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Potent lipid peroxidation inhibitors from *Withania somnifera* fruits

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Abstract—A bioassay-guided purification of the methanolic extract of *Withania somnifera* fruits yielded novel withanamides A–I (**1**–**9**) and withanolides (**10**–**13**). Among the withanolides, compound **10** is novel. The structures of these compounds were determined by using FABMS, HRFABMS, 1D and 2D NMR spectral and chemical methods. The withanamides possess novel chemical structures and consisted of serotonin, glucose and long-chain hydroxyl fatty acid moieties. The stereochemistry of the hydroxyl group in the long-chain fatty acid moiety in compound **1** was determined by the modified Mosher's ester method. Compounds **1**–**13** were tested for their ability to inhibit lipid peroxidation in a model system using large unilamellar vesicles. Withanamides **1**–**5** and **9** inhibited lipid peroxidation by 98, 93, 79, 94, 81 and 86%, respectively, at 1 μg/mL. However, compounds **6**–**8** inhibited the lipid peroxidation by 85, 82 and 90%, respectively, at 0.5 μg/mL. Withanolides **10**–**13** were also tested and only compound **12** inhibited the lipid peroxidation by 82% at 10 μg/mL. To evaluate the structure activity relationships of withanamides A–I, compounds **14**–**16** were purchased and their lipid peroxidation activity determined as in the case of compounds **1**–**9**. Commercial antioxidants, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ), were also tested in this assay at 1 μg/mL and showed 80, 81 and 85% of inhibition, respectively. Our results suggest that the potent antioxidant activity exhibited by novel withanamides is probably due to the hydroxylated long-chain acyl group. This is the first report of withanamides, unique serotonin conjugates, from *W. somnifera* fruits.

1. Introduction

The life-supporting oxygen becomes toxic to most aerobic organisms when exposed to greater concentrations. Reasons for this toxicity are due to the formation of superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (–OH·) during the conversion of oxygen to water in the mitochondria. The free radicals generated from environmental contaminants and by exogenous factors such as drugs, toxins and stress cause oxidative damage to biological macromolecular structure and function.[1] It results in the progression of many disease processes including atherosclerosis, cardiovascular diseases and cancer. Several studies linked the aging process to the generation of reactive oxygen and nitrogen.[2] The oxidative stress also damages the function of pancreatic β-cells function and results in diabetes.[3] The singlet oxygen reacts with unsaturated fatty acids to form lipid peroxides which in turn decompose to initiate the formation of mutagens. Therefore, natural products or chemicals with the potential to scavenge singlet species could reduce biological disorders that limit the progression of various aging related diseases. Many epidemiological studies show that diets rich in antioxidants play a major role in the prevention of heart disease, cancer, diabetes, and Alzheimers's disease.[4]

Some of the pharmaceuticals prescribed for depression or anxiety contain natural antioxidants. Mixtures of ascorbic acid, pyridoxine, carotene, vitamin E, Zn, nicotinamide, and Se are used to treat depression or anxiety. Also, natural antioxidants are used as food additives to inhibit lipid peroxidation and to maintain the nutritional qualities of food. It is also reported that antioxidants decrease the side effects of chemotherapy during cancer treatment.[5] The commonly used synthetic antioxidants to prevent the lipid peroxidation in food are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tert-butylhydroquinone (TBHQ). However, these antioxidants are considered to be potential carcinogens[6] and hence, there is considerable interest in developing safe and natural antioxidants.

Withania somnifera L Dunal, a member of the Solanaceae family and is well known for its medicinal uses. It is an annual herb, and the major constituents reported from its

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roots are withanolides and alkaloids.[7] In Ayurvedic medicine, commonly practiced in India, the fruits of *W. somnifera* are used as an emetic, sedative, diuretic and for the treatment of asthma, atherosclerosis, intestinal and liver disorders.[8] Our recent study on the leaves of this plant yielded several withanolides with cyclooxygenase enzyme inhibitory activity.[9] Also, unsaturated fatty acids[10–12] and withanolides[13,14] were reported from the seeds of *W. somnifera*. However, the components of *W. somnifera* fruits were not investigated for biological activities. In this study, we report the bioassay guided extraction and purification of withanamides, novel serotonin conjugates, and a novel withanolide with potent antioxidant activity from *W. somnifera* fruits.

2. Results and discussion

The fruits were collected from *W. somnifera* plants grown in the greenhouses of the Bioactive Natural Products and Phytoceutical Laboratory at Michigan State University. Dried fruits were ground and extracted at room temperature sequentially with hexane, EtOAc, MeOH and ammoniacal MeOH. Hexane and EtOAc extracts were analyzed by TLC and GCMS and found to contain mainly β-carotene and fatty acids. These extracts did not exhibit antioxidant activities and were not further investigated. Fractionation of the MeOH extract by MPLC yielded five fractions, and all inhibited lipid peroxidation at 10 ppm. Purifications of these active fractions by CC, reverse phase HPLC and prep. TLC yielded compounds **1**–**9**, the withanamides, and **10**–**13**, the withanolides.

Withanamide A (**1**) was obtained as a pale brown amorphous powder. The HRFABMS of **1** displayed an $[M+H]^+$ ion at *m/z* 779.4329 (calcd 779.4330) and indicated its molecular formula as $C_{40}H_{62}N_2O_{13}$. The IR spectrum gave bands at 1633, 3413 cm^{-1} and suggested the presence of amide carbonyl and hydroxyl groups in the molecule. The ^1H NMR spectrum displayed three doublets of doublets at δ 7.15, 6.92, and 6.66, respectively, and a singlet at δ 6.99. Also, it showed two triplets at δ 2.84 and 3.42, respectively. These signals clearly indicated the presence of a substituted tryptamine[15] moiety in the molecule. Two doublets at δ 4.38 and 4.30, integrated for one proton each, were assigned to two anomeric protons, respectively, and indicated that compound **1** contained a disaccharide moiety. A broad singlet at δ 1.27, a triplet at δ 2.12 and a multiplet, integrated for four protons, at δ 5.31 were assigned to the presence of an unsaturated fatty acid moiety in compound **1**. A methyl signal at δ 1.19 appeared as a doublet was indicative of a methine carbon in the fatty acid moiety and was assigned to H-18‴. Also, a one-proton multiplet at δ 3.79 confirmed the presence of a hydroxyl moiety at C-17. Three signals observed at δ 2.04 and 2.75 were typical of allylic methylene protons, and the corresponding carbon signals were at δ 28.2, 28.1 and 26.5, respectively. The ^{13}C NMR shift values indicated that the geometry of double bonds in compound **1** as *Z* since the allylic carbons in the *E* isomer would appear at around 32 ppm.[16,17]

The ^{13}C NMR signals observed in compound **1** at δ 112.4, 112.6, 112.5, 151.0 and 133.0 were assigned to C-6, C-7, C-3, C-5 and C-9, respectively. The signal at δ 176.2 indicated an amide linkage in the molecule. The signals at δ 77.7 and 22.1 were attributable to hydroxyl and methyl carbons, respectively, of the fatty acid moiety in **1**.

Acid hydrolysis of compound **1** gave glucose as the only sugar in addition to serotonin and a fatty acid as products. The identity of glucose was confirmed by NMR spectral data and was further supported by the TLC comparison of the sugar resulting from the hydrolysis with an authentic sample of glucose. The downfield shifts observed for C-6′ (δ 69.7) by 7 ppm as compared to the C-6″ (δ 62.7) in the ^{13}C NMR spectrum of compound **1** suggested a 1″→6′ linkage of the two glucose moieties. The ^1H and ^{13}C NMR data confirmed that the sugar unit in compound **1** was a diglucoside and was also in agreement with published spectral data of diglucosides.[9]

Additional evidence for the structural assignment of compound **1** was obtained from its MS fragmentation, NOESY, HMBC and COSY studies. The ion at *m/z* 617 observed in its MS confirmed the loss of one of the glucose units from the molecular ion. The fragment at *m/z* 455 was assigned to the aglycone moiety and showed that the hydroxyl fatty acid side chain contained 18-carbons. The diglucoside unit was placed at C-5 based on the NOESY correlation of H-1′ to H-4. Also, the HMBC correlations between the H-1″ at δ 4.30 and C-6′ at δ 69.7 confirmed a 1″→6′ linkage of glucose moieties (Fig. 1). The COSY and TOCSY spectral data of compound **1** confirmed the positions of double bonds at C-6‴ and C-9‴, respectively (Fig. 1). The methine proton of the oxygenated carbon at δ 3.79 in the side chain was correlated to the terminal methyl group in the molecule as indicated by its COSY spectrum. It was also supported by HMBC correlations (Fig. 1) and confirmed the −OH group at C-17‴.

The absolute configuration of the −OH group at C-17‴ in compound **1** was determined by Mosher ester method.[18] Compound **1** was reacted separately with *R* (−) and *S* (+) α-methoxytrifluorophenylacetyl chlorides (MTPA) in anhydrous pyridine. Purification of the reaction mixtures yielded the *R* and *S*-MTPA esters. The ^1H NMR analyses of the resulting esters revealed that the terminal methyl in the *S*-MTPA ester appeared at a lower field than in the *R*-MTPA ester. Similarly, H-16‴ in *S*-MTPA ester appeared at higher field than the corresponding proton in the *R*-MTPA ester. The $\Delta\delta$ ($\delta_S-\delta_R$) value for H-18‴ and H-16‴ were+0.03 and −0.02, respectively, and confirmed the configuration at C-17‴ as *R*.[18]

Withanamide B (**2**), a colorless amorphous powder with an $[\alpha]_D$ of −34°, gave the $[M+H]^+$ at *m/z* 755.4330 and confirmed its molecular formula as $C_{38}H_{63}N_2O_{13}$ (calcd 755.4331). The ^1H and ^{13}C NMR spectra of **2** were very similar to those of compound **1** except that it lacked olefinic protons signals. Molecular ion of **2** was 24 amu less than that of compound **1**. This showed that side chain in **2** was saturated and contained only sixteen carbons. The linkage of the glucose moieties was evidenced as 1″→6′ by the downfield shift of C-6′ to δ 69.7 and HMBC correlations observed between C-1″ and H-6′ (Fig. 2). The methyl

