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→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→2 : 1→1 : 1→1 : 2, v/v) → 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 ($\log\varepsilon$)]: 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 ($\log\varepsilon$)]: 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 1 h. 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, 4.6 mm i.d.×250 mm (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 1.0 mL/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) 8.9 min (negative optical rotation) and (ii) 18.7 min (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—35 g) by collagenase perfusion method. A cell suspension at 4×10^4 cells in 100 μL William's E medium containing FCS (10%), penicillin G (100 units/mL), and streptomycin (100 μg/mL) was inoculated in a 96-well microplate and precultured for 4 h at 37°C under a 5% CO_2 atmosphere. The medium was added with 100 μL of the fresh medium containing D-GalN (2 mM) with or without the test sample and the hepatocytes were cultured for 44 h. The medium was exchanged with 100 μL of the fresh medium, and 10 μL of MTT [5 mg/mL in phosphate buffered saline (PBS)] solution was added to the medium. After 4 h of cultivation, the medium was removed, and 100 μL of isopropanol containing 0.04 M 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 570 nm (reference: 655 nm). 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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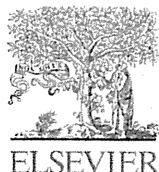
Bioorganic & Medicinal Chemistry 20 (2012) 832–840



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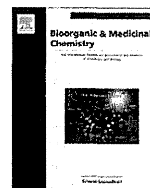

Antidiabetogenic oligostilbenoids and 3-ethyl-4-phenyl-3,4-dihydroisocoumarins from the bark of *Shorea roxburghii*

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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).

