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Cycloartane Triterpenes Isolated from *Combretum quadrangulare* in a Screening Program for Death-Receptor Expression Enhancing Activity

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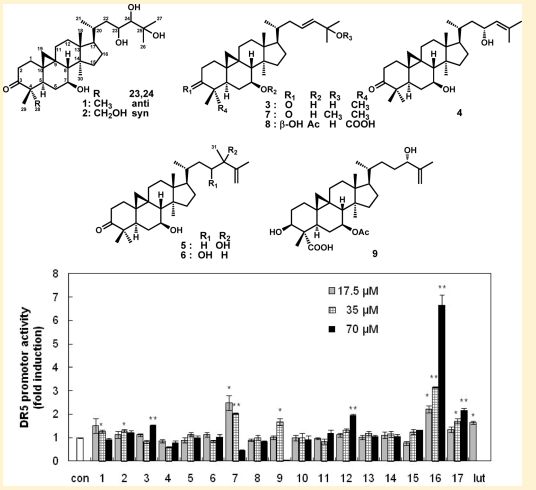
Supporting Information

ABSTRACT: In our screening program for natural products that increase DR5 (death-receptor 5) expression, nine new cycloartane triterpenes, combretanones A−G (**1**−**7**), combretic acid A (**8**), and combretic acid B (**9**), were isolated from a MeOH extract of *Combretum quadrangulare* leaves. The known oleanane triterpenes (**10**, **11**) and six known flavonols (**12**−**17**) were also isolated. The structures of **1**−**9** were elucidated by spectroscopic studies. Compounds **7**, **9**, **12**, **16**, and **17** enhanced DR5 expression, and **16** showed TRAIL-resistance abrogating activity.



During our search for bioactive natural products we investigated the constituents of various terrestrial plants and isolated several death-receptor 5 promoter-enhancing isoflavones.[1] Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF superfamily, is a promising agent for anticancer therapy since it triggers apoptosis in a variety of cancer cells but not in many normal cells.[2] 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 leading to apoptosis.[3] However, studies have shown that some cancer cells display intrinsic or acquired resistance to apoptosis induced by TRAIL, which may restrict its use in treatment.[4,5] Therefore, for the clinical use of TRAIL in cancer therapy, it is extremely important to overcome TRAIL-resistance. TRAIL-resistance has been attributed to a loss of TRAIL receptors, the upregulation of TRAIL decoy receptors, the enhanced expression of cellular FLICE-like inhibitory protein (cFLIP) and cellular inhibitor of apoptosis protein (cIAP), and changes in the expression of Bcl-2 family proteins. In many cases, overcoming TRAIL-resistance involved the up-regulation of the expression of death receptors, especially DR5. Therefore, compounds that enhance DR5 expression are considered to have potential for overcoming TRAIL-resistance. During our search for bioactive natural products with tumor selective apoptosis inducing activity,[6] we examined MeOH extracts of medicinal plants collected in Thailand in order to find compounds that enhance DR5 promoter expression. We previously reported that eight xanthone derivatives isolated from *Garcinia mangostana* (mangosteen), and teleocidin A-2, which was isolated from an actinomycete, enhance DR5 promoter expression.[7] We suggested that these compounds overcome TRAIL-resistance by up-regulating DR5 expression. In this paper, we describe the isolation and structure elucidation of nine new cycloartane triterpenes (**1**−**9**) and eight known compounds (**10**−**17**) from *Combretum quadrangulare* Kurz (Combretaceae) leaves and report the DR5-enhancing activities of these compounds.

RESULTS AND DISCUSSION

In our screening study for tumor-selective anticancer agents, a MeOH extract of *C. quadrangulare* leaves exhibited a potent effect on DR5 promoter enhancement (2.8-fold increase in

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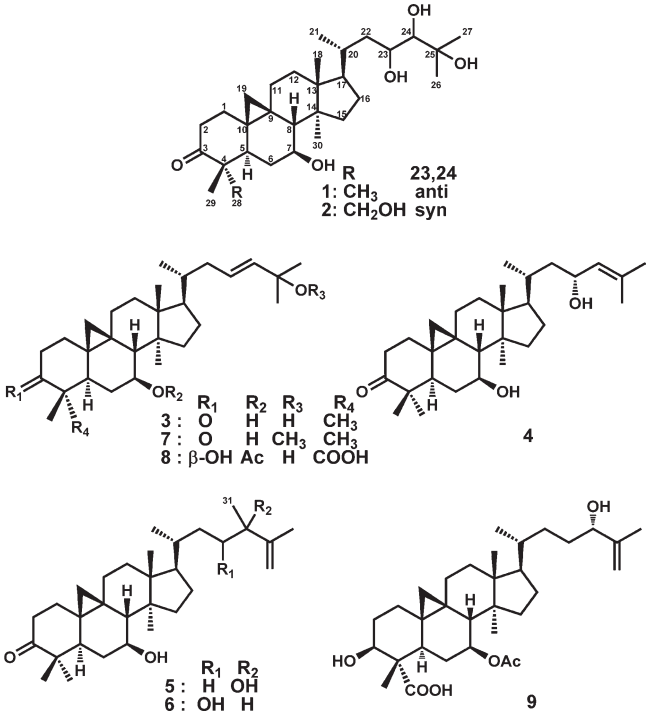
activity at 100 μg/mL). The extract was subjected to Diaion HP20 column chromatography (CC) to yield three fractions. One fraction eluted with MeOH/acetone (1:1), which had a potent effect on DR5 enhancement (1.9-fold increase in activity at 50 μg/mL), was partitioned into hexane, EtOAc, *n*-BuOH, and aqueous layers, and DR5 promoter activity was found to be particularly strong in the *n*-BuOH- and EtOAc-soluble fractions (4.8- and 1.8-fold increase, respectively, at 50 μg/mL). Activity-guided fractionation of the *n*-BuOH- and EtOAc-soluble fractions by CC yielded nine new cycloartane triterpenes (**1**−**9**) and eight known compounds (**10**−**17**).



