Notes

Cardenolide Glycosides of *Thevetia peruviana* and Triterpenoid Saponins of *Sapindus emarginatus* as TRAIL Resistance-Overcoming Compounds

Takashi Miyagawa,[†] Takashi Ohtsuki,[†] Takashi Koyano,[‡] Thaworn Kowithayakorn,[§] and Masami Ishibashi*[,†]

Graduate School of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan, Temko Corporation, 4-27-4 Honcho, Nakano, Tokyo 164-0012, Japan, and Faculty of Agriculture, Khon Kaen University, Khon Kaen 40002, Thailand

Received March 30, 2009

A screening study for TRAIL resistance-overcoming activity was carried out, and activity-guided fractionations of *Thevetia peruviana* and *Sapindus emarginatus* led to the isolation of four cardenolide glycosides (**1**−**4**) and four triterpenoid saponins (**5**−**8**), respectively. In particular, cardenolide glycosides (**1** and **2**) from *T. peruviana* were shown to have a significant reversal effect on TRAIL resistance in human gastric adenocarcinoma cells, and real-time PCR showed that thevefolin (**2**) enhanced mRNA expression of death receptor 4 (DR4) and DR5. In addition, ^{1}H and ^{13}C NMR characterizations are shown for thevefolin (**2**) for the first time.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF superfamily, is a promising agent for new anticancer therapy, since it triggers apoptosis in a variety of cancer cells but not in many normal cells.[1] TRAIL is a death ligand and is known to bind to death receptors such as DR5 (death receptor 5 = TRAIL-R2) or DR4 (death receptor 4 = TRAIL-R1), resulting in the activation of caspase-signaling pathways leading to apoptosis. However, it has become a problem that considerable numbers of cancer cells, especially some highly malignant tumors, are resistant to apoptosis induction by TRAIL. A search for compounds capable of abrogating TRAIL resistance has thus become an important protocol for anticancer drug discovery.[2] During our search for bioactive natural products from various natural resources,[3] we recently identified several natural products that exhibited activities related to TRAIL signaling, such as a cadinane-sesquiterpene dimer with DR5 expression enhancement activity[4] and new isoflavones with TRAIL-mediated ability of apoptosis induction.[5] In our screening program for TRAIL resistance-overcoming substances from natural resources,[6] the plants *Thevetia peruviana* and *Sapindus emarginatus* were selected for further studies of their active constituents. A number of cardiac and flavonol glycosides having unique bioactivity such as HIV-1 reverse transcriptase have been isolated from *T. peruviana*,[7] while several triterpene saponins and flavonoids are known from *S. emarginatus*.[8] TRAIL resistance-overcoming activity of the extracts of these two plants was assessed by comparing cell viability in the presence and absence of TRAIL against TRAIL-resistant human gastric adenocarcinoma (AGS) cell lines. Bioassay-guided fractionation of the extracts led to the isolation of four cardenolide glycosides (**1**−**4**) and four triterpenoid saponins (**5**−**8**), respectively. Here, we report the isolation, structure identification, and the TRAIL resistance-overcoming activity of the isolated compounds.

The MeOH extract of the bark of *T. peruviana* (Apocynaceae) was partitioned successively with *n*-hexane, EtOAc, *n*-BuOH, and H_2O. The EtOAc layer, which showed the most potent activity (39% decrease in cell viability in the presence of TRAIL compared with that in the absence of TRAIL at 2.1 μg/mL), was subjected to silica gel column chromatography, followed by Sephadex LH-20 and ODS-HPLC, to yield four cardenolide glycosides, neriifolin (**1**),[9] thevefolin (**2**),[10] peruvoside (**3**),[11] and (20*S*)-18,20-epoxydigitoxigenin α-L-thvetoside (**4**).[12] ^{1}H and ^{13}C NMR data of compounds **1**,[9] **3**,[11] and **4**[13] are available in the literature, and comparison of these data confirmed the structures of the compounds. On the other hand, ^{1}H and ^{13}C NMR data of compound **2** were not sufficiently available in the literature;[10] however, the structure of compound **2** [thevefolin[10] = 14-hydroxy-3β-(α-L-thevetopyranosyl)-5α-card-20(22)-enolide] could be identified by comparison of the ^{1}H and ^{13}C NMR data of **2** with those of the aglycone (= uzarigenin) and sugar (= α-thevetopyranose) parts. ^{1}H and ^{13}C NMR chemical shifts of the aglycone of **2** were consistent with those of the aglycone of uzarigenin β-D-diginoside[13] and 5α-cardenolides,[14] while those of the sugar were identical to those of the thevetosyl moiety of compounds **1**, **3**, and **4**.[9,11,12] 2D NMR experiments including HMQC and HMBC spectra of compound **2** provided further structural support of compound **2**. The ^{1}H and ^{13}C NMR chemical shift data of **2** are summarized in the Experimental Section, and these data have not been previously described.

