ISOLATION, STRUCTURES, AND ANTIFUNGAL ACTIVITIES OF NEW AUREOBASIDINS

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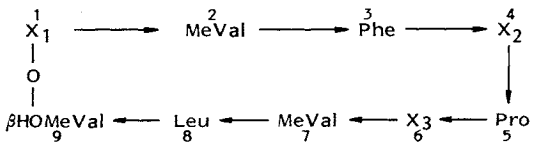
Aureobasidins are a group of cyclic depsipeptides with antifungal activity and are produced by *Aureobasidium pullulans*. Aureobasidins are composed of eight amino acids and one hydroxy acid such as 2-hydroxy-3-methylpentanoic acid (Hmp), and highly lipophilic. Five new aureobasidins, S_1, S_{2a}, S_{2b}, S_3 and S_4, which have higher hydrophilicity in reversed phase HPLC than the known aureobasidins A~R, were discovered in a fermentation broth of *A. pullulans* R106 by means of on-line liquid chromatography/mass spectrometry with electrospray ionization. We identified the structures of the compounds and studied their antifungal activities. Three of the new aureobasidins, S_{2b}, S_3 and S_4, which have hydroxylated Hmp as the hydroxy acid, were highly active against *Candida* spp. and *Cryptococcus neoformans*.

Aureobasidins are a group of antifungal antibiotics produced by *Aureobasidium pullulans* R106.[1] The main component, aureobasidin A (Fig. 1), has high activity against many pathogenic fungi *in vitro* and *in vivo*, and low toxicity,[1,2] and so it is a promising antifungal agent. Seventeen minor components of aureobasidins, B~R, have been isolated so far. All aureobasidins are cyclic depsipeptides consisting of eight L-form α-amino acids, three or four of which are *N*-methylated, and one hydroxy acid, 2-hydroxy-3-methylpentanoic acid (Hmp) or 2-hydroxy-3-methylbutanoic acid,[3,4] and highly lipophilic.

On-line liquid chromatography/MS (LC/MS) with electrospray ionization is a conventional technique on the primary structure analysis of complex mixtures of peptides.[5] Aureobasidins are fragmented systematically by FAB-MS, making possible the identification of their structure.[4] Electrospray MS/MS fragmentations of aureobasidins are also systematic, so we screened for new aureobasidins with high hydrophilicity in a fermentation broth by means

Fig. 1. Structures of aureobasidins A, S_1, S_{2a}, S_{2b}, S_3 and S_4.



Aureobasidin	X_1	X_2	X_3
A	Hmp	MePhe	aIle
S_1	Hmp	MePhe	Met(O)
S_{2a}	Hmp	MeTyr	aIle
S_{2b}	2,5-Dihydroxy-3-methylpentanoyl	MePhe	aIle
S_3	2-Hydroxy-3-hydroxymethylpentanoyl	MePhe	aIle
S_4	2,4-Dihydroxy-3-methylpentanoyl	MePhe	aIle

Abbreviations: Hmp, 2-hydroxy-3-methylpentanoic acid; MeVal, *N*-methylvaline; Phe, phenylalanine; MePhe, *N*-methylphenylalanine; Pro, proline; aIle, allo-isoleucine; Leu, leucine; βHOMeVal, β-hydroxy-*N*-methylvaline; Met(O), methionine sulfoxide; MeTyr, *N*-methyltyrosine.

of LC/MS with electrospray ionization.

In this paper, we report the method used to discover new aureobasidins, and the structures and antifungal activities of the newly isolated aureobasidins, S_1, S_{2a}, S_{2b}, S_3 and S_4.

