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Cytotoxic Principles from the Formosan Milkweed, *Asclepias curassavica*

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A series of cardenolides and related compounds have been isolated from the aerial parts and roots of the ornamental milkweed, *Asclepias curassavica*. Their structures were determined by spectroscopic and chemical methods. Among them, three derivatives of calactinic acid methyl ester (**13**−**15**), 19-*nor*-16α-acetoxy-10*β*-hydroxyasclepin (**16**), 20*β*,21-dihydroxypregna-4,6-dien-3-one (**19**), and 3,4-*seco*-urs-20(30)-en-3-oic acid (**22**) are new compounds. The relative configuration of calactinic acid methyl ester (**12**) has been confirmed by X-ray diffraction analysis on its derivative **13**. Most of the cardenolides obtained showed pronounced cytotoxicity against four cancer cell lines (IC_{50} 0.01 to 2.0 *μ*g/mL).

The common garden plant *Asclepias curassavica* L. belongs to the family Asclepiadaceae (milkweeds) and is a good source of the cardenolide cardiac glycosides.[1] These cardioactive compounds have also been isolated from the monarch butterfly (*Danaus plexippus* L.), which feeds on the *Asclepias* genus, including *A. curassavica*.[2] Butterflies and other sucking insects sequester these noxious chemicals from host plants for their protection from vertebrate predators. From an ecological point of view, this host−guest−predator relationship has been well established.[3,4] However, only limited research has been carried out concerning the cytotoxic constituents of *A. curassavica*. This plant is used as a cancer treatment in traditional medical practice.[5] Calotropin isolated from this plant family has been reported as a potent cytotoxic agent against KB cells (IC_{50} 15 ng/mL).[6] In a search for cytotoxic compounds from Formosan plants, this plant was chosen for chemical investigation. This report deals with the isolation and the structure elucidation of the new derivatives (**13**−**16**, **19**, and **22**), together with the known compounds (**1**−**12**, **17**, **18**, **20**, and **21**), and the cytotoxic activity of the cardenolides from *A. curassavica*.

Results and Discussion

The plant material was collected and separated into its roots and aerial parts. The dried plant materials were extracted separately with MeOH. After concentration, each MeOH extract was partitioned between EtOAc and water to give the organic-soluble material. Chromatographic separation of the lipophilic extracts followed by HPLC purification yielded 19 compounds. Cardenolides **1**−**13**, 19-*nor*-cardenolides **14**−**16**, cardenolide genins **17** and **18**, a pregnane (**19**), an androstane (**20**), a triterpene (**21**), and a 3,4-*seco*-urs-20(30)-en-3-oic acid (**22**) were characterized by analysis of their spectroscopic data. Compounds **13**−**15** are oxidation products of **12**.

Thirteen known compounds were identified as calotropin (**1**),[7] 16α-acetoxycalotropin (**2**),[2] 15*β*-hydroxycalotropin (**3**),[8] calactin (**4**),[7] 15*β*-hydroxycalactin (**5**),[9] 16α-acetoxycalactin (**6**),[10] asclepin (**7**),[11] 16α-acetoxyasclepin (**8**),[2] 16α-hydroxyasclepin (**9**),[2] uscharidin (**10**),[7] uscharin (**11**),[7] uzarigenin (**17**),[8,11] and afrogenin (**18**)[11] by comparison of their NMR and MS data with those reported in the literature.

Compound **12** was isolated as a colorless glass. It was assigned the molecular formula $C_{30}H_{42}O_{10}$, as deduced from the HRESIMS (*m/z* 563.2857 $[M + H]^+$, Δ +0.1 mmu). The ^1H and ^{13}C NMR spectra of **12** showed the typical signals for a calotropagenin: an aldehyde [δ_H 9.79 (s), δ_C 207.1], an α,*β*-unsaturated-*γ*-lactone [δ_H 5.73 (brs), 4.90 (dd, J = 18.0, 2.0 Hz), 4.67 (dd, J = 18.0, 2.0 Hz), δ_C 174.5 s, 174.4 s, 117.3 d, 73.1 t], two secondary oxymethines [δ_H 3.31 (ddd, J = 13.0, 12.0, 5.0 Hz), 3.14 (ddd, J = 13.0, 12.0, 5.0 Hz), δ_C 85.2 d, 70.0 d], and one quaternary oxygenated carbon (δ_C 83.8). In addition, an anomeric signal [δ_H 4.88 (s), δ_C 108.5 d], together with resonances for two oxygenated carbons [δ_H 4.42 (m), δ_C 76.1 d, 84.1 s] and a methyl doublet [δ_H 1.24 (d, J = 6.0 Hz), δ_C 21.8], indicated the presence of a sugar moiety in **12**, as found in compounds **1**−**11**. The sugar part was attached to C-3 by an acetal linkage, as evidenced by an HMBC cross-peak (H-1′/C-3). Moreover, the sugar was in a furanose form instead of a pyranose form, which is the common sugar form in cardenolides (e.g., **1**−**11**). The furanose unit was supported by the observation of HMBC correlations from H-1′ to C-2′ and C-5′ and from H-4′ to C-1′, C-2′, and C-5′. A methyl ester group (COOMe, δ_C 171.2 s, 51.5 q) was placed at C-2′ on the basis of HMBC cross-peaks (H-1′/C-3′, H-4′/C-3′, and Me/C-3′). A large coupling constant (12 Hz) between protons H-2 and H-3 suggested their stereochemistry to be 2α and 3*β*, respectively. NOE correlations between the protons H-19/Heq-1, H-2, H-8; H-18/H-8, H-21, H-22; and H-3/H-5 revealed the relative stereostructure of the genin portion of **12**, as shown. However, the configuration of the sugar unit was ambiguous.

Autoxidation of **12** gave an oxidation product, **13**. Its NMR spectra showed similarities with those of **12**. The major difference in **13** was that a carboxyl group (δ_C 175.7) was present instead of an aldehyde group (δ_C 207.1). This was also supported by the ESIMS, which showed a molecular ion at *m/z* 579.5 $[M + H]^+$, corresponding to the molecular formula $C_{30}H_{42}O_{11}$. Careful analysis of the 2D NMR data of **13** revealed that the aldehyde group (C-19) of **12** was oxidized to give the corresponding carboxylic group in **13**. A single-crystal X-ray diffraction analysis revealed the relative configuration of **13** to be determined as shown in Figure 1. Therefore, the relative configuration of **12** was also confirmed.

