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# 17$\beta$-Hydroxywithanolides as Sensitizers of Renal Carcinoma Cells to Tumor Necrosis Factor-$\alpha$ Related Apoptosis Inducing Ligand (TRAIL)–Mediated Apoptosis: Structure–Activity Relationships

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**ABSTRACT:** Renal cell carcinoma (RCC) is a cancer with poor prognosis and the 5-year survival rate of patients with metastatic RCC is 5–10%. Consequently, treatment of metastatic RCC represents an unmet clinical need. Screening of a 50,000-member library of natural and synthetic compounds for sensitizers of RCC cells to TRAIL-mediated apoptosis led to identification of the 17$\beta$-hydroxywithanolide (17-BHW), withanolide E (**1**), as a promising lead. To explore structure–activity relationships, we obtained natural and semi-synthetic withanolides **1**, **2a**, **2c**, and **3**–**36** and compared their ability to sensitize TRAIL-mediated apoptosis in a panel of renal carcinoma cells. Our findings revealed that 17-BHWs with a $\alpha$-oriented side-chain are superior to known TRAIL-sensitizing withanolides belonging to withaferin A class with a $\beta$-oriented side-chain and demonstrated that the 17-BHW scaffold can be modified to enhance sensitization of RCCs to TRAIL-mediated apoptosis, thereby assisting development of natural product-inspired drugs to treat metastatic RCC.

## ■ INTRODUCTION

Renal carcinoma is among the ten most common cancers in the US and according to the American Cancer Society's most recent estimates, about 63,990 new cases of kidney cancer will occur and over 14,400 people will die from this disease in 2017.[1] There are over 338,000 new renal cancer cases worldwide each year[2] and it is often diagnosed with poor prognosis at the metastatic stage.[3] Approximately 90% of renal cancers arise in the renal parenchyma and are termed renal cell carcinoma (RCC). The current standard of care, immunotherapy using interferon or interleukin-2, has had some success with responses in a small fraction of patients with metastatic RCC.[4] Although some anti-angiogenic therapies including sunitinib and sorafenib (Supporting Information, Figure S1) have provided modest responses, it remains unclear which patients would have durable responses.[5] Thus, the treatment of advanced RCC represents an unmet clinical need and the discovery of innovative approaches for this purpose is urgently required.

Current strategies for cancer therapy aim to overcome two key hallmarks of cancer including excessive proliferation and avoidance of apoptosis.[6] In contrast to inhibiting proliferation, which will mostly achieve stable disease by limiting tumor outgrowth, induction of apoptosis has the potential to eliminate cancer cells, which could provide an opportunity for cure. Consequently, methods of inducing apoptosis have become an important approach in the design of effective cancer therapies. TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) is a protein predominantly derived from immune cells, that functions as a ligand to induce cell death by apoptosis.[7] TRAIL binding to death receptors (DR4 and DR5) assembles the death-inducing signaling complex (DISC) through recruitment of FAS-associated protein with death domain (FADD) and caspase-8, and autocatalytic activation of caspase-8 causes a cascade of caspase activation ultimately resulting in cell death.[8] TRAIL has a significant advantage in its

selectivity for targeting cancer cells due to their relatively higher expression of death receptors than normal cells, which in contrast highly express decoy receptors, DcR1 and DcR2.[9] TRAIL binds to decoy receptors, but these complexes are unable to activate the apoptotic signaling pathway. Thus, TRAIL has little effect on apoptosis of normal cells. Furthermore in preclinical models of renal cancer, TRAIL produced by immune cells has been shown to limit cancer progression.[10] However, many cancers acquire resistance to TRAIL therapy by down-regulating DRs and up-regulating anti-apoptotic proteins including cellular FLICE-like inhibitory protein (*c*FLIP).[11] Since many cancer cells, particularly those freshly isolated from cancer patients, are resistant to TRAIL-mediated apoptosis, the search for novel sensitizers to overcome TRAIL resistance is considered an effective approach for anticancer drug discovery and the future improvement of TRAIL-based therapies.

Historically, plants have proven to be a valuable source of clinically useful anticancer agents and they continue to play a major role in drug discovery as evidenced by the number of promising new agents in clinical development based on their selective activity against cancer-related molecular targets.[12] Several plant-derived natural products are also known to enhance TRAIL-induced apoptosis through modulation of diverse signaling pathways suggesting that combination therapy involving TRAIL and appropriate natural products might offer an attractive strategy for treatment of cancer.[13] Among the natural products capable of promoting TRAIL-induced apoptosis of renal carcinoma cells are the well-known withanolide, withaferin A (**2a**) derived from *Withania somnifera*,[14] and its new analogue **2b** (Figure 1) recently encountered in *Physalis pubescens*.[15] In our search for sensitizers of renal carcinoma cells to TRAIL-induced apoptosis, we previously used a cell-based high-throughput assay to screen a 50,000-member library of natural products and synthetic compounds.[16] The most promising compound identified in this study was withanolide E (**1**), a constituent of the plant, *Physalis peruviana*.[17] It was also

found that this 17$\beta$-hydroxywithanolide (17-BHW) was about 5-fold more potent than **2a** in sensitizing cancer cells to TRAIL-mediated apoptosis. In addition, **1** was found to induce TRAIL-mediated apoptotic signaling via a rapid decline in the levels of *c*FLIP proteins, and animal studies revealed that it sensitized human renal carcinoma cells to apoptosis at concentrations that did not promote any obvious manifestations of toxicity in mice.[17] More importantly, in contrast to **2a**, **1** did not cause growth inhibition, dramatic changes in cell morphology or trigger a cell stress response at concentrations that sensitized cancer cells to undergo apoptosis, suggesting that biological activity of 17-BHWs with a $\alpha$-oriented side chain (e.g. **1**) may differ considerably from those with a $\beta$-oriented side chain (e.g. **2a** and **2b**).[18] Based on its unique and promising activity, **1** was considered a lead molecule suitable for structure–activity relationship (SAR) studies to identify natural product-based compounds that can selectively promote apoptosis in renal cancer cells and therefore potentially be used to treat renal carcinoma.

## ■ RESULTS AND DISCUSSION

**Isolation and Semi-synthesis of Withanolides 1–36.** Naturally occurring withanolides **1**–**5**, **20**, **27**–**31**, and **34**–**36** for biological evaluation and preparation of semisynthetic 17-BHWs were obtained from aeroponically cultivated *Withania somnifera*,[19] *Physalis crassifolia*,[20] and *P. peruviana*.[21] 17-BHWs encountered in these plant species belonged to three distinct structural sub-classes based on their oxygenation pattern and were grouped as analogues of withanolide E (WE) (**5**–**14**; Figure 2), physachenolide C (PCC) (**15**–**26**; Figure 3), and physachenolide D (PCD) (**27**–**36**; Figure 4). 3$\beta$-Azido-4$\beta$-hydroxywithanolide E (**6**) was obtained by treating 4$\beta$-hydroxywithanolide E (**5**) with $Me_3SiN_3$/AcOH/$Et_3N$.[22] Acetyl derivatives **7**, **12**, **32** and **33** were prepared by acetylation of

their corresponding hydroxy analogues with $Ac_2O$/pyridine. Treatment of **5** with 1,1′-carbonyldiimidazole gave **9** which on methanolysis afforded **8**. Oxidation of **5** with $MnO_2$ yielded withaperuvin E (**10**). Reduction of **10** with $NaBH_4/CeCl_3$ afforded 4$\alpha$-hydroxywithanolide E (**11**) as the major product formed as a result of regio- and stereo-specific reduction of its ring A ene-dione moiety.[23] When treated with $Ph_3P/I_2$, **10** underwent deoxygenation to afford withaperuvin M (**13**) which on $NaBH_4/CeCl_3$ reduction afforded 4-*epi*-physapruin A (**14**). Catalytic hydrogenation of **3** with Pd/C in EtOH containing $Et_3N$ afforded 2,3-dihydrophysachenolide C (**15**) and its desacetyl analogue **24**. Michael adducts of **3** at C-3, 2,3-dihydro-3$\beta$-azidophysachenolide C (**16**), 2,3-dihydro-3$\beta$-imidazolylphysachenolide C (**17**), and 2,3-dihydro-3$\beta$-methoxyphysachenolide C (**18**) were obtained by reacting **3** with $Me_3SiN_3$/AcOH/$CH_2Cl_2$,[22] imidazole/$CH_2Cl_2$, and MeOH/$Et_3N$, respectively. Epoxidation of **4** with *m*-CPBA yielded **3**, its epimer, 5,6-*epi*-physachenolide C (**19**), and 24$\alpha$,25$\alpha$-epoxyphysachenolide C (**26**) whereas similar treatment of **36** afforded 15$\alpha$-hydroxyphysachenolide C (**21**) and 15$\alpha$-hydroxy-5,6-*epi*-physachenolide C (**23**).[24] Treatment of **3** with *t*-BuOK/*t*-BuOH/$CH_2Cl_2$/–5 $^{o}$C afforded its18-desacetyl analogue **25**. All new analogues were characterized using their spectroscopic data (see Experimental Section and Supporting Information, Figures S2–S60).

**TRAIL-Induced Apoptosis.** We have previously demonstrated that a combination of WE (**1**) and TRAIL eliminated long-term survival of ACHN renal carcinoma cells while either agent alone had no effect.[17] In this study we first determined the ability of each of the withanolides **1**, **2a**, **2c**, and **3**–**36** to sensitize ACHN cells to apoptosis in the presence of TRAIL (50.0 ng/mL) (Table 1 and Supporting Information, Figure S61). Of these, the 17-BHW analogues **3**–**5**, **7**–**9**, **11**–**14**, **16** and **17** had activities similar or superior to **1** (Figure 5) suggesting that those belonging to WE and PCC sub-classes of 17-BHWs (Figures 2 and 3, respectively) bearing 5$\beta$,6$\beta$-epoxy moieties exhibited

potent activity. Among those active, **3** and the novel WE analogue, 4$\alpha$-hydroxywithanolide E (**11**), were selected for further evaluation and **1** and **2a** were included for comparison purposes. Analysis of a panel of human renal carcinoma cells (Caki-1, SN12C, and UO-31) for sensitization to TRAIL-induced apoptosis demonstrated that the relative activities of these analogues were similar for all renal cancer cells tested other than ACHN, with **3** and **11** being significantly more active than **1**; **2a** displayed no activity at the concentrations tested (Figure 6). Since our previous studies have shown that treatment of ACHN cells with **1** resulted in a drop in levels of the anti-apoptotic *c*FLIP proteins thus promoting enhanced caspase-8 activation upon subsequent exposure of cells to TRAIL,[17] we assessed the effects of these 17-BHWs on levels of *c*FLIP and caspase activation. As depicted in Figure 7, **1** caused some reduction of *c*FLIP, but this was much more pronounced in cells treated with the same concentration of **3** or **11**. Interestingly other active withanolides (**7** and **8**) also caused a reduction of *c*FLIP levels (Supporting Information, Figure S62). On subsequent exposure of cells to TRAIL, the conversion of procaspase-8 to the active form the enzyme, and the subsequent proteolytic activation of the executioner caspase-3 was also more dramatic in cells treated with **3** and **11** compared to **1** with weak activity and **2a** with no activity (Figure 8)**.** This suggested that reduction of the anti-apoptotic *c*FLIP proteins in renal carcinoma cells by these withanolides, resulted in an enhanced activation of caspase-8 on subsequent exposure to TRAIL, and this is likely an important component of their molecular mechanism of action. Previous studies have reported that reducing levels of *c*FLIP with these same RCC cell lines using siRNA was sufficient to sensitize them to TRAIL-mediated apoptosis.[11b] Therefore the efficient reduction of *c*FLIP levels by **3** and **11** is likely to be critical for apoptosis sensitization of RCC cells. Thus these decreases in *c*FLIP proteins result in an amplification of proximal TRAIL-induced extrinsic apoptosis signaling by increasing caspase-8 activation. Kinetic studies showed that a 6 h treatment of cells with **3** was sufficient to enhance caspase-8 activation following a short (90 min) exposure to TRAIL (Supporting Information, Figure

S63). For these RCCs a contribution of the intrinsic or mitochondrial signaling pathway might not be required for apoptosis to occur.

It is known that **2a**[14] and its analogue **2b**[15] could sensitize RCC cells to TRAIL-sensitized apoptosis at high concentrations (1.2 $\mu$M and 1.0–2.0 $\mu$M, respectively). Data presented here suggests that **2a** and its analogue 2,27-diacetyl-4-*epi*-withaferin A (**2c**)[23] with a $\beta$-oriented side chain are much less potent as TRAIL-sensitizers and are less effective in reducing *c*FLIP levels than 17-BHWs with a $\alpha$-oriented side chain (Supporting Information, Figure S64). In contrast to the effects on TRAIL sensitization, the active 17-BHWs (**1**, **3** and **11**) were less directly cytotoxic to ACHN cells than **2a** and **2c** (Supporting Information, Figure S65). In addition, **1** and **3** in contrast to **2a** and **2c** did not significantly increase the cellular levels of proteins CHOP, BIP and HSP70 that are elevated in response to cell stress (Supporting Information, Figure S66). Many compounds that have been proposed as TRAIL sensitizers, including the chemotherapeutic agent, bortezomib[25] (Supporting Information, Figure S1), and natural products such as cucurbitacin A,[13b] triptolide[13d] and **2a**[14] as well a novel small-molecule, 7-benzyl-4-(2-methylbenzyl)-2,4,6,7,8,9-hexahydroimidazo[1,2-a]pyrido[3,4-e]pyrimidin-5(1H)-one (ONC201)[26] (see Supporting Information, Figure S1), are known to induce significant levels of cell stress. Furthermore, this cell stress induction has been proposed to contribute to increased TRAIL-induced apoptosis, since increases in CHOP levels are reported to increase the levels of DR5 on cancer cells resulting in a stronger apoptosis response following exposure to TRAIL.[27] Interestingly, the17-BHWs found to be active in this study did not increase levels of DR5 on RCC (data not shown). The lack of direct cytotoxicity or induction of cell stress by active 17-BHWs suggests that in contrast to many other TRAIL-sensitizers, induction of a cell stress response is not a part of their mechanism of action, and they may have a novel and unique molecular mechanism of action that differs from that of many other TRAIL-sensitizing agents.

**Cytotoxicity and Thiol Reactivity.** The ability of **2a** to sensitize TRAIL-induced apoptosis of renal carcinoma cells has been attributed to ROS-mediated upregulation of DR5 and downregulation of *c*FLIP.[14] Withanolide **2a** has also been reported to exert its cytotoxicity and cellular stress induction activities by depletion of intracellular glutathione (GSH).[28] Thus it was of interest to determine if sensitization of TRAIL-induced apoptosis of ACHN cells by active 17-BHWs is associated with cytotoxicity and/or cellular stress. The $IC_{50}$ data (Figure 9) for **1**, **3**, and those 17-BHWs (**5**, **7**, **8**, **11** and **12**) with TRAIL-sensitization activity superior or comparable to that of **1** suggested the order of cytotoxic activity to be **1** = **3** < **5** < **11** < **8** < **12** < **7**. The 17-BHWs, **7**, **8**, **11**, and **12** with strong activity as sensitizers of ACHN cells to TRAIL-induced apoptosis were found to be comparatively more cytotoxic than **1** and **3** (Figure 9). Furthermore, testing of a larger panel of various human cancer cell lines showed no significant differences between **3** and **11** for TRAIL sensitizing activity (data not shown). However, **11** was found to undergo decomposition on storage suggesting that it may not be a good candidate for further development. Therefore **3** was selected for further testing in studies currently underway in mouse pre-clinical cancer models.

