ACTA CHEMICA SCANDINAVICA 18 (1964) 83–97

Pimaricin

II *. High Pressure — High Temperature Hydrogenation Studies

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Catalytic hydrogenations, performed at 200 atm, and 250–300° on the polyene antibiotic pimaricin, yielded 2-tetradecyltridecandioic acid, *2*, hexacosanoic acid, *1*, 9-oxohexacosanoic acid, *3*, and 5,9-epoxyhexacosanoic acid, *4*. These findings lead to partial structure *6* for pimaricin.

In the preceding paper [1] it was shown that pimaricin has a carbon skeleton that cannot be derived from the structure considered by Patrick *et al.*[2]

In this communication we wish to describe four crystalline compounds obtained when pimaricin was subjected to catalytic reduction under high pressure and elevated temperature. Their structures verify the new carbon skeleton and enable us to place two carboxyl groups, one ketonic carbonyl group, and one oxygen function in pimaricin.

* Part I, *Acta Chem. Scand.* 18 (1964) 77.

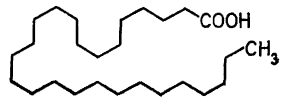
During the isolation work [3] it was found that the antibiotic was amphoteric and formed water soluble crystalline alkali salts. On the basis of decarboxylation experiments the Lederle group assumed the presence of a β-keto acid. We have verified the amphoteric character by electrometric titrations. In methyl cellosolve—water pimaricin showed one acidic and one basic function with pK_{MCS} of 4.92 and 9.62. The corresponding values for dodecahydropimaricin (see below) were 5.17 and 8.92. These values are in agreement with the presence of a free amino group and a free carboxyl group [4] in both the antibiotic and its dodecahydro derivative. Although attempts to liberate carbon dioxide by warming pimaricin with dilute acids were not successful, large amounts of carbon dioxide were generated during a chromium trioxide—sulfuric acid oxidation.

A systematic study of the catalytic hydrogenation of pimaricin under different conditions yielded some remarkable results. In methanol or glacial acetic acid solution with pure platinum or pure palladium, or with palladium on carbon or on alumina as catalysts, six moles of hydrogen were absorbed at room temperature and atmospheric pressure. Dodecahydropimaricin resulted. The same compound was isolated from reductions performed at 60° and 30 atm. In order to achieve a more complete hydrogenation as well as hydrogenolysis, pimaricin was dissolved in glacial acetic acid and reduced at 200 atm. and 250—300°, using palladium on alumina or palladium on carbon as catalysts. After esterification of the products with diazomethane, investigation by thin layer chromatography indicated the presence of a large number of compounds. Four of them, here called ZHHI-low, ZHHI-high, Fa-Fr-high and Fa-Fr-low, could be obtained in a crystalline form. Their isolation is described in the experimental section.

ZHHI-low is a saturated compound without any high intensity ultraviolet absorption. Its infrared spectrum showed bands typical of a long, straight-chain fatty acid (714; singlet, 1695; broad absorption between 2400—3500 cm^{-1}). Titrations gave an equivalent weight of 402. Treatment with diazomethane gave a crystalline methyl ester, shown to be homogeneous by thin layer and gas chromatography. Oxidation of ZHHI-low with chromium trioxide in glacial acetic acid [5] gave a large number of monobasic acids. Gas chromatographic analysis of their methyl esters showed a pattern characteristic of that obtained by degradation of a straight-chain fatty acid.[6] The mass spectrum of the original methyl ester showed a molecular weight of 410, corresponding to $C_{25}H_{51}COOCH_3$, and was identical with that of authentic hexacosanoic acid methyl ester.[7] Infrared spectra and mixed melting points of the methyl esters also established their identity.* ZHHI-low is therefore the straight chain monocarboxylic acid obtained from pimaricin by loss of one tertiary carbon atom. This is in agreement with the basic carbon skeleton being 12-methylhexacosane.

Fa-Fr-high was a saturated compound without absorption above 210 mμ in the ultraviolet. Its infrared spectrum was very similar to that of ZHHI-low with skeletal methylene vibrations at 720 cm^{-1}. The relative intensity of the carbonyl band (1685 cm^{-1}) was considerably higher than that of ZHHI-low.

* Authentic hexacosanoic acid was kindly supplied by Professor E. Stenhagen, Gothenburgh.



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The acid properties of Fa-Fr-high were demonstrated by the preparation of a potassium salt and by electrometric titrations. An equivalent weight of 236 and a $pK_{MCS} = 7.54$ were found. Molecular weight determination by the Rast method using 1,4-endomethylene dehydropiperidazine [8] gave a value of 464. We therefore assumed Fa-Fr-high to be a dicarboxylic acid of high molecular weight, hopefully containing the complete carbon skeleton of pimaricin.

With diazomethane a crystalline methyl ester (1732 cm^{-1}) was formed; it was purified by distillation. Thin layer and high temperature gas chromatography proved it to be homogeneous. The molecular weight of the ester was found to be 468 by mass spectrometry. From elemental analyses a molecular formula of $C_{29}H_{56}O_4$ followed, corresponding to $C_{27}H_{52}O_4$ for the free acid. The nuclear magnetic resonance spectrum of the methyl ester (*cf.* Fig. 1) shows six protons at $\delta = 3.65$ corresponding to two $\mathbf{CH_3O}$—CO-groups and centered at $\delta = 0.92$, as a characteristically distorted triplet,[9] one $\mathbf{CH_3}$ adja-

