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Pimaricin

V *. Determination of the Size of the Macrocyclic Lactone Ring and the Position of Mycosamine

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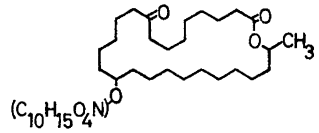
Catalytic hydrogenation of pimaricin at 150° and 150 atm. yields two compounds, HH2, *1*, and HH1, *2*, whose structures imply that the antibiotic contains a twenty-six membered ring lactone and has mycosamine attached to C–15.

In a preceding paper [1] we have described the isolation and structural determination of four nitrogen-free, fatty acid-like, crystalline compounds. They were obtained when pimaricin was subjected to high pressure catalytic hydrogenation at elevated temperatures.

It seemed conceivable that the use of milder conditions would yield recognizable products retaining the glycosidic linkage, and possibly the lactone ring. Indeed this supposition proved correct, and the present communication

* Part IV, *Acta Chem. Scand.* 18 (1964) 103.

concerns structural investigations of the following two compounds, designated as HH2, *1*, and HH1, *2*.



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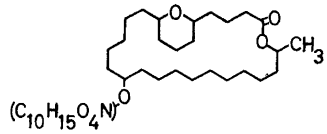

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The compounds were obtained (among other products) by hydrogenations of pimaricin in glacial acetic acid at 150° and 150 atm. with palladium on alumina as catalyst. They have very similar chemical and physical properties, but could be separated by fractional crystallization. Their formulas imply that pimaricin contains a twenty-six membered lactone ring, and that mycosamine is attached to the nucleus *via* a glycosidic linkage at C—15.

HH2 is optically active ($[\alpha]_D = -51°$) and contains one atom of nitrogen calculated on the basis of a molecular weight of 625—650. Its infrared spectrum and other physical and chemical properties proved HH2 to be different from dodecahydropimaricin. Consequently the pimaricin molecule had been reductively degraded without breaking the glycosidic bond.

HH2 represents another case where the empirical formula cannot be unambiguously calculated from the analytical data. We therefore attempted to determine the molecular weight directly by mass spectrometry. HH2 showed a molecular ion peak at $m/e = 625-650$. The exact position of this peak was determined to 637 from the spectrum of a mixture of HH2 and a high molecular weight "fluorolube residues",* using the high intensity fluorine-containing ions at $m/e = 631$, 641, and 643 as references.[2] It then follows from analytical data that the empirical formula is $C_{36}H_{63}NO_8$.

As expected from the mode of preparation, HH2 is a saturated compound. It does not exhibit any high intensity ultraviolet absorption and is recovered unchanged from ozonolysis in chloroform at −50°. Solubility and chromatographic properties indicate that HH2 is neutral and not extensively hydroxylated. During the hydrogenation, the free carboxyl group in pimaricin has either been eliminated or formed a new lactone, because after treatment with diazomethane HH2 is recovered unchanged.

The infrared spectrum of HH2 shows a band at 1725 cm^{-1} with a shoulder at 1710 cm^{-1}. At 1418 cm^{-1} absorption typical of a $-CH_2-CO$-grouping appears. These findings are in accord with a lactone structure containing a nonconjugated carbonyl group. Reduction of HH2 with potassium borohydride produces the crystalline "dihydro HH2", whose infrared spectrum shows only a single band at 1725 cm^{-1}. The shoulder at 1710 cm^{-1} can therefore be assigned to a ketonic carbonyl group. We then proceeded to determine the location of this function. Treatment of HH2 with concentrated hydroiodic acid and red phosphorus produced an oil that was catalytically reduced and then esterified with diazomethane. After chromatography on silica gel a crystalline

* Kindly supplied by Dr. J. H. Beynon, Manchester, England.

compound resulted. Its gas and thin layer chromatographic behavior, melting point, infrared, and mass spectra were identical with those of the methyl ester of 9-oxohexacosanoic acid.[1]

Esterification with diazomethane of the oil obtained from the phosphorus-hydriodic acid treatment yielded a semicrystalline product. Thin layer and gas chromatographic investigations showed it to be a mixture of two very similar substances that could be only partially separated in preparative experiments. Mass spectrometric studies revealed that it consisted of two compounds with molecular weights of 420 and 422. Hydrogenation converted the mixture to pure methyl-9-oxohexacosanoate, which has the molecular weight of 424. The formation of a mono- and a diolefin on hydroiodic acid treatment indicated, that the central part of HH2 is a 9-oxohexacosanoic acid substituted in two positions by oxygen functions. Probably one of these links the nitrogen-containing portion to the nucleus, while the other is lactonized.

Quantitative Kuhn-Roth determinations indicated the presence of three C-alkyl groups in HH2. This result was unexpected, as pimaricin and its dodecahydro derivative contain only two C-methyl groups. Paper chromatographic investigation of the volatile acids revealed the presence of acetic acid only. The nature of the new methyl group was determined from a study of the nuclear magnetic resonance and infrared spectra using deuterium exchange. The nuclear magnetic resonance spectrum of HH2 (*cf.* Fig. 1) displays a three-

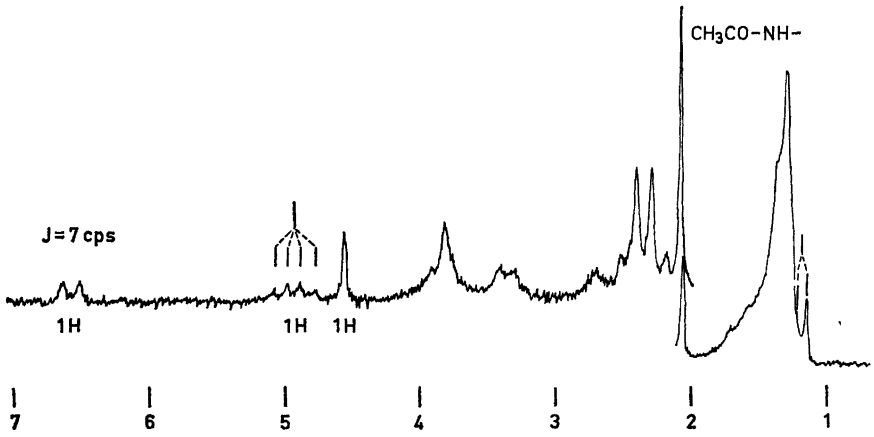


Fig. 1. NMR spectrum of HH2.

