

## Bioactive Withanolides from *Withania obtusifolia*

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### Abstract:

Seven withanolides were isolated from the leaves of *Withania obtusifolia*. Of these, one was new [obtusifonolide (**1**)], five were new to the species [sitoindoside IX (**2**), 6 $\alpha$ -chloro-5 $\beta$ -hydroxy withaferin A (**3**), isowithanone (**4**), 2,3-dihydro-3-ethoxywithaferin A (**5**), and daturataturin A (**6**)], and one was reported previously from *W. obtusifolia* [withaferin A (**7**)]. The structures were elucidated using a set of spectroscopic and spectrometric techniques. Compounds (**1**–**7**) were evaluated for cytotoxicity against a human cancer cell panel and for antimicrobial activity in an array of bacteria and fungi. Compound **7** showed cytotoxic activity against the MDA-MB-435 (human melanoma) and SW-620 (human colon cancer) cell lines with IC<sub>50</sub> values of 1.7 and 0.3  $\mu$ M, respectively. The *in vitro* activity of **7** on 17 $\beta$ -hydroxysteroid dehydrogenase and 5 $\alpha$ -reductase was also investigated.

**Keywords:** *Withania obtusifolia* | Cytotoxicity | Withanolides | Withaferin A | 17 $\beta$ -Hydroxysteroid dehydrogenase | 5 $\alpha$ -Reductase

### Article:

#### 1. Introduction

The withanolides are a group of naturally occurring polyoxygenated C-28 ergostane-type steroids (Chen et al., 2011, Lavie et al., 1965). A common feature among most of them is the oxidation at C-1, C-22, and C-26 (Chen et al., 2011). Withanolides occur largely, but not exclusively, in genera belonging to the plant family Solanaceae, including *Withania*, *Lycium*, *Datura*, *Dunalia*, *Acnistus*, *Jaborosa*, *Nicandra*, and *Physalis* (Misico et al., 2011). Withaferin A, isolated in 1965, was the first withanolide to be characterized from *Withania somnifera* (Lavie

et al., 1965) and from *Acnistus arborescens* (Kupchan et al., 1965). It showed *in vitro* and *in vivo* cytotoxic activity against an array of tumor cells (Glotter, 1991, Samadi et al., 2010).

Withanolides can be divided into two types, those with a  $\delta$ -lactone or  $\delta$ -lactol, resulting from appropriate oxidation of C-22 and C-26, and those with a  $\gamma$ -lactone or  $\gamma$ -lactol involving C-23 and C-26; most of the withanolides belong to the former type (Chen et al., 2011, Glotter, 1991, Misico et al., 2011). Biogenetic transformations of the steroidal skeleton and the side chain have diversified the structures of withanolides (Chen et al., 2011, Glotter, 1991, Misico et al., 2011). Withanolides have attracted attention due to their wide range of biological activities, including antitumor, anti-inflammatory, antifeedant, antimicrobial, cytotoxic, immunomodulating, and cancer chemopreventive activities (Chen et al., 2011, Glotter, 1991, Misico et al., 2011). Moreover, recent studies have suggested that withanolides may also act as growth regulators due to their common biosynthetic origin with brassinosteroids, a well-known class of growth regulators (Sangwan et al., 2008).

As part of an ongoing project to explore medicinal plants of Jordan for anticancer leads (Alali et al., 2005, Alali et al., 2008, Alali et al., 2010), seven withanolides (**1–7**), of which one was new, were isolated and characterized from an ethanolic extract of the leaves of *Withania obtusifolia*.

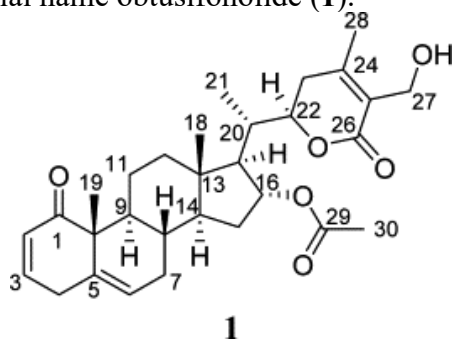
## 2. Results and discussion

Dried leaves of *W. obtusifolia* were extracted with EtOH and partitioned with organic solvents to yield five fractions (F01–F05). Fraction F05 was purified using silica gel column chromatography to yield 241 sub-fractions, and similar ones were combined into 11 pools. Pools P08 and P10 were subjected to gel filtration on Sephadex LH-20 to yield a total of 7 and 5 sub-fractions, respectively. Sub-fractions P08-5 and P10-3 were found to be rich in withanolides as evidenced by thin layer chromatography (TLC), and were purified further using preparative and semipreparative reversed-phase high performance liquid chromatography (RP-HPLC) to yield seven compounds (**1–7**) with >95% purity as evidenced by ultra-performance liquid chromatography (UPLC) (Fig. S1, Supplementary data).

Six known withanolides were identified by 1- and 2-dimensional nuclear magnetic resonance (1- and 2D-NMR), high-resolution mass spectrometry (HRMS) analyses, and comparisons to literature data: sitoindoside IX (**2**) (Ghosal et al., 1988), 6 $\alpha$ -chloro-5 $\beta$ -hydroxy withaferin A (**3**) (Nittala et al., 1981), isowithanone (**4**) (Lala et al., 2006), 2,3-dihydro-3-ethoxywithaferin A (**5**) (Pelletier et al., 1979), daturaturin A (**6**) (Shingu et al., 1990), and withaferin A (**7**) (Lavie et al., 1965). This is the first report of compounds **2–6** from *W. obtusifolia*.

