Induction of Quinone Reductase by Withanolides Isolated from *Physalis philadelphica* (Tomatillos)

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The fruits of *Physalis philadelphica*, known commonly as tomatillos, are an ingredient of the condiment “salsa verde”. As part of an ongoing project to discover natural product cancer chemopreventive agents, an ethyl acetate-soluble extract of the commercially available fresh fruits of *P. philadelphica* was found to induce quinone reductase activity in cultured Hepa 1c1c7 murine hepatoma cells. Bioassay-directed fractionation of an EtOAc extract of the fruits, aided by LC/MS, led to the isolation of a series of structurally related withanolides. One novel substance, 2,3-dihydro-3-methoxywithaphysacarpin (**1**), and two known compounds, withaphysacarpin (**2**) and 24,25-dihydrowithanolide D (**3**), were isolated, with the structure of **1** characterized spectroscopically. All three withanolides significantly induced quinone reductase activity in Hepa 1c1c7, TAPclBP^{r}c1, and BP^{r}cl murine hepatoma cells, suggesting that these compounds are monofunctional inducers, specifically elevating phase II enzymes responsible for detoxification, while not influencing phase I enzymes that may activate carcinogens.

Keywords: *Physalis philadelphica; Solanaceae; tomatillo; 2,3-dihydro-3-methoxywithaphysacarpin; withaphysacarpin; 24,25-dihydrowithanolide D; withanolides; quinone reductase induction; cancer chemoprevention*

INTRODUCTION

Epidemiological studies have found that persons who consume a high proportion of green and yellow vegetables in their diet have a decreased risk of developing some types of cancer (Colditz *et al.*, 1985; Graham, 1983). Subsequent laboratory work has led to the isolation of various compounds from fruits and vegetables that reduce the incidence of experimental carcinogenesis in animal models, such as sulforaphane from broccoli and other cruciferous vegetables (Zhang *et al.*, 1992), β-carotene from a variety of vegetables and fruits (Peto *et al.*, 1981), and the monoterpenes D-limonene and D-carvone from various food plants, including *Citrus* species (Wattenberg *et al.*, 1989).

In our current work on cancer chemoprevention, a battery of mechanism-based *in vitro* assays is employed to detect potential cancer chemopreventative agents, and this is comprised of procedures associated with the inhibition of tumor initiation, tumor promotion, and/or tumor progression (Pezzuto, 1995). One such *in vitro* procedure employs Hepa 1c1c7 cells. Induction of quinone reductase (QR) is monitored, and this response is indicative of a generalized elevation of phase II enzyme levels. It is generally agreed that phase II enzymes are primarily responsible for the metabolic detoxification of chemical carcinogens and other harmful oxidants. Therefore, induction of QR is suggestive of cancer prevention at the tumor initiation stage. The Hepa 1c1c7 cell culture model has been used previously to direct the isolation of sulforaphane from broccoli (Zhang *et al.*, 1992); the cancer chemopreventive activity of this agent is considered very promising.

We presently report that extracts derived from the fruits of another dietary plant, *Physalis philadelphica* Lam. (Solanaceae), commonly known as the tomatillo, also induces quinone reductase activity with cultured Hepa 1c1c7 cells. These commercially available fruits are about 5−7 cm in diameter, green, and edible. They are used as an ingredient of foods such as enchiladas and salsas in certain countries in Latin America, and are also employed in North American sauces and relishes, being used as an acid source in place of tomatoes (Bock *et al.*, 1995). A nutritional analysis of tomatillos found that these fruits contain 11% protein, 18% fat, 13% ash, and 5% total dietary fiber on a dry weight basis, and an average of about 31 kcal/100 g (Bock *et al.*, 1995).

Previous phytochemical work on *P. philadelphica* Lam., formerly known as *P. ixocarpa* Brot. (Waterfall, 1967), has resulted in the isolation of several withanolides, including ixocarpalactones A and B (Kirson *et al.*, 1979), ixocarpanolide (Abdullaev *et al.*, 1986), physalin B (Subramanian and Sethi, 1973), and withaphysacarpin (Subramanian and Sethi, 1973). Withanolides have a limited distribution, having been first isolated from *Withania somnifera* (L.) Dunal, and subsequently being found primarily in 12 genera of the Solanaceae (Glotter, 1991; Raffauf *et al.*, 1991; Ray and Gupta, 1994). The alkaloid phygrine was identified as a constituent of the roots and aerial parts of *P. philadelphica* (Basey *et al.*, 1992), while the steroidal alkaloid α-tomatine (Freidman and Levin, 1995) and the vitamin ascorbic acid (Mahna and Singh, 1974) were detected

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in the fruits. Previous research has indicated that different chemotypes (chemical races) in a given *Physalis* species can produce different withanolides (Maslennikova *et al.*, 1977).

