Constituents from the Stem Wood of *Euphorbia quinquecostata* with Phorbol Dibutyrate Receptor-Binding Inhibitory Activity

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Received April 23, 1996[⊗]

Bioassay-guided fractionation of an EtOAc extract of the stem wood of *Euphorbia quinquecostata* using a phorbol dibutyrate receptor-binding assay system as a monitor yielded four inhibitory isolates (**1**−**4**), inclusive of one new compound, 17-hydroxyingenol 20-hexadecanoate (**1**), and three previously known compounds, ingenol 20-hexadecanoate (**2**), *ent*-16α,17-dihydroxyatisan-3-one (**3**), and *ent*-3*β*,16α,17-trihydroxyatisane (**4**). Also isolated from this extract were 10 constituents inactive in this bioassay, namely, 2,2′-dihydroxy-4,6-dimethoxy-3-methylacetophenone (**5**), a new structure, and nine known compounds identified as *ent*-13*S*-hydroxyatis-16-ene-3,14-dione; 2-hydroxy-4,6-dimethoxyacetophenone (xanthoxylin); 2-hydroxy-4,6-dimethoxy-3-methylacetophenone; 6-hydroxy-7-methoxycoumarin; lupeol acetate; *β*-sitosterol; sitosterol *β*-D-glucopyranoside; 6,7,8-trimethoxycoumarin; and 3,3′,4′-tri-*O*-methyl-4-*O*-[α-L-rhamnopyranosyl (1‴→6″)-*β*-D-glucopyranosyl]ellagic acid. The structures of compounds **1** and **5** were established by chemical and spectroscopic methods.

Euphorbia quinquecostata Volk. (Euphorbiaceae) is a tree found in certain regions of Tanzania and Kenya.[1] Based on interviews with traditional healers in Tanzania, it has been established that preparations of the stem wood of this plant are used to accelerate wound healing and to treat stomach pains. Thus far there appears to have been no phytochemical or biological studies on this plant. In a search for naturally occurring cancer chemopreventive agents, we have investigated the chemical constituents of the stem wood of *E. quinquecostata*, because an EtOAc-soluble extract of a MeOH percolate demonstrated significant inhibition in a phorbol dibutyrate receptor-binding (PDBu) bioassay system.[2] Bioassay-guided chromatographic fractionation using this test system led to the isolation of four active compounds (**1**−**4**) constituted by the ingenane esters, **1** and **2**, and the *ent*-atisane derivatives, **3** and **4**. Also obtained in this investigation were ten inactive compounds including a novel benzenoid (**5**). The structure elucidation of **1** and **5** and the biological evaluation of **1**−**4** are the subject of this report.

