JOURNAL OF NATURAL PRODUCTS

Article

pubs.acs.org/jnp

Cytotoxicity and Modulation of Cancer-Related Signaling by (*Z*)- and (*E*)-3,4,3′,5′-Tetramethoxystilbene Isolated from *Eugenia rigida*

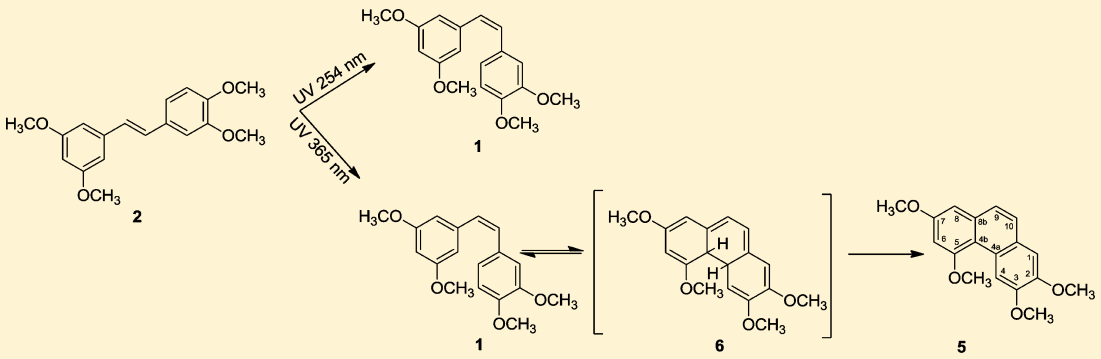
Mohamed A. Zaki,[†,‡] Premalatha Balachandran,[†] Shabana Khan,[†,§] Mei Wang,[†] Rabab Mohammed,[‡] Mona H. Hetta,[‡] David S. Pasco,*[,†,§] and Ilias Muhammad*[,†]

[†]National Center for Natural Products Research, Research Institute of Pharmaceutical Sciences, School of Pharmacy, The University of Mississippi, University, Mississippi 38677, United States

[‡]Department of Pharmacognosy, School of Pharmacy, Beni-Suef University, Beni-Suef, Egypt

[§]Department of Pharmacognosy, Research Institute of Pharmaceutical Sciences, School of Pharmacy, The University of Mississippi, University, Mississippi 38677, United States

Supporting Information



ABSTRACT: Bioassay-guided fractionation of the leaves of *Eugenia rigida* yielded three stilbenes, (*Z*)-3,4,3′,5′-tetramethoxystilbene (**1**), (*E*)-3,4,3′,5′-tetramethoxystilbene (**2**), and (*E*)-3,5,4′-trimethoxystilbene (**3**). Their structures were determined using 1D- and 2D-NMR spectroscopy and HRESIMS. The sterically hindered *Z*-stereoisomer **1**, a new natural product, was prepared by time-dependent photoisomerization of the *E*-isomer (**2**) under UV irradiation at λ_{254} nm, while 2,3,5,7-tetramethoxyphenanthrene (**5**) was identified at λ_{365} nm by UHPLC/APCI-MS and NMR spectroscopy. Compounds **1**−**3** were tested against a panel of luciferase reporter gene assays that assess the activity of many cancer-related signaling pathways, and the *Z*-isomer (**1**) was found to be more potent than the *E*-isomer (**2**) in inhibiting the activation of Stat3, Smad3/4, myc, Ets, Notch, and Wnt signaling, with IC_{50} values between 40 and 80 μM. However, both compounds showed similar inhibition against Ap-1 and NF-κB signaling. In addition, **1** demonstrated cytotoxic activity toward human leukemia cells, solid tumor cells of epidermal, breast, and cervical carcinomas, and skin melanoma, with IC_{50} values between 3.6 and 4.3 μM, while **2** was weakly active against leukemia, cervical carcinoma, and skin melanoma cells. Interestingly, **2** showed antioxidant activity by inhibition of ROS generation to 50% at 33.3 μM in PMA-induced HL-60 cells, while **1** was inactive at 100 μM (vs Trolox 1.4 μM).

Stilbenes are naturally occurring phytoalexins that generally exist as their more stable *E*-isomers. The most well-known stilbene is resveratrol [(*E*)-3,5,4′-trihydroxystilbene], which is present in a wide range of plants, notably *Vitis vinifera* L. (Vitaceae) and *Arachis hypogaea* L. (Fabaceae).[1] During the past decade, an array of important biological activities, including the potential cancer chemopreventive effect of resveratrol, has prompted investigations on the modulation of cancer-related signaling pathways[2] as well as the development of potent natural and synthetic anticancer analogues.[3] Since transcription factors and the signaling pathways that control their activity are involved in modulating the expression of many cancer-related genes, the identification of molecular targets within these pathways for such compounds or analogues is required to understand how they affect the chemoprevention process. Resveratrol is postulated as a potential modulator of signal transduction pathways for cancer and the carcinogen response; therefore, this collective activity may contribute an important role in the anticancer properties of resveratrol[2b] and its analogues.

