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Prolyl Endopeptidase Inhibitors from Caryophylli Flos

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Three prolyl endopeptidase inhibitors were isolated and identified as luteolin, quercetin and β-sitosterol-3-O-β-D-glucopyranoside with IC_{50} of 0.17, 0.19 and 27.5 ppm, respectively. The inhibition of two flavonoids were non-competitive with substrate. Twenty authentic flavonoids were tested in order to investigate structure-activity relationship. No significant relationship was found in them, however, catechol moiety of B-ring and 7-OH group in flavonoid skeleton were seemed to be responsible for the stronger activity.

Key words : Prolyl endopeptidase inhibitor, *Eugenia caryophyllata,* Caryophylli Flos, Luteolin, Quercetin, Flavonoids, β-sitosterol-3-O-β-D-glucopyranoside

INTRODUCTION

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 (Yoshimoto *et al.,* 1977; Koida *et al.,* 1976). In the central nervous system, PEP degrades proline-containing neuropeptides such as vasopressin, substrate P, and tyrosine-releasing hormone (TRH) which have been suggested to play an important role in learning and memory (Burbach *et al.,* 1983; De Wied *et al.,* 1983; Weingartner *et al.,* 1981). In addition, recent studies suggested that PEP could be implicated in the processing the C-terminal portion of the amyloid precursor protein in Alzheimer's disease (Ishiura *et al.,* 1990). It is also reported that cognitive deficits in Alzheimer's patients show improvement with TRH (Kovacs *et al.,* 1975). 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 (Yoshimoto *et al.,* 1987; Portevin *et al.,* 1996). Peptide analogues such as eurystatin (Toda *et al.,* 1992), poststatin (Aoyagi *et al.,* 1991), staurosporine (Kimura *et al.,* 1990), SNA-8073-B (Kimura *et al.,* 1997a), propetin (Kimura *et al.,* 1997b) and polyozellin (Hwang *et al.,* 1997) have been isolated as PEP inhibitors from microbial origin but PEP inhibitors have been rarely investigated from plant material. In the course of screening for PEP inhibitors from 176 kinds of oriental crude drugs, we found that EtOAc soluble fraction of Caryophylli Flos showed significant activity (Lee *et al.,* 1997). In this paper, isolation, structure determination, Lineweaver-Burk plot of inhibitors and the structure-activity relationship of related compounds will be discussed.

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MATERIALS AND METHODS

General

Caryophylli Flos was purchased from crude drug store located at Taegu, Korea. OD was measured with ELISA autoreader (Bio-TEK ELX 808). ^1H and ^{13}C NMR spectra were recorded on a Varian Unity Plus 300 spectrometer at 300 and 75.5 MHz, respectively. Chemical shifts were given in δ (ppm) from TMS. IR spectrum was measured in KBr disc on Bruker IFS120HR/FRA106 spectrophotometer. EI-MS was measured on VG QUATTRO II spectrometer. Authentic flavonoids were those which had been isolated and identified in our laboratory.

Biological activity

Inhibitory activity of samples against prolyl endopeptidase (PEP) was determined using the method of Yoshimoto *et al.,* 1980. Prolyl endopeptidase (from *Flavobacterium meningosepticum*) and substrate (*Z*-Gly-Pro-pNA) was purchased from Seikagaku Co. (Japan). *Z*-Pro-Prolinal was used as a positive control and syn-

thesized according to Bakker *et al.,* 1990.

Extraction and isolation

Caryophylli Flos (2 Kg) was refluxed with 80% MeOH for 3 hr and the MeOH extract was partitioned with CH_2Cl_2, EtOAc, consecutively. EtOAc fraction (43 g) was chromatographed on SiO_2 column (5×47 cm, CH_2Cl_2-MeOH=30:1→1:1, total volume of mobile phase was *ca* 18 l). Ten fractions (Fr A~Z) were obtained and among them, Fr G and H showed significant activity (above 80% of inhibition at 40 and 80 ppm, respectively). Re-chromatography of Fr G on Sephadex-LH 20 column (3×105 cm, 80% MeOH) afforded yellow powder (compound **1**, 69 mg) and deep yellow powder (compound **2**, 65 mg). From Fr H, colorless plate crystal (compound **3**, 12 mg) was obtained from MeOH solution.

