Prolyl Endopeptidase Inhibitors from the Underground Part of Rhodiola sacra S. H. Fu

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Prolyl endopeptidase (PEP, EC 3.4.21.26) is an enzyme which plays a role in the metabolism of proline-containing neuropeptides, e.g., vasopressin, substance P and thyrotropin-releasing hormone (TRH), which have been suggested to be involved in learning and memory processes. In our systematic screening for PEP inhibitors from traditional Chinese medicines, we found that MeOH extract from the underground part of Rhodiola sacra S. H. Fu shows significant inhibitory activity against PEP from Flavobacterium meningosepticum. Examination of the constituents of the extract resulted in the isolation of nineteen known compounds, identified as hydroquinone (1), 4-hydroxybenzoic acid (2), caffeic acid (3), 4-hydroxycinnamic acid (4), suberic acid (5), protocatechuic acid (6), gallic acid (7), (+)-epigallocatechin 3-O-gallate (8), 2-phenylethyl β-D-glucopyranoside (9), 3-O-galloyl epigallocatechin-(4β–38)epigallocatechin 3-O-gallate (10), 2-phenylethyl α-L-arabinopyranosyl-(1→6)-β-D-glucopyranoside (11), sacranose A (12), β-D-glucopyranosyl 4-hydroxybenzoate (13), rhodiocyanoside A (14), rhodocyanoside (15), sarmentosin (16), heterodendrin (17), arbutin (18) and 4-O-(β-D-glucopyranosyl)-gallic acid (19). Among these, 1, 2, 5, 6–10, 13, 16, 18 and 19 have been isolated for the first time from R. sacra, among which 5, 9, 10, 13, 16, 18 and 19 have been isolated from Rhodiola plants for the first time. On the PEP inhibition, seven compounds (6–8, 10, 12, 18, 19) showed inhibition with an IC₅₀ of 27.8, 487, 1.47, 0.437, 348, 391 and 215 μM, respectively. The kinetic study of these inhibitors indicated that they are noncompetitive inhibitors, except for 6 which is a competitive inhibitor.

Key words Prolyl endopeptidase (PEP) inhibitor; Rhodiola sacra; Crassulaceae; (+)-epigallocatechin 3-O-gallate; 3-O-galloyl epigallocatechin-(4β–38)epigallocatechin 3-O-gallate; protocatechuic acid

Prolyl endopeptidase (PEP, EC 3.4.21.26) was first discovered in the human uterus as an oxytocin-degrading enzyme and specifically cleaves the peptide bonds at the carboxyl side of proline residues. 1) This enzyme is widely distributed in various organs, particularly in the human brain, and prolyl endopeptidase-like immunoreactivity has been detected in the hippocampus of senescence-accelerated mouse. 2) In the central nervous system, PEP is suspected to hydrolyze proline-containing neuropeptides, e.g., vasopressin, substance P and thyrotropin-releasing hormone (TRH), which are suggested to participate in learning and memory processes. 3)

Recently, one of the proteinases, which cleaves A4 peptide from a larger precursor protein in Alzheimer’s patients, has been identified as PEP 4) and PEP activity has been reported to be significantly high in Alzheimer’s patients. 5) In addition, an abnormal PEP level may be related to neuropathological disorders, such as major depression, mania, schizophrenia and senile dementia of the Alzheimer’s type. 6) Thus, a specific inhibitor of PEP is expected to have anti-amnesic effect, and many inhibitors have been synthesized as candidates for such neuropathological disorders. 7)

On the other hand, through medical practice over more than thousands years, many traditional Chinese medicines have been confirmed to be effective for treating amnesia and/or dementia. 8) We thus made a screening for PEP inhibitors from the medicinal plants selected based on the traditional medicine theory, and found that a MeOH extract of the underground part of Rhodiola sacra S. H. Fu (Sheng-di-hong-jing-tian, 聖地紅景天) shows significant inhibitory activity. 9) In this paper, we wish to report the isolation, structural determination and PEP inhibitory activity of the constituents of the MeOH extract.

MATERIALS AND METHODS

General 1H- and 13C-NMR spectra were recorded on a JEOL GX-400 spectrometer with tetramethylsilane (TMS) as an internal standard. IR spectra were measured in a KBr disk on a Shimadzu UV-160A spectrophotometer. PEP activity was measured with a Perkin-Elmer HTS 7000 Bio Assay reader.