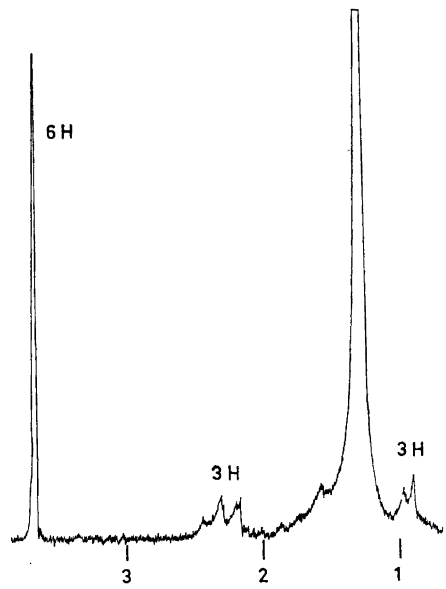


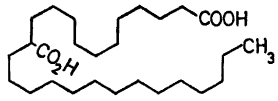
Fig. 1. NMR spectrum of Fa-Fr-high dimethyl ester.

cent to a methylene group. A triplet at $\delta = 2.30$ corresponding to three protons was characteristic of a CH_2—$\mathbf{CH_2}$—CO-grouping overlapping a CH_2—$\mathbf{CH}$—$COOCH_3$. A large number of methylene protons appeared at $\delta = 1.27$. The nuclear magnetic resonance and mass spectra narrowed the structure to a dimethyl ester of a saturated 2-alkyl dicarboxylic acid contain-

ing twenty-seven carbon atoms. The compound was expected to be either 2-tetradecyldodecandioic acid or 2-undecylhexadecandioic acid.

We first wanted to show that Fa-Fr-high contained the same carbon skeleton as found earlier. Reduction of the dimethyl ester with lithium aluminum hydride gave a crystalline diol lacking carbonyl absorption in the infrared. The diol was converted to a hydrocarbon with red phosphorus and iodine followed by reduction with lithium aluminum hydride[10]. High and low temperature gas chromatography proved it to be homogeneous. Its mass spectrum was identical with that of synthetic 12-methylhexacosane.

Oxidation of Fa-Fr-high with potassium permanganate in acetone [8] gave a mixture of acids from which the monobasic acids were isolated by steam distillation. Gas chromatographic analysis of their methyl esters showed pentadecanoic acid to be the highest acid present. This is the expected result from structure *2*:



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Fa-Fr-high is in a formal sense both a high dibasic acid and a high α-substituted monobasic acid. The mass spectrum of its dimethyl ester, reproduced in Fig. 2, exhibited the peaks expected from these two types of com-

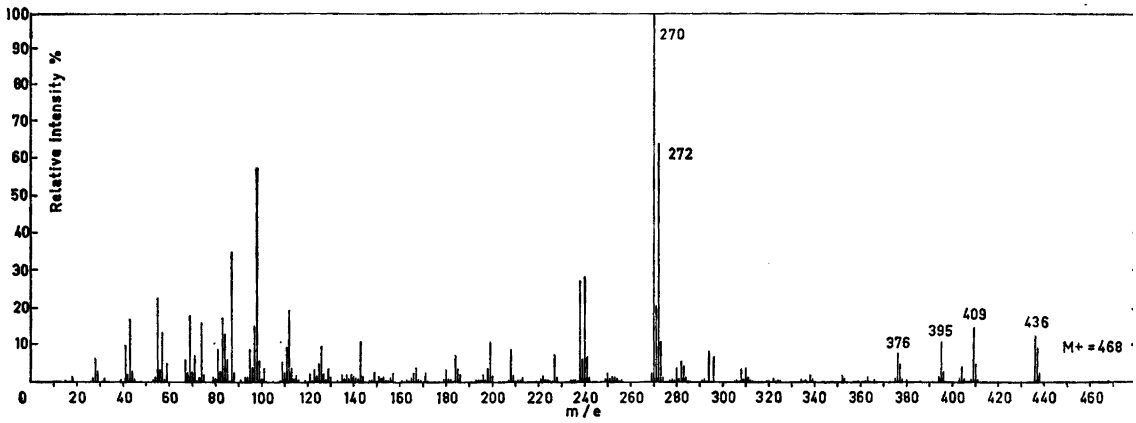
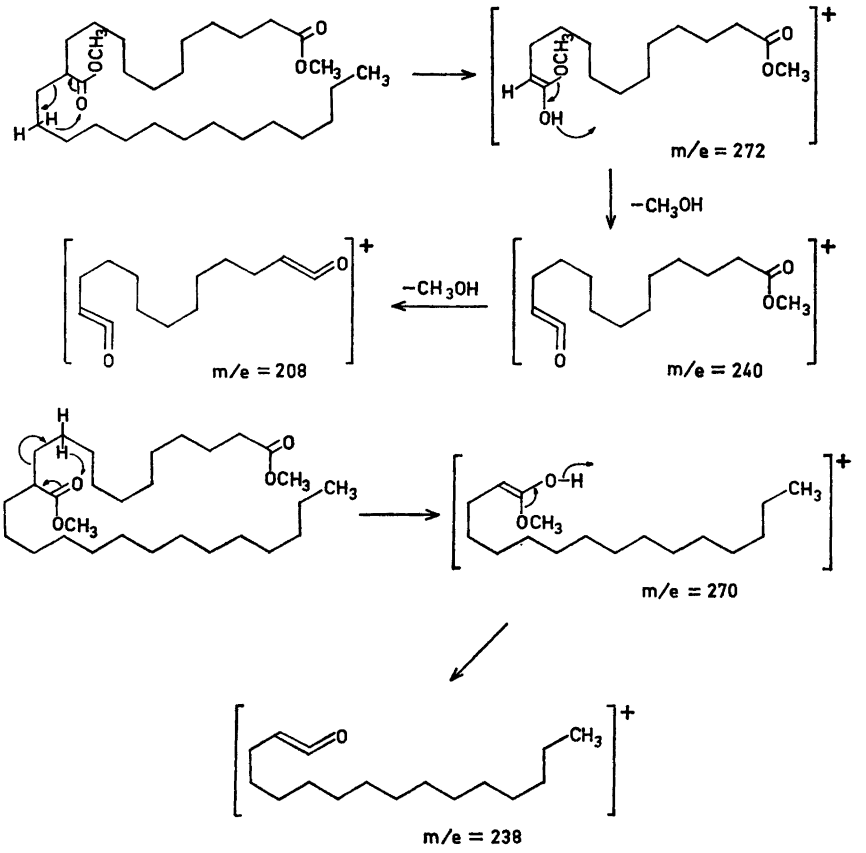