The chemical structures of **2a** and 17-BHWs used in this study contain three potential electrophilic sites (2,3-en-1-one in ring A, 5,6-oxirane in ring B, and $\alpha,\beta$-unsaturated lactone in the side chain), but it is known that among these reactive sites ring A enone moiety is the most susceptible for nucleophilic attack,[28,29] and we have recently shown that cellular stress caused by **2a** is due to the formation of Michael adducts of its ring A enone moiety with GSH and that cytotoxicity of **2a** was counteracted by the thiol-based antioxidant, N-acetylcysteine (NAC).[28] Thus, it was of interest to compare thiol reactivity (thiophilicity) of some withanolides used in this study with their ability to affect TRAIL sensitization. Previous work has shown that the reaction of GSH with enones proceeded rapidly at basic pH (pH = 8), but extremely slowly and irreversibly at low pH.[29] However, after some experimentation with reaction conditions, we

found that the addition of GSH to **2a** took place at a measurable rate at the physiological pH (pH = 7.4). Thus, the 17-BHWs **1**, **3**, **5**, **7**, **8**, **11** and **12** in DMSO were treated with aqueous solutions of GSH and NAC at pH 7.4 and the amount of unreacted withanolide was monitored by HPLC for 24 h. The cytotoxic withanolide, **2a**,[23] and comparatively less cytotoxic, 5,6-*epi*-PCC (**19**),[24] were included for comparison purposes. As shown in Figure 10, these withanolides varied considerably in their relative ability for irreversible thiol capture and suggested that they belong to 3 distinct classes, those with high (**2a**, **5**, **7**, **8**, **11**, and **12**), medium (**1** and **3**), and low (**19**) thiophilicities. Intriguingly, for 17-BHWs the observed thiophilicity was found to parallel their cytotoxic activity (Figure 9). Withanolides (**2a**, **5** and **11**) with relatively high Michael acceptor capacity all contain a hydroxy group at the allylic position (C-4) which is known to accelerate thiol addition.[30] We have previously shown that the $\beta$-hydroxy group at C-4 in **5** caused enhancement of its cytotoxic activity compared to **1** lacking this hydroxy group, and had weak activity for sensitization of ACHN cells to TRAIL[17] The least active withanolide **19** displays an enone moiety in a *trans*-decalin ring system compared to other 17-BHWs which contain this moiety in a *cis*-decalin system. Based on reported studies on related molecules,[31] it is likely that the *trans*-decalin-type enone in **19** has a less favorable equilibrium for thiol capture than the *cis*-decalin-type enones in **1**, **3**, **5**, **7**, **8**, **11**, and **12**.

**Structure-Activity Relationships.** In order to identify structural features of withanolides responsible for sensitizing renal carcinoma cells to TRAIL-induced apoptosis, we examined thirty four natural and semi-synthetic 17-BHWs (**3**–**36**) bearing an $\alpha$-oriented side chain and compared their activities with those of **1** and withanolides with a $\beta$-oriented side chain, **2a** and its analogue **2c**. It was found that the 17-BHWs had superior desirable activity as TRAIL-sensitizers and therefore we focused our attention to structure-activity relationships (SARs) within this group of withanolides.

Summary of SAR data for 17-BHWs evaluated is depicted in Figure 11. These data suggested that in addition to the $\alpha$-oriented side-chain and/or 17$\beta$-hydroxy group, enone moiety in ring A and 5$\beta$,6$\beta$-oxirane functionality in ring B are essential for activity and that $\alpha$- and $\beta$-acetoxylation/$\alpha$-hydroxylation/$\beta$-methoxycarbonylation at C-4, and acetoxylation at C-18 lead to enhancement of activity, $\beta$-hydroxylation at C-4 has no effect on activity, whereas hydroxylation at C-15, C-18, C-26 and C-27 and $\alpha$-epoxidation of the double bond at C-24(25), all lead to decrease or loss of activity.

## ■ CONCLUSIONS

Withanolides, a class of steroidal lactones occurring in some plants used in traditional medicines, continue to attract attention due to their intriguing biological activities.[32] Among all withanolides, the most extensively studied is **2a** with a $\beta$-oriented side chain at C-17 that has been evaluated for its anticancer activity.[28,33] Of over 650 withanolides encountered to date, only about 50 contain a $\alpha$-oriented side chain at C-17 which is not a part of a cyclic system, and most of these bear a $\beta$-hydroxy group at this carbon.[32] These 17-BHWs have been reported to have in vitro cytotoxic activity against breast,[34] and prostate[35] cancers. Our recent studies demonstrated that **1** was able to sensitize TRAIL to induce apoptosis of ACHN renal carcinoma cells[17] and **3** had potent and selective cytotoxic activity to prostate cancer cells,[24] suggesting the future promise of this class of withanolides as potential anticancer agents.

Although this study demonstrated the ability of active 17-BHWs to reduce levels of *c*FLIP and that increased caspase-8 activation to be critical for sensitization of human RCC to TRAIL-induced apoptosis, this may not be sufficient for apoptosis sensitization in other cell types. Interestingly, resistance to TRAIL apoptosis can be due to multiple redundant pathways for normal cells, whereas many cancer cells may rely on a single non-redundant resistance mechanism.[36] Therefore it is likely for RCC that the overexpression of *c*FLIP proteins may

constitute a single dominant apoptosis resistance mechanism. However, genetic and pharmacological screens have also identified multiple inhibitors of TRAIL-mediated cell death in certain cancer cells.[37] Thus, for TRAIL-sensitizing compounds such as the kinase inhibitors sorafenib[38] (Supporting Information, Figure S1) and (N-(5-(((5-(*tert*-butyl)oxazol-2-yl)methyl)thio)thiazol-2-yl)piperidine-4-carboxamide (SNS-032)[39] (see Supporting Information, Figure S1), their molecular mechanism of action seems to involve the reduction in cellular levels of a number of other anti-apoptotic proteins including various Bcl-2 family members and IAPs. As such, these compounds promote not only increases in extrinsic apoptosis signaling but also intrinsic apoptosis signaling, both of which may be necessary for optimal TRAIL apoptosis in many cancer cells. The lack of direct cytotoxicity or induction of cell stress by 17-BHWs **1** and **3** suggests that in contrast to many other TRAIL-sensitizers, induction of cell stress response is not a part of their mechanism of action, and these may have a novel and unique molecular mechanism of action that differs from that of many other TRAIL-sensitizing agents. Thus, taken together these findings suggest that **3** represents a promising natural product-based molecule to be developed as an effective and non-toxic agent to treat metastatic renal carcinomas. Since some active 17-BHWs can promote apoptosis in cancer cells other than RCC cells,[40] we are currently investigating in more detail if they can have additional effects on other apoptosis modifying proteins in addition to reducing levels of *c*FLIP.

## ■ EXPERIMENTAL SECTION

**Chemistry.** Optical rotations were measured in MeOH or $CHCl_3$ with a Jasco DIP-370 digital polarimeter. 1D and 2D NMR spectra were recorded in $CDCl_3$, unless otherwise stated, using residual solvents as internal standards on Bruker Avance III 400 spectrometer at 400 MHz for $^1$H NMR, and 100 MHz for $^{13}$C NMR, respectively. The chemical shift values ($\delta$) are given in parts per

million (ppm), and the coupling constants (*J* values) are in Hz. LR-MS were recorded using Shimadzu LCMS-QP8000α and HR-MS were recorded using JEOL HX110A or Agilent TOF mass spectrometers. Analytical thin-layer chromatography was carried out on silica gel 60 $F_{254}$ Aluminum-backed TLC plates (Merck). Preparative thin-layer chromatography (TLC) was performed on Analtech silica gel 500 $\mu$m glass plates. Compounds were visualized with short-wavelength UV and by spraying with anisaldehyde-sulfuric acid spray reagent and heating until the spots appeared. Silica gel column chromatography (CC) was accomplished using 230–400 mesh silica gel. Sephadex LH-20 was obtained from Amersham Biosciences. HPLC was carried out on a Phenomenex Luna 5$\mu$ C18 (2) column (10 × 250 mm) or Alltech Econosil normal phase silica column (250 × 10 mm, 10 $\mu$) with Waters Delta Prep system consisting of a PDA 996 detector. All yields refer to yields of isolated compounds. Unless otherwise stated, chemicals and solvents were of reagent grade and used as obtained from commercial sources without further purification. HPLC analysis was conducted for all compounds that were subjected to bioassays on a Hitachi L-6200A LC system equipped with Hitachi AS-4000 Intelligent Auto Sampler, Hitachi L-4500 Photodiode array detector and Shimadzu ELSD-LT detector using a Phenomenex Luna 5$\mu$ C18 (2) 100 Å (4.6 mm x 250 mm) column and a gradient solvent system of MeOH/$H_2O$ [from 40:60 to 100:0 (v/v) in 30 min]. All the assayed compounds displayed a chemical purity of >95% by both ELSD and UV (at 230 nm) detection methods.

### Isolation of Naturally Occurring Withanolides 1, 2a, 3–5, 20, 27–31, and 34–36.

Withanolide E (**1**) and 4$\beta$-hydroxywithanolide E (**5**) were obtained from *Physalis peruviana*,[21] withaferin A (**2a**) from *Withania somnifera*,[19] and physachenolide C (**3**), physachenolide D (**4**), 15$\alpha$-acetoxyphysachenolide C (**20**), 2,3-dihydrophysachenolide D-3$\beta$-*O*-sulfate (**27**), 15$\alpha$-acetoxyphysachenolide D (**28**), 15$\alpha$-acetoxy-2,3-dihydrophysachenolide D-3$\beta$-*O*-sulfate (**29**), 15$\alpha$-acetoxy-28-hydroxyphysachenolide D (**30**), 15$\alpha$-acetoxy-27-hydroxyphysachenolide D (**31**), 15$\alpha$-acetoxy-28-*O*-$\beta$-glucopyranosylphysachenolide D (**34**), 15$\alpha$-acetoxy-27-*O*-$\beta$-

glucopyranosylphysachenolide D (**35**), and 15$\alpha$-hydroxyphysachenolide D (**36**) were obtained from *Physalis crassifolia*[20] as previously described.

**General Procedure for Acetylation of Withanolides.** To a solution of the withanolide (2.0 mg) in anhydrous pyridine (0.2 mL) was added $Ac_2O$ (0.5 mL), and the mixture was stirred at 25 °C until the reaction was complete (judged by the disappearance of the starting material by TLC). The reaction mixture was evaporated under reduced pressure and the resulting crude product was purified by RP-HPLC or preparative TLC to yield the corresponding acetyl derivative.

**Preparation of Withanolide Analogues 2c, 21 and 23.** 2,27-Diacetyl-4-*epi*-withaferin A (**2c**) was obtained from withaferin A (**2a**) as described previously.[23] Epoxidation of 15$\alpha$-hydroxyphysachenolide D (**36**) with *m*-chloroperbenzoic acid (*m*-CPBA) afforded 15$\alpha$-hydroxyphysachenolide C (**21**) and 15$\alpha$-hydroxy-5,6-*epi*-physachenolide C (**23**).[24]

**Preparation of 2,3-Dihydro-3$\beta$-azido-4$\beta$-hydroxywithanolide E (6).** To a solution of 4$\beta$-hydroxywithanolide E (**5**, 7.0 mg) in anhydrous $CH_2Cl_2$ (0.8 mL) were added AcOH (19.7 $\mu$L), $Et_3N$ (1.93 $\mu$L), and $Me_3SiN_3$ (45.5 $\mu$L) and stirred at 25 $^oC$. After 20 h, the reaction was quenched with aqueous $NH_4Cl$ and extracted with EtOAc (3 x 10 mL), combined organic layer was washed with brine, dried over $Na_2SO_4$, and evaporated under reduced pressure. The residue thus obtained was purified by silica gel preparative TLC using hexanes/EtOAc (2:8) to give **6** (6.2 mg, 82%, $R_f$ = 0.47) as a white powder; $[\alpha]_D^{25}$ –159 (*c* 0.11, $CHCl_3$); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 4.82 (1H, dd, *J* = 10.0, 6.1 Hz, H-22), 4.00 (1H, dt, *J* = 8.2, 3.9 Hz, H-3), 3.43 (1H, d, *J* = 3.9 Hz, H-4), 3.32 (1H, t, *J* = 1.7 Hz, H-6), 2.91 (1H, dd, *J* = 15.7, 7.3 Hz, Ha-2), 2.69 (1H, ddd, *J* = 14.5, 11.0, 8.5 Hz, Ha-16), 2.62 (1H, dd, *J* = 15.7, 4.2 Hz, Hb-2), 2.51-2.46 (2H, m, $H_2$-23), 2.27 (1H, dt, *J* = 16.9, 4.6 Hz, Ha-12), 2.13 (1H, dd, *J* = 14.5, 10.8 Hz, Ha-7),

2.03 (1H, ddd, $J$ = 14.5, 6.5, 1.7 Hz, Hb-7), 1.91 (3H, s, $H_3$-28), 1.86 (3H, s, $H_3$-27), 183 (1H, m, H-8), 1.72 (1H, dt, $J$ = 11.5, 3.7 Hz, H-9), 1.64 (1H, dt, $J$ = 12.0, 3.7 Hz, Ha-15), 1.56 (1H, dt, $J$ = 12.0, 3.4 Hz, Hb-15), 1.42 (2H, dd, $J$ = 14.5, 8.2 Hz, Ha-11, Hb-16), 1.40 (3H, s, $H_3$-21), 1.35 (1H, dd, $J$ = 15.0, 5.5 Hz, Hb-11), 1.29 (1H, m, Hb-12), 1.26 (3H, s, $H_3$-19), 1.00 (3H, s, $H_3$-18); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 208.1 (C, C-1), 165.8 (C, C-26), 150.6 (C, C-24), 121.5 (C, C-25), 87.7 (C, C-17), 81.5 (C, C-14), 79.5 (CH, C-22), 79.1 (C, C-20), 75.7 (CH, C-4), 64.0 (C, C-5), 60.3, (CH, C-6), 58.7 (C, C-13), 54.5 (CH, C-3), 50.4 (C, C-10), 38.8 ($CH_2$, C-2), 37.9 ($CH_2$, C-16), 35.6 (CH, C-8), 34.2 ($CH_2$, C-23), 33.8 (CH, C-9), 32.2 ($CH_2$, C-15), 29.3 ($CH_2$, C-12), 25.5 ($CH_2$, C-7), 21.0 ($CH_2$, C-11), 20.6 ($CH_3$, C-28), 20.1 ($CH_3$, C-18), 19.7 ($CH_3$, C-21), 15.0 ($CH_3$, C-19), 12.4 ($CH_3$, C-27); HRESI-MS $m/z$ 568.2634 $[M+Na]^+$ (calcd for $C_{28}H_{29}N_3NaO_8$ 568.2634).

**Preparation of 4*β*-Acetoxywithanolide E (7).** Acetylation of withanolide E (**1**) by the general procedure afforded **7**.

*4β-Acetoxywithanolide E (**7**).* White powder; $[\alpha]_D^{25}$ +136 ($c$ 0.1, $CHCl_3$); $^1$H and $^{13}$C NMR data were consistent with those reported.[41]

**Preparation of 4*β*-Methoxycarbonyloxywithanolide E (8).** A solution of **9** (3.0 mg) in MeOH (0.5 mL) was stirred at 25 $^o$C. After 1 h solvent was evaporated under reduced pressure and the crude product was purified by preparative TLC (silica gel) using $CH_2Cl_2$/MeOH (94:6) as eluent to give **8** (1.2 mg, 43%).