proton singlet at $\delta = 2.00$, characteristic of an acetyl group attached to either a nitrogen or an oxygen atom. The infrared spectrum, measured in potassium bromide, shows very strong bands at 1566 and 1625 cm^{-1}, typical of a secondary amide. In ethanol-free chloroform solution two sharp bands of equal intensity are found at 3460 and 3520 cm^{-1}. If the solution is shaken with one drop of deuterium oxide for 2 min (*cf.* Ref.[3]), the hydroxyl band at 3520 cm^{-1} disappears and the amide absorption at 3460 cm^{-1} remains, while a new band at 2640 cm^{-1} characteristic of OD vibrations develops. These findings show that HH2

contains one acetylamino group and one or more hydroxyl groups. Apparently acetylation of the amino group is effected by the solvent at the temperature used during the hydrogenation.

Acetylation of HH2 with acetic anhydride in pyridine produced a crystalline compound having NH absorption at 3350 cm^{-1} and amide bands at 1533 and 1676 cm^{-1} in the infrared. The nuclear magnetic resonance spectrum (*cf.* Fig. 2) showed three singlets at $\delta = 1.90$, 2.07, and 2.15, each representing three

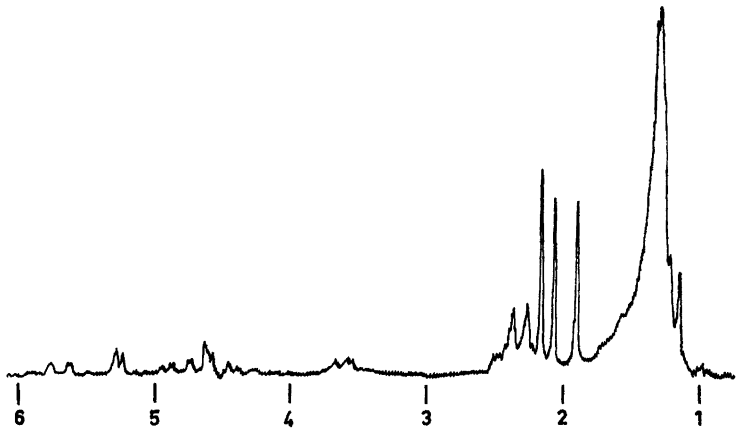


Fig. 2. NMR spectrum of HH2-diacetate.

protons. We conclude that HH2 contains two acetylatable hydroxyl groups. In the nuclear magnetic resonance spectrum of HH2 only one hydroxyl proton can be clearly detected at $\delta = 4.66$. This fact and the differences in chemical shift between the corresponding acetyl functions show that the two hydroxyl groups are nonequivalent; probably one is hydrogenbonded. If both were situated on a six-membered ring they could have an axial-equatorial relation.

Attempts to prepare an acetonide of HH2 were unsuccessful; even after prolonged reaction times and the use of all the standard catalysts only starting material could be detected by infrared spectroscopy and thin layer chromatography. Column chromatography on silica gel, however, gave a small amount of a crystalline compound of lower polarity than HH2. In the new product the amide bands at 1575 and 1650 cm^{-1} had vanished. The rest of the infrared spectrum was very similar to that of HH2 but the absorption in the 3400—3500 cm^{-1} region was less intense. We suspected that cleavage of the glycosidic linkage had occurred. Consequently, HH2 was hydrolyzed in refluxing acetone containing a small amount of dilute aqueous hydrochloric acid. The progress of the reaction was followed by thin layer chromatography. After 20—30 h, most of the HH2 had been converted to the new compound which we named HH2-aglycone.

Mass spectrometry proved its molecular weight to be 424. In combination with combustion analysis, its empirical formula is then $C_{26}H_{48}O_4$. The fact that degradation of HH2 with hydroiodic acid gives 9-oxohexacosanoic acid

accounts for all carbon atoms in the aglycone. Infrared absorption at 1704 cm^{-1} verifies the presence of the 9-oxogroup, and at 1725 cm^{-1} that of a lactone. The methylene groups adjacent to these functions appear in the nuclear mag-

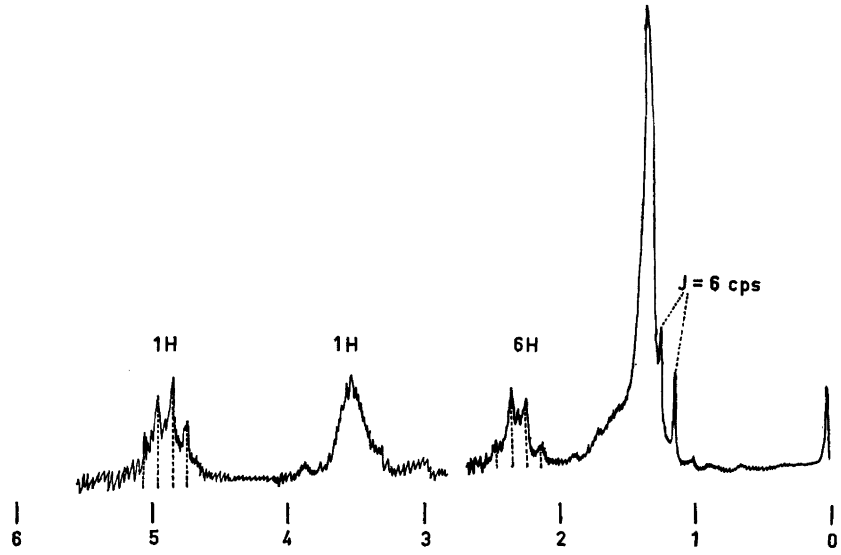
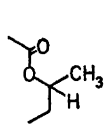


