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NOTE

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Generation of New Benanomicin Analogues by Biotransformation Using *Escherichia coli* Expressing Actinomycete Cytochrome P450

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Abstract Benanomicins were found as antifungal antibiotics from the culture of an actinomycete with potent antifungal activities *in vitro* and *in vivo*. We aimed to generate derivatives superior to benanomicin A by biotransformation using *Escherichia coli* constructed with bacterial P450 expression system. We found transformation of benanomicin A into two derivatives, 10-hydroxybenanomicin A and 11-*O*-demethylbenanomicin A by one of the P450-expressed strains which harbored a plasmid carrying a CYP105C1-homologous gene. Unexpectedly, the biotransformed compounds showed weak antifungal activities *in vitro* compared with those of benanomicin A.

Keywords benanomicin, biotransformation, P450, actinomycete, *E. coli*

Benanomicin A, possessing a benzo(a)naphthacenequinone skeleton, was isolated from the culture of *Actinomadura spadix* MH193-16F4 from a soil sample, and represented the broad antimicrobial spectrum against a wide range of fungi including pathogens of endemic and opportunistic mycoses [1~3]. Benanomicin A showed the potent antifungal activities in animal models using *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans* [4, 5]. Moreover, benanomicin A showed antiviral activities [6, 7]. The mechanism of action of benanomicin A was deduced to bind to fungal cell wall, but it has not been confirmed clearly [8, 9]. On the other hand, pradimicins were found as antifungal compounds at almost the same time of the discovery of benanomicins [10~12]. The structures of pradimicins are closely related to those of benanomicins and the mechanism of action in their antifungal activity was clarified to bind specifically to D-mannoside of the fungal cell surface in the presence of calcium [13, 14].

Benanomicin A was obtained from the culture broth of *Actinomadura spadix* MH193-16F4 using the methods mentioned in the previous paper [1].

In our previous study, we developed the versatile expression system for bacterial P450 genes using *Escherichia coli* BL21(DE3) as a host. Based on the system, we created the bacterial P450 expression library which serves the rapid biotransformation screening with help of co-expressed *Pseudomonas* redox partner genes *cam*AB [15]. In our attempt to obtain benanomicin derivatives with improved biological activities, we took an approach to test P450-catalyzed transformation of benanomicin A by the strain of the bacterial P450 expressed *E. coli*, since the P450s are able to catalyze monooxygenation (principally hydroxylation) to a diverse set of organic compounds. For the expression of the P450 genes in the *E. coli* BL21(DE3) strains, 25 ml of M9 mix-ampicillin medium (6.78 g/liter Na_2HPO_4, 3.0 g/liter

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KH_2PO_4, 0.5 g/liter NaCl, 1.0% casamino acid, 0.4% D-glucose, 0.1 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.1 mM $FeSO_4$, 20 μg/ml thymine and 50 μg/ml ampicillin) was inoculated with 0.5 ml of the culture over night, and was cultured further at 37°C. When the optical density at 660 nm reached 0.7 to 0.8, isopropyl-thio-β-D-galactoside (IPTG) and 5-aminolevulinic acid were added to the culture at the final concentration of 0.1 and 0.5 mM respectively, and the cells were grown for a further 20 hours at 22°C. The cells were harvested by centrifugation at 6,000 *g* for 10 minutes at 4°C, and the pellet was resuspended in 5.0 ml of CV2 buffer (2.0% glycerol, 50 mg/ml ampicillin, 0.1 mM IPTG and 50 mM phosphate buffer at pH 7.4). One hundred microgram of benanomicin A was dissolved in 20 μl of H_2O, and was added to the 0.5 ml of the cell suspension at a final concentration of 200 μg/ml. After incubation using a rotary shaker under 200 rpm at 28°C for 24 hours, the reaction products were extracted with 0.5 ml of 1-BuOH at pH 2.5. The organic layer was evaporated to dryness under reduced pressure using a centrifugal evaporator. The residue was dissolved in 0.2 ml of MeOH for HPLC analysis (Waters 2695 HPLC system, CAPCELL PAK UG120 5.0 μm, column 4.6×100 mm, Shiseido Co. LTD., Japan, flow rate 1.0 ml/minute, column temperature 40°C, diode array detection in 15 minutes) with acetonitrile concentration linear-gradient in water containing 0.1% TFA from 20% (v/v) to 90% (v/v).

