

Cytotoxic Withanolide Constituents of *Physalis longifolia*

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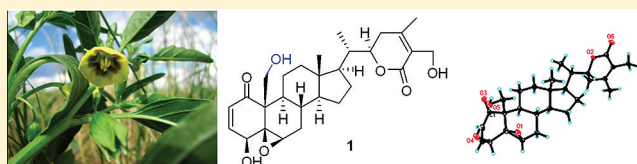
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Supporting Information

ABSTRACT: Fourteen new withanolides, **1–14**, named withalongolides A–N, respectively, were isolated from the aerial parts of *Physalis longifolia* together with eight known compounds (**15–22**). The structures of compounds **1–14** were elucidated through spectroscopic techniques and chemical methods. In addition, the structures of withanolides **1**, **2**, **3**, and **6** were confirmed by X-ray crystallographic analysis. Using a MTS viability assay, eight withanolides (**1**, **2**, **3**, **7**, **8**, **15**, **16**, and **19**) and four acetylated derivatives (**1a**, **1b**, **2a**, and **2b**) showed potent cytotoxicity against human head and neck squamous cell carcinoma (JMAR and MDA-1986), melanoma (B16F10 and SKMEL-28), and normal fetal fibroblast (MRC-5) cells with IC₅₀ values in the range between 0.067 and 9.3 μM.



Classically defined withanolides are a group of C₂₈ ergostane-type steroids with a C-22,26 δ-lactone group, first isolated from the genus *Withania*.¹ They are present primarily in the Solanaceae family, which includes the genera *Acnistus*, *Datura*, *Dunalia*, *Jaborosa*, *Nicandra*, *Physalis*, and *Withania*.^{2–9} Withanolides have attracted interest in recent years mainly due to their exhibition of significant biological activities, inclusive of antimicrobial, antitumor, anti-inflammatory, immunomodulatory, and insect-antifeedant activities.^{2,3,6} It has been reported that those withanolides displaying the most promising antitumor characteristics contain an α,β-unsaturated ketone in ring A, a 5β,6β-epoxy group in ring B, and a nine-carbon side chain with an α,β-unsaturated δ-lactone group.¹⁰ The typical withanolide withaferin A (**16**) (Figure 1) contains these three moieties and has been shown in vitro and in vivo to suppress the growth of an array of tumor cells, including breast, pancreatic, prostate, lung, leukemia, and head and neck squamous cell carcinoma (HNSCC), by inducing apoptosis,¹¹ thus possessing potential application as an antiproliferative agent. As part of an ongoing study of withanolides from plant sources,^{11,12} a library of 224 native plant extracts from the U.S. Great Plains was evaluated for cytotoxic activities against HNSCC and melanoma cell lines using the MTS viability assay. One of the most promising leads, *Physalis longifolia* Nutt. (Solanaceae), commonly known as “long leaf groundcherry”, was subjected to a phytochemical investigation, and the results are presented herein, including the details of the isolation and structure elucidation of 14 new withanolides (**1–14**), four acetylated derivatives (**1a**, **1b**, **2a**, and **2b**), and eight known compounds (**15–22**). Their cytotoxicity was determined against HNSCC (JMAR and MDA-1986), melanoma (B16F10 and SKMEL-28), and normal

fetal fibroblast (MRC-5) cells. This constitutes the first report of a phytochemical and bioactivity study of *P. longifolia*.

■ RESULTS AND DISCUSSION

The CH₂Cl₂–MeOH (1:1) extract of the aerial parts of the title plant and the EtOAc-soluble and *n*-BuOH-soluble fractions showed cytotoxicity against the above-mentioned cells with IC₅₀ values in the range between 0.7 and 9.8 μg/mL using a MTS assay. All compounds (**1–22**) were isolated from the EtOAc-soluble or *n*-BuOH-soluble fractions (see Experimental Section).

Compound **1** was isolated as colorless cuboid crystals obtained from a CH₂Cl₂–CH₃CN mixture, a major metabolite in the EtOAc-soluble fraction. Its molecular formula, C₂₈H₃₈O₇, was determined by HRESIMS and NMR experiments, equating to 10 double-bond equivalents. Its IR absorptions revealed the presence of hydroxy (3431 and 3233 cm⁻¹), keto (1671 cm⁻¹), and ester (1706 cm⁻¹) groups. The ¹H NMR spectrum (Table 1) showed the presence of three methyl groups at δ 0.60 (3H, s), 0.90 (3H, d, *J* = 6.6 Hz), and 1.97 (3H, s), seven protons attached to oxygenated carbons at δ 3.18 (1H, brs), 3.52 (1H, d, *J* = 6.1 Hz), 3.65 (1H, d, *J* = 9.6 Hz), 4.17 (1H, d, *J* = 9.6 Hz), 4.23 (1H, d, *J* = 12.5 Hz), 4.28 (1H, d, *J* = 12.5 Hz), and 4.33 (1H, dt, *J* = 13.3, 3.4 Hz), and two olefinic methine groups at δ 6.16 (1H, d, *J* = 10.0 Hz) and 6.95 (1H, dd, *J* = 10.0, 6.1 Hz). The ¹³C NMR (APT) and HSQC spectra for **1** (Table 2) displayed 28 carbon signals differentiated as three CH₃, eight CH₂ (including two oxygenated at δ 61.0 and 56.7), 10 CH (including two olefins at δ 145.7 and 132.9, three oxygenated at

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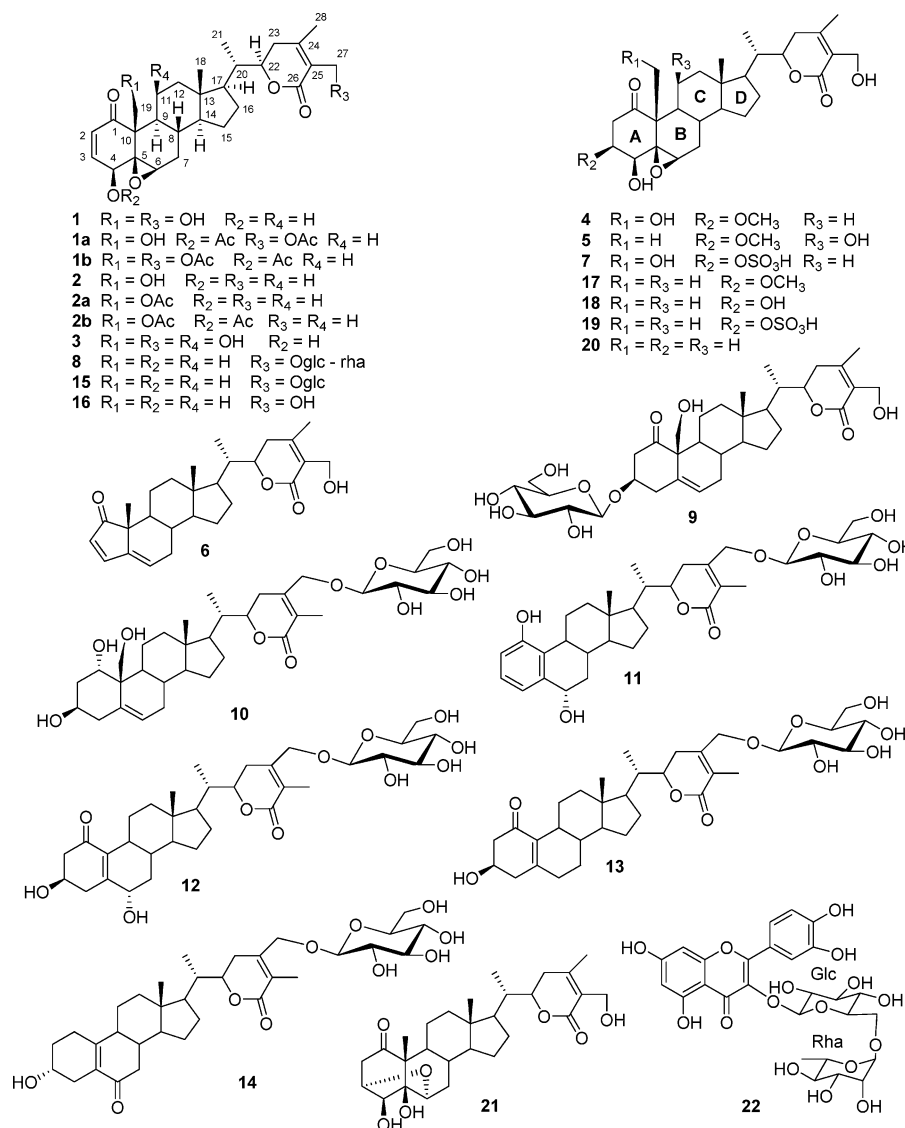


Figure 1. Withanolides 1–21 and rutin (22) isolated from *Physalis longifolia*.

δ 78.8, 68.0, and 61.8), and seven C (including one keto carbonyl at δ 200.7, one ester carbonyl at δ 167.4, two olefins at δ 154.3 and 125.6, and one oxygenated at δ 61.5), corresponding to C₂₈H₃₅. The remaining three hydrogen atoms were therefore assigned to three OH groups, indicating that six rings must be present in the structure.

The NMR data of **1** were very close to those obtained for withaferin A **16**,^{1,13} a six-ring withanolide isolated as another major compound in this study (Tables 1–3 and Figure 1). Compound **1** was found to contain the following moieties also observed in **16**: an α,β -unsaturated ketone in ring A (¹³C: δ 200.7 (C-1), 132.9 (C-2), 145.7 (C-3); ¹H: δ 6.16 (H-2, d, J = 10.0 Hz), 6.95 (H-3, dd, J = 10.0, 6.1 Hz)); an epoxy group in ring B [¹³C: δ 61.5 (C-5), 61.8 (C-6); ¹H: δ 3.18 (brs, H-6)]; and a nine-carbon side chain with an α,β -unsaturated δ -lactone group [¹³C: δ 78.8 (C-22), 154.3 (C-24), 125.6 (C-25), 167.4 (C-26); ¹H: δ 4.33 (H-22, dt, J = 13.3, 3.4 Hz)], as supported by ¹H–¹H COSY and HMBC experiments. The obvious differences between **1** and **16** were the presence of an oxygenated methylene [C-19, ¹³C: δ 61.0; ¹H: δ 3.65 (1H, d, J = 9.6 Hz), 4.17 (1H, d, J = 9.6 Hz)] in **1** and a methyl carbon [C-19, ¹³C: δ 17.6; ¹H: 1.38 (3H, s)] in the latter, suggesting

that **1** is a 19-hydroxy derivative of **16**. This observation was supported by the high-frequency shift of C-10 (δ 54.3 in **1** and δ 47.9 in **16**), the low-frequency shifts of C-1 (δ 200.7 in **1** and δ 202.5 in **16**), C-5 (δ 61.5 in **1** and δ 64.1 in **16**), and C-9 (δ 43.9 in **1** and δ 44.3 in **16**) in the ¹³C NMR spectra, and the HMBC correlations between H₂-19 [δ 3.65 (1H, d, J = 9.6 Hz), 4.17 (1H, d, J = 9.6 Hz)] and C-1, C-5, C-9, and C-10.

Acetylation of **1** with acetic anhydride in pyridine gave two derivatives, the 4,19,27-triacetate (**1a**) and the 4,27-diacetate (**1b**) (Tables 1 and 2), which proved the presence of hydroxy groups at C-4, C-19, and C-27 by a high-frequency shift of H-4 (from δ 3.52 in **1** to δ 4.79 in **1a** and to δ 4.73 in **1b**), of H₂-19 (from δ 3.65, 4.17 in **1** to δ 4.32, 5.07 in **1a**), and of H₂-27 (δ 4.23, 4.28 in **1** to δ 4.84, 4.88 in **1a** and to δ 4.85, 4.88 in **1b**) and by HMBC correlations between H-4 and the ester carbonyl, between H₂-27 and the ester carbonyl, and between H₂-19 and the ester carbonyl in **1a** and **1b**.

