NEW ANTIFUNGAL ANTIBIOTICS, PRADIMICINS D AND E

GLYCINE ANALOGS OF PRADIMICINS A AND C

Yosuke Sawada, Maki Nishio, Haruaki Yamamoto, Masami Hatori, Takeo Miyaki, Masataka Konishi and Toshikazu Oki

Bristol-Myers Research Institute, Ltd., Tokyo Research Center,
2-9-3 Shimo-meguro, Meguro-ku, Tokyo 153, Japan

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New antifungal antibiotics pradimicins D and E were isolated from the culture filtrates of *Actinomadura hibisca* P157-2 (ATCC 53557) and its mutant A2660 (ATCC 53762). The structure of pradimicin D is *N*-[[(5*S*,6*S*)-5-*O*-[4,6-dideoxy-4-(methylamino)-3-*O*-(β-D-xylopyranosyl)-β-D-galactopyranosyl]-5,6,8,13-tetrahydro-1,6,9,14-tetrahydroxy-11-methoxy-3-methyl-8,13-dioxobenzo[*a*]naphthacen-2-yl]carbonyl]glycine, based on spectral analyses compared to pradimicin A. Pradimicin E is the des-*N*-methyl analog of pradimicin D. Pradimicins D and E were equal in activity to pradimicin A *in vitro* against a variety of fungi and *in vivo* against *Candida albicans* A9540 in mice.

Pradimicin, a complex of novel antifungal antibiotics, was found in the culture filtrate of *Actinomadura hibisca* P157-2 (ATCC 53557). In addition to a principal component pradimicin A, two accessory components pradimicins B and C have been isolated from the culture filtrate as described in the previous reports.[1~4] Recently, the closely related antifungal antibiotics benanomicins A and B produced by an actinomycete strain, MH193-16F4, have been reported.[5,6]

Our efforts to isolate other congeners from the culture filtrate of *A. hibisca* P157-2 resulted in the discovery of two minor components (designated pradimicins D and E). Also improvement of the producing strain has been investigated. A mutant strain A2660 (ATCC 53762) was isolated after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) treatment of *A. hibisca* P157-2. The mutant produced substantial amounts of pradimicins D and E. These new congeners were isolated and their structures were determined by comparative spectral analyses with pradimicin A. Pradimicins D and E contained glycine as the amino acid moiety instead of D-alanine found in pradimicin A. *In vitro* and *in vivo* antifungal activities of these congeners were tested against various yeasts and fungi. This paper deals with the production, isolation, structural determination, and antifungal activity of pradimicins D and E.

Materials and Methods

Mutation

The spores of *A. hibisca* P157-2 were grown on a modified Bennett's agar medium composed of soluble starch 0.5%, glucose 0.5%, fish meat extract 0.1%, yeast extract 0.1%, NZ-case 0.2%, NaCl 0.2%, $CaCO_3$ 0.1% and agar 1.6%, pH 7.0, for 10 days at 28°C. They were suspended in saline, dispersed by ultrasonic vibration in an ice-bath into single cell units, harvested by centrifugation at 5,000 rpm for 10 minutes at 25°C, and resuspended in 10 mM Tris-HCl, pH 9.0. The spore suspension was mixed with MNNG (5,000 μg/ml) and gently shaken for 1 hour at 28°C. The MNNG-treated spores were harvested by centrifugation, resuspended in saline, spread on a fresh agar plate, and then incubated at 28°C for 7 days. Each colony was transferred to a fresh agar medium and incubated at 28°C for 7 days.

