THE STRUCTURE OF FATTY ACIDS FROM THE ANTIBIOTIC AMPHOMYCIN

MIKLOS BODANSZKY, NISHITH C. CHATURVEDI and JAMES A. SCOZZIE

Department of Chemistry, Case Western Reserve University, Cleveland, Ohio, U.S.A., 44106

(Received for publication July 3, 1969)

From acid hydrolysates of amphomycin (+) 3-anteisotridecenoic acid, (+) 4-hydroxy-anteisotridecanoic acid lactone and 4-hydroxy-isododecanoic acid lactone were isolated. While the unsaturated acid is a genuine building component of the antibiotic, the lactones are formed from unsaturated acids during hydrolysis.

Amphomycin, a peptide antibiotic, was described by HEINEMANN, KAPLAN, MUIR and HOOPER[1] in 1953. While it is known that the molecule of this microbial peptide resembles those of aspartocin[2], glumamycin[3], zaomycin[4], crystallomycin[5], and tsushimycin[6], to our best knowledge no detailed structural study on amphomycin appeared in the literature so far*. We wish to report here the isolation of 3-anteisotridecenoic acid (10-methyl-3-dodecenoic acid), and the lactone of 4-hydroxy-anteisotridecanoic acid (4-hydroxy-10-methyl-dodecanoic acid) from hydrolysates of amphomycin. A smaller amount of a second lactone, that of 4-hydroxy-isodecanoic acid (4-hydroxy-10-methyl-hendecanoic acid), was also obtained.

Hydrolysis of the antibiotic with hydrochloric acid, extraction of the fatty acids (and lactones) with ether and their separation by countercurrent distribution in the system *n*-heptane-acetonitrile-acetic acid-methanol (4 : 1 : 1 : 1)[7] led to the isolation of three compounds, with distribution coefficients 0.53 (compound I), 0.22 (compound II), and 0.18 (compound III). The presence of several minor components could be observed. However, those were incompletely separated from each other and their amounts were too small to warrant further investigation.

Compound I was distilled *in vacuo* and was shown to be homogeneous by vapor phase chromatography. The ir spectrum (Fig. 1 a) corresponds to that of a fatty acid. Unsaturation was indicated by the rapid addition of bromine and revealed even more definitely by the nmr spectra (Figs. 2a and 2b) of the acid (I) and its methyl ester (Ia). These spectra show considerable similarity to those of the unsaturated C_{15} fatty acids from aspartocin[8] with the notable exception in the ratio of the area of the $-CH_2-$ region to the area of the methyl protons. Moreover, while a mixture of iso and anteiso fatty acids was obtained from aspartocin, the pattern of the signals

* The authors express their gratitude to Mr. L. SZABO and H. Lundbeck & Co. of Copenhagen-Valby, Denmark, for the generous sample of amphomycin which made it possible to carry out such a detailed study.

