Cytotoxic Compounds from the Stems of *Cinnamomum tenuifolium*

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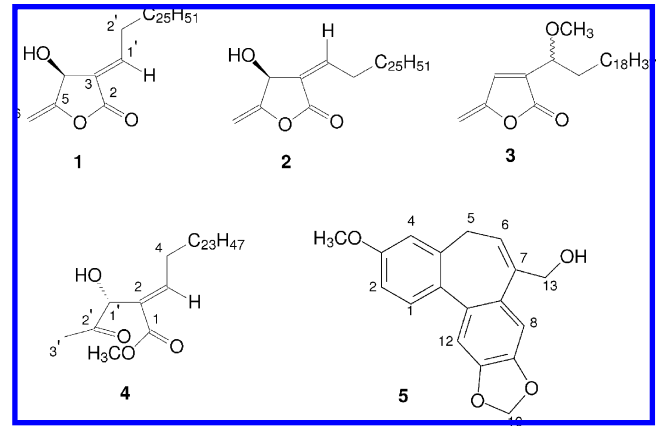
Three new butanolides, tenuifolide A (**1**), isotenuifolide A (**2**), and tenuifolide B (**3**), a new secobutanolide, secotenuifolide A (**4**), and one new sesquiterpenoid, tenuifolin (**5**), along with 16 known compounds were isolated from the stems of *Cinnamomum tenuifolium*. Their structures were determined by spectroscopic analyses. Compound **4** was found to induce apoptotic-related DNA damage, increase sub-G1 cells, and inhibit the growth of human prostate cancer cells, DU145. In addition, treatment with **4** significantly increased intracellular H_2O_2 and/or peroxide. The results show that **4** induced (a) noticeable reduction of mitochondrial transmembrane potential ($\Delta\Psi_m$); (b) significant increase in the ratio of cytochrome *c* concentration (cytosol/mitochondria); and (c) subsequent activation of caspase-9/caspase-3. Antiproliferation caused by **4** was found to markedly decrease when pretreated with caspase-9/caspase-3 inhibitor. In ROS scavenging, antioxidant, NADPH oxidase, and NO inhibitor studies, pretreatment of DU145 cells with either DPI, dexamethasone, L-NAME, or mannitol decreased **4**-induced intracellular DCF fluorescence of ROS. These results suggest that an increase of H_2O_2 and/or peroxide by **4** is the initial apoptotic event and **4** has anticancer effects on DU145 cells.

Cinnamomum tenuifolium Sugimoto form. nervosum (Meissn.) Hara. (Lauraceae) is a medium-sized evergreen tree endemic to the Lanyu Island of Taiwan, all plant parts being conspicuously free of cinnamon odor.[1] The chemical constituents and the biological activity of this plant have not yet been reported. In continuation of some studies of chemotaxonomy and biologically active metabolites from Formosan Lauraceous plants,[2−16] a methanol extraction of the stems of *C. tenuifolium* afforded three new butanolides, tenuifolide A [(4*S*,3*Z*)-4-hydroxy-5-methylene-3-heptacosylidenedihydrofuran-2-one] (**1**), isotenuifolide A [(4*S*,3*E*)-4-hydroxy-5-methylene-3- heptacosylidenedihydrofuran-2-one] (**2**), and tenuifolide B [3-(1-methoxyeicosyl)-5-methylene-5*H*-furan-2-one] (**3**), a new secobutanolide, secotenuifolide A {methyl[(2*E*)-2-[(1*R*)-1-hydroxy-2-oxopropyl]heptacos-2-enoate]} (**4**), one new sesquiterpenoid, tenuifolin (**5**), and 17 known compounds, including two butanolides, obtusilactone A[17] and isoobtusilactone A,[17] one sesquiterpenoid, alpinenone,[18] two flavan-3-ols, catechin[19] and *epi*-catechin,[20] two lignans, (+)-sesamin[21] and (+)-syringaresinol,[22] five benzenoids, 4-allylcatechol,[23] myristicin,[23] eugenol 4-*O*-methyl ether,[23] ferulic acid,[24] and *p*-hydroxybenzaldehyde,[25] three steroids, β-sitosterol,[26] β-sitostenone,[27] and β-sitosteryl-D-glucoside,[27] and two aliphatic compounds, palmitic acid[28] and stearic acid.[29] This study reports the structural elucidation of **1**−**5** and the apoptotic inducing capability of secotenuifolide A (**4**) on a human prostate cancer cell line, DU145.



Results and Discussion

Tenuifolide A (**1**) was isolated as a pale yellowish liquid. Its molecular formula, $C_{32}H_{58}O_3$, was established by HRFABMS. The UV absorption at 225 nm was similar to that of obtusilactone A,[17] suggesting the presence of a β-hydroxy-γ-methylene-α,β-unsaturated-γ-lactone unit.[30] The IR spectrum showed absorption bands of a hydroxy group at 3440 cm^{-1} and an α,β-unsaturated γ-lactone moiety at 1770 and 1670 cm^{-1}. The ^1H NMR spectrum of **1** was similar to that of obtusilactone A,[17] indicating that **1** has the same β-hydroxy-γ-methylene-α,β-unsaturated-γ-lactone skeleton and the same *Z* geometry of the trisubstituted double bond (see Table 1). The presence of a broad singlet δ 1.29 was attributed to protons in an aliphatic chain in **1**. The exocyclic olefinic protons appeared at δ 4.57 and 4.72, and one hydroxymethine proton was located at δ 5.16. Compound **1** is levorotatory, which indicates an *S*-configuration at C-4.[31] Thus, the structure of tenuifolide A is (4*S*,3*Z*)-4-hydroxy-5-methylene-3-heptacosylidenedihydrofuran-2-one, as represented in **1**.

Isotenuifolide A (**2**), a pale yellowish liquid, also had the molecular formula $C_{32}H_{58}O_3$ as deduced from HRFABMS. Its spectroscopic data (IR, UV, ^1H and ^{13}C NMR) were similar to those of **1**. The difference in chemical shift of H-1′ and its coupling constant supported an *E*-configuration for $\Delta^{3(1')}$ in **2**. The ^1H NMR spectrum of **2** was similar to that of isoobtusilactone A,[17] indicating that **2** has the same β-hydroxy-γ-methylene-α,β-unsaturated-γ-lactone skeleton and the same *E*-geometry of the trisubstituted double bond. A broad singlet at δ 1.28 was attributed to the 46 protons in the aliphatic chain in **2**. The exocyclic olefinic protons appeared at δ 4.72 and 4.95, and one hydroxymethine proton was

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Table 1. ^{1}H NMR Data of Butanolides **1** and **2** (400 MHz, δ in ppm, J in Hz, $CDCl_3$)

proton	tenuifolide A (**1**)	isotenuifolide A (**2**)
4	5.11 (1H, br s)	5.25 (1H, br s)
6a	4.67 (1H, dd, J = 2.8, 1.6)	4.72 (1H, dd, J = 2.8, 1.2)
6b	4.89 (1H, dd, J = 2.8, 1.6)	4.94 (1H, dd, J = 2.8, 1.2)
1′	6.68 (1H, td, J = 7.6, 2.0)	7.07 (1H, td, J = 7.6, 2.4)
2′	2.77 (2H, m)	2.45 (2H, m)
3′	1.48 (2H, m)	1.53 (2H, m)
4′−26′	1.25 (46H, br s)	1.25 (46H, br s)
27′	0.88 (3H, t, J = 7.2)	0.87 (3H, t, J = 6.8)

Table 2. ^{13}C NMR Data of Butanolides **1** and **2** (100 MHz, δ in ppm, $CDCl_3$)

proton	tenuifolide A (**1**)	isotenuifolide A (**2**)
2	165.5 (s)	165.4 (s)
3	126.7 (s)	127.3 (s)
4	68.8 (d)	66.6 (d)
5	157.5 (s)	157.7 (s)
6	90.4 (t)	91.5 (t)
1′	151.3 (d)	150.2 (d)
2′−24′	28.3−29.8 (t)	28.2−29.7 (t)
25′	31.9 (t)	32.0 (t)
26′	22.6 (t)	22.8 (t)
27′	14.0 (q)	14.1 (q)

located at δ 5.25. Compound **2** was levorotatory, which indicates that the configuration at C-4 is *S* by comparison with reported compounds.[32] Thus, the structure of isotenuifolide A is represented as **2** and elucidated as (4*S*,3*E*)-4-hydroxy-5-methylene-3-heptacosylidenedihydrofuran-2-one. The ^{1}H and ^{13}C NMR data of **2** were assigned by comparison with those of **1** (Tables 1 and 2).