Screening for Pradimicins D and E Producing Mutants

An agar plug of each colony was transferred to medium A consisting of glucose 3%, soybean meal 3%, Pharmamedia 0.5%, yeast extract 0.1% and $CaCO_3$ 0.3%, pH 7.0, (10 ml/50 ml-Erlenmeyer flask) and incubated at 28°C for 6 days at 200 rpm on a rotary shaker. Identification of antibiotics was carried out by silica gel TLC (Kieselgel $60F_{254}$, 0.25 mm thick, Merck), solvent system: Methyl acetate - propanol - 28% ammonia (3 : 7 : 4) and HPLC (Waters M600, column: YMC A-301-3, ODS, 4.6 mm i.d. × 100 mm, 3 μm, Yamamura Chemical Lab.), elution: CH_3CN - 0.15% KH_2PO_4, pH 3.5 (1 : 3), flow rate: 0.7 ml/minute, detection: 254 nm. Samples for TLC were prepared as follows: The fermentation broth was adjusted to pH 2.0 with 6 N HCl and mixed with the same volume of BuOH; the BuOH layer was separated by centrifugation at 10,000 rpm for 10 minutes. The BuOH layer (10 μl) was applied on a silica gel plate. Rf values of pradimicins A: 0.45, C: 0.40, D: 0.36, E: 0.31. Samples for HPLC were prepared as follows: The broth was adjusted to pH 2.0 and centrifuged to remove impurities. The supernate was adjusted to pH 5.0 with 6 N NaOH and centrifuged. The sediment obtained was dissolved in water at pH 3.5 and passed through a Sep-Pak C_{18} cartridge filter (Millipore-Waters) which was eluted with a mixture of CH_3CN - 0.15% KH_2PO_4, pH 3.5 (1 : 1). The filtrate was mixed with an equal volume of DMSO and passed through a HV filter (Millipore, 0.45 μm). A few μl of the sample was analyzed. Retention time (minutes) of pradimicins A: 22.14, C: 19.60, D: 16.21, E: 14.37.

Production of Pradimicins D and E

A. hibisca P157-2 or the mutant strain A2660 was maintained on a agar slant medium composed of the modified BENNETT's medium as above described. A small piece of the microorganisms on the slant culture was inoculated to a 500-ml Erlenmeyer flask containing 100 ml of medium B consisting of glucose 1.0%, soluble starch 2.0%, NZ-amine A 0.5%, yeast extract 0.5% and $CaCO_3$ 0.1% (pH 7.2) and incubated at 28°C for 4 days at 200 rpm. A 5-ml aliquot of this seed culture was transferred to a 500-ml Erlenmeyer flask containing 100 ml of medium A and shake cultured at 28°C. Fermentation period throughout this paper was 6 days. Total antibiotic production was determined by the UV method as follows: After the supernate of the culture broth was diluted to an appropriate concentration with 0.1 N NaOH - MeOH (1 : 1), the optical density was measured at 500 nm. The antibiotic concentration was expressed in μg/ml, defined as the amount of pradimicin A. The component ratio of the antibiotics was determined by the HPLC method described previously.

Isolation and Purification of Pradimicins D and E

The fermentation broth (60 liters) of *A. hibisca* P157-2 (or the mutant strain A2660 (15 liters)) was centrifuged and the supernate was adjusted to pH 2.0 with 6 N HCl. After the resultant brown precipitate was removed by filtration, the filtrate was adjusted to pH 5.0 with 6 N NaOH to deposit a dark red solid of pradimicins. This solid was thoroughly stirred with a mixture of BuOH - MeOH - 1% NaCl, pH 2.0 (3 : 1 : 4, 20 liters). The organic layer was separated and was extracted with 6.5 liters of water adjusted to pH 8.0 with NaOH. The aqueous layer was adjusted to pH 2.0 with 1 N HCl and subjected to column chromatography on Diaion HP-20 eluted with 60% aqueous acetone (pH 3.0). The red eluate was concentrated *in vacuo* to afford a semi-pure solid of the antibiotic (5.6 g). Half of the solid was subjected to reversed-phase silica gel chromatography (ODS-A60, Yamamura Chemical Lab., 8 × 100 cm) developed with CH_3CN - 0.15% KH_2PO_4, pH 3.5 (21 : 79). Components in the eluate were examined by HPLC and pale orange-colored fractions appearing before pradimicin C-fractions were pooled and desalted using Diaion HP-20 chromatography. These chromatographic steps were repeated with the other half of the semi-pure solid. The product thus obtained was enriched in the two components D and E. The pooled solid (128 mg) was charged on a column of the reversed phase silica gel (ODS-A60, 8 × 90 cm) and eluted with the solvent mixture described above; the eluate was examined by HPLC. Pradimicin E was eluted first followed by pradimicin D. The relevant fractions were pooled, concentrated *in vacuo* and desalted by Diaion HP-20 chromatography to yield nearly homogeneous pradimicin E hydrochloride (19 mg from strain P157-2 or 471 mg from strain A2660) and pradimicin D hydrochloride (9 or 193 mg from respective strains). In order to convert the hydrochloride to the free form and also to remove contaminating inorganic salts, an aqueous solution of each hydrochloride salt was adjusted to pH 5.0 with 0.1 N NaOH to deposit a pure zwitterionic form.