The MeOH extract of fruits of *S. emarginatus* (Sapindaceae) was partitioned successively with *n*-hexane, EtOAc, *n*-BuOH, and H_2O. The most active EtOAc layer (40% decrease in cell viability in the presence of TRAIL compared with that in the absence of TRAIL at 50 μg/mL) was subjected to silica gel column chromatography, followed by purification with ODS-HPLC to afford four triterpenoid saponins, hederagenin 3-*O*-(2-*O*-acetyl-β-D-xylopyranosyl)-(l→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (**5**),[15] hederagenin 3-*O*-(3,4-di-*O*-acetyl-β-D-xylopyranosyl)-(l→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (**6**),[16] hederagenin 3-*O*-(2,4-di-*O*-acetyl-β-D-xylopyranosyl)-(l→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (**7** = sapinmusaponin N),[17] and hederagenin 3-*O*-(4-*O*-acetyl-β-D-xylopyranosyl)-(l→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (**8**).[16] Identification of these known triterpenoid saponins was confirmed by comparing ^{1}H and ^{13}C NMR data with literature data.[15−17]

Compounds **1**−**8** were evaluated for their activity in overcoming TRAIL resistance in AGS cells through cell viability tests using FMCA methods,[18] and the assay results are summarized in Figure 1. Luteolin was used as a positive control, which produced about

* To whom correspondence should be addressed. Tel: +81-43-290-2913. Fax: +81-43-290-2913. E-mail: mish@p.chiba-u.ac.jp.
[†] Chiba University.
[‡] Temko Corporation.
[§] Khon Kaen University.

