Phytochemistry 69 (2008) 3062–3069

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Flavonol glucuronides and *C*-glucosidic ellagitannins from *Melaleuca squarrosa*

Morio Yoshimura [a], Hideyuki Ito [b], Kyoko Miyashita [b], Tsutomu Hatano [b], Shoko Taniguchi [b], Yoshiaki Amakura [a], Takashi Yoshida [a,*]

[a] College of Pharmaceutical Sciences, Matsuyama University, Bunkyo-cho, Matsuyama 790-8578, Japan
[b] Department of Pharmacognosy, Okayama University Graduate School of Medicine, Dentistry, Pharmaceutical Sciences, Tsushima, Okayama 700-8530, Japan

ARTICLE INFO

Article history:
Received 25 February 2008
Received in revised form 2 April 2008
Available online 22 May 2008

Keywords:
Melaleuca squarrosa
Myrtaceae
Flavonol glucuronide
C-Glucosidic ellagitannin
Squarrosanin A
Squarrosanin B
Squarrosanin C
Antioxidant

ABSTRACT

Two flavonoids and three ellagitannins, squarrosanins A, B, and C, were isolated from the leaves of *Melaleuca squarrosa*. The flavonoids were characterized structurally as kaempferol-3-*O*-(2″-*O*-galloyl)-glucuronide and herbacetin-3-*O*-glucuronide, while the ellagitannins were characterized as monomeric and dimeric *C*-glucosidic ellagitannins by application of spectroscopic and chemical methods. The antioxidant effect of the polyphenolic constituents of the *M. squarrosa* leaves was also examined *in vitro*, and *C*-glucosidic tannins including oligomers were shown to be more effective radical scavengers against 1,1-diphenyl-2-picrylhydrazyl (DPPH) than flavonoids and ordinary ellagitannins.

© 2008 Elsevier Ltd. All rights reserved.

1. Introduction

Melaleuca squarrosa Donn ex Sm. (family Myrtaceae) is a small evergreen tree that grows in New Zealand and the southern and southeastern parts of Australia. In our continuing study of natural polyphenolic products from myrtaceous plants (Okuda et al., 1982b; Yoshida et al., 1992, 1996; Lee et al., 1997; Ito et al., 2000, 2004), we reported the isolation of four novel *C*-glucosidic ellagitannin oligomers, melasquanins A–D, together with many other hydrolyzable tannins from the water-soluble portion of an aqueous acetone extract of *M. squarrosa* leaves (Yoshida et al., in press). *C*-glucosidic ellagitannin oligomers of this plant, including cowaniin (Ito et al., 2007; Yoshida et al., in press), a complex tannin dimer, were found to be composed of two or three molecules of casuarinin and/or stachyurin (Okuda et al., 1983; Nonaka et al., 1990), which are linked to each other by a wide variety of C–C coupling mode(s) (Yoshida et al., in press). Further examination of the nonpolar and polar fractions of the plant has led to the isolation of two new flavonoids (**1** and **2**) and three additional ellagitannins, designated squarrosanins A (**3**), B (**4**), and C (**5**). This paper describes the elucidation of their structures and their antioxidant activity.

2. Results and discussion

The aqueous acetone homogenate of air-dried leaves of *M. squarrosa* was successively extracted with ether, ethyl acetate, and *n*-butanol. Each extract obtained after concentration in vacuo was separately submitted to a combination of chromatographic steps over silica gel, Diaion HP-20, Toyopearl HW-40, and MCI GEL CHP-20P to yield compounds **1**–**5**. Five known compounds, grandis-3 (Crow et al., 1971), messagenic acid I (Macías et al., 1998), oleanolic acid, betulinic acid, and angophorol (Wollenweber et al., 2000) from the ether extract and six polyphenols, catechin, ellagic acid, valoneic acid dilactone, strictinin, 1,2,3,6-tetragalloylglucose, and casuarictin (Okuda et al., 1983) in addition to *C*-glucosidic ellagitannin monomers (casuarinin (**6**) and stachyurin (**7**)) from the ethyl acetate extract, and dimers reported in the preceding paper (Yoshida et al., in press) from the *n*-butanol and aqueous extracts were also obtained. These were identified based on comparisons of the respective spectroscopic data with that of data reported in the literature.

Compound **1** was obtained as a yellow amorphous powder. High resolution electrospray ionization mass spectra (HRESIMS) and ^{13}C NMR spectra established the molecular formula of **1** as $C_{28}H_{22}O_{16}$. The UV spectrum showed maxima characteristic of a flavonoid at 267 and 349 nm. The ^{1}H NMR spectrum displayed A_2B_2 signals at δ 6.95 and 8.08 (2H each d, J = 9.0 Hz) and two meta-coupled signals at δ 6.22 and 6.44 (each J = 2.5 Hz), indicating the presence of a kaempferol skeleton. The spectrum also implied

* Corresponding author. Tel.: +81 89 926 7128.
E-mail address: tyoshida@cc.matsuyama-u.ac.jp (T. Yoshida).

0031-9422/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2008.04.004

the presence of a galloyl group and a glucuronyl moiety as suggested from the 2H-singlet at δ 7.22 and the five sequential spin-system of the aliphatic proton region. These structural components were confirmed by the production of kaempferol, gallic acid, glucurono-6,3-lactone, and glucuronic acid upon acid hydrolysis of **1**. The HMBC experiment showed correlations from anomeric H-1″ (δ 6.00) to C-3 (δ 133.8) and from a downfield shifted H-2″ (δ 5.21) to ester carbonyl carbon (δ 166.6), which in turn showed a three-bond correlation with a galloyl proton signal, indicating the location of the glucuronyl residue at O-3 and the galloyl group at O-2″. The structure of compound **1** was thus established as kaempferol-3-*O*-(2″-*O*-galloyl)-glucuronide (see Fig. 1).

Compound **2** was assigned the molecular formula $C_{21}H_{18}O_{13}$ based on HRESIMS. The NMR features of **2** closely resembled those of **1**, except for a lack of galloyl protons and one of the A-ring protons. Acid hydrolysis of **2** yielded glucuronic acid, glucurono-6,3-lactone, and an aglycone. The aglycone showed an HMBC correlation between a chelated 5-OH proton at δ 11.96 and a hydrogen-bearing aromatic carbon at δ 98.9, indicating it to be 8-hydroxykaempferol, which was confirmed by direct comparison of the ^{1}H NMR spectrum and HPLC co-chromatography with an authentic sample (herbacetin) (Sikorska et al., 2004). The location of the sugar residue was established by HMBC correlation between H-1″ and C-3. Therefore, compound **2** was assigned as herbacetin-3-*O*-glucuronide.

Squarrosanins A (**3**), B (**4**), and C (**5**) were shown to be ellagitannins by their spectroscopic features as well as via the production of gallic and ellagic acids upon acid hydrolysis.

