THE STRUCTURE OF LIENOMYCIN, A PENTAENE MACROLIDE ANTITUMOR ANTIBIOTIC

I. THE STRUCTURE OF THE CARBON SKELETON AND THE LOCATION OF FUNCTIONALITIES

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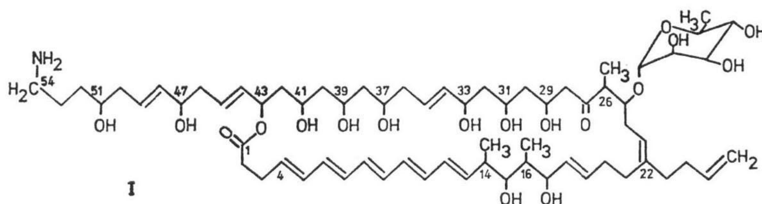
The structure of a novel type of pentaene macrolide named lienomycin (I) was established by selected chemical transformations of I followed by MS examination of the products. This report provides evidence on the structure of the carbon skeleton of I and on the location of functionalities.

Lienomycin, a polyene macrolide antibiotic produced by *Actinomyces diastatochromogenes* var. *lienomycini*[1], is a pentaene with glycosidically bound rhamnose[2] of the now established L-configuration. Besides antifungal activity, typical for polyene macrolides, lienomycin is characterized by antibacterial[2] and antitumor[3] activity as well. It also exhibits, exceptional among polyene macrolides, high mitochondrial membrane activity, which is a result of a novel biological property of lienomycin, the ability to interact with phospholipid components of biological membranes[4].

The structure elucidation studies were performed by running selected chemical transformations of the antibiotic molecule followed by MS and ^{1}H NMR examination of the products. The results thus obtained prove the complete structure of the lienomycin molecule (Fig. 1)[5,6]. This report provides evidence for the structure of the carbon skeleton of I and on the location of most functional groups.

Fig. 1. The structure of the antibiotic lienomycin (I).



The structure of the carbon skeleton of the fragment C-1 to C-28 of I was established by the formation of methyl 14,16,26-trimethyl-22-*n*-butyloctacosanoate (II) from I by the following reaction sequence: (1) hydrogenation in the presence of palladium, (2) treating with barium hydroxide to give hydrolysis of a lactone bond, retroaldol cleavage of the bond between C-28 and C-29 of I and elimination of rhamnose, (3) hydrogenation in the presence of palladium of the double bond resulting from the elimination of rhamnose, (4) reduction of a keto group with sodium borohydride, (5) substitution of three hydroxyl groups by iodine atoms with trimethylsilyl iodide, (6) reduction of the iodides formed with RANEY nickel and finally, (7) esterification with diazomethane. The end-product (II) was examined by

MS, and the fragmentation pattern (Fig. 2), typical for long-chain carboxylic acid methyl esters[7] provides evidence for its structure. Elemental composition of the following ions: 227.20198 ($C_{14}H_{27}O_2$), 255.23297 ($C_{16}H_{31}O_2$), 269.24847 ($C_{17}H_{33}O_2$), 297.27934 ($C_{19}H_{37}O_2$), 367.35809 ($C_{24}H_{47}O_2$), 437.43574 ($C_{29}H_{57}O_2$), 479.48133 ($C_{32}H_{63}O_2$), 493.49811 ($C_{33}H_{65}O_2$), 507.51473 ($C_{34}H_{67}O_2$) and 536.55386 ($C_{36}H_{72}O_2$), as determined by HRMS, is in agreement with the proposed fragmentation pattern. Thus, three methyl groups are attached to C-14, C-16 and C-26 in **II**, while a *n*-butyl group is present at C-22 in **II**.

The structure of the carbon skeleton between C-1 and C-54 of **I** and the location of oxygen and nitrogen functions in this skeleton were established by MS analysis of the O-methyl derivative of N-methyl-N-acetyloctacosahydrolienomycinolide (**III**) obtained from **I** by the following reaction sequence: (1) hydrogenation of double bonds in the presence of palladium, with docosahydrolienomycin as the product, (2) acetylation of the amino group, (3) reduction with lithium borohydride of the keto group and of the lactone bond, with N-acetyloctacosahydrolienomycin as the product, (4) methylation with methyl iodide in the presence of sodium hydride, (5) acid hydrolysis of a glycosidic bond and (6) methylation of the hydroxyl group formed in reaction (5). The mass spectrum of **III** (Fig. 3) shows a molecular ion at *m/e* 1347 and three series of elimination ions:

