EFFECT OF STEREOCHEMISTRY AT THE C-17 POSITION ON THE ANTIFUNGAL ACTIVITY OF PRADIMICIN A

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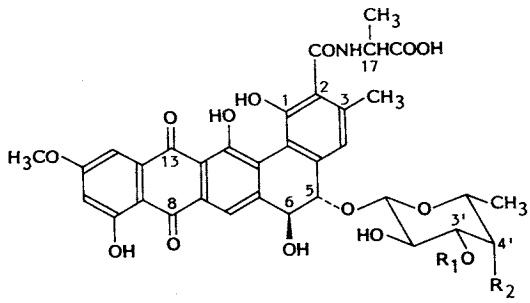
Pradimicin A (**1**) is a novel antifungal antibiotic produced by *Actinomadura hibisca* No. P157-2 (ATCC 53557)[1,2], along with minor components, pradimicins B (**2**) and C (**3**)[3~5]. Pradimicin A (**1**) was found to be active *in vitro* against a wide variety of fungi and yeasts, and highly effective against systemic fungal infections in mice[5,6]. Comparative *in vitro* studies showed that there was no cross-resistance to other antifungal agents and that **1** inhibited the growth of 5-fluorocytosine- and amphotericin B-resistant *Candida albicans*[1,5]. However, as pradimicin A (**1**) is hardly soluble in phosphate-buffered saline (PBS) at physiological pH's, it is difficult to develop **1** as an injectable drug. As part of the program aimed at identifying pharmaceutically acceptable structures, we embarked on chemical modification studies of **1** which has a novel 5,6-dihydrobenzo[*a*]naphthacene chromophore substituted with D-alanine and 2 sugars. We initially focused on the D-alanine moiety of **1**, and report here the synthesis and physico-chemical properties of 17-epipradimicin A (**4**), and the effect of change in stereochemistry at the C-17 position on the activity of pradimicin A (**1**).



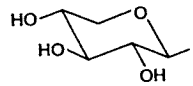
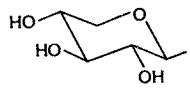
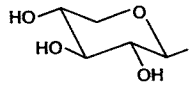
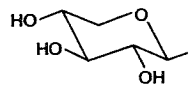
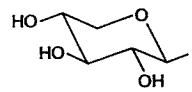
	R_1	R_2	Configuration at C-17
Pradimicin A (**1**)		$NHCH_3$	*R*
Compound **5a**		$N(CH_3)COOCH_2Ph$	*R*
Pradimicin B (**2**)	H	$NHCH_3$	*R*
Pradimicin C (**3**)		NH_2	*R*
17-Epipradimicin A (**4**)		$NHCH_3$	*S*
Compound **5b**		$N(CH_3)COOCH_2Ph$	*S*

Table 1. Effect of stereochemistry at the C-17 position on the activity of pradimicin A (**1**).

Compound	Solubility[a] (μg/ml)	MIC[b] (μg/ml)		Binding[c] (μg/mg dried cells)
	PBS	*Candida albicans* A9540	*Aspergillus fumigatus* IAM 2034	to *C. albicans* A9540
1	22	12.5	1.6	75.8
4	43	>100	>100	1.7

[a] Each sample was taken up in PBS containing 0.9 mM of $CaCl_2$ and 0.5 mM of $MgCl_2$, pH 7.2, sonicated at 30°C for 10 minutes and the insoluble material was removed by centrifugation at 12,000 rpm for 10 minutes. The supernatant was diluted 5-fold with 0.01 N NaOH and the OD at 500 nm was read. Concentration of each compound was calculated from the OD reading compared with the standard curve of each compound.

[b] The MICs were determined by the serial 2-fold agar dilution method in yeast morphology agar medium buffered with 0.067 M phosphate, pH 7.0.

[c] Each sample was mixed with acetone-dried cells of *C. albicans* A9540 (1 mg/ml) in 0.1 M phosphate buffer containing 0.2 mM of $CaCl_2$, 2% polyvinylpyrrolidone and 1% DMSO, pH 7.2, for 15 minutes at 25°C and the cells were removed by centrifugation. The quantity of material bound to the cells was calculated from the total amount used and the amount in the supernatant.

Synthesis

Transformation of pradimicin A (**1**) into 17-epipradimicin A (**4**) was carried out through the following steps. Upon treatment with benzyl chloroformate (5.0 ml) and Na_2CO_3 (7.5 g) in a mixture of acetone (400 ml) and water (400 ml) followed by hydrolysis, **1** (2.5 g) was converted to *N*-(benzyloxycarbonyl)pradimicin A (**5a**) (2.2 g (76% yield); UV (0.01 N NaOH - methanol (1 : 1)) λ_{max} nm (ε) 319.2 (15,400), 498.4 (14,500); IR (KBr) cm^{-1} 3450, 1735, 1630, 1605, 700). Treatment of **5a** (200 mg) with formic acetic anhydride (20 ml) at 60°C for 30 minutes followed by hydrolysis with NaOH gave a mixture of **5a** and **5b** which was hydrogenolyzed over 5% Pd-C (200 mg) in a mixture of methanol (40 ml), ethanol (10 ml) and water (20 ml). The catalyst was removed by filtration and washed with acetone - water (1 : 1). The filtrate and washings were combined and concentrated to a small volume. HPLC analysis on a column of Microsorb Short One C18 (4.6 mm diameter × 10 cm, 3 μm, Rainin Instrument Co.) eluting with acetonitrile - 0.15% phosphate buffer (7 : 17) (pH 3.5) showed that the product contained a nearly 1 : 1 mixture of **1** and **4**. These isomers were chromatographed on a column of Lichroprep RP-18 (40 ~ 63 μm, 4 cm diameter × 45 cm) eluting with acetonitrile - 0.15% phosphate buffer (3 : 7) (pH 3.5). The first orange eluate was concentrated, adsorbed on a column of Diaion HP-20 and eluted with acetone - water (4 : 1) (pH 2.5) to afford 39 mg of **1**·HCl. The second orange eluate was similarly desalted to afford 32 mg of **4**·HCl (>99.5% pure by HPLC). Zwitterionic **4** was obtained by adjusting a solution of **4**·HCl in water to pH 5.5 with 1 N NaOH. The resulting precipitate was collected by centrifugation, washed successively with methanol and acetone, and dried at 60°C under vacuum for 24 hours.

