Minor Withanolides of *Physalis longifolia*: Structure and Cytotoxicity

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In our recent publication on bioactive guided isolation of compounds from *Physalis longifolia* (Solanaceae) novel anti-proliferative agents withalongolides A (4) and B (5), and their highly cytotoxic analogues, withalongolide A 4,19,27-triacetate (4a) and withalongolide B 4,19-diacetate (5a) were elucidated. In this study, the two lead compounds (4, 5) were re-isolated in gram quantities for the purpose of further analogue preparation and *in vivo* testing that would continue to probe structure–activity relationships. During this process, two additional withanolides, named withalongolides O (1) and P (2), were elucidated. Their structures were determined by spectroscopic techniques with 1 being subsequently confirmed by X-ray crystallographic analysis. Utilizing a MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] viability assay, withalongolide O (1) and its 4,7-diaceatate (1a), both containing the functionalities of Δ^2-1-oxo- in A ring, a 5*β*,6*β*-epoxy in B ring, and a lactone ring in the nine-carbon side chain, exhibited potent cytotoxicity against human head and neck squamous cell carcinoma (JMAR and MDA-1986), melanoma (B16F10 and SKMEL-28), and normal fetal lung fibroblast (MRC-5) cells with IC_{50} values in the range between 0.15 and 2.95 μM. In addition, the previously reported *α* orientation of 7-acetate group in acnistins C and D should be revised to the *β* orientation on the basis of NMR data comparison.

Key words *Physalis longifolia*; withanolide; cytotoxicity; withalongolide O; withalongolide P; acnistin C

Withanolides are classified as modified ergostane-type C28 steroidal lactones, present mainly in 25 genera of the Solanaceae family, which includes *Acnistus*, *Datura*, *Dunalia*, *Jaborosa*, *Nicandra*, *Physalis*, and *Withania*.[1–3] Approximately 770 withanolides, exhibiting more than 22 different carbon frameworks, have been reported over the past five decades.[4] Among them, the classically-defined withanolides or the so-called unmodified withanolides, boasting a four-ring steroid nucleus and a nine-carbon side chain with a lactone moiety, are the most abundant forms discovered in nature. Within this category alone, nearly 550 compounds were reported thus far in the literature. Furthermore, unmodified withanolides that display the most promising anti-proliferative characteristics contain an A ring Δ^2-1-oxo-functionality, a B ring 5*β*,6*β*-epoxy group, and a nine-carbon side chain incorporating a *δ*-lactone, such as withaferin A (**6**)[3] (Fig. 1). Recently, we explored the anti-proliferative potential of compounds present in several members of the Solanaceae: *Physalis longifolia* Nutt.,[4] *Vassobia breviflora* (Sendtn.) Hunz,[5] and *Withania somnifera* (L.) Dunal.[6] Each extract, fraction and isolated compound from the three genera were evaluated *via* a series of selected cell lines that probed epithelial tumor response, specifically the head and neck squamous cell carcinoma (HNSCC) cell lines (JMAR and MDA-1986) and melanoma cell lines (B16F10 and SKMEL-28), coupled with the toxicity gauging non-malignant fetal lung fibroblast cell line (MRC5). This work resulted in the isolation, characterization, and cytotoxic evaluation of 35 withanolides.[4–6] Two of the most promising withanolides, withalongolide A 4,19,27-triacetate (**4a**) and withalongolide B 4,19-diacetate (**5a**) (Fig. 1), showed IC_{50} values less than 1 μM