(a) $[M-n\times MeOH]^+$ with *m/e* values: 1315 (n=1), 1283 (n=2), 1251 (n=3) *etc.*; (b) $[M-Me-n\times MeOH]^+$ with *m/e* values: 1332 (n=0), 1300 (n=1), 1268 (n=2) *etc.*; (c) $[M-Ac-n\times MeOH]^+$ of the *m/e* values: 1304 (n=0), 1272 (n=1), 1240 (n=2) *etc.* The presence of the ions formed by fragmentation patterns typical for methoxy compounds (cleavage of the carbon-carbon bond next to methoxy group) with *m/e* values: 86,

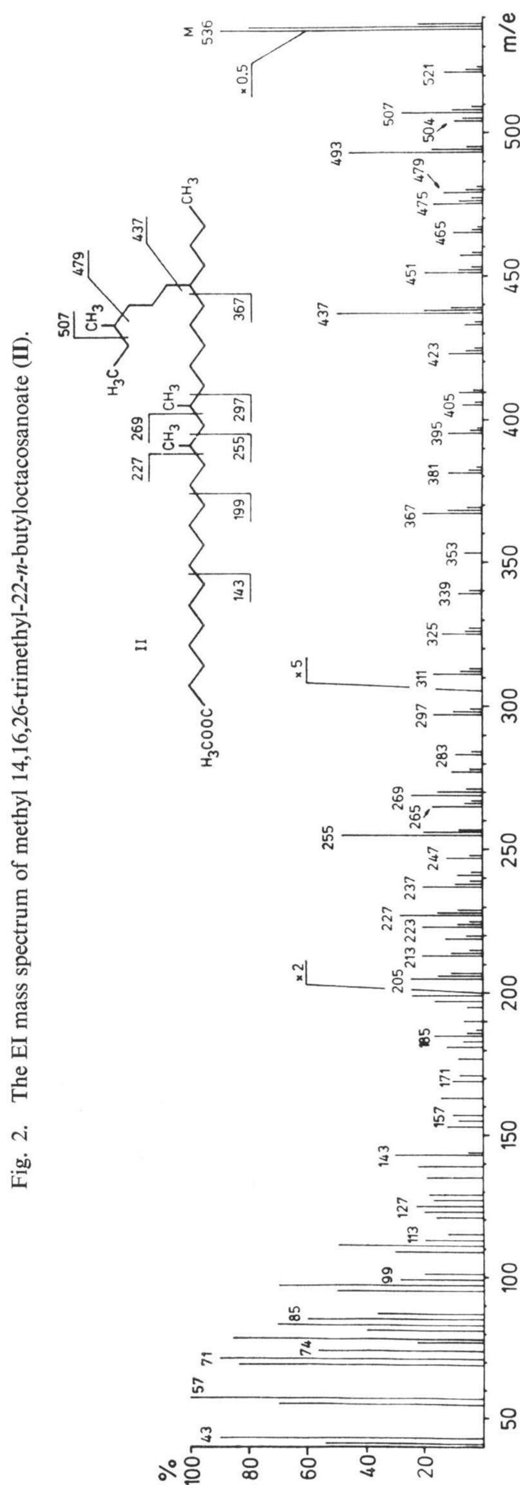


Fig. 2. The EI mass spectrum of methyl 14,16,26-trimethyl-22-*n*-butyloctacosanoate (**II**).

Fig. 3. The EI mass spectrum of N-acetyl-N-methyl-tetradeca-O-methyl-octacosahydrolienomycin (III).

