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Isolation and Structural Characterization of Siderophores, Madurastatins, Produced by a Pathogenic *Actinomadura madurae*

KEN-ICHI HARADA[a,b,*], KOJI TOMITA[a], KIYONAGA FUJII[a], KATSUYOSHI MASUDA[c], YUZURU MIKAMI[d], KATSUKIYO YAZAWA[d] and HISAYUKI KOMAKI[e]

[a] Faculty of Pharmacy, Meijo University, Tempaku, Nagoya 468-8503, Japan
[b] Graduate School of Environmental and Human Sciences, Meijo University, Tempaku, Nagoya 468-8503, Japan
[c] Suntory Institute for Bioorganic Research, 1-1-1 Wakayamadai, Shimamoto-chou, Mishimagun, Osaka 618-8503, Japan
[d] Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, 1-8-2 Inohana, Chuo-ku, Chiba 260-8673, Japan
[e] R & D Department, Higeta Shoyu Co., Ltd., Chuo-cho, Choshi, Chiba 288-8680, Japan

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Madurastatins A1 (**1**), A2 (**2**) and A3 (**3**), novel pentapeptides that were acylated with salicylic acid at the *N*-terminus, were isolated from the culture broth of a pathogenic *Actinomadura madurae* IFM 0745 strain. These structures were mainly determined by 2D NMR and MS/MS spectral techniques. The strain produced simultaneously madurastatins B1 (**4**) and B2 (**5**) consisting of Ser and salicylic acid moieties. Compounds **1** and **4** had an antibacterial activity against *Micrococcus luteus*, indicating that the presence of the aziridine ring is essential for such activity. Because **1** has a strong affinity with ferric ion due to the presence of two hydroxamic acids and a salicylic acid, it is considered to be a siderophore that is a low molecular weight iron chelater. The production of siderophores may be one of the characteristics of pathogenic microorganisms.

Pathogenic actinomycetes are distributed on a limited basis in the natural environment and it is difficult to isolate them. Therefore, they can be recognized as one of rare actinomycetes and recognized as an additional unexplored source of new bioactive compounds. We have collected many pathogenic actinomycetes from clinical materials, which belong mainly to the genera of *Nocardia*. They produce several biologically active compounds such as antitumor and immunosuppressive ones[1~14]. In 1997, we also isolated three actinomycetes from clinical materials and they were identified as *Actinomadura madurae*. The culture broths of these strains showed microbial activity against Gram-positive bacteria such as *Micrococcus luteus*. In this report, we describe the isolation and structure determination of new peptides (designated as madurastatins, Fig. 1) produced by one of the three *A. madurae*, the IFM 0745 strain, which shows an interesting siderophoric activity.

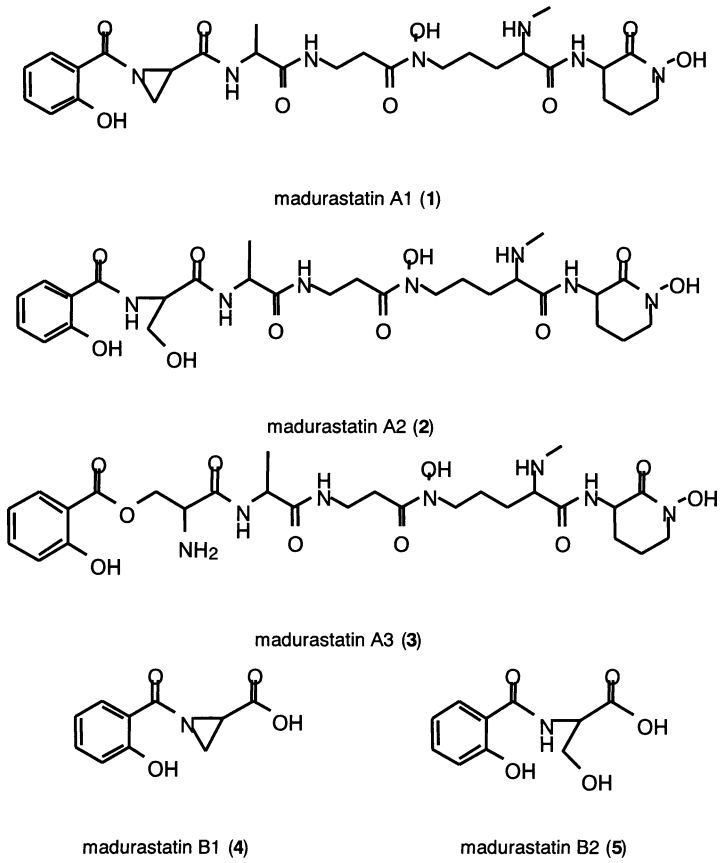
Results

Taxonomy and Fermentation

An actinomycete IFM 0745 strain was isolated from the sputum of a patient at the Chiba University Hospital on September 5, 1997. This strain did not contain mycolic acid that is characteristic of *Nocardia* sp. but contained madurose as a taxonomically important cell wall sugar component, and MK-9 (H6) was a major menaquinone component[15], indicating that this strain belongs to a species of *Actinomadura*. Our further phenotypic and phylogenetic characterization studies showed that *Actinomadura* IFM

* Corresponding author: kiharada@ccmfs.meijo-u.ac.jp

Fig. 1. Structures of madurastatins.



