Chemical Constituents of Propolis from Myanmar and Their Preferential Cytotoxicity against a Human Pancreatic Cancer Cell Line

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A methanolic extract of propolis obtained in Myanmar was found to inhibit PANC-1 human pancreatic cancer cells preferentially under nutrient-deprived conditions (NDM), with a PC_{50} value of 9.3 μg/mL. Bioactivity-guided fractionation of the extract led to the isolation of two new cycloartane-type triterpenes, (22*Z*,24*E*)-3-oxocycloart-22,24-dien-26-oic acid (**1**) and (24*E*)-3-oxo-27,28-dihydroxycycloart-24-en-26-oic acid (**2**), together with 13 cycloartanes (**3**−**13**) and four known prenylated flavanones (**14**−**17**). Among these, compound **1** exhibited the most potent preferential cytotoxicity (PC_{50} 4.3 μM) in a concentration- and time-dependent manner. Furthermore, **1** induced apoptosis-like morphological changes of PANC-1 cells within 24 h of treatment.

Propolis, a natural resinous hive product produced by honeybees from various plant sources, has a long history of being used in folk medicine.[1,2] Recently, propolis has gained increasing popularity as an alternative medicine and as a dietary supplement for health amelioration and disease prevention in many parts of the world because of a broad spectrum of biological activities such as antioxidant,[3] antibacterial,[4,5] antiviral,[5] antifungal,[5,6] anti-inflammatory,[7] and anticancer effects.[8,9] In our ongoing study on propolis samples of various geographical origins,[10−19] we have reported on the constituents of the methanol extract of Brazilian red propolis and their preferential cytotoxicity against PANC-1 human pancreatic cancer cells in a nutrient-deprived medium (NDM).[18] In a continued investigation, we have examined the preferential cytotoxicity of the methanolic extract of propolis, collected from Shan State in Myanmar, and found the specimen to exhibit potent preferential cytotoxicity (PC_{50} 9.3 μg/mL). Thus, we carried out a phytochemical investigation on the propolis sample and obtained 17 compounds, including two new cycloartane-type triterpenes (**1** and **2**). In this paper, we report the isolation and structure elucidation of the new compounds and the preferential cytotoxicity of the constituents obtained against PANC-1 cells in a NDM.

Results and Discussion

The MeOH extract of the propolis from Myanmar, which showed potent preferential cytotoxicity (PC_{50} 9.3 μg/mL) against PANC-1 cells in a NDM, was subjected to a series of chromatographic separations and resulted in the isolation of two new cycloartane-type triterpenes, (22*Z*,24*E*)-3-oxocycloart-22,24-dien-26-oic acid (**1**) and (24*E*)-3-oxo-27,28-dihydroxycycloart-24-en-26-oic acid (**2**), together with 15 known compounds: mangiferonic acid (**3**),[20] 28-hydroxymangiferonic acid (**4**),[21] 27-hydroxymangiferonic acid (**5**),[22] (24*E*)-3-oxo-23-hydroxycycloart-24-en-26-oic acid (**6**),[20] (24*E*)-3β-hydroxycycloart-24-en-26-al (**7**),[23] isomangiferolic acid (**8**),[20] mangiferolic acid (**9**),[20] (24*E*)-3α,27-dihydroxycycloart-24-en-26-oic acid (**10**),[24] (24*E*)-3β,27-dihydroxycycloart-24-en-26-oic acid (**11**),[25] (24*E*)-3α,22-dihydroxycycloart-24-en-26-oic acid (**12**),[24] (24*E*)-3β,23-dihydroxycycloart-24-en-26-oic acid (**13**),[20] (2*S*)-5,7-dihydroxy-4′-methoxy-8,3-diprenylflavanone (**14**),[26] (2*S*)-5,7,4′-trihydroxy-8,3′-diprenylflavanone (**15**),[27] (2*S*)-5,7-dihydroxy-4′-methoxy-8-prenylflavanone (**16**),[28] and (2*S*)-5,7,4′-trihydroxy-8-prenylflavanone (**17**).[27] The identities of these known compounds were determined by analyzing their spectroscopic data and confirmed by comparing their values with those in the literature.