Fig. 2. Mass spectrum of Fa-Fr-high dimethyl ester.

pounds.[7,11] It should be mentioned that fragments occurred at M−31, M−32, M−64, M−91, and M−92, all characteristic for dimethyl esters of dibasic acids. Due to a structural coincidence, the two most intense peaks appeared only two mass units apart, at 270 and 272. They are formed through a rearrangement of type H according to the nomenclature of Biemann.[12] When the C-11—C-12 bond is cleaved the ion will contain the C_{14} alkyl side chain and give

the 270 fragment. The 272 ion is formed by cleavage of the C-12—C-13 bond. The same type of fragmentation has been found earlier in α-methyl substituted monobasic acids.[13] The alkylated ion is the base peak in the spectrum. The lower intensity of the 272 ion probably depends on the ease with which it can undergo secondary fragmentation processes. The correctness of these assignments is supported by the fragments with m/e = 240, 238, and 208 formed in the following way:



The "270 fragment" can lose only *one* CH_3OH, therefore no ion at m/e = 206 is formed. The mass spectrum of the dimethyl ester exhibits a very clear fragmentation pattern and would alone be sufficient to deduce the structure. Fa-Fr-high is therefore the dicarboxylic acid obtained from pimaricin by complete hydrogenation of the molecule followed by elimination of the mycosamine moiety and hydrogenolysis of all hydroxyl groups. Under these conditions the carbon skeleton remained unchanged.

ZHHI-high was by titration shown to be a carboxylic acid with an equivalent weight of 412. Molecular weight determination by the Rast method

using 1,4-endomethylene dehydropiperidazine as solvent gave a value of 456. The ultraviolet spectrum of ZHHI-high showed no absorption at low concentrations. Its infrared spectrum exhibited characteristic methylene vibrations at 712 cm^{-1} (singlet) and strong carboxyl absorption at 1693 and at 2500—3500 cm^{-1}. The crystalline methyl ester was homogeneous to thin layer and high temperature gas chromatography. Its molecular weight as determined by mass spectrometry (424) in combination with elemental analysis required the formula $C_{26}H_{51}O_3$ for the free acid. The infrared spectrum of the methyl ester displayed two carbonyl bands, while only one was shown by the free acid. The stronger band (1736 cm^{-1}) can safely be assigned to the ester group and the slightly weaker one (1708 cm^{-1}) is probably due to a ketonic carbonyl group. This assignment was supported by absorption in the methylene region at 1416 cm^{-1} in the ester and at 1412 cm^{-1} in the free acid. Conclusive evidence for the carbonyl group was obtained from the nuclear magnetic resonance spectrum. At $\delta = 2.1$, the region for methylene groups adjacent to carbonyl groups, six protons forming a multiplet appeared. The $\mathbf{CH_3}-CH_2$ triplet fell at $\delta = 0.90$ and the $\mathbf{CH_3}-O-CO$ singlet at $\delta = 3.65$. The spectrum with assignments is reproduced in Fig. 3.

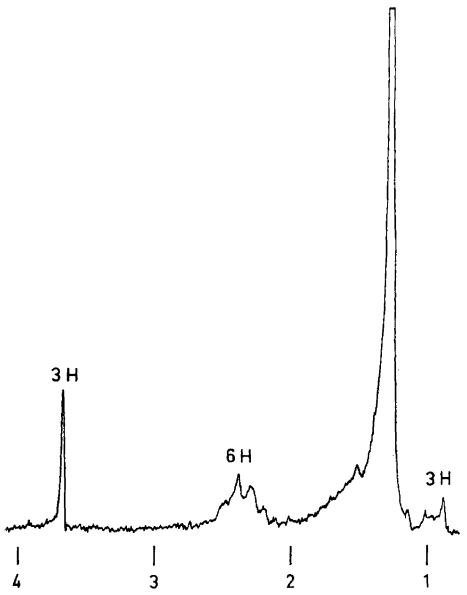


Fig. 3. NMR spectrum of ZHHI-high methyl ester.

ZHHI-high is therefore a C_{26}-monoketo monobasic acid. The position of the carbonyl group was determined by oxidation with chromium trioxide in glacial acetic acid and separation of the monobasic acids by steam distillation. Gas chromatographic analysis of their methyl esters showed octadecanoic acid to be the highest acid formed. It was likely therefore that the carbonyl group was located on C—18, counted from the methyl end of the C_{26} acid.

