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Preparation and Antimicrobial Activity of Micacocidin

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Micacocidin (**3**), a Zn-free derivative of micacocidin A (**1**), was prepared to evaluate its antimicrobial activity in comparison with **1** and to obtain a starting material for chemical modification of **1**. The structure of **3**, quite unlike those of any previously known antimicrobial agents, was elucidated by 1-D and 2-D homonuclear and heteronuclear NMR and mass spectroscopy.

Micacocidin (**3**) thus prepared exhibited weak or no antibacterial activity except against *Mycoplasma* species, *i.e.* **3** showed stronger activity than **1**. It is noteworthy that **3** displayed high activity against fungi such as *Candida*, *Aspergillus* and *Trichophyton* species.

During the course of a screening program to search for new antibiotics from bacteria, we discovered micacocidin A (**1**) and two minor congeners, micacocidins B and C, in the culture filtrate of *Pseudomonas* sp. No. 57-250. These new antibiotics were characterized by their metal chelating properties and heterocyclic structures with five chiral centers. They showed specific and potent growth-inhibitory effects against *Mycoplasma* species.[1~3] Mycoplasmas have attracted considerable attention as causative agents of pneumonia and upper respiratory infection, and recently a close relationship has been noted between mycoplasma infection and AIDS.[4,5]

Micacocidin (*i.e.* **3**) is composed of two thiazolines and one thiazolidine headed by a pentylated phenol moiety. The terminal thiazoline-4-carboxylic acid moiety is connected to the central thiazolidine ring across a two-carbon unit containing an asymmetric secondary hydroxyl group. Most of the oxygen and nitrogen atoms in these moieties were shown to participate in the octahedral coordination with Zn^{2+}, Cu^{2+}, and Fe^{3+} ions in micacocidins A, B and C, respectively. The taxonomy, fermentation, isolation, physicochemical properties, biological activity and the absolute chemical structures of these micacocidins were reported in our previous papers[1~3].

To assess the biological potential of the desmetallated compound, micacocidin (**3**), and to provide a starting material for chemical modification of the functional groups in **3**, *i.e.* carboxyl, phenolic hydroxyl and secondary alcoholic hydroxyl group, we investigated the preparation, physicochemical properties, biological activity and the mode of action of **3**.

Materials and Methods

Spectroscopic Measurements

UV spectra were recorded with a Hitachi U-3200 spectrophotometer, IR spectra with a JASCO FT/IR-700 spectrometer, and optical rotations with a Perkin-Elmer 241 polarimeter. Liquid secondary ion mass spectra (LSI-MS) were obtained using a Hitachi M-90 mass spectrometer (BEE geometry) equipped with a cesium ion gun. Samples were dispersed in 3-nitrobenzyl alcohol and introduced into the mass spectrometer on an LSI-MS target. 1-D and 2-D NMR spectra were recorded with a Varian UNITY-600 spectrometer at 20°C in $CDCl_3$ solution using TMS as an internal reference. Preparative HPLC was carried out on a Develosil ODS-HG-5 column (i.d. 20 mm×250 mm) and

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guard column (i.d. 20 mm×50 mm) (Nomura Chemical) with detection by UV absorption at 250 nm (Shimadzu SPD 6AV).