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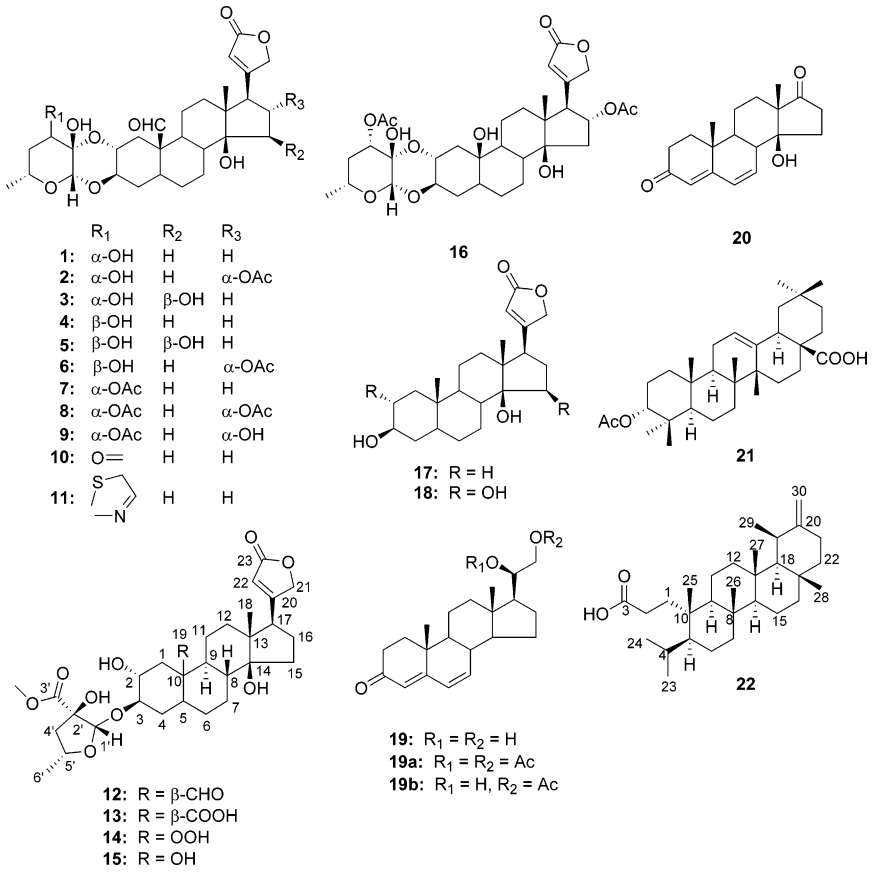
Chart 1



Compound **12** was assigned as calactinic acid methyl ester, reported as a reaction product of uscharidin (**10**),[12,13] which was also isolated in this study. However, this is the first report of the isolation of **12** from a natural source, and detailed spectroscopic data are provided herein.

Decarboxylation followed by oxidation of **12** gave two minor products, **14** and **15**. The products were identified by MS, as shown. In the ESIMS, both **14** and **15** showed two pseudomolecular ions at *m/z* 567.4 $[M + H]^+$, 589.4 $[M + Na]^+$ and 551.4 $[M + H]^+$, 573.4 $[M + Na]^+$, respectively. It is also worth mentioning that autoxidation of **10** in the presence of aqueous methanol gave both **12** and **13**, along with the minor products **14** and **15**.

Compound **16** was obtained as a colorless glass. Its molecular formula, $C_{32}H_{44}O_{12}$, was deduced from the HRESIMS (*m/z* 621.2916 $[M + H]^+$, Δ +0.5 mmu), which is 12 amu less than **8**. Both the ^1H and ^{13}C NMR spectra of **16** showed signals for an α,β-unsaturated-γ-lactone [δ_H 5.92 (s), 4.92 (dd, J = 18.0, 1.6 Hz), 4.84 (dd, J = 18.0, 1.6 Hz), δ_C 174.2 s, 171.0 s, 118.3 d, 73.2 t], an anomeric proton [δ_H 4.59 (s), δ_C 96.1 d], five oxymethines [δ_H 5.23 (td, J = 8.0, 4.0 Hz), 4.78 (dd, J = 12.0, 5.0 Hz), 4.14 (ddd, J = 10.0, 8.0, 4.4 Hz), 3.98 (ddd, J = 10.0, 10.0, 4.4 Hz), 3.69 (m), δ_C 78.2 d, 75.7 d, 71.4 d, 68.8 d, 67.7 d], two acetyls [δ_H 2.16 (s), 2.02 (s), δ_C 172.6 s, 170.6 s, 21.2 q, 21.0 q], and two methyls [δ_H 1.29 (d, J = 6.0 Hz), 0.89 (s), δ_C 20.9 q, 15.6 q]. These data were quite similar to those of **8** except for an aldehyde signal. The similarity of the NMR data with **8** and the absence of an aldehyde group suggested that **16** is a 19-*nor*-calotropagenin derivative. Thus, the aldehyde group (δ_C 206.7 d) of **8** was replaced by a hydroxyl group in **16**. The hydroxyl group was placed at C-10 (δ_C 83.2 s), since no extra oxygenated signal other than the aforementioned signals was observed in the ^1H NMR spectrum of **16**. This was further supported by the HMBC cross-peaks (H-2/C-3, C-4, and C-10). Comparable coupling constants and patterns of the corresponding proton signals in **8** and **16** suggested that both had an identical relative configu-

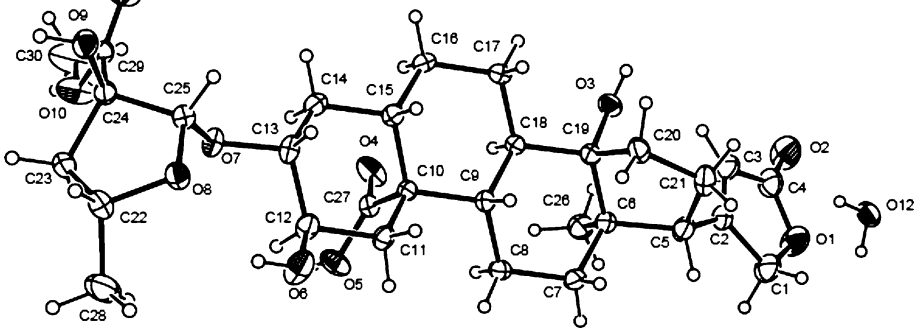


Figure 1. ORTEP drawing of the X-ray crystallographic structure of **13**.

ration, as shown. Observation of the NOEs from the hydroxyl signal (δ_H 4.54) at C-10 to the proton signals at H-4 (δ_H 1.55) and H-11ax (δ_H 1.48) indicated that the hydroxyl group was β. Therefore, **16** was assigned as 19-*nor*-16α-acetoxy-10β-hydroxyasclepin.