*4β-Methoxycarbonyloxywithanolide E (**8**).* White amorphous solid; $[\alpha]_D^{25}$ +108 ($c$ 0.5, $CHCl_3$); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 6.99 (1H, dd, $J$ = 10.1, 6.0 Hz, H-3), 6.27 (1H, d, $J$ = 10.1 Hz, H-2), 4.83 (1H, dd, $J$ = 11.4, 5.5 Hz, H-22), 4.54 (1H, d, $J$ = 6.0 Hz, H-4), 3.74 (3H, s, $OCH_3$), 3.28 (1H, t, $J$ = 1.7 Hz, H-6), 2.69 (1H, ddd, $J$ = 15.0, 11.0, 8.5 Hz, Ha-16), 2.52-2.45 (2H, m, $H_2$-23), 2.21 (1H, m, Ha-12), 2.06-2.01 (2H, m, $H_2$-7), 1.92 (3H, s, $H_3$-28), 1.86 (3H, s,

$H_3$-27), 1.81 (1H, m, H-8), 1.67-1.52 (5H, m, H-9, $H_2$-11, $H_2$-15), 1.41 (dd, *J* = 15.0, 8.5 Hz, 1H, Hb-16), 1.39 (3H, s, $H_3$-21), 1.37 (3H, s, $H_3$-19), 1.29 (1H, brd, *J* = 12.5 Hz, Hb-12), 1.04 (3H, s, $H_3$-18); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 200.6 (C, C-1), 165.7 (C, C-26), 155.0 (C, $OCOOCH_3$), 150.6 (C, C-24), 134. 3 (C, C-3), 135.0 (C, C-2), 121.5 (C, C-25), 87.5 (C, C-17), 81.6 (C, C-14), 79.4 (CH, C-22), 79.1 (C, C-20), 75.4 (CH, C-4), 61.2 (CH, C-6), 61.1 (C, C-5), 55.1 ($CH_3$, $OCH_3$), 54.5 (C, C-13), 48.0 (C, C-10), 37.9 ($CH_2$, C-16), 36.7 (CH, C-8), 34.3 ($CH_2$, C-23), 34.0 (CH, C-9), 32.3 ($CH_2$, C-15), 29.4 ($CH_2$, C-12), 25.6 ($CH_2$, C-7), 20.9 ($CH_2$, C-11), 20.6 ($CH_3$, C-28), 20.2 ($CH_3$, C-18), 19.6 ($CH_3$, C-21), 15.2 ($CH_3$, C-18), 12.4 ($CH_3$, C-27); HRESI-MS (*m*/*z*) 583.2514 $[M+Na]^+$ (calcd for $C_{30}H_{40}NaO_{10}$ 583.2514).

**Preparation of 4*β*-Imidazolylcarbonyloxywithanolide E (9).** To a solution of 4*β*-hydroxywithanolide E (**5**, 10.0 mg) in anhydrous $CH_2Cl_2$ (1.0 mL) was added 1,1'-carbonyldiimidazole (8.0 mg) and stirred at 25 $^{o}$C. After 1 h, reaction mixture was filtered through a short bed of silica gel (0.5 mg) using EtOAc as eluent to give **9** (8.1 mg, 68%).

*4β-Imidazolylcarbonyloxywithanolide E (**9**).* White amorphous solid; $[\alpha]_D^{25}$ +108 (*c* 0.5, $CHCl_3$); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 8.04 (1H, s, N-CH=N), 7.31 (1H, t, *J* = 1.6 Hz, N-CH), 7.04 (1H, m, N-CH), 7.04 (1H, dd, *J* = 9.8, 6.1 Hz, H-3), 6.37 (1H, d, *J* = 9.8 Hz, H-2), 4.89 (1H, d, *J* = 6.1 Hz, H-4), 4.83 (1H, dd, *J* = 11.5, 5.5 Hz, H-22), 3.37 (1H, t, *J* = 2.0 Hz, H-6), 2.69 (1H, ddd, *J* = 15.0, 11.0, 8.5 Hz, Ha-16), 2.54–2.42 (2H, m, $H_2$-23), 2.24 (1H, dt, *J* = 11.8, 5.7 Hz, Ha-12), 2.13–2.05 (2H, m, $H_2$-7), 1.92 (3H, s, $H_3$-28), 1.86 (3H, s, $H_3$-27), 1.81 (1H, dd, *J* = 11.0, 6.3 Hz, H-8), 1.70–1.52 (5H, m, H-9, $H_2$-11, $H_2$-15), 1.43 (1H, m, Hb-16), 1.42 (3H, s, $H_3$-19), 1.39 (3H, s, $H_3$-21), 1.29 (1H, brd, *J* = 11.8 Hz, Hb-12), 1.05 (3H, s, $H_3$-18); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 200.1 (C, C-1), 165.7 (C, C-26), 150.6 (C, C-24), 147.9 (C, NC=O), 137.1 (CH, N-CH=N), 136.9 (CH, C-3), 136.0 (CH, C-2), 131.0 (CH, N-CH), 121.5 (C,

C-25), 117.1 (CH, N-CH), 87.6 (C, C-17), 81.6 (C, C-14), 79.4 (CH, C-22), 79.1 (C, C-20), 76.1 (CH, C-4), 61.5 (CH, C-6), 60.9 (C, C-5), 54.5 (C, C-13), 48.0 (C, C-10), 37.8 ($CH_2$, C-16), 36.8 (CH, C-9), 34.3 ($CH_2$, C-23), 33.9 (CH, C-8), 32.3 ($CH_2$, C-15), 29.4 ($CH_2$, C-12), 25.6 ($CH_2$, C-7), 20.9 ($CH_2$, C-11), 20.7 ($CH_3$, C-28), 20.2 ($CH_3$, C-18), 19.7 ($CH_3$, C-21), 15.4 ($CH_3$, C-19), 12.4 ($CH_3$, C-27); APCI-MS (+) *m*/*z* 597 $[M+1]^+$; APCI-MS (–) *m*/*z* 595 $[M–1]^–$; HRESI-MS *m*/*z* 619.2624 $[M+Na]^+$ (calcd for $C_{32}H_{40}N_2NaO_9$ 619.2632).

**Oxidation of 4$\beta$-Hydroxywithanolide E (5) to Withaperuvin E (10)**. To a solution **5** (10.0 mg) in $CHCl_3$/EtOAc (1:1, 3.0 mL) was added activated $MnO_2$ (60.0 mg) and stirred at 25 $^oC$. After 6 h, reaction mixture was passed through a short column of silica gel (1.0 g) using $CH_2Cl_2$/MeOH (94:6) as eluent to give **10** (8.1 mg, 81%) as a pale yellow solid; $[\alpha]_D^{25}$ –74 (*c* 0.5, $CH_3CN$) [lit.[42] –72.7 (c 0.7, $CH_3CN$]; $^1H$ and $^{13}C$ NMR data were consistent with those reported.[42]

**Reduction of Withaperuvin E (10) to 4$\alpha$-Hydroxywithanolide E (11).** To stirred solution of **10** (4.0 mg) in MeOH (1.0 mL) was added $CeCl_3.7H_2O$ (30.0 mg). The reaction mixture was cooled to 0 $^oC$ and stirred for 5 min. To this mixture was added $NaBH_4$ (ca 0.5 mg) and stirred at 0 $^oC$. After 5 min, the reaction mixture was quenched with ice, evaporated under reduced pressure and extracted with EtOAc (3 x 5 mL). Combined organic layer was washed with brine, dried over anhydrous $Na_2SO_4$ and evaporated under reduced pressure and the resulting residue was chromatographed over a column of silica gel (500.0 mg) made up in $CH_2Cl_2$ and eluted with $CH_2Cl_2$ containing increasing amounts of MeOH. Fractions eluted with $CH_2Cl_2$/MeOH (94:6) were combined and evaporated under reduced pressure and the crude product was further purified by RP-HPLC using MeOH/$H_2O$ (70:30) as eluent to give **11** (3.0 mg, 75%, $t_R$ = 15 min).

*4α-Hydroxywithanolide E (**11**).* White amorphous solid; $[\alpha]_D^{25}$ +65 (*c* 0.2, $CHCl_3$); $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 6.78 (1H, dd, *J* = 10.1, 1.7 Hz, H-3), 5.99 (1H, dd, *J* = 10.1, 2.6 Hz, H-2), 4.84 (1H, dd, *J* = 11.2, 5.6, Hz, H-22), 4.65 (1H, brs, H-4), 3.71 (1H, brt, *J* = 1.9 Hz, H-6), 2.69 (1H, ddd, *J* = 15.1, 11.0, 8.0 Hz, Ha-16), 2.52–2.42 (2H, m, $H_2$-23), 2.24 (1H, dt, *J* = 12.5, 4.9 Hz, Ha-12), 2.13 (1H, d, *J* = 2.8 Hz, $D_2O$ exchangeable, OH-4), 1.98 (2H, m, $H_2$-7), 1.91 (3H, s, $H_3$-28), 1.85 (3H, s, $H_3$-27), 1.83 (2H, m, H-8, Ha-11), 1.69–1.53 (4H, m, H-9, Hb-11, $H_2$-15), 1.42 (1H, dd, *J* = 15.1, 8.3 Hz, Hb-16), 1.39 (3H, s, $H_3$-21), 1.27 (1H, brd, *J* = 12.5 Hz, Hb-12), 1.20 (3H, s, $H_3$-19), 1.06 (3H, s, $H_3$-18); $^{13}C$ NMR (100 MHz, $CDCl_3$) $\delta$ 200.6 (C, C-1), 165.7 (C, C-26), 150.5 (C, C-24), 146.9 (CH, C-3), 128.7 (CH, C-2), 121.5 (C, C-25), 87.6 (C, C-17), 81.8 (C, C-14), 79.5 (CH, C-22), 79.1 (C, C-20), 65.6 (C, C-5), 64.7 (CH, C-4), 56.2 (CH, C-6), 54.5 (C, C-13), 47.5 (C, C-10), 37.9 ($CH_2$, C-16), 37.8 (CH, C-9), 34.2 ($CH_2$, C-23), 34.1 (CH, C-8), 32.3 ($CH_2$, C-15), 29.7 ($CH_2$, C-12), 25.6 ($CH_2$, C-7), 21.6 ($CH_2$, C-11), 20.6 ($CH_3$, C-28), 20.4 ($CH_3$, C-18), 19.7 ($CH_3$, C-21), 13.2 ($CH_3$, C-19), 12.4 ($CH_3$, C-27); HRESI-MS *m*/*z* 525.2463 $[M+Na]^+$ (calcd for $C_{28}H_{38}NaO_8$ 525.2463).

*4α-Acetoxywithanolide E (**12**).* Acetylation of **11** by the general procedure afforded **12** as an amorphous white solid; $[\alpha]_D^{25}$ +100 (*c* 0.1, $CHCl_3$); $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 6.62 (1H, dd, *J* = 10.3, 1.9 Hz, H-3), 6.05 (1H, dd, *J* = 10.3, 2.8 Hz, H-2), 5.85 (dd, *J* = 2.8, 1.9 Hz. 1H, H-4), 4.85 (1H, dd, *J* = 11.4, 5.1 Hz, H-22), 3.58 (1H, brs, H-6), 2.69 (1H, dt, *J* = 12.8, 10.6 Hz, Ha-16 ), 2.54–2.42 (2H, m, $H_2$-23), 2.25 (1H, dt, *J* = 13.0, 5.0 Hz, Ha-12), 2.09 (3H, s, OAc), 2.03–1.93 (2H, m), 1.92 (3H, s, $H_3$-28), 1.90–1.82 (2H, m), 1.86 (3H, s, $H_3$-27), 1.73 (1H, m), 1.67–1.53 (3H, m), 1.41 (1H, dd, *J* = 15.1, 8.3 Hz, Hb-16), 1.39 (3H, s, $H_3$-21), 1.28 (1H, m, Hb-12), 1.27 (3H, s, $H_3$-19), 1.06 (3H, s, $H_3$-18); $^{13}C$ NMR (100 MHz, $CDCl_3$) $\delta$ 200.3 (C, C-1), 169.8 (C, OAc), 165.7 (C, C-26), 150.3 (C, C-24), 144.4 (CH, C-3), 129.6 (CH, C-2), 121.5 (C, C-25), 87.6 (C, C-17), 81.7 (C, C-14), 79.4 (CH, C-22), 79.1 (C, C-20), 65.6 (C, C-5), 63.2 (CH,

C-4), 56.5 (CH, C-6), 54.5 (C, C-13), 47.9 (C, C-10), 37.8 ($CH_2$, C-16), 37.7 (CH, C-9), 34.3 ($CH_2$, C-23), 34.0 (CH, C-8), 32.4 ($CH_2$, C-15), 29.8 ($CH_2$, C-12), 25.5 ($CH_2$, C-7), 21.9 ($CH_2$, C-11), 20.8 ($CH_3$, C-28), 20.6 ($CH_3$, C-18), 20.4 ($CH_3$, OAc), 19.7 ($CH_3$, C-21), 13.9 ($CH_3$, C-19), 12.4 ($CH_3$, C-27); HRESI-MS *m*/*z* 567.2565 $[M+Na]^+$ (calcd for $C_{30}H_{40}NaO_9$ 567.2563).

**Conversion of Withaperuvin E (10) to Withaperuvin M (13)**. To a stirred solution **10** (23.0 mg) in anhydrous $CH_2Cl_2$ (2.0 mL) at 0 $^oC$ was added a solution of $Ph_3P$ (14.6 mg) and $I_2$ (14.0 mg) in $CH_2Cl_2$ (0.3mL). Ice bath was removed and reaction mixture was stirred at 25 $^oC$. The reaction was monitored by TLC and after disappearance of the starting material, the reaction mixture was diluted with $CH_2Cl_2$ (25.0 mL), washed with aq. $Na_2S_2O_3$ solution, $H_2O$, dried over anhydrous $Na_2SO_4$ and evaporated under reduced pressure to give crude product mixture (35.0 mg). This was separated by RP-HPLC using MeOH/$H_2O$ (65:35) to give **13 (**9.8 mg, 44%). $^1H$ and $^{13}C$ NMR data were consistent with those reported;[43] APCI-MS (+) (*m*/*z*) 485 $[M+1]^+$.

**Conversion of Withaperuvin M (13) to *4-Epi*-physapruin A (14).** To a solution of **13** (9.5 mg) in MeOH (1.0 mL) and THF (0.2 mL) at 0 $^oC$ was added $CeCl_3.7H_2O$ (100.0 mg) and stirred at 0 $^oC$ for 5 min. To this solution was added $NaBH_4$ (ca 0.5 mg) and continued stirring at 0 $^oC$. After 5 min, the reaction mixture was quenched with ice, solvents were evaporated under reduced pressure and extracted with EtOAc (3 x 5.0 mL). Combined organic layer was washed with brine, dried over anhydrous $Na_2SO_4$ and evaporated under reduced pressure to give the crude product (9.8 mg) which was purified by silica gel preparative TLC using $CH_2Cl_2$/MeOH (93:7, double elution) to give **14** (5.8 mg, 59%, $R_f$ = 0.34).

*4-Epi-physapruin A (**14**).* White amorphous solid; $[\alpha]_D^{25}$ +118 (*c* 0.2, $CHCl_3$); $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 6.72 (1H, dd, *J* = 10.5, 2.1 Hz, H-3), 6.01 (1H, dt, *J* = 6.3, 2.1 Hz, H-6), 5.80 (1H, dd, *J* = 10.5, 2.5 Hz, H-2), 4.91 (1H, m, H-4), 4.87 (1H, dd, *J* = 11.6, 5.2 Hz, H-22),

2.67 (1H, ddd, $J$ = 14.5, 11.0, 8.5 Hz, Ha-16), 2.50-2.45 (2H, m, $H_2$-23), 2.42 (1H, m, Ha-12), 2.21 (1H, m, Ha-11), 2.28 (1H, m, H-8), 2.14 (1H, m, Ha-7), 1.92 (1H, m, Hb-7), 1.90 (3H, s, $H_3$-28), 1.83 (3H, s, $H_3$-27), 1.77 (1H, dt, $J$ = 11.2, 5.6 Hz, H-9), 1.66 (1H, m, Ha-15), 1.58 (1H, m, Hb-11), 1.55 (1H, m, Hb-15), 1.40 (1H, dd, $J$ = 14.5, 7.8 Hz, Hb-16), 1.35 (3H, s, $H_3$-21), 1.28 (1H, brd, $J$ = 14.5 Hz, Hb-12), 1.21 (3H, s, $H_3$-19), 1.08 (3H, s, $H_3$-18); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 204.2 (C, C-1), 151.2 (C, C-24), 148.6 (CH, C-3), 139.6 (C, C-5), 127.6 (CH, C-2), 121.8 (CH, C-6), 121.3 (C, C-25), 87.8 (C, C-17), 82.1 (C, C-14), 80.6 (CH, C-22), 78.6 (C, C-20), 66.9 (CH, C-4), 54.4 (C, C-13), 50.8 (C, C-10), 37.6 ($CH_2$, C-16), 36.7 (CH, C-9), 36.2 (CH, C-8), 34.3 ($CH_2$, C-23), 32.4 ($CH_2$, C-15), 30.3 ($CH_2$, C-12), 25.2 ($CH_2$, C-7), 22.6 ($CH_2$, C-11), 20.6 ($CH_3$, C-28), 20.5 ($CH_3$, C-18), 19.5 ($CH_3$, C-21), 19.2 ($CH_3$, C-19), 12.2 ($CH_3$, C-27); HRESI-MS $m/z$ 509.2500 $[M+Na]^+$ (calcd for $C_{28}H_{38}NaO_7$ 509.2515).

