Withangulatin I, a New Cytotoxic Withanolide from *Physalis angulata*

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A new withanolide, withangulatin I (2), was isolated from the whole plant of *Physalis angulata*. Its structure was established as (20*S*,22*R*)-15α-acetoxy-5β,6β-epoxy-14α-hydroxy-1,4-dioxo-witha-2,16,24-trienolide on the basis of chemical and spectroscopic methods including 2D-NMR and circular dichroism (CD) experiments. Withangulatin A (1) and withangulatin I (2) were tested for their cytotoxic activities against two human cancer cell lines, colorectal carcinoma COLO 205 and gastric carcinoma AGS, *in vitro*. Compounds 1 and 2 exhibited inhibitory activities against these two human cancer cells with IC_{50} values of 16.6 and 1.8 and 53.6 and 65.4 μM, respectively.

Key words *Physalis angulata*; Solanaceae; withanolide; withangulatin; cytotoxic activity

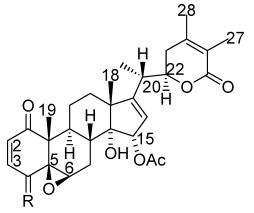
Physalis angulata L. is an annual herb belonging to the family Solanaceae and is widely distributed in tropical and temperate regions. It is a common herbal plant, known in Chinese as kuzhi, which has been used as a folk medicine with antiinflammatory, antitussive, antipyretic, diuretic, antidotal, and antitumor effects in Taiwan.[1)] The extracts and components of this plant were found to have antitumor,[2,3)] cytotoxic,[4—8)] inhibition of the ubiquitin-proteasome pathway,[9)] immunomodulatory,[10)] antimycobacterial,[11)] antinociceptive, antiinflammatory, and antiallergic activities.[12)]

Physalis angulata is known to contain a wide variety of pharmacologically important steroidal compounds, such as withasteroids.[4)] To date, phytochemical studies of this plant have described the isolation of numerous phytochemicals, including withasteroids,[4—6,8,13—17)] flavonoid glycoside,[18)] and alkaloids.[19)] In a previous study, we isolated a withanolide, withangulatin A (**1**),[15)] with significant biological activities[20)] from this plant as a part of our search for bioactive constituents from natural sources. Here, we report the isolation and structural elucidation of a minor withanolide, withangulatin I (**2**), from this plant. Its structure was established as (20*S*,22*R*)-15α-acetoxy-5β,6β-epoxy-14α-hydroxy-1,4-dioxo-witha-2,16,24-trienolide on the basis of extensive spectroscopic data interpretation, chemical transformation, and circular dichroism (CD) experiments. The cytotoxic activities of compounds **1** and **2** against human colorectal carcinoma COLO 205 and human gastric carcinoma AGS cell lines were also evaluated *in vitro*.

Results and Discussion

As in the previously reported procedure,[15)] the fractions containing withanolides were combined. The combined fractions were further purified by repeated column chromatography and preparative TLC to afford a minor withanolide, withangulatin I (**2**). The structure of **2** was established based on detailed spectral analyses, chemical transformation, and comparison with the spectral data reported in the literature.

