Matrix Metalloproteinase-2 Inhibitors from *Clinopodium chinense* var. *parviflorum*

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Received December 9, 2008

From the aerial parts of *Clinopodium chinense* var. *parviflorum*, nine new phenylpropanoids, clinopodic acids A−I (**2**−**10**), were isolated together with a known phenylpropanoid, rosmarinic acid (**1**). The structures of these new compounds were elucidated on the basis of spectroscopic analysis. Clinopodic acid C (**4**) showed MMP-2 inhibitory activity (IC_{50} 3.26 μM).

In the course of our research on the saponins from *Clinopodium* species,[1,2] we reported the structures of oleanane-type triterpene saponins related to the antihepatotoxic[3] saikosaponins from *Clinopodium chinense* (Benth.) O. Kuntze var. *parviflorum* (Kudo) Hara. (Labiatae). The ethanol extract of *C. chinense* Kuntze showed antihyperglycemic effect.[4] The water extract of *C. vulgare* L. showed strong antitumor activity in an *in vitro* screening.[5] In a search for bioactive compounds from the water extract of *C. chinense* var. *parviflorum*, we now report the isolation and the structural elucidation of eight new phenylpropanoids, named clinopodic acids A−I (**2**−**10**), and a known phenylpropanoid, rosmarinic acid (**1**). Clinopodic acid C (**4**) showed MMP-2 inhibitory activity (IC_{50} 3.26 μM).

Results and Discussion

Clinopodic acid A (**2**) was isolated as a colorless, amorphous solid. Its molecular formula was established as $C_{18}H_{16}O_7$ on the basis of a pseudomolecular ion peak at *m/z* 345.0975 ($[M + H]^+$). The ^{1}H NMR spectrum was similar to that of rosmarinic acid (**1**) except for the presence of AA′BB-type aromatic proton signals at δ 6.90 (2H, d, J = 8.0 Hz) and 7.54 (2H, d, J = 8.0 Hz). In the ROE experiment, an ROE was observed at the aromatic proton (δ 7.54) on irradiation at an olefinic proton at δ 7.63 (d, J = 15.5 Hz). The CD spectrum showed negative Cotton effects at 230 and 282 nm and positive Cotton effects at 300 and 329 nm and were similar to those of **1**. From these data, the structure of clinopodic acid A was elucidated as **2**. Compound **2** is assumed to be a precursor of rosmarinic acid.[6]

Clinopodic acid B (**3**) was isolated as a colorless, amorphous solid, and the ^{1}H NMR spectrum of **3** was also similar to that of **1** except for the presence of a methoxy proton singlet at δ 3.83. In the ROE experiment, ROEs were observed at the aromatic proton [δ 6.97 (1H, d, J = 2.0 Hz)] on irradiation at the *O*-methyl signal and on irradiation at two methylene protons [δ 3.09 (dd, J = 14.0 and 9.0 Hz); 3.19 (dd, J = 14.0 and 4.0 Hz)]. Therefore the position of the *O*-methyl group was decided to be C-3′. The absolute configuration was determined from the dextrorotatory specific rotation and the CD spectrum, which was similar to those of **1**.

The HRFABMS spectrum of clinopodic acid C (**4**) showed a pseudomolecular ion $[M + H]^+$ at *m/z* 539.1218, consistent with the molecular formula $C_{27}H_{23}O_{12}$. The ^{1}H NMR spectrum showed ABC-type aromatic protons at δ 6.81 (1H, d, J = 8.0 Hz), 6.84 (1H, dd, J = 8.0 and 2.0 Hz), and 6.96 (1H, d, J = 2.0 Hz) and AB-type aliphatic proton signals at δ 5.10 (1H, d, J = 3.5 Hz) and 5.48 (1H, d, J = 3.5 Hz) in addition to a set of proton signals due to a rosmarinic acid moiety. Irradiating at the proton doublet at δ 5.48 showed ROEs at two aromatic protons at δ 6.84 and 6.96, which were coupled with each other, and at an aliphatic proton doublet at δ 5.10, which was coupled with a proton at δ 5.48. In the HMBC spectrum, the aliphatic proton at δ 5.48 was long-range coupled with a carbon at δ 146.5, which was assigned to C-4 of the caffeoyl moiety because of the long-range coupling with the aromatic proton at δ 7.24 (1H, d, J = 8.0 Hz) due to H-6. These NMR data suggested that this compound had a phenyl-benzodioxane moiety.[7] The CD spectrum showed a positive Cotton effect at 244 nm, suggesting that the absolute configuration of the benzylic carbon was *S*.[8] The C-8′ configuration was established as *R* from the retention time of the amide with (*S*)-2-phenylglycine methyl ester, which was synthesized after alkaline hydrolysis.[8] From these data the structure of **4** was determined as indicated.

The ^{1}H NMR spectrum of clinopodic acid D (**5**) was similar to that of **4** except for the presence of an *O*-methyl singlet at δ 3.83 (3H, s). ROEs were observed at the aromatic proton signal at δ 6.88 (1H, brs) on irradiation at the *O*-methyl signal, and at the *O*-methyl and the methine protons at δ 5.25 (1H, dd, J = 8.0 and 4.5 Hz) on irradiation at the aromatic proton at δ 6.88. In the HMBC spectrum, an oxygenated aromatic carbon signal at δ 148.8 was long-range coupled with the *O*-methyl proton and the aromatic proton at δ 6.88. The configuration of the benzodioxane moiety was established as 7″*S*, 8″*S* from the CD data and that at C-8′ to be *R* from the retention time of the amide derivative as in the case of **4**.

The ^{1}H, ^{13}C NMR and MS data of clinopodic acid E (**6**) were similar to those of **4**. In the CD spectrum, **6** showed a negative Cotton effect at 231 nm, suggesting a 7″*R* absolute configuration.

The NMR spectra of clinopodic acids F (**7**) and G (**8**) were similar to those of clinopodic acid D (**5**) and had one and two *O*-methyl groups, respectively.

The ^{1}H NMR spectrum of clinopodic acid F (**7**) showed an *O*-methyl signal at δ 3.84 (3H, s), which was located at C-3′ from the ROE data.

The ^{1}H NMR spectrum of clinopodic acid G (**8**) showed two *O*-methyl signals at δ 3.83 and 3.84 (each 3H, s) located at C-3″ and C-3′, respectively. These locations were confirmed by ROE and HMBC spectra.

The HRFABMS spectrum of clinopodic acid H (**9**) showed a molecular ion peak at *m/z* 566.1435 (calcd for $C_{29}H_{26}O_{12}$ 566.1424).

