A NEW ORNITHINE-CONTAINING LIPID FROM Actinomyces NO. 660-15

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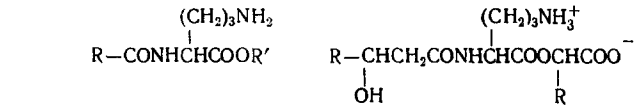
In 1963, Asselineau and his colleagues [1] isolated from the cells of a mycobacterium a lipo-amino acid containing no phosphorus and constructed from fatty acid and ornithine residues. Subsequently, similar ornithine-containing lipids were found in other microorganisms [2-9]. However, only in two cases has their structure been shown completely: for siolipin B (I) isolated from Streptomyces sioyaensis [5-8] and its analog (II), obtained from Rhodopseudomonas spheroides [9].



I. R = alkyl or 2-hydroxyalkyl
R′ = hydroxyalkyl
II. R = R′ = alkyl

III. R = alkyl

In the present paper we describe the isolation and determination of the structure of a lipo-amino acid (III) which we have found in the cells of Actinomyces No. 660-15.* The lipo-amino acid (III) is structurally related to the ornithine-containing lipids mentioned above but differs from them by the fact that as the alcoholic component its molecule contains a residue of an aliphatic α-hydroxy acid, thanks to which this lipid acquires the nature of a bipolar ion. Lipid (III), isolated from the total lipids by chromatography on silica gel in the form of a white powder with mp 147-148°C, $[\alpha]_D^{20} - 14.9°$, gives a positive ninhydrin reaction and does not contain phosphorus. Its IR spectrum (Fig. 1) has absorption bands of an alcoholic HO-

*Producer of the antibiotic albofungin [10, 11].

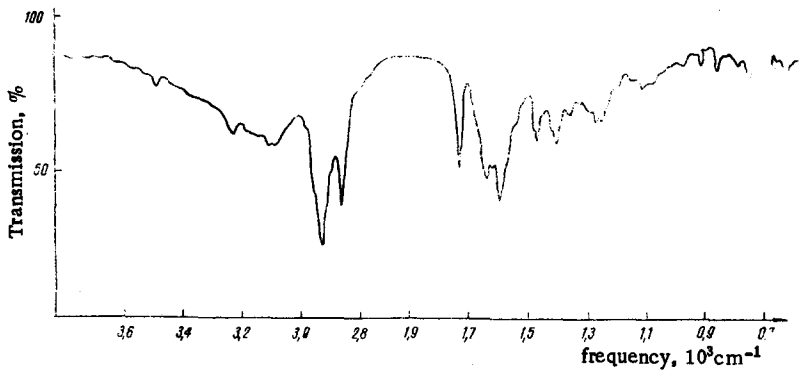


Fig. 1. IR spectrum of the lipo-amino acid (II) ($CHCl_3$).

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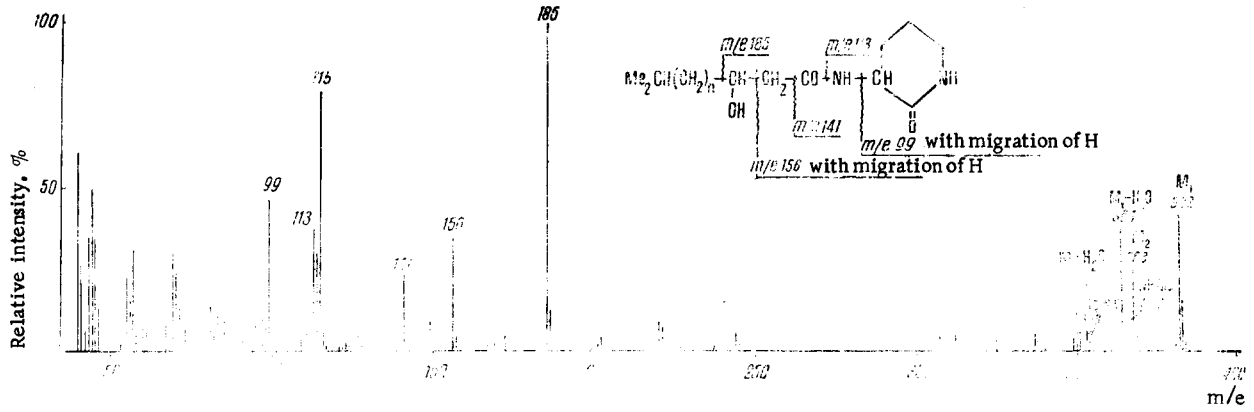


