Cytotoxic Terpenoids and Flavonoids from *Artemisia annua*

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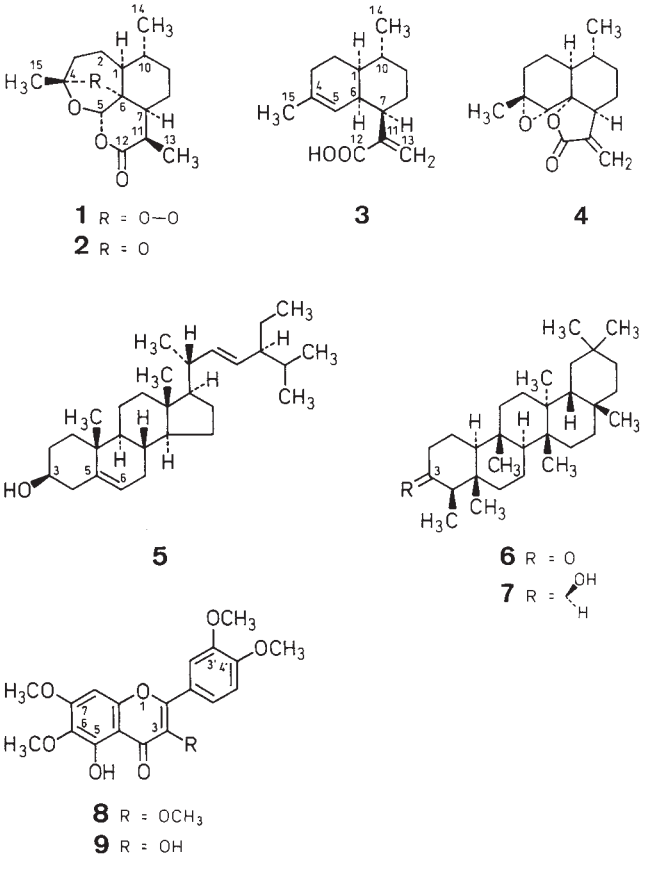
Abstract

The cytotoxic activity of nine terpenoids and flavonoids isolated from *Artemisia annua* was tested *in vitro* on several human tumor cell lines. These compounds are artemisinin, deoxyartemisinin, artemisinic acid, arteannuin-B, stigmasterol, friedelin, friedelan-3β-ol, artemetin, and quercetagetin 6,7,3′,4′-tetramethyl ether. Friedelane-type triterpenoids were isolated for the first time from this plant. Artemisinin and quercetagetin 6,7,3′,4′-tetramethyl ether showed significant cytotoxicity against P-388, A-549, HT-29, MCF-7, and KB tumor cells.

Key words

Artemisia annua, Compositae, sesquiterpene lactones, friedelane triterpenes, flavonols, cytotoxicity, human tumor cells.



Introduction

As a continuation of our search for antitumor agents from plants, we investigated the active constituents of *Artemisia annua* L. (Compositae) using *in vitro* cytotoxicity assays. *A. annua* has been used as a medicinal herb (Qinghao) for the treatment of malaria for many centuries in Chinese folk medicine (1). In this paper, the isolation and cytotoxic activity of nine terpenoids and flavonoids from the dried leaves of *A. annua* is reported. This includes artemisinin (**1**) deoxyartemisinin (**2**), artemisinic acid (**3**), arteannuin-B (**4**), stigmasterol (**5**), friedelin (**6**), friedelan-3β-ol (**7**), artemetin (**8**), and quercetagetin 6,7,3′,4′-tetramethyl ether (**9**).

Materials and Methods

Instruments

All melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. IR and UV spectra were recorded on a Laser Precision Analytical RFX-40 FTIR and Beckman UV-5260 spectrophotometers, respectively. Low and high resolution mass spectra (HRMS) were determined on a Kratos MS-30 mass spectrometer. ^{1}H- and ^{13}C-NMR including 2D-NMR and DEPT spectra were recorded on a Bruker AM-500 NMR spectrometer. Specific rotation was determined on a Perkin-Elmer 241 digital polarimeter.

Extraction, separation, and identification of active compounds

The above-ground portions of *Artemisia annua* used in this study were collected from Maryland in August 1988. Its identity was confirmed by Dr. Richard W. Spjut of the World Botanical Associates, Laurel, Maryland. The voucher specimen is deposited in the United States National Herbarium. The air-dried leaves of *A. annua* (200 g) were ground and extracted with boiling hexane for 48 h. The extract was then dissolved in 20 ml of $CHCl_3$ and to this solution was added 180 ml of CH_3CN. The insoluble ma-

terial was removed, and the filtrate was evaporated to give 5.8 g of gummy residue. A portion of the residue (4.5 g) was subjected to flash column (5 × 75 cm) chromatography on silica gel (200 g, 230–400 mesh). Gradient elution of the column started with hexane, followed by increasing percentage of EtOAc in hexane (1%, 1.5 l; 2%, 1.2 l; 5%, 1.2 l; 10%, 1.8 l; 20%, 0.5 l; 50%, 1.5 l) to yield 30 major fractions. Fractions were analyzed by silica gel TLC (solvent system A: 5% EtOAc in hexane; B: 10% EtOAc in hexane; C: 20% EtOAc in hexane). Compounds were detected by UV light (254 nm), I_2 absorption, or vanillin-H_2SO_4 spray reagent.

