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Kynapcin-13 and -28, New Benzofuran Prolyl Endopeptidase Inhibitors from *Polyozellus multiplex*

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Two new benzofurans, 5,6-dihydroxybenzofuran-2,3-dicarboxylic acid dimethyl ester (kynapcin-13) and 5,6,5′,6′-tetrahydroxy[3,3′]bibenzofuranyl-2,2′-dicarboxylic acid 2′-methyl ester (kynapcin-28) were isolated from *Polyozellus multiplex*, and shown to non-competitively inhibit prolyl endopeptidase (PEP), with the IC_{50} values of 76.80 and 0.98 μM, respectively. Kynapcin-13 and -28 were less inhibitory to other serine proteases such as chymotrypsin, trypsin, and elastase.

A major histopathological characteristics of Alzheimer's disease (AD) is the deposition of amyloid protein in the parenchyma of the amygdala, hippocampus, and neocortex[1]. The major 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 [PEP; EC 3.4.21.26] could be involved in the processing of the *C*-terminal portion of the APP in AD[5].

The PEP 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 AD patients is significantly higher than that of the normal person[9]. It has been suggested 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 developing PEP inhibitors as anti-dementia drugs. PEP inhibitors such as thelephoric acid, kynapcin-9, -12, -24 and polyozellin have been isolated from *Polyozellus multiplex*[12~15]. During further investigation of the mushroom, two new benzofuran derivatives having PEP inhibitory activity were isolated from the methanolic extract of *P. multiplex*. In this report, the isolation, physicochemical properties, structure elucidation, and inhibitory activity of the compounds are described.

Results and Discussion

The fruiting bodies of *P. multiplex* 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 Sephadex LH-20 columns to yield **1** and **2**.

1 was obtained as a pale brown powder that was positive to $FeCl_3$ reagent, suggesting that it had phenolic OH group(s) in its structure. The broad band near 3414 cm^{-1} and the strong band at 1711 cm^{-1} in the IR spectrum

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Table 1. Physico-chemical properties of kynapcin-13 and -28.

	Kynapcin-13	Kynapcin-28
Appearance	pale brown powder	light green powder
MP (℃)	186℃	>340℃
EI-MS *m/z*	266 $[M]^+$, 234 $[M - CH_3OH]^+$, 149 $[M - (COOCH_3 \times 2 + H)]^+$	400 $[M]^+$, 369 $[M - OMe]^+$, 353 $[M - OMe - OH]^+$
HRFAB-MS *m/z*		
found:	266.0499	400.0432
calcd.:	266.0504 (for $C_{12}H_{11}O_7$)	400.0430 (for $C_{19}H_{12}O_{10}$)
Molecular formula	$C_{12}H_{11}O_7$	$C_{19}H_{12}O_{10}$
UV λ_{max}(MeOH)nm(log ε)	339 (4.07), 273 (4.19), 210 (4.31)	381 (3.95), 272 (4.07), 219 (4.09)
IR ν(KBr)cm^{-1}	3414, 3291, 1711, 1680, 1294	3424, 2955, 1711, 1624, 1385
Color reaction (positive)	$FeCl_3$ and H_2SO_4	$FeCl_3$ and H_2SO_4

Table 2. NMR data of kynapcin-13 and -28 (δ in ppm).

Position	Kynapcin-13		Kynapcin-28	
	^1H (int., multi.)[a]	^{13}C (multi.)[a]	^1H (int., multi., *J*)[a]	^{13}C (multi.)[a]
2		142.5 (*s*)		146.0 (*s*)
3		118.6 (*s*)		114.4 (*s*)
3a		116.5 (*s*)		117.2 (*s*)
4	7.11 (1H, *s*)	105.0 (*d*)	7.39 (1H, *s*)	106.0 (*d*)
5		145.4 (*s*)		144.2 (*s*)
6		148.9 (*s*)		146.5 (*s*)
7	7.06 (1H, *s*)	98.0 (*d*)	7.04 (1H, *s*)	97.7 (*d*)
7a		149.1 (*s*)		148.7 (*s*)
2'				145.7 (*s*)
3'				113.5 (*s*)
3a'				117.5 (*s*)
4'			7.33 (1H, *s*)	105.6 (*d*)
5'				144.3 (*s*)
6'				146.5 (*s*)
7'			7.03 (1H, *s*)	97.6 (*d*)
7a'				148.6 (*s*)
5(5')-OH	9.56 (1H, *brs*)		9.25 (2H, *brs*)	
6(6')-OH	9.92 (1H, *brs*)		9.51 (2H, *brs*)	
2-C=O		158.5 (*s*)		164.1 (*s*)
2'-C=O				163.0 (*s*)
3-C=O		162.9 (*s*)		162.9 (*s*)
2-OCH_3	3.87 (3H, *s*)	52.6 (*q*)		
2'-OCH_3			3.77 (3H, *s*)	51.3 (*q*)
3-OCH_3	3.90 (3H, *s*)	52.7 (*q*)		

[a]Integral, multiplicity, and coupling constants.

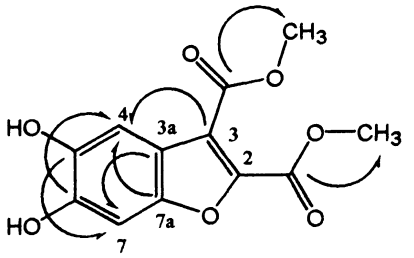
NMR spectra were measured in methanol-d_4.

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

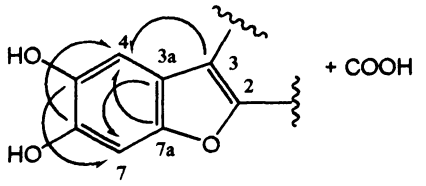
indicated the presence of hydroxyl and carbonyl groups, respectively. Its molecular formula was determined as $C_{12}H_{10}O_7$ on the basis of high resolution FABMS. Two major fragment ions at *m/z* 234 [M^+-CH_3OH] and 149 [$M^+-(COOCH_3\times2+H)$] in EIMS revealed the presence of two methyl ester groups. The UV absorption pattern and NMR data were very similar to those of 5,6-dimethoxybenzofuran-2,3-dicarboxylic acid[16] and 3-carboxymethylbenzofuran-2-carboxylic acids[17]. In the ^{1}H-NMR spectrum, two aromatic singlets (δ 7.06 and 7.11, 1H each), two heteroatomic protons (δ 9.56 and 9.92), and two methoxyl signals (δ 3.87 and 3.90, 3H each) were evident. In the ^{13}C-NMR spectrum, two signals corresponding to α,β-unsaturated carboxyl carbons (δ 158.5 and 162.9), six aromatic quaternary carbons (δ 149.1, 148.9, 145.4, 142.5, 118.6, and 116.5), and two aromatic methine carbons (δ 105.0 and 98.0) were observed. The NMR data are very similar to those of kynapcin-24 (5,6,5′,6′-tetrahydroxy-[3,3′]bibenzofuranyl-2,2′-dicarboxylic acid dimethyl ester)[14], which had been isolated from *P. multiplex*. Considering these data, **1** was finally identified as 5,6-dihydroxybenzofuran-2,3-dicarboxylic acid dimethyl ester (trivial name was given as kynapcin-13). The NMR assignments were made by ^{13}C-^{1}H and ^{13}C-^{1}H long-range COSY analyses, in which the carbon at δ 118.6 (C-3) correlated only with the proton at δ 7.11 (H-4) (Fig. 1).

2 was obtained as a light green powder and positive to $FeCl_3$ reagent. Hydroxyl (3424 cm^{-1}) and carbonyl stretching (1711 cm^{-1}) were observed in the IR spectrum. The molecular formula was determined as $C_{19}H_{12}O_{10}$ from the HR FABMS. In ^{1}H NMR spectrum, four exchangeable protons (δ 9.25 and 9.51), four aromatic singlets (δ 7.03, 7.04, 7.33 and 7.39) and a methoxyl signal (δ 3.77) were detected. Four aromatic methine carbons (δ 97.7, 97.6, 105.6 and 106.0), twelve aromatic quaternary carbons (δ 113.5 to 148.7), one methoxyl carbon (δ 51.3), and two carbonyl carbons (δ 163.0 and 164.1) were observed in the ^{13}C NMR spectrum. All aromatic and carbonyl carbons appeared as a pair, which had almost the same chemical shifts each other. In addition, the carbon chemical shifts and UV spectrum of **2** were almost identical to those of kynapcin-24, a benzofuran carboxylic acid dimer which had been isolated from *P. multiplex*[14]. From these observations, **2** was postulated as a mono-methyl ester of kynapcin-24. The partial structure **2a** and **2b** were established by COLOC analysis (Fig. 1), in which the carbon resonance at δ 146.0 and 145.7, which did not show any correlation spots in COLOC, could be assigned to C-2 or -2′. The partial structure **2a** could be connected to **2b** through either 3-3′ or 2-2′ linkage. If **2a** was connected through C-2 and

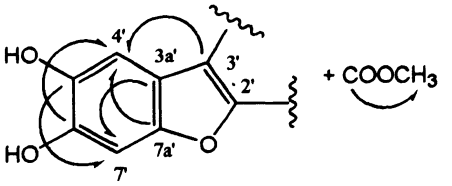
Fig. 1. Structures, partial structures, and summarized ^{13}C-^{1}H long-range correlations.