Fig. 3. NMR spectrum of HH2-aglycone.

netic resonance spectrum of the aglycone (*cf.* Fig. 3) as an apparent doublet, corresponding to six hydrogens, centered at $\delta = 2.10$. Oxidation of the aglycone (and also of HH2) with chromium trioxide in acetic acid gave a mixture of dibasic acids. Gas chromatographic analysis of their methyl esters revealed that undecandioic acid was the highest one formed. This finding places two hydroxyl functions on C—15 and C—25, and it remains to determine which one is lactonized. At $\delta = 1.20$ in the nuclear magnetic resonance spectrum a three-proton doublet appears ($J = 6$ cps). It partly overlaps the absorption of the methylene hydrogens. The chemical shift corresponds to a methyl group on a carbon atom carrying an oxygen function. The proton on the same carbon appears at $\delta = 4.95$ as a quartet with fine structure. Its chemical shift is typical of arrangement *3*.[4,5] This structural unit requires that the lactone is formed by the C—25 hydroxyl group and leads to the following formula for the aglycone of HH2, *4*:



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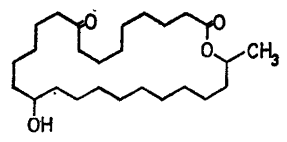

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We can now conclude that the nitrogen-containing portion of HH2 is attached to C—15 *via* a glycosidic linkage. These findings imply that pimaricin contains a twenty-six membered ring lactone and has mycosamine linked to C—15.

The aglycone of HH2 is of stereochemical interest. If during the hydrolysis a carbonium ion is formed on C—15, the resulting alcohol is racemic at that center. The optical activity of the aglycone, $[\alpha]_D = +1.5°$, is hence due to the asymmetry at C—25. These circumstances open the possibility of determining the absolute configuration of the macrocyclic lactone.

We now wish to present a more complete discussion and comparison of the nuclear magnetic resonance spectra of HH2, its diacetate, and aglycone, which support our earlier conclusions. The first indication that the macrocyclic ring was closed at C—25 was obtained from the spectrum of HH2. Centered at $\delta = 4.94$, it displays a symmetrical one-proton quartet ($J = 6$ cps) with traces of fine structure. Acetylation did not change the situation and in the spectrum of the diacetate an identical quartet occurs with the same chemical shift. In the spectrum of the aglycone the quartet shows a stronger tendency to fine structure. One would expect a more complicated pattern because H—25 is also adjacent to a methylene group. An open chain example of a proton in the same environment, producing a quartet with very little fine structure is found in β-acetoxymethylbutyrate.[8] The doublet of the C—25 methyl group occurs in the aglycone and HH2 at $\delta = 1.18$. The shoulder at $\delta = 1.35$ in the spectrum of HH2, absent in that of the aglycone, is due to the methyl group in the "mycosamine" moiety. The normal position for a 3—ABQ hydrogen * seems to be slightly lower [8] which originally misled us to make the reverse assignments. Between $\delta = 2.2$ and 2.5, the spectra of HH2 and HH2-aglycone show bands representing the six protons adjacent to keto groups. In dihydro-HH2, obtained by borohydride reduction of HH2, only a two-proton triplet, due to the methylene group next to the lactone carbonyl, remains in the same region. Four protons are therefore located α to the 9-carbonyl group. This splitting pattern looked superficially like a doublet, but closer examination revealed a quartet probably derived from the four strongest lines of an A_2X_2 system. In the aglycone spectrum the quartet is distorted, and in the middle of it a new peak appears. This is probably due to the triplet of the lactone methylene group which has been slightly displaced. Finally, a comparison with the corresponding regions in the spectra of methyl-N-acetylmycosaminide and tetraacetylmycosamine [6] indicated that the amide band at $\delta = 6.56$ (1 H; doublet, $J = 7$ cps) in the spectrum of HH2 is displaced to $\delta = 5.70$ ($J = 8$ cps) on acetylation.

A study of the mass spectra of HH2, its diacetate, and its aglycone confirms that the aglycone has structure *4*. The extreme importance of mass spectrometry in this entire investigation cannot be overemphasized. In the early stages, after other data had given us incorrect ideas, the mass spectra enabled us to discover this and indicated the right course.

The spectrum of HH2 (*cf.* Fig. 4) displays a striking intensity pattern. The peaks between $m/e = 424$ and 637, the latter representing the molecular ion, are hardly detectable on the reproduced chart. The situation changes at

* For nomenclature *cf.* Ref.[8]