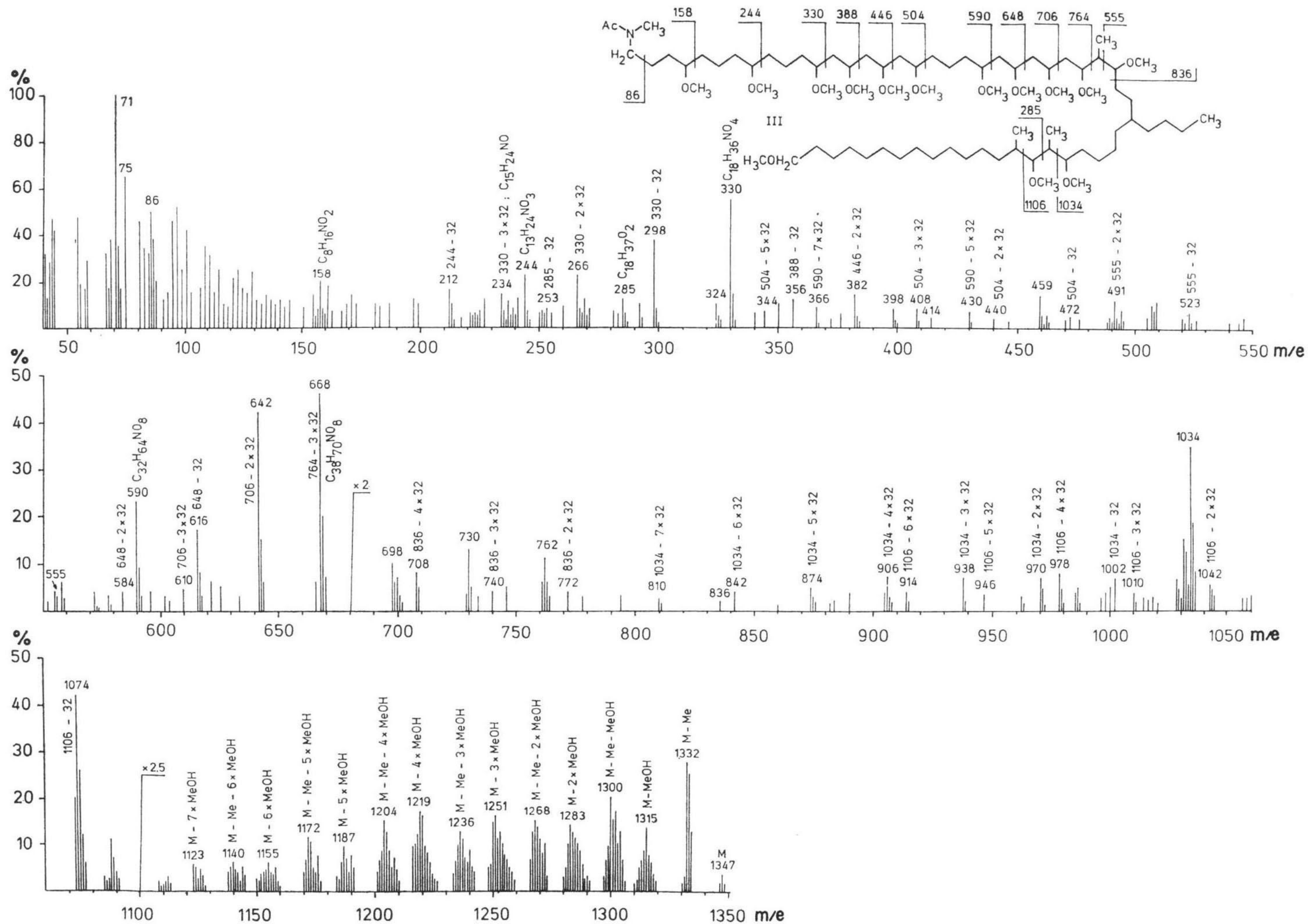


Fig. 4. The EI mass spectrum of N-acetyl-N-methyl-hexadeca-O-methyl-octacosahydrolienomycin (**IV**).

