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Texas Native Plants Yield Compounds with Cytotoxic Activities against Prostate Cancer Cells

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Abstract

There remains a critical need for more effective therapies for the treatment of late-stage and metastatic prostate cancers. Three Texas native plants yielded three new and three known compounds with antiproliferative and cytotoxic activities against prostate cancer cells with IC₅₀ values in the range 1.7–35.0 μM. A new sesquiterpene named espadalide (**1**), isolated from *Gochnatia hypoleuca*, had low micromolar potency and was highly effective in clonogenic assays. Two known bioactive germacranolides (**2** and **3**) were additionally isolated from *G. hypoleuca*. *Dalea frutescens* yielded two new isoprenylated chalcones, named sanjuanolide (**4**) and

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Dedicated to Professors John Blunt and Murray Munro, of the University of Canterbury, for their pioneering work on bioactive marine natural products.

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Supporting Information

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¹H, ¹³C, COSY, HSQC and HMBC NMR spectra of **1–5**; HRESIMS spectra for **1–3**; chiral HPLC analysis profiles of compounds **4** and **5**; concentration response curves of **1–6** in PC-3 and DU 145 cells; effects of **1–3** on purified tubulin polymerization; compound **2** and **3** systematic name (PDF)

Crystallographic data (CIF)

Notes

The authors declare no competing financial interest.

sanjoseolide (**5**), and the known sesquiterpenediol verbesindiol (**6**) was isolated from *Verbesina virginica*. Mechanistic studies showed that **1–4** caused G₂/M accumulation and the formation of abnormal mitotic spindles. Tubulin polymerization assays revealed that **4** increased the initial rate of tubulin polymerization, but did not change total tubulin polymer levels, and **1–3** had no effects on tubulin polymerization. Despite its cytotoxic activity, compound **6** did not initiate changes in cell cycle distribution and has a mechanism of action different from the other compounds. This study demonstrates that new compounds with significant biological activities germane to unmet oncological needs can be isolated from Texas native plants.

Graphical abstract

Prostate cancer is the most commonly diagnosed cancer and a leading cause of cancer-related deaths in men, second only to lung and bronchial cancers.¹ The probability of a man in the United States developing prostate cancer in his lifetime is 1 in 7.¹ While the overall incidence and mortality due to prostate cancer are decreasing, it is estimated that 27540 men in the United States will die of prostate cancer in 2015.¹ Most prostate cancers are diagnosed at an early stage and are managed by active surveillance and/or watchful waiting.² Androgen deprivation therapy is used for men diagnosed with later stage disease; however 10–20% will develop castration-resistant prostate cancer (CRPC) within five years of their initial diagnosis.³ The microtubule stabilizer docetaxel is a mainstay in the treatment of CRPC and provides survival advantages for patients.⁴ A third-generation taxane, cabazitaxel, was approved in 2010 for use in prostate cancer.⁴ Cabazitaxel overcomes multidrug resistance mechanisms, and it has utility for patients who have become unresponsive to docetaxel.^{5, 6} Recent studies suggest that these taxanes can have activity in patients with earlier stage disease and in hormone-sensitive disease.⁴ In spite of the utility of the taxanes, there remains a need to identify new and improved treatments for late-stage, hormone refractory, and metastatic prostate cancers.

The development of docetaxel and cabazitaxel was only possible following the discovery and clinical development of paclitaxel, the first taxane that was originally isolated from the bark of the Pacific yew.⁷ Paclitaxel provides an excellent example of the value of plants as sources of potential anticancer compounds because plants have a long history of yielding pharmaceutically useful compounds.⁸ In oncology, natural products remain the most valuable source of new drug entities, with over 60% of cancer therapeutics derived directly from natural products, semisynthesized from natural products, or based on a natural product pharmacophore.⁹ The continuing value of plants as important sources for drug discovery is due in part to the fact that plant-derived secondary metabolites occupy a distinct region of chemical space that has little overlap with most synthetically derived molecules.¹⁰ While plant-based biosynthetic processes might explain a large portion of their chemical

uniqueness, caution is needed since recent findings implicate endophytes as the biosynthetic source of certain plant-derived compounds.¹¹

In this investigation, we tested the hypothesis that plants that thrive in Texas would provide new bioactive compounds with activity against prostate cancer cells. A total of 332 plants, both native and introduced to Texas, were collected and extracted using supercritical fluid and aqueous extraction methods to yield 1086 extracts. These extracts were tested for activity against PC-3 and DU 145 prostate cancer cell lines, which are androgen independent and provide a model of CRPC.¹² In prior studies, we reported the isolation and cytotoxic activities of four other new compounds from Texas plants with activities against prostate cancer cells.^{13, 14} These compounds include three *O*-prenylated flavonoids, amyrisins A–C, isolated from *Amyris madrensis* and the tricyclic sesquiterpene meleucanthin from *Melampodium leucanthum*.^{13, 14} The compounds presented in this report were purified from three plants that are native to South Texas: *Gochnatia hypoleuca*, *Dalea frutescens*, and *Verbesina virginica*. The supercritical CO₂ extract of each of these plants demonstrated activity at concentrations less than 10 µg/mL in one or both of the prostate cancer cell lines. Bioassay-guided fractionation of these extracts yielded a new sesquiterpene, espadalide (**1**), and two known sesquiterpenes,¹⁵ germacranolide analogues **2** and **3**, from *Gochnatia hypoleuca* A. Gray (Asteraceae), two new isoprenylated chalcones, sanjuanolide (**4**) and sanjoseolide (**5**), from *Dalea frutescens* A. Gray (Leguminosae), and one known sesquiterpenediol, verbesindiol^{16, 17} (**6**) from *Verbesina virginica* L. (Asteraceae). We propose the trivial names espadalide, sanjuanolide, and sanjoseolide for new compounds **1**, **4**, and **5**, respectively, to honor the recent selection of the San Antonio missions as UNESCO World Heritage Sites. This study demonstrates that the evaluation of Texas native plants in tandem with a focused bioassay-guided fractionation process can yield bioactive compounds that are active in models of advanced prostate cancers.

