A GLUCURONOSYLDIGLYCERIDE FROM AN ACTINOMYCETE

A MASS-SPECTROMETRIC INVESTIGATION OF GLYCEROL GLYCOSIDES

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Glycosyldiglycerides are a widely distributed class of microbial lipids [1]. The glycosidic moieties of the molecules of these compounds contain, as a rule, the residues of three sugars: glucose, galactose, and mannose. Glycosyldiglycerides containing other carbohydrates are a comparatively rare phenomenon. They include, in particular, the uronosyldiglycerides. The first glycolipids of this type were reported by Wilkinson, who isolated from Pseudomonas rubescens [2] a 1-O-(β-D-glucopyranuronosyl)-2,3-diglyceride, and from Pseudomonas diminuta [3] 1-O-(α-D-glucopyranuronosyl)- and 1-O-[4-O-(β-D-glucopyranosyl)-α-D-glucopyranuronosyl]-2,3-diglycerides. However, the identification of these uronosides was not brought to completion, since the position of the fatty acid residues was assumed solely on the basis of analogy with known diglyceryl hexosides.

The isolation from an unidentified strain of moderately halophilic bacteria of a glycolipid which, according to an analysis of its degradation products, may be a glucuronosyldiglyceride has recently been reported [4, 5]. Nevertheless, the authors found a considerable discrepancy between the chromatographic behavior of this lipid and that of a sample of a glucuronodiglyceride obtained from Wilkinson.

We have previously reported the isolation from Streptomyces LA 7017 of two new representatives of the class of uronosyldiglycerides – a 1-O-[4-O-(α-D-glucopyranosyl)-2-O-acyl-α-D-galactopyranuronosyl]-2,3-diglyceride [6] and a 1-O-[4-O-(α-D-glucopyranosyl)-α-D-glucopyranuronosyl]-2,3-diglyceride [7]. The present paper gives the results of a study of the glucuronosyldiglyceride (I) from the cells of a strain of Streptomyces producing the antibiotic aureolic acid [8, 9].

The glucuronosyldiglyceride (I) was isolated by chromatographing the combined cell lipids obtained by extracting the lyophilized mycelium with mixtures of chloroform and methanol (2:1 and 1:1).

On thin-layer chromatograms, (I) gives the coloration characteristic for glycolipids on treatment with diphenylamine reagent [10] or with periodate and Schiff's reagent [11]. The IR spectrum of (I) contains intense absorption bands of alcoholic OH groups (3380 cm^{-1}), an ester carbonyl group (1742 cm^{-1}), an ionized carboxy group (1608 cm^{-1}), and C–O bonds (1100 cm^{-1}).

Under the conditions of acid methanolysis (Scheme 1), compound (I) forms a mixture of methyl esters of fatty acids and a mixture of water-soluble products. The former were analyzed by a combination of gas–liquid chromatography (GLC) and mass spectrometry; the results of the analysis are given below:

Fatty acids	Amount, %	Fatty acids	Amount, %
iso-$C_{14:0}$	0.7	iso-$C_{16:0}$	23.8
n-$C_{14:0}$	1.0	n-$C_{16:0}$	13.6
iso-$C_{15:0}$	22.7	iso-$C_{17:0}$	24.5
n-$C_{15:0}$	0.1	n-$C_{18.1}$	13.7

In the hydrophilic fraction of the methanolyzate the presence of glycerol and of a mixture of methyl esters of anomeric methyl glucuronosides was found by the GLC method.

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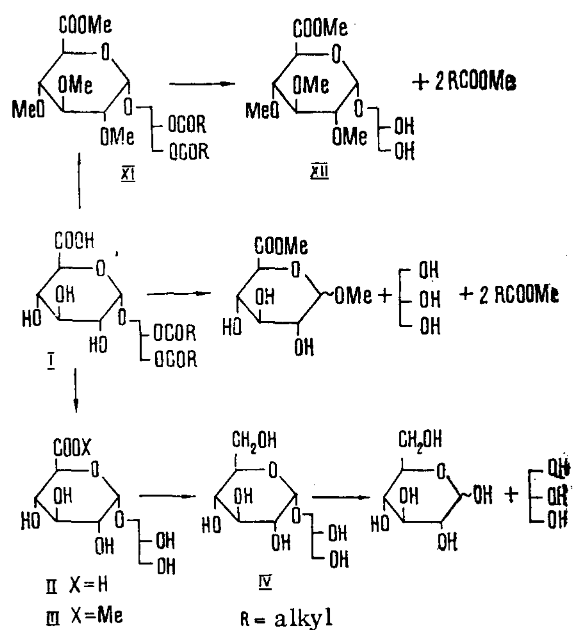
Scheme 1



Alkaline methanolysis cleaved (I) into methyl esters of fatty acids and a single water-soluble product which in its behavior on paper chromatography (PC) proved to be similar to 1-O-(α-D-glucopyranuronosyl)glycerol (II); we obtained the latter compound previously by the degradation of 1-O-[4-O-(α-D-glucopyranosyl)-α-D-glucopyranuronosyl]-2,3-diglyceride [7]. The methyl ester (III) formed by the treatment of (II) with diazomethane was reduced with sodium tetrahydroborate. The reduction product – the glucosylglycerol (IV) – on acid methanolysis gave glycerol and glucose in a molar ratio of 1 : 1. The results of an investigation of the "reduced" glycoside (IV) by the GLC method showed that, with respect to its

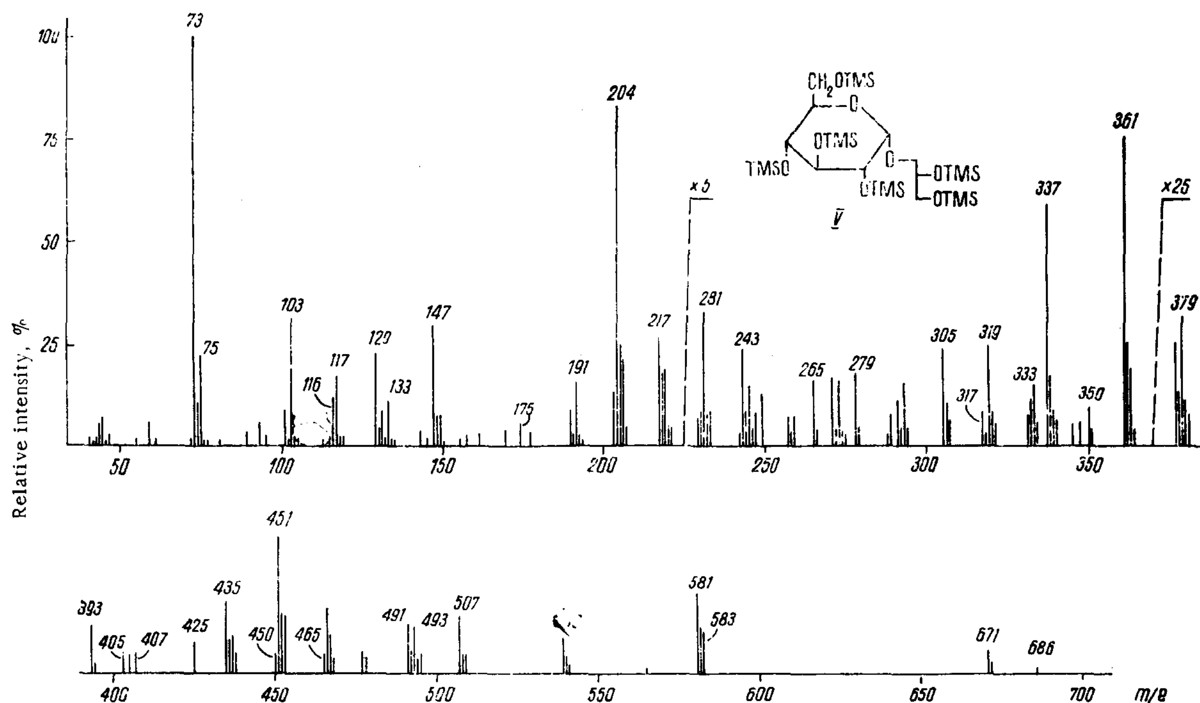
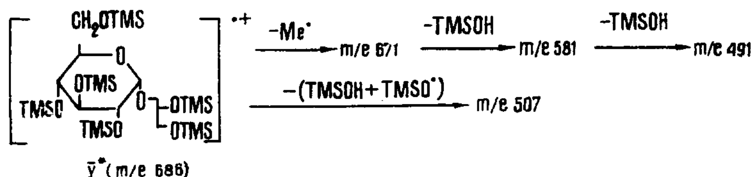


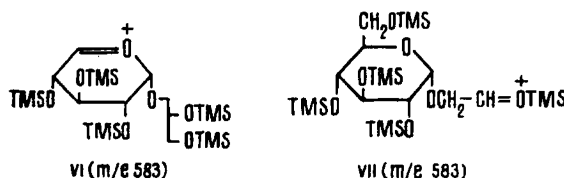
Fig. 1. Mass spectrum of the TMS derivative of (V).

retention volume, it was identical with 1-O-(α-D-glucopyranosyl)glycerol and differed from the corresponding β anomer. The mixture of 1-O-(α- and β-D-glucopyranosyl)glycerols was prepared by the methodof Brundish and Baddiley [12].

