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Studies of Cirratiomycins. Part II. The Structures of Cirratiomycin A and B, the New Peptide Antibiotics†

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In the preceding paper, we described the production, isolation, physico-chemical properties and biological activities of cirratiomycin A (**1**) and B (**2**) produced by *Streptomyces cirratus* 248-Sq2. These two antibiotics are very closely related to each other in structure. This report presents the structural elucidation of cirratiomycin A and B in detail.

The 400 MHz ^{1}H-NMR spectrum of **1** (Fig. 1) revealed 40 well-resolved unexchangeable proton signals. Based on the chemical shift trends, these were classified as follows ; 6 methyls, with one allylic methyl (δ 1.82 ppm); 7 methylenes, of which three were oxygenated ; 7 aliphatic methines and one olefinic methine. Exhaustive spin decoupling experiments proved the partial structures shown in the nexf page.

The 100 MHz ^{13}C-NMR spectrum of **1** (Table I) showed a total of 30 carbon signals, with one of double intensity. All protonated carbons were analyzed by selective proton decoupling experiments. The other carbons were as follows ; seven carbonyl carbons

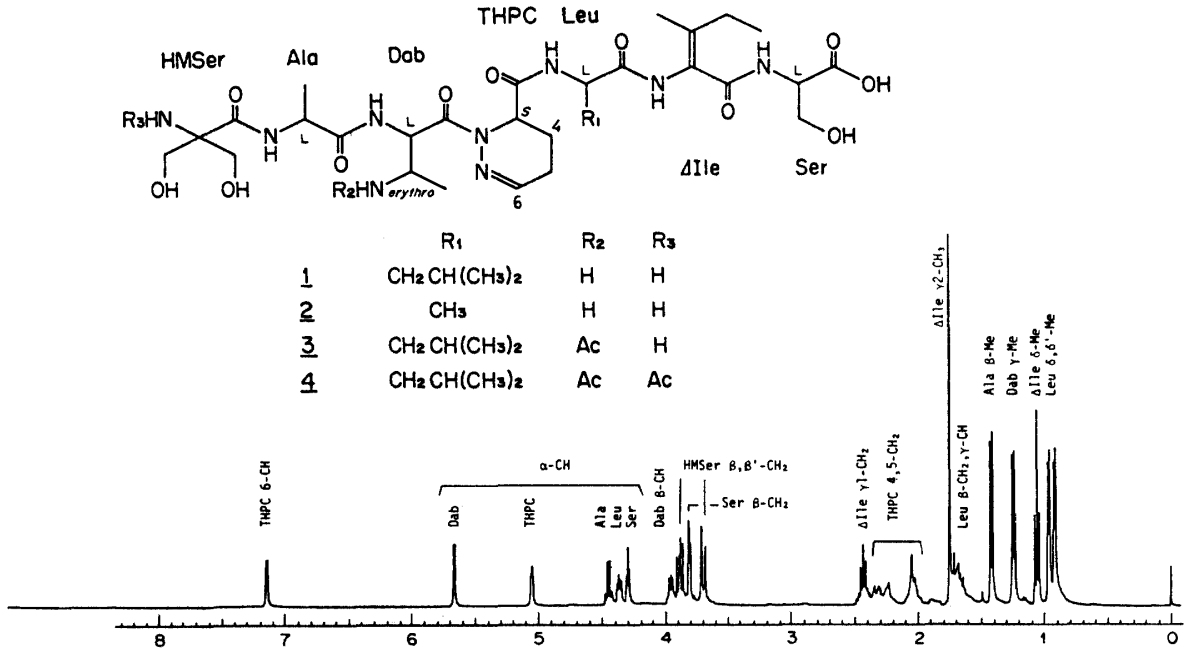

Fig. 1. 400MHz ^{1}H-NMR Spectrum of Cirratiomycin A in D_2O

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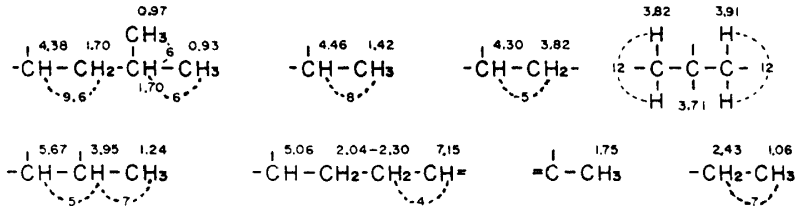
Table I. ^{13}C-Chemical Shift of Cirratiomycin A (dioxane as an internal standard, 67.4ppm)