Table 1. ^{1}H and ^{13}C NMR Data for Compounds **1** and **2** in $CDCl_3$ (*J* values in parentheses)

	1		**2**	
position	δ_H	δ_C	δ_H	δ_C
1α	1.86 tdd (12.7, 4.2, 1.0)	33.4	1.87 tdd (12.3, 4.2, 1.0)	33.6
1β	1.56 m[a]		1.56 m[a]	
2α	2.31 ddd (14.2, 4.2, 2.7)	37.5	2.29 ddd (13.9, 4.2, 2.4)	38.2
2β	2.76 td (14.2, 6.6)		2.72 td (13.9, 6.4)	
3		216.6		218.6
4		50.2		55.0
5	1.72 m[a]	48.4	2.15 dd (12.5, 4.4)	42.8
6α, 6β	1.58 m[a]; 0.94 m	21.5	1.56 m[a]; 0.97 m	21.2
7α, 7β	1.15 m[a]; 1.38 m	25.8	1.91 m; 1.34 m	28.2
8	1.61 dd (12.0, 4.4)	47.8	1.61 m[a]	47.8
9		21.0		21.0
10		26.1		25.7
11α, 11β	2.08 m; 1.19 m	26.7	2.05 m; 1.21 m	26.7
12	1.69 m (2H)	32.9	1.67 m (2H)	32.8
13		45.6		45.4
14		49.0		48.8
15	1.28 m (2H)	35.6	1.29 m (2H)	35.5
16	1.72 m[a], 1.15 m[a]	28.3	1.39 m; 1.25 m	25.5
17	1.75 m	52.0	1.61 m[a]	52.2
18	1.09 s	18.5	0.99 s	18.2
19α,19β	0.59 d (4.4); 0.82 d (4.4)	29.6	0.61 d (4.2); 0.82 d (4.2)	29.7
20	2.77 m	35.7	1.45 m	36.0
21	1.01 d (6.4)	19.9	0.93 d (6.4)	18.1
22	5.69 t (11.4)	147.7	1.60 m[a] (2H)	35.2
23	6.19 t (11.4)	121.0	2.37 m; 2.19 m	25.7
24	7.66 d (11.9)	135.5	7.02 t (7.8)	148.9
25		125.9		129.9
26		173.4		171.2
27	1.26 s	12.1	4.37 s (2H)	57.1
28	1.05 s	22.2	3.75 d (11.7); 3.46 d (11.7)	65.2
29	1.10 s	20.8	1.08 s	16.0
30	0.92 s	19.3	0.91 s	19.3

[a] Chemical shifts were deduced on the basis of the cross-peaks in the COSY, ROESY, and HMQC spectra.

Compound **1** was isolated as a white, waxy substance, and its molecular formula was determined by HRFABMS to be $C_{30}H_{44}O_3$. The IR spectrum of **1** showed absorptions for a conjugated acid [3400 (br), 1680, 1630 cm^{-1}] and a ketone carbonyl (1710 cm^{-1}) group. The ^{1}H NMR spectrum of **1** (Table 1) displayed a set of AB doublets at δ_H 0.59 and 0.82 (J = 4.4 Hz), characteristic of a cyclopropane methylene,[29−31] together with signals of five tertiary methyls (δ_H 0.92, 1.05, 1.09, 1.10, 1.26, each s), a secondary methyl

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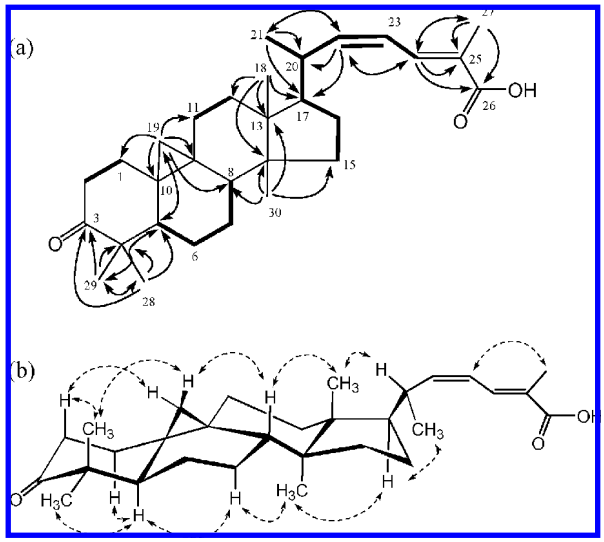


Figure 1. Connectivities (bold lines) deduced by the COSY and HMQC spectra and significant HMBC correlations (solid arrows) (a) and selected ROESY correlations (dashed arrows) (b) observed in the ROESY spectrum of **1**.

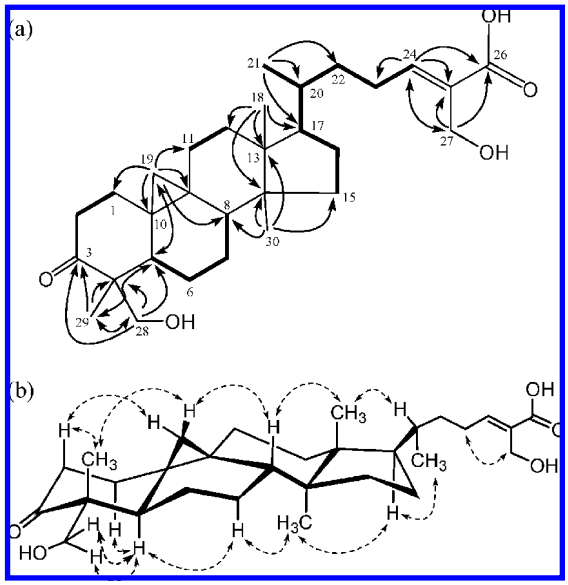


Figure 2. Connectivities (bold lines) deduced by the COSY and HMQC spectra and significant HMBC correlations (solid arrows) (a) and selected ROESY correlations (dashed arrows) (b) observed in the ROESY spectrum of **2**.

(δ_H 1.01, d, J = 6.4 Hz), and three olefinic protons (δ_H 5.69, t, J = 11.4 Hz; δ_H 6.19, t, J = 11.4 Hz; δ_H 7.66, d, J = 11.9 Hz). In turn, the ^{13}C NMR spectrum (Table 1) of **1** exhibited signals for six methyls (δ_C 12.1, 18.5, 19.3, 19.9, 20.8, 22.2), a cyclopropane methylene (δ_C 29.6), four olefinic carbons (δ_C 121.0, 125.9, 135.5, 147.7), an acid carbonyl carbon (δ_C 173.4), and a ketone carbonyl carbon (δ_C 216.6). The ^{1}H and ^{13}C NMR spectra of **1** were similar to those of mangiferonic acid (**3**),[20] isolated from the same extract, except for the presence of additional signals due to two olefinic methines (δ_H 5.69, δ_C 147.7; δ_H 6.19, δ_C 121.0) instead of the signals of two methylenes in **3** (δ_C 22.2, 34.9). The additional double bond was located between C-22 and C-23 by the analysis of the COSY and HMQC spectra of **1**, together with the HMBC correlations of H-22 with C-17 (δ_C 52.0), C-20 (δ_C 35.7), and C-24 (δ_C 135.5) and of H-24 with C-22 (δ_C 147.7), C-25 (δ_C 125.9), C-26 (δ_C 173.4), and C-27 (δ_C 12.1) (Figure 1a). The coupling constant (11.4 Hz) between H-22 and H-23 suggested their *cis* relationship,[32,33] while the ROESY correlation between H-23 and H_3-27 indicated the double bond between C_{24} and C_{25} as having an *E* configuration. Finally, the relative configuration of **1** was established to be the same as that of **3**, on the basis of ROESY correlations between H-5/H-1α, H-5/H_3-28, H-5/H-7α, H-7α/H_3-30, H_3-30/H-17, H_3-29/H-2β, H-2β/H-19α, H-19β/H-8, H-8/H_3-18, and H_3-18/H-20 (Figure 1b). Therefore, **1** was concluded to be (22*Z*,24*E*)-3-oxocycloart-22,24-dien-26-oic acid.

