FERROCINS, NEW IRON-CONTAINING PEPTIDE ANTIBIOTICS PRODUCED BY BACTERIA

ISOLATION, CHARACTERIZATION AND STRUCTURE ELUCIDATION

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Novel iron-containing peptide antibiotics, ferrocins A, B, C and D, have been isolated from the culture filtrate of *Pseudomonas fluorescens* YK-310. These antibiotics were purified by butanol extraction, followed by column chromatography using adsorption resin, silica gel and preparative reverse-phase HPLC. The structures of ferrocins were elucidated using spectroscopic and degradative methods. Ferrocins contain three hydroxamate moieties per ferric ion which forms an octahedral iron complex.

In the course of screening for new antibiotics that show antibacterial activity against *Pseudomonas aeruginosa*, we discovered ferrocins A, B, C and D in the culture filtrate of *Pseudomonas fluorescens* YK-310. Ferrocins were found to be iron chelating cyclic decapeptides having strong therapeutic effects against *P. aeruginosa* infection[1,2]. This paper deals with the isolation, characterization and structure elucidation of ferrocins.

Isolation

To isolate ferrocins A (**1**), B (**2**), C (**3**) and D (**4**), the culture broth was filtered using Hyflo Super Cel (Johns Manville). The filtrate (1,300 liters) was chromatographed on a column of Diaion HP-20 (<50 mesh, 50 liters) eluting with 80% aqueous methanol (350 liters). The concentrate of this eluate (30 liters) was extracted with isobutyl alcohol (20 liters) at pH 7. The extract was washed with 2% sodium bicarbonate and 0.05 N hydrochloric acid and concentrated. The concentrate was applied to a column of silica gel (1.5 liters). After washing with isobutyl alcohol (4.5 liters), 2-propanol (4.5 liters) and 2-propanol-methanol (1 : 1, 4.5 liters), the bioactive fraction was eluted with methanol (4.5 liters). The solvent was evaporated to afford a crude powder (10.6 g).

The crude powder was applied to a column of Diaion HP-20 (100 ~ 200 mesh, 340 ml). After washing with 50% methanol (1 liter) and 60% methanol (340 ml), the bioactive substances were eluted with 70% methanol (1.7 liters). The bioactive fractions were concentrated and lyophilized to afford the ferrocin complex as a powder (2.5 g). This complex was purified by repetitive preparative reverse-phase HPLC using an ODS column of YMC-Pack R-355 or a YMC-Pack S-363 I-15 with a mobile phase of 32% aqueous acetonitrile. The pure fractions as analyzed by HPLC were concentrated and freeze-dried to give **1** (1.1 g), **2** (69 mg), **3** (23 mg) and **4** (50 mg) as reddish-orange powders.

Physico-chemical Properties

Ferrocins were obtained as reddish-orange powders. They showed positive color reactions to BARTON's

reagent[3] and potassium permanganate, and negative reactions to ninhydrin, Dragendorff and Sakaguchi reagents. They are soluble in water, dimethyl sulfoxide and methanol and sparingly soluble in hexane and ether.

The physico-chemical properties of **1**~**4** are summarized in Table 1. The characteristic UV absorptions at 422~423 nm (ε 2,700~3,300) suggested the presence of iron, which was confirmed by elemental analysis. The IR absorptions at 1750 and 1660 cm^{-1} are consistent with lactone and amide bonds. Amino acid analysis of hydrolysates obtained by treatment with 6 N hydrochloric acid gave the following data (Table 2): valine, alanine, serine and glycine were detected in molar ratios of 1:1:2:3 in **1**, **3** and **4** and 1:0:3:3 in **2**. The absolute configurations of valine, alanine and serine were determined to be D, L and L, respectively, by HPLC using a chiral mobile phase[4]. These data suggest that ferrocins are iron-chelating peptides like albomycins[5].

