A cell wall component of a smooth variant of Gordona hydrophobica 1775/15 was isolated and purified, and its structure was determined by various chemical methods, including chemical synthesis of part structures, Edman degradation, gas chromatography/mass spectrometry analysis, matrix-assisted laser desorption ionization-post-source decay (MALDI-PSD) tandem mass spectrometry, and 1H and 13C NMR using one- and two-dimensional, homo- and heteronuclear correlated spectroscopy. The cell wall component was found to be a (mono-)glycosylated peptidolipid (GPL) consisting of a tridec-apeptide interlinked by a β-hydroxylated fatty acid (3-hydroxyeicosanoic acid, 20:0 (3-OH)) to form a cyclic lactone ring structure. The main fraction of GPL, for which we propose the name gordonin, was identified as 3-hydroxyeicosanoyl-L-seryl-L-phenylalanyl-L-seryl-L-seryl-D-alanyl-L-(O-D-glucopyranosyl)-threonyl-glycyl-D-leucyl-L-valyl-L-seryl-L-phenylalanyl-glycyl-L-valyl lactone. The other GPLs constitute structural variations within the nature of the β-hydroxylated fatty acid (20:0 (3-OH) versus 22:1 (3-OH)) in a ratio of about 1:0.9 as well as within one amino acid (D-Leu versus L-Phe) in about 30%. Sequence information was obtained in part by Edman degradation as well as gas chromatography/mass spectrometry analysis of di- and tripeptide fragments. However, the complete amino acid sequence could only be established by MALDI-PSD from the linear molecule, i.e. after ring opening of the lactone.

In contrast, rough variants of G. hydrophobica 1775/15 lack these peptidolipids or synthesize them to a much lesser extent indicating that gordonin contributes significantly to the physicochemical character of the cell surface.

Biofiltration is the method of choice for the treatment of waste gases with high volumes and low concentrations of organic pollutants. This technology requires low investment and operation costs and guarantees high reliability. Although biofiltration is already widely used, knowledge about the biology underlying this technology is lacking. Only a few studies have so far been conducted on the microorganisms involved in the process of exhaust-air treatment. Recently, Bendinger et al. (1) isolated coryneform bacteria from a biofilter that was loaded with the waste gas from an animal-rendering plant. On the basis of chemotaxonomic differentiation they were able to identify strains of the genus Corynebacterium, Gordona, Mycobacterium, and Arthrobacter. Some biofilter isolates of Gordona hydrophobica (2) showed colony morphologies, which gave rise to different physicochemical properties of their cell surface. The rough variants possess an extremely hydrophobic cell surface due to a hydrophobic mycolic acid layer. In contrast, the smooth variants exhibit a hydrophilic to moderate hydrophobic surface (3), although they are also surrounded by a hydrophilic mycolic acid layer. Obviously, the smooth ones incorporate into their cell wall an additional surface component, which exposes its hydrophilic part toward the medium. The observation that rough colonies of Gordona only gave rise to rough colonies whereas smooth colonies gave rise to smooth as well as rough ones when grown on complex media indicated that the rough variants lost the ability to cover their extremely hydrophobic surface with a more hydrophilic compound.

A similar change in colony morphology was reported for mycobacterial species that are closely related to the genus Gordona. Camphausen et al. (4) found a trehalose containing lipooligosaccharide to be responsible for the observed change in Mycobacterium paratuberculosis. Fregnan et al. (5) showed that smooth variants of an unclassified scotochromogenic Mycobacterium possessed a mycoside D that is absent in the rough variants. However, Barrow and Brennan (6) pointed out that mycoside D is likely to be a mixture of polar and apolar C mycoside glycopolidolipids (GPLs). Belisle and Brennan (7) showed that the smooth variants of Mycobacterium kansasi possess trehalose-containing lipooligosaccharides, whereas the rough variants were devoid of such surface components.

Such a change of morphology within the nocardioform actinomycetes has so far only been described for mycobacteria and not for other members of this taxon. Therefore, it was of interest to establish whether the morphological change observed with G. hydrophobica was due to the loss of mycosides or lipooligosaccharides or whether a different cell wall component is responsible for the more hydrophilic cell surface. Our studies revealed that the smooth variant of G. hydrophobica carries an additional glycosylated peptidolipid, which we named gordonin. Such peptidolipids have been described as surfactants or antibiotics (8–10), but so far not as constituents of bacterial cell walls.

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New Glycosylated Lipopeptide Incorporated into the Cell Wall

Materials and Methods

Bacterial Strains

Gordonia bronchialis DSM 43247T and Gordonia rubropertinctus DSM 43197T were obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH (DSM), Braunschweig, Germany. The isolation and classification of G. hydrophobica DSM 44015T has been published previously (1, 2). A detailed description of chemotaxonomical and physiological markers of G. hydrophobica

G. hydrophobica DSM 44015T (=1610/1b), G. hydrophobica DSM 44015r (=1610/1a), G. hydrophobica

G. hydrophobica 1775/15s was cultivated in 2 liters of brain-heart infusion broth (Difco) at 30 °C (pH 7.4) for 3–4 days. The cells were pelleted by centrifugation (3000 × g, 30 min), washed twice with deionized water, and lyophilized.

