A DIGLYCERIDE URONOSIDE FROM STREPTOMYCES LA 7017

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UDC 576.809.8+547.915

In an analysis of the cell lipids of Streptomyces LA 7017 (the producing agent of the antibiotic LA 7017) by thin-layer chromatography (TLC), we found that with various reagents [1-3] that two lipid fractions give a coloration characteristic for glycolipids. We have described the isolation and the determination of the structure of one of these lipids – α1-O-[4-O](α-D-glucopyranosyl)-α-D-(2- or 3-O-acyl)galactopyranuronosyl]-2,3-diglyceride (I) – in a preceding paper [4]. The present paper gives the results of the isolation and the determination of the structure of the second glycolipid (II), which has a somewhat lower mobility on TLC in silica gel than the glycolipid I.

The combined cell lipids obtained by the extraction of the lyophilized mycelium with mixtures of chloroform and methanol (2 : 1 and 1 : 1) was subjected to preliminary fractionation on a column of silica gel, and then the fractions containing the glycolipids I and II were chromatographed on silica gel impregnated with boric acid. The glycolipid I was isolated in the chromatographically pure state. For the final purification of lipid II from phospholipids, preparative TLC was used. After reprecipitation with methanol from chloroform, this lipid was obtained in the form of a white powder with mp 234-235°C and $[\alpha]_D^{20}$ + 67.7°.

The glycolipid II, like I, does not contain phosphorus, sulfur, or nitrogen; but, in addition to carbon, oxygen, and hydrogen, it contains sodium and potassium. The IR spectrum of II is similar to that of I (Fig. 1) and has absorption bands at, cm^{-1}: 3380 (alcoholic HO groups), 1730 (ester groups), 1600 (ionized carboxyl), disappearing after treatment of the lipid with Dowex-50 cation-exchange resin in the H^+ form, 1278 and 1180 (stretching vibrations of a C–O bond), 1050 (deformation vibrations of an H–O bond). There is no selective absorption in the UV region in the 216-300 nm region. (See Scheme on following page.)

Under the conditions of acid hydrolysis (Scheme 1), the glycolipid II forms a mixture of fatty acids and water-soluble products. The composition of the fatty acids of the glycolipid II, studied by the gas-liquid chromatography (GLC) of their methyl esters, is given below:

Fatty acids	V_{rel}^*	Content, %
$C_{14:0}$	0.29	3.3
$C_{15:0}$	0.39	45.2
$C_{16:0}$	0.58	8.2
Unidentified	0.50	22.3
"	0.69	21.2

It was concluded previously [4] on the basis of the IR, mass, and NMR spectra of the mixture of methyl esters of the acids of the glycolipid (I) and the results of chromatography that the two unidentified fatty acids present in this lipid were saturated branched acids with two or more methyl groups in the carbon chain, one of these acids being a $C_{17:0}$ acid. Since the fatty-acid compositions of the glycolipids I and

*V_{rel} refers to the retention volume of the methyl esters of the fatty acids relative to methyl stearate.

Institute of the Chemistry of Natural Compounds, Academy of Sciences of the USSR. Translated from Khimiya Prirodnykh Soedinenii, No. 6, pp. 664-671, November-December, 1970. Original article submitted July 28, 1970.