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10.1021/np800781t CCC: $40.75 © 2009 American Chemical Society and American Society of Pharmacognosy
Published on Web 08/03/2009

The ^{1}H NMR spectrum showed two *O*-methyl signals at δ 3.80 and 3.82 and two aliphatic doublet proton signals at δ 4.57 (1H, d, $J = 5.0$ Hz) and 6.02 (1H, d, $J = 5.0$ Hz) coupling with each other in the H−H COSY spectrum. These protons were assigned to the aliphatic proton of a dihydrobenzofuran moiety. The coupling constant of these protons suggested *trans* fusion.[9] The positive Cotton effect at 253 nm in the CD spectrum of **9** suggested a 7″*S* absolute configuration.[10] The location of two *O*-methyl groups was decided to be C-3′ and C-3″ by an ROE experiment. Alkaline hydrolysis gave a dextrorotary optically active 3-(3-methoxy-4-hydroxyphenyl)-2-hydroxypropanoic acid similar to (2*R*)-3-(3,4-dihydroxyphenyl)-2-hydroxypropanoic acid from rosmarinic acid (**1**) and 8-epibechnic acid.[9]

The absolute configuration of the 3-(3,4-dihydroxyphenyl)-2-hydroxypropanoic acid moiety in compound **6** and the 3-(3-methoxy-4-hydroxyphenyl)-2-hydroxypropanoic acid moiety in compounds **5** and **7**−**9** were decided to be both *R* in the same way as for **4**. Compound **9** has the same structure as lithospermic acid,[11] but their NMR data are not identical.

The HRFABMS spectrum of clinopodic acid I (**10**) showed a pseudomolecular ion peak at *m/z* 717.1476 (calcd for $C_{36}H_{29}O_{16}$ 717.1455). The ^{1}H NMR spectrum was similar to that of **9** except for signals of an additional 3-(3,4-dihydroxyphenyl)-2-hydroxypropanoic acid moiety. In the ROE spectra, ROEs were observed at two aromatic protons at δ 6.57 (1H, dd, $J = 8.0$ and 2.0 Hz) and 6.79 (1H, d, $J = 2.0$ Hz) on irradiation at an aliphatic proton at δ 5.33 (1H, dd, $J = 8.0$ and 4.5 Hz), at three protons at δ 6.74 (1H, dd, $J = 8.0$ and 2.0 Hz) and 6.90 (1H, d, $J = 2.0$ Hz) on irradiation at δ 5.78 (1H, d, $J = 5.0$ Hz), at four protons at δ 3.01 (1H, dd, $J = 14.5$ and 7.0 Hz), 3.10 (1H, dd, $J = 14.5$ and 4.5 Hz), 4.53 (1H, d, $J = 5.0$ Hz), and 6.70 (1H, d, $J = 8.0$ Hz) on irradiation at δ 5.28 (1H, dd, $J = 7.0$ and 4.5 Hz), at two protons at δ 3.01 and 5.28 on irradiation at δ 6.70, and at two aromatic protons at δ 6.94 (1H, dd, $J = 8.0$ and 2.0 Hz) and 7.07 (1H, d, $J = 2.0$ Hz) on irradiation at δ 7.53 (1H, d, $J = 16.0$ Hz). The absolute configurations of the two 3-(3,4-dihydroxyphenyl)-2-hydroxypropanoic acid moieties were established as *R*, from the ^{1}H NMR chemical shifts of H-7′ and H-7″ (Table 2) of the amides with (*S*)-phenylglycine methyl ester and (*R*)-phenylglycine methyl ester.[8]

Matrix metalloproteinase-2 (MMP-2) inhibitory activity was measured for these compounds. Rosmarinic acid (**1**), clinopodic acid C (**4**), and lithospermic acid[12] showed inhibitory activity, (IC_{50} 27.2, 3.26, and 10.2 μM, respectively). Series of MMPs play an important part in the regulation of both cell−cell and cell−extracellular matrix interactions. Especially, the activation of MMP-2 contributes to proteolytic activity and the degrading of denatured collagens, gelatins, and extracellular matrix.[13] This can be related to the phenomena of tissue regeneration and/or various diseases such as cancer invasion or metastasis, arthritis, and blood vessel impairment.[13,14] Moreover, the relationship between antioxidants and MMP inhibitory activity of rosmarinic acid (**1**) had been reported.[15] Thus, some MMP-2 inhibitors observed in this paper would play a role not only in ameliorating the diseases described above but also in a possible cosmetic for skin aging.[16]

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO DIP-1000 digital polarimeter. UV spectra were measured in MeOH on a Hitachi U-2010 spectrophotometer. CD spectra were measured on a JASCO J-20A spectrometer. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a JEOL JNM α-400 FT-NMR spectrometer, and chemical shifts are given as δ values with TMS as an internal standard at 35 °C. Inverse-detected heteronuclear correlations were measured using HMQC (optimized for $^1J_{C-H} = 145$ Hz) and HMBC (optimized for $^nJ_{C-H} = 8$ Hz) pulse sequences with a pulse field gradient. HRFABMS data were obtained on a JEOL JMS 700 mass spectrometer in the positive mode and on a JEOL JMS SX 102 mass spectrometer in the negative mode using a *m*-nitrobenzyl alcohol matrix. Preparative HPLC was performed on a JASCO 800 instrument.

Plant Material. *C. chinense* var. *parviflorum* was harvested on September 2006 from our medicinal botanical garden. The plant was authenticated by Prof. A. Ueno, School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka, Japan. A voucher specimen (200609030) has been deposited at the Herbarium of the University of Shizuoka.

Materials. *p*-Aminophenylmercuric acetate was purchased from Sigma (St. Louis, MO). The reagents used for SDS-PAGE were purchased from Bio-Rad Co. Ltd. (Hercules, CA).