Chemicals PEP (Flavobacterium meningosepticum origin) was purchased from Seikagaku Corporation (Tokyo, Japan), and Z-Gly-Pro-pNA was from Bachem Fine Chemical Co. (Switzerland). Two positive controls, Z-Pro–prolinal and Z-Pro–prolinol, were synthesized by the method in the literature. 10)

PEP Inhibitory Assay PEP activity was assayed by the method of Yoshimoto et al., 12) in which 0.1 ml Tris–HCl (pH 7.0, 840 μl), PEP (50 μl of 0.1 unit/ml) and a test sample solution (10 μl) were mixed in a well of a 24-well microplate and pre-incubated for 5 min at 30°C. The reaction was started by adding 100 μl of 2 mM Z-Gly–Pro-pNA (in 40% 1,4-dioxane), and after incubation at 30°C for 30 min, the amount of released p-nitroaniline was determined colorimetrically with a microplate autoreader at 410 nm. The percent inhibition was calculated by the formula [(A–B)/A]×100, where A is the p-nitroaniline liberated by the enzyme in the system without an inhibitor and B is that with an inhibitor.

The inhibitor constant, Kᵢ, was estimated by the method of Dixon using 0.1 U/ml enzyme, various concentrations of inhibitors and 0.1, 0.15 and 0.2 mM substrates. As the reaction proceeds, a linear correlation was obtained between absorbance and time, and the initial velocity was calculated from the curve at 350 s.

Extraction and Fractionation Dried underground part

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of *Rhodiola sacra* S. H. Fu (Crassulaceae) was supplied by Prof. J. Huang of Tibet Plateau Institute of Biology, People’s Republic of China. The plant material was identified by Prof. J. Hung and the voucher specimen (TMPW16956) is preserved at the Museum of Materia Medica, Analytical Research Center for Ethnomedicines of Our Institute.

The underground part of *R. sacra* (2.0 kg) was pulverized and extracted three times with MeOH under reflux (each 41, 2 h), and the removal of MeOH in *vacuo* yielded a MeOH extract (566 g). The MeOH extract (400 g) was suspended on water and partitioned successively with CHCl₃ (500 ml×2), EtOAc (500 ml×2) and BuOH (500 ml×2). Each extract was evaporated to dryness in *vacuo*, and the remaining water layer was freeze-dried to give CHCl₃-soluble (42 g), EtOAc-soluble (15 g), BuOH-soluble (105 g) and H₂O-soluble (220 g) fractions, respectively. Among them, the EtOAc-soluble and BuOH-soluble fraction showed significant inhibitory activity with IC₅₀ of 0.33 and 0.46 µg/ml, respectively.

**Treatment of EtOAc-soluble Fraction** A portion (14 g) of the EtOAc-soluble fraction was chromatographed over a silica gel (600 g) column and eluted with increasing amounts of MeOH in CHCl₃ (5:95→30:70) to afford seven fractions (fr. 1, 192 mg; fr. 2, 569 mg; fr. 3, 1.2 g; fr. 4, 1.4 g; fr. 5, 755 mg; fr. 6, 2.3 g; fr. 7, 3.0 g). Among the fractions, fraction 3 was identified as gallic acid (7, 1.2 g) by a comparison with authentic sample, while fraction 4 was determined to be (−)-epigallocatechin 3-O-gallate (8, 1.4 g) by a comparison of the NMR data with those in the literature.¹⁵

Fraction 1 (100 mg) was separated by reverse-phase preparative TLC with MeOH–H₂O (50:50) to give hydroquinone (1, 7.0 mg), 4-hydroxybenzoic acid (2, 8.0 mg), caffeic acid (3, 7.0 mg) and 4-hydroxycinnamic acid (4, 1.7 mg). Fraction 2 (569 mg) was subjected to silica gel (20 g) column chromatography with EtOAc–Hexane (5:95→0:100) to give 1 (33.1 mg), 2, (8.0 mg), suberic acid (5, 11.1 mg) and protocatechuic acid (6, 2.4 mg). Fraction 5 (755 mg) was separated by silica gel (30 g) column chromatography with MeOH–CHCl₃ (5:95→30:70) to furnish 8 (81.9 mg) and 2-phenylethyl β-D-glucopyranoside (4) (9, 20.4 mg). Fractions 6 and 7 were separately subjected to Sephadex LH-20 (120 g) column chromatography with H₂O–MeOH (10:90→0:100), and fraction 6 gave 3-O-galloylepigallocatechin-(4β→8)-epigallocatechin 3-O-gallate (10, 78.8 mg) and 2-phenylethyl α-L-arabinopyranosyl-(1→6)-β-D-glucopyranoside (11, 19.6 mg), while fraction 7 yielded 8 (49.6 mg) and 10 (99.2 mg).

**Treatment of BuOH-soluble Fraction** A portion (51.4 g) of the BuOH-soluble fraction was subjected to silica
gel (1.0 kg) column chromatography with MeOH–CHCl₃ (5:95–50:50), and eluates were monitored by TLC and finally divided into five fractions (fr. 1, 340 mg; fr. 2, 572 mg; fr. 3, 1.1 g; fr. 4 17.5 g; fr. 5, 16.9 g).