The biotransformation was carried out with over 200 strains of the P450 library. Subsequent HPLC analysis of the extracts from the reaction mixture revealed that one strain BL21(DE3)/pCP55-camAB converted benanomicin A to new compounds, and the UV spectra in HPLC analysis showed new peaks similar to those of benanomicin A (Fig. 1). Then, we performed a large-scale microbial conversion using this strain to clarify the structure and biological activities of the newly formed compounds.

The P450-expressing strain, BL21(DE3)/pCP55-camAB, was inoculated into 2.0 ml of M9 medium in test tube and cultured under 200 rpm at 37°C for 3 hours to give an optical density reading of about 0.8 at 660 nm. Two hundred microliters of the culture were inoculated into 225 ml of M9 medium in a 500-ml Erlenmyer flask and cultured under 125 rpm at 37°C for 3 hours to give an optical density reading of about 0.8 at 660 nm. IPTG and 5-aminolevulinic acid were added to the culture at the final concentration of 0.1 and 0.5 mM respectively, and cultured at 22°C for 24 hours. Ten milliliters of benanomicin A (5.0 mg/ml in H_2O) and 12 ml of glycerin were added to the culture to 200 μg/ml and 5.0% respectively, and cultured at 28°C for 24 hours. The culture supernatant obtained by centrifugation of the culture at 12,000 rpm at 4°C for 15 minutes was adjusted to pH 2.5 with 1.0 M HCl and extracted with equal volume of 1-BuOH. The extract was evaporated to dryness under reduced pressure, and the resultant residue was dissolved in DMF. The solution was placed in a refrigerator over night, and the resultant DMF-insoluble materials such as proteins were removed by centrifugation at 3,000 rpm for 20 minutes. The supernatant was evaporated to dryness under reduced pressure and dissolved in the solvent composed with 1-BuOH - MeOH -

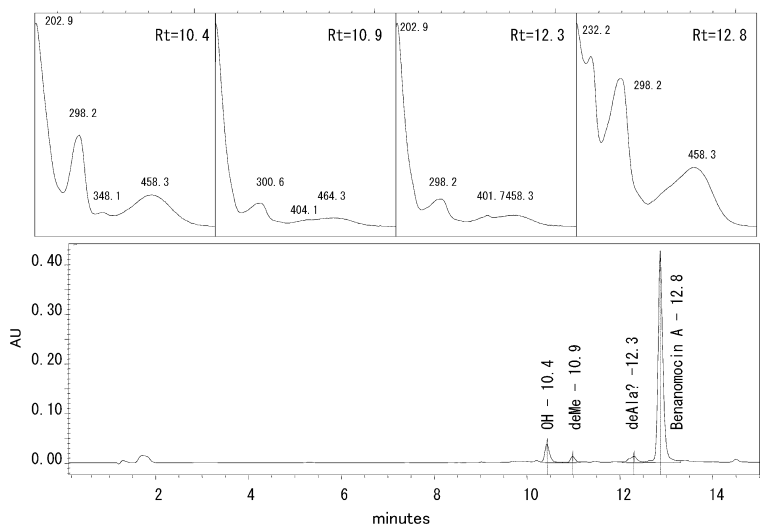

Fig. 1 HPLC analysis of the extract from bioconversion of benanomicin A using a *Escherichia coli* transfected an actonomycete P450 gene.

UV spectra for each of the four compounds are shown at the top of the figure.