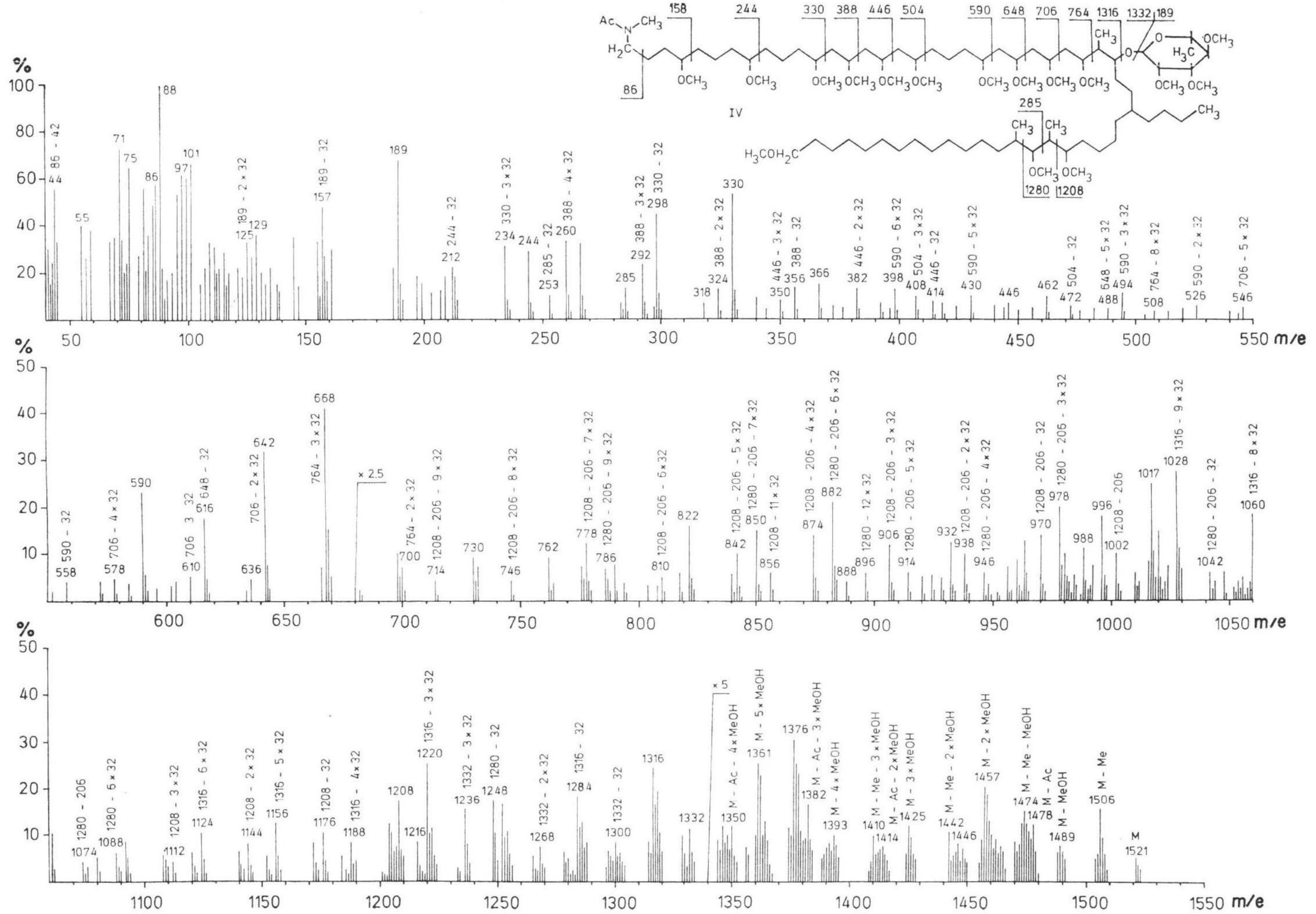
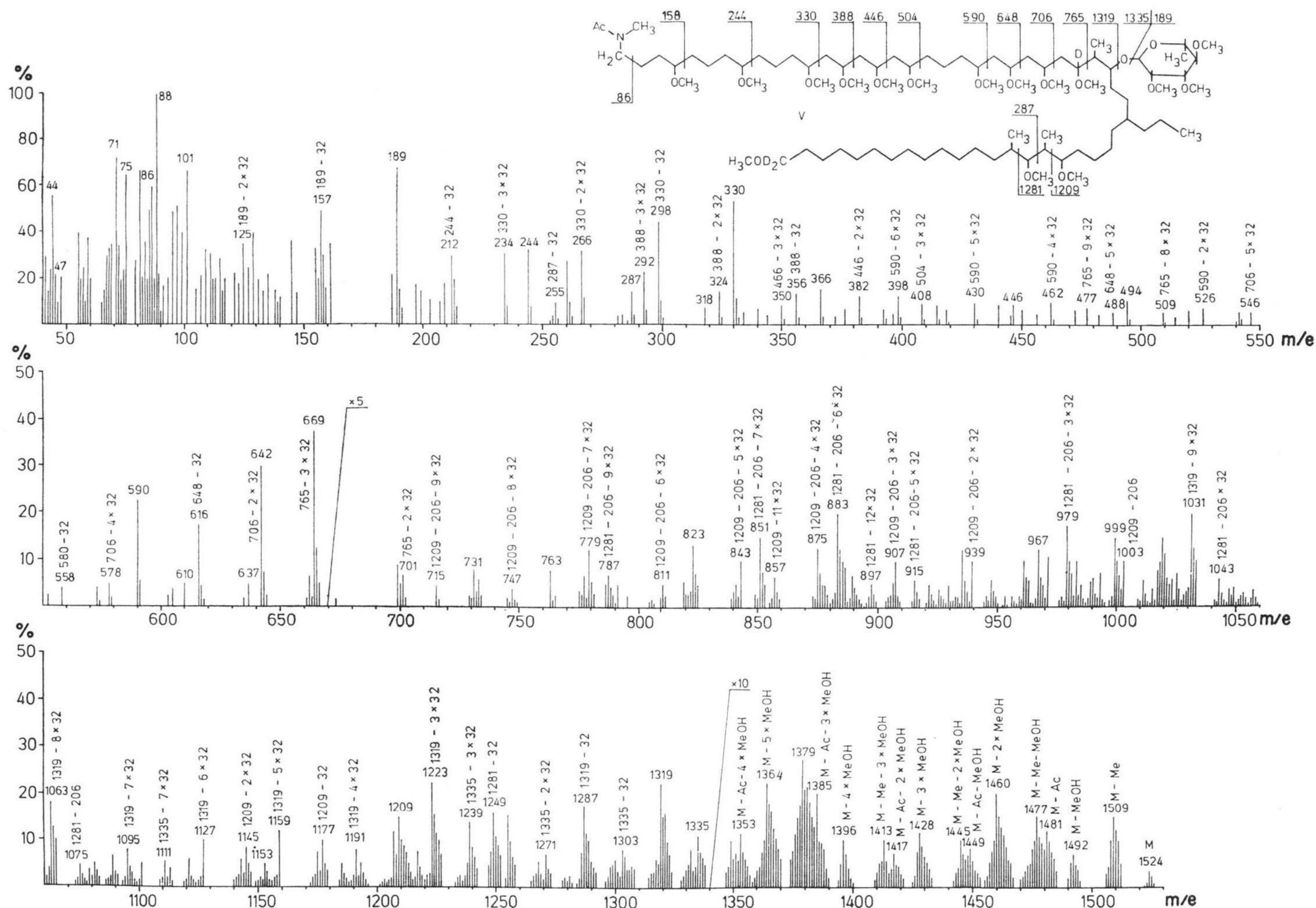