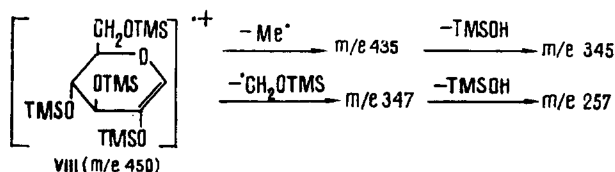
We obtained the final proof of the structure of (IV) by a mass-spectrometric study of its trimethylsilyl (TMS) derivative (V). The spectrum of (V) (Fig. 1) can be interpreted satisfactorily on the basis of the available information on the fragmentation under electron impact of the TMS derivatives of α-D-glucopyranose and methyl α-D-glucopyranoside [13]. The peak of the molecular ion of (V) (V*, m/e 686) possesses a very low intensity, and the loss by the molecular ion of Me· TMSOH (TMS = $SiMe_3$) and TMSO· leads to the formation of the following ions:



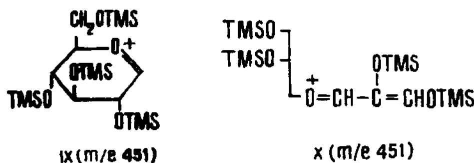
The ion $[M-CH_2OTMS]^+$ observed in the spectra of the TMS derivatives of the above-mentioned carbohydrates can arise in the case of (V) by two routes: by the splitting of the C_5-C_6 bond of the glucose residue and by the cleavage of the C_2-C_3 bond of the glycerol moiety of the molecule; these directions of decomposition are represented by the structures of the isomeric ions (VI) and (VII).



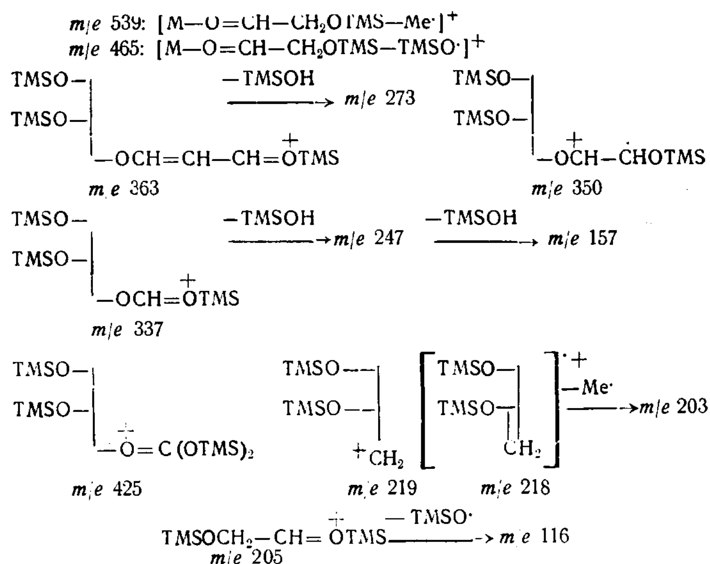
The splitting out of one and of two molecules of TMSOH from (VI) and (VII) leads to the appearance of ions with m/e 493 and 403. The elimination from the molecular ion of bis-O-(trimethylsilyl)glycerol gives rise to an ion radical (VIII, m/e 450) from which ions with m/e 435, 347, 345, and 257 arise.