Squarrosanin A (**3**), a pale brownish amorphous powder, was shown to have the formula $C_{55}H_{32}O_{33}$ from its HRESIMS and ^{13}C NMR spectroscopic data. Compound **3** showed ^{1}H and ^{13}C NMR spectra characteristic of *C*-glucosidic ellagitannins such as casuarinin (**6**) and stachyurin (**7**). That is, a 2H-singlet (δ 7.21) and three 1H-singlets (δ 6.56, 6.59, 6.85) in the aromatic region of the ^{1}H NMR spectrum were accounted for by the presence of a galloyl and two hexahydroxydiphenoyl (HHDP) groups among which one HHDP group participates biogenetically in the formation of a C–C bond arising from a phenol–aldehyde coupling (Okuda et al., 1995). The presence of an ellagic acid moiety in the molecule was also indicated by the ^{1}H NMR (δ 7.57, 1H, s) and ^{13}C NMR (see Section 3) spectra along with UV (λ_{max} 367 nm) data. Open-chain glucosyl signals in the ^{13}C NMR spectrum of **3** were similar to those of **7** except for a remarkable upfield shift (δ 65.5 in **7** → δ 41.4 in **3**) for C-1 resonance, suggesting that the hydroxyl group at C-1 in **7** is replaced by ellagic acid through an additional C–C bond in **3**. The allocation of the ellagic acid moiety in **3** was substantiated from the long-range correlations of the glucose H-1 and H-2 signals with ellagic acid carbons (C-4, C-5, and C-6) in the HMBC spectrum. Other HMBC correlations illustrated in Fig. 2 were consistent with the proposed structure. The H-1 signal {δ 5.79 (d, J = 1.0 Hz)} in **3** showed an unusual, larger downfield shift than that of **7** {δ 4.82 (d, J = 1.5 Hz)} in the ^{1}H NMR spectrum, despite the absence of an oxygen function. This may be explained by a deshielding effect caused from the coplanar H-1 and ellagic acid planes. The assignment of the (*S*)-configuration at the chiral HHDP groups was determined from a strong positive Cotton effect at 235 nm in the circular dichroism (CD) spectrum (Okuda et al., 1982a). From the above data, the structure of squarrosanin A (**3**) was established as depicted.

Squarrosanin B (**4**) was isolated as a pale brownish amorphous powder, and its molecular formula was determined as $C_{41}H_{28}O_{26}$ by HRESIMS. The ^{1}H and ^{13}C NMR spectra of **4** (Table 1) were similar to those of **6** and **7** except for a remarkable downfield shift of an aromatic proton and upfield shifts of the H-2 and H-3 signals (δ 4.04 and 4.15) of a glucose residue. The low-field aromatic proton signal at δ 7.56, along with characteristic UV absorption (358 nm), indicated the presence of an ellagic acid moiety as found in **3**. Considering a Cotton effect at 234 nm with amplitude for one HHDP group in the CD spectrum, the structure of squarrosanin B was deduced as **4,** which was substantiated by HMQC and HMBC correlations (Fig. 2). The presence of a glucose core and an ellagic acid moiety linked at C-1 in **4** was chemically verified by treatment with acid in boiling water bath, giving a hydrolyzate (**8**), which was identified with that obtained from casuarinin (**6**) of known structures in a similar manner. The pyranose structure of **8** was assigned on the basis of a large coupling constant of the H-1 signal (d, J = 10.0 Hz) at δ 6.46 in the ^{1}H NMR spectrum and of the molecular formula $C_{20}H_{16}O_{13}$ determined by HRESIMS. The derivative **8** was also obtained from the C-1 epimer of **6,** stachyurin (**7**), thereby indicating that the pyranose formation occurred through a benzylic cation during the reaction to have a stable β-configuration for the anomeric center. Thus, although the reaction did not provide an expected evidence for the C-1 configuration in **4**, the 1*R*-configuration was tentatively assigned by the small coupling constant of the H-1 signal (δ 6.39, br d, J = 3.0 Hz) in ^{1}H NMR and a clear ROE correlation for H-1/H-3 in the ROESY (Fig. 3). Namely, among possible rotations for C1–C2 bond, anti-conformation in which the two bulky substituents (ellagic acid and C3–C6 chain) are as far apart as possible (Fig. 3) would likely be adoptable. Thus, the most plausible structure of squarrosanin B was assigned as **4**.

Squarrosanin C (**5**) was indicated to be a dimeric congener of **4** by ESIMS giving a pseudomolecular ion peak at *m/z*: 1872 $[M+NH_4]^+$ corresponding to the molecular formula $C_{82}H_{54}O_{51}$, characteristic UV absorption at 367 nm, and a proton signal at δ 7.54 indicative of the presence of ellagic acid. The ^{1}H and ^{13}C NMR spectra indicated that **5** is a dimer composed of **4** and stachyurin (**7**), in which the C-1 of **7** and an HHDP group of **4** participate in the formation of a C–C bond linking the monomers, as indicated by an upfield shift of H-1′ at δ 4.69 (s) and four singlets due to the HHDP protons. Note that one of the H-6 methylene proton signals of the glucose core shifted to a remarkably higher field (δ 2.97, d, J = 13.0 Hz) than that of **4** (δ 4.03, d, J = 13.0 Hz), which is a diagnostic shift for alienanin B-type *C*-glucosidic dimers (Yoshida et al., in press). The proposed structure (**5**) was supported by good correspondence of the glucose carbon resonances of **5** with those of **4** and the glucose-II (Glc-II) core in alienanin B (**9**) (Nonaka et al., 1991) (Table 1). The positions of the acyl groups were secured by HMBC correlations among aromatic and glucose protons through three-bond coupling with carbonyl carbons as illustrated in Fig. 2. The atropisomerism of the chiral HHDP groups in **5** was determined to be all *S*-series by a strong positive Cotton effect at 231 nm in CD. The C-1 configuration was also tentatively assigned to be *R* by the small coupling constant of H-1 (δ 6.36, br s) similar to that of **4**, leading to the gross structure **5** for squarrosanin C.

Present and previous studies (Yoshida et al., in press) indicated that *M. squarrosa* is a rich source of *C*-glucosidic ellagitannins including oligomers. Although hydrolyzable tannins are well documented to exhibit antioxidative activity (Yoshida et al., 1989) as well as diverse biological properties such as anti-tumor promotion (Ito et al., 1999), host-mediated antiproliferative effects against Sarcoma 180 in mice (Miyamoto et al., 1993), and antiviral effects (Sakagami et al., 1999), the antioxidant properties of the *M. squarrosa* polyphenols have not been examined. Polyphenolic compounds together with previously isolated ones from this plant were thus evaluated for their radical scavenging effect against 1,1-diphenyl-2-picrylhydrazyl (DPPH). All of the tested polyphenolic constituents exhibited stronger activity than a positive control, trolox (Table 2). The *C*-glucosidic ellagitannins, including complex tannins and oligomers (EC_{50} 1.6–3.6 μM), were found to be more effective radical scavengers than flavonoids (EC_{50} 14.8–30.4 μM) and ordinary ellagitannins (EC_{50} 5.5–8.0 μM) such as tellimagrandins I and II as shown in Table 2. The activity increased depending

