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Withaferin A and Withanolide D Analogues with Dual Heat-Shock-Inducing and Cytotoxic Activities: Semisynthesis and Biological Evaluation

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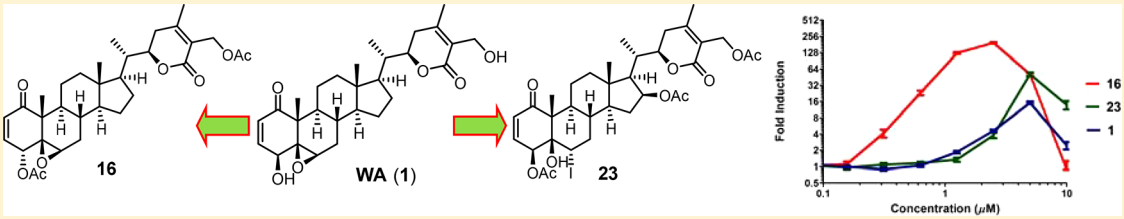
Supporting Information



ABSTRACT: Withanolides constitute a valuable class of bioactive natural products because some members of the class are known to exhibit cytotoxic activity and also induce a cytoprotective heat-shock response. In order to understand the relationship between their structures and these dual bioactivities of the withanolide scaffold, we obtained 25 analogues of withaferin A (WA) and withanolide D (WD) including 17 new compounds by semisynthesis involving chemical and microbial transformations. Hitherto unknown 16*β*-hydroxy analogues of WA and WD were prepared by their reaction with triphenylphosphine/iodine, providing unexpected 5*β*-hydroxy-6*α*-iodo analogues (iodohydrins) followed by microbial biotransformation with *Cunninghamella echinulata* and base-catalyzed cyclization of the resulting 16*β*-hydroxy iodohydrins. Evaluation of these 25 withanolide analogues for their cytotoxicity and heat-shock-inducing activity (HSA) confirmed the known structure−activity relationships for WA-type withanolides and revealed that WD analogues were less active in both assays compared to their corresponding WA analogues. The 5*β*,6*β*-epoxide moiety of withanolides contributed to their cytotoxicity but not HSA. Introduction of a 16*β*-OAc group to 4,27-di-*O*-acetyl-WA enhanced cytotoxicity and decreased HSA, whereas introduction of the same group to 4-*O*-acetyl-WD decreased both activities.

Plants used in traditional medicines constitute valuable resources for the identification of therapeutic leads with a variety of structural scaffolds and biological activities.[1] *Withania somnifera* (L.) Dunal (Indian ginseng, "Ashwagandha" in Ayurveda) is a prominent plant in traditional Indian medicine,[2] with claimed anticancer,[3] neuroprotective,[4] anti-inflammatory,[5] antioxidant,[2] and immunomodulatory[6] activities. Molecular pharmacological studies have tied many of these biological activities of *W. somnifera* to its constituent withanolides, a group of steroidal lactones, including withaferin A (**1**, WA) and withanolide D (**2**, WD) (Figure 1).[7] WA, the most extensively studied among withanolides, has been reported to have cytotoxic activity against breast carcinoma,[8] cervical cancer,[9] cutaneous melanoma,[10] glioma,[11] leukemia,[12] ovarian carcinoma,[13] pancreatic cancer,[14] prostate adenocarcinoma,[15] and renal carcinoma[16] cell lines. Intriguingly, WA and some of its analogues have also been found to induce the cytoprotective heat-shock response.[17,18] Biological activities reported for WD (**2**) include cytotoxicity against lymphoma,[19] pancreatic adenocarcinoma,[20] and leukemia[21] cell lines. Studies in mice have demonstrated anticancer activity for WA against brain,[17] breast,[8] prostate,[15] and pancreatic[14a] cancers and immunomodulatory activity for WD.[22]

Structurally, WA (**1**) and WD (**2**) are isomeric withanolides that feature 2(3)-en-1-one, 4*β*-hydroxy, and 5*β*,6*β*-epoxy moieties in their A and B rings. They differ from each other only in the position of the additional hydroxy group present in their side chain (C-27 in **1** and C-20 in **2**) (see Figure 1). Although **1** has been subjected to a variety of chemical and

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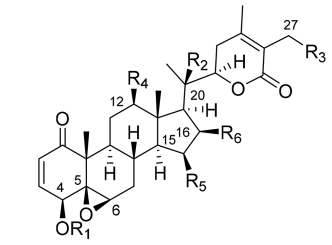

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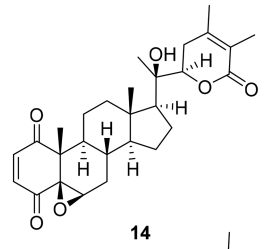
4 $R_1 = R_2 = R_4 = R_6 = H, R_3 = R_5 = OH$
5 $R_1 = R_2 = R_4 = R_5 = H, R_3 = R_6 = OH$
6 $R_1 = Ac, R_2 = R_4 = R_5 = R_6 = H, R_3 = OAc$
7 $R_1 = Ac, R_2 = R_5 = R_6 = H, R_3 = R_4 = OAc$
8 $R_1 = Ac, R_2 = R_4 = R_5 = H, R_3 = R_6 = OAc$
9 $R_1 = R_3 = R_4 = R_6 = H, R_2 = R_5 = OH$
10 $R_1 = R_3 = R_4 = R_5 = H, R_2 = R_6 = OH$
11 $R_1 = Ac, R_2 = OH, R_3 = R_4 = R_5 = R_6 = H$
12 $R_1 = Ac, R_2 = OH, R_3 = R_4 = R_6 = H, R_5 = OAc$
13 $R_1 = Ac, R_2 = OH, R_3 = R_4 = R_5 = H, R_6 = OAc$



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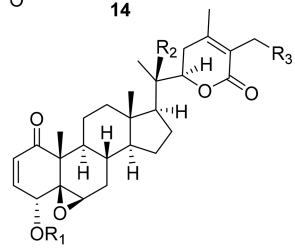
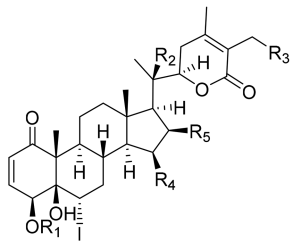


15 $R_1 = R_2 = H, R_3 = OH$
16 $R_1 = Ac, R_2 = H, R_3 = OAc$
17 $R_1 = R_3 = H, R_2 = OH$
18 $R_1 = Ac, R_2 = OH, R_3 = H$



19 $R_1 = R_2 = R_4 = R_5 = H, R_3 = OH$
20 $R_1 = R_2 = R_4 = H, R_3 = R_5 = OH$
21 $R_1 = R_2 = R_5 = H, R_3 = R_4 = OH$
22 $R_1 = Ac, R_2 = R_4 = R_5 = H, R_3 = OAc$
23 $R_1 = Ac, R_2 = R_4 = H, R_3 = R_5 = OAc$
24 $R_1 = Ac, R_2 = R_5 = H, R_3 = R_4 = OAc$
25 $R_1 = R_3 = R_4 = R_5 = H, R_2 = OH$
26 $R_1 = R_3 = R_4 = H, R_2 = R_5 = OH$
27 $R_1 = Ac, R_2 = OH, R_3 = R_4 = R_5 = H$

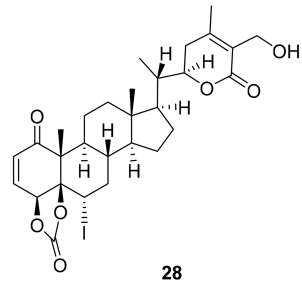


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Figure 1. Structures of withaferin A (**1**), withanolide D (**2**), their analogues **3**−**27**, and the 4,5-cyclic carbonate derivative (**28**) of **19**.

microbial transformations yielding an array of structural analogues, similar studies have not been reported for **2**. Significant chemical transformations of WA (**1**) have involved (i) conversion of the 2(3)-en-1-one to a 1-one functionality;[18] (ii) preparation of 3-substituted 2,3-dihydro analogues by Michael addition of nucleophiles (e.g., azide and alkyl/aryl thiols);[18,23] (iii) preparation of spiro-pyrrolizidine-indole adducts across the 2,3-double bond;[24] (iv) derivatization of 4β-OH and/or 27-OH groups to their corresponding acetates[18] and silyl ethers;[25] (v) oxidation of the 4β-OH to a 4-oxo group followed by reduction leading to the 4α-OH analogue and its derivatization to the acetate;[18] (vi) conversion of the 5β,6β-epoxy moiety into its corresponding 1,4-oxathiane analogues,[26] thiiranes, amino alcohols, and alcohols;[27] and (vii) substitution of the 27-OH group by an azido group.[28] When subjected to microbial biotransformation using the fungus *Cunninghamella echinulata*, **1** afforded its 12β-hydroxy (**3**) and 15β-hydroxy (**4**) analogues,[18,29] which have been converted to their corresponding acetates.[18]

Despite the availability of a considerable number of naturally occurring and semisynthetic analogues of WA (**1**), only a few of these have been subjected to any systematic structure−activity relationship (SAR) studies, and almost all of these have focused on cytotoxic activity. We recently reported evaluation of 24 structural analogues of **1** for cytotoxicity and activation of the cellular heat-shock response, leading to a conclusion that the withanolide scaffold is readily amenable to structural modifications that modulate these activities.[18] Herein we report the preparation and characterization of 25 withanolide analogues derived from **1** and **2** including 17 new compounds. Evaluating the influence of structural changes on the cytotoxic and heat-shock induction activities of these compounds provided interesting new SAR insights. Certain structural modifications of **1** and **2** enhanced cytotoxicity over heat-shock induction activity, while other modifications were found to have an opposite effect.

RESULTS AND DISCUSSION

Withaferin A (**1**) was obtained from aeroponically grown *W. somnifera*.[30] Upon microbial biotransformation with *C. echinulata*, it provided the expected hydroxylation products, 12β-hydroxywithaferin A (**3**) and 15β-hydroxywithaferin A (**4**).[18,29] Other known analogues of WA and their acetates **6**, **7**, **15**, and **16** were obtained as described previously.[18] New analogues **5**, **8**−**10**, **12**, **13**, and **17**−**27** of WA (**1**) and WD (**2**)

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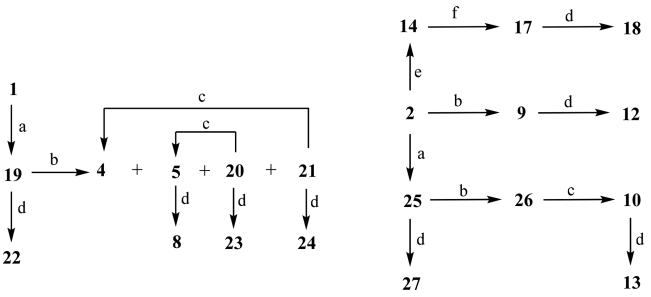
were prepared by the chemical reactions and microbial biotransformations depicted in Scheme 1. Because 5,6-

Scheme 1. Preparation of New Analogues and Derivatives of Withaferin A (1) and Withanolide D (2)[a]

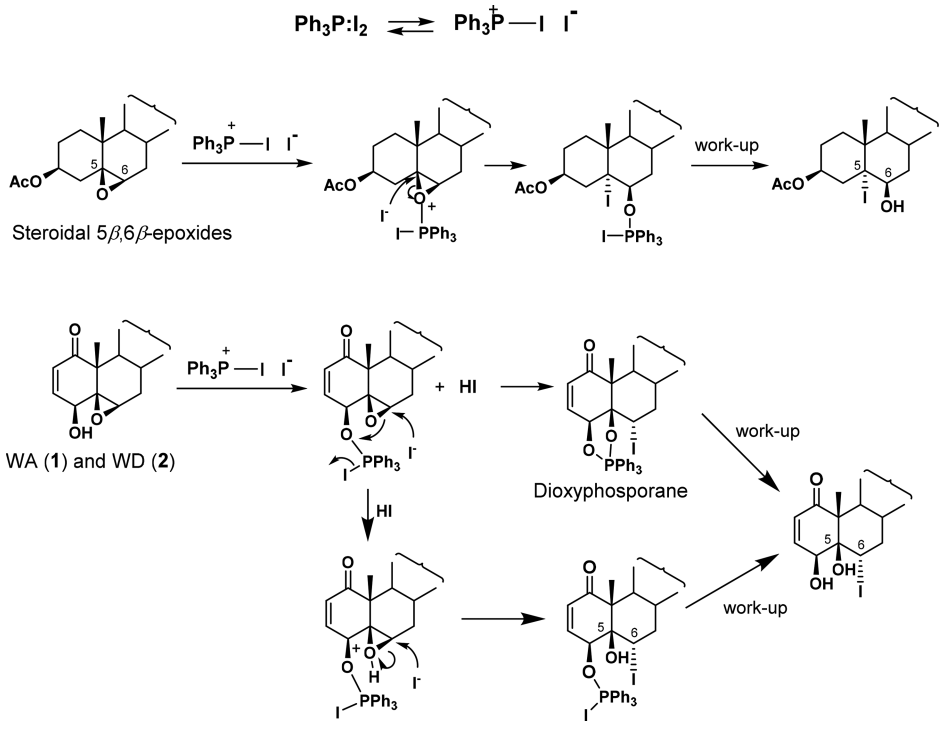


[a]Reagents and conditions: (a) Ph_3P-I_2, CH_2Cl_2, 25 °C, 2 h; (b) microbial biotransformation with *Cunninghamella echinulata*; (c) *N,N*-diisopropylethylamine, CH_2Cl_2, 25 °C, 72 h; (d) Ac_2O, pyridine, 25 °C, 16 h; (e) MnO_2, $CHCl_3$–EtOAc, 25 °C, 6 h; (f) $NaBH_4$, $CeCl_3 \cdot 7H_2O$, MeOH, THF, 0 °C, 5 min.