Results and Discussion

Compound **1** was assigned the protonated molecular formula of $C_{36}H_{59}O_7$ from its HRFABMS (*m*/*z* 603.4260) $[M + 1]^+$. A molecular weight of 602 Da was confirmed using ESMS, in which a sodiated quasimolecular ion was observed at *m*/*z* 625. This compound displayed diagnostic IR absorptions at 3499−3371 (OH), 1726 (ester carbonyl), 1710 (C=O), and 1640 (C=C) cm^{-1}. The ^{1}H-NMR spectrum of **1** revealed a broad singlet at *δ* 1.25 indicative of the presence of a long-chain fatty acid moiety, as well as four methyl groups at *δ* 0.88 (3H, t, *J* = 6.8 Hz, terminal Me group of side chain), 0.97 (3H, d, *J* = 6.9 Hz, CH_3-18), 1.18 (3H, s, CH_3-16), and 1.85 (3H, d, *J* = 1.2 Hz, CH_3-19). Furthermore, the ^{1}H-NMR spectrum showed two separate AB systems at *δ* 3.86 (1H, d, *J* = 12.0 Hz, H-17$_A$) and 3.76 (1H, d, *J* = 12.0 Hz, H-17$_B$), and at *δ* 4.69 (1H, d, *J* = 13.2 Hz, H-20$_A$) and 4.56 (1H, d, *J* = 13.2 Hz, H-20$_B$). Compound **1** formed a triacetylated derivative (**6**) on acetylation under standard conditions. When compared to the ^{1}H-NMR spectrum of a known close analogue also obtained in this investigation, ingenol 20-hexadecanoate (**2**),[3,4] it was apparent from its ^{1}H-NMR spectrum that **1** was an ingenane diterpenoid with a C-17 hydroxymethyl rather than a C-17 methyl functionality. The structure and stereochemistry of the ingenane diterpenoids have been confirmed by the single crystal X-ray crystallography of ingenol 3,5,20-triacetate.[5] The occurrence of esters of 17-hydroxyingenol as opposed to 16-hydroxyingenol has been established previously by a combination of 1D- and 2D-dimensional NMR methods.[6,7] Analysis of the ^{1}H−^{1}H COSY NMR spectrum of **1** showed that H-7 at *δ* 6.06 (d, *J* = 4.2 Hz) was coupled to H-8 at *δ* 4.25 (dd, *J* = 12.0, 4.2 Hz) which was in turn coupled to H-14, a multiplet at *δ* 1.09. This coupling pattern included CH_3-18 at *δ* 0.97 (d, *J* = 6.9 Hz), which coupled to H-11 (*δ* 2.45, m). In turn, H-11 coupled to H_2-12 at *δ* 2.50 and 1.90, which exhibited geminal coupling and coupling to the H-13 multiplet (*δ* 1.58). The latter showed coupling to H-14 at *δ* 1.09. A separate spin system was apparent between H-1 at *δ* 5.89, which exhibited long-range coupling with CH_3-19 at *δ* 1.85 (d, *J* = 1.2 Hz). The ^{13}C-NMR spectrum of **1** exhibited among other resonances, two carbonyl signals at *δ* 206.46 (C-9) and *δ* 174.32 (C-1′), and two carbinol carbons resonating at *δ* 63.77 (C-17) and 66.53 (C-20).

Confirmation of the structure of **1** in the present investigation was obtained from a ^{1}H−^{13}C FLOCK NMR experiment[8] in which a clear correlation ($^3J_{C-H}$) was demonstrated between the CH_3-16 (*δ* 1.18, s) ^{1}H-NMR signal and the ^{13}C-NMR resonance of C-17 at *δ* 63.77 (three-bond coupling). The position of the second carbinol carbon at *δ* 66.53 was confirmed as being at C-20,

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⊗ Abstract published in *Advance ACS Abstracts*, November 1, 1996.

S0163-3864(96)00412-0 CCC: $12.00

through a selective INEPT experiment[9] in which irradiation ($^3J_{CH}$ = 6 Hz) of the two methylene protons at δ 4.69 (d, J = 13.2 Hz, H-20$_A$) and at 4.56 (1H, d, J = 13.2 Hz, H-20$_B$) resulted in signal enhancements at chemical shift values corresponding to the ester carbonyl C-1′ (δ 174.32), C-6 (δ 137.70), and C-7 (δ 127.14). Similarly, irradiation ($^3J_{CH}$ = 6 Hz) of H-7 at δ 6.06 (1H, d, J = 4.2 Hz) resulted in signal enhancements of carbons C-8 (δ 43.43), C-9 (δ 206.46), and C-20 (δ 66.53). These observations proved both the assignment of C-20 and the fact that the C-20 carbon bears the fatty acid side chain in **1**. On alkaline hydrolysis, derivatization, and GC−MS, the fatty acid side chain of **1** was confirmed as being hexadecanoic acid. Thus, on the basis of the foregoing observations coupled with a detailed analysis of its ^{1}H−^{13}C (HETCOR) 2D-NMR spectrum, the structure of **1** was established as 17-hydroxyingenol 20-hexadecanoate.

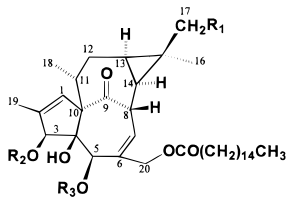
Three known compounds were isolated in this study as additional inhibitors of PDBu-receptor binding and identified by comparison with published physical and spectral data as ingenol 20-hexadecanoate (**2**),[3,4] *ent*-16α,17-dihydroxyatisan-3-one (**3**),[10,11] and *ent*-3*β*,16α,-17-trihydroxyatisane (**4**).[12]