Compound **2** was isolated as a pale yellow, amorphous powder. The IR spectrum of **2** displayed absorption bands of hydroxyl (3450 cm^{-1}), α,β-unsaturated δ-lactone (1710 cm^{-1}), and α,β-unsaturated ketone (1690 cm^{-1}) functions. The UV spectrum of **2** showed absorption at λ_{max} (MeOH) 227 nm, which also implied the presence of α,β-unsaturated δ-lactone and α,β-unsaturated ketone moieties.[21)] The molecular formula of **2** was determined to be $C_{30}H_{36}O_8$ by high-resolution (HR)-EI-MS at *m*/*z* 524.2413 (M^+, Calcd 524.2410). The ^{1}H- and ^{13}C-NMR spectral data (Table 1) showed four methyl singlets (δ 1.09, 1.38, 1.85, 1.92), a secondary methyl (δ 1.12, d, J=7.0 Hz), a 5β,6β-epoxy group [δ_H 3.56 (1H, br d, J=2.2 Hz); δ_C 63.5 (s), 64.0 (d)], an acetoxyl group [δ_H 1.97 (Me, s); δ_C 21.4 (q), 169.6 (s)], an α,β-unsaturated δ-lactone [δ_C 148.5 (C-24), 122.2 (C-25), 166.3 (C-26)], two oxygenated methines [δ_H 5.22 (d, J=2.6 Hz, H-15); δ_C 83.6 (C-15)] and [δ_H 4.23 (ddd, J=12.5, 7.0, 3.0 Hz, H-22); δ_C 79.2 (C-22)], a trisubstituted double bond [δ_H 5.73 (d, J=2.6 Hz, H-16); δ_C 121.2 (C-16), 162.3 (C-17)], and a Δ^2-1,4-dione moiety [δ_H 6.85, 6.89 (each 1H, d, J=10.3 Hz, H-2 and H-3, respectively); δ_C 202.2 (C-1), 139.4 (C-2), 141.2 (C-3), 193.6 (C-4)].[22)] The EI-MS spectrum showed a base peak at *m*/*z* 125 which confirmed the presence of the α,β-unsaturated δ-lactone moiety.[23)] These data revealed that compound **2** is a typical withanolide bearing an acetoxyl group and a Δ^2-1,4-dione moiety. The deshielding proton



(**1**) R = β-OH , α-H
(**2**) R = O

Fig. 1. Structures of Withangulatin A (**1**) and Withangulatin I (**2**)

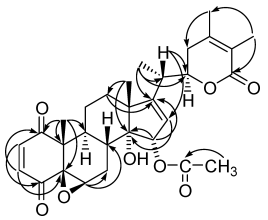


Fig. 2. Key HMBC Correlations Observed in **2**

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Table 1. ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) Spectral Data of Withangulatin I (**2**) in $CDCl_3$

Position	^{13}C	DEPT	^{1}H	HMBC (^{1}H→^{13}C)	NOESY
1	202.2	C			
2	139.4	CH	6.85 (d, J=10.3 Hz)	C-4	
3	141.2	CH	6.89 (d, J=10.3 Hz)	C-1, 5	
4	193.6	C			
5	63.5	C			
6	64.0	CH	3.56 (br d, J=2.2 Hz)	C-7, 8	H-7α, 7β
7	24.5	CH_2	2.65 (m, 7β)		H-6, 7α, 8, 15
			1.67 (m, 7α)		H-6, 7β, 9
8	34.5	CH	1.97 (m)		H-7β, 11β, 15, 18, 19
9	38.3	CH	2.36 (m)		H-7α, 11α
10	50.0	C			
11	23.0	CH_2	2.02 (m, 11α)		H-9, 11β, 12β
			1.41 (m, 11β)		H-8, 11α,18, 19
12	37.6	CH_2	1.80 (m, 12β)		H-11β, 18
			1.64 (m, 12α)		H-9
13	52.1	C			
14	81.1	C			
15	83.6	CH	5.22 (d, J=2.6 Hz)	C-13, 14, 17	H-8, 16, 18
16	121.2	CH	5.73 (d, J=2.6 Hz)	C-13, 14, 15	H-15, 21, 22
17	162.3	C			
18	16.0	CH_3	1.09 (s)	C-12, 13, 14, 17	H-8, 11β, 12β, 15
19	19.2	CH_3	1.38 (s)	C-1, 5, 9, 10	H-8, 11β
20	35.4	CH	2.51 (m)	C-16, 17, 21, 22	H-21, 22
21	17.6	CH_3	1.12 (d, J=7.0 Hz)	C-17, 20, 22	H-16, 20, 22
22	79.2	CH	4.23 (ddd, J=12.5, 7.0, 3.0 Hz)		H-16, 20, 21, 23α
23	33.0	CH_2	2.42 (m, 23β)		H-23α, 28
			2.18 (dd, J=17.6, 3.0 Hz, 23α)		H-22, 23β, 28
24	148.5	C			
25	122.2	C			
26	166.3	C			
27	12.4	CH_3	1.85 (s)	C-24, 25, 26, 28	
28	20.4	CH_3	1.92 (s)	C-23, 24, 25	H-23α, 23β
15-OAc	21.4	CH_3	1.97 (s)	15-C=O	
	169.6	C			