chlorohydrin analogues of WA have been reported to have potent cytotoxic activity[31] and some iodine-containing compounds have found applications in targeted cancer therapy,[32] it was of interest to prepare and evaluate the new 5,6-iodohydrin analogues of **1** and **2**. Steroidal 5β,6β-epoxides are known to react with phosphine–iodine complexes to yield their corresponding iodohydrin (5α-iodo-6β-hydroxy) analogues (Scheme 2).[33] Treatment of **1** with a Ph_3P-I_2 complex in CH_2Cl_2 at 25 °C afforded its iodohydrin (**19**) in 93% yield. The ^{13}C NMR chemical shift for C-6 (δ_C 38.6) of **19** indicated that it is a 5-hydroxy-6-iodo analogue and not the anticipated 5-iodo-6-hydroxy analogue of WA. Exposure of **19** to Ac_2O–pyridine for an extended period of time afforded the diacetate **22** and not the corresponding triacetate, suggesting that the OH group of **19** resided at C-5 and not C-6. This was confirmed by the formation of the 4,5-cyclic carbonate (**28**) on treatment of **19** with carbonyl diimidazole.[34] The orientation of H-6 in **19** was determined as β based on its ^1H NMR data (δ_H 4.65, dd, J = 13.7, 4.3 Hz)[31b] and the observed NOE association between H-6 and CH_3-19 (Figure S39, Supporting Information). These findings established the structure of **19** as (22*R*)-4β,5β,27-trihydroxy-6α-iodo-1-oxowitha-2,24-dienolide. As noted above, the reaction of steroidal 5β,6β-epoxides with Ph_3P-I_2 has been reported to afford 5α-iodo-6β-hydroxy analogues as major products,[33] and formation of these has been explained as due to the attack by the nucleophile (I^-) at the more substituted position (C-5) of the epoxide ring via a borderline S_N2 mechanism (Scheme 2).[35] However, during the reaction of **1** with Ph_3P-I_2, it is possible that this reagent first reacts with the hydroxy group at C-4.[36] Introduction of the bulky Ph_3PI moiety at this position would then result in steric crowding, causing I^- to react at the less substituted position (C-6) of the epoxide with or without participation of a cyclic dioxyphosphorane that subsequently undergoes ready hydrolysis[37] to afford 5β-hydroxy-6α-iodo analogue **19** as the major product (Scheme 2).

Interestingly, microbial biotransformation of the iodohydrin **19** with *C. echinulata* afforded **20** (27%) and three minor products, **21** (8%), **5** (7%), and **4** (3%). The difference in molecular mass of 16 Da between **19** and its biotransformation products **20** and **21** suggested that these were monohydroxylated analogues of **19**. This was confirmed by the presence of signals due to hydroxymethine groups in the NMR spectra of **20** [δ_H 4.26 (ddd, J = 11.8, 7.4, 4.0 Hz); δ_C 71.2] and **21** [δ_H 4.17 (t, J = 6.3 Hz); δ_C 69.1]. The newly introduced OH groups in **20** and **21** were located at C-16 and C-15,

Scheme 2. Proposed Mechanisms for the Reaction of Triphenylphosphine–Iodine Complex with Steroidal 5β,6β-Epoxides and 4β-Hydroxywithanolides, Withaferin A (1), and Withanolide D (2)



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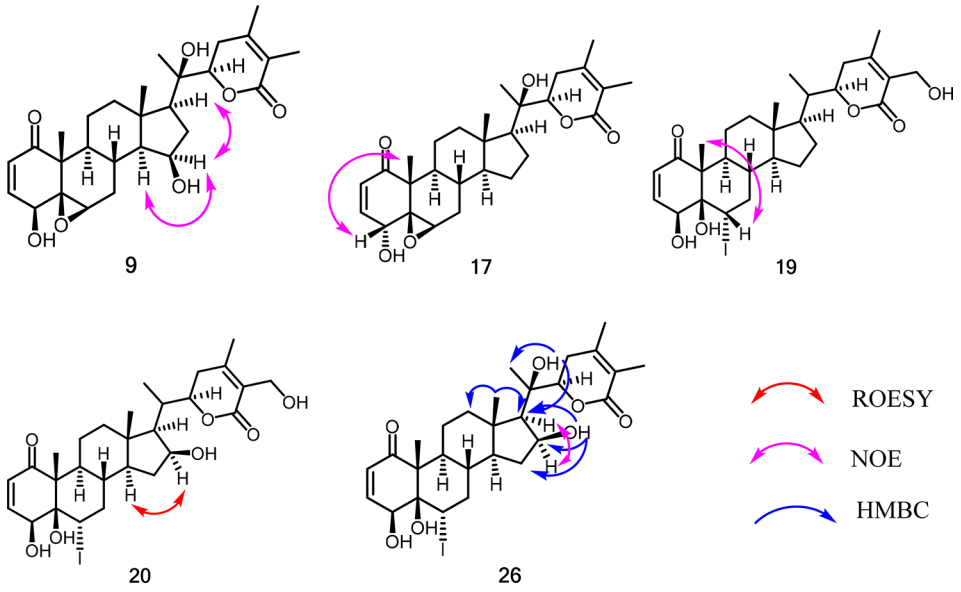

Figure 2. Selected NOE correlations for **9**, **17**, **19**, and **26**, ROESY correlations for **20**, and HMBC correlations for **26**.

respectively, by comparison of their ^{13}C NMR data with those of **19**. Thus, the hydroxymethine carbon of **20** was assigned to C-16 on the basis of the downfield shifts of C-17 ($\Delta\delta_C$ 6.0, β-effect) and C-15 ($\Delta\delta_C$ 12.8, β-effect) and upfield shift of C-14 ($\Delta\delta_C$ −2.7, γ-effect). The orientation of OH-16 in **20** was confirmed as β by the upfield shift of C-20 ($\Delta\delta_C$ −5.2, γ-gauche effect) due to the *syn*-configuration of OH-16 and C-20[38] and the observed ROESY correlation between H-14 (δ_H 0.97) and H-16 (Figure 2). Based on this evidence, the structure of **20** was elucidated as (22*R*)-4β,5β,16β,27-tetrahydroxy-6α-iodo-1-oxowitha-2,24-dienolide. The hydroxymethine carbon of the second biotransformation product (**21**) was assigned to C-15 on the basis of the downfield shifts observed for C-14 ($\Delta\delta_C$ 4.4, β-effect) and C-16 ($\Delta\delta_C$ 13.6, β-effect) compared to chemical shifts observed in the ^{13}C NMR spectrum of **19**.[35] The orientation of OH-15 of **21** was determined as β by its conversion to the known WA analogue 15β-hydroxywithaferin A (**4**).[18] Thus, the structure of **21** was established as (22*R*)-4β,5β,15β,27-tetrahydroxy-6α-iodo-1-oxowitha-2,24-dienolide. The formation of two minor products, 15β-hydroxywithaferin A (**4**)[18] and 16β-hydroxywithaferin A (**5**) (see below), during the biotransformation reaction of the iodohydrin **19** was suspected to be due to the elimination of a molecule of HI from **20** and **21** to form their corresponding 5β,6β-epoxides. This observation was exploited for the chemical conversion of the biotransformation products, **20** to **4** and **5** to **21**. Treatment of **21** with *N*,*N*-diisopropylethylamine in CH_2Cl_2 gave a product identical to 15β-hydroxywithaferin A (**4**) previously obtained as a biotransformation product of WA (**1**).[18,29] Similar treatment of (22*R*)-4β,5β,16β,27-tetrahydroxy-6α-iodo-1-oxowitha-2,24-dienolide (**20**) gave a product that was identified as 16β-hydroxywithaferin A (**5**) by the analysis of its HRMS and NMR data (Figures S1−S4, Supporting Information). To the best of our knowledge, this finding constitutes the first report of microbial biotransformation of a substrate containing an iodine substituent and the ability of *C. echinulata* to hydroxylate at C-16 of a withanolide. As noted above, microbial biotransformation of WA (**1**) with *C. echinulata* has previously afforded 12β-hydroxywithaferin A (**3**) and 15β-hydroxywithaferin A (**4**) as the only products.[21,33] It is possible that the conformational change caused by the absence of the 5β,6β-epoxide moiety in iodohydrin **19** of WA allowed the hydroxylating enzyme of *C. echinulata* to hydroxylate at C-15 and C-16 instead of C-15 and C-12.

Withanolide D (**2**) was obtained from *Acnistus arborescens* (L.) Schlecht (Solanaceae)[39] and *W. somnifera*.[40] Upon microbial biotransformation with *C. echinulata,* it afforded **9** as the only product. The molecular weight difference of 16 Da between **2** and **9** observed by MS and the presence of signals due to a hydroxymethine group [δ_H 4.20 (m); δ_C 69.5] in the NMR spectra of **9** suggested that it was a monohydroxylated product of **2**. The downfield shifts of the signals due to C-14 ($\Delta\delta_C$ 4.7) and C-16 ($\Delta\delta_C$ 12.8) in the ^{13}C NMR spectrum of **9** compared to those of **2** suggested that the microbial hydroxylation has occurred at C-15.[38] The orientation of OH-15 in **9** was determined as α based on strong NOESY correlations observed for H-15 (δ_H 4.20) with H-14 (δ_H 0.78) and H-17 (δ_H 1.54) (see Figure 2 and Figure S15, Supporting Information). Thus, the structure of **9** was elucidated as 15β-hydroxywithanolide D.

Semisynthesis of 4-*epi*-withanolide D (**17**) was efficiently achieved by the MnO_2 oxidation[18,41] of withanolide D (**2**) to 4-dehydrowithanolide D (**14**) followed by regio- and stereoselective reduction of the carbonyl group at C-4 with $NaBH_4$−$CeCl_3$[18] (Scheme 1). The NOE data for **17** confirmed an α-orientation for the OH group at C-4 (Figure S32, Supporting Information). The treatment of WD (**2**) with Ph_3P−I_2 in CH_2Cl_2 at 25 °C afforded **25**. Comparison of its NMR data with those of **19** suggested that the opening of the 5β,6β-epoxide ring of **2** resulted in the formation of an iodohydrin (5β-hydroxy-6α-iodo analogue) similar to the product formed from WA (**1**) upon treatment with Ph_3P−I_2 (Scheme 2). Analysis of its HRMS and NMR data (Figures S61−S64, Supporting Information) identified **25** as (22*R*)-4β,5β,20-trihydroxy-6α-iodo-1-oxowitha-2,24-dienolide. Microbial biotransformation of **25** with *C. echinulata* afforded **26**. The molecular weight difference of 16 Da between **25** and **26** in their mass spectra and the presence of an oxygen-bearing carbon at δ_C 73.6 in the ^{13}C NMR spectrum of **26** suggested it to be a monohydroxylated product of **25**. The proton of this

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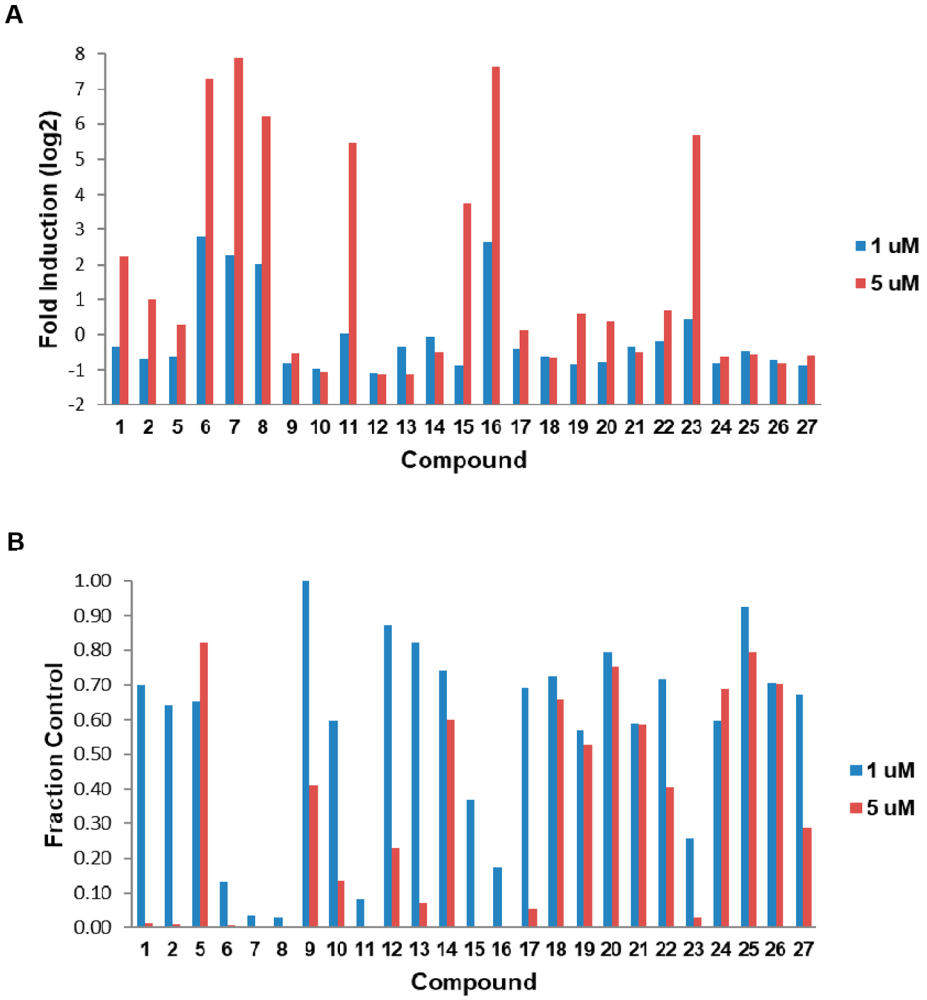

Figure 3. Heat-shock-inducing and cytotoxic activities of withaferin A (**1**), withanolide D (**2**), and their analogues, **5**−**27**. (A) Heat-shock reporter induction in confluent 293T cells after overnight exposure to the indicated compounds at 1.0 and 5.0 μM. (B) Inhibition of 293T cell growth and survival as measured by resazurin dye reduction after 72 h of exposure to the indicated compounds at 1.0 and 5.0 μM. In both panels, the mean of two biological replicates for each determination is depicted.

newly introduced OH group of **26** (δ_H 4.21) showed HMBC correlations with C-15 (δ_C 36.1), C-16 (δ_C 73.6), and C-17 (δ_C 58.0) locating the OH group at C-16 (Figure S69, Supporting Information). The orientation of this OH group was determined to be β based on the observed NOE correlation between H-17 (δ_H 1.27) and H-16 (Figure S70, Supporting Information). Thus, the biotransformation product of **25** was identified as (22*R*)-4β,5β,16β,20-tetrahydroxy-6α-iodo-1-oxo-witha-2,24-dienolide (**26**). As expected, treatment of **26** with *N*,*N*-diisopropylethylamine in CH_2Cl_2 afforded 16β-hydroxywithanolide D (**10**) as the only product. The *O*-acetyl analogues **6**−**8**, **11**−**13**, **16**, **18**, **22**−**24**, and **27** were obtained by the acetylation (Ac_2O−pyridine) of the corresponding hydroxywithanolides (see Experimental Section).

