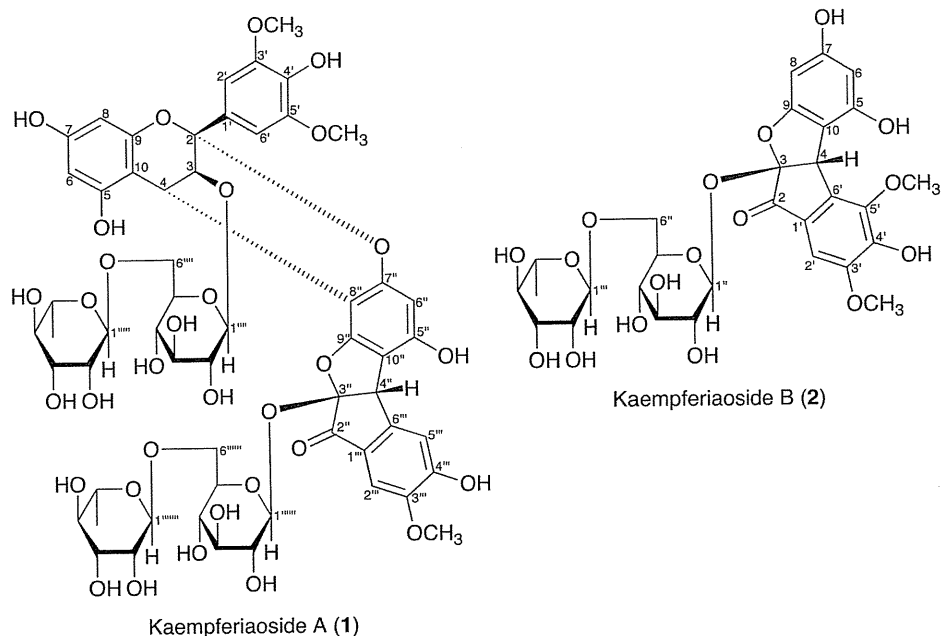

Chart 1

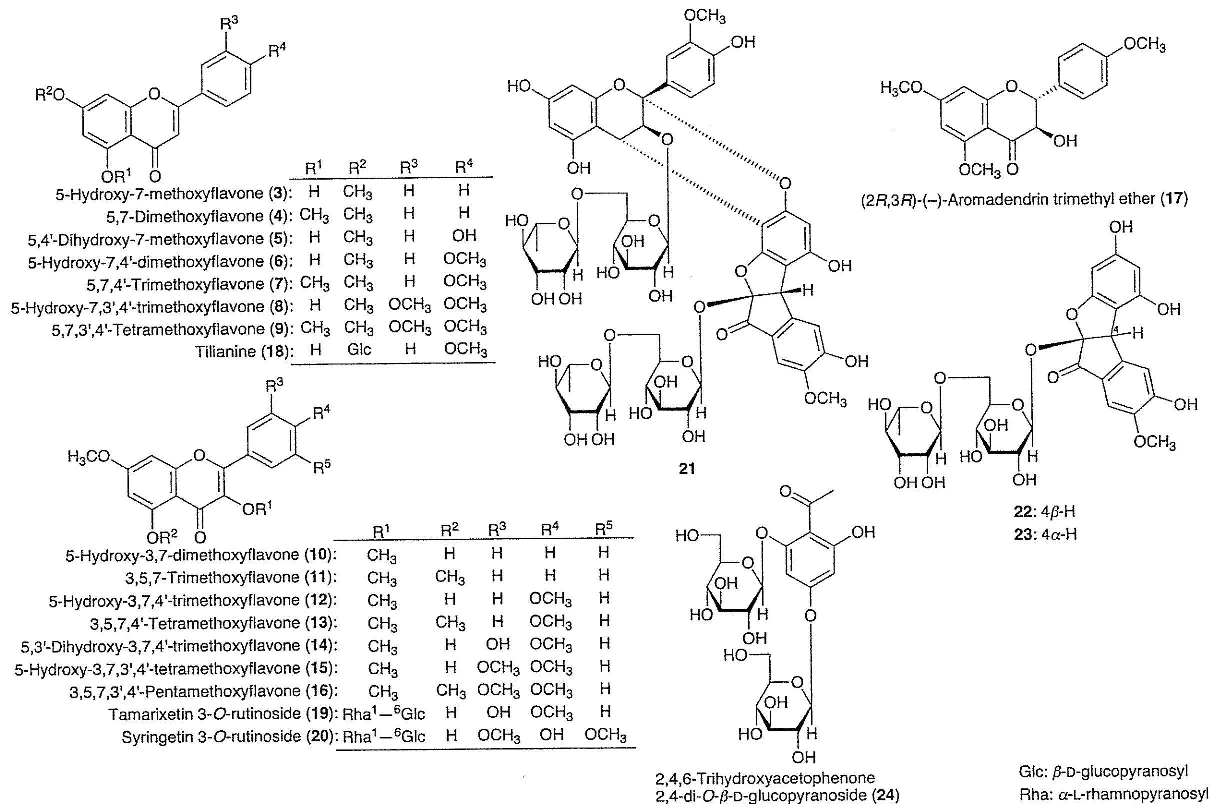

Chart 2

rotation detector.[42] The ^{1}H- and ^{13}C-NMR spectra (CD_3OD, Table 2) of **1**, which were assigned by various NMR experiments,[43] showed signals assignable to three methoxy groups [δ 3.50 (3H, br s, 3‴-OCH_3), 3.89 (6H, s, 3′,5′-OCH_3)], three methines [δ 4.53, 4.93 (1H each, both d, J=3.5 Hz, 3, 4-H), 5.33 (1H, br s, 4″-H)], and seven aromatic protons [δ 5.82, 5.83 (1H each, d, J=2.3 Hz, 8, 6-H), 6.18 (1H, s, 6″-H), 7.01 (3H, s, 2′,6′, 2‴-H), 7.32 (1H, br s, 5‴-H)] together with two

Table 1. Inhibitory Effects of the Methanolic Extract from the Rhizomes of *K. parviflora* and Its Fractions on D-GalN-Induced Cytotoxicity in Primary Cultured Mouse Hepatocytes

	Inhibition (%)				
	0 μg/mL	3 μg/mL	10 μg/mL	30 μg/mL	100 μg/mL
MeOH extract	0.0±1.9	11.5±0.6**	18.4±1.3**	16.1±1.7**	—
EtOAc-soluble fraction	0.0±2.6	12.4±2.1**	23.3±2.3**	—	—
MeOH-eluted fraction	0.0±1.4	4.4±0.3	10.4±1.3**	19.6±1.7**	42.5±2.6**
H_2O-eluted fraction	0.0±2.7	−0.8±0.7	−1.9±0.9	−2.1±1.4	2.2±2.3

Each value represents the mean±S.E.M. (n=4). Significantly different from the control, **p<0.01.

Table 2. ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) Data for **1**

Position	δ_H (J Hz)	δ_C	Position	δ_H (J Hz)	δ_C
2		100.1	3-*O*-Glc		
3	4.53 (1H, d, 3.5)	70.1	1''''	4.57 (1H, d, 7.5)	99.7
4	4.93 (1H, d, 3.5)	25.0	2''''	2.98 (1H, dd, 7.5, 9.2)	75.3
5		156.5	3''''	3.57 (1H, m)	77.3
6	5.83 (1H, d, 2.3)	97.5	4''''	3.12 (1H, dd like)	71.9
7		158.6	5''''	3.48 (1H, m)	77.2
8	5.82 (1H, d, 2.3)	95.4	6''''	3.48 (1H, m)	68.3
9		154.1		3.92 (1H, m)	
10		105.4	6''''-*O*-Rha		
1'		131.0	1'''''	4.66 (1H, br s)	102.4
2',6'	7.01 (2H, s)	106.2	2'''''	3.71 (1H, m)	72.2
3',5'		148.5	3'''''	3.58 (1H, m)	72.4
4'		137.1	4'''''	3.31 (1H, m)	74.0
2'		195.7	5'''''	3.55 (1H, m)	69.9
3'		113.0	6'''''	1.18 (3H, d, 6.3)	18.0
4'	5.33 (1H, br s)	49.4	3'-*O*-Glc		
5''		154.1	1''''''	4.72 (1H, d, 7.8)	99.4
6''	6.18 (1H, s)	97.6	2''''''	3.35 (1H, m)	75.5
7''		155.1	3''''''	3.68 (1H, m)	76.5
8''		100.2	4''''''	3.36 (1H, m)	71.2
9''		158.6	5''''''	3.31 (1H, m)	76.5
10''		107.4	6''''''	3.52 (1H, m)	67.2
1'''		124.5		3.93 (1H, m)	
2'''	7.01 (1H, s)	106.4	6''''''-*O*-Rha		
3'''		150.7	1'''''''	4.67 (1H, br s)	101.9
4'''		158.6	2'''''''	3.94 (1H, m)	72.2
5'''	7.32 (1H, br s)	113.8	3'''''''	3.71 (1H, m)	72.4
6'''		152.5	4'''''''	3.34 (1H, m)	74.2
3',5'-OCH_3	3.89 (6H, s)	57.1	5'''''''	3.65 (1H, m)	69.7
3'''-OCH_3	3.50 (3H, br s)	56.1	6'''''''	1.24 (3H, d, 6.3)	18.1

Measured in CD_3OD.

rhamnopyranosyl [δ 1.18 (3H, d, J=6.3 Hz, 6'''''-H_3), 1.24 (3H, d, J=6.3 Hz, 6'''''''-H_3), 4.66, 4.67 (1H each, both br s, 1''''', 1'''''''-H)] and two glucopyranosyl moieties [δ 4.57 (1H, d, J=7.5 Hz, 1''''-H), 4.72 (1H, d, J=7.8 Hz, 1''''''-H)]. As shown in Fig. 1, the ^{1}H–^{1}H correlation spectroscopy (COSY) experiments on **1** indicated the presence of three partials written in bold lines. In the heteronuclear multiple bond connectivity (HMBC) experiment, long range correlations were observed between the following proton and carbon pairs: 3-H and 2-C; 4-H and 2, 9, 10, 8'', 9''-C; 6-H and 8, 10-C; 8-H and 6, 10-C; 2',6'-H and 2, 4'-C; the 3',5'-methoxy methyl protons and 3',5'-C; 4''-H and 3'', 5'', 9'', 10'', 1''', 6'''-C; 6''-H and 8'', 10''-C; 2'''-H and 4''', 6'''-C; 5'''-H and 1''', 3'''-C; the 3'''-methoxy proton and 3'''-C; 1''''-H and 3-C; 1'''''-H and 6''''-C; 1''''''-H and 3''-C; 1'''''''-H and 6''''''-C (Fig. 1). The proton and carbon signals in the ^{1}H- and ^{13}C-NMR spectra of **1** were similar to those of **21**, except for the signals due to the B ring in the flavan-3-ol unit. The relative stereochemistry of **1** was determined on the basis of the coupling constants in the ^{1}H-NMR spectrum and the rotating frame nuclear Overhauser effect spectroscopy (ROESY) experiment. The 2,3-*trans* form in the flavan-3-ol unit was identified by comparison of the coupling constant ($J_{3,4}$=3.5 Hz) with that of **21** ($J_{3,4}$=3.7 Hz).[21] The rotating frame Overhauser effect (ROE) correlations were observed between following proton pairs: 6-H and 2'''-H; 2',6'-H and 3',5'-OCH_3; 2'''-H and the 3'''-OCH_3; and 4''-H and 1''''-H (Fig. 1). Finally, the absolute stereochemistry of **1** was elucidated by circular dichroism (CD) spectrum, which showed a negative Cotton effect in the