Fractions 2 (500 mg) and 4 (17.5 g) were separated by silica gel (30 g) column chromatography with MeOH–CHCl₃ (10:90), followed by reverse-phase preparative TLC with MeOH–H₂O (50:50). The former fraction gave 7 (61.1 mg), β-D-glucopyranosyl 4-hydroxybenzoate(27) (13, 7.0 mg), rhodiocyanoside A(8) (14, 6.0 mg), sarmentosin(19) (16, 10.8 mg) and heterodendrin(20) (17, 6.5 mg), while the latter fraction yielded 11 (234 mg), sacranoside A(21) (12, 20.4 mg), 14 (4.0 mg), rhodioctanoside(18) (15, 2.0 mg) and 17 (10.8 mg). Fraction 3 (1.1 g) was subjected to Sephadex LH-20 (50 g) column chromatography with H₂O–MeOH (20:80), followed by reverse-phase preparative TLC with MeOH–H₂O (50:50), to afford 11 (2.5 mg), 12 (30.3 mg), arbutin(22) (18, 8.4 mg) and 4-O-(β-D-glucopyranosyl)-gallic acid(23) (19, 9.1 mg).

RESULTS AND DISCUSSION

PEP is known to hydrolyze biologically active peptides, which contain a proline residue and participate in learning and memory processes. In the course of our search for anti-amnesic drugs from Chinese traditional medicines, we examined the PEP inhibitory constituents of the underground part of Rhodiola sacra (Sheng-di-hong-jing-tian, 圣地红景天) and isolated nineteen known compounds 1–19. The structures of 1–7 were identified by direct comparison with authentic samples, and the others were based on analyses of the spectroscopic data and comparisons of them with results in the literature. Previously, umbelliferone, tyrosol, kaempferol, rhodioloside, β-D-stigmaster, cyanoglycosides (14 and rhodiocyanoside D), monoterpene glycosides (12 and sacranoside B), cyanogenic glycosides (17 and iotatualalin), phenethyl glycosides (rhodioloside and 11), aliphatic glycosides (2-methyl-3-buten-2-yl β-D-glucopyranoside, kenposide A and 15) and organic acids were reported as constituents of R. sacra.22,24 However, 1, 2, 5, 8–10, 13, 16, 18 and 19 have been isolated for the first time from R. sacra, and 5, 9, 10, 13, 16, 18 and 19 have been isolated from Rhodiola plants for the first time.25

The inhibitory activities of the nineteen compounds against Flavobacterium PEP are listed in Table 1, together with those of positive controls, Z-Pro–prolinol and Z-Pro–prolinol. The IC₅₀ and Kᵢ values are also presented in Table 1. Among them, seven compounds (6–8, 10, 12, 18, 19) showed inhibitory activities concentration-dependently with IC₅₀ of 27.8, 487, 1.47, 0.437, 348, 391 and 215 μM, respectively.

Until now, many PEP inhibitors have been reported but almost all are synthetic substrate mimetics,4 and natural PEP inhibitors are only thirteen: six peptides29 and seven non-peptides.27–29 On the inhibitory activity, synthetic inhibitors show stronger inhibition and have an IC₅₀ value of nanomolar order (e.g., IC₅₀ of Z-Pro–prolinol is 2.55 mM). The inhibitors obtained from R. sacra have IC₅₀ of 0.437–487 μM, which are equally matched to the natural inhibitors in activity, and the activities of 8 (IC₅₀ 1.47 μM) and 10 (IC₅₀ 0.437 μM) are similar to that reported for the strongest natural inhibitory, staurosporine (IC₅₀ 0.77 μM).27

On the other hand, the inhibition mode of all the peptide inhibitors is competitive, while that of non-peptide inhibitors is non-competitive. Lineweaver–Burk plots for all seven inhibitors obtained from R. sacra indicated that six, except for 6, were noncompetitive inhibitors, but interestingly, 6 showed competitive inhibition (Fig. 1), which is the first example of competitive inhibitor from a natural source. In addition, it should be interesting to note here that 12, showing weak (IC₅₀ 348 μM) but concentration-dependent inhibition, is the first example of an inhibitor of monoterpene glycoside.