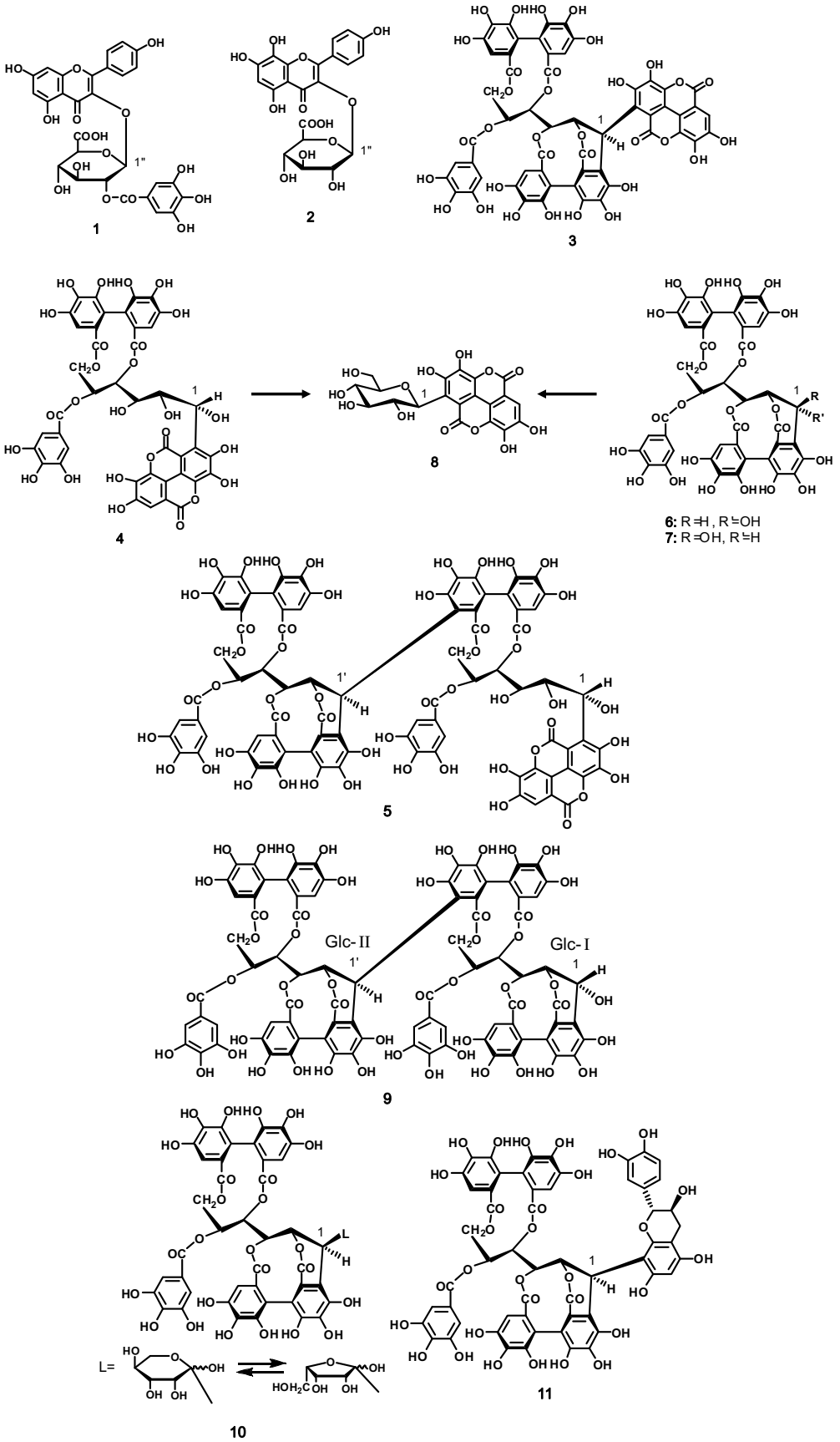

Fig. 1. Structures of compounds **1**–**11**.

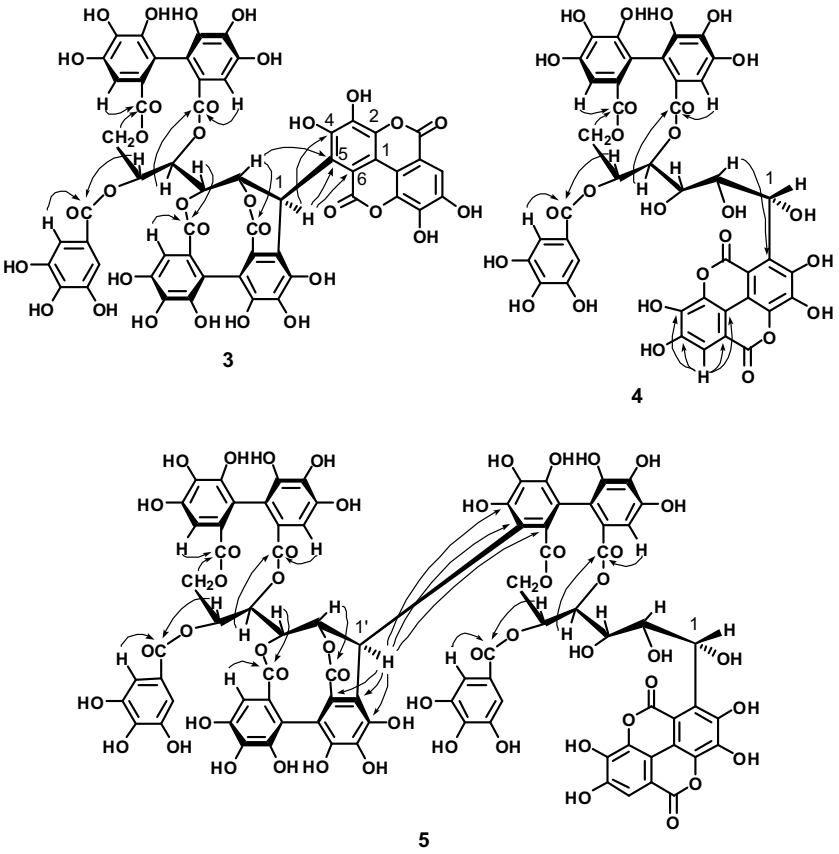

Fig. 2. HMBC correlations of compounds **3**–**5**.

on the molecular size (trimers > dimers > monomers), but the difference in potency was small. Interestingly, squarrosanins A (**3**), B (**4**), and C (**5**), which have a C–C linked ellagic acid moiety at C-1 of the open-chain glucose residue, showed less potency than their congeners having an HHDP or catechin unit instead of the ellagic acid (e.g., stenophyllanin A (**11**) (complex tannins)), casuarinin (**6**) (*C*-glucosidic tannin monomer), and alienanin B (**9**) (dimer), respectively, suggesting that the ellagic acid unit has less contribution toward the radical scavenging potency than the HHDP unit.

