Structures and Antifungal Activities of New Aureobasidins

NAOYUKI AWAZU, KATSUSHIGE IKAI, JUNKO YAMAMOTO, KAZUKO NISHIMURA, SHIGETOSHI MIZUTANI, KAZUTOH TAKESAKO and IKUNOSHIN KATO

Biotechnology Research Laboratories, Takara Shuzo Co., Ltd., 3-4-1 Seta, Otsu, Shiga 520-21, Japan

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Aureobasidins (Ab's) are a group of antifungal antibiotics produced by a black yeast, *Aureobasidium pullulans* R106.[1~4] Besides its main product AbA, there are over 20 congeners of Ab's and their structures characterized as a cyclic depsipeptide consisted of eight amino acids, three or four of which are *N*-methylated, and one hydroxy acid. In the course of our search for new Ab's, we discovered six new congeners designated as AbT_1, AbT_2, AbT_3, AbT_4, AbU_1, and AbU_2 (Table 1). Here we report their isolation, structures and antifungal activities.

The fermentation broth (3,700 liters) prepared as described previously[4] was treated with an equal volume of 95% ethanol. After mixing and centrifugation, the supernatant obtained was applied to a HP-40 (Mitsubishi Chemical Industries Co., Ltd.) column (400 liters), and the column was washed with 50% ethanol in water (1,000 liters) and eluted with ethanol (2,900 liters). Fractions having antifungal activity against *Candida albicans* were collected and concentrated *in vacuo* to obtain a mixture of Ab's (2,600 g). The mixture was dissolved in chloroform, applied on a silica-gel column (30 × 100 cm, 30~70 μm), and eluted with a solvent of hexane - 2-propanol - acetonitrile (85 : 6 : 9). A fraction containing mainly AbA, a hydrophobic fraction, and a fraction eluted slower than AbA, a hydrophilic fraction, were separately collected, concentrated *in vacuo* and dissolved in ethanol. One tenth of the respective solutions was separately applied on a ODS-silica HPLC column

Table 1. Structures and HPLC data of aureobasidins A, T_1~T_4, U_1 and U_2.

Position 1 2 3 4 5 6 7 8 9

—X1—X2—Phe—X3—Pro—X4—X5—Leu—X6—

Compound	HPLC* α value	Position					
		X1	X2	X3	X4	X5	X6
AbA	10.0	(2*R*,3*R*)Hmp	MeVal	MePhe	aIle	MeVal	βHOMeVal
AbT_1	10.6	(2*R*,3*S*)Hmp	·	·	·	·	·
AbT_2	11.2	·	·	·	·	MeLeu	·
AbT_3	11.8	D-Hiv	·	βHOMePhe	·	·	MeVal
AbT_4	12.3	·	·	·	·	MeaIle	·
AbU_1	5.2	·	Val	·	·	·	·
AbU_2	6.5	D-Hiv	·	·	Val	·	·

* The α-value is defined as relative retention time $[(t_{R,1}-t_0)/(t_{R,2}-t_0)] \times 10$, where $t_{R,1}$ and $t_{R,2}$ mean the retention times of the new aureobasidins and AbA, respectively, and t_0 is the dead retention time.

Dots (·) indicate identity with the amino acids or hydroxy acid of AbA.

Abbreviations: Hmp, 2-hydroxy-3-methylpentanoic acid; D-Hiv, D-2-hydroxyisovaleric acid; MeVal, *N*-methylvaline; Val, valine; Phe, phenylalanine; MePhe, *N*-methylphenylalanine; βHOMeVal, β-hydroxy-*N*-methylvaline; Pro, proline; aIle, alloisoleucine; MeaIle, *N*-methylalloisoleucine; MeLeu, *N*-methylleucine; Leu, leucine; βHOMePhe, β-hydroxy-*N*-methylphenylalanine.

Table 2. Amino acid composition, HRFAB-MS data, and molecular formulas of aureobasidins T_1~T_4, U_1 and U_2.

