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Absolute configuration of (+)-pinoresinol 4-*O*-[6″-*O*-galloyl]-*β*-D-glucopyranoside, macarangiosides E, and F isolated from the leaves of *Macaranga tanarius*

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ABSTRACT

A lignan glucoside, (+)-pinoresinol 4-*O*-[6″-*O*-galloyl]-*β*-D-glucopyranoside (**1**), and two megastigmane glucosides, named macarangiosides E and F (**2, 3**), together with 15 known compounds (**4**–**18**) were isolated from leaves of *Macaranga tanarius* (L.) Müll.-Arg. (Euphorbiaceae). Their structures were elucidated by spectroscopic and chemical analyses. In addition, the absolute stereochemistry of macarangiosides B and C isolated previously from the same plant was also determined for the first time. Compounds **1** and **2** were galloylated on glucose and possessed potent DPPH radical-scavenging activity.

1. Introduction

In our continuing studies on the subtropical plants collected in Okinawa Island, we have phytochemically investigated the chemical constituents of the leaves of *Macaranga tanarius* (L.) Müll.-Arg. (Euphorbiaceae). It is well known as a pioneer tree that grows first ahead of other plants on impoverished soil (Blattner et al., 2001), and also known as an ant-plant defended by ants against herbivores by producing the ant-attracting food body (Heil et al., 2001). It is a small evergreen tree of about 4–5 m in the height found in the bush layer throughout eastern and southern Asia, especially in southern China, Korea and Okinawa, Japan. In China, the root and bark of this plant are used as a folk medicine for hemoptysis and dysentery, respectively (Primary Chinese Herbs Pictorial Illustrated Editorial Committee, 1986). In our previous papers, we described the isolation and structure elucidations of four new megastigmane glucosides, named macarangiosides A–D, and seven prenylated flavanones, macaflavanones A–G, from the leaves of *M. tanarius* (Matsunami et al., 2006; Kawakami et al., 2008). As a result of further investigation of the same plant, we successfully isolated three new glucosides (**1**–**3**), together with 15 known compounds (**4**–**18**). Their structures were elucidated on the basis of spectroscopic and chemical analyses. In addition, the absolute stereochemistry of macarangiosides B and C was also determined for the first time in this study by chemical conversion and application of the modified Mosher's method. Finally, DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging activity and cytotoxicity of these compounds were also investigated.

2. Results

Air-dried leaves (12.1 kg) of *M. tanarius* were extracted with MeOH at room temperature, then defatted with *n*-hexane, and partitioned with EtOAc and 1-BuOH to give EtOAc- (801 g) and 1-BuOH- (374 g) soluble fractions, respectively. Part of the 1-BuOH-soluble fraction (181 g) was subjected to various kinds of column chromatography to give three new glucosides (**1**–**3**) (Fig. 1), and 15 known compounds (**4**–**18**).

(+)-Pinoresinol 4-*O*-[6″-*O*-galloyl]-*β*-D-glucopyranoside (**1**) was obtained as an off-white amorphous powder and its elemental composition was determined to be $C_{33}H_{36}O_{15}$ by HR-electrospray-ionization (ESI)-time-of-flight (TOF)-MS. Its IR spectrum indicated the presence of hydroxyl groups (3367 cm^{-1}), carbonyl functional group(s) (1703 cm^{-1}) and aromatic rings (1606 and 1515 cm^{-1}). Its UV spectrum also suggested the presence of aro-

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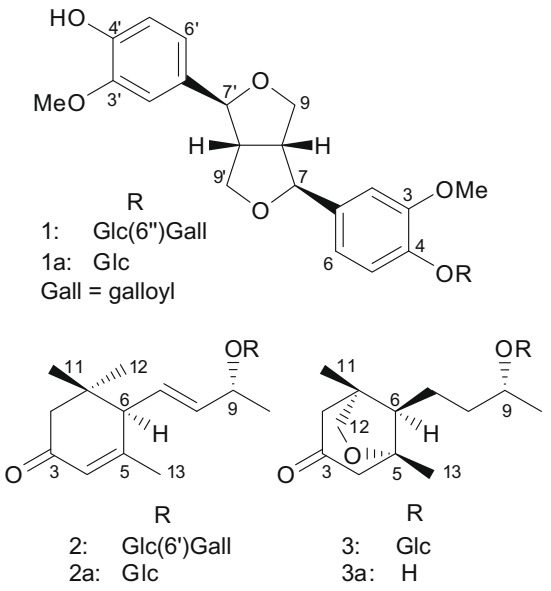


Fig. 1. Structures of new compounds from *Macaranga tanarius* (L.) Müll.-Arg.

Table 1
^{13}C and ^{1}H NMR spectroscopic data for compound (**1**).

	C		H
1	137.6		
2	111.9	6.96	(1H, *d*, *J* = 1.9 Hz)
3	150.7		
4	147.3		
5	118.1	7.02	(1H, *d*, *J* = 8.4 Hz)
6	119.8	6.61	(1H, *dd*, *J* = 8.4, 1.9 Hz)
7	87.1	4.71	(1H, *d*, *J* = 5.2 Hz)
8	55.5	3.05	(1H, *m*)
9	73.0	4.26	(1H, *dd*, *J* = 9.1, 7.1 Hz)
		3.83	(1H, overlapped with H-9′)
1′	133.9		
2′	111.3	6.98	(1H, *d*, *J* = 1.9 Hz)
3′	149.2		
4′	147.5		
5′	116.2	6.78	(1H, *d*, *J* = 8.1 Hz)
6′	120.3	6.83	(1H, *dd*, *J* = 8.1, 1.9 Hz)
7′	87.7	4.68	(1H, *d*, *J* = 5.8 Hz)
8′	55.4	3.12	(1H, *m*)
9′	72.6	4.16	(1H, *dd*, *J* = 9.1, 7.0 Hz)
		3.86	(1H, overlapped with H-9)
3-OMe	56.9	3.84	(3H, *s*)
3′-OMe	56.6	3.87	(3H, *s*)
1″	102.7	4.83	(1H, *d*, *J* = 7.6 Hz)
2″	75.0	3.54	(1H, *dd*, *J* = 9.1, 7.6 Hz)
3″	78.0	3.50	(1H, *t*, *J* = 9.1, 8.6 Hz)
4″	72.2	3.41	(1H, *dd*, *J* = 9.7, 8.6 Hz)
5″	75.8	3.73	(1H, *ddd*, *J* = 9.7, 7.8, 1.8 Hz)
6″	65.0	4.56	(1H, *dd*, *J* = 11.8, 1.8 Hz)
		4.44	(1H, *dd*, *J* = 11.8, 7.8 Hz)
1‴	121.5		
2‴,6‴	110.5	7.10	(2H, *s*)
3‴,5‴	146.7		
4‴	140.1		
C=O	168.2		

Measured in CD_3OD.

matic rings (276 nm). Close inspection of ^{1}H and ^{13}C NMR spectra (Table 1) suggested the presence of a pinoresinol aglycone (Inouye et al., 1973), a 6-*O*-acylated glucopyranose (Matsunami et al., 2006) and a galloyl moiety at the position-6 of glucose. From the above evidence, compound **1** was proposed to be a pinoresinol glucoside with a galloyl moiety on C-6″. The COSY, HMQC and HMBC spectra well supported the predicted structure shown in Fig. 1. The absolute stereochemistry was determined to be same as (+)-pinoresinol by comparison of circular dichroism (CD) spectrum (Xie et al., 2003). Finally, mild alkaline methanolysis of **1** afforded (+)-pinoresinol 4-*O*-*β*-D-glucopyranoside (**1a**) (Abe and Yamauchi, 1989). From these results, compound **1** was elucidated to be (+)-pinoresinol 4-*O*-[6″-*O*-galloyl]-*β*-D-glucopyranoside as a new compound.