2.1. Concluding remarks

These findings may provide the basis for further clarifying the antioxidant-related actions of the *M. squarrosa* leaves, which are rich in *C*-glucosidic tannins, and may contribute toward the development of a new resource of agents beneficial to human health.

3. Experimental

3.1. General

UV spectra on a HITACHI U-2000-10 spectrophotometer and CD spectra on a JASCO J-720W spectrometer were measured. Optical rotations were recorded on a JASCO DIP-1000 polarimeter. Elemental analyses were recorded on a Yanaco CHN corder MT-5. NMR spectra were recorded on a Varian VXR-500, Bruker AVANCE500 (500 MHz for ^{1}H and 126 MHz for ^{13}C) or Varian INOVA 600 instrument (600 MHz for ^{1}H and 150 MHz for ^{13}C), and chemical shifts are given in δ (ppm) values relative to those of the solvent [acetone-d_6 (δ_H 2.04; δ_C 29.8)] on a tetramethylsilane scale. In the HMBC experiments, the J_{CH} value was set at 6 Hz. ESIMS spectra were recorded on a Micromass Auto Spec OA-Tof mass spectrometer (solvent: 50% aqueous MeOH + 0.1% NH_4OAc for positive mode; CH_3CN for negative mode). Normal phase HPLC was conducted on a YMC-Pack SIL A-003 (YMC Co., Ltd.) column (4.6 i.d. × 250 mm) developed with *n*-hexane–MeOH–tetrahydrofuran–HCO_2H (55:33:11:1) containing oxalic acid (450 mg/1.0 L) (flow rate: 1.5 mL/min; 280 nm UV detection) at room temperature. Reversed-phase HPLC was performed on a YMC-Pack ODS-A A-302 (YMC Co., Ltd.) column (4.6 i.d. × 150 mm) developed with 10 mM H_3PO_4–10 mM KH_2PO_4–CH_3CN (44:44:12) (solvent A) and MeOH–H_2O (4:1, v/v) (solvent B), with each at a flow rate of 1.0 mL/min; detection, 280 nm UV or diode array detector (DAD) (HITACHI L-7445) 200–600 nm at 40 °C. Column chromatography

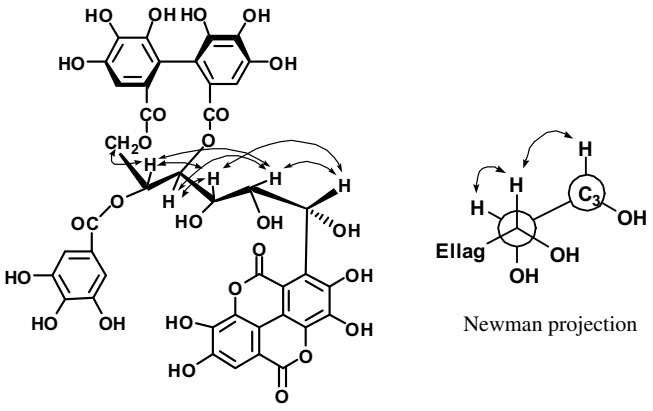

Fig. 3. ROESY correlations of compound **4**.

Table 1
^1H, ^{13}C NMR spectroscopic data for the glucose moieties of **4**–**7** and **9** in acetone-d_6 + D_2O

Position	NMR									
	4[a]		**5**[a]		**6**[a]		**7**[a]		**9**[b]	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	6.39 br d (3.0)	75.0	6.36 br s	74.6	5.58 d (5.0)	67.6	4.82 d (1.5)	65.5	5.62 d (4.8)	67.4
2	4.15 dd (2.0, 3.0)	74.4	4.14 br s	74.6	4.63 dd (2.5, 5.0)	76.7	4.90 t (1.5)	81.0	4.61 dd (1.8, 4.8)	77.0
3	4.04 br s	71.8	3.92 br s	71.2	5.42 t (2.5)	69.8	5.00 dd (1.5, 2.5)	72.0	5.37 t (1.8)	69.2
4	5.35 dd (3.0, 8.5)	73.3	5.26 dd (3.0, 8.5)	73.8	5.44 dd (2.5, 8.5)	74.2	5.69 dd (2.5, 8.5)	73.3	5.28 dd (1.8, 9.0)	74.6
5	5.68 dd (3.0, 8.5)	71.7	5.68 br dd (2.0, 8.5)	70.2	5.31 dd (3.0, 8.5)	71.2	5.30 dd (3.0, 8.5)	70.9	5.18 dd (3.6, 9.0)	69.4
6	4.03 d (13.0) 4.85 dd (3.0, 13.0)	64.8	2.97 d (13.0) 4.03 br dd (13.0)	65.9	4.04 d (13.0) 4.84 dd (3.0, 13.0)	64.6	4.01 d (13.0) 4.91 dd (3.0, 13.0)	64.5	2.99 d (12.6) 4.03 br d (12.6)	65.6
1′			4.69 br s	40.5					4.75 d (1.8)	40.3
2′			4.92 br dd (1.0, 2.0)	80.9					4.92 t (1.8)	80.8
3′			4.98 t (2.0)	74.7					4.98 t (1.8)	74.6
4′			5.62 dd (2.0, 8.5)	73.5					5.63 dd (1.8, 9.0)	73.3
5′			5.29 br dd (3.0, 8.5)	71.6					5.27 dd (3.0, 9.0)	71.4
6′			4.03 d (13.0) 4.78 dd (3.0, 13.0)	64.4					4.03 br d (12.6) 4.79 dd (3.0, 12.6)	64.3

J values (Hz) are in parenthesis.
[a] ^1H NMR: 500 MHz and ^{13}C NMR: 126 MHz.
[b] ^1H NMR: 600 MHz and ^{13}C NMR: 150 MHz.

was carried out on a Diaion HP-20, MCI GEL CHP-20P (Mitsubishi Chemical Co.), and Toyopearl HW-40 (coarse grade; Tosoh Co.).

3.2. Plant material

The leaves of *M. squarrosa* were supplied from the herbal garden of POLA Chemical Industries, Inc. A voucher specimen (OKP-MY98005) is deposited at the Medicinal Herbal Garden of Okayama University Graduate School of Medicine, Dentistry, Pharmaceutical Sciences.