Amino acid		ppm	Amino acid		ppm
HMSer	α-C	66.2	Leu	α-CH	53.6
	β1, β2-CH_2	62.4		β-CH_2	40.4
				γ-CH	25.2
Ala	α—CH	51.0		δ 1-CH_3	22.8
	β-CH_3	17.3		δ 2-CH_3	21.7
Dab	α-CH	53.0	ΔIle	α-C	122.5
	β-CH	48.8		β-C	149.3
	γ-CH_3	14.0		γ1-CH_2	27.5
				γ2-CH_3	18.7
THPC	C-3	53.7		δ-CH_3	12.9
	C-4	19.9*			
	C-5	20.6*	Ser	α-CH	57.8
	C-6	149.1		β-CH_2	63.2
CO		167.7			170.0
		171.3			172.6
		174.6			176.0
		176.7			

* Interchangeable.

Partial structcres



(δ 167—177 ppm), two olefinic (δ 122, 149 ppm) and one aliphatic (δ 66.2 ppm) quaternary carbon. The existence of one C=C and one C=N bond, which are suggested by the three olefinic carbon signals, and seven C=O bonds indicate the presence of a ring structure in **1** (10-degrees of unsaturation).

Since the IR spectrum of **1** suggested the presence of peptide bonds, ν_{max}^{KBr} 1650 cm^{-1}, **1** (0.5 mg) was hydrolyzed with 6 N HCl, 120°C for 16 hours and an amino acid analysis was carried out using an amino acid autoanalyzer. As a result, one mol each of Leu, Ala and Ser were detected together with two unidentified peaks. One was observed shortly before Asp and the other shrotly before NH_3.

In order to isolate these amino acids, 10 mg of **1** was hydrolyzed (6 N HCl, 120°C, 16 hours) in a sealed tube. After removal of the HCl *in vacuo*, the residue was passed through a column of Dowex 50 W-X2 (0.5 M pyridine-AcOH buffer, pH 6.4) to separate a neutral amino acid fraction and one basic amino acid which was identified as a diastereomeric mixture of α,β-diaminobutyric acid (abbreviated as Dab) by ^1H-NMR. it has been

reported that under this hydrolysis condition, racemization occurs at the α-carbon atom of Dab. The different magnetic environments of the two protons, Hα and Hβ, between the diastereomric isomers are reflected in the signals having different coupling constants (7 Hz *erythro* and 4 Hz *threo*). The absolute configuration of Dab in the native antibiotic will be discussed later.

Then, the neutral amino acid fraction was successively separated into four amino acids by Sephadex G-25 partition column chromatography (*n*-BuOH-AcOH-water, 4 : 1 : 2). The first three were identified as L-Leu, L-Ala and L-Ser, and the last unknown amino acid was determined as hydroxymethylserine (HMSer) by direct comparison (^{1}H-NMR, amino acid analysis) with an authentic sample.

In the ^{1}H-NMR spectrum, the resonances due to these five amino acids which showed the presence in an equimolar ratio were easily assigned, while the ^{13}C-NMR spectrum suggested further the existence of two acid-labile amino acids, temporarily designated as X and Y, whose partial structures were as follows ;

$$\text{Two CO},\quad =CH\text{-}CH_2\text{-}CH_2\text{-}\dot{C}H,\quad -CH_3,\quad -CH_2\text{-}CH_3,\quad -\dot{C}=,\quad =\dot{C}-$$

The ^{1}H-NMR spectrum of **1** in DMSO-d_6 (Fig. 2) afforded four doublet amide protons as well as a singlet. Spin decoupling experiments irradiating at each α-methine proton permitted us to assign these doublet α-amide protons as Leu, Ala, Ser and Dab (δ 8.49, 8.68, 7.30 and 8.22 ppm, respectively).

The dissociation constants, p*K*a', of **1** were determined as 3.4, 6.7 and 8.8 by potantiometric titration. These values suggested the presence of three functional groups, *i.e.* one carboxylic and two amino groups which were well reflected in *N*-acetylation experiments. Treatment of **1** (10 mg) with acetic anhydride in MeOH (0°C, 30 min) gave 7 mg of *N*-monoacetyl cirratiomycin A (**3**) and 3 mg of *N*-diacetyl cirratiomycin A (**4**). Repeated acetylation of **3** under the same conditions gave **4**. In the ^{1}H-NMR spectrum of **3** (in D_2O), the β-methine proton of Dab was shifted downfield from 4.0 ppm for the parent antibiotic to 4.4 ppm, at the same time as an upfield shift of Dab γ-Me (from 1.24 to 1.08 ppm), indicating the β-amino group of Dab to be free. On the other hand, the ^{1}H-NMR spectrum of **4** closely resembled that of **3** except for signals assignable to HM-Ser, suggesting that the second acetylation took place at a free amino group attached to the quaternary carbon. In agreement with this, a new singlet amide proton (7.70 ppm) ppeared in the spectrum of **4** in DMSO-d_6 (Fig. 3), together with the doublet β-amide proton of Dab (7.10 ppm). These results are compatible with the p*K*a' values of 8.8 and 6.4 which were assigned to β-NH_2 of Dab and NH_2-C$\lessgtr$, respectively. Treatment of **1** with dansyl chloride (DNS-Cl) at pH 9.5 only dansylated the β-amino group of Dab but did not give a bis DNS derivative.

Because **1** contained acid-labile amino acids, enzymatic reactions (endo and exo peptidases) were tried to obtain X and Y as intact. However, these degradations were

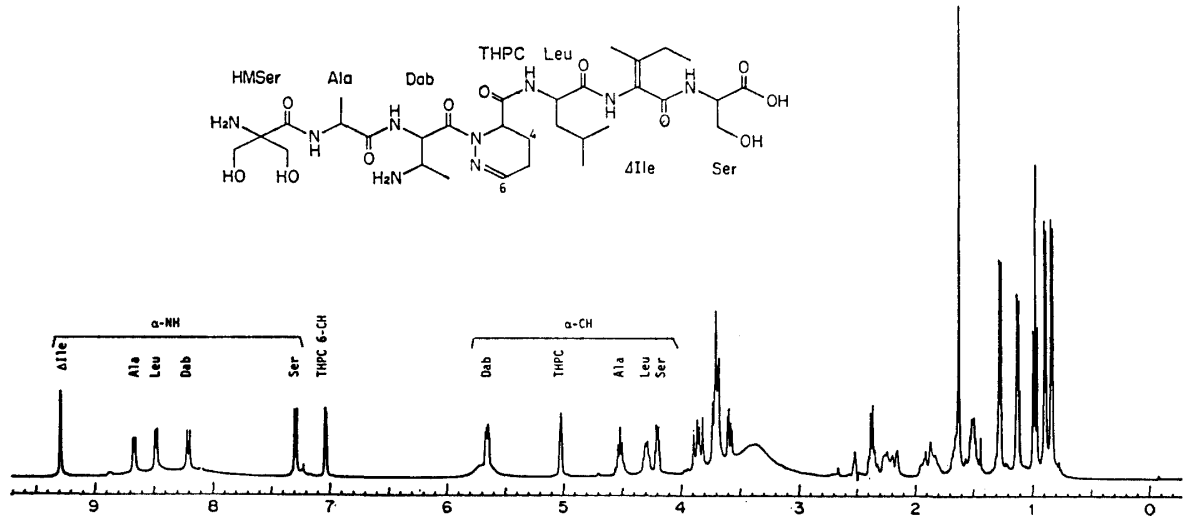


Fig. 2. ^{1}H-NMR Spectrum of Cirratiomycin A in DMSO-d_6.

