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Thelephoric acid and Kynapcin-9 in Mushroom *Polyozellus multiflex* Inhibit Prolyl Endopeptidase *In Vitro*

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Abstract Prolyl endopeptidase [PEP; EC 3.4.21.26], a serine protease which is known to cleave peptide bonds on the carboxy side of a proline residue, plays an important role in the degradation of proline-containing neuropeptides that have been suggested to participate in learning and memory processes. An abnormal increase in the level of PEP, which can lead to generation of Aβ, is also suggested to be involved in Alzheimer's type senile dementia. In the course of screening PEP inhibitors from Basidiomycetes, the mushroom *Polyozellus multiplex* exhibited a high inhibitory activity against PEP. Two active compounds were isolated from the ethyl acetate soluble fraction by consecutive purification, using silica gel, Sephadex LH-20, and Lobar RP-18 chromatography. The chemical structures of these compounds were identified as thelephoric acid and 12-acetyl-2,3,7,8-tetrahydroxy-[12*H*]-12-hydroxymethylbenzobis[1.2*b*,3.4*b*'] benzofuran-11-one (kynapcin-9) by spectral data including UV, IR, MS, HR-MS, ^{1}H-, ^{13}C-, and 2D-NMR. The IC_{50} values of the thelephoric acid and kynapcin-9 were 0.157 ppm (446 nM) and 0.087 ppm (212 nM) and their inhibitor constants (K_i) were 0.73 ppm (2.09 μM) and 0.060 ppm (146 nM), respectively. Furthermore, they were non-competitive with a substrate in Dixon plots.

Key words: Prolyl endopeptidase, inhibitor, *Polyozellus multiflex*, thelephoric acid, 12-acetyl-2,3,7,8-tetrahydroxy-[12*H*]-12-hydroxymethylbenzobis[1.2*b*,3.4*b*'] benzofuran-11-one (Kynapcin-9)

The deposition of the amyloid β protein (Aβ) is an extracellular plaque, and cerebrovascular amyloid is one of the major histopathological features of Alzheimer's disease [22]. Aβ(1-42) with an alanine *C*-terminus is derived from the proteolytic cleavage of a large protein, known as an amyloid precursor protein (APP), by the action of a yet unidentified endoproteolytic enzyme 'secretase' [6]. The neurotoxicity of Aβ has been detected in several cell systems, including primary-cultured neurons [17]. Accordingly, in the development of anti-dementia drugs, it would seem to be important to identify agents that can prevent the formation of Aβ. Prolyl endopeptidase [PEP; EC 3.4.21.26] is a serine protease which is known to cleave peptide substrates on the *C*-terminal side of proline residues. PEP also plays an important role in the degradation of proline-containing neuropeptides such as oxytocin, vasopressin, substance P, neurotensin, and angiotensin, which have been suggested to be participants in learning and memory processes [21, 27]. It was previously found that the PEP activity of Alzheimer's patients was significantly higher than that of a normal person [3]. In addition, recent studies suggested that PEP could be involved in the processing of the *C*-terminal portion of the amyloid precursor protein in Alzheimer's disease [12]. As a result, it has been postulated that specific PEP inhibitors could prevent memory loss and increase attention span in patients suffering from senile dementia. Some natural and synthetic PEP inhibitors have been reported to show dose-dependant cognition-enhancing activity in rats with scopolamine-induced amnesia [18, 26]. Eurystatin [24], poststatin [2], staurosporine [15], SNA-8073-B [13], propeptin [14], and lipohexin [7] have been isolated as PEP inhibitors from microbial origin, and the modification of poststatin to non-peptidyl analogues has also been investigated [25]. Previously, in the course of screening for PEP inhibitors from Basidiomycetes, polyozellin was isolated from *Polyozellus multiplex* [10]. During further investigation, two more compounds were isolated from the methanolic extract of *P. multiplex*. This report discusses the isolation, physicochemical properties, structure determination, and inhibitory activities of these two compounds.

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MATERIALS AND METHODS

General

Optical density was measured with an ELISA autoreader (Bio-TEK ELX 808, U.S.A.). ^{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 spectra were measured in KBr disc on a Bruker IFS120HR/FRA106 spectrophotometer (Germany). EI-MS and HR-FAB-MS were measured on VG QUATTRO II (VG, U.K.) and JMS HX-110/110A (JEOL, Japan) spectrometers, respectively. UV spectra were analyzed by a Varian CARY5G spectrometer (Australia). Melting points were measured by a Gallenkamp melting point apparatus (Sanyo, Japan).