This was confirmed by the mass spectrum of the ZHHI-high methyl ester, which would alone furnish sufficient evidence for the structural assignment. It

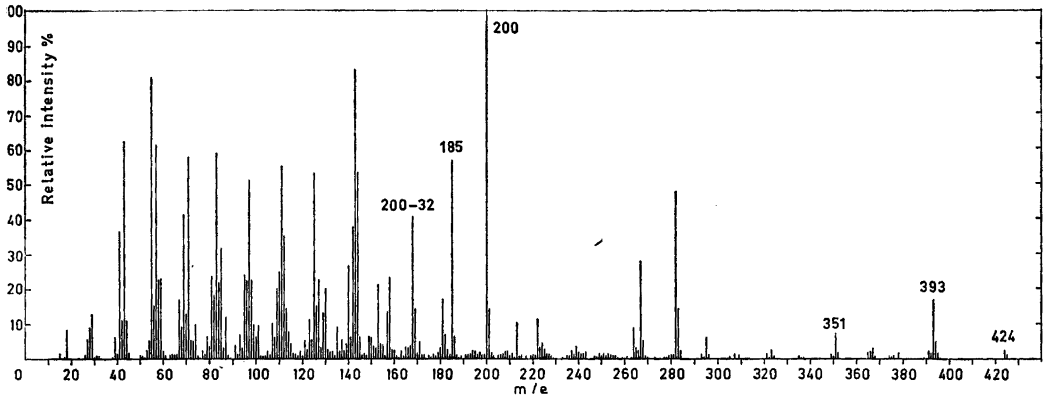
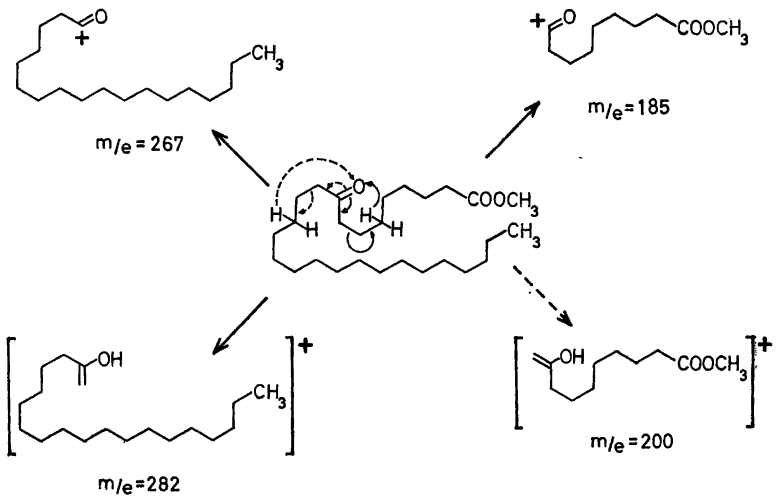


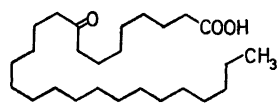
Fig. 4. Mass spectrum of ZHHI-high methyl ester.

is reproduced in Fig. 4. The mass spectrum of 9-oxooctadecanoate has been discussed earlier;[14] that of ZHHI-high is strictly analogous. The M + 1 peak at 425 is higher than expected from the isotope ratio and the peak at $m/e = 393$ is typical of a methyl ester. Decisive for the structural assignment are fragments at $m/e = 282$, 267, 200, and 185, which are formed in the following way:



They constitute examples of type H and C rearrangements according to Biemann's conventions.[14] The chemical and physical evidence presented conclusively shows that ZHHI-high is 9-oxohexacosanoic acid, *3*.

The acid properties of Fa-Fr-low were demonstrated by preparation of a potassium salt. Electrometric titration gave a pK_{MCS} of 7.83. The infrared



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spectrum was again characteristic of a high molecular straight-chain fatty acid with bands at 1688 and 715 cm^{-1} (singlet). The ultraviolet spectrum did not show any characteristic absorption. The crystalline methyl ester with a single carbonyl absorption at 1735 cm^{-1} was proved to be homogeneous by gas and thin layer chromatographic analysis. The ester was purified by distillation. From analytical data and from the molecular weight by mass spectrometry (424) the formula $C_{28}H_{50}O_3$ was obtained for the free acid. The infrared spectra of the acid and of its methyl ester showed no hydroxyl absorption but a strong band at 1080 cm^{-1}, suggesting a five- or six-membered ring ether. The nuclear magnetic resonance spectrum of the methyl ester, reproduced in Fig. 5, showed

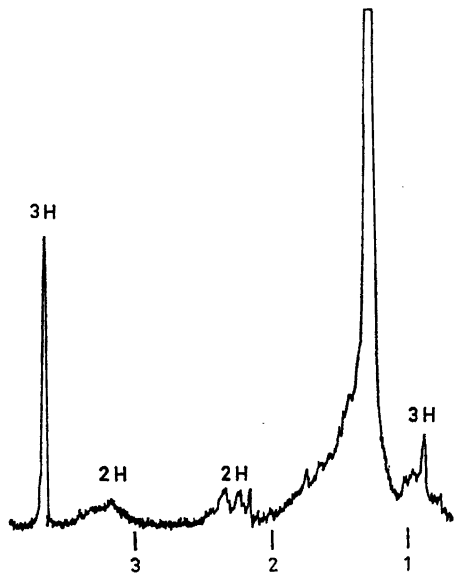


Fig. 5. NMR spectrum of Fa-Fr-low methyl ester.

three methyl ester protons at $\delta = 3.65$, the distorted triplet of the CH_3-CH_2 group at $\delta = 0.88$, and a large number of methylene groups around $\delta = 1.27$. The methylene group α to the carboxyl group formed a triplet centered at $\delta = 2.23$. A broad band at $\delta = 3.18$, corresponding to two hydrogens, supported the presence of a cyclic ether in Fa-Fr-low.

