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Diastereomers of lithospermic acid and lithospermic acid B from *Monarda fistulosa* and *Lithospermum erythrorhizon*

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ABSTRACT

Monardic acids A (**1**) and B (**2**), which are (7*R*,8*R*) diastereomers of lithospermic acid (LA) and lithospermic acid B, respectively, were isolated from *Monarda fistulosa*. A (7*S*,8*R*) isomer (**3**) of LA was also isolated from this plant, and a (7*R*,8*S*) isomer (**7**) of LA was obtained from *Lithospermum erythrorhizon*. The absolute configuration of **1** was confirmed by analysis of its hydrolysates, 7-epiblechnic acid and 2*R*-3-(3,4-dihydroxyphenyl)-2-hydroxypropanoic acid. The configuration in the dihydrobenzofuran moieties of **2**, **3**, and **7** was extrapolated by using the phenylglycine methyl ester method and a Cotton effect at approximately 250–260 nm in their electronic circular dichroism spectra. Diastereomers (**1**–**3** and **7**) displayed moderate hyaluronidase inhibitory and histamine release inhibitory activities.

1. Introduction

Lithospermic acid (LA) [1,2] and lithospermic acid B (LAB) [3,4] are major phenylpropanoid oligomers present in various Lamiaceae and Boraginaceae plants. LA and LAB are the bioactive components in medicinal herbs, including sage (*Salvia officinalis* L.), danshen (*S. miltiorrhiza* Bunge), and shikon (*Lithospermum erythrorhizon* Siebold et Zuccarini). Previously, LA and LAB have been shown to possess antioxidative properties and biological activities, including HIV-1 integrase inhibition, hyaluronidase inhibition, aldose reductase inhibition, and improvement of uremic symptoms, resulting in significant decreases in blood urea nitrogen, creatinine, methylguanidine, and guanidinosuccinic acid levels [5–8]. Furthermore, the entire synthesis route of LA has been previously demonstrated [9–12].

The dihydrobenzofuran moiety, with the (7*S*,8*S*) configuration, is a common structural feature in both LA and LAB [1,2,4]. To date, the (7*R*,8*R*) isomers of LA and LAB have not been reported as natural products. Although salvianolic acid B was initially assigned a (7*R*,8*R*) configuration [3], Watzke et al. reassigned its absolute configuration as (7*S*,8*S*) and demonstrated that it was identical to LAB [13].

Monarda fistulosa L. (Lamiaceae) is used in traditional medicines, and Native Americans have been known to use it for treating vomiting, headaches, and rheumatic pains [14,15]. Herein, we report the isolation of monardic acids A, B, and C (**1–3**) together with rosmarinic acid (**4**) from the acetone–H_2O (8:2) extract of whole *M. fistulosa* plants. In order to compare the steric structures of the isolates (**1–3**), we isolated LA (**5**), LAB (**6**), and compound **7** from the acetone–H_2O (8:2) extract of the roots of *L. erythrorhizon*

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(Boraginaceae). Compounds **1** and **2** have a *trans*-oriented dihydrobenzofuran moiety similar to that in LA and LAB; **3** and **7** are *cis* isomers of LA (Fig. 1).

Alkaline hydrolysis of **1** gave **8** and **9**, and the absolute configuration of **1** was determined by identification of the hydrolysates. On the other hand, it is known that the phenylglycine methyl ester (PGME) method and electronic circular dichroism (ECD) are available for determining the absolute configuration of chiral compounds. It was confirmed that the PGME method was applicable for determinating the configuration of C-8 in the dihydrobenzofuran based on examples applied to **1** (8*R*) and **5** (8*S*). The absolute configurations at C-7 and C-8 in the dihydrobenzofuran moieties of **2**, **3**, and **7** were extrapolated using the PGME method and ECD spectra.

Isolated diastereomers (**1–3** and **7**) were expected to show biological activities similar to those of LA and LAB. The hyaluronidase inhibitory activity and histamine release inhibitory activity of these diastereomers were evaluated in order to identify potential anti-allergic and anti-inflammatory agents [6].

2. Experimental

2.1. General experimental procedures

Optical rotations were measured on a JASCO P-2300 polarimeter. ECD spectra were recorded on a JASCO J-700 spectropolarimeter. A JEOL JNM-AL400 spectrometer was used to record ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra, and chemical shifts are given as δ values with TMS as an internal standard at 30 °C. Inverse-detected heteronuclear correlations were measured using HMQC (optimized for $^{1}J_{C-H} = 145$ Hz) and HMBC (optimized for $^{n}J_{C-H} = 8$ Hz) pulse sequences with a pulsed field gradient. HRFABMS data were obtained on a JEOL JMS700 mass spectrometer by using *m*-nitrobenzyl alcohol or glycerol matrix. Preparative HPLC was performed on a JASCO 2089 instrument (detector: UV 210 nm).

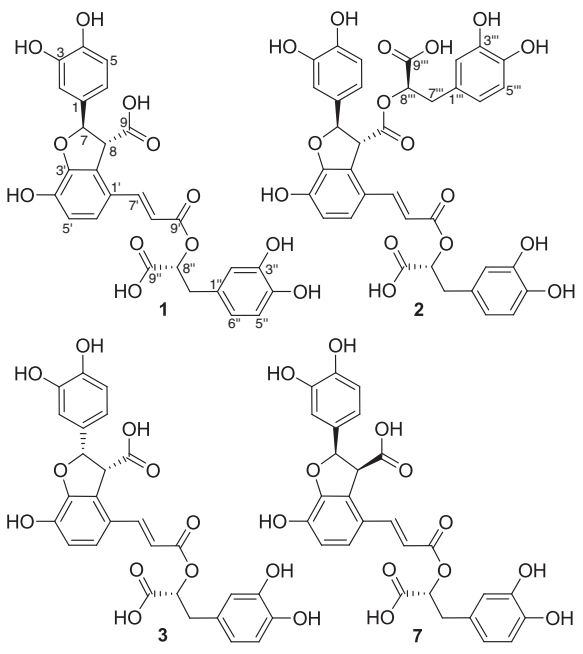


Fig. 1. Structures of **1–3** and **7.**

2.2. Plant materials

Monarda fistulosa L. was harvested from the medicinal plant garden of Tohoku Pharmaceutical University, Sendai, Japan. The young plants were purchased from Tamagawa engei, Nagano, Japan. The plant was identified according to advice from Dr. Goro Kokata, the Plant Identification Unit of the National Museum of Nature and Science, Tsukuba Botanical Garden, Tsukuba, Japan [14–16]. A voucher specimen was deposited at the herbarium of Tohoku Pharmaceutical University, No. 20081101. Dried roots of *Lithospermum erythrorhizon* Siebold et Zuccarini were purchased from Tochimoto Tenkaido Co., Ltd., Osaka, Japan, as a medicinal Shikon, which was identified according to microscopic observation of the roots by the company [17]. A voucher specimen was deposited at the herbarium of Tohoku Pharmaceutical University, No. 20120803.