The spectrum of (V) contains a series of peaks (m/e 73, 75, 89, 103, 117, 129, 133, 147, 189, 191, 204, 217, 221, 231, 243, 265, 271, 279, 291, 305, 317, 319, 332, 333, 361, 379, 393, 407, 451), that are characteristic for the mass spectra of the TMS derivatives of hexapyranoses and their glycosides; in all these cases, the natures of the corresponding ions are probably identical. The peak of the ion with m/e 204 is the second in intensity, and this fact unambiguously shows the pyranose form of the glucose residue in the molecule of (V) [and, consequently, in the molecules of (I-IV), as well]. In the spectrum of (V), the peak of the ion with m/e 451 is slightly stronger than in the spectra of the TMS derivatives of methyl glycoside [13] and of disaccharides [14, 15]. This is probably the consequence of the fact that in (V) this peak may represent two ions: (IX), arising from the carbohydrate residue, and (X), including the glycerol residue. The subsequent loss by these ions of two molecules of TMSOH corresponds to the peaks of ions with m/e 361 and 271.



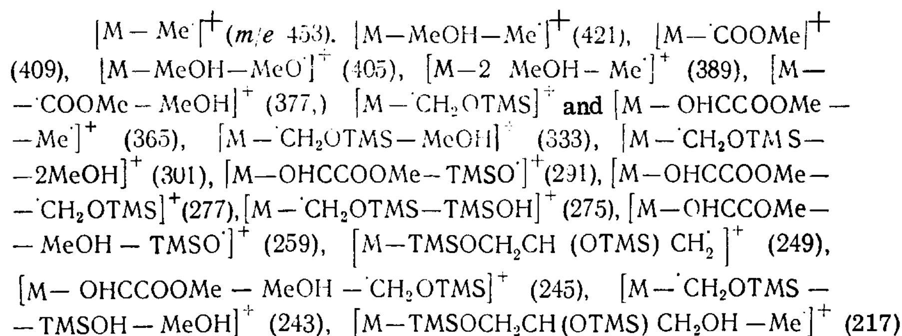
By analogy with the fragmentation of the TMS derivative of the methyl glycoside, other ions containing the aglycone residue (or part of it) are also formed:



The spectrum considered is practically identical with the mass spectrum of the TMS derivative of synthetic 1-O-(α-D-glucopyranosyl)glycerol, which was obtained by the condensation of D-acetobromoglucose with 1,2-O-propylideneglycerol by the method of Wulf et al. [16] and subsequent hydrolysis of the condensation product; slight differences are observed only in the intensities of some peaks.

The results of the investigation of the glycoside (IV) unambiguously show that it is 1-O-(α-D-glucopyranosyl)glycerol; the D configuration of the glucose residue follows from a calculation of the molecular angles of rotation of the lipid (I) by Klyne's rule. Thus, the water-soluble product (II) of the alkaline methanolysis of (I) must have the structure of 1-O-(α-D-glucopyranuronosyl)glycerol.

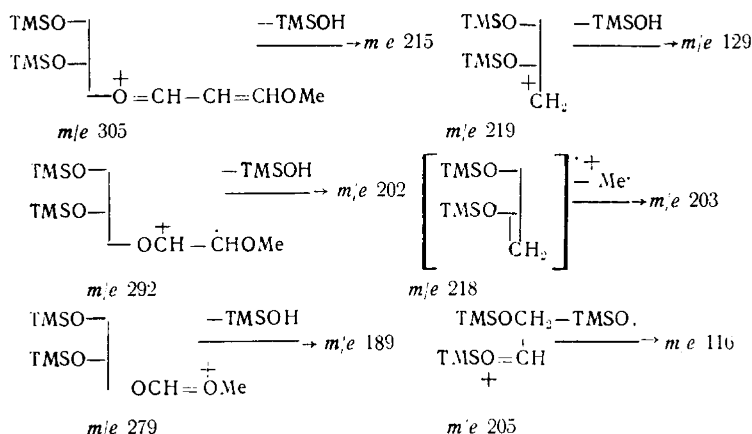
We determined the number and position distribution of the fatty acid residues in the molecule of (I) by the exhaustive methylation of this substance followed by alkaline methanolysis. The O-methyl derivative (XI) (Scheme 1) was obtained in satisfactory yield by the treatment of (I) with methyl iodide in dimethylformamide in the presence of silver oxide. The alkaline methanolysis of (XI) led to the formation of methyl esters of fatty acids and the glycoside (XII), the structure of which we proved on the basis of the mass spectrum (Fig. 2) of its TMS derivative (XIII). The peak of the molecular ion in the spectrum of (XIII) is absent and the peaks of the following ions have very low intensities:


$[M-Me^{\cdot}]^{+}$ (m/e 453), $[M-MeOH-Me^{\cdot}]^{+}$ (421), $[M-{}^{\cdot}COOMe]^{+}$ (409), $[M-MeOH-MeO^{\cdot}]^{+}$ (405), $[M-2\,MeOH-Me^{\cdot}]^{+}$ (389), $[M-{}^{\cdot}COOMe-MeOH]^{+}$ (377,) $[M-{}^{\cdot}CH_2OTMS]^{+}$ and $[M-OHCCOOMe-Me^{\cdot}]^{+}$ (365), $[M-{}^{\cdot}CH_2OTMS-MeOH]^{+}$ (333), $[M-{}^{\cdot}CH_2OTMS-2MeOH]^{+}$ (301), $[M-OHCCOOMe-TMSO^{\cdot}]^{+}$(291), $[M-OHCCOOMe-{}^{\cdot}CH_2OTMS]^{+}$(277), $[M-{}^{\cdot}CH_2OTMS-TMSOH]^{+}$ (275), $[M-OHCCOMe-MeOH-TMSO^{\cdot}]^{+}$ (259), $[M-TMSOCH_2CH\,(OTMS)\,CH_2^{\cdot}]^{+}$ (249), $[M-OHCCOOMe-MeOH-{}^{\cdot}CH_2OTMS]^{+}$ (245), $[M-{}^{\cdot}CH_2OTMS-TMSOH-MeOH]^{+}$ (243), $[M-TMSOCH_2CH(OTMS)\,CH_2OH-Me^{\cdot}]^{+}$ (217)

The spectrum of (XIII) contains almost all the characteristic peaks observed in the fragmentation of completely methylated glucuronic acid [17] (exceptions, of course, are the peaks corresponding to the ions in the structure of which a residue of the aglycone is present): m/e 233,† 201,† 173, 169,† 163, 141, 131, 111, 101, 88, 85, 75, 73, 45. It follows from this that there are methoxy groups only at C_2, C_3, and C_4 of

† m/e 233 $\xrightarrow[m^{*}173.4]{-MeOH}$ m/e 201 $\xrightarrow[m^{*}142.8]{-MeOH}$ m/e 169.

the carbohydrate moiety of the molecule of (XIII), while TMS groupings are attached to the oxygen atoms in positions 2 and 3 of the glycerol residue. The structures of the ions including a glycerol residue are apparently analogous to the structures of the corresponding ions formed in the fragmentation of (V):



Thus, the molecule of (I) contains two fatty acid residues (confirmed by the results of quantitative analysis), both being attached to oxygen atoms of the glycerol residue, while the OH groups of the glycosidic moiety are free. Consequently, the lipid (I) is a 1-O-(α-D-glucopyranuronosyl)-2,3-diglyceride.

Since the isolation of the diglyceryl uronosides from the cell lipids of actinomycetes is difficult and requires the use of special methods of chromatography in each individual case (see [6, 7]), we decided to determine the possibility of identifying these glycolipids in the chromatographic fractions by investigating the products of their alkaline deacylation by the method of combined GLC and mass spectrometry. For this purpose we studied the mass spectra of the TMS derivatives of (XIV) and (XV) obtained, respectively, from the glyceryl uronoside (II) and its methyl ester (III). The fragmentation of the molecular ions of (XIV) (XIV*) and (XV) (XV*) is largely analogous to the fragmentation of (V*): the scheme of decomposition given for the first two compounds [Scheme 2, and the mass spectrum of (XV), Fig. 3] does not require detailed consideration (the m/e values of the ions formed in the decomposition of (XIV) are given in parentheses and the mass numbers of the ions the peaks of which are absent from the spectra are given in square brackets).

A series of peaks observed in the spectra of (XIV) and (XV) is also present in the spectrum of (V). These include the peaks of ions in the structures of which a glycerol residue or part of it is included – m/e 539, 465, 451, 363, 350, 337, 273, 247, 219, 218, 205, 203, 157, 129, 116; the peaks of ions arising from the C_1–C_5 part of the glycoside residue–m/e 379, 305, 279, 265, 217, 204, 189; and the peaks

