Precursor Directed Biosynthesis of Aureobasidins

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The antifungal antibiotic aureobasidin A (AbA) is a cyclic depsipeptide composed of eight amino acids and a hydroxy acid. New Ab analogs were produced by feeding various amino acids to Aureobasidium pullulans R106 c-712 in a chemically-defined medium containing glucose and ammonium sulfate. The constituent amino acids of AbA at positions 3 (L-phenylalanine), 4 (N-methyl-L-phenylalanine), 5 (L-proline), 6 (L-a/fo-isoleucine) and 8 (L-leucine) were replaced by respective analogous amino acids such as o-fluoro-L-phenylalanine, 4-hydroxy-L-proline, L-norleucine and L-norvaline, resulting in the production of eight new Ab analogs. This is the first paper to describe amino acid replacements at positions 3, 5 and 8. L-[l-13C]-Valine exogenously added was incorporated into the three valine-related moieties of AbA at positions 2, 7 (both N-methyl-L-valine) and 9 (β-hydroxy-N-methyl-L-valine), but these moieties were never replaced by exogenous amino acid analogs. The comparative antifungal activities of AbA and the eight new Ab analogs were determined.

Aureobasidins (Abs) are a group of potent antifungal antibiotics produced by the black yeast Aureobasidium pullulans R106.1'5) AbA (Fig. 1), the major Ab, has a moderate spectrum of antifungal activity including Candida albicans in vitro.2) AbA was orally effective in a murine infection model of candidiasis and showed very low toxicity.2) Consequently, AbA is a promising agent for use against systemic fungal infections. In addition to AbA, we have isolated over 20 closely related congeners from the fermentation broth.1'3~5) Abs are cyclic depsipeptides composed of a hydroxy acid and eight amino acids, three or four of which are N-methylated. Among the known Abs produced by A. pullulans R106, variations in the hydrox or amino acids have been observed in moieties 1, 4, 6, 7 and 9, while variations in the rest (moieties 2, 3, 5 and 8) have not been previously described.1'3~5) Studies of the structure-activity relationships of these Abs revealed that the amino acid at position 9 should be β-hydroxy-N-methyl-L-valine (βHOMEVal) for potent antifungal activity.5) Many new analogs have been synthesized using precursor-directed biosynthesis.6)

Fig. 1. Structure of aureobasidin A.

Abbreviations: D-Hmp, 2(R)-hydroxy-3(R)-methylpentanoic acid; MeVal, N-methyl-L-valine; Phe, L-phenylalanine; MePhe, N-methyl-L-phenylalanine; Pro, L-proline; afe, L-2-allo-isoleucine; Leu, L-leucine; βHOMEVal, β-hydroxy-N-methyl-L-valine.
been made by precursor-directed biosynthesis (e.g., cyclosporin analogs). Replaceability of the original amino acid moiety by exogenous amino acid analogs in depsipeptide antibiotics is thought to largely depend upon the relative pool sizes of that amino acid and exogenous analogs, and the broad substrate specificity of the non-ribosomal biosynthetic pathway. This paper describes precursor-directed biosynthesis and antifungal properties of eight new Ab analogs.