Compound	Amino acids	HRFAB-MS (*m/z*) found, calcd for M+H	Molecular formula
AbT_1	βHOMeVal 0.3, MeVal 2, Pro 1, aIle 1, MePhe 1, Leu 1, Phe 1, methylamine 0.4	1,101.697, 1,101.696	$C_{60}H_{92}N_8O_{11}$
AbT_2	βHOMeVal 0.5, MeVal 1, Pro 1, MeLeu 1, aIle 1, MePhe 1, Leu 1, Phe 1, methylamine 0.4	1,115.717, 1,115.712	$C_{61}H_{94}N_8O_{11}$
AbT_3	MeVal 3, Pro 1, aIle 1, Leu 1, Phe 1, methylamine 0.6	1,087.683, 1,087.681	$C_{59}H_{90}H_8O_{11}$
AbT_4	βHOMeVal 0.4, MeVal 1, Pro 1, MeaIle 1, aIle 1, MePhe 1, Leu 1, Phe 1, methylamine 0.3	1,115.717, 1,115.712	$C_{61}H_{94}N_8O_{11}$
AbU_1	βHOMeVal 0.6, MeVal 1, Pro 1, Val 1, aIle 1, MePhe 1, Leu 1, Phe 1, methylamine 0.2	1,087.674, 1,087.681	$C_{59}H_{90}N_8O_{11}$
AbU_2	βHOMeVal 0.6, MeVal 2, Pro 1, Val 1, MePhe 1, Leu 1, Phe 1, methylamine 0.2	1,073.667, 1,073.665	$C_{58}H_{88}N_8O_{11}$

(10×50 cm, $15 \sim 30$ μm) with 70% acetonitrile in water. Ab's $T_1 \sim T_4$ were isolated from the hydrophobic fraction by the repetitive HPLC procedures, yielding pure AbT_1 (30 mg), AbT_2 (10 mg), AbT_3 (76 mg), and AbT_4 (15 mg). Ab's U_1 and U_2 were isolated from the hydrophilic fraction by the HPLC procedures, yielding pure AbU_1 (15 mg) and AbU_2 (24 mg).

Amino acid analyses of the amino acids and *N*-methylated amino acids of the six new Ab's were carried out as described previously[2] and molecular formulas of them were determined by HRFAB-MS with Jeol-JMS DX-302 (Table 2).

AbT_1 had the same amino acid composition and molecular formula (Table 2) as AbA, and further showed the same fragment ions in its FAB-MS (Table 3) as AbA, suggesting a difference in the stereochemistry of the constituent amino acids or hydroxy acid. The stereochemistry of each amino acid residue was found to be L-form, the same as that of AbA, by HPLC with the chiral column.[2] To determine the absolute configuration of the hydroxy acid residue, Hmp of AbT_1 was purified from its hydrolysate by Dowex 50W and analyzed by ODS-silica column (Capcell Pak, 4.6×250 mm; 4% acetonitrile in 0.05% trifluoroacetic acid; UV detection at 210 nm) and Chiralpak WH column.[2] The retention time in ODS-silica HPLC of the Hmp from AbT_1 was 27.8 minutes, whereas those of synthesized (2*R*,3*R*)- or (2*S*,3*S*)-Hmp and (2*R*,3*S*)- or (2*S*,3*R*)-Hmp were 27.0 and 28.0 minutes, respectively,[2] indicating the Hmp of AbT_1 to be (2*R*,3*S*) or (2*S*,3*R*). The analysis with Chiralpak showed the configuration of 2-position of Hmp to be *R*, resulting that AbT_1 had (2*R*,3*S*)-Hmp, differing from (2*R*,3*R*)-Hmp of AbA. The structure of AbT_1 was determined as [(2*R*,3*S*)-Hmp^1]-AbA.

The molecular formula of AbT_2 and its fragment ions (*m/z* 338, 499) in the FAB-MS were larger than those of AbA by 14 daltons, a methylene unit. Further, the amino acid analysis indicated presence of MeLeu instead of MeVal of AbA (Table 2). These results revealed the structure of AbT_2 as [MeLeu^7]-AbA. AbT_4 had the same molecular formula and FAB-MS fragment ions with AbT_2. The amino acid analysis indicated presence of MeAlle instead of MeVal of AbA, indicating the structure of AbT_4 as [MeAlle^7]-AbA.

The pattern of FAB-MS fragment ions of AbT_3 was similar to that of AbE, [βHOMePhe^4]-AbA. The amino acid analysis of AbT_3 indicated absence of βHOMeVal and MePhe, and presence of 3 moles of MeVal. These results suggested substitutions of βHOMeVal with MeVal and MePhe with βHOMePhe. The hydroxy acid purified from its acid hydrolysate by Dowex 50W was identified as D-Hiv by Chiralpak WH column.[2] Thus, the structure of AbT_3 was identified as [D-Hiv^1, βHOMePhe^4, MeVal^9]-AbA.

