Structural Elucidation of a Novel Family of Acyltrehaloses from *Mycobacterium tuberculosis*†

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ABSTRACT: Analysis of the lipids of *Mycobacterium tuberculosis* H37Rv, by both normal- and reverse-phase thin-layer chromatography, revealed a series of novel glycolipids based on 2,3-di-*O*-acyltrehalose. The structures of these acylated trehaloses were elucidated by a combination of gas chromatography–mass spectrometry, ^1H, ^{13}C, two-dimensional ^1H–^1H, and ^1H–^{13}C nuclear magnetic resonance spectrometry. The fatty acyl substituents were mainly of three types: saturated straight-chain C_{16}–C_{19} acids; C_{21}–C_{25} "mycosanoic acids"; and C_{24}–C_{28} "mycolipanolic acids." Analysis of one of the major 2,3-di-*O*-acyltrehaloses by two-dimensional ^1H-chemical shift correlated and ^1H-detected heteronuclear multiple-bond correlation spectroscopy established that the C_{18} saturated straight-chain acyl group was located at the 2 position and that the C_{24} mycosanoyl substituent was at the 3 position of the same "right-hand" glucosyl residue. At least six molecular species differing only in their fatty acid content comprised this family of di-*O*-acylated trehaloses. We regard these acyltrehaloses as elemental forms of the multiglycosylated acyltrehaloses (the lipooligosaccharides) perhaps due to an inability of the majority of isolates of virulent tubercle bacilli to glycosylate core acyltrehaloses. The acyltrehaloses are minor but consistent components of virulent *M. tuberculosis* and apparently the basis of the specific serological activity long associated with its lipid fractions.

Tuberculosis still remains a serious public health problem with an estimated three million deaths per annum worldwide (Styblo, 1989). The search continues for serological tools capable of identifying individuals infected with *Mycobacterium tuberculosis*. Precedent demonstrates that most, if not all, of the nontuberculosis mycobacterial species are endowed with large quantities of a variety of antigenic glycolipids, either the trehalose-containing lipooligosaccharides (LOS)[1] (Hunter et al., 1983, 1985), the glycopeptidolipids (GPL) (Brennan & Goren, 1979; Brennan, 1984), or the so-called phenolic glycolipids (PGL) (Brennan, 1988). The variable oligosaccharide constituents of these three classes of glycolipids are usually of sufficient antigenicity as to evoke corresponding specific antibodies and thereby allow serodiagnosis of individual mycobacterioses (Brennan, 1988).

Isolates of the tubercle bacillus are generally devoid of these specialized glycolipid classes and their corresponding unusual sugars (Daffé et al., 1991a,b). They do, however, have simple acyl trehaloses, quite apart from the mycolyltrehaloses (the cord factors). Two distinct classes of such acylated trehaloses were identified in *M. tuberculosis* (Dobson et al., 1985; Minnikin et al., 1985). The nonpolar class was heavily acylated with the unsaturated mycolipenic (phthienoic) and other long-chain acids, and the polar class was acylated with the methyl-branched mycolipanolic and mycosanoic acids and straight-chain fatty acids. It was predicted that members of the polar class were potential antigens (Minnikin et al., 1985). The nonpolar members, also described later by Daffé et al (1988), were shown not to be strongly antigenic, but the polar glycolipids were strongly antigenic (Minnikin et al., 1987). We have now returned to the challenge of a more complete resolution of these glycolipids and a more thorough structural definition, all as a prelude to an analysis of the serological potency of individual members.

EXPERIMENTAL PROCEDURES

Purification of Acylated Trehaloses. Apolar and polar lipids are extracted from freeze-dried harvests of *M. tuberculosis* H37Rv using a biphasic mixture of aqueous methanol and petroleum ether (bp 60–80 °C) to yield apolar lipids in the upper organic layer. The remaining cells and aqueous methanol phase were then treated according to the established lipid extraction procedure of Bligh and Dyer (1959) using monophasic $CHCl_3$–CH_3OH–H_2O, the extracts being diluted with additional $CHCl_3$ and H_2O to give biphasic mixture with polar lipids in the lower organic layer (Minnikin et al., 1985). Previously, the polar lipid fraction, when subjected to TLC, yielded a series of glycolipids, which are now designated as diacyltrehaloses, DAT_1, DAT_2, DAT_3, and DAT_4; and DAT_3 and DAT_4 were minor components (Minnikin et al., 1985). In the present instance, the two major components, DAT_1 and DAT_2, were purified by preparative TLC on silica gel (Merck 5735 silica gel $60F_{254}$, Darmstadt, Germany), developed six