Combretanone A (**1**) was shown to have the molecular formula $C_{30}H_{50}O_5$ on the basis of HREIMS data (*m*/*z* 490.3652, calcd for $C_{30}H_{50}O_5$, M^+), which indicated six degrees of unsaturation. The IR spectrum exhibited absorption bands at ν_{max} 3390 and 1700 cm^{-1} due to OH and carbonyl groups, respectively. The ^1H NMR spectrum of **1** (Table 1) showed seven methyl groups [δ_H 1.31 (3H, s), 1.28 (3H, s), 1.10 (3H, s), 1.05 (9H, s), and 0.96 (3H, d, *J* = 6.4 Hz)], three oxymethine protons [δ_H 3.84 (1H, ddd, *J* = 10.7, 6.5, 1.6 Hz), 3.64 (1H, td, *J* = 9.5, 4.0 Hz), and 3.31 (1H, d, *J* = 6.5 Hz)], and characteristic methylene protons displaying high-field resonances [δ_H 0.94 and 0.56 (each 1H, d, *J* = 4.6 Hz)]. The ^{13}C NMR spectrum of **1** showed 30 carbon signals: one carbonyl carbon (δ_C 215.8), three oxymethines (δ_C 79.5, 70.7, and 70.4), one O-bearing sp^3 quaternary carbon (δ_C 73.4), five sp^3 quaternary carbons (δ_C 49.6, 48.3, 46.0, 26.5, and 20.8), four sp^3 methines (δ_C 54.5, 52.1, 46.6, and 32.2), nine sp^3 methylenes (δ_C 40.2, 37.2, 37.0, 32.8, 32.5, 31.1, 28.8, 27.8, and 26.9), and seven methyl groups (δ_C 26.8, 25.9, 22.2, 20.7, 18.9, 18.1, and 17.4) by DEPT and HMQC analysis. A carbonyl and five rings accounted for six degrees of unsaturation. Except for its side chain, the ^1H and ^{13}C NMR data of **1** were similar to those of known cycloartane-type triterpenes.[8,9] Detailed analysis of its $^1H-^1H$ COSY and HMBC experiments established the planar structure of **1**. A ketone group at C-3 was confirmed by HMBC correlations from H-2, H_3-28, and H_3-29 to C-3 (δ_C 215.8). $^1H-^1H$ COSY cross-peaks for H-5/H-6, H-6/H-7, and H-7/H-8 and the HMBC cross-peaks from H-5, H-6, and H-8 to C-7 (δ_C 70.7) indicated the presence of an OH at C-7. The 23-, 24-, and 25-OH substituents were confirmed by the $^1H-^1H$ coupling between H-23 [δ_H 3.84 (1H, ddd, *J* = 10.7, 6.5, 1.6 Hz)] and H-24 [δ_H 3.31 (1H, d, *J* = 6.5 Hz)] and the HMBC correlations from H_3-26 and H_3-27 to C-24 (δ_C 79.5) and C-25 (δ_C 73.4) (as shown in Figure S1, Supporting Information). Thus, the planar structure of **1** was concluded to be as shown. Axial orientations of H-7 and H-8 were deduced by their large coupling constant value ($J_{H\text{-}7/H\text{-}8}$ 9.5 Hz).

The HRESIMS data of combretanone B (**2**) exhibited a quasi-molecular ion peak at *m*/*z* 529.3491 (calcd for $C_{30}H_{50}O_6Na$ $[M + Na]^+$), indicating the molecular formula $C_{30}H_{50}O_6$. The ^1H and ^{13}C NMR spectra of **2** (Table 1) closely resembled those of **1**. The ^1H and ^{13}C NMR spectra of **2** showed six methyl signals [δ_H 0.99 (3H, s)/δ_C 17.9, δ_H 0.95 (3H, d, *J* = 6.4 Hz)/δ_C 16.9, δ_H 1.25 (3H, s)/δ_C 26.1, δ_H 1.27 (3H, s)/δ_C 27.3, δ_H 0.95 (3H, s)/δ_C 19.8, and δ_H 0.97 (3H, s)/δ_C 19.2] and a set of oxymethylene signals [δ_H 3.85 and 3.28 (each 1H, d, *J* = 11.4 Hz)/δ_C 64.4]. The oxymethylene signal was assigned to C-28 on the basis of the HMBC cross-peaks for H_3-29/C-3, H_3-29/C-28, H_2-28/C-3, and H_2-28/C-5. The remaining moiety of **2** had the same structure as in **1** on the basis of its $^1H-^1H$ COSY and HMBC correlation data and comparison of ^1H and ^{13}C chemical shifts (Table 1). Thus, the planar structure of **2** corresponded to a 28-hydroxy derivative of **1**. Axial orientations of H-7 and H-8 were deduced by their large coupling constant value ($J_{H\text{-}7/H\text{-}8}$ 11.0 Hz). In the NOE experiment, correlations observed between H-2β (δ_H 2.67), H_3-29, and H-19β (δ_H 0.95) indicated that the methyl group (C-29) at C-4 had a β-axial orientation and that the CH_2OH group (C-28) at C-4 was α-equatorial.

To assess the relative configurations of the side chains of compounds **1** and **2**, we focused on their $^1H-^1H$ coupling constant values. Piscidinol A,[10] which possesses the same side-chain moiety as **1** with a *syn* configuration between H-23 and H-24, demonstrated the following coupling constant: $J_{H\text{-}23/H\text{-}24} \approx 0$ Hz. Meanwhile, 24-*epi*-piscidinol A,[10] which possesses an *anti* configuration between H-23 and H-24, displayed a coupling constant of $J_{H\text{-}23/H\text{-}24}$ = 8.0 Hz, and their structures were established by X-ray crystallographic analysis.[10] Correspondingly, 23,24,25-trihydroxycycloartan-3-one[11] and alisol E,[12] which have *syn* and *anti* configurations between H-23 and H-24, showed coupling constants of $J_{H\text{-}23/H\text{-}24} \approx 0$ and 6.0 Hz, respectively. Thus, the coupling constants of **1** and **2** ($J_{H\text{-}23/H\text{-}24}$ = 6.5 Hz and $J_{H\text{-}23/H\text{-}24}$ = 0 Hz, respectively) indicated that their relative configurations between H-23 and H-24 were *anti* and *syn*, respectively.

Combretanone C (**3**) had the molecular formula $C_{30}H_{48}O_3$ (HRESIMS). The ^1H and ^{13}C NMR spectra of **3** were similar to those of **1**, except for the absence of the oxymethine signals at C-23 and C-24 observed in **1** and the presence of alternative olefin signals [δ_H 5.59 (2H, m); δ_C 125.3 and δ_C 139.5] in **3** instead of the 1,2-diol group. The double bond was located between C-23 and C-24 by the HMBC cross-peaks from H_2-22 to C-23, from H_2-22 to C-24, from H-23/H-24 (δ_H 5.59, 2H) to C-25, C-26, and C-27, and from H_3-26/H_3-27 (δ_H 1.31, 6H) to C-25 and C-24. Axial orientations of H-7 and H-8 were indicated by the large coupling constant value ($J_{H\text{-}7/H\text{-}8}$ = 9.9 Hz).

