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A New Dehydrogeranylgeraniol Antioxidant from *Saururus cernuus* that Inhibits Intracellular Reactive Oxygen Species (ROS)-Catalyzed Oxidation within HL-60 Cells

Ira Rajbhandari, Satoshi Takamatsu, and Dale G. Nagle*

Department of Pharmacognosy and National Center for Natural Products Research, Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi, University, Mississippi 38677-1848

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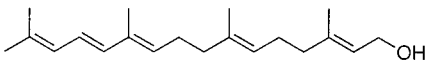
Abstract: A new triene, 12,13-dehydrogeranylgeraniol (**1**), was isolated from the aquatic plant *Saururus cernuus* and its structure determined spectroscopically. Compound **1** inhibits PMA-induced peroxide-catalyzed oxidation of 2′,7′-dichlorodihydrofluorescein dye (DCFH) by reactive oxygen species (ROS) within human promyelocyctic HL-60 cells.

As part of an ongoing examination of aquatic and wetland plants that grow in the swamps and marshes of the southeastern United States, *Saururus cernuus* L. (Saururaceae) was found to be among the most chemically rich. This emergent species (commonly called lizard's tail) has been used in folk medicine as a sedative and as a treatment for tumors.[1,2] The nature of the chemical constituents responsible for these properties has not been clearly defined. However, many biologically active natural products, including more than 20 lignans and cinnamic acid dimers, have been isolated from *S. cernuus*. The dineolignans manassantins A and B have been reported to be responsible for neuroleptic activity in mice.[3−7] Two perhydroxylated derivatives of dihydroguaiaretic acid, sauriols A and B, and other lignans have been demonstrated to protect *S. cernuus* from herbivory.[8]

Lyophilized *S. cernuus* stems and leaves were extracted exhaustively with 50% CH_2Cl_2 in MeOH (v/v). Analysis by 2D TLC revealed this extract to be chemically rich, relative to several hundred other Mississippi plants examined. This extract contained a variety of UV-absorbing secondary metabolites that produced a char reaction upon treatment with ethanolic H_2SO_4 (heat). Compound **1** was isolated from *S. cernuus* by bioassay-guided fractionation using two 2D TLC autography bioassays simultaneously. Compound **1** was readily observable by both a modified 2D TLC DPPH antioxidant autography assay for detecting natural products that act as radical scavengers[9,10] and a newly described antifungal bioautography bioassay.[11] A portion of the crude extract was fractionated by sequential Si gel vacuum flash chromatography and Sephadex LH-20 chromatography. Final purification by RP-HPLC provided the pure compound **1** (0.31% w/w yield).



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Compound **1** was a clear oil. Analysis by ^{13}C NMR and HREIMS provided a molecular mass for $C_{20}H_{32}O$, indicating five degrees of unsaturation. The presence of a triene moiety was indicated by the characteristic UV absorbance pattern (λ_{max} 266, 275, 286 nm). All proton spin systems of a linear diterpene chain with five methyl singlets were connected from analysis of $^1H-^{13}C$ HMBC spectra. The configuration of double bonds was identified on the basis of the carbon chemical shifts of methyl groups in isomeric geranylgeraniol standards.[12] The characteristic signals of the methyl carbon branches of *trans*-configured linear terpenes resonate at approximately 16.29 ppm, while those in the *cis* configuration resonate near 23.44 ppm.[12] The ^{13}C NMR chemical shifts for the methyl branches attached at C-3, C-7, and C-15 in **1** all resonated between 16.42 and 18.70 ppm, indicating that all are in the *trans* configuration. The chemical shift of the C-11 methyl group in **1** was upfield and shielded relative to that of other geranylgeraniol isomers.[12] The C-12−C-13 olefin was assigned *E* configuration on the basis of the large vicinal coupling constant ($^3J_{HH} = 15.2$ Hz).

Compound **1** was shown to be moderately antifungal at 100 nmol against the plant pathogens *Colletotrichum*

* To whom correspondence should be addressed. Tel: 662-915-7143. Fax: 662-915-6975. E-mail: dnagle@olemiss.edu.

