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Withanolides from the Rhizomes of *Dioscorea japonica* and Their Cytotoxicity

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Supporting Information

ABSTRACT: Edible yams are tropical crops that serve as important staple foods in many parts of the world. The rhizome of *Dioscorea japonica*, well-known as "Japanese yam", is a food and medicinal source known as "San Yak" in Korea. Bioassay-guided fractionation and chemical investigation of the extract of this yam resulted in the identification of two new withanolides, named dioscorolide A (**1**) and dioscorolide B (**2**). The structures of these new compounds were determined by spectroscopic methods, including 1D and 2D nuclear magnetic resonance (NMR) techniques, high-resolution mass spectrometry (HRMS), and chemical methods. The cytotoxic activities of the isolates (**1** and **2**) were evaluated by determining their inhibitory effects on four human tumor cell lines (A549, SK-OV-3, SK-MEL-2, and HCT15) and a human normal cell line (HUVEC) using a sulforhodamine B (SRB) bioassay. Compounds **1** and **2** showed cytotoxicity against tumor cell lines (A549, SK-OV-3, SK-MEL-2, and HCT15) with IC_{50} values ranging from 6.3 to 26.9 μM and exhibited lower activity against the normal cell line (HUVEC) with IC_{50} values ranging from 27.1 to 28.8 μM, suggesting selective toxicity among tumor and normal cells.

KEYWORDS: *Dioscorea japonica*, Dioscoreaceae, withanolides, structural elucidation, cytotoxicity

INTRODUCTION

Edible yams are tropical crops that serve as important staple foods in many parts of the world. There are several species in the genus *Dioscorea* that are known as yams. The two most important ones in the Pacific are *Dioscorea alata* (greater yam, water yam) and *Dioscorea esculenta* (lesser yam, potato yam).[1] These yams have continued to make an important contribution to nutrition and food security in most Pacific islands. In particular, *D. alata* is an important prestige food in Papua New Guinea, Fiji, Tonga, Vanuata, Samoa, and the Federated States of Micronesia.[2] The rhizome of *Dioscorea japonica* Thunb. (Dioscoreaceae), naturally distributed in East Asia, China, Japan, and Korea, is well-known as "Japanese yam". In Korea, this is a food and medicinal source known as "San Yak". This yam has been used to strengthen stomach function, improve anorexia, eliminate diarrhea, dilute sputum, and moisturize skin in traditional Chinese medicine.[3] Previous phytochemical investigations on *D. japonica* revealed the presence of active hypoglycemic compounds (dioscorans A−F),[4] sesquiterpene, and acetophenone.[5] The main secondary metabolites of *Dioscorea* species were well-known for steroidal components,[6] but these are rarely reported from this yam. Although this famous yam is plentiful in East Asia, there are few reports of its biologically active components.

In our continuing search for bioactive constituents from Korean natural sources, we have investigated the active constituents of this yam and reported the identification of several active constituents including steroidal saponins and their effects on NGF induction.[7] In our screening procedures, an EtOH extract of the rhizome of *D. japonica* showed considerable cytotoxic activity against some human tumor cell lines using a sulforhodamine B (SRB) bioassay. Our interest in further research on cytotoxic constituents from this yam led us to investigate this source in the present study. We describe the isolation and identification of two new withanolides and their in vitro antitumor activity in the present paper.

MATERIALS AND METHODS

General Experimental Procedures. Optical rotations were measured on a Jasco P-1020 polarimeter (Jasco, Easton, MD). IR spectra were recorded on a Bruker IFS-66/S FT-IR spectrometer (Bruker, Karlsruhe, Germany). Circular dichroism (CD) spectra were measured on a Jasco J-715 spectropolarimeter (Jasco). Ultraviolet (UV) spectra were recorded with a Shimadzu UV-1601 UV−visible spectrophotometer (Shimadzu, Tokyo, Japan). High-resolution (HR) electrospray ionization (ESI) mass spectra were recorded on an SI-2/LCQ DecaXP liquid chromatograph (LC)−mass spectrometer (Thermo Scientific, West Palm Beach, FL). Fast-atom bombardment (FAB) and high-resolution (HR) FAB mass spectra were obtained on a JEOL JMS700 mass spectrometer (JEOL, Peabody, MA). Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Unity INOVA 500 NMR spectrometer (Varian, Palo Alto, CA) operating at 500 MHz (^{1}H) and 125 MHz (^{13}C), with chemical shifts given in ppm (δ). Preparative high-performance liquid chromatography (HPLC) used a Gilson 306 pump (Gilson, Middleton, WI) with a Shodex refractive index detector (Shodex, New York, NY). Low-pressure liquid chromatography (LPLC) was carried out over a LiChroprep Lobar-A Si 60 column (240 mm × 10 mm i.d.; Merck, Darmstadt, Germany) with a FMI QSY-0 pump

