NOVEL ASPARTYL PROTEASE INHIBITORS, YF-0200R-A and B

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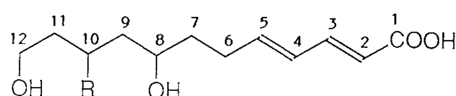
Novel pepstatin A-sensitive *Candida albicans* aspartyl protease inhibitors, named YF-0200R-A and B, were isolated by column chromatography and preparative HPLC from the fermentation broth of *Streptomyces* sp. YF-0200R. The structures of YF-0200R-A and B were elucidated by spectroscopic analysis as α, β and γ, δ unsaturated fatty acids with two or three hydroxyl groups. YF-0200R-A and B inhibit aspartyl protease from *Candida albicans* with IC_{50} values of 6.5×10^{-4} M and 6.2×10^{-4} M, respectively.

Fungal infection can be divided into two types according to the site of pathogen. One type is superficial and can be treated with azole group drugs such as bifonazole or clotrimazole, many fungal diseases belong to this type. The other type is systemic and difficult to treat. Systemic opportunistic infections include mycoses such as candidiasis, aspergillosis, cryptococcosis and phycomycosis. They commonly occur in immunosuppressed patients with leukemia, lymphomas, diabetes mellitus or AIDS. Pepstatin A, which is an aspartyl protease inhibitor, shows a stronger antifungal effect than that of clotrimazole when *Candida albicans* is cultivated in liquid medium containing human stratum corneum as sole nitrogen source[1]. The positive correlation between virulence of *Candida albicans* and the amount of secreted aspartyl protease suggested that aspartyl protease is one of virulence factors of *Candida albicans*[2]. The secretion of protease at the infected site of *Candida albicans* was recognized[3]. Pepstatin A-sensitive aspartyl protease is produced by *Aspergillus niger* and *Mucor rouxii*[4] as well. RÜCHEL *et al.* showed that pepstatin A has a protective effect when administered intravenously to mice as crystal suspension[5]. These results suggested that pepstatin A is possibly a new type of antifungal agent[6]. But pepstatin A is not clinically used because it is distributed and metabolized in the liver and quickly cleared from the blood after iv administration[5]. Therefore it would be useful for systemic candidiasis to search for new, water soluble and non-peptide inhibitors of *Candida albicans* aspartyl protease. In our screening program we found and purified new inhibitors, YF-0200R-A and B, from the culture broth of *Streptomyces* sp. YF-0200R which was isolated from a soil sample from South Sumatra, Indonesia. The structures of YF-0200R-A and B were elucidated mainly by the NMR spectral analysis and are shown in Fig. 1. In this paper, we describe the fermentation profile

Fig. 1. Structures of YF-0200R-A and B.



YF-0200R-A (1): R=H
YF-0200R-B (2): R=OH

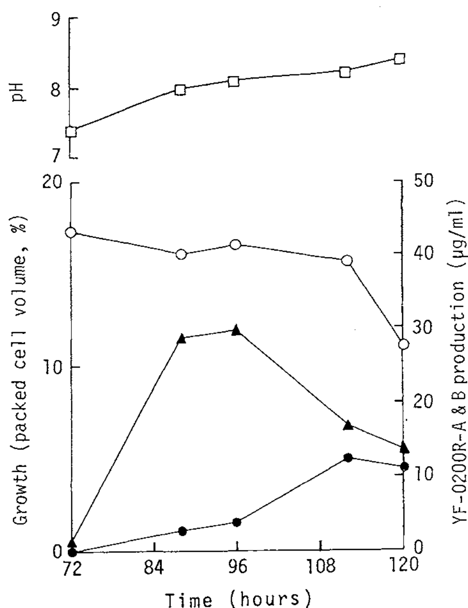
for production of YF-0200R-A and B, the isolation procedure, the physico-chemical properties, the structural elucidation and biological activities.