Extraction and Isolation. Powdered aerial parts of *C. chinense* var. *parviflorum* (1.13 kg) were extracted with hot H_2O. The H_2O extract was dissolved in H_2O, and the aqueous solution was passed through a porous polymer gel column (Mithubishi Diaion HP-20, 9 × 45 cm). The adsorbed material was eluted with 30% MeOH (5 L), 50% MeOH (5 L), and MeOH (8 L) to give 30% MeOH eluate (26.7 g), 50% MeOH eluate (16.9 g), and MeOH eluate (19.3 g). Some of the 30% MeOH eluate (3.0 g) was subjected to HPLC [column, YMC ODS, 5 × 100 cm; solvent, H_2O-CH_3CN (80:20) → (78: 22) linear gradient; flow rate, 45 mL/min; detection, UV 320 nm] to afford 12 fractions. Fractions 4−12 were subjected further to preparative HPLC to give compounds **1** (152 mg), **2** (3 mg), **4** (20 mg), **6** (15 mg), **7** (8 mg), and **10** (13 mg). The 50% MeOH eluate (16.9 g) was subjected to a silica gel column [Fuji Silysia PSQ-100B, 7 × 85 cm; solvent, $CHCl_3-MeOH-H_2O$ (80:18:2) → + MeOH gradient] to afford eight fractions. Fraction 2 (2.3 g) was subjected to HPLC [column, YMC ODS, 5 × 100 cm; solvent, H_2O-CH_3CN (80:20) → (70:30) linear gradient; flow rate, 45 mL/min; detection, UV 320 nm] to afford 10 fractions. Fractions 5−10 were subjected further to preparative HPLC to give compounds **2** (20 mg), **3** (24 mg), **5** (19 mg), **7** (57 mg), **8** (13 mg), and **9** (228 mg).

Rosmarinic acid (1): colorless, amorphous solid; $[\alpha]^{23}_D$ +37.2 (*c* 2.92, MeOH); CD (*c* 0.030, MeOH) λ_{max} nm ([θ]) 218 (−2.40 × 10^3), 232 (−9.36 × 10^3), 280 (−2.88 × 10^3), 298 (+4.80 × 10^3), 320 (+3.12 × 10^3).

Clinopodic acid A (2): colorless, amorphous solid; $[\alpha]^{23}_D$ +83.8 (*c* 1.60, MeOH); UV (MeOH) λ_{max} (log ϵ) 230 (4.84), 279 (4.23), 308 (4.01), 319 (4.02); CD (*c* 0.050, MeOH) λ_{max} nm ([θ]) 230 (−7.91 × 10^3), 250 (+5.50 × 10^3), 282 (−3.44 × 10^3), 300 (+6.88 × 10^3), 325 (+4.47 × 10^3); ^{1}H and ^{13}C NMR, Table 1; HRFABMS *m/z* 345.0975 $[M + H]^+$ (calcd for $C_{18}H_{17}O_7$, 345.0974), 367.0783 $[M + Na]^+$ ($C_{18}H_{16}O_7Na$, 367.0794).

Clinopodic acid B (3): colorless, amorphous solid; $[\alpha]^{23}_D$ +79.3 (*c* 1.91, MeOH); UV (MeOH) λ_{max} (log ϵ) 220sh (4.19), 232sh (4.13), 288 (4.07), 326 (4.17); CD (*c* 0.050, MeOH) λ_{max} nm ([θ]) 230 (−7.33 × 10^3), 250 (+6.43 × 10^3), 275 (−2.99 × 10^3), 295 (+5.54 × 10^3), 325 (+4.19 × 10^3); ^{1}H and ^{13}C NMR, Table 1; HRFABMS *m/z* 375.1077 $[M + H]^+$ (calcd for $C_{19}H_{19}O_8$, 375.1080), 397.0903 $[M + Na]^+$ ($C_{19}H_{18}O_8Na$, 397.0899).

Clinopodic acid C (4): colorless, amorphous solid; $[\alpha]^{23}_D$ −14.0 (*c* 1.10, MeOH); UV (MeOH) λ_{max} (log ϵ) 221sh (4.13), 233sh (4.01), 289 (3.96), 326 (3.95); CD (*c* 0.050, MeOH) λ_{max} nm ([θ]) 214 (−2.15 × 10^4), 229 (+7.09 × 10^3), 244 (+1.88 × 10^4), 294 (−1.26 × 10^4), 306 (−9.39 × 10^3), 322 (−1.21 × 10^4); ^{1}H and ^{13}C NMR, Table 1; HRFABMS *m/z* 539.1218 $[M + H]^+$ (calcd for $C_{27}H_{23}O_{12}$, 539.1190).

Clinopodic acid D (5): colorless, amorphous solid; $[\alpha]^{23}_D$ −56.5 (*c* 0.46, MeOH); UV (MeOH) λ_{max} (log ϵ) 223sh (4.46), 233sh (4.38), 288 (4.29), 324 (4.31); CD (*c* 0.050. MeOH) λ_{max} nm ([θ]) 213 (−2.83 × 10^4), 227 (+1.35 × 10^3), 233 (+1.24 × 10^4), 244 (+2.45 × 10^4), 300 (−1.30 × 10^4), 306 (−1.10 × 10^4), 321 (−1.57 × 10^4); ^{1}H and ^{13}C NMR, Table 1; HRFABMS *m/z* 575.1127 $[M + Na]^+$ (calcd for $C_{28}H_{24}O_{12}Na$, 575.1165).

Clinopodic acid E (6): colorless, amorphous solid; $[\alpha]^{23}_D$ +4.4 (*c* 0.46, MeOH); UV (MeOH) λ_{max} (log ϵ) 223sh (4.11), 234sh (3.98), 288 (3.91), 326 (3.81); CD (*c* 0.052, MeOH) λ_{max} nm ([θ]) 231 (−1.08 × 10^4), 250 (−1.45 × 10^3), 282 (−6.21 × 10^3), 320 (+5.38 × 10^3); ^{1}H and ^{13}C NMR, Table 1; HRFABMS *m/z* 539.1167 $[M + H]^+$ (calcd for $C_{27}H_{23}O_{12}$, 539.1190).

Clinopodic acid F (7): colorless, amorphous solid; $[\alpha]^{23}_D$ +38.4 (*c* 0.35, MeOH); UV (MeOH) λ_{max} (log ϵ) 222sh (4.36), 233sh (4.28), 288 (4.22), 323 (4.21); CD (*c* 0.050, MeOH) λ_{max} nm ([θ]) 218 (+1.41 × 10^4), 238 (−7.29 × 10^3), 259 (−1.32 × 10^3), 275 (−3.75 × 10^3), 296 (−1.02 × 10^4), 315 (+8.39 × 10^3); ^{1}H and ^{13}C NMR, Table 1; HRFABMS *m/z* 551.1198 $[M - H]^-$ (calcd for $C_{28}H_{23}O_{12}$, 551.1189).

Clinopodic acid G (8): colorless, amorphous solid; $[\alpha]^{23}_{D}$ +59.8 (*c* 0.31, MeOH); UV (MeOH) λ_{max} (log ϵ) 224sh (4.43), 233sh (4.38), 288 (4.28), 325 (4.28); CD (*c* 0.050, MeOH) λ_{max} nm ([θ]) 223 (+1.52 × 10^4), 237 (−5.66 × 10^3), 273 (−3.17 × 10^3), 293 (+1.11 × 10^4), 320 (+1.02 × 10^4); ^{1}H and ^{13}C NMR, Table 1; HRFABMS *m/z* 565.1340 [M + H]$^+$ (calcd for $C_{29}H_{25}O_{12}$, 565.1346).

