Chemical constituents from *Daphne tangutica* and their cytotoxicity against nasopharyngeal carcinoma cells

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

Two new sesquiterpenoids (1–2), together with 30 known compounds including one sesquiterpenoid (3), six diterpenoids (4–9), fourteen lignans (10–23), and nine other kinds of compounds (24–32), were isolated from the stems of *Daphne tangutica* Maxim. Their structures were determined through extensive spectroscopic analyses, and the absolute configuration of daphnoid A (1) and B (2) were determined by the experimental and calculated circular dichroism (ECD) spectra. All the isolates were evaluated against two human nasopharyngeal carcinoma cell lines (HONE-1 and SUNE-1). Compound 25 (daphnene) showed potent cytotoxicity toward HONE-1 and SUNE-1 with IC_{50} values of 2.23 and 1.43 μM, respectively. Further studies indicated that compound 25 exhibited cytotoxic effects by inducing tumor cell apoptosis and arresting the cell cycle at G2/M phases in HONE-1 cells.

1. Introduction

*Daphne* genus (Thymelaeaceae) comprises of almost 90 species, of which 44 species are distributed in West of China. Besides being grown for ornamental purposes, *D. tangutica* is traditional used for the treatment of rheumatoid arthritis and apoplexia [1,2]. Previous phytochemical work resulted in the isolation of sesquiterpenoids, diterpenoids, flavonoids, coumarins, and lignans [2–5]. These secondary metabolites have been reported having a broad range of biological activity [6], including anti-tumor [7], anti-HIV [4], and anti-inflammatory activity [5].

Nasopharyngeal carcinoma (NPC) is the most common malignant tumor of thensopharynx. Geographically, Southeast Asia, Southern China, and North African countries have the highest prevalence of NPC. There are 80,000 new cases per year, making NPC the 23rd most common of all new cancers worldwide [8]. Although cisplatin-based concurrent chemoradiotherapy is currently considered to be the standard treatment regimen for patients with advanced NPC. Recently, it was reported that cisplatin does not improve survival after concurrent chemoradiotherapy in patients [9]. It is necessary to develop novel agents for the treatment of NPC. Natural products play a key role in looking for lead compounds [10]. Herein, we report the isolation of 32 compounds from the stems of *D. tangutica* and elucidation of their structures, as well as evaluation of their cytotoxicity toward a poorly differentiated human nasopharyngeal carcinoma (HONE-1) cell line and a highly metastatic human nasopharyngeal carcinoma (SUNE-1) cell line.

2. Results and discussion

Chromatographic separation of the EtOAc extract from the stems of *D. tangutica* yielded two new guaiane-type sesquiterpenoids (1–2) and 30 known compounds (3–32), anhydrogerigin (3) [11], daphnetoxin (4) [12], 9,13,14-ortho-(2,4,6-decatrienioate) of 5β-hydroxyxeriferonol-6α,7α-oxide (5) [13], excoecarioxin (6) [13], g niditirin (7) [14], yuanchuanin (8) [15], yuanchujian (9) [16], (8S,8′R,8″S)-4,4″-dihydroxy-3,3″-9-trimetoxy-9,9″-epoxy lignan (10) [17], (8S,8′R)-4,4″-dihydroxy-3,3″-9-trimetoxy-9,9″-epoxy lignan (11) [17], (7S,8R,8″R)-isolariciresinol (12) [18], secosolariciresinol-9,9″-acetonide (13) [19], matairesinol (14) [20], (-)-nortrachelogenin (15) [21], 3′,3′-bis(3,4-dihydro-6-methoxy-2H-1-benzopyran) (16) [22], acuminatin (17) [23], 4,4″-dihydroxy-3,3″-dimethoxy-9-ethoxy-9,9″-epoxy lignan (18) [24], daphnetone (19) [25], pluvialolide (20) [26], piperitol (21) [27], isocubebin (22) [24], epipinoniresinol (23) [28], daphnelone (24) [25], daphnenine (25) [29], daphneone (26) [29], 3′-hydroxy-3,3″-dimethoxy-9-ethoxy-9,9″-epoxy lignan (27) [30], threo-1,5-dihydropent-1-ene-1,3-diol (28) [31], (-)-(25)sakuranetin (29) [32], daphnetin (30) [33], aurantiamide acetate (31) [34], and syringaldehyde (32) [35] Fig. 1.

Compound 1 (daphnoid A) was isolated as yellow oil. Its molecular...
The formula was established as C_{15}H_{18}O_{4} based on HREIMS ion at m/z 262.1201 [M]+ (calcd 262.1200). The IR spectrum showed absorption bands for hydroxy (3401 cm\(^{-1}\)), conjugated carbonyl (1701 cm\(^{-1}\), and formyl (1757, 2766 cm\(^{-1}\)) groups. In the \(^1\)H NMR spectrum, a singlet proton appeared at \(\delta_H 9.76\), and this signal exhibited a correlation with a carbonyl group signal at \(\delta_C 207.3\) in the HSQC spectrum, which implied the presence of a formyl group. The \(^13\)C NMR spectrum displayed 15 carbon signals, including one aldehyde carbonyl group (\(\delta_C 207.3\)), two ketone carbonyl groups (\(\delta_C 204.8\) and 215.0), two double-bond carbons (\(\delta_C 142.6\) and 175.0), one hydroxylated tertiary carbon (\(\delta_C 81.3\)), two quaternary carbons (\(\delta_C 42.6\) and 60.6), one methine (\(\delta_C 34.5\)), three methylenes (\(\delta_C 36.3, 45.2,\) and 50.7), and three methyl groups (\(\delta_C 9.0, 15.0,\) and 18.8). The above mentioned revealed a modified guaiane-type sesquiterpenoid [3]. The NMR data of compound 1 were similar to those of the known compound wiksphyllamin A [36], except for the presence of an olefinic bond (\(\delta_C 142.6,\) C-1; 175.0, C-5) instead of two methines at C-1 (\(\delta_C 60.1\)) and C-5 (\(\delta_C 36.7\)) in wiksphyllamin A. The planar structure of compound 1 was further confirmed through HMBC correlations from H-3, H-4, H-6, and H-9 to C-1 (\(\delta_C 142.6\)), and from H-3, H-4, H-6, and H-15 to C-5 (\(\delta_C 175.0\)). Thus, the planar structure of compound 1 was determined as shown in Fig. 2.

Biogenetically, compound 1 had the same stereo centers at C-4, C-7, C-10, and C-11 as those of wiksphyllamin A. Hence, the absolute configuration of compound 1 was assigned as 4S, 7S, 10R, and 11S, which was further confirmed by comparing the experimental and calculated electron circular dichroism (ECD) spectra (Fig. 3).

Compound 2 (daphnoid B) was obtained as a yellow oil. Its molecular
formula was assigned as C_{18}H_{26}O_{5} based on the [M + Na]^+ ion peak at m/z 345.16723 (calcd 345.16725) in the HRESIMS. The IR spectrum of compound 2 exhibited characteristic absorptions of hydroxy (3394 cm\(^{-1}\)), carboxyl (1739 cm\(^{-1}\)), and olefinic bond (1644 cm\(^{-1}\)) groups. The \(^1\)H NMR spectrum displayed four methyl groups at \(\delta_{H} 0.95, 1.68, 2.02, \) and 3.24, and two olefinic protons at \(\delta_{H} 4.86\) and 5.16. The \(^{13}\)C NMR data of compound 2 showed 18 carbon signals including four olefinic bond carbons (\(\delta_{C} 103.1, 122.3, 141.9, \) and 154.4), one carbonyl carbon (\(\delta_{C} 171.0\)), two oxygenated sp\(^3\) carbons (\(\delta_{C} 81.5\) and 103.6), one oxygenated tertiary carbon (\(\delta_{C} 87.8\)), two methines (\(\delta_{C} 39.4\) and 41.2), four methylenes (\(\delta_{C} 34.8, 37.1, 37.1, \) and 67.7), one methoxy carbon (\(\delta_{C} 48.1\)), and three methyl carbons (\(\delta_{C} 10.4\), 21.5, and 22.4). The NMR data of compound 2 were comparable to those of auranticanol F, isolated from the stems of Daphne aurantiaca [4,37]. Comparison of the 1D and 2D NMR data of compound 2 with those of auranticanol F revealed major changes at C-3 and C-8. And the chemical shift of C-3 at \(\delta_{C} 33.6\) being downfield to \(\delta_{C} 78.7\) in compound 2. The \(^{13}\)C NMR data of compound 2 also revealed an acetoxyl group (\(\delta_{C} 21.5\) and 171.0), which was connected to C-3 based on the HMBC correlation from H-3 (\(\delta_{H} 5.15, 1H, m\)) to 171.0 (carbon of acetoxyl). The methoxy group (\(\delta_{C} 48.1\)) substituted at C-8 was deduced from the HMBC correlation of the methoxy group hydrogen (\(\delta_{H} 3.24, 3H, s\)) with C-8 (\(\delta_{C} 103.6\)) (Fig. 4).

The NOESY cross peaks of H-3/H-15 and H-5/H-15 suggested that CH3-15, H-3, and H-5 are co-facial and assigned as \(\beta\)-oriented. The NOESY cross peaks of H-15/H-6 (\(\delta_{H} 1.73, 1H, m\)), H-6a (\(\delta_{H} 1.43, 1H, m\)), H-13α (\(\delta_{H} 4.42, 1H, m\)), and H-13β (\(\delta_{H} 4.29, 1H, m\))/H2-OMe (\(\delta_{H} 3.24, 3H\)) indicated that H2-OMe was \(\beta\)-oriented. The \(\beta\)-orientation of 7-OH was determined by ECD (Fig. 3). Thus, the absolute configuration of compound 2 was defined as 3R, 4R, 5R, 7R, and 8S.

Bioisosteric pathways for compounds 1 and 2 were proposed in Scheme 1. Since the guaiane-type sesquiterpenoids are the main sesquiterpenes of Daphne genus [4], the precursor of compounds 1 and 2 may be the fundamental sesquiterpene precursor farnesyl diphosphate (FPP). FPP can produce the guaiyl cation by biological catalysis. Compound 1 is formed from the guaiyl cation by electrophilic addition, dehydration, and oxidation (Scheme 1, route i). Compound 2 is also formed from guaiyl cation by series of dehydration, oxidation, aetherization, oxidation, methylation, and acetylation (Scheme 1, route ii) [37,38].

Thirty-two compounds, including two new guaiane-type sesquiterpenoids, were isolated from the stems of D. tangutica. Compound 1 had a rare distorted guaiane skeleton. Among of them, six compounds (4, 6, 7, 8, 9, and 25, Table 2) showed cytotoxic activity against two human nasopharyngeal carcinoma cells HONE-1 and SUNE-1 (IC\(_{50}\) < 20 \(\mu\)M). Specially, compound 25 (IC\(_{50}\) = 2.23 \(\mu\)M) exhibited better activity than the positive control cisplatin (IC\(_{50}\) = 5.16 \(\mu\)M) in HONE-1 cells. Compounds 24-28 possess C6-C5-C6 scaffold. In comparison to 24 and 26-28, compound 25, having an unsaturated ketone moiety, showed the most potent cytotoxic activity. These results indicated the unsaturated ketone moiety plays a key role for cytotoxicity. Annexin V-FITC/PI analysis showed compound 25 significantly induced apoptosis in HONE-1 cells (Fig. 5). Furthermore, compound 25 interfered with cell cycle progression and led to an increasing number of cells in the G2/M region in HONE-1 cells (Fig. 6). These findings indicated that compound 25 (daphnenone) may be a promising lead compound for development of anti-NPC drug.