Fig. 2. Mass spectrum of the lactam (VII).

group and of the N−H bond of an amide group (3452 and 3238 cm^{-1}), of an NH_3^+ group (3080 cm^{-1}), of an ester carbonyl (1730 cm^{-1}), of an amide carbonyl (1638 cm^{-1}), and of an ionized carboxy group (1596 cm^{-1}). It shows no selective absorption in the UV spectrum in the 220-320 nm region. Under the action of N-acetoxysuccinimide in the presence of triethylamine, the lipo-amino acid (III) forms a N-acetate (IV) which, after treatment with diazomethane, gives the corresponding methyl ester (V). On severe acid hydrolysis, lipid (III) is cleaved (scheme 1) into a lipophilic moiety, which consists, according to thin-layer chromatography (TLC), of a mixture of aliphatic α- and β-hydroxy acids (and also the products of transformation of the latter), and a single hydrophilic fragment identified by paper chromatography (PC) and also by a study on an amino-acid analyzer as ornithine.

Under the conditions of mild acid methanolysis, the lipid (III) is cleaved into a mixture of methyl esters of aliphatic α-hydroxy acids and a ninhydrin-positive substance having the structure of the methyl ester (VI), which is converted into the lactam (VII) when the methanolysate is treated with an anion exchanger, followed by chromatography on silica gel. The IR spectrum of the lactam (VII) is similar to the IR spectrum of the lactam (VIII), which we synthesized by the condensation of racemic α-hydroxypalmitic acid and N^{δ}-benzyloxycarbonyl-L-ornithine followed by removal of the protective benzyloxycarbonyl group and the cyclization of the condensation product (scheme 2). On TLC in various systems of solvents, the two lactams had the same mobility. A final proof of the structure of the lactam (VII) was obtained by mass spectrometry (Fig. 2).

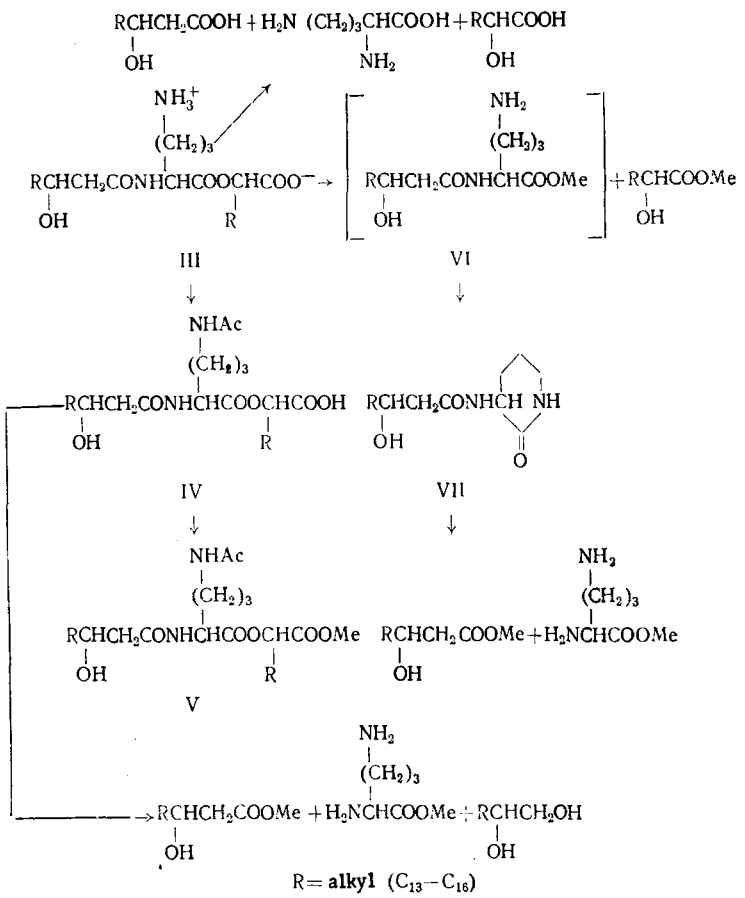
An intense peak with m/e 115 probably corresponds to the ion $HO^+ = CHCH_2CONHCHCH_3$ formed as the result of the cleavage of the lactam ring of the ion with m/e 185; the formation of the former from the latter is confirmed by the presence in the spectrum of the peak of a metastable ion with m/e 71.5.

