Prolyl Endopeptidase Inhibitors from the Roots of *Lindera strychnifolia* F. VILL

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Prolyl endopeptidase (PEP, EC 3.4.21.26) has been proposed to play a role in degradation of proline-containing neuropeptides involved in the processes of learning and memory, *e.g.*, vasopressin, substance P, and thyrotropin-releasing hormone (TRH). In the course of our search for bioactive constituents in medicinal plants, we studied the PEP inhibitory constituents of the roots of *Lindera strychnifolia* F. VILL and isolated two known tannins, epicatechin (1) and aesculitannin B (2), and four known sesquiterpenes, linderene (3), linderene acetate (4), linderalactone (5) and isolinderalactone (6) as inhibitors. On the inhibitory activities of six compounds against PEP from *Flavobacterium meningosepticum* and that from rat brain supernatant, compounds 1, 2 and 4 inhibited the enzyme from *Flavobacterium* more strongly than that from rat brain supernatant. However, compounds 3, 5 and 6 inhibited the enzymes from both origins to the same extent and furthermore, compound 6 was the strongest natural inhibitor against PEP from rat brain supernatant. The kinetic study of these inhibitors indicated that compounds 1, 2 are noncompetitive inhibitors and compounds 3—6 are competitive inhibitors. This is the first example of non-phenolic constituents showing significant competitive inhibitory activity being isolated from natural medicines.

Key words isolinderalactone; prolyl endopeptidase (PEP) inhibitor; *Lindera strychnifolia*; sesquiterpene; rat brain supernatant

Prolyl endopeptidase (PEP, EC 3.4.21.26) is a serine protease cleaving peptide bond at the carboxyl side of proline residues and was first isolated as an oxytocin-inactivating enzyme from the human uterus.[1)] This enzyme is distributed in a wide range of species, especially in human brain.[2)] In the central nervous system, PEP degrades proline-containing neuropeptides involved in the processes of learning and memory, *e.g.*, vasopressin, substance P, and thyrotropin-releasing hormone (TRH),[3,4)] and abnormal levels of PEP activity are associated with neurodegenerative disorders.[5,6)] In addition, PEP-like immunoreactivity was detected in the hippocampus of senescence-accelerated mouse,[7)] and the PEP activity of Alzheimer's patients was significantly higher than the control.[8)] Thus specific inhibitors for PEP are believed to have anti-amnesic effects[9)] and many inhibitors have been chemically synthesized or identified from natural resources.[10)]

In general, almost all PEP inhibitors derived from natural medicines are phenolic compounds containing a catechol or pyrogallol group, and there is no report concerning the actions of natural inhibitors against bacterial or mammalian enzymes.[11—14)] So we developed a screening for PEP inhibitors from natural medicines in order to identify non-phenolic inhibitors from natural medicines and to determine the inhibitory potency against PEP originating from a bacterium and a mammal. After the PEP inhibition test of 129 kinds of natural medicines, we found that a MeOH extract of the roots of *Lindera strychnifolia* showed significant inhibitory activity. *L. strychnifolia* (Lauraceae) is widely distributed in Japan and the People's Republic of China and its roots, Linderae Radix, are used as a traditional medicine as an analeptic, an analgesic and a digestive.[15)] From the roots of *L. strychnifolia*, monoterpenes, sesquiterpenes and alkaloids were reported but phenolic constituents such as tannins, have not been reported so far.[16,17)] Thus we studied the PEP inhibitory constituents of the roots of *L. strychnifolia* and isolated four known sesquiterpenes and two known tannins. In this paper, we report the isolation and structural identification of the constituents of the roots of *L. strychnifolia* and inhibitory activity against PEP from *Flavobacterium* and rat brain supernatant.

MATERIALS AND METHODS

General ^{1}H- (400 MHz) and ^{13}C-NMR (100 MHz) spectra were recorded on a JEOL α-400 FT-NMR spectrometer with tetramethylsilane as an internal standard. Positive-mode FAB-MS were recorded on a JEOL JMS-SX102 spectrometer, using a *m*-nitrobenzyl alcohol matrix. HPLC was performed using a JASCO System 800. PEP activity was measured with a Tosoh MPR-A4i II microplate reader and a BIO-TEK FL500.

Materials PEP (*Flavobacterium meningosepticum* origin) was purchased from Seikagaku Co. (Tokyo, Japan). Z-Gly-Pro-*p*NA and Z-Gly-Pro-AMC were purchased from Bachem Fine Chemical Co. (Switzerland). Z-Pro-prolinal was purchased from Biomol Research Laboratories Co. (U.S.A.), and brain samples from male Wistar rats were purchased from Funakoshi Co. (Tokyo). Epigallocatechin gallate (EGCG) was isolated in our laboratory.