Macarangioside E (**2**) was isolated as an off-white amorphous powder and a molecular-related ion peak was observed at *m*/*z* 545.1990 $[M+Na]^+$ ($C_{26}H_{34}O_{11}Na$) on positive-ion HR-ESI-TOF-MS. In the IR spectrum, strong absorptions at 3303 and 1706 cm^{-1} suggested the presence of hydroxyl and carbonyl functional groups, respectively. The absorption maxima in the UV spectrum (244sh and 275 nm) also suggested presence of aromatic ring and conjugated ketone moieties. The ^{13}C NMR spectrum showed the presence of six signals assignable to a 6-*O*-acylated glucopyranose and five resonances to a galloyl moiety (Table 2), which indicated the presence a of 6-*O*-galloyl glucopyranose moiety in its structure. The remaining 13 carbon signals corresponded to a compound having a megastigmane skeleton. The ^{1}H NMR spectra displayed two olefinic protons at δ_H 5.69 (*J* = 15, 7, 1 Hz) and 5.53 (*J* = 15, 9, 1 Hz), indicative of the presence of a *trans* double bond (Table 3). A ketonic carbonyl signal at δ_c 202.2 (s) and two olefinic carbons [δ_c 166.0 (s) and 126.1 (d)] established the presence of a conjugated ketone functional group. The COSY spectrum clarified connections from H-6 to H_3-10, and HMBC correlations between H_3-13 and C-4, 5 and 6 confirmed the location of the conjugated ketone function, and the correlations between H_3-11 and 12 and C-1, 2 and 6 also clarified the six-membered ring moiety of **2** as in Fig. 2. The attachments of glucopyranosyl and galloyl moieties were confirmed by analysis of HMBC correlations (Fig. 2). The ^{13}C chemical shift value of C-9 [δ_c 78.6 (d)] was diagnostic in assigning the absolute configuration at C-9 as 9*R*, when compared with the the reported deshielded signal of C-9 for 9*R*-configuration (Pabst et al., 1992). The Cotton effects observed in the CD spectrum [$\Delta\varepsilon$

Table 2
^{13}C NMR spectroscopic data for macarangiosides E (**2**), F (**3**), and degalloylmacarangioside B (**19a**).

	2	3	**19a**
1	37.0	44.9	45.4
2	48.5	49.6	49.9
3	202.2	212.4	211.6
4	126.1	50.7	51.1
5	166.0	85.6	85.0
6	56.3	54.9	59.6
7	129.5	22.2	124.9
8	138.0	37.8	141.3
9	78.6	76.0	76.9
10	21.6	20.1	21.2
11	27.4	20.9	20.6
12	27.9	79.5	80.0
13	23.7	25.2	24.4
Glc			
1′	103.3	102.4	102.6
2′	75.3	75.3	75.3
3′	78.1	78.3	78.2
4′	72.1	72.0	71.7
5′	75.6	78.0	78.1
6′	65.2	63.1	62.8
Gall			
1″	121.7		
2″,6″	110.4		
3″,5″	146.7		
4″	140.0		
C=O	168.3		

Measured in CD_3OD.

Table 3
^{1}H NMR spectroscopic data for macarangiosides E **(2)**, F **(3)** and degalloylmacarangioside B **(19a)**.

	2	3	19a
2	1.94 (1H, *d*, *J* = 16.6 Hz) 2.31 (1H, *d*, *J* = 16.6 Hz)	2.16 (1H, *dt*, *J* = 17.3, 1.6 Hz, eq) 2.54 (1H, *dd*, *J* = 17.3, 2.4 Hz, ax)	2.23 (1H, *dt*, *J* = 17.4, 1.7 Hz, eq) 2.57 (1H, *dd*, *J* = 17.4, 2.7 Hz, ax)
4	5.79 (1H, br *s*)	2.21 (1H, *dt*, *J* = 17.1, 1.6 Hz, eq) 2.57 (1H, *d*, *J* = 17.1 Hz, ax)	2.27 (1H, *dt*, *J* = 17.7, 1.7 Hz, eq) 2.56 (1H, *d*, *J* = 17.7 Hz, ax)
6	2.41 (1H, br *d*, *J* = 9.0 Hz)	1.74 (1H, *m*)	2.41 (1H, br *dt*, *J* = 9.5, 1.7 Hz)
7	5.53 (1H, *ddd*, *J* = 15.4, 9.0, 0.7 Hz)	1.49 (1H, *m*, pro-*S*) 1.84 (1H, *m*, pro-*R*)	5.80 (1H, *ddd*, *J* = 15.1, 9.5, 1.2 Hz)
8	5.69 (1H, *ddd*, *J* = 15.4, 7.0, 0.5 Hz)	1.70 (1H, *m*) 1.78 (1H, *m*)	5.95 (1H, *ddd*, *J* = 15.1, 6.3, 0.6 Hz)
9	4.29 (1H, *m*)	3.94 (1H, *m*)	4.45 (1H, *qdd*, *J* = 6.3, 6.3, 1.2 Hz)
10	1.28 (1H, *d*, *J* = 6.4 Hz)	1.22 (3H, *d*, *J* = 6.2 Hz)	1.32 (3H, *d*, *J* = 6.3 Hz)
11	0.87 (3H, *s*)	1.09 (3H, *s*)	1.04 (3H, *s*)
12	0.86 (3H, *s*)	3.55 (1H, *dd*, *J* = 7.9, 2.4 Hz, pro-*R*) 3.57 (1H, *d*, *J* = 7.9 Hz, pro-*S*)	3.60 (1H, *dd*, *J* = 8.0, 2.7 Hz, pro-*R*) 3.68 (1H, *d*, *J* = 8.0 Hz, pro-*S*)
13	1.82 (3H, *d*, *J* = 1.1 Hz)	1.31 (3H, *s*)	1.23 (3H, *s*)
Glc			
1′	4.37 (1H, *d*, *J* = 7.9 Hz)	4.35 (1H, *d*, *J* = 7.8 Hz)	4.38 (1H, *d*, *J* = 7.8 Hz)
2′	3.21 (1H, *dd*, *J* = 8.8, 7.9 Hz)	3.15 (1H, *dd*, *J* = 9.2, 7.8 Hz)	3.19 (1H, *dd*, *J* = 9.1, 7.8 Hz)
3′	3.37 (1H, *t*, *J* = 8.8 Hz)	3.36 (1H, *m*)	3.35 (1H, *t*, *J* = 9.1 Hz)
4′	3.35 (1H, *t*, *J* = 8.8 Hz)	3.26 (1H, overlapped)	3.18 (1H, *t*, *J* = 9.1 Hz)
5′	3.51 (1H, *ddd*, *J* = 8.8, 6.8, 2.2 Hz)	3.26 (1H, overlapped)	3.23 (1H, *ddd*, *J* = 9.1, 5.6, 2.4 Hz)
6′	4.57 (1H, *dd*, *J* = 11.8, 2.2 Hz) 4.30 (1H, *dd*, *J* = 11.8, 6.8 Hz)	3.63 (1H, *dd*, *J* = 11.6, 5.8 Hz) 3.87 (1H, *dd*, *J* = 11.6, 2.0 Hz)	3.65 (1H, *dd*, *J* = 11.8, 5.6 Hz) 3.82 (1H, *dd*, *J* = 11.8, 2.4 Hz)
Gall			
2″,6″	7.10 (2H, *s*)		

Measured in CD_3OD.

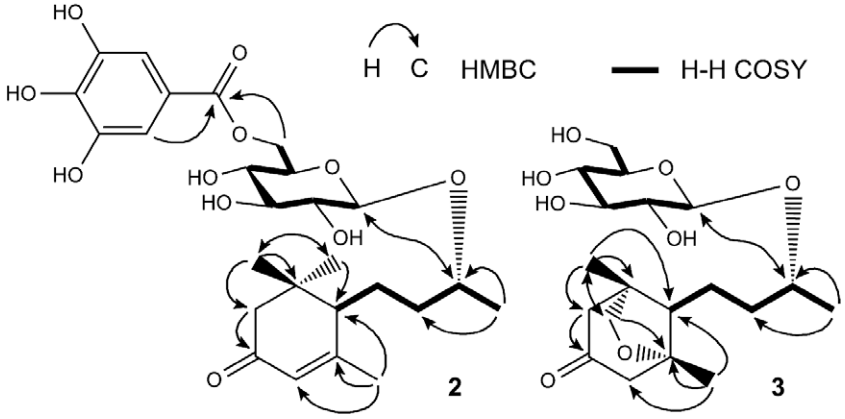


Fig. 2. COSY and HMBC correlations of macarangiosides E (**2**) and F (**3**).