It has been suggested that the 19-*nor*-calotropagenins are derived via autoxidation of the aldehyde (C-19) group followed by decarboxylation.[14] This proposal was supported by the isolation of **13**−**15** from the oxidation mixture derived from **12**.

Compound **19** was isolated as a colorless solid. It showed an absorption maximum at 284 nm in the UV spectrum, which indicated the presence of a dienone system in the molecule. Its ^{1}H and ^{13}C NMR spectra exhibited signals for a conjugated dienone [δ_H 6.11 (2H, overlapped), 5.67 (1H, s), δ_C 199.7 s, 163.9 s, 141.2 d, 127.9 d, 123.5 d], one primary and one secondary alcohol [δ_H 3.69 (1H, dd, J = 11.2, 2.6 Hz), 3.67 (1H, dd, J = 8.0, 2.6 Hz), 3.40 (1H, dd, J = 11.2, 8.0 Hz), δ_C 74.4 d, 66.3 t], and two angular methyls [δ_H 1.12 (s), 0.86 (s), δ_C 16.2, 12.3]. A total of 21 resonances, including four quaternaries, seven methines, eight methylenes, and two methyls, were observed in the ^{13}C NMR spectrum of **19** and suggested that it was a pregnane derivative. Analysis of the 2D NMR data established the gross structure of **19**, as shown.

Acetylation of **19** gave diacetylated (**19a**) and monoacetylated (**19b**) products, which supported the presence of a glycol moiety (C-20−C-21) in the molecule. A positive molecular rotation difference ($\Delta[\phi]_D$ = +254°) between the 20,21-di-*O*-acetate **19a** and the 20-hydroxy-21-*O*-acetate **19b** indicated that the configuration of the hydroxy group at C-20 was β.[15] Thus, **19** was elucidated as 20β,21-dihydroxypregna-4,6-dien-3-one.

Compound **20** was isolated as a white powder. The ^{1}H NMR spectrum of **20** showed three olefinic signals [δ_H 6.49 (dd, J = 10.0, 2.0 Hz), 6.23 (dd, J = 10.0, 2.0 Hz), 5.68 (s)] and two methyl singlets (δ_H 1.09 and 1.08). Its ^{13}C NMR spectrum revealed 19 carbon resonances, including two ketones (δ_C 219.9, 199.1), four olefinic carbons (δ_C 161.9 s, 136.3 d, 129.7 d, 124.2 d), and a quaternary oxygenated carbon (δ_C 81.3). These data suggested that it also contained a conjugated 4,6-dien-3-one moiety, as in **19**. This moiety was further supported by the UV maximum at 282 nm. Detailed analysis of the 2D NMR data (COSY, NOESY, HMQC, HMBC) was used to establish the structure of **20** as an androstane derivative, as shown. HMBC correlations from H_3-18 to one of the ketone groups (δ_C 219.9) and to the oxygenated carbon (δ_C 81.3) indicated that the ketone and the hydroxy groups are located at C-17 and C-14, respectively. The hydroxy group at C-14 was assigned tentatively as β, on the basis of the C-14 chemical shift.[9] Thus, **20** was established as 14β-hydroxyandrosta-4,6-dien-3,17-dione.[16]

Compound **22** was isolated as fine needles. The molecular formula, $C_{30}H_{50}O_2$ (six degrees of unsaturation), was deduced from the HREIMS (*m/z* 442.3808 M^+, Δ −0.2 mmu). The ^{1}H NMR spectrum of **22** exhibited resonances for seven methyls (δ_H 0.99 d, 0.96 s, 0.90 s, 0.89 d, 0.86 s, 0.80 s, 0.74 d) and terminal olefinic signals (δ_H 4.73 s, 4.68 s). Its ^{13}C NMR spectrum revealed a total of 30 carbon signals including a carbonyl (δ_C 176.6 s) and two alkene carbons (δ_C 154.6 s, 107.3 t). These data suggested that **22** is a triterpene. Since the carbonyl group and the double bond account for two of the six degrees of unsaturation required by the molecular formula, **22** must be tetracyclic. Analysis of the 2D NMR data (COSY, HMQC, HMBC, and NOESY) enabled the structure of **22** to be determined, as shown. The HMBC cross-peaks from H-19 and H-21 to both C-20 and C-30 indicated the position of the terminal double bond at C-20(30). Similarly, an isopropyl group was placed at C-5 and the other methyl group (C-29) at C-19 (HMBC cross-peaks H-4/C-5, C-6, C-10; H-29/C-18, C-19, C-20). A propionic acid group (C-1−C-3) was attached at C-10 by the observed HMBC correlations (H-25/C-1, C-10; H-1/C-10; H-2/C-10). The relative configuration of **22** was assigned by the NOESY correlations observed among the protons H-5/H-1, H-2, H-9; H-14/H-18; H-25/H-26; H-26/H-27; H-27/H-28; and H-28/H-29, H-30. Accordingly, **22** was assigned as 3,4-*seco*-urs-20(30)-en-3-oic acid.

Table 1. Cytotoxic Activity of Cardenolides against Four Cancer Cell Lines

	IC_{50} (μg/mL)			
compound	A549	MDA-MB-231	MCF-7	HepG2
1	0.02	0.16	0.08	0.11
2	0.11	0.49	0.28	0.39
3	0.10	0.98	0.48	0.15
4	0.0029	0.03	0.01	0.02
5	0.07	0.45	0.38	0.16
7	0.01	0.06	0.02	0.05
8	0.02	0.39	0.17	0.13
9	0.14	1.52	1.00	0.51
10	0.01	0.09	0.04	0.04
11	0.01	0.10	0.04	1.10
17	0.24	1.90	1.69	0.12
doxorubicin[a]	0.79	0.65	0.56	0.18

[a] Positive control.