Results and Discussion

Plant Collections, Compound Isolations and Structure Determination

The extracts from three plants (*Gochnatia hypoleuca*, *Dalea frutescens*, and *Verbesina virginica*) that demonstrated inhibitory activities against two prostate cancer cell lines were subjected to a sequence of orthogonal chromatography steps (including silica gel flash chromatography and C₁₈ semipreparative HPLC), yielding compounds **1**–**6**.

Compound **1** was obtained as colorless, needle-shaped crystals. The molecular formula was established as C₁₉H₂₂O₅ based on HRESIMS data ([M + Na]⁺ ion at *m/z* 353.1360, calcd 353.1359), indicating nine degrees of unsaturation. The 1D and 2D NMR data (Table 1) together with the proposed molecular formula revealed that **1** had two methyl groups, five methylenes (two of which were olefinic), six methines (two of which were olefinic and one was incorporated in an aldehyde), and six quaternary carbons (including four olefinic carbons and two carbonyl groups). HMBC analysis established that **1** is similar structurally to the known germacranolide eupahualin C.¹⁸ The ¹H and ¹³C NMR shift data revealed that the most substantial differences between the known metabolite and **1** were traceable to resonance arising from the 4-hydroxytigloyl moiety of eupahualin C. Specifically, there was

no evidence from the NMR data for the requisite *O*-linked methylene carbon or proton resonances of this group. This was in agreement with the proposed molecular formula for **1**, which was deficient for CH₂O compared to eupahualin C. Instead, a new olefinic methylene was detected [δ_{C} 127.2 (C-3); δ_{H} 6.33 and 5.77 (H-3a,b)], which was rationalized to show that the 4-hydroxytigloyl unit in eupahualin C is replaced by a 2-methylacryloyl group in metabolite **1** (Figure 1). During the course of our studies, crystals of **1** were obtained, which enabled us to determine the absolute configuration by refinement of the X-ray crystallographic Hooft parameters.¹⁹ Accordingly, the structure of **1** was established and this compound was named espadalide in honor of the San Antonio mission San Francisco de la Espada.

Compounds **2** and **3** were both obtained as amorphous, white powders and were determined to be structurally similar to one another. Structure determination utilizing 1D and 2D NMR (Table 1 and Figure 2), and HRESIMS data revealed that the planar structure of compound **2** matched an unnamed germacranolide for which the relative configuration had been described in 1986.¹⁵ However, during the course of our dereplication analysis, it became apparent that the *4S**, *5R**, *6S**, *7S**, *8S** configuration for **2** inferred from the bond-line drawing presented in the published paper was different from the *4R**, *5R**, *6R**, *7R**, *8R** configuration reported in the SciFinder database. To address this discrepancy and determine the compound's correct relative configuration, an analysis of its coupling constant data was performed (Table 1), along with an examination of results from a ROESY experiment (Figure 2). After considering the merits of the two suspected diastereomers, as well as additional configurational isomers, it was concluded that compound **2** possesses a *4R**, *5R**, *6R**, *7R**, *8R** configuration. Likewise, compound **3** yielded very similar results and was assigned the same relative configuration. The only difference between these two compounds was determined to be the reduction of the ^{2',3'} bond of the 2-methylacryloyl group in **2** that generated new methine and methyl carbon and proton resonances (C-2': δ_{C} 34.1 CH, δ_{H} 2.55 m; C-3': δ_{C} 18.8 CH₃, δ_{H} 1.18 d, 7.0) in **3**. Although the absolute configurations of **2** and **3** were not assessed via independent experimental measurements, it is interesting to note that the isopropyl subunit of the germacrane cores in **1–3** appears to be installed early in their biosynthesis, thereby generating a rather consistent trend in the resulting C-7 configuration. Thus, it is tempting, though speculative, to suggest that **1–3** share identical C-7 absolute configurations in light of their shared biogenetic origins.

Compound **4** was obtained as a yellow powder, and its molecular formula (C₂₀H₂₀O₄) was ascertained by HRESIMS [*m/z* 325.1449 (calcd 325.1440)]. The 1D and 2D NMR spectroscopic data revealed the presence of units of one monosubstituted benzene, one tetrasubstituted benzene, one carbonyl, and one *trans*-substituted olefin, which could be combined to form the core (C-3'-substituted-2',4'-dihydroxychalcone) portion of **4**. The bonding of the remaining atoms (C₅H₉O) in **4** was analyzed from the HMBC spectrum to establish that they form a 2-hydroxy-3-methyl-3-butenyl group. The attachment of this hydroxyisoprenyl group to the chalcone was confirmed by HMBC correlations from H-1''a, H-1''b, and H-2'' to C-3'. Compound **4** was given the trivial name sanjuanolide in honor of the San Antonio mission San Juan Capistrano.

The configuration of the C-2'' stereocenter in **4** was examined next. The freshly purified material was deemed >90% pure, and it generated a large, negative specific rotation value ($[\alpha]_{\text{D}}^{20}$ -40) that was in agreement with a 2''*R* configuration (calcd $[\alpha]_{\text{D}}$ -27 in the gas phase using Gaussian'09).²⁰ However, after several weeks of freezer storage of the sample with its intermitant removal for biological assays and spectroscopic analysis, the magnitude of the specific rotation value for compound **4** dropped to near zero. Reanalysis of the ¹H NMR data for **4** showed no discernible changes in the sample, but analysis by chiral HPLC revealed that the sample was now a virtual (~1:1) racemic mixture (Figure S27, Supporting Information). The precise chemical basis for the instability of **4** in DMSO and/or CDCl₃ remains undetermined at this time.