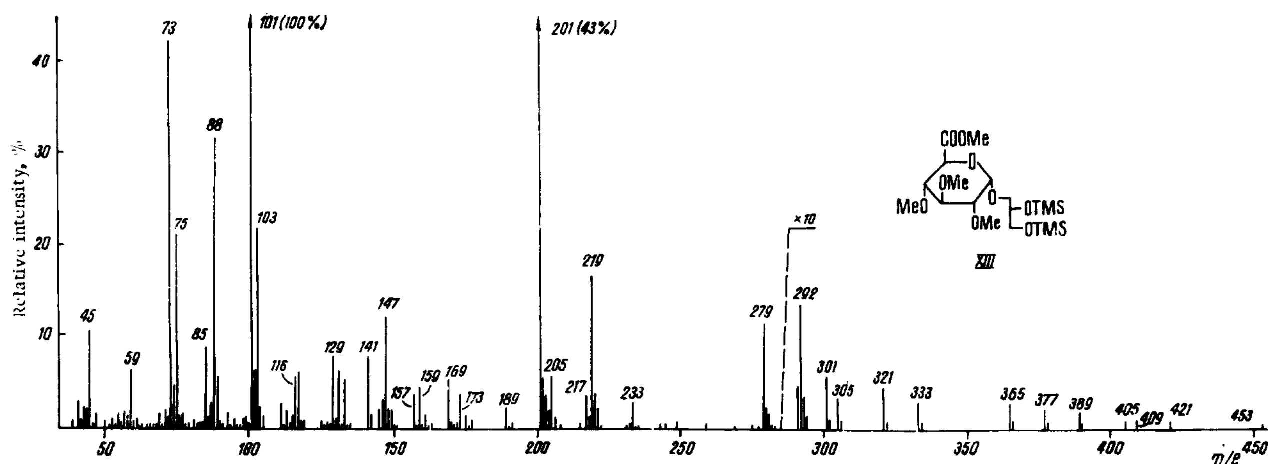


Fig. 2. Mass spectrum of the TMS derivative of (XIII).

Fig. 3. Mass spectrum of the TMS derivative (XV).

usually present in the spectra of TMS derivatives of polyols – m/e 191, 147, 133, 103, 89, 75, 73. The peaks of the ions including the C_3-C_6 and C_2-C_6 atoms of the glycoside residue are present in the spectra of all three TMS derivatives mentioned: the mass numbers of these ions differ according to the nature of the substituent at C_5:

$$R-\overset{5}{C}H=\overset{4}{C}(OTMS)-\overset{3}{C}H=\overset{+}{O}TMS$$

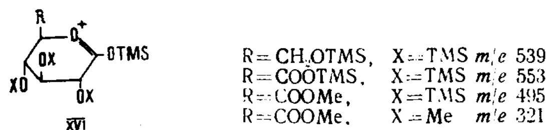
(from V*) R=CH₂OTMS *m/e* 319
(from XIV*) R=COOTMS *m/e* 333
(from XV*) R=COOMe *m/e* 275

$$R=\overset{5}{C}H-\overset{4}{C}H\overset{OTMS}{=}\overset{3}{C}H-\overset{2}{C}H=\overset{+}{O}TMS$$

(from V*) R=CH_2 *m/e* 243
(from XIV* and XV*) R=CO *m/e* 257

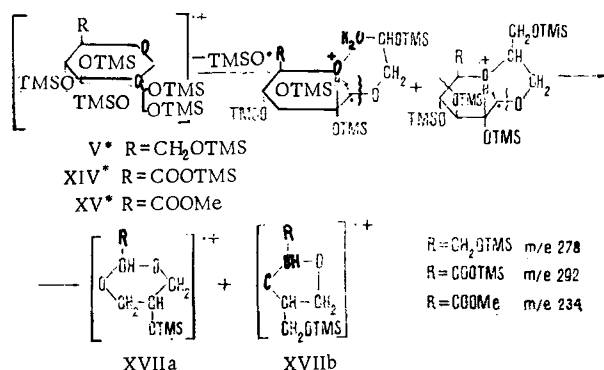
Scheme 2

In conclusion, we must dwell on some features of the mass spectra of (V, XIII-XV) that are apparently connected with the specific structure of the aglycone. In the first place, the spectra of all these compounds contain the peaks of the ions $[M-147]^+$. For (V), this ion has been ascribed the structure $[M - OHCCH_2OTMS - Me\cdot]^+$ (m/e 539), since the peak of an ion of analogous origin is present in the spectrum of the TMS derivative of an α-D-glucopyranoside. However, such an analogy cannot be extended to (XIII-XV). It may be assumed that the ion $[M-147]^+$ arises from (V*, and XIII*-XV*) by the cleavage of the glycosidic bond with the simultaneous loss of a hydrogen atom and the migration of the TMSO group of the glycerol residue to the C_1 atom of the glycoside part of the molecular ion. In this case, the ion mentioned must have the structure (XVI)