0745 should belong to a strain of *A. madurae*. The strain *A. madurae* IFM 0745 grows well in BHI (brain heart infusion) medium containing 2% glucose at 32°C for 7 days. The cultured broth was inoculated in nutrient broth (2 times concentrated) containing each 1% glucose and glycerol at a 1% concentration and the culturing continued for 6 days.

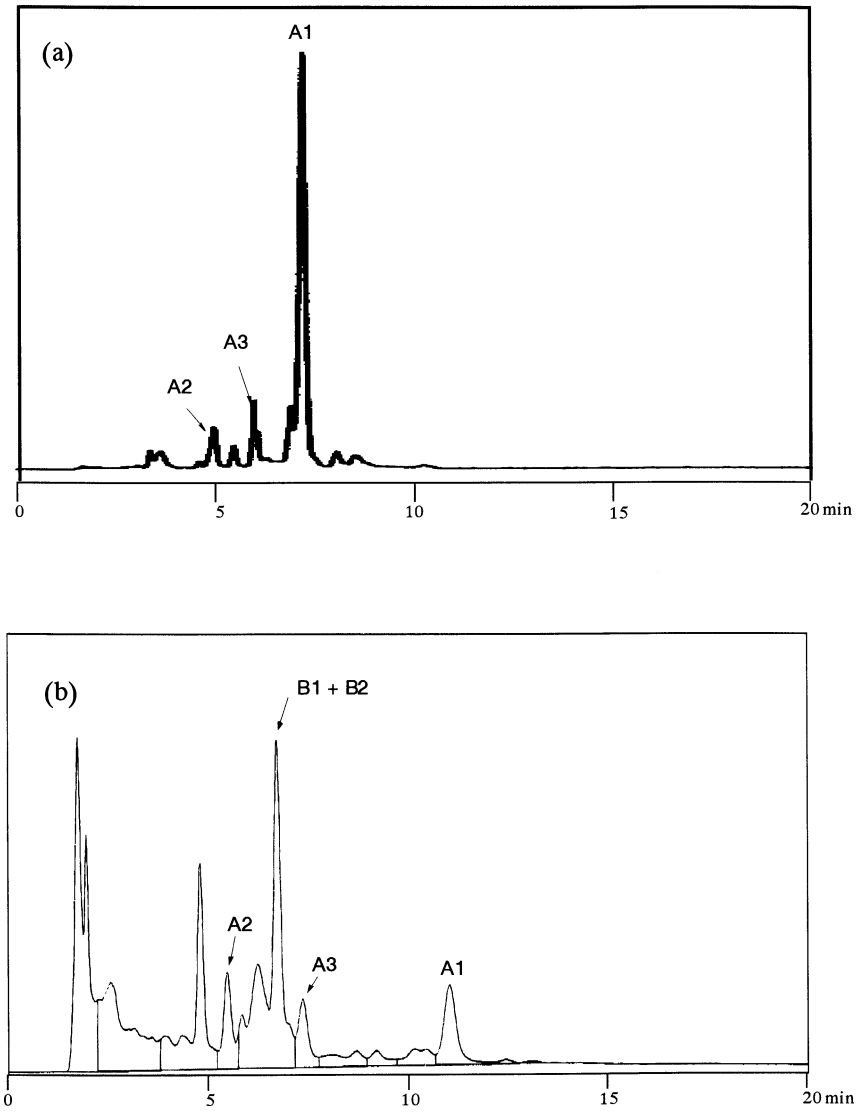
Isolation of Madurastatins

Because the cultured broth of *A. madurae* IFM 0745 exhibited activity against *M. luteus*, the separation of the active compounds from this strain was performed under the guidance of this microbiological activity. The active and related compounds were retained on HP-20 and were eluted with 50% methanol followed by 100% methanol. The HPLC chromatograms of the 100% methanol and 50% methanol fractions are shown in Fig. 2(a) and (b), respectively. The 100% methanol fraction contained mainly three compounds (madurastatins (Fig. 1)), of which the main peak (A1 (**1**)) at 7 minutes exhibited activity against *M. luteus*. Two minor components (A2 (**2**) and A3 (**3**)) were eluted prior to **1** (Fig. 2(a)) and did not show any activity. The preparative HPLC of the 100% methanol fractions gave 60.5 mg of **1**, 3.0 mg of **2** and 6.5 mg of **3**. The 50% methanol fraction contained several compounds in addition to the madurastatins mentioned above (Fig. 2(b)). The peak at 7 minutes was composed of two components (madurastatins B1 (**4**) and B2 (**5**)) and **4** exhibited activity against *M. luteus*. These compounds were isolated by a combination of silica gel column chromatography and preparative HPLC to give 15.2 mg of **4** and 8.2 mg of **5**.