Finally, the structure of **1** was confirmed through a single-crystal X-ray diffraction experiment (Figure 2). Thus, **1** (withalongolide A) was established as 19-hydroxywithaferin A. The full assignments of NMR data of **1**, measured in CDCl₃ with a trace amount of CD₃OD and in C₅D₅N (Tables 1

Table 1. ¹H NMR Data for Withanolides **1**, **1a**, **1b**, **2**, **2a**, **2b**, and **3** (400 or 500 MHz)

pos.	1 ^a	1 ^b	1a ^c	1b ^c	2 ^c	2a ^c	2b ^c	3 ^c
2	6.16 d (10.0)	6.46 d (10.0)	6.23 d (10.0)	6.21 d (10.0)	6.23 d (10.4)	6.23 d (9.9)	6.23 d (9.9)	6.22 d (10.0)
3	6.95 dd (10.0, 6.1)	7.26 dd (10.0, 6.1)	7.00 dd (10.0, 6.1)	7.00 dd (10.0, 6.1)	7.01 dd (10.4, 6.4)	7.00 dd (9.9, 6.0)	7.01 dd (9.9, 6.0)	6.96 dd (10.0, 6.5)
4	3.52 d (6.1)	4.01 d (6.1)	4.79 d (6.1)	4.73 d (6.1)	3.63 d (6.4)	4.79 d (6.0)	4.74 d (6.0)	3.78 d (6.5)
6	3.18 brs	3.42 brs	3.12 brs	3.20 brs	3.25 brs	3.16 brs	3.20 brs	3.23 brs
7	2.14 m, 1.25 m	2.15 ddd (2.2, 4.0, 14.5)	2.15 m	2.01 m, 1.96 m	2.19 ddd (2.2, 4.2, 15.0)	2.15 m	2.17 m	2.26 ddd (2.4, 4.7, 15.1)
		1.37 dd (11.1, 14.5)	1.25 m		1.30 m	1.25 ddd (1.4, 11.3, 15.0)	1.28 m	1.29 m
8	1.44 m	1.64 dq (4.0, 11.1)	1.77 dq (4.2, 11.1)	1.85 m	1.51 dq (4.2, 11.1)	1.76 dq (4.2, 11.3)	1.85 m	1.87 dq (4.5, 10.8)
9	0.85 m	1.15 m	1.00 m	0.91 m	0.94 m	1.00 m	0.92 m	1.25 dd (3.3, 10.8)
11	1.90 m, 1.32 m	2.31 m, 1.55 m	1.96 m, 1.62 m	1.94 m, 1.85 m	1.94 m, 1.39 m	1.96 m, 1.62 m	1.94 m, 1.84 m	4.15 (brs)
12	1.86 m, 0.95 m	1.82 m, 0.99 m	1.97 m, 1.08 m	1.96 m, 1.05 m	1.93 m, 1.01 m	1.97 m, 1.08 m	1.98 m, 1.05 m	2.17 dd (13.8, 2.8), 1.38 m
14	0.81 m	0.83 m	0.87 m	0.83 m	0.85 m	0.86 m	0.85 m	0.92 m
15	1.57 m, 1.05 m	1.49 m, 0.92 m	1.62 m, 1.14 m	1.58 m, 1.13 m	1.61 m, 1.12 m	1.61 m, 1.12 m	1.61 m, 1.12 m	1.64 m, 1.22 m
16	1.59 m, 1.29 m	1.49 m, 1.12 m	1.67 m, 1.36 m	1.65 m, 1.36 m	1.65 m, 1.35 m	1.66 m, 1.36 m	1.65 m, 1.36 m	1.63 m, 1.37 m
17	0.99 m	0.99 m	1.07 m	1.05 m	1.04 m	1.07 m	1.06 m	1.04 m
18	0.60 s	0.53 s	0.73 s	0.73 s	0.66 s	0.72 s	0.73 s	0.90 s
19	4.17 d, 3.65 d (9.6)	4.79 d, 4.21 d (9.4)	5.07 d, 4.32 d (11.5)	4.36 d, 4.07 d (11.5)	4.32 d, 3.77 d (9.7)	5.07 d, 4.32 d (11.5)	4.37 d, 4.09 d (11.6)	1.63 s
20	1.91 m	1.88 m	2.00 m	1.98 m	1.95 m	1.96 m	1.96 m	1.99 m
21	0.90 d (6.6)	0.95 d (6.7)	0.98 d (6.7)	0.97 d (6.6)	0.95 d (6.6)	0.97 d (6.6)	0.97 (6.6)	1.00 d (6.6)
22	4.33 dt (13.3, 3.4)	4.37 dt (13.3, 3.5)	4.38 dt (13.3, 3.4)	4.36 dt (13.3, 3.4)	4.31 dt (13.3, 3.4)	4.31 dt (13.3, 3.4)	4.32 dt (13.3, 3.4)	4.39 dt (13.2, 3.5)
23	2.42 dd (17.0, 13.3)	2.49 dd (18.0, 13.3)	2.50 dd (17.9, 13.3)	2.48 dd (18.2, 13.3)	2.40 dd (18.0, 13.3)	2.41 dd (18.0, 13.3)	2.41 dd (18.0, 13.3)	2.47 dd (13.2, 17.0)
	1.91 m	2.17 m	1.99 m	2.16 m	1.87 m	1.88 m	1.88 m	1.94 dd (3.5, 17.0)
27	4.28 d, 4.23 d (12.5)	4.86 d, 4.76 d (11.8)	4.88 d, 4.84 d (11.8)	4.88 d, 4.85 d (11.9)	1.85 s	1.85 s	1.85 s	4.34 br
28	1.97 s	2.11 s	2.05 s	2.04 s	1.90 s	1.91 s	1.90 s	2.01 s
4-OAc			2.09 s	2.07 s		2.09 s	2.08 s	
19-OAc			2.06 s			2.06 s		
27-OAc			2.03 s	2.02 s				

^aIn CDCl₃ with trace amount of CD₃OD. ^bIn C₅D₅N. ^cIn CDCl₃.

and **2**), were obtained by 2D-NMR methods including ¹H–¹H COSY, HSQC, HMBC, and ROESY spectra.

Compound **2** was isolated as colorless rod crystals from a CH₂Cl₂–CH₃C₆H₅ mixture and was also a major metabolite from the EtOAc-soluble part. Its molecular formula, C₂₈H₃₈O₆, was determined by HRESIMS and NMR experiments. The IR and NMR (¹H and ¹³C) data (Tables 1 and 2) were similar to those of **1**. Analysis of the 1D- and 2D-NMR data for **2** identified resonances consistent with an α,β -unsaturated ketone in ring A [¹³C: δ 200.2 (C-1), 133.1 (C-2), 145.4 (C-3); ¹H: δ 6.23 (H-2, d, J = 10.4 Hz), 7.01 (H-3, dd, J = 10.4, 6.4 Hz)], an epoxy in ring B [¹³C: δ 61.7 (C-5), 62.1 (C-6); ¹H: δ 3.25 (brs, H-6)], a nine-carbon side chain with a δ -lactone group [¹³C: δ 78.4 (C-22), 149.2 (C-24), 122.2 (C-25), 167.3 (C-26); ¹H: δ 4.31 (H-22, dt, J = 13.3, 3.4 Hz)], and an oxygenated C-19 [¹³C: δ 62.1; ¹H: δ 4.32, 3.77 (d, J = 9.7 Hz)]. The obvious differences between **2** and **1** were the presence of a 27-methyl carbon [¹³C: δ 12.7; ¹H: 1.85 (3H, s)] in **2** and an oxygenated methylene [C-27, ¹³C: δ 56.7; ¹H: δ 4.28 (1H, d, J = 12.5 Hz), 4.23 (1H, d, J = 12.5 Hz)] in **1**, suggesting that **2** is a 27-deoxy derivative of **1**. This observation was supported by the ¹³C NMR chemical shift values of the δ -lactone moiety [δ 149.2 (C-24), 122.2 (C-25), 167.3 (C-26) in **2** and δ 154.3 (C-24), 125.6 (C-25), 167.4 (C-26) in **1**], by the NMR data comparison

of the side chain moiety of **2** to those of 27-deoxywithanolides,^{14,15} and by HMBC correlations of H₃-27/C-24, C-25, and C-26.

Acetylation of **2** with acetic anhydride in pyridine yielded the 4,19-diacetate (**2a**) and the 4-monoacetate (**2b**) (Tables 1 and 2), which confirmed the presence of hydroxy groups at C-4 and C-19 by a high-frequency shift of H-4 (from δ 3.63 in **2** to δ 4.79 in **2a** and to δ 4.74 in **2b**) and H₂-19 (from δ 3.77, 4.32 in **2** to δ 4.32, 5.07 in **2a**).

Finally, the structure of **2** was confirmed through a single-crystal X-ray diffraction experiment (Figure 3). Thus, **2** (withalongolide B) was determined as 27-deoxy-19-hydroxywithaferin A.

Compound **3** was isolated as colorless cube crystals from a CH₂Cl₂–CH₃CN mixture, a minor component from the EtOAc-soluble fraction. Its molecular formula, C₂₈H₃₈O₇, was determined by HRESIMS and NMR experiments. The NMR data of **3** (Tables 1 and 2) were also akin to those of withaferin A, **16**.^{1,13} Analysis of the 1D- and 2D-NMR data of **3** identified resonances consistent with an α,β -unsaturated ketone in ring A [¹³C: δ 204.0 (C-1), 131.6 (C-2), 143.2 (C-3); ¹H: δ 6.22 (H-2, d, J = 10.0 Hz), 6.96 (H-3, dd, J = 10.0, 6.5 Hz)], an epoxy in ring B [¹³C: δ 64.5 (C-5), 63.0 (C-6); ¹H: δ 3.23 (brs, H-6)], and a nine-carbon side chain with a δ -lactone group [¹³C: δ 78.9 (C-22), 153.0 (C-24), 125.9 (C-25), 167.2 (C-26); ¹H: δ 4.39 (H-22, dt, J = 13.2, 3.5 Hz)]. The obvious differences

Table 2. ¹³C NMR Data for Withanolides 1–7 and 16–20 (125 MHz)

pos.	1 ^c	1 ^b	1a ^a	1b ^a	2 ^a	2a ^a	2b ^a	3 ^a	4 ^a	5 ^a	6 ^a	7 ^a	16 ^a	17 ^a	18 ^b	19 ^b	20 ^b
1	200.7	201.4	198.3	198.9	200.2	198.3	198.9	204.0	208.1	213.3	211.6	208.0	202.5	210.1	210.4	209.7	210.5
2	132.9	133.2	133.7	134.4	133.1	133.8	134.5	131.6	40.5	39.5	129.4	42.0	132.5	39.8	44.4	42.0	32.2
3	145.7	147.5	141.0	140.2	145.4	140.9	140.1	143.2	77.7	76.2	155.2	73.6	142.1	77.7	67.1	73.1	27.1
4	68.0	69.0	71.2	71.7	68.5	71.2	71.8	69.7	72.8	74.4	---	75.4	70.1	75.1	78.7	76.9	72.4
5	61.5	61.7	59.4	61.1	61.7	59.4	61.1	64.5	62.5	66.0	147.4	63.0	64.1	65.2	65.5	65.1	65.9
6	61.8	62.0	59.2	59.9	62.1	59.2	59.9	63.0	57.6	59.5	123.8	57.2	62.7	60.5	57.4	57.2	58.5
7	31.1	31.9	31.0	30.4	31.2	30.9	30.4	31.7	31.1	32.2	31.9	31.4	31.3	31.3	32.3	31.9	29.4
8	30.5	30.3	30.9	31.4	30.6	31.0	31.4	27.0	30.1	27.2	32.8	30.7	29.9	29.5	30.5	30.4	31.0
9	43.9	44.8	44.8	44.6	44.0	44.9	44.7	48.1	42.1	47.1	42.8	42.3	44.3	42.9	43.7	43.1	42.7
10	54.3	55.6	51.2	52.2	54.3	51.2	52.3	49.0	56.1	51.3	47.6	55.7	47.9	50.6	49.7	49.9	50.2
11	22.1	23.0	22.3	21.9	22.3	22.3	21.9	69.5	21.6	69.1	22.0	22.1	22.3	21.8	21.8	21.7	21.3
12	39.7	40.3	39.6	39.7	39.7	39.6	39.7	47.8	39.3	47.4	39.5	39.8	39.5	39.3	39.7	39.5	38.9
13	42.5	42.9	42.9	43.1	42.6	42.8	43.0	42.1	42.8	42.0	43.6	43.1	42.8	42.9	43.2	43.2	42.5
14	56.7	57.1	56.7	57.0	56.9	56.7	57.1	58.2	56.7	58.0	56.5	56.7	56.2	56.2	56.6	56.4	56.1
15	24.2	24.7	24.5	24.3	24.3	24.5	24.4	24.4	24.3	24.5	24.6	24.7	24.5	24.5	24.9	24.9	24.0
16	27.3	27.6	27.5	27.5	27.4	27.5	27.5	27.2	27.5	27.2	27.4	27.7	27.5	27.5	27.7	27.7	27.5
17	51.9	52.4	52.0	52.0	52.1	52.1	52.1	52.7	52.0	52.5	52.1	52.3	52.1	52.5	52.4	52.4	51.6
18	11.7	12.1	11.8	12.0	11.8	11.8	11.8	14.2	11.7	13.6	12.1	12.0	11.8	11.7	11.9	11.9	11.5
19	61.0	61.7	64.4	65.6	62.1	64.4	65.6	21.3	60.4	19.0	19.9	59.9	17.6	15.9	16.1	15.9	15.2
20	38.8	39.5	39.0	39.0	39.0	39.0	39.0	39.0	38.9	39.0	39.1	39.5	39.0	38.9	39.5	39.6	38.3
21	13.3	13.9	13.5	13.5	13.6	13.6	13.6	13.6	13.6	13.6	13.6	14.0	13.5	13.5	14.0	14.0	13.5
22	78.8	78.7	78.4	78.4	78.4	78.4	78.4	78.9	78.8	78.9	79.0	78.9	78.9	78.9	78.8	78.9	78.4
23	29.9	30.3	30.3	30.3	29.8	29.8	29.8	30.1	30.0	30.1	30.0	30.3	30.0	30.0	30.4	30.3	29.1
24	154.3	154.4	157.2	157.1	149.2	149.2	149.1	153.0	153.0	152.9	153.1	154.6	153.1	153.1	154.3	154.6	152.2
25	125.6	127.9	122.1	122.1	122.2	122.2	122.2	125.9	125.9	125.9	125.9	127.7	125.8	125.5	127.9	127.7	125.4
26	167.4	166.8	165.5	165.5	167.3	167.2	167.2	167.2	167.2	167.2	167.3	167.0	167.3	167.2	166.8	167.1	167.2
27	56.7	56.6	58.2	58.2	12.7	12.7	12.7	57.7	57.6	57.7	57.7	56.6	57.7	57.6	56.6	56.6	57.6
28	20.2	20.6	20.8	20.8	20.7	20.7	20.7	20.2	20.2	20.2	20.2	20.5	20.3	20.3	20.6	20.5	20.2
OCOCH ₃ -4			170.4	170.0		170.4	170.1										
OCOCH ₃ -19			21.1	21.1		21.0	21.1										
OCOCH ₃ -27			170.6			170.7											
			21.0			21.4											
OCOCH ₃ -27			171.1	171.1													
OCOCH ₃ -3			21.4	21.0													
CH ₃ -3																	
^a In CDCl ₃ , ^b In C ₅ D ₅ N, ^c In CDCl ₃ with trace amount of CD ₃ OD.									57.6	57.4							57.0

