A Prolyl Endopeptidase-Inhibiting Benzofuran Dimer from *Polyozellus multiflex*

Kyung-Sik Song*,† and Ilya Raskin‡

Department of Agricultural Chemistry, Kyungpook National University, 1370 Sankyuk-Dong, Taegu 702-701, Korea, and Biotech Center, Foran Hall, Cook College, Rutgers, 59 Dudley Road, New Brunswick, New Jersey 08901

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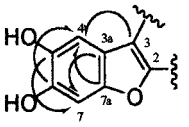
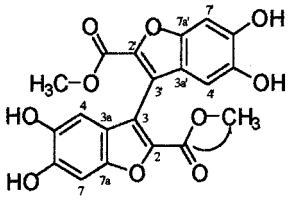
A new benzofuran dimer, 5,6,5′,6′-tetrahydroxy[3,3′]bibenzofuranyl-2,2′-dicarboxylic acid dimethyl ester (kynapcin-24), was isolated from *Polyozellus multiflex* and shown to noncompetitively inhibit prolyl endopeptidase (PEP), with an IC_{50} value of 1.14 μM. Kynapcin-24 was less inhibitory to other serine proteases such as chymotrypsin, trypsin, and elastase.

Prolyl endopeptidase [PEP; EC 3.4.21.26] is a serine protease, which is known to cleave peptide substrates in the *C*-terminal side of proline residues.[1] It plays an important role in degradation of the proline-containing neuropeptides such as oxytocin, vasopressin, substance P, neurotensin, and angiotensin, which have been speculated to participate in learning and memory processes.[2,3] The PEP activity of Alzheimer's patients has been found to be significantly higher than that of the normal person.[4] It has been postulated that specific PEP inhibitors could prevent memory loss and increase attention span in patients suffering from senile dementia. For example, some natural and synthetic PEP inhibitors have been reported to show dose-dependent cognition-enhancing activity in rats with scopolamine-induced amnesia.[5,6] Therefore, much effort has been devoted to searching for PEP inhibitors as leads to develop anti-dementia drugs. PEP inhibitors such as kynapcin-9[7] and -12,[8] polyozellin,[9] and thelephoric acid[7,10] have been previously isolated from the mushroom *Polyozellus multiplex* Murr. In this report, the isolation, physicochemical properties, structure elucidation, and PEP inhibitory activity of a new compound, kynapcin-24, are described.

The fruiting bodies of *P. multiplex* (Therephoraceae) were air-dried in a well-ventilated fume hood and extracted with MeOH. The MeOH extract was partitioned with EtOAc, and the EtOAc soluble fraction was repeatedly chromatographed on silica gel and Lobar RP-18 columns to yield **1** as a dark yellow powder that was positive to $FeCl_3$ reagent. Hydroxyl (3229 cm^{-1}) and carbonyl stretching (1709 cm^{-1}) bands were observed in the IR spectrum. Its molecular formula was determined as $C_{20}H_{14}O_{10}$ by high-resolution FABMS. In the ^1H NMR spectrum, two exchangeable protons (δ 9.30 and 9.57), two aromatic singlets (δ 7.01 and 7.31), and a methoxyl signal (δ 3.74) were evident. In the ^{13}C NMR spectrum, two aromatic methine carbons (δ 97.7 and 105.7), six aromatic quaternary carbons (δ 113.3 to 148.7), one methoxyl carbon (δ 51.4), and a carbonyl carbon at δ 163.0 were observed. To accommodate the molecular formula and the number of carbon signals observed in the ^{13}C NMR spectrum, **1** must have a symmetrical structure. The chemical shifts of the aromatic signals in the ^1H and ^{13}C NMR were very similar to those of 5,6-dihydroxybenzofurans.[11] The NMR assignment and a partial structure **1a** were established by COLOC analysis, in which the carbon resonance at δ 113.3 (C-3) was correlated with the proton signal at δ 7.31 (H-4). The signal at δ 145.8, which did not show any correlations in COLOC, was assigned to C-2. The partial structure **1a** could be connected as a dimer through either a 3−3′ or 2−2′ linkage. If **1a** was connected through C-2 and C-2′, the chemical shift of C-2, which is β to the carbonyl group, should be downfield about 10 ppm relative to the reported data.[11] However, the chemical shift of C-2 is very similar to those of benzofuran 2-carboxylic acids,[12−14] thus supporting the location of the dimeric linkage as 3−3′. From these observations, the structure of kynapcin-24 was identified as 5,6,5′,6′-tetrahydroxy[3,3′]-bibenzofuranyl-2,2′-dicarboxylic acid dimethyl ester and given the trivial name kynapcin-24 (**1**). 5,6-Dihydroxybenzofurans or their dimers have never been isolated from natural sources, although some related benzofuran carboxylic acid monomers have been synthesized.[11−14]



