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Three new prenylated flavonoids from *Macaranga denticulata* and their anticancer effects

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**A B S T R A C T**

One rare flavonoid–diterpene heterodimer, denticulatain C (1), one modified geranyl-type side chain substituted flavonoid, denticulatain D (2) and one geranylated flavonoid, denticulatain E (3), as well as 11 known compounds (4–14) were isolated from the fronds of *Macaranga denticulata*. Their structures were elucidated on the basis of extensive spectroscopic interpretation. Compounds 4 and 8 inhibited the proliferation of A-549 cell line with IC\(_{50}\) values of 48.6 and 20.2 \(\mu\)g/mL, respectively. Compounds 3, 6, and 8 exhibited significant antiangiogenic activity on a zebrafish model with IC\(_{50}\) values of 9.78, 0.34, and 2.55 \(\mu\)g/mL, respectively.

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**1. Introduction**

Nature relies on an intricate network of biosynthetic pathways to produce the cornucopia of small organic molecules needed to support life, among which combinatorial chemical synthesis plays an important role. Prenylated flavonoids, a combination of terpenoid and flavonoid, are extraordinarily diverse in chemistry and bioactivity [1]. Due to the differences of prenylation position, various lengths of prenyl chain, and further modifications of the prenyl moiety such as cyclization and hydroxylation, prenylation has greatly enriched the structural diversity of flavonoid. Studies have showed that the presence of isoprenoid chain is a major determinant of the bioactivity of prenylated flavonoids and the remarkable properties of these compounds are thought to reside in their enhanced interaction with biological membranes and increased affinity for target proteins [2].

The genus *Macaranga* (Euphorbiaceae), which is widely distributed in the tropical regions, is a rich source of prenylated flavonoids and stilbenes, and the biological activities of those metabolites encompass virtually all fields of pharmacological sciences [1,3]. In Chinese traditional medicine, the roots of *Macaranga denticulata* have been used for the treatment of icteric hepatitis. Previous studies on this plant have resulted in the isolation of prenylated flavonoids [4] and stilbene–diterpene heterodimers [5]. As a part of our ongoing research on structurally diverse and potentially anticancer prenylated aromatic products from genus *Macaranga*, one rare flavonoid–diterpene heterodimer, denticulatain C (1), one modified geranyl-type side chain substituted flavonoid, denticulatain D (2) and one geranylated flavonoid, denticulatain E (3), as well as 11 known compounds (Fig. 1) were isolated from the fronds of *M. denticulata*. Their cytotoxicity against A549 cancer cell line
and the antiangiogenic activity against zebrafish embryos were tested. Herein, we describe the isolation, structural elucidation and anticancer effects of these compounds.

Generally speaking, flavonoids are rarely substituted by isoprenoid residue longer than the geranyl [1], especially those isoprenoid residues with further intricate cyclization. The complex structures and sufficient biological activities of those compounds have attracted scientists’ greater attention to study their structures, bioactivities, and synthesis. Previously studies have led to the isolation and characterization of the first example of flavonoid–isoprene heterodimer, denticulaflavonol [4], which have been further synthesized along with three other derivatives [6]. Thus, the discovery of denticulatin C (1), the second example flavonoid–isoprene heterodimer from nature has enriched the diversity of prenylated flavonoid class.

2. Experimental

2.1. General

Optical rotations were recorded using a Jasco P-1020 digital polarimeter. UV spectra were recorded using a Shimadzu UV-2401PC spectrophotometer. IR spectra were recorded on a Bruker Tenor 27 spectrophotometer with KBr pellets. 1D and 2D NMR experiments were recorded on Bruker AM-400 or DRX-1000 spectrometers with TMS as internal standard. Chemical shifts (δ) are expressed in ppm with reference to the solvent signals. ESI-MS were recorded using a Finngan MAT 90 instrument and HR-ESI-MS was performed on an API QSTAR time-of-flight spectrometer. Spectroscopic chromatography (CC) was performed on Sephadex LH-20 (Amer sham Biosciences, Piscataway, USA), silica gel (200–300 mesh, Qingdao Marine Chemical Inc., Qingdao, China), RP-18 gel (LiChroprep, 40–63 μm, Merck, Darmstadt, Germany), and MCI gel CHP20P (75–150 μm, Mitsubishi Chemical Corporation, Tokyo, Japan). Semipreparative HPLC was performed on an Agilent 1200 (column: Zorbax SB, 4.6 × 250 mm, DAD detector). Fractions were monitored by TLC and visualized by heating plates sprayed with 15% H2SO4 in EtOH.