Table 2. Preferential Cytotoxicity of Compounds **1**−**17** for PANC-1 Cells in Nutrient-Deprived Medium (NDM)

compound	PC_{100} (μM)[a]	PC_{50} (μM)[b]
1	6.3	4.3
5	50	38.5
6	50	28.0
8	25	13.7
10	25	15.5
12	25	13.4
14	12.5	7.9
15	25	19.8
16	50	36.7
17	50	39.4
2−**4**, **7**, **9**, **11**, **13**	>100	>100
paclitaxel[c]	>100	>100
5-fluorouracil[c]	>100	>100
arctigenin[d]	1.0	0.4

[a] Concentration at which 100% cells died preferentially in NDM. [b] Concentration at which 50% cells died preferentially in NDM. [c] Conventional anticancer drug in clinical use. [d] Positive control.

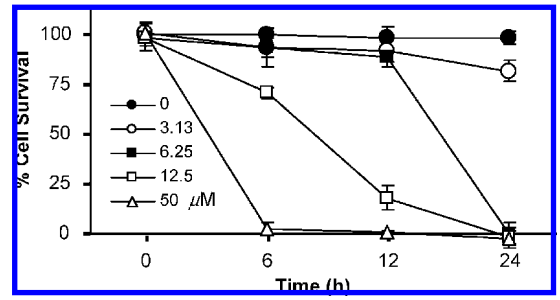


Figure 3. Effect of **1** on cell survival of PANC-1 cells in NDM.

Compound **2** was obtained as a white, amorphous solid, and its molecular formula, $C_{30}H_{46}O_5$, was established by HRFABMS. Absorption bands at 3500 and 1690 cm^{-1} in the IR spectrum of **2** indicated the presence of hydroxy and carbonyl groups, respectively. The ^{1}H NMR spectrum of **2** displayed signals due to a cyclopropane methylene (δ_H 0.61, 0.82, both d, J = 4.2 Hz), three tertiary methyls (δ_H 0.91, 0.99, 1.08, each s), a secondary methyl (δ_H 0.93, d, J = 6.4 Hz), a hydroxymethyl (δ_H 3.46, 3.75, ABq, J = 11.7 Hz), a hydroxylated allylic methyl (δ_H 4.37, s, 2H), and a conjugated olefin (δ_H 7.02, t, J = 7.8 Hz). Its ^{13}C NMR spectrum revealed signals of four methyls (δ_C 16.0, 18.1, 18.2, 19.3), a cyclopropane methylene (δ_C 29.7), two hydroxymethyls (δ_C 57.1, 65.2), two olefinic carbons (δ_C 129.9, 148.9), an acid carbonyl carbon (δ_C 171.2), and a ketone carbonyl carbon (δ_C 218.6). These ^{1}H and ^{13}C NMR data (Table 1) closely resembled those of 28-hydroxymangiferonic acid (**4**),[21] except for the appearance of the signals due to a hydroxymethyl (δ_H 4.37; δ_C 57.1) in **2**, instead of a vinyl methyl (C-27) in **4**. The hydroxymethyl group was confirmed to be at C-27 on the basis of the HMBC correlations of H_2-27 (δ_H 4.37, 2H) with the olefinic carbons at δ_C 148.9 (C-24) and 129.9 (C-25) and the carbonyl carbon at δ_C 171.2 (C-26) (Figure 2a). The relative configuration of **2** was determined to be the same as that of **4** on the basis of ROESY correlations depicted in Figure 2b. Further, the ROESY correlation between H_2-23 and H_2-27 indicated the configuration of the C_{24}−C_{25} as *E*. Thus, **2** was determined to be (24*E*)-3-oxo-27,28-dihydroxycycloart-24-en-26-oic acid.

