

## Minor Withanolides from *Physalis philadelphica*: Structures, Quinone Reductase Induction Activities, and Liquid Chromatography (LC)-MS-MS Investigation as Artifacts

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As a result of a bioactivity-guided search for novel, plant-derived cancer chemopreventive agents, ixocarpalactone A (5) was isolated previously as a potent quinone reductase inducer from the leaves and stems of *Physalis philadelphica*. In the present study, this promising lead compound was reisolated in gram quantities for *in vivo* biological testing. During the course of this work, four additional minor new withanolides were also obtained and characterized, namely, 2,3-dihydro-3 $\beta$ -methoxyixocarpalactone A (1), 2,3-dihydro-3 $\beta$ -methoxyixocarpalactone B (2), 2,3-dihydroixocarpalactone B (3), and 4 $\beta$ ,7 $\beta$ ,20R-trihydroxy-1-oxowitha-2,5-dien-22,26-olide (4). However, compounds 1 and 2 were determined using liquid chromatography (LC)-MS-MS to be artifacts generated during the extraction and isolation procedure. Ixocarpalactone A was detected in the fresh fruits (tomatillos) of *P. philadelphica* by LC-MS-MS analysis at a concentration of 143 $\pm$ 4.53 ppb.

**Key words** *Physalis philadelphica*; withanolide; ixocarpalactone A; quinone reductase induction; artifact; liquid chromatography (LC)-MS-MS

The withanolides are a group of C-28 steroidal lactones which occur primarily in 12 genera of the plant family Solanaceae.<sup>1–3</sup> Representatives of this compound class have been previously evaluated for their antifeedant, anti-inflammatory, antitumor, cytotoxic, and immunomodulatory activities and for protection against CCl<sub>4</sub>-induced hepatotoxicity.<sup>4,5</sup> Recently, over 30 withanolides have been investigated in our laboratory for their potential to induce the phase II enzyme quinone reductase in cultured Hepa 1c1c7 mouse hepatoma cells.<sup>6–8</sup> One of the most promising compounds to emerge from this study is ixocarpalactone A (5), which was isolated and characterized from the aerial parts of *Physalis philadelphica* LAM. (Solanaceae) (formerly known as *P. ixocarpa* BROT.).<sup>8,9</sup> The fruits of this species, known as “tomatillos”,<sup>6</sup> are consumed in enchiladas, salsas and other foods in North America.<sup>10</sup> As part of our ongoing project on the discovery and evaluation of naturally occurring cancer chemopreventive agents,<sup>11,12</sup> it was decided to reisolate ixocarpalactone A (5) in gram quantities for the evaluation of its *in vivo* biological activity in a full-term animal tumorigenesis model. Background information on the botany, ethnobotany, and biological activity of *P. philadelphica* has been provided in our previous contributions on this plant.<sup>6–8</sup>

In the current work, fractionation of a methanolic extract of the leaves and stems of *P. philadelphica* led to the isolation of four new withanolides, 2,3-dihydro-3 $\beta$ -methoxyixocarpalactone A (1), 2,3-dihydro-3 $\beta$ -methoxyixocarpalactone B (2), 2,3-dihydroixocarpalactone B (3), and 4 $\beta$ ,7 $\beta$ ,20R-trihydroxy-1-oxowitha-2,5-dien-22,26-olide (4), along with gram quantities of ixocarpalactone A (5) (Fig. 1). The structure elucidation of the new compounds was carried out through extensive spectral data interpretation, and the isolates were evaluated for potential to induce quinone reductase in Hepa 1c1c7 cells.<sup>7,13</sup>

More than ten methoxylated and ethoxylated withanolides (such as 2,3-dihydro-3 $\beta$ -methoxywithaferin A, 2,3-dihydro-

3 $\beta$ -ethoxywithaferin A, and 4,5-dihydro-5 $\alpha$ -methoxyjaborosolactone B) have been hypothesized to be Michael addition adducts due to treatment with alcohols in the extraction and purification processes of these compounds.<sup>2,14–21</sup> This hypothesis was confirmed experimentally, for example, by heating withaferin A in MeOH or treating this compound with sodium acetate in MeOH, to afford 2,3-dihydro-3 $\beta$ -methoxywithaferin A in either case.<sup>18–20</sup> Thus, it was considered of interest to determine if the methoxylated compounds 1 and 2 are extraction artifacts or naturally occurring substances.

A larger number of withanolides have been isolated from the aerial parts of *P. philadelphica* than from the edible fruits.<sup>3,6,8,9</sup> So far, ixocarpalactone A (5) has not been detected in the fruits of *P. philadelphica*. Thus, the presence of this compound in a tomatillo sample was also investigated in the current study. We report herein the isolation from the aerial parts of *P. philadelphica* and the structural determination

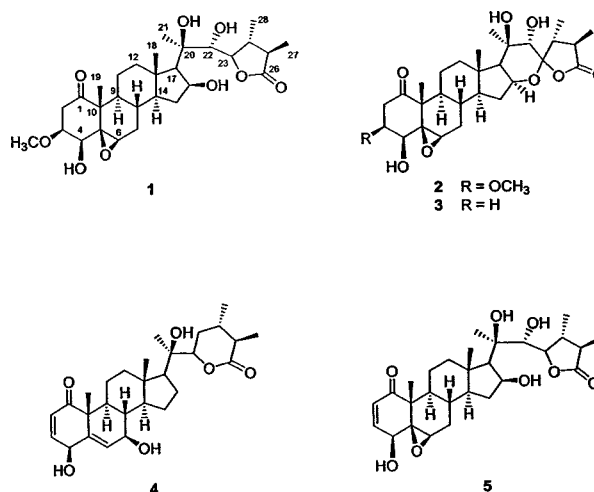


Fig. 1. Structures of Minor Withanolides (1–4) and Ixocarpalactone A (5) Isolated from *Physalis philadelphica*

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of compounds **1**–**4**, their biological evaluation in terms of the induction of quinone reductase in Hepa 1c1c7 cells, an investigation into their possible artifactual nature, and the quantification of ixocaralactone A (**5**) in the fresh fruits of *P. philadelphica*.

## Results and Discussion

Ixocaralactone A (**5**) was the major compound found in the EtOAc-soluble extract of the leaves and stems of *P. philadelphica* in the present investigation, and was obtained in a yield of ca. 0.0088% w/w (88 ppm) of the dried plant material. Compound **5** was crystallized in CHCl<sub>3</sub>–MeOH (20:1) and identified by comparison of its physical and spectroscopic data with those of an authentic sample and reported values.<sup>8)</sup> This compound was originally reported by Kirson *et al.* in 1979,<sup>9)</sup> and its relative and absolute configurations were recently confirmed by X-ray crystallography and Mosher ester methodology, respectively.<sup>8)</sup>

Compound **1** was isolated as a white amorphous powder, mp 240–243 °C, and  $[\alpha]_D^{20} -39^\circ$  ( $c=0.088$ , CH<sub>3</sub>CN), and its molecular formula was determined to be C<sub>29</sub>H<sub>44</sub>O<sub>9</sub> by high-resolution (HR)-MS (obsd  $m/z$  537.3068 [M+H]<sup>+</sup>). The <sup>1</sup>H-NMR spectrum (in CDCl<sub>3</sub>) of compound **1** (Table 1) displayed characteristic signals for five methyl groups [ $\delta_H$  1.11 (3H, s, CH<sub>3</sub>-18), 1.17 (3H, d,  $J=7.1$  Hz, CH<sub>3</sub>-27), 1.25 (3H, d,  $J=6.8$  Hz, CH<sub>3</sub>-28), 1.29 (3H, s, CH<sub>3</sub>-19), 1.30 (3H, s, CH<sub>3</sub>-21)], and one methoxyl group [ $\delta_H$  3.36 (3H, s, OCH<sub>3</sub>-3)]. The <sup>13</sup>C-NMR and distortionless enhancement by polarization transfer (DEPT) spectra of **1** (Table 3) disclosed 29

carbons, which were indicative of a ketone [ $\delta_C$  210.9 (C-1)], a  $\gamma$ -lactone carbonyl [ $\delta_C$  181.8 (C-26)], five oxygenated methines [ $\delta_C$  72.8 (C-16), 73.1 (C-22), 74.5 (C-4), 77.8 (C-3), 79.0 (C-23)], an epoxide functional group [ $\delta_C$  58.9 (C-6), 65.0 (C-5)], one methoxyl [ $\delta_C$  57.0 (OCH<sub>3</sub>-3)], one oxygenated quaternary carbon [ $\delta_C$  79.4 (C-20)], in addition to five methyls, five methylenes, six other methines, and two additional quaternary carbons. Comparison of the <sup>1</sup>H- and <sup>13</sup>C-NMR spectral data of **1** with analogous data for the known withanolide, ixocaralactone A (**5**),<sup>8)</sup> indicated that **1** has the same substituent patterns and relative configuration in rings B–D and in the lactone ring as **5**, with the differences being only in the substituents in ring A. The <sup>13</sup>C-NMR spectrum of **1** exhibited additional signals for a methoxyl group at C-3, a secondary carbon (C-2), and an oxygenated tertiary carbon (C-3) in ring A, instead of the resonances observed for a double bond at C-2 and C-3 in **5**. This was confirmed by observed correlations in the two dimensional (2D) NMR spectra [<sup>1</sup>H–<sup>1</sup>H correlation spectroscopy (COSY), <sup>1</sup>H-detected heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond connectivity (HMBC), and rotating frame Overhauser enhancement spectroscopy (ROESY)]. The methoxyl group in **1** was located at C-3 as a result of the observed HMBC correlations (OCH<sub>3</sub>/C-3; H-2/C-1, -3, -4, -10; H-4/C-2, -3, -5, -10).

