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17 β -Hydroxywithanolides as Sensitizers of Renal Carcinoma Cells to Tumor Necrosis Factor- α 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 β -hydroxywithanolide (17-BHW), withanolide E (**1**), as a promising lead. To explore structure–activity relationships, we obtained natural and semisynthetic 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 α -oriented side chain are superior to known TRAIL-sensitizing withanolides belonging to withaferin A class with a β -oriented side chain and

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmed-chem.7b00069. Additional experimental methods, describing details of growth inhibition assays and immunoblotting; structures of sorafenib, sunitinib, bortezomib, ONC201, and SNS-032; ¹H, ¹³C, and 2D NMR spectra of **6**, **8**, **9**, **11**, **14–18**, **21–26**, ¹H and ¹³C NMR spectra of **12**, and ¹H 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 β -acetoxywithanolide E (**7**), 4 β -methoxycarbonyloxywithanolide E (**8**), and 4 α -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)

Molecular formula strings and some data (CSV)

The authors declare no competing financial interest.

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.

Graphical Abstract

INTRODUCTION

Renal carcinoma is among the 10 most common cancers in the U.S., 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 antiangiogenic 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 antiapoptotic proteins including cellular FLICE-like inhibitory protein (cFLIP).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 β -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 cFLIP 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 α -oriented side chain (e.g., **1**) may differ considerably from those with a β -oriented side chain (e.g., **2a** and **2b**).¹⁸ On the basis of 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 Semisynthesis 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 subclasses 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 β -Azido-4 β -hydroxywithanolide E (**6**) was obtained by treating 4 β -hydroxywithanolide E (**5**) with Me₃SiN₃/AcOH/Et₃N.²² Acetyl derivatives **7**, **12**, **32**, and **33** were prepared by acetylation of their corresponding hydroxy analogues with Ac₂O/pyridine. Treatment of **5** with 1,1'-carbonyldiimidazole gave **9** which on methanolysis afforded **8**. Oxidation of **5** with MnO₂ yielded withaperuvine E (**10**). Reduction of **10** with NaBH₄/CeCl₃ afforded 4 α -hydroxywithanolide E (**11**) as the major product formed as a result of regio- and stereospecific reduction of its ring A enedione moiety.²³ When treated with Ph₃P/I₂, **10** underwent deoxygenation to afford withaperuvine M (**13**) which on NaBH₄/CeCl₃ reduction afforded 4-*epi*-physapruvins A (**14**). Catalytic hydrogenation of **3** with Pd/C in EtOH containing Et₃N afforded 2,3-dihydrophysachenolide C (**15**) and its desacetyl analogue **24**. Michael adducts of **3** at C-3, 2,3-dihydro-3 β -azidophysachenolide C (**16**), 2,3-dihydro-3 β -imidazolylphysachenolide C (**17**), and 2,3-dihydro-3 β -methoxyphysachenolide C (**18**) were obtained by reacting **3** with Me₃SiN₃/AcOH/CH₂Cl₂,²² imidazole/CH₂Cl₂, and MeOH/Et₃N, respectively. Epoxidation of **4** with *m*-CPBA yielded **3**, its epimer, 5,6-*epi*-physachenolide C (**19**), and 24 α ,25 α -epoxyphysachenolide C (**26**), whereas similar treatment of **36** afforded 15 α -hydroxyphysachenolide C (**21**) and 15 α -hydroxy-5,6-*epi*-physachenolide C (**23**).²⁴ Treatment of **3** with *t*-BuOK/*t*-BuOH/CH₂Cl₂/–5 °C afforded its 18-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 subclasses of 17-BHWs (Figures 2 and 3, respectively) bearing 5 β ,6 β -epoxy moieties exhibited potent activity. Among those active, **3** and the novel WE analogue 4 α -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 antiapoptotic α 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 α FLIP and caspase activation. As depicted in Figure 7, **1** caused some reduction of α 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 α FLIP levels (Supporting Information, Figure S62). On subsequent exposure of cells to TRAIL, the conversion of procaspase-8 to the active form of the enzyme and the subsequent proteolytic activation of the executioner caspase-3 were 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 antiapoptotic α 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 α FLIP with these same RCC cell lines using siRNA was sufficient to sensitize them to TRAIL-mediated apoptosis.^{11b} Therefore, the efficient reduction of α FLIP levels by **3** and **11** is likely to be critical for apoptosis sensitization of RCC cells. Thus, these decreases in α 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 μ M and 1.0–2.0 μ M, respectively). Data presented here suggest that **2a** and its analogue 2,27-diacetyl-4-*epi*-withaferin A (**2c**)²³ with a β -oriented side chain are much less potent as TRAIL-sensitizers and are less effective in reducing α FLIP levels than 17-BHWs with a α -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 bortezomib²⁵ (Supporting Information, Figure S1) and natural products such as cucurbitacin A,^{13b} triptolide,^{13d} and **2a**¹⁴ as well as 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(1*H*)-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, the 17-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 α 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 IC₅₀ 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 preclinical 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 α,β -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 three 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 β -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. On the basis of 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 34 natural and semisynthetic 17-BHWs (**3–36**) bearing an α -oriented side chain and compared their activities with those of **1** and withanolides with a β -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 α -oriented side chain and/or 17 β -hydroxy group, enone moiety in ring A and 5 β ,6 β -oxirane functionality in ring B are essential for activity and that α - and β -acetoxylation/ α -hydroxylation/ β -methoxycarbonylation at C-4 and acetoxylation at C-18 lead to enhancement of activity, β -hydroxylation at C-4 has no effect on activity, whereas hydroxylation at C-15, C-18, C-26, and C-27 and α -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 β -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 α -oriented side chain at C-17 which is not a part of a cyclic system, and most of these bear a β -hydroxy group at this carbon.³² These 17-BHWs have been reported to have in vitro cytotoxic activity against breast³⁴ and prostate³⁵ cancers. Our recent studies demonstrated that **1** was able to sensitize TRAIL to induce apoptosis of ACHN renal carcinoma cells¹⁷ 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 cFLIP 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 nonredundant resistance mechanism.³⁶ Therefore, it is likely for RCC that the overexpression of cFLIP 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(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 antiapoptotic 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 nontoxic 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 α FLIP.