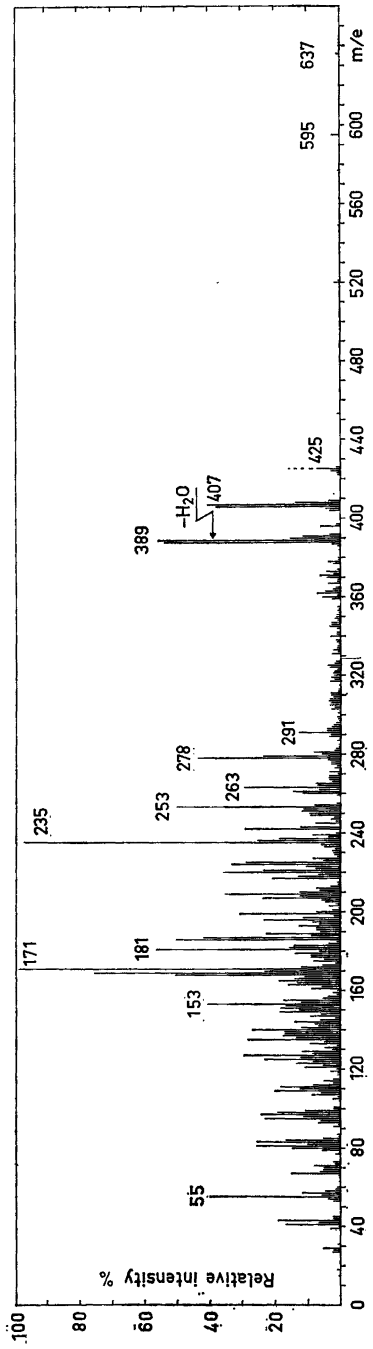


Fig. 4. Mass spectrum of HH2.

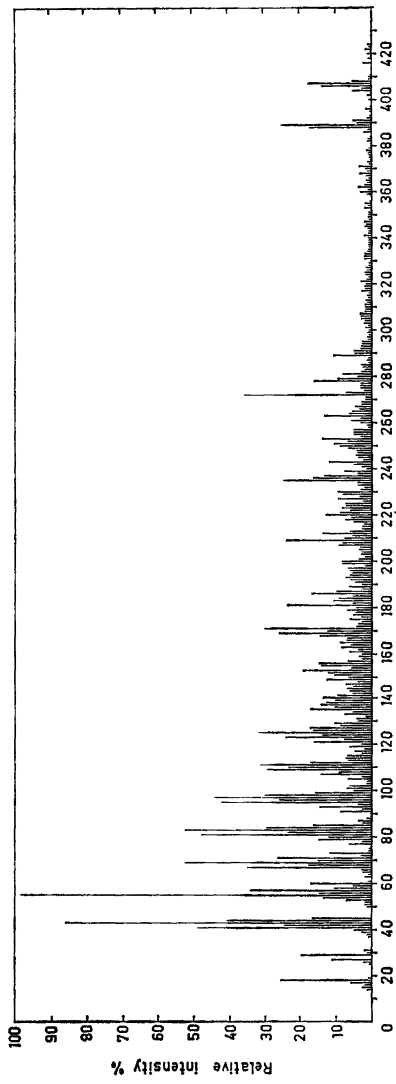
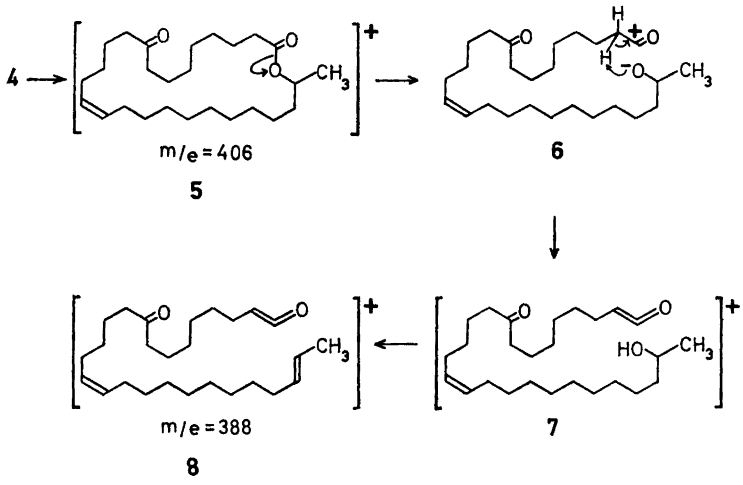


Fig. 5. Mass spectrum of HH2-diacetate (between $m/e = 0-425$).

m/e = 407. This is readily explicable in terms of the proposed structure; the molecular weight of the central nucleus of HH2, excluding the glycosidic oxygen, is 407. The formula of the nitrogen-containing portion, including the glycosidic oxygen, is then $C_{10}H_{16}NO_5$, determined by difference. All ions occurring between m/e = 425 and 637 must contain the macrocyclic ring plus atoms from the "sugar moiety"; hence their low stability is not unexpected. The most intense peak in the upper region falls at m/e = 595 and corresponds to loss of the N-acetyl group in form of ketene. The resulting ion can expel water and give rise to the m/e = 577 fragment. At present there is no obvious explanation for the ions at m/e = 549, 520, and 453.

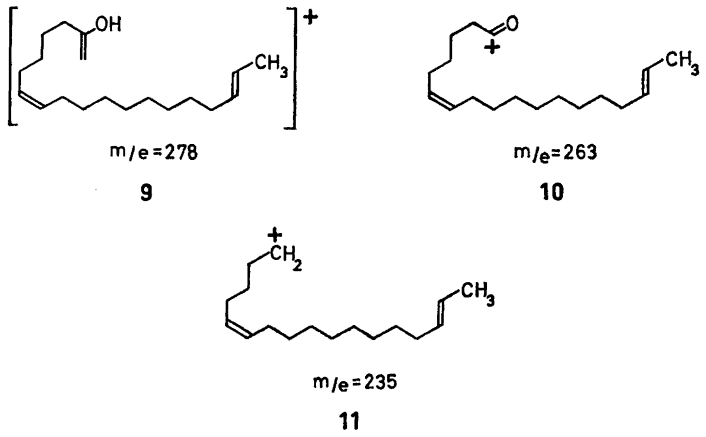
The mass spectrum of the aglycone turned out to be virtually identical with the lower region of that of HH2, except for small intensity differences. This greatly simplified the interpretation of the mass spectrum of HH2 since we could conclude that the nitrogen-containing portion gives rise to very few peaks of significant intensity below m/e = 407. The fragments at m/e = 406 and 388 indicate that the aglycone readily loses two moles of water, as shown below:



Cleavage of the carbon-oxygen bond can also occur, resulting in the formation of the ion with m/e = 407. When it loses water, the fragment at 389 is formed according to mechanism *6*.

