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Neolignan and megastigmane glucosides from the aerial parts of *Isodon japonicus* with cell protective effects on BaP-induced cytotoxicity

Takahiro Matsumoto, Seikou Nakamura, Souichi Nakashima, Tomoe Ohta, Keiko Ogawa, Masashi Fukaya, Junko Tsukioka, Tomohiro Hasei, Tetsushi Watanabe, Hisashi Matsuda*

Kyoto Pharmaceutical University, Misasagi, Yamashina-ku, Kyoto, 607-8412, Japan

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ABSTRACT

Six neolignan glucosides, named isodonosides I–VI, and a megastigmane glucoside named isodonmegastigmane I, were isolated together with 15 known compounds from the methanolic extract of aerial parts of *Isodon japonicus* cultivated in Tokushima, Japan. The chemical structures of the compounds were elucidated based on their MS and NMR spectroscopic analysis. The absolute configurations of the neolignan and megastigmane glucosides were determined by derivatizations, by ECD (electronic circular dicroism) Cotton effect approximation, and by the modified Mosher's method. In addition, a significant cell protective effects of neolignan glucosides on benzo[a]pyrene-induced cytotoxicity was found.

1. Introduction

Isodon japonicus (Burm. f.) H. Hara (Lamiacaeae) is a perennial plant with a wide distribution in China and Japan (Lim et al., 2010). The aerial parts of *I. japonicus* have been used as a traditional medicine for treatment of gastrointestinal disorders, tumors, and inflammatory diseases (Tanaka et al., 2014). Previous reports described the structures of diterpenoids (Takeda et al., 1990), flavonoids (Hong et al., 2009), and lignans (Agata et al., 1989) as constituents of *I. japonicus*. More than 40 *ent*-kaurane diterpenoids were identified in phytochemical studies, and some of these compounds have cytotoxic and antibacterial activities (Hou et al., 2012). In contrast, there have been few reports about glycosides as constituents of *I. japonicus* except for only a few flavonoid glycosides and a diterpene glycoside (Xiang et al., 2015). Based on this information and as a part of our ongoing research program for discovery of bioactive constituents of Asian traditional medicines (Nakamura et al., 2012, 2013; Matsumoto et al., 2013, 2014a, 2014b; Matsuda et al., 2016), the glycoside constituents of the aerial parts of *I. Japonicus* were examined. Herein, the isolation of glucosides **1**–**22** and the structure elucidation of six new neolignan glucosides, named isodonosides I (**1**), II (**2**), III (**3**), IV (**4**), V (**5**), and VI (**6**), and a new megastigmane glucoside named isodonmegastigmane I (**7**), as well as the protective effects of neolignan glucosides on benzo[a]pyrene-induced cytotoxicity in HT1080 cells are described.

2. Results and discussion

2.1. Isolation of constituents from the aerial parts of I. japonicus

A methanolic extract of the dried aerial parts (8.36%) of *I. japonicus* (cultivated in Tokushima, Japan) was partitioned between EtOAc and H_2O (1:1, v/v) to furnish an EtOAc-soluble fraction (3.45%) and an aqueous layer (4.91%). The latter was further extracted with *n*-BuOH to give *n*-BuOH- (0.24%) and H_2O- (4.67%) soluble fractions. The *n*-BuOH soluble fraction was subjected to normal- and reversed-phase silica-gel column chromatography and repeated HPLC. From the *n*-BuOH-soluble fraction, six new neolignan glucosides, isodonosides I (**1**), II (**2**), III (**3**), IV (**4**), V (**5**), and VI

* Corresponding author.
E-mail address: matsuda@mb.kyoto-phu.ac.jp (H. Matsuda).

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(**6**), and a megastigmane glucoside, isodonmegastigmane I (**7**), were isolated together with fifteen known compounds, (7*R*,8*S*)-*erythro*-guaiacylglycerol-*β*-*O*-4′-sinapyl ether 9-*O*-*β*-D-glucopyranoside (**8**) (Machida et al., 2014), (7*S*,8*S*)-*threo*-4,9,9′-trihydroxy-8-*O*-4′-neolignan-7-*O*-*β*-D-glucopyranoside (**9**) (Matsuda and Kikuchi, 1996), (7*S*,8*S*)-*threo*-4,9,9′-trihydroxy-8-*O*-4′-neolignan-9′-*O*-*β*-D-glucopyranoside (**10**) (Matsuda and Kikuchi, 1996), dehydrodiconiferyl-glucoside D (**11**) (Binns et al., 1987), dihydrodehydrodiconiferyl alcohol-9-*O*-*β*-D-glucopyranoside (**12**) (Otsuka et al., 2000), (7*S*,8*R*)-dihydrodehydrodiconiferyl alcohol-3-*O*-*β*-D-glucopyranoside (**13**) (Yang et al., 2012), (7*S*,8*S*)-dihydrodehydrodiconiferyl alcohol-9-*O*-*β*-D-glucopyranoside (**14**) (Lee et al., 2014), (−)-secoisolariciresinol-9′-*O*-β-D-glucopyranoside (**15**) (Inoshiri et al., 1987), (6*R*,9*S*)-3-oxo-α-ionol-9-*O*-*β*-D-glucopyranoside (**16**) (Pabst et al., 1992), (6*R*,9*R*)-3-oxo-α-ionol-9-*O*-*β*-D-glucopyranoside (**17**) (Pabst et al., 1992), (6*S*,9*S*)-9-*O*-*β*-D-glucopyranosyloxy-6-hydroxy-3-oxo-*α*-ionol (**18**) (Calis et al., 2002), 3-hydroxy-5,6-epoxy-*β*-ionol-9-*O*-*β*-D-glucopyranosyloxy-6-hydroxy-3-oxo-*α*-ionol (**19**) (Harput et al., 2002), (4*R*)-(*Z*)-3-methyl-2-(pent-2-en-1-yl)cyclopent-2-en-1-one-4-*O*-*β*-D-glucopyranoside (**20**) (Yamamura et al., 1998), phenylethyl-2-*O*-*β*-D-glucopyranoside (**21**) (Miyase et al., 1982), and methylsalicylate-2-*O*-*β*-D-glucopyranoside (**22**) (Karrer and Weidmann, 1920), respectively. The ^{1}H NMR and ^{13}C NMR spectra and optical rotation of the known compounds were identical with reported data.