Compound 1 (luteolin); $FeCl_3$ positive, $C_{15}H_{10}O_6$ (M. W. 286); EI-MS *m/z* (rel. int.) 286 (M^+, 100.0), 258 (M^+-CO, 20.4), 153 (M^+-$C_8H_6O_2$+H, 39.1), 134 (M^+-$C_7H_4O_4$, 21.5); IR ν_{max} cm^{-1} 3432 (-OH), 1716 (C=O), 1618 (C=C), 1363 (C-O); ^{1}H NMR δ ppm (DMSO-d_6) 13.0 (1H, *s,* 5-OH), 10.90 (1H, *brs,* -OH), 9.80 (3H, *brs,* -OH x 3), 7.42 (1H, d, *J*=7.4 Hz, H-6'), 7.41 (1H, *s,* H-2'), 6.90 (1H, *d J*=7.4 Hz, H-5'), 6.68 (1H, *s,* H-3), 6.45 (1H, d, *J*=1.8 Hz, H-8), 6.20 (1H, *J*=1.8 Hz, H-6); ^{13}C NMR δ ppm (DMSO-d_6) 182.1 (C-4, *s*), 164.5 (C-7, *s*), 164.3 (C-2, *s*), 161.9 (C-5, *s*), 157.7 (C-9, *s*), 150.1 (C-4', *s*), 146.1 (C-3', *s*), 121.9 (C-1', *s*), 119.4 (C-6', *d*), 116.4 (C-5', *d*), 113.8 (C-2', *d*), 104.1 (C-10, *d*), 103.3 (C-3, *d*), 99.2 (C-6, *d*), 94.3 (C-8, *d*).

Compound 2 (quercetin); $FeCl_3$ positive, $C_{15}H_{10}O_7$ (M.W. 302); EI-MS *m/z* (rel. int.) 302 (M^+, 100.0), 285 (M^+-H_2O, 1.9), 153 (M^+-$C_8H_6O_2$+H, 5.7); IR ν_{max} cm^{-1} 3303~3503 (-OH), 1696 (C=O), 1618 (C=C), 1314 (C-O); ^{1}H NMR δ ppm (DMSO-d_6) 12.50 (1H, *s,* 5-OH), 10.76 (1H, *brs,* -OH), 9.35 (3H, *brs,* -OH x 3), 7.68 (1H, *d, J*=0.9Hz, H-2'), 7.59 (1H, *dd, J*=8.7, 0.9 Hz, H-6'), 6.90 (1H, *d, J*=8.7 Hz, H-5'), 6.42 (1H, *d, J*=1.0 Hz, H-8), 6.20 (1H, *J*=1.0 Hz, H-6); ^{13}C NMR δ ppm (DMSO-d_6) 176.0 (C-4, *s*), 164.1 (C-7, *s*), 160.9 (C-5, *s*), 156.3 (C-9, *s*), 147.9 (C-4', *s*), 147.0 (C-2, *s*), 145.2 (C-3', *s*), 135.9 (C-3, *s*), 122.1 (C-1', *s*), 120.1 (C-6', *d*), 115.8 (C-5', *d*), 115.2 (C-2', *d*), 103.2 (C-10, *d*), 98.4 (C-6, *d*), 93.5 (C-8, *d*).