Antifungal Activity *In Vitro* and *In Vivo*

The MICs against various yeasts and fungi were determined by the serial agar dilution method using Sabouraud dextrose agar, pH 7.0. Approximately 3 μl of fungal suspension containing 10^6 cells/ml was applied to the surface of agar plates containing the test samples. The MIC values were recorded after the cultures had been incubated for 3 days at 28°C. *In vivo* antifungal activity was evaluated against intravenous infections with *Candida albicans* A9540 in mice. The test strain was cultured for 18 hours at 28°C in YGP medium (yeast extract 0.2%, glucose 1.5%, peptone 0.5%, K_2HPO_4 0.05% and $MgSO_4$ 0.05%) and suspended in saline. Male ICR mice weighing 20 to 24 g were infected intravenously with about 10 times the median lethal dose of the fungi (1×10^6 cells/mouse). Various doses of the test compounds were administered to groups of 5 mice each intravenously just once after fungal challenge. The 50% protective dose (PD_{50}) was calculated from survival rates recorded on the 20th day after the fungal challenge.

General

MP's were measured by a Yanagimoto micro melting point apparatus type MP-S3. The IR absorption spectra were obtained with a Jasco IR-810 spectrometer (KBr pellet) and the UV spectra with a Jasco UVIDEC-610C spectrometer. The ^{1}H and ^{13}C NMR spectra were recorded on a Jeol JMN-GX 400 spectrometer operated in Fourier transform mode using tetramethylsilane as the internal standard. SI-MS were obtained with a Hitachi M80B mass spectrometer. HRFAB-MS were run with a Jeol JMS-AX 505H spectrometer. Optical rotations were determined with a Jasco Model DIP-140. CD spectrum was recorded using a Jasco J-500A spectrometer.

Results

Production of Pradimicins D and E

During the course of our mutation study of *A. hibisca* P157-2 by MNNG treatment, a strain which produced increased amounts of pradimicins D and E was isolated from approximately 3,000 mutant strains tested and designated strain A2660. Ratio of pradimicin components produced by the parent strain and the mutant strain in medium A is shown in Table 1. The mutant strain A2660 produced almost equal amounts of the four components, while the parent strain P157-2 produced predominantly pradimicin A with only small amounts of pradimicins D and E. Morphological characteristics and carbohydrate utilization of the mutant strain A2660 were identical with those of the parent strain P157-2.

Physico-chemical Properties and Structures of Pradimicins D and E

Pradimicins D and E exhibited physico-chemical properties similar to those of pradimicin A as summarized in Table 2. These components are distinguished from each other and from other pradimicin components by their HPLC and TLC mobilities (see Materials and Methods). Pradimicins D and E are soluble in dimethyl sulfoxide, *N,N*-dimethylformamide, and acidic or alkaline water, slightly soluble in ethanol, methanol and neutral water, but practically insoluble in common organic solvents. The UV and visible spectra of pradimicins D and E at various pHs are nearly identical with the corresponding spectra of pradimicin A. The molecular formulas of pradimicins D and E were determined to be $C_{39}H_{42}N_2O_{18}$ and $C_{38}H_{40}N_2O_{18}$, respectively, based on their HRFAB-MS and ^{13}C NMR spectra. The structures of

Table 1. Production of pradimicins D and E by strains P157-2 and A2660.

Strain	Total production (μg/ml)	Ratio (%)			
		Pradimicin A	Pradimicin C	Pradimicin D	Pradimicin E
P157-2 (parent)	830	91.6	7.6	0.6	0.2
A2660 (mutant)	740	36.1	21.6	22.7	19.6

Table 2. Physico-chemical properties of pradimicins D and E.

	Pradimicins	
	D	E
Nature	Dark red amorphous powder	Dark red amorphous powder
MP (°C, dec)	220~225	187~192
$[\alpha]_D^{23}$ (*c* 0.1, 0.1 N HCl)	+409°	+606°
SI-MS ($(M+H)^+$ *m/z*)	827	813
HRFAB-MS	Obsd: 827.2513	Obsd: 813.2361
	Calcd for $C_{39}H_{43}N_2O_{18}$: 827.2511	Calcd for $C_{38}H_{41}N_2O_{18}$: 813.2354
UV λ_{max} nm (ε)		
in 50% MeOH	218 (30,000), 280 (23,600), 495 (12,100)	223 (22,300), 280 (18,700), 496 (8,800)
in 0.02 N HCl-MeOH (1:1)	233 (32,300), 298 (28,500), 460 (12,800)	234 (36,500), 298 (31,700), 459 (13,100)
in 0.02 N NaOH-MeOH (1:1)	240 (31,000), 318 (14,300), 500 (14,500)	240 (32,900), 318 (14,200), 500 (14,900)
IR (KBr) cm^{-1}	3400, 2920, 1605, 1385, 1295, 1260, 1160, 1055	3380, 2900, 1605, 1385, 1295, 1255, 1160, 1040

Table 3. ^1H NMR spectra of pradimicins A, D and E (400 MHz, DMSO-d_6).