Fermentation of *Streptomyces* sp. YF-0200R

A 500-ml Erlenmeyer flask containing 60 ml medium was inoculated from an agar slant culture of *Streptomyces* sp. YF-0200R. The flask was incubated at 27°C on a rotary shaker for 96 hours. The medium was composed of 1.0% glucose, 2.0% potato starch, 0.5% yeast extract, 0.5% peptone and 0.4% $CaCO_3$ and adjusted to pH 7.0 before autoclaving. 1.8 ml of this seed culture was used to inoculate fifty 500-ml Erlenmeyer flasks containing 60 ml of the same medium. The fermentation was carried out at 27°C for 108 hours. As shown in Fig. 2, the production of YF-0200R-A was maximal at 112 hours (12.5 μg/ml) and YF-0200R-B at 96 hours with 29.7 μg/ml.

Fig. 2. Fermentation profile for the production of YF-0200R-A and B.

(□) pH, (○) growth, (●) YF-0200R-A production, (▲) YF-0200R-B production.



Isolation and Physico-chemical Properties of YF-0200R-A and B

The culture broth (3 liters) was adjusted to pH 3.0 and filtered. The filtrate was extracted twice with EtOAc (5 liters) and then extracted twice with *n*-BuOH. YF-0200R-A was extracted with EtOAc from the filtrate and purified with silica gel column chromatography and finally by HPLC (μBondasphere ODS C18, 19 i.d. × 150 mm) with MeOH - H_2O - AcOH (35 : 65 : 0.01) as the solvent. The flow rate was 10 ml/minute and detection was at 254 nm. The peak at 22 minutes was collected. After evaporation of MeOH, YF-0200R-A was extracted with EtOAc, dried *in vacuo* and 8.6 mg was obtained as a white powder. YF-0200R-B was extracted with *n*-BuOH from the waste fluid of EtOAc extraction and purified by silica gel column chromatography and finally by HPLC in the same way as YF-0200R-A but the solvent was MeOH - H_2O - AcOH (30 : 70 : 0.01). YF-0200R-B was eluted at 15 minutes. After evaporation of MeOH, YF-0200R-B was extracted with *n*-BuOH and dried to needles. Twenty-one mg of YF-0200R-B was obtained. The purification procedures of YF-0200R-A and B are shown in Fig. 3. The physico-chemical properties are summarized in Table 1.

Structures of YF-0200R-A and B

In the structural elucidation, YF-0200R-B was studied first because it was obtained in higher purity and greater quantity than YF-0200R-A. The molecular formula of YF-0200R-B was determined to be $C_{12}H_{20}O_5$ by HRFAB-MS and ^{13}C NMR spectrum. The carboxyl group was suggested by the chemical shift of the ^{13}C NMR spectrum (170.9 ppm) and the acidity of YF-0200R-B. Three hydroxyl groups were

Fig. 3. Purification procedure of YF-0200R-A and B.