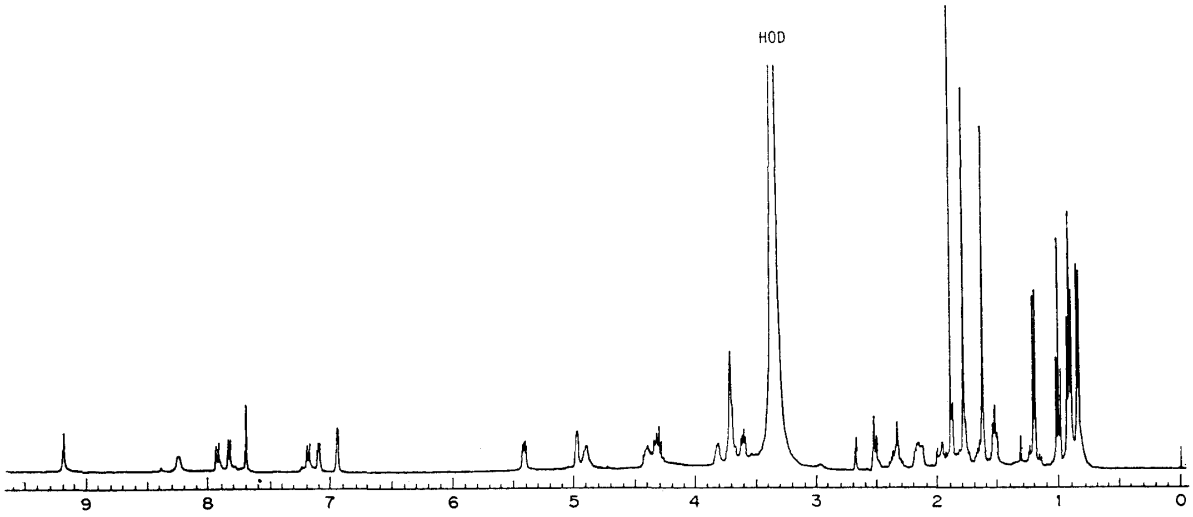


Fig. 3. ^{1}H-NMR Spectrum of *N*-Diacetyl Cirratiomycin A in DMSO-d_6.

not successful due to the presence of unusual amino acids. Then, efforts were made to isolate small peptide fragments under several hydrolysis conditions. Mild acid hydrolysis of **1** (6 N HCl, 37°C, 7 days) and subsequent purification by column chromatography using Toyopearl HW 40 F and Dowex 50 W-X 2 (0.5 M pyridine-AcOH buffer, pH 6.4) afforded four peptide fragments. The *N*-terminal residues of these fragments were determined by the DNS method. The ^{1}H-NMR spectra (**6**, **7**) are shown in Fig. 4a, b.