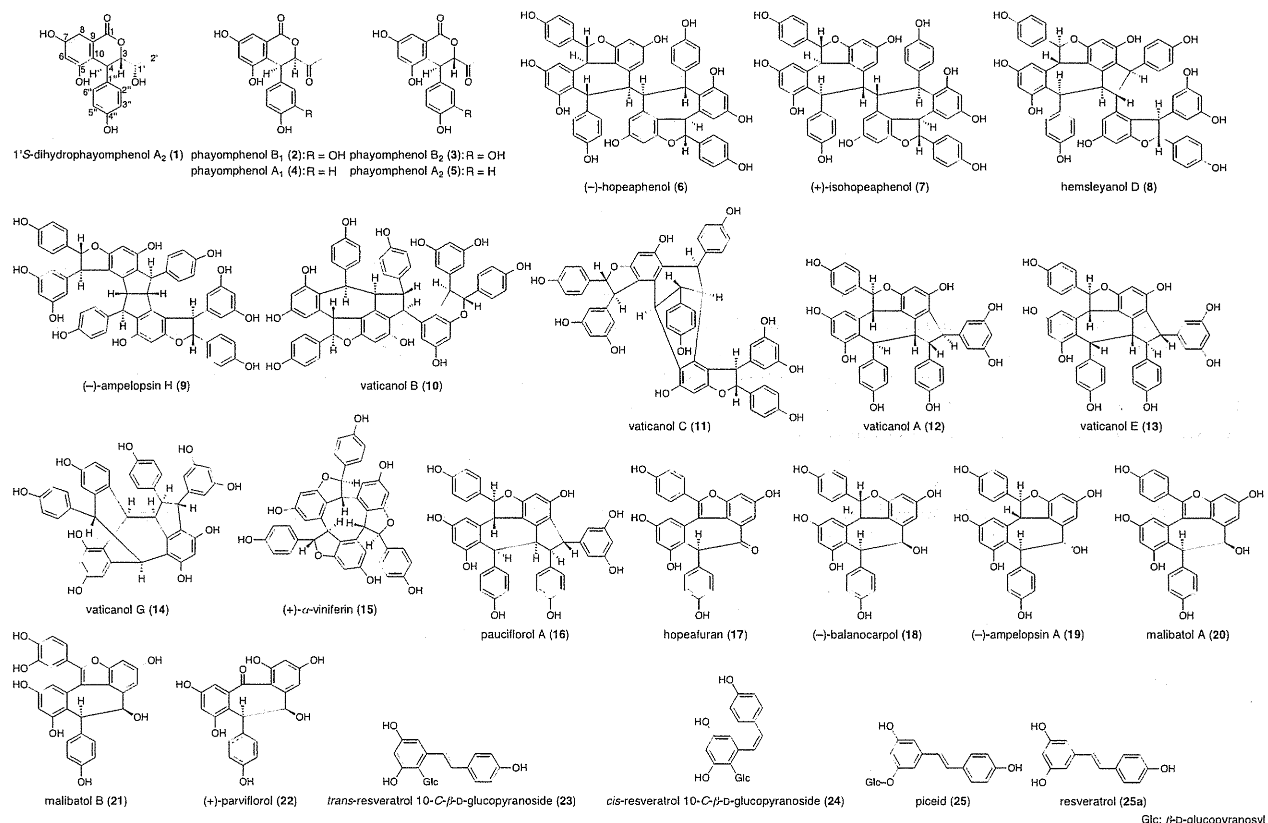

Chart 1. Constituents from the bark of *S. roxburghii*.

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-*OCH*$_3$			55.7
7-*OCH*$_3$			56.1
4″-*OCH*$_3$			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-*OCH*$_3$			3.72 (3H, s)	3.68 (3H, s)	3.77 (3H, s)
7-*OCH*$_3$			3.86 (3H, s)	3.89 (3H, s)	3.90 (3H, s)
4″-*OCH*$_3$			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 ^{1}H 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 ^{1}H–^{1}H 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 ^{1}H 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 ^{1}H NMR and HMBC spectra, respectively.

Figure 1. ^{1}H–^{1}H 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; ^{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; 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}. ^{1}H 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}. ^{1}H 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}. ^{1}H 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 (log ε)]: 258 (3.67), 319 (3.45). IR (KBr): 3400, 1717, 1609, 1512, 1458, 1362, 1252, 1132, 1046 cm^{-1}. ^1H 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

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

3.6.2. Compound 1c

^1H 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:

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

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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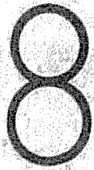

ヒトES・iPS細胞に由来する再生医療製品の造腫瘍性をどう見るか？

中島啓行，安田 智，佐藤陽治

ヒト多能性幹細胞を加工して製造される再生医療製品（ヒト多能性幹細胞加工製品）は，従来の方法では治療困難な疾病・損傷に対するブレークスルーとして期待を集めている．その開発においては想定されるリスク評価や品質・安全性確保に対する方策が求められる．特に造腫瘍性の評価が重要な課題であるが，ヒト多能性幹細胞加工製品の造腫瘍性試験のガイドラインは今のところ存在しない．ヒト多能性幹細胞加工製品の造腫瘍性試験は製造工程上の目的別に３つに分けられ，その目的に応じて各種造腫瘍性関連試験法を選択する必要がある．本項では，製品の品質・安全性評価における造腫瘍性関連試験の考え方とその適用について概説する．

はじめに

ヒト多能性幹細胞に分化誘導などの加工を施した**再生医療製品**は，従来の方法では治療が困難な疾病・損傷に対するブレークスルーとして期待されており，国内外で研究開発が盛んに行われている．このような，一昔前には想定されていなかった全く新しい製品の開発においては，想定されるリスクの評価法や品質・安全性確保のための基盤技術の整備が必須である．ヒト由来の胚性幹細胞（ES細胞）や人工多能性幹細胞（iPS細胞）といった多能性幹細胞は**造腫瘍性**をもっていることから，多能性幹細胞を用いて製造される製品においては造腫瘍性の評価と品質管理が重要な課題となっている．しかしながら，移植による治療目的で患者に投与するヒト由来の生細胞を対象にした造腫瘍性試験のガイドラインは，今のところ存在しない．本項では，ヒト多能性幹細胞を用いた再生医療の実現において不可避である造腫瘍性評価の現状と課題について概説する．