EXPERIMENTAL SECTION

Chemistry.

Optical rotations were measured in MeOH or CHCl₃ with a Jasco DIP-370 digital polarimeter. 1D and 2D NMR spectra were recorded in CDCl₃, unless otherwise stated, using residual solvents as internal standards on Bruker Avance III 400 spectrometer at 400 MHz for ¹H NMR and 100 MHz for ¹³C NMR, respectively. The chemical shift values (δ) are given in parts per million (ppm), and the coupling constants (J values) are in Hz. LR-MS were recorded using Shimadzu LCMS-QP8000A and HR-MS were recorded using JEOL HX110A or Agilent TOF mass spectrometer. Analytical thin-layer chromatography was carried out on silica gel 60 F₂₅₄ aluminum-backed TLC plates (Merck). Preparative thin-layer chromatography (TLC) was performed on Analtech silica gel 500 μ 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 μ m C18 (2) column (10 mm $\times$ 250 mm) or Alltech Econosil normal phase silica column (250 mm $\times$ 10 mm, 10 μ m) 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 autosampler, Hitachi L-4500 photodiode array detector, and Shimadzu ELSD-LT detector using a Phenomenex Luna 5 μ m C18 (2) 100 Å (4.6 mm $\times$ 250 mm) column and a gradient solvent system of MeOH/H₂O [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 β -hydroxywithanolide E (**5**) were obtained from *Physalis peruviana*,²¹ withaferin A (**2a**) from *Withania somnifera*,¹⁹ and physachenolide C (**3**), physachenolide

D (**4**), 15 α -acetoxyphysachenolide C (**20**), 2,3-dihydrophysachenolide D 3 β -O-sulfate (**27**), 15 α -acetoxyphysachenolide D (**28**), 15 α -acetoxy-2,3-dihydrophysachenolide D-3 β -O-sulfate (**29**), 15 α -acetoxy-28-hydroxyphysachenolide D (**30**), 15 α -acetoxy-27-hydroxyphysachenolide D (**31**), 15 α -acetoxy-28-*O*- β -glucopyranosylphysachenolide D (**34**), 15 α -acetoxy-27-*O*- β -glucopyranosylphysachenolide D (**35**), and 15 α -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 Ac₂O (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 α -hydroxyphysachenolide D (**36**) with *m*-chloroperbenzoic acid (*m*-CPBA) afforded 15 α -hydroxyphysachenolide C (**21**) and 15 α -hydroxy-5,6-*epi*-physachenolide C (**23**).²⁴

Preparation of 2,3-Dihydro-3 β -azido-4 β -hydroxywithanolide **6**.

To a solution of 4 β -hydroxywithanolide E (**5**, 7.0 mg) in anhydrous CH₂Cl₂ (0.8 mL) were added AcOH (19.7 μ L), Et₃N (1.93 μ L), and Me₃SiN₃ (45.5 μ L) and stirred at 25 °C. After 20 h, the reaction was quenched with aqueous NH₄Cl and extracted with EtOAc (3 $\times$ 10 mL), and combined organic layer was washed with brine, dried over Na₂SO₄, 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; [α]_D²⁵ – 159 (*c* 0.11, CHCl₃); ¹H NMR (400 MHz, CDCl₃) δ 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, H₂-23), 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, H₃-28), 1.86 (3H, s, H₃-27), 1.83 (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, H₃-21), 1.35 (1H, dd, *J* = 15.0, 5.5 Hz, Hb-11), 1.29 (1H, m, Hb-12), 1.26 (3H, s, H₃-19), 1.00 (3H, s, H₃-18); ¹³C NMR (100 MHz, CDCl₃) δ 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 (CH₂, C-2), 37.9 (CH₂, C-16), 35.6 (CH, C-8), 34.2 (CH₂, C-23), 33.8 (CH, C-9), 32.2 (CH₂, C-15), 29.3 (CH₂, C-12), 25.5 (CH₂, C-7), 21.0 (CH₂, C-11), 20.6 (CH₃, C-28), 20.1 (CH₃, C-18), 19.7 (CH₃, C-21), 15.0 (CH₃, C-19), 12.4 (CH₃, C-27); HRESI-MS *m/z* 568.2634 [M + Na]⁺ (calcd for C₂₈H₂₉N₃NaO₈ 568.2634).

Preparation of 4 β -Acetoxywithanolide E (7).

Acetylation of withanolide E (**1**) by the general procedure afforded **7**.