HMSer-Ala	**5**
Dab-X-Leu	**6**
Leu-Y	**7**
Leu-Y-Ser	**8**

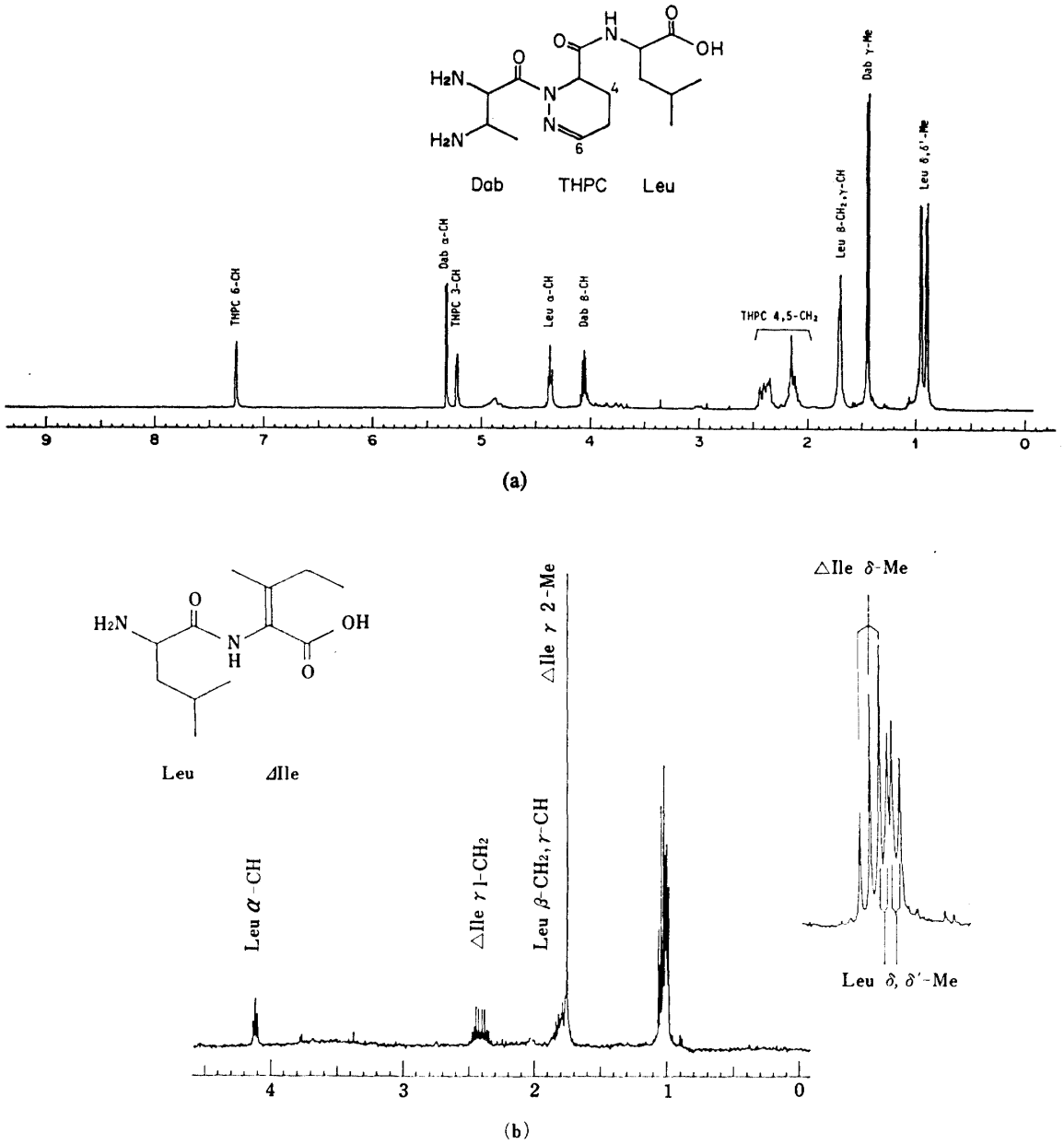


Fig. 4. ^{1}H-NMR Spectra of Tripeptide **6** (a) and Dipeptide **7** (b) in D_2O.

In addition to these fragments, Dab was isolated without any recamization and identified to take an L-*erythro* configuration by ORD and ^{1}H-NMR spectra ($J_{H\alpha-H\beta}=8$ Hz).

The ^{1}H-NMR spectrum of **5** revealed the signals attributed to HMSer (δ 3.73 ppm, 2 H, 3.92 ppm, 1 H, 3.94 ppm, 1 H) and Ala (δ 1.39 ppm, 3 H, 4.23 ppm, 1 H), but dansylated **5** could not be obtained, probably due to steric hindrance around the amino group of HMSer. This result indicated the structure of **5** as HMSer-Ala.

From the ^{1}H-NMR spectra of **6** and **7**, the following partial structures are suggested for the two unknown amino acids X and Y.

