Notes

NOVEL *Candida albicans* ASPARTYL PROTEASE INHIBITOR. II. A NEW PEPSTATIN-AHPATININ GROUP INHIBITOR, YF-044P-D

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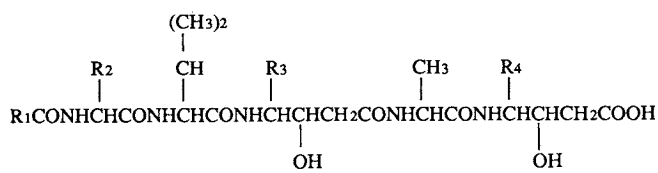
Pepstatin A[1,2], an aspartyl protease inhibitor, is a potent new type of antifungal antibiotic[3]. However, it is not clinically used because it is metabolized in the liver and rapidly cleared from the blood[4]. As a result of our screening program for the search of *Candida albicans* aspartyl protease inhibitors, we reported on the new inhibitors, YF-0200R-A and B in a preceding paper[5]. Subsequently we have isolated a new highly potent inhibitor, YF-044P-D (Fig. 1) which is produced by *Streptomyces* sp. YF-044P together with ahpatinin E, F and G[6]. In this paper, we describe the fermentation conditions for YF-044P-D production, isolation procedure, physico-chemical properties, structural elucidation and biological activity of YF-044P-D. The producing strain was isolated from a soil sample collected at Hatoma island in Okinawa prefecture, Japan. YF-044P-D is an acidic compound of pepstatin-ahpatinin group.

A loopful spores of the strain was inoculated into a 500-ml Erlenmeyer flask containing 100 ml medium composed of glucose 1.0%, potato starch 2.0%, yeast extract 0.5%, peptone 0.5% and $CaCO_3$ 0.4%, pH 7.0 before sterilization. The flask was incubated at 28°C for 3 days on a rotary shaker. Three ml of the culture broth was transferred into 500-ml Erlenmeyer flasks containing 100 ml of fermentation medium composed of glucose 3.0%, dextrin 3.0%, defatted soybean meal 1.5%, roasted wheat germ 1.5%, K_2HPO_4 0.06%, KH_2PO_4 0.025% and $CoCl_2 \cdot 6H_2O$ 0.0004%, pH 7.0 before sterilization. The fermentation was carried out at 28°C for 4 days.

The culture broth (20 liters) was adjusted to pH 3.0 with 4N HCl and filtered. YF-044P-D was extracted from the broth filtrate and mycelia with ethylacetate (EtOAc). Both EtOAc extracts were combined, concentrated *in vacuo* and fractionated by silica gel column chromatography. YF-044P-D was finally purified by preparative reverse phase HPLC and obtained as a white powder (2.2 mg) together with ahpatinin E (0.6 mg), F (0.7 mg) and G (1.5 mg). The isolation procedure and physico-chemical properties are summarized in Fig. 2 and Table 1, respectively.

By the IR and NMR spectra, one ester bond and

Fig. 1. Structures of YF-044P-D and related compounds.



	R_1	R_2	R_3	R_4
YF-044P-D	$C_6H_5CH_2$	$(CH_3)_2CH$	$C_6H_5CH_2$	$C_6H_5CH_2$
L-174580	$C_6H_5CH_2$	$(CH_3)_2CH$	$(CH_3)_2CHCH_2$	$C_6H_5CH_2$
Pepstatin A	$(CH_3)_2CHCH_2$	$(CH_3)_2CH$	$(CH_3)_2CHCH_2$	$(CH_3)_2CHCH_2$
Ahpatinin E	$(CH_3)_2CHCH_2$	$(CH_3)_2CH$	$(CH_3)_2CHCH_2$	$C_6H_5CH_2$
Ahpatinin F	$(CH_3)_2CHCH_2$	C_6H_5	$(CH_3)_2CHCH_2$	$(CH_3)_2CHCH_2$
Ahpatinin G	$(CH_3)_2CHCH_2$	$(CH_3)_2CH$	$C_6H_5CH_2$	$C_6H_5CH_2$

five amido bonds were suggested (1710, 1640 and 1550 cm^{-1} in the IR spectrum, δ 177.2, 175.5, 172.0, 170.6, 170.6, 170.5 and 170.3 ppm in the ^{13}C NMR spectrum in DMSO-d_6, δ 8.14; 1H, 7.85; 2H, 7.63; 1H, 7.58 ppm; 1H in the ^1H NMR spectrum). Two oximethines (δ 67.6 and 67.4 ppm in the ^{13}C NMR spectrum, δ 4.12~4.22; 3H, 3.88~3.97 ppm; 4H in the ^1H NMR spectrum, together with five α methines), three phenyl groups and five methyl groups (δ 7.15~7.26; 15H, 1.10; 3H and 0.71~0.85 ppm; 12H in the ^1H NMR spectrum) were also suggested by the NMR spectra. Taking the negativity of the Ninhydrin color reaction into account, YF-044P-D was considered to belong to the pepstatin-ahpatinin group. Its molecular weight was determined to be 787 by FAB-MS of YF-044P-D and its methyl ester. The FAB MS-MS spectrum of YF-044P-D methyl ester was measured and compared with that of pepstatin A methyl ester. The amino acid sequence was determined from the *N* terminal to be *N*-acylamino acid, valine, 4-amino-3-hydroxy-5-phenylpentanoic acid (AHPPA) whose 3-hydroxy position was assured by the fragmentation in EI-MS[6], alanine and AHPPA by peaks of *m/z* 218, 317, 508 and 579. The fragmentation pattern is shown in Fig. 3. Acid degradation was carried out to determine the *N* terminal amino acid and acyl group. After degradation with 6 N HCl at

Fig. 2. Purification procedure of YF-044P-D.