2.3. Extraction and isolation

Whole plants (980 g) of *M. fistulosa* were extracted with acetone–H_2O (8:2) (2 × 10 L) at 60 °C. The extract (124.5 g) was concentrated at reduced pressure, suspended in H_2O (1.5 L), and extracted with Et_2O (3 × 1 L). The aqueous extract (78.4 g) was a red–brown syrup. It was dissolved in H_2O, and the aqueous solution was passed through a porous polymer gel column (Mitsubishi Diaion HP-20, 70 × 180 mm) and eluted with H_2O, 10, 80% MeOH, and MeOH. The 80% MeOH eluate (22.4 g) was chromatographed on a reversed-phase column using ODS (Cosmosil 140C_{18}-OPN, 150 g; Nacalai Tesque) and was eluted with 20, 40, 60, and 80% MeOH (fractions 1A–1D). Fraction 1A (16.4 g) was subjected to preparative column chromatography [Yamazen, Ultra Pack ODS-SM-50C-M, 37 × 100 mm; solvent, MeOH–H_2O containing 0.2% TFA (20:80)–(40:60); detector, UV 210 nm] to give 16 fractions (Frs. 2A–2P). Fractions 2 F, 2G, and 2H (755 mg) were subjected to preparative HPLC [columns, Cosmosil, AR-II, 20 × 250 mm; solvent, CH_3CN–H_2O containing 0.2% TFA; Cosmosil, 5PE-MS, 20 × 250 mm; solvent, CH_3CN–H_2O containing 0.2% TFA (25:75)] to yield compounds **1** (261.2 mg) and **4** (23.1 mg). Fractions 2I and 2 J (767 mg) were subjected to preparative HPLC [columns, Cosmosil, AR-II, 20 × 250 mm; solvent, CH_3CN–H_2O containing 0.2% TFA (20:80); Cosmosil, 5PE-MS, 20 × 250 mm; solvent, CH_3CN–H_2O containing 0.2% TFA (25:75); and Kanto Chemical, Mightysil RP-18 GP, 10 × 250 mm; solvent, CH_3CN–H_2O containing 0.2% TFA (25:75)] to yield compounds **2** (5.3 mg) and **3** (11.2 mg).

The roots (480 g) of *L. erythrorhizon* were extracted with acetone–H_2O (8:2) (4 × 3 L) at room temperature for 1 week. The extract (236.0 g) was concentrated at reduced pressure, suspended in H_2O (1.5 L), and extracted with Et_2O (3 × 1 L). The aqueous extract (206.6 g) was a black syrup. It was dissolved in H_2O, and the aqueous solution was passed through a porous polymer gel column (Mitsubishi Diaion HP-20, 70 × 180 mm) and eluted with H_2O, 10, 20, 40, 60, 80% MeOH, and MeOH. The 80% MeOH eluate (1.38 g) was subjected to preparative column chromatography [Yamazen, Ultra Pack ODS-SM-50C-M, 37 × 100 mm; solvent, MeOH–

H_2O containing 0.2% TFA (20:80)–(30:70); detector, UV 210 nm] to give nine fractions (Frs. 3A–3I). Fractions 3D, 3E, and 3 F (375 mg), which were subjected to preparative HPLC [columns, Cosmosil, AR-II, 20 × 250 mm; solvent, CH_3CN–H_2O containing 0.2% TFA (25:75); Cosmosil, 5PE-MS, 20 × 250 mm; solvent, CH_3CN–H_2O containing 0.2% TFA (25:75); and Kanto Chemical, Mightysil RP-18 GP, 10 × 250 mm; solvent, CH_3CN–H_2O containing 0.2% TFA (25:75)] to yield compounds **5** (234.6 mg), **6** (27.6 mg), and **7** (5.3 mg).

*2.3.1. Monardic acid A (**1**)*

Colorless amorphous solid; $[\alpha]^{25}_D$ −86.2 (*c* 2.49, MeOH); UV (MeOH) λ_{max} (log ε) 256 (4.28), 291 (4.24), 311 (4.26); ECD (*c* 0.05, MeOH) nm ([θ]) 230 (−52,700), 252 (−75,100), 288 (5700), 333 (−5400) nm; ^{1}H NMR (CD_3OD, 400 MHz) and ^{13}C NMR: (CD_3OD, 100 MHz) see Table 1; HRFABMS (positive) *m/z* 539.1196 $[M + H]^+$ (Calcd for $C_{27}H_{23}O_{12}$: 539.1189).

*2.3.2. Monardic acid B (**2**)*

Colorless amorphous solid; $[\alpha]^{24}_D$ −43.5 (*c* 0.46, MeOH); UV (MeOH) λ_{max} (log ε) 254 (4.25), 288 (4.26), 307 (4.19); ECD (*c* 0.05, MeOH) nm ([θ]) 234 (−49,100), 252 (−67,000), 289 (20,100), 338 (−3500) nm; ^{1}H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz) see Table 1; HRFABMS (positive) *m/z* 741.1429 $[M + Na]^+$ (Calcd for $C_{36}H_{30}O_{16}Na$: 741.1430).

*2.3.3. Monardic acid C (**3**)*

Colorless amorphous solid; $[\alpha]^{25}_D$ +41.4 (*c* 0.88, MeOH); UV (MeOH) λ_{max} (log ε) 256 (4.19), 290 (4.18), 313 (4.21); ECD (*c* 0.05, MeOH) nm ([θ]) 233 (−72,500), 254 (61,900), 278 (6700), 298 (39,900) nm; ^{1}H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz) see Table 1; HRFABMS (positive) *m/z* 561.1012 $[M + Na]^+$ (Calcd for $C_{27}H_{22}O_{12}Na$: 561.1008).

*2.3.4. Lithospermic acid C (**7**)*

Colorless amorphous solid; $[\alpha]^{25}_D$ +40.7 (*c* 0.54, MeOH); UV (MeOH) λ_{max} (log ε) 257 (4.21), 290 (4.19), 311 (4.23); ECD (*c* 0.05, MeOH) nm ([θ]) 230 (99,800), 255 (−46,600), 291 (−7700), 336 (11,000) nm; ^{1}H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz) see Table 1; HRFABMS (positive) *m/z* 561.1012 $[M + Na]^+$ (Calcd for $C_{27}H_{22}O_{12}Na$: 561.1008).

