WATER-SOLUBLE PRADIMICIN DERIVATIVES, SYNTHESIS AND ANTIFUNGAL EVALUATION OF *N,N*-DIMETHYL PRADIMICINS

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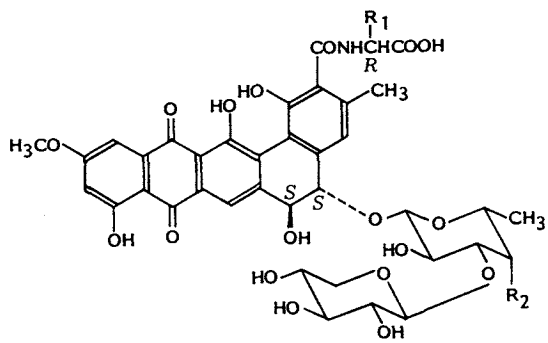
Three *N,N*-dimethyl pradimicins were synthesized by reductive alkylation of pradimicins A, E and FA-2 and evaluated for antifungal activity, water solubility and acute toxicity in mice. They showed *in vitro* antifungal activity superior to pradimicin A. *N,N*-Dimethylpradimicins E and FA-2 showed great improvement in water solubility and animal tolerance. *N,N*-Dimethylpradimicin FA-2 was effective in 3 experimental *in vivo* fungal infection models.

Pradimicins A, B and C[1~4] and benanomicins A and B[5,6] are produced by *Actinomadura hibisca* P157-2 (ATCC 53557) and an actinomycete numbered MH193-16F4, respectively. They belong to a novel group of antifungal antibiotics possessing a core structure of glycosylated dihydrobenzo[*a*]naphthacenequinone substituted with D-alanine at C-15 (Fig. 1). Pradimicins D and E (glycine analogs of pradimicins A and C) which have also been isolated from fermentations of *A. hibisca* P157-2 and its mutant strains, showed antifungal activity similar to pradimicin A[7]. Biosynthetically, the aglycone of pradimicin A is

Fig. 1. Structures of pradimicins and their derivatives.



	R_1	R_2
Pradimicin A	CH_3	$NHCH_3$
Pradimicin C (Benanomicin B)	CH_3	NH_2
Benanomicin A	CH_3	OH
Pradimicin D	H	$NHCH_3$
Pradimicin E	H	NH_2
Pradimicin FA-1	CH_2OH	$NHCH_3$
Pradimicin FA-2	CH_2OH	NH_2
N,N-Dimethylpradimicin C (**1**)	CH_3	$N(CH_3)_2$
N,N-Dimethylpradimicin E (**2**)	H	$N(CH_3)_2$
N,N-Dimethylpradimicin FA-2 (**3**)	CH_2OH	$N(CH_3)_2$

derived from acetate and alanine, as shown by feeding studies with ^{13}C-enriched precursors[8]. Efficient incorporation of both D- and L-alanines into the D-alanine side chain of pradimicin A suggested that D-alanine might act as the direct precursor for the side chain. Recently, through the addition of D-serine to antibiotic fermentations, the formation of new active metabolites, pradimicins FA-1 and FA-2, has been accomplished[9]. Pradimicins FA-1 and FA-2 (D-serine analogs of pradimicins A and C) showed antifungal activity similar to pradimicin A. Although pradimicins A, B, C, D, E, FA-1 and FA-2 exhibited significant *in vivo* activity against *Candida albicans* A9540 systemic infections in mice[4,7,9], their limited solubility in aqueous media posed some problems in further development. In order to obtain water-soluble analogs that retain the antifungal activity of pradimicin A, a program of chemical modification was undertaken, using the fermentation products as starting materials. We anticipated that incorporation of a second methyl group at the basic nitrogen atom in the sugar moiety would change the physico-chemical properties of the parent molecule without impairing the antifungal activity. This report describes the synthesis and evaluation of *N,N*-dimethyl pradimicins (**1**, **2** and **3**).

Chemistry

The reductive alkylation of pradimicins A, E, and FA-2 with formaldehyde yielded *N,N*-dimethylpradimicin C (**1**), *N,N*-dimethylpradimicin E (**2**) and *N,N*-dimethylpradimicin FA-2 (**3**), respectively. The progress of the reaction was monitored by HPLC on a YMC A-301-3 ODS column using an acetonitrile concentration gradient in 0.15% phosphate buffer (pH 3.5) from 15% to 55%. The *N*-H_2 pradimicins eluted first, then the *N*-methyl pradimicins, and finally the *N,N*-dimethyl derivatives. The products were purified by Diaion HP-20 column chromatography and isolated in an analytically pure zwitterionic form.

The structures of *N,N*-dimethyl pradimicins (**1**, **2** and **3**) were confirmed by FAB-MS, combustion analysis and ^1H NMR spectroscopy.

In Vitro Antifungal Evaluation

Table 1 summarizes the MICs determined by the agar dilution method on Sabouraud dextrose agar medium for **1**, **2**, **3**, pradimicin A (PRD-A), amphotericin B (AMP-B) and ketoconazole (KCZ) against a panel of 12 yeasts and 7 filamentous fungi. *N,N*-Dimethyl pradimicins (**1**, **2** and **3**) showed significant improvement in activity against yeasts. The MICs against 3 isolates of *C. albicans* ranged from 3.1~12.5 μg/ml for **1**, **2**, and **3** as compared to MICs of 12.5~50 μg/ml for PRD-A. *N,N*-Dimethyl pradimicins (**1**, **2** and **3**) also have better activity against isolates of *Candida tropicalis*, *Candida parapsilosis* and *Candida krusei* than PRD-A, demonstrating an expansion of the PRD-A spectrum. The MIC against AMP-B-resistant *C. albicans* (ATCC 38247) ranged from 0.8~3.1 μg/ml for **1**, **2**, **3** and PRD-A, indicating that there is no cross resistant between pradimicins and AMP-B. *N,N*-Dimethyl pradimicins (**1** and **3**) retained the pradimicin activity against filamentous fungi, while the activity of **2** was significantly reduced against them when compared to PRD-A.

Water Solubility and Animal Tolerance

Table 2 lists data for the solubility in phosphate buffered saline containing Ca^{2+} and Mg^{2+} and animal tolerance of *N,N*-dimethyl pradimicins (**1**, **2** and **3**) and PRD-A. No acute toxicity, as measured by mortality, was observed with the water-soluble derivatives (**2** and **3**) at doses up to 600 mg/kg in mice after an intravenous administration. Ataxia was observed with **2** at a dose of 600 mg/kg, while

Table 1. Spectra of antifungal activity for pradimicin A (PRD-A), *N,N*-dimethyl pradimicins (**1**, **2** and **3**), amphotericin B (AMP-B) and ketoconazole (KCZ).

Test organism	MIC (μg/ml)[a]					
	PRD-A	**1**	**2**	**3**	AMP-B	KCZ
Saccharomyces cerevisiae ATCC 9763	12.5	3.1	1.6	3.1	0.2	50
Candida albicans IAM 4888	50	6.3	6.3	6.3	0.8	12.5
C. albicans A9540	50	12.5	3.1	6.3	0.8	25
C. albicans ATCC 38247	3.1	1.6	1.6	0.8	25	12.5
C. albicans ATCC 32354 (B311)	12.5	6.3	3.1	6.3	0.8	25
C. tropicalis IFO 10241	>100	>100	>100	50	0.8	12.5
C. tropicalis CS-07	25	6.3	6.3	6.3	1.6	50
C. parapsilosis CS-08	>100	6.3	6.3	3.1	1.6	<0.05
C. krusei A15052	25	6.3	6.3	3.1	1.6	50
Cryptococcus neoformans D49	1.6	0.8	1.6	1.6	0.4	0.2
C. neoformans IAM 4514	0.8	0.8	1.6	1.6	0.4	0.2
C. neoformans CS-01	1.6	1.6	3.1	1.6	1.6	0.8
Aspergillus fumigatus IAM 2034	3.1	3.1	12.5	3.1	0.8	1.6
A. fumigatus IAM 2530	1.6	3.1	12.5	3.1	1.6	3.1
A. flavus FA 21436	6.3	6.3	25	6.3	3.1	0.4
A. flavus CS-18	25	>100	>100	100	6.3	1.6
Sporothrix schenckii IFO 8158	1.6	1.6	50	3.1	3.1	6.3
Trichophyton mentagrophytes No. 4329	6.3	12.5	25	12.5	0.8	0.4
T. mentagrophytes D155	3.1	6.3	25	12.5	1.6	1.6

[a] MICs determined by the agar dilution method on Sabouraud dextrose agar medium, pH 7.0; inoculum size: 10^6 cells/ml (5 μl/spot).

Table 2. Solubility and acute toxicity of pradimicin derivatives.

Compound	Solubility[a] (mg/ml)	LD_{50} (mg/kg)
PRD-A	0.02	120
1	<0.01	85
2	2.0	>600
3	>20.0	>600
AMP-B	NT	4.5

[a] Solubility in DULBECCO's phosphate buffered saline containing Ca^{2+} and Mg^{2+}.
NT: Not tested.

N,N-dimethylpradimicin FA-2 (**3**) appeared to be well tolerated; no apparent side effects following injections of **3** up to 600 mg/kg were noted.

Table 3. *In vivo* activity of pradimicin derivatives against *Candida*, *Cryptococcus* and *Aspergillus* systemic infections in mice (n=5).

Compound	PD_{50} (mg/kg)[a]		
	C. albicans A9540	*C. neoformans* IAM 4514	*A. fumigatus* IAM 2034
PRD-A	8.9	11	16
1	3.5	NT	NT
2	11	45	42
3	9	11	36
AMP-B	0.35	0.36	0.28

[a] PD_{50} value was determined 20 days after the fungal challenge.
Inoculum size (cells/mouse): *C. albicans*, 1×10^6, *C. neoformans*, 1×10^6, *A. fumigatus*, 1×10^7.
NT: Not tested.

In Vivo Antifungal Evaluation

The *in vivo* activity of *N,N*-dimethylpradimicins E (**2**) and FA-2 (**3**) against experimental systemic infections with *C. albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* is shown in Table 3. Both **2** and **3** showed excellent activity against *C. albicans* and somewhat reduced activity against *A. fumigatus*, when compared to PRD-A by a single intravenous administration in normal mice.

Discussion

The results of the present study have provided a novel series of *N,N*-dimethyl pradimicin derivatives

which have excellent activity against *C. albicans* as well as other pathogenic fungi. The data presented in Table 2 suggest a positive correlation between the water solubility and acute toxicity; the water-soluble derivatives (**2**) and (**3**) showed superior tolerance in mice. The results thus appear to indicate that incorporation of a second methyl group at the basic nitrogen atom in the sugar moiety improved the properties of the glycine and serine analogs of PRD-A in aqueous media. *N,N*-Dimethylpradimicin C (**1**) was more toxic than PRD-A. *N,N*-Dimethylpradimicin FA-2 (**3**) has been selected for development as a therapeutic agent for systemic fungal infections based on its spectrum of *in vitro* activity, solubility, *in vivo* activity and tolerance in mice. Further evaluation of **3** is in progress.

Experimental

General Methods

All mp's were determined with a Yanaco micro melting point apparatus and are uncorrected. UV and IR spectra were recorded on a Jasco UVIDEC-610C spectrometer and a Jasco IR-810 spectrometer, respectively. ^{1}H NMR spectra were recorded on a Jeol JNM-GX400 spectrometer. Mass spectra were obtained with a Jeol JMS-AX505H spectrometer. AMP-B (Fungizone, sodium deoxycholate complex) was obtained from Nippon Squibb Co. KCZ was obtained from Janssen Pharmaceuticals.

Reductive Methylation of Pradimicins

The following procedure is an example for preparation of *N,N*-dimethyl pradimicins.

A solution of pradimicin E hydrochloride (485 mg) in water (40 ml) was adjusted to pH 8.0 with 1 N NaOH at 25°C, diluted with acetonitrile (40 ml), and HCHO (37%, 1.6 ml) and $NaBH_3CN$ (240 mg) were sequentially added. The mixture was stirred for 15 hours and the solvent was removed *in vacuo*. The residue was dissolved in water (50 ml), adjusted to pH 11.0, and added dropwise to acetone (300 ml) while stirring. The resulting precipitate was collected by centrifugation and redissolved in water. The solution was adjusted to pH 2.0 with 6 N HCl and chromatographed on a column of Diaion HP-20 eluted with 3:2, acetone-water (pH 3.0). The orange eluate was concentrated *in vacuo* to 5 ml and adjusted to pH 5.5. The resulting solid was collected, washed successively with water and acetone, and dried at 60°C under vacuum for 40 hours to afford 364 mg (73% yield) of *N,N*-dimethylpradimicin E (**2**): MP 214~218°C (dec); UV (0.01 N NaOH) λ_{max} nm (ε) 233.6 (32,900), 319.2 (15,500), 497.6 (15,100); IR (KBr) cm^{-1} 3400, 1600, 1385, 1295, 1050; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.23 (3H, br, 5'-CH_3), 2.29 (3H, s, 3-CH_3), 2.78 (6H, br, 4'-N(CH_3)$_2$), 3.1~3.2 (4H, m), 3.3~3.4 (1H, m), 3.50 (1H, m, 2'-H), 3.73 (1H, dd, J = 5.1 and 11.1 Hz, 5''-H), 3.9~4.0 (4H, m, 3'-H, 5'-H and 17-H_2), 3.91 (3H, s, 11-OCH_3), 4.39~4.48 (3H, m), 4.69 (1H, br, 1'-H), 5.0~5.2 (3H, m, exchangeable with D_2O), 5.8~5.9 (2H, m, exchangeable with D_2O), 6.57 (2H, s, exchangeable with D_2O), 6.77 (1H, d, J = 2.6 Hz, 10-H), 6.90 (1H, s, 4-H), 7.17 (1H, d, J = 2.6 Hz, 12-H), 7.81 (1H, s, 7-H), 8.65 (1H, t, J = 5.8 Hz, 16-H, exchangeable with D_2O), 12.98 (1H, s, exchangeable with D_2O), 13.12 (1H, s, exchangeable with D_2O); FAB-MS (3-nitrobenzyl alcohol (NBA)) m/z 841 $(M+H)^+$.

Anal Calcd for $C_{40}H_{44}N_2O_{18}\cdot 1\frac{1}{2}H_2O$: C 55.36, H 5.46, N 3.23.
Found: C 55.26, H 5.45, N 3.19.

The derivatives prepared by this procedure and characterizing data are listed below.

N,N-Dimethylpradimicin C (**1**) from PRD-A: MP 190~195°C (dec); UV (0.01 N NaOH) λ_{max} nm (ε) 233.6 (31,400), 319.2 (14,700), 498.4 (14,600); IR (KBr) cm^{-1} 3400, 1605, 1380, 1295, 1255, 1050; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.27 (3H, br, 5'-CH_3), 1.34 (3H, d, J = 7.3 Hz, 17-CH_3), 2.29 (3H, s, 3-CH_3), 2.83 (6H, br, 4'-N(CH_3)$_2$), 3.1~3.2 (5H, m), 3.50 (1H, m, 2'-H), 3.74 (1H, dd, J = 5.3 and 11.3 Hz, 5''-H), 3.90~4.03 (2H, m, 3'-H and 5'-H), 3.93 (3H, s, 11-OCH_3), 4.39 (1H, m, 17-H), 4.45~4.50 (3H, m, 5-H, 6-H and 1''-H), 4.73 (1H, br, 1'-H), 5.00 (2H, br s, exchangeable with D_2O), 5.08 (1H, s, exchangeable with D_2O), 5.77 (1H, s, exchangeable with D_2O), 5.85 (1H, s, exchangeable with D_2O), 6.80 (1H, d, J = 2.4 Hz, 10-H), 6.91 (1H, s, 4-H), 7.18 (1H, d, J = 2.4 Hz, 12-H), 7.86 (1H, s, 7-H), 8.72 (1H, d, J = 6.8 Hz, 16-H, exchangeable with D_2O), 13.06 (1H, s, exchangeable with D_2O); FAB-MS (NBA) m/z 855 $(M+H)^+$.

Anal Calcd for $C_{41}H_{46}N_2O_{18}\cdot 2H_2O$: C 55.28, H 5.66, N 3.15.
Found: C 55.58, H 5.43, N 3.11.