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[1] Abbreviations: PGL, phenolic glycolipid; ELISA, enzyme-linked immunosorbent assay; 2D COSY, two-dimensional chemical shift correlated spectroscopy; GC/MS, gas chromatography-mass spectrometry; R_f, retardation factor; TLC, thin-layer chromatography; Glc, glucose; Glc*p*, glucopyranose; 2D HMBC, two-dimensional heteronuclear multiple-bond correlation spectroscopy; DAT, di-*O*-acyltrehalose; PBS, phosphate-buffered saline; LOS, lipooligosaccharide, GPL, glycopeptidolipid; TMS, trimethylsilyl; GC, gas chromatography; NMR, nuclear magnetic resonance; FID, free induction decay; TFA, trifluoroacetic acid.

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times with $CHCl_3$–$(CH_3)_2CO$–CH_3OH–H_2O (50:60:2.5:3) to afford 46 and 25 mg of purified DAT_1 (R_f 0.67) and DAT_2 (R_f 0.62), respectively, from 120 g of dried cells. Further fractionation of DAT_1 and DAT_2 by preparative TLC on reverse-phase silica gel (Merck 13724 reverse-phase silica gel C_{18}, Darmstadt, Germany) developed three times with $CHCl_3$–CH_3OH–H_2O (6:15:0.1) allowed further resolution based on fatty acyl composition and yielded DAT_{1a} (10.7 mg; R_f 0.30), DAT_{1b} (19.0 mg; R_f 0.28), and DAT_{1c} (10.6 mg; R_f 0.25) from DAT_1, and DAT_{2a} (3.1 mg; R_f 0.36), DAT_{2b} (6.2 mg; R_f 0.30), and DAT_{2c} (5.7 mg; R_f 0.26) from DAT_2, respectively.

Antigenicity of Glycolipids. Polyclonal sera against whole cells of four different strains of *M. tuberculosis* (the type strain and three strains isolated from Swedish patients with tuberculosis) and two of *Mycobacterium bovis* BCG were produced in rabbits as described (Ridell et al., 1992). ELISA was conducted as described previously (Ridell et al., 1992).

NMR Analysis. Routine ^1H, ^{13}C, and 2D ^1H–^1H, and ^1H–^{13}C NMR spectra were recorded on a Bruker ACE-300 or a Bruker-AM 500 NMR spectrometer at Colorado State University, Department of Chemistry. Diacyltrehalose, DAT_{1a} (~10 mg), was dissolved in C^2HCl_3–$C^2H_3O^2H$ (2:1) and then subjected to NMR spectroscopy at the University of Georgia. $^1H(^{13}C)$-HMBC experiments were performed in absolute-value mode (Bax & Summers, 1986; Halbeek, 1990) without ^{13}C decoupling during acquisition. The spectral width was set to 7246.38 Hz in the ^1H dimension and to 31 240 Hz in the ^{13}C dimension. Ninety-two FIDs of 2048 complex data points were acquired. The spectra were processed off-line using the FELIX 2.0 software package (Hare Research, Bothell, WA) on a SUN-4 workstation.

Gas Chromatography and Mass Spectrometry. GC/MS of acetylated glycolipids were performed on a Hewlett-Packard 5890 gas chromatograph connected to a Hewlett-Packard 5970 mass selective detector. Samples were injected in the splitless mode. The injection port temperature was 290 °C and the transfer line 280 °C. The column used was a 12-m HP-1 (Hewlett-Packard, Avondale, PA). For the analysis of acetylated trehaloses, the oven was programmed to hold at 50 °C for 1 min followed by a 30 °C/min rise to 200 °C, an 8 °C/min rise to 320 °C, and an 8-min hold at this temperature (program 1). The temperature program that was applied to all trimethylsilyl derivatives of butyl glycosides involved a 50°C hold for 1 min followed by a 30 °C/min rise to 140 °C, an 6 °C/min rise to 260 °C, a 30 °C/min rise to 280 °C, and a final 2-min hold at this temperature (program 2). A modified version of program 2 was used in the fatty acid ester analysis, where the final temperature was 325 °C, held for 20 min (program 3). The mass spectrometer was set to scan from 50 to 800 amu with 0.81 scans per second.

