ORNITHINE-CONTAINING LIPID FROM Actinomyces globisporus

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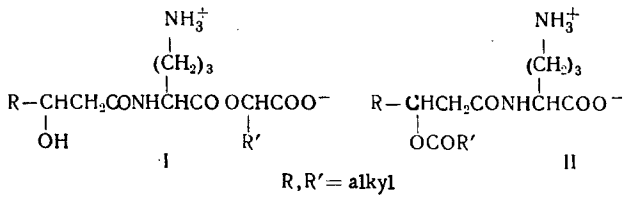
In one of our preceding communications [1] we described the process of isolating from the cells of Actinomyces No. 660-15 an ornithine-containing lipid for which structure (I) was proposed. According to a quantitative analysis, the lipo amino acid amounted to about 35% of the sum of the polar lipids of the actinomycete. This fact, and also some features of the chemical structure of (I) – its bipolar nature and the presence in the molecule of two fatty chains – permitted the assumption that the lipid (I) formed part of the cell membranes of the given microorganism as one of its main structural components.

Continuing a study of the lipids of actinomycetes, we have found that the cells of Actinomyces globisporus 1131* also contain a considerable amount of a lipo amino acid which includes an ornithine residue. The present paper gives the results of the isolation and identification of this lipid (II).



To isolate the (II), the total lipids obtained by the extraction of the freeze-dried mycelium with mixtures of $CHCl_3$ and MeOH (2 : 1 and 1 : 1) were chromatographed on a column of silica gel. In this way it was possible to isolate part of the (II) in the chromatographically pure state in the form of an amorphous

* The producing agent of the antibiotic actinoxanthin [2].

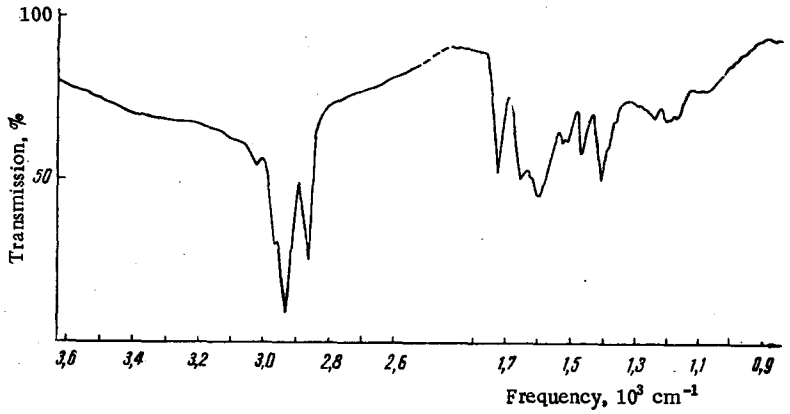


Fig. 1. IR spectrum of the lipo amino acid (II) in chloroform.

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TABLE 1. Fatty-Acid Composition of the Lipo Amino Acid (II)

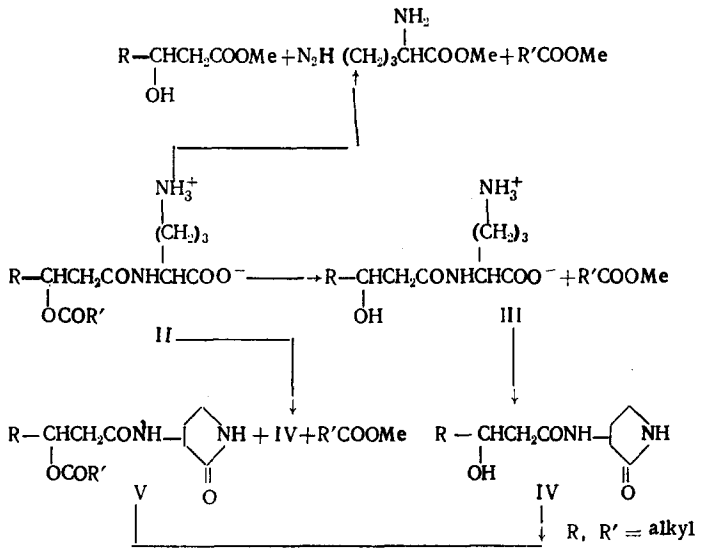
Fatty-Acid		β-Hydroxy fatty acids	
acids	content, %	acids	content, %
iso-$C_{14:0}$	3.6	iso-$C_{15:0}$	6.4
n-$C_{14:0}$	Traces	iso-$C_{16:0}$	51.6
iso-$C_{15:0}$	84.5	iso-$C_{17:0}$	42.0
n-$C_{15:0}$	5.1	–	–
iso-$C_{16:0}$	4.8	–	–
n-$C_{17:0}$	2.0	–	–

mass with $[\alpha]_D^{20}$ equal to −2.4°. For the quantitative isolation of the lipid (II), the fraction containing it was rechromatographed under the same conditions.