(nm) −0.9 (317) and +18.5 (245)] were well in accord with those of (6*R*,9*R*)-3-oxo-α-ionol β-D-glucopyranoside. In addition, the degalloylated compound (**2a**) obtained in mild alkaline methanolysis was fully identical with (6*R*,9*R*)-3-oxo-α-ionol β-D-glucopyranoside on the bases of ^{1}H, and ^{13}C NMR spectra and the optical rotation value (Pabst et al., 1992; Mohamed et al., 1999). As a result, macarangioside E (**2**) was elucidated to be (6*R*,9*R*)-3-oxo-α-ionol 9-*O*-[6′-*O*-galloyl]-β-D-glucopyranoside.

Macarangioside F (**3**) was obtained as a colorless amorphous powder, whose molecular formula was determined to be $C_{19}H_{32}O_8$ based on its positive-ion HR-ESI-TOF-MS. Its IR spectrum suggested the presence of hydroxyl groups (3396 cm^{-1}) and a carbonyl functional group (1712 cm^{-1}) in its structure. In the ^{1}H and ^{13}C NMR spectra (Tables 2 and 3), an anomeric proton signal and six carbon signals assignable to a glucopyranose moiety were observed. The chemical shift values of the remaining 13 carbon signals suggested a 9-hydroxy-3-oxo-megastigmane skeleton, of which one of the methyl signals was oxygenated [δc 79.5 (t)]. The index of hydrogen deficiency calculated from the molecular formula indicated the presence of an additional ring system in its structure. The HMBC correlation between H_2-12 and C-5 clarified the presence of an oxirane ring system between C-5 and C-12 (Fig. 2). Thus the aglycone of macarangioside F (**3**) was considered to be a 5,12-epoxymegastigmane. The COSY correlations from H-6 to H_3-10, and the HMBC correlations between H_3-13 and C-4, 5 and 6, and between H_3-11 and C-1, 2, 6 and 12 established the structure of aglycone as shown in Fig. 2. The attachment of glucose was also confirmed by analysis of the HMBC correlation. The relative stereochemistry of the aglycone moiety was suggested to be of Fig. 2 and 3 from the proton–proton coupling pattern data especially "W"-type long-range coupling, i.e., H-2 eq was coupled with H-4 eq and H-6, H-2ax with pro-*R* H-12, and H-4 eq also both with H-2 eq and H-6. The NOESY experiment also supported these observations (Fig. 3). The absolute stereochemistry at C-9 [δ_c 76.0 (d)] was suggested to be 9*R* by comparison of the chemical shift value of myrsinionoside A (δc 75.9) (Otsuka et al., 2001). The absolute configuration of glucose was determined to be in D-series on HPLC analysis with optical rotation detector after enzymatic hydrolysis of **3**. Thus the structure of macarangioside F (**3**) was tentatively

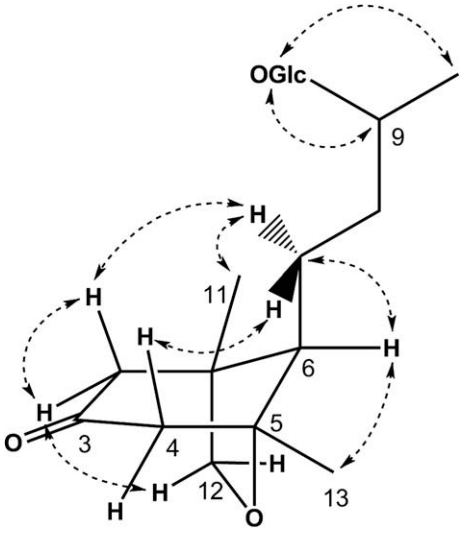


Fig. 3. Phase-sensitive NOESY correlations of macarangioside F (**3**).

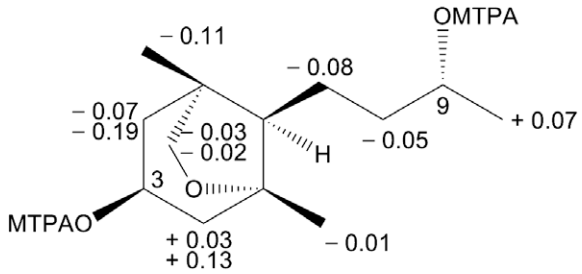
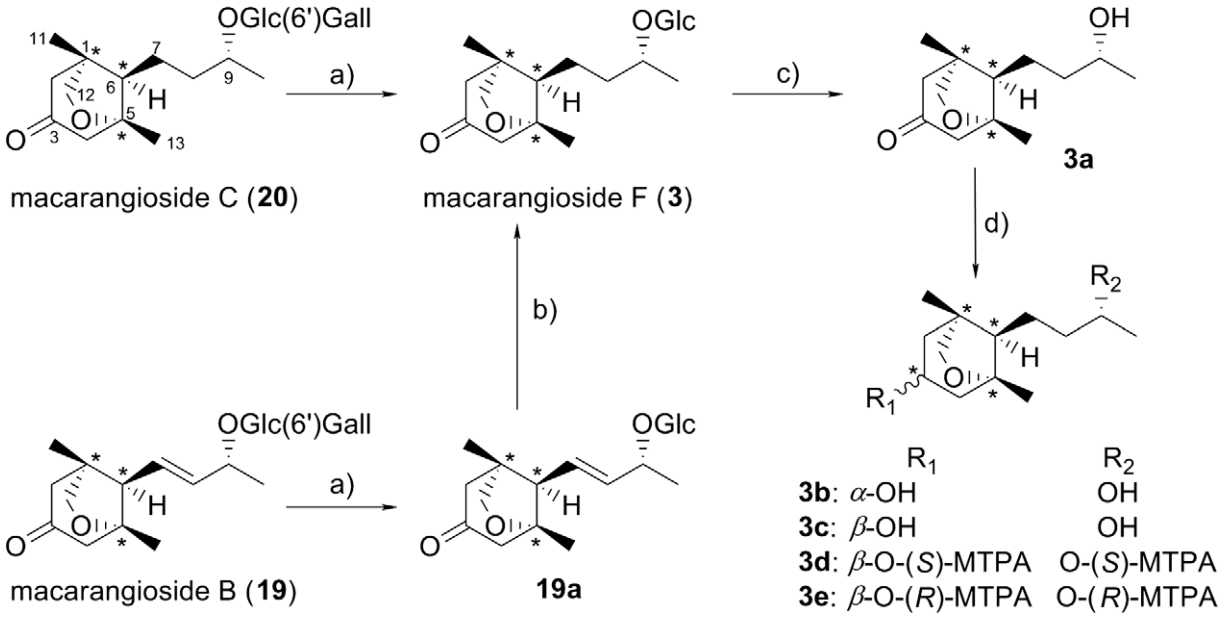


Fig. 4. Results with the modified Mosher's method for **3c**. $\Delta\delta$ values ($\delta_S - \delta_R$) were shown in ppm.

elucidated to be (1*S**,5*R**,6*R**,9*R*)-megastigman-3-on-5,12-epoxy-9-ol 9-*O*-*β*-D-glucopyranoside.

