STUDIES ON TSUSHIMYCIN. II

THE STRUCTURES OF CONSTITUENT FATTY ACIDS

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The constituent fatty acids of the antibiotic tsushimycin were examined by gas-liquid chromatography, mass spectrometry, nuclear magnetic resonance and infrared spectroscopy. Also, periodate-permanganate oxidation unequivocally indicated the position of double bonds. The structures of two major constituent fatty acids were determined as *cis*-3-isotetradecenoic acid and *cis*-3-anteisopentadecenoic acid. As a result of parallel experiments, the main constituent fatty acid of the antibiotic amphomycin was also elucidated as *cis*-3-anteisotridecenoic acid.

In the previous paper[1], the isolation and characterization of the acidic acylpeptide antibiotic tsushimycin have been reported. It has also been reported that the antibiotic contains isotetradecenoic acid and isopentadecenoic acid as tentatively determined from only a G. L. C. study. The present study includes further experiments on these fatty acids, showing their structures to be *cis*-3-isotetradecenoic acid and *cis*-3-anteisopentadecenoic acid, respectively. The latter acid has already been proved to be in the hydrolysate of the antibiotic aspartocin[2].

On the fatty acid composition of amphomycin, no confirmatory information has been available to date except one brief description[3]. The previous paper[1] has suggested that amphomycin contains a C_{16} fatty acid as determined in a preliminary G.L.C. experiment. However, the present study carried out with amphomycin samples from two other batches, indicated that the main constituent fatty acid of amphomycin is *cis*-3-anteisotridecenoic acid (these samples of amphomycin were kindly donated by Dr. BERNARD HEINEMANN, Bristol Laboratories, for the purpose of this study).

The antibiotics tsushimycin and amphomycin were hydrolyzed with hydrochloric acid. Ethereal extracts were methylated and analyzed by G. L. C. The total fatty acid compositions of these antibiotics are shown in chromatographic patterns (Figs. 1 and 2). The composition of tsushimycin was estimated as approximately 38 % of i-$C_{14:1}$ acid, 5 % of n-$C_{14:1}$ acid*, 8 % of i-$C_{15:1}$ acid* and 49 % of a-$C_{15:1}$ acid (For convenience the abbreviations** of the fatty acids as determined subsequently are employed

* Those fatty acids which occurred as minor components were tentatively determined only from G. L. C. data on the acid esters as well as on their hydrogenated and oxidized products.

** The abbreviations used are : a-$C_{15:1}$, anteisopentadecenoic acid; i-$C_{15:1}$, isopentadecenoic acid; i-$C_{14:1}$, isotetradecenoic acid; n-$C_{14:1}$, tetradecenoic acid; a-$C_{13:1}$, anteisotridecenoic acid; i-$C_{13:1}$, isotridecenoic acid; i-$C_{12:1}$, isododecenoic acid; a-C_{15}, anteisopentadecanoic acid; i-C_{15}, isopentadecanoic acid; and so on.

throughout this paper). Those of amphomycin were also found to be approximately 8 % of i-$C_{12:1}$ acid*, 20 % of i-$C_{13:1}$ acid* and 72 % of a-$C_{13:1}$ acid. However, it is likely that these compositions are somewhat variable depending on the cultural conditions of the antibiotic-producing organisms.

Fig. 1. Gas-liquid chromatography of the methyl esters of constituent fatty acids of tsushimycin.

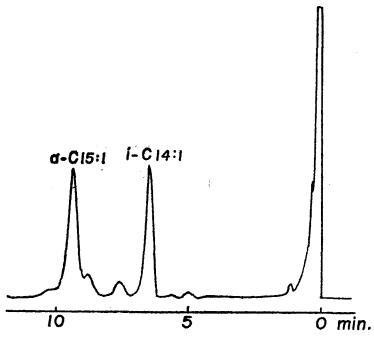


Fig. 2. Gas-liquid chromatography of the methyl esters of constituent fatty acids of amphomycin.

