THE STRUCTURE OF ANTRIMYCIN

Sir:

In the previous paper[1], we reported the isolation of a new peptide antibiotic, antrimycin. In this communication, the structure determination of antrimycin is reported.

^{13}C and ^{1}H NMR Spectra

As described in the previous paper, the molecular formula was established as $C_{28}H_{47}N_9O_{11}$ by elemental analysis and mass spectrometry. The ^{13}C NMR spectrum in D_2O (Fig. 1) also indicated the presence of 28 carbons. The off-resonance spectrum showed that in the sp^3-carbon region (δ 13.0~64.8) there are five methyls, six methylenes, six methines and one non-proton-bearing carbon (δ 64.6), and in the sp^2-carbon region (δ 122.5~176.8) there are seven carbons (δ 167.7~176.8) tentatively assigned to carbonyl, one methine (δ 149.2) and two non-proton-bearing carbons (δ 122.5, 149.3). The multiplicity of the signals in the off-resonance spectrum is recorded in Fig. 1. The well-resolved 250 MHz ^{1}H NMR spectrum in D_2O (Fig. 2) indicated the presence of 34 non-exchangeable protons, which was in accord with the proton number counted from the off-resonance ^{13}C NMR spectrum. The ^{1}H-^{1}H

Fig. 1. ^{13}C NMR spectrum of antrimycin in D_2O at 25.2 MHz.
(pHm 7.0, internal dioxane as δ 67.4)

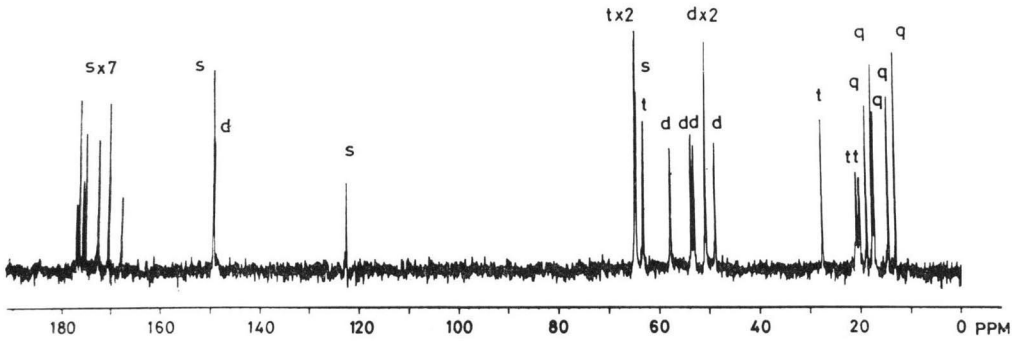
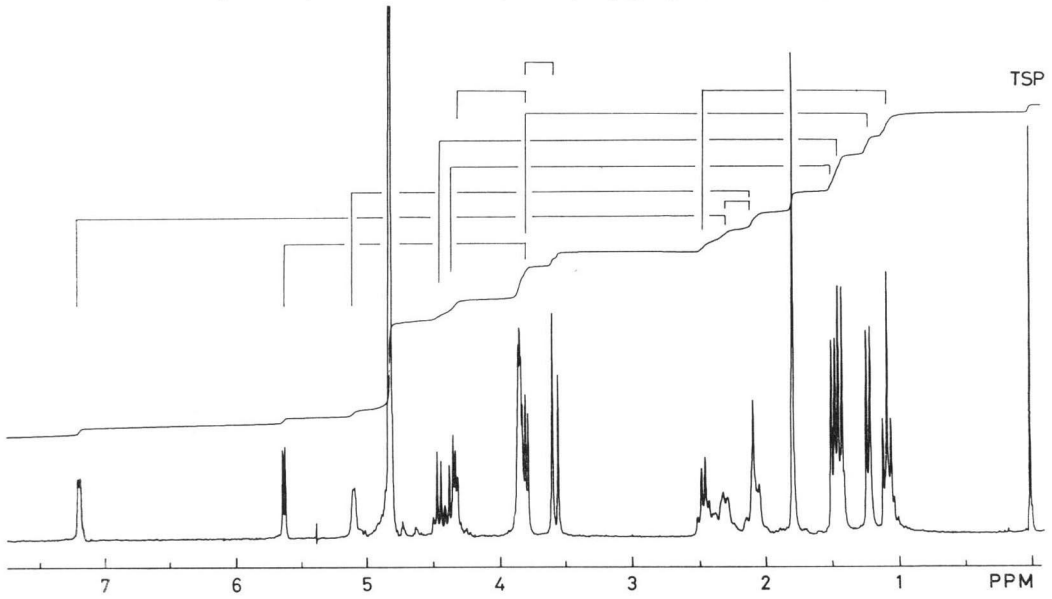