Materials and Methods

Strain

A high AbA producer, strain c-712 derived from the original strain A. pullulans R106 (FERM BP-1938), was used in this study. The strain was obtained from the producing organism by repeated single colony isolation. The original strain was cultured in 500-ml Erlenmeyer flasks each containing 100 ml of GN medium (glucose 2%, ammonium sulfate 0.5%, KH₂PO₄ 0.15%, MgSO₄·7H₂O 0.05%, CaCl₂·2H₂O 0.01%, NaCl 0.1%, FeCl₃·6H₂O 0.5 μg/ml and ZnSO₄·7H₂O 0.5 μg/ml) for 4 days with shaking. Then the flasks were supplemented with 10 ml each of 10 x GN medium and 10% polypeptide four times at an interval of 4 days and cultured totally for 20 days. From each culture broth at days-12, -16 and -20 containing 260, 457 and 600 μg/ml of AbA, respectively, 80 ~ 100 strains were isolated by conventional single colony isolation. Each isolate was cultured in 7 ml of GN medium in a test tube for 4 days, supplemented once with 0.7 ml each of 10 x GN medium and 10% polypeptide. After shaking for a further 4 days, the amount of AbA in the culture was measured by HPLC. The best isolate was cultivated in 500-ml Erlenmeyer flasks and supplemented twice with nutrients as described above for the next round of colony isolation. From day-8 and -12 culture broths containing 320 and 440 μg/ml of AbA, respectively, about 200 strains were isolated. Each isolate was cultured in a test tube as described above and the amount of AbA produced was determined by HPLC. The best isolate was cultivated in 500-ml Erlenmeyer flasks and supplemented twice with nutrients as described above for the next round of colony isolation. From day-8 and -12 culture broths containing 320 and 440 μg/ml of AbA, respectively, about 200 strains were isolated. Each isolate was cultured in a test tube as described above and the amount of AbA produced was determined by HPLC. The best isolate was cultivated in 500-ml Erlenmeyer flasks and supplemented twice with nutrients as described above for the next round of colony isolation.

Search for New Ab Analogs by Precursor Directed Biosynthesis

A loopful of cells from a slant culture of strain c-712 on potato dextrose agar medium (Nissui Pharmaceuticals, Japan) was inoculated into a tube containing 7 ml of YNBG medium (0.67% Difco yeast nitrogen base and 2% glucose) and shaken for 2 days at 25°C. The generated seed culture (0.1 ml) was transferred to a tube containing 7 ml of GN medium and shaken for 4 days. To the culture, 0.7 ml of 10 x GN medium and 8 mg of an amino or hydroxy acid listed below (Nakalai Tesque Inc., Japan) were added. As the control fermentation, only 0.7 ml of 10 x GN medium was added to a tube. The tubes were shaken for a further 4 days in duplicate. The culture broth was thoroughly mixed with an equal volume of ethanol and centrifuged. The supernatant was analyzed by reversed phase HPLC [column: Capcell Pak C₁₈ (Shiseido Co., Ltd., Japan), 6 mm i.d. x 250 mm; column temperature: 50°C; mobile phase: acetonitrile-water (7:3), 1.0 ml/minute; detection: UV 220 nm].

The additives were (i) hydroxy acids for moiety 1 including DL-2-hydroxy-3-methylpentanoic acid (dt-Hmp), DL-2-hydroxyisovaleric acid (dt-Hiv), DL-2-hydroxybutyric acid, DL-2-hydroxyisobutyric acid, DL-2-hydroxyvaleric acid and DL-2-hydroxy-4-methylpentanoic acid; (ii) valine-related amino acids for moieties 2, 7 and 9 including D-valine (D-Val), DL-norvaline (DL-Nva), DL-norleucine (DL-Nle), L-isoleucine (Ile), L- allo-isoleucine (allo), L-leucine (Leu), d-Leu, glycine and L-allo-glycine; (iii) aromatic amino acids for moieties 3 and 4 including L-tyrosine (Tyr), o-fluoro-L-phenylalanine (dt-oFPhe), m-fluoro-dL-phenylalanine (dt-mFPhe), p-fluoro-dL-phenylalanine and cyclohexyl-dL-alanine; (iv) proline analogs for moiety 5 including 4-hydroxy-L-proline (4HyP) and L-thiopropionyl (SPPr); (v) alphabetic amino acids for moieties 6 and 8 including the valine-related amino acids described above; (vi) hydroxyamino acids for moiety 9 including L-threonine, L-serine, β-hydroxy-dL-norvaline and β-hydroxy-L-valine; (vii) other unrelated amino acids including L-methionine (Met), l-cysteine, L-histidine, L-arginine, L-aspatic acid, L-lysine and L-tryptophane.