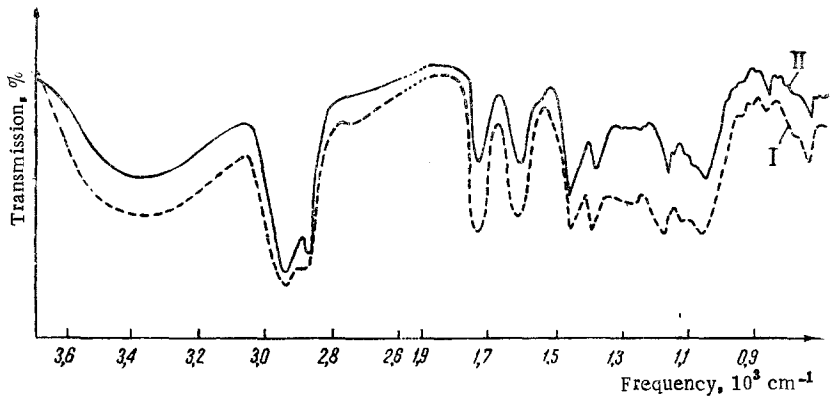
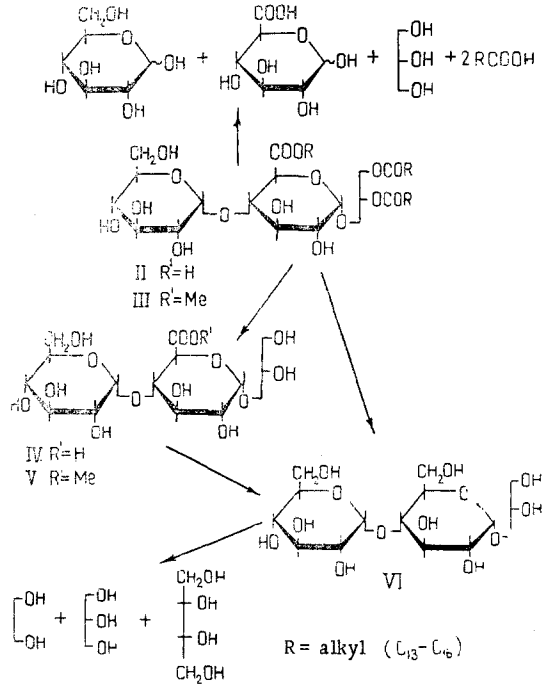


Fig. 1. IR spectra of glycolipids I and II (in chloroform).

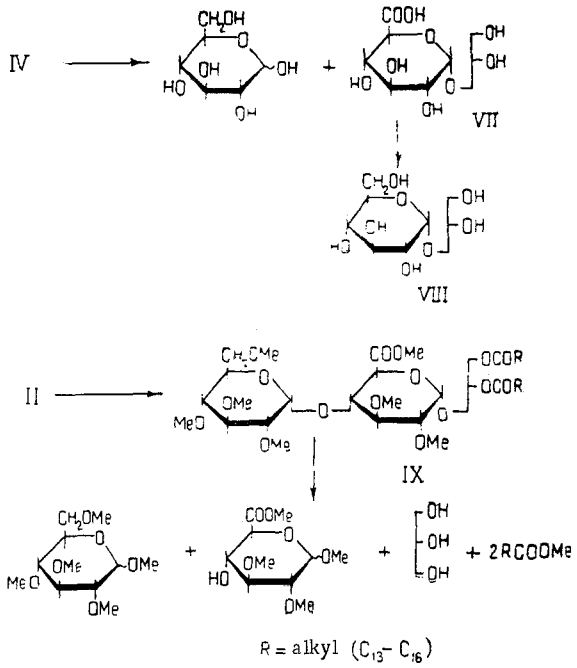


Scheme 1

II are identical qualitatively, all that has been said above apparently applies also to the unidentified acids of the latter.

By paper chromatography (PC), TLC on silica gel, and GLC, the water-soluble fraction of the hydrolysate was found to contain glycerol, glucose, and a fairly polar compound coinciding on PC and TLC with glucuronic acid. To determine the chemical nature of this fragment, we subjected the glycolipid II to mild alkaline hydrolysis. The glycoside IV that was formed was methylated with diazomethane, and the methylation product V was reduced with sodium borohydride to the diglucosylglycerol VI. The same glycoside was also obtained by the reduction of the methyl ester III of the glycolipid II with lithium aluminum hydride. Since the acid hydrolysis of the diglucoside VI gave only glycerol and glucose and the content of the latter in VI proved to be approximately twice as great as in the uronoside IV, it is obvious that the molecule of the glycolipid II contains a glucoronic acid residue. Thus, the molecule of the glycolipid II is based on the residues of fatty acids, glycerol, glucose, and glucuronic acid. A quantitative analysis of these components showed that they are present in a ratio of 2 : 1 : 1 : 1, their sequence in the molecule of II following from the experimental results (Scheme 2).