Compound **1** (5.43 mg) was obtained as an off-white powder. The molecular formula was deduced as C<sub>30</sub>H<sub>40</sub>O<sub>6</sub> via high-resolution electrospray ionization mass spectrometry (HRESIMS), establishing an index of hydrogen deficiency of 11. The (+)-atmospheric pressure chemical ionization mass spectrum (APCIMS) displayed peaks at *m/z* 479.53 [M+H–H<sub>2</sub>O]<sup>+</sup>, 437.60 [M+H–OHAc]<sup>+</sup>, 419.38 [M+H–OHAc–H<sub>2</sub>O]<sup>+</sup>, 401.41 [M+H–OHAc–2H<sub>2</sub>O]<sup>+</sup>, 383.35 [M+H–OHAc–3H<sub>2</sub>O]<sup>+</sup>, 267.55 [M–OHAc–lactone]<sup>+</sup>, and 249.34 [M–OHAc–lactone–H<sub>2</sub>O]<sup>+</sup>, a characteristic withanolide fragmentation pattern (Atta-ur-Rahman et al., 2003, Llanos et al., 2010). The <sup>1</sup>H NMR data of **1** revealed the presence of three olefinic protons ( $\delta_{\text{H}}$  5.87,

dd,  $J = 10.3, 2.3$  Hz; 6.78, m; and 5.55, d,  $J = 5.7$  Hz, for H-2, H-3, and H-6, respectively), two oxymethines ( $\delta_{\text{H}}$  4.89, t,  $J = 7.5$  Hz; and 4.24, dt,  $J = 13.2, 3.4$  Hz, for H-16 and H-22, respectively), one oxymethylene ( $\delta_{\text{H}}$  4.34, d,  $J = 5.7$  Hz, for H<sub>2</sub>-27), five methines ( $\delta_{\text{H}}$  1.43, m; 1.69, m; 1.41, m; 1.46, m; and 2.16, m, for H-8, H-9, H-14, H-17, and H-20, respectively), six methylenes ( $\delta_{\text{H}}$  2.83, dd,  $J = 21.2, 5.2$  Hz; 3.28, dd,  $J = 21.2, 1.7$  Hz, for H-4 $\alpha$  and H-4 $\beta$ , respectively; 1.57, m; 1.92, m, for H-7 $\alpha$  and H-7 $\beta$ , respectively; 1.48, m; 2.23, m, for H-11 $\alpha$  and H-11 $\beta$ , respectively; 1.48, m; 2.01, m, for H-12 $\alpha$  and H-12 $\beta$ , respectively; 1.51, m; 1.83, m, for H-15 $\alpha$  and H-15 $\beta$ , respectively; and 2.25, m; 2.51, dd,  $J = 13.8, 13.2$  Hz, for H-23 $\alpha$  and H-23 $\beta$ , respectively), five methyls ( $\delta_{\text{H}}$  0.79, s; 1.23, s; 1.06, d,  $J = 6.3$  Hz; 2.03, s; and 1.96, s, for CH<sub>3</sub>-18, CH<sub>3</sub>-19, CH<sub>3</sub>-21, CH<sub>3</sub>-28, and CH<sub>3</sub>-30, respectively), and one exchangeable proton ( $\delta_{\text{H}}$  2.88, t,  $J = 5.7$  Hz, for 27-OH) (Table 1). The <sup>13</sup>C NMR data revealed 30 carbons, consistent with the HRMS data and indicative of three carbonyls ( $\delta_{\text{C}}$  204.5, 167.0, and 170.3, for C-1, C-26, and C-29, respectively), six olefinic carbons ( $\delta_{\text{C}}$  127.9, 145.4, 136.1, 124.5, 152.7, and 125.9, for C-2, C-3, C-5, C-6, C-24, and C-25, respectively), two oxymethines ( $\delta_{\text{C}}$  79.9 and 78.8, for C-16 and C-22, respectively), one oxymethylene ( $\delta_{\text{C}}$  57.7 for C-27), five methines ( $\delta_{\text{C}}$  32.6, 42.8, 53.8, 57.4, and 37.5, for C-8, C-9, C-14, C-17, and C-20, respectively), six methylenes ( $\delta_{\text{C}}$  33.5, 30.7, 23.2, 39.8, 34.8, and 30.0, for C-4, C-7, C-11, C-12, C-15, and C-23, respectively), and five methyls ( $\delta_{\text{C}}$  13.3, 19.1, 13.6, 20.1, and 21.2, for C-18, C-19, C-21, C-28, and C-30, respectively), and two quaternary carbons ( $\delta_{\text{C}}$  50.5 and 43.6, for C-10 and C-13, respectively) (Table 1). The three double bonds and the three carbonyl groups accounted for six degrees of unsaturation, indicating that five rings must exist in the structure. Correlation spectroscopy (COSY) data identified two spin systems H-2/H-3/H-4 $\alpha$  and H-6/H-7/H-8/(H-9/H-11/H-12)/H-14/H-15/H-16/H-17/H-20/(H-21)/H22/H-23. A heteronuclear multiple-bond correlation (HMBC) was observed from H-16 to C-29, indicating the connectivity of the acetoxy group, while the HMBC correlations observed from CH<sub>2</sub>-27 to C-24 and C-26 indicated the connectivity of the hydroxymethyl to C-25 (Fig. 2). In all known withanolides, the configuration of C-22 was assigned as *R*, which was deduced from the splitting pattern of H-22. Herein, H-22 appeared as a doublet of triplets with coupling constant of 13.2 and 3.4 Hz, which was consistent with the *R*-configuration as reported previously. For example, the H-22 in withalongolide A, a withanolide that was isolated from the aerial parts of *Physalis longifolia*, appeared as a doublet of triplets with coupling constants of 13.3 and 3.4 Hz (Atta-ur-Rahman et al., 1998, Misra et al., 2005, Ray and Gupta, 1994, Zhang et al., 2011). The relative configuration at position 16 was established as *R* via nuclear overhauser effect spectroscopy (NOESY) correlations that were observed between H-16 and the protons at C-8 and C-18 (Fig. 2), indicating that these protons were in close proximity in space and that the orientation of the proton at C-16 should be beta. These data suggested the structure of **1** as 16 $\alpha$ -acetoxy-27-hydroxy-1-oxo(22*R*)witha-2,5,24-trienolide (Fig. 1), which was ascribed the trivial name obtusifonolide (**1**).