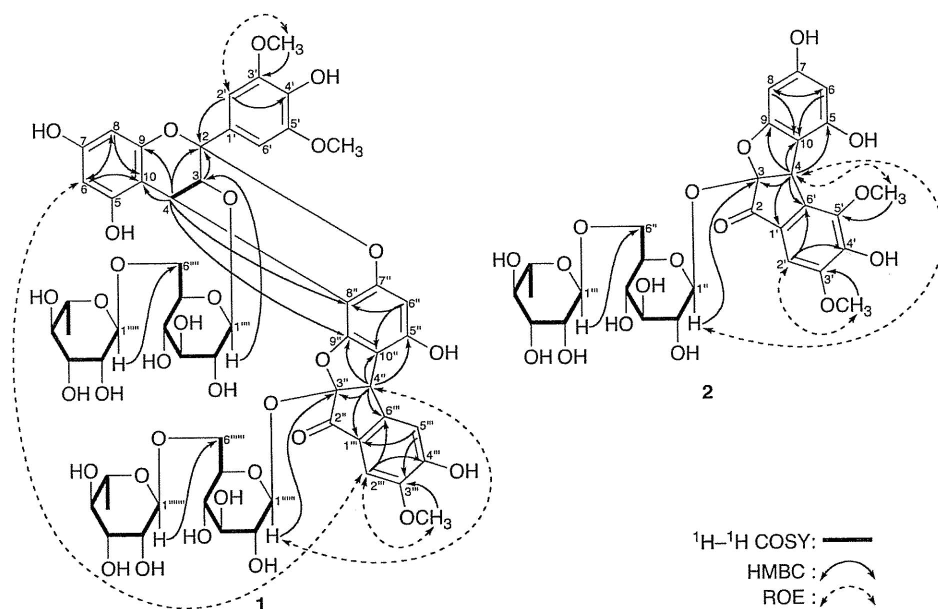

Fig. 1. ^{1}H–^{1}H COSY, HMBC, and NOESY Correlations for **1** and **2**

Table 3. ^{1}H-NMR (700 MHz) and ^{13}C-NMR (175 MHz) Data for **2**

Position	δ_H (J Hz)	δ_C	Position	δ_H (J Hz)	δ_C
2		194.9	3-*O*-Glc		
3		112.1	1″	4.57 (1H, d, 7.4)	99.2
4	5.01 (1H, s)	45.1	2″	3.02 (1H, m)	73.3
5		154.2	3″	3.11 (1H, m)	76.9
6	5.83 (1H, d, 2.0)	96.7	4″	3.11 (1H, m)	69.2
7		159.4	5″	2.98 (1H, m)	75.8
8	5.79 (1H, d, 2.0)	89.4	6″	3.39 (1H, m)	66.3
9		160.2		3.68 (1H, dd, 2.1, 11.0)	
10		103.8	6″-*O*-Rha		
1′		123.5	1‴	4.52 (1H, d, 1.9)	100.9
2′	6.89 (1H, s)	102.0	2‴	3.65 (1H, m)	70.4
3′		152.0	3‴	3.41 (1H, m)	70.8
4′		148.5	4‴	3.16 (1H, m)	72.2
5′		142.7	5‴	3.39 (1H, m)	68.4
6′		139.1	6‴	1.07 (3H, d, 6.2)	17.8
3′-OCH_3	3.76 (3H, s)	56.0			
5′-OCH_3	3.90 (3H, s)	60.3			

Measured in DMSO-d_6.

short wavelength region ($\Delta\varepsilon$ −37.54 at 214 nm), indicating that the orientation of the 4-position in the flavan-3-ol unit is *S* configuration.[21,44—47] This evidence indicated that the absolute configuration of **1** was determined to be 2*R*, 3*S*, 4*S*, 3″*S*, and 4″*S* orientations. Consequently, the absolute stereostructure of **1** was determined to be as shown.

Kaempferiaoside B (**2**) was also obtained as an amorphous powder with negative optical rotation ($[\alpha]_D^{23}$ −166.8° in MeOH). The molecular formula, $C_{29}H_{34}O_{17}$, of **2** was determined by high-resolution positive-ion FAB-MS measurement. Acid hydrolysis of **2** with 1 M HCl liberated L-rhamnose and D-glucose, which were identified by HPLC using an optical rotation detector. The proton and carbon signals in the ^{1}H- and ^{13}C-NMR spectra (DMSO-d_6, Table 3) of **2** were superimposable on those of **22**, except for the signals due to an additional 5′-methoxy group: two methoxy groups [δ 3.76, 3.90 (3H each, both s, 3′, 5′-OCH_3)], a methine [δ 5.01 (1H, s, 4-H)], and three aromatic protons [δ 5.79, 5.83 (1H each, d, J=2.0 Hz, 8, 6-H), 6.89 (1H, s, 2′-H)] together with a rhamnopyranosyl [δ 1.07 (3H, d, J=6.2 Hz, 6‴-H_3), 4.52 (1H, d, J=1.9 Hz, 1‴-H)] and a glucopyranosyl moieties [δ 4.57 (1H, d, J=7.4 Hz, 1″-H)]. In the HMBC experiment on **2**, long-range correlations were observed between the following protons and carbons: 4-H and C-3, 5, 9, 10, 1′, 6′-C; 6-H and 8, 10-C; 8-H and 6, 10-C; 2′-H and 4′, 6′-C; the 3′-methoxy proton and 3′-C; the 5′-methoxy proton and 5′-C; 1″-H and 3-C; 1‴-H and 6″-C (Fig. 1). The ROE correlations were observed as shown in Fig. 1 (4-H, 1″-H, 5′-methoxy proton; 2′-H, 3′-methoxy proton). Consequently,

the structure of **2** was elucidated to be the 5′-methoxy derivative of **22**.

Effects of the Chemical Constituents on D-GalN-Induced Cytotoxicity in Primary Cultured Mouse Hepatocytes Previously, we have reported the isolation and structure elucidation of several constituents with protective effects on D-GalN-induced cytotoxicity in primary cultured mouse hepatocytes.[30,48—57] As a continuing exploratory study for hepatoprotective agents from medicinal plants, the constituents from the rhizomes of *K. parviflora* were examined. As shown in Table 4, 5,3′-dihydroxy-3,7,4′-trimethoxyflavone (**14**, IC_{50}=18.4 μM) showed higher activity than silybin (38.8 μM),[49,50,53—56] a commercial hepatoprotective agent as a positive control.[58,59]

Experimental

The following instruments were used to obtain physical data: specific rotations, Horiba SEPA-300 digital polarimeter (l=5 cm); CD spectra, JASCO J-720WI spectrometer; UV spectra, Shimadzu UV-1600 spectrometer; IR spectra, Shimadzu FTIR-8100 spectrometer; FAB-MS and high-resolution MS, JEOL JMS-SX 102A mass spectrometer; ^{1}H-NMR spectra, JEOL JNM-ECA700 (700 MHz), JNM-ECA500 (500 MHz), and JNM-ECS400 (400 MHz) spectrometers; ^{13}C-NMR spectra, JEOL JNM-ECA700 (175 MHz), JNM-ECA500 (125 MHz), and JNM-ECS400 (100 MHz) spectrometers with tetramethylsilane as an internal standard; HPLC detector, Shimadzu SPD-10A*vp* UV–VIS detector; HPLC column, Cosmosil $5C_{18}$-MS-II and πNAP (250×4.6 mm i.d. and 250×20 mm i.d. for analytical and preparative purposes, respectively).

The following experimental conditions were used for chromatography (CC): ordinary-phase silica gel column chromatography, silica gel 60N (Kanto Chemical Co., Tokyo, Japan; 63—210 mesh, spherical, neutral); reverse-phase silica gel CC, Diaion HP-20 (Nippon Rensui, Tokyo, Japan) and Chromatorex ODS DM1020T (Fuji Silysia Chemical, Aichi, Japan; 100—200 mesh); normal-phase TLC, pre-coated TLC plates with silica gel $60F_{254}$ (Merck, Darmstadt, Germany; 0.25 mm); reversed-phase TLC, pre-coated TLC plates with silica gel RP-18 F_{254S} (Merck, 0.25 mm); reversed-phase HPTLC, pre-coated TLC plates with silica gel RP-18 WF_{254S} (Merck, 0.25 mm), detection was carried out by spraying 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4 on the plates, followed by heating.

Plant Material The rhizomes of *Kaempferia parviflora* WALL. *ex* BAKER cultivated at Loei province, Thailand were purchased from a Thai traditional drug store in Nakhonsithammarat province, Thailand on May 2008. The plant material was identified by one of the authors (Y. P.). A voucher specimen (2008.05. Raj-03) of this plant is on file in our laboratory.

Extraction and Isolation The dried rhizomes of *K. parviflora* (1.8 kg) were finely cut and extracted four times with MeOH under reflux for 4 h. Evaporation of the combined extract under reduced pressure provided a MeOH extract (128.0 g, 7.10%). An aliquot (108.0 g) was partitioned into an EtOAc–H_2O (1 : 1, v/v) mixture to furnish the EtOAc-soluble fraction (58.10 g, 3.82%) and an aqueous phase. The aqueous phase was subjected to Diaion HP-20 CC (1.5 kg, $H_2O \rightarrow$ MeOH) to give H_2O-eluted (30.98 g, 2.04%) and MeOH-eluted (18.66 g, 1.23%) fractions, respectively.

The EtOAc-soluble fraction (48.00 g) was subjected to normal-phase silica gel CC [1.2 kg, *n*-hexane–EtOAc (10 : 1 $\rightarrow$ 2 : 1 $\rightarrow$ 1 : 1 $\rightarrow$ 1 : 2, v/v) $\rightarrow$ MeOH] to give 14 fractions {Fr. 1 (330.9 mg), Fr. 2 (282.1 mg), Fr. 3 (177.9 mg), Fr. 4 (75.3 mg), Fr. 5 [=5-hydroxy-3,7-dimethoxyflavone (**10**, 1216.0 mg, 0.0968%)], Fr. 6 (1001.5 mg), Fr. 7 (491.4 mg), Fr. 8 (4168.0 mg),

Table 4. Inhibitory Effects of the Constituents from *K. parviflora* on D-GalN-Induced Cytotoxicity in Primary Cultured Mouse Hepatocytes

	Inhibition (%)				
	0 μM	3 μM	10 μM	30 μM	100 μM
Kaempferiaoside A (**1**)	0.0±3.8	2.3±2.6	14.7±3.1**	17.5±3.2**	23.7±4.9**
5-Hydroxy-7-methoxyflavone (**3**)	0.0±1.4	1.6±1.5	9.8±1.0**	24.7±2.8**	17.8±0.8**
5,7-Dimethoxyflavone (**4**)	0.0±2.7	8.1±2.3	16.1±2.5**	28.3±1.3**	—
5,4′-Dihydroxy-7-methoxyflavone (**5**)	0.0±1.9	8.7±1.5*	21.1±2.2**	23.3±3.1**	15.6±1.6**
5-Hydroxy-7,4′-dimethoxyflavone (**6**)	0.0±2.1	8.3±1.4*	36.7±2.8**	27.5±1.5**	16.9±1.4**
5,7,4′-Trimethoxyflavone (**7**)	0.0±4.0	5.6±3.3	7.7±2.4	18.3±1.7**	—
5,7,3′,4′-Tetramethoxyflavone (**9**)	0.0±3.0	5.2±5.0	17.7±2.9**	39.5±1.8**	27.1±3.4**
5-Hydroxy-3,7-dimethoxyflavone (**10**)	0.0±0.6	8.1±0.9**	23.6±0.6**	39.5±2.1**	21.6±1.5**
3,5,7-Trimethoxyflavone (**11**)	0.0±3.2	7.6±3.0	20.1±2.4**	34.3±1.0**	37.3±2.1**
5-Hydroxy-3,7,4′-trimethoxyflavone (**12**)	0.0±5.7	18.2±1.9**	35.1±2.4**	34.4±1.4**	28.4±0.8**
3,5,7,4′-Tetramethoxyflavone (**13**)	0.0±2.5	10.4±3.6*	26.8±1.6**	47.0±1.5**	41.6±1.0**
5,3′-Dihydroxy-3,7,4′-trimethoxyflavone (**14**)	0.0±3.1	12.0±6.1*	24.6±5.5**	68.4±2.7**	38.4±3.3**
5-Hydroxy-3,7,3′,4′-tetramethoxyflavone (**15**)	0.0±4.5	21.2±1.6**	16.7±2.6**	41.6±4.5**	32.0±3.2**
3,5,7,3′,4′-Pentamethoxyflavone (**16**)	0.0±3.2	12.9±3.9**	33.0±3.7**	48.8±0.7**	32.7±0.7**
(2*R*,3*R*)-(−)-Aromadendrin trimethyl ether (**17**)	0.0±6.3	15.6±6.9	19.3±4.3*	14.6±5.4*	107.1±3.7**
Tilianine (**18**)	0.0±3.1	5.7±2.8	7.2±3.8	21.4±0.6**	39.5±0.9**
21	0.0±2.9	5.7±2.2	9.1±2.3*	14.0±2.0**	11.0±1.6**
22	0.0±4.5	7.4±1.7	9.3±3.8	0.6±4.9	9.8±2.1
23	0.0±2.4	7.7±5.5	7.8±4.7	−1.2±3.8	−1.6±2.8
2,4,6-Trihydroxyacetophenone 2,4-di-*O*-Glc (**24**)	0.0±5.4	4.6±3.1	8.3±2.0	5.5±8.1	29.4±2.1**
Silybin[a]	0.0±0.3	4.8±1.1	7.7±0.7	45.2±8.8**	77.0±5.5**

Each value represents the mean±S.E.M. (n=4). Significantly different from the control, *p<0.05, **p<0.01. *a*) Commercial silybin was purchased from Funakoshi Co., Ltd. (Tokyo, Japan).