The molecular formula of the inactive compound **5** was established as $C_{11}H_{14}O_5$ from its HREIMS (*m*/*z* 226.0849). The IR spectrum of **5** exhibited diagnostic absorptions at 3312 (OH), 1633 (C=O), and 1597 (C=C) cm^{-1}. Analysis of the ^{1}H-NMR spectrum of **5** revealed the presence of a hydrogen-bonded proton (δ 13.42), two methoxyl singlets at δ 3.90 and 3.91, a methyl group attached to an aromatic ring at δ 2.02, two methylene alcohol protons at δ 4.70, with only one aromatic proton at δ 5.95. Comparison of the ^{13}C-NMR data of **5** with those of 2-hydroxy-4,6-dimethoxyacetophenone (xanthoxylin)[11] and 2-hydroxy-4,6-dimethoxy-3-methylacetophenone,[13] which were both isolated in the present study, indicated that an additional hydroxyl group was present in **5** when compared with the latter compound. Further investigation of the structure of **5** was performed using a selective INEPT NMR experiment[9] in which irradiation ($^3J_{CH}$ = 7 Hz) of the two carbinol protons at δ 4.70 (d, J = 4.2 Hz) resulted in the signal enhancement of the C-1′ (δ 201.83) carbonyl resonance, while irradiation ($^3J_{CH}$ = 7 Hz) of the C-3 methyl protons at δ 2.02 resulted in the enhancements of C-2 (δ 163.18), C-3 (δ 105.99), and C-4 (δ 164.42). Under the same conditions, irradiation ($^3J_{CH}$ = 7 Hz) of H-5 (δ 5.95, s) gave enhancements of C-1 (δ 103.03), C-3 (δ 105.99), C-4 (δ 164.42), and C-6 (δ 161.18), whereas irradiation ($^3J_{CH}$ = 7 Hz) of the methoxy group protons at δ 3.90 and δ 3.91 enhanced the C-4 (δ 164.42) and C-6 (δ 161.18) resonances only. Because irradiation of the C-3 methyl protons at δ 2.02 enhanced signals corresponding to carbons C-2 through C-4, it was thus concluded that the only possible positions for the two methoxylated aromatic carbons were C-4 (δ 164.42) and C-6 (δ 161.18). On acetylation under standard conditions, compound **5** yielded a diacetyl derivative (**7**). From all of the observations made, compound **5** was assigned as 2,2′-dihydroxy-4,6-dimethoxy-3-methylacetophenone.

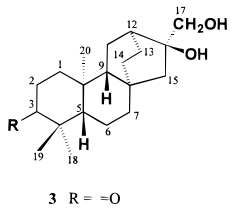
The PDBu receptor-binding inhibitory activity data for compounds **1**−**4** are summarized in Table 1. Within the two basic structural types represented by **1**−**4** (ingenol ester and hydroxylated *ent*-atisane diterpene), it may be seen that strict requirements are necessary for activity with this assay, based on the comparison with inactive and/or less active analogues also obtained in the present investigation. The novel ingenol ester **1** (17-hydroxyingenol 20-hexadecanoate) (IC$_{50}$ 0.023 μM) was greater than two orders of magnitude more potent in the PDBu assay than ingenol 20-hexadecanoate (**2**) (IC$_{50}$ 5.3 μM), indicating the importance of the 17-hydroxyl group for the mediation of this type of activity. The acetylated derivative (**6**) of **1** was inactive in this test system (IC$_{50}$ >275 μM). Although considerably less potent than in the case of the ingenanes, hydroxylation at C-16 and/or C-17 appears to be important for activity in this bioassay among the *ent*-atisane diterpenoids, because compounds **3** and **4** (*ent*-16α,17-dihydroxyatisan-3-one and *ent*-3*β*,16α,17-trihydroxyatisane; IC$_{50}$ values of 66 μM and 73 μM, respectively) were considered active, while *ent*-13*S*-hydroxyatis-16-ene-3,14-dione, a related compound, was inactive (IC$_{50}$ >630 μM). The biological ramifications of this receptor interaction by **1** and **2**, in terms of tumor promotion, will be considered elsewhere. From a phytochemical point of view, the present recognition of PDBu receptor-binding inhibitory activity by two *ent*-atisane diterpenes (**3** and **4**) from *E. quinquecostata* may explain in part a previous report[14] of PDBu binding by extracts of plants in subfamilies of the Euphorbiaceae that are not known thus far to produce phorbol and biogenetically related polycyclic diterpene esters, since *ent*-atisane diterpenoids may be more widespread in this family than presently known.