3. Experimental section

3.1. General experimental procedures

Optical rotations data were obtained with the Anton Paar MCP 200 automatic polarimeter. UV spectra were recorded using a Shimadzu UV-2450 spectrophotometer. ECD spectra were acquired on an Applied Photophysics Chirascan spectrometer. IR spectra were determined from KBr pellets on a Fourier transformation infrared spectrometer coupled with infrared microscope. The \(^1\)H (400 MHz), \(^{13}\)C (100 MHz), and 2D NMR spectra were obtained on a Bruker AM-400 NMR spectrometer with TMS as an internal reference. HREIMS were measured on a Thermo MAT95XP high-resolution mass spectrometer and EIMS on a Thermo DSQ1MS spectrometer. HRESIMS were acquired on a Shimadzu LCMS-IT-TOF, and the ESIMS data were measured on an Agilent 1200 series LC-MS/MS system. RP-C\(_{18}\) silica gel (Fuji, 40-75 \(\mu\)m), MCI gel (CHP20P, 75-100 \(\mu\)m), Mitsubishi Chemical Corporation, Tokyo, Japan), silica gel (200–300 Mesh Marine Chemical Ltd., Qingdao, People’s Republic of China), and Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Sweden) were used for column chromatography (CC). Preparative and semi-preparative HPLC separations were carried out on a LC-20AT Shimadzu liquid chromatography system with a YMC ODS-A column (250 × 4.6 mm, 5 \(\mu\)m) connected with an SPD-M20A diode array detector. TLC analysis was carried out on silica gel plates (Marine Chemical Ltd., Qingdao, China). Fractions were monitored by TLC and visualized by heating plates sprayed with 5%
The human nasopharyngeal carcinoma cell lines (HONE-1) and (SUNE-1) were cultured on RPMI-1640 medium (Gibco, China) containing 5% FBS (Gibco, America), 100 IU/mL penicillin, and 100 mg/mL streptomycin (Hyclone, Shanghai). MTT (Sigma, America) assay was measured on Microplate reader (Thermo, Electroncorporation, China). Cell apoptosis assay, using Annexin V-FITC Apoptosis Detection Kit (Beyotime, China). And cell circle assay, detecting with Cell Cycle and Apoptosis Analysis Kit (Beyotime, China) were analyzed on a Flow cytometry (Beckman Coulter, EPICS XL, USA).

3.2. Plant material

The stems of *D. tangutica* (8.0 kg) were collected on August 11, 2016 in Puan District, Guizhou Province, People's Republic of China, and identified by Dr. Chunyan Han from Kunming Institute of Botany, the Chinese Academy of Sciences. A voucher specimen (2016002G) was deposited at the School of Pharmaceutical Sciences, Sun Yat-sen University.

3.3. Extraction and isolation

The air-dried and powdered stems of *D. tangutica* (8.0 kg) were extracted with 90% EtOH to obtain an extract that was partitioned with EtOAc (3 x 3L) and H_2O. The EtOAc fraction (380 g) was subjected to silica gel column chromatography (CC) using dichloromethane/acetone (1:0, 50:1, 9:1, 3:1, 0:1 v/v) to afford fractions A−E. Fraction B (21 g) was purified using an MCI gel column with MeOH−H_2O (60%–100%) as eluting solvent to give three subfractions B1−B3. Fraction B1 (5.4 g) was loaded onto a Sephadex LH-20 column and eluted with CH_2Cl_2/MeOH (1:1) to yield two subfractions B1A1−B1A2. B1A1 (1.8 g)...