Fr. 9 (1012.5 mg), Fr. 10 (1098.9 mg), Fr. 11 (1910.4 mg), Fr. 12 (489.4 mg), Fr. 13 (3852.3 mg), and Fr. 14 (30.83 g)}. The fraction 6 (250.5 mg) was purified by HPLC [Cosmosil $5C_{18}$-MS-II, MeOH–1% aqueous AcOH (70:30, v/v)] to give 5-hydroxy-7-methoxyflavone (**3**, 72.3 mg, 0.0230%) and **10** (144.5 mg, 0.0460%). The fraction 8 (250.4 mg) was separated by HPLC [Cosmosil $5C_{18}$-MS-II, MeOH–1% aqueous AcOH (70:30, v/v)] to give **3** (59.6 mg, 0.0790%), **10** (5.8 mg, 0.0077%), and 5-hydroxy-3,7,4'-trimethoxyflavone (**12**, 83.6 mg, 0.111%). The fraction 9 (250.0 mg) was purified by HPLC [Cosmosil $5C_{18}$-MS-II, MeOH–1% aqueous AcOH (80:20, v/v)] to give **3** (24.6 mg, 0.0079%) and **12** (171.9 mg, 0.0554%). The fraction 10 (250.5 mg) was separated by HPLC [Cosmosil $5C_{18}$-MS-II, MeOH–1% aqueous AcOH (70:30, v/v)] to give **3** (8.2 mg, 0.0029%), 5-hydroxy-7,4′-dimethoxyflavone (**6**, 96.0 mg, 0.0335%), and **12** (112.9 mg, 0.0394%). The fraction 11 (250.1 mg) was purified by HPLC [Cosmosil $5C_{18}$-MS-II, MeOH–1% aqueous AcOH (70:30, v/v)] to give 5,3′-dihydroxy-3,7,4′-trimethoxyflavone (**14**, 36.3 mg, 0.0221%), 5-hydroxy-3,7,3′,4′-tetramethoxyflavone (**15**, 73.2 mg, 0.0445%), **6** (53.4 mg, 0.0325%), and **12** (8.5 mg, 0.0052%). The fraction 12 (489.4 mg) was separated by HPLC [Cosmosil πNAP, CH_3CN–1% aqueous AcOH (45:55, v/v)] to give (2*R*,3*R*)-(−)-aromadendrin trimethyl ether (**17**, 12.9 mg, 0.0010%), 5,4'-dihydroxy-7-methoxyflavone (**5**, 34.6 mg, 0.0028%), **14** (68.6 mg, 0.0055%), 5-hydroxy-7,3′,4′-trimethoxyflavone (**8**, 6.0 mg, 0.00048%), and **15** (21.0 mg, 0.0017%). The fraction 13 (500.1 mg) was purified by HPLC [Cosmosil $5C_{18}$-MS-II, isopropanol–1% aqueous AcOH (30:70, v/v)] to give 3,5,7,4′-tetramethoxyflavone (**13**, 234.3 mg, 0.144%) and 3,5,7-trimethoxyflavone (**11**, 137.0 mg, 0.0840%). The fraction 14 (500.5 mg) was separated by HPLC [Cosmosil πNAP, CH_3CN–1% aqueous AcOH (35:65, v/v)] to give 5,7-dimethoxyflavone (**4**, 109.2 mg, 0.535%), 5,7,4′-trimethoxyflavone (**7**, 168.1 mg, 0.824%), 5,7,3′4′-tetramethoxyflavone (**9**, 19.2 mg, 0.0941%), **13** (28.9 mg, 0.142%), and 3,5,7,3′,4′-pentamethoxyflavone (**16**, 148.3 mg, 0.727%).

The MeOH-eluted fraction (13.60 g) was subjected to normal-phase silica gel CC [600 g, $CHCl_3$–MeOH–H_2O (10:3:0.4→7:3:0.5→6:4:1, v/v/v) →MeOH→acetone] to give 10 fractions [Fr. 1 (1113.3 mg), Fr. 2 (588.8 mg), Fr. 3 (606.2 mg), Fr. 4 (268.0 mg), Fr. 5 (519.8 mg), Fr. 6 (985.6 mg), Fr. 7 (634.1 mg), Fr. 8 (1850.0 mg), Fr. 9 (3.50 g), and Fr. 10 (3.50 g)]. The fraction 1 (1113.3 mg) was subjected to reversed-phase silica gel CC [35 g, MeOH–H_2O (70:30, v/v)→MeOH] to afford four fractions [Fr. 1-1 (40.0 mg), Fr. 1-2 (22.2 mg), Fr. 1-3 (798.4 mg), and Fr. 1-4 (6.7 mg)]. The fraction 1-3 (798.4 mg) was further purified by HPLC [Cosmosil $5C_{18}$-MS-II, MeOH–1% aqueous AcOH (60:40, v/v)] to give **4** (142.6 mg, 0.0129%), **7** (145.1 mg, 0.0131%), **9** (16.1 mg, 0.0015%), **11** (37.2 mg, 0.0034%), **13** (117.5 mg, 0.0106%), and **16** (189.1 mg, 0.0171%). The fraction 2 (588.8 mg) was subjected to reversed-phase silica gel CC [18 g, MeOH–H_2O (10:90→40:60→50:50→70:30, v/v)→MeOH] to afford seven fractions [Fr. 2-1 (37.5 mg), Fr. 2-2 (49.0 mg), Fr. 2-3 (97.8 mg), Fr. 2-4 (105.4 mg), Fr. 2-5 (71.9 mg), Fr. 2-6 (30.6 mg), and Fr. 2-7 (19.3 mg)]. The fraction 2-2 (49.0 mg) was purified by HPLC [Cosmosil $5C_{18}$-MS-II, MeOH–1% aqueous AcOH (20:80, v/v)] to give adenosine (8.9 mg, 0.00080%). The fraction 2-4 (105.4 mg) was separated by HPLC [Cosmosil $5C_{18}$-MS-II, MeOH–1% aqueous AcOH (25:75, v/v)] to give tilianine (**18**, 16.7 mg, 0.0015%). The fraction 2-5 (71.9 mg) was purified by HPLC [Cosmosil $5C_{18}$-MS-II, MeOH–1% aqueous AcOH (35:65, v/v)] to give tamarixetin 3-*O*-rutinoside (**19**, 3.9 mg, 0.00035%) and syringetin 3-*O*-rutinoside (**20**, 6.0 mg, 0.00054%). The fraction 2-6 (30.6 mg) was purified by HPLC [Cosmosil $5C_{18}$-MS-II, MeOH–1% aqueous AcOH (60:40, v/v)] to give **4** (4.0 mg, 0.00030%), **7** (4.1 mg, 0.00030%), and **16** (5.6 mg, 0.00040%). The fraction 3 (606.2 mg) was subjected to reversed-phase silica gel CC [19 g, MeOH–H_2O (20:80→50:50→70:30, v/v)→MeOH] to afford six fractions [Fr. 3-1 (97.7 mg), Fr. 3-2 (97.3 mg), Fr. 3-3 (155.6 mg), Fr. 3-4 (137.1 mg), Fr. 3-5 (11.5 mg), and Fr. 3-6 (16.6 mg)]. The fraction 3-3 (155.6 mg) was separated by HPLC [Cosmosil $5C_{18}$-MS-II, MeOH–1% aqueous AcOH (15:85, v/v)] to give L-phenylalanine (7.4 mg, 0.00070%). The fraction 5 (519.8 mg) was subjected to reversed-phase silica gel CC [19 g, MeOH–H_2O (10:90→30:70, v/v)→MeOH] to afford four fractions {Fr. 5-1 (9.7 mg), Fr. 5-2 (81.9 mg), Fr. 5-3 [=**22** (123.5 mg, 0.011%)], and Fr. 5-4 (76.1 mg)}. The fraction 5-4 (76.1 mg) was purified by HPLC [Cosmosil $5C_{18}$-MS-II, MeOH–1% aqueous AcOH (25:75, v/v)] to give kaempferiaoside B (**2**, 5.9 mg, 0.00050%) and **22** (8.7 mg, 0.00080%). The fraction 6 (985.6 mg) was subjected to reversed-phase silica gel CC [30 g, MeOH–H_2O (10:90→20:80→50:50, v/v)→MeOH] to afford eight fractions [Fr. 6-1 (41.6 mg), Fr. 6-2 (33.6 mg), Fr. 6-3 (127.1 mg), Fr. 6-4 (98.9 mg), Fr. 6-5 [=**23** (44.7 mg, 0.0040%)], Fr. 6-6 (30.1 mg), Fr. 6-7 (51.3 mg), and Fr. 6-8 (327.0 mg)]. The fraction 6-3 (127.1 mg) was separated by HPLC [Cosmosil $5C_{18}$-MS-II, MeOH–1% aqueous AcOH (10:90, v/v)] to give 2,4,6-trihydroxyacetophenone 2,4-di-*O*-β-D-glucopyranoside (**24**, 4.7 mg, 0.00043%). The fraction 6-4 (98.9 mg) was purified by HPLC [Cosmosil $5C_{18}$-MS-II, MeOH–1% aqueous AcOH (20:80, v/v)] to give **22** (26.6 mg, 0.0024%). The fraction 7 (634.1 mg) was subjected to reversed-phase silica gel CC [19 g, MeOH–H_2O (20:80→40:60, v/v)→MeOH] to afford four fractions [Fr. 7-1 (196.7 mg), Fr. 7-2 (35.6 mg), Fr. 7-3 (20.5 mg), and Fr. 7-4 (196.7 mg)]. The fraction 7-4 (196.7 mg) was separated by HPLC [Cosmosil $5C_{18}$-MS-II, MeOH–1% aqueous AcOH (25:75, v/v)] to give kaempferiaoside A (**1**, 28.5 mg, 0.0026%) and **21** (48.3 mg, 0.0044%).

Kaempferiaoside A (**1**): An amorphous powder, $[\alpha]_D^{23}$ −153.8° (*c*=0.23, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{57}H_{66}O_{32}Na$ $(M+Na)^+$: 1285.3435. Found: 1285.3438. CD [MeOH, nm ($\Delta\varepsilon$)]: 214 (−37.54), 245 (+19.28), 281 (−4.26), 292 (−2.60), 321 (−7.45). UV [MeOH, nm (logε)]: 234 (4.67), 280 (4.12), 328 (4.04). IR (KBr): 3400, 1624, 1458, 1339, 1277, 1225, 1094, 1069, 1015 cm^{-1}. ^{1}H-NMR (500 MHz, CD_3OD) δ: given in Table 2. ^{13}C-NMR data (125 MHz, CD_3OD) δ_C: given in Table 2. Positive-ion FAB-MS *m/z*: 1285 $(M+Na)^+$.

Kaempferiaoside B (**2**): An amorphous powder, $[\alpha]_D^{23}$ −166.8° (*c*=0.55, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{29}H_{34}O_{17}Na$ $(M+Na)^+$: 677.1694. Found: 677.1699. CD [MeOH, nm ($\Delta\varepsilon$)]: 213 (+10.67), 260 (−0.55), 318 (−4.58), 359 (−2.54). UV [MeOH, nm (logε)]: 259 (4.13), 369 (4.53). IR (KBr): 3400, 1638, 1541, 1509, 1474, 1350, 1281, 1142, 1073 cm^{-1}. ^{1}H-NMR (700 MHz, DMSO-d_6) δ: given in Table 3. ^{13}C-NMR data (175 MHz, DMSO-d_6) δ_C: given in Table 3. Positive-ion FAB-MS *m/z*: 677 $(M+Na)^+$.

Acid Hydrolysis of Kaempferiaosides A (1) and B (2) Solutions of **1** (1.2 mg) and **2** (1.0 mg) in 1 M HCl (1.0 mL)

were stirred at 80°C for 1h. After cooling, the reaction mixture was neutralized with Amberlite IRA-400 (OH^- form), and the resin was removed by filtration. On removal of the solvent from the filtrate under reduced pressure, the residue was partitioned in an EtOAc–H_2O (1 : 1, v/v) mixture, and the solvent was removed *in vacuo* from the EtOAc- and H_2O-soluble fractions. The H_2O-soluble fraction was subjected to HPLC analysis under the following conditions: HPLC column, Kaseisorb LC NH_2-60-5, 4.6mm i.d.×250mm (Tokyo Kasei Co., Ltd., Tokyo, Japan); detection, optical rotation [OR-2090 Plus (JASCO, Tokyo, Japan); mobile phase, CH_3CN–H_2O (80:20, v/v); flow rate 1.0mL/min]. Identification of L-rhamnose (i) and D-glucose (ii) from **1** and **2** present in the H_2O-soluble fraction was carried out by comparison of their retention time and optical rotation with those of authentic samples. t_R: (i) 8.9min (negative optical rotation) and (ii) 18.7min (positive optical rotation).