4 β -Acetoxywithanolide E (7).—White powder; $[\alpha]_{\text{D}}^{25} + 136$ (*c* 0.1, CHCl₃); ¹H and ¹³C 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 °C. After 1 h solvent was evaporated under reduced pressure and the crude product was purified by preparative TLC (silica gel) using CH₂Cl₂/MeOH (94:6) as eluent to give **8** (1.2 mg, 43%).

4 β -Methoxycarbonyloxywithanolide E (8).—White amorphous solid; $[\alpha]_{\text{D}}^{25} + 108$ (*c* 0.5, CHCl₃); ¹H NMR (400 MHz, CDCl₃) δ 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, OCH₃), 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, H₂-23), 2.21 (1H, m, Ha-12), 2.06–2.01 (2H, m, H₂-7), 1.92 (3H, s, H₃-28), 1.86 (3H, s, H₃-27), 1.81 (1H, m, H-8), 1.67–1.52 (5H, m, H-9, H₂-11, H₂-15), 1.41 (dd, *J* = 15.0, 8.5 Hz, 1H, Hb-16), 1.39 (3H, s, H₃-21), 1.37 (3H, s, H₃-19), 1.29 (1H, brd, *J* = 12.5 Hz, Hb-12), 1.04 (3H, s, H₃-18); ¹³C NMR (100 MHz, CDCl₃) δ 200.6 (C, C-1), 165.7 (C, C-26), 155.0 (C, OCOOCH₃), 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 (CH₃, OCH₃), 54.5 (C, C-13), 48.0 (C, C-10), 37.9 (CH₂, C-16), 36.7 (CH, C-8), 34.3 (CH₂, C-23), 34.0 (CH, C-9), 32.3 (CH₂, C-15), 29.4 (CH₂, C-12), 25.6 (CH₂, C-7), 20.9 (CH₂, C-11), 20.6 (CH₃, C-28), 20.2 (CH₃, C-18), 19.6 (CH₃, C-21), 15.2 (CH₃, C-18), 12.4 (CH₃, C-27); HRESI-MS (*m/z*) 583.2514 [*M* + Na]⁺ (calcd for C₃₀H₄₀NaO₁₀ 583.2514).

Preparation of 4 β -Imidazolylcarbonyloxywithanolide E (9).

To a solution of 4 β -hydroxywithanolide E (**5**, 10.0 mg) in anhydrous CH₂Cl₂ (1.0 mL) was added 1,1'-carbonyldiimidazole (8.0 mg) and stirred at 25 °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%).

4 β -Imidazolylcarbonyloxywithanolide E (9).—White amorphous solid; $[\alpha]_{\text{D}}^{25} + 108$ (*c* 0.5, CHCl₃); ¹H NMR (400 MHz, CDCl₃) δ 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, H₂-23), 2.24 (1H, dt, *J* = 11.8, 5.7 Hz, Ha-12), 2.13–2.05 (2H, m, H₂-7), 1.92 (3H, s, H₃-28), 1.86 (3H, s, H₃-27), 1.81 (1H, dd, *J* = 11.0, 6.3 Hz, H-8), 1.70–1.52 (5H, m, H-9, H₂-11, H₂-15), 1.43 (1H, m, Hb-16), 1.42 (3H, s, H₃-19), 1.39 (3H, s, H₃-21), 1.29 (1H, brd, *J* = 11.8 Hz, Hb-12), 1.05 (3H, s, H₃-18); ¹³C NMR (100 MHz, CDCl₃) δ 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 (CH₂, C-16), 36.8 (CH, C-9), 34.3 (CH₂, C-23), 33.9 (CH, C-8), 32.3 (CH₂, C-15), 29.4 (CH₂, C-12), 25.6 (CH₂, C-7), 20.9 (CH₂, C-11), 20.7 (CH₃, C-28), 20.2 (CH₃, C-18), 19.7 (CH₃, C-21), 15.4 (CH₃, C-19), 12.4 (CH₃, 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 C₃₂H₄₀N₂NaO₉ 619.2632).

Oxidation of 4β-Hydroxywithanolide E (5) to Withaperuvine E (10).

To a solution **5** (10.0 mg) in CHCl₃/EtOAc (1:1, 3.0 mL) was added activated MnO₂ (60.0 mg) and stirred at 25 °C. After 6 h, reaction mixture was passed through a short column of silica gel (1.0 g) using CH₂Cl₂/MeOH (94:6) as eluent to give **10** (8.1 mg, 81%) as a pale yellow solid; [α]_D²⁵ − 74 (*c* 0.5, CH₃CN) [lit.⁴² −72.7 (*c* 0.7, CH₃CN)]; ¹H and ¹³C NMR data were consistent with those reported.⁴²

Reduction of Withaperuvine E (10) to 4α-Hydroxywithanolide E (11).

To stirred solution of **10** (4.0 mg) in MeOH (1.0 mL) was added CeCl₃·7H₂O (30.0 mg). The reaction mixture was cooled to 0 °C and stirred for 5 min. To this mixture was added NaBH₄ (~0.5 mg) and stirred at 0 °C. After 5 min, the reaction mixture was quenched with ice, evaporated under reduced pressure, and extracted with EtOAc (3 × 5 mL). Combined organic layer was washed with brine, dried over anhydrous Na₂SO₄, and evaporated under reduced pressure, and the resulting residue was chromatographed over a column of silica gel (500.0 mg) made up in CH₂Cl₂ and eluted with CH₂Cl₂ containing increasing amounts of MeOH. Fractions eluted with CH₂Cl₂/MeOH (94:6) were combined and evaporated under reduced pressure and the crude product was further purified by RP-HPLC using MeOH/H₂O (70:30) as eluent to give **11** (3.0 mg, 75%, *t*_R = 15 min).

4α-Hydroxywithanolide E (11).—White amorphous solid; [α]_D²⁵ + 65 (*c* 0.2, CHCl₃); ¹H NMR (400 MHz, CDCl₃) δ 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, H₂-23), 2.24 (1H, dt, *J* = 12.5, 4.9 Hz, Ha-12), 2.13 (1H, d, *J* = 2.8 Hz, D₂O exchangeable, OH-4), 1.98 (2H, m, H₂-7), 1.91 (3H, s, H₃-28), 1.85 (3H, s, H₃-27), 1.83 (2H, m, H-8, Ha-11), 1.69–1.53 (4H, m, H-9, Hb-11, H₂-15), 1.42 (1H, dd, *J* = 15.1, 8.3 Hz, Hb-16), 1.39 (3H, s, H₃-21), 1.27 (1H, brd, *J* = 12.5 Hz, Hb-12), 1.20 (3H, s, H₃-19), 1.06 (3H, s, H₃-18); ¹³C NMR (100 MHz, CDCl₃) δ 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 (CH₂, C-16), 37.8 (CH, C-9), 34.2 (CH₂, C-23), 34.1 (CH, C-8), 32.3 (CH₂, C-15), 29.7 (CH₂, C-12), 25.6 (CH₂, C-7), 21.6 (CH₂, C-11), 20.6 (CH₃, C-28), 20.4 (CH₃, C-18), 19.7 (CH₃, C-21), 13.2 (CH₃, C-19), 12.4 (CH₃, C-27); HRESI-MS *m/z* 525.2463 [M + Na]⁺ (calcd for C₂₈H₃₈NaO₈ 525.2463).

4 α -Acetoxywithanolide E (12).—Acetylation of **11** by the general procedure afforded **12** as an amorphous white solid; $[\alpha]_D^{25} + 100$ (c 0.1, CHCl₃); ¹H NMR (400 MHz, CDCl₃) δ 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, H₂-23), 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, H₃-28), 1.90–1.82 (2H, m), 1.86 (3H, s, H₃-27), 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, H₃-21), 1.28 (1H, m, Hb-12), 1.27 (3H, s, H₃-19), 1.06 (3H, s, H₃-18); ¹³C NMR (100 MHz, CDCl₃) δ 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 (CH₂, C-16), 37.7 (CH, C-9), 34.3 (CH₂, C-23), 34.0 (CH, C-8), 32.4 (CH₂, C-15), 29.8 (CH₂, C-12), 25.5 (CH₂, C-7), 21.9 (CH₂, C-11), 20.8 (CH₃, C-28), 20.6 (CH₃, C-18), 20.4 (CH₃, OAc), 19.7 (CH₃, C-21), 13.9 (CH₃, C-19), 12.4 (CH₃, C-27); HRESI-MS m/z 567.2565 [M + Na]⁺ (calcd for C₃₀H₄₀NaO₉ 567.2563).

Conversion of Withaperuvine E (10) to Withaperuvine M (13).

To a stirred solution **10** (23.0 mg) in anhydrous CH₂Cl₂ (2.0 mL) at 0 °C was added a solution of Ph₃P (14.6 mg) and I₂ (14.0 mg) in CH₂Cl₂ (0.3 mL). Ice bath was removed, and reaction mixture was stirred at 25 °C. The reaction was monitored by TLC, and after disappearance of the starting material, the reaction mixture was diluted with CH₂Cl₂ (25.0 mL), washed with aq Na₂S₂O₃ solution, H₂O, dried over anhydrous Na₂SO₄, and evaporated under reduced pressure to give crude product mixture (35.0 mg). This was separated by RP-HPLC using MeOH/H₂O (65:35) to give **13** (9.8 mg, 44%). ¹H and ¹³C NMR data were consistent with those reported;⁴³ APCI-MS (+) (m/z) 485 [M + 1]⁺.

Conversion of Withaperuvine 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 °C was added CeCl₃·7H₂O (100.0 mg) and stirred at 0 °C for 5 min. To this solution was added NaBH₄ (~0.5 mg), and the mixture continued stirring at 0 °C. After 5 min, the reaction mixture was quenched with ice, and solvents were evaporated under reduced pressure and extracted with EtOAc (3 × 5.0 mL). Combined organic layer was washed with brine, dried over anhydrous Na₂SO₄, and evaporated under reduced pressure to give the crude product (9.8 mg) which was purified by silica gel preparative TLC using CH₂Cl₂/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, CHCl₃); ¹H NMR (400 MHz, CDCl₃) δ 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, H₂-23), 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, H₃-28), 1.83 (3H, s, H₃-27), 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, H₃-21), 1.28 (1H, brd, J = 14.5 Hz, Hb-12), 1.21 (3H, s, H₃-19), 1.08 (3H, s,

H₃-18); ¹³C NMR (100 MHz, CDCl₃) δ 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 (CH₂, C-16), 36.7 (CH, C-9), 36.2 (CH, C-8), 34.3 (CH₂, C-23), 32.4 (CH₂, C-15), 30.3 (CH₂, C-12), 25.2 (CH₂, C-7), 22.6 (CH₂, C-11), 20.6 (CH₃, C-28), 20.5 (CH₃, C-18), 19.5 (CH₃, C-21), 19.2 (CH₃, C-19), 12.2 (CH₃, C-27); HRESI-MS *m/z* 509.2500 [M + Na]⁺ (calcd for C₂₈H₃₈NaO₇ 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 Et₃N (20 μL) was added 10% Pd/C (0.5 mg), and the mixture was stirred under an atmosphere of H₂ 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 CHCl₃/MeOH (95:5) to afford **15** (2.6 mg) and **24** (1.5 mg).