Most of the cardenolides showed strong cytotoxicity against the four cancer cell lines tested (Table 1, human lung carcinoma A549, two human breast carcinomas MCF-7 and MDA-MB-231, and hepatoma HepG2). Among them, calactin (**4**) showed the most potent activity, with an IC_{50} of 2.9 ng/mL against A549 cells. Its 3′α-isomer (calotropin, **1**) and acetoxyl derivatives (**7**−**9**) showed weaker activity.

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a JASCO polarimeter (P-1020), and IR spectra were measured on a Genesis II (Mattson) FT-IR instrument. UV spectra were obtained on a UV−vis spectrophotometer (JASCO V-530). ^{1}H and ^{13}C NMR spectra were run on Varian Unity 400 and JEOL A-600 instruments. LRMS was measured on an API 3000 (Applied Bioscience) and the HRMS on Bruker APEX II and Waters Q-TOF Ultima API mass spectrometers. HPLC was performed with a JASCO 980 pump equipped with a RI detector (JASCO RI-930) and a Shimadzu (LC 10AT VP) pump with a diode array detector (Shimadzu, SPD-M10A VP) using ODS (Hibar Purospher STAR, RP-18e, 250 × 10 mm) and silica gel (Thermo Hypersil silica, 250 × 10 mm) columns. Silica gel Si 60 (40−63 μm) was used for column chromatography. Precoated aluminum sheets (silica gel 60 F_{254} and RP-18 F_{254}) were used for thin-layer chromatography (TLC). All reagents and solvents used were reagent grade. HPLC grade solvents (Merck KGaA, Taiwan) were used for HPLC.

Plant Material. *Asclepias curassavica* (20 kg) was collected at Tainan, Taiwan, in August 2003. The plant material was identified by Dr. Hsin-Fu Yen (senior botanist and researcher, National Museum of Natural Science, Taichung, Taiwan). A voucher specimen (Asclepias 1) was deposited at the Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung, Taiwan.

Extraction and Isolation. After collection, the plants were separated into their aerial and root parts. Each part was chopped into small pieces (0.5−1 cm) and air-dried indoors. The dried plant materials (roots 5 kg and aerial parts 15 kg)

were extracted separately with MeOH (20 L × 3) at room temperature overnight. After concentration, the MeOH extract gave a black slurry, which was extracted with EtOAc (2.5 L × 3). After removal of solvent, the EtOAc extract yielded a crude solid mass (25 g from the roots and 118 g from the aerial parts). A portion of the root extract (13 g) was fractionated over a silica gel column (450 g), using (a) CH_2Cl_2, (b) CH_2Cl_2−EtOAc (1, 5, 10, 20%), and (c) CH_2Cl_2−MeOH (5, 10, 20, 100%) as solvent systems (each 1 L), to give a total of 13 fractions. The combined fourth and fifth fractions (626 mg), eluted with 5% EtOAc−CH_2Cl_2, were further purified by HPLC (silica gel, CH_2Cl_2−EtOAc, 95:5) to give 3*β*-acetyloleanolic acid (**21**, 9 mg),[17] **22** (15 mg), 3*β*-acetyl-*β*-amyrin,[18] phytol,[19] *β*-sitosterol,[20] and stigmasterol.[20] The polar 12th fraction (3.48 g), eluted with 10% MeOH−CH_2Cl_2, was further fractionated over a silica gel column (100 g) using EtOAc−MeOH (0, 5, 10, 100%, each 1 L) to furnish 11 fractions. The second and third fractions (1.69 g) yielded **1** (6.5 mg), **2** (10 mg), **4** (19 mg), **6** (3.3 mg), **7** (7 mg), **8** (30.5 mg), **16** (1.5 mg), and **20** (1.1 mg) by repeated ODS HPLC, using the solvent systems (i) MeCN−H_2O (60:40), (ii) MeCN−H_2O (50:50), and (iii) MeCN−H_2O (30:70). These fractions also gave steroidal glycosides.

Similarly, the EtOAc extract (81 g) of the aerial part was separated by silica gel column chromatography followed by HPLC. Fractionation of the extract over a silica gel column (2.5 kg) using the above solvent systems (a−c, each 2.5 L) gave 18 fractions. The combined eighth and ninth fractions (14 g), eluted with 10% MeOH−CH_2Cl_2, contained cardenolides and were rechromatographed over silica gel (400 g) using EtOAc−MeOH (0, 5, 10, 100%, each 1 L) to give nine fractions. Most of the cardenolides were concentrated in the second (6.11 g) and third (4.07 g) fractions. A portion of the second fraction (1.0 g) was further purified by ODS HPLC (solvents i−iii) to yield **4** (41 mg), **7** (79 mg), **8** (35 mg), **10** (40.5 mg), **11** (12 mg), and **17** (15 mg). Purification of the third fraction (1.0 g) by HPLC (ODS, solvents i−iii) gave **1**−**5** (47, 22, 18, 41, and 25 mg, respectively), **9** (14 mg), **12** (22.5 mg), **18** (14 mg), and **19** (19 mg).