Piceatannol [(*E*)-3,4,3′,5′-tetrahydroxystilbene], a natural resveratrol analogue with antileukemic activity,[4] was isolated from *Euphorbia lagascae* Spreng. (Euphorbiaceae)[1] and, like resveratrol, is also present in grapes and red wine.[1] Piceatannol was reported as a metabolite of resveratrol, transformed with the hydroxylation by the CYP1B1 enzyme, which is overexpressed in a wide range of human tumors.[5] Piceatannol and

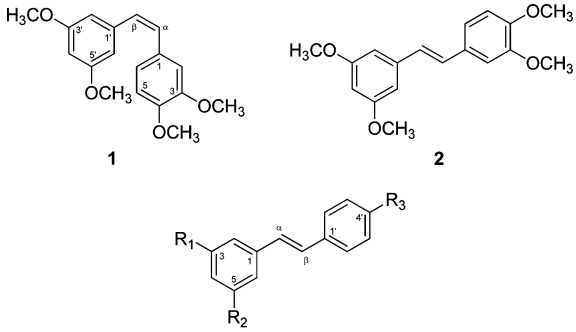
Received: December 21, 2012

ACS Publications © XXXX American Chemical Society and American Society of Pharmacognosy

dx.doi.org/10.1021/np300893n | *J. Nat. Prod.* XXXX, XXX, XXX−XXX

(*E*)-3,4,3′,5′-tetramethoxystilbene were also found to be equally effective as etoposide against the gastric carcinoma cell line with the classical MDR phenotype EPG85-257RDB, while piceatannol was more potent than (*E*)-3,4,3′,5′-tetramethoxystilbene against the pancreatic carcinoma cell line with the classical EPP85-181RDB6MDR phenotype.[6] On the other hand, the *Z*-form of 3,4,3′,5′-tetramethoxystilbene showed >10-fold more activity than the *E*-form against the SW480 human colorectal tumor cell line.[7]

The genus *Eugenia* is the largest in the family Myrtaceae, with up to 2000 species distributed from the south of Mexico, Cuba, and the Antilles, to Uruguay and Argentina, with a small number of species in Africa.[8] Some species, with edible fruits, have been cultivated in tropical and subtropical regions.[9] *Eugenia rigida* DC. is a shrub or small tree producing characteristic brown and green fruits, which turn black when mature. This plant, used folklorically for leukemia in Argentina, has not been subjected previously to either chemical or biological investigations, but the genus has exhibited a wide array of secondary metabolites on phytochemical analysis. However, the only stilbene reported from the family Myrtaceae is piceatannol, isolated from *Callistemon rigidus* R. Br.[9] In this study, we report the isolation of (*Z*)- (**1**) and (*E*)-3,4,3′,5′-tetramethoxystilbene (**2**) and (*E*)-3,5,4′-trimethoxystilbene (**3**) from *E. rigida* and the photoisomerization of **2** to **1**. Their cytotoxicity is described against selected human tumor cells, as well as their effects on modulation of cancer-related signaling pathways and inhibition of reactive oxygen species (ROS) and nitrite generation.



RESULTS AND DISCUSSION

Bioassay-guided fractionation, based on luciferase reporter assays, of the active *n*-hexane extract of *E. rigida*, using centrifugal preparative thin-layer chromatography with custom-made C_{18} and normal-phase silica gel rotors (see Experimental Section), resulted in the isolation of three stilbenes (**1**−**3**), of which **1**[10] was found to be more cytotoxic than **2** and **3**. Compounds **2** and **3** have been isolated previously,[11,12] and their structures were suggested by comparison of spectroscopic data with those published.[11−13] The HRESIMS of compound **1** showed a molecular ion at *m*/*z* 301.1357 $[M + H]^+$ (calcd for $C_{18}H_{21}O_4$, 301.1362), attributed to the molecular formula $C_{18}H_{20}O_4$. The ^{1}H NMR data of **1** were reported previously as a synthetic compound,[10] but only at low field, and the ^{13}C NMR data were not published. In order to assign the ^{13}C NMR data unambiguously to substantiate the structure of **1**, a complete set of 2D-NMR experiments was carried out, with the data compared with those assigned for **2** (see Table S1, Supporting Information). Thus, the HMBC spectrum of **1** confirmed the unambiguous assignments of carbon signals by showing correlations between H-α (δ 6.52) and C-1 (δ 129.7), C-2 (δ 111.8), C-6 (δ 122.0), C-β (δ 128.8), and C-1′ (δ 139.5) and between H-β (δ 6.47) and C-1′, C-2′/6′ (δ 106.6), C-α (δ 130.3), and C-1. In addition, the signal at δ 6.46 (H-2′/6′) showed correlations with resonances at δ 99.6 (C-4′) and 128.8 (C-β), and that at δ 6.32 (H-4′) with the C-2′/6′ signal (δ 106.6), indicating the presence of methoxy groups at the C-3′ and C-5′ positions. Finally, the ^{1}H−^{1}H NOESY spectrum revealed cross-peaks between δ 6.75 (H-5) and 3.86 (OMe-4) and between δ 6.83 (H-2) and 3.64 (OMe-3), confirming the position of the methoxy groups at C-3 and C-4.