In our previous paper, closely related compounds, macarangiosides B (**19**) and C (**20**) (Scheme 1), were isolated from the same plant (Matsunami et al., 2006). When comparing the structures of **19** and **20** to that of **3**, the differences were a galloyl moiety on glucose for **19** and **20**, and a *trans* double bond at C-7 and 8 for **19** (Scheme 1). However the absolute stereochemistry at C-1, 5 and 6 was not determined. Therefore, in this study, we have elucidated the absolute stereochemistry of these compounds by chemical conversion and application of the modified Mosher's method (Scheme 1). Macarangiosides B (**19**) and C (**20**) were first hydrolyzed with 0.1 M NaOMe at room temperature to afford **19a** and **20a**, respectively. The physical data including optical rotation value of **20a** were essentially identical to those of **3**. The double bond at C-7 and 8 of **19a** was then reduced by catalytic hydrogenation to afford **19b**. The physical data of **19b** were fully identical to those of **3**. Thus, macarangiosides B (**19**), C (**20**) and F (**3**) had the same relative stereochemistry at their chiral centers. Then, macarangioside F (**3**) was enzymatically hydrolyzed to afford the aglycone (**3a**), and reduced with $NaBH_4$ to give a mixture of 3*α* and 3*β*,9*α*-diols (**3b** and **3c**). After preparative TLC separation, the 3*β*,9*α*-diol (**3c**) having an equatorial hydroxyl group was esterified with (*S*)- and (*R*)- α-methoxy-α-trifluoromethylphenylacetic acids (MTPAs) to afford diesters, **3d** and **3e**, respectively. The $\Delta\delta$ values from their ^{1}H NMR spectra indicated that compound **3c** had 3*S* configuration (Fig. 4). Therefore, **3**, **19** and **20** were elucidated to be (1*S*,5*R*,6*R*,9*R*)-megastigman-3-on-5,12-epoxy-9-ol 9-*O*-*β*-D-glucopyranoside, (1*S*,5*R*,6*R*,7*E*,9*R*)-megastigman-7-en-3-on-5,12-epoxy-9-ol 9-*O*-[6′-*O*-galloyl]-*β*-D-glucopyranoside, and (1*S*,5*R*,6*R*,9*R*)-megastigman-3-on-5,12-epoxy-9-ol 9-*O*-[6′-*O*-galloyl]-*β*-D-glucopyranoside, respectively.

The known compounds (**4**–**18**) were identified by comparison of spectroscopic data with those reported in the literature as follows. Roseoside (**4**), amorphous powder, $[\alpha]_D^{25}$ +135 (*c* 0.45, MeOH) (Otsuka et al., 1995), icariside B_5 (**5**), amorphous powder, $[\alpha]_D^{24}$ −1.0 (*c* 0.32, MeOH), CD (*c* 4.12 × 10^{-5} M, MeOH) Δε (nm):+0.59 (329), −1.47 (254), +3.06 (219) (Andersson and Lundgren, 1988), (6*R*,9*R*)-3-oxo-α-ionol *β*-D-glucoside (**6**), amorphous powder, $[\alpha]_D^{25}$ +154 (*c* 1.08, MeOH), CD (*c* 2.92 × 10^{-5} M, MeOH) Δε (nm): −0.64 (319),+20.0 (244) (Pabst et al., 1992), (6*R*,9*S*)-3-oxo-α-ionol *β*-D-glucoside (**7**), amorphous powder, $[\alpha]_D^{26}$ +27.7 (*c* 0.84, MeOH)



Scheme 1. (a) 0.1 M NaOMe in MeOH, rt, 2 h, (b) PtO_2/H_2, rt, 1 h, (c) crude hesperidinase, 37 °C, 12 h, (d) $NaBH_4$ in MeOH, 0–25 °C, 30 min, (e) EDC, DMAP, (*R*) and (*S*)-MTPA in CH_2Cl_2, rt, 30 min.

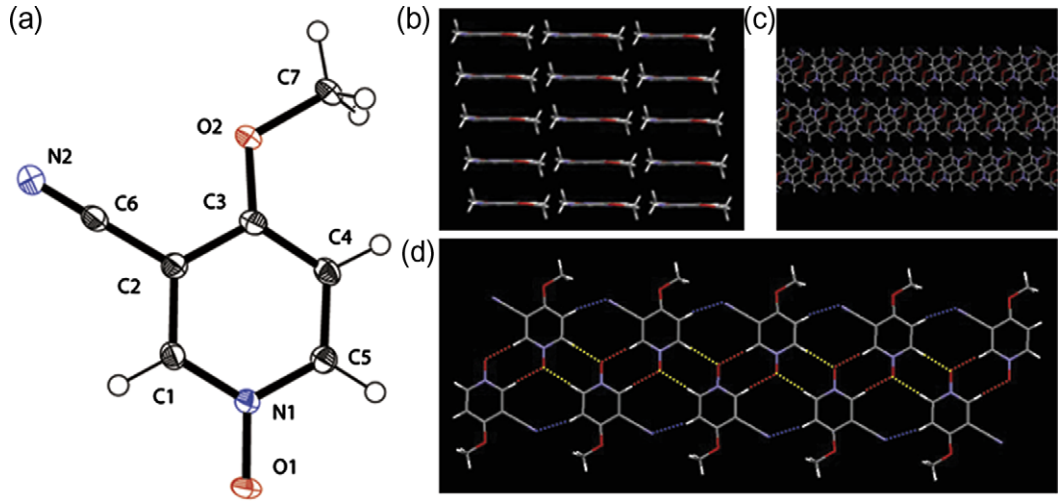


Fig. 5. X-ray crystallographic analysis of **18.** (a) ORTEP drawing of **18** with crystallographic numbering, (b) lateral view, (c) bird's-eye view and (d) inter-molecule hydrogen bonding.

(Pabst et al., 1992), (2*S*,3*R*)-dihydrodehydrodiconiferyl alcohol *β*-D-glucoside (**8**), amorphous powder, $[\alpha]_D^{25}$ −27.6 (*c* 0.9, MeOH), CD (*c* 1.72 × 10^{-5} M, MeOH) Δε (nm): −0.48 (289), −0.92 (242), +1.16 (223) (Matsuda et al., 1996), (+)-pinoresinol 4-*O*-*β*-D-glucopyranoside (**9**), amorphous powder, $[\alpha]_D^{25}$ +0.25 (*c* 0.8, MeOH) (Deyama, 1983), scopolin (**10**), amorphous powder, $[\alpha]_D^{26}$ −45.2 (*c* 0.65, MeOH) (Tsukamoto et al., 1985), quercetin 3-*O*-rutinoside (**11**), amorphous powder, $[\alpha]_D^{25}$ −23.1 (*c* 6.65, MeOH) (Harborne and Mabry, 1982), quercetin 3-*O*-galactopyranoside (**12**), amorphous powder, $[\alpha]_D^{25}$ −30.3 (*c* 0.07, MeOH) (Harborne and Mabry, 1982), quercetin 3-*O*-arabinopyranoside (**13**), amorphous powder, $[\alpha]_D^{25}$ −102 (*c* 0.20, MeOH) (Harborne and Mabry, 1982), isovitexin (**14**), amorphous powder, $[\alpha]_D^{24}$ +19.6 (*c* 0.51, MeOH) (Harborne and Mabry, 1982), methyl gallate (**15**), amorphous powder, (*Z*)-3-hexenyl *β*-D-glucoside (**16**), amorphous powder, $[\alpha]_D^{25}$ −13.1 (*c* 0.32, MeOH) (Mizutani et al., 1988), (*E*)-2-hexenyl *β*-D-glucoside (**17**), amorphous powder, $[\alpha]_D^{25}$ −39.2 (*c* 0.53, MeOH) (Mizutani et al., 1988), malloapeltine (**18**), colorless rods, mp 203–205 (Cheng et al., 1998).

The pyridine *N*-oxide **18** is rare and its isolation was the second time from Nature. It is noteworthy that the calculated specific density value from the X-ray crystallographic analysis was unusually large (1.461 g/cm^3) due to tight stacking of molecules by π–π interaction of the pyridine rings as shown in Fig. 5.

The MeOH extract of *M. tanarius* possessed anti-oxidative activity (Lim et al., 2009). Thus, the free radical-scavenging activity of new compounds **1**–**3** was evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH). Trolox was used as a reference compound. Compounds **1** and **2** showed potent radical-scavenging activities. The activity of compounds **1** and **2** were 1.07 and 0.55 times of Trolox, respectively. Considering the structural feature, the radical-scavenging activity was interpreted as dependent on their galloyl and phenolic hydroxyl moieties. Cytotoxicity of the new compounds (**1**–**3**) was also assayed against human lung adenocarcinoma cell line A549. Even at the highest concentration employed (100 μM), compounds **1**–**3** did not exhibit any significant cytotoxicity (30.3%, 11.3% and 23.8% inhibition, respectively). Thus, compounds **1** and **2** can be considered as non-toxic safe antioxidant agents.