3.3. Extraction and isolation

Dried leaves of *M. squarrosa* (1.4 kg) were homogenized in H_2O–acetone (3:7, v/v, 11 L) at room temperature. The filtered homogenate was concentrated *in vacuo* below 40 °C to 1 L and extracted with Et_2O (1 L × 3), EtOAc (1 L × 3), and *n*-BuOH (1 L × 3) successively to give Et_2O (53.7 g), EtOAc (35.4 g), *n*-BuOH (82.9 g), and H_2O (135.8 g) extracts. Fractionations of the respective extracts were achieved by monitoring either normal- or reversed-phase HPLC. The Et_2O extract (22.0 g) was applied to a silica gel column (63–200 μm; Merck, 5.0 i.d. × 50 cm) with $CHCl_3$ → $CHCl_3$–MeOH (95:5 → 9:1 → 4:1 → 1:1) as eluants. The $CHCl_3$–MeOH (95:5, v/v) eluate was further subjected to Toyopearl HW-40 (coarse grade, 2.2 i.d. × 60 cm) followed by preparative HPLC to yield grandis-3 (4.9 mg), angophorol (5.4 mg), betulinic acid (8.9 mg), messagenic acid I (11.0 mg), and oleanolic acid (47.6 mg). A part (2.00 g) of the EtOAc extract was subjected to Toyopearl HW-40 (coarse grade, 2.2 i.d. × 45 cm) with aqueous MeOH (30% → 40% → 50% → 60% → 100% MeOH) → H_2O–acetone (3:7, v/v) as eluants. The H_2O–MeOH (6:4, v/v) eluate afforded (+)-catechin (35.5 mg). The H_2O–MeOH (1:1, v/v) eluate was further subjected to column chromatography over MCI GEL CHP-20P (1.1 i.d. × 16 cm) eluted with H_2O → aqueous MeOH (10% → 20% → 30% → 40% → 50%) → 100% MeOH to yield strictinin (15.9 mg) and kaempferol-3-*O*-(2″-*O*-galloyl)-glucuronide (**1**) (22.0 mg). The H_2O–MeOH (4:6, v/v) eluate was divided into three portions, and each portion was separately subjected to column chromatography over MEGA BOND ELUT C18 with H_2O-MeOH (9:1, v/v) giving casuarinin (**6**) (327.6 mg), 1,2,3,6-tetragalloylglucose (12.2 mg), and valoneic acid dilactone (4.3 mg). The MeOH eluate gave ellagic acid (16.4 mg), whereas H_2O–acetone (3:7, v/v) eluate was purified by column chromatography over MCI GEL CHP-20 (1.1 i.d. × 17 cm) with aqueous MeOH (30% → 100% MeOH) → H_2O–acetone (3:7, v/v) as eluates to give casuarictin (22.1 mg).

The *n*-BuOH extract (82.9 g) was applied to a Diaion HP-20 (6.5 i.d. × 70 cm) column, eluated with H_2O → aqueous MeOH (10% → 20% → 30% → 50%) → MeOH → H_2O–acetone (3:7, v/v). An aliquot of the H_2O–MeOH (8:2, v/v) eluate (3.00 g) was further subjected to column chromatography over Toyopearl HW-40 (coarse grade, 2.2 i.d. × 60 cm) eluted with aqueous MeOH (50% → 60% → 70%) → 100% MeOH → H_2O–acetone (3:7, v/v) and MCI GEL CHP-20P (1.1 i.d. × 40 cm) eluted with H_2O → aqueous MeOH (10% → 20% → 30% → 50%) → 100% MeOH, to yield strictinin (28.9 mg), pterocarinin A (**10**) (120.4 mg), stachyurin (**7**) (35.3 mg), casuarinin (**6**) (482.6 mg), alienanin B (**9**) (96.8 mg), pedunculagin (23.0 mg), and stenophyllanin A (**11**) (47.2 mg), respectively. An aliquot of the H_2O–MeOH (7:3, v/v) eluate (3.00 g) was further subjected to column chromatography over Toyopearl HW-40 (coarse grade, 2.2 i.d. × 60 cm) eluted with aqueous MeOH (50% → 60% → 70%) → 100% MeOH → H_2O–acetone (3:7, v/v) and MCI GEL CHP-20P (1.1 i.d. × 40 cm) eluted with H_2O → aqueous MeOH (10% → 20% → 30% → 50%) → MeOH, to yield herbacetin-3-*O*-glucuronide (**2**) (62.5 mg), strictinin

Table 2
Radical scavenging effect of polyphenols against DPPH

	EC_{50} (μM)[a]	
Reference compound		
Trolox	36.5	
Flavonoids		
Kaempferol-3-*O*-(2″-*O*-G)-glucuronide (**1**)	14.8	
Herbacetin-3-*O*-glucuronide (**2**)	30.4	
Ellagitannins and related polyphenols		
Ellagic acid	18.4	
Gallic acid	15.5	
Monomers		Composing unit
Strictinin	5.9	HHDP + 1 Galloyl
Tellimagrandin I	8.0	HHDP + 2 Galloyl
Tellimagrandin II	7.6	HHDP + 3 Galloyl
Pedunculagin	5.5	2 HHDP
C-glucosidic tannin monomers		Composing unit (monomer)
Casuarinin (**6**)	3.3	2 HHDP + Galloyl
Pterocarinin A (**10**)	2.5	Stachyurin (**7**) + Lyxose
Stenophyllanin A (**11**)	3.6	Stachyurin (**7**) + Catechin
C-glucosidic tannin oligomers		
Alienanin B (**9**) (dimer)	2.5	Stachyurin (**7**) + **6**
Casuglaunin A (dimer)	2.6	Stachyurin (**7**) + **6**
Casuglaunin B (dimer)	2.2	Stachyurin (**7**) + Catechin + **6**
Melasquanin A (dimer)	2.5	Stachyurin (**7**) + **6**
Melasquanin B (trimer)	1.8	(Stachyurin (**7**)) × 2 + **6**
Melasquanin C (trimer)	2.3	(Stachyurin (**7**)) × 2 + **6**
Melasquanin D (trimer)	1.6	(Stachyurin (**7**)) × 2 + **6**
C-glucosidic tannin with C_1-ellagic acid		(Related congener)
Squarrosanin A (**3**) (monomer)	4.2	(**10**), (**11**)
Squarrosanin B (**4**) (monomer)	6.0	(**6**)
Squarrosanin C (**5**) (dimer)	3.2	(**9**)

[a] Each value is a mean value of triplicate data.

(64.3 mg), casuarinin (**6**) (394.7 mg), stenophyllanin A (**11**) (66.6 mg), alienanin B (**9**) (121.7 mg), casuglaunin A (14.0 mg), cowaniin (7.0 mg), melasquanin A (13.9 mg), and casuglaunin B (14.8 mg).

The water-soluble portion (37.58 g) was subjected to column chromatography over Diaion HP-20 (6.5 i.d. × 45 cm) with H_2O → aqueous MeOH (10% → 20% → 30% → 50%) → 100% MeOH → H_2O–acetone (3:7, v/v). The H_2O–MeOH (1:1, v/v) eluate was further applied to Toyopearl HW-40 (coarse grade, 2.2 i.d. × 60 cm) with 70% MeOH → MeOH → MeOH–H_2O–acetone (7:2:1) → MeOH–H_2O–acetone (7:1:2) → H_2O–acetone (3:7, v/v), MCI GEL CHP-20P (1.1 i.d. × 40 cm) with aqueous MeOH (10% → 20% → 30% → 50%) → MeOH → H_2O–acetone (3:7, v/v) and YMC GEL ODS AQ12S50 (1.1 i.d. × 40 cm) with aqueous MeOH (10% → 20% → 30% → 50%) → MeOH to give alienanin B (**9**) (265.8 mg), casuglaunin B (236.1 mg), and squarrosanins A (**3**) (20.0 mg), B (**4**) (43.3 mg), and C (**5**) (22.6 mg).