0.2 M NH_4OH (4 : 1 : 5). The solution was subjected to a Centrifugal Partition Chromatography (CPC). The chromatography was performed using CPC apparatus (Senshu Scientific Co. LTD., Japan) with a solvent system of 1-BuOH - MeOH - H_2O (4 : 1 : 5) by ascending mode to remove untransformed benanomicin A from the extract. The fractions containing the compound which was eluted at 12.3 minutes in the HPLC analysis were collected. Its structure was presumed to be dealaninyl derivative of benanomicin A which was known previously [16], since this compound showed *m/z* 755 $(M-H)^-$ in the mass spectrum smaller than 827 of molecular weight of benanomicin A. We supposed that this compound was converted from benanomicin A not by P450 but by enzymes such as peptidases of *E. coli*. The partially purified benanomicin A analogues were further purified using the CPC apparatus with a solvent system of $CHCl_3$ - MeOH - 0.1% AcOH aq (5 : 6 : 4) by ascending mode. Two analogues of benanomicin A were purified and collected as free forms. These structures of the benanomicin analogues were clarified as 10-hydroxybenanomicin A (**1**, retention time was 10.4 minutes) and 11-*O*-demethylbenanomicin A (**2**, retention time was 10.9 minutes) as mentioned below. The conversion ratios of the two analogues from benanomicin A were 1.5 and 3.0%, respectively. The P450 gene on the plasmid pCP55-camAB, which was responsible for the conversion, had been amplified by PCR from the genomic DNA of an unidentified actinomycete strain previously isolated from a soil sample in our laboratory. As primers designed based on the sequence of a streptomycete P450 gene *choP* encoding CYP105C1 [17] had been used for the amplification of the gene, the gene showed a high homology to *choP* (>98% identify at the amino acid sequence level) encoding CYP105C1. We designated the P450 gene *cphP* (*choP*-homologous-P450). The sequence data of *cphP* can be found in the DNA database of Gen Bank/EMBL/DDBJ with the accession number AB425822.

^{1}H- and ^{13}C-NMR, DEPT, HMQC and HMBC spectra of **1** were measured using a JEOL JNM-ECA600 apparatus (JEOL Ltd., Japan) and compared with those of benanomicin A. The assignments established by these spectra were summarized in Table 1. The chemical shifts of ^{1}H- and ^{13}C-NMR of **1** and benanomicin A were quite similar except for those of A-ring. Among them, the C-10 was completely different from each other; C-10 (δ 106.9) of benanomicin A was shifted downfield (δ 141.4) and an exchangeable proton at δ 10.4 newly observed in **1**. The molecular formula of **1**, $C_{39}H_{41}NO_{20}$, was established by HRESI-MS (JMS-T100LC apparatus, JEOL Ltd., Japan), which possesses one more oxygen atom compared with benanomicin A. These results suggested that **1** was oxygenated at position 10 of benanomicin A. The results of the HMBC experiments shown in Fig. 3 also supported the structure.

The structure of **2** was determined by the same procedure described above. The molecular formula of **2**, $C_{38}H_{39}NO_{19}$, was established by HRESI-MS, which differed from that of

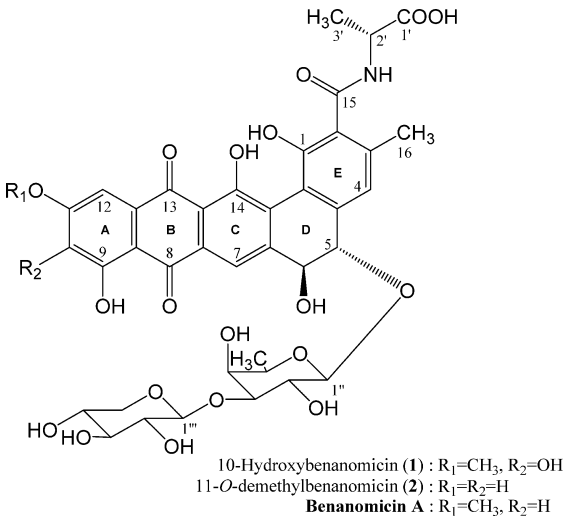

Fig. 2 Structures of benanomicin A and its analogues.

