

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 anti-cancer 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'')-flavone¹⁰⁾ (2), (–)-isolonchocarpin¹¹⁾ (3), pongamol¹²⁾ (4), pongapin¹³⁾ (5), [2'',3'':7,8]furanoflavone¹³⁾ (6), and isopongaflavone¹⁴⁾ (7), re-

spectively, by comparing their spectral data with values in the literature.

1 was shown to have the molecular formula C₁₇H₁₂O₃ on the basis of high resolution-electrospray ionization-mass spectra (HR-ESI-MS) at *m/z* 287.0680 (M+Na)⁺ (Calcd for C₁₇H₁₂O₃Na, 287.0679, Δ +0.1 mmu) together with its ¹H- and ¹³C-NMR spectroscopic data (Table 1). The ¹³C-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 ¹H-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, H₂-3, respectively) of the C-ring, suggesting

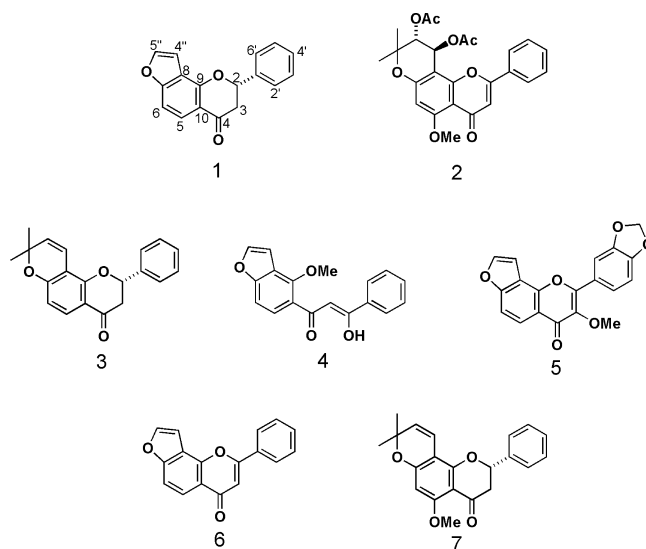
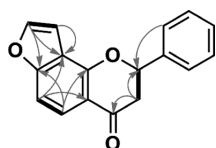


Fig. 1. Structure of Compounds 1–7 from *P. pinnata*

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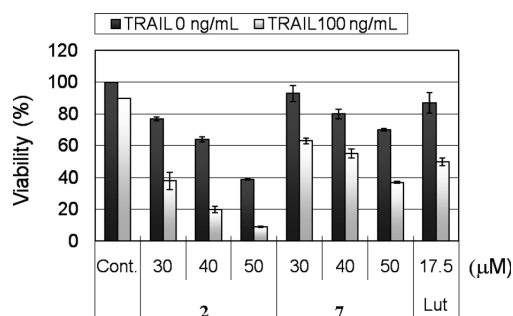
Table 1. ^1H - and ^{13}C -NMR Data of **1** in CDCl_3

Positions	^{13}C -NMR	^1H -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)

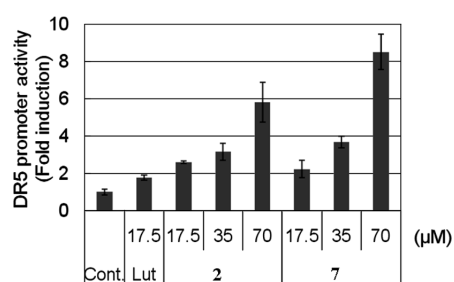
Fig. 2. Key COSY (Bold Line) and HMBC (Arrows) Correlations of **1**

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 ^1H - 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 $^nJ_{\text{CH}}$ 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 $^3J_{\text{CH}}$ -correlations are more clearly observed than $^2J_{\text{CH}}$ -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 ($91 \pm 4.5\%$), while luteolin,²⁰⁾ used as a positive control, produced about 35% more inhibition along with TRAIL than the agent alone at $17.5 \mu\text{M}$. With 30 and $40 \mu\text{M}$ 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 ($n=3 \pm \text{S.D.}$).

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

DLD1/*SacI* cells were treated with the indicated concentrations of **2** and **7**, and the activity was measured by luciferase assay. The bars represent the mean ($n=3 \pm \text{S.D.}$).

