Kynapcin-12, a New *p*-Terphenyl Derivative from *Polyozellus multiplex*, Inhibits Prolyl Endopeptidase

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A major histopathological hallmark of Alzheimer's disease (AD) is the presence of amyloid deposits in the parenchyma of the amygdala, hippocampus, and neocortex[1]. The principal component of the amyloid is the β-amyloid protein (Aβ), a 39~43 amino acid peptide composed of a portion of the transmembrane domain and the extracellular domain of the amyloid precursor protein (APP)[2]. The neurotoxicity of the Aβ has been detected in several cell systems, including primary cultured neurons[3]. The Aβ having an alanine *C*-terminus is derived from the proteolytic cleavage of the APP by the action of the yet unidentified endoproteolytic enzymes, β- and γ-secretase[4]. Recent studies have suggested that prolyl endopeptidase could be involved in the processing of the *C*-terminal portion of the APP in AD[5].

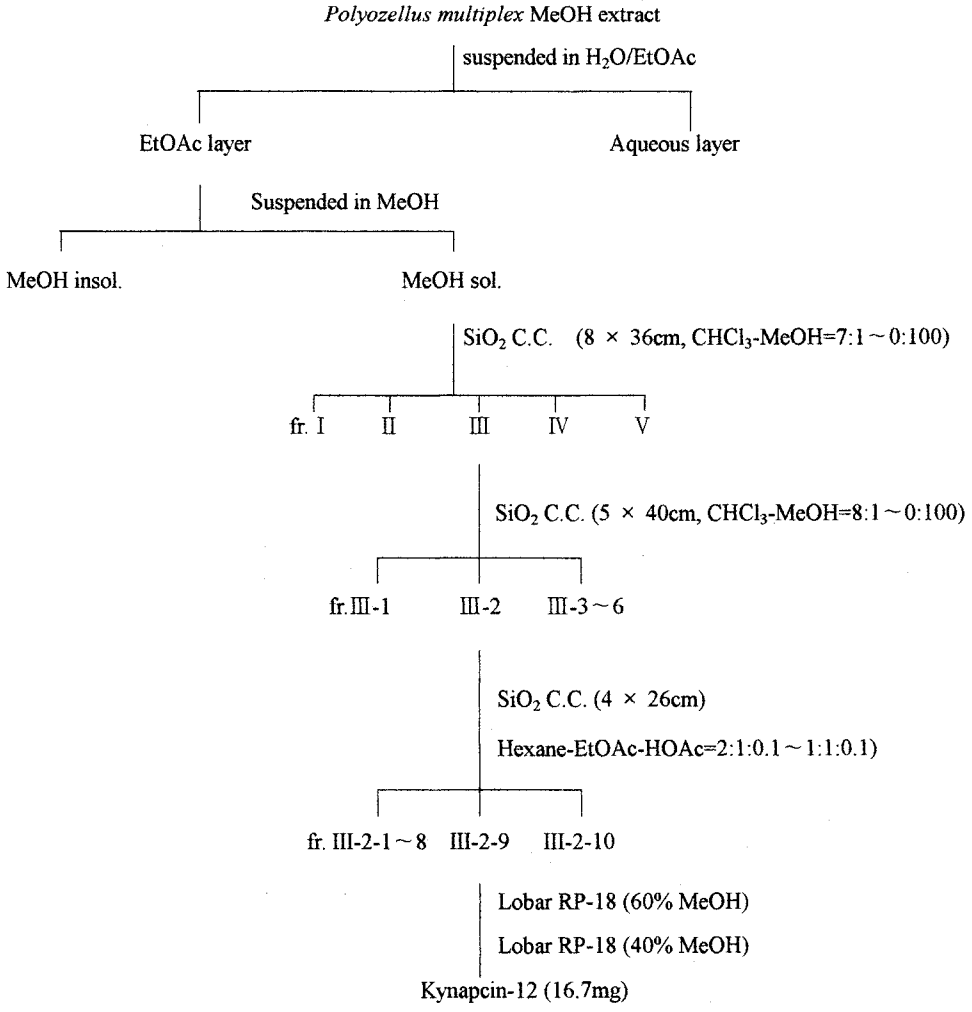
The prolyl endopeptidase [PEP; EC 3.4.21.26] is a serine protease, which is known to cleave peptide substrates in the *C*-terminal side of proline residues[6]. It plays an important role in degradation of the proline-containing neuropeptides such as oxytocin, vasopressin, substance P, neurotensin and angiotensin, which were suggested to participate in learning and memory processes[7,8]. It was found that the PEP activity of Alzheimer's patients is significantly higher than that of the normal person[9]. 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-dependant cognition-enhancing activity in rats with scopolamine-induced amnesia[10,11]. Therefore, much effort has been devoted to search for PEP inhibitors in order to develop anti-dementia drugs. PEP inhibitors such as thelephoric acid, kynapcin-9, and polyozellin have been isolated from *Polyozellus multiplex*[12,13]. During further investigation of the mushroom, a new *p*-terphenyl derivative having PEP inhibitory activity was isolated from the methanolic extract of *P. multiplex*. In this report, the isolation, physicochemical properties, structure elucidation and inhibitory activity of the compound are described.