Prolyl endopeptidase (from *Flavobacterium meningosepticum*) and a 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.* [5].

Chymotrypsin, trypsin, and elastase were purchased from Sigma and assayed according to the protocol described in the Sigma catalogue using *N*-benzoyl-L-Arg-*p*NA, *N*-benzoyl-L-Tyr-*p*NA, and *N*-succinyl-Ala-Ala-Ala-*p*NA as substrates, respectively. Polyozellin was purified from *P. multiflex* by the previous method [3].

PEP Assay

The PEP activity and inhibition percent of the samples were determined according to the method of Toda *et al.* [24]. 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, 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). Control was made by adding 10 μl of distilled water instead of the sample solution to 240 μl of the buffer. The % of inhibition was calculated by the following equation.

$$\text{Inhibition } (\%) = \frac{A_{410} \text{ of Control} - (A - B)}{A_{410} \text{ of Control}} \times 100$$

Microorganism, Extraction, Purification, and Isolation

Fruiting bodies of *Polyozellus multiplex* (1 kg) were collected from Mt. Odae, Korea, and identified by previous report [11]. After drying in a well-ventilated place, they were refluxed three times in 3 l methanol (MeOH). The extract was then evaporated to dryness, and the residue (96.4 g) was suspended in water and partitioned three times with 2.5 l ethyl acetate (EtOAc). The EtOAc soluble fraction (60.7 g) was suspended in 500 ml MeOH and subsequently filtered through a suction flask and Büchner funnel. The MeOH insoluble fraction was washed more than ten times with MeOH to produce compound **1** (50 mg) as a black powder. A part of the MeOH soluble portion (31.2 g) was chromatographed on a silica gel column (Merck Art. 7734, 8×36 cm, chloroform-MeOH = 7:1 to 100% MeOH) to give rise to fr. I to fr. V. The active fr. III (2.82 g) was then applied to a silica gel column (Merck Art. 7734, 5×40 cm, chloroform-MeOH = 8:1 to 100% MeOH). As a result, Fr. III-1 to III-6 were obtained and fr. III-2 (1.54 g) was then further purified by silica gel column (Merck Art. 9385, 4×26 cm, *n*-hexane-EtOAc-acetic acid = 2:1:0.1 to 1:1:0.1) chromatography. Two times of Lobar RP-18 (Merck LiChroprep RP-18, 40-63 μm, 2.5×25 cm, 2.2 ml min^{-1}, 1st.; 60%, 2nd.; 40% MeOH), followed by Sephadex LH-20 (Sigma, 5×64 cm, 60% MeOH to 100% MeOH) chromatography produced compound **2** (32 mg) as a red brown powder.

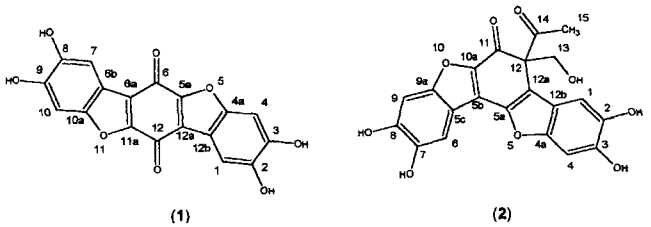
Deacetylation of Polyozellin

Polyozellin (80.7 mg) was added to 20 ml of 0.2 N HCl in 80% dioxane and the mixture was heated in a water bath at 50°C for 7 h. After evaporation, the dark residue was repeatedly washed with MeOH and water. The final black powdered material was dried in a vacuum desiccator to produce 54.6 mg of thelephoric acid.

RESULTS AND DISCUSSION

Structure Determination

Compound **1** was positive to $FeCl_3$ reagent, suggesting that it includes a phenolic OH group(s) in its structure. A broad band near 3380 cm^{-1} in the IR spectrum indicated the presence of a hydroxyl group(s). In ^{13}C-NMR (in DMSO-d_6), an α,β-unsaturated ketone signal appeared at δ 176.58 ppm with six aromatic quarternary carbons (δ 151.88 to 115.82 ppm) and two aromatic methine carbons (δ 105.46 and 99.81 ppm). Only two aromatic methine proton singlets were detected in ^{1}H-NMR (in DMSO-d_6). The NMR data are very similar to those of polyozellin except for the absence of an acetyl signal. The UV spectrum showed typical thelephoric acid absorption bands at 248 and 479 nm [9]. From these observations, compound **1** was suggested to be thelephoric acid, a deacetylated form of polyozellin, which has been found in several species of



(1) (2)

Fig. 1. Strucutures of thelephoric acid (**1**) and kynapcin-9 (**2**).