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Table 1. ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) Data of Compounds 1 and 2 in $CDCl_3$ [δ, J Values (Hertz) in Parentheses][a]

	1		2	
position	δ_H	δ_C	δ_H	δ_C
1		203.3		209.5
2α	5.80 dd (10.0, 2.5)	128.9	2.35 dd (16.0, 3.0)	44.0
2β			3.20 dd (16.0, 8.0)	
3	6.55 ddd (10.0, 5.0, 2.5)	139.7	4.34 m	36.1
4α	2.50 dd (19.0, 5.0)	36.7	2.01 dd (15.0, 2.5)	37.7
4β	2.65 dd (19.0, 2.5)		2.70 dd (15.0, 7.5)	
5		73.2		74.0
6	3.01 d (3.5)	56.3	2.97 d (4.0)	56.3
7	3.28 dd (3.5, 1.5)	57.2	3.24 dd (4.0, 2.0)	57.2
8	1.79 m	35.7	1.79 m	35.3
9	1.77 m	35.68	1.77 m	35.5
10		50.9		52.7
11α	2.69 m	21.9	2.30 m	21.8
11β	1.36 m		1.35 m	
12α	1.34 m	39.8	1.33 m	39.7
12β	2.00 m		1.98 m	
13		43.4		43.6
14	1.15 m	51.8	1.17 m	51.7
15α	1.80 m	23.5	1.79 m	23.4
15β	1.20 m		1.20 m	
16α	1.79 m	27.1	1.78 m	27.1
16β	1.27 m		1.27 m	
17	1.40 m	51.5	1.39 m	51.2
18	0.72 s	12.1	0.70 s	12.1
19	1.13 s	14.7	1.13 s	15.3
20	2.01 m	39.0	2.00 m	38.9
21	0.91 d (7.0)	12.6	0.90 d (7.0)	12.6
22	4.72 dt (12.0, 3.5)	77.9	4.70 dt (12.0, 3.5)	77.9
23α	1.73 m	35.63	1.74 m	35.6
23β	1.52 m		1.49 m	
24		69.9		70.0
25	2.30 q (7.0)	45.9	2.29 q (7.0)	45.9
26		174.0		173.8
27	1.27 d (7.0)	9.2	1.28 d (7.0)	9.2
28	1.33 s	28.5	1.33 s	28.5
SAc				196.3
			2.25 s	30.1

[a] The assignments were based on DEPT, $^{1}H,^{1}H-COSY$, HMQC, and HMBC experiments.

(Teledyne Isco, Lincoln, NE). Column chromatography was performed with a silica gel 60 (Merck, 70−230 and 230−400 mesh) and Sephadex LH-20 (Pharmacia, Uppsala, Sweden). Merck precoated silica gel F_{254} plates and reversed-phase (RP)-18 F_{254s} plates (Merck) were used for thin-layer chromatography (TLC). Spots were detected on TLC under UV light or by heating after spraying with 10% H_2SO_4 in EtOH (v/v).

Plant Material. The rhizomes of *D. japonica* were imported from Hubei, China, in March 2008, and the plant was identified by one of the authors (K.R.L.). A voucher specimen (SKKU 2008-3) was deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