Results and Discussion

The fruiting bodies of *P. multiplex* (1 kg) were air-dried in the well-ventilated fume hood. The MeOH extract was partitioned with EtOAc and the EtOAc soluble fraction was repeatedly chromatographed on silica gel and Lobar RP-18 columns. This purification procedure afforded a dark brown powdered inhibitor, named kynapcin-12. The isolation protocol is summarized in Fig. 1.

Kynapcin-12 was positive to $FeCl_3$ reagent, suggesting that it had phenolic OH group(s) in its structure. The broad band near 3394 cm^{-1} and the strong band at 1763 cm^{-1} in the IR spectrum indicated the presence of hydroxyl and ester carbonyl groups, respectively. In the ^{13}C-NMR (in methanol-d_4) spectrum, a signal corresponding to an α,β-unsaturated ketone appeared at δ 170.6 ppm along with signals for five aromatic quaternary carbons (δ 123.8, 125.0, 134.8, 142.5, and 158.1 ppm) and two aromatic methine carbons (δ 116.0 and 132.6 ppm). The ^{1}H-NMR spectrum (in methanol-d_4) showed signals of typical *p*-substituted benzene [doublets at δ 6.83 (J=8.4 Hz) and 7.16 (J=8.4 Hz) ppm], and an acetyl group at δ 1.90 (3H, s) ppm. The molecular formula of kynapcin-12 was determined to be $C_{22}H_{18}O_8$ (MW 410.3801) on the basis of high resolution FAB-MS. Taking into account the molecular formula and the number of carbon signals in the ^{13}C-NMR spectrum, kynapcin-12 should have a symmetrical structure. The partial structure **1a** was established by HMBC analysis, in which δ 6.83 and 7.16 ppm proton signals were correlated with δ 125.0 and 123.8 ppm carbon resonances, respectively (Fig. 2). In addition, the methyl signal at δ 1.90 ppm exhibited correlation spots with the carbonyl signal at δ 170.6 and the quaternary carbon resonance at δ 134.8 ppm. From all these observations, the structure of kynapcin-12 was postulated as **1** or **2**. Many analogous structures of **1** had been isolated from *Hydnum aurantiacum*[14], while those of **2** were found in *Sarcodon leucopus*[15]. Compound **3**, which had an

Fig. 1. Isolation procedure of kynapcin-12.