On hydrolysis under mild acid conditions, the glycoside IV split into two fragments: glucose and compound VII, which obviously is a glyceryl glucuronoside, since free glycerol and glucuronic acid are absent



Scheme 2

from the hydrolysate. Consequently, the polyol residues of the glycolipid must be arranged in the sequence glucose → glucuronic acid → glycerol; the uronic acid must be bound to a primary hydroxyl of the glycerol: the product of the alkaline hydrolysis of the glycolipid II – the glycoside IV – forms one mole of formaldehyde on oxidation with periodate, while the glycolipid II itself does not form formaldehyde on periodate oxidation. This fact also shows the pyranose form of the glucose residue. The pyranose form of the glucuronic acid residue, also, follows from the fact that the product of the reduction of the glycoside VII – the glyceryl glucoside VIII – forms only one mole of formaldehyde on oxidation with periodate (by the oxidation of the glycerol residue).

To determine the positions of the acyl groups, we exhaustively methylated the glycolipid II (see Scheme 2). On acid hydrolysis, the O-methyl derivative IX gave a mixture of products among which free glycerol and a mixture of α- and β-1,2,3,4,6-penta-O-methylglucopyranosides were identified by PC and TLC. The presence of the former in the methanolysate shows that the glycerol residue in the glycolipid molecule has no free HO groups and, consequently, is acylated with two fatty acids. In addition to this, the formation of completely methylated glucosides once again shows the terminal position of the glucose residue.

In order to determine the nature of the bond between the carbohydrates present in the glucolipid, the periodate oxidation of the "reduced" glycoside VI was performed. The oxidation product was reduced with sodium borohydride and was then hydrolyzed under acid conditions. The hydrolysate was shown by PC and GLC to contain ethylene glycol, glycerol, and erythritol, which shows a 1 → 4 linkage between the glucose and the uronic acid.

In Scheme 1, the glycolipid II is shown with an α,α configuration of the glycosidic bonds. We adopted this configuration on the basis of a calculation of molecular rotations according to Klyne for all four possible anomeric D-glycopyranosyl-D-glucopyranuronosyldiglycerides.

Glycoside	$[\alpha]_D^0$	$[M]_D^0$
Methyl α-D-glucopyranoside [5]	+158.2	+306.1
Methyl β-D-glucopyranoside [6]	− 34.2	− 66.3
Methyl α-D-glucopyranuronoside [7]	+129.0	+268.3
Methyl β-D-glucopyranuronoside [8]	− 60.0	−124.8
D-1,2-Di-O-stearoylglycerol [9]	− 2.6	− 16.2

Glycoside	$[\alpha]_D^0$	$[M]_D^0$
Calculated for D-glucopyranosyl-D-glucopyranuronosyl-2,3-diglycerides with the following configurations of the glycosidic linkages:		
α, α		+558.2
α, β		+173.5
β, α		+185.7
β, β		−199.0
Found for the glycolipid II	+67.7	+594.4

Thus, the glycolipid II is a 1-O-[4-O-(α-D-glucopyranosyl)-α-D-glucopyranuronosyl]-2,3-diglyceride.

A diglycosyldiglyceride containing glucose and glucronic acid residues has been isolated previously from the cells of Pseudomonas diminuta [10]. The glycolipid II described in the present paper differs from this uronoside by the configuration at the anomeric C atom of the glucose residue.

EXPERIMENTAL

Materials and General Methods. The growth of the culture of Str. LA 7017, the extraction of the cell lipids, and the preparation of the adsorbents and plates for TLC were carried out by the methods described previously [4].