The lipo amino acid (II) gives a positive reaction with ninhydrin; its IR spectrum (Fig. 1) shows the absorption bands of an NH_3^+ group (3030 cm^{-1}), of ester and amide carbonyls (1730 and 1648 cm^{-1}, respectively), and of an ionized carboxy group (1598 cm^{-1}). Under the conditions of severe acid methanolysis, (II) is cleaved (Scheme 1) into a lipophilic fraction which consists, according to thin-layer chromatography (TLC), of a mixture of methyl esters of fatty acids and β-hydroxy fatty acids, and a single hydrophilic fragment – the methyl ester of ornithine, which we identified chromatographically on paper (PC) and (in the form of trimethylsilyl and N,N-diacetyl derivatives) by gas–liquid chromatography (GLC). The methyl esters of fatty acids and of β-hydroxy fatty acids were separated by preparative TLC and were analyzed by the method of combined GLC and mass spectrometry (Table 1).

In the mass spectra of the methyl esters of the β-hydroxy acids there are characteristic intense peaks of ions with m/e 74 and 103 and also the peaks of the ions M^+, $[M-1]^+$ (both with a very low intensity), $[M-H_2O]^+$, $[M-H_2O-MeOH]^+$, $[M-CH_2COOOMe]^+$, and $[M-CH_2COOMe-H_2O-1]^+$.

SCHEME 1



The structure of the carbon chains of the acids listed in Table 1 is confirmed by the fact that in the NMR spectra of the methyl esters of both the fatty acids and of the β-hydroxy fatty acids, the C-methyl groups are represented by a doublet signal at 0.92 ppm (J = 6.5 Hz), which shows that the molecules of at least the main components of these mixtures have a terminal isopropyl grouping. The β-hydroxy fatty acids give a positive optical rotatory dispersion curve, which shows the L configuration at the asymmetric C_3 atom [3]. As quantitative analysis showed, the esters of the fatty acids, of the β-hydroxy fatty acids, and of the ornithine were present in the methanolyzate in a molar ratio of 1 : 1 : 1.