Table 1. ^{1}H NMR Spectroscopic Data for Combretanones A−G (1−7)

	1	2	3	4	5	6	7
position	δ_H[a] (*J* in Hz)	δ_H[b] (*J* in Hz)	δ_H[a] (*J* in Hz)	δ_H[a] (*J* in Hz)	δ_H[a] (*J* in Hz)	δ_H[a] (*J* in Hz)	δ_H[a] (*J* in Hz)
1	1.56 m, 1.81 m	1.55 m, 1.85 m	1.55 m, 1.85 m	1.55 m, 1.83 m	1.55 m, 1.85 m	1.55 m, 1.85 m	1.55 m, 1.85 m
2	2.32 ddd (13.7, 4.0, 2.7)	2.30 ddd (14.6, 4.0, 3.0)	2.31 ddd (14.0, 4.6, 2.5)	2.31 ddd (14.0, 4.6, 2.7)	2.31 ddd (14.0, 4.3, 2.5)	2.31 ddd (14.0, 4.3, 2.5)	2.32 ddd (14.0, 4.0, 2.7)
	2.70 td (13.7, 6.4)	2.67 td (14.6, 6.3)	2.69 td (14.0, 6.4)	2.69 td (14.0, 6.4)	2.69 td (14.0, 6.4)	2.70 td (14.0, 4.6)	2.70 td (14.0, 6.4)
5	1.91 m	2.50 dd (12.8, 3.7)	1.89 m	1.88 m	1.88 m	1.87 m	1.90 m
6	1.13 m, 1.70 m	1.13 m, 1.70 m	1.15 m, 1.70 m	1.13, 1.70 m	1.13 m, 1.70 m	1.13 m, 1.70 m	1.15 m, 1.70 m
7	3.64 td (9.5, 4.0)	3.60 td (11.0, 4.3)	3.63 td (9.9, 4,3)	3.63 td (9.8, 4.0)	3.63 td (9.9, 4.3)	3.63 td (9.9, 4.3)	3.63 td (9.8, 4.0)
8	1.71 d (9.5)	1.55 d (11.0)	1.68 d (9.9)	1.68 m	1.68 m	1.66 m	1.66 m
11	1.93 m	1.30 m, 1.90 m	1.25 m, 1.88 m	1.30, 1.88 m	1.25, 1.88 m	1.30, 1.88 m	1.25 m, 1.88 m
12	1.70 m	1.40−1.60 m	1.65 m	1.70 m	1.65 m	1.65 m	1.65 m
15	1.48−1.63 m	1.50−1.80 m	1.45 m, 1.60 m	1.45 m, 1.60 m	1.45 m, 1.60 m	1.45 m, 1.58 m	1.45 m, 1.60 m
16	1.98 m	1.30 m, 1.98 m	1.35 m, 1.95 m	1.35 m, 1.95 m	1.35 m, 1.95 m	1.35 m, 1.95 m	1.38 m, 1.95 m
17	1.56 m	1.75 m	1.51 m	1.52 m	1.52 m	1.52 m	1.55 m
18	1.05 s	0.99 s	1.01 s	1.03 s	1.00 s	1.04 s	1.02 s
19	0.56 d (4.6)	0.57 d (4.6)	0.56 d (4.6)	0.56 d (4.9)	0.56 d (4.9)	0.56 d (4.6)	0.57 d (4.9)
	0.94 d (4.6)	0.95 d (4.6)	0.93 d (4.6)	0.92 d (4.9)	0.93 d (4.9)	0.93 d (4.6)	0.93 d (4.9)
20	1.74 m	1.50−1.80 m	1.53 m	1.65 m	1.53 m	1.65 m	1.53 m
21	0.96 d (6.4)	0.95 d (6.4)	0.87 d (6.1)	0.96 d (6.4)	0.88 d (6.4)	0.92 d (6.4)	0.89 d (6.1)
22	1.45−1.65 m	1.60−1.90 m	1.75 m, 2.20 m	1.01 m, 1.55 m	1.35−1.45 m	1.50 m	1.78 m, 2.21 m
23	3.84 ddd (10.7, 6.5, 1.6)	4.04 bd (10.4)	5.59 m	4.48 td (2.7, 8.7)	1.40 m, 1.68 m	3.65 m	5.52 m
24	3.31 d (6.5)	3.02 br s	5.59 m	5.19 d (8.7)		2.09 quint. (6.7)	5.40 d (15.6)
26	1.28 s	1.25 s	1.31 s	1.70 s	4.82 br s, 4.96 br s	4.75 br s, 4.81 br s	1.25 s
27	1.31 s	1.27 s	1.31 s	1.68 s	1.74 s	1.72 s	1.25 s
28	1.05 s	3.28 d (11.4), 3.85 d (11.4)	1.05 s	1.04 s	0.94 s	0.95 s	0.93 s
29	1.10 s	0.95 s	1.09 s	1.09 s	1.05 s	1.05 s	1.05 s
30	1.05 s	0.97 s	0.93 s	0.94 s	1.10 s	1.10 s	1.10 s
31					1.31 s	1.07 d (6.7)	
							OCH_3 3.15 s

[a] In $CDCl_3$. [b] In CD_3OD.

The geometry of the disubstituted double bond at C-23 could not be determined by the vicinal coupling constant of the olefinic signals due to overlapping. However, comparison of the ^{1}H and ^{13}C NMR chemical shifts of the side chain of **3** [$\delta_{H\text{-}23 \text{ and } H\text{-}24}$ 5.59 (2H, m), $\delta_{H\text{-}26 \text{ and } H\text{-}27}$ 1.31 (6H, s), $\delta_{C\text{-}22}$ 39.0, $\delta_{C\text{-}23}$ 125.3, and $\delta_{C\text{-}24}$ 139.5] with those of both synthesized *E*/*Z*-isomers of cycloart-23-ene-3,25-diol[13] [$\delta_{H\text{-}23 \text{ and } H\text{-}24}$ 5.60 (2H, m), $\delta_{H\text{-}26}$ 1.31 (3H, s), $\delta_{H\text{-}27}$ 1.32 (3H, s), $\delta_{C\text{-}22}$ 39.0, $\delta_{C\text{-}23}$ 125.6, and $\delta_{C\text{-}24}$ 139.4 for the *E*-isomer and $\delta_{H\text{-}23}$ 5.31 (1H, ddd, *J* = 12.1, 8.8, 6.1), $\delta_{H\text{-}24}$ 5.53 (1H, ddd, *J* = 12.1, 1.7, 1.7), $\delta_{H\text{-}26}$ 1.33 (3H, s), $\delta_{H\text{-}27}$ 1.33 (3H, s), $\delta_{C\text{-}22}$ 34.6, $\delta_{C\text{-}23}$ 130.2, and $\delta_{C\text{-}24}$ 137.4 for the *Z*-isomer] allowed assignment of *E*-geometry to the $\Delta^{23,24}$-double bond of **3**.