Extraction and Isolation. Dried and pulverized rhizomes of *D. japonica* (25 kg) were extracted with 50% aqueous EtOH (3 × 4 L every 3 days) at room temperature and filtered. The filtrate was evaporated under vacuum to obtain an EtOH extract (2.5 kg), which we suspended in distilled water (8 L) and then successively partitioned with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH, yielding 9, 9, 5, and 78 g of residue, respectively. The remaining water layer was evaporated under vacuum to give a residue, which was extracted with acetone to afford the acetone-soluble fraction (47 g). To identify the active ingredients responsible for the cytotoxic activity, each fraction was evaluated for cytotoxicity against some human tumor cell lines using an SRB bioassay. The active fraction, the $CHCl_3$-soluble fraction (9 g), was chromatographed on a silica gel (230−400 mesh, 300 g) column and eluted with $CHCl_3$/MeOH (15:1 → 1:1, gradient system) to yield nine fractions (A−I). Fraction B (1.9 g) was chromatographed further on a Sephadex LH-20 column (CH_2Cl_2/MeOH, 1:1) and applied to low-pressure liquid chromatography (LPLC) on a LiChroprep Lobar-A Si 60 column (240 mm × 10 mm i.d., 40−63 μm, Merck) eluted with $CHCl_3$/MeOH (40:1) to give three subfractions (B1−B3). Compounds **1** (6 mg, t_R = 16.0 min) and **2** (4 mg, t_R = 13.5 min) were obtained from subfraction B2 (45 mg) by semipreparative normal-phase HPLC with a Shodex refractive index detector (Shodex, New York, NY), using an Apollo Silica column (250 mm × 10 mm i.d., 5 μm, Alltech, Nicholasville, KY) with a solvent system of $CHCl_3$/MeOH (45:1).

Dioscorolide A (1). Compound **1** was obtained as a white amorphous powder: $[\alpha]_D^{25}$ +74.4 (*c* 0.20, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 217 (3.9) nm; CD (MeOH) λ_{max} ($\Delta\varepsilon$) 340 (−13.9) nm; IR (KBr) ν_{max} 3497, 2920, 1714, 1687, 1382, 1221, 1123 cm^{-1}; ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1; fast-atom bombardment mass spectrometry (FABMS) (positive-ion mode) *m/z* 473 $[M + H]^+$; high-resolution (HR)-FABMS (positive-ion mode) *m/z* 473.2896 $[M + H]^+$ (calcd for $C_{28}H_{41}O_6$, 473.2903).

Dioscorolide B (2). Compound **2** was obtained as a white amorphous powder: $[\alpha]_D^{25}$ +12.7 (*c* 0.08, $CHCl_3$); IR (KBr) ν_{max} 3485, 2921, 1710, 1685, 1378, 1255, 1098 cm^{-1}; ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1; FABMS (positive-ion mode) *m/z* 549 $[M + H]^+$; HR-electrospray ionization (ESI)-MS (positive-ion mode) *m/z* 571.2709 $[M + Na]^+$ (calcd for $C_{30}H_{44}NaO_7S$, 571.2705).

Synthesis of 2. A solution of **1** (2.3 mg, 0.0049 mmol) in tetrahydrofuran (THF) (3.5 mL) was treated with thiolacetic acid (1.87 μL; 2.0 mg, 0.026 mmol), and the mixture was stirred at room temperature for 1 h and then diluted with 3.5 mL of EtOAc. After quenching by careful addition of saturated sodium bicarbonate solution, the reaction mixture was transferred to a separatory funnel, extracted with EtOAc, and evaporated under reduced pressure to give the crude extract (2.8 mg).[8] The synthesized **2** was confirmed by a silica gel TLC by comparison with the isolated compound **2** [solvent system ($CHCl_3$/MeOH, 10:1), TLC R_f 0.51]. The crude extract was purified by using semipreparative normal-phase HPLC, using an Apollo Silica column (250 mm × 10 mm i.d., 5 μm, Alltech) with a solvent system of $CHCl_3$/MeOH (45:1) to give the synthesized **2** (0.8 mg, t_R = 13.5 min). The synthesized **2** was identified by comparison of its ^{1}H NMR, MS, and $[\alpha]_D$ value with those of isolated compound **2**.

Tumor and Normal Cell Lines. The cell lines used were A549 (non-small-cell lung adenocarcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma), HCT15 (colon cancer cells), and HUVEC (human umbilical cord endothelial cells). The cancer cell lines A549, SK-OV-3, SK-MEL-2, and HCT15 were provided by the National Cancer Institute (NCI). A normal cell line, HUVEC cells, was purchased from American Type Cell Culture.