2.2. Structures of isodonosides I–VI (*1*–*6*) and isodonmegastigmane I (7)

Isodonosides I (**1**) and II (**2**) were isolated as white powders with negative optical rotations (**1**: $[\alpha]^{25}_{D}$ −46.1, **2**: $[\alpha]^{25}_{D}$ −35.3 in MeOH). Their IR spectra showed absorption bands due to hydroxy groups, aromatic rings, and ether functionalities (**1**: 3400, 1583 and 1034 cm^{-1}, **2**: 3395, 1583 and 1034 cm^{-1}). Their molecular formulae ($C_{27}H_{38}O_{13}$ of **1**, $C_{26}H_{36}O_{12}$ of **2**) were determined from the quasimolecular ion peaks (*m/z* 593 $[M+Na]^+$ for **1**, 563 $[M+Na]^+$ for **2**) in the positive-ion FABMS and by HRMS measurement. Acid hydrolysis of **1** and **2** with 5% aqueous H_2SO_4 in 1,4-dioxane yielded D-glucose. D-Glucose was identified by HPLC of the tolylthiocarbamoyl thiazolidine derivatives (Tanaka et al., 2007). The ^{1}H NMR (methanol-d_4) spectra of **1** and **2**, which were assigned by various NMR experiments, showed signals assignable to **1**: a 1,3,4-trisubstituted benzenring, 1,3,4,5-tetrasubstituted benzene ring, three methoxy groups, two oxymethines, two oxymethylenes, two methylenes, and a glucose moiety. **2**: two 1,3,4-trisubstituted benzene rings, two methoxy groups, two oxymethines, two oxymethylenes, two methylenes, and a glucose moiety. According to the DQF COSY and HMBC spectroscopy, the overall structure of **1** was elucidated as a 7-glucose-substituted bursenolignan (Jutiviboonsuk et al., 2005). Enzymatic hydrolysis of **1** and **2** afforded the aglycones **1a** and **2a**. ^{1}H NMR signals, HR-EI MS, and optical rotation of **1a** were in agreement with that of bursenolignan (Jutiviboonsuk et al., 2005). The *erythro* configuration of **1** and **2** was also confirmed by the coupling constants (**1a**: $J_{7,8}$ = 5.0 in methanol-d_4, **2a**: $J_{7,8}$ = 4.5 in chloroform-*d*) (Miyase et al., 1987; Machida et al., 2014). In addition, the absolute configurations of the neolignan moiety of **1** and **2** at C-7 and C-8 were also confirmed to be *7S*, *8R* on the basis of the negative electronic circular dichroism (ECD) Cotton effects of **1** and **2** [**1**: 241 nm (Δε −4.3) **2**: 240 nm (Δε −1.1)] (Huo et al., 2008; Arnoldi and Merlini, 1985). Consequently, the chemical structures of isodonosides I (**1**) and II (**2**) were characterized as shown. There are many reports about 7- or 9′-glucose-substituted 8-*O*-4′ neolignans without a double bond on the sidechains such as **9** and **10**. On the other hand, there is only one report on 9-glucose-substituted 8-*O*-4′ neolignans without a double bond on the sidechain (Lundgren et al., 1985) (see Fig. 1).

Isodonosides III (**3**), IV (**4**), and V (**5**) were determined as $C_{26}H_{32}O_{11}$ on the basis of their HRMS results. The presence of a D-glucose moiety was indicated from acid hydrolysis of **3**, **4**, and **5** as same as **1** and **2**. By comparison of the ^{1}H NMR and ^{13}C NMR (Table 2) spectra of **3**, **4**, and **5** with previous reports, the overall structure of the neolignan moieties of **3**, **4**, and **5** were determined to be the same as the known compound 4-*O*-methylcedrusin (Pieters et al., 1993). The relative configurations of **3**, **4**, and **5** at the C-7 and C-8 positions were also determined as *S*′, *R*′ by the NOESY experiment (Fig. 2). The absolute configurations of the neolignan moiety of **3**, **4**, and **5** at C-7 and C-8 were also confirmed on the basis of the ECD Cotton effects (Schramm et al., 1979). The ECD spectrum (MeOH) of **3** [203 nm (Δε +13.9), 210 nm (Δε −15.3), 228 nm (Δε +3.3), 239 nm (Δε −1.9)], **4** [203 nm (Δε +14.4), 212 nm (Δε −7.1), 225 nm (Δε +0.4), 239 nm (Δε −4.8)], and **5** [202 nm (Δε +20.6), 211 nm (Δε −16.5), 227 nm (Δε +3.5), 242 nm (Δε −1.8)] were identical to that of the known compound (*7R*, *8S*)-dihydrodehydrodiconiferyl alcohol 4-*O*-*β*-D-glucopyranoside [203 nm (Δε +6.9), 211 nm (Δε −5.3), 226 nm (Δε +2.3), 243 nm (Δε −1.1)] (Machida et al., 2009). In addition, the ECD maxima of **3**, **4**, and **5** appear to be opposite to those of the *7S*, *8R* known compounds **12** [204 nm (Δε −1.6), 212 nm (Δε +1.8), 223 nm (Δε −0.4), 240 nm (Δε +0.6)] and **13** [203 nm (Δε −12.5), 212 nm (Δε +0.6), 226 nm (Δε −1.5), 242 nm (Δε +1.8)]. Thus, the absolute configurations of the neolignan moiety at the C-7 and C-8 positions were determined to be *7R*,*8S*. Based on this evidence, the chemical structures of isodonosides III (**3**), IV (**4**), and V (**5**) were characterized as shown. Among the isolated dihydrobenzofuran neolignans, 4-methoxy-substituted neolignans, such as **3**, **4**, and **5**, are less common than 4-hydroxy-substituted neolignans.

Isodonoside VI (**6**) was isolated as a white powder with a negative optical rotation ($[\alpha]^{25}_{D}$ −18.2 in MeOH). Its molecular formula ($C_{25}H_{32}O_{11}$) was determined from the positive-ion FABMS and by HRMS measurement. From the ^{1}H NMR and ^{13}C NMR (Table 2) spectra of **6**, the overall structure was determined to be the same as **3**, except for the methoxy group at the C-4 position. NOESY correlations were observed between H-7 and H-9 (Fig. 2). Therefore, the relative configuration of **6** at the 7 and 8-positions were determined as *S*′,*R*′. Next, the ECD (MeOH) maxima of **6** [202 nm (Δε −5.2), 210 nm (Δε +10.0), 223 nm (Δε −1.4), 240 nm (Δε +1.8)] were identical to that of the known compounds **12** and **13**. In addition, the ECD maxima of **6** appears to be opposite to those of compounds **3**, **4**, and **5**. Thus, the absolute configurations of the neolignan moiety at C-7 and C-8 positions were determined to be *7S*,*8R*. From this evidence, the chemical structures of isodonoside VI (**6**) were characterized as shown. The structural differences of **6** from known compound **13** were in the position of the glucose moiety.