3.4. Kaempferol 3-O-(2″-O-galloyl)-glucuronide (*1*)

A yellow amorphous powder, $[\alpha]_D$ −102.6 (*c* = 1.0, MeOH). UV λ_{max} (MeOH) nm (log ε): 218 (sh 4.63), 267 (4.41), 349 (4.14). ^{1}H NMR (500 MHz, acetone-d_6 + D_2O): δ 3.80 (1H, t, *J* = 9.5 Hz, H-4″), 3.89 (1H, t, *J* = 9.5 Hz, H-3″), 3.94 (1H, t, *J* = 9.5 Hz, H-5″), 5.21 (1H, dd, *J* = 8.0, 9.5 Hz, H-2″), 6.00 (1H, d, *J* = 8.0 Hz, H-1″), 6.22 (1H, d, *J* = 2.5 Hz, H-6), 6.44 (1H, d, *J* = 2.5 Hz, H-8), 6.95 (2H, d, *J* = 9.0 Hz, H-3′, 5′), 7.22 (2H, s, galloyl-H), and 8.08 (2 H, d, *J* = 9.0 Hz, H-2′, 6′). ^{13}C NMR (126 MHz, acetone-d_6 + D_2O): δ 72.8 (C-4″), 74.8 (C-2″), 74.9 (C-3″), 76.5 (C-5″), 94.5 (C-8), 99.5 (C-6), 99.7 (C-1″), 105.2 (C-4a), 110.2 (galloyl C-2, 6), 116.1 (C-3′, 5′), 121.4, 122.0 (C-1′, galloyl C-1), 131.9 (C-2′, 6′), 133.8 (C-3), 139.0 (galloyl C-4), 145.9 (galloyl C-3, 5), 157.7, 157.8, 161.1, 162.4 (C-2, C-5, C-8a, C-4′), 165.2 (C-7), 166.6 (galloyl C-7), 170.6 (C-6″), and 178.3 (C-4). ESIMS *m/z*: 615 $[M+H]^+$. HRESIMS *m/z*: 637.0787 $[M+Na]^+$ and calcd for $C_{28}H_{22}O_{16}$ + Na, 637.0806.

3.5. Herbacetin 3-O-glucuronide (*2*)

A yellow amorphous powder, $[\alpha]_D$ −20.0 (*c* = 1.0, MeOH). UV λ_{max} (MeOH) nm (log ε): 213 (sh 4.55), 278 (4.27), and 328 (3.99). ^{1}H NMR (600 MHz, DMSO-d_6): δ 3.25 (1H, t, *J* = 9.0 Hz, H-2″), 3.26 (1H, t, *J* = 9.0 Hz, H-3″), 3.36 (1H, br dd, *J* = 9.0, 9.6 Hz, H-4″), 3.58 (1H, d, *J* = 9.6 Hz, H-5″), 5.47 (1H, d, *J* = 9.0 Hz, H-1″), 6.28 (1H, s, H-6), 6.88 (2 H, d, *J* = 9.0 Hz, H-3′, 5′), 8.12 (2H, d, *J* = 9.0 Hz, H-2′, 6′), 8.67 (1H, s, 8-OH), 10.19 (1 H, s, 4′-OH), 10.51 (1H, s, 7-OH), and 11.96 (1H, s, 5-OH). ^{13}C NMR (150 MHz, DMSO-d_6): δ 71.6 (C-4″), 74.1 (C-2″), 75.9 (C-3″), 76.2 (C-5″), 98.9 (C-6), 101.4 (C-1″), 103.8 (C-4a), 115.3 (C-3′, 5′), 121.1 (C-1′), 125.1 (C-8), 131.2 (C-2′, 6′), 132.9 (C-3), 145.0 (C-2), 152.9 (C-8a), 153.6 (C-5), 156.4 (C-7), 160.3 (C-4′), 169.9 (C-6″), 177.7 (C-4). ESIMS *m/z*: 479 $[M+H]^+$. HRESIMS *m/z*: 479.0898 $[M+H]^+$ and calcd for $C_{21}H_{18}O_{13}$ + H, 479.0826.

3.6. Hydrolysis of (*1*) and (*2*)

Compound **1** (5 mg) was treated with 1% HCl (2 mL) in a boiling water bath for 12 h to yield kaempferol, gallic acid, glucuronic acid, and glucurono-6,3-lactone, which were identified by comparison with authentic samples on HPLC (UV or RI). Compound **2** was similarly treated, and the products were identified as herbacetin, glucuronic acid, and glucurono-6,3-lactone on HPLC.

3.7. Squarrosanin A (*3*)

A pale brownish amorphous powder, $[\alpha]_D$ +30.3 (*c* = 1.0, MeOH). UV λ_{max} (MeOH) nm (log ε): 217 (4.78), 272 (4.51), and 366 (3.80). CD (MeOH) [θ] (nm): $+16.6 \times 10^4$ (235), -6.3×10^4 (261), and $+4.5 \times 10^4$ (284). ^{1}H NMR (500 MHz, acetone-d_6 + D_2O): δ 4.07 (1H, d, *J* = 13.0 Hz, H-6), 4.92 (1H, br t, H-2), 4.99 (1H, dd, *J* = 3.0, 13.0 Hz, H-6), 5.35 (1H, t, *J* = 2.0 Hz, H-3), 5.39 (1H, dd, *J* = 3.0, 8.5 Hz, H-5), 5.79 (1H, d, *J* = 1.0 Hz, H-1), 5.92 (1H, dd, *J* = 2.0, 8.5 Hz, H-4), 6.56, 6.59, 6.85 (each 1H, s, HHDP-H), 7.21 (2H, s, galloyl-H), and 7.57 (1H, s, aromatic-H). ^{13}C NMR (126 MHz, acetone-d_6 + D_2O): δ 41.1 (C-1), 64.5 (C-6), 71.1 (C-5), 72.8 (C-4), 75.1 (C-3), 82.0 (C-2), 106.0, 107.4, 108.7, 127.9 (each 1C, HHDP C-3), 110.0 (2C, galloyl C-2, 6), 106.9, 109.6, 111.7, 113.4, 114.6, 122.4, 137.2, 137.8, 139.5 (2C), 148.8 (2C), 159.9, 160.7 (ellagic acid moiety), 115.4, 115.5, 116.3, 116.7 (each 1C, HHDP C-1), 120.6 (galloyl C-1), 123.0, 125.0, 126.7, 126.8 (each 1C, HHDP C-2), 135.1, 136.0, 136.4, 137.8 (each 1C, HHDP C-5), 139.4 (galloyl C-4), 142.8, 142.9, 144.27, 144.30 (2C), 145.1, 145.2, 145.7 (HHDP C-4, 6), 146.1 (2C, galloyl C-3, 5), 166.3, 167.7, 168.5, 169.0, 169.8 (each 1C, ester carbonyls). ESIMS *m/z*: 1221 $[M+H]^+$, 1238 $[M+NH_4]^+$, 1243 $[M+Na]^+$. HRESIMS *m/z*: 1243.0722 $[M+Na]^+$ and calcd for $C_{55}H_{32}O_{33}$ + Na, 1243.0724.