2 g of lyophilized cells were extracted twice with 60 ml of chloroform/methanol (2:1, by volume) and once with chloroform/methanol (1:1, by volume). 40 ml of chloroform and 40 ml of 0.3% NaCl were added to the combined extracts. After gentle shaking the phases were separated by centrifugation (3000 × g, 20 min). The organic phase was evaporated to dryness on a rotary evaporator, and the pellet was redissolved in 50 ml of chloroform. This fraction was applied to a DEAE-cellulose column (20 × 2 cm) that was equilibrated with chloroform. The column was washed with chloroform, and gordonin eluted with chloroform/methanol (9:1, by volume). The eluate was evaporated to dryness by rotary evaporation, and the pellet was redissolved in 30 ml of chloroform/methanol/glacial acetic acid/water (186:34:5:3). It was further purified by column chromatography (50 × 1 cm) on silica gel 60 (Merck, Darmstadt, Germany). Gordonin was eluted by a linear gradient of chloroform/methanol/glacial acetic acid/water (90:40:15:9) as solvent B. The gordonin-containing fractions were collected and dried by rotary evaporation.

Analytical Thin Layer Chromatography

2–5 µg of a sample were dissolved in an appropriate solvent and spotted on a silica 60 plate (Merck). Plates were developed in chloroform/glacial acetic acid/methanol/water (80:15:12:4). After drying, lipids were detected by wetting the plate with 20% sulfuric acid in ethanol and heating at 120 °C for 2–5 min. Sugar residues were detected by spraying the plate with 0.1% orcinol in 40% sulfuric acid followed by heating at 120 °C for 15 min. For detection of amino groups, plates were sprayed with ninhydrin solution (Riedel de Haen, Seelze, Germany) and heated at 100 °C for 10 min.

GC/MS Analysis

An aliquot (2 mg) of gordonin was methanolyzed with either 0.5 M HCl/MeOH (65 °C, 45 min) or 2 M HCl in methanol (85 °C, 16 h) followed by per-acetylation. Gas chromatography/mass spectrometry (GC/MS) of resulting fragments of gordonin was carried out with a Hewlett-Packard 5890 instrument. GLC was performed with a Varian model 3700 chromatograph equipped with a capillary column of SPB-5 using a temperature gradient 150–320 °C at 5 °C/min. GLC-MS was carried out with a Hewlett-Packard model 5891 instrument equipped with a capillary column of HP-1 under the same chromatographic conditions as in GLC. Electron impact spectra were recorded at 70 keV, and chemical ionization was done using ammonia as reactant gas.

Ring Opening of Gordinin by Mild Transsterification

1 mg of gordonin was dissolved in 500 µl of 100 mM sodium methyate and incubated at 37 °C for 16 h. After neutralization with 2 M HCl in methanol the solvent was evaporated under reduced pressure, and the saponified gordonin was dissolved in chloroform. The precipitated NaCl was discarded.

Fatty Acid Analysis

An aliquot (100 µg) of gordonin was methanolyzed with 2 M HCl in methanol (85 °C, 16 h). GC/MS of the resulting fragments was carried out with a Hewlett-Packard model 5890 series II gas chromatograph equipped with a capillary column of HP-5 (0.25 mm × 30 m) and a Hewlett-Packard model 5972 MSD. The temperature gradient was 120–300 °C at 5 °C/min. Electron impact spectra were recorded at 70 keV. Fatty acid methyl esters were identified by comparison of retention times and mass spectra using standards from Sigma (Germany).

Amino Acid Analysis

Gordonin was hydrolyzed in a gas-phase hydrolyzer with 6 M HCl at 150 °C for 1–4 h. The amino acid composition was analyzed with a ABI type 421 amino acid analyzer (Applied Biosystems, Foster, CA).

Absolute Configuration Analysis of Amino Acids

100 nmol gordonin were hydrolyzed as described above. The separation of enantiomeric amino acids was achieved after derivatization with N-(2,4-dinitro-5-fluorophenyl)-l-alaninamide. Derivatization was performed as described (11). Amino acid derivatives were separated at 50 °C by HPLC on a C18 reversed-phase column (Hewlett-Packard ODS Hypersil, 5 µm, 250 × 4 mm) fitted with a LiChrospher®100 RP-18 guard column (5 µm, 4 × 4 mm). The separation was achieved by a linear gradient of 50 mM triethylammonophosphate, pH 2.9 (buffer A), and acetoniitre (buffer B). The gradient increased from 15 to 50% buffer B in 35 min. The flow rate was 500 µl/min and amino acid derivatives were detected at 340 nm. Peaks were recorded and quantified with the Hewlett-Packard 3D Chem Station software.

Synthesis of Gordinin Part Structures as GC/MS and NMR Reference Compounds

Synthesis of (R)-3-Acetoxytetradecanamido-L-valine Methyl Ester (2)—For the synthesis of (R)-3-hydroxytetradecanamido-L-valine methyl ester (2), 24 mg (0.1 mmol) of optically pure (R)-3-hydroxytetradecanoic acid (14:03-0H)) was dissolved in 6 ml of 1,2-dimethoxyethane (Fluka), and 10 mg (0.9 mmol) of N-hydroxysuccinimide (Aldrich) and 29 mg of N,N,N-tricyclohexylcarbodiimide (0.14 mmol, Sigma) were added to the solution. The reaction was allowed to proceed for 36 h at 4 °C, and precipitating material was removed by filtration. The solution was evaporated to dryness under a stream of nitrogen, and the product was dissolved in 1 ml of pyridine. 10 mg of N-valine methyl ester (freshly prepared from L-valine (Sigma) and diazomethane in ether) were added to the solution, and the reaction was allowed to proceed for 1 h at room temperature. The product (2) was applied to a small silica gel column (0.5 × 5 cm, Kiesel gel 60, 230–400 mesh, Merck) and stepwise eluted with ether/hexane (10:90, by volume), chloroform, chloroform/methanol (95:5 and 90:10, by volume). The product (2) eluted in the chloroform fraction (yield 3 mg, 0.01 mmol, 10%) and was further characterized by GC/MS and NMR spectroscopy.