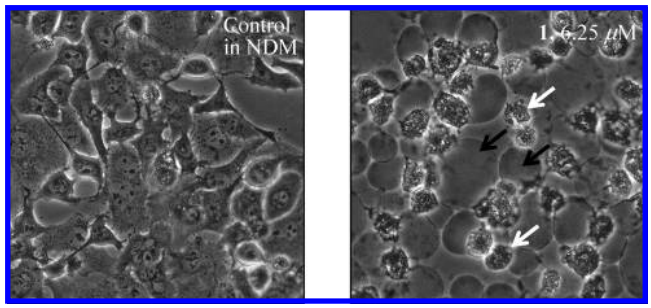


Figure 4. Morphological changes of PANC-1 human pancreatic cancer cells (white arrow: nucleus fragmentation and condensation; black arrow: membrane bleb) in NDM after 24 h exposure with 6.25 μM of **1**.

Altogether, we isolated 13 cycloartanes (**1**−**13**) and four prenylated flavanones (**14**−**17**) from the MeOH extract of the Myanmar propolis sample. Among them, compounds **3**, **5**, **9**, and **11** were major constituents and have been reported as constituents of *Mangifera indica*.[20,22,25] The other seven cycloartanes, **4**, **6**−**8**, **10**, **12**, and **13**, occur also in *M. indica*.[20−25] Moreover, *M. indica* is widely distributed in Shan State of Myanmar,[34] where the propolis used in this study was collected. Thus, the predominating plant source of the Myanmar propolis sample might be *M. indica*. Propolis from temperate zones, especially of the European type, contains mainly cinnamic acid derivatives and flavonoids, and poplar tree (*Populus nigra*) was reported to be its dominant plant source.[35] In contrast, prenylated *p*-coumaric acids and diterpenes were found to be the main constituents in Brazilian green propolis, and its main plant source was identified as *Baccharis dracunculifolia*.[36] In addition, we also reported both Nepalese propolis and Brazilian red propolis to originate from plants of the genus *Dalbergia*.[16,18] However, cycloartane-type triterpenes have been isolated from propolis for the first time, which indicates the uniqueness of propolis from Myanmar.

Pancreatic cancer cells such as PANC-1 show a marked tolerance to nutrition starvation that enables them to survive under hypovascular conditions.[37] Development of drugs countering this resistance to nutrition starvation is considered as a novel approach to anticancer drug discovery.[38] In the present work, all the isolated compounds (**1**−**17**) were further tested for their preferential cytotoxicity against PANC-1 human pancreatic cancer cells in a NDM. Among the compounds tested, (22*Z*,24*E*)-3-oxocycloart-22,24-dien-26-oic acid (**1**) displayed the most potent preferential activity (PC_{100} 6.3 μM; PC_{50} 4.3 μM) (Table 2). As shown in Figure 3, **1** remarkably eliminated the resistance of PANC-1 cells to nutrient starvation in a concentration- and time-dependent manner. Cells exposed to **1** at 6.25 μM exhibited 100% cell death within 24 h of starvation. This sensitivity was more obvious when **1** was added at 50 μM, at which 100% of the cells were killed within 6 h of treatment. Phase-contrast microscopic observation indicated that **1** induced apoptosis-like morphological changes for PANC-1 cells within 24 h at 6.25 μM in NDM (Figure 4). Interestingly, two conventional anticancer drugs in clinical use, paclitaxel and 5-fluorouracil, were inactive (PC_{100} > 100 μM). By comparing the PC_{100} or PC_{50} values of the compounds tested, the presence of the conjugated double bond in **1** seems to be important for the activity increase (**1** ≫ **3**), and the hydroxy group at C-23 or C-27 appears to enhance the activity (**6** > **3**; **5** > **3**). At C-3, the α-hydroxy group may be more favorable than a β-hydroxy group for the activity (**8** > **9**, **10** > **11**, **12** > **13**). In the flavanone skeleton, an increase in the number of prenyl groups enhances the activity (**14** > **16**, **15** > **17**), while at C-4′, a methoxy group is more favorable than a hydroxy group (**14** > **15**).

