# THE CONSTITUENT AMINO ACIDS AND FATTY ACID OF ANTIBIOTIC 333–25

## (STUDIES ON ANTIBIOTICS FROM THE GENUS *BACILLUS*. XII[1])

JUN'ICHI SHOJI, HIROSHI HINOO and RYUZI SAKAZAKI

Shionogi Research Laboratory, Shionogi & Co., Ltd.,
Fukushima-ku, Osaka 553, Japan

(Received for publication January 23, 1976)

Measurement of the optical rotational activities of the constituent amino acids of antibiotic 333–25 identified 2,4-diaminobutyric acid (D-form 1, L-form 4), L-leucine (2) and D-phenylalanine (1). The fatty acid consituent was determined to be β-hydroxy anteisononanoic acid by gas chromatography, nuclear magnetic resonance and mass spectra. Differentiation from the structure of antibiotic EM 49 is discussed.

Antibiotic 333–25, active against Gram-positive and -negative bacteria, is produced by a strain of *Bacillus circulans*. The fact that the antibiotic is a basic acylpeptide containing 2,4-diaminobutyric acid (5), leucine (2) and phenylalanine (1) with close structural similarity to antibiotic EM 49[2,3,4,5] has been reported.[1]

2,4-Diaminobutyric acid (Dab) and leucine were isolated by preparative paper chromatography, and phenylalanine was isolated from the hydrolyzate using a column containing the porous polymer, XAD-2.

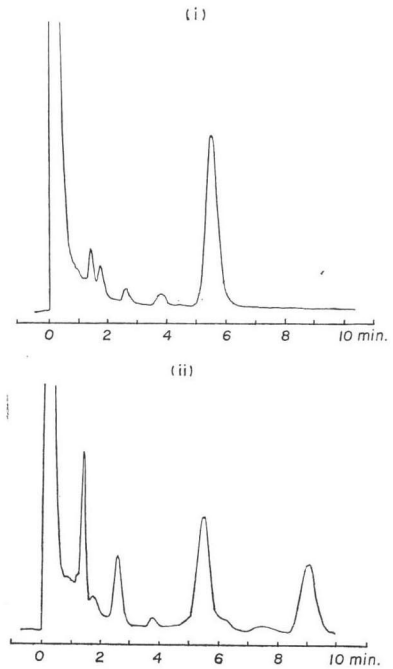
The measured molecular rotation of the Dab isolated was near to that calculated for a mixture of one D-form and four L-forms as illustrated in Table 1. From that, the presence of one D-Dab and four L-Dab residues is deduced as in the case of antibiotic EM 49. The leucine isolated was deduced to be the L-form and the phenylalanine to be the D-form from the molecular rotations (Table 1) and the ORD

Table 1. Comparison of molecular rotations

| Amino acid | $[M]_D$ (in 5 N HCl) |
|---|---|
| Isolated Dab | +18.9±0.8° |
| L-Dab | +39.0±1.1° |
| Calcd. for a mixture of | |
| D-Dab (1), L-Dab (4) | +23.4° |
| D-Dab (2), L-Dab (3) | + 7.8° |
| Isolated Leu | +16.1±2.1° |
| L-Leu | +21.0°* |
| Isolated Phe | + 8.4±2.3° |
| L-Phe | −7.4°* |

* Cited from the literature[6].

Fig. 1. Gas chromatograms of fatty acid methyl ester from antibiotic 333–25.
(i) Preparation from 1-hour hydrolysis
(ii) Preparation from 20-hour hydrolysis



curves.[7] In antibiotic EM 49, the presence of D- and L-leucine and L-phenylalanine has been reported.

For preliminary analysis of the constituent fatty acids the ethereal extract of the hydrolyzate of the antibiotic was methylated and examined with gas chromatography. When the hydrolyzate obtained by the usual hydrolysis conditions for amino acid analysis, *e.g.* in constant boiling hydrochloric acid at 110°C for 20 hours, was used for the analysis, several peaks were observed (Fig. 1). However, the sample prepared by hydrolysis for 1 hour gave primarily one main peak (Fig. 1). It has already been stated in the report on antibiotic EM 49,[8] that longer hydrolysis times resulted in extensive destruction of β-hydroxy fatty acids and formation of a mixture consisting largely of α,β-unsaturated fatty acids and butyrolactones. When the ethereal extract from the hydrolysis for 20 hours was hydrogenated and analyzed, methyl anteisononanoate was tentatively identified from the retention time. Therefore, the constituent fatty acid of antibiotic 333–25 was assumed to be β-hydroxy anteisononanoic acid.

The fatty acid methyl ester was isolated by preparative gas chromatography from the methylated product of the ethereal extract obtained from a one-hour hydrolyzate. The n.m.r. spectrum of the ester provides the evidence that the ester is indeed methyl β-hydroxy anteisononanoate. Assignment of the signals are cited in the figure (Fig. 2).