10.1021/np900202n CCC: $40.75
Published on Web 07/13/2009

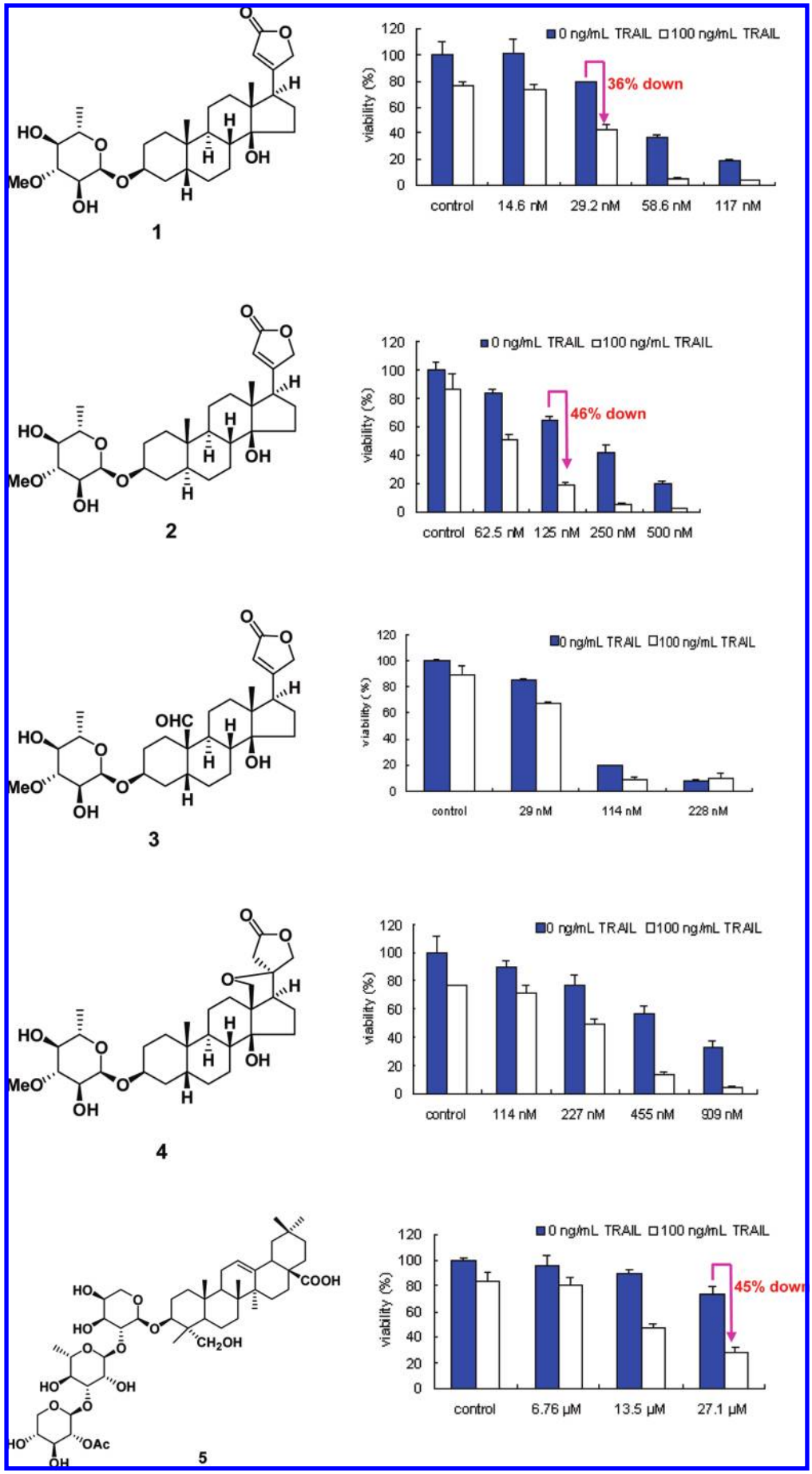

Figure 1. Continued.