Structures of Madurastatins

The physicochemical properties of the madurastatins are summarized in Table 1. These compounds seemed to contain hydroxamic acid or phenolic moiety because they produced a purple color with $FeCl_3$ on a thin layer plate. It was found that **1** and **4** have the same chromophore owing

Fig. 2. HPLC chromatogram of (a) the methanol and (b) the 50% methanol fraction.



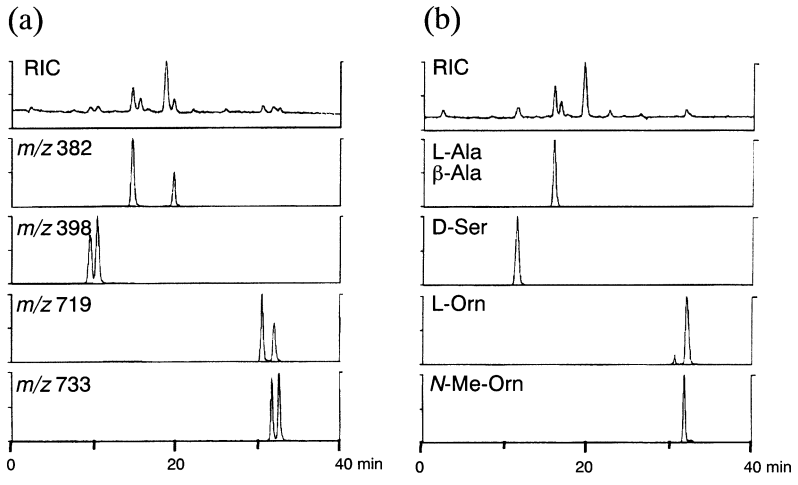
(a) HPLC conditions: column; Cosmosil 5C18-AR (150×4.6 mm i.d., Nacalai Tesque), mobile phase; methanol : 0.1% TFA=30 : 70, flow rate; 1.0 ml/minute, detection; 254 nm and (b) HPLC conditions: column; Cosmosil 5C18-AR (150×4.6 mm i.d., Nacalai Tesque), mobile phase; methanol : 0.1% TFA=25 : 75, flow rate; 1.0 ml/minute, detection; 254 nm.

to the identical UV absorption spectra. In order to determine the constituent amino acids of the components of the A series, acidic hydrolysis followed by the analysis of the resulting hydrolysate using advanced Marfey's method[16,17] were performed. Fig. 3 shows the mass chromatograms monitored at the *m/z* values of the deprotonated molecules of (a) the D,L- and (b) the L-DLA (2,4-dinitrophenyl-5-L-leucinamide) derivatives of each constituent amino acid in the hydrolysate of **1**. There are four peaks of the constituent amino acids in the Fig. 3(b), in which the ion peak at *m/z* 382 corresponds to Ala and *β*-Ala and the peaks for Orn, *N*-Me-Orn are detected at *m/z* 719 and 733, respectively, which were reductively derived from *N*-hydroxy-Orn and *N'*-Me-*N*-hydroxy-Orn under acid hydrolysis. The peak of the remaining constituent amino acid can be detected at *m/z* 398 that corresponds to the derivative of Ser and its absolute stereochemistry can be determined to be D by comparison of the authentic sample. The same experiments for **4** and **5** indicated that they include only D-Ser as the amino acid moiety.

Table 1. Physicochemical properties of madurastatins.

	madurastatin			madurastatin	
	A1 (**1**)	A2 (**2**)	A3 (**3**)	B1 (**4**)	B2 (**5**)
FABMS (*m/z*)	606 $[M+H]^+$	624 $[M+H]^+$	624 $[M+H]^+$	208 $[M+H]^+$	226 $[M+H]^+$
Molecular formula	$C_{27}H_{39}N_7O_9$	$C_{27}H_{41}N_7O_{10}$	$C_{27}H_{41}N_7O_{10}$	$C_{10}H_9NO_4$	$C_{10}H_{11}NO_5$
HR-FABMS (*m/z*)					
Obsd. ($[M+H]^+$)	606.2859	624.3015	624.3001	208.0491	226.0663
Calcd.	606.2831	624.2997	624.2997	208.0499	226.0652
$[\alpha]_D^{27}$ (*c* 0.1, MeOH)	-4.1	-13.2	-10.5	-5.6	-8.3
UV λ_{max} nm	254, 323	236, 297	237, 306	254, 323	236, 297
antibacterial activity (*M. luteus*)	○	×	×	○	×

Fig. 3. Mass chromatograms monitored at the *m/z* values of the deprotonated molecules of (a) the D,L- and (b) the L-DLA (2,4-dinitrophenyl-5-L-leucinamide) derivatives of each constituent amino acid in the hydrolysate of madurastatins A1 (**1**).