We have previously used the heat-shock response (HSR) as a biosensor to discover potential anticancer agents targeting protein homeostasis. Using this approach, it was found that WA (**1**) and several of its analogues strongly activated the heat-shock factor 1 (HSF1)-dependent stress response as a prominent component of their anticancer activity.[17,18] Because WA and its analogues have also been reported to have potent cytotoxic activity,[18,25,42] it was of interest to investigate the specific structural determinants that might contribute to these distinctive bioactivities for withanolides. Thus, WA (**1**), WD (**2**), and their analogues **5**−**27** were evaluated for their ability to activate the HSR using a cell-based reporter system, and their cytotoxic activity was measured in the same reporter cell line (293T), initially at screening concentrations of 1.0 and 5.0 μM. WA (**1**) was used as a positive control and internal standard for comparison between assays because it possesses both these activities. WA analogues **3** and **4** were previously shown to have no heat-shock-inducing activity (HSA) and therefore were not evaluated in this study.[18] To measure concentration-dependent activation of the HSR of **1**, **2**, and **5**−**27**, serial dilutions of each compound were applied in a 384-well format to human 293T reporter cells stably expressing a green fluorescent protein−firefly luciferase fusion protein under transcriptional control of classical heat-shock promoter elements.[43] After overnight incubation, luciferase activity was determined as a quantitative measure of relative heat-shock activation. Depicted in Figure 3A is Log_2 of the maximal HSA (fold induction) for each of the compounds when tested at concentrations of 1.0 and 5.0 μM. These data suggested that WA analogues were more active than their corresponding WD analogues. WA analogues **6**−**8**, **16**, and **23** exhibited enhanced heat-shock activation compared to WA (**1**) at both concentrations, whereas **15** showed higher

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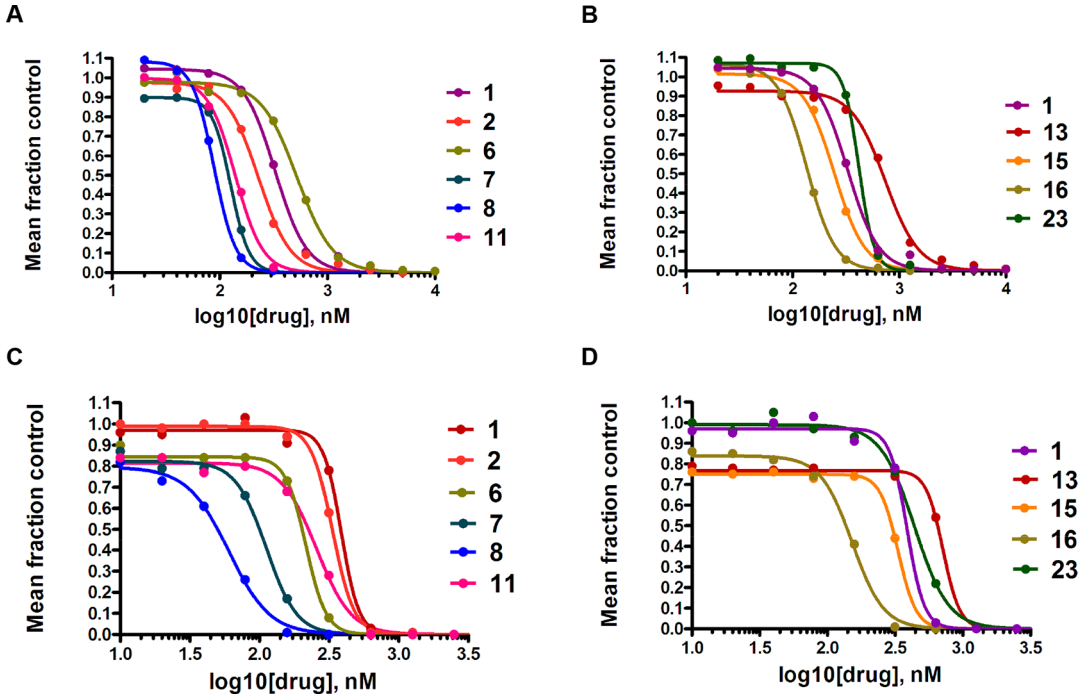

Figure 4. Concentration-dependent inhibition of growth and proliferation of 293T cells (A and B) and CHP-100 cells (C and D) by WA (**1**), WD (**2**), and their active analogues, **6**–**8**, **11**, **13**, **15**, **16**, and **23**, at 10 concentrations. All determinations were performed in 384-well format using quadruplicate replicates.

activity than **1** at 5.0 μM. Although WD (**2**) was less active, its analogues **11**, **13**, and **14** showed the same or higher HSA in comparison to WA (**1**) at a concentration of 1.0 μM.

Next, the cytotoxicity of WA (**1**), WD (**2**), and their analogues **5**–**27** was determined using the same reporter cell line (293T) at 1.0 and 5.0 μM concentrations. Relative cell proliferation and survival were measured by resazurin dye reduction after 72 h of compound exposure.[44] As evident in Figure 3B, the cytotoxic activity of WA analogues was more pronounced than that of the corresponding WD analogues. WA analogues **6**–**8**, **15**, **16**, **19**, and **23**, as well as WD (**2**) and its analogue **11**, were found to be more cytotoxic than WA (**1**). In follow-up experiments, the concentrations resulting in 50% inhibition (IC_{50}'s) were determined for those cytotoxic compounds (**6**–**8**, **11**, **15**, **16**, and **23**) that exhibited enhanced HSA compared to **1**. WA (**1**) and WD (**2**) were included for comparison purposes. The IC_{50}'s of these withanolides for the human Ewing's sarcoma cell line CHP-100 were also determined using the same resazurin dye reduction assay.[44] This rapidly proliferating cell line was chosen to maximize sensitivity over a short period (24 h) of compound exposure and for the purpose of comparing data obtained for WA analogues in our previous studies.[17,18] Concentration-dependent growth inhibition curves for these withanolides when applied to the 293T and CHP-100 cell lines are presented in Figure 4. IC_{50} data derived from these curves are summarized in Table 1. These data established the following order of cytotoxic potency for withanolide analogues included in this study. For the CHP-100 cell line it was **8** > **7** > **16** > **6** > **11** > **15** > **2** > **1** > **23**, whereas for the 293T cell line the order of potency was found to be **8** > **7** > **11** > **16** > **6** > **15** > **1** > **23** > **2**, suggesting some degree of cell-line-selective cytotoxicity of these compounds by mechanism(s) that remain to be defined in future work.

Concentration-dependent HSA of WA (**1**), WD (**2**), and their analogues **6**–**8**, **11**, **13**, **15**, **16**, and **23** was determined by incubating heat-shock reporter 293T cells overnight in a 384-well format followed by measurement of luciferase activity on a microplate luminometer. Heat-shock-inducing activity for **1**, **2**, **6**–**8**, **11**, **13**, **15**, **16**, and **23** as depicted in Figure 5 demonstrates that reporter response was not a monotonic function but rather peaked over a relatively limited concentration range for each active compound and then declined, presumably as toxicity compromised the ability of cells to respond. Presented in Table 1 is the area under the curve (AUC in arbitrary units) as a measure of relative activity for each of these withanolides together with those for the classical heat-shock activators MG-132 (proteasome inhibitor) and STA-9090 (Ganetespib) (HSP90 inhibitor) (Figure S78, Supporting Information). These compounds were included because the proteasome and HSP90 have been reported as potential targets for WA (**1**).[14a]

To determine whether the dual heat-shock induction and cytotoxic activities of withanolides **1**, **2**, **6**–**8**, **11**, **13**, **15**, **16**, and **23** could be dissected on the basis of their structural features, potential correlation of the two activities was examined across these analogues. The ratio of AUC for heat-shock induction divided by cytotoxicity (IC_{50}) was calculated for each analogue when applied to the 293T reporter line (Table 1). Analogues **6**–**8**, **11**, **16**, and **23** enhanced heat-shock-inducing potential compared to WA (**1**) in the order **16** > **7** > **8** > **6** > **23** > **11**. To better visualize the relationship between activities for these compounds, a scatter plot is provided (Figure 6). The absence of a linear correlation between activities, especially for WA analogues, supports the reported conclusion that heat-shock induction by withanolides is not a simple consequence of cytotoxicity nor solely a result of impairment of the proteasome, HSP90, or any other single mediator of protein homeostasis. As is evident in Figure 6, there was actually

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Table 1. Concentration-Dependent Heat-Shock Induction and Cytotoxicity Data for WA (1), WD (2), and Their Analogues 6−8, 11, 15, 16, and 23

	cell line			
	CHP-100	293T		
compound	cytotoxicity, IC_{50} (nM)[a]	cytotoxicity, IC_{50} (nM)[b]	heat-shock induction (reporter AUC)[c]	ratio (heat-shock induction/ cytotoxicity)
1	424	299	68	0.23
2	324	710	13	0.02
6	232	180	351	1.95
7	120	116	419	3.61
8	59	88	196	2.23
11	292	141	71	0.50
15	311	250	44	0.18
16	142	154	685	4.45
23	466	417	235	0.56
MG-132[d]	ND[f]	ND[f]	332	
STA-9090[e]	ND[f]	ND[f]	59	

[a]Concentration resulting in 50% reduction in relative viable cell number after 24 h of compound exposure based on four-parameter curve fit of dose−response data using GraphPad Prism 6 software; R^2 for all curve fits ≥ 0.95; all determinations were performed in 384-well format using quadruplicate replicates. [b]Concentration resulting in 50% reduction in relative viable cell number after 72 h of compound exposure based on four-parameter curve fit of dose−response data using GraphPad Prism 6 software; R^2 for all curve fits ≥ 0.95. All determinations were performed in 384-well format using quadruplicate replicates. [c]Heat-shock reporter 293T cells were incubated in serial dilutions of the indicated compounds overnight in 384-well format followed by measurement of luciferase activity on a microplate luminometer; data were plotted over a 4-Log_{10} concentration range as the mean ratio of treated to control, with all determinations performed in quadruplicate wells. The area under the curve (AUC in arbitrary units) for each plot was calculated using GraphPad Prism 6 software. [d]Proteasome inhibitor. [e]HSP90 inhibitor. [f]ND = not determined.

relatively poor correlation between heat-shock induction and cytotoxic activities across the panel of bioactive withanolides. Indeed, analogues **16**, **23**, and **6**−**8** all showed substantially higher HSA than WA (**1**) while demonstrating varying degrees of cytotoxic activity. Our previous studies have shown that 4,27-di-*O*-acetyl-4-*epi*-withaferin A (**16**) was capable of inducing the heat-shock response in cell culture at subcytotoxic concentrations and in mice at systemically tolerable doses.[18] It is

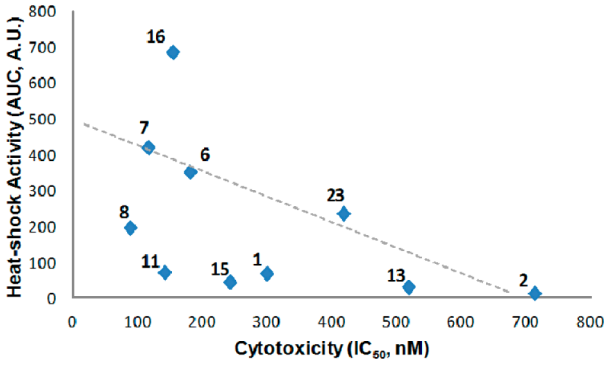

Figure 6. Correlation of heat-shock-inducing activity with cytotoxicity for WA (**1**), WD (**2**), and their analogues, **6**−**8**, **11**, **13**, **15**, **16**, and **23**. The dashed line depicts an idealized linear relationship between bioactivities. Heat-shock induction and cytotoxic activities were determined in 293T cells as described in Table 1.

particularly interesting that the 5,6-iodohydrin (**23**) of 4,27-di-*O*-acetylwithaferin A (**6**) showed 3.5-fold higher HSA, but 1.4-fold less cytotoxicity compared to WA (**1**). The remaining analogues **7** and **8** with higher HSA were found to be more cytotoxic than **1**. Comparison of HSA and cytotoxic potencies of WA analogues **6** and **23** suggested that the presence of the 5*β*,6*β*-epoxide moiety in **6** may be responsible for its enhanced cytotoxic activity.