Table 1. Inhibition of PDBu Binding to Partially Purified PKC Mediated by Compounds **1**−**4**

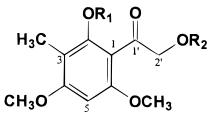
compound	IC$_{50}$ (μM)
1	0.023
2	5.3
3	66
4	73



1 R_1 = OH, R_2 = R_3 = H
2 R_1 = R_2 = R_3 = H
6 R_1 = OAc, R_2 = R_3 = Ac



3 R = =O
4 R = αOH, βH



5 R_1 = R_2 = H
7 R_1 = R_2 = Ac

Experimental Section

General Experimental Procedures. Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer model 241 polarimeter. UV and IR spectra were taken on Beckman DU-7 and Midac

Collegian FT-IR spectrometers, respectively. ^{1}H-NMR spectra were recorded using a Varian XL-300 spectrometer operating at 300 MHz. ^{13}C-NMR spectra were recorded on this same instrument (75.4 MHz) and on a Nicolet NMC-360 (90.8 MHz) spectrometer. NMR spectra were obtained in $CDCl_3$, C_5D_5N, and DMSO-d_6, with TMS as internal standard. EIMS and HREIMS were obtained on a Varian MAT-112S spectrometer. FABMS and HRFABMS were determined on a Finnigan MAT-90 spectrometer. ESMS were obtained on a Hewlett-Packard HP-59987A instrument. GC/MS was carried out on a Finnigan GC/MS 4510 equipped with an INCOS data system. Column chromatography was performed on Merck Si gel 60 (70−230 mesh), Si gel H (10−40 μm), and Sephadex LH-20. Precoated Kieselgel 60 F_{254} (thickness 1 mm, E. Merck, Darmstadt, Germany, Art. No. 13895) was used for preparative TLC. Spot detection in TLC was conducted by spraying with 10% aqueous H_2SO_4 followed by heating at 110 °C for 10 min. GC/MS was carried out using a DB-1 column (J & W Scientific, Folsom, CA; 30 m × 0.25 mm i.d. × 0.25 μm film thickness).

Plant Material. The stem wood of *E. quinquecostata* Volk. was collected in Handeni district, Tanzania, in September 1989, and identified by Dr. R. L. Mahunnah, Traditional Medicine Research Institute, Muhimbili Medical Center, University of Dar-es-Salaam, Dar-es-Salaam, Tanzania. A voucher specimen (MM 6267) has been deposited in the herbarium of the Traditional Medicine Research Institute, Muhimbili Medical Center, University of Dar-es-Salaam, Dar-es-Salaam, Tanzania.

Extraction and Isolation. The air-dried powdered stem wood of *E. quiquecostata* (6 kg) was exhaustively extracted with MeOH (2 × 20 L) for 24 h by maceration, and the resultant extracts were combined and concentrated *in vacuo* at 37 °C to afford 270 g of a residue. This residue was defatted with hexane (2 × 1 L), and then partitioned between H_2O (500 mL) and EtOAc (1 L) to give, on evaporation *in vacuo*, 16 g of an EtOAc-soluble extract. When evaluated against a PDBu binding assay,[2] the EtOAc extract exhibited an IC_{50} value of 1.2 μg/mL. Fractionation of this extract was initiated by column chromatography over Si gel (800 g) eluting with mixtures of hexane, EtOAc, and MeOH of increasing polarity to obtain a total of ten major fractions. The biological activities were found to concentrate in fractions 5 and 7−10. Further fractionation of fractions 5, 7−8, and 9−10 resulted in the purification of bioactive compounds **1**−**4**. Thus, when fraction 5 was subjected to flash column chromatography using petrolum ether−EtOAc (1:1) followed by preparative TLC with the same solvent system, ingenol 20-hexadecanoate (**2**) (59 mg; 0.0009% w/w) was isolated. Combined fractions 7 and 8 were subjected to flash column chromatography over Sephadex LH-20 using MeOH as eluent followed by flash column chromatography over Si gel H using EtOAc and 2% MeOH−EtOAc to give 17-hydroxyingenol 20-hexadecanoate (**1**) (48 mg; 0.0008% w/w) and *ent*-16α,-17-dihydroxyatisan-3-one (**3**) (260 mg; 0.0043% w/w). Combined fractions 9 and 10 were subjected to column chromatography using 5−10% MeOH−EtOAc to afford *ent*-3β,16α,17-trihydroxyatisane (**4**) (26 mg; 0.00043% w/w). Column chromatography of fraction 3 using petroleum ether−EtOAc (7:3), afforded a new compound, 2,2′-dihydroxy-4,6-dimethoxy-3-methylacetophenone (**5**) (12 mg; 0.00026% w/w), which was inactive in the PDBu assay.