ヒト多能性幹細胞の造腫瘍性

多能性幹細胞は無限の自己複製能とあらゆる種類の細胞へと分化できる分化多能性によって定義される．その能力は，免疫不全マウスに移植した場合にテラトーマ（奇形腫）と呼ばれる腫瘍を形成することによって確認されるが，これは同時に，ヒト多能性幹細胞を製造基材とする再生医療製品（ヒト多能性幹細胞加工製品）は，未分化なヒト多能性幹細胞

再生医療製品：再生医療・細胞治療に使用されることが目的とされている物のうち，ヒトまたは動物の細胞に培養その他の加工を施したもの．細胞・組織加工製品とも呼ばれる．

造腫瘍性：動物に移植された細胞集団が増殖することにより，良性または悪性の腫瘍を形成する能力．

表1　多能性幹細胞に由来する再生医療製品の造腫瘍性に影響を及ぼす要因の例

多能性幹細胞に起因する要因	その他の要因
・目的細胞への分化の難しさ ・原材料となる体細胞の種類* ・初期化の方法* ・初期化因子の残存* ・細胞増殖の条件（培地・添加物など） ・ゲノムの安定性およびインテグリティ	・投与部位 ・投与細胞数 ・目的細胞の種類（特定の液性因子の分泌など） ・製造工程における処理（分化誘導・純化など） ・患者の免疫状態 ・共時投与物（マトリゲルなど）の有無

*iPS細胞の場合のみ

の残留・混入により腫瘍を形成する可能性があることを示している．ヒトES細胞を用いた研究では，線維芽細胞と懸濁したわずか数百個のES細胞の投与によって免疫不全マウス（SCIDマウス）に腫瘍が形成されることが報告されている[1]．

現在，高効率の分化誘導法や残存する多能性幹細胞の除去法などが精力的に研究されているが，100％の純度で目的細胞を調製・製造することは非常に困難である．したがって，製品にどれくらい未分化な多能性幹細胞が残存しているのか，最終製品は投与部位で造腫瘍性をもつのか，といった点を適切な試験系を用いて評価することが，実用化に向けての必須事項である．

1．造腫瘍性の2つのリスク

「造腫瘍性のリスク」は，安全性上の視点から大きく2つ，すなわち「腫瘍による物理的障害のリスク」と「悪性腫瘍形成のリスク」に分けられる．「腫瘍による物理的障害」とは，腫瘍形成により周辺組織が圧迫などを受けることによる障害で，関節再生・脊髄損傷再生などのケースで問題となる．この場合はたとえ良性であっても腫瘍自体がリスクファクターとなる．一方，「悪性腫瘍形成」は，腫瘍の悪性度がリスクファクターとなる．

実は，ヒトES/iPS細胞が免疫不全マウス内で増殖分化して形成される奇形腫は多くの場合，良性であり，正常2倍体のヒトES細胞を免疫不全マウスに移植して悪性腫瘍が発生したという報告はない．しかしながら，ヒト由来iPS細胞に関しては，免疫不全マウスに投与した場合に悪性腫瘍が形成されたという報告が存在する[2]．再生医療製品の製造基材としてのヒト多能性幹細胞に内在する奇形腫悪性化にかかわる因子・機序の詳細は明らかではないが，iPS細胞樹立時の細胞初期化過程は，悪性形質転換の研究で従来用いられてきた発がんフォーカス形成試験（*in vitro*での遺伝子導入による悪性肉腫形成試験）との類似性が指摘され，共通の機序の存在が提唱されている[3]．

2．造腫瘍性に影響を及ぼす要因

ヒト多能性幹細胞由来の再生医療製品の中に残存する未分化細胞の造腫瘍性には，さまざまな要素，すなわち，目的細胞への分化の難しさのほか，ヒトiPS細胞の場合には，原材料となる体細胞の種類や初期化因子残存の有無[2]など製造基材としての多能性幹細胞に付随する要因と，投与部位，投与細胞数，製造工程における処理，患者の免疫状態，マトリゲルなどの共時投与物の有無といった要因とが影響しうる（表1）．したがって，**最終製品**