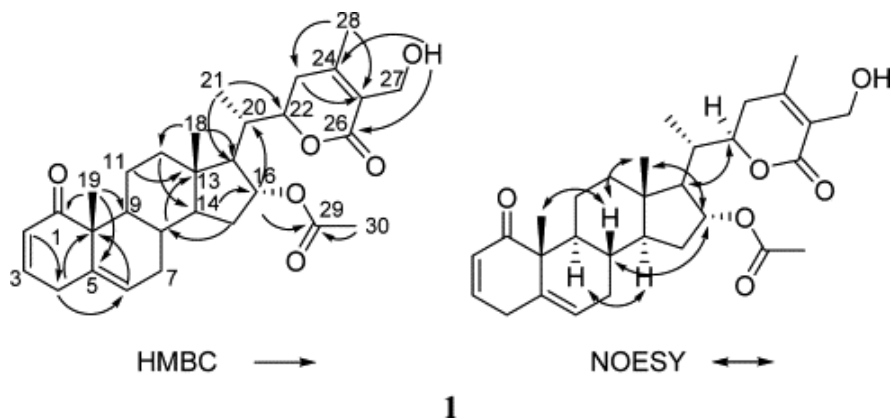
**Fig. 1.** Structure of compound **1**.

**Table 1.**  $^1\text{H}$  (500 MHz) and  $^{13}\text{C}$  (125 MHz) NMR data for obtusifonolide (**1**) in  $\text{CDCl}_3$ .

Position	$\delta_{\text{C}}$	$\delta_{\text{H}}$ , mult ( <i>J</i> in Hz)
1	204.5	–
2	127.9	5.87, dd (10.3, 2.3)
3	145.4	6.78, m
4	33.5	2.83, dd (21.2, 5.2); 3.28, dd (21.2, 1.7)
5	136.1	–
6	124.5	5.55, d (5.7)
7	30.7	1.57, m
		1.92, m
8	32.6	1.43, m
9	42.8	1.69, m
10	50.5	–
11	23.2	1.48, m
		2.23, m
12	39.8	1.48, m
		2.01, m
13	43.6	–
14	53.8	1.41, m
15	34.8	1.51, m
		1.83, m
16	79.9	4.89, t (7.5)
17	57.4	1.46, m
18	13.3	0.79, s
19	19.1	1.23, s
20	37.5	2.16, m
21	13.6	1.06, d (6.3)
22	78.8	4.24, dt (13.2, 3.4)
23	30.0	2.25, m
		2.51, dd (13.8, 13.2)
24	152.7	–
25	125.9	–
26	167.0	–
27	57.7	4.34, d (5.7)
28	20.1	2.03, s
29	170.3	–
30	21.2	1.96, s
27-OH		2.88, t (5.7)

Compounds **1–7** were tested for cytotoxicity against two cancer cell lines, MDA-MB-435 (human melanoma) and SW-620 (human colon cancer). Compound **7** showed cytotoxic activity against the MDA-MB-435 and SW-620 cell lines with  $\text{IC}_{50}$  values of 1.7 and 0.3  $\mu\text{M}$ , respectively. Compounds **1–3** showed cytotoxic activities against the SW-620 cell line with  $\text{IC}_{50}$  values of 7.3, 5.5, and 7.1  $\mu\text{M}$ , respectively, while the other compounds were inactive (Table 2). As predicted from previous structure–activity relationship studies (Zhang et al., 2011), compound **7**, with the enone moiety in ring A, the  $5\beta,6\beta$ -epoxy group in ring B, and the side chain lactone ring was the most potent among the isolates. Withanolide glycoside **2** displayed less cytotoxic activity relative to its aglycone **7**. When compounds **1–7** were tested for antimicrobial

activity in an array of bacteria, yeast, and fungi, none of the compounds, except 7, demonstrated any activity (Table S1, Supplementary data).



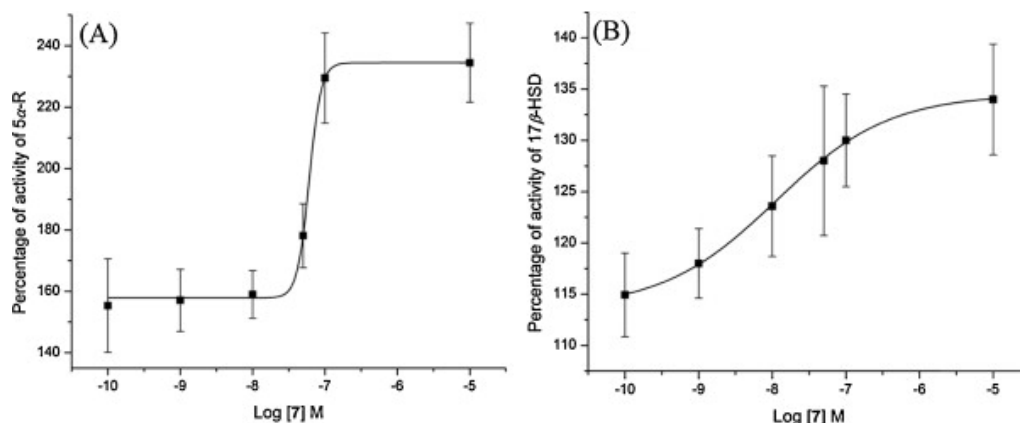
**Fig. 2.** Key HMBC and NOESY correlations for **1**.