Structure Elucidation

The iron of ferrocins could be removed by treating with 8-hydroxyquinoline[6]. The ^1H NMR spectral data of the iron-free compounds (**5**~**8**) derived from ferrocins A~D are shown in Table 3. The ^1H-^1H correlation spectroscopy (COSY) experiment with **5** confirmed a valyl, an alanyl, two seryl and three glycyl

Table 1. Physico-chemical properties of ferrocins A (**1**), B (**2**), C (**3**) and D (**4**).

Property	A (1)			B (2)		C (3)		D (4)	
Appearance	Reddish orange powder			Reddish orange powder		Reddish orange powder		Reddish orange powder	
$[\alpha]_D$[a]	+170° (*c* 0.1)			+164° (*c* 0.1)		+187° (*c* 0.1)		+176° (*c* 0.1)	
SI-MS[b]: *m/z*	1,237 $(M+H)^+$			1,253 $(M+H)^+$		1,251 $(M+H)^+$		1,251 $(M+H)^+$	
Molecular formula	$C_{51}H_{82}N_{13}O_{19}Fe$ $(5H_2O)$			$C_{51}H_{82}N_{13}O_{20}Fe$ $(6H_2O)$		$C_{52}H_{84}N_{13}O_{19}Fe$ $(4H_2O)$		$C_{52}H_{84}N_{13}O_{19}Fe$ $(4H_2O)$	
Analysis (%)		Found	Calcd	Found	Calcd	Found	Calcd	Found	Calcd
	C:	45.86	46.15	45.07	45.00	47.29	47.20	47.10	47.20
	H:	6.67	6.99	6.88	6.96	7.12	7.01	7.07	7.01
	N:	13.68	13.72	13.47	13.38	13.88	13.76	13.80	13.76
	Fe:	5.0	4.21	3.0	4.10	4.3	4.22	4.2	4.22
UV: λ nm (ε)[a]	423 (3,300)			422 (2,700)		423 (3,200)		423 (2,900)	
IR: ν cm^{-1}, KBr	1750, 1660, 1530			1750, 1660, 1530		1750, 1660, 1540		1750, 1660, 1540	
HPLC[c]: Rt (minutes)	5.3			4.7		5.8		6.2	

[a] All data were obtained in water at 23~25°C.
[b] The secondary ion mass spectra (SI-MS) were measured with a Hitachi M-80A mass spectrometer with xenon ion beam source.
[c] Column: YMC-Pack A-312. Mobile phase: 36% CH_3CN. Flow rate: 2 ml/minute.

Table 2. Amino acid analysis of ferrocin hydrolysates.

Amino acid	HCl				HI			
	A	B	C	D	A	B	C	D
D-Val	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
L-Ala	1.0	—	0.9	0.9	1.0	—	1.1	1.2
L-Ser	2.1	2.9	2.1	2.1	1.9	2.7	2.0	2.1
Gly	3.1	2.8	2.8	2.9	3.0	2.9	2.9	3.0
Orn[a]	—	—	—	—	2.9	2.7	2.7	3.0

Condition of hydrolysis; 6 N HCl or 57% HI, 110°C, 16 hours.
[a] L:D=2:1.

Table 3. ^{1}H NMR spectral data of iron-free ferrocins (300 MHz, in D_2O).

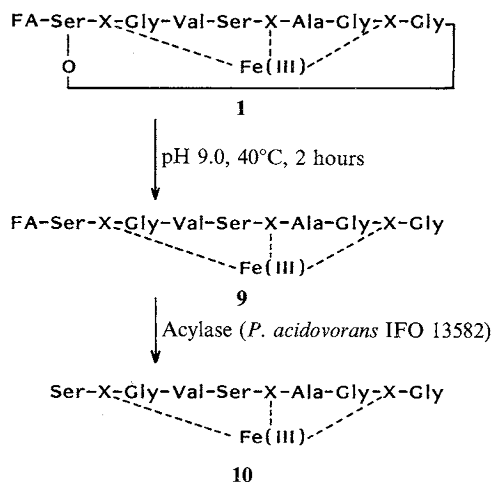
Assignment	5	6	7	8
DA-CH_3	0.84 (3H, t, 6.6)	0.84 (3H, t, 6.8)	0.84 (3H, t, 6.6)	0.84 (3H, t, 6.5)
Val-CH_3	0.94 (6H, d, 6.6)	0.92 (6H, d, 6.5)	0.93 (6H, d, 6.5)	0.93 (6H, d, 6.5)
PHO-CH_3	—	—	1.06 (3H, t, 7.5)	1.06 (3H, t, 7.5)
DA-CH_2	1.22~1.33 (6H, m)	1.19~1.29 (6H, m)	1.20~1.30 (6H, m)	1.20~1.31 (6H, m)
DA-CH_2	1.33~1.45 (2H, m)	1.29~1.41 (2H, m)	1.30~1.41 (2H, m)	1.31~1.44 (2H, m)
Ala-CH_3	1.38 (3H, d, 7.2)	—	1.38 (3H, d, 7.1)	1.38 (3H, d, 7.0)
AHO(PHO)-CH_2	1.55~1.97 (12H, m)	1.55~1.95 (12H, m)	1.58~1.95 (12H, m)	1.55~1.97 (12H, m)
Val-CH, DA-CH_2	2.00~2.15 (3H, m)	1.99~2.15 (3H, m)	2.00~2.15 (3H, m)	2.00~2.14 (3H, m)
AHO-CH_3	2.13 (9H, s)	2.13 (9H, s)	2.13 (6H, s)	2.13 (6H, s)
PHO-CH_2	—	—	2.50 (2H, q, 7.5)	2.50 (2H, q, 7.5)
DA-CH_2	3.15 (2H, d, 6.7)	3.15 (2H, m)	3.14 (2H, m)	3.14 (2H, m)
AHO(PHO)-CH_2	3.55~3.72 (6H, m)	3.55~3.71 (6H, m)	3.56~3.72 (6H, m)	3.55~3.73 (6H, m)
Ser-CH_2, Gly-CH_2	3.80~4.10 (8H, m)	3.78~4.10 (10H, m)	3.80~4.10 (8H, m)	3.80~4.09 (8H, m)
Val-CH	4.14 (1H, d, 7.8)	4.14 (1H, d, 7.0)	4.14 (1H, d, 7.0)	4.14 (1H, d, 7.0)
AHO(PHO)-CH, Ala-CH, Ser-CH, Ser-CH_2	4.23~4.50 (6H, m)	4.25~4.50 (6H, m)	4.25~4.50 (6H, m)	4.23~4.50 (6H, m)
Ser-CH_2	4.58 (1H, m)	4.60 (1H, m)	4.59 (1H, m)	4.59 (1H, m)
Ser-CH	4.74 (1H, m)	4.74 (1H, m)	4.73 (1H, m)	4.74 (1H, m)
DA-CH=	5.50 (1H, td, 6.7, 10.5)	5.50 (1H, td, 8.0, 11.0)	5.50 (1H, td, 8.0, 11.0)	5.50 (1H, td, 7.0, 10.5)
DA-CH=	5.72 (1H, td, 7.0, 10.5)	5.72 (1H, td, 8.0, 11.0)	5.72 (1H, td, 8.0, 11.0)	5.72 (1H, td, 7.0, 10.5)

DA: (*Z*)-3-Decenoic acid. AHO: N^5-acetyl-N^5-hydroxyornithine. PHO: N^5-propionyl-N^5-hydroxyornithine. The δ values of ^{1}H NMR spectra were recorded in ppm downfield from 3-(trimethylsilyl)propionic acid-d_4 sodium salt using a Bruker AC-300.

moieties which were proposed from amino acid analysis. The downfield shift of a methylene signal in one of two serine residues indicated the acylation of the hydroxyl group. Upon hydroiodic acid hydrolysis, three moles of ornithine were obtained in addition to the amino acids detected in hydrochloric acid hydrolysates (Table 2). The absolute configurations of three moles of ornithine were determined that two moles were L and one mole was D by HPLC. Ornithine was also observed in the case of albomycins[5] which contain N^5-acetyl-N^5-hydroxyornithine (N-Ac-N-OH-Orn) moiety. Nine acetyl protons at δ 2.13 ppm in D_2O and three exchangeable protons at δ 9.70 ppm in DMSO-d_6 confirmed the presence of three N-Ac-N-OH-Orn moieties. Furthermore, a 3-*Z*-decenoyl moiety was indicated by ^{1}H NMR.

Fig. 1. Degradation pathway of ferrocin A (**1**).

FA: (*Z*)-3-Decenoic acid. X: N^5-Acetyl-N^5-hydroxyornithine.



By comparing the ^{1}H NMR data, the partial structures of **6**~**8** were easily deduced. In **2**, the alanine moiety common to the other members of this complex is replaced by serine, and in **3** and **4**, one of the N-Ac-N-OH-Orn moieties is replaced by N^5-propionyl-N^5-hydroxyornithine (N-Pr-N-OH-Orn).

Table 4. Amino acid sequences of degradation products.

Compound	1		2		3		4		5		6		7		8		9		10
10	Ser	–	*	–	Gly	–	Val	–	Ser	–	*	–	Ala	–	Gly	–	*	–	Gly
11	Ser	–	*	–	Gly	–	Val	–	Ser	–	*	–	Ser	–	Gly	–	*	–	Gly
12	Ser	–	*	–	Gly	–	Val	–	Ser	–	*	–	Ala	–	Gly	–	*	–	Gly
13	Ser	–	*	–	Gly	–	Val	–	Ser	–	*	–	Ala	–	Gly	–	*	–	Gly

* Not detected.
The Edmann degradations were performed by a Protein Sequencer 477A (Applied Biosystem).

Fig. 2. NOESY spectrum of **5** (in DMSO-d_6).

FA: (*Z*)-3-Decenoic acid. X: N^5-Acetyl-N^5-hydroxyornithine. *: Lactone-forming serine.

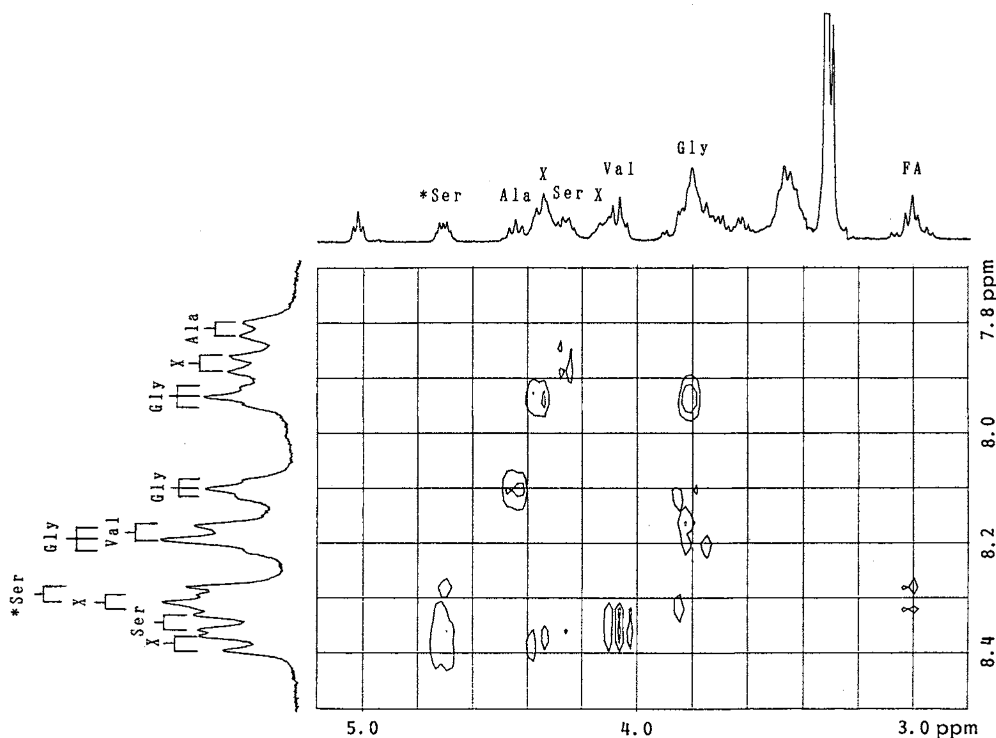
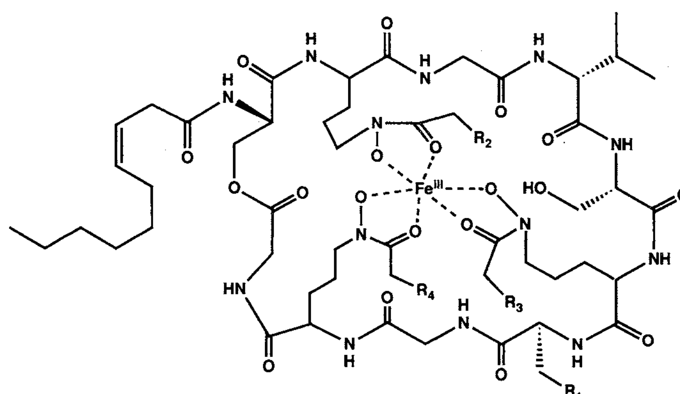


Fig. 1 shows the degradation pathway of **1**. On mild alkaline hydrolysis (pH 9.0), **1** gave the iron coordinated linear peptide (**9**). The cleavage of the lactone was deduced by the loss of absorption at 1750 cm^{-1} in the IR spectrum and the difference in the molecular ion peaks between **1** and **9** in SI-MS.

Upon incubation with cells of *Pseudomonas acidovorans* IFO 13582 having acylase activity[7], **9** afforded the deacylated compound **10**. By the same procedure, **2**, **3** and **4** also gave deacyl compounds **11**, **12** and **13**, respectively. The amino acid sequences of **10**～**13** were determined by the Edmann degradation method using a protein sequencer (Table 4). As the 2nd, 6th and 9th amino acids did not coincide with any common amino acids, there must be three moles of N-Ac-N-OH-Orn in **10** and **11** and two moles of N-Ac-N-OH-Orn and one mole of N-Pr-N-OH-Orn in **12** and **13**.

A nuclear Overhauser and exchange spectroscopy (NOESY) experiment was used to determine which serine residue was part of the lactone (Fig. 2). The ^{1}H NMR signals of **5** in DMSO-d_6 were assigned by

Fig. 3. Structures of ferrocins A (**1**), B (**2**), C (**3**) and D (**4**).



Ferrocin	R_1	R_2, R_3, R_4
A (**1**)	H	H×3
B (**2**)	OH	H×3
C (**3**)	H	H×2, CH_3×1
D (**4**)	H	H×2, CH_3×1

^1H-^1H COSY experiment. The 2-methine protons of two serines were observed at δ 4.70 and 4.26. As the signal at δ 4.70 coupled with a methylene at δ 4.09 and 4.36 and the signal at δ 4.26 coupled with a methylene at δ 3.66 in the COSY spectrum, the former signal was found to be derived from the acylated serine. A cross peak was found between an amide proton (δ 8.29) of the lactone serine and 2-methylene protons (δ 3.00) of the 3-decenoic acid moiety in the NOESY spectrum. Furthermore, a cross peak between an amide proton (δ 8.35) of the other serine and 2-methine proton (δ 4.08) of valine was detected. These findings suggested that the lactone ring was formed at the *N*-terminal serine. In addition, the sequence of **10** determined by the Edmann degradation method was clarified by the NOESY experiment. Therefore, the structures of **1**~**4** were finally deduced as shown in Fig. 3. However, it is not clarified which ornithine residue is D-form. Moreover, the position of the N-Pr-N-OH-Orn in **3** and **4** remains to be elucidated.

Ferrocins are active against Gram-negative bacteria *in vitro*. They show strong therapeutic effects selective against *P. aeruginosa* in experimentally infected mice at a dose of less than 1 mg/kg (ED_{50}) when administered subcutaneously. The iron-free ferrocin A (**5**) also showed therapeutic effect in mice at a similar dose. The antibacterial activity of the lactone-cleaved and deacylated compounds (**10**~**13**) was not tested. The preliminary acute toxicity (LD_{50}) of ferrocin A in mice upon intraperitoneal injection or oral aministration is more than 1,000 mg/kg[2].

Experimental

Assay of Ferrocins

Ferrocins were detected by a paper-disk method using *P. aeruginosa* C141[8] or NS[9] as the test organism and assayed by reverse-phase HPLC using a YMC-pack A-312 (Yamamura Chem. Lab.) with a mobile phase of 36% aqueous CH_3CN.

Iron-free Ferrocins (5~8)

A methanol solution of 8-hydroxyquinoline (2 ml, 20 mg/ml) was added to a solution of **1** (40 mg) in

H_2O (2 ml). The mixture was allowed to stand at 4°C for 15 hours. The black precipitate was removed by filtration, and the filtrate was concentrated. The concentrate was diluted with H_2O (20 ml) and washed with $CHCl_3$ (10 ml × 4). The aqueous layer was concentrated and lyophilized to yield a white powder of **5** (29 mg).

By a similar method, **2** (20 mg), **3** (10 mg) and **4** (20 mg) gave **6** (20 mg), **7** (8 mg) and **8** (19 mg), respectively.

5: SI-MS *m/z* 1,184 $(M+H)^+$.
Anal Calcd for $C_{51}H_{85}N_{13}O_{19} \cdot 3H_2O$: C 49.47, H 7.41, N 14.70.
Found: C 49.33, H 7.45, N 14.78.

6: SI-MS *m/z* 1,200 $(M+H)^+$.
Anal Calcd for $C_{51}H_{85}N_{13}O_{20} \cdot \frac{5}{2}H_2O$: C 49.19, H 7.28, N 14.62.
Found: C 49.03, H 7.24, N 14.59.

7: SI-MS *m/z* 1,198 $(M+H)^+$.
Anal Calcd for $C_{52}H_{87}N_{13}O_{19} \cdot \frac{7}{2}H_2O$: C 49.51, H 7.35, N 14.38.
Found: C 49.36, H 7.21, N 14.26.

8: SI-MS *m/z* 1,198 $(M+H)^+$.
Anal Calcd for $C_{52}H_{87}N_{13}O_{19} \cdot 3H_2O$: C 49.87, H 7.48, N 14.54.
Found: C 49.71, H 7.22, N 14.41.

Deacyl Ferrocins (**10**~**13**)

A solution of **1** (150 mg) in 0.05 M phosphate buffer (pH 9, 150 ml) was stirred at 40°C for 2 hours. The reaction mixture was chromatographed on a column of Diaion HP-20 (20 ml) eluting with 50% MeOH. The eluate was concentrated and freeze-dried to afford **9** as the sodium salt (136 mg).
SI-MS *m/z* 1,277 $(M+H)^+$; $C_{51}H_{83}N_{13}O_{20}FeNa$.

To a solution of **9** (150 mg) in 0.05 M phosphate buffer (pH 7, 150 ml) the cells of *Pseudomonas acidovorans* IFO 13582 (1.5 g) (recently this strain was reclassified as *Comamonas acidovorans*[10]) were added. The mixture was shaken at 37°C for 18 hours. The reaction mixture was centrifuged, and the supernatant was adjusted to pH 2.5. The solution was washed with EtOAc, and the aqueous layer was chromatographed on a column of Diaion HP-20 (50~100 mesh, 30 ml) eluting with 5~20% MeOH. The eluate was concentrated and lyophilized to give **10** (97 mg).

By a similar method, **2** (10 mg), **3** (2 mg) and **4** (10 mg) yielded **11** (5 mg), **12** (1.5 mg) and **13** (5.7 mg), respectively. SI-MS *m/z* $(M+H)^+$ **10**; 1,103, **11**; 1,119, **12**; 1,117, **13**; 1,117.

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