Bioassays

Minimal inhibitory concentrations (MICs) of **1** and **3** for the test organisms were determined by the agar or liquid dilution methods designated by the Society of Chemotherapy in Japan[6]. Several different media were used for antibacterial evaluation: PPLO Liquid Medium (Difco) supplemented with 12% horse serum, 1% glucose and 0.025% phenol red adjusted to pH 7.7 for *Mycoplasma gallisepticum*[7]; Hanks Liquid Medium (Nissui) supplemented with 0.5% lacto-albumin, 25% yeast extract, 1% glucose, 10% horse serum and 0.025% phenol red adjusted to pH 7.7 for *M. hyopneumoniae*; PPLO Liquid Medium supplemented with 2% horse serum, 1% glucose, 25% yeast extract and 0.025% phenol red adjusted to pH 7.7% for *M. pneumoniae*; Frey medium (Gibco) supplemented with 10% swine serum, 0.01% β-NAD and 1% glucose for *M. synoviae*. The respective organisms were incubated at 37°C for 120~168 hours. Brain Heart Infusion agar (Difco) supplemented with 2% NaCl was used for *Pasteurella piscida*, and Sensitivity-disc N medium (Nissui) for *Streptococcus* sp. These organisms were incubated at 25°C for 20~24 hours. Trypticase soy agar supplemented with 5% defibrinized sheep blood was used for *Serpulina hyodysenteriae*, which was incubated at 37°C for 96~120 hours. GAM Medium (Nissui), used for *Clostridium perfringens*, was incubated in a gas-pack anaerobic chamber at 37°C for 24 hours.[8] Mueller-Hinton Agar (Difco), used for *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus agalactiae*, *Bordetella bronchiseptiae*, *Actinobacillus pleuropneumoniae*, *Salmonella enteritidis*, *Pasteurella multocida* or *Klebsiella pneumoniae*, was incubated at 35°C for 20~24 hours.

The inoculum size for MIC determination was approximately 10^6 CFU/ml for liquid medium and agar medium. After incubation, the MICs were defined as the minimum drug concentrations that completely inhibited the growth of test organisms.

The antifungal activity was determined using the micro-broth dilution method with RPMI 1640 medium (RPMI, Nissui) supplemented with 0.01 M MOPS at 35°C for 24 hours under 5% CO_2 incubator. Another medium such as Yeast Nitrogen Base (YNB, Difco) was used for *Candida* and *Aspergillus* sp. and Sabouraud Dextrose Broth (SDB, Eiken) was used for *Trichophyton* sp. The inoculum size for MIC determination was approximately 10^5 CFU/ml. The 50% inhibition concentration (IC_{50}) was defined as the dose of sample to prevent the growth 50% of control growth using a spectrometer at 595 nm after incubation at 35°C for 24 hours.

Results and Discussion

Chemistry

Micacocidin (**3**) was conveniently obtained by treatment of micacocidin A (**1**) with diluted aq. acid and methylene chloride at room temperature under similar conditions to those reported for the pyochelin-zinc complex.[9] The product obtained from the organic phase was 90~95% pure **3** according to HPLC analysis. An alternative method for preparing **3** was a combination of acylation and subsequent deacylation procedures (Fig. 1). That is, acetylation of **1** with acetic anhydride in pyridine at room temperature gave a crude diacetate, which was purified by column or thin layer chromatography to remove the liberated zinc ion. In the LSI-mass spectrum of the zinc-free diacetate (**2**), the protonated molecular ion ($[M+H]^+$) observed at *m/z* 650 confirmed the presence of two acetyl groups. The fragment ion peaks at *m/z* 359 and 391 were consistent with the one acetyl group substituted on the aliphatic hydroxyl group attached to the methine carbon and the other on the phenolic hydroxyl group. Then, deacetylation of **2** was carried out with dilute aqueous sodium hydroxide in methyl alcohol at room temperature to obtain the desired product (**3**). Structure **3** was substantiated primarily by means of spectroscopy and chemical conversion to the parent substance **1**.

The UV spectrum of **3** exhibited absorption maxima at 202 (ε=26,900), 213 (18,600), and 250 (8,800) nm. The IR spectrum showed absorption peaks at 3396, 2928, 1583, 1454 and 1397 cm^{-1}. IR studies of **3** in chloroform solution indicated the existence of some strong hydrogen bonds, which suggested that **3** had a quasi-cyclic structure[10]. In LSI-MS, the protonated molecular ion peak and fragment ion peaks were observed at *m/z* 566, 317 and 349 (Fig. 2). The ion peak at *m/z* 379 was assigned to an oxonium ion, presumably formed through bond cleavage between the methinyl carbon and the *gem*-dimethyl carbon. A summary of the fragmentation is shown in Fig. 3.

1D and 2D NMR spectra were recorded at 20°C. The complete assignment of the ^1H and ^{13}C NMR spectra is shown in Table 1 together with that of **1** for comparison.