**Table 2.** Cytotoxicity of compounds **1–7** against two cancer cell lines.<sup>a</sup>

Compound	MDA-MB-435	SW-620
<b>1</b>	>10	7.3
<b>2</b>	>10	5.5
<b>3</b>	>10	7.1
<b>4</b>	>10	>10
<b>5</b>	>10	>10
<b>6</b>	>10	>10
<b>7</b>	1.7	0.3

<sup>a</sup> Results were expressed as IC<sub>50</sub> values (μM). Positive control was vinblastine tested at 1 nM for MDA-MB-435 and 10 nM for SW620, which had 48% and 30% viable cells after treatment, respectively.

The biological effects of withaferin A (**7**) on the activity of 17β-hydroxysteroid dehydrogenase (17β-HSD) and 5α-reductase (5α-R) were investigated. These enzymes have an important role in the biosynthesis of 5α-dihydrotestosterone (DHT) and pathologies associated with the prostate gland (*i.e.* hyperplasia and prostate cancer) (Bonkhoff et al., 1996, Geissler et al., 1994, Marberger, 2006, Thomas et al., 2005). Type 5 17β-HSD catalyzes the conversion of androstenedione into testosterone (Peltoketo et al., 1999). On the other hand, 5α-R converts testosterone into the more potent androgen, dihydrotestosterone. The hyperplasia of the prostate gland and prostate cancer has been associated with high levels of serum DHT (Bonkhoff et al., 1996, Marberger, 2006, Thomas et al., 2005). Since different plant-derived 5α-cholestane molecules have been identified as selective inhibitors or anabolic agents with minimal or no androgenic side effects (Esposito et al., 2011), it was of interest to study the *in vitro* effect of withaferin A on the enzymes 17β-HSD and 5α-R. Withaferin A (**7**) stimulated the activity of 17β-HSD and 5α-R enzymes with half maximal effective concentration (EC<sub>50</sub>) values of 63 ± 8.7 and 20 ± 6.5 nM, respectively (Fig. 3).



**Fig. 3.** Stimulatory withaferin A (7) EC<sub>50</sub> curves against (A) 5 $\alpha$ -R and (B) 17 $\beta$ -HSD enzymes.

### 3. Experimental

#### 3.1. General experimental procedures

Optical rotations and UV spectra were acquired on a Rudolph Autopol III automatic polarimeter and a Varian Cary 100 Bio UV–Vis spectrophotometer. NMR experiments were conducted in either CDCl<sub>3</sub> or methanol-*d*<sub>4</sub> with TMSi as a reference *via* a Bruker NMR spectrometer operating at 400 MHz for <sup>1</sup>H and 100 MHz for <sup>13</sup>C and a JEOL ECA-500 NMR spectrometer operating at 500 MHz for <sup>1</sup>H and 125 MHz for <sup>13</sup>C. Low-resolution ESIMS data were measured with PE Sciex API 3200 mass spectrometer, while high-resolution ESIMS was performed on a Thermo LTQ Orbitrap XL mass spectrometer. UPLC was carried out on a Waters Acquity system with data collected and analyzed using Empower software. HPLC was carried out using either a Varian Prostar HPLC system equipped with ProStar 210 pumps and a Prostar 335 photodiode array detector (PDA), with data collected and analyzed using Galaxie Chromatography Workstation software (version 1.9.3.2) or on a Lachrom Merck-Hitachi, equipped with a quaternary gradient L-7150 pump, L-7455 diode-array detector, L-7200 autosampler, and D-7000 interface. For preparative HPLC, a Phenomenex Gemini-NX C<sub>18</sub> (4  $\mu$ m; 250 mm  $\times$  21.2 mm) column was used at a 21 ml/min flow rate, or a Hibar Merck prepacked column RT 250-25, Lichrosorb RP-18 (7  $\mu$ m) at a flow rate of 10 ml/min. For the semi-preparative HPLC, a Phenomenex Gemini-NX C<sub>18</sub> (4  $\mu$ m; 250 mm  $\times$  10 mm) column was used at a 4.72 ml/min flow rate. For UPLC analysis, a Waters BEH C<sub>18</sub> column (1.7  $\mu$ m; 50 mm  $\times$  2.1 mm) was used with a 0.6 ml/min flow rate. Column chromatography was performed using silica gel 60 (0.06–0.2 mm; 70–230 mesh) and Sephadex<sup>®</sup> LH-20. For thin layer chromatography, silica gel 60 with gypsum and pigment addition for UV or silica gel 60 with 15% calcium sulfate and fluorescent indicator were used. TLC spots were visualized using a UV lamp at 254 nm (Vilber Lourmat, 4 W-254 nm tube). All other reagents and solvents were obtained from either Fisher Scientific or Sigma–Aldrich and were used without further purification.

#### 3.2. Plant material

Leaves, stems and fruits of *W. obtusifolia* were collected in October 2010 in the central part of Jordan from Madaba, near the Dead Sea, at 31.7°18'53" N and 035.6°58'59" E. The plant material was identified by Mohammad Gharaibah, Plant Taxonomist, Faculty of Agriculture,

Jordan University of Science and Technology. A voucher specimen (PHS-117) was deposited in the herbarium of the Faculty of Pharmacy, Jordan University of Science and Technology, Irbid, Jordan.