R = CH_2OTMS, X = TMS m/e 539
R = COOTMS, X = TMS m/e 553
R = COOMe, X = TMS m/e 495
R = COOMe, X = Me m/e 321

The second feature of the spectrum of (V, XIV, and XV) consists in the presence of fairly strong peaks of ions with m/e 278, 292, and 234, respectively. These peaks are absent from the spectra of the TMS derivatives of hexoses and hexosides [13]; we have found that they are not present, either, in the spectra of the TMS derivatives of methyl α-D-glucosiduronic acid and its methyl ester. The results of a comparison of the mass numbers of the ions under consideration show that they differ according to the chemical nature of the substituent at C_5 of the glycosidic residue. On this basis it may be assumed that the ions include a fragment of the glycerol residue and the C_6 carbon atom. The following route for the formation of the above-mentioned ions is possible:



The elimination of R· from (XVIIa,b) leads to the formation of ions with m/e 175, the peaks of which have a considerable intensity in the spectra of (V, XIV, and XV). A proof of the structure of the ions (XVI) and (XVIIa,b) will be given in a separate communication.

EXPERIMENTAL

Column chromatography and thin-layer chromatography (TLC) were performed with type KSK silica gel (100-150 and 150-200 mesh, respectively). The chromatographic plates were prepared by the method of Bergel'son et al. [18]. Paper chromatography (PC) was performed on Leningrad C "medium" paper by the descending method in the following solvent systems: 1) benzene–n-BuOH–pyridine–water (1 : 5 : 3 : 3; upper phase); 2) EtOAc–pyridine–water (5 : 2 : 5; upper phase), and 3) n-PrOH–EtOAc–water (7 : 1 : 2). To reveal the substances in TLC we used 50% H_2SO_4 with subsequent heating at 180-200°C, a 0.1% ethanolic solution of morin, the diphenylamine reagent [10], and periodate–Schiff's reagent [11]. On paper, the substances were revealed with ammoniacal silver and with KIO_4–benzidine.

All the polyols in the form of their TMS derivatives were subjected to GLC. The TMS derivatives were obtained by the action of diethyl(trimethylsilyl)amine at 20-25°C for 12 h or at 100°C for 1 h. The TMS derivatives and the methyl esters of the fatty acids were analyzed on an LKB-9000 instrument; chromatographic separation was performed in a column (3 × 3000 mm) containing 3% of SE-30 on Chromosorb W (40-60 mesh) with He as the carrier gas (20 ml/min); the temperature for the esters was 190°C,

for the TMS derivatives of the glyceryl glycosides 235-240°C, and for the other TMS derivatives 100-200°C (4°C/min). The fractions corresponding to individual peaks on the chromatograms were introduced directly into the ion source; the energy of the ionizing electrons was 70 eV. The methyl esters of fatty acids were additionally analyzed on a column containing 10% of poly(ethylene succinate) on Chromosorb W with Ar as the carrier gas (60 ml/min) at 180°C. In the quantitative determination of the esters, methyl stearate was used as the standard, and in the determination of glucuronic acid and glycerol the standard was myo-inositol.

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

The culture of the actinomycete was grown on a medium containing 1.5% of soya flour, 2.5% of starch, 0.3% of NaCl, and 0.3% of technical chalk (pH 6.9-7.0) at 28°C for 96 h. The mycelium was separated by centrifuging and was freeze-dried.

Extraction of the Combined Lipids. The freeze-dried mycelium (820 g) was extracted as described previously [6]. This gave 252 g of total cell lipids (30% of the weight of the dry cells).

Isolation of the Glycolipid (I). A solution of 48 g of the combined lipids in 200 ml of $CHCl_3$ was added to a column containing 300 g of silica gel. The column was washed with 1.5 liter of $CHCl_3$, after which elution was continued with mixtures of $CHCl_3$ and MeOH with gradually increasing concentrations of the latter (from 30 : 1 to 1 : 4); elution was completed with pure MeOH. The fractions, of 20 ml each, were analyzed by TLC in the $CHCl_3$–MeOH–water (65 : 25 : 4) system. The fractions containing the (I) (185 mg) were chromatographed on 15 g of silica gel previously kept for 12 h in a mixture of $CHCl_3$–MeOH–AcOH (20 : 1 : 0.5), elution being performed first with the same mixture and then in systems with increasing amounts of MeOH (up to 20 : 4 : 0.5). This gave 33 mg (0.66%) of the total lipids of (I) α_D^{20} +28° (c 0.53; $CHCl_3$); TLC: R_f 0.50, R_{PE} (mobility with respect to phosphatidylethanolamine) 0.80 $CHCl_3$–MeOH–water (65 : 25 : 4) R_f 0.43, R_{PE} 0.71 $CHCl_3$–MeOH–AcOH–water (80 : 13 : 8 : 0.3), R_f 0.40 $CHCl_3$–MeOH–7N NH_4OH (17 : 7 : 1).

