Activity of Mangosteen Xanthones and Teleocidin A-2 in Death Receptor Expression Enhancement and Tumor Necrosis Factor Related Apoptosis-Inducing Ligand Assays[#]

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A screening study using a luciferase assay to identify natural products that enhance death receptor 5 (DR5) expression was carried out, and bioassay-guided fractionation of two organisms, the pericarp of *Garcinia mangostana* (mangosteen) and actinomycete CKK609 strain, led to the isolation of eight xanthone derivatives (**1**−**8**) and teleocidin A-2 (**9**). Among them, compounds **1**, **2**, and **5**, isolated from *G. mangostana*, and **9**, from the actinomycete, showed potent DR5 promoter activity. Furthermore, we revealed that combined treatment with gartanin (**5**) and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) showed a potentiation effect in sensitizing TRAIL-resistant human gastric adenocarcinoma (AGS) cells. Thus, the present results suggested that **5** has the ability to overcome TRAIL resistance via the up-regulation of DR5 and may be an effective sensitizer of TRAIL-resistant cells.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF superfamily, may have significant potential as an antitumor agent due to its ability to selectively trigger apoptosis in a variety of cancer cells as opposed to most normal cells.[1−5] TRAIL is known to bind to death receptors, such as DR5 (death receptor 5 = TRAIL-R2) and DR4 (death receptor 4 = TRAIL-R1), resulting in the activation of caspase-signaling pathways and leading to apoptosis.[6] However, recent studies showed that many types of cancer cells have intrinsic or acquired resistance to TRAIL-induced apoptosis, which potentially restricts its use in treatment.[7,8] Therefore, for the clinical use of TRAIL in cancer therapy, it is extremely important to overcome TRAIL resistance.

Recently, it was reported that TRAIL-resistant cancer cells can be sensitized by combined treatment with TRAIL and chemotherapeutic drugs or natural products, such as PS-341 (bortezomib),[9] tunicamycin,[10] withaferin A,[11] sodium butyrate,[12] and silibinin.[13] In many cases, overcoming TRAIL resistance involved up-regulation of the expression of death receptors, especially DR5; therefore, discovering additional compounds that enhance DR5 expression represents an important strategy to abrogate TRAIL resistance.

During a search for bioactive natural products that activate the DR5 promoter, we have isolated some promising natural products, such as a cadinane-sesquiterpene dimer[14] and flavonoids.[15,16]

In a continuing search for natural products with DR5 promoter activity from plants and actinomycetes, we found that MeOH extracts of the pericarp of *Garcinia mangostana* L. (Guttiferae) (mangosteen) and an actinomycete CKK609 strain were potently active. Therefore, bioassay-guided fractionation was performed on these two active materials. *G. mangostana* L. is a tropical tree widespread in Southeast Asia, and its extracts and xanthone constituents have demonstrated interesting biological effects, such as antioxidant, cytotoxic, antiallergic, anti-inflammatory, antiviral, and antibacterial activities.[17] Previous chemical studies of this plant have shown a variety of oxygenated and prenylated xanthones.[18]

Activity-guided isolation of two organisms, *G. mangostana* and the actinomycete CKK609 strain, led to the isolation of eight xanthones (**1**−**8**) and one alkaloid (**9**), respectively. Using a TRAIL-resistant AGS cell line, we evaluated the TRAIL resistance-overcoming activity of those compounds (**1**, **2**, **5**, and **9**), which showed the most potent DR5 promoter activity among the isolates.

The MeOH extract of the pericarp of *G. mangostana* fruits had a potent effect as a DR5 promoter (4.6-fold increase in activity at 100 μg/mL). The extract was successively partitioned between hexane, EtOAc, and *n*-BuOH, along with the aqueous layer, and DR5 promoter activity was found in the EtOAc-soluble fraction (4.5-fold at 50 μg/mL). The EtOAc-soluble fraction was subjected to column chromatography, followed by repeated reversed-phase HPLC, to give α-mangostin (**1**),[19] 1,6-dihydroxy-7-methoxy-8-(3-methylbut-2-enyl)-6′,6′-dimethylpyrano(2′,3′:3,2)xanthone (**2**),[20] β-mangostin (**3**),[21] 8-deoxygartanin (**4**),[22] gartanin (**5**),[23] 1,5,8-trihydroxy-3-methoxy-2-(3-methyl-2-butenyl)xanthone (**6**),[24] γ-mangostin (**7**),[19] and garcinone D (**8**).[25]