Clinopodic acid H (9): colorless, amorphous solid; $[\alpha]^{23}_{D}$ +131.0 (*c* 2.59, MeOH); UV (MeOH) λ_{max} (log ϵ) 229 (4.53), 254 (4.42), 288 (4.35), 310 (4.39), 333 (4.33); CD (*c* 0.050, MeOH) λ_{max} nm ([θ]) 212 (−3.85 × 10^4), 217 (−1.81 × 10^4), 226 (+1.87 × 10^4), 234 (+9.62 × 10^3), 253 (+5.60 × 10^4), 280 (−6.79 × 10^3), 295 (+6.79 × 10^4), 334 (+1.25 × 10^4); ^{1}H and ^{13}C NMR, Table 1; HRFABMS *m/z* 566.1435 [M]$^+$ (calcd for $C_{29}H_{26}O_{12}$, 566.1424), 567.1500 [M + H]$^+$ (calcd for $C_{29}H_{27}O_{12}$, 567.1503), 589.1313 [M + Na]$^+$ (calcd for $C_{29}H_{26}O_{12}$Na, 589.1322).

Clinopodic acid I (10): colorless, amorphous solid; $[\alpha]_{D}^{23}$ −76.6 (*c* 0.55, MeOH); UV (MeOH) λ_{max} (log ϵ) 231sh (4.35), 289 (4.21), 331 (4.17); CD (*c* 0.050, MeOH) λ_{max} nm ([θ]) 225 (−3.52 × 10^4), 248 (+4.30 × 10^3), 293 (−3.04 × 10^4), 328 (−8.04 × 10^3); ^{1}H and ^{13}C NMR, Table 1; HRFABMS *m/z* 717.1476 [M − H]$^-$ (calcd for $C_{36}H_{29}O_{16}$, 717.1455).

Table 1. NMR Spectroscopic Data (400 MHz) of Compounds **2**−**10**

	2[a]				**3**[a]				**4**[a]			
position	δ_H (*J* in Hz)	δ_C	HMBC (H to C)	ROE (H to H)	δ_H (*J* in Hz)	δ_C	HMBC (H to C)	ROE (H to H)	δ_H (*J* in Hz)	δ_C	HMBC (H to C)	ROE (H to H)
1		126.9				127.5				129.2		
2	7.54, d (8.0)	116.7	4		7.16, d (2.0)	115.3	4, 7		7.33, d (2.0)	117.4	3, 4	
3	6.90, d (8.0)	131.1	1			148.1				143.7		
4		146.2				148.9				146.5		
5	6.90, d (8.0)	131.1	1, 7		6.87, d (8.0)	116.4	4, 7		6.97, d (8.0)	118.4	3	
6	7.54, d (8.0)	116.7			7.03, dd (8.0, 2.0)	122.6	4′, 7		7.24, dd (8.0, 2.0)	123.5	4	
7	7.63, d (15.5)	146.2	3, 5, 9	2, 6	7.58, d (16.0)	146.5	1, 2, 6, 9	2, 6	7.63, d (16.0)	145.7	1, 2, 6, 9	2′, 6′
8	6.36, d (15.5)	115.0	1		6.30, d (16.0)	114.9	1, 9		6.45, d (16.0)	116.7	1, 7, 9	
9		166.9				166.8				166.6		
OMe					3.83, s	56.3	3′	2′				
1′		129.3				128.9				129.2		
2′	6.85, d (2.0)	117.3	4′		6.97, d (2.0)	113.9	1′, 4′		6.87, d (2.0)	117.3	4′	
3′		145.7				146.5				145.7		
4′		144.7				146.4				144.8		
5′	6.75, d (8.0)	116.0	1′, 3′		6.76, d (8.0)	115.6	4′		6.76, d (8.0)	115.9	3′	
6′	6.68, dd (8.0, 2.0)	121.7	4′		6.79, dd (8.0, 2.0)	122.9	4′		6.69, dd (8.0, 2.0)	121.7	4′	
7′	3.04, dd (13.5, 8.0)	37.5	2′, 6′, 9′		3.09, dd (14.0, 9.0)	37.7	1′, 2′, 6′, 8′, 9′	2′, 6′, 8′	3.05, dd (13.5, 8.0)	37.5	1′, 2′, 6′	2′, 6′
	3.14, dd (13.5, 4.0)		2′, 6′, 9′		3.19, dd (14.0, 4.0)		1′, 2′, 6′, 8′ 9′	2′, 6′, 8′	3.14, dd (13.5, 4.0)		1′, 2′, 6′	2′, 6′
8′	5.24, dd (8.0, 4.0)	73.8			5.26, dd (9.0, 4.0)	73.8	1′, 9, 9′	2′, 6′, 7′	5.25, dd (8.0, 4.0)	73.8	1′	
9′		171.3				171.3				171.0		
OMe												
1″										127.8		
2″									6.96, d (2.0)	115.0	4″	
3″										145.8		
4″										146.3		
5″									6.81, d (8.0)	116.0	3″	
6″									6.84, dd (8.0, 2.0)	119.8	4″, 7″	
7″									5.48, d (3.5)	76.0	1″, 2″, 4, 6″	2″, 6″, 8″
8″									5.10, d (3.5)	75.7	1″, 3	
9″										168.0		