Compound **5** was obtained as a pale yellow powder that had the molecular formula C₂₀H₂₂O₅ [determined by HRESIMS *m/z* 343.1548 (calcd 343.1545)]. The ¹H NMR and HMBC data for **5** were very similar to those for **4**, with the major exceptions noted among the proton and carbon signals attributed to the hydroxyisoprenyl group. The loss of the alkene carbon signals for the hydroxyisoprenyl group when compared to **4**, combined with the gain of H₂O in the molecular formula of **5**, indicated that a process equivalent to the addition of water across the C-3''/C-4'' double bond had occurred. This assertion was substantiated by the appearance of a new hydroxylated quaternary carbon (δ_{C} 78.2, C-3'') (Table 2) in **5**. An examination of the optical rotation data for **5** revealed a specific rotation value near zero and chiral HPLC confirmed that the sample was composed of a racemic mixture (Figure S27, Supporting Information). Compound **5** was given the trivial name sanjoseolide in honor of the San Antonio mission San José y San Miguel de Aguayo.

Biological Evaluation of the Isolated Plant Metabolites

The antiproliferative and cytotoxic activities of the compounds were evaluated in PC-3 and DU 145 prostate cancer cells using the sulforhodamine B (SRB) assay. Concentration response curves were generated, and the IC₅₀ values were determined from these curves (Table 3). The IC₅₀ values for compounds **1**–**6** were all in the low micromolar range for both prostate cancer cell lines, with **1** being the most potent and **5** the least potent. Among the sesquiterpenes isolated from *G. hypoleuca*, **1** was the most potent in PC-3 cells, with an IC₅₀ of 3.0 μM , and **1** and **3** were the most potent in DU 145 cells, with IC₅₀ values of 1.8 and 1.7 μM , respectively. Compound **2** was the least potent of the sesquiterpenes, with 2.6- and 2.2-fold lower potencies as compared to **3** in PC-3 and DU 145 cells, respectively. The only difference between these compounds was the reduction of the 2',3' double bond in the 2-methylacryloyl group in **3**, suggesting that the appendage of the macrocycle may contribute to its biological activity. Additionally, compound **1** was 6.5–10-fold more potent in the prostate cancer cell lines as compared to the structurally related compound eupahaulin C in other human cancer cell lines.¹⁸ Comparing the structurally related chalcones **4** and **5** from *D. frutescens*, it was noted that compound **4** was 3.2- and 3.6-fold more potent than **5** against PC-3 and DU 145 cells, respectively. These compounds differ only by the replacement of a 2,3-dihydroxy-3-methyl-3-butenyl group in **5** by a 2-hydroxy-3-methyl-3-butenyl group in **4**. This again points to the importance of minor structure modifications in determining the biological potencies of these natural products. The previously identified sesquiterpene diol **6** exhibited activity against both prostate cancer cell lines in the low micromolar range. It is

interesting to note that compound **6** yielded results that differed from those of the other metabolites and the positive control. Specifically, compounds **1–5** and paclitaxel exhibited slightly greater antiproliferative activity (1.4–5.3-fold increased potency) toward the DU 145 cell line versus the PC-3 cells. In contrast, **6** consistently exhibited greater potency against PC-3 cells versus DU 145 cells (IC_{50} values were 1.3-fold higher). This result indicates the possibility that **6** has a mechanism of action that is different from the other compounds and paclitaxel.

In addition to the antiproliferative effects, the ability of each of the compounds to initiate cytotoxicity was evaluated. Cytotoxicity was assessed by calculating the change in cell density from the time of compound addition.²¹ The data were plotted with a dashed line to indicate cell density at time of compound addition and cytotoxicity indicated when the curves drop below this line (Figure S28, Supporting Information). The results show that compounds **1–3** have moderate, 20–50%, cytotoxic activities in the PC-3 and DU 145 cells at the concentrations tested. In particular, the effects of **2** and **3** plateau at 30–40% cytotoxicity in DU 145 cells. In contrast, **4** and **6** were able to cause 84–97% cytotoxicity in both prostate cancer cell lines at 61.7 μ M and 12.5 μ M, respectively. Cytotoxicity of 46% was observed with 61.3 μ M **5** in the DU 145 cell line. However, the limited amounts of material available precluded testing at concentrations high enough to detect cytotoxicity in the PC-3 cells.

Clonogenic assays can be used to identify whether a short-term exposure to test compounds impacts the long-term clonogenic growth of cancer cells. The ability of compounds to exert long-term effects following drug wash-out demonstrates cellular persistence, which can be helpful in predicting in vivo efficacy.^{22, 23} DU 145 cells were treated with the respective IC_{90} concentrations of **1–4**, and **6** for 4 h, after which the compounds were removed, the cells washed, and colonies allowed to grow in the absence of compound for 7–9 days. The results of these experiments are shown in Figure 3. The most pronounced effect was caused by **1**, which caused a 98% reduction in colony number relative to vehicle-treated controls. While **2** and **3** are structurally related to **1**, both compounds were substantially less effective at inhibiting colony formation in these assays, with 23% and 42% reductions in the number of colonies formed, respectively. Compound **4** caused a 44% reduction in the number of colonies, and **6** showed no detectable effects on colony-forming ability. These data indicate that **1** has strong cellular persistence that can almost completely inhibit DU 145 colony formation following a short exposure.