の造腫瘍性に影響する製造基材（多能性幹細胞）の品質特性プロファイルは目的とする最終製品ごとに異なり，不適格な製造基材をどのような評価法で事前に排除するか，その方策も最終製品ごとに明らかにする必要がある．

造腫瘍性試験国際ガイドライン

前述の通り，多能性幹細胞加工製品の造腫瘍性評価は，再生医療の実現における重要な課題であるが，現在，再生医療製品を対象とした造腫瘍性試験ガイドラインは存在しない．細胞の造腫瘍性試験に関する国際的なガイドラインとして唯一存在するのは，世界保健機関（WHO）の生物薬品標準化専門委員会第47次報告（1998）（Technical Report Series No. 878：TRS 878）にあるAnnex I「生物薬品製造用の*in vitro*基材としての動物細胞の使用の要件」[4)5)]である（以下，WHO TRS 878とする）．

WHO TRS 878にある造腫瘍性試験の目的は，セル・バンクの造腫瘍性の程度を品質特性指標として把握し，その変化を細胞特性上の異常発生の検知のために利用することにある．ただし，ここで注意しなければならないのは，この試験の適用対象は生物薬品（ワクチンやタンパク質製剤など）を製造する際に用いられる動物由来細胞株であり，ヒトに投与される再生医療製品およびその製造基材は対象としていない点である．**WHO TRS 878の試験は，あくまで細胞株のセル・バンクという均一な細胞集団の造腫瘍性評価を対象にしているため，ごくわずかに混入する未分化・造腫瘍性細胞に起因する再生医療製品の造腫瘍性の評価を目的とした場合，そのまま転用することには感度などの面で無理がある．**混入するごく少数の未分化・造腫瘍性細胞に起因する再生医療製品の造腫瘍性を評価するにはWHO TSR 878よりも感度を上げるなど，目的に応じた適切な評価系の開発が必要となる．

ヒト多能性幹細胞加工製品の造腫瘍性試験

ヒト多能性幹細胞加工製品の製造における造腫瘍性試験は，目的別に次の3つが存在しうる（図）．

①製造基材となる細胞の品質管理のための造腫瘍性試験
②製造工程（中間製品）評価のための造腫瘍性試験
③最終製品の安全性評価のための造腫瘍性試験

①および②は品質試験，③は非臨床安全性試験という位置づけとなる．

細胞集団の造腫瘍性あるいは細胞集団中の造腫瘍性細胞の検出には，表2に示す*in vitro*/*in vivo*試験法が知られており，これらを組み合わせることで，それぞれの目的に応じた評価が可能であると考えられる．次に，上記3種の造腫瘍性試験の特徴と方法について，WHO

WHO TRS 878にある造腫瘍試験の概要：「ヌードマウスなどの動物10匹に10^7個の細胞を投与して16週間（1998年版では12週間）観察する．陽性対照としてはHeLa細胞などを用いる」というもの

