THE STRUCTURE OF ANTRIMYCIN

Sir:

In the previous paper, 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 $sp^2$-carbon region ($\delta$ 13.0 - 64.8) there are five methyls, six methylenes, six methines and one non-proton-bearing carbon ($\delta$ 64.6), and in the $sp^2$-carbon region ($\delta$ 122.5 - 176.8) there are seven carbons ($\delta$ 167.7 - 176.8) tentatively assigned to carbonyl, one methine ($\delta$ 149.2) and two non-proton-bearing carbons ($\delta$ 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 spectrum showed that in the $sp^2$-carbon region ($\delta$ 13.0 - 64.8) there are five methyls, six methylenes, six methines and one non-proton-bearing carbon ($\delta$ 64.6), and in the $sp^2$-carbon region ($\delta$ 122.5 - 176.8) there are seven carbons ($\delta$ 167.7 - 176.8) tentatively assigned to carbonyl, one methine ($\delta$ 149.2) and two non-proton-bearing carbons ($\delta$ 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 $\delta$ 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 $\delta$ 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 $^{13}\text{C}$-$^1\text{H}$ decoupling (Data are not shown).

Amino Acids Obtained by Total Acid Hydrolysis

Total acid hydrolysis ($6\text{ N HCl, } 110^\circ\text{C, } 16\text{ hours}$) 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, $[\alpha]_D^{13}=+13.2^\circ$ (c 0.5, 6 N HCl), and L-alanine, $[\alpha]_D^{13}=+14.0^\circ$ (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). Calcd. for C$_4$H$_7$NO$_4$ (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)$^+$. $^1\text{H}$ NMR in D$_2$O; 3.95 (2H, d, 12.3 Hz) and 4.10 (2H, d, 12.3 Hz), external TMS reference at 100 MHz. $^{13}\text{C}$ NMR in D$_2$O; 173.7 (s), 68.1 (s), 62.3 $\times$ 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 $^1\text{H}$ and $^{13}\text{C}$ NMR, and FD mass spectrometry ($m/z$ 119 (M$^-$H)$^+$). The major component was isolated by crystallization from aqueous ethanol. It was identified as erythro-1-$\alpha$-$\delta$-diaminobutanoic acid, that is (2S,3S)-DABA, by $^1\text{H}$ NMR ($^1\text{H}_\alpha$-$^1\text{H}_\beta$ coupling constant 6.5 Hz)$^3$ and ORD spectrometry (peak at 226 nm 2400'). It is already known that DABA is partially epimerized during acid hydrolysis.$^3$ Therefore, the (2S,3S)-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 $^1\text{H}$ 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 (p$K_a$ 3.4, 6.4 and 8.5). Hydrazinolysis of antrimycin liberated serine. Therefore, the p$K_a$ 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 $^1\text{H}$ NMR of antrimycin measured in DMSO-$d_6$, the $\alpha$-methine proton of DABA appeared as doublets of doublet at $\delta$ 5.56 ($J$, 9.0 (C$_6$H$_4$-NH) and 5.5 (C$_6$H$_4$-C$_6$H) Hz). It means that the $\alpha$-amino function of DABA is concerned in the peptide bond formation and the mono-DNP-DABA described above is $\beta$-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 $\beta$-amino group of DABA and the amino group of BHMG are free in the antrimycin molecule. By the pH-dependent $^{13}\text{C}$ chemical shift, p$K_a$ 6.4 is assigned to the amino group of BHMG and p$K_a$ 8.5 to the $\beta$-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$_1$N$_2$-containing moiety could not be obtained. Therefore, mild alkaline hydrolysis ($1\text{ N NaOH, } 37^\circ\text{C, } 5\text{ days}$) 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 $^1\text{C}$ NMR spectrum in D$_2$O showed the presence of 5 carbon atoms ($\delta$ 22.1, 22.2, 56.3, 147.3 and 179.8). The result of the $^1\text{H}$ NMR study ($\delta$ 7.45 (1H, m), 2.76 (2H, m), 2.55 (2H, m), 4.10 (1H, m)) together with the $^{13}\text{C}$ NMR study suggested a ring structure having the following partial structure:
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 C10H12N2O4 (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 NaBH₄CN in methanol at room temperature overnight to give a sticky dihydro-THPC (m/z 130). The ¹H NMR spectrum showed the presence of -CH₂-CH₂-CH₂-CH₂- 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 atmospher 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 ¹⁴C NMR spectrum in D₂O 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 ¹³C 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 J-Ile (dehydroisoleucine). The results described above indicate that antrimycin is a linear heptapeptide, of which the N-terminus is BHMG and...
the C-terminus is serine, with the free β-aminofunction of DABA.

The 1H and 13C NMR spectra of Peptide-B2 suggested that Peptide-B2 is the C-terminal tetrapeptide of antrimycin: THPC-Ala-J-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, J-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.

Acknowledgement

The authors are grateful to Messrs. T. Shimazu and S. Seto, Nippon Kayaku Co. Ltd., for amino acid analysis.

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(Received September 9, 1981)

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