TLC was carried out with the following systems of solvents: 1) propan-1-ol–water (7 : 1) [11] for carbohydrates, glycosides, and other polyols, and 2) chloroform–methanol–water (65 : 25 : 4) for lipids. Paper chromatography was performed on Leningrad S paper by the descending method with the following solvent systems: 3) ethyl acetate–pyridine–water (5 : 2 : 5, upper phase), 4) butan-1-ol–pyridine–water (6 : 4 : 3), 5) benzene–butan-1-ol–pyridine–water (1 : 5 : 3 : 3, upper phase), 6) ethyl acetate–pyridine–water–acetic acid (5 : 5 : 3 : 1), 7) propan-1-ol–ethyl acetate–water (7 : 1 : 2). The spots were revealed in TLC by means of a 0.1% ethanolic solution of morin, by 50% H_2SO_4 with subsequent heating at 180°C, by diphenylamine [1], by orsin [2], by periodate–Schiff's reagent [3], and by ammoniacal silver nitrate. The substances were revealed on paper with ammoniacal silver nitrate, aniline hydrogen phthalate, KIO_4–benzidine, $NaIO_4$–Schiff's reagent, and the p-anisidine reagent.

The methyl esters of the fatty acids were analyzed by GLC in a column containing 10% of poly(ethylglycol succinate) on Chromosorb W (40-60 mesh) at 160°C, the carbohydrates in the form of the trimethylsilyl derivatives of the corresponding methyl glycosides [12] in a column containing 3% of SE-30 on Chromosorb W at 146°C, and the simple polyols (in the form of acetates) in a column containing 10% of Reoplex-400 on Chromosorb W at 140°C; in all cases the carrier gas was argon (60 ml/min).

In addition, the phenol method was used for the quantitative determination of glucose [13], the carbazole method for glucuronic acid [14], and the chromotropic acid method for formaldehyde [15]. When glucose and glucuronic acid were present simultaneously in the glucosides to be analyzed (and the amounts of both were determined), suitable corrections were introduced. The amount of glycerol was determined from the formaldehyde formed in its periodate oxidation.

The IR spectra were recorded on a UR-10 spectrograph (Zeiss, GDR).

Isolation of the Glycolipid II. 20 g of the total lipids in 60 ml of chloroform was deposited on a column containing 1.5 kg of silica gel. The column was eluted with 3 liters of chloroform and then elution was continued with mixtures of chloroform and methanol with increasing contents of the latter (from 25 : 1 to 1 : 1) and, finally, with pure methanol. 25-ml fractions were collected. They were analyzed by TLC in system 2 and the fractions containing the glycolipids I and II (to a total amount of 3.5 g) were combined and chromatographed on a column containing 200 g of silica gel impregnated with boric acid. The column was washed with 1 liter of a mixture of chloroform and methanol (19 : 1) and then with 1 liter of a mixture of chloroform and methanol (7 : 1). 1.2 g of the glycolipid I was eluted. Further elution with 1 liter of a mixture of chloroform and methanol (2 : 1 and 1 : 1) gave 500 mg of a fraction enriched in the glycolipid II. The latter was isolated by preparative TLC (20 × 20 cm plates with a 0.3-0.4 mm thick layer of adsorbent) in system 2; not more than 45 mg of mixture was deposited on each plate. A total of 250 mg (1.25% of the weight of the dry mycelium) of the glycolipid II was obtained with R_f 0.17, R_I^* 0.49 in system 2, R_f 0.24, R_I 0.73

*Mobility relative to the glycolipid I.

[chloroform–methanol–acetic acid–water (80 : 13 : 8 : 0.3)]; R_f 0.23, R_I 0.59 [chloroform–acetone–methanol–acetic acid–water (5 : 2 : 1 : 1 : 0.5)]; R_f 0.19, R_I 0.45 [chloroform–methanol–7 N aqueous ammonia (17 : 7 : 1)]. The oily substance obtained was dissolved in 2 ml of chloroform and the solution was treated with 8 ml of methanol and left at 0°C for 2 days, after which 180 mg of the glycolipid II was filtered off in the form of a white powder with mp 234-235°C, $[\alpha]_D^{20}$ + 67.7° (c 0.89; chloroform).

