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# Chemical structure of glycosphingolipids isolated from *Sphingomonas paucimobilis*

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Two novel glycosphingolipids were isolated from *Sphingomonas paucimobilis* and their structures were completely elucidated. The glycosyl portion of the glycosphingolipid consists of an α-D-Man*p*-[1→2)-α-D-Gal*p*-(1→6)-α-D-Glc*pN*-(1→4)-α-D-Glc*p*A-R tetrasaccharide. The hydrophobic residue R was found to be heterogeneous with respect to the dihydrosphingosine residue. *Erythro*-1,3-dihydroxy-2-amino-octadecane and *erythro*-1,3-dihydroxy-2-amino-*cis*-13,14-methyleneoctadecane were identified in comparable amounts. Both dihydrosphingosine derivatives were quantitatively substituted by an (*S*)-2-hydroxymyristic acid in amide linkage.

Chemical structure; Glycosphingolipid; Dihydrosphingosine; Lipopolysaccharide; *Sphingomonas paucimobilis*

## 1. INTRODUCTION

*Sphingomonas paucimobilis* (formerly *Pseudomonas paucimobilis*) is a strictly aerobic, yellow-pigmented and non-fermentative Gram-negative rod [1]. The new genus name *Sphingomonas* was recently proposed for this bacterium [2] because many taxonomical data have been accumulated which distinguished it from typical *Pseudomonas* species. Gram-negative bacteria, in general, carry an endotoxin in the outer leaflet of the outer membrane lipopolysaccharide (LPS), which is of considerable biomedical interest [3]. Earlier studies directed towards the extraction of LPS from *P. paucimobilis* by the phenol/water or the phenol/chloroform/petroleum ether (PCP) procedure [4], were unsuccessful. Instead, a glycolipid was obtained with unexpected and unusual structural features. Although this 'lipid A-type' glycolipid carried the expected D-glucosamine (Glc*p*N), (*R*)-3-hydroxylated (long chain) fatty acids were absent. Instead, a 2-hydroxylated myristic acid, 14:0(2-OH) in amide linkage [4] was identified in amounts expected for those of (*R*)-3-hydroxylated fatty acids usually present in lipid A. This unexpected finding led us to assume that *S. paucimobilis* harbors an unusual lipid A (and probably also an unusual LPS) giving further evidence for the revised taxonomical classification of *S. paucimobilis*. In this paper, we describe the purification and complete structural analysis of two new glycosphingolipids isolated from *S. paucimobilis*.

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## 2. MATERIALS AND METHODS

### 2.1. *Bacterial strain and culture condition*

*S. paucimobilis* IAM12576 (originally NCTC11030, type strain) was grown as described [4].

### 2.2. *Extraction of glycosphingolipid*

Glycosphingolipids were extracted with chloroform/methanol (C/M 1:3, v/v) at 80°C for 1 h under reflux from the material obtained after C/M 2:1 extraction. The resulting lipids were successively eluted from the silica gel column by the stepwise increase in methanol content from a C/M vol. ratio of 2:1 to 1:3.

### 2.3. *Methylation analysis and chromatographic procedures*

Methylation analysis was carried out as described [5]. TLC was carried out on silica 60 plates (Merck) with chloroform/methanol/acetic acid/water (25:15:4:2, v/v) and high-voltage paper electrophoresis (HVPE) was done as described in [5,6].

### 2.4. *Analysis of 3-hydroxylated fatty acids in whole cells*

Lyophilized cells (50 mg) were subjected to acidic methanolysis (2 M HCl, 65 h, 120°C) in the presence of heptadecanoic acid (17:0, 200 μg) and 2,2,3,4,4-pentadeutero-(*R*)-3-hydroxy-tetradecanoic acid (5 μg) which served as internal reference standards.

### 2.5. *Chemical analysis*

GlcN and neutral sugars were determined according to [7] and [8] as described in [5]. Fatty acids were quantified by GLC as methyl esters after hydrolysis (4 M HCl, 100°C, 5 h) and methylation (5% HCl/MeOH). Uronic acid was determined after hydrolysis with 1 M $H_2SO_4$ at 100°C according to [9].

