Polyozellin, a New Inhibitor of Prolyl Endopeptidase from *Polyozellus multiplex*

JI-SOOK HWANG, KYUNG-SIK SONG[†], WON-GON KIM, TAE-HO LEE[††], HIROYUKI KOSHINO[†††] and ICK-DONG YOO*

Korea Research Institute of Bioscience and Biotechnology, KIST, P. O. Box 115, Yusong, Taejon 305-600, Korea
[†] Dept. of Agricultural Chemistry, College of Agriculture, Kyungpook National University, 1370, Sankyuk-Dong, Taegu 702-701, Korea
[††] Department of Microbiology, College of Natural Sciences, Pusan National University, Pusan 609-735, Korea
[†††] The Institute of Physical and Chemical Research (RIKEN), Hirosawa 2-1, Wako, Saitama 351-01, Japan

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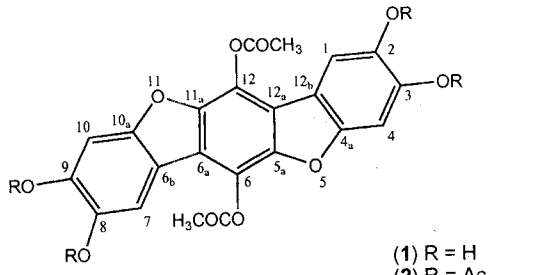
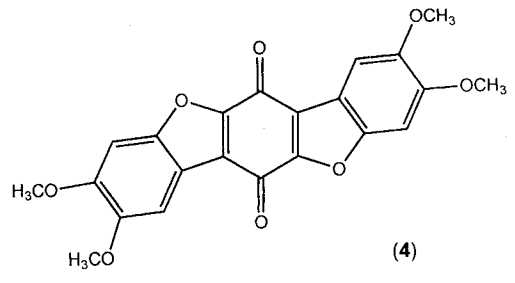
Prolyl endopeptidase (PEP, EC 3.4.21.26) is a serine protease which is known to cleave a peptide substrate in the *C*-terminal side of a proline residue[1,2]. In the central nervous system, PEP degrades proline-containing neuropeptides such as vasopressin, substance P, and thyrotropin-releasing hormone (TRH) which has been suggested to play an important role in learning and memory[3~5]. In addition, recent studies suggest that PEP could be implicated in the processing the *C*-terminal portion of the amyloid precursor protein in ALZHEIMER's disease[6]. It is also reported that cognitive deficits in ALZHEIMER's patients show improvement with TRH[7]. Therefore, it has been postulated that PEP inhibitors could prevent memory loss and increase attention span in patients suffering from senile dementia. Some PEP inhibitors have been reported to show dose-dependant cognition-enhancing activity in rats with scopolamine-induced amnesia[8,9]. Eurystatin[10], poststatin[11], staurosporine[12], SNA-8073-B[13], and propeptin[14] have been isolated as PEP inhibitors of microbial origin and the modification of poststatin to non-peptidyl analogues have been studied[15]. In the course of screening for PEP inhibitors, we found a new non-peptidyl inhibitor named polyozellin (**1**) from the Korean mushroom *Polyozellus multiplex*. In this paper, we report the isolation, physico-chemical properties, structure determination, and biological activities of **1** (Fig. 1).

Fresh fruiting body of the mushroom (700 g) was refluxed in 3 liters MeOH for 3 hours at 70°C. The extract was evaporated to dryness and the residue (134 g) was suspended in water to partition with benzene, chloroform and EtOAc, consecutively. EtOAc soluble layer was evaporated *in vacuo* and the resultant residue (1.1 g) was washed with 100 ml EtOAc. The residue (592 mg) was suspended in 300 ml MeOH, then the suspension was centrifuged at 3,000*g* for 5 minutes. The precipitate was discarded and the supernatant was concentrated *in vacuo*. The resultant residue was further purified on Senshu Pak ODS HPLC. Elution with MeOH-H_2O (65:10) containing 1% acetic acid afforded **1** (387 mg) as dark green powder.

Fig. 1. Chemical structures of polyozellin (**1**) and related compounds.



(**1**) R = H
(**2**) R = Ac
(**3**) R = Me



(**4**)

Table 1. Physico-chemical properties of **1**.