Withanolides have been studied previously for their antifeedant, anti-inflammatory, antitumor, cytotoxic, and immunomodulating activity, and for protection against CCl_4-induced hepatoxicity (Glotter, 1991). For example, an insect-antifeedant property of withanolide E isolated from *Physalis peruviana* has been demonstrated against *Spodoptera littoralis* larvae (Ascher *et al.*, 1980). From *P. angulata*, the withanolide physalin F displayed cytotoxicity against five human cancer cell lines, namely, Calu-1 (lung), Colo-205 (colon), HA225 (hepatoma), HeLa (cervix uteri), and KB (nasopharynx) (Chiang *et al.*, 1992). In addition, physalin F also showed *in vivo* antitumor activity in the murine P388 lymphocytic leukemia test system (Chiang *et al.*, 1992). Withangulatin A, also isolated from *P. angulata*, has been found to promote Type II DNA topoisomerase-mediated DNA damage with *in vitro* systems, similar to the action of epipodophyllotoxin (Juang *et al.*, 1989; Lee *et al.*, 1991). Withanolide E and 4β-hydroxywithanolide E have been tested pre-clinically as anticancer agents by the National Cancer Institute, but their activity was not sufficient to warrant subsequent clinical development (Cassady and Suffness, 1980; Glotter, 1991).

On the basis of induction of quinone reductase activity, we currently report the isolation and identification of one novel (**1**) and two known withanolides (**2**, **3**) from the fruits of *P. philadelphica*.

MATERIALS AND METHODS

^{1}H NMR and ^{13}C NMR (including APT) spectra were measured on a Varian XL-300 instrument operating at 300 MHz and 75.6 MHz, respectively. Compounds were analyzed in $CDCl_3$, with tetramethylsilane (TMS) as internal standard. A General Electric Omega 500 NMR spectrometer, operating at 499.9 MHz, was used to perform ^{1}H-^{1}H COSY, ROESY, HMQC, HMBC, and homonuclear NMR decoupling experiments. ^{13}C NMR multiplicity was determined using APT and DEPT experiments. The DEPT experiments were conducted on a Nicolet NMC-360 instrument, operating at 90.8 MHz for ^{13}C. Chemical-ionization mass spectra (CIMS) were measured on a Finnigan MAT-90 mass spectrometer, using methane as reactant gas. Low-resolution fast-atom bombardment mass spectra (FABMS) and high-resolution (HR) FABMS were obtained on a Finnigan MAT-90 instrument, with samples being dispersed in glycerol and bombarded with a beam of Xe atoms with an acceleration of 8 kV. IR spectra were taken on a Midac Collegian FT-IR spectrometer; UV spectra were measured on a Beckman DU-7 spectrometer. Melting points were determined using a Fisher-Johns melting point apparatus, and are uncorrected, and optical rotations were obtained on a Perkin-Elmer model 241 polarimeter.

Thin-layer chromatographic (TLC) analysis was performed on Kieselgel 60 F_{254} (Merck, Darmstadt, Germany) plates (silica gel, 0.25 mm layer thickness), with compounds visualized by spraying with 10% (v/v) H_2SO_4 followed by charring at 110 °C for 10 min. Silica gel (Merck 60 A, 230−400 mesh ASTM), Sephadex LH-20 (25−100 μm; Pharmacia Fine Chemicals, Piscataway, NJ), and Sorbisil C_{18} reversed-phase silica gel (Phase Separations, Ltd., Deeside, Clywd, U.K.) were used for column chromatography. Vacuum-liquid chromatography (VLC) was carried out using Merck 60 A, 70−230 mesh silica gel. All solvents used for chromatographic separations were purchased from Fisher Scientific (Fair Lawn, NJ), and distilled before use.

For liquid chromatography/mass spectrometry (LC/MS), a Hewlett-Packard Electrospray System equipped with a 1090 Series II L HPLC, a photodiode array detector, a 5987A Electrospray source, and a 5989B quadrupole mass spectrometer were used. A description of this system has been published previously (Constant and Beecher, 1995). A Technikrom Kromasil C_{18} column (octadecylsilyl silica gel, 0.32 × 25 cm, 5 mm packing material) was employed for HPLC separation.

Plant Material. The fruits of *P. philadelphica* (20 kg fresh weight) were purchased commercially from a local fruit and vegetable market (Stanley's Fruits and Vegetables, Chicago, IL). The plant material was identified taxonomically by Dr. D. Doel Soejarto, Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago. A voucher sample has been deposited at the Field Museum of Natural History, Chicago, IL, under the acquisition number *Soejarto and Perez, 9777*.