The mass spectrum of methyl-9-oxohexacosanoate demonstrated that prominent peaks were obtained from cleavage on both sides of the 9-keto group.[1] In analogy herewith HH2-aglycone yields characteristic fragments with the masses 278, 263, and 235. They are formed by well-documented mechanisms [7,8] and have structures *9*, *10* and *11*.

In the hope of shedding some light on the structure of the "sugar moiety" of HH2, the mass spectrum of the diacetate was determined. A molecular ion peak was not detected, and the spectrum in the region above 425 displays an intensity pattern similar to that of HH2. In the region below 425 (Fig. 5)



the spectra are identical, except for two additional peaks at $m/e = 212$ and 272 in the acetate, certainly derived from the sugar portion. We inferred earlier that the formula of the nitrogen-containing fragment should be $C_{10}H_{16}NO_5$; hence its diacetate would be $C_{14}H_{19}NO_7$. No ion corresponding to this fragment ($m/e = 313$ or 314) was present but the one at 272 corresponds to loss of one acetyl group as ketene.[9] Elimination of a second mole of ketene and one mole of water yields the ion at 212. A good correlation is therefore obtained between the spectra of HH2 and the diacetate with respect to the composition of the nitrogen-containing portion. Its composition, $C_{10}H_{17}NO_5$, shows that it contains three units of unsaturation. Two are accounted for in the N-acetyl group and a pyranose ring. As HH2 is not attacked by ozone, it is likely that the third unit is present in form of a ring, and the addition of the C_2-fragment to mycosamine during the hydrogenation has resulted in a bicyclic structure. Since the structure of this fragment is not necessary for the structure proof of pimaricin and could not readily be deduced, the problem was not further pursued.

The second crystalline compound, HH1, also contains one atom of nitrogen, based on a molecular weight of 625—650. It has the same specific rotation (−51°) as HH2. The mass spectra of HH1 and HH2 are identical between $m/e = 407$ and 637. As their analytical data are the same, HH1 also has the empirical formula $C_{36}H_{63}NO_8$. Kuhn-Roth determination, from which only acetic acid was obtained, indicated the presence of three C-alkyl groups. By the same methods as those described for HH2 it was shown that HH1 also contained an N-acetyl group, introduced during the hydrogenation. The nuclear magnetic resonance spectrum (*cf.* Fig. 6) displayed a three-proton singlet at $\delta = 2.07$. The infrared spectrum (in KBr) showed two amide bands at 1640 and 1548 cm^{-1}. In ethanol-free chloroform solution a hydroxyl band at 3610 cm^{-1} and an NH band at 3450 cm^{-1} were found. After deuterium exchange,[5] the band at 3610 cm^{-1} disappeared and an OD band at 2645 cm^{-1} developed. With acetic anhydride in pyridine, an oily acetate was obtained. As it required several months to solidify, a 2,4-dinitrobenzoate, which readily crystallized,

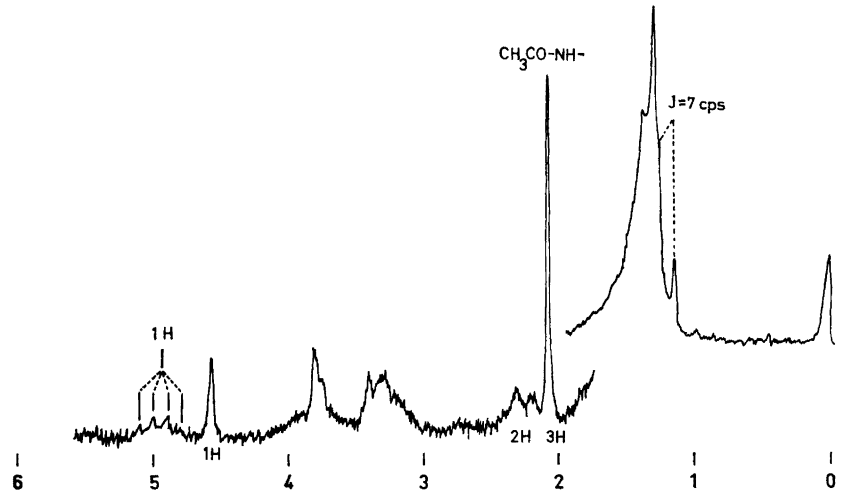


Fig. 6. NMR spectrum of HH1.

was prepared instead. Its nitrogen content indicated the presence of two hydroxyl groups in HH1.

The ultraviolet spectrum of HH1 showed no characteristic absorption, and the infrared spectrum showed a single sharp band at 1725 cm^{-1}. Treatment with diazomethane led to quantitative recovery of the starting material. The nuclear magnetic resonance spectrum showed only two hydrogens between $\delta = 2.0-2.2$. We therefore conclude that no ketonic carbonyl group is present in HH1. As it is a saturated isomer of HH2, it must contain one additional ring. Since the number of hydroxyl groups is the same as in HH2 we expected a cyclic ether grouping. This is supported by the presence of the broad four-