Appearance	Dark green powder
MP (°C)	>245 (dec)
FAB-MS	439 (M^++1, 48.6%), 396 (M^+−H_3CO, 69.5), 376 (396-H_2O, 100.0), 354 (M^+−2Ac+H, 93.3), 337 (354-OH, 81.0)
HRFAB-MS (*m/z*)	
Found	439.0618 $(M+H)^+$
Calcd.	439.0660 (for $C_{22}H_{15}O_{10}$)
Molecular formula	$C_{22}H_{14}O_{10}$
UV λ_{max} nm (log ε) (MeOH)	360 (4.69), 346 (4.99), 331 (4.81), 278 (4.55), 268 (4.42), 245 (4.78), 224 (4.87)
IR (KBr) γ cm^{-1}	3392, 3276, 1780,1753, 1614, 1201
Solubility	
Soluble	DMSO, pyridine, EtOAc
Slightly soluble	MeOH, EtOH
Insoluble	$CHCl_3$, *n*-Hexane, benzene, H_2O
Rf value[a] 1)	0.18
2)	0.66
Color reaction positive	$FeCl_3$, H_2SO_4, I_2

[a] Silica gel TLC (Kieselgel $60F_{254}$, Merck) was used with developing solvent 1) $CHCl_3$-MeOH-HOAc (13:1:0.5), 2) EtOAc-MeOH-HOAc (25:1:0.2).

Table 2. ^{1}H and ^{13}C NMR data for **1**~**4**.

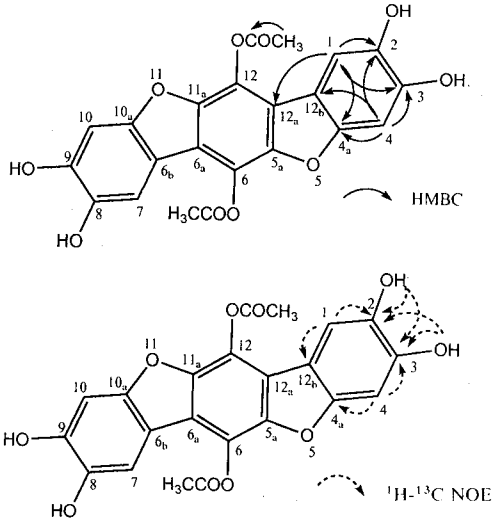
No.	**1** (DMSO-d_6-$CDCl_3$, 1:4)		**2** (pyridine-d_5)		**3** ($CDCl_3$)		**4** ($CDCl_3$)	
1, 7	7.21 (2H, s)	106.17 d	8.19 (2H, s)	116.38 d	7.22 (2H, s)	103.19 d	7.51 (2H, s)	102.01 d
2, 8		142.85 s		140.10[b] s		146.64 s		149.84[j] s
3, 9		146.58 s		143.08[b] s		150.24[f] s		151.70[j] s
4, 10	7.14 (2H, s)	98.76 d	7.86 (2H, s)	108.25 d	7.20 (2H, s)	95.90 d	7.09 (2H, s)	95.39 d
4a, 10a		150.74 s		154.01 s		151.77[f] s		152.85[i] s
5a, 11a		137.59[a] s		134.97[c] s		138.38[g] s		152.26i s
6, 12		130.80[a] s		133.20[c] s		131.27[g] s		180.20 s
6a, 12a		116.94 s		120.72 s		117.34 s		123.56 s
6b, 12b		113.51 s		118.56 s		114.44 s		115.27 s
2,8-OH	8.78 (2H, br s)							
3,9-OH	9.23 (2H, br s)							
2,8-$OCCH_3$				173.34[d] s				
3,9-$OCCH_3$				168.40[d] s				
2,8-$OCCH_3$			2.31 (6H, s)	20.34[e] q				
3,9-$OCCH_3$			2.23 (6H, s)	20.36[e] q				
2,8-OCH_3					4.00 (6H, s)	56.54[h] q	4.01 (6H, s)	56.45 q
3,9-OCH_3					3.97 (6H, s)	56.36[h] q	3.99 (6H, s)	56.45 q
6,12-$OCCH_3$		168.02 s		168.72[d] s		168.04 s		
6,12-$OCCH_3$	2.54 (6H, s)	20.42 q	2.49 (6H, s)	20.43 q	2.53 (6H, s)	20.55 q		

Chemical shift assignments of **1** were aided by ^{13}C-^{1}H NOE, DEPT, HMQC, and HMBC experiments. Chemical shift assignments of **2**~**4** were based on comparison with those of **1**. Assignments of a~i were interchangeable.

The physico-chemical properties of **1** are summarized in Table 1. **1** is soluble in dimethlylsulfoxide, pyridine and ethyl acetate, but insoluble in chloroform, *n*-hexane, benzene and water. **1** showed brilliant blue fluorescence under UV 365 nm and was visualized as a greenish blue spot after spraying with ferric chloride reagent and heating to 80°C for 10 minutes. UV spectrum showed typical absorption pattern of dihydrothelephoric acid[16]. In IR spectrum, bands at 1780 cm^{-1} and at 3392 cm^{-1} indicated the presence of the ester carbonyl and the hydroxyl group, respectively.