Bioassay. Reagents LPS (from *Salmonella enteritidis*), minimum essential medium (MEM), and William's E medium were purchased from Sigma-Aldrich Chemical (St. Louis, MO, U.S.A.); fetal calf serum (FCS) was from Life Technologies (Rockville, MD, U.S.A.); and other chemicals were from Wako Pure Chemical Industries, Co., Ltd. (Osaka, Japan). 96-Well microplates were purchased from Sumitomo Bakelite Co., Ltd. (Tokyo, Japan).

Effects on D-GalN-Induced Cytotoxicity in Primary Cultured Mouse Hepatocytes The hepatoprotective effect of the constituents were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay using primary cultured mouse hepatocytes.[48—51,53—56] Hepatocytes were isolated from male ddY mice (30—35g) by collagenase perfusion method. A cell suspension at 4×10^4 cells in 100μL William's E medium containing FCS (10%), penicillin G (100 units/mL), and streptomycin (100μg/mL) was inoculated in a 96-well microplate and precultured for 4h at 37°C under a 5% CO_2 atmosphere. The medium was added with 100μL of the fresh medium containing D-GalN (2mM) with or without the test sample and the hepatocytes were cultured for 44h. The medium was exchanged with 100μL of the fresh medium, and 10μL of MTT [5mg/mL in phosphate buffered saline (PBS)] solution was added to the medium. After 4h of cultivation, the medium was removed, and 100μL of isopropanol containing 0.04M HCl was then added to dissolve the formazan produced in the cells. The optical density (OD) of the formazan solution was measured by microplate reader at 570nm (reference: 655nm). Inhibition (%) was obtained by following formula.

$$\text{inhibition } (\%) = [(\text{OD(sample)} - \text{OD(control)})/(\text{OD(normal)} - \text{OD(control)})] \times 100$$

Statistics Values are expressed as means±S.E.M. One-way analysis of variance (ANOVA) followed by Dunnett's test was used for statistical analysis. Probability (p) values less than 0.05 were considered significant.

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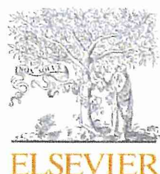

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Antidiabetogenic oligostilbenoids and 3-ethyl-4-phenyl-3,4-dihydroisocoumarins from the bark of *Shorea roxburghii*

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ABSTRACT

A methanol extract of the bark of *Shorea roxburghii* (Dipterocarpaceae) was found to inhibit plasma glucose elevation in sucrose-loaded mice. From the extract, three new 3-ethyl-4-phenyl-3,4-dihydroisocoumarins, 1′*S*-dihydrophayomphenol A_2 (**1**) and phayomphenols B_1 (**2**) and B_2 (**3**), were isolated together with 24 known compounds including 20 stilbenoids and oligostilbenoids. The structures of **1–3** were determined on the basis of their spectroscopic properties as well as of chemical evidences. Among the isolates, (−)-hopeaphenol (**6**), hemsleyanol D (**8**), (+)-α-viniferin (**15**), and (−)-balanocarpol (**18**) showed inhibitory activity against plasma glucose elevation in sucrose-loaded rats at doses of 100–200 mg/kg, p.o. To clarify the mode of action of the antihyperglycemic property, effects of these oligostilbenoids on gastric emptying in mice, those on glucose uptake in isolated intestinal tissues as well as inhibitory activities against rat intestinal α-glucosidase and rat lens aldose reductase were examined.

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1. Introduction

Shorea roxburghii G. DON (Dipterocarpaceae) is widely distributed in Thailand and its neighboring countries such as Cambodia, India, Laos, Malaysia, Myanmar, and Vietnam, etc. The bark of *S. roxburghii* ('Phayom' in Thailand) has been used as an astringent or a preservative for traditional beverages in Thailand. In Indian folk medicine, they have been used for treatments of dysentery, diarrhea, and cholera, etc.[1] In the course of our exploratory studies on bioactive constituents in Thai natural medicines,[2–13] we have reported isolation and structural elucidation of dihydroisocoumarins, stilbenoids, and oligostilbenoids from a methanol extract of the bark of *S. roxburghii*.[2] We also revealed that the methanol extract and several oligostilbenoid constituents showed antihyperlipidemic effect in olive oil-loaded mice and pancreatic lipase inhibitory activity.[2] As a continuing study, we conducted further evaluations of the extract and/or constituents, and found that the methanol extract showed inhibitory activity against increase in plasma glucose levels in sucrose-loaded mice. By the intensive fractionalization of the extract, three new 3-ethyl-4-phenyl-3,4-dihydroisocoumarins named 1′*S*-dihydrophayomphenol A_2 (**1**) and phayomphenols B_1 (**2**) and B_2 (**3**) have been isolated as minor constituents. This paper deals with the structural elucidation of these new dihydroisocoumarins (**1–3**) as well as inhibitory effects of the principal oligostilbenoid constituents on increase in plasma glucose levels in sucrose-loaded mice. To clarify the mechanism of action of the antidiabetogenic activity observed, effects of these oligostilbenoids on gastric emptying in mice, those on glucose uptake in isolated intestinal tissues as well as their inhibitory activities against rat intestinal α-glucosidase and rat lens aldose reductase were also examined.

2. Results and discussion

2.1. Effect of methanol extract of the bark of *S. roxburghii* on plasma glucose elevation in sucrose-loaded mice

The bark of *S. roxburghii* (collected in Phatthalung Province, Thailand) was extracted with methanol under reflux to yield a methanol extract (15.6% from the dried bark). The methanol extract was subjected to Diaion HP-20 column chromatography ($H_2O \rightarrow$ MeOH) to give H_2O- and MeOH-eluted fractions (3.2% and 10.6%, respectively). As shown in Table 1, the methanol extract and its MeOH-eluted fraction showed inhibitory effect against increase in plasma glucose levels in sucrose-loaded mice at a dose of 500 mg/kg, p.o.

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Table 1
Inhibitory effects of the methanolic extract from the bark of *S. roxburghii* and its methanol- and H_2O-eluted fractions on plasma glucose levels in sucrose-loaded mice

Treatment	Dose (mg/kg, p.o.)	*n*	Plasma glucose (mg/dL)[a]		
			0.5 h	1.0 h	2.0 h
Normal	–	4	121.4 ± 14.7[c]	124.9 ± 7.2[c]	106.5 ± 3.9[b]
Control	–	6	225.1 ± 12.5	229.9 ± 16.2	145.2 ± 7.2
MeOH extract	125	4	207.6 ± 22.6	213.4 ± 19.2	156.0 ± 7.4
	250	4	188.1 ± 17.9	180.0 ± 23.2	146.9 ± 12.6
	500	4	154.4 ± 9.8[c]	179.3 ± 12.1	167.6 ± 6.9
MeOH-eluted fraction	125	4	198.0 ± 13.9	185.8 ± 14.4	152.5 ± 3.4
	250	4	159.9 ± 17.2	172.7 ± 6.1	152.8 ± 3.4
	500	4	137.3 ± 4.4[c]	160.5 ± 14.4[a]	162.3 ± 8.0
H_2O-eluted fraction	250	4	176.7 ± 12.6	178.6 ± 14.0	142.7 ± 14.4
	500	4	230.6 ± 34.3	210.0 ± 20.9	142.3 ± 14.6
Normal	–	6	124.8 ± 7.3[c]	143.0 ± 5.4[c]	131.8 ± 6.4[c]
Control	–	9	218.7 ± 4.0	208.9 ± 6.8	163.7 ± 3.7
Acarbose	10	6	162.4 ± 11.7[c]	183.8 ± 3.8[b]	151.5 ± 6.3
	20	6	153.8 ± 10.2[c]	185.4 ± 8.1[b]	152.8 ± 3.8

[a] Each value represents the mean ± S.E.M.
[b] Significantly different from the control, $p < 0.05$.
[c] Significantly different from the control, $p < 0.01$.

2.2. Chemical constituents from the bark of *S. roxburghii*

In the preceding paper,[2] we reported the isolation and structure elucidation of two new dihydroisocoumarins, phayomphenols A_1 (**4**) and A_2 (**5**), relatively as the major constituents, the yield of which from the dried bark being 0.29% and 0.11%, respectively. By the intensive fractionation of the MeOH-eluted fraction in this study, three related compounds, 1′*S*-dihydrophayomphenol A_2 (**1**, 0.0061%) and phayomphenols B_1 (**2**, 0.0005%) and B_2 (**3**, 0.0006%) were isolated as new constituents. Twenty kinds of stilbenoids and oligostilbenoids, (−)-hopeaphenol (**6**, 0.63%), (+)-isohopeaphenol (**7**, 0.53%), hemsleyanol D (**8**, 0.30%), (−)-ampelopsin H (**9**, 0.015%), vaticanols B (**10**, 0.031%), C (**11**, 0.032%), A (**12**, 0.28%), E (**13**, 0.30%), and G (**14**, 0.042%), (+)-α-viniferin (**15**, 0.10%), pauciflorol A (**16**, 0.014%), hopeafuran (**17**, 0.012%), (−)-balanocarpol (**18**, 0.070%), (−)-ampelopsin A (**19**, 0.012%), malibatols A (**20**, 0.0029%) and B (**21**, 0.0007%), (+)-parviflorol (**22**, 0.0029%), *trans*-resveratrol 10-*C*-β-D-glucopyranoside (**23**, 0.90%), *cis*-resveratrol 10-*C*-β-D-glucopyranoside (**24**, 0.0081%), and piceid (**25**, 0.0098%), and a flavonol glycoside, quercetin 3-*O*-α-L-rhamnopyranoside (0.0050%), and a megastigmane glycoside, (6*S*,9*S*)-roseoside (0.0017%), were also isolated from the extracts of this plant (Chart 1).

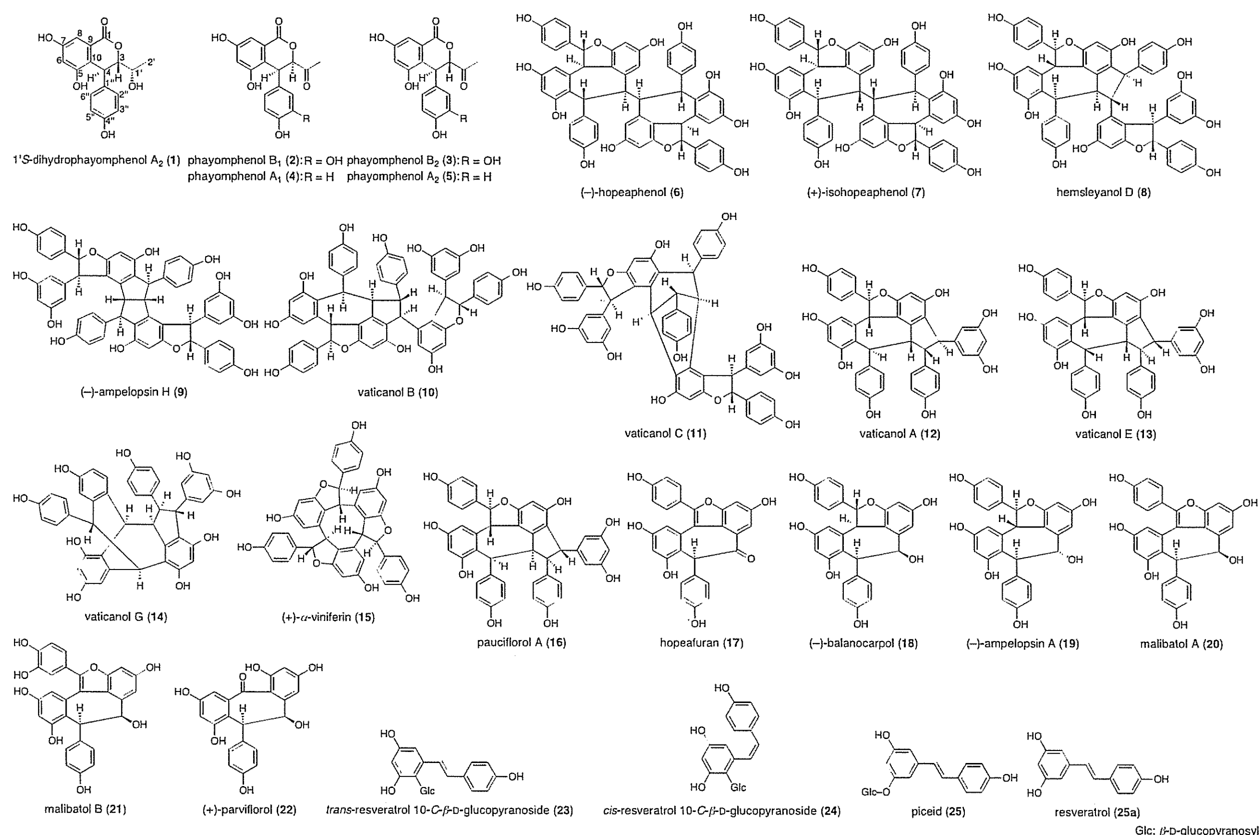

Chart 1. Constituents from the bark of *S. roxburghii*.