Isodonmegastigmane I (**7**), ($[\alpha]^{25}_{D}$ −39.3 in MeOH) was isolated as a white powder. Its IR spectrum gave absorption bands at 3350, 1259 and 1038 cm^{-1}, suggesting the presence of hydroxy, carbonyl, and ether functionalities, respectively. Its molecular formula was established to be $C_{19}H_{40}O_7$ by the positive-ion FABMS and by HRMS measurement. Acid hydrolysis of **7** with 5% aqueous H_2SO_4-1,4-dioxane yielded D-glucose. The ^{1}H NMR (methanol-d_4) spectra of **7** suggested its megastigmane moiety (an olefin, an oxymethine, two methylenes, four methyl groups), and a glucose moiety. From the DQF COSY and HMBC spectroscopy of **7**, the overall structure was determined to be the same as the known compound 9-hydroxy-5,7-megastigmadien-4-one, except for the glucose moiety at the C-9 position (Prelog and Meier, 1950). The absolute configuration of the 9-position in **7** was characterized by the application of the modified Mosher's method. Namely, enzymatic hydrolysis of **7** afforded the aglycone **7a**, treatment of which

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Table 1
^{1}H NMR Spectroscopic data for compounds **1**–**6**.

Position	**1**	**2**	**3**	**4**	**5**	**6**
	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)
2	6.96 (d, 2.0)	7.00 (d, 2.0)	7.05 (d, 2.0)	6.97 (d, 1.7)	7.00 (d, 2.0)	7.01 (d, 2.0)
5	6.70 (d, 8.0)	6.70 (d, 8.0)	6.90 (d, 8.5)	6.90 (d, 6.9)	6.78 (d, 6.5)	6.74 (d, 8.0)
6	6.74 (dd, 8.0, 2.0)	6.82 (dd, 8.0, 2.0)	7.00 (dd, 8.5, 2.0)	6.95 (dd, 6.9, 1.7)	6.90 (dd, 6.5, 2.0)	6.86 (dd, 8.0, 2.0)
7	4.84 (d, 4.5)	4.83[a]	5.63 (d, 6.0)	5.53 (d, 5.2)	5.51 (d, 6.0)	5.56 (d, 6.5)
8	4.42 (m)	4.49 (m)	3.60 (m)	3.50 (m)	3.43 (m)	3.62 (m)
9	3.31 (dd, 10.0, 5.5)	4.10 (dd, 11.0, 3.0)	3.74 (t-like, 9.5)	3.76 (m)	3.74 (t-like, 7.5)	4.10 (t-like, 9.5)
				3.82 (m)		3.85 (m)
2′	6.53 (s)	6.77 (d, 2.0)	6.63 (s)	6.91 (s)	6.59 (s)	6.55 (s)
5′		6.90 (d, 8.0)				
6′	6.53 (s)	6.65 (dd, 8.0, 2.0)	6.56 (s)	6.79 (s)	6.62 (s)	6.66 (s)
7′	2.63 (t, 7.5)	2.59 (t, 8.0)	2.55 (t, 9.5)	2.60 (t, 6.9)	2.60 (t, 8.0)	2.55 (t, 8.0)
8′	1.82 (m)	1.78 (m)	1.76 (m)	1.79 (t-like, 6.9)	1.86 (t-like, 8.0)	1.78 (m)
9′	3.57 (t, 6.5)	3.54 (t, 8.0)	3.54 (t, 6.5)	3.55 (t, 5.2)	3.59 (t, 6.5)	3.54 (t, 6.5)
3-OMe	3.82 (s)	3.79 (s)	3.80 (s)	3.79 (s)[b]	3.74 (s)	3.82 (s)
4-OMe			3.81 (s)	3.80 (s)[b]	3.79 (s)	
3′-OMe	3.81 (s)	3.76 (s)				
5′-OMe	3.81 (s)					
1″	4.29 (d, 7.5)	4.32 (d, 8.0)	4.35 (d, 7.5)	5.00 (d, 6.3)	4.23 (d, 7.5)	4.34 (d, 7.5)
2″	3.17 (dd, 8.0, 7.5)	3.20 (t, 8.0)	3.23 (t, 7.5)	3.42 (m)	3.18 (dd, 9.0, 7.5)	3.22 (t, 7.5)
3″	3.30 (m)	3.34 (m)	3.27 (m)	3.42 (m)	3.34 (m)	3.28 (m)
4″	3.29 (m)	3.27 (m)	3.28 (m)	3.38 (m)	3.26 (m)	3.29 (m)
5″	3.30 (m)	3.34 (m)	3.27 (m)	3.78 (m)	3.35 (m)	3.34 (m)
6″	3.63 (m)	3.82 (m)	3.85 (m)	3.68 (m)	3.65 (m)	3.68 (m)
	3.79 (m)	3.84 (m)	3.87 (m)	3.83 (m)	3.78 (m)	3.87 (m)

Measured in methanol-d_4.
[a] Overlapped signal.
[b] Interchangeable.

Table 2
^{13}C NMR Spectroscopic data for compounds **1**–**6**.

Position	**1**	**2**	**3**	**4**	**5**	**6**	Position	**1**	**2**	**3**	**4**	**5**	**6**
1	133.2	133.9	136.5	135.8	136.5	134.9	1′	139.9	138.0	136.8	137.2	136.7	136.8
2	111.4	111.9	110.8	110.9	110.7	110.8	2′	106.9	114.1	116.9	118.2	116.9	116.9
3	148.7	148.7	150.5	150.7	150.6	149.0	3′	154.3	151.8	142.0	142.4	141.9	141.9
4	146.8	147.0	150.1	150.5	150.2	147.3	4′	134.6	147.0	146.4	147.5	146.5	146.4
5	115.7	115.6	112.8	112.9	112.9	116.0	5′	154.3	119.6	129.3	130.4	129.6	129.6
6	120.7	121.1	119.4	119.8	119.4	119.8	6′	106.9	121.8	117.1	119.6	117.2	117.1
7	73.8	73.8	88.4	89.2	88.4	88.9	7′	33.4	32.7	32.7	32.7	32.7	32.7
8	86.1	85.0	53.7	55.2	55.8	53.2	8′	35.4	35.5	35.8	35.6	32.9	35.8
9	69.6	69.8	72.6	64.9	65.2	72.4	9′	62.2	62.2	62.3	62.2	69.9	62.3
1″	105.0	105.1	104.6	102.8	104.4	104.2	3-OMe	56.4	56.4	56.5	56.5	56.5	56.4
2″	75.2	75.2	75.2	74.9	75.2	75.2	4-OMe			56.5	56.5	56.4	
3″	77.8	77.9	78.1	77.8	77.9	78.1	3′-OMe	56.7	56.5				
4″	71.5	71.6	71.7	71.4	71.7	71.7	5′-OMe	56.7					
5″	78.0	78.0	78.3	78.2	78.1	78.2							
6″	62.6	62.7	62.8	62.5	62.8	62.8							