In order to confirm the orientation of OH-4, the <sup>1</sup>H, <sup>13</sup>C, DEPT, <sup>1</sup>H–<sup>1</sup>H COSY, HMQC, HMBC and ROESY NMR spectra of **1** were recorded in both C<sub>5</sub>D<sub>5</sub>N and CDCl<sub>3</sub>. Pyridine can interact with hydroxyl groups to form weak hydro-

Table 1. <sup>1</sup>H-NMR Data for Compounds **1** and **2**<sup>a)</sup>

Position	<b>1</b> (C <sub>5</sub> D <sub>5</sub> N)	<b>1</b> (CDCl <sub>3</sub> )	$\delta_{\text{CDCl}_3} - \delta_{\text{C}_5\text{D}_5\text{N}}$	<b>2</b> (C <sub>5</sub> D <sub>5</sub> N)	<b>2</b> (CDCl <sub>3</sub> )	$\delta_{\text{CDCl}_3} - \delta_{\text{C}_5\text{D}_5\text{N}}$
2	3.05 <sup>b)</sup> , 3.21 dd (7.7, 15.5)	2.63 dd (3.6, 15.5), 2.90 dd (7.5, 15.5)		2.98 dd (3.3, 15.5), 3.14 dd (7.5, 15.5)	2.61 dd (3.6, 15.3), 2.95 dd (7.0, 15.3)	
3	3.97 m	3.67 ddd (3.0, 3.6, 7.5)	–0.30	3.95 <sup>b)</sup>	3.69 m	–0.26
4	3.95 d (2.7)	3.45 d (3.0)	–0.50	3.95 <sup>b)</sup>	3.48 d (3.0)	–0.47
6	3.45 brs	3.21 brs	–0.24	3.46 br s	3.22 br s	–0.24
7	1.46 br d (12.9), 2.26 br d (12.9)	1.34 br d (14.9), 2.19 br d (14.9)		1.45 <sup>b)</sup> , 2.25 <sup>b)</sup>	1.35 m, 2.17 br d (14.8)	
8	1.71 m	1.54 m (4.2, 10.9)	–0.17	1.70 <sup>b)</sup>	1.48 <sup>b)</sup>	–0.22
9	1.34 dt (4.4, 11.8)	1.11 <sup>b)</sup>	–0.23	1.41 <sup>b)</sup>	1.16 <sup>b)</sup>	–0.25
11	2.32–2.46 m	1.45 dd (2.5, 13.5), 1.28 <sup>b)</sup>		1.51 <sup>b)</sup> , 1.71 <sup>b)</sup>	1.26 <sup>b)</sup> , 1.41 <sup>b)</sup>	
12	1.03 brt (12.7), 2.15 br d (12.7)	1.11 <sup>b)</sup> , 2.10 td (3.0, 12.5)		1.26 <sup>b)</sup> , 2.65 br d (12.6)	1.10 m, 2.07 br d (12.3)	
14	0.77 m	0.85 m	+0.08	0.83 m	0.90 m	+0.07
15	1.54–1.64 <sup>b)</sup>	1.39 <sup>b)</sup> , 2.31 <sup>b)</sup>		1.41 <sup>b)</sup> , 2.25 <sup>b)</sup>	1.33 <sup>b)</sup> , 2.20 m	
16	4.80 m	4.45 m	–0.35	4.71 m	4.49 m	–0.22
17	1.57 d (7.3)	1.33 <sup>b)</sup>	–0.24	1.68 <sup>b)</sup>	1.49 <sup>b)</sup>	–0.19
18	1.43 s	1.11 s	–0.32	1.26 s	1.02 s	–0.24
19	1.74 s	1.29 s	–0.45	1.78 s	1.29 s	–0.49
21	1.74 s	1.30 s	–0.44	1.95 s	1.47 s	–0.48
22	4.59 s	4.04 s	–0.55	4.61 br s	4.14 br s	–0.47
23	4.93 d (8.3)	4.49 d (8.4)	–0.44			
24	2.32–2.46 <sup>b)</sup>	2.33 m (7.1)		2.21 <sup>b)</sup>	2.01 <sup>b)</sup>	–0.20
25	3.05 <sup>b)</sup>	2.69 m (7.1)	–0.36	3.10 m	2.65 m (7.0)	–0.45
27	1.20 d (7.1)	1.17 d (7.1)	–0.03	1.23 d (7.0)	1.18 d (7.0)	–0.05
28	1.21 d (7.1)	1.25 d (6.8)	+0.04	1.33 d (7.0)	1.20 d (7.0)	–0.13
OCH <sub>3</sub> -3	3.36 s	3.36 s	0	3.36 s	3.35 s	–0.01
OH-4	7.98 s					
OH-16	7.81 d (2.9)					
OH-20	6.21 s					
OH-22	6.00 s					

a) Spectra taken at 360 MHz; chemical shift values presented in ppm;  $J$  values in parentheses given in Hz; TMS used as internal standard. b) Overlapped with other proton signals.

gen bonds and collision complexes, and thereby produces shielding and deshielding cones and influences the chemical shift of the neighboring protons.<sup>8,9</sup> The shift differences observed in different solvents are then used to determine the position and orientation of hydroxyl groups.<sup>8,9</sup> A difference of the <sup>1</sup>H-NMR chemical shift ( $\Delta = \delta_{\text{CDCl}_3} - \delta_{\text{C}_5\text{D}_5\text{N}}$ ) (Table 1) of the most diagnostic signal was found for CH<sub>3</sub>-19 ( $\Delta = -0.45$ ), which suggested that both OH-4 and CH<sub>3</sub>-19 have a  $\beta$ -orientation in **1**. Additionally, the relative stereochemistry at H-3 $\alpha$  and H-4 $\alpha$  was confirmed by the observed coupling constant values in CDCl<sub>3</sub> ( $J_{2\alpha,3\alpha} = 3.6$  Hz,  $J_{2\beta,3\alpha} = 7.5$  Hz,  $J_{3\alpha,4\alpha} = 3.0$  Hz), and the observed ROESY correlations (H-2 $\alpha$ /H-3 $\alpha$ ; H-4 $\alpha$ /H-6 $\alpha$ ). The absolute configurations of the stereogenic centers at C-3 and C-4 in **1** were both assigned as *S* from circular dichroism (CD) spectroscopic evidence. It was previously reported that the 4 $\beta$ -hydroxy-5 $\beta$ ,6 $\beta$ -epoxy-1-one structure of withanolides gives a strong negative Cotton effect at 290 nm based on the  $n \rightarrow \pi^*$  transition of the non-conjugated ketone.<sup>21</sup> The identical CD spectrum of **1** ( $\Delta\epsilon_{290} - 21027$ ) to that of (20*R*,22*R*,24*S*,25*R*)-4 $\beta$ ,20 $\beta$ -dihydroxy-5 $\beta$ ,6 $\beta$ -epoxy-3 $\beta$ -methoxy-1-oxowithanolide ( $\Delta\epsilon_{290} - 15100$ ) indicated that the absolute configuration of the ring A/B part of **1** was the same as that of the latter compound.<sup>21</sup> In this manner, compound **1** was assigned as 2,3-dihydro-3 $\beta$ -methoxyixocarpalactone A.