Quinone Reductase (QR) Assay. This assay was modified from a previously described method (Zhang *et al.*, 1992). Cultured Hepa 1c1c7 mouse hepatoma cells were plated at a density of 2×10^4 cells/mL in 96-well plates, and incubated for 24 h. The medium was then changed, and test compounds, dissolved in 10% dimethyl sulfoxide (DMSO), were introduced and serially diluted to a concentration range of 0.15−20 μg/mL. The cells were incubated for an additional 48 h. Quinone reductase activity was measured by the NADPH-dependent menadiol-mediated reduction of 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue formazan. Protein levels were determined in a duplicate set of plates using crystal violet staining, and subsequent measurement at 595 nm (Prochaska and Santamaria, 1988). Enzyme activity was expressed as a "CD" value, the concentration of test material needed to double the specific activity of quinone reductase (in micromolar for pure compounds and μg/mL for extracts). Another endpoint used in the present investigation was "CQ", the concentration of a test compound (micromolar) required to quadruple the specific activity of quinone reductase. For pure compounds, IC_{50} values (half-maximal inhibitory concentration of cell viability) (micromolar) were determined. Chemoprevention Index (CI) values were determined by dividing IC_{50} values by CD values (Gerhäuser *et al.*, 1997).

The isolates from *P. philadelphica* were also analyzed for induction potential with cultured TAOc1BP^{r}c1 cells. Because of a defect in *Ah*-mediated nuclear translocation, activity with this cell line is suggestive of induction of phase II enzymes that is independent of phase I enzyme induction (i.e., monofunctional enzyme induction). The assay procedure was repeated with a third cell line designated BP^{r}c1, which is derived from Hepa 1c1c7 cells, but does not produce a functional aryl hydrocarbon (*Ah*) receptor. Activity with this cell line is again suggestive of monofunctional induction (Gerhäuser *et al.*, 1997).

Extraction and Isolation Procedures. The fresh fruits of *P. philadelphica* were blended and extracted with MeOH, and filtered through cheese cloth and Celite. The resulting MeOH extract was concentrated and partitioned with EtOAc, resulting in two extracts, the EtOAc (19 g) and aqueous methanolic. A portion of the EtOAc extract (17 g) was adsorbed onto silica gel and separated over additional silica gel (100 g) by VLC using a gradient of 1−20% MeOH in $CHCl_3$, and eluates containing materials of similar polarity were combined to provide 10 pooled fractions (fractions A−J). Fractions B−F were active in the quinone reductase assay (CD values of 0.8, 5.3, 8.7, 9.1, and 9.8 μg/mL, respectively). Additional chromatographic separation of bioactive fraction B (*ca.* 3 g) over silica gel with a gradient of 2−10% MeOH/$CHCl_3$ yielded nine subfractions, with subfractions B_2−H_2 being active in the quinone reductase assay (CD values of 1.4, 0.9, 0.7, 1.0, 1.7, 3.2, and 11.4 μg/mL, respectively). Further chromatography of subfraction D_2 (421 mg) over silica gel with 25−40% acetone/hexane yielded two pure withanolides (**1** and **2**; 15 mg and 8 mg, respectively), both being eluted with about 30% acetone in hexane. A third pure withanolide (**3**; 5 mg) was obtained from fraction C using silica gel flash chromatography (20−40% acetone/hexane), Sephadex LH-20 (MeOH), and reversed-phase low-pressure liquid chromatography over C_{18} silica gel (45−55% MeOH in H_2O).

Table 1. NMR Data for Compound 1

position	δ_C[a]	δ_H multiplicity (J, Hz)[b]	HMBC[b]
1	209.8 s		
2	39.4 t	3.00 d,d (15, 6)	209.8 (C-1), 77.3 (C-3), 75.1 (C-4), 50.4 (C-10)
		2.57 d,d (15, 4)	209.8 (C-1), 77.3 (C-3), 75.1 (C-4)
3	77.3 d	3.70 d,d,d (6, 4, 1.5)	209.8 (C-1), 75.1 (C-4), 64.9 (C-5), 56.8 (OMe)
4	75.1 d	3.48 d (1.5)	77.3 (C-3), 50.4 (C-10), 39.4 (C-2)
5	64.9 s		
6	60.5 d	3.21 brs	75.1 (C-4), 28.4 (C-8)
7	31.2 t	2.17 m	64.9 (C-5), 60.5 (C-6), 42.7 (C-9)
		1.31 m	
8	28.4 d	1.52 m	
9	42.7 d	1.30 m	
10	50.4 s		
11	21.4 t	1.42 m	
		(H_b not observed)	
12	39.9 t	2.05 m	
		1.12 m	
13	43.3 s		
14	54.1 d	0.77 m	
15	36.8 t	2.22 m	58.4 (C-17), 54.1 (C-14), 43.3 (C-13)
		1.37 m	
16	73.5 d	4.54 m	58.4 (C-17), 36.8 (C-15)
17	58.4 d	1.19 m	
18	14.4 q	1.10 s	58.4 (C-17), 54.1 (C-14), 43.3 (C-13), 39.9 (C-12)
19	15.9 q	1.41 s	209.8 (C-1), 64.9 (C-5), 50.4 (C-10) 42.7 (C-9)
20	78.3 s		
20-OH		4.00 brs	78.3 (C-20), 58.4 (C-17), 22.4 (C-21)
21	22.4 q	1.30 s	82.3 (C-22), 78.3 (C-20), 58.4 (C-17)
22	81.3 d	4.55 d,d (10.5, 3.5)	78.3 (C-20), 31.0 (C-24), 22.4 (C-21)
23	30.9 t	1.97 m	
		1.53 m	
24	31.0 d	1.82 m	
25	40.7 d	2.23 m	176.0 (C-26), 31.0 (C-24), 21.1 (C-28), 14.1 (C-27)
26	176.0 s		
27	14.1 q	1.23 d (6.5)	176.0 (C-26), 40.7 (C-25), 31.0 (C-24)
28	21.1 q	1.14 d (6.5)	40.7 (C-25), 31.0 (C-24)
OMe	56.8 q	3.32 s	77.3 (C-3)