Preparation of New Ab Analogs

One ml of the seed culture was transferred to 20 ~ 70 500-ml Erlenmeyer flasks containing 100 ml of GN medium, which were shaken for 4 days at 25°C. To each flask, 10 ml of 10 x GN medium and 50 ~ 500 mg of a precursor amino or hydroxy acid were added. After shaking for 4 days, each flask was again supplemented with 10 ml of 10 x GN medium and 50 ~ 500 mg of the additive, and shaken for a further 4 days. The culture broth (2 ~ 7 liters) was collected and centrifuged. The mycelial cake was extracted with 400 ~ 1200 ml of acetone, and the extract was concentrated under reduced pressure. The residue was extracted with 100 ~ 300 ml of ethyl acetate. The ethyl acetate extract was concentrated to dryness under reduced pressure. The residue was dissolved in methanol (4 ~ 10 ml) and applied to a preparative HPLC column [column: YMC Pak C₁₈ (YMC Inc., Japan), 20 mm i.d. x 250 mm; mobile phase, acetonitrile-water (65:35), 1.0 ml/minute; detection at UV 230 nm]. The following new Abs were produced by feeding dt-oFPhe, dt-mFPhe, Tyr, 4HyP, SPPr, DL-Nle, Met, allo, and DL-Nva: [oFPhe³, oFPMePhe⁴]- (13 mg),
[mFPhe³, mFMePhe⁴]- (7 mg), [MeTyr⁴]- (3 mg),
[4Hyp⁵]- (14 mg), [SPro⁵]- (16 mg), [Nle⁶]- (48 mg),
[Met⁶]- (8 mg), [aIle⁸]- (8 mg) and [Nva⁸]-AbA (12 mg),
respectively.

Incorporation of 13C-labeled Val to AbA
One ml of the seed culture was transferred into a 500-ml flask
containing 100 ml of GN medium, which was shaken for
4 days at 25°C. Thereafter, 10 ml each of 10 × GN medium
and 10% polypeptone were added to the culture. The flask
was shaken for 2 days, then supplemented with 100 mg of [1-13C]Val
(99 atom %, Nippon Sanso Corp., Japan) and incubated for a further 2 days.
The culture broth was centrifuged and the mycelial cake obtained
was extracted with 10 ml of methanol. The extract was
concentrated to dryness under reduced pressure and the
residue was dissolved in methanol (500 μl). The solution
was applied to a preparative HPLC column as described
above, to yield 10 mg of [1-13C]Val-fed AbA.

Characterization of New Abs and 13C-labeled AbA
Structures of the new Abs were determined by FAB-MS
and amino acid analysis, and by NMR if needed.
FAB-MS spectra were obtained on a Jeol JMS DX-302
spectrometer. The Abs were hydrolyzed with 6 N HCl at
110°C for 24 hours in sealed tubes, and the amino acids
in the hydrolysates were determined by autoanalysis
using a Jeol JCL-300 amino acid autoanalyzer and by
HPLC with post-column derivatization for N-methyl
amino acids as described. 3) 13C NMR of AbA labeled
with 13C-Val was measured on a Jeol JNM FX-200
spectrometer (50 MHz).

Measurement of Antifungal Activity
Antifungal activities of the new Abs were determined
by serial two-fold dilution on Sabouraud-dextrose agar
medium. 13

Results and Discussion
Incorporation of 13C-Val into AbA
Fig. 2 shows the 13C NMR spectrum of [1-13C]Val-fed AbA.
Twice as many carbon signals were detected for carbon-13-labeled AbA because there were two conformations of AbA depending on the cis-trans rotation
of the MePhe-Pro peptide bond. 8) [1-13C]Val-fed AbA had four enriched carbonyl carbon signals at the chemical
shifts (ppm) of 169.8 (MeVal7), 169.3 and 168.2 (both
MeVal2), and 168.0 (jSHOMeVal9), showing that
exogenous 13C-Val was incorporated at positions 2, 7
and 9.

Precursor-directed Biosynthesis and
Structures of New Abs
When strain c-712 was cultured in a medium fed
simultaneously with five amino acids (Val, Phe, Pro,
Leu, Ile) as additives to GN medium, it produced almost
the same amount of AbA and other Abs as when
cultured in a medium supplemented with polypeptone
(Table 1). Val was incorporated into moieties 2, 7 (both
MeVal) and 9 (βHOMeVal), Phe into moieties 3 (Phe)
and 4 (MePhe), Pro into moiety 5, Leu into moiety 8,
and Ile into moieties 1 (β-Hmp) and 6 (aIle). The
concentration of these amino acids yielding the best
productivity was 0.05~0.2%. Each amino acid was
necessary to give high productivity of AbA and the other
Abs. These results indicate that amino acids synthesized
from glucose and ammonium sulfate in GN medium are
used to produce 100~150 μg/ml AbA, and exogenous
amino acids are incorporated into cells and used to
produce more AbA.