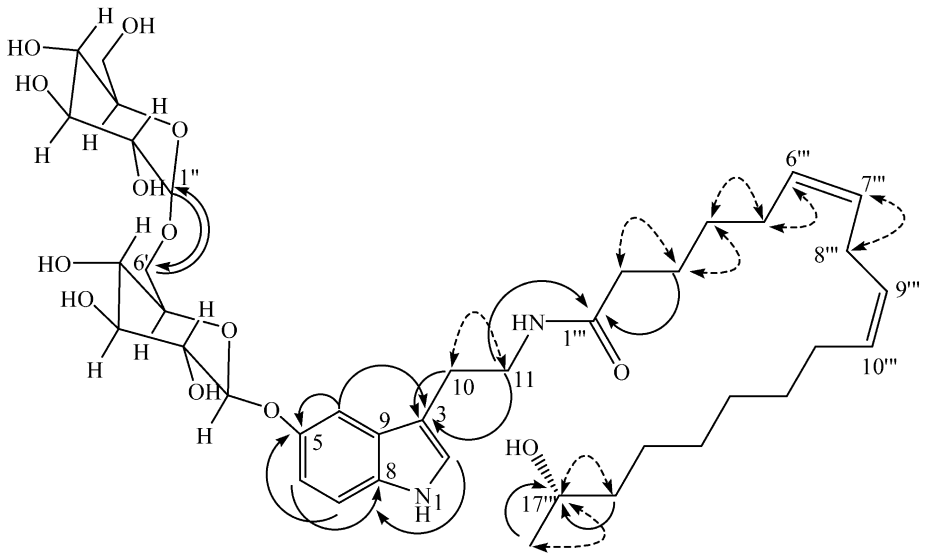


Figure 1. Selected HMBC (→) and COSY (↔) correlations observed in compound **1**.

protons (3H, d, J=6.5 Hz, H-16‴) were correlated to the carbon at δ 77.7 in its HMBC spectrum and confirmed the −OH substitution at C-15‴ (Fig. 2). The proposed structure of **2** was confirmed by HMQC, HMBC, DEPT and NOESY experiments.

The ^{1}H NMR of withanamide C (**3**) was similar to compound **2** except for the presence of a methyl triplet at δ 0.91 instead of a doublet at δ 1.20. In addition, it gave the molecular formula as $C_{38}H_{63}N_2O_{13}$ similar to that of compound **2**. The major difference in the ^{13}C NMR spectrum of **3** was the up-field shift of one of the methylene groups and appeared at δ 26.3. The appearance of a methyl carbon at δ 10.1, as compared to those of the regular fatty acids (14.0 ppm) and the downfield shift of a long chain hydroxyl carbon (δ 82.0) suggested the position of an −OH group at C-14‴. The triplet at δ 0.91 showed a COSY correlation to the methylene protons at δ 1.56, which in turn

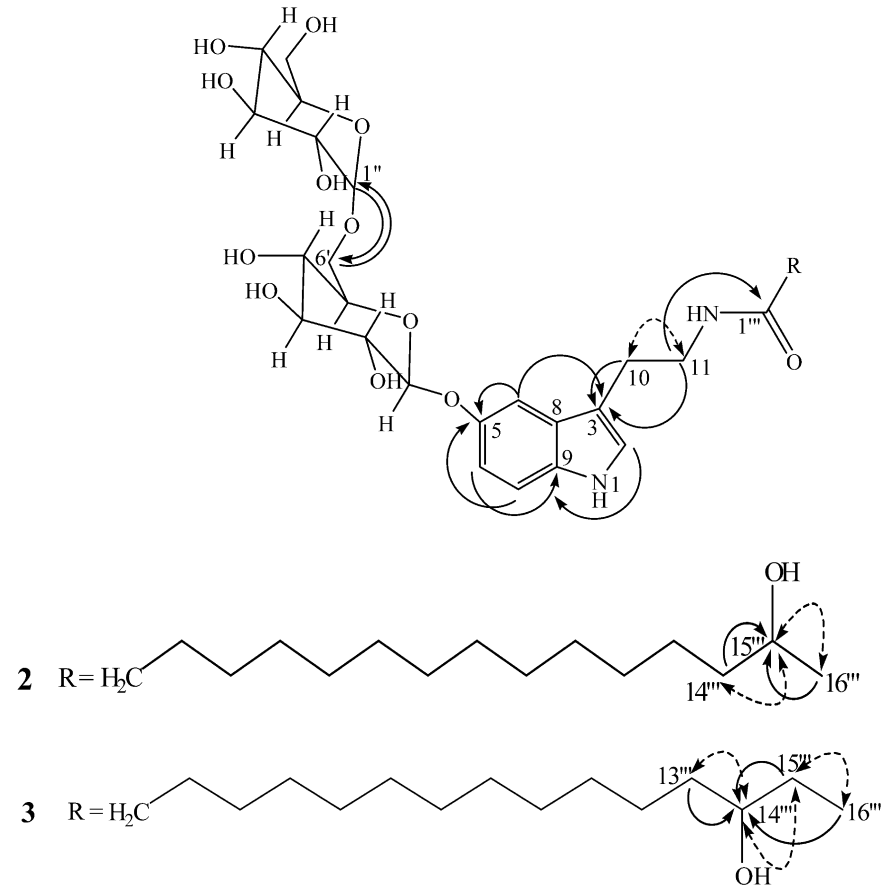


Figure 2. Significant HMBC (→) and COSY (↔) correlations of **2** and **3**.

correlated to the proton at δ 3.63, further confirming the $-$OH moiety at C-14‴ (Fig. 2). The presence of $-$OH at C-14‴ was also substantiated by HMBC correlations of the methyl triplet at δ 0.91 to the hydroxyl carbon at δ 82.0 (Fig. 2). Therefore, compound **3** was confirmed as a positional isomer of **2**.

The HRMS of withanamide D (**4**) gave an $[M+Na]^+$ ion at *m*/*z* 805.4462 that indicated its molecular formula as $C_{40}H_{66}O_{13}N_2$. The ^1H and ^{13}C NMR spectral data of **4** were similar to those of compound **2** and indicated that it contained a saturated side chain with hydroxyl group. Since the methyl signal appeared as a doublet at δ 1.12, the position of the hydroxyl in 4 was assigned at C-17‴. In addition, the MS data confirmed that the side chain in compound **4** consisted of eighteen carbons.

Withanamide E (**5**), a pale brown solid, gave the $[M+H]^+$ ion at *m*/*z* 783.4645 that indicated its molecular formula as $C_{40}H_{66}N_2O_{13}$. The ^1H NMR spectral data of **5** was similar to those of withanamide C (**3**) and indicated the presence of a saturated side chain in the molecule. The methyl triplet at δ 0.91 suggested that the terminal carbons in compound **5** had a similar substitution pattern as in **3**. The difference in the molecular weight by 28 amu, as compared to **3**, indicated the presence of two additional $-CH_2$ groups in **5**. This confirmed that compound **5** contained an 18-carbon side chain with the $-$OH group at C-16‴. Therefore, compound **5** was characterized as a positional isomer of **4**.

The ^1H NMR data of compound **6** were similar to those of withanamide A (**1**), with the exception that it gave a 2H multiplet at δ 5.33 assigned to the olefinic protons in the molecule. The corresponding carbons signals appeared at δ 130.9 and 130.8, respectively. The appearance of a methyl triplet at δ 0.91 together with the signal for a carbon at δ 82.0 in compound **6** indicated that the terminal carbon in the fatty acid moiety had a similar substitution pattern as in compounds **3** and **5**. Therefore, the olefinic moiety was assigned to C-9‴ since the chemical shift of these two olefinic carbons differed by 0.2 ppm.[17] The geometry of the double bond was deduced as *Z* since C-8‴ and C-11‴ appeared at δ 28.1 and 28.2, respectively.[17] The HRFABMS of **6** gave a molecular ion at *m*/*z* 803.4304 $[M+Na]^+$ and further supported a C-18 fatty acid moiety in its structure.

Withanamide G (**7**) gave a molecular ion at *m*/*z* 753.4173, $[M+H]^+$, which was two mass units less than the molecular weight of compound **2** (755.4331). Therefore, it suggested that the fatty acid moiety present in it contained 16-carbons with one olefinic moiety. A 2H multiplet at δ 5.34 supported this assignment. The doublet appeared at δ 1.21, assigned to methyl protons, indicated that the $-$OH moiety present in the side chain had similar substitution to that of compounds **1** and **2**. The ^{13}C NMR displayed signals for serotonin diglucoside and unsaturated hydroxy fatty acid side chain moieties. The olefinic carbon signals in **7** appeared at δ 130.9 and 130.8, similar to that of compound **6**, were assigned to C-9‴ and 10‴, respectively.[17] The geometry of the double bond in compound **7** was therefore deduced as *Z* based on the chemical shifts of the allylic carbons C-8‴ and C-11‴ at δ 28.0 and 28.1, respectively.

Withanamide I (**8**) gave a molecular ion at *m*/*z* 775.4013. The ^1H and ^{13}C NMR spectra of compound **8** were similar to those of withanamide A (**1**) except for the 8H multiplet at δ 5.34. The corresponding carbon signals appeared at δ 132.6, 132.2, 131.4, 130.1, 128.7 and 128.5, respectively, and indicated the presence of four double bonds in the hydroxyl fatty acid moiety. One of the double bonds was assigned at C-15‴ based on correlations observed in its TOCSY spectrum (Fig. 3). A signal at δ 2.82, integrated for 6H, was assigned to three methylene groups adjacent to the double bonds at C-9‴, C-12‴ and C-15‴. As in withanamide A, the geometry of the double bonds was deduced to be *Z* based on the chemical shifts of allylic carbons at δ 26.6, 27.0 and 28.2, respectively, in its ^{13}C NMR spectrum.[17]

Withanamide I (**9**) also gave a similar ^1H NMR spectral data to that of withanamide A (**1**) as indicated by the chemical shifts for the serotonin and hydroxyl-fatty acid moieties in it. The $[M+H]^+$ ion at *m*/*z* 941.4857 confirmed that the molecular formula of compound **9** was $C_{46}H_{72}O_{18}N_2$. The presence of two double bonds in the fatty acid moiety was confirmed by 4H multiplet at 5.33 ppm. In addition, the presence of three anomeric protons at δ 4.32, 4.36 and 4.39

