Induction of the Phase II Enzyme, Quinone Reductase, by Withanolides and Norwithanolides from Solanaceous Species

Bao-Ning Su, Jian-Qiao Gu[†], Young-Hwa Kang, Eun-Jung Park[$], John M. Pezzuto[§] and A. Douglas Kinghorn[*]

Program for Collaborative Research in the Pharmaceutical Sciences and Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, Chicago, IL 60612, USA

Abstract: The induction of the phase II drug-metabolizing enzyme, NAD(P)H:quinone reductase (QR), using Hepa 1c1c7 hepatoma cells, is currently used as a key member of a panel of *in vitro* bioassays in our program directed towards the discovery of new plant-derived cancer chemopreventive agents. Among a group of natural products, which have been studied as QR inducers are the withanolides, which constitutes about 400 C_{28} ergostane-type steroids found mainly in approximately 10 genera of the plant family Solanaceae. New withanolides have been isolated and characterized in our recent work on *Physalis philadelphica* (tomatillo), which is used as a vegetable and condiment in Mexican and Central American cuisine. A further study on *Deprea subtriflora* has led to the discovery of a new class of C-18 norwithanolides with only 27 carbons in their skeleton. Preliminary structure-activity relationships have been determined for the *in vitro* induction of QR by members of the withanolide and norwithanolide classes.

INTRODUCTION

Cancer is a complicated group of diseases characterized by the uncontrolled growth and spread of abnormal cells, and it is considered an extremely onerous health problem afflicting the general population worldwide. In recent years, although much progress has been made in cancer research, 5-year survival rates for many types of cancer remain poor. Cancer chemoprevention is a strategy for reducing cancer mortality and involves the prevention, delay, or reversal of cancer by the ingestion of dietary or pharmaceutical agents capable of modulating the process of carcinogenesis [1-4]. Cancer chemoprevention has been applied to some apparently healthy persons at risk of cancer to prevent the subsequent development of invasive disease [5]. Since the establishment of our cancer chemoprevention program project entitled "Natural Inhibitors of Carcinogenesis" in 1991, funded by the United States National Cancer Institute, Bethesda, Maryland, organic-soluble extracts of several thousand plants collected from countries throughout the world have been screened using a panel of short-term *in vitro* bioassays. Plants showing potency in one or more of these assays were then chosen for detailed bioassay-guided fractionation, and the cancer chemopreventive potential of the isolates obtained was then evaluated [6-10].

One key *in vitro* bioassay employed in our study is the induction of quinone reductase (QR) with cultured Hepa 1c1c7 (mouse hepatoma) cells, which is indicative of a generalized elevation of phase II enzyme levels. QR is a convenient representative enzyme because it is widely distributed in mammalian tissues, is easily measured, and shows a large inducer response [11]. A highly suitable and robust cell line for studying the induction of this enzyme is the Hepa 1c1c7 murine hepatoma line [12]. 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 potential cancer prevention at the tumor initiation stage. Recently, QR has been reported to reduce the levels of prooxidative metabolites of dopamine in nerve cells, raising the possibility that QR might play a role in protection against chronic neurodegenerative diseases [13, 14].

In the course of our cancer chemoprevention research, several hundred compounds with different types of *in vitro* bioactivity have been isolated and evaluated [6-10]. Besides a number of new and previously known withanolides isolated from the fruits [15] and the leaves and stems [16, 17] of *Physalis philadelphica* Lam., a number of new withanolides and norwithanolides were recently isolated and characterized from another Solanaceous plant, *Deprea subtriflora* (Ruiz & Pavon) D'Arcy [18, 19].

In 1962, Lavie and Yarden reported the isolation of three crystalline compounds (A_1, A_2, and A_3) from the leaves of *Withania somnifera* Dun (Solanaceae), which was growing in Israel [20]. The partial structure of one of those compounds (the major component, A_2) was then proposed by investigation of its 60 MHz ^{1}H NMR, UV, and IR spectra, the elemental analysis data, and especially by a series of chemical transformations [21, 22]. The authors gave the trivial name, withaferin A, to this compound [22]. Later,

*Address correspondence to this author at the Program for Collaborative Research in the Pharmaceutical Sciences and Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, Chicago, IL 60612, USA; Tel.: +1-312-996-0914; Fax: +1-312-996-7107; E-mail: kinghorn@uic.edu

[†]Present address: Department of Pharmacology and Toxicology, College of Pharmacy, University of Arizona, Tucson, AZ 85721, USA

[$]Present address: DTP-Tumor Hypoxia Laboratory, SAIC-Frederick, P.O. Box B, Bldg. 432, Rm.224, NCI-Frederick, Frederick, MD 21702, USA

[§]Present address: Heine Pharmacy Building, Purdue University, West Lafayette, IN 47907, USA