The fermentation broth of the actinomycete strain CKK609 exhibited a potent effect as a DR5 promoter (4.0-fold increase in activity at 500 μg/mL). It was centrifuged and the mycelium was extracted with MeOH for 1 day. The extracts were filtered, concentrated in vacuo, and partitioned with EtOAc, and DR5 promoter activity was concentrated in the EtOAc-soluble fraction (4.5-fold at 50 μg/mL). The EtOAc fraction was subjected to column chromatography, followed by repeated reversed-phase HPLC, to give teleocidin A-2 (**9**).[26]

The isolated compounds (**1**−**9**) were tested for DR5 promoter activity using a luciferase assay in DLD-1/*Sac*I cells, and luteolin,[27,28] which has been previously reported to increase DR5 expression, was used as a positive control at 17.5 μM. As shown in Figure 2, compounds **1**, **2**, **5**, and **9** increased DR5 promoter activity by 3.8-, 4.0-, 3.1-, and 4.7-fold, respectively, as compared with control cells at the indicated concentrations. Although it is known that some natural products or synthetic small molecules show enhancement of DR5 expression, this is the first report in which xanthones and teleocidins (or lyngbiatoxins[29]) showed enhancement of DR5 expression.

Compounds **1**, **2**, **5**, and **9** were next investigated in their ability to overcome TRAIL resistance in AGS cells. Recently, AGS human gastrointestinal tract cancer cells have been reported to be refractory to apoptosis induction by TRAIL.[30,31] To assess the possible combination effect of each of these compounds and TRAIL on cell

[#] Dedicated to the late Dr. Richard E. Moore of the University of Hawaii at Manoa for his pioneering work on bioactive natural products.

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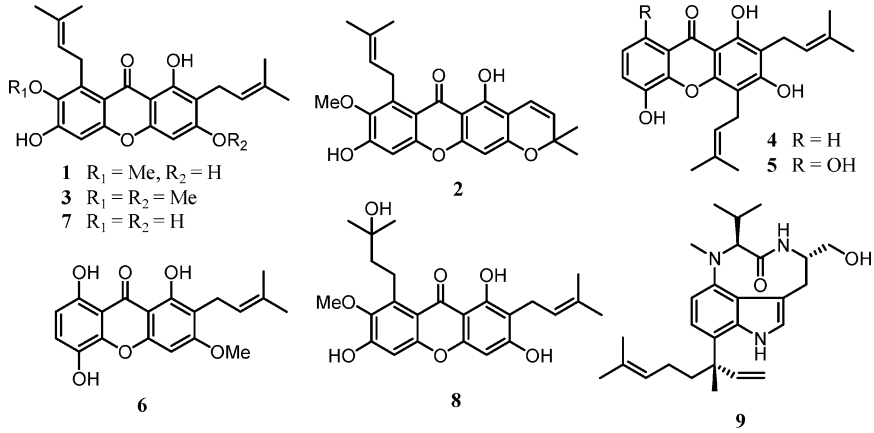

Figure 1. Structures of compounds isolated from *Garcinia mangostana* (**1**−**8**) and an actinomycete CKK609 strain (**9**).

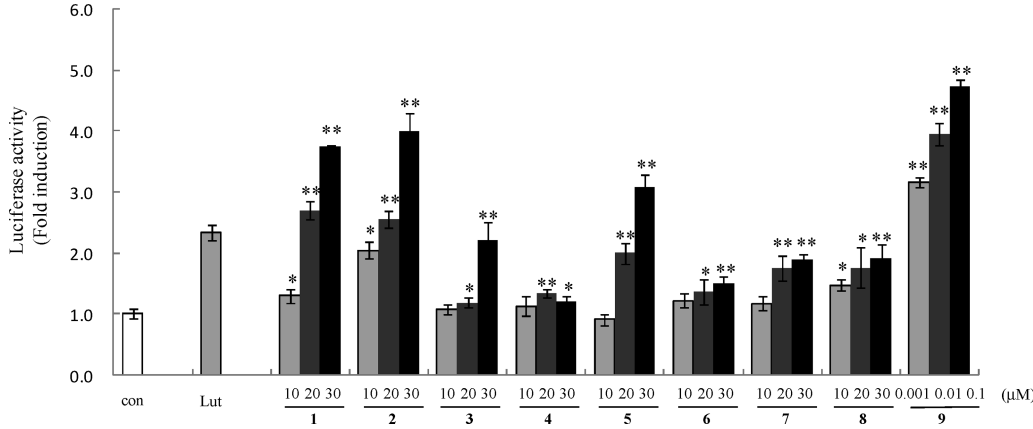

Figure 2. Activation of the DR5 promoter by compounds **1**−**9**, luteolin (positive control: Lut), and DMSO (negative control: Con) in DLD-1/*Sac*I cells. Samples were tested at 10.0 to 30.0 μM for xanthones (**1**−**8**), 0.001 to 0.1 μM for teleocidin A-2 (**9**), and 17.5 μM for luteolin. Each value represents the mean ± SD (n = 3), with significance determined with the Student's t-test (*p < 0.05, **p < 0.01 vs control).

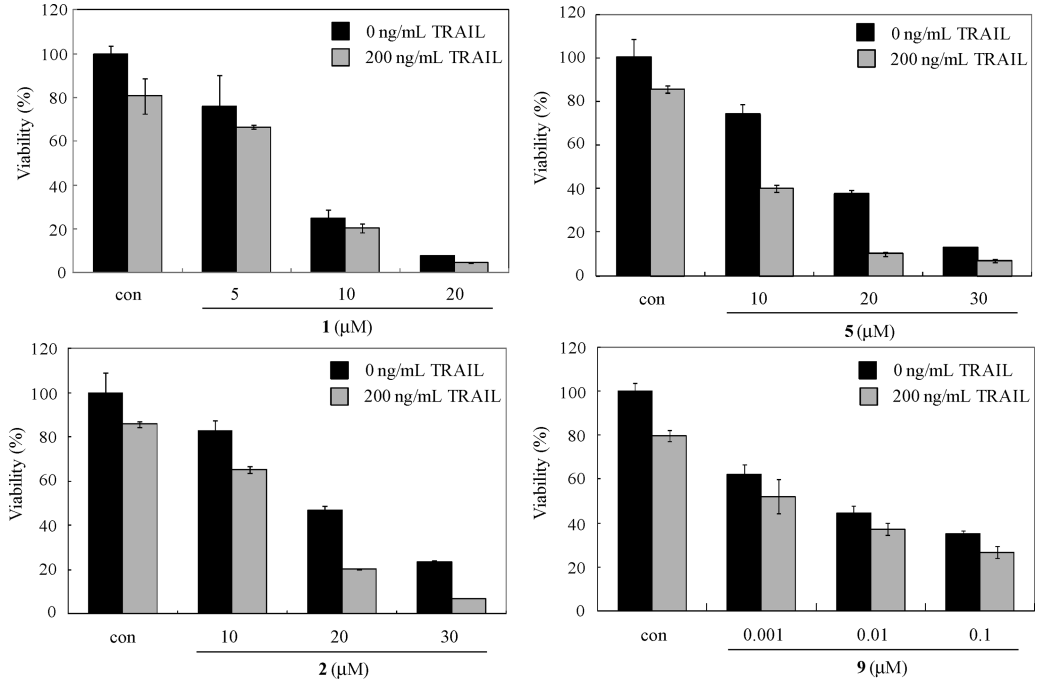

Figure 3. Viability of TRAIL-resistant AGS cells incubated with compounds **1**, **2**, **5**, and **9** and/or TRAIL. AGS cells were treated at the indicated concentration of test samples and/or 200 ng/mL of TRAIL for 24 h. Cell viability was determined after 24 h by a FMCA method. The bars represent means ± SD (n = 3).

viability, AGS cells were treated with the isolates alone, TRAIL alone (200 ng/mL), and a combination treatment with TRAIL for 24 h, and subjected to a FMCA procedure.[32] As shown in Figure 3, the combination of **5** and TRAIL exhibited 34% and 27%

decreases in cell viability at 10 and 20 μM when compared with that in the absence of TRAIL, which showed that this compound together with TRAIL has a potentiation effect on AGS cells. Compound **2** also showed appreciable activity. However, combined treatment of compounds **1** and **9** with TRAIL did not show any change in cell viability compared to the TRAIL treatment alone.