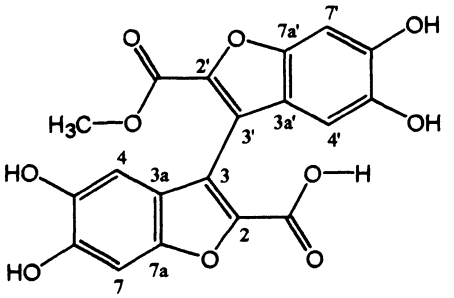
1



2a



2b

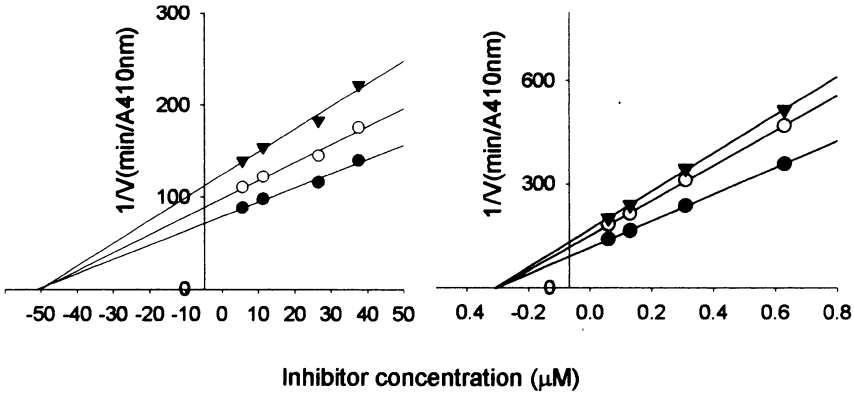


2

Arrows indicate the correlations between ^{1}H and ^{13}C in COLOC analyses. **1**, Kynapcin-13; **2a** and **2b**, partial structures of kynapcin-28; **2**, kynapcin-28.

C-2′, the chemical shift of C-2 (or C-2′), which is β to the carbonyl group, should be down field-shifted to *ca* 10 ppm relative to the reported data[19]. However, the chemical shift of C-2 (or C-2′) is very close to those of benzofuran

Fig. 2. Dixon plots of the inhibition of PEP by kynapcin-13 and 28.



Left, kynapcin-13. Right, kynapcin-28. Concentration of substrate: 0.5 mM (—▲—), 0.75 mM (—○—), 1.0 mM (—●—). 1/V was indirectly estimated by taking reciprocal value of the changes in OD at 410 nm per minute.

Table 3. Inhibitory activities[a] of kynapcin-13 and -28 against PEP and other serine proteases.

Conc. (μM)	Chymotrypsin	Trypsin	Elastase	PEP
Kynapcin-13				
2.4	0.6	3.3	10.2	15.3
12.2	2.4	1.0	13.7	32.8
97.6	2.8	10.1	22.2	80.1
Kynapcin-28				
2.4	7.3	1.6	21.0	97.5
12.2	7.2	4.5	23.6	99.5
97.6	10.6	10.9	24.0	98.7
Control[b]	4.4	2.9	8.1	3.1

[a]The activities (%) are calculated as described in the experimental section. [b]Ten μl of MeOH was added to the reaction mixture instead of sample solution.

2-carboxylic acids[17,20,21]. From all these observations, the structure of kynapcin-28 was identified as 5,6,5′,6′-tetrahydroxy[3,3′]bibenzofuranyl-2,2′-dicarboxylic acid 2′-methyl ester and given the trivial name kynapcin-28. The carbon signal at δ 113.5 was assigned to C-2′ by comparing its chemical shift with that of kynapcin-24[14]. The other assignments were done by ^{13}C-^{1}H COSY and COLOC analyses.