1a



Kynapcin-24 (1)

Arrows indicate the correlations in COLOC

The PEP inhibitory activity of kynapcin-24 (IC_{50} = 1.14 ± 0.16 μM) was less than that of the positive control *Z*-Pro-Prolinal (IC_{50} = (5.16 ± 0.11) × 10^{-2} μM) but similar to those of polyozellin (IC_{50} = 2.72 μM)[9] and kynapcin-12 (IC_{50} = 1.25 μM),[8] which have been previously isolated from *P. multiflex*. Kynapcin-24 was noncompetitive with a substrate in a Dixon plot (Figure 1). Data points in the Dixon plot represent the mean values of 10 replications. The inhibition constant (K_i) was 0.29 μM. To check the enzyme specificity, the inhibitory activity on other serine proteases such as chymotrypsin, trypsin, and elastase was compared with that of PEP. At 12.2 μM, kynapcin-24 inhibited 97.5 ± 0.4% of PEP activity, but showed no

* To whom correspondence should be addressed. Tel: +82-53-950-5715. Fax: +82-53-953-7233. E-mail: kssong@bh.knu.ac.kr.
† Kyungpook National University.
‡ Biotech Center, Rutgers.

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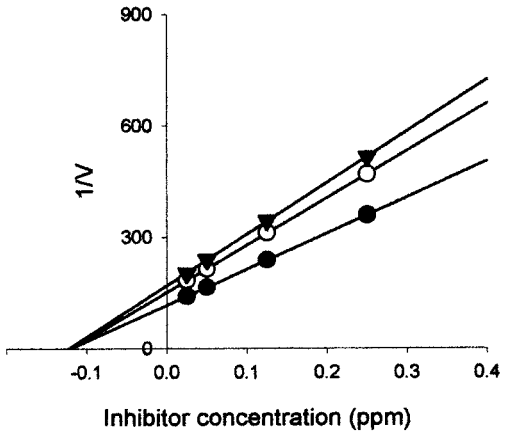

Figure 1. Dixon plot of the inhibition of PEP by kynapcin-24. Concentration of substrate 207 ppm (0.5 mM, −▲−), 310.5 ppm (0.75 mM, −○−), 414 ppm (1.0 mM, −●−).

Table 1. Inhibitory Activity[a] of Kynapcin-24 against PEP and Other Serine Proteases

conc (μM)	chymotrypsin	trypsin	elastase	PEP
2.4	3.7(±2.1)	3.1(±1.3)	22.0(±4.3)	67.5(±0.6)
12.2	6.6(±2.4)	5.6(±1.7)	27.6(±5.2)	97.5(±0.4)
97.6	4.0(±2.0)	5.9(±0.9)	29.0(±3.7)	98.0(±0.1)
control[b]	4.4(±1.9)	2.9(±1.1)	8.1(±4.4)	3.1(±0.2)

[a] The activities are presented as means of five experimental data points and are relative to percent of solvent-treated control. Numbers in parentheses are standard errors of the mean. [b] 10 μL of MeOH was added to the reaction mixture instead of sample solution.