*2.4. Alkaline hydrolysis of compounds **1–3** and **7**, and condensation with (S)-PGME*

Each compound [compounds **1** (20.0 mg), **2**, **3**, and **7** (each 1.0 mg)] was dissolved in 10% NaOH (0.5 mL) and stirred for 2 h. The reaction mixtures were passed through a Dowex 50 W × 2 column (1.0 × 7.0 cm) and eluted with H_2O (100 mL). The residue from **1** was subjected to HPLC [column, Mightysil RP18-GP (10 × 250 mm); solvent, CH_3CN–H_2O containing 0.2% TFA (20:80); flow rate, 0.8 mL/min; detector, UV 210 nm] to yield 7-epiblechnic acid (**8**: 1.9 mg) and (2*R*)-3-(3,4-dihydroxyphenyl)-2-hydroxypropanoic acid (**9**: 4.6 mg) [18,19]. The residues from **2**, **3**, **7** and **9** from **1** were dissolved in DMF and (*S*)-PGME, benzotriazol-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP), 1-hydroxybenzotriazole (HOBT), and *N*-methylmorpholine were added as reported previously [18]. The mixtures were then stirred for 10 h at room temperature to give (*S*)-PGME amide; t_R = 33.9 min in the HPLC analysis [column, Mightysil RP18-GP (6.0 × 250 mm); solvent, CH_3CN–H_2O containing 0.2% TFA (25:75); flow rate, 0.8 mL/min; detector, UV 210 nm]. The retention time of (*S*)-PGME amide of (2*R*)-3-(3,4-dihydroxyphenyl)-2-hydroxypropanoic acid was 33.9 min and that of (*R*)-PGME amide of (2*R*)-3-(3,4-dihydroxyphenyl)-2-hydroxypropanoic acid was 34.7 min, which corresponded with authentic samples.

*2.4.1. 7-Epiblechnic acid (**8**)*

Colorless amorphous solid; $[\alpha]^{24}_D$ −144.0(*c* 0.18, MeOH); ECD (*c* 0.02, MeOH) nm ([θ]) 230 (−32,000), 250 (−44,400), 283 (2100), 326 (−9900) nm; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.77 (1H, d, *J* = 16.0 Hz), 7.16 (1H, d, *J* = 9.0 Hz), 6.79 (1H, d, *J* = 2.0 Hz), 6.79 (1H, d, *J* = 9.0 Hz), 6.75 (1H, d, *J* = 8.0 Hz), 6.72 (1H, dd, *J* = 8.0, 2.0 Hz), 6.26 (1H, d, *J* = 16.0 Hz), 5.87 (1H, d, *J* = 5.0 Hz), 4.30 (1H, d, *J* = 5.0 Hz); FABMS (positive) *m/z* 359 $[M + H]^+$.

*2.5. (S)-PGME and (R)-PGME amides of compounds **1**, **3**, **5**, and **7** for determining the configuration of C-8*

Separately, (*S*)- or (*R*)-PGME (20 mg) was added to **1** and **5** (10 mg each) in DMF (0.8 mL), and then PyBOP (20 mg), HOBT (20 mg), and *N*-methylmorpholine (20 μL) were added, and the mixtures were stirred for 10 h at room temperature. The reactions produced the (*S*)-PGME amide of **1** (**10**: 11.5 mg) and the (*R*)-PGME amide of **1** (**11**: 8.1 mg) and the (*S*)-PGME amide of **5**: (**12**: 7.0 mg) and the (*R*)-PGME amide of **5** (**13**: 7.0 mg), respectively. Separately, (*S*)- or (*R*)-PGME (20 mg) was added to **3** (2.0 mg) and **7** (1.0 mg) in DMF (0.8 mL), and then PyBOP (8 mg), HOBT (8 mg), and *N*-methylmorpholine (20 μL) were added, and the mixtures were stirred for 10 h at room temperature. The reactions of **3** produced the (*S*)-PGME amide: **12** (1.2 mg) and **14** (0.8 mg) and the (*R*)-PGME amide: **13** (1.2 mg) and **15** (0.4 mg), respectively. The reactions of **7** produced the (*S*)-PGME amide (**10**: 0.6 mg) and the (*R*)-PGME amide (**11**: 0.4 mg), respectively.

*2.5.1. (S)-PGME amide of **1** (**10**)*

Colorless amorphous solid; $[\alpha]^{24}_D$ −63.5 (*c* 0.34, MeOH); UV (MeOH) λ_{max} (log ε) 256 (4.31), 290 (4.28), 314 (4.29); ECD (*c* 0.02, MeOH) nm ([θ]) 218 (96,500), 253 (−43,300), 344 (−7600) nm; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.77 (1H, d, *J* = 16.0 Hz), 7.27–7.40 (8H, overlapping), 7.21 (2H, overlapping), 7.19 (1H, d, *J* = 8.5 Hz), 6.81 (1H, d, *J* = 8.5 Hz), 6.78 (1H, d, *J* = 2.0 Hz), 6.72 (1H, d, *J* = 8.0 Hz), 6.70 (1H, d, *J* = 2.0 Hz), 6.61 (1H, d, *J* = 8.0 Hz), 6.61 (1H, dd, *J* = 8.0, 2.0 Hz), 6.53 (1H, dd, *J* = 8.0, 2.0 Hz), 6.39 (1H, d, *J* = 16.0 Hz), 5.64 (1H, d, *J* = 6.0 Hz), 5.48 (1H, dd, *J* = 3.5, 3.0 Hz), 5.45 (1H, dd, *J* = 3.5, 3.0 Hz), 5.35 (1H, t, *J* = 6.5 Hz), 4.53 (1H, d, *J* = 6.0 Hz), 3.66 (3H, s), 3.54 (3H, s), 2.98 (2H, d, *J* = 6.5 Hz); FABMS (positive) *m/z* 833 $[M + H]^+$, 855 $[M + Na]^+$.