Physico-chemical Properties

17-Epipradimicin A (**4**): MP 197 ~ 201°C (dec); UV (0.01 N NaOH - methanol (1 : 1)) λ_{max} nm (ε) 240 (32,300), 319 (13,300), 498 (12,800): IR (KBr) cm^{-1} 3400, 1620, 1600, 1440, 1255; ^1H NMR (400 MHz, DMSO-d_6) δ 1.21 (3H, d, J=6.5 Hz, 5'-CH_3), 1.34 (3H, d, J=7.3 Hz, 17-CH_3), 2.28 (3H, s, 3-CH_3), 2.53 (3H, s, 4'-NCH_3), 3.1 ~ 3.2 (5H, m), 3.58 (1H, m, 2'-H), 3.71 (1H, dd, J=5.2 and 11.3 Hz, 5''-H), 3.75 (2H, m, 3'-H and 5'-H), 3.91 (3H, s, 11-OCH_3), 4.34 (1H, m, 17-H), 4.41 ~ 4.47 (3H, m, 1''-H, 5-H and 6-H), 4.69 (1H, d, J=7.7 Hz, 1'-H), 5.03 (2H, br s, exchangeable with D_2O), 5.07 (1H, s, exchangeable with D_2O), 5.74 (1H, s, exchangeable with D_2O), 5.87 (1H, s, exchangeable with D_2O), 6.72 (1H, d, J=2.4 Hz, 10-H), 6.89 (1H, s, 4-H), 7.12 (1H, d, J=2.4 Hz, 12-H), 7.71 (1H, s, 7-H), 8.67 (1H, d, J=6.1 Hz, 16-H, exchangeable with D_2O), 13.18 (1H, s, exchangeable with D_2O); FAB-MS (NBA) m/z 841 (MH^+); HRFAB-MS (NBA) m/z 841.2661 (MH^+), molecular formula $C_{40}H_{44}N_2O_{18}$. CD (0.01 N HCl) λ_{ext} nm (Δε) 210 (+6.4), 237 (−14.4), 296 (+4.5), 306 (+3.2), 313 (+4.5), 347 (−3.2), 511 (+6.4). The solubility in PBS was given in Table 1.

Stereochemistry

The spectral data did not allow us to distinguish between the natural product (**1**)[3] and its epimer (**4**). The stereochemistry of **4** was determined to be 5*S*,6*S*,17*S* by CD analysis comparing with **1**[3,4] and by degradation study as described below. Compound **4** (13 mg) was hydrolyzed in a mixture of

dioxane (0.5 ml) and 6 N HCl (4.5 ml) in a sealed tube at 115°C for 16 hours and the supernatant was collected by filtration and then passed through a column of Diaion HP-20. HPLC analysis of the ninhydrin positive eluate on a column of MCI Gel ODS 1HU (4.6 mm diameter × 150 mm, 5 μm, Mitsubishi Kasei) eluting with 2 mM *N,N*-dipropyl-L-alanine and 1 mM cupric acetate, pH 5.7, at a flow rate of 0.8 ml/minute (UV detection:230 nm) showed that the alanine derived from **4** was L-alanine (Rt 6.18 minutes) instead of D-alanine (Rt 4.26 minutes)[3].

Antifungal Activity and Discussion

17-Epipradimicin A (**4**) had no antifungal activity *in vitro* as shown in Table 1, indicating that the change in stereochemistry at the C-17 position resulted in a complete loss of antifungal activity. For other isolates of *C. albicans* and *Candida tropicalis*, similar results were obtained (data not shown). These results demonstrate that the D-alanine moiety of **1** plays an important role in the expression of antifungal activity.

In addition, we have found an interesting phenomenon which seems to be consistent with the antifungal activity. While active **1** bound to the cell surface of *C. albicans* in the presence of Ca^{2+}, inactive **4** had virtually no binding activity under the same conditions (Table 1). The binding of **1** to the cell surface appears to be the first event which subsequently induces an alteration in cellular permeability, ultimately leading to death of the cells[7]. One can speculate that the amino acid moiety is one of the key structural elements for binding to the cell surface and that the inactivity of **4** may have resulted from the inability of the L-alanine side chain to assume a geometry required for binding to the cell surface due to steric effects of the two neighboring substituents (a hydroxy group at C-1 and a methyl group at C-3).

Interestingly, both the D- and L-alanines fed to growing cultures of *A. hibisca* were efficiently incorporated into the D-alanine side chain of **1** whereas **4** could not be detected in the crude fermentation broth[8]. This raises a possibility that pradimicin analogs which have a different D-amino acid chain may be produced by feeding an appropriate precursor to growing cultures of *A. hibisca*.

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