Evaluating the effects of compounds on cell cycle distribution by flow cytometry provides a method to rapidly identify potential mechanisms of action. DU 145 cells were treated with **1–4** or **6** at 5-times their respective IC_{50} values for 16–23 h. The data are plotted as the number of events versus propidium iodide intensity as a measure of DNA content. A representative cell cycle distribution profile for vehicle-treated DU 145 cells is presented in Figure 4A. The results show that **2**, **3**, and **4** increased the percentage of cells in G_2/M . The flow cytometry data were further analyzed, and percentages of cells in each phase of the cell cycle determined using the ModfitLT program; these data are presented in Figure 4B. In vehicle-treated cells, 21.8% of cells were in the G_2/M phase and this increased slightly, to 26.4%, in cells treated with **1**. Compounds **2**, **3**, and **4** each caused a 50% increase in the

percentage of cells in the G₂/M phase (Figure 4B). Compound **6** caused a change in the shape of the cell cycle curve; however, analysis of multiple experiments indicated that **6** did not cause a change in cell cycle distribution. These data indicate that **6** has a mechanism of action different from the other compounds, consistent with the reversed sensitivity of the two prostate cell lines to this compound.

The effects of **1–4** on G₂/M accumulation suggested the possibility that they interrupt the formation and function of mitotic spindles, leading to delayed mitotic progression. The effects of the compounds on mitotic spindles and interphase microtubules were evaluated by indirect immunofluorescence in DU 145 cells. Vehicle-treated cells undergoing normal mitosis with bipolar spindles orienting the DNA at the metaphase plate are shown in Figure 5. Cells treated with **1–4** exhibited incorrect alignment of DNA at the metaphase plate, suggesting mitotic spindle defects. Indeed, the mitotic spindles looked abnormal, with elongated and curved morphologies, suggesting the possibility that the microtubule-kinetochore interactions were not stabilized. These results suggest that these compounds have the ability to disrupt mitotic spindle structures, leading to delayed mitotic progression and the accumulation of cells in G₂/M. These effects of **1** were not unexpected, because it is an analogue of parthenolide, a sesquiterpene lactone that has been shown to interfere with normal microtubule formation.²⁴ However, it is important to note that the mitotic accumulation and spindle defects observed in cells treated with **1–4** were much less pronounced than those observed in the presence of tubulin-binding drugs, suggesting the relatively minor defects observed in spindle morphology can be overcome, allowing progression through mitosis, albeit at a slower rate, leading to moderate G₂/M accumulation. Compound **6** had no effects on mitotic or interphase microtubules, consistent with a mechanism of action different from the other compounds.

The ability of **1–4** to initiate changes in mitotic spindles prompted an evaluation of the extent to which these compounds could interact directly with tubulin to alter its polymerization (Figure 6). In these studies, the positive controls were paclitaxel, which stimulated the polymerization of tubulin, and combretastatin A-4, which inhibited tubulin polymerization relative to vehicle. The addition of 2 μ M or 20 μ M of **1–3** did not alter the rate or extent of tubulin polymerization (Figure S29, Supporting Information). Parthenolide has been shown to inhibit tubulin carboxypeptidase, thus decreasing stabilized deetyrosinated microtubules.²⁵ The inability of **1** to affect the polymerization of purified tubulin is also consistent with an indirect mechanism of altering microtubules. A 2 μ M concentration of **4** also had no effect on tubulin polymerization; however at 20 μ M, **4** increased the initial rate of tubulin polymerization in each of four independent experiments. While the rate of tubulin polymerization was increased with the 20 μ M concentration of **4**, the amount of total tubulin polymer formed was not different from vehicle-treated cells after 1 h. These data indicate that **4** can interact directly with tubulin to affect its polymerization, which could be expected to lead to the mitotic spindle defects observed in cells treated with this compound. These results also indicate different mechanisms of action for the sesquiterpene **1** and the isoprenylated chalcone **4**. This is consistent with published reports that indicate that the sesquiterpene parthenolide inhibits tubulin carboxypeptidase²⁴ and the chalcones interact directly with tubulin and depolymerize microtubules.^{25, 26}

An intracellular signaling antibody array was used to evaluate short-term effects of the compounds on cell signaling pathways. The results indicated that **1–3** and **6** caused activation of Erk1/2, indicated by increased phosphorylation of Thr202/Tyr204, within 4 h of treatment. This initial finding was further evaluated by Western blot analysis over a 2–18 h time course. The results show that **1** and **2** caused a rapid and substantial increase in the levels of phospho-Erk1/2 within 2 h of treatment, which decreased over the later time points (Figure 7). Compound **3** caused a slight but transient increase in Erk1/2 activation and **6** had no effects on Erk1/2 activation. Activation of the MAPK pathway is associated with a cellular stress response to a wide variety of stimuli, including treatment with a variety of cytotoxic drugs including microtubule depolymerizers.^{26, 27} This is consistent with our previous results with microtubule-targeting agents, indicating that disruption of cellular microtubules, either depolymerization or stabilization, can lead to Erk1/2 activation.^{27, 28} Interestingly, sustained activation of Erk1/2 was initiated by microtubule-binding agents^{27, 28} as opposed to the transient activation observed here, further demonstrating that these compounds affect microtubule structures and cause mitotic accumulation in a distinct manner.

In summary, we have isolated three new and three known compounds from the Texas native plants *Gochnatia hypoleuca*, *Dalea frutescens*, and *Verbesina virginica*. Each of the compounds has antiproliferative and cytotoxic effects against prostate cancer cell lines with potencies ranging from the low to midmicromolar range. Compounds **1–4** are able to inhibit colony formation after short periods of drug treatment, causing moderate G₂/M accumulation and the formation of abnormal mitotic spindles. Purified tubulin polymerization studies indicate that **4** interacts directly with tubulin to cause a change in the rate of tubulin polymerization, while **1–3** disrupt cellular mitotic structures independently of a direct effect on tubulin polymerization. It is interesting that this unbiased approach to identifying compounds with activity against prostate cancer cells yielded four compounds that affected mitotic spindles and cell cycle progression, effects shared with the microtubule stabilizers docetaxel and cabazitaxel, drugs that provide a survival advantage in the treatment of prostate cancer. These studies confirm that new and known compounds with significant biological activities can be identified from Texas native plants using bioassay-guided fractionation.