In order to supplement the low yield of the sterically hindered *Z*-isomer (**1**), an additional quantity of this compound was prepared from the more stable **2** by photoisomerization at λ_{254} nm.[14] A time-dependent UV irradiation was carried out to study the conversion of (*E*)-**2** to the (*Z*)-**1** isomer over a time range of 0−1000 min, where the yield of product **1** was quantified by UHPLC/APCI-MS. A linear increase in the *Z*-isomer, with 80% conversion, was noted at λ_{254} nm during the first 250 min of irradiation (Figure 2A). Figure 2D depicts a first-order rate plot of (*E*)-2 photoisomerization within a 250 min time interval, using data from Figure 2A, which showed clearly good linearity, as reflected by an R^2 of 0.9937. In contrast, a sharp increase of **1** was noted in the first 30 min of irradiation at λ_{365} nm, followed by a steady decrease of **1** (Figure 2B) with the formation of **5**, which was

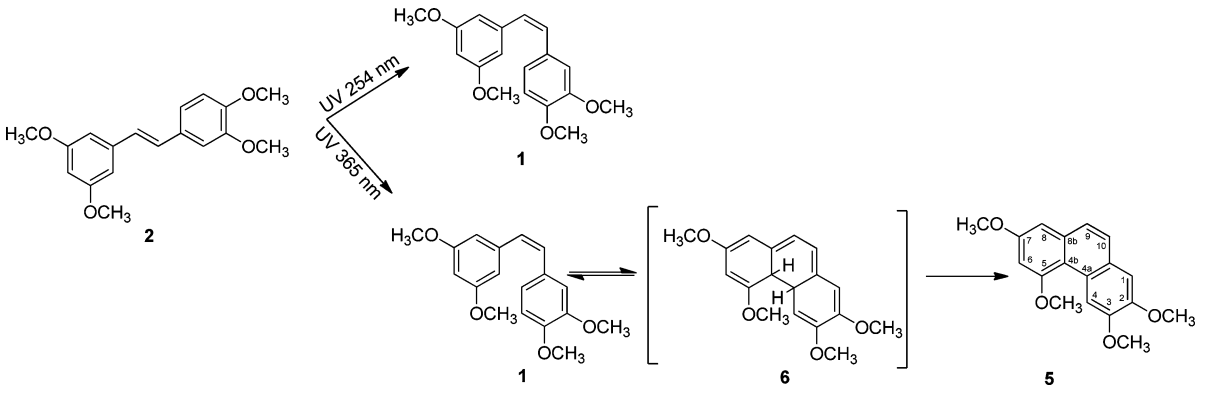


Figure 1. Photoisomerization of (*E*)-**2** into (*Z*)-**1** and compound **5** by UV irradiation at λ_{254} nm and λ_{365} nm, respectively, in tetrahydrofuran.

dx.doi.org/10.1021/np300893n | *J. Nat. Prod.* XXXX, XXX, XXX−XXX

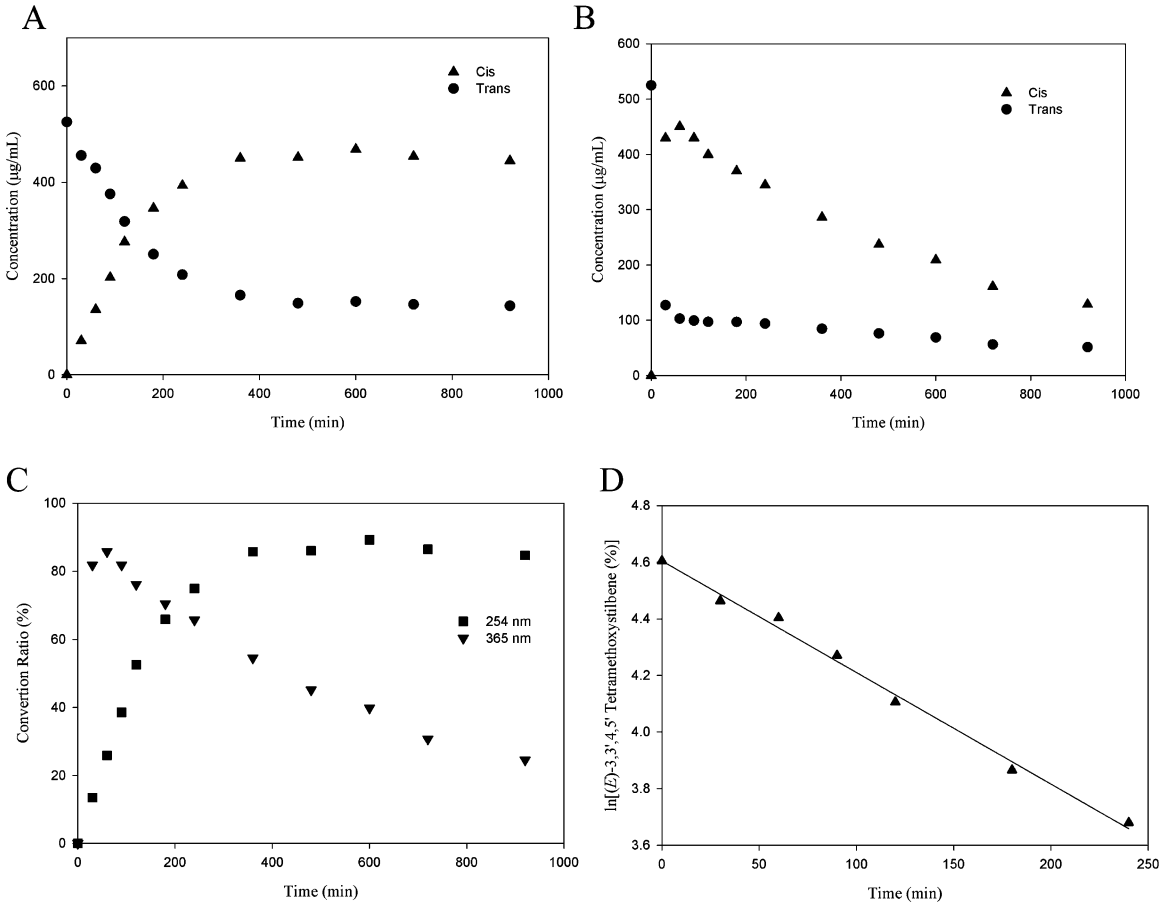


Figure 2. Photoisomerization of (*E*)-**2** into (*Z*)-**1** by UV irradiation in tetrahydrofuran (A) at λ_{254} nm and (B) at λ_{365} nm. (C) Assessment of the ratio of (*E*)-**2** into (*Z*)-**1** inversion by UHPLC at λ_{254} nm or λ_{365} nm. (D) Determination of apparent rate constant (k_z) and half-life ($t_{1/2}$) of (*E*)-**2** photoisomerization based on pseudo-first-order kinetics by UHPLC.