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the full structure for withaferin A was elucidated based on additional chemical transformation evidence [23]. At about the same time, withaferin A was isolated from the leaves of another plant in the Solanaceae, *Acnistus arborescens*, and found to be a significant tumor inhibitor against sarcoma 180 in mice [24]. The structure of withaferin A was confirmed unambiguously by the single-crystal X-ray analysis of its 4-monoacetate-27-*p*-bromobenzoate derivative [24]. Then, several more steroids structurally similar to withaferin A were isolated and identified from *Withania somnifera* [25, 26]. Hence, the class name "withanolide" was proposed for this type of steroid, with most of these compounds found to possess an α,β-unsaturated δ-lactone ring in the side chain of the molecule [25, 26]. This class of steroid derivative is largely restricted in distribution to the genera *Acnistus*, *Datura*, *Discopodium*, *Dunalia*, *Jaborosa*, *Lycium*, *Nicandra*, *Physalis*, *Solanum*, and *Withania*, all belonging to the plant family Solanaceae [27-30]. To date, a few other withanolides have been isolated from *Ajuga parviflora* and *Tacca chantrieri*, which belong to the families Labiatae and Taccaceae, respectively [31-37]. Progress in the investigation of the chemistry [27-30] and bioactivity [38, 39] of the withanolides and related compounds has been reviewed. To date, over 400 withanolide derivatives have been reported, including some novel withanolides with new carbon skeletons derived biogenetically from the basic skeletons. However, all previously reported withanolides have skeletons made up of 28 carbons, with the exception of the C_{27} norwithanolides we have reported recently from *D. subtriflora* [18, 19]. In the following paragraphs, we will first describe the structure elucidation of a few key compounds isolated recently in our laboratory, and then discuss structural aspects among the withanolides and norwithanolides for inducing activity in the *in vitro* quinone reductase assay we have used.

MINOR WITHANOLIDES ISOLATED FROM THE LEAVES AND STEMS OF *PHYSALIS PHILADELPHICA*

The former name for *Physalis philadelphica* Lam. is *Physalis ixocarpa* Brot., and this Solanaceous plant is cultivated in Mexico and Guatemala and originates from Mesoamerica [40]. The fruits of *P. philadelphica* are commonly known as the tomatillo, husk-tomato, jamberry, or ground cherry, and their use in the diet of the Mexican people dates back to pre-Columbian times. The tomatillo is incorporated into sauces prepared with ground chilies to improve the flavor of meals and stimulate the appetite, and as an acid source in place of tomatoes [41]. The whole plants of *P. philadelphica* have been used for the treatment of gastrointestinal disorders in Guatemala [42] and for treating leprosy, purifying the blood, and as a poison antidote in Mexico [43]. Previous phytochemical studies on the leaves and epigeal parts of this plant resulted in the isolation of the withanolides, 4-acetylixocarpalactone B, ixocarpalactone A (**1**), ixocarpanolide, physalin B, and withaphysacarpin [44-46]. In our initial work, the fruits of *P. philadelphica* were investigated, and three withanolides, 2,3-dihydro-3-methoxywithaphysacarpin, 24,25-dihydrowithanolide D, and withaphysacarpin, were found to show significant induction of quinone reductase in Hepa 1c1c7 cells [15]. Then, we investigated the leaves and stems of *P. philadelphica*, leading to the isolation of three active known withanolides, ixocarpalactone A (**1**), withaphysacarpin, and 18-hydroxywithanolide D, as well as two new 17-hydroxywithanolides, philadelphicalactones A (**2**) and B, one new spiro-acetal withanolide, ixocarpalactone B (**5**), which was previously isolated as its 4-monoacetate from the same plant [45], one new ceramide, (2*S*,3*S*,4*R*,9*E*)-1,3,4-trihydroxy-2-[(2'*R*)-2'-hydroxytetracosanoylamino]-9-octadecene, two known ceramides, (2*S*,3*S*,4*R*)-2-[(2'*R*)-2'-hydroxytetracosanoylamino]-1,3,4-octadecanetriol and (2*S*,3*S*,4*R*)-2-tetracosanoylamino-1,3,4-octadecanetriol, and the known porphyrin derivative, chlorophyllide *a* [16]. The QR-inducing activity of all these isolates was determined, and the withanolide, ixocarpalactone A (**1**), was demonstrated to be a promising QR inducer (Table 1) [10, 16]. Therefore, a large-scale reisolation of this lead compound in gram quantities for the evaluation of its *in vivo* biological activity in a full-term animal tumorigenesis study has been undertaken [17]. In addition to ixocarpalactone A, four new minor withanolides, 2,3-dihydro-3β-methoxyixocarpalactone A (**3**), 4β,7β,20*R*-trihydroxy-1-oxowitha-2,5-dien-22,26-olide (**4**), 2,3-dihydroixocarpalactone B (**6**), and 2,3-dihydro-3β-methoxyixocarpalactone B (**7**) were also obtained from the leaves and stems of *P. philadelphica* by reversed-phase HPLC purification [17].

The identification of ixocarpalactone A (**1**) and the structure elucidation of philadelphicalactone A (**2**) and ixocarpalactone B (**5**) were carried out based on their obtained 1D- and 2D-NMR spectral and high-resolution MS data. The absolute stereochemistry of C-4 was determined as *S* for both **1** and **2** by the Mosher ester method, and their structures and stereochemistry were confirmed by single-crystal X-ray diffraction [16].