*2.5.2. (R)-PGME amide of **1** (**11**)*

Colorless amorphous solid; $[\alpha]^{24}_D$ −126.9 (*c* 0.35, MeOH); UV (MeOH) λ_{max} (log ε) 257 (4.25), 289 (4.39),

Table 1
1H and 13C NMR spectroscopic data (methanol-d_4) for compounds **1–3** and **7.**

	1			2			3			7		
Position	δ_H (J in Hz)	δc	HMBC (H to C)	δ_H (J in Hz)	δc	HMBC (H to C)	δ_H (J in Hz)	δc	HMBC (H to C)	δ_H (J in Hz)	δc	HMBC (H to C)
1		133.7			133.9			129.3			129.4	
2	6.80 (d, 2.0)	113.5	7	6.75 (d, 2.0)	113.3	4,6,7	6.98 (d, 2.0)	115.1	1,6,7	6.98 (d, 2.0)	115.1	4,7
3		146.6			146.2			146.1			146.0	
4		146.7			146.7			146.5			146.5	
5	6.77 (d, 8.5)	116.4	3	6.74 (d, 8.0)	116.4		6.76 (d, 8.0)	115.9	1,3	6.76 (d, 8.0)	115.9	1,3
6	6.73 (dd, 8.5, 2.0)	118.3	4,7	6.62 (dd, 8.0 2.0)	118.4	2,7	6.86 (dd, 8.0, 2.0)	119.6	4,7	6.86 (dd, 8.0, 2.0)	119.6	4,7
7	5.90 (d, 4.5)	88.8	1,2,6,8,9,2',3'	5.85 (d, 4.5)	88.2	1,2,6,8,9,2',3'	5.94 (d, 9.5)	88.4	1,2,6,8,9,2',3'	5.94 (d, 9.5)	88.4	1,2,6,8,9,2',3'
8	4.36 (d, 4.5)	57.5	1,7,9,1',2',3'	4.42 (d, 4.5)	57.3	1,7,9,1',2',3'	4.61 (d, 9.5)	55.2	1,7,9,2',3'	4.63 (d, 9.5)	55.4	1,7,9,2',3'
9		175.1			172.5			173.6			173.8	
1'		124.6			124.9			124.3			124.3	
2'		127.6			126.3			129.3			129.4	
3'		148.8			149.1			149.6			149.6	
4'		145.2			145.3			145.3			145.2	
5'	6.81 (d, 8.5)	118.3	1'	6.82 (d, 8.5)	118.5	1',3'	6.81 (d, 8.5)	118.1	1',3'	6.81 (d, 8.5)	118.1	1',3'
6	7.21 (d, 8.5)	121.7	2',4',7'	7.19 (d, 8.5)	122.0	2',4',7'	7.15 (d, 8.5)	122.6	2',4',7'	7.15 (d, 8.5)	122.7	2',4',7'
7'	7.81 (d, 16.0)	144.0	1',2',6',9'	7.69 (d, 16.0)	144.3	1',2',6',9'	7.58 (d, 16.0)	143.8	1',2',6',9'	7.61 (d, 16.0)	143.8	1',2',6',9'
8'	6.34 (d, 16.0)	116.4	1',9'	6.30 (d, 16.0)	116.6	1',9'	6.30 (d, 16.0)	116.4	1',9'	6.32 (d, 16.0)	116.4	1',9'
9'		168.2			168.2			168.1			168.0	
1''		129.3			129.4			129.3			129.3	
2''	6.76 (d, 2.0)	117.5	7''	6.74 (d, 2.0)	117.6	4'',6''	6.76 (d, 2.0)	117.6	1''	6.75 (d, 2.0)	117.7	4'',7''
3''		146.1			146.6			146.0			146.0	
4''		145.2			145.3			145.4			145.4	
5''	6.68 (d, 8.0)	116.3	3''	6.65 (d, 8.0)	116.4		6.70 (d, 8.0)	116.5	1''	6.68 (d, 8.0)	116.7	3''
6''	6.62 (dd, 8.0, 2.0)	121.9	4'',7''	6.60 (dd, 8.0, 2.0)	122.0	4''	6.62 (dd, 8.0, 2.0)	121.9	4''	6.60 (dd, 8.0, 2.0)	122.0	2'',4'',7''
7''	2.99 (dd, 14.0, 8.0)	37.9	1'',2'',6'',8'',9''	2.99 (dd, 14.0, 8.5)	37.9	1'',2'',6'',8'',9''	2.97 (dd, 14.0, 8.5)	37.9	1'',2'',6'',8'',9''	2.98 (dd, 14.0, 8.0)	38.0	1'',2'',6'',8'',9''
	3.08 (dd, 14.0, 4.5)		1'',2'',6'',8'',9''	3.07 (dd, 14.0, 4.5)		1'',2'',6'',8'',9''	3.08 (dd, 14.0, 4.0)		1'',2'',6'',8'',9''	3.06 (dd, 14.0, 4.5)		1'',2'',6'',8'',9''
8''	5.14 (dd, 8.0, 4.5)	74.9	9',1'',7'',9''	5.15 (dd, 8.5, 4.5)	74.9		5.11 (dd, 8.5, 4.0)	74.9	9',1'',7'',9''	5.16 (dd, 8.0, 4.5)	74.9	1'',7'',9''
9''		173.4			173.5			173.3			173.5	

315 (4.39); ECD (*c* 0.02, MeOH) nm ([θ]) 221 (101,600), 256 (–37000), 337 (–3700) nm; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.59 (1H, d, *J* = 16.0 Hz), 7.25–7.40 (8H, overlapping), 7.18 (2H, overlapping), 7.14 (1H, d, *J* = 8.5 Hz), 6.86 (1H, d, *J* = 2.0 Hz), 6.78 (1H, d, *J* = 8.5 Hz), 6.78 (1H, d, *J* = 8.0 Hz), 6.78 (1H, dd, *J* = 8.0, 2.0 Hz), 6.70 (1H, d, *J* = 2.0 Hz), 6.65 (1H, d, *J* = 8.0 Hz), 6.55 (1H, dd, *J* = 8.0, 2.0 Hz), 6.30 (1H, d, *J* = 16.0 Hz), 5.71 (1H, d, *J* = 5.5 Hz), 5.45 (1H, dd, *J* = 3.5, 3.0 Hz), 5.43 (1H, dd, *J* = 3.5, 3.0 Hz), 5.27 (1H, dd, *J* = 8, 4.5 Hz), 4.51 (1H, d, *J* = 5.5 Hz), 3.69 (3H, s), 3.67 (3H, s), 3.01 (1H, dd, *J* = 14.0, 4.5 Hz), 2.95 (1H, dd, *J* = 14.0, 8.0 Hz); FABMS (positive) *m/z* 833 $[M + H]^+$, 855 $[M + Na]^+$.

2.5.3. (S)-PGME amide of **5** *(***12***)*

Colorless amorphous solid; $[\alpha]^{25}_D$ + 134.5 (*c* 0.84, MeOH); UV (MeOH) λ_{max} (log ε) 257 (4.34), 291 (4.28), 314 (4.31); ECD (*c* 0.02, MeOH) nm ([θ]) 221 (116,500), 253 (47,000), 334 (9900) nm; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.63 (1H, d, *J* = 16.0 Hz), 7.23–7.35 (8H, overlapping), 7.15 (2H, overlapping), 7.15 (1H, d, *J* = 8.5 Hz), 6.88 (1H, d, *J* = 2.0 Hz), 6.79 (1H, d, *J* = 8.5 Hz), 6.79 (1H, d, *J* = 8.0 Hz), 6.79 (1H, dd, *J* = 8.0, 2.0 Hz), 6.62 (1H, d, *J* = 2.0 Hz), 6.61 (1H, d, *J* = 8.0 Hz), 6.46 (1H, dd, *J* = 8.0, 2.0 Hz), 6.30 (1H, d, *J* = 16.0 Hz), 5.72 (1H, d, *J* = 6.0 Hz), 5.42 (1H, br s), 5.40 (1H, br s), 5.29 (1H, t, *J* = 7.5 Hz), 4.54 (1H, d, *J* = 6.0 Hz), 3.72 (3H, s), 3.65 (3H, s), 2.82 (2H, d, *J* = 7.5 Hz); FABMS (positive) *m/z* 833 $[M + H]^+$, 855 $[M + Na]^+$.