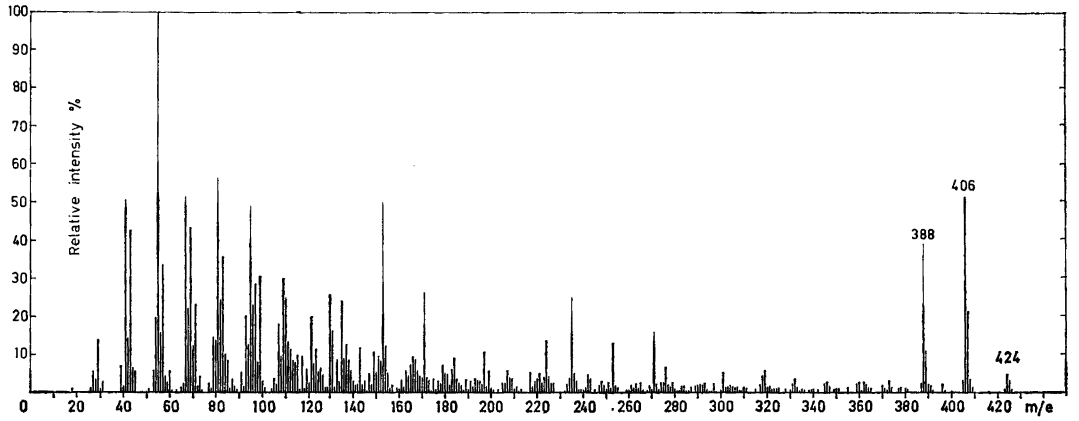
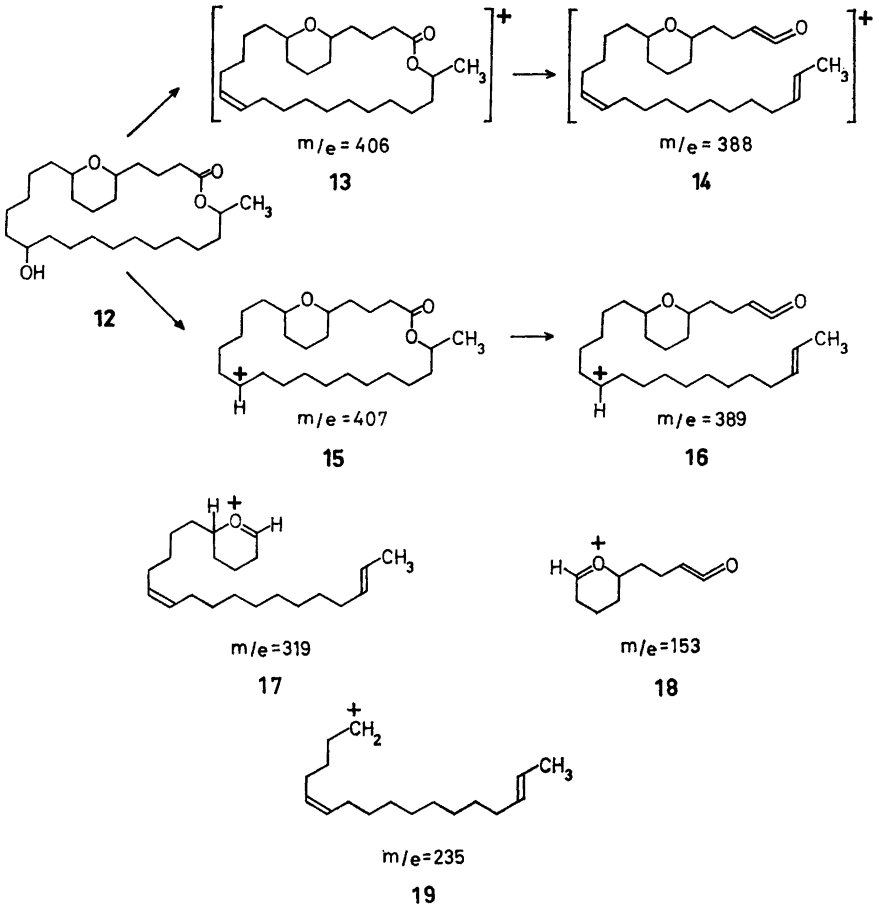


Fig. 7. Mass spectrum of HH1 aglycone.

proton absorption at $\delta = 3.3$ in the nuclear magnetic resonance spectrum of HH1. The same region in HH2 contains only two hydrogens.

Oxidation of HH1 with chromium trioxide in acetic acid produced a number of dibasic acids of which undecandioic acid was the highest formed. The nuclear magnetic resonance spectrum shows a one-proton quartet at $\delta = 4.9$, characteristic of grouping *3*. The evidence presented makes it likely that HH1, like HH2, is composed of a nitrogen-containing $C_{10}H_{17}NO_4$ portion and a central C_{26} nucleus.

Hydrolysis of HH1 in acetone, containing dilute aqueous hydrochloric acid, yielded after chromatography an aglycone which could not be obtained crystalline. We have therefore determined its structure entirely by mass spectrometry (*cf.* Fig. 7). The molecular weight is 424. Like the HH2 aglycone, it loses two molecules of water and gives four fragments at $m/e = 406$, 407, 388, and 389. As 5,9-epoxyhexacosanoic acid was earlier isolated from a similar reduction procedure we suspected that the HH1 aglycone had an analogous



structure. The fragmentation pattern of 5,9-epoxyhexacosanoate showed prominent peaks for cleavage on both sides of the tetrahydropyran ring.[1] The ions at m/e = 319, 235, and 153 derived from HH1 appear to be due to such a fragmentation.

The ions at m/e = 301, 252, 171, and 135 differ from the principal peaks by one molecule of water. The fragmentation pattern is satisfactorily explained in terms of structure *12*.

The nitrogen-containing C_{10}-portion is then attached to C—15 by a glycosidic bond, and HH1 is a second example supporting the assumption that in pimaricin mycosamine is attached to C—15. The fact that HH1 and HH2 have the same specific rotations suggests in addition that the asymmetric centers in both compounds have the same absolute configuration.