adjacent phenolic OH group in the central benzene ring of *p*-terphenyl, had been known to form phenylboronate derivative[15]. If structure **2** were correct for kynapcin-12, the phenylboronate derivative should be formed and the molecular ion peak of it should be detected at *m/z* 496 in EI-MS. To verify the structure, an attempt was made to form a phenylboronate derivative of kynapcin-12. However, the intact kynapcin-12 did not form a phenylboronate, neither did its deacetylated tetramethoxy derivative, which was synthesized to protect hydroxyl groups in the outer benzene rings. The colorimetric reaction, which had been known to be specific to *ortho*-dihydroxybenzene (catechol)[16], was negative to both of kynapcin-12 and its tetramethoxy derivative. In addition, IR bands corresponding to hydroxyl (3394 cm^{-1}) and carbonyl group (1763 cm^{-1}) of kynapcin-12 were quite different from those of **3** (3420 and 1750 cm^{-1}, respectively)[15]. Consequently, the chemical structure of kynapcin-12 was identified as 2,5-diacetoxy-3,6-di(*p*-hydroxyphenyl)hydroquinone (**1**) (Fig. 2). The physico-chemical properties of kynapcin-12 are presented in Table 1 and the NMR data are listed in Table 2.

The PEP inhibitory activity of the kynapcin-12 was measured as described in the 'Experimental' section. The inhibitory activity of kynapcin-12 (IC_{50} value, 1.25 μM) was lower than that of a positive control, *Z*-Pro-Prolinal (5.16×10^{-2} μM) but similar to that of polyozellin (2.72 μM)[13]. It was shown to be non-competitive with a substrate in a Dixon plot (Fig. 3) as in the cases of other *p*-terphenyls which had been isolated from *P. multiplex*[12,13]. The inhibition constant (*Ki*) of kynapcin-12 was 24.7 μM. To check the enzyme specificity of kynapcin-12, its inhibitory activity on other serine proteases such as

Table 1. Physico-chemical properties of kynapcin-12.

Appearance	Dark brown powder
MP (℃)	184-185℃
EI-MS *m/z*	410[M^+], 368[M^+ − Ac+H], 326[368 − Ac+H]$^+$
HRFAB-MS *m/z*	found, 410.3808
	calcd., 410.3801 (for $C_{22}H_{18}O_8$)
Molecular formula	$C_{22}H_{18}O_8$
UV λ_{max}nm(logε)(MeOH)	407 (1.89), 261 (3.13), 210 (3.34)
IR νcm^{-1} (KBr)	3394, 1763, 1612, 1525, 1454, 1371, 1219
Color reaction	positive to $FeCl_3$ and H_2SO_4

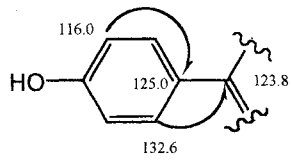
Table 2. NMR data of kynapcin-12 (δ in ppm).

No.	^{1}H	^{13}C
1,4		134.8 (*s*)
2,5		142.5 (*s*)
3,6		123.8 (*s*)
1',1"		125.0 (*s*)
2',2",6',6"	7.16 (*d*, 8.4 Hz)	132.6 (*d*)
3',3",5',5"	6.83 (*d*, 8.4 Hz)	116.0 (*d*)
4',4"		158.1 (*s*)
C=O		170.6 (*s*)
CH_3	1.90 (*s*)	20.1 (*q*)

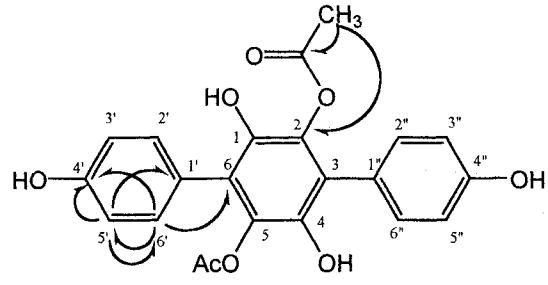
NMR spectra were measured in methanol-d_4.

Assignments were aided by DEPT, ^{13}C-^{1}H HMQC and HMBC.