Found, %: C 58.06; H 9.04. Calculated for $C_{45}H_{81}O_{16}Na$*, % C 59.9; H 9.10.

Acid Hydrolysis of the Glycolipid II. Lipid II (19 mg) was hydrolyzed in a similar manner to the glycolipid I [4], but the time of hydrolysis was increased to 20 h. The lipophilic fraction was methylated with diazomethane and the methylated product (10.4 mg), consisting, according to TLC, of a mixture of fatty esters [R_f 0.08 in the hexane–ether (85 : 15) system] was analyzed by GLC (for details, see preceding page). The contents of glucose and glycerol in the aqueous layer were determined colorimetrically. The yields were 3.8 and 1.98 mg, respectively. The molar ratio of fatty acid to glucose to glycerol was 1.90 : 0.98 : 1.0.

Alkaline Hydrolysis of the Glycolipids II. The glycolipid II (14.2 mg) was hydrolyzed with caustic potash in chloroform–methanol [4], the lipophilic part of the hydrolysate was treated with an ethereal solution of diazomethane, and the mixture of methyl esters of fatty acids (4.20 mg) was analyzed by GLC. The amounts of glucose and glucuronic acid in the water-soluble fraction of the hydrolysate were determined colorimetrically (found 1.51 and 1.55 mg, respectively). The molar ratio of fatty acids to glucose to glucuronic acid was 2.05 : 1.05 : 1.0.

Evaporation of the aqueous solution gave the glycoside IV; on PC, R_{glu} (mobility relative to glucose) 0.18 (system 3), 0.24 (4), 0.33 (6). The action of an ethereal solution of diazomethane on a solution of IV in methanol gave the methyl ester of V; R_{glu} on PC 0.65 (system 3), 0.62 (4), 0.79 (6).

The Glycoside VI. A solution of 8.2 mg of the methyl ester V in 1.5 ml of water was treated with 10 mg of $NaBH_4$ and the mixture was left at 20°C for 1.5 h. Then the excess of hydride was decomposed by the addition of acetic acid. The mixture was shaken with Dowex-50 (H^+), the resin was filtered off, the filtrate was evaporated to dryness, and the boric acid was removed in the form of trimethyl borate by distillation with methanol (3 × 10 ml). This gave the glycoside VI with mp 173-176°C (from ethanol–methanol); R_{glu} on PC 0.62 (system 3), 0.60 (4), 0.77 (6); R_{glu} on TLC 0.81 (system 1).

Periodate Oxidation of the Glycoside VI. A solution of 10 moles of the glycoside VI in 1 ml of water was treated with 4 ml of 0.025 M $NaIO_4$ solution. The mixture was left in the dark at 20°C until the consumption of periodate ceased (72 h), which was determined colorimetrically [16]. A total of 3.92 moles of periodate (per mole of glycoside) was consumed. The periodate and the iodate ions were precipitated with a saturated solution of barium acetate and the precipitate was separated off by centrifuging. The solution was then treated with 10 mg of $NaBH_4$ and the mixture was left at 20°C for 24 h. Then the excess of hydride was decomposed with acetic acid and the mixture was treated with Dowex-50 (H^+), the resin was filtered off, the filtrate was evaporated to dryness, and the boric acid was eliminated in the form of trimethyl borate by distillation with methanol (3 × 10 ml). The residue after evaporation was hydrolyzed with 1.5 ml of 2 N hydrochloric acid at 105°C for 2 h. The hydrolysate was neutralized with Amberlite XE-58 (CO_3^-), concentrated, and analyzed by PC in systems 1 and 7, by TLC in system 1, and by GLC. The ratio of glycerol to erythritol was 0.91 : 1.0.