Tenuifolide B (**3**) was isolated as a colorless oil. The molecular formula was determined as $C_{26}H_{46}O_3$ by EIMS ([M]$^+$, *m*/*z* 406) and HREIMS. The presence of an α,β-unsaturated γ-lactone moiety was apparent from a UV absorption at 265 nm.[33] The IR peaks at 1780 and 1680 cm^{-1} supported the presence of the α,β-unsaturated γ-lactone moiety. The ^{1}H NMR spectrum of **3** indicated the presence of an exomethylene group and another downfield alkene proton, which is located on the β-carbon of the unsaturated lactone from its chemical shift. In addition, it showed the signals corresponding to a methoxy functionality at δ 3.35, an oxymethine proton at δ 4.12, and long-chain aliphatic protons at δ 1.26 (32H, br s) and 1.42−1.68 (4H, m). The structure of **3** is similar to that of the known butanolide 3-(1-methoxyoctadecyl)-5-methylene-5*H*-furan-2-one.[30] Thus, the structure of **3** was elucidated as 3-(1-methoxyeicosyl)-5-methylene-5*H*-furan-2-one. The configuration at C-1′ remains undefined, since the specific rotation could not be correlated to known compounds.

Secotenuifolide A (**4**), a pale yellowish liquid, has the molecular formula $C_{31}H_{58}O_4$, as deduced from HRFABMS. The UV absorption at 210 nm is similar to that of secolincomolide A, indicating the presence of a secobutanolide skeleton.[34] The IR spectrum of **4** showed characteristic absorption bands due to the presence of hydroxy (3500 cm^{-1}), ester (1735 cm^{-1}), and ketone (1710 cm^{-1}) groups. The ^{1}H NMR spectrum of **4** was similar to that of secomahubanolide,[30] except for the *E*-geometry of the trisubstituted double bond [δ 7.06 (1H, t, J = 7.6 Hz, H-3)] in **4** instead of the *Z*-geometry [δ 6.34 (1H, t, J = 7.6 Hz, H-3)] in secomahubanolide.[30] Secomahubanolide has four additional methylene units compared to secotenuifolide A (**4**). An acetyl and one *O*-methyl group were observed at δ 2.13 and 3.71, respectively. Compound **4** showed a negative specific rotation {$[\alpha]^{25}_D$ −22.5 (*c* 0.05, $CHCl_3$)} indicating the 1′*R*-configuration, similar to that of secomahubanolide {$[\alpha]^{25}_D$ −11.2 (*c* 0.029, $CHCl_3$)},[30] but contrary to that of secoisolancifolide {$[\alpha]^{25}_D$ +102.7 (*c* 0.49, $CHCl_3$)}.[33] From the above data, compound **4** was established as methyl (2*E*)-2-[(1*R*)-1-hydroxy-2-oxopropyl]heptacos-2-enoate.

Tenuifolin (**5**) was isolated as a white, amorphous powder with a molecular formula of $C_{18}H_{16}O_4$, as determined by HREIMS (obsd [M]$^+$ at *m*/*z* 296.1048; calcd [M]$^+$ 296.1049). This formula agrees with deductions from the ^{1}H and ^{13}C NMR data and corresponds to 11 degrees of unsaturation. The UV spectrum contained absorption bands typical of 5*H*-dibenzo[*a*,*c*]cycloheptene derivatives.[35,36] IR absorption peaks at 920, 1070, and 3300 cm^{-1} indicated the presence of methylenedioxy and hydroxy functionalities, respectively.[36] The ^{1}H NMR resonances of **5** were well dispersed in $CDCl_3$ and displayed an ABX pattern (H-4 at δ 6.76, H-2 at 6.84, and H-1 at 7.38), singlets at δ 7.08 and 7.14 for H-12 and H-8, respectively, and the methylenedioxy protons at δ 6.02, accounting for seven protons. A three-proton singlet at δ 3.84 indicated the presence of the methoxy group. The C-6 olefinic proton (δ 6.16, tq, J = 8.0, 1.4 Hz) coupled with the C-5 and C-13 methylene protons, the latter four hydrogens resonating at δ 2.77 (dd, J = 12.8, 6.2 Hz, H-5a), 3.08 (dd, J = 12.8, 8.0 Hz, H-5b), 4.32 (d, J = 13.0 Hz, H-13a), and 4.49 (dt, J = 13.0, 1.4 Hz, H-13b), respectively. The ^{13}C NMR and DEPT spectra of **5** showed 18 resonances comprising one methyl, three methylene, six methine, and eight quaternary carbons. Structure **5** was also confirmed by 2D NMR experiments. A COSY correlation was observed between H-1 and H-2 and between H-5 and H-6. A triplet of quartets at δ 6.16 was assigned to H-6 and showed coupling to the nearby C-5 and C-13 methylene protons, which appeared at δ 2.77 and 3.08, and at δ 4.49, respectively. Thus, the structure of **5** was elucidated as (3-methoxy-5*H*-9,11-dioxabenzo[3,4]cyclohepta[1,2-*f*])inden-7-yl)methanol, which was further confirmed by NOESY and HMBC experiments.

The cytotoxicities of **1**−**5**, obtusilactone A, and alpinenone were tested against two human prostate cancer epithelial cell lines, DU145 and LNCaP. Compound **4** showed antiproliferative activity against both lines, with EC_{50} values of less than 7 μM (equal to 3.45 μg/mL), close to the value of EC_{50} < 4 μg/mL, as established for the potent cytotoxic agent prepared by Geran et al.[37] Compounds **2** and **3** had moderate activities in this assay. Compound **1** showed only weak antiproliferative activities, while compound **5** was weakly active only against DU145.

Our previous study reported that isokotomolide A, an isomer of kotomolide A (the alkylidene side chain of isokotomolide A is *trans* to the carbonyl group, whereas it is *cis* in kotomolide A), causes cell cycle arrest and induces apoptosis through a p53/p21 manner in A549 cells. The bioactivities of pure chemical compounds are affected by changes in the geometric structure of the compound.[4,6] Our previous study reported that secokotomolide A-induced DNA damage was found to markedly decrease when HeLa cells were pretreated with an intracellular glutathione supplement. These results suggest that an increase of H_2O_2 and/or peroxide by secokotomolide A is the initial apoptotic event. The intracellular GSH depletion is a critical event in secokotomolide A-induced apoptosis in HeLa cells.[12]

This study examined the effects of **4** on antiproliferation and cytotoxicity in DU145 cells. In the first series of experiments, an XTT test was used to study the ability of **4** to alter antiproliferation of a human prostate cancer cell line, DU145. As shown in Figure 3A, the proliferative inhibitory effects of **4** were observed as dose- and time-dependent. The maximum proliferative inhibition of 100 μM **4** occurred at 72 h. Similar results were obtained when we evaluated the cytotoxic effect of **4** by the TB dye exclusion assay (Figure 3B). In DU145 cells, the IC_{50} values (μM) were, on average, lower than those observed by the XTT assay IC_{50} (μM).

The DNA content of **4**-treated DU145 cells was determined by staining with propidium iodide (PI) and flow cytometry. The DNA histograms and the percentages of cells in each phase of the cell cycle are shown in Figure 4A, as compared with vehicle (1% DMSO in sterilized distilled H_2O). The DU145 cells on treatment with 1, 5, 10, and 100 μM of **4** for 24 h gave a dose-responsive

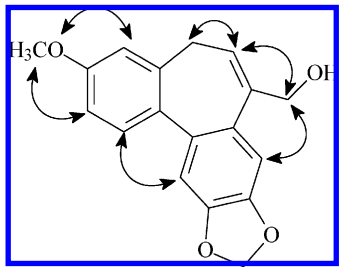

Figure 1. NOESY correlations of **5**.