For the purpose of illustration, the 600 MHz NMR spectra of **1** and **3** are shown in Fig. 4; the NMR spectral characteristics of **3** were similar to but could be clearly distinguished from those of **1**. In the ^{13}C NMR spectrum

Fig. 1. Preparation of micacocidin (**3**) from micacocidin A (**1**).

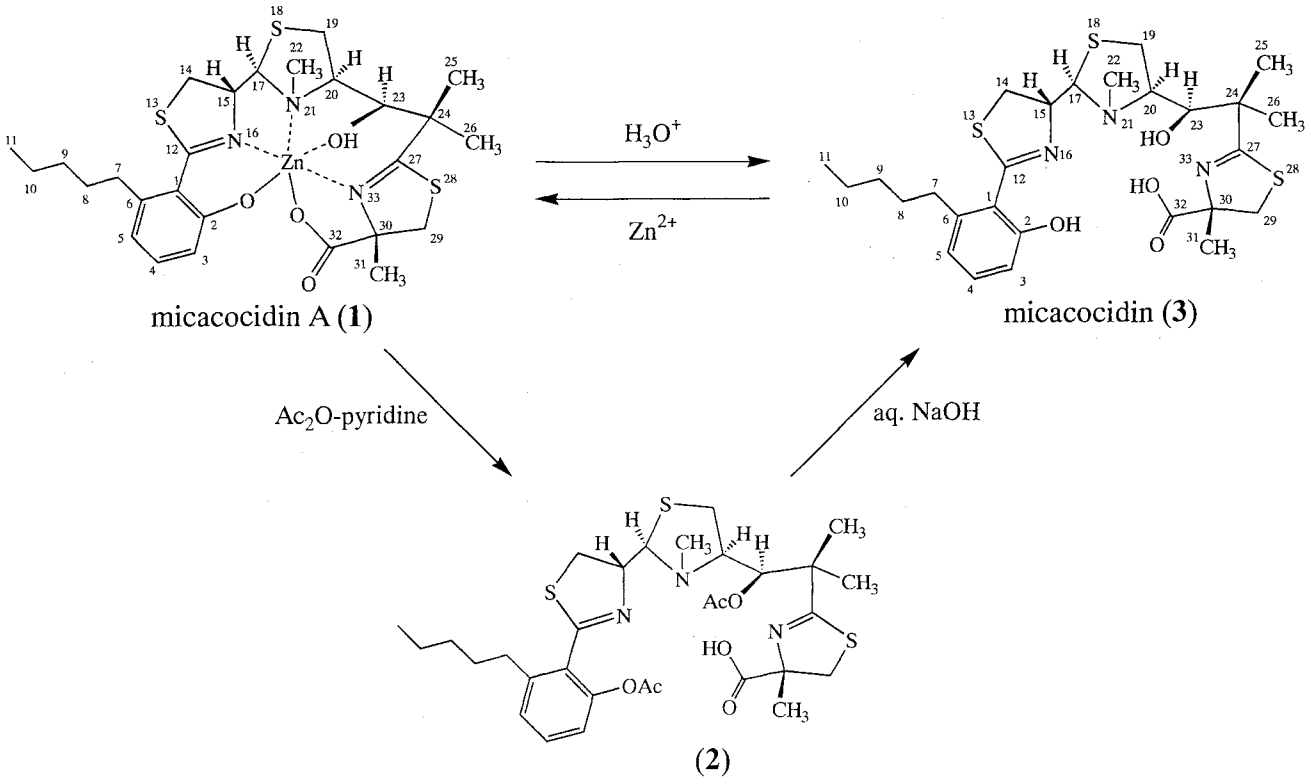
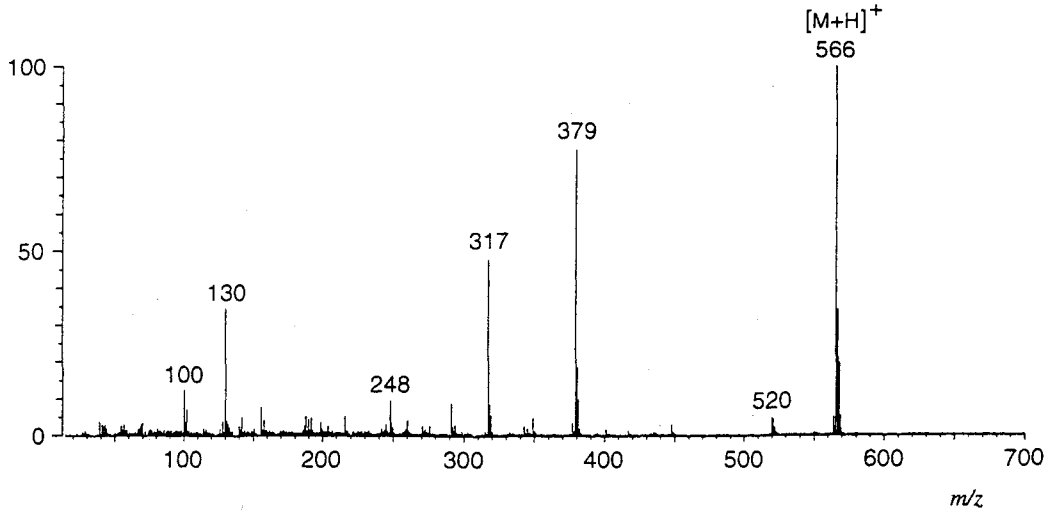