In the mass spectrum of the ester, the molecular ion peak was not obtained. However, peaks at *m/e*, 170 ($M-H_2O$), 141 ($M-H_2O$, $C_2H_5$), 139 ($M-H_2O$, $OCH_3$), 103 (base peak), 74 *etc.* were observed (Fig. 3). The base peak, *m/e*, 103, is attributed to be the fragment ion caused by β,γ-fragmentation, and is thought to be the common base peak of β-hydroxy fatty acids from survey of our other experiments.

From the results of the above and the previous paper,[1] the constituents of antibiotic 333–25 are concluded to be Dab (D-form 1, L-form 4), L-leucine (2) and D-phenylalanine (1) and β-hydroxy anteisononanoic acid. Like antibiotic EM 49, hydrolysis of 2,4-dinitrophenylated 333–25 afforded approximately 1 mole of free Dab in addition to release of γ-DNP-Dab. In contrast to antibiotic

Fig. 2. N.m.r. spectrum of methyl β-hydroxy anteisononanoate from antibiotic 333–25. The spectrum was recorded with a Varian A-60 spectrometer on solution in $CCl_4$ containing TMS. The signal at *ca.* 2.95 ppm disappeared by addition of $D_2O$.

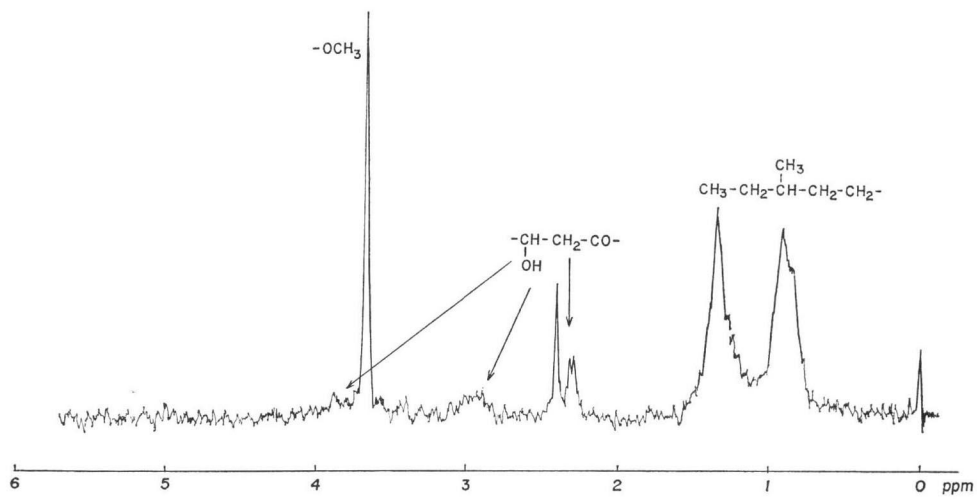
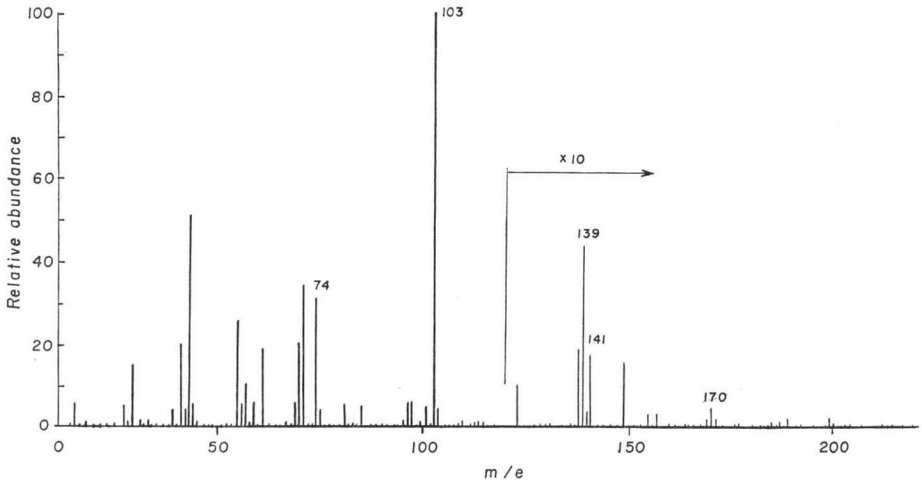


Fig. 3. Mass spectrum of methyl β-hydroxy anteisononanoate from antibiotic 333–25.