Base-Catalyzed De-O-acylation of Acylated Trehaloses. Purified glycolipids (250 μg) were dissolved in 100 μL of $CHCl_3$–CH_3OH (2:1) and treated with 100 μL of 0.2 M NaOH in CH_3OH at 40 °C for 30 min. The base was neutralized with an equivalent amount of glacial acetic acid, the entire reaction mixture was dried and resuspended in $CHCl_3$–CH_3OH–H_2O (8:4:2), and the upper aqueous layer containing the acyl-free carbohydrate was dried and acetylated. Acetylations were accomplished using 50 μL of acetic anhydride, 50 μL of pyridine, and heating at 100 °C for 20 min. The resulting acetylated trehalose was dried, redissolved in acetone, and examined by GC/MS (program 1). An authentic standard of trehalose was acetylated and analyzed using the same procedure.

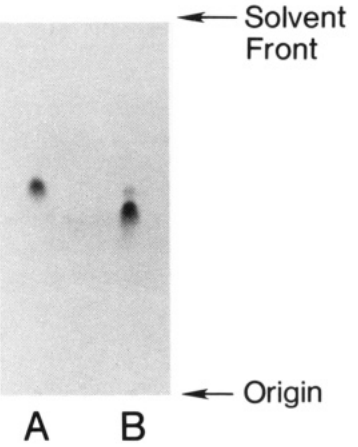

FIGURE 1: Thin-layer chromatogram of purified diacyltrehaloses isolated from *M. tuberculosis* H37Rv; (lane A) DAT_1, (lane B) DAT_2; solvent, $CHCl_3$–$(CH_3)_2CO$–CH_3OH–H_2O (50:60:2.5:3) developed six times. The plate was sprayed with 10% H_2SO_4 in ethanol and heated at 110 °C for 5 min.

Formation of Trimethylsilylated Fatty Acid Methyl Esters. The glycolipids (200 μg) were treated with 3 M HCl in methanol at 80 °C overnight. The sample was blown dry and trimethylsilylated using 130 μL of pyridine–hexamethyldisilazane (Supelco, Bellefonte, PA)–trimethylchlorosilane (Sigma, St. Louis, MO) (100:20:10) at 80 °C for 20 min, blown dry, and redissolved in hexane and examined by GC/MS (program 3).

Glycosyl Group Analysis. Purified glycolipids were hydrolyzed in 250 μL of 2 M TFA at 120 °C for 1 h as described (McNeil et al., 1987). Glycosyl composition, including absolute configuration of glycosyl residues, was determined by GC/MS analysis of the TMS derivatives of glycosides of (*R*)-(–)- and (*S*)-(+)-2-butanol as described (Gerwig et al., 1978). Per-*O*-methylation and glycosyl linkage analysis were achieved using a modification of the Hakomori procedure (York et al., 1986; Stellner et al., 1973).

RESULTS

Purification and Antigenicity of the Acylated Trehaloses. The complex mixture of what proved to be a family of 2,3-di-*O*-acyl trehalose glycolipids was purified as described under Experimental Procedures by extensive normal-phase and reverse-phase preparative TLC. Normal-phase TLC in $CHCl_3$–$(CH_3)_2CO$–CH_3OH–H_2O (50:60:2.5:3) developed six times yielded a series of four glycolipids, of which DAT_1 and DAT_2 were the most prominent and were chosen for further fractionation (Figure 1). Reverse-phase TLC of DAT_1 with repeated development in $CHCl_3$–CH_3OH–H_2O (6:15:0.1) yielded DAT_{1a}, DAT_{1b}, and DAT_{1c}. When DAT_2 was subjected to the same resolution protocol, DAT_{2a}, DAT_{2b}, and DAT_{2c} emerged. ELISA showed that both DAT_{1b} and DAT_{2a} reacted readily with rabbit antisera raised against the homologous strain and showed definite reaction with sera raised against three isolates from three different tuberculosis patients (Table I). No reactions were seen against anti-BCG sera.

Chemical Characteristics of the Acylated Trehaloses. ^1H NMR spectroscopy of the eight glycolipids showed similar resonances and suggested that all of the glycolipids had the same basic structure but differed in the fatty acyl population associated with each glycolipid. Specifically, the ^1H NMR spectrum of glycolipid DAT_{1a} (Figure 2) revealed the presence of two characteristic anomeric protons centered at 5.25 ppm ($J_{1,2} < 1.5$ Hz) and 5.05 ppm ($J_{1,2} < 1.5$ Hz). ^{13}C NMR was

Table I: Antigenicity of Diacyl Trehaloses from *M. tuberculosis*

rabbit serum to[a]	antigen,[b] relative reactivity in ELISA[c] DAT_{1b}	DAT_{2a}
type strain of *M. tuberculosis* H37Rv	+++	++
strain 9829/77 from tuberculosis patient	+	+
strain 10097/87 from tuberculosis patient	+	+
strain 141/89 from tuberculosis patient	+	+
Swedish substrain of BCG	–	–
Danish substrain of BCG	–	–

[a] Preparation of polyclonal sera against whole cells of the different isolates has been described (Ridell et al., 1992). All sera were applied at a dilution of 1 in 100. [b] The DAT antigens were applied to plates at a concentration of 1 μg/mL. [c] The conditions for ELISA have been described (Ridell et al., 1992). The absorption values were read after 20 min and scored as follows: 0.10–0.29, +; 0.30–1.00, ++; 1.01–2.00, +++.