In summary, while confirming the previous SAR data for the cytotoxic activity and HSA of some analogues of WA (**1**), we now show that (i) analogues of WD (**2**) exhibit reduced cytotoxicity and heat-shock induction activity compared to their corresponding WA analogues; (ii) the 5*β*,6*β*-epoxide moiety of withanolides contribute to their cytotoxicity but not HSA; and (iii) introduction of a 16*β*-OAc group to 4,27-di-*O*-acetyl-WA enhances cytotoxicity and decreases HSA, whereas introduction of a 16*β*-OAc group to 4-*O*-acetyl-WD decreases both activities. From these SAR data and those from the previous studies,[18] it may be concluded that the withanolide scaffold is amenable to structural modifications, leading to modulation of one or both of its dual heat-shock induction and cytotoxic activities. The more potent bioactivities found for withanolide analogues **7**, **8**, and **16** suggest that further translational investigation and/or in vivo evaluation of these compounds should be considered.

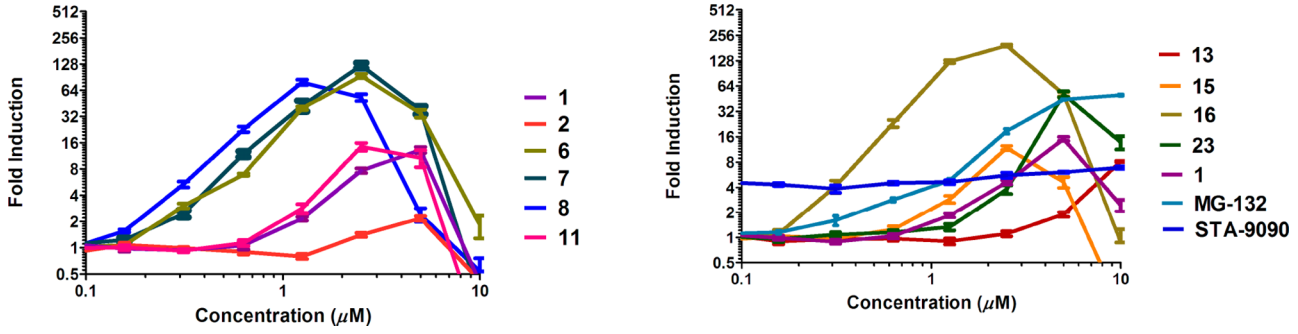

Figure 5. Concentration-dependent induction of heat-shock reporter activity by WA (**1**), WD (**2**), and their active analogues, **6**−**8**, **11**, **13**, **15**, **16**, and **23**, and the controls, MG-132 (proteasome inhibitor) and STA-9090 (HSP90 inhibitor). Heat-shock reporter cells were incubated with the indicated compound overnight in a 384-well format followed by measurement of luciferase activity on a microplate luminometer. Data are plotted as the mean ratio of treated to control, with all determinations performed in quadruplicate wells. Error bars, SD.

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EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were determined in capillary tubes using an Electrothermal melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO Dip-370 digital polarimeter using $CHCl_3$ or MeOH as solvent. 1D and 2D NMR spectra were recorded in $CDCl_3$ with a Bruker AVANCE III instrument at 400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR using residual $CHCl_3$ as the internal standard. The chemical shift (δ) values are given in parts per million (ppm), and the coupling constants (J values) are in Hz. Low-resolution mass spectra were obtained using a Shimadzu LCMS-QP8000α mass spectrometer, whereas high-resolution mass spectra were recorded on Shimadzu LCMS-IT-TOF (225-07100-34 equipped with ESI and APCI as ionization sources) and Agilent G6224A TOF mass spectrometers. All reagents for chemical and microbial transformations were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Fisher Scientific (Pittsburgh, PA, USA). All solvents used were distilled before use. The progress of all reactions was monitored by TLC on silica gel 60 F_{254} plates (Merck, Darmstadt, Germany), and spots were visualized under UV and using a solution of anisaldehyde in H_2SO_4 and HOAc followed by heating. Column chromatographic separations were performed on silica gel 40 μm flash chromatography packing (J. T. Baker, Jackson, TN, USA) or Sephadex LH-20 (25−100 μm; GE Healthcare AB, Uppsala, Sweden). Analytical and preparative TLC were performed on precoated 0.20 mm thick plates of silica gel 60 F_{254} (Merck, Darmstadt, Germany). Preparative HPLC was performed on a Waters Delta Prep 4000 system equipped with a Waters 996 photodiode array detector and a Waters Prep LC controller utilizing Empower Pro software and using a reversed-phase (RP) column (Phenomenex, Luna 5 μm, C_{18}, 100 Å, 250 × 10 mm); chromatograms were acquired at 254 and 270 nm.

Isolation of Withaferin A (1) and Withanolide D (2). Withaferin A (**1**) was obtained from the aerial parts of aeroponically grown *W. somnifera*,[30] whereas withanolide D (**2**) was isolated from the leaves of soil-cultivated *A. arborescens*[39] and aerial parts of aeroponically grown *W. somnifera*,[40] as reported previously.

Microbial Biotransformation of Withanolide D (2). A spore solution (1.0 mL, OD_{600} 0.6) of *C. echinulata* (ATCC 10028B) was transferred to four Erlenmeyer flasks (250 mL), each holding 50 mL of soybean meal−glucose medium. After shaking for 48 h (28 °C and 160 rpm), the mycelium in each flask was separated from the liquid medium by filtration, washed twice with 10 mL portions of phosphate buffer solution (PBS, pH 7), and transferred to Erlenmeyer flasks (4 × 250 mL) each holding 50 mL of PBS (pH 7) and withanolide D (**2**) (5.0 mg in 0.5 mL of dimethylformamide (DMF)). After shaking for 48 h (28 °C and 160 rpm), mycelium was separated by filtration and the combined filtrate was extracted with EtOAc (3 × 1 L). The resulting organic layer was washed with H_2O (3 × 500 mL), dried over anhydrous Na_2SO_4, and evaporated under reduced pressure, providing the crude product (28.0 mg). This was separated by preparative RP-TLC (RP-18) and elution with MeOH−H_2O (70:30) to give partially pure product (7.8 mg), which was further purified by RP-HPLC [MeOH−H_2O (75:25)], affording 15β-hydroxywithanolide D (**9**) (5.9 mg, 16%, t_R 6.0 min) as a white powder: mp 268−270 °C; $[\alpha]_D^{25}$ +148 (c 0.1, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz) δ 6.92 (1H, dd, J = 9.9, 6.3 Hz, H-3), 6.18 (1H, d, J = 9.9 Hz, H-2), 4.20 (1H, m, H-15), 4.16 (1H, dd, J = 13.3, 3.5 Hz, H-22), 3.70 (1H, d, J = 6.3 Hz, H-4), 3.23 (1H, brs, H-6), 2.47 (1H, dd, J = 17.1, 13.3 Hz, H-23a), 2.37 (1H, dt, J = 14.8, 3.3 Hz, H-7a), 2.13 (1H, ddd, J = 14.8, 8.6, 2.0 Hz, H-16a), 2.07 (1H, dd, J = 17.1, 3.5 Hz, H-23b), 1.91 (3H, s, H_3-28), 1.90 (1H, m, H-8), 1.89 (1H, m, H-16b), 1.84 (3H, s, H_3-27), 1.83 (1H, m, H-12a), 1.74 (1H, brd, J = 14.7, 4.5 Hz, H-11a), 1.54 (1H, dd, J = 11.4, 8.6 Hz, H-17), 1.42 (1H, m, H-11b), 1.40 (3H, s, H_3-19), 1.35 (1H, dd, J = 14.8, 5.0 Hz, H-7b), 1.21 (3H, s, H_3-21), 1.14 (1H, dt, J = 12.6, 4.0 Hz, H-12b), 1.01 (3H, s, H_3-18), 0.96 (1H, dd, J = 11.7, 5.0 Hz, H-9), 0.78 (1H, dd, J = 11.7, 5.0 Hz, H-14); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 201.3 (C, C-1), 166.0 (C, C-26), 149.2 (C, C-24), 142.1 (CH, C-3), 132.6 (CH, C-2), 121.8 (C, C-25), 80.6 (CH, C-22), 74.7 (C, C-20), 69.9 (C, C-4), 69.5 (CH, C-15), 64.3 (CH, C-5), 61.8 (CH, C-6), 60.9 (CH, C-14), 55.0 (CH, C-17), 47.8 (C, C-10), 44.2 (CH, C-9), 41.9 (C, C-13), 40.6 (CH_2, C-12), 34.6 (CH_2, C-16), 31.1 (CH_2, C-23), 30.5 (CH_2, C-7), 25.2 (CH, C-8), 21.3 (CH_2, C-11), 20.5 (CH_3, C-28), 20.3 (CH_3, C-21), 17.0 (CH_3, C-19), 16.2 (CH_3, C-18), 12.4 (CH_3, C-27); HRESIMS m/z 509.2510 $[M + Na]^+$ (calcd for $C_{28}H_{38}NaO_7$, 509.2547).

*Preparation of 4-Dehydrowithanolide D (**14**).* To a solution of withanolide D (**2**, 10.0 mg) in $CHCl_3$−EtOAc (75:25, 3.0 mL) was added MnO_2 (18.5 mg), and the mixture was stirred at 25 °C. After 16 h (TLC control), the reaction mixture was filtered, the filtrate was evaporated under reduced pressure, and the residue was subjected to silica gel (1.0 g) CC using CH_2Cl_2−MeOH (96:4) to afford 4-dehydrowithanolide D (**14**) (9.6 mg, 96%) as a white powder: mp 273−274 °C (lit.[44] 272−275 °C); $[\alpha]_D^{25}$ +112 (c 0.8, MeOH) [lit.[44] +113.5 (c 0.22)]; APCI-MS (+) m/z 469 $[M + 1]^+$; ^1H NMR data were consistent with those reported.[45]

*Preparation of 4-Epi-withanolide D (**17**).* To a stirred solution of 4-dehydrowithanolide D (**14**) (9.0 mg) in MeOH (0.5 mL) and THF (0.5 mL) was added $CeCl_3 \cdot 7H_2O$ (71.7 mg). The reaction mixture was cooled to 0 °C in an ice bath, and $NaBH_4$ (1.0 mg) was added. The reaction was monitored by TLC, and after the disappearance of the starting material ice-cold water (5.0 mL) was added, concentrated under reduced pressure, and extracted with EtOAc (3 × 10 mL). The combined EtOAc extracts were washed with H_2O, died over anhydrous Na_2SO_4, and evaporated under reduced pressure to provide the crude product mixture. Purification by RP-HPLC (MeOH−H_2O, 65:35) yielded **17** (6.2 mg, 69%, t_R 22.2 min) as a white powder: mp 210−212 °C; $[\alpha]_D^{25}$ +65 (c 0.1, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz) δ 6.83 (1H, dd, J = 10.1, 1.8 Hz, H-3), 6.00 (1H, dd, J = 10.1, 2.5 Hz, H-2), 4.69 (1H, dd, J = 2.5, 1.8 Hz, H-4), 4.17 (1H, dd, J = 13.1, 3.9 Hz, H-22), 3.65 (1H, brd, J = 2.1 Hz, H-6), 2.41, (1H, dd, J = 15.2, 13.1 Hz, H-23a), 2.16 (1H, m, H-7a), 2.06 (1H, dd, J = 15.2, 3.9 Hz, H-23b), 1.96 (1H, m, H-12a), 1.93 (3H, s, H_3-28), 1.92 (2H, m, H-11a and H-16a), 1.86 (3H, s, H_3-27), 1.62 (1H, m, H-15a), 1.51 (1H, m, H-8), 1.50 (1H, m, H-11b), 1.43 (1H, m, H-17), 1.24 (3H, s, H_3-21), 1.22 (1H, m, H-7b), 1.20 (3H, s, H_3-19), 1.17 (1H, m, H-15b), 1.16 (1H, dd, J = 12.6, 5.5 Hz, H-12b), 0.90 (1H, dd, J = 10.1, 6.9 Hz, H-14), 0.88 (1H, dd, J = 5.2, 2.4 Hz, H-9), 0.84 (3H, s, H_3-18); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 201.1 (C, C-1), 166.0 (C, C-26), 148.9 (C, C-24), 147.4 (CH, C-3), 128.6 (CH, C-2), 122.0 (C, C-25), 80.9 (CH, C-22), 77.1 (C, C-20), 65.6 (C, C-5), 64.9 (CH, C-4), 56.4 (CH, C-14), 55.2 (CH, C-6), 54.6 (CH, C-17), 47.5 (C, C-10), 45.5 (CH, C-9), 42.8 (C, C-13), 39.8 (CH_2, C-12), 31.6 (CH_2, C-23), 30.6 (CH_2, C-7), 29.2 (CH, C-8), 23.7 (CH_2, C-15), 22.9 (CH_2, C-16), 21.9 (CH_2, C-11), 20.9 (CH_3, C-21), 20.6 (CH_3, C-28), 13.6 (CH_3, C-18), 13.5 (CH_3, C-19), 12.5 (CH_3, C-27); HRESIMS m/z 493.2547 $[M + Na]^+$ (calcd for $C_{28}H_{38}NaO_6$, 493.2561).