Calactinic Acid Methyl Ester (12): colorless glass; $[\alpha]_D^{25}$ −22.0° (*c* 0.45, CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 217 (4.04), 265 (3.41) nm; IR (film) ν_{max} 3470, 2973, 2878, 1723, 1631 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$−C_5D_5N, 9:1) δ 9.79 (1H, s, H-19), 5.73 (1H, s, H-22), 4.90 (1H, dd, J = 18.0, 2.0 Hz, H-21a), 4.88 (1H, s, H-1′), 4.67 (1H, dd, J = 18.0, 2.0 Hz, H-21b), 4.42 (1H, m, H-5′), 3.58 (3H, s, COOCH_3-3′), 3.31 (1H, ddd, J = 13.0, 12.0, 5.0 Hz, H-2), 3.14 (1H, ddd, J = 13.0, 12.0, 5.0 Hz, H-3), 2.62 (1H, dd, J = 9.6, 4.8 Hz, H-17), 2.50 (1H, dd, J = 13.0, 5.0 Hz, H-1a), 2.21 (1H, dd, J = 13.0, 10.0 Hz, H-4′a), 2.19 (1H, m, H-6a), 2.06 (1H, dd, J = 13.0, 5.6 Hz, H-4′b), 1.97 (1H, m, H-16a), 1.87 (1H, m, H-15a), 1.75 (1H, m, H-16b), 1.59 (2H, m, H-15b, H-7a), 1.50 (2H, m, H-8, H-4a), 1.30 (1H, m, H-12a), 1.24 (3H, d, J = 6.0 Hz, H-6′), 1.20 (1H, m, H-12b), 1.17 (1H, m, H-9), 1.14 (1H, m, H-5), 1.11 (1H, m, H-4b), 1.10 (2H, m, H-6b, H-7b), 0.83 (1H, t, J = 13.0 Hz, H-1b), 0.69 (3H, s, H-18); ^{13}C NMR (100 MHz, $CDCl_3$−C_5D_5N, 9:1) δ 207.1 (d, C-19), 174.5 (s, C-20), 174.4 (s, C-23), 171.2 (s, C-3′), 117.3 (d, C-22), 108.5 (d, C-1′), 85.2 (d, C-3), 84.1 (s, C-2′), 83.8 (s, C-14), 76.1 (d, C-5′), 73.1 (t, C-21), 70.0 (d, C-2), 51.8 (s, C-10), 51.5 (q, COOCH_3-3′C), 50.4 (d, C-17), 49.2 (s, C-13), 48.0 (d, C-5), 42.3 (d, C-9), 41.8 (d, C-8), 40.2 (t, C-4′), 38.9 (t, C-12), 37.9 (t, C-1), 34.0 (t, C-4), 31.7 (t, C-15), 27.3 (t, C-11), 26.9 (t, C-6), 26.4 (t, C-16), 21.8 (q, C-6′), 21.5 (t, C-7), 15.4 (q, C-18); LRESIMS *m/z* 585.4 $[M + Na]^+$ (100), 563.3 $[M + H]^+$ (80), 411 (25), 405 (25), 353 (15), 181 (25); HRESIMS *m/z* 563.2857 $[M + H]^+$ (calcd for $C_{30}H_{43}O_{10}$, 563.2856).

19-*nor*-16α-Acetoxy-10*β*-hydroxyasclepin (16): colorless glass; $[\alpha]_D^{25}$ +46.6° (*c* 0.03, CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 216 (4.00), 265 (3.15) nm; IR (film) ν_{max} 3472, 2921, 2850, 1710, 1631, 1105 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$) δ 5.92 (1H, s, H-22), 5.23 (1H, td, J = 8.0, 4.0 Hz, H-16), 4.92 (1H, dd, J = 18.0, 1.6 Hz, H-21a), 4.84 (1H, dd, J = 18.0, 1.6 Hz, H-21b), 4.78 (1H, dd, J = 12.0, 5.0 Hz, H-3′), 4.59 (1H, s, H-1′), 4.14 (1H, ddd, J = 10.0, 8.0, 4.4 Hz, H-2), 3.98 (1H, ddd, J = 10.0, 10.0, 4.4 Hz, H-3), 3.69 (1H, m, H-5′), 2.73 (1H, dd, J = 13.2, 4.2 Hz, H-1a), 2.64 (1H, d, J = 4.0 Hz, H-17), 2.23 (1H, dd, J = 14.0, 8.0 Hz, H-15a), 2.16 (3H, s, OCOCH_3), 2.04 (1H, m, H-15b), 2.02 (3H, s, OCOCH_3), 2.01 (1H, m, H-7a), 1.94 (1H, m, H-11a), 1.86 (1H, m, H-4′a), 1.83 (1H, m, H-8), 1.73 (1H, m, H-4′b), 1.71 (1H, m, H-6a), 1.62 (1H, m, H-12a), 1.55 (2H, m, H-4), 1.52 (1H, m, H-12b), 1.51 (1H, m, H-6b), 1.48 (1H, m, H-11b), 1.29 (3H, d, J = 6.0 Hz, H-6′), 1.25 (2H, m, H-5, H-7b), 1.20 (1H, m, H-1b), 1.06 (1H, m, H-9), 0.89 (3H, s, H-18); ^{13}C NMR (150 MHz, $CDCl_3$) δ 174.2 (s, C-23), 172.6 (s, O*C*OCH_3-3′C), 171.0 (s, C-20), 170.6 (s, O*C*OCH_3-16C), 118.3 (d, C-22), 96.1 (d, C-1′), 90.9 (s, C-2′), 85.1 (s, C-14), 83.2 (s, C-10), 78.2 (d, C-16), 75.7 (d, C-3′), 73.2 (t, C-21), 71.4 (d, C-3), 68.8 (d, C-2), 67.7 (d, C-5′), 57.8 (d, C-17), 48.8 (s, C-13), 48.5 (d, C-5), 44.0 (d, C-9), 40.9 (d, C-8), 40.3 (t, C-12), 39.3 (t, C-15), 35.4 (t, C-4′), 33.9 (t, C-1), 31.9 (t, C-4), 27.0 (t, C-6), 26.8 (t, C-11), 22.5 (t, C-7), 21.2 (q, OCO*C*H_3-3′C), 21.0 (q, OCO*C*H_3-16C), 20.9 (q, C-6′), 15.6 (q, C-18); LRESIMS *m/z* 659.5 $[M + K]^+$ (20), 643.4 $[M + Na]^+$ (100), 621.5 $[M + H]^+$ (90), 432 (60), 381 (85), 353 (95), 337 (50), 304 (50), 295 (40), 282 (80), 249 (80), 217 (35); HRESIMS *m/z* 621.2916 $[M + H]^+$ (calcd for $C_{32}H_{45}O_{12}$, 621.2911).