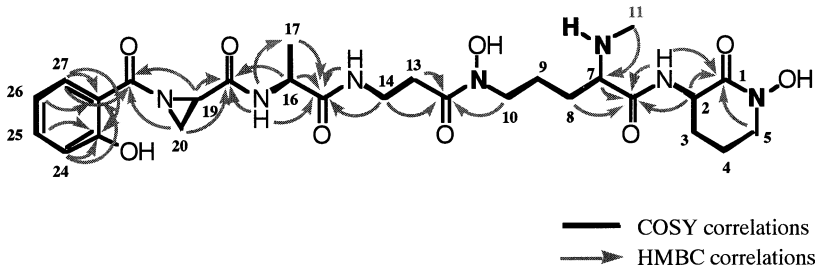


The molecular formula of **1** was determined as $C_{27}H_{39}N_7O_9$ by HRFAB-MS, which gave the $(M+H)^+$ ion at *m/z* 606 in the positive mode and the $(M-H)^-$ ion at *m/z* 604 in the negative mode. In the ^{1}H-NMR spectrum, **1** showed a doublet signal at 1.21 ppm due to the methyl group and a singlet signal at 2.19 ppm due to the *N*-methyl group. Four aromatic protons were assigned as shown in Table 2, indicating the presence of the *ortho* disubstituted benzene. A few α-protons from the amino acid moieties were also observed. In the ^{13}C-NMR spectrum, a methyl

Table 2. ^{1}H- and ^{13}C-NMR spectral data for madurastatin A1 (**1**).

position	δ_H, J (Hz)	δ_C	position	δ_H, J (Hz)	δ_C
1		164.9	16	4.27 q (7.2)	48.3
2	4.30 m	49.4	17	1.21 d (6.8)	18.4
3	1.66 m	27.7	18		169.2
	1.88 m		19	4.98 dd (10.2, 7.8)	67.2
4	1.90 m	20.3	20	4.47 t (8.0)	69.4
5	3.51 m	51.2		4.60 dd (10.4, 8.4)	
6		173.6	21		165.9
7	2.83 t (6.6)	63.7	22		110.0
8	1.45 m	30.2	23		159.0
9	1.55 m	22.8	24	6.98 dt (8.2, 0.6)	116.5
10	3.50 m	47.0	25	7.44 dt (7.8, 1.6)	134.0
11	2.19 s	34.2	26	6.96 dt (7.4, 1.0)	119.0
12		170.9	27	7.62 dd (7.6, 1.6)	128.0
13	2.51 t (4.8)	31.9	NH	8.03 d (5.6)	
14	3.25 m	34.7	NH	7.91 t (8.0)	
15		171.6	NH	8.34 d (7.6)	

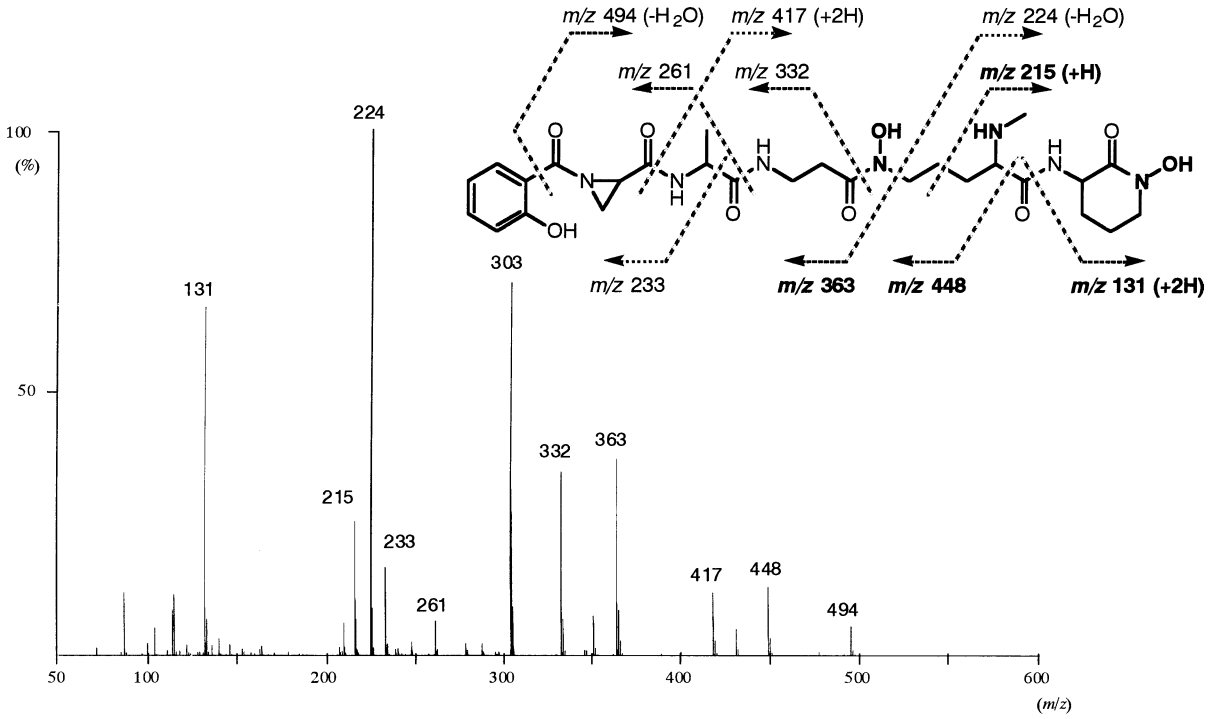
Fig. 4. COSY and HMBC correlations of madurastatins A1 (**1**).