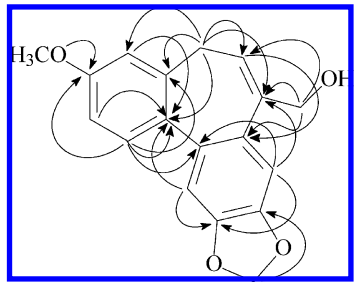

Figure 2. HMBC correlations of **5**.

increase in the sub-G1 population, extending from 3.22 to 6.56, 19.02, 19.72, and 24.88%, respectively. In addition, this effect was enhanced when DU145 cells were treated with **4** for 72 h (Figure 4A). Disorganization of the nucleus with chromatin changes induced by **4** in DU145 cells was characterized using Hoechst 33342 staining. The DU145 cells were exposed to 1 and 100 μM **4** for 24 h. Compared to cells cultured in the vehicle (1% DMSO), most of which contained intact genomic DNA, cells cultured with **4** had numerous cells with condensed chromatin, nuclear fragmentation, and apoptotic body formation (Figure 4B). Granulation of the nucleus appeared as fluorescent blue in the detail of the Hoechst 33342 stained cells (those from which vehicle was absent). Since nucleus granulation is a feature of the morphological change in apoptosis, these results suggest that **4** may induce apoptosis. Cells that exhibited reduced nuclear size, chromatin condensation, and nuclear fragmentation are considered apoptotic.[38]

A decrease in mitochondrial transmembrane potential ($\Delta\Psi_m$) has been reported as an early event in apoptosis and the generation of ROS and may contribute to mitochondrial damage, leading to cell death, by acting as an apoptotic signaling molecule.[39] Hence, this study evaluated the effect of **4** on changes in $\Delta\Psi_m$, and the release of cytochrome *c*. As shown in Figure 5A, **4** induced mitochondrial transmembrane depolarization, represented as the decrease of mitochondrial membrane potential, which was represented by a fall of fluorescent intensity of rhodamine 123 staining. In addition, **4** induced a significant increase in the ratio of cytochrome *c* concentration (concentration of cyt *c* in cytosol/concentration of cyt *c* in mitochondria) in DU145 cells (Figure 5B). These data suggest that loss of mitochondrial membrane potential may be required for a **4**-induced release of cytochrome *c* into cytosol, which would later trigger the cleavage and activation of mitochondrial downstream caspases and the onset of apoptosis.

This study further examined the effect of **4**-induced apoptosis through caspase-9 and caspase-3 activity and poly-(ADP-ribose) polymerase (PARP) cleavage. Caspases, a group of cysteine proteases, have been demonstrated to play a pivotal role in the induction of apoptosis.[40] To detect the caspase-9 and caspase-3 activities, this study explored whether **4**-induced apoptosis of DU145 cells may be the result of a caspase-dependent mechanism. As shown in Figure 6A, caspase-9 activity was obviously maximized at 24 h and significantly activated in a time-dependent manner. In addition, when the cells were treated with 10 μM **4**, caspase-3 activity was significantly activated (Figure 6B). In

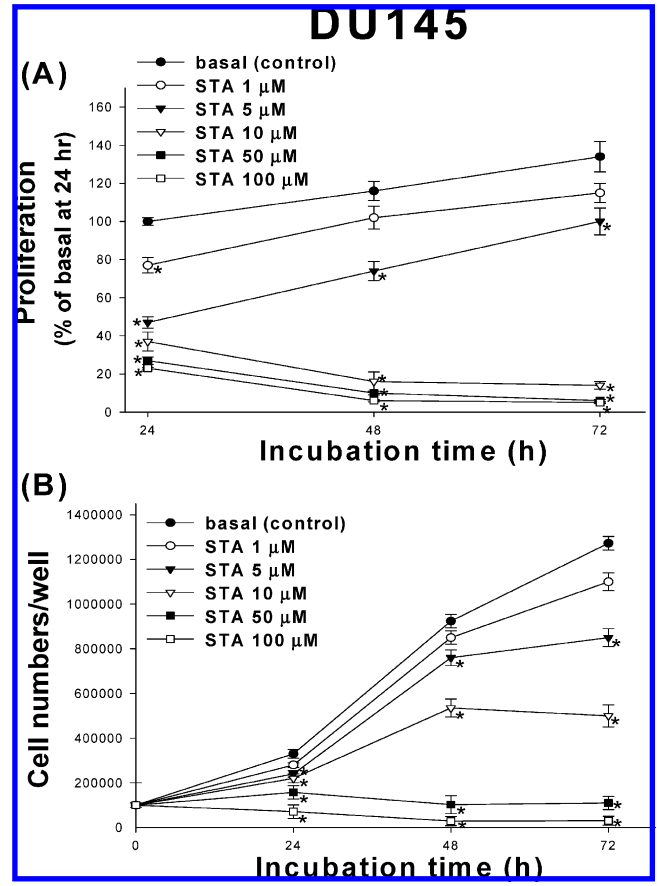

Figure 3. Antiproliferative and cytotoxic effect of secotenuifolide A (**4**) (STA) on DU145 cells. (A) Cell proliferation was determined by XTT assay after incubation for various concentrations of secotenuifolide A (**4**) at 24, 48, and 72 h, respectively. Results are expressed as the percentage of cell proliferation of basal group at 24 h. (B) Cell viability under different concentrations of secotenuifolide A (**4**) for 24, 48, and 72 h, respectively. Cell numbers were calculated using a hemocytometer. Each value represents the mean ± SE. Statistically significant, $*p < 0.05$ compared to basal group. ANOVA followed by Dunnett's test.

addition **4** (100 μM) significantly induced PARP (116 kDa), known as an endogenous substrate for caspase-3 and a marker for apoptosis, which is cleaved to an 85 kDa C-terminal fragment in 24 h (data not shown), similar to caspase-3 activation. Therefore, this study further investigated the effects of a caspase inhibitor, such as caspase-9 inhibitor (Ac-LEHD-CMK)[41] and caspase-3 inhibitor (Ac- DEVD-CMK),[42] on **4**-induced antiproliferation in DU145 cells (Figure 6C). Antiproliferation was observed in **4**-treated cells, but not those treated with Ac-DEVD-CMK or Ac-LEHD-CMK alone. On the other hand, **4**-induced antiproliferation was significantly inhibited by pretreatment with the 50 μM Ac-DEVD-CMK and Ac-LEHD-CMK, respectively. In summary, the **4**-induced DU145 cells released cytochrome *c* to the cytosol, which subsequently mediated caspase-9 and caspase-3 activation and cell apoptosis. The cleavage of PARP, a 116 kDa nuclear enzyme downstream of caspase-3, produces an 85 kDa fragment, and the decrease of **4**-induced antiproliferation by pretreatment with caspase-9 and caspase-3 inhibitors provides further evidence that treatment of DU145 cells with **4** induces activation of caspase-9 and caspase-3.

Many drugs induce apoptosis through an increased intracellular ROS.[43,44] This study further examined whether **4** affected the intracellular ROS, including superoxide, H_2O_2, and peroxide of DU145 cells. Thus, the production of intracellular H_2O_2 and/or peroxide was determined at 6, 12, 18, and 24 h after the addition of **4** by spectrofluorimetry and DCFH-DA staining.[3] The intracel-