Table 1. Cytotoxic Activities of Compounds 1−4

	cytotoxic activity (IC_{50} in μM)[a]							
compound	HL-60[b]	KB[c]	BT-549[d]	SK-OV-3[e]	SK-MEL[f]	HeLa[g]	LLC-PK-1[h]	Vero[i]
1	4.3	4.3	4.0	NA[j]	4.3	3.6	6.0	>33.3
2	33.3	NA	NA	NA	60.0	8.0	NA	NA
3	NT[k]	17.7	15.6	55.5	12.2	13.3	20	26.7
4	31.8	NA	NA	113.0	47.3	NA	52.5	73.6
doxorubicin	0.2	3.8	3.7	2.6	1.5	3.9	1.6	>9.2

[a]IC_{50} is the concentration that affords 50% inhibition of cell growth. [b−g]Human cell lines of leukemia, epidermal carcinoma, breast carcinoma, ovarian carcinoma, skin melanoma, cervical carcinoma. [h]Pig kidney epithelial cells. [i]African green monkey kidney cell line. [j]No activity at 100 μM. [k]Not tested.

identified from the mixture as 2,3,5,7-tetramethoxyphenanthrene by UHPLC/MS (M^+ 298; $C_{18}H_{19}O_4$), UV, and ^{1}H NMR spectra[15] (Figures S22−S27, Supporting Information). The ^{1}H NMR spectrum showed doublets at δ 7.53 and 7.62 (each J = 8.5 Hz; H-9, H-10) and δ 6.88 and 6.74 (each J = 2.0 Hz; H-6, H-8) and singlets at δ 7.21 and 9.11 (H-1, H-4), thereby suggesting the position of cyclization at C-4a and C-4b. Four methoxy groups at δ 3.95, 4.03, 4.08, and 4.10 were assigned at the C-2, -3, -5, and -7 positions, respectively, of **5**. The formation of **5** suggested that **1** was likely cyclized to an unstable intermediate, 2,3,5,7-tetramethoxy-4a,4b-dihydrophenanthrene (**6**), which rapidly undergoes oxidative transformation to yield **5** (Figure 1).[16] Finally, since the geometrical isomerism of (*E*)-**1** or (*Z*)-**2** can influence bioactivity (vide infra), knowledge of photostability together with methods to determine their isomer composition and/or byproduct (**5**, **6**) formation is relevant to their therapeutic promise.

Compound **1** showed cytotoxic activity against human leukemia (HL-60) cells, human solid tumor cells of epidermal (KB), breast (BT-459), and cervical (HeLa) carcinomas, and skin melanoma (SK-MEL), with IC_{50} values of 4.3, 4.3, 4.0, 3.6, and 4.3 μM, respectively, while **2** was weakly active against HL-60, HeLa, and SK-MEL cells (IC_{50} values of 33.3, 8.0, and 60.0 μM) (Table 1). In addition, **3** was also found to be weakly cytotoxic toward KB, BT-459, HeLa, and SK-MEL cells (IC_{50} 17.7, 15.6, 13.3, and 12.2 μM, respectively). Compound **1** was inactive against a noncancerous cell line (monkey kidney fibroblast; Vero) up to 33.3 μM, thus exhibiting selectivity toward the tumor cells. Since compound **1** was more potent than **2** with respect to human cancer cell line cytotoxicity, it was

dx.doi.org/10.1021/np300893n | J. Nat. Prod. XXXX, XXX, XXX−XXX

Table 2. Activity of Compounds 1, 2, and 4 (IC_{50} values in μM) against Cancer-Related Signaling Pathways in HeLa Cells[a]

	Stat3 IL-6	Smad3/4 TGF-β	Ap-1 PMA	NF-κB PMA	E2F PMA	Myc PMA	Ets PMA	Notch PMA	FoxO	Wnt wnt3a	Hedgehog PMA	pTK
1	50/77	40/55	80/80	80/80	−/−	60/65	40/65	40/40	−/−	40/50	−/−	−/−
2	−/−	100/77	80/100	90/-	−/−	−/−	−/−	−/−	−/−	80/80	−/−	−/−
4	70/53	50/33	−/−	50/60	−/−	60/55	50/55	40/40	−/−	60/70	40/40	−/−

[a]Values (from two independent experiments) are the IC_{50} or lowest concentration (both in μmol/L) that maximally inhibited luciferase induction by 50−60%. A dash indicates that luciferase induction was not inhibited more than 40% at 100 μM. Compounds (final concentrations of 40, 60, 80, or 100 μM) were added to cells 30 min before the addition of the indicated inducer and were harvested for the luciferase assay 4 or 6 h (Notch, FoxO, Wnt, and Hedgehog) later. No inducer was added to cells transfected with FoxO vector or pTK control vector.

