STUDIES ON TSUSHIMYCIN. I

ISOLATION AND CHARACTERIZATION OF AN ACIDIC ACYLPEPTIDE CONTAINING A NEW FATTY ACID

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A new antibiotic, tsushimycin, was isolated from cultures of a *Streptomyces* strain. The antibiotic is an acidic acylpeptide related to the amphomycin-glumamycin group of antibiotics. However, fatty acid analysis by means of gas-liquid chromatography revealed that the antibiotic contains isotetradecenoic acid which has not been found in known antibiotics. The constituent fatty acids as well as the amino acid composition were compared with those of the above group of antibiotics.

Tsushimycin was isolated from the culture broth of a *Streptomyces* strain Z-237 by *n*-butanol extraction at acid pH. The crude material contained two active substances which were separable on thin-layer chromatograms. The slower moving one was named tsushimycin. The faster moving one was a minor product and was judged to be a similar antibiotic from amino acid analysis.

Tsushimycin was purified by charcoal decolorization and column chromatography on silica gel. A free acid form of tsushimycin was obtained as a colorless amorphous powder, m. p. 230~240°C (dec.). A possible molecular formula $C_{59}H_{93}O_{20}N_{13}\cdot 6H_2O$ was indicated from elemental analysis and molecular weight determination. The optical rotation was $[\alpha]_D^{23}$ $+11.9\pm0.5°$ in methanol, $+12.2\pm0.5°$ in ethanol, and $-5.6\pm0.5°$ in M phosphate buffer, pH 6.0.

The antibiotic is soluble in lower alcohols, acid and alkaline water, but not or slightly soluble in acetone, ethyl acetate, chloroform, ether and petroleum ether. Amphoteric nature with an isoelectric point at approximately pH 3.0 was indicated by paper electrophoresis carried out in several buffer solutions of different pHs. It shows weakly positive ninhydrin and biuret reactions and decolorizes potassium permanganate and bromine solutions. In the ultraviolet absorption spectrum measured in methanol, only an absorption at 207 mμ was observed. The infrared absorption spectrum on a KBr tablet showed the following absorption bands (cm^{-1}): 3300, 3040, 2920, 1725 (carboxyl), 1655, 1530 (peptide bond), 1450, and 1015.

The acid hydrolysate of tsushimycin was subjected to two-dimensional paper chromatography and automatic amino acid analysis. The following amino acids were found: aspartic acid, glycine, proline, pipecolic acid, valine, and trace amounts of ammonia as well as two unknown amino acids, which, however, can be deduced to

be β-methylaspartic acid and α,β-diaminobutyric acid from parallel run of a hydrolysate of aspartocin.

Constituent fatty acids were analyzed by gas-liquid chromatography as their methyl esters. Isotetradecenoic acid and isopentadecenoic acid were identified from direct comparisons of their hydrogenated products with authentic specimens. The ratio of their contents was found to be approximately 1.00 : 1.27.

Among many antibiotics isolated from *Streptomyces* species, amphomycin[1], zaomycin[2], crystallomycin[3], aspartocin[4], and glumamycin[5] have been considered to be closely related to each other. Studies of their constituent amino acids as well as fatty acids have been made with crystallomycin[6] (partly with amphomycin), aspartocin[7,8] and glumamycin[9,10]. Two possible structural formulas have been presented for glumamycin[11]. Recently, laspartomycin[12] has been reported to be a new member of this group, but has a considerably different amino acid composition.

In comparing tsushimycin with these antibiotics, amphomycin and aspartocin are easily differentiated in their optical rotations : amphomycin, $[\alpha]_D^{25}+7.5\pm0.5°$ (*c* 1, water at pH 6)[1] ; and aspartocin, $[\alpha]_D^{25}+26.4°$ (*c* 2.1, methanol)[4].