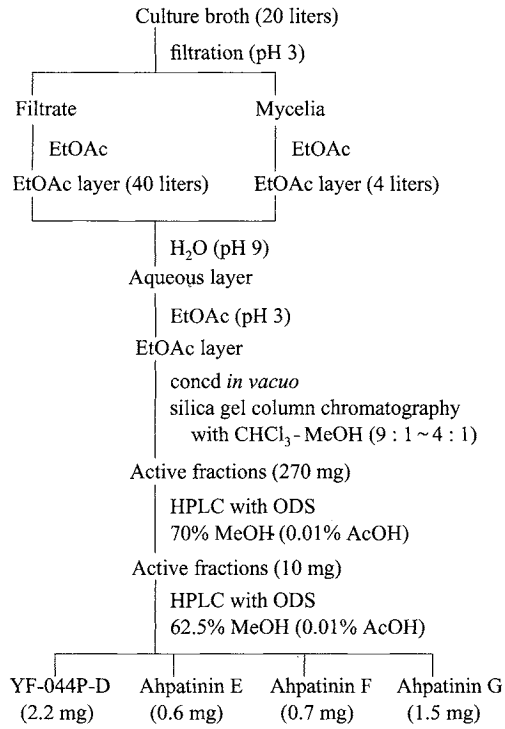


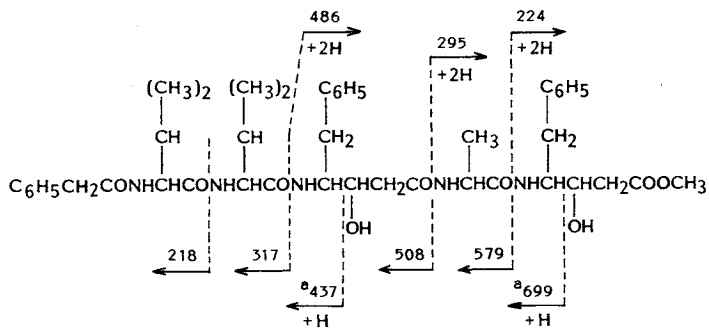
Table 1. Physico-chemical properties of YF-044P-D.

Molecular formula	$C_{43}H_{57}N_5O_9$
Molecular weight	787
UV λ_{max}^{MeOH} nm	203, 228 (sh)
IR ν_{max}^{KBr} cm	3400, 3280, 2930, 1710, 1640, 1550, 1450, 1380, 1100
^1H NMR δ ppm (in DMSO-d_6)	0.71~0.85; 12H, 1.10; 3H, 1.48; 2H, 1.87~1.93; 2H, 2.17; 2H, 2.64; 2H, 2.76~2.83; 2H, 3.44~3.57; 2H, 3.33; 3H, 3.88~3.97; 4H, 4.12~4.22; 3H, 7.15~7.26; 15H, 7.58; 1H, 7.63; 1H, 7.85; 2H, 8.14; 1H
$[\alpha]_D^{25}$ (*c* 0.1, MeOH)	−24.5
Rf[a] (silica gel plate $60F_{254}$)	0.61
Rt[b] HPLC (minutes)	6.20
Solubility (soluble in)	MeOH, EtOAc, $CHCl_3$

[a] $CHCl_3$-MeOH (1 : 2).

[b] μBondasphere C-18, 3.9 i.d. × 150 mm, 67.7% MeOH (0.01% AcOH), 1 ml/minute.

Fig. 3. Fragmentation of YF-044P-D methyl ester in the FAB MS-MS spectrum.



[a]: Detected in the EI-MS spectrum.

110°C for 20 hours, phenylacetic acid was detected from the EtOAc extract of the degradation mixture in GC-MS with DB-1, J & W Scientific, as a column. Two moles of valine and 1 mole of alanine were detected as known amino acids in the amino acid analysis with HPLC. As a result, the *N* terminal amino acid is valine and the acyl group is the phenylacetyl group. Valine and alanine are determined as L form by HPLC after reaction with MARFEY's reagent[7]. The structure of YF-044P-D is shown in Fig. 1.

Inhibitory activity of YF-044P-D against *Candida albicans* aspartyl protease was measured in the same way as that of YF-0200R-A and B[5]. The IC_{50} values of YF-044P-D, ahpatinin E, F and G are 6.4×10^{-7} M, 6.8×10^{-7} M, 8.1×10^{-7} M and 6.5×10^{-7} M, respectively. YF-044P-D showed much stronger inhibitory activity than YF-0200R-A and B, but it showed almost equal activity to that of ahpatinin E, F and G. Among another aspartyl proteases, YF-044P-D showed the strongest inhibitory activity against cathepsin D (cathepsin D; bovine spleen, purchased from Sigma $\gg$ *Candida albicans* aspartyl protease, partially purified in our laboratory $>$ pepsin; porcine stomach mucosa, Sigma) (data not shown).

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