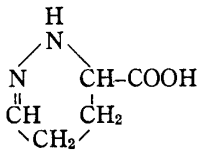
X; $-\overset{|}{C}H-CH_2-CH_2-CH=$

Y; $=\overset{|}{C}-CH_3$, $-CH_2-CH_3$

Isolation of these peptide fragments, together with the fact that the α-amino group of Dab was protected in the parent antibiotic, established the amino acid sequence of **1** as

HMSer-Ala-Dab-X-Leu-Y-Ser

The FD mass spectrum of tripeptide **6** exhibited $(M+H)^+$ and $(M+Na)^+$ ion peaks at *m/z* 342 and 364, respectively, and further spectral data allowed us to assign the molecular formula of X as $C_5H_8N_2O_2$. Catalytic hydrogenation of **6** over PtO_2 in 1 N AcOH (room temperature, 5 days), followed by acid hydrolysis, liberated Leu, Dab and L-ornithine which were identified by TLC, amino acid analysis and ORD. This result proved the unknown amino acid, X, to be 2,3,4,5-tetrahydropyridazine-3-carboxylic acid (THPC, **9**). Thus the structure of **6** was elucidated as L-*erythro* Dab-(S)-THPC-L-Leu. Reductive cleavage of the 4-hydroxy-2,3,4,5-tetrahydropyridazine-3-carboxylic acid to β-hydroxyornithine has been described.



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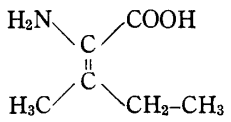

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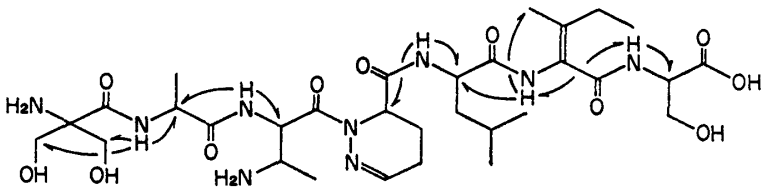

The EI mass spectrum of an *N*-acetyl derivative of **7** (M^+ *m/z* 284) suggested the molecular formula of $C_6H_{11}NO_2$ for Y and the ^{1}H-NMR spectral analysis of **7** revealed the structure of Y as 2,3-didehydroisoleucine (*⊿*Ile, **10**). Hydrogenation of 8 over PtO_2 in 1 N AcOH followed by acid hydrolysis gave Leu, Ser and Ile, confirming the above conclusion and the sequence of 8 as Leu-*⊿*Ile-Ser.

In order to determine the stereochemistry of the *⊿*Ile moiety, an NOE experiment was carried out. Upon irradiation of a singlet amide proton of **1** (δ 9.31 ppm, assignable to the amide proton of *⊿*Ile) in DMSO-d_6, the negative NOE was observed with singlet methyl protons (δ 1.65 ppm) of *⊿*Ile indicating its configuration to be *E*. By irradiating four other amide protons separately, negative NOEs were also observed with relevant protons, and the amino acid sequence proposed was again confirmed as shown below.

These experimental results elucidated the linear structure for **1**, having the free amino group of HMSer which could not be dansylated because of its presumable steric hindrance

and Ser as the C-terminal.

Cirratiomycin B (**2**) has properties closely related with **1** and its ^{1}H- and ^{13}C-NMR spectra were essentially superimposable with those of **1**, except for the presence of Ala residue instead of Leu. Acid hydrolysis of **2** liberated two moles of L-Ala and one mol of L-Ser along with HMSer and Dab. *N*-Acetylation of **2** also afforded *N*-monoacetyl cirratiomycin B and *N*-diacetyl cirratiomycin B. Mild acid hydrolysis of **2** gave amino acid segments HMSer-Ala, Dab-THPC-Ala, Ala-*allo*Ile and Ala-*allo*Ile-Ser and confirmed the structure of cirratiomycin B as shown in **2**.

Recently, Dr. Umezawa and his associates have reported independently the structures of antrimycin A-Dv, in which the identity of antrimycin A with cirratiomycin B and antrimycin D with cirratiomycin A were confirmed, respectively.