Oxidation of Fa-Fr-low with chromium trioxide in acetic acid gave a mixture of acids which was separated into mono- and dibasic acids by steam distillation. The highest volatile acid was found to be octadecanoic acid by gas chromatographic analysis of the methyl esters. The nonvolatile acids consisted of a

mixture of approximately equal amounts of succinic and glutaric acids. These results lead to structure *2* for Fa-Fr-low. The presence of a five-membered ring would on oxidation yield adipic acid, of which no trace could be found.

The mass spectrum of the methyl ester of Fa-Fr-low is reproduced in Fig. 6. The peak for the molecular ion at 424 is relatively strong. At $m/e = 74$ and

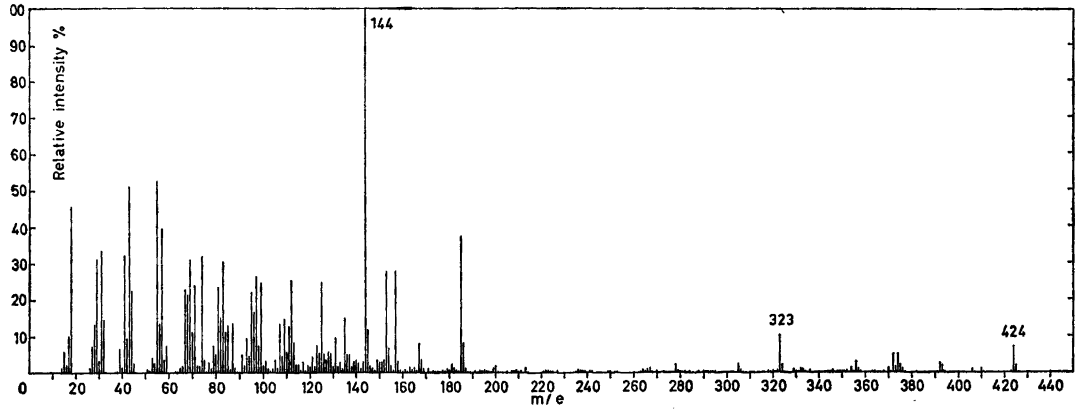
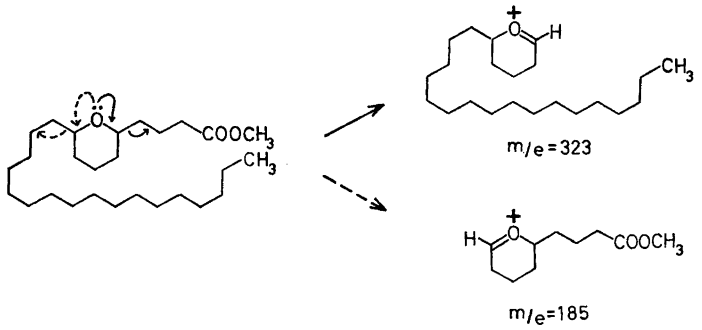


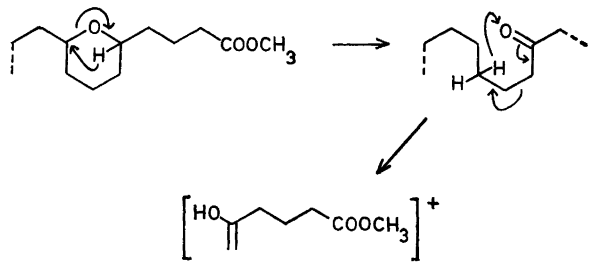
Fig. 6. Mass spectrum of Fa-Fr-low methyl ester.

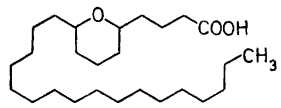
M−31, ions characteristic of methyl esters appear. The two peaks at $m/e = 323$ and 185 are significant for the structural assignment. They are formed by loss of the side chains of the tetrahydropyran ring in the following way:



Analogies to this type of cleavage can be found in the mass spectra of monoterpene cyclic ethers.[15] The base peak in the spectrum ($m/e = 144$) corresponds to $C_7H_{12}O_3^+$. The likely formation and structure of this fragment is outlined below. Fa-Fr-low is therefore 5,9-epoxyhexacosanoic acid, *4*.

Fa-Fr-high and Fa-Fr-low could be obtained in a pure state by recrystallization, while ZHHI-low and ZHHI-high always formed mixtures. Gas chromatography revealed, that samples recrystallized to constant melting point con-





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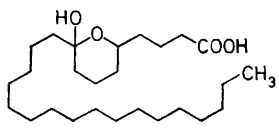
tained about 20 % of the other component. Successful separation of ZHHI-low and ZHHI-high was first achieved by chromatography of the free acids or their methyl esters on finely powdered urea using isooctane as eluant. This convenient method[16] does not seem to have been used widely. It was later found (by thin layer chromatography) that faster separation could be performed on silica gel.

We now want to discuss the implication of the four hydrogenation products on the structure of pimaricin. Based on the formula for Fa-Fr-high two carboxyl functions can be placed in the antibiotic, one at the end of the carbon chain and the other on C—12. Electrometric titrations have demonstrated the presence of only one acidic function in pimaricin and its dodecahydro derivative. Functional group analysis did not reveal the presence of an ester. Therefore one carboxyl group is lactonized and the second is free. Biogenetic analogies leave no doubt that the primary carboxyl group forms the macrocyclic ring, but, as yet, no evidence has been given to support this assumption. We shall present such facts in a following publication.

Several observations had indicated the presence of a keto group in pimaricin, but the isolation of ZHHI-high was the first conclusive demonstration that the carbonyl function is located on C—9.