Compound **2** was obtained as a white amorphous powder, with mp 190–192 °C and  $[\alpha]_{\text{D}}^{20} - 117^\circ$  ( $c = 0.076$ , CH<sub>3</sub>CN). The molecular formula of **2** was determined to be C<sub>29</sub>H<sub>42</sub>O<sub>9</sub> based on HR-MS ( $m/z$  535.2931 [M+H]<sup>+</sup>). Both the <sup>1</sup>H- and <sup>13</sup>C-NMR spectral data of compound **2** (Tables 1, 3) were very similar to those of the withanolide, ixocarpalactone B, which was obtained in our previous work on *P. philadelphica* aerial parts,<sup>8</sup>) but differences were evident between these substances in terms of the ring A substituents. In the <sup>1</sup>H-NMR spectrum of **2**, four methyl singlets and two methyl doublets

were apparent. The <sup>13</sup>C-NMR spectrum in CDCl<sub>3</sub> of **2** exhibited additional signals for an additional methoxyl group ( $\delta_{\text{C}}$  56.9), a secondary carbon [ $\delta_{\text{C}}$  39.9 (C-2)], and an oxygenated tertiary carbon [ $\delta_{\text{C}}$  77.1 (C-3)] in ring A, but minus the resonances for a double bond at C-2 and C-3, when compared to ixocarpalactone B. The methoxyl group in **2** was located at C-3 as a result of the observed HMBC correlations (OCH<sub>3</sub>/C-3; H-2/C-1, -3, -4, -10; H-4/C-2, -3, -5, -6, -10). A pyridine-induced shift ( $\Delta = \delta_{\text{CDCl}_3} - \delta_{\text{C}_5\text{D}_5\text{N}}$ ) experiment for compound **2** (Table 1) gave analogous results to those obtained for **1**, and indicated that OH-4 also has a  $\beta$ -orientation. The relative stereochemistry of H-3 $\alpha$  and H-4 $\alpha$  in **2** was also confirmed by the similar *J* values and ROESY correlations, as observed for the ring A moiety of **1**. The similar CD spectrum of **2** ( $\Delta\epsilon - 17992$ ) at 290 nm to that observed for **1** indicated that the absolute configuration of the AB-ring portion of **2** was the same as that of **1**. Hence, compound **2** was determined to be 2,3-dihydro-3 $\beta$ -methoxyixocarpalactone B.

A molecular formula of C<sub>28</sub>H<sub>40</sub>O<sub>8</sub> was determined for **3** based on HR-MS ( $m/z$  505.2816 [M+H]<sup>+</sup>). Comparison of the <sup>1</sup>H- and <sup>13</sup>C-NMR spectral data of **3** (Tables 2, 3) with those of ixocarpalactone B,<sup>8</sup>) indicated that compound **3** possesses a non-conjugated ketone [ $\delta_{\text{C}}$  212.0 (s, C-1)], instead of an  $\alpha,\beta$ -unsaturated ketone. The relative stereochemistry of OH-4 $\beta$  was confirmed by the pyridine-induced shift ( $\Delta = \delta_{\text{CDCl}_3} - \delta_{\text{C}_5\text{D}_5\text{N}}$ ) for CH<sub>3</sub>-19 ( $\Delta = -0.47$ ) (Table 2), and the observed ROESY correlations between H-4 $\alpha$  and H-6 $\alpha$ . The absolute configuration of C-4 of **3** was established as *S* as a result of the observed negative Cotton effect ( $\Delta\epsilon - 9840$ ) at 295 nm from a CD experiment. Thus, compound **3** was assigned as 2,3-dihydroixocarpalactone B.

Compound **4**,  $[\alpha]_{\text{D}}^{20} - 26^\circ$  ( $c = 0.082$ , CH<sub>3</sub>CN), was obtained as an amorphous solid. The molecular formula was determined to be C<sub>28</sub>H<sub>40</sub>O<sub>6</sub> based on HR-MS (obsd  $m/z$  495.2757

Table 2. <sup>1</sup>H-NMR Data for Compounds **3** and **4**<sup>a)</sup>

Position	<b>3</b> (C <sub>5</sub> D <sub>5</sub> N)	<b>3</b> (CDCl <sub>3</sub> )	$\delta_{\text{CDCl}_3} - \delta_{\text{C}_5\text{D}_5\text{N}}$	<b>4</b> (C <sub>5</sub> D <sub>5</sub> N)	<b>4</b> (CDCl <sub>3</sub> )	$\delta_{\text{CDCl}_3} - \delta_{\text{C}_5\text{D}_5\text{N}}$
2	2.67 <sup>b)</sup> , 2.83, ddd (5.0, 5.5 16.3)	2.51–2.60 m		6.08 d (9.9)	6.00 d (10.0)	-0.08
3	2.18 m	2.02–2.12 m		7.04 dd (4.4, 9.9)	6.79 dd (4.4, 10.0)	-0.25
4	3.80 brt (2.7)	3.50 dd (3.1, 3.4)	-0.30	4.97 d (4.4)	4.69 d (4.4)	-0.28
6	3.24 brs	3.13 brs	-0.11	6.43 d (5.4)	6.12 d (5.5)	-0.31
7	1.45 <sup>b)</sup> , 2.25 <sup>b)</sup>	1.29–1.39 <sup>b)</sup> , 2.19 <sup>b)</sup>		4.20 dd (5.4, 5.4)	3.98 dd (5.5, 5.5)	-0.22
8	1.69 <sup>b)</sup>	1.51 m	-0.18	1.68 <sup>b)</sup>	1.55 <sup>b)</sup>	-0.13
9	1.28 <sup>b)</sup>	1.07–1.12 <sup>b)</sup>		2.52 <sup>b)</sup>	1.88 <sup>b)</sup>	-0.64
11	1.69 <sup>b)</sup> , 1.48 <sup>b)</sup>	1.42 m, 1.23 <sup>b)</sup>		1.75 <sup>b)</sup> , 2.41 <sup>b)</sup>	1.60 <sup>b)</sup> , 2.00 <sup>b)</sup>	
12	1.20 <sup>b)</sup> , 2.65 <sup>b)</sup>	1.07–1.12 <sup>b)</sup> , 2.02–2.12 <sup>b)</sup>		1.52 <sup>b)</sup> , 2.17 <sup>b)</sup>	2.05 <sup>b)</sup> , 2.15 <sup>b)</sup>	
14	0.92 m	0.87 m	-0.05	2.09 <sup>b)</sup>	1.52 <sup>b)</sup>	-0.57
15	1.41 <sup>b)</sup> , 2.27 <sup>b)</sup>	1.29–1.39 <sup>b)</sup> , 2.20 <sup>b)</sup>		2.58 <sup>b)</sup>	1.30 <sup>b)</sup> , 1.80 <sup>b)</sup>	
16	4.71 m (3.0)	4.48 m	-0.23	2.22 <sup>b)</sup>	1.42 <sup>b)</sup> , 2.22 <sup>b)</sup>	
17	1.69 d (5.8)	1.48 d (6.1)	-0.21	1.72 <sup>b)</sup>	1.50 <sup>b)</sup>	-0.22
18	1.25 s	1.01 s	-0.24	1.25 s	0.93 s	-0.32
19	1.76 s	1.29 s	-0.47	1.79 s	1.45 s	-0.34
21	1.94 s	1.47 s	-0.47	1.46 s	1.30 s	-0.16
22	4.61 brs	4.14 brs	-0.47	4.43 br d (10.0)	4.19 dd (2.0, 10.0)	-0.24
23				1.41 <sup>b)</sup> , 1.57 <sup>b)</sup>	1.50 <sup>b)</sup> , 1.75 <sup>b)</sup>	
24	2.23 <sup>b)</sup>	2.02 dq (7.1)	-0.21	1.82 <sup>b)</sup>	1.82 <sup>b)</sup>	0
25	3.09 dq (7.1)	2.64 dq (7.1)	-0.45	2.17 <sup>b)</sup>	2.15 <sup>b)</sup>	-0.02
27	1.23 d (7.1)	1.17 d (7.1)	-0.06	1.22 d (6.6)	1.23 d (6.6)	+0.01
28	1.33 d (7.0)	1.19 d (7.0)	-0.14	1.00 d (6.7)	1.15 d (6.6)	+0.15
OH-22		2.76 brs				

a) Spectra taken at 500 MHz; chemical shift values presented in ppm; *J* values in parentheses given in Hz; TMS used as internal standard. b) Overlapped with other proton signals.