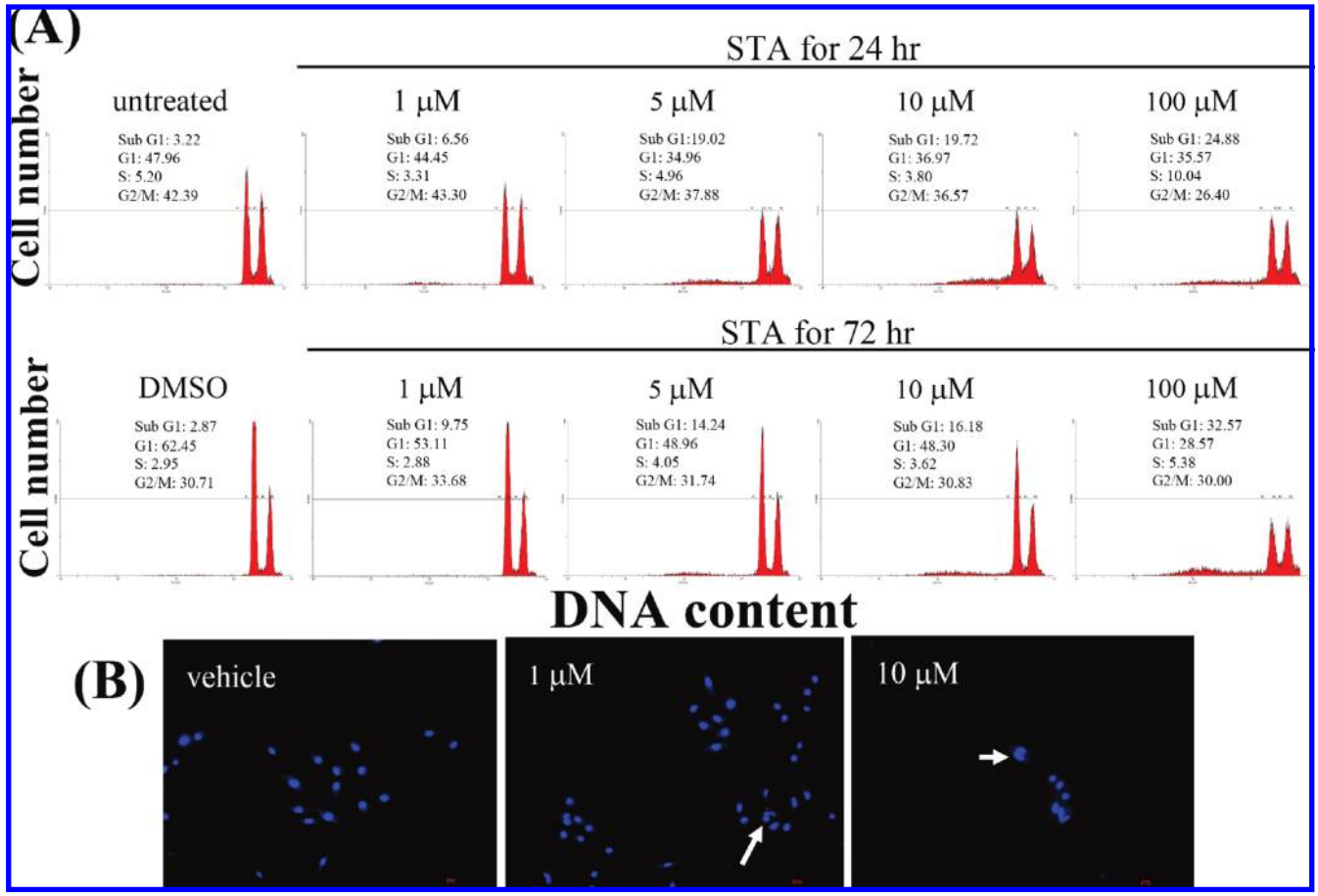

Figure 4. Effect of secotenuifolide A (**4**) (STA) on cell cycle of DU145 cells. (A) DU145 cells were treated with STA (1, 5, 10, and 100 μM), for 24 and 72 h, respectively. After treatment, cells were collected, fixed with methanol, stained with propidium iodide, and analyzed by flow cytometry. Data on each sample represent the percentage of cells in the G1, S, G2/M, and sub-G1 phases of the cell cycle, respectively. These experiments were performed at least three times. A representative experiment is presented. (B) Induction of apoptosis in DU145 by STA. The morphology of DU145 cells was assayed by Hoechst 33342 stain. The cells were incubated in the absence or presence of 1 and 100 μM STA, for 24 h (vehicle: 1% DMSO).

lular DCF fluorescence significantly increased, as compared with basal DU145 cells, in 1, 10, and 50 μM **4** at 6, 12, 18, and 24 h, respectively (Figure 7A). In order to determine the chief component of ROS generation and the oxidative status of antioxidants and NO inhibitor on treatment with **4**, we evaluated the effect of several ROS scavengers (catalase and mannitol),[45] antioxidants (*N*-acetylcysteine (NAC) and trolox),[46] an NADPH oxidase (PHOX) inhibitor (DPI),[47,48] and NO inhibitors (dexamethasone and L-NAME)[49] on **4**-induced intracellular DCF fluorescence in DU145 cells. As shown in Figure 7B, DU145 cells treated with 50 μM secotenuifolide A had an approximately 2.5-fold increase in DCF fluorescence intensity after 24 h treatment, compared to the untreated cells. DPI, dexamethasone, L-NAME, and mannitol significantly decrease intracellular DCF fluorescence in DU145 cells treated with **4**. However, pretreatment with NAC and trolox did not decrease intracellular DCF fluorescence in the treated DU145 cells. To characterize **4**-induced intracellular DCF fluorescence in DU145 cells, immunohistochemical analysis was performed to confirm that **4** induced apoptosis through increased intracellular ROS levels. As shown in Figure 7C, the vehicle cells showed no fluorescent staining, while the cells treated with 10 μM **4** showed strong fluorescent staining, indicating the presence of significant intracellular ROS expression.

The results facilitate several important conclusions. The therapeutic potential of **4** has been illustrated for DU145 cells because **4** has antiproliferative and apoptotic properties in DU145 cells. Secotenuifolide A can disrupt the function of mitochondria and subsequently activate caspase-9 and caspase-3 through the release of cytochrome *c*. STA-induced cell antiproliferation and apoptotic effects in DU145 cells are mediated by the production of ROS. These findings describe a basic mechanism whereby **4** has promise for the treatment of prostate cancer, and further investigation along these lines should be given high priority.

Experimental Section

General Experimental Procedures. Melting points were determined using a Yanagimoto micromelting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. UV spectra were obtained in MeCN, using a JASCO V-530 spectrophotometer. The IR spectra were measured on a Hitachi 260-30 spectrophotometer. ^{1}H (400 MHz, using $CDCl_3$ as solvent), ^{13}C (100 MHz), DEPT, HETCOR, COSY, NOESY, and HMBC NMR spectra were obtained on a Unity Plus Varian spectrometer. LRFABMS and LREIMS spectra were obtained with a JEOL JMS-SX/SX 102A mass spectrometer or a Quattro GC-MS spectrometer with a direct inlet system. HRFABMS and HREIMS were measured on a JEOL JMS-HX 110 mass spectrometer. Silica gel 60 (Merck, 230−400 mesh) was used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F-254, 0.20 mm) were used for analytical TLC, and precoated silica gel plates (Merck, Kieselgel 60 F-254, 0.50 mm) were used for preparative TLC. Spots were detected by spraying with 50% H_2SO_4 and then heating on a hot plate. Flow cytometry analysis was done by a Coulter Epics XL-MCL (Beckman Coulter, Fullerton, CA) flow cytometer. Labeling dyes, such as propidium iodide (PI), were used to investigate the events involved in apoptosis.

Plant Material. The stems of *C. tenuifolium* were collected from Lanyu Island, Taiwan, in March 2007. Plant material was identified by Dr. Fu-Yuan Lu (Department of Forestry and Natural Resources

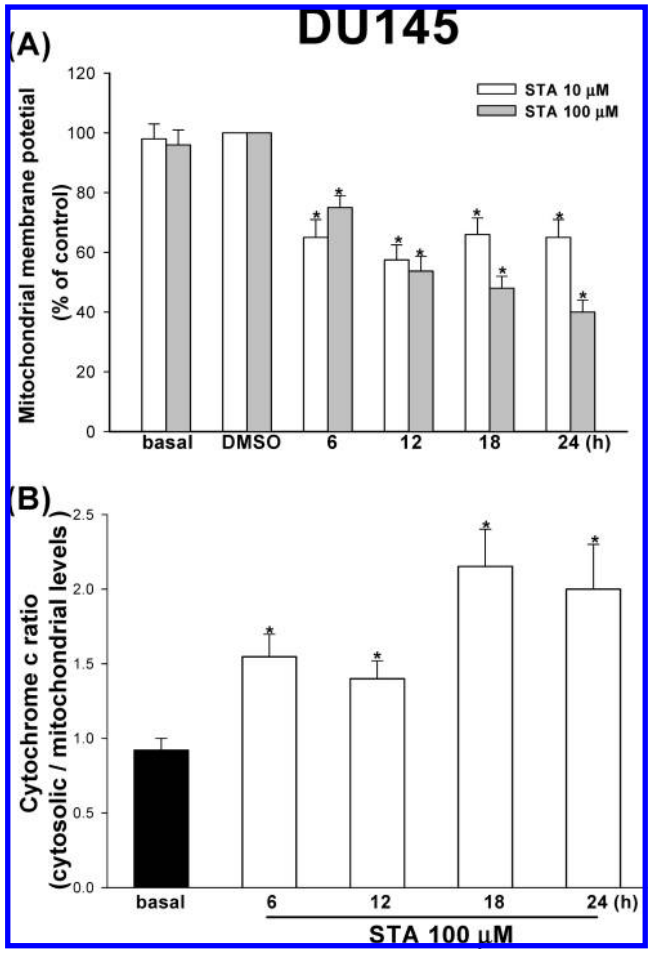

Figure 5. Effect of secotenuifolide A (**4**) (STA) on $\Delta\Psi_m$ reduction and cytochrome *c* release of DU145 cells. (A) Cells exposed to 10 and 100 μM STA, or not, for 6, 12, 18, and 24 h were incubated with rhodamine 123. Then, the fluorescence intensity was measured. (B) Level of cytochrome *c* protein ratio (concentration of cyt *c* in cytosol/concentration of cyt *c* in mitochondria) in DU145 cells. DU145 cells were treated with 100 μM STA. Lysates were prepared from these cells, and cytochrome *c* levels were determined by a cytochrome *c* ELISA kit. Each value represents the mean ± SE of three individual experiments. Statistically significant, $^*p < 0.05$ compared to basal group. ANOVA followed by Dunnett's test.

