Structural characterization of gentiobiosyl diglycerides from *Bacillus pumilus* associated with ascidia *Halocynthia aurantium*

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A mixture of 1(3),2-di-*O*-acyl-3(1)-*O*-β-gentiobiosylglycerols was isolated from a sea isolate of *Bacillus pumilus*. The components of the mixture were structurally characterized by mass spectrometry and ^1H and ^{13}C NMR spectroscopy data for the native compounds and their derivatives. The predominant component contains two C_{15} acyl groups, while the second component contains C_{15} and C_{17} fatty acids. Six minor components differ in residues of fatty acids and/or their combinations.

Key words: glycolipids, gentiobiosyl diglycerides, fatty acids, sea isolates of bacteria, *Bacillus pumilus*, ascidia *Halocynthia aurantium*.

In the last few years, it was shown that sea microorganisms are promising sources of new biologically active compounds.[1] In a search for surfactant-producing bacteria among sea microorganisms,[2] we found that a sea isolate of the bacterium *Bacillus pumilus* associated with the ascidia *Halocynthia aurantium* produces not only surface-active depsipeptides but also glycolipids. It is known that the glycolipids of many bacteria possess antigenic properties and seem to determine the serologic characteristics of a microorganism.[3] Among glycolipids, glycosyl diglycerides occur widely as membrane components of plant[4–6] and animal cells,[7,8] Gram-positive eubacteria and archaebacteria,[5,6,9,10] and, much less often, in Gram-negative bacteria.[5,10,11] The glycolipids of sea microorganisms have been studied insufficiently. The present work is devoted to isolation of a glycolipid fraction from a sea isolate of *B. pumilus* and structural determination of its components by chemical and spectroscopic methods.

Bacterial cells were extracted with a $CHCl_3$—MeOH mixture, the extract was concentrated *in vacuo*, and the residue was chromatographed on SiO_2. This treatment gave a fraction producing a color characteristic of glycolipids in a reaction with anisaldehyde (TLC, SiO_2). The ^1H NMR spectrum (in C_5D_5N) of a homogeneous (TLC data) glycolipid fraction (**1**) contains, along with signals for carbohydrate protons at δ 3.90—5.11, characteristic resonance signals for terminal CH_3 protons of fatty acids (δ 0.82—0.90), a broad intense singlet for methylene protons (δ 1.26), two overlapping triplets (δ 2.39, α-CH_2), a broad singlet (δ 1.67, β-CH_2), and a multiplet at δ 1.50 relating to the methine proton of an isobutyl fragment. The ^{13}C NMR spectrum exhibits signals for CO (δ 172.9 and 172.8) and those for terminal methyl groups (δ 11.4 and 19.3, *anteiso*-, and δ 22.7, *iso*-), which indicate the presence of two isomeric acyl fragments. The IR spectrum of fraction **1** contains absorption bands corresponding to OH (3650—3100 cm^{-1}); CH, CH_2, and Me (2926—2855 cm^{-1}); and ester stretching vibrations (1738 cm^{-1}); methylene bending vibrations (1466 cm^{-1}); and C—O vibrations (1168 cm^{-1}). All this corresponds to the IR spectra of glycoglycerolipids.[12] A low-intensity band at 900 cm^{-1} indicates the presence of β-glycoside bonds, and a minor absorption band at 725 cm^{-1} suggests the skeletal vibrations of a nonbranched CH_2 chain composed of at least four carbon atoms. Because no bands appear in the regions characteristic of sulfate or amide absorption, glycosphingolipids can be excluded from consideration.[13]

The products of acid hydrolysis of fraction **1** were analyzed by paper chromatography and GLC-MS in the form of polyol acetates; glucose and glycerol were identified. The absolute configuration of glucose (D) was established from determination of optic rotation; the equilibrium value of $[\alpha]_D^{20}$ is +51° (*c* 0.2, H_2O). Two signals for anomeric protons in the ^1H NMR spectrum of glycolipid **1** (Table 1) at δ 4.83 and 5.11 (J = 8.0 Hz) and the chemical shifts of anomeric C atoms in the ^{13}C NMR spectrum (δ 105.2 and 104.6) are typical of a β-glucoside bond.[14] Identification of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl- and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl glucitols, the products of full methylation of

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glycolipid **1** with subsequent acid hydrolysis, reduction with $NaBH_4$, and acetylation, indicates that the glucose fragments are linked by a 1→6-bond. The presence of the 1→6-glucoside bond finds additional support in ^1H NMR spectroscopic data (in $CDCl_3$ and C_5D_5N, see Table 1) for acetylated glycolipid (**1'**). Signals were assigned with the use of selective homonuclear $^1H\{^1H\}$ double resonance. In the ^1H NMR spectrum in $CDCl_3$, the positions of signals for the H(2')—H(6') and H(1")—H(6") protons are close to those of signals for the corresponding protons in the spectrum of acetylated gentiobiose.[15] The positions of signals for the terminal glucose fragment in the ^1H NMR spectrum of glycolipid **1'** in C_5D_5N are much like those in the spectrum of methyl β-D-glucopyranoside tetraacetate (**2**) (see Table 1). Moreover, NOEs were observed for the H(2"), H(3"), H(5"), and H(6a') protons upon irradiation of the H(1") proton (δ 5.10), whereas pre-irradiation of H(1') (δ 5.02) gave, along with NOEs for the signals of H(2'), H(3'), and H(5'), an NOE for the H(3a) glycerol proton. ^{13}C NMR spectroscopic data for glycolipid **1** (see Table 1) also indicate the presence of a 1→6-glucoside bond. Thus, signals for C(5') and C(6') are shifted from their positions in the spectrum of methyl β-D-glucopyranoside[16] by values corresponding to β- and α-effects of glycosylation at C(6').[17] Hence, the glycolipid contains β-gentiobiose linked to the C(3(1)) atom of glycerol. The ^1H NMR spectra of glycolipids **1** and **1'** in C_5D_5N (see Table 1) and the ^{13}C NMR spectrum of glycolipid **1** suggests that acylation occurs at the C(1(3)) and C(2) glycerol atoms (if C(6") had been acylated, a signal for this carbon atom would have been found at about δ 64.6[18]).

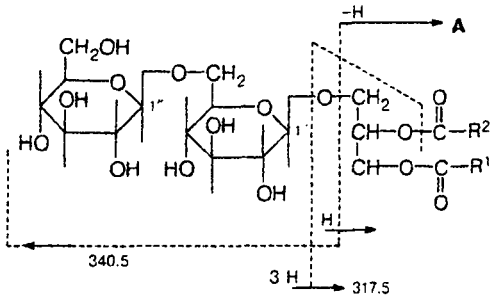
The FAB mass spectrum of glycolipid **1** exhibits peaks of cluster ions $[M + Na]^+$ at *m/z* 873.9, 887.9, 885.9, 901.9, 899.9, 915.9, 913.9, and 943.9. These data indicate that fraction **1** contains a mixture of biosyl diacylglycerols differing in fatty acid residues and/or their combinations. Basically, the mass spectra of fraction **1** is a series of ion peaks that differ from each other by two CH_2 groups and characterize two main glycolipids with molecular weights of 864 (51%, $C_{45}H_{84}O_{15}$) and 892 (21%, $C_{47}H_{88}O_{15}$). Thus, predominant diagnostic ion peaks with *m/z* 523.6 and 551.7 in FAB MS seem to be a result of breaking a glycoside bond linking disaccharide with glyceride. The same pair of peaks for subsidiary ions with *m/z* 522.7 and 550.8 (Scheme 1) is observed in MS EI, thus characterizing elimination of a disaccharide fragment with *m/z* 340.5.[19] An ion with *m/z* 317.5 is probably formed upon rupture of the C(1')—O bond, the protons being abstracted from the disaccharide $[299 + OH + H]^+$. Thus, high-molecular secondary ions correspond to the diacylglycerol fragment, which confirms ^1H and ^{13}C NMR spectroscopic

Table 1. ^1H and ^{13}C NMR spectral data for compounds **1**, **1'**, and **2**

Atom	^{13}C NMR, δ, **1** (C_5D_5N)	Proton	^1H NMR, δ (*J*/Hz)[a] **1** (C_5D_5N)	**1'** (C_5D_5N)	**1'** ($CDCl_3$)	**2** ($CDCl_3$)
C(1)	63.2 t	H(1a)	4.58 dd (7.0; 12.5)	4.48 dd (6.6; 12.1)	4.12 dd (6.1; 12.2)	
		H(1b)	4.75 dd (3.5; 12.5)	4.66 dd (3.4; 12.1)	4.30 dd (3.7; 12.2)	
C(2)	70.9 d[b]	H(2)	5.70 m	5.61 m	5.20 m	
C(3)	68.1 t	H(3a)	4.05 dd (6.0; 11.5)	4.02 dd (5.5; 11.0)	3.64 dd (5.8; 11.0)	
		H(3b)	4.46 dd (6.0; 11.5)	4.30 dd (5.2; 11.0)	3.97 dd (5.2; 11.0)	
C(1')	105.2 d[c]	H(1')	4.83 d (8.0)	5.02 d (7.9)	4.48 d (8.0)	
C(2')	75.0 d	H(2')		5.44 dd (7.9; 9.6)	4.93 dd (8.0; 9.8)	
C(3')	78.1 d	H(3')		5.71 t (9.6)	5.18 t (9.8)	
C(4')	71.7 d[c]	H(4')	4.87—3.90	5.38 t (9.6)	4.90 t (9.8)	
C(5')	77.1 d	H(5')		4.14 m	3.65 m	
C(6')	70.1 t	H(6a')		3.96 dd (6.4; 11.3)	3.62 m	
		H(6b')		4.26 dd (2.2; 11.3)	3.87 (br.d, 11.0)	
C(1")	104.6 d[c]	H(1")	5.11 d (8.0)	5.10 d (8.0)	4.58 (7.9)	4.83 d
C(2")	74.7 d	H(2")		5.50 dd (8.0; 9.5)	4.98 dd (7.9; 9.5)	5.46 dd
C(3")	78.1 d	H(3")		5.74 t (9.5)	5.19 t (9.5)	5.73 t
C(4")	71.5 d[c]	H(4")	4.87—3.90	5.50 t (9.5)	5.08 t (9.5)	5.51 t
C(5")	78.1 d	H(5")		4.14 m	3.71 m	4.10 m
C(6")	62.8 t	H(6a")		4.38 dd (3.1; 12.3)	4.13 dd (2.7; 12.2)	4.40 dd
		H(6b")		4.58 dd (4.7; 12.3)	4.28 dd (4.9; 12.2)	4.58 dd

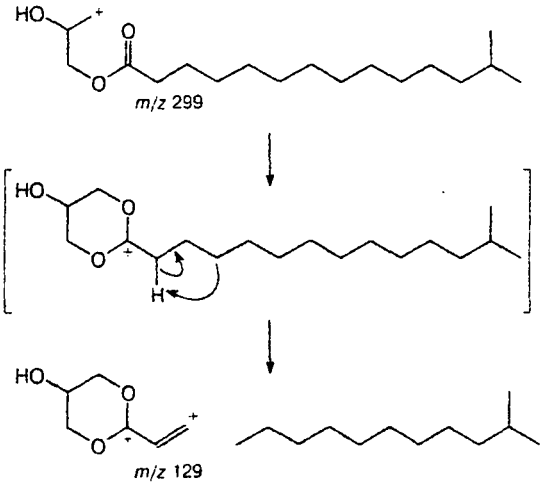
[a] Integral intensities of signals in ^1H NMR spectra correspond to the expected values for the given structures.
[b] The position of the signal was determined by $^{13}C\{^1H\}$ selective heteronuclear double resonance.
[c] Assignments of signals may be interchanged.

Scheme 1



A:	*m/z*	R^1	R^2
	522.7	$C_{14}H_{29}$	$C_{14}H_{29}$
	550.8	$C_{16}H_{33}$	$C_{14}H_{29}$
	508.7	$C_{13}H_{27}$	$C_{14}H_{29}$
	536.8	$C_{15}H_{31}$	$C_{14}H_{29}$

Scheme 2



data excluding acylated disaccharide. Minor ion peaks with *m/z* 508.7 and 536.8 in MS EI are the lowest and highest homologs of an ion with *m/z* 522.7, which differ from it by one CH_2 group.