General Procedure for Preparation of 5β-Hydroxy-6α-iodowithanolides. To a stirred solution of the withanolide (**1** or **2**, 50.0 mg) in CH_2Cl_2 (1.0 mL) at 0 °C was added a solution of Ph_3P (31.0 mg, 1.1 mol equiv) and I_2 (30.0 mg, 1.1 mol equiv) in CH_2Cl_2. The ice bath was removed, and the reaction mixture was stirred at 25 °C and monitored by TLC. After disappearance of the starting material, the reaction mixture was diluted with CH_2Cl_2 (25 mL), washed with aqueous $Na_2S_2O_3$ solution and H_2O, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure to yield the crude product mixture. The crude product mixtures derived from **1** (95.1 mg) and **2** (98.3 mg) on purification by silica gel (3 g) CC (Et_2O−MeOH, 97:3) afforded **19** (59.1 mg, 93%) and **25** (61.0 mg, 96%), respectively.

*(22R)-4β,5β,27-Trihydroxy-6α-iodo-1-oxowitha-2,24-dienolide (**19**):* white powder; mp >220 °C dec; $[\alpha]_D^{25}$ +64 (c 0.2, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz) δ 6.45 (1H, dd, J = 10.5, 2.3 Hz, H-3), 5.99 (1H, dd, J = 10.5, 1.6 Hz, H-2), 4.95 (1H, m, H-4), 4.65 (1H, dd, J = 13.7, 4.3 Hz, H-6), 4.39 (1H, dd, J = 13.3, 3.6 Hz, H-22), 4.37 (1H, dd, J = 12.5, 6.2 Hz, H-27a), 4.32 (1H, dd, J = 12.5, 6.2 Hz, H-27b), 2.56 (1H, dt, J = 13.9, 4.4 Hz, H-7a), 3.05 (1H, d, J = 5.9 Hz, D_2O exchangeable, OH-4), 2.46, (1H, dd, J = 17.5, 13.4 Hz, H-23a), 2.82 (1H, t, J = 6.2 Hz, D_2O exchangeable, OH-27), 2.02 (3H, s, H_3-28), 1.99 (1H, m, H-7b), 1.96 (1H, m, H-20), 1.95 (1H, m, H-23b), 1.89

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(1H, dt, J = 12.1, 3.3 Hz, H-12a), 1.71 (1H, m, H-8), 1.67 (1H, m, H-16a), 1.63 (1H, m, H-15a), 1.36 (1H, m, Hb −16), 1.32 (1H, m, H-9), 1.31 (1H, m, H-11a), 1.25 (3H, s, H_3-19), 1.17 (1H, m, H-15b), 1.10−1.00 (3H, m, H-12b, H-14, H-17), 0.95 (3H, d, J = 6.9, H_3-21), 0.87 (1H, m, H-11b), 0.65 (3H, s, H_3-18); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 200.3 (C, C-1), 166.9 (C, C-26), 152.3 (C, C-24), 142.7 (CH, C-3), 127.6 (CH, C-2), 125.8 (C, C-25), 78.6 (CH, C-22), 77.5 (C, C-5), 66.5 (CH, C-4), 57.5 (CH_2, C-27), 56.9 (C, C-10), 55.1 (CH, C-14), 51.7 (CH, C-17), 46.3 (CH, C-9), 43.3 (CH_2, C-7), 43.1 (C, C-13), 38.9 (CH_2, C-12), 38.7 (CH, C-20), 38.6 (CH, C-6), 37.4 (CH, C-8), 29.8 (CH_2, C-23), 27.2 (CH_2, C-16), 23.9 (CH_2, C-15), 22.9 (CH_2, C-11), 20.0 (CH_3, C-28), 13.3 (CH_3, C-21), 11.8 (CH_3, C-18), 9.6 (CH_3, C-19); HRESIMS m/z 621.1673 $[M + Na]^+$ (calcd for $C_{28}H_{39}NaIO_6$, 621.1684).

*(22R)-4β,5β,20-Trihydroxy-6α-iodo-1-oxowitha-2,24-dienolide (**25**):* white powder; mp >164 °C dec; $[\alpha]_D^{25}$ +51 (c 0.8, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.44 (1H, dd, J = 10.5, 3.2 Hz, H-3), 5.97 (1H, dd, J = 10.5, 1.9 Hz, H-2), 4.94 (1H, m, H-4), 4.63 (1H, dd, J = 13.4, 5.0 Hz, H-6), 4.16 (1H, dd, J = 13.0, 3.6 Hz, H-22), 3.10 (1H, d, J = 5.8 Hz, D_2O exchangeable, OH-4), 2.54 (1H, dt, J = 13.4, 4.2 Hz, H-7a), 2.43 (1H, brt, J = 13.0 Hz, H-23a), 2.05 (1H, m, H-23b), 1.99 (1H, m, H-7b), 1.91 (3H, s, H_3-28), 1.87 (1H, m, H-12a), 1.84 (3H, s, H_3-27), 1.65 (1H, m, H-15a), 1.62 (1H, m, H-8), 1.56 (1H, m, H-17), 1.55 (1H, m, H-11a), 1.34−1.31 (3H, m, H-9, H-11b, H-16a), 1.24 (3H, s, H_3-19), 1.19 (3H, s, H_3-21), 1.18 (1H, m, H-12b), 1.15 (1H, m, H-15b), 1.08 (1H, ddd, J = 12.5, 10.3, 6.3 Hz, H-14), 0.85 (1H, m, H-16b), 0.78 (3H, s, H_3-18); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 200.4 (C, C-1), 166.0 (C, C-26), 149.0 (C, C-24), 142.8 (CH, C-3), 127.6 (CH, C-2), 121.9 (C, C-25), 80.6 (CH, C-22), 77.6 (C, C-5), 74.9 (C, C-20), 66.4 (CH, C-4), 56.9 (C, C-10), 55.5 (CH, C-14), 54.4 (CH, C-17), 46.2 (CH, C-9), 43.2 (C, C-13), 43.0 (CH_2, C-7), 39.1 (CH_2, C-12), 38.6 (CH, C-6), 36.8 (CH, C-8), 31.2 (CH_2, C-23), 23.4 (CH_2, C-15), 22.7 (CH_2, C-16), 21.9 (CH_2, C-11), 20.6 (CH_3, C-28), 20.4 (CH_3, C-21), 13.7 (CH_3, C-18), 12.4 (CH_3, C-27), 9.8 (CH_3, C-18); HRESIMS m/z 621.1711 $[M + Na]^+$ (calcd for $C_{28}H_{39}NaIO_6$, 621.1684).

General Procedure for Microbial Biotransformations of Withanolide Iodohydrins 19 and 25. Small-scale fermentation of *C. echinulata* (ATCC 10028B) was performed in an Erlenmeyer flask (125 mL) containing soybean meal−glucose medium (25 mL) on a rotary shaker operating at 160 rpm at 28 °C for 24 h. Biotransformation was performed under the same conditions in eight Erlenmeyer flasks (250 mL) each holding 50 mL of soybean meal−glucose medium and inoculated with 5.0 mL of the above small-scale culture of *C. echinulata*. A solution of the substrate (5 mg in 0.5 mL of DMF; a total amount of 40 mg of **19** or **25**) was added to each flask containing the inoculum (72 h old for **19** and 24 h old for **25**). After disappearance of the substrate in the culture broth (TLC control), fermentation broths resulting from each substrate were combined, mycelium was filtered off and washed with H_2O (100 mL), and the washings were combined with the filtrate and extracted with EtOAc (3 × 300 mL). The combined organic extracts was washed with H_2O (3 × 200 mL), dried over anhydrous Na_2SO_4, and evaporated under reduced pressure to give the crude extract. Gel-permeation chromatography of the EtOAc extract (48.5 mg, derived from the biotransformation of **19**) over a column of Sephadex LH-20 (1.5 g) followed by RP-HPLC [MeOH−H_2O (60:40)] purification afforded **20** (11.2 mg, 27%, t_R 14.5 min) and a mixture of three compounds with the same retention time (t_R 11.3 min). This mixture was separated by preparative TLC (silica gel) using Et_2O−MeOH (92:8) to give **4** (0.8 mg, 3%, R_f 0.29), **5** (2.1 mg, 7%, R_f 0.43), and **21** (3.1 mg, 8%, R_f 0.64). Gel-permeation chromatography of the EtOAc extract (42.3 mg) derived from the biotransformation of **25** over a column of Sephadex LH-20 (1.5 g) followed by RP-HPLC [MeOH−H_2O (65:35)] purification afforded **26** (3.8 mg, 10%, t_R 14.5 min).

*(22R)-4β,5β,16β,27-Tetrahydroxy-6α-iodo-1-oxowitha-2,24-dienolide (**20**):* white powder; mp >212 °C dec; $[\alpha]_D^{25}$ +148 (c 0.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.45 (1H, dd, J = 10.4, 2.6 Hz, H-3), 5.92 (1H, dd, J = 10.4, 1.9 Hz, H-2), 4.94 (1H, dd, J = 2.6, 1.9 Hz, H-4), 4.65 (1H, dd, J = 13.6, 4.5 Hz, H-6), 4.62 (1H, dt, J = 13.4, 4.2 Hz, H-22), 4.38 (1H, d, J = 12.7 Hz, H-27a), 4.32 (1H, d, J = 12.7 Hz, H-27b), 4.26 (1H, ddd, J = 11.8, 7.4, 4.0 Hz, H-16), 3.05 (1H, d, J = 5.1 Hz, D_2O exchangeable, OH-4), 2.55 (1H, dt, J = 13.4, 4.2 Hz, H-7a), 2.47 (1H, dd, J = 17.2, 12.3 Hz, H-23a), 2.46 (1H, m, H-20), 2.26 (1H, dt, J = 12.9, 7.6 Hz, H-15a), 2.12 (1H, m, H-23b), 2.03 (1H, m, H-7b), 2.02 (3H, s, H_3-28), 1.89 (1H, dt, J = 12.8, 3.2 Hz, H-12a), 1.75 (1H, m, H-8), 1.33 (1H, m, H-9), 1.31 (1H, m, Ha-11), 1.26 (3H, s, H_3-19), 1.23 (1H, m, H-15b), 1.02 (1H, m, H-12b), 1.00 (1H, m, H-17), 0.99 (3H, d, J = 6.4 Hz, H_3-21), 0.97 (1H, m, H-14), 0.87 (3H, s, H_3-18), 0.88 (1H, m, H-11b); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 200.2 (C, C-1), 166.6 (C, C-26), 152.6 (C, C-24), 142.8 (CH, C-3), 127.7 (CH, C-2), 125.8 (C, C-25), 79.3 (CH, C-22), 77.5 (C, C-5), 71.2 (CH, C-16), 66.5 (CH, C-4), 57.7 (CH, C-17), 57.4 (CH_2, C-27), 56.9 (C, C-10), 53.4 (CH, C-14), 46.3 (CH, C-9), 43.3 (CH_2, C-7), 43.0 (C, C-13), 39.3 (CH_2, C-12), 38.3 (CH, C-6), 36.9 (CH, C-8), 36.7 (CH_2, C-15), 33.5 (CH, C-20), 31.6 (CH_2, C-23), 22.5 (CH_2, C-11), 20.1 (CH_3, C-28), 13.4 (CH_3, C-18), 13.0 (CH_3, C-21), 9.8 (CH_3, C-19); HRESIMS m/z 615.1833 $[M + H]^+$ (calcd for $C_{28}H_{39}IO_7$, 615.1774).

*(22R)-4β,5β,15β,27-Tetrahydroxy-6α-iodo-1-oxowitha-2,24-dienolide (**21**):* white powder; mp >224 °C dec; $[\alpha]_D^{25}$ +156 (c 0.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.46 (1H, dd, J = 10.4, 2.6 Hz, H-3), 5.97 (1H, dd, J = 10.4, 1.8 Hz, H-2), 4.93 (1H, dd, J = 2.6, 1.8 Hz, H-4), 4.68 (1H, dd, J = 13.9, 4.8 Hz, H-6), 4.35 (1H, dt, J = 13.4, 3.6 Hz, H-22), 4.34 (1H, d, J = 12.3 Hz, H-27a), 4.28 (1H, d, J = 12.3 Hz, H-27b), 4.17 (1H, t, J = 6.3 Hz, H-15), 3.35 (1H, d, J = 5.8 Hz, D_2O exchangeable, OH-4), 2.87 (1H, dt, J = 13.8, 4.4 Hz, H-7a), 2.44 (1H, dd, J = 17.1, 13.4 Hz, H-23a), 2.22 (1H, dt, J = 14.2, 9.0 Hz, H-16a), 2.08 (1H, m, H-8), 2.05 (1H, m, H-7b), 2.02 (1H, m, H-20), 2.03 (1H, m, H-7b), 2.01 (3H, s, H_3-28), 1.90 (1H, dd, J = 17.1, 3.6 Hz, H-23b), 1.84 (1H, dt, J = 12.7, 3.1 Hz, H-12a), 1.42 (1H, m, H-16b), 1.35 (1H, m, H-11a), 1.34 (1H, m, H-9), 1.26 (3H, s, H_3-19), 1.01 (1H, m, H-14), 1.00 (1H, m, H-12b), 0.94 (3H, d, J = 6.7 Hz, H_3-21), 0.92 (3H, s, H_3-18), 0.86 (1H, m, H-17), 0.85 (1H, m, Hb-11); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 200.8 (C, C-1), 167.0 (C, C-26), 153.1 (C, C-24), 143.2 (CH, C-3), 127.4 (CH, C-2), 125.8 (C, C-25), 78.6 (CH, C-22), 77.0 (C, C-5), 69.1 (CH, C-15), 66.3 (CH, C-4), 59.5 (2 x CH, C-10,C-14), 57.0 (CH_2, C-27), 52.2 (CH, C-17), 46.7 (CH, C-9), 43.0 (C, C-13), 42.1 (CH_2, C-7), 40.9 (CH_2, C-16), 40.3 (CH_2, C-12), 38.5 (2 x CH, C-6, C-8), 33.6 (CH, C-20), 30.0 (CH_2, C-23), 22.9 (CH_2, C-11), 20.1 (CH_3, C-28), 14.3 (CH_3, C-18), 13.4 (CH_3, C-21), 9.9 (CH_3, C-19); HRESIMS m/z 613.1676 $[M - H]^-$ (calcd for $C_{28}H_{38}IO_7$, 613.1662).