From the least polar fraction obtained by column chromatography of the active fraction, lupeol acetate (240 mg; 0.004% w/w) and β-sitosterol (120 mg; 0.002% w/w) were isolated using gradient mixtures of petroleum ether and EtOAc. The second major fraction obtained from the EtOAc fraction was subjected to another column chromatographic separation using mixtures of petroleum ether and EtOAc to afford *ent*-13*S*-hydroxyatis-16-ene-3,14-dione (40 mg; 0.00066% w/w) and 6,7,8-trimethoxycoumarin (46 mg; 0.00076% w/w). The third major fraction was subjected to repeated column chromatography, eluting with petroleum ether and $CHCl_2$, to afford two closely related compounds, 2-hydroxy-4,6-dimethoxyacetophenone (xanthoxylin) (10 mg; 0.0002% w/w) and 2-hydroxy-4,6-dimethoxy-3-methylacetophenone (18 mg; 0.0003% w/w). Column chromatography coupled with separation over Sephadex LH-20 of the fourth major fraction by elution with petroleum ether and EtOAc (2:3) and MeOH, respectively, afforded sitosterol β-D-glucopyranoside (52 mg; 0.00087% w/w) and 6-hydroxy-7-methoxycoumarin (isoscopoletin) (26 mg; 0.00043% w/w). Combined major fractions 8 and 9 gave a precipitate upon standing at room temperature. Column chromatography using Sephadex LH-20 of this precipitate eluting with MeOH afforded 3,3′,4′ tri-*O*-methyl-4-*O*-[α-L-rhamnopyranosyl (1‴→6″)-β-D-glucopyranosyl]ellagic acid (30 mg; 0.0005% w/w).

17-Hydroxyingenol 20-hexadecanoate (**1**): colorless oil, $[\alpha]^{20}_D$ −16° (*c* 0.1, EtOH); UV (MeOH) λ max (log ϵ) 208 (3.99) nm; IR (dry film) ν max 3499−3371 (OH), 2990, 2858, 1726 (C=O), 1710 (C=O), 1640 (C=C), 1458, 1381, 1070, 1016 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.06 (1H, d, J = 4.2 Hz, H-7), 5.89 (1H, q, J = 1.2 Hz, H-1), 4.69 (1H, d, J = 13.2 Hz, H-20$_A$), 4.56 (1H, d, J = 13.2 Hz, H-20$_B$), 4.43 (1H, s, H-3), 4.25 (1H, dd, J = 12.0, 4.2 Hz, H-8), 3.86 (1H, d, J = 12.0 Hz, H-17$_A$), 3.76 (1H, d, J = 12.0 Hz, H-17$_B$), 3.64 (1H, s, H-5), 2.50, 1.90 (2H, m, H_2-12), 2.45 (1H, m, H-11), 1.85 (3H, d, J = 1.2 Hz, CH_3-19), 1.58 (1H, m, H-13), 1.25 (br s, fatty acid side chain), 1.18 (3H, s, CH_3-16), 1.09 (1H, m, H-14), 0.97 (3H, d, J = 6.9 Hz, CH_3-18), 0.88 (3H, t, J = 6.8 Hz, CH_3-16′); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 206.46 (s, C-9), 174.32 (C-1′), 139.62 (s, C-2), 137.70 (s, C-6), 129.44 (d, C-1), 127.14 (d, C-7), 84.84 (s, C-4), 80.59 (d, C-3), 74.23 (d, C-5), 73.65 (s, C-10), 66.53 (t, C-20), 63.77 (t, C-17), 43.43 (d, C-8), 40.04 (d, C-11), 34.22 (t, C-2′), 30.65 (t, C-12), 29.31 (t, fatty acid side chain), 25.32 (d, C-13), 24.55 (s, C-15), 24.45 (q, C-16), 23.87 (d, C-14), 17.48 (q, C-18), 15.74 (q, C-19), 14.05 (q, C-16′); EIMS *m*/*z* 346 (<1), 328 (3), 310 (3), 256 (4), 161 (7), 135 (11), 121 (19), 95 (19), 69 (25), 55 (52), 43 (100); ESMS *m*/*z* 625 $[M + Na]^+$ (100); FABMS *m*/*z* $[M + 1 + Na]^+$ 626 (2), $[M + 1]^+$, 603 (1); HRFABMS *m*/*z* 603.4260 (calcd for $C_{36}H_{59}O_7$, 603.4251).