Fig. 1. IR spectra (in KBr)
a. Compound I b. Compound II

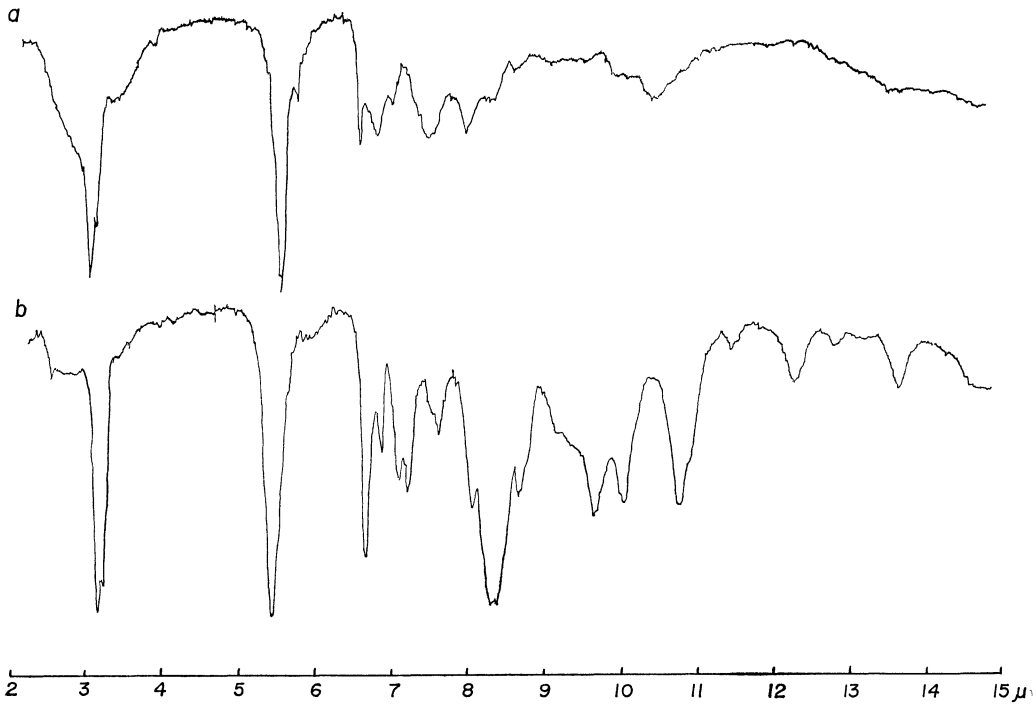


Fig. 2. NMR spectra (60 Mc) in $CDCl_3$
a. Compound I

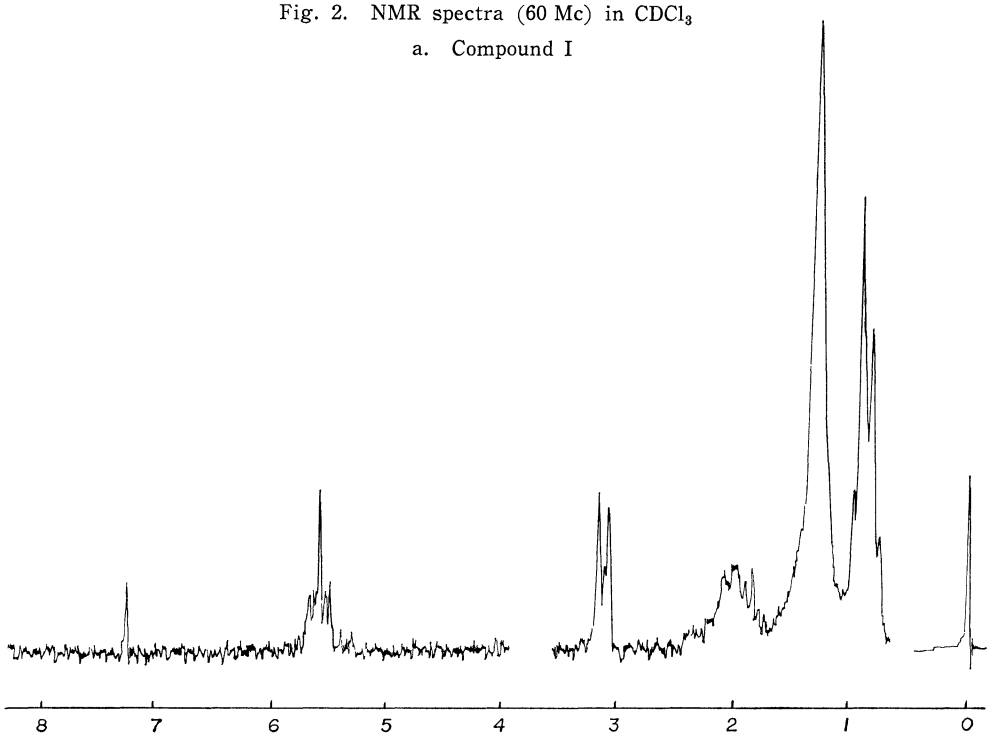


Fig. 2. b. Compound Ia

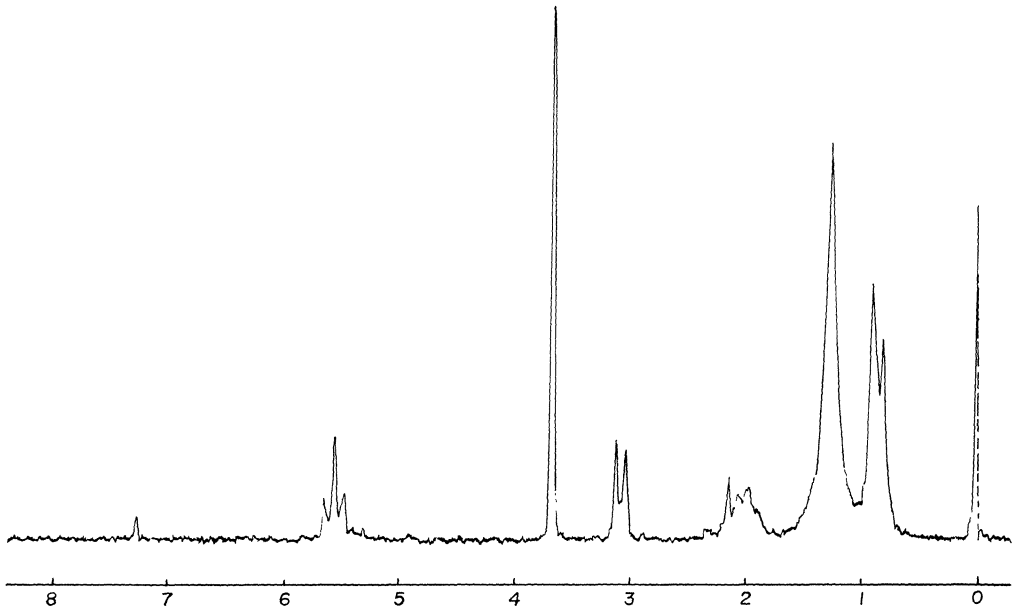


Fig. 2. c. Compound Ib

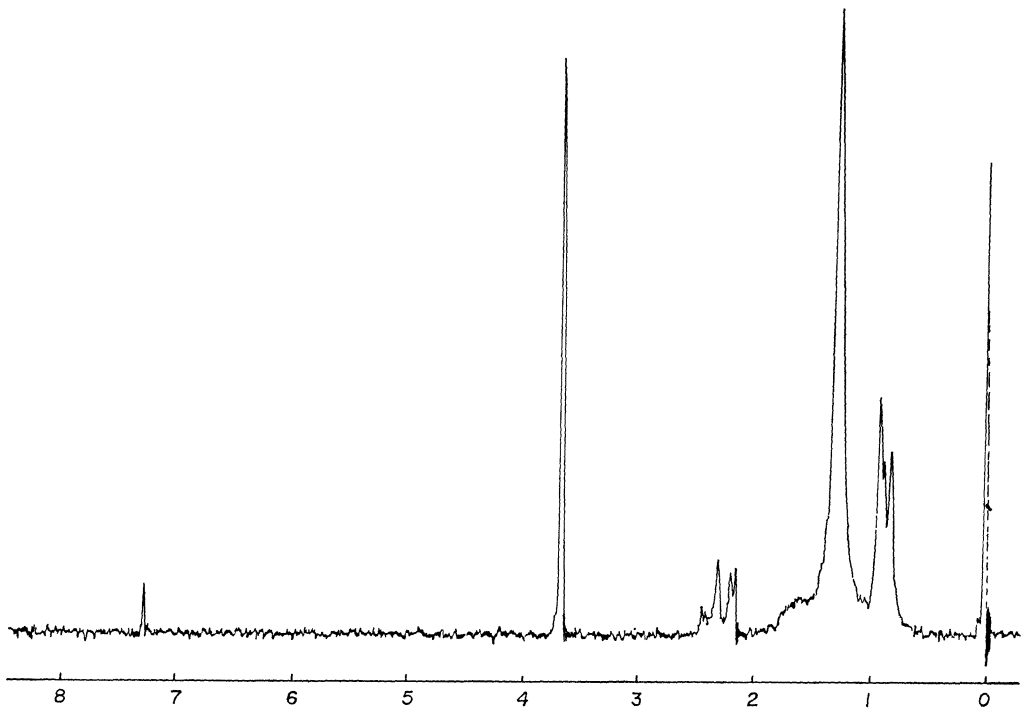


Fig. 2. d. Compound II

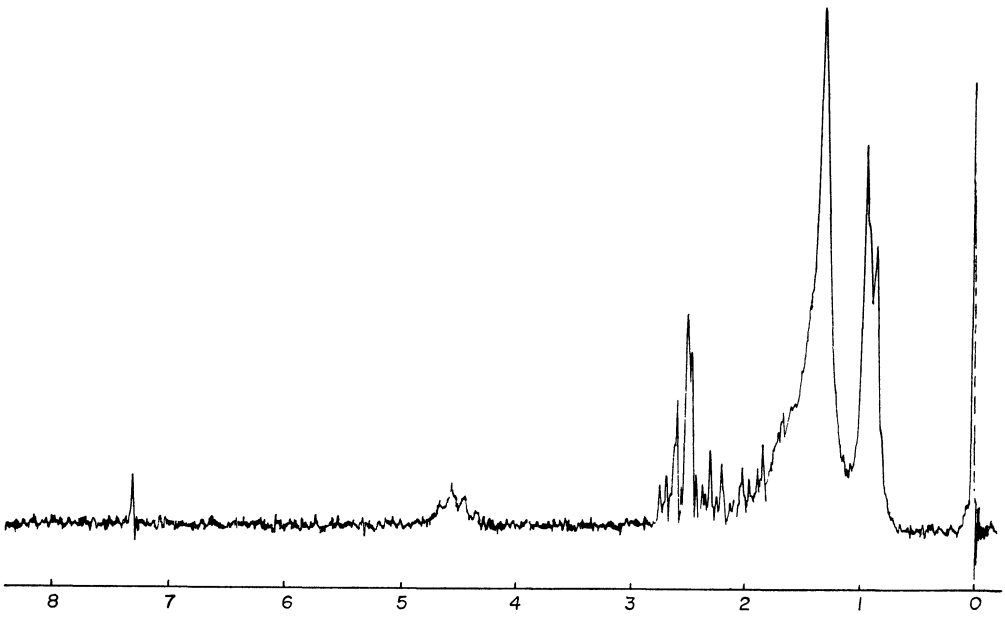
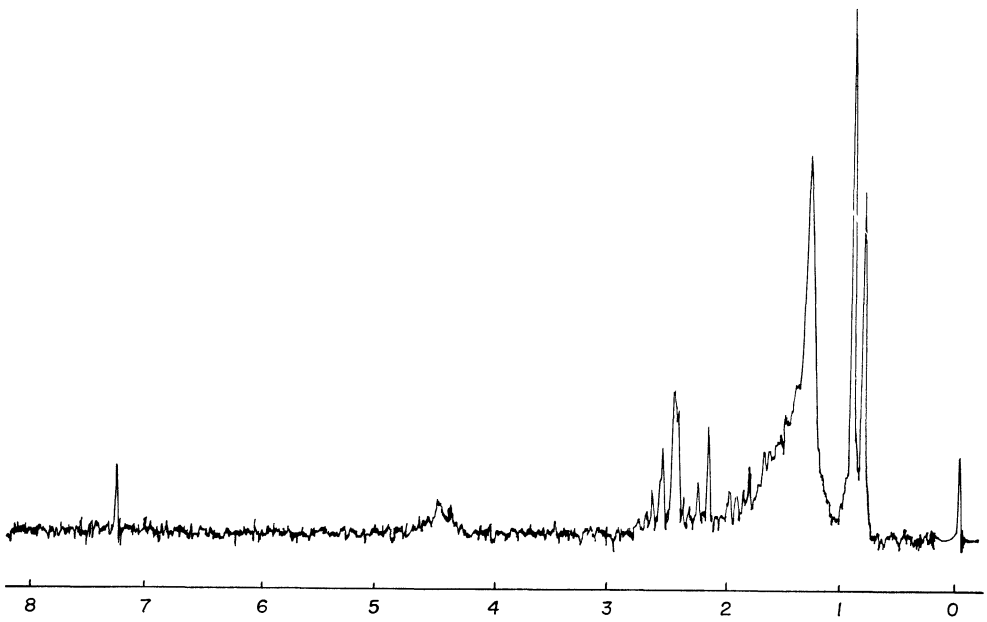
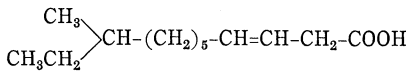


Fig. 2. e. Compound III



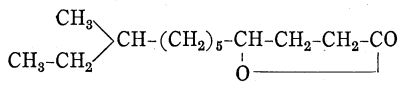
of the methyl protons in compound I corresponds to that of two methyl groups in an anteiso compound. The specific rotation of compound I, $[\alpha]_D^{25}$ +5.2 (*c* 9.07, $CHCl_3$), is in good agreement with the rotation expected[9] for a C_{13} anteiso fatty acid. From the data of elemental analysis and from the neutralization equivalent and mass spectrum, the formula of an unsaturated fatty acid, $C_{13}H_{24}O_2$, could be calculated