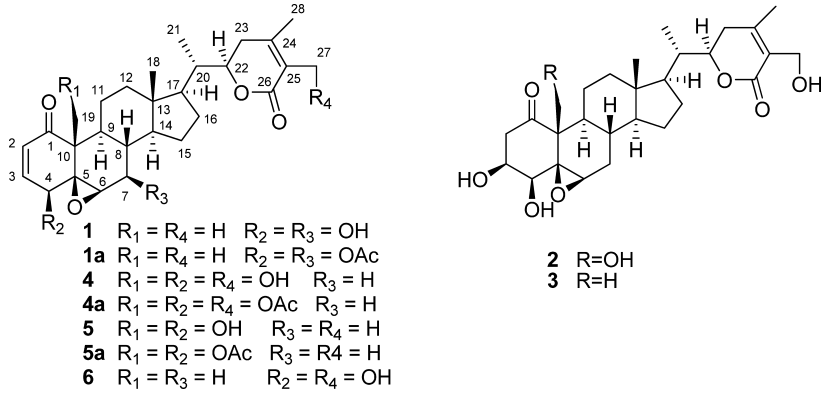

Fig. 1. Structures of Minor Withanolides (**1**–**3**) and Major Withanolides (**4**–**6**) of *Physalis longifolia*

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against all the cells tested. These two compounds were synthesized from two rare 19-OH withanolides, withalongolides A (**4**) and B (**5**) (Fig. 1), which originated from the aerial parts of *Physalis longifolia*.[4] It became apparent that to effectively probe the structure activity relationships of these analogues and in order to fulfill the requirements of an *in vivo* biological activity study in a full-term animal tumorigenesis model, a re-isolation of gram quantities of **4** and **5** was warranted.

The resulting fractionation of a dichloromethane-methanolic extract of the aerial parts of *P. longifolia* led to the isolation of two new minor withanolides: withalongolides O (**1**) and P (**2**) along with gram quantities of **4** and half gram quantities of **5** (Fig. 1). The structure elucidation of the new compounds was carried out through extensive spectroscopic data interpretations and chemical methods. Furthermore, the isolates themselves were evaluated for their anti-proliferative activities against cell lines B16F10, JMAR, MDA-1986, SKMEL-28, and MRC5.

Results and Discussion

In this present investigation, 2.97 kg dried aerial parts of *P. longifolia* were extracted with a mixture of dichloromethane and methanol (1 : 1). The extract obtained was suspended in water and subsequently partitioned with hexane, EtOAc and *n*-BuOH, respectively. The majority of the compounds isolated from the EtOAc-soluble fraction were withalongolides A (**4**), B (**5**), and withaferin A (**6**), obtained in quantities of 1.8 g (*ca.* 0.06% yield), 0.5 g (*ca.* 0.017% yield), and 1.5 g (*ca.* 0.05% yield) respectively. All of these compounds were crystallized in a hexane and acetone solution, and identified by physical data comparisons *versus* the authentic samples obtained previously in our laboratory.

Compound **1** was obtained as colorless needle-like crystals by EtOAc re-crystallization. Its molecular formula was determined to be $C_{28}H_{38}O_6$ by high resolution-electrospray ionization (HR-ESI)-MS. The ^{1}H-NMR spectrum (in $CDCl_3$) of **1** (Table 1) displayed characteristic signals for five methyl

Table 1. ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) Data for Withanolides **1**, **1a**, and **2**

Pos.	**1**[a,b]		**1a**[a]		**2**[c,d]	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		201.5		200.5		209.6
2	6.20 d (9.9)	132.8	6.24 d (9.8)	134.0	3.39 dd (16.2, 7.8), 3.17 dd (16.2, 2.7)	44.7
3	6.92 dd (9.9, 5.9)	141.7	6.99 dd (9.8, 6.0)	139.8	4.74 m	69.6
4	3.77 d (5.9)	69.6	4.66 d (6.0)	71.2	3.97 br s	77.8
5		68.2		64.6		63.7
6	3.28 d (2.0)	65.6	3.33 d (2.0)	60.6	3.75 s	58.9
7	3.56 m	73.3	4.81 dd (2.0, 9.5)	74.7	2.29 m, 1.62 m	32.0
8	1.41 m	38.8	1.74 m	34.6	1.66 m	31.0
9	1.11 td (12.0, 4.2)	43.6	1.08 m	43.8	1.58 m	43.1
10		47.2		47.5		57.2
11	1.90 m, 1.45 m	27.4	1.62 m, 1.40 m	26.1	1.73 m, 1.45 m	22.6
12	1.92 m, 1.06 m	39.3	1.93 m, 1.03 m	39.1	1.80 td (3.3, 12.7), 0.96 m	40.0
13		43.5		43.5		43.1
14	1.07 m	55.7	1.05 m	55.2	0.80 m	56.9
15	1.78 qd (3.7, 16.0), 1.45 m	22.3	1.75 m, 1.49 m	21.7	1.50 m, 0.93 m	24.7
16	1.68 m, 1.36 m	27.9	1.62 m, 1.33 m	27.8	1.50 m, 1.13 m	27.6
17	1.02 m	51.4	1.01 m	51.3	1.00 m	52.3
18	0.70 s	11.9	0.69 s	11.8	0.51 s	12.0
19	1.39 s	17.1	1.40 s	15.9	4.91 dd (9.3, 2.7), 4.22 dd (9.3, 3.2)	60.3
20	1.95 m	39.0	1.94 m	38.9	1.88 m	39.4
21	0.97 d (6.6)	13.7	0.96 d (6.6)	13.6	0.95 d (6.7)	13.9
22	4.36 td (13.3, 3.4)	78.5	4.31 td (13.3, 3.4)	78.4	4.38 td (13.3, 3.4)	78.8
23	2.42 t (15.2), 1.90 m	29.8	2.39 t (15.2), 1.85 m	29.8	2.37 dd (13.2, 17.0), 2.04 (3.1, 17.0)	30.4
24		149.2		149.2		154.4
25		122.2		122.2		127.9
26		167.3		167.2		166.8
27	1.87 s	12.7	1.85 s	12.7	4.87 dd (11.7, 3.1), 4.77 dd (11.7, 2.0)	56.6
28	1.91 s	20.8	1.90 s	20.7	2.11 s	20.6
4-OAc				170.0		
			2.03 s	20.9		
7-OAc				171.4		
			2.07 s	21.7		

a) In $CDCl_3$. *b*) OH-4 δ 2.56 br s, OH-7 δ 1.60 br s. *c*) In C_5D_5N. *d*) OH-19 δ 8.20 br s, OH-4 δ 7.75 br s, OH-3 δ 7.22 br s, OH-27 δ 6.50 br s.

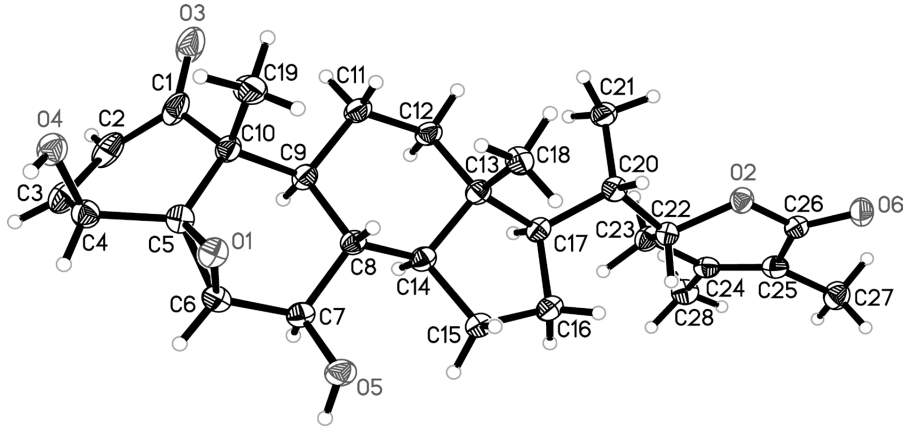

Fig. 2. X-Ray ORTEP Drawing of Withalongolide O (**1**)