2.5.4. (R)-PGME amide of **5** *(***13***)*

Colorless amorphous solid; $[\alpha]^{25}_D$ + 55.5 (*c* 0.84, MeOH); UV (MeOH) λ_{max} (log ε) 257 (4.33), 290 (4.27), 314 (4.29); ECD (*c* 0.02, MeOH) nm ([θ]) 219 (−82,500), 254 (46,200), 334 (12500) nm; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.77 (1H, d, *J* = 16.0 Hz), 7.28–7.37 (10H, overlapping), 7.20 (1H, d, *J* = 8.5 Hz), 6.81 (1H, d, *J* = 8.5 Hz), 6.77 (1H, d, *J* = 2.0 Hz), 6.71 (1H, d, *J* = 8.0 Hz), 6.71 (1H, d, *J* = 2.0 Hz), 6.65 (1H, d, *J* = 8.0 Hz), 6.61 (1H, dd, *J* = 8.0, 2.0 Hz), 6.55 (1H, dd, *J* = 8.0, 2.0 Hz), 6.37 (1H, d, *J* = 16.0 Hz), 5.63 (1H, d, *J* = 6.0 Hz), 5.48 (1H, br s), 5.47 (1H, br s), 5.34 (1H, t, *J* = 6.5 Hz), 4.52 (1H, d, *J* = 6.0 Hz), 3.64 (3H, s), 3.54 (3H, s), 3.02 (2H, d, *J* = 6.5 Hz); FABMS (positive) *m/z* 833 $[M + H]^+$, 855 $[M + Na]^+$.

2.5.5. (S)-PGME amide of **3** *(***14***)*

Colorless amorphous solid, $[\alpha]^{21}_D$ +46.3 (*c* 0.06, MeOH); UV (MeOH) λ_{max} (log ε) 257 (4.30), 290 (4.19), 311 (4.20); ECD (*c* 0.02, MeOH) nm ([θ]) 219 (67,700), 234 (−35,800), 254 (34,400), 297 (21,900) nm; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.64 (1H, d, *J* = 16.0 Hz), 7.22–7.32 (8H, overlapping), 7.16 (1H, d, *J* = 8.5 Hz), 6.93 (2H, overlapping), 6.89 (1H, d, *J* = 2.0 Hz), 6.81 (1H, d, *J* = 8.5 Hz), 6.74 (1H, d, *J* = 2.0 Hz), 6.73 (1H, dd, *J* = 8.0, 2.0 Hz), 6.64 (1H, d, *J* = 8.0 Hz), 6.63 (1H, d, *J* = 8.0 Hz), 6.56 (1H, dd, *J* = 8.0, 2.0 Hz), 6.37 (1H, d, *J* = 16.0 Hz), 5.82 (1H, d, *J* = 9.0 Hz), 5.50 (1H, m), 5.33 (1H, t, *J* = 7.0 Hz), 4.61 (1H, d, *J* = 9.0 Hz), 3.66 (3H, s), 3.50 (3H, s), 3.01 (2H, d, *J* = 7.0 Hz); FABMS (positive) *m/z* 833 $[M + H]^+$, 855 $[M + Na]^+$.

2.5.6. (R)-PGME amide of **3** *(***15***)*

Colorless amorphous solid; $[\alpha]^{22}_D$ –40.0(*c* 0.34, MeOH); UV (MeOH) λ_{max} (log ε) 257 (4.30), 290 (4.29), 314 (4.30); ECD (*c* 0.02, MeOH) nm ([θ]) 223 (−130,700), 256 (47,400), 299 (26,700) nm; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.57 (1H, d, *J* = 16.0 Hz), 7.10–7.40 (10H, overlapping), 7.14 (1H, d, *J* = 8.5 Hz), 6.93 (1H, d, *J* = 2.0 Hz), 6.81 (1H, d, *J* = 8.0 Hz), 6.78 (1H, dd, *J* = 8.5, 2.0 Hz), 6.73 (1H, d, *J* = 8.0 Hz), 6.68 (1H, d, *J* = 2.0 Hz), 6.64 (1H, d, *J* = 8.0 Hz), 6.53 (1H, dd, *J* = 8.0, 2.0 Hz), 6.32 (1H, d, *J* = 16.0 Hz), 5.88 (1H, d, *J* = 9.0 Hz), 5.48 (1H, m), 5.27 (1H, dd, *J* = 8.0, 5.0 Hz), 5.08 (1H, m), 4.73 (1H, d, *J* = 9.0 Hz), 3.71 (3H, s), 3.58 (3H, s), 2.95 (1H, dd, *J* = 14.0, 8.0 Hz), 3.02 (1H, dd, *J* = 14.0, 5.0 Hz); FABMS (positive) *m/z* 833 $[M + H]^+$, 855 $[M + Na]^+$.

2.6. Hyaluronidase inhibitory assay

The inhibitory activity of hyaluronidase was determined by the Morgan–Elson method, which was modified by Davidson and Aronson. The assay was carried out in accordance with the procedure reported previously [6]. Samples dissolved in 0.1 M acetate buffer (0.2 mL) and hyaluronidase (Type IV-S: from bovine testes, Sigma Chemical Co., St. Louis, USA) in buffer (final concentration: 400 unit/mL, 0.1 mL) were mixed and the mixture was incubated at 37 °C for 20 min. Then, compound 48/80 (Sigma Chemical Co.) in buffer (final concentration: 0.3 mg/mL, 0.2 mL) was added and incubated at 37 °C for 20 min. After hyaluronic acid potassium salt, from rooster comb (Sigma Chemical Co.) in buffer (final concentration: 0.4 mg/mL, 0.5 mL) had been added, the mixture was incubated at 37 °C for 40 min. Then, the reaction was stopped by adding 0.4 M NaOH and borate solution and then boiling the mixture in a H_2O bath for 3 min. An acetone solution of dimethylaminobenzaldehyde (Wako Pure Chemical Industries Ltd., Osaka, Japan) (6 mL) was then added and incubated at 37 °C for 20 min. Acetate buffer was added in place of the sample as a control, and the buffer was added in place of hyaluronidase in buffer as a blank. The enzyme inhibitory activity (%) was calculated as follows: Inhibitory activity (%) = [(Control Abs $_{600\ nm}$ − Control $_{blank}$ Abs $_{600\ nm}$) − (Sample Abs $_{600\ nm}$ − Sample $_{blank}$ Abs $_{600\ nm}$)]/(Control Abs $_{600\ nm}$ − Control $_{blank}$ Abs $_{600\ nm}$) × 100.