Results and Discussion

Search for New Aureobasidins

An electrospray MS/MS spectrum of aureobasidin A by an atmospheric pressure ionization triple-quadrupole mass spectrometer was similar to the fragmentation pattern obtained by FAB-MS (Fig. 2A). The only difference was that the ion peak at *m/z* 536 was detected instead of that at *m/z* 518 in FAB-MS, which is a dehydrated product of the fragment at *m/z* 536. All known aureobasidins had similar fragmentation patterns (data not shown). A linear peptide, **A1a**, which is a methyl ester derivative of the alkaline hydrolysate of aureobasidin A,[3,4] produced characteristic fragment ion peaks (Fig. 2B): the parent ion at *m/z* 1,076 produced daughter ion peaks of high intensity at *m/z* 536 and 859. This fragmentation pattern was the same as those of linear peptides obtained from all aureobasidins.[4]

A crude fermentation broth was assayed for new aureobasidins by LC/MS. The total ion-current chromatogram (*m/z* 800 to 1,300) showed that there were seven new peaks in the fractions that were eluted faster than aureobasidin F in reversed-phase HPLC (Fig. 3). The MS/MS spectra suggested that three of the peaks, 1, 2 and 4, were linear peptides and four peaks, 3 (S_1), 5 (S_2), 6 (S_3) and 7(S_4), were cyclic depsipeptides (Table 1). The MS/MS spectra of the $(M+H)^+$ ion peaks at *m/z* 1,268 (peak 1), 1,238 (peak 2) and 1,102 (peak 4), gave fragment ion peaks at *m/z* 536 and 859, which suggested that these compounds were linear peptides containing an amino acid sequence Hmp-MeVal-Phe-MePhe-Pro-aIle-MeVal (abbreviations were listed in Fig. 1). The MS/MS fragmentation pattern of the $(M+H)^+$ ion peak at *m/z* 1,136 of S_1 was similar to that of aureobasidin A, but S_1 had the ion peaks at *m/z* 358 and 519, which were larger by 34 mass units than those at *m/z* 324 and 485 of aureobasidin A. The MS/MS fragmentation of the ion peak at *m/z* 1,118 of S_2 was complicated because of a mixture of two cyclic peptides, which produced two type fragment ion peaks, for example, *m/z* 210 and 226 corresponding to Hmp-MeVal part. S_2 was separated into S_{2a} and S_{2b} by silica gel HPLC. S_3 and S_4 gave the same fragment ion peaks by MS/MS of the $(M+H)^+$ ion.

Structure of Aureobasidin S_1 (S_1)

