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New Stilbenoids from Peanut (*Arachis hypogaea*) Seeds Challenged by an *Aspergillus caelatus* Strain

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Four new stilbene derivatives, termed arahypins, have been isolated from peanut seeds challenged by an *Aspergillus caelatus* strain, along with two known stilbenoids that have not been previously reported in peanuts. The structures of these new putative phytoalexins were determined by analysis of NMR, MS, and UV data. Together with other known peanut stilbenoids that were also produced in the challenged seeds, these new compounds may play a defensive role against invasive fungi.

KEYWORDS: Peanuts; *Arachis hypogaea*; arahypin; groundnuts; stilbenes; prenylated stilbenes; stilbenoids; structure elucidation; NMR; HPLC-MS

INTRODUCTION

Peanut is a plant endemic to South America that has been introduced around the world and has become an economically and nutritionally important crop (*1*). Peanuts are host to approximately 50 genera of fungi (*2*), among which *Aspergillus flavus* and *Aspergillus parasiticus* are of particular agricultural significance due to their ability to produce carcinogenic aflatoxins (*3*). Under favorable conditions, the peanut plant can resist fungal attacks by promptly producing stilbene-derived phytoalexins (*2, 4*−*6*). Such a natural phytoalexin-based mechanism of peanut resistance attracts the attention of researchers because, once understood, this mechanism of resistance could be manipulated to obtain a greater peanut resistance to pests. Such knowledge may be crucial for breeding new fungi-resistant peanut cultivars.

A number of stilbene phytoalexins from peanuts have been reported (*7*−*12*). The pathway of formation of stilbenoids (*12*−*16*) as well as examples of stilbenoids isolated from other plants (*17*−*21*) suggests that peanuts may be capable of producing several other important bioactive stilbenoids. The purpose of this research was to isolate and characterize further new and/or known peanut seed stilbenoids that may act as phytoalexins.

MATERIALS AND METHODS

Reagents, Materials, and Basic Apparatus. HPLC-grade solvents used in the preparation of mobile phases and separations on silica gel were obtained from Fisher (Suwanee, GA). HPLC-grade H_2O was prepared with a ZD20 four-bowl Milli-Q water system (Millipore). ACS-grade methanol used for seed extraction was purchased from VWR (Suwanee, GA). Silica gel for column chromatography (silica gel 60, 0.063−0.200 mm) was purchased from EM Science (Gibbstown, NJ).

Reference Compounds. *trans*-Resveratrol (approximately 99%) was purchased from Sigma. Pure individual peanut stilbenes (*trans*-arachidin-1, *trans*-arachidin-2, *trans*-arachidin-3, *trans*-3′-isopentadienyl-3,5,4′-trihydroxystilbene, and SB-1) were obtained as previously described (*12, 22*), with the exception that preparative HPLC was used in the final step instead of preparative TLC. The identities of the reference compounds as well as known stilbenes extracted from challenged peanuts were confirmed by APCI-MS/MS (MS^2) and UV spectroscopy. These data are given in parentheses as $[M + H]^+$ values followed by UV absorption maxima: *trans*-resveratrol (*m*/*z* 229; 305 and 317 nm), *trans*-arachidin-1 (*m*/*z* 313; 339 nm), *trans*-arachidin-2 (*m*/*z* 297; 308 and 322 nm), *trans*-arachidin-3 (*m*/*z* 297; 335 nm), *trans*-3′-isopentadienyl-3,5,4′-trihydroxystilbene (*m*/*z* 295; 295 nm), and SB-1 (*m*/*z* 345; 363 nm). The above results were in agreement with published data (*7*−*10, 12, 22*).

Fungal Culture. Spores of *Aspergillus caelatus* NRRL 25528 (ex type) were used to elicit phytoalexin production in peanuts.

Plant Material and Processing. The 31-1314 peanut runner breeding line, 2007 harvest, from the National Peanut Research Laboratory (Dawson, GA) was used. Peanut seeds were allowed to imbibe distilled water for 16 h at room temperature. They were then chopped with a sharp hand cutter into 3−6 mm pieces (**Figure 1**), washed with distilled water, blotted with a paper towel, air-dried to the condition where sliced peanuts did not leave water spots on filter paper, and placed on stainless steel trays so that the thickness of the layer did not exceed 1 cm. The trays were evenly sprayed with the fungal spores, placed into autoclave bags, and incubated at 30 °C for 96 h. To obtain nonviable peanut material as a control, a portion of the sliced seeds was placed into distilled boiling water for 90 s, blotted with a paper towel, and air-dried under sterile conditions. Viable peanut material, as a second control, was prepared as previously described (*12*); whole axenic peanut seeds were aseptically sliced. Nonviable (inoculated and uninoculated) and viable (inoculated and uninoculated) peanut material was placed into Petri dishes in triplicate (12 dishes total) and incubated in the same autoclave bag at 30 °C for 96 h.

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Figure 1. Sliced peanut seeds after 96 h of incubation with *A. caelatus* NRRL 25528 at 30 °C: (**A**) nonviable seeds; (**B**) viable seeds.

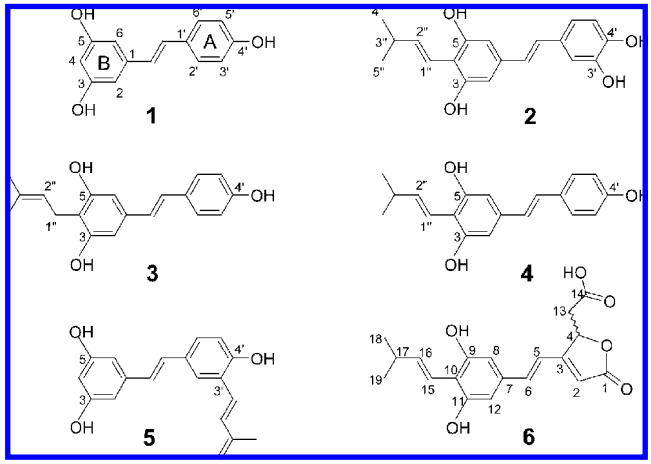

Figure 2. Structures of known stilbenoids from peanut seeds: **1**, *trans*-resveratrol; **2**, *trans*-arachidin-1; **3**, *trans*-arachidin-2; **4**, *trans*-arachidin-3; **5**, *trans*-3′-isopentadienyl-3,5,4′-trihydroxystilbene; **6**, *trans*-SB-1.

Nonviable and viable inoculated material after 96 h of incubation is shown in **Figure 1**. A portion of untreated sliced peanuts, as a third control, was frozen at −20 °C and kept frozen until analysis.

Extraction and Purification. For analytical purposes, 6 g of inoculated and incubated peanut seeds was aseptically collected from the trays every 24 h and extracted with 30 mL of MeOH in a high-speed blender for 1 min. Filtered aliquots of the extracts were used for direct determination of target constituents by HPLC. A similar extraction procedure was applied to the nonviable and viable control sliced seeds.

For preparative isolation of stilbenoids, 1.2 kg of inoculated and incubated peanut seeds was extracted with 6.0 L of MeOH in a high-speed blender for 1 min (300 mL for each portion of 60 g of seeds). The combined mixture was filtered through a paper filter in a Büchner-type funnel under reduced pressure. The solid residue was resuspended in 1.5 L of MeOH, and the extraction procedure was repeated twice. The combined extract solutions were filtered through a filter paper and defatted three times with 0.5 L of *n*-hexane. The MeOH layer was evaporated to dryness. The residue was redissolved in $CHCl_3$ and applied to a chromatographic column (34 mm i.d.) packed with silica gel to the height of 350 mm. The column was subsequently eluted with 0.3 L of $CHCl_3$, 1.2 L of EtOAc, 1.2 L of acetone, and 1.2 L of MeOH. Six fractions were collected from the column and analyzed by HPLC. Fractions containing stilbenoids **1**−**12** (**Figures 2** and **3**) were separately combined, evaporated to dryness with a rotary evaporator, and subjected to further purification on a similar silica gel column. The column was subsequently eluted with 0.3 L of $CHCl_3$, 1.2 L of $CHCl_3$/EtOAc (1:1), 1.5 L of EtOAc, 0.7 L of acetone, and 0.3 L of MeOH. Nineteen fractions were collected. Combined fractions containing compounds **7** and **8**, **9** and **10**, and **11** were evaporated to dryness on a rotary evaporator, redissolved in MeOH, filtered, and subjected to final purification with a preparative HPLC column using isocratic mobile

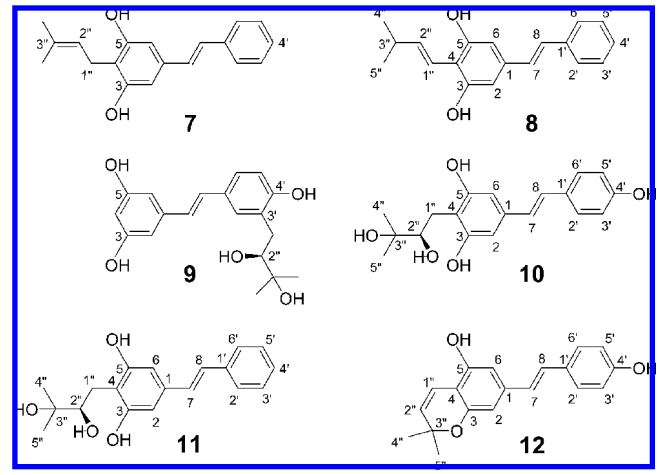

Figure 3. Structures of stilbenoids newly described here from peanut seeds: **7**, chiricanine A (*trans*-4′-deoxyarachidin-2); **8**, arahypin-1 (*trans*-4′-deoxyarachidin-3); **9**, arahypin-2 [*trans*-3′-(2″,3″-dihydroxy-3″-methylbutyl)resveratrol]; **10**, arahypin-3 [*trans*-4-(2″,3″-dihydroxy-3″-methylbutyl)resveratrol]; **11**, arahypin-4 [*trans*-4-(2″,3″-dihydroxy-3″-methylbutyl)-4′-deoxyresveratrol]; **12**, arahypin-5.

phase 1 for compounds **7** and **8**, mobile phase 2 for compounds **9** and **10**, and mobile phase 3 for compound **11**, respectively. Combined fractions containing compound **12** were evaporated to dryness on a rotary evaporator, redissolved in $CHCl_3$, and subjected to purification on a similar (350 mm × 34 mm) silica gel column using the following solvents: $CHCl_3$ (500 mL), hexane/acetone (250 + 50 mL), hexane/acetone (250 + 70 mL), hexane/acetone (500 + 200 mL), hexane/acetone (250 + 250 mL), and acetone/MeOH (250 + 250 mL). Combined fractions containing purified compound **12** were evaporated to dryness, redissolved in MeOH, and finally purified on a preparative HPLC column using mobile phase 1. Pure fractions obtained from HPLC were separately evaporated with a rotary evaporator to a point where almost all of the organic solvent was removed. The remaining aqueous mixtures containing the target compounds were separately extracted four times with EtOAc (H_2O/EtOAc ratio 1:1, v/v). The combined EtOAc layers were evaporated nearly to dryness with a rotary evaporator. The residue was transferred into a 15 mL vial with MeOH and evaporated to dryness with a stream of N_2. The residues were redissolved in acetone or EtOAc, filtered, and transferred into 4 mL vials. The vials were placed into a lyophilizer for 2 h at room temperature to remove traces of the solvents.

HPLC-DAD-MS Analyses. Analyses were performed using an HPLC system equipped with a model LC-10ATvp pump (Shimadzu), a model SPD-M10Avp DAD covering the 200−600 nm range with Shimadzu Client/Server software, version 7.3, and a model 717 plus autosampler (Waters). Separations of seed extracts and purified stilbenoids were performed on a 50 mm × 4.6 mm i.d., 2.5 μm, XTerra MS C18 analytical column (Waters). H_2O (A), MeOH (B), 2% HCOOH in H_2O (C), and CH_3CN (D) were used in the following gradient (mobile phase 4): initial conditions, 67% A/30% B/3% C, changed linearly to 2% A/95% B/3% C in 12 min, held isocratic for 4 min, and then changed to initial conditions in 0.01 min. The flow rate was 1.3 mL/min. The column was maintained at 40 °C in a model 105 column heater (Timberline Instruments, Boulder, CO). For tandem HPLC-MS analyses, a Surveyor HPLC system equipped with MS Pump Plus, Autosampler Plus, a PDA Plus Detector (Thermo Electron Corp., San Jose, CA), and a 50 mm × 4.6 mm i.d., 2.5 μm, XTerra MS C18 analytical column (Waters) was used. The column was maintained at 40 °C. The same solvents that were used for mobile phase 4 were used for the HPLC-MS analyses. The solvents were mixed in the following gradient: mobile phase 5, initial conditions, 67% A/30% B/3% C, changed linearly to 2% A/95% B/3% C in 12 min, held isocratic for 2 min, changed to initial conditions in 0.01 min; mobile phase 6, initial conditions, 70% A/27% D/3% C, changed linearly to 2% A/95% D/3% C in 12 min, held isocratic for 2 min, changed to initial conditions in 0.01 min. The flow rate for both systems was 0.8 mL/min.

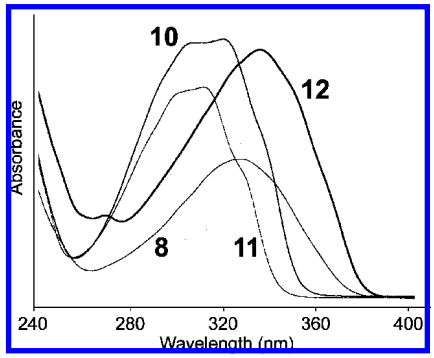

Figure 4. UV spectra of new stilbenoids in mobile phase 4: **8**, arahypin-1; **10**, arahypin-3; **11**, arahypin-4; **12**, arahypin-5.

Preparative HPLC Separations. Preparative HPLC separations were performed using a 100 mm × 19 mm i.d., 5 μm XTerra Prep RP_{18} OBD column (Waters). The column temperature was 40 °C. The following isocratic mobile phases were used: mobile phase 1, 74% CH_3CN, 3% of 2% HCOOH in H_2O, and 21% of H_2O; mobile phase 2, 55% MeOH, 5% of 2% HCOOH in H_2O, and 40% of H_2O; mobile phase 3, 55% CH_3CN, 3% of 2% HCOOH in H_2O, and 42% of H_2O. The flow rate was 8.0 mL/min.

Spectroscopic Measurements. UV measurements were performed with a Varian Cary 100 Bio UV−vis spectrophotometer. Optical rotation measurements employed a Rudolph automatic polarimeter, model APIII. ^{1}H, ^{13}C NMR, and DEPT experiments were performed on a Bruker DRX-400 instrument. HMBC data for compound **12** were obtained using a Bruker AVANCE 600 MHz instrument. Chemical shift values were referenced to the solvent signals for CD_3OD (δ_H 3.31/δ_C 49.1) or $CDCl_3$ (δ_H 7.24/δ_C 77.0). HRESIMS data were recorded on a Waters Q-Tof Premier instrument in negative ion mode. APCI-MS^n data were obtained on a Finnigan LCQ Advantage MAX ion trap mass spectrometer equipped with an APCI interface and operated with Xcalibur version 1.4 software (Thermo Electron Corp.). All data were acquired in the full-scan positive polarity mode from *m/z* 100 to 2000. Capillary temperature was 165 °C, APCI vaporizer temperature 240 °C, sheath gas flow 55 units, auxiliary/sweep gas flow 5 units, source voltage 6 kV, and source current 6 μA. In MS^2 analyses, the $[M + H]^+$ ions observed for each chromatographic peak in full-scan analyses were isolated and subjected to source collision-induced dissociation (CID) using He buffer gas. In all CID analyses, the isolation width, relative fragmentation energy, relative activation *Q*, and activation time were *m/z* 2.8, 35%, 0.25, and 30 ms, respectively. Concentrations of compounds of interest were determined by reference to peak areas of corresponding pure compounds. The results of MS^2 experiments are represented throughout the text as follows: *m/z aaa@bb*: *aaa*, *ccc*, *ddd*, where *aaa* is parent ion, *bb* is normalized collision energy (%), and *ccc* and ddd are fragment ions.

All manipulations of purified compounds were carried out under minimal lighting conditions to avoid any possible photoisomerization of the stilbenoid olefinic double bond.

Chiricanine A (7): slightly yellowish oil; UV (mobile phase 4) λ_{max} (nm) 209, 301sh, 312, 326sh; APCI-MS, *m/z* 281 ($[M + H]^+$; rel int 100), 225 (17); APCI-MS^2, *m/z* 281@35: 281 ($[M + H]^+$; rel int 4), 225 (100). The UV, MS, and NMR data agree with published values (*17*).

Arahypin-1 (8): yellowish oil; UV (mobile phase 4) λ_{max} (nm) 217, 327 (**Figure 4**); APCI-MS, *m/z* 281 ($[M + H]^+$; rel int 100), 225 (21); APCI-MS^2, *m/z* 281@35: 281 ($[M + H]^+$; rel int 3), 225 (100); HRESIMS, obsd *m/z* 279.1387, calcd for $C_{19}H_{20}O_2$ − H, 279.2385.

Arahypin-2 (9): yellowish oil; UV (mobile phase 4) λ_{max} (nm) 213, 229sh, 296sh, 306, 317; $[\alpha]_D$ +8.1 (*c* 0.45; MeOH); APCI-MS, *m/z* 331 ($[M + H]^+$; rel int 100), 327 (38), 313 (68), 295 (58), 241 (4); APCI-MS^2, *m/z* 331@35: 313 ($[M + H - H_2O]^+$; rel int 100), 295 (11). The UV, MS, and NMR data agree with published values (*18*).

Arahypin-3 (10): yellowish crystals from EtOAc; mp 213−216 °C; UV (mobile phase 4) λ_{max} (nm) 210, 230sh, 298sh, 306, 320, 335sh (**Figure 4**); $[\alpha]_D$ +20 (*c* 0.49; MeOH); APCI-MS, *m/z* 331 ($[M + H]^+$; rel int 100), 313 (28), 295 (5), 241 (18); APCI-MS^2 *m/z* 331@35: 313 ($[M + H - H_2O]^+$; rel int 100), 241 (11); HRESIMS, obsd *m/z* 329.1390, calcd for $C_{19}H_{22}O_5$ − H, 329.1389.

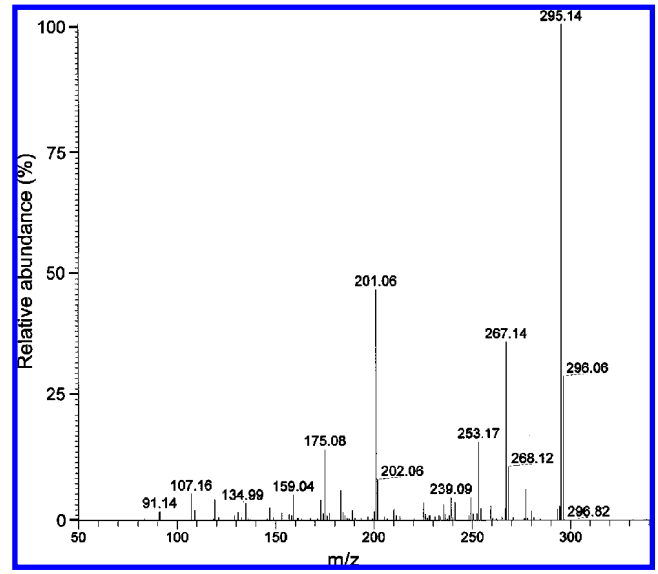

Figure 5. APCI-MS^2 data for arahypin-5 (**12**).

Arahypin-4 (11): yellowish oil; UV (mobile phase 4) λ_{max} (nm) 206sh, 226sh, 234sh, 301sh, 311, 327sh (**Figure 4**); $[\alpha]_D$ +9.5 (*c* 0.25; MeOH); APCI-MS, *m/z* 315 ($[M + H]^+$; rel int 100), 297 (28), 279 (7), 225 (15); APCI-MS^2 *m/z* 315@35: 297 ($[M + H - H_2O]^+$; rel int 100), 225 (7); HRESIMS, obsd *m/z* 313.1439, calcd for $C_{19}H_{22}O_4$ − H, 313.1440.

Arahypin-5 (12): off-white oil; UV (mobile phase 4) λ_{max} (nm) 217, 270, 339, 346sh (**Figure 4**); APCI-MS, *m/z* 295 ($[M + H]^+$; rel int 100); key HMBC correlations, H-2 → C-3, C-4, C-6, and C-7; H-6 → C-2, C-3, C-4, C-5, and C-7; H-7 → C-1, C-2, and C-6; H-8 → C-1′, C-2′, and C-6′; H-1″ → C-3, C-4, C-5, C-3″, C-4″, and C-5″; H-2″ → C-3, C-4, C-3″, C-4″, and C-5″; H_3-4″ → C-1″, C-2″, C-3″, and C-5″; H_3-5″ → C-1″, C-2″, C-3″, and C-4; APCI-MS^2 *m/z* 295@35: 295 ($[M + H]^+$; rel int 100), 267 (36), 253 (16), 201 (47), 175 (14), 159 (5), 107 (5) (**Figure 5**); HRESIMS, obsd *m/z* 293.1175, calcd for $C_{19}H_{18}O_3$ − H, 293.1178.

^{1}H and ^{13}C NMR data for **8**, **10**, **11**, and **12** are given in **Tables 1** and **2**.

RESULTS AND DISCUSSION

Structure Elucidation. Challenged peanuts were expected to synthesize stilbene phytoalexins (*7*−*12*). Three major peaks that were detected in the seed extracts after 96 h of incubation (**Figure 6**) were identified as SB-1 (**6**), arachidin-1 (**2**), and arachidin-3 (**4**). Other known phytoalexins, resveratrol (**1**), arachidin-2 (**3**), and (**5**), were detected at lower concentrations. Several other unidentified compounds (**7**−**12**) were suggested to be stilbