Characterization and in vivo production of three glycolipids from *Candida bogoriensis*: 13-glucopyranosylglucopyranosyloxydocosanoic acid and its mono- and diacetylated derivatives

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Abstract Three glycosides of 13-hydroxydocosanoic acid isolated from *Candida bogoriensis* were characterized by quantitating the amount of carbohydrate, acetate, and hydroxy acid in each, and by gas–liquid chromatography and mass spectrometry of their methyl ester, trimethylsilyl ether derivatives. One of the glycosides was the diacetylated derivative of 13-glucosylglucosyloxydocosanoic acid previously characterized by Tulloch, Spencer, and Deinema (*Can. J. Chem.*, **46:** 345 [1968]), in which the disaccharide had the $\beta(1 \rightarrow 2)$ sophorose linkage and the acetyl groups were attached to the 6′ and 6″ positions of the glucose residues. The other two glycosides were 13-glucosylglucosyloxydocosanoic acid and its monoacetylated derivative. A comparison of the mass spectra of derivatives indicates that the acetyl group of the monoacetyl lipid is on the internal glucose. Methyl 13-glucosyloxydocosanoate was produced by acid hydrolysis of the methyl ester of the unacetylated glycolipid and was characterized by the same techniques as the other glycolipids.

Time course of production of the three glycolipids is consistent with the diacetylated derivative being the first extracellular product and the other two glycolipids being formed by deacetylation. 13-Hydroxy[13-^{3}H]docosanoic acid, methyl 13-hydroxy[13-^{3}H]docosanoate, and 9-hydroxy[11,12-^{3}H]-stearic acid were each incorporated into the glycolipid fraction.

Supplementary key words mass spectrometry · 13-hydroxydocosanoic acid · hydroxy acid glycosides

An extracellular glycolipid produced by *Candida bogoriensis* was characterized by Tulloch, Spencer, and Deinema (1) as a hydroxy fatty acid sophoroside with the structure 13-[(2′-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]docosanoic acid 6′,6″-diacetate (Ia, Ac_2-Glc_2HDA). In addition, they observed another compound which they postulated to be the corresponding monoacetate ($AcGlc_2$HDA) based on its R_F on thin-layer chromatography and the relative intensity of its acetoxy signal in the NMR spectrum. A slightly different hydroxy acid sophoroside mixture has been characterized in the extracellular oil produced by a species of *Torulopsis* (2). This lipid mixture contained sophorose linked glycosidically to 17-L-hydroxystearic acid and 17-L-hydroxyoleic acid with acetate groups in the 6′ and 6″ positions (1, 3). A macrocyclic lactone formed between the 4″ hydroxyl of the terminal glucose and the hydroxy acid carboxyl group was also found (3).

In this report, we describe the occurrence of a third, unacetylated glycolipid in *C. bogoriensis* (Glc_2HDA) and give partial characterization of the structures of the three *C. bogoriensis* glycolipids and in vivo studies on their biosynthesis.

Abbreviations: HDA, 13-hydroxydocosanoic acid; Ac_2Glc_2-HDA, 13-glucopyranosylglucopyranosyloxydocosanoic acid diacetate; $AcGlc_2$HDA, 13-glucopyranosylglucopyranosyloxydocosanoic acid monoacetate; Glc_2HDA, 13-glucopyranosylglucopyranosyl-oxydocosanoic acid; GlcHDA, 13-glucopyranosyloxydocosanoic acid; TLC, thin-layer chromatography; GLC, gas–liquid chromatography. Abbreviations prefixed by "methyl" refer to methyl esters.

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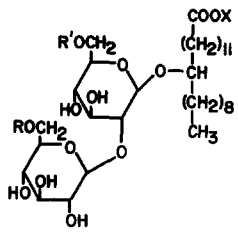
Formula I



Ia R = R′ = CH_3CO; X = H(Ac_2Glc_2HDA)

Ib R = R′ = CH_3CO; X =

CH_3 (methyl Ac_2Glc_2HDA)

EXPERIMENTAL PROCEDURE

Materials

13-Hydroxy[13-^{3}H]docosanoic acid was prepared as previously described (4). 9-Hydroxy[11,12-^{3}H]stearic acid was a generous gift of Professor Konrad Bloch and was purified prior to use by chromatography on silicic acid (eluting solvent, 2% methanol in chloroform). Scintillation materials and Cab-O-Sil were obtained from Packard Instrument Co., Downers Grove, Ill. Thin-layer plates were prepared from Adsorbosil-1 (Applied Science Laboratories, State College, Pa.), and silicic acid column chromatography was performed on Mallinckrodt SilicAR CC-4, 100–200 mesh. BF_3–methanol reagent (14%), trimethylchlorosilane, hexamethyldichlorosilazane, OV-1, diethylene glycol succinate, and Gas-Chrom Q were obtained from Applied Science Laboratories. Methyl-α-D-glucopyranoside was obtained from Eastman Organic Chemicals, Rochester, N.Y.

Analytical methods

GLC was carried out on an F&M model 700 instrument with dual 4 ft × 0.25 inch glass columns and dual hydrogen flame detectors using a helium carrier gas flow of 60 ml/min. For GLC of fatty acid methyl esters the columns were packed with 10% diethylene glycol succinate on Gas-Chrom Q. For GLC of the methyl ester, trimethylsilyl ether derivative of the glycolipids, columns were packed with 1.6% OV-1 on Gas-Chrom Q. Combined GLC–mass spectrometry was carried out on the LKB 9000 instrument in the laboratory of Professor Bengt Samuelsson, Stockholm. Low resolution mass spectrometry of methyl HDA was obtained on a Nuclide instrument (Nuclide Analysis Associates, State College, Pa.) at 70 ev. Methyl esters were prepared with BF_3–methanol reagent (5) or with diazomethane (6). Trimethylsilyl ether derivatives of glycolipid methyl esters and methyl HDA were prepared by treatment with trimethylchlorosilane and hexamethyldichlorosilazane in pyridine by the method of Sweeley et al. (7). Radioactive samples were analyzed in a Packard Tri-Carb model 3214 liquid scintillation spectrometer in 15 ml of either dioxane (8 g of 2,5-diphenyloxazole and 100 g of naphthalene per liter of dioxane) or toluene (4 g of 2,5-diphenyloxazole and 50 mg of 1,4-[bis(2,5-phenyloxazolyl)]benzene per liter of toluene) scintillator solution. Counting efficiency for ^{3}H was 25% in dioxane and 33% in toluene. Radioactivity from thin-layer plates was determined by suspending the Adsorbosil scraped from the plates in the scintillator solution with Cab-O-Sil. Lipids on thin-layer plates were visualized by exposure of the plates to iodine vapor. Paper chromatography of sugars was carried out on Whatman no. 1 chromatography paper, using as a developing solvent isopropanol–water–acetic acid 3:1:1 (v/v/v). Sugars were located by use of the periodate–benzidine spray (8). Glucose content of glycolipids was determined by the anthrone reaction (9), using glucose as a standard. Methyl acetate obtained from methanolysis of the glycolipids was quantitated by the method of Ludowieg and Dorfman (10). NMR spectra were obtained on a 90-MHz Bruker NMR spectrometer (Bruker Scientific, Inc., Elmsford, N.Y.) with tetramethylsilane as an internal standard.

Culturing methods

C. bogoriensis (NRRL no. Y5980) cells were grown either in 10-l fermentor cultures as previously described (11) or in Erlenmeyer flasks on a rotary shaker (1-inch stroke, about 150 rpm) at room temperature (25°C).

Isolation of lipids

The extracellular glycolipids from *C. bogoriensis* sedimented with yeast cells when centrifuged for 10 min at 27,000 *g* in the cold. Acetone extraction of the sedimented cells (four 20-ml portions/2 g wet wt of cells) yielded more than 85% of the total glycolipids in the culture. For preparative purification of the three glycolipid components as their methyl esters, an acetone extract was obtained from a 400-ml shaking culture of cells grown for 25 days in a medium containing 5% glucose and 0.15% yeast extract. The crude lipid (900 mg) was treated with diazomethane and applied to a 120-g silicic acid column prepared in chloroform. Nonpolar lipids were eluted with 1.5 l of chloroform. The glycolipid methyl esters were eluted as follows: methyl Ac_2Glc_2HDA (85.4 mg) with 2 l of 2.5% methanol in chloroform; methyl $AcGlc_2HDA$ (203.0 mg) with 1.8 l of 5% methanol in chloroform; and methyl Glc_2HDA (280.6 mg) with 1 l of 10% methanol in chloroform. Column fractions were assayed by TLC (developing solvent, chloroform–methanol–acetic acid 90:10:2 [v/v/v]), and fractions containing a single iodine-staining spot were pooled.

Methyl GlcHDA was prepared by partial acid hydrolysis of 111.3 mg of methyl Glc_2HDA in 35 ml of 3% methanolic HCl at 50°C for 2 hr. The reaction was terminated by addition of 41 ml of 1 N NaOH. 20 ml of water was added, and the mixture was extracted with ether (300 ml), yielding, after evaporation of the ether, 101 mg of residue, which was applied to a 10-g silicic acid column prepared in chloroform. The three ether-soluble components were then eluted as follows: methyl HDA with 300 ml of chloroform; methyl GlcHDA with 200 ml of 2.5% methanol in chloroform; and methyl Glc_2HDA with 150 ml of 10% methanol in chloroform. The combined methyl GlcHDA fraction contained 31.5 mg of a clear yellow gum which produced a single iodine-staining spot on TLC (developing solvent, chloroform–methanol–acetic acid 93:7:2 [v/v/v]).

For characterization of the hydroxy acid component of the glycolipids, the crude acetone extract (348 mg) was treated with BF_3–methanol in a closed tube for 15 min at 90°C, and the fatty acid methyl esters (192 mg) were extracted into hexane. These were applied to a 30-g silicic acid column prepared in hexane, and 200 ml of 3% ether in hexane was used to elute the less polar compounds. Methyl HDA eluted from the column with 200 ml of 20% ether in hexane, and this material was subsequently crystallized from hexane, giving 74 mg of white flakes, mp 69.5–70.5°C, reported mp 69–70°C (1).

For the Beckmann degradation, a sample (22.9 mg) of recrystallized methyl HDA was oxidized to the keto ester by CrO_3 in acetic acid. The resulting keto ester (21.6 mg) was converted to the oxime by refluxing for 2 hr with 23.1 mg of hydroxylamine hydrochloride and 27.6 mg of sodium acetate in 3 ml of 80% ethanol. The oxime was heated at 100°C for 1 hr in 2 ml of concentrated sulfuric acid, and the resulting amides were extracted into ether, giving 13.2 mg of oily material. This mixture was subjected to acid methanolysis (BF_3–methanol) in a closed tube at 80–90°C for 5 min, giving 5 mg of a mixture of mono- and dicarboxylic acid methyl esters, which were subsequently examined by GLC at 80°C and 140°C, respectively.

In vivo labeling studies

Yeast cells obtained from 100-ml shake cultures grown for 4 days were centrifuged in a Sorvall refrigerated centrifuge (0°C, 27,000 *g*, 10 min), washed twice with distilled water, and suspended in 20 ml of an 8% glucose solution. These operations were performed under sterile conditions. Duplicate flasks were inoculated with the appropriate ^3H-labeled precursor dissolved in 20 µl of ethanol, and the suspensions were incubated on the shaker at room temperature for 24 hr. The contents of each flask were extracted with four 50-ml portions of chloroform; the cells were then centrifuged (0°C, 27,000 *g*, 10 min) and extracted with two 60-ml portions of chloroform–methanol 2:1 (v/v). Both extracts were combined. A 30-g silicic acid column was used to separate the hydroxy fatty acids (eluted with 2.5% methanol in chloroform) from the glycolipids (eluted with 20% methanol in chloroform). The glycolipids were finally purified by preparative TLC (developing solvent, chloroform–methanol–acetic acid 90:10:2 [v/v/v]). The radioactivity in each glycolipid area was determined by scraping appropriate portions of the Adsorbosil-1 into scintillation vials and counting in the Packard Tri-Carb spectrometer. Structural location of radioactivity in the glycolipid products was determined by acid methanolysis in 3% methanolic HCl at reflux for 22 hr. The solution was concentrated, water was added, and methyl esters were extracted into hexane. The radioactive glycolipids formed from 9-hydroxy[11,-12-^3H]stearic acid (31.2 mg, 5.76×10^4 dpm) yielded 17.5 mg (5.6×10^4 dpm) of hexane-soluble material, of which less than 5% migrated with methyl HDA on TLC (developing solvent, chloroform). The radioactive glycolipids formed from [13-^3H]HDA (38.2 mg, 1.16×10^5 dpm) yielded 18.2 mg (1.05×10^5 dpm) of hexane-soluble material, all of which migrated with authentic methyl HDA on TLC (developing solvent, chloroform). An aliquot of this product (11.6 mg, 7.0×10^4 dpm) was dissolved in 1.0 ml of glacial acetic acid, and 16 mg of CrO_3 in 1 ml of acetic acid was added. The reaction was allowed to proceed at room temperature for 1 hr. Water (3 ml) was added and the keto ester was extracted into hexane. This procedure yielded 10.7 mg of hexane-soluble material containing only 9.0×10^2 dpm of ^3H, demonstrating that more than 99% of the ^3H remained in the 13-position of HDA during incorporation into glycolipids.

RESULTS AND DISCUSSION

Characterization of 13-hydroxydocosanoic acid (HDA)

Crystalline Ac_2Glc_2HDA was shown by Tulloch et al. (1) to yield on methanolysis a fatty acid methyl ester which, after crystallization, was characterized as methyl 13-HDA by comparison with an authentic sample and by conversion to the oxo ester and the free acid. This procedure would not have detected minor amounts of homologous or isomeric hydroxy fatty acids. In the current work the total crude acetone extract was subjected to acidic methanolysis with BF_3–methanol, followed by extraction with hexane. GLC analysis (180°C) of the hexane-soluble fraction showed one major peak with a retention time of 47.8 min which was identical to that of authentic methyl 13-HDA (4). When the sample was

treated with trimethylchlorosilane and hexamethyldisilazane (7), this major peak disappeared and a new one appeared with a retention time of 8.1 min. It was the only peak in the spectrum which shifted, and hence the only hydroxy compound which could be converted to a trimethylsilyl ether.

Purified methyl HDA was degraded via the Beckmann rearrangement of the oxime. GLC analysis of the mono- and dicarboxylic esters produced in the degradation showed the major fragments with retention times corresponding to methyl decanoate and dimethyl tridecanedioate. Minor homologous peaks, each less than 10% of the major peaks, could indicate minor amounts of HDA isomers, but the 13-hydroxy isomer is certainly the major one.

The structure of methyl HDA was further confirmed by its mass spectrum. The fragmentation pattern was consistent with those reported for other long-chain hydroxy acid methyl esters (12). No molecular ion was seen. The base peak was at *m/e* 211 (relative intensity, 100), which is attributed to an ion of the structure $[HOC^{+}H(CH_2)_{10}CH{=}CO]$, corresponding to cleavage between C_{13} and C_{14} along with loss of methanol. Cleavage between C_{13} and C_{14} with no loss of methanol would produce an ion seen at *m/e* 243, relative intensity, 40.5 $[HOC^{+}H(CH_2)_{11}{-}COOCH_3]$. Cleavage between C_{12} and C_{13} would produce an ion seen at *m/e* 214, relative intensity, 39.8 $[H{\cdot}C^{+}H_2(CH_2)_{10}COOCH_3]$. Homologous peaks of the type C_nH_{2n-1} were also prevalent at *m/e* 41, *m/e* 55, *m/e* 69, *m/e* 83, and *m/e* 97, with relative intensities of 20.9, 37.0, 54.6, 89.5, and 17.4, respectively.

It should be pointed out that Tulloch (13) has characterized HDA isolated from this organism as having the L configuration by comparison of its optical rotation with those of synthetic L- and D-HDA isomers.

Characterization of *C. bogoriensis* glycolipids

Three glycolipids made up 60% of the crude acetone extract of centrifuged *C. bogoriensis* cells. Ether extraction of the supernatant culture medium yielded a slight additional amount of the same three glycolipids. Acid degradation of the purified glycolipid methyl esters yielded methyl HDA as the only hexane-soluble product and a water-soluble substance which cochromatographed on paper with authentic methyl-α-D-glucopyranoside. A volatile ester, methyl acetate, was detected in the degradation of only two of the glycolipids. Table 1 shows the quantitative data for degradation of these glycolipids. The data support their structures as methyl Ac_2Glc_2-HDA, methyl $AcGlc_2HDA$, and methyl Glc_2HDA.

Further support for these compositions came from their NMR spectra. Samples were run as 8% solutions in deuterated dimethyl sulfoxide. Methyl Ac_2Glc_2HDA showed a sharp singlet at δ 1.99 ppm containing 5.6 protons (relative to a value of 3.0 for the OCH_3 protons at 3.58 ppm), with an expected value of 6 for two acetyl groups. Methyl $AcGlc_2HDA$ showed a sharp singlet at 1.99 ppm containing 2.7 protons, with an expected value of 3 for one acetyl group, and methyl Glc_2HDA gave no signal in this region of the spectrum. Other features of the NMR spectra were very similar to the spectrum reported by Tulloch et al. (1) for crystalline methyl Ac_2-Glc_2HDA (Ib), but we did not obtain sufficient resolution of the anomeric protons in this solvent to confirm assignment of the β configuration.

Fig. 1 shows the time course of acid methanolysis of methyl Glc_2HDA. An intermediate glycolipid was formed which was less polar on TLC than methyl Glc_2HDA. Acid degradation of this compound yielded methyl HDA and glucose in a molar ratio of 1.0:1.2. Hence, its structure is presumed to be the methyl ester of GlcHDA (IIb). Since the initial rate of production of IIb is

TABLE 1. Quantitation of hydrolytic products of *C. bogoriensis* glycolipids

R_F[a] of Glycolipid	Component Analyzed			Empirical Ratio[b]			Identification
	Acetate[c]	Glucose[d]	Methyl HDA[e]	Acetate[c]	Glucose[d]	Methyl HDA[e]	
	μmoles/mg glycolipid						
0.84	2.84	2.92	1.55	1.95	2.0	1.1	Methyl Ac_2Glc_2HDA
0.35	1.44	2.94	1.62	0.98	2.0	1.1	Methyl $AcGlc_2HDA$
0.17	0.15	3.30	1.82	0.09	2.0	1.1	Methyl Glc_2HDA

The glycolipid methyl esters were isolated as described under Experimental Procedure.

[a] R_F on thin-layer chromatogram; developing solvent, chloroform–methanol–acetic acid 90:10:2 (v/v/v).

[b] Based on 2 moles of glucose.

[c] Methyl acetate was quantitated by the method of Ludowieg and Dorfman (10) as a volatile ester produced when 5 mg of each glycolipid was heated in 1 N methanolic HCl in a sealed tube at 100°C for 2 hr.

[d] Glucose was determined by heating 100–150 μg of each glycolipid with the anthrone reagent (9), producing a resultant absorbance of 0.2–0.4 at 620 nm when compared with a water-containing blank. Glucose was used as a standard.

[e] Methyl HDA was quantitated gravimetrically as hexane-soluble material formed from methanolysis of 10–15 mg of each glycolipid with BF_3–methanol in a sealed tube at 85°C for 5 min.

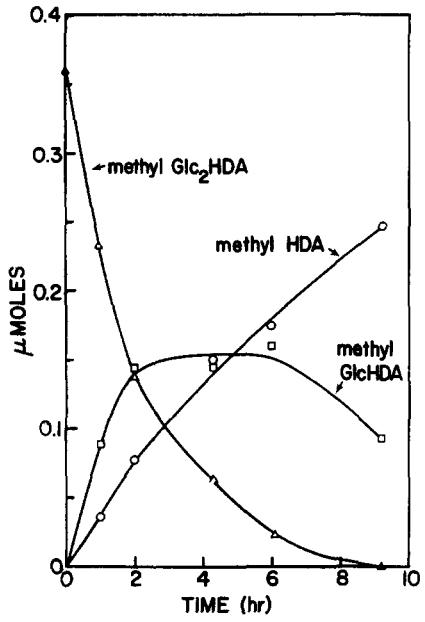
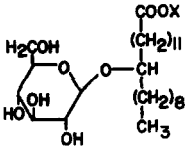


FIG. 1. Time course of methanolysis of methyl Glc_2HDA. A series of tubes each containing 248 μg of methyl Glc_2HDA were subjected to hydrolysis in 0.5 ml of 4% methanolic HCl at 50°C. At the times indicated, the reaction was terminated, and the glycolipids were extracted into ether. Methyl Glc_2HDA and methyl GlcHDA were separated by preparative TLC (developing solvent, chloroform–methanol–acetic acid 93:7:2 [v/v/v]) and quantitated by the anthrone reaction (9). The methyl HDA production curve was calculated by difference.

greater than that of methyl HDA, one can conclude that the disaccharide linkage is more labile to methanolysis than the hydroxy acid glycoside bond. GlcHDA was not detected in crude extracts of *C. bogoriensis* but may have been present in concentrations much lower than the other three glycolipids.

Formula II



IIa X = H (GlcHDA)
IIb X = CH_3 (methyl GlcHDA)

The glycolipid methyl esters were analyzed by GLC of their trimethylsilyl ethers. Fig. 2 shows the chromatographic recorder tracings and gives an indication of the purity of each fraction. The relative retention times of the glycolipid derivatives are consistent with their proposed structures. Substitution of each acetyl group for a trimethylsilyl group produced an increase in the retention time by a factor of 1.2.

Using a combined gas–liquid chromatograph–mass spectrometer it was possible to obtain partial mass spectra of the major peak fractions of each trimethylsilyl ether sample. The spectra are recorded in Table 2. At

TABLE 2. Partial mass spectra of *C. bogoriensis* glycolipids as their methyl ester, trimethylsilyl ether derivatives

Mass	Relative Intensity	Mass	Relative Intensity	Mass	Relative Intensity	Mass	Relative Intensity
Ac_2Glc_2HDA (methyl ester, trimethylsilyl ether) mol wt = 1138							
42	3.1	117	5.3	218	2.9	331	17.3
44	3.7	129	12.1	219	2.0	332	4.9
55	2.5	145	12.4	221	5.7	333	2.6
56	2.8	147	8.0	223	2.6	349	5.4
57	4.0	155	6.2	233	3.9	353	2.3
69	2.3	169	4.3	241	2.0	355	5.9
73	15.0	175	5.9	243	4.8	357	2.9
74	3.7	188	5.3	245	2.3	361	11.1
75	25.2	191	4.3	259	2.8	362	3.7
83	2.8	199	8.3	271	25.3	363	3.2
96	2.8	204	100.0	272	5.7	415	2.3
97	4.0	205	24.7	273	3.1	421	3.6
101	2.9	206	8.3	281	3.2	429	2.0
103	2.6	208	2.3	289	4.3	431	4.0
109	2.9	217	21.8	295	3.4	504	2.2
AcGlc2HDA (methyl ester, trimethylsilyl ether) mol wt = 1168							
43	2.1	129	6.9	204	100.0	271	4.6
69	2.2	145	5.0	205	21.2	295	2.0
73	12.2	147	5.1	206	10.5	331	3.0
74	4.0	148	2.0	217	19.2	349	4.3
75	12.1	155	2.0	218	7.0	353	3.1
83	2.4	169	5.1	219	2.3	361	26.3
103	3.4	191	3.4	221	2.4	362	9.0
109	2.0	199	2.9	243	2.3	363	4.3
117	2.7	203	3.1	259	2.0		
Glc_2HDA (methyl ester, trimethylsilyl ether) mol wt = 1198							
73	10.2	169	6.9	219	4.0	353	3.6
74	2.4	191	4.8	221	2.7	357	2.6
75	10.2	199	2.3	233	2.0	361	40.2
103	8.4	204	100.0	243	2.9	362	14.8
129	4.7	205	18.6	271	4.6	363	6.3
147	9.0	206	8.6	289	3.2	379	6.9
149	2.4	217	22.8	295	2.0	380	2.1
161	2.4	218	8.4	319	3.2		
GlcHDA (methyl ester, trimethylsilyl ether) mol wt = 820							
73	5.1	191	3.2	206	9.4	219	2.3
75	2.9	204	100.0	217	20.5	353	2.4
147	2.9	205	19.7	218	6.5	361	2.9

Spectra were recorded on the gas–liquid chromatograph–mass spectrometer, LKB 9000, equipped with a 2% OV-1 column. The column temperature was 300°C, and the electron energy was 22.5 ev. Each spectrum was taken from the major peak seen on the gas chromatograph recorder. Background spectra obtained on column bleed were subtracted. Each spectrum was obtained from about 0.1–0.5 μg of injected sample. Relative intensities are expressed in % of base peak, and only peaks with relative intensities greater than 2% are shown.

the concentration of sample used (about 0.1–0.5 μg of injected sample), ions of mass greater than those shown in the table were not visible in the recorder tracing. The base peak in the spectrum of each glycolipid was at *m/e* 204; this fragment is characteristic of trimethylsilyl ether derivatives of carbohydrates with the pyranose ring structure (14). It retains the C_2-C_3 or C_3-C_4 ring carbon atoms and has been assigned the structure $[(CH_3)_3SiOC^+$-$HCHOSi(CH_3)_3]$. Other peaks characteristic of car-

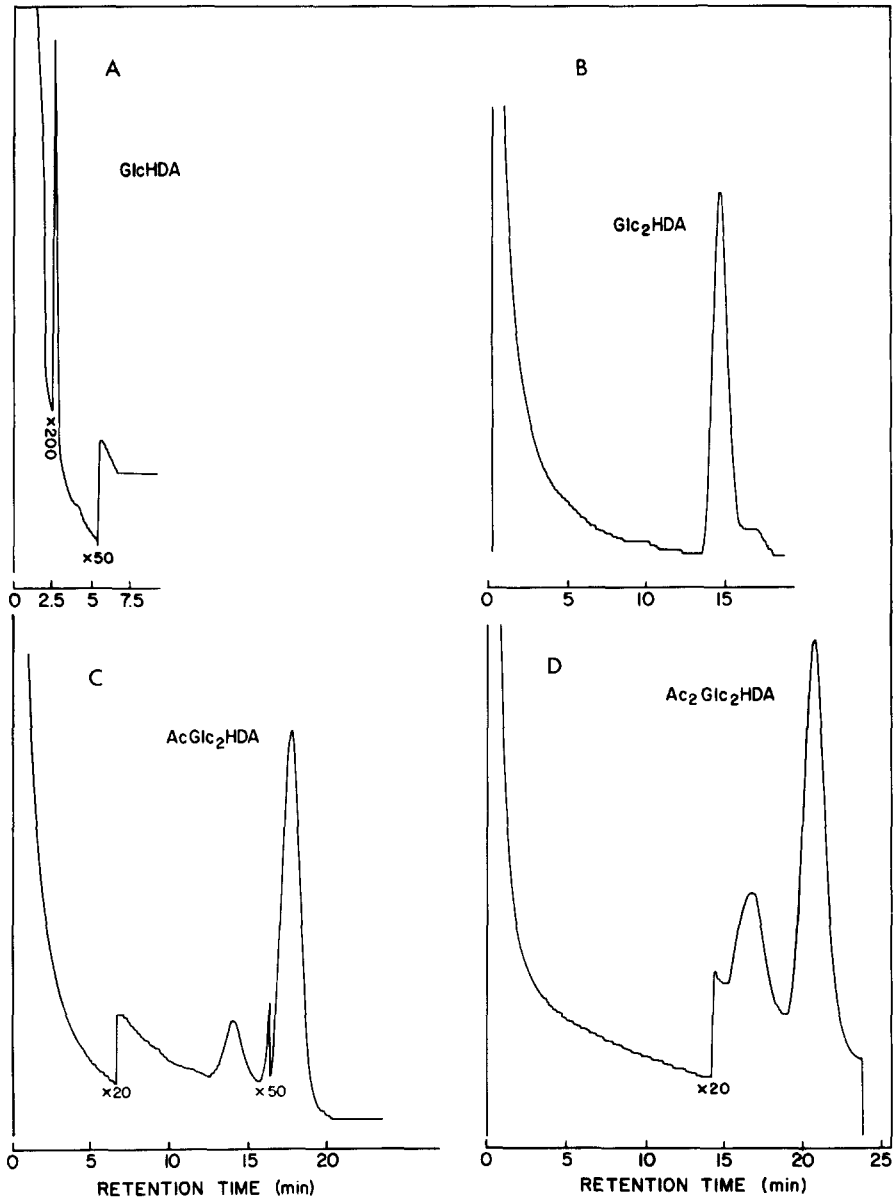


FIG. 2. GLC of *C. bogoriensis* glycolipids as their methyl ester, trimethylsilyl ether derivatives. The 5-ft columns were packed with 1.6% OV-1 on Gas-Chrom Q, and the instrument was operated isothermally at 275°C. The methyl ester, trimethylsilyl ether derivatives were prepared as described in Experimental Procedure.

bohydrates were found in all four spectra: *m/e* 73 [$^{+}Si(CH_3)_3$], *m/e* 217 [$(CH_3)_3SiOCH{=}CHC^{+}HOSi(CH_3)_3$], and *m/e* 129 [$(CH_3)_3SiOC^{+}HCH{=}CH_2$]. A small peak at *m/e* 353 in all four spectra can be assigned to cleavage of the fatty acid glycosidic bond with charge retention on the fatty acid. This fragment is not usually found in simple carbohydrates.