The molecular formula of combretanone D (**4**) was revealed to be $C_{30}H_{48}O_3$, the same as that of **3**. The ^{1}H and ^{13}C NMR spectra of **4** were similar to those of **3**, except for one less sp^2 methine [δ_H 5.19 (1H, d, *J* = 8.7 Hz)/δ_C 128.9], one more sp^2 quaternary carbon (δ_C 133.8) signal in **4** than in **3**, and an oxymethine signal [δ_H 4.48 (1H, td, *J* = 8.7, 2.7 Hz)/δ_C 65.9] in **4** instead of the O-bearing sp^3 quaternary carbon seen in **3**. HMBC cross-peaks of H-23/C-20, H-23/C-24, H-23/C-25, H-24/C-26, H-24/C-27, H_3-26/C-24, and H_3-27/C-24 indicated that the oxymethine and a trisubstituted double bond were located at C-23 and C-24/C-25, respectively. Thus, the structure of compound **4** was revealed to be as shown. Axial orientations of H-7 and H-8 were deduced from the coupling constant ($J_{H\text{-}7/H\text{-}8}$ 9.8 Hz). The relative configuration of C-23 was deduced to be 23α-OH by comparison of the ^{1}H and ^{13}C NMR data with two epimers of related compounds possessing the same side chain. The ^{13}C NMR chemical shift values of the C-23, C-24, and C-25 positions of **4** ($\delta_{C\text{-}23}$ 65.9, $\delta_{C\text{-}24}$ 128.9, and $\delta_{C\text{-}25}$ 133.8) more closely resembled those of 23α-cycloarta-24-ene-3β,23-diol ($\delta_{C\text{-}23}$ 66.1, $\delta_{C\text{-}24}$ 129.1, and $\delta_{C\text{-}25}$ 133.8)[14] than those of 23β-cycloarta-24-ene-3β,23-diol ($\delta_{C\text{-}23}$ 67.3, $\delta_{C\text{-}24}$ 128.4, and $\delta_{C\text{-}25}$ 135.6).[14]

Combretanone E (**5**) and combretanone F (**6**) had the same molecular formula ($C_{31}H_{50}O_3$), one more carbon atom and two more hydrogen atoms than **4**. The ^{1}H and ^{13}C NMR spectra of **5** and **6** were again similar to those of other cycloartanes. However, the ^{13}C NMR spectra of **5** and **6** showed 31 carbon signals. In the ^{1}H and ^{13}C NMR spectra of **5**, the signals of one sp^2 methylene [δ_H 4.82 (1H, br s) and δ_H 4.96 (1H, br s)/δ_C 109.6], one sp^3

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O-bearing quaternary carbon [δ_C 75.5], and an sp^2 quaternary carbon [δ_C 150.3] were observed. Detailed analysis of the HMBC cross-peaks of H_2-26/C-27, H_2-26/C-24, H_3-27/C-24, H_3-27/C-25, H_3-27/C-26, H_3-31/C-23, H_3-31/C-24, H_3-31/C-25, H_2-23/C-24, and H_2-23/C-25 indicated that a tertiary OH and a tertiary CH_3 group were located at C-24 and the double bond between C-25 and C-26. In the ^1H and ^{13}C NMR data of **6**, an sp^2 methylene signal [δ_H 4.75 (1H, br s) and δ_H 4.81 (1H, br s)/δ_C 111.4] and an sp^2 quaternary carbon (δ_C 148.1) were observed, similar to **5**; however, signals for oxymethine [δ_H 3.65 (1H, m)/δ_C 69.9] and sp^3 methine [δ_H 2.09 (1H, quint., J = 6.7 Hz)/δ_C 47.9] were detected instead of the sp^3 O-bearing quaternary carbon observed in **5**. HMBC cross-peaks of H_2-26/C-24, H_2-26/C-25, H_2-26/C-27, H_3-27/C-24, H_3-27/C-25, H_3-27/C-26, H_2-23/C-25, and H_2-23/C-31 indicated a secondary OH at C-23 and a secondary methyl group at C-24. Thus, the structures of **5** and **6** were elucidated to be as shown. Their large coupling constant values confirmed axial orientations of H-7 and H-8.

Combretanone G (**7**) also had the molecular formula $C_{31}H_{50}O_3$. The ^1H and ^{13}C NMR spectra of **7** closely resembled those of **3**; however, one additional OCH_3 signal [δ_H 3.15/δ_C 50.2] was observed. The OCH_3 group was determined to be at C-25 by the HMBC correlations of H_3CO/C-25, H_3-26/C-25, and H_3-27/C-25. Thus, the planar structure of **7** was concluded to be as shown.

The CD spectra of **1**−**7** all showed a negative Cotton effect at 295 nm, as does cycloartan-3-one-16,23,24,25-tetrol,[9] indicating that **1**−**7** and cycloartan-3-one-16,23,24,25-tetrol ($[\theta]_{295}$ −3000)[9] have the same cycloartane skeleton.

Combretic acid A (**8**) was obtained as colorless needle crystals (mp 190 °C from MeOH), and its molecular formula was determined to be $C_{32}H_{50}O_6$, indicating that it possessed two more carbons, two more hydrogens, and three more oxygens than **3**. The ^1H and ^{13}C NMR data of **8** closely resembled those of **3**, especially in its side chain; however, the ^1H and ^{13}C NMR spectra of **8** showed signals for two carbonyls [δ_C 172.1 and 180.2] and two oxymethines [δ_H 4.02 (1H, dd, J = 11.6, 4.3 Hz)/δ_C 75.9 and δ_H 4.78 (1H, m)/δ_C 73.7]. Comparison of the ^1H and ^{13}C NMR data of **8** and **3** and detailed analysis of the COSY cross-peaks of H_2-6/H-7/H-8 and the HMBC cross-peaks of H_3-29/C-3 and H_3-29/C-28 revealed an OAc group at C-7, an OH at C-3, and a carboxylic acid at C-28. The relative configuration of **8** was suggested by $^1H-^1H$ coupling constants and NOE experiments. The large vicinal coupling constant for H-2β and H-3 ($J_{H\text{-}2\alpha/H\text{-}3}$ = 11.6 Hz) indicated that they were oriented in a *trans*-diaxial manner. The axial orientations of H-7 and H-8 were deduced by their large coupling constant value ($J_{H\text{-}7/H\text{-}8}$ 8.3 Hz). In the NOE experiment, correlations observed between H-2β, H_3-29, and H-19β indicated that the methyl group (C-29) at C-4 was β-oriented and revealed the α-equatorial orientation of the carboxyl group (C-28) at C-4.