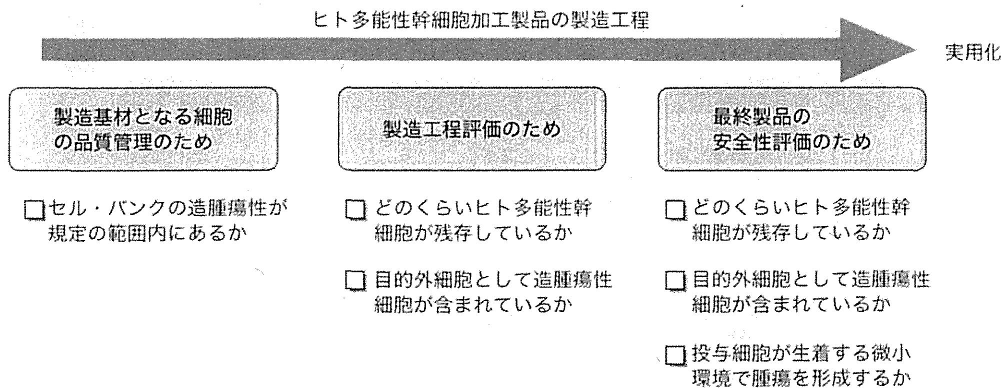

図　造腫瘍性試験が必要な３つの目的と各段階での懸念事項

TRS 878との関連も含めて述べる．

1．製造基材となる細胞の品質管理のための造腫瘍性試験

製造基材となる多能性幹細胞の品質管理のための造腫瘍性における懸念事項は，「セル・バンクの造腫瘍性が規定の範囲内にあるか」という点にある．多能性幹細胞加工製品の製造基材であるヒトES・iPS細胞バンクの造腫瘍性の程度に大幅な変化が生じた場合，既知あるいは未知のウイルス感染，変異原性物質やストレスによる遺伝子変異・発がん遺伝子活性化など，原因はいずれにせよ，細胞特性に何らかの異常が起こったということが示唆される．つまり，ヒトES・iPS細胞バンクの造腫瘍性を細胞特性指標の１つとして評価すれば，ヒトES・iPS細胞バンクの異常を検出し，品質管理に活用することができる．その評価方法については，セル・バンクという均一な細胞集団を対象とするため，WHO TRS 878の方法を準用することが可能であると考えられる．

2．製造工程（中間製品）評価のための造腫瘍性試験

ヒト多能性幹細胞加工製品の中間製品となる細胞集団には，「目的細胞」，「目的細胞の前駆細胞」，「残存多能性幹細胞」および「その他の目的外細胞」の４種類が含まれている可能性がある．したがって，

①どのくらいヒト多能性幹細胞が残存しているか
②目的外細胞として造腫瘍性細胞が含まれているか

という２点が，製造工程（中間製品）評価における造腫瘍性の懸念事項となる．

1）どのくらいヒト多能性幹細胞が残存しているか

①については，未分化多能性幹細胞特異的なマーカーを指標としたフローサイトメトリーや定量RT-PCRによる評価が可能である．この評価法の利点は感度が高い点にあり，われわれは初代培養ヒト体細胞中にヒトiPS細胞を添加して評価した結果，フローサイトメトリーでは0.1 %，定量RT-PCRの場合には0.002 %の存在比のヒトiPS細胞を検出するこ

表2 主な造腫瘍性関連試験の能力と限界

in vivo 試験法

試験法	測定事項	目的	利点	欠点
ヌードマウスへの移植	腫瘍形成	造腫瘍性細胞の検出	定量化の方策が整備（WHO TRS 878）	時間（数週間〜数カ月）・費用がかかる 膵がん，乳がん，グリア細胞腫，リンパ腫，白血病細胞に由来する細胞株は腫瘍を形成しない わずかに含まれる造腫瘍性細胞を検出できない
NOD-SCIDマウスへの移植			ヌードマウスよりも高感度	時間（数週間〜数カ月）・費用がかかる 定量化の方策が未整備 胸腺腫を自然発症
NOG/NSGマウスへの移植			INOD-SCIDよりも高感度／胸腺腫なし	時間（数週間〜数カ月）・費用がかかる 定量化の方策が未整備