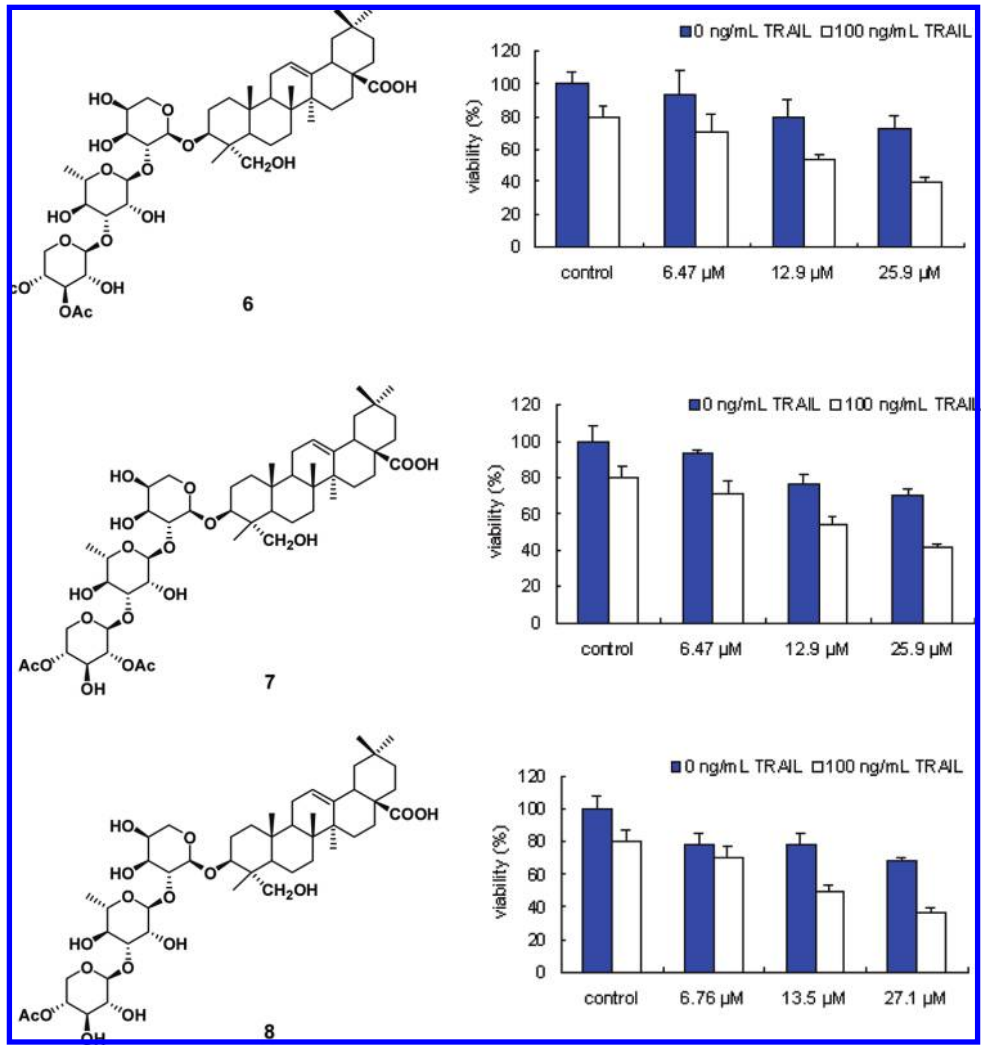

Figure 1. Effect of compounds **1**−**8** on the cell viability of AGS cells in the presence and absence of TRAIL.

70% more inhibition along with TRAIL than the TRAIL alone.[6,19] Among these compounds, cardenolide glycosides neriifolin (**1**) and thevefolin (**2**) exhibited significant TRAIL resistance-overcoming activity at submicromolar concentrations. Compounds **1** (29.2 nM) and **2** (125 nM) showed 36% and 46% decreases, respectively, in cell viability in the presence of TRAIL (100 ng/mL) compared with in the absence of TRAIL, and at the same concentration, **1** or **2** alone still showed high cell viability (79% and 65%, respectively), implying that these compounds (**1** and **2**) had a synergistic effect in combination with TRAIL against AGS cells. Triterpenoid saponins **5**−**8** also exhibited appreciable TRAIL resistance-overcoming activity. Particularly, treatment with compound **5** (27.1 μM) and TRAIL decreased cell viability 45% more than with compound **5** alone. It was revealed that cardenolide glycosides **1**−**4** showed a synergistic effect at lower concentrations than triterpenoid saponins **5**−**8**.

The resistance of cancer cells to TRAIL may occur at different points in the TRAIL-mediated apoptotic pathways including, for example, the increase of apoptosis inhibitors or decrease of apoptosis inducers or death receptors. Thus, up-regulation of the expression of death receptors, such as DR4 or DR5, may be one of the mechanisms of overcoming TRAIL resistance. Known cardenolides, such as oleandrin or digitoxin, were found to exhibit TRAIL resistance-overcoming activity through enhancement of the expression of DR4 and DR5.[20] We examined the effect of thevefolin (**2**) on the mRNA expression of death receptors DR4 and DR5 by real-time PCR analysis. Thevefolin (**2**) actually increased the mRNA expression of DR4 and DR5 in a dose-dependent manner, as shown in Figure 2.

In conclusion, we investigated a screening program targeting TRAIL resistance-overcoming activity since compounds capable of abrogating TRAIL resistance have become an important protocol for anticancer therapy. As a result of our screening study on extracts of various natural resources, we selected two plant extracts, *T. peruviana* and *S. emarginatus*, as active samples and isolated cardenolide glycosides from *T. peruviana* and triterpenoid saponins from *S. emarginatus* through activity-guided fractionations. The isolated compounds have synergistic activity in sensitizing TRAIL-resistant AGS cells, and the mechanism of the TRAIL resistance abrogating activity of thevefolin (**2**) was suggested through increase of expression of death receptors, thereby suggesting their possible use in combination with TRAIL against human gastric adenocarcinoma.

Experimental Section

General Experimental Procedures. NMR spectra were recorded on a JEOL JNM ECP600 spectrometer with a deuterated solvent ($CDCl_3$ and CD_3OD) whose chemical shift was taken as an internal standard.

Plant Materials. *T. peruviana* and *S. emarginatus* plants were collected in Khon Kaen, Thailand, and were identified by T. Kowithayakorn. Voucher specimens (*T. peruviana*, 7-213; *S. emarginatus*, 7-150) have been deposited in our laboratory.

Extraction and Isolation from *T. peruviana.* Air-dried bark (400 g) was extracted with MeOH. The combined extract (35.5 g) suspended in 10:90 MeOH/H_2O (300 mL) was partitioned with *n*-hexane (300

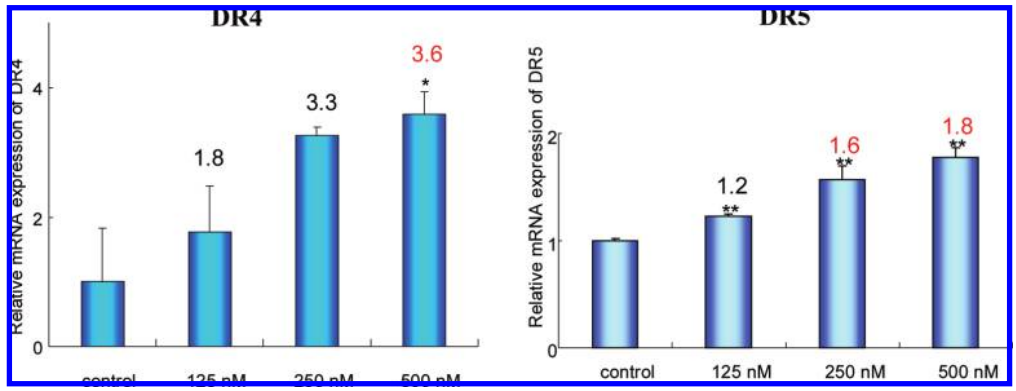

Figure 2. Effect of compound **2** on mRNA expression of DR4 and DR5 in AGS cells by real-time RT-PCR. The significance of differences was determined with Student's *t*-test (**<0.01, *<0.05 vs control.)

mL × 3), EtOAc (300 mL × 3), and *n*-BuOH (300 mL × 3). The EtOAc-soluble fraction (2.1 g) was subjected to silica gel column chromatography (3.2 × 20 cm) using a gradient of increasing MeOH (0−100%) in $CHCl_3$. The fraction (0.15 g) eluted with 10:90 MeOH/$CHCl_3$ was further separated by Sephadex LH-20 (1.7 × 55 cm) eluted with MeOH, followed by reversed-phase HPLC with 65:35 MeOH/H_2O (Inertsil ODS-3, 10 × 250 mm; flow rate, 2.5 mL/min; UV detection at 230 nm) to give compounds **1** (2.1 mg, t_R = 32 min), **2** (1.5 mg, t_R = 35 min), **3** (0.9 mg, t_R = 16 min), and **4** (1.2 mg, t_R = 20 min).