and this was consistent with the integration values of the nmr spectrum. Catalytic hydrogenation followed by esterification with an ethereal solution of diazomethane gave the saturated acid methyl ester (IIb) which was characterized by its nmr spectrum (Fig. 2 c), and mass spectrum, both corresponding to a $C_{14}H_{28}O_2$ compound. The position of the double bond was deduced from the nmr spectra (Figs. 2 a and 2 b) including double resonance studies. From the above evidence, the structure of (+) 3-anteisotridecenoic acid (I)** is proposed for compound I.



I

Compound II showed no unsaturation and because of a strong single CO band at 5.62 μ in its ir spectrum (Fig. 1 b) was tentatively identified as a γ-lactone. Elemental analysis, saponification number and mass spectrum revealed it to be a second $C_{13}H_{24}O_2$ compound. Inspection of the nmr spectrum (Fig. 2 d), especially after comparison with the spectrum of γ-valerolactone, left no doubt about the γ-lactone nature of this material. The formation of an amide (IIa) with methanolic ammonia and of a hydrazide (IIb) with a methanolic solution of hydrazine furnished conclusive evidence for the lactone structure. Treatment with warm alkali converts compound II to the salt of a hydroxy acid; from the latter, however, the lactone reforms on treatment with acids. Furthermore, during distillation of the amide and of the hydrazide, partial elimination of ammonia or hydrazine and the concommitant formation of the lactone could be observed. The specific rotation of the lactone, and also its optical rotatory dispersion, were identical to those of compound I. Based on these experiments, compound II could be identified as (+) 4-hydroxy-anteisotridecanoic acid lactone (II).