3.8. Squarrosanin B (*4*)

A pale brownish amorphous powder, $[\alpha]_D$ −11.7 (*c* = 1.0, MeOH). UV λ_{max} (MeOH) nm (log ε): 215 (4.66), 256 (4.49), and 358 (3.79). CD (MeOH) [θ] (nm): $+7.8 \times 10^4$ (234), -3.1×10^4 (259), and $+1.5 \times 10^4$ (281). ^{1}H NMR (500 MHz, acetone-d_6 + D_2O): δ 6.52, 6.84 (each 1H, s, HHDP-H), 6.96 (2H, s, galloyl-H), and 7.56 (1H, s, aromatic-H); for sugar protons, see Table 1; ^{13}C NMR (126 MHz, acetone-d_6 + D_2O): δ 107.2, 108.9 (each 1C, HHDP C-3), 109.6 (2C, galloyl C-2, 5), 105.8, 109.4, 111.6, 113.0, 114.3, 125.2, 135.8, 136.8, 139.3, 140.3, 148.5, 149.1, 159.9, 160.3 (each 1C, ellagic acid moiety), 115.4, 116.0 (each 1C, HHDP C-1), 120.3 (galloyl C-1), 125.8, 126.0 (each 1C, HHDP C-2), 136.2, 136.4 (each 1C,

HHDP C-5), 139.0 (galloyl C-4), 144.2, 144.3 (each 1C, HHDP C-6), 145.0, 145.1 (each 1C, HHDP C-4), 145.7 (2C, galloyl C-3, 5), 166.4, 168.0, 169.2 (each 1C, ester carbonyls); for sugar carbons, see Table 1; ESIMS *m/z*: 954 $[M+NH_4]^+$, 959 $[M+Na]^+$. HRESIMS *m/z*: 959.0704 $[M+Na]^+$ and calcd for $C_{41}H_{28}O_{26}$ + Na, 959.0767.

3.9. Squarrosanin C (**5**)

A pale brownish amorphous powder, $[\alpha]_D$ +10.4 (*c* = 1.0, MeOH). UV λ_{max} (MeOH) nm (log ε): 214 (4.48), 272 (4.24), and 367 (3.41). CD (MeOH) $[\theta]$ (nm): $+17.3 \times 10^4$ (231), -2.8×10^4 (257), and $+3.5 \times 10^4$ (282). ^{1}H NMR (500 MHz, acetone-d_6 + D_2O): δ 6.44, 6.54, 6.76, 6.83 (each 1H, s, HHDP-H), 7.04, 7.08 (each 2H, s, galloyl-H), and 7.54 (1H, br s, aromatic-H); for sugar protons, see Table 1; ^{13}C NMR (126 MHz, acetone-d_6 + D_2O): δ 106.1, 107.5, 108.7, 109.8, 114.4, 114.7, 115.5 (2C), 115.7, 116.1, 116.3, 116.6 (HHDP C-1, 1′, 3, 3′), 109.9 (2C, galloyl C-2, 5), 110.1 (2C, galloyl C-2′, 5′), 105.8, 109.0, 111.9, 113.2, 125.3, 134.4, 135.7, 139.3 (2C), 148.6, 149.3, 159.7, 160.3 (ellagic acid moiety), 121.0, 121.6 (each 1C, galloyl C-1, C-1′), 125.1 (2C), 125.3, 126.7, 127.0, 127.1 (HHDP C-2, 2′), 136.1, 136.5, 136.8, 136.9, 137.0, 137.1 (each 1C, HHDP C-5, 5′), 138.8, 140.4, 143.5, 143.9, 144.1, 144.3, 144.4, 144.5, 145.1, 145.2, 145.3, 145.8 (each 1C, HHDP C-4, 6, 4′, 6'), 145.6, 146.0 (each 2C, galloyl C-3, 5, 3′, 5′), 166.0, 166.1, 166.5, 168.1, 168.2, 168.5, 169.4, 169.5 (each 1C, ester carbonyls); for sugar carbons, see Table 1; ESIMS *m/z*: 1872 $[M+NH_4]^+$. *Anal.* Found: C, 45.11; H, 3.82. $C_{82}H_{54}O_{51}\cdot 17.5\ H_2O$ requires: C, 47.37; H, 4.10%.

3.10. Acid hydrolysis of casuarinin (**6**) and stachyurin (**7**)

Casuarinin (**6**) (50.8 mg) was treated with 1% HCl (10 ml) in a boiling water bath for 7 h. After cooling followed by removal of insoluble material (ellagic acid), the solution was subjected to column chromatography over a Sep-pak C18 cartridge (900 mg) with H_2O → aqueous MeOH (30% → 50%) → MeOH as eluants. The H_2O–MeOH (7:3, v/v) eluate was further applied to MCI GEL CHP-20P (1.1 i.d. × 20 cm) with H_2O → aqueous MeOH (10% → 20% → 30% → 50%) → MeOH to give **8** (3.5 mg) from 30% MeOH eluate.

Stachyurin (**7**) was also treated in a similar manner to show the production of **8** (*Rt* 20.2 min) on the reversed-phase HPLC (solvent A).

3.11. Compound **8**

A yellow amorphous powder. ^{1}H NMR (500 MHz, MeOH-d_4): δ 3.57 (1H, m, H-5), 3.59 (1H, m, H-3), 3.60 (1H, m, H-4), 3.80 (1H, br t, *J* = 10.0 Hz, H-2), 3.87 (1H, dd, *J* = 3.5, 12.0 Hz, H-6), 3.94 (1H, d, *J* = 12.0 Hz, H-6), 6.46 (1H, d, *J* = 10.0 Hz, H-1), 7.47 (1H, s, aromatic-H). ^{13}C NMR (126 MHz, MeOH-d_4): δ 61.9 (C-6), 71.2 (C-4), 73.7 (C-2), 78.1 (C-1), 79.5 (C-3), 82.5 (C-5), 107.8, 109.4, 111.7, 113.7, 115.1, 124.2, 137.4, 137.6, 140.6, 141.6, 148.5, 149.5, 160.9, 161.2 (each 1C, ellagic acid moieties). ESIMS *m/z*: 463 $[M-H]^-$. HRESIMS *m/z*: 463.0505 $[M-H]^-$ and calcd for $C_{20}H_{16}O_{13}$ − H, 463.0513.

3.12. Acid hydrolysis of **3**, **4**, and **5**

A solution of each compound (1–2 mg) in 0.5% H_2SO_4 (1.0 mL) was heated in a boiling water bath for 8 h. After cooling, the reaction mixture was adsorbed on an ODS Mega Bond Elut cartridge (1 g). After washing with H_2O, the product was eluted with MeOH, and the concentrated solution was analyzed by reversed-phase HPLC (solvent A) to detect ellagic (*Rt* 38.7 min) and gallic (*Rt* 2.6 min) acids, which were identified by co-chromatography with authentic specimens.