determined if the *Z*-isomer (**1**) was also a more effective modulator of cancer-related signaling pathways than the *E*-isomer (**2**). Modulation of the activity of these pathways was assessed using a battery of luciferase reporter gene vectors, in which luciferase expression is driven by the binding of transcription factors to multiple copies of synthetic enhancers within each vector. Compound **1** was more potent than **2** at inhibiting the activation of Stat3, Smad3/4, myc, Ets, Notch, and Wnt signaling. Ap-1 and NF-κB signaling were inhibited by both compounds similarly, and neither compound inhibited E2F or Hedgehog pathway activation at the tested concentrations. Similarly, the activation of the apoptotic mediator FoxO was not observed with either compound. Compound **1** was similar in potency to (*E*)-resveratrol (**4**) for inhibiting signaling mediated by Stat3, Smad3/4, myc, Ets, and Notch, while resveratrol was more potent for NF-κB and Hedgehog. Compound **3** was also tested and found inactive up to 100 μM. None of the compounds at the concentrations tested inhibited luciferase expression driven by the minimal thymidine kinase promoter (pTK), indicating the lack of general cytotoxicity or luciferase enzyme inhibition (Table 2). Finally, the inhibition of intracellular generation of reactive oxygen and nitrite species was measured to determine the potentials of the test compounds against oxidative and inflammatory stress in the cellular environment. The results obtained showed that the *E*-isomer (**2**) exhibited an inhibition of 50% in ROS generation at 33.3 μM in phorbol-12-myristate-13-acetate (PMA)-induced HL-60 cells, while the *Z*-isomer (**1**) did not exhibit any effect at the doses tested (100 μM), which might be due to the enhanced conjugation of the olefinic bond with the aromatic ring in the *E*-isomer (**1**), compared to the *Z*-isomer. However, compounds **1** and **2** did not show any effect on inducible nitric oxide synthase (*i*NOS) activity in LPS-induced macrophages (RAW264.7) up to 33.3 μM (vs parthenolide at 8 μM).

This is the first report of (*Z*)-3,4,3′,5′-tetramethoxystilbene (**1**) from a natural source, although it has been synthesized previously together with its *E*-isomer,[7,10] and also the first report of stilbenoids from the genus *Eugenia*. Interestingly, the potent inhibitory activity of **1** against HL-60 cells supports the reported use of *E. rigida* in Argentina for leukemia,[17] although this needs to be confirmed by additional experimental work.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were determined on a Mettler FP 51 apparatus. UV spectra were obtained in MeOH using a Hewlett-Packard 8453 UV/vis spectrometer. IR spectra were obtained using a Bruker Tensor 27 instrument. NMR spectra were acquired on a Varian Mercury 400 MHz spectrometer at 400 (^{1}H) and 100 (^{13}C) MHz in $CDCl_3$, using the residual solvent as an internal standard. Multiplicity determinations (DEPT) and 2D-NMR spectra (HMQC, HMBC, NOESY) were obtained using standard Bruker pulse programs. HRESIMS were obtained by direct injection using a Bruker Bioapex-FTMS with electrospray ionization (ESI). UHPLC/APCI-MS was performed using an Agilent 1290 UHPLC system coupled with an Agilent 6120 single quadrupole mass spectrometer. UV irradiation was carried out with a Spectroline UV lamp ENF 240C (Spectromics Corporation, NY, USA) in a sealed chamber. TLC was conducted on precoated silica gel 60 F_{254} (EMD Chemicals Inc., Darmstadt, Germany) using toluene−EtOAc (9:1) as solvent. Centrifugal preparative TLC (CPTLC, using a Chromatotron, Harrison Research Inc., Palo Alto, CA, USA; model 8924, tagged with a fraction collector) was carried out on 6 mm reversed-phase C_{18} silica gel (Chromatorotor)[18] or a 2 mm silica gel P_{254} (Analtech) disk, using H_2O in MeOH, and Me_2CO in *n*-hexane as eluents, respectively. Samples were dried using a Savant Speed Vac Plus SC210A concentrator. The compounds were visualized by spraying the TLC plates with 1% vanillin−H_2SO_4 as spray reagent. The reference compounds (*E*)-resveratrol and (*E*)-3,5,4′-trimethoxystilbene were purchased from TCI America (Waltham, MA, USA; purity >98.0%, GC), and reference standard doxorubicin (purity ≥98.0%) was procured from Sigma-Aldrich. Details of vectors for transfection and luciferase assays are listed in Table S2, Supporting Information.

Plant Material. The leaves of *Eugenia rigida* were collected from Guanica, Puerto Rico, in March 2006. The sample was identified by Mr. F. Axelrod, and a voucher specimen (no. 3008783; collection no. Gust 1116) was deposited at the Herbarium of the Missouri Botanical Garden, St. Louis, MO, USA.

Extraction and Isolation. Air-dried, powdered leaves (107 g) were soaked in *n*-hexane and sonicated (600 mL × 3 × 2 h each). The combined extract was filtered and dried (2.5 g), and the residue was extracted with CH_2Cl_2, followed by EtOAc and MeOH, yielding extracts of 3.8 g (CH_2Cl_2), 1.1 g (EtOAc), and 14.0 g (MeOH). The *n*-hexane extract (2 g) was subjected to centrifugal preparative TLC (CPTLC, Chromatotron), using a 6 mm custom-made reversed- phase Chromatorotor packed with binder-free C_{18} silica gel.[18] The rotor was mounted on a Chromatotron and packed under slow rotation (100 rpm) by applying a slurry of C_{18} silica gel (100 g) impregnated with UV 254 and 365 nm fluorescent indicators (0.5% each) in H_2O−MeOH (1:9; 300 mL). The sample, dissolved in Me_2CO, was applied to the rotor under a rotation of 700 rpm, and then the rotor was removed from the instrument and left to dry in a desiccator. The dried rotor was mounted on a Chromatotron and eluted with H_2O−MeOH (3:7), which afforded fr. 12−21 (174 mg) containing a mixture of compounds **1**−**3**. This fraction was subjected to CPTLC, using a 2 mm silica gel rotor, and eluted with 0.5−20% Me_2CO in *n*-hexane, which furnished compound **1** (3 mg), followed by **2** (30 mg) and **3** (1 mg), respectively, as monitored and pooled by TLC analysis (silica gel; solvent: toluene−EtOAc, 9:1).