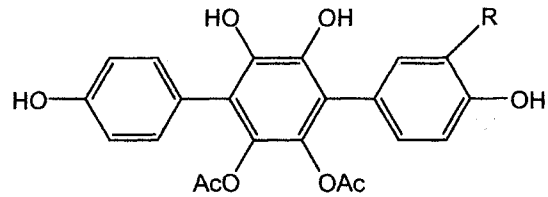
Fig. 2. Kynapcin-12 (**1**) and related structures (**1a**, **2** and **3**).



1a



1



2 R=H
3 R=OH

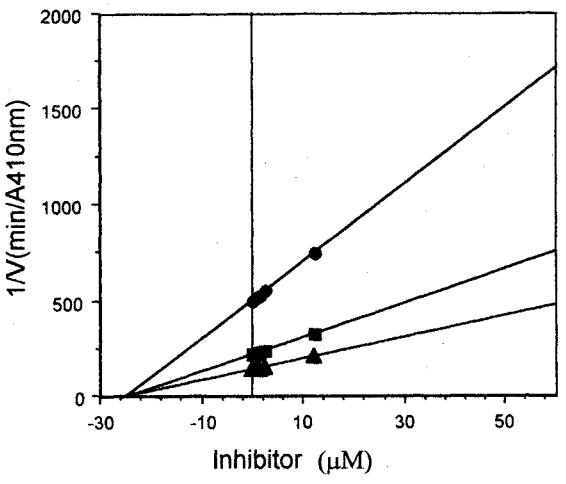
Arrows indicate the correlations between ^{1}H and ^{13}C in HMBC. **1a**, partial structure of kynapcin-12. **2**, presumable alternative structure of kynapcin-12. **3**, the most related congener of kynapcin-12 isolated from *S. leucopus* (refer reference 15).

chymotrypsin, trypsin, and elastase were compared with that of PEP. It inhibited more than 90% of PEP activity at 40 ppm (97.6 μM), while it inhibited less than 26% of the chymotrypsin and trypsin. Although kynapcin-12 showed a mild inhibition against the elastase, it was not so significant compared to PEP (Table 3). Thus, kynapcin-12 was thought to be a relatively specific inhibitor of PEP as in the case of other *p*-terphenyl inhibitors such as polyozellin[13] and thelephoric acid[12].

Many pyrrolidine derivatives such as *Z*-Pro-Prolinal and JTP-4819 had been synthesized[17]. On the other hand, staurosporine[18], poststatin[19], eurystatin[20], lipohexin[21], propeptin[22] and SNA-8073-B[23] were isolated from

microbial sources. The flavonoids containing a catechol ring[24] and tannins having a pyrogallol moiety[25] from plant sources have been reported to effectively inhibit the activity of PEP. Even though propeptin (1.1 μM) has a similar activity to kynapcin-12, it is a large molecular-weight peptide containing hydrophilic moiety and this character might make it difficult for it to penetrate into the blood-brain barrier. The non-peptidyl and small molecular-weight kynapcin-12, isolated from the edible mushroom *Polyozellus multiplex*, is expected to be used in the prevention and treatment of Alzheimer's disease.

Fig. 3. Dixon plot of the inhibition of PEP by kynapcin-12.



Concentration of substrate; 0.25 mM (—●—), 0.5 mM (—■—), 0.75 mM (—▲—). Five concentration (0, 0.6, 1.2, 2.4 and 12.2 μM) of kynapcin-12 were used for plotting.

Experimental

General

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 disc on Bruker IFS120HR/FRA106 spectrophotometer (Germany). EI-MS and HR-FAB-MS were recorded on VG QUATTRO II (VG, UK) and JMS HX-110/110A (JEOL, Japan) spectrometer, respectively. UV scanning was made by Varian CARY5G spectrometer (Australia). Melting point was measured with Gallenkamp melting point apparatus (Sanyo, Japan).