The most intense peak in the EI and FAB mass spectra (*m/z* 299.4) can correspond to the monoacylated glycerol fragment containing pentadecanoic acid. Then, homologous ions with *m/z* 285.4, 313.4, and 327.4 have myristoyl (C_{14}), palmitoyl (C_{16}), and stearoyl (C_{17}) as an acyl fragment. This type of acylglyceride cations is well known in EI MS,[20] electrospray (ESI) MS,[21] and FAB MS/MS.[22] Scheme 2 illustrates a plausible way of fragmentation of an ion from *m/z* 299 to *m/z* 129. A similar mechanism was proposed for the fragmentation of triglycerides upon EI ionization.[23] An intense peak with *m/z* 225.3 in EI MS indicates the loss of a pentadecanoyl group. No other peaks of $[RCO]^+$ homologous ions are observed in the spectrum. The CI MS spectrum (NH_3) exhibits a moderate ion peak with *m/z* 558, probably, caused by breaking of the C(1′)—O bond $[523 + OH + NH_4]^+$ and a very intense peak with *m/z* 334.5 resulting from a subsequent detachment of the pentadecanoyl fragment $[558 - 225 + H]^+$. Homologous ions give minor peaks with *m/z* 362 and 376. An ion with *m/z* 318 seems to be derived from an ion with *m/z* 334 *via* elimination of oxygen. Therefore, the fragmentation of glycolipid **1** confirms that disaccharide is bonded to glycerol esterified with fatty acids. All mass-spectrometric data are indirect evidence that the C(2)—OH glycerol group is esterified with a C_{15} fatty acid, whereas the other acyl groups are at the C(1(3)) atom. An ion with *m/z* 915.9 corresponds to the presence of C_{15} and C_{17} fatty acids, while an ion with *m/z* 887.9 suggests two C_{15} fatty acids. Methyl esters of fatty acids obtained by acid hydrolysis of glycolipid **1** were identified by GLC to confirm the presence of C_{14}—C_{19} fatty acids (Table 2), branched C_{15} and C_{17} acids being predominant.

The absolute configuration of the glycerol part of glycolipids **1** was not determined. By analogy with other bacterial diglucosyl diglycerides, one may believe that gentiobiosyl diglycerides of *B. pumilus* are derivatives of 3-*O*-biosyl-*sn*-glycerol.[24]

Hence, the glycolipids isolated were assigned the structures of 1(3),2-di-*O*-acyl-3(1)-*O*-β-gentiobiosyl-glycerols (Scheme 3). Distribution of acyl fragments at the C(1(3)) and C(2) atoms requires a special study.

Previously, β-gentiobiosyl diglycerides were isolated and characterized as secondary metabolites of *Staphylococcus lactis* 13.[25] Their fatty-acid composition was not fully established and differs from that determined by us. Later,[3] related β-gentiobiosyl diglycerides produced by *Mycobacterium tuberculosis* were described. A comparison of FAB MS showed that their composition is also different from that of glycolipids in *Bacillus pumilus*. The fatty-acid composition of diglucosyl diglycerides in *Mycobacterium tuberculosis* is less diversified.

Although a β-(1→6)-glucoside bond is frequently present in other important nonlipid plant compounds, it

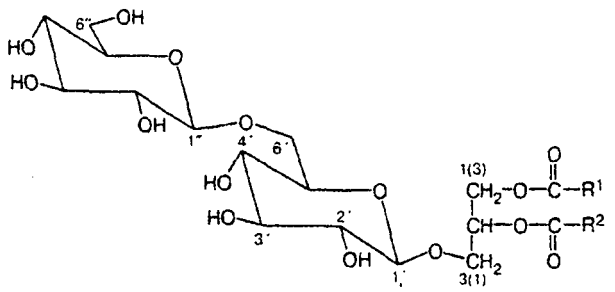
Table 2. Fatty-acid composition of diglucosyl diglycerides **1** from *Bacillus pumilus*