Artemisinin (**1**). Fraction 17 (47.2 mg, from 10 % EtOAc in hexane) yielded a white solid which was then recrystallized in $CHCl_3$-hexane (1 : 1) to give **1** (22.4 mg, R_f = 0.20, solvent system B) as colorless needles. The physical (m.p., $[\alpha]_D$) and spectral data (IR, mass, ^{1}H-NMR) were consistent with those of artemisinin (2).

Deoxyartemisinin (**2**). Column chromatography of fraction 15 (77.6 mg, from 10% EtOAc in hexane) on silica gel (5 g) eluted with 2% (0.5 l) and 10% (0.5 l) EtOAc in hexane afforded **2** (1.1 mg, R_f = 0.25, solvent system B) as colorless needles. The physical (m.p., $[\alpha]_D$) and spectral data (IR, mass, ^{1}H-NMR) were consistent with those of deoxyartemisinin (2–4).

Hydrogenation of **1**. Artemisinin (**1**, 6.5 mg) in MeOH (2 ml) was hydrogenated in the presence of 5% Pd-$CaCO_3$ (2 mg) for 10 h. The catalyst was removed by filtration through a Celite pad. The filtrate was evaporated and the residue was crystallized with Me_2CO-hexane. After storing in a refrigerator for 3 days, colorless needles were obtained (5.4 mg, 88% yield). This compound was identified as **2** by identical spectral data and TLC behavior.

Artemisinic acid (**3**). Fraction 16 (286.2 mg, from 10% EtOAc in hexane) was chromatographed on a silica gel column (5 g) eluted with 10% (0.5 l) and 15% (0.7 l) EtOAc in hexane to give **3** (32.6 mg, R_f = 0.26, solvent system B) as colorless needles. The physical (m.p., $[\alpha]_D$) and spectral data (IR, mass, ^{1}H-NMR) were consistent with those of artemisinic acid (3, 5).

Arteannuin-B (**4**). Fraction 24 (106.8 mg, from 20% EtOAc in hexane) was separated by column chromatography on silica gel (2 g) eluted with 10% (0.5 l) and 20% (0.5 l) EtOAc in hexane and then crystallized in hexane to yield **4** (32.8 mg, R_f = 0.09, solvent system B) as colorless needles. The physical (m.p., $[\alpha]_D$) and spectral data (IR, mass, ^{1}H-NMR) were consistent with those of arteannuin-B (4, 6).

Stigmasterol (**5**). Fraction 13 (36.9 mg, from 5 % EtOAc in hexane) gave a white solid, which was purified by silica gel (1 g) column chromatography eluted with 5% EtOAc in hexane (0.35 l) and by recrystallization with Me_2CO to afford **5** (3.7 mg, R_f = 0.14, solvent system A) as white needles. The physical (m.p., $[\alpha]_D$) and spectral data (IR, mass, ^{1}H-NMR) were consistent with those of stigmasterol (3).

Acetylation of **5**. The acetyl derivative (2.1 mg) prepared as routine was crystallized in EtOAc and obtained as colorless needles. The physical (m.p.) and spectral data (IR, mass, ^{1}H-NMR) were consistent with those of stigmasterol acetate (3).

Friedelin (**6**). Fraction 8 (72.3 mg, from 2 % EtOAc in hexane) afforded the crude crystals, which were then recrystallized in $CHCl_3$ to give **6** (10.1 mg, R_f = 0.26, solvent system A) as colorless needles. The physical (m.p., $[\alpha]_D$) and spectral data (IR, mass, ^{1}H- and ^{13}C-NMR) were consistent with those of friedelin (7–9).

Friedelan-3β-ol (**7**). Fraction 19 (15.3 mg, from 20% EtOAc in hexane) was purified by column chromatography on silica gel (1 g) eluted with 15% EtOAc in hexane (0.5 l), followed by recrystallization in MeOH to give **7** (0.3 mg, R_f = 0.10, solvent system A) as white prisms. The physical (m.p., $[\alpha]_D$) and spectral data (IR, mass, ^{1}H-NMR) were consistent with those of friedelan-3β-ol (7, 10).