Both the ^{1}H- and ^{13}C-NMR spectral data of compound **3** (2,3-dihydro-3β-methoxyixocarpalactone A) were closely comparable to those of ixocarpalactone A (**1**) [16]. The evident differences between these two compounds were the chemical shifts of the protons and carbons of the A-ring. When compared to ixocarpalactone A, the ketone signal of **3** showed a significant downfield shift (8.2 ppm in pyridine-d_5 and 8.0 ppm in $CDCl_3$), which suggested the ketone group in **3** was not conjugated to a double bond. This was consistent with the absence of the olefinic resonance signals in the ^{1}H- and ^{13}C-NMR spectra of **3**. However, an aliphatic methoxyl group was apparent in the 1D-NMR spectra (δ_H 3.36, 3H, s; δ_C 55.9, q) of **3**. This, in combination with the observed 2D-NMR (^{1}H-^{1}H COSY, HMQC, and HMBC) data, indicated **3** is a methanol adduct of ixocarpalactone A (**1**). The orientation of the OMe-3 group in the molecule of **3** was assigned as β by analysis the coupling constants from H-3α to H_2-2 and from H-3α to H-4α, which was further confirmed by the observed ROESY correlations [17].

Compound **4** (4β,7β,20*R*-trihydroxy-1-oxowitha-2,5-dien-22,26-olide) was isolated as a very minor component (4 mg from 36.4 kg plant material) [17], and a molecular formula of $C_{28}H_{40}O_6$ was provided by HRTOFMS. The ^{13}C-NMR spectrum of **4** displayed four olefinic signals at δ_C 146.0, 142.0, 132.5 and 128.5, and the locations of these two double bonds were determined to be at C-2/C-3 and C-5/C-6 based on the observed ^{1}H-^{1}H COSY correlations from

Table 1. Quinone Reductase (QR)-Inducing Activity of Compounds 1–25

compound code	compound name	CD (μM)[a]	IC_{50} (μM)[b]	CI[c]
1	ixocarpalactone A	0.32	7.5	24.0
2	philadelphicalactone A	0.04	0.47	11.8
3	2,3-dihydro-3β-methoxyixocarpalactone A	18.6	>20	>1.1
4	4β,7β,20*R*-trihydroxy-1-oxowitha-2,5-dien-22,26-olide	1.12	23.9	21.3
5	ixocarpalactone B	0.21	1.42	6.8
6	2,3-dihydroixocarpalactone B	3.81	96.9	25.4
7	2,3-dihydro-3β-methoxyixocarpalactone B	10.2	106.6	10.5
8	subtrifloralactone A	0.55	35.5	64.5
9	subtrifloralactone B	>11.0	>11.0	ND
10	subtrifloralactone C	0.72	4.9	6.8
11	subtrifloralactone D	0.18	1.3	7.2
12	subtrifloralactone E	>11.0	>11.0	ND
13	13β-hydroxymethylsubtrifloralactone E	>10.3	>10.3	ND
14	subtrifloralactone F	0.42	>42.4	101.0
15	subtrifloralactone G	>21.2	>42.4	ND
16	subtrifloralactone H	>9.8	>9.8	ND
17	subtrifloralactone I	3.5	>9.8	>2.8
18	subtrifloralactone J	0.62	3.6	5.8
19	subtrifloralactone K	0.36	4.8	13.3
20	subtrifloralactone L	>10.6	>10.6	ND
21	jaborosalactone 1	0.28	8.08	28.9
22	jaborosalactone O	1.52	>42.3	>27.8
23	jaborosalactone P	0.75	>42.7	>57.0
24	trechonolide A	0.27	7.74	28.7
25	withaphysalin J	0.39	11.0	28.1
positive control	sulforaphane	0.49	11.7	23.9
positive control	4-bromoflavone	0.013	>166	>17000

[a]Concentration required to double QR activity. [b]Concentration required to inhibit cell growth by 50%. [c]Chemopreventive index (CI) = IC_{50}/CD. [d]Sulforaphane[75] and 4-bromoflavone[94] were used as positive control substances.

H-3 to both H-2 and H-4, and HMBC correlations from H-4 to C-2, C-3, C-5, and C-6. Besides C-4, C-20 and C-22, another oxygenated carbon (δ_C 64.1, DEPT 135 spectrum indicated it was a methine) signal was shown in the ^{13}C-NMR spectrum of **4**. The position of this oxygenated methine carbon was assigned at C-7 by analysis of the observed ^{1}H-^{1}H COSY and HMBC data of **4**, and the β-oriented relative stereochemistry of the OH-7 group was determined from the ROESY correlation between H-7 and H-9.

Compound **6** (2,3-dihydroixocarpalactone B) was another very minor metabolite purified by reversed-phase HPLC [17], and its molecular formula was determined as $C_{28}H_{40}O_8$ by HRTOFMS, two hydrogen atoms greater than that of ixocarpalactone B (**5**) [16]. The ^{1}H- and ^{13}C-NMR spectra of **6** were very similar to those of the parent compound, **5** [16], and the ^{13}C-NMR spectrum of **6** displayed two additional methylene carbon signals (δ_C 32.0 and 27.0) when compared to ixocarpalactone B (**5**). In the HMBC spectrum, the H-4 signal correlated with both of these two methylene carbons, which suggested the structure of **6** to be 2,3-dihydroixocarpalactone B [17].

The ^{1}H- and ^{13}C-NMR spectra of compound **7** (2,3-dihydro-3β-methoxyixocarpalactone B) were also very similar to those of ixocarpalactone B (**5**) [16], and the spectral differences between these two compounds were

analogous to those between 2,3-dihydro-3β-methoxyixocarpalactone A (**3**) and ixocarpalactone A (**1**) as mentioned above. The structure of **7** was elucidated as 2,3-dihydro-3β-methoxyixocarpalactone B by detailed investigation of its ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H COSY, HMQC, HMBC, ROESY data, as well as its HRTOFMS data [17].