In Vitro Cytotoxicity Test. A sulforhodamine B (SRB) bioassay was used to determine the cytotoxicity of each compound against the cell lines mentioned above.[9] The assays were performed at the Korea

Research Institute of Chemical Technology. Doxorubicin (Sigma Chemical Co., ≥98%) was used as a positive control.

NGF and Cell Viability Assay. We used C6 glial cells to measure NGF release into the medium.[10] C6 cells were purchased from the Korean Cell Line Bank (Seoul, Korea). To measure NGF content in medium and cell viability, C6 cells were seeded into 24-well plates (1×10^5 cells/well). After 24 h, the cells were treated with Dulbecco's modified Eagle medium (DMEM) containing 2% fetal bovine serum (FBS) and 1% streptomycin (PS) with 20 μM of each sample for 1 day. Medium supernatant was used for the NGF assay using an ELISA development kit (R&D System, Minneapolis, MN). Cell viability was assessed by a 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay.[11]

RESULTS AND DISCUSSION

Isolation and Structural Elucidation of Compounds. Dried and pulverized rhizomes of *D. japonica* were extracted with 50% aqueous EtOH. The hydroethanolic extract showed considerable cytotoxicity against some human tumor cell lines using an SRB bioassay in our screening procedures. Bioassay-guided fractionation and chemical investigation of the extract using successive column chromatography over silica gel and Sephadex LH-20 and preparative HPLC resulted in the isolation and identification of two new withanolides, dioscorolides A (**1**) and B (**2**) (Figure 1).

Dioscorolide A (**1**), obtained as a white amorphous powder, possessed a molecular formula of $C_{28}H_{40}O_6$ (9 degrees of unsaturation) as determined by the positive-ion HRFABMS and the ^{13}C NMR spectrum. The IR spectrum of **1** displayed absorption bands of hydroxy (3497 cm^{-1}), α,β-unsaturated ketone (1687 cm^{-1}), and δ-lactone (1714 cm^{-1}) functional groups. The UV spectrum of **1** showed absorption at λ_{max} (MeOH) 217 nm, which also implied the presence of an α,β-unsaturated ketone moiety. The ^{1}H and ^{13}C NMR spectra of **1** (Table 1) showed signals for three tertiary methyl groups at δ_H 0.72, 1.13, and 1.33 (each 3H, s) and δ_C 12.1, 14.7, and 28.5 and for two secondary methyl groups at δ_H 0.91 and 1.27 (each 3H, d, J = 7.0 Hz) and δ_C 12.6 and 9.2. Furthermore, signals for an α,β-unsaturated ketone at δ_H 5.80 (1H, dd, J = 10.0, 2.5 Hz) and 6.55 (1H, ddd, J = 10.0, 5.0, 2.5 Hz) and δ_C 203.3 (C-1), 128.9 (C-2), and 139.7 (C-3) and for one epoxy group at δ_H 3.01 (1H, d, J = 3.5 Hz) and 3.28 (1H, dd, J = 3.5, 1.5 Hz) and δ_C 56.3 (C-6) and 57.2 (C-7) could be assigned, indicating the characteristic signals of a 1-oxo-2,3-ene-5-hydroxy-6,7-epoxywithanolide for the A- and B-ring substitution pattern.[12] This partial structure was confirmed by the ^{1}H−^{1}H correlation spectroscopy (COSY) correlations starting at H-2, via H-3, and ending at H-4, and the heteronuclear multiple bond correlation (HMBC) correlations between H-2 and C-1, C-4, and C-10, between H-3 and C-1, C-2, and C-5, and between H-6 and C-4, C-5, and C-10 (Figure 2). Overall, the ^{1}H and ^{13}C NMR data were similar to those of related withanolides,[12,13] but differences were evident at the δ-lactone side chain in the E-ring in terms of the proton splitting pattern and the carbon chemical shifts. The α,β-unsaturated δ-lactone moiety of the E-ring observed in most withanolides was saturated in **1** with the existence of one oxygenated quaternary carbon (δ_C 69.9). HMBC correlations of H-22 at δ_H 4.72 (1H, dt, J = 12.0, 3.5 Hz), H-25 at δ_H 2.30 (1H, q, J = 7.0 Hz), H-27 at δ_H 1.27 (3H, d, J = 7.0 Hz), and H-28 at δ_H 1.33 (3H, s) with C-24 at δ_C 69.9 indicated that the additional hydroxy group was attached to C-24 in **1** (Figure 2). This partial structure was confirmed by the identical ^{13}C NMR chemical shifts of the δ-lactone moiety of **1** with those of philadelphicalactone B.[14] The configuration of **1** was established by analyses of the nuclear Overhauser effect spectroscopy (NOESY) spectrum (Figure 2), the proton coupling constants, and CD spectroscopic data. The NOESY correlation between H-6 and CH_3-19 and the coupling constant (J = 3.5 Hz) between H-6 and H-7 indicated that the A/B ring conformation was *trans*, because a *cis*-junction shows a very small coupling constant value for H-6 (0−2 Hz).[13] The proton signal for CH_3-19 showed correlations to H-6, H-7, and H-8 in the NOESY spectrum, which was consistent with the 6α,7α-epoxy group. The stereochemistry of 5α-hydroxy-6α,7α-epoxy-2-en-1-one was confirmed by a CD spectrum showing a negative Cotton effect at 340 nm.[15] NOESY correlations from H-20 to CH_3-18 and H-23β and from CH_3-21 to H-14, H-17, and H-23α indicated an α-orientated methyl group (C-21) at C-20 (Figure 2). From literature reports, H-22α shows two different coupling constants (0.5−4.0 and 9.0−13.8 Hz); however, H-22β shows two similar coupling constants (2.5−7.0 and 2.0−5.0 Hz).[16] Thus, the H-22 appearing as double triplet (J = 12.0, 3.5 Hz) was defined as α-orientation and the center as *R*. This point was supported by NOESY correlations from H-22 to H-16α and H-17 (Figure 2). Finally, the stereochemistry of the δ-lactone moiety was confirmed by a NOESY experiment showing the correlations between H-22 and H-23α and between H-23β and CH_3-27,

