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Pimaricin

VI *. Complete Structure of the Antibiotic

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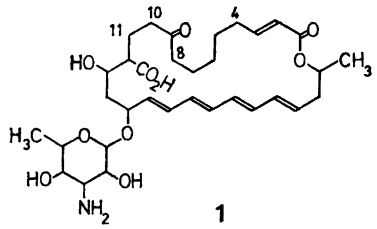
Data leading to structure *9* for pimaricin are presented.

In five preceding papers [1–5] it has been demonstrated that pimaricin has partial structure *1*:



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As the antibiotic has the empirical formula $C_{34}H_{47-49}NO_{14}$,[4] four oxygens remain to be placed. A satisfactory structure should not contain two or more adjacent methylene groups in order to accommodate the finding that pimaricin does not yield any saturated dicarboxylic acids on vigorous oxidation.[4] The possible sites are therefore C—4 to C—8, C—10, and C—11.

On catalytic reduction, pimaricin absorbs six moles of hydrogen. Five of these are accounted for by the tetraene and the α,β-unsaturated lactone systems. The Lederle group assumed that the sixth mole was consumed by an epoxide function.[6] We have reached the same conclusion and offer additional data in support of it. Treatment of ethylene oxides adjacent to a carbonyl function is known to remove the oxygen atom and introduce a double bond.[7] This reaction was used to prove the presence of system *2* in the macrolide magnamycin.[8] The reaction product, *3*, contains an $\alpha,\beta,\gamma,\delta$-unsa-

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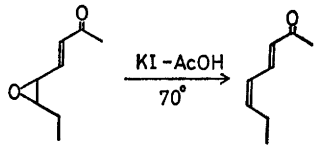
turated ketone system, readily detectable by its characteristic ultraviolet absorption at 275 mμ ($\varepsilon = 25\,000$):



2 3

On treatment of pimaricin with the same reagent at 60—70° large amounts of iodine were liberated. This is in itself not unexpected since we are dealing with a highly unsaturated and hydroxylated compound. Attempts to isolate a crystalline reaction product were unsuccessful. The ultraviolet spectrum (*cf.* Fig. 1) reveals, however, that a new chromophore has resulted from the

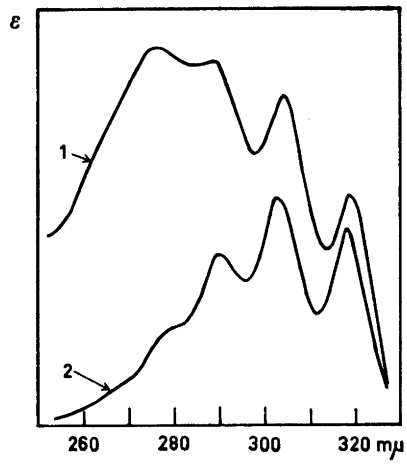
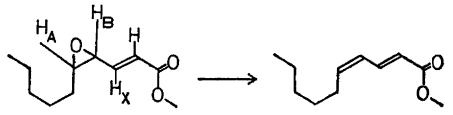


Fig. 1. Ultraviolet spectra of pimaricin (2) and of pimaricin after treatment with potassium iodide — acetic acid at 70° (1).

potassium iodide—acetic acid treatment. The new absorption overlaps the tetraene bands, but a difference spectrum shows a single, strong maximum at 269 mμ ($\varepsilon = 27\,000$). Since the ultraviolet spectrum of the new chromophore displays no fine-structure it is due to a doubly conjugated carbonyl system, *5*. We therefore assume that the epoxide is adjacent to the already existing α,β-unsaturated lactone:



4 5

Conceivably the epoxide function could be at C—10, C—11 or at C—12, C—13 and the elimination reaction be initiated by the C—9 keto group and/or the C—12 carboxyl group. In either case formation of an α,β,γ,δ-unsaturated

ketone would in addition require elimination of a hydroxyl group and possibly decarboxylation. These possibilities seem remote as the ultraviolet spectrum is not affected by changes of pH and remains unaltered after reduction with sodium borohydride.

