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

Tsutomu Sato, Mitsuyoshi Shibazaki, Hiroshi Yamaguchi and Kenji Abe
Drug Serendipity Research Laboratories, Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., 1-1-8 Azusawa, Itabashi-ku, Tokyo 174, Japan

Hisao Matsumoto and Minoru Shimizu
Molecular Chemistry Research Laboratories, Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., 21 Miyukigaoka, Tsukuba City, Ibaraki 305, Japan

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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\(_2\)HPO\(_4\) 0.06%, KH\(_2\)PO\(_4\) 0.025% and CoCl\(_2\)-6H\(_2\)O 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...
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 ¹³C NMR spectrum in DMSO-d₆, 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 ¹³C 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-MS⁶), 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.](image)

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

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
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<tbody>
<tr>
<td>Molecular formula</td>
<td>C₄₃H₅₇N₅O₉</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>787</td>
</tr>
<tr>
<td>UV λ_max (nm)</td>
<td>203, 228 (sh)</td>
</tr>
<tr>
<td>IR ν_max cm</td>
<td>3400, 3280, 2930, 1710, 1640, 1550, 1450, 1380, 1100</td>
</tr>
<tr>
<td>¹H NMR δ ppm (in DMSO-d₆)</td>
<td>0.71<del>0.85; 12H, 1.10; 3H, 1.48; 2H, 1.87</del>1.93; 2H, 2.17; 2H, 2.64; 2H, 2.76<del>2.83; 2H, 3.44</del>3.57; 2H, 3.33; 3H, 3.88<del>3.97; 4H, 4.12</del>4.22; 3H, 7.15~7.26; 15H, 7.58; 1H, 7.63; 1H, 7.85; 2H, 8.14; 1H</td>
</tr>
<tr>
<td>[α]D²⁵ (c 0.1, MeOH)</td>
<td>-24.5</td>
</tr>
<tr>
<td>RP³ (silica gel plate 60F₂₅₄)</td>
<td>0.61</td>
</tr>
<tr>
<td>Rf⁴ HPLC (minutes)</td>
<td>6.20</td>
</tr>
<tr>
<td>Solubility (soluble in)</td>
<td>MeOH, EtOAc, CHCl₃</td>
</tr>
</tbody>
</table>

(x) CHCl₃-MeOH (1:2).  
³ (Luxorbosphere C-18, 3.9 i.d. x 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.](image)

* 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. 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. The IC₅₀ 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; Candida albicans aspartyl protease, partially purified in our laboratory; pepsin; porcine stomach mucosa, Sigma) (data not shown).

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References