Micacocidin A, B and C, Novel Antimycoplasma Agents from *Pseudomonas* sp.

II. Structure Elucidation

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Metal-containing novel heterocyclic antibiotics, micacocidin A (**1**), B (**2**), and C (**3**) have been isolated from the culture filtrate of *Pseudomonas* sp. No. 57-250. The structure and absolute configuration of micacocidin A, an octahedral Zn^{2+} complex, was determined by X-ray crystallographic analysis. And then, the structures of the two congeners, micacocidin B (Cu^{2+} complex) and C (Fe^{3+} complex) were investigated by employing one dimensional and two dimensional homonuclear and heteronuclear NMR spectroscopies and mass spectrometry.

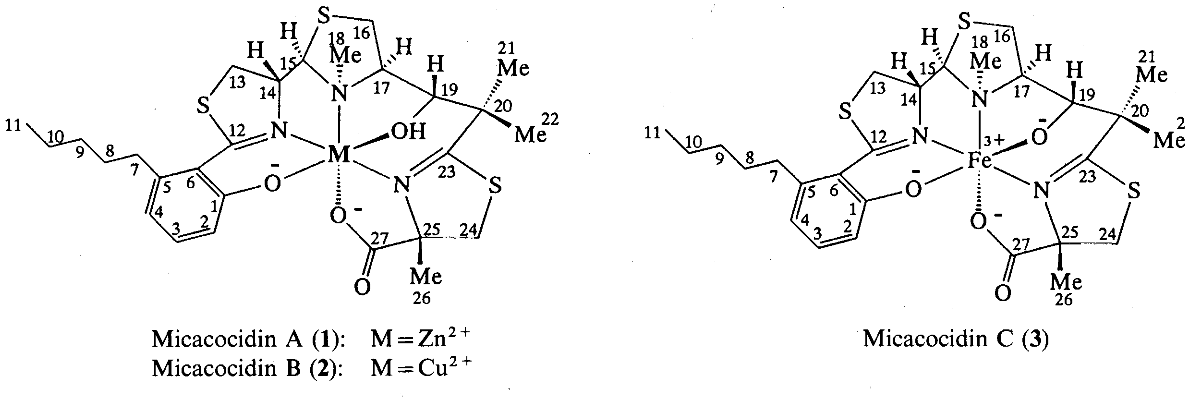
In the accompanied paper[1)], the taxonomy, fermentation, isolation, physico-chemical properties, and biological activities of micacocidin A, B, and C were reported. Herein, we describe the account of the structure determination of micacocidin A (**1**) and two congeners (**2** and **3**), metal chelated antibiotics having specific and excellent activity against *Mycoplasma* species (Fig. 1).

Materials and Methods

Spectroscopic Studies

Liquid secondary ion mass spectra (LSI-MS) were obtained by using a Hitachi M-90 mass spectrometer (BEE geometry) equipped with cesium ion gun. Samples were dispersed in 3-nitrobenzyl alcohol and introduced into the mass spectrometer on a LSI-MS target. One dimensional (1D) and two dimensional (2D) NMR were recorded with Varian UNITY-600 spectrometer at 20°C,

Fig. 1. Structure of micacocidin A (1), B (2) and C (3).



Micacocidin A (**1**): M = Zn^{2+}
Micacocidin B (**2**): M = Cu^{2+}

Micacocidin C (**3**)

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in $CDCl_3$ solution using tetramethylsilane as an internal standard. X-ray diffraction data were collected on a Rigaku AFC-5R diffractometer. All crystallographic calculations were performed on a VAX3100 computer using the Texsan crystallographic software package of Molecular Structure Corporation.

Results and Discussion

Spectroscopic Properties and Structures

High resolution LSI-MS was utilized to determine the molecular formulas of **1** and its congeners (**2**, **3**) and confirm the structures of them. X-ray crystallographic analysis was utilized to determine the molecular structure and formula of **1**. Finally, 1D and 2D homonuclear and heteronuclear NMR spectroscopy was used to confirm the structure of **1**.