Supporting evidence for the presence of an 1,2-epoxide group is provided by the nuclear magnetic resonance spectra of the N-acetyl derivatives of pimaricin (*cf.* Fig. 2) and dodecahydropimaricin (*cf.* Fig. 3). Methine protons on sub-

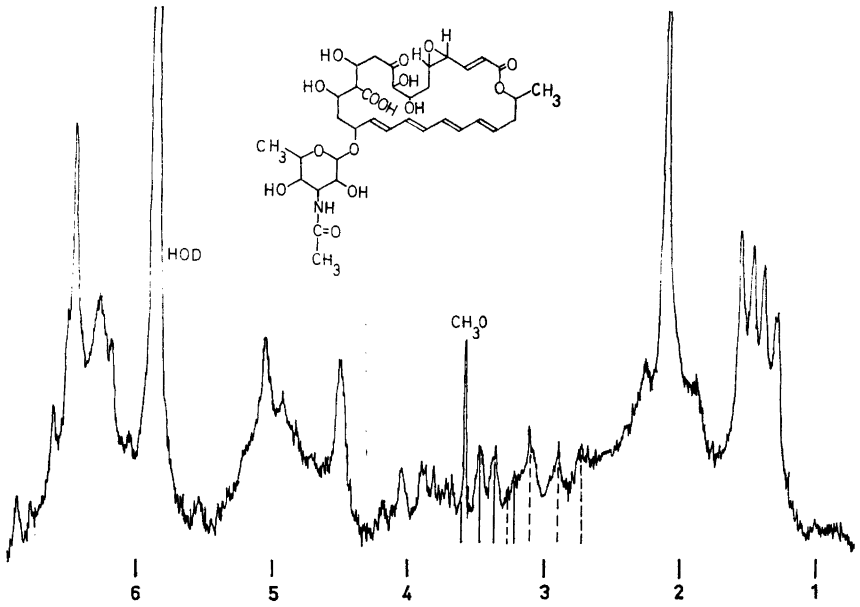


Fig. 2. NMR spectrum of N-acetylpimaricin.

stituted epoxides are reported to appear at $\delta = 3.0$.[9] The spectrum of N-acetylpimaricin displays between $\delta = 2.6$ and 3.6 several signals together representing three protons. One of these is exchanged by shaking the sample with deuterium oxide.[10] The two remaining ones then give rise to two slightly overlapping quartets centered at $\delta = 3.00$ and 3.38, the latter representing the allylic epoxy proton. The pattern is in agreement with the AB part of an ABX system. The spectrum of the dodecahydro compound does not show any absorption in this region. It should be mentioned that the absorption at $\delta = 3.38$, described as a quartet, in fact has a more complicated splitting pattern. A spectrum of N-acetylpimaricin measured at 100 Mc in pentadeuteropyridine displays at least eight lines. This is not unexpected since coupling with one or two protons on C—6 also should occur. Reductive opening of the epoxide probably creates a new hydroxyl group on C—5 and explains the presence of a 5,9-epoxy ring in Fa-Fr-low [2] and HH1.[5]

Turning now to the substitution on C—10 and C—11, we already discussed that at least one of them must carry an oxygen function. It was shown earlier [3] that no trace of the pentaene aldehyde could be detected when borohydride-