Some methanol or ethanol Michael addition products of withanolides have been reported previously [47-55]. Compounds **3** and **7** possess a methoxy group at C-3, and they were isolated together with ixocarpalactones A and B from the leaves and stems of *P. philadelphica*. Thus, it was considered of interest to determine if the methoxylated **3** and **7** are extraction artifacts or naturally occurring substances. Two small portions of the leaves and stems of *P. philadelphica* were extracted separately with methanol and acetonitrile, respectively, and the extracts obtained were then subjected to LC-MS-MS analysis after each extract was stored at room temperature for one week. As expected, both **3** and **7** were detected in the methanol extract but not in the acetonitrile extract. This result clearly verified that compounds **3** and **7** were formed, at least in part, as a result of the MeOH extraction procedure used in our work [17].

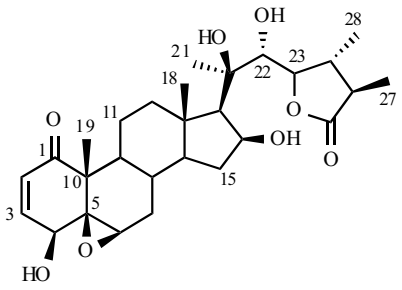
NOVEL C-18 NORWITHANOLIDES WITH A C_{27} NEW CARBON SKELETON FROM *DEPREA SUBTRIFLORA*

In the course our ongoing plant screening work, an extract of the whole plants of *Deprea subtriflora* (Ruiz & Pavon) D'Arcy (Solanaceae), collected in Peru, was found to show significant QR-inducing activity with cultured Hepa 1c1c7 cells. There have been no previous laboratory investigations on this plant to date. The dried and milled whole plant (1.1 kg) was extracted by maceration with MeOH, and a $CHCl_3$-soluble partition extract showed significant inducing activity (CD <2.5 μg/mL and IC_{50} >20 μg/mL) in the cell-based QR assay. Therefore, this extract was further purified by repeated silica gel chromatography and preparative thin-layer chromatography (PTLC), and led to the isolation of 12 novel C-18 norwithanolides based on a new C_{27} skeleton, subtrifloralactones A–E (**8**–**12**) and F–L (**14**–**20**), a new C-18 oxygenated withanolide, 13β-hydroxymethylsubtrifloralactone E (**13**), a new α-ionone derivative, (+)-7α,8α-epoxyblumenol B, and five known compounds, philadelphicalactone A (**2**), (2*S*,3*S*,4*R*)-2-[(2*R*)-2-hydroxytetracosanoylamino]-1,3,4-octadecanetriol, trans-*N*-feruloyltyramine, cis-*N*-feruloyltyramine, and (*S*)-coriolic acid [18,19]. The active known compounds philadelphicalactone A and (2*S*,3*S*,4*R*)-2-[(2*R*)-2-hydroxytetracosanoylamino]-1,3,4-octadecanetriol were previously isolated from the leaves and stems of *P. philadelphica* [10,16].

A molecular formula of $C_{27}H_{34}O_6$, indicating 11 degrees of unsaturation, was assigned to **8** on the basis of its HRTOFMS. Twenty-seven carbon signals including the characteristic resonances for an α,β-unsaturated ketone (δ_C 203.6) and a γ-lactone carbonyl carbon (δ_C 178.9) were evident from the ^{13}C-NMR and DEPT spectral data of **8**. The most notable feature in the ^{13}C-NMR spectrum of **8** was the observation of a doubly oxygenated quaternary carbon signal in a downfield region (δ_C 105.4, C-12), which suggested the presence of a hemiketal or ketal functionality [56-59]. The structures of rings A, B and F could be easily assigned by interpretation of the 2D-NMR data (^{1}H-^{1}H COSY, HMQC, HMBC and NOESY) of **8**. Further inspection of the chemical shifts of the remaining carbon signals indicated that four carbons (δ_C 105.4, C-12; 79.3, C-16; 69.3, C-20; 79.8, C-22) were linked to oxygen atoms. However, it was clear that only three oxygen atoms could be attached to these four oxygenated carbons based on the determined molecular formula. Therefore, the presence of two oxygen ether bridges and only one hydroxyl group in the molecule of **8** could be deduced. In the HMBC spectrum of **8**, correlations from both H-16 and H-22 to C-12 were observed. Thus, two oxygen ether bridges could be proposed for **8** between C-12 and C-16, and between C-12 and C-22. Accordingly, the signal at δ_C 105.4 represented a ketal rather than a hemiketal carbon. Compound **8** reacted with trichloroacetyl isocyanate to afford a monocarbamate [60, 61], and both the CH_3-21 and H-17 signals of the obtained monocarbamate showed significant downfield shifts with values of 0.40 and 1.16 ppm, respectively, when compared with analogous data for **8** [18]. This permitted the placement of the only hydroxyl group in compound **8** at C-20. Finally, the structure of **8** was confirmed unambiguously by a single-crystal X-ray analysis [18]. Furthermore, the X-ray data set collected at room temperature with CuKα radiation determined the absolute configuration of this compound as shown in Figure 1 based on the Flack absolute structure parameter [18]. The structures of subtrifloralactone B (**9**) and subtrifloralactone C (**10**) were assigned by comparison their 1D- and 2D-NMR spectral data with those of subtrifloralactone A (**8**).