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. Polyozellin and thelephoric acid were obtained according to the previous report[12,13].

Enzyme Assays

PEP activity and inhibition percent of samples were determined according to the method of YOSHIMOTO *et al.*[27]. Chymotrypsin, trypsin and elastase were assayed by the

Table 3. Inhibitory activity[a] of polyozellin, thelephoric acid and kynapcin-12 against PEP and other serine proteases.

Conc. (μM)	Chymotrypsin	Trypsin	Elastase	PEP
Polyozellin				
2.4	1.6	4.6	17.3	33.5
12.2	2.7	10.9	35.3	83.5
97.6	4.9	21.2	41.1	99.8
Thelephoric acid				
2.4	2.8	3.6	47.9	91.5
12.2	4.5	3.8	50.8	94.5
97.6	4.8	4.8	65.7	97.7
Kynapcin-12				
2.4	1.1	9.1	30.6	56.6
12.2	2.3	23.9	43.6	72.6
97.6	5.1	25.4	53.0	93.0

[a]Presented as a relative percent over water-treated control.

protocol described in 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.

Microorganism, Extraction, Purification and Isolation

The fruiting bodies of *P. multiplex* (1 kg) were collected at Mt. Odae, Kangwon-Do, Korea and identified by the previous report[28]. After being air-dried in the fume hood at room temperature, they were refluxed in 3 liters of MeOH, thrice. The extract was evaporated to dryness and the residue (96.4 g) was suspended in water to be partitioned with 2.5 liters of EtOAc. The EtOAc soluble fraction (60.7 g) was suspended in 500 ml MeOH, subsequently filtered with the suction flask and Büchner funnel. A part of the MeOH soluble fraction (31.2 g) was chromatographed on a silica gel column [Merck Art. 7734, 8×36 cm, chloroform - MeOH (7 : 1, v/v) to 100% MeOH] to give fr. I to V. Subsequent silica gel column [Merck Art. 7734, 5×40 cm, chloroform - MeOH (8 : 1, v/v) to 100% MeOH] chromatography of the fr. III (2.82 g) afforded fr. III-1 to III-6. Fr. III-2 (1.54 g) was further separated into ten frs. (Fr. III-2-1 to III-2-10) by a silica gel column [Merck Art. 9385, 4×26 cm, *n*-hexane - EtOAc - AcOH (2 : 1 : 0.1 to 1 : 1 : 0.1, v/v)] chromatography. Finally, Lobar RP-18 chromatography (Merck LiChroprep RP-18, 40~63 μm, 2.5×25 cm, 2.2 ml min^{-1}, 1st., 60% and 2nd., 40% MeOH) of fr. III-2-9 afforded kynapcin-12 (16.7 mg).

Colorimetric Assay and Synthesis of Kynapcin-12 Derivatives

The colorimetric assay specific to catechol was carried out according to PRAKASA SASTRY and REDDY[16]. Permethylation of kynapcin-12 (12 mg) was performed with dimethylsulfate and K_2CO_3 in a routine way. ^{1}H-NMR of permethylated kynapcin-12 (δ ppm, methanol-d_4); 7.26 (2H, d, 8.4 Hz), 6.98 (2H, d, 8.4 Hz), 3.84 (3H, s), 3.58 (3H, s), 1.95 (3H, s). The permethylated kynapcin-12 (10 mg) was deacetylated with 0.1 M KOH in 40% dioxane, overnight at room temperature. After neutralization, nine mg of 2,5-dimethoxy-3,6-di(*p*-methoxyphenyl)hydroquinone [alternatively, 4,5-dimethoxy-3,6-di(*p*-methoxyphenyl)-hydroquinone] was obtained from the EtOAc soluble fraction. The molecular ion peak was found at *m/z* 382 in EI-MS. The phenylboronate formation was tried by the reported method using phenylboronic acid[15].

Acknowledgments

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