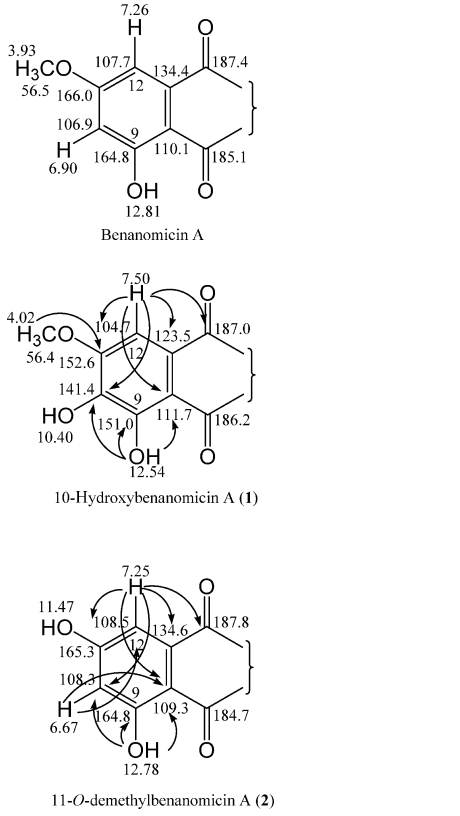

Fig. 3 Partial long-range coupling of benanomicin A and its analogues.

Table 1 ^{1}H- and ^{13}C-NMR data of benanomicin A and its analogues

Position	10-Hydroxybenanomicin A (**1**)		11-*O*-demethylbenanomicin A (**2**)		Benanomicin A	
	^{13}C	^{1}H (multiplicity, *J* Hz)	^{13}C	^{1}H (multiplicity, *J* Hz)	^{13}C	^{1}H (multiplicity *J* Hz)
1-OH		8.60 (br)		8.65 (br)		8.70 (br)
1	151.0		150.9		151.2	
2	127.8		127.5		127.6	
3	137.4		137.3		137.5	
4	118.2	7.19 (br)	118.3	7.19 (br s)	118.6	7.20 (br s)
4a	138.1		138.0		138.1	
5	82.0	4.51 (br d 9.8)	82.0	4.51 (br d, 10.2)	81.7	4.52 (br d, 10.4)
6	72.0	4.56 (br)	71.9	4.56 (br)	71.9	4.57 (br)
6a	147.0		147.6		147.7	
7	115.4	8.06 (br)	115.5	8.07 (br s)	115.4	8.05 (s)
7a	131.3		131.4		131.4	
8	186.2		184.7		185.1	
8a	111.7		109.3		110.1	
9	151.0		164.8		164.8	
9-OH		12.54 (s)		12.78 (s)		12.81 (s)
10	141.4		108.3	6.67 (d, 2.3)	106.9	6.90 (dd, 1.2, 2.3)
10-OH		10.40 (br s)				
11	152.6		165.3		166.0	
11-OH				11.47 (br s)		
11-OCH_3	56.4	4.02 (s)			56.5	3.93 (s)
12	104.7	7.50 (s)	108.5	7.25 (d, 2.3)	107.7	7.26 (dd, 1.2, 2.3)
12a	123.5		134.6		134.4	
13	187.0		187.8		187.4	
13a	114.6		115.5		115.6	
14	156.7		156.8		157.1	
14-OH		14.36 (br)		13.87 (br)		13.82 (br)
14a	125.8		125.2		125.6	
14b	113.7		113.6		113.7	
15	166.9		166.9		162.0	
16	19.1	2.31 (s)	19.1	2.32 (s)	19.2	2.33 (s)
1′	174.1		174.0		174.2	
1′-OH				12.50 (br)		12.53 (br)
2′	47.7	4.40 (quintet, 7.2)	47.6	4.40 (quintet, 7.2)	47.7	4.41 (quintet, 7.3)
2′-NH		8.53 (d, 7.2)		8.53 (d, 7.2)		8.54 (d, 7.3)
3′	16.9	1.33 (d, 7.2)	16.8	1.33 (d, 7.2)	16.9	1.34 (d, 7.3)
1″	104.7	4.64 (br d, 6.9)	104.4	4.64 (br d, 8.0)	104.5	4.65 (d, 7.0)
2″	70.1	3.70 (br)	70.1	3.72 (br)	70.1	3.74 (br)
3″	83.2	3.54 (m)	83.1	3.53 (m)	83.2	3.55 (m)
4″	70.4	3.60 (m)	70.1	3.61 (m)	70.4	3.61 (m)
5″	70.1	3.59 (m)	70.3	3.60 (m)	70.2	3.61 (m)
6″	16.4	1.11 (d, 6.3)	16.4	1.11 (d, 6.3)	16.5	1.12 (d, 6.3)
1‴	105.4	4.41 (br d, 7.3)	105.3	4.41 (d, 7.8)	105.4	4.42 (d, 7.3)
2‴	73.7	3.11 (dd, 7.3, 9.0)	73.6	3.11 (dd, 7.8, 9.0)	73.7	3.11 (dd, 7.3, 8.8)
3‴	76.1	3.14 (t, 9.0)	76.0	3.14 (t, 9.0)	76.1	3.15 (t, 8.8)
4‴	69.4	3.29 (m)	69.4	3.29 (ddd, 5.6, 9.0, 10.8)	69.5	3.30 (ddd, 5.4, 8.8, 11.0)
5‴	65.9	3.07 (t, 10.8)	65.6	3.07 (t, 10.8)	65.7	3.08 (t, 11.0)
		3.70 (dd, 5.3, 10.8)		3.70 (dd, 5.6, 10.8)		3.71 (dd, 5.4, 11.0)

Chemical shifts in ppm with TMS as an internal standard. Solvent: DMSO-d_6 (25°C).

Table 2 Antifungal activities of benanomicin A and its analogues

Strains	Benanomicin A	10-OH benanomicin A	11-*O*-deMe benanomicin A
Candida albicans 3147	6.25	>100	25
Candida albicans NCCLS QC strain	6.25	>100	12.5
Candida albicans flucytosine-R NCCLS QC strain	6.25	>100	25
Candida albicans azole-R	6.25	>100	25
Candida sp. YU-1200	12.5	100	25
Candida tropicalis	12.5	>100	25
Candida pseudotropicalis	3.13	25	6.25
Candida krusei	6.25	>100	50
Cryptococcus neoformans v. *neof.sero* A	1.56	25	25
Cryptococcus neoformans	1.6	>100	25
Saccharomyces cerevisiae	1.6	>100	3.13
Trichophyton rubrum (IFO 9185)	25	>100	>100
Trichophyton rubrum (TIMM 2659)	25	25	>100
Trichophyton asteroides 429	25	>100	>100
Trichophyton mentagrophytes	25	>100	>100
Aspergillus niger	3.13	>100	50
Aspergillus fumigatus (IFO 9733)	3.13	>100	>100
Aspergillus fumigatus (TIMM 2905)	6.25	>100	>100
Cochliobolus miyabeanus	12.5	>100	>100
Pyricularia oryzae	25	>100	>100
Pellicularia filamentoza sasakii	6.25	25	25

benanomicin A in CH_2 unit. The difference of the NMR data between **2** and benanomicin A was absence of methoxy group at position 11 in benanomicin A and was to observe an exchangeable proton at δ 11.47 in **2** newly. The other chemical shifts of ^{1}H- and ^{13}C-NMR of **2** and benanomicin A were almost same. These data showed that the structure of **2** was 11-*O*-demethylbenanomicin A. The correlations derived from the HMBC experiment shown in Fig. 3; from H-10 (δ 6.67) to C-8a (δ 109.3), C-12 (δ 108.5), from H-12 (δ 7.25) to C-8a, C-10 (δ 108.3), C-11 (δ 165.3), C-12a (δ 134.6), C-13 (δ 187.8), from 9-OH (δ 12.78) to C-8a, C-9 (δ 164.8), C-10, supported the structure.

Antifungal activities of benanomicin A and its analogues were evaluated using an agar dilution method, and were summarized in Table 2. Benanomicin A exerted antifungal activities against a wide range of fungi. The analogues of benanomicin A failed to exert the antifungal activities superior to those of benanomicin A. Antifungal activities of 11-*O*-demethylbenanomicin A was weak compared with those of benanomicin A, especially against *Trichophyton*, *Aspergillus*, *Cochliobolus* and *Pyricularia*. Antifungal activities of 10-hydroxy-benanomicin A were weaker than those of 11-*O*-demethylbenanomicin A, and showed weak antifungal activities against some fungi such as *Candida pseudotropicalis*. These results suggest that the hydrogen and methoxy groups at the position 10 and 11 are necessary for exerting the potent antifungal activities in benanomicin A.

In this paper, we described that two analogues of benanomicins were produced by the biotransformation system using *E. coli* expressing actinomycete cytochrome P450, and showed antimicrobial activities against some fungi. While we could not generate new compound superior to benanomicin A in this study, this approach should be useful for a development of pharmaceuticals from antibiotics which are not in clinical use.

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