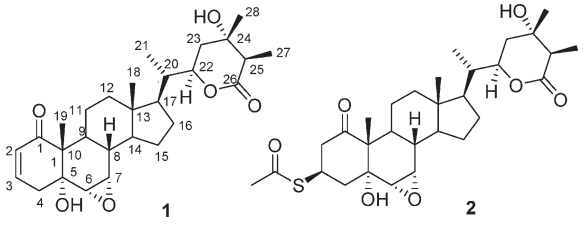

Figure 1. Chemical structures of compounds **1** and **2**.

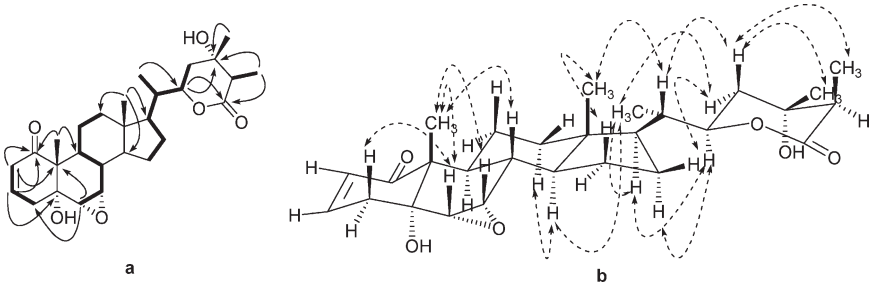

Figure 2. COSY (bold lines) and key HMBC correlations (arrow) of **1** (a); key NOESY correlations (dashed arrow) of **1** (b).

Table 2. Cytotoxicity of Compounds 1 and 2 against Four Cultured Human Tumor Cell Lines Using the SRB Assay in Vitro

	IC_{50}[a] (μM)				
compound	A549	SK-OV-3	SK-MEL-2	HCT15	HUVEC
1	20.4 ± 0.7	7.1 ± 1.3	6.3 ± 0.5	26.9 ± 2.1	28.8 ± 1.4
2	12.6 ± 1.8	25.6 ± 0.4	19.7 ± 2.5	13.5 ± 0.8	27.1 ± 0.2
doxorubicin[b]	0.01 ± 0.003	0.09 ± 0.002	0.02 ± 0.007	0.06 ± 0.008	
cisplatin[c]	1.7 ± 0.1	1.3 ± 0.2	1.0 ± 0.1	1.6 ± 0.4	0.9 ± 0.3

[a] IC_{50} value of compounds against each cancer cell line, which was defined as the concentration (μM) that caused 50% inhibition of cell growth in vitro. Data are expressed as the mean ± SD of three distinct experiments. [b] Doxorubicin as a positive control. [c] Cisplatin as a reference compound.