Table 3.  $^{13}\text{C}$ -NMR Spectra Data for Compounds 1–4<sup>a)</sup>

Position	1 <sup>b)</sup>	1 <sup>c)</sup>	2 <sup>b)</sup>	2 <sup>c)</sup>	3 <sup>b)</sup>	3 <sup>c)</sup>	4 <sup>b)</sup>	4 <sup>c)</sup>
1	210.7 s	210.9 s	209.6 s	210.5 s	211.0 s	212.0 s	203.4 s	202.1 s
2	42.3 t	40.2 t	41.2 t	39.9 t	32.0 t	31.6 t	128.5 d	129.3 d
3	80.1 d	77.8 d	78.8 d	77.1 d	27.0 t	26.1 t	146.0 d	142.4 d
4	75.9 d	74.5 d	74.7 d	74.5 d	73.0 d	72.8 d	69.2 d	69.1 d
5	66.4 s	65.0 s	65.2 s	65.1 s	67.4 s	66.8 s	142.0 s	143.0 s
6	59.3 d	58.9 d	58.0 d	59.2 d	57.1 d	58.4 d	132.5 d	131.4 d
7	33.0 t	31.2 t	31.8 t	30.9 t	31.9 t	31.3 t	64.1 d	64.4 d
8	30.5 d	28.8 d	29.5 d	28.7 d	29.6 d	28.8 d	38.3 d	37.2 d
9	44.5 d	43.0 d	43.3 d	42.8 d	43.8 d	43.3 d	36.2 d	35.0 d
10	52.2 s	50.7 s	51.1 s	50.4 s	51.1 s	50.6 s	50.7 s	50.0 s
11	22.6 t	21.0 t	21.2 t	20.6 t	21.2 t	20.8 t	22.4 t	21.7 t
12	41.2 t	39.8 t	40.0 t	39.1 t	40.1 t	39.3 t	40.6 t	39.7 t
13	44.9 s	43.4 s	43.4 s	42.7 s	43.4 s	42.9 s	43.2 s	42.9 s
14	58.0 d	54.5 d	54.5 d	54.2 d	54.6 d	54.5 d	50.8 d	50.0 d
15	39.3 t	37.1 t	34.2 t	33.5 t	34.0 t	33.6 t	23.5 t	23.8 t
16	73.8 d	72.8 d	75.6 d	75.1 d	75.7 d	75.1 d	24.5 t	22.6 t
17	59.3 d	57.4 d	63.5 d	62.3 d	63.5 d	62.4 d	55.5 d	55.4 d
18	16.2 q	14.5 q	15.2 q	14.7 q	15.1 q	14.8 q	14.3 q	13.4 q
19	16.7 q	14.8 q	15.4 q	15.0 q	15.4 q	15.2 q	21.8 q	21.7 q
20	80.9 s	79.4 s	74.3 s	74.7 s	74.3 s	74.8 s	75.2 s	75.5 s
21	24.0 q	21.9 q	27.3 q	26.6 q	27.2 q	26.9 q	21.0 q	20.7 q
22	75.2 d	73.1 d	72.1 d	71.5 d	72.1 d	71.8 d	81.2 d	80.4 d
23	80.6 d	79.0 d	109.0 s	108.0 s	109.0 s	107.7 s	31.8 t	31.2 t
24	43.6 d	42.0 d	50.2 d	49.2 d	50.2 d	49.3 d	31.8 d	31.3 d
25	41.9 d	40.3 d	43.5 d	42.4 d	43.3 d	42.4 d	40.4 d	40.5 d
26	181.8 s	181.7 s	177.8 s	178.1 s	177.8 s	177.8 s	176.2 s	176.0 s
27	15.8 q	14.1 q	14.5 q	13.9 q	14.5 q	13.9 q	14.5 q	14.2 q
28	14.4 q	12.9 q	12.3 q	12.0 q	12.3 q	12.1 q	21.1 q	21.4 q
OCH <sub>3</sub> -3	55.9 q	57.0 q	56.8 q	56.9 q				

a) TMS was used as internal standard; the data of compounds 1 and 2 were obtained at 90 MHz, while the spectra of 3 and 4 were taken at 125 MHz; all data were assigned based on their  $^1\text{H}$ - $^1\text{H}$  COSY, HMQC and HMBC spectra. b)  $\text{C}_2\text{D}_3\text{N}$  was used as solvent. c)  $\text{CDCl}_3$  was used as solvent.

[M+Na]<sup>+</sup>). The  $^1\text{H}$ -NMR spectrum (in  $\text{CDCl}_3$ ) of compound 4 (Table 2) displayed characteristic signals for five methyl groups [ $\delta_{\text{H}}$  0.93 (3H, s, CH<sub>3</sub>-18), 1.15 (3H, d,  $J=6.6$  Hz, CH<sub>3</sub>-28), 1.23 (3H, d,  $J=6.6$  Hz, CH<sub>3</sub>-27), 1.30 (3H, s, CH<sub>3</sub>-21), 1.45 (3H, s, CH<sub>3</sub>-19)], and a pair of  $\alpha,\beta$ -unsaturated olefinic protons [ $\delta_{\text{H}}$  6.00 (1H, d,  $J=10.0$  Hz, H-2), 6.79 (1H, dd,  $J=4.4, 10.0$  Hz, H-3)], and an additional olefinic proton [ $\delta_{\text{H}}$  6.12 (1H, d,  $J=5.5$  Hz, H-6)]. In the  $^1\text{H}$ - $^1\text{H}$  COSY spectrum of 4, H-3 correlated to H-2 and the oxygenated methine proton at  $\delta_{\text{H}}$  4.69 (1H, d,  $J=4.4$  Hz, H-4), and indicated the presence of a 4 $\beta$ -hydroxy-2-en-1-one unit in the molecule. The  $^{13}\text{C}$ -NMR and DEPT spectra (in  $\text{CDCl}_3$ ) of 4 (Table 3) disclosed 28 carbons, which were indicative of an  $\alpha,\beta$ -unsaturated ketone [ $\delta_{\text{C}}$  202.1 (C-1), 129.3 (C-2), 142.4 (C-3)], a further double bond [ $\delta_{\text{C}}$  131.4 (C-6), 143.0 (C-5)], a  $\delta$ -lactone carbonyl [ $\delta_{\text{C}}$  176.0 (C-26)], three oxygenated methines [ $\delta_{\text{C}}$  64.4 (C-7), 69.1 (C-4), 80.4 (C-22)], one oxygenated quaternary carbon [ $\delta_{\text{C}}$  75.5 (C-20)], as well as an additional five methyls, five methylenes, six methines, and two quaternary carbons. These NMR data were closely comparable to those of the recently reported withanolide, philadelphicalactone A,<sup>8)</sup> and suggested that compound 4 is also a withanolide. Comparison of the  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectral data (Tables 2, 3) of 4 with analogous data of philadelphicalactone A indicated that compound 4 exhibited additional signals for a double bond between C-5 and C-6, an oxygenated methine at C-7, and another methine at C-17, instead of the resonances observed for an epoxide group, a methylene, and an oxygenated quaternary carbon in the latter compound, respectively. One double bond was located at C-5 and C-6 as a re-

sult of the observed HMBC correlations (H-3/C-1, -5; H-4/C-3, -6, -10; H-6/C-4, -7, -8, 10). The fact that one hydroxyl group in 4 was translocated from C-17 to C-7 as compared to philadelphicalactone A was evidenced by the observed  $^1\text{H}$ - $^1\text{H}$  COSY correlations (H-7/H-6, -8) and HMBC correlations (H-6/C-4, -7, -8, -10; H-7/C-5) in 4, as well as the decreased pyridine-induced shift effect ( $\Delta=-0.24$ ) of H-22 in 4 as compared to the shift ( $\Delta=-0.63$ ) in the latter compound.<sup>22)</sup> The relative stereochemistry of H-4 $\alpha$  and H-7 $\alpha$  was confirmed by the observed coupling constant values in  $\text{CDCl}_3$  ( $J_{3,4\alpha}=4.4$  Hz,  $J_{6,7\alpha}=5.5$  Hz,  $J_{7\alpha,8\beta}=5.5$  Hz), and the observed ROESY correlations (H-6/H-4 $\alpha$ , -7 $\alpha$ ; H-7 $\alpha$ /H-6, -9 $\alpha$ ). Thus, compound 4 was elucidated as 4 $\beta,7\beta,20R$ -tri-hydroxy-1-oxowitha-2,5-dien-22,26-olide.

Isolates 1–4 obtained from the leaves and stems of *P. philadelphica* were evaluated for their potential to induce quinone reductase (QR) activity in murine Hepa 1c1c7 hepatoma cells.<sup>7,13)</sup> The results (Table 4) showed that compounds 3 and 4 significantly induced QR activity, with observed concentrations to double induction (CD) values of 3.81 and 1.12  $\mu\text{M}$ , while compounds 1 and 2 were not regarded as active (CD > 10  $\mu\text{M}$ ). These results support the previously proposed structure–activity relationships for withanolides, in that the presence of 4 $\beta$ -hydroxy-2-en-1-one and/or 5 $\beta,6\beta$ -epoxy units in the withanolides is associated with quinone reductase-inducing activity.<sup>8)</sup> Ixocarपालactone A (5) has been selected for ongoing *in vivo* biological testing in our program of research on naturally occurring cancer chemopreventive agents due to its relatively high content in the leaves and stems of *P. philadelphica* and its promising *in vitro* activ-

ities (e.g., induction of quinone reductase; inhibition of JB6 cell transformation).<sup>8</sup>) This compound is included in Table 4 for comparison purposes. Sulforaphane was used as a positive control substance.<sup>7</sup>)