Table 1. Physicochemical properties of thelephoric acid and kynapcin-9.

	Thelephoric acid (**1**)	Kynapcin-9 (**2**)
Appearance	Black powder	Reddish brown powder
MP (°C)	>300°C (dec.)	200°C (dec.)
EI-MS *m/z*	520 [M^+] (as a tetra acetate) 478 [M^+-Ac+H], 436 [M^+-2Ac+H]	410 [M^+], 396 [M^+-CH_3+H)], 380 [M^+-CH_3O+H], 338 [380-Ac+H]
HR-FAB-MS *m/z* found/calcd.	-	410.3372/410.3368 (for $C_{21}H_{14}O_9$)
Molecular formular	$C_{18}H_8O_8$	$C_{21}H_{14}O_9$
UV λ_{max}^{MeOH} nm (log ε)	587 (2.02), 479 (2.01), 248 (2.09), 207 (2.35)	456 (3.07), 303 (3.13), 264 (3.19), 210 (3.29)
IR (KBr) ν cm^{-1}	3379, 2924, 1656, 1626, 1467, 1444, 1294	3319, 1630, 1483, 1294, 1248

Thelephora and chemically synthesized [9]. To verify the structure of compound **1**, polyozellin was deacetylated with 2 N HCl. The deacetylated form of polyozellin can be readily auto-oxidized in air to form the terphenyl quinone, thelephoric acid. The spectral data of the resulting compound were absolutely identical to those of compound **1**. Therefore, compound **1** was confirmed as thelephoric acid (**1**) (Fig. 1). The physicochemical properties of thelephoric acid are presented in Table 1 and the NMR data are shown in Table 2.

Compound **2** was found to be positive to $FeCl_3$. The molecular formula was determined as $C_{21}H_{14}O_9$ using high resolution fast atom bombardment mass spectroscopy (HR-FAB-MS) and NMR data. The broad IR band near 3,320 cm^{-1} suggested the presence of a hydroxyl moiety. In ^{1}H-NMR (in methanol-d_4), four singlet aromatic proton resonances were detected at δ 7.35 (1H), 7.16 (1H), 7.08 (1H), and 7.04 (1H) ppm. In addition, an oxymethylene signal at δ 3.61 (2H) ppm was detected with an acetyl proton signal at δ 2.01 (3H) ppm. In ^{13}C-NMR, a total of twenty one carbon signals appeared, out of which fifteen were detected as quarternary, four methine, one methylene, and one methyl carbon using DEPT (distortionless enhancement by polarization transfer) analysis. The chemical shift patterns were similar to those of *p*-terphenyls [1, 9, 23] yet quite different in that they had three more skeletal carbons. In addition, they should have an asymmetrical structure, when considering the molecular weight and the numbers of carbon signals in the ^{13}C-NMR spectrum. In order to assign NMR signals and deduce the chemical structure of compound **2**, HMQC (heteronuclear multiple quantum correlation) and HMBC (heteronuclear multiple bond correlation) analyses were carried out. The partial structure of (**2a**) was established by chemical shifts, spin-spin splitting patterns (all singlets), and two- or three-bond correlations of aromatic protons with carbons in HMBC analysis. The partial structure of (**2c**) was deduced by almost the same methods used with those in (**2a**). In HMBC, methyl protons at δ 2.01 ppm was correlated with δ 208.14 ppm carbonyl and δ 75.52 ppm quarternary carbon. In addition, oxymethylene proton at δ 3.61 ppm showed correlation spots with δ 75.52 and 127.49 (*s*) ppm. Accordingly, the partial structure of (**2b**) was established (Fig. 2).

Partial structures (**2b**) and (**2c**) were connected as the partial structure (**2d**) since the δ 7.15 ppm proton was correlated with the δ 127.49 ppm (*s*) carbon and this carbon showed a correlation spot with the δ 3.61 ppm methylene protons in HMBC. The structure of (**2d**) could be fused with (**2a**) in two possible ways as shown in (**2e**) and (**2**) (Figs. 1 and 2). However, in the case of (**2e**), the chemical shift at δ 156.51 was too high field-shifted to be assigned as a β-carbon to ketone, when compared to previously reported data [20]. The biosynthetic pathway of terphenyls, as discussed in a pevious report [19], provides another indirect evidence on the structure of **2**, as it suggests that

Table 2. NMR data[a] of thelephoric acid and kynapcin-9.