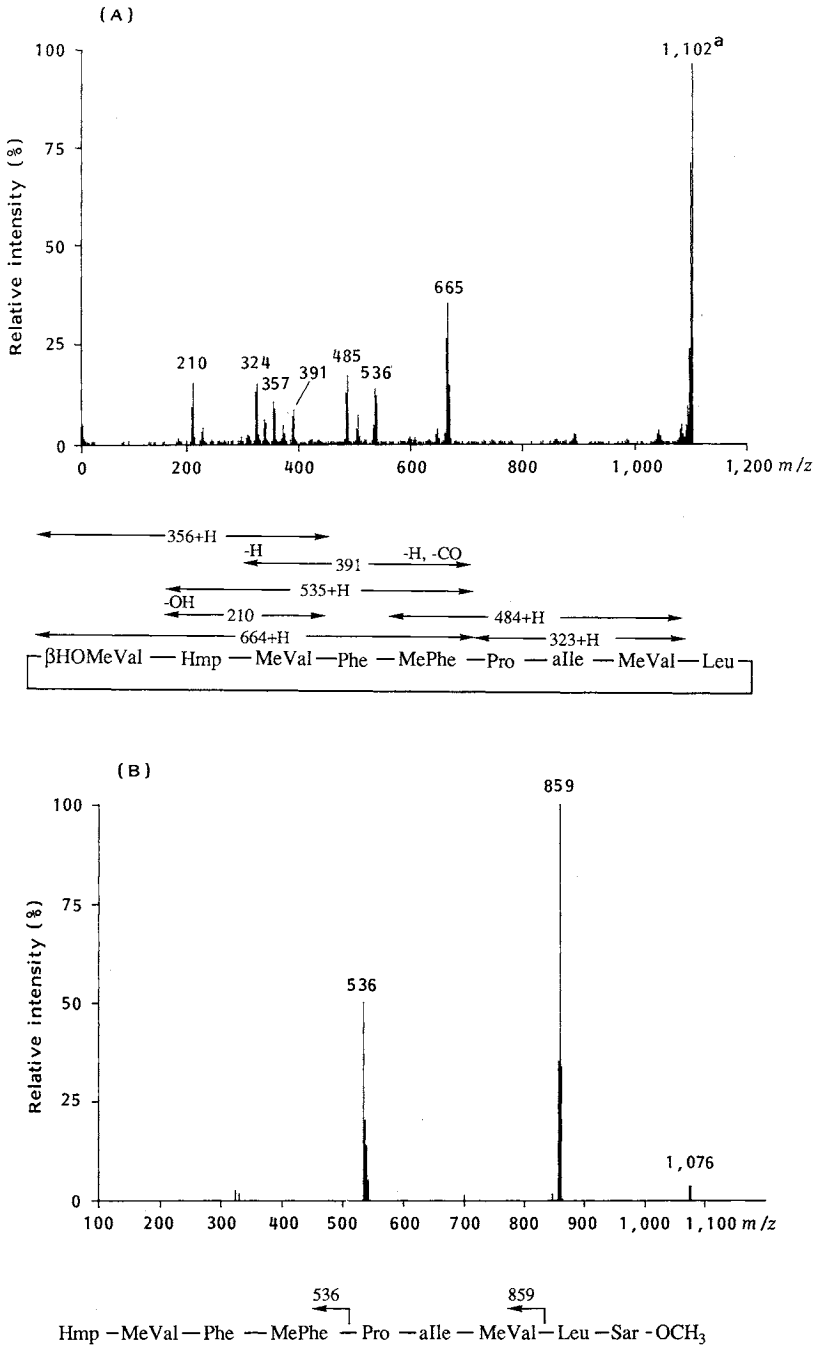
The MW of S_1 was determined to be 1,134, which was 34 larger than aureobasidin A, by LC/MS and FAB-MS. Amino acid analysis of its acid hydrolysate (6 N HCl, 110°C, 22 hours) showed to contain 0.6 mol of βHOMeVal, 2 mol of MeVal, 1 mol each of Pro, MePhe, Leu and Phe, and 0.4 mol of methionine (Met). Aureobasidin A is composed of aIle (MW 131) instead of Met (MW 149). Aureobasidin S_1 was hydrolyzed with 3 M *p*-toluenesulfonic acid (110°C, 22 hours)[6] and this hydrolysate contained 1 mol of Met(O), the MW of which is 34 larger than that of aIle. FAB-MS fragment ion peaks at *m/z* 210, 391 and 665 were the same as those of aureobasidin A, which suggested presence of the sequence βHOMeVal-Hmp-MeVal-Phe-MePhe in S_1. Fragment ion peaks at *m/z* 358 and 519, corresponding to those at *m/z* 324 and 485 of aureobasidin A, were assigned as Pro-Met(O)-MeVal(+H) and MePhe-Pro-Met(O)-MeVal(+H), respectively. Thus, the structure of S_1 was as shown in Fig. 1.

Structure of Aureobasidin S_{2a}

Amino acid analysis of the acid hydrolysate of S_{2a} showed to contain 0.5 mol of βHOMeVal, 2 mol

Fig. 2. Electrospray MS/MS spectrum.