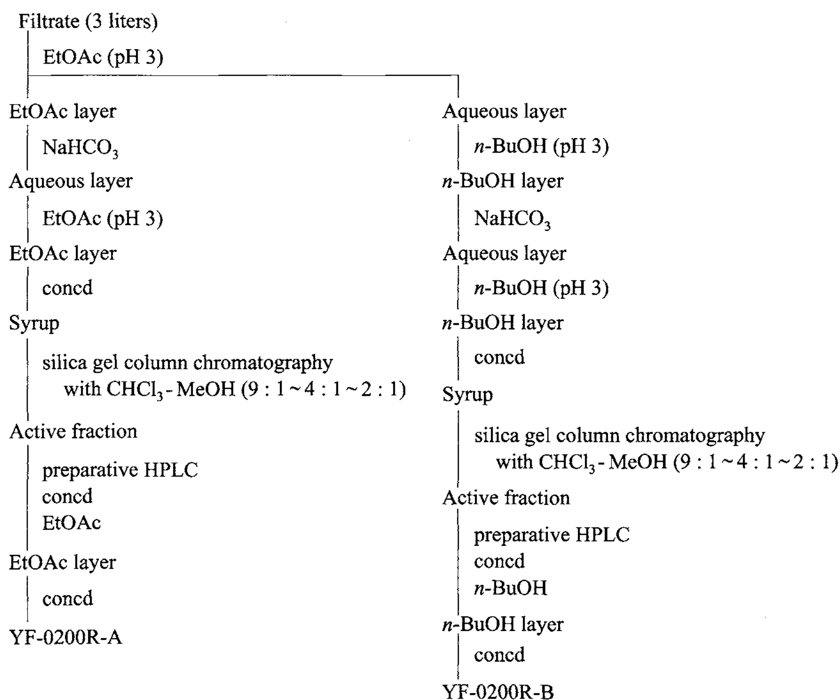


Table 1. Physico-chemical properties of YF-0200R-A and B.

	YF-0200R-A	YF-0200R-B
Molecular formula	$C_{12}H_{20}O_4$	$C_{12}H_{20}O_5$
Molecular weight	228	244
HRFAB-MS $(M+H)^+$		
Calcd:	—	245.1389
Found:	—	245.1356
UV λ_{max}^{MeOH} nm	253	256
IR ν_{max}^{KBr} cm^{-1}	3400, 1690, 1640, 1260, 1010	3470, 3340, 1690, 1640, 1260, 1060
$[\alpha]_D^{25}$ (*c* 0.2, MeOH)	4.0	6.5
MP (°C)	—	143 ~ 145
Rf[a]	0.36	0.32
(silica gel plate 60 F_{254})		
Rt[b] HPLC (minute)	18	5.2
Solubility (soluble in)	EtOAc, MeOH	*n*-BuOH, MeOH

[a] $CHCl_3$ - MeOH (2:1).
[b] MeOH - H_2O - AcOH (30:70:0.01).

suggested by chemical shifts of the ^{13}C and ^1H NMR spectra (C-12; 60.2 ppm, 12-H_2; 3.70 ppm, C-10; 67.0 ppm, 10-H; 4.00 ppm and C-8; 68.6 ppm, 8-H; 3.82 ppm). Two double bonds were suggested by the chemical shifts of the ^{13}C NMR spectrum (120.6, 129.9, 145.5 and 146.9 ppm) and the index of hydrogen deficiency of the molecule. Whole planar structure was easily elucidated by the ^1H-^1H COSY spectrum. The position of the carboxyl group was assured by the HMBC spectrum, *i.e.* couplings between C-1 and 2-H and between C-1 and 3-H were observed. Other connections were ascertained by the HMBC spectrum. As for double bonds, the coupling constants between H-2 and H-3 and between H-4 and H-5 were both

Table 2. NMR data for **1** and **2** in $CDCl_3$ (δ: ppm).

Position No.	YF-0200R-A			YF-0200R-B		
	$^{13}C^a$	$^1H^b$	^{13}C–C-^1H or ^{13}C–C–C-$^1H^c$	$^{13}C^a$	$^1H^b$	^{13}C–C-^1H or ^{13}C–C–C-$^1H^c$
1	170.4	—	2-H, 3-H	170.9	—	2-H, 3-H
2	120.0	5.78	3-H, 4-H	120.6	5.78	3-H, 4-H
3	146.2	7.25	2-H, 4-H, 5-H	146.9	7.24	2-H, 4-H, 5-H
4	129.1	6.22	2-H, 3-H, 5-H, 6-H	129.9	6.28	2-H, 3-H, 5-H, 6-H
5	144.8	6.16	3-H, 4-H, 6-H, 7-H	145.5	6.21	3-H, 4-H, 6-H, 7-H
6	29.7	2.25, 2.35	4-H, 5-H, 7-H	30.2	2.27, 2.33	4-H, 5-H, 7-H, 8-H
7	36.6	1.56	5-H, 6-H	38.1	1.57	5-H, 6-H, 8-H, 9-H
8	71.0	3.60	6-H, 7-H, 9-H	68.6	3.82	6-H, 7-H, 9-H, 10-H
9	37.5	1.48	7-H, 10-H	45.9	1.52	7-H, 8-H, 10-H, 11-H
10	22.4	1.52	8-H, 9-H, 11-H, 12-H	67.0	4.00	8-H, 9-H, 11-H, 12-H
11	32.9	1.58	10-H, 12-H	41.5	1.67	9-H, 10-H, 12-H
12	62.4	3.59	—	60.2	3.70	10-H, 11-H

[a] ^{13}C NMR spectra were measured at 125 MHz.
[b] ^1H NMR spectra were measured at 500 MHz.
[c] Couplings were recognized in the HMBC experiment.