Table 3. ^1H NMR Data for Withanolides 4–7 and 16–19 (400 or 500 MHz)

pos.	4 ^a	5 ^a	6 ^a	7 ^b	16 ^a	17 ^a	18 ^{b,c}	19 ^b
2	H β 2.86 dd (8.3, 16.4) H α 2.56 dd (2.6, 16.4)	H β 2.86 dd (6.5, 17.5) H α 2.56 dd (2.2, 17.5)	5.93 d (6.4)	H β 3.65 dd (8.1, 18.0) H α 3.22 dd (6.2, 18.0)	6.18 d (10.0)	H β 2.99 dd (6.3, 15.0) H α 2.56 dd (2.2, 15.0)	H β 3.62 dd (10.0, 17.1) H α 3.02 dd (5.5, 17.1)	H β 3.62 dd (9.7, 17.6) H α 3.30 dd (2.0, 17.6)
3	3.70 ddd (8.3, 3.4, 2.6)	3.75 ddd (6.5, 4.0, 2.2)	7.57 d (6.4)	5.61 brt (8.1)	6.91 dd (10.0, 5.9)	3.68 ddd (6.3, 3.2, 2.2)	4.67 br	5.62 ddd (9.7, 6.5, 2.0)
4	3.42 d (3.4)	3.56 d (4.0)		4.54 br	3.74 d (5.9)	3.46 d (3.2)	4.05 br	4.51 br
6	3.22 brs	3.15 brs	5.76 t (3.5)	3.37 brs	3.21 s	3.19 s	3.30 br	3.62 br
7	2.25 m 1.42 m	2.31 dd (5.2, 14.8) 1.34 m	2.37 ddd (3.5, 8.8, 15.0) 1.70 m	2.35 m 2.11 m	2.12 ddd (2.2, 3.7, 14.6) 1.30 m	2.14 ddd (2.2, 3.7, 14.6) 1.31 m	2.22 ddd (1.7, 4.3, 15.0) 1.40 ddd (2.0, 10.2, 15.0)	2.07 m 1.23 m
8	1.41 m	1.70 dq (4.5, 10.8)	1.75 m	1.50 m	1.39 m	1.39 m	1.50 m	1.41 m
9	1.14 m	1.28 m	1.17 m	1.16 m	1.14 m	1.16 m	1.17 m	1.04 m
11	1.27 m, 1.18 m	3.88 m	2.24 m, 1.58 m	1.57 m, 1.34 m	1.27 m, 1.18 m	1.35 m	1.52 m	1.44 m
12	1.90 m, 1.02 m	2.15 dd (13.9, 2.4), 1.38 m	1.99 m, 1.18 m	1.76 m, 1.02 m	1.88 m, 1.08 m	1.88 m, 1.08 m	1.85 m, 1.05 m	1.80 m, 1.05 m
14	0.95 m	0.94 m	1.03 m	0.91 m	0.95 m	0.94 m	0.93 m	0.92 m
15	1.64 m, 1.13 m	1.66 m, 1.21 m	1.61 m, 1.19 m	1.52 m, 0.90 m	1.64 m, 1.13 m	1.61 m, 1.12 m	1.54 m, 0.96 m	1.54 m, 0.92 m
16	1.66 m, 1.35 m	1.64 m, 1.38 m	1.64 m, 1.35 m	1.57 m, 1.13 m	1.66 m, 1.35 m	1.64 m, 1.31 m	1.54 m, 1.15 m	1.57 m, 1.15 m
17	1.07 m	1.04 m	1.10 m	1.13 m	1.07 m	1.05 m	1.07 m	1.14 m
18	0.62 s	0.85 s	0.75 s	0.47 s	0.67 s	0.64 s	0.55 s	0.52 s
19	4.38 d, 3.61 d (10.2)	1.49 s	1.15 s	4.80 d, 4.01 d (9.4)	1.38 s	1.28 s	1.76 s	1.69 s
20	1.96 m	1.99 m	1.99 m	1.86 m	1.97 m	1.97 m	1.90 m	1.90 m
21	0.95 d (6.7)	1.00 d (6.7)	1.01 d (6.6)	0.95 d (6.6)	0.97 d (6.6)	0.96 d (6.6)	0.97 d (6.6)	0.97 d (6.6)
22	4.39 dt (13.2, 3.5)	4.39 dt (13.2, 3.5)	4.42 dt (13.3, 3.4)	4.38 dt (13.2, 3.3)	4.39 dt (13.2, 3.5)	4.39 dt (13.2, 3.5)	4.39 dt (13.2, 3.3)	4.40 dt (13.2, 3.3)
23	2.46 dd (13.2, 17.0) 1.93 dd (3.5, 17.0)	2.47 dd (13.7, 17.8) 1.94 dd (3.2, 17.8)	2.48 dd (13.6, 17.6) 1.98 dd (3.2, 17.6)	2.35 dd (13.9, 18.0) 2.07 m	2.47 dd (13.6, 17.4) 1.94 dd (3.2, 17.4)	2.47 dd (13.6, 17.4) 1.94 dd (3.2, 17.4)	2.35 dd (13.4, 17.7) 2.01 dd (3.3, 17.7)	2.36 dd (13.7, 17.8) 2.07 m
27	4.36 d, 4.32 d (12.5)	4.37 d, 4.31 d (12.6)	4.37 d, 4.32 d (12.5)	4.85 d, 4.75 d (11.7)	4.36 d, 4.31 d (12.6)	4.36 d, 4.31 d (12.6)	4.80 d, 4.78 d (11.8)	4.85 d, 4.75 d (11.7)
28	2.02 s	2.01 s	2.01 s	2.09 s	2.01 s	2.01 s	2.10 s	2.09 s
3-OMe	3.35 s	3.34 s				3.32 s		

^aIn CDCl₃. ^bIn C₅D₅N. ^cOH-4 signal 7.75 s, OH-3 signal 6.91 brs, OH-27 signal 6.61 brs.

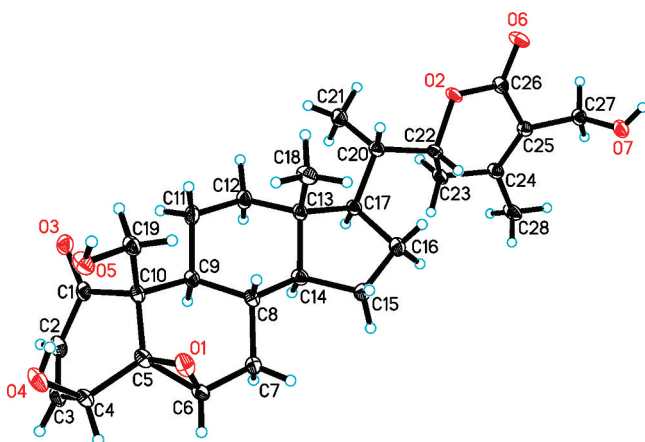


Figure 2. X-ray ORTEP drawing of withalongolide A (1).

between 3 and 16 were the presence of an oxygenated methine [C-11, ^{13}C : δ 69.5; ^1H : 4.15 (1H, brs)] in 3 and a low-frequency methylene [C-11, ^{13}C : δ 22.3; ^1H : δ 1.27 (1H, m), 1.18 (1H, m)] in 16, implying that 3 is 11-hydroxywithaferin A. This observation was supported by the high-frequency shift of C-9 (δ 48.1 in 3 and δ 44.3 in 16) and C-12 (δ 47.8 in 3 and δ 39.5 in 16) and the low-frequency shift of C-8 (δ 27.0 in 3 and

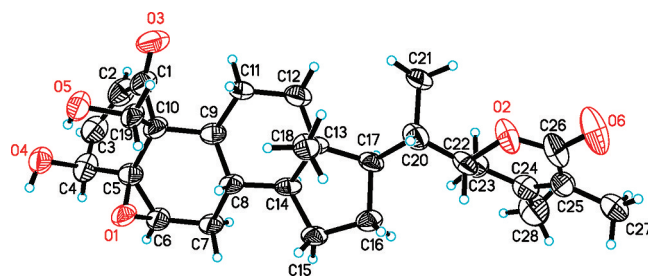


Figure 3. X-ray ORTEP drawing of withalongolide B (2).

δ 29.9 in 16) in the ^{13}C NMR spectrum; by the presence of the fragment $-\text{CH}_2-\text{CH}(\text{OH})-\text{CH}-\text{CH}(\text{CH}_2)-\text{CH}-$ (starting with C-12 and ending with C-14) deduced from $^1\text{H}-^1\text{H}$ COSY and HSQC experiments; and by the HMBC correlations between H $_{\alpha}$ -12 (δ 2.17, dd, J = 13.8, 2.8 Hz) and C-9 (δ 48.1), C-11 (δ 69.5), C-13 (δ 42.1), and C-14 (δ 58.2). The orientation of the hydroxy group at C-11 was deduced as β due to the broad single-peak pattern of H-11 (δ 4.15, brs), the small coupling constant of 3.3 Hz between H-9 (δ 1.25, dd, J = 10.8, 3.3 Hz) and H-11, and ROESY correlations of H-11/H-9 and H-11/H $_{\alpha}$ -12. Finally, the structure of 3 was confirmed through a single-crystal X-ray diffraction experiment (Figure 4). Thus,

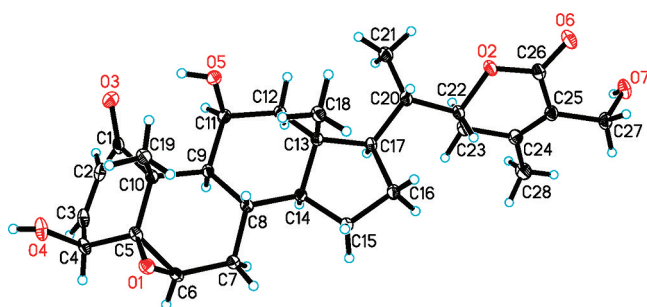


Figure 4. X-ray ORTEP drawing of withalongolide C (3).

the new withanolide 3 (withalongolide C) was established as 11 β -hydroxywithaferin A.

Compounds 4 and 5 were isolated as two presumed artifacts. These two compounds were probably formed from withalongolide A (1) and withalongolide C (3), respectively, by a Michael-type addition due to the use of CH₃OH during the extraction procedure. It is possible that these compounds are formed in a similar fashion to 2,3-dihydro-3 β -methoxywithaferin A (17)¹ (Tables 2 and 3), which was most likely derived from withaferin A (16) during this study. Comparing the NMR data of the methoxy group in 17 [–OCH₃ group at C-3: ¹H: δ 3.32 (3H, s); ¹³C: δ 57.0; H-3: δ 3.68 (1H, ddd, J = 6.3, 3.2, 2.2 Hz); C-3: δ 77.7] with both 4 and 5 [–OCH₃: ¹H: δ 3.35 (3H, s); ¹³C: δ 57.6; H-3: δ 3.70 (1H, ddd, J = 8.3, 3.4, 2.6 Hz); C-3: δ 77.7 in 4 and –OCH₃: ¹H: δ 3.34 (3H, s); ¹³C: δ 57.4; H-3: δ 3.75 (1H, ddd, J = 6.5, 4.0, 2.2 Hz); C-3: δ 76.2 in 5] suggested the presence of a methoxy group at the C-3 positions in 4 and 5. This was confirmed by the presence of a ¹H–¹H COSY fragment of –CH₂–CH(OCH₃)–CH(OH)– in ring A and HMBC correlation of OCH₃/C-3 in both 4 and 5. The structures of 4 (withalongolide D) and 5 (withalongolide E) were determined by spectroscopic methods, and complete assignments of their NMR data are listed in Tables 2 and 3.

Compound 6 was isolated as colorless cube crystals obtained from a CH₂Cl₂–CH₃CN mixture. Its molecular formula, C₂₇H₃₄O₄, was ascertained by HRESIMS and NMR experiments (Tables 2 and 3). Similar to withaferin A (16), it showed signals for four methyl groups [¹³C: δ 20.2 (C-28), 19.9 (C-19), 13.6 (C-21), 12.1 (C-18); ¹H: δ 2.01 (H₃-28, s), 1.15 (H₃-19, s), 1.01 (H₃-21, d, J = 6.6 Hz), 0.75 (H₃-18, s)], an α,β -unsaturated ketone in ring A [¹³C: δ 211.6 (C-1), 129.4 (C-2), 155.2 (C-3); ¹H: δ 5.93 (H-2, d, J = 6.4 Hz), 7.57 (H-3, d, J = 6.4 Hz)], and a nine-carbon side chain with an α,β -unsaturated δ -lactone group [¹³C: δ 57.7 (C-27), 79.0 (C-22), 153.1 (C-24), 125.9 (C-25), 167.3 (C-26); ¹H: δ 4.42 (H-22, dt, J = 13.3, 3.4 Hz), 4.37 (H-27, d, J = 12.5 Hz), 4.32 (H-27, d, J = 12.5)]. A detailed comparison of the NMR data of 6 to those of 16 indicated that both compounds share identical ring C, D and side chain moieties, but are different in their A and B rings. A five-membered ring A for 6 was proposed on the basis of the following evidence: (1) the unusual chemical shift value of the conjugated ketone carbon (C-1, δ 211.6); (2) the coupling pattern of H-3 (δ 7.57, d, J = 6.4 Hz) and the small coupling constant of 6.4 Hz between the olefinic protons H-2 and H-3 when compared to those of H-3 (6.91, dd, J = 10.0, 5.9 Hz) in 16, showing C-3 to be linked with a quaternary carbon in 6. This five-membered ring A and a C-5,6 double bond in ring B were supported by the HMBC correlations of H₃-19/C-1, C-5 (quaternary carbon, δ 147.4), C-9 (δ 42.8), and C-10 (δ 47.6);

of H-2/C-1, C-3, C-5, and C-10; and of H-3/C-1, C-2, C-5, C-6 (methine, δ 123.8), and C-10. Finally, the observation was confirmed through a single-crystal X-ray diffraction experiment (Figure 5). Thus, 6 (withalongolide F) was deduced to be

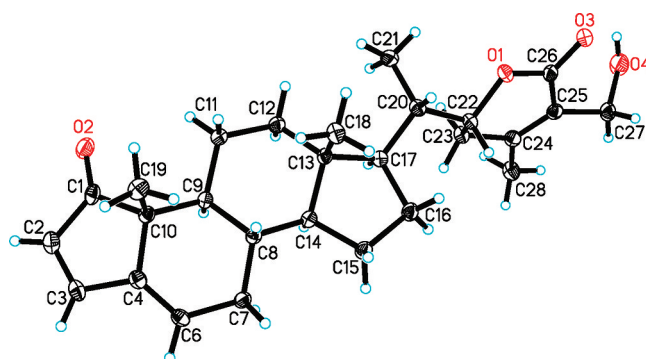


Figure 5. X-ray ORTEP drawing of withalongolide F (6).

A-nor-27-hydroxy-1-oxowitha-2,5,24-trienolide. This 4-norwithanolide with a 2,5-dien-1-one system was reported previously as a semisynthetic product derived from withaferin A by an acid-catalyzed rearrangement.¹⁶

Compound 7 was a major compound isolated from the BuOH-soluble fraction. Its molecular formula, C₂₈H₄₀O₁₁S, was determined by HRESIMS and NMR experiments. The NMR data of 7 (Tables 2 and 3) were similar to those of 2,3-dihydro-3 β -O-sulfate-withaferin A (19) (Tables 2 and 3),¹⁷ another major withanolide isolated from the BuOH-soluble fraction during this study (Figure 1). The obvious differences between 7 and 19 were the presence of an oxygenated methylene [C-19, ¹³C: δ 59.9; ¹H: δ 4.80 (1H, d, J = 9.4 Hz), 4.01 (1H, d, J = 9.4 Hz)] in 7 and a methyl carbon [C-19, ¹³C: δ 15.9; ¹H: δ 1.69 (3H, s)] in the latter, suggesting that 7 is a 19-hydroxy derivative of 19. This observation was supported by the high-frequency shift of C-10 (δ 55.7 in 7 and δ 49.9 in 19), the low-frequency shift of C-1 (δ 208.0 in 7 and δ 209.7 in 19), C-5 (δ 63.0 in 7 and δ 65.1 in 19), and C-9 (δ 42.3 in 7 and δ 43.1 in 19) in the ¹³C NMR spectrum, and the HMBC correlations of H₂-19 [δ 4.01 (1H, d, J = 9.4 Hz), 4.80 (1H, d, J = 9.4 Hz)]/C-1, C-5, C-9, and C-10 in 7. Thus, 7 (withalongolide G) was determined as 2,3-dihydro-19-hydroxy-3 β -O-sulfate-withaferin A.

Compound 8 was isolated as a major component from the BuOH-soluble fraction. Its molecular formula, C₄₀H₅₈O₁₅, was ascertained by HRESIMS and NMR experiments. The NMR data of 8 (Tables 4 and 5) showed similarities to those of 27-O- β -D-glucopyranosylwithaferin A (15) (sitoindoside IX)¹⁸ (Tables 4 and 5) isolated during this study, suggesting 8 to be a withanolide saponin. The aglycone of 8 was determined to be withaferin A, as both 8 and 15 possess the superimposable ¹H and ¹³C NMR signals of the steroid aglycone moieties and both showed the same main LC-MS/MS fragments of m/z 471 and 281 due to the presence of a withaferin A moiety. Differing in the presence of only one glucose residue in 15, two sugar residues were observed in 8 on the basis of the signals of two anomeric carbons [methines, δ 105.3 (C-1') and 103.1 (C-1'')] and their corresponding anomeric protons [δ 4.97 (H-1', 1H, d, J = 7.8) and 5.94 (H-1'', 1H, s)]. Furthermore, the data for 8 suggested that the compound had, besides a glucose unit, an additional five oxygenated carbons (five methines) and one low-frequency methyl group [¹³C: δ 19.0; ¹H: δ 1.74 (3H, d,

Table 4. ¹H NMR Data for Withanolides 8–15 in C₅D₅N (500 MHz)

pos.	8 ^a	9 ^b	10 ^c	11 ^d	12 ^e	13 ^f	14 ^g	15 ^h
1			4.61 m				H α 2.74 m, H β 2.10 m	
2	6.43 d (10.0)	H β 3.48 dd (11.0, 12.4) H α 3.26 ddd (1.5, 5.3, 12.4)	H α 2.73 d (18.8) H β 2.35 dd (10.2, 18.8)	7.14 d (7.7)	3.01 dd (7.1, 15.5) 2.91 dd (3.7, 15.5)	2.94 dd (8.1, 15.2) 2.91 dd (4.1, 15.2)	H α 1.96 m, H β 1.80 m	6.43 d (10.0)
3	7.25 dd (10.0, 6.3)	4.29 m	4.84 m	7.34 t (7.7)	4.58 m	4.47 m	4.33 br	7.25 dd (10.0, 6.3)
4	4.05 dd (6.3, 3.8)	H β 3.07 dt (2.0, 12.2) H α 2.99 ddd (1.5, 5.5, 12.2)	H β 2.95 t (12.5) H α 2.88 dd (12.5, 5.0)	7.87 d (7.7)	3.63 td (3.2, 18.0) 2.92 m	2.68 dd (5.9, 16.8) 2.58 td (2.7, 16.8)	H α 2.90 d (15.8) H β 2.81 dd (2.2, 15.8)	4.05 dd (6.3, 3.7)
6	3.28 s	5.75 brd (5.3)	5.92 brd (4.5)	5.26 dt (11.0, 6.5)	4.69 m	H α 2.26 m, H β 2.09 m		3.28 s
7	2.13 dt (13.0, 1.8) 1.30 dd (11.5, 13.0)	1.92 m, 1.53 m	H α 1.66 dd (11.0, 16.0) H β 2.02 m	1.63 q (11.0) 2.32 dd (6.5, 11.0)	2.23 m	1.98 m, 1.53 m	2.47 dd (3.6, 16.0) 2.00 m	2.13 dt (13.0, 1.8) 1.30 dd (11.5, 13.0)
8	1.58 m	1.74 dt (5.3, 10.7)	2.20 m	1.73 q (11.0)	1.48 m	1.24 m	1.62 m	1.57 m
9	1.06 m	1.94 m	2.32 m	2.71 t (11.0)	2.20 m	2.04 m	1.86 m	1.06 m
11	2.02 m, 1.57 m	2.37 m, 1.96 m	2.15 m, 1.98 m	3.68 m, 1.46 m	2.96 m, 1.17 m	2.80 m, 2.34 m	1.84 m, 1.24 m	2.02 m, 1.57 m
12	1.83 m, 1.03 m	1.95 m, 1.26 m	1.95 m, 1.02 m	1.95 m, 1.30 m	1.83 m, 1.17 m	1.82 m, 1.13 m	1.85 m, 1.03 m	1.85 m, 1.02 m
14	0.86 m	0.97 m	0.86 m	1.10 m	0.95 m	0.90 m	0.88 m	0.86 m
15	1.50 m, 0.96 m	1.50 m, 1.02 m	1.49 m, 1.06 m	1.52 m, 1.12 m	1.45 m, 1.02 m	1.44 m, 1.02 m	1.33 m, 0.88 m	1.50 m, 0.95 m
16	1.49 m, 1.11 m	1.50 m, 1.14 m	1.91 m, 1.24 m	1.98 m, 1.27 m	1.95 m, 1.23 m	1.95 m, 1.23 m	1.91 m, 1.21 m	1.49 m, 1.11 m
17	0.99 m	1.05 m	1.04 m	1.12 m	1.07 m	1.07 m	1.06 m	0.99 m
18	0.57 s	0.67 s	0.80 s	0.70 m	0.59 s	0.58 s	0.50 s	0.57 s
19	1.87 s	4.30 m, 4.23 dd (3.9, 12.3)	4.32 d, 4.11d (11.0)					1.86 s
20	1.88 m	1.90 m	1.98 m	2.01 m	1.96 m	1.96 m	1.96 m	1.88 m
21	0.96 d (6.6)	0.97 d (6.6)	0.99 d (6.6)	1.01 d (6.6)	0.97 d (6.6)	0.96 d (6.6)	0.99 d (6.6)	0.95 d (6.6)
22	4.34 m	4.40 dt (13.2, 3.4)	4.25 m	4.55 dt (13.2, 3.4)	4.53 dt (13.2, 3.4)	4.53 dt (13.2, 3.4)	4.53 dt (13.2, 3.4)	4.34 dt (13.2, 3.4)
23	2.27 dd (13.3, 18.0) 1.98 dd (3.1, 18.0)	2.34 dd (18.0, 14.0) 2.03 dd (3.1, 18.0)	2.78 m, 2.33 m	2.83 dd (18.0, 2.2) 2.34 dd (18.0, 14.0)	2.81 dd (18.0, 2.2) 2.35 dd (18.0, 14.0)	2.81 dd (18.0, 2.2) 2.35 dd (18.0, 14.0)	2.81 dd (18.0, 2.2) 2.37 dd (18.0, 14.0)	2.28 dd (13.3, 18.0) 1.98 dd (3.1, 18.0)
27	5.02 d, 4.80 d (10.8)	4.88 d, 4.78 d (11.0)	2.04 s	2.05 s	2.05 s	2.05 s	2.06 s	5.07 d, 4.86 d (10.8)
28	2.07 s	2.14 s	4.87 d, 4.49 d (13.5)	4.89 d, 4.47 d (13.5)	4.89 d, 4.49 d (13.5)	4.89 d, 4.49 d (13.5)	4.90 d, 4.50 d (13.5)	2.10 s
1'	4.97 d (7.8)	5.08 d (7.8)	4.84 d (7.8)	4.84 d (7.8)	4.84 d (7.8)	4.84 d (7.8)	4.85 d (7.8)	5.06 d (7.8)
2'	4.01 td (7.8, 3.6)	4.05 t (7.8)	4.10 m	4.10 m	4.10 m	4.10 m	4.11 dd (7.8, 7.0)	4.08 t (7.8)
3'	4.23 m	4.30 m	4.25 m	4.25 m	4.25 m	4.25 m	4.25 m	4.31 m
4'	4.51 t (9.0)	4.31 m	4.24 m	4.24 m	4.24 m	4.24 m	4.24 m	4.30 m
5'	3.75 ddd (2.5, 5.0, 9.0)	3.97 ddd (2.5, 5.0, 9.0)	3.99 m	4.00 m	4.00 m	4.00 m	4.00 m	4.00 ddd (2.5, 5.6, 9.0)
6'	4.31 m, 4.16 m	4.54 ddd (2.5, 5.0, 11.6) 4.42 td (5.0, 11.6)	4.62 m 4.42 m	4.62 ddd (2.0, 5.6, 11.6) 4.42 td (5.6, 11.6)	4.62 ddd (2.0, 5.6, 11.6) 4.42 td (5.6, 11.6)	4.62 ddd (2.0, 5.6, 11.6) 4.42 td (5.6, 11.6)	4.62 ddd (2.0, 5.6, 11.6) 4.42 td (5.6, 11.6)	4.60 ddd (2.5, 5.6, 11.5) 4.44 td (5.6, 11.5)