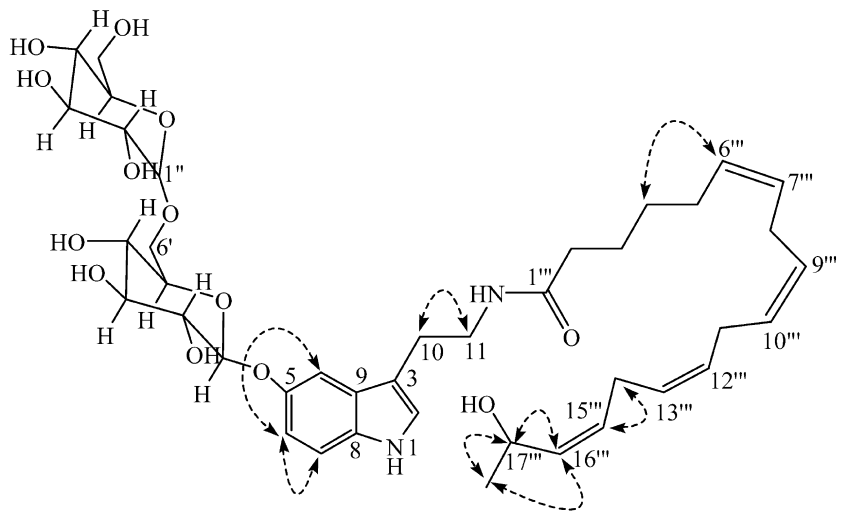
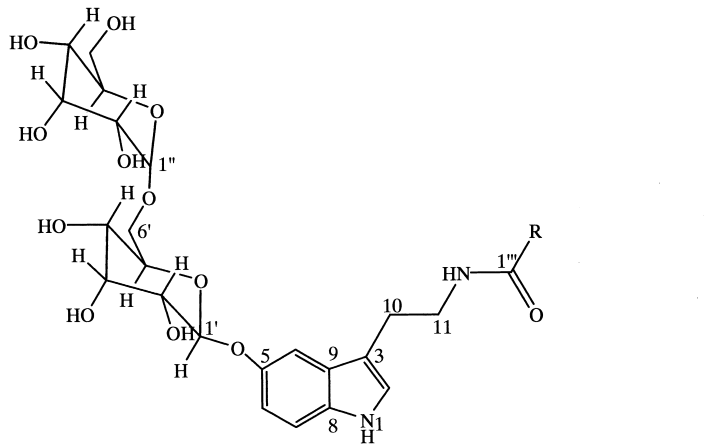
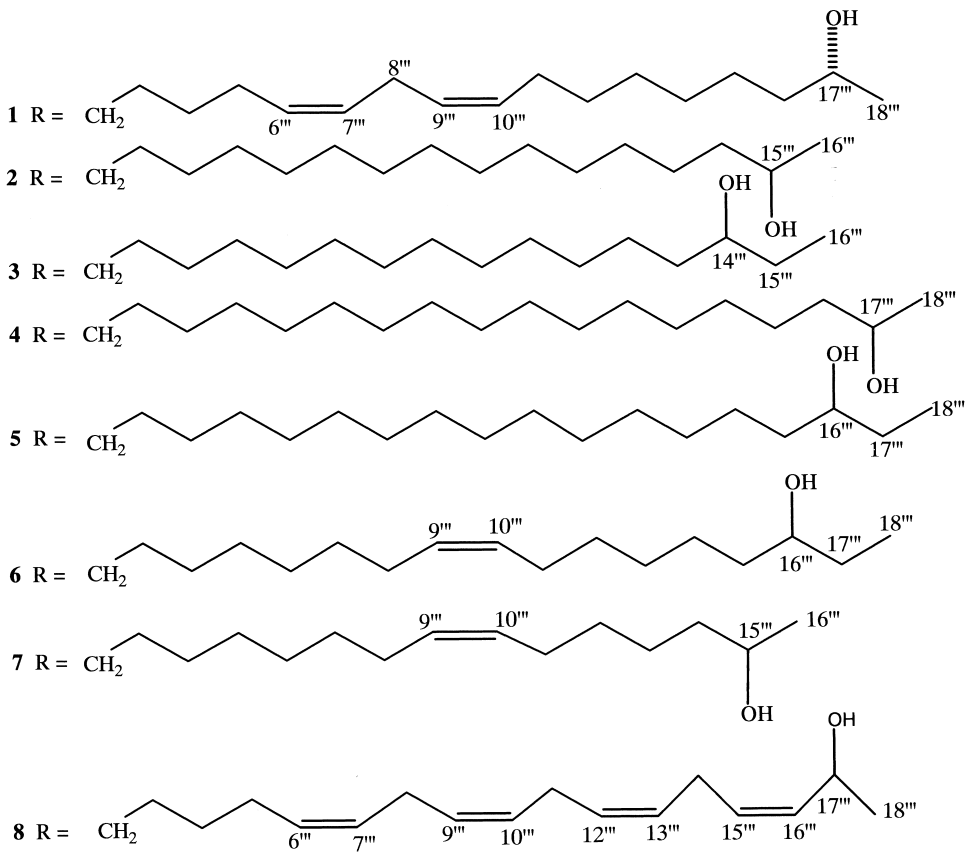


Figure 3. Selected TOCSY (→) correlations of **8**.

indicated that withanamide I (**9**) was a triglucoside. The linkage of two glucose units, as in the case of withanamide A (**1**), was established as C-1″→C-6′as indicated by the downfield shift of H-6′ protons. A third glucose unit present in **9** was also assigned a linkage of 1‴→6″, as confirmed by the downfield shift of H-6″. Therefore, the polysaccharide unit in compound **9** was established as β-D-glucopyranosyl (1″→6′)-β-D-glucopyranosyl (1‴→6″)-β-D-glucopyranoside. The spectral data of compound **1** and **9** were very similar except that compound **9** showed signals for an additional glucose moiety. Also, the appearance of the methyl group as a doublet in the ^{1}H NMR of compound **9** was identical to that of the terminal substitution of long chain fatty acid moiety present in withanamide A (**1**).

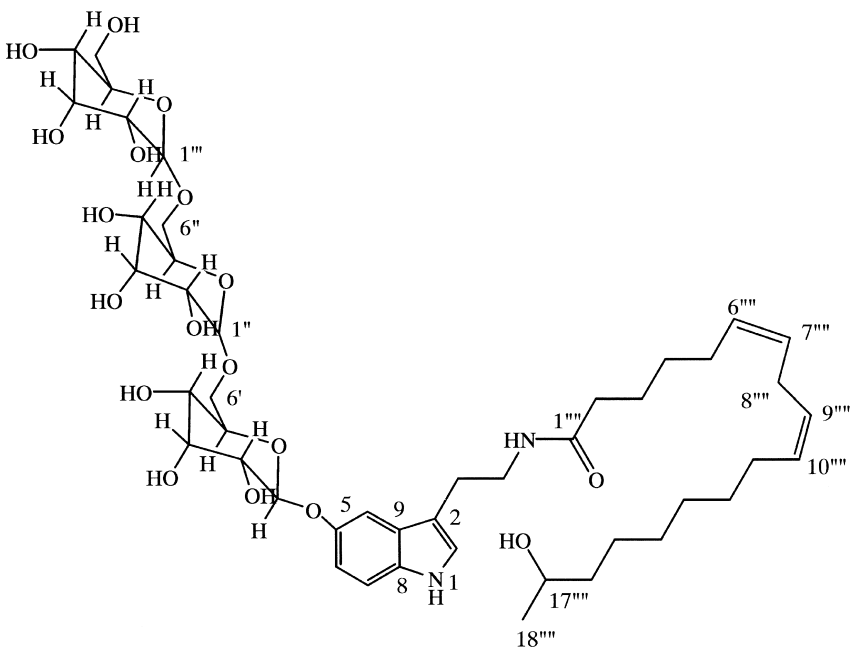
Compound **10** was isolated as a colorless amorphous powder and displayed a molecular ion at *m*/*z* 785 in its FABMS spectrum. The IR absorption bands at 3421, 1724 and 1663 cm^{-1} in **10** suggested the presence of an −OH and a saturated lactone in the molecule. The HRFABMS confirmed its molecular formula as $C_{40}H_{65}O_{15}$ (M+H$^+$ 785.4325; calcd 785.4323). Three singlets at δ 0.89, 1.01, 1.25 and two doublets at δ 1.17 and 1.15 observed in its ^{1}H NMR spectrum were assigned to the methyl groups at 18, 19, 21, 27 and 28, respectively. The broad doublet at δ 5.52 and doublets at δ 4.39 and 4.36, which integrated for one proton each, were assigned to olefinic and anomeric protons, respectively. The doublet of doublets at δ 4.24 and a multiplet at δ 4.00 were assigned to H-22 and H-3, respectively. Although compounds **10** and withanoside VI (**11**) showed similar ^{1}H NMR chemical shifts,[19] the appearance of two methyl doublets in **10** indicated that a





double bond in the α,β-unsaturated δ-lactone ring was absent. In addition, the signal at δ 178.9, assigned to C=O, confirmed a saturated lactone moiety in compound **10**. Two signals at δ 104.8 and 103.1, assigned to anomeric carbons, supported a diglucosidic moiety in the molecule. The downfield shift of H-6′ protons as compared to H-6″ indicated a 1″→6′ linkage of the two glucose moieties in compound **10**. Also, the downfield shift of C-6 (δ 69.7) of one of the glucose units further confirmed the linkage as 1″→6′ similar to the results in withanamides. The carbon signals at δ 81.9, 58.1, 56.1, 139.1, 125.5, 75.1 and 73.6 were assigned to C-22, C-14, C-17, C-5, C-6, C-1 and C-3, respectively. Other carbon signals, which appeared at δ 14.2, 14.4, 19.9, 20.5 and 21.2, were assigned to methyls at C-18, C-28, C-19, C-27 and C-21, respectively. The position of the diglucoside moiety was assigned at C-3 by comparison of its spectral data to the spectral data of withanolides **11**–**13**. The molecular ion at *m*/*z* 784 indicated that it is two mass units higher than the withanoside VI and further supported the proposed structure for compound **10**. From the spectral data, the structure of compound **10** was derived as 24, 25-dihydrowithanolide VI.

Serotonin, a neurotransmitter, is the aglycone moiety in all the withanamides A–I (**1**–**9**) characterized from *W. somnifera* fruits. Therefore, to compare the structure and activity of these compounds, tryptamine (**14**), 5-methoxy-serotonin (**15**) and serotonin (**16**) were assayed along with the withanamides. Compounds **1**–**16** and commercial antioxidants BHT, BHA and TBHQ were tested for the inhibition of lipid peroxidation by using large unilamellar vesicles (LUVs) model system.[20] A dose response study was performed for all compounds and Fig. 4 represents the activity profiles of withanamides compared to the commercial antioxidants evaluated at 1 ppm concentration. BHA, BHT and TBHQ inhibited lipid peroxidation by 80, 81 and



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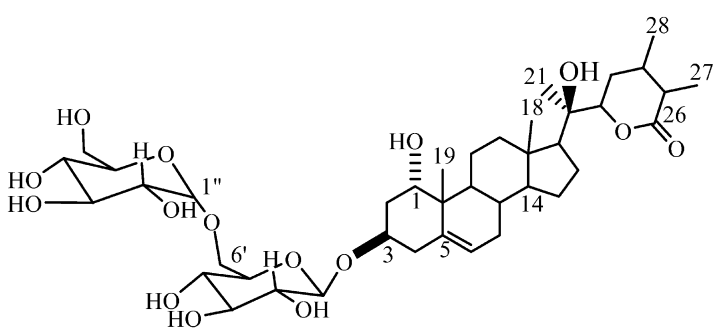

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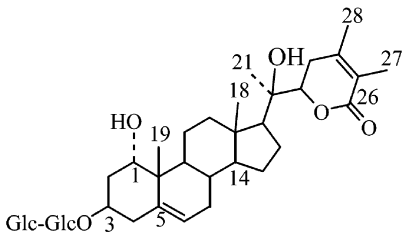

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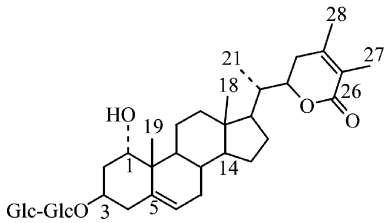

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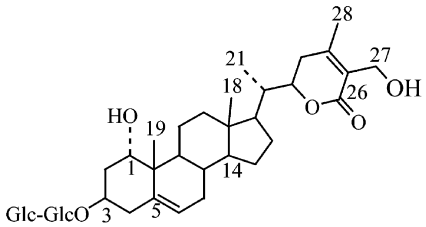

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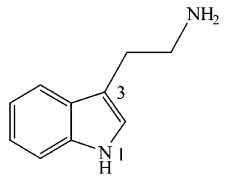

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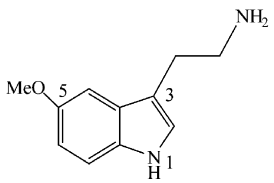

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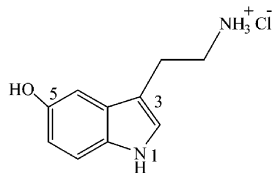

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85%, respectively, at 1 μg/mL (Fig. 4). Withanamide B (**2**) contained a saturated side chain and inhibited lipid peroxidation by 93% at 1 μg/mL (Fig. 4) whereas withanamide C (**3**), a positional isomer of **2**, showed 79% inhibition. Similarly, the inhibitions observed with withanamide D (**4**) and E (**5**) were 94 and 81%, respectively, at 1 μg/mL. Interestingly, compounds **6** and **7** with one double bond in the acyl moiety showed 85 and 82% inhibition, respectively, at 0.5 μg/mL. Similarly, withanamide H (**8**) with four double bonds in its fatty acid moiety exhibited 90% inhibition at 0.5 μg/mL. However, Withanamide A (**1**), a digluoside with two double bonds in its fatty acid moiety, inhibited lipid peroxidation by 98% whereas withanamide I (**9**), a triglucoside, gave 86% inhibition at 1 μg/mL (Fig. 4).

Tryptamine (**14**) inhibited lipid peroxidation by 40% at 100 μg/mL. However, 5-methoxytryptamine or 5-methylserotonin, compound **15**, inhibited lipid peroxidation by 30% at 50 μg/mL compared to serotonin (5-hydroxy tryptamine) hydrochloride which inhibited by 44% at 10 μg/mL. 5-Methoxytryptamine (**15**) gave a higher activity than tryptamine (**7**) and indicated that 5-oxygenation contributed to the increased lipid peroxidation activity. Increased peroxidation inhibition was observed for serotonin hydrochloride when compared to its 5-methoxyl derivative and suggested that the free hydroxyl at the 5-position was significant to account for the increased lipid peroxidation activity. Withanamides A–I, (**1**–**9**), exhibited excellent lipid peroxidation inhibitory activity equal to or better than the commercial antioxidants and far better than serotonin (Fig. 4). The serotonin and hydroxyl fatty acid moieties were therefore contributing to the strong lipid peroxidation inhibitory activity displayed by these compounds. Among withanamides, the unsaturation in the acyl moiety further contributed to higher lipid peroxidation inhibitory activity. Compounds **2** and **4**, with hydroxyl groups at C-15‴ and C-17‴, respectively, were more active than their isomers **3** and **5**. This indicated that the position of the hydroxyl groups in the acyl moieties also played an important role in the antioxidant activity.

Withanolides isolated from the fruits *W. somnifera* in our study also inhibited lipid peroxidation (Fig. 5). Withanoside V (**12**), one of the major compounds isolated from *W. somnifera* fruits, showed 82.5% inhibition of lipid peroxidation at 10 ppm whereas the inhibition of withanoside IV (**13**) was only 25% at 100 μg/mL. Withanolide VI (**11**) gave 86% lipid peroxidation inhibitory activity at 50 ppm and its 24, 25-dihydroderivative (**10**) showed similar activity at 100 ppm (Fig. 5). The saturation of the lactone moiety in compound **10** decreased the activity as compared to its dehydroderivative **11** and indicated that the α,β-unsaturated δ-lactone moiety was significant for the

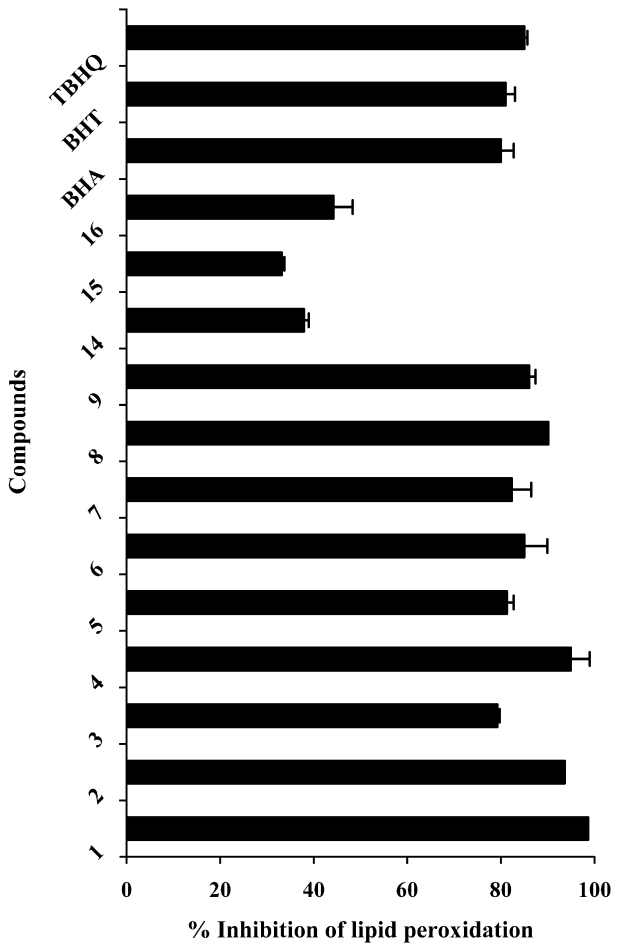

Figure 4. Inhibition of lipid peroxidation by compounds **1**–**9** and **14**–**16**. Fluorescence intensity was monitored for 21 min at intervals of 3 min. The percentage of inhibition represented was calculated with respect to DMSO control at 21 min. Fe^{2+} was used to induce the peroxidation. The concentrations of compounds tested were **1**–**5** and **9** at 1 μg/mL; **6**–**8** at 0.5 μg/mL; **15** at 100 μg/mL; **16** at 50 μg/mL; **17** at 10 μg/mL. Commercial antioxidants BHA, BHT and TBHQ were tested at 1 μg/mL. Data represented indicates the mean±one standard deviation (n=2).

lipid peroxidation inhibitory activity of withanolides. Hydroxylation at C-27 in compound **13** decreased the activity than the hydroxylation at C-20 in compound **11**. This may be due to hydrogen bonding between the C-27 hydroxyl and the carbonyl group of the lactone moiety in compound **13**.

Withanamides A–C (**1**–**3**) were also tested in the 2,2-azobis-(2-amidinopropane) dihydrochloride (AAPH) induced lipid peroxidation (Fig. 6) assay[20] using LUVs under identical conditions with LUVs and Fe^{2+}. Withanamides **1**–**3** and TBHQ exhibited 71, 60, 63 and 67% of inhibition at 1 μg/mL, respectively. This confirmed that the lipid peroxidation inhibitory activity observed for withanamides was not due to the chelation effect with Fe^{2+}.

Cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) enzymes inhibitory activities of withanamides A–C (**1**–**3**) and withanoside V (**12**) were found to be inactive when assayed at 100 μg/mL.[9] Compounds **1**–**3** were also tested for the inhibition of tumor cell proliferation on NCI-H460 (Lung), HCT-116 (colon), SF-268 (Central Nervous System; CNS) and MCF-7 (breast) human tumor cell lines using MTT assay[21] and found to be inactive. This implied that withanamides possessed little or no cytotoxicity. These compounds were not tested against corresponding normal cells due to the unavailability of normal cells.

The lipid peroxidation inhibition by these withanamides at 0.5–1 μg/mL was similar to or better than that of BHA, BHT and TBHQ, suggesting that these compounds could be used as natural antioxidants and a potential substitute for BHA, BHT and TBHQ. It is important to note that these compounds did not exhibit cellular toxicity in our human tumor cell assays. Therefore, *W. somnifera* fruits or the withanamides are potential candidates for the development of natural antioxidants for food and health applications.