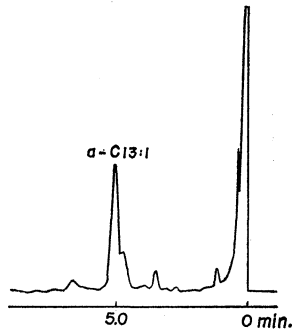


Fig. 3. Retention time and carbon number.
1 : anteiso monounsaturated fatty acid esters
2 : iso monounsaturated fatty acid esters
3 : normal fatty acid esters
4 : anteiso fatty acid esters
5 : iso fatty acid esters

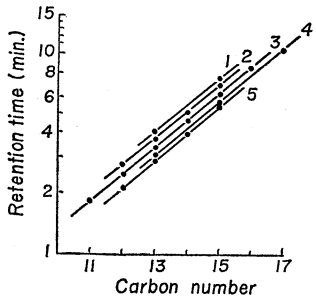
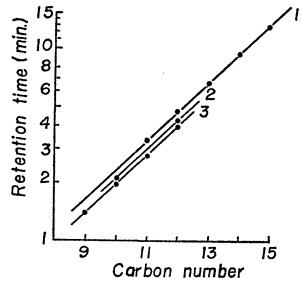


Fig. 4. Retention time and carbon number
1 : normal fatty acid esters
2 : anteiso fatty acid esters
3 : iso fatty acid esters



The retention times measured on a polyester column of the above fatty acid methyl esters and their hydrogenated products were compared with the retention times of appropriate reference fatty acid esters.*** Well paralleled linear relationships were observed with each series of homologous fatty acid esters (Fig. 3). The linear relationship with homologous unsaturated fatty acids has been shown by ACKMAN[5]. Later the relationship based on separation factors has also been shown to allow inclusion of the methyl esters of iso, anteiso, neo and cyclopentenyl fatty acids for tentative identification of their structures[6].

These esters were then oxidized with periodate-permanganate reagent[7], and the acids thus produced with shorter chains were examined by G. L. C. after methylation. As shown in Fig. 4, the newly formed acid esters fell directly on the assumed lines of each series of homologous fatty acids at carbon number corresponding to those of the respective parent unsaturated acid less three carbon numbers. This indicated unequivocally that the positions of double bonds in the naturally occurring unsaturated fatty acids lie between C-3 and C-4.

Three fatty acid esters which occurred as major constituents and their hydrogenated products were isolated by G. L. C. in a semi-preparative manner. Thus, methyl esters of a-$C_{15:1}$, i-$C_{14:1}$, a-$C_{13:1}$, a-C_{15}, i-C_{14} and a-C_{13} acid were available for

*** A commercial preparation of n-C_{11}, n-C_{12}, n-C_{13}, n-C_{14} and n-C_{15} acid methyl esters and a mixture of i-C_{14}, a-C_{15}, i-C_{16}, n-C_{16} and a-C_{17} acid methyl esters prepared from *Bacillus subtilis*[4] were used.

Fig. 5. N. m. r. spectrum of anteisopentadecenoic acid methyl ester.

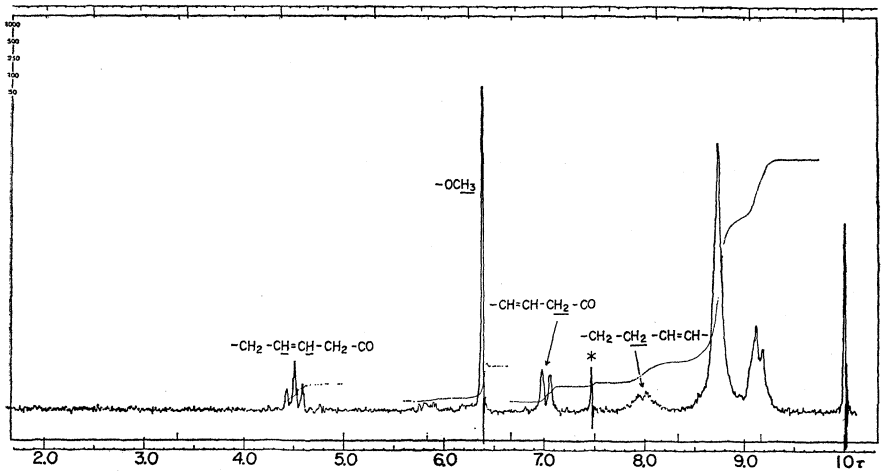


Fig. 6. N. m. r. spectrum of isotetradecenoic acid methyl ester.