Fig. 5. The EI mass spectrum of 1,1,27-trideuterio-N-acetyl-N-methyl-hexadeca-O-methyl-octacosahydrolienomycin (V).



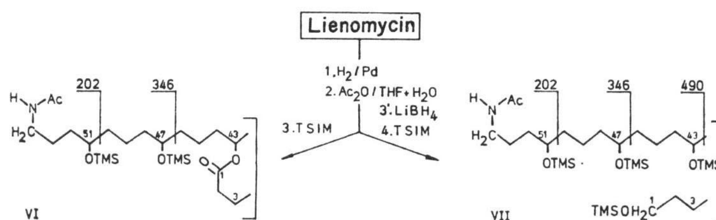
158, 244, 285, 330, 388, 446, 504, 555, 590, 648, 706, 764, 836, 1034 and 1106 implies the locations of the N-methylacetamide and methoxy groups at the indicated carbon atoms of **III** (Fig. 3). Although the fragment ions at *m/e*: 388, 504, 648, 706, 764 and 1106 exhibited low abundance in the mass spectrum of **III**, (Fig. 3), the presence of ions at *m/e*: 356, 440, 616, 642, 668 and 1074 (Fig. 3) formed by elimination of methanol molecule(s) from the former fragment ions confirms an ether-type cleavage at the indicated carbon atoms (Fig. 3). Elemental composition of the following ions: 158.11851 ($C_8H_{16}NO_2$), 234.18571 ($C_{15}H_{24}NO$), 244.19182 ($C_{13}H_{26}NO_3$), 285.27843 ($C_{18}H_{37}O_2$), 330.26442($C_{18}H_{36}NO_4$), 555.53505 ($C_{35}H_{71}O_4$), 590.46332 ($C_{32}H_{64}NO_8$), and 668.51113 ($C_{39}H_{70}NO_8$), as determined by HRMS, is in agreement with the proposed fragmentation pattern. The structure of the aglycone of octacosahydrolienomycin was established upon both the information from the mass spectrum of **III** and the evidence on the location of the lienomycin carbon skeleton branches obtained from the studies on **II**.