2.2. Plant material

The fronds of M. denticulata were collected from Xishuangbanna of Yunnan province, PR China, in March 2008. A voucher specimen (Yangyp–20080316) was deposited in the Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences, which was identified by Prof. Yong-Ping Yang.

2.3. Extraction and isolation

The air-dried and powdered fronds of M. denticulata (11 kg) were extracted with 90% EtOH (3 × 25 L) at room temperature and concentrated in vacuo to yield a residue, which was partitioned between H2O and EtOAc. The EtOAc portion was decolorized on MCI gel (eluting with 95% EtOH), and the residue (185 g) was subjected to silica gel CC with a gradient elution of CHCl3/acetone (10:0 to 3:7) to afford five fractions (Fr. A–E). Fr. A was purified by Sephadex LH-20 (CHCl3–MeOH, 1:1) and then subjected to CC over silica gel eluted with petroleum ether–EtOAc (1:0 to 2:1) to yield compounds 10 (3.1 mg) and 13 (4.3 mg). Fr. B was separated by Sephadex LH-20 (CHCl3–MeOH, 1:1) to obtain 12 (13.2 mg) and a major fraction which was subjected to CC over silica gel eluted with petroleum ether–EtOAc (4:1 to 1:1), to give 4 (42.5 mg) and 5 (9.7 mg). Fr. C was purified over a Sephadex LH-20 (CHCl3–MeOH, 1:1) and then fractionated by RP-18 with a gradient elution of MeOH–H2O (2:8 to 10:0) to yield subfractions C1–C6. Subfraction C1 was repeatedly purified by CC over silica gel eluted with petroleum ether–EtOAc (9:1 to 1:1) to afford 11 (21.6 mg) and 14 (7.4 mg). Compounds 1 (8.5 mg), 8 (10.7 mg) and 9 (9.2 mg) were isolated from subfraction C3 by repeatedly subjected to CC over silica gel eluted with petroleum ether–EtOAc (9:1 to 3:1). Fr. D was fractionated by Sephadex LH-20 (CHCl3–MeOH, 1:1) to yield subfractions D1–D3. Subfraction D1 was purified by semipreparative HPLC eluted by 87% MeOH–H2O, to afford 2 (12.8 mg) and 3 (10.3 mg). Subfraction D3 was purified by semipreparative HPLC eluted by 82% MeOH–H2O, to afford 6 (5.6 mg) and 7 (16.4 mg).

2.4. Spectroscopic data

2.4.1. Denticulatin C (1)

Yellow oil; [α]D580 +1.4 (c 0.14, MeOH); UV (MeOH) λ max (log ε) 202 (4.64), 237 (4.36), 370 (4.38) nm; IR (KBr) ν max 2343, 2925, 2845, 1647, 1626, 1564, 1483, 1443, 1367, 1319, 1268, 1196, 1157, 1089, 1035, 959, 886, 812 cm−1; 1H NMR (acetone-d6, 400 MHz) data, see Table 1; negative ESIMS m/z 575 [M + H]−; HR-ESI-MS m/z 575.2995 [M + H]− (calcd. for C35H43O7, 575.3008).

2.4.2. Denticulatin D (2)

Yellow amorphous powder; [α]D8 −2.5 (c 0.31, MeOH); UV (MeOH) λ max (log ε) 202 (4.88), 222 (4.79), 272 (4.51), 348 (4.02), 428 (4.48) nm; IR (KBr) ν max 3377, 2920, 1651, 1622, 1607, 1564, 1483, 1367, 1316, 1267, 1228, 1182, 1086, 1024, 854, 839, 805 cm−1; 1H NMR (acetone-d6, 500 MHz) and 13C NMR (acetone-d6, 100 MHz) data, see Table 1; negative ESIMS m/z 437 [M − H]−; HR-ESI-MS m/z 437.1957 [M − H]− (calcld. for C25H25O7, 437.1600).

2.4.3. Denticulatin E (3)

Yellow oil; [α]D580 −4.0 (c 0.15, MeOH); UV (MeOH) λ max (log ε) 202 (4.72), 220 (4.72), 286 (4.15), 346 (3.82) nm; IR (KBr) ν max 3454, 2967, 2821, 1657, 1620, 1552, 1438, 1373, 1311, 1254, 1199, 1158, 1110, 1093, 1027, 824 cm−1; 1H NMR (acetone-d6, 400 MHz) and 13C NMR (acetone-d6, 125 MHz) data, see Table 1; negative ESIMS m/z 437 [M − H]−; HR-ESI-MS m/z 437.1598 [M − H]− (calcld. for C25H25O7, 437.1600).