(*Z*)-3,4,3′,5′-Tetramethoxystilbene (1): yellow oil; UV (MeOH) λ_{max} (log ε) 286 (0.80) nm; IR (KBr) ν_{max} 2999, 1588, 1456, 1203 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table S1, Supporting Information; HRESIMS *m*/*z* 301.1357 $[M + H]^+$ (calcd for $C_{18}H_{21}O_4$, 301.1362).

(*E*)-3,4,3′,5′-Tetramethoxystilbene (2): colorless solid ($CHCl_3$); mp 67−71 °C (lit.[13] mp 67−68 °C); UV (MeOH) λ_{max} (log ε) 323 (1.49) nm; IR (KBr) ν_{max} 2932, 1591, 1458, 1203 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table S1, Supporting Information; HRESIMS *m*/*z* 301.1358 $[M + H]^+$ [calcd for $C_{18}H_{21}O_4$, 301.1362]).

(*E*)-3,5,4′-Trimethoxystilbene (3): colorless solid; ^{1}H and ^{13}C NMR spectroscopic data were in agreement with those reported for

dx.doi.org/10.1021/np300893n | J. Nat. Prod. XXXX, XXX, XXX−XXX

(*E*)-3,5,4′-trimethoxystilbene.[12] The identity of compound **3** was confirmed by direct comparison with an authentic sample of (*E*)-3,5,4′-trimethoxystilbene.

Photoisomerization of 2. Compound **2** (10 mg) was dissolved in tertrahydrofuran (THF; 5 mL) and subjected to UV irradiation at λ_{254} nm at room temperature. The reaction mixture was monitored by running TLC of aliquots at 30 min intervals. The irradiation was stopped after 250 min (i.e., initially determined by a time-dependent study, see below), and the solution was dried and then dissolved in CH_2Cl_2 (5 mL). The sample was loaded on a Chromatotron, using a 1 mm silica gel rotor to separate the *Z*-isomer (**1**) from the residual *E*-isomer (**2**), following a similar procedure to that described above to afford 8 mg of **1**, showing an 80% conversion.

UHPLC/APCI-MS Analysis. Analysis was performed on an Agilent 1290 Infinity liquid chromatograph coupled with an Agilent 6120 single quadrupole mass spectrometer. The LC column was a Waters Acquity UPLC BEH RP-C_{18} column (1.7 μm, 2.1 × 150 mm). The mobile phase consisted of A (acetonitrile with 0.05% formic acid) and B (water with 0.05% formic acid) at a flow rate of 0.2 mL/min. The gradient elution started with 55% A for 8 min, and then it was increased linearly to 100% A in 9 min and held for 3 min. The column temperature was maintained at 30 °C. The compounds of interest were analyzed by ESI and APCI in both the positive and negative modes. The APCI positive mode was selected for analysis because it produced a better ion signal for the test compounds than the other ionization mode. The drying gas flow was 10 L/min, and the nebulizer pressure was 30 psi. The drying gas temperature and vaporizer temperature were set to 250 and 200 °C, respectively. The capillary voltage was 3000 V, and the corona current was 4.0 μA. The MS was operated in a selected ion monitoring (SIM) mode; *m*/*z* 301 $[M + H]^+$ was selected to monitor compounds **1** and **2**, and *m*/*z* 299 $[M + H]^+$ was selected for compound **5**.

Stock solutions of compounds **1** and **2** were prepared separately at a concentration of ∼1.0 mg/mL in THF. A series of calibration standard solutions within the concentration range of 10−550 μg/mL was prepared. The calibration curves were linear over the full concentration range. Compound **2** (2.0 mg) was dissolved in 4 mL of THF, and the solution was divided into two vials. One was exposed to short- (λ_{254} nm) and the other to long-wavelength (λ_{365} nm) UV light. An aliquot (100 μL) from each reaction mixture was taken at 30 min intervals and analyzed by UHPLC/MS as discussed above. The last aliquot was taken from λ_{254} nm UV irradiation reaction after 1000 min, which showed compounds **1** (t_R 6.929 min; $[M + H]^+$ *m*/*z* 301) and **2** (t_R 6.397 min; $[M + H]^+$ *m*/*z* 301). Compound **5** (t_R 6.593 min; $[M + H]^+$ *m*/*z* 299; $C_{18}H_{19}O_4$) was identified as the major product from the reaction mixture, irradiated at λ_{365} nm, in addition to residual amounts of compounds **1** and **2**. The ^{1}H NMR spectroscopic data of compound **5** were in agreement with those reported for 2,3,5,7-tetramethoxyphenanthrene.[15]

Cytotoxicity Assays. Cytotoxic activity was determined against six human cancer cell lines (HL-60, SK-MEL, KB, BT-549, SKOV-3, and HeLa) and two noncancerous kidney cell lines (LLC-PK1 and Vero). All cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Each assay was performed in 96-well tissue culture-treated microplates. Cells were seeded at a density of 25 000 cells/well and incubated for 24 h (except HL-60 cells, which were incubated for 3 h). Samples at different concentrations were added, and cells were again incubated for 48 h. At the end of incubation, the cell viability was determined using Neutral Red dye according to a modification of the procedure of Borenfreund et al.[19] In the case of HL-60 cells, viability was determined by an XTT method, as described earlier.[20] IC_{50} values were determined from dose−response curves of percent growth inhibition against test concentrations. Doxorubicin was used as a positive control, while DMSO was used as the negative (vehicle) control.