*(22R)-4β,5β,16β,20-Tetrahydroxy-6α-iodo-1-oxowitha-2,24-dienolide (**26**):* white powder; mp >228 °C dec; $[\alpha]_D^{25}$ +152 (c 0.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.44 (1H, dd, J = 10.2, 1.9 Hz, H-3), 5.97 (1H, dd, J = 10.0, 2.8 Hz, H-2), 4.93 (1H, m, H-4), 4.65 (1H, dd, J = 13.4, 4.5 Hz, H-6), 4.60 (1H, m, H-16), 4.47 (1H, dd, J = 13.0, 3.4 Hz, H-22), 4.21 (1H, d, J = 4.5 Hz, D_2O exchangeable, OH-16), 4.05 (1H, brs, D_2O exchangeable, OH-20), 3.10 (1H, d, J = 5.6 Hz, D_2O exchangeable, 1H, OH-4), 2.55 (2H, m, Ha-7, H-23a), 2.20 (1H, dt, J = 13.3, 7.6 Hz, H-15a), 2.12 (1H, dd, J = 17.4, 3.5 Hz, H-23b), 2.00 (1H, m, H-12a), 1.99 (1H, dd, J = 13.6, 3.5 Hz, H-7b), 1.93 (1H, s, 1H, H_3-28), 1.84 (3H, s, H_3-27), 1.75 (1H, dq, J = 10.2, 4.3 Hz, H-8), 1.40 (1H, m, H-11a), 1.39 (1H, m, H-15b), 1.33 (1H, dt, J = 13.9, 2.8 Hz, H-9), 1.28 (3H, s, H_3-19), 1.27 (3H, s, H_3-21), 1.27 (1H, m, H-17), 1.10 (1H, m, H-12b), 1.08 (3H, s, H_3-18), 0.88 (2H, m, H-11b, H-14); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 200.3 (C, C-1), 165.9 (C, C-26), 149.3 (C, C-24), 142.9 (CH, C-3), 127.6 (CH, C-2), 121.8 (C, C-25), 81.9 (CH, C-22), 77.5 (C, C-5), 77.4 (C, C-20), 73.6 (CH, C-16), 66.5 (CH, C-4), 58.0 (CH, C-17), 56.8 (C, C-10), 53.0 (CH, C-14), 46.3 (CH, C-9), 43.8 (C, C-13), 42.9 (CH_2, C-7), 39.7 (CH_2, C-12), 38.2 (CH, C-6), 36.4 (CH, C-8), 36.1 (CH_2, C-15), 31.3 (CH_2, C-23), 22.6 (CH_2, C-11), 22.8 (CH_3, C-21), 20.7 (CH_3, C-28), 14.8 (CH_3, C-18), 12.4 (CH_3, C-27), 9.8 (CH_3, C-19); HRESIMS m/z 637.1646 $[M + Na]^+$ (calcd for $C_{28}H_{39}NaIO_7$, 637.1633).

Reaction of Withanolide Iodohydrins with N,N-Diisopropylethylamine. To separate solutions of the iodohydrin **20** (2.0 mg), **21** (1.9 mg), or **26** (2.5 mg) in CH_2Cl_2 (0.2 mL) was added *N,N*-diisopropylethylamine (0.03 mL), and the mixtures were stirred at

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25 °C. The reaction was monitored by TLC, and after the disappearance of the starting material (120 h), the solvent was evaporated under reduced pressure and the resulting crude products were purified by RP-HPLC (MeOH−H_2O, 65:35) to afford epoxides **5** (1.6 mg, 98%), **4** (1.5 mg, 99%), and **10** (1.9 mg, 96%), respectively.

*16β-Hydroxywithaferin A (**5**):* white powder; mp 256−258 °C; $[\alpha]_D^{25}$ +78 (*c* 0.1, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz) δ 6.91 (1H, dd, *J* = 10.0, 5.7 Hz, H-3), 6.18 (1H, d, *J* = 10.0 Hz, H-2), 4.67 (1H, dt, *J* = 13.4, 4.2 Hz, H-22), 4.37 (1H, dd, *J* = 12.9, 6.3 Hz, H-27a), 4.32 (1H, dd, *J* = 12.9, 6.3 Hz, H-27b), 4.22 (1H, m, H-16), 3.74 (1H, d, *J* = 5.7 Hz, H-4), 3.22 (1H, brs, H-6), 2.86 (1H, t, *J* = 6.3 Hz, D_2O exchangeable, OH-27), 2.54 (1H, brd, *J* = 2.9 Hz, D_2O exchangeable, OH-16), 2.51 (1H, dd, *J* = 17.0, 4.1 Hz, H-23a), 2.49 (1H, m, H-20), 2.26 (1H, dt, *J* = 13.3, 8.2 Hz, H-15a), 2.13 (1H, ddd, *J* = 14.8, 6.5, 3.4 Hz, H-7a), 2.09 (1H, dd, *J* = 17.0, 13.4 Hz, H-23b), 2.02 (3H, s, H_3-28), 1.93 (1H, dt, *J* = 12.8, 3.7 Hz, H-12a), 1.80 (1H, m, H-11a), 1.61 (1H, dd, *J* = 14.8, 5.1 Hz, H-8), 1.46 (1H, dd, *J* = 13.0, 3.7 Hz, H-11b), 1.40 (3H, s, H_3-19), 1.24 (1H, m, H-7b), 1.18 (1H, dd, *J* = 13.3, 4.2 Hz, H-15b), 1.07 (1H, dd, *J* = 12.8, 3.7 Hz, H-12b), 1.02 (3H, d, *J* = 6.8 Hz, H_3-21), 1.02−0.95 (2H, m, H-9, H-14), 0.90 (3H, s, H_3-18), 0.80 (1H, m, H-17); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 202.3 (C, C-1), 166.8 (C, C-26), 152.7 (C, C-24), 142.9 (CH, C-3), 132.2 (CH, C-2), 125.7 (C, C-25), 79.2 (CH, C-22), 77.7 (C, C-5), 71.6 (CH, C-16), 69.8 (CH, C-4), 62.6 (CH, C-6), 57.7 (CH, C-14), 57.5 (CH_2, C-27), 54.2 (CH, C-17), 47.7 (C, C-10), 44.1 (CH, C-9), 42.6 (C, C-13), 39.7 (CH_2, C-12), 37.3 (CH_2, C-15), 33.5 (CH, C-20), 31.4 (CH_2, C-23), 31.1 (CH_2, C-7), 29.4 (CH, C-8), 21.9 (CH_2, C-11), 20.0 (CH_3, C-28), 17.5 (CH_3, C-19), 13.3 (CH_3, C-21), 12.9 (CH_3, C-18); HRESIMS *m*/*z* 487.2648 $[M + H]^+$ (calcd for $C_{28}H_{38}O_7$, 487.2690).

*16β-Hydroxywithanolide D (**10**):* white powder; mp 278−280 °C; $[\alpha]_D^{25}$ +136 (*c* 0.1, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz) δ 6.92 (1H, dd, *J* = 9.9, 6.3 Hz, H-3), 6.17 (1H, dd, *J* = 9.9, 2.4 Hz, H-2), 4.59 (1H, ddd, *J* = 12.6, 7.8, 4.7 Hz, H-16), 4.52 (1H, dd, *J* = 13.0, 3.5 Hz, H-22), 4.25 (1H, d, *J* = 2.4 Hz, D_2O exchangeable, OH-16), 4.06 (1H, s, D_2O exchangeable, OH-20), 3.75 (1H, dd, *J* = 6.1, 2.4 Hz, H-4), 3.23 (1H, brs, H-6), 2.57 (1H, dd, *J* = 17.1, 13.0 Hz, H-23a), 2.50 (1H, d, *J* = 2.4 Hz, D_2O exchangeable, OH-4), 2.20 (1H, dt, *J* = 14.6, 5.5 Hz, H-7a), 2.19 (1H, m, H-15a), 2.13 (1H, dd, *J* = 17.1, 3.5 Hz, H-23b), 2.06 (1H, dt, *J* = 12.7, 3.5 Hz, H-12a), 1.94 (3H, s, H_3-28), 1.87 (3H, s, H_3-27), 1.83 (1H, dd, *J* = 11.4, 4.4 Hz, H-11a), 1.62 (1H, dd, *J* = 11.2, 3.5 Hz, H-8), 1.51 (1H, m, H-11b), 1.41 (3H, s, H_3-19), 1.37 (1H, dd, *J* = 14.6, 5.0 Hz, H-7b), 1.36 (1H, dd, *J* = 13.7, 5.2 Hz, H-15b),1.31 (3H, s, H_3-21), 1.24 (1H, m, H-17), 1.12 (3H, s, H_3-18), 1.08 (1H, m, H-12b), 0.99 (1H, dt, *J* = 11.7, 4.4 Hz, H-9), 0.73 (1H, m, H-14); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 202.3 (C, C-1), 165.9 (C, C-26), 149.2 (C, C-24), 142.0 (CH, C-3), 132.2 (CH, C-2), 122.1 (C, C-25), 83.8 (C, C-5), 82.0 (CH, C-22), 77.7 (C, C-20), 73.8 (CH, C-16), 69.8 (CH, C-4), 62.7 (CH, C-6), 58.3 (CH, C-17), 54.1 (CH, C-14), 47.7 (C, C-10), 44.1 (CH, C-9), 43.2 (C, C-13), 40.2 (CH_2, C-12), 36.7 (CH_2, C-15), 31.4 (CH_2, C-23), 31.0 (CH_2, C-7), 28.9 (CH, C-8), 22.8 (CH_3, C-21), 22.0 (CH_2, C-11), 20.6 (CH_3, C-28), 17.5 (CH_3, C-19), 14.7 (CH_3, C-18), 12.4 (CH_3, C-27); HRESIMS *m*/*z* 509.2540 $[M + Na]^+$ (calcd for $C_{28}H_{38}NaO_7$, 509.2510).

General Procedure for the Preparation of *O*-Acetylwithanolides 6−8, 11−13, 16, 18, 22−24, and 27. 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). Excess EtOH was added to the reaction mixture and evaporated under reduced pressure. The crude product was purified by preparative TLC (CH_2Cl_2−MeOH, 96:4) or RP-HPLC (MeOH−H_2O, 75:25) to yield the corresponding *O*-acetyl derivative. Application of this procedure afforded the known acetates 4,27-di-*O*-acetylwithaferin A (**6**),[18] 4,12,27-tri-*O*-acetyl-12β-hydroxywithaferin A (**7**),[18] 4-*O*-acetylwithanolide D (**11**),[46] and 4,27-di-*O*-acetyl-4-*epi*-withaferin A (**16**)[18] and the new *O*-acetyl derivatives 4,16,27-tri-*O*-acetyl-16β-hydroxywithaferin A (**8**), 4,15-di-*O*-acetyl-15β-hydroxywithanolide D (**12**), 4,16-di-*O*-acetyl-16β-hydroxywithanolide D (**13**), 4-*O*-acetyl-4-*epi*-withanolide D (**18**), (22*R*)-4β,27-diacetoxy-5β-hydroxy-6α-iodo-1-oxowitha-2,24-dienolide (**22**), (22*R*)-4β,16β,27-triacetoxy-5β-hydroxy-6α-iodo-1-oxowitha-2,24-dienolide (**23**), (22*R*)-4β,15β,27-triacetoxy-5β-hydroxy-6α-iodo-1-oxowitha-2,24-dienolide (**24**), and (22*R*)-4β-acetoxy-5β,20-dihydroxy-6α-iodo-1-oxowitha-2,24-dienolide (**27**).

*4,16,27-Tri-O-acetyl-16β-hydroxywithaferin A (**8**):* white powder; mp 168−170 °C; $[\alpha]_D^{25}$ +108 (*c* 0.1, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz) δ 7.02 (1H, dd, *J* = 9.9, 6.1 Hz, H-3), 6.22 (1H, d, *J* = 9.9 Hz, H-2), 4.95 (1H, ddd, *J* = 11.8,7.5, 4.1 Hz, H-16), 4.88 (1H, dd, *J* = 11.8 Hz, Ha-27), 4.81 (1H, dd, *J* = 11.8 Hz, Hb-27), 4.65 (1H, d, *J* = 6.1 Hz, H-4), 4.23 (1H, dt, *J* = 13.2, 3.2 Hz, H-22), 3.20 (1H, brs, H-6), 2.53 (1H, dd, *J* = 17.5, 13.2 Hz, H-23a), 2.50 (1H, m, H-20), 2.41 (1H, dt, *J* = 13.8, 7.4 Hz, H-15a), 2.11 (1H, m, H-7), 2.04 (9H, s, 3 x OAc), 2.02 (3H, s, H_3-28), 1.97 (1H, dd, *J* = 17.5, 3.3 Hz, H-23b), 1.95 (1H, m, H-12a), 1.71 (1H, dt, *J* = 10.7, 4.0 Hz, H-11a), 1.53 (1H, m, H-8), 1.49 (1H, m, H-11b), 1.38 (3H, s, H_3-19), 1.26 (1H, dd, *J* = 14.4, 11.1 Hz, H-7b), 1.13 (1H, m, H-17), 1.12 (1H, m, H-15b), 1.05 (1H, m, H-12b), 1.02 (3H, d, *J* = 6.8 Hz, H_3-21), 0.89 (3H, s, H_3-18), 0.85 (1H, m, H-14); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 201.1 (C, C-1), 170.9 (C, OAc), 170.8 (C, OAc), 170.1 (C, OAc), 165.1 (C, C-26), 156.6 (C, C-24), 140.0 (CH, C-3), 133.8 (CH, C-2), 122.0 (C, C-25), 77.2 (CH, C-22), 77.1 (C, C-5), 74.1 (CH, C-16), 72.0 (CH, C-4), 60.2 (CH, C-6), 57.9 (CH_2, C-27), 55.0 (CH, C-17), 54.4 (CH, C-14), 48.1 (C, C-10), 44.1 (CH, C-9), 42.8 (C, C-13), 39.2 (CH_2, C-12), 35.3 (CH_2, C-15), 33.5 (CH, C-20), 31.0 (CH_2, C-23), 30.4 (CH_2, C-7), 29.2 (CH, C-8), 21.4 (CH_3, C-28), 21.1 (CH_2, C-11), 20.9 (CH_3, OAc), 20.8 (CH_3, OAc), 20.7 (CH_3, OAc), 15.8 (CH_3, C-19), 12.9 (CH_3, C-21), 12.2 (CH_3, C-18); HRESIMS *m*/*z* 635.2833 $[M + Na]^+$ (calcd for $C_{34}H_{44}NaO_{10}$, 635.2832).