Acid Methanolysis of (I). A solution of 11.3 mg of (I) in 1 ml of $CHCl_3$ and 1 ml of MeOH was boiled with 3 ml of a 5% solution of HCl in MeOH for 24 h. After cooling, the mixture was neutralized with Amberlite XE-58 (OH^-), the MeOH was distilled off, and the residue was treated with 2 ml of water and was extracted with petroleum ether–ether (1 : 1) (3× 2 ml). The organic phase yielded a mixture of methyl esters of fatty acids (according to TLC and GLC) and the aqueous phase a mixture of glycerol and methyl esters of methyl glycosiduronic acids (according to GLC). The molar ratio of fatty acid esters to glycosiduronates to glycerol was 1.92 : 1 : 0.95.

The alkaline methanolysis of (I) was performed as described by Wilkinson [2]. The lipophilic fraction of the methanolyzate was treated with an ethereal solution of CH_2N_2 and the resulting mixture of methyl esters of fatty acids was analyzed by GLC. The amount of glucuronic acid in the water-soluble fraction of the methanolyzate, which contained a glyceryl glucuronoside (II) (PC: $R_{glucose}$ 0.14 in system 1) by the carbazole method [19]. The molar ratio of esters to glucuronic acid was 2.04 : 1.

Reduction of (II). A solution of (II) in MeOH was treated with an ethereal solution of CH_2N_2, and the methyl ester (III) was reduced with sodium tetrahydroborate by a method described previously [7]. The glyceryl glycoside (IV) that was formed was hydrolyzed by being heated with 2 N hydrochloric acid at 105°C for 3 h. The hydrolyzate was neutralized with Amberlite XE-58 (OH^-). By the PC method in systems 1-3 and by GLC the hydrolyzate was found to contain glycerol and glucose; their molar ratio was 1 : 0.92 (by GLC) or 1 : 0.85 (from the results of colorimetric methods; the phenol method [20] for the glucose, and periodate oxidation with subsequent determination of the formaldehyde [21] for the glycerol).

O-Methyl Derivative (XI). A mixture of 30 mg of (I), 1 ml of MeI, 2 ml of dimethylformamide, and 150 mg of Ag_2O was stirred at 50°C for 20 h. After cooling, the precipitate was filtered off and washed with $CHCl_3$, the combined filtrate was evaporated to dryness in vacuum, and the residue was subjected to preparative TLC in the benzene–EtOAc (5 : 1) system. This gave 20 mg of (XI), R_f 0.63. IR spectrum (CCl_4): ν_{max} 1749 cm^{-1} (ester carbonyl), 1102 cm^{-1} (C–O bond); the absorption bands of an OH group were absent.

The Glycoside (XII). A solution of 5 mg of (XI) in 0.5 ml of $CHCl_3$ was heated at 40°C for 2.5 h with 1.5 ml of a 0.5 N solution of MeONa in MeOH. After cooling, the mixture was neutralized with Dowex 50 (H^+), the MeOH was distilled off, and the residue was deposited on a column containing 1.5 g of silica gel, from which $CHCl_3$ eluted a mixture of methyl esters of fatty acids, after which MeOH eluted the glycoside (XII).

SUMMARY

1. From the sum of the cell lipids of a strain of Streptomyces producing the antibiotic aureolic acid a glycoside has been isolated which is a 1-O-(α-D-glucopyranuronosyl)-2,3-diacylglycerol.

2. The trimethylsilyl derivatives of glycerol glycosides have been studied by mass spectrometry. Features of the fragmentation under electron impact of the trimethylsilyl derivatives of the glyceryl glucosides have been determined; the results obtained enable the method of combined gas–liquid chromatography and mass spectrometry to be used for the identification of diacylglyceryl uronosides in lipid fractions.

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