A molecular formula of $C_{27}H_{36}O_6$ was determined for subtrifloralactone D (**11**) from its HRTOFMS. The ^{1}H- and ^{13}C-NMR data of **11** were also closely comparable to those of **8**–**10**, but one difference evident for **11** compared to **8**–**10** was a signal for a nonconjugated ketone (δ_C 209.8) group in the^{13}C NMR spectrum of **11** instead of the ketal group (δ_C 101.6-105.4) present in **8**–**10**. This nonconjugated ketone was placed at C-12 based on the observed HMBC correlations from H-9, H_2-11, H-13, H-14, and H-17 to the carbon signal of this ketone. In addition, the chemical shift of the lactone carbonyl carbon (δ_C 175.7) of **11** showed an upfield shift compared to those of **8**–**10**, which suggested that a six-membered lactone ring rather than a five-membered lactone ring was present [16]. Single-crystal X-ray crystallographic analysis was used to confirm the structure of subtrifloralactone D (**11**) [18]. Compound **12**, subtrifloralactone E, was an isomer of subtrifloralactone D (**11**), with the double bond located at C-3/C-4 in the A ring, by NMR spectral data comparison with model withanolides.

A molecular formula of $C_{28}H_{38}O_7$ was determined for compound **13** by HRTOFMS, and the ^{13}C-NMR spectrum of **13** displayed 28 carbon signals [19]. An oxygenated methylene was observed at δ_C 62.6 (t, C-18) and δ_H 4.34 (H_2-18) in the 1D-NMR spectra of **13**. The other ^{1}H- and ^{13}C-NMR spectral data of **13** were closely comparable to those of **11**. The observed HMBC correlations from δ_H 4.34 (H_2-18) to C-12, C-13, C-14 and C-17 permitted the assignment of the hydroxymethyl group to be located at C-



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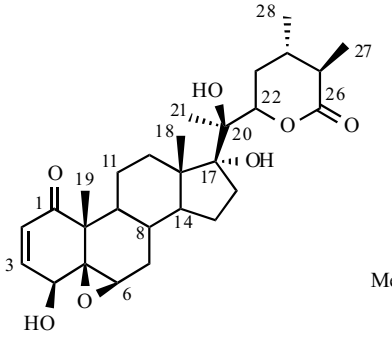

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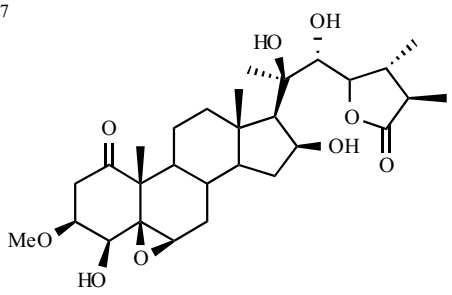

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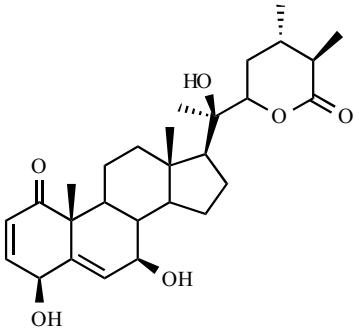

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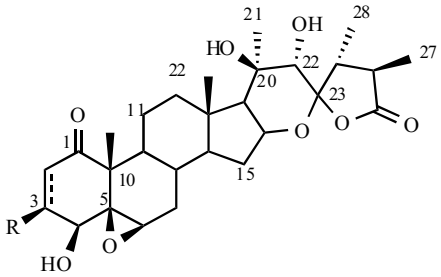

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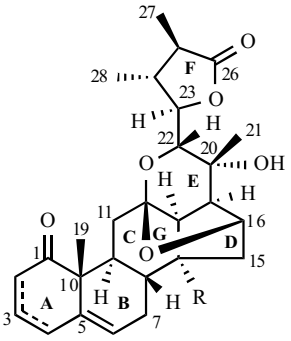
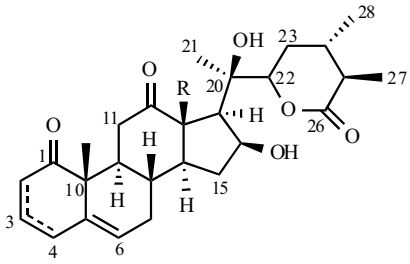
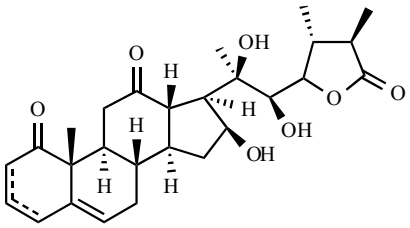
6 2,3-dihydro R = H
7 2,3-dihydro R = OMe



