,4,6-tetra-*O*-CH_3-glucitol = 1.0) of 0.92 (*m/z* 203, 161, 143, 117, 101, and 43) and 1.00 (*m/z* 208, 164, 148, 132, 128, 104, and 43). This data is in agreement with 1,4,5-tri-*O*-Ac-2,3,-di-*O*-CH_3-rhamnitol and 1,5-di-*O*-Ac-3,6-di-*O*-CH_3-2,4-di-*O*-CD_3-glucitol (18, 19). The same sequence of reactions described for compound VIII with D-glucose as the starting

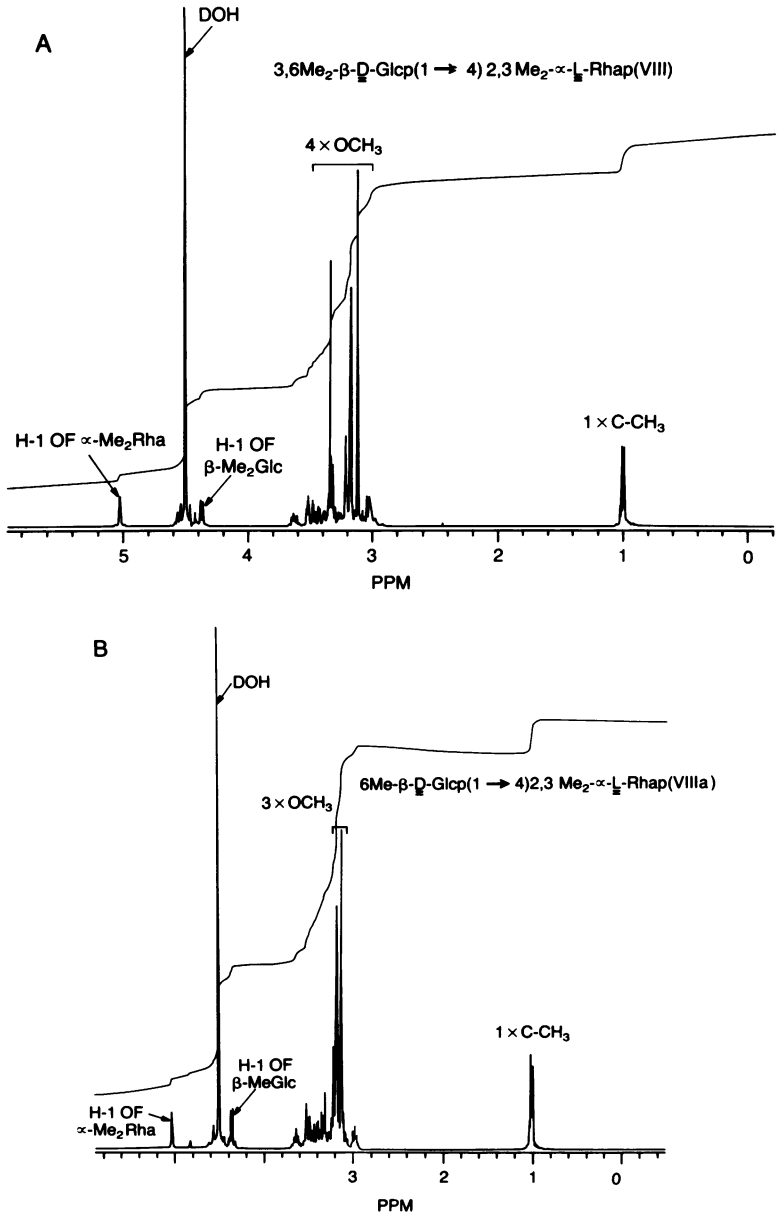

FIG. 3. A 360-Hz ^{1}H-NMR spectrum of: A, synthetic 2,3-di-*O*-methyl-4-*O*-(3,6-di-*O*-methyl-β-D-glucopyranosyl)-α-L-rhamnopyranose (compound VIII) and B, 2,3-di-*O*-methyl-4-*O*-(6-*O*-methyl-β-D-glucopyranosyl)-α-L-rhamnopyranose (compound VIIIa). DOH ($^2HO^1H$) signal was set at 4.50 ppm.

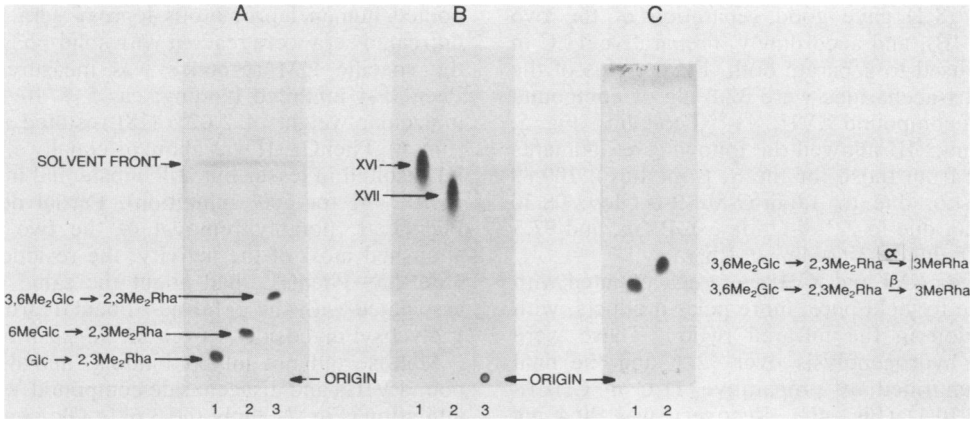

FIG. 4. A, TLC in $CHCl_3$-CH_3OH-H_2O (60:10:1) of the three synthetic disaccharides. B, TLC in CH_2Cl_2-acetone (8:1) of the two acetylated, benzylated trisaccharides. Compound XVI, benzyl 3-*O*-methyl-4-*O*-benzyl-2-*O*-[2,3-di-*O*-methyl-4-*O*-(2,4-di-*O*-acetyl-3,6-di-*O*-methyl-β-D-glucopyranosyl)-β-L-rhamnopyranosyl]-α-L-rhamnopyranoside; compound XVII, benzyl 3-*O*-methyl-4-*O*-benzyl-2-*O*-[2,3-di-*O*-methyl-4-*O*-(2,4-di-*O*-acetyl-3,6-di-*O*-methyl-β-D-glucopyranosyl)-α-L-rhamnopyranosyl]-α-L-rhamnopyranoside. C, TLC in $CHCl_3$-CH_3OH-H_2O (60:10:1) of the two synthetic trisaccharides. All plates were sprayed with 0.1% orcinol in H_2SO_4.

material led to the synthesis of compound VIIIa (Fig. 1). The overall yield was 6.6%. ^{1}H-NMR spectroscopy of this product (Fig. 3B) showed: two anomeric protons at 5.02 ppm ($J_{1,2}$ = 1.55) and 4.37 ppm ($J_{1,2}$ = 7.59); and three OCH_3 groups at 3.12, 3.17, and 3.18 ppm. Likewise, again with D-glucose as the starting material, but with omission of the tritylation step, compound VIIIb was produced (Fig. 1). The yield was 7.1%. TLC of the three disaccharides in $CHCl_3$-CH_3OH-H_2O (60:10:1) is shown in Fig. 4A.

Starting with 15 g of L-rhamnose, ca. 1.5 g of compound XV was recovered (Fig. 2). To confirm the position of the OCH_3 group, compound XV was hydrogenolized, reduced with $NaBH_4$, and the alditol acetate was subjected to GLC/EI-MS on an OV-225 SCOT column. A single peak with a retention time of 1.62 (relative to the di-*O*-Ac-tetra-*O*-CH_3-glucitol) was detected with fragments at *m/z* 203, 189, 143, 129, 101, 87, and 43, demonstrating that the product was a 3-*O*-methyl-6-deoxyhexose derivative (18, 19).

Synthesis of a mixture of the acetylated, benzylated 3,6-di-*O*-Me-D-Glc*p*-containing trisaccharides is summarized in Fig. 2. Silica gel column chromatography yielded 177 mg of the acetylated, benzylated products (compounds XVI and XVII). TLC of the final product in benzene-acetone (4:1) showed one spot (R_f, 0.48). However, ^{13}C-NMR showed four anomeric carbon signals (96.61, 97.98, 98.30 and 99.61 ppm) present in equal amounts and one anomeric carbon signal (100.89 ppm) in about twice the amount. The ^{13}C-^{1}H coupled spectrum showed coupling constants of ca. 174 Hz for the signals at 96.61, 97.88, and 98.30 ppm, 158 Hz for that at 99.61 ppm, and 166 Hz for that at 100.89 ppm. Therefore, the purified derivatized trisaccharide consisted of a mixture of the Rha-Rha α and β anomeric forms. TLC in dichloro-

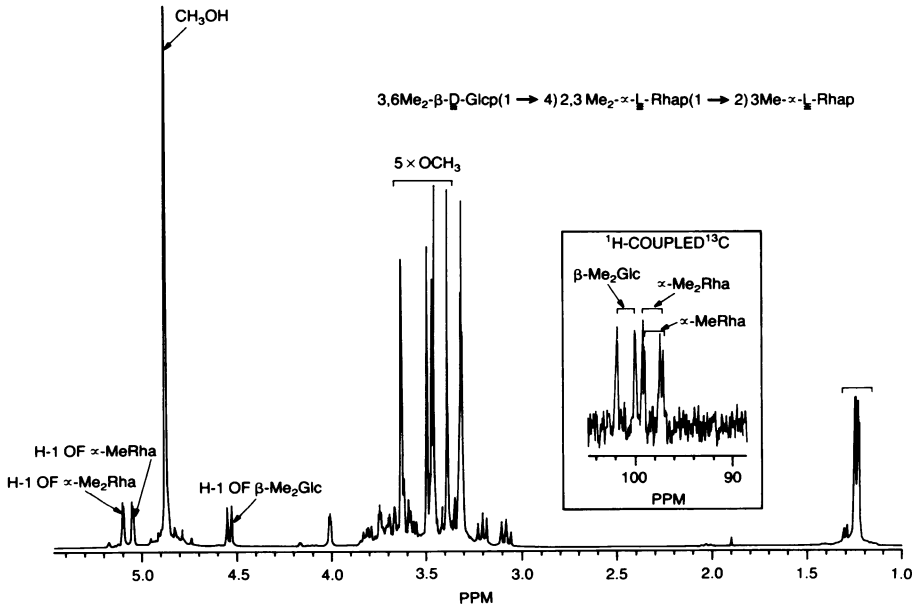

FIG. 5. A 360-MHz ^{1}H-NMR of 3,6-di-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3-di-*O*-methyl-α-L-rhamnopyranosyl-(1→2)-3-*O*-methyl-α-L-rhamnopyranose. Inset, ^{1}H-^{13}C coupling of benzyl-3-*O*-methyl-4-*O*-benzyl-2-*O*-[2,3-di-*O*-methyl-4-*O*-(2,4-di-*O*-acetyl-3,6-di-*O*-methyl-β-D-glucopyranosyl)-α-L-rhamnopyranosyl]-α-L-rhamnopyranoside.

methane-acetone (8:1) gave good separation of the two anomers (Figure 4B), and accordingly, preparative TLC in this solvent was used to separate both. Final yields of the two derivatized trisaccharides were 32.8 mg of compound XVI and 16 mg of compound XVII. ^{1}H-^{13}C coupling (Fig. 5, inset) of compound XIII allowed the following assignments (slightly different from those for the α, β mixture): 100.89 ppm, $J_{C\text{-}H}$ = 164.6, due to 3,6-di-*O*-Me-β-D-Glc*p*; 98.30 ppm, $J_{C\text{-}H}$ = 168.6, due to 2,3-di-*O*-Me-α-L-Rha*p*; and 97.5 ppm, $J_{C\text{-}H}$ = 173.8 due to 3-*O*-Me-α-L-Rha*p*.

All of compounds XVI and XVII were deacetylated with sodium methoxide to yield pure, more polar products, with no ester absorption in the infrared region. These were debenzylated by hydrogenolysis over 72 h and the final products were repurified by preparative TLC in $CHCl_3$-CH_3OH-H_2O (60:10:1) (Fig. 4C). Recovery was 20.4 mg from treatment of compound XVI and 10 mg arising from compound XVII. ^{1}H-NMR (Fig. 5) of compound XVII showed signals which may be assigned as follows: three protons at 5.09 ppm ($J_{1,2}$ = 1.73) due to the anomeric proton of the 3-*O*-Me-α-L-Rha*p* or 2,3-di-*O*-Me-α-L-Rha*p*; 5.04 ppm ($J_{1,2}$ = 1.65) due to the anomeric proton of 2,3-di-*O*-Me-α-L-Rha*p* or 3-*O*-Me-α-L-Rha*p*; 4.53 ppm ($J_{1,2}$ = 7.78) due to the anomeric proton of 3,6-di-*O*-Me-β-D-Glc*p*; five OCH_3 groups in the 3.35 to 3.65 ppm region; and two C-CH_3 groups at 1.22 and 1.26 ppm. Thus the evidence supports a structure corresponding to the natural trisaccharide 3,6-di-*O*-Me-β-D-Glc*p*-(1→4)-2,3-di-*O*-Me-α-L-Rha*p*-(1→2)-3-*O*- Me-α-L-Rha*p*. A similar approach to deacylated debenzylated compound XVI supported the structure, 3,6-di-*O*-Me-β-D-Glc*p*-(1→4)-2,3-di-*O*-Me-β-L-Rha*p*-(1→2)-3-*O*- Me-α-L-Rha*p*.