Synthesis of (R)-3-O-[2-N-(Acetamido)-D-valinoxy]-tetradecanoic Acid Methyl Ester (3)—(R)-3-O-[2-N-(Acetamido)-D-valinoxy]-tetradecanoic acid methyl ester (3) was synthesized starting from 15 mg of N-val (0.1 mmol) that were N-acetylated as described (12). To the resulting 2-N-(acetamido)-N-valine, 1 ml of trifluoroacetic acid anhydride was added, and the reaction was kept at 40 °C for 24 h. After removal of trifluoroacetic acid anhydride under a stream of nitrogen, 5 mg (0.02 mmol) of (R)-3-hydroxytetradecanoic acid methyl ester were dissolved together with 0.5 ml of pyridine containing catalytic amounts of 2,6-dichlorophenolindophenol. The reaction was allowed to proceed for 16 h at 85 °C. GC/MS analysis revealed that the product obtained was (R)-3-O-[2-N-(trifluoroaceticamido)-D-valinoxy]-tetradecanoic acid methyl ester from which the N-trifluoroacetamidino residue was removed by treatment with 0.25 ml of methanol/water (4:1, by volume) at room temperature. After the product was dried in vacuo, it was dissolved in 1 ml of pyridine/acetylhydridine (2:1, by volume) thus yielding the desired (R)-3-O-[2-N-(acetamido)-D-valinoxy]-tetradecanoic acid methyl ester (3), which was purified on a small silica gel column as mentioned above (yield 3 mg, 0.01 mmol, 50%).

Amino Acid Sequence Analysis by Edman Degradation

Gordonin was partially hydrolyzed with 2 M HCl (90 °C, 2–4 h) or with 1 M HCl (37 °C, 4–12 h). The hydrolysates were purified by C18 reversed-phase HPLC. The main peaks (monitored at 220 nm) were collected and analyzed with a peptide sequencer ABI 476A (Applied Biosystems, Foster, CA). Since peptides resulting from the hydrolysis were short and hydrophobic, an arylamine-modified membrane (Sevlon AA; Millipore Corp., Bedford, MA) was used to covalently bind the carboxyl-terminal amino acid.

Amino Acid Sequence Analysis by GC/MS

Partial hydrolysis of gordonin was carried out after saponification as described above. Preparation of trifluoroacetylated peptide esters was performed as described elsewhere (13). The resulting trifluoroacetyl peptide methyl esters were identified by performing gas chromatography-mass spectrometry using a Hewlett-Packard model 5890 series II gas chromatograph equipped with a 5% phenyl methyl silicone capillary column (0.25 mm by 30 m) and a model 5972 mass selective detector.
Helium was used as carrier gas; the injection volume was 5 μl, and the injector temperature was 250 °C. Split was set at 1:37. The initial column temperature was 70 °C, which was held for 2 min and then increased to 300 °C at a rate of 5 °C/min. The gas chromatography-mass spectrometry transfer line temperature was 280 °C.

**Time-of-Flight-Secondary Ion Mass Spectrometry (TOF-SIMS)**

The TOF-SIMS determinations were carried out with a TOF II instrument developed at the University of Münster (14). It consists of a primary ion source with a pulsed 90° deflector, a reflectron TOF analyzer, and a single ion counting registration system. The primary ions generated by the ion source bombard the surface as short mass separated ion packets (0.8 ns, 10 keV Ar⁺) in an area of about 70 nm diameter. The generated secondary ions are accelerated to 1.8 keV, mass-separated in a field free flight path, and then detected with an ion electron-photon conversion system. For the insulating surfaces, the TOF-mass spectrometer is a reflectron-type instrument employing a gridded two-stage reflector at the end of a first field drift path of 204 cm in length. Its second field drift path (reflector to detector) extends over 175 cm. Standard acceleration voltage in this instrument is 10 kV. For ion detection a 75-mm diameter dual microchannel plate is employed. The ion source in the instrument is a coaxial optical/ion optical device which simultaneously serves for laser focusing, sample imaging, and optical beam collimation, all in a common axis strictly perpendicular to the surface plane of the sample.

The acceleration stage is a split device (2/6 mm) with the option to apply delayed extraction conditions. This means that desorbed ions are initially (for 50–500 μs) allowed to expand against a slightly retarding field before the accelerating field is turned on (fast HV switch). If the delay time and the ratio of field strength in the two acceleration stages are properly chosen (settings dependent on the mass range of interest), so-called velocity focusing conditions can be fulfilled, resulting in a dramatic improvement of mass resolution. In the present investigation delay times between the desorption pulse and the onset of the extraction field of 230–440 μs were chosen, and a ratio of 0.47 for the field strength in the first and second acceleration stages, respectively, was found to perform best with respect to mass resolution of the quasimolecular ion species. Typical values obtained under these conditions were

**MALDI and MALDI-PSD Mass Spectrometry**

MALDI specimens were prepared by dissolving 50–200 pmol of the analyte in 10 μl of chloroform/glacial acetic acid/methanol/water (80:15:12:4). To this solution 10 μl of acetone saturated with 2,5-dihydroxybenzoic acid was added. A 5–10μl aliquot of this mixture was pipetted on the surface of a slightly preheated sample holder, on which the solvent evaporated within a few seconds. Transfer of the sample into the instrument was performed as quickly as possible.