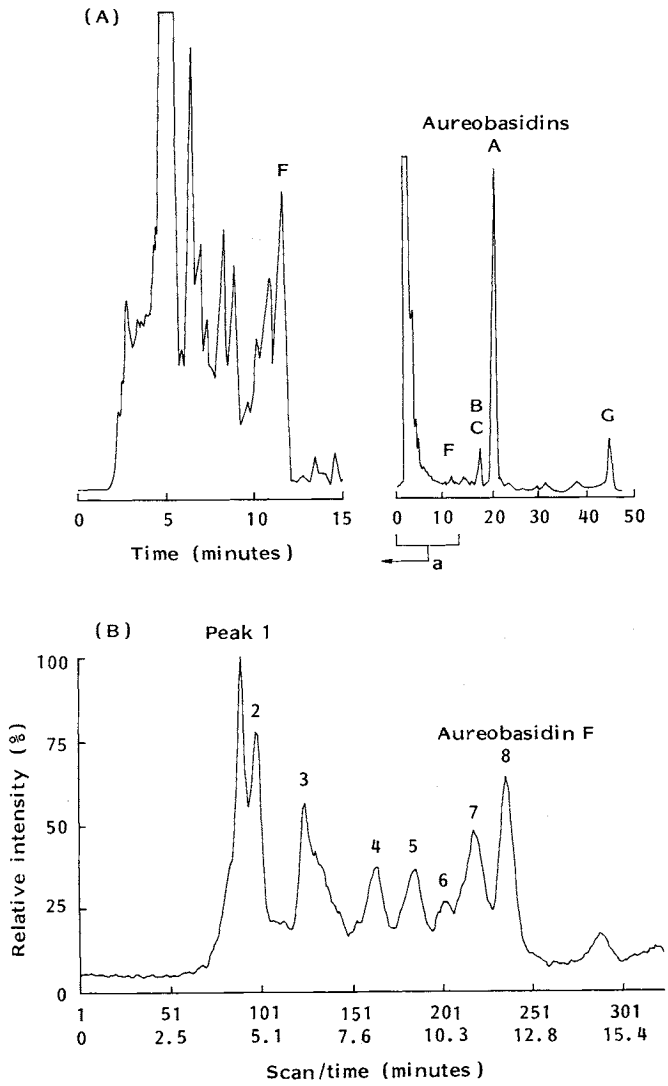
(A) Aureobasidin A, (B) **A1a**.



[a] Because the monoisotopic MW of aureobasidin A is 1,100.68,[3] the protonated molecular ion $(M+H)^+$ by electrospray MS was detected at *m/z* 1,102.

Fig. 3. LC/MS of the fermentation broth.

(A) UV220, (B) total ion current. m/z 800~1,300.



[a] The fractions eluted faster than aureobasidin F were collected.

of MeVal, 1 mol each of Pro, aIle, Leu and Phe, and an undefined amino acid analogue, which was identified as MeTyr by comparison with a synthesized product in amino acid analysis. FAB-MS of S_{2a} gave $(M+H)^+$ at m/z 1,117, which was 16 mass units larger than that of aureobasidin A. Fragment ion peaks at m/z 210 and 324, which were the same as those of aureobasidin A, were assigned as Hmp-MeVal(−OH) and Pro-aIle-MeVal(+H), respectively. Ion peaks at m/z 681, 501 and 407 were 16 mass units larger than the corresponding peaks of aureobasidin A. These results suggested that the MePhe at position 4 of aureobasidin A was replaced to a 16 mass units larger amino acid, MeTyr, in S_{2a} (Fig. 1).

Structures of Aureobasidins S_{2b}, S_3 and S_4

The amino acid compositions of S_{2b}, S_3 and S_4 were the same as that of aureobasidin A. By FAB-MS,

Table 1. Electrospray MS/MS fragmentation of $(M+H)^+$ ions.

Peak No.	$(M+H)^+$	Fragment ion peaks *m/z*	Type[a]
1	1,268	536,[b] 859[b]	Linear
2	1,238	536,[b] 859[b]	Linear
3 (S_1)	1,136	210, 357, 358, 391, 519, 536, 665	Cyclic
4	1,102	536,[b] 859[b]	Linear
5 (S_2)	1,118	210, 226, 324, 391, 407, 485, 501, 552, 681	Cyclic
6 (S_3)	1,118	226, 324, 373, 391, 485, 552, 681	Cyclic
7 (S_4)	1,118	226, 324, 373, 391, 485, 552, 681	Cyclic
8 (Aureobasidin F)	1,088	210, 310, 357, 391, 471, 536, 665	Cyclic
Aureobasidin A	1,102	210, 324, 357, 391, 485, 536, 665	Cyclic