Transfection and Luciferase Assays. HeLa cells (ATCC, Bethesda, MD, USA) were maintained in Dulbecco's modified Eagle's medium (Gibco Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (Atlanta Biologicals Inc., Atlanta, GA, USA) and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. Luciferase vectors are summarized in Table S2, Supporting Information. Cells were grown to 60−80% confluence, trypsinized with 0.05% trypsin-EDTA (Gibco Life Technologies, Grand Island, NY, USA), and plated in 96-well plates at a density of 0.015×10^6 cells/well in 100 μL of growth medium with 10% fetal bovine serum (FBS). After 24 h, the growth medium was replaced with DMEM containing 1% FBS. The cells were transfected with the appropriate plasmid DNA(s) using X-tremeGENE HP transfection reagent (Roche Applied Science, Indianapolis, IN, USA). After 24 h of transfection, the agents to be tested were added to the transfected cells, followed 30 min later by an inducing agent (IL-6 and TGF-beta were from R&D Systems, Inc., Minneapolis, MN, USA; m-wnt3a was from Peprotech Corporation, Rocky Hill, NJ, USA; and phorbol 12-myristate 13-acetate was from Sigma Chemical Company, St. Louis, MO, USA). After 4 or 6 h of induction, the medium was aspirated and the cells were lysed by the addition of a 1:1 mixture of One-Glo luciferase assay system (Promega Corporation, Madison, WI, USA) and phosphate-buffered saline (PBS). The light output was detected in a Glomax Multi+ detection system with Instinct Software (Promega Corporation, Madison, WI, USA).

Antioxidant Assays. Antioxidant activity was assessed by the DCFH-DA (2′,7′-dichlorodihydrofluorescein diacetate) method, as described earlier.[20] Myelomonocytic HL-60 cells (0.8×10^6 cells/mL; ATTC, Manassas, VA, USA) were suspended in RPMI-1640 medium containing 10% fetal bovine serum, penicillin (50 units/mL), and streptomycin (50 μg/mL). The cell suspension (150 μL) was added to the wells of a 96-well plate. After treatment with different concentrations (0.4−100 μM) of the test compounds for 30 min, cells were treated with phorbol-12-myristate-13-acetate (PMA, 100 ng/mL) for 30 min. DCFH-DA (5 μg/mL) was added, and cells were further incubated for 15 min. Levels of fluorescent DCF [produced by ROS-catalyzed oxidation of DCFH] were measured on a SpectraMax plate reader with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. DCFH-DA is a nonfluorescent probe that diffuses into the cells, where cytoplasmic esterases hydrolyze it to the nonfluorescent 2′,7′-dichlorodihydrofluorescein (DCFH). ROS generated within HL-60 cells oxidize DCFH to the fluorescent dye 2′,7′-dichlorofluorescein (DCF). The ability of the test compounds to inhibit ROS-mediated oxidation of DCFH in PMA-treated HL-60 cells was measured in comparison to the vehicle control. Trolox (IC_{50} 1.4 μM) was used as a positive control. The cytotoxicity to HL-60 cells was also determined after incubation for 48 h of cells (2×10^4 cells/well in 225 μL) with test samples by the XTT method, as described earlier.[20]

Assay for Inhibition of *i*NOS. Inhibition of intracellular NO production as a result of *i*NOS activity was assayed in mouse macrophages (RAW 264.7 cells), as described before.[21] Cells were seeded at a density of 50 000 cells/well in 96-well plates and grown for 24 h. Test samples were added to the cells after incubating with samples for 30 min, LPS (5 μg/mL) was added, and cells were further incubated for 24 h. The activity of *i*NOS was determined by measuring the level of nitrite in the cell culture supernatant with Griess reagent. The degree of inhibition of nitrite production was calculated in comparison to the vehicle control. IC_{50} values were obtained from dose−response curves. Parthenolide was used as a positive control (IC_{50} 8 μM). Cytotoxicity of test samples to macrophages was also determined in parallel to check if the inhibition of *i*NOS was due to cytotoxic effects.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Spectroscopic data of compounds **1** and **2**; ^{1}H and ^{13}C NMR spectra of **3**; and photoisomerization data. This material is available free of charge via the Internet at http://pubs.acs.org.

dx.doi.org/10.1021/np300893n | *J. Nat. Prod.* XXXX, XXX, XXX−XXX

AUTHOR INFORMATION

Corresponding Author

*(I.M.) Tel: (662) 915-1051. Fax: (662) 915-7989. E-mail: milias@olemiss.edu. (D.S.P.) Tel: (662) 915-7130. Fax: (662) 915-7062. E-mail: dpasco@olemiss.edu.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors sincerely thank Dr. V. Raman, Dr. B. Avula, Mr. F. Wiggers, Ms. K. Martin, and Mr. J. Trott, NCNPR, School of Pharmacy, for providing information on the plant, recording mass spectra, running NMR spectra, and performing the cytotoxicity assays, respectively. This work was supported in part by a contract from the University of Mississippi Medical Center Cancer Institute and the USDA Agricultural Research Service Specific Cooperative Agreement No. 58-6408-2-0009. M.A. Zaki is thankful to the Egyptian Government for a scholarship.