### 2.6. *Determination of* S-*configuration of 14:0(2-OH), the* erythro-*configuration and the position of the cyclopropane ring in the dihydrosphingosines*

For the analysis of dihydrosphingosine(s), the sphingolipid (10 mg) was carboxyl-reduced (esterified, $CH_2N_2$ and reduced, $NaBH_4$) prior to acidic methanolysis (0.2 M HCl/MeOH, 65°C, 5 h). The dihydrosphingosines and fatty acid liberated were extracted with chloroform/water (3 × 5 ml). The organic layers were combined, dried ($Na_2SO_4$) and used for the following analyses; one aliquot of the methanolysate

was used for the determination of the *S*-configuration of 14:0(2-OH) according to [10]; for the determination of the *erythro/threo*-configuration another aliquot was per-*O*-acetylated ($Ac_2O$/pyridine, 1:1, 100°C); the position of the cyclopropane ring in the dihydrosphingosine was analyzed by dissolving the third aliquot (8/10) in chloroform (5 ml) and adding 2 mg of $Pb(OAc)_4$ under stirring. After 2 h, water (6 ml) and 8 M HCl (100 μl) were added, the product was extracted three times (chloroform/water), the dried aldehyde was reduced with $LiAlH_4$ (5 mg) in diethylether (2 ml) under stirring for 2 h at room temperature and the solvent was evaporated under a stream of nitrogen. Water was added and the alcohol extracted (chloroform/water), dried and derivatized to the nicotinyl ester for GLC analysis [11].

The reference compound *cis*-11,12-methyleneoctadecanoic acid methyl ester from L-α-phosphatidylethanolamine of *E. coli* (Type V, Sigma) served as a reference compound for NMR and GLC-MS analysis.

### 2.7. *Laser-desorption mass spectrometry and NMR analysis*

Laser desorption mass spectrometry (LD-MS) was performed under conditions described [12]. $^1H$ NMR and $^{13}C$ NMR spectra were recorded by a Bruker AM-360 spectrometer with the carboxyl-methylated ($CH_2N_2$)glycosphingolipid derivatives in $CDCl_3/CD_3OD/D_2O$ 3:1:0.1 (by vol.) at 35°C, using TMS as an internal standard (360.13 MHz for $^1H$, 90.5 MHz for $^{13}C$).

## 3. RESULTS

### 3.1. *Absence of LPS in* S. paucimobilis

As previously reported [4,13], 2-hydroxytetradecanoic acid, 14:0(2-OH) (93 nmol/mg), was the only hydroxyl fatty acid in the lyophilized bacterial cells. The glycolipid material obtained by phenol/water extraction [14] of *S. paucimobilis* contained Glc (317 nmol/mg), GlcN (56 nmol/mg), Man (64 nmol/mg), Gal (30 nmol/mg) and 14:0(2-OH) (45 nmol/mg), and was devoid of characteristic LPS components such as 3-hydroxy fatty acid, 3- deoxy-D-*manno*-2-octulosonic acid (KDO), or L-*glycero*-D-*manno*-heptose (L,D-Hep). The PCP method [15] gave lower yields of glycolipid and neither 3-hydroxy fatty acid nor KDO or L,D-Hep were found.

### 3.2. *Chemical composition of the purified glycolipid*

Cellular lipids of *S. paucimobilis* obtained either by the phenol/water, PCP or by the C/M extraction procedure contained phospholipids and two major glycolipids. Upon TLC one glycolipid expressed higher $R_f$ value (0.39) which was later identified as the glucuronosyl ceramide already described [16] and the structure of which is now completely elaborated as a partial structure of **1** and **2** (Fig. 1). The more polar compound ($R_f$ = 0.07) corresponded to the unknown lipid reported earlier [4]. This lipid contained 14:0(2-OH), GlcN, Man, and Gal in approximately equimolar amounts. Uronic acid was also detected (0.38 μmol/mg). Methanolysis of the carboxyl-reduced sphingolipid revealed 14:0(2-OH) and two slightly separated ninhydrin-positive substances ($R_f$ = 0.7–0.8) with $R_f$-value similar to that of *erythro*- and *threo*-dihydrosphingosine ($R_f$ = 0.78). Analysis of the (*R*)- and (*S*)-configuration of the (*L*)-phenylethylamide derivative of 14:0(2-OH) by GLC revealed that the (*S*)-configuration was exclusively