20*β*,21-Dihydroxypregna-4,6-dien-3-one (19): colorless solid; $[\alpha]_D^{25}$ +12.0° (*c* 1.0, CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 282 (3.40) nm; IR (film) ν_{max} 3400, 2900, 2880, 1620 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.11 (2H, overlapped, H-6, H-7), 5.67 (1H, s, H-4), 3.69 (1H, dd, J = 11.2, 2.6 Hz, H-21a), 3.67 (1H, dd, J = 8.0, 2.6 Hz, H-20), 3.40 (1H, dd, J = 11.2, 8.0 Hz, H-21b), 2.57 (1H, ddd, J = 18.0, 14.4, 5.6 Hz, H-2a), 2.42 (1H, ddd, J = 18.0, 5.6, 2.0 Hz, H-2b), 2.23 (1H, t, J = 10.4 Hz, H-14), 2.18 (1H, dt, J = 9.6, 3.2 Hz, H-12a), 2.01 (1H, ddd, J = 13.2, 5.2, 2.0 Hz, H-1a), 1.87 (1H, m, H-16a), 1.72 (1H, m, H-1a), 1.68 (2H, m, H-15), 1.56 (1H, m, H-11a), 1.50 (1H, m, H-8), 1.45 (1H, m, H-11b), 1.34 (1H, m, H-16b), 1.32 (1H, m, H-12b), 1.26 (1H, m, H-9), 1.25 (1H, m, H-17), 1.12 (3H, s, H-19), 0.86 (3H, s, H-18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.7 (s, C-3), 163.9 (s, C-5), 141.2 (d, C-7), 127.9 (d, C-6), 123.5 (d, C-4), 74.4 (d, C-20), 66.3 (t, C-21), 52.6 (d, C-17), 52.0 (d, C-8), 50.7 (d, C-9), 43.5 (s, C-13), 39.3 (t, C-12), 37.5 (d, C-14), 36.0 (s, C-10), 33.9 (t, C-2), 33.8 (t, C-1), 24.5 (t, C-16), 24.0 (t, C-15), 20.5 (t, C-11), 16.2 (q, C-19), 12.3 (q, C-18); LRESIMS *m/z* 331.1 $[M + H]^+$ (10), 312.0 $[M - H_2O]^+$ (100), 297 (100), 294 (60), 279 (60); HRESIMS *m/z* 331.2273 $[M + H]^+$ (calcd for $C_{21}H_{31}O_3$, 331.2273).

14*β*-Hydroxyandrosta-4,6-dien-3,17-dione (20): white powder; $[\alpha]_D^{25}$ +75.4° (*c* 0.11, CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 281 (3.37) nm; IR (film) ν_{max} 3466, 2918, 2845, 1718, 1630, 1105 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.49 (1H, dd, J = 10.0, 2.0 Hz, H-7), 6.23 (1H, dd, J = 10.0, 2.0 Hz, H-6), 5.68 (1H, s, H-4), 2.58 (1H, dd, J = 14.4, 5.2 Hz, H-2a), 2.42 (1H, dd, J = 14.4, 5.2 Hz, H-2b), 2.42 (1H, m, H-16), 2.39 (1H, m, H-8), 2.04 (1H, ddd, J = 14.0, 7.6, 3.6 Hz, H-15a), 2.01 (1H, m, H-1a), 1.80 (1H, ddd, J = 14.0, 7.6, 3.6 Hz, H-15b), 1.70 (1H, td, J = 13.6, 5.2 Hz, H-1b), 1.62 (1H, m, H-12a), 1.50 (1H, m, H-11a), 1.32 (2H, td, J = 10.0, 2.0 Hz, H-11b, H-12b), 1.43 (1H, m, H-9), 1.09 (3H, s, H-18), 1.08 (3H, s, H-19); ^{13}C NMR (100 MHz, $CDCl_3$) δ 219.9 (s, C-17), 199.1 (s, C-3), 161.9 (s, C-5), 136.3 (d, C-7), 129.7 (d, C-6), 124.2 (d, C-4), 81.3 (s, C-14), 53.5 (s, C-13), 46.7 (d, C-9), 43.8 (d, C-8), 36.0 (s, C-10), 33.9 (t, C-2), 33.7 (t, C-16), 33.2 (t, C-1), 32.0 (t, C-11), 27.1 (t, C-15), 19.5 (t, C-12), 16.1 (q, C-19), 13.2 (q, C-18); LRESIMS *m/z* 323.3 $[M + Na]^+$ (30), 301.2 $[M + H]^+$ (90), 282.5 $[M - H_2O]^+$ (100), 249 (40).

3,4-*seco*-Urs-20(30)-en-3-oic acid (22): colorless fine needles; $[\alpha]_D^{25}$ +34.0° (*c* 0.4, MeOH); UV (MeOH) λ_{max} (log ϵ) 208 (3.33), 270 (2.33) nm; IR (film) ν_{max} 3400, 2930, 1650 cm^{-1}; ^1H NMR (400 MHz, C_5D_5N) δ 4.73 (1H, brs, H-30a), 4.68 (1H, brs, H-30b), 2.45 (2H, t, J = 8.0 Hz, H-2), 2.40 (1H, m, H-21a), 2.17 (1H, m, H-21b), 2.07 (1H, pent., J = 6.4 Hz, H-19), 1.97 (2H, m, H-1), 1.89 (1H, m, H-4), 1.60 (1H, m, H-15a), 1.60 (1H, m, H-14), 1.58 (2H, m, H-11), 1.50 (1H, m, H-9), 1.33 (2H, m, H-22), 1.30 (3H, m, H-6, H-7a), 1.22 (1H, m, H-7b), 1.18 (1H, m, H-16a), 1.15 (1H, m, H-15b), 1.09 (1H, m, H-5), 1.08 (1H, m, H-16b), 0.99 (3H, d, J = 6.4 Hz, H-29), 0.96 (3H, s, H-26), 0.90 (3H, s, H-27), 0.90 (3H, overlapped, H-12, H-18), 0.89 (3H, d, J = 6.4 Hz, H-24), 0.86 (3H, s, H-28), 0.80 (3H, s, H-25), 0.74 (3H, d, J = 6.4 Hz, H-23); ^{13}C NMR (100 MHz, C_5D_5N) δ

176.6 (s, C-3), 154.6 (s, C-20), 107.3 (t, C-30), 48.4 (d, C-18), 47.1 (d, C-5), 42.3 (s, C-13), 40.7 (d, C-14), 40.6 (s, C-8), 39.9 (s, C-10), 39.3 (both d, C-9, C-19), 38.8 (t, C-22), 38.2 (t, C-16), 34.4 (s, C-17), 33.7 (t, C-1), 32.7 (t, C-7), 29.2 (t, C-2), 26.7 (t, C-12), 26.2 (t, C-11), 25.6 (t, C-21), 25.4 (d, C-4), 25.3 (q, C-29), 24.6 (q, C-27), 22.1 (t, C-15), 19.8 (q, C-25), 19.6 (q, C-28), 18.8 (q, C-23), 18.3 (t, C-6), 15.8 (q, C-26), 14.5 (q, C-24); LREIMS *m/z* 442.2 M^+ (10), 382 (20), 373 (10), 354 (10), 342 (20), 341 (100), 340 (10); HREIMS *m/z* 442.3808 M^+ (calcd for $C_{30}H_{50}O_2$, 442.3810).