Experimental Section

General Experimental Procedures

Optical rotation data were determined on a Rudolph Research AUTOPOL III automatic polarimeter. UV data were collected on a Hewlett-Packard 8452A diode array spectrophotometer. NMR data were collected on Bruker Avance 500 or 700 MHz instruments equipped with CryoProbe and Varian 500 MHz instruments. LCMS analyses were carried out on a Waters Alliance 2695 HPLC module, 996 photodiode array detector, and Micromass Quattro triple quadrupole mass spectrometer equipped with ESI under the positive mode. Accurate mass data were collected on an Agilent 6244 or 6538 QTOF MS coupled to an Agilent 1290 HPLC. Semipreparative HPLC was performed using a Waters 1525 binary pump system with a Waters 2998 photodiode array detector and with

Phenomenex Luna 5 μm C₁₈(2) columns (110 Å, 250 × 4.6 mm, 1 mL/min; 110 Å, 250 × 10 mm, 4 mL/min). Chiral HPLC analysis was performed on a Waters 1525 HPLC system using a Phenomenex Lux 5 μm Cellulose-3 column (250 × 4.6 mm, 1 mL/min). X-ray data were collected using a diffractometer with a Difractis CMOS area detector on a Rigaku goniometer and Cu K α radiation ($\lambda = 1.54178$ Å). All solvents were of ACS grade or better.

Plant Material

The leaves and stems of the chomonque, *Gochnatia hypoleuca*, a Texas native shrub, the white crownbeard, *Verbesina virginica*, and the black dalea or black prairie clover, *Dalea frutescens*, were originally collected from the San Antonio Botanical Gardens (SABG), San Antonio, Texas, USA, in the summer of 2005. Re-collections were made at the SABG as well as other locations around San Antonio by Mr. Paul Cox, the former superintendent of the SABG. The plant material was transported to the laboratory and then frozen at -20 °C prior to lyophilization. Voucher specimens SLM147 (*D. frutescens*), SLM176 (*G. hypoleuca*), and SLM269 (*V. virginica*) were deposited in the Mooberry laboratory herbarium and authenticated by Mr. Paul Cox. Lyophilized and pulverized plant material was extracted with supercritical CO₂.

Extraction and Isolation

The CO₂ supercritical fluid extract of *G. hypoleuca* (4 g) demonstrated activity against PC-3 and DU 145 cell lines with IC₅₀ values of 2.8 and 4.2 $\mu\text{g/mL}$ respectively, and was applied to flash chromatography over silica gel and eluted with a gradient of hexane and EtOAc, which yielded further active fractions. Fraction 68, which demonstrated activity at less than 1 $\mu\text{g/mL}$, was further processed on C₁₈ semipreparative HPLC (MeCN–H₂O, 55:45) to yield compounds **1** (1.0 mg), **2** (1.5 mg), and **3** (1.0 mg).

The CO₂ supercritical fluid extract of *D. frutescens* was active against PC-3 and DU 145 cells with IC₅₀ values of 8.2 and 12.0 $\mu\text{g/mL}$, respectively. This extract was subjected to flash chromatography on silica gel columns by elution with increasingly polar mixtures of hexane and EtOAc. Fractions were tested for activity against the prostate cancer cell lines, and fraction 82 turned out to be active, with an IC₅₀ of approximately 7 $\mu\text{g/mL}$. Fraction 82 was further processed by reversed-phase HPLC with gradient elution (MeCN–H₂O from 40:60 to 100% organic mobile phase over 50 min) to yield compounds **4** (1.7 mg) and **5** (0.6 mg).

The crude extract of *V. virginica* (1.76 g), which generated IC₅₀ values of 5.7 and 7.5 $\mu\text{g/mL}$ against PC-3 and DU 145 cells, respectively, was subjected to silica gel flash chromatography and eluted using increasingly polar mixtures of hexane and EtOAc. Fraction 79 was ascertained to be the active fraction against the test cells at an IC₅₀ of approximately 2 $\mu\text{g/mL}$, and this fraction was then further purified using silica gel chromatography and reversed-phase chromatography, leading to the isolation of compound **6** (15.4 mg). Compound **6** was identified as verbesindiol by comparison of its physical and spectroscopic data with literature values.^{16, 29}

Espadalide (1): colorless, needle-shaped crystals; $[\alpha]_D^{20} +112$ (c 0.05, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 212 (4.2) nm; ^1H and ^{13}C NMR, see Table 1; HRESIMS $[\text{M} + \text{Na}]^+$ m/z 353.1360 (calcd for $\text{C}_{19}\text{H}_{22}\text{O}_5\text{Na}$, 353.1359).

Compound 2: amorphous, white powder; $[\alpha]_D^{20} +202$ (c 0.075, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 212 (4.2) nm; ^1H and ^{13}C NMR, see Table 1; HRESIMS $[\text{M} + \text{Na}]^+$ m/z 371.1466 (calcd for $\text{C}_{19}\text{H}_{24}\text{O}_6\text{Na}$, 371.1465).

Compound 3: amorphous, white powder; $[\alpha]_D^{20} +44$ (c 0.05, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 210 (4.2) nm; ^1H and ^{13}C NMR, see Table 1; HRESIMS $[\text{M} + \text{Na}]^+$ m/z 373.1627 (calcd for $\text{C}_{19}\text{H}_{26}\text{O}_6\text{Na}$, 373.1622).

rac-Sanjuanolid (4): yellow powder; UV (from HPLC PDA) 225, 340 nm; ^1H and ^{13}C NMR, see Table 2; HRESIMS $[\text{M} + \text{H}]^+$ m/z 325.1449 (calcd for $\text{C}_{20}\text{H}_{21}\text{O}_4$, 325.1440).

rac-Sanjoeseolid (5): pale yellow powder; UV (from HPLC PDA) 225, 342 nm; ^1H and ^{13}C NMR, see Table 2; HRESIMS $[\text{M} + \text{H}]^+$ m/z 343.1548 (calcd for $\text{C}_{20}\text{H}_{23}\text{O}_5$, 343.1545).

X-ray Crystal Structure Analysis of Espadalide (1)

A colorless, needle-shaped crystal of dimensions $0.35 \times 0.02 \times 0.02$ mm was selected for structural analysis. Intensity data for this compound were collected using a diffractometer with a Difractis CMOS area detector on a Rigaku goniometer and Cu K α radiation ($\lambda = 1.54178$ Å). The sample was cooled to 100(2) K. Cell parameters were determined from a nonlinear least-squares fit of 802 peaks in the range $6.2^\circ < \theta < 124.4^\circ$. A total of 2863 data points were measured in the range $6.024^\circ < \theta < 66.251^\circ$ using ϕ and ϕ oscillation frames. The data were corrected for absorption by the empirical method,³⁰ giving minimum and maximum transmission factors of 0.781 and 0.985. The data were merged to form a set of 2863 independent data points and coverage of 98.7%. The monoclinic space group $P2_1$ was determined by systematic absences and statistical tests and verified by subsequent refinement. The structure was solved by direct methods and refined by full-matrix least-squares methods on F^2 .³¹ The positions of hydrogens bonded to carbons were initially determined by geometry and were refined using a riding model. Non-hydrogen atoms were refined with anisotropic displacement parameters. Hydrogen atom displacement parameters were set to 1.2 (1.5 for methyl) times the isotropic equivalent displacement parameters of the bonded atoms. A total of 220 parameters were refined against 193 restraints and 2863 data to give $wR(F^2) = 0.2975$ and $S = 1.098$ for weights of $w = 1/[\sigma^2(F^2) + (0.0900 P)^2 + 8.0000 P]$, where $P = [Fo^2 + 2Fc^2]/3$. The final $R(F)$ was 0.1117 for the 2741 observed, $[F > 4\sigma(F)]$, data. The largest shift/s.u. was 0.004 in the final refinement cycle. The final difference map had maxima and minima of 0.593 and -0.806 e/Å³, respectively. The absolute structure was determined by refinement of the Hooft (Hooft $y = -0.18(8)$, $P2$ (true) = 1.00, $P3$ (true) = 1.00, $P3$ (rac-twin) = 0.0, $P3$ (false) = 0) parameter.¹⁹ The polar axis restraints were taken from Flack and Schwarzenbach.³² The X-ray crystallographic data for espadalide (1) have been deposited with the Cambridge Crystallographic Data Center under accession number CCDC 1422019. The data can be accessed free of charge at <http://www.ccdc.cam.ac.uk/>.

Cell Culture

The DU 145 (ATCC HTB-81) and PC-3 (ATCC CRL 1435) cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained at 37 °C with 5% CO₂. The DU 145 cells were grown in Improved Minimal Essential Medium (IMEM) (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, GE Life Sciences, Logan, UT, USA) and 25 µg/mL gentamycin (Life Technologies, Waltham, MA, USA). PC-3 cells were maintained in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS (Hyclone) and 50 µg/mL gentamycin (Life Technologies).

Antiproliferative and Cytotoxicity Assays

The antiproliferative and cytotoxic activities of the compounds were evaluated using the sulforhodamine B assay, as previously described.^{14, 21, 33} The inclusion of a time zero point allows determination of cytotoxicity as well as antiproliferative effects.²¹ Briefly, cells were plated at predetermined densities, allowed to adhere overnight, and then treated with a range of concentrations of each compound. The cells were exposed to the compounds or vehicle (DMSO) for 48 h then the cellular protein was fixed in 10% trichloroacetic acid. Fixed cells were stained with SRB and washed to remove excess dye. Bound dye was dissolved in Tris, and the absorbance at 560 nm was measured using a Spectramax 384 Plus microplate reader (Molecular Devices, Chicago, IL, USA). The IC₅₀, the concentration that caused 50% decrease in the number of cells as compared to vehicle-treated cells, was determined using nonlinear regression (GraphPad Prism 6). The data were expressed as the means ± SEM. Cytotoxicity was defined by the ability of the compounds to decrease cellular protein below that measured at the time of compound addition.

Flow Cytometry

The effects of the compounds on cell cycle distribution were evaluated using flow cytometry of propidium iodide stained cells. DU 145 cells were treated with vehicle (DMSO), **1–4**, or **6** at 5-times the respective IC₅₀ concentrations for 16–23 h. Cells were harvested on ice and stained with Krishan's reagent.³⁴ The DNA content was analyzed using a BD LSRII flow cytometer (BD Biosciences, San Jose, CA, USA). The ModFitLT 3.3 program (Verity Software House, Topsham, ME, USA) was used to determine the percentage of cells in each phase of the cell cycle.

Clonogenic Assays

DU 145 cells were plated at a density of 150 cells per 60 mm cell culture plate and allowed to adhere for approximately 24 h. The cells were then treated for 4 h with the IC₉₀ concentration of **1–4** or **6** and then washed with fresh medium. Colonies were allowed to form for an additional 7–9 days in drug-free medium then stained with 0.5% crystal violet in 20% methanol. Pictures of the colonies were taken on a Geliance 600 using GeneSnap and the number of colonies on each plate was counted using GeneTools software (PerkinElmer, Waltham, MA, USA).