3.13. Antioxidant activity

The antioxidant activity was evaluated by DPPH radical in the usual way (Yoshida et al., 1989). Briefly, a 2 mL portion of extract or trolox (12.5, 25, 50, 100, 200 μM) in MeOH was added to a 15 × 105 mm assay tube containing 0.5 mL of freshly prepared DPPH solution (1 mM) in MeOH. The tubes were vigorously vortex mixed, covered, and left in the dark at room temperature. After 30 min, the absorbance at 520 nm was measured on a spectrophotometer. The degree of discoloration of the solution indicates the scavenging efficacy of the added substance. All experiments were performed in triplicate.

Antioxidant activity (after 30 min) was defined as the percent discoloration of a DPPH solution and was calculated by the following equation:

$$\text{Antioxidant effect } (30) = 100 \times (A_{\text{sample}}/A_{\text{control}}).$$

Acknowledgements

We thank Mr. Y. Kitada of the Central Research Laboratory of the Pola Chemical Industries Inc., for kindly supplying the plant material. The NMR experiments were performed at the SC-NMR Laboratory of Okayama and Matsuyama Universities.

References

Crow, W.D., Nicholls, W., Sterns, M., 1971. Root inhibitors in *Eucalyptus grandis*. Naturally occurring derivatives of the 2,3-dioxabicyclo[4.4.0]decane system. Tetrahedron Letters 18, 1353–1356.

Ito, H., Miyake, M., Nishitani, E., Mori, K., Hatano, T., Okuda, T., Konoshima, T., Takasaki, M., Kozuka, M., Mukainaka, T., Tokuda, H., Nishino, H., Yoshida, T., 1999. Anti-tumor promoting activity of polyphenols from *Cowania mexicana* and *Coleogyne ramosissima*. Cancer Letters 143, 5–13.

Ito, H., Koreishi, M., Tokuda, H., Nishino, H., Yoshida, T., 2000. Cypellocarpins A–C, phenol glycosides esterified with oleuropeic acid, from *Eucalyptus cypellocarpa*. Journal of Natural Products 63, 1253–1257.

Ito, H., Iwamori, H., Kasajima, N., Kaneda, M., Yoshida, T., 2004. Kunzeanones A, B, and C: novel alkylated phloroglucinol metabolites from *Kunzea ambigua*. Tetrahedron 60, 9971–9976.

Ito, H., Miyake, M., Nishitani, E., Miyashita, K., Yoshimura, M., Yoshida, T., Takasaki, M., Konoshima, T., Kozuka, M., Hatano, T., 2007. Cowaniin, a *C*-glucosidic ellagitannin dimer linked through catechin from *Cowania mexicana*. Chemical and Pharmaceutical Bulletin 55, 492–494.

Lee, M., Nishimoto, S., Yang, L., Yen, K., Hatano, T., Yoshida, T., Okuda, T., 1997. Two macrocyclic hydrolysable tannin dimers from *Eugenia uniflora*. Phytochemistry 44, 1343–1349.

Macías, F.A., Simonet, A.M., Galindo, J.C.G., Pacheco, P.C., Sánchez, J.A., 1998. Bioactive polar triterpenoids from *Melilotus messanensis*. Phytochemistry 49, 709–717.

Miyamoto, K., Nomura, M., Maruyama, T., Ito, H., Hatano, T., Yoshida, T., Koshiura, R., Okuda, T., 1993. Antitumor activities of ellagitannins against sarcoma-180 in mice. Biological and Pharmaceutical Bulletin 16, 379–387.

Nonaka, G., Sakai, T., Tanaka, T., Mihashi, K., Nishioka, I., 1990. Structure revision of *C*-glycosidic ellagitannins, castalagin, vescalagin, casuarinin and stachyurin, and related hydrolyzable tannins. Chemical and Pharmaceutical Bulletin 38, 2151–2156.

Nonaka, G., Sakai, K., Mihashi, K., Nishioka, I., 1991. Isolation of alienanins A and B, novel C–C linked ellagitannin dimers from *Quercus aliena* BLUME. Chemical and Pharmaceutical Bulletin 39, 884–888.

Okuda, T., Yoshida, T., Hatano, T., Koga, T., Toh, N., Kuriyama, K., 1982a. Circular dichroism of hydrolysable tannins – I. Ellagitannins and gallotannins. Tetrahedron Letters 23, 3937–3940.

Okuda, T., Yoshida, T., Hatano, T., Yazaki, K., Ashida, M., 1982b. Ellagitannins of the Casuarinaceae, Stachyuraceae and Myrtaceae. Phytochemistry 21, 2871–2874.

Okuda, T., Yoshida, T., Ashida, M., Yazaki, K., 1983. Structures of pedunculagin, casuarictin, strictinin, casuarinin, casuariin, and stachyurin. Journal of the Chemical Society, Perkin Transactions 1, 1765–1772.

Okuda, T., Yoshida, T., Hatano, T., 1995. Hydrolyzable tannins and related polyphenols. Progress in the Chemistry of Organic Natural Products 66, 1–117.

Sakagami, H., Satoh, K., Ida, Y., Koyama, N., Premanathan, M., Arakaki, R., Nakashima, H., Hatano, T., Okuda, T., Yoshida, T., 1999. Induction of apoptosis and anti-HIV activity by tannin- and lignin-related substances. Basic Life Sciences 66, 595–611.

Sikorska, M., Matlawska, I., Franski, R., 2004. 8-Hydroxyflavonoid glucuronides of *Malope trifida*. Acta Physiologiae Plantarum 26, 291–297.

Wollenweber, E., Wehde, R., Dorr, M., Lang, G., Stevens, J.F., 2000. *C*-methyl flavonoids from the leaf waxes of some Myrtaceae. Phytochemistry 55, 965–970.

Yoshida, T., Mori, K., Hatano, T., Okumura, T., Uehara, I., Komagoe, K., Fujita, Y., Okuda, T., 1989. Radical-scavenging effects of tannins and related polyphenols on 1,1-diphenyl-2-picrylhydrazyl radical. Chemical and Pharmaceutical Bulletin 37, 1919–1921.

Yoshida, T., Maruyama, T., Nitta, A., Okuda, T., 1992. Eucalbanins A, B and C, monomeric and dimeric hydrolyzable tannins from *Eucalyptus alba* Reinw. Chemical and Pharmaceutical Bulletin 40, 1750–1754.

Yoshida, T., Maruyama, T., Nitta, A., Okuda, T., 1996. A hydrolysable tannin and accompanying polyphenols from *Melaleuca leucadendron*. Phytochemistry 42, 1171–1173.

Yoshida, T., Ito, H., Yoshimura, M., Miyashita, K., Hatano, T., in press. *C* -glucosidic ellagitannin oligomers from *Melaleuca squarrosa* Donn ex Sm., Myrtaceae. Phytochemistry 69, 3070–3079.