II

Analytical data, nmr (Fig. 2 e) and mass spectra showed that compound III is the lactone of a 4-hydroxy-dodecanoic acid. The nmr spectrum (Fig. 2 e) and the low value of the specific rotation ($[\alpha]_D^{25}$ 0.35, c 9, $CHCl_3$) suggested that in this case an iso rather than anteiso compound was isolated.

Homologs of fatty acids are known to occur in antibiotics in such a way that different members of an antibiotic family contain different members of the homologous series[10]. However, it was puzzling to find both an unsaturated acid and the lactone of a related hydroxy acid in the hydrolysis mixture of amphomycin. The lack of contribution to the optical activity of compound II by the asymmetric center at carbon 4 suggested that the latter is present in racemic form. Therefore, it seemed likely that while the unsaturated acid (I) is a genuine building component of the antibiotic amphomycin, the lactones are formed from unsaturated acids during hydrolysis.

** The tentative identification by SHOJI, *et al.*[6], of the fatty acid constituent of amphomycin as a C_{16} compound is not confirmed by our study.

The presence of the unsaturated acid in amphomycin could be seen in the nmr spectrum of the antibiotic (in deuteroacetic acid), which clearly shows the vinyl protons. Therefore, the possibility that the unsaturated acid would be a secondary degradation product formed by dehydration can be excluded. Futhermore, catalytic hydrogenation of amphomycin followed by hydrolysis yielded a saturated C_{13} fatty acid, but none of the lactones were present in the hydrolysate. The formation of the lactone from the unsaturated acid could also be demonstrated if compound I was heated in a mixture of acetic acid and 6 N hydrochloric acid. Subsequently, samples of the antibiotic were hydrolyzed in a similar mixture and it was observed that with increasing time the amount of unsaturated acid in the hydrolysate decreases with the concommitant increase in the amount of the lactones.

Though all this evidence points to unsaturated fatty acids as building components of amphomycin, it was still intriguing to see that while from the related antibiotics[2~6] similar unsaturated branched fatty acids were isolated, no lactones were found among their degradation products. The excellent yields of fatty acids from aspartocin[8] in conjunction with the remarkable care and elegance in the work-up of the fatty acid mixture makes it unlikely that any appreciable amounts of lactones could have been overlooked by the authors of ref. 8. It is even more interesting to note that no lactone formation was observed in the degradation of glumamycin. (According to INOUE[11], on addition of sodium bicarbonate, the oily ether extract of the hydrolysates became completely soluble in water.) From this antibiotic a C_{13} unsaturated fatty acid was obtained and identified as 3-isotridecenoic acid. Based on the reported[11] optical activity of this material and the results of the KUHN-ROTH oxidation, it follows that the fatty acid from glumamycin is an anteiso rather than iso acid and therefore should be indentical with compound I.

The relationship of compound I and II is clear and as discussed above, II must have formed from I during hydrolysis. The lack of formation of lactones from the hydrogenated antibiotic unequivocally demonstrates that compound III, too, is a conversion product and makes it obvious that the corresponding unsaturated fatty acid, 3-isododecenoic acid is a constituent of amphomycin. The lactone corresponding to this C_{12} acid was isolated in substantially lower amounts than the total amounts of compounds I and II, and hence the conclusion should be drawn that the C_{13} anteiso and the C_{12} iso acid are constituents of different members of the amphomycin family.***

Experimental

Isolation of the mixture of fatty acids and lactones from a hydrolysate of amphomycin: A sample (15 g) of the antibiotic (calcium salt) was dissolved in 6 N hydrochloric acid (300 ml) and the solution was heated to relux in an atmosphere of nitrogen for 24 hours.

*** In the case of the closely related antibiotic aspartocin, the isolation of equimolar amounts of two C_{15} unsaturated fatty acids (one iso, the other anteiso) was interpreted (ref. 8) as an indication for the occurrence of *two* fatty acids in one molecule of the antibiotic. The assumption of *one* fatty acid residue per antibiotic molecule seems to be more consistent with our findings.

During this time the separation of an oily layer was observed. The oily material could be separated from the mixture by steam distillation, or alternatively by extraction with ether (or hexane). The ether extract was dried over $MgSO_4$, the solvent removed *in vacuo* leaving an oily residue (1.5 g). In the ir spectrum two strong CO bands were observed, at 5.62 μ and 5.88 μ. Treatment of a sample with methanolic ammonia led to the disappearance of the 5.62 μ absorption band while treatment of a second sample with an ethereal solution of diazomethane resulted in the shift of the 5.88 μ band to 5.78 μ. Therefore, the 5.62 μ band could be assigned to that of a lactone carbonyl, the 5.88 μ to a carboxyl group. The nmr spectrum of the crude oil in $CDCl_3$ showed the presence of vinyl protons (multiplet at 5.56 ppm).