CH_3-28, suggesting that the CH_3-27 and CH_3-28 were both in β-orientation. Thus, the structure of **1** was determined to be (20*S*,22*R*,24*S*,25*R*)-5α,24α-dihydroxy-6α,7α-epoxy-1-oxowitha-2-en-26,22-olide, trivially named dioscorolide A.

Dioscorolide B (**2**) was obtained as a white amorphous powder with the molecular formula of $C_{30}H_{44}O_7S$, deduced by the positive-ion HRESIMS. Compound **2** also contained an epoxy group linked at C-6/7, on the basis of NMR signals at δ_H 2.97 (1H, d, J = 4.0 Hz) and 3.24 (1H, dd, J = 4.0, 2.0 Hz), which was confirmed by HMBC analysis. Evidence for a δ-lactone moiety containing an OH group at C-24 was suggested by NMR signals for H-27 at δ_H 1.28 (3H, d, J = 7.0 Hz), H-28 at δ_H 1.33 (3H, s), C-24 at δ_C 70.0, and C-26 at δ_C 173.8 and further supported by HMBC analysis. The ^{1}H and ^{13}C NMR data of **1** and **2** were very similar, with the major difference being the presence of an additional acetylthio group (δ_H 2.25; δ_C 30.1, 196.3) at the A-ring in **2**.[17−19] The signals for C-2 at δ_H 5.80 (dd, J = 10.0, 2.5 Hz) and δ_C 128.9 and for C-3 at δ_H 6.55 (ddd, J = 10.0, 5.0, 2.5 Hz) and δ_C 139.7 in **1** were shifted to δ_H 2.35 (dd, J = 16.0, 3.0 Hz), 3.20 (dd, J = 16.0, 8.0 Hz) and δ_C 44.0 and to δ_H 4.34 (m) and δ_C 36.1 in **2**, respectively, suggesting that the α,β-unsaturated ketone in **1** was replaced by a saturated ketone in **2**. The signal for H-3 (δ_H 4.34) shifted to a lower field supported the assignment of the acetylthio group at C-3. This A-ring structure was confirmed by ^{1}H−^{1}H COSY correlations starting at H-2 via H-3 and ending at H-4 in combination with heteronuclear multiple quantum coherence (HMQC) and HMBC correlation between H-3 at δ_H 4.34 and C-3-S$\underline{C}$O (δ_C 196.3). The coupling constant values for $^3J_{2\beta,3}$ (8.0 Hz), $^3J_{4\beta,3}$ (7.5 Hz), $^3J_{2\alpha,3}$ (3.0 Hz), and $^3J_{4\alpha,3}$ (2.5 Hz) revealed that the acetylthio group at C-3 is equatorial in β-orientation. Usually, the J values in the substituted cyclohexanes are expected to be in the ranges of ∼10 Hz for axial−axial and ∼5 Hz for axial−equatorial if the substitution is equatorial and in the order of ∼2−3 Hz for both axial−equatorial and equatorial−equatorial if the substituent is axial.[20] Analysis of the NOESY experiment confirmed the assignment of a β-orientated acetylthio group at C-3. The full assignment of all NMR signals of **2** was performed by the ^{1}H−^{1}H COSY, MHQC, HMBC, and NOESY experiments (Table 1), in agreement with **1** except for major differences in the A-ring of **2**. Withanolides containing an acetylthio group are rarely found in natural sources. Finally, the structure of **2** was confirmed by synthesis from dioscorolide A (**1**) and thiolacetic acid (CH_3COSH).[8] The synthesized product was identified as compound **2** by comparison of the ^{1}H NMR, MS, and $[\alpha]_D$ values of the synthetic compound with those of **2**. Accordingly, the structure of **2** was concluded to be (20*S*,22*R*,24*S*,25*R*)-3β-acetylthio-5α,24α-dihydroxy-6α,7α-epoxy-1-oxowithan-26,22-olide, trivially named dioscorolide B.