Measured in methanol-d_4.

with (−)-α-methoxy-α-(trifluoromethyl) phenylacetyl chloride [(−)-MTPA-Cl] in pyridine yielded (*S*)-MTPA ester **7b**. The (*R*)-MTPA ester **7c** was derived from **7** by treatment with (+)-MTPA-Cl in pyridine. As shown in Fig. 3, the signals due to protons attached to the 10-positions in **7b** were observed at lower fields compared with those of **7c** [$\Delta\delta$: positive], while the signals due to protons attached to the 4, 5, 7, 8, 11, 12, and 13-positions in the (*S*)-MTPA ester (**7b**) were observed at higher field compared with those of the (*R*)-MTPA ester (**7c**) [$\Delta\delta$: negative]. Thus, the absolute configuration at the 9-position in **7** was determined to be *R*. The absolute configulation of **7a** was different from known compound with same overall structure (*S*)-9-hydroxy-5,7-megastigmadien-4-one (D'Abrosca et al., 2004). Based on this evidence, the chemical structure of isodonmegastigmane I (**7**) was characterized as shown.

2.3. The protective effects of constituents on benzo[a]pyrene-induced cytotoxicity

Benzo[a]pyrene (BaP) is a well-known environmental carcinogen that promotes oxidative stress and DNA damage and plays a critical role in lung carcinogenesis (Stephen and Steven, 1993). Chemopreventive agents against BaP can decrease the level of DNA damage, and should therefore decrease the risk of cancer (Zhu et al., 2014). In the present study, to find chemopreventive agents, cell protective effects of glucoside constituents from *I. japonicus* were evaluatedon human fibroblasts fibrosarcoma cell line (HT1080). Among the isolates, neolignan glucosides **4** [recovery: 23.2% ($P < 0.01$) at 10 μM], **9** [recovery: 37.0% ($P < 0.01$) at 10 μM], **10** [recovery: 14.1% ($P < 0.05$) at 5.0 μM], **16** [recovery: 51.9% ($P < 0.01$) at 10 μM], and **19** [recovery: 75.3% ($P < 0.01$) at 10 μM] showed significant protective against benzo[a]pyrene-

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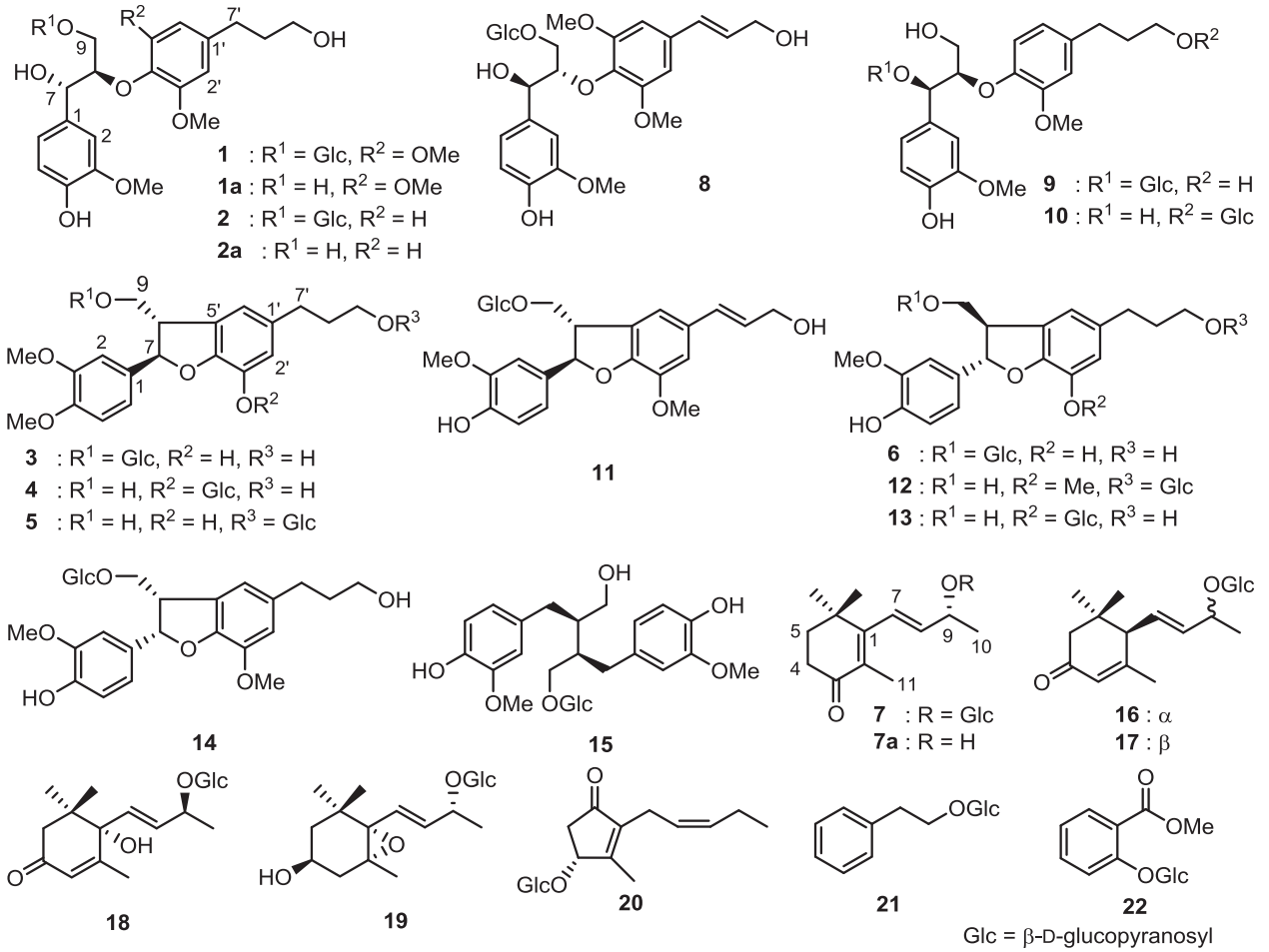

Fig. 1. Structures of compounds isolated from *I. japonicus*.

induced cytotoxicity (Supporting information S5). In this study, no structure-activity relationship was clarified among the isolated compounds.