[a] "Cyclic" means cyclic depsipeptide and "linear" means linear peptide.
[b] This fragment ion peak was higher in intensity than that of the parent $(M+H)^+$ ion.

all of these compounds gave $(M+H)^+$ at *m/z* 1,117, which were 16 mass units larger than that of aureobasidin A, and similar fragmentation patterns. Their fragment ion peaks at *m/z* 324, 391 and 485 were also observed in the fragmentation of aureobasidin A, and ion peaks at *m/z* 226, 534 and 681 were 16 mass units larger than the corresponding peaks in aureobasidin A, all of which are assigned as the sequences containing Hmp. These results suggested that S_{2b}, S_3 and S_4 had a 16 mass units larger component instead of Hmp at position 1.

The ^{13}C NMR spectra (Table 2) of S_{2b}, S_3 and S_4 showed that these compounds were independently a mixture of two isomers, as is aureobasidin A; such isomerism seems to be associated with *cis-trans* rotation of the amide bond between MePhe and Pro.[7] In the ^{13}C NMR spectrum of S_{2b}, the δ-carbon (methyl carbon, 11.3 and 11.3 ppm) of Hmp of aureobasidin A was replaced to a methylene carbon (59.7 and 60.0 ppm), which indicated that S_{2b} had δ-hydroxylated Hmp (2,5-dihydroxy-3-methylpentanoic acid) at position 1 (Fig. 1). The signals of the γ- and ε-carbons of the hydroxy acid were shifted to lower fields and the β-carbon was to a higher field in S_{2b}. These findings fit the general rule about chemical shifts of carbons in ^{13}C NMR spectra when a methyl group of an alkane is hydroxylated.[8]

Table 2. ^{13}C NMR chemical shift of the hydroxy acid (position 1) of aureobasidins A, S_{2b}, S_3 and S_4.

Position 1 $\overset{\delta}{CH_2}-\overset{\gamma}{CH}(R_2)-\overset{\beta}{CH}(\overset{\varepsilon}{CH_2}OH)(R_1)-\overset{\alpha}{CH}(R_3)-COOH$

Hmp: $R_1=R_2=R_3=H$

Position of carbon atom	Chemical shift (ppm) and multiplicity			
	Aureobasidin			
	A	S_{2b}	S_3	S_4
α	73.5 (d) 73.5	73.3 (d) 73.3	69.17 (d) 69.22	73.5 (d) 73.5
β	37.0 (d) 37.1	32.3 (d) 32.5	44.7 (d) 45.0	39.6 (d) 39.9
γ	22.9 (t) 22.8	33.1 (t) 33.3	18.1 (t) 18.1	66.3 (d) 66.3
δ	11.3 (q) 11.3	59.7 (t) 60.0	11.7 (q) 11.7	20.0 (q) 20.0
ε	16.4 (q) 16.3	17.3 (q) 17.4	61.6 (t) 61.7	9.3 (q) 9.3

In the ^{13}C NMR spectrum of S_3, the ε-carbon (methyl carbon) of Hmp in aureobasidin A was changed to a methylene carbon and shifted to 61.6 (61.7) ppm, which suggested that hydroxylation had occurred at the ε-carbon of Hmp. As a consequence, a downfield shift of the β-carbon and upfield shifts of the α- and γ-carbons were observed. Thus, position 1 of S_3 was 2-hydroxy-3-hydroxymethylpentanoic acid (Fig. 1).

In S_4, the introduction of a hydroxy group at the γ-carbon of Hmp in aureobasidin A was evidenced

Table 3. Antifungal activities of aureobasidins A, S_1, S_{2a}, S_{2b}, S_3 and S_4.

Organism	TIMM No.	MIC (μg/ml)					
		Aureobasidin					
		A	S_1	S_{2a}	S_{2b}	S_3	S_4
Candida albicans	0136	0.05	25	0.4	0.2	0.1	0.1
C. albicans	0144	0.2	25	1.6	0.8	0.8	0.8
C. albicans	0171	0.05	25	0.4	0.2	0.1	0.1
C. albicans	1768	3.1	>25	3.1	3.1	6.3	3.1
C. kefyr	0301	0.8	25	0.8	0.4	0.4	0.4
C. glabrata	1062	0.2	>25	1.6	0.8	0.4	0.8
Cryptococcus neoformans	0354	0.8	>25	>25	6.3	3.1	1.6
Saccharomyces cerevisiae	9763[a]	0.4	>25	3.1	1.6	0.8	1.6

[a] ATCC No.

by the chemical shift of the carbon signal in ^{13}C NMR. The signals of adjacent β- and δ-carbons were shifted to lower fields and the signal of the ε-carbon was shifted to a higher field. Therefore, position 1 of S_4 was 2,4-dihydroxy-3-methylpentanoic acid (Fig. 1).

Biological Activity

Structure-activity relationship studies of aureobasidins A~R and their derivatives indicate that the cyclic structure and the hydroxy group at position 9 play an important role.[9] Linear peptides, peaks 1, 2 and 4 gave no growth inhibition zone against *Candida albicans* TIMM 0136 by disk diffusion assay with disks containing 2.5 μg of one of these compounds. Aureobasidins S_{2b}, S_3 and S_4, all cyclic depsipeptides containing βHOMeVal at position 9 and hydroxylated Hmp, were highly active against *Candida* spp. and *Cryptococcus neoformans*, but less active than aureobasidin A (Table 3). Aureobasidin S_{2a}, which is a cyclic depsipeptide having βHOMeVal and hydroxylated MePhe, MeTyr, showed high activity against *Candida* spp., but no activity against *C. neoformans*. Hydroxylation of aureobasidin A seems to cause some reduction of its antifungal activity depending on the position of hydroxylation; hydroxylation of Hmp caused less reduction than that of aromatic carbon of MePhe did, however hydroxylation of β-methylene of MePhe (aureobasidin E) causes little reduction.[1,4] The hydroxy group of MeTyr is a phenolic one, but that of the latter is not. The difference of antifungal activity between aureobasidin S_{2a} and E might depend on the nature of these hydroxy groups. The conformation of aureobasidin E in a crystal is formed by three intramolecular NH···O=C hydrogen bonds and two intramolecular OH···O=C hydrogen bonds formed by βHOMeVal.[10] The conformation is probably important for the activity of aureobasidins and might be disturbed by hydroxy groups of hydroxylated Hmp and MeTyr, especially by a phenolic hydroxy group. Aureobasidin S_1, a cyclic depsipeptide containing βHOMeVal and Met(O), did not have obvious antifungal activity. A compound that has Met at position 6 has as much antifungal activity as S_{2b}, S_3 and S_4 (unpublished data). Therefore, the introduction of an sulfoxide in Met as a polar group might disturb the conformation.

Experimental

General

FAB-MS and HRFAB-MS data were obtained on a JEOL JMS DX-302 spectrometer. NMR spectra were recorded in $CDCl_3$ on a JEOL JNM-GX 400 spectrometer; chemical shifts are given in ppm relative

to TMS as internal standard.

LC/MS

HPLC was done with a 4.6×250 mm Capcell Pak C_{18} column (5 μm, Shiseido Co., Ltd.) at a flow rate of 1.0 ml/minute with a Waters 650S system. The solvent for elution was acetonitrile-water-TFA (70:30:0.05). The column effluent was split 49:1 with a Valco tee to give a flow rate of 20 μl/minute into a triple-stage quadrupole mass spectrometer (Sciex API-III) equipped with ion spray source (Perkin-Elmer Sciex). The ion source was operated at 5000 V and the interface plate and the orifice voltages were adjusted to 630 and 90 V, respectively. The *m/z* of the protonated molecular ion $(M+H)^+$ observed by API-III was shown by rounding to the nearest whole number. In MS/MS, mass spectra were obtained at the collision energy of 100 eV. Argon was used as the collision gas at the density of 3.5×10^{14} atoms/cm^2.