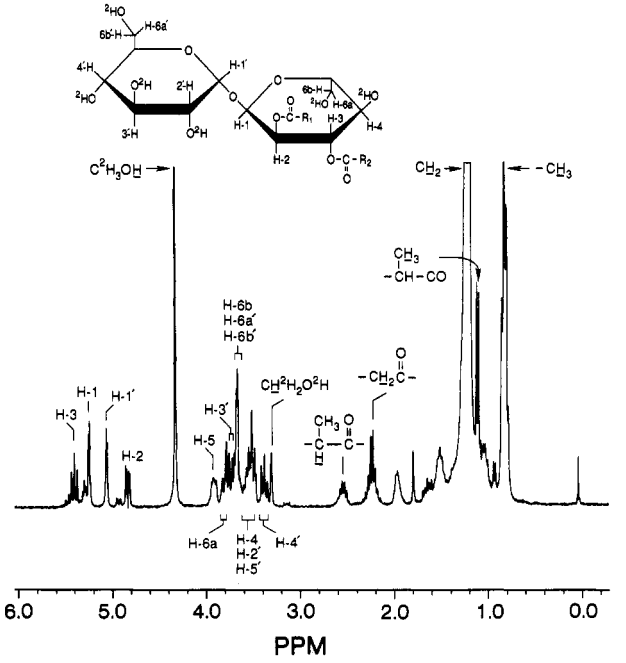

FIGURE 2: ^1H NMR of one of the diacylated trehaloses, DAT_{1a}, at 300 MHz in C^2HCl_3–$C^2H_3O^2H$ (4:1). The acylated trehalose was purified by preparative normal-phase and reverse-phase C_{18} silica gel TLC and dissolved in C^2HCl_3–$C^2H_3O^2H$ (4:1) for analysis at a concentration of 10 mg of DAT_{1a}/0.5 mL. The various signals were assigned as follows: H-1, 5.25; H-2, 4.83; H-3, 5.40; H-4, 3.46; H-5, 3.90; H-6a, 3.65; H-6b, 3.80; H-1′, 5.05; H-2′, 3.47; H-3′, 3.75; H-4′, 3.35; H-5′, 3.55; H-6a′, 3.68; H-6b′, 3.68. The prime (e.g., H-2′) numerals refer to the nonacylated glucosyl ring protons and the nonprime numerals to the acylated glucosyl ring protons of the trehalose moiety.

the most informative in revealing the general nature of the glycolipids as a family of di-*O*-acyltrehaloses. For example, the ^{13}C NMR spectrum of glycolipid DAT_{1a} (Figure 3) showed characteristic resonances of δ 95.0 and δ 92.0 associated with the two C-1s of trehalose (Bradbury & Jenkins, 1984). Also, all eight of the ring carbons of the trehalose were visible between δ 69 and δ 74, while the two primary C-6 resonated at the same frequency, δ 61.8. The two carbonyl carbons at δ 173.8 and δ 177.8 support the designation of a diacyl substitution. The fatty acyl carbons are seen in the upfield region of the spectrum centered at δ 30.1 (Bradbury & Jenkins, 1984).

Glycosyl Composition of the Acylated Trehaloses. The presence of trehalose in the glycolipids, as suggested by ^{13}C

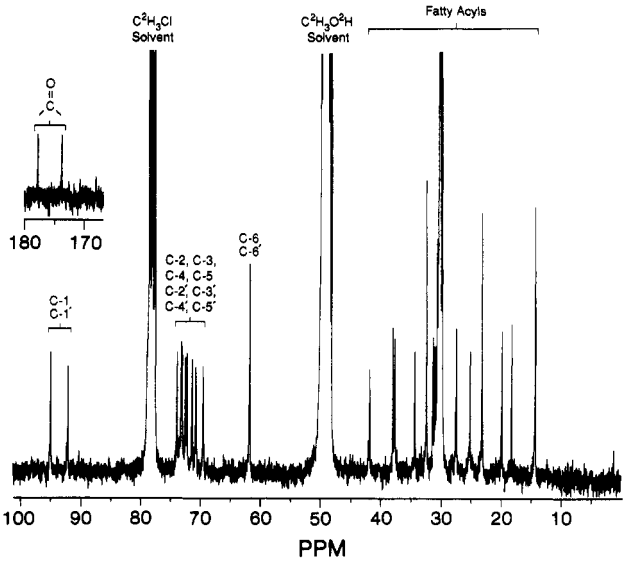

FIGURE 3: ^{13}C NMR of the diacylated trehalose, DAT_{1a}, at 125 MHz in C^2HCl_3–$C^2H_3O^2H$ (4:1). The assignments of the various signals are discussed in the text.