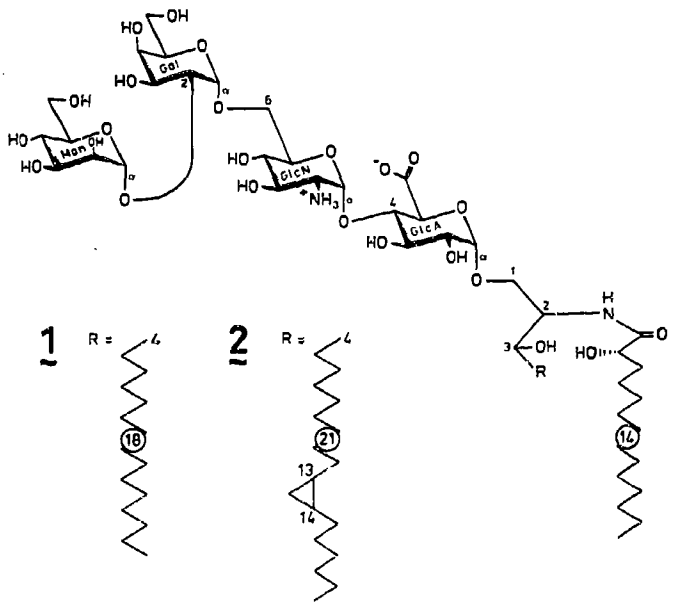


Fig. 1. Chemical structure of the two major glycosphingolipids isolated from *S. paucimobilis* IAM12576.

present. Upon GLC-MS, the dihydrosphingosines were identified as *erythro*-2-amino-1,3-dihydroxy-octadecane. A dihydrosphingosine homologue with a molecular mass of 40 kDa higher was also detected (see below).

### 3.3. *Methylation analysis of oligosaccharide*

Strong acid hydrolysis of the glycolipid (4 M HCl, 100°C, 5 h) and preparative HVPE revealed an unknown ninhydrin-positive substance ($M_{GlcN}$ = 0.2) which was *N*-acetylated, reduced, and permethylated. The molecular weight of the permethylated disaccharide was $M_r$ = 526 (CI-MS) and its EI-MS spectrum gave fragment ions (*m/z* = 250 and 260→228) of a reduced and permethylated $Hex_pNAc$-HexA disaccharide. For the identification of the hexuronic acid, the permethylated disaccharide was carboxyl-reduced ($NaBH_4$), hydrolyzed and acetylated. Characteristic fragments (*m/z* = 233, 201, 134, 117) and the comparison of retention times on GLC-MS analysis with authentic standards revealed 4,6-di-*O*-acetyl-1,2,3,5-tetra-*O*-methylglucitol. Therefore, the disaccharide released from the glycolipid was identified as D-Glc*p*N-(1→4)-D-Glc*p*A.

The hydrazinolysate of the glycolipid was subjected to methylation analysis. GLC-MS analysis revealed 1,5- di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol, 1,2,5-tri-*O*-acetyl-3,4,5-tri-*O*-methyl-D-galactitol and 1,5,6-tri-*O*-acetyl-2-deoxy-3,4-di-*O*-methyl-2-(*N*-methylacetamido)-D-glucitol indicating D-Man*p* as terminal sugar, and →2)-D-Gal*p* as well as →6)-D-Glc*p*N as sugar components of the glycosphingolipid.

The glycolipid was permethylated [17] and the product directly applied to GLC-MS. In this way one trisaccharide ($M_r$ = 655) was identified by means of charac-

teristic fragments (*m/z* = 219, 187) which were assigned to a terminal Man*p*, and GlcN (*m/z* = 232) at the reducing end. Therefore, the structure of the trisaccharide was deduced to be Man*p*-Gal*p*-GlcN(Me)$_2$.