Reduction of **6**. A solution of **6** (2.0 mg) in dry MeOH (2 ml) was treated with $NaBH_4$ (5 mg) and stirred overnight. Water (5 ml) was added to the mixture and the solution was acidified with 1 N HCl. The product was extracted with CH_2Cl_2. The CH_2Cl_2 extract was washed with water, dried, and evaporated. The product (1.4 mg) was crystallized from hexane as colorless needles. This compound was identified as **7** by identical spectral data and TLC behavior.

Artemetin (**8**). Fraction 25 (32.3 mg, from 20 % EtOAc in hexane) was separated on a silica gel column (1 g) eluted with 20% EtOAc in hexane (0.3 l), followed by crystallization with MeOH to afford **8** (4.3 mg, R_f = 0.21, solvent system C) as yellowish needles. The physical (m.p.) and spectral data (IR, UV, mass, ^{1}H- and ^{13}C-NMR) were consistent with those of artemetin (11, 12). The UV spectra with various reagents (MeOH, NaOMe, $AlCl_3$, $AlCl_3$/HCl, NaOAc, NaOAc/H_3BO_3) were determined according to the standard procedures (13).

Acetylation of **8**. The acetyl derivative (3.0 mg) prepared as routine was crystallized in MeOH and obtained as white needles, m.p. 155–157 °C. HRMS: *m/z* 430.1263 $[M]^+$ (calcd. for $C_{22}H_{22}O_9$, 430.1264). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.69 (1H, dd, J = 8.4 Hz, 2.2 Hz, H-6′), 7.66 (1H, d, J = 2.2 Hz, H-2′), 6.99 (1H, d, J = 8.4 Hz, H-5′), 6.86 (1H, s, H-8), 3.99 (3H, s, 7-OMe), 3.97 (3H, s, 3-OMe), 3.97 (3H, s, 3′- or 4′-OMe), 3.86 (3H, s, 6-OMe), 3.79 (3H, s, 4′- or 3′-OMe), 2.52 (3H, s, 5-OAc).

Quercetagetin 6,7,3′,4′-tetramethyl ether (**9**). Fraction 28 (56.2 mg, from 50% EtOAc in hexane) gave a yellow solid, which was then purified by column chromatography on silica gel (1 g) eluted with 20% (0.2 l), 30% (0.2 l), and 50% (0.1 l) EtOAc in hexane, respectively. Crystallization of **9** with MeOH afforded yellowish needles (30.2 mg, R_f = 0.12, solvent system C). The physical (m.p.) and spectral data (IR, UV, mass, ^{1}H- and ^{13}C-NMR) were consistent with those of quercetagetin 6,7,3′,4′-tetramethyl ether (4, 14). The UV spectra with the reagents were measured according to the standard procedures (13).

Cytotoxicity assays

The *in vitro* cytotoxicity assays were carried out according to the standard protocols established by the National Cancer Institute for P-388 (murine lymphocytic leukemia) (15), A-549 (human lung carcinoma) (16), MCF-7 (human breast adenocarcinoma) (17), HT-29 (human colon adenocarcinoma) (18), and KB (human nasopharynx carcinoma) (15) tumor cell lines. Adriamycin was used as a positive control. ED_{50} values ≤ 4 μg/ml for pure compounds are considered significant (15).

Results and Discussion

The ground leaves of *A. annua* were extracted with hexane. Treatment of the hexane extract with CH_3CN removed much of the accompanying waxes. Column chromatography of the extract afforded compounds **1**–**9**. These compounds were reported earlier, however, with varying quality of the spectroscopic data. Thus, their structures were confirmed on the basis of chemical correlation and spectral analysis including 2D-NMR spectroscopic techniques.

Compound **1** showed a molecular ion at *m/z* 282.1470 in the HRMS corresponding to the formula $C_{15}H_{22}O_5$. The IR spectrum exhibited the characteristic bands for lactone (1740 cm^{-1}) and peroxide function (1115, 880, 830 cm^{-1}) (2). The identity of **1** with an authentic sample of artemisinin was confirmed by comparative m.p., $[\alpha]_D$, co-TLC, and superimposable IR spectra. The molecular formula ($C_{15}H_{22}O_4$) of **2** differed from that of **1** by one oxygen. The IR spectrum showed the lactone absorption, but no peroxide bands. These data suggested that **2** might be a deoxy-analog of artemisinin. The structural correlation of **2** with **1** by hydrogenation confirmed the assignment. Compound **2** was thus identified as deoxyartemisinin (2–4).

The IR and UV spectra of **3** indicated the presence of an α,β-unsaturated carboxylic acid. The proton signals in the NMR spectrum were assigned by using 2D-COSY analysis. Based on these data, **3** was characterized as artemisinic acid (3, 5). The IR and NMR spectra of **4** indicated the presence of γ-lactone, olefinic bond, and epoxide function. Comparison of spectral data of **4** with those of arteannuin-B (4, 6) confirmed that they were the same compound.