significant inhibition of chymotrypsin and trypsin (Table 1). Even at the higher concentration of 97.6 μM, kinapcin-24 inhibited less than 5.9 ± 0.9% of chymotrypsin and trypsin and 29.0 ± 3.0% of elastase activity. The inhibition of PEP was almost saturated at this concentration. Thus, kynapcin-24 appears to be a relatively specific inhibitor of PEP, as is the case with other natural inhibitors.[7−10,23]

Many pyrrolidine derivatives such as *Z*-Pro-Prolinal and JTP-4819 have been synthesized as potent PEP inhibitors.[15] On the other hand, staurosporine,[16] poststatin,[17] eurystatin,[18] lipohexin,[19] propeptin,[20] and SNA-8073-B[21] were isolated from microbial sources. Plant-derived flavonoids containing a catechol ring[22] and tannins with a pyrogallol moiety[23] have also been reported to effectively inhibit the activity of PEP. The presence of a carbonyl group with a catechol or pyrogallol moiety has been suggested as the essential structural feature for PEP inhibitory activity.[22,24] Although propeptin (IC_{50}, 1.1 μM) has activity similar to kynapcin-24, it is a large molecular weight peptide with a hydrophilic moiety, which may make it difficult to penetrate the blood-brain barrier. The non-peptidyl and small molecular weight kynapcin-24, isolated from the edible mushroom *P. multiplex*, may have potential use in the prevention and treatment of Alzheimer's disease.

Experimental Section

General Experimental Procedures. Optical density was measured with an ELISA autoreader (Bio-TEK ELX 808, USA). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer (Germany) at 400 and 100 MHz, respectively. Chemical shifts were given in δ (ppm) from TMS. IR was measured in KBr disk on a Bruker IFS120HR/FRA106 spectrophotometer (Germany). EIMS and high-resolution FABMS were recorded on VG QUATTRO II (VG, UK) and JMS HX-110/110A (JEOL, Japan) spectrometers, respectively. UV/vis scanning was made on a Varian CARY5G spectrometer (Australia). Melting points were measured with a Gallenkamp melting point apparatus (Sanyo, Japan). TLC was performed on precoated silica gel plates (Merck, Art. 5715). Si gel column chromatography was carried out on Merck Art. 7734.

Material, Extraction, and Isolation. The fruiting bodies of *P. multiplex* (8 kg) were collected at Mt. Odae, Kangwon-Do, Korea, and identified as previously reported.[25] The specimen (voucher no. knunpc-98-pm03) is stored at the Department of Agricultural Chemistry, Kyungpook National University, Taegu, Korea. After being air-dried in the fume hood at room temperature, the mushrooms were refluxed in MeOH. The MeOH extract (674.8 g) was suspended in water and partitioned with *n*-hexane and EtOAc, consecutively. The EtOAc-soluble fraction (31.2 g out of 90.68 g) was chromatographed on a Si gel column [8 × 36 cm, $CHCl_3$−MeOH (7:1) → $CHCl_3$−MeOH−H_2O (60:20:1) → $CHCl_3$−MeOH−HOAc (5:2:0.3)] to give five fractions. The third fraction (2.8 g) was rechromatographed on a Si gel (5 × 40 cm) using a stepwise gradient of $CHCl_3$ and MeOH as eluent (8:1 to 1:1). Four fractions were collected, and the second fraction (580.4 mg) was applied on a Si gel column (3 × 40 cm, *n*-hexanes−EtOAc−AcOH (2:1:0.1 to 1:1:0.1) to yield three fractions. Finally, two repetitions of Lobar RP-18 chromatography (Merck LiChroprep RP-18, 40−63 μm, 2.5 × 25 cm, 2.2 mL min^{-1}; first, eluted with 60% MeOH then the active fraction was rechromatographed with 40% MeOH by the same column and flow rate) of the second fraction (152.9 mg) afforded **1** (7.7 mg).