The prolyl endopeptidase (PEP) inhibitory activities of kynapcin-13 and -28 (IC_{50}=76.80 and 0.98 μM, respectively) were less than that of a positive control, *Z*-Pro-Prolinal ($IC_{50}=5.16\times10^{-2}$ μM) but similar to that of polyozellin (IC_{50}=2.72 μM)[15], kynapcin-12 (IC_{50}=1.25 μM)[13], and kynapcin-24 (IC_{50}=1.14 μM)[14], which have been previously isolated from *P. multiplex*. Kynapcin-13 and -28 were non-competitive with a substrate in Dixon plots (Fig. 2) and the inhibition constants (*Ki*) were 52.80 and 0.35 μM, respectively. To check the enzyme specificity, the inhibitory activities on other serine proteases such as chymotrypsin, trypsin, and elastase were compared with that of PEP. At 97.6 μM, kynapcin-13 and -28 inhibited more than 80% of PEP activity, but showed no significant inhibition of chymotrypsin and trypsin (Table 3). Although they showed mild inhibition against the elastase, they were not as significant as PEP. Thus, they appeare to be relatively specific inhibitors of PEP, as is the case with other natural inhibitors[12~15,29,30].

Many pyrrolidine derivatives such as *Z*-Pro-Prolinal and JTP-4819[22] have been synthesized as potent PEP inhibitors. On the other hand, staurosporine[23], poststatin[24],

eurystatin[25], lipohexin[26], propeptin[27], and SNA-8073-B[28] were isolated from microbial sources. Plant-derived flavonoids containing a catechol ring[29] and tannins with a pyrogallol moiety[30] have also been reported to effectively inhibit the activity of PEP. The presence of carbonyl group with a catechol or pyrogallol moiety has been suggested as the essential structural feature for PEP inhibitory activity[29,31]. Although propeptin (IC_{50}=1.1 μM) has activity similar to kynapcin-28, it maybe difficult to penetrate the blood-brain barrier since it is a large molecular-weight peptide with a hydrophilic moiety. The non-peptidyl and small molecular-weight kynapcin-13 and 28, isolated from the edible mushroom *P. multiplex*, may have potential use in the prevention and treatment of AD.

Experimental

General

Optical density was measured with a Bio-TEK ELISA autoreader ELX 808 (USA). ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker Avance Digital 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). EIMS and high resolution FABMS were recorded on VG QUATTRO II (UK) and JEOL JMS HX-110/110A (Japan) spectrometer, respectively. UV/VIS scanning was made on a Varian CARY5G spectrophotometer (Australia). Melting point was measured with a Sanyo Gallenkamp melting point apparatus (Japan). TLC was performed on a precoated silica gel plate (Merck, Art. 5715). Silica gel column chromatography was carried out using Merck Art. 7734.

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.*[32]. Chymotrypsin, trypsin, and elastase were purchased from Sigma (USA). PEP activity and inhibition percent of samples were determined according to the reported method[14]. 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 minutes, 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 MeOH instead of the sample solution to 240 μl of the buffer. The percent inhibition was calculated by the following equation: inhibition (%)=[{A_{410} of the control−(A−B)}/A_{410} of the control]×100. Chymotrypsin, trypsin, and elastase were assayed according to the protocols described in Sigma catalog (Sigma, USA) using *N*-benzoyl-L-Arg-*p*NA, *N*-benzoyl-L-Tyr-*p*NA, and *N*-succinyl-Ala-Ala-Ala-*p*NA as substrates, respectively.

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[33]. The specimen (voucher no. knunpc-pm03) is stored at the Department of Agricultural Chemistry, Kyungpook National University, Daegu, Korea. After being air-dried in the fume hood at room temperature, the mushrooms were refluxed in 36 liters of MeOH thrice. The extract was evaporated to dryness and the residue (674.8 g) was suspended in water to be partitioned with *n*-hexane and EtOAc, consecutively. The EtOAc-soluble fraction (31.2 g out of 90.68 g) was chromatographed on a silica gel column [Merck Art. 7734, 8×36 cm, $CHCl_3$-MeOH (7 : 1)→$CHCl_3$-MeOH-H_2O (60 : 20 : 1)→$CHCl_3$-MeOH-HOAc (50 : 20 : 3)]. Subsequent repeated silica gel [1st, Merck Art. 7734, 5×40 cm, $CHCl_3$-MeOH (8 : 1→1 : 1), 2nd, Merck Art. 9385, 4×26 cm, *n*-hexane-EtOAc-AcOH (20 : 10 : 1→10 : 10 : 1)] and Sephadex LH-20 [1.5×30 cm, 1st, 80%→100% MeOH, 2nd, $CHCl_3$-MEOH (1 : 1)] chromatography gave 6.3 mg of kynapcin-13 (**1**) and 15.8 mg of kynapcin-28 (**2**).

Acknowledgments

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