The location of glycosidically bound L-rhamnose, the keto group and the acyl component of the lactone bond was established by the formation of N-methyl-N-acetyl-hexadeca-O-methyl-octacosahydrolienomycin (**IV**) and its 1,1,27-trideuterio analogue (**V**) obtained from **I** by carrying out the first four reactions of the sequence applied to obtain **III**, and using lithium borodeuteride therein at step 3 to give **V**. The mass spectrum of **IV** (Fig. 4) shows a molecular ion at *m/e* 1521 and five series of elimination ions:

(a) $[M-n\times MeOH]^+$ with *m/e* values: 1489 (n=1), 1457 (n=2), 1425 (n=3) *etc.*; (b) $[M-Me-n\times MeOH]^+$ with *m/e* values: 1506 (n=0), 1474 (n=1), 1442 (n=2) *etc.*; (c) $[M-Ac-n\times MeOH]^+$ with *m/e* values: 1478 (n=0), 1446 (n=1), 1414 (n=2) *etc.*; (d) $[M-189-n\times MeOH]^+$ with *m/e* values: 1332 (n=0), 1300 (n=1), 1268 (n=2) *etc.*; (e) $[M-205-n\times MeOH]^+$ with *m/e* values 1316 (n=0), 1284 (n=1), 1252 (n=2) *etc.* The last two series (d and e) of the elimination ions resulted from the successive elimination of methanol molecules from a fragment ion formed by typical cleavage of a glycosidic bond[8] by the loss of tri-O-methyl-rhamnosyl or tri-O-methylrhamnosyloxy radicals from the molecular ion.

The presence of ions at *m/e* 125, 157 and 189 (Fig. 4) proves the pyranose ring structure of glycosidically bound tri-O-methylrhamnose[8]. The location of the N-methylacetamide moiety and the methoxy groups at the appropriate carbon atoms in the fragment C-27 to C-54 of **IV** (Fig. 4) is indicated by the presence of ions at *m/e* 86, 158, 244, 330, 388, 446, 504, 590, 648, 706 and 764 (Fig. 4) which are identical with the ions seen in the mass spectrum of **III** (*cf.* also the above discussion of the fragmentation pattern shown in Fig. 3). The ion at *m/e* 285 provides evidence that the methoxy group at C-15 of **IV** is present. The fragment ions at *m/e* 1208 and 1280 are formed by an ether-type cleavage at carbon atoms C-15 and C-17, respectively, and each of them gives rise to the formation of two series of elimination ions (Fig. 4). The first series $(p-n\times 32)^+$ is formed by the successive loss of methanol molecules from the appropriate parent fragment ion, while the second series $(p-206-n\times 32)^+$ begins with elimination of tri-O-methylrhamnose (206 m.u.). The presence of the same fragment ion at *m/e* 764 and of two pairs of related ions at *m/e* 1034, 1106 and 1208, 1280 in the mass spectra of **III** (Fig. 3) and **IV** (Fig. 4), respectively, points to the location of glycosidically bound tri-O-methylrhamnose at C-25 of **IV**. Since the mass spectrum of **V** (Fig. 5) shows a molecular ion at *m/e* 1524, three mass units greater than the molecular ion observed for **IV** (Fig. 4), three deuterium atoms are present in **V**. Comparison of the values of fragment ions observed in the mass spectra of **IV** and **V** enables one to recognize deuterium atoms in **V**. Thus, the ions at *m/e* 285 (Fig. 4) and *m/e* 287 (Fig. 5) locate at C-1 of **V** two deuterium

Fig. 6. The diagnostic difference in the structures of the N-acetyl-trideca-O-trimethylsilyldocosahydrolienomycin (**VI**) and N-acetyl-hexadeca-O-trimethylsilyloctacosahydrolienomycin (**VII**) obtained from lienomycin by the reaction sequences in the text, designed to locate a lactone bond in the antibiotic molecule.



atoms resulting from the reduction of the lactone bond in **I** with lithium borodeuteride and evidence thereby on the location of the acyl component of the lactone of **I**. The presence of ions at *m/e* 642 ($706-2\times$MeOH), 764, 1208 and 1280 (Fig. 4) and at *m/e* 642 ($706-2\times$MeOH), 765, 1209 and 1281 (Fig. 5) points to the location at C-27 of **V** of one deuterium atom resulting from the reduction of a keto group in **I** with lithium borodeuteride.

