Constituents of *Pongamia pinnata* Isolated in a Screening for Activity to Overcome Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand-Resistance

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In a search for natural products with activity to overcome tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-resistance, we performed the bioassay-guided fractionation of a semi mangrove, *Pongamia pinnata*, collected from Bangladesh, and isolated a new compound, (2*S*)-(2″,3″:7,8)-furanoflavanone (1), along with six known flavonoids (2—7). Two of the compounds significantly overcame TRAIL-resistance in human gastric adenocarcinoma (AGS) cell lines.

Key words *Pongamia pinnata*; flavanone; screening

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a promising anticancer agent, as it can kill tumor cells selectively.[1)] TRAIL-induced apoptosis initiated by the death-receptor pathway involves the engagement of death receptors (DRs), formation of a death-inducing signaling complex (DISC), proteolytic activation of caspase-8, and, consequently, activation of caspase-3. However, considerable numbers of cancer cells are resistant to TRAIL. Overcoming TRAIL-resistance and understanding the mechanisms underlying such resistance are therefore very important in anticancer drug discovery.[2)]

During research on natural products with activity to overcome TRAIL-resistance,[3)] we previously isolated several active compounds from plant materials collected in Thailand.[4,5)] Recently, we further investigated plant materials of Bangladesh[6,7)] and found that the MeOH extract of fruits of *Pongamia pinnata* (Fabaceae) was potently active in our assay for activity against TRAIL-resistant human gastric adenocarcinoma (AGS) cell lines. *Pongamia pinnata* is a tall tree that grows in coastal and limestone areas of Taiwan, India, and Southeast Asian countries. Its fruits and sprouts are used in folk remedies for tumors.[8)] Previous chemical investigation of the tree's bark and fruits have led to the isolation of flavonoid derivatives.[9,10)] Bioassay-guided fractionation of the MeOH extracts of the fruits resulted in the isolation of one new compound (**1**) along with six known compounds (**2**—**7**). Here, we report the isolation and structural elucidation of **1** together with the activity of the isolated compounds on resistance to TRAIL.

Results and Discussion

The MeOH extract of fruits of *Pongamia pinnata* was chromatographed over Diaion HP-20 to remove the chlorophyll, and subjected to repeated column chromatography and preparative HPLC to afford a new compound **1**, together with known compounds **2**—**7** (Fig. 1). The known compounds were identified as 5-methoxy-(3″,4″-dihydro-3″,4″-diacetoxy)-2″,2″-dimethylpyrano-(7,8:5″,6″)-flavone[10)] (**2**), (−)-isolonchocarpin[11)] (**3**), pongamol[12)] (**4**), pongapin[13)] (**5**), [2″,3″:7,8]furanoflavone[13)] (**6**), and isopongaflavone[14)] (**7**), respectively, by comparing their spectral data with values in the literature.

1 was shown to have the molecular formula $C_{17}H_{12}O_3$ on the basis of high resolution-electrospray ionization-mass spectra (HR-ESI-MS) at *m*/*z* 287.0680 $(M+Na)^+$ (Calcd for $C_{17}H_{12}O_3Na$, 287.0679, Δ +0.1 mmu) together with its ^{1}H- and ^{13}C-NMR spectroscopic data (Table 1). The ^{13}C-NMR spectrum of **1** showed signals due to six quaternary carbons, one sp^3 methylene (δ_C 44.4), one sp^3 oxymethine (δ_C 80.5), and nine sp^2 methines. The ^{1}H-NMR spectrum showed signals for two furan ring hydrogens [δ_H 7.58 (1H, d, J=2.2 Hz, H-5″) and 6.96 (1H, dd, J=2.2, 0.9 Hz, H-4″)] and two aromatic hydrogens [δ_H 7.87 (1H, d, J=8.7 Hz, H-5) and 7.19 (1H, dd, J=8.7, 0.9 Hz, H-6)]; these signals were reminiscent of a (2″,3″:7,8)-furanoflavone structure such as **4**—**6**. However, **1** showed signals of the ABX system [δ_H 5.60 (1H, dd, J=13.1, 3.1 Hz), 2.90 (1H, dd, J=16.9, 3.1 Hz) and 3.13 (1H, dd, J=16.9, 13.1 Hz)], which were assignable to three hydrogens (H-2, H_2-3, respectively) of the C-ring, suggesting

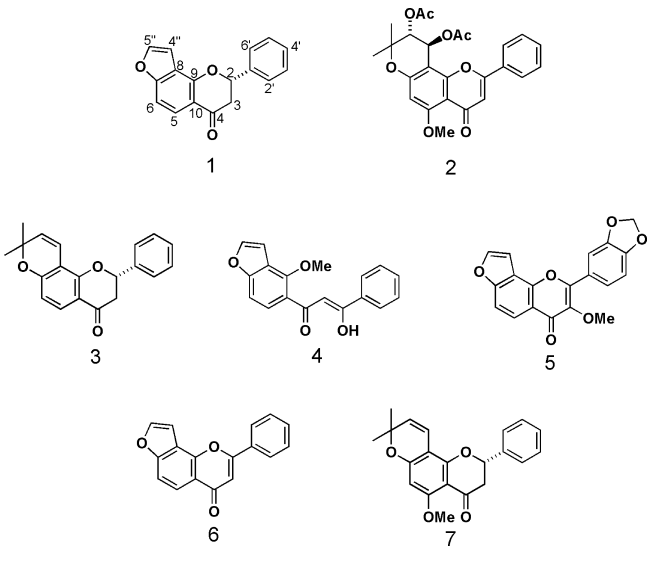

Fig. 1. Structure of Compounds **1**—**7** from *P. pinnata*

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Table 1. ^{1}H- and ^{13}C-NMR Data of **1** in $CDCl_3$

Positions	^{13}C-NMR	^{1}H-NMR
2	80.5	5.60 (1H, dd, 13.1, 3.1)
3	44.4	2.90 (1H, dd, 16.9, 3.1)
		3.13 (1H, dd, 16.9, 13.1)
4	191.3	
5	123.8	7.87 (1H, d, 8.7)
6	106.7	7.19 (1H, dd, 8.7, 0.9)
7	160.2	
8	117.4	
9	156.8	
10	115.7	
1′	129.0	
2′	126.7	7.50—7.53 (m)
3′	129.2	7.40—7.47 (m)
4′	129.2	7.40—7.47 (m)
5′	129.2	7.40—7.47 (m)
6′	126.7	7.50—7.53 (m)
4″	105.1	6.96 (1H, dd, 2.2, 0.9)
5″	145.4	7.58 (1H, br d, 2.2)

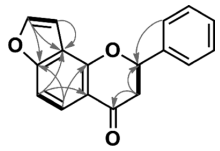

Fig. 2. Key COSY (Bold Line) and HMBC (Arrows) Correlations of **1**

that the compound had a (2″,3″:7,8)-furanoflavanone structure.[15] The heteronuclear multiple bond connectivity (HMBC) correlations observed from H-5″ to C-7 and C-8 and from H-4″ to C-8 (Fig. 2) supported the structure of the benzofuran moiety. The ^{1}H- and ^{13}C-NMR spectra indicated the presence of a phenyl group for the B-ring [δ_H 7.50—7.53 (2H, m, H-2′, H-6′) and 7.40—7.47 (3H, m, H-3′, H-4′, H-5′); δ_C 126.7 (C-2′, C-6′) and 129.2 (C-3′, C-4′, C-5′)]. The circular dichroism (CD) spectrum of **1** showed a positive Cotton effect at 337 nm and a negative effect at 305 and 286 nm, which were consistent with the 2*S*-configuration.[16,17] The long-range coupling between H-6 and H-4″ (J=0.9 Hz) were characteristically observed for other (2″,3″:7,8)-furanoflavones such as **4**—**6**.[12,13] The (2″,3″:6,5)-furanoflavone structure is therefore unlikely for **1**. In addition, in the HMBC spectrum (long-range delay of 62.5 ms corresponding to $^{n}J_{CH}$ of 8 Hz) of **1** (Fig. 2), the correlation from H-5 to C-9 (δ_C 156.8) was observed clearly while no cross-peak was observed between H-5 and C-10 (δ_C 115.7). In aromatic rings, generally $^{3}J_{CH}$-correlations are more clearly observed than $^{2}J_{CH}$-correlations in HMBC spectra,[18] further supporting the (2″,3″:7,8)-furanoflavone structure for **1**. Based on these results, **1** was identified as (2*S*)-(2″,3″:7,8)-furanoflavanone.

The isolated compounds (**1**—**7**) were evaluated for effects on TRAIL-resistance in AGS cells by comparing cell viability in the presence and absence of TRAIL (100 ng/ml) using the fluorometric microculture cytotoxicity assay (FMCA) method.[19] As shown in Fig. 3, treatment with 100 ng/ml of TRAIL for 24 h resulted in only a slight decrease in cell viability (91±4.5%), while luteolin,[20] used as a positive control, produced about 35% more inhibition along with TRAIL than the agent alone at 17.5 μM. With 30 and 40 μM of **2**, cell

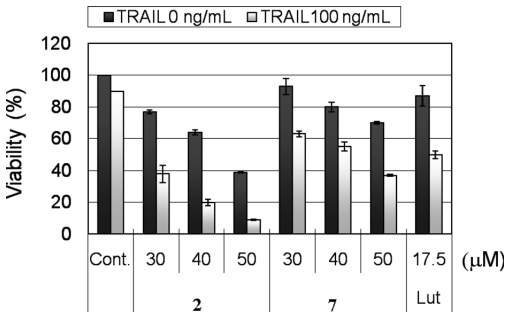

Fig. 3. Effect of **2**, **7**, and Luteolin (Positive Control: Lut) in the Presence and Absence of TRAIL on the Viability of AGS Cells

Cells were seeded in a 96-well culture plate (6×10^3 cells per well) for 24 h and then treated with indicated concentrations of the compounds and TRAIL (100 ng/ml) for 24 h. Cell viability was determined by the fluorometric microculture cytotoxicity assay (FMCA). The bars represent the mean (n=3±S.D.).

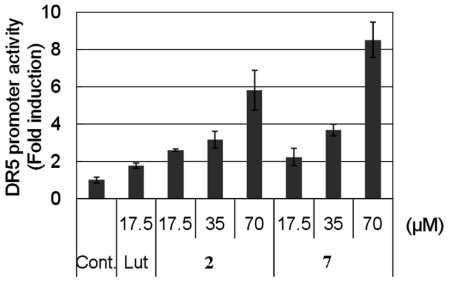

Fig. 4. Effect of **2** and **7** on DR5 Promoter Activity in DLD1/*Sac*I Cells

DLD1/*Sac*I cells were treated with the indicated concentrations of **2** and **7**, and the activity was measured by luciferase assay. The bars represent the mean (n=3±S.D.).

viability was 77% and 64%, respectively, whereas the same concentration of **2** in the presence of 100 ng/ml TRAIL reduced cell viability to 38 and 20% of control levels, respectively, which was 39 and 44% lower than the agent alone, implying that **2** significantly overcame TRAIL-resistance. **7** at 30 and 40 μM caused 30 and 25% decreases, respectively, in cell viability in the presence of TRAIL (100 ng/ml), implying that **7** had moderate activity. Compounds **1**, **3**, **4**, **5**, and **6**, however, did not produce any significant reduction in cell viability with TRAIL.

Since several compounds with TRAIL-resistance-overcoming activity caused an enhancement of the expression of death receptors,[1,21] we investigated death receptor 5 (DR5) promoter activity using a luciferase assay in DLD-1/*sac*I cells, a human colon cancer cell line stably transfected with the pDR5/*Sac*I plasmid.[22] As shown in Fig. 4, **2** enhanced DR5 promoter activity 2.6 and 5.8-fold at 17.5 and 70 μM, respectively, while **7** enhanced it 2.2 and 8.5-fold at 17.5 and 70 μM, respectively. These results suggested the enhanced expression of death receptors such as DR5 to be related to the activity of **2** and **7** to overcome resistance to TRAIL.[23,24]

Experimental

General Optical rotations were determined on a JASCO P-1020 polarimeter. UV spectra were obtained on a Shimadzu UV mini-1240 spectrometer. IR spectra were measured on ATR in a JASCO Fourier transform (FT)-IR 230 spectrophotometer. The NMR spectra were recorded on a JEOL ECP600 spectrometer with deuterated solvents, the chemical shift of which was used as an internal standard. HR-ESI-MS was measured on a JEOL JMS-T100LP.

Plant Material The fruits of *P. pinnata* were collected from Khulna, Bangladesh, in November, 2008 and taxonomically identified by Prof. A. K.

Fazlul Huq, Forestry and Wood Technology Discipline, Khulna University, Bangladesh. A voucher specimen (KKB53) has been deposited in our laboratory.

Extraction and Isolation Dried fruits (279.2 g) of *P. pinnata* were treated with MeOH for 2 d at room temperature, then subjected to homogenization, filtration, evaporation, and vacuum desiccation to give the crude extract (5.93 g). The extract was chromatographed over Diaion HP-20 (4.3×26 cm; particle size 250—850 μm) to remove the chlorophyll. The chlorophyll-free fraction (2.55 g) was subjected to silica gel 60N column chromatography (4.2×23 cm) using a hexane–EtOAc solvent system with increasing polarity to afford fractions 5A—5O and compound **2** (120.2 mg) was obtained in fraction 5N. Fraction 5D (41.0 mg) eluted with hexane/EtOAc (7 : 1) was subjected to preparative HPLC [YMC-Pack ODS-AM, 1.0×25 cm; MeOH : H_2O (78 : 22); flow rate: 2.0 ml/min; RI and UV detection at 254 nm] to give compounds **1** (2.2 mg, t_R 14 min) and **3** (5.5 mg, t_R 28 min). Fraction 5E (42 mg) eluted with hexane/EtOAc (5 : 1) was subjected to octadecylsilane (ODS) flash column chromatography using a MeOH–H_2O solvent system, followed by preparative HPLC [Develosil C30, 1.0×25 cm; MeOH : H_2O (83 : 17); flow rate: 2.0 ml/min; RI and UV detection at 254 nm] to give compounds **1** (0.3 mg, t_R 7 min), **3** (1.6 mg, t_R 14 min), and **4** (5.7 mg, t_R 24 min). Fraction 5I (453 mg) eluted with hexane/EtOAc (1 : 3) was subjected to ODS flash column chromatography using the MeOH–H_2O solvent system to afford **5** (16.8 mg), followed by preparative HPLC [SHISEIDO CAPCELL PAK C18, 1.0×25 cm; MeOH : H_2O (77 : 23); flow rate: 2.0 ml/min; RI and UV detection at 254 nm] to afford compounds **6** (9.6 mg, t_R 16 min) and **7** (8.5 mg, t_R 28 min).

Compound **1**: Yellow solid; $[\alpha]_D^{19}$ −111 (c=0.1, MeOH); UV (MeOH) λ_{max} 239 nm (ε 28000); CD (MeOH) λ_{ext} nm ($\Delta\varepsilon$) 337 (+2.8), 305 (−3.5), 286 (−2.0); IR (ATR) ν_{max} 2919 (br), 1684, and 1613 cm^{-1}; For ^{1}H- and ^{13}C-NMR data, see Table 1; HR-ESI-MS m/z 287.0680 $[M+Na]^+$ (Calcd for $C_{17}H_{12}N_2O_3Na$, 287.0679, Δ +0.1 mmu).

Cell Cultures AGS cells were derived from the Institute of Development, Aging and Cancer, Tohoku University; Cells were cultured in RPMI-1640 medium (Wako) with 10% fetal bovine serum (FBS). Cultures were maintained in a humidified incubator at 37 °C in 5% CO_2/95% air.

TRAIL-Resistance Test TRAIL-resistance was assessed by comparison of cell viability in the presence and absence of TRAIL with TRAIL-resistant human gastric adenocarcinoma (AGS) cell lines.[3,18] AGS cells were seeded in a 96-well culture plate (6×10^3 cells per well) in 200 μl of RPMI medium containing 10% FBS. Cells were incubated at 37 °C in a 5% CO_2 incubator for 24 h. Then the test samples with or without TRAIL (100 ng/ml) at different doses were added to each well. After 24 h of incubation, the cells were washed with phosphate buffered saline (PBS), and 200 μl of PBS containing fluorescein diacetate (10 μg/ml) was added to each well. The plates were then incubated at 37 °C for 1 h, and fluorescence was measured in a 96-well scanning spectrofluorometer at 538 nm with excitation at 485 nm.

DR5 Promoter Activity Test The assay procedure was as described previously.[21] Briefly, DLD-1/*Sac*I cells (2×10^5 cells per well), a human colon cancer cell line stably transfected with the DR5 promoter-luciferase reporter plasmid pDR5/*Sac*I,[22] were treated with different concentrations of each compound for 24 h at 37 °C. After medium containing the compound was removed, cells were lysed in a Cell Culture Lysis Reagent (Promega). The lysate was measured for 10 s as relative light units by a luminometer, and DR5 promoter activity was evaluated using the relative light units of the sample compared with the control (cells treated with EtOH).

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