PEP Inhibition Assay (*Flavobacterium meningosepticum*) PEP activity was assayed by the method of Yoshimoto *et al.*,[18)] in which 840 μl of 0.1 M phosphate buffer (pH 7.0), 50 μl of 0.1 unit/ml PEP in 50 mM phosphate buffer (pH 7.0) and 10 μl test sample solution in EtOH were mixed and pre-incubated for 5 min at 30 °C. The reaction was started by adding 100 μl of 2 mM Z-Gly-Pro-*p*NA in 40% 1,4-dioxane. After incubation at 30 °C for 30 min, 500 μl of 1 M acetate buffer (pH 4.0) was added and the absorbance of the solution was measured at 415 nm. The percentage of inhibition can be calculated from the absorbance with (A) and without (B) inhibitor, by the following equation: percentage of inhibition=

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$[(B-A)/B]\times100$.

PEP Inhibition Assay (Rat Brain Supernatant) PEP activity was measured by a modification of the method of Kato *et al.*[19] In brief, the brain of a rat was homogenized in 3 volumes of 0.1 M phosphate buffer (pH 7.0). After centrifugation at $10000\times g$ for 20 min, the supernatant was used for the enzyme assay. The PEP activity of this enzyme solution was 1.17×10^{-2} unit/mg protein. Ten microliters of enzyme solution was pre-incubated with 960 μl of 0.1 M phosphate buffer (pH 7.0) and 10 μl of test sample solution in EtOH at 30 °C for 5 min. The reaction was initiated by adding 20 μl of 2 mM Z-Gly-Pro-AMC and incubated at 30 °C for 30 min. The reaction was stopped by adding 2 ml of 1 M acetate buffer (pH 4.0), and the fluorescence intensity of the solution was measured at 460 nm with excitation at 360 nm. The percentage of inhibition can be calculated from the fluorescence intensity with (A) and without (B) inhibitor, by the following equation: Percentage of inhibition=$[(B-A)/B]\times100$.

PEP Inhibitory Activity of Natural Medicines The PEP inhibitory activities of 129 kinds of natural, mainly traditional anti-amnesic medicines, stimulants and tonics, were examined. As shown in Table 1, sixteen natural medicines showed significant PEP inhibitory activity. Among them, twelve, excluding *Epimedium grandiflorum*, *Lindera strychnifolia*, *Momordica cochinchinensis* and *Polygala tenuifolia*, were reported to contain phenolic compounds.[20,21] There are many reports concerning the PEP inhibitory activity of phenolic compounds,[11—14] and it is known that tannins interact with proteins and inhibit various enzymes.[20] So it seemed that the PEP inhibitory activities of these twelve natural medicines were caused by phenolic compounds. The PEP inhibitory activities of the other four natural medicines were investigated, and that of *L. strychnifolia* was the strongest, so we studied the PEP inhibitory constituents of the roots of *L. strychnifolia*.

Extraction and Fractionation The commercial available roots of *L. strychnifolia* (4.7 kg) supplied by Niiya (Shizuoka, Japan) were extracted three times with MeOH under reflux. After evaporation of the solvent under reduced pressure, the MeOH extract (135 g) was suspended in H_2O and extracted successively with $CHCl_3$ and EtOAc. Each layer was concentrated *in vacuo* to give $CHCl_3$ soluble (52 g), EtOAc soluble (4.7 g) and H_2O soluble (75 g) fractions, respectively. Among them, the EtOAc soluble and $CHCl_3$ soluble fraction showed significant PEP inhibitory activity with IC_{50} of 2.9 and 7.9 μg/ml, respectively.

Separation of EtOAc Soluble Fraction EtOAc soluble fraction (2.0 g) was subjected to preparative HPLC [ODS 5×100 cm; CH_3CN–H_2O (8 : 92)→(12 : 88) linear gradient and CH_3CN] to afford fractions A—H. Fractions B and G showed PEP inhibitory activity and fraction B was identified as epicatechin (**1**, 9 mg) by direct comparison with that of authentic sample; G was elucidated as aesculitannin B (**2**, 26 mg) by a comparison of NMR data with those in the literature.[22]

Separation of $CHCl_3$ Soluble Fraction The $CHCl_3$ soluble fraction (50 g) was suspended in 80% MeOH aq. and extracted with hexane–benzene (1 : 1). Each layer was evaporated *in vacuo* to give an 80% MeOH aq. layer (27 g, IC_{50}= 32.6 μg/ml) and a hexane–benzene (1 : 1) layer (21 g, IC_{50}= 3.9 μg/ml). The hexane–benzene (1 : 1) layer (20 g) was chromatographed on alumina (25 g) column and eluted with $CHCl_3$, MeOH and AcOH–MeOH (5 : 95), successively. Each eluate was concentrated *in vacuo* to give $CHCl_3$ eluate (13 g, IC_{50}=3.2 μg/ml), MeOH eluate (1.0 g, IC_{50}=42.1 μg/ml) and AcOH–MeOH (5 : 95) eluate (5.1 g, IC_{50}=22.6 μg/ml). The $CHCl_3$ eluate (6.0 g) was subjected to preparative HPLC [ODS 5×100 cm; CH_3CN–H_2O (47 : 53)→(63 : 37) linear gradient and CH_3CN] to afford fractions a—h. Fractions c—f showed significant PEP inhibitory activity and faction c was identified as linderalctone (**5**, 330 mg), fraction d as linderene (**3**, 218 mg), fraction e as isolinderalactone (**6**,

Table 1. MeOH Extracts of Natural Medicines Used in This Study and Their PEP Inhibitory Activity at 100 μg/ml

Inhibition (%)	MeOH extract
>80%	*Anemopaegma mirandum*, *Bixa orellana*, *Camellia sinensis*, *Casearia sylvestris*, *Crataegus cuneata*, *Epimedium grandiflorum*, *Filipendula purpurea*, *Fraxinus japonica*, *Lindera strychnifolia*, *Momordica cochinchinensis*, *Nelumbo nucifera*, *Paeonia suffruticosa*, *Polygala tenuifolia*, *Rhus javanica*, *Sanguisorba officinalis*, *Uncaria gambir*
>50%	*Arnica montata*, *Asiasarum sieboldii*, *Curculigo orchioides*, *Echinodorus grandiflorus*, *Thuja orientalis*
50%>	*Achyranthes bidentata*, *Acorus calamus*, *Acorus gramineus*, *Agaricus blazei*, *Akebia quinata*, *Albizzia julibrissin*, *Alpinia officinarum*, *Alpinia oxyphylla*, *Amomum xanthioides*, *Anemarrhena asphodeloides*, *Angelica acutiloba*, *Angelica decursiva*, *Arctium lappa*, *Arctostaphylos uva-ursi*, *Artemisia capillaris*, *Aster tataricus*, *Astragalus membranaceus*, *Atractylodes lancea*, *Atractylodes ovata*, *Baccharis dracunculifolia*, *Baccharis genistelloides*, *Bauhinia forticata*, *Boswellia carterii*, *Buddleja brasiliensis*, *Buddleja japonica*, *Bupleurum scorzoneraefolium*, *Cannabis sativa*, *Cassia acutifolia*, *Cassia obtusifolia*, *Cetraria islandica*, *Cimicifuga simplex*, *Cistanche salsa*, *Citrus aurantium*, *Citrus unshiu*, *Clematis chinensis*, *Coptis japonica*, *Cordia verbenacea*, *Coreopsis drummondii*, *Cuscuta japonica*, *Cyathula officinalis*, *Cynanchum caudatum*, *Cynanchum glaucescens*, *Cynomorium songaricum*, *Cyperus rotundus*, *Dioscorea japonica*, *Euphorbia jolkinii*, *Euphorbia lathyris*, *Euphorbia pekinensis*, *Euphoria longan*, *Evodia rutaecarpa*, *Forsythia suspensa*, *Fortunella japonica*, *Ginkgo biloba*, *Houttuynia cordata*, *Humulus scandens*, *Hydrangea macrophylla*, *Imperata cylindrica*, *Isodon japonicus*, *Lonicera japonica*, *Lycium chinense*, *Magnolia biondii*, *Magnolia obovata*, *Maytenus ilicifolia*, *Melia toosendan*, *Nothosmyrnium japonicum*, *Ophiopogon japonicus*, *Panax ginseng*, *Periploca sepium*, *Pharbitis nil*, *Phellodendron amurense*, *Phyllanthus niruni*, *Phytolacca americana*, *Picrasma quassioides*, *Picrorrhyza kurrooa*, *Pieris japonica*, *Plantago asiatica*, *Pogostemon cablin*, *Polygonatum falcatum*, *Polygonum multiflorum*, *Psoralea corylifolia*, *Pueraria lobata*, *Rauwolfia serpentina*, *Rehmannia glutinosa*, *Rhamnus purshiana*, *Rheum undulatum*, *Rosa multiflora*, *Salvia miltiorrhiza*, *Sambucus sieboldiana*, *Santalum album*, *Saussurea lappa*, *Schisandra chinensis*, *Scrophularia ningpoensis*, *Scutellaria baicalensis*, *Sinomenium acutum*, *Smallanthus sonchifolia*, *Sophora angustifolia*, *Sophora japonica*, *Stevia rebaudiana*, *Stryphnodendron adstringens*, *Swertia japonica*, *Tabebuia impetiginoea*, *Trigonella foenum-graecum*, *Tussilago farfara*, *Uncaria sinensis*, *Valeriana fauriei*, *Withania somnifera*, *Zingiber officinale*, *Zizyphus jujuba*