The molecular formula of **1** was determined to be $C_{22}H_{14}O_{10}$ on the basis of high resolution FAB-MS. The ^{1}H NMR spectrum (Table 2) showed signals attributable to two broad singlet phenolic hydroxyl protons at 9.23 and 8.78 ppm, two singlet aromatic methine protons at 7.21 (H-1) and 7.14 ppm (H-4), and singlet acetyl protons at 2.54 ppm. The ^{13}C NMR spectrum in combination with DEPT and HMQC spectral data revealed signals due to a carbonyl carbon at 168.02 ppm; seven sp^2 quaternary carbons at 150.74 (C-4_a), 146.58 (C-3), 142.85 (C-2), 137.59 (C-5_a), 130.80 (C-6), 116.94 (C-6_a), and 113.47 ppm (C-6_b); two aromatic methine carbons at 106.17 (C-1) and 98.76 ppm (C-4); and an acetyl carbon at 20.4 ppm. Taking into account the molecular formula, these spectral data suggested that **1** was a symmetric structure. In PFG-HMBC experiment using duration time of 60 msec (Fig. 2), strong long

Fig. 2. PFG-HMBC and NOE data of polyozellin (**1**).



range correlations were observed from the proton of H-1 to carbons of C-4_a and C-3, and from the proton of H-4 to carbons of C-12_b and C-2. In addition, relatively weak long range correlations were observed from the proton of H-1 to carbons of C-12_a and C-2, and from the proton of H-4 to carbons of C-3 and C-4_a. In NOE study (Fig. 2), ^{1}H-^{13}C NOE effects were observed from both protons at 8.78 and 9.23 ppm to carbons of C-2 and C-3, respectively, indicating that C-2 and C-3 were

Fig. 3. Inhibitory activity of **1**~**4** against prolyl endopeptidase.

1 (■), **2** (◇), **3** (△), **4** (□), poststatin (○).

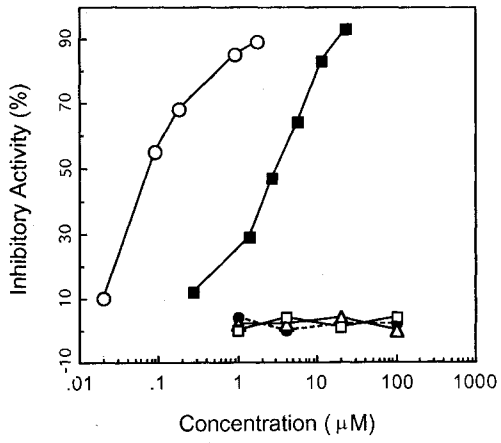
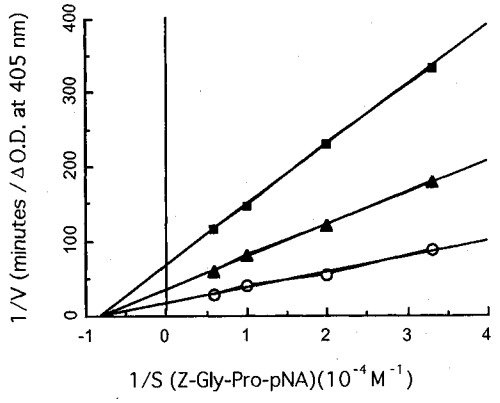


Fig. 4. Lineweaver-Burk plot of inhibition of PEP by polyozellin.

I=0 μg/ml (○), I=2.5 μg/ml (▲), I=5.0 μg/ml (■).



hydroxylated. The protons of acetyl group at 2.54 ppm had long-range correlation with the carbonyl carbon at 168.0 ppm. The presence of four hydroxyl groups in **1** was confirmed by acetylation. The obtained compound (**2**) showed two additional acetyl signals at 2.31 and 2.23 ppm in the ^{1}H NMR spectrum. These data were in good agreement with the reported values of thelephoric acid leuco-peracetate[16,17]. Based on these spectral data, the structure of **1** was speculated to be thelephoric acid leuco-6,12-acetate, *i.e.* 6,12-diacetoxy-2,3,8,9-tetrahydroxybenzo[1,2-b;4,5-b']bisbenzofuran. To confirm the proposed structure of **1**, **1** was methylated with dimethyl sulfate to yield tetramethoxy derivative (**3**). The physicochemical properties of **3** prepared from **1** were completely identical to those of synthetic **3** prepared by reductive acetylation from 2,3,8,9-tetramethyl thelephoric acid (**4**)[16]. Therefore, the structure of **1** was determined as shown in Fig. 1.