**FIG. 1. Identification of an additional glycolipid synthesized by smooth colony-forming variants of Gordona biofilter isolates.** Analytical TLC was carried out as described under "Materials and Methods." The most polar fractions of the following strains have been analyzed: lane 1, Gordona bronchialis DSM 43247T; lane 2, G. rubropertinctus DSM 43197T; lane 3, G. hydrophobica DSM 44015T; lane 4, G. hydrophobica DSM 44015T; lane 5, G. hydrophobica 1775/15r; lane 6, G. hydrophobica 1775/15r (s = smooth variants, r = rough variants).

**TABLE I**

Calculated molecular weight of gordonin and its homologue

| Residue | M⁺ | Number of residues | Molecular weight |
|---------|----|-------------------|-----------------|
| Serine  | 105| 4                 | 420             |
| Glycine | 75 | 2                 | 150             |
| Threonine| 119| 1                 | 119             |
| Alanine | 89 | 1                 | 89              |
| Valine  | 117| 2                 | 234             |
| Leucine | 131| 1                 | 131             |
| Phenylalanine | 165| 2     | 330             |
| Glucose | 180| 1                 | 180             |
| FA C90–3OH | 328| 1     | 328             |
| FA C22:1–3OH | 354| 1     | 354             |
| Sum     | 1981| 2007         | 1981            |
| H₂O subtracted | 15 | 270          | 270             |
| Calculated molecular weight | 1711| 1737          | 1711            |

**FIG. 2. Electron impact-mass spectrum of [(R)-3-hydroxypentadecanamide]-D-valine methyl ester (2) (a), [(R)-3-O-[2-N-(acetylamido)-n-valinoyl]tetradecanoic acid methyl ester (2) (b), and of the compound isolated and derivatized in a similar way from gordonin (c).** 2 and 3 have been synthesized as described under "Materials and Methods."
New Glycosylated Lipopeptide Incorporated into the Cell Wall

Identification, Isolation, and Purification of Gordonin

A systematic analysis of complex lipids in G. hydrophobica 1775/15s and G. hydrophobica 1775/15r according to the method of Dobson et al. (16) revealed that an additional compound was present in the fraction of the most polar lipids of the smooth variants compared with that of the rough ones. The less polar lipids were identical between smooth and rough variants (data not shown). In TLC analysis this compound showed an \( R_f \) value of 0.23 (Fig. 1). In contrast to the rough variants, all investigated smooth ones synthesize this compound. The ninhydrin reaction was negative, whereas the reaction with orcinol was positive. This indicated that the lipid contains no free amino group but instead carries a sugar moiety. We therefore tentatively concluded that the isolated compound is a glycolipid, which was named gordonin. For structural analysis we used the glycolipid of the strain 1775/15s, which was confirmed to be G. hydrophobica by DNA-DNA hybridization with G. hydrophobica DSM 44015\(^5\) and that was the object of further physiological investigations.\(^2\)

Structural Analysis of Gordonin

Compositional Analysis

After weak methanolysis and subsequent peracetylation of the purified gordonin, glucose could be detected as peracetylated methyl glycoside by GC/MS analysis to be the only sugar component (Table I). Strong methanolysis released glucose, serine, and two fatty acids. Two further fragments, in which the valine was bound to two different fatty acids, could be identified, but it was not possible to decide whether the amino acid was amide- or ester-linked to the fatty acid.

Amino Acid Analysis

After strong hydrolysis and subsequent peracetylation of the purified gordonin, alanine, leucine, threonine, glycine, valine, phenylalanine, and serine could be identified in a molar ratio of 2:2:2:2:2:1.5:0.23 (Fig. 1). In contrast to the rough variants, all investigated smooth ones synthesize this compound. The ninhydrin reaction was negative, whereas the reaction with orcinol was positive. This indicated that the lipid contains no free amino group but instead carries a sugar moiety. We therefore tentatively concluded that the isolated compound is a glycolipid, which was named gordonin. For structural analysis we used the glycolipid of the strain 1775/15s, which was confirmed to be G. hydrophobica by DNA-DNA hybridization with G. hydrophobica DSM 44015 and that was the object of further physiological investigations.\(^2\)

NMR Spectroscopy

\(^1\)H and \(^13\)C NMR spectra were obtained with a Bruker AM-360 spectrometer for solutions in Me\(_2\)SO-\(_d_6\) containing 2% D\(_2\)O at room temperature referenced to dimethyl sulfoxide (H 2.49, C 39.5). For the detection of NH protons and correlated signals, additional one-dimensional and two-dimensional \(^1\)H spectra were run containing 2% water (by volume) under the same conditions. Standard Bruker software was used for two-dimensional COSY, relayed COSY, homo- and heteronuclear correlated experiments.