Compound **1** (4 mg) was acetylated with a mixture of pyridine−$(Ac)_2O$ (1 mL) by overnight treatment at room temperature to afford 3,5,17-triacetyl-17-hydroxyingenol 20-hexadecanoate (**6**) (2.6 mg): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.23 (1H, d, J = 3.8 Hz, H-7), 6.08 (1H, q, J = 1.2 Hz, H-1), 5.38 (1H, br s, H-3), 4.95 (1H, s, H-5), 4.57 (1H, d, J = 12.5 Hz, H_A-20), 4.48 (1H, J = 12.5 Hz, H_B-20), 4.20 (1H, d, J = 11.9 Hz, H_A-17), 4.13 (1H, d, J = 11.9 Hz, H_B-17), 2.23 (3 H, s, Ac), 2.13 (3 H, s, Ac), 2.08

(3 H, s, Ac), 1.75 (3 H, d, J = 1.4 Hz, CH_3-19), 1.25 (br s, fatty acid side chain), 1.14 (3H, s, CH_3-16), 1.01 (3H, d, J = 6.9 Hz, CH_3-18), 0.88 (3H, d, J = 7.0 Hz, CH_3-16′); CIMS m/z $[M + 1]^+$ 729 (11), 353 (100).

Alkaline hydrolysis of **1** (5 mg) was performed at room temperature with 0.5 M KOH/MeOH (2 mL) for 1 h, according to conditions used in a previous report.[15] This procedure resulted in the isolation of a minor rearranged compound as a result of the apparent instability of the diterpene portion of the molecule. The structure of this rearranged product was not determined due to paucity of the starting compound. The fatty acid ester group obtained after hydrolysis of **1** was silylated by treating 2 mg of the dried residue with 100 μL of Sigma-Sil-A in a sealed vial in waterbath at 65 °C for 30 min. Analysis of the silylated derivative by GC−MS was carried out by injecting a 1-μL aliquot of the silyl ester sample on to a DB-1 fused silica capillary column. The column was held at an ion source temperature of 180 °C for 3 min then programmed at 10 °C/min to 280 °C. The injector and interface temperatures were maintained at 200 °C and 300 °C, respectively. The mass spectrometer was used in the EI mode. The other parameters were as follows: voltage, 70 eV; current, 0.25 mA; scan time, 1 s; and mass range 40−500 amu. EIMS m/z $[M + 1]^+$ 329 (<1), 313 (26), 269 (1), 201 (1), 145 (13), 132 (23), 117 (36), 97 (1), 73 (100), 43 (39). When compared with a standard this silylated product corresponded to hexadecanoic acid trimethyl silyl ester ($[M]^+$ m/z 328).