Countercurrent distribution: Separation of the major components of the crude oil was carried out by distribution in the solvent system *n*-heptane – acetonitrile – acetic acid – methanol (4 : 1 : 1 : 1)[7]. The oil (from 25 g amphomycin) was dissolved in the lower layer and placed into the first 5 tubes of a 520 tube automatic CRAIG apparatus (with 3 ml upper and 3 ml lower layers). After 1,000 transfers, the contents of the tubes were scanned by weight-analysis. Two major bands with distribution coefficients (K) of 0.53 and about 0.2, and several minor components were detected. The distribution curve of the faster moving band was in fair agreement with a curve calculated for K=0.53 and therefore, the contents of tubes 325~375 were pooled, the solvents removed *in vacuo*, leaving a residue (0.60 g), compound I. The frontal slope of the second major peak was consistent with a calculated curve, but the trailing part of the peak indicated significant heterogeneity. Vapor phase chromatographic analysis (discussed below) showed the frontal half of the peak to be homogeneous and therefore, the contents of tubes 165~190 were pooled. From this fraction compound II (0.32 g) was secured by evaporation of the solvents *in vacuo*. Distribution was continued until a total of 2,525 transfers were completed, when two major peaks with K=0.22 and 0.18 could be detected. The distribution curve of the faster peak was in good agreement with one for K=0.22 and from the corresponding tubes 435~500 an additional quantity of compound II (0.30 g) was isolated. Comparison of the distribution curve of the K=0.18 peak showed some minor deviation from the curve calculated for this distribution coefficient. The bulk of the corresponding material was collected from tubes 375~410. Evaporation of the solvents left a residue (0.18 g), compound III. Vapor phase chromatography revealed the presence of minor amounts of impurities in this sample.

Compound I: The homogeneity of this material was shown by vpc (Varian Aerograph, Model 202B, 5 foot diethylglycol succinimide (DEGS) on 60/80 chromasorb W column, 175°, flow rate 80 ml/min, retention time 13.2 minutes). In the ir spectrum (Fig. 1 a) only one (5.88 μ) carbonyl band is present. A solution of I in $CHCl_3$ instantaneously decolorizes a solution of Br_2 in $CHCl_3$. For analysis a sample was distilled at about 105°C and 0.025 mm. $[\alpha]_D^{25}$ +5.22 (*c* 9.07, $CHCl_3$).

Anal.: Found: C 73.41, H 11.27, neutr. equiv. 209.
Calc'd for $C_{13}H_{24}O_2$: C 73.54, H 11.39, neutr. equiv. 212.3.

In the nmr spectrum of I taken in $CDCl_3$ at 60 Mc (Fig. 2 a), a 6 proton multiplet centered 0.87 ppm corresponds to the methyl protons in an anteiso acid. The pattern is consistent with that shown by isoleucine. A broad singlet (10 protons) at 1.28 ppm represents the $-CH_2-$ groups in normal aliphatic positions. A broad multiplet (3 protons) at 2.05 ppm reveals an aliphatic CH– proton and the protons of an allylic CH_2 group. A two proton perturbed doublet at 3.14 ppm corresponds to a second allylic CH_2- group (between a carboxyl and a vinyl group). A two proton multiplet at 5.56 ppm stems from the vinyl protons. Double resonance on a 100 Mc instrument demonstrated coupling between the 5.56 ppm signals and the signals at 2.05 and 3.14 ppm, respectively. In the mass spectrum (Varian M66 mass spectrometer), the molecular ion peak at 212 amu could be observed.

Methyl (+) 3-anteisotridecenoate (Ia): An ethereal solution of diazomethane was added to a solution of compound I in ether until the yellow color persisted. Several hours later the solvents and the excess reagent were removed *in vacuo* and the residue distilled at 85°C and 0.05 mm. In vpc the material gave a single peak with a retention time of 6.1 minutes at 135°C, flow rate 75 ml/min. The mass spectrum gave the molecular ion peak at 226 amu and also a peak at M–31.

Anal.: Calc'd for $C_{14}H_{26}O_2$: C 74.29, H 11.58, CH_3O 13.7.
Found: C 74.40, H 11.80, CH_3O 13.3.

The nmr spectrum (Fig. 2 b) in addition to the signals discussed for compound I, shows also the expected 3 proton singlet ($-OCH_3$) at 3.7 ppm.