REFERENCES

(1) Ferreira, M.; Duarte, N.; Gyémánt, N.; Radics, R.; Cherepnev, G.; Varga, A.; Molnár, J. *Anticancer Res.* **2006**, *26*, 3541−3546.

(2) (a) Lin, H.; Xiong, W.; Zhang, Y.; Cheng, J.; Huang, H. *Oncol. Rep.* **2011**, *26*, 925−930. (b) Whitlock, N. C.; Baek, S. *J. Nutr. Cancer* **2012**, *64*, 493−502.

(3) Lee, H. S.; Lee, B. W.; Kim, M. R.; Jun, J. G. *Bull. Korean Chem. Soc.* **2010**, *31*, 971−975.

(4) Gautam, S. C.; Xu, Y. X.; Dumaguin, M.; Janakiraman, N.; Chapman, R. A. *Bone Marrow Transplant.* **2000**, *25*, 639−645.

(5) Potter, G. A.; Patterson, L. H.; Wanogho, E.; Perry, P. J.; Butler, P. C.; Ijaz, T.; Ruparelia, K. C.; Lamb, J. H.; Farmer, P. B.; Stanley, L. A.; Burke, M. D. *Br. J. Cancer* **2002**, *86*, 774−778.

(6) Duarte, N.; Lage, H.; Abrantes, M.; Ferreira, M. *Planta Med.* **2010**, *76*, 975−980.

(7) Mazue, F.; Colin, D.; Gobbo, J.; Wegner, M.; Rescifina, A.; Spatafora, C.; Fasseur, D.; Delmas, D.; Meunier, P.; Tringali, C.; Latruffe, N. *Eur. J. Med. Chem.* **2010**, *45*, 2792−2780.

(8) Mazine, F. F.; Souza, V. C. *Bot. J. Linn. Soc.* **2008**, *158*, 775−777.

(9) Kobashi, K.; Ishihara, T.; Khono, E.; Miyase, T.; Yoshizaki, F. *Biol. Pharm. Bull.* **2006**, *29*, 1275−1277.

(10) Sun, H.; Xiao, C.; Cai, Y.; Chen, Y.; Wei, W.; Liu, X.; Lv, Z.; Zou, Y. *Chem. Pharm. Bull.* **2010**, *58*, 1492−1496.

(11) Dawidar, A. M.; Jakupovic, J.; Abdel-Mogib, M.; Mashaly, I. A. *Phytochemistry* **1994**, *36*, 803−806.

(12) Macrae, W. D.; Towers, G. H. N. *Phytochemistry* **1985**, *24*, 561−566.

(13) (a) Shibutani, S.; Samejima, M.; Doi, S. *J. Wood Sci.* **2004**, *50*, 439−444. (b) Botella, L.; Najera, C. *Tetrahedron* **2004**, *60*, 5563−5570.

(14) (a) Minezawa, N.; Gordon, M. S. *J. Phys. Chem. A* **2011**, *115*, 7901−7911. (b) Waldeck, D. H. *Chem. Rev.* **1991**, *91*, 415−436.

(15) Majumder, P. L.; Banerjee, S.; Sen, S. *Phytochemistry* **1996**, *42*, 347−352.

(16) Mallory, F. B.; Mallory, C. W. In *Organic Reactions*, Vol. *30*; Dauben, W. G., Ed.; John Wiley & Sons, Inc.: New York, 1984; pp 1−456.

(17) Johnson, T. *CRC Ethnobotany Desk Reference*; CRC Press: Boca Raton, FL, 1999.

(18) Ilias, M.; Samoylenko, V.; Gillum, V. Int. Appl. No. PCT/US12/54220, 2011.

(19) (a) Borenfreund, E.; Babich, H.; Martin-Alguacil, N. *In Vitro Cell. Dev. Biol.* **1990**, *26*, 1030. (b) Samoylenko, V.; Jacob, M. R.; Khan, S. I.; Zhao, J.; Tekwani, B. L.; Midiwo, J. O.; Walker, L. A.; Muhammad, I. *Nat. Prod. Commun.* **2009**, *4*, 791−796.

(20) (a) Choi, Y. W.; Takamatsu, S.; Khan, S. I.; Srinivas, P. V.; Ferreira, D.; Zhao, J. *J. Nat. Prod.* **2006**, *69*, 356−359. (b) Reddy, M. K.; Gupta, S. K.; Jackob, M. R.; Khan, S. I.; Ferreira, D. *Planta Med.* **2007**, *73*, 461−467.

(21) Quang, D. N.; Harinantenaina, L.; Nishizawa, T.; Hashimoto, T.; Kohchi, C.; Soma, G.; Asakawa, Y. *Biol. Pharm. Bull.* **2006**, *29*, 34−37.

(22) Chang, C. C.; Zhang, J.; Lombardi, L.; Neri, A.; Dalla-Favera, R. *Oncogene* **1994**, *9*, 923−933.

(23) Subbaramaiah, K.; Bulic, P.; Lin, Y.; Dannenberg, A. J.; Pasco, D. S. *J. Biomol. Screen.* **2001**, *6*, 101−110.

(24) Laherty, C. D.; Yang, W. M.; Sun, J. M.; Davie, J. R.; Seto, E.; Eisenman, R. N. *Cell* **1997**, *89*, 349−356.