Teleocidins are well known as protein kinase C (PKC) activators, and PKC plays crucial roles in the signal transduction pathways affecting physiological activities. Recently, it was reported that rottlerin, which is known as a specific inhibitor of PKC δ, induces apoptosis via DR5 up-regulation, which has a PKC δ-independent mechanism.[33] To clarify the underlying mechanism of DR5 expression enhancement activity by teleocidin A-2 (**9**), further studies are needed.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO P-1020 polarimeter. The NMR spectra were recorded on JEOL JNM ECP 600 spectrometers with a deuterated solvent, the chemical shift of which was used as an internal standard. EIMS was measured on a JEOL GC-Mate spectrophotometer and high-resolution fast-atom bombardment mass spectra (HRFABMS) on a JEOL HX-110A spectrometer.

Plant Material. The pericarp of *G. mangostana* fruits was collected in Khon Kaen, Thailand, in August 2006 and identified by one of the authors (T. Kowithayakorn). A voucher specimen (7-110) is maintained in our laboratory.

Microorganism. Spores of the CKK609 strain collected form a soil sample in Chiba, Japan, in June 2007, grown on solid Waksman medium, were inoculated into a 500 mL Sakaguchi flask containing 100 mL of liquid medium and cultured for 4 days at 28 °C with reciprocal shaking at 200 rpm to produce seed culture. A 10 mL sample of the seed culture was transferred into a 5 L flask containing 500 mL of the same medium and cultured for 5 days at 28 °C with reciprocal shaking at 100 rpm to obtain 32 L of fermentation broth.

Extraction and Isolation. The pericarp of *G. mangostana* (31.8 g) was extracted with MeOH at room temperature. The MeOH extract (9.5 g) was suspended in 10:90 MeOH−H_2O (300 mL) and partitioned with hexane (300 mL × 3), EtOAc (300 mL × 3), and *n*-BuOH (300 mL × 3). The EtOAc-soluble fraction (5.3 g) was subjected to silica gel flash column chromatography (25 × 250 mm) and eluted successively with hexane−acetone (from 20% to 100% acetone) to give seven fractions (1A−1G). Fraction 1B (30.3 mg), eluted with 70% hexane in acetone, was subjected to reversed-phase HPLC (YMC-Pack ODS-AM, 250 × 10 mm; flow rate, 2.0 mL/min) with 100% MeOH to give two fractions (2A, 2B). Fraction 2B (8.0 mg) was purified by reversed-phase HPLC (Chromatorex ODS, 250 × 4.6 mm; flow rate, 1.1 mL/min) with 84% MeOH to afford compounds **2** (4.1 mg, t_R 35 min) and **3** (1.8 mg, t_R 46 min). Fraction 1C (129.7 mg), eluted with 60% hexane in acetone, was applied to reversed-phase HPLC (YMC-Pack ODS-AM, 250 × 10 mm; flow rate, 2.0 mL/min) with 92% MeOH, to yield fractions 3A and 3B and compound **3** (9.3 mg, t_R 45 min). Fraction 3B was further purified by reversed-phase HPLC (YMC-Pack ODS-AM, 250 × 10 mm; flow rate, 2.0 mL/min), with 90% MeOH, to yield compounds **4** (5.6 mg, t_R 17 min), **5** (8.9 mg, t_R 22 min), and **6** (1.5 mg, t_R 24 min). Compound **1** (34.4 mg, t_R 30 min) was obtained by separating fraction 1D (857.3 mg) using reversed-phase HPLC (YMC-Pack ODS-AM, 250 × 10 mm; flow rate, 2.0 mL/min) with 90% MeOH. Fraction 1F (328.4 mg), eluted with 20% hexane in acetone, was subjected to reversed-phase HPLC (YMC-Pack ODS-AM, 250 × 10 mm; flow rate, 2.0 mL/min) with 85% MeOH to give compounds **7** (1.3 mg, t_R 18 min) and **8** (21.9 mg, t_R 24 min).

The cultured broth (32 L) was centrifuged at 3000 rpm for 10 min to separate the mycelium and supernatant. The mycelium was extracted with MeOH for 1 day. The extracts were filtered and concentrated in vacuo and then partitioned with EtOAc (1 L × 3). The EtOAc-soluble fraction (11.8 g) was subjected to silica gel flash column chromatography (25 × 250 mm) and successively eluted with $CHCl_3$−MeOH (from 0% to 100% MeOH) to give nine fractions (1A−1I). Fraction 1E (2.0 g), eluted with 94% $CHCl_3$ in MeOH, was subjected to ODS flash column chromatography (5 × 250 mm) and successively eluted with MeOH−H_2O (from 70% to 100% MeOH) to give 10 fractions (2A−2K). Fraction 2E (18.3 mg) was purified by reversed-phase HPLC (Chromatorex ODS, 250 × 4.6 mm; flow rate, 1.0 mL/min) with 80% MeOH to afford compound **9** (6.0 mg, t_R 35 min).