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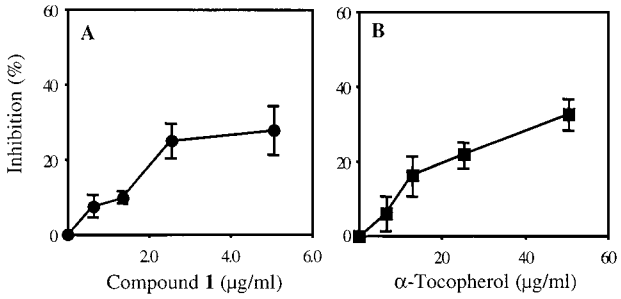

Figure 1. Inhibition of compound **1** (A) and α-tocopherol (B) on DCFH-DA oxidation by PMA-stimulated hydrogen peroxide in HL-60 cells. The data are presented as percentage of inhibition based upon measurement of fluorescence at 530 nm. Values are means of three independent determinations ± SD. Statistical analysis was performed by using Student's *t*-test.

acutatum, *C. fragariae*, and *C. gloeosporioides* in a direct bioautography assay.[11] The triene moiety may impart some additional weak antifungal activity, relative to geranylgeraniol.

Many studies of plant-derived antioxidants have examined the reduction potential or radical-scavenging effects of natural products in solution-based chemical assays.[13−17] Researchers have recently begun to look at the antioxidant effects of natural products in living systems.[18,19] A cell-based method to directly examine the ability of natural products to penetrate living human cells and inhibit reactive oxygen species (ROS)-catalyzed oxidation was used to evaluate **1** for its ability to scavenge exogenous ROS induced by PMA (phorbol 12-myristate 13-acetate) in HL-60 cells. Compound **1** showed an inhibition of 28% at 5.0 μg/mL (1.74×10^{-5} M) without cytotoxicity (Figure 1). Furthermore, compound **1** inhibited the activity of endogenous ROS by 8%. Vitamin E (α-tocopherol) showed 32.4% inhibition against ROS at 50 μg/mL (1.16×10^{-4} M), but had no effect on ROS activity. The relative potency of **1**, nearly 7 times that of vitamin E, suggests that further investigation of the interaction between **1** and the generation of hydrogen peroxide and other reactive oxygen species is warranted.

Experimental Section

General Experimental Procedures. The UV spectrum was obtained using a Hewlett-Packard 8453. The IR spectrum was obtained using an AATI Mattson Genesis Series FTIR. NMR spectra (^{1}H, ^{13}C, COSY, HMQC, and HMBC) of **1** were recorded in C_6D_6 on a Bruker DRX 500 spectrometer operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C, running gradients and using residual solvent peaks as internal references. The HREIMS data were acquired on a Finnigan MAT 95 (University of Minnesota Department of Chemistry, MS Service Laboratory), and the HRESIMS data were acquired on a Bruker BioAPEX 30es (NCNPR, University of Mississippi). DPPH (1,1-diphenyl-2-picrylhydrazyl) was purchased from Sigma Chemical Co.

Plant Material. The plant material was collected from Benton County, Mississippi, near Michigan City (GPS 34° 58′ 33″ N; 89° 15′ 42″ W). A voucher specimen (DN-075) was placed on file with the University of Mississippi Herbarium, Department of Biology.

Extraction and Isolation. Lyophilized stems and leaves (155.4 g marc) were extracted with 50% CH_2Cl_2 in MeOH (v/v) and dried under vacuum to yield 16.57 g of an oil. A portion of the crude extract (11.0 g) was fractionated by Si gel vacuum flash chromatography with a hexanes−EtOAc−MeOH gradient. The second nonpolar fraction (1.66 g) that eluted with 5% EtOAc in hexanes (v/v) was fractionated by Si gel vacuum flash chromatography (hexanes−EtOAc gradient). The combined nonpolar fractions (1.06 g) that eluted with 5% and 10% EtOAc in hexanes (v/v), respectively, were separated by Sephadex LH-20 chromatography [50% CH_2Cl_2 in MeOH (v/v)]. Elution of a 200 mg fraction was shown to contain a UV-active, charring (H_2SO_4, heat) nonpolar secondary metabolite by TLC analysis. Final purification by RP-HPLC (Econosil C_{18}, 10 μM, 22 × 250 mm, 10% H_2O in MeOH (v/v), 3.0 mL/min, photodiode-array detection monitored at 254 nm) provided compound **1** (7.4 mg).