In order to determine if compounds **1** and **2** are artificial in nature, two sample solutions were prepared by extracting the dried and milled leaves and stems of *P. philadelphica* with MeOH and CH<sub>3</sub>CN, respectively, and then subjecting them to LC-MS-MS analysis, as described in the Experimental Section. Tandem mass spectrometric analysis of **1** and **2** was carried out to select pairs of precursor/product ions for multiple reaction monitoring (MRM) during LC-MS-MS. The collision-induced dissociation (CID) product ion tandem mass spectra of the [M+H]<sup>+</sup> ions of *m/z* 537 (**1**) and 535 (**2**) are shown in Fig. 2. The presence of abundant fragment ions of **1** and **2** provided many choices for their selective detec-

tion during LC-MS-MS analysis of the complex crude plant extract. Precursor/product ion pairs selected for MRM in LC-MS-MS analysis are listed in Table 5. Optimized collision energy and electrospray cone voltage, parameters which affect additional internal energy input into precursor ions within the ion source, as well as the transmission of ions for each of the MRM channels, are also listed in Table 5. The fragment ions chosen in Table 5 were among the most abundant observed (Fig. 2) and represent structural characteristics of each compound of interest. Standards **1** and **2** were mixed and analyzed by LC-MS-MS using these optimized MRM conditions (Table 5). A typical LC-MS-MS MRM chromatogram of the standards over 30 min is shown in Fig. 3. MRM channels of *m/z* 537→451, 537→519, and 537→501 were used for the detection of **1**, and *m/z* 535→143, 535→517, and 535→211 for **2**. The total ion chromatogram (TIC) of these six MRM channels is provided at the bottom of the figure. Under the HPLC separation conditions selected, compounds **1** and **2** showed baseline separation (Fig. 3), with retention times of 16.8 and 20.7 min, respectively, and the limit of detection (LOD) was *ca.* 200 pg (S/N >3) for each compound in the mass spectrometer. Two sets of methanolic extracts of the dried leaves and stems of *P. philadelphica* (*ca.* 50 mg/ml) were analyzed using MRM LC-MS-MS. During the sample preparation and analysis steps, great care was used to prevent cross contamination or sample carry over. An example is shown in Fig. 4. Only compound **2** was detected in trace amounts in the extract. For comparison, when an CH<sub>3</sub>CN extract of the sample at concentration of 260 mg/ml was subjected to LC-MS-MS analysis, neither **1** nor **2** was

Table 4. Induction of Quinone Reductase (QR) Activity by Withanolides (**1**–**5**)<sup>a)</sup>

Compound	CD±S.D. (μM)	IC <sub>50</sub> ±S.D. (μM)	CI
<b>1</b>	18.63±4.29	>20	n.c. <sup>b)</sup>
<b>2</b>	10.15±4.18	106.6±0.2	10
<b>3</b>	3.81±1.47	96.9±2.4	20
<b>4</b>	1.12±0.46	23.9±5.2	21
Ixocarpalactone A ( <b>5</b> )	0.22±0.08	4.1±2.9	19
Sulforaphane <sup>c)</sup>	0.36±0.17	9.9±2.1	28

a) QR activity was determined with murine Hepa 1c1c7 cells as described in the Experimental Section. CD, concentration required to double QR activity; IC<sub>50</sub>, concentration to inhibit cell viability by 50%; CI, chemopreventive index (IC<sub>50</sub>/CD) (expressed to nearest whole number). b) Not calculated. c) Positive control.

Table 5. Precursor/Product Ion Pairs Selected for Multiple Reaction Monitoring (MRM) of Compounds **1** and **2**

Compound	Precursor ion ( <i>m/z</i> )	Product ion ( <i>m/z</i> )	ESI cone voltage (V)	Collision energy (eV)
<b>1</b>	[M+H] <sup>+</sup> 537	[M+H-H <sub>2</sub> O] <sup>+</sup> 519	30	7
	[M+H] <sup>+</sup> 537	[M+H-2H <sub>2</sub> O] <sup>+</sup> 501	30	7
	[M+H] <sup>+</sup> 537	[M+H-3H <sub>2</sub> O-MeOH] <sup>+</sup> 451	30	7
<b>2</b>	[M+H] <sup>+</sup> 535	[M+H-H <sub>2</sub> O] <sup>+</sup> 517	30	25
	[M+H] <sup>+</sup> 535	211	30	25
	[M+H] <sup>+</sup> 535	143	30	25

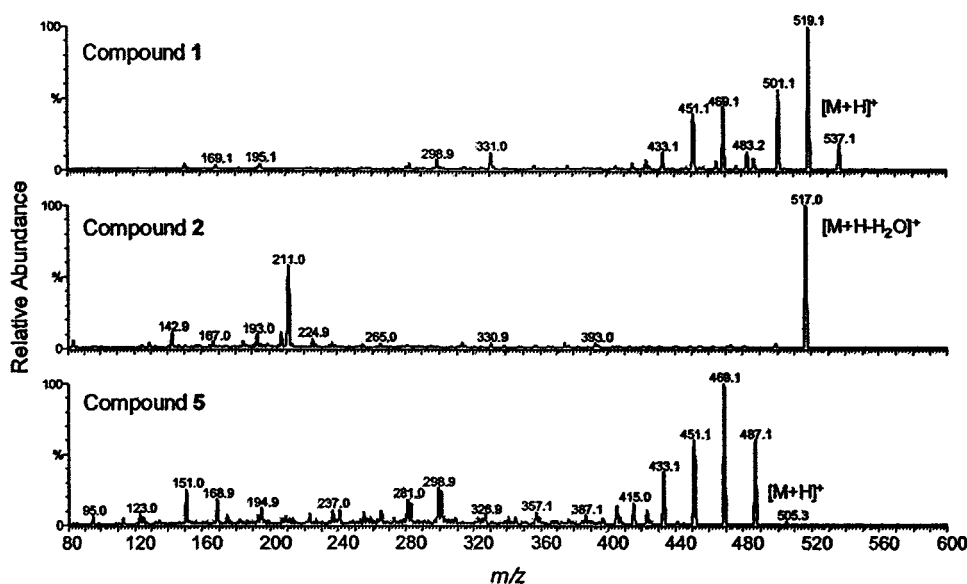


Fig. 2. Positive Ion Electrospray Product Ion Tandem Mass Spectra with CID of Compounds **1**, **2**, and **5**

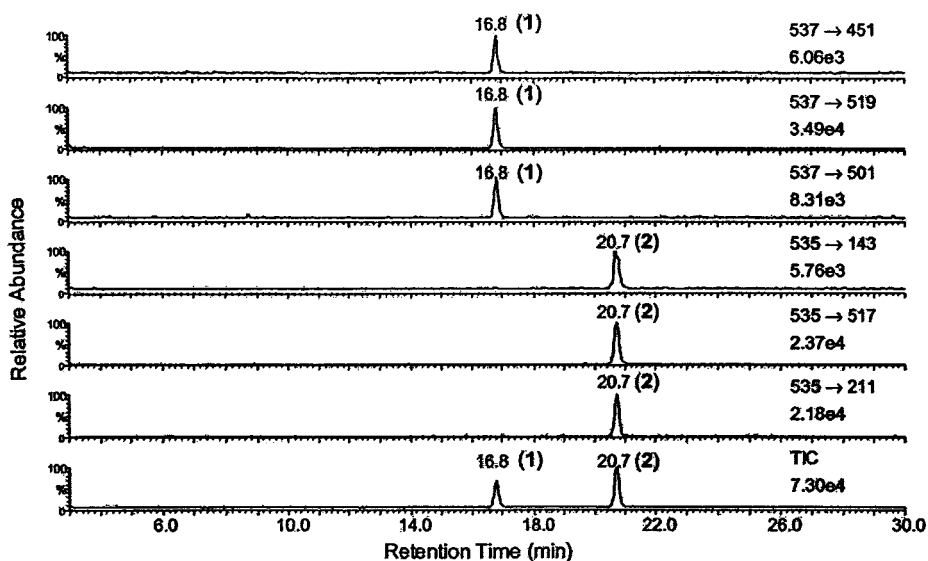


Fig. 3. LC-MS-MS MRM Chromatograms of a Reference Standard Mixture of 1 and 2  
The retention times of 1 and 2 were observed at 16.8 and 20.7 min, respectively.