No.	Thelephoric acid (**1**)		Kynapcin-9 (**2**)	
	^{1}H (*multi.*)	^{13}C (*multi.*)	^{1}H (*multi.*)	^{13}C (*multi.*)
1	7.13 (*s*)	99.81 (*d*)	7.15 (*s*)	105.78 (*d*)
2		147.47 (*s*)		145.51 (*s*)
3		141.59 (*s*)		148.04 (*s*)
4	7.23 (*s*)	105.46 (*d*)	7.04 (*s*)	99.88 (*d*)
4a		115.82 (*s*)		153.26 (*s*)
5a		116.07 (*s*)		141.76 (*s*)
5b				156.51 (*s*)
5c				117.39 (*s*)
6		176.58 (*s*)	7.35 (*s*)	106.92 (*d*)
7	7.13 (*s*)	99.81 (*d*)		146.27 (*s*)
8		147.47 (*s*)		146.98 (*s*)
9		141.59 (*s*)	7.08 (*s*)	99.94 (*d*)
9a				151.77 (*s*)
10	7.23 (*s*)	105.46 (*d*)		
10a		115.82 (*s*)		156.51 (*s*)
11				198.21 (*s*)
11a		116.07 (*s*)		
12		176.58 (*s*)		75.52 (*s*)
12a				127.49 (*s*)
12b				119.74 (*s*)
13			3.61 (*s*)	55.07 (*t*)
14				208.14 (*s*)
15			2.01 (*s*)	31.07 (*q*)

[a]Presented in ppm from TMS and measured in DMSO-d_6.

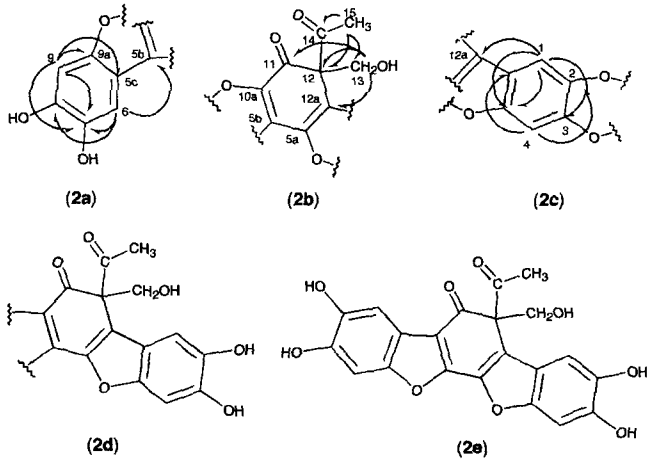


Fig. 2. Partial structures of kynapcin-9 (**2**) and summarized HMBC data.
Arrows indicate the correlations between proton and carbons in HMBC.

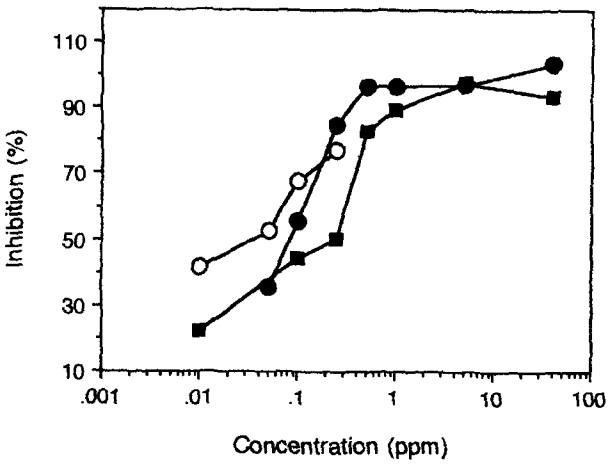


Fig. 3. Concentration-dependant inhibition of prolyl endopeptidase by thelephoric acid (**1**) and kynapcin-9 (**2**).
○, Z-Prolyl-prolinal used as a positive control; ●, thelephoric acid; ■, kynapcin-9.

terphenyls may be synthesized through condensation of two phenylpyruvic acids. Considering the above facts, compound **2** was identified as a new compound, 12-acetyl-2,3,7,8-tetrahydroxy[12*H*]12-hydroxymethylbenzobis[1.2*b*,3.4*b*'] benzofuran-11-one (designated as kynapcin-9).