59 mg), and fraction f as linderene acetate (**4**, 1.3 g) by comparison of NMR data with those in the literature.[16)]

RESULTS AND DISCUSSION

In the course of our search for bioactive constituents in medicinal plants, we examined PEP inhibitory constituents of the roots of *L. strychnifolia* and isolated six known compounds **1**—**6** as inhibitors. Compound **1** was determined to be epicatechin by direct comparison with authentic sample, and the others were identified by comparison of spectral data with reported data as aesculitannin B (**2**), linderene (**3**), linderene acetate (**4**), lindera lactone (**5**) and isolindera lactone (**6**) (Fig. 1). Compounds **3**—**6** were earlier reported to be constituents of the roots of *L. strychnifolia*, but this was the first time tannins **1** and **2** were isolated from this plant.[16)]

PEP suggests that there is no difference among mammalia,[23)] but there are some differences between bacteria and mammalia, *e.g.*, isoelectric point and sensitivity to *p*-chloromercuribenzoic acid (PCMB).[18)] In addition, bacterial (*Flavobacterium*) and mammalian (bovine brain) enzyme genes were cloned and their amino acid sequences had 48% identity[24)]; we therefore investigated the inhibitory activities of six compounds by two different assays. One is the method of Yoshimoto *et al.*,[9)] which is the most popular *Flavobacterium* PEP inhibition assay, and the other is the method of Kato *et al.*,[19)] which measures inhibitory activity against PEP from rat brain supernatant. The concentrations of these compounds causing 50% inhibition are shown in Table 2, together with the positive controls, EGCG and Z-Pro-prolinal. Compound **1** is a known inhibitor against PEP from *Flavobacterium*,[13)] however, compounds **2**—**6** are new PEP inhibitors. Against PEP from *Flavobacterium*, compound **2** showed the same inhibitory effect as other tannins reported to date,[11—14)] and compounds **3**—**6** were first examples of sesquiterpenic inhibitors. Compound **6** showed significant inhibitory activity as a non-phenolic compound. Against PEP from rat brain supernatant, all compounds including positive controls showed weak inhibition, in comparison with *Flavobacterium* PEP inhibitory activity, and those of the tannins **1**, **2** and EGCG, declined considerably. It seemed that these results were caused by tannins interacting with other proteins in rat brain supernatant or tannins inhibiting only *Flavobacterium* PEP specifically but not inhibiting mammalian PEP. However, interestingly, these sesquiterpenic inhibitors exhibit little differences in PEP inhibitory activity between *Flavobacterium* and rat brain supernatant and compound **6** was the strongest natural inhibitor in this report.

On the other hand, the inhibition mode of almost all non-peptidic inhibitors is noncompetitive and the only competitive inhibitor from natural sources is protocatechuic acid.[11)] Lineweaver–Burk plots for these inhibitors obtained from the roots of *L. strychnifolia* indicated that conpounds **1** and **2** were noncompetitive inhibitors, but interestingly, compounds **3**—**6** showed competitive inhibition; Lineweaver–Burk plots of **6** are shown in Fig. 2. These sesquiterpenes are thus considered new type of inhibitors in contrast to non-phenolic competitive inhibitors.