The optical rotatory dispersion curves of the lactams (VII) and (VIII) are practically identical (Fig. 3) which shows the L configuration of the ornithine residue in the lactam (VII) and also in the lipo-amino acid (III). The methyl esters of aliphatic α-hydroxy acids obtained by the methanolysis of the lipid (III) were analyzed by a combination of gas-liquid chromatography (GLC) and mass spectrometry (Table 1). The mass spectra of the hydroxy esters contain intense peaks with m/e 90 and 103, which are characteristic for the methyl esters of α-hydroxy acids [12], and also peaks corresponding to the M^+, $[M-H_2O]^+$, $[M-MeOH]^+$, $[M-CH_2CH_2OH]^+$, and $[M-COOMe]^+$. The mixture of α-hydroxy acids gave a positive optical rotatory dispersion curve, on the basis of which we have ascribed the L configuration to it [13].

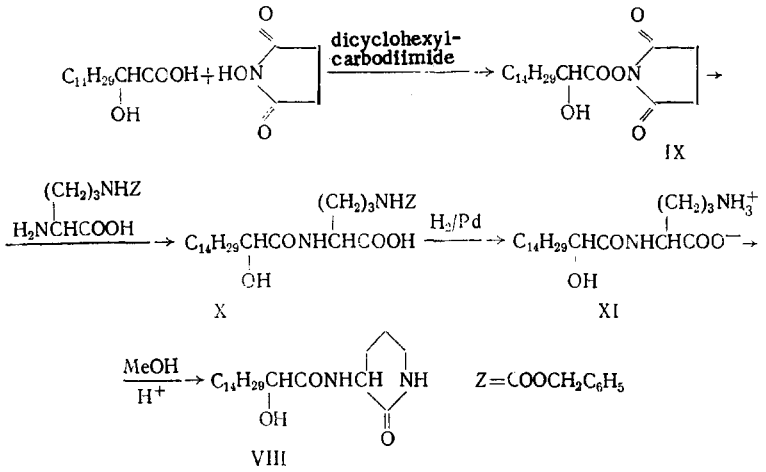
Methanolysis of the lactam (VII) under severe conditions led to the formation of the methyl ester of ornithine and a mixture of the methyl esters of aliphatic β-hydroxy acids (see Table 1). The mass spectra of the latter contained characteristic peaks with m/e 74 and 103 [12], and also peaks corresponding to the ions M^+, $[M-1]^+$ (both of very low intensity), $[M-H_2O]^+$, $[M-H_2O-MeOH]^+$, $[M-CH_2COOMe]^+$, and $[M-CH_2-COOMe-H_2O-1]^+$. The mixture of β-hydroxy acids had a positive optical rotatory dispersion curve, showing the L configuration at the asymmetric carbon atom.

The structures of the carbon chains of the α- and β-hydroxy acids entering into the composition of the lipid (III) follow from the following facts: in the NMR spectrum of the acetate of (IV) the methyl groups of the fatty-acid residues are represented by a doublet signal (τ 9.11; J = 6.3 Hz), which is characteristic

Scheme 1



Scheme 2



for an isopropyl group; on this basis it may be considered that at least the main acids of the lipid contain a terminal isopropyl grouping and have no other branching points. This is also confirmed by the fact that the ratio of the areas of the methylene protons (τ 8.72) and of the protons of the methyl groups in the NMR spectrum found (3.44) agree well with the calculated ratio (3.50). Furthermore, in the mass spectra of the methyl esters of the hydroxy acids, shown in Table 1 as iso-acids, the peak with m/e 43 ($[Me_2CH]^+$) is one of the strongest, unlike the methyl ester of β-hydroxy-n-hexadecanoic acid.