^aH-1" 5.94 s, H-2" 4.73 s, H-3" 4.60 br, H-4" 4.38 dd (4.1, 9.9), H-5" 5.06 m, H-6" 1.74 d (6.1), OH-4 7.87 d (3.8), OH-2' 7.36 d (3.5), OH-2" 6.84 s, OH-4" 6.83 s, OH-3' 6.83 s, OH-6" 6.82 m, OH-3" 6.55 d (5.5). ^bOH-2' 7.27 s, OH-19 7.27 d (3.9), OH-6' 6.56 t (5.0), OH-27 6.49 brs. ^cOH-2' 7.51, OH-3' 7.36 s, OH-4' 7.24 s, OH-6' 6.52 t (5.0), OH-3 6.20 d (5.0), OH-1 5.97 d (5.0), OH-19 5.96 brs. ^dOH-1 11.33 s, OH-2' 7.45 d (4.5), OH-3' 7.35 brs, OH-4' 7.25 brs, OH-6 6.81 d (6.5), OH-6' 6.53 t (5.6). ^eOH-2' 7.54 d (4.3), OH-3' 7.34 brs, OH-4' 7.26 brs, OH-6 6.93 d (6.5), OH-3 6.84 (4.0), OH-6' 6.53 t (5.6). ^fOH-2' 7.54 d (4.2), OH-3' 7.34 brs, OH-4' 7.26 brs, OH-3 6.81 d (4.1), OH-6' 6.53 t (5.5). ^gOH-2' 7.49 brs, OH-3' 7.38 brs, OH-4' 7.28 brs, OH-6' 6.53 t (5.6), OH-3 6.28 brs. ^hOH-4 7.86 d (3.7), OH-3' 7.22 brs, OH-4' 7.28 brs, OH-2' 7.21 brs, OH-6' 6.47 t (5.6), OH-3 6.28 brs.

$J = 6.1$ Hz)], corresponding to a rhamnose in the pyranose form. The rhamnose moiety was deduced by the detailed comparison of the NMR data of **8** with those of rutin **22** {3-O-[α -L-rhamnopyranosyl-(1 \rightarrow 6)]- β -D-glucopyranosylquercetin} also isolated in this study and confirmed from the ¹H–¹H COSY,

HSQC, and HMBC spectra when starting with the characteristic methyl group [C-6", ¹³C: δ 19.0; ¹H: δ 1.74 (3H, d, $J = 6.1$ Hz)]. The α -anomeric configuration of the rhamnose unit was assigned from the small coupling constant between H-1" (1H, δ 5.94, s) and H-2" (1H, δ 4.73, s). Furthermore, the rhamnose

Table 5. ^{13}C NMR Data for Withanolides 8–15 in $\text{C}_5\text{D}_5\text{N}$ (125 MHz)

pos.	8 ^a	9	10	11	12	13	14	15
1	203.1	208.9	69.5	157.8	199.4	198.3	25.6	203.1
2	132.8	49.6	41.7	115.1	49.4	49.7	30.6	132.8
3	145.6	75.9	66.6	127.4	66.3	66.1	64.7	145.6
4	70.8	39.9	44.4	119.3	36.9	41.9	32.9	70.8
5	65.0	132.8	137.8	146.2	156.4	153.6	130.4	65.0
6	60.6	129.1	127.0	70.7	71.2	33.8	198.6	60.6
7	32.2	31.9	32.6	38.3	37.3	26.4	44.0	32.2
8	30.7	33.5	33.8	39.0	37.5	39.9	40.0	30.7
9	45.1	43.6	42.4	46.6	44.3	43.6	46.6	45.1
10	49.0	60.2	48.2	127.8	136.5	136.1	158.7	49.0
11	22.2	24.1	22.3	27.1	26.4	26.6	25.1	22.2
12	39.9	40.6	40.7	41.5	41.0	41.0	40.2	39.9
13	43.1	43.5	43.6	44.2	43.9	44.1	43.3	43.1
14	56.5	57.0	57.5	54.9	54.2	54.9	54.8	56.5
15	24.9	25.0	25.2	24.7	24.5	24.5	24.0	24.9
16	27.6	27.6	27.8	27.7	27.6	27.7	27.5	27.6
17	52.4	52.5	52.6	52.9	52.7	52.7	52.3	52.4
18	12.0	12.5	12.6	12.9	12.6	12.7	12.2	12.0
19	17.7	63.7	63.9					17.7
20	39.5	39.6	39.9	40.0	39.9	39.9	39.8	39.5
21	13.9	13.9	14.1	13.9	13.9	13.9	14.0	13.9
22	78.6	78.9	79.7	79.6	79.6	79.6	79.5	78.7
23	30.4	30.3	25.2	25.2	25.2	25.2	25.2	30.4
24	157.4	154.5	148.8	148.7	148.8	148.7	148.7	157.4
25	124.3	127.8	125.2	125.3	125.2	125.2	125.2	124.4
26	166.3	166.9	167.1	167.1	167.1	167.1	167.1	166.4
27	64.0	56.6	13.0	12.9	12.9	12.9	12.9	63.9
28	21.0	20.6	67.1	66.9	67.0	67.0	67.0	21.0
1'	105.3	103.5	103.5	103.4	103.5	103.5	103.5	105.4
2'	75.9	75.6	75.4	75.4	75.4	75.4	75.4	75.7
3'	77.1	79.1	79.0	79.0	79.0	79.0	79.1	79.0
4'	78.5	71.9	72.1	72.1	72.1	72.1	72.1	72.1
5'	77.8	79.1	79.3	79.3	79.3	79.3	79.3	79.1
6'	61.9	63.0	63.3	63.3	63.3	63.3	63.3	63.2

^a1" (103.1), 2" (73.1), 3" (73.3), 4" (74.5), 5" (70.8), 6" (19.0).

was confirmed to be attached at C-4' (δ 78.5) on the basis of HMBC correlations of H-1"/C-4' and H-4'/C-1", also supported by the glycosylation shifts of C-4' (δ 78.5 in **8** and δ 72.1 in **15**), C-3' (δ 77.1 in **8** and δ 79.0 in **15**), and C-5' (δ 77.8 in **8** and δ 79.1 in **15**). Thus, the structure of **8** (withalongolide H) was determined as 27-O- $[\alpha$ -L-rhamnopyranosyl(1 \rightarrow 4)]- β -D-glucopyranosylwithaferin A.

Compounds **9**–**14** were isolated as the minor components from the BuOH-soluble fraction. Withanolide **9** was assigned a molecular formula of $\text{C}_{34}\text{H}_{50}\text{O}_{11}$ by HRESIMS and NMR experiments. Its NMR data (Tables 4 and 5) exhibited a close resemblance to those of withalongolide A (**1**), possessing the same nine-carbon side chain with an α,β -unsaturated δ -lactone, identical rings C and D, and an oxygenated C-19 methylene group. In addition, the remaining rings A and B showed similarities to those of 3-O- β -D-glucopyranosyl-20,27-dihydroxy-1-oxowitha-5,24-dienolide, a withanolide saponin reported from *Physalis peruviana*,¹⁹ with the following signals: (1) the occurrence of a ketone (δ 208.9, C-1) and a double bond [^{13}C : C-5 quaternary carbon, δ 132.8, and C-6 methine, δ 129.1; ^1H : δ 5.75 (1H, brd, J = 5.3 Hz, H-6)]; (2) a glucose moiety attached to C-3 in ring A [^{13}C : δ 75.9 (CH, C-3); characteristic signals for glucose: δ 103.5 (CH, C-1'), 79.1 (CH, C-5'), 79.1 (CH, C-3'), 75.6 (CH, C-2'), 71.9 (CH, C-4'), and

63.0 (CH₂, C-6')]; (3) a fragment of $-\text{CH}_2-\text{CH}(\text{O})-\text{CH}_2-$ in ring A deduced from the $^1\text{H}-^1\text{H}$ COSY and HSQC spectra; (4) HMBC correlations of H-3/C-1' and H-1'/C-3; of H₂-19/C-1, C-5, C-9, and C-10; of H₂-2/C-1, C-3, C-4, and C-10; and of H β -4/C-2, C-3, C-5, C-6, and C-10. Thus, the structure of **9** (withalongolide I) was determined as 3-O- β -D-glucopyranosyl-19,27-dihydroxy-1-oxo-witha-5,24-dienolide.

Compound **10** was assigned a molecular formula of $\text{C}_{34}\text{H}_{52}\text{O}_{11}$ by HRESIMS and NMR experiments. Similar to those of **9**, the NMR data of **10** (Tables 4 and 5) displayed the presence of an oxygenated C-19 methylene group [^{13}C : δ 63.9 CH₂; ^1H : δ 4.32 (1H, d, J = 11.0 Hz), 4.11 (1H, d, J = 11.0 Hz)] and three methyl groups [^{13}C : δ 12.6, 13.0, 14.1; ^1H : δ 0.80 (s), 0.99 (d, J = 6.6 Hz), 2.04 (s)]. The obvious differences between **10** and **9** were the presence of an oxygenated methine (δ 69.5) in **10** instead of the keto carbon (C-1, δ 208.9) in **9**, implying that a hydroxy group is attached to C-1. This was supported by the presence of a $^1\text{H}-^1\text{H}$ COSY fragment of $-\text{CH}(\text{O})-\text{CH}_2-\text{CH}(\text{O})-\text{CH}_2-$ assigned as a C-1 to C-4 moiety in ring A and confirmed by HMBC correlations of H₂-19/C-1, C-5, C-9, and C-10 and of H₂-4/C-2, C-3, C-5, C-6, and C-10. The orientation of the hydroxy group at C-1 was assigned as α on the basis of the small coupling constant of H-1 (δ 4.61, s)/H₂-2. Furthermore, it was determined that the

glucose was attached to C-28 by the HMBC correlations of H₂-28/C-23, 24, 25, 1' and of H-1'/C-28, as well as the chemical shifts of C-23 (δ 25.2), C-24 (δ 148.8), C-25 (δ 125.2), C-26 (δ 167.1), C-27 (δ 13.0), and C-28 (δ 67.1) and detailed comparison to those of withanolides with a 28-O-glucoside moiety.²⁰ Thus, the structure of **10** (withalongolide J) was determined as 28-O- β -D-glucopyranosyl-1 α ,3 β ,19-trihydroxywitha-5,24-dienolide.

Similar to withalongolide J (**10**), compounds **11–14** were shown to possess the same nine-carbon side chain with an α,β -unsaturated δ -lactone and a glucose moiety at C-28, on the basis of their superimposable NMR signals assigned to the side chain (Tables 4 and 5).