Acid[a]	Content[b] (%)	Acid[a]	Content[b] (%)
14 : 0 (*iso*)	0.9	16 : 1	0.8
14 : 0	1.3	17 : 0 (*iso*)	3.5
15 : 0 (*iso*)	30.6	17 : 0 (*anteiso*)	9.9
15 : 0 (*anteiso*)	39.9	17 : 1 (*iso*)	0.6
15 : 0	0.9	18 : 0 (*anteiso*)	1.3
16 : 0 (*iso*)	1.6	18 : 0	1.1
16 : 0 (*anteiso*)	1.1	18 : 1	0.3
16 : 0	4.7	19 : 0 (*anteiso*)	1.5

[a] Hydrocarbon chain length : number of double bonds in the chain.

[b] The content of fatty acid with respect to the overall fatty-acid fraction.

Scheme 3



$[M]^+$	*m/z*	R^1	R^2
$[M_1 + Na]^+$	873.9	$C_{13}H_{27}$	$C_{14}H_{29}$
$[M_2 + Na]^+$	887.9	$C_{14}H_{29}$	$C_{14}H_{29}$
$[M_3 + Na]^+$	885.9	$C_{13}H_{27}$	$C_{15}H_{29}$
$[M_4 + Na]^+$	901.9	$C_{15}H_{31}$	$C_{14}H_{29}$
		$C_{13}H_{27}$	$C_{16}H_{33}$
$[M_5 + Na]^+$	899.9	$C_{15}H_{29}$	$C_{14}H_{29}$
$[M_6 + Na]^+$	915.9	$C_{16}H_{33}$	$C_{14}H_{29}$
$[M_7 + Na]^+$	913.9	$C_{16}H_{31}$	$C_{14}H_{29}$
		$C_{15}H_{31}$	$C_{15}H_{29}$
		$C_{17}H_{33}$	$C_{13}H_{27}$
$[M_8 + Na]^+$	943.9	$C_{16}H_{33}$	$C_{16}H_{33}$
		$C_{17}H_{35}$	$C_{15}H_{31}$
		$C_{18}H_{37}$	$C_{14}H_{29}$
		(*anteiso*)	

Note. The combination of fatty acids is based on the relative intensity of molecular ions in FAB MS of the glycolipid fraction of **1**.

is unique for glycoglycerolipids. There are few papers concerned with the structures of glycolipids in microorganisms associated with sea bodies.[26] The present work initiates investigations in this field. Such glycoglycerolipids can be interesting in terms of studying the relationship between the structure and surface activity of compounds.

Experimental

The KMM 1364 strain was isolated from the purple ascidia *Holocynthia aurantium* (August, 1989, Troitsa Bay, Petr Velikii Gulf of the Sea of Japan, marine experimental station) and identified as *Bacillus pumilus*.

Thin-layer chromatography of glycolipids was performed on Chemapol silica gel (Czechia), 5/40 μm, in a $CHCl_3$—MeOH—H_2O system (65 : 25 : 4). Chromatograms were developed using reagents containing anisaldehyde[27] and a mixture of phenol with sulfuric acid.[28]

EI, FAB, and CI mass spectra were obtained with a Varian MAT 95 (70 eV) instrument. Low-resolution EI MS were obtained with an LKB 9000s mass spectrometer (70 eV, direct inlet) and GLC-MS, 3% QF-1. ^1H and ^{13}C NMR spectra were recorded on a Bruker WM-250 instrument (250 and 75 MHz, respectively) with Me_4Si as the internal standard. Specific rotation was determined on a Perkin—Elmer 141 polarimeter at 578 nm. IR spectra were recorded on a Specord M-82 spectrometer (Karl Zeiss, Jena). GLC analysis of polyol acetates was performed on a Pye Unicam-104 chromatograph with the use of an UZDN-2T ultrasound disintegrator. Methyl esters of fatty acids were analyzed by GLC on a GC-9A chromatograph (flame ionization detector; a Chromatopak C-R3A integrator (Shimadzu); a Supelcowax 10 capillary column (Supelco), 30000 × 0.25 mm; He as the carrier gas; separation temperature 200 °C). Fatty acids were identified with the use of standards and the known tabulated carbon numbers (equivalent chain length[29]).