It follows from the figures given that the molecule of the lipid (III) is constructed of L-α-hydroxy acid, L-β-hydroxy acid, and L-ornithine residues. As a quantitative analysis showed, these components are present in a ratio of 1:1:1. The formation of the lactam (VII) and of α-hydroxy esters of mild acid methanolysis of the lipo-amino acid shows that the L-β-hydroxy acids in the molecule of the latter have an amide link with the α-amino group of the L-ornithine. In this case, the L-α-hydroxy acid must be attached

TABLE 1. Composition of the Fatty Hydroxy Acids of the Lipo-amino Acid (III)

Acid	α-Hydroxy		β-Hydroxy	
	V^*_{rel}	relative amount, %	V^*_{rel}	relative amount, %
iso-$C_{14:0}$	0,196	< 1	—	—
iso-$C_{15:0}$	0,292	68,0	0,315	5,2
iso-$C_{16:0}$	0,393	22,5	0,462	33,0
n-$C_{16:0}$	—	—	0,520	11,3
iso-$C_{17:0}$	0,618	9,4	0,670	50,5

*V_{rel} represents the retention volume of the methyl esters of the hydroxy acids relative to methyl α-hydroxystearate.

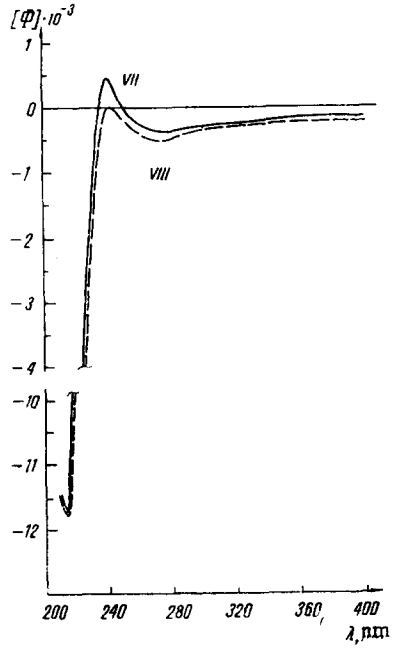


Fig. 3. Optical rotatory dispersion curves of the lactams (VII) and (VIII).

by an ester bond either to the ornithine residue or to the β-hydroxy acid residue. In order to make a choice between these two possible structures, we subjected the N-acetyl derivative (IV) to hydroboration. Severe acid methanolysis of the hydroboration product gave the methyl ester of ornithine, the methyl esters of β-hydroxy acids, and a mixture of aliphatic α-diols; methyl esters of α-hydroxy acids and 2,5-diaminopentan-1-ol, the product of the reduction of ornithine, were absent from the methanolysate. It follows from this that the free carboxy group of the lipid belongs to the α-hydroxy acid residue and, thus, the structure (III) is the only possible one for it.

The lipo-amino acid (III) amounts to about 35% of the polar cell lipids of Actinomyces No. 660-15; this fact, and also the bipolar nature of the lipid and the presence of two fatty chains in its molecule, permits the assumption that it is present in the cell membranes as one of their structural components.

EXPERIMENTAL

Type KSK silica gel was used for column chromatography and for TLC. The TLC plates were prepared by a published method [14] and the following solvent systems were used: 1) $CHCl_3$–MeOH–water (65 : 25 : 4), 2) $CHCl_3$–MeOH–AcOH–water (80 : 13 : 8 : 0.3), 3) $CHCl_3$–MeOH–water–conc. aqueous NH_3 (65 : 36 : 8 : 1), 4) $CHCl_3$–MeOH (7 : 1), and 5) benzene–EtOAc (8 : 1). The substances on the chromatograms were revealed with 50% H_2SO_4 followed by heating at 180-200°C, by a 0.1% ethanolic solution of morin, and by a 0.3% solution of ninhydrin in ethanol. Paper chromatography was performed on Leningrad S ["medium"] paper in the following systems: 6) n-BuOH–AcOH–water (4 : 1 : 5, upper phase), 7) n-BuOH–HCOOH–water (75 : 15 : 10), 8) water-saturated phenol, and 9) n-BuOH–3% aqueous NH_3 (3 : 1, upper phase). The spots of the substances were revealed with the ninhydrin reagent.