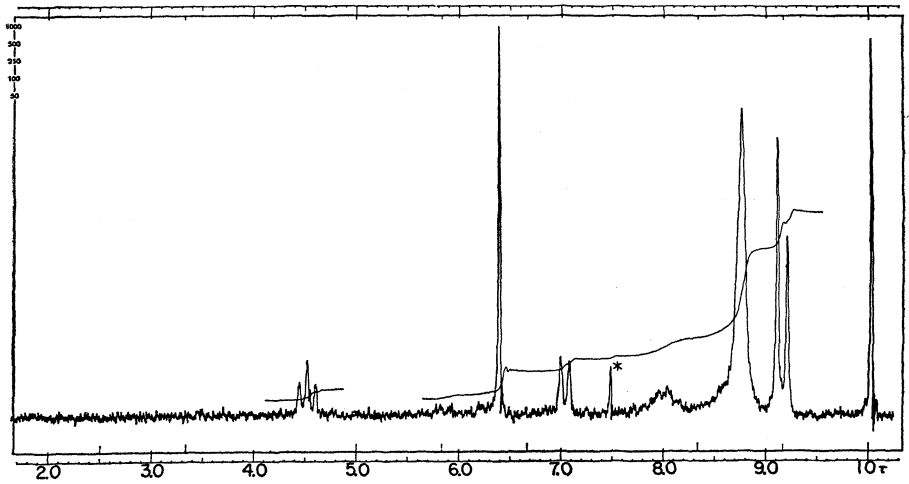
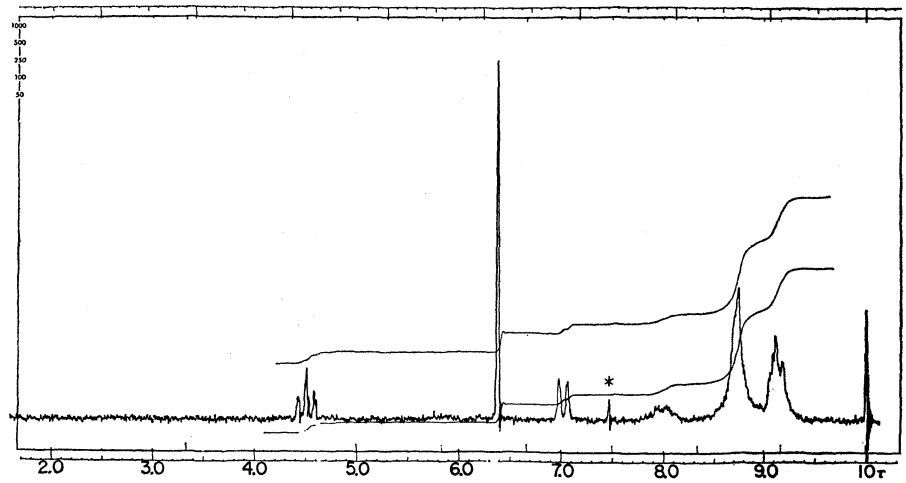


Fig. 7. N. m. r. spectrum of anteisotridecenoic acid methyl ester.



These spectra were taken with a Varian A-60 spectrometer on solutions in CCl_4 containing tetramethylsilan.
* This signal was probably caused by a trace amount of impurity derived from a G.L.C. column.

the following measurements.

The n. m. r. spectra of a-$C_{15:1}$, i-$C_{14:1}$ and a-$C_{13:1}$ acid esters (Figs. 5, 6 and 7) were quite similar except for the shapes of signals corresponding to the six methyl protons appearing at high field.