RESULTS

Identification, Isolation, and Purification of Gordonin

A systematic analysis of complex lipids in G. hydrophobica 1775/15s and G. hydrophobica 1775/15r according to the method of Dobson et al. (16) revealed that an additional compound was present in the fraction of the most polar lipids of the smooth variants compared with that of the rough ones. The less polar lipids were identical between smooth and rough variants (data not shown). In TLC analysis this compound showed an \( R_f \) value of 0.23 (Fig. 1). In contrast to the rough variants, all investigated smooth ones synthesize this compound. The ninhydrin reaction was negative, whereas the reaction with orcinol was positive. This indicated that the lipid contains no free amino group but instead carries a sugar moiety. We therefore tentatively concluded that the isolated compound is a glycolipid, which was named gordonin. For structural analysis we used the glycolipid of the strain 1775/15s, which was confirmed to be G. hydrophobica by DNA-DNA hybridization with G. hydrophobica DSM 44015 and that was the object of further physiological investigations.\(^2\)

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Amino Acid Analysis

After strong hydrolysis and subsequent peracetylation amino acid analysis the amino acids alanine, leucine, threonine, glycine, valine, phenylalanine, and serine could be identified in a molar ratio of 1:1:1:2:2:2:4, respectively (Table I). The determination of the absolute configuration of the amino acids was achieved by separation of diastereomeric \(N\)-(2,4-dinitro-5-fluorophenyl)-l-alaninamide-amino acid derivatives by HPLC (data not shown)
and showed that only leucine and alanine were present in the \(L\) configuration.

**Fatty Acid Analysis**

The fatty acids were isolated, methylated, and analyzed by GC/MS. Two main peaks were detected. One of them belonged to a saturated 3-OH fatty acid since its basal peak at \(m/z \ 103\) originated from \([\text{CH(OH)}\text{CH}_{2}\text{COOCH}_{3}]^{-}\). The other one was an unsaturated 3-OH fatty acid with a significant peak at \(m/z \ 103\) and a basal peak at \(m/z \ 55\) due to \([\text{HC} \text{H}_{2}\text{CH}_{2}]^{-}\). The parental peaks of the methyl esters were difficult to detect by this ionization method and were only observed for the unsaturated fatty acid, but their molecular weight could be obtained from the peaks at \(m/z \ = \ M-50\), which are of significant height in the case of 3-OH fatty acids (17). The data indicated that the fatty acid fraction was a mixture of two components, 20:0 (3-OH) and 22:1 (3-OH) (Table I).

![Diagram of PSD fragment ion spectrum of prompt 1020 atomic mass unit precursor.](image_url)

**Isolation and Characterization of Part Structures of Gordonin**

**GC/MS Analysis of Val-20:0(3-OH) After Strong Methanolysis**—Using strong methanolysis and subsequent peracylation, GC/MS analysis revealed that valine was attached to 20:0 (3-OH) as a major compound. It was expected that ester-bound valine would not have resisted the strong methanolysis conditions applied (2 \(M\) HCl/MeOH, 85 °C, 2 h). As judged by GLC/MS analysis it was difficult to decide whether valine is ester- or amide-linked to the 20:0 (3-OH), since both derivatives exhibit identical molecular weights. To solve this problem, the peracetylated derivatives \([R]-3\)-hydroxy-tetradecanamido-D-valine methyl ester (2) and \([R]-3-O-[2-N-(acetamido)-D-valinooxy]-tetradecanoic acid methyl ester (3) have been synthesized whereby the \(R\)-3-hydroxymyristic acid (14:0 (3-OH)) was used instead of 3-hydroxyeicosanoic acid (20:0 (3-OH)), since it was the only \(R\)-configured 3-hydroxylated fatty acid available.

As compared with GC/MS analysis, compounds 2 and 3 displayed in the electron impact-mass spectrum characteristic fragment ions for either the amide- or the ester-linked valine to the \(\beta\)-hydroxylated fatty acid (compounds 2 and 3, Figs. 2, a and b). Comparing the electron impact-mass spectra of 2 and 3 with that of the compound isolated and derivatized in a similar way from gordonin, it was unequivocally proven that valine is ester linked to the \(\beta\)-hydroxy group of the \(\beta\)-hydroxylated fatty acid (Fig. 2c). Therefore, the valine residue in the intact gordonin is either amino-terminally blocked or gordonin is a cyclic depsipeptide, because it is ninhydrin-negative.

**Amino Acid Sequence Analysis**—To determine the amino acid sequence of gordonin, the compound was partially hydrolyzed. After separation on C18 reversed-phase HPLC, resulting
fragments were analyzed by Edman degradation. The separation pattern of partially hydrolyzed peptides (a–f) and their amino acid sequences are shown in Fig. 3. Edman degradation of the other fragments has been unsuccessful because of amino-terminal blocking. Hydrolysis, trifluoracetylation, and GC/MS analysis revealed that besides the monomeric amino acids, dimeric fragments being composed of Thr-Gly, Gly-Val, Val-Ser, Leu-Val, Ser-Phe, Phe-Ser, and Phe-Val and also a trimeric Ser-Ser-Ala fragment could be found (Fig. 4).

**Determination of Molecular Weight by TOF-SIMS**

The molecular weight of gordonin was determined by TOF-SIMS. This analysis further revealed that gordonin is not homogeneous (Fig. 5). Besides two main masses with m/z 1711 and 1737, additional masses with m/z 1745, 1755, and 1771 were detected. The assignment of the signals shown in Fig. 5 could be confirmed by thin layer chromatography and thin layer chromatography-SIMS (18) of gordonin. The main molecule (gordonin) with m/z 1711 could only be explained when 15 mol of water per molecule of gordonin are released (Table I). The same holds true for the gordonin homologue (m/z 1737) containing the 22:1 (3-OH) fatty acid. This argues strongly in favor of a cyclic structure.