3. Discussion

There were several literature references concerning 5,12-epoxymegastigmane. However, the absolute stereochemistry of the 3-keto-type 5,12-epoxymegastigmane, such as asysgangoside (Kanchanapoom and Ruchirawat, 2007), spionoside B (Calis et al., 2002), and annuionone E (Macías et al., 2004) have never been fully determined, because of a lack of reliable reference data such as CD spectra. In this study, both macarangioside F (**3**) and degalloylmacarangioside B (**19a**) showed similar CD values to those reported for asysgangoside and spionoside B. Thus, asysgangoside and spionoside B are considered to must have the same absolute stereochemistry as that of **3** and **19a**. In addition, the physical data of annuionone E including its optical rotation value were superimposable on those of the aglycone of macarangioside F (**3a**), which meant that annuionone E also has the same absolute stereostructure as that of **3a**.

The plant hormone, abscisic acid (ABA), is mainly oxidized at C-8′ (equivalent to C-12 of megastigmane) by ABA 8′-hydroxylase (cytochrome P450 monooxygenase) in plant (Krochko et al., 1998). 8′-Hydroxy ABA spontaneously isomerizes to phaseic acid (PA) by formation of a 5-membered ether ring between C-8′ and C-2′ (equivalent to C-5 of megastigmane). Thus the same mechanism may contribute to the biosynthesis of 5,12-epoxymegastigmane. However, another oxidation pathway of ABA has discovered recently (Zhou et al., 2004). This minor pathway involved the hydroxylation of 9′-methyl group (equivalent to C-11 methyl group of megastigmane) of ABA resulting in 9'-hydroxy ABA, which isomerizes to neo-PA. Therefore, 5,11-epoxymegastigmane might be isolated in future.

4. Concluding remarks

Three new glucosides, (+)-pinoresinol 4-*O*-[6″-*O*-galloyl]-*β*-D-glucopyranoside (**1**), macarangioside E and F (**2**, **3**), and 15 known compounds (**4**–**18**) were isolated from the leaves of *Macaranga tanarius*. The absolute stereochemistries of them were fully established using both spectroscopic and chemical approaches. Galloylated compounds (**1** and **2**) possessed potent anti DPPH radical-scavenging activity.

5. Experimental

5.1. General experimental procedures

A highly porous synthetic resin (Diaion HP-20) was purchased from Mitsubishi Chemical Co., Ltd. (Tokyo, Japan). Column chromatography

(CC) was performed using silica gel 60 (Merck, Darmstadt, Germany), and reversed-phase [octadecyl silica gel (ODS)] open CC (RPCC) on Cosmosil 75C_{18}-OPN (Nacalai Tesque, Kyoto, Japan) [Φ = 5 cm, L = 25 cm, linear gradient: MeOH–H_2O (1:9, 1 L) → (1:1, 1 L), then (1:1, 0.5 L) → (7:3, 0.5 L), fractions of 10 g being collected]. Droplet Counter-Current Chromatography (DCCC) (Tokyo Rikakikai, Tokyo, Japan) was equipped with 300 glass columns (Φ = 2 mm, L = 40 cm), and the lower and upper layers of a solvent mixture of $CHCl_3$–MeOH–H_2O-1–PrOH (9:12:8:2) were used as stationary and mobile phases, respectively. Five-gram fractions were collected and numbered according to their order of elution with the mobile phase. HPLC was performed on ODS (Inertsil ODS-3; GL Science, Tokyo, Japan; Φ = 6 mm, L = 250 mm), and the eluate was monitored with a UV detector at 254 nm and a refractive index monitor. Optical rotations were measured on a JASCO P-1030 polarimeter. IR spectra were recorded on a Horiba FT-710 Fourier transform infrared spectrophotometer and UV spectra on a JASCO V-520 UV/Vis spectrophotometer. ^1H and ^{13}C NMR spectra were obtained on JEOL JNM α-400, λ-500 and ECA-600 spectrometers at 400, 500, and 600 MHz for ^1H, and 100, 125 and 150 MHz for ^{13}C, respectively, with tetramethylsilane as an internal standard. Positive-ion HR-ESI-TOF-MS was recorded on an Applied Biosystem QSTAR XL spectrometer. CD spectra were obtained on a JASCO J-720 spectropolarimeter. VERSA max (Molecular Device) was used as a microplate reader.

5.2. Plant material

Leaves of *M. tanarius* Müll.-Arg (Euphorbiaceae) were collected in Okinawa, Japan, in June, 2003, and a voucher specimen was deposited in the herbarium of the Department of Pharmacognosy, Division of Medicinal Chemistry, Graduate School of Biomedical Sciences, Hiroshima University (03-MT-OKINAWA-0630).

5.3. Extraction and isolation

The 1-BuOH soluble material (801 g) was prepared by the procedure described previously (Matsunami et al., 2006). A portion of the 1-BuOH-soluble fraction (181 g) was subjected to highly porous synthetic resin (Diaion HP-20) CC (Mitsubishi Chemical Co., Ltd.; Φ = 8 cm, L = 40 cm), using H_2O–MeOH (4:1, 6 L), (2:3, 6 L), (3:2, 6 L) and (1:4, 6 L) and MeOH (6 L), 1 L fractions were collected. The residue (24.9 g in fractions 9–12) of the MeOH–H_2O (40:60, v/v) eluate obtained on Diaion HP-20 CC was subjected to silica gel (Φ = 6 cm, L = 50 cm) CC with increasing amounts of MeOH in $CHCl_3$ [$CHCl_3$ (2 L), and $CHCl_3$–MeOH (49:1, 3 L), (19:1, 3 L), (47:3, 3 L), (23:2, 3 L), (9:1, 3 L), (17:3, 3 L), (4:1, 3 L), (3:1, 3 L), and (7:3, 3 L)], $CHCl_3$–MeOH–H_2O (70:30:4, 3 L) and MeOH (3 L). 500 mL fractions were collected. The residue (0.45 g in fractions 22–31) of the MeOH–$CHCl_3$ (1:9) eluate obtained on silica gel CC was subsequently subjected to RPCC with gradient mixture of MeOH–H_2O (1:9, 1 L; 1:1, 1 L, and then 1:1, 500 mL; 3:7, 500 mL). 10 g fractions were collected. The residue (24.0 mg in fractions 1–55 of RPCC) was then purified by HPLC (H_2O–CH_3CN, 19:1) to give compound **18** (10.2 mg), whereas the residue (42.2 mg in fractions 65–88 of RPCC) was purified by HPLC (H_2O–CH_3CN, 9:1) to afford compound **10** (6.5 mg). The residue (65.4 mg in fractions 89–102 of RPCC) was purified by HPLC (H_2O–CH_3CN, 19:1) to give compounds **3** (5.1 mg), **4** (4.5 mg) and **5** (3.2 mg), with residue (77.2 mg in fractions 123–145 of RPCC) purified by HPLC (H_2O–CH_3CN, 41:9) to afford compounds **6** (10.8 mg), **7** (12.6 mg), **16** (3.2 mg) and **17** (5.3 mg). The residue (3.15 g in fractions 45–59) of the MeOH–$CHCl_3$ (1:3, v/v) eluate obtained on silica gel CC was subsequently subjected to RPCC with a gradient mixture of MeOH–H_2O (1:9, 1 L; 1:1, 1 L, and then 1:1, 500 mL; 3:7, 500 mL). 10 g fractions were collected. The residue (1.44 g in fractions 71–110 of RPCC) was then subjected to DCCC to give five fractions. The residue (23.8 mg in fractions 85–100 of DCCC) was further purified by HPLC (H_2O–CH_3CN, 4:1) to give compound **15** (5.2 mg). The residue (23.1 g in fractions 13–17) of the 60% MeOH eluate obtained on Diaion HP-20 CC was subjected to silica gel (Φ = 5 cm, L = 50 cm) CC with increasing amounts of MeOH in $CHCl_3$ [$CHCl_3$–MeOH (100:1, 6 L), (25:1, 3 L), (10:1, 3 L), (5:1, 3 L), (2:1, 3 L), (1:1, 3 L)]. The residue (7.66 g) of the $CHCl_3$–MeOH (2:1) eluate obtained on silica gel CC was subsequently subjected to RPCC with gradient mixture of MeOH–H_2O (1:9, 1 L; 1:1, 1 L, then 1:1, 500 mL; 3:7, 500 mL). 10 g fractions were collected. The residue (2.47 g in fractions 126–144 of RPCC) was then subjected to DCCC to give five fractions. The residue (670 mg in fractions 26–36 of DCCC) was further purified by HPLC (H_2O–CH_3CN, 41:9) to afford compound **11** (66.5 mg). The residue (206 mg in fractions 54–64 of DCCC) was further purified by HPLC (H_2O–CH_3CN, 83:17) to afford compounds **12** (13.1 mg) and **14** (20.3 mg). The residue (154 mg in fractions 86–100 of DCCC) was further purified by HPLC (H_2O–CH_3CN, 4:1) to afford compounds **8** (9.0 mg) and **2** (0.9 mg). The residue (3.54 g) of the $CHCl_3$-MeOH (1:1) eluate obtained on silica gel CC was subsequently subjected to RPCC with gradient mixture of MeOH–H_2O (1:9, 1 L; 1:1, 1 L, then 1:1, 500 mL; 3:7, 500 mL). 10 g fractions were collected. The residue (306 mg in fractions 51–105 of RPCC) was then subjected to DCCC to afford five fractions, whereas residue (82.8 mg in fractions 32–42 of DCCC) was further purified by HPLC (H_2O–CH_3CN, 4:1) to give compound **13** (40.8 mg). The residue (97.6 mg in fractions 43–60 of DCCC) was further purified by HPLC (ODS, H_2O–CH_3CN, 4:1, v/v) to give compound **1** (7.6 mg), residue (114 mg in fractions 61–85 of DCCC) was further purified by HPLC (H_2O–CH_3CN, 4:1) to afford compound **9** (8.0 mg).