Fig. 2. LSI-mass spectrum of micacocidin (**3**).



the signals assignable to C-2, C-3 and C-25 of **3** were shifted considerably compared with those of **1**, while signals due to other carbons in **3** had a lesser shift.

The structure of **3** appears to be constructed biosynthetically from one molecule of an alkylated salicylic acid analogue of polyketide-origin, an isobutyric acid unit,

Fig. 3. Mass spectral fragmentation of micacocidin (**3**) (upper) and micacocidin diacetate (**2**) (lower).

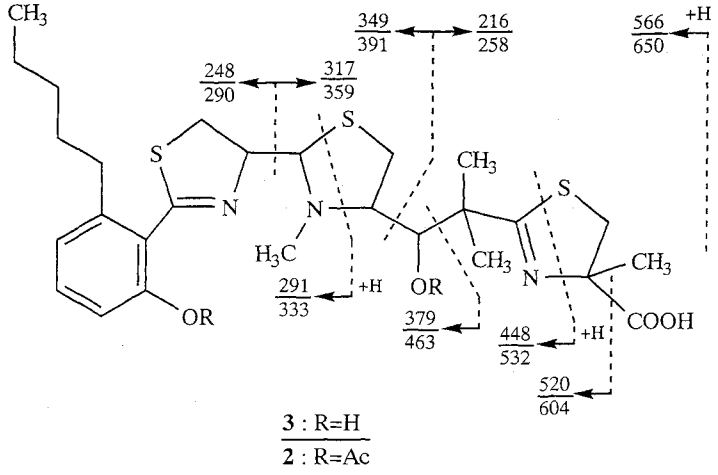
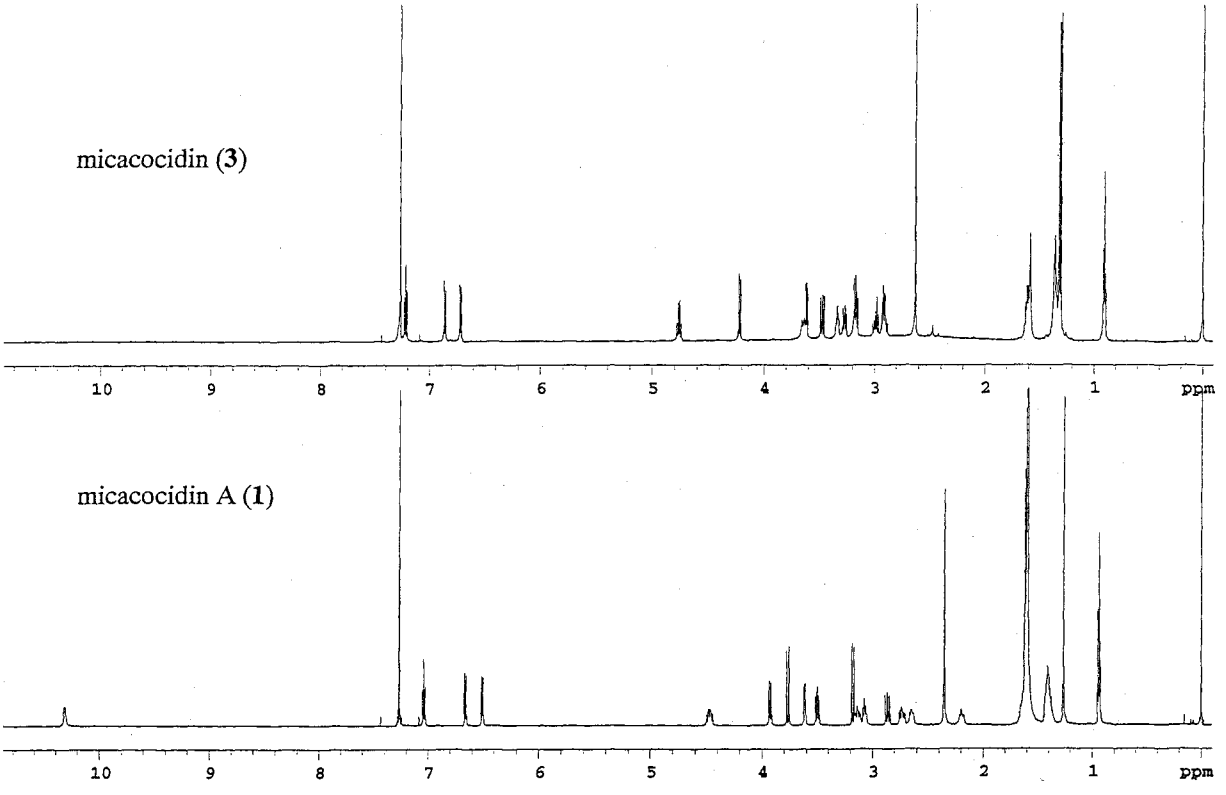