carbon and an *N*-methyl carbon appeared at 18.4 and 34.2 ppm, respectively, and aromatic carbons, α-carbons of the amino acid moieties and carbonyl carbons also were observed (Table 2). The C-H connectivity and assignment of the signals were confirmed by 2D-NMR experiments using COSY and HMBC techniques (Fig. 4). The long range couplings from the α-proton (4.98 ppm) and the β-methine proton (4.47 and 4.60 ppm) to the two carbonyl carbons (165.9 and 169.2 ppm) confirmed the presence of an aziridine ring (abbreviated as Azi) of the *N*-terminus, and from the δ-methylene proton (3.51 ppm) to the carbonyl carbon (164.9 ppm) confirmed that a lactam is formed at Orn of the *C*-terminus (Fig. 4 and Table 2). Consequently, **1** was a pentapeptide acylated with salicylic acid at the *N*-terminus.

There are three hydroxyl groups in this molecule based on its molecular formula and the results from the NMR experiment. Because they were not observed in this NMR experiment, the MS/MS technique using Q-TOF (time of flight) was applied. The doubly charged protonated molecule $(M+2H)^{++}$ at *m/z* 303 was selected as the precursor ion and the obtained product ion spectrum is shown in Fig. 5. These product ions can be assigned as shown in Fig. 5 and this experiment can reject completely two of the three candidates. This conclusion was also supported by an experiment of the corresponding acetylated derivatives by FABMS (data not shown). Consequently, **1** has one phenolic hydroxyl and two hydroxamic acid moieties, in two of which the hydroxyl groups are located at the δ-amino groups of Orn (Fig. 1).

Fig. 5. Product ion spectrum of the doubly charged protonated molecule $(M+2H)^+$ at *m/z* 303 of madurastatin A1 (**1**) and assignment of the resulting product ions.



The molecular formulae of **2** and **3** were determined as $C_{27}H_{41}N_7O_{10}$ by HRFAB-MS, which were 18 mass units larger than that of **1**, suggesting that they are compounds with the addition of one mole of water to **1**. In the ^{1}H-NMR spectrum of **2**, the three signals at 3.74, 4.52 and 5.10 ppm appeared, whereas the signals of the β-protons (4.47 and 4.60 ppm) for Azi in **1** disappeared. In the ^{13}C-NMR spectrum of **2**, the signals at 67.2 and 69.5 ppm for Azi in **1** were upfield shifted to 55.6 and 61.5 ppm, respectively. The signal of a methine proton observed at 4.98 ppm in **1** was upfield shifted to 4.36 ppm in the ^{1}H-NMR spectrum of **3**. In the ^{13}C-NMR spectrum of **3**, the signals at 67.2 and 69.5 ppm for Azi in **1** were upfield shifted to 51.3 and 63.0 ppm, respectively. These results indicated that the structural difference between **1** from **2** and **3** is derived from the *N*-terminus moiety. The final conclusive evidence was obtained for the structural relationship among the three components from the following 2D experiments: the COSY correlations between the β-proton of Ser (3.74 ppm) and the hydroxyl proton (5.10 ppm), and the long range couplings from the α-proton of Ser (4.52 ppm) and the amino proton (8.84 ppm) to the two carbonyl carbons (167.0 and 169.4 ppm) confirmed the presence of a peptide bond at Ser on the *N*-terminus in **2** (Fig. 1). In **3**, the long-range coupling from the β-protons of Ser (4.58 and 4.73 ppm) to the two carbonyl carbons (165.1 and 167.6 ppm) confirmed the presence of an ester bond at Ser on the *N*-terminus (Fig. 1).