EM 49, which is a complex of acylpeptides comprising a "replaceable" leucine residue and three fatty acid residues: 8-methyl-3(R)-hydroxynonanoic acid, 3(R)-hydroxydecanoic acid and 8(S)-methyl-3(R)-hydroxydecanoic acid, antibiotic 333–25 is a homogeneous acylpeptide. Other than the difference in the fatty acid residue, antibiotic 333–25 contains one mole of D-phenylalanine and two moles of L-leucine, whereas antibiotic EM 49 contains *ca.* 0.5 moles of L-phenylalanine, *ca.* 1.5 moles of L-leucine and one mole of D-leucine. Our attempt to deacylate antibiotic 333–25 was unsuccessful with the enzyme preparation, Polymyxin Acylase, by whose action we had previously succeeded in deacylating the acylpeptide antibiotics, cerexin A[8] and brevistin.[9] Though we have not any information on the sequence of the amino acid residues of antibiotic 333–25, it is thought to be likely that the positions of the phenylalanine and leucine residues in the sequence differ from those of antibiotic EM 49.[5]

## Experimental

ORD curves were recorded with a JASCO Model ORD/UV-5 spectropolarimeter. $[\alpha]_D$ was measured with a Perkin-Elmer Model 141 polarimeter. Mass spectra were measured with a Hitachi RMU-6 mass spectrometer.

Isolation of constituent amino acids:

Some 500 mg of hydrochloric acid salt of antibiotic 333–25 were hydrolyzed with constant boiling hydrochloric acid at 110°C for 20 hours. The hydrolyzate was evaporated to dryness and the residue was applied to two sheets of Toyo Roshi No. 527, 60×60 cm. The papers were developed with *n*-butanol - acetic acid - water (4: 1: 2). Zones of leucine, phenylalanine and Dab, those of the former two overlapping extensively, were detected by ninhydrin coloration. The zones were cut out and extracted with water. By concentrating the extracts, a fraction of Dab (216 mg), a leucine-rich fraction (95 mg) and a phenylalanine-rich fraction (74 mg) were obtained.

By crystallization with water and ethanol, 2,4-diaminobutyric acid monohydrochloride was obtained as colorless needles (180 mg).

*Anal.* Found: C, 30.94; H, 7.30; N, 17.93; O, 20.90; Cl, 22.79.
Calcd. for $C_4H_{10}N_2O_2 \cdot HCl$: C, 31.07; H, 7.17; N, 18.12; O, 20.70; Cl, 22.94.

$[\alpha]_D^{24.0}$ +12.2±0.5° (*c* 1.020, 5 N HCl).

ORD: $[\phi]_{250}$ +400, $[\phi]_{225}$ +1030, $[\phi]_{215}$ +430 (*c* 0.3587, 0.5 N HCl).

A commercial specimen of L-2,4-diaminobutyric acid dihydrochloride purchased from Tokyo

Kasei Kogyo Co., Ltd., gave: $[\alpha]_D^{26.0}+20.4\pm0.6°$ (*c* 0.982, 5 N HCl), ORD: $[\phi]_{250}+650$, $[\phi]_{225}+1790$, $[\phi]_{215}+810$ (*c* 0.3890, 0.5 N HCl).

The leucine-rich fraction (50 mg) was dissolved in water (5 ml) and adjusted to pH 7.0. The solution was placed on top of an XAD-2 column (1.8×40 cm), that was washed slowly with water. Leucine was eluted in the fraction of 100~130 ml. Concentration of the fraction gave a colorless residue (20 mg), which was again adsorbed on a small column of Dowex 50×8 ($NH_4$) and eluted with 0.3 N $NH_4OH$. Concentration of the eluate and crystallization with water and ethanol gave leucine as colorless plates (11 mg).

*Anal.* Found: C, 55.23; H, 10.21; N, 10.83.
Calcd. for $C_6H_{13}NO_2$: C, 54.94; H, 9.99; N, 10.67.

$[\alpha]_D^{22.5}+12.3\pm1.5°$ (*c* 0.351, 5 N HCl).

ORD: $[\phi]_{250}+880$, $[\phi]_{224}+2970$, $[\phi]_{215}+1470$ (*c* 0.0698, 0.5 N HCl).

The phenylalanine-rich fraction (30 mg) was treated with the XAD-2 column in essentially the same manner. Phenylalanine was eluted in the fraction of 272~440 ml. The eluate was concentrated to dryness. Crystallization with water and ethanol gave phenylalanine as colorless plates (12 mg).

*Anal.* Found: C, 65.86; H, 6.93; N, 8.49; O, 18.72.
Calcd. for $C_9H_{11}NO_2$: C, 65.43; H, 6.71; N, 8.48; O, 19.37.

$[\alpha]_D^{23.0}+5.1\pm1.4°$ (*c* 0.323, 5 N HCl).