2.3. Structures of 1′S-dihydrophayomphenol A_2 (1) and phayomphenols B_1 (2) and B_2 (3)

1′S-Dihydrophayomphenol A_2 (**1**) was obtained as a white powder with positive optical rotation ($[\alpha]_D^{25}$ +79.5 in MeOH). Its IR spectrum showed absorption bands at 3390, 1698, 1615, 1514, 1474, 1362, and 1258 cm^{-1} ascribable to hydroxyls, lactone carbonyl functions, and an aromatic ring. The positive-ion FABMS spectrum of **1** showed a quasimolecular ion peak at *m/z* 339 $(M+Na)^+$, and the molecular formula was determined as $C_{17}H_{16}O_6$, a two hydrogen homolog to compound **5**, by high-resolution positive-ion FABMS measurement. The ^1H and ^{13}C NMR spectral properties of **1**[14] (Tables 2 and 3) were quite similar to those of **5** (Tables 4 and 5), except for signals due to 1′ and 2′ positions. Instead of a signal due to the carbonyl carbon [(δ_c 205.4 in CD_3OD)] observed in the ^{13}C NMR spectrum of **5**, a signal of methine carbon bearing an oxygen appeared at δ_c 69.9 (in CD_3OD) in that of **1**. In the ^1H NMR spectrum of **1** (pyridine-d_5, Tables 2 and 3), an additional methine signal appeared at δ 4.43 (1H, dq, *J* = 6.2, 6.6 Hz, H-1′), and thus the signal of the adjacent methyl appeared as a doublet at δ 1.52 (3H, d, *J* = 6.6 Hz, H_3-2′). Therefore, the structure of **1** was speculated to be the reduced product of **5** at C-1′ position. Actually oxidation of **1** with chromium trioxide (CrO_3) in pyridine afforded phayomphenol A_2 (**5**). The CD spectrum of the oxidized product was completely in accord with that of **5**, thus the absolute stereochemistry at C-3 and C-4 being approved to be *S* and *S*, respectively.

The absolute configuration of the C-1′ position in **1** was elucidated by the application of the modified Mosher's method.[15] Namely, 5,7,4″-trimethyl ester (**1a**), which was obtained from **1** upon reaction with trimethylsilyldiazomethane ($TMSCHN_2$), was derived to 1′-(*R*)-MTPA ester (**1b**) by treatment with (*R*)-2-methoxy-2-trifluoromethylphenylacetic acid [(*R*)-MTPA] in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC·HCl) and 4-dimethylaminopyridine (4-DMAP). On the other hand, 1′-(*S*)-MTPA ester (**1c**) was obtained from **1a** using (*S*)-MTPA under the same conditions. As shown in Figure 2, signals due to the protons at C-3 and C-4 in **1c** were observed at lower field compared with those of **1b** [$\Delta\delta$: positive], while the signal due to the proton at C-1′ in **1c** was observed at higher field compared with that of **1b** [$\Delta\delta$: negative]. Thus, the absolute configuration at C-1′ of **1a** was determined to be *S*. Consequently, the absolute stereostructure of **1** was elucidated to be as shown. ^1H–^1H COSY, HMQC, HMBC, and NOESY spectral properties also supported the depicted structure.

Phayomphenol B_1 (**2**), $[\alpha]_D^{25}$ +162.9 (in MeOH), was obtained as a white powder. Its IR spectrum showed absorption bands at 3400, 1717, 1698, 1617, 1362, 1250, and 1125 cm^{-1} ascribable to hydroxyls, a carbonyl, and a lactone carbonyl functions, and an aromatic ring. The molecular formula determined by means of high-resolution EIMS was $C_{17}H_{14}O_7$, a one oxygen homolog of **4**. Instead of *ortho*-coupled A_2B_2-type aromatic protons observed in the ^1H NMR spectrum of **4** [δ 6.64, 6.82 (2H each, both d, *J* = 8.6 Hz, H-3″,5″, 2″,6″)], *ortho*- and *meta*-coupled ABC-type aromatic protons

Table 3
^{13}C NMR (125 MHz) data on 1′S-dihydrophayomphenol A_2 (**1**) and **1a**

Position	**1**[a] δ_C	**1**[b] δ_C	**1a**[b] δ_C
1	165.2	166.9	166.4
3	90.3	90.7	90.3
4	38.0	37.9	38.3
5	157.0	156.8	158.7
6	109.1	109.3	105.6
7	159.0	158.8	161.6
8	107.4	107.4	104.5
9	128.6	128.0	127.9
10	119.7	119.7	123.0
1′	69.5	69.9	70.1
2′	20.8	20.0	20.0
1″	134.2	134.2	135.3
2″,6″	129.5	129.6	129.5
3″,5″	116.5	116.3	115.0
4″	157.8	157.3	160.1
5-*OCH₃*			55.7
7-*OCH₃*			56.1
4″-*OCH₃*			56.4

[a] Measured in pyridine-d_5.
[b] Measured in CD_3OD.

Table 4
^1H NMR (CD_3OD) data on phayomphenols B_1 (**2**), B_2 (**3**), A_1 (**4**), and A_2 (**5**)

Position	**2**[a] δ_H (J Hz)	**3**[a] δ_H (J Hz)	**4**[b] δ_H (J Hz)	**5**[b] δ_H (J Hz)
3	5.11 (d, 3.9)	5.27 (d, 1.2)	5.14 (d, 4.0)	5.28 (d, 1.5)
4	4.54 (d, 3.9)	4.74 (br s)	4.61 (d, 4.0)	4.80 (br s)
6	6.57 (d, 2.2)	6.55 (d, 2.2)	6.57 (d, 2.3)	6.55 (d, 2.3)
8	7.04 (d, 2.2)	6.98 (d, 2.2)	7.04 (d, 2.3)	6.99 (d, 2.3)
2′	1.88 (3H, s)	2.22 (3H, s)	1.86 (3H, s)	2.33 (3H, s)
2″	6.45 (d, 2.2)	6.54 (d, 2.2)	6.82 (d, 8.6)	6.96 (d, 8.6)
3″	6.62 (d, 8.2)	6.70 (d, 8.2)	6.64 (d, 8.6)	6.71 (d, 8.6)
5″	6.62 (d, 8.2)	6.70 (d, 8.2)	6.64 (d, 8.6)	6.71 (d, 8.6)
6″	6.33 (dd, 2.2, 8.2)	6.51 (dd, 2.2, 8.2)	6.82 (d, 8.6)	6.96 (d, 8.6)

[a] Measured at 700 MHz.
[b] Measured at 500 MHz.

Table 2
^1H NMR (500 MHz) data on 1′S-dihydrophayomphenol A_2 (**1**) and **1a–1c**

Position	**1**[a] δ_H (J Hz)	**1**[b] δ_H (J Hz)	**1a**[b] δ_H (J Hz)	**1b**[b] δ_H (J Hz)	**1c**[b] δ_H (J Hz)
3	5.01 (br d, ca. 6)	4.39 (dd, 1.2, 6.9)	4.42 (dd, 1.2, 5.8)	4.58 (dd, 1.2, 6.9)	4.64 (dd, 1.2, 8.0)
4	5.10 (br s)	4.39 (br s)	4.43 (br s)	4.38 (br s)	4.47 (br s)
6	7.11 (d, 2.4)	6.58 (d, 2.3)	6.81 (d, 2.3)	6.68 (d, 2.3)	6.86 (d, 2.3)
8	7.42 (d, 2.4)	7.01 (d, 2.3)	7.21 (d, 2.3)	7.22 (d, 2.3)	7.22 (d, 2.3)
1′	4.43 (dq, 6.2, 6.6)	3.79 (dq, 6.9, 6.6)	3.82 (m)	5.29 (m)	5.25 (m)
2′	1.52 (3H, d, 6.6)	1.19 (3H, d, 6.6)	1.17 (3H, d, 6.6)	1.45 (3H, d, 6.6)	1.31 (3H, d, 6.3)
2″,6″	7.46 (2H, d, 8.6)	6.89 (2H, d, 8.6)	6.96 (2H, d, 8.6)	6.91 (2H, d, 8.6)	6.95 (2H, d, 8.6)
3″,5″	7.09 (2H, d, 8.6)	6.68 (2H, d, 8.6)	6.81 (2H, d, 8.6)	6.80 (2H, d, 8.6)	6.82 (2H, d, 8.6)
5-*OCH₃*			3.72 (3H, s)	3.68 (3H, s)	3.77 (3H, s)
7-*OCH₃*			3.86 (3H, s)	3.89 (3H, s)	3.90 (3H, s)
4″-*OCH₃*			3.73 (3H, s)	3.73 (3H, s)	3.74 (3H, s)
1′-*OMTPA*				3.50 (3H, s)	3.50 (3H, s)
				7.36–7.41 (3H, m)	7.39–7.42 (3H, m)
				7.45–7.47 (2H, m)	7.47–7.48 (2H, m)

[a] Measured in pyridine-d_5.
[b] Measured in CD_3OD.

Table 5
^{13}C NMR (CD_3OD) data on phayomphenols B_1 (**2**), B_2 (**3**), A_1 (**4**), and A_2 (**5**)

Position	**2**[a]	**3**[a]	**4**[b]	**5**[b]
	δ_C	δ_C	δ_C	δ_C
1	166.9	166.7	166.9	166.7
3	86.9	89.3	86.7	89.1
4	40.5	38.7	40.2	38.5
5	156.5	156.8	156.4	156.7
6	109.8	109.2	109.7	109.2
7	159.2	159.3	159.1	159.3
8	107.6	107.5	107.5	107.5
9	126.7	127.8	126.9	127.8
10	121.7	117.6	121.6	117.5
1′	207.8	205.4	207.6	205.4
2′	27.7	26.0	27.6	26.0
1″	129.1	133.2	128.3	132.4
2″	117.3	115.9	131.2	129.8
3″	146.2	146.4	116.1	116.3
4″	145.8	145.5	157.9	157.6
5″	116.2	116.5	116.1	116.3
6″	121.7	120.1	131.2	129.8

[a] Measured at 175 MHz.
[b] Measured at 125 MHz.

[δ 6.33 (1H, dd, J = 2.2, 8.2 Hz, H-6″), 6.45 (1H, d, J = 2.2 Hz, H-2″), 6.62 (1H, d, J = 8.2 Hz, H-5″)] were detected in the ^1H NMR spectrum of **2**. As a HMBC cross-peak was observed between the signals due to C-4 (δ_C 40.5) and a signal at δ 6.45, which was assigned to H-2″, the position of the newly introduced OH group was deduced to be at C-3″. The ^1H–^1H COSY, HMBC and NOESY (Fig. 1) spectra supported well the structure of **2**.

Phayomphenol B_2 (**3**) was also isolated as a white powder with positive optical rotation ($[\alpha]_D^{25}$ +212.1 in MeOH). The molecular formula, $C_{17}H_{14}O_7$, obtained by the positive-ion high-resolution FABMS measurement, was found to be the same as that of **2**. The ^1H and ^{13}C NMR spectroscopic properties of **3** (CD_3OD, Tables 4 and 5) were quite similar to those of **2**, except for the signal due to the methyl group [δ 2.22 (3H, s, H_3-2′)]. The position of the newly introduced OH was determined in the same manner as was applied in the case for **2**, the *ortho*- and *meta*-coupled ABC-type aromatic protons [δ 6.51 (1H, dd, J = 2.2, 8.2 Hz, H-6″), 6.54 (1H, d, J = 2.2 Hz, H-2″), 6.70 (1H, d, J = 8.2 Hz, H-5″)] and a cross-peak between the signal due to C-4 (δ_C 38.7) and the signal at δ 6.54 being observed in the ^1H NMR and HMBC spectra, respectively.