Fermentation

A loopful cells of a slant culture of *A. pullulans* R106 was inoculated into 100 ml of medium containing 0.67% yeast nitrogen base (Difco Co.) and 2% glucose in a 500-ml Erlenmeyer flask and the flask was shaken at 25°C for 2 days to give a seed culture. The seed culture (150 ml) was transferred into a 30-liter jar fermentor containing 15 liters of medium A and was cultured at 25°C for 56 hours with aeration (15 liters/minute) and agitation (150 rpm). Then 2 liters of medium B was supplemented and fermentation was further continued at 25°C for 88 hours.

Medium A: Glucose 4%, skim milk 3%, soybean flour 3%, $(NH_4)_2SO_4$ 0.5%, KH_2PO_4 0.15%, $MgSO_4\cdot7H_2O$ 0.05%, $CaCl_2\cdot2H_2O$ 0.01%, NaCl 0.01%, $FeCl_3\cdot6H_2O$ 0.5 μg/ml and $ZnSO_4\cdot7H_2O$ 0.5 μg/ml.

Medium B: Glucose 10%, Polypepton 5%, KH_2PO_4 0.75%, $MgSO_4\cdot7H_2O$ 0.25%, $CaCl_2\cdot2H_2O$ 0.05%, NaCl 0.05%, $FeCl_3\cdot6H_2O$ 2.5 μg/ml and $ZnSO_4\cdot7H_2O$ 2.5 μg/ml.

Isolation and Purification

The fermentation broth (17 liters) was extracted with an equal volume of ethanol and then centrifuged to obtain the supernatant. The supernatant was applied to a HP-40 (Mitsubishi Chemical Industries Co., Ltd.) column (7.5×120 cm) equilibrated with 50% ethanol and eluted with 95% ethanol. The elute was diluted twice with water, applied to a ODS-W (74/150 μm, Soken Chemicals Co., Ltd.) column (4.5×60 cm) equilibrated with 40% ethanol and eluted with 60% ethanol. The fractions containing compounds eluted faster than aureobasidin F were collected and concentrated under reduced pressure. The residue was dissolved in acetonitrile and studied by LC/MS. The solution was chromatographed on a preparative ODS-silica gel column (Capcell Pak C_{18}, 10×250 mm, 5 μm) with 60% acetonitrile containing 0.1% TFA to separate peaks 1~7. Peak 3 was purified by the C_{18} column with 55% acetonitrile to isolate aureobasidin S_1 (21 mg). Peaks 6 and 7 were separately purified by a silica gel HPLC column (Soken Pak, 6×150 mm) with a solvent of hexane-2-propanol-acetonitrile (80:12:8) to obtain aureobasidins S_3 (12 mg) and S_4 (15 mg), respectively. Peak 5 was purified by the silica gel column with a solvent of hexane-2-propanol-acetonitrile (77.5:13.5:9) to obtain aureobasidins S_{2a} (6 mg) and S_{2b} (12 mg).

Aureobasidin S_1: HRFAB-MS Calcd for $C_{59}H_{91}N_8O_{12}S$: 1,135.648, Found: *m/z* 1,135.652 (M+H); FAB-MS *m/z* 1,157 (M+Na), 1,135 (M+H), 665, 536, 519, 391, 358, 210.

Aureobasidin S_{2a}: HRFAB-MS Calcd for $C_{60}H_{93}N_8O_{12}$: 1,117.691, Found: *m/z* 1,117.689 (M+H); FAB-MS *m/z* 1,139 (M+Na), 1,117 (M+H), 681, 534, 501, 407, 324, 210.