Immunofluorescence Microscopy

Cellular microtubules and DNA were visualized in DU 145 cells by indirect immunofluorescence, as previously described.¹⁴ The cells were treated with 5 times the IC₅₀ of **1**, **2**, **3**, or **6** or 2 times the IC₅₀ concentration of **4** for 16–18 h; then the cells were fixed with ice-cold methanol, and microtubules were visualized with a β -tubulin antibody (T4026, Sigma- Aldrich) and a FITC-conjugated secondary antibody (F3008, Sigma-Aldrich). The DNA was visualized with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). Images were obtained using a Nikon Eclipse 80i microscope and NIS Elements Advanced Research imaging software (Nikon Instruments, Melville, NY, USA).

Tubulin Polymerization

The turbidimetric tubulin polymerization assay was performed as previously described.¹⁴ Briefly, 2 mg/mL purified porcine brain tubulin (Cytoskeleton, Inc., Denver, CO, USA) in GPEM buffer containing 1 mM GTP was polymerized in the presence of vehicle (1% DMSO), paclitaxel, combretastatin A-4, or **1–4** at 2 and 20 μ M for 1 h at 37 °C, and the absorbance was measured at 340 nm.

Lysate Preparation and Western Blot Analysis

DU 145 cells were treated with the IC₉₀ concentration of each compound for 2, 4, 8, and 18 h prior to lysis. Cells were lysed with cell extraction buffer (Invitrogen) supplemented with protease inhibitors. Samples containing equal protein were resolved by SDS-PAGE, and the protein was transferred to an Immobilon-P membrane (Millipore, Billerica, MA, USA). Primary antibodies against phospho-P44/42 MAPK (phospho-Erk1/2) and P44/42 MAPK (Erk1/2) (Cell Signaling Technologies, Danvers, MA, USA; 4370, 4695, respectively) were used at 1:1000 concentration, and respective secondary antibodies conjugated to horseradish peroxidase were used at 1:5000 concentration. The phospho-P44/42 MAPK blot was stripped using mild stripping buffer according to the suggested protocol (Abcam, Cambridge, MA, USA) and then reprobed for total P44/42 MAPK. Membranes were developed using SuperSignal West Pico stable peroxide solution and SuperSignal West Pico luminal enhancer solution (ThermoFisher Scientific) and imaged on Geliance 600 using GeneSnap with densitometry quantification using GeneTools software (PerkinElmer).

Intracellular Signaling Array

A PathScan intracellular signaling array kit with fluorescent readout (Cell Signaling Technologies) was used according to the manufacturer's protocol. DU 145 cells were treated with **1**, **2**, **3**, or **6** for 4 h. Cell lysates were prepared as described above for Western blot analyses. The cell lysates were added to the array slide, followed by incubation with antibody detection cocktail, then DyLight 680-linked streptavidin detection. Images were taken using an Odyssey infrared imaging system and analyzed using Image Studio version 4.0 (Li-Core, Lincoln, NE, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Key ^1H - ^1H COSY and HMBC correlations (left) and X-ray-derived ORTEP rendering (right) for **1**

Figure 2.
Selected ^1H - ^1H COSY and HMBC correlations (left) and ROESY correlations (right) for **2**

Figure 3.

Effects of compounds on clonogenic growth. DU 145 cells were treated with the IC₉₀ concentration of the indicated compounds for 4 h and then the compounds were removed, the cells rinsed, and colonies allowed to grow for 7–9 days. The colonies were then fixed, stained, and counted. (A) Number of colonies in each of the treatment groups. (B) Representative pictures of colonies at the conclusion of the experiment. Results represent $n = 2$ experiments, with each performed in duplicate.

Figure 4.

Effects of the compounds on cell cycle distribution. (A) Representative graphs of cell cycle distribution of cells treated with 5 times the IC₅₀ concentration of each compound. (B) Percentage of the cells in each phase of the cell cycle. (C) Effects of the compounds on accumulation of cells in G₂/M. Results represent $n = 2$ for **4**, $n = 3$ for **1–3**, and **6**, and $n = 5$ for vehicle control. Error bars represent the standard error of the mean.

Figure 5. Effects of the compounds on mitotic spindles. Indirect immunofluorescence of β -tubulin (green) was used to visualize microtubules and DAPI staining (blue) was used to visualize the DNA in DU 145 cells treated with 2 or 5 times the IC_{50} concentration of the indicated compounds.

Figure 6. Effects of **4** on tubulin polymerization. The effects of paclitaxel, combretastatin A-4, vehicle, and **4** on the polymerization of purified tubulin were measured turbidimetrically.

Figure 7.

Effects of compounds on Erk1/2 activation. DU 145 cells were treated with the IC₉₀ concentration of indicated compounds or vehicle control (DMSO) for the indicated times prior to lysis, SDS-PAGE, immunoblotting and quantification. (A) Image of the Western blot used for quantification by densitometry. β-Tubulin was used as the protein loading control. (B) Ratio of the densitometry for phospho-Erk1/2 levels divided by the total Erk1/2 levels for each condition.