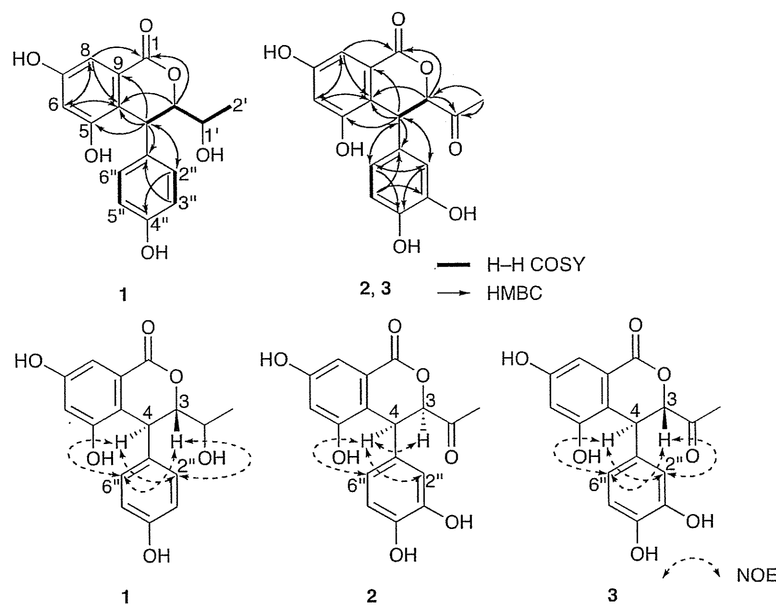

Figure 1. ^1H–^1H COSY, HMBC, and NOESY correlations of **1**–**3**.

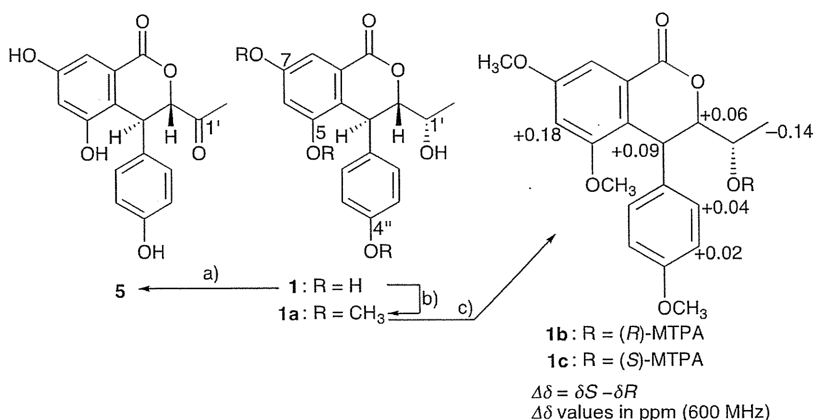

Figure 2. Determination of absolute stereochemistry of **1**. Reagents and conditions: (a) CrO_3/pyridine, rt, 8 h, 29%. (b) $TMSCHN_2$/MeOH, rt, 8 h, quant. (c) (R)- or (S)-MTPA, EDC·HCl,4-DMAP/CH_2Cl_2, Δ, 15 h, **1b**: 43%, **1c**: 66%.

Finally, the absolute stereochemistry of **2** and **3** were confirmed by their CD spectra, which showed a negative ($\Delta\varepsilon$ −0.14 at 296 nm) and a positive Cotton effect ($\Delta\varepsilon$ +4.37 at 294 nm), respectively. According to published CD data for 3,4-dihydroisocoumarins, these Cotton effects were assignable to $n \rightarrow \pi^*$ transition of the lactone carbonyl group of dihydroisocoumarin chromophore.[2,16,17] This indicated that the 3-acetyl group in **3** was in α-axial orientation, and accordingly the absolute configuration at C-3 of **3** was concluded to be *S*.[18–23] In the same manner, the absolute configuration at C-3 of **2** was confirmed to be *R*. These results indicated that the 3,4-dihydroxyphenyl moieties at C-4 in **2** and **3** were both in β-axial orientation, and their absolute configurations were also concluded to be *S*. On the basis of the foregoing evidences, the absolute stereostructures of **2** and **3** were confirmed as (3*R*,4*S*)- and (3*S*,4*S*)-3-acetyl-5,7-dihydroxy-4-(3,4-dihydroxyphenyl)-3,4-dihydroisocoumarin, respectively.

2.4. Antihyperglycemic effects of the chemical constituents from the bark of *S. roxburghii*

With respect to the principal constituents of *S. roxburghii*, phayomphenols A_1 (**4**) and A_2 (**5**), (−)-hopeaphenol (**6**), (+)-isohopeaphenol (**7**), hemsleyanol D (**8**), (+)-α-viniferin (**15**), (−)-balanocarpol (**18**), *trans*-resveratrol 10-*C*-β-D-glucopyranoside (**23**), and resveratrol (**25a**),[24–27] effects on increase in plasma glucose levels in sucrose-loaded mice were examined. As shown in Table 6, oligostilbenoids, **6**, **8**, **15**, and **18** showed significant inhibitory activity against increase in plasma glucose levels at a dose of 100 or 200 mg/kg, p.o., and at 200 mg/kg, p.o. (p = 0.070), **7** tended to inhibit the elevation. The inhibitory effects of **8** and **15** were stronger than those of **6**, **7**, and **18**, while a stilbene monomer (**25a**) and its glycoside (**23**) showed weak effects. In this experiment, an intestinal α-glucosidase inhibitor, acarbose, was employed as a positive control, which showed reasonable inhibition as was reported in the literature (Table 1).[28]

Next, the mode of action for the antihyperglycemic effects of the stilbenoids was studied. Plasma glucose levels are known to be regulated by many factors such as transport of sugar in the digestive tract, the secretion and release of hormones, and absorption of glucose through membranes of the small intestine.[29] Previously, we reported that inhibition of gastric emptying markedly inhibited plasma glucose and triglyceride elevations in sucrose- and olive oil-loaded mice or rats, respectively.[3,30–37] Thus, effects of the oligostilbenes (**6–8**, **15**, and **18**) on the gastric emptying rate were examined. As shown in Table 7, compounds **6**, **7**, and **18** significantly suppressed the gastric emptying in mice at a dose of 200 mg/kg, p.o., and **15** also tended to suppress gastric emptying (p = 0.063) at the same dose. These findings suggest that these oligostilbenoids inhibited the plasma glucose elevation in orally sucrose-loaded mice possibly in part by suppressing the transfer of sucrose from the stomach to the small intestine.

Next, effects of these constituents on glucose uptake in rat small intestinal tissues[38,39] and also those on inhibitory activities[28,31,40–42] against rat small intestinal α-glucosidases, maltase and sucrase, were examined. As shown in Table 8, principal oligostilbenoids (**6–8**, **15**, and **18**) did not inhibit glucose uptake at a concentration of 1 mM. On the other hand, most of the oligostilbenoids (**6–13**, **15**, **17**, **20**, and **21**) moderately inhibited the α-glucosidase activities as shown in Table 9. These results suggest that the intestinal α-glucosidase inhibitory activity is also involved in the inhibitory effects of the oligostilbenoids (**6–8** and **15**, but not **18**) against blood glucose elevation in sucrose-loaded mice.

Finally, inhibitory effects of these constituents including minor contents on rat lens aldose reductase were examined. Aldose reductase is known to be a key enzyme which catalyzes the reduction of glucose to sorbitol in the polyol processing pathway. Sorbitol dose not readily diffuse across cell membranes, and the intracellular accumulation of sorbitol has been implicated in the chronic complications of diabetes such as cataracts.[27,43] As shown in Table 9, several dihydroisocoumarins (**2–5**) and stilbenoids (**6**,

Table 6
Inhibitory effects of the constituents from tfhe bark of *S. roxburghii* on plasma glucose levels in sucrose-loaded mice

Treatment	Dose (mg/kg, p.o.)	*n*	Plasma glucose (mg/dL)[a]		
			0.5 h	1.0 h	2.0 h
Normal	–	6	126.6 ± 5.2[c]	139.8 ± 5.6[c]	116.0 ± 3.2
Control	–	7	220.5 ± 16.9	221.4 ± 9.1	160.8 ± 3.5
Phayomphenol A_1 (**4**)	100	7	189.3 ± 10.1	199.5 ± 10.1	159.2 ± 5.9
	200	7	184.1 ± 15.5	178.1 ± 11.0[b]	166.2 ± 7.0
Phayomphenol A_2 (**5**)	100	6	231.2 ± 22.6	226.7 ± 19.8	179.4 ± 12.9
	200	7	177.0 ± 10.4	197.3 ± 6.0	166.9 ± 8.0
Normal	–	7	115.7 ± 9.4[c]	125.2 ± 10.3[c]	129.9 ± 10.2[c]
Control	–	10	232.9 ± 11.8	229.0 ± 9.6	172.0 ± 11.3
(−)-Hopeaphenol (**6**)	100	6	191.6 ± 13.9	194.9 ± 5.9	160.6 ± 7.3
	200	7	158.3 ± 5.6[c]	187.9 ± 6.1	179.4 ± 4.4
(+)-Isohopeaphenol (**7**)	100	6	182.5 ± 19.1	204.9 ± 7.3	191.6 ± 10.8
	200	7	179.7 ± 8.2	209.4 ± 11.0	189.1 ± +6.8
Hemsleyanol D (**8**)	100	6	173.5 ± 16.8[b]	211.8 ± 24.3	174.1 ± 16.6
	200	7	142.4 ± 22.7[c]	201.7 ± 24.9	177.4 ± 15.2
(+)-α-Viniferin (**15**)	100	6	151.2 ± 21.2[c]	219.8 ± 6.1	173.3 ± 9.1
	200	7	153.5 ± 19.2[c]	184.2 ± 7.7	167.2 ± 6.9
(−)-Balanocarpol (**18**)	100	6	227.9 ± 12.6	210.6 ± 12.0	157.4 ± 5.1
	200	7	169.2 ± 14.5[c]	192.0 ± 10.8	156.4 ± 9.7
Normal	–	8	119.4 ± 4.8[c]	131.9 ± 2.7[c]	114.7 ± 3.9[c]
Control	–	8	226.5 ± 17.3	200.9 ± 4.3	148.8 ± 6.3
trans-Resveratrol 10-*C*-Glc (**23**)	100	8	184.3 ± 17.8	183.4 ± 10.0	140.9 ± 6.0
	200	8	188.7 ± 10.1	202.3 ± 12.4	147.9 ± 3.1
Normal	–	5	137.5 ± 7.2[c]	142.1 ± 11.0[c]	135.9 ± 8.1[c]
Control	–	10	230.9 ± 15.7	203.0 ± 9.3	174.5 ± 7.1
Resveratrol (**25a**)	100	7	218.3 ± 12.2	210.9 ± 14.0	181.4 ± 4.5
	200	6	205.6 ± 8.0	192.7 ± 7.7	158.7 ± 7.5

[a] Each value represents the mean ± S.E.M.
[b] Significantly different from the control, p <0.05.
[c] Significantly different from the control, p <0.01.

Table 7
Inhibitory effects of the constituents from the bark of *S. roxburghii* on gastric emptying in CMC-Na-loaded mice

Treatment	Dose (mg/kg, p.o.)	*n*	Gastric emptying (%)[a]	Inhibition (%)
Control	–	9	82.0 ± 3.1	–
(–)-Hopeaphenol (**6**)	200	8	58.2 ± 2.7[b]	29.0
(+)-Isohopeaphenol (**7**)	200	8	64.7 ± 5.1[b]	21.1
Hemsleyanol D (**8**)	200	4	74.9 ± 6.5	8.7
(+)-α-Viniferin (**15**)	200	8	69.4 ± 3.4	15.4
(–)-Balanocarpol (**18**)	200	7	61.7 ± 2.3[b]	24.8

[a] Each value represents the mean ± S.E.M.
[b] Significantly different from the control, $p < 0.01$.

Table 8
Inhibitory effects of the constituents from the bark of *S. roxburghii* on glucose uptake in rats intestinal tissues

	Conc. (mM)	*n*	Glucose uptake[a] (dpm/100 mg tissue)	Inhibition (%)
Control	–	6	3172 ± 419	0.0
Phrolizin	1	6	1606 ± 103[b]	100.0
(–)-Hopeaphenol (**6**)	1	6	2830 ± 259	21.8
(+)-Isohopeaphenol (**7**)	1	6	3038 ± 240	8.5
Control	–	10	3992 ± 370	0.0
Phrolizin	1	5	1642 ± 128[b]	100.0
Hemsleyanol D (**8**)	1	5	3595 ± 311	16.9
(+)-α-Viniferin (**15**)	1	5	3564 ± 254	18.2
(–)-Balanocarpol (**18**)	1	5	4088 ± 396	–4.1

[a] Each value represents the mean ± S.E.M.
[b] Significantly different from the control, $p < 0.01$.