signals at δ 5.22 (d, J=2.6 Hz, H-15) showed NOESY cross-peaks with signals at δ 1.97 (H-8) and 1.09 (H-18) indicating a 15β-proton and the acetoxyl group was located at C-15 with the α-orientation.[15] The above ^{1}H-NMR data and the ^{13}C-NMR chemical shifts for C-14, C-15, C-16, and C-17 at δ 81.1, 83.6, 121.2, and 162.3, respectively, indicated that **2** comprises a 14α-hydroxy-15α-acetoxy-16-ene system in ring D.[6,8,13,15] The structure of the Δ^2-1,4-dione moiety in ring A was further confirmed on the basis of the following HMBC correlations: H-2 with C-4; H-3 with C-1 and C-5; H-19 with C-1, C-5, C-9, and C-10; and H-6 with C-4, C-7, and C-8. The ^{1}H- and ^{13}C-NMR spectral data of **2** were similar to those of withangulatin A (**1**), except for the signals due to ring A. The Cotton effects of **2** ($\Delta\varepsilon$+6.03 at 248 nm and $\Delta\varepsilon$−0.87 at 352 nm) were similar to those of withaperuvin E,[22] bearing the same structure of rings A, B, and the α,β-unsaturated δ-lactone moiety, indicating a 22*R* configuration and *cis*-fusion of rings A and B. Based on the above evidence, compound **2** was assumed to be 4-oxowithangulatin A, which was confirmed by manganese dioxide oxidation[21] of **1** to **2**. Therefore the structure of withangulatin I (**2**) was considered to be (20*S*,22*R*)-15α-acetoxy-5β,6β-epoxy-14α-hydroxy-1,4-dioxo-witha-2,16,24-trienolide.

Biological Studies The cytotoxic activities of compounds **1** and **2** isolated from *P. angulata*, along with doxorubicin and camptothecin (as the positive control) against two human cancer cell lines, COLO 205 (colorectal carcinoma) and AGS (gastric carcinoma) were evaluated *in vitro*. The results (as shown in Table 2) were expressed as the concentration inhibiting 50% of the cell growth (IC_{50}). Compounds **1** and **2** exhibited inhibitory activities against COLO 205 and AGS cancer cells with IC_{50} values of 16.6 and 1.8 and 53.6 and 65.4 μM, respectively. Previous structure–activity studies of withanolides indicated the importance of the 4β-hydroxy-2-ene-1-one and 5β,6β-epoxy moieties for biological activities.[24,25] Compound **1**, bearing the same moieties mentioned above, showed stronger cytotoxic activities compared with **2**, bearing Δ^2-1,4-dione and 5β,6β-epoxy moieties. These findings reveal that the 4-hydroxy group is a cytotoxic activity contributor to withanolide and the presence of 4-oxo group

Table 2. Cytotoxic Activities of Compounds **1** and **2** against Colorectal Carcinoma COLO 205 and Gastric Carcinoma AGS Cell Lines

Compounds	IC_{50} [μM][a]	
	COLO 205	AGS
Withangulatin A (**1**)	16.6±0.5	1.8±0.1
Withangulatin I (**2**)	53.6±0.5	65.4±4.2
Doxorubicin[b]	4.8±0.5	0.9±0.1
Camptothecin[b]	>100	1.2±0.1

a) IC_{50} is defined as the concentration that required to reduce the number of viable cells by 50% and the results are means±standard deviation of three independent experiments. *b*) Positive control compound.

appears to induce a decrease in the cytotoxic activity of withanolide.