**Conversion of Physachenolide C (3) to 2,3-Dihydrophysachenolide C (15) and 18-Deacetyl-2,3-dihydrophysachenolide C (24).** To a solution of **3** (5.0 mg) in EtOH (2.0 mL) and $Et_3N$ (20 μL) was added 10% Pd/C (0.5 mg) and the mixture was stirred under an atmosphere of $H_2$ for 7 h. The reaction mixture was filtered, and the filtrate was concentrated under reduced pressure to give the product, which was separated by silica gel preparative TLC $CHCl_3$/MeOH (95:5) to afford **15** (2.6 mg) and **24** (1.5 mg).

*2,3-Dihydrophysachenolide C (**15**).* Off-white amorphous powder; $[\alpha]_D^{25}$ +3 ($c$ 0.1, MeOH); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 4.83 (1H, dd, $J$ = 8.8, 8.0 Hz, H-22), 4.34 (1H, d, $J$ = 11.6 Hz, Ha-18), 4.27 (1H, d, $J$ = 11.6 Hz, Hb-18), 3.17 (1H, brs, H-6$\alpha$), 2.05 (3H, s, 18-OAc), 1.91 (3H, s, $CH_3$-28), 1.87 (3H, s, $CH_3$-27), 1.39 (3H, s, $CH_3$-21), 1.12 (3H, s, $CH_3$-19); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 213.2 (C, C-1), 169.9 (C, 18-OAc), 165.5 (C, C-26), 149.8 (C, C-24), 121.9 (C, C-25), 87.9 (C, C-17), 81.2 (C, C-14), 79.3 (CH, C-22), 78.9 (C, C-20), 64.8 ($CH_2$, C-18), 64.3 (C, C-5), 60.9 (CH, C-

6), 57.6 (C, C-13), 52.4 (C, C-10), 38.2 ($CH_2$, C-16), 35.6 (CH, C-9), 34.7 ($CH_2$, C-2), 33.9 ($CH_2$, C-23), 33.7 (CH, C-8), 32.9 ($CH_2$, C-15), 30.0 ($CH_2$, C-4), 26.6 ($CH_2$, C-7), 25.2 ($CH_2$, C-12), 21.4 ($CH_2$, C-11), 21.3 ($CH_3$, 18-OAc), 20.7 ($CH_3$, C-28), 19.1 ($CH_3$, C-21), 17.8 ($CH_2$, C-3), 12.5 ($CH_3$, C-19), 12.4 ($CH_3$, C-27); APCI-MS (+) *m/z* 529 $[MH–H_2O]^+$.

*18-Deacetyl-2,3-dihydrophysachenolide C (**24**).* Off-white amorphous powder; $[\alpha]_D^{25}$ –20 (*c* 0.04, MeOH); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 4.85 (1H, dd, *J* = 12.8, 4.0 Hz, H-22), 3.90 (1H, d, *J* = 10.8 Hz, Ha-18), 3.81 (1H, d, *J* = 10.8 Hz, Hb-18), 3.17 (1H, brs, H-6$\alpha$), 1.91 (3H, s, $CH_3$-28), 1.86 (3H, s, $CH_3$-27), 1.39 (3H, s, $CH_3$-21), 1.12 (3H, s, $CH_3$-19); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 213.1 (C, C-1), 165.6 (C, C-26), 150.2 (C, C-24), 121.6 (C, C-25), 89.0 (C, C-17), 80.9 (C, C-14), 79.0 (CH, C-22), 78.8 (C, C-20), 64.4 (C, C-5), 63.4 ($CH_2$, C-18), 60.9 (CH, C-6), 58.7 (C, C-13), 52.5 (C, C-10), 37.5 ($CH_2$, C-16), 35.6 (CH, C-9), 34.6 ($CH_2$, C-2), 34.1 ($CH_2$, C-23), 33.4 (CH, C-8), 33.1 ($CH_2$, C-15), 30.0 ($CH_2$, C-4), 26.5 ($CH_2$, C-7), 24.1 ($CH_2$, C-12), 21.3 ($CH_2$, C-11), 20.7 ($CH_3$, C-28), 19.0 ($CH_3$, C-21), 17.7 ($CH_2$, C-3), 12.5 ($CH_3$, C-19), 12.4 ($CH_3$, C-27); APCI-MS (+) *m/z* 527 $[M+Na]^+$.

**Preparation of 2,3-Dihydro-3*β*-azidophysachenolide C (16).** To a solution of **3** (7.0 mg) in anhydrous $CH_2Cl_2$ (0.8 mL) were added AcOH (13.35 µL), $Et_3N$ (1.82 µL), and TMS-$N_3$ (30.8 µL) and stirred at 25 $^o$C. After 20 h, the reaction mixture was quenched with aqueous $NH_4Cl$ and extracted with EtOAc (3 x 10 mL). Combined organic layer was washed with brine, dried over $Na_2SO_4$, and evaporated under reduced pressure and the resulting residue was purified by silica gel preparative TLC using hexanes/EtOAc (2:8) as eluent to afford 1**6** (3.1 mg, 67%, $R_f$ = 0.58) and unreacted **3** (2.7 mg).

*2,3-Dihydro-3β-azidophysachenolide C (**16**).* White powder; $[\alpha]_D^{25}$ –145 (*c* 0.11, $CHCl_3$); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 4.83 (1H, t, *J* = 9.1 Hz, H-22), 4.34 (1H, d, *J* = 11.6 Hz,

Ha-18), 4.23 (1H, d, *J* = 11.6 Hz, Hb-18), 4.03 (1H, dt, *J* = 7.5, 6.6 Hz, H-3), 3.30 (1H, brs, H-6), 2.76 (1H, dd, *J* = 14.1, 6.2 Hz, Ha-2), 2.73 (1H, dd, *J* = 14.1, 7.6 Hz, Hb-2), 2.70 (1H, m, Ha-16), 2.49 (2H, brd, *J* = 9.2 Hz, $H_2$-23), 2.28 (1H, dd, *J* = 14.6, 5.8 Hz, Ha-4), 2.20 (1H, dd, *J* = 10.1, 5.4 Hz, Ha-7), 2.13 (1H, d, *J* = 11.6, Ha-12), 2.06 (3H, s, OAc), 2.04 (1H, d, *J* = 11.6, Hb-12), 1.93 (1H, m, H-9), 1.91 (3H, s, $H_3$-28), 1.87 (3H, s, $H_3$-27), 1.74 (1H, dd, *J* = 10.9, 4.1 Hz, H-8), 1.68 (2H, m, Hb-7, Ha-15), 1.59-1.50 (2H, m, Hb-15, Hb-16), 1.44 (1H, dd, *J* = 14.6, 2.2 Hz, Hb-4), 1.39 (3H, s, $H_3$-21), 1.37 (1H, m, Ha-11), 1.26 (1H, m, Hb-11), 1.13 (3H, s, $H_3$-19); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 209.2 (C, C-1), 170.0 (C, OAc), 165.5 (C, C-26), 149.7 (C, C-24), 121.9 (C, C-25), 87.9 (C, C-17), 81.1 (C, C-14), 79.3 (CH, C-22), 78.9 (C, C-20), 64.8 ($CH_2$, C-18), 61.6 (C, C-5), 61.4 (CH, C-6), 57.5 (C, C-13), 53.8 (CH, C-3), 52.1 (C, C-10), 41.4 ($CH_2$, C-2), 38.2 4 ($CH_2$, C-16), 36.3 ($CH_2$, C-4), 35.4 (CH, C-9), 33.9 ($CH_2$, C-23), 33.6 (CH, C-8), 32.9 ($CH_2$, C-15), 26.1 ($CH_2$, C-12), 25.2 ($CH_2$, C-6), 21.5 ($CH_2$, C-11), 21.3 ($CH_3$, OAc), 20.7 ($CH_3$, C-28), 19.1 ($CH_3$, C-21), 13.1 ($CH_3$, C-19), 12.4 ($CH_3$, C-27); HRESI-MS *m*/*z* 610.2735 $[M+Na]^+$ (calcd for $C_{30}H_{41}N_3NaO_9$ 610.2740).

**Preparation of 2,3-Dihydro-3*β*-imidazolylphysachenolide C (17).** To a solution of **3** (5.0 mg) in anhydrous $CH_2Cl_2$ (1.0 mL) was added imidazole (8.0 mg) and stirred at 25 $^{o}$C. After 6 h, reaction mixture was diluted with EtOAc (15.0 mL), washed with brine (3 x 10.0 mL), dried over anhydrous $Na_2SO_4$ and evaporated under reduced pressure. The resulting residue was then passed through a short column of Sephadex LH-20 (0.5 g) made up in hexanes/$CH_2Cl_2$ (1:4) and eluted with hexanes/$CH_2Cl_2$ (1:4) followed by $CH_2Cl_2$/acetone (3:2) to give **17** (4.3 mg, 76%).

*2,3-Dihydro-3β-imidazolylphysachenolide C (**17**).* While amorphous solid; $[\alpha]_D^{25}$ –128 (*c* 0.2, $CHCl_3$); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 7.63 (1H, s, NCHN), 7.08 (1H, s, NCH), 6.93 (1H, s, NCH), 4.82 (1H, dd, *J* = 10.3, 6.3 Hz, H-22), 4.68 (1H, m, H-3), 4.37 (1H, d, *J* =

11.4 Hz, Ha-18), 4.24 (1H, d, *J* = 11.4 Hz, Hb-18), 3.16 (1H, dd, *J* = 14.0, 8.8 Hz, Ha-2), 3.10 (1H, brs, H-6), 2.88 (1H, dd, *J* = 14.0, 8.1 Hz, Hb-2), 2.71 (1H, dt, *J* = 17.6, 3.2 Hz, Ha-16), 2.55 (1H, dd, *J* = 15.4, 6.6 Hz, Ha-4), 2.52-2.48 (2H, m, $H_2$-23), 2.27 (1H, dd, *J* = 16.4, 3.7 Hz, Ha-7), 2.13 (1H, dd, *J* = 12.3, 6.3 Hz, Ha-12), 2.07 (3H, s, OAc), 2.04 (1H, m, Hb-12), 1.96 (1H, m, H-9), 1.91 (3H, s, $H_3$-28), 1.86 (3H, s, $H_3$-27), 1.78 (1H, dd, *J* = 11.2, 4.1 Hz, H-8), 1.76 (1H, dd, *J* = 11.2, 4.1 Hz, Hb-7), 1.72 (1H, m, Ha-15), 1.65 (1H, m, Hb-15), 1.58 (1H, dd, *J* = 17.6, 2.0 Hz, Hb-16), 1.40 (3H, s, $H_3$-21), 1.34-1.24 (2H, m, $H_2$-11), 1.19 (3H, s, $H_3$-19); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 208.8 (C, C-1), 170.0 (C, OAc), 165.7 (C, C-26), 149.8 (C, C-24), 136.1 (CH, NCN), 130.2 (CH, NCH), 121.9 (C, C-25), 116.8 (C, NC), 88.0 (C, C-17), 81.1 (C, C-14), 79.5 (CH, C-22), 78.6 (C, C-20), 64.7 ($CH_2$- C-18), 61.5 (C, C-5), 61.2 (CH, C-6), 57.5 (C, C-13), 52.0 (C, C-10), 49.2 (CH, C-3), 42.1 ($CH_2$, C-2), 38.3 ($CH_2$, C-4), 38.1 ($CH_2$, C-16), 35.6 (CH, C-9), 33.9 ($CH_2$, C-23), 33.5 (CH, C-8), 32.9 ($CH_2$, C-15), 26.2 ($CH_2$, C-12), 25.2 ($CH_2$, C-7), 21.5 ($CH_2$, C-11), 21.4 ($CH_3$, OAc), 20.7 ($CH_3$, C-28), 19.1 ($CH_3$, C-21), 13.1 ($CH_3$, C-19), 12.4 ($CH_3$, C-27); HRESI-MS *m*/*z* 635.2940 $[M+Na]^+$ (calcd for $C_{33}H_{44}N_2NaO_9$ 635.2945).

**Preparation of 2,3-Dihydro-3*β*-methoxyphysachenolide C (18).** To a solution of **17** (2.8 mg) in MeOH (0.5 mL) was added $Et_3N$ (5.0 $\mu$L) and stirred at 60 $^{o}$C. After 1h reaction mixture was evaporated under reduced pressure and the resulting residue was purified by RP-HPLC using MeOH/$H_2O$ (1:1) as eluent and the product was acetylated by the general procedure to give **18** (1.1 mg, 42%).

*2,3-Dihydro-3β-methoxyphysachenolide C (**18**).* White amorphous solid; $[\alpha]_D^{25}$ –142 (*c* 0.1, $CHCl_3$); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 4.82 (1H, dd, *J* = 10.3, 6.3 Hz, H-22), 4.34 (1H, d, *J* = 11.8 Hz, Ha-18), 4.25 (1H, d, *J* = 11.8 Hz, Hb-18), 3.70 (1H, m, H-3), 3.27 (1H, brs, H-6), 3.25 (3H, s, $OCH_3$), 2.79 (1H, dd, *J* = 13.6, 5.6 Hz, Ha-2), 2.71 (1H, dd, *J* = 13.6, 8.1 Hz, Hb-2),

2.67 (1H, m, Ha-16), 2.49 (2H, brd, $J$ = 8.5 Hz, $H_2$-23), 2.20 (1H, m, Ha-7), 2.15 (1H, ddd, $J$ = 14.4, 5.6, 1.8 Hz, Ha-4), 2.10 (1H, m, Ha-12), 2.06 (3H, s, OAc), 2.00 (1H, dt, $J$ = 14.8, 3.2 Hz, Hb-12), 1.92 (1H, m, H-9), 1.91 (3H, s, $H_3$-28), 1.87 (3H, s, $H_3$-27), 1.75 (1H, dt, $J$ = 11.1, 4.2 Hz, H-8), 1.67 (1H, m, Hb-7), 1.60 (1H, dd, $J$ = 12.2, 3.7 Hz, Ha-15), 1.57 (1H, dd, $J$ = 12.2, 8.5 Hz, Hb-15), 1.50 (1H, m, b-16), 1.39 (3H, s, $H_3$-21), 1.30-1.27 (2H, m, $H_2$-11), 1.12 (3H, s, $H_3$-19); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 210.7 (C, C-1), 169.9 (C, OAc), 165.2 (C, C-26), 149.6 (C, C-24), 121.9 (C, C-25), 87.9 (C, C-17), 81.2 (C, C-14), 79.2 (CH, C-22), 78.9 (C, C-20), 72.5 (CH, C-3), 64.8 ($CH_2$, C-18), 62.1 (C, C-5), 61.5 (CH, C-6), 57.6 (C, C-13), 56.0 ($CH_3$, $OCH_3$), 52.0 (C, C-10), 42.7 ($CH_2$, C-2), 38.2 ($CH_2$, C-16), 36.4 ($CH_2$, C-4), 35.4 (CH, C-9), 33.9 ($CH_2$, C-23), 33.6 (CH, C-8), 32.9 ($CH_2$, C-15), 26.3 ($CH_2$, C-12), 25.2 ($CH_2$, C-7), 21.5 ($CH_2$, C-11), 21.3 ($CH_3$, OAc), 20.7 ($CH_3$, C-28), 19.2 ($CH_3$, C-21), 13.0 ($CH_3$, C-19), 12.4 ($CH_3$, C-27); HRESI-MS $m/z$ 599.2829 $[M+Na]^+$ (calcd for $C_{31}H_{44}NaO_{10}$ 599.2832).

**Epoxidation of 15*α*-Hydroxyphysachenolide D**. To a solution of 15*α*-hydroxyphysachenolide D (**36**)[20] (5.5 mg) in anhydrous $CH_2Cl_2$ (0.5 mL) at 0 $^o$C was added *m*-CPBA (4.8 mg) and stirred for 30 min. Ice bath was then removed and reaction mixture was stirred at 25 $^o$C for additional 2 h, after which $CH_2Cl_2$ was evaporated under reduced pressure and the resulting residue was separated by silica gel preparative TLC using $CH_2Cl_2$/MeOH (94:6, double elution) as eluent to give **21** (2.3 mg, 41%, $R_f$ = 0.62) and **23** (1.3 mg, 23%, $R_f$ = 0.54).

*15α-Hydroxyphysachenolide C (**21**)*. White amorphous solid; $[\alpha]_D^{25}$ +68 ($c$ 0.2, $CHCl_3$); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 6.66 (1H, ddd, $J$ = 10.1, 4.9, 2.3 Hz, H-3), 5.86 (1H, dd, $J$ = 10.1, 2.8 Hz, H-2), 4.80 (1H, brd, $J$ = 12.0 Hz, H-22), 4.43 (1H, d, $J$ = 11.3 Hz, Ha-18), 4.10 (1H, d, $J$ = 11.3 Hz, Hb-18), 3.92 (1H, t, $J$ = 8.5 Hz, H-15), 3.03 (1H, dt, $J$ = 14.5, 2.9 Hz, Ha-4), 3.02 (1H, t, $J$ = 2.8 Hz, H-6), 2.54 (1H, brd, $J$ = 18.0 Hz, Ha-23), 2.39 (1H, m, Hb-23), 2.38

(1H, m, Ha-11), 2.29 (1H, m, Ha-12), 2.27 (1H, m, Ha-16), 2.25 (1H, m, H-9), 2.08 (2H, m, $H_2$-7), 2.03 (3H, s, OAc), 1.91 (1H, dd, $J$ = 15.5, 8.5 Hz, Hb-16), 1.87 (3H, s, $H_3$-28), 1.81 (3H, s, $H_3$-27), 1.77 (1H, dd, $J$ = 14.5, 4.9 Hz, Hb-4), 1.75 (1H, m, H-8), 1.61 (1H, brd, $J$ = 11.7 Hz, Hb-12), 1.29 (3H, s, $H_3$-19), 1.28 (3H, s, $H_3$-21), 1.17 (1H, ddd, $J$ = 18.7, 13.2, 5.5 Hz, Hb-11); $^{13}$C NMR (100 MHz, $CDCl_3$) 203.0 (C, C-1), 170 (C, $OCOCH_3$), 167.2 (C, C-26), 150.9 (C, C-24), 142.6 (CH, C-3), 128.6 (CH, C-2), 121.4 (C, C-25), 84.6 (C, C-20), 80.7 (C, C-14), 79.1 (CH, C-22), 78.4 (C, C-17), 74.0 (CH, C-15), 64.7 (C, C-5), 64.5 ($CH_2$, C-18), 58.9 (C, C-13), 56.8 (CH, C-6), 48.6 (C, C-10), 47.3 (C, C-16), 35.5 (CH, C-8), 33.9 ($CH_2$, C-23), 33.8 ($CH_2$, C-4), 31.8 (CH, C-9), 25.5 ($CH_2$, C-12), 23.9 ($CH_2$, C-7), 21.9 ($CH_2$, C-11), 21.2 ($CH_3$, OAc), 20.6 ($CH_3$, C-28), 15.2 ($CH_3$, C-19), 12.1 ($CH_3$, C-27); APCI-MS (+) *m/z* 561 $[M+H]^+$; APCI-MS (–): *m/z* 559 $[M–H]^-$; HRESIMS *m/z* 583.2541 $[M+Na]^+$ (calcd for $C_{30}H_{40}NaO_{10}$ 583.2519).

*15α-Hydroxy-5,6-epi-physachenolide C (**23**)*. White amorphous solid; $[\alpha]_D^{25}$ +73 (*c* 0.1, $CHCl_3$); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 6.81 (1H, ddd, $J$ = 10.0, 6.1, 2.4 Hz, H-3), 6.00 (1H, dd, $J$ = 10.0, 2.7 Hz, H-2), 4.86 (1H, t, $J$ = 8.5 Hz, H-22), 4.47 (1H, d, $J$ = 11.1 Hz, Ha-18), 4.25 (1H, d, $J$ = 11.1 Hz, Hb-18), 4.09 (1H, m, H-15), 3.14 (1H, d, $J$ = 2.8 Hz, H-6), 2.93 (1H, dt, $J$ = 18.5, 3.2 Hz, Ha-4), 2.49 (2H, m, $H_2$-23), 2.47 (1H, m, Ha-12), 2.36 (1H, m, Ha-16), 2.30 (1H, m, Ha-7), 2.12 (1H, m, Ha-11), 2.10 (3H, s, OAc), 2.00 (1H, m, Hb-16), 1.96 (1H, m, H-9), 1.93 (1H, m, H-8), 1.92 (3H, s, $H_3$-28), 1.87 (3H, s, $H_3$-27), 1.86 (1H, m, Hb-4), 1.85 (1H, m, Hb-7), 1.65 (1H, m, Hb-12), 1.45 (1H, m, Hb-11), 1.36 (3H, s, $H_3$-21), 1.22 (3H, s, $H_3$-19); $^{13}$C NMR (100 MHz, $CDCl_3$) 203.1 (C, C-1), 170 (C, OAc), 167.2 (C, C-26), 150.9 (C, C-24), 144.2 (CH, C-3), 129.4 (CH, C-2), 121.8 (C, C-25), 84.8 (C, C-20), 80.1 (C, C-14), 79.2 (CH, C-22), 77.7 (C, C-17), 73.7 (CH, C-15), 65.3 ($CH_2$, C-18), 61.6 (C, C-5), 63.9 (CH, C-6), 57.2 (C, C-13), 48.6 (C, C-10), 48.3 (C, C-16), 36.8 (CH, C-8), 34.6 (CH, C-9), 34.0 ($CH_2$, C-23), 32.8 ($CH_2$, C-4), 26.3 ($CH_2$, C-12), 26.0 ($CH_2$, C-7), 22.9 ($CH_2$, C-11), 21.3 ($CH_3$, OAc), 20.7 ($CH_3$, C-28),

14.9 ($CH_3$, C-19), 12.3 ($CH_3$, C-27); APCI-MS (+) *m/z* 561 $[M+H]^+$; APCI-MS (–) *m/z* 559 $[M–H]^-$; HRESI-MS *m/z* 583.2525 $[M+Na]^+$ (calcd for $C_{30}H_{40}NaO_{10}$ 583.2519).

**Epoxidation of 15*α*-Acetoxyphysachenolide C (28).** To a stirred solution of **28** (10.0 mg) in $CHCl_3$ (1.0 mL) was added *m*-CPBA (6.0 mg), and the mixture was stirred at 25 $^oC$ for 4 h (TLC control). The reaction mixture was concentrated under reduced pressure and the crude product was purified by silica gel preparative TLC using $CHCl_3$/MeOH (95:5) as eluent to afford epoxides **20** (3.8 mg) and **22** (2.6 mg) of which **20** was identified as 15*α*-acetoxyphysachenolide C by comparison of its NMR and MS data with those reported.[20]

*15α-Acetoxy-5,6-epi-physachenolide C (**22**).* Off-white amorphous powder; $[\alpha]_D^{25}$ +83 (*c* 0.11, MeOH); $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 6.68 (1H, ddd, *J* = 10.0, 4.8, 2.0 Hz, H-3), 5.91 (1H, dd, *J* = 10.0, 2.0 Hz, H-2), 5.15 (1H, dd, *J* = 9.2, 8.8 Hz, H-15*β*), 4.84 (1H, m, H-22), 4.66 (1H, d, *J* = 11.6 Hz, H-18), 4.18 (1H, d, *J* = 11.6 Hz, H-18), 3.05 (1H, dt, *J* = 19.6, 2.4 Hz,, H-4*β*), 3.01 (1H, d, *J* = 4.8 Hz,, H-6*β*), 2.13 (3H, s, 18-OAc), 2.07 (3H, s, 15-OAc), 1.92 (3H, s, $CH_3$-28), 1.87 (3H, s, $CH_3$-27), 1.38 (3H, s, $CH_3$-21), 1.33 (3H, s, $CH_3$-19); $^{13}C$ NMR (100 MHz, $CDCl_3$) $\delta$ 202.4 (C, C-1), 171.3 (C, 15-OAc), 170.1 (C, 18-OAc), 165.5 (C, C-26), 149.9 (C, C-24), 142.2 (CH, C-3), 128.9 (CH, C-2), 121.8 (C, C-25), 85.0 (C, C-17), 79.5 (C, C-22), 79.3 (C, C-14), 79.0 (CH, C-20), 65.0 ($CH_2$, C-18), 64.2 (C, C-5), 58.5 (CH, C-6), 57.5(C, C-13), 48.5 (C, C-10), 43.6 ($CH_2$, C-16), 35.6 (CH, C-9), 33.9 (2x$CH_2$, C-4 and C-23), 31.7 (CH, C-8), 25.9 ($CH_2$, C-12), 23.2 ($CH_2$, C-7), 21.8 ($CH_2$, C-11), 21.5 ($CH_3$, 15-OAc), 21.3 ($CH_3$, 18-OAc), 20.6 ($CH_3$, C-28), 19.6 ($CH_3$, C-21), 15.2 ($CH_3$, C-19), 12.4 ($CH_3$, C-27); APCI-MS (+) *m/z* 603 $[MH]^+$.

**Deacetylation of Physachenolide C (3).** To a solution of **3** (10.2 mg) in *t*-BuOH (1.0 mL) and $CH_2Cl_2$ (0.4 mL) at -5 $^oC$ was added a 1.0 M solution of *t*-BuOK in *t*-BuOH (60 µL) and stirred at -5 $^oC$. After 45 min, reaction mixture was diluted with EtOAc (15 mL) and

washed with $H_2O$ (3 x 10 mL). Organic layer was evaporated under reduced pressure and the residue was separated by silica gel preparative TLC using $CH_2Cl_2$/MeOH (90:10) as eluent to give **25** (2.6 mg, 35%, $R_f$ = 0.66) and unreacted **3** (2.2 mg, 22%, $R_f$ = 0.72).

*18-Desacetylphysachenolide C (**25**).* White amorphous solid; $[\alpha]_D^{25}$ +87 (*c* 0.1, $CHCl_3$); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 6.80 (1H, ddd, *J* = 10.0, 6.4, 2.4 Hz, H-3), 6.00 (1H, dd, *J* = 10.0, 2.7 Hz, H-2), 4.88 (1H, dd, *J* = 12.8, 4.0 Hz, H-22), 3.99 (1H, d, *J* = 10.8 Hz, Ha-18), 3.83 (1H, d, *J* = 10.8 Hz, Hb-18), 3.16 (1H, brs, H-6), 2.92 (1H, dt, *J* = 18.8, 2.8 Hz, Ha-4), 2.63 (1H, m, Ha-16), 2.51 (1H, m, Ha-23), 2.43 (1H, m, Hb-23), 2.17 (1H, dt, *J* = 12.5, 2.8 Hz, Ha-12), 2.08 (1H, m, Ha-11), 1.98-1.92 (2H, m, $H_2$-7), 1.91 (3H, s, $H_3$-28), 1.90 (1H, m, H-9), 1.87 (3H, s, $H_3$-27), 1.84 (2H, m, Hb-4 and Hb-12), 1.75 (1H, dt, *J* = 11.6, 2.4 Hz, H-8), 1.62-1.58 (3H, m, $H_2$-15, Hb-16), 1.53 (1H, dt, *J* = 14.5, 2.1 Hz, Hb-11), 1.38 (3H, s, $H_3$-21), 1.22 (3H, s, $H_3$-19); $^{13}$C NMR (100 MHz, $CDCl_3$) 202.9 (C, C-1), 165.8 (C, C-26), 150.3 (C, C-24), 143,8 (CH, C-3), 129.8 (CH, C-2), 122.6 (C, C-25), 89.2 (C, C-20), 81.2 (C, C-14), 79.3 (CH, C-22), 78.8 (C, C-14), 64.1 (CH, C-6), 63.6 ($CH_2$, C-18), 62.2 (C, C-5), 58.7 (C, C-13), 48.5 (C, C-10), 37.5 ($CH_2$, C-16), 36.8 (CH, C-9), 34.2 ($CH_2$, C-23), 34.0 (CH, C-8), 33.3 ($CH_2$, C-15), 32.6 ($CH_2$, C-4), 26.3 ($CH_2$, C-7), 24.6 ($CH_2$, C-12), 22.7 ($CH_2$, C-11), 20.7 ($CH_3$, C-28), 19.0 ($CH_3$, C-21), 14.7 $CH_3$, C-19), 12.4 $CH_3$, C-27); HRESI-MS *m*/*z* 525.2461 $[M+Na]^+$ (calcd for $C_{28}H_{38}NaO_8$ 525.2464).

**Epoxidation of Physachenolide D (4).** To a stirred solution of **4** (20.0 mg) in $CHCl_3$ (2.0 mL) was added *m*-CPBA (12.0 mg) and the mixture was stirred at 25 $^o$C for 4 h (TLC control). The reaction mixture was concentrated under reduced pressure and the product mixture was separated by $C_{18}$ RP HPLC (60% aq. MeOH, 3 mL/min, UV detection at 230 nm) to afford physachenolide C (**3**) (8.5 mg, $t_R$ = 23.4 min) and a mixture of two compounds ($t_R$ = 17.7 min). This mixture ($t_R$ = 17.7 min) was further separated by silica gel preparative TLC using $CHCl_3$/MeOH (92:8) as eluent to

afford **19** (5.0 mg) and **26** (1.6 mg). Compounds **3** and **19** were identified by comparison of their spectroscopic data (NMR and MS) with those reported.[24]

*24α,25α-Epoxyphysachenolide C (**26**).* Off-white amorphous powder; $[\alpha]_D^{25}$ +54 (*c* 0.85, MeOH); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 6.80 (1H, ddd, *J* = 2.4, 6.4, 10.0 Hz, H-3), 6.01 (1H, dd, *J* = 2.8, 10 Hz, H-2), 5.07 (1H, dd, *J* = 2.8, 12.0 Hz, H-22), 4.39 (1H, d, *J* = 11.6 Hz, H-18), 4.32 (1H, d, *J* = 11.6 Hz, H-18), 3.15 (1H, brs, H-6α), 2.93 (1H, dt, *J* = 18.8, 2.4 Hz, H-4), 2.64 (1H, m, H-23), 2.58 (1H, dd, *J* = 15.2, 2.8 Hz, H-23), 2.10 (3H, s, 18-OAc), 1.56 (3H, s, $CH_3$-28), 1.45 (3H, s, $CH_3$-27), 1.29 (3H, s, $CH_3$-21), 1.22 (3H, s, $CH_3$-19); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 202.8 (C, C-1), 170.1 (C, OAc), 168.9 (C, C-26), 143.7 (CH, C-3), 129.7 (CH, C-2), 87.9 (C, C-17), 81.5 (C, C-14), 78.7 (CH, C-22), 77.4 (C, C-20), 64.9 ($CH_2$, C-18), 63.8 (CH, C-6), 62.8 (C, C-25), 62.1 (C, C-24), 59.4 (C, C-5), 57.5 (C, C-13), 48.5 (C, C-10), 38.1 ($CH_2$, C-16), 36.9 (CH, C-9), 34.2 (CH, C-8), 32.9 ($CH_2$, C-4), 32.8 ($CH_2$, C-15), 29.7 ($CH_2$, C-23), 26.4 ($CH_2$, C-7), 25.4 ($CH_2$, C-12), 22.8 ($CH_2$, C-11), 21.3 ($CH_3$, OAc), 18.8 ($CH_3$, C-21), 17.9 ($CH_3$, C-28), 14.6 ($CH_3$, C-19), 13.5 ($CH_3$, C-27); HRESI-MS *m/z* 583.2511 $[M+Na]^+$ (calcd for $C_{30}H_{40}O_{10}Na$, 583.2519).

**Preparation of 15α,28-Diacetoxyphysachenolide D (32).** Acetylation of 15α-acetoxy-28-hydroxyphysachenolide D (**30**) by the general procedure afforded **32** as an off-white amorphous powder; $[\alpha]_D^{25}$ +61 (*c* 0.02, MeOH); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 6.74 (1H, ddd, *J* = 10.0, 5.2, 2.8 Hz, H-3), 5.84 (1H, dd, *J* = 10.0, 2.0 Hz, H-2), 5.52 (1H, d, *J* = 6.0 Hz, H-6), 5.20 (1H, dd, *J* = 9.2, 8.4 Hz, H-15*β*), 4.89 (1H, brd, *J* = 12.8 Hz, H-22), 4.77 (1H, d, *J* = 13.6 Hz, H-28), 4.73 (1H, d, *J* = 13.6 Hz, H-28), 4.69 (1H, d, *J* = 11.6 Hz, H-18), 4.21 (1H, d, *J* = 11.6 Hz, H-18), 3.24 (1H, brd, *J* = 21.6 Hz,, H-4*β*), 2.80 (1H, dd, *J* = 21.2, 4.8 Hz,, H-4*α*), 2.14 (3H, s, 18-OAc), 2.08 (3H, s, 27-OAc), 2.07 (3H, s, 15-OAc), 1.95 (3H, s, $CH_3$-27), 1.40 (3H, s, $CH_3$-21), 1.21 (3H, s, $CH_3$-19); APCI-MS (+) *m/z* 667 $[M+Na]^+$.

**Preparation of 15α,27-Diacetoxyphysachenolide D (33).** Acetylation of 15α-acetoxy-27-hydroxyphysachenolide D (**31**) by the general procedure afforded **33** as an off-white amorphous powder; $[\alpha]_D^{25}$ +51 (*c* 0.04, MeOH); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 6.74 (1H, ddd, *J* = 10.0, 5.2, 2.8 Hz, H-3), 5.84 (1H, dd, *J* = 10.0, 2.0 Hz, H-2), 5.52 (1H, d, *J* = 5.6 Hz, H-6), 5.18 (1H, dd, *J* = 8.8, 8.4 Hz, H-15*β*), 4.92 (1H, m, H-22), 4.89 (1H, d, *J* = 12.0 Hz, H-27), 4.85 (1H, d, *J* = 12.0 Hz, H-27), 4.73 (1H, d, *J* = 12.0 Hz, H-18), 4.23 (1H, d, *J* = 11.6 Hz, H-18), 3.24 (1H, brd, *J* = 21.2 Hz,, H-4*β*), 2.80 (1H, dd, *J* = 21.2, 4.8 Hz,, H-4*α*), 2.15 (3H, s, 18-OAc), 2.08 (3H, s, 15-OAc), 2.06 (6H, s, 27-OAc and $CH_3$-28), 1.39 (3H, s, $CH_3$-21), 1.21 (3H, s, $CH_3$-19); APCI-MS (+) *m/z* 667 $[M+Na]^+$.

**TRAIL-Sensitization Assay.** The renal cancer cell lines ACHN, Caki-1, SN12C, TK10 and UO-31 were obtained from the NCI Frederick. Cells were plated at 5000 cells per well in 96 well flat-bottomed microtiter plates. Plates were incubated overnight at 37 $^o$C. Cells were treated with compound for 2 h, 50 ng/mL of TRAIL was then added to appropriate wells and the plates were incubated for a further 18 h at 37 $^o$C. Viable cell number was determined by the addition of CellTiter 96® AQueous One Solution Cell Proliferation Assay solution (MTS) and plates were incubated for 2 h and then absorbance at 490 nM was measured. Growth inhibition was calculated as in the following GI =[(Media-Treatment)/Media)]*100. The assay was performed in RPMI with 5% FCS, 2.0 mM L-glutamine, 1 x nonessential amino acids, 1.0 mM sodium pyruvate, 100 U/mL penicillin, 100 $\mu$g/mL streptomycin, 10 mM HEPES and 5 x $10^{-5}$ M 2-mercaptoethanol.

**Immunoblotting.** ACHN cells were grown in RPMI (RPMI, 5% FBS, Pen-strep, NEAA, HEPES, Glutamax, Sodium Pyruvate, 2ME), 1.5 x $10^6$ cells/well were plated in Costar 6-well plates and then incubated overnight at 37 $^o$C . On day 2 compounds were added, followed by overnight incubation. On day 3 either cells were treated with media or TRAIL (200 ng/mL)

for 90 min then lysed in RIPA buffer (25 mM Tris•HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with Pierce Halt Protease and Phosphatase Inhibitor and 40 $\mu$M zVAD-FMK, and Pierce Universal Nuclease. Protein concentrations were measured using the Pierce BCA Protein Assay (Thermo Fisher Scientific, Waltham, MA). Twenty $\mu$g of each sample was run on a Bolt Gel (Thermo Fisher Scientific), transferred to a PVDF membrane using the BioRad Trans-Blot Turbo Transfer System (BioRad, Hercules, California), briefly placed in Methanol, dried, rehydrated, and blocked in Pierce Start Block buffer. Blots were probed with either an anti-Caspase 8 cocktail (Cleaved Caspase-8 (Asp391) (18C8) Rabbit mAb #9496 1:1000, Cleaved Caspase-8 (Asp384) (11G10) Mouse mAb 1:5000, Caspase-8 (1C12) Mouse mAb #9746 1:5000, All from Cell Signaling Technology, Danvers, MA), anti-FLIP (7F10 1.0 $\mu$g/mL) (Enzo Life Sciences, Farmingdale, NY) or anti-GAPDH (GAPDH (D16H11) XP® Rabbit mAb (HRP Conjugate, 1:10,000, Cell Signaling) prepared in TBST (Tris Buffered Saline) containing 0.1% Tween 20 and 1% BSA. Blots were washed with TBST containing 0.5% Tween 20, probed with appropriate HRP labeled secondary (Pierce poly-HRP anti-rabbit and/or mouse at 1:50,000), washed again, treated with Pierce SuperSignal West Femto Maximum Sensitivity Substrate and imaged with a Licor Odyssey C-Digit.

**Thiol reactivity.** Solution A (0.5 mL) containing 2.12 $\mu$M of the withanolide (**1**, **2a**, **3**, **5**, **7**, **8**, **11**, **12** or **19**) in DMSO was mixed with solution B (0.5 mL) containing 4.24 $\mu$M GSH (or NAC), and the mixture was incubated at 37 ºC. The test samples (100 $\mu$L/each) were withdrawn at the time points 0.0, 0.5 2.0, 4.0, 8.0, and 24.0 h, and were stored at -80 ºC for subsequent HPLC analysis. All analyses were performed on a HPLC system equipped with a Hitachi AS-4000 intelligent auto sampler, Hitachi L-6200A intelligent pump, Hitachi L-4500 photodiode array detector, Shimadzu ELSD-LT detector, and a Phenomenex Luna 5$\mu$ C18 (2) 100 Å HPLC

column (4.6 X 250 mm) with gradient elution using $H_2O$ (containing 40 mM $NH_4OAc$)-MeOH from 60:40 to 0:100 (v/v) over a period of 30 min and UV detection at 230 nm. HPLC peak area method was used to calculate the concentration of withanolide in each of the sampled aliquots.[44]

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge via the internet at http://pubs.acs.org

Additional Experimental Methods, describing details of growth inhibition assays and immunoblotting; structures of sorafenib, sunitinib, bortezomib, ONC201 and SNS-032; $^1$H, $^{13}$C, and 2D NMR spectra of **6**, **8**, **9**, **11**, **14**–**18**, **21**–**26**, $^1$H and $^{13}$C NMR spectra of **12** and $^1$H spectra of **32** and **33**; results of TRAIL-sensitized apoptosis of ACHN cells by withanolides **1**, **2a**, and **3**–**36**; immunoblot showing reduction of $cFLIP_L$ and $cFLIP_S$ levels by WE (**1**), 4$\beta$-acetoxywithanolide E (**7**), 4$\beta$-methoxycarbonyloxywithanolide E (**8**) and 4$\alpha$-hydroxywithanolide E (**11**); kinetic study showing that 6 h exposure of ACHN cells to PCC (**3**) is sufficient to enhance caspase-8 activation; immunoblot of **2a** showing levels of $cFLIP_L$ and $cFLIP_S$; growth inhibitory effects of **1**, **2a**, **2c**, and **3** on ACHN cells; immunoblot of **1**, **2a**, **2c**, and **3** showing induction of proteins associated with cell stress (PDF)

Compound data (CSV)

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## Notes

The authors declare no competing financial interests.

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## ■ ABBREVIATIONS USED

17-BHW, 17$\beta$-hydroxywithanolide; DcR, decoy receptor; ELSD, evaporative light scattering detector; *c*FLIP, cellular FLICE-like inhibitory protein; DISC, death-inducing signaling complex; DMSO, dimethyl sulfoxide; DR, death receptor; FADD, Fas-associated death domain protein; Fas, a

cell surface death receptor involved in apoptosis; FCS, fetal calf serum; HMBC, hetero nuclear multi-bond correlation; HPLC, high-pressure liquid chromatography; IAP, Inhibitor of apoptosis proteins; NOE, nuclear Overhauser effect; PBS, phosphate saline buffer; PCC, physachenolide C; PCD, physachenolide D; RCC, renal cell carcinoma; RP, reversed phase; SAR, structure–activity relationship; TLC, thin-layer chromatography; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; WA, withaferin A; WE, withanolide E.

## ■ REFERENCES

(1) Key Statistics about Kidney Cancer. American Cancer Society Home Page. http://www.cancer.org/cancer/kidney-cancer/about/key-statistics.html (accessed February 18, 2017).

(2) Kidney Cancer Statistics. World Cancer Research Fund International Home Page. http://www.wcrf.org/int/cancer-facts-figures/data-specific-cancers/kidney-cancer-statistics (accessed February 18, 2017).

(3) Survival Statistics for Kidney Cancer. Cancer Research UK Home Page. http://www.cancerresearchuk.org/about-cancer/kidney-cancer/survival (accessed February 18, 2017).

(4) McDermott, D. I.; Atkins, M. B. Interleukin-2 therapy for metastatic renal cell carcinoma–predictors of response. *Semin. Oncol.* **2006**, *33*, 583–587.

(5) Cohen, R. B.; Oudard, S. Antiangiogenic therapy for advanced renal cell carcinoma: management of treatment–related toxicities. *Invest. New Drugs* **2012**, *30*, 2066–2079.

(6) Hanahan, D.; Weinberg, R. A. The hallmarks of cancer: the next generation. *Cell* **2011**, *100*, 57–70.

(7) (a) Sayers, T. J. Targeting the extrinsic apoptosis signaling pathway for cancer therapy. *Cancer Immunol. Immunother.* **2011**, *60*, 1173–1180. (b) Martinez-Lostao, L.; Marzo, I.; Anel, A.; Naval, J. Targeting the Apo2L/TRAIL system for the therapy of autoimmune diseases and cancer. *Biochem. Pharmacol.* **2012**, *83*, 1475–1483. (c) Micheau, O.; Shirley, S.; Dufour, F. Death receptors as targets in cancer. *Br. J. Pharmacol.* **2013**, *169*, 1723–1744. (d) Fulda, S. Tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL). *Adv. Exp. Med. Biol.* **2014**, *818*, 167–180. (e) Lemke, J.; von Karstedt, S.; Zinngrebe, J.; Walczak, H. Getting TRAIL back on track for cancer therapy. *Cell Death Differ*. **2014**, *21*, 1350–1364.

(8) Muzio, M.; Chinnaiyan, A. M.; Kischkel, F. C.; O'Rourke, K.; Shevchenko, A.; Ni, J.; Scaffidi, C.; Bretz, J. D.; Zhang, M.; Gentz, R.; Mann, M.; Krammer, P. H.; Peter, M. E.; Dixit, V. M. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell* **1996**, *85***,** 817–827.

(9) Bucur, O.; Stancu, A. L.; Khosravi-Far, R.; Almasan, A. Analysis of apoptosis methods recently used in Cancer Research and Cell Death & Disease publications. *Cell Death Dis.* **2012**, *3***,** e263; doi: 10.1038/cddis.2012.2.

(10) Takeda, K.; Smyth, M. J.; Cretney, E.; Hayakawa, Y.; Kayagaki, N.; Yagita, H.; Okumura, K. Critical role for tumor necrosis factor-related apoptosis-inducing ligand in immune surveillance against tumor development. *J. Exp. Med*. **2002**, *195*, 161–169.

(11) (a) Sayers, T. J.; Brooks, A. D.; Koh, C. Y.; Ma, W.; Seki, N.; Raziuddin, A.; Zhang, X.; Elliott, P. J.; Murphy, W. J. The proteasome inhibitor PS-341 sensitizes neoplastic cells to TRAIL-mediated apoptosis by reducing levels of c-FLIP. *Blood* **2003**, *102*, 303–310. (b) Brooks, A. D.; Sayers, T. J. Reduction of the antiapoptotic protein cFLIP enhances the susceptibility of human renal cancer cells to TRAIL apoptosis. *Cancer Immunol.*

*Immunother.* **2005**, *54*, 499–505. (c) Azijli, K.; Weyhenmeyer, B.; Peters, G. J.; deJong, S.; Kruyt, F. A. E. Non-canonical kinase signaling by the death ligand TRAIL in cancer cells: discord in the death receptor family. *Cell Death Differ*. **2013**, *20*, 858–868.

(12) Grothaus, P. G.; Cragg, G. M.; Newman, D. J. Plant natural products in anticancer drug discovery. *Curr. Org. Chem.* **2010**, *14*, 1781–1791.

(13) (a) Whitson, E. L.; Thomas, C. L.; Henrich, C. J.; Sayers, T. J.; McMahon, J. B.; McKee, T. C. Clerodane diterpenes from *Casearia arguta* that act as synergistic TRAIL sensitizers. *J. Nat. Prod.* **2010**, *73*, 2013–2018. (b) Henrich, C. J.; Thomas, C. L.; Brooks, A. D.; Booth, N. L.; Lowery, E. M.; Pompei, R. J.; McMahon, J. B.; Sayers, T. J. Effects of cucurbitacins on cell morphology are associated with sensitization of renal carcinoma cells to TRAIL-induced apoptosis, *Apoptosis* **2012**, *17*, 79–89. (c) Whitson, E. L.; Sun, H.; Thomas, C. L.; Henrich, C. J.; Sayers, T. J.; McMahon, J. B.; Griesinger, C.; McKee, T. C. Synergistic TRAIL sensitizers from *Barleria alluaudii* and *Diospyros maritima*. *J. Nat. Prod*. **2012**, *75*, 394–399. (d) Brincks, E. L.; Kucaba, T. A.; James, B. R.; Murphy, K. A.; Schwertfeger, K. L.; Sangwan, V.; Banerjee, S.; Saluja, A. K.; Griffith, T. S. Triptolide enhances the tumoricidal activity of TRAIL against renal cell carcinoma. *FEBS J.* **2015**, *282*, 4747–4765. (e) Dai, X.; Zhang, J.; Arfuso, F.; Chinnathambi, A.; Zayed, M. E.; Alharbi, S. A.; Kumar, A. P.; Ahn, K. S.; Sethi, G. Targeting TNF-related apoptosis-inducing ligand (TRAIL) receptor by natural products as a potential therapeutic approach for cancer therapy. *Exp. Biol. Med.* **2015**, *240*, 760–773.

(14) Lee, T.-J.; Um, H. J.; Min, D. S.; Park, J.-W.; Choi, K. S.; Kwon, T. K. Withaferin A sensitizes TRAIL-induced apoptosis through reactive oxygen species-mediated up-regulation of death receptor 5 and down-regulation of c-FLIP. *Free Radical Biol. & Med.* **2009**, *46*, 1639–1649.

(15) Chen, L.-X.; Xia, G.-Y.; He, H.; Huang, J.; Qiu, F.; Zi, X.-L. New withanolides with TRAIL-sensitizing effect from *Physalis pubescens* L. *RSC Adv.* **2016**, *6*, 52925–52936.

(16) Booth, N. L.; Sayers, T. J.; Brooks, A. D.; Thomas, C. L.; Jacobsen, K.; Goncharova, E. I.; McMahon, J. B.; Henrich, C. J. A cell-based high-throughput screen to identify synergistic TRAIL sensitizers. *Cancer Immunol. Immunother*. **2009**, *58*, 1229–1244.