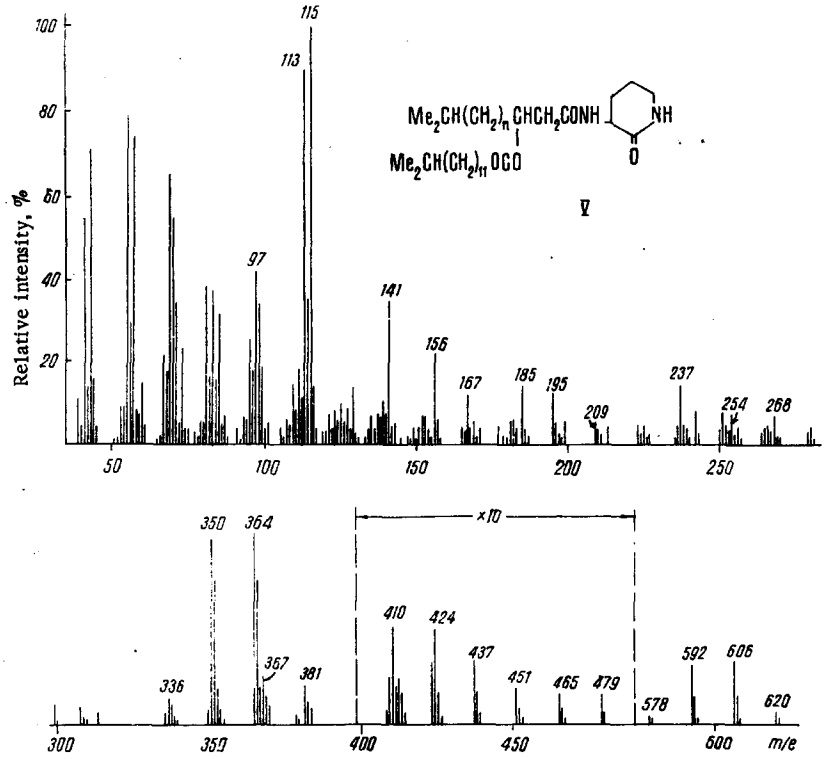
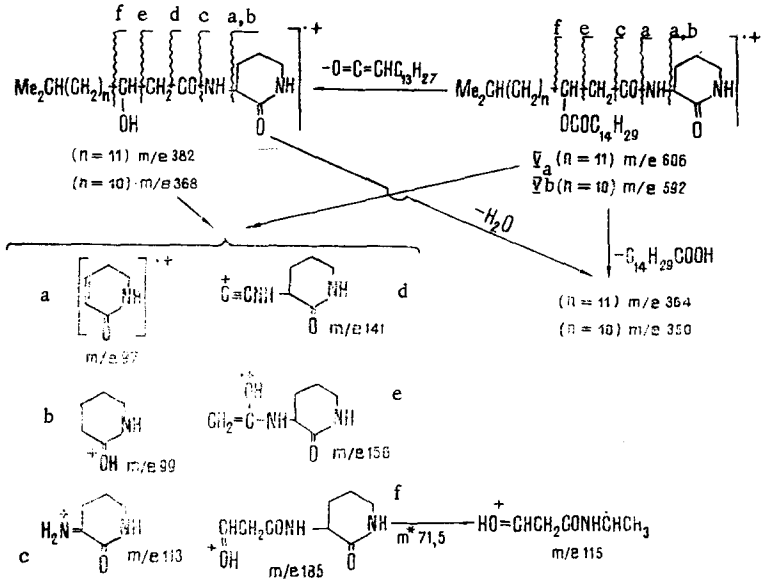


Fig. 2. Mass spectrum of the lactum (V) (70 eV).

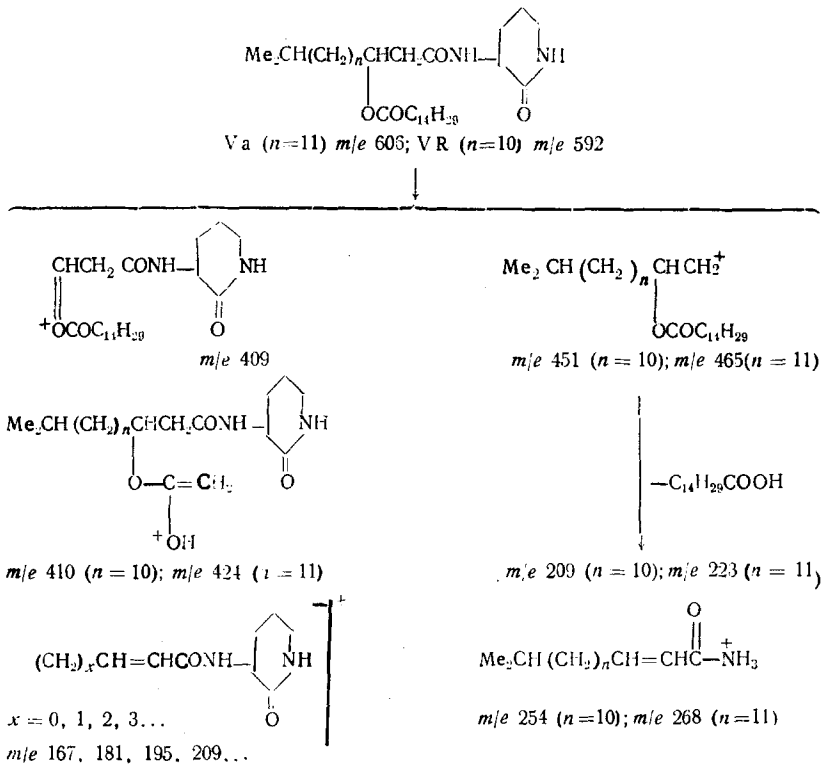
The alkaline methanolysis of (II) formed a mixture of methyl esters of fatty acids (the composition of which did not differ from that given in Table 1) and a ninhydrin-positive product (III) the structure of which follows from the facts given below. The IR spectrum of (III) showed the absorption bands of an alcoholic OH group (3380 cm^{-1}), of an NH_3^+ group (3050 cm^{-1}), of an amide carbonyl (1652 cm^{-1}), and of an ionized carboxyl (1600 cm^{-1}). Severe acid methanolysis of (III) led to the formation of methyl esters of β-hydroxy fatty acids and the methyl ester of ornithine in a molar ratio of 1:1. The result of the action of methanolic HCl at 20°C on (III) followed by treatment with a weak anion-exchange resin formed a product similar in its behavior on TLC in various solvent systems to the lactam (IV) which we have obtained previously by the degradation of the lipo amino acid (I) [1]. The two substances mentioned have practically identical IR and mass spectra and optical rotatory dispersion curves. This shows that the ornithine residue in the molecule of (II) possesses the L configuration, since the L configuration of the same residue in the molecule of the product of the degradation of (I) was shown strictly [1].