Serological activity of natural phenolic glycolipids, degradation products, and synthetic oligosaccharides. The activities of the three natural and two partially deglycosylated phenolic glycolipids against pooled human lepromatous leprosy sera were compared (Table 1). The two 3,6-di-*O*-Me-Glc*p*-containing glycolipids, PhenGL-I and PhenGL-II, were about equally active; however, the 6-*O*-Me-Glc*p*-containing PhenGL-III showed only about one-third the activity. Moreover, the absence of 3,6-di-*O*-Me-Glc*p* in the two degradation products of PhenGL-I resulted in loss of much of the activity. The phenolic glycolipids from *M. kansasii* and *M. bovis* are completely inactive against human lepromatous leprosy sera (4).

The synthetic di- and trisaccharides were inactive in direct ELISA, and therefore, an inhibition assay was developed to compare their activities. Glycolipids were emulsified by direct sonication in a high pH carbonate coating buffer, whereas the oligosaccharides were dissolved directly in the buffer. Suspensions or solutions were incubated with the pooled human lepromatous leprosy sera. The treated and untreated sera were reacted with solid-phase PhenGL-I, and the specific IgM response was measured. As expected, PhenGL-I inhibited binding; ca. 5×10^{-7} M (based on a molecular weight of 2,025) (18) resulted in 50% inhibition (Fig. 6). PhenGL-II was about as equally effective. PhenGL-III resulted in lesser but still substantial inhibition (ca. 12.5×10^{-7} M for 50% inhibition). Partial deglycosylation of PhenGL-I, notably removal of the two terminal sugars, abolished most of the activity; the residue from the latter, 3MeRha→PhenGL, had about the same marginal activity associated with the 2MeRha→PhenGL from *M. bovis*. The triglycosyl mycoside A from *M. kansasii* had little activity.

Maltose did not inhibit binding, and disaccharide compound VIIIa and disaccharide compound VIIIb showed little inhibition (Fig. 7). Only the 3,6Me_2Glc-containing disaccharide compound VIII brought about substantial inhibition of binding of PhenGL-I to anti-PhenGL IgM; 10^{-6} M caused 50% inhibition. Substitution of the disaccharide with the "natural" trisaccharide 3,6Me_2Glc→2,3Me_2Rha$\overset{\alpha}{\Rightarrow}$3MeRha did little to enhance inhibition (1.1×10^{-6} M caused 50% inhibition). From a comparison of the antagonistic effects of the 3,6Me_2Glc→2,3Me_2Rha$\overset{\alpha}{\Rightarrow}$3MeRha and 3,6$Me_2$Glc→2,3$Me_2Rha\overset{\beta}{\Rightarrow}$3MeRha, it was obvious that the anomeric configuration of the 2,3Me_2Rha-(1→2)-3MeRha linkage was not important for antigen-antibody binding.

Serological activity of 3,6-di-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3-di-*O*-methyl-L-rhamnose-BSA conjugate. Analysis of the BSA conjugate showed 4.67 μmol of glucose equivalents per micromole of BSA (Table 2). Incorporation of the conjugate into direct ELISA and comparison with PhenGL-I showed high activity against human lepromatous leprosy sera, and the overall results were qualitatively similar to those obtained when PhenGL-I was used as the solid-phase antien (4), except for some slight cross-reactivity with the tuberculosis sera. It may be possible in future experiments to remove this cross-reactivity by use of other protein carriers.