5.4. Characterization data

5.4.1. (+)-Pinoresinol 4-O-[6″-O-galloyl]-β-D-glucopyranoside (1)

Amorphous powder; $[\alpha]_D^{25}$ +18.1 (*c* 0.44, MeOH); IR ν_{max} (film) cm^{-1}: 3367, 2961, 1703, 1606, 1515, 1266, 1226, 1073, 1034; UV λ_{max} (MeOH) nm (log ε): 218 (4.42), 276 (4.05); for ^{13}C- and ^1H NMR (CD_3OD) spectroscopic data, see Table 1; CD Δε (nm): +1.74 (280), +1.79 (256), −0.23 (235), +1.27 (223), (*c* 1.18×10^{-5} M, MeOH); HR-ESI-TOF-MS (positive-ion mode) *m*/*z*: 695.1949 $[M+Na]^+$ (Calcd. for $C_{33}H_{36}O_{15}Na$: 695.1946).

5.4.2. Macarangioside E (2)

Amorphous powder; $[\alpha]_D^{25}$ +78.4 (*c* 0.19, MeOH); IR ν_{max} (film) cm^{-1}: 3303, 2961, 1706, 1648, 1514, 1449, 1259, 1076, 1032; UV λ_{max} (MeOH) nm (log ε): 219 (4.54), 244sh (4.25), 275 (4.12); for ^{13}C and ^1H NMR (CD_3OD) spectroscopic data, see Tables 2 and 3; CD Δε (nm): −0.90 (317), +18.5 (245) (*c* 1.85×10^{-5} M, MeOH); HR-ESI-TOF-MS (positive-ion mode) *m*/*z*: 545.1990 $[M+Na]^+$ (Calcd. for $C_{26}H_{34}O_{11}Na$: 545.1993).

5.4.3. Macarangioside F (3)

Amorphous powder; $[\alpha]_D^{26}$ −6.0 (*c* 0.34, MeOH); IR ν_{max} (film) cm^{-1}: 3396, 2928, 1712, 1380, 1275, 1077, 1037; for ^{13}C and ^1H NMR (CD_3OD) spectroscopic data, see Table 2 and 3; CD Δε (nm): +0.31 (362), −0.06 (298), +0.53 (250) (*c* 5.03×10^{-5} M, MeOH); HR-ESI-TOF-MS (positive-ion mode) *m*/*z*: 411.1987 $[M+Na]^+$ (Calcd. for $C_{19}H_{32}O_8Na$: 411.1989).

5.4.4. Mild alkaline methanolysis of 1

A mixture of **1** (3.3 mg) and 0.1 M NaOMe in MeOH (1.0 mL) was allowed to stand at room temperature for 1 h under N_2. Liberation of degalloylated compound **1a** was monitored by TLC analysis ($CHCl_3$: MeOH: H_2O, 15:6:1, R_f values, **1**: 0.48 and **1a**: 0.57). The reaction mixture was neutralized with Amberlite IR-120B (Organo) and subjected to silica gel CC [7 g, Φ = 1 cm, L = 15 cm, linear gra-

dient, $CHCl_3$–MeOH (10:1, 100 mL) → $CHCl_3$–MeOH–H_2O (15:6:1, 100 mL), 8 g fractions being collected]. Degalloylated compound **1a** (1.7 mg) was recovered in fractions 10–14 as (+)-pinoresinol *O*-*β*-D-glucopyranoside, which was identified analysis of ^{1}H- and ^{13}C NMR spectroscopic data in C_5D_5N (Abe and Yamauchi, 1989) and optical rotation value, $[\alpha]_D^{26}$ +13.1 (*c* 0.11, MeOH) [Ref. for (+)-pinoresinol *O*-*β*-D-glucopyranoside, +8.0 (*c* 0.1, MeOH) (Deyama, 1983)]. ^{13}C NMR (CD_3OD, 150 MHz) δ_C: 151.2 (C-3), 149.2 (C-3′), 147.6 (2 × C, C-4 & 4′), 137.7 (C-1), 133.9 (C-1′), 120.1 (C-6′), 119.9 (C-6), 118.3 (C-5), 116.2 (C-5′), 111.9 (C-2), 111.2 (C-2′), 103.1 (C-1″), 87.6 (C-7′), 87.2 (C-7), 78.3 (C-3″), 78.0 (C-5″), 75.0 (C-2″), 72.8 (2 × C, C-9 & 9′), 71.5 (C-4″), 62.6 (C-6″), 56.9 (3-OMe), 56.6 (3′-OMe), 55.6 (C-8), 55.4 (C-8′).

5.4.5. Mild alkaline methanolysis of *2*

In a similar to that described above, compound **2** (1.5 mg) liberated degalloylated compound **2a** (0.72 mg) (TLC, $CHCl_3$: MeOH: H_2O, 15:6:1, R_f values, **2**: 0.42 and **2a**: 0.58). **2a**: Amorphous powder; $[\alpha]_D^{26}$ +84.2 (*c* 0.048, MeOH) and CD $\Delta\varepsilon$ (nm): −0.9 (311),+11.0 (244) (*c* 0.97 × 10^{-5} M, MeOH) [Ref. for (6*R*,9*R*)-3-oxo-*α*-ionol 9-*O*-*β*-D-glucopyranoside; $[\alpha]_D^{22}$ +95.2 (*c* 1.33, MeOH), (Mohamed et al., 1999), CD rel.int. (nm): −8 (327), +207 (243) (EtOH) (Pabst et al., 1992)].

5.4.6. Enzymatic hydrolysis of *3*

Compound **3** (4.1 mg) was hydrolyzed with crude hesperidinase at 37 °C in H_2O (1 mL) with reciprocal shaking for 12 h. Liberation of glucose was monitored by TLC analysis ($CHCl_3$: MeOH: H_2O, 15:6:1, R_f values, **3**: 0.47, aglycone **3a**: 0.75, and D-glucose: 0.12). The reaction mixture was concentrated and subjected to silica gel CC [7 g, Φ = 1 cm, *L* = 15 cm, linear gradient, $CHCl_3$–MeOH (10:1, 100 mL) → $CHCl_3$–MeOH–H_2O (15:6:1, 100 mL) → EtOH 30 mL, 8 g fractions were collected]. The aglycone **3a** (1.8 mg, 80%) and glucose were recovered in fractions 6–11 and 28–29, respectively. (1*S*,5*R*,6*R*,9*R*)-megastigman-3-on-5,12-epoxy-9-ol (**3a**): Amorphous powder; $[\alpha]_D^{24}$ +6.1 (*c* 0.11, MeOH) [Ref. for annuionone E: $[\alpha]_D$ + 4.2 (*c* 0.1, MeOH) (Macías et al., 2002)], IR ν_{max} (film) cm^{-1}: 3423, 2925, 1712, 1457, 1377, 1261, 1019; ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C: 209.3 (C-3), 83.5 (C-5), 78.5 (C-12), 68.1 (C-9), 53.8 (C-6), 49.5 (C-4), 48.8 (C-2), 43.5 (C-1), 38.7 (C-8), 24.9 (C-13), 23.9 (C-10), 21.5 (C-7), 20.9 (C-11); ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H: 3.83 (1H, sextet, *J* = 6 Hz, H-9), 3.64 (1H, d, *J* = 8 Hz, pro-*S* H-12), 3.58 (1H, dd, *J* = 8, 2 Hz, pro-*R* H-12), 2.42–2.32 (3H, overlapped, H-2β, 4α and 4β), 2.23 (1H, dt, *J* = 18, 2 Hz, H-2α), 1.74 (1H, dq, *J* = 16, 6 Hz, pro-*R* H-7), 1.68 (1H, m, H-6), 1.63 (2H, q, *J* = 6 Hz, H_2-8), 1.37 (1H, dq, *J* = 16, 6 Hz, pro-*S* H-7), 1.33 (3H, s, H_3-13), 1.24 (3H, d, *J* = 6 Hz, H_3-10), 1.08 (3H, s, H_3-11); CD $\Delta\varepsilon$ (nm): +0.04 (357), −0.25 (281), −0.70 (211) (*c* = 4.78 × 10^{-5} M, MeOH); HR-ESI-TOF-MS (positive-ion mode) *m*/*z*: 249.1464 $[M+Na]^+$ (Calcd. for $C_{13}H_{22}O_3Na$: 249.1461).

The absolute configuration of glucose was determined to be D by the sign of its positive optical rotation and retention time (14.8 min) following HPLC separation [JASCO OR-2090 plus: Optical rotation detector, SHODEX Asahipak NH2P-50: Φ = 4.5 mm, *L* = 25 cm, 80% CH_3CN aq., 1 mL/min]. Peaks were identified by co-chromatography with authentic D-glucose.

5.4.7. Mild alkaline methanolysis of macarangioside B (*19*)

In a similar manner to that described above, compound **19** (2.0 mg) liberated degalloylated compound **19a** (1.2 mg) (TLC, $CHCl_3$: MeOH: H_2O, 15:6:1, R_f values, **19**: 0.25 and **19a**: 0.51). Degalloylmacarangioside B (**19a**): Amorphous powder; $[\alpha]_D^{24}$ −29.5 (*c* = 0.078, MeOH); IR ν_{max} (film) cm^{-1}: 3395, 2925, 1712, 1652, 1455, 1266, 1072, 1037; ^{13}C NMR (CD_3OD): Table 2; ^{1}H NMR (CD_3OD): Table 3; CD $\Delta\varepsilon$ (nm): +0.33 (354), −0.72 (284), +0.62 (243), −0.73 (211) (*c* = 2.02 × 10^{-5} M, MeOH); HR-ESI-TOF-MS (positive-ion mode) *m*/*z*: 409.1835 $[M+Na]^+$ (Calcd. for $C_{19}H_{30}O_8Na$: 409.1832).

5.4.8. Catalytic hydrogenation of *19a*

An aliquot of **19a** (1.1 mg) was dissolved in MeOH (0.5 mL), then 1 mg of Adams' catalyst (PtO_2) was added and stirred at room temperature for 1 h under H_2 atmosphere. Hydrogenation of **19a** was monitored by TLC analysis [$CHCl_3$: MeOH: H_2O, 15:6:1, R_f values, **19a**: 0.45 and dihydrodegalloylmacarangioside B (**19b**): 0.49]. The spot of **19b** was confirmed to be identical to **3** by TLC co-chromatography. The reaction mixture was filtered and concentrated to afford **19b** (0.82 mg). The ^{1}H and ^{13}C NMR, MS and specific optical rotation were also identical to **3** [**19b**: $[\alpha]_D^{25}$ −8.4 (*c* = 0.055, MeOH)].

5.4.9. $NaBH_4$ reduction of *3a*

A solution of **3a** (1.62 mg) in EtOH (0.5 mL), 1.5 mg of $NaBH_4$ dissolved in EtOH (0.5 mL) was added dropwise at 0 °C and then stirred for 1 h at rt with the progress of the reaction was monitored by TLC analysis [$CHCl_3$: MeOH, 5:1, R_f values, **3a**: 0.58, 3α,9α-diol (**3b**): 0.49, and 3β,9α-diol (**3c**): 0.37]. The reaction mixture was quenched by addition of 0.5 mL of 1% CH_3COOH at 0 °C, concentrated and then purified by preparative TLC [$CHCl_3$: MeOH, 5:1] to afford 3α,9α-diol (**3b**) (0.64 mg) and 3β,9α-diol (**3c**) (0.78 mg). 3α,9α-diol (**3b**): Amorphous powder; ^{1}H NMR (CD_3OD, 500 MHz) δ_H: 3.99 (1H, d, *J* = 7 Hz, pro-*R* H-12), 3.97 (1H, m, H-3), 3.71 (1H, m, H-9), 3.40 (1H, dd, *J* = 7, 2 Hz, pro-*S* H-12), 1.88–1.81 (2H, m, H-2ax and H-4ax), 1.61–1.54 (2H, m, H-2 eq and H-4 eq), 1.54–1.51 (3H, m, H_2-8 and pro-*R* H-7), 1.37 (1H, m, H-6), 1.23 (3H, s, H_3-13), 1.18 (1H, m, pro-*S* H-7), 1.16 (3H, d, *J* = 6 Hz, H_3-10), 0.97 (3H, s, H_3-11); ^{1}H NMR ($CDCl_3$, 500 MHz) δ_H: 3.95 (1H, d, *J* = 8 Hz, pro-*R* H-12), 3.90 (1H, m, H-3), 3.78 (1H, m, H-9), 3.47 (1H, dd, *J* = 8, 2 Hz, pro-*S* H-12), 1.84 (1H, dd, *J* = 15, 5 Hz, H-4ax), 1.78 (1H, ddd, *J* = 15, 5, 2 Hz, H-2ax), 1.68 (1H, br d, *J* = 15 Hz, H-2 eq or H-4 eq), 1.66 (1H, br d, *J* = 15 Hz, H-2 eq or H-4 eq), 1.56–1.49 (3H, m, H_2-8 and pro-*R* H-7), 1.41 (1H, m, H-6), 1.27 (3H, s, H_3-13), 1.22 (3H, d, *J* = 6 Hz, H_3-10), 1.15 (1H, m, pro-*S* H-7), 0.97 (3H, s, H_3-11); HR-ESI-TOF-MS (positive-ion mode) *m*/*z*: 251.1614 $[M + Na]^+$ (Calcd. for $C_{13}H_{24}O_3Na$: 251.1617). 3β,9α-diol (**3c**): Amorphous powder; $[\alpha]_D^{24}$ + 18.5 (*c* 0.052, MeOH); ^{1}H NMR (CD_3OD, 500 MHz) δ_H: 3.95 (1H, tt, *J* = 10, 7 Hz, H-3), 3.62 (1H, d, *J* = 8 Hz, pro-*R* H-12), 3.73 (1H, sextet, *J* = 6 Hz, H-9), 3.42 (1H, dd, *J* = 8, 2 Hz, pro-*S* H-12), 1.80 (1H, m, H-4ax), 1.66 (1H, m, pro-*R* H-7), 1.64 (1H, m, H-2ax), 1.54 (2H, m, H_2-8), 1.46 (2H, m, H-2 eq and H-4 eq), 1.35 (1H, m, H-6), 1.32 (1H, m, pro-*S* H-7), 1.23 (3H, s, H_3-13), 1.18 (3H, d, *J* = 6 Hz, H_3-10), 1.00 (3H, s, H_3-11); HR-ESI-TOF-MS (positive-ion mode) *m*/*z*: 251.1616 $[M+Na]^+$ (Calcd. for $C_{13}H_{24}O_3Na$: 251.1617).