Fig. 4. ^{1}H NMR spectra of micacocidin A (**1**) and micacocidin (**3**).



and three molecules of cysteine. The structure of micacocidin (**3**) elucidated here is similar to that of yersiniabactin (**5**)[11], an iron-siderophore produced by the Gram-negative bacterium *Yersinia enterocolitica*.

Micacocidin (**3**) can readily be converted to the original chelated form, micacocidin A (**1**), by addition of zinc ions; *i.e.* treatment of **3** in methyl alcohol with a large excess of 0.1 M aq. $ZnCl_2$ or aq. $ZnSO_4$ at room temperature, followed

Table 1. ^{1}H and ^{13}C NMR data for micacocidin (**3**) in comparison with micacocidin A (**1**).

	micacocidin A (**1**)		micacocidin (**3**)	
Carbon No.	^{13}C shift	^{1}H shift (J in Hz)	^{13}C shift	^{1}H shift (J in Hz)
1	119.65		116.90	
2	167.98		158.72	
3	121.44	6.66 (d, J=7.4)	115.19	6.86 (dd, J=8.3, 1.2)
4	132.94	7.04 (t, J=7.5)	131.95	7.19 (dd, J=8.3, 7.8)
5	120.21	6.51 (d, J=6.4)	121.24	6.71 (dd, J=7.8, 1.2)
6	146.27		143.50	
7	35.71	2.73 (m), 3.14 (m)	35.08	2.87 (m)
8	33.62	~1.58 (m), ~1.66 (m)	32.05	1.61 (m)
9	22.63	~1.43 (m)	22.53	1.35 (m)
10	31.96	~1.40 (m)	31.91	1.35 (m)
11	14.18	0.94 (t, J=6.9)	14.09	0.90 (t, J=7.1)
12	172.74		171.71	
14	35.48	2.87 (t, J=12.0)	35.44	3.17 (dd, J=11.2, 7.8)
		3.50 (t, J=9.1)		3.50 (dd, J=11.2, 8.8)
15	75.11	4.47 (td, J=10.8, 8.6)	78.38	4.75 (ddd, J=8.8, 8.7, 7.8)
17	81.31	3.94 (d, J=10.4)	79.33	4.21 (d, J=8.7)
19	38.19	2.19 (t, J=10.8)	36.79	2.91 (m)
		2.64 (t, J=8.9)		3.26 (m)
20	72.35	3.07 (t, J=7.6)	72.64	3.32 (m)
22	48.16	2.35 (s)	~ 45.8	2.60 (m)
23	~ 77.2	3.61 (s)	78.14	3.65 (d, J=11.5)
24	44.92		45.47	
25	29.04	1.62 (s)	23.66	1.29 (s)
26	25.41	1.26 (s)	23.66	1.28 (s)
27	182.37		180.60	
29	38.19	3.20 (d, J=12.0)	41.23	3.14 (dd, J=11.2, 7.8)
		3.76 (d, J=12.0)		3.66 (d, J=11.5)
30	86.04		84.34	
31	24.27	1.60 (s)	24.24	1.59 (s)
32	177.12		176.33	