Cell Cultures. AGS cells were obtained from the Institute of Development, Aging and Cancer, Tohoku University. DLD-1/*SacI* cells were constructed by T.S., one of our group. Both cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37 °C under an atmosphere of 5% CO_2.

Luciferase Assay to Assess DR5 Promoter Activation. The experimental details of this assay system were described in a previous report.[15]

Cell Viability. To assess the sensitivity to TRAIL, cell viability was evaluated using a fluorometric microculture cytotoxicity assay (FMCA).[31] Briefly, AGS cells were seeded in a 96-well plate (6×10^3 cells per well) in 200 μL of RPMI medium containing 10% FBS. Cells were cultured in 24 h and then treated with test samples in the absence or presence of TRAIL (200 ng/mL), and control cells were treated with culture medium containing 0.1% DMSO. After 24 h incubation, the cells were washed with PBS, and 200 μL of PBS containing fluorescein diacetate (10 μg/mL) was added to each well. After 1 h incubation at 37 °C, fluorescence was measured in a 96-well scanning spectrofluorometer (Thermo Electron Corporation) at 538 nm, following excitation at 485 nm.

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References and Notes

(1) Ishibashi, M.; Ohtsuki, T. *Med. Res. Rev.* **2008**, *28*, 688–714.
(2) Wiley, S. R.; Schooley, K.; Smolak, P. J.; Din, W. S.; Huang, C. P.; Nicholl, J. K.; Sutherland, G. R.; Smith, T. D.; Rauch, C.; Smith, C. A.; Goodwin, R. G. *Immunity* **1995**, *3*, 673–682.
(3) Pitti, R. M.; Marsters, S. A.; Ruppert, S.; Donahue, C. J.; Moore, A.; Ashkenazi, A. *J. Biol. Chem.* **1996**, *271*, 12687–12690.
(4) Walczak, H.; Miller, R. E.; Ariail, K.; Gliniak, B.; Griffith, T. S.; Kubin, M.; Chin, W.; Jones, J.; Woodward, A.; Le, T.; Smith, C.; Smolak, P.; Goodwin, R. G.; Rauch, C. T.; Schuh, J. C.; Lynch, D. H. *Nat. Med.* **1999**, *5*, 157–163.
(5) Ashkenazi, A.; Pai, R. C.; Fong, S.; Leung, S.; Lawrence, D. A.; Marsters, S. A.; Blackie, C.; Chang, L.; McMurtrey, A. E.; Hebert, A.; DeForge, L.; Koumenis, I. L.; Lewis, D.; Harris, L.; Bussiere, J.; Koeppen, H.; Shahrokh, Z.; Schwall, R. H. *J. Clin. Invest.* **1999**, *104*, 155–162.
(6) LeBlanc, H. N.; Ashkenazi, A. *Cell Death Differ.* **2003**, *10*, 66–75.
(7) Zhang, L.; Fang, B. *Cancer Gene Ther.* **2005**, *12*, 228–237.
(8) Lee, T.; Lee, J.; Park, J.; Kwon, T. *Biochem. Biophys. Res. Commun.* **2006**, *351*, 1024–1030.
(9) Liu, X.; Yue, P.; Chen, S.; Hu, L.; Lonial, S.; Khuri, F.; Sun, S. *Cancer Res.* **2007**, *67*, 4981–4988.
(10) Shiraishi, T.; Yoshida, T.; Nakata, S.; Horinaka, M.; Wakada, M.; Mizutani, Y.; Miki, T.; Sakai, T. *Cancer Res.* **2005**, *65*, 6364–6370.
(11) Lee, T. J.; Um, H. J.; Min, D. S.; Parl, J. W.; Choi, K. S.; Kwon, T. K. *Free Radical Biol. Med.* **2009**, *46*, 1639–1649.
(12) Kim, Y.; Park, J.; Lee, J.; Kwon, T. *Carcinogenesis* **2004**, *25*, 1813–1820.
(13) Son, Y.; Kim, E.; Kim, J.; Kim, S.; Kwon, T.; Yoon, A.; Yon, C.; Choi, K. *Cancer Res.* **2007**, *67*, 8274–8284.
(14) Ohtsuki, T.; Tamaki, M.; Toume, K.; Ishibashi, M. *Bioorg. Med. Chem.* **2008**, *16*, 1756–1763.
(15) (a) Kikuchi, H.; Ohtsuki, T.; Koyano, T.; Kowithayakorn, T.; Sakai, T.; Ishibashi, M. *J. Nat. Prod.* **2007**, *70*, 1910–1914. (b) Kikuchi, H.; Ohtsuki, T.; Koyano, T.; Kowithayakorn, T.; Sakai, T.; Ishibashi, M. *Bioorg. Med. Chem.* **2009**, *17*, 1181–1186.
(16) Ohtsuki, T.; Hiraka, T.; Kikuchi, H.; Koyano, T.; Kowithayakorn, T.; Sakai, T.; Ishibashi, M. *Heterocycles* **2009**, *77*, 1379–1388.
(17) Pedraza-Chaverri, J.; Cardenas-Rodriguez, N.; Orozco-Ibarra, M.; Perez-Rojas, J. *Food Chem. Toxicol.* **2008**, *46*, 3227–3239.
(18) Matsumoto, K.; Akao, Y.; Kobayashi, E.; Ohguchi, K.; Ito, T.; Tanaka, T.; Iinuma, M.; Nozawa, Y. *J. Nat. Prod.* **2003**, *66*, 1124–1127.
(19) Mahabusarkam, W.; Wiriyachitra, P. *J. Nat. Prod.* **1987**, *50*, 474–478.
(20) San, A. K.; Sarkar, K. K.; Mazumder, P. C.; Banerji, N.; Uusvuori, R.; Hase, T. A. *Phytochemistry* **1980**, *19*, 2223–2225.