*4,15-Di-O-acetyl-15β-hydroxywithanolide D (**12**):* white powder; mp 236−238 °C; $[\alpha]_D^{25}$ +116 (*c* 0.1, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz) δ 7.00 (1H, dd, *J* = 9.9, 6.1 Hz, H-3), 6.23 (1H, d, *J* = 9.9 Hz, H-2), 5.05 (1H, ddd, *J* = 8.0, 5.6, 2.5 Hz, H-15), 4.67 (1H, d, *J* = 6.1 Hz, H-4), 4.14 (1H, dd, *J* = 13.2, 3.3 Hz, H-22), 3.21 (1H, brt, *J* = 1.8 Hz, H-6), 2.40 (1H, t, *J* = 15.0 Hz, H-23a), 2.22 (1H, dt, *J* = 15.2, 6.9 Hz, H-16a), 2.10 (1H, m, H-7a), 2.04 (1H, m H-23b), 1.95 (1H, m, H-12a), 1.92 (3H, s, H_3-28), 1.91 (1H, m, Hb-16), 1.85 (3H, s, H_3-27), 1.83 (1H, dd, *J* = 11.3, 4.2 Hz, H-8), 1.71 (1H, ddd, *J* = 14.2, 6.9, 3.3, Hz, H-11a), 1.49 (1H, m, H-11b), 1.44 (1H, m, H-17), 1.40 (3H, s, H_3-19), 1.31 (1H, m, H-7b), 1.25 (3H, s, H_3-21), 1.15 (1H, dt, *J* = 11.3, 6.3 Hz, H-12b), 1.03 (3H, s, H_3-18), 0.96 (1H, dd, *J* = 11.3, 6.3 Hz, H-14), 0.88 (1H, dd, *J* = 6.9, 3.7 Hz, H-9); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 201.1 (C, C-1), 170.9 (C, OAc), 170.0 (C, OAc), 165.8 (C, C-26), 148.7 (C, C-24), 139.9 (CH, C-3), 133.8 (CH, C-2), 122.1 (C, C-25), 80.7 (CH, C-22), 76.8 (C, C-20), 72.0 (CH, C-15), 71.9 (C, C-4), 61.1 (CH, C-5), 60.3 (CH, C-6), 59.2 (CH, C-14), 54.7 (CH, C-17), 48.1 (C, C-10), 44.4 (CH, C-9), 42.8 (C, C-13), 40.5 (CH_2, C-12), 33.3 (CH_2, C-16), 31.4 (CH_2, C-23), 30.2 (CH_2, C-7), 25.4 (CH, C-8), 21.3 (CH_2, C-11), 20.5 (CH_3, C-28), 20.8 (CH_3, C-21), 15.4 (CH_3, C-19), 15.3 (CH_3, C-18), 12.4 (CH_3, C-27); HRESIMS *m*/*z* 593.2723 $[M + Na]^+$ (calcd for $C_{32}H_{42}NaO_9$, 593.2727).

*4,16-Di-O-acetyl-16β-hydroxywithanolide D (**13**):* white powder; mp 234−236 °C; $[\alpha]_D^{25}$ +166 (*c* 0.1, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz) δ 7.02 (1H, dd, *J* = 10.0, 6.0 Hz, H-3), 6.21 (1H, d, *J* = 10.0 Hz, H-2), 5.39 (1H, ddd, *J* = 11.8, 7.6, 4.4 Hz, H-16), 4.63 (1H, dd, *J* = 6.0 Hz, H-4), 4.18 (1H, dd, *J* = 12.8, 3.5 Hz, H-22), 3.20 (1H, brs, H-6), 2.41 (1H, dt, *J* = 13.7, 6.9 Hz, H-23a), 2.14−2.07 (4H, m, H-7, H-12a, H-15a, H-23a), 2.05 (3H, s, OAc), 2.03 (3H, s, OAc), 1.92 (3H, s, H_3-28), 1.85 (3H, s, H_3-27), 1.68 (1H, dd, *J* = 14.9, 4.2 Hz, H-11a), 1.62 (1H, dd, *J* = 11.2, 3.5 Hz, H-8), 1.51 (1H, m, H-11b), 1.44 (1H, m, H-15b), 1.36 (3H, s, H_3-19), 1.31 (3H, s, H_3-21), 1.24 (1H, m, H-17), 1.25 (1H, m, H-7b), 1.16 (1H, dd, *J* = 13.0, 4.2 Hz, H-12b), 1.03 (3H, s, H_3-18), 0.96−0.80 (2H, m, H-9, H-14); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 200.9 (C, C-1), 170.0 (C, OAc), 169.9 (C, OAc), 165.6 (C, C-26), 149.0 (C, C-24), 139.9 (CH, C-3), 133.8 (CH, C-2), 121.8 (C, C-25), 81.3 (CH, C-22), 76.4 (C, C-20), 75.5 (CH, C-16), 72.1(CH, C-4), 61.0 (C, C-5), 60.1 (CH, C-6), 57.9 (CH, C-17), 54.5 (CH, C-14), 48.1 (C, C-10), 43.9 (CH, C-9), 42.8 (C, C-13), 39.1 (CH_2, C-12), 34.8 (CH_2, C-15), 30.8 (CH_2, C-23), 30.7 (CH_2, C-7), 28.8 (CH, C-8), 22.5 (CH_3, OAc), 21.6 (CH_2, C-11), 20.8 (CH_3, C-21), 20.7 (CH_3, OAc), 20.6 (CH_3, C-28), 15.6 (CH_3, C-19), 14.7 (CH_3, C-18),

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12.4 (CH_3, C-27); HRESIMS m/z 593.2725 $[M + Na]^+$ (calcd for $C_{32}H_{42}NaO_9$, 593.2727).

*4-O-Acetyl-4-epi-withanolide D (**18**):* white powder; mp 184−186 °C; $[\alpha]_D^{25}$ +73 (c 0.1, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz) δ 6.65 (1H, dd, J = 10.2, 1.8 Hz, H-3), 6.05 (1H, dd, J = 10.2, 2.6 Hz, H-2), 5.86 (1H, dd, J = 2.6, 1.8 Hz, H-4), 4.17 (1H, dd, J = 13.4, 3.4 Hz, H-22), 3.53 (1H, brd, J = 2.7 Hz, H-6), 2.42 (1H, brt, J = 15.1 Hz, H-23a), 2.11 (1H, m, H-7a), 2.09 (3H, s, OAc), 2.04 (1H, m, H-23b), 1.98−1.93 (3H, m, H-11a, Ha-12, H-16a), 1.92 (3H, s, H_3-28), 1.86 (3H, s, H_3-27), 1.62 (1H, m, H-15a), 1.52−1.45 (4H, m, H-8, H-11b, H-16b, H-17), 1.27 (3H, s, H_3-21), 1.23 (3H, s, H_3-19), 1.19−1.12 (3H, m, H-7b, H-12b, H-15b), 0.98−0.88 (2H, m, H-9, H-14), 0.84 (3H, s, H_3-18); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 200.9 (C, C-1), 169.7 (C, OAc), 166.0 (C, C-26), 148.8 (C, C-24), 144.8 (CH, C-3), 129.4 (CH, C-2), 122.0 (C, C-25), 80.9 (CH, C-22), 77.7 (C, C-20), 75.1 (CH, C-4), 65.8 (C, C-5), 56.4 (CH, C-14), 55.8 (CH, C-6), 54.7 (CH, C-17), 48.1 (C, C-10), 45.4 (CH, C-9), 42.8 (C, C-13), 39.8 (CH_2, C-12), 31.5 (CH_2, C-23), 30.6 (CH_2, C-7), 29.1 (CH, C-8), 23.8 (CH_2, C-15), 22.5 (CH_2, C-16), 21.9 (CH_2, C-11), 20.8 (CH_3, C-21), 20.7 (CH_3, C-28), 20.5 (CH_3, OAc), 14.1 (CH_3, C-18), 13.6 (CH_3, C-19), 12.5 (CH_3, C-27); HRESIMS m/z 535.2678 $[M + Na]^+$ (calcd for $C_{30}H_{40}NaO_7$, 535.2672).

*(22R)-4β,27-Diacetoxy-5β-hydroxy-6α-iodo-1-oxowitha-2,24-dienolide (**22**):* white powder; mp >179 °C dec; $[\alpha]_D^{25}$ +76 (c 0.2, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz) δ 6.46 (1H, dd, J = 10.5, 2.3 Hz, H-3), 6.11 (1H, dd, J = 2.3, 2.0 Hz, H-4), 6.03 (1H, dd, J = 10.5, 2.0 Hz, H-2), 4.61 (1H, dd, J = 13.4, 4.2 Hz, H-6), 4.87 (1H, d, J = 12.0 Hz, H-27a), 4.83 (1H, d, J = 12.0, H-27b), 4.37 (1H, dt, J = 13.1, 3.5 Hz, H-22), 2.44 (1H, dt, J = 13.9, 4.4 Hz, H-7a), 2.44 (1H, dd, J = 17.7, 13.1 Hz, H-23a), 2.19 (3H, s, OAc), 2.06 (3H, s, H_3-28), 2.02 (3H, s, OAc), 2.01 (1H, m, H-7b), 1.98 (1H, m, H-23b), 1.96 (1H,m, H-20), 1.88 (1H, dt, J = 12.8, 3.1 Hz, H-12a), 1.69 (1H, m, H-16a), 1.64 (1H, 1H, m, H-8), 1.61 (1H, m, H-15a), 1.39 (1H, m, H-9), 1.35 (1H, m, H-16b), 1.31 (1H, m, H-11a), 1.25 (3H, s, H_3-19), 1.13 (1H, m, H-15b), 1.14 (1H, m, H-14), 1.08 (1H, m, H-17), 1.03 (1H, dd, J = 12.8, 3.7 Hz, H-12b), 0.94 (3H, d, J = 6.8, H_3-21), 0.90 (1H, m, H-11b), 0.65 (3H, s, H_3-18); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 199.3 (C, C-1), 170.9 (C, OAc), 170.1 (C, OAc), 165.2 (C, C-26), 156.9 (C, C-24), 140.4 (CH, C-3), 128.0 (CH, C-2), 121.9 (C, C-25), 78.0 (CH, C-22), 77.6 (C, C-5), 67.8 (CH, C-4), 58.4 (C, C-10), 58.0 (CH_2, C-27), 54.8 (CH, C-14), 51.7 (CH, C-17), 46.1 (CH, C-9), 43.3 (C, C-13), 42.1 (CH_2, C-7), 39.0 (CH_2, C-12), 38.7 (CH, C-20), 37.6 (CH, C-8), 34.7 (CH, C-6), 30.0 (CH_2, C-23), 27.2 (CH_2, C-16), 23.8 (CH_2, C-15), 23.0 (CH_2, C-11), 22.4 (CH_3, OAc), 20.9 (CH_3, OAc), 20.6 (CH_3, C-28), 13.2 (CH_3, C-21), 11.8 (CH_3, C-18), 9.3 (CH_3, C-19); HRESIMS m/z 705.1894 $[M + Na]^+$ (calcd for $C_{32}H_{43}INaO_8$, 705.1900).