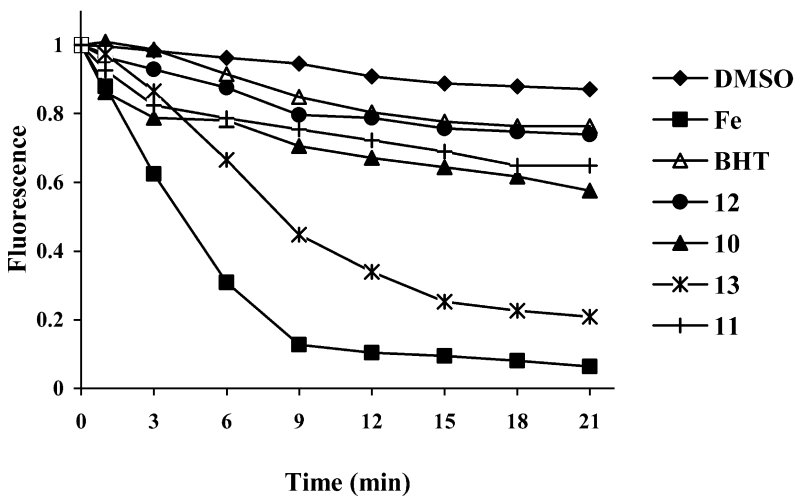

Figure 5. The fluorescence intensity was monitored over 21 min of Fe^{2+} induced lipid peroxidation by withanolides **10**–**13** at intervals of 3 min. BHT was used as positive control in this assay at 1 ppm. Compounds tested were **10** and **13** at 100 μg/mL; **12** and **11** were at 10 and 50 μg/mL, respectively. Data represented indicates the mean±one standard deviation (n=2).

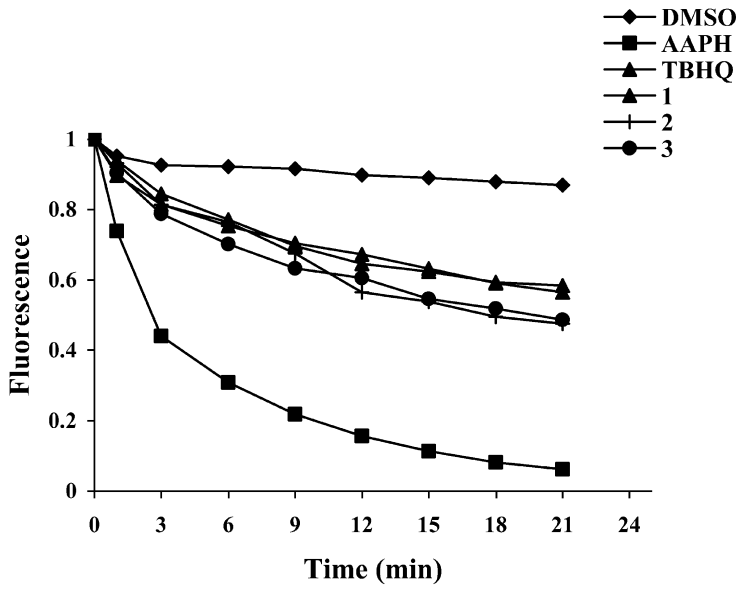

Figure 6. Effect of withanamides **1**–**3** on AAPH induced lipid peroxidation at 1 ppm. Fluorescence intensity was monitored for 21 min at intervals of 3 min. The percentage of inhibition was with respect to control (DMSO). TBHQ was used as positive control in this assay at 1 ppm. The concentration of AAPH was 2.5 mM at 37 °C.

3. Experimental

3.1. General

The HRFAB and FAB (positive ion mode) mass spectra were measured on JEOL MX 110 mass spectrometer at the Mass Spectrometry Facility Center, Michigan State University. ^{1}H (500 MHz) and ^{13}C (125 MHz) and 2D NMR experiments were carried out on an INOVA VARIAN VRX 500 instrument using standard pulse sequences. The chemical shifts were measured in CD_3OD and expressed in δ (ppm). HMBC was optimized for J=8 Hz. IR spectra were recorded on Mattson Galaxy Series FTIR 300 using WinFIRST software (Thermo Nicoloet, Madison, WI) spectrometer. $[\alpha]_D$ was measured in MeOH at 20 °C using a Polarimeter (Perkin Elmer Model 341, Shelton, CT). ACS grade solvents were used for the isolation and purification. The silica gel used for MPLC was Merck Silica gel 60 (35–70 μm particle size). Si gel PTLC plates (20×20, 500 μm) were purchased from Analtech, Inc. (Newark, DE). Recycling preparative HPLC (Japan Analytical Industry Co. model LC-20) was used with JAIGEL-ODS-C_{18} Column for separation of compounds. Positive controls butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butylhdroquinone (TBHQ), serotonine, 5-methoxyserotonine, and tryptamine were purchased from sigma-aldrich Co (St Louis, MO). The lipid, 1-stearoyl 2-linoleoyl *sn*-glycerol 3-phosphocholine (SLPC), was purchased from Avanti Polar Lipids (Alabaster, AL). The fluorescent probe, 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl]-phenylpropionic acid was purchased from Molecular Probes (Eugene, OR) and *R*- and *S*-methoxy-(trifluoromethyl)-phenylacetyl (MTPA) chlorides from Sigma-Aldrich Co.

3.2. Plant material

The *W. somnifera* plants were grown in the greenhouses of Bioactive Natural Products and Phytoceutical Laboratory at Michigan State University. Plants were grown under 12 h photoperiod at 75 °F in 1:1 mixture of loamy sand and bacto mix in 6″-plastic pots. The plants were watered and fertilized daily using 20:20:20 (N–P–K). The fully ripened fruits were collected, dried at room temperature and extracted immediately.

3.3. Extraction and isolation

The dried and ground fruits (100 g) of *W. somnifera* were sequentially extracted with *n*-hexane (3×500 mL), EtOAc (3×500 mL), MeOH (5×500 mL) and ammoniacal MeOH (3×500 mL). Evaporation of the solvent under reduced pressure yielded *n*-hexane (8 g), EtOAc (2 g), MeOH (8 g) and ammoniacal MeOH (2 g) crude extracts. The MeOH extract (7 g) was defatted (1.5 g) with *n*-hexane (5×150 mL) and fractionated by silica gel medium pressure liquid chromatography under gradient conditions with 70% $CHCl_3$ to 80% MeOH. The 70% $CHCl_3$ eluates were collected in 10 fractions of each 40 mL, similar on TLC, pooled and concentrated to yield fractions I (300 mg). The similar fractions (8 fractions, 50 mL each) obtained from $CHCl_3$–MeOH (1:1) elution were combined and concentrated to give fraction II (100 mg). The $CHCl_3$–MeOH (40:60) eluates gave 15 fractions (50 mL each) were similar, pooled, evaporated to afford fraction III (2 g). Concentration of six similar fractions (each 45 mL) from $CHCl_3$–MeOH (30:70) elution gave fraction IV (1.8 g). The 80% MeOH eluates were pooled and evaporated to afford V (200 mg).

The fractions I and II contained predominantly fatty acids as indicated by TLC. Fraction III (1.8 g) was purified by prep. HPLC using JAIGEL-ODS-C_{18} column and MeOH–H_2O (75:25, v/v) as mobile phase at 3 mL/min. Fractions collected were A (15–30 min, 500 mg), B (31–41 min, 200 mg), C (42–56 min, 500 mg), D (58–70 min, 200 mg) and E (71–95 min, 50 mg). Fraction C was further purified by prep. HPLC using MeCN–H_2O (62.5:37.5, v/v) and yielded pure compounds **1** (81.95 min, 62 mg), **2** (92.0 min, 71 mg) and a fraction (104 min, 35 mg). Compounds **1** and

2 were purified again by prep. HPLC using MeCN–H_2O (1:1, v/v) and yielded pure compounds **1** (35 min, 50 mg) and **2** (38.0, 70 mg). The fraction at 104 min was further purified on prep. TLC using EtOAc–MeOH (9:1, v/v) and developed three times in the same mobile phase yielded pure compound **3** (R_f=0.5, 12 mg). Fraction D was purified by HPLC using MeOH–H_2O (76:24, v/v) and gave pure compound **12** (67.3 min, 150 mg). Fraction E was purified by HPLC using MeOH–H_2O (75:25) and yielded three fractions F (71 min, 14 mg), G (101 min, 5 mg), H (112 min, 4.0 mg).

The fraction G was purified by preparative TLC (PTLC) ($CHCl_3$–MeOH, 4:1) and gave compound **4** (R_f=0.6, 2.5 mg). Purification of fractions F and H on PTLC using $CHCl_3$–MeOH (5:1) on the mobile phase gave **5** (R_f=0.65, 8 mg) and **6** (R_f=0.58, 3.0 mg). Fraction II was subjected to HPLC using ACN–H_2O (34:66, v/v) to yield five fractions fr.1 (37.0 min, 38.1 mg), fr.2 (45–70 min, 68.8 mg), fr.3 (84.4 min, 19.8 mg) and fr.4 (94.9 min, 11.4 mg).

Fr.1 was purified by prep.TLC using the mobile phase ($CHCl_3$–MeOH, 1:1, v/v) and afforded a pure withanolide **13** (R_f=0.40, 7.0 mg). Repeated purification of fr.4 by PTLC ($CHCl_3$–MeOH; 75:25, v/v) yielded pure compound **8** (R_f=0.72, 2 mg). Similarly, fr.3 was purified by PTLC ($CHCl_3$–MeOH, 70:30, v/v) yielded compounds **7** (R_f=0.61, 1.0 mg) and **9** (R_f=0.8, 0.7 mg). Purification of fr.2 by PTLC ($CHCl_3$–MeOH, 1:1, v/v) gave band of R_f=0.5 (25.0 mg) and further purified by prep. HPLC using MeCN–H_2O (33:67) as mobile phase to yield withanolides **10** (62.4 min, 6.0 mg) and **11** (70.8 min, 4.0 mg).