The methyl esters of the α- and β-hydroxy acids were analyzed on an LKB-9000 instrument; chromatographic separation was performed on a column (3000×3 mm) with 3% of SE-30 on Chromosorb W (40-60 mesh), temperature 190°C, carrier gas helium (60 ml/min). The fractions corresponding to individual peaks on the chromatogram were introduced directly into the ion source; the energy of the ionizing electrons was 70 eV. The mass spectra of the other compounds were recorded on an MKh-1309 mass spectrometer.

The amino-acid analysis was effected on a Unichrom (Beckman) analyzer. The IR spectra were recorded on a UR-10 (Zeiss) spectrograph and the NMR spectra on a JEOL-4H-100 (Hitachi) spectrometer at 100 MHz in $CDCl_3$ with Me_4Si as internal standard, and the optical rotatory dispersion curves (and $[\alpha]_D$) on a Cary-60 spectropolarimeter in MeOH (for the lactams) or in heptane (for the hydroxy acids).

The results of the elementary analyses of the substances corresponded to the calculated figures. The culture of Actinomyces No. 660-15 was grown for four days at 28°C on a medium containing 1.5% of starch, 0.5% of maize extract, 1% of glucose, 0.5% of $CaCO_3$, 0.5% of NaCl, and 0.3% of $(NH_4)_2SO_4$; pH 6.8-7.0. The mycelium was separated by centrifuging, washed with water, and freeze-dried.

Extraction of the Total Lipids. The freeze-dried mycelium (200 g) was extracted with $CHCl_3$–MeOH (2 : 1; 2×2.5 liters) and then with $CHCl_3$–MeOH (1 : 1; same volume). The combined extracts were evaporated

to dryness and the residue was dissolved in 800 ml of $CHCl_3$–MeOH (2:1) and washed with 160 ml of a 0.58% solution of NaCl. The lower phase, after the elimination of the solvent, gave 30.4 g of the total cell lipids.

Isolation of the Lipo-amino Acid (III). Onto a column of 450 g of silica gel was deposited 30.4 g of the total lipids in 100 ml of $CHCl_3$. The column was washed with 1.5 liter of $CHCl_3$ and, after this, elution was continued with mixtures of $CHCl_3$ and MeOH with a gradually increasing concentration of the latter (from 35:1 to 1:1), elution being completed with pure MeOH. The eluates (20-ml fractions) were analyzed by TLC in system 1. The fraction (1.2 g) obtained on elution with $CHCL_3$–MeOH (3:1, 2:1, and 1:1) and with MeOH was rechromatographed on 400 g of silica gel; mixtures of $CHCl_3$ and MeOH (2:1 and 1:1) eluted 750 mg of a fraction enriched in the lipo-amino acid (III). The latter was isolated by two or three repetitions of preparative TLC on 20×20-cm plates with a thickness of the layer of absorbent of 0.4 mm; not more than 40 mg of the mixture was deposited on each plate. A total of 65 mg of the lipo-amino acid (III) was obtained, with mp 147-148°C, $[\alpha]_D$ −14.5° ($CHCl_3$–MeOH, 2:1; c 0.27), R_f 0.40 (in system 1), 0.15 (2), 0.44 (3).

The Acetate (IV). At 0°C, 0.2 ml of Et_3N and then 6 mg of N-acetoxysuccinimide was added to a suspension of 23 mg of the lipid (III) in 3 ml of $CHCL_3$–MeOH. The mixture was left at 20°C for 15 h, and then it was diluted with 10 ml of $CHCl_3$ and was treated with Dowex 50 (H^+). The resin was filtered off, the filtrate was evaporated, and the residue was subjected to TLC in system 1. This gave 17 mg of the acetate (IV) with $[\alpha]_D^{20}$ +6.5° ($CHCl_3$; c 1.6); R_f 0.43 (system 1), 0.61 (2), 0.72 (3). IR spectrum (in $CHCl_3$): ν_{max} 3322 cm^{-1} (alcoholic HO group and amide NH groups), 1728 cm^{-1} (ester and acid carbonyls), and 1655 and 1556 cm^{-1} (amide I and II bands). Treatment of the acetyl derivative (IV) with an ethereal solution of diazomethane gave the methyl ester (V), R_f 0.79 (system 1), 0.65 (system 4).