The location of the lactone bond in **I** was established by MS analysis of OTMS derivatives of N-acetyldocosahydrolienomycin and N-acetyloctacosahydrolienomycin (**VI** and **VII**, Fig. 6) obtained from **I** by the following reaction sequence: (1) hydrogenation in the presence of palladium, (2) acetylation of the amino group, and (3) silylation with N-trimethylsilylimidazole (TSIM) to give **VI** or reduction with lithium borohydride followed by treatment with TSIM to give **VII**. Diagnostic for the location of a lactone bond between C-1 and C-43 in **I** is the presence only in the mass spectrum of **VII** of an additional fragment ion at *m/e* 490 due to the ether-type cleavage fragmentation (Fig. 6 and the mass spectra in Experimental section).

Experimental

The mass spectra were obtained on a Varian MAT 711 spectrometer: (a) by a direct introduction probe and electron impact (electron energy, 70 eV; emission current, 0.8 mA; ion source temperature, 300°C; resolution 1,800 (10,000 for exact mass determination)) or (b) by field desorption (wire heating current, 18 mA; ion source temperature, 70°C; accelerating voltage, 8 kV extraction voltage, −4 kV).

General

Lienomycin (prepared in the Institute of New Antibiotics, Acad. Med. Sci., Moscow, USSR) exhibited $E^{1\%}_{1cm}$ 1,220 at 333 nm in methanol. Column chromatography was carried out on silica gel ($\leq$0.08 nm, Merck). Solvents were removed under reduced pressure.

Methylation (procedure a)

The compound to be methylated (15 mg), dissolved in THF (3 ml) and stirred, was treated at room temperature with sequentially added sodium hydride (20 mg) and methyl iodide (0.1 ml). After 16 hours *n*-hexane (20 ml) was added, the mixture was centrifuged, the supernatant was evaporated to dryness and the residue was purified by column chromatography.

Silylation (procedure b)

The compound to be silylated (12 mg), suspended in *n*-hexane (0.3 ml) reacted overnight at room temperature with N-trimethylsilylimidazole (0.2 ml), *n*-hexane (10 ml) was added, the pellet was centrifuged, the supernatant was evaporated to dryness and the residue was dried under reduced pressure (1×10^{-3} mm Hg) at 50°C.

Docosahydrolienomycin

Lienomycin (240 mg) dissolved in THF-H_2O (5: 1, v/v, 15 ml) was hydrogenated (12 hours, room temperature) over 10% Pd-$BaSO_4$ (60 mg) under slight hydrogen pressure, the catalyst was centrifuged

and 1-butanol (15 ml) was added to the supernatant. The solution was concentrated (to 5 ml), the product was precipitated with ethyl ether, purified by column chromatography in 2-propanol-water (5: 1, v/v) and crystallized from ethanol-acetone (9: 1, v/v); yield, 60 mg of docosahydrolienomycin, m.p. 92~93°C, N. eq. 1230 (by titration with perchloric acid).

Anal.: Calcd for $C_{54}H_{129}NO_{18}$; C 65.10; H 10.53; N 1.13.
Found: C 64.90; H 10.70; N 1.14.

Methyl 14,16,26-trimethyl-22-*n*-butyl-octacosanoate (**II**)

Stirred methanol solution (25 ml) of docosahydrolienomycin (600 mg) was treated (60°C, 1 hour) with 2N barium hydroxide (20 ml), barium ions were removed with Dowex 50 W×8 (H^+) and the mixture was evaporated to dryness. The residue was dissolved in methanol (20 ml) and hydrogenated (12 hours, room temperature) over 10% PdO_2-$BaSO_4$ (20 mg), the catalyst was centrifuged and methanol was evaporated. The product, dissolved in THF-H_2O (1: 1, v/v; 5 ml), reacted (5 min, 5°C) with sodium borohydride (20 mg in 2 ml of water), sodium ions were removed with Dowex 50 W×8 (H^+) and the solvents was evaporated.