Oxidation of 10 and 12. While in solution in the NMR tube, **12** gradually started oxidizing within 48 h even when kept at low temperature (4 °C). After 72 h, its NMR spectra clearly showed that it contained a 1:1 mixture of two components. Within a few days, the ratio changed to 3:2. Finally, purification of this mixture by HPLC (ODS, MeCN−H_2O, 30:70) gave **13** (9.7 mg, t_R = 21.9 min), **14** (ca. 0.1 mg, t_R = 24.2 min), **15** (ca. 0.1 mg, t_R = 17.0 min), and **12** (4.5 mg, t_R = 33.2 min).

Initially, uscharidin (**10**) showed homogeneity by NMR and HPLC analysis. However, it readily converted into a mixture in the presence of aqueous MeOH within 72 h at room temperature. HPLC analysis of this mixture indicated that it contained **12** and **13** as major products along with **14** and **15** as minor products.

Compound 13: colorless crystals (EtOH−MeOH, 1:1); $[\alpha]_D^{25}$ −18.0° (*c* 0.97, MeOH); UV (MeOH) λ_{max} (log ϵ) 218 (4.06) nm; IR (film) ν_{max} 3400, 2950, 2820, 1657 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$−C_5D_5N, 9:1) δ 5.82 (1H, s, H-22), 5.12 (1H, dd, *J* = 18.0, 1.6 Hz, H-21a), 4.78 (1H, dd, *J* = 18.0, 1.6 Hz, H-21b), 5.05 (1H, s, H-1′), 4.54 (1H, m, H-5′), 3.67 (3H, s, COOC*H*$_3$-3′C), 3.76 (1H, ddd, *J* = 13.0, 12.0, 5.0 Hz, H-2), 3.31 (1H, ddd, *J* = 13.0, 12.0, 5.0 Hz, H-3), 2.83 (1H, dd, *J* = 12.8, 4.8 Hz, H-1a), 2.73 (1H, dd, *J* = 9.6, 4.8 Hz, H-17), 2.34 (1H, dd, *J* = 13.0, 10.0 Hz, H-4′a), 2.18 (1H, dd, *J* = 13.0, 4.8 Hz, H-4′b), 2.22 (1H, m, H-6a), 2.08 (1H, m, H-16a), 2.04 (1H, m, H-15a), 1.95 (1H, m, H-8), 1.82 (1H, m, H-16b), 1.75 (2H, m, H-7a, H-15b), 1.57 (2H, ddd, *J* = 13.2, 5.2, 2.4 Hz, H-4), 1.47 (1H, m, H-12a), 1.40 (2H, m, H-7b, H-12b), 1.35 (3H, d, *J* = 6.4 Hz, H-6′), 1.22 (1H, m, H-9), 1.16 (1H, m, H-5), 1.10 (1H, m, H-6b), 0.96 (1H, t, *J* = 12.0 Hz, H-1b), 0.79 (3H, s, H-18); ^{13}C NMR (100 MHz, $CDCl_3$−C_5D_5N, 9:1) δ 175.7 (s, C-19), 174.7 (s, C-20), 174.1 (s, C-23), 171.1 (s, C-3′), 116.9 (d, C-22), 108.6 (d, C-1′), 85.8 (d, C-3), 84.1 (both s, C-2′ and C-14), 75.8 (d, C-5′), 73.0 (t, C-21), 71.1 (d, C-2), 51.6 (q, COO*C*H$_3$-3′C), 50.4 (d, C-17), 50.1 (s, C-13), 49.3 (s, C-10), 47.3 (d, C-5), 43.4 (d, C-9), 40.5 (d, C-8), 40.2 (t, C-4′), 39.0 (t, C-12), 41.0 (t, C-1), 34.3 (t, C-4), 32.0 (t, C-15), 27.7 (t, C-11), 26.7 (t, C-6), 26.4 (t, C-16), 22.5 (t, C-7), 21.7 (q, C-6′), 15.5 (q, C-18); LRESIMS *m/z* 601.4 $[M + Na]^+$ (90), 579.5 $[M + H]^+$ (100), 421 (20), 384 (20), 169 (70); HRESIMS *m/z* 579.2815 $[M + H]^+$ (calcd for $C_{30}H_{43}O_{11}$, 579.2805).

Compound 14: colorless glass; UV (MeOH) λ_{max} (log ϵ) 218 (3.55), 275 (2.60) nm; IR (film) ν_{max} 3400, 2950, 2820, 1657 cm^{-1}; LRESIMS *m/z* 589.4 $[M + Na]^+$ (100), 567.4 $[M + H]^+$ (50), 422 (20), 405 (15), 384 (10), 181 (20); HRESIMS *m/z* 567.2793 $[M + H]^+$ (calcd for $C_{29}H_{43}O_{11}$, 567.2805).

Compound 15: colorless glass; UV (MeOH) λ_{max} (log ϵ) 218 (3.88) nm; IR (film) ν_{max} 3400, 2950, 2820, 1657 cm^{-1}; LRESIMS *m/z* 573.4 $[M + Na]^+$ (100), 551.4 $[M + H]^+$ (90), 348 (15), 195 (20), 181 (20); HRESIMS *m/z* 551.2848 $[M + H]^+$ (calcd for $C_{29}H_{43}O_{10}$, 551.2856).

Acetylation of 19. Compound **19** (5.5 mg) was treated with pyridine (0.3 mL) and Ac_2O (0.1 mL) in the usual way to give the acetylated products. The products were separated by HPLC (ODS, MeCN−H_2O, 60:40) to furnish **19a** (3.6 mg) and **19b** (0.5 mg).