Experimental

General Optical rotation was measured on a JASCO DIP-360 digital polarimeter. UV spectra were measured on a Hitachi 200 spectrophotometer. IR spectra were recorded on a Perkin Elmer 781 infrared spectrophotometer. CD spectra were recorded with a Jasco J-720 spectropolarimeter. ^{1}H-NMR (500 MHz), ^{13}C-NMR (125 MHz), and 2D-NMR spectra were recorded on a Bruker AV-500 spectrometer. EI-MS and HR-EI-MS were recorded on a JMS-700 mass spectrometer. Column chromatography was performed on silica gel 60 (Merck, 70—230 mesh), and TLC and preparative TLC were performed on precoated silica gel plates (Merck, Kieselgel 60 F_{254}, 0.25 mm and 1.00 mm, respectively).

Plant Material The entire plant of *P. angulata* was collected in Puli, Nantou county, Taiwan, and authenticated by Prof. Chang-Sheng Kuoh, Department of Biology, National Cheng Kung University. A voucher specimen was deposited in the natural product laboratory of the Department of Applied Chemistry, Chia-Nan University of Pharmacy and Science, Tainan, Taiwan.

Extraction and Isolation The dried, powdered whole plant of *P. angulata* (1.0 kg) was extracted with MeOH (9.0 l) under room temperature for 1 week. The concentrated MeOH extract (95 g) was chromatographed on a silica gel column with a $CHCl_3$/acetone mixture of increasing polarity as eluents, and each fraction was monitored by TLC. The fractions eluted with $CHCl_3$/acetone (5 : 3) were combined and evaporated to give a residue (1.8 g) that was further chromatographed on a silica gel column with *n*-hexane/EtOAc/MeOH (8 : 5 : 0.5) as eluent. The fractions containing withanolides were collected and further purified with preparative TLC and developed with *n*-hexane/EtOAc/MeOH (4 : 3 : 0.3) to give compound **2** (2.1 mg).

Withangulatin I (**2**): Pale yellow, amorphous powder; $[\alpha]_D^{27}$ +200.1° (c=0.050 $CDCl_3$); UV (MeOH) λ_{max} (log ε) 227 (4.30) nm; IR (KBr) ν_{max} 3450, 3020, 1740, 1710, 1690, 1465, 1380, 1260, 1250, 1140, 790 cm^{-1}; CD (MeOH, c 1.21×10^{-4}) $\Delta\varepsilon$ (nm): +6.03 (248), −0.87 (352); ^{1}H-NMR ($CDCl_3$, 500 MHz): see Table 1; ^{13}C-NMR ($CDCl_3$, 125 MHz): see Table 1; EI-MS *m/z* (75 eV, rel. int. %): 524 (M^+, 6), 509 (8), 482 (20), 464 (10), 449 (7), 260 (12), 125 (100); HR-EI-MS: *m/z* 524.2413 (M^+, Calcd for $C_{30}H_{36}O_8$ 524.2410).

Oxidation of 1 Compound **1** (5.1 mg) in 15 ml of chloroform was stirred with activated MnO_2 (30 mg) for 48 h. The mixture was filtered and the filtrate was evaporated to dryness. The dried residue was subjected to preparative TLC with *n*-hexane/EtOAc/MeOH (4 : 3 : 0.3) as eluent to afford a pale yellow powder (2.6 mg), which was identical to withangulatin I (**2**) (^{1}H-NMR, IR, and MS).

Cytotoxicity Assay Standard natural product anticancer agents (doxorubicin and camptothecin) were obtained from commercial sources. The cytotoxicities of compounds **1**, **2**, and the two anticancer agents against the two human cancer cell lines COLO 205 and AGS were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.[26] Human cancer cells and selected cells were plated at a density of 1×10^5 cells/ml into 24-well plates. After overnight culture, cells were pretreated with a series of concentrations of compounds **1** and **2** for 24 h. The final concentration of dimethyl sulfoxide in the culture medium was <0.05%. At the end of treatment, 30 μl of MTT was added, and the cells were incubated for an additional 4 h. Cell viability was determined by scanning with an enzyme-linked immunosorbent assay reader with a 570-nm filter.

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