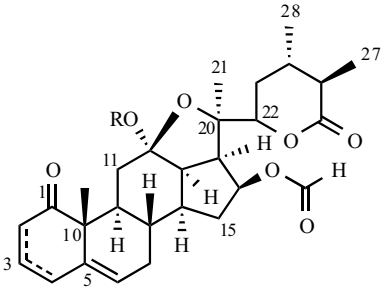
8 Δ^2 R = H
9 Δ^3 R = H
10 Δ^2 R = OH



11 Δ^2 R = H
12 Δ^3 R = H
13 Δ^2 R = CH_2OH



14 Δ^2
15 Δ^3



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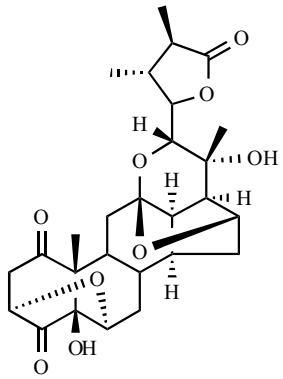
17 Δ^2 R = CH_2CH_3
18 Δ^2 R = CH_3



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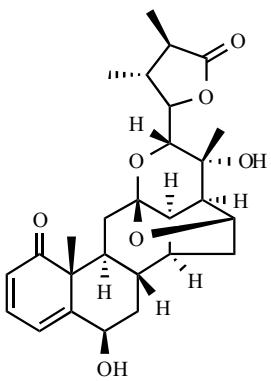

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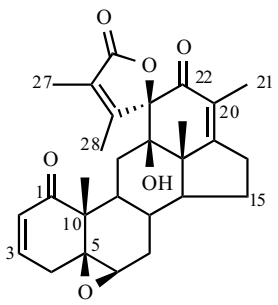

21

(Fig. 1) contd.....

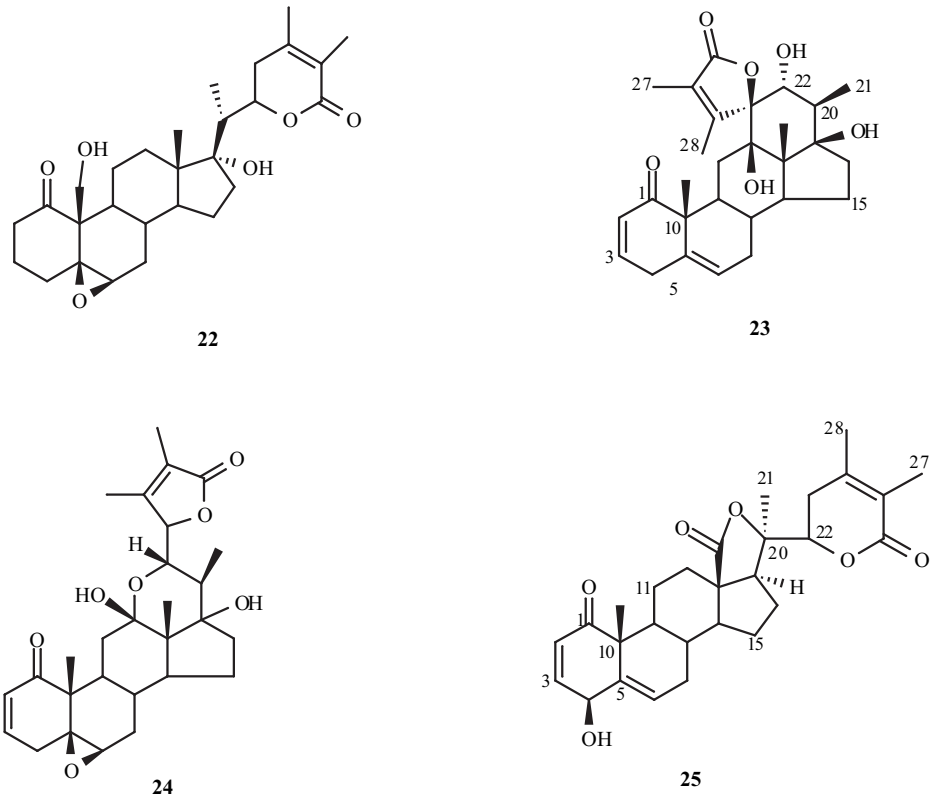

Fig. (1). Structures of selected withanolides isolated from Solanaceous species.

13. A β-orientation of this hydroxymethyl was determined from the strong NOESY correlation between δ_H 4.34 (H_2-18) and δ_H 2.07 (H-8). Accordingly, compound **13** was assigned as 13β-hydroxymethylsubtrifloralactone E. This was the only C_{28} withanolide among the thirteen new withanolides isolated during our work on *Deprea subtriflora* [18, 19].

The HRTOFMS data of **14** and **15** (subtrifloralactones F and G) gave the same molecular formula of $C_{27}H_{37}O_7$ for each compound, and, when evaluated in combination with their ^{1}H- and ^{13}C-NMR data, this indicated that they are a further pair of isomers with an enone and a nonconjugated ketone in their A ring, respectively. The chemical shifts of the lactone carbonyl carbons of **14** and **15** showed downfield shifts comparable to those of **11** and **12** [18], and suggested the presence of a γ-lactone ring rather than a δ-lactone ring in both the molecules of **14** and **15**. The side chains of compounds **14** and **15** were both assigned as the same as that present in the known compound, ixocarpalactone A (**1**) [16], as a result of detailed analysis of their 2D NMR spectral data.