(17) Henrich, C. J.; Brooks, A. D.; Erickson, K. L.; Thomas, C. L.; Bokesch, H. R.; Tewary, P.; Thompson, C. R.; Pompei, R. J.; Gustafson, K. R.; McMahon, J. B.; Sayers, T. J. Withanolide E sensitizes renal carcinoma cells to TRAIL-induced apoptosis by increasing cFLIP degradation. *Cell Death and Dis.* **2015**, *6*, e1666; doi:10.1038/cddis.2015.38.

(18) Tewary, P.; Gunatilaka, A. A. L.; Sayers, T. J. Using natural products to promote caspase-8-dependent cancer cell death. *Cancer Immunol. Immunother*. **2017**, *66*, 223–231.

(19) (a) Xu, Y.; Marron, M. T.; Seddon, E.; McLaughlin, S. P.; Ray, D. T.; Whitesell, L.; Gunatilaka, A. A. L. 2,3-Dihydrowithaferin A-3*β*-*O*-sulfate, a potential prodrug of withaferin A from aeroponically grown *Withania somnifera*. *Bioorg. Med. Chem*. **2009**, *17*, 2210–2214. (b) Xu, Y.; Gao, S.; Bunting, D. P.; Gunatilaka, A. A. L. Unusual withanolides from aeroponically grown *Withania somnifera*. *Phytochemistry* **2011**, *72*, 518–522.

(20) Xu, Y.; Bunting, D. P.; Liu, M. X.; Bandaranayake, H. A.; Gunatilaka, A. A. L. 17*β*-Hydroxy-18-acetoxywithanolides from aeroponically grown *Physalis crassifolia* and their potent and selective cytotoxicity for prostate cancer cells. *J. Nat. Prod*. **2016**, *79*, 821–830.

(21) Xu, Y.; Babyak, A. I.; Marks, H. R.; Wijeratne, E. M. K.; Brooks, A. D.; Tewary, P.; Xuan, L.-J.; Wang, W.-Q.; Sayers, T. J.; Gunatilaka, A. A. L. unpublished results.

(22) Yousuf, S. K.; Majeed, R.; Ahmad, M.; Sangwan, P. L.; Purnima, B.; Saxsena, A. K.; Suri, K. A.; Mukherjee, D.; Taneja, S. C. Ring A structural modified derivatives of withaferin A and the evaluation of their cytotoxic potential. *Steroids* **2011**, *76*, 1213–1222.

(23) Wijeratne, E. M. K.; Xu, Y.; Scherz-Shouval, R.; Marron, M. T.; Rocha, D. D.; Liu, M. X.; Costa-Lotufo, L. V.; Santagata, S.; Lindquist, S.; Whitesell, L.; Gunatilaka, A. A. L. Structure-activity relationships for withanolides as inducers of the cellular heat-shock response. *J. Med. Chem*. **2014**, *57*, 2851–2863.

(24) Xu, Y.; Liu, M. X.; Grunow, N.; Wijeratne, E. M. K.; Paine-Murrieta, G.; Felder, S.; Kris, R. M.; Gunatilaka, A. A. L. Discovery of potent 17*β*-hydroxywithanolides for castration-resistant prostate cancer by high-throughput screening of a natural products library for androgen-induced gene expression inhibitors. *J. Med. Chem.* **2015**, *58*, 6984–6993.

(25) De Wilt, L. H. A. M.; Kroon, J.; Jansen, G.; de Jong, S.; Peters, D. J.; Kruyt, F. A. E. Bortezomib and TRAIL: a perfect match for apoptotic elimination of tumor cells? C*rit. Rev. Oncol. Hematol.* **2013**, *85*, 363–372.

(26) Kline, C. L. B.; Van den Heuvel, A. P. J.; Allen, J. E.; Prabhu, V. V.; Dicker, D. T.; El-Deiry, W. S. ONC201 kills solid tumor cells by triggering an integrated stress response dependent on ATF4 activation by specific elF2α kinases. *Sci. Signal.* **2016**, *9* (415) ra18 (doi: 10.1126/scisignal.aac4374).

(27) Trivedi, R.; Mishra, D. P. Trailing TRAIL resistance: Novel targets for TRAIL sensitization in cancer cells. *Front. Oncol*. **2015**, *5*, 69. (doi: 10.3389/fonc.2015.00069).

(28) Santagata, S.; Xu, Y.; Wijeratne, E. M. K.; Kontnik, R.; Rooney, C.; Perley, C. C.; Kwon, H.; Clardy, J.; Kesari, S.; Whitesell, L.; Lindquist, S.; Gunatilaka, A. A. L. Using heat-shock response to discover anticancer compounds that target protein homeostasis. *ACS Chem. Biol*. **2012**, *7*, 340–349.

(29) Shi, B.; Greaney, M. F. Reversible Michael addition of thiols as a new tool for dynamic combinatorial chemistry. *Chem. Commun*. **2005**, 886–888.

(30) Antony, M. I.; Lee, J.; Hahm, E.-K.; Kim, S.-H.; Marcus, A. I.; Kumari, V.; Ji, X.; Yang, Z.; Vowell, C. I.; Wipf, P.; Uechi, G. T.; Yates, N. A.; Romero, K.; Sarkar, S. N.; Singh, S. V. Growth arrest by the antitumor steroidal lactone withaferin A in human breast cancer cells is associated with down-regulation and covalent binding at cysteine 303 of β-tubulin. *J. Biol. Chem.* **2014**, *389*, 1852–1865.

(31) Rosenker, C. J.; Krenske, E. H.; Houk, K. N.; Wipf, P. Influence of base and structure in reversible covalent conjugate addition of thiol to polycyclic enone scaffold. *Org. Lett.* **2013**, *15*, 1076–1079.

(32) (a) Chen, L.-X.; He, H.; Qiu, F. Natural withanolides: an overview. *Nat. Prod. Rep*. **2011**, *28*, 705–740. (b) Yang, B.-Y.; Xia, Y.-G.; Pan, J.; Liu, Y.; Wang, Q.-H.; Kuang, H.-X. Phytochemistry and biosynthesis of δ-lactone withanolides. *Phytochem. Rev.* **2016**, *15*, 771–797.

(33) (a) Xu, Y.; Marron, M. T.; Seddon, E.; McLaughlin, S. P.; Ray, D. T.; Whitesell, L.; Gunatilaka, A. A. L. 2,3-Dihydrowithaferin A-3*β*-*O*-sulfate, a new potential prodrug of withaferin A from aeroponically grown *Withania somnifera*. *Bioorg. Med. Chem*. **2009**, *17*, 2210–2214. (b) Yu, Y.; Hamza, A.; Zhang, T.; Gu, M.; Zou, P.; Newman, B.; Li, Y.; Gunatilaka, A. A. L.; Zhan, C.-G.; Sun, D. Withaferin A targets heat shock protein 90 in pancreatic cancer cells. *Biochem. Pharmacol*. **2010**, *79*, 542–551. (c) Liu, X.; Qi, W.;

Cooke, L. S.; Wijeratne, E. M. K.; Xu, Y.; Marron, M. T.; Gunatilaka, A. A. L.; Mahadevan, D. An analog of withaferin A activates the MAPK and glutathione "stress" pathways and inhibits pancreatic cancer cell proliferation. *Cancer Invest*. **2011**, *29*, 668–675. (d) Nagalingam, A.; Kuppusamy, P.; Singh, S. V.; Sharma, D.; Saxena, N. K. Mechanistic elucidation of the antitumor properties of withaferin A in breast cancer, *Cancer Res.* **2014**, *74*, 2617–2629.

(34) Machin, R. P.; Veleiro, A. S.; Nicotra, V. E.; Oberti, J. C.; Padrón, J. M. Antiproliferative activity of withanolides against human breast cancer cell lines. *J. Nat. Prod*. **2010**, *73*, 966–968.

(35) Damu, A. G.; Kuo, P.-C.; Su, C.-R.; Kuo, T.-H.; Chen, T.-H.; Bastow, K. F.; Lee, K.-H.; Wu, T.-S. Isolation, structures, and structure-cytotoxic activity relationships of withanolides and physalins from *Physalis angulata*. *J. Nat. Prod*. **2007**, *70*, 1146–1152.

(36) van Dijk, M.; Halpin-McCormik, A.; Sessler, T.; Samali, A.; Szegezdi, E. Resistance to TRAIL in non-transformed cells is due to multiple redundant pathways. *Cell Death and Dis.* **2013**, *4*, e702; doi:10.1038/cddis.2013.214.

(37) Allen, J. E.; Prabhu, V. V.; Talekar, M.; van den Heuvel, A. P.; Lim, B.; Dicker, D. T.; Fritz, J. L.; Beck, A.; El-Deiry, W. S. Genetic and pharmacological screens converge in identifying FLIP, BCL2, and IAP proteins as key regulators of sensitivity to the TRAIL-inducing anticancer agent ONC201/TIC10. *Cancer Res*. **2015**, *75*, 1668–1674.

(38) Ricci, M. S.; Kim, S. H.; Ogi, K.; Plastaras, J. P.; Ling, J.; Wang, W.; Jin, Z.; Liu, Y. Y.; Dicker, D. T.; Chiao, P. J.; Flaherty, K. T.; Smith, C. D.; El-Deiry, W. S. Reduction of TRAIL-induced Mcl-1 and cIAP2 by c-Myc or sorafenib sensitizes resistant human cancer cells to TRAIL-induced death. *Cancer Cell* **2007**, *12*, 66–80.

(39) Lemke, J.; von Karstedt, S.; Abd El Hay, M.; Conti, A.; Arce, F.; Montinaro, A.; Papenfuss, K.; El-Bahrawy, M. A.; Walczak, H. Selective CDK9 inhibition overcomes TRAIL resistance by concomitant suppression of cFlip and Mcl-1. *Cell Death Differ.* **2014**, *21*, 491–502.

(40) Tewary, P., Brooks, A. D.; Xu, Y.; Wijeratne, E. M. K.; Gunatilaka, A. A. L.; Sayers, T. J. unpublished results.

(41) Gottlieb, H. E.; Kirson, I. $^{13}C$ NMR spectroscopy of the withanolides and other highly oxygenated $C_{28}$ steroids. *Org. Magn. Reson.* **1981**, *16*, 20–25.

(42) Bagchi, A.; Neogi, P.; Sahai, M.; Ray, A. B.; Oshima, Y.; Hikino, H. Withaperuvin E and nicandrin B, withanolides from *Physalis peruviana* and *Nicandra physaloides*. *Phytochemistry* **1984**, *23*, 853–855.

(43) Fang, S.-T.; Liu, J.-K.; Li, B. Ten new withanolides from *Physalis peruviana*. *Steroids* **2012**, *77*, 36–44.

(44) Pryde, A.; Gilbert, M. T. Application of High Performance Liquid Chromatography; Chapman and Hall: New York, 1979; pp 24–33.

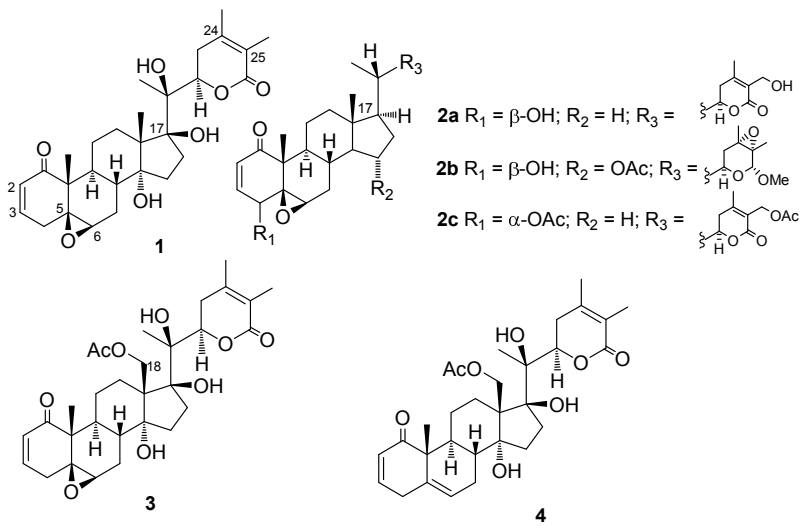
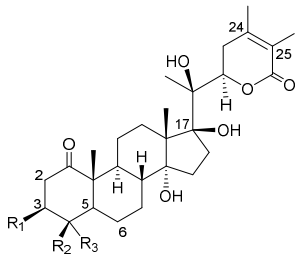



**Figure 1**. Structures of withanolide E (**1**), withaferin A analogues (**2a**, **2b** and **2c**), physachenolide C (**3**), and physachenolide D (**4**).



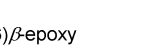
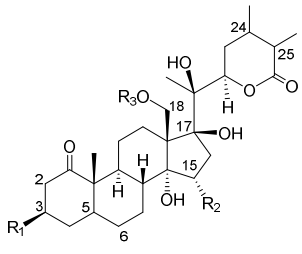
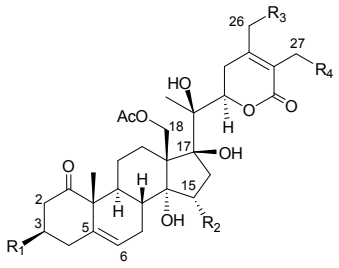
**5** $R_1 = R_3 = H$; $R_2 = OH$; $\Delta^{2,3}$; 5(6)$\beta$-epoxy
**6** $R_1 = N_3$; $R_2 = OH$; $R_3 = H$; 5(6)$\beta$-epoxy
**7** $R_1 = R_3 = H$; $R_2 = OAc$; $\Delta^{2,3}$; 5(6)$\beta$-epoxy
**8** $R_1 = R_3 = H$; $R_2 = OCO_2Me$; $\Delta^{2,3}$; 5(6)$\beta$-epoxy
**9** $R_1 = R_3 = H$; $R_2$ =  ; $\Delta^{2,3}$; 5(6)$\beta$-epoxy
**10** $R_1 = H$; $R_2,R_3 = O$; $\Delta^{2,3}$; 5(6)$\beta$-epoxy
**11** $R_1 = R_2 = H$; $R_3 = OH$; $\Delta^{2,3}$; 5(6)$\beta$-epoxy
**12** $R_1 = R_2 = H$; $R_3 = OAc$; $\Delta^{2,3}$; 5(6)$\beta$-epoxy
**13** $R_1 = H$; $R_2,R_3 = O$; $\Delta^{2,3}$; $\Delta^{5,6}$
**14** $R_1 = R_2 = H$; $R_3 = OH$; $\Delta^{2,3}$; $\Delta^{5,6}$

**Figure 2**. Structures of withanolide E analogues (**5–14**).



**15** $R_1 = R_2 = H$; $R_3 = Ac$; 5(6)$\beta$-epoxy; $\Delta^{24,25}$
**16** $R_1 = N_3$; $R_2 = H$; $R_3 = Ac$; 5(6)$\beta$-epoxy; $\Delta^{24,25}$
**17** $R_1$ =  ; $R_2 = H$; $R_3 = Ac$; 5(6)$\beta$-epoxy; $\Delta^{24,25}$
**18** $R_1 = OMe$; $R_2 = H$; $R_3 = Ac$; 5(6)$\beta$-epoxy; $\Delta^{24,25}$
**19** $R_1 = R_2 = H$; $R_3 = Ac$; $\Delta^{2,3}$; 5(6)$\alpha$-epoxy; $\Delta^{24,25}$
**20** $R_1 =$ H; $R_2 = OAc$; $R_3 = Ac$; $\Delta^{2,3}$; 5(6)$\beta$-epoxy; $\Delta^{24,25}$
**21** $R_1 = H$; $R_2 = OH$; $R_3 = Ac$; $\Delta^{2,3}$; 5(6)$\beta$-epoxy; $\Delta^{24,25}$
**22** $R_1 = H$; $R_2 = OAc$; $R_3 = Ac$; $\Delta^{2,3}$; 5(6)$\alpha$-epoxy; $\Delta^{24,25}$
**23** $R_1 = H$; $R_2 = OH$; $R_3 = Ac$; $\Delta^{2,3}$; 5(6)$\alpha$-epoxy; $\Delta^{24,25}$
**24** $R_1 = R_2 = R_3 = H$; 5(6)$\beta$-epoxy; $\Delta^{24,25}$
**25** $R_1 = R_2 = R_3 = H$; $\Delta^{2,3}$; 5(6)$\alpha$-epoxy; $\Delta^{24,25}$
**26** $R_1 = R_2 =$ H; $R_3 = Ac$; $\Delta^{2,3}$; 5(6)$\beta$-epoxy; 24(25)$\alpha$-epoxy