Kochetkov, Chizhov, and Molodtsov (15) found that fragments resulting from cleavage of the nonreducing residue of disaccharide trimethylsilyl ether derivatives depend only on the structure of that residue. Many of the resulting peaks come from the loss of one or more $(CH_3)_3SiOH$ molecules (90 mass units) from the original fragment and hence appear 90 mass units apart. The position of the acetyl group in $AcGlc_2HDA$ is unknown, so comparison of the fragments resulting from the terminal glucose residue of this compound with those resulting from the other two compounds should show if the acetyl group is bonded to the terminal glucose. Table 3 summarizes some mass spectral peaks which can be assigned to these terminal glucose fragments and which would be important in assigning their structures. The terminal glucose residue from fragmentation of the trimethylsilyl ether derivative of methyl Ac_2Glc_2HDA

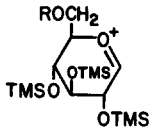
TABLE 3. Some fragment ions assigned to the terminal glucose from the partial mass spectra of trimethylsilyl ether derivatives of glycolipid methyl esters

Ion	Relative Abundance[a] Ac_2Glc_2-HDA	$AcGlc_2$-HDA	Glc_2-HDA	Assignment
m/e	*% of base peak*			
421	3.6	0	0	
361	11.1	26.3	40.2	421 − 60 or 451 − 90
331	17.3	3.0	1.1	421 − 90
271	25.3	4.6	4.6	421 − 60 − 90 or 451 − 90 − 90

[a] Data have been taken from Table 2.

would produce fragment ion IIIa, assuming structure I assigned to this glycolipid by Tulloch et al. (1). The terminal fragment ion would be

Formula III



IIIa R = CH_3CO; *m/e* = 421
IIIb R = TMS; *m/e* = 451
TMS = $(CH_3)_3Si$-

at *m/e* 421 regardless of the position of the acetyl group on this terminal glucose residue. An ion was found at *m/e* 421 for this compound, as well as ions at 421 − 60, 421 − 90, and 421 − 60 − 90. If both acetyl groups of Ac_2Glc_2HDA were on the terminal glucose, ions at 391 and 391 − 90 should be seen, and they are not. If neither acetyl group were on the terminal glucose, the terminal fragment would be IIIb, and intensities of the ions at *m/e* 361 (451 − 90) and *m/e* 271 (451 − 90 − 90) should be similar to the intensities of these ions in the spectrum of the Glc_2HDA derivative, and they are not. (An ion at *m/e* 451 was not found in any of the spectra.) Location of the acetyl group in the $AcGlc_2HDA$ derivative can then be deduced by comparing its spectrum with those of the diacetylated and unacetylated compounds, as is also shown in Table 3. A peak at *m/e* 331 (421 − 90) was not expected in the unacetylated glycolipid, and indeed it was much smaller in this compound and in the monoacetyl derivative than in the diacetyl derivative. Intensities of peaks at *m/e* 361 (421 − 60 or 451 − 90) and at *m/e* 271 (421 − 60 − 90 or 451 − 90 − 90) in the spectrum of the $AcGlc_2HDA$ derivative were much more like those from the unacetylated glycolipid than from the diacetylated glycolipid. These data then support an assignment of the single acetyl group to the internal glucose residue, and hence the structure Glc(Ac)GlcHDA for this lipid. The presence of a small percentage of the alternative monoacetyl isomer in the sample could not be ruled out, of course, if both monoacetyl isomers had identical chromatographic properties.

Tulloch et al. (1) presented NMR evidence that the two acetyl groups in Ac_2Glc_2HDA were on the 6′ and 6″ positions of the glucose residues. The glycosidic linkages were established as β from the coupling constants of the anomeric protons of the deacetylated lipid and from optical rotation, and the glucosylglucosyl linkage as 1→2 by periodate oxidation, by methylation and hydrolysis, and by isolation of sophorose octaacetate. The characterizations reported here establish the composition of the three HDA glycosides we have isolated but do not establish the position or stereochemistry of the chemical linkages. Our Ac_2Glc_2HDA is very likely the same as that isolated by Tulloch from the same organism, however. The biosynthetic relationship suggested below, together with an in vitro study of glucosyl- and acetyltransferases involved in the biosynthesis of these glycolipids (11), supports a biochemical, and hence a structural, similarity for the three glycolipids.

In vivo production of glycolipids

Fig. 3 shows the time course of production of the three glycolipids in relation to the growth of *C. bogoriensis*. Glycolipid production began during the period of rapid growth and continued into the early part of the stationary period. The first glycolipid to appear was Ac_2Glc_2HDA, which gradually disappeared after 3.5 days. At the same time that Ac_2Glc_2HDA was disappearing, $AcGlc_2HDA$ and Glc_2HDA were accumulating. This time course is suggestive of the precursor–product relationship

$$Ac_2Glc_2HDA \rightarrow AcGlc_2HDA \rightarrow Glc_2HDA$$

in which the diacetylated glycolipid is formed first, and the acetyl groups are subsequently removed to produce the monoacetylated and unacetylated glycolipids. Tulloch et al. (1) reported that the extracellular glycolipid from *C. bogoriensis* sometimes appeared as crystals and sometimes as viscous droplets, and that the total yield of Ac_2Glc_2HDA was somewhat variable. Both observations can be explained in terms of the changes in composition shown in Fig. 3. Notice also that in much older cultures the glycolipids disappear from the medium, as originally reported by Deinema (16). If the disappearance is the result of glycolipid degradation rather than incorporation into other molecular species, then the timing of the disappearance would require that older cultures develop or release hydrolytic enzymes for the glycosidic linkage at some time later than the appearance of hydrolytic enzymes for the acetyl groups. Demonstration of such enzymes would confirm our conclusions concerning the biosynthetic relationship of these three glycolipids and might provide valuable clues to the