[a] Spectrum was measured in $CDCl_3$, and values are reported in parts per million relative to TMS. Spectrum was run at 90.8 MHz; multiplicity determined by DEPT ^{13}C NMR experiment. Multiplicity is designated as follows: s, singlet; d, doublet; t, triplet; q, quartet. [b] Spectra were run in $CDCl_3$ at 499.9 MHz. Coupling constants are reported in Hz. Multiplicities are as follows: dd, doublet of doublets; m, multiplet; brs, broad singlet.

LC/MS. Test samples were dissolved in DMSO, to a concentration of about 1 mg/mL. A solvent gradient of acetonitrile and water was designed as follows: 100% water from 0 to 1 min; linear gradient to 50% acetonitrile from 1 to 8 min; linear gradient to 100% acetonitrile from 8 to 20 min; and held at 100% acetonitrile from 20 to 30 min (flow rate 0.75 mL min^{-1}). The injected sample was split unevenly, with 2% (about 15 μL) of the effluent going to the MS detector and the remainder collected into 96-well microtiter plates for biological activity testing. Approximately 200 μL was collected per well. The eluent for mass spectral analysis was post-column treated with 0.2% triethylamine in 10% aqueous MeOH, and the mass spectrometer was adjusted to observe negative ions. After elution, 96-well plates were placed in a laminar flow hood and the solvent permitted to evaporate to dryness. Using the same plates, 10 μL of DMSO was added to each well, and the quinone reductase induction assay was performed with Hepa 1c1c7 cells, as described above. Since each well corresponded to a specific elution time, activity could be correlated with LC and MS data. The actual concentration of extract in each well was not determined, but it was possible to streamline the isolation process by utilizing this approach.

2,3-Dihydro-3-methoxywithaphysacarpin (1). Amorphous gum: $[\alpha]^{22}_D$ +47.4° (c 0.1 $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 222 nm (3.72); IR ($CHCl_3$) ν_{max} 3418, 2917, 2849, 1753, 1683, 1462, 1367, 1188, 1041, 756 cm^{-1}; ^1H and ^{13}C NMR data are presented in Table 1; CIMS (CH_4) m/z (rel int) $[M + H]^+$ 521 (21), 503 (100), 485 (80), 467 (33); HRFABMS m/z found 521.31119, calculated for $C_{29}H_{45}O_8$, 521.31144.

Withaphysacarpin (2). Crystalline colorless powder: mp 270−273 °C [reported mp 275−278 °C (Subramanian and Sethi, 1973)]; $[\alpha]^{22}_D$ + 20° (c 0.05, $CHCl_3$) [reported $[\alpha]_D$ +20° (Subramanian and Sethi, 1973)]; ^1H and ^{13}C NMR data are presented in Table 2; CIMS (CH_4) m/z (rel int) $[M + H]^+$ 489 (35), 471 (100), 453 (69), 435 (38).

24,25-Dihydrowithanolide D (3). Crystalline colorless powder: mp 273−275 °C [reported mp 275 °C (Kirson *et al.*, 1970)]; $[\alpha]_D$ + 11° (c 0.05, $CHCl_3$) [reported $[\alpha]_D$ + 14° (Kirson *et al.*, 1970)]; ^1H NMR data, consistent with published values (Eastwood *et al.*, 1980); CIMS (CH_4) m/z (rel int) $[M + H]^+$ 473 (16), 455 (100), 437 (86), 419 (45).

Small-Scale Extraction of *P. philadephica* Using Alternative Procedures. Commercially available tomatillos (*P. philadelphica*) were extracted by a variety of methods, using in turn room temperature H_2O, boiling H_2O, EtOH, and MeOH. Approximately 75 g of fruits was used for each extraction. These extracts were then partitioned into EtOAc, and dried under reduced pressure.

Two preparations of "salsa verde" were also extracted with MeOH and then partitioned into EtOAc in the manner described above. First, raw salsa verde was prepared from 100 mL chopped onions, three cloves of minced garlic, one teaspoon of honey, two chopped jalapenos, 25 mL chopped cilantro, and 1000 mL tomatillos. These ingredients were combined and refrigerated before use. Second, cooked salsa verde was prepared from 100 mL of chopped onions, 1.5 teaspoons of honey, two chopped jalapenos, 20 mL lemon juice, 10 mL olive oil, 25 mL chopped cilantro, and 500 mL tomatillos. The onions and garlic were sauteed in the olive oil, and the remaining ingredients were then added. The mixture was brought to the boil, and simmered for 25 min, producing a final volume of about 400 mL.

RESULTS AND DISCUSSION

The fresh fruits of *P. philadelphica* were blended to a fine pulp, extracted with MeOH, and filtered. The

Table 2. NMR Data for Compound 2

position	δ_C[a]	δ_H multiplicity (*J*, Hz)[b]	HMBC[b]
1	202.3 s		
2	132.1 d	6.16 d (10.0)	69.8 (C-4), 47.6 (C.10)
3	142.0 d	6.94 dd (10.0, 5.5)	202.3 (C-1), 63.8 (C-5), 69.8 (C-4)
4	69.8 d	3.73 d (5.5)	47.6 (C-10), 62.5 (C-6), 63.8 (C-5), 132.1 (C-2), 142.0 (C-3)
5	63.8 s		
6	62.5 d	3.20 m	69.8 (C-4), 31.2 (C-7), 28.9 (C-8)
7	31.2 t	2.18 m	
		1.24 m	
8	28.9 d	1.53 m	
9	44.1 d	1.01 m	202.3 (C-1), 47.6 (C-10), 28.9 (C-8), 21.9 (C-11), 17.5 (C-19)
10	47.6 s		
11	21.9 t	1.82 m	
		1.40 m	
12	40.1 t	2.07 m	
		1.11 m	
13	43.2 s		
14	54.1 d	0.72 m	44.1 (9), 40.1 (12), 36.8 (15), 28.9 (8), 14.5 (18)
15	36.8 t	2.20 m	
		1.38 m	
16	73.5 d	4.53 m	
17	58.4 d	1.17 m	
18	14.5 q	1.12 s	58.4 (C-17), 54.1 (C-14), 43.2 (C-13)
19	17.5 q	1.41 s	202.3 (C-1), 63.8 (C-5), 47.6 (C-10), 44.1 (C-9)
20	78.2 s		
21	22.4 q	1.30 s	58.4 (C-17), 78.2 (C-20), 81.3 (C-22)
22	81.3 d	4.52 m	176.1 (C-26), 78.2 (C-20), 58.4 (C-17), 30.9 (C-23), 22.4 (C-21)
23	30.9 t	2.00 m	
		1.52 m	
24	31.0 d	1.81 m	
25	40.6 d	2.20 m	
26	176.1 s		
27	14.2 q	1.22 d (6.5)	176.1 (C-26), 40.6 (C-25), 31.0 (C-24)
28	21.1 q	1.14 d (6.5)	40.6 (C-25), 31.0 (C-24)

[a] Spectrum was measured in $CDCl_3$, and values are recorded in parts per million relative to TMS. Spectrum was run at 90.8 MHz; multiplicity determined by DEPT ^{13}C NMR experiment. Multiplicity is designated as follows: s, singlet; d, doublet; t, triplet; q, quartet. [b] Spectra were run in $CDCl_3$ at 500 MHz. Coupling constants are reported in Hz. Multiplicities are as follows: dd, doublet of doublets; m, multiplet; bs, broad singlet.

resulting MeOH/H_2O extract was concentrated under reduced pressure and partitioned with EtOAc. On biological testing, the EtOAc extract was found to be active in the quinone reductase (QR) induction assay, exhibiting a concentration to double the activity of the enzyme (CD) of 2.8 mg/mL. The EtOAc extract (19 g) was subjected to VLC and eluates of similar polarity were pooled based on their TLC profiles to yield 10 major fractions. These fractions were tested in the quinone reductase assay, with fractions B−E being the most active. Analysis of fraction B (*ca.* 3 g), which showed the most potent activity in the QR assay of the 10 initial subfractions (CD 0.8 μg/mL), was carried out by LC/MS. Under the chromatographic conditions described above, and after testing the eluent distributed in 96-well microtiter plates in the QR assay, the biological activity was associated with fractions eluted 11−13 min after sample injection. The compounds being eluted during this time period exhibited only end absorption in their UV spectra, but prominent MS ions in this region of the chromatogram occurred at *m/z* 469 (R_t 11.9 min), 471 (t_R 12.4 min), 487 (t_R 11.0 min), and 519 (t_R 11.3 min). Based on the MS and UV data obtained from this dereplication experiment, it appeared most likely that the QR-active compounds present in the EtOAc extract of *P. philadelphica* belonged to the withanolide group, which are known to occur in this species (Subramanian and Sethi, 1973; Tursunova *et al.*, 1977; Kirson *et al.*, 1979; Ascher *et al.*, 1980; Abdullaev *et al.*, 1986).