15.2 Hz, thus they are *trans*. The structure of YF-0200R-B is shown in Fig. 1 and the results of the NMR spectra are summarized in Table 2. The structure of YF-0200R-A was elucidated by comparison with YF-0200R-B of the spectroscopic data. From the FAB-MS spectrum, its MW was determined to be 228, 16 mass units smaller than that of YF-0200R-B. In the ^1H and ^{13}C NMR spectra, one methylene was recognized instead of the oxymethine in YF-0200R-B. Therefore YF-0200R-A is thought to be the deoxy derivative of YF-0200R-B. In the ^1H-^1H COSY spectrum, oxymethine in C-10 has been changed into methylene in YF-0200R-A. The whole C–C connections were ascertained by the HMBC spectrum. Since the coupling constants between 2-H and 3-H and between 4-H and 5-H were the same as those of YF-0200R-B, they are *trans*.

Biological Activities of YF-0200R-A and B

Inhibitory activity for *Candida albicans* aspartyl protease was measured in a reaction mixture which consisted of 0.80 ml of 1% bovine serum albumin in a 0.05 M citrate buffer (pH 3.2), 0.06 ml of protease (partially purified in our laboratories by Sephadex G-75 from the broth filtrate of *Candida albicans* isolated from human sputum), 0.05 ml of sample and 0.09 ml of 0.05 M citrate buffer (pH 3.2). After the reaction was carried out at 37°C for 60 minutes, it was stopped by the addition of 2.0 ml of 5% TCA. The reaction mixture was centrifuged at 3,000 rpm for 15 minutes and the absorbance of the supernatant was measured at 280 nm in a spectrophotometer. The IC_{50} values of YF-0200R-A and B were 6.5×10^{-4} M and 6.2×10^{-4} M, respectively. In Sabouraud agar medium, neither YF-0200R-B nor pepstatin A showed any antifungal activity against *Candida albicans*, *Saccharomyces sake*, *Aspergillus niger*, *Mucor hiemalis* and *Trichophyton mentagrophytes* at 100 μg/ml.

Discussion

YF-0200R-A and B are α, β and γ, δ unsaturated hydroxy fatty acids. Though the activity of YF-0200R-A and B is weak, compared with that of pepstatin A (1.1×10^{-7} M) and ahpatinin E (7.0×10^{-7} M)

which was obtained in our laboratory, YF-0200R-A and B are the first unsaturated fatty acids that inhibit aspartyl protease from *Candida albicans*. In our study, the minimum partial structure exhibiting inhibitory activity is α, β unsaturated carboxylic acid with the exception of acrylic acid (data not shown). The positions substituted with hydroxyl groups are C-8, C-10 and C-12 in YF-0200R-B and C-8 and C-12 in YF-0200R-A, all of these carbons would be biosynthetically derived from C-2 of acetic acid. Generally carbons which are substituted with hydroxyl group derive from C-1 of acetic acid, or C-2 through oxidation of double bond such as ipurolic acid (3,11-dihydroxy tetradecanoic acid), 9,10-dihydroxystearic acid or ustilic acid (2,15,16-trihydroxy hexadecanoic acid), so the structures of YF-0200R-A and B are biosynthetically unusual. Among bioactive unsaturated fatty acid analogues, fumifungin[7] and sphingofungins[8] are antifungal antibiotics, but the latter are serine palmitoyl transferase inhibitors. As aspartyl protease inhibitors[9], pepstatins[10,11] and ahpatinins[12] have been found in the screening program for pepsin inhibitors. Pepstatin A and its synthetic analogues are active against renin,[13] cathepsin D[14] and HIV protease.[15,16]

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