3. Conclusion

Six new neolignan glucosides isodonosides I (**1**), II (**2**), III (**3**), IV (**4**), V (**5**), and VI (**6**) and a new megastigmane glucoside, isodonmegastigmane I (**7**), were isolated from aerial parts of *I. japonicus* cultivated in Tokushima, Japan. The absolute configurations of the new compounds were elucidated by derivatization, by ECD Cotton effect approximations, and by modified Mosher's method. In this study, the *erythro* type (**1** and **2**) and *threo* type (**8**–**10**), the *trans* configuration at the benzofuran ring (**3**–**6** and **11**–**13**), as well as the *cis* configuration of the benzofuran ring (**14**) neolignans were isolated. Aglycones of new compounds **1** and **2** were elucidated as enantiomers of aglycones of known compound **8**. From these results, it is concluded that the absolute stereo structure of these neolignans resulted from non-specified phenoxy radical couplings in this plant. On the other hand, because of steric effects, neolignans with a *cis*-dihydrobenzofuran ring were comparatively difficult to be biosynthesized in plants. In addition, significant protective effects of neolignan glucosides on benzo[a]pyrene-induced cytotoxicity were found.

4. Experimental section

4.1. General experimental procedures

The following instruments were used to obtain physical data: specific rotations, a Horiba SEPA-300 digital polarimeter (l = 5 cm); IR spectra, a Thermo Electron Nexus 470; FABMS and HRFABMS, a JEOL JMS-SX 102A mass spectrometer; EIMS and HREIMS, a JEOL JMS-GCMATE mass spectrometer; ^{1}H-NMR spectra, JEOL JNM-LA 500 (500 MHz) and JNM-ECA 600K (600 MHz) spectrometers; ^{13}C-NMR spectra, JEOL JNM-LA 500 (125 MHz) and JNM-ECA 600K (150 MHz) spectrometers; NOESY spectra, JEOL JNM-ECA 600K (600 MHz) spectrometer; ECD spectra, a Jasco J-1500 circular dichroism spectrometer; HPLC, a Shimadzu SPD-10AVP UV-VIS detector. YMC Triart PFP (250 × 4.6 mm i.d. and 250 × 10 mm i.d.), YMC Pack Ph (250 × 4.6 mm i.d. and 250 × 20 mm i.d.), YMC Triart PFP (250 × 4.6 mm i.d. and 250 × 10 mm i.d.), COSMOSIL 5PFP (250 × 4.6 mm i.d. and 250 × 20 mm i.d.), COSMOSIL 5C18-MS-II (250 × 4.6 mm i.d., 250 × 10 mm i.d. and 250 × 20 mm i.d.) and COSMOSIL 5C18-PAQ (250 × 4.6 mm i.d. and 250 × 20 mm i.d.) columns were used for analytical and preparative purposes.

The following experimental materials were used for chromatography: normal-phase silica gel column chromatography (CC), silica gel BW-200 (Fuji Silysia Chemical, Ltd., 150–350 mesh); reversed-phase silica gel CC, Chromatorex ODS DM1020T (Fuji

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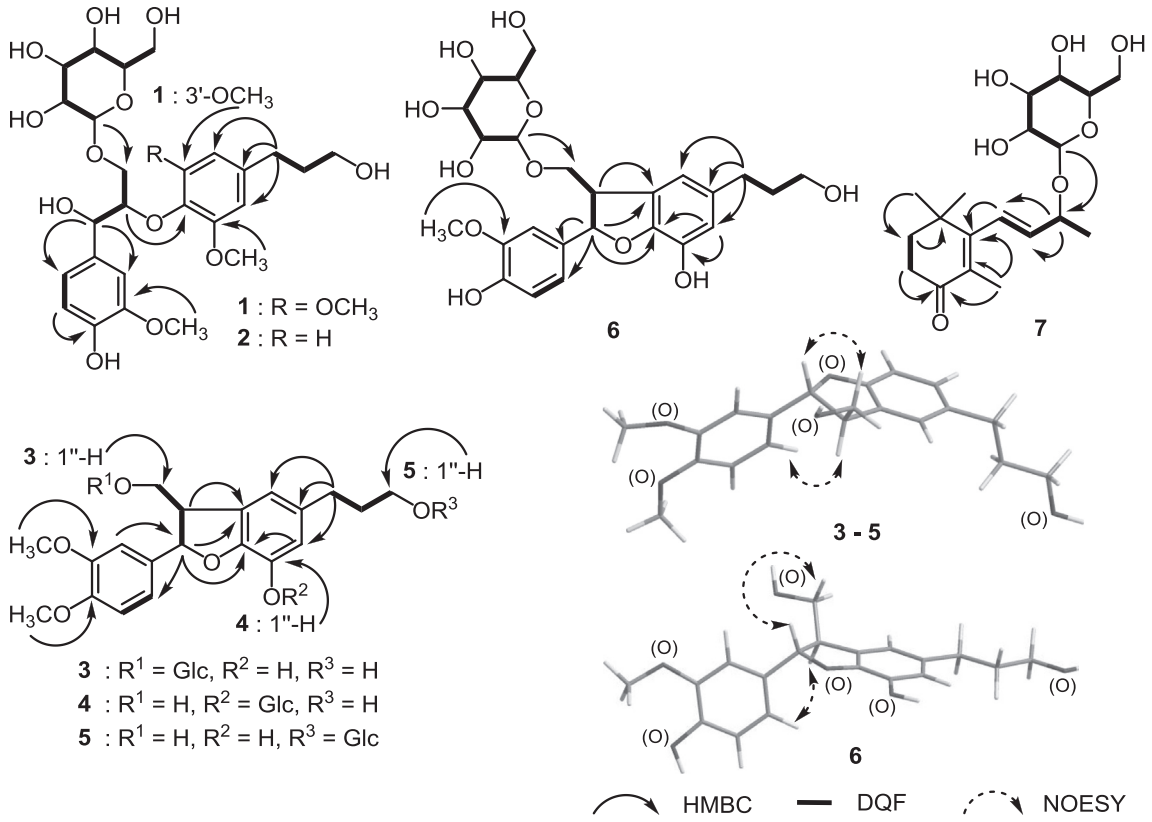

Fig. 2. Important 2D NMR correlations of compounds **1**–**7**.

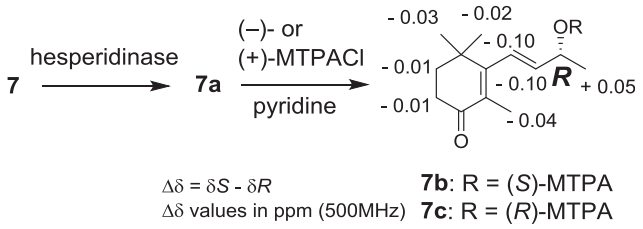

Fig. 3. Determination of the absolute configurations of 9-position in **7**.