A triplet of two protons of an olefinic group, and a doublet of a methylene which from its chemical shift must be adjoined to the olefinic group and to a carboxyl group, is common in these Figures. Additional evidence for the location of the double

Fig. 8. Mass spectrum of anteisopentadecanoic acid methyl ester.

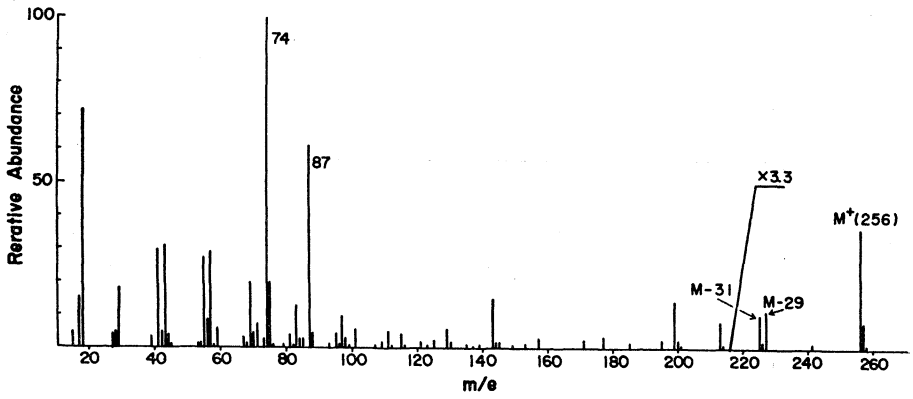


Fig. 9. Mass spectrum of isotetradecanoic acid methyl ester.

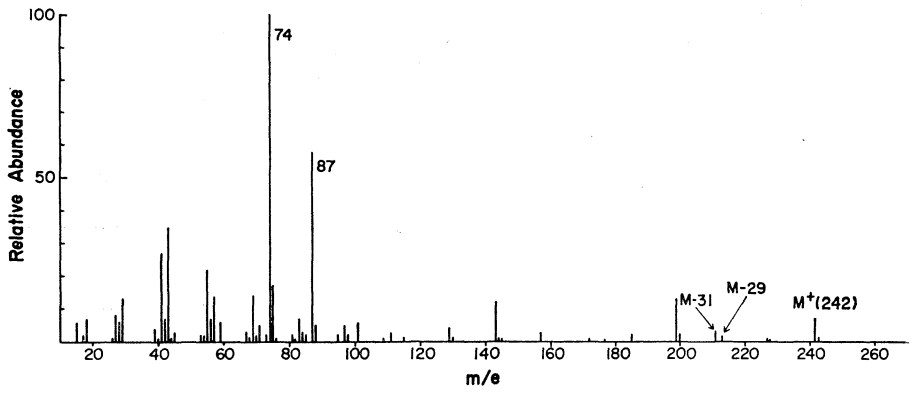
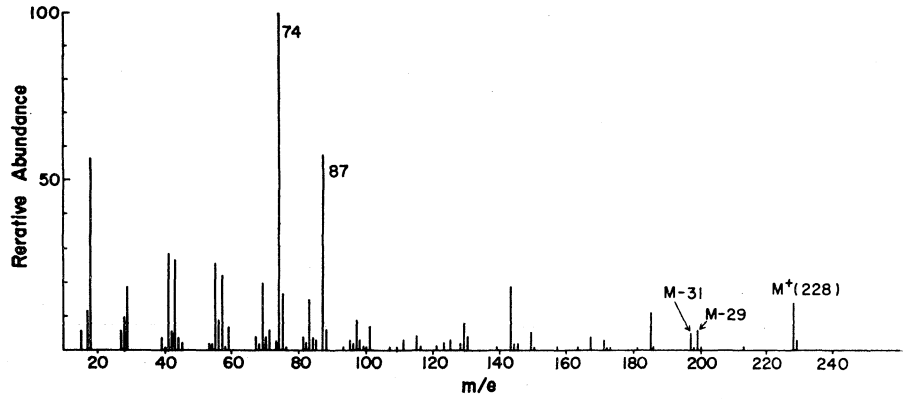