Methyl (+) anteisotridecanoate (Ib): To a solution of compound I (65 mg) in a mixture of ethanol (10 ml) and acetic acid (1 ml) a 10 % palladium on charcoal catalyst (100 mg) was added and the mixture was stirred in an atmosphere of hydrogen overnight. After removal of the catalyst and of the solvents, the residue was esterified as described for Ia. The methyl ester was distilled *in vacuo*. The mass spectrum revealed the molecular ion peak at 228 amu, and peaks at M–29, M–31 and M–57. The nmr spectrum (Fig. 2 c) shows neither vinyl protons nor the 3.1 ppm signal of the allylic protons in the spectrum of I.

λ (mμ)	$[\alpha]^{25}$	
	I	II
600	4.0	5.5
550	6.5	5.9
500	8.0	7.0
450	9.0	8.7
400	11.3	12.2
350	16.0	17.2
300	25.5	24.5

(+) 4-Hydroxy-anteisotridecanoic acid lactone (II): Isolation of II was described above at the counter-currrent distribution of the crude oil. The lactone was distilled at 90°C and 0.05 mm. On addition of bromine (in $CHCl_3$) to a chloroform solution of II, no decolorization was observed. A strong CO band is shown at 5.62 μ in the ir spectrum (Ib). On vpc a single peak was found with 13 minutes retention time at 175°C and an 80 ml/min flow rate. The optical rotatory dispersion (taken in $CHCl_3$ on a Cary 60 spectropolarimeter) follows a one term DRUDE equation. The values for compound II are close to those for I.

The mass spectrum revealed the molecular ion peak at 212 amu.

Anal.: Found: C 73.39, H 11.31, saponif. number 204.
Calc'd for $C_{13}H_{24}O_2$: C 73.54, H 11.39, saponif. number 212.3.

The nmr spectrum (Fig. 2 d) reveals no vinyl protons. A six proton multiplet at 0.85 ppm corresponds to the methyl protons in an anteiso acid. A broad, *ca.* 10 proton singlet at 0.28 ppm is that of the aliphatic $-CH_2-$ protons, the complex pattern between 1.7~2.4 ppm is practically identical with the corresponding CH_2 region in the spectrum of γ-valerolactone. A multiplet at 4.5 ppm originates from the proton on the γ-carbon atom.

4-Hydroxy-anteisotridecanoic acid amide (IIa): A sample (0.1 g) of compound II was dissolved in a 15 % solution (5 ml) of ammonia in methanol. After about 5 days at room temperature the ammonia and methanol were removed with a stream of nitrogen leaving a solid residue. The ir spectrum indicated partial reconversion into the lactone during distillation. The semi-solid product was washed with ether to remove the lactone. The product melts at 80~81°C; its ir spectrum shows the expected amide bands at 6.0 and 6.25 μ.

Anal.: Calc'd for $C_{13}H_{27}NO_2$: C 68.08, H 11.87, N 6.11.
Found: C 68.31, H 11.61, N 6.08.

4-Hydroxy-anteisotridecanoic acid hydrazide (IIb): This compound was prepared as described for the amide. M. p. 76~77°C.

Anal.: Calc'd for $C_{13}H_{28}N_2O_2$: C 63.89, H 11.15, N 11.46.
Found: C 63.63, H 11.74, N 11.43.

4-Hydroxy-isododecanoic acid lactone (III): The material with K=0.18 isolated by countercurrent distribution through 2,525 transfers (*cf.* above) was distilled *in vacuo* and analyzed by vpc. In addition to the main component (9.4 minutes, at 175°C, 80 ml/min), the presence of a minor (*ca.* 5 %) component was also detected (10.8 minutes). $[\alpha]_D^{25}$ 0.35 (*c* 8.97, $CHCl_3$). In the ir spectrum a strong carbonyl band is shown at 5.62 μ.

Anal.: Found: C 72.31, H 11.55, saponif. number 203.
Calc'd for $C_{12}H_{22}O_2$: C 72.68, H 11.18, saponif. number 198.3.

The nmr spectrum (Fig. 2 e) is similar to that of II except that the pattern of the methyl protons is that of an iso fatty acid (*e. g.*, isomyristic acid).

Opening of the lactone ring in compound II: A sample (30 mg) of II was heated with 0.6 N NaOH (0.5 ml) to about 60°C. A clear solution was obtained within a few minutes. The solution was cooled and acidified with N HCl (0.4 ml). An oil separated and was extracted with hexane. The organic layer was decanted, filtered through cotton, and the solvent evaporated leaving an oily residue (31 mg). The ir spectrum shows the absence of the lactone carbonyl, and the appearance of a new band at 5.88 μ. The hydroxy acid was treated with 6 N HCl in an evacuated sealed ampoule at 110°C for 16 hours. The oily material, extracted with ether, showed the characteristic γ-lactone ir spectrum of II.