NMR, was confirmed chemically as follows. The glycolipids DAT_{2a} and DAT_{1a} were hydrolyzed with 2 M TFA at 120 °C for 1 h to produce free sugars (McNeil et al., 1987). The liberated sugars were butanolyzed with (*S*)-(+)-2-butanol and trimethylsilylated by the method of Gerwig et al. (1978). GC demonstrated the presence of D-Glc as the only glycosyl residue. The fact that the D-Glc existed as an α,α′,1,1-diglucoside was confirmed by deacylating DAT_{1a} with 0.2 M NaOH in methanol, neutralizing the mixture, removing the fatty acids with chloroform, and then acetylating the disaccharide. Analysis by GC/MS yielded a 2,2′,3,3′,4,4′,6,6′-octa-*O*-acetyl trehalose as identified by GC retention time and characteristic mass spectrum (Figure 4) Thus, the carbohydrate portion of the glycolipids was shown to be α-D-Glc*p*-(1↔1)-α-D-Glc*p* (α,α′-D-trehalose).

Fatty Acid Composition of the Acylated Trehaloses. The fatty acyl composition of the entire family of acylated trehaloses was examined by methanolysis of the intact glycolipid to form fatty acid methyl esters and then trimethylsilylation of any free hydroxyl groups. The resulting products were analyzed by GC and GC/MS (Ryhage & Stenhagen, 1963). Three families of fatty acid methyl esters were identified (Figure 5). Straight chain fatty acid methyl esters (I) were readily identifiable through a McLafferty ion at *m/z* 74; α-methyl branched fatty acid methyl esters (II) were marked by a McLafferty ion at *m/z* 88 (Ryhage & Stenhagen, 1963). The β-hydroxy-α-methyl branched fatty acid methyl esters (III) showed the base ion at *m/z* 189, corresponding to a C3–C4 bond cleavage of the trimethylsilylated derivative. The fatty acid methyl ester population for the DAT_1 series of glycolipids, i.e., DAT_{1a}, DAT_{1b}, and DAT_{1c}, consistently yielded *n*-C_{18} saturated acids and varying amounts of *n*-C_{16},C_{19} fatty acids. Also, this series contained predominantly 2,4-dimethyldocosanoic acid (C_{24} mycosanoic acid) and variable amounts of C_{25} and C_{26} analogues. The ratio of the branched analogues to the linear chain fatty acids was approximately 1:1. The analysis of DAT_{1a} shown in Figure 6 serves to corroborate these conclusions. The DAT_2 series of glycolipids, DAT_{2a}, DAT_{2b}, and DAT_{2c}, contained linear straight-chain fatty acids, *n*-C_{16}, *n*-C_{18}, and *n*-C_{19}. The mycosanoic acids were absent, and, instead, the glycolipids were characterized by a predominant 3-hydroxy-2,4,6-trimethyltetracosanoic (mycolipanolic) acid as well as minor