The HRTOFMS of **16** (subtrifloralactone H) provided a molecular formula of $C_{30}H_{40}O_7$, indicating eleven degrees of unsaturation. In the ^{13}C-NMR spectrum of **16**, a ketal group at δ_C 110.0 (C-12) similar to those in **8**–**10** and a six-membered lactone carbonyl carbon at δ_C 175.6 (C-26) were evident, as in **11** and **12**. The presence of an ethoxyl group could be easily deduced from the 1D- and 2D-NMR data of **16**. In addition, the ^{1}H- and ^{13}C-NMR spectra of **16** exhibited two downfield signals of a formate ester group [62,63] at δ_H 8.46 and δ_C 161.5. The observed HMBC correlations from δ_H 4.18 and 3.90 (O$\underline{CH_2}$CH$_3$) to δ_C 110.0 (C-12), from δ_H 8.46 (1H, s, OCOH) to δ_C 76.9 (C-16), and from δ_H 5.53-5.57 (H-16) to δ_C 161.5 (OCOH), indicated that the ethoxyl and formate ester groups were attached to C-12 and C-16, respectively. The presence of an oxygen ether bridge from C-12 to C-20 could be deduced by analysis of the molecular formula and the determined partial structural units of **16**. The double bond position of the A ring in the molecule of subtrifloralactone I (**17**) was readily assigned at C-2/C-3 by the observed ^{1}H-^{1}H COSY and HMBC correlations. The NMR and HRTOFMS data of the third formate ester group-possessing withanolide, subtrifloralactone J (**18**), indicated that a methoxyl group (δ_H 3.52 and δ_C 48.9) had replaced the ethoxyl group in **17**, and its structure was confirmed by 2D-NMR spectral measurements.

The HRTOFMS of subtrifloralactone K (**19**) provided a molecular formula of $C_{27}H_{34}O_9$, suggesting it was another C_{27} withanolide. The ring C-G portions of **19** could be assigned as the same as those in **8** and **9** by interpretation of its 1D- and 2D-NMR data. The remaining oxygenated carbons were found to comprise two nonconjugated ketone signals at δ_C 208.5 (C-1) and 208.1 (C-4), two methine signals at δ_C 74.8 (C-3) and 72.6 (C-6), and a quaternary carbon signal at δ_C 77.7 (C-5). The locations of the two ketones at C-1 and C-4 and a hydroxyl group at C-5 were assigned in turn on the basis of the observed HMBC correlations from the hydroxyl group (δ_H 3.64, OH-5) to C-4, C-5, C-6 and C-10, and from CH$_3$-19 (δ_H 0.96) to C-1, C-5, C-9 and C-10. The presence of an oxygen ether bridge from C-3 to C-6 was then indicated by the determined molecular formula of **19**, and it was confirmed by the

observed weak but diagnostic HMBC correlation from H-3 to C-6. The β-orientation of OH-5 was determined by the NOESY correlation between the signals of CH_3-19 and OH-5. The relative configuration of the C-3/C-6 oxygen ether functionality in the molecule of subtrifloralactone K (**19**) was assigned based on the comparison of the splitting patterns and coupling constants of H_2-2, H-3 and H-6 of **19** with those of previously reported analogs [64, 65].

Inspection of the 1D- and 2D-NMR data of subtrifloralactone L (**20**) suggested that the substituent pattern and relative stereochemistry of rings C-G of **20** were the same as those of compounds **8**, **9**, and **19**. The ^{1}H- and ^{13}C-NMR signals of rings A and B of **20** were consistent with the presence of an α,β-unsaturated ketone, two double bonds, and an oxygenated methine. The positions of these functionalities were established by the observed 2D-NMR correlations. The signal for H-6 was obtained as a broad triplet in both $CDCl_3$ (2.6 Hz) and pyridine-d_5 (2.4 Hz), which was suggestive of the β-orientation of OH-6 [66-72] in this isolate.

INDUCTION OF QUINONE REDUCTASE WITH CULTURED MOUSE HEPATOMA CELLS BY WITHANOLIDES AND NORWITHANOLIDES

The cell-based quinone reductase induction assay is one of the important *in vitro* assays used in our research program on cancer chemoprevention. For the evaluation of our pure withanolides and norwithanolides as inducers of QR, cultured mouse Hepa 1c1c7 cells were used as described previously [73, 74]. In brief, 96-well plates were seeded at a density of 4000 cells/mL (200 μL/well) and incubated for 24 h at 37 °C in a CO_2 incubator. The medium was then changed, and test compounds, dissolved in 10 μL of 10% DMSO, were introduced and serially diluted in 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 MTT [3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide] to a blue formazan. Protein levels were determined in a duplicate set of plates using crystal violet staining and subsequent measurement at 595 nm [74]. Induction of QR activity was calculated from the ratio of specific enzyme activities of compound-treated cells in comparison with a solvent control. Enzyme activity was expressed as: (a) the CD, the concentration required to double the specific activity of QR; (b) the IC_{50}, the half-maximal inhibitory concentration of cell viability; and (c) the CI (chemoprevention index), IC_{50}/CD.