Ingenol 20-hexadecanoate (2): light-yellow oil; $[\alpha]^{20}_D$ −7° (*c* 0.1 $CHCl_3$), lit.[4] $[\alpha]^{23}_D$ −24° (*c* 0.3 $CHCl_3$), which exhibited comparable spectral (UV, IR, ^{1}H-NMR, ^{13}C-NMR, EIMS) data to published values.[3,4]

***ent*-16α,17-Dihydroxyatisan-3-one (3):** white crystals ($CHCl_3$); mp 135 °C; $[\alpha]^{20}_D$ −39° (*c* 0.2, $CHCl_3$), lit.[10] $[\alpha]^{20}_D$ −86° (*c* 0.7, $CHCl_3$), which exhibited comparable spectral (UV, IR, ^{1}H-NMR, ^{13}C-NMR, EIMS) data to published values.[10,11]

***ent*-3β,16α,17-Trihydroxyatisane (4):** white fluffy crystals (MeOH); mp 226−228 °C; $[\alpha]^{20}$D −28° (*c* 0.3, MeOH), which exhibited comparable spectral (UV, IR, ^{1}H-NMR, ^{13}C-NMR, EIMS) data to published values.[12]

2,2′-Dihydroxy-4,6-dimethoxy-3-methylacetophenone (5): colorless needles ($CHCl_3$); mp 164−166 °C; UV (MeOH) λ max (log ϵ) 211 (4.32), 290 (4.38) nm; IR (dry film) ν max 3312, 1633, 1597, 1500, 1473, 1462, 1286, 1219, 1140, 1057, 870, 788 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 13.42 (1H, s, O*H*-2), 5.95 (1H, s, H-5), 4.70 (2H, d, J = 4.2 Hz, H_2-2′), 3.91 (3H, s, OMe-4), 3.90 (3H, s, OMe-6), 2.02 (3H, s, Me-3); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 201.83 (s, C-1′), 164.42 (s, C-4), 163.18 (s, C-2), 161.18 (s, C-6), 105.99 (s, C-3), 103.03 (s, C-1), 85.65 (d, C-5), 68.69 (t, C-2′), 55.55 (q, OMe-4), 55.55 (q, OMe-6), 7.18 (q, Me-3); EIMS m/z $[M]^+$ 226 (14), 195 (100); HREIMS m/z 226.0849 (calcd for $C_{11}H_{14}O_5$, 226.0841).

Compound **5** (2 mg) was acetylated in a mixture of pyridine−$(Ac)_2O$ (1:2) (1.5 ml) by overnight treatment at room temperature. The product of acetylation was allowed to crystallize in $CHCl_3$ to afford fine crystals of 2,2′-diacetoxy-4,6-dimethoxy-3-methylacetophenone (**7**) (1.8 mg): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.36 (1H, s, H-5), 5.07 (2H, br s, H_2-2′), 3.90 (3H, s, OMe-4), 3.89 (3H, s, OMe-6), 2.32 (3H, s, Ac), 2.19 (3H, s, Ac), 1.96 (3H, s, Me-3); EIMS m/z $[M]^+$ 310 (7), 268 (8), 237 (27), 195 (100), 180 (3), 149 (2), 95 (3), 69 (3), 57 (4), 43 (23).

***ent*-13*S*-Hydroxyatis-16-ene-3,14-dione**: white crystals ($CHCl_3$); mp 170 °C, $[\alpha]^{20}_D$ +36° (*c* 0.1 $CHCl_3$), lit.[10] mp 175−177 °C, $[\alpha]^{20}_D$ +44° (*c* 0.03, $CHCl_3$), which exhibited comparable spectral (UV, IR, ^{1}H NMR, ^{13}C NMR, EIMS) data to published values.[10,16]

2-Hydroxy-4,6-dimethoxyacetophenone (xanthoxylin): light yellow needles ($CHCl_3$); mp 78−80 °C, lit.[11] 72−74 °C, which exhibited comparable spectral (UV, IR, ^{1}H NMR, ^{13}C NMR, EIMS) data to published values.[11]

2-Hydroxy-4,6-dimethoxy-3-methylacetophenone: light yellow prisms ($CHCl_3$); mp 130−131 °C, lit.[13] 142−143 °C, which exhibited comparable spectral (UV, IR, ^{1}H NMR, ^{13}C NMR, EIMS) data to published values.[13]

6-Hydroxy-7-methoxycoumarin (isoscopoletin): white crystals (2% MeOH− $CHCl_3$); mp 208−210 °C, lit.[17] mp 208 °C, which exhibited comparable spectral (UV, IR, ^{1}H NMR, ^{13}C NMR, EIMS) data to published values.[17]