Peptidic inhibitors contain an electrophilic center such as an α-keto-β-amine group and this group is considered to be an active center. On the other hand, non-peptidic inhibitors are grouped into two types, one containing a catechol or pyrogallol group or not. In the former compounds, the catechol or pyrogallol group is supposed to be related to its PEP inhibition.26 In our case, 8 and its dimer 10, showing strong inhibitory activity, both contain pyrogallol groups, but 7, also containing a pyrogallol group, showed only weak activity.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration (μM)</th>
<th>Inhibition (%)</th>
<th>IC₅₀ (μM)</th>
<th>Kᵢ (μM)</th>
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<tbody>
<tr>
<td>1</td>
<td>1000</td>
<td>14.6 ± 6.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1000</td>
<td>13.4 ± 2.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1000</td>
<td>1.5 ± 0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1000</td>
<td>6.0 ± 2.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1000</td>
<td>15.9 ± 8.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1000</td>
<td>97.2 ± 1.1</td>
<td>27.8</td>
<td>26.6 ± 0.6</td>
</tr>
<tr>
<td>7</td>
<td>1000</td>
<td>57.8 ± 2.8</td>
<td>487</td>
<td>264 ± 60</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>93.7 ± 3.4</td>
<td>1.47</td>
<td>0.222 ± 0.053</td>
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<td>9</td>
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<td>11.5 ± 2.3</td>
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</tr>
<tr>
<td>10</td>
<td>10</td>
<td>97.3 ± 1.8</td>
<td>0.437</td>
<td>0.174 ± 0.028</td>
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<tr>
<td>11</td>
<td>1000</td>
<td>71.5 ± 9.6</td>
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<tr>
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<td>1000</td>
<td>34.5 ± 7.5</td>
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<td>1000</td>
<td>21.8 ± 3.8</td>
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<td>4.9 ± 2.0</td>
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<td>18.9 ± 7.1</td>
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<td>17</td>
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<td>9.6 ± 5.6</td>
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<tr>
<td>18</td>
<td>1000</td>
<td>64.8 ± 1.4</td>
<td>391</td>
<td>205 ± 44</td>
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<tr>
<td>19</td>
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<td>80.7 ± 7.6</td>
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<td>133 ± 33</td>
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<td>20</td>
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<td>34.8 ± 4.5</td>
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<tr>
<td>21</td>
<td>1000</td>
<td>24.2 ± 8.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>1000</td>
<td>89.3 ± 1.3</td>
<td>2.55 nm</td>
<td>0.421 ± 0.173 nm</td>
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</table>

a) The values are the means of triplicate experiments. b) Kᵢ values were obtained from Dixon plot.
Fig. 1. Kinetic Analysis of the Inhibition by 6, 8, and 10

a) Lineweaver-Burk plot of inhibition by 6, [I]=0 µM ( ), 20 µM ( ● ) and 30 µM ( ● ● ),
    Lineweaver-Burk plot of inhibition by 8, [I]=0 µM ( ), 0.125 µM ( ● ) and 0.25 µM ( ● ● ),
    Lineweaver-Burk plot of inhibition by 10, [I]=0 µM ( ), 0.15 µM ( ● ) and 0.20 µM ( ● ● ).

b) Dixon plot of inhibition by 6, [S]=0.10 mM ( ● ), 0.15 mM ( ● ● ) and 0.25 mM ( ● ● ● ).

c) Dixon plot of inhibition by 8, [S]=0.10 mM ( ● ), 0.15 mM ( ● ● ) and 0.20 mM ( ● ● ● ).

d) Dixon plot of inhibition by 10, [S]=0.10 mM ( ● ), 0.15 mM ( ● ● ) and 0.20 mM ( ● ● ● ).

e) Dixon plot of the inhibition by 6, [S]=0.10 mM ( ● ), 0.15 mM ( ● ● ) and 0.20 mM ( ● ● ● ).

( IC₅₀ 487 µM ). Moreover, 6 revealed mild competitive inhibition ( IC₅₀ 27.8 µM ), but 3 did not inhibit even at 1 mM. Thus, in addition to the presence of a pyrogallol or catechol group, an electric and/or steric factor may be related to the inhibitory activity.

Rhodiola sacra is a perennial plant belonging to the family Crassulaceae and is mainly distributed in the high cold region of Tibet and Sichuan Province in the People's Republic of China. The underground part of this and other Rhodiola plants (Hong-jing-tian, 紅景天 ) is used as a hemostatic, tonic and curative in traditional Chinese medicines, and recently it has been reported that Rhodiola plants demonstrate anti-anoxia, anti-fatigue, anti-toxic and anti-radiation effects and improve learning and memory. In this paper, we reported seven PEP inhibitors from the underground part of R. sacra. Among them, 8 was suggested to be absorbed from the digestive tract and reach the brain in rats. On the other hand, Yoshimoto et al. reported that a relatively weak PEP inhibitor such as Z-Gly-Pro-CH₂Cl ( Kᵣ value, 70 µM ) also had a strong anti-amnesic effect and that Z-Gly-Pro ( Kᵣ value, 620 µM ) and Z-Val-Pro ( Kᵣ value, 120 µM ) showed the same tendency in animal models. Thus, although more detailed examination will be needed, it is probable that the inhibitors isolated from R. sacra inhibit PEP metabolism, to a different extent, and contribute to the improvement of learning and memory by R. sacra.

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REFERENCES AND NOTES


25) Reports on the constituents of other Rhodiola plants are as follows:


