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Pimaricin

IV *. Investigations on the Pattern of Unsaturation and Oxygenation and the Empirical Formula

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Based on oxidative and retroaldol degradations the partial formula of the polyene antibiotic pimaricin may be expanded to *5*.

The isolation of the aminohexose mycosamine from pimaricin [1,2] and the finding that 12-methylhexacosane represents its parent carbon skeleton [3] provide a reliable basis for further investigations of the gross structure. The present communication reports experiments that locate the tetraene system, three oxygen functions, and one conjugated double bond.

It has been shown that the chromophore is an all-*trans* conjugated tetraene [4] and that the antibiotic [5] and its N-acetyl derivative [1] absorb six moles of hydrogen on catalytic hydrogenation, forming the saturated dodecahydro compounds.

We first wish to describe a series of oxidation experiments performed on pimaricin and its dodecahydro derivative, defining the positions of the polyene chromophore and two oxygen functions enclosing it. Quantitative Kuhn-Roth oxidations indicated that pimaricin and dodecahydropimaricin contain more than one and probably two C-methyl groups. Gas and paper chromatographic [6] investigations of the steam volatile acids revealed the presence of only acetic acid. It was also ascertained by routine group analysis that alkoxy, alkylimino, and acyl groups were absent. In an earlier communication [5] we reported that catalytic hydrogenation of pimaricin under very forcing conditions produced 2-tetradecylundecandioic acid. Since the parent hydrocarbon obtained from pimaricin by a milder and different procedure [3] also exhibits the same branching pattern, C—25 is the only possible position for a C-methyl group in the aglycone. A second C-methyl group is located in the mycosamine moiety. Kuhn-Roth oxidations of both pimaricin and dodecahydropimaricin yielded only acetic acid; consequently C—25 must carry one oxygen function in addition

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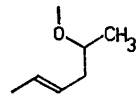
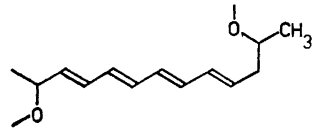
to the methyl group. Ozonolysis of pimaricin followed by steam distillation of the ozonide yielded crotonaldehyde, identified as the 2,4-dinitrophenylhydrazone. The double bond in crotonaldehyde could be formed by elimination of a free or protected hydroxyl group on C—25 during the work-up of the ozonide. These considerations lead to partial formula, *1*:



1

We next proceeded to investigate the dibasic acids resulting from chromic acid oxidations of pimaricin and dodecahydropimaricin. The structure proposed by Patrick *et al.*[1] implies that pimaricin on vigorous oxidation should yield glutaric acid. However, numerous attempts in this laboratory to obtain glutaric acid have been in vain. We have also been unable to isolate succinic acid or any of its higher homologues from oxidations with chromium trioxide in sulfuric acid as well as with nitric acid of different concentrations, red fuming nitric acid, and alkaline permanganate. Controlled oxidations under milder conditions gave on the other hand good yields of fumaric acid, identified as the dimethyl ester and its corresponding pyrazoline.

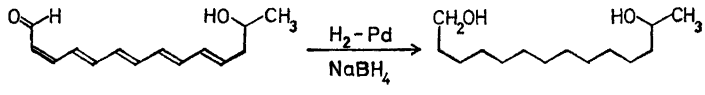
Oxidation of dodecahydropimaricin with chromium trioxide in sulfuric or acetic acid gave a number of dibasic acids. Gas chromatographic analysis of their methyl esters proved undecandioic acid to be the highest acid formed. These findings lead to structure *2* for the tetraene system and its immediate environment:



2

Patrick and his co-workers reported[1] that chromium trioxide—sulfuric acid oxidation of N-acetyldodecahydropimaricin gave sebacic acid. This fact is not entirely satisfactory for their structure proposal. Gas chromatographic investigations of our reaction mixtures have shown sebacic acid under certain conditions to be the main product. Undecandioic acid was formed in minor amounts, not likely to be detected without the aid of chromatographic methods. In view of these results the claim that the isolation of pimelic acid from the chromic acid oxidation of dodecahydropimaricin "completes the minimum proof for the presence of a large ring" seems irrelevant.

Treatment of pimaricin with strong aqueous base gave the hydroxylated conjugated pentaene aldehyde *3*. It was first isolated and described by the



3 4

Lederle group.[1] We wish to present additional evidence for the structure of this remarkable compound. It was obtained in 10 % yield when pimaricin was heated to 90° in 5 % sodium hydroxide solution. After extraction with ether and chromatography of the extract, an amorphous powder with a very intense absorption at 378 mμ, characteristic of a conjugated pentaene carbonyl chromophore,[7] resulted. Its infrared spectrum showed hydroxyl and aldehyde CH stretching absorption at 3500, 2850, and 2750 cm^{-1} and a band for a conjugated carbonyl function at 1690 cm^{-1}. The nuclear magnetic resonance spectrum

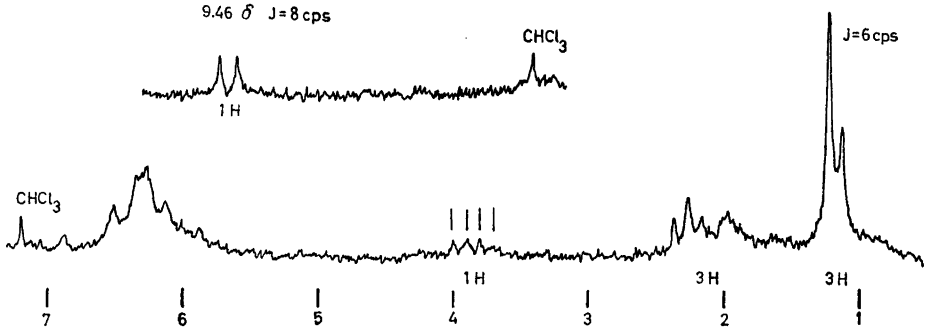
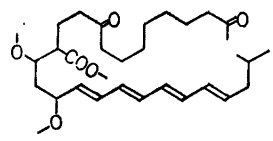


Fig. 1. NMR spectrum of 13-hydroxy-2,4,6,8,10-dodecapentaene-1-al.

with assignments is reproduced in Fig. 1. It is in complete agreement with the proposed structure. The one-proton doublet at $\delta = 9.46$ ($J = 8$ cps) is conclusive proof for the presence of an aldehyde group. The import of the pentaene aldehyde for the complete structure of pimaricin made it desirable to determine its carbon skeleton beyond doubt. Catalytic hydrogenation followed by reduction with sodium borohydride produced a saturated diol which was converted to the iodide with red phosphorus and iodine. Reduction with lithium aluminum hydride then yielded a saturated hydrocarbon. It was gas chromatographically homogeneous and its mass spectrum was identical with that of tetradecane. Finally ozonolysis of the pentaene aldehyde followed by steam distillation produced crotonaldehyde, identified as the 2,4-dinitrophenylhydrazone. These results established the structure as 13-hydroxy-2,4,6,8,10-dodecapentaene-1-al. The Lederle group based their structural proof on the observation that hypobromite oxidation of the saturated C_{14}-diol, *4*, gave tridecandioic acid. The oxidation of a primary hydroxyl to a carboxyl group under these circumstances seemed unusual.

The pentaene aldehyde is formed under conditions inducing retroaldol reactions. We may assume that C—13 in pimaricin carries a hydroxyl group and the cleavage of the bond between C—12 and C—13 is triggered by a real or potential carbonyl function in a β-position. The presented evidence allows partial formula, *5*, for pimaricin:



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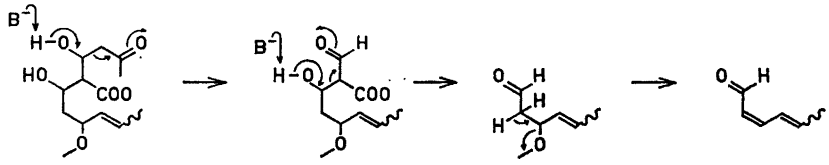
The retroaldol cleavage persuaded the Lederle group to place the keto function erroneously on C—11. We have found, however that if pimaricin is reduced with sodium borohydride before the treatment with sodiumhydroxide, no pentaene aldehyde can be detected. This indicates instead that the C—9 keto group is the origin of a retroaldol reaction proceeding in two steps. Such a process would require a hydroxyl group on C—11:



6 7 8 3

The above mechanism supports the biogenetically attractive assumption that the carboxyl group on C—12 is not lactonized in pimaricin; the carboxylate anion which would prevail in basic medium would not promote the retroaldol cleavage.

Treatment of pimaricin with strong base resulted in the liberation of ammonia.[1] On these grounds, Patrick and co-workers assumed that mycosamine is attached to C—15. Beta-elimination of the sugar produces the fifth double bond of the pentaene aldehyde; the free aminohexose is then susceptible to alkaline degradation. This conclusion is probably correct, but the elimination of mycosamine only requires the glycosidic linkage to be in a β-position to a real or potential carbonyl group. Other possibilities are therefore still open at the present stage.

Pimaricin contains at least five double bonds, of which four have been assigned to the chromophore. The antibiotic exhibits strong ultraviolet absorption at 221 mμ (ε = 20 000), which disappears on hydrogenation. The absence of carbonyl bands below 1710 cm^{-1} in its infrared spectrum excludes the presence of a conjugated aldehyde or ketone group. Hence the fifth double bond must be present as part of an α,β-unsaturated acid or lactone system. From results of the retroaldol cleavage we have placed an oxygen atom on C—11. Therefore only the C—1 carboxyl group can be α,β-unsaturated. The position of attachment of the lactone ring (whether at the C—1 or the C—12 carboxyl) will be considered in a subsequent paper.

At this stage of the structural determination it seems appropriate to discuss the empirical formula of pimaricin. The analytical values for carbon, hydrogen, nitrogen, and oxygen total 100 %; hence no other elements are present. We have already reported that functional group analysis proved the absence of

alkoxy, alkylimino and acyl groups. The isolation of mycosamine and 12-methylhexacosane therefore accounts for all carbon atoms in the pimaricin molecule, a total of thirty-three. This information in combination with the elemental analyses leads to a molecular formula of $C_{33}H_{47-49}NO_{14}$ for the antibiotic, where the number of hydrogens still remains in doubt. We should like to emphasize here that determination of the molecular formulas of similar compounds requires a knowledge of the exact number of carbon atoms before a single molecular composition may be decided upon from the result of microanalyses. The molecular weight determination is not always a statistical problem; a large number of analyses do not necessarily lead to a more reliable value. Structural work in the macrolide group has clearly demonstrated the failure of this approach,[8] when it is applied to compounds in the C_{25} to C_{40} range. In this case, for example, on the basis of the mean values of seventeen analyses the Lederle group assigned pimaricin the composition $C_{34}H_{49}NO_{14}$. The present investigations have shown that this formula is incorrect. Our preliminary results based on twenty-four analyses seemed to agree with a C_{33}-formulation but a C_{34} could not be entirely excluded. It was noteworthy that even after several recrystallizations all samples invariably contained 0.2—3.0 % of ash, most likely due to the amphoteric character of pimaricin. When the antibiotic was purified by chromatography on ion-exchange resins, ash-free samples, affording consistent analytical data, not in conflict with a C_{33}-formula, were obtained after recrystallization. Complete agreement could not be achieved due to the extreme difficulty encountered in the preparation of solvent-free samples.[9]

Examination of partial formula *5* for pimaricin shows that four oxygen functions and one carbon-carbon double bond remain to be placed on C—2 to C—8 and C—10 to C—11. We have reported that no dicarboxylic acids could be found among the oxidation products of pimaricin. This observation excludes structures of the antibiotic containing two or more adjacent methylene groups.

EXPERIMENTAL

Ultraviolet spectra were determined in ethanol solution on a Beckman DK 2 instrument and infrared spectra on a Perkin-Elmer Model 21 spectrograph. The nuclear magnetic resonance spectrum was recorded on a Varian A—60 spectrometer using deuterochloroform as solvent and tetramethylsilane as internal standard. The gas chromatographic analyses were performed with a Pye Argon Chromatograph. The mass spectrum was determined using a CEC 21—103 C type instrument. The elemental analyses were performed by Dr. A. Bernhardt, Mülheim, Ruhr, Mr. M. van Leeuwen, Delft, and Mr. P. J. Hubers, Amsterdam.

Kuhn-Roth oxidations of pimaricin and of dodecahydropimaricin. 2 mg of pimaricin was oxidized following the modified procedure of Garbers, Schmid, and Karrer.[5] The paper chromatogram showed only one large spot for acetic acid.

When 2 mg of dodecahydropimaricin was oxidized in an identical manner, the paper chromatogram again showed only a large spot for acetic acid.

Ozonolysis of pimaricin. A solution of 0.200 g of pimaricin in 10 ml of methanol was ozonized with a stream of oxygen containing 3 % of ozone for 15 min at −50°. After evaporation of the solvent, the ozonide was decomposed by steam distillation. The distillate was led into a saturated solution of 2,4-dinitrophenylhydrazine in hydrochloric acid. The red precipitate formed was proved to be crotonaldehyde-2,4-dinitrophenylhydrazone by comparison of infrared spectra and by mixed melting points with authentic material.