Combretic acid B (**9**) was obtained as colorless needle crystals (mp 190 °C from MeOH), and the molecular formula was $C_{32}H_{50}O_6$ from its HRESIMS data, the same as that of **8**. The ^1H and ^{13}C NMR spectra of **9** closely resembled those of **3**, especially in its cycloartane moiety; however, signals due to an exo-olefin group [δ_H 4.97 (1H, br s) and 5.22 (1H, br s)/δ_C 110.4] and an additional oxymethine [δ_H 4.35 (1H, t, J = 6.4 Hz)/δ_C 76.2] were also observed. The HMBC cross-peaks of H_2-26/C-24, H_2-26/C-25, H_3-27/C-24, H_3-27/C-25, H_3-27/C-26, H_2-22/C-24, and H_2-23/C-24 indicated that an OH group

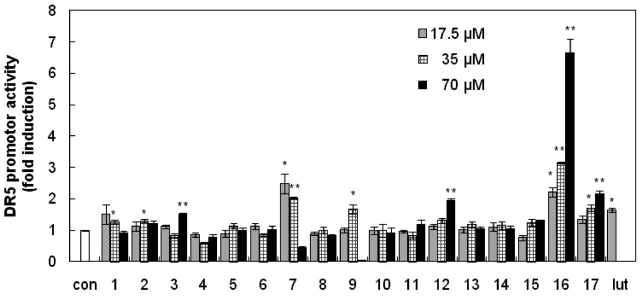

Figure 1. Activation of the DR5 promoter by compounds **1**−**17**, luteolin (positive control: lut), and DMSO (negative control: con) in DLD-1/*Sac*I cells. The samples were tested at concentrations of 17.5 and 35 to 70 μM for compounds (**1**−**17**) and 17.5 μM for luteolin. Each value represents the mean ± SE (n = 3), with significance determined with the Student's t-test (*p < 0.05, **p < 0.01 vs control).

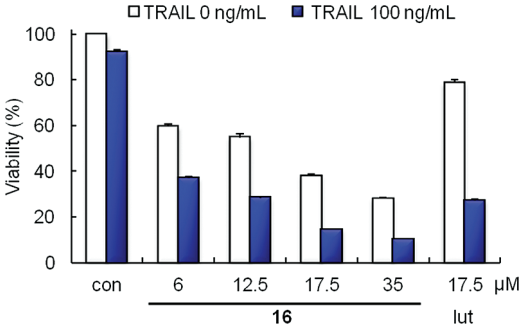

Figure 2. Effects of compound **16**, luteolin (positive control: lut), and DMSO (negative control: con) in the presence or absence of TRAIL on the viability of AGS cells. The cells were seeded in a 96-well culture plate (6×10^3 cells per well) for 24 h and then treated with the indicated concentrations of the compounds and TRAIL (100 ng/mL) for 24 h. Cell viability was determined by the fluorometric microculture cytotoxicity assay (FMCA). Bars represent the mean ± SE (n = 3).

was located at C-24 and that the exo-olefin group was located at C-25. This was supported by comparison of the ^{13}C NMR data of **9** with that of 24-epiquadrangularic acid M,[15] which has the same side-chain moiety. The relative configuration of **9** was indicated by $^1H-^1H$ coupling constants and NOE experiments. Axial orientations of H-7 and H-8 were indicated by the large coupling constant ($J_{H\text{-}7/H\text{-}8}$ 8.5 Hz). NOE correlations observed between H-2β, H_3-29, and H-19β indicated a β-axial orientation of the methyl group (C-29) at C-4 and α-equatorial orientation of the carboxyl group (C-28) at C-4. The configuration at C-24 was determined to be 24α by comparison of the ^{13}C NMR chemical shift values of **9** ($\delta_{C\text{-}24}$ 76.2, $\delta_{C\text{-}25}$ 149.6, $\delta_{C\text{-}26}$ 110.4, and $\delta_{C\text{-}27}$ 17.9), 24-epiquadrangularic acid M ($\delta_{C\text{-}24}$ 76.1, $\delta_{C\text{-}25}$ 149.6, $\delta_{C\text{-}26}$ 110.4, and $\delta_{C\text{-}27}$ 17.7), which has a 24α-configuration, and quadrangularic acid M ($\delta_{C\text{-}24}$ 75.6, $\delta_{C\text{-}25}$ 149.6, $\delta_{C\text{-}26}$ 110.0, and $\delta_{C\text{-}27}$ 18.2), which displays a 24β-configuration.

Compounds **10**−**17** were revealed to be arjunolic acid[16] (**10**), asiatic acid[17] (**11**), 5,7,3′,5′-tetrahydroxy-3,4′-dimethyoxyflavone[18] (**12**), combretol[19] (**13**), 3′,5-dihydroxy-3,4,′5′,7-tetramethoxyflavone[20] (**14**), pachypodol (5,4′-dihydroxy-3,7,3′-trimethoxyflavone)[21] (**15**), quercetin 3,4′-dimethyl ether (5,7,3′-trihydroxy-3,4′-dimethoxyflavone)[22] (**16**), and myricetin 3,3′,4′-trimethyl ether[23] (**17**) by comparison of their NMR and MS data with those in the literature.

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Table 2. ^{13}C NMR Spectroscopic Data for Combretanones A−G (1−7)