A comparison of the products obtained from hydrogenations performed at 250° and at 150° reveals striking similarities. Due to the simplicity of the compounds resulting from the more forcing conditions, we were able to isolate four hydroxyl-free acids. The close solubility and chromatographic properties of the HH-products make them more difficult to separate. We believe, however, that a very careful investigation of the reaction mixtures would yield HH-compounds analogous to Fa-Fr-high (2-tetradecylundecandioic acid) and ZHHI-low (hexacosanoic acid). Thus the high pressure hydrogenation may form basis of a general method for proofs of the structures of complex polyhydroxy macrocyclic antibiotics. Further variation of reaction conditions, followed by careful investigation of the mixtures of products, would likely yield compounds at any desired stage of degradation. It would therefore be possible to obtain the complete carbon skeleton of a macrolide antibiotic, its oxygenation pattern and the position of the sugar by the use of a single degradative method.

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,[10] and an Atlas CH4 spectrometer, both equipped with heated inlet system, kept at 200—225°. Thin layer chromatograms were carried out on Silica gel G (Merck) according to Stahl.[11] The spots were made visible either with iodine[12] or by spraying with chromium trioxide in sulfuric acid,[13] 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 HH1. 60 g of pimaricin and 33 g of 5 % palladium on alumina were suspended in 1400 ml of glacial acetic acid. The mixture was hydrogenated with shaking in an autoclave at 100 atm and 150° for 4 h. The hydrogen uptake was 12.5 moles per mole of pimaricin. The catalyst was removed by filtration and the straw-colored filtrate was concentrated to a sirup under reduced pressure. In order to remove the last traces of acetic acid, the sirup was dissolved in ethyl alcohol and evaporated to dryness; this process was repeated several times. After drying under high vacuum 62.5 g of a brown sirup remained. This product was dissolved in 800 ml of 0.1 N sodium hydroxide. Some warming was necessary to achieve solution. After filtration of the warm solution and cooling of the filtrate, 6.9 g of crude HH1 crystallized; m.p. 150—160°. Recrystallization of

13.1 g of crude HH1 from 400 ml of acetone gave 6.1 g of HH1 with m.p. 157–160°. (Found: C 66.71; H 10.05; N 2.37; O 20.91; C–CH_3 6.79; CH_3CO 8.65. Calc. for $C_{35}H_{63}NO_8$: C 67.78; H 9.95; N 2.20; O 20.07; 3 C–CH_3 7.04; CH_3CO 6.76). Mol. wt. = 637 (mass spec.); $[\alpha]_D^{20} = -51.5°$ (c = 1, glacial acetic acid).

Isolation of HH2. Recrystallization of 26.5 g of crude HH1 from 600 ml of ethyl acetate gave 14.5 g of a product, m.p. 148–154°. Concentration of the mother liquor to 200 ml gave 3 g of another fraction, m.p. 130–136°. After recrystallization from 40 ml of ethyl acetate 2.44 g of HH2, m.p. 134–136°, was obtained. (Found: C 67.15; H 10.20; N 2.41; O 21.19. Calc. for $C_{36}H_{63}NO_8$: *cf.* HH1.) Mol. wt. = 637 (mass spec.); $[\alpha]_D^{20} = -51.0°$ (c = 2.0, glacial acetic acid).

Ozonolysis of HH2. A solution of 89 mg of HH2 in 3 ml of chloroform was ozonized at −50°. A blue color developed almost immediately. After 10 min the ozonolysis was interrupted and nitrogen was passed through the solution for 30 min. The chloroform was evaporated and the crystalline residue, 89 mg, was shown by thin layer chromatography on silica gel (benzene-methanol, 9:1) to contain only one component having an R_F-value (0.20) and an infrared spectrum identical with that of HH2.

Treatment of HH2 and HH1 with diazomethane. 30 mg of HH2 was dissolved in 2 ml of methanol and esterified with an ethereal solution of diazomethane. After evaporation of the solvent under reduced pressure, the residue crystallized. Its infrared spectrum was identical with that of HH2, and thin layer chromatography on silica gel (benzene-methanol, 9:1) showed the presence of a single component having the same R_F-value as HH2.

When 30 mg of HH1 in 2 ml of methanol was treated with diazomethane the methods used above proved that the starting material was completely recovered.

Reduction of HH2 with potassium borohydride. To a solution of 70 mg of HH2 in 3 ml of methanol was added 50 mg of potassium borohydride at + 4°. After 24 h at 20° the mixture was deionized over an ion-exchange resin (Amberlite MB–3). Evaporation of the solvent gave 70 mg of a crystalline product. Thin layer chromatography on silica gel (benzene-methanol, 50:6), proved it pure and free from starting material. R_F (HH2) = 0.29; R_F (dihydro-HH2) = 0.23. The crystalline product was absorbed on 7 g of silica gel. Elution with chloroformm–ethanol gave 70 mg of dihydro-HH2 melting at 120°. (Found: C 67.12; H 9.88. Calc. for $C_{36}H_{65}NO_8$: C 67.57; H 10.25).

Isolation of 9-oxohexacosanoic acid from HH2. 50 mg of HH2 was dissolved in 1 ml of glacial acetic acid and added to 10 ml of refluxing 48 % hydroiodic acid containing excess of red phosphorus. After 15 h water was added and the mixture extracted with ether. The combined extracts were washed with sodium thiosulfate and water, and evaporated to dryness. Esterification of the resulting colorless oil (32 mg) with diazomethane yielded a semicrystalline product. Thin layer chromatography on silica gel [petroleum ether (b.p. 60–80°)–diethyl ether, 9:1] revealed the presence of two components with R_F-values of 0.44 and 0.47. Gas chromatography at 250° showed two components with relative retention times 1:1.05. A mass spectrum showed two molecular ion peaks at m/e = 420 and 422. The ester mixture, 18 mg, was chromatographed on 1 g of silica gel with petroleum ether (b.p. 40–60°) – diethyl ether, 9:1. Fractions of 15–20 drops were collected and were shown to be mixtures by gas and thin layer chromatography as described above. A solution of 18 mg of oily ester in 5 ml of ethanol was hydrogenated for 2 h using 10 mg of 5 % palladium on carbon as catalyst. After separation of the catalyst and evaporation of the solvent a crystalline residue was obtained. After recrystallization from acetone, 5 mg of a compound melting at 68–70° resulted. It was identified as methyl 9-oxohexacosanoate by gas and thin layer chromatography and by comparison of infrared spectra and mixed melting points with authentic material.