Exhaustive oxidations of pimaricin. (*a*) 5 ml of fuming nitric acid ($d = 1.52$) was added dropwise to 0.300 g of pimaricin and the mixture was heated on the steam bath for 1 h. The solution was diluted with water and extracted with ether. The extracted material, 15 mg, was esterified with diazomethane.

(*b*) A solution of 0.240 g of pimaricin in 15 ml of concentrated nitric acid and 5 ml of water heated for 1 h on the steam bath and then worked up as described under (*a*) yielded 13 mg.

(*c*) A mixture of 0.100 g of pimaricin and 6.5 ml of 20 % sulfuric acid containing 1.3 g of chromium trioxide was heated on the steam bath for 15 h. Work-up as described under (*a*) yielded 10 mg.

(*d*) A solution of 0.100 g of primaricin in 10 ml of glacial acetic acid containing 0.400 g of chromium trioxide was kept at 60° for 2 h. After dilution with water the acidic material was extracted with ether and the combined fractions evaporated to dryness under reduced pressure. The residue, 10 mg, was esterified with diazomethane.

(*e*) A solution of 50 mg of pimaricin in 10 ml of water containing 200 mg of potassium carbonate was oxidized with 480 mg of potassium permanganate at 20° for 2 days. The mixture was then acidified with 2 N sulfuric acid, and sodium bisulfate was added to dissolve the manganese dioxide. The clear solution was extracted with ether, the combined extracts washed with a small amount of water, dried over sodium sulfate and evaporated to dryness. The residue, 10 mg, was esterified with diazomethane.

The methylated fractions obtained in (*a*)—(*e*) were analyzed by gas chromatography at 100—150° (5 % SE—30 on celite). In no case could peaks corresponding to the dimethylesters of succinic, glutaric, or adipic acid be detected.

Isolation of fumaric acid from pimaricin. A solution of 2.07 g of pimaricin in 20 ml of 20 % sulfuric acid was oxidized with 7.2 g of chromium trioxide for 10 min at 60°. The mixture was directly extracted with 300 ml of ether and the combined extracts were washed three times with 5 ml of water. After evaporation of the solvent, 0.520 g of a white powder remained.

81 mg of this material was esterified in 3 ml of absolute methanol containing 3 drops of concentrated sulfuric acid under reflux for 3 h. The solution was deionized by passing it over an ion exchange resin (Amberlite—MB3). Evaporation of the solvent gave crystalline dimethylfumarate, m.p. 100—101°, identified by comparison of infrared spectra and by mixed melting points with authentic material.

Treatment of the dimethylester and of the free acid with diazomethane gave the crystalline, m.p. 98°, dimethylester of pyrazoline-3,4-dicarboxylic acid [9] identified by comparison of infrared spectra and by mixed melting points with authentic material.

Isolation of dibasic acids from dodecahydropimaricin. Dodecahydropimaricin, 62 mg, was oxidized with 320 mg of chromium trioxide in 3 ml of 20 % sulfuric acid at 70° for 15 min. The acidic material was extracted with ether, the combined extracts were washed with water, dried over sodium sulfate and evaporated to dryness. After esterification with diazomethane, a petroleum ether solution of the esters was filtered over alumina (Woelm, activity III). The purified mixture was analyzed by gas chromatography at 160° (5 % SE—30 on celite). The gas chromatogram showed a number of peaks due to a series of homologous dimethyl esters. Dimethylundecandioate was found to be the highest one present; no trace of dimethyldodecanoate could be detected.

Isolation of 13-hydroxy-2,4,6,8,10-dodecapentaene-1-al (3) from pimaricin. A solution of 250 mg of pimaricin in 10 ml of water containing 100 mg of sodium hydroxide was heated on the steam bath for 15 min; ammonia was liberated. The cooled mixture was extracted with three 20 ml portions of ether. After drying over sodium sulfate, the ether layer was filtered through an alumina column (Woelm, activity II) and evaporated to dryness yielding 14 mg of a yellow non-crystalline powder; $\lambda_{max} = 378$ mμ, $\varepsilon \approx 30\,000$.