	5[b]				**6**[a]				**7**[a]			
position	δ_H (*J* in Hz)	δ_C	HMBC (H to C)	ROE (H to H)	δ_H (*J* in Hz)	δ_C	HMBC (H to C)	ROE (H to H)	δ_H (*J* in Hz)	δ_C	HMBC (H to C)	ROE (H to H)
1		129.4				129.3				129.2		
2	7.27, d (2.0)	117.6	3, 4, 6	7, 8	7.29, d (2.0)	117.4	3, 4		7.29, d (2.0)	117.3	6	7, 8
3		144.2				143.6				143.6		
4		147.0				146.2				146.2		
5	6.93, d (8.5)	118.6	1, 3, 4		6.96, d (8.0)	118.6	3		6.96, d (8.0)	118.5	1	
6	7.15, dd (8.5, 2.0)	124.0	2, 4		7.24, dd (8.0, 2.0)	123.4	4		7.22, dd (8.0, 2.0)	123.4	2	
7	7.62, d (16.0)	146.7	2, 6, 9		7.62, d (15.5)	116.8	1, 2, 6	2, 6	7.63, d (15.0)	116.7	1, 9	2, 6
8	6.39, d (16.0)	116.4	1, 7, 9		6.44, d (15.5)	145.6	1		6.46, d (15.0)	145.7	1, 2, 6, 9	
9		168.0				166.6				166.6		
OMe												
1′		129.3				129.3				128.8		
2′	6.88, brs	114.3	1′, 3′, 4′, 7′	OMe, 9′	6.86, d (2.0)	117.4	3′, 4′		6.97, brs	113.9	1′, 6′, 7′	
3′		148.8				145.6				148.1		
4′		146.6				144.9				146.3		
5′	6.74, overlapped	116.2	3′, 4′		6.75, d (8.0)	116.0	1′, 3′		6.76, d (8.0)	115.6	1′	
6′	6.73, overlapped	123.1	1′, 2′, 7′		6.68, dd (8.0, 2.0)	121.7	4′		6.80, dd (8.0, 2.0)	122.9	1′, 7′	
7′	3.09, dd (14.5, 8.0)	38.1	1′, 2′, 6′, 8′		3.04, dd (13.5, 8.0)	37.5		2′, 6′	3.10, dd (14.0, 8.0)	37.7	1′, 2′, 6′, 8′	
	3.17, dd (14.5, 4.5)		1′, 2′, 6′, 8′		3.14, dd (13.5, 4.0)			2′, 6′	3.20, dd (14.0, 3.5)		1′, 2′, 6′, 8′	
8′	5.25, dd (8.0, 4.5)	74.7	1′, 9, 9′	2′, 6′	5.24, dd (8.0, 4.0)	73.8	1′, 7′, 9, 9′	2′, 6′	5.27, dd (8.0, 3.5)	73.8	1′, 9, 9′	
9′		173.4				171.0				171.0		
OMe	3.83, s	56.5	3′	2′					3.84, s	56.3		2′
1″		128.3				128.5				128.4		
2″	6.83, d (1.5)	115.4	3″, 4″, 6″, 7″	7″, 8″	6.97, brs	115.3	4″		6.97, brs	115.3	1″, 6″, 7″	OMe, 8′
3″		146.2				146.0				145.9		
4″		146.8				146.6				146.5		
5″	6.70, d (8.0)	116.1	1″		6.84, brs	116.1	3″		6.84, brs	116.1	1″, 7″	7″, 8″
6″	6.71, overlapped	120.1	7″		6.84, brs	120.1			6.84, brs	120.0	1″, 2″, 7″	
7″	5.45, d (3.0)	76.9	1″, 2″, 4, 6″, 8″	2″, 6″, 8″	5.32, d (5.0)	76.7		2″, 6″, 8″	5.31, d (4.5)	76.6	1″, 2″, 4, 6″, 8″	2″, 6″
8″	4.95, d (3.0)	76.5	1″, 3, 7″, 9″	7″	4.99, d (5.0)	76.6	3		4.98, d (4.5)	76.6	1″, 3, 7″, 9″	2″, 6″
9″		170.5				168.9				168.8		

Table 1. Continued

	8[a]				9[a]				10[a]			
position	δ_H (J in Hz)	δ_C	HMBC (H to C)	ROE (H to H)	δ_H (J in Hz)	δ_C	HMBC (H to C)	ROE (H to H)	δ_H (J in Hz)	δ_C	HMBC (H to C)	ROE (H to H)
1		129.3				124.4				127.5		
2	7.29, d (2.0)	117.3	1, 3, 4			127.3			7.07, d (2.0)	115.4	3, 4, 6	
3		143.6				148.4				145.6		
4		146.4				144.6				148.8		
5	6.98, d (8.0)	118.6	1, 3		6.89, d (8.5)	118.2	1	6	6.88, d (8.0)	116.5	1, 4	
6	7.23, dd (8.0, 2.0)	123.4	4		7.27, d (8.5)	121.5	2, 8″	5, 7, 8	6.94, dd (8.0, 2.0)	122.8		2, 4
7	7.62, d (16.0)	145.6	1, 2, 6, 9	2, 6	7.85, d (16.0)	143.2	1, 2, 6, 8, 9	6, 8″	7.53, d (16.0)	146.9	1, 2, 6, 9	2, 6
8	6.45, d (16.0)	116.8	1, 9		6.39, d (16.0)	116.6	1, 7, 9		6.12, d (16.0)	114.6	1, 9	
9		166.6				166.6				166.9		
1′		128.9				128.8				126.1		
2′	6.98, d (2.0)	113.9	6′, 8′		6.92, d (2.0)	113.9	6′, 7′	OMe, 7′, 7′, 8′		125.6		
3′		148.1				148.1				148.6		
4′		146.2				146.4				141.2		
5′	6.76, d (8.0)	115.7	1′, 3′		6.75, d (8.0)	115.7	1′		6.75, d (8.0)	117.2	1′, 3′	
6′	6.80, dd (8.0, 2.0)	122.9	2′, 4′, 7′		6.79, dd (8.0, 2.0)	123.0	2′, 7′		6.70, d (8.0)	123.6	2′, 4′, 7′	7′, 8′
7′	3.09, dd (14.0, 8.5)	37.7	1′, 2′, 6′, 8′, 9′	2′, 6′	3.08, dd (14.5, 8.5)	37.7	1′, 2′, 6′, 8′, 9′		3.01, dd (14.5, 7.0)	34.2	1′, 2′, 6′, 9′	
	3.20, dd (14.0, 4.0)		1′, 2′, 6′, 8′, 9′	2′, 6′	3.16, dd (14.5, 4.0)		1′, 2′, 6′, 8′, 9′		3.10, dd (14.5, 4.5)		1′, 2′, 6′, 9′	
8′	5.27, dd (8.5, 4.0)	73.9	1′, 7′, 9, 9′		5.24, dd (8.5, 4.0)	73.9	1′, 9, 9′		5.28, dd (7.0, 4.5)	73.1	1′, 7′, 9, 9′	6′, 7′, 7′, 8″
9′		171.1				171.1				170.8		
OMe	3.84, s	56.4	3′	2′	3.80, s	56.3	3′	1′				
1″		128.1				132.9				134.3		
2″	7.13, d (2.0)	111.9	1″, 3″, 6″, 7″		7.09, d (2.0)	110.4	1″, 6″	OMe, 7″, 8″	6.90, d (2.0)	113.4	6″, 7″	
3″		148.5				148.5				146.0		
4″		148.1				147.8				145.8		
5″	6.83, d (8.0)	115.6	1″, 3″, 4″, 6″		6.84, d (8.0)	115.9	1″, 7″		6.82, d (8.0)	116.3	1″	
6″	6.95, dd (8.0, 2.0)	121.3	2″, 4″		6.91, dd (8.0, 2.0)	119.4	2″		6.74, dd (8.0, 2.0)	118.3	2″, 7″	
7″	5.35, d (5.0)	76.8	1″, 2″, 4, 8″	2″, 6″	6.02, d (5.0)	88.3	1″, 2, 2″, 3, 6″, 8″, 9″	2″, 6″	5.78, d (5.0)	87.8	1″, 2′, 2″, 3′, 6″, 9″	2″, 6″
8″	5.05, d (5.0)	76.7	3, 7″, 9″		4.57, d (5.0)	56.7	1, 1″, 2, 3, 7″, 9″		4.53, d (5.0)	57.3	1″, 2′, 3′, 9″	
9″		168.9				172.8				172.3		
OMe	3.83, s	56.3	3″	2″	3.82, s	56.4	3″					
1‴										128.7		
2‴									6.79, d (2.0)	117.2	6‴, 7‴	
3‴										145.9		
4‴										144.8		
5‴									6.72, d (8.0)	116.1	1‴	
6‴									6.57, dd (8.0, 2.0)	121.9	2‴, 4‴, 7‴	
7‴									3.02, dd (14.5, 8.0)	37.1	1‴, 2‴, 6‴, 9‴	
									3.15, dd (14.5, 4.5)		1‴, 2‴, 6‴, 9‴	
8‴									5.33, dd (8.0, 4.5)	74.6	7‴, 9″, 9‴	2‴, 6‴, 8″
9‴										170.5		

[a] In acetone-d_6. [b] In methanol-d_4.

Table 2. NMR Data (400 MHz, Acetone-d_6) for (*S*)- and (*R*)-PGME Amide of Clinopodic Acid I (**10**)

(*S*)-PGME amide	(*R*)-PGME amide	$\delta_{(S)\text{-PGME amide}} - \delta_{(R)\text{-PGME amide}}$
H-7′ and H-7″ δ_H (J in Hz)	H-7′ and H-7″ δ_H (J in Hz)	
3.007, 2H, dd (14.5, 7.5)	2.880, 1H, dd (14.5, 7.0)	+
	2.932, 1H, dd (14.0, 8.0)	+
3.130, 1H, dd (14.5, 5.5)	3.078, 1H, dd (14.0, 4.5)	+
3.151, 1H, dd (14.5, 4.5)	3.108, 1H, dd (14.5, 5.5)	+

Table 3. MMP-2 Inhibitory Activity of Compounds **1**−**4**, **6**, **7**, and **9**, Methyl Ester of **1**, and Lithospermic Acid

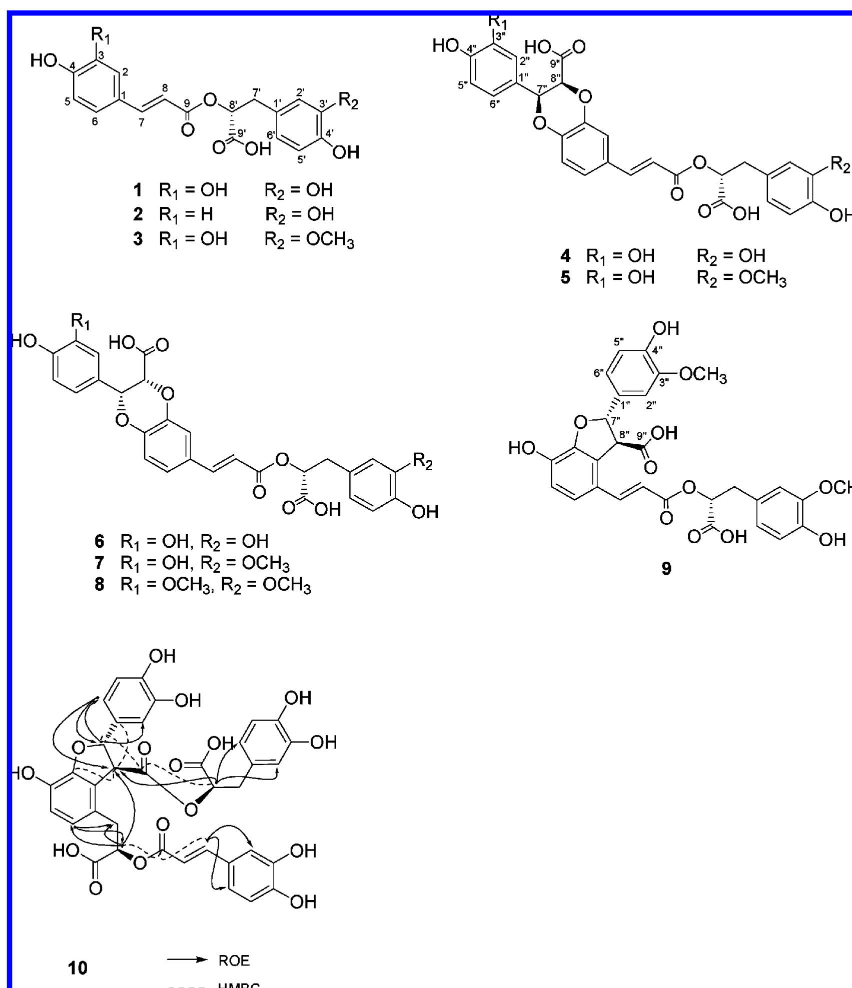
compound	IC_{50} (μM)	compound	IC_{50} (μM)
1	27.2	**6**	>100
methyl ester of **1**	>100	**7**	>100
2	>100	**9**	>100
3	>100	lithospermic acid	10.2
4	3.26		

Alkaline Hydrolysis of Rosmarinic Acid (1). Rosmarinic acid (**1**) (100 mg) was dissolved in 10% NaOH (5 mL), and the mixture was stirred for 1 h at room temperature under N_2. The reaction mixture was passed through an Amberlite IR-120B column (2 × 10 cm) and eluted with H_2O (100 mL). The eluate was concentrated under reduced pressure to give a brown residue (60 mg). The eluate was purified by HPLC [column, Inertsil ODS-3, 3 × 50 cm; solvent, H_2O-CH_3CN (93:7) + 0.1% TFA; flow rate, 9.5 mL/min; 280 nm) to give (2*R*)-3-(3,4-dihydroxyphenyl)-2-hydroxypropanoic acid (12 mg) as a colorless, amorphous solid: $[\alpha]^{23}_D$ +13.9 (*c* 2.54, acetone); ^{1}H NMR (acetone-d_6) δ 2.78 (1H, dd, J = 14.0, 7.5 Hz), 2.97 (1H, dd, J = 14.0, 4.5 Hz), 4.31 (1H, dd, J = 7.5, 4.5 Hz), 6.60 (1H, dd, J = 8.0, 2.0 Hz), 6.71 (1H, d, J = 8.0 Hz), 6.79 (1H, d, J = 2.0 Hz).

Condensation of the Acid Moiety of Rosmarinic Acid (1) with Phenylglycine Methyl Ester. The acid moiety (5 mg) and (*S*)-phenylglycine methyl ester (PGME) (10 mg) were dissolved in dry DMF (0.5 mL), and to the solution were added benzotriazol-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP) (20 mg), 1-hydroxybenzotriazole (HOBT) (7 mg), and *N*-methylmorpholine (30 μL). The reaction mixture was stirred overnight. EtOAc (2 mL) was added, and resulting diluted solution was successively washed with dilute HCl, saturated $NaHCO_3$, and saturated NaCl solutions. The EtOAc layer was separated by HPLC [column, TOSOH TSKgel ODS-80Ts, 0.46 × 25 cm; H_2O-CH_3CN (80:20) + 0.05% TFA; flow rate, 1 mL/min; detector, UV 280 nm] to give (*S*)-amide (0.5 mg) as a colorless, amorphous solid, t_R 25.5 min; ^{1}H NMR (acetone-d_6) δ 2.682 (1H, dd, J = 14.0, 7.5 Hz), 2.992 (1H, dd, J = 14.0, 3.5 Hz), 4.234 (1H, dd, J = 7.5, 3.5 Hz), 5.52 (1H, m), 6.58 (1H, dd, J = 8.0, 2.0 Hz), 6.70 (1H, d, J = 8.0 Hz), 6.76 (1H, d, J = 2.0 Hz), 7.38 (5H, m). By the same method, (*R*)-amide (0.5 mg) was obtained as a colorless, amorphous solid, t_R 27.4 min; ^{1}H NMR (acetone-d_6) δ 2.688 (1H, dd, J = 14.0, 8.0 Hz), 2.952 (1H, dd, J = 14.0, 3.5 Hz), 4.274 (1H, dd, J = 7.5, 3.5 Hz), 5.50 (1H, m), 6.64 (1H, dd, J = 8.0, 2.0 Hz), 6.67 (1H, d, J = 8.0 Hz), 6.74 (1H, d, J = 2.0 Hz), 7.27−7.38 (5H, m).

Alkaline Hydrolysis of Clinopodic Acids C (4) and E (6) and Condensation with (S)-PGME. Each compound (1 mg) was dissolved in 10% NaOH solution (1 mL), and the solution was stirred for 1 h at

Chart 1



room temperature under N_2. The reaction mixture was passed through an Amberlite IR-120B column (15 × 40 mm) and eluted with H_2O (30 mL). The eluate was concentrated under reduced pressure to give a brown residue (0.5 mg). The residue (0.5 mg) was treated the same as above. The EtOAc layer was analyzed by HPLC [column, TOSOH TSKgel ODS-80Ts, 0.46 × 25 cm; solvent, H_2O-CH_3CN (80:20) + 0.05% TFA; flow rate, 1 mL/min; 280 nm] to give amide peak, t_R 25.5 min.

Alkaline Hydrolysis of Clinopodic Acid H (9) and Condensation with (*S*)- and (*R*)-PGME. Clinopodic acid H (**9**) (50 mg) was dissolved in 10% NaOH (0.5 mL), and the mixture was stirred for 5 h at room temperature under N_2. The reaction mixture was passed through an Amberlite IR-120B column (1 × 15 cm) and eluted with H_2O (100 mL). The eluate was concentrated under reduced pressure to give a brown residue (50 mg). The eluate was purified by HPLC (column, Cosmosil $5C_{18}$-AR-II, 2 × 25 cm; solvent, H_2O-CH_3CN (85:15) + 0.05% TFA; flow rate, 6.5 mL/min; detector, UV 280 nm) to give (2*R*)-3-(3-methoxy-4-hydroxyphenyl)-2-hydroxypropanoic acid (15 mg) as a colorless, amorphous solid, $[\alpha]^{23}_D$ +25.8 (*c* 1.50, acetone); ^{1}H NMR (acetone-d_6) δ 2.84 (1H, dd, J = 14.0, 7.5 Hz), 3.02 (1H, dd, J = 14.0, 4.5 Hz), 3.82 (3H, s), 4.34 (1H, dd, J = 7.5, 4.5 Hz), 6.72 (2H, brs), 6.90 (1H, brs), and 4-(2-carboxyethenyl)-2-(3-methoxy-4-hydroxyphenyl)-2,3-dihydro-7-hydroxy-3-benzofurancarboxylic acid (17 mg), as a colorless, amorphous solid, $[\alpha]^{23}_D$ +173.9 (*c* 1.66, acetone); ^{1}H NMR (acetone-d_6) δ 3.83 (3H, s), 4.56 (1H, d, J = 5.5 Hz), 6.01 (1H, d, J = 5.5 Hz), 6.32 (1H, d, J = 16.0 Hz), 6.83 (1H, d, J = 8.0 Hz), 6.89 (1H, d, J = 8.0 Hz), 6.90 (1H, brs), 6.91 (1H, dd, J = 8.0, 2.0 Hz), 7.10 (1H, d, J = 2.0 Hz), 7.25 (1H, d, J = 8.0 Hz), 7.80 (1H, d, J = 16.0 Hz); ^{13}C NMR (acetone-d_6) δ 56.4, 56.7, 88.3, 110.4, 115.8, 117.6, 118.1, 119.5, 121.3, 124.7, 127.2, 132.9, 142.5, 144.3, 147.7, 148.4, 148.5, 167.9, 172.9.

(2*R*)-3-(3-Methoxy-4-hydroxyphenyl)-2-hydroxypropanoic acid (5 mg) was condensed with (*S*)-PGME and (*R*)-PGME by the same method as for (2*R*)-3-(3,4-dihydroxyphenyl)-2-hydroxypropanoic acid and gave (*S*)-amide, t_R 37.2 min, and (*R*)-amide, t_R 38.8 min, in HPLC analysis [column, TOSOH TSKgel ODS-80Ts, 0.46 × 25 cm; solvent, H_2O-CH_3CN (77:23) + 0.05% TFA; flow rate, 1 mL/min; detector, UV 280 nm].