The residue was purified by column chromatography in benzene - ethyl acetate- -ethanol (300: 200: 5, v/v). The product obtained (43 mg) was dissolved in methylene chloride (1 ml), treated (12 hours, room temperature) under nitrogen atmosphere with trimethylsilyl iodide (0.2 ml)[9] and evaporated to dryness. The residue, dissolved in 20% NaOH-50% methanol (0.5 ml), reacted with RANEY nickel (50 mg) by stepwise heating (2 hours) to 100°C, and the mixture was acidified with 20% H_2SO_4 to pH 3 and extracted into ethyl ether. The organic phase was washed with water, dried over $MgSO_4$, diazomethane in ether (0.2 ml) was added dropwise and after 5 minutes the reaction mixture was evaporated to dryness. The product was purified by column chromatography in *n*-heptane-benzene (2:1, v/v); yield, 11 mg of **II**.

N-Methyl-N-acetyl-hexadeca-O-methyl-octacosahydrolienomycin (**IV**)

Docosahydrolienomycin (120 mg), dissolved in THF-H_2O (5: 1, v/v; 6 ml), reacted (10 min, 5°C) with acetic anhydride (0.011 ml), 1-butanol (5 ml) was added and the resulted mixture was concentrated to 2 ml.

The product (N-acetyldocosahydrolienomycin) was precipitated with ethyl ether, suspended in THF (10 ml) and added dropwise into $LiBH_4$ solution (44 mg in 10 ml THF) to react (1 hour) at the boiling temperature of THF. The reaction mixture was ice-cooled, excess of $LiBH_4$ was decomposed and the solvents was evaporated to dryness. The residue was dissolved in water (10 ml), and extracted into 1-butanol and the organic phase was concentrated to 2 ml. The product was precipitated with ethyl ether and purified by column chromatography in chloroform - methanol - water (300: 100: 5, v/v); yield, 25 mg of N-acetyloctacosahydrolienomycin, whose field desorption mass spectrum gave ions at *m/e* (abund.): 1284 (18%), $(M+H)^+$ and 1306 (100%), $(M+Na)^+$. This compound was methylated by procedure (a) and the resulting product (**IV**) was purified by column chromatography in benzene - ethyl acetate - ethanol (10: 10: 1, v/v); yield, 12 mg of **IV**. The 1,1,27-trideuterio analogue (**V**) was obtained by the procedure applied to yield **IV**, with $LiBD_4$ instead of $LiBH_4$.

N-Methyl-N-acetyl-tetradeca-O-methyl-octacosahydrolienomycinolide (**III**)

Compound **IV** (10 mg) was dissolved in 10% H_2SO_4-50% methanol (0.5 ml) left at 50°C for 1 hour, sulphate ions were removed with Dowex 50 1×8 (OH^-) and the solvents was evaporated to dryness. The residue was methylated by procedure (a), and the product formed was purified by column chromatography in benzene - ethyl acetate - ethanol (40: 40: 5, v/v); yield, 6 mg of **III**.

OTMS-Derivative of N-acetyldocosahydrolienomycin (**VI**)

N-Acetyldocosahydrolienomycin, one of the intermediates formed during the procedure applied to obtain **IV**, was silylated by procedure (b). The product obtained (**VI**) was examined by MS, and major ions were found in the diagnostic ion range of the mass spectrum at the following *m/e* (abund.) values: 73 (88%), 75 (42%), 103 (21%), 117 (28%), 129 (56%), 147 (21%), 183 (14%), 202 (14%), 204 (88%), 217 (100%), 256 (11%), 273 (42%), 346 (14%) and 363 (56%).

OTMS-Derivative of N-acetyloctacosahydrolienomycin (**VII**)

N-Acetyloctacosahydrolienomycin, one of the intermediates formed during the procedure applied

to obtain **IV**, was silylated by procedure (b). The product obtained (**VII**) was examined by MS and major ions were found in the diagnostic ion range of the mass spectrum at the following *m/e* (abund.) values: 73 (91%), 75 (42%), 103 (21%), 117 (35%), 129 (63%), 147 (35%), 183 (14%), 202 (14%), 204 (100%) 217 (56%), 256 (11%), 273 (49%), 310 (7%), 346 (14%), 363 (63%), 400 (10%) and 490 (21%).

L-Rhamnose

Lienomycin (1.2 g) was hydrolized (2 hours, 60°C) with 0.1 N H_2SO_4 (20 ml), sulphate ions were removed with barium hydroxide and water was evaporated. The dried residue was separated by column chromatography in chloroform - methanol (5: 1, v/v); yield, 105 mg of L-rhamnose, $[\alpha]^{20}_{589}$ +8.9° c=1, H_2O, (JEOL J-20 spectropolarimeter).

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