3.3.1. Withanamide A (1). Amorphous powder; $[\alpha]_D$=−35° (C 0.0125); UV (MeOH) λ_{max} nm (log ε) 276 (3.50), 300 sh (3.40); IR ν_{max} (KBr) 3413 (–OH), 2926, 2854, 1633 (–CONH), 1458, 1071, 1033, 626. ^{1}H NMR (500 MHz, CD_3OD) δ 7.15 (1H, dd, J=8.5, 1.0 Hz, H-7), 6.99 (1H, s, H-2), 6.92 (1H, dd, J=2.5, 0.5 Hz, H-4), 6.66 (1H, ddd, J=9.0, 2.0, 0.5 Hz, H-6), 5.31 (4H, m, H-6‴, 7‴, 9‴, 10‴), 4.38 (1H, d, J=8.0 Hz, H-1′), 4.30 (1H, d, J=7.5 Hz, H-1″), 4.10 (1H, dd, J=11.5, 2.0 Hz, H-6′b), 3.85 (1H, dd, J=12.0, 2.5 Hz, H-6″b), 3.79 (1H, m, H-17‴), 3.77 (1H, dd, J=11.5, 5.0 Hz, H-6′a), 3.65 (1H, dd, J=12.0, 5.5 Hz, H-6″a), 3.42 (2H, t, J=7.5 Hz, H-11), 3.40 (1H, m, H-5′), 3.39 (2H, m, H-4′, 4″), 3.27–3.38 (3H, m, H-5″, 3″, 3′), 3.20 (1H, d, J=9.0, 8.0 Hz, H-2″), 3.15 (1H, dd, J=9.0, 8.0 Hz, H-2′), 2.84 (2H, t, J=7.0 Hz, H-10), 2.75 (2H, t, J=6.5 Hz, H-8‴), 2.12 (2H, t, J=7.5 Hz, H-2‴), 2.04 (4H, m, H-5‴, 11‴), 1.54 (2H, m, H-3‴), 1.42 (2H, m, H-4‴), 1.32 (2H, m, H-16‴), 1.27 (8H, br. s, H-12‴-H-15‴), 1.19 (3H, d, J=6.0 Hz, H-18‴). ^{13}C NMR (125 MHz, CD_3OD) δ 176.2 (C-1‴), 151.0 (C-5), 133.0 (C-8), 130.9 (C-10‴), 130.8 (C-7‴), 129.4 (C-9), 129.2 (C-9‴), 129.0 (C-6‴), 124.2 (C-2), 112.6 (C-7), 112.5 (C-3), 112.4 (C-6), 104.7 (C-1″), 104.0 (C-1′), 103.5 (C-4), 77.9 (C-3′, 3″, 5″), 77.7 (C-17‴), 76.8 (C-5′), 75.2 (C-2″), 75.0 (C-2′), 71.6 (C-4″), 71.4 (C-4′), 69.7 (C-6′), 62.7 (C-6″), 41.2 (C-11), 37.3 (C-16‴), 37.2 (C-2‴), 30.6-30.1 (C-12‴-15‴), 30.0 (C-4‴), 28.2 (C-11‴), 28.1 (C-5‴), 27.0 (C-3‴), 26.5 (C-8‴), 26.3 (C-10), 22.1 (C-18‴). HRFABMS 779.4329 (calcd for $C_{40}H_{63}N_2O_{13}$ $(M+H)^+$, 779.4330). FABMS (m/z) 779 $[M+H]^+$, 778 $[M]^+$, 617, 455, 437, 175, 160, 159, 146.

3.3.2. Withanamide B (2). Amorphous powder; $[\alpha]_D$=−34° (C 0.0125); UV (MeOH) λ_{max} nm (log ε) 277 (3.32), 300 sh (3.22); IR ν_{max} (KBr) 3372 (–OH), 2924, 2853, 1632 (–CONH), 1463, 1371, 1071, 1031, 631. ^{1}H NMR (500 MHz, CD_3OD) δ 7.15 (1H, dd, J=8.5, 0.5 Hz, H-7), 7.0 (1H, s, H-2), 6.94 (1H, dd, J=2.5, 0.5 Hz, H-4), 6.66 (1H, dd, J=9.0, 2.5 Hz, H-6), 4.40 (1H, d, J=8.0 Hz, H-1″), 4.32 (1H, d, J=7.5 Hz, H-1′), 4.11 (1H, dd, J=12.0, 2.0 Hz, H-6′b), 3.87 (1H, dd, J=12.0, 2.0 Hz, H-6″b), 3.79 (1H, m, H-15‴), 3.78 (1H, dd, J=12.0, 5.5 Hz, H-6′a), 3.67 (1H, dd, J=12.0, 5.5 Hz, H-6″a), 3.44 (2H, t, J=7.0 Hz, H-11), 3.41 (2H, m, H-4″, 5′), 3.40 (1H, m, H-4′), 3.25–3.38 (3H, m, H-5″, 3″, 3′), 3.24 (1H, dd, J=9.0, 8.0 Hz, H-2″), 3.17 (1H, dd, J=9.0, 8.0 Hz, H-2′), 2.85 (2H, t, J=8.0 Hz, H-10), 2.13 (2H, t, J=7.0 Hz, H-2‴), 1.55 (2H, m, H-3‴), 1.39 (4H, m, H-4‴, H-14‴), 1.26 (18H, br. s, H-5‴-H-13‴), 1.20 (3H, d, J=6.5 Hz, H-16‴). ^{13}C NMR (125 MHz, CD_3OD) δ 176.2 (C-1‴), 151.0 (C-5), 133.0 (C-8), 129.4 (C-9), 124.2 (C-2), 112.6 (C-7), 112.4 (C-3), 112.3 (C-6), 104.7 (C-1″), 103.9 (C-1′), 103.5 (C-4), 77.9 (3″, 5″), 77.8 (C-3′), 77.7 (C-15‴), 76.8 (C-5′), 75.2 (C-2″), 75.0 (C-2′), 71.5 (C-4″), 71.4 (C-4′), 69.7 (C-6′), 62.7 (C-6″), 41.2 (C-11), 37.6 (C-14‴), 37.2 (C-2‴), 30.8-30.2 (C-4‴-13‴), 27.0 (C-3‴), 26.3 (C-10), 22.0 (C-16‴). HRFABMS 755.4331 (calcd for $C_{38}H_{63}N_2O_{13}$ $(M+H)^+$, 755.4330). FABMS (m/z) 777 $[M+Na]^+$, 755 $[M+H]^+$, 754 $[M]^+$, 593, 431, 413, 396, 160, 146.

3.3.3. Withanamide C (3). Amorphous powder; $[\alpha]_D$=−34° (C 0.01); UV (MeOH) λ_{max} nm (log ε) 276 (3.53), 300 sh (3.42); IR ν_{max} (KBr) 3422 (–OH), 2924, 2853, 1633 (–CONH), 1459, 1071, 1032, 631. ^{1}H NMR (500 MHz, CD_3OD) δ 7.15 (1H, dd, J=8.5, 0.5 Hz, H-7), 6.99 (1H, s, H-2), 6.92 (1H, dd, J=2.0, 0.5 Hz, H-4), 6.65 (1H, dd, J=8.5, 2.0 Hz, H-6), 4.40 (1H, d, J=8.0 Hz, H-1″), 4.30 (1H, d, J=8.0 Hz, H-1′), 4.10 (1H, dd, J=12.0, 2.0 Hz, H-6′b), 3.86 (1H, dd, J=12.0, 2.5 Hz, H-6″b), 3.79 (1H, dd, J=12.0, 6.0 Hz, H-6′a), 3.66 (1H, dd, J=12.0, 5.5 Hz, H-6″a), 3.63 (1H, m, H-14‴), 3.44 (2H, t, J=7.0 Hz, H-11), 3.40 (1H, m, H-4′), 3.39 (1H, t, J=7.5 Hz, H-5′), 3.25–3.37 (4H, m, H-5″, 4″, 3′, 4′), 3.20 (1H, dd, J=9.0, 7.5 Hz, H-2″), 3.16 (1H, dd, J=9.0, 7.5 Hz, H-2′), 2.85 (2H, t, J=7.5 Hz, H-10), 2.14 (2H, t, J=7.5 Hz, H-2‴), 1.56 (4H, m, H-3‴, 15‴), 1.52 (2H, m, H-13‴), 1.27 (18H, br. s, H-4‴-H-12‴), 0.91 (3H, t, J=7.5 Hz, H-16‴). ^{13}C NMR (125 MHz, CD_3OD) δ 176.3 (C-1‴), 151.1 (C-5), 133.1 (C-8), 129.5 (C-9), 124.2 (C-2), 112.6 (C-7), 112.5 (C-3), 112.4 (C-6), 104.9 (C-1″), 103.6 (C-1′), 103.5 (C-4), 82.0 (C-14‴), 78.1 (C-5″), 78.0 (C-3′, 3″), 77.0 (C-5′), 75.3 (C-2″), 75.2 (C-2′), 71.7 (C-4″), 71.6 (C-4′), 69.9 (C-6′), 62.8 (C-6″), 41.2 (C-11), 37.2 (C-2‴), 34.5 (C-13‴), 31.0-28.6 (C-4‴-12‴), 27.0 (C-3‴), 26.3 (C-15‴), 26.0 (C-10), 10.1 (C-16‴). HRFABMS 755.4331 (calcd for $C_{38}H_{63}N_2O_{13}$ $(M+H)^+$, 755.4330). FABMS (m/z) 777 $[M+Na]^+$, 755 $[M+H]^+$, 754 $[M]^+$, 431, 413, 396, 160, 159, 146.

3.3.4. Withanamide D (4). Amorphous powder; UV (MeOH) λ_{max} nm (log ϵ) 277 (3.43), 301 sh (3.30); IR ν_{max} (KBr) 3402 (–OH), 2923, 2852, 1636 (–CONH), 1464, 1381, 1071, 1040, 630. ^{1}H NMR (500 MHz, CD_3OD)

δ 7.14 (1H, dd, J=9.0, 0.5 Hz, H-7), 6.99 (1H, s, H-2), 6.93 (1H, dd, J=2.5, 0.5 Hz, H-4), 6.65 (1H, dd, J=9.0, 2.5 Hz, H-6), 4.39 (1H, d, J=8.0 Hz, H-1″), 4.32 (1H, d, J=8.0 Hz, H-1′), 4.10 (1H, dd, J=11.5, 2.0 Hz, H-6′b), 3.86 (1H, dd, J=12.0, 2.5 Hz, H-6″b), 3.79 (1H, m, H-17‴), 3.79 (1H, dd, J=12.0, 5.5 Hz, H-6′a), 3.66 (1H, dd, J=12.0, 5.5 Hz, H-6″a), 3.44 (2H, t, J=7.0 Hz, H-11), 3.41 (2H, m, H-4″, 5′), 3.40 (1H, m, H-4′), 3.25–3.36 (4H, m, H-3′, 3″, 5′, 5″), 3.20 (1H, dd, J=9.0, 8.0 Hz, H-2″), 3.15 (1H, dd, J=9.0, 8.0 Hz, H-2′), 2.86 (2H, t, J=7.0 Hz, H-10), 2.14 (2H, t, J=7.0 Hz, H-2‴), 1.57 (2H, m, H-3‴), 1.40 (2H, m, H-16‴), 1.28 (24H, br. s, H-4‴-H-15‴), 1.21 (3H, d, J=6.0 Hz, H-18‴). ^{13}C NMR (125 MHz, CD_3OD) δ 176.3 (C-1‴), 151.1 (C-5), 133.1 (C-8), 129.5 (C-9), 124.2 (C-2), 112.6 (C-7), 112.5 (C-3), 112.4 (C-6), 104.8 (C-1″), 104.0 (C-1′), 103.5 (C-4), 78.0 (C-3′, 3″, 5″), 77.8 (C-17‴), 77.0 (C-5′), 75.3 (C-2″), 75.1 (C-2′), 71.6 (C-4′), 71.5 (C-4″), 69.8 (C-6′), 62.8 (C-6″), 41.2 (C-11), 37.8 (C-16‴), 37.2 (C-2‴), 30.9-30.2 (C-4‴-15‴), 27.0 (C-3‴), 26.3 (C-10), 22.1 (C-18‴). HRFABMS 805.4462 (calcd for $C_{40}H_{67}N_2O_{13}Na$, 805.4463). FABMS (m/z) 805 $[M+Na]^+$, 783 $[M+H]^+$, 643, 459, 441, 371, 363, 347, 160, 159.