in vitro 試験法

試験法	測定事項	目的	利点	欠点
細胞増殖特性解析（所定培養期間を超えた培養）	細胞増殖速度	不死化細胞の検出	簡便・安価 ときにはヌードマウスよりも高感度 （不死化していても腫瘍形成のないケース）	わずかな不死化細胞の混入の検出には時間がかかる
フローサイトメトリー	細胞マーカータンパク質発現	造腫瘍性細胞・未分化細胞の検出	短時間（〜1日）・簡便 ときには軟寒天コロニー試験よりも高感度 細胞を識別・分離・回収できる	特定のマーカー発現細胞だけしか検出できない（＝マーカー（−）の造腫瘍性細胞を見逃すおそれ） ゲートの掛け方で結果がばらつく
qRT-PCR	細胞マーカー遺伝子発現		短時間（〜1日）・簡便 ときにはフローサイトメトリーよりも高感度	特定のマーカー発現細胞だけしか検出できない（＝マーカー（−）の造腫瘍性細胞を見逃すおそれ）
軟寒天コロニー形成試験	コロニー形成	足場非依存的増殖の検出	*in vivo* 試験より短時間（数週間〜1カ月程度） 安価 ときにはヌードマウスよりも高感度	浮遊系細胞に使用できない わずかに含まれる造腫瘍性細胞を検出できない ヒトES/iPS細胞は検出不能（分散誘導性細胞死）
核型分析	染色体の数・サイズ・形	染色体異常の検出	技術的に確立	相関性の問題（染色体異常⇔造腫瘍性） わずかに含まれる造腫瘍性細胞を検出できない
染色体CGHおよびアレイCGH	ゲノムDNAのコピー数異常			
蛍光 *in situ* ハイブリダイゼーション（FISH）分析	特定遺伝子の位置・コピー数			

ができることを明らかにしている[6]．

2）目的外細胞として造腫瘍性細胞が含まれているか

一方，②を検出するための試験系としては，細胞増殖特性解析（増殖曲線による不死化細胞の検出）や，軟寒天コロニー形成試験による足場非依存性増殖細胞の検出が挙げられる．われわれは軟寒天コロニー形成試験において，ヒトテラトカルシノーマ細胞を1％の検出限界で検出可能であることを報告している．しかしながら，ヒト多能性幹細胞はシングルセルにまで分散させるとアポトーシスを起こすという特異な性質をもつため，残存するヒト多能性幹細胞の検出（①）に，軟寒天コロニー形成試験は不向きである[4]．

また，②の評価に *in vivo* の方法を活用することも可能である．しかしながら，均一な細

胞集団を対象としたWHO TRS 878にある造腫瘍性試験では，正常細胞中にわずかに混入する未分化・造腫瘍性細胞を検出するには感度が低く，結果が偽陰性になってしまう恐れが高いため，より感度の高い系を用いる必要がある．そこで有力な選択肢として挙げられるのが，Rag2-γC double-knockout（DKO）[7]，NOD/SCID/γC^{null}（NOG）[8]，NOD/SCID/IL2rgKO（NSG）[9]などの重度免疫不全マウス系統を利用する検出系である．これらのマウスはT細胞，B細胞およびNK細胞を欠失しており，ヌードマウスなどの従来の免疫不全マウスと比べてヒトの細胞や組織の生着性が高く，ヒトがん細胞を非常に高い効率で生着させることが可能といわれている[10][11]．われわれがNOGマウスにマトリゲルと懸濁したHeLa細胞を皮下投与し，腫瘍形成に必要な細胞数を検討した結果，WHO TRS 878にある造腫瘍性試験に比べ，2,000倍以上の感度の上昇が認められた（投稿準備中）．重度免疫不全マウス系統を利用した試験系開発における課題としては，a）試験系の検出限界・感度・精度，b）陽性・陰性コントロールのあり方，c）投与細胞数，d）観察期間，e）投与経路，f）投与方法，g）ヌードマウスとの比較，などを検討していく必要がある．

3．最終製品の安全性評価のための造腫瘍性試験

ヒト多能性幹細胞加工製品の最終製品には，中間製品と同じく，「目的細胞」，「目的細胞の前駆細胞」，「残存多能性幹細胞」，および「その他の目的外細胞」の4種類が含まれている可能性がある．ただし中間製品の場合とは異なり，最終製品の造腫瘍性試験においては，生着部位での腫瘍形成能を考察できることが要求される．そのため，

①どのくらいヒト多能性幹細胞が残存しているか
②目的外細胞として造腫瘍性細胞が含まれているか
③投与細胞が，生着する微小環境で腫瘍を形成するか

ということが最終製品における造腫瘍性の懸念事項となる．①，②については，中間製品評価の場合と同様，多能性幹細胞のマーカータンパク質/マーカー遺伝子の検出（①），不死化細胞の検出や足場非依存性増殖細胞の検出（②）などでそれぞれ評価が可能であると考えられる．