### 3.4. *Anomeric configuration of glycosyl residues*

Four distinct signals of anomeric protons were recorded by $^1$H NMR analysis (Table I). The scarcely resolved broad doublet at 5.03 ppm ($J_{1,2}\approx$1.5 Hz) was assigned to H-1 of Man*p*. The coupling constants ($J_{1,2}$) of the other anomeric protons were found in the range of 3–4 Hz indicating α-configuration. The H-1 signals were assigned on the basis of the characteristic cross-peaks in the $^1$H,$^1$H- and $^1$H,$^{13}$C-COSY spectra. The sphingolipid described [19] served as a reference compound expressing only one anomeric proton signal at 4.80 ppm with $J_{1,2}$ = 3.7 Hz. From this result we could assign the signal 4.87 to H-1 of GlcA. Consequently the signal at 5.21 was assigned to H-1 of Gal. Anomeric C-1 signals of $^{13}$C-NMR were assigned based on the $^1$H- and $^{13}$C-COSY spectrum (Table I). Heteronuclear coupling constants ($^1J_{H,C}$) were found in the range from 170 to 176 ppm (Table I) indicating that all sugars including Man*p* had α-configuration and further suggesting that GlcA was α-glycosidically linked to the lipid (dihydrosphingosine) region.

### 3.5. *Laser-desorption/mass analysis*

The LD-mass spectrum with $Cs^+$ as cation contained only two abundant ion signals which were assigned to two quasimolecular ions $[M+2Cs]^+$ with *m/z* = 1455 and 1495, respectively, and thus two molecular species with $M_r$ of 1189 ($C_{56}O_{24}N_2H_{104}$) and 1229 ($C_{59}O_{24}N_2H_{108}$), indicating structural heterogeneity of the glycosphingolipid. The LD-mass spectrum in the presence of $Na^+$ contained, besides the two quasimolecular ions $[M+2NA]^+$ at *m/z* = 1235 and 1275, characteristic ions lacking the Man-Gal-GlcN trisaccharide at *m/z* = 749 and 789 (each carrying two $Na^+$) and lacking a Man-Gal-GlcN-GlcA tetrasaccharide at *m/z* = 551 and 591 (each carrying one $Na^+$), the latter representing the lipid moiety of the glycosphingolipid.

Table I

Selected $^1$H and $^{13}$C NMR data (relative to TMS) and coupling constants obtained from the anomeric regions of the carboxymethylated *S. paucimobilis* glycosphingolipid (35°C; $CDCl_3/CD_3OD/D_2O$, 3:1:0.1, by vol.)

| Anomeric signal of | Chemical shift and coupling constants obtained with | | | |
|---|---|---|---|---|
| | $^1$H | | $^{13}$C | |
| | ppm | $J_{1,2}$(Hz) | ppm | $J_{C,H}$(Hz) |
| Glc*p*N | 5.73 | 3.7 | 95.41 | 175.31 |
| Gal*p* | 5.21 | 3.1 | 96.67 | 170.93 |
| Man*p* | 5.03 | ≈1.5 | 97.63 | 170.70 |
| Glc*p*A | 4.87 | 3.7 | 99.67 | 170.44 |

### 3.6. *Analysis of the lipid portion*

GC-MS analysis of the peracetylated dihydrosphingosines revealed a mixture of *erythro*-2-acetamido-1,3-di-*O*-acetyl-dihydroxy-octadecane [$M_r$ = 427, $[M + H]^+$ = 428, CI-[ammonia]-mode) and a homologue derivative with comparable EI-mass spectrum but with a $M_r$ = 467 of 40 kDa higher ([M + H] = 468) being present in almost comparable amounts.