The physicochemical properties of kynapcin-9 are summarized in Table 1 and the NMR data are listed in Table 2. The HMBC data of kynapcin-9 are summarized in Fig. 2 and the structure of kynapcin-9 is presented in Fig. 1.

Biological Activity

The inhibitory activities of thelephoric acid and kynapcin-9 against PEP were measured as described in Materials and Methods. The IC_{50} values of thelephoric acid and kynapcin-9 were 0.157 ppm (446 nM) and 0.087 ppm (212 nM), respectively (Fig. 3). Their activities were lower than that of *Z*-Pro-prolinal (22 ppb), used as a positive control. They were also found to be noncompetitive with a substrate in Dixon plots (Fig. 4). The inhibitor constants (K_i) were 0.737 ppm (2.09 μM; thelephoric acid) and 0.060 ppm (146 nM; kynapcin-9). In order to define the specificity of the isolated compounds, their inhibitory activities on other serine proteases such as chymotrypsin, trypsin, and elastase were examined. The compounds were not able to inhibit more than 33% of chymotrypsin and trypsin activity at 40 ppm. In the case of elastase, they showed a slight inhibition, however, not so much in the case of PEP (Table 3). Accordingly, thelephoric acid and kynapcin-9 are believed to be relatively specific inhibitors of PEP.

Many pyrrolidine derivatives like Z-Pro-Prolinal and JTP-4819 have been synthesized [4], whereas staurosporine [15], poststatin [2], eurystatin [24], lipohexin [7], propeptin [14], and SNA-8073-B [13] were all isolated from microbial sources. Flavonoids containing a catechol ring [16] and tannins with a pyrogallol moiety [8] from plant sources

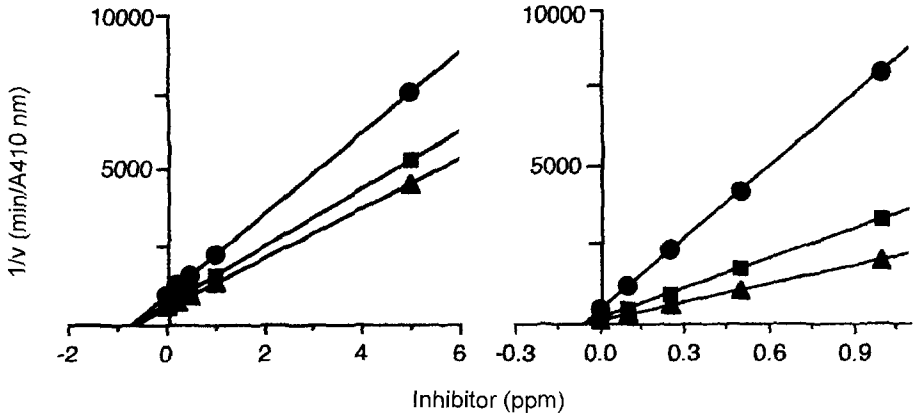


Fig. 4. Dixon plots of thelephoric acid (**1**) and kynapcin-9 (**2**).
Left: thelephoric acid; ●, 0.50 mM; ■, 0.75 mM; ▲, 1.00 mM. Right: kynapcin-9; ●, 0.10 mM; ■, 0.25 mM; ▲, 0.50 mM.

Table 3. Enzyme specificity of thelephoric acid and kynapcin-9[a].

Enzyme	Thelephoric acid (**1**)			Kynapcin-9 (**2**)		
	1 ppm	5 ppm	40 ppm	1 ppm	5 ppm	40 ppm
Chymotrypsin	2.8	4.4	4.6	0.8	0.5	8.3
Trypsin	3.2	3.6	4.6	13.2	32.8	21.0
Elastase	47.8	50.7	65.5	48.7	70.6	68.1

[a]Presented as inhibition %.

have been reported to effectively inhibit PEP. The catechol or pyrogallol ring is supposedly essential for a stronger activity [8, 16]. Thelephoric acid and kynapcin-9 showed a slightly higher inhibitory activity than staurosporine (IC_{50} = 0.7 μM) and propeptin (1.1 μM), and a much higher activity than lipohexin (25 μM), SNA-8073B (3.1 ppm), and eurystatin A (3.7 ppm) and B (2.1 ppm). Propeptin and poststatin are large molecular weight peptides containing a hydrophilic moiety. This characteristic could make them difficult to penetrate the blood-brain barrier. As a result, a non-peptidyl and small-molecular thelephoric acid and kynapcin-9, purified from *Polyozellus multiplex*, are expected to be successfully used in the prevention and treatment of Alzheimer's disease.

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