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 $40 \mu\text{M}$ 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/*sacI* cells, a human colon cancer cell line stably transfected with the pDR5/*SacI* plasmid.²²⁾ As shown in Fig. 4, **2** enhanced DR5 promoter activity 2.6 and 5.8-fold at 17.5 and $70 \mu\text{M}$, respectively, while **7** enhanced it 2.2 and 8.5-fold at 17.5 and $70 \mu\text{M}$, 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 μ m) 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:H₂O (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–H₂O solvent system, followed by preparative HPLC [Develosil C30, 1.0×25 cm; MeOH:H₂O (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–H₂O solvent system to afford **5** (16.8 mg), followed by preparative HPLC [SHISEIDO CAPCELL PAK C18, 1.0×25 cm; MeOH:H₂O (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 ($\Delta\epsilon$) 337 (+2.8), 305 (–3.5), 286 (–2.0); IR (ATR) ν_{max} 2919 (br), 1684, and 1613 cm^{-1} ; For ¹H- and ¹³C-NMR data, see Table 1; HR-ESI-MS m/z 287.0680 [$M+Na$]⁺ (Calcd for C₁₇H₁₂N₂O₃Na, 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% CO₂/95% 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 (6×10³ cells per well) in 200 μ l of RPMI medium containing 10% FBS. Cells were incubated at 37 °C in a 5% CO₂ 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 μ l of PBS containing fluorescein diacetate (10 μ g/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/*SacI* cells (2×10⁵ cells per well), a human colon cancer cell line stably transfected with the DR5 promoter-luciferase reporter plasmid pDR5/*SacI*,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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References

- 1) Ishibashi M., Ohtsuki T., *Med. Res. Rev.*, **28**, 688–714 (2008).
- 2) Zhang L., Fang B., *Cancer Gene Ther.*, **12**, 228–237 (2005).
- 3) Ahmed F., Ohtsuki T., Aida W., Ishibashi M., *J. Nat. Prod.*, **71**, 1963–1966 (2008).
- 4) Miyagawa T., Ohtsuki T., Koyano T., Kowithayakorn T., Ishibashi M., *Tetrahedron Lett.*, **50**, 4658–4662 (2009).
- 5) Miyagawa T., Ohtsuki T., Koyano T., Kowithayakorn T., Ishibashi M., *J. Nat. Prod.*, **72**, 1507–1511 (2009).
- 6) Ahmed F., Sadhu S. K., Ishibashi M., *J. Nat. Med.*, **64**, 393–401 (2010).
- 7) Ahmed F., Toume K., Sadhu S. K., Ohtsuki T., Arai M. A., Ishibashi M., *Org. Biomol. Chem.*, **8**, 3696–3703 (2010).
- 8) Hartwell J. L., *Lloydia*, **34**, 30–34 (1971).
- 9) Yadav P. P., Ahmed G., Maurya R., *Phytochemistry*, **65**, 439–443 (2004).
- 10) Carcache-Blanco E. J., Kang Y., Park E. J., Su B., Kardono L. B., Riswan S., Fong H. H., Pezzuto J. M., Kinghorn A. D., *J. Nat. Prod.*, **66**, 1197–1202 (2003).
- 11) Rao E. V., Raju N. R., *Phytochemistry*, **18**, 1581–1582 (1979).
- 12) Parmar V. S., Rathore J. S., Jain R., Henderson D. A., Malone J. F., *Phytochemistry*, **28**, 591–593 (1989).
- 13) Tanaka T., Iinuma M., Yuki K., Fujii Y., Mizuno M., *Phytochemistry*, **31**, 993–998 (1992).
- 14) Camele G., Monache F. D., Monache G. D., Bettolo G. B. M., *Phytochemistry*, **19**, 707–709 (1980).
- 15) Magalhães A. F., Tozzi A. M., Magalhães E. G., Nogueira M. A., Queiroz S. C., *Phytochemistry*, **55**, 787–792 (2000).
- 16) Gaffield W., *Tetrahedron*, **26**, 4093–4108 (1970).
- 17) Yenesew A., Midiwo J. O., Miessner M., Heydenreich M., Peter M. G., *Phytochemistry*, **48**, 1439–1443 (1998).
- 18) Shintani A., Yamazaki H., Yamamoto Y., Ahmed F., Ishibashi M., *Chem. Pharm. Bull.*, **57**, 894–895 (2009).
- 19) Larsson R., Kristensen J., Sandberg C., Nygren P., *Int. J. Cancer*, **50**, 177–185 (1992).
- 20) Horinaka M., Yoshida T., Shiraishi T., Nakata S., Wakada M., Nakanishi R., Nishino H., Matsui H., Sakai T., *Oncogene*, **24**, 7180–7189 (2005).
- 21) Kikuchi H., Ohtsuki T., Koyano T., Kowithayakorn T., Sakai T., Ishibashi M., *J. Nat. Prod.*, **70**, 1910–1914 (2007).
- 22) Yoshida T., Maeda A., Tani N., Sakai T., *FEBS Lett.*, **507**, 381–385 (2001).
- 23) Horinaka M., Yoshida T., Shiraishi T., Nakata S., Wakada M., Sakai T., *Mol. Cancer Ther.*, **5**, 945–951 (2006).
- 24) Hasegawa H., Yamada Y., Komiyama K., Hayashi M., Ishibashi M., Yoshida T., Sakai T., Koyano T., Kam T., Murata K., Sugahara K., Tsuruda K., Akamatsu N., Tsukasaki K., Masuda M., Takasu N., Kamihira S., *Blood*, **107**, 679–688 (2006).