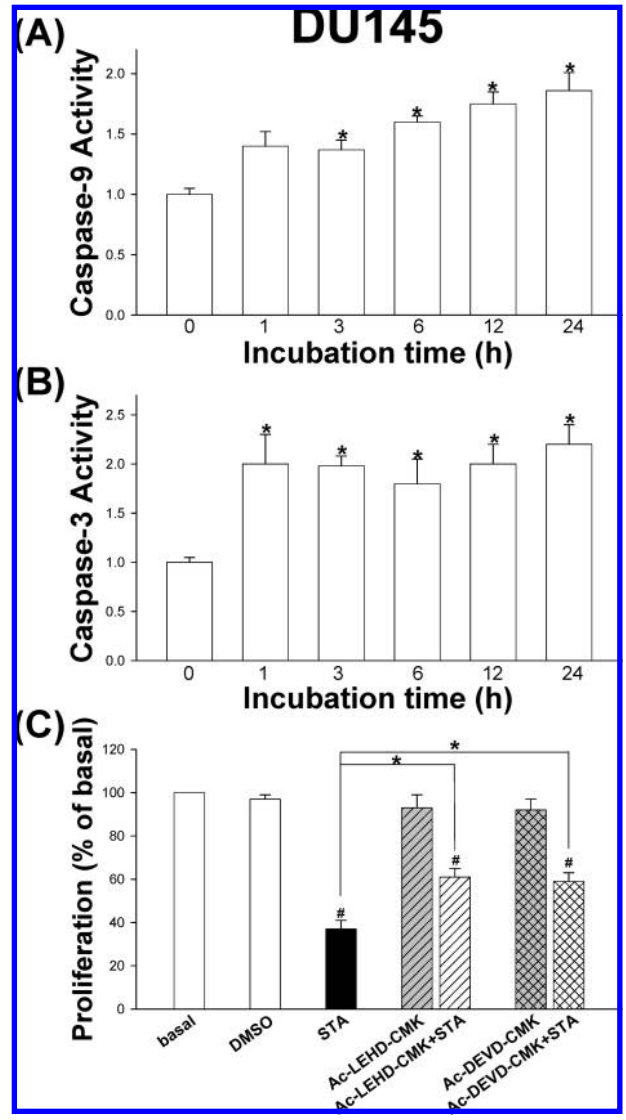

Figure 6. Effect of 10 μM secotenuifolide A (**4**) (STA) on time-dependent activation of caspase-9 and caspase-3 of DU145 cells. Enzymatic activity assay of caspase-9 (A) and caspase-3 (B) was determined by incubation, with a colorimetric peptide substrate, Ac-LEHD-pNA, and a fluorogenic peptide substrate, Ac-DEVD-pNA, respectively, as described in the Experimental Section. (C) In STA-induced antiproliferation, DU145 cells were pretreated with 50 μM caspase-3 inhibitor (Ac-DEVD-CMK) and 50 μM casapse-9 inhibitor (Ac-LEHD-CMK) prior to STA for 60 min, followed by 10 μM STA treatment for 24 h. Cell survival was determined by XTT assay (1% DMSO: vehicle). Each value represents the mean ± SE of three individual experiments, using the 0 h group as control. Statistically significant, $^*p < 0.05$ compared to 0 h group (parts A and B), and $^*p < 0.05$ compared to only STA group (part C); $^{\#}p < 0.05$ compared to basal group. ANOVA followed by Dunnett's test.

College of Agriculture, National Chiayi University). A voucher specimen (Cinnamo. 6) was deposited at the School of Medical and Health Sciences, Fooyin University, Kaohsiung County, Taiwan.

Extraction and Isolation. The air-dried stems of *C. tenuifolium* (4.2 kg) were extracted with MeOH (80 L × 6) at room temperature. The MeOH extract (84.9 g) was obtained by concentration under reduced pressure. The MeOH extract, suspended in H_2O (1 L), was partitioned with $CHCl_3$ (2 L × 5) to give fractions soluble in $CHCl_3$ (52.4 g) and H_2O (11.3 g). The $CHCl_3$-soluble fraction (52.4 g) was chromatographed over silica gel (800 g, 70−230 mesh) using *n*-hexane−EtOAc−acetone as eluent to produce five fractions. Part of fraction 1 (8.56 g) was subjected to Si gel chromatography by eluting with *n*-hexane−EtOAc (40:1), then enriched with EtOAc to furnish 10 fractions (1-1−1-10). Fraction 1-1 (4.65 g) was resubjected to Si gel chromatography, eluted with *n*-hexane−EtOAc (50:1) and gradually enriched with EtOAc, to obtain five fractions (1-1-1−1-1-5). Fraction 1-1-1 (0.17 g) was further purified by silica gel CC using *n*-hexane−EtOAc to obtain stearic acid (11 mg) and palmitic acid (12 mg). Fraction 1-1-2 (0.71 g) was further purified by silica gel CC using *n*-hexane−EtOAc to obtain tenuifolide B (**3**) (9 mg) and secotenuifolide A (**4**) (26 mg). Fractions 1-1-3 (0.15 g) and 1-1-4 (0.21 g) were resubjected to Si gel CC and purified by preparative TLC to yield 4-allylcatechol (34 mg), myristicin (18 mg), and eugenol 4-*O*-methyl ether (9 mg). Fraction 1-2 (0.64 g) was further separated using silica gel CC (*n*-hexane−EtOAc (50:1) and preparative TLC (*n*-hexane−EtOAc (100:1), giving tenuifolide A (**1**) (21 mg) and isotenuifolide A (**2**) (42 mg). Fraction 1-4 (2.88 g) was resubjected to Si gel chromatography, eluted with *n*-hexane−EtOAc (40:1), and gradually enriched with EtOAc to obtain four fractions (1-4-1−1-4-4). Fraction 1-4-2 (1.53 g) was further separated using silica gel CC (*n*-hexane−EtOAc (40:1)) and preparative TLC (*n*-hexane−EtOAc (30:1)) and gave isoobtusilactone A (52 mg) and obtusilactone A (12 mg). A part of fraction 2 (5.11 g) was subjected to Si gel chromatography by eluting with *n*-hexane−EtOAc (30:1), then enriched with EtOAc to furnish five fractions (2-1−2-5). Fraction 2-1 (1.29 g) was resubjected to Si gel chromatography, eluted with $CHCl_3$−MeOH (100:1), and gradually