Kynapcin-24 (1): dark yellow powder, mp 257 °C (dec); UV (EtOH) λ_{max} (log ϵ) 215.4 (4.09), 271.9 (4.05), 387.6 (3.85) nm; IR (KBr) ν_{max} 3229 (OH), 2955 (aliphatic C−H), 1709 (C=O), 1300 (C−O) cm^{-1}; ^{1}H NMR [DMSO-d_6−$CDCl_3$ (1:1), 400 MHz] δ 3.74 (3H, s, −OC*H*$_3$), 7.01 (1H, s, H-7,7′), 7.31 (1H, s, H-4,4′), 9.30 (1H, brs, −O*H*), 9.57 (1H, brs, −O*H*); ^{13}C NMR [DMSO-d_6−$CDCl_3$ (1:1) 100 MHz] δ 51.4 (q, O*C*H$_3$), 97.7 (d, C-7,7′), 105.7 (d, C-4,4′), 113.3 (s, C-3,3′), 117.0 (s, C-3a,3a′), 144.4 (s, C-5,5′), 145.8 (s, C-2,2′), 146.7 (s, C-6,6′), 163.0 (s, *C*OO); EIMS (70 eV) *m/z* 414 [M]$^+$, 383 [M − OCH_3]$^+$, 356 [M − $COOCH_3$ + H]$^+$; positive ion HRFABMS *m/z* 415.0567 [M + 1]$^+$ (calcd for $C_{20}H_{15}O_{10}$, 415.0586).

Enzyme Assays. Prolyl endopeptidase (from *Flavobacterium meningosepticum*) and its substrate (*Z*-Gly-Pro-*p*NA) were purchased from Seikagaku Co (Japan). *Z*-Pro-Prolinal was used as a positive control and synthesized according to Bakker et al.[26] Chymotrypsin, trypsin, and elastase were purchased from Sigma. PEP activity and inhibition percent of samples were determined according to the method of Yoshimoto et al.[27] Briefly, a mixture of 210 μL of 0.1 M Tris-HCl buffer (pH 7.0), 20 μL of 2 mM *Z*-Gly-Pro-*p*NA (in 40% dioxane), 10 μL of the sample solution (in MeOH), and 10 μL of 0.1 unit/mL PEP was incubated at 30 °C for 30 min, and A_{410} of the reaction mixture was then measured (A). The A_{410} of the mixture containing 240 μL of 0.1 M Tris-HCl (pH 7.0) and 10 μL of the sample was separately measured as above (B). A control was made by adding 10 μL of solvent instead of the sample solution to 240 μL of the buffer. The percent inhibition was calculated by the following equation: inhibition % = [{A_{410} of control − (A − B)}/A_{410} of control] × 100. Chymotrypsin, trypsin, and elastase were assayed according to the protocols described in the Sigma catalog (Sigma Chemical Company, MO) using *N*-benzoyl-L-Arg-*p*NA, *N*-benzoyl-L-Tyr-*p*NA, and *N*-succinyl-Ala-Ala-Ala-*p*NA as substrates, respectively.

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References and Notes

(1) Yaron, A.; Naider, F. *Critic. Rev. Biochem., Mol. Biol.* **1993**, *28*, 31−81.
(2) Rennex, D.; Hemmings, B. A.; Hofsteenge, J.; Stone, S. R. *Biochemistry* **1991**, *30*, 7195−2203.
(3) Yoshinoto, T.; Nishimura, T.; Kita, T.; Tsuru, D. *J. Biochem.* **1893**, *94*, 1179−1190.
(4) Aoyagi, T.; Wada, T.; Nagai, M.; Kojima, F.; Harada, S.; Takeuchi, T.; Takahashi, H.; Hirokawa, K.; Tsumita, T. *Experientia* **1990**, *46*, 94−97.