Sugars containing carbonyl groups exist to a large extent as cyclic hemiacetals. One may therefore expect that a hydroxyl group properly placed in pimaricin would form a hemiketal with the C—9 carbonyl group, *5*:



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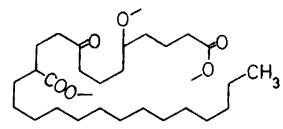

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Elimination of the ketalic hydroxyl group, either by direct hydrogenolysis or by dehydration followed by reduction, would then lead to Fa-Fr-low. Both processes are conceivable under the conditions used. Therefore we believe that pimaricin carries an hydroxyl group on C—5 and propose partial structure, *6*.

In this investigation and in earlier ones on macrolides the overriding problem has been that of the molecular weight. The physical and analytical methods usually applied have not furnished information about the presence of impurities of similar structural type, *e.g.*, another homologue differing in molecular composition by one or a few carbon atoms. It is not inconceivable that in the biosynthesis a propionate could be accidentally used instead of an acetate unit. Thus it is worthy of note here that gas chromatographic analysis of the high-temperature high-pressure reduction products in no case revealed the presence of a similar compound differing in molecular weight by one or a few carbon atoms. These admittedly negative results seem to support the present view that a microorganism is unlikely to make errors of that type and that the macrolides produced by a specific strain always contain the same carbon skeleton. Different oxygenation states, on the other hand, are well exemplified by members in the methymycin, erythromycin, and magnamycin family.[17]

As demonstrated in this communication, the high-temperature high-pressure hydrogenation has been of great value in the structural elucidation of pimaricin. We anticipate its usefulness as a general method in structural investigations of macrocyclic antibiotics.

EXPERIMENTAL

Ultraviolet spectra were determined in ethanol solution on a Beckman DK 2 spectrophotometer and infrared spectra on a Perkin-Elmer Model 21 instrument. Nuclear magnetic resonance spectra were recorded on a Varian A—60 spectrometer using deuterochloroform as solvent and tetramethylsilane as internal standard. Gas chromatographic analyses were performed with a Pye Argon chromatograph using a 90 × 1 cm column packed with 5% silicone rubber SE—30 (General Electric Co.) on 60—65 mesh celite. The mass spectra were determined with a 180° instrument of the semicircular type previously described [18] and an Atlas CH4 spectrometer, both equipped with heated inlet systems, kept at 200—225°. Thin layer chromatograms were carried out on Silica gel G (Merck) according to Stahl.[19] The spots were made visible either with iodine [20] or by spraying with chromium trioxide in sulfuric acid [21] followed by heating to 120—140° for 5 min. The analyses were performed by Dr. A. Bernhardt, Mülheim, Ruhr, Mr. M. van Leeuwen, Delft, and Mr. P. J. Hubers, Amsterdam.

Preparation of dodecahydropimaricin. To a suspension of 100 mg of prereduced platinum oxide in 500 ml of methanol 1 g of pure pimaricin was added. The mixture was hydrogenated for 12 h. Pimaricin, very slightly soluble in methanol, gradually went into solution as the reduction proceeded. Uptake of hydrogen = 208 ml at 20° (5.9 moles). Dodecahydropimaricin was obtained as an amorphous powder on evaporation of the methanol under reduced pressure. (Found: C 57.69; H 8.61; N 2.09. Calc. for $C_{33}H_{59}NO_{14}$: C 57.13; H 8.57; N 2.02).

Decarboxylation experiments on pimaricin and dodecahydropimaricin. A solution of 1 g of pimaricin in 10 ml of 20 % sulfuric acid was heated for 5 min on the steam bath and then to boiling on a hot plate. No gas evolution could be detected. The same result was obtained with dodecahydropimaricin.

On oxidation of 0.254 g of pimaricin with 1.07 g of chromium trioxide in 20 % sulfuric acid at 70° large amounts of carbon dioxide were formed during the first few minutes.

Dodecahydropimaricin, oxidized under the same conditions, also gave carbon dioxide.

*Preparation of crude ZHHI-high.** To a solution of 60 g of pimaricin in 1200 ml of glacial acetic acid 33 g of 5 % palladium on alumina was added. The suspension was introduced into a high pressure hydrogenation apparatus which was filled with hydrogen to a pressure of 100 atm at 20°. Over a period of 3.5 h the temperature was raised to 300° after which the heating was discontinued. The following day the catalyst cake, in which crystals had formed, was separated and extracted with 3 l of boiling ethanol. On cooling, 12.5 g of crystalline material melting at 92.5–95° precipitated. Repeated recrystallizations from alcohol, heptane, and acetone did not raise the melting point.

Preparation of crude ZHHI-low. A solution containing 60 g of pimaricin and 33 g of 5 % palladium on alumina in 800 ml of glacial acetic acid was hydrogenated as described above. At 30° the initial pressure was 100 atm. and the temperature was raised to 295° during 2.5 h. The heating was then discontinued. The following day the catalyst cake was extracted with 2 l of boiling ethanol. On cooling, 7.5 g crystalline material melting at 85.5–86.5° precipitated. The yield of this compound was found to decrease when solutions containing lower concentrations of pimaricin were reduced. If during the "high concentration" conditions the heating was discontinued when the pressure reached 200 atm., only 4.3 g of crude ZHHI-low was obtained from 60 g of pimaricin. When instead the temperature and pressure were maintained at their maximum values for 4.5 h, up to 11.5 g of crude ZHHI-low could be isolated from 60 g of pimaricin.