groups [δ_H 0.70 (3H, s), 0.97 (3H, d, J=6.6 Hz), 1.39 (3H, s), 1.87 (3H, s), and 1.91 (3H, s)]. The ^{13}C-NMR (APT) and heteronuclear single quantum coherence (HSQC) spectra of **1** (Table 1) disclosed 28 carbons differentiated as five CH_3, five CH_2, eleven CH (including two olefins at δ 141.7 and 132.8, four oxygenated at δ 78.5, 73.3, 69.6, 65.6), and seven C (including one keto carbonyl at δ 201.5, one ester carbonyl at δ 167.3, two olefins at δ 149.2 and 122.2, and one oxygenated at δ 68.2). Its NMR data were closely related to those of the two previously isolated isomers: withalongolide B (**5**) and withaferin A (**6**).[4] Detailed comparison of the ^{1}H- and ^{13}C-NMR spectral data of **1** with those of the two analogues indicated that **1** contained the same A ring with a Δ^2-1-oxo-4-hydroxy functionality as compound **6**, and that it yielded identical substituent patterns in C, D rings, the side chain with a δ-lactone ring as in **5**. This suggested the only difference present was the substitution pattern in the B ring. The obvious difference observed among **1**, **5**, and **6** was the presence of an oxygenated methine (δ 73.3, CH) in **1** and an oxygenated methylene in **5** (C-19: δ 62.1, CH_2) and **6** (C-27: δ 57.7, CH_2),[4] implying that **1** was either a 27-deoxy-7-hydroxy derivative of **6** or a 19-deoxy-7-hydroxy derivative of **5**. This observation was supported by the high-frequency shifts of C-6 (δ 65.6 in **1** and δ 62.7 in **6**) and C-8 (δ 38.8 in **1** and δ 29.9 in **6**) in the ^{13}C-NMR spectrum of **1** and ^{13}C-NNR data comparison with previously reported withanolides.[7] It was also supported by the ^{1}H–^{1}H correlation spectroscopy (COSY) and HSQC spectra, showing a fragment of $C(O_{epoxide})$–$CH(O_{epoxide})$–CH(OH)–CH–CH of ring B in **1** and a fragment of $C(O_{epoxide})$–$CH(O_{epoxide})$–CH_2–CH–CH of ring B in **6**. Further corroboration was done by heteronuclear multiple bond connectivity (HMBC) experiments which exhibited correlations between H-6 (δ 3.28, d, J=2.0 Hz) and C-4 (δ 69.6) and C-6 (δ 65.6); between H-4 (δ 3.77, d, J=5.9 Hz) and C-5 (δ 68.2) and C-6 (δ 65.6) in **1**. The orientation of the hydroxyl group at C-7 was deduced as β due to the large coupling constant (J=9.5 Hz) present between H-7α and H-8[8,9] with a *trans*-axial relationship and nuclear Overhauser effect spectroscopy (NOESY) correlations between H-7 and H-14.

Acetylation of **1** with acetic anhydride in pyridine yielded the 4,7-diacetate derivative **1a** (Table 1), which confirmed the presence of hydroxy groups at C-4 and C-7 by a high frequency shift of H-4 (from δ 3.77 in **1** to δ 4.66 in **1a**) and H-7 (from δ 3.56 in **1** to δ 4.81 in **1a**) and the disappearance of the signals of the labile protons of 4-OH (δ 2.56 br s) and 7-OH (δ 1.60 br s) in **1** (Table 1).

Finally, the structure of **1** was confirmed through a single crystal X-ray diffraction experiment (Fig. 2). Thus, **1** (named as withalongolide O) was determined as 27-deoxy-7β-hydroxywithaferin A. All of the material obtained was used to obtain spectroscopic, crystallographic, and biological data and to prepare the derivative **1a**; doing so unfortunately did not leave enough material for a melting point and UV data determination of **1**.

X-Ray diffraction analysis of **1** showed that the dihedral angles of H_6–C_6–C_7–H_7 and H_7–C_7–C_8–H_8 were 57° and 166° respectively, which explains the observed small coupling constant of $J_{H6,H7}$=2.0 Hz [equatorial (H-6)-axial (H-7) relationship] and the large coupling constant of 9.5 Hz ($J_{H7,H8}$, *trans*-axial relationship). In our previous X-ray diffraction study of withalongolide A (**4**), B (**5**) and withaferin A (**6**),[4] it was presented that the dihedral angles of H_6–C_6–C_7–$H_{7\alpha}$ and H_6–C_6–C_7–$H_{7\beta}$ were both close to 57° while those of $H_{7\alpha}$–C_7–C_8–H_8 and $H_{7\beta}$–C_7–C_8–H_8 were within the range of 167–174° and 50–59°, respectively. These results are consistent with the small coupling constant of approximately 2.0 Hz ($J_{H6,7\alpha}$ or $J_{H6,7\beta}$), the medium coupling constant of 4.0 Hz ($J_{H7\beta,8}$), and the large coupling constant of 14.5 Hz ($J_{H7\alpha,8}$) observed for withanolides **4**–**6**. It is evident that, in withanolides with an α,β-unsaturated ketone in the A ring and a 5β,6β-epoxy functionality (such as in **1**, **1a**, **4**, **5**, **6**), the small coupling constant between H-6 and H-7 (either H-7α or H-7β) could not be used to propose the orientation of the functional group at C-7, but the large coupling constant between H-7 and H-8 can be reliably used for the determination of the β orientation of the C-7 functional group due to the presence of the axially oriented H-8 in the four-ring withanolide moiety. A structurally related withanolide acnistin C (Fig. 3), with an α,β-unsaturated ketone in the A ring and a 5β,6β-epoxy functionality in B ring, was incorrectly proposed in 1994 to have an α orientation of the acetoxy group at C-7 based on the small coupling constant of $J_{H6,7}$=1.3 Hz ["which establishes a *trans* diequatorial relationship between H-6 (δ 3.21, d, J=1.3 Hz) and H-7 (δ 4.87, d, J=10.0 Hz)"].[10] The orientation of the 7-OAc group of acnistin C should be revised to be β (akin to values observed in **1a**) due to the originally reported large coupling constant of $J_{H7,8}$=10.0 Hz for acnistin C. Relative to acnistin C, the α orientation of the 7-OAc group in acnistin D (Fig. 3) [H-6: δ

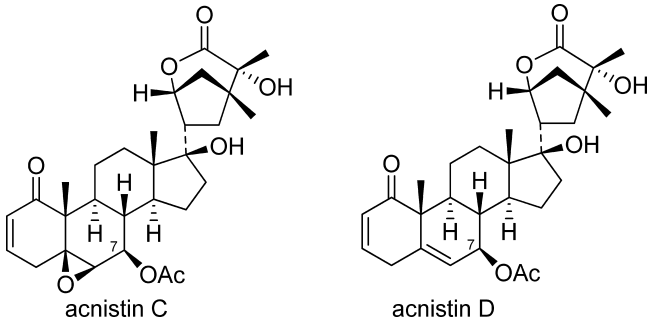

Fig. 3. Orientation of the Acetoxy Group at C-7 in Acnistins C and D Revised from α to β

5.34, s; H-7: δ 5.01, d, J=8.5 Hz][10] should be revised to be β as it exhibited a large coupling constant of $J_{H7\alpha,8}$=8.5 Hz.

Compound **2** was isolated as a white amorphous powder. Its molecular formula was determined to be $C_{28}H_{40}O_8$ by HR-ESI-MS. The ^{1}H-NMR spectrum (in C_5D_5N) of **2** (Table 1) displayed signals for three methyl groups [δ_H 0.51 (3H, s), 0.95 (3H, d, J=6.7 Hz), and 2.11 (3H, s)]. The ^{13}C-NMR (APT) and HSQC spectra of **2** (Table 1) disclosed 28 carbons differentiated as three CH_3, nine CH_2 (including two oxygenated at δ 56.6, 60.3), nine CH (including four oxygenated at δ 78.8, 77.8, 69.7, 58.9), and seven C (including one keto carbonyl at δ 209.6, one ester carbonyl at δ 166.8, two olefins at δ 154.4, 127.9, and one oxygenated at δ 63.7). Its NMR data are similar to those we obtained for the previously isolated viscosalactone B (**3**) (in C_5D_5N).[4,11] The obvious differences between **2** and **3** were the presence of an oxygenated methylene [C-19, ^{13}C: δ 60.3; ^{1}H: δ 4.91 (1H, dd, J=9.3, 2.7 Hz), 4.22 (1H, dd, J=9.3, 3.2 Hz)] in **2** and a methyl carbon [C-19, ^{13}C: δ 16.1; ^{1}H: 1.76 (3H, s)] in **3**, suggesting that **2** is a 19-hydroxy derivative of **3**. This observation was supported by the high-frequency shift of C-10 (δ 57.2 in **2** and δ 49.7 in **3**), the low-frequency shifts of C-1 (δ 209.6 in **2** and δ 210.5 in **3**), C-5 (δ 63.7 in **2** and δ 65.5 in **3**), and C-9 (δ 43.1 in **2** and δ 43.7 in **3**) in the ^{13}C-NMR spectra, and the HMBC correlations between H_2-19 [δ 4.91 (1H, dd, J=9.3, 2.7 Hz), 4.22 (1H, dd, J=9.3, 3.2 Hz) and C-1, C-5, C-9, and C-10. Thus, **2** (named as withalongolide P) was determined as 19-hydroxy viscosalactone B. This is the 7th example of a withanolide with an oxygenated C-19 group isolated from *P. longifolia*.