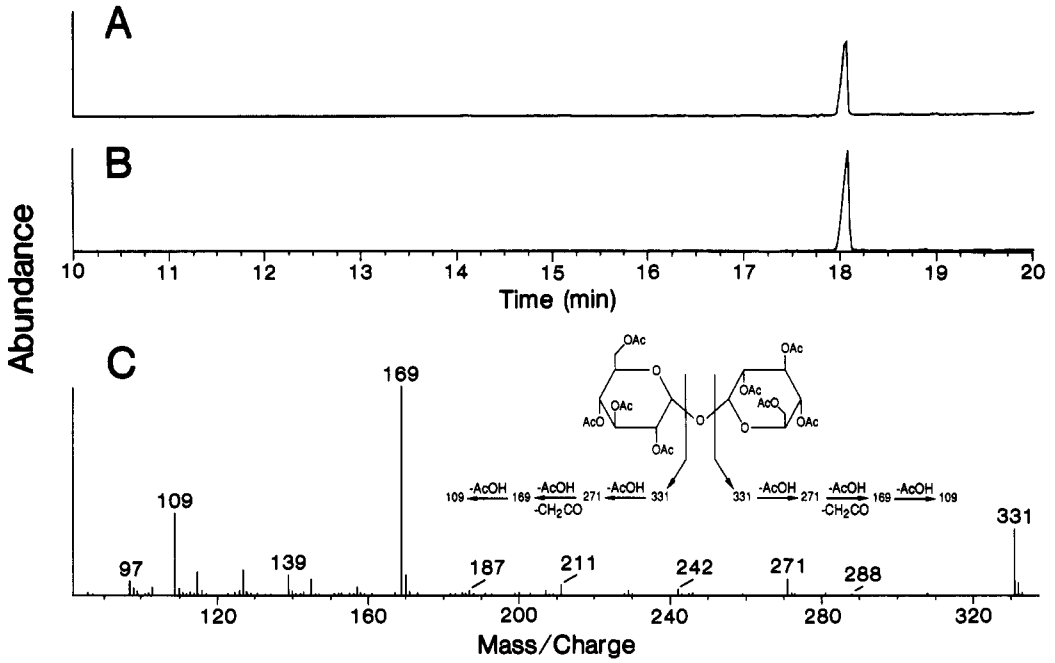

FIGURE 4: GC/MS analysis of one of the acetylated trehaloses. The total ion chromatograms resulting from the analysis of standard trehalose (A) and of trehalose prepared from DAT_{1a} (B) are illustrated as is the mass spectrum of the acetylated trehalose from DAT_{1a} (C).

I. $CH_3(CH_2)_{14\text{-}17}COOCH_3$

II. $CH_3(CH_2)_{16\text{-}18}\left[CH_2\text{-}\underset{CH_3}{CH}\right]_{1\text{-}3}COOCH_3$

III. $CH_3(CH_2)_{16\text{-}18}\left[CH_2\text{-}\underset{CH_3}{CH}\right]_{1\text{-}3}\text{-}\underset{OH}{CH}\text{-}\underset{CH_3}{CH}\text{-}COOCH_3$

FIGURE 5: Deduced structures of the range of fatty acid methyl esters obtained from the family of the 2,3-di-*O*-acyltrehaloses of *M. tuberculosis*. The acylated trehaloses were subjected to methanolysis in 3 M methanolic HCl at 80 °C overnight followed by trimethylsilylation of the fatty acid methyl esters. The resulting fatty acid derivatives were analyzed by GC and GC/MS.

C_{24}, C_{26}, and C_{28} homologues. The ratio of the nonhydroxylated to the hydroxylated fatty acids was approximately 1:1 (results not shown).

Complete Structure of the Acylated Trehaloses. ^{1}H-detected heteronuclear multiple-bond correlation (HMBC) spectroscopy NMR experiments helped establish the location of the fatty acyl groups of glycolipid DAT_{1a} through multiple-bond transglycosidic correlations. The 2D HMBC spectrum (Figure 7) of glycolipid DAT_{1a} revealed the α-CH_2 proton signal of the *n*-C_{18} saturated straight chain fatty acyl group at 2.25 ppm, which showed a $^2J_{HC}$ connectivity to the upfield carbonyl CO signal at 173.8 ppm in the ^{13}C spectrum. This CO signal, in turn, showed a $^3J_{HC}$ connectivity to the proton signal at 4.83 ppm, which is assigned to H-2 of the acylated glucose residue, on the basis of 2D COSY. On the other hand, the α-C*H* proton of the C_{24} mycosanoic fatty acyl group, resonating at 2.55 ppm, showed a $^2J_{HC}$ connectivity to the CO signal at 177.8 ppm; the latter is clearly connected to H-3 of the acylated glucose residue, at 5.40 ppm. The combination of these connectivity trails clearly established that the *n*-C_{18} saturated straight-chain and the C_{24} mycosanoic fatty acyl groups are located at the 2 and 3 positions of the same glucosyl residue, respectively. All of the other glycolipids yielded very similar ^{1}H NMR spectra (results not shown). The presence generally of an *O*-acyl group at H-2 was shown by the characteristic doublet of doublet signal at δ 4.83. The presence