5.4.10. Preparation of (*S*)- and (*R*)-MTPA diesters (*3d* and *3e*) from *3c*

A solution of **3c** (0.39 mg) in dry CH_2Cl_2 (0.5 mL) was reacted with (*S*)-MTPA (14.3 mg) in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (7.7 mg) and 4-*N*,*N*′-dimethylaminopyridine (DMAP) (5.7 mg), and stirred at 25 °C for 30 min, then 40 °C for 5 min. After addition of H_2O (0.5 mL) and $CHCl_3$ (0.5 mL) the solution was successively washed with 1 M HCl (1 mL), $NaHCO_3$-saturated H_2O (1 mL), and saturated brine (1 mL). The organic layer was dried (Na_2SO_4), and evaporated under reduced pressure. The resulting residue was then purified by preparative TLC (silica gel (0.25 mm thickness, applied for 18 cm and developed with $CHCl_3$–$(CH_3)_2CO$ (20: 1) for 9 cm and eluted with $CHCl_3$–MeOH (2:1)) to furnish a diester, **3d** (0.7 mg, 66%). Through a similar procedure, **3e** (0.8 mg, 75%) was also prepared from **3c** (0.39 mg) using (*R*)-MTPA (10.3 mg), EDC (7.9 mg) and DMAP (3.9 mg). (1*S*,3*S*,5*R*,6*R*,9*R*)-megastigman-5,12-epoxy-3,9-diol 3,9-di-(*S*)-MTPA ester (**3d**): Amorphous powder; ^{1}H NMR

($CDCl_3$, 500 MHz) δ_H: 7.52–7.49 (4H, m, aromatic protons), 7.42–7.32 (6H, m, aromatic protons), 5.33 (1H, m, H-3), 5.12 (1H, m, H-9), 3.72 (1H, d, J = 8 Hz, pro-*S* H-12), 3.54 (3H, m, OMe), 3.52 (3H, m, OMe), 3.44 (1H, dd, J = 8, 2 Hz, pro-*R* H-12), 1.97 (1H, m, H-4 eq), 1.75 (1H, m, H-2 eq), 1.72 (2H, m, H_2-8), 1.50 (1H, m, H-4ax), 1.36 (3H, d, J = 6 Hz, H_3-10), 1.31 (1H, m, H-2ax), 1.15 (1H, m, H-7a), 1.20 (3H, s, H_3-13), 0.97 (3H, s, H_3-11); HR-ESI-TOF-MS (positive-ion mode) m/z: 683.2406 $[M+Na]^+$ (Calcd. for $C_{33}H_{38}O_7F_6Na$: 683.2413). (1*S*,3*S*,5*R*,6*R*,9*R*)-megastigman-5,12-epoxy-3,9-diol 3,9-di-(*R*)-MTPA ester (**3e**): Amorphous powder; ^1H NMR ($CDCl_3$, 500 MHz) δ_H: 7.52–7.49 (4H, m, aromatic protons), 7.41–7.35 (6H, m, aromatic protons), 5.35 (1H, m, H-3), 5.11 (1H, m, H-9), 3.74 (1H, d, J = 8 Hz, pro-*S* H-12), 3.52 (3H, m, OMe), 3.49 (3H, m, OMe), 3.47 (1H, dd, J = 8, 2 Hz, pro-*R* H-12), 1.94 (1H, m, H-2 eq), 1.84 (1H, m, H-4 eq), 1.77 (2H, m, H-8), 1.47 (1H, m, H-4ax), 1.38 (1H, m, H-2ax), 1.29 (3H, d, J = 6 Hz, H_3-10), 1.27 (1H, m, H-7a), 1.21 (3H, s, H_3-13), 0.97 (3H, s, H_3-11);HR-ESI-TOF-MS (positive-ion mode) m/z: 683.2419 $[M+Na]^+$ (Calcd. for $C_{33}H_{38}O_7F_6Na$: 683.2413).

5.4.11. Single-crystal X-ray structure analysis of **18**

A suitable crystal (0.30 × 0.10 × 0.10 mm) was used for analysis. The data were measured using a Bruker SMART 1000 CCD diffractometer, using Mo$K\alpha$ graphite-monochromated radiation (λ = 0.71073 Å). The structure was solved by a direct method using the program SHELXTL-97 (Sheldrick, 2008). The refinement and all further calculations were carried out using SHELXTL-97. The absorption correction was carried out utilizing the SADABS routine (Sheldrick, 1996). The H atoms were included at calculated positions and treated as riding atoms using the SHELXTL default parameters. The non-H atoms were refined anisotropically, using weighted full-matrix least-squares on F^2. Fig. 5 was drawn with ORTEP32 (Farrugia, 1997). Crystal Data: $C_7H_6N_2O_2$, M = 150.14 g mol^{-1}, triclinic, $P\bar{1}$, a = 5.508(2) Å, b = 7.561(3) Å, c = 8.741(4) Å, α = 91.908(6)°, β = 96.676(6)°, γ = 108.788(5)°, V = 341.4(2) Å^3, T = 90 K, Z = 2, Dc = 1.461 g cm^{-3}, μ(MoKα) = 0.110 mm^{-1}, F(0 0 0) = 156; 3721 reflections measured, 1557 were unique (R_{int} = 0.0352) and used in all calculations. Final goodness-of-fit = 1.129, R_1 = 0.0467, wR_2 = 0.1298 based on $I > 2\sigma(I)$. CCDC deposit contains the supplementary crystallographic data. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html, or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge, CB2 1EZ, UK; fax: +44 1223-336033; or e-mail: deposit@ccdc.cam.ac.uk.

5.4.12. DPPH radical assay

Trolox and DPPH were purchased from Aldrich Chemical Co., and DPPH radical-scavenging activities of isolated new compounds were tested according to the previously described method (Matsunami et al., 2006). Reaction mixtures containing various concentrations of test compounds dissolved in MeOH and 200 μM DPPH solution in a 96-well microtiter plate were incubated at 37 °C for 30 min, with absorbance measured at 515 nm. The activity was expressed in Trolox equivalents (μM), i.e., the data was converted to activity in terms of μM of Trolox by using standard curves for the reaction of Trolox with DPPH.

5.4.13. MTT cytotoxicity assay

The cytotoxicity of the new compounds (**1**–**3**) was investigated against the human lung adenocarcinoma cell line A549 (RCB0098, Riken Cell Bank, Tsukuba, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 100 mg/L of kanamycin sulfate and 5.6 mg/L of amphotericin B at 37 °C with 5% CO_2. The test compounds were dissolved in DMSO. Cells (1×10^4 cells/well) were seeded in 96-well plates in 100 μL of medium containing various amount of compounds (final concentrations were ranging from 100 to 1 μM with 1% DMSO). Etoposide (3 μM) was used as a positive control. Negative control cells were incubated in the same medium only with 1% DMSO (vehicle). After 72 h incubation, the medium in each well was replaced by fresh medium (100 μL) containing 0.5 mg/mL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT). Two hours later, the medium was removed, and the precipitated MTT formazan was dissolved in DMSO. The absorbance at 540 nm was measured using a microplate reader. The viability was calculated by the following equation.

$$\% \text{ cytotoxicity} = 1 - (A_{\text{sample}} - A_{\text{background}})/(A_{\text{vehicle}} - A_{\text{background}}) \times 100$$

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