All the withanolides (**1**–**6**) and the acetylated derivative (**1a**) were tested against the HNSCC (JMAR, MDA-1986), melanoma (B16F10 and SKMEL-28), and normal fetal lung fibroblast (MRC-5) cells for their anti-proliferative activities. As shown in Table 2, withanolides **1**, **1a**, **4**, **5**, **6** exhibited cytotoxic effects against the cells tested with IC_{50} values in the range 0.15–12.7 μM, while **2** and **3** were inactive when tested with a concentration of 20 μM. As consistent with previous observations,[3,4] **1** and **1a** containing the functionalities of Δ^2-1-oxo- in A ring, a 5β,6β-epoxy in B ring, and a lactone ring in the side chain, were active, which is consistent with expectations that these three groups are required for activity. The esterification of the hydroxy groups at C-4 and C-7 increased the resultant cytotoxicity when comparing the IC_{50} values of **1** and **1a**. On the other hand, **2** and **3**, lacking a Δ^2-1-oxo-functionality in A ring, were inactive. It is interesting to note that the three isomers **1**, **5**, and **6** demonstrated potent cytotoxicity although the position of the hydroxyl groups in the three withanolides differed (**1**, **5**, and **6** have a 7β-hydroxy, a 19-hydroxy, or a 27-hydroxy group, respectively). This observation not only revealed the significance of the above-mentioned functionalities, but also supported the hypothesis that the presence of a OH group at either C-7, or C-19, or C-27 is not critically responsible for the observed anti-proliferative activity.[3]

Experimental

General Melting point was obtained using an MPA100 melting point apparatus. UV-vis measurement was conducted with a Varian Cary 50 UV-vis spectrophotometer. Optical rotations were measured with a Rudolph RS Autopol IV automatic polarimeter. IR data were obtained with a Thermo Nicolet Avatar 360 FT-IR spectrometer or a Perkin-Elmer Spectrum 100 FT-IR instrument. NMR spectra were recorded with a Bruker AV-500 instrument with a cryoprobe. Chemical shift values are given in δ (ppm) using the peak signals of the solvent $CDCl_3$ (δ_H 7.24 and δ_C 77.23) or C_5D_5N (δ_H 8.74, 7.58, 7.22; and δ_C 150.35, 135.91, 123.87) as references, and coupling constants are reported in Hz. HR-ESI-MS data were collected with a LCT Premier time-of-flight mass spectrometer (Waters Corp., Milford, MA, U.S.A.). Column chromatography was performed on silica gel (particle size 12–25 μm) (Sorbent Technologies, Atlanta, GA, U.S.A.). Normal-phase silica gel G TLC plates (w/UV 254) (Sorbent Technologies) were used for fraction/compound detection. The spots were visualized using UV light at 254 nm and 10% EtOH–sulfuric acid spray reagent. Semi-preparative HPLC was performed on an Agilent 1200 unit equipped with a DAD detector, utilizing a Luna RP-18 column (250×10 mm, 5 μm). Preparative thin layer chromatography was carried out using Analtech (Newark, DE) TLC plates (silica gel GF with UV 254 nm, 1000 microns). A CombiFlash® (Teledyne Isco, Lincoln, NE, U.S.A.) apparatus was used for some compound purifications (24 g normal phase RediSep® Rf flash column, 33–35 mL/min, maximum pressure 350 psi).