As shown in Table 1, although the molecular weights of **4** and **5** were much smaller than those of the madurastatin A series, the UV spectral behavior was quite similar to that of **1** and **2**, respectively. The observed signal pattern including the aromatic and α and β protons of the Ser moiety in the ^{1}H-NMR spectra of **4** and **5** was similar to that of the *N*-terminus of **1** and **2**, respectively. Therefore, **4** has an aziridine ring on the *N*-terminus as shown in Fig. 1 and **5** is D-Ser combined with salicylic acid at the *N*-terminus.

Complex Formation of **1** with Ferric Ion

As shown above, the madurastains produced a purple color with $FeCl_3$ on a thin layer plate, suggesting that they form a complex with ferric ion. The reaction of **1** with a ferric chloride aqueous solution gave a complex whose FAB mass spectrum is shown in Fig. 6. The protonated

Fig. 6. FAB mass spectrum of a complex of madurastatin A1 (**1**) and ferric ion.

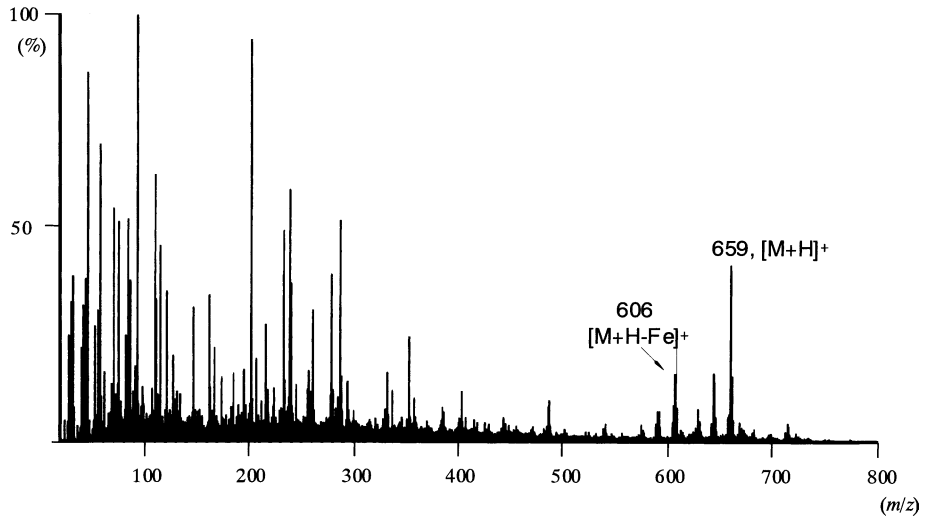
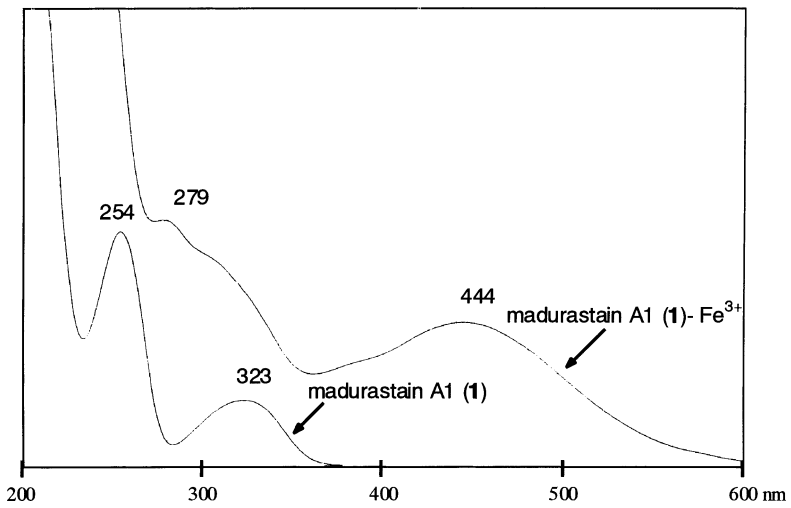


Fig. 7. UV spectra of madurastatin A1 (**1**) and its complex with ferric ion.



molecule of the complex was observed at *m/z* 659 together with the protonated molecule of **1** corresponding to the deferric ion of the complex in the spectrum. The formation of the complex was also supported by the UV spectrum shown in Fig. 7, in which the characteristic absorption maxima at 254 and 323 nm in **1** were changed to those at 279 and 444 nm. These experiments suggested that **1** formed a complex with ferric ion using a phenolic and two hydroxamic acid moieties in the molecule. The resulting complex did not show any microbial activity against *M. luteus*.