L. strychnifolia belongs to the family Lauraceae and its roots are used as an analeptic, an analgesic and a digestive. These roots are proposed to improve palsy and prevent stroke,[15)] however, the principles of these medicinal effects are not yet clear. In this paper, we reported that the roots of *L. strychnifolia* showed significant PEP inhibitory activity. It is inferred that constituents of these roots inhibit PEP and contribute to its medicinal effects. On the other hand, most of the PEP inhibitors reported so far are synthetic substrate mimetics and only a few have been reported from natural sources.[10)] Almost all PEP inhibitors from medicinal plants are phenolic compounds, and there has been no report concerning a natural inhibitor which is not a peptide or a phenolic compound.[10—14)] In this paper, we reported six PEP inhibitors from the roots of *L. strychnifolia*, compounds **3**—**6** among them being non-phenolic inhibitors. Compound **6** showed strong competitive inhibitory activity against PEP both from *Flavobacterium* and from rat brain supernatant. These sesquiterpenes are also lipophilic compounds. Since PEP is an intracellular enzyme and distributed in brain, these characteristics are meaningful for a PEP inhibitor. These re-

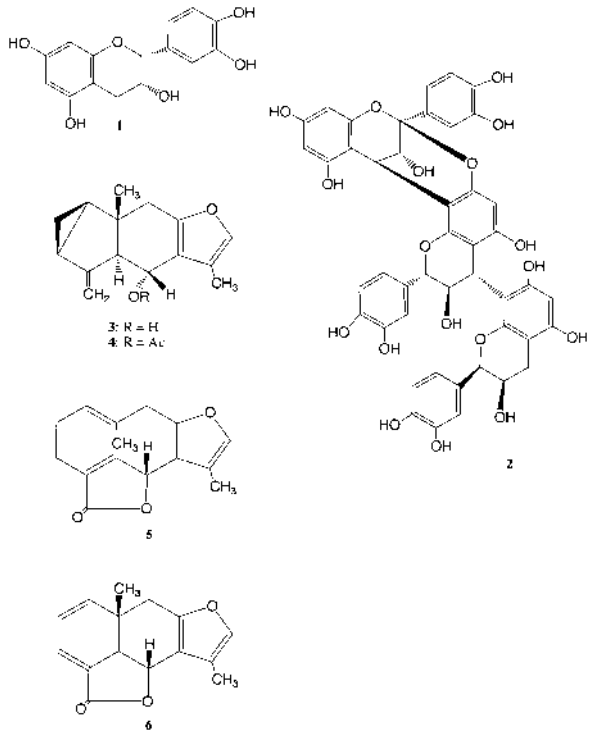

Fig. 1. Chemical Structures of PEP Inhibitors Isolated from *Lindera strychnifolia*

Table 2. PEP Inhibitory Activity of Isolated Compounds

Compound	IC_{50} (μM; mean±S.E.)[a)]	
	Flavobacterium[b)]	Rat brain supernatant[c)]
1	26.2±0.97	>500
2	4.0±0.08	60.3±6.74
3	147.0±3.67	164.6±1.64
4	65.4±1.37	>500
5	102.2±1.02	134.2±2.29
6	8.8±0.03	19.8±7.31
EGCG	1.7±0.02	32.3±7.42
Z-Pro-prolinal	0.5±0.21 nM	1.9±0.14 nM

a) Data are expressed as the mean±S.E. of three independent experiments. *b*) The substrate used was Z-Gly-Pro-*p*NA. *c*) The substrate used was Z-Gly-Pro-AMC.

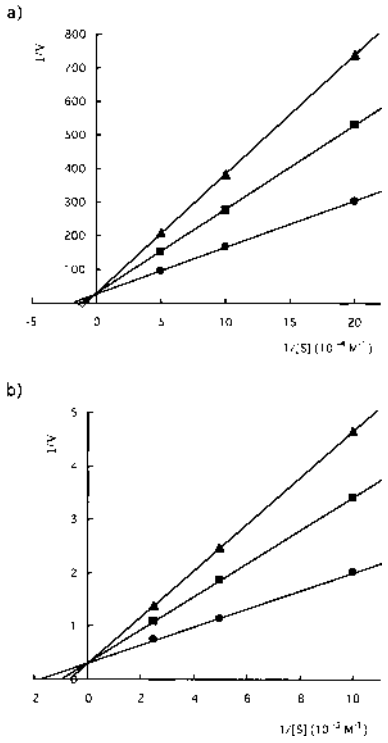

Fig. 2. Lineweaver–Burk Plots of the Inhibition by **6**

a) Lineweaver–Burk plot of inhibition against PEP from *Flavobacterium* by **6**, [I]=0 μM (●), 2.5 μM (■), 5 μM (▲). 1/V was defined as min/ΔA_{415}. b) Lineweaver–Burk plot of inhibition against PEP from rat brain supernatant by **6**, [I]=0 μM (●), 15 μM (■), 30 μM (▲). 1/V was defined as min/Δ460 nm with excitation at 360 nm.

sults might thus be useful in investigating PEP inhibition mechanisms and developing of a new type of PEP inhibitor from natural products for use in future.

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