Proton	Pradimicins		
	A	D	E
5'-CH_3	1.25 (3H, d, *J*=6.2)	1.24 (3H, d, *J*=7.3)	1.14 (3H, d, *J*=6.3)
17-CH_3	1.35 (3H, d, *J*=7.0)	—	—
3-CH_3	2.29 (3H, s)	2.29 (3H, s)	2.29 (3H, s)
4'-NCH_3	2.61 (3H, s)	2.59 (3H, s)	—
4',2'',3'' 4'',5''-H	3.1~3.2 (5H, m)	3.1~3.2 (5H, m)	3.05~3.15 (3H), 3.41 (2H)
2'-H	3.53 (1H, m)	3.52 (1H, m)	3.53 (1H, m)
5''-H	3.74 (1H, dd, *J*=5.3, 11.2)	3.74 (1H, dd, *J*=5.4, 11.2)	3.70 (1H, dd, *J*=4.9, 11.2)
5'-H	3.84 (1H, q, *J*=6.2)	3.85 (2H, m)	3.88 (2H, m)
3'-H	3.91 (1H, m)		
11-OCH_3	3.91 (3H, s)	3.90 (3H, s)	3.91 (3H, s)
17-H	4.39 (1H, qui, *J*=7.0)	3.85 (2H, d-like)	3.88 (2H, d-like)
1''-H	4.44 (1H, d, *J*=7.3)	4.43 (1H, d, *J*=6.8)	4.48 (1H, d, *J*=7.3)
5-H	4.46 (1H, d, *J*=10.3)	4.44 (1H, d, *J*=9.0)	4.46 (1H, d, *J*=11.0)
6-H	4.49 (1H, br d, *J*=10.3)	4.49 (1H, dd, *J*=9.0, 2.4)	4.53 (1H, dd, *J*=11.0, 2.4)
1'-H	4.73 (1H, d, *J*=7.7), 5.02 (2H, br s)[a], 5.08 (1H, s)[a], 5.68 (1H, s)[a], 5.91 (1H, s)[a]	4.72 (1H, d, *J*=7.3), 5.02 (2H, br s)[a], 5.08 (1H, s)[a], 5.70 (1H, s)[a], 5.91 (1H, s)[a]	4.72 (1H, d, *J*=7.8), 4.90 (1H, br s)[a], 5.01 (1H, d, *J*=3.9)[a], 5.09 (1H, s)[a], 5.69 (1H, s)[a]. 5.93 (1H, s)[a]
10-H	6.71 (1H, d, *J*=2.4)	6.71 (1H, d, *J*=2.0)	6.72 (1H, d, *J*=2.4)
4-H	6.85 (1H, s)	6.85 (1H, s)	6.88 (1H, s)
12-H	7.11 (1H, d, *J*=2.4)	7.11 (1H, d, *J*=2.0)	7.12 (1H, d, *J*=2.4)
7-H	7.69 (1H, s)	7.68 (1H, s)	7.68 (1H, s)
16-NH	8.78 (1H, d, *J*=7.0)[a], 13.16 (1H, s)[a]	8.66 (1H, br t)[a], 13.17 (1H, s)[a], 15.30 (1H, br s)[a]	8.75 (1H, br t)[a], 13.17 (1H, s)[a], 15.40 (1H, br s)[a]

[a] Disappeared upon D_2O addition. *J* in Hz.

pradimicins D and E were determined by analyzing their ^1H and ^{13}C NMR spectra in comparison with those of pradimicin A. The ^1H NMR spectrum of pradimicin D was very similar to that of pradimicin A except for the signals of the D-alanine moiety. The 17-CH_3 signals at δ 4.39 ppm (1H) and 1.35 ppm (3H)

Table 4. ^{13}C NMR spectra of pradimicins A, D and E (100 MHz, DMSO-d_6).