The results shown above suggested possible preparation
of new Abs by precursor-directed biosynthesis. To
Table 1. Production of AbA by a chemically-defined medium composed of constituent amino acids.

<table>
<thead>
<tr>
<th>Additives (%)</th>
<th>AbA production (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Val</td>
<td>Ile</td>
</tr>
<tr>
<td>0.1</td>
<td>-*</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* - no addition, +: 0.1% addition.

One ml of the seed culture was inoculated to 100 ml of GN medium in a 500-ml Erlenmeyer flask. After 4 days shaking, the culture (8 ml) was transferred to test tubes containing 10 x GN medium (0.8 ml) and additives (each 8 mg), and incubated for a further 4 days. The amount of AbA produced in the culture broth was determined by HPLC. Experiments were carried out in triplicate.

Table 2. The production of aureobasidin analogs by precursor-directed biosynthesis.

<table>
<thead>
<tr>
<th>Ab</th>
<th>HPLC a-value*</th>
<th>Production (µg/ml)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>[oFPhe3, oFMePhe4]-AbA</td>
<td>11.9</td>
<td>21/70</td>
</tr>
<tr>
<td>[mFPhe3, mFMePhe4]-AbA</td>
<td>10.4</td>
<td>6/75</td>
</tr>
<tr>
<td>[MeTyr4]-AbA (* AbS2a)</td>
<td>4.0</td>
<td>3/85</td>
</tr>
<tr>
<td>[4Hyp5]-AbA</td>
<td>6.4</td>
<td>5/88</td>
</tr>
<tr>
<td>[SPro5]-AbA</td>
<td>11.1</td>
<td>6/91</td>
</tr>
<tr>
<td>[Nle6]-AbA</td>
<td>9.3</td>
<td>39/81</td>
</tr>
<tr>
<td>[Met6]-AbA</td>
<td>6.6</td>
<td>5/92</td>
</tr>
<tr>
<td>[alle8]-AbA</td>
<td>10.4</td>
<td>8/87</td>
</tr>
<tr>
<td>[Nva8]-AbA</td>
<td>7.9</td>
<td>6/95</td>
</tr>
</tbody>
</table>

Abbreviations: oFPhe, o-fluoro-L-phenylalanine; oFMePhe, o-fluoro-N-methyl-L-phenylalanine; mFPhe, m-fluoro-L-phenylalanine; mFMePhe, m-fluoro-N-methyl-L-phenylalanine; 4Hyp, 4-hydroxy-L-proline; SPro, L-thioproline; Nle, L-norleucine; Nva, L-norvaline.

* The a-value is defined as the relative retention time
K = (tR1 - t0) / (tR2 - t0) x 10, where tR1 and tR2 mean the retention times of a new Ab and AbA, respectively, and t0 is the dead retention time. As reference compound, AbA is taken (a=10.0).

** The production of new Abs and AbA was determined by comparing the HPLC with that of a AbA standard solution.

Thus, difficulty in hydroxylation of the valine analogs may be the reason that we did not discover such Ab analogs.

Amino acids at positions 3 and 4 are probably derived from Phe, and thus aromatic amino acids, Tyr, oFPhe and mFMePhe were incorporated (Table 2). Interestingly, oFPhe and mFMePhe replaced both Phe3 and MePhe4, whereas Tyr replaced only MePhe4 to produce [MeTyr4]-AbA, AbS2a.

Pro at position 5 in AbA was substituted with the analog amino acids, 4Hyp and SPro.