^{1}H and ^{13}C NMR Data of Compound 2: ^{1}H NMR ($CDCl_3$) δ_H 1.73 (1H, dt, *J* = 13.6 and 3.6 Hz; H-1a), 0.98 (1H, m; H-1b), 1.83 (1H, m; H-2a), 1.50 (1H, m; H-2b), 3.53 (1H, m; H-3), 1.64 (1H, m; H-4a), 1.23 (1H, m; H-4b), 1.05 (1H, m; H-5), 1.36 (1H, m; H-6a), 1.23 (1H, m; H-6b), 1.91 (1H, dd, *J* = 12.8 and 3.6 Hz; H-7a), 1.05 (1H, m; H-7b), 1.50 (1H, m; H-8), 0.90 (1H, m; H-9), 1.50 (1H, m; H-11a), 1.23 (1H, m; H-11b), 1.50 (1H, m; H-12 a), 1.36 (1H, m; H-12b), 2.08 (1H, m; H-15a), 1.64 (1H, m; H-15b), 2.08 (1H, m; H-16a), 1.83 (1H, m; H-16b), 2.76 (1H, m; H-17), 0.86 (3H, s; H_3−18), 0.79 (3H, s; H_3−19), 4.96 (1H, dd, *J* = 18.0 and 1.6 Hz; H-21a), 4.78 (1H, dd, *J* = 18.0 and 2.0 Hz; H-21b), 5.85 (1H, br s; H-22), 4.88 (1H, d, *J* = 4.4 Hz; H-1′), 3.53 (1H, dd, *J* = 8.8 and 4.4 Hz; H-2′), 3.22 (1H, t, *J* = 8.8 Hz; H-3′), 3.11 (1H, t, *J* = 8.8 Hz; H-4′), 3.74 (1H, dq, *J* = 9.2 and 6.4 Hz; H-5′), 1.23 (3H, d, *J* = 6.4 Hz; H_3-6′), and 3.65 (3H, s; MeO-3′); ^{13}C NMR ($CDCl_3$) δ_C 37.1 (C-1), 29.3 (C-2), 77.2 (C-3), 34.2 (C-4), 44.2 (C-5), 28.5 (C-6), 27.3 (C-7), 41.6 (C-8), 49.7 (C-9), 35.8 (C-10), 21.1 (C-11), 39.8 (C-12), 49.5 (C-13), 85.5 (C-14), 33.1 (C-15), 26.8 (C-16), 50.8 (C-17), 15.7 (C-18), 12.2 (C-19), 174.4 (C-20), 73.4 (C-21), 117.7 (C-22), 174.4 (C-23), 97.0 (C-1′), 72.8 (C-2′), 84.4 (C-3′), 74.9 (C-4′), 67.3 (C-5′), 17.5 (C-6′), and 60.8 (MeO-3′).

Extraction and Isolation from *S. emarginatus*. Air-dried fruit (40 g) was extracted with MeOH. The combined extract (16.6 g) suspended in 10:90 MeOH/H_2O (200 mL) was partitioned with *n*-hexane (200 mL × 3), EtOAc (200 mL × 3), and *n*-BuOH (200 mL × 3). The EtOAc-soluble fraction (3.6 g) was subjected to silica gel column chromatography (column A, 2.3 × 550 cm) using a gradient of increasing MeOH (0−100%) in $CHCl_3$. A fraction (0.33 g) of column A eluted with 17:83 MeOH/$CHCl_3$ was further separated by ODS open column chromatography (1.7 × 60 cm) using a gradient of increasing MeOH (29−100%) in H_2O, followed by Diol-HPLC [Inertsil Diol, 6.0 × 250 mm; flow rate, 0.9 mL/min; RI detection; eluant, 90:10:1 $CHCl_3$/MeOH/H_2O] to give compound **5** (4.9 mg, t_R = 18 min). A fraction (0.14 g) of column A eluted with 9:91 MeOH/$CHCl_3$ was purified by reversed-phase HPLC [YMC-Pack C18 Pro, 10 × 250 mm; flow rate, 1.5 mL/min; UV detection at 210 nm; eluant, 55:45 CH_3CN/H_2O] to give compound **6** (9.4 mg, t_R = 32 min). A fraction (0.13 g) of column A eluted with 10% MeOH in $CHCl_3$ was purified by reversed-phase HPLC [YMC-Pack C18 Pro, 10 × 250 mm; flow rate, 1.8 mL/min; UV detection at 210 nm; eluant, 45:55 CH_3CN/H_2O] to give compound **7** (13.5 mg, t_R = 72 min). Another fraction (0.19 g) of column A eluted with 10:90 MeOH/$CHCl_3$ was purified by reversed-phase HPLC [Develosil C30-UG-5, 10 × 250 mm; flow rate, 1.3 mL/min; UV detection at 210 nm; eluant, 50:50 CH_3CN/H_2O] to give compound **8** (6.1 mg, t_R = 48 min).

Fluorometric Microculture Cytotoxicity Assay (FMCA).[19] AGS cells were seeded in a 96-well culture plate (6 × 103 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, and then the test samples with or without TRAIL (100 ng/mL) at different concentrations were added to each well. 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. The plates were then incubated at 37 °C for 1 h, and fluorescence was measured in a 96-well scanning spectrofluorometer at 538 nm, following excitation at 485 nm.

Real-Time RT-PCR Analysis.[4] RNA samples, obtained from cultured AGS cells treated with three concentrations (125, 250, and 500 μM) of compound **2**, were reverse transcribed using RT-PCR SuperScript III Platinum Two-step qRT-PCR Kit (Invitrogen) and TaKaRa PCR Thermal Cycler Dice (TaKaRa). The template cDNA thus obtained was incubated with 200 nM gene-specific primers (Fasmac) and a Platinum SYBR Green SuperMix-UDG (Invitrogen) in a Mx3000 QPCR System (Stratagene). The thermal cycling program was initial incubation (50 °C for 2 min), initial denaturation (95 °C for 2 min), and 40 cycles of denaturation (95 °C for 15 s), annealing, and extension (60 °C for 30 s). The primer sets were as follows: glyceraldehydes-3-phosphate dehydrogenase (GAPDH), 5′-ATGGGGAAGGTGAAGGTCG-3′ and 5′-TAAAAGCAGCCCTGGTGACC-3′; DR5 (TRAIL-R2), 5′-GAGCTAAGTCCCTGCACCAC-3′ and 5′-AATCACCGACCTTGACCATC-3′; DR4 (TRAIL-R1), 5′-CCGCGGCCACACCCAGAAAGT-3′ and 5′-GTACATGGGAGGCAAGCAAACAAA-3′. A fluorescence signal was collected at the end of each cycle. After the reactions were terminated, the signal at each temperature, from 60 to 95 °C, was also collected for dissociation curve analysis. All reactions were performed in triplicate to confirm reproducibility, and the amount of target mRNA in each sample was normalized with that of mean GAPDH, an endogenous control.

Acknowledgment. This work was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan and by a Grant-in-Aid from the Japan Science and Technology Agency Innovation Branch Chiba (JST).

References and Notes

(1) Johnstone, R. W.; Frew, A. J.; Smyth, M. J. *Nat. Rev. Cancer* **2008**, *8*, 782–798.
(2) Ishibashi, M.; Ohtsuki, T. *Med. Res. Rev.* **2008**, *28*, 688–714.
(3) (a) Arai, M. A.; Tateno, C.; Hosoya, T.; Koyano, T.; Kowithayakorn, T.; Ishibashi, M. *Bioorg. Med. Chem.* **2008**, *16*, 9420–9424. (b) Aida, W.; Ohtsuki, T.; Li, X.; Ishibashi, M. *Tetrahedron* **2009**, *65*, 369–373. (c) Li, X.; Ohtsuki, T.; Koyano, T.; Kowithayakorn, T.; Ishibashi, M. *Chem. Asian J.* **2009**, *4*, 540–547.
(4) Ohtsuki, T.; Tamaki, M.; Toume, K.; Ishibashi, M. *Bioorg. Med. Chem.* **2008**, *16*, 1756–1763.
(5) (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.