2.7. Histamine release inhibitory assay

Degranulation of KU812F cells was monitored by measuring the release of histamine, which was carried out in accordance with the reported procedure [20]. KU812F cells (Japan Human Sciences Foundation, Tokyo, Japan) were stimulated with 1 ng/mL of IL-4 for 2 weeks to prepare mature basophils. KU812F cells (6.25×10^6 cells/mL) were resuspended in 400 μL Tyrode buffer at pH 7.2. Then, 50 μM A23187 (BioVision, Inc., California, USA) (50 μL) was incubated with each sample (50 μL) as the test solution and was then added to the cell suspension (Test Sample). Tyrode buffer (50 μL) was added in place of the sample as a positive control (PC), buffer (100 μL) was added in place of the sample and A23187 as a negative control (NC), and buffer (50 μL) was added in place of the A23187 as a negative control-2 (NC-2). After adding the mixture to the cells, the mixture was incubated at 37 °C for 20 min, and reaction was terminated by cooling at 4 °C for 15 min. The cell suspension was then centrifuged at 1000 rpm for 3 min, and the supernatant moved to a 50 μL microtiter plate. It was mixed

with 50 μL of 1 M NaOH and 50 μL of 0.2% *o*-phthalaldehyde (Wako, Osaka, Japan), and kept for 5 min at room temperature. The reaction was terminated by adding 50 μL of 3 N HCl, and then the fluorescence intensity was measured using a spectrofluorophotometer with excitation at 355 nm and emission at 460 nm. The inhibitory percentage histamine release was calculated as follows: inhibitory activity (%) = [1 − (Test Sample − NC-2)/(PC − NC)] × 100. The significance of the differences was examined by Tukey's test.

2.8. HPLC analysis of compounds 1, 2, 5, and 6 using reversed-phase columns

The methanol solutions of compounds **1**, **2**, **5**, and **6** were subjected to HPLC using a C_{18} column [column, Cosmosil 5C_{18}-AR-II (4.6 × 250 mm); solvent, CH_3CN–H_2O containing 0.2% TFA (22.5:77.5); flow rate, 1.0 mL/min; detector, UV 310 nm]. The retention times were as follows: **1** (15.3 min), **2** (26.5 min), **5** (16.3 min), and **6** (24.0 min). When the C_{30} column was used [column, Develosil C30-UG-5 (4.6 × 250 mm); solvent, CH_3CN–H_2O containing 0.2% TFA (25:75); flow rate, 1.0 mL/min; detector, UV 310 nm], the retention times were as follows: **1** (14.1 min), **2** (23.1 min), **5** (15.4 min), and **6** (22.3 min).

3. Results and discussion

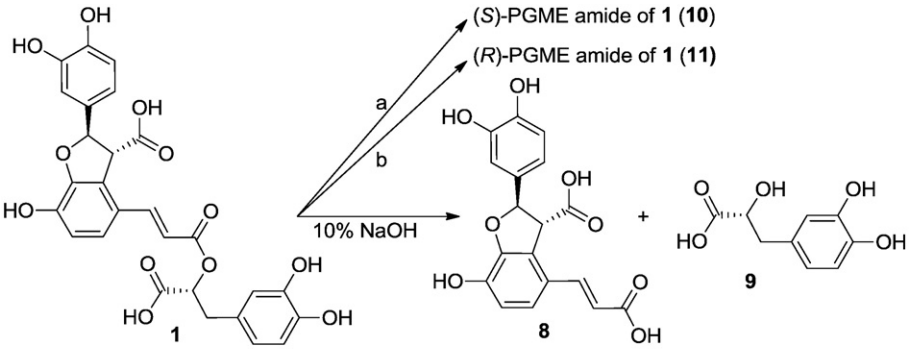
Whole plants of *M. fistulosa* were extracted with acetone–H_2O (8:2). The extract was concentrated at reduced pressure, suspended in H_2O, and extracted with Et_2O. The aqueous extract was subjected to column chromatography and preparative HPLC to afford compounds **1–4**. Similarly, compounds **5–7** were isolated from the acetone–H_2O (8:2) extract of the roots of *L. erythrorhizon*. Rosmarinic acid (**4**) [18], LA (**5**) [9], and LAB (**6**) [4] were identified by comparing their observed and reported spectroscopic data.

The molecular formula of **1** ($C_{27}H_{22}O_{12}$, *m/z* 539.1196 $[M + H]^+$) was established using HRFABMS. In the ^{13}C NMR spectra (Table 1), 27 carbons, including three carbonyl carbons (δc 175.1, C-9; 168.2, C-9′; 173.4, C-9″), suggested that **1** was a phenylpropanoid trimer. In the ^1H NMR spectrum (Table 1), a –CH(O–)–CH_2– unit [δ_H 2.99 (dd, J = 14.0, 8.0 Hz, H-7″), 3.08 (dd, J = 14.0, 4.5 Hz, H-7″), 5.14 (dd, J = 8.0, 4.5 Hz, H-8″)] and the aromatic spin system [δ_H 6.76 (d, J = 2.0 Hz, H-2″), 6.68 (d, J = 8.0 Hz, H-5″), 6.62 (dd, J = 8.0, 2.0 Hz, H-6″)] suggested the presence of a 3-(3,4-dihydroxyphenyl)-2-hydroxypropanoyl moiety. The NMR spin system at δ_H 5.90, d, J = 4.5 Hz, H-7; 4.36, d, J = 4.5 Hz, H-8; δ_C 88.8, C-7; 57.5 C-8 indicated the presence of a dihydrobenzofuran moiety. The ^1H and ^{13}C NMR resonances of **1** were close to those of **5** [9]. In fact, 2D NMR data suggested that the molecular structure of **1** was the same as that of **5**. The $^3J_{7,8}$ coupling constant (4.5 Hz) suggested that the methine protons were in a *trans* configuration (7*S*,8*S* or 7*R*,8*R*) [19,21]. Alkaline hydrolysis of **1** gave 7-epiblechnic acid (**8**) [19], indicating the (7*R*,8*R*) configuration of the dihydrobenzofuran moiety (Scheme 1). The absolute configuration of C-8″ of **1** was *R*, as determined from its hydrolysate (**9**), which was identical to that of (2*R*)-3-(3,4-dihydroxyphenyl)-2-hydroxypropanoic acid in our previous report (Scheme 1) [18]. From these data, the structure of **1** was determined to be monardic acid A, which is the (7*R*,8*R*) isomer of LA.