Acid Hydrolysis of the Lipid (III). A mixture of 20 mg of the lipid (III) and 1 ml of 6 N hydrochloric acid was heated at 105°C for 24 h. After cooling, it was diluted with 4 ml of water and extracted with ether (3× 5 ml). The combined extract was washed with 2 ml of water and treated with an ethereal solution of diazomethane; the ether was distilled off and the residue was found by TLC in system 5 to contain methyl esters of α- and β-hydroxy fatty acids. The combined aqueous phase was found by TLC in system 8, PC in systems 6-8, and also by analysis on an amino-acid analyzer to contain ornithine (3.46 mg, 17% of the weight of the initial lipid).

Mild Acid Methanolysis of the Lipid (III). A mixture of 20 mg of the lipid (III) and 3 ml of a 5% solution of HCl in MeOH was left at 20°C for 20 h and was then neutralized with Amberlite XE-58 (OH^-), the MeOH was distilled off, and the residue was subjected to TLC in system 5 to give 7.9 mg (40% of the weight of the initial lipid) of methyl esters of α-hydroxy acids, R_f 0.48, and 9.5 mg of the lactam (VII), R_f 0.00; mp 96-98°C, $[\alpha]_D^{20}$ −19.3° ($CHCl_3$; c 0.16), R_f 0.72 (system 1), 0.69 (2), 0.53 (4). IR spectrum of the lactam (VII) (in $CHCl_3$): ν_{max} 3364 cm^{-1} (alcoholic OH and amide NH groups), 1660 and 1562 cm^{-1} (amide I and II bands).

Methanolysis of the Lactam (VII). A solution of 7 mg of the lactam (VII) in 2 ml of 5% HCl in MeOH was heated in a sealed tube at 105-110°C for 30 h. After cooling, the mixture was neutralized with Amberlite XE-58 (OH^-), the MeOH was distilled off, and the residue was subjected to TLC in system 5, giving 2.8 mg [40% of the weight of the initial lipid (III)] of methyl esters of β-hydroxy acids, R_f 0.41. Paper chromatography in system 9 showed the presence in the methanolysate of the methyl ester of ornithine.

Hydroboration of the Acetate (IV). Over 1 h, an excess of diborane was passed into a solution of 5 mg of the acetyl derivative (IV) in 2 ml of tetrahydrofuran. The mixture was left in an atmosphere of argon at 20°C for 48 h, after which 3 ml of MeOH was added. The solvents were distilled off and the residue was heated with 2 ml of 5% HCl in MeOH at 105°C for 30 h. After cooling, the mixture was neutralized with Amberlite XE-58 (OH^-), and TLC in systems 1, 4, and 5 showed the presence in the methanolysate of the methyl esters of β-hydroxy fatty acids and aliphatic α-diols (octadecane-1,2-diol was used as a marker for the latter). Paper chromatography in system 9 showed the presence of the methyl ester of ornithine in the methanolysate.

N^{δ}-Benzyloxycarbonyl-N^{α}-(rac-2'-hydroxypalmitoyl)-L-ornithine (X). This compound was obtained by the method described previously for N^{ε}-benzyloxycarbonyl-N^{α}-(rac-2'-hydroxysteroyl)-L-lysine [15].

A. N-(2-Hydroxypalmitoyl)succinimide (IX). To a suspension of 7.9 g of α-hydroxypalmitic acid in 500 ml of EtOAc were added 3.35 g of N-hydroxysuccinimide and then 6.0 g of dicyclohexylcarbodiimide.