2,3-Dihydrophysachenolide C (15).—Off-white amorphous powder; [α]_D²⁵ + 3 (c 0.1, MeOH); ¹H NMR (400 MHz, CDCl₃) δ 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-6a), 2.05 (3H, s, 18-OAc), 1.91 (3H, s, CH₃-28), 1.87 (3H, s, CH₃-27), 1.39 (3H, s, CH₃-21), 1.12 (3H, s, CH₃-19); ¹³C NMR (100 MHz, CDCl₃) δ 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 (CH₂, C-18), 64.3 (C, C-5), 60.9 (CH, C-6), 57.6 (C, C-13), 52.4 (C, C-10), 38.2 (CH₂, C-16), 35.6 (CH, C-9), 34.7 (CH₂, C-2), 33.9 (CH₂, C-23), 33.7 (CH, C-8), 32.9 (CH₂, C-15), 30.0 (CH₂, C-4), 26.6 (CH₂, C-7), 25.2 (CH₂, C-12), 21.4 (CH₂, C-11), 21.3 (CH₃, 18-OAc), 20.7 (CH₃, C-28), 19.1 (CH₃, C-21), 17.8 (CH₂, C-3), 12.5 (CH₃, C-19), 12.4 (CH₃, C-27); APCI-MS (+) *m/z* 529 [MH – H₂O]⁺.

18-Deacetyl-2,3-dihydrophysachenolide C (24).—Off-white amorphous powder; [α]_D²⁵ – 20 (c 0.04, MeOH); ¹H NMR (400 MHz, CDCl₃) δ 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-6a), 1.91 (3H, s, CH₃-28), 1.86 (3H, s, CH₃-27), 1.39 (3H, s, CH₃-21), 1.12 (3H, s, CH₃-19); ¹³C NMR (100 MHz, CDCl₃) δ 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 (CH₂, C-18), 60.9 (CH, C-6), 58.7 (C, C-13), 52.5 (C, C-10), 37.5 (CH₂, C-16), 35.6 (CH, C-9), 34.6 (CH₂, C-2), 34.1 (CH₂, C-23), 33.4 (CH, C-8), 33.1 (CH₂, C-15), 30.0 (CH₂, C-4), 26.5 (CH₂, C-7), 24.1 (CH₂, C-12), 21.3 (CH₂, C-11), 20.7 (CH₃, C-28), 19.0 (CH₃, C-21), 17.7 (CH₂, C-3), 12.5 (CH₃, C-19), 12.4 (CH₃, C-27); APCI-MS (+) *m/z* 527 [M + Na]⁺.

Preparation of 2,3-Dihydro-3β-azidophysachenolide C (16).

To a solution of **3** (7.0 mg) in anhydrous CH₂Cl₂ (0.8 mL) were added AcOH (13.35 μL), Et₃N (1.82 μL), and TMS-N₃ (30.8 μL), and the mixture was stirred at 25 °C. After 20 h, the reaction mixture was quenched with aqueous NH₄Cl and extracted with EtOAc (3 × 10 mL).

Combined organic layer was washed with brine, dried over Na₂SO₄, 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 **16** (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, CHCl₃); ¹H NMR (400 MHz, CDCl₃) δ 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, H₂-23), 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, H₃-28), 1.87 (3H, s, H₃-27), 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, H₃-21), 1.37 (1H, m, Ha-11), 1.26 (1H, m, Hb-11), 1.13 (3H, s, H₃-19); ¹³C NMR (100 MHz, CDCl₃) δ 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 (CH₂, 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 (CH₂, C-2), 38.2 4 (CH₂, C-16), 36.3 (CH₂, C-4), 35.4 (CH, C-9), 33.9 (CH₂, C-23), 33.6 (CH, C-8), 32.9 (CH₂, C-15), 26.1 (CH₂, C-12), 25.2 (CH₂, C-6), 21.5 (CH₂, C-11), 21.3 (CH₃, OAc), 20.7 (CH₃, C-28), 19.1 (CH₃, C-21), 13.1 (CH₃, C-19), 12.4 (CH₃, C-27); HRESI-MS *m/z* 610.2735 [M + Na]⁺ (calcd for C₃₀H₄₁N₃NaO₉ 610.2740).

Preparation of 2,3-Dihydro-3β-imidazolylphysachenolide C (17).