**MALDI and MALDI-PSD Analysis**

To obtain the amino acid sequence of gordonin, MALDI and MALDI-PSD mass analysis were employed. This could be best achieved after mild alkaline treatment of the cyclic gordonin molecule and its homologues with sodium methylate and subsequent methylation, by which the cyclic lactone ring was transferred to the linear methyl ester, and the glucose was released from the molecule. The derivatives, although complex in structure, were suitable for MALDI and MALDI-PSD time-of-flight mass analysis. The MALDI spectra contained a large variety of parent ions up to the expected mass range, where abundant parent ion signals could be recorded at 1254 and 1286 atomic mass units, respectively. However, both precursors did not match the expected molecular masses based on the amino acid analysis by −18 atomic mass units.

On a 1020 atomic mass unit parent ion, a MALDI-PSD fragment, an ion analysis was performed (Fig. 6), which unambiguously confirmed (by sets of consecutive b_n and y_m signals) the presence of two carboxy-terminal sequence ladders: GLVSFGV-OCH_3 and GFVSFGV-OCH_3. Since the mass increment of 83 atomic mass units between y_7 and y_8 cannot represent any standard amino acid, a dehydrothreonine (101 − 18 atomic mass units) was suggested for this position. This assumption was further corroborated by the −18 atomic mass unit differences between the calculated and measured masses of the full stretch precursor ions. Alkaline treatment of gordonin causes obviously detachment of glucose by β-elimination. This observation supports the notion that the Glc residue was attached to Thr and not to any other of the putative hydroxyl groups in the four serine residues (compare also NMR analysis below).

Subsequent analysis of the full prompt precursor ion spectra (data not shown) revealed that the missing amino-terminal stretch had the following sequences SFSSAT(−18)GL and SFSSAT(−18)GF. Based on these sets of complementary information and in full agreement with the sequence information derived from partial hydrolysis (see above), Sequence 1 as determined by MALDI and MALDI-PSD-MS could be compiled as shown.

**NMR Spectrometric Analysis**

In the 1H NMR spectrum of gordonin (Fig. 7) one characteristic doublet for the anomeric proton of a hexose (H-1, δ 4.22...
ppm) was present at high field with a high coupling constant \( J_{1,2} 7.2 \text{ Hz} \) characteristic for \( \beta \)-anomeric-linked pyranoses. Although the other signals from the glucose were not resolved completely, they could be assigned following the contour plot in the two-dimensional COSY spectrum to be a \( \beta \)-glycosidically linked Glc residue. Adding traces of water to the sample, NH signals of the amino acids were detected thus providing further help for the assignment of the various characteristic H-2 proton signals of the 13 amino acids. Therefore, the \( ^{13} \text{C} \) NMR spectrum as well as the distortionless-enhancement by polarization-transfer (DEPT) spectrum (Fig. 8, a and b) could be completely assigned by the help of the \( ^{13} \text{C}, ^{1} \text{H} \) COSY.

The \( ^{13} \text{C} \) NMR spectrum contained 14 carbonyl carbons, including those for 13 amino acids and 1 for a fatty acid (Table II). Characteristic signals for a \( \beta \)-linked terminal pyranosidic glucose residue were also identified. From the low field shift of the C-3Thr signal (\( \delta 72.73 \) versus \( 67.1 \text{ ppm} \); Ref. 19) it was deduced that Glc was linked to C-3 of Thr.

The linkages of 20:0 (3-OH) in the cyclic gordonin were investigated with the help of two synthetic reference com-

![Fig. 8. \( ^{13} \text{C} \) NMR spectrum (a) and \( ^{13} \text{C} \) DEPT spectrum (b) of gordonin.](image)

### Table II

\( ^{13} \text{C} \) NMR chemical shifts of gordonin

| Component | C-1 (ppm) | C-2 (ppm) | C-3 (ppm) | C-4 (ppm) | C-5 (ppm) | C-6 (ppm) | C-7 (ppm) |
|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| Amino acids |           |           |           |           |           |           |           |
| Ala       | 172.98    | 48.80     | 16.36     |           |           |           |           |
| Gly       |           |           |           |           |           |           |           |
| I         | 170.30    | 42.37     |           |           |           |           |           |
| II        | 170.11    | 41.73     |           |           |           |           |           |
| Leu       | 170.30    | 50.86     | 41.38     | 24.22     | 21.77     | 23.22     |           |
| Phe       |           |           |           |           |           |           |           |
| I         | 171.03    | 54.18     | 37.60     | 137.79    | 128.08    | 126.26    | 129.39    |
| II        | 170.79    | 53.96     | 37.60     | 137.63    | 128.08    | 126.28    | 129.11    |
| Ser       |           |           |           |           |           |           |           |
| I         | 169.85    | 55.02     | 62.31     |           |           |           |           |
| II        | 169.72    | 54.93     | 62.02     |           |           |           |           |
| III       | 169.49    | 54.90     | 62.02     |           |           |           |           |
| IV        | 169.23    | 54.83     | 61.89     |           |           |           |           |
| Thr       | 170.53    | 57.51     | 72.75     | 18.43     |           |           |           |
| Val       |           |           |           |           |           |           |           |
| I         | 171.16    | 58.31     | 31.00     | 18.30     | 19.18     |           |           |
| II        | 171.16    | 58.09     | 29.71     | 18.27     | 19.03     |           |           |
| Sugar     |           |           |           |           |           |           |           |
| \( \beta \)-Glc \(_p\) | 100.45    | 73.29     | 76.97     | 70.21     | 76.48     | 61.35     |           |
| Fatty acid |           |           |           |           |           |           |           |
| 20:0 (3-OH) | 168.53   | 40.70     | 71.94     | 36.50     | 29.80     | 24.67/24.22 | 31.40     | 22.21     | 14.05     |

\(^a\) Assignment could be interchanged.
\(^b\) Other signals derived from 22:1 (3-OR): \(-\text{CH=CH}–, 129.74\) and \(\text{CH}_2\text{CH=CH}–, 26.65 \text{ ppm}\).
pounds [(R)-3-hydroxytetradecanoylamide]-d-valine methyl ester (2) and [2-N-(acetamido)-d-valinamido)-(R)-3-hydroxytetradecanoyl amide methyl ester (3). The $^{13}$C NMR of the synthetic compound 3 was very similar to that of gordonin (1) (Table III) but different to that of compound 2 (data not shown). These data support the result of the GC/MS analysis and are also in good agreement with the data from the MALDI-PSD analysis of the linear molecule (see above).

In conclusion, chemical analysis, MALDI-TOF-mass spectrometry, and NMR spectroscopy revealed that gordonin is a cyclic tridecapeptidolipid carrying a fatty acid (20:0 (3-OR)) as part of the ring structure and a pyranosidic Glc in $\beta$-anomeric linkage attached to Thr (1). Besides gordonin (1) (Fig. 9), at least three additional GPL molecules were identified being structurally related to gordonin. These molecules carry either l-Phe instead of d-Leu or 22:1 (3-OR) instead of 20:0 (3-OR).

**DISCUSSION**

The plasma membrane of mycobacteria and related actinomycetes like *Rhodococcus, Corynebacterium, Nocardia*, and *Gordonia* is not very different from that of the plasma membrane of other bacteria, but the cell envelope is very distinct (20–23). The peptidoglycan of mycobacteria and other *Nocardiaceae* is linked to characteristic arabinogalactan polysaccharides (24), which are esterified with high molecular weight mycic acids. A range of complex free lipids are associated with the mycelic acid matrix in the mycobacterial cell envelope. These complex free lipids are composed of very apolar lipids like dimycocerosates of the phtiocerol family and lipooligosaccharides. All of these free lipids contain fatty acids or long chain alcohols, which are structurally different from those constituting the hydrophobic section of the plasma membrane’s amphiphatic polar lipids. These long chain components are thought to intercalate with the mycic acid layer by hydrophobic interaction resulting in the exposition of the more polar groups of the free lipids to the hydrophilic exterior. Recently, Ortalo-Magné *et al.* (25) identified the surface-exposed lipids of several mycobacterial species. They isolated the lipids of *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Mycobacterium kansasi*, *Mycobacterium gastri*, *Mycobacterium smegmitis*, and *Mycobacterium aurum* by gently treating the cells with glass beads thereby demonstrating a selective location of classes of ubiquitous lipids on the surface of mycobacteria. Phosphatidylethanolamine and phosphatidylinositol mannosides were exposed in all species examined, whereas dimycoceryl trehalose (“cord factor”) was only found at the surface of *M. aurum*. Monomycoceryl trehaloses and triacylglycerides were exposed in *M. avium* and *M. smegmitis* but not in other mycobacterial species studied so far. Phenolic glycolipids, dimycocerosates of phtiocerols and lipooligosaccharides were identified at the surface of *M. tuberculosis* (Canetti), *M. kansasi*, and *M. gastri*, whereas glycopeptidolipids were identified in the outermost layer of *M. avium* and *M. smegmitis* (25). However, a glycosylated N-acyl peptide on the cell surface of *G. hydrophobica* has so far not been isolated as a constituent of a cell envelope.

N-Acyl peptides can be structurally divided into two classes: those having a linear peptide moiety like stenothricin (13, 26), fortuitin (27), and amphomyxin (28) and cyclic peptides. The latter group can be further subdivided in several subgroups depending on the functional groups of the molecule responsible for the ring formation such as lactones and lactams. The lactones can be further subdivided depending on the involvement of the hydroxyl group of the fatty acid in the ring formation or not. Finally, also glycosides of cyclic peptidolipids have been found. A comprehensive synopsis of bacterial lipids containing amino acids or peptides linked by amide bonds is given by Asselineau (29).

Gordonin described here was found to be a glycoside of an N-acyltridecapeptide containing a hydroxy fatty acid thus forming a cyclic lactone ring structure. The glucose is bound to $\alpha$-acyl-L-threonine, similar to one of the four most polar peptidolipids of *Rhodococcus erythropolis* isolated by Koronelli (30). In two other polar peptidolipids glucose is bound to serine, and the fourth is lacking glucose. Furthermore, these peptidolipids contain 1 mol of a normal chain fatty acid and 1 mol of a mycolic acid residue. In addition to this, Koronelli (30) found a different amino acid pattern compared with gordonin. A cyclic structure was only found for one of the three apolar peptidolipids. The low polarity lipids are N-acyl-t-threonyl-t-valyl-t-valyl-t-leucinolide (I), N-acyl-t-(O-acyl)-threonyl-t-valyl-t-valyl-t-leucinolide (II), and N-acyl-t-(O-mycocetyl)-threonyl-t-valyl-t-valyl-t-leucinolide (III). In all cases the N-acyl units and the O-acyl unit in lipid II are moieties of $C_{20}$, $C_{22}$, $C_{24}$, $C_{26}$, and $C_{28}$ saturated and monoenoic acids.