Table 9
Inhibitory effects of the methanolic extract from the bark of *S. roxburghii* and its methanol- and H_2O-eluted fractions and the constituents on enzyme activities of α-glucosidases, and aldose reductase activities

	α-Glucosidase IC_{50}[a] (μg/mL)		Aldose reductase IC_{50}[a] (μg/mL)
	Maltase	Sucrase	
MeOH extract	150	128	19.6
MeOH-eluted fraction	235	183	17.9
H_2O-eluted fraction	>400 (4.8)[c]	>400 (6.5)[c]	>400 (0.9)[c]
	α-Glucosidase IC_{50}[a] (μM)		Aldose reductase IC_{50}[a] (μM)
	Maltase	Sucrase	
1′*S*-Dihydrophayomphenol A_2 (**1**)	>400 (31.4)[c]	>400 (30.3)[c]	>100 (18.8)[b]
Phayomphenol B_1 (**2**)	>400 (20.9)[c]	ca. 400	32.5
Phayomphenol B_2 (**3**)	>400 (40.4)[c]	211	26.6
Phayomphenol A_1 (**4**)	>400 (4.0)[c]	>400 (6.4)[c]	39.8
Phayomphenol A_2 (**5**)	>400 (19.6)[c]	>400 (3.7)[c]	47.7
(–)-Hopeaphenol (**6**)	338	195	69.0
(+)-Isohopeaphenol (**7**)	216	90.0	>100 (34.4)[b]
Hemsleyanol D (**8**)	266	218	29.4
(–)-Ampelopsin H (**9**)	178	97.1	50.2
Vaticanol B (**10**)	233	294	30.0
Vaticanol C (**11**)	140	94.2	21.2
Vaticanol A (**12**)[31]	218	148	23.7
Vaticanol E (**13**)[31]	342	88.9	30.8
Vaticanol G (**14**)[31]	>400 (32.2)[c]	>400 (37.8)[c]	>100 (46.7)[b]
(+)-α-Viniferin (**15**)	172	234	7.8
Pauciflorol A (**16**)	>400 (44.0)[c]	55.7	29.5
Hopeafuran (**17**)	142[c]	105	6.9
(–)-Balanocarpol (**18**)	>400 (29.4)[c]	>400 (36.6)[c]	30.0
(–)-Ampelopsin A (**19**)	> 400 (–6.3)	>400 (0.4)[c]	68.8
Malibatol A (**20**)	362	231	35.6
Malibatol B (**21**)	262	143	10.0
(+)-Parviflorol (**22**)	>400 (6.8)[c]	>400 (-0.8)[c]	>100 (43.7)[b]
trans-Resveratrol 10-C-Glc (**23**)	>400 (20.1)[c]	>400 (14.7)[c]	>100 (30.3)[b]
cis-Resveratrol 10-C-Glc (**24**)	>400 (36.6)[c]	>400 (26.8)[c]	>100 (39.2)[b]
Piceid (**25**)	>400 (38.2)[c]	>400 (48.0)[c]	ca. 100
Resveratrol (**25a**)	>400 (36.9)[c]	>400 (35.7)[c]	25.0[27]
Acarbose[31]	2.0	1.7	
Epalrestat[31]			0.072

[a] Each value represents the mean of 2–4 experiments.
[b,c] Values in parentheses present inhibition % at 100 or 400 μM.

8–13, **15–21**, and **25a**) moderately inhibited the activity of rat lens aldose reductase.

In conclusion, the methanol extract of the bark of *S. roxburghii* was found to inhibit plasma glucose elevation in sucrose-loaded mice. Three new 3-ethyl-4-phenyl-3,4-dihydroisocoumarins, 1′*S*-dihydrophayomphenol A_2 (**1**) and phayomphenols B_1 (**2**) and B_2 (**3**), were isolated together with 24 known compounds including 20 stilbenoids and oligostilbenoids. Among the isolates, (−)-hopeaphenol (**6**), hemsleyanol D (**8**), (+)-α-viniferin (**15**), and (−)-balanocarpol (**18**) significantly inhibited plasma glucose elevation in sucrose-loaded mice at doses of 100–200 mg/kg, p.o., and (+)-isohopeaphenol (**7**) tended to inhibit it at a dose of 200 mg/kg, p.o. As the possible mechanism of action responsible for the antihyperglycemic effect of the extract, following multiple modes were recursively induced on the basis of the observed activities of the constituents; (1) inhibition of gastric emptying: constituents **6**, **7**, and **18** significantly suppressed the gastric emptying, and **15** also acted moderately, (2) α-glucosidase inhibitory activity: constituents **6–8** and **15** moderately inhibited enzyme activities of rat small intestinal sucrose, (3) aldose reductase inhibitory activity: constituents **2–6**, **8–13**, **15–21**, and **25a** moderately inhibited the rat lens aldose reductase. Inhibition of glucose uptake was excluded as a mode of the action because major constituents **6–8**, **15**, and **18** did not inhibit glucose uptake in rat small intestinal tissues.

3. Experimental

3.1. General

The following instruments were used to obtain spectral and physical data: specific rotations, Horiba SEPA-300 digital polarimeter (l = 5 cm); CD spectra, JASCO J-720WI spectrometer; UV spectra, Shimadzu UV-1600 spectrometer; IR spectra, Shimadzu FTIR-8100 spectrometer; ^1H NMR spectra, JEOL JNM-ECA700 (700 MHz), JNM-ECA500 (500 MHz), and JNM-ECS400 (400 MHz) spectrometers; ^{13}C NMR spectra, JEOL JNM-ECA700 (175 MHz), JNM-ECA500 (125 MHz), and JNM-ECS400 (100 MHz) spectrometers with tetramethylsilane as an internal standard; EIMS and HREIMS, JEOL JMS-GCMATE mass spectrometer; FABMS and HRFABMS, JEOL JMS-SX 102A mass spectrometer; HPLC detector, Shimadzu SPD-10A UV–VIS detectors; HPLC column, Cosmosil $5C_{18}$-MS-II and πNAP (250 × 4.6 mm i.d. and 250 × 20 mm i.d. for analytical and preparative purposes, respectively).

The following experimental conditions were used for chromatography: normal-phase silica gel column chromatography (CC), silica gel 60 N (Kanto Chemical Co., Ltd, 63–210 mesh, spherical, neutral); reversed-phase silica gel CC, Diaion HP-20 (Nippon Rensui) and Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd, 100–200 mesh); TLC, pre-coated TLC plates with silica gel $60F_{254}$ (Merck, 0.25 mm) (normal-phase) and silica gel RP-18 F_{254S} (Merck, 0.25 mm) (reversed-phase); reversed-phase HPTLC, pre-coated TLC plates with silica gel RP-18 WF_{254S} (Merck, 0.25 mm), detection was achieved by spraying with 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4, followed by heating.

3.2. Plant material

The bark of *S. roxburghii* was collected in Phatthalung Province, Thailand on September 2006. The plant material was identified by one of the authors (Y.P.). A voucher specimen (2006.09. Raj-02) of this plant is on file in our laboratory.

3.3. Extraction and isolation

Dried barks of *S. roxburghii* (3.7 kg) were finely cut and extracted three times with MeOH under reflux for 3 h. Evaporation of the combined extracts under reduced pressure provided a MeOH extract (575.7 g, 15.6%). An aliquot (525.7 g) was subjected to Diaion HP-20 CC (3.0 kg, H_2O → MeOH, twice) to give H_2O-eluted (107.0 g, 3.2%) and MeOH-eluted (358.5 g, 10.6%) fractions. An aliquot (180.0 g) of the MeOH-eluted fraction was subjected to normal-phase silica gel CC [3.0 kg, $CHCl_3$–MeOH–H_2O (10:3:0.4 → 7:3:0.5 → 6:4:1, v/v/v) → MeOH] to give eight fractions [Fr. 1 (2.82 g), Fr. 2 (8.20 g), Fr. 3 (9.20 g), Fr. 4 (66.38 g), Fr. 5 (18.29 g), Fr. 6 (20.11 g), Fr. 7 (25.93 g), and Fr. 8 (31.25 g)]. The fraction 2 (8.20 g) was subjected to reversed-phase silica gel CC [250 g, MeOH–H_2O (20:80 → 40:60 → 50:50, v/v) → MeOH] to afford seven fractions [Fr. 2-1 (4.8 mg), Fr. 2-2 (41.1 mg), Fr. 2-3 (55.3 mg), Fr. 2-4 (774.3 mg), Fr. 2-5 (1256.3 mg), Fr. 2-6 (5540.5 mg), and Fr. 2-7 (1081.9 mg)] as reported previously.[2] The fraction 2-4 (500.4 mg) was purified by HPLC [Cosmosil $5C_{18}$-MS-II, MeOH–1% aqueous AcOH (20:80, v/v)] to give 1′*S*-dihydrophayomphenol A_2 (**1**, 22.5 mg, 0.0021%) and phayomphenols B_1 (**2**, 5.1 mg, 0.0005%) and B_2 (**3**, 6.7 mg, 0.0006%) together with phayomphenols A_1 (**4**, 6.4 mg, 0.0006%) and A_2 (**5**, 442.6 mg, 0.040%). The fraction 2–5 (500.7 mg) was purified by HPLC [Cosmosil $5C_{18}$-MS-II, MeOH–1% aqueous AcOH (30:70, v/v)] to give **1** (9.9 mg, 0.0015%) together with **4** (123.0 mg, 0.018%) and **5** (165.4 mg, 0.025%).

3.3.1. 1′*S*-Dihydrophayomphenol A_2 (1)

A white powder, $[\alpha]_D^{25}$ +79.5 (*c* 0.17, MeOH). Positive-ion FABMS: *m/z* 339 $(M+Na)^+$. High-resolution positive-ion FABMS: Calcd for $C_{17}H_{16}O_6Na$ $(M+Na)^+$: 339.0845. Found: 339.0849. CD [MeOH, nm (Δε)]: 228 (−22.20), 265 (+2.86), 330 (+1.25). UV [MeOH, nm (log ε)]: 261 (3.72), 327 (3.45). IR (KBr): 3390, 1698, 1615, 1514, 1474, 1362, 1258, 1223, 1123, 1017 cm^{-1}. ^1H NMR (500 MHz, pyridine-d_5 and CD_3OD) δ: given in Table 2. ^{13}C NMR (125 MHz, pyridine-d_5 and CD_3OD) δ_C: given in Table 3.

3.3.2. Phayomphenol B_1 (2)

A white powder, $[\alpha]_D^{25}$ +162.9 (*c* 0.10, MeOH). EIMS *m/z* (%): 330 $(M^+, 11)$, 43 (100). High-resolution EIMS: Calcd for $C_{17}H_{14}O_7$ (M^+): 330.0740. Found: 330.0733. CD [MeOH, nm (Δε)]: 228 (−6.79), 267 (+2.56), 296 (−0.14). UV [MeOH, nm (log ε)]: 262 (3.53), 328 (3.30). IR (KBr): 3400, 1717, 1698, 1617, 1362, 1250, 1125 cm^{-1}. ^1H NMR (700 MHz, CD_3OD) δ: given in Table 2. ^{13}C NMR (175 MHz, CD_3OD) δ_C: given in Table 3.

3.3.3. Phayomphenol B_2 (3)

A white powder, $[\alpha]_D^{25}$ +212.1 (*c* 0.08, MeOH). Positive-ion FABMS: *m/z* 353 $(M+Na)^+$. High-resolution positive-ion FABMS: Calcd for $C_{17}H_{14}O_7Na$ $(M+Na)^+$: 353.0637. Found: 353.0630. CD [MeOH, nm (Δε)]: 227 (−13.63), 270 (+3.83), 294 (+4.37). UV [MeOH, nm (log ε)]: 260 (3.52), 324 (3.28). IR (KBr): 3400, 1732, 1717, 1541, 1509, 1362, 1123 cm^{-1}. ^1H NMR (700 MHz, CD_3OD) δ: given in Table 2. ^{13}C NMR (175 MHz, CD_3OD) δ_C: given in Table 3.

3.4. Chromium trioxide (CrO_3) oxidation of 1

To a solution of **1** (3.5 mg) in pyridine (0.2 mL) was added CrO_3 (6.6 mg)–pyridine (0.8 mL), and the mixture was stirred at 60 °C for 8 h. The reaction mixture was poured into saturated aqueous NaCl and the resulting mixture was extracted with EtOAc. The extract was washed with brine, and dried over anhydrous $MgSO_4$. Removal of the solvent under reduced pressure gave a pale yellow oil, which was purified by HPLC [Cosmosil $5C_{18}$-MS-II, MeOH–1% aqueous AcOH (70:30, v/v)] to furnish **5** (1.0 mg, 29%) as a white powder.

3.5. Methylation of 1

To a solution of **1** (8.5 mg) in MeOH (1.0 mL) was added trimethylsilyldiazomethane ($TMSCHN_2$, 10% in hexane, ca. 0.5 mL).