Carbon	Pradimicins			Carbon	Pradimicins		
	A	D	E		A	D	E
5'-CH_3	16.4 q[a]	16.2 q	16.4 q	8a	110.5 s	110.2 s	110.3 s
17-CH_3	17.6 q	—	—	7	111.6 d	111.4 d	111.2 d
3-CH_3	20.0 q	20.0 q	20.1 q	4	116.9 d	116.6 d	117.0 d
4'-NCH_3	36.6 q	36.6 q	—	14b	119.0 s	118.7 s	118.8 s
17	48.2 d	41.0 t	41.4 t	13a	119.3 s	118.9 s	119.1 s
11-OCH_3	56.2 q	55.8 q	56.0 q	2	126.9 s	126.0 s	127.1 s
4'	63.4 d	63.1 d	54.3 d	7a	132.2 s	131.7 s	131.9 s
5"	66.0 t	65.7 t	65.8 t	14a	133.1 s	132.7 s	133.3 s
5'	67.9 d	68.2 d	67.7 d	3	136.5 s	136.5 s	136.4 s
4"	69.6 d	69.3 d	69.4 d	4a	137.7 s	137.4 s	137.5 s
2'	70.2 d	70.0 d	69.7 d	12a	138.0 s	137.8 s	137.9 s
6	71.9 d	71.6 d	71.8 d	6a	143.7 s	143.3 s	143.5 s
2"	73.8 d	73.5 d	73.5 d	1	157.6 s	158.1 s	158.0 s
3"	76.1 d	75.8 d	76.0 d	9	164.1 s	163.7 s	163.9 s
3'	80.4 d	80.9 d	79.8 d	11	166.0 s	165.6 s	165.8 s
5	82.7 d	81.9 d	82.5 d	14	166.4 s	165.7 s	166.4 s
10	104.4 d	104.0 d	104.2 d	15	168.9 s	168.7 s	168.9 s
1'	104.5 d	104.0 d	104.4 d	18	174.6 s	171.1 s	171.6 s
1"	105.3 d	105.0 d	105.1 d	13	180.5 s	179.9 s	180.0 s
12	106.3 d	105.7 d	106.0 d	8	187.5 s	187.1 s	187.3 s

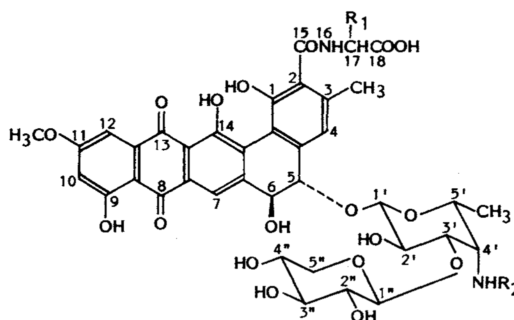
[a] Multiplicities determined from DEPT spectra.

Table 5. *In vitro* antifungal activity in Sabouraud dextrose agar (pH 7.0).

Test organism	MIC (μg/ml)				
	Pradimicins			Amphotericin B	Ketoconazole
	A	D	E		
Candida albicans IAM 4888	6.3	6.3	3.1	1.6	50
C. albicans A9540	12.5	6.3	6.3	3.1	50
Cryptococcus neoformans D49	0.8	1.6	0.8	1.6	0.4
C. neoformans IAM 4514	0.8	0.8	0.8	1.6	0.4
Aspergillus fumigatus IAM 2530	1.6	6.3	1.6	3.1	6.3
A. fumigatus IAM 2034	3.1	6.3	6.3	3.1	6.3
A. flavus FA 21436	6.3	3.1	6.3	6.3	0.8
Fusarium moniliforme A2284	3.1	6.3	12.5	3.1	3.1
Trichophyton mentagrophytes D155	6.3	3.1	12.5	1.6	1.6
T. mentagrophytes No. 4329	6.3	6.3	12.5	3.1	1.6
Blastomyces dermatitidis D40	6.3	6.3	3.1	>50	0.8
Sporothrix schenckii IFO 8158	1.6	1.6	6.3	3.1	1.6
Petriellidium boydii IFO 8078	6.3	25	>100	>50	1.6
Mucor spinosus IFO 5317	>100	>100	>100	3.1	50

of pradimicin A were missing, while a new signal at δ 3.85 ppm (2H) was observed in pradimicin D (Table 4). This was further substantiated by the ^{13}C NMR spectrum of pradimicin D which exhibited 39 carbon signals: The spectrum lacked a methyl carbon at δ 17.6 ppm which had been assigned to the 17-CH_3 (methyl of alanine moiety) in pradimicin A. Most of the other signals appeared at positions nearly identical

Fig. 1. Structures of pradimicins A, B, C, D and E.