### 3.3. Extraction and isolation

Air-dried leaves of *W. obtusifolia* were ground to a powder using a Retsch Mühle mill (RETSCH GmbH, Haan, Germany). About 540 g of powdered leaves were extracted exhaustively with EtOH using a Soxhlet apparatus. The solvent was evaporated under reduced pressure to yield 70.01 g of EtOH extract (F01), which was reconstituted in 1.34 L of CHCl<sub>3</sub> and partitioned 5 times with water (1:1) using a separatory funnel. The CHCl<sub>3</sub> fraction (F03) was evaporated to dryness and the residue (26.15 g) was dissolved in 90% MeOH and partitioned 5 times with n-hexane (1:1) to yield 15.59 g of the defatted MeOH fraction (F05). F05 was subjected to chromatography over silica gel using a gradient of 100% hexane to 100% CH<sub>2</sub>Cl<sub>2</sub> to 25% MeOH in CH<sub>2</sub>Cl<sub>2</sub> to yield 11 pools (P01–P11). P08 and P10 eluted with 25% MeOH in CH<sub>2</sub>Cl<sub>2</sub> was found to be rich in withanolides as evidenced from TLC and hence subjected to further purification using gel filtration on Sephadex LH-20. The columns were eluted with 100% ethanol to afford 7 (P08–C01 to P08–C07) and 5 pools (P10–C01 to P10–C05), from P08 and P10, respectively. P08–C05 (~165 mg) was subjected to preparative HPLC using a gradient system of 35:65 to 45:55 of CH<sub>3</sub>CN–H<sub>2</sub>O (acidified with 0.1% formic acid) over 20 min at a flow rate of 21.24 ml/min to yield eleven sub-fractions. Sub-fractions 1, 5, 6, and 10 yielded compounds **7** (62.49 mg), **3** (6.07 mg), **4** (9.08 mg), and **1** (5.43 mg), which were eluted at 11.2, 14.5, 16.0, and 27.4 min, respectively. Sub-fraction 7 was subjected to semipreparative HPLC using a gradient system of 60:40 to 65:35 of MeOH–H<sub>2</sub>O (acidified with 0.1% formic acid) over 20 min at a flow rate of 4.72 and yielded compound **5** (3.73 mg), which was eluted at 15.8 min. P10–C03 from the dextran column (~125 mg) was subjected to further purification using preparative HPLC using a gradient system of 20:80 to 50:50 of CH<sub>3</sub>CN–H<sub>2</sub>O (acidified with 0.1% formic acid) over 30 min at a flow rate of 21.24 ml/min to yield seven sub-fractions. Sub-fractions 2, 5, and 6 yielded compounds **2** (3.57 mg), **6** (2.89 mg), and **7** (4.88 mg), which were eluted at 14.5, 21.1, and 22.8 min, respectively. The purity of the isolated compounds were evaluated using UPLC with a gradient solvent system that initiated with 20:80 CH<sub>3</sub>CN–H<sub>2</sub>O to 100% CH<sub>3</sub>CN over 4.5 min; all compounds were >95% pure (Fig. S1, Supplementary data).

#### 3.3.1. Obtusifonolide (**1**)

Off-white powder (5.43 mg);  $\alpha$ D<sub>26</sub>=+7.05 (*c* 0.23, CHCl<sub>3</sub>); UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 226 (3.54) nm; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 500 MHz) and <sup>13</sup>C NMR (CDCl<sub>3</sub>, 125 MHz), see Table 1; HR-ESI-MS *m/z* 497.2891 [M+H]<sup>+</sup> (calcd for C<sub>30</sub>H<sub>41</sub>O<sub>6</sub> 497.2898).

### 3.4. Cytotoxicity assay

Human cancer cell lines designated MDA-MB-435 (melanoma) and SW-620 (colon) were purchased from the American Type Culture Collection (Manassas, VA). The lines were propagated at 37 °C in an atmosphere of 5% CO<sub>2</sub> in air. The medium was Roswell Park Memorial Institute (RPMI) 1640 supplemented with fetal bovine serum (10%), penicillin (100 units/ml), and streptomycin (100 µg/ml). Cells in log phase growth were harvested by

trypsinization and washed twice with phosphate-buffered saline (PBS) to remove all traces of enzyme. A total of 5000 cells were seeded per well of a 96-well clear, flat-bottom plate (Microtest 96<sup>®</sup>, Falcon) and incubated overnight (37 °C in 5% CO<sub>2</sub>). Samples dissolved in DMSO were then diluted and added to the appropriate wells (concentrations: 25 µg/ml, 5 µg/ml, 1 µg/ml, 0.2 µg/ml, 0.04 µg/ml; total volume: 100 µL; DMSO: 0.5%). The cells were incubated in the presence of test substance for 72 h at 37 °C and evaluated for viability with a commercial absorbance assay (CellTiter 96<sup>®</sup> AQueous One Solution Cell Proliferation Assay, Promega Corp, Madison, WI) that measured viable cells. Activity was expressed by molarity relative to the negative (solvent) control. The positive control was vinblastine tested at 1 nM in MDA-MB-435 cells, which had 48% viable cells after treatment; 10 nM in SW-620, which had 30% viable cells after treatment.