The brief acid methanolysis of (II) at 20°C followed by treatment of the methanolyzate with an anion-exchange resin yielded three lipophilic fractions: methyl esters of fatty acids, the lactam (IV), and a ninhydrin-negative fraction more mobile on TLC than (IV) but less mobile than the methyl esters of the fatty acids. On more prolonged methanolysis, this product was split into a mixture of methyl esters of fatty acids and the lactam (IV), and we therefore proposed for it the structure (V). This was confirmed by the results of a study of its mass spectrum (Fig. 2 and Schemes 2 and 3). In the mass spectrum there are four peaks in the region of high mass numbers which correspond to homologous molecular ions with m/e 620, 606, 592, and 578. Of these, only the peaks of the ions with m/e 606 and 592 (Va and Vb, respectively) possess appreciable intensity, which is in full agreement with the relative amounts of the fatty acids found in the lipid (II) [the molecular weights of the homologous lactams (V) containing fatty-acid residues in the combinations $C_{15:0}$-β-hydroxy-$C_{17:0}$ and $C_{15:0}$-β-hydroxy-$C_{16:0}$ are, respectively, 606 and 592]. With the loss by the molecular ions (Va) and (Vb) of the molecule of 13-methyl tetradecanoic acid, ions with m/e 364 and 350 (or, for the minor components, m/e 378 and 336) are formed, the loss of the corresponding acyl radical gives ions with m/e 381 and 367, and the loss of the hydroxy acyl radical gives ions with m/e 365 and 351. The spectrum of (V) contains all the main peaks observed in the mass spectrum of (IV) (see [1]): 382, 368, 364, 350, 185, 156, 141, 115, 113, 99, 97. It can be seen from Scheme 2 that the nature of the ions to which the peaks mentioned correspond is the same in both cases; Scheme 3 shows other directions of the fragmentation of (Va, b).

SCHEME 2

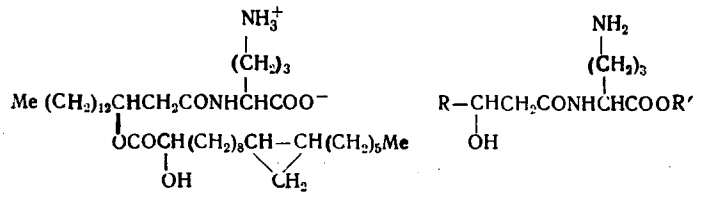


SCHEME 3



It is interesting to note that the mass spectrum of the product formed by the treatment of the lipo amino acid (II) with N-(trimethylsilyl)diethylamine proved to be completely identical with the mass spectrum of the lactam (V). It is difficult to state whether the closure of the lactam ring takes place during the silylation of the lipid or whether the formation of (V) is the consequence of the thermal cyclization of the trimethylsilyl derivative of (II) directly in the ion source of the mass spectrometer (150°C); however, it is not a matter of doubt that the molecule of the lipo amino acid that we isolated does not contain other groupings apart from residues of fatty acids, β-hydroxy fatty acids, and ornithine. Thus, the lipo amino acid (II) is an N^2-[L-(3'-acyloxy)acyl]-L-ornithine.

A lipo amino acid of the type of (II) has recently been discovered in the cells of Pseudomonas rubescens [4], and a lipid of similar structure (VI) has been found in Thiobacillus thiooxidans [5], but the stereochemistry of these compounds was not established.