Aydin *et al.* (8) isolated two antimicrobial agents from *Erwinia herbicola* called herbicolin A and herbicolin B. Both

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**Table III**

$^{13}$C NMR chemical shifts of gordonin compared with part structure 3

| Component | C-1 | C-2 | C-3 | C-4 | C-5 | C-6/C-17 | C-18 | C-19 | C-20 |
|-----------|-----|-----|-----|-----|-----|----------|------|------|------|
| Val       |     |     |     |     |     |          |      |      |      |
| Gordonin  |     |     |     |     |     |          |      |      |      |
| I         |  171.16$^a$ |  58.31 |  31.00 |  18.30 |  19.18 |          |      |      |      |
| II        |  171.16$^a$ |  58.09 |  29.71 |  18.27 |  19.03 |          |      |      |      |
| 3         |  171.03 |  57.53 |  31.37 |  18.03 |  18.96 |          |      |      |      |

**3-Hydroxy fatty acids$^a$**

| Gordonin | 20:0 (3-OR) | 168.53$^a$ | 40.70 | 71.94 | 36.50 | 29.80 | 24.67/24.22 | 31.40 | 22.21 | 14.05 |
|-----------|-------------|-----------|------|------|------|------|-------------|------|------|------|
| 3         | 14:0 (3-OR) | 170.63 | 38.23 | 70.92 | 33.10 | 29.06 | 24.35/24.10 | 29.61 | 22.16 | 14.03 |

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$^a$ 20:0 (3-OR) in gordonin and 14:0 (3-OR) in 3 are compared.
compounds have the same fatty acid and amino acid composition with the following sequence: dehydro-Abu-\(L\)-Thr-\(d\)-allo-Thr-\(d\)-Leu-Gly-\(d\)-Gln-Gly-N-Me-\(L\)-allo-Thr-L-Arg (dehydro-Abu is 2,3-dehydro-\(\alpha\)-aminobutyric acid). This peptide is \(N\)-acylated by a 3-hydroxytetradecanoic acid. It is interesting to note that only herbicolin A carries \(d\)-glucose. This carbohydrate residue is linked in an \(\alpha\)-glycosidic bond to the hydroxy group of the 3-hydroxytetradecanoic acid. The carboxyl-terminal arginine residue forms a lactone ring with the hydroxy group of \(L\)-threonine.

Globomycin and surfactin are well-known \(N\)-acyl peptides containing a hydroxy fatty acid as part of a lactone ring (9, 10). Globomycin exhibits antibiotic activity against Gram-negative bacteria affecting cell wall synthesis. Surfactin was isolated and characterized by Arima \textit{et al.} (10). They found that this bacterial peptidolipid remarkably extents the time necessary for fibrin clot formation by inhibiting the conversion of fibrin monomers to fibrin polymers. In addition, surfactin was found to lower the surface tension of 0.1 M NaHCO\(_3\) from 71.6 to 27.0 millinewton/m. An even better surface activity was found for arthrofactin, a lipopeptide biosurfactant isolated from \textit{Arthrobacter} species strain MIS38 (31). The minimum surface tension of arthrofactin was 24 millinewton/m above the critical micelle concentration.

The amphiphilic character of many peptidolipids give rise to several biological functions of these molecules like hemolytic properties, antibiotic activities, or enzyme-inhibiting properties (29). The amphiphilic nature of gordonin is caused by the 20:0 (3-OH) fatty acid and several hydrophobic amino acids clustered at one side of the proposed structure (Fig. 9), and the glucose and several \(L\)-serine residues, which are located on the opposite side. Therefore, gordonin is able to intercalate with the very hydrophobic mycolic acid layer of the cell wall by the hydrophobic tail of the 3-hydroxy fatty acid and to expose the hydrophilic head of the molecule to the exterior to make the cell surface more hydrophilic. This hydrophilic cell surface enables the smooth variants of \textit{G. hydrophobica} to grow in suspension in liquid culture. In contrast, the rough variants, lacking gordonin, grow in large flocks resulting in anaerobic microenvironments. This was shown by demonstrating that the rough variants in contrast to the smooth ones show nitrate dissimilation despite aerobic culture conditions (32).

It is also conceivable that gordonin is partially released to the environment during growth to dissolve hydrophobic substrates. Therefore, gordonin could act as a surfactant, like surfactin or arthrofactin, and this would allow \textit{G. hydrophobica} to use pollutants of the animal-rendering plant waste gas as carbon source, which are hardly soluble in water. Currently, we are investigating if gordonin is able to reduce interfacial tension between air and water or water and organic solvents, respectively.

**Fig. 9.** Proposed structure of gordonin (I). The heterogeneity in the other GPL structures related to gordonin comprises 22:1 (3-OH) instead of 20:0 (3-OH) as well as \(L\)-Phe instead of \(d\)-Leu.
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