Plant Material Fresh aerial parts of *P. longifolia* were collected in the Kanopolis wildlife area (latitude: 37.17318°;

Table 2. Cytotoxicity IC_{50} of Withanolides (μM) against Five Cell Lines[a]

Compound	B16F10	SKMEL-28	JMAR	MDA-1986	MRC-5
Withalongolide O **1**	1.82±0.21	0.33±0.06	3.2±0.62	1.5±0.08	1.3±0.11
Withalongolide O 4,7-diacetate **1a**	0.69±0.14	0.18±0.03	0.83±0.14	0.54±0.07	0.53±0.12
Withalongolide A **4**	11.6±0.19	5.6±0.60	5.1±0.23	3.10±0.46	12.1±0.42
Withalongolide B **5**	0.26±0.07	3.3±0.48	0.25±0.14	1.7±0.08	0.38±0.03
Withaferin A **6**	0.27±0.04	3.5±0.15	1.5±0.23	0.86±0.25	0.32±0.06
Cisplatin (positive control)	1.4±0.35	1.7±0.32	1.5±0.41	1.9±0.58	9.1±0.25

a) For cell lines used, see text. Withalongolide P **2** and viscosalactone B **3** were inactive for all cell lines used (IC_{50}>20 μM).

longitude: 100.45146°) of Meade County, KS, U.S.A., in August 2010. It was identified by plant taxonomist Dr. Kelly Kindscher at the Kansas Biological Survey, University of Kansas. A voucher specimen (Hillary Loring 4095) was deposited in the R.L. McGregor Herbarium of the University of Kansas.

Cytotoxicity Bioassay The cytotoxicity assays were performed as previously described.[5] In general, ten concentrations ranging from 50 nM to 20 μM were tested for each withanolide. Statistical analysis was carried out by one-way analysis of variance (ANOVA) on ranks test using GraphPad Prism 5 (GraphPad Software, San Diego, CA, U.S.A.). IC_{50} values were obtained from cell viability plots fitted with a sigmoidal dose–response function with variable slope using GraphPad Prism 5 software.

Extraction and Isolation The collected biomass was air dried at room temperature. The dried material was ground to a coarse powder (2.97 kg), and extracted three times with CH_2Cl_2–MeOH (1 : 1, 10.0 L) at room temperature. After removing the solvents under vacuum, the extract (350 g) was suspended in 1 L H_2O, followed by partitions with *n*-hexane, EtOAc, and *n*-butanol (3×1 L). The resulting ethyl acetate fraction (50 g) was applied to silica gel flash CC (column chromatography), and eluted subsequently with hexane–acetone mixtures of increasing polarities. The fraction obtained on elution with hexane–acetone (4 : 1) (1.0 g), was again subjected to silica gel CC [eluted with CH_2Cl_2–CH_3COCH_3 (9 : 1)] to afford compound **5** (510 mg). The fraction obtained on elution with hexane–acetone (70 : 30) afforded **6** (1.0 g) and crude withaferin A (3.2 g). 1.52 g crude withaferin A was subjected to CombiFlash® purification, eluted by CH_2Cl_2–MeOH (97 : 3), to yield **6** (0.45 g) and by CH_2Cl_2–MeOH (95 : 5) to afford a mixture containing **1**. The latter residue was subjected to multiple runs of preparative TLC using CH_2Cl_2–MeOH (97 : 3) and CH_2Cl_2–EtOAc (80 : 20) to afford an off-white solid that was recrystallized from EtOAc to afford a sample of pure **1** (7.0 mg). The fraction acquired on elution with hexane–acetone (3 : 2) (2.2 g), was applied to silica gel CC [eluted with hexane–acetone (3 : 2)] to afford compound **4** (1.8 g) and crude **2** (50 mg). This crude **2** sample was subjected to semi-preparative HPLC, with the mobile phase CH_3CN–H_2O (26 : 74), to afford compound **2** (20 mg).