*Preparation of Fa-Fr-high and Fa-Fr-low.*** Fa-Fr-high and Fa-Fr-low were formed simultaneously and isolated from the acetic acid solutions from which the catalyst cake, crude ZHHI-high, and crude ZHHI-low had been separated. Fa-Fr-high and Fa-Fr-low were obtained during both high and low concentration conditions. The following account describes a typical experiment: 60 g of pimaricin in 800 ml of glacial acetic acid were reduced as described above. A temperature of 300° was kept for 4.75 h and 9.7 g of ZHHI-low was separated. The filtrate was then evaporated in a nitrogen atmosphere under reduced pressure. The residue, 39 g, was dissolved in 200 ml of 2 N aqueous sodium hydroxide and heated on the steam bath for 18 h. The solution was then cooled, acidified with 40 ml of concentrated hydrochloric acid, and extracted with 750 ml of ether. Evaporation of the solvent gave 26 g of material which was recrystallized twice from 40 ml of ethanol, yielding 17.9 g of material which was recrystallized once more from 100 ml of heptane. Yield: 8.3 g of a substance melting at 65–82°. From the mother liquor 3.5 g of material melting at 58.5–61.5° was obtained as well as 0.8 g of a fraction melting at 50–54°.

34 g of combined fractions, m.p. 65–82°, was esterified by treatment with 200 ml of refluxing 2.25 N methanolic hydrochloric acid for 23 h. After standing at 0° for several days 34.7 g of a crystalline product had precipitated. This material was subjected to fractional distillation and the following fractions were collected at 2×10^{-4} mm.

Fract. No.	temp. °C	weight, g
1	100–140	1.68
2	141–148	4.6
3	150–153	21.9
4	residue	4.3

Fraction No. 3 was saponified for 4.5 h in 180 ml of refluxing 1 N aqueous potassium hydroxide. After cooling, 6.5 g of the crude potassium salt precipitated. (Found: K 8.40. Calc. for $C_{27}H_{51}O_4K$: K 8.16).

To the mother liquor which was concentrated under reduced pressure, 100 ml of water and 20 ml of concentrated hydrochloric acid were added. The precipitate formed, 7.7 g, was recrystallized from 200 ml of hexane yielding 6.9 g of Fa-Fr-high melting at 85–87°. (Found: C 73.90; H 11.99. Calc. for $C_{27}H_{52}O_4$: C 73.58; H 11.89). $pK_{MCS} = 7.54$; equiv. wt. = 236. With diazomethane a methyl ester, m.p. 37–38°, was obtained; mol. wt. = 468 (mass spec.) (Found: C 74.04; H 12.32. Calc. for $C_{29}H_{56}O_4$: C 74.30; H 12.04).

* ZHHI is the Dutch abbreviation for: "Zeer Hooge Hydrering Indeed"; "high" and "low" refer to the melting points.

** "Fa-Fr" = *Fa*tty acid *fr*action; "high" and "low" refer to the melting points.

8 g of combined fractions, m.p. 58.5—61.5°, were esterified by boiling with 50 ml of 2.25 M methanolic hydrochloric acid for 24 h; 7.4 g of the crude methyl ester was obtained. It was subjected to fractional distillation. The major fraction, 5.3 g, distilled at 170°/3 × 10^{-4} mm; it was hydrolyzed in 50 ml of 1 N ethanolic potassium hydroxide. On cooling, 4.9 g of crude potassium salt crystallized. After recrystallization from 150 ml of ethanol, 4 g of Fa-Fr-low potassium salt was obtained. (Found: K 8.03. Calc. for $C_{26}H_{49}O_3K$: K 8.71).

This salt was dissolved in a mixture of 50 ml of ethanol, 30 ml of concentrated hydrochloric acid, and 100 ml of water; slight warming was necessary to dissolve all the material. On cooling, 3.5 g of the free acid was obtained; after recrystallization from 60 ml of acetone, and then from a small amount of acetone, it melted at 61—63°. (Found: C 75.50; H 12.35. Calc. for $C_{26}H_{50}O_3$: C 76.03; H 12.28). pK_{MCS} = 7.83.

With diazomethane a methyl ester, m.p. 28—29°, was obtained; mol. wt. = 424 (mass spec.). (Found: C 76.85; H 12.34. Calc. for $C_{27}H_{52}O_3$: C 76.35; H 12.34).

Gas chromatographic analysis of crude reduction products. The ZHHI fractions, melting at 92—95° and 85—86°, both formed crystalline products when esterified with ethereal diazomethane. Gas chromatography at 250° showed, however, that the high melting fraction consisted of two components in the ratio 82:18 with the relative retention times of 1.52:1. The lower-melting fraction was by the same method shown to be a 28:72 mixture of two components having the same absolute retention times as the two compounds of the higher-melting fraction. The methyl esters of Fa-Fr-high and Fa-Fr-low, analyzed under the same conditions, proved to be pure. In comparative runs of the methyl esters, the relative retention times were found to be:

$$t_{\text{ZHHI-low}} : t_{\text{Fa-Fr-low}} : t_{\text{ZHHI-high}} : t_{\text{Fa-Fr-high}} = 1.00 : 1.00 : 1.52 : 1.95$$

The retention times of ZHHI-low and Fa-Fr-low methyl esters are identical and mixtures of them gave only one peak in a gas chromatogram.

Thin layer chromatography of the methyl esters of ZHHI-low, ZHHI-high, Fa-Fr-low, and Fa-Fr-high. The four compounds could be separated by thin layer chromatography on silica gel G using petroleum ether (b.p. 60—80°)—diethyl ether, 10:2.