The LSI-MS spectrum of **1** is shown in Fig. 2. The most abundant protonated molecular ion ($[M+H]^+$) was observed at *m/z* 628 and the sodiated molecular ion ($[M+Na]^+$) was observed at *m/z* 650 by addition of sodium iodide, accordingly the nominal molecular weight of **1** was determined 627. The complicated pattern of $[M+Na]^+$ ion which was characteristic of zinc atom indicated the presence of one zinc atom. In fact, the accurate masses of 628.1320 and 650.1133 yielded from the high resolution LSI-MS for the $[M+H]^+$ and $[M+Na]^+$ ions matched the elemental formulas $C_{27}H_{38}N_3O_4S_3{}^{64}Zn$ (calcd 628.1314) and $C_{27}H_{37}N_3O_4S_3{}^{64}ZnNa$ (calcd 650.1133), respectively. Therefore, the molecular formula was established $C_{27}H_{37}N_3O_4S_3Zn$ which was also supported by a single crystal X-ray analysis.

Colorless rhombic plate crystals of **1** were grown from MeOH / AcOEt solution. A single crystal with dimensions $0.05 \times 0.15 \times 0.40\ mm^3$ was used for data collection. Diffraction measurements were carried out using graphite-monochromated Mo K_α radiation ($\lambda = 0.7107$ Å). The crystal data are as follows: triclinic, space group $P1$, $a = 12.118(2)$ Å, $b = 12.651(3)$ Å, $c = 10.811(4)$ Å, $\alpha = 115.27(2)°$, $\beta = 93.32(2)°$, $\gamma = 86.45(2)°$, $V = 1495.1(7)$ Å^3, $Z = 2$. A total of 11132 reflections, include friedel pairs, in the range $2\theta < 51°$ were measured and corrected for Lorentz and polarization factors, but not for adsorption effects.

The structure was solved by heavy-atom method, and finally the structure was determined as $C_{27}H_{37}N_3O_4S_3Zn$. The positional and anisotropic thermal parameters of the non-hydrogen atoms were refined by full-matrix least-squares with observed 7749 reflections [$I > 1.0\sigma(I)$], include friedel pairs, and the final R and weighted R value were 0.068 and 0.038, respectively. The absolute configuration of **1** was also determined by X-ray analysis based on anomalous scatterings of zinc atom.

A perspective view with atom numbering system of the molecule is depicted in Fig. 3. Configurations of the chiral carbon atoms were 14*R*, 15*R*, 17*R*, 19*S*, and 25*S*. The donor groups of the ligand were as follows: the

Fig. 2. LSI-MS of micacocidin A (**1**) using the matrix of (a) *m*-nitrobenzyl alcohol (NBA) and (b) NBA containing NaI.

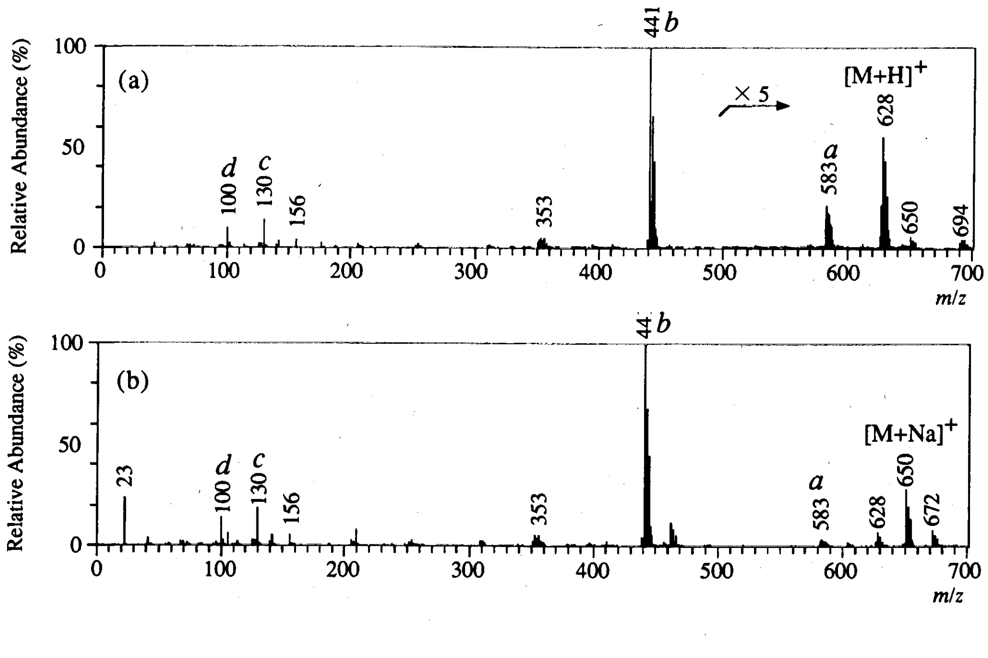


Fig. 3. ORTEP drawing of the crystallographic structure of micacocidin A.