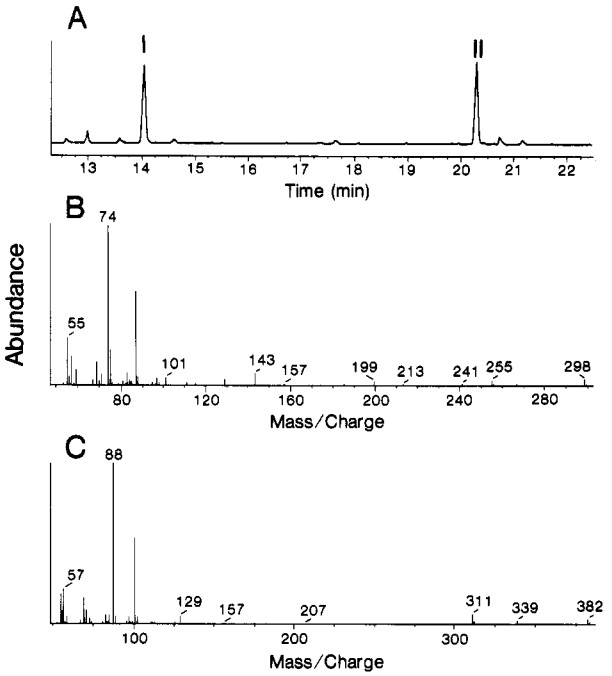

FIGURE 6: GC/MS analysis of the trimethylsilylated fatty acid methyl esters prepared from diacyltrehalose DAT_{1a}. (A) Total ion chromatogram; I, *n*-C_{18}, straight chain; II, C_{24}, α-methyl branched fatty acid (mycosanoic acid). (B) Mass specrum of I showing characteristic fragment ions. (C) Mass spectrum of II showing characteristic fragment ions.

of 3-*O*-acyl substitution on the same glucosyl residue was demonstrated in all instances by decoupling experiments as follows. The signal at δ 4.83 was selectively irradiated, and, in all cases, this resulted in the collapse of the signal at δ 5.40, demonstrating that this signal invariably arose from H-3 which was necessarily on an *O*-acylated carbon. These results along with data on fatty acid composition demonstrated that all of the glycolipids are based on trehalose with an H-2 and H-3 acyl substitution. Thus, this family of 2,3-di-*O*-acyltrehaloses was found to exist in at least six molecular species differing only in their fatty acyl substituents as discussed earlier. The structure of the diacyltrehalose DAT_{1a} is shown in Figure 8.

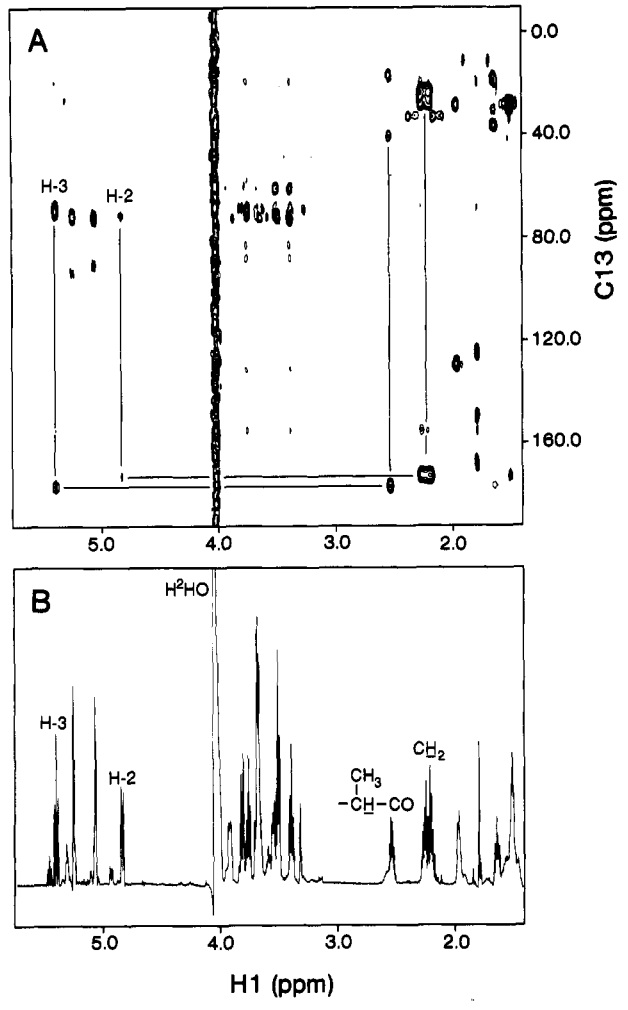

FIGURE 7: Two-dimensional $^1H\{^{13}C\}$ HMBC spectrum (600 MHz, C^2HCl_3–$C^2H_3O^2H$, 25 °C) (A) and high-resolution one-dimensional ^1H NMR spectrum (B) of diacyltrehalose DAT_{1a}. The assignments of the various signals are discussed in the text.