Compound 1: clear oil; UV (MeOH) λ_{max} (log ϵ) 266 (4.33), 275 (4.44), 286 (4.33) nm; IR (film) ν_{max} 3405, 2922, 1667, 1443, 1379, 967 cm^{-1}; ^{1}H NMR (C_6D_6, 500 MHz) δ 1.59 (3H, s, Me-3), 1.67 (3H, s, Me-7), 1.78 (3H, s, Me-15), 1.80 (3H, s, Me-16), 1.90 (3H, s, Me-11), 2.09 (2H, t, J = 7.2 Hz, H-4), 2.18 (2H, t, J = 7.4 Hz, H-8), 2.23 (2H, q, J = 7.5 Hz, H-5), 2.37 (2H, q, J = 7.3 Hz, H-9), 4.11 (2H, d, J = 6.6 Hz, H-1), 5.30 (1H, br t, J = 5.9, 1.0 Hz, H-6), 5.51 (1H, br t, J = 6.6, 1.1 Hz, H-2), 5.65 (1H, br t, J = 7.2, 0.9 Hz, H-10), 6.13 (1H, br d, J = 10.7 Hz, H-14), 6.45 (1H, d, J = 15.2 Hz, H-12), 6.60 (1H, dd, J = 15.2, 10.8 Hz, H-13); ^{13}C NMR (C_6D_6, 125 MHz) δ 12.99 (q, Me-11), 16.42 (q, Me-7), 16.55 (q, Me-3), 18.70 (q, Me-15), 26.50 (q, C-16), 27.08 (t, C-5), 27.80 (t, C-9), 40.18 (t, C-4), 40.18 (t, C-8), 59.73 (t, C-1), 123.74 (d, C-13), 125.15 (d, C-6), 125.30 (d, C-2), 127.09 (d, C-14), 132.20 (d, C-10), 134.07 (s, C-15), 134.97 (s, C-11), 135.30 (s, C-7), 136.18 (d, C-12), 138.40 (s, C-3); HREIMS m/z 288.2442 (calcd for $C_{20}H_{32}O$, 288.2453); HRESIMS m/z 287.2367 $[M - H]^-$ (calcd for $C_{20}H_{31}O$, 287.2375).

TLC Autographic Assay for DPPH Radical-Scavenging Effect. The radical-scavenging effect of natural products was detected by 2D TLC analysis, using a spray reagent composed of a 0.2% (w/v) solution of DPPH in MeOH.[9,10] Plates were observed 30 min after spraying. Active compounds are observed as yellow spots against a purple background.

Determination of ROS-Catalyzed Oxidation of DCFH in Flow Cytometer. Promyelocyctic HL-60 cells (1×10^6 cells/mL, ATCC) were suspended in RPMI 1640 medium with 10% FBS and antibiotics at 37 °C (5% CO_2:95% air). After treatment with test solution for 30 min, cells were stimulated with 100 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma) for 30 min. Furthermore, cells were incubated for 15 min after the addition of 5 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, Molecular Probes). DCFH-DA is a nonfluorescent probe that diffuses into cells. Cytoplasmic esterases hydrolyze DCF-DA to 2′,7′-dichlorodihydrofluorescein (DCFH). Reactive oxygen species (ROS) generated within HL-60 cells oxidize DCFH to the fluorescent dye 2′,7′-dichlorofluorescein (DCF). Cells were washed twice with phosphate-buffered saline (PBS, pH 7.2) and analyzed using a flow cytometer (FACSCalibur, Becton Dickinson). Fluorescence was measured at 530 nm using a FL1 filter. The ability of **1** to inhibit exogenous cytoplasmic ROS-catalyzed oxidation of DCFH in HL-60 cells was measured by PMA-treated control incubations with and without **1**.[20,21] Vitamin E (α-tocopherol) was used as a positive control at maximal effective concentrations.

XTT Assay for Cytotoxicity. The 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) assay was performed using the methods described by Scudiero et al.[22] Briefly, 25 μL of XTT-PMS solution (1 mg/mL XTT solution supplement by 25 μM phenazine methosulfate) was added to the cells in each well of the microplates. After incubating for 4 h at 37 °C, absorbance at 450 nm was measured using a microplate reader (reference absorbance at 650 nm).[22]

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