Lactone formation from compound I: A sample of the unsaturated acid (20 mg) was dissolved in acetic acid (*ca.* 0.6 ml) and 6 N HCl (*ca.* 0.4 ml) was added to the solution. The solution was heated in an evacuated sealed ampoule at 110°C for 16 hours. The mixture was extracted with ether, the ether layer dried with $MgSO_4$, and the solvent removed. The residue shows a strong carbonyl band at 5.62 μ; a somewhat weaker carbonyl band is present at 5.88 μ.

Samples of amphomycin Ca-salt (40 mg each) were treated similarly for different reaction times. Heating for two hours already results in considerable lactone formation; after 65 hours there is more lactone present than acid. However, no further increase in the relative intensity of the lactone carbonyl band was observed when the reaction time was extended to 5 days.

Catalytic reduction of amphomycin followed by hydrolysis: A sample (1.0 g) of the antibiotic was dissolved in a mixture of ethanol (20 ml) and acetic acid (20 ml), a 10 % palladium on charcoal catalyst (0.20 g) was added and the mixture was stirred in an atmosphere of hydrogen overnight. After the removal of the catalyst and of the solvents the residue was examined by nmr spectroscopy (in CD_3COOD). The vinyl protons (5.56 ppm) present in the spectrum of amphomycin before hydrogenation were not seen in the reduced material. Hydrolysis with constant boiling hydrochloric acid followed by extraction with ether yielded an oil which had only one CO band (5.88 μ) in the ir spectrum. The lactone CO band was absent.

Acknowledgement

The authors express their gratitude to Professor DAVID PEARLMAN of the University of Wisconsin for suggesting the structure of amphomycin as an interesting objective, and to Mr. JOSEPH ALICINO for the microanalysis herein reported. This study was supported by a grant from the U. S. Public Health Service (NIH AI-07515-04). One of the authors (J.A.S.) is an NIH predoctoral fellow.

References

1) HEINEMAN, B.; M. A. KAPLAN, R. D. MUIR & I. R. HOOPER: Amphomycin, a new antibiotic. Antibiot. & Chemoth. 3 : 1239~1242, 1953.
2) SHAY, A. J.; J. ADAM, J. H. MARTIN, W. K. HAUSMANN, P. SHU & N. BOHONOS: Aspartocin. I. Production, isolation, and characteristics. Antibiot. Ann. 1959/1960 : 194~198, 1960.
3) SHIBATA, M.; T. KANZAKI, K. NAKAZAWA, M. INOUE, H. HITOMI, K. MIZUNO, M. FUJINO & A. MIYAKE: On glumamycin, a new antibiotic. J. Antibiotics, Ser. A 15 : 1~6, 1962.

4) HINUMA, Y.: Zaomycin, a new antibiotic from *Streptomyces* sp. Studies on the antibiotic substances from actinomyces. XXXIII. J. Antibiotics, Ser. A 7 : 134～136, 1954.
5) GAUSE, G. F.; T. P. PREOBRAZHENSKAYA, V. K. KOVALENKOVA, N. P. ILYICHEVA, M. G. BRAZHNIKOVA. N. N. LOMAKINA, I. N. KOVSHAROVA, V. A. CHORIN, I. A. KUNRAT & S. P. SHAPOVALOVA: Crystallomycin, a new antibacterial antibiotic. Antibiotiki 2(6) : 9～14, 1957.
6) SHOJI, J.; S. KOZUKI, S. OKAMOTO, R. SAKAZAKI & H. OTSUKA: Studies on tsushimycin. I. Isolation and characterization of an acidic acylpeptide containing a new fatty acid. J. Antibiotics 11 : 439～443, 1968.
7) AHRENS, E. H., Jr., & L. C. CRAIG: Separation of the higher fatty acids. J. Biol. Chem. 195 : 299～310, 1952.
8) HAUSMANN, W. K.; A. H. STRUCK, J. H. MARTIN, R. H. BARRITT & N. BOHONOS: Structure determination of fatty acids from the antibiotic aspartocin. Antimicr. Agents & Chemoth. -1963 : 352～359, 1964.
9) MILBURN, A. H. & E. V. TRUTER: The components of wool wax. II. Synthesis of the acids and alcohols of the iso and (+)-anteiso-*series*. J. Chem. Soc. 1954 : 3344～3351, 1954.
10) BODANSZKY, M.; I. MURAMATSU & A. BODANSZKY: Fatty acid constituents of the antifungal antibiotic stendomycin. J. Antibiotics, Ser. A 20 : 384～385, 1967.
11) INOUE, M.: On glumamycin, a new antibiotic. III. Fatty acid, a constituent of the antibiotic. Bull. Chem. Soc. Jap. 35 : 1255～1257, 1962.