Saponin **11** was assigned a molecular formula of C₃₃H₄₆O₁₀ by HRESIMS and NMR experiments. Excluding the six carbons corresponding to the glucose moiety, the 27-carbon aglycon implied that one carbon in the C₂₈ withanolide scaffold must be lost. The NMR data of its aglycon were similar to those of 1,6,27-trihydroxy-19-norwitha-1,3,5(10),24-tetraenolide (a 19-norwithanolide with an aromatic ring A).²¹ A trisubstituted aromatic ring A in **11** was observed from the ¹H NMR (H-2: δ 7.14, 1H, d, J = 7.7 Hz; H-3: δ 7.34, 1H, t, J = 7.7 Hz; and H-4: δ 7.87, 1H, d, J = 7.7 Hz) and ¹³C NMR (C-1: δ 157.8 C; C-2: δ 115.1, CH; C-3: δ 127.4 CH; C-4: δ 119.3 CH; C-5: δ 146.2 C; and C-10: δ 127.8, C) experiments. This was confirmed by ¹H–¹H COSY and HSQC experiments and HMBC correlations of H-2/C-4 and C-10, H-3/C-1 and C-5, and H-4/C-2 and C-10. Moreover, the HMBC correlations of H-4/C-6 (δ 70.7 CH), of OH-6 (δ 6.81, d, J = 6.5 Hz)/C-5 and C-6, and of H-7 β /C-5 and the ¹H–¹H COSY fragment –CH(OH)–CH₂–CH–CH–CH₂–CH₂– (corresponding to –C₆–C₇–C₈–C₉–C₁₁–C₁₂–) showed that both the aglycone of **16** and 1,6,27-trihydroxy-19-norwitha-1,3,5(10),24-tetraenolide have the same planar structural moieties in rings A and B. However, the orientation of the hydroxy group at C-6 was assigned as α because of the large coupling constants (11.0 Hz) between H-6 (δ 5.26, dt, J = 11.0, 6.5 Hz) and H-7 α (δ 1.63, q, J = 11.0 Hz) and (6.5 Hz) between H-6 and H-7 β (δ 2.32, dd, J = 11.0, 6.5 Hz). Thus, the structure of **11** (withalongolide K) was determined as 28-O- β -D-glucopyranosyl-1,6 α -dihydroxy-19-norwitha-1,3,5(10),24-tetraenolide.

Compound **12** was assigned a molecular formula of C₃₃H₄₈O₁₁ by HRESIMS and NMR experiments. Its NMR data (Tables 4 and 5) were similar to those of withalongolide K (**11**), containing the same rings B, C, and D because of their superimposable ¹H and ¹³C NMR signals. The differences observed between **12** and **11** were caused by changes in the ring A moiety. Unlike the aromatic ring A in **11**, a conjugated 5(10)-en-1-one system in **12** was revealed by the chemical shifts of quaternary carbons at δ 199.4 (C-1), 156.4 (C-5), and 136.5 (C-10). A ¹H–¹H COSY fragment of –CH₂–CH(OH)–CH₂– was assigned as –C₂–C₃–C₄– in ring A and confirmed by the HMBC correlations of H₂-2/C-1, C-3, and C-4 and of H₂-4/C-2, C-5, and C-10. Thus, the structure of **12** (withalongolide L) was determined as 28-O- β -D-glucopyranosyl-3 β ,6 α -dihydroxy-1-oxo-19-norwitha-5(10),24-dienolide.

Compound **13** was assigned a molecular formula of C₃₃H₄₈O₁₀ by HRESIMS and NMR experiments. Its NMR data were similar to those observed for withalongolide L (**12**), containing a conjugated 5(10)-en-1-one system [¹³C: δ 198.3 (C-1), 153.6 (C-5), 136.1 (C-10)] in ring A. The obvious difference between **13** and **12** was the presence of a methylene (¹³C: δ 33.8; ¹H: δ 2.30, 2H, m) instead of an oxygenated

methine (¹³C: δ 71.2; ¹H: δ 4.69), suggesting that **13** is a 6-deoxy derivative of **12**. This observation was supported by the ¹³C NMR high-frequency shift of C-4 (δ 41.9 in **13** and δ 36.9 in **12**) and C-8 (δ 39.9 in **13** and δ 37.5 in **12**), the low-frequency shift of C-7 (δ 26.4 in **13** and δ 37.3 in **12**), and HMBC correlations of OH-3 (δ 6.81, 1H, d, J = 4.1 Hz)/C-2 (δ 49.7), C-3 (δ 66.1), and C-4 (δ 41.9), of H₂-4 (δ 2.68, dd, J = 5.9, 16.8 Hz and δ 2.58, dd, J = 2.7, 16.8 Hz)/C-2, C-3, C-5 (δ 153.6), C-6 (δ 33.8), and C-10 (δ 136.1), and of H-6 β (δ 2.09, m)/C-4, C-5, C-7 (δ 26.4), C-8 (39.9), and C-10 in **13**. Thus, the structure of **13** (withalongolide M) was determined as 28-O- β -D-glucopyranosyl-3 β -hydroxy-1-oxo-19-norwitha-5(10),24-dienolide.

Compound **14** was assigned a molecular formula of C₃₃H₄₈O₁₀ by HRESIMS and NMR experiments and as an isomer of withalongolide M (**13**). The NMR data of these two compounds (Tables 4 and 5) were similar to each other, having the same functional groups and the same multiplicities for all other carbons present. A conjugated 5(10)-en-6-one system [¹³C: δ 198.6 (C-6), 158.7 (C-10), 130.4 (C-5)] in **14** was proposed instead of the 5(10)-en-1-one one in **13** on the basis of the following observations: (1) a ¹H–¹H COSY fragment of –CH₂–CH₂–CH(OH)–CH₂– (from C-1 to C-4) in ring A of **14** replaced the ring A fragment –CH₂–CH(OH)–CH₂– (from C-2 to C-4) in **13**; (2) a ¹H–¹H COSY fragment of –CH₂–CH–CH– (from C-7 to C-9) in ring B of **14** replaced the ring B fragment –CH₂–CH₂–CH–CH– (from C-6 to C-9) in **13**; (3) HMBC correlations of H-2/C-1 (δ 25.6), C-3 (δ 64.7), and C-4 (δ 32.9); of H₂-7/C-5 (δ 130.4), C-6 (δ 198.6), C-8 (δ 40.0), and C-9 (δ 46.6); and of H-1/C-3, C-5, and C-10 (δ 158.7). Furthermore, the orientation of the hydroxy group at C-3 was determined as α due to the small coupling constant (J = 2.2 Hz) between H-3 (δ 4.33, brs)/H-4 β (δ 2.81, dd, J = 2.2, 15.8 Hz) and the NOESY correlations of H-3/H-1 β , H-2 β , and H-4 β . Thus, the structure of **14** (withalongolide N) was assigned as 28-O- β -D-glucopyranosyl-3 α -hydroxy-6-oxo-19-norwitha-5(10),24-dienolide.

Eight known compounds were identified by comparison of their data with those published in the literature, as seven withanolides, sitoindoside IX (**15**),¹⁸ withaferin A (**16**),^{1,13} 2,3-dihydro-3 β -methoxywithaferin A (**17**),¹⁵ viscosalactone B (**18**),²² 2,3-dihydro-3 β -O-sulfate withaferin A (**19**),¹⁷ 2,3-dihydrowithaferin A (**20**),¹⁵ and 3 α ,6 α -epoxy-4 β ,5 β ,27-trihydroxy-1-oxowitha-24-enolide (**21**),²³ and a flavonoid glucoside, rutin (**22**).²⁴ The full assignments of the NMR data of **15**, **16**, **17**, **18**, and **19** are listed in Tables 2–5, as these data were either unavailable, incomplete, or in need of revision within the published literature.

The classically defined withanolide-type steroids (**1–21**) isolated from the title plant showed a diversity of oxygenation patterns that may be summarized as follows: (1) Six withanolides (**1**, **2**, **4**, **7**, **9**, and **10**) have an oxygenated C-19 group, which is rare in Nature. A literature investigation showed that from the approximately 520 unmodified withanolides only nine C-19 oxygenated withanolides have been reported so far. They are as follows: jaborosalactones O,²⁵ V, W, X,²⁶ 46, 47, and 48,²⁷ cinerolide,²⁸ and bracteosin B.²⁹ (2) Compounds **3** and **5** are rare examples of unmodified withanolides having an oxygenated C-11, although withasomniferanolid (with an 11 β -OH group),³⁰ somniferanolid (with an 11 β -OH group),³⁰ and witharifeen (with an 11 α -OH group)³¹ have been previously reported. (3) Saponins **10–14** have a sugar constituent attached at C-28. Only two previously published withanolide

saponins (physagulins E and G)²⁰ have been shown to have a sugar moiety at C-28 thus far. (4) Compounds **11–14** are the first reported examples of C-19 norwithanolide saponins. It should be noted, however, that there are only four C-19 norwithanolides (jaborosalactones **Q**,²¹ **7**,³² and **45**; 12-*O*-methyljaborosalactone **45**²⁷) reported in the literature. (5) Most withanolides have an oxygenated C-1 in ring A, but **15** is the exception by not being oxidized at C-1. In addition, the presence of a 3-*O*-sulfate group in naturally occurring withanolides is extremely rare. Besides withanolides **7** and **19**, there are only five other 3-*O*-sulfate withanolides previously reported from *Datura metel*,³³ *Solanum cilistum*,³⁴ and *Withania somnifera*.³⁵

All the withanolides (**1–21**) and the four acetylated derivatives (**1a**, **1b**, **2a**, and **2b**) were tested against the HNSCC (JMAR, MDA-1986), melanoma (B16F10 and SKMEL-28), and/or normal fetal fibroblast (MRC-5) cells for their cytotoxicity. As summarized in Table 6, withanolides **1–5**, **7**,

Table 6. Cytotoxicity (IC₅₀) of Isolated Withanolides (μM) against Five Cell Lines^a

compound	B16F10	SKMEL-28	JMAR	MDA-1986	MRC-5
1	>10	5.1	5.3	3.3	>10
1a	0.067	0.54	0.16	0.91	0.58
1b	0.098	0.81	0.14	2.2	0.41
2	0.20	3.9	0.17	1.3	0.40
2a	0.13	0.27	0.24	0.11	0.51
2b	0.19	0.64	0.12	0.49	0.16
3	0.49	3.0	0.77	2.6	3.6
4	3.2	9.3	4.7	>10	6.5
5	5.6	>10	>10	8.3	7.3
7	1.3	4.8	2.3	2.0	3.3
8	>10	>10	8.2	8.1	8.7
15	3.7	8.3	4.2	>10	5.2
16	0.29	4.0	2.0	0.80	0.20
19	0.18	5.1	0.48	0.27	1.4
cisplatin (positive control)	1.0	1.1	1.1	1.6	8.9

^aFor cell lines used, see text. Withanolides **6**, **9–14**, **17**, **18**, **20**, and **21** were inactive for all cell lines used (IC₅₀ > 10 μM).

8, **15**, **16**, and **19** and the four derivatives (**1a**, **1b**, **2a**, and **2b**) showed cytotoxic effects against the cells tested with IC₅₀ values in the range 0.067–9.3 μM, while the other withanolides were inactive. Similar to withaferin A (**16**), withanolides **1–3**, containing the functional groups of a 2-en-1-one in ring A, a *Sβ,6β*-epoxy in ring B, and a lactone ring in the side chain, were active, showing the importance of these three groups. The activity of the 3-*O*-sulfate withanolides **7** and **19** was due to their transformations to **1** and **16**, respectively. Withanolide glycosides **8** and **15** displayed less cytotoxicity relative to their aglycone withaferin A (**16**). However, the esterification of the hydroxy groups at C-4, C-19, and C-27 increased the resultant cytotoxicity, as shown for the acetylated derivatives **1a** and **2a** with IC₅₀ values less than 1 μM against all the cells tested. These results are in agreement with previous structure–activity relationship reports.^{10,36,37} In addition, it should be noted that withalongolide A (**1**), withalongolide B (**2**), and withaferin A (**16**) are most likely responsible for the cytotoxic activities of the extract prepared from the title plant due to their relatively high abundance levels (0.16% for **1**, 0.10% for **16**, and 0.03% for **2**).

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were obtained using an MPA100 melting point apparatus. 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. NMR spectra were recorded with a Bruker AV-400 or AV-500 instrument with a cryoprobe for ¹H, APT, COSY/DQF-COSY, HSQC, HMBC, and NOESY/ROESY. Chemical shift values are given in δ (ppm) using the peak signals of the solvent C₅D₅N (δ_H 8.74, 7.58, and 7.22; and δ_C 150.35, 135.91, and 123.87) or CDCl₃ (δ_H 7.24 and δ_C 77.23) as references, and coupling constants are reported in Hz. ESIMS data were measured with an Agilent 1200 Series LC-MS/MS ion trap 6300 mass spectrometer. HRESIMS data were collected with a LCT Premier time-of-flight mass spectrometer (Waters Corp., Milford, MA, USA). Column chromatography was performed on silica gel (particle size 12–25 μm) (Sorbent Technologies, Atlanta, GA, USA), MCI CHP20P (particle size 75–150 μm) (Sigma-Aldrich, Saint Louis, MO, USA), or Sephadex LH-20 (GE Healthcare, Piscataway, NJ, USA) or C₁₈ reversed-phase silica gel (particle size 40–65 μm) (Sigma-Aldrich). Normal-phase silica gel G TLC plates (w/UV 254) and reversed-phase C₁₈ TLC plates (w/UV 254) (Sorbent Technologies) were used for fraction detection. The spots were visualized using UV light at 254 nm and spraying with 10% EtOH–sulfuric acid reagent. Semipreparative HPLC was performed on an Agilent 1200 unit equipped with a DAD detector, utilizing a Lichrospher RP-18 column (250 × 10 mm, 5 μm).