### 3.5. Microbial strains, culture conditions, and preparation of inocula for susceptibility testing

Strains of *Escherichia coli* strain C (ATCC 13706), *Staphylococcus aureus* (ATCC 6538) and *Mycobacterium smegmatis* (ATCC 607) were obtained from the American Type Culture Collection (ATCC). *Saccharomyces cerevisiae*, *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus niger* and *Micrococcus luteus* strains were obtained from the Virginia Tech Microbiology teaching culture collection. Colonies of *E. coli*, *S. aureus*, *M. luteus*, *S. cerevisiae*, *C. albicans* and *C. neoformans* were grown on 1/10-strength Brain Heart Infusion Broth (BBL Microbiology Systems, Cockeysville, MD, USA) containing 0.2% (w/v) sucrose (BHIB + S) and 1.5% (w/v) agar. *M. smegmatis* was grown on Middlebrook 7H10 agar (BBL Microbiology Systems) and *A. niger* on potato dextrose agar (PDA; BBL Microbiology Systems). Streaked plates were incubated at 37 °C for 3–7 days, except for that of *A. niger*, which was incubated in the dark at 30 °C. A single colony for each microbe except *A. niger* was used to inoculate 5 mL of 1/10-strength BHIB + S (*E. coli*, *M. luteus*, and *S. aureus*), Middlebrook 7H9 broth (*M. smegmatis*) or yeast extract peptone maltose broth (*S. cerevisiae*, *C. albicans* and *C. neoformans*) and incubated at 37 °C (*S. cerevisiae* and *M. luteus* at 30 °C) for 4–7 days. After growth, the resulting broth cultures were diluted with buffered saline gelatin [BSG; gelatin (0.1 g/L), NaCl (8.5 g/L), KH<sub>2</sub>PO<sub>4</sub> (0.3 g/L), Na<sub>2</sub>HPO<sub>4</sub> (0.6 g/L)] to equal the turbidity of a no. 1 McFarland standard. To check for viability and contamination, broth cultures were streaked on plate count agar (BBL Microbiology Systems); the plates were incubated at 37 °C for 3–4 days. Plates for *M. luteus* and *S. cerevisiae* were grown at 30 °C. Spores of *A. niger* were scraped from the surface of the PDA and suspended in 5 mL of 1/10-strength BHIB + S and that suspension transferred to a sterile test tube. The turbidity was adjusted to be equivalent to that of a no. 1 McFarland standard by dilution with BSG. To check for viability and contamination, those spore suspensions were streaked on PDA and incubated at 37 °C for 3–4 days.

### 3.6. Measurement of antimicrobial activity

The minimal inhibitory concentrations (MICs) of the isolated compounds were measured by broth microdilution in 96-well microtitre plates. A 2-fold dilution series of the compounds was prepared in 96-well microtitre plates in a 50 µL volume of 1/10-strength BHIB + S and the dilution series was inoculated with 50 µL of each cell suspension. The resulting inoculated dilution series were incubated at either 30 or 37 °C (same as growth temperature) and growth, as turbidity, scored visually and recorded on the fourth day. MIC of each compound was measured



in triplicate and was defined as the lowest concentration of drug resulting in a complete absence of turbidity compared with the drug-free control.

### 3.7. *In vitro* human prostatic 17 $\beta$ -hydroxysteroid dehydrogenase and 5 $\alpha$ -reductase assays of withaferin A

The *in vitro* 17 $\beta$ -HSD and 5 $\alpha$ -R activity assays were carried out using the membrane fraction obtained from human prostate homogenates, as described previously (Cabeza et al., 2009, Cabeza et al., 2011, Hirosumi et al., 1995).

## 4. Conclusions

Phytochemical investigation of the ethanolic extract of the leaves of *W. obtusifolia* resulted in the isolation of seven withanolides (1–7), namely obtusifonolide (1), sitoindoside IX (2), 6 $\alpha$ -chloro-5 $\beta$ -hydroxy withaferin A (3), isowithanone (4), 2,3-dihydro-3-ethoxywithaferin A (5), daturaturin A (6), and withaferin A (7). The cytotoxic activity of compounds 1–7 was assessed against two cancer cell lines, MDA-MB-435 and SW-620. Compound 7 was the most potent with IC<sub>50</sub> values of 1.7 and 0.3  $\mu$ M, respectively. Moreover, withaferin A (7) stimulated the activity of 17 $\beta$ -hydroxysteroid dehydrogenase and 5 $\alpha$ -reductase *in vitro*.

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## Appendix A. Supplementary data

### Bioactive withanolides from *Withania obtusifolia*

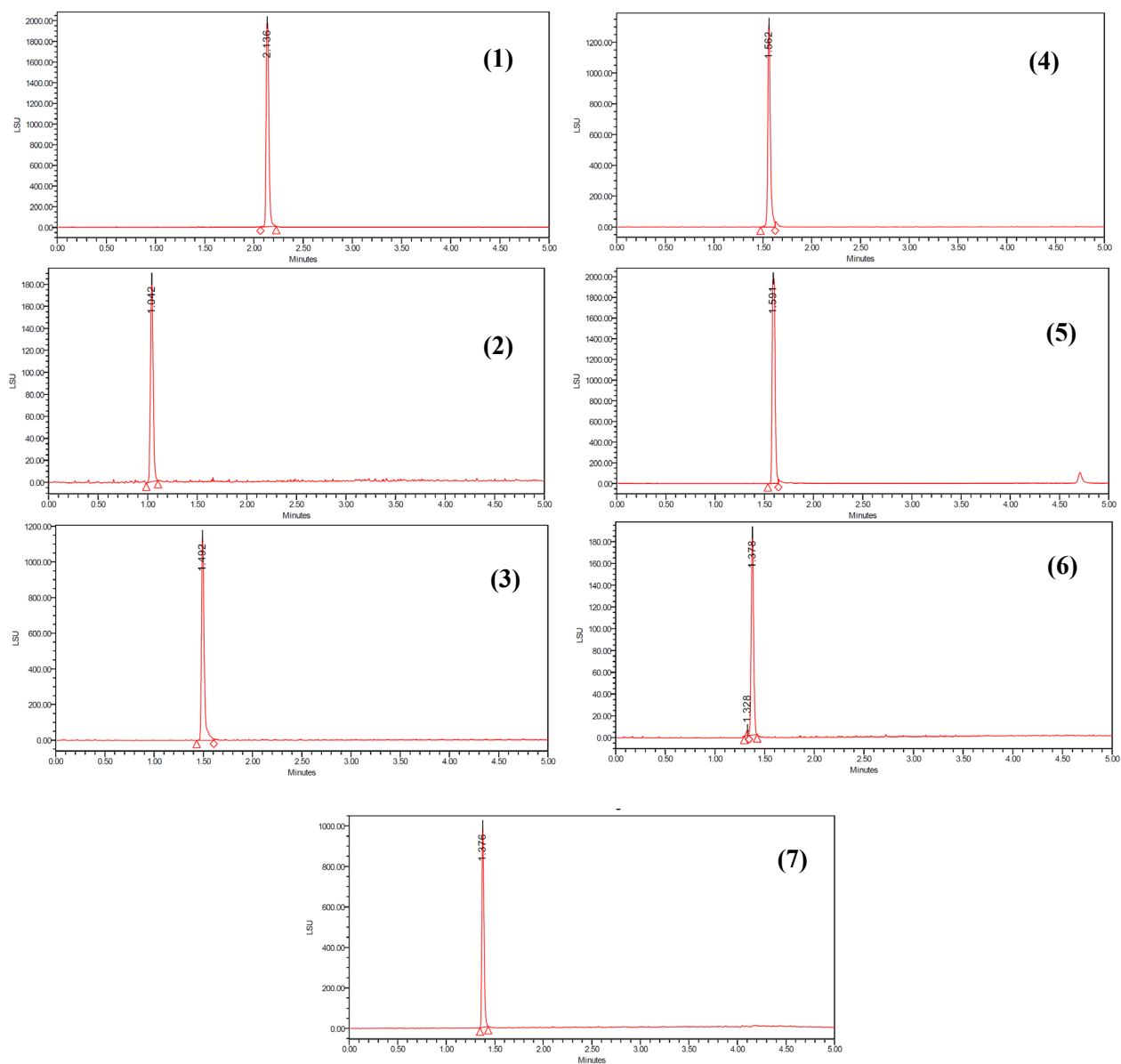
Feras Q. Alali, Chiraz Soumia M. Amrine, Tamam El-Elimat, Ahmad Alkofahi, Khaled Tawaha, Mohammad Gharaibah, Steven M. Swanson, Joseph O. Falkinham III, Marisa Cabeza, Araceli Sánchez, Mario Figueroa, and Nicholas H. Oberlies