Supplementation with Val specifically enhanced the production of AbC ([Val6]-AbA) several-fold. Addition of dl-Nle caused production of a large amount of a new Ab analog, [Nle6]-AbA (Table 2). Interestingly, Met, which differs from other aliphatic amino acids of moiety 6 and was used as the N-methyl donor of the four N-methylated moieties of AbA (data not shown), was incorporated into this position.

Leu8, which is analogous to alle at position 6 of AbA,
Table 3. The amino acid composition and FAB-MS of new aureobasidins.

<table>
<thead>
<tr>
<th>Ab</th>
<th>Amino acid composition*</th>
<th>FAB-MS ** m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>[oFPhe₃] AbA</td>
<td>8HOMEVal (0.6), MeVal (2), Pro (1), alle (1), Leu (1), oFPhe (1)</td>
<td>210, 324, 427, 503, 554, 701</td>
</tr>
<tr>
<td>[MeFPhe₄] AbA</td>
<td>8HOMEVal (0.5), MeVal (2), Pro (1), alle (1), Leu (1), oFPhe (1)</td>
<td>1137 (M+H), 1159 (M+Na)</td>
</tr>
<tr>
<td>[mFPhe₃] AbA</td>
<td>Pro (1), alle (1), Leu (1), mFPhe (1)</td>
<td>210, 324, 427, 503, 554, 701</td>
</tr>
<tr>
<td>[4Hyp⁵] AbA</td>
<td>4Hyp (1), alle (1), Leu (1), Phe (1)</td>
<td>1137 (M+H), 1159 (M+Na)</td>
</tr>
<tr>
<td>[SPro⁵] AbA</td>
<td>SPro (1), alle (1), Leu (1), Phe (1)</td>
<td>210, 324, 391, 503, 518, 665</td>
</tr>
<tr>
<td>[Nle⁶] AbA</td>
<td>Phe (1), Leu (1), Nle (1), Phe (1)</td>
<td>1119 (M+H)</td>
</tr>
<tr>
<td>[Met⁶] AbA</td>
<td>8HOMEVal (0.5), MeVal (2), Pro (1), alle (1), Met (1), Leu (1), Phe (1)</td>
<td>1119 (M+H), 1141 (M+Na)</td>
</tr>
<tr>
<td>[alle⁷] AbA</td>
<td>8HOMEVal (0.2), MeVal (2), Pro (1), alle (1), MePhe (1), Phe (1)</td>
<td>1101 (M+H), 1123 (M+Na)</td>
</tr>
<tr>
<td>[Nva⁸] AbA</td>
<td>Pro (1), Nva (1), alle (1), Phe (1)</td>
<td>1087 (M+H), 1109 (M+Na)</td>
</tr>
<tr>
<td>AbA</td>
<td>8HOMEVal (0.5), MeVal (2), Pro (1), alle (1), Leu (1), oFPhe (1), oFMePhe (1)</td>
<td>1101 (M+H), 1123 (M+Na)</td>
</tr>
</tbody>
</table>

* Acid hydrolysates of [mFPhe₄], [mFMePhe₄], [4Hyp⁵], [SPro⁵], [Nle⁶], and [Nva⁸]-AbAs were examined only with an amino acid autoanalyzer. The βHOMEVal residue of Abs was decomposed to afford 0.3~0.5 mol of methylamine in each Ab acid hydrolysate.

** The fragment ions of AbA are assigned as follows40:

was substituted by exogenous Nva but alle⁶ was not. Exogenous L-allylglycine also produced [Nva⁸]-AbA, presumably due to incorporation after the enzymatic reduction of the olefinic bond. Addition of alle yielded [alle⁶]-AbA containing two alle residues.