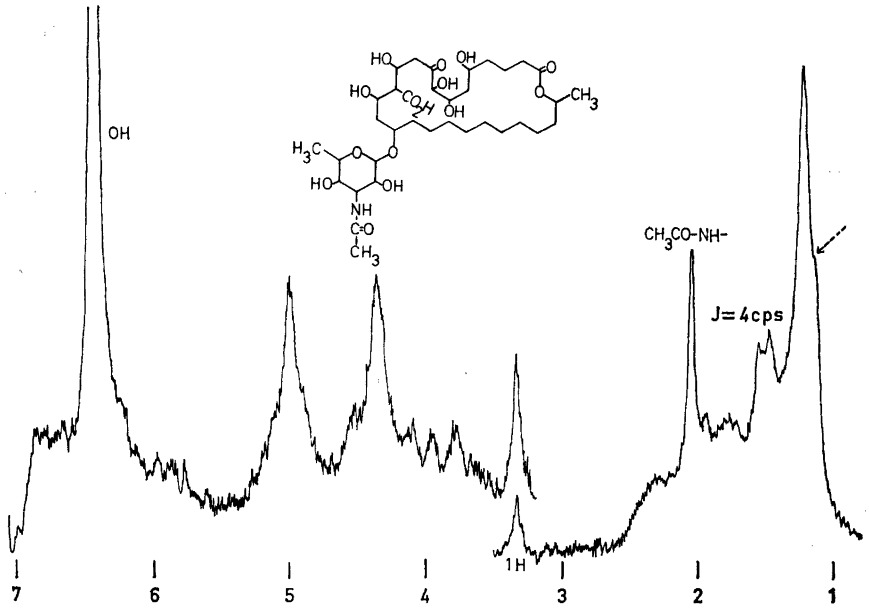
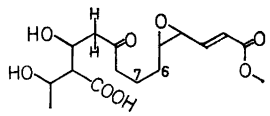


Fig. 3. NMR spectrum of N-acetyldodecahydropimaricin.

reduced pimaricin was subjected to strong base treatment. This observation favored a two-step retroaldol reaction and required a hydroxyl group on C—11. We believe, further, that C—10 is unsubstituted because a hydroxyl group in this position would be able to form a γ-lactone with the free carboxyl group on C—12. The infrared spectra of pimaricin, dodecahydropimaricin and their N-acetyl derivatives in no case display absorption characteristic of a five-membered ring lactone system. Furthermore, electrometric titrations have proved that a free carboxyl group is present in pimaricin and its dodecahydro derivative.[3]

The partial formula for pimaricin can now be extended to *6*:



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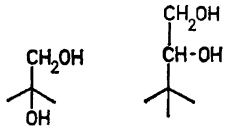
leaving two oxygen atoms to be placed on C—6, C—7 or C—8.

N-Acetylpimaricin and N-acetyldodecahydropimaricin each consume two equivalents of periodate in neutral medium, proving the presence of two 1,2- or one 1,2,3-glycol, or equivalent systems. A hydroxyl group on C—6 is not in accord with these requirements and leaves C—7 and C—8 as the only possible sites. This arrangement agrees well with the observations that one mole of periodate is consumed immediately and the second after two hours,[6] and that

after complete oxidation the solution is strongly acidic. Further support for the assumption that two hydroxyl groups are located on C—7 and C—8 is obtained from the fact that periodate-permanganate oxidations [11] of N-acetyl dodecahydropimaricin followed by basic hydrolysis in no case yielded a saturated and hydroxyl-free dicarboxylic acid.

Patrick *et al.* claimed that formaldehyde was a product of the periodate oxidation of N-acetylpimaricin,[6] a finding we have not been able to verify. Numerous experiments involving careful column, paper, and thin layer chromatography of the 2,4-dinitrophenylhydrazone mixtures never resulted in the isolation of the formaldehyde derivative. Its formation would require arrangements *7* or *8*:



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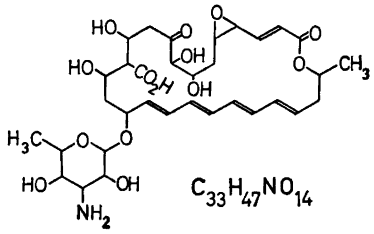
and it is apparent that no such grouping can be accommodated in the established carbon skeleton of the antibiotic. The complete structure of pimaricin is therefore *9*:



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The nuclear magnetic resonance spectra of N-acetylpimaricin and its dodecahydro derivative (measured in pyridine solution, see Figs. 2 and 3) should now be considered in terms of the established structure. In N-acetylpimaricin the N-acetyl signal appears as a three-proton singlet at $\delta = 2.07$. The two doublets ($J = 6$ cps), centered at $\delta = 1.45$ and 1.17, conclusively show the presence of only two methyl groups each on a carbon carrying one hydrogen atom. The absorption at $\delta = 2.20$ is probably due to the methylene group adjacent to the carbonyl group on C—9. The signal at $\delta = 3.22$ which disappears after exchange with deuterium oxide could be due to a hydrogen bonded proton in the acyloin grouping or to the carboxyl proton. The sharp band at $\delta = 3.57$ is derived from residual methanol, a demonstration of the difficulty of eliminating traces of solvents even by long drying. The region between $\delta = 3.5$ and 5.5 is unchanged after deuterium exchange and represents the protons on the carbon atoms bearing hydroxyl or ether oxygens.

Although it is quite complex, one can distinguish between $\delta = 3.6$ and 4.2, the same pattern that in the mycosamine spectrum is due to the hydrogens on C—2, C—3, C—4, and C—5. The olefinic and anomeric protons appear be-

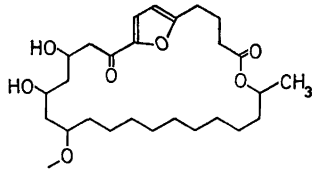
tween $\delta = 6.0$ and 6.7. The hydroxyl protons are not visible in the spectrum, but after deuterium exchange the HOD band appears at $\delta = 5.85$.

N-Acetyldodecahydropimaricin shows strong absorption at $\delta = 6.42$ which on deuteration moves to $\delta = 5.80$ and consequently is due to the hydroxyl protons. The large number of methylene protons introduced by hydrogenation appear as a very strong signal at $\delta = 1.25$ dividing and partly overlapping one of the methyl doublets. At $\delta = 3.37$ a new one-proton singlet, non-exchangeable with deuterium, has formed. We are uncertain of its origin.

With the structure of pimaricin established we wish to report a few findings unrelated to the structural proof. Elimination reactions creating conjugated systems when a macrolide is treated with sodium alkoxides in alcohol have been reported for fungichromin,[12] oleandomycin,[13] magnamycin,[8] and neomethymycin.[14] We have found that similar treatment of pimaricin and dodecahydropimaricin does not produce a conjugated system.

Very useful structural information has been obtained from the isolation of volatile carbonyl compounds formed in retroaldol reactions.[12,15] Under similar conditions pimaricin and its dodecahydro derivative yielded only acetaldehyde, probably formed by degradation of the mycosamine moiety.

Patrick *et al.* reported the isolation of a crystalline compound from acid-treated N-acetyldodecahydropimaricin to which they assigned the structure of a furylketone based on its ultraviolet absorption.[6] We have verified this finding but have not succeeded in obtaining the compound in a crystalline state. In terms of the revised formula one can visualize a ketone of that type with the following structure:



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It should be kept in mind, however, that an $\alpha,\beta,\gamma,\delta$-unsaturated ketone displays the same ultraviolet absorption.

The difficulty of reconciling the structure proposed for pimaricin by the Lederle group with an acetate biogenesis was one of the main reasons for this reinvestigation. The oxygenation pattern of the revised formula is in complete agreement with the assumption of an acetate origin. In only one site, C—8, an extra oxygenation occurs. The presence of a carboxyl group on C—12 is a new feature not earlier encountered in macrolides of known structure, although limited information published on amphotericin B [16] indicates that it also contains a free carboxyl group.

It has been demonstrated that the presence of oleic acid in the fermentation medium considerably increases the yields of polyene antibiotics.[17] The carbon chain C—26, C—15 to C—27 formally represents a C_{16} monocarboxylic acid which is condensed with a C_{11} fragment. A β-oxidation of oleic acid could possibly be the starting point in the biosynthesis of the polyene macrolides.