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

by extraction with CH_2Cl_2 to obtain **1**. The NMR spectrum, mass spectrum and HPLC of the product were completely superimposable with those of naturally obtained **1**. More recently, the total synthesis of **3** was successfully achieved by INO *et al.*[12~14] Studies of the chemical modifications of **3** in view of structure-activity relationships are currently underway.

Biological Properties

Table 2 shows the MIC values of micacocidin (**3**) in comparison with those of micacocidin A (**1**) against Gram-

Table 2. Antibacterial spectrum for micacocidin (**3**) and micacocidin A (**1**).

Organisms	MIC (μg/ml)	
	micacocidin A (**1**)	micacocidin (**3**)
Mycoplasma pneumoniae Mac	0.025	0.00313
M. hyopneumoniae ST-11	0.1	0.025
M. gallisepticum S6	0.1	0.1
M. gallisepticum T-7T (Tylosin resistant)	0.2	0.2
M. synoviae 1853	0.78	NT
Staphylococcus aureus FDA 209P JC-1	>25	25
Pasteurella multocida D-6	>25	25
Bordetella bronchiseptica H-16	>25	25
Escherichia coli NIHJ JC-2	>25	>25
Salmonella enteritidis 8966 (PT34)	>25	>25
Klebsiella pneumoniae ATCC 27736	>25	>25
Streptococcus sp. SN86119	6.25	>25
S. agalactiae ATCC 9925	>25	>25
Actinobacillus pleuropneumoniae NB-001 (1)	25	6.25
Clostridium perfringens ATCC 13124	25	6.25
Serpulina hyodysenteriae ATCC 27164	12.5	12.5

positive and Gram-negative bacteria and *Mycoplasma* species.

The MIC values were determined by using the agar dilution method authorized by the Japan Society of Chemotherapy,[6] and for mycoplasma, the liquid dilution method was used. Among the bacteria tested, *Serpulina hyodysenteriae* and *Clostridium perfringens* were anaerobically cultured using the gas-pack method. **3** showed weak or no antibacterial activity, except for the *Mycoplasma* species, for which **3** exhibited slightly better activity than **1**. A preliminary *in vivo* evaluation of **3** after oral administration was performed in comparison with chlorotetracycline against an experimental infection of chickens caused by *Mycoplasma gallisepticum*. Both **1** and **3** were superior to chlorotetracycline, which will be reported and discussed elsewhere.

Antifungal activity was determined by the micro-broth dilution method using 96-well micro plate with RPMI, YNB and SDB medium at 35°C. Table 3 shows the IC_{50} values of **1** and **3** against *Candida*, *Aspergillus* and *Trichophyton* species. It is noteworthy that only **3** exhibited high activity against *Candida albicans*, *C. glabrata*, *C. krusei*, *Aspergillus fumigatus*, *Trichophyton rubrum*, *T. asteroides*, and *T. mentagrophytes*. Furthermore, the activity of **3** against these fungi were higher in RPMI than in YNB or SDB. These difference may occur from the fact that synthetic medium (RPMI) includes less contamination of metal ions than natural (SDB) or semi-synthetic (YNB) medium.