Repeated silica gel flash column chromatography of fraction B led to the isolation of two major withanolides, the novel 2,3-dihydro-3-methoxywithaphysacarpin (**1**) and the known compound withaphysacarpin (**2**). An additional withanolide, 24,25-dihydrowithanolide D (**3**), was obtained from further chromatography over silica gel, Sephadex LH-20, and C_{18} reversed-phase silica gel. Withaphysacarpin (**2**), previously isolated from *P. philadelphica*, was identified through physical and spectroscopic comparison with literature values (Subramanian Sethi, 1973). Compound **3**, with a mol wt of 472, $[\alpha]_D$ + 11°, and mp 273−275 °C was in agreement with published values (Kirson *et al.*, 1970). Compound **3** has been isolated previously from two chemotypes (chemical races) of *Withania somnifera* (Kirson *et al.*, 1970; Eastwood *et al.*, 1980). The structures of compounds **1**−**3** are shown in Figure 1.

The CIMS of **1** showed a $[M + H]^+$ ion at *m/z* 521, with the HRFABMS at *m/z* 521.31119 $[M + H]^+$ indicating a molecular formula of $C_{29}H_{44}O_8$. The ^{13}C NMR spectrum of **1** suggested the presence of two carbonyls [cyclic ketone (δ 209.8, s) and a δ lactone (δ 176.0, s)], an epoxide (δ 64.9, s; 60.5, d), and three hydroxylated carbons [two secondary alcohols (δ 75.1, d and 73.5, d) and one tertiary alcohol (δ 78.3, s)], all functionalities in common with compound **2**. However, neither the ^1H nor the ^{13}C NMR spectrum of **1** displayed any olefinic chemical shifts, as present in the NMR spectra of **2**, and the ^{13}C NMR spectrum of **1** displayed an additional resonance at δ 56.8 (q), indicative of a methoxy group. The position of this methoxy functional group in **1** was established through long-range ^1H-^{13}C NMR experiments. Thus, the HMBC NMR spectrum of **1** showed three-bond connectivities between the methoxy protons (δ 3.32) and C-3 (δ 77.3) and between H-3 (δ 3.70) and C-1 (δ 209.8), C-5 (δ 64.9) and OMe

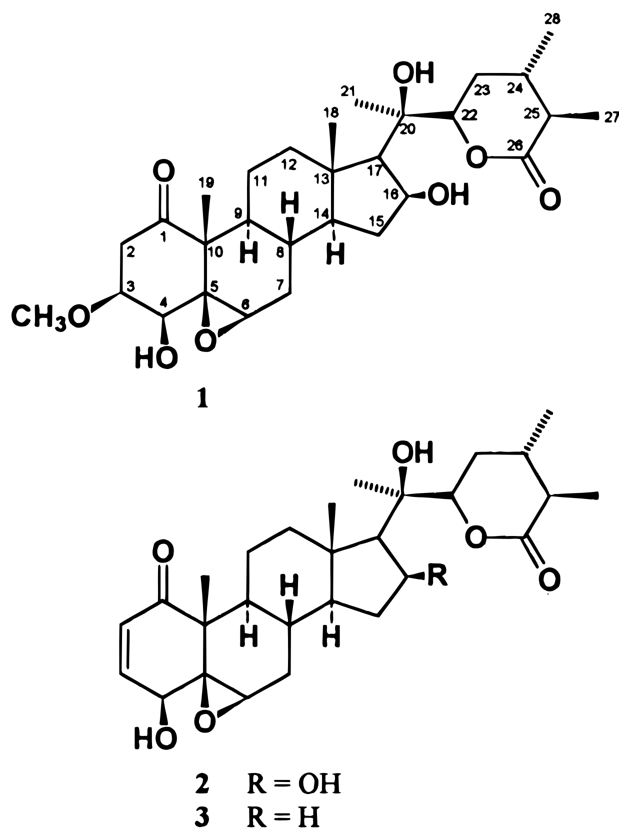

Figure 1. Structures of withanolides **1**−**3** isolated from *Physalis philadelphica*.

(δ 56.8). In this manner it was possible to establish that the methoxy group was affixed to C-3, and that the A-ring of this compound was saturated. The β-orientation of the methoxy group was determined from the ^{1}H NMR coupling constants [$J_{2,3}$ (4 and 6 Hz), and $J_{3,4}$ (1.5 Hz)]. Full ^{1}H and ^{13}C NMR assignments for **1** were carried out using appropriate HMQC and HMBC NMR experiments (Table 1).