Two macrolides have been found to contain side chains of a higher oxidation state than that of an alcohol. In oleandomycin [12] an ethylene oxide system is present and in magnamycin [8] an aldehyde group. A recent investigation demonstrated the latter to be derived from the glucose in the fermentation medium.[18] Conceivably in pimaricin C—27 could also have a carbohydrate origin. The oxygenation pattern between C—7 and C—11 would not be in disagreement with such a theory.

It is tempting to speculate whether or not the biosynthesis of the polyene macrolides and the saturated macrocyclic lactones follow a common pathway. If this were the case, the conjugated system could be formed by elimination reactions from hydroxylated macrocyclic precursors and the polyenes would represent final stages of macrolide biosynthesis. However, other facts, in particular the larger ring size of the polyenes and the effect of oleic acid on their formation seem to indicate that the two classes are synthesized by different routes.

The problem of the formation of the polyene system is also intriguing. Since a glycosidic hydroxyl would be a more facile leaving group than a free one, a function of the sugar moiety may be to introduce a double bond. Pimaricin has mycosamine in a position suitable for elimination to form a pentaene. It is noteworthy that the only macrolides as yet found lacking a carbohydrate moiety (filipin,[19] fungichromin,[12] and lagosin [20]) belong to the polyene group.

If the carbohydrate elimination hypothesis is correct, a number of intermediates containing more than one or two sugar moieties might be present during earlier stages of polyene biosynthesis. Isolation of such intermediates might be difficult but would serve to improve our understanding of the biogenesis of macrolides.

EXPERIMENTAL

Ultraviolet spectra were determined in ethanol solution on a Beckman DK 2 spectrophotometer. Nuclear magnetic resonance spectra were recorded with a Varian A—60 spectrometer using pyridine as solvent and with a Varian HR—100 using pentadeuteropyridine as solvent. Gas chromatographic analyses were performed with a Pye Argon chromatograph using a 90 × 1 cm column packed with silicone rubber (General Electric Co.) on 60—65 mesh celite. Thin layer chromatograms were carried out on silica gel (Merck) according to Stahl.[21] The spots were made visible either with iodine or by spraying with chromium trioxide in sulfuric acid followed by heating to 120—140° for 5 min.

Treatment of pimaricin with potassium iodide—acetic acid. To a solution of 6.0 mg of pimaricin in 2 ml of glacial acetic acid a few crystals of potassium iodide were added. Kept at a temperature of 60—70° for 5 min, the solution turned yellowish brown due to liberation of iodine. After cooling and dilution with methanol the iodine was removed by passing the solution over an ion exchange resin (Dowex 2—Cl^-). An aliquot of the eluate was taken for determination of the ultraviolet spectrum (*cf.* Fig. 1), which displayed a new maximum at 269 mμ (ε = 27 000) overlapping the tetraene bands. The position and intensity of the absorption of the additional chromophore was obtained by subtracting the curve of pure pimaricin from the spectrum.

The above experiment was carried out with 100—500 mg samples of pimaricin. After deionizing (Amberlite MB 3) the reaction mixture and evaporating the solvent an amorphous powder remained. Its ultraviolet spectrum was identical with that obtained in the small scale experiment. The extinction coefficient at 269 mμ had a value of 20 000 —26 000.

Periodate titrations. The consumption of sodium metaperiodate was determined by the arsenite method.[22] Water was used as solvent. After complete oxidation usually no material remained undissolved.

Compound	Moles of $NaIO_4$ consumed
N-Acetylpimaricin	2.10
N-Acetyldodecahydropimaricin	1.95
Pimaricin	5.86
Dodecahydropimaricin	2.00

Oxidation of N-acetyldodecahydropimaricin with periodate — permanganate.[11] 500 mg of N-acetyldodecahydropimaricin were oxidized with 8.56 g of sodium metaperiodate and 0.106 g of potassium permanganate in 1.5 l of water containing 2.10 g of potassium carbonate. After stirring for 22 h in the dark the solution was acidified with hydrochloric acid. Extraction with chloroform yielded 0.221 g of oily material. A part of it was directly esterified with diazomethane and investigated by gas and thin layer chromatography. The rest was saponified with aqueous sodium hydroxide, the solution acidified and extracted with ether. The extracted material was esterified with diazomethane and investigated by thin layer and gas chromatography. In no case could the methyl esters of succinic, glutaric, or adipic acid be found.

Attempts to isolate formaldehyde from periodate oxidations of N-acetylpimaricin and N-acetyldodecahydropimaricin. (*a*) A suspension of 77 mg (0.104 mmole) of N-acetylpimaricin in 2.25 ml of water containing 22.5 mg (0.104 mmole) of sodium metaperiodate was stirred for 2 h in the dark. The solution was passed over an ion exchange resin (Amberlite IRA−400, acetate form [23]) and the eluate steam-distilled. The distillate was led into a solution of dimedone and left over night. No precipitate formed.

The same experiment was repeated and the distillate led into 2,4-dinitrophenylhydrazine reagent. No precipitate could be detected. The weight of 0.1 mmole of formaldehyde 2,4-dinitrophenylhydrazone is 22 mg.

(*b*) 32 mg (0.043 mmole) of N-acetylpimaricin was oxidized with 71 mg (0.332 mmole) of sodium metaperiodate in 7 ml of water as described under (*a*). No precipitate could be detected in the 2,4-dinitrophenylhydrazine solution.

(*c*) 500 mg of N-acetylpimaricin was oxidized with 1.68 g of periodic acid ($HIO_4 \cdot 2H_2O$) in 15 ml of water. The temperature was slowly raised to 80° (*cf.* Ref.[24]). Through the solution a slow stream of nitrogen was led into a trap containing 2,4-dinitrophenylhydrazine reagent. After 3 h the precipitate formed was separated.

The solutions of 2,4-dinitrophenylhydrazine from (*a*)−(*c*) were extracted with chloroform and the extracted material chromatographed on bentonite-celite (4:1) [25] with chloroform−3 % ethanol. The extracts were also investigated by paper chromatography (heptane-methanol [26]) and by thin layer chromatography on silica gel (carbon tetrachloride-ether, 4:1 [27]). In no case could formaldehyde 2,4-dinitrophenylhydrazone be found.

The precipitate from (*c*) was investigated with the same methods as the chloroform extracts. It was found to consist of only acetaldehyde 2,4-dinitrophenylhydrazone.

The same oxidations carried out on N-acetyldodecahydropimaricin gave identical results.

Treatment of pimaricin with sodium methoxide. 10 mg of pimaricin was dissolved in 10 ml of 0.2 M sodium methoxide in absolute methanol. After standing for 12 h the ultraviolet spectrum of the solution was determined; the tetraene absorption was unchanged and no additional maxima could be detected.

The same result was obtained when the experiment was repeated with 2 M sodium methoxide in absolute methanol.

Retroaldol reactions on pimaricin and dodecahydropimaricin. Pimaricin (200 mg) was added to a solution of 150 mg of sodium hydroxide in 10 ml of water, and the suspension was steam-distilled into a solution of 2,4-dinitrophenylhydrazine in aqueous hydrochloric acid. The precipitate was extracted with chloroform and the combined extracts chromatographed on bentonite-celite (4:1).[25] Elution with chloroform containing increasing amounts of ethanol gave 10 mg of crystalline acetaldehyde 2,4-dinitrophenylhydrazone. It was identified by mixed melting points and paper chromatography (heptane-methanol [26]) using authentic material as references. No other 2,4-dinitrophenylhydrazone could be detected.

The same result was obtained when the retroaldol experiment was repeated with 200 mg of dodecahydropimaricin.

Preparation of "furylketone".[6] A suspension of 110 mg of N-acetyldodecahydropimaricin in 5 ml of 1 N sulfuric acid was kept at 90° for 5 min. Extraction with chloroform yielded 26 mg of an amorphous product, showing strong absorption at 281 mμ ($E^{1\,\%}_{1\,cm} = 120$) The extinction coefficient is 35 % of the value reported.[6] Chromatography on silica gel did not yield any crystalline material.

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