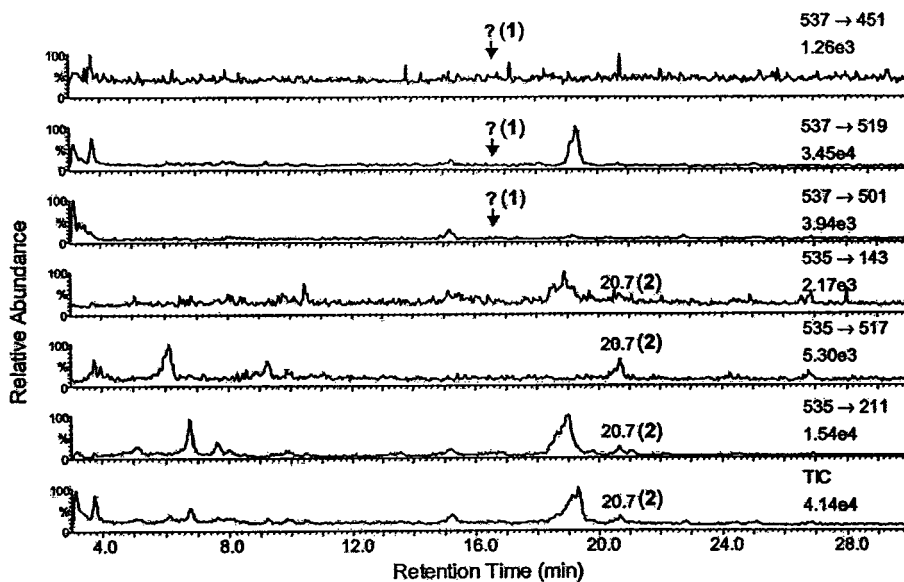


Fig. 4. LC-MS-MS MRM Chromatograms of a Methanol Extract of the Dried Leaves and Stems of *P. philadelphica*  
A question mark indicates that at the retention time, the compound of interest was not detected (LOD ≈ 200 pg on-column).

detected (Fig. 5). Moreover, both 1 and 2 were detected in the same MeOH extract (Fig. 6) but not an CH<sub>3</sub>CN extract (data not shown) after each extract was stored at room temperature for one week. The identification of 1 and 2 in the MeOH extract was based on co-elution with standards, the simultaneous appearance of diagnostic MRM signals during LC-MS-MS and the comparison of ratios of the MRM peak areas for 1 (1:7.62:1.77, corresponding to *m/z* 537→451, 537→519, 537→501, respectively) and for 2 (1:4.59:3.53, corresponding to *m/z* 535→143, 535→517, 535→211, respectively) with those obtained for the purified standards (1:7.68:1.69 for 1; 1:4.55:3.76 for 2). Accordingly, 1 and 2 were determined to be artifacts generated from 5 and ixocarपालactone B, respectively, due to the use of MeOH in the extraction and purification of these withanolides. These observations provide further evidence that withanolides with

a 2-en-1-one unit will form Michael addition adducts in MeOH. Therefore, the use of MeOH as a solvent in the isolation of withanolides should be avoided. In contrast, compounds 3–5 were verified as authentic natural withanolides because they were detected in both MeOH and CH<sub>3</sub>CN extracts of the dried leaves and stems of *P. philadelphica* using the same strategies applied to 1 and 2 (data not shown).

For the qualitative and quantitative analysis of ixocarपालactone A (5) in commercially available fresh tomatillos (the fruits of *P. philadelphica*), an CH<sub>3</sub>CN-soluble extract was prepared and subjected to LC-MS-MS analysis. Prior to LC-MS-MS quantification, standard 5 was introduced into the electrospray ionization source of the mass spectrometer via infusion in H<sub>2</sub>O–CH<sub>3</sub>CN (1:1, v/v, containing 0.1% formic acid). CID experiments on 5 were carried out to optimize instrumental operating conditions of the mass spec-

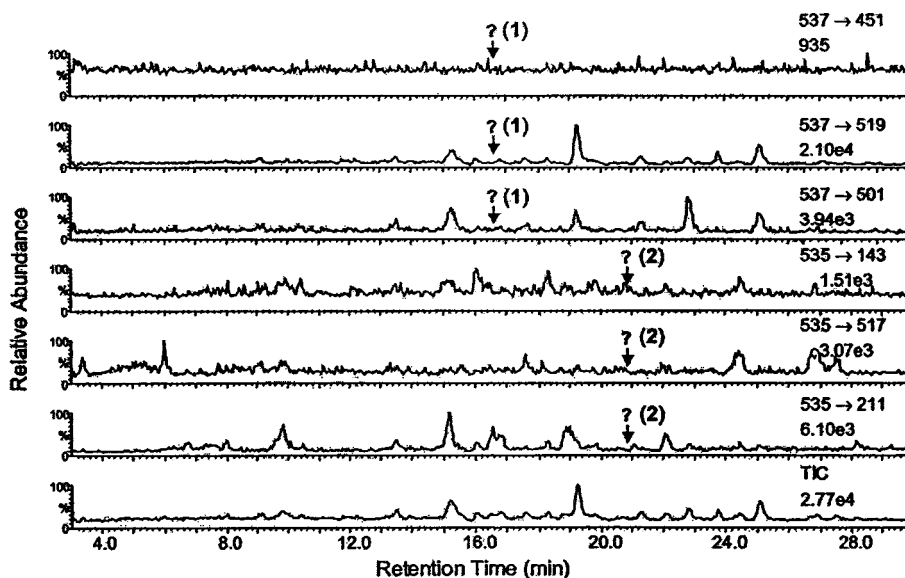


Fig. 5. LC-MS-MS MRM Chromatograms of an Acetonitrile Extract of the Dried Leaves and Stems of *P. philadelphica*

A question mark indicates that at the retention time, the compound of interest was not detected (LOD  $\approx$  200 pg on-column).

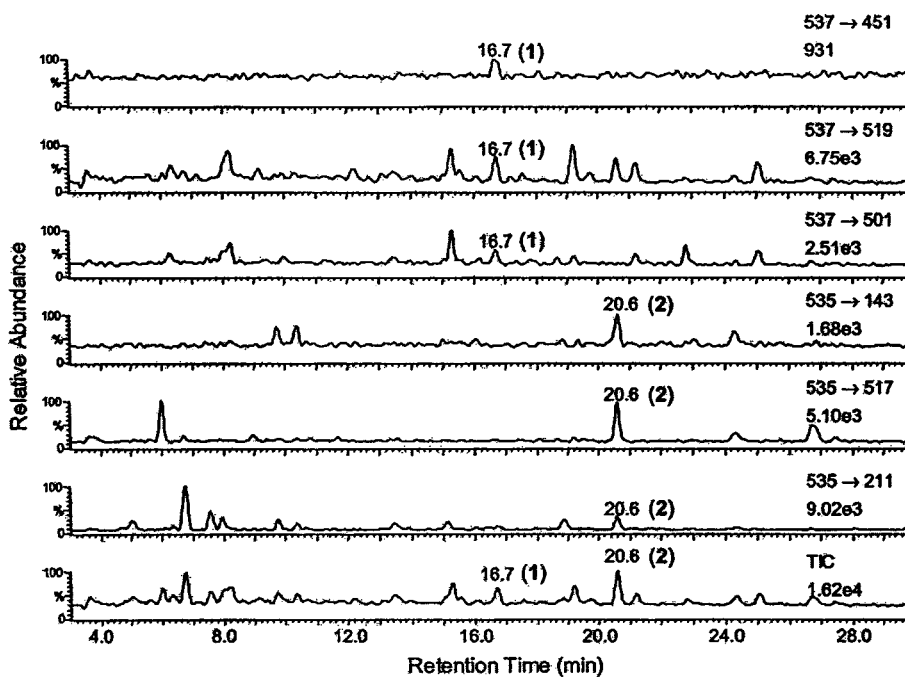


Fig. 6. LC-MS-MS MRM Chromatograms of a Methanol Extract of the Dried Leaves and Stems of *P. philadelphica* After Storage for 7 Days

Compounds 1 and 2 were observed with retention times of 16.7 and 20.6 min, respectively.

trometer and to select precursor/product ion pairs to be used for MRM during LC-MS-MS. The CID product ion mass spectrum of the  $[M+H]^+$  ion at  $m/z$  505 is shown in Fig. 2. The most abundant ion pair ( $m/z$  505 $\rightarrow$ 469) was chosen for LC-MS-MS quantification. Eight non-zero working standard solutions were prepared to produce a LC-MS-MS calibration curve containing **5** in concentrations corresponding to 0.5–90 ng per HPLC injection, with 4-androstene-3,17-dione (MW 286) as the internal standard. As shown in Fig. 7, the standard calibration curve for **5** was linear over the range of 0.5–90 ng with a correlation coefficient ( $R^2$ ) of 0.994. The LOD of the LC-MS-MS analysis for **5** was 200 pg on-column

( $S/N > 3$ , see the insert of Fig. 7) and the limit of quantification was 400 pg on-column (data not shown). A typical LC-MS-MS chromatogram of an  $CH_3CN$  extract of commercially available fresh tomatillos is shown in Fig. 8. The results showed that the sample of the fresh fruits of *P. philadelphica* investigated contained ixocarpalactone A (**5**) at a concentration of  $143 \pm 4.53$  ppb. Should ixocarpalactone A (**5**) ultimately prove to be a promising cancer chemopreventive agent, its detection as a constituent of the edible tomatillo fruit in the present study will be of significance.