1 inhibited PEP in a dose dependant fashion with an IC_{50} value of 2.72 μM although its activity was lower than that of poststatin (IC_{50}=0.07 μM), used as a positive control (Fig. 3). The inhibition of **1** was noncompetitive with substrate (Fig. 4). The *Ki* and *Km* values for PEP are 2.46×10^{-5} M and 1.26×10^{-4} M, respectively. The inhibitory activity of the related compounds of **1** was also investigated (Fig. 3). Deacetylated derivative (**4**) of **1** showed no activity even at 100 μM. Acetylated and methylated derivatives (**2** and **3**, respectively) of **1** was also inactive at 100 μM. Therefore, it is suggested that phenolic hydroxyl group or catechol group are important for its PEP inhibitory activity. **1** exhibited no antimicrobial activities at 1 mg/ml against the following bacteria and yeast: *Escherichia coli* AB1157, *Escherichia coli* BE 1186, *Salmonella typhimurium* TV119, *Salmonella typhimurium* SL1102, *Pseudomonas aeruginosa* IFO13130, *Staphylococcus aureus* IFO12732, *Staphylococcus aureus* R-209, *Mycobacterium phlei* IFO3158, or *Candida albicans* IFO1594. Thelephoric acid derivatives had been reported as fungal pigments from several species of *Thelephora* and *Boletopsis leucomelaena*[16,17]. Terphenyl derivatives had been isolated, identified and characterized as 5-lipoxygenase inhibitors[18], 3',5'-monophosphate phosphodiesterase inhibitors[19], fungal pigments and antibiotics.

Experimental

General

^{1}H NMR and ^{13}C NMR were recorded on a JEOL JNM A600 spectrometer at 600 and 150 MHz, respectively. Chemical shifts were given in δ (ppm) from TMS. IR was measured in KBr disk on Laser Precision Analect RFX-65 spectrophotometer. FAB-MS and HRFAB-MS were measured on HITACHI M-80 and JEOL JMS-HX 110A spectrometers, respectively. UV was detected on Kontron Unicon 903 UV spectrophotometer.

Producing Organism

The strain, which was identified as *P. multiplex*[20,21] by macro- and microscopic methods, was collected in mountain Odae, Korea and deposited in Dept. of Agricultural Chemistry, Col. of Agriculture, Kyungpook National Univ., Taegu, Korea.

Biological Activity

Inhibitory effects of polyozellin on prolyl endopeptidase (PEP) were determined using the method of YOSHIMOTO *et al.*[22]. Antimicrobial activity was tested by agar plate diffusion assay using nutrient agar (Difco) media. Ten μl of the test solution in a concentration of 1 mg/ml were applied onto filter disc (6 mm diameter), and the test plates were incubated for 24 hours at 37°C for bacteria and 28°C for yeast.

Acetylation and Methylation of **1**

Once precipitation was formed, an acetylated derivative of **1** (2,3,6,8,9,12-hexaacetoxybenzo[1,2-b;4,5-b']bisbenzofuran) (**2**) was hardly soluble in any commercial NMR solvents, therefore, acetylation was performed directly in NMR tube with pyridine-d_5 and molar equivalent of acetic anhydride. **1** was methylated by refluxing the 50 ml anhydrous acetone containing **1** (80 mg), 2 g dimethylsulfate and 2 g K_2CO_3 for 3 hours. After cooling the reaction mixture was extracted with $CHCl_3$ and the organic layer was chromatographed on Sephadex LH 20 column (1.2 × 25 cm, $CHCl_3$ - MeOH = 1 : 1). Crystallization in $CHCl_3$ - MeOH (1 : 1) of major fractions afforded a methylated compound of **1** (6,12-diacetoxy-2,3,8,9-tetramethoxybenzo[1,2-b;4,5-b']bisbenzofuran) (**3**).

Synthesis of **3** and **4**

Thelephoric acid tetramethyl ether (2,3,8,9-tetramethoxybenzo[1,2-b;4,5-b']bisbenzofuran 6,12-dione) (**4**) was synthesized by reported method[15]. Synthetic **3** was prepared by reductive acetylation of **4**. **4** (50 mg) suspended in 4 ml 1 : 1 mixture of $CHCl_3$ and acetic anhydride was treated with a few drops of pyridine and a little Zn powder. After the color was disappeared (2 to 3 hours), the reaction mixture was filtered and the filtrate was cooled to give **3** (57 mg) as the precipitated white needles.

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