AbU_1 was a methylene smaller than AbA, coincidently with the amino acid analysis indicating substitution of 1 mole MeVal with Val. The fragment ions at *m/z* 210 and 391 derived from the fragments containing MeVal^2 in AbA were little observed, showing presence of Val instead of MeVal^2. These results indicated the structure of AbU_1 as [Val^2]-AbA.

The molecular formula of AbU_2 was smaller than AbA by two methylene units, which was also indicated

Table 3. FAB-MS data of aureobasidins A, E, $T_1 \sim T_4$, U_1 and U_2.

Compound	$(M+H)^+$	Fragment ion peaks *m/z*
AbA[a]	1,101	210, 324, 391, 485, 518, 665
AbE[b]	1,117	210, 324, 393, 428, 501, 575, 1,011
AbT_1	1,101	210, 324, 391, 485, 518, 665
AbT_2	1,115	210, 338, 391, 499, 518, 665
AbT_3	1,087	196, 324, 393, 414, 501, 545, 981
AbT_4	1,115	210, 338, 391, 499, 518, 665
AbU_1	1,087	324, 485, 504, 651
AbU_2	1,073	196, 310, 391, 471, 504, 651

[a,b] The assignment of the fragment ions are as follows[2]:

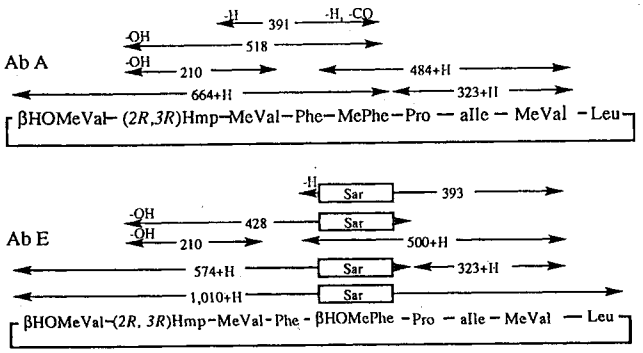

Table 4. Antifungal activity of aureobasidins A, $T_1 \sim T_4$, U_1 and U_2.

Organism*	MIC (μg/ml)						
	AbA	AbT_1	AbT_2	AbT_3	AbT_4	AbU_1	AbU_2
C.a. 0136	0.05	0.0125	0.10	0.39	0.10	0.78	0.025
C.a. 0171	0.05	0.0125	0.20	0.39	0.20	0.78	0.05
C.k. 0301	0.78	0.39	0.78	6.25	1.56	0.39	0.39
C.g. 1062	0.20	0.20	0.78	12.5	0.78	>25	0.78
Cr.n. 0354	0.78	1.56	1.56	>25	1.56	>25	6.25
S.c. 9763	0.39	0.39	0.78	25	0.39	>25	0.78

* *C.a.* 0136: *Candida albicans* TIMM 0136; *C.a.* 0171: *Candida albicans* TIMM 0171; *C.k.* 0301: *Candida kefyr* TIMM 0301; *C.g.* 1062: *Candida glabrata* TIMM 1062; *Cr.n.* 0354: *Cryptococcus neoformans* TIMM 0354; *S.c.* 9763: *Saccharomyces cerevisiae* ATCC 9763. MIC's were determined by the serial two-fold dilution method on Sabouraud-dextrose agar medium.[4]

by the amino acid analysis showing presence of Val instead of aIle, and by the hydroxy acid analysis with the chiral column indicating presence of D-Hiv instead of Hmp. Thus, the structure of AbU_2 was identified as [D-Hiv[1], Val[6]]-AbA.

The antifungal activities of the new Ab's are shown in Table 4. AbT_1, [(2*R*,3*S*)-Hmp[1]]-AbA showed a little higher activity against *C. albicans* than AbA. AbU_1, [Val[2]]-AbA, showed lowest activity among the Ab's having βHOMeVal at position 9 and was active as much as AbF, [Val[7]]-AbA, indicating importance of the *N*-methyl groups of the amino acids at positions 2 and 7 to the high activity of Ab's. The activity of AbT_3 was highest among the Ab's having no βHOMeVal at position 9, which may suggest contribution of βHOMePhe in place of βHOMeVal but contradict the activity of AbR, [βHOMePhe[4], MeVal[9]]-AbA showing little antifungal activity.[1)]

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