Aluminum Hydride Reduction of the Glycolipid II. A solution of 16 mg of the glycolipid II in 2 ml of tetrahydrofuran was shaken with an excess of Dowex-50 (H^+) at 20°C for 15 min, the resin was filtered off, and the filtrate was treated with an ethereal solution of diazomethane. The solvent was distilled off and the residue was reduced with $LiAlH_4$ in tetrahydrofuran in a similar manner to the glycolipid I [4]. The lipophilic fraction of the reduction products consisted of a mixture of fatty alcohols (6.4 mg according to GLC). According to TLC and PC in systems 1, 3, 4, and 6, the aqueous solution contained the glycoside VI as the sole product. The latter was hydrolyzed by heating at 100°C with 1.5 ml of 2 N H_2SO_4 for 4 h. The hydrolysate was neutralized with Amberlite XE-58 (CO_3^-) and was then concentrated and was analyzed by PC. The content of glucose in the hydrolysate was 4.8 mg. The molar ratio of fatty alcohols to glucose was 1.0 : 1.05.

*In the calculation of the C and H contents expected theoretically, it was assumed that the lipid contained only the $C_{15:0}$ acid; the presence of $C_{16:0}$ and $C_{17:0}$ acids does not introduce an appreciable error.

Mild Acid Hydrolysis of the Glycoside IV. A solution of 6 mg of the glycoside IV in 1 ml of 0.1 M hydrochloric acid was heated at 104°C for 50 min. After cooling, the mixture was neutralized with Amberlite XE-58 (CO_3^-), and was then evaporated to a volume of ~0.2 ml and was deposited on paper. The chromatogram was run in system 7, the band with R_{glu} 0.16 was cut out, and the glyceryl glucuronoside (VII) was eluted with 15 ml of methanol (content of uronic acid 1.08 mg and of glycerol 0.51 mg). The eluate was evaporated to a volume of 1 ml and treated with an ethereal solution of CH_2N_2; the mixture was evaporated to dryness and the residue was treated with a solution of 5 mg of $NaBH_4$ in 1 ml of water; after 2 h, as in the case of VI, the glucoside VIII was isolated, and this was oxidized with periodate as in the case of VI.

The Methyl Derivative IX. A solution of 20 mg of the glycolipid II in 2 ml of a mixture of dimethylformamide and methyl iodide (1 : 1) was heated with 60 mg of silver oxide with stirring at 50°C for 15 h, after which another 100 mg of silver oxide was added; stirring was continued for 20 h. After cooling, the mixture was treated with 7 ml of chloroform, the precipitate was eliminated by centrifuging, the supernatant liquid was washed with water (3 × 5 ml), and the solvent was distilled off. By TLC the residue yielded 14 mg of the O-methyl derivative IX with R_f 0.41 in the chloroform–ethyl acetate (5 : 1) system. IR spectrum (in CCl_4): ν_{max} 1745 cm^{-1} (ester groups), 1166, 1111, and 1047 cm^{-1} (C–O bonds).

Methanolysis of the Methyl Derivative IX. A mixture of 5 mg of the methyl derivative IX and 3 ml of 5% HCl in methanol was boiled for 10 h. After cooling, the mixture was neutralized with Amberlite XE-58 (CO_3^-). Glycerol was detected in the methanolysate by paper chromatography in systems 3, 5, and 7 and by TLC in system 1; 1,2,3,4,6-penta-O-methylglucose was detected by TLC on the methanolysate in the chloroform–ethyl acetate (2.5 : 1) and ethyl acetate–tetrahydrofuran (5 : 1) systems.

SUMMARY

A new glycolipid, a 1-O-[4-O-(α-D-glucopyranosyl)-α-D-glucopyranuronosyl]-2,3-diglyceride has been isolated from the total cell lipids of Streptomyces LA 7017.

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