VI

VII (R= alkyl)

Descriptions have also appeared in the literature of ornithino lipids of the general structure (VII) in the molecules of which the carboxy group of the ornithine residue is esterified either with a fatty alcohol (R' = alkyl) [6, 7], with a β-(acyloxy)ethanol (R' = $CH_2CH_2OCO(CH_2)_8CHMe(CH_2)_7Me$ [8], R' = $CH_2CH_2OCOCH(OH)(CH_2)_{10}CHMe_2$ [9]), or with a monoacyloxyisopropanol [R' = $CHMeCH_2OCO(CH_2)_9CH-CH(CH_2)_5Me$] [8]. Neither monohydric alcohols nor diols were found in the products of the methanolysis of the lipo amino acid (II) that we isolated. A structure of type (VII) is excluded for this lipid also because the lactam (V) formed from it retains both fatty-acid residues of the initial molecule.

EXPERIMENTAL

Type KSK silica gel was used for column chromatography and TLC (100-150 and 150-200 mesh, respectively). The plates for TLC were prepared as described previously [10] and were run in the following solvent systems: 1) $CHCl_3$–MeOH–water (65 : 25 : 4); 2) $CHCl_3$–MeOH–water (60 : 30 : 5); 3) $CHCl_3$–MeOH–concentrated aqueous NH_3 (60 : 30 : 5); 4) $CHCl_3$–Me_2CO–MeOH–AcOH–water (80 : 40 : 20 : 20 : 10); 5) $CHCl_3$–MeOH–water–concentrated aqueous NH_3 (130 : 70 : 8 : 2); 6) $CHCl_3$–MeOH (9 : 1); and 7) hexane–ether (85 : 15). The substances on the chromatograms were revealed by treatment with 50% H_2SO_4 followed by heating to 180-200°C, with a 0.1% ethanolic solution of morin, and with a 0.3% solution of ninhydrin in ethanol.

Paper chromatography (PC) was performed on Leningrad C ["medium"] paper by the descending method in the systems 8) n-BuOH–AcOH–water (4 : 1 : 5, upper phase), 9) water-saturated phenol, and 10) phenol–water (78 : 22) + 1% of concentrated aqueous NH_3. The spots of the substances were detected by the ninhydrin reagent.

The methyl esters of fatty acids of β-hydroxy fatty acids were analyzed on an LKB-9000 instrument, and for the chromatographic separation we used a column (3000 × 3 mm) containing 3% of SE-30 on Chromosorb W (40-60 mesh) with helium as the carrier gas (20 ml/min) at column temperatures of 190°C for the methyl esters of fatty acids (standard – methylstearate) and 205°C for the methyl esters of β-hydroxy fatty acids (standard – methyl α-hydroxystearate). The fractions corresponding to the individual peaks on the chromatograms were introduced directly into the ion source; the energy of the ionizing electrons was 70 eV. The mass spectra of the lactams were taken on the same instrument.

Ornithine and its methyl ester were analyzed by the GLC method in the form of the trimethylsilyl derivatives [11]: the N, N-diacetate of ornithine methyl ester was obtained by the action of Ac_2O in pyridine at 70-80°C for 4 h. In the quantitative determination of ornithine, the corresponding lysine derivative was used as standard.

The IR spectrum were taken on a UR-10 spectrograph (Zeiss, GDR) and the optical rotatory dispersion curves on a Cary-60 spectropolarimeter in MeOH (for the lactams) or in heptane (for the β-hydroxy acids).

The culture of Actinomyces globisporous 1131 was grown at 28°C for 39 h in a medium containing 1% of maize extract, 1% of starch, 0.5% of glucose, 0.5% of NaCl, 0.35% of $(NH_4)_2SO_4$, and 0.4% of $CaCO_3$ (pH 7 before sterilization). The mycelium was separated by centrifuging, washed with distilled water, and freeze-dried.

The extraction of the total cell lipids was performed by the method described previously [1]. From 200 g of freeze-dried mycelium the yield of total lipids was 26.4 g (13.2%).