*(22R)-4β,16β,27-Triacetoxy-5β-hydroxy-6α-iodo-1-oxowitha-2,24-dienolide (**23**):* white powder; mp >168 °C dec; $[\alpha]_D^{25}$ +127 (c 0.1, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz) δ 6.27 (1H, dd, J = 10.4, 2.7 Hz, H-3), 6.09 (1H, dd, J = 2.7, 2.0 Hz, H-4), 6.04 (1H, dd, J = 10.4, 2.0 Hz, H-2), 4.95 (1H, ddd, J = 11.1, 7.4, 3.4 Hz, H-16), 4.88 (1H, d, J = 12.0 Hz, H-27a), 4.81 (1H, d, J = 12.0 Hz, H-27b), 4.59 (1H, dd, J = 13.2, 4.2 Hz, H-6), 4.23 (1H, dt, J = 13.2, 3.2 Hz, H-22), 2.51 (1H, m, H-23a), 2.50 (1H, m, H-20), 2.43 (1H, m, H-15a), 2.42 (1H, m, H-7a), 2.20 (3H, s, OAc), 2.05 (3H, s, H_3-28), 2.04 (3H, s, OAc), 2.02 (3H, s, OAc), 1.99 (1H, m, H-23b), 1.92 (1H, dd, J = 13.0, 3.0 Hz, H-7b), 1.91 (1H, m, H-12a), 1.73 (1H, m, H-8), 1.41 (1H, m, H-9), 1.38 (1H, m, H-11a), 1.26 (3H, s, H_3-19), 1.17 (1H, dd, J = 12.4, 7.1 Hz, H-15b), 1.16 (1H, m, H-14), 1.07 (1H, m, H-12b), 1.10 (1H, m, H-17), 0.94 (1H, m, H-11b), 0.86 (3H, s, H_3-18); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 199.1 (C, C-1), 170.8 (C, OAc), 170.7 (C, OAc), 170.1 (C, OAc), 165.0 (C, C-26), 156.6 (C, C-24), 140.4 (CH, C-3), 128.1 (CH, C-2), 122.0 (C, C-25), 77.6 (C, C-5), 76.9 (CH, C-22), 73.8 (CH, C-16), 67.4 (CH, C-4), 57.9 (CH_2, C-27), 54.8 (CH, C-14), 57.0 (C, C-10), 53.1 (CH, C-17), 46.1 (CH, C-9), 41.9 (CH_2, C-7), 43.5 (C, C-13), 38.9 (CH_2, C-12), 37.1 (CH, C-8), 34.9 (CH_2, C-15), 33.8 (CH, C-6), 33.4 (CH, C-20), 30.3 (CH_2, C-23), 22.7 (CH_2, C-11), 22.4 (CH_3, OAc), 21.3 (CH_3, OAc), 20.8 (CH_3, OAc), 20.7 (CH_3, C-28), 12.8 (CH_3, C-21), 12.5 (CH_3, C-18), 9.4 (CH_3, C-19); HRESIMS m/z 763.1956 $[M + Na]^+$ (calcd for $C_{34}H_{45}INaO_{10}$, 763.1955).

*(22R)-4β,15β,27-Triacetoxy-5β-hydroxy-6α-iodo-1-oxowitha-2,24-dienolide (**24**):* white powder; mp >145 °C dec; $[\alpha]_D^{25}$ +98 (c 0.1, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz) δ 6.28 (1H, dd, J = 10.1, 2.1 Hz, H-3), 6.10 (1H, dd, J = 2.1, 1.9 Hz, H-4), 6.05 (1H, dd, J = 10.1, 2.0 Hz, H-2), 4.91 (1H, m, H-15), 4.88 (1H, d, J = 11.8 Hz, H-27a), 4.83 (1H, d, J = 11.8 Hz, H-27b), 4.63 (1H, dd, J = 13.2, 4.7 Hz, H-6), 4.33 (1H, dt, J = 13.1, 3.4 Hz, H-22), 2.49 (1H, dd, J = 17.7, 13.1 Hz, H-23a), 2.42 (1H, m, H-7a), 2.40 (1H, m, H-16a), 2.20 (3H, s, OAc), 2.06 (3H, s, H_3-28), 2.08−2.02 (3H, m, H-7b, H-8, H-20), 2.05 (3H, s, OAc), 2.04 (3H, s, OAc), 1.94 (1H, dd, J = 17.7, 3.4 Hz, H-23b), 1.90 (1H, m, H-12a), 1.45 (1H, m, H-9), 1.38 (1H, m, H-11a), 1.37 (1H, m, H-16b), 1.30 (3H, s, H_3-19), 1.09 (3H, m, H-12b, H-14, H-17), 0.96 (3H, d, J = 6.5 Hz, H_3-21), 0.94 (1H, m, H-11b), 0.88 (3H, s, H_3-18); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 199.1 (C, C-1), 171.0 (C, OAc), 170.8 (C, OAc), 170.7 (C, OAc), 165.0 (C, C-26), 156.5 (C, C-24), 140.4 (CH, C-3), 128.1 (CH, C-2), 122.2 (C, C-25), 77.7 (CH, C-22), 77.6 (C, C-5), 72.7 (CH, C-15), 67.7 (CH, C-4), 57.9 (CH_2, C-27), 57.4 (CH, C-14), 57.0 (C, C-10), 51.8 (CH, C-17), 46.6 (CH, C-9), 43.6 (C, C-13), 41.1 (2 x CH_2, C-7, C-16), 40.2 (CH_2, C-12), 38.2 (CH, C-8), 33.9 (CH, C-8), 33.8 (CH, C-20), 30.1 (CH_2, C-23), 23.0 (CH_2, C-11), 22.4 (CH_3, OAc), 21.3 (CH_3, OAc), 20.9 (CH_3, OAc), 20.6 (CH_3, C-28), 13.8 (CH_3, C-18), 13.3 (CH_3, C-21), 9.5 (CH_3, C-19); HRESIMS m/z 763.1956 $[M + Na]^+$ (calcd for $C_{34}H_{45}INaO_{10}$, 763.1955).

*(22R)-4β-Acetoxy-5β,20-dihydroxy-6α-iodo-1-oxowitha-2,24-dienolide (**27**):* white powder; mp 147−149 °C; $[\alpha]_D^{25}$ +63 (c 0.2, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz) δ 6.26 (1H, dd, J = 10.5, 2.3 Hz, H-3), 6.12 (1H, dd, J = 2.3, 1.9 Hz, H-4), 6.03 (1H, dd, J = 10.5, 1.9 Hz, H-2), 4.63 (1H, dd, J = 13.6, 4.5 Hz, H-6), 4.16 (1H, dd, J = 13.2, 3.3 Hz, H-22), 2.46 (1H, dt, J = 13.7, 4.3 Hz, H-7a), 2.36 (1H, brt, J = 16.9 Hz, H-23a), 2.20 (3H, s, OAc), 2.07 (1H, m, H-7b), 2.06 (1H, m, H-23b), 1.99 (1H, m, H-11a), 1.96 (1H, m, H-12), 1.93 (3H, s, H_3-28), 1.87 (3H, s, H_3-27), 1.65 (1H, m, H-8), 1.62 (1H, m, H-15a), 1.55 (1H, dd, J = 12.7, 3.6 Hz, H-11b), 1.49 (1H, m, H-17), 1.40 (1H, m, H-9), 1.25 (3H, s, H_3-21), 1.33 (1H, dd, J = 12.7, 3.6 Hz, H-16a), 1.22 (3H, s, H_3-19), 1.17 (2H, m, H-14, H-15b), 1.16 (1H, m, H-12b), 0.91 (1H, m, H-16b), 0.81 (3H, s, H_3-18); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 199.4 (C, C-1), 170.0 (C, OAc), 166.0 (C, C-26), 148.8 (C, C-24), 140.4 (CH, C-3), 128.1(CH, C-2), 122.0 (C, C-25), 80.8 (CH, C-22), 77.5 (C, C-5), 75.0 (C, C-20), 67.7 (CH, C-4), 57.1 (C, C-10), 55.4 (CH, C-14), 54.4 (CH, C-17), 46.2 (CH, C-9), 43.5 (C, C-13), 42.1 (CH_2, C-7), 39.4 (CH_2, C-12), 37.0 (CH, C-8), 34.8 (CH, C-6), 31.5 (CH_2, C-23), 23.4 (CH_2, C-15), 22.7 (CH_2, C-16), 22.5 (CH_3, OAc), 21.8 (CH_2, C-11), 20.8 (CH_3, C-21), 20.6 (CH_3, C-28), 13.7 (CH_3, C-18), 12.5 (CH_3, C-27), 9.3 (CH_3, C-19); HRESIMS m/z 663.1769 $[M + Na]^+$ (calcd for $C_{30}H_{41}INaO_7$, 663.1795).

*(22R)-4β,5β,27-Trihydroxy-6α-iodo-1-oxowitha-2,24-dienolide 4,5-carbonate (**28**).* To a stirred solution of **19** (5.0 mg, 8.4 μmol) in dry CH_2Cl_2 (0.5 mL) was added 1,1′-carbonyldiimidazole (3.25 mg, 20.2 μmol), and the mixture was stirred at 25 °C. After stirring for 16 h, the mixture was concentrated and the residue was partially purified using a short column of silica gel and elution with EtOAc to provide a crude product. This was further purified by silica gel preparative TLC [CH_2Cl_2−MeOH (95:5)] to afford a white solid (2.6 mg, 43%, R_f 0.63), which on hydrolysis with LiOH (0.5 mg) in THF (0.4 mL) and H_2O (0.02 mL) at 0 °C for 3 h followed by usual workup afforded a crude product. This was purified by silica gel preparative TLC [CH_2Cl_2−MeOH (95:5)] to afford **28** (2.0 mg, R_f 0.57) as a white solid: ^1H NMR (400 MHz, $CDCl_3$) δ 6.70 (1H, dd, J = 10.4, 3.7 Hz, H-3), 6.20 (1H, d, J = 10.4 Hz, H-2), 5.16 (1H, d, J = 3.7 Hz, H-4), 4.69 (1H, dd, J = 13.5, 4.5 Hz, H-6), 4.38 (1H, dt, J = 17.7, 13.5 Hz, H-22), 4.37 (1H, d, J = 13.0 Hz, H-27a), 4.32 (1H, d, J = 13.0 Hz, H-27b), 2.58 (1H, dt, J = 13.5, 4.2 Hz, H-7a), 2.45 (1H, dd, J = 13.5, 3.6 Hz, H-23a), 2.02 (3H, s, H_3-28), 1.97 (1H, m, H-20), 1.93 (1H, m, H-23b), 1.91 (1H, m, H-12a), 1.79 (1H, m, H-7b), 1.68 (1H, m, H-16a), 1.65 (1H, m, H-15a), 1.59 (1H, m, H-8), 1.37 (1H, m, H-16b), 1.34 (1H, m, H-11a), 1.32 (3H, s, H_3-19), 1.25 (1H, m, H-9), 1.18 (1H, m, H-15b), 1.09 (1H, m, H-17), 1.06 (1H, m, H-12b), 0.96 (1H, m, H-

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14), 0.95 (3H, d, J = 6.4 Hz, H_3-21), 0.86 (1H, m, Hb-11), 0.66 (3H, s, H_3-18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 197.3 (C, C-1), 166.8(C, C-26), 152.5 (C, C-24), 151.4 [C, O(C = O)O], 135.6 (CH, C-3), 129.3 (CH, C-2), 125.8 (C, C-25), 87.6 (C, C-5), 78.5 (CH, C-22), 72.9 (CH, C-4), 57.5 (CH_2, C-27), 54.7 (CH, C-14), 54.6 (C, C-10), 51.7 (CH, C-17), 48.5 (CH, C-9), 43.1 (CH_2, C-7), 42.7 (C, C-13), 38.6 (CH_2, CH, C-12 and C-20), 36.9 (CH, C-8), 29.9 (CH_2, C-23), 28.2 (CH, C-6), 27.1 (CH_2, C-16), 23.9 (CH_2, C-15), 22.3 (CH_2, C-11), 20.0 (CH_3, C-28), 13.3 (CH_3, C-21), 11.7 (CH_3, C-18), 10.2 (CH_3, C-19); HRESIMS m/z 647.1474 $[M + Na]^+$ (calcd for $C_{29}H_{37}INaO_7$, 647.1482).

Cytotoxicity Assays. Ewing's sarcoma cell line CHP-100 was cultured at 37 °C under 6% CO_2 in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), while 293T (American Type Culture Collection) kidney cancer cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS. All cell lines were tested and found negative for *Mycoplasma* contamination. Cultures were passaged twice weekly, and cells in exponential growth were used for experiments. Stock solutions of compounds were formulated in DMSO and maintained at −20 °C protected from light. To measure acute toxicity, CHP-100 cells were seeded in flat-bottom 96-well plates (7500 cells/well) and allowed to adhere overnight. Serial dilutions of compounds or DMSO vehicle control (not exceeding 0.2%) were added, and the relative viable cell number was determined 24 h later by the dye reduction assay using resazurin (Alamar Blue) as previously described.[18] Cytotoxicity over 3 days against 293T cells was measured in 384-well format (3000 cells/well), and the relative viable cell number was determined by the same (Alamar Blue) assay.[18]

Heat-Shock Reporter Assays. Reporter cells were generated by infecting 293T cells with a reported lentiviral vector encoding a fusion protein consisting of enhanced green fluorescent protein (GFP) fused to firefly luciferase under control of *HSP70B′* promoter elements.[47] The plasmid encoding the fusion protein was generously provided by Khalid Shah (Massachusetts General Hospital, Boston, Massachusetts, USA). To isolate a homogeneous population of high-responding cells, a transduced culture was heat shocked at 42 °C for 1 h and processed 8 h later by fluorescence activated cell sorting (FACS). Prior to use, cells were reverse selected by FACS to eliminate a minority population of cells constitutively expressing the reporter in the absence of induction. To evaluate compounds, cells were seeded in white 384-well plates (20 000 cells/well). The following day, serial compound dilutions were added to quadruplicate wells and incubation continued overnight. Measurement of relative luciferase activity was achieved using an Envision plate luminometer (PerkinElmer) and Steady-Glo reagent (Promega) per manufacturer's recommendations. As a complementary assay for some compounds, 3T3-Y9-B12 reporter cells were seeded in black flat-bottom 96-well plates (20 000/well) and allowed to adhere overnight as previously reported.[30a] Cells were incubated for 24 h in the presence of withanolide analogues (1.0 or 5.0 μM). After washing with PBS, fluorescence was quantified using an Analyst AD (LJL Biosystems) plate reader with excitation and emission filters set at 485 and 530 nm, respectively.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00918.

^{1}H, ^{13}C, and 2D NMR spectra of withanolides **5**, **8**, **9**, **10**, **12**, **17**, **19**−**23**, and **25**−**28**; ^{1}H and ^{13}C NMR spectra of withanolides **13**, **18**, and **24**; structures of MG-132 and STA-9090 (PDF)

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Notes

The authors declare no competing financial interest.

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