Mode of Action

The growth-inhibitory activity of both **1** and **3** against the *Mycoplasma* species was not antagonized in the presence of Zn^{2+}, Cu^{2+} and Fe^{3+} ions. Therefore, although the precise mechanism is not yet clear, these findings suggest that the mode of action of **1** and **3** for the *Mycoplasma* species is not related to its metal ion chelation activity. Conversely, the antifungal activity of **3** was antagonized in the medium containing the above metal ions, which suggests a different mode of action for the antimycoplasma activity. These results strongly suggest that the mode of action of **3** against fungi is related to its ability to chelate metal ions.

Detailed studies of the mechanism of action are currently underway in our laboratory.

Table 3. Antifungul activity of micacocidin (**3**) and micacocidin A (**1**).

Strain	Medium	IC_{50} μg/ml	
		Micacocidin A(**1**)	Micacocidin(**3**)
Candida albicans Ca-15	YNB	>100	6.3
	RPMI	>100	0.8
Candida glabrata IFO 0005	YNB	>25	6.3
	RPMI	>25	0.8
Candida krusei IFO 0841	YNB	>25	12.5
	RPMI	>25	1.6
Aspergillus fumigatus IFO 4400	YNB	>100	3.2
	RPMI	>100	0.2
Trichophyton rubrum IFO 6203	SDB	>100	3.1
	RPMI	>100	0.8
T. asteroides SM 110	SDB	>100	3.2
	RPMI	>100	0.2
T. mentagrophytes IFO 5809	SDB	>100	3.1
	RPMI	>100	0.4

Experimental

Preparation of Micacocidin (**3**)

Method A: Acetylation

To a solution of micacocidin A (**1**, 199 mg) in pyridine (4 ml) was added acetic anhydride (2.5 ml), and the mixture was stirred at room temperature for 16 hours. The reaction mixture was then concentrated *in vacuo*, and the oily residue was purified by PTLC (Kieselgel 60 F_{254} Art, 5744, Merck, $CHCl_3$: MeOH=9 : 1) or column chromatography (Kieselgel 60, 70~230 mesh, Merck) to afford the diacetate (**2**) (165 mg, 81.3%).

^{1}H NMR ($CDCl_3$): δ 2.30 (3H, s, C-2 CO*CH*$_3$), 2.11 (3H, s, C-23 CO*CH*$_3$), 5.30 (1H, d, J=6.0 Hz, 23-H), ^{13}C NMR ($CDCl_3$): δ 20.80 (C-2 CO*C*H$_3$), 21.19 (C-23 CO*C*H$_3$), 79.04 (C-23), 169.30 (C-2 *C*OCH$_3$), 170.34 (C-23 *C*OCH$_3$).

A solution of **2** (145 mg) in MeOH (4 ml) was hydrolyzed by addition of 1 N aq. NaOH (3 ml) under ice cooling. The mixture was stirred at room temperature for 16 hours and then acidified (pH 5~6) with 2 N aq. HCl under ice cooling. The reaction mixture was concentrated to *ca.* half the volume *in vacuo*, diluted with sat. aq. NaCl, and extracted with EtOAc. The organic layer was washed with sat. aq. NaCl and evaporated to give oily **3** (109 mg, 82.6%). A portion of **3** was purified by preparative HPLC [elution with 81% of MeOH containing phosphate buffer (1 mM, pH 7.0) at 10 ml/minute] for analysis and bioassay to yield a pale yellow oil, $[\alpha]^{22}$ -65.3 ± 1.1 (c 0.93, MeOH); UV λ_{max} (ε) 202 (26,900), 213 (18,600), 250 (8,800); IR ν_{max} (film) cm^{-1} 3396, 2928, 1583, 1454, 1397.

Method B: Treatment with dilute aq. HCl

To a solution of **1** (2 mg) in CH_2Cl_2 (2 ml) was added 1 N aq. HCl or 5% aq. $KHSO_4$ (4 ml) with stirring for 5 minutes, and the whole mixture was extracted with CH_2Cl_2. The organic phase was washed with H_2O and evaporated *in vacuo* to afford oily **3** (1.8 mg) in almost quantitative yield. This was subjected to preparative HPLC as described above to prepare the analytical specimen. The product was identified as **3** based on completely superimposable NMR and HPLC curves.

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