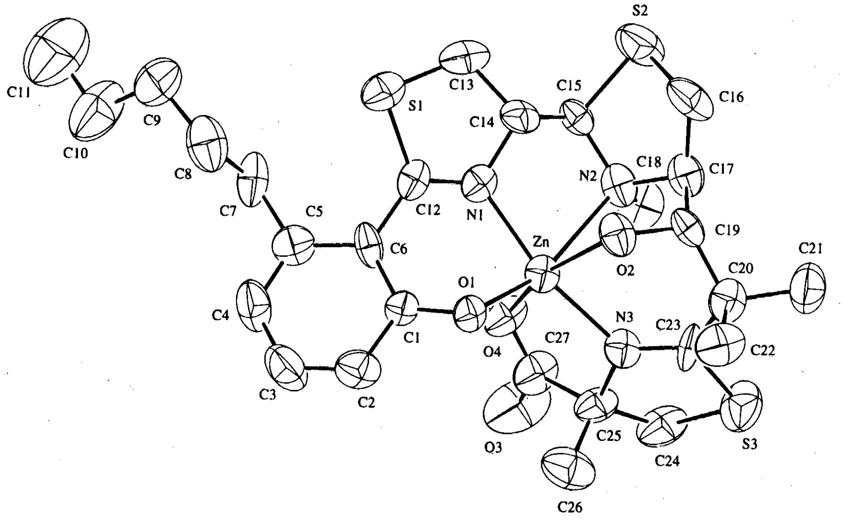
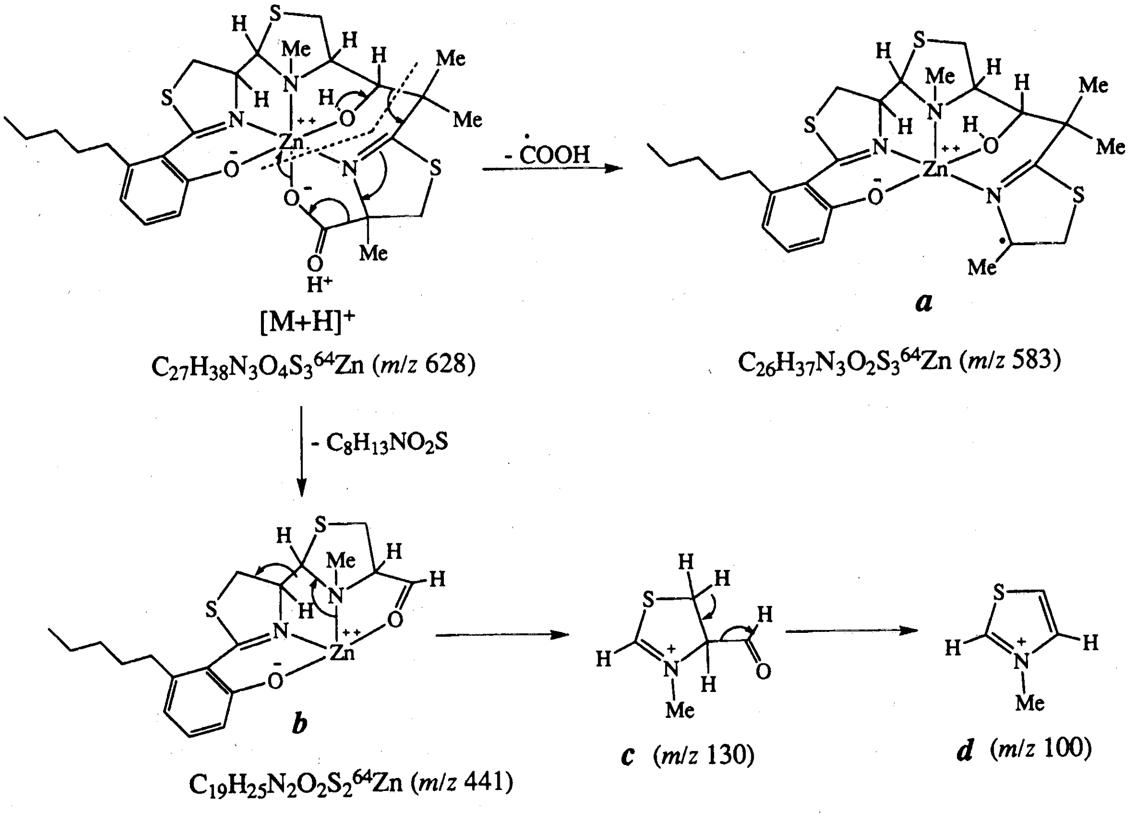