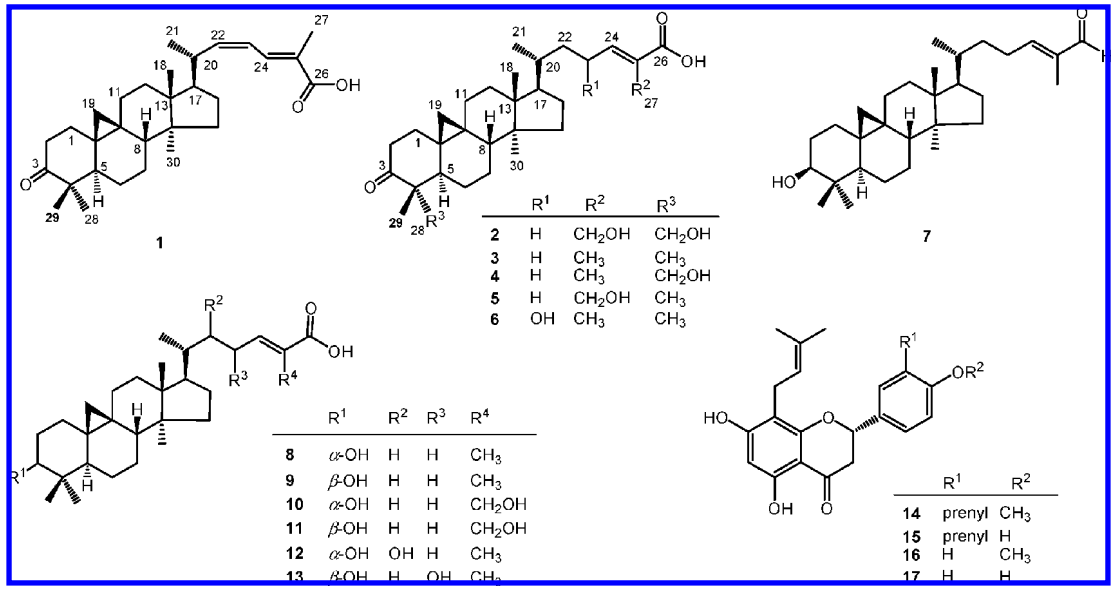
Experimental Section

General Experimental Procedures. Optical rotations were recorded on a JASCO DIP-140 digital polarimeter. IR spectra were measured with a Shimadzu IR-408 spectrophotometer. NMR spectra were taken on a JEOL JNM-LA400 spectrometer with tetramethylsilane (TMS) as an internal standard, and chemical shifts are expressed in δ values. FABMS and HRFABMS measurements were carried out on a JEOL JMS-700T spectrometer, and glycerol was used as a matrix. Column chromatography was performed with normal-phase silica gel (silica gel 60N, spherical, neutral, 40−50 μm, Kanto Chemical Co., Inc.) and reversed-phase silica gel (Cosmosil 75C_{18}-OPN, Nacalai Tesque Inc.). Medium-pressure liquid chromatography (MPLC) was performed with a Buchi pump module C-650 system. Preparative TLC was carried out on precoated silica gel 60F_{254} and RP-18F_{254} plates (Merck, 0.25 or 0.50 mm thickness).

Plant Material. Propolis was collected at Ywar Taw village, Shan State of Myanmar, in December 2006. A voucher specimen (TMPW 26484) was deposited at the Museum of Materia Medica, Research Center for Ethnomedicines, Institute of Natural Medicine, University of Toyama, Japan.

Extraction and Isolation. Propolis (100 g) was extracted with MeOH under sonication (90 min, ×3) at room temperature, and the solvent was evaporated under reduced pressure to give 15.5 g of a MeOH extract. The MeOH extract (15.0 g) was chromatographed on silica gel with hexane and then MeOH−CH_2Cl_2 solvent systems to give

Chart 1



five fractions [fr. 1: hexane eluate, 3.8 g; fr. 2: CH_2Cl_2 eluate, 2.2 g; fr. 3: MeOH−CH_2Cl_2 (5:95) eluate, 1.8 g; fr. 4: MeOH−CH_2Cl_2 (10:90) eluate, 2.1 g; fr. 5: MeOH−CH_2Cl_2 (30:70) eluate, 1.1 g].