To a solution of **3** (5.0 mg) in anhydrous CH₂Cl₂ (1.0 mL) was added imidazole (8.0 mg), and the mixture was stirred at 25 °C. After 6 h, reaction mixture was diluted with EtOAc (15.0 mL), washed with brine (3 × 10.0 mL), dried over anhydrous Na₂SO₄, 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/CH₂Cl₂ (1:4) and eluted with hexanes/CH₂Cl₂ (1:4) followed by CH₂Cl₂/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, CHCl₃); ¹H NMR (400 MHz, CDCl₃) δ 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, H₂-23), 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, H₃-28), 1.86 (3H, s, H₃-27), 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, H₃-21), 1.34–1.24 (2H, m, H₂-11), 1.19 (3H, s, H₃-19); ¹³C NMR (100 MHz, CDCl₃) δ 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

(CH₂, 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 (CH₂, C-2), 38.3 (CH₂, C-4), 38.1 (CH₂, C-16), 35.6 (CH, C-9), 33.9 (CH₂, C-23), 33.5 (CH, C-8), 32.9 (CH₂, C-15), 26.2 (CH₂, C-12), 25.2 (CH₂, C-7), 21.5 (CH₂, C-11), 21.4 (CH₃, OAc), 20.7 (CH₃, C-28), 19.1 (CH₃, C-21), 13.1 (CH₃, C-19), 12.4 (CH₃, C-27); HRESI-MS *m/z* 635.2940 [M + Na]⁺ (calcd for C₃₃H₄₄N₂NaO₉ 635.2945).

Preparation of 2,3-Dihydro-3β-methoxyphysachenolide C (18).

To a solution of **17** (2.8 mg) in MeOH (0.5 mL) was added Et₃N (5.0 μL) and stirred at 60 °C. After 1 h reaction mixture was evaporated under reduced pressure and the resulting residue was purified by RP-HPLC using MeOH/H₂O (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;

[α]_D²⁵ − 142 (*c* 0.1, CHCl₃); ¹H NMR (400 MHz, CDCl₃) δ 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, OCH₃), 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, H₂-23), 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, H₃-28), 1.87 (3H, s, H₃-27), 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, H₃-21), 1.30–1.27 (2H, m, H₂-11), 1.12 (3H, s, H₃-19); ¹³C NMR (100 MHz, CDCl₃) δ 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 (CH₂, C-18), 62.1 (C, C-5), 61.5 (CH, C-6), 57.6 (C, C-13), 56.0 (CH₃, OCH₃), 52.0 (C, C-10), 42.7 (CH₂, C-2), 38.2 (CH₂, C-16), 36.4 (CH₂, C-4), 35.4 (CH, C-9), 33.9 (CH₂, C-23), 33.6 (CH, C-8), 32.9 (CH₂, C-15), 26.3 (CH₂, C-12), 25.2 (CH₂, C-7), 21.5 (CH₂, C-11), 21.3 (CH₃, OAc), 20.7 (CH₃, C-28), 19.2 (CH₃, C-21), 13.0 (CH₃, C-19), 12.4 (CH₃, C-27); HRESI-MS *m/z* 599.2829 [M + Na]⁺ (calcd for C₃₁H₄₄NaO₁₀ 599.2832).

Epoxidation of 15α-Hydroxyphysachenolide D.

To a solution of 15α-hydroxyphysachenolide D (**36**)²⁰ (5.5 mg) in anhydrous CH₂Cl₂ (0.5 mL) at 0 °C was added *m*-CPBA (4.8 mg), and the mixture was stirred for 30 min. Ice bath was then removed and reaction mixture was stirred at 25 °C for additional 2 h, after which CH₂Cl₂ was evaporated under reduced pressure and the resulting residue was separated by silica gel preparative TLC using CH₂Cl₂/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; [α]_D²⁵ + 68 (*c* 0.2, CHCl₃); ¹H NMR (400 MHz, CDCl₃) δ 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, H₂-7), 2.03 (3H, s, OAc), 1.91 (1H, dd, J = 15.5, 8.5 Hz, Hb-16), 1.87 (3H, s, H₃-28), 1.81 (3H, s, H₃-27), 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, H₃-19), 1.28 (3H, s, H₃-21), 1.17 (1H, ddd, J = 18.7, 13.2, 5.5 Hz, Hb-11); ¹³C NMR (100 MHz, CDCl₃) 203.0 (C, C-1), 170.8 (C, OCOCH₃), 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 (CH₂, 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 (CH₂, C-23), 33.8 (CH₂, C-4), 31.8 (CH, C-9), 25.5 (CH₂, C-12), 23.9 (CH₂, C-7), 21.9 (CH₂, C-11), 21.2 (CH₃, OAc), 20.6 (CH₃, C-28), 15.2 (CH₃, C-19), 12.1 (CH₃, 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 C₃₀H₄₀NaO₁₀ 583.2519).

15 α -Hydroxy-5,6-epi-physachenolide C (23).—White amorphous solid; $[\alpha]_D^{25} + 73$ (c 0.1, CHCl₃); ¹H NMR (400 MHz, CDCl₃) δ 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, H₂-23), 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, H₃-28), 1.87 (3H, s, H₃-27), 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, H₃-21), 1.22 (3H, s, H₃-19); ¹³C NMR (100 MHz, CDCl₃) 203.1 (C, C-1), 169.8 (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 (CH₂, 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 (CH₂, C-23), 32.8 (CH₂, C-4), 26.3 (CH₂, C-12), 26.0 (CH₂, C-7), 22.9 (CH₂, C-11), 21.3 (CH₃, OAc), 20.7 (CH₃, C-28), 14.9 (CH₃, C-19), 12.3 (CH₃, C-27); APCI-MS (+) m/z 561 [M + H]⁺; APCI-MS (–) m/z 559 [M – H][–]; HRESIMS m/z 583.2525 [M + Na]⁺ (calcd for C₃₀H₄₀NaO₁₀ 583.2519).