Table 1

¹H and ¹³C Spectroscopic Data of Compounds 1–3 in CDCl₃^a

no.	1			2			3		
	δ_{C} , mult.	δ_{H} , mult., <i>J</i> in Hz		δ_{C} , mult.	δ_{H} , mult., <i>J</i> in Hz		δ_{C} , mult.	δ_{H} , mult., <i>J</i> in Hz	
1	151.1 CH	6.60, dd (8.1, 8.6)		133.8 CH	5.56, brs		133.6 CH	5.56, brs	
2	26.4 CH ₂	2.50, m		24.3 CH ₂	2.44, m		24.2 CH ₂	2.44, brs	
		2.21, m							
3	36.6 CH ₂	2.38, m		38.1 CH ₂	2.17, m		38.1 CH ₂	2.18, m	
		2.14, m			1.25, m			1.25, m	
4	139.6 C			61.0 C			61.1 C		
5	124.6 CH	5.11, d (10.5)		64.8 CH	2.78, brs		64.8 CH	2.79, brs	
6	76.8 CH	4.67, dd (10.0, 10.1)		68.6 CH	4.12, dd (10.9, 2.9)		68.5 CH	4.13, dd (3.2, 10.8)	
7	48.5 CH	2.89, m		45.8 CH	3.11, brd (10.9)		45.8 CH	3.12, brd (10.7)	
8	72.7 CH	5.26, m		77.5 CH	4.59, m		77.2 CH	4.59, m	
9	29.2 CH ₂	2.89, m		not detected	2.99, dd (3.7, 14.3)		not detected	2.97, d (11.3)	
		2.21, m			2.12, dd (13.9, 12.4)			2.10, brdd (11.6, 12.8)	
10	143.0 C			128.3 C			128.5 C		
11	136.3 C			134.6 C			134.6 C		
12	169.7 C			169.6 C			169.6 C		
13	123.7 CH ₂	6.25, d (3.3)		128.3 CH ₂	6.44, brs		128.3 CH ₂	6.46, s	
		5.73, d (3.0)			6.08, brs			6.13, s	
14	193.7 CH	9.57, s		62.8 CH ₂	4.75, d (12.6)		62.5 CH ₂	4.69, d (12.5)	
					4.64, d (12.7)			4.56, d (12.6)	
15	18.2 CH ₃	1.92, s		15.6 CH ₃	1.47, s		15.5 CH ₃	1.48, s	
1'	166.4 C			167.1 C			176.7 C		
2'	136.0 C			135.7 C			34.1 CH	2.55, m	
3'	127.2 CH ₂	6.33, brs		126.3 CH ₂	6.07, brs		18.8 CH ₃	1.18, d (7.0)	
		5.77, brs			5.59, brs				
4'	18.7 CH ₃	2.05, s		18.4 CH ₃	1.93, s		18.8 CH ₃	1.17, d (6.9)	
OH-6					2.24, s			2.22, s	

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The ^1H COSY, HSQC, and HMBC were obtained from a Varian 500 MHz instrument, ^{13}C data of compound **2** was obtained from a Varian 100 MHz instrument, ^{13}C data of compounds **1** and **3** was extracted from HSQC and HMBC spectra

Table 2¹H and ¹³C NMR Spectroscopic Data of Sanjuanolid (4) and anjoseolid (5) in CDCl₃^a

no.	sanjuanolid (4)		sanjoseolid (5)	
	δ _C , mult.	δ _H , mult., <i>J</i> in Hz	δ _C , mult.	δ _H , mult., <i>J</i> in Hz
1	135.1 C		134.8 C	
2	128.6 CH	7.65, m	128.3 CH	7.65, m
3	129.1 CH	7.42, m	128.7 CH	7.43, m
4	130.7 CH	7.42, m	130.5 CH	7.43, m
5	129.1 CH	7.42, m	128.7 CH	7.43, m
6	128.6 CH	7.65, m	128.3 CH	7.65, m
7	144.2 CH	7.87, d (15.5)	144.0 CH	7.88, d (15.5)
8	120.8 CH	7.61, d (15.5)	120.4 CH	7.59, d (15.5)
9	192.2 C		191.9 C	
1'	113.7 C		113.4 C	
2'	164.6 C		164.7 C	
3'	113.3 C		107.2 C	
4'	163.7 C		159.8 C	
5'	109.3 CH	6.54, d (9.0)	108.8 CH	6.44, d (9.0)
6'	130.1 CH	7.77, d (9.0)	128.9 CH	7.74, d (9.0)
1''	28.6 CH ₂	3.22, dd (15.0, 1.7) 2.89, dd (14.9, 8.4)	25.9 CH ₂	2.99, dd (17.2, 5.0) 2.78, dd (17.2, 5.5)
2''	77.8 CH	4.42, brd (8.3)	69.1 CH	3.89, t (4.9)
3''	146.9 C		78.2 C	
4''	110.6 CH ₂	5.01, s 4.88, s	22.6 CH ₃	1.41, s
5''	18.7 CH ₃	1.88, s	24.6 CH ₃	1.36, s
OH-2'		13.8, brs		13.8, brs

^aThe ¹H NMR spectrum was obtained on Bruker Avance 700 MHz instrument. ¹³C NMR data for compound **4** was on Bruker Avance 125 MHz instrument, and the ¹³C NMR data for compound **5** were extracted from HSQC and HMBC experiments

Table 3IC₅₀ Values $\pm$ SEM (μ M) of 1–6 and Paclitaxel^a

compound	PC-3	DU 145
espadalide (1)	3.0 $\pm$ 0.2	1.8 $\pm$ 0.3
(2)	12 $\pm$ 3	3.7 $\pm$ 0.7
(3)	4.7 $\pm$ 0.1	1.7 $\pm$ 0.2
sanjuanolide (4)	11 $\pm$ 4	7 $\pm$ 3
sanjoseolide (5)	35.0	25.5
verbesindiol (6)	4.0 $\pm$ 0.6	5.2 $\pm$ 0.6
paclitaxel	0.020 $\pm$ 0.006	0.0038 $\pm$ 0.0007

^aCompounds 1–4, 6, and paclitaxel, $n = 3$ –5; compound 5, $n = 1$.