一方，③については，*in vivo* 造腫瘍性試験による評価が必要となる．その場合に考慮すべき点として，a）試験系の検出限界，b）陽性・陰性コントロールのあり方，c）投与細胞数，d）観察期間，e）投与部位，f）例数などが挙げられる．特に，投与部位に関しては，生着部位の違いによって腫瘍形成能や，腫瘍のタイプが異なる恐れがあるため，可能な限りヒトでの投与部位に相当する部位にするべきである（表2，図）．

4．新技術による造腫瘍性評価の可能性

ヒト多能性幹細胞は，細胞株と培養条件によっては遺伝子・染色体に異常が生じることが報告されている[12][13]．そのため，ヒト多能性幹細胞加工製品および製造基材であるヒトES・iPS細胞の造腫瘍性評価に次世代シーケンサーを使えないかという議論がある．

1）次世代シーケンサーによる造腫瘍性評価

全ゲノムシーケンスや全エキソンシーケンスのデータを用いて遺伝子変異を網羅的に検出し，造腫瘍性細胞の混入を検知する，というのがその狙いである．しかしながら，こうしたアプローチは現実的にはあまり用をなさない．主な理由は，**ヒト多能性幹細胞加工製品の安全性との因果関係が明瞭な遺伝子変異の具体例は乏しく，個々の最終製品の安全性の指標としてどのような変異の検出が有用なのか明らかではない**からである．感度面でも，次世代シーケンサーでは細胞集団中の1％未満のみが保持しているようなマイナーな変異を検出するのは難しく，充分とはいえない．

また，ヒトES・iPS細胞由来製品の造腫瘍性を評価するうえでは，「製造基材となる幹細胞の造腫瘍性と最終製品の造腫瘍性との相関・因果関係は未解明である」という点に最大の注意が必要である．すなわち，**臨床適用に際しては，原材料や製造基材ではなくあくまで最終製品としてのヒトES・iPS細胞由来製品の造腫瘍性評価が最も重要であることに常に留意しなければならない**．したがって，製造基材としての多能性幹細胞のシーケンスデータ中のどの遺伝子を確認対象にするかによっては，最終製品による腫瘍形成への寄与がきわめて低い遺伝子変異しか含まないような多能性幹細胞までも不適切として排除してしまうことになり，合理性が失われる恐れもある．

2）先端技術による評価における注意点

この例のように，新しい技術が開発されても，「先端的技術だから」という理由のみでは，それをただちに製品の品質・安全性評価に適用することはできない．その技術による試験の結果を受けた後に，製品開発，製造および臨床の場において具体的にどういう判断が可能なのかが明らかでなければ，「手元にある当該製品の安全対策」としては意味をなさないということに注意が必要である．つまり現状では，遺伝子変異を指標にして造腫瘍性細胞の混入を検知しようとするならば，発がんリスクと非常に高い相関があることが既知である特定の遺伝子変異に限定し，より高感度かつ高精度で検出する方法を開発する方がむしろ有用である．

なお，再生医療製品の開発における次世代シーケンサーの可能性としては他に例えば，製造基材であるES・iPS細胞の同一性評価を目的とした利用（STRなどの代替としての利用）や，製造基材ES・iPS細胞および製品中の細胞のゲノム不安定性の評価を目的とした利用（CGHなどの代替としての利用）が考えられる．それぞれにおける有用性を議論するためには，レギュラトリー・サイエンス研究，すなわち，各目的に応じた試験系の性能と限界についての科学的な理解が必須である．

おわりに

ヒト多能性幹細胞加工製品を含む再生医療製品を対象にした造腫瘍性試験ガイドラインはいまだに存在しない．現時点では，再生医療製品の中でも特に造腫瘍性に関して懸念の強い製品について，本項で挙げたタイプの異なる試験を複数実施し，総合的に判断すべき