Epoxidation of 15 α -Acetoxypysachenolide C (28).—To a stirred solution of **28** (10.0 mg) in CHCl₃ (1.0 mL) was added *m*-CPBA (6.0 mg), and the mixture was stirred at 25 °C 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 CHCl₃/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 -acetoxypysachenolide C by comparison of its NMR and MS data with those reported.²⁰

15 α -Acetoxy-5,6-epi-physachenolide C (22).—Off-white amorphous powder; $[\alpha]_D^{25} + 83$ (c 0.11, MeOH); ¹H NMR (400 MHz, CDCl₃) δ 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, CH₃-28), 1.87 (3H, s, CH₃-27), 1.38 (3H, s, CH₃-21), 1.33 (3H, s, CH₃-19); ¹³C NMR (100 MHz, CDCl₃) δ 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 (CH₂, C-18), 64.2 (C, C-5), 58.5 (CH, C-6), 57.5 (C, C-13), 48.5 (C, C-10), 43.6 (CH₂, C-16), 35.6 (CH, C-9), 33.9 (2 × CH₂, C-4 and C-23), 31.7 (CH, C-8), 25.9 (CH₂, C-12), 23.2 (CH₂, C-7), 21.8 (CH₂, C-11), 21.5 (CH₃, 15-OAc), 21.3 (CH₃, 18-OAc), 20.6 (CH₃, C-28), 19.6 (CH₃, C-21), 15.2 (CH₃, C-19), 12.4 (CH₃, 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 CH₂Cl₂ (0.4 mL) at −5 °C was added a 1.0 M solution of *t*-BuOK in *t*-BuOH (60 μL), and the mixture was stirred at −5 °C. After 45 min, the reaction mixture was diluted with EtOAc (15 mL) and washed with H₂O (3 × 10 mL). Organic layer was evaporated under reduced pressure and the residue was separated by silica gel preparative TLC using CH₂Cl₂/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; [α]_D²⁵ + 87 (*c* 0.1, CHCl₃); ¹H NMR (400 MHz, CDCl₃) δ 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, H₂-7), 1.91 (3H, s, H₃-28), 1.90 (1H, m, H-9), 1.87 (3H, s, H₃-27), 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, H₂-15, Hb-16), 1.53 (1H, dt, *J* = 14.5, 2.1 Hz, Hb-11), 1.38 (3H, s, H₃-21), 1.22 (3H, s, H₃-19); ¹³C NMR (100 MHz, CDCl₃) 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 (CH₂, C-18), 62.2 (C, C-5), 58.7 (C, C-13), 48.5 (C, C-10), 37.5 (CH₂, C-16), 36.8 (CH, C-9), 34.2 (CH₂, C-23), 34.0 (CH, C-8), 33.3 (CH₂, C-15), 32.6 (CH₂, C-4), 26.3 (CH₂, C-7), 24.6 (CH₂, C-12), 22.7 (CH₂, C-11), 20.7 (CH₃, C-28), 19.0 (CH₃, C-21), 14.7 CH₃, C-19), 12.4 CH₃, C-27); HRESI-MS *m/z* 525.2461 [M + Na]⁺ (calcd for C₂₈H₃₈NaO₈ 525.2464).

Epoxidation of Physachenolide D (4).

To a stirred solution of **4** (20.0 mg) in CHCl₃ (2.0 mL) was added *m*-CPBA (12.0 mg), and the mixture was stirred at 25 °C for 4 h (TLC control). The reaction mixture was concentrated under reduced pressure and the product mixture was separated by C₁₈ 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 CHCl₃/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

24 α ,25 α -Epoxyphysachenolide C (26).—Off-white amorphous powder; [α]_D²⁵ + 54 (*c* 0.85, MeOH); ¹H NMR (400 MHz, CDCl₃) δ 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-6 α), 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, CH₃-28), 1.45 (3H, s, CH₃-27), 1.29 (3H, s, CH₃-21), 1.22 (3H, s, CH₃-19); ¹³C NMR (100 MHz, CDCl₃) δ 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 (CH₂, 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 (CH₂, C-16), 36.9 (CH, C-9), 34.2 (CH, C-8), 32.9 (CH₂, C-4), 32.8 (CH₂, C-15), 29.7 (CH₂, C-23), 26.4 (CH₂, C-7), 25.4 (CH₂, C-12), 22.8 (CH₂, C-11), 21.3 (CH₃, OAc), 18.8 (CH₃, C-21), 17.9 (CH₃, C-28), 14.6 (CH₃, C-19), 13.5 (CH₃, C-27); HRESI-MS m/z 583.2511 [M + Na]⁺ (calcd for C₃₀H₄₀O₁₀Na, 583.2519).

Preparation of 15 α ,28-Diacetoxypsychachenolide D (32).

Acetylation of 15 α -acetoxy-28-hydroxypsychachenolide D (30) by the general procedure afforded 32 as an off-white amorphous powder; $[\alpha]_D^{25} + 61$ (c 0.02, MeOH); ¹H NMR (400 MHz, CDCl₃) δ 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, CH₃-27), 1.40 (3H, s, CH₃-21), 1.21 (3H, s, CH₃-19); APCI-MS (+) m/z 667 [M + Na]⁺.