The PGME method has been used to define the absolute configuration of natural products [22]. In this study, the PGME method was employed to determine the absolute configuration at C-8 of **1** and its analogues. Separate treatment of **1** with (*S*)-PGME and (*R*)-PGME produced **10** and **11**, respectively. Similarly, treatment of **5** with (*S*)-PGME and (*R*)-PGME produced **12** and **13**, respectively. For **1**, the values of the ^1H NMR chemical shift differences [$\Delta\delta$ (ppm) = $\delta_{(S)\text{-PGME amide (10)}} - \delta_{(R)\text{-PGME amide (11)}}$] adjacent to C-8 were consistent with the (8*R*) absolute configuration [22], except for the negative values of H-5″ and H-6″ affected by the second PGME moiety at C-8″ (Fig. 2). Furthermore, the values around C-8 had signs opposite to those of **5** suggesting (8*S*) configuration [$\Delta\delta$ (ppm) = $\delta_{(S)\text{-PGME amide (12)}} - \delta_{(R)\text{-PGME amide (13)}}$] (Fig. 2). These data suggested that the PGME method is applicable to the analysis of the dihydrobenzofuran moiety and supported the (8*R*) configuration of **1**.

The molecular formula of **2** was established as $C_{36}H_{30}O_{16}$ on the basis of HRFABMS (*m/z* 741.1429 $[M + Na]^+$), which, when compared with **1**, corresponded to an additional phenylpropanoid unit of formula $C_9H_8O_4$. The molecular formula revealed **2** to be a phenylpropanoid tetramer. The ^1H and ^{13}C NMR resonances of **2** (Table 1) were close to those of **6** [4,13]. The resonances of the dihydrobenzofuran moiety (δ_H 5.85, d, J = 4.5 Hz, H-7; 4.42, d, J = 4.5 Hz, H-8; δ_C 88.2, C-7; 57.3, C-8) suggested that the methine protons of **2** were in a *trans* configuration (7*S*,8*S* or 7*R*,8*R*) [19,21]. The ECD spectrum of **2** showed that its Cotton effects (CEs) were similar to those of **1** and opposite to those of **5** and **6** [13], suggesting that the absolute configuration of the dihydrobenzofuran moiety of **2** was also (7*R*,8*R*) (Fig. 3) [18]. In the ^1H NMR spectrum, two –CH(O–)–CH_2– units [δ_H 2.99 (dd, J = 14.0, 8.5 Hz, H-7″), 3.07 (dd, J = 14.0, 4.5 Hz, H-7″), 5.15 (dd, J = 8.5, 4.5 Hz, H-8″)] and [δ_H 3.00 (dd, J = 14.0, 7.5 Hz, H-7‴), 3.09 (dd, J = 14.0, 4.5 Hz, H-7‴), 5.20 (dd, J = 7.5, 4.5 Hz, H-8‴)] and two aromatic spin systems [δ_H 6.74 (d, J = 2.0 Hz, H-2″), 6.65 (d, J = 8.0 Hz, H-5″), 6.60 (dd, J = 8.0, 2.0 Hz, H-6″)] and [δ_H 6.67 (d, J = 2.0 Hz, H-2‴), 6.62 (d, J = 8.0 Hz, H-5‴), 6.48 (dd, J = 8.0, 2.0 Hz, H-6‴)] suggested the presence of two 3-(3,4-dihydroxyphenyl)-2-hydroxypropanoyl moieties. The configurations of C-8″ and C-8‴ of **2** were determined to be (*R*) from the retention time by performing HPLC analysis of the (*S*)-PGME amide of 3-(3,4-dihydroxyphenyl)-2-hydroxypropanoic acid from the alkaline hydrolysis of **2** [18]. From these data, the structure of **2** was determined as monardic acid B, which is the (7*R*,8*R*) isomer of LAB.

Compounds **3** and **7** also had the molecular formula $C_{27}H_{22}O_{12}$ (HRFABMS; **3**: *m/z* 561.1012 $[M + Na]^+$ and **7**: *m/z* 561.1012 $[M + Na]^+$) suggesting a phenylpropanoid trimer. In their ^1H NMR spectra (Table 1), the coupling constants between H-7 and H-8 (J = 9.5 Hz) suggested that they were *cis*-related (7*S*,8*R* or 7*R*,8*S*) [19,23]. The resonances of H-8 (**3**: δ_H 4.61, **7**: δ_H 4.63) were downfield shifted relative to those of the *trans* isomers, which also supported the *cis*-dihydrobenzofuran moiety [10]. These data indicated that one has (7*S*,8*R*) configuration and the other a (7*R*,8*S*) configuration. Some *cis*-oriented LA analogues have been reported from natural sources, e.g., *cis*-lithospermic acid from *S. yunnanensis* C. H. Wright [23] and clinopodic acid N from *Clinopodium gracile* (Benth.) O. Kuntze [24]. However, there was insufficient discussion about the absolute configuration, and ECD data



[a]Key: (a) (*S*)-PGME, benzotriazol-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP), 1-hydroxybenzotriazole (HOBT), and *N*-methylmorpholine in DMF; (b) (*R*)-PGME, PyBOP, HOBT, and *N*-methylmorpholine in DMF.

Scheme 1. PGME amidations and alkaline hydrolysis of compound **1**[a].

from these analogues in previous reports. Compounds **3** and **7** showed nearly identical specific optical rotations and NMR resonances, and it is difficult to distinguish these compounds using these data alone. Although the PGME method is an empirical method similar to Mosher's methods, the applied models of **1** and **5** suggested that the method is applicable to compounds comprising dihydrobenzofurans. According to this method, the (*S*)-PGME and (*R*)-PGME amidation of **3** produced **14** and **15**, and the values of the ^{1}H NMR chemical shift differences [$\Delta\delta$ (ppm) = $\delta_{(S)\text{-PGME amide (14)}} - \delta_{(R)\text{-PGME amide (15)}}$]