Biological Evaluation of Compounds. Compounds **1** and **2** were evaluated for cytotoxicity against four human tumor cell lines including A549 (non-small-cell lung carcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma), and HCT15 (colon cancer) using the SRB bioassay in vitro.[9] The results (Table 2) showed that the tested withanolides (**1** and **2**) had consistent cytotoxicity against the above tested cell lines with IC_{50} values ranging from 6.3 to 26.9 μM. Compound **1** showed significant cytotoxicity against all of the cell lines tested with IC_{50} values of 20.4 ± 0.7, 7.1 ± 1.3, 6.3 ± 0.5, and 26.9 ± 2.1 μM, respectively, for the A549, SK-OV-3, SK-MEL-2, and HCT15 cell lines. Compound **2** also exhibited cytotoxicity against the A549, SK-OV-3, SK-MEL-2, and HCT15 cell lines with IC_{50} values of 12.6 ± 1.8, 25.6 ± 0.4, 19.7 ± 2.5, and 13.5 ± 0.8 μM, respectively. Withanolides have been reported to show potent or moderate cytotoxicity against various tumor cell lines according to literature reports.[13,16,21,22] It was also verified that an α,β-unsaturated ketone unit in ring A is necessary for the cytotoxic activity of withanolides.[23,24] On the basis of this evidence, the relatively weak cytotoxicity of compound **2** against the SK-OV-3 and SK-MEL-2 cells can be explained by the absence of the double bond between C-2 and C-3. However, it seems that the presence of an acetylthio group at C-3 in **2** increases the activity against the A549 and HCT-15 cell lines in consideration of the above obtained data even though it lacks the 2-en-1-one system in ring A. The discovery of the cytotoxic withanolides **1** and **2** suggested that they might be involved in the antitumor activity of the rhizome of *D. japonica*. To establish whether the cytotoxicity exhibited by compounds **1** and **2** was selective between tumor and normal cells, these compounds were tested on a normal human cell line, HUVEC. The results (Table 2) showed that the cytotoxic effects of **1** and **2** were more active against tumor cells than normal cells, indicating that compounds **1** and **2** possess selective toxicity among tumor and normal cells. In particular, compound **1** showed the highest selective cytotoxicity for the SK-MEL-2 cell line; it exhibited a selectivity index (SI) value of 4.6, greater than that of cisplatin, a well-known anticancer agent (SI, 0.9). The SI value was obtained by dividing the IC_{50} value for the normal cell line (HUVEC) by the IC_{50} value for the tumor cell line (SK-MEL-2).[25] Compound **1** also displayed high selective toxicity (SI, 4.1) against the SK-OV-3 cell line. We next evaluated whether compounds **1** and **2** could increase NGF release from C6 glial cells because we found that this yam (*D. japonica*) induced increases in endogenous NGF levels.[7] NGF influences neuronal survival and differentiation and may have therapeutic potential for neurodegenerative diseases and diabetic polyneuropathy triggered by dysfunction of neurons.[26−28] Unfortunately, compounds **1** and **2** did not affect NGF release effectively at concentrations below 20 μM (Supporting Information, Table S1).

In conclusion, the structures of two new withanolides (**1** and **2**) isolated from the rhizomes of *D. japonica* were identified. With regard to bioactivity, anticancer effects of the withanolides showing selective toxicity among tumor and normal cells were confirmed. From the results of the cytotoxicity evaluation of the withanolides, it appears that these compounds may be valuable anticancer agents. Furthermore, dioscorolide A (**1**), which displayed high selective toxicity against the SK-MEL-2 and SK-OV-3 cell lines, may be especially promising for developing an effective drug for melanoma and ovarian cancer in this regard.

This study shows that they can be considered as contributors to the antitumor activity of this yam.

■ ASSOCIATED CONTENT

Supporting Information. 1D (^{1}H and ^{13}C NMR), 2D NMR (^{1}H−^{1}H COSY, HMQC, and HMBC) data of **1** and **2** and effects of compounds **1** and **2** on NGF secretion in C6 cells. This information is available free of charge via the Internet at http://pubs.acs.org.

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