In regards to amino acid composition, tsushimycin is thought to be similar or identical to aspartocin[7] and/or glumamycin[9]. Crystallomycin[6] (and amphomycin) are different from the above three antibiotics in that they contain a basic amino acid $C_7H_{14}N_2O_2$ instead of α,β-diaminobutyric acid. The amino acid composition of zaomycin was similar to that of tsushimycin by amino acid analysis carried out in the present experiment.

In the literature[8], it has been reported that aspartocin contains 3-isopentadecenoic acid and 3-anteisopentadecenoic acid in equimolar ratio, and it has been suggested that both C_{15} acids are constituents of the same molecule. The presence of a saturated fatty acid in crystallomycin[6], and 3-isotridecenoic acid in glumamycin[10] has also been reported. In a preliminary experiment (unpublished data), it was suggested that amphomycin contains a C_{16} fatty acid and zaomycin contains only isoundecenoic acid. Thus, tsushimycin is clearly differentiated from any of the above antibiotics.

As mentioned in the preceding part of this paper, tsushimycin was not further separable by thin-layer chromatographic technique. However, the content of the two constituent fatty acids was not eqaul, suggesting that these two fatty acids might be derived from different molecules. A trial by mass spectrometric analysis of the methyl ester of this antibiotic was not successful because of difficulty in obtaining a proper vapour pressure.

Experimental

Fermentation : *Streptomyces* strain Z-237 was shake-cultured for 48 hours at 27°C with a medium consisting of glucose 2.0 %, meat extract 0.5 %, peptone 0.5 %, sodium chloride 0.5 %, and calcium carbonate 0.35 %, pH 7.0. A 450 ml portion of the culture was then transferred to a 30-liter jar fermenter containing 20 liters of the same medium. Fermentation was carried out for 72 hours at 27°C under agitation of 250 r.p.m. and aeration of 20 liters per minute. Production of tsushimycin was approximately 100 mcg/ml as an average of several fermentations.

Preparation of crude material: About 60 liters of the cultured broth obtained as above was adjusted to pH 9.5 by dilute sodium hydroxide under vigorous stirring and filtered by the aid of Celite (600 g). The mycelial cake was washed with 15 liters of water adjusted to pH 9.5. The filtrate and the washing were combined and extracted with 15 liters of *n*-butanol at pH 2.0. The antibiotic contained in the *n*-butanol solution was then extracted with 5 liters of water adjusted to pH 9.5 twice, and again transferred into 3 liters of *n*-butanol by adjusting to pH 2.0. The final *n*-butanol solution was then concentrated under reduced pressure to a syrup, which produced a brown powder by addition of ethyl acetate. Dissolution into a minimal volume of methanol and precipitation by addition of ethyl acetate afforded 6.2 g of crude material (670 mcg*/mg).

The crude material contained a minor component in addition to tsushimycin. Thin-layer chromatographic studies revaled the Rf values of the minor component and tsushimycin as 0.43~0.46 and 0.37~0.40 on silica gel G with *n*-butanol - acetic acid - water (3 : 1 : 1), and 0.36~0.39 and 0.16~0.19 on silica gel G with ethanol - 14 % aq. ammonia (4 : 1) as detected by bioautography and also sulfuric acid.

Isolation of tsushimycin:

(a) Decolorization with active carbon. Twenty-five grams of active carbon (Darco G-60) was slurried with methanol and placed on a glass filter to form a layer of approximately 2 cm in thickness. Five grams of the crude material dissolved in 50 ml of methanol was passed through the carbon layer. The eluate and subsequent methanol washings were combined and concentrated. Addition of ethyl acetate gave a colorless powder (2.7 g, 860 mcg/ml).

(b) Silica gel column chromatography. The above preparation (1.5 g) was applied on a silica gel column (Merck 0.5~0.8 mm, 300 g ; column size, 3.2×70 cm) and developed with ethanol - 1.4 % aq. ammonium hydroxide (4 : 1). Tsushimycin was eluted partly over-lapping with the minor component. The fractions containing tsushimycin were collected and processed by alternate concentration and ethanol-addition. This resulted in the precipitation of tsushimycin ammonium salt (1.14 g, 940 mcg/mg) as a fine crystalline powder.

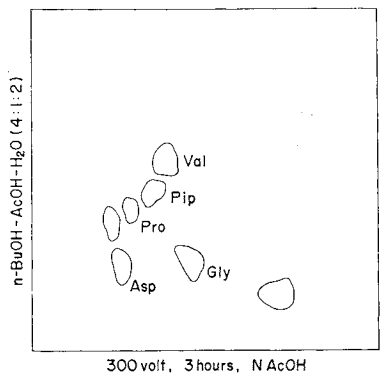
(c) Preparation of tsushimycin free acid. The ammonium salt (500 mg) was suspended in 50 ml of water and shaken with *n*-butanol after adjusting to pH 2.0 with dilute hydrochloric acid. The *n*-butanol layer was washed with water and then concentrated. The resulting residue was dissolved in methanol and precipitated by addition of ethyl acetate. Tsushimycin free acid (348 mg) was obtained as a colorless amorphous powder. m. p. 230~240°C (dec.), $[\alpha]_D^{23}+11.9\pm0.5°$ (*c* 0.992, methanol), $+12.2\pm0.5°$ (*c* 0.998, ethanol), $-5.6\pm0.5°$ (*c* 0.787, M phosphate buffer pH 6.0).

Anal. Found: C 49.98, H 7.53, N 12.76, H_2O 7.37, MW 1296 (Osmometry in 95 % methanol), EW** 445.

Calcd. for $C_{59}H_{93}O_{20}N_{13}\cdot 6H_2O$: C 50.53, H 7.51, N 12.77, H_2O 7.58 %, MW 1425.

Amino acid analysis: Tsushimycin was hydrolyzed with 6 N HCl at 105°C for 24 hours and also for 48 hours. The hydrolysates were examined by two-dimensional paper chromatography. One-dimension was developed with *n*-butanol - acetic acid - water (4 : 1 : 2), and subsequently the other dimension migrated by electrophoresis at 300 volt for 3 hours in N AcOH. As illustrated in Fig. 1, aspartic acid, glycine, proline, pipecolic acid and valine were detected by ninhydrin

Fig. 1. Two-dimensional paper-grams of the hydrolysate of tsushimycin.



* Tsushimycin free acid was used as a standard for the bioassay.

** Titrated in 50 % aq. methanol.

Fig. 2. The relationships between retention times and carbon numbers of fatty acids.

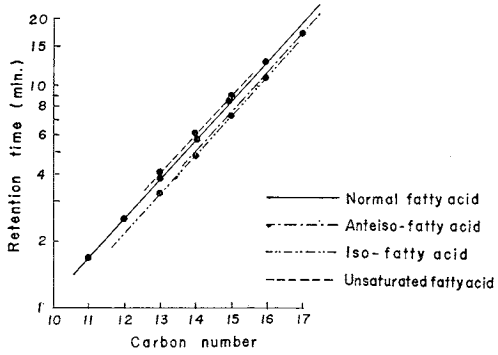
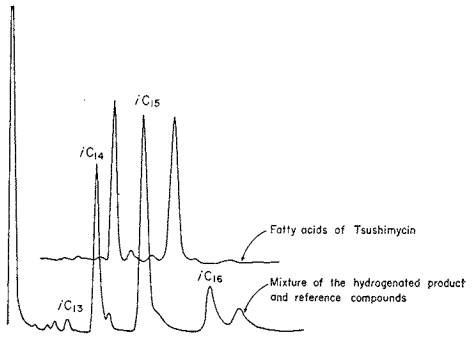


Fig. 3. Gas-liquid chromatography of the constituent fatty acids of tsushimycin and the hydrogenated products.



and isatin solution. In addition to the above, an unknown acidic and an unknown basic amino acid were found. By a Hitachi automatic amino acid analyzer, the following amino acids were measured (Fig. 1): aspartic acid (3.98*), proline (1.03), glycine (2.00**), valine (0.98), pipecolic acid (not measured), unknown basic amino acid (not measured) and ammonia (*ca.* 0.35). Nearly identical peaks were obtained by parallel runs of samples of aspartocin, and zaomycin***. Therefore, the unknown amino acids found in tsushimycin were considered to be β-methylaspartic acid and α,β-diaminobutyric acid, previously known as constituents of aspartocin[7] and glumamycin[9].

Fatty acid analysis: The hydrolysate (for 24 hours) was extracted with ether. The ether extract was methylated with diazomethane in the usual way and subjected to gas-liquid chromatography. The chromatography was carried out on a Shimadzu Gas Chromatograph GC-1B with 15% diethyleneglycol succinate polymer on Chromosorb W (acid washed, siliconized) at 160°C. The hydrolysate of tsushimycin gave two peaks at retention times of 5.80 and 8.90 minutes, whose peak areas were estimated as 1.00 : 1.27. As reference, normal fatty acids of C_{11}, C_{12}, C_{13}, C_{14} and C_{15} purchased from commercial sources, and iso-fatty acids of C_{14}, C_{15} and C_{16} and anteiso-fatty acid of C_{17}, obtained from a neutral lipid fraction of *Streptomyces sioyaensis*[14] as well as 3-isotridecenoic acid obtained from glumamycin[10] were run in a similar way. On the basis of the relationships between retention times and carbon numbers (Fig. 2), the two peaks observed with tsushimycin were assigned to isotetradecenoic acid and isopentadecenoic acid, respectively. After hydrogenation on platinum black, the two peaks shifted to retention times of 4.90 and 7.55 minutes and were identified with those of isotetradecanoic acid and isopentadecanoic acid by direct comparison with the authentic specimens (Fig. 3).

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References

1) HEINEMANN, B.; M. A. KAPLAN, R. D. MUIR & I. R. HOOPER: Amphomycin, a new antibiotic. Antibiot. & Chemoth. 3 : 1239~1242, 1953.
2) HINUMA, Y.: Zaomycin, a new antibiotic from a *Streptomyces* sp. Studies on the antibiotic substances from actinomyces. XXXIII. J. Antibiotics, Ser. A 7 : 134~136, 1954.

* This value may involve that of β-methylaspartic acid.
** Molar ratios were expressed in relative values to 2 moles of glycine.
*** The sample of zaomycin was prepared in a similar way from *Streptomyces zaomyceticus*.

3) 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.
4) 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.
5) 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.
6) LOMAKINA, N.N. & M.G. BRAZHNIKOVA : The chemical composition of crystallomycin. Biokhimiia 24 : 425～431, 1959.
7) MARTIN, J.H. & W.K. HAUSMANN : Isolation and identification of D-α-pipecolic acid, α[L],β-methylaspartic acid and α,β-diaminobutyric acid from the polypeptide antibiotic aspartocin. J. Am. Chem. Soc. 82 : 2079, 1960.
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) INOUE, M. : On glumamycin, a new antibiotic. II. Isolation and identification of amino acids constituting glumamycin. Bull. Chem. Soc. Jap. 35 : 1249～1254, 1962.
10) INOUE, M. : On glumamycin, a new antibiotic. III. Fatty acid, a constituent of the antibiotic. Bull. Chem. Soc. Jap. 35 : 1255～1257, 1962.
11) FUJINO, M. : On glumamycin, a new antibiotic. VI. An approach to the amino acid sequence. Bull. Chem. Soc. Jap. 38 : 517～522, 1965.
12) NAGANAWA, H. ; M. HAMADA, K. MAEDA, Y. OKAMI, T. TAKEUCHI & H. UMEZAWA : Lasparto-mycin, a new anti-staphylococcal peptide. J. Antibiotics 21 : 55～62, 1968.
13) KIMURA, A. ; J. KAWANAMI & H. OTSUKA : Lipids of *Streptomyces sioyaensis*. II. Lipid composition of *Streptomyces sioyaensis*. Agr. & Biol. Chem. 31 : 1434～1440, 1967.