20*β*,21-Diacetylpregna-4,6-dien-3-one (19a): colorless glass; $[\alpha]_D^{25}$ +86.5° (*c* 0.36, CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 284 (4.32) nm; IR (film) ν_{max} 3500, 2900, 2825, 1620 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.10 (2H, overlapped, H-6, H-7), 5.67 (1H, s, H-4), 5.11 (1H, ddd, *J* = 10.4, 6.0, 2.4 Hz, H-20), 4.34 (1H, dd, *J* = 12.4, 2.4 Hz, H-21a), 3.90 (1H, dd, *J* = 12.4, 6.0 Hz, H-21b), 2.57 (1H, ddd, *J* = 18.0, 14.4, 5.6 Hz, H-2a), 2.42 (1H, ddd, *J* = 18.0, 5.6, 2.4 Hz, H-2b), 2.21 (2H, t, *J* = 10.4 Hz, H-12a, H-14), 2.06 (3H, s, OCOC*H*$_3$), 2.05 (3H, s, OCOC*H*$_3$), 1.99 (1H, ddd, *J* = 12.8, 5.6, 2.4 Hz, H-1a), 1.88 (1H, m, H-16a), 1.78 (1H, m, H-1b), 1.71 (2H, m, H-15), 1.56 (1H, m, H-11a), 1.52 (1H, m, H-8), 1.45 (1H, m, H-11b), 1.36 (1H, m, H-16b), 1.30 (1H, m, H-12b), 1.28 (1H, m, H-9), 1.26 (1H, m, H-17), 1.10 (3H, s, H-19), 0.75 (3H, s, H-18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.6 (s, C-3), 170.8 (s, *O*C*O*CH$_3$), 170.3 (s, *O*C*O*CH$_3$), 163.6 (s, C-5), 140.7 (d, C-7), 128.0 (d, C-6), 123.7 (d, C-4), 73.0 (d, C-20), 65.1 (t, C-21), 52.6 (d, C-8), 50.6 (d, C-9), 49.5 (d, C-17), 43.3 (s, C-13), 38.6 (t, C-12), 37.5 (d, C-14), 36.0 (s, C-10), 33.9 (t, C-2), 33.8 (t, C-1), 24.7 (t, C-16), 23.7 (t, C-15), 21.2 (q, OCO*C*H$_3$), 20.8 (q, OCO*C*H$_3$), 20.5 (t, C-11), 16.2 (q, C-19), 12.3 (q, C-18); LREIMS *m/z* 415.0 $[M + H]^+$ (5), 312 (10), 294 (100), 279 (50), 226 (50).

20*β*-Hydroxy-21-acetylpregna-4,6-dien-3-one (19b): colorless glass; $[\alpha]_D^{25}$ +28.0° (*c* 0.05, CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 283 (4.04) nm; IR (film) ν_{max} 3500, 2900, 2825, 1620 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.11 (2H, overlapped, H-6, H-7), 5.67 (1H, s, H-4), 4.18 (1H, dd, *J* = 11.2, 2.4 Hz, H-21a), 3.91 (1H, dd, *J* = 11.2, 7.2 Hz, H-21b), 3.81 (1H, ddd, *J* = 10.4, 7.2, 2.4 Hz, H-20), 2.57 (1H, ddd, *J* = 18.0, 14.4, 5.6 Hz, H-2a), 2.42 (1H, ddd, *J* = 18.0, 5.6, 2.4 Hz, H-2b), 2.23 (1H, t, *J* = 12.0 Hz, H-14), 2.20 (1H, m, H-12a), 2.10 (3H, s, OCOC*H*$_3$), 2.00 (1H, m, H-1a), 1.86 (1H, m, H-16a), 1.75 (1H, m, H-1b), 1.74 (2H, m, H-15), 1.56 (2H, m, H-8, H-11a), 1.45 (1H, m, H-11b), 1.36 (1H, m, H-16b), 1.30 (1H, m, H-12b), 1.28 (1H, m, H-9), 1.26 (1H, m, H-17), 1.12 (3H, s, H-19), 0.86 (3H, s, H-18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.7 (s, C-3), 170.3 (s, *O*C*O*CH$_3$), 163.8 (s, C-5), 141.0 (d, C-7), 127.9 (d, C-6), 123.6 (d, C-4), 72.5 (d, C-20), 68.7 (t, C-21), 52.7 (d, C-17), 52.0 (d, C-8), 50.7 (d, C-9), 43.6 (s, C-13), 39.2 (t, C-12), 37.5 (d, C-14), 36.0 (s, C-10), 33.9 (t, C-2), 33.8 (t, C-1), 24.7 (t, C-16), 24.1 (t, C-15), 20.9 (q, OCO*C*H$_3$), 20.5 (t, C-11), 16.2 (q, C-19), 12.3 (q, C-18); LREIMS *m/z* 372.0 M^+ (5), 312 (20), 294 (100), 279 (50), 226 (50).

X-ray Diffraction of 13.[21] Suitable colorless crystals of **13** were obtained by recrystallization (95% EtOH−MeOH, 1:1). The crystal (0.6 × 0.6 × 0.4 mm) belonged to the orthorhombic system, space group $P2_12_12_1$, with a = 7.9800(16) Å, b = 11.290(2) Å, c = 33.220(7) Å, V = 2993(1) Å^3, Z = 4, D_{calcd} = 1.324 g/cm^3, λ(Mo Kα) = 0.71073 Å. Intensity data were measured on a Rigaku AFC7S diffractometer up to 2θ of 52°. A total of 3360 reflections were collected. The structure was solved by the direct method (SIR 92) and refined by the full-matrix least-squares procedure. The non-hydrogen atoms were given anisotropic thermal parameters. The refinement converged to a final R = 0.034, R_w = 0.084 for 2706 observed reflections [$I > 2\sigma(I)$], and 406 variable parameters.

Cytotoxicity Assay. Compounds were assayed for cytotoxicity against A549, MCF-7, MDA-MB-231, and HepG2 cancer cell lines using the MTT method.[22] Freshly trypsinized cell suspensions were seeded in 96-well microtiter plates at densities of 5000−10 000 cells per well with tested compounds added from DMSO stock solution. After 3 days in culture, attached cells were incubated with MTT (0.5 mg/mL, 1 h) and subsequently solubilized in DMSO. The absorbance was measured at 550 nm using a microplate reader. The IC_{50} is the concentration of agent that reduced cell growth by 50% under the experimental conditions.

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