	1	2	3	4	5	6	7
position	δ_C[a], mult.	δ_C[b], mult.	δ_C[a], mult.	δ_C[a], mult.	δ_C[a], mult.	δ_C[a], mult.	δ_C[a], mult.
1	32.8, CH_2	32.8, CH_2	32.8, CH_2	32.8, CH_2	32.8, CH_2	32.8, CH_2	32.8, CH_2
2	37.2, CH_2	38.7, CH_2	37.2, CH_2	37.2, CH_2	37.2, CH_2	37.2, CH_2	37.2, CH_2
3	215.8, qC	217.3[c], qC	215.9, qC	215.9, qC	215.9, qC	215.9, qC	215.9[c], qC
4	49.6, qC	55.5, qC	49.6, qC	49.6, qC	49.6, qC	49.6, qC	49.6, qC
5	46.6, CH	40.2, CH	46.5, CH	46.6, CH	46.6, CH	46.6, CH	46.5, CH
6	31.1, CH_2	31.8, CH_2	31.0, CH_2	31.0, CH_2	31.0, CH_2	31.0, CH_2	31.1, CH_2
7	70.7, CH	70.8, CH	70.6, CH	70.6, CH	70.6, CH	70.6, CH	70.6, CH
8	54.5, CH	53.9, CH	54.6, CH	54.6, CH	54.6, CH	54.5, CH	54.6, CH
9	20.8, qC	22.2, qC	20.8, qC	20.8, qC	20.8, qC	20.8, qC	20.8, qC
10	26.5, qC	26.9, qC	26.5, qC	26.4, qC	26.5, qC	26.4, qC	26.5, qC
11	26.9, CH_2	28.2, CH_2	26.9, CH_2	26.9, CH_2	27.0, CH_2	27.0, CH_2	26.9, CH_2
12	32.5, CH_2	37.6, CH_2	32.3, CH_2	32.5, CH_2	32.4, CH_2	32.5, CH_2	32.4, CH_2
13	46.0, qC	47.0, qC	45.8, qC	45.9, qC	45.8, qC	45.9, qC	45.8, qC
14	48.3, qC	49.8, qC	48.2, qC	48.2, qC	48.2, qC	48.3, qC	48.2, qC
15	37.0, CH_2	34.0, CH_2	37.0, CH_2	37.0, CH_2	37.0, CH_2	37.0, CH_2	37.0, CH_2
16	28.8, CH_2	29.4, CH_2	28.3, CH_2	28.6, CH_2	28.4, CH_2	28.7, CH_2	28.3, CH_2
17	52.1, CH	55.1, CH	51.1, CH	51.9, CH	51.2, CH	52.0, CH	51.0, CH
18	17.4, CH_3	17.9, CH_3	17.4, CH_3	17.4, CH_3	17.3, CH_3	17.4, CH_3	17.4, CH_3
19	27.8, CH_2	27.8, CH_2	27.7, CH_2	27.7, CH_2	27.7, CH_2	27.7, CH_2	27.7, CH_2
20	32.2, CH	33.5, CH	36.3, CH	32.6, CH	36.1, CH	32.9, CH	36.2, CH
21	18.1, CH_3	16.9, CH_3	18.3, CH_3	18.4, CH_3	18.4, CH_3	18.3, CH_3	18.4, CH_3
22	40.2, CH_2	43.4, CH_2	39.0, CH_2	44.3, CH_2	29.9, CH_2	41.9, CH_2	39.3, CH_2
23	70.4, CH	68.5, CH	125.3, CH	65.9, CH	36.8, CH_2	69.9, CH	128.5, CH
24	79.5, CH	80.5, CH	139.5, CH	128.9, CH	75.5, qC	47.9, CH	136.7, CH
25	73.4, qC	74.6, qC	70.7, qC	133.8, qC	150.3, qC	148.1, qC	74.9, qC
26	26.8, CH_3	26.1, CH_3	29.9, CH_3	25.7, CH_3	109.6, CH_2	111.4, CH_2	25.7, CH_2
27	25.9, CH_3	27.3, CH_3	30.0, CH_3	18.1, CH_3	19.4, CH_3	20.9, CH_3	26.2, CH_3
28	22.2, CH_3	64.4, CH_2	22.1, CH_3	22.1, CH_3	18.9, CH_3	18.8, CH_3	18.9, CH_3
29	20.7, CH_3	19.8, CH_3	20.6, CH_3	20.6, CH_3	22.2, CH_3	22.1, CH_3	22.2, CH_3
30	18.9, CH_3	19.2, CH_3	18.9, CH_3	18.8, CH_3	20.6, CH_3	20.6, CH_3	20.6, CH_3
31					27.9, CH_3	14.3, CH_3	
OCH_3							50.2, CH_3

[a] In $CDCl_3$. [b] In CD_3OD. [c] Assignment was based on the HMBC spectrum.

Compounds **1**−**17** were evaluated for DR5 promoter activity using a luciferase assay in DLD-1/SacI cells, and luteolin,[24,25] which had been reported to increase DR5 expression, was used as a positive control at 17.5 μM. As shown in Figure 1, compounds **7**, **9**, **12**, **16**, and **17** increased DR5 promoter activity by 2.5-, 1.7-, 2.0-, 6.7-, and 2.2-fold, respectively, as compared with the control cells at the indicated concentrations, and the flavonol **16** produced the most potent enhancement of DR5 expression. Although it is known that some natural products and small synthetic molecules induce enhanced DR5 expression, this is the first report in which cycloartane triterpenes produced enhanced DR5 expression.

In the TRAIL signaling pathway, DR5 enhancement induces TRAIL-mediated apoptosis in TRAIL-resistant cell lines. Therefore, we next investigated the TRAIL-resistance abrogating activity of the flavonol quercetin 3,4′-dimethyl ether (5,7,3′-trihydroxy-3,4′-dimethoxyflavone) (**16**), which possessed the most potent activity, in TRAIL-resistant human gastric adenocarcinoma (AGS) cell lines by comparing cell viability in the presence and absence of TRAIL (100 ng/mL) using the FMCA method.[26] As shown in Figure 2, treatment with 100 ng/mL of TRAIL for 24 h resulted in a slight decrease in cell viability (92%), while luteolin[23] at 17.5 μM, which was used as a positive control, produced about 51% stronger inhibition when administered in combination with TRAIL than TRAIL alone. Treating the cells with **16** alone produced some toxicity, and treating the cells with 12.5 and 17.5 μM of **16** in combination with TRAIL (100 ng/mL) produced 27% and 24% more inhibition than treatment with **16** alone, implying that **16** significantly overcame TRAIL-resistance. These results suggested that **16** produced its TRAIL-resistance abrogating activity by activating the TRAIL pathway via the enhancement of DR5 expression.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured with a JASCO P-1020 polarimeter. Melting points were determined with a Yanako MP-500P micro melting point apparatus and are uncorrected. IR spectra were measured using the ATR method in a JASCO FT-IR 230 spectrophotometer. UV spectra were assessed in a Shimadzu UV mini-1240 spectrometer. CD spectra were measured in a JASCO J-720WI spectropolarimeter. NMR spectra were recorded on

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JEOL JNM-A500 and JEOL JNM-ECP600 spectrometers with a deuterated solvent, the chemical shift of which was used as an internal standard. High-resolution electron impact mass spectra (EIMS) were measured on a JEOL GC mate, and high-resolution electrospray ionization mass spectra (ESIMS) were obtained on a Thermo Scientific Exactive spectrometer.

Plant Material. The leaves of *C. quadrangulare* were collected in Khon Kaen, Thailand, in August 2006, and identified by T. Kowithayakorn. A voucher specimen (7-129) has been deposited in our laboratory.