The $^{13}$C NMR analysis (DEPT-mode) of the native glycosphingolipid revealed a methylene carbon signal (11.28 ppm) and two overlapping methine signals (16.25 ppm) which were assigned to a cyclopropyl residue in the dihydrosphingosine. These were identical to those signals derived from 11,12-*cis*-methylene-octadecanoic acid methyl ester isolated from *E. coli* which served as a reference compound. Heteronuclear $^1$H,$^{13}$C NMR revealed two cross-peaks to the $^1$H NMR methylene signal at 0.56 ppm (>$CH_{2a}$) and −0.34 ppm (>$CH_{2b}$) with $^1J$ = −4.1 Hz characteristic for geminal protons in cyclopropyl residues.

In order to localize the cyclopropyl residue and to determine its configuration (*cis* or *trans*) the nicotinyl ester of the alcohol derived from the dihydrosphingosine was analyzed. Upon GLC and EI-MS it was found identical as compared with the nicotinyl ester of *cis*-11,12-methyleneoctadecanol isolated from the L-α-phosphatidylethanolamine of *E. coli* (diagnostic fragment at *m/z* = 275). This result shows that in the parent dihydrosphingosine the cyclopropyl ring is located between carbon atoms 13 and 14. Moreover, from the identical retention time in GLC analysis of the nicotinyl ester of *cis*-11,12-methyleneoctadecanol found for both derivatives isolated either from *E. coli* or *S. paucimobilis*, as well as from the identical chemical shifts and coupling constants in the $^1$H and $^{13}$C NMR spectra, it can be deduced that also in **2** (Fig. 1) the configuration of the cyclopropyl ring is *cis*.

As a summary of the data described in the present study, the chemical structure of the glycosphingolipid is proposed as shown in Fig. 1.

## 4. DISCUSSION

Glycosphingolipids are ubiquitous components of the plasma membrane of eukaryotic cells. They are located in the outer leaflet of the membrane and function in cellular interactions, differentiation, and the immune response [18]. Glycosphingolipids are usually not found in prokaryotic cells. The only exception so far known is a glucuronosyl ceramide from *Flavobacterium devorans* ATCC10829 [16]. This bacterium was classified into the new genus *Sphingomonas* and Yabuuchi et al. postulated that glucuronosyl ceramide is commonly

contained in bacteria of this genus as one of the cellular lipid components [2].

Similar to the plasma membrane of eukaryotic cells, the outer membrane of Gram-negative bacteria also contains various amphiphilic molecules, an essential one being LPS [3]. However, *Sphingomonas paucimobilis* (type species) and other bacteria of the genus *Sphingomonas* were reported to lack 3-hydroxy fatty acids [2] and therefore they are likely to lack a typical LPS.

In this study we show that *S. paucimobilis* IAM12576 (type strain) contained cellular lipids differing significantly from other Gram-negative bacteria. The most hydrophilic lipid, previously assumed to be a 'lipid A-type lipid' [4], is now proven to represent a glycolipid and to be composed of an equimolar mixture of **1** and **2** (Fig. 1), the structure of which was established. Based on the amounts of the glycophospholipids present we propose that the outer membrane of *S. paucimobilis* is constructed from phospholipids and glycosphingolipids in which glycosphingolipids are playing a similar role as LPS of other Gram-negative bacteria. Two kinds of physicochemical and biochemical similarities between LPS and the bacterial glycosphingolipids deserve attention. Firstly, like the core-lipid A region (KDO and lipid A), the glycosphingolipids have an α-linked negatively-charged pyranosidic glycosyl component adjacent to the lipid portion. Secondly, like rough (R)-type LPS (Re through Ra), they also express a variation of the sugar portion in both molecules. We studied also the glucuronosyl ceramide [16] and found that it possesses an identical structure to the glycosphingolipid described here (ceramide and GlcA moiety). Therefore, these lipids can be considered in analogy to R-type LPS.

To our best knowledge, the present paper is the first report demonstrating that certain Gram-negative bacteria are devoid of LPS and have other amphiphilic molecules able to replace LPS in the membrane. Further work with emphasis on gaining deeper knowledge on the physicochemical and immunological properties of this molecule, as well as towards the taxonomical and evolutionary relationship of the genus *Sphingomonas* as compared with other genera of Gram-negative bacteria, is in progress.

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