3.3.5. Withanamide E (5). Amorphous powder; UV (MeOH) λ_{max} nm (log ε) 275 (3.30), 300 sh (3.20). ^{1}H NMR (500 MHz, CD_3OD) δ 7.15 (1H, dd, J=8.5, 1.0 Hz, H-7), 7.0 (1H, s, H-2), 6.93 (1H, dd, J=2.0, 0.5 Hz, H-4), 6.65 (1H, dd, J=8.5, 2.0 Hz, H-6), 4.40 (1H, d, J=8.0 Hz, H-1″), 4.30 (1H, d, J=8.0 Hz, H-1′), 4.10 (1H, dd, J=12.0, 2.0 Hz, H-6′b), 3.86 (1H, dd, J=12.0, 2.5 Hz, H-6″b), 3.78 (1H, dd, J=12.0, 6.0 Hz, H-6′a), 3.66 (1H, dd, J=12.0, 5.5 Hz, H-6″a), 3.63 (1H, t, J=6.0, H-16‴), 3.44 (2H, t, J=7.5 Hz, H-11), 3.40 (1H, m, H-4′), 3.39 (1H, t, J=7.5 Hz, H-5′), 3.25–3.37 (4H, m, H-5″, 4″, 3′, 4′), 3.20 (1H, dd, J=9.0, 7.5 Hz, H-2″), 3.16 (1H, dd, J=9.0, 7.5 Hz, H-2′), 2.85 (2H, t, J=7.5 Hz, H-10), 2.14 (2H, t, J=7.5 Hz, H-2‴), 1.56 (4H, m, H-3‴, 17‴), 1.52 (2H, m, H-15‴), 1.27 (22H, br. s, H-4‴-H-14‴), 0.91 (3H, t, J=7.5 Hz, H-18‴). HRFABMS 783.4645 (calcd for $C_{40}H_{67}O_{13}N_2$ 783.4644). FABMS (m/z) 805 $[M+Na]^+$, 783 $[M+H]^+$, 765, 621, 459, 441, 282, 202, 175, 160, 159, 146.

3.3.6. Withanamide F (6). Amorphous powder; UV (MeOH) λ_{max} nm (log ε) 276 (3.31), 301 sh (3.21); IR ν_{max} (KBr) 3402 (–OH), 2926, 2853, 1635 (–CONH), 1456, 1368, 1069, 1036, 615. ^{1}H NMR (500 MHz, CD_3OD) δ 7.14 (1H, dd, J=8.5, 0.5 Hz, H-7), 6.99 (1H, s, H-2), 6.92 (1H, dd, J=2.5, 0.5 Hz, H-4), 6.65 (1H, dd, J=8.5, 2.5 Hz, H-6), 5.33 (2H, m, H-9‴, 10‴), 4.39 (1H, d, J=7.0 Hz, H-1″), 4.30 (1H, d, J=7.5 Hz, H-1′), 4.09 (1H, dd, J=11.5, 2.0 Hz, H-6′b), 3.86 (1H, dd, J=11.5, 2.0 Hz, H-6″b), 3.78 (1H, dd, J=11.5, 5.5 Hz, H-6′a), 3.66 (1H, dd, J=11.5, 5.5 Hz, H-6″a), 3.62 (1H, t, J=6.0 Hz, H-16‴), 3.44 (2H, t, J=7.5 Hz, H-11), 3.41 (2H, m, H-4″, 5′), 3.40 (1H, m, H-4′), 3.25–3.36 (4H, m, H-3′, 3″, 5′, 5″), 3.20 (1H, dd, J=9.0, 8.0 Hz, H-2″), 3.15 (1H, dd, J=9.0, 8.0 Hz, H-2′), 2.85 (2H, t, J=7.0 Hz, H-10), 2.14 (2H, t, J=7.5 Hz, H-2‴), 2.02 (4H, m, H-8‴, 11‴), 1.55 (8H, m, H-3‴, 17‴, 7‴, 12‴), 1.28 (12H, br. s, H-4‴-6‴, H-13‴-15‴), 0.91 (3H, t, J=7.5 Hz, H-18‴). ^{13}C NMR (125 MHz, CD_3OD) δ 176.3 (C-1‴), 151.2 (C-5), 133.1 (C-8), 130.9 (C-10‴), 130.8 (C-9‴), 129.5 (C-9), 124.2 (C-2), 112.6 (C-7), 112.5 (C-3), 112.4 (C-6), 104.9 (C-1″), 103.6 (C-1′), 103.5 (C-4), 82.0 (C-16‴), 78.0 (C-3′, 3″, 5″), 77.0 (C-5′), 75.3 (C-2″), 75.1 (C-2′), 71.7 (C-4″), 71.6 (C-4′), 69.9 (C-6′), 62.8 (C-6″), 41.2 (C-11), 37.2 (C-2‴), 30.8-30.1 (C-5‴-7‴, C-12‴-C15‴), 30.2 (C-4‴), 28.2 (C-11‴), 28.1 (C-8‴), 27.0 (C-3‴), 26.5 (C-17‴) 26.3 (C-10), 10.2 (C-18‴). HRFABMS 803.4304 (calcd for $C_{40}H_{64}O_{13}N_2Na$, 803.4306). FABMS (m/z) 803 $[M+Na]^+$, 781 $[M+H]^+$, 641, 619, 457, 439, 393, 347, 160, 159, 146.

3.3.7. Withanamide G (7). Amorphous powder; UV (MeOH) λ_{max} nm (log ε) 277 (3.34), 301 sh (3.23). ^{1}H NMR (500 MHz, CD_3OD) δ 7.15 (1H, d, J=9.0 Hz, H-7), 7.0 (1H, s, H-2), 6.92 (1H, d, J=2.0 Hz, H-4), 6.65 (1H, dd, J=9.0, 2.0 Hz, H-6), 5.34 (2H, m, H-9‴, 10‴), 4.39 (1H, d, J=7.5 Hz, H-1″), 4.31 (1H, d, J=7.5 Hz, H-1′), 4.10 (1H, dd, J=12.0, 2.0 Hz, H-6′b), 3.85 (1H, dd, J=12.0, 2.5 Hz, H-6″b), 3.79 (1H, m, H-15‴), 3.78 (1H, dd, J=12.0, 5.0 Hz, H-6′a), 3.66 (1H, dd, J=12.0, 5.0 Hz, H-6″a), 3.44 (2H, t, J=7.5 Hz, H-11), 3.41 (2H, m, H-4″, 5′), 3.40 (1H, m, H-4′), 3.25–3.38 (4H, m, H-5″, H-3″, H-3′), 3.24 (1H, dd, J=9.0, 8.0 Hz, H-2″), 3.16 (1H, dd, J=9.0, 8.0 Hz, H-2′), 2.85 (2H, t, J=7.0 Hz, H-10), 2.14 (2H, t, J=7.5 Hz, H-2‴), 1.55 (2H, m, H-3‴, H-14‴), 2.03 (4H, m, H-8‴, 11‴), 1.55 (4H, m, H-3‴, H-14‴), 1.39 (2H, m, H-4‴), 1.28 (16H, br. s, H-5‴-7‴, 12‴, 13‴), 1.21 (3H, d, J=6.5 Hz, H-16‴). ^{13}C NMR[†] (125 MHz, CD_3OD) δ 151.3 (C-5), 130.9 (C-10‴), 130.8 (C-9‴), 129.5 (C-9), 124.2 (C-2), 112.7 (C-7), 112.5 (C-3), 112.3 (C-6), 104.9 (C-1″), 104.0 (C-1′), 103.5 (C-4), 77.9 (C-15‴), 78.0 (C-3′, 3″, 5″), 77.0 (C-5′), 75.3 (C-2″), 75.1 (C-2′), 71.6 (C-4″), 71.5 (C-4′), 69.9 (C-6′), 62.8 (C-6″), 41.2 (C-11), 37.2 (C-2‴), 30.9-30.1 (C-5‴-7‴, 12‴-13‴), 30.2 (C-4‴), 28.1 (C-11‴), 28.0 (C-8‴), 27.0 (C-3‴), 26.3 (C-10), 22.1 (C-16‴). HRFABMS 753.4173 (calcd for $C_{38}H_{61}O_{13}N_2$, 753.4174). FABMS (m/z) 775 $[M+Na]^+$, 753 $[M+H]^+$, 596, 155, 114.