Aureobasidin S_{2b}: HRFAB-MS Calcd for $C_{60}H_{93}N_8O_{12}$: 1,117.691, Found: *m/z* 1,117.688 (M+H); FAB-MS *m/z* 1,139 (M+Na), 1,117 (M+H), 681, 534, 485, 391, 324, 226.

Aureobasidin S_3: HRFAB-MS Calcd for $C_{60}H_{93}N_8O_{12}$: 1,117.691, Found: *m/z* 1,117.689 (M+H); FAB-MS *m/z* 1,139 (M+Na), 1,117 (M+H), 681, 534, 485, 391, 324, 226.

Aureobasidin S_4: HRFAB-MS Calcd for $C_{60}H_{93}N_8O_{12}$: 1,117.691, Found: *m/z* 1,117.685 (M+H); FAB-MS *m/z* 1,139 (M+Na), 1,117 (M+H), 681, 534, 485, 391, 324, 226.

Acid Hydrolysis and Amino Acid Analysis

Aureobasidins S_1, S_{2a}, S_{2b}, S_3 and S_4 (1 mg each) were hydrolyzed with the vapor of 6 N HCl at 110°C for 22 hours in a reaction vial in which air was replaced by N_2 gas. In a sealed tube, S_1 (1 mg) was also

hydrolyzed with 10 μl of 3 M *p*-toluenesulfonic acid at 110°C for 22 hours. The reaction mixture was neutralized with 15 μl of 2 N NaOH. The hydrolysates were examined in an amino acid autoanalyzer (JEOL JCL-300) and the *N*-methylated amino acids were analyzed by HPLC as described for post-column derivatization in a previous paper.[3]

Aureobasidin S_1: βHOMeVal 0.56, MeVal 2.43, Pro 0.87, Met(O) 0.72, MePhe 1.15, Leu 1.08, Phe 1.00.
Aureobasidin S_{2a}: βHOMeVal 0.51, MeVal 2.49, Pro 0.76, aIle 0.79, MeTyr 0.97, Leu 1.34, Phe 1.00.
Aureobasidin S_{2b}: βHOMeVal 0.52, MeVal 2.17, Pro 1.06, aIle 1.09, MePhe 0.98, Leu 1.13, Phe 1.00.
Aureobasidin S_3: βHOMeVal 0.53, MeVal 1.81, Pro 1.05, aIle 1.07, MePhe 0.98, Leu 1.02, Phe 1.00.
Aureobasidin S_4: βHOMeVal 0.63, MeVal 1.89, Pro 1.02, aIle 1.04, MePhe 0.96, Leu 1.13, Phe 1.00.

Synthesis of L-MeTyr

To a solution of L-Tyr (181 mg) in 0.1 N NaOH (15 ml) was added formaldehyde (0.2 ml), and the mixture was stirred for 2 hours at room temperature. To this mixture, $NaBH_4$ (70 mg) was added with cooling on ice and the mixture was stirred for one hour at room temperature. The reaction mixture was acidified with 1 N HCl and chromatographed on a Nucleosil $5C_{18}$ column (Macherey Nagel Co.) with H_2O, giving L-MeTyr (30 mg) as a colorless powder; ^{1}H NMR (ND_4OD) δ 2.35 (3H, s, NCH_3), 2.86 (2H, d, J=6.6 Hz), 3.30 (1H, t, J=6.6 Hz), 6.69 (2H, d, J=8.6 Hz), 7.07 (2H, d, J=8.6 Hz).

Antifungal Activity

The MICs against fungi were determined by the agar dilution method on Sabouraud-dextrose agar medium. Compounds to be tested were dissolved in ethanol, the solution was diluted with 50% aqueous ethanol, and the solution was added to the medium at a final concentration of 5% to prepare agar plates. A loopful of a fungal cell suspension in sterile saline (2×10^7 cells/ml) prepared from the slant culture was streaked on the surface of the agar plates containing a test compound. After incubation of the plates at 30°C for 3 days, the lowest concentration of the test compound causing virtually complete growth inhibition was determined as the MIC.

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