Isolation of the Lipo Amino Acid (II). On a column containing 500 g of silica gel was deposited 26.4 g of the combined lipids in 150 ml of $CHCl_3$. The column was washed with two liters of $CHCl_3$, after which elution was performed with mixtures of $CHCl_3$ and MeOH with a gradual increase in the proportion of the latter (from 30 : 1 to 1 : 1). The eluates (15-ml portions) were analyzed by TLC in systems 2 and 3,. The fraction of the eluates obtained on elution with $CHCl_3$–MeOH (7 : 1) contained chromatographically pure (II) (64 mg). The remaining fractions containing (II) (in admixture with phosphatidylethanolamine) were combined and chromatographed on 100 g of silica gel. Elution with mixtures of $CHCl_3$ and MeOH (10 : 1, 8 : 1, 7 : 1, 6 : 1, and 5 : 1) gave an additional 50 mg of (II) $[\alpha]_D^{20}$–2.4° (c 2.9; $CHCl_3$), R*f* 0.39 (in system 1), 0.61 (2), 0.52 (3), 0.49 (4), 0.45 (5).

Severe Acid Methanolysis of the Lipid (II). A mixture of 10 mg of (II) and 1.5 ml of a 7% solution of HCl in MeOH was heated in a tube at 105°C for 35 h. After cooling, the mixture was neutralized with Amberlite XE-58 (OH^-), the MeOH was distilled off, the residue was treated with 1.5 ml of water, and the mixture was extracted with $CHCl_3$ (3 × 2 ml). The substance from the organic phase was subjected to TLC in system 7. This gave 3.7 mg of the methyl esters of fatty acids with R*f* 0.80 and 3.9 g of the methyl esters of β-hydroxy fatty acids with R*f* 0.12. In the aqueous phase, the methyl ester of ornithine was detected by the GLC method. The molar ratio of methyl esters of fatty acids to methyl esters of β-hydroxy fatty acids to the methyl ester of ornithine was 1.0 : 0.92 : 0.90.

An aliquot of the hydrophilic fraction was hydrolyzed by heating with 2 N hydrochloric acid at 100°C for 15 h, and ornithine was found in the hydrolyzate by PC (in systems 8-10) and GLC.

Alkaline Methanolysis of the Lipid (II). To a solution of 20 mg of (II) in 1 ml of $CHCl_3$–MeOH (1 : 1) was added 1 ml of a 0.5 N solution of MeONa in MeOH. The mixture was kept at 25°C for 1 h and was neutralized with Dowex-50 (H^+) at 0°C. The solvents were distilled off, and the residue was chromatographed on 1.5 g of silica gel; $CHCl_3$ eluted 7.6 mg of methyl esters of fatty acids, and $CHCl_3$–MeOH (1 : 1) eluted 10 mg of the hydroxyacylornithine (III), R*f* 0.29, R_{II}* 0.47 (in system 2); R*f* 0.21, R_{II} 0.40 (3); R*f* 0.26, R_{II} 0.54 (4).

By the method described above, aliquots of (III) yielded methyl esters of β-hydroxy fatty acids and ornithine in a molar ratio of 1 : 1.1 (according to GLC).

The Lactam (IV). A solution of 10 mg of (III) in 1.5 ml of a 5% solution of HCl in MeOH was left at 20°C for 24 h. The mixture was neutralized with Amberlite XE-58 (OH^-), the solvent was distilled off, and the residue was subjected to TLC in system 6. This gave 7.2 mg of (IV) with R*f* 0.65. IR spectrum ($CHCl_3$): ν_{max} 3364 cm^{-1} (alcoholic OH and amide NH groups), 1662 and 1564 cm^{-1} (amide bands I and II).

Mild Acid Methanolysis of the Lipid (II). A mixture of 17 mg of (II) and 2 ml of a 5% solution of HCl in MeOH was left at 35°C for 4 h, after which it was neutralized with Amberlite XE-58 (OH^-), the MeOH was distilled off, and the residue was investigated by TLC in system 6. This gave 3.9 mg of methyl esters of fatty acids migrating with the solvent front, 5.2 mg of the lactam (IV) with R*f* 0.78.

A solution of (V) (~ 1-1.5 mg) in 0.5 ml of a 5% solution of HCl in MeOH was left at 25°C for 48 h. After the working up of the reaction mixture described above, methyl esters of fatty acids and a substance identical with the lactam (IV) in its mobility on TLC and in its mass spectrum were obtained.

CONCLUSIONS

From the cell lipids of Actinomyces globisporus 1131 a lipo amino acid has been isolated which is a N^2-[L-(3'-acyloxy)acyl]-L-ornithine. The configurations of all the asymmetric centers in an ornithino lipid have been established for the first time.

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* R_{II} is the mobility relative to the lipid (II).

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