Extraction and Isolation. The leaves of *C. quadrangulare* (300 g) were extracted with MeOH (5 L) at room temperature to give 77.8 g of crude extract after evaporation of the MeOH. The extract was subjected to a Diaion HP-20 CC (8 × 30 cm; particle size 250−850 μm) eluted with MeOH and acetone. The fraction (19.3 g) eluted with an equal mixture of MeOH and acetone was then suspended in 10% aqueous MeOH (100 mL) and partitioned into hexane-, EtOAc-, and BuOH-soluble fractions (300 mL × 3). The BuOH-soluble fraction (2.7 g) was subjected to silica gel (PSQ100B) CC (5 × 23 cm) eluted with a $CHCl_3$/MeOH solvent system to give nine fractions (2A to 2I). Fraction 2E (206 mg), which was eluted with $CHCl_3$/MeOH (95:5), was purified by preparative HPLC [YMC Pro C18, 1.0 × 25 cm; MeOH/H_2O (75:15); flow rate: 2.5 mL/min; RI and UV detection at 254 nm] to give compounds **1** (4.7 mg, t_R 18 min), **2** (4.1 mg, t_R 16 min), **10** (7.4 mg, t_R 31 min), **11** (7.3 mg, t_R 32 min), and **12** (1.3 mg, t_R 8 min). Fraction 2D (99 mg), eluted with $CHCl_3$/MeOH (95:5), was subjected to ODS CC (3 × 25 cm) eluted with a MeOH/H_2O solvent system, followed by preparative HPLC [YMC ODS-AM, 1.0 × 25 cm; MeOH/H_2O (85:15); flow rate: 2.5 mL/min; RI and UV detection at 254 nm] to give compound **8** (14.0 mg, t_R 43 min) and a crude fraction (27.9 mg, t_R 40 min), which was further separated by preparative HPLC [YMC ODS-AM, 1.0 × 25 cm; MeCN/H_2O (55:45); flow rate: 2.5 mL/min; RI and UV detection at 254 nm] to give compound **9** (3.7 mg, t_R 42 min). Fractions 2B and 2C (764 mg) eluted with $CHCl_3$/MeOH (95:5) were subjected to silica gel (PSQ100B) CC (5 × 23 cm) eluted with a $CHCl_3$/MeOH solvent system, followed by preparative HPLC [YMC ODS-AM, 1.0 × 25 cm; MeOH/H_2O (75:25); flow rate: 2.5 mL/min; RI and UV detection at 254 nm] to give compounds **13** (6.4 mg, t_R 14 min), **3** (53.4 mg, t_R 20 min), **4** (56.7 mg, t_R 22 min), **5** (23.9 mg, t_R 25 min), **6** (20.7 mg, t_R 28 min), and **7** (7.5 mg, t_R 38 min) and a crude fraction (90.2 mg, t_R 11 min), which was further purified by preparative HPLC [Inertsil Diol, 0.6 × 25 cm; hexane/*i*-PrOH (85:15); flow rate: 1.3 mL/min; RI and UV detection at 254 nm] to give compounds **14** (2.6 mg, t_R 31 min) and **15** (6.0 mg, t_R 36 min). The EtOAc-soluble fraction (12.8 g) was subjected to silica gel CC (8 × 23 cm) eluted with a $CHCl_3$/MeOH solvent system to give five fractions (3A to 3E). Fraction 3B eluted with $CHCl_3$/MeOH (95:5) was subjected to silica gel (PSQ100B) CC (5 × 20 cm) eluted with a $CHCl_3$/MeOH solvent system, then purified by ODS CC (4 × 17 cm) eluted with a MeOH/H_2O solvent system and preparative HPLC [YMC ODS-AM, 1.0 × 25 cm; MeCN/H_2O (35:65); flow rate: 2.5 mL/min; RI and UV detection at 254 nm] to give compounds **16** (2.8 mg, t_R 38 min) and **17** (7.0 mg, t_R 44 min).

Combretanone A (***1***)*:*. colorless, amorphous solid; $[\alpha]_D^{24}$ +37.0 (*c* 1.0, $CHCl_3$); IR (ATR) ν_{max} 3390, 2970, and 1700 cm^{-1}; CD (MeOH) λ_{ext} ($[\theta]$) 295 nm (−800); ^1H and ^{13}C data, see Tables 1 and 2; HREIMS *m*/*z* 490.3652 (M^+), calcd for $C_{30}H_{50}O_5$, 490.3658.

Combretanone B (***2***)*:*. colorless, amorphous solid; $[\alpha]_D^{25}$ +13.9 (*c* 1.0, $CHCl_3$); IR (ATR) ν_{max} 3410, 2940, and 1700 cm^{-1}; CD (MeOH) λ_{ext} ($[\theta]$) 295 nm (−1500); ^1H and ^{13}C data, see Tables 1 and 2; HRESIMS *m*/*z* 529.3491 $[M + Na]^+$, calcd. for $C_{30}H_{50}O_6Na$, 529.3505.

Combretanone C (***3***)*:*. colorless, amorphous solid; $[\alpha]_D^{25}$ +14.6 (*c* 1.0, $CHCl_3$); IR (ATR) ν_{max} 3410, 2970, and 1700 cm^{-1}; CD (MeOH) λ_{ext} ($[\theta]$) 295 nm (−1800); ^1H and ^{13}C data, see Tables 1 and 2; HRESIMS *m*/*z* 479.3496 $[M + Na]^+$, calcd for $C_{30}H_{48}O_3Na$, 479.3501.

Combretanone D (***4***)*:*. colorless, amorphous solid; $[\alpha]_D^{25}$ +25.6 (*c* 1.0, $CHCl_3$); IR (ATR) ν_{max} 3470, 2970, and 1700 cm^{-1}; CD (MeOH) λ_{ext} ($[\theta]$) 295 nm (−1300); ^1H and ^{13}C data, see Tables 1 and 2; HRESIMS *m*/*z* 479.3481 $[M + Na]^+$, calcd for $C_{30}H_{48}O_3Na$, 479.3501.

Combretanone E (***5***)*:*. colorless, amorphous solid; $[\alpha]_D^{25}$ +17.3 (*c* 1.0, $CHCl_3$); IR (ATR) ν_{max} 3550, 2970, and 1700 cm^{-1}; CD (MeOH) λ_{ext} ($[\theta]$) 295 nm (−1400); ^1H and ^{13}C data, see Tables 1 and 2; HRESIMS *m*/*z* 493.3639 $[M + Na]^+$, calcd for $C_{31}H_{50}O_3Na$, 493.3658.