(21) Likhitwitayawuid, K.; Phadungcharoen, T.; Krungkrai, J. *Planta Med.* **1998**, *64*, 70–72.
(22) Nguyen, L. D.; Vo, H. T.; Pham, H. D.; Connolly, J. D.; Harrison, L. D. *Phytochemistry* **2003**, *63*, 467–470.
(23) Bennett, G. J.; Lee, H. H.; Lee, L. P. *J. Nat. Prod.* **1990**, *53*, 1463–1470.
(24) Parveen, M.; Khan, N. U. *Phytochemistry* **1998**, *27*, 3694–3696.
(25) Bennett, G. J.; Harrison, L. J.; Sia, G. L.; Sim, K. Y. *Phytochemistry* **1993**, *32*, 1245–1251.
(26) Sakai, S.; Hitotsuyanagi, Y.; Aimi, N.; Fujiki, H.; Suganuma, M.; Sugimura, T.; Endo, Y.; Shudo, K. *Tetrahedron Lett.* **1986**, *27*, 5219–5220.
(27) Horinaka, M.; Yoshida, T.; Shiraishi, T.; Nakata, S.; Wakada, M.; Nakanishi, R.; Nishino, H.; Matsui, H.; Sakai, T. *Oncogene* **2005**, *24*, 7180–7189.
(28) Horinaka, M.; Yoshida, T.; Shiraishi, T.; Nakata, S.; Wakada, M.; Nakanishi, R.; Nishino, H.; Sakai, T. *Biochem. Biophys. Res. Commun.* **2005**, *333*, 833–838.
(29) Cardellina, J. H., II; Marner, F.-J.; Moore, R. E. *Science* **1979**, *204*, 193–195.
(30) Jin, C.; Park, C.; Cheong, J.; Choi, B.; Lee, T.; Lee, J.; Lee, W.; Kim, G.; Ryu, C.; Choi, Y. *Cancer Lett.* **2007**, *257*, 56–64.
(31) Srivastava, R. *Neoplasia* **2001**, *3*, 535–546.
(32) Ohtsuki, T.; Sato, M.; Koyano, T.; Kowithayakorn, T.; Kawahara, N.; Goda, Y.; Ishibashi, M. *Bioorg. Med. Chem.* **2006**, *14*, 659–665.
(33) Lim, J.; Park, J.; Choi, K.; Park, Y.; Kwon, T. *Carcinogenesis* **2009**, *30*, 729–736.

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