Silysia Chemical, Ltd., 100–200 mesh); Sephadex LH-20 (GE Healthcare); TLC, precoated TLC plates with silica gel $60F_{254}$ (Merck, 0.25 mm) (ordinary phase) and silica gel RP-18 F_{254S} (Merck, 0.25 mm) (reversed phase); reversed-phase HPTLC, precoated TLC plates with silica gel RP-18 WF_{254S} (Merck, 0.25 mm). Detection of compounds was achieved by UV irradiation and by spraying with 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4 followed by heating.

4.2. Reagents for bioassay

Minimum essential medium Eagle's (MEM) and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (MO, U.S.A.). Other reagents were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 96-Well microplates were purchased from Greiner Japan Co., Ltd. (Tokyo, Japan).

4.3. Plant material

Dried aerial parts of *I. japonicus* cultivated in Tokushima, Japan, were obtained as commercial products purchased from Tochimoto Tenkaido Co. Ltd (Osaka, Japan) in September 2015. A voucher specimen is on file in our laboratory (KPU IJ-2016-1).

4.4. Extraction and isolation

Dried aerial parts of *I. japonicus* (11.4 kg) were extracted with MeOH (90 L × 3) under conditions of reflux for 3 h. Evaporation of the combined extracts under reduced pressure provided a MeOH extract (953.4 g, 8.36%). The latter was partitioned into an EtOAc-H_2O (1:1, v/v) mixture to furnish an EtOAc- soluble fraction (393.3 g, 3.45%) and an aqueous phase. The latter was further extracted with *n*-BuOH to give a *n*-BuOH-soluble fraction (27.4 g, 0.24%) and an H_2O-soluble fraction (532.3 g, 4.67%). The *n*-BuOH-soluble fraction was subjected to normal phase silica gel CC [1.2 kg, $CHCl_3$ → $CHCl_3$–MeOH (10:1 → 5:1 → 4:1 → 3:1 → 2:1 → 1:1 → 1:2 → 1:4, v/v) →MeOH] to give seven fractions [Fr.B1–Fr.B8]. Fraction B4 (1.6 g) was further separated by reversed phase silica gel CC [40.0 g, MeOH:H_2O (2:8 → 3:7 → 4:6 → 5:5 → 6:4 → 7:3 → 8:2 → 9:1 v/v) → MeOH] to give twelve fractions [Fr.B4-1–Fr.B4-12]. Fraction B4-4 (131.9 mg) was separated by HPLC, {mobile phase: H_2O:MeCN (85:15, v/v) [YMC Pack Ph (250 × 20 mm i.d.)]} followed by {mobile phase: H_2O:MeCN (85:15, v/v) [COSMOSIL 5C18-MS-II (250 × 10 mm i.d.)]} to give **21** (3.7 mg, 0.000032%) and **22** (0.9 mg, 0.0000079%). Fraction B4-6 (227.2 mg) was separated by HPLC, {mobile phase: H_2O:MeCN (80:20, v/v) [COSMOSIL 5C18-MS-II (250 × 20 mm i.d.)]} and {mobile phase: H_2O:MeCN (77:23, v/v) [YMC triart PFP (250 × 10 mm i.d.)]}to give **7** (4.5 mg, 0.000039%), **16** (15.6 mg, 0.00014%), **17** (7.1 mg, 0.000062%), and **20** (1.2 mg, 0.000011%). Fraction B5 (4.5 g) was further separated by reversed phase silica gel CC [150.0 g, MeOH:H_2O (1:9 →2:8 → 3:7 → 4:6 → 5:5 → 6:4 → 7:3 → 8:2 → 9:1 v/v) → MeOH] to give fifteen fractions [Fr.B5-

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1−Fr.B5-15]. Fraction B5-3 (407.3 mg) was further separated by size exclusion CC (10.0 g, MeOH) to give six fractions [Fr.B5-3-1−Fr.B5-3-6]. Fraction Fr.B5-3-1 (44.4 mg) and Fr.B5-3-2 (281.8 mg) were individually purified by HPLC [COSMOSIL 5C18-MS-II (250 × 20 mm i.d.)] to give **2** [H_2O:MeCN (87:13, v/v), 3.0 mg, 0.000026%], **18** [H_2O:MeCN (87:13, v/v), 3.0 mg, 0.000026%], **19** [H_2O:MeCN (87:13, v/v), 15.5 mg, 0.00014%], and eleven fractions [Fr.B5-3-2-1−Fr.B5-3-2-11]. Fraction Fr.B5-3-2-11 (9.2 mg) was further purified by HPLC {mobile phase: H_2O:MeCN (8:92, v/v) [COSMOSIL HILIC (250 × 10 mm i.d.)]} to give **13** (2.1 mg, 0.000018%). Fraction Fr.B5-4 (239.8 mg) was applied to HPLC, {mobile phase: H_2O:MeCN (85:15, v/v) [COSMOSIL 5PFP (250 × 20 mm i.d.)]} and {mobile phase: H_2O:MeCN (85:15, v/v) [YMC Triart 5C18 (250 × 10 mm i.d.)]} to give **10** (1.2 mg, 0.000011%), **6** (2.7 mg, 0.000024%), and **9** (1.2 mg, 0.000011%). Fraction Fr.B5-5 (332.1 mg) was subjected to HPLC {mobile phase: H_2O:MeCN (80:20, v/v) [COSMOSIL 5C18-MS-II (250 × 20 mm i.d.)]} to give **12** (13.0 mg, 0.00011%), **14** (11.6 mg, 0.0001%), and six fractions [Fr.B5-5-1−Fr.B5-5-6]. Fraction Fr.B5-5-1 (21.9 mg) and Fr.B5-5-2 (34.1 mg) were individually subjected to HPLC [YMC Triart PFP (250 × 10 mm i.d.)] to give **1** [H_2O:MeCN (83:17, v/v), 5.1 mg, 0.000045%], **8** [H_2O:MeCN (83:17, v/v), 2.9 mg, 0.000025%], and **4** [H_2O:MeCN (81:19, v/v), 9.6 mg, 0.000084%]. Fraction Fr.B5-6 (340.7 mg) was purified by HPLC {mobile phase: H_2O:MeCN (80:20, v/v) [COSMOSIL 5C18-MS-II (250 × 20 mm i.d.)]}, {mobile phase: H_2O:MeCN (80:20, v/v) [YMC Triart PFP (250 × 10 mm i.d.)]}, and {mobile phase: H_2O:MeCN (80:20, v/v) [COSMOSIL 5C18-MS-II (250 × 10 mm i.d.)]}to give **3** (1.0 mg, 0.0000088%), **5** (2.5 mg, 0.000022%), **11** (1.1 mg, 0.0000096%), and **15** (13.5 mg, 0.00012%).