Kuhn-Roth oxidation of HH2 and HH1. 2 mg of HH2 was oxidized following the procedure of Garbers, Schmid, and Karrer.[14] The paper chromatograms showed only one large spot for acetic acid. When 2 mg of HH1 was oxidized in an identical manner, the paper chromatogram showed only one large sport for acetic acid.

Deuterium exchange in HH2 and HH1. 10 mg of HH2 was dissolved in 1 ml of ethanol-free chloroform and an infrared spectrum was determined, using a 0.025 mm sodium chloride cell. Two strong bands, representing OH and NH absorption, were present at 3620 and 3460 cm^{-1}. The solution was then shaken with one drop of deuterium oxide for 2 min and the chloroform solution transferred to the same cell. In the spectrum of the deuterated HH2, the OH band at 3620 cm^{-1} had disappeared and a new OD band at

2640 cm^{-1} had formed. The intensity of the NH band at 3460 cm^{-1} was slightly lower, but no significant exchange had taken place.

When HH1 was subjected to the same spectroscopical investigation, identical results were obtained.

The ethanol-free solvent was prepared by passing stabilized chloroform over alumina (Woelm, activity I).

Acetylation of HH2. 100 mg of HH2 was dissolved in 1 ml of pyridine, and 1 ml of acetic anhydride was added at 0°. After 24 h the excess of anhydride was destroyed with ice, the reaction mixture acidified with sulfuric acid and extracted with chloroform. The combined extracts were washed with water and dried over sodium sulfate. Evaporation of the solvent under reduced pressure gave 95 mg of oily HH2-diacetate which crystallized on standing; m.p. 100—102°. (Found: C 65.98; H 9.31. Calc. for $C_{40}H_{67}NO_{10}$: C 66.54; H 9.37).

Attempt to prepare HH2-acetonide. A solution of 160 mg of HH2 and 16 mg of *p*-toluene sulfonic acid in 15 ml of acetone was refluxed for 2 days. The mixture was then passed over a short column of silica gel to absorb the catalyst. Evaporation of the solvent gave 160 mg of a crystalline residue, which was chromatographed on 10 g of silica gel (each fraction had a volume of 5 ml).

Solvent	Fraction	Weight, (mg)
Chloroform	1	0
Chloroform—methanol		
9:1	2	5 (m.p. 60—62°)
»	3	1
»	4	0
»	5	150 (m.p. 133—134°)
»	6	3

Infrared spectra and thin layer chromatograms showed fractions 4—6 to be unchanged HH2. The infrared spectrum of fraction 2 contained no amide bands at 1575 and 1650 cm^{-1}.

Preparation of HH2-aglycone. A solution of 89 mg of HH2 in 30 ml of acetone containing 3 drops of concentrated hydrochloric acid and 7 drops of water was refluxed for 25 h. The mixture was deionized with an ion-exchange resin (Amberlite MB—3) and evaporated to dryness. Yield: 90 mg. Thin layer chromatography on silica gel (benzene-methanol, 9:1) showed two spots, R_F = 0.20 and 0.47. The slower moving compound was unchanged HH2. The mixture was absorbed on 5 g of silica gel. Elution with benzene-methanol, 85:15, gave 31 mg of crystalline HH2-aglycone, m.p. 60—62°. (Found: C 73.62; H 11.29. Calc. for $C_{26}H_{48}O_4$: C 73.52; H 11.39.) Mol. wt. = 424 (mass spec.); $[\alpha]_D^{22} = -4.1°$ (c = 2.1 in chloroform).

Oxidation of HH2 and HH1 with chromium trioxide. 27 mg of HH2 was dissolved in 1 ml of glacial acetic acid and oxidized at 70° with 80 mg of chromium trioxide. The mixture was diluted with water and extracted with ether. The combined extracts were washed with water and then evaporated to dryness. The residue was subjected to steam distillation to separate any higher monobasic acids; none were, however, found in the distillate. After evaporation to dryness the residue was esterified with diazomethane and analyzed by gas chromatography. At 149° the homologous esters up to dimethylundecandioate were detected. No trace of higher diesters was found.

25 mg of HH1 was oxidized in an identical experiment. Also here dimethylundecandioate was the highest homologue found.

Acetylation of HH1. 104 mg of HH1 was acetylated as described under HH2 diacetate. Chromatography gave 85 mg of oily HH1 diacetate, which was not further investigated. After 4 months part of the oil had crystallized.

HH1-3,5-dinitrobenzoate. A 3,5-dinitrobenzoate was prepared from 3,5-dinitrobenzoyl chloride and 300 mg of HH1 using benzene-pyridine as solvent. The crude product was recrystallized three times from benzene-petroleum ether; m.p. 148—150°. (Found: C 57.93; H 6.75; N 6.97. Calc. for $C_{48}H_{65}O_{18}N_5$: C 57.65; H 6.55; N 7.00).

Preparation of HH1-aglycone. A solution of 0.153 g of HH1 in 40 ml of acetone containing two drops of concentrated hydrochloric acid and 7 drops of water was heated under reflux for 25 h. The product, 0.102 g of an oil, was isolated as described under HH2-aglycone: Thin layer chromatography in benzene-methanol, 9:1, gave the following R_F-values. HH1 = 0.18, HH1-aglycone = 0.47. Mol. wt. = 424 (mass spec.).

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