Using the above-described methodology, the potential of compounds **1**–**20** to induce QR was evaluated, and the data obtained are shown in Table 1. Among the withanolides (**1**–**7**) obtained from *Physalis philadelphica*, the most potent compounds were found to be ixocarpalactone A (**1**), philadelphicalactone A (**2**), 4β,7β,20*R*-trihydroxy-1-oxowitha-2,5-dien-22,26-olide (**4**), and ixocarpalactone B (**5**), which all contain a 4β-hydroxy-2-en-1-one structural unit. The Michael addition products **3** and **7** lost the QR inducing activities compared to their precursors, ixocarpalactones A (**1**) and B (**5**) [16], respectively. While compound **6**, 2,3-dihydroixocarpalactone B, still showed evident QR inducing activity, its CD value (3.82 μM) was almost 20-times higher than that of ixocarpalactone B (**5**) (0.21 μM).

Among the seven active compounds (**8**, **10**, **11**, **14**, and **17**–**19**) obtained from *Deprea subtriflora*, with the exception of compound **19** (an unusual withanolide possessing a 1,4-diketo unit and a C-3/C-6 oxygen ether bridge), all other active compounds contain a ring A α, β-unsaturated ketone structural unit. However, although compounds **13** and **20** also possess this structural characteristic, they were found to be inactive in the QR assay (CD >5 μg/mL). Compound **13** was the only isolate with a hydroxymethyl group at C-13 obtained from *D. subtriflora*, while **20** was the only ring A dienone-possessing withanolide. In our previous detailed study on the potential to induce QR assay by withanolides [75], jaborosalactone 1 (**21**), jaborosalactone O (**22**), jaborosalactone P (**23**), trechonolide A (**24**), and withaphysalin J (**25**), isolated from various South American plants by our collaborators in Argentina, were demonstrated to be significant QR inducers with CD values in the range of 0.27-1.52 μM (Table 1). Four (**21**, **23**–**25**) of these five most active compounds also possess an α,β-unsaturated ketone unit (Figure 1), with the only exception being the 19-hydroxyl group-possessing withanolide, jaborosalactone O (**22**). We have previously proposed that an α,β-unsaturated ketone unit in withanolides is important for inducing activity in the cell-based QR induction assay [16, 75]. However, in addition to this functional group, other structural features may also mediate the QR induction activity of the withanolides.

CONCLUSIONS

A number of new withanolides have been isolated and characterized as inducers of the phase II enzyme, quinone reductase, during our recent work on *Physalis philadelphica*, of which the fruits are used in North American as a vegetable (tomatillo). Several of these compounds, which are methoxylated at C-3, have been shown to be extraction artifacts.

In addition, a series of highly oxygenated C-18 norwithanolides (**8**–**12**, **14**–**20**) with a new C_{27} skeleton has been isolated and identified from the $CHCl_3$-soluble extract of *D. subtriflora* [18, 19]. All these novel withanolides possess either a ketone or a ketal group at C-12. Furthermore, an unusual formate ester structure unit was present in the molecules of **16**–**18**. Since these norwithanolides (**8**–**12**, **14**–**20**) have 27 carbons with their skeleton, it is possible that they are derived from the more typical withanolides by oxidation of CH_3-18. For most withanolides, a methyl group is attached to C-13, but this methyl is sometimes oxidized to a hydroxymethyl (like in present isolate **13**), an aldehyde, or a carbonyl ester group, which could be converted to a carbonyl acid, followed by the loss of CO_2. However, as shown in Figure 1, the rings C and D are *cis*-fused in compounds **8**–**10** and **16**–**20**, which possess a ketal group at C-12. In contrast, rings C and D are *trans*-fused in compounds **11**–**15**, which possess a ketone group at C-12. Thus, studies of the biosynthetic pathways of these norwithanolides are suggested.

It is worth noting that the ^{13}C-NMR resonances for C-17 were weak or even absent (both in $CDCl_3$ and pyridine-d_5)

for all four 12-keto norwithanolides (**11**, **12**, **14**, and **15**) obtained [17], when general instrument operating parameters were used. In contrast, the C-17 signal was clearly observed at δ_C 51.7 (d, C-17) for another 12-keto compound, 13β-hydroxymethylsubtrifloralactone E (**13**) [19], which is a C_{28} withanolide. This spectral observation is worthy of more detailed examination.

Withanolides have been studied previously for their antitumor [76-78], antifeedant [79-82], antiulcer [83], antistress [84, 85], antifungal [86, 87], cytotoxicity [88], immunosuppressive [88-90], antibacterial [87], antimicrobial [76, 91], and anti-inflammatory [92] activities. Very recently, the inhibitory activity against cyclooxygenase-1 (COX-1) and -2 (COX-2) by a number of withanolides isolated from the leaves of *Withania somnifera* Dun was reported [93]. However, a literature survey has revealed that the potential to induce quinone reductase of withanolides has been reported only by our laboratory at this point in time [15-19, 75]. Ixocarpalactone A (**1**), one of the most promising compounds obtained in the course of our program project research on cancer chemoprevention agents, is a known compound previously isolated and characterized from the aerial parts of *P. philadelphica* (formerly known as *P. ixocarpa* Brot.) [16, 17, 45]. The presence of ixocarpalactone A in the edible fresh fruits (tomatillo) of *P. philadelphica* at a concentration level of 143±4.53 ppb was detected by LC-MS-MS analysis [17]. Based on the excellent activity profile and comparative lack of cellular toxicity in the QR assay, and its relatively high abundance in the aerial parts of its plant of origin, testing with more advanced biological models for evaluating the potential cancer chemopreventive activity of ixocarpalactone A (**1**) is currently being conducted.

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