DISCUSSION

This paper describes the complete synthesis of the di- and trisaccharide portions of the specific phenolic glycolipid antigens of the leprosy bacillus. Demonstration of their serological activities against human lepromatous leprosy sera tends to support the earlier arbitrary assignment of enantiomeric configurations to derivatives of D-glucose and L-rhamnose residues in the natural glycolipids (18). Synthesis of the disaccharides was particulary satisfactory, although yields at 4 to 6% have yet to be optimized. The correctness of the structure of the trisaccharide corresponding to that in the natural antigen follows from confirmations of the structures of intermediate compounds used in the

TABLE 1. Activity of PhenGL-I, -II, -III, and partially deglycosylated PhenGL-I in binding specific IgM from human lepromatous leprosy sera[a]

Glycolipid	Activity at A_{488} (±SD) for	
	Leprosy sera	Normal sera
PhenGL-I (3,6Me_2Glc→2,3Me_2Rha→3MeRha→PhenGL)	1.520 ± 0.071	0.062 ± 0.011
Mono-deglycosylated PhenGL-I (2,3Me_2Rha→3MeRha→PhenGL)	0.192 ± 0.037	0.082 ± 0.016
Di-deglycosylated PhenGL-I (3MeRha→PhenGL)	0.038 ± 0.010	0.042 ± 0.005
PhenGL-II (3,6Me_2Glc→3MeRha→3MeRha→PhenGL)	1.410 ± 0.075	0.061 ± 0.004
PhenGL-III (6MeGlc→2,3Me_2Rha→3MeRha→PhenGL)	0.590 ± 0.054	0.071 ± 0.003

[a] Polystyrene wells were coated with 50 μl of a sonicate of each glycolipid (5 μg per ml of buffer). Pooled human lepromatous leprosy sera was diluted 1:300 with carbonate-bicarbonate buffer. Blocking was conducted with 5% BSA. Goat anti-human IgM peroxidase conjugate was diluted 1:1,000 in 20% normal goat serum. Assays were conducted in triplicate.

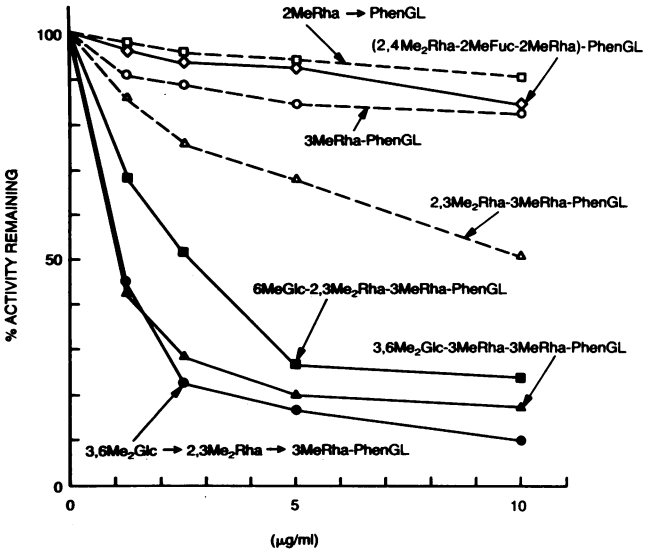

FIG. 6. Activity in inhibition assay of native and deglycosylated PhenGL-I, the native PhenGL-II and PhenGL-III from *M. leprae* and the native products from *M. bovis* and *M. kansasii*. Glycolipids were incubated with human lepromatous leprosy sera (diluted 1:300) at 37°C for 3 h and subsequently at 4°C for 18 h, and the activity of the sera against solid-phase PhenGL-I (2 μg/ml; 100 nmol per well) by using the IgM conjugate was tested.

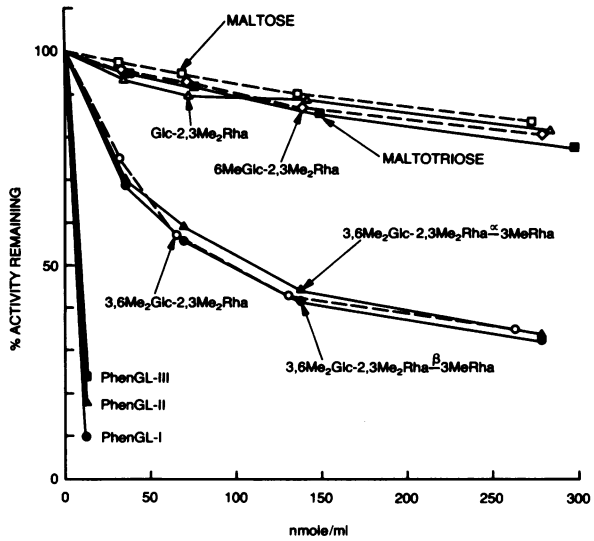

FIG. 7. ELISA inhibition activity of the synthetic disaccharides and trisaccharides against human lepromatous leprosy sera. The general experimental protocol is described in the legend to Fig. 6.