Regarding the hydroxy acid at position 1, exogenous DL-Hiv seemed to be incorporated in place of D-Hmp, because addition of DL-Hiv enhanced the amount of AbB, [D-Hiv¹]-AbA, up to 20% of total Abs and decreased that of AbA to 38%, whereas normally AbA and AbB are 89 and 3% of the total, respectively. In contrast, DL-2-hydroxybutyric acid, DL-2-hydroxyisobutyric acid, DL-2-hydroxyvaleric acid and DL-2-hydroxy-4-methylpentanoic acid were not incorporated. The hydroxy acids were inhibitory to growth of the producing organism and the production of Abs: specifically DL-2-hydroxy-4-methylpentanoic acid showed strong growth inhibition and 70% inhibition of AbA production. However, the result shown in Table 1 suggests the possibility of incorporation and conversion of other related amino acids to position 1. Among the known Abs, position 1 is only occupied by D-Hmp, its hydroxylated derivatives, or D-Hiv. These few variations may indicate the restricted substrate specificity of the responsible biosynthetic pathway at the step of Hmp uptake as is true of other depsipeptide antibiotics.¹⁰,¹¹

Antifungal Activities of the New Abs

All the new Ab analogs had potent antifungal activity (Table 4). The Abs having a replacement at position 8 were highly active: [Nva⁸]-AbA was as active as AbA and [alle⁶]-AbA was the most active among the currently
related amino acids caused little decrease of antifungal activity against all the fungi tested.

### Table 4. Antifungal activities of new aureobasidins.

<table>
<thead>
<tr>
<th>Ab</th>
<th>MIC (µg/ml)</th>
<th>C.a.</th>
<th>C.k.</th>
<th>C.g.</th>
<th>Cr.n.</th>
<th>S.c.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AbA</td>
<td></td>
<td>0.025</td>
<td>0.39</td>
<td>0.20</td>
<td>0.78</td>
<td>0.39</td>
</tr>
<tr>
<td>[oFPhe³, oFMePhe⁴]-AbA</td>
<td>0.025</td>
<td>0.39</td>
<td>0.39</td>
<td>6.25</td>
<td>1.56</td>
<td></td>
</tr>
<tr>
<td>[oFPhe³, oFMePhe⁴]-AbA</td>
<td>0.025</td>
<td>0.78</td>
<td>0.78</td>
<td>6.25</td>
<td>1.56</td>
<td></td>
</tr>
<tr>
<td>[4Hyp⁵]-AbA</td>
<td>0.10</td>
<td>0.39</td>
<td>0.39</td>
<td>1.56</td>
<td>0.78</td>
<td></td>
</tr>
<tr>
<td>[SPro⁵]-AbA</td>
<td>0.05</td>
<td>0.78</td>
<td>0.39</td>
<td>1.56</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>[Nle⁶]-AbA</td>
<td>0.05</td>
<td>0.39</td>
<td>0.78</td>
<td>6.25</td>
<td>1.56</td>
<td></td>
</tr>
<tr>
<td>[Met⁶]-AbA</td>
<td>0.10</td>
<td>0.78</td>
<td>1.56</td>
<td>6.25</td>
<td>1.56</td>
<td></td>
</tr>
<tr>
<td>[alle⁸]-AbA</td>
<td>&lt;0.006</td>
<td>0.20</td>
<td>0.05</td>
<td>0.78</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>[Nva⁸]-AbA</td>
<td>0.025</td>
<td>0.39</td>
<td>0.20</td>
<td>0.78</td>
<td>0.20</td>
<td></td>
</tr>
</tbody>
</table>

C.a., Candida albicans TIMM 0136; C.k., Candida kefyr TIMM 0301; C.g., Candida glabrata TIMM 1062; Cr.n., Cryptococcus neoformans TIMM 0354; S.c., Saccharomyces cerevisiae ATCC 9763.

known Abs. Since the β-hydroxy group of βHOMeVal is important for antifungal activity, even a slight change in the alkyl chain of moiety 8, the adjacent amino acid, may significantly affect the interaction between the hydroxy group and the target molecule of fungi and thus influence the antifungal activity.

Replacement of alle⁸ with Nle or Met decreased the activity specifically against *Cryptococcus neoformans*. [oFPhe³, oFMePhe⁴]- and [mFPe³, mFMePhe⁴]-AbA were as active as AbA against *Candida* sp., but less so against *C. neoformans* and *Saccharomyces cerevisiae*. The anti-cryptococcal activity was more easily reduced than the anti-candidal activity by a subtle change in constituent amino acids.¹⁴) [alle⁸]-AbA was more active against *Candida* sp. than AbA and was as potent as AbA against *C. neoformans*. Replacement of Pro³ by

### References