Withalongolide O (**1**): A colorless needle crystal (EtOAc); IR (neat) ν_{max} 3418 (br), 2937, 1684, 1397, 1128, 1034, 916, 731 cm^{-1}; $[\alpha]_{546}^{25}$ +29.5 [*c*=0.15, CH_2Cl_2–MeOH (9 : 1)]; HR-MS (ESI) *m/z* 471.2749 $[M+H]^+$ (Calcd for $C_{28}H_{39}O_6$, 471.2741); ^{1}H- and ^{13}C-NMR data, see Table 1.

Single-Crystal X-Ray Structure Determination of Withalongolide O (**1**): Crystal analysis was performed with a colorless triangular plate crystal (dimensions 0.34×0.26×0.09 mm^3) obtained from EtOAc using Cu*Kα* radiation (*λ*=1.54178 Å) on a Bruker APEX2 diffractometer equipped with a Bruker MicroStar microfocus rotating anode X-ray source and Helios multilayer optics. Crystal data for **1**: empirical formula $C_{32}H_{46}O_8$ ($C_{28}H_{38}O_6$+$CH_3COOCH_2CH_3$, formula weight 558.69), monoclinic, space group $P2_1$, *T*=100(2) K, crystal cell parameters *a*=10.830(19) Å, *b*=12.273(2) Å, *c*=11.693(2) Å, *β*=112.283(3)°, *V*=1438.0 (4) $Å^3$, D_c=1.29 Mg/m^3, *Z*=2, *F*(000)=604, absorption coefficient μ=0.743 mm^{-1}. A total of 15638 reflections were collected in the range $4.09<\theta<67.39°$, with 4754 independent reflections [$R_{(int)}$=0.0235] and 4741 with $I>2\sigma(I)$, completeness to θ_{max} was 98.7%. Multi-scan absorption correction applied; full-matrix least-squares refinement on F^2, the number of data/restraints/parameters were 4754/1/533; goodness-of-fit on F^2=1.031; final *R* indices [$I>2\sigma(I)$], R_1=0.0257, ωR_2=0.0678; *R* indices (all data), R_1=0.0258, ωR_2=0.0679; largest difference peak and hole, 0.190 and −0.154 e/$Å^{-3}$.

Acetylation of Withalongolide O (1) A solution of **1** (4.2 mg) and 4-(dimethylamino)pyridine (*ca.* 0.2 mg) in pyridine (0.1 mL) and acetic anhydride (0.1 mL) was stirred at room temperature under nitrogen atmosphere for 1.5 h. Pyridine and acetic anhydride were removed under reduced pressure. Absolute ethanol was added to the residue and subjected to evaporation to remove the residual reagents. The pale yellow residue obtained was dissolved in CH_2Cl_2, applied on a preparative TLC plate, and developed twice by 2% in CH_2Cl_2–MeOH (98 : 2). The band corresponding to the product was scraped and eluted with 5% MeOH in CH_2Cl_2. Evaporation of elution solvent under reduced pressure gave 4.9 mg of **1a** as a colorless residue.

Withalongolide O 4,7-Diacetate (**1a**): IR (neat) 2926, 1735, 1702, 1372, 1229, 1125, 1022, 914, 731 cm^{-1}; HR-ESI-MS *m/z* 555.2944 $[M+H]^+$ (Calcd for $C_{32}H_{43}O_8$, 555.2952); ^{1}H- and ^{13}C-NMR data, see Table 1.

Withalongolide P (**2**): mp 227–228°C; UV (MeOH) λ_{max} (log *ε*) 220 (4.10) nm; IR (neat) 3375 (br), 2940, 1690, 1390, 1184, 1000, 922, 794 cm^{-1}; $[\alpha]_D^{25}$ +6.7 (*c*=0.15, MeOH); HR-ESI-MS *m/z* 505.2811 $[M+H]^+$ (Calcd for $C_{28}H_{41}O_8$, 505.2801); ^{1}H- and ^{13}C-NMR data, see Table 1.

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