Except for the A-ring, the rest of the molecule of **1** was seen to be identical to that of **2**. The positions of the remaining functionalities in **1** were confirmed by the HMBC NMR experiment (see Table 1), inclusive of 5β,6β-epoxy, δ-lactone, 4β-OH, and 16β-OH groups. In 5,6-epoxywithanolides, the β-orientation of the epoxide functional group is favored almost exclusively, except in the presence of a 7α-OH directing group, which **1** does not contain (Bessalle *et al.*, 1987). The 4β-hydroxy-5β,6β-epoxy configuration was further corroborated by examination of the chemical shifts for H-4 and H-6 (δ 3.48 and 3.21, respectively) and by comparison with a model compound (2,3-dihydro-3-methoxywithaferin A) (Fuska *et al.*, 1986). The 4α-hydroxy-5α,6α-epoxy configuration, based on model cholestane compounds, would be expected to have chemical shifts of δ 3.98 and 3.63 for H-4 and H-6, respectively (Maslennikova *et al.*, 1977). The position of the OH-16 group was confirmed by ^{1}H-^{1}H COSY correlations: H-14 (δ 0.77) with both H-8 (δ 1.52) and H-15 (δ 2.22 and 1.37), and H-16 (δ 4.54) with both H-17 (δ 1.19) and H-15 (δ 2.22 and 1.37). The β-configuration of this functionality was confirmed by an NOE observation between H-14 (δ 0.77) and H-16 (δ 4.54). In the structure elucidation of withaphysacarpin (**2**), the stereochemistry of the methyl lactones was not addressed (Subramanian and Sethi, 1973). Through a homonuclear decoupling experiment (^{1}H NMR, 500 MHz), irradiation of Me-27 resulted in H-25 being displayed as a doublet ($J_{24,25}$ = 9.5 Hz) rather than as a multiplet, therefore suggesting the *trans* stereochemistry of the lactone methyls by comparison with model compounds (Eastwood *et al.*, 1980). To date, in all withanolides containing a saturated δ-lactone in the side chain, the asymmetric centers have been of the 24*S* and 25*R* configurations (Glotter, 1991).

Previous researchers have isolated a 2,3-dihydro-3-methoxy withanolide similar to **1**, namely, physalactone, from *P. alkekengi* growing in former Soviet Turkmenia (Maslennikova *et al.*, 1977). Physalactone has also been semi-synthesized by refluxing 4β-hydroxywithanolide E with MeOH (Abdullaev *et al.*, 1984), which indicates that this compound could be an extraction artifact. A similar inference has been made for the origin of 3-methoxy-2,3-dihydrowithanolide D, which is a possible Michael adduct of MeOH with withanolide D (Raffauf *et al.*, 1991), and another example is 2,3-dihydro-3-methoxywithaferin A from *Acnistus arborescens*, which was produced by mixing a solution of withaferin A in 0.05 N methanolic sodium acetate for 17 h at room temperature, and then refluxing for 2 h (Kupchan *et al.*, 1969). Thus, it has been postulated that such 3-methoxy-2,3-dihydro-compounds appear to be artifacts of extraction especially since 3-ethoxy-2,3-dihydro-withanolides have been produced when EtOH is used for extraction (Kupchan *et al.*, 1969). However, there is a possibility that some withanolide methyl esters may occur naturally as well (Maslennikova *et al.*, 1977; Glotter, 1991). To determine whether **1** was an extraction artifact, an aqueous rather than methanolic extraction of *P. philadelphica* was conducted on the fresh fruits. A solvent partition was carried out using EtOAc, and the EtOAc extract was analyzed using LC/MS, under the same conditions as described above. The chromatogram displayed an ion *m/z* 519, having the same retention time as **1**. However, the total ion abundance was low. This suggests that **1** does occur naturally, but was generated to a larger extent as a result of the extraction method employed in the present investigation.

Only limited ^{1}H NMR data, and no ^{13}C NMR data, have been published for **2**. Therefore, the ^{1}H and ^{13}C NMR assignments for **2**, based on HMQC, HMBC, and COSY NMR experiments, are presented in Table 2. As previously mentioned, the stereochemistry of the lactone methyl groups was not addressed in the original structure elucidation (Subramanian and Sethi, 1973). In a homonuclear decoupling experiment (^{1}H NMR, 500 MHz), irradiation of Me-27 resulted in the appearance of H-25 as a doublet ($J_{24,25}$ = 9.1 Hz) rather than as a multiplet, again suggesting the *trans* stereochemistry of the lactone methyls by comparison with model compounds, and consistent with the stereochemistry assigned for **1**.