Alkaline Hydrolysis of Clinopodic Acids D and F−H (5, 7−9) and Condensation with (*S*)-PGME. Each compound (1 mg) was treated as for the clinopodic acids C (**4**) and E (**6**) to give (*S*)-amide, t_R 37.2 min, in the HPLC analysis [column, TOSOH TSKgel ODS-80Ts, 0.46 × 25 cm; solvent, H_2O-CH_3CN (77:23) + 0.05% TFA; flow rate, 1 mL/min; detector, UV 280 nm].

Condensation of Clinopodic Acid I (10) with (*S*)-PGME. To a stirred solution of a mixture of clinopodic acid I (**10**) (5.3 mg) and (*S*)-PGME (12.8 mg) in DMF (700 μL) was successively added benzotriazol-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP, 11.1 mg), 1-hydroxybenzotriazole (HOBT, 7.4 mg), and *N*-methylmorphorine (50 μL) at 0 °C. After the mixture was stirred at room temperature overnight, EtOAc (20 mL) was added, and the resulting diluted solution was successively washed with dilute HCl, saturated $NaHCO_3$, and saturated NaCl solutions. The EtOAc layer was dried and concentrated to give a residue, which was purified by preparative HPLC [column, YMC-ODS 1 × 25 cm; solvent, H_2O-CH_3CN (72.5:37.5); flow rate, 3.5 mL/min; detector, UV 280 nm] to give (*S*)-PGME amide (2.3 mg): ^{1}H NMR (acetone-d_6) δ 3.007 (2H, dd, J = 14.5, 7.5 Hz, H-7′ or H-7″), 3.130 (1H, dd, J = 14.5, 5.5 Hz, H-7′ or H-7″), 3.151 (1H, dd, J = 14.5, 4.5 Hz, H-7′ or H-7″), 5.402 (1H, dd, J = 7.5, 4.5 Hz, H-8′ or H-8″), 5.422 (1H, dd, J = 7.5, 5.5 Hz, H-8′ or H-8″).

Condensation of Clinopodic Acid I (10) with (*R*)-PGME. Clinopodic acid I (**10**) (5.4 mg) was treated with (*R*)-PGME (12.9 mg) by the procedure described above to obtain the (*R*)-PGME amide (2.4 mg) as a colorless, amorphous solid: ^{1}H NMR (acetone-d_6) δ 2.880 (1H, dd, J = 14.5, 7.0 Hz, H-7′ or H-7″), 2.932 (1H, dd, J = 14.0, 8.0 Hz, H-7′ or H-7″), 3.078 (1H, dd, J = 14.0, 4.5 Hz, H-7′ or H-7″), 3.108

(1H, dd, $J = 14.5, 5.5$ Hz, H-7′ or H-7″), 5.387 (1H, dd, $J = 7.0, 5.5$ Hz, H-8′ or H-8″), 5.427 (1H, dd, $J = 8.0, 4.5$ Hz, H-8′ or H-8″).

Preparation of Tissue Homogenate. Male Wistar rats age 9−10 weeks and weighing 200−220 g (Nihon SLC, Hamamatsu, Japan) were housed in groups of two in stainless-steel cages with free access to food (Japan CLEA; CE-2, Tokyo, Japan) and H_2O. They were kept in a room maintained at ambient temperature and humidity (25 ± 5 °C, 55 ± 5%) under a day/night regime (day 7:00−19:00 and night 19:00−7:00). All animals were maintained in the laboratory for a minimum of 1 week prior to the start of the experiment. Lung was collected from nine naive rats by decapitation followed by immediately freezing in liquid nitrogen and maintained at −80 °C until use. Animal treatment and maintenance were conducted in accordance with the Guidelines for Animal Experimentation of Tohoku Pharmaceutical University, Japan.

Lung tissue was homogenized with five volumes of homogenate medium composed of 50 mM Tris-HCl buffer, pH 7.5, at 4 °C followed by centrifuge (9000*g*, 4 °C, 20 min) to obtain a supernatant fraction as an enzyme source maintained as aliquots for repeated experiments at −80 °C until use.

SDS-PAGE Gelatin Zymography. Substrate gelatin-gel zymography was performed by nonreducible SDS-PAGE conditions with modification to the literature.[17] Briefly, a lung 9000*g* supernatant fraction was activated by incubation at 37 °C for 1 h with 0.5 mM APMA in a buffer containing 50 mM Tris-HCl, pH 7.5, and 50 mM NaCl. The protein content was measured by the method of Bradford,[18] and equal amounts (0.5 mg/mL) of protein were used. The effect of acid derivatives on gelatinolytic activity was tested as follows. The activated supernatant fraction was incubated with 10 μL of each acid solution and 5 μL of Tris-HCl, pH 7.5, for 30 min at 37 °C. The final concentration of each acid derivative was 5−100 μM prepared in DMSO solution (5%). Thereafter, these were resuspended in laemmli sample buffer with the exception of 2-mercaptoethanol and boiling followed by separation with SDS-PAGE gel containing 1.0 mg/mL of gelatin. EDTA at 10 mM was used as a control for inhibition. The electrophoresis was carried out by loading onto 7.5% acrylamide/bisacrylamide (29:1) separating gel and applied for SDS-PAGE performed with a Mini-Protein III apparatus (Bio-Rad, Hercules, CA). Electrophoresis was carried out with molecular mass maker of myosin (200 kDa), β-galactosidase (116 kDa), and bovine serum albumin (66 kDa), at a constant voltage of 200 V for 40 min. Proteins were stained by Rapid Stain Coomassie Brilliant Blue kit (Nacalai Tesque, Kyoto, Japan). After electrophoresis, the gel was soaked in 0.25% Triton X-100 (30 min twice) at room temperature and rinsed in H_2O following incubation at 37 °C for 18 h in the incubation buffer containing 50 mM Tris-HCl (pH 7.6), 20 mM NaCl, 5 mM $CaCl_2$, and 0.02% Brij-58. The gel was then stained with Rapid Stain Coomassie Brilliant Blue kit. The inhibitory activities are collected as the % of EDTA-control value. Each experiment was performed independently in triplicate, and the data are indicated as mean ($n = 3$), followed by calculated IC_{50} values (μM).

Quantification of Gelatinolytic Activity by SDS-PAGE. The SDS-PAGE gel was digitized using a PC scanner (Epson ES-2200, Epson Co. Ltd., Nagano, Japan) operating on the image acquisition and analysis program L Process V2.0 and Image Gauge V4.0 (Fuji Photofilm Co., Ltd., Tokyo, Japan).

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NP800781T