(5) Portevin, B.; Benoist, A.; Remond, G.; Herve, Y.; Vincent, M.; Lepagnol, J.; De Nanteuil, G. *J. Med. Chem.* **1996**, *39*, 2379−2391.
(6) Yoshimoto, T.; Kado, K.; Matubara, F.; Kpriyama, N.; Kaneto, H.; Tsuru, D. *J. Pharmacobio-Dyn.* **1987**, *10*, 730−735.
(7) Kwak, J.-Y.; Rhee, I.-K.; Lee, K.-B.; Hwang, J.-S.; Yoo I.-D.; Song, K.-S. *J. Microbiol., Biotechnol.* **1999**, *9*, 798−803.
(8) Lee, H.-J.; Rhee, I.-K.; Lee, K.-B.; Song, K.-S. *J. Antibiot.* **2000**, *53*, 714−719.
(9) Hwang, J.-S.; Song, K.-S.; Kim, W.-G.; Lee, T.-H.; Koshino, H.; Yoo, I.-D. *J. Antibiot.* **1997**, *50*, 773−777.
(10) Gripenberg, J. *Acta Chem. Scand.* **1958**, *12*, 1411−1414. Tringali, C.; Piattelli, M. *Can. J. Chem.* **1987**, *65*, 2369−2372.
(11) Brinkmeier, E.; Geiger, H.; Zinsmeister, H. D. *Phytochemistry* **1999**, *52*, 297−302.
(12) Williams, L. A. D.; Anderson, M.; Jackson Y. A. *Pestic. Sci.* **1994**, *42*, 167−171.
(13) Redondo, J.; Sanchez-Ferrando, F.; Valls, M.; Virgili, A. *Magn. Reson. Chem.* **1988**, *26*, 511−517.
(14) Traulsen, T.; Friedrichsen, W. *J. Chem. Soc., Perkin Trans. 1* **2000**, 1387−1398.
(15) Arai, H.; Nishioka, H.; Niwa, S.; Yamanaka, T.; Tanaka, Y.; Yoshinaga, K.; Kobayashi, N.; Miura, N.; Ikeda, Y. *Chem. Pharm. Bull.* **1993**, *41*, 1583−1588.
(16) Kimura, K.; Kanou, F.; Koshino, H.; Uramoto, M.; Yoshihara, M. *J. Antibiot.* **1997**, *50*, 291−296.
(17) Aoyagi, T.; Nagai, M.; Ogawa, K.; Kojima, F.; Okada, M.; Ikeda, T.; Hamada, M.; Takeuchi, T. *J. Antibiot.* **1991**, *44*, 949−955.
(18) Toda, S.; Obi, Y.; Numata, K.; Hamagishi, Y.; Tomita, K.; Komiyama, N.; Kotake, C.; Furumai, T.; Oki, T. *J. Antibiot.* **1992**, *45*, 1573−1579.
(19) Christner, C.; Zerlin, M.; Gräfe, U.; Heinze, S.; Küllertz, G.; Fisher, G. *J. Antibiot.* **1997**, *50*, 384−385.
(20) Kimura, K.; Kanou, F.; Takahashi, H.; Esumi, Y.; Uramoto, M.; Yoshihara, M. *J. Antibiot.* **1997**, *50*, 373−378.
(21) Kimura, K.; Kanou, F.; Koshino, H.; Uramoto, M.; Yoshihara, M. *J. Antibiot.* **1997**, *50*, 291−296.
(22) Lee, K.-H.; Kwak, J.-H.; Lee, B.-K.; Song, K.-S. *Arch. Pharm. Res.* **1998** *21*, 207−211.
(23) Fan, W.; Tezuka, Y.; Komatsu, K.; Namaba, T.; Kadata, S. *Biol. Pharm. Bull.* **1999**, *22*, 157.
(24) Kim, S.-I.; Song, K.-S. *J. Agric. Chem., Biotechnol.* **2000**, *43*, 158−161.
(25) Hwang, J.-S.; Song, K.-S.; Kim, Y.-S.; Seok, S.-J.; Lee, T.-H.; Yoo, I.-D. *Kor. J. Appl. Microbiol. Biotechnol.* **1996**, *24*, 591−596.
(26) Bakker, A. V.; Jung, S.; Spencer; R. W.; Vinick, F. J.; Faraci, W. S. *Biochem. J.* **1990**, *271*, 559−562.
(27) Yoshimoto, T.; Walter, R.; Tsuru, D. *J. Biol. Chem.* **1980**, *225*, 4786−4792.

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