Fig. 4. Mass fragmentation of micacocidin A (1).



phenolate anion, carboxylate anion, secondary hydroxyl group, and three nitrogen atoms. The details of the determinations will be published elsewhere.[2] The fragment ions shown in Fig. 4 supported the structure 1 determined by the X-ray analysis.

The signals in the ^{1}H NMR spectrum and the ^{13}C NMR spectrum accounted for the 37 protons and 27 carbons, respectively. Assignments of the signals shown in Table 1 were carried out by double-quantum filterd correlated spectroscopy (DQF-COSY), total-correlation spectroscopy (TOCSY), rotating frame overhauser effect spectroscopy (ROESY), heteronuclear multiple-quan-

Table 1. ^1H and ^{13}C NMR data of micacocidin A in $CDCl_3$.

Carbon No.	Carbon shift	Proton shift(*J* in Hz)
1	121.44	6.66 (d, *J*=7.4)
2	132.94	7.04 (t, *J*=7.5)
3	120.21	6.51 (d, *J*=6.4)
4	146.27	
5	119.65	
6	167.98	
7	35.71	2.73 (m), 3.14 (m)
8	33.62	~1.58(m),~1.64(m)
9	22.63	~1.43(m)
10	31.96	~1.40(m)
11	14.18	0.94 (t, *J*=6.9)
12	172.74	
13	35.48	2.87 (t, *J*=12.0) 3.50 (t, *J*=9.1)
14	75.11	4.47 (td, *J*=10.8, 8.6)
15	81.31	3.94 (d, *J*=10.4)
16	38.19	2.19 (t, *J*=10.8) 2.64 (t, *J*=8.9)
17	72.35	3.07 (t, *J*=7.6)
18	48.16	2.35 (s)
19	~77.20	3.61 (s)
20	44.92	
21	29.04	1.62 (s)
22	25.41	1.26 (s)
23	182.37	
24	38.19	3.20 (d, *J*=12.0) 3.76 (d, *J*=12.0)
25	86.04	
26	24.27	1.60 (s)
27	177.12	

Chemical shift values are ppm from internal TMS in $CDCl_3$.

tum coherence (HMQC) and heteronuclear multiple-band correlation (HMBC) experiments. Connectivity in ligand was revealed by C–H long-range couplings in HMBC spectrum shown in Fig. 5. The relative configuration was determined by the observations of nuclear overhauser effects in ROESY spectrum (Fig. 6).

In the case of **2**, the $[M+H]^+$ ion and $[M+Na]^+$ ions were observed at *m/z* 627 and 649, respectively. The isotopic distribution patterns of them indicated the presence of a copper atom. The molecular formula $C_{27}H_{37}N_3O_4S_3Cu$ was determined from the accurate masses 627.1322 for the $[M+H]^+$ ion (calcd 627.1319 for $C_{27}H_{38}N_3O_4S_3{}^{63}Cu$) and 649.1145 for $[M+Na]^+$ ion (calcd 649.1138 for $C_{27}H_{37}N_3O_4S_3{}^{63}CuNa$) by high resolution LSI-MS. Therefore, it was revealed that compound **2** was an analog of **1**, with the zinc atom replaced by a copper atom. This structure was supported by chemical conversion of **1** to **2** with Cu^{2+} sources. That is, a large excess of 0.1 M of aq. $CuCl_2$ or aq. $CuSO_4$

Fig. 5. Long-range ^1H-^{13}C coupling observed in HMBC of micacocidin A in $CDCl_3$.

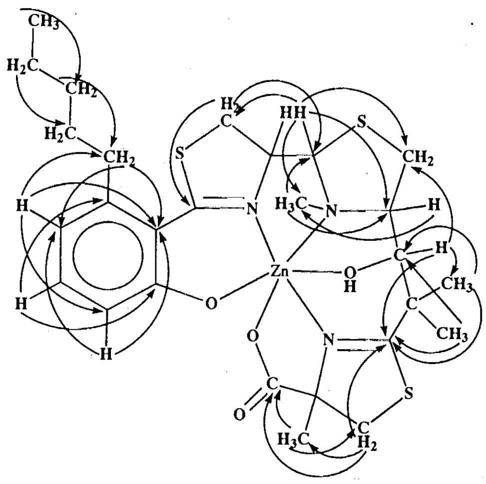
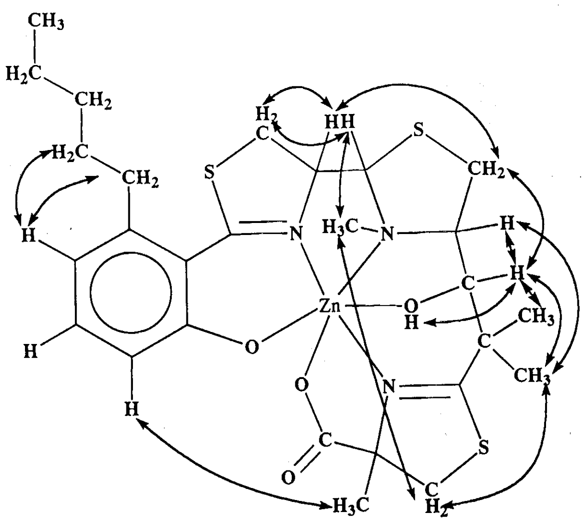


Fig. 6. NOE of micacocidin A in $CDCl_3$.



was added to **1** in MeOH at room temperature, followed by extraction with methylene dichloride to get the expected product, which was identified with **2** on the basis of the LSI-MS, HPLC, and TLC.

In LSI-MS spectrum of compound **3**, the $[M+H]^+$ and $[M+Na]^+$ ions were observed at *m/z* 619 and 641, respectively. The presence of an iron atom was presumed from the isotopic distribution patterns of them. The molecular formula $C_{27}H_{36}N_3O_4S_3Fe$ was determined by high resolution LSI-MS include measured values. The numbers of hydrogen atom were less by one than in **1** and **2**, which indicated the iron atom to be a trivalent cation (Fe^{3+}) matching three negative-charged groups, *i.e.*, phenolate anion, carboxylate anion, and hydroxylate anion. This structure was also confirmed by chemical conversion of **1** to **3** with aq. $Fe_2(SO_4)_3$ or aq. $FeCl_3$, in the same manner as described above.

Conclusion

Micacocidin A (**1**), B (**2**), and C (**3**) were elucidated to be a zinc(II)-, copper(II)- and iron(III)-complexes with same metal-free ligand, respectively. The metal-free ligand, *i.e.*, (*S*)-2-[(*S*)-2[(2*R*,4*R*,4′*R*)-2,3,4,4′,5,5′-hexahydro-2′-(2-hydroxy-6-pentylphenyl)-3-methyl-2,4′-bithiazoyl-4-yl]-2-hydroxy-1,1-dimethylethyl]-4,5-dihydro-4-methylthiazole-4-carboxylic acid resembled the thiazoline-containing pyochelin[3,4] and aeruginoic acid.[5]

To the best our knowledge this is the first report of antimycoplasma agents having chelate structures from natural sources. They are different in both structure and activity from the known antibiotics. Thus, these new compounds may be regarded as a novel class. During preparation of the manuscript it came to our attention that DRECHSEL *et al.*[6] has isolated and elucidated a similar structure, yersiniabactin, iron-transporting compound.

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