Fig. 10. Mass spectrum of anteisotridecanoic acid methyl ester.



bonds was thus provided (possible assignments of signals are described in the figures). Moreover, these n. m. r. data provided important information on the positions of branched-methyls. As shown in Fig. 6, i-$C_{14:1}$ acid ester demonstrated a typical doublet (τ=9.15, J=6 c.p.s.) of a isopropyl group. On the other hand, a-$C_{15:1}$ and a-$C_{13:1}$ acid ester (Figs. 5 and 7) demonstrated a combined methyl band with a rather ill-defined appearance. The shapes of the methyl signals of branched-chain fatty acids have been examined by CASON and LANGE[8].

In the IR spectra of a-$C_{15:1}$, i-$C_{14:1}$ and a-$C_{13:1}$ acid esters, there was a common band observed at 3020 cm^{-1} corresponding to C-H streching vibration of a *cis*-double bond[9]. No distinct absorption was observed near 965~975 cm^{-1} or 690 cm^{-1}. It has been described[9] that a strong band at 965~975 cm^{-1} is associated with the *trans*-substituted ethylene structure and is used to differentiate between *cis*- and *trans*-substituted fatty acids. A band near 690 cm^{-1} has been associated with the vibration of the *cis* group, but this has been proved to be less certain[9] especially when measured on liquid film or in solutions.

The mass spectra of hydrogenated products of the above three fatty acid esters (a-C_{15}, i-C_{14} and a-C_{13}) are shown in Figs. 8, 9 and 10. There is a molecular ion peak 256 for a-C_{15} acid ester, 242 for i-C_{14} acid ester and 228 for a-C_{13} acid ester. All spectra showed a base peak at m/e=74 and a moderately intense peak at m/e=87, both of which are indicative of typical cleavage of saturated fatty acid methyl esters. In the anteiso acid esters (Figs. 8 and 10), it is characteristic that a peak at m/e=M-29 is higher than a peak at m/e=M-31 attributable to the acylium ion. The former peak (M-29) is attributed to the ion formed by the loss of an ethyl group at the tertiary carbon atom[10]. On the other hand, i-C_{14} acid ester (Fig. 9) produced a peak at m/e=M-29 smaller than a peak at m/e=M-31, although this is similar to those of normal fatty acid esters[10].

The results described above allow the determination of the structures of the two major constituent fatty acids of tsushimycin as *cis*-3-anteisopentadecenoic acid and *cis*-3-isotetradecenoic acid. Similarly, the structure of the main constituent fatty acid of amphomycin was determinad to be *cis*-3-anteisotridecenoic acid.

Some actinomycetes produce acidic acylpeptide antibiotics which contain a variety of fatty acids as their constituents. The structures of these fatty acids so far elucidated are: (+)-12- and 13-methyl-3-tetradecenoic acids in aspartocin[2] and 3-isotridecenoic acid in glumamycin[11]. Recently, the basic peptide antibiotic stendomycin[12] has been shown to contain two major fatty acid constituents which were identified as isomyristic acid and iso-tridecanoic acid.

Including the results of this paper, the double bonds in all of these monounsaturated fatty acids lie between C-3 and C-4. This may be indicative of the close similarity of the biosynthetic routes of these fatty acids in the microorganisms. The present study was carried out with strict consideration for the elucidation of the positions of branched methyls. It is of interest to note that the predominant occurrence of anteiso fatty acids with an odd number and of iso fatty acids with an even number of carbon atoms are confirmed in both the antibiotics examined in this study.