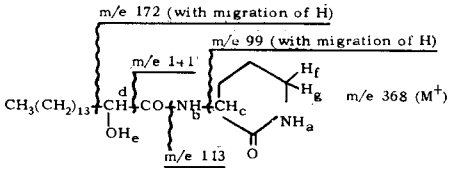
The mixture was stirred at 20°C for 12 h. Then the precipitate was filtered off, the filtrate was evaporated to dryness, and the residue was recrystallized from MeOH. This gave 7.9 g (74%) of the ester (IX) with mp 76-77°C.

Found, %: C 65.28; H 9.47; N 3.35. $C_{20}H_{35}NO_5$. Calculated %: C 65.01; H 9.55; N 3.54.

B. The Benzyloxycarbonyl Derivative (X). A solution of 4.02 g of the ester (IX) in 20 ml of tetrahydrofuran was added to a solution of 2.9 g of N^{δ}-benzyloxycarbonyl-L-ornithine and 2.5 g of $NaHCO_3$ in 300 ml of 50% aqueous tetrahydrofuran, and the mixture was left at 20°C for 12 h, after which the tetrahydrofuran was distilled off and the residue was acidified to pH ~1 and extracted with $CHCl_3$ (3× 150 ml). The combined extract was washed with water (2×150 ml), the $CHCl_3$ was distilled off, and the residue was recrystallized from EtOAc. This gave 3.35 g (68%) of the benzyloxycarbonyl derivative (X) with the composition $C_{29}H_{48}N_2O_6$, mp 78-85°C, R_f 0.35 (in system 1), 0.74 (2). IR spectrum (in KBr): ν_{max} 3318 cm^{-1} (HO and NH groups), 1694 cm^{-1} (carboxy carbonyl), and 1672 and 1562 cm^{-1} (amide I and II bands).

N^{α}-(rac-2'-Hydroxypalmitoyl)-L-ornithine (XI). At 25°C and atmospheric pressure, 1.2 g of the benzyloxycarbonyl acid (X) in 25 ml of tetrahydrofuran was hydrogenated in the presence of 1 ml of glacial AcOH over 0.3 g of palladium black. After the absorption of hydrogen had ceased, the precipitate was filtered off and was extracted with boiling MeOH (2× 10 ml). The residue obtained after the evaporation of the extract was recrystallized from MeOH. This gave 0.79 g (90%) of the amino acid (XI), composition $C_{21}H_{42}N_2O_4$, mp 146-148°C; R_f 0.18 (in system 1). IR spectrum (in paraffin oil): ν_{max} 3324 cm^{-1} (HO and NH groups), 1654 and 1570 cm^{-1} (amide I and II bands), 1596 cm^{-1} (ionized carboxy group and NH_3).

The Lactam (VIII). A mixture of 200 mg of the amino acid (XI) and 15 ml of 5% HCl in MeOH was boiled for 30 min and was then left at 25°C for 12 h. After this, the MeOH was distilled off, the residue was dissolved in 20 ml of $CHCl_3$, and the solution was washed with saturated $NaHCO_3$ solution and with water. The residue obtained after the distillation of the $CHCl_3$ was recrystallized from aqueous MeOH. This gave 166 mg (87%) of the lactam (VIII), composition $C_{21}H_{40}NO_3$, mp 140-142°C, $[\alpha]_D^{20}$ −16.9° (MeOH; c 0.17). IR spectrum ($CHCl_3$): ν_{max} 3364 cm^{-1} (HO and NH groups), 1660 and 1562 cm^{-1} (amide I and II bands). NMR spectrum (see formula A): τ 2.37 (quadruplet, 1 H, H_a), 2.99 (doublet, 1 H, H_b), 5.59 (multiplet, 1 H, H_c), 5.88 (triplet, 1 H, H_d), 6.66 (2 H, H_e and H_f or H_g), 7.68 (multiplet, 1 H, H_f or H_g), 8.78 (singlet, CH_2 in a chain), 9.08 (triplet, 3 H, CH_3). Mass spectrum:



SUMMARY

From the sum of the cell lipids of Actinomyces No. 660-15 we have isolated a new ornithine-containing lipid the molecule of which is constructed of residues of L-α and L-β-hydroxy fatty acids and L-ornithine.

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