Plant Material. Fresh aerial parts of the plant *P. longifolia* were collected from the Kanopolis wildlife area (latitude: 38.74206°; longitude: 97.98467°), Ellsworth County, Kansas, USA, in August 2009. It was identified by plant taxonomist Dr. Kelly Kindscher at the Kansas Biological Survey, University of Kansas. A voucher specimen (Hillary Loring 3583) was deposited in the R.L. McGregor Herbarium of the University of Kansas.

Extraction and Isolation. The collected biomass was air-dried at room temperature. The dried material was then ground to a coarse powder (930 g) and extracted three times with CH₂Cl₂–MeOH (50:50, 4.0 L) at room temperature. After removing the solvents under vacuum, the extract (107 g) was suspended in 400 mL of H₂O, followed by partitions with *n*-hexane, EtOAc, and *n*-butanol (3 × 500 mL). The resulting ethyl acetate fraction (22 g) collected 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 (80:20) (1.0 g) was again subjected to silica gel CC [eluted with CH₂Cl₂–CH₃COCH₃ (90:10)] to afford compound **2** (280 mg). The fraction (3.0 g) obtained on elution with hexane–acetone (70:30) was subjected to silica gel CC [eluted with CH₂Cl₂–CH₃COCH₃ (80:20)] to yield compounds **16** (730 mg), **17** (7 mg), and **20** (10 mg). The fraction (700 mg) obtained on elution with hexane–acetone (65:35) was subjected further to silica gel CC [eluted with hexane–acetone (65:35)] to afford compounds **3** (30 mg) and **5** (5 mg). The fraction (2.2 g) acquired on elution with hexane–acetone (60:40) was applied to silica gel CC [eluted with hexane–acetone (60:40)] to afford compounds **1** (1200 mg) and **4** (15 mg). The *n*-butanol fraction (29 g) obtained was subjected to a MCI CHP20P gel CC (500 g) eluted with a mixture of H₂O–MeOH (100:0, 80:20, 60:40, 40:60, 85:15, 0:100), in order of increasing concentrations of methanol. The 85% MeOH fraction (3.5 g) was subjected to silica gel CC, eluted with CH₂Cl₂–CH₃COCH₃ with increasing amounts of acetone to afford compounds **1** (240 mg), **6** (40 mg), **16** (200 mg), **18** (250 mg), and **21** (220 mg). The 60% MeOH fraction (4.2 g) was subjected to silica gel CC, eluted with CH₂Cl₂–MeOH–H₂O (7:1:0.1) with increasing amounts of MeOH–H₂O (10:1). Then the fractions were further subjected to reversed-phase C₁₈ Si gel column chromatography (200 g, particle size 40–63 μm), eluted with MeOH–H₂O (40:60, 50:50, 60:40, 65:35). The fractions obtained were subjected to semipreparative HPLC, with the mobile phase CH₃CN–H₂O (26:74; 28:72), to afford compounds **7** (40 mg), **8** (35 mg), **9** (9 mg), **10** (12 mg), **11** (6 mg), **12** (5 mg), **13** (6 mg), **14** (7 mg), **15** (22 mg), **19** (35 mg), and **22** (80 mg).

Withalongolide A (1): colorless cuboid crystals (CH_3CN); mp 216–217 °C; $[\alpha]_D^{25} +14.2$ (c 0.16, CHCl_3); IR (neat) ν_{\max} 3431 (br), 3233 (br), 2922, 1706, 1671, 1400, 1037, 962 cm^{-1} ; ^1H NMR and ^{13}C NMR, see Tables 1 and 2; ESIMS (positive-ion mode) m/z 487 ($M + \text{H}$, 6), 469 ($M - \text{H}_2\text{O} + \text{H}$, 100), 451 ($M - 2 \text{H}_2\text{O} + \text{H}$, 6); HRESIMS m/z 509.2489 [$M + \text{Na}$] $^+$ (calcd for $\text{C}_{28}\text{H}_{38}\text{O}_7\text{Na}$, 509.2471), m/z 487.2674 [$M + \text{H}$] $^+$ (calcd for $\text{C}_{28}\text{H}_{39}\text{O}_7$, 487.2696), m/z 469.2585 [$M - \text{H}_2\text{O} + \text{H}$] $^+$ (calcd for $\text{C}_{28}\text{H}_{37}\text{O}_6$, 469.2590).

Single-Crystal X-ray Structure Determination of Withalongolide A (1). Crystal analysis was performed with a colorless plate crystal (dimensions $0.42 \times 0.35 \times 0.21 \text{ mm}^3$) obtained from CH_2Cl_2 – CH_3CN (1:1) using Mo $K\alpha$ radiation ($\lambda = 0.71073 \text{ \AA}$) on a Bruker SMART APEX diffractometer equipped with a sealed-tube X-ray source and a graphite monochromator. Crystal data for 1: $\text{C}_{28}\text{H}_{38}\text{O}_7$ (formula weight 486.58), monoclinic, space group $P2_1$, $T = 100(2) \text{ K}$, crystal cell parameters $a = 8.370(2) \text{ \AA}$, $b = 10.523(3) \text{ \AA}$, $c = 14.280(3) \text{ \AA}$, $\beta = 104.552(4)^\circ$, $V = 1217.4(5) \text{ \AA}^3$, $D_c = 1.327 \text{ Mg/m}^3$, $Z = 2$, $F(000) = 524$, absorption coefficient $\mu = 0.094 \text{ mm}^{-1}$. A total of 11 335 reflections were collected in the range $2.43^\circ < \theta < 29.21^\circ$, with 5809 independent reflections [$R_{\text{int}} = 0.050$] and 5478 with $I > 2\sigma(I)$; completeness to θ_{\max} was 93.1%. Multiscan absorption correction applied; full-matrix least-squares refinement on F^2 , the numbers of data/restraints/parameters were 5809/1/468; goodness-of-fit on $F^2 = 1.015$; final R indices [$I > 2\sigma(I)$], $R_1 = 0.045$, $wR_2 = 0.098$; R indices (all data), $R_1 = 0.048$, $wR_2 = 0.099$; largest difference peak and hole, 0.37 and -0.21 e/\AA^{-3} .

Acetylation of Withalongolide A (1). A solution of 1 (50 mg) in pyridine (8 mL) and acetic anhydride (2 mL) was stirred for 24 h at room temperature. Then 50 mL of water was added to the solution. The precipitate (70 mg) was subjected to semipreparative HPLC, eluted with the mobile phase CH_3CN – H_2O (45:55), to afford triacetate 1a (40 mg) and diacetate 1b (10 mg).

Withalongolide A 4,19,27-triacetate (1a): IR (neat) ν_{\max} 2953 (br), 1731, 1702, 1674, 1366, 1208, 1023, 966 cm^{-1} ; ^1H NMR and ^{13}C NMR, see Tables 1 and 2; ESIMS (positive-ion mode) m/z 630 ($M + \text{H}_2\text{O}$, 30), 613 ($M + \text{H}$, 100); HRESIMS m/z 635.2829 [$M + \text{Na}$] $^+$ (calcd for $\text{C}_{34}\text{H}_{44}\text{O}_{10}\text{Na}$, 635.2832).

Withalongolide A 4,27-diacetate (1b): IR (neat) ν_{\max} 3536 (br), 2922, 1736, 1701, 1674, 1376, 1215, 1021, 967 cm^{-1} ; ^1H NMR and ^{13}C NMR, see Tables 1 and 2; ESIMS (positive-ion mode) m/z 1163 ($2 M + \text{Na}$, 6), 588 ($M + \text{H}_2\text{O}$, 10), 571 ($M + \text{H}$, 100); HRESIMS m/z 593.2740 [$M + \text{Na}$] $^+$ (calcd for $\text{C}_{32}\text{H}_{42}\text{O}_9\text{Na}$, 593.2727).

Withalongolide B (2): colorless plate crystals (toluene); mp 197–198 °C; $[\alpha]_D^{25} +12.3$ (c 0.15, CHCl_3); IR (neat) ν_{\max} 3260 (br), 3006, 2946, 2887, 1693, 706, 1675, 1383, 1082, 763 cm^{-1} ; ^1H NMR and ^{13}C NMR, see Tables 1 and 2; ESIMS (positive-ion mode) m/z 963 ($2 M + \text{Na}$, 40), 941 ($2 M + \text{H}$, 70), 493 ($M + \text{Na}$, 10), 471 ($M + \text{H}$, 4), 453 ($M - \text{H}_2\text{O} + \text{H}$, 100), 435 ($M - 2 \text{H}_2\text{O} + \text{H}$, 45%); HRESIMS m/z 493.2554 [$M + \text{Na}$] $^+$ (calcd for $\text{C}_{28}\text{H}_{38}\text{O}_6\text{Na}$, 493.2566).

Single-Crystal X-ray Structure Determination of Withalongolide B (2). Crystal analysis was performed with a colorless plate (dimensions $0.21 \times 0.16 \times 0.15 \text{ mm}^3$) obtained from CH_2Cl_2 – CH_3COCH_3 –toluene (1:1:1) using Cu $K\alpha$ radiation ($\lambda = 1.54178 \text{ \AA}$) on a Bruker APEX2 diffractometer equipped with a Bruker MicroStar microfocussing rotating anode X-ray source and Helios multilayer optics. Crystal data for 2: $\text{C}_{28}\text{H}_{38}\text{O}_6$ · C_7H_8 (formula weight 562.72), orthorhombic, space group $P2_12_12_1$, $T = 100(2) \text{ K}$, crystal cell parameters $a = 7.1844(3) \text{ \AA}$, $b = 26.0678(10) \text{ \AA}$, $c = 49.0852(18) \text{ \AA}$, $V = 9192.8(6) \text{ \AA}^3$, $D_c = 1.220 \text{ Mg/m}^3$, $Z = 12$, $F(000) = 3648$, absorption coefficient $\mu = 0.65 \text{ mm}^{-1}$. A total of 32 032 reflections were collected in the range $1.80^\circ < \theta < 69.15^\circ$, with 13 806 independent reflections [$R_{\text{int}} = 0.035$] and 12 653 with $I > 2\sigma(I)$; completeness to θ_{\max} was 90.8%. Multiscan absorption correction applied; full-matrix least-squares refinement on F^2 , the numbers of data/restraints/parameters were 13 806/0/1108; goodness-of-fit on $F^2 = 1.083$; final R indices [$I > 2\sigma(I)$], $R_1 = 0.096$, $wR_2 = 0.233$; R indices (all data), $R_1 = 0.102$, $wR_2 = 0.238$; largest difference peak and hole, 0.84 and -0.32 e/\AA^{-3} .

Acetylation of Withalongolide B (2). A solution of 2 (50 mg) in pyridine (8 mL) and acetic anhydride (2 mL) was stirred for 24 h at

room temperature. Then 50 mL of water was added to the solution. The precipitate (65 mg) was subjected to semipreparative HPLC, eluted with the mobile phase CH_3CN – H_2O (43:57), to afford diacetate 2a (41 mg) and monoacetate 2b (11 mg).

Withalongolide B 4,19-diacetate (2a): IR (neat) ν_{\max} 2943 (br), 1738, 1698, 1368, 1220, 1127, 1043, 1019, 762 cm^{-1} ; ^1H NMR and ^{13}C NMR, see Tables 1 and 2; ESIMS (positive-ion mode) m/z 1109 ($2 M + \text{H}$, 60), 577 ($M + \text{Na}$, 40), 555 ($M + \text{H}$, 30), 495 ($M - \text{HOAc} + \text{H}$, 100), 435 ($M - 2 \text{HOAc} + \text{H}$, 30), 296 (70); HRESIMS m/z 577.2764 [$M + \text{Na}$] $^+$ (calcd for $\text{C}_{32}\text{H}_{42}\text{O}_8\text{Na}$, 577.2727).

Withalongolide B 4-acetate (2b): IR (neat) ν_{\max} 3536 (br), 2922, 1736, 1701, 1674, 1376, 1215, 1021, 967 cm^{-1} ; ^1H NMR and ^{13}C NMR, see Tables 1 and 2; ESIMS (positive-ion mode) m/z 1047 ($2 M + \text{Na}$, 30), 1025 ($2 M + \text{H}$, 75), 513 ($M + \text{H}$, 100), 453 ($M - \text{HOAc} + \text{H}$, 6); HRESIMS m/z 535.2670 [$M + \text{Na}$] $^+$ (calcd for $\text{C}_{30}\text{H}_{40}\text{O}_7\text{Na}$, 535.2672).