2.5. Cytotoxicity assay

Compounds 1–14 were tested for their cytotoxicity against human lung cancer cell line A-549 by the MTT method, 5-FU was used as a positive control. Briefly, 100 μL of cell suspension (1 × 105 cells/mL) was seeded into 96-well microtiter plates and cultured for 24 h before the compound was added. Then, different concentrations of the compounds were added to the plates, the cells were cultivated for 48 h, and 10 μL of MTT (5 mg/mL) was added to each well. After 4 h, the culture medium was removed and
the formazan crystals were completely dissolved with 150 μL DMSO in each well by vigorously shaking the plate. Finally, formazan absorbance was assessed by a BioRad microplate reader at 570 nm.

2.6. Antiangiogenesis assay

Stock solutions (20 mg/mL) of all samples were prepared by dissolving the compounds 1–14 in DMSO. These solutions were diluted in sterile salt water (5 mM NaCl, 0.17 mM KCl, 0.4 mM CaCl₂, 0.16 mM MgSO₄) to obtain final solutions of various concentrations in 0.2% DMSO. Aliquots were placed into 24-well plates, and the embryos (TG[VEGFR2:GRCFP]) at 24 hpf (hours post-fertilization) were also transferred randomly into the above wells. Control embryos were treated with the equivalent amount of DMSO solutions. All embryos were incubated at 28.5 °C. After 48 h treatment, the intersegmental vessels of embryos were visualized with green fluorescent protein labeling and endogenous alkaline phosphatase staining. The antiangiogenic activities of compounds were calculated from the inhibition ratio of antiangiogenesis.

3. Results and discussion

Denticulatain C (1) was obtained as yellow oil. HR-ESI-MS analysis of 1 provided a molecular formula of C₃₅H₄₂O₇,
corresponding to 15 unsaturation degrees. The IR spectrum showed typical absorption bands for OH (3423 cm\(^{-1}\)), \(\alpha\beta\)-unsaturated carbonyl (1647 cm\(^{-1}\)) and aromatic ring (1602 and 1483 cm\(^{-1}\)) moieties. The UV spectrum showed absorption maxima at \(\lambda_{\text{max}}\) 257 (4.36) and 380 (4.38) nm, which indicated the presence of a flavonol skeleton \([7]\). The \(^1\)H and \(^13\)C NMR spectra of \(1\) showed the existence of C-6 substituted quercetin \([\delta_c 136.7 (s), 176.5 (s), 93.8 (d), 116.3 (d), 121.3 (d); \delta_h 6.60 (1H, s), 6.97 (1H, d, \(J = 8.4\) Hz), 7.66 (2H, d, \(J = 8.4\) Hz), 7.79 (1H, s)]\)[7,8] and the remaining moiety possessed 20 carbons including one pair of terminal double bond \([\delta_c 106.5 (t), 149.4 (s); \delta_h 4.47 (1H, s), 4.74 (1H, s)]; one pair of trisubstituted double bond \([\delta_c 123.5 (d), 135.6 (s)]; 4\) tertiary methyls \((\delta_h 0.61, 0.71, 0.73, 1.78, 3H each, s); 8\) methylenes; 2 methines and 2 quaternary carbons, which can be identified as a bicyclic labdane diterpenoid by comparing the NMR data with those of denticulaflavonol \([4]\) and this deduction was confirmed by the observed 2D NMR correlations (Fig. 2). The HMBC correlations of H-15\(^{\prime}\)/C-5, C-6, C-7 and H-14\(^{\prime}\)/C-5 demonstrated that labdanyl unit was connected to quercetin at C-6 of ring A. The observed ROESY (Fig. 3) correlations of H-5\(^{\prime}\)/H-9\(^{\prime}\), Me-18\(^{\prime}\), Me-20\(^{\prime}\)/H-11\(^{\prime}\), Me-19\(^{\prime}\); H-12\(^{\prime}\)/H-14\(^{\prime}\); H-15\(^{\prime}\)/H-16\(^{\prime}\) suggested that the stereochemistry of labdane skeleton in \(1\) was the same as denticulaflavonol \([5]\). Therefore, the structure of denticulatain C \((1)\) was determined as 6-[(5\(^{\prime}\)S,9\(^{\prime}\)S,10\(^{\prime}\)S)-15\(^{\prime}\)-labd-8\(^{\prime}\)(17\(^{\prime}\))]-diennyl]quercetin, and it is the second example flavonoid–diterpene heterodimer from nature.

Denticulatain D \((2)\) was obtained as optically active amorphous powder \((\lbrack \alpha \rbrack)_{D}^{18} − 2.5, c 0.31, \text{MeOH}) and its molecular formula of \(C_{25}H_{26}O_7\) was established by HR-ESI-MS at \(m/z\) 437.1597 \([M – H]^{−}\), requiring 13 degrees of unsaturation. Analysis of the \(^1\)H and \(^13\)C NMR (Table 1) data of \(2\) aided by HSQC revealed resonances for one 6-substituted kaempferol \([\delta_c 135.5 (s), 176.5 (s), 93.8 (d), 116.3 (d), 130.4 (d); \delta_h 6.59 (1H, s), 6.99 (2H, d, \(J = 8.6\) Hz), 8.12 (2H, d, \(J = 8.6\) Hz)]\)[4,9].
one modified geranyl-type side chain (6-hydroxy-3,7-dimethyl-2E,7-octadienyl) [one pair of trisubstituted double bond (δC 122.9 d, 136.6 s); one pair of terminal double bond (δC 110.3 t, 149.3 s); one oxygenated methine (δC 75.2); three alkyl methylenes (δC 21.9, 34.5, 36.4) and two tertiary methyls (δH 1.65, 1.78, 3H each, s)] [10], which was confirmed by the observed 1H-1H COSY correlation of H-6″/H-7″ and the HMBC correlations of H-6″/C-8″; H-9″/C-7″; Me-10″/C-7″. The HMBC correlations of H-1″/C-5, C-6, C-7 and H-2″/C-6 indicated the attachment of the modified geranyl-type side chain at C-6 of ring A. Thus, the structure of denticulatain D was determined as 6-(6-hydroxy-3,7-dimethyl-2E,7-octadienyl)kaempferol.

The molecular formula of denticulatain E (3) was found to be C25H26O7, as deduced from HR-ESI-MS and NMR data, indicative of 13 indices of hydrogen deficiency. The strong IR absorptions implied the presence of hydroxy (3454 cm⁻¹), conjugated carbonyl (1657 cm⁻¹), and aromatic ring (1620 and 1438 cm⁻¹) functionalities. The 1H and 13C NMR (Table 1) displayed characteristic signals corresponding to a ring B-substituted quercetin [δC 136.6 s, 176.5 s, 94.4 d, 99.1 d, 113.3 d, 121.8 d; δH 6.24 (1H, d, J = 1.7 Hz), 6.44 (1H, d, J = 1.7 Hz), 7.61 (s), 7.66 (s)] [11] and a geranyl substituent [three methyls (δC 16.2, 17.6, 25.7); three methylenes (δC 27.4, 28.8, 40.4); two pair of trisubstituted double bond (δC 123.2 d, 125.0 d, 131.7 s, 136.6 s)]. The observed HMBC correlations of H-1″/C-5′, C-6′ and H-2″/C-5′ indicated the location of the geranyl group at C-5′ of ring B. Accordingly, the structure of denticulatain E (3) was determined as 5′-geranylquercetin.

The known compounds (Fig. 1) were identified as 5-hydroxy-2-(4-hydroxyphenyl)-7-methoxy-6-(3-methylbut-2-enyl)chroman-4-one (4) [12], sophoraflavanone A (5) [13], bonanniol A (6) [14], diplacol (7) [15], 5,7,3′,4′-tetrahydroxy-6-geranylflavonol (8) [7], 5,7,3′,4′-tetrahydroxy-3-methoxy-6-geranylflavone (9) [7], 3β-hydroxy-7α-ethoxy-24β-ethylcholest-5-ene (10) [16], (24R)-6β-hydroxy-24-ethylcholest-4-en-3-one (11) [17], epitaraxerol (12) [18], α-tocopherolquinone (13) [19] and boehmenan (14) [20] by comparison of their experimental and reported spectroscopic data. All the compounds were isolated from M. denticulata for the first time.

Since prenylated flavonoids are reported to have modest or strong anticancer activities [1,9], the cytotoxicity of compounds 1–14 were evaluated against human lung cancer cell line A549 by the MTT method [21], with 5-FU used as a positive control (IC50 22.48 μg/mL). The results showed that compounds 4 and 8 inhibit the proliferation of A-549 cell line with IC50 values of 48.6 and 20.2 μg/mL, respectively, and this is the first time to report the cytotoxicity of known compounds 4 and 8. The antiangiogenic activities of compounds 1–14 were further evaluated using a zebrafish model in terms of the inhibition on the growth of intersegmental vessels, using PTK787 as...
positive control (IC$_{50}$ 0.10 µg/mL) [22]. The results showed that intersegmental vessels of embryos treated with compounds 3, 6, and 8 were significantly fewer than those of the control (0.2% DMSO in sterile salt water) and the reduction was dose dependent with an IC$_{50}$ value of 9.78, 0.34 and 2.55 µg/mL, respectively. The angiogenic activities of known compounds 6 and 8 were reported for the first time.

4. Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fitoter.2015.04.001.

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