Table 3. NMR Spectroscopic Data for Combretic Acids A (8) and B (9)

	8		9	
position	δ_C[a], mult.	δ_H[a] (*J* in Hz)	δ_C[b], mult.	δ_H[b] (*J* in Hz)
1	31.8, CH_2	1.55 m, 1.85 m	33.0, CH_2	1.50 m, 1.83 m
2	29.7, CH_2	1.63, 1.90 m	29.6, CH_2	1.98 m, 2.12 m
3	75.9, CH	4.02 dd (11.6, 4,3)	75.3, CH	4.66 m
4	55.1, qC		55.0, qC	
5	42.8, CH	2.19 m	42.3, CH	2.79 m
6	30.1, CH_2	1.15 m, 1.40 m	30.5, CH_2	1.40 m, 2.10 m
7	73.7, CH	4.78 m	73.0, CH	5.14 m
8	51.3, CH	2.00 d (8.3)	50.9, CH	2.11 d (8.5)
9	21.0, qC		20.1, qC	
10	26.8, qC		26.5, qC	
11	27.9, CH_2	1.75−1.85 m	27.2, CH_2	1.80, 1.90 m
12	33.6, CH_2	1.60 m	32.9, CH_2	1.60 m
13	46.7, qC		46.1, qC	
14	49.5, qC		48.8, qC	
15	36.8, CH_2	1.20 m, 1.55 m	36.3, CH_2	1.33 m, 1.73 m
16	29.0, CH_2	1.40−1.60 m	28.6, CH_2	1.40−1.90 m
17	52.3, CH	1.58 m	52.0, CH	1.58 m
18	17.3, CH_3	1.01 s	17.3, CH_3	1.05 s
19	27.6, CH_2	0.37 d (4.9) 0.83 d (4.9)	27.6, CH_2	0.40 d (4.6) 0.96 d (4.6)
20	37.7, CH	1.50 m	36.5, CH	1.50 m
21	19.0, CH_3	0.89 d (6.4)	19.0, CH_3	1.01 d (6.4)
22	40.2, CH_2	1.78 m, 2.16 m	32.9, CH_2	1.25 m
23	125.9, CH	5.56 m	31.5, CH_2	1.77, 1.90 m
24	140.8, CH	5.56 d (14.9)	76.2, CH	4.35 t (6.4)
25	71.2, qC		149.6, qC	
26	30.1, CH_3	1.25 s	110.4, CH_2	4.97 br s, 5.22 br s
27	30.0, CH_3	1.25 s	17.9, CH_3	1.94 s
28	172.1, qC		179.9, qC	
29	19.5, CH_3	0.90 s	19.4, CH_3	0.98 s
30	9.6, CH_3	1.08 s	10.3, CH_3	1.65 s
(CO)	180.2, qC		170.0, qC	
(CH_3)	21.6, CH_3	1.95 s	21.6, CH_3	1.96 s

[a] In CD_3OD. [b] In C_5D_5N.

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Combretanone F (**6**):. colorless, amorphous solid; $[\alpha]_D^{25}$ +26.4 (*c* 1.0, $CHCl_3$); IR (ATR) ν_{max} 3570, 2970, and 1700 cm^{-1}; CD (MeOH) λ_{ext} ($[\theta]$) 295 nm (−1300); ^1H and ^{13}C data, see Tables 1 and 2; HRESIMS *m*/*z* 493.3641 $[M + Na]^+$, calcd for $C_{31}H_{50}O_3Na$, 493.3658.

Combretanone G (**7**):. colorless, amorphous solid; $[\alpha]_D^{25}$ +26.4 (*c* 1.0, $CHCl_3$); IR (ATR) ν_{max} 3460, 2970, and 1710 cm^{-1}; CD (MeOH) λ_{ext} ($[\theta]$) 295 nm (−1100); ^1H and ^{13}C data, see Tables 1 and 2; HRESIMS *m*/*z* 493.3638 $[M + Na]^+$, calcd for $C_{31}H_{50}O_3Na$, 493.3658.

Combretic acid A (**8**):. colorless needles; mp 190 °C (from MeOH); $[\alpha]_D^{20}$ +83.6 (*c* 1.0, MeOH); IR (ATR) ν_{max} 3450, 2970, and 1720 cm^{-1}; ^1H and ^{13}C data, see Table 3; HRESIMS *m*/*z* 553.3504 $[M + Na]^+$, calcd for $C_{32}H_{50}O_6Na$, 553.3505.

Combretic acid B (**9**):. colorless needles; mp 190 °C (from MeOH); $[\alpha]_D^{20}$ +56.9 (*c* 1.0, MeOH); IR (ATR) ν_{max} 3420, 2950, and 1720 cm^{-1}; ^1H and ^{13}C data, see Table 3; HRESIMS *m*/*z* 553.3500 $[M + Na]^+$, calcd for $C_{32}H_{50}O_6Na$, 553.3505.

Cell Cultures. DLD-1/*Sac*I cells were kindly provided by Prof. Toshiyuki Sakai (Kyoto Prefectural University of Medicine). AGS cells were obtained from the Institute of Development, Aging and Cancer, Tohoku University. Both cell lines were cultured in RPMI-1640 medium (Wako) with 10% FBS. All cultures were maintained in a humidified incubator at 37 °C in 5% CO_2/95% air.

DR5 Promoter Activity Assay. The assay procedure was as described previously.[27] Briefly, DLD-1/*Sac*I cells (2×10^5 cells per well), a human colon cancer cell line that had been stably transfected with the DR5 promoter-luciferase reporter plasmid pDR5/*Sac*I,[28] were treated with different concentrations of each compound for 24 h at 37 °C. After the medium containing the compound had been removed, the cells were lysed in Cell Culture Lysis Reagent (Promega). The fluorescence of the lysate was then measured for 10 s as relative light units using a luminometer, and DR5 promoter activity was evaluated by comparing the fluorescence of the sample with that of the control (cells treated with EtOH).

TRAIL-Resistance Overcoming Activity Assay. TRAIL-resistance abrogating activity was assessed by a comparison of cell viability in the presence and absence of TRAIL using TRAIL-resistant human gastric adenocarcinoma (AGS) cell lines.[26,29] The 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. The cells were then incubated at 37 °C in a 5% CO_2 incubator for 24 h. Then, test samples with or without TRAIL (100 ng/mL) at different doses 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 with excitation at 485 nm.

■ ASSOCIATED CONTENT

Supporting Information. ^1H and ^{13}C NMR spectra of compounds **1**−**9** are available free of charge via the Internet at http://pubs.acs.org.

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