The mixture was stirred at room temperature for 8 h. Removal of the solvent under reduced pressure gave **1a** (9.6 mg, quant.).

3.5.1. Compound 1a

A white powder, $[\alpha]_D^{25}$ +69.6 (*c* 0.10, MeOH). Positive-ion FABMS: *m/z* 381 (M+Na)$^+$. High-resolution positive-ion FABMS: Calcd for $C_{20}H_{22}O_6Na$ (M+Na)$^+$: 381.1314. Found: 381.1309. UV [MeOH, nm (logε)]: 258 (3.67), 319 (3.45). IR (KBr): 3400, 1717, 1609, 1512, 1458, 1362, 1252, 1132, 1046 cm^{-1}. ^{1}H NMR (500 MHz, CD_3OD) δ: given in Table 2. ^{13}C NMR (125 MHz, CD_3OD) δ_C: given in Table 3.

3.6. Preparation of (*R*)-MTPA ester (1b) and (*S*)-MTPA ester (1c) from 1a

A solution of **1a** (1.9 mg) in CH_2Cl_2 (1.0 mL) was treated with (*R*)-2-methoxy-2-trifluoromethylphenylacetic acid [(*R*)-MTPA, 6.5 mg] in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl, 5.6 mg) and 4-dimethylaminopyridine (4-DMAP, 2.0 mg), and the mixture was heated under reflux for 15 h. The reaction mixture was poured into ice-water and extracted with EtOAc. The extract was successively washed with 5% aqueous HCl, saturated aqueous $NaHCO_3$, and brine, then dried over anhydrous $MgSO_4$ and filtered. Removal of the solvent from the filtrate under reduced pressure furnished a pale yellow oil, which was purified by HPLC [Cosmosil 5C_{18}-MS-II, MeOH–1% aqueous AcOH (70:30, v/v)] to give **1b** (1.3 mg, 43%). According to the similar procedure, **1c** (2.0 mg, 66%) was obtained from **1a** (1.9 mg) by using (*S*)-MTPA (6.5 mg), EDC·HCl (5.6 mg), and 4-DMAP (2.0 mg).

3.6.1. Compound 1b

^{1}H NMR (500 MHz, CD_3OD) δ: given in Table 2.

3.6.2. Compound 1c

^{1}H NMR (500 MHz, CD_3OD) δ: given in Table 2.

3.7. Bioassay

3.7.1. Animals

Male ddY mice were purchased from Kiwa Laboratory Animal Co., Ltd, (Wakayama, Japan). The animals were housed at a constant temperature of 23 ± 2 °C and were fed a standard laboratory chow (MF, Oriental Yeast Co., Ltd, Tokyo, Japan). The animals were fasted for 20–24 h prior to the beginning of experiments, but were allowed free access to a tap water. All of the experiments were performed with conscious mice unless otherwise noted. The experimental protocol was approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University.

3.7.2. Effects on plasma glucose elevation in sucrose-loaded mice

The experiments were performed according to the method as described in our previous reports with a slight modification.[3,30,31] Thus, each test sample was administrated orally to the fasted male ddY mice (body weight 24–27 g), and 20% (w/v) sucrose solution (10 mL/kg, p.o.) was administrated 30 min thereafter. Blood samples (ca. 0.1 mL) were collected from the infraorbital venosus plexus under ether anesthesia 0.5, 1, and 2 h after the oral administration of sucrose. The collected blood was immediately mixed with heparin sodium (5 units/tube). After centrifugation of the blood samples, the plasma glucose level was determined enzymatically by the Glucose CII test Wako (Wako Pure Chemical Industries Ltd, Osaka, Japan). An intestinal α-glucosidase inhibitor acarbose was used as a reference compound.

3.7.3. Effect on gastric emptying in mice

The experiments were performed according to the method as described in our previous reports.[3,30–37] A solution of 1.5% CMC-Na containing 0.05% phenol red as a marker was given intragastrically (0.3 mL/mouse) to conscious mice. Thirty minutes latter, the mice were killed by cervical dislocation. The abdominal cavity was opened, the gastroesophageal junction and the pylorus were elamped, and then the stomach was removed, weighed, placed in 10 mL of 0.1 M NaOH, and homogenized. The suspension was allowed to settle for 1 h at room temperature, 1 mL of the supernatant was added to 0.1 mL of 20% (w/v) trichloroacetic acid, and then centrifuged at 3000 rpm for 20 min. The supernatant (0.1 mL) was mixed with 0.1 mL of 0.5 M NaOH, and the amount of the phenol red was determined on the basis of the optical density (OD) at 560 nm using a microplate reader (SH-1000 Lab., Corona Electric Co., Ltd). A phenol red solution (0.3 mL) was used as the standard (0% emptying). Test samples were given orally via a metal orogastric tube 30 min prior to the administration of the test meals. Gastric emptying rate (%) in the 30-min period was calculated according to the following equation:

$$\text{Gastric emptying rate (\%)} \\ = (1 - \text{amount of the test sample/amount of the standard}) \times 100$$

3.7.4. Effects on glucose uptake in rat small intestinal tissues

The experiments were performed according to the method as described in our previous reports with a slight modification.[38,39] Thus, small fragments (0.10–0.15 g) of everted rat intestine were placed in 1 mL of modified Krebs–Henseleit solution, pH 7.4, with ^{14}C-U-glucose (2 mM, 1 × 10^5 cpm/mL) with or without a test sample. Incubation was carried out at 30 °C for 6 min, then the pieces were washed two times for 3–5 s with the medium containing 1 mM phlorizin without ^{14}C-U-glucose, and placed on a filter paper to absorb the water from the tissue. The tissue was then weighed and dissolved using 2 M NaOH. After neutralization by 2 M HCl, the radioactivity was examined. Each test sample was dissolved in dimethyl sulfoxide (DMSO) and added to an incubation solution (final DMSO concentration was 0.5%). Inhibitions of the uptake by the vehicle only and 1 mM phrolizin were calculated to be 0% and 100%, respectively.

3.7.5. Effects on rat intestinal α-glucosidase

The experiments were performed according to the method as described in our previous reports with a slight modification.[18,27–30] Thus, a rat small intestinal brush border membrane fraction was prepared and its suspension in 0.1 M maleate buffer (pH 6.0) was used to determine the small intestinal α-glucosidase activity of maltase and sucrase. A mixture of a substrate (maltose: 37 mM, sucrose: 37 mM), a test compound, and the enzyme in 0.1 M maleate buffer (pH 6.0, 0.1 mL) were incubated at 37 °C. After 30 min of incubation, 0.4 mL of water was added to the test tube, and the tube was immediately immersed in boiling water for 2 min to stop the reaction and then cooled with water. The glucose concentration was determined using the enzymatic method. Each test sample was dissolved in DMSO and the measurements were performed in duplicate, and IC_{50} values were determined graphically. The concentration of each enzyme suspension was adjusted to produce ca. 0.30 and 0.15 μmol/tube of D-glucose from the substrate maltose and sucrose, respectively. The intestinal α-glucosidase inhibitor acarbose was used as a reference compound.

3.7.6. Effects on rat lens aldose reductase

The experiments were performed according to the method as described in our previous reports.[27,43] Thus, the supernatant fluid

of a rat lens homogenate was used as a crude enzyme. The incubation mixture contained 135 mM phosphate buffer (pH 7.0), 100 mM Li_2SO_4, 0.03 mM NADPH, 1 mM DL-glyceraldehyde as a substrate, and 100 μL of enzyme fraction, with 25 μL of sample solution, in a total volume of 0.5 mL. The reaction was initiated by the addition of NADPH at 30 °C. After 30 min, the reaction was stopped by the addition of 150 μL of 0.5 M HCl. Then, 0.5 mL of 6 M NaOH containing 10 mM imidazole was added, and the mixture was heated at 60 °C for 20 min to convert NADP into a fluorescent product. The fluorescence intensity was measured using a fluorescence microplate reader (FLUOstar OPTIMA, BMG Labtechnologies) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Each test sample was dissolved in DMSO and the measurements were performed in duplicate, and IC_{50} values were determined graphically. The concentration of enzyme suspension was adjusted to produce ca. 10 nmol/tube of β-nicotinamide adenine dinucleotide phosphate (NADP). An aldose reductase inhibitor epalrestat was used as a reference compound.

3.8. Statistics

Values are expressed as means ± S.E.M. One-way analysis of variance (ANOVA) followed by Dunnett's test was used for statistical analysis. Probability (p) values less than 0.05 were considered significant.

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ヒト（自己）体性幹細胞加工医薬品等の品質及び安全性の確保について

ヒト由来の細胞・組織を加工した医薬品又は医療機器の品質及び安全性を確保するための基本的な技術要件については、平成20年２月８日付け薬食発第0208003号厚生労働省医薬食品局長通知「ヒト（自己）由来細胞や組織を加工した医薬品又は医療機器の品質及び安全性の確保について」の別添及び平成20年９月12日付け薬食発第0912006号厚生労働省医薬食品局長通知「ヒト（同種）由来細胞や組織を加工した医薬品又は医療機器の品質及び安全性の確保について」の別添（以下、「平成20年２指針」という。）により通知したところである。

今般、ヒト由来の体性幹細胞うち、自己由来体性幹細胞を加工した医薬品又は医療機器の品質及び安全性の確保のための基本的な技術要件については、平成20年２指針に代えて、新たな指針を別添「ヒト（自己）体性幹細胞加工医薬品等の品質及び安全性の確保に関する指針」のとおりとりまとめたので、御了知の上、貴管内関係業者等が自己由来体性幹細胞を加工した医薬品又は医療機器を開発する際等に参考として利用できるよう周知願いたい。

ヒト（自己）体性幹細胞加工医薬品等の品質及び安全性の確保に関する指針

はじめに

1．　本指針は、ヒト由来の体性幹細胞のうち、自己由来体性幹細胞を加工した医薬品又は医療機器(以下「ヒト(自己)体性幹細胞加工医薬品等」という)の品質及び安全性の確保のための基本的な技術要件について定めるものである。
　しかしながら、体性幹細胞加工医薬品等の種類や特性、臨床上の適用法は多種多様であり、また、本分野における科学的進歩や経験の蓄積は日進月歩である。本指針を一律に適用したり、本指針の内容が必要事項すべてを包含しているとみなしたりすることが必ずしも適切でない場合もある。したがって、個々の医薬品等についての試験の実施や評価に際しては本指針の目的を踏まえ、その時点の学問の進歩を反映した合理的根拠に基づき、ケース・バイ・ケースで柔軟に対応することが必要であること。

2．　薬事戦略相談あるいは治験相談におけるヒト体性幹細胞加工医薬品等の治験を開始するに当たっての基本的留意点は、当該製品にヒトへの適用により支障となる品質及び安全性上の明らかな問題が存在するか否か、臨床で得られた知見との関係性を照合できる程度に品質特性が把握され、その一定範囲の恒常性が確保されているか否かを確認することにある。その際、明らかに想定される製品のリスクを現在の学問・技術を駆使して排除し、その科学的妥当性を明らかにした上で、なお残る「未知のリスク」と、重篤で生命を脅かす疾患、身体の機能を著しく損なう疾患、身体の機能や形態を一定程度損なうことによりQOLを著しく損なう疾患などに罹患し、従来の治療法では限界があり、克服できない患者が「新たな治療機会を失うことにより被るかもしれないリスク」とのリスクの大小を勘案し、かつ、これらすべての情報を開示した上で患者の自己決定権に委ねるという視点を持つこと、すなわち、リスク・期待されるベネフィットの情報を開示した上で、治験に入るかどうかの意思決定は患者が行うという視点を入れて評価することも重要である。したがって、治験開始の場合、その届出に当たって添付するべき資料について本指針に示された要件や内容をすべて充たすことを必ずしも求めている訳ではない。製造販売承認申請時における品質及び安全性の確保のための資料は治験の進行とともに本指針に沿って充実整備されることを前提に、治験開始時点でその趣旨に適う条件を充たし、合理的に作成された適切な資料を提出すること。
　また、治験開始に必要とされる資料の範囲及び程度については、当該製品の由来、対象疾患、対象患者、適用部位、適用方法及び加工方法等により異なり、本指針では具体的に明らかでないことも少なくないので、個別に独立行政法人医薬品医療機器総合機構に相談することが望ましい。

3．　本指針に記述された事項、試験方法、基準その他の技術要件は、それぞれの目的に適う内容と程度をもとに考慮、選択、適用、及び評価されるべきことを意図して

おり、必ずしも常に同一（最高）水準での解釈、運用を求めている訳ではない。この趣旨を踏まえ、申請者は、考慮した背景、選択、適用、及び評価した内容と程度がそれぞれの目的に相応しく、科学的合理性からみて妥当であることを明らかにすること。

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