Fraction 2 (2.2 g) was rechromatographed on silica gel with MPLC using an EtOAc−hexane gradient system to give four subfractions (fr. 2-1: 952 mg; fr. 2-2: 300 mg; fr. 2-3: 320 mg; fr. 2-4: 210 mg). Subfraction 2-2 (300 mg) was further purified by normal-phase preparative TLC with C_6H_6−$CHCl_3$ (3:7) to give 3-oxo-cycloart-24*E*-en-26-oic acid (**3**, 125 mg) and 3-oxo-28-hydroxycycloart-24*E*-en-26-oic acid (**4**, 22.5 mg). Subfractions 2-3 (320 mg) and 2-4 (210 mg) were separately subjected to preparative TLC with C_6H_6−$CHCl_3$−MeOH (30:70:2) to give 3-oxo-27-hydroxycycloart-24*E*-en-26-oic acid (**5**, 121 mg) and 3-oxo-23-hydroxycycloart-24*E*-en-26-oic acid (**6**, 17.5 mg), and 3β-hydroxycycloart-24*E*-en-26-aldehyde (**7**, 5.8 mg), respectively.

Fraction 3 (1.8 g) was rechromatographed on silica gel with MPLC using a MeOH−$CHCl_3$ gradient system to afford three subfractions (fr. 3-1: 352 mg; fr. 3-2: 151 mg; fr. 3-3: 723 mg). Subfraction 3-1 (352 mg) gave crystals of 3-oxo-27-hydroxycycloart-24*E*-en-26-oic acid (**5**, 251 mg) upon being left overnight. The mother liquor from subfraction 3-1 (81 mg) was combined with subfraction 3-2 (151 mg) and subjected to normal-phase preparative TLC with EtOAc−hexane (1:3), followed by reversed-phase preparative TLC with H_2O−CH_3CN−acetone (1:2:2), to give 3-oxocycloart-22*Z*,24*E*-dien-26-oic acid (**1**, 12.5 mg), 3β-hydroxycycloart-24*E*-en-26-aldehyde (**7**, 10.8 mg), and 5,7-dihydroxy-4′-methoxy-8,3′-diprenylflavanone (**14**, 2.3 mg). Subfraction 3-3 (723 mg) was chromatographed on reversed-phase silica gel with MPLC using an H_2O−acetone system (4:6 → 3:7 → 1:9) to afford three fractions (fr. 3-3-1: 123 mg; fr. 3-3-2: 235 mg; fr. 3-3-3: 31 mg). Fraction 3-3-1 was purified by normal-phase preparative TLC with 35% acetone−benzene to give 5,7-dihydroxy-4′-methoxy-8-prenylflavanone (**16**, 4.3 mg) and 5,7,4′-trihydroxy-8-prenylflavanone (**17**, 1.8 mg), while fraction 3-3-2 was separated by normal-phase preparative TLC with 50% EtOAc−hexane and then with 7% MeOH−CH_2Cl_2 to give 3-oxo-27,28-dihydroxycycloart-24*E*-en-26-oic acid (**2**, 21.2 mg), 3β-hydroxycycloart-24*E*-en-26-aldehyde (**7**, 5.8 mg), and 5,7,4′-trihydroxy-8,3′-diprenylflavanone (**15**, 2.5 mg).