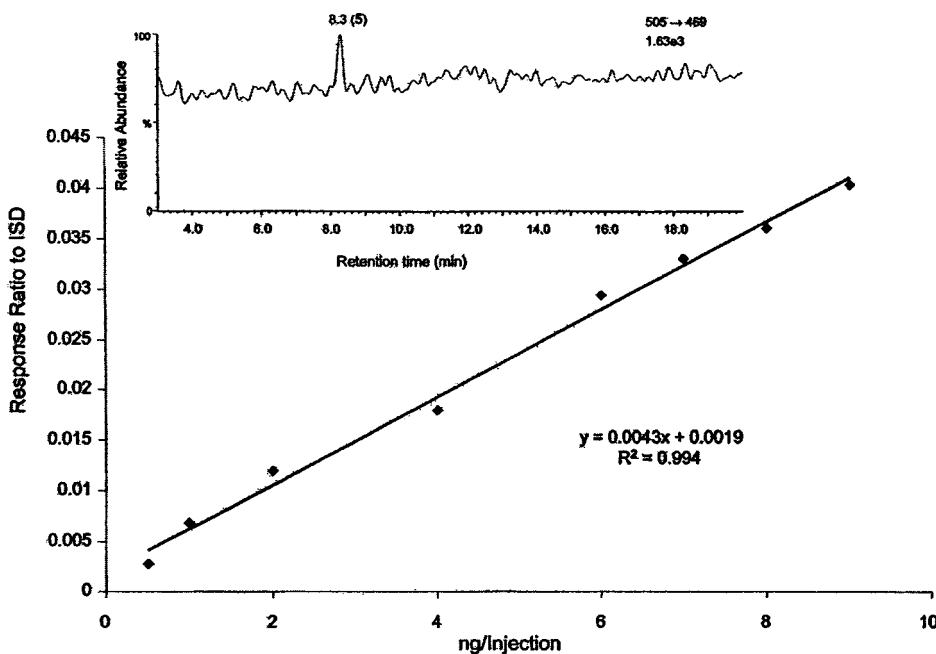


Fig. 7. LC-MS-MS Calibration Curve for 5 Using a Precursor/Product Ion Pair of  $m/z$  505→469 (for 5) and 287→109 (for the Internal Standard, 4-Androstene-3,17-dione)

The calibration curve was constructed using a ratio of 5 to 4-androstene-3,17-dione throughout the concentration range. The insert shows LC-MS-MS chromatograms of the compound at the limit of detection of 200 pg on column.

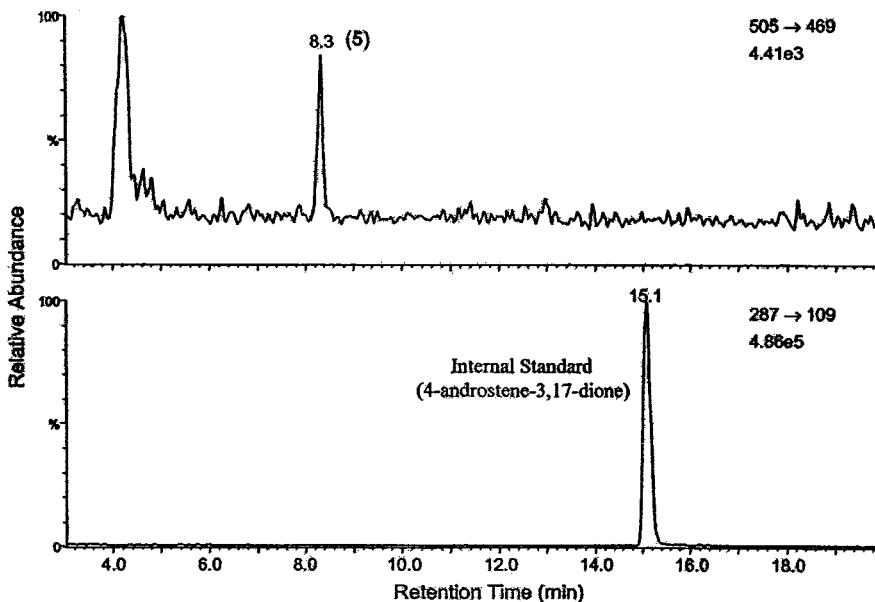


Fig. 8. LC-MS-MS MRM Chromatograms of an Acetonitrile Extract of Commercially Available Fresh Tomatillos (*P. philadelphica* Fruits)

The retention times of 5 and the internal standard, 4-androstene-3,17-dione, were observed at retention times of 8.3 and 15.1 min, respectively.

**Experimental**

**General** Melting points were determined using a Fisher-Johns melting point apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer 241 automatic polarimeter. UV spectra were obtained with a Beckman DU-7 spectrometer. CD measurements were performed using a JASCO-810 CD spectropolarimeter. IR spectra were run on an ATI Mattson Genesis Series FT-IR spectrophotometer. NMR spectral data were recorded at room temperature on Bruker DPX-360 and DRX-500 MHz spectrometers with tetramethylsilane (TMS) as internal standard. High-resolution electrospray exact mass measurements were obtained on a Micromass (Manchester, U.K.) QTOF II mass spectrometer. Semi-preparative HPLC was performed on a Waters (Milford, MA, U.S.A.) HPLC system consisting of two model 515 pumps and a 996 photodiode array detector (monitoring at 202 nm and

217 nm) with a 20×250 mm, 5- $\mu$ m, YMC-Pack ODC-AQ column (YMC, Wilmington, NC, U.S.A.) at 8 ml/min. Column chromatography was carried out with silica gel G (Merck, 230–400 mesh). Analytical thin-layer chromatography (TLC) was performed on precoated 250  $\mu$ m thick silica gel 60 F<sub>254</sub> aluminum plates (Merck, Germany). Compounds were visualized by dipping TLC plates into phosphomolybdic acid reagent (Aldrich, Milwaukee, WI, U.S.A.) following by charring at 110 °C for 5–10 min.

**Plant Material** The leaves and stems (36.4 kg) of *P. philadelphica* were harvested before fruiting in the summer of 2000 from plants grown at the University of Illinois Pharmacognosy Field Station, Downers Grove, IL, U.S.A. Harvesting was performed approximately every two weeks starting at the end of July and ending in the middle of September. A voucher specimen (PC3070) of leaves and stems was deposited at the University of Illinois



Pharmacognosy Field Station.

The fruits of *P. philadelphica* (ca. 200 g fresh weight) were purchased commercially from a local supermarket (Treasure Island Foods, Clyborne Street, Chicago, IL, U.S.A.) in July 2002. The plant material was compared with a taxonomically identified sample deposited in the Field Museum of Natural History, Chicago, IL, under the acquisition number *Soejarto and Perez, 9777*.

**Biological Assays for the Induction of Quinone Reductase (QR) with Cultured Mouse Hepatoma Cells** For the evaluation of plant extracts, fractions, and pure compounds as inducers of QR, cultured murine hepatoma 1c1c7 cells were used as described previously.<sup>7,13</sup> Enzyme specific activity was expressed as CD, the concentration required to double the induction of QR. IC<sub>50</sub> (half-maximal inhibitory concentration of cell viability) and CI (chemoprevention index, IC<sub>50</sub>/CD) values were also determined.

**Extraction and Isolation** The dried and milled leaves and stems (36.4 kg) of *P. philadelphica* were extracted by maceration with MeOH–H<sub>2</sub>O (9 : 1) three times (3×90 l) at room temperature, for three days each. After filtration and evaporation of the solvent under reduced pressure, the combined crude methanolic extract was partitioned in turn with *n*-hexane (3×2 l) and EtOAc (4×2 l), to afford a dried EtOAc-soluble extract (180 g), which was subjected to silica gel column chromatography by elution with increasing concentrations of MeOH in CHCl<sub>3</sub> to give six major fractions (F01–F06). Fraction F03 (13 g, SiO<sub>2</sub>, CHCl<sub>3</sub>–MeOH, 30 : 1) was subjected to silica gel column chromatography by elution with mixtures of *n*-hexane–2-propanol (85 : 15) to give **2** (440 mg). In turn, ixocarpalactone A (**5**, 3.0 g) was crystallized in CHCl<sub>3</sub>–MeOH from fraction F05 (25 g, SiO<sub>2</sub>, CHCl<sub>3</sub>–MeOH, 20 : 1),<sup>8</sup> and the mother liquor extract was further fractionated over a silica gel column by elution with gradient mixtures of *n*-hexane–2-propanol to afford **5** (150 mg, *n*-hexane–2-propanol, 3 : 1), and a subfraction (300 mg; *n*-hexane–2-propanol, 4 : 1), which afforded **1** (90 mg), **3** (15 mg), **4** (4 mg), and an additional amount of **5** (50 mg) by reversed-phase HPLC (YMC-Pack ODC-AQ column, CH<sub>3</sub>CN–H<sub>2</sub>O, 4.8 : 3.2; *t*<sub>R</sub> 19.6, 21.9, 23.2, and 29.5 min, respectively).