The HRMS of **5** gave the formula as $C_{29}H_{48}O$. Analysis of 2D-COSY spectrum of **5** and comparison with the data reported in the literature indicated that **5** was stigmasterol (3). The formula of **6** was determined as $C_{30}H_{50}O$ by HRMS. The IR spectrum showed a strong ketone absorption (1715 cm^{-1}). The ^1H and ^{13}C NMR spectra were assigned by using 2D-COSY and 2D-HETCOR techniques. The formula of **7** differed from that of **6** by one H_2 unit. Its IR showed a hydroxy band (3480 cm^{-1}). These data suggested that **7** could be a carbonyl-reduced derivative of **6**, which was then supported by chemical correlation. Finally, by comparison of their physical data with those reported (7–10), compounds **6** and **7** were identified as friedelin and friedelan-3β-ol, respectively. Friedelin-type triterpenes were isolated for the first time from *A. annua*.

The UV, IR and proton NMR spectra of **8** exhibited flavonoid characteristics. The methoxy positions were determined by the methods of UV-reagents (13) and NOE. Based on the spectral analysis, **8** was identified as artemetin or artemisetin (11, 12). The molecular formula of **9** differed from that of **8** by one methylene. The UV-reagent (MeOH/NaOMe) spectra of **9** indicated the presence of 3-OH (13). Finally, consistency of the spectral data of **9** with those of quercetagetin 6,7,3′,4′-tetramethyl ether (4, 14) supported the structural assignment. It has been reported that the antimalarial activity of artemisinin can be enhanced by the presence of methoxylated flavones such as artemetin (19).

These compounds were tested for their cytotoxicity against several tumor cell lines (Table **1**). In the sesquiterpene series, only artemisinin exhibited potent cytotoxicity against P-388 (murine lymphocytic leukemia), A-549 (human lung carcinoma) and HT-29 (human colon adenocarcinoma) tumor cells with ED_{50} values of 9.62×10^{-2}, 4.16, and 4.41 μg/ml, respectively. The endoperoxide function appeared to be responsible for such high activity.

Table 1 Cytotoxicities of compounds **1**–**9** against tumor cell lines.

Compound[a]	ED_{50} (μg/ml) P-388[b]	A-549[c]	MCF-7[d]	HT-29[e]	KB[f]
Artemisinin (**1**)	9.62×10^{-2}	4.16	> 10	4.41	> 10
Deoxyartemisinin (**2**)	7.58	> 10	> 10	> 10	> 10
Artemisinic acid (**3**)	> 10	> 10	> 10	> 10	> 10
Arteannuin-B (**4**)	> 10	> 10	> 10	> 10	> 10
Stigmasterol (**5**)	> 10	> 10	> 10	> 10	> 10
Friedelin (**6**)	> 10	> 10	> 10	> 10	> 10
Friedelan-3β-ol (**7**)	> 10	> 10	> 10	> 10	> 10
Artemetin (**8**)	> 10	> 10	> 10	> 10	> 10
Quercetagetin 6,7,3′,4′-tetramethyl ether (**9**)	4.90×10^{-1}	4.81×10^{-1}	2.47	1.25	6.80×10^{-1}

[a] The cytotoxicity of reference adriamycin was in the range of ED_{50} 10^{-2}-10^{-3} μg/ml in these tests.
Significant activity is generally considered when pure compounds show $ED_{50} \leq 4$ μg/ml (15).
[b] P-388: murine lymphocytic leukemia.
[c] A-549: human lung carcinoma.
[d] MCF-7: human breast adenocarcinoma.
[e] HT-29: human colon adenocarcinoma.
[f] KB: human nasopharynx carcinoma.

Reduction of the peroxide bond to an ether bond greatly reduced cytotoxicity. Deoxyartemisinin showed marginal cytotoxicity against P-388 (ED_{50} = 7.58 μg/ml) and no activity in A-549 and HT-29 tumor cells. Neither compound **3** nor **4** was cytotoxic against these tumor cell lines at the level of ED_{50} = 10 μg/ml. For the flavonols, the 3-OH appeared to be critical for the activity. Compound **9** showed significant cytotoxicity against P-388, A-549, MCF-7 (human breast adenocarcinoma), HT-29, and KB (human nasopharynx carcinoma) cell lines with ED_{50} values of 4.90×10^{-1}, 4.81×10^{-1}, 2.47, 1.25, and 6.80×10^{-1} μg/ml, respectively, while its 3-*O*-methyl analog **8** showed no activity in any of these tumor cells. No cytotoxicity was shown by triterpenes **5**–**7** at 10 μg/ml against the above tumor cell lines.

Acknowledgements

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