Experimental

Preparation of fatty acid methyl esters from antibiotics:

Some 10 mg of the antibiotic (tsushimycin or amphomycin) was hydrolyzed with 1 ml of 6 N HCl at 105°C for 24 hours. The hydrolysate was extracted three times with 2 ml portions of ether. The ethereal extract was dried with anhydrous sodium sulfate and filtered into a 10 ml glass-stoppered centrifuge tube. The tube was dipped in a cold water bath (*ca.* 15°C), and the ether was evaporated by a stream of nitrogen. To the concentrate, approximately 0.4 ml of ethereal slution of diazomethane was added and the solution was allowed to stand at room temperature for a short time. Excess diazomethane was removed by a stream of nitrogen at *ca.* 15°C, and the ethereal solution was ready for G. L. C. analysis.

Hydrogenation of unsaturated fatty acid methyl esters:

A portion of the ethereal solution of fatty acid methyl esters obtained from the antibiotic as described above was evaporated by a stream of nitrogen to an oily residue. The residue was washed into a 25 ml glass-stoppered Erlenmeyer flask with 3 ml of methanol. After hydrogenation on platinum oxide, the solution was filtered into a centrifuge tube, and the methanol was evaporated with a rotary evaporator at bath temperature of *ca.* 30°C. The ethereal solution of the residue was employed for G. L. C. analysis.

Periodate-permanganate oxidation:

The mixture of fatty acid methyl esters derived from approximately 20 mg of the antibiotic (tsushimycin or amphomycin) was mixed with 4 ml of RUDLOFFS' oxidant (0.018 M sodium periodate and 0.0025 M potassium permanganate), 2 ml of 0.1 M sodium carbonate and 3 ml of *t*-butanol. The reaction mixture was placed in a 25 ml flask and shaken for 6 hours at 27°C. Then 3 mg of sodium metabisulfite was added to the mixture. After the addition of a pellet of potassium hydroxide, the solution was concentrated to 0.5 ml under reduced pressure. The solution was then acidified with N sulfuric acid and extracted four times with ether (2 ml). The ether extract was dehydrated with sodium sulfate, filtered into a centrifuge tube, and concentrated by a stream of nitrogen at *ca.* 15°C. Ethereal solution of diazomethane was added and the excess diazomethane was removed by a stream of nitrogen before G. L. C. analysis.

Gas-liquid chromatography:

(a) For analytical purposes a Perkin-Elmer model 881 equipped with a hydrogen flame detector was used. A specially prepared steel column (3 m in length, 4 mm in inner diameter) was packed with 15 % diethylene glycol succinate polymer on Chromosorb W (AW-DMCS) 80~100 mesh. The column size and the concentration of the polyester were previously examined for good separation, *e. g.* when a smaller column or a lower concentration was used, apparent separation of anteiso and iso fatty acids was not attained. Chromatography was carried out under the following conditions: carrier gas (N_2), 5.0 kg/cm², approximately 40 ml/min; temperature, 170°C and 150°C. The results as shown in Figs. 1, 2 and 3 were that of the run at 170°C, and those as shown in Fig. 4 at 150°C.

(b) For preparative purposes a Shimazu Gaschromatograph GC-1B epuipped with a thermal conductivity detector was used on an U-shaped steel column (3 m in length, 6 mm in inner diameter) which was packed with the same adsorbent as above. Conditions were: carrier gas (He), 1.0 kg/cm², approximately 60 ml/min; temperature, 170°C. Approximately 4 μl of a mixture of fatty acid methyl esters was applied. A trapping tube was inserted into the outlet of the detector chamber at the appropriate time to catch the desired peak.

Preparation of methyl esters of a-$C_{15:1}$, i-$C_{14:1}$ and a-$C_{13:1}$ acid:

The hydrolysate of tsushimycin (600 mg) was extracted with ether (20 ml portions, four times). The ethereal extract was processed in a similar manner to that described in the previous paragraph, and the mixture of fatty acid methyl esters was obtained as an oily material. Each 4 μl of the oil was run repeatedly in the semi-preparative manner as

described above. From that, 20 mg of i-$C_{14:1}$ acid methyl ester and 22 mg of a-$C_{15:1}$ acid methyl ester were obtained as oily materials.

Similarly, from the hydrolysate of amphomycin (500 mg), 20 mg of a-$C_{13:1}$ acid methyl ester was obtained.

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