Conversion of 3 to tetradecane. The pentaene aldehyde, 14 mg, was added to 5 ml of ethanol containing 10 mg of prereduced platinum oxide and hydrogenated for 2 h. After separation of the catalyst and evaporation of the solvent the noncrystalline residue was dissolved in 5 ml of absolute ether and reduced with excess lithium aluminum hydride at 20° for 15 h. The unchanged hydride was destroyed with ethyl acetate, and the mixture evaporated to dryness. The inorganic material was dissolved in 2 N sulfuric acid and extraction with ether gave after drying (sodium sulfate) and evaporation of the solvent 11 mg of an oily polyol. This oil was treated with 18 mg of red phosphorus and 60 mg of iodine according to the procedure of Downing, Kranz, and Murray.[10] Reduction with

lithium aluminum hydride, then with hydrogen and platinum, and subsequent chromatography on alumina (Woelm, activity I) yielded 5 mg of a hydrocarbon which was shown to be homogeneous by gas chromatography (20 % silicone grease, 210°). A sample was collected from the same column and its mass spectrum proved identical with that of tetradecane.

Ozonolysis of 3. 20 mg of the pentaene aldehyde 3 was dissolved in 3 ml of chloroform and ozonized at −50° for 10 min. The solvent was evaporated and the ozonide was steam-distilled into a solution of 2,4-dinitrophenylhydrazine reagent. The red precipitate formed showed an infrared spectrum (KBr) identical with that of authentic crotonaldehyde-2,4-dinitrophenylhydrazone and a mixed melting point showed no depression.

Treatment of sodium borohydride-reduced pimaricin with sodium hydroxide. 500 mg of pimaricin was reduced with excess sodium borohydride in 10 ml of methanol. After 15 h the solution was deionized by passing it over an ion exchange resin (Amberlite-MB3). After evaporation of the solvent under reduced pressure the residue was dissolved in

Table 1.

Calculated for	% C	% H	% N
$C_{33}H_{47}NO_{14}$	58.14	6.95	2.06
$C_{33}H_{47}NO_{14}\cdot H_2O$	56.64	7.06	2.00
$C_{33}H_{47}NO_{14}\cdot CH_3OH$	57.21	7.20	1.96
$C_{34}H_{49}NO_{14}$	58.69	7.09	2.02
$C_{34}H_{49}NO_{14}\cdot H_2O$	57.21	7.20	1.96
$C_{34}H_{49}NO_{14}\cdot CH_3OH$	57.76	7.35	1.93

Table 2.

No.	Method of purification	% C	% H	% N	% Ash
1	Dissolved in glacial acetic acid, then precipitated with alkali	57.62	7.25	2.05	1.38
2	Same as No. 1	57.77	7.27	1.95	0.35
3	Same as No. 1	57.79	7.25	2.00	—
4	Dissolved in dimethylformamide, then precipitated with water	57.00	7.29	1.87	3.77
5	Washed with methanol containing acetic acid	57.32	7.34	1.91	—
6	Recrystallized from methanol containing aqueous calcium chloride	57.48	7.28	2.25	0.6
7	Same as No. 6	58.45	7.47	2.10	0.54
8	Liberated from Na-salt, then recrystallized from methanol	57.80	7.24	2.10	—
9	Liberated from HCl-salt	57.98	7.22	2.04	—
10	Chromatography on Amberlite IRC−50	57.79	7.30	1.87	—
11	Recrystallized from water	58.02	7.28	2.03	0.9
12	Recrystallized from methanol—water	57.50	7.34	2.06	1.07
13	Same as No. 12	57.50	7.32	2.04	1.02
14	Recrystallized repeatedly from methanol-water	58.02	7.31	1.93	—
15	Same as No. 14	58.01	7.44	1.88	—
16	Same as No. 14	57.79	7.68	2.11	—

Table 2 reports analytical results obtained with samples of pimaricin purified in different ways. The samples were dried at 20−70°/0.2 mm for *ca.* 12 h.

The mean values of analyses Nos. 3, 8, 9, 10, 14, 15, and 16, as of seventeen more were: C 57.90, H 7.33 and N 2.08.

20 ml of water containing 200 mg of sodium hydroxide and heated on a steam bath for 15 min. Extraction with ether did not yield any solid material, and an ultraviolet spectrum of the reaction mixture did not reveal any absorption at 378 mμ.

Elemental analyses of pimaricin. Table 1 lists C, H, and N values calculated for C_{33} and C_{34} formulas, assuming that pimaricin contains (*i*) no solvent of crystallization, (*ii*) one mole of water, and (*iii*) one mole of methanol.

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