N,N-Dimethylpradimicin FA-2 (**3**) from Pradimicin FA-2: MP 214~218°C (dec); UV (0.01 N NaOH) λ_{max} nm (ε) 232.8 (32,900), 320.0 (15,500), 498.4 (15,200); IR (KBr) cm^{-1} 3400, 1610, 1385, 1295, 1260, 1060; ^1H NMR (400 MHz, DMSO-d_6) δ 1.26 (3H, br, 5'-CH_3), 2.31 (3H, s, 3-CH_3), 2.81 (6H, br, 4'-N(CH_3)$_2$), 3.06~3.18 (3H, m), 3.24~3.43 (2H, m), 3.48 (1H, m, 2'-H), 3.69~3.78 (3H, m), 3.9~4.1 (2H, m), 3.91 (3H, s, 11-OCH_3), 4.41~4.49 (4H, m), 4.71 (1H, br, 1'-H), 4.9~5.2 (4H, m, exchangeable with D_2O), 5.78~5.90 (2H, br, exchangeable with D_2O), 6.77 (1H, d, J = 2.4 Hz, 10-H), 6.89 (1H, s, 4-H), 7.16 (1H, d, J = 2.4 Hz, 12-H), 7.83 (1H, s, 7-H), 8.61 (1H, br, 16-H, exchangeable with D_2O), 13.11 (1H, s, exchangeable with D_2O); FAB-MS (NBA) m/z 871 $(M+H)^+$.

Anal Calcd for $C_{41}H_{46}N_2O_{19}\cdot 5H_2O$: C 53.77, H 5.61, N 3.06.
Found: C 53.62, H 5.41, N 2.87.

Determination of Water Solubility

The water solubility of each compound was assessed in DULBECCO's phosphate buffered saline containing 0.9 mM of $CaCl_2$ and 0.5 mM of $MgCl_2$, pH 7.2. Each sample (1~20 mg/ml) was sonicated at 30°C for 10 minutes and allowed to stand at 25°C for 2 hours. The supernatant was collected by centrifugation at 12,000 rpm for 10 minutes, diluted 50-fold with 0.01 N NaOH, and the absorption value at 500 nm was read. On the basis of the absorption value, the solubility of the compound was calculated.

Test Strains

A panel of 12 yeasts and 7 filamentous fungi was used for comparative studies. Yeasts were cultured at 28°C in YGP medium (yeast extract (0.2%), glucose (1.5%), peptone (0.5%), K_2HPO_4 (0.05%), $MgSO_4$ (0.05%)). The inocula for the susceptibility test were broth dilutions or suspensions of mid-exponentially growing cells. Fungi were cultured at 28°C on YGP agar medium for 14 days and a suspension of spores in 0.2% Tween 80 was filtered through a gauze.

Antifungal Spectrum of Activity

The susceptibility of yeasts and filamentous fungi was determined by the agar dilution method on Sabouraud dextrose agar medium (pH 7.0). Test compounds and AMP-B were dissolved in 10% DMSO at a concentration of 1,000 μg/ml; 2-fold serial dilutions thereof were made in water to give final concentrations ranging from 0.05 to 100 μg/ml. KCZ was dissolved in 10% DMSO containing a trace of 1 N HCl at a concentration of 1,000 μg/ml and diluted with water for use at concentrations ranging from 0.05 to 100 μg/ml. One part of each solution was mixed with nine parts of molten agar medium in Petri dishes. A final inoculum of 10^6 cells/ml (0.005 ml of cell suspension/spot) was used. All the organisms were incubated at 28°C for 40 hours. The MIC in each case was defined as the lowest concentration of antibiotic showing no distinct growth or less than five discrete colonies/spot.

Acute Toxicity

Compounds were dissolved in 10% DMSO and administered once intravenously to groups of 5 male ICR mice at the dosed volume of 0.1 ml per 10 g body weight. The animals were observed daily for 10 days for physical and behavioral signs of toxicity.

In Vivo Antifungal Activity

The *in vivo* activity against systemic infections with *C. albicans* A9540 (10^6 cells/mouse), *C. neoformans* IAM 4514 (10^6 cells/mouse) and *A. fumigatus* IAM 2034 (10^7 cells/mouse) was examined in normal mice. Male ICR mice weighing 20~24 g were infected intravenously. Groups of five mice at each dose level were treated with the test compounds in 10% DMSO. The 50% protection dose (PD_{50}) was calculated by the method of LITCHFIELD and WILCOXON[10] from the survival rate 20 days after the fungal challenge. Untreated animals died 7 to 15 days post infection.

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