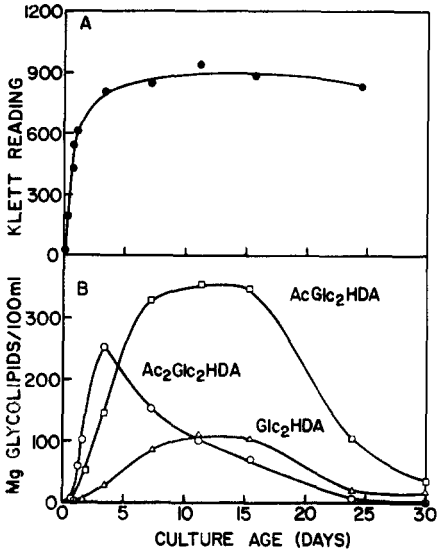


FIG. 3. Glycolipid production as a function of culture age. *C. bogoriensis* cells were grown in a fermentor culture as described in Experimental Procedure. Aliquots were removed for the determination of growth (*A*) and of glycolipid content (*B*). Growth was measured by turbidity on a Klett-Summerson colorimeter with a 540-nm filter. Cells were extracted with acetone and the glycolipids were separated by preparative TLC (developing solvent, chloroform–methanol–acetic acid 90:10:2 [v/v/v]). Separated fractions were eluted from the appropriate area of the thin-layer plate and quantitated by the anthrone reagent (9). After day 9, the entire remaining culture was transferred from the fermentor to Erlenmeyer flasks and incubation was continued at room temperature on the rotary shaker.

function of the lipids. It should be pointed out that rhamnolipid, a hydroxy acid containing glycolipid found in *Pseudomonas aeruginosa*, once synthesized, remained unchanged in cultures for up to 33 days (17).

Incorporation of hydroxy acids into the glycolipids

Incubation of *C. bogoriensis* cells with [13-^{3}H]HDA resulted in incorporation of tritium into the glycolipids as shown in Table 4. Hydrolysis of the glycolipid mixture, followed by chromic acid oxidation of the hexane-soluble product, demonstrated that at least 99% of the incorporated tritium was still in the 13-position of HDA. Incubation of the methyl ester of [13-^{3}H]HDA resulted in a lower total incorporation into glycolipid, but the distribution among the three forms was the same. It is interesting to note that the products in this case co-chromatographed on thin-layer plates with the corresponding glycolipid free acids, and hence hydrolysis of the methyl ester had occurred. Incubation of cells with 9-hydroxy[11,12-^{3}H]stearic acid resulted in an incorporation of the tritium into the glycolipid fraction. The glycolipids were not further separated or characterized since no standards were available for chromatographic studies, but hydrolysis of the glycolipid fraction followed by a thin-layer study of the fatty acid

TABLE 4. Incorporation of hydroxy acids into glycolipids

Labeled Precursor	Incorporation			
	Glc_2-HDA	$AcGlc_2$-HDA	Ac_2Glc_2-HDA	Total
	% of added radioactivity[a]			
[13-^{3}H]HDA[b]	0.8	3.1	2.7	6.6
Methyl [13-^{3}H]HDA[c]	0.2	0.9	0.7	1.8
9-Hydroxy[11,12-^{3}H]-stearic acid[d]				8.7[e]

Each labeled precursor was incubated with a suspension of *C. bogoriensis* cells for 24 hr, and the radioactive glycolipids formed were isolated and purified as described in Experimental Procedure.

[a] Aliquots of the glycolipids containing 2700–4000 dpm were applied to thin-layer plates, which were developed with chloroform–methanol–acetic acid 85:15:2 (v/v/v). Areas corresponding to the standard glycolipids were scraped from the plates, suspended in Cab-O-Sil, and counted in dioxane solvent. At least 94% of the applied radioactivity was recovered from the thin-layer plates.

[b] 78 μg of specific activity 15 μCi/mg.

[c] 71 μg of specific activity 19 μCi/mg.

[d] 2 μg of specific activity 110 μCi/mg.

[e] Distribution among various forms was not determined.

methyl esters showed only negligible amounts (< 5%) of the radioactivity cochromatographing with methyl HDA. Therefore, elongation to HDA had not occurred prior to attachment of the sugar residues.

These experiments indicate that externally added hydroxy acid can serve as an acceptor for the glucosyl residues in glycolipid biosynthesis and, therefore, that the glucosyl residues are not involved in the hydroxylation or chain elongation reactions leading to HDA. A similar conclusion could be drawn from the studies of Heinz, Tulloch, and Spencer (18, 19) on the formation of 17-hydroxystearate and 17-hydroxyoleate in *Torulopsis* in which hydroxylation could be separated from the subsequent formation of the sophorosyl derivatives of these hydroxy acids. Incorporation of 9-hydroxystearate into the *Candida* glycolipids indicates some latitude in the specificity of the biosynthetic system. An in vitro study of glucosyltransferases from *Candida* (11) bears out these conclusions.

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