Compound 3 (β-sitosterol-3-O-β-D-glucopyranoside); $FeCl_3$ negative, $C_{35}H_{60}O_6$ (M.W. 576); EI-MS *m/z* (rel. int.) 415 (aglycon+H, 8.5), 397 (aglycon-OH, 100.0), 382 (397-Me, 16.5); ^{1}H NMR δ ppm (pyridine-d_5) 5.34 (1H, *brs,* olefinic), 5.04 (1H, *d,* anomeric, *J*=7.8 Hz), 0.65 (3H, s, 29-Me); ^{13}C NMR δ ppm (pyridine-d_5) 140.8 (C-5, *s*) 121.8 (C-6, *d*), 102.5 (C-1', *d*), 78.5 [C-5', *d,* (assignment may be exchange with C-3 or C-5')], 78.4 (C-3', *d*), 78.1 (C-3, *d*), 75.2 (C-2', *d*), 71.6 (C-4', *d*), 62.8 (C-6', *t*), 56.8 (C-14, *d*), 56.2 (C-17, *d*), 50.3 (C-9, *d*), 46.0 (C-24, *d*), 42.4 (C-13, *s*), 39.9 (C-12, *t*), 39.3 (C-4, *t*), 37.4 (C-1, *t*), 36.9 (C-10, *s*), 36.3 (C-20, *d*), 34.1 (C-22, *t*), 32.1 (C-7, *t*), 32.0 (C-8, *d*), 30.2 (C-2, *t*), 29.4 (C-25, *d*), 28.5 (C-16, *t*), 26.4 (C-23, *t*), 24.4 (C-15, *t*), 23.3 (C-28, *t*), 21.2 (C-11, *t*), 19.9 (C-27, *q*), 19.3 (C-19, *q*), 19.1 (C-26), 18.9 [C-21, *q,* (assignment may be exchange with C-19 or 26)], 12.1 [C-29, *q,* (assignment may be exchange with C-18)], 11.9 (C-18, *q*).

Acid Hydrolysis of Compound 3 (Woo *et al.,* 1996)

Compound **3** (8 mg) in 5% H_2SO_4 (in 60% dioxane) was refluxed for 3 hr. Extraction of reaction mixture with EtOAc afforded genin **3a** (5 mg). Sugar moiety was identified by TLC (RP-18 F_{254s}, Merck, Art. 5628, *n*-BuOH-C_6H_6-C_5H_5N-H_2O) after treatment with saturated HCl vapor.

Compound 3a (β-sitosterol); $FeCl_3$ negative, $C_{29}H_{50}O$ (M.W. 414); ^{13}C NMR δ ppm ($CDCl_3$) 140.8 (C-5, *s*) 121.7 (C-6, *d*), 71.8 (C-3, *d*), 56.8 (C-14, *d*), 56.2 (C-17, *d*), 50.2 (C-9, *d*), 46.0 (C-24, *d*), 42.4 (C-13, *s*), 39.7 (C-12, *t*), 42.3 (C-4, *t*), 37.1 (C-1, *t*), 36.5 (C-10, *s*), 36.3 (C-20, *d*), 34.1 (C-22, *t*), 31.9 (C-7, *t*), 31.7 (C-8, *d*), 31.9 (C-2, *t*), 29.1 (C-25, *d*), 28.4 (C-16, *t*), 26.1 (C-23, *t*), 24.4 (C-15, *t*), 23.1 (C-28, *t*), 21.1 (C-11, *t*), 19.8 (C-26, *q*), 19.4 (C-19, *q*), 19.1 [C-27, *q,* (assignment may be exchange with C-18, 21, 27)], 18.8 (C-21, *q*), 12.1 (C-29, *q*), 11.9 (C-18, *q*).

RESULTS AND DISCUSSION

Compound **1** was positive to $FeCl_3$ reagent, indicating it was a phenolic compound. In EI-MS spectrum, molecular ion peak was observed at *m/z* 286 and fragment ions at *m/z* 153, 134 which were characteristic to flavone (Mabry and Ulubelen, 1980). In ^{1}H NMR, a sharp hydroxyl proton signal at δ 13.0 ppm (which might be hydrogen bonded one) and three broad phenolic OH signals (δ 10.90, 9.80 ppm) were observed. Two doublets at δ 7.42 and 6.90 and a singlet at δ 7.41 ppm showed typical resonance of B-ring containing catechol moiety in flavonoids. The proton signal at δ 6.68 ppm strongly suggested that **1** was a flavone which do not have a substituent at C-3 position. From these observations and ^{13}C NMR data, **1** was assumed to be a luteolin and finally identified by comparing these data with those of authentic sample (Agrawal *et al.,* 1988).

Compound **2** was positive to $FeCl_3$ reagent and showed [M^+] at *m/z* 302 in EI-MS spectrum. ^{1}H NMR pattern of **2** was very similar to that of **1** except for the presence of one more phenolic OH proton signal and absence of a singlet at δ 6.68 ppm. This fact indicate that **2** is a flavonol derivative. Resonances at δ 7.68, 7.59, 6.90 should be originated from B-ring of flavonol and two meta-coupled doublets (*J*=1.0 Hz) at δ

6.42 and 6.20 ppm should be assigned to protons at C-6 and C-8 position, respectively. These data suggested that **2** was a quercetin and finally confirmed by comparing them with those of authentic sample (Agrawal *et al.*, 1988).

Compound **3** was negative to $FeCl_3$ and positive to phenol-sulfuric acid. EI-MS fragmentation pattern revealed **3** was a kind of steroid or terpenoid (Silberstein *et al.*, 1991, Budzikiewicz, 1980). In ^1H NMR spectrum, signals at δ 3.80~5.20 ppm were postulated to be originated from sugar moiety. An olefinic proton was observed at δ 5.34 ppm. A doublet signal at δ 5.35 ppm should be an anomeric proton having β-configuration considering its coupling constant (J=7.8 Hz). Total thirty five carbon signals were detected from ^{13}C NMR and three of them were identified as quaternary carbon, fourteen were as methine, twelve were as methylene and six were as methyl carbon by DEPT spectrum. These spectral data suggested that **3** was a β-sitosterol-β-glycoside. To confirm the structure of aglycon and sugar, **3** was acid-hydrolysed and the hydrolysate was analysed. Sugar was identified as glucose by TLC comparison and aglycon was identified as β-sitosterol by direct comparison with authentic material. The chemical shift of C-3 of **3** was shifted to down field about 7 ppm compared to that of β-sitosterol, verifying the glycosylated position was C-3. From these data, **3** was postulated as β-sitosterol-3-O-β-D-glucopyranoside and confirmed by comparing them with reference (Woo *et al.*, 1996).

All Three compounds inhibited PEP in a dose-dependant manner. Although their activity were lower than that of Z-Pro-Prolinal (IC_{50}, *ca* 22 ppb) used as a positive control, the IC_{50} value of **1**, **2**, **3** were 0.17, 0.19 and 27.5 ppm, respectively (Fig. 1). Lineweaver-Burk plots were drawn for two active flavonoids. Both

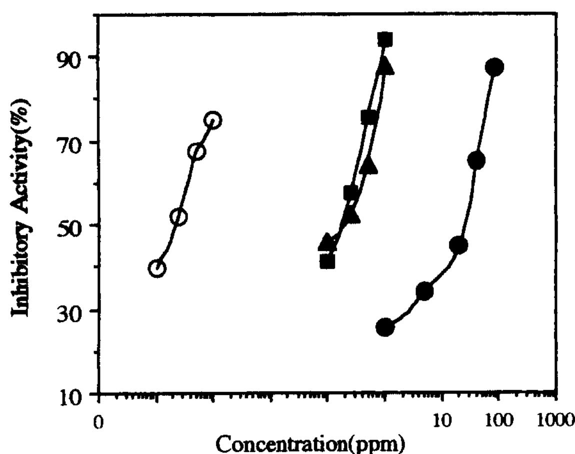

Fig. 1. Inhibitory activity of compounds 1~3 against prolyl endopeptidase. ○: Positive control (*Z*-Pro-Prolinal), ■: luteolin (1), ▲: quercetin (2), ●: β-sitosterol-3-O-β-D-glucopyranoside (3).

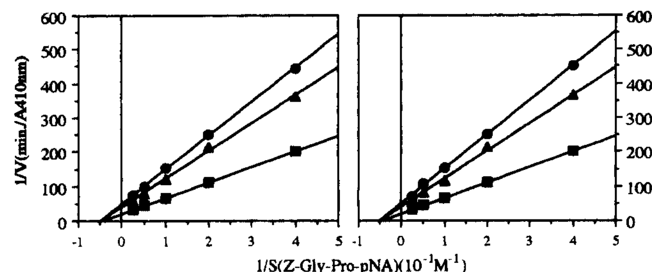

Fig. 2. Lineweaver-Burk plot of inhibition by luteolin and quercetin. Left: luteolin (1), right: quercetin (2). ■: [I]=0 ppm, ▲: [I]=0.1 ppm, ●: [I]=0.25 ppm.

two were non-competitive with substrate (Fig. 2). The *Km* value for PEP was 2.04×10^{-4} and Ki value of luteolin and quercetin were 1.19×10^{-6} and 1.13×10^{-6} M, respectively.

In order to understand structure-activity relationship in flavonoids, the inhibitory activity of twenty authentic flavonoids were tested. The structures of flavonoids used were presented in Fig. 3. Flavonoids having catechol moiety such as quercetin, luteolin exhibited strong activity except for orientin which belongs to flavone-8-C-glycoside. When luteolin was glycosylated at C-7 position, activity was remarkably reduced while glycosylation at C-3 position of quercetin did not affect on the activity, Therefore, catechol moiety of B-ring and 7-OH were seemed to be responsible for the stronger inhibitory activity (Table 1).

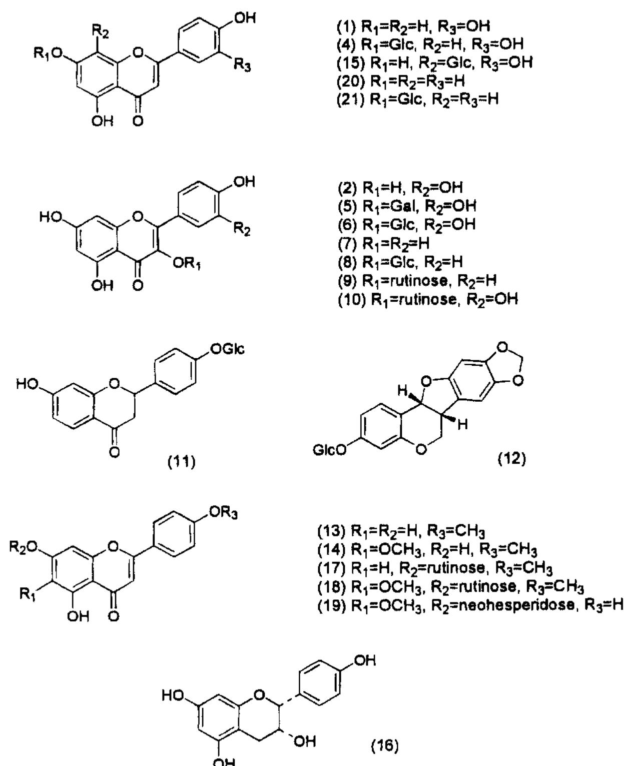

Fig. 3. Structures of tested flavonoids.

Table I. IC_{50} values of flavonoids

Flavonoids	IC_{50} (ppm)	Flavonoids	IC_{50} (ppm)
Luteolin (**1**)	0.17	Trifolirhizin (**12**)	87.9
Luteolin-7-O-Glc (**4**)	45.0	Acacetin (**13**)	14.3
Quercetin (**2**)	0.19	Pectolinarigenin (**14**)	8.10
Quercetin-3-O-Gal (**5**)	0.10	Orientin (**15**)	38.5
Quercetin-3-O-Glc (**6**)	0.15	(-)Epiafzelechin (**16**)	45.9
Kaempferol (**7**)	1.72	Linarin (**17**)	>120
Kaempferol-3-O-Glc (**8**)	108	Pectolinarin (**18**)	70.9
Kaempferol-3-O-rutinoside (**9**)	92.4	Hispidulin-7-O-neohesperidoside (**19**)	86.6
Rutin (**10**)	>120	Apigenin (**20**)	9.66
Liquiritin (**11**)	120	Apigenin-7-O-Glc (**21**)	66.5

Many inhibitors were isolated from microorganisms (Toda *et al.*, 1992; Aoyagi *et al.*, 1991) or synthesized chemically (Bakker *et al.*, 1990; Nakajima *et al.*, 1992). Most of them were peptide analogues and because of their hydrophilic nature or toxicity, it was difficult to penetrate blood-brain barrier (Nakajima *et al.*, 1992) and to apply clinical use. Lipophilic and little toxic compounds such as flavonoids are expected to solve such problems.

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