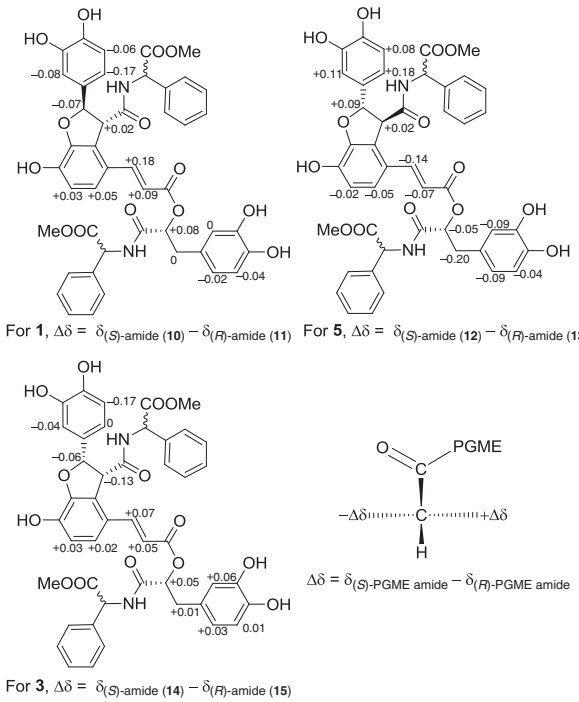


Fig. 2. The values (in ppm) of the ^{1}H NMR chemical shift differences for **1**, **3**, and **5** and the model of the PGME method.

indicated that C-8 was in the (*R*) configuration (Fig. 2) [22]. That is, **3** had the (7*S*,8*R*) configuration of the dihydrobenzofuran moiety and **7** had the (7*R*,8*S*) configuration of this moiety. The absolute configuration of C-8″ in **3** and **7** was determined to be (*R*) using the same HPLC method of the (*S*)-PGME amide as used for **2**. Thus, **3** was identified as the (7*S*,8*R*) *cis* isomer of LA, and **7** was determined to be the (7*R*,8*S*) *cis* isomer of LA, hereafter referred to as monardic acid C and lithospermic acid C, respectively.

Using the PGME method, amidation of **3** with (*S*)-PGME and (*R*)-PGME produced not only **14** and **15** but also **12** and **13**. Each of these products (**12** and **13**) had a C-8 configuration that was opposite to the C-8 configuration in **3**. These products revealed that the *cis* dihydrobenzofuran moiety of **3** changed to the *trans* dihydrobenzofuran moiety during the reaction. Similarly, the main products of the (*S*)-PGME and (*R*)-PGME amidation of **7** were **10** and **11**. One hypothesis to explain these unexpected products was their epimerization at C-8 through enolization via a sterically hindered condensation reaction during PGME amidation. Thus, attention to epimerization is required when the PGME method is used.

In the ECD spectrum of **3**, a negative CE at 230 nm and a positive CE at 254 nm were observed, and these effects were opposite for **7** (Fig. 3). In many compounds with

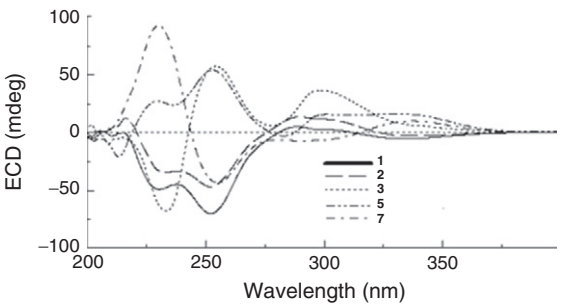


Fig. 3. ECD spectra of **1–3**, **5**, and **7**.

dihydrobenzofuran moieties, the configurations at C-7 were determined from the 220–240 nm region [24,25]. However, it has not been demonstrated whether this empirical rule is applicable to the C-7 configuration in the dihydrobenzofuran moieties of isolates (**1–3** and **7**). Specific CEs were observed around 220–240 nm and 250–260 nm in the ECD spectra of derivatives obtained here. In the region around 250–260 nm, compounds with the (7*R*) configuration (**1**, **2**, **7**, **8**, **10**, and **11**) had negative values, and in contrast, compounds with the (7*S*) configuration (**3**, **5**, **6**, and **12–15**) had positive values. On the other hand, signals around 220–240 nm were not related to the configuration of C-7 or were not observed. These ECD data suggested that the values around 250–260 nm were responsible for the absolute configuration at C-7 in the dihydrobenzofuran moiety [3,4,13].

The diastereomers isolated in this research are expected to have a variety of biological activities similar to LA and LAB. To identify anti-allergic and anti-inflammatory agents [6], the hyaluronidase inhibitory activity of these diastereomers was measured (Table 2). The compounds (**1–3** and **7**) showed potent activities (IC_{50} 381–406 μM) similar to **5** and **6**. The positive control was disodium cromoglycate (DSCG). Moreover, **1**, **4**, and **5** had moderate inhibitory effects on histamine release on the degranulation process in a human basophilic cell line (KU812F) similar to the positive control epigallocatechin-3-*O*-(3-*O*-methyl)gallate (Fig. 4) [20]. These results suggested that these compounds possibly have anti-allergic effects.

The biosynthetic pathways of LA and LAB are unknown, but it is expected that they are produced from rosmarinic acid [26]. Compound **1** was obtained as the main phenylpropanoid oligomer, and **5** and **6** were not detected in our investigation of *M. fistulosa*. Compounds **1**, **2**, **5**, and **6** showed different retention times of the observed peaks by HPLC using reversed-phase columns (Experimental Section). For the four diastereomers, only peaks of the (7*R*,8*R*) diastereomers (**1** and **2**) were detected in the chromatograms of the Et_2O and aqueous extracts of *M. fistulosa*, and only peaks of the (7*S*,8*S*) diastereomers (**5** and **6**) were detected in the chromatograms of the Et_2O and aqueous extracts of *L.* erythrorhizon. From these observations, each plant species may have a stereoselective biosynthetic pathway to produce either the (7*R*,8*R*) or (7*S*,8*S*) dihydrobenzofuran moieties. The *cis* isomers (**3** and **7**) were C-7 epimers of **1** and **5**, respectively. It is assumed that **3** and **7** are epimerized from **1** and **5** through the ring-opening intermediate [19].

Table 2
Hyaluronidase inhibitory activity of **1–7** and DSCG.

Compound	Hyaluronidase inhibition
	IC_{50} (μM)
1	406
2	398
3	404
4	1360
5	379
6	373
7	381
DSCG	1020

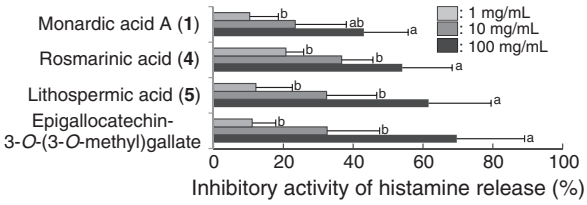


Fig. 4. Inhibitory effect of **1**, **4**, and **5** on the degradation of KU812F cells. Results are means ± SD for $n = 5$. Values not sharing a common letter (a, ab, or b) are significantly different between groups ($p < 0.05$).

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