4.5. Isodonoside I (**1**)

White powder; $[\alpha]_D^{25}$ -46.1 (*c* 0.4, MeOH); IR(KBr): ν_{max} 3400, 1583, and 1034 cm^{-1}; For ^1H NMR (methanol-d_4, 500 MHz) and ^{13}C NMR (125 MHz) spectroscopic data, see Tables 1 and 2; UV (MeOH) λ_{max} 230.0 nm (log ε 3.3), 280.2 nm (log ε 2.9); ECD: $\Delta\varepsilon$ (nm) −4.3 (241) (MeOH); positive-ion FABMS: *m/z* 593 $[M+Na]^+$; HRFABMS: *m/z* 593.2213 (Calcd for $C_{27}H_{38}O_{13}Na$ $[M+Na]^+$: *m/z* 593.2210).

4.6. Isodonoside II (**2**)

White powder; $[\alpha]_D^{25}$ -35.3 (*c* 0.3, MeOH); IR(KBr): ν_{max} 3395, 1583, and 1034 cm^{-1}; For ^1H NMR (methanol-d_4, 500 MHz) and ^{13}C NMR (125 MHz) spectroscopic data, see Tables 1 and 2; UV (MeOH) λ_{max} 230.4 nm (log ε 3.6), 280.4 nm (log ε 3.2); ECD: $\Delta\varepsilon$ (nm) −1.1 (240) (MeOH); positive-ion FABMS: *m/z* 563 $[M+Na]^+$; HRFABMS: *m/z* 563.2108 (Calcd for $C_{26}H_{36}O_{12}Na$ $[M+Na]^+$: *m/z* 563.2104).

4.7. Isodonoside III (**3**)

White powder; $[\alpha]_D^{25}$ -135.6 (*c* 0.1, MeOH); IR(KBr): ν_{max} 3350, 1606, and 1033 cm^{-1}; For ^1H NMR (methanol-d_4, 500 MHz) and ^{13}C NMR (125 MHz) spectroscopic data, see Tables 1 and 2; UV (MeOH) λ_{max} 281.4 nm (log ε 2.3); ECD: $\Delta\varepsilon$ (nm) +13.9 (203), −15.3 (210), +3.3 (228), −1.9 (239) (MeOH); positive-ion FABMS: *m/z* 545 $[M+Na]^+$; HRFABMS: *m/z* 545.1993 (Calcd for $C_{26}H_{34}O_{11}Na$ $[M+Na]^+$: *m/z* 545.1999).

4.8. Isodonoside IV (**4**)

White powder; $[\alpha]_D^{25}$ -24.1 (*c* 0.48, MeOH); IR(KBr): ν_{max} 3390, 3305, 1068, 1033, 1604and 1517 cm^{-1}; For ^1H NMR (methanol-d_4, 500 MHz) and ^{13}C NMR (125 MHz) spectroscopic data, see Tables 1 and 2; UV (MeOH) λ_{max} 281.0 nm (log ε 3.1); ECD: $\Delta\varepsilon$ (nm) +14.4 (203), −7.1 (212), +0.4 (225), −4.8 (239) (MeOH); positive-ion FABMS: *m/z* 545 $[M+Na]^+$; HRFABMS: *m/z* 545.2005 (Calcd for $C_{26}H_{34}O_{11}Na$ $[M+Na]^+$: *m/z* 545.1999).

4.9. Isodonoside V (**5**)

White powder; $[\alpha]_D^{25}$ -43.4 (*c* 0.25, MeOH); IR(KBr): ν_{max} 3390, 3305, 1604, and 1080 cm^{-1}; For ^1H NMR (methanol-d_4, 500 MHz) and ^{13}C NMR (125 MHz) spectroscopic data, see Tables 1 and 2; UV (MeOH) λ_{max} 281.6 nm (log ε 3.2); ECD: $\Delta\varepsilon$ (nm) +20.6 (202), −16.5 (211), +3.5 (227), −1.8 (242) (MeOH); positive-ion FABMS: *m/z* 545 $[M+Na]^+$; HRFABMS: *m/z* 545.2005 (Calcd for $C_{26}H_{34}O_{11}Na$ $[M+Na]^+$: *m/z* 545.1999).

4.10. Isodonoside VI (**6**)

White powder; $[\alpha]_D^{25}$ -18.2 (*c* 0.27, MeOH); IR(KBr): ν_{max} 3400, 1607, and 1031 cm^{-1}; For ^1H NMR (methanol-d_4, 500 MHz) and ^{13}C NMR (125 MHz) spectroscopic data, see Tables 1 and 2; UV (MeOH) λ_{max} 282.8 nm (log ε 3.0); ECD: $\Delta\varepsilon$ (nm) −5.2 (202), +10.0 (210), −1.4 (223), +1.8 (240) (MeOH); positive-ion FABMS: *m/z* 531 $[M+Na]^+$; HRFABMS: *m/z* 531.1846 (Calcd for $C_{25}H_{32}O_{11}Na$ $[M+Na]^+$: *m/z* 531.1842).

4.11. Isodonmegastigmane I (**7**)

White powder; $[\alpha]_D^{25}$ -39.3 (c 0.4, MeOH); IR(KBr): ν_{max} 3350, 1259, and 1038 cm^{-1}; For ^1H NMR (methanol-d$_4$, 500 MHz) and ^{13}C NMR (125 MHz) spectroscopic data, see Table 3; positive-ion FABMS: *m/z* 393 $[M+Na]^+$; HRFABMS: m/z 393.1892 (Calcd for $C_{19}H_{40}O_7Na$ $[M+Na]^+$: *m/z* 393.1889).

4.12. Enzymatic hydrolysis of **1** and **2**

To the individual solution of **1** (2.0 mg) or **2** (2.0 mg) in 20 mM acetate buffer (2.5 mL, pH = 5.0) was added β-glucosidase (1 mg, from Sweet almond), and cellulase (1 mg). The mixtures were then individually stirred for 12 h at 50 °C. Each supernatant solution was concentrated under vacuum to give the correspond residues, which were subjected to normal phase silica gel CC [0.5 g, $CHCl_3$ → MeOH] to give **1a** (0.8 mg) or **2a** (0.7 mg).

Table 3
^1H and ^{13}C NMR spectroscopic data for compounds **7** and **7a**.