Methyl esters of	R_F
ZHHI-low	0.74
ZHHI-high	0.42
Fa-Fr-low	0.58
Fa-Fr-high	0.53

Isolation of pure ZHHI-low and ZHHI-high methyl esters by chromatography on silica gel. 100 mg of crude ZHHI-low methyl ester was chromatographed on 5 g of silica gel (dried at 130°). With petroleum ether, b.p. 40—60°, 64 mg of material, melting at 61—62°, was obtained. Further elution with petroleum ether—5 % diethyl ether gave 25 mg of a compound melting at 69—70°. Thin layer and gas chromatographic analysis, as described above, proved the two compounds to be pure. ZHHI-low methyl ester was by a mixed melting point determination, infrared and mass spectrometry, and gas and thin layer chromatography shown to be identical with authentic methylhexacosanoate.

Larger amounts of pure ZHHI-high methyl ester were obtained by chromatography of crude ZHHI-high methyl ester fractions. (Found: C 75.77; H 12.43. Calc. for $C_{27}H_{52}O_3$: C 76.35; H 12.34). Mol. wt. = 424 (mass spec.).

Separation of ZHHI-low and ZHHI-high methyl esters by chromatography on urea. 5 g of urea, ground in a ball mill and passed through a 100 mesh screen, was firmly dry-packed in a column. A concentrated solution of 100 mg of crude ZHHI-low methyl ester in isooctane, purified by distillation, was applied at the top of the column. Passing 50 ml of the same solvent through the column without the use of over-pressure gave a fraction containing 24 mg of ZHHI-high methyl ester. It was by gas chromatography at 250° shown to be free from ZHHI-low methyl ester. The latter was isolated by dissolving the urea column in water and extracting with ether. Gas chromatography as described above showed that it contained *ca.* 5 % of ZHHI-high methyl ester. By repeating the chromatography on 5 g of urea, 75 mg of pure ZHHI-low methyl ester was obtained.

Fractions of crude ZHHI-high methyl ester were separated into gas chromatographically pure components by the same procedure.

Preparation of 12-methylhexacosane from Fa-Fr-high. 100 mg of Fa-Fr-high was heated under reflux with 150 mg of lithium aluminum hydride in 7 ml of absolute ether for 15 h. The excess of hydride was destroyed with ethyl acetate and the mixture evaporated to dryness. The inorganic material was dissolved in 2 N hydrochloric acid and extracted with ether giving 95 mg of a crystalline diol, m.p. 75–76°. (Found: C 78.85; H 13.51. Calc. for $C_{27}H_{56}O_2$: C 78.57; H 13.68).

Following the procedure of Downing, Kranz, and Murray,[12] 33 mg of the diol was treated with 100 mg of red phosphorus and 20 mg of iodine. Subsequent reduction with 100 mg of lithium aluminum hydride gave 15 mg of an oil. Gas chromatography at 230° revealed the presence of only one component. Its mass spectrum proved to be identical with that of synthetic 12-methylhexacosane.

Oxidation of Fa-Fr-high with potassium permanganate. 10 mg of Fa-Fr-high was oxidized with 150 mg of potassium permanganate in 5 ml of acetone following the procedure of Murray.[8] Before esterification, however, the monobasic acids were separated from the dibasic acids by steam distillation. Gas chromatography at 163° showed the presence of approximately equal amounts of homologous mono-methyl esters up to pentadecanoic acid. No trace of methyl hexacosanoate could be detected.

Oxidation of Fa-Fr-low with chromium trioxide. 17 mg of Fa-Fr-high was oxidized with 35 mg of chromium trioxide in 2 ml of glacial acetic acid for 2 h at 65°. The green solution was diluted with 15 ml of water and extracted with ether. The combined extracts were washed with a small amount of water and the solvent removed at 50°. The mixture of acids was steam distilled and the volatile acids were extracted with ether and esterified with diazomethane. Gas chromatography of the mixture at 195° proved the presence of methyl octadecanoate and lower homologues but no trace of methyl nonadecanoate. The distillation residue was dissolved in ether and esterified with diazomethane. Gas chromatography at 115° proved the presence of equal amounts of dimethyl succinate and dimethyl glutarate. No trace of dimethyl adipate could be detected.

Oxidation of ZHHI-high methyl ester with chromium trioxide. A solution of 35 mg of ZHHI-high methyl ester in 2 ml of glacial acetic acid was oxidized with 70 mg of chromium trioxide for 2 h at 65°. The steam-volatile acids were isolated as described under the oxidation of Fa-Fr-low. Gas chromatography of the methyl esters at 195° proved the presence of a homologous series of esters up to methyl octadecanoate. No trace of methyl nonadecanoate was detected.

Oxidation of ZHHI-low methyl ester with chromium trioxide. 10 mg of ZHHI-low methyl ester was oxidized with 20 mg of chromium trioxide in 1 ml of glacial acetic acid at 65° for 1.5 h. The steam-volatile acids were isolated as described under the oxidation of Fa-Fr-low. Gas chromatography at 258° showed the presence of a homologous series of methyl esters up to hexacosanoate.

Acknowledgements. The nuclear magnetic resonance spectra have been obtained through the courtesy of Dr. S. Forsén.

The electrometric titrations reported in this paper were kindly performed by Dr. W. Simon, E.T.H., Zürich.

We are indebted to Miss Gurli Hammarberg for infrared and ultraviolet spectra.

We express our appreciation to Professor H. Erdtman and Dr. J. C. Hoogerheide for their interest.

This work has been supported by a grant from the *Swedish Natural Science Research Council.*

The cost of operating the mass spectrometers has been defrayed by grants received from the *Swedish Medical Research Council*, the *Swedish Natural Science Research Council*, and the *Swedish Technical Research Council.*

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Received August 5, 1963.