**2,3-Dihydro-3β-methoxyixocarpalactone A (1):** White amorphous powder, mp 240–243 °C, [α]<sub>D</sub><sup>20</sup> –39° (*c*=0.088, CH<sub>3</sub>CN). IR (film) cm<sup>-1</sup>: 3352, 1737, 1692, 1455, 1371, 1352, 1185, 1093. UV λ<sub>max</sub> (CH<sub>3</sub>CN) nm (log ε): 197 (3.91), 221 sh (3.21), 273 (2.70). CD (CH<sub>3</sub>CN) nm: Δε<sub>200</sub> –21027. HR-MS *m/z*: 537.3068 [M+H]<sup>+</sup> (Calcd for C<sub>29</sub>H<sub>45</sub>O<sub>9</sub>, 537.3064). <sup>1</sup>H- and <sup>13</sup>C-NMR: see Tables 1 and 3, respectively.

**2,3-Dihydro-3β-methoxyixocarpalactone B (2):** White amorphous powder, mp 190–192 °C, [α]<sub>D</sub><sup>20</sup> –117° (*c*=0.076, CH<sub>3</sub>CN). IR (film) cm<sup>-1</sup>: 3463, 1762, 1708, 1459, 1219, 1093, 1018. UV λ<sub>max</sub> (CH<sub>3</sub>CN) nm (log ε): 198 (3.82), 236 (3.08), 283 (2.76). CD (CH<sub>3</sub>CN) nm: Δε<sub>217</sub> –10335, Δε<sub>290</sub> –17992. HR-MS *m/z*: 535.2931 [M+H]<sup>+</sup> (Calcd for C<sub>29</sub>H<sub>43</sub>O<sub>9</sub>, 535.2907). <sup>1</sup>H- and <sup>13</sup>C-NMR: see Tables 1 and 3, respectively.

**2,3-Dihydroixocarpalactone B (3):** White amorphous powder, mp 178–180 °C, [α]<sub>D</sub><sup>20</sup> –93° (*c*=0.089, CH<sub>3</sub>CN). IR (film) cm<sup>-1</sup>: 3442, 1763, 1697, 1457, 1211. UV λ<sub>max</sub> (CH<sub>3</sub>CN) nm (log ε): 196 (3.90), 221 (3.25). CD (CH<sub>3</sub>CN) nm: Δε<sub>217</sub> –8614, Δε<sub>295</sub> –9840. HR-MS *m/z*: 505.2816 [M+H]<sup>+</sup> (Calcd for C<sub>28</sub>H<sub>41</sub>O<sub>8</sub>, 505.2801). <sup>1</sup>H- and <sup>13</sup>C-NMR: see Tables 2 and 3, respectively.

**4β,7β,20R-Trihydroxy-1-oxowitha-2,5-dien-22,26-olide (4):** White amorphous solid, [α]<sub>D</sub><sup>20</sup> –26° (*c*=0.082, CH<sub>3</sub>CN). IR (film) cm<sup>-1</sup>: 3425, 1729, 1679, 1456, 1378, 1197. UV λ<sub>max</sub> (CH<sub>3</sub>CN) nm (log ε): 201 (4.28), 261 (3.46). HR-MS *m/z*: 495.2757 [M+Na]<sup>+</sup> (Calcd for C<sub>28</sub>H<sub>40</sub>O<sub>6</sub>Na 495.2723). <sup>1</sup>H- and <sup>13</sup>C-NMR: see Tables 2 and 3, respectively.

**Ixocarpalactone A (5):** White amorphous powder, mp 290–292 °C, [α]<sub>D</sub><sup>20</sup> +83° (*c*=0.15, CH<sub>3</sub>CN). (lit. mp 291–292 °C and 294–295 °C, [α]<sub>D</sub><sup>25</sup> +84° and +68.9°, CH<sub>3</sub>CN),<sup>9,23</sup> CD (CH<sub>3</sub>CN) nm: Δε<sub>290</sub> –1294, Δε<sub>340</sub> +6210. HR-MS *m/z*: 505.2864 [M+H]<sup>+</sup> (Calcd for C<sub>28</sub>H<sub>41</sub>O<sub>8</sub>, 505.2801). UV, <sup>1</sup>H- and <sup>13</sup>C-NMR: consistent with published values.<sup>8</sup>

**LC-MS-MS** All organic solvents and chemicals used for this part of study were of HPLC grade (Fisher Scientific, Fair Lawn, NJ, U.S.A.). The withanolide standards were obtained from the repository of natural product isolates in our own laboratory, and their identities and purities were verified using LC-MS-MS, HPLC, and NMR. Deionized water was generated using an in-house Nano-pure water system (Barnstead, Newton, MA, U.S.A.).

The reference standards were dissolved individually in an appropriate volume of CH<sub>3</sub>CN to yield 100 μg/ml stock solutions. An aliquot each of compounds **1** and **2** (1 ml) was mixed and diluted using 50% CH<sub>3</sub>CN to give a standard working solution containing each standard at 10 μg/ml. By dilution of the standard working solution with 50% CH<sub>3</sub>CN, a series of standard working solutions was obtained at levels of 2–100 ng/ml. For quantification of ixocarpalactone A (**5**), the standard working solutions were prepared by

diluting the stock (100 μg/ml) with 50% CH<sub>3</sub>CN to give a concentration of 1 μg/ml. On dilution of the standard working solution with 50% CH<sub>3</sub>CN (v/v), calibrated working solutions at levels of 10–900 ng/ml were prepared by introducing 4-androstene-3,17-dione (MW 286) as the internal standard at a concentration of 1 μg/ml. All stock solutions and working solutions were stored at –20 °C and brought to room temperature before use.

The dried and milled leaves and stems (0.5 g) of *P. philadelphica* were sonicated in 15 ml of HPLC MeOH at 25–30 °C for 60 min. After cooling, the mixture was filtered through Whatman (Clifton, NJ, U.S.A.) No. 1 filter paper into a 250-ml round-bottom flask. The marc was extracted twice more in the same manner, with the final residue being washed with MeOH. The combined extract was evaporated under reduced pressure, and the resulting residue was re-dissolved and transferred using MeOH into a 10-ml volumetric flask and made up to volume with MeOH. Duplicate sample solutions were prepared for analysis. For comparison, the sample solution (260 mg/ml) was prepared in the same way using CH<sub>3</sub>CN instead MeOH. Both the MeOH and CH<sub>3</sub>CN extracts were centrifuged at 5000 rpm and then analyzed using LC-MS-MS.

Commercially available fresh tomatillos (50 g) were blended, extracted with CH<sub>3</sub>CN for 10 min (3×80 ml), and then filtered through Whatman No. 5 filter paper into a 250-ml round-bottom flask. The combined extract was evaporated under reduced pressure, and the resulting residue was re-dissolved and transferred using CH<sub>3</sub>CN into a 100-ml volumetric flask and made up to volume with CH<sub>3</sub>CN. In the same manner as mentioned above, the CH<sub>3</sub>CN extract was centrifuged and then analyzed using LC-MS-MS.

For verification of the origin of **1** and **2**, LC-MS-MS was carried out using a Waters Alliance 2690 HPLC system interfaced to a Micromass Quattro II electrospray triple quadrupole mass spectrometer. A Supelco (Bellefonte, PA, U.S.A.) C<sub>18</sub> column (4.6×250 mm, 5-μm, Serial #504971) was used for HPLC separations. The sample injection volume was 10 μl. The solvent system consisted of a 30 min linear gradient from 20–50% CH<sub>3</sub>CN in water containing 0.5% acetic acid at a flow rate of 1 ml/min. The column effluent was split so that approximately 20% was transferred to the mass spectrometer.

For the quantification of **5**, the sample solution was injected onto a Waters Xterra MS C<sub>18</sub> column (2.1×100 mm, 3.5-μm, Serial #T10731E-14). The solvent system consisted of a 20 min linear gradient from 20–55% CH<sub>3</sub>CN in water containing 0.1% formic acid. In this case, the entire column effluent was transferred into the mass spectrometer without splitting.

During LC-MS-MS, the electrospray ion source of the mass spectrometer was operated at 140 °C in the positive ion mode to generate [M+H]<sup>+</sup> ions of the compounds of interest. Nitrogen was used as both nebulizing and drying gas at a flow rate of 20 l/h and 450 l/h, respectively. Following the selection of the protonated precursor ions by the first quadrupole mass analyzer, collision-induced dissociation (CID) was carried out using argon as the collision gas at 1×10<sup>-3</sup> mbar with a collision energy of 7–25 eV. Both the first and last quadrupole mass analyzers were operated at unit-mass resolution. During multiple reaction monitoring (MRM), the dwell time was set at 0.2 s for each of precursor/product ion pairs.

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