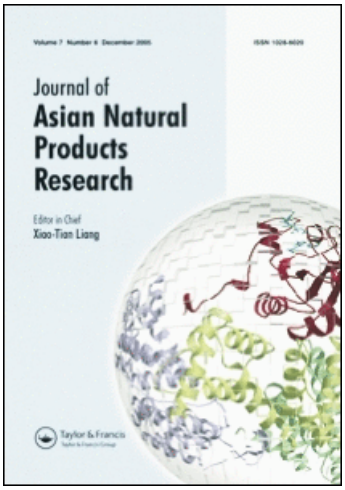

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[a] Department of Agriculture and Forestry, Taiwan Endemic Species Research Institute, Nantou County, Taiwan [b] National Research Institute of Chinese Medicine, Taipei, Taiwan [c] Institute of Life Science, National Taitung University, Taitung, Taiwan [d] Graduate Institute of Pharmaceutical Sciences, School of Pharmacy, National Taiwan University, Taipei, Taiwan

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[a]*Department of Agriculture and Forestry, Taiwan Endemic Species Research Institute, Nantou County 552, Taiwan;* [b]*National Research Institute of Chinese Medicine, Taipei 112, Taiwan;* [c]*Institute of Life Science, National Taitung University, Taitung 950, Taiwan;* [d]*Graduate Institute of Pharmaceutical Sciences, School of Pharmacy, National Taiwan University, Taipei 100, Taiwan*

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A new dimeric phenylpropanoid namely podonaka A (**1**), along with the 13 known compounds including diterpenes (**2** and **3**), norditerpenes (**4** and **5**), benzenoids (**6**–**10**), steroids (**11** and **12**), chalcone (**13**), and megastigmane (**14**), was isolated from the EtOH extract of *Podocarpus nakaii* Hayata. The structure of **1** was elucidated on the basis of spectroscopic analysis including 1D and 2D NMR and MS techniques. Biological evaluation showed that norditerpenes, inumakilactone B (**4**), and podolactone E (**5**) have potent cytotoxic activities against Daoy, WiDr, KB, and HeLa tumor cell lines. Moreover, low dosage of **5** may induce early apoptosis in KB cells before 12 h.

Keywords: *Podocarpus nakaii*; podonaka A; norditerpene; cytotoxicity; apoptosis

1. Introduction

In our continuing research for bioactive constituents from Taiwanese plants for developing an antitumor drug, we found that the EtOH extract of *Podocarpus nakaii* exhibited cytotoxicity against human tumor cells. *P. nakaii*, a dicotyledonous shrub plant in the family Podocarpaceae, is distributed in the middle area of Taiwan [1]. It was reported that a number of nor- and bisnorditerpene dilactones were isolated from the plants of the genus *Podocarpus* and they showed cytotoxicity against a panel of human cancer cells [2–5]. We reported herein that the bioassay-directed fractionations from the EtOH extract led to the isolation of a new dimeric phenylpropanoid, podonaka A (**1**), along with the 13 known compounds. The structural elucidation of these isolated compounds was mainly based on the spectroscopic analyses. Biological evaluation for these isolates (**1**–**14**) against human medulloblastoma (Daoy), colon adenocarcinoma (WiDr), oral epidermoid carcinoma (KB), and cervix epitheloid carcinoma (HeLa) tumor cell lines, as well as the investigation of apoptosis by the change of chromatin loop for the most potent cytotoxic compound was also reported. The EtOH extract of the stem bark of *P. nakaii* was extracted successively with *n*-hexane, $CHCl_3$, and H_2O, respectively. The $CHCl_3$ layer of EtOH

*Corresponding author. Email: kuoyh@nricm.edu.tw

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extract was concentrated and separated by a series of column chromatography and HPLC to yield compounds **1**–**14**. Structural elucidation of the new isolates was based on the spectroscopic analysis, including 1D and 2D NMR techniques (^{1}H–^{1}H COSY, HMQC, HMBC, TOCSY, and NOESY) and chemical hydrolysis.

2. Results and discussion

Compound **1** was given a molecular formula of $C_{18}H_{16}O_4$, as determined from the HR-FAB-MS data which gave a quasi-molecular ion peak at *m/z* 319.0926 $[M+Na]^+$. The IR spectrum showed the presence of hydroxyl (3300 cm^{-1}) and aromatic (1641 and 1511 cm^{-1}) groups. The signals for the two aromatic moieties [δ_H 7.55 (2H, d, $J = 8.5$ Hz), 7.27 (2H, d, $J = 8.5$ Hz), 6.85 (2H, d, $J = 8.5$ Hz), and 6.75 (2H, d, $J = 8.5$ Hz)] displayed by two AA′BB′ patterns, two pairs of *trans* double bonds [δ_H 6.64 (1H, d, $J = 16.0$ Hz), 5.96 (1H, dd, $J = 16.0$, 5.5 Hz), 7.60 (1H, d, $J = 16.0$ Hz), and 6.62 (1H, d, $J = 16.0$ Hz)], and an aldehydic proton at δ_H 9.55 were found in the ^{1}H NMR spectrum (Table 1). Additionally, the ^{13}C NMR and DEPT spectra indicated the other characteristic signals including two oxygenated aromatic carbons at δ_C 162.3 and 158.8, one dioxygenated carbon at δ_C 105.1, two pairs of olefinic carbons at δ_C 156.0, 134.7, and 126.4, 123.5, and one carbonyl carbon of aldehyde at δ_C 196.2. These findings, together with the ^{1}H–^{1}H COSY spectral analysis, suggested that **1** possessed two *p*-hydroxy phenylpropanoid units (Figure 1).

Based on HMBC correlations (H-α with C-4,β,γ; H-β with C-4; H-α with C-α; H-α′ with C-3′,5′,γ′; H-β′ with C-γ′; and H-γ′ with C-β′), the linkages of olefinic carbons for α and β and aromatic C-4, and the dioxygenated γ carbon were determined. Also, the protons for α′ and β′ were correlated with the other aromatic ring and aldehydic γ′ carbon in the HMBC spectrum. By the coupling constant at 8 Hz in HMBC spectrum, most of the two or three bond correlations for the C—H of **1** would be found, except for the correlation between dioxygenated H-γ and aromatic carbon at 1′. Further, adjusting the coupling constant by 5 Hz and a detailed checking of the HMBC spectrum of **1**, the cross-peak of H-γ and C-1′ was therefore observed. Moreover, due to the deshielding effect influenced by an aldehyde, the chemical shift of α′ carbon (δ_C 156.0) was much larger than that of α′ carbon (δ_C 134.7). According to these evidences, together with the mass spectrum of **1** showing the characteristic fragment ion at *m/z* 148, the linkage of two phenylpropanoid units was deduced (Figures 1 and 2). Based on the above corroborations, the structure of **1** was unambiguously confirmed and named as podonaka A.

Other known compounds (**2**–**14**) including two diterpenes: lambertic acid (**2**) and 4β-carboxy-17-hydroxy-19-nortotarol (**3**) [6], two norditerpenes: inumakilactone B (**4**) and podolactone E (**5**) [7], five benzenoids: evofolin-B (**6**) [8], isovanillin (**7**) [9], 4-hydroxy-3-methoxycinnamaldehyde (**8**) [10], vanillin acid (**9**) [11], and 4-ethoxy-3-hydroxybenzoic acid (**10**) [10], and two steroids: β-sitosterol (**11**) and β-sitosteryl glucoside (**12**) [12], in addition to 4,4′-dihydroxychalcone (**13**) [13] and vomifoliol (**14**) [14] were also isolated from the $CHCl_3$ layer derived from the EtOH extract. Their structures were identified by comparing the data with those of reported papers or authentic samples.

Compounds **1**–**14** were evaluated for cytotoxicity against several human tumor cell lines: HeLa, WiDr, KB, and Daoy. The cytotoxicity data (Table 2) revealed that **4**, **5**, **7**, **10**, **13**, and **14** exhibited significant cytotoxicity against one to four kinds of the above-mentioned tumor cell lines. Of these active compounds, norditerpenes (**4** and **5**) had the most potent cytotoxic effects ($ED_{50} = 1.05$–3.18 μg/ml). Moreover,

Table 1. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data, and HMBC and COSY correlations of **1**.

Position	δ_H (J in Hz)	δ_C	HMBC	^{1}H–^{1}H COSY
1		158.8		
2	6.75 (1H, d, $J = 8.5$)	116.4	C-1, C-3, C-6	H-3
3	7.27 (1H, d, $J = 8.5$)	129.1	C-1, C-4, C-5, C-α	H-2
4		129.0		
5	7.27 (1H, d, $J = 8.5$)	129.1	C-1, C-3, C-4, C-α	H-6
6	6.75 (1H, d, $J = 8.5$)	116.4	C-1, C-2, C-5	H-5
α	6.64 (1H, d, $J = 16.0$)	134.7	C-4, C-β	H-β
β	5.96 (1H, dd, $J = 16.0, 5.5$)	123.5	C-4	H-α,γ
γ	4.88 (1H, d, $J = 5.5$)	105.1	C-α, C-1′	H-β
1′		162.3		
2′	6.85 (1H, d, $J = 8.5$)	117.0	C-1′, C-4′, C-6′	H-3′
3′	7.55 (1H, d, $J = 8.5$)	132.0	C-1′, C-5, C-α′	H-2′
4′		127.1		
5′	7.55 (1H, d, $J = 8.5$)	132.0	C-1′, C-3′, C-α′	H-6′
6′	6.85 (1H, d, $J = 8.5$)	117.0	C-1′, C-2′, C-4′	H-5′
α′	7.60 (1H, d, $J = 16.0$)	156.0	C-5′, C-γ′	H-β′
β′	6.62 (1H, dd, $J = 16.0$)	126.4	C-γ′	H-α′, H-γ′
γ′	9.55 (1H, d, $J = 7.5$)	196.2	C-β′	H-β′

The ^{1}H chemical shifts were assigned by a combination of HMQC and HMBC experiments.

podolactone E (**5**) was further measured for apoptosis using ELISA-based apoptosis assay. As shown in Figure 3, the amount of chromatin loop treating with **5** obviously increased than that of control, after both 12 and 24 h. These results implied that **5** could induce early apoptosis in KB cells before 12 h. Due to the promising cytotoxicity of

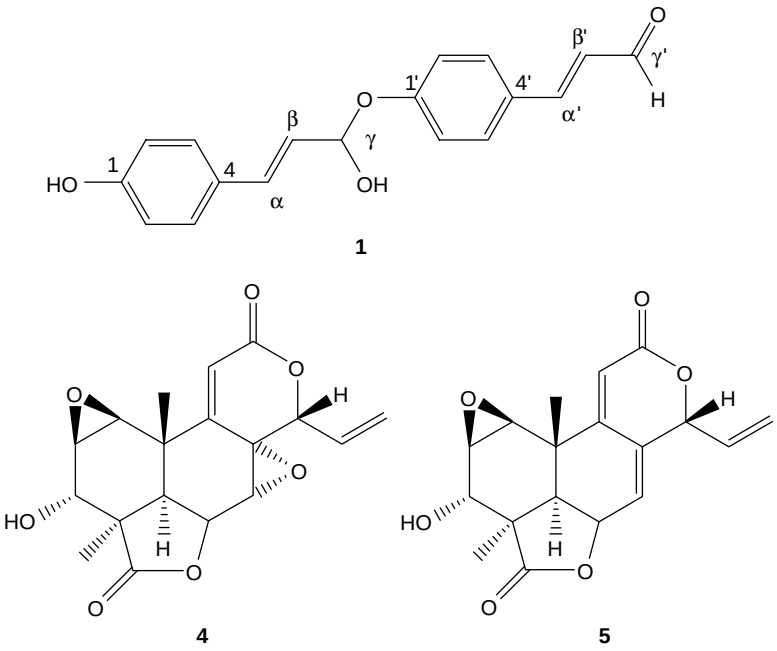

Figure 1. Chemical structures of **1**, **4**, and **5**.

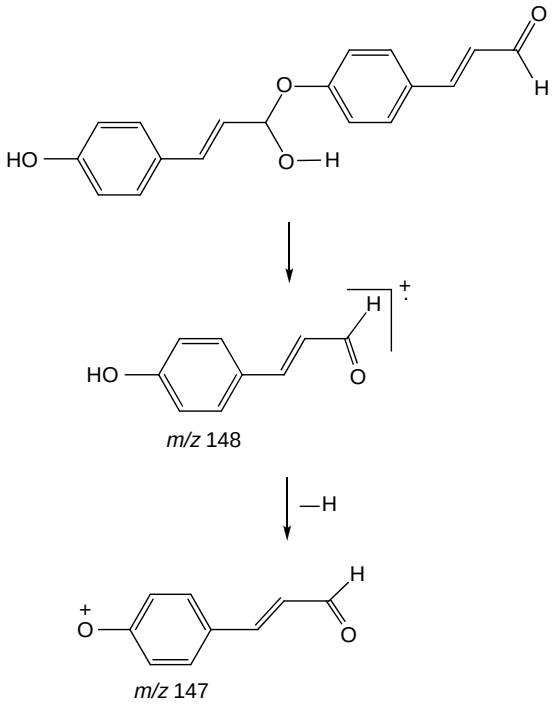

Figure 2. The EI-MS fragment ion at *m/z* 148.

4 and **5**, and apoptosis for **5**, norditerpene dilactones seem to be the major cytotoxic components in the plants of *Podocarpus* sp. and might potentially be developed as antitumor agents.

3. Experimental

3.1 General experimental procedures

Optical rotations were measured by a JASCO P-1020 polarimeter. IR spectra were measured with a Mattson Genesis II spectrophotometer. FAB-MS data were performed on a Jeol SX-102A instrument. HR-FAB-MS were measured on a Finnigan (Sunnyvale, CA, USA)/Thermo Quest MAT mass spectrometer. The 1D and 2D NMR spectral measurements were performed on a Bruker NMR spectrometer (Avance 400 MHz) and Varian NMR spectrometer (Unity Plus 500 MHz) using CD_3OD and $CDCl_3$ as solvent for measurement. Sephadex LH-20 and silica gel (70–230 and 230–400 mesh, respectively;

Table 2. Cytotoxicity data of **1**–**14** against human tumor cells (ED_{50}, μg/ml).

	Cell line			
Compound	HeLa	WiDr	KB	Daoy
1	–	–	–	NT
2	–	–	–	–
3	–	–	–	–
4	1.09	1.12	3.01	3.18
5	1.05	1.54	1.07	1.51
6	19.00	–	NT	–
7	–	13.98	NT	8.45
8	–	–	–	–
9	–	–	–	–
10	8.74	9.92	8.32	–
11	–	–	–	–
12	–	–	–	17.19
13	–	19.11	–	8.38
14	11.18	13.69	NT	11.01
Mytomycin C	0.26	0.19	0.11	0.18

Key to human cell lines used: human HeLa (cervix epitheloid carcinoma), WiDr (colon adenocarcinoma), KB (oral epidermoid carcinoma), and Daoy (medulloblastoma); –, inactive, $ED_{50} > 20$ μg/ml; NT, not tested.

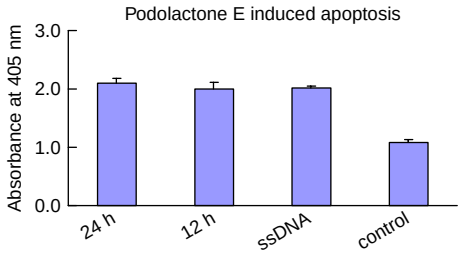

Figure 3. Effect of podolactone E on early apoptosis analyzed by ssDNA apoptosis ELISA kit. KB cells were treated with podolactone E (10 μg/ml) for 12 and 24 h. As a positive control, ssDNA was added to mimic the structure of chromatin loop in early apoptosis. The existence of chromatin loop or ssDNA was measured by the absorbance at 405 nm.

Merck, Darmstadt, Germany) were used for column chromatography and pre-coated silica gel (Merck 60 F-254) plates were used for TLC. The spots on TLC were detected by spraying with 95% H_2SO_4 then heating on a hotplate. HPLC separations were performed on a Shimadzu LC-6AD series apparatus with a RID-10A refractive index, equipped with a 250 × 20 mm i.d. and 250 × 10 mm i.d. preparative Cosmosil 5C18-AR II column.

3.2 Plant material

The stems and barks of *P. nakaii* were collected from Nantou County, Taiwan, in July 2001. The plant was taxonomically identified by Professor Muh-Tsuen Kao and a voucher specimen (No. NRICM200607A1) is deposited at the Herbarium of National Institute of Chinese Medicine, Taipei, Taiwan.

3.3 Extraction and isolation

The dried stems and barks of *P. nakaii* (15 kg) were extracted thrice with 95% ethanol at room temperature. After removal of the solvent *in vacuo*, the ethanol extract was successively partitioned between H_2O, *n*-hexane, and $CHCl_3$. Removal of $CHCl_3$ under reduced pressure left a dark residue (150 g). The residue was subjected to silica gel column chromatography, eluting with *n*-hexane/EtOAc (100:1 → 0:1) and EtOAc/MeOH (100:1 → 3:1), to yield five fractions (fractions 1–5) after combination by TLC method. Fraction 2 was subjected to silica gel column chromatography, eluting with *n*-hexane/EtOAc/MeOH (50:1:1 → 2:2:1), successively, to yield five fractions (fractions 2.1–2.5). Fraction 2.2 was further separated by chromatography on a Sephadex LH-20 column with $CHCl_3$/MeOH (1:1) as the eluent to yield seven fractions (fractions 2.2.1–2.2.7). Fraction 2.2.3 was recrystallized to afford **11** (176.0 mg). Fraction 2.2.7 was separated by HPLC on a Si 60 column (250 × 10.0 mm) with *n*-hexane/EtOAc (6:1) as the eluent to afford **3** (25.0 mg) and **4** (7.0 mg). Fraction 2.4 was further subjected to column chromatography over Sephadex LH-20 using $CHCl_3$/MeOH (1:1) to give five fractions (fractions 2.4.1–2.4.5). Fraction 2.4.2 was purified by HPLC with *n*-hexane/EtOAc (8:1) as the eluent to afford **8** (27.0 mg) and **2** (6.0 mg). Compound **7** (7.5 mg) was obtained by recrystallization of fraction 2.4.4. Fraction 3 was separated by chromatography on a silica gel column with CH_2Cl_2/MeOH from 100:1 to 4:1 to yield five fractions (fractions 3.1–3.5). Fraction 3.2 was further separated by chromatography on a Sephadex LH-20 column with $CHCl_3$/MeOH (1:1) as the eluent to yield three fractions (fractions 3.2.1–3.2.3). Compounds **5** (10.0 mg) and **6** (8.0 mg) were obtained from the recrystallization of fraction 3.2.2. Fraction 3.2.3 was further purified by HPLC eluting with *n*-hexane/EtOAc/MeOH (25:10:1) to afford **1** (6.0 mg). Fraction 3.4 was further purified by chromatography on a Sephadex LH-20 column with $CHCl_3$/MeOH (1:1) as the eluent to give three fractions (fractions 3.4.1–3.4.3). Fraction 3.4.2 was subjected to column chromatography over a silica gel column, eluting with CH_2Cl_2/MeOH

(50:1 → 4:1), to yield five fractions (fractions 3.4.2.1–3.4.2.5). Fraction 3.4.2.3 was purified by HPLC with *n*-hexane/ EtOAc/MeOH (20:10:1) to afford **14** (5.0 mg). Fraction 3.4.2.4 was further purified by recrystallization to afford **12** (210.0 mg). Fraction 4 was chromatographed over a Sephadex LH-20 column eluting with $CHCl_3$/MeOH (3:1) to yield five fractions (fractions 4.1–4.5). Fraction 4.4 was purified by PTLC eluting with *n*-hexane/EtOAc/MeOH (3:3:1) to afford **9** (13.0 mg) and **10** (4.0 mg).

*3.3.1 Podonaka A (**1**)*

A yellow oil; $[\alpha]_D^{24} - 40$ ($c = 0.1$, MeOH); IR ν_{max} (neat) 3300, 1641, 1511, 1378, 1284 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) spectral data, see Table 1; HR-FAB-MS: *m/z* 319.0926 $[M+Na]^+$ (calcd for $C_{18}H_{16}O_4Na$, 319.0946).

3.4 Cytotoxicity assay

MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] agent, against human HeLa, KB, Daoy, and WiDr cells, was based on the literature procedure [15]. In brief, the cells were cultured in RPMI-1640 medium and test samples were prepared at four concentrations. After these cell lines seeding in a 96-well microplate for 4 h, 20 μl of a sample was placed in each well. The microplate was incubated at 37°C for 3 days, and then 20 μl of MTT was added for 5 h. After removing the medium and adding DMSO (200 μl/well) into the microplate with shaking for 10 min, the formazan crystals were re-dissolved and their absorbance was measured on a microtiter plate reader (Dynatech, MR 7000), at a wavelength of 550 nm.

3.5 Detection of apoptosis

The assay was designed for detection of early apoptosis using ssDNA apoptosis ELISA kit (Cat. No. APT225; Chemicon, Temecula, CA, USA) [16]. KB cells (about 10,000 cells per well) were transferred into a 96-well microplate, which was then treated with 10 μg/ml of respective tested samples in 200 μl of RPMI-1640 medium (5% FBS) and incubated at room temperature for 12–24 h. Removing the medium and adding 200 μl of fixative solution (80% methanol in PBS), the microplate was incubated for 30 min. Then the fixative solution was removed and the microplate was heated at 37°C until thoroughly dry. After adding 10 μl of formamide into each well under room temperature for 10 min, the microplate was heated at 75°C for 10 min to denature DNA in the apoptotic cells and then was chilled at −20°C for 5 min. The formamide was removed and washed with PBS thrice; 200 μl of nonfat milk was then added into each well to block nonspecific-binding sites. After an hour, the milk was removed and 100 μl of an antibody mixture (recognizing for ssDNA) was added at 37°C for 2 h. Subsequently, each well was washed with 1× wash buffer three times and 100 μl of ABST solution was then added into the well under dark conditions at 37°C for an hour. Finally, 100 μl of stop solution was added into each well and the microplate was measured in a reader with the absorbance at 405 nm.

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