**Scheme 1.** Plausible biosynthetic pathway of 1 and 2.

**Fig. 5.** Effects of compound 25 on cell apoptosis in HONE-1 cells. (A) Cells were treated with compound 25 (0, 1, 3, and 10 μM) for 48 h, and stained with Annexin-V FITC/PI, then analyzed by flow cytometry to evaluate apoptosis. (B) The histogram analysis of (A).
chromatographed over silica gel CC with cyclohexane/EtOAc (from 4:1 to 1:2) as the eluent to give six subfractions, B1A1C1−B1A1C6. B1A1C1 (120 mg) was purified with semi-preparative HPLC (70% MeCN in H2O, 2 mL/min) to yield 25 (32 mg), 26 (2 mg), 29 (5 mg). Fraction B1A1C2 (54 mg) was purified with semi-preparative HPLC (50% MeCN in H2O, 2 mL/min) to yield 1 (4 mg), 3 (4 mg), 19 (5 mg). B1A2 (3.4 g) chromatographed over silica gel CC with cyclohexane/EtOAc (from 5:1 to 1:2) as the eluent to give sixteen subfractions, B1A1C1−B1A1C16. B1A1C12 (93 mg) was purified with semi-preparative HPLC (70% MeOH in H2O, 2 mL/min) to yield 17 (15 mg), 18 (5 mg), B1A1C13 (120 mg) was purified with semi-preparative HPLC (70% MeOH in H2O, 2 mL/min) to yield 20 (2 mg), 32 (20 mg). B1A1C14 (50 mg) was purified with semi-preparative HPLC (70% MeOH in H2O, 2 mL/min) to yield 27 (7 mg), B1A1C15 (144 mg) was purified with semi-preparative HPLC (70% MeCN in H2O, 2 mL/min) to yield 21 (4 mg), 22 (55 mg). B1A1C16 (132 mg) was purified with semi-preparative HPLC (70% MeCN in H2O, 2 mL/min) to yield 16 (24 mg), 23 (15 mg). Fraction C (13 g) was further fractionated over silica gel CC (CH2Cl2/MeOH, 300:1 to 95:5) to give two subfractions, C1−C2. Fraction C1 (10 g) was further fractionated over silica gel CC (CH3Cl/MeOH, 300:1 to 95:5) to give two subfractions C1A1−C1A3. C1A1 (900 mg) was chromatographed over Sephadex LH-20 with MeOH as eluent and further purified by semi-preparative HPLC with 85% MeCN−H2O to yield 2 (4 mg). C1A2 (3.3 g) was chromatographed over silica gel CC with cyclohexane/acetone (from 20:1 to 0:1) as the eluent to give twelve subfractions C1A2B1−C1A2B12, C1A2B1 (44 mg) finally purified by HPLC (55% MeOH in H2O, 2 mL/min) to obtain 31 (8 mg). C1A2B2 (80 mg) was purified with semi-preparative HPLC (90% MeCN in H2O, 2 mL/min) to yield 7 (14 mg), C1A2B3 (112 mg) was purified with semi-preparative HPLC (90% MeCN in H2O, 2 mL/min) to yield 4 (14 mg), 5 (12 mg), 6 (8 mg). C1A2B4 (32 mg) was purified with semi-preparative HPLC (90% MeCN in H2O, 2 mL/min) to yield 8 (7 mg), 9 (4 mg). C1A2B7 (50 mg) was purified with semi-preparative HPLC (65% MeCN in H2O, 2 mL/min) to yield 28 (8 mg). E (32 g) was purified using an MCI gel column with MeOH−H2O (60%−100%) as eluting solvent to give three subfractions E1−E3. Fraction E2 (7.3 g) was loaded onto a Sephadex LH-20 column and eluted with CH2Cl2/MeOH (1:1) to yield two subfractions E1A1−E1A2. E1A1 (3.2 g) was chromatographed over silica gel CC with hexane/EtOAc (from 5:1 to 1:2) as the eluent to give ten subfractions, E1A1B1−E1A1B10. E1A1B1 (87 mg) was purified with semi-preparative HPLC (60% MeCN in H2O, 2 mL/min) to yield 10 (12 mg), 11 (8 mg), 12 (2 mg). E1A1B3 (77 mg) was purified with semi-preparative HPLC (60% MeCN in H2O, 2 mL/min) to yield 5 (6 mg), 24 (10 mg). E1A1B6 (180 mg) was purified with semi-preparative HPLC (65% MeCN in H2O, 2 mL/min) to yield 13 (8 mg), 14 (10 mg), 15 (12 mg).

3.3.1. Daphnoid A (1)

Yellow oil; [α]D − 17.3 (c 0.1, MeOH); UV (MeOH) λmax (log ε) 237 (1.033) nm; CD (MeOH) λmax (log ε) 209 (+ 2.68), 236 (− 2.87) nm; IR (KBr) νmax 3401, 3001, 2965, 2921, 2876, 2852, 2766, 1757, 1701, 1662, 1622, 1514, 1452, 1364, 1312, 1293, 1197, 1150, 1091, 1038, 991, 934 and 899 cm−1; 1H and 13C NMR data, see Table 1;
reading at 492 nm. All IC50 values were calculated by nonlinear regression analysis (GraphPad Prism).

3.4.2. Cell cycle and cell apoptosis assay
HONE-1 cells were seeded into 6-well cell culture plates at a density of 2 × 10^4 cells/well overnight. After 48 h treatment with compound 25 (0, 1, 3 and 10 μM), HONE-1 cells were harvested. To measure the ratio of apoptotic cells, the cells stained with Annexin V-FITC/PI at room temperature in the dark for 25 min and analyzed by flow cytometry. To examine the cellular DNA content, the cells fixed with 1 mL 70% ETOH overnight then stained with propidium iodide (PI) solution, at 4 °C in the dark for 30 min and then analyzed by flow cytometry.

Conflict of interest
The authors declare no competing financial interests.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2018.08.012.

References