Position	**7**		**7a**	
	δ C	δ H (*J* in Hz)	δ C	δ H (*J* in Hz)
1	163.7		160.5	
2	130.7		130.0	
3	201.8		199.4	
4	35.1	2.48 (t, 6.5)	34.3	2.50 (t, 6.5)
5	38.2	1.85 (t, 6.5)	37.2	1.85 (t, 6.5)
6	36.6		35.4	
7	127.5	6.29 (d, 16.0)	125.3	6.20 (d, 16.0)
8	140.4	5.75 (dd, 16.0, 6.0)	140.4	5.70 (dd, 16.0, 6.0)
9	77.2	4.50 (m)	68.7	4.45 (m)
10	21.0	1.35 (d, 6.0)	23.6	1.35 (d, 6.0)
11	13.7	1.78 (s)	13.4	1.78 (s)
12	27.7	1.17 (s)	27.3	1.17 (s)
13	27.7	1.18 (s)	27.3	1.18 (s)
1′	102.7	4.39 (d, 8.0)		
2′	75.3	3.19 (m)		
3′	78.0	3.24 (m)		
4′	71.5	3.34 (m)		
5′	78.1	3.35 (m)		
6′	62.6	3.65 (dd, 11.0, 5.5)		
		3.89 (d-like, 11.0)		

Measured in methanol-d_4.

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1a: Colorless oil; $[\alpha]_D^{25}$ +40.7 (*c* 0.2, MeOH); positive-ion EIMS *m/z* 408 $[M]^+$; HREIMS m/z 593.2213 (calcd for $C_{27}H_{38}O_{13}$ $[M]^+$, 593.2210).

2a: Colorless oil; $[\alpha]_D^{25}$ +15.8 (*c* 0.07, MeOH); positive-ion EIMS *m/z* 378 $[M]^+$; HREIMS m/z 378.1671 (calcd for $C_{26}H_{36}O_{12}$ $[M]^+$, 378.1679).

4.13. Enzymatic hydrolysis of **7**

To a solution of **7** (3.8 mg) in 20 mM acetate buffer (2 mL, pH = 5.0) was added hesperidinase (2 mg, from *Penicillium* sp. Sigma-Aldrich) and the mixture was stirred for 12 h at 50 °C. The supernatant solution was concentrated under vacuum to give a residue, which was subjected to normal phase silica gel CC [0.5 g, $CHCl_3$ → MeOH] to give **7a** (1.6 mg).

7a: Colorless oil; $[\alpha]_D^{25}$ -46.1 (*c* 0.4, MeOH); For ^{1}H NMR (methanol-d_4, 500 MHz) and ^{13}C NMR (125 MHz) spectroscopic data, see Table 3; positive-ion EIMS: *m/z* 208 $[M]^+$; HREIMS *m/z* 208.1458 (calcd for $C_{13}H_{20}O_2$ $[M]^+$, 208.1463).

4.14. Preparation of the (S)- and (R)-MTPA esters (**7b** and **7c**) of **7a**

A solution of **7a** (0.8 mg) in pyridine (0.5 ml) was treated with (−)-MTPA-Cl (0.02 mL), and the mixture was stirred at r.t. for 12 h. Removal of the solvent from the reaction mixture under reduced pressure furnished a residue, which was subjected to normal phase silica gel CC [0.5 g, $CHCl_3$ → MeOH] to give (*S*)-MTPA ester (**7b**, 0.8 mg). Employing a similar procedure, (*R*)-MTPA ester (**7c**, 0.9 mg) was obtained from **7a** (0.8 mg, 0.0059 mmol) using (+)-MTPA-Cl.

(*S*)-MTPA ester (**7b**). ^{1}H-NMR (chloroform-*d*, 500 MHz) δ 2.49 (t, J = 6.4, H-4), 1.83 (t, J = 6.4, H-5), 6.22 (d, J = 16.8, H-7), 5.51 (m, H-8), 3.76 (m, H-9), 1.49 (d, J = 6.0, H-10), 1.72 (s, H-11), 1.07 (s, H-12), 1.08 (s, H-13); EIMS: m/z 424 $[M]^+$; HREIMS m/z 424.1864 (calcd for $C_{23}H_{27}O_4F_3$ $[M]^+$, m/z 424.1862).

(*R*)-MTPA ester (**7c**). ^{1}H-NMR (chloroform-*d*, 500 MHz) δ 2.50 (t, J = 6.4, H-4), 1.84 (t, J = 6.4, H-5), 6.33 (d, J = 15.2, H-7), 5.62 (m, H-8), 3.76 (m, H-9), 1.44 (d, J = 6.0, H-10), 1.76 (s, H-11), 1.10 (s, H-12), 1.10 (s, H-13); EIMS: m/z 424 $[M]^+$; HREIMS m/z 424.1864 (calcd for $C_{23}H_{27}O_4F_3$ $[M]^+$, m/z 424.1862).

4.15. Acid hydrolysis of **1**–**7**

To determine the absolute configurations of the constituent monosaccharids of **1** and **2**, the reported method by Tanaka et al. was used with slight modifications as follows. Compounds **1**–**7** (0.4 mg) were dissolved in 5% aqueous H_2SO_4 in 1,4-dioxane (1:1, v/v, 2.0 mL), then each solution heated at 90 °C for 3 h. After extraction with EtOAc (× 3), the solutions were individually neutralized with Amberlite IRA-400 (OH^- form). After drying *in vacuo*, each residue was dissolved in pyridine (0.1 mL) containing L-cysteine methyl ester hydrochloride (0.5 mg) and heated at 60 °C for 1 h. Each solution of *o*-tolylisothiocyanate (0.5 mg) in pyridine (0.1 mL) was added to each mixture and heated at 60 °C for 1 h. The reaction mixture was analyzed by reversed-phase HPLC [column: COSMOSIL 5C18-MS-II (Nacalai Tesque), 250 × 4.6 mm i.d. (5 μm); mobile phase: MeCN:H_2O (18:82, v/v); detection: UV (254 nm); flow rate: 1.0 mL/min; column temperature: 25 °C] to identify the derivatives of D-glucose in **1**–**7** by comparison of their retention times with those of authentic samples (t_R: D-glucose; 50.0 min, L-glucose; 44.5 min).

4.16. Cell culture

Human fibrosarcoma HT1080 cells (Cell No. JCRB9113) were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Cells were maintained in MEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

4.17. MTT assay

Human fibrosarcoma HT1080 cells were seeded into 96-well plates (5.0×10^4 cells/100 μL/well). Cells were treated with 50 μM BaP alone or with test samples for 20 h. After that, MTT solution (10 μL, 5 mg/mL) was added to the well and incubated for 4 h at 37 °C. After removal of the culture medium, the cells were dissolved by *i*-PrOH including 0.04 M HCl and absorbance was measured at 570 nm with a reference wavelength at 655 nm. Results expressed the percent of recovery rate compared to the control- and normal- group.

4.18. Statistical analyses

Values are expressed as mean ± S.E.M. (n = 4) One-way analysis of variance followed by Dunnett's test was used for statistical analyses. Probability (p) values less than 0.05 were considered significant.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.phytochem.2017.02.007.

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