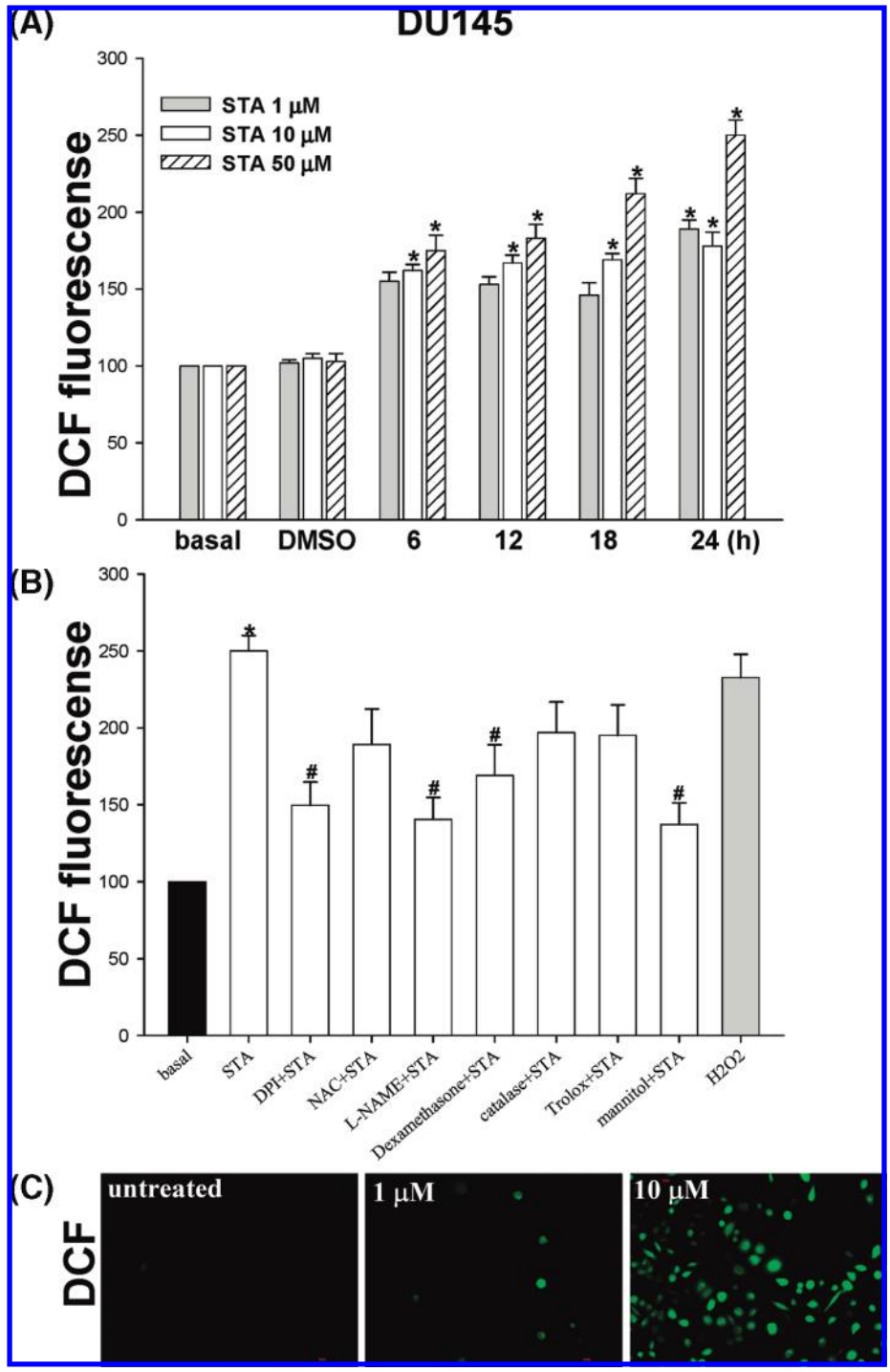

Figure 7. Effect of secotenuifolide A (**4**) (STA) on intracellular H_2O_2 and/or peroxide of DU145 cells. (A) Intracellular DCF fluorescence significantly increased, as compared with basal DU145 cells in 1, 10, and 50 μM STA, at 6, 12, 18, and 24 h, respectively. (B) DPI (1 μM), catalase (200 U/mL), mannitol (50 mM), dexamethasone (10 μM), NAC (1 mM), trolox (50 μM), and L-NAME (100 μM) were used to treat cells 60 min before treatment with STA and treated with 50 μM STA for 24 h. (C) For immunohistochemical analysis, this study used treatment with STA (1 and 100 μM) for 24 h, respectively, and the cells were fixed with 3.7% paraformaldehyde, in 1× PBS (pH 7.4), at 4 °C for 10 min, washed twice in PBS, and then incubated with DCFH-DA (10 μM) in the dark at 37 °C for 30 min. The expression of intracellular H_2O_2 and/or peroxide (ROS) was detected by fluorescent microscope. The datum in each panel represents the DCF fluorescence intensity within the cells. Each value represents the mean ± SE of 5 individual experiments. Statistically significant, $^*p < 0.05$ compared to basal group (control), and $^{\#}p < 0.05$ compared to STA group only. ANOVA followed by Dunnett's test.

enriched with MeOH, to obtain 10 fractions (2-1-1−2-1-10). Fractions 2-1-1 (0.12 g) and 2-1-2 (0.28 g) were resubjected to Si gel CC and purified by preparative TLC to yield ferulic acid (5 mg) and *p*-hydroxybenzaldehyde (13 mg). Fraction 2-2 (1.36 g) was resubjected to Si gel chromatography, eluted with *n*-hexane−EtOAc (40:1), and gradually enriched with EtOAc, to obtain four fractions (2-2-1−2-2-4). Fraction 2-2-2 (0.79 g) was further separated using silica gel CC (*n*-hexane−EtOAc (20:1)) and preparative TLC (*n*-hexane−EtOAc (40:1)), giving tenuifolin (**5**) (16 mg) and alpinenone (27 mg). Fraction 2-5 (1.11 g) was further purified on a silica gel column (300 g, 230−400 mesh) using $CHCl_3$−MeOH to obtain β-sitosterol (93 mg), and β-sitostenone (245 mg). Part of fraction 3 (7.11 g) was subjected to Si gel chromatography by eluting with *n*-hexane−EtOAc (1:1), then enriched with EtOAc, to furnish five fractions (3-1−3-5). Fraction 3-1

(2.78 g) was further purified by another silica gel column, using a $CHCl_3$−MeOH system, to obtain *epi*-catechin (23 mg). Fraction 3-2 (1.75 g) was further purified on a silica gel column, using $CHCl_3$−MeOH, to obtain (+)-sesamin (8 mg) and (+)-syringaresinol (20 mg). Fraction 3-3 (0.78 g) was purified on a silica gel column, using $CHCl_3$−MeOH, to obtain catechin (5 mg). Part of fraction 4 (13.54 g) was subjected to Si gel chromatography by eluting with *n*-hexane−EtOAc (1:3), then enriched with EtOAc, to furnish four fractions (4-1−4-4). Fraction 4-3 (2.41 g) was further purified on a silica gel column (300 g, 230−400 mesh), using $CHCl_3$−MeOH, to obtain β-sitosteryl-D-glucoside (45 mg). Known compounds were characterized by comparison of their spectroscopic data with literature values.[17−29]

Tenuifolide A [(4*S*,3*Z*)-4-hydroxy-5-methylene-3-heptacosylidenedihydrofuran-2-one] (1): pale yellowish liquid; $[\alpha]^{25}_D$ −32.1 (*c* 0.05, $CHCl_3$); UV λ_{max} (MeCN, log ε) 225 (4.10) nm; IR (neat) ν_{max} 3440 (br, OH), 1770, 1670 (α,β-unsaturated γ-lactone), 1465, 1365, 1090 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; FABMS *m*/*z* 491 $[M + H]^+$ (7), 211 (15), 167 (6), 149 (13), 139 (10), 125 (35), 111 (100), 97 (68), 83 (80), 69 (92); HRFABMS *m*/*z* 491.4471 $[M + H]^+$ (calcd for $C_{32}H_{59}O_3$, 491.4464).

Isotenuifolide A [(4*S*,3*E*)-4-hydroxy-5-methylene-3-heptacosylidenedihydrofuran-2 -one] (2): pale yellowish liquid; $[\alpha]^{25}_D$ −25.2 (*c* 0.05, $CHCl_3$); UV λ_{max} (MeCN, log ε) 225 (4.13) nm; IR (neat) ν_{max} 3440 (br, OH), 1770, 1670 (α,β-unsaturated γ-lactone), 1465, 1270, 1025 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; FABMS *m*/*z* 491 $[M + H]^+$ (6), 211 (14), 167 (3), 149 (16), 139 (11), 125 (33), 111 (100), 97 (70), 83 (81), 69 (91); HRFABMS *m*/*z* 491.4468 $[M + H]^+$ (calcd for $C_{32}H_{59}O_3$, 491.4464).

Tenuifolide B [3-(1-methoxyeicosyl)-5-methylene-5*H*-furan-2-one] (3): colorless oil; $[\alpha]^{25}_D$ −28.9 (*c* 0.05, $CHCl_3$); UV λ_{max} (MeCN, log ε) 265 (4.05) nm; IR (neat) ν_{max} 3455 (br, OH), 1780, 1680 (α,β-unsaturated γ-lactone), 1290 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.87 (3H, t, J = 6.8 Hz, H-20′), 1.26 (32H, br s, H-4′−19′), 1.43−1.68 (4H, m, H-2′, 3′), 3.35 (3H, s, OMe-1′), 4.12 (1H, dd, J = 7.4, 4.8 Hz, H-1′), 4.88, 5.20 (each 1H, d, J = 2.6 Hz, H-6a,b), 7.22 (1H, br s, H-4); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.2 (C-20′), 22.6 (C-19′), 25.5 (C-3′), 29.1−30.3 (C-4′−17′), 31.6 (C-18′), 35.1 (C-2′), 57.5 (OMe-1′), 77.4 (C-1′), 98.0 (C-6), 137.7 (C-4), 137.7 (C-3), 155.0 (C-5), 169.8 (C-2); EIMS *m*/*z* 406 $[M]^+$ (1), 280 (10), 267 (3), 179 (7), 165 (14), 149 (16), 142 (100), 123 (27), 111 (31), 97 (45), 83 (33), 69 (71), 55 (90); HREIMS *m*/*z* 406.3449 $[M]^+$ (calcd for $C_{26}H_{46}O_3$, 406.3447).

Secotenuifolide A {methyl[(2*E*)-2-[(1*R*)-1-hydroxy-2-oxopropyl]heptacos-2-enoate]} (4): pale yellowish liquid; $[\alpha]^{25}_D$ −22.5 (*c* 0.05, $CHCl_3$); UV λ_{max} (MeCN, log ε) 210 (3.80) nm; IR (neat) ν_{max} 3500 (br, OH), 1735 (ester), 1710 (ketone) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.88 (3H, t, J = 6.8 Hz, H-27), 1.28 (42H, br s, H-6−26), 1.47 (2H, m, H-5), 2.13 (3H, s, H-3′), 2.33 (2H, q, J = 7.4 Hz, H-4), 3.71 (3H, s, OMe-1), 4.88 (1H, br d, J = 3.2 Hz, H-1′), 7.06 (1H, t, J = 7.6 Hz, H-3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.1 (C-27), 22.5 (C-26), 24.5 (C-3′), 28.6 (C-4), 28.8 (C-5), 29.0−30.0 (C-6−24), 31.9 (C-25), 52.0 (OMe-1), 74.1 (C-1′), 129.2 (C-2), 149.5 (C-3), 166.1 (C-1), 206.2 (C-2′); FABMS *m*/*z* 495 $[M + H]^+$ (1), 323 (1), 309 (5), 297 (70), 265 (77), 247 (8), 237 (10), 219 (18), 191 (10), 167 (14), 155 (16), 149 (24), 125 (43), 115 (61), 97 (80), 83 (92), 69 (90), 55 (100); HRFABMS *m*/*z* 495.4418 $[M + H]^+$ (calcd for $C_{31}H_{59}O_4$, 495.4413).

Tenuifolin {(3-methoxy-5*H*-9,11-dioxabenzo[3,4]cyclohepta[1,2-*f*])inden-7-yl) methanol} (5): white, amorphous powder; $[\alpha]^{25}_D$ 0.0 (*c* 0.01, $CHCl_3$); UV λ_{max} (MeCN, log ε) 235 (3.23), 255 (2.65), 290 (2.11) nm; IR (neat) ν_{max} 3300 (br, OH), 3000, 1700, 1250, 1070, 920 (methylenedioxy) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 2.77 (1H, dd, J = 12.8, 6.2 Hz, H-5a), 3.08 (1H, dd, J = 12.8, 8.0 Hz, H-5b), 3.84 (3H, s, OMe-3), 4.32 (1H, d, J = 13.0 Hz, H-13a), 4.49 (1H, dt, J = 13.0, 1.4 Hz, H-13b), 6.02 (each 1H, d, J = 1.6 Hz, H-10), 6.16 (1H, tq, J = 8.0, 1.4 Hz, H-6), 6.76 (1H, d, J = 2.8 Hz, H-4), 6.84 (1H, dd, J = 8.4, 2.8 Hz, H-2), 7.08 (1H, s, H-12), 7.14 (1H, s, H-8), 7.38 (1H, d, J = 8.4 Hz, H-1); ^{13}C NMR (100 MHz, $CDCl_3$) δ 33.1 (C-5), 55.3 (OMe-3), 66.2 (C-13), 101.2 (C-10), 106.0 (C-8), 109.6 (C-12), 111.5 (C-4), 111.8 (C-2), 127.7 (C-6), 129.7 (C-7a), 130.6 (C-1), 131.0 (C-12b), 134.7 (C-12a), 137.4 (C-7), 143.6 (C-4a), 146.5 (C-8a), 146.5 (C-11a), 159.2 (C-3); EIMS *m*/*z* 296 $[M]^+$ (15), 285 (5), 279 (8), 265 (24), 255 (4), 239 (20), 236 (6), 222 (3), 192 (5), 185 (8), 167 (8), 154 (10), 149 (14), 137 (10), 134 (37), 129 (25), 116 (18), 111 (52), 109 (41), 97 (100), 83 (86), 69 (90); HREIMS *m*/*z* 296.1048 $[M]^+$ (calcd for $C_{18}H_{16}O_4$, 296.1049).

Cell Culture. Two human prostate cancer cell lines, DU145 and LNCaP (American Type Culture Collection, ATCC), were routinely grown at 37 °C and 5% CO_2 in RPMI-1640 medium, supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B.

Drugs and Chemicals. 2,3-Bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5- carboxyanilide (XTT), DMSO, ribonuclease (RNase), propidium iodide (PI), *N*-acetyl-L-cysteine (NAC), mannitol, dexamethasone, catalase, diphenyleneiodonium chloride (DPI), and *N*-nitro-L-arginine methyl ester (L-NAME) were obtained from the Sigma-Aldrich Chemical Co. (St. Louis, MO). RPMI-1640, fetal bovine serum (FBS), penicillin G, streptomycin, amphotericin B, trypan blue (TB), and all other cell culture reagents were obtained from GIBCO BRL Life Technologies (Invitrogen, Carlsbad, CA). 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR). Caspase-9 substrate (Ac-LEHD-pNA), caspase-3 substrate (Ac-DEVD-pNA), Ac-DEVD-CMK, and Ac-LEHD-CMK were purchased from Calbiochem (San Diego, CA). Cytochrome *c* ELISA kits were purchased from Assay Designs (Ann Arbor, MI). All drugs and reagents were dissolved in distilled H_2O, unless otherwise noted. Secotenuifolide A (**4**) (STA) was dissolved in DMSO at 1 M stock and serially diluted with distilled H_2O and vehicle (containing 1% DMSO in sterilized distilled H_2O).

Cell Proliferation Assay. The XTT assay was used to measure the proliferation response. In order to measure the proliferation response, the (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxyanilide inner salt (XTT) assay was used. Tetrazolium salt, XTT, is especially useful in quantifying viable cells. This assay is designed for the spectrophotometric quantification of cell growth and viability, without the use of radioactive isotopes, and is based on the cleavage of yellow tetrazolium salt, XTT, to form an orange formazan dye by metabolically active cells. XTT cleavage into an orange formazan dye by the mitochondrial enzyme dehydrogenase occurs exclusively in living cells. Cells were grown in growth medium, plus 10% FBS in 96-well plates (until 70−80% confluence), then treated with the various concentrations of test compounds (0.1−100 μM), and incubated for 24, 48, and 72 h, respectively. An XTT assay was performed at the end of incubation. Briefly, 50 μL of XTT labeling mixture solution was added to each well, and the cells were incubated at 37 °C for 4 h. The formazan dye formed is soluble in aqueous solutions, and the optical density at 450 nm was compared with that of control wells with a screening multiwell spectrophotometer enzyme-linked immunosorbent assay (ELISA) reader. The reference wavelength was 650 nm. Taxol and cisplatin were used as positive controls, and IC_{50} values were calculated from the mean of data from the eight wells.

Trypan Blue Dye Exclusion Assay. DU145 cells (1×10^5) were seeded onto a 60 mm culture dish with RPMI-1640 medium plus 10% FBS. The next day, cells were treated with 1−100 μM **4** for 24, 48, and 72 h, respectively. The treated cells were then harvested, washed twice with phosphate-buffered saline (PBS), concentrated to 150 μL, and transferred to a 20 μL cell suspension stained with 20 μL of trypan blue. This method is based on the principle that live (viable) cells do not take up certain dyes, whereas dead (nonviable) cells do. The percentage of unstained viable cells was counted using a microscope.

Propidium Iodide (PI) Staining of Cellular DNA. After treatment with **4**, adherent and floating DU145 cells were pooled, washed with PBS, fixed in PBS−MeOH (1:2, v/v) solution, and maintained at 4 °C for at least 18 h. After an additional wash with PBS, the cell pellets were stained with the fluorescent probe solution containing PBS, 50 μg of propidium iodide/mL, and 50 μg of DNase-free RNaseA/mL, for 30 min at room temperature in the dark. Cells were then analyzed using a Coulter Epics XL-MCL flow cytometer (Beckman Coulter), with excitation at 488 nm and gating out of doublets and clumps using pulse processing and collection of fluorescence emission above 580 nm. The percentage of cells undergoing DNA damage was defined as the percentage of cells in the subdiploid region (subG1) of the DNA distribution histograms.

Morphological Changes (Chromatin Condensation). To access the DNA, chromatin morphologic features were detected by Hoechst 33342 staining. Briefly, cells were treated with secotenufolide A (1 and 100 μM) for 24 h, fixed with 3.7% paraformaldehyde (pH 7.4), washed twice in PBS, and incubated with Hoechst 33342 (5 μg/mL) at 37 °C for 1 h. After washing with PBS, the stained nuclei were observed

Table 3. Cytotoxicity of Compounds **1**−**5**, Obtusilactone A, and Alpinenone Obtained from the Stems of *C. tenuifolium*

	IC_{50} (μM)[a]	
	DU145[b]	LNCaP[b]
tenuifolide A (**1**)	211 ± 9	450 ± 11
isotenuifolide A (**2**)	84.1 ± 4.7	42.7 ± 5.2
tenuifolide B (**3**)	246 ± 8	22.2 ± 0.8
secotenuifolide A (**4**)	5.20 ± 0.11	6.76 ± 0.12
tenuifolin (**5**)	>500	223 ± 11
obtusilactone A	68.4 ± 2.5	36.4 ± 1.3
alpinenone	267 ± 12	67.1 ± 2.4
cisplatin[d]	54.1 ± 0.8	ND[c]
taxol[d]	4.84 ± 0.15	6.32 ± 0.12

[a] Cells were treated with various concentrations of test compound for 1 day. Cell growth was determined by XTT assay. Each value represents the mean ± SE. [b] DU145 and LNCaP as human prostate cancer cell lines. [c] ND, undetermined. [d] Reference compound.

and photographed, using an Olympus fluorescence microscope (Olympus IX 70, Japan) at 480 nm. Cells exhibiting reduced nuclear size, chromatin condensation, and nuclear fragmentation were considered apoptotic.

Measurement of Mitochondrial Membrane Potential ($\Delta\Psi_m$). Mitochondrial membrane potential ($\Delta\Psi_m$) was measured by the incorporation of cationic fluorescent dye, rhodamine 123. After 6, 12, 18, and 24 h incubation in normal medium with or without treatment with **4** (10 and 100 μM), the normal medium was changed to a serum-free medium containing 10 μM rhodamine 123 and incubated for 15 min at 37 °C. The cells were then collected and the fluorescence intensity was analyzed within 15 min by a spectrophotofluorimeter (FLUOstar OPTIMA, Germany, 495 nm excitation and 520 nm emission).

Measurement of Cytochrome *c* Levels on Cytosolic and Mitochondrial Fractions. Cytochrome *c* ELISA kits were used to detect cytochrome *c*. Briefly, DU145 cells were treated with **4** (100 μM) or with DMSO (for a vehicle control) for 6, 12, 18, and 24 h, respectively, at 37 °C. Cells were harvested and centrifuged briefly at 800*g*. The supernatant was discarded. The cell pellet was resuspended, washed with PBS, and centrifuged at 1000*g* for 5 min, after which the supernatant was discarded. The cell pellet was resuspended with digitonin cell permeabilization buffer (250 mM sucrose, 137 mM NaCl, 70 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM K_2HPO_4, 0.2 mg/mL digitonin, and 0.1% hydorol M), vortexed, and incubated on ice for 5 min. Cells were then centrifuged at 1000*g* for 5 min at 4 °C. The supernatants were saved, as these contained the cytosolic fraction of cytochrome *c*. The remaining pellet was then resuspended with cell lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS), vortexed, and incubated on ice for 5 min. The lysate was vortexed and centrifuged at 10000*g* for 10 min at 4 °C as mitochondrial fractions. According to the ELISA assay methods, two fractions were run, using a modification of the manufacturer's directions for performing the assay.[3]

Measurement of Caspase-9 and Caspase-3 Activity. To measure the enzymatic activity of caspase-9 and caspase-3, DU145 cells were treated with 10 μM **4** for 1, 3, 6, 12, and 24 h, respectively, then washed with PBS, and collected by centrifugation. The cell pellets were lysed in a lysis buffer (1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/μL aprotinin, 1 mM leupetin, 2 mM dithiothreitol, and 10 mM Tris-HCl), incubated on ice for 1 h, and then centrifuged for 30 min at 13200*g*. The assay was based on the ability of the active enzyme to cleave the chromophore from the enzyme substrates, Ac-DEVD-pNA and Ac-LEHD-pNA, respectively. The cell lysates (60 μg) were incubated with peptide substrate in an assay buffer (100 mM NaCl, 20 mM PIPES, 10 mM dithiothreitol (DTT), 1 mM EDTA, 0.1% CHAPS, 10% sucrose, pH 7.2), at 37 °C for 1 h in the dark. The Ac-DEVD-pNA and Ac-LEHD-pNA cleavage (paranitroaniline) was measured in a multiwell spectrophotometer ELISA reader, at a wavelength of 405 nm. Results are represented as the percentage change of the activity, as compared to the untreated control. To explore the possibility that **4** induced apoptosis in antiproliferation, the DU145 cells were pretreated with 50 μM caspase-3 inhibitor (Ac-DEVD-CMK) and 50 μM casapse-9 inhibotor (Ac-LEHD-CMK), 60 min before treatment with **4**, and followed by treatment with 10 μM **4** for 24 h. DU145 cells' proliferation response was determined by XTT assay, as described above.

Measurement of Intracellular H_2O_2 and/or Peroxide (ROS). Production of intracellular H_2O_2 and/or peroxide was detected using the cell-permeable probe DCFH-DA. This dye is a stable compound that readily diffuses into cells and yields dichlorodihydrofluorescein (DCFH) in the presence of H_2O_2 and/or peroxide. DU145 cells (1×10^4) were cultured in a 96-well plate. After 6, 12, 18, and 24 h incubation in normal medium with or without treatment with **4** (1, 10, and 50 μM), the normal medium was changed to a serum-free medium containing 10 μM DCFH-DA for another 30 min in the dark at 37 °C. Then plate was washed twice with PBS, and the intracellular H_2O_2 and/or peroxide (ROS), as indicated by the fluorescence of dichlorofluorescein (DCF) intensity, was analyzed within 15 min by a spectrophotofluorimeter (FLUOstar OPTIMA, Germany, 495 nm excitation and 530 nm emission). For antioxidant studies, DU145 cells were first pretreated with either DPI (1 μM), NAC (1 mM), catalase (200 U/mL), mannitol (50 mM), dexamethasone (10 μM), trolox (50 μM), or L-NAME (100 μM) for 60 min, followed by incubation with 50 μM **4** for another 24 h, and then analyzed as described above. For immunohistochemical analysis, we used treatment with **4** (1 and 100 μM) for 24 h, and then the cells were fixed with 3.7% paraformaldehyde in 1× PBS (pH 7.4) at 4 °C for 10 min, washed twice in PBS, and incubated with DCFH-DA (10 μM) in the dark at 37 °C for 30 min. The expression of intracellular H_2O_2 and/or peroxide (ROS) was detected by fluorescent microscope.

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Supporting Information Available: ^1H and ^{13}C NMR spectra of compounds **1**−**5** in pdf format are available free of charge via the Internet at http://pubs.acs.org.

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