**Fig. S1.** UPLC chromatograms of compounds **1-7** (ELSD detection).

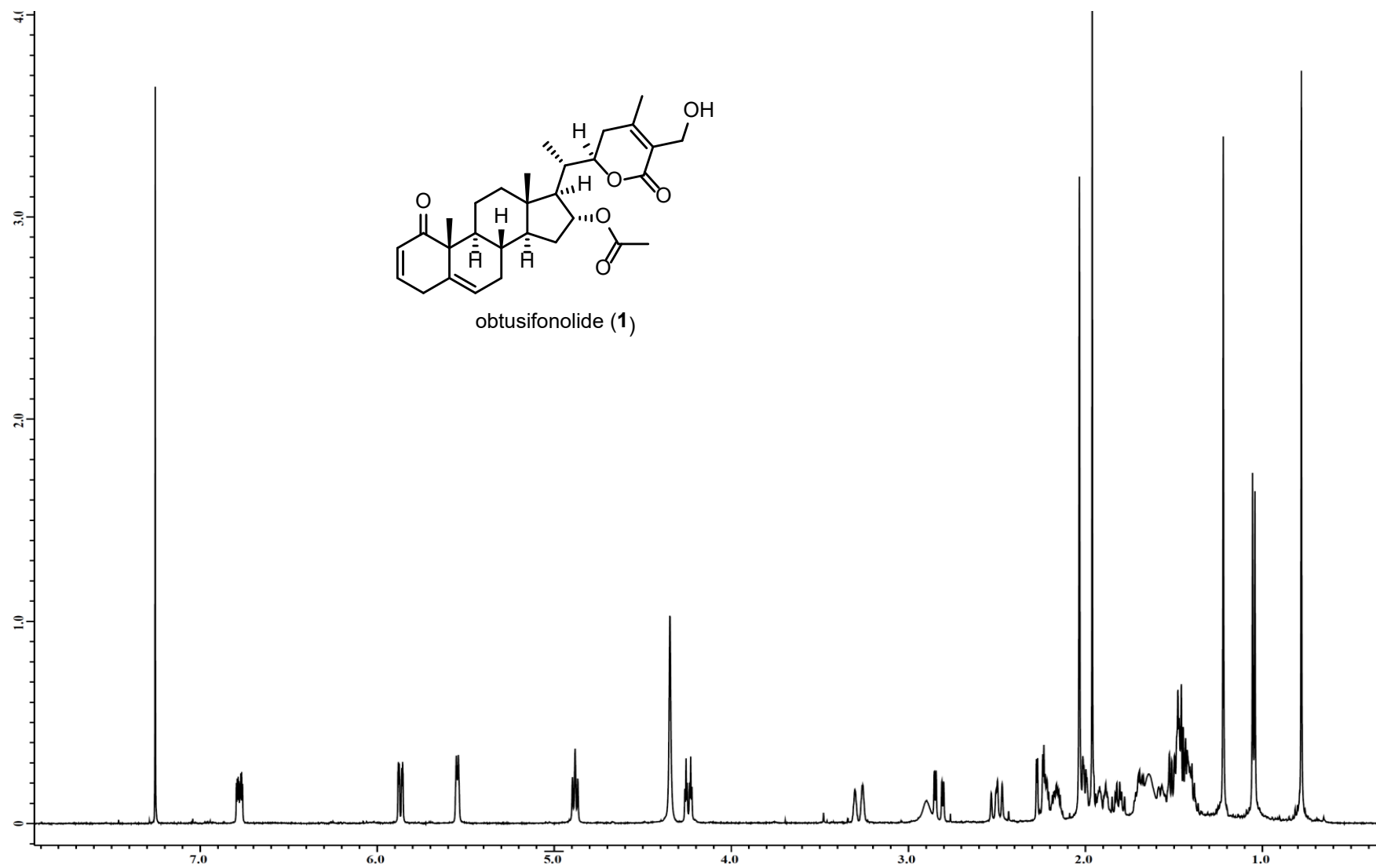
**Fig. S2.**  $^1\text{H}$  NMR spectrum of obtusifonolide (**1**) [500 MHz,  $\text{CDCl}_3$ ].