The potential of compounds **1**−**3** to induce quinone reductase activity is summarized in Table 3. Similar to sulforaphane (Zhang *et al.*, 1992), withanolides **1**−**3** appear to function as monofunctional inducers, since activity of similar magnitude was observed with each of the three cell lines. The unhindered ring-A enone structural feature of compounds **2** and **3** led to greater potency in terms of enzyme induction relative to **1**. This functionality also leads to a more intense cytotoxic response, but nonetheless CI values greater than 1.0 were obtained for **2** and **3**, clearly indicative of selectivity. Of the three isolates tested, the potency of withaphysacarpin (**2**) was comparable with or superior to the

Table 3. Induction of QR Activity by Withanolides (1−3) from *P. philadelphica* and Sulforaphane[a]

compound tested	cell line	CD (μM)	CQ (μM)	IC_{50} (μM)	CI
1	Hepa 1c1c7	7.8 ± 0.31	37.5 ± 1.6	46.9	6.0
	TAOc1BP^{r}cl	5.8 ± 0.03	29.5 ± 0.07	47.5	8.2
	BP^{r}cl	4.2 ± 0.15	21.0 ± 0.57	41.3	9.8
2	Hepa 1c1c7	0.43 ± 0.00	1.9 ± 0.13	4.8	11.1
	TAOc1BP^{r}cl	0.28 ± 0.02	1.2 ± 0.17	4.4	15.7
	BP^{r}cl	0.22 ± 0.01	0.79 ± 0.14	3.5	15.9
3	Hepa 1c1c7	0.70 ± 0.07	3.8 ± 0.14	5.5	7.8
	TAOc1BP^{r}cl	0.65 ± 0.13	3.0 ± 0.05	5.2	8.0
	BP^{r}cl	0.54 ± 0.01	2.0 ± 0.10	3.5	6.5
sulforaphane	Hepa 1c1c7	0.49 ± 0.05	10.6 ± 0.03	11.7	23.9
	TAOc1BP^{r}cl	0.40 ± 0.01	5.0 ± 0.01	10.6	26.5
	BP^{r}cl	0.43 ± 0.06	3.8 ± 0.02	11.4	26.5

[a] QR activity was determined with the designated cell lines as described in Materials and Methods. CD, concentration required to double QR activity; CQ, concentration required to increase QR activity four-fold; IC_{50}, concentration inhibiting cell growth by 50%; CI, Chemoprevention Index (IC_{50}/CD). Ratios are expressed as means (duplicate determinations, ≥5 concentrations) ± SD. Control enzyme levels were as follows (nmol/min/mg protein): 205 ± 7 (Hepa 1c1c7); 156 ± 4 (Bp^{r}c1); 187 ± 5 (TAOc1BP^{r}c1).

Table 4. Induction of QR Activity and Cytotoxic Activity Mediated by Various *P. philadelphica* Extracts and by Salsa Verde Preparations with Cultured Hepa 1c1c7 Cells[a]

sample	QR activity (CD, μg/mL)	IC_{50} (μg/mL)
water extract (room temperature)	0.66	11.9
water extract (boiled)	0.66	13.9
EtOH extract	15.4	>20
MeOH extract	1.9	10.0
Salsa verde (raw)[b]	18.7	>20
Salsa verde (cooked)[b]	9.2	>20

[a] For details of extraction preparation methods used, and definitions of the terms CD and IC_{50}, see Materials and Methods. [b] Methanol extract tested.

potency of sulforaphane in terms of CD or CQ, but an enhancement of cytotoxicity by a factor of approximately 2 led to a less favorable CI. This value is arbitrarily assigned, however, and an equally valid definition would be CI = IC_{50}/CQ. In this context, compounds **1** and **3** were comparable to sulforaphane, and compound **2** would be judged superior.

The data obtained in this investigation may be of physiological relevance, since withanolides are found in the diets of humans. In preliminary studies, small-scale extractions of tomatillos were performed using various extraction techniques that more closely mimic those used in the preparation of the fruits for human consumption than MeOH extraction (Table 4). Extraction with H_2O, either boiling or at room temperature, did not inactivate the QR activity of tomatillos. In fact, relative to cytotoxicity, the activity profile was very favorable. The activity of extracts was not improved by utilizing EtOH or MeOH as the solvent. Salsa verde was also tested in the QR assay (Table 4) and both raw and cooked salsa preparations displayed activity, with CD values of 18.7 and 9.2 mg/mL, respectively. Thus, it appears relatively certain that the biologically active withanolides of tomatillos (*P. philadephica*) are consumed by humans, although when taken in the form of the condiment salsa verde these compounds would be ingested in very small amounts because of the low percent w/w yields of compounds **1**−**3** in these fruits.

It is known that sulforaphane and other compounds capable of inducing phase II enzymes are effective cancer chemopreventive agents (Zhang *et al.*, 1992). The precise mechanism is unknown, although it is well-established that metabolic detoxification of chemical carcinogens or diminution of reactive oxygen species, as can be facilitated by phase II enzymes, can prevent tumorigenesis at the stage of DNA damage (initiation). Since phase II enzyme induction is not tissue specific, in principle, various types of cancers should be prevented by substances capable of functioning by this mechanism. Tissue specific cancer chemopreventive efficacy is not a predictable phenomenon, however, and more advanced tests are currently underway to more fully characterize the activity of withanolides **1**−**3**.

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