ORD: $[\phi]_{250}-1090$, $[\phi]_{225}-5710$, $[\phi]_{220}-2520$ (*c* 0.1752, 0.5 N HCl).

Gas chromatographic examination:

For analytical purpose a Perkin-Elmer Model 881 equipped with a hydrogen flame detector and a steel column (6 feet, 1/8-inch diameter) packed with 15% diethylene glycol succinate polymer on Chromosorb W 80~100 mesh was used. Conditions were: carrier gas ($N_2$), 5.0 kg/cm$^2$, approximately 30 ml/min, temperature, 160°C or 120°C.

Some 10 mg each of antibiotic 333–25 were hydrolyzed with constant boiling hydrochloric acid at 110°C for 1 hour or 20 hours. The hydrolyzate was extracted with 2 ml of ether three times. 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 was evaporated by a stream of nitrogen. To the concentrate, a few drops of an ethereal solution of diazomethane was added. After a few minutes, excess diazomethane was removed by a stream of nitrogen and the ethereal solution was ready for GC analysis. The chromatograms of the two samples (1- and 20-hour hydrolysis) run at 160°C are illustrated in Fig. 1.

Another preparation of the ethereal extract from 20-hour hydrolysis was evaporated to an oily film, dissolved in 2 ml of methanol and hydrogenated for 1 hour in the presence of platinum oxide. The methanol was carefully removed by a stream of nitrogen and the hydrogenated product was methylated as above.

When the sample was analyzed by the GC at 120°C, a main peak at a retention time of 2.45 minutes was obtained. Identification of the peak with that of methyl anteisononanoate was made by the use of the authentic specimen prepared from the hydrolyzate of polymyxin E.

Isolation of methyl β-hydroxy anteisononanoate:

Some 500 mg of antibiotic 333–25 dissolved in 10 ml of constant boiling hydrochloric acid in a sealed tube was heated at 110°C for 1 hour. The hydrolyzate was extracted with 20 ml of ether three times. The residual solution was concentrated to a residue, which was again hydrolyzed in the same manner. The hydrolysis and extraction with ether were repeated three times. The ethereal extracts were combined, dried ($Na_2SO_4$), concentrated carefully by a stream of nitrogen and methylated.

For preparative purpose of gas chromatography a Varian Aerograph Model 1520-IB equipped with a thermal conductivity detector and an aluminum column (10 feet, 3/8-inch outer diameter) packed with 5% diethylene glycol succinate polymer on Chromosorb Q 60~80 mesh was used. Conditions were: carrier gas (He), 4 kg/cm$^2$, approximately 200 ml/min; temperature, 180°C.

About ten runs were carried out with the above preparation and the main peak was collected in a trapping tube. The fatty acid methyl ester was obtained as a colorless oil. The n.m.r. and mass

spectra are illustrated in Figs. 2 and 3.

## References


1) SHOJI, J.; H. HINOO, Y. WAKISAKA, K. KOIZUMI, M. MAYAMA, S. MATSUURA & K. MATSUMOTO: Isolation of a new antibiotic 333–25, related to EM 49. (Studies on antibiotics from the genus *Bacillus*. XI). J. Antibiotics 29: 516~520, 1976
2) MEYERS, E.; W. E. BROWN, P. A. PRINCIPE, M. L. RATHNUM & W. L. PARKER: EM 49, a new peptide antibiotic.I. Fermentation, isolation, and preliminary characterization. J. Antibiotics 26: 444~448, 1973
3) PARKER, W. L. & M. L. RATHNUM: EM 49, a new peptide antibiotic. II. Chemical characterization. J. Antibiotics 26: 449~456, 1973
4) MEYERS, E.; F. E. PANSY, H. I. BASCH & R. J. MCRIPLEY: EM 49, a new peptide antibiotic. III. Biological characterization *in vitro* and *in vivo*. J. Antibiotics 26: 457~462, 1973
5) PARKER, W. L. & M. L. RATHNUM: EM 49, a new peptide antibiotic. IV. The structure of EM 49. J. Antibiotics 28: 379~389, 1975
6) GREENSTEIN, J. P. & M. WINITZ: Chemistry of the Amino Acids, Vol. 1, p. 86, John Wiley & Sons, Inc., New York, 1961
7) JENNINGS, J. P.; W KLYNE & P M. SCOPES: Optical rotatory dispersion. X. Amino acids. J. Chem. Soc. 1965: 294~296, 1965
8) SHOJI, J. & T. KATO: The amino acid sequence of cerexin A. (Studies on antibiotics from the genus *Bacillus*. VII). J. Antibiotics 28: 764~769, 1975
9) SHOJI, J. & T. KATO: The structure of brevistin. (Studies on antibiotics from the genus *Bacillus*. X). J. Antibiotics 29: 380~389, 1976