(6) Ahmed, F.; Ohtsuki, T.; Aida, W.; Ishibashi, M. *J. Nat. Prod.* **2008**, *71*, 1963–1966.
(7) (a) Tewtrakul, S.; Nakamura, N.; Hattori, M.; Fujiwara, T.; Supavita, T. *Chem. Pharm. Bull.* **2002**, *50*, 630–635. (b) Abe, F.; Chen, R.-F.; Yamauchi, T. *Phytochemistry* **1996**, *43*, 161–163. (c) Abe, F.; Wase, Y.; Yamauchi, T.; Yahara, S.; Nohara, T. *Phytochemistry* **1995**, *40*, 577–581. (d) Abe, F.; Chen, R.-F.; Yamauchi, T.; Ohashi, H. *Chem. Pharm. Bull.* **1995**, *43*, 499–500. (e) Abe, F.; Yamauchi, T.; Yahara, S.; Nohara, T. *Phytochemistry* **1995**, *38*, 793–794. (f) Begum, S.; Adil, Q.; Siddiqui, B. S.; Siddiqui, S. *J. Nat. Prod.* **1993**, *56*, 613–617. (g) Venkata Rao, E. *Ind. J. Pharm.* **1973**, *35*, 107–113.
(8) (a) Samy, R. P.; Thwin, M. M.; Gopalakrishnakone, P.; Ignacimuthu, S. *J. Ethnopharm.* **2008**, *115*, 302–312. (b) Kanchanapoom, T.; Kasai, R.; Yamasaki, K. *Chem. Pharm. Bull.* **2001**, *49*, 1195–1197. (c) Jain, S. C. *Ind. J. Pharm.* **1976**, *38*, 141–142.
(9) Jolad, S. D.; Hoffmann, J. J.; Cole, J. R.; Tempesta, M. S.; Bates, R. B. *J. Org. Chem.* **1981**, *46*, 1946–1947.
(10) Frerejacque, M.; Durgeat, M. C. R. *Acad. Sci. Hebd. Seances. Acad. Sci. D* **1971**, *272*, 2620–2621.
(11) Siddiqui, S.; Siddiqui, B. S.; Adil, Q.; Begum, S. *Phytochemistry* **1992**, *31*, 3541–3546.
(12) Abe, F.; Yamauchi, T.; Wan, A. S. C. *Phytochemistry* **1992**, *31*, 3189–3193.
(13) Abe, F.; Yamauchi, T. *Chem. Pharm. Bull.* **1978**, *26*, 3023–3027.
(14) Zhao, M.; Bai, L.; Wang, L.; Toki, A.; Hasegawa, T.; Kikuchi, M.; Abe, M.; Sakai, J.; Hasegawa, R.; Bai, Y.; Mitsui, T.; Ogura, H.; Kataoka, T.; Oka, S.; Tsushima, H.; Kiuchi, M.; Hirose, K.; Tomida, A.; Tsuruo, T.; Ando, M. *J. Nat. Prod.* **2007**, *70*, 1098–1103.
(15) Kanchanapoom, T.; Kasai, R.; Yamasaki, K. *Chem. Pharm. Bull.* **2001**, *49*, 1195–1197.
(16) (a) Kojima, K.; Zhu, X.-B.; Ogihara, Y. *Phytochemistry* **1998**, *48*, 885–888. (b) Hamburger, M.; Slacanin, I.; Hostettmann, K.; Dyatmiko, W.; Sutarjadi, *Phytochem. Anal.* **1992**, *3*, 231–237.
(17) Huang, H.-C.; Wu, M.-D.; Tsai, W.-J.; Liao, S.-C.; Liaw, C.-C.; Hsu, L.-C.; Wu, Y.-C.; Kuo, Y.-H. *Phytochemistry* **2008**, *69*, 1609–1616.
(18) Larsson, R.; Kristensen, J.; Sandberg, C.; Nygren, P. *Int. J. Cancer* **1992**, *50*, 177–185.
(19) Horinaka, M.; Yoshida, T.; Shiraishi, T.; Nakata, S.; Wakada, M.; Nakanishi, R.; Nishino, H.; Sakai, T. *Biochem. Biophys. Res. Commun.* **2005**, *333*, 833–838.
(20) Frese, S.; Frese-Schaper, M.; Andres, A.-C.; Miescher, D.; Zumkehr, B.; Schmid, R. A. *Cancer Res.* **2006**, *66*, 5867–5874.

NP900202N