**Figure 3**. Structures of physachenolide C analogues (**15–26**).




**27** $R_1 = OSO_3H$; $R_2 = R_3 = R_4 = H$
**28** $R_1 = R_3 = R_4 = H$; $R_2 = OAc$; $\Delta^{2,3}$
**29** $R_1 = OSO_3H$; $R_2 = OAc$; $R_3 = R_4 = H$
**30** $R_1 = R_4 = H$; $R_2 = OAc$; $R_3 = OH$; $\Delta^{2,3}$
**31** $R_1 = R_3 = H$; $R_2 = OAc$; $R_4 = OH$; $\Delta^{2,3}$
**32** $R_1 = R_4 = H$; $R_2 = R_3 = OAc$; $\Delta^{2,3}$
**33** $R_1 = R_3 = H$; $R_2 = R_4 = OAc$; $\Delta^{2,3}$
**34** $R_1 = R_4 = H$; $R_2 = OAc$; $R_3 = OGlu$; $\Delta^{2,3}$
**35** $R_1 = R_3 = H$; $R_2 = OAc$; $R_4 = OGlu$; $\Delta^{2,3}$
**36** $R_1 = R_3 = R_4 = H$; $R_2 = OH$; $\Delta^{2,3}$

**Figure 4**. Structures of physachenolide D analogues (**27–36**).

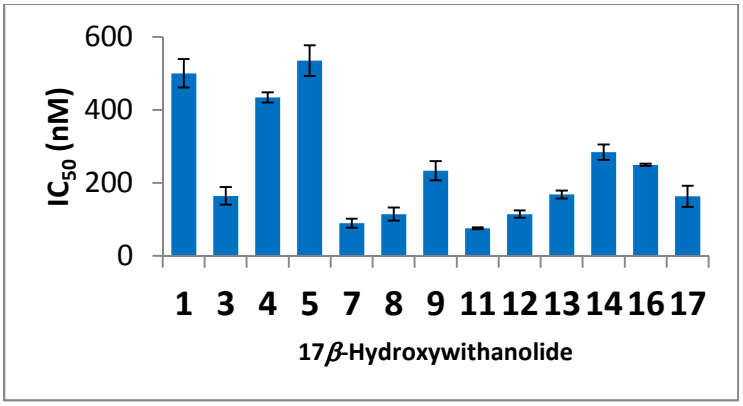



**Figure 5**. TRAIL sensitization activity of **1** and its active analogues. Renal carcinoma cells (ACHN) were pretreated with each compound for 2 h after which TRAIL (50.0 ng/mL) was added and cells were incubated overnight at 37 °C. Viable cell numbers were then estimated using an MTS assay. All determinations were performed in triplicate. The $IC_{50}$s (concentrations required to reduce cell number by 50%) were determined from 3 independent experiments using Microsoft Excel software with +/- referring to the standard deviation.

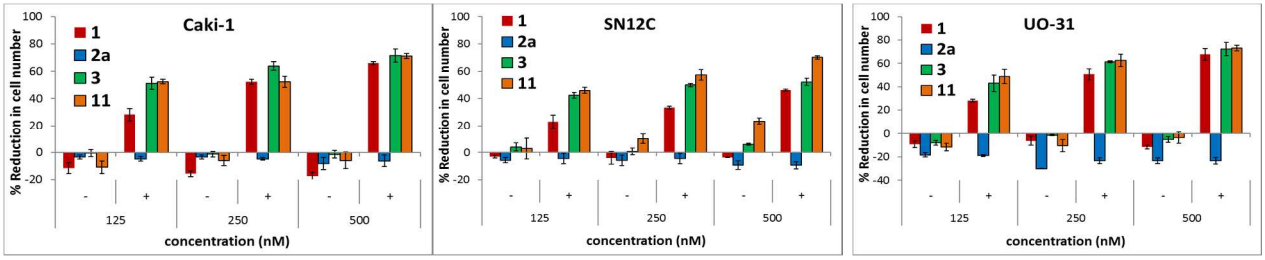



**Figure 6**. Sensitization of renal cancer cell lines Caki-1, SN12C, and UO-31 to TRAIL by WE (**1**), WA (**2a**), PCC (**3**), and 4$\alpha$-hydroxywithanolide E (**11**). Cells were incubated with various concentrations of the compounds in the presence or absence of TRAIL (50 ng/mL) as described in the Experimental Section. All determinations were performed in triplicate. Error bars, +/– SD. At least 3 experiments with similar findings were performed.

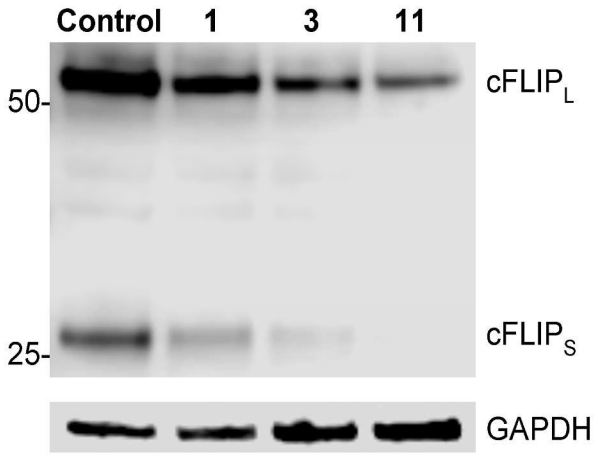



**Figure 7.** Effects of WE (**1**), PCC (**3**), and 4$\alpha$-hydroxywithanolide E (**11**) on *c*FLIP levels. ACHN cells were incubated for 18 h with the compounds (500 nM) and *c*FLIP levels were then determined by western blotting. GAPDH was used as the loading control.

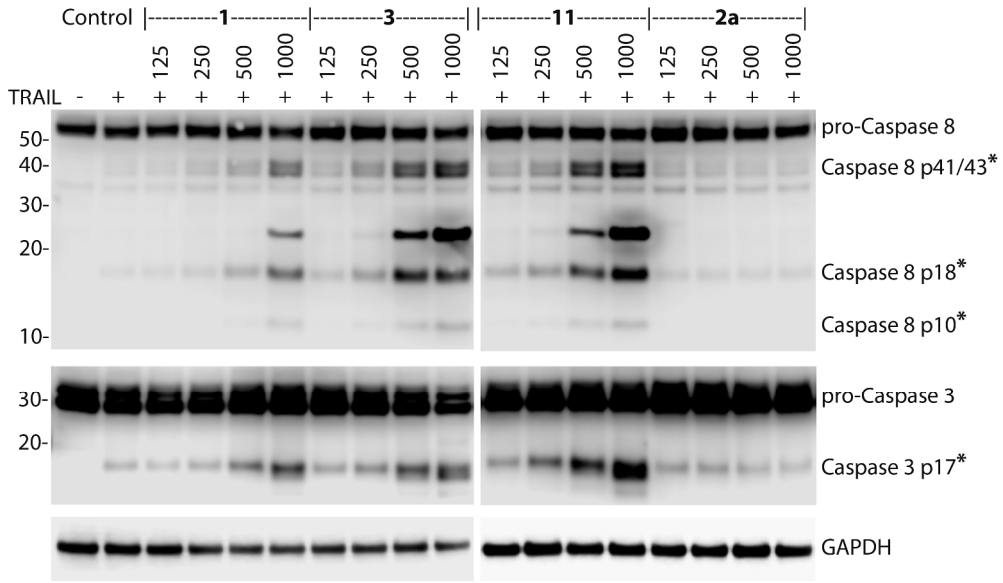



**Figure 8**. Effects of WE (**1**), PCC (**3**), 4$\alpha$-hydroxywithanolide E (**11**) and WA (**2a**) (at concentrations ranging from 125–1000 nM for 18 h) on cleavage of pro-caspase-3 and pro-caspase-8 following exposure to TRAIL (50 ng/mL for 90 min). The cleaved forms of pro-caspase-3 and pro-caspase-8 (marked with *) are the active forms of the enzymes. GAPDH was used as the loading control.

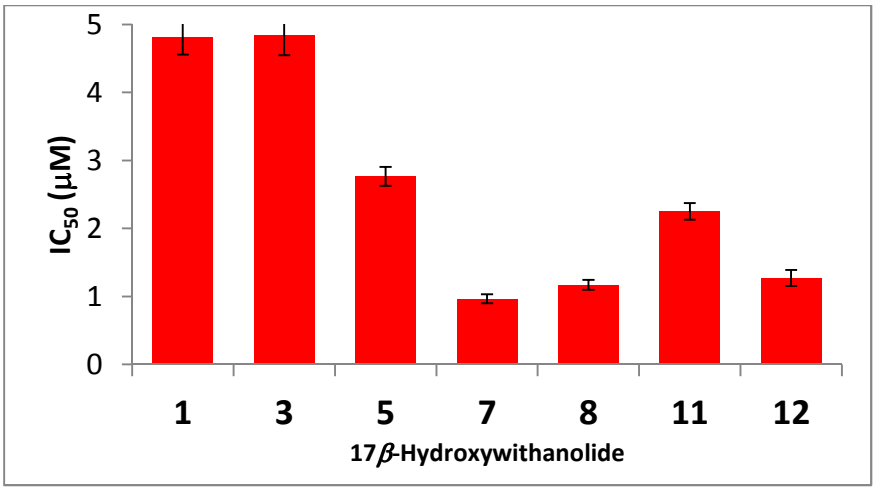



**Figure 9**. Cytotoxic activity of the 17-BHWs **1**, **3**, **5**, **7**, **8**, **11**, and **12** vs. renal carcinoma cells (ACHN). The cells were incubated with each compound for 48 h at 37 °C and viable cell numbers were estimated using an MTS assay. All determinations were performed in triplicate. The $IC_{50}$s (concentrations required to reduce cell number by 50%) were determined from 3 independent experiments using Microsoft Excel software with +/- referring to the standard deviation.

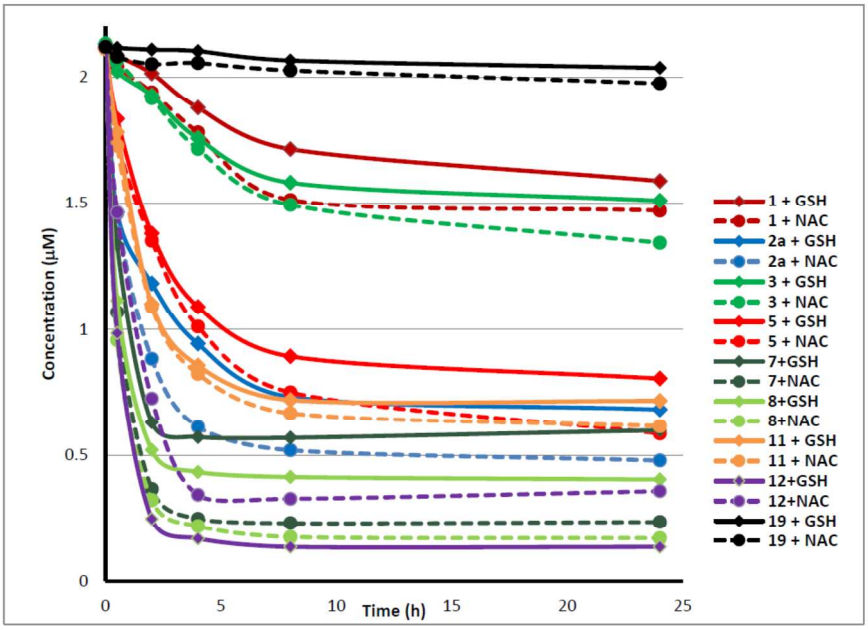



**Figure 10**. Reactivity of thiols [glutathione (GSH) and N-acetylcysteine (NAC)] toward withanolide E (**1**), withaferin A (**2a**), physachenolide C (**3**), 4$\beta$-hydroxywithanolide E (**5**), 4$\beta$-acetoxywithanolide E (7) 4$\beta$-methoxycarbonyloxywithanolide E (**8**), 4$\alpha$-hydroxywithanolide E (**11**) 5,6-*epi*-physachenolide C (**19**) monitored by HPLC analysis.

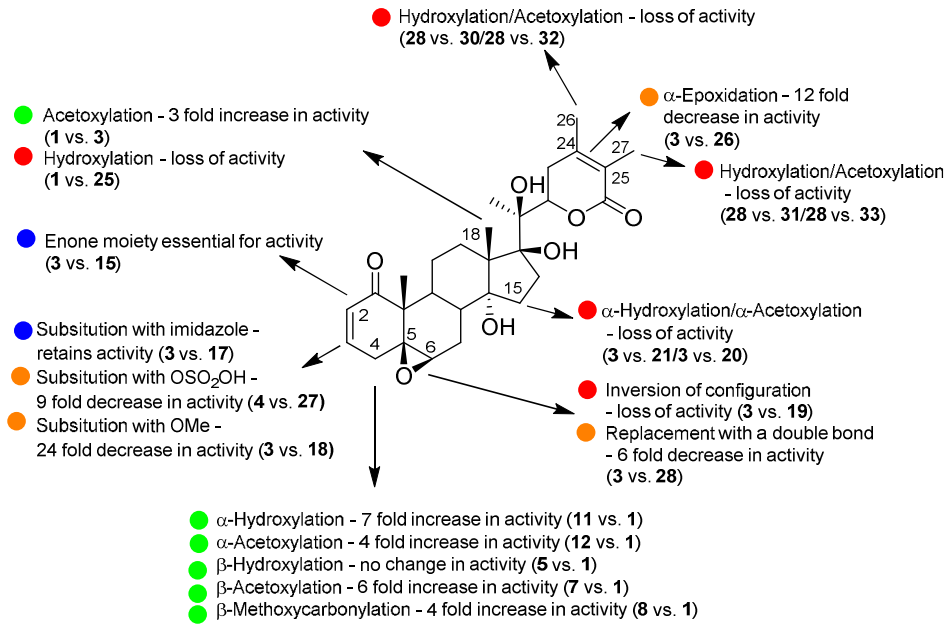



**Figure 11**. Summary of the structure-activity relationships of 17-BHWs for in vitro TRAIL sensitization in ACHN cells.

**Table 1. TRAIL sensitizing activity of withanolides 1, 2a, and 3–36**

| withanolide | $IC_{50}$[a] | withanolide | $IC_{50}$[a] | withanolide | $IC_{50}$[a] | withanolide | $IC_{50}$[a] |
|---|---|---|---|---|---|---|---|
| **1** | 489 ± 53 | **10** | >4000 | **19** | >4000 | **28** | >4000 |
| **2a** | >2000 | **11** | 67 ± 15 | **20** | >4000 | **29** | >4000 |
| **3** | 164 ± 24 | **12** | 90 ± 32 | **21** | >4000 | **30** | >4000 |
| **4** | 564 ± 55 | **13** | 168 ± 11 | **22** | >4000 | **31** | >4000 |
| **5** | 563 ± 56 | **14** | 284 ± 22 | **23** | 1650 ± 70 | **32** | >4000 |
| **6** | 1800 ± 28 | **15** | >4000 | **24** | >4000 | **33** | >4000 |
| **7** | 76 ± 21 | **16** | 251 ± 32 | **25** | 1950 ± 70 | **34** | >4000 |
| **8** | 111 ± 24 | **17** | 164 ± 29 | **26** | 1250 ± 70 | **35** | >4000 |
| **9** | 233 ± 26 | **18** | >4000 | **27** | >4000 | **36** | >2000 |

[a]Concentration of withanolide (nM) that in combination with TRAIL (50 ng/mL) reduced ACHN cell number by 50% after 18 h exposure. $IC_{50}$ values were determined from dose-response curves using Microsoft Excel software. Values from at least 3 independent experiments; ± refers to standard deviation.

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