Lupeol acetate: white crystals ($CHCl_3$): mp 210−212 °C; $[\alpha]^{20}_D$ +20° (*c* 0.5 $CHCl_3$), lit.[18] mp 215−216 °C; $[\alpha]^{30}_D$ +46° (*c* 1.5 $CHCl_3$), which exhibited comparable spectral (UV, IR, ^{1}H NMR, ^{13}C NMR, EIMS) data to published values.[18,19]

β-Sitosterol: white crystals (hexane−EtOAc, 4:1; mp 144−145 °C, lit.[20,21] 144 °C; which exhibited comparable spectral (UV, IR, ^{1}H NMR, ^{13}C NMR, EIMS) data to published values.[20,21]

Sitosterol 3-*O*-β-D-glucopyranoside: white precipitate (MeOH) (52 mg; 0.00087% w/w): mp 263−265 °C (browning), 288−290° C (dec), lit.[22] 257−259 °C (browning), 280−283 °C (dec); $[\alpha]^{20}_D$ −33° (*c* 0.3, DMSO); lit.[22] $[\alpha]^{20}_D$ −42° (*c* 0.57, pyridine), which exhibited comparable spectral (UV, IR, ^{1}H NMR, ^{13}C NMR, and FABMS) data to published values.[22]

6,7,8-Trimethoxycoumarin: light brown crystals ($CHCl_3$−MeOH (49:1); mp 99−100 °C, lit.[23,24] 103−104 °C, which exhibited comparable spectral (UV, IR, ^{1}H NMR, ^{13}C NMR, EIMS) data to published values.[23,24]

3,3′,4′-Tri-*O*-methyl-4-*O*-[α-L-rhamnopyranosyl-(1‴→6″)-β-D-glucopyranosyl]ellagic acid: white yellow precipitate (EtOAc-MeOH (9:1): mp 275−277 °C; $[\alpha]^{20}_D$ −63° (*c* 0.75, DMSO), which exhibited comparable spectral (UV, IR, ^{1}H NMR, ^{13}C NMR, FABMS) data to published values.[25]

Biological Testing

Protein Kinase C (PKC)-binding (PDBu) Assay. The PDBu binding assay was performed in 96-well microtiter plates using calf brain homogenate as a source of soluble PKC.[2] Each reaction mixture (200 μL) contained calf brain homogenate (25 μg protein), 20 mM Tris−HCl buffer (pH 7.4), and 1 μL of [20−^{3}H]phorbol 12,13-dibutyrate (PDBu) (20 Ci $mmol^{-1}$, 20 nCi DuPont/NEN, Wilmington, DE), diluted with EtOH, final concentration 5 nM. Various concentrations of test substances (10 μL, dissolved in DMSO) were added to the reaction mixture, in duplicate, at half-log serial dilutions. Incubations were performed for 1 h at 37 °C with shaking. Unbound [^{3}H]PDBu was removed by filtration with 50 mM Tris−HCl buffer (pH 7.4) through glass fiber filter mats (Wallac Inc., Gaithersberg, MD) using

a Tomtec Mach III 96-well plate harvester (Tomtec, Orange, CT). Radioactivity was determined by scintillation counting with a Wallac 1450 MicroBeta liquid scintillation counter (Wallac Inc.). The amount of [^{3}H]-PDBu bound with the presence of nonradioactive PDBu (4 μM) was used to measure nonspecific binding. Specific binding was calculated from the difference between total and nonspecific binding, and results obtained in the presence of test compounds were expressed as a percentage relative to the DMSO control group. Finally, dose−response curves were used for the calculation of IC_{50} values.

Acknowledgment. This research was supported by Program Project P01 CA48112, funded by the National Cancer Institute, National Institute of Health, Bethesda, MD. We acknowledge the Nuclear Magnetic Resonance Laboratory of the Research Resources Center (RRC), University of Illinois at Chicago, for providing expert assistance and spectroscopic equipment used in this study. We thank Mr. E. Robbins of RRC for recording the EIMS and GCMS, and Mr. R. B. Dvorak, Dr. R. B. Van Breemen, and Dr. K. Zaw of the Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago, for recording FABMS, ESMS, and for providing assistance with the NMR experiments, respectively.

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NP960412E