Withalongolide C (3): colorless cuboid crystals, mp 197–198 °C; $[\alpha]_D^{25} +10.3$ (c 0.12, CHCl_3); IR (neat) ν_{\max} 3550 (br), 3419 (br), 2952, 2879, 1686, 1663, 1394, 1227, 1026, 957 cm^{-1} ; ^1H NMR and ^{13}C NMR, see Tables 1 and 2; ESIMS (positive-ion mode) m/z 469 ($M - \text{H}_2\text{O} + \text{H}$, 100); HRESIMS m/z 509.2481 [$M + \text{Na}$] $^+$ (calcd for $\text{C}_{28}\text{H}_{38}\text{O}_7\text{Na}$, 509.2471).

Single-Crystal X-ray Structure Determination of Withalongolide C (3). Crystal analysis was performed with a colorless irregular chunk (dimensions $0.45 \times 0.32 \times 0.25 \text{ mm}^3$) obtained from CH_2Cl_2 – CH_3CN (1:1) and measured using Mo $K\alpha$ radiation ($\lambda = 0.71073 \text{ \AA}$) on a Bruker APEX diffractometer equipped with a sealed-tube X-ray source and a graphite monochromator. Crystal data for 3: $\text{C}_{28}\text{H}_{38}\text{O}_7$ (formula weight 486.58), orthorhombic, space group $P2_12_12_1$, $T = 100(2) \text{ K}$, crystal cell parameters $a = 10.679(4) \text{ \AA}$, $b = 12.245(5) \text{ \AA}$, $c = 18.674(7) \text{ \AA}$, $V = 2442(2) \text{ \AA}^3$, $D_c = 1.324 \text{ Mg/m}^3$, $Z = 4$, $F(000) = 1048$, absorption coefficient $\mu = 0.094 \text{ mm}^{-1}$. A total of 22 352 reflections were collected in the range $2.53^\circ < \theta < 29.12^\circ$, with 6145 independent reflections [$R_{\text{int}} = 0.055$] and 5925 with $I > 2\sigma(I)$; completeness to θ_{\max} was 95.6%. Multiscan absorption correction applied; full-matrix least-squares refinement on F^2 , the numbers of data/restraints/parameters were 6145/0/468; goodness-of-fit on $F^2 = 1.080$; final R indices [$I > 2\sigma(I)$], $R_1 = 0.045$, $wR_2 = 0.109$; R indices (all data), $R_1 = 0.046$, $wR_2 = 0.113$; largest difference peak and hole, 0.63 and -0.21 e/\AA^{-3} .

Withalongolide D (4): $[\alpha]_D^{25} +2.7$ (c 0.08, CHCl_3); IR (neat) ν_{\max} 3411 (br), 2944 (br), 1693, 1393, 1211, 1023 cm^{-1} ; ^1H NMR and ^{13}C NMR, see Tables 2 and 3; ESIMS (positive-ion mode) m/z 1059 ($2 M + \text{Na}$, 6), 519 ($M + \text{H}$, 25), 501 ($M - \text{H}_2\text{O} + \text{H}$, 100), 483 ($M - 2 \text{H}_2\text{O} + \text{H}$, 4); HRESIMS m/z 541.2798 [$M + \text{Na}$] $^+$ (calcd for $\text{C}_{28}\text{H}_{38}\text{O}_7\text{Na}$, 541.2777).

Withalongolide E (5): $[\alpha]_D^{25} +2.2$ (c 0.12, CHCl_3); IR (neat) ν_{\max} 3550 (br), 2940 (br), 1690, 1390, 1200, 1020 cm^{-1} ; ^1H NMR and ^{13}C NMR, see Tables 2 and 3; ESIMS (positive-ion mode) m/z 1059 ($2 M + \text{Na}$, 20), 541 ($M + \text{Na}$, 18), 501 ($M - \text{H}_2\text{O} + \text{H}$, 100); HRESIMS m/z 541.2777 [$M + \text{Na}$] $^+$ (calcd for $\text{C}_{28}\text{H}_{38}\text{O}_7\text{Na}$, 541.2777).

Withalongolide F (6): colorless cuboid crystals, mp 190–191 °C; $[\alpha]_D^{25} +8.9$ (c 0.16, CHCl_3); IR (neat) ν_{\max} 2887, 1683, 1659, 1393, 1002, 851 cm^{-1} ; ^1H NMR and ^{13}C NMR, see Tables 2 and 3; ESIMS (positive-ion mode) m/z 871 ($2 M + \text{Na}$, 25), 425 ($M + \text{H}$, 100); HRESIMS m/z 447.2503 [$M + \text{Na}$] $^+$ (calcd for $\text{C}_{27}\text{H}_{38}\text{O}_4\text{Na}$, 447.2511).

Single-Crystal X-ray Structure Determination of Withalongolide F (6). Crystal analysis was performed with a colorless rectangular parallelepiped (dimensions $0.39 \times 0.37 \times 0.20 \text{ mm}^3$) obtained from CH_2Cl_2 – CH_3CN (1:1) and measured using Mo $K\alpha$ radiation ($\lambda = 0.71073 \text{ \AA}$) on a Bruker APEX diffractometer equipped with a sealed-tube X-ray source and graphite monochromator. Crystal data for 6: $\text{C}_{27}\text{H}_{36}\text{O}_4$ (formula weight 424.56), monoclinic, space group $P2_1$, $T = 100(2) \text{ K}$, crystal cell parameters $a = 10.873(5) \text{ \AA}$, $b = 9.233(4) \text{ \AA}$, $c = 12.271(6) \text{ \AA}$, $\beta = 113.273(7)^\circ$, $V = 1132(9) \text{ \AA}^3$, $D_c = 1.246 \text{ Mg/m}^3$, $Z = 2$, $F(000) = 460$, absorption coefficient $\mu = 0.082 \text{ mm}^{-1}$. A total of 10 347 reflections were collected in the range $2.85^\circ < \theta < 29.06^\circ$, with 5283 independent reflections [$R_{\text{int}} = 0.041$] and 5133 with $I > 2\sigma(I)$; completeness to θ_{\max} was 93.8%. Multiscan absorption correction

applied; full-matrix least-squares refinement on F^2 , the numbers of data/restraints/parameters were 5283/1/424; goodness-of-fit on $F^2 = 1.055$; final R indices [$I > 2\sigma(I)$], $R_1 = 0.045$, $wR_2 = 0.107$; R indices (all data), $R_1 = 0.046$, $wR_2 = 0.108$; largest difference peak and hole, 0.43 and -0.21 e/Å³.

Withalongoide G (7): $[\alpha]_D^{25} -2.3$ (c 0.11, MeOH); IR (neat) ν_{\max} 3385 (br), 2950 (br), 1686, 1399, 1234, 992 cm⁻¹; ¹H NMR and ¹³C NMR, see Tables 2 and 3; ESIMS (positive-ion mode) m/z 1031 [2 (M - SO₃) + Na, 20], 585 (M + H, 50), 505 (M - SO₃ + H, 100); (negative-ion mode) m/z 583 (M - H, 100); HRESIMS m/z 607.2169 [M + Na]⁺ (calcd for C₂₈H₄₀O₁₁SNa, 607.2189).

Withalongoide H (8): $[\alpha]_D^{25} -0.9$ (c 0.12, MeOH); IR (neat) ν_{\max} 3369 (br), 2929 (br), 1686, 1398, 1018, 916 cm⁻¹; ¹H NMR and ¹³C NMR, see Tables 4 and 5; ESIMS (positive-ion mode) m/z 796 [M + H₂O, 40], 471 (M - rha - glc + H, 100); LC-MS/MS fragments of m/z 796 peak: m/z 471 (M - rha - glc + H, 100), 281 (10); LC-MS/MS fragments of m/z 471 peak: m/z 281 (100); HRESIMS m/z 801.3683 [M + Na]⁺ (calcd for C₄₀H₅₈O₁₅Na, 801.3673).

Withalongoide I (9): $[\alpha]_D^{25} -7.1$ (c 0.08, MeOH); IR (neat) ν_{\max} 3381 (br), 2921 (br), 1686, 1398, 1007 cm⁻¹; ¹H NMR and ¹³C NMR, see Tables 4 and 5; ESIMS (positive-ion mode) m/z 652 (M + H₂O, 6), 635 (M + H, 5), 473 (M - glc + H, 100); HRESIMS m/z 657.3267 [M + Na]⁺ (calcd for C₃₄H₅₀O₁₁Na, 657.3251).

Withalongoide J (10): $[\alpha]_D^{25} -1.0$ (c 0.13, MeOH); IR (neat) ν_{\max} 3370 (br), 2931 (br), 1687, 1396, 1017 cm⁻¹; ¹H NMR and ¹³C NMR, see Tables 4 and 5; ESIMS (positive-ion mode) m/z 1273 (2 M + H, 100), 619 (M - H₂O + H, 20), 601 (M - 2 H₂O + H, 15), 583 (M - 3 H₂O + H, 10), 475 (M - glc + H, 6); HRESIMS m/z 659.3411 [M + Na]⁺ (calcd for C₃₄H₅₂O₁₁Na, 657.3407).

Withalongoide K (11): $[\alpha]_D^{25} +3.1$ (c 0.17, MeOH); IR (neat) ν_{\max} 3381 (br), 2921 (br), 1686, 1398, 1007 cm⁻¹; ¹H NMR and ¹³C NMR, see Tables 4 and 5; ESIMS (positive-ion mode) m/z 460 (M - glc + H, 40) 603 (M + H, 35), 1205 (2 M + H, 100); HRESIMS m/z 625.33007 [M + Na]⁺ (calcd for C₃₃H₄₆O₁₀Na, 625.2989).

Withalongoide L (12): $[\alpha]_D^{25} -3.1$ (c 0.09, MeOH); IR (neat) ν_{\max} 3350 (br), 2931 (br), 1687, 1396, 1018 cm⁻¹; ¹H NMR and ¹³C NMR, see Tables 4 and 5; ESIMS (positive-ion mode) m/z 1241 (2 M + H, 15), 1079 (2 M - glc + H, 30), 638 (M + H₂O, 80), 459 (M - glc + H, 100); LC-MS/MS fragments of the m/z 638 peak: m/z 621 (M + H, 80), 459 (M - glc + H, 100); HRESIMS m/z 643.3101 [M + Na]⁺ (calcd for C₃₃H₄₈O₁₁Na, 643.3094).

Withalongoide M (13): $[\alpha]_D^{25} -4.4$ (c 0.08, MeOH); IR (neat) ν_{\max} 3380 (br), 2932 (br), 1687, 1650, 1388, 1070 cm⁻¹; ¹H NMR and ¹³C NMR, see Tables 4 and 5; ESIMS (positive-ion mode) m/z 1209 (2 M + H, 20), 1048 (2 M - glc + H, 30), 622 (M + H₂O, 30), 605 (M + H, 10), 443 (M - glc + H, 100); HRESIMS m/z 627.3156 [M + Na]⁺ (calcd for C₃₃H₄₈O₁₀Na, 627.3145).

Withalongoide N (14): $[\alpha]_D^{25} -2.0$ (c 0.14, MeOH); IR (neat) ν_{\max} 3368 (br), 2931 (br), 1688, 1650, 1384, 1072, 1017 cm⁻¹; ¹H NMR and ¹³C NMR, see Tables 4 and 5; ESIMS (positive-ion mode) m/z 1209 (2 M + H, 35), 605 (M + H, 100); LC-MS/MS fragments of m/z 1209 peak: m/z 605 (M + H, 100), 443 (M - glc + H, 100); LC-MS/MS fragments of m/z 605 peak: m/z 443 (M - glc + H, 100); HRESIMS m/z 627.3146 [M + Na]⁺ (calcd for C₃₃H₄₈O₁₀Na, 627.3145).

Cytotoxicity Bioassay. The cytotoxicity assays were performed as previously described.¹¹ In general, five concentrations ranging from 0.1 to 100 μg/mL were tested for the extracts, and 10 concentrations ranging from 50 nM to 20 μM were tested for pure compounds. Statistical analysis was carried out by one-way ANOVA on ranks test using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). IC₅₀ values were obtained from cell viability plots fitted with a sigmoidal dose-response function with variable slope using GraphPad Prism 5 software.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

¹H and ¹³C NMR spectra of withanolides 1–21 and the bioassay data of the samples (CH₂Cl₂-MeOH crude extract,

hexane-soluble fraction, EtOAc-soluble fraction, and *n*-BuOH-soluble fraction) are available free of charge via the Internet at <http://pubs.acs.org>. Crystallographic data for the structures of 1, 2, 3, and 6 reported in this paper have been deposited with the Cambridge Crystallographic Data Centre, under reference numbers CCDC 840311, CCDC 840312, CCDC 840313, and CCDC 840314, respectively. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

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