synthesis and is supported by spectroscopic data for the complete trisaccharide and its derivatives. Nevertheless, there is room for improvement in the final condensation step in view of the instability of the *O*-acetyl glycobiosyl bromide (X in Fig. 2) which underwent considerable nonproductive hydrolysis. Furthermore, this condensation proceeded with a lack of stereoselectivity. An alternative pathway involving condensation of 3,6-di-*O*-acetyl-2,4-di-*O*-benzyl-D-glucosyl bromide with a suitably substituted rhamnobiose derivative is proving to be much more satisfactory.

Structural features of oligosaccharides are known to be responsible for the antigenic specificity of many glycoconjugates such as streptococcal, klebsiella, pneumonococcal, and mycoplasma polysaccharides (1, 6, 14, 22), lipopolysaccharides (6), and blood groups (20). Glycolipids of bacterial (25) and human (23) origin have recently been added to this list. One of these shows exquisite positional isomeric specificity (23). Both IgM and IgG classes responding to glycolipids have been recognized (23, 26).

TABLE 2. Human IgM seroreactivity to disaccharide-BSA conjugate

Subject	No. positive[a]/ negative	A_{488} (mean ± SD)
Healthy controls	0/23	0.038 ± 0.011
Tuberculosis	3/9	0.058 ± 0.035
Atypical mycobacterial infections	0/15	0.024 ± 0.017
Tuberculoid (TT, BT) leprosy[b]	8/5	0.175 ± 0.159
Lepromatous (LLp, LLs, BL)[c] leprosy	30/3	0.543 ± 0.559
with <2 yr therapy	23/1	0.684 ± 0.594
with ≥2 yr therapy	7/2	0.169 ± 0.132

[a] With >3 standard deviations from mean A_{488} of healthy controls. The conjugate contained 140 pmol/ml of glucose equivalents and 35 pmol/ml of protein; 50 μl was applied to wells.

[b] TT, Tuberculoid; BT, borderline.

[c] LLp, Polar lepromatous; LLs, subpolar lepromatous; BL, borderline lepromatous.

The major purpose of the present investigation was to define which sugar residues on the phenolic glycolipids serve as antigen determinants. We used direct ELISA and an inhibition assay with partially deglycosylated PhenGL-I, two very minor less methylated relatives of PhenGL-I, and synthetic oligosaccharides. In the inhibition assay the glycolipids were much more active than the synthetic products suggesting greater avidity for antibodies. Nevertheless, it was clearly established that most of the activity resides in the terminal 3,6-di-*O*-methyl-β-D-glucopyranoside with a small contribution from the penultimate 2,3-di-*O*-methyl-α-L-rhamnopyranoside. The absence of a 2-*O*-methyl group on the penultimate rhamnosyl substituent seems to make little difference, although the presence of a second *O*-methyl substituent on the D-glucose end group was clearly necessary for optimum antibody binding. The reducing-end sugar had little bearing on antigenic activity, and neither had the configuration of the glycosidic linkage in the rhamnobiose unit. Broadly similar conclusions have emerged from structural analysis of the tetrasaccharide entities of the "C-mycoside" glycopeptidolipid antigens of some atypical mycobacteria (2, 3); all contain a common α-L-rhamnopyranosyl-(1→2)-6-deoxy-L-talose-reducing terminus, but each has a type-specific nonreducing mono- or disaccharide terminus which is responsible for serological specificity.

Although in the present work not all structural features possibly involved in antigen-antibody binding have been explored, sufficient information on the specificity of the reaction emerged to warrant conjugation of the natural disaccharide to BSA by the most facile of available procedures, namely reductive amination. The serological results clearly indicated that the probe was highly sensitive in detecting antibodies in serum from leprosy patients. Since, in the reductive amination step, the structural integrity of the terminal sugar, only, was maintained, this evidence also implicates 3,6-di-*O*-methyl-D-glucoside as the primary determinant of PhenGL-I. The synthetic glycoconjugate has the

potential of a specific probe with the sensitivity to detect presymptomatic leprosy and to provide base-line data for upcoming vaccine trials (21).

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