Preparation of 15 α ,27-Diacetoxypsychachenolide D (33).

Acetylation of 15 α -acetoxy-27-hydroxypsychachenolide D (31) by the general procedure afforded 33 as an off-white amorphous powder; $[\alpha]_D^{25} + 51$ (c 0.04, MeOH); ¹H NMR (400 MHz, CDCl₃) δ 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 CH₃-28), 1.39 (3H, s, CH₃-21), 1.21 (3H, s, CH₃-19); 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 °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 °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: $GI = [(media - treatment)/media] \times 100$. The assay was performed in RPMI with 5% FCS, 2.0 mM L-glutamine, 1 $\times$ nonessential amino acids, 1.0 mM sodium pyruvate, 100 U/mL penicillin, 100 μ g/mL streptomycin, 10 mM HEPES, and 5×10^{-5} M 2-mercaptoethanol.

Immunoblotting.

ACHN cells were grown in RPMI (RPMI, 5% FBS, Pen-strep, NEAA, HEPES, Glutamax, sodium pyruvate, 2ME), and 1.5×10^6 cells/well were plated in Costar 6-well plates and then incubated overnight at 37 °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 and 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 μ M zVAD-FMK and Pierce universal nuclease. Protein concentrations were measured using the Pierce BCA protein assay (Thermo Fisher Scientific, Waltham, MA). 20 μ 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 no. 9496 1:1000, cleaved caspase-8 (Asp384) (11G10) mouse mAb 1:5000, caspase-8 (1C12) mouse mAb no. 9746 1:5000, all from Cell Signaling Technology, Danvers, MA), anti-FLIP (7F10 1.0 μ 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 μ M withanolide (**1**, **2a**, **3**, **5**, **7**, **8**, **11**, **12**, or **19**) in DMSO was mixed with solution B (0.5 mL) containing 4.24 μ M GSH (or NAC), and the mixture was incubated at 37 °C. The test samples (100 μ 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 °C for subsequent HPLC analysis. All analyses were performed on a HPLC system equipped with a Hitachi AS-4000 Intelligent autosampler, Hitachi L-6200A Intelligent pump, Hitachi L-4500 photodiode array detector, Shimadzu ELSD-LT detector, and a Phenomenex Luna 5 μ m C18 (2) 100 Å HPLC column (4.6 mm $\times$ 250 mm) with gradient elution using H₂O (containing 40 mM NH₄OAc)–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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

17-BHW	17 β -hydroxywithanolide
DcR	decoy receptor
ELSD	evaporative light scattering detector
cFLIP	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	heteronuclear multibond correlation
HPLC	high-pressure liquid chromatography
IAP	inhibitor of apoptosis proteins
NOE	nuclear Overhauser effect
PBS	phosphate buffered saline
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

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

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

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

- 27** $R_1 = \text{OSO}_3\text{H}; R_2 = R_3 = R_4 = \text{H}$
28 $R_1 = R_3 = R_4 = \text{H}; R_2 = \text{OAc}; \Delta^{2,3}$
29 $R_1 = \text{OSO}_3\text{H}; R_2 = \text{OAc}; R_3 = R_4 = \text{H}$
30 $R_1 = R_4 = \text{H}; R_2 = \text{OAc}; R_3 = \text{OH}; \Delta^{2,3}$
31 $R_1 = R_3 = \text{H}; R_2 = \text{OAc}; R_4 = \text{OH}; \Delta^{2,3}$
32 $R_1 = R_4 = \text{H}; R_2 = R_3 = \text{OAc}; \Delta^{2,3}$
33 $R_1 = R_3 = \text{H}; R_2 = R_4 = \text{OAc}; \Delta^{2,3}$
34 $R_1 = R_4 = \text{H}; R_2 = \text{OAc}; R_3 = \text{OGlu}; \Delta^{2,3}$
35 $R_1 = R_3 = \text{H}; R_2 = \text{OAc}; R_4 = \text{OGlu}; \Delta^{2,3}$
36 $R_1 = R_3 = R_4 = \text{H}; R_2 = \text{OH}; \Delta^{2,3}$

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 IC₅₀ values (concentrations required to reduce cell number by 50%) were determined from three independent experiments using Microsoft Excel software with $\pm$ 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 α -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 indicate $\pm$ SD. At least three experiments with similar findings were performed.

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

Figure 8.

Effects of WE (**1**), PCC (**3**), 4α-hydroxywithanolide E (**11**), and WA (**2a**) (at concentrations ranging from 125 to 1000 nM for 18 h) on cleavage of procaspase-3 and procaspase-8 following exposure to TRAIL (50 ng/mL for 90 min). The cleaved forms of procaspase-3 and procaspase-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 IC₅₀ values (concentrations required to reduce cell number by 50%) were determined from three 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 β -hydroxywithanolide E (**5**), 4 β -acetoxywithanolide E (**7**), 4 β -methoxycarbonyloxywithanolide E (**8**), 4 α -hydroxywithanolide E, and (**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

^aConcentration of withanolide (nM) that in combination with TRAIL (50 ng/mL) reduced ACHN cell number by 50% after 18 h of exposure. IC₅₀ values were determined from dose–response curves using Microsoft Excel software. Values are from at least three independent experiments; ± refers to standard deviation.