**Fig. S3.**  $^{13}\text{C}$  NMR spectrum of obtusifonolide (**1**) [125 MHz,  $\text{CDCl}_3$ ].

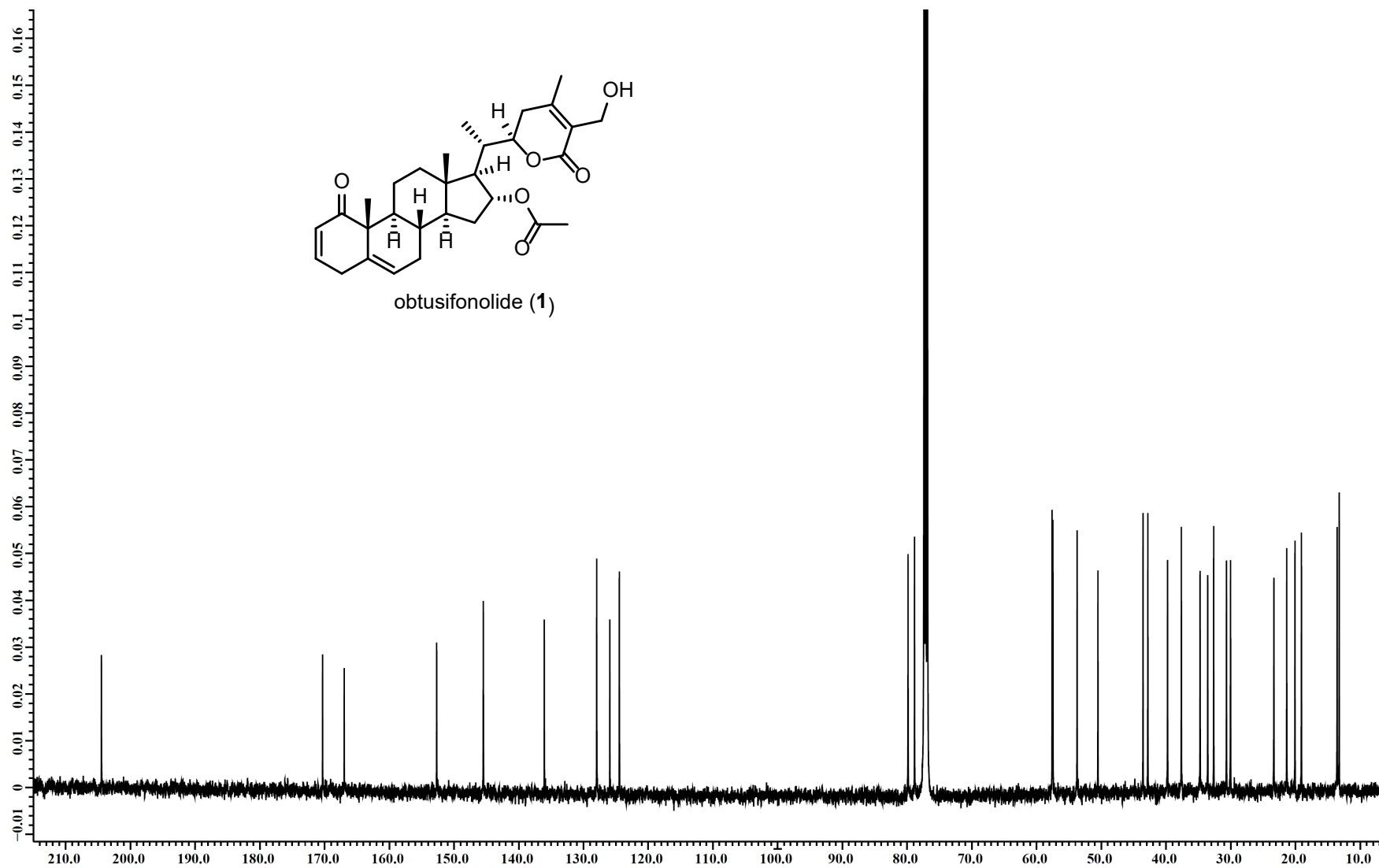
**Table S1.** Antimicrobial activities of compounds (**1-7**).



**Fig. S1.** UPLC chromatograms of compounds **1–7** (ELSD detection).



**Fig. S2.** <sup>1</sup>H NMR spectrum of obtusifonolide (1) [500 MHz, CDCl<sub>3</sub>].



**Fig. S3.** <sup>13</sup>C NMR spectrum of obtusifonolide (1) [125 MHz, CDCl<sub>3</sub>].

**Table S1**

Antimicrobial activities of compounds (1-7)

compound	Minimal inhibitory activity ( $\mu\text{g/ml}$ )							
	<i>M. luteus</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>M. smegmatis</i>	<i>S. cerevisiae</i>	<i>C. albicans</i>	<i>C. neoformans</i>	<i>A. niger</i>
<b>1</b>	> 60	> 60	> 60	> 60	> 60	> 60	> 60	> 60
<b>2</b>	> 95	> 95	> 95	> 95	> 95	> 95	> 95	> 95
<b>3</b>	> 75	> 75	> 75	> 75	> 75	> 75	> 75	> 75
<b>4</b>	> 70	> 70	> 70	> 70	> 70	> 70	> 70	> 70
<b>5</b>	> 55	> 55	> 55	> 55	> 55	> 55	> 55	> 55
<b>6</b>	> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128
<b>7</b>	19	19	> 75	> 75	> 75	> 75	> 75	> 75
Vancomycin	NT	0.25	NT	NT	NT	NT	NT	NT
Ampicillin	NT	NT	8	NT	NT	NT	NT	NT
Ciprofloxacin	NT	NT	NT	2.0	NT	NT	NT	NT
Amphotericin B	NT	NT	NT	NT	NT	25	25	100

NT: not tested