Fraction 4 (2.1 g) was rechromatographed on reversed-phase silica gel by MPLC using an H_2O−acetone system (6:4 → 4:6 → 2:8 → 0:10) to afford four subfractions (fr. 4-1: 35 mg; fr. 4-2: 82 mg; fr. 4-3: 823 mg; fr. 4-4: 405 mg). Subfraction 4-2 was separated by normal-phase preparative TLC with MeOH−CH_2Cl_2−CF_3COOH (6:94:0.5) to give 3α,22-dihydroxycycloart-24*E*-en-26-oic acid (**12**, 2.3 mg) and 3β,23-dihydroxycycloart-24*E*-en-26-oic acid (**13**, 10.1 mg). Subfractions 4-3 and 4-4 were dissolved in $CHCl_3$−MeOH (9:1) and left overnight to give crystals of 3β,23-dihydroxycycloart-24*E*-en-26-oic acid (**11**, 253 mg) and 3β,27-dihydroxycycloart-24*E*-en-26-oic acid (**9**, 81.3 mg), respectively. Their mother liquors were combined and further purified by normal-phase preparative TLC with MeOH−CH_2Cl_2−CF_3COOH (8:92:0.5) followed by reversed-phase preparative TLC with H_2O−CH_3CN−acetone−CF_3COOH (20:20:60:0.5) to afford isomangiferolic acid (**8**, 8.2 mg), mangiferolic acid (**9**, 52.5 mg), 3α,27-dihydroxycycloart-24*E*-en-26-oic acid (**10**, 12.1 mg), and 3β,27-dihydroxycycloart-24*E*-en-26-oic acid (**11**, 83.2 mg).

(22*Z*,24*E*)-3-Oxocycloart-22,24-dien-26-oic acid (1): white, waxy substance; $[\alpha]^{22}_D$ −3.9 (*c* 1.0, $CHCl_3$); IR (KBr) ν_{max} 3400 (br), 1710, 1680, 1630, 1460 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRFABMS *m*/*z* 453.3384 (calcd for $C_{30}H_{45}O_3$ $[M + H]^+$, 453.3369).

(24*E*)-3-Oxo-27,28-dihydroxycycloart-24-en-26-oic acid (2): white, amorphous powder; $[\alpha]^{22}_D$ +25.1 (*c* 1.0, $CHCl_3$); IR (KBr) ν_{max} 3510, 3400 (br), 1690, 1640, 1460 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRFABMS *m*/*z* 487.3405 (calcd for $C_{30}H_{47}O_5$ $[M + H]^+$, 487.3424).

Preferential Cytotoxicity in Nutrient-Deprived Medium (NDM). The preferential cytotoxicity of propolis extract and the isolated compounds was determined by a procedure described previously.[37] Briefly, PANC-1 human pancreatic cancer cells were seeded in 96-well plates (2×10^4 per well) and incubated in fresh Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceuticals, Tokyo, Japan) at 37 °C under 5% CO_2 and 95% air for 24 h. After the cells were washed with PBS (Nissui Pharmaceuticals, Tokyo, Japan), the medium was changed to either DMEM or NDM [the composition of the NDM was as follows: 265 mg/L $CaCl_2(2H_2O)$, 0.1 mg/L $Fe(NO_3)(9H_2O)$, 400 mg/L KCl, 200 mg/L $MgSO_4(7H_2O)$, 6400 mg/L NaCl, 700 mg/L $NaHCO_3$, 125 mg/L NaH_2PO_4, 15 mg/L phenol red, 25 mmol/L HEPES buffer (pH 7.4), and MEM vitamin solution (Life Technologies, Inc., Rockville, MD); the final pH was adjusted to 7.4 with 10% $NaHCO_3$],[38] and serial dilutions of the test samples were added. The cell viability in each medium was then measured after 24 h of incubation. For time-dependent preferential cytotoxicity, the cells were incubated with the test compounds for 0, 6, 12, and 24 h. At the end of incubation, the morphological changes were recorded by photomicrograph using a phase-contrast microscope under 200× magnification (Olympus D-340 L/C-840 L digital camera, Tokyo, Japan). Then, the cells were washed with PBS, and 100 μL of DMEM containing 10% WST-8 (Dojindo; Kumamoto, Japan) was added to the wells. After 3 h incubation, the absorbance at 450 nm was measured. Cell viability was calculated from the mean values of data from three wells by using the following equation:

$$(\%)\ \text{Cell viability} = [(\text{Abs}_{(\text{test sample})} - \text{Abs}_{(\text{blank})})/\text{Abs}_{(\text{control})} - \text{Abs}_{(\text{blank})}] \times 100$$

The preferential cytotoxicities were expressed as PC_{100} (the concentration at which 100% cells died preferentially in NDM) and PC_{50} (the concentration at which 50% cells died preferentially in NDM) values.

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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