Fig. 2. ^{1}H NMR spectrum of antrimycin in D_2O at 250 MHz.
(pHm 7.9, internal sodium 3-(trimethylsilyl)propionate-d_4 as δ 0)



coupling relations are also shown in Fig. 2. All of the proton signals can be correlated to the proton-bearing carbon signals by the aid of selective ^{13}C-^{1}H decoupling (Data are not shown).

Amino Acids Obtained by Total Acid Hydrolysis

Total acid hydrolysis (6 N HCl, 110°C, 16 hours) of antrimycin gave four ninhydrin positive products. They were isolated by Dowex 50-X8 column chromatography developed with pyridine-formate (pH 3.0) followed by pyridine-acetate (pH 4.6 and 4.9) buffers. The two amino acids eluted secondly and thirdly were identified as L-serine, $[\alpha]_D^{20}+13.2°$ (*c* 0.5, 6 N HCl), and L-alanine, $[\alpha]_D^{21}+14.0°$ (*c* 0.5, 6 N HCl), respectively, but the other two were found to be uncommon.

The first eluted unusual amino acid was determined to be 2,2-bis(hydroxymethyl)glycine (BHMG).[2] Calcd. for $C_4H_9NO_4$ (MW 135.12): C, 35.55; H, 6.71; N, 10.37. Found: C, 36.04, H, 6.64; N, 10.20. FDMS, *m/z* 136 $(M+H)^+$. ^{1}H NMR in D_2O; 3.95 (2H, d, 12.3 Hz) and 4.10 (2H, d, 12.3 Hz), external TMS reference at 100 MHz. ^{13}C NMR in D_2O; 173.7 (s), 68.1 (s), 62.3×2 (t).

The last eluted basic amino acid was found to be a diastereoisomeric mixture (*ca.* 2: 1) of 2,3-diaminobutanoic acid (DABA)[3,4] by ^{1}H and ^{13}C NMR, and FD mass spectrometries [*m/z* 119 $(M+H)^+$]. The major component was isolated by crystallization from aqueous ethanol. It was identified as *erythro*-L-α,β-diaminobutanoic acid, that is (2*S*, 3*S*)-DABA, by ^{1}H NMR ($^{1}H_\alpha$-$^{1}H_\beta$ coupling constant 6.5 Hz)[3] and ORD spectrometry (peak at 226 nm 2400°)[3,4]. It is already known that DABA is partially epimerized during acid hydrolysis.[4] Therefore, the (2*S*,3*S*)-isomer should exist originally in antrimycin.

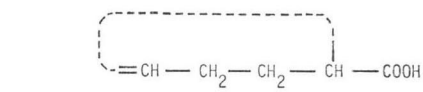
The molar ratio of the four amino acids in antrimycin was shown to be: BHMG - Ser - Ala - DABA, 1: 1: 2: 1, by ^{1}H NMR spectrometry of antrimycin. The molar ratio of Ala to Ser was also confirmed by amino acid analysis with a Hitachi 835 amino acid analyzer.

Terminal Analysis

Potentiometric titration of antrimycin indicated the presence of three dissociable functions (*pKa* 3.4, 6.4 and 8.5). Hydrazinolysis of antrimycin liberated serine. Therefore, the *pKa* 3.4 can be assigned to the carboxyl group of the serine. Treatment of antrimycin with dinitrofluorobenzene followed by acid hydrolysis gave mono-DNP-DABA and a trace of DNP-BHMG. In the ^{1}H NMR of antrimycin measured in DMSO-d_6, the α-methine proton of DABA appeared as doublets of doublet at δ 5.56 [*J*, 9.0 (C_αH-NH) and 5.5 (C_αH-C_βH) Hz]. It means that the α-amino function of DABA is concerned in the peptide bond formation and the mono-DNP-DABA described above is β-DNP-DABA. The poor yield of DNP-BHMG was suggested to be due to steric hindrance by vicinal bis(hydroxymethyl) groups. In fact, BHMG itself was hardly dinitrophenylated. Thus, it was concluded that the β-amino group of DABA and the amino group of BHMG are free in the antrimycin molecule. By the pH-dependent ^{13}C chemical shift, *pKa* 6.4 is assigned to the amino group of BHMG and *pKa* 8.5 to the β-amino group of DABA (data are not shown).

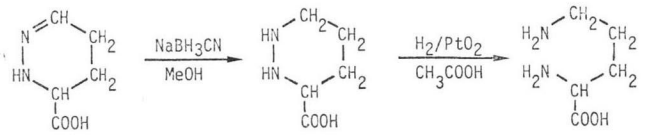
Mild Alkaline Hydrolysis Products

From the total acid hydrolysate of antrimycin, information on the structure of the remaining $C_{11}N_3$-containing moiety could not be obtained. Therefore, mild alkaline hydrolysis (1 N NaOH, 37°C, 5 days) was carried out to minimize decomposition and racemization. The alkaline hydrolysate was separated into two fractions: the effluent from Diaion HP-20 column and the eluate from the column with 50% aqueous acetone. The effluent contained a substance (THPC) which gave a yellow color by ninhydrin reaction. THPC was isolated as a sticky colorless material by Avicel column chromatography developed with 95% ethanol. The ^{13}C NMR spectrum in D_2O showed the presence of 5 carbon atoms (δ 22.1, 22.2, 56.3, 147.3 and 179.8). The result of the ^{1}H NMR study [δ 7.45 (1H, m), 2.76 (2H, m), 2.55 (2H, m), 4.10 (1H, m)] together with the ^{13}C NMR study suggested a ring structure having the following partial structure:



^{1}H (δ)		7.45	2.76	2.55	4.10
^{13}C (δ)	147.3	22.1*	22.2*	56.3	179.8

*may be exchanged



THPC L-ornithine

The presence of 2 nitrogen atoms in THPC was suggested by the N/C atom ratio in the elemental analysis. The FD mass spectrum gave M^+ ion at m/z 128. Thus, the molecular formula was shown to be $C_5H_8N_2O_2$ (MW 128). From this molecular formula and the partial structure described above, the structure of THPC was supposed to be 2,3,4,5-tetrahydropyridazine-3-carboxylic acid.

To confirm this proposed structure, the transformation of THPC into ornithine was carried out. First, THPC was reduced with $NaBH_3CN$ in methanol at room temperature overnight to give a sticky dihydro-THPC (m/z 130). The ^1H NMR spectrum showed the presence of $-CH_2-CH_2-CH_2-\overset{1}{C}H-$ sequence [δ 3.68 (2H, m), 2.60 (2H, m), 2.36 (2H, m) and 4.15 (1H, m)]. The dihydro-THPC was hydrogenated under 4 atomospher pressure of hydrogen with platinum catalyst for 2 days at room temperature in acetic acid to yield ornithine. It was identified by TLC, high voltage paper electrophoresis and amino acid analysis. The ORD spectrum in 1 N HCl showed a peak at 226 nm (1400°); authentic L-ornithine (226 nm, 2870°). From the result, the stereochemistry of the derived ornithine was shown to be partially racemized L-isomer. Thus, the presence of an (*S*)-2,3,4,5-tetrahydropyridazine-3-carboxylic acid moiety in antrimycin was disclosed.

The eluate fraction from Diaion HP-20 of the alkaline hydrolysate contained two ninhydrin positive products as the major components: one gave a violet color (Peptide-B1) and the other a yellow color (Peptide-B2) with the ninhydrin reaction. They were isolated by preparative Avicel TLC. Peptide-B1 was also found as a minor component in the effluent from the Diaion HP-20 column.

Acid hydrolysis of Peptide-B1 yielded one mole each of alanine and serine. The N-terminus was determined to be alanine by DNP-method. The ^{13}C NMR spectrum in D_2O showed the presence of 6 carbon signals [δ 13.0 (q), 18.1 (q), 27.5 (t), 122.7 (s), 147.2 (s) and one of three carbonyl signals], which remained to be solved in antrimycin molecule, in addition to 6 signals ascribed to alanine and serine. The ^1H NMR spectrum also showed the presence of the unclarified ethyl [δ 1.60 (3H, t, 7.5 Hz), 2.93 (2H, q, 7.5 Hz)] and methyl [δ 2.32 (3H, s)] signals in addition to the signals of alanine and serine. Thus, Peptide-B1 was supposed to be a tripeptide, alanyl-dehydroisoleucyl-serine (MW 287). In fact, the FD mass spectrum of Peptide-B1 gave a peak at m/z 288 $(M+H)^+$. To confirm the structure, Peptide-B1 was hydrogenolyzed under 4 atomospher pressure of hydrogen with platinum catalyst for 2 days at room temperature in 1 N acetic acid. The product was hydrolyzed in 6 N HCl at 110°C overnight. Amino acid analysis indicated the presence of almost one mole each of alanine, serine, and isoleucine, and a trace amount of *allo*-isoleucine. Thus, the structure of Peptide-B1 was determined to be L-alanyl-(*E*)-(2,3-didehydroisoleucyl)-L-serine.

All of the signals of ^1H and ^{13}C NMR spectra of antrimycin can be explained by the seven amino acids hitherto characterized: 2 moles of Ala, one mole each of Ser, BHMG, DABA, THPC and *⊿*-Ile (dehydroisoleucine). The results described above indicate that antrimycin is a linear heptapeptide, of which the N-terminus is BHMG and

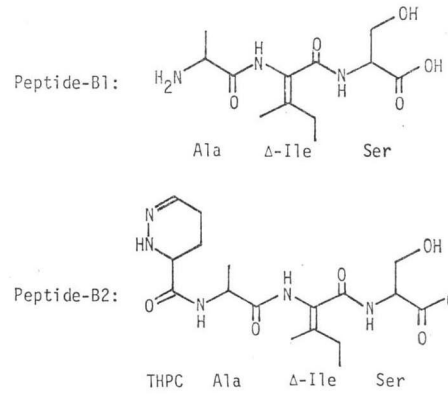
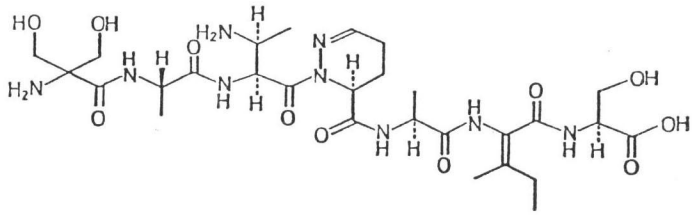


Fig. 3. Structure of antrimycin.



the C-terminus is serine, with the free β-amino function of DABA.

The ^{1}H and ^{13}C NMR spectra of Peptide-B2 suggested that Peptide-B2 is the C-terminal tetrapeptide of antrimycin: THPC-Ala-Δ-Ile-Ser.

Mild Acidic Hydrolysis Products

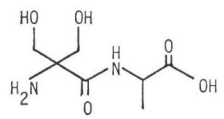
In order to determine the total amino acid sequence in antrimycin, partial acidic hydrolysis of antrimycin (6 N HCl, 37°C, 6 days) was carried out. The hydrolysates were separated by Dowex 50-X8 column chromatography developed with pyridine-formate (pH 3.0) followed by pyridine-acetate (pH 4.6 and 4.9) buffers. Four segment peptides were isolated and named Peptides-A1, -A2, -A3 and -A4 in order of the elution.

Peptide-A1 was found to be identical with Peptide-B1. Peptide-A2 was found to be the N-terminal dipeptide, BHMG-Ala, by total acid hydrolysis and NMR studies. Peptide-A3 was found to be a pentapeptide composed of one mole each of DABA, THPC, Ala, Δ-Ile and Ser by NMR studies. Peptide-A4 was found to be a tripeptide, DABA-THPC-Ala, by total acid hydrolysis, N-terminal analysis (DNP-method), FDMS m/z 300 $(M+H)^+$, and NMR studies.

From the experimental results described above, the structure of antrimycin, including the absolute configuration, has been determined to be that shown in Fig. 3.

Peptide-A1 = Peptide-B1

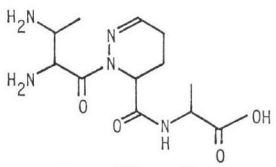
Peptide-A2:



BHMG Ala

Peptide-A3: (DABA-THPC-Ala-Δ-Ile-Ser)

Peptide-A4:



DABA THPC Ala

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