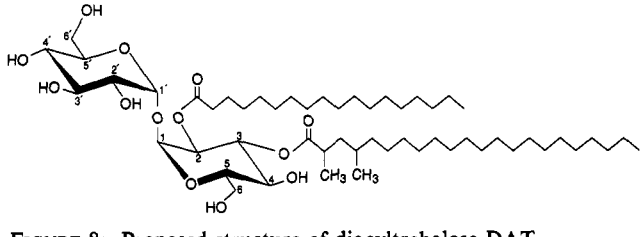

FIGURE 8: Proposed structure of diacyltrehalose DAT_{1a}.

DISCUSSION

The search within "field" isolates of *M. tuberculosis* for species-specific, highly antigenic glycolipids, similar to those from other species of *Mycobacterium* (Hunter et al., 1983, 1985; Brennan, 1984, 1988), has had mixed results. Of the more definitive works, Reggiardo et al. (1981) described a series of phosphorus-free glycolipids (and thus obviously not the trans-species phosphatidylinositol mannosides; Brennan, 1988) that were highly antigenic and showed potential as serodiagnostic tools for tuberculosis. Minnikin et al. (1985) showed catagorically that these were polar acyltrehaloses but were not of the dimycoyltrehalose/cord factor class. The fatty acyl composition of these is quite complex and consists of three major types: n-C_{16}–C_{19} saturated fatty acids; C_{21}–C_{25} α-methyl branched (mycosanoic) fatty acids; and the C_{24}–C_{28} α-methyl branched, β-hydroxy (mycolipanolic) fatty acids. The DAT_1 series contains fatty acids of the n-C_{16}–C_{19} and C_{21}–C_{25} α-methyl branched classes, whereas the DAT_2 series was comprised of both the n-C_{16}–C_{19} and the α-methyl, β-hydroxy types. The use of 2D ^1H–^1H COSY NMR established that these fatty acyl residues uniformly occupy the 2 and 3 positions of the same glucosyl residue of the trehalose moiety, thus establishing that this family of glycolipids is based on a 2,3-di-*O*-acyl trehalose core. Two-dimensional heteronuclear multiple-bond correlation spectroscopy established that, for glycolipid DAT_{1a}, the n-C_{18}-saturated straight-chain and the C_{24}-mycosanoic fatty acyl substituents are located at the *O*-2 and *O*-3 positions of the same glucosyl residue, respectively.

Although the matter of the serological activity of these glycolipids was not pursued extensively in this present work, the 2,3-di-*O*-acyl trehalose family reacted readily in ELISA with rabbit antibodies raised against whole *M. tuberculosis*, and the limited results reported here combined with previous information (Ridell et al., 1992) indicated that DAT_{1b} and DAT_{2a} are specific to *M. tuberculosis*, both the type strain H37Rv and recent isolates from patients. Thus, these acylated trehaloses, although not major components of *M. tuberculosis*, may provide useful serological tools for the diagnosis of tuberculosis. Interestingly, an acylated trehalose once thought to be sulfated (Daffé et al., 1989) and corresponding to one of the sulfolipids described by Goren (1979) also showed appreciable specific seroreactivity. It now seems clear (Lemassu et al., 1991) that this product is not sulfated and probably corresponds to the diacyltrehaloses described here.

The detailed structure of the diacyltrehaloses (Figure 8) is reminiscent of the "core" acyltrehalose of many of the multiglycosylated trehalose-containing lipooligosaccharides (Camphausen et al., 1987). This observation leads us to suggest that a phenotypic trait of the true etiological agent of tuberculosis is the inability to glycosylate an elemental core diacyltrehalose. In fact, the atypical Canetti variants of *M. tuberculosis*, marked by smooth colony morphology as distinct from the typical granular appearance of the usual forms of *M. tuberculosis*, produces a highly glycosylated, highly characteristic member of the lipooligosaccharide class of glycolipids (Daffé et al., 1991a). We proposed that this is an aberrant, "glycosylation-competent" strain of *M. tuberculosis*. The genetic and biochemical basis of this attribute and its pathogenic consequences are now being investigated. As of now, the realization of a relationship between the simple, basic acyltrehaloses and the multiglycosylated forms leads us to propose that many of the known mycobacterial strains, including those of *M. tuberculosis*, may be spontaneous mutants of one another as found among the rough and smooth variants of *M. avium* (Belisle et al., 1991), resulting from spontaneous genetic deletions resulting in loss of the ability to extend glycosyl chains on several of the known glycolipids.

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