Pradimicin A	$R_1=CH_3$	$R_2=CH_3$
Pradimicin B	$R_1=CH_3$	$R_2=CH_3$ (Desxylosylpradimicin A)
Pradimicin C	$R_1=CH_3$	$R_2=H$
Pradimicin D	$R_1=H$	$R_2=CH_3$
Pradimicin E	$R_1=H$	$R_2=H$

with the corresponding signals of pradimicin A except that the signals at δ 48.2 ppm (d, C-17) of pradimicin A appeared at δ 41.0 ppm (t) in pradimicin D, with concomitant shift of a carbonyl carbon at δ 174.6 ppm (s, C-18 in pradimicin A) to δ 171.1 ppm (s, in pradimicin D) (Table 5). These data, with the assigned molecular formula, clearly indicated that the D-alanine of pradimicin A is replaced by a glycine in pradimicin D. The CD spectrum of pradimicin D ($\lambda_{\text{extreme}}^{0.01\,\text{N-HCl}}$ nm ($\Delta\varepsilon$) 209 (+16.8), 233 (−30.9), 293 (+5.9), 306 (+4.7), 311 (+5.1), 345 (−4.4), 502 (+4.2)) corresponded well to that of pradimicin A. This fact indicated that the two plane rings of pradimicin D have the same orientation as that of pradimicin A and therefore the absolute configuration of pradimicin D was assigned to be 5*S*, 6*S*.[2,3]

Table 6. *In vivo* activity against *Candida albicans* A9540 systemic infection in mice.

Compound	PD_{50} mg/kg (iv)
Pradimicin A	8.9
Pradimicin D	9.0
Pradimicin E	8.9
Amphotericin B	0.21
Ketoconazole	45.0

The molecular formula of pradimicin E differs from that of pradimicin D by CH_2 unit. The ^1H and ^{13}C NMR spectra of pradimicin E corresponded well to those of pradimicin D (Tables 4 and 5). The only difference was that the *N*-CH_3 of pradimicin D was missing in the spectrum of pradimicin E. Thus, the ^{13}C NMR of pradimicin E lacked a methyl carbon at δ 36.6 ppm which had been assigned to 4'-NCH_3 in pradimicin D. Also, the C-4'-signal of pradimicin D at δ 63.1 ppm moved upfield to δ 54.3 ppm in the spectrum of pradimicin E. The ^1H NMR of pradimicin E was clearly devoid of *N*-CH_3 protons observed at δ 2.59 ppm in the spectrum of pradimicin D. Consequently, pradimicin E has 4,6-dideoxy-4-amino-D-galactose in place of the 4,6-dideoxy-4-(methylamino)-D-galactose of pradimicin D (Fig. 1).

Antifungal Activity of Pradimicins D and E

In vitro antifungal activity of pradimicins D and E was tested in comparison with those of pradimicin A. Pradimicins D and E showed activity similar to pradimicin A against a variety of yeasts and fungi (Table 5). Also, the *in vivo* anticandidal activity of pradimicins D and E was almost the same as that of pradimicin A (Table 6).

Discussion

Two glycine substituted congeners of pradimicin A, pradimicins D and E, have been isolated from the fermentation broths of both the parent strain P157-2 and a mutant strain A2660. These compounds result from substitution with glycine instead of D-alanine at a certain step of their biosyntheses. We have reported that the D-alanine moiety of pradimicin A was derived from L-alanine in *A. hibisca* P157-2.[7] Thus, it was expected that exogeneously added glycine would be incorporated into the molecules with high efficiency. However, when glycine was exogeneously added to the culture medium of either strain P157-2 or strain A2660, production of pradimicins D and E was somewhat inhibited in strain P157-2, and significantly inhibited in strain A2660. We attempted to examine the intracellular glycine pools in both strains P157-2 and A2660 grown in medium A at various periods of fermentation time. Both strains have almost the same levels of glycine pool during pradimicins D and E biosynthesis (data not shown). Further characterization of mutant strain A2660 is under way.

Pradimicins D and E showed *in vitro* and *in vivo* antifungal activities comparable to pradimicin A. Glycine in the molecules, therefore, may mimic D-alanine in pradimicin A in terms of antifungal mode of action.

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