3.3.8. Withanamide H (8). Amorphous powder; UV (MeOH) λ_{max} nm (log ε) 276 (3.53), 301 sh (3.38), 330 sh (3.20). ^{1}H NMR (500 MHz, CD_3OD) δ 7.14 (1H, d, J=8.5 Hz, H-7), 6.99 (1H, s, H-2), 6.92 (1H, d, J=2.5 Hz, H-4), 6.65 (1H, dd, J=8.5, 2.5 Hz, H-6), 5.34 (8H, m, H-6‴, 7‴, 9‴, 10‴, 12‴, 13‴, 15‴, 16‴), 4.33 (1H, d, J=8.0 Hz, H-1″), 4.27 (1H, d, J=8.0 Hz, H-1′), 4.10 (1H, dd, J=12.0, 2.0 Hz, H-6′b), 3.85 (1H, dd, J=12.0, 2.5 Hz, H-6″b), 3.79 (1H, m, H-15‴), 3.78 (1H, dd, J=12.0, 5.0 Hz, H-6′a), 3.66 (1H, dd, J=12.0, 5.0 Hz, H-6″a), 3.44 (2H, t, J=7.5 Hz, H-11), 3.41 (2H, m, H-4″, 5′), 3.40 (1H, m, H-4′), 3.25–3.38 (3H, m, H-5″, H-3″, H-3′), 3.24 (1H, dd, J=9.0, 8.0 Hz, H-2″), 3.16 (1H, dd, J=9.0, 8.0 Hz, H-2′), 2.85 (2H, t, J=7.0 Hz, H-10), 2.82 (6H, m, H-8‴, 11‴, 14‴), 2.14 (2H, t, J=7.5 Hz, H-2‴), 2.07 (2H, m, 5‴), 1.55 (2H, m, H-3‴), 1.28 (2H, br. s, H-4‴), 1.24 (3H, d, J=6.5 Hz, H-18‴). ^{13}C NMR (125 MHz, CD_3OD) δ 176.3 (C-1‴), 151.2 (C-5), 133.2 (C-8), 132.6 (C-9‴), 132.2 (C-6‴), 131.4 (C-7‴, 10‴), 130.1 (C-15‴), 129.5 (C-9), 128.7 (C-12‴, 13‴), 128.5 (C-15‴, 16‴), 124.2 (C-2), 112.6 (C-7), 112.5 (C-3), 112.4 (C-6), 104.9 (C-1″), 100.9 (C-1′), 103.5 (C-4), 78.0 (C-3′, 3″, 5″), 77.7 (C-17‴), 76.8 (C-5′), 75.0 (C-2″), 74.9 (C-2′), 71.6 (C-4″), 71.3 (C-4′), 69.6 (C-6′), 62.8 (C-6″), 41.2 (C-11), 37.2 (C-2‴), 30.7 (C-4‴), 28.2 (C-5‴), 27.0 (C-3‴),

[†] ^{13}C NMR signals for C-8 and C-1‴ were not observed due to insufficient quantity of sample.

26.6 (C-8‴, 11‴, 14‴), 26.3 (C-10), 21.9 (C-18‴). HRFABMS 775.4013 (calcd for $C_{40}H_{59}O_{13}N_2$, 775.4017). FABMS (*m/z*) 799 $[M+Na]^+$, 775 $[M+H]^+$, 591, 435, 411, 160, 159, 146.

3.3.9. Withanamide I (9). Amorphous powder; UV (MeOH) λ_{max} nm (log ε) 278 (3.43), 302 sh (3.30). ^1H NMR (500 MHz, CD_3OD) δ 7.14 (1H, dd, *J*=8.5, 0.5 Hz, H-7), 6.99 (1H, s, H-2), 6.93 (1H, dd, *J*=2.5, 0.5 Hz, H-4), 6.65 (1H, dd, *J*=8.5, 2.0 Hz, H-6), 5.33 (4H, m, H-6‴, 7‴, 9‴, 10‴), 4.39 (1H, d, *J*=7.5 Hz, H-1″), 4.36 (1H, d, *J*=8.0 Hz, H-1‴), 4.32 (1H, d, *J*=8.0 Hz, H-1′), 4.15 (1H, bd, *J*=12.0 Hz, H-6″b), 4.09 (1H, br. d, *J*=12.0 Hz, H-6′b), 3.86 (1H, dd, *J*=12.0, 2.0 Hz, H-6‴b), 3.79 (1H, m, H-17‴), 3.78 (1H, dd, *J*=12.0, 5.5 Hz, H-6″a), 3.75 (1H, dd, *J*=11.0, 6.0 Hz, H-6′a), 3.66 (1H, dd, *J*=12.0, 5.0 Hz, H-6‴a), 3.44 (2H, t, *J*=7.5 Hz, H-11), 3.41 (3H, m, H-4″, 4‴, 5′), 3.40 (1H, m, H-4′), 3.25-3.38 (5H, m, H-5″, 5‴, 3‴, 3″, 3′), 3.24 (1H, dd, *J*=9.0, 8.0 Hz, H-2″, 2‴), 3.16 (1H, dd, *J*=9.0, 8.0 Hz, H-2′), 2.85 (2H, t, *J*=7.0 Hz, H-10), 2.77 (2H, t, *J*=6.0 Hz, H-8⁗), 2.15 (2H, t, *J*=7.5 Hz, H-2⁗), 2.04 (4H, m, H-5⁗, 11⁗), 1.42 (2H, m, H-4⁗), 1.28 (8H, br s, H-12⁗-H-15⁗), 1.56 (2H, m, H-3⁗), 1.21 (3H, d, *J*=6.5 Hz, H-18⁗). HRFABMS 941.4857 (calcd for $C_{46}H_{73}O_{18}N_2$, 941.4859). FABMS (*m/z*) 963 $[M+Na]^+$, 941 $[M+H]^+$, 617, 455, 437, 316, 160, 159, 146.

3.3.10. 23,24-Dihydrowithanolide VI (10). Colorless, amorphous powder; IR ν_{max} (KBr) 3421 (–OH), 2936, 1724, 1663, 1460, 1384, 1073, 1043. ^1H NMR (500 MHz, CD_3OD) δ 5.52 (1H, br d, *J*=5.0 Hz, H-6), 4.39 (1H, d, *J*=8.0 Hz, H-1″), 4.36 (1H, d, *J*=8.0 Hz, H-1′), 4.24 (1H, dd, *J*=11.5, 2.5 Hz, H-22), 4.12 (1H, dd, *J*=11.5, 2.5 Hz, H-6′b), 4.0 (1H, m, H-3), 3.86 (1H, dd, *J*=11.5, 2.0 Hz, H-6″b), 3.80 (1H, m, H-1), 3.76 (1H, dd, *J*=11.5, 6.0 Hz, H-6′a), 3.66 (1H, dd, *J*=12.0, 6.0 Hz, H-6″a), 3.41 (2H, m, H-4″, 5′), 3.40 (1H, m, H-4′), 3.25–3.38 (3H, m, H-5″, H-3″, H-3′), 3.24 (1H, dd, *J*=9.0, 8.0 Hz, H-2″), 3.16 (1H, dd, *J*=9.0, 8.0 Hz, H-2′), 1.24 (3H, s, Me-21), 1.17 (3H, d, *J*=6.5 Hz, Me-27), 1.15 (3H, d, *J*=6.5 Hz, Me-28), 1.01 (3H, s, Me-19), 0.89 (3H, s, Me-18). ^{13}C NMR (125 MHz, CD_3OD) δ 178.9 (C-26), 139.2 (C-5), 125.5 (C-6), 104.8 (C-1″), 103.1 (C-1′), 81.9 (C-22), 78.0 (C-3′, 3″), 77.9 (C-5″), 77.0 (C-5′), 76.5 (C-20), 75.5 (C-2″), 75.2 (C-2′), 75.1 (C-1), 73.6 (C-3), 71.7 (C-4″), 71.6 (C-4′), 69.7 (C-6′), 62.8 (C-6″), 58.1 (C-14), 56.1 (C-17), 44.0 (C-24), 42.7 (C-13), 42.5 (C-10), 41.4 (C-9), 41.1 (C-12), 39.2 (C-4), 37.8 (C-2), 32.8 (C-25), 32.7 (C-23), 32.6 (C-7), 32.0 (C-8), 25.0 (C-15), 23.0 (C-16), 21.3 (C-11), 21.2 (C-21), 20.5 (C-27), 19.9 (C-19), 14.4 (C-28), 14.2 (C-18). HRFABMS 785.4325 (calcd for $C_{40}H_{65}O_{15}$, 785.4323). FABMS *m/z* 807 $[M+Na]^+$, 785, 623, 605, 587, 443, 425, 407, 255.

3.3.11. Compounds 11–13. The structures of compounds **11–13** were elucidated by ^1H and ^{13}C NMR experiments and their identity was confirmed by comparing the spectral data with the published results.[9,19]

3.4. Preparation of *R*- and *S*-MTPA esters of compound 1

A mixture of compound **1** (1.5 mg) and *R*-(−)-Methoxy trifluorophenyl acetyl chyloride (*R*-MTPA) in pyridine was stirred with dimethylaminopyridine (DMAP) (5 h) at room temperature. The solvent was evaporated and residue obtained was purified over PTLC using $CHCl_3$–MeOH (9:1, v/v) to yield *R*-MTPA ester (1.0 mg). Similarly, compound **1** (1.2 mg) was treated with *S*-(+)-methoxytrifluorophenyl acetyl chloride and the purification of the resulting product gave *S*-MTPA ester (0.9 mg).

3.5. Fe^{2+} induced lipid peroxidation assay

Compounds **1–16** were tested for their inhibition of lipid peroxidation using LUVs (Liposome suspension) according to the published procedure.[20] The liposome suspension was prepared by mixing the phospholipid 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocoline (SLPC) and a fluorescence probe [3-[*p*-(6-phenyl)-1,3,5-hexatrienyl]phenylpropionic acid (DPH-PA). The final assay volume was 2 mL and consisted HEPES (100 μL), 1 M NaCl (200 μL), N_2-sparged water (1.64 mL), test sample or DMSO (20 μL) and liposome suspension (20 μL). The peroxidation was initiated by the addition of 20 μL of $FeCl_2$. $4H_2O$ (0.5 mM). The decrease in fluorescence intensity over time (21 min) indicated the rate of peroxidation. The percentage of lipid peroxidation was calculated with respect to DMSO solvent control. Stock solutions of the samples were prepared at 100 μg/mL and diluted further for the assay. Commercial standards BHA, BHT and TBHQ were tested at 1 μg/mL.

3.6. AAPH $^+$ induced lipid peroxidation assay

The AAPH (2,2-azobis (2-amidinopropane) dihydrochloride) induced lipid peroxidation was carried out using LUVs[20] at 37 °C. The HEPES (100 μL), 1 M NaCl (200 μL), N_2-sparged water (1.64 mL) and test sample or DMSO (20 μL) were mixed and 20 μL of liposome added. The peroxidation was initiated by the addition of 2.5 mM APPH. The flourescence was monitored for 21 min for every three minutes and percentage inhibition was calculated as in the case of Fe^{+2} induced lipid peroxidation assay.

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