The cultivation of the KMM 1364 strain of *Bacillus pumilus* was described earlier.[2]

Isolation of glycolipids. A culture liquid (6 L) was centrifuged at 500 *g* for 30 min. The cells obtained were suspended in 50 mL of distilled water and then deeply frozen. The frozen cells were destroyed with ultrasound for 2 min at 20-second intervals. The suspension of destroyed cells was extracted three times with a $CHCl_3$—MeOH mixture (3 : 1). The organic layer was concentrated to dryness, and the residue (250 mg) chromatographed on SiO_2 (40/100 μm, Chemapol, 1.5×20 cm) in a hexane—AcOEt gradient system (3 : 1, 2 : 1, 1 : 1, 1 : 3), AcOEt, and AcOEt—MeOH (95 : 5, 9 : 1). The last fraction (TLC data showed it to be homogeneous) was glycolipid **1** (25 mg).

Complete acid hydrolysis of fraction 1. Fraction **1** (1 mL) was hydrolyzed with 2 *M* TFA at 100 °C for 5 h. The hydrolyzate was concentrated to dryness in a flow of nitrogen. The residue was dissolved in water and analyzed by paper chromatography together with the reference samples (Whatman paper N 1, Bu^nOH—Py—H_2O (6 : 4 : 3), visualization with an alkaline solution of $AgNO_3$).

Preparative hydrolysis of compound **1** (8 mg) was carried out with TFA (2 mL) at 100 °C for 5 h. Preparative paper chromatography in a Bu^nOH—Py—H_2O system (6 : 4 : 3) gave D-glucose (2 mg), $[\alpha]_D^{20} \rightarrow +51°$ (*c* 0.2, H_2O).

Polyol acetates. The water-soluble part of hydrolyzate **1** (200 μg) was reduced with $NaBH_4$ (10 μL, 20 mg mL^{-1} in 0.05 *M* NaOH) at ~20 °C for 16 h. The reaction was terminated by adding 10 μL of AcOH. The H_3BO_3 that formed was removed by evaporation with a MeOH—AcOH mixture (9 : 1). Alditols were acetylated with a Ac_2O—Py mixture (1 : 1, 60 μL), extracted with $CHCl_3$ (2×1 mL), and analyzed by GLC (column 3% QF-1, 150→230 °C, 5 °C min^{-1}). Hexa-*O*-acetylglucitol (*m/z* 103, 115, 127, 128, 139, 145, 153, 157, 170, 187, 217, 230, 259, 289, 315, 361, 375) and triacetylglycerol (*m/z* 61, 73, 86, 103, 116, 145) were identified by MS EI.

Methyl esters of fatty acids. Hydrolyzate **1** was diluted with water and extracted with CH_2Cl_2 (3×1 mL). The organic phase was concentrated to dryness and dissolved in a MeOH—ether mixture (1 : 1). An ethereal diazomethane solution (1 mL) was added, and the reaction mixture was left at ~20 °C for 30 min. The obtained methyl esters of fatty acids were analyzed by GLC.

Acetylated glycolipid 1' was obtained upon treatment of compound **1** (15 mg) with a Ac_2O—Py mixture (1 : 1, 700 μL) at 25 °C for 18 h.

Analysis of glycolipid 1 by methylation. Diglycosyl diglyceride **1** (1 mg) was methylated,[2] hydrolyzed with 2 *M* TFA (0.5 mg, 100 °C, 5 h), concentrated to dryness, and dissolved in 0.5 mL of MeOH. $NaBH_4$ (20 μL, 20 mg mL^{-1} in 1 *M* NH_4OH) was added (–20 °C, 8 h). The reaction was terminated by adding 20 μL of AcOH. The borate that formed was removed by evaporation with a MeOH—AcOH mixture (9 : 1). The reac-

tion products were acetylated with a Ac_2O—Py mixture (1 : 1, 200 μL, ~20 °C, 10 h) and identified by GLC-MS (EI) as 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol (*m/z*: 71, 87, 101, 117, 129, 145, 161, 173, 205) and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol (*m/z*: 87, 99, 101, 117, 129, 159, 161, 173, 189, 233).

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