Discussion

During continuing studies on biologically active compounds from pathogenic actinomycetes, we isolated several peptides called madurastatins from *Actinomadura madurae* IFM 0745 isolated from the sputum of a patient at the Chiba University Hospital in the present study. The

main component **1** is a pentapeptide acylated with salicylic acid at the *N*-terminus and has an aziridine ring, and a phenolic and two hydroxamic acid moieties. The absolute configurations of four of the constituent amino acids can be determined by the advanced Marfey's method[16,17], of which the aziridine-containing amino acid was detected as D-Ser. However, it was hard to apply the method to the case of *N*-Me Orn because its separation mechanism has not yet been elucidated. This is due to the different predominant conformation of the DLA derivatives of usual amino acid[16] and *N*-Me amino acid. Although **1** is unstable to some extent probably due to the presence of the aziridine ring in the molecule, it is interesting that **1** and **4** have an antibacterial activity. This indicates that the presence of the aziridine ring is essential for this activity.

The main component **1** shows another characteristic behavior that has a strong affinity with ferric ion, indicating that it is an ion chelater, a so-called siderophore[18]. The siderophores produced by microorganisms are structurally classified into four groups, hydroxamate, catecholate, salicylate and carboxylate. Bacteria produce the four types of siderophores and fungi produce the hydroxamate. The siderophores produced by actinomycetes has both hydroxamate and salicylate moieties[18]. *Actinomadura* sp. produced only one siderophore, maduraferrin[19] whose structure is similar to that of **1**. Similar siderophores such as formobactin[20], nocobactin NA[21], mycobactin[22] and exochelin[23] were also produced by pathogenic *Nocardia* and *Mycobacterium*.

Iron is an essential element for all living cells. It is difficult to take up iron into cells due to its poor solubility. Therefore, microorganisms had to evolve methods of solubilization and uptake of mineral iron. For the iron supply of living cells, some microorganisms produce an iron chelater, siderophore, outside the cells and the siderophore is chelated with ferric ion. The complex is recognized by a specific receptor in the outer cell membrane and transported into the periplasm. The complex is then stored and ferric ions are utilized after the decomposition and reduction of the complex as the need arises[18].

The ability of the pathogenic microorganisms to grow in its animal and human hosts, especially in iron-limited extracellular spaces, such as respiratory cavities, is dependent on its ability to scavenge iron. Like many other pathogenic microorganisms, pathogenic *Nocardia* species synthesize and secrete iron chelators known as siderophores. Therefore, siderophores have been considered to play an important role as virulence factors in the iron-limiting environments of their vertebrate hosts in order to compete for ferric iron. Very recently, a siderophore, asterobactin, was also isolated from the culture broth of a pathogenic *Nocardia asteroides*[24]. *Actinomadura* strains have been used as the sources of antibiotic production, but among the *Actinomadura* species, two species *A. madurae* and *A. pelletieri* have been reported as pathogenic species. Therefore, madurastatin was also considered to be a virulent factor because this strain was isolated from a Japanese patient with bronchitis.

We have demonstrated that some antibiotics such as rifampicin, fusidic acid and macrolide antibiotics become inactive by pathogenic *Nocarida* through the modification of the structures by phosphorylation, glycosylation or oxidation[25~30]. In this experiment, it was found that the pathogenic *Actinomadura* produced a siderophore. Based on the results accumulated by many researchers including us, the inactivation of antibiotics and the production of siderophores seem to be closely related to the pathogenicity of a microorganism.

Materials and Method

Instrumentations

NMR experiments were performed using JEOL-A400 and -A600 spectrometers in DMSO-d_6 or CD_3OD at 40°C. The MS spectra were measured on a JEOL-HX110 spectrometer for FABMS and Finnigan MAT TSQ700 for ESI LC/MS. High resolution MS spectra were obtained using a JEOL-HX110 under FAB conditions. The MS/MS spectra were measured with a Micromass Q-TOF mass spectrometer. UV spectra were recorded with a Shimadzu UV-2100. Optical rotations were measured with a JASCO DIP-370 polarimeter. HPLC was carried out using the following two systems: pump; Shimadzu LC-9A, detector, Shimadzu SPD-10A (UV-VIS) or Shimadzu SPD-M10A (diode array) and pump; TOSOH CCPS, detector; TOSOH UV-8020.

Microorganism, Taxonomy and Fermentation

A. madurae IFM 0745 isolated from a Japanese patient with bronchitis in Japan was used. Whole cell hydrolysates were analyzed for diaminopimelic acid (DAP) isomers and sugars using thin layer chromatography (TLC). Mycolic acid and menaquinones were prepared and analyzed[31]. Physiological characterisitics including the decomposition and acid production profiles of various carbohydrates, growth temperature, and other phenotypic characteristics were determined by the methods of TRUJILLO and GOODFELLOW, and GORDON *et al.*[15,32]. The seed broth was

prepared by inoculating mycelial elements of the producing strain (*A. madurae* IFM 0745) grown on a Mueller Hinton II agar slant (Difco, Detroit) in 10 ml of Brain Heart Infusion broth (BHI, Difco) with 2% glucose in a 50-ml Erlenmeyer shake flask. The culture was inoculated on a rotary shaker at 259 rpm for 6 days. One percent inoculum was transferred to a 500-ml Erlenmeyer flask containing 150-ml of the production medium composed of 2 times concentrated Nutrient broth (Difco) with 1% glucose. The culture was incubated on a rotary shaker at 250 rpm for 6 days.

Isolation

After centrifugation of the culture broth, the supernatant was subjected to an HP-20 column. After washing the column with water, the desired products were eluted with 50% methanol (15 liters) followed by 100% methanol (15 liters) to give the fractions of 12.5 g and 15.5 g, respectively. The 100% methanol fraction was separated by the following preparative HPLC to give **1** (256 mg), **2** (13.8 mg) and **3** (23.2 mg): column; Cosmosil 5C18-ARII (250×10 mm i.d., Nacalai Tesque), mobile phase; methanol : 0.1% TFA=25 : 75, flow rate; 4.0 ml/minute, detection; 254 nm. The 50% methanol fraction was applied to a silica gel column (680×4.5 mm i.d.) chromatography using ethyl acetate : 2-propanol : water (4 : 3 : 7, upper phase) as the mobile phase to give **4** (82.1 mg) and **5** (18.1 mg) fractions. The following preparative HPLC of these fractions gave **4** (20.3 mg) and **5** (10.4 mg): column; Cosmosil 5C18-ARII (250×10 mm i.d., Nacalai Tesque), mobile phase; methanol : 0.1% TFA=25 : 75, flow rate; 3.0 ml/minute, detection; 254 nm. These five fractions were neutralized with 7% ammonium hydroxide solution and then subjected to ODS cartridges (Waters, Sep-pac, 500 mg). After washing these cartridges with water (10 ml), the desired components were eluted with 50% methanol (10 ml) to give purified **1** (60.5 mg), **2** (3.0 mg), **3** (6.5 mg), **4** (15.2 mg) and **5** (8.2 mg).

Advanced Marfey's Method

One mg of the madurastains was dissolved in 400 μl of 6 M HCl, and heated at 110°C for 24 hours under nitrogen. The reaction solution was evaporated to dryness and 50 μl of water, 40 μl of 1 M $NaHCO_3$ and 100 μl of 1% FDLA (1-fluoro-2,4-dinitrophenyl-leucinamide) acetone solution were added to the residue. The solution was heated at 40°C for 1 hour and the reaction was quenched by the addition of 20 μl of 1 M HCl. After dilution with 220 μl of acetonitrile, 0.1 and 1 μl of the DLA derivatives of all the samples were analyzed by the following HPLC and ESILC/MS, respectively: column; TSK gel ODS-80Ts (150×4.6 mm i.d., TOSOH), mobile phase; acetonitril : 0.1% TFA, gradient condition; acetonitrile 30→70%, flow rate; 1.0 ml/minute, detection; 340 nm, column; Develosil ODS-HG5 (150×2 mm i.d., TOSOH), mobile phase; acetonitril : 0.1% TFA, gradient condition; acetonitrile 30→70%, flow rate; 0.2 ml/minute, split ratio; 1 : 40, detection; MS (negative ion mode).

MS/MS Experiment

ESI (electrospray ionization)-MS/MS spectra were acquired using a Q (quadrupole)-TOF (time-of-flight) instrument and Mass Lynx data acquisition. This instrument was a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer, with a Z-spray nanoflow electrospray ion source. The mass spectrometer was operated in the positive-ion mode. Purified samples were dissolved in 50% aqueous methanol solution containing 0.2% formic acid and loaded into a nanoflow tip. A high voltage (1.0 kV) was applied to the nanoflow tip of the capillary. The collision energy was increased to an optimum value of 25～35 V using an argon collision gas.

Preparation of Complex of **1** with Ferric Ion

Compound **1** (5.0 mg) was dissolved in 10% $FeCl_3$ (aq.), and was neutralized with 7% NH_3 (aq.). The solution was evaporated to dryness and the residue was purified by the following preparative HPLC to gave a complex of **1** (3.8 mg): column; Cosmosil 5C18-ARII (4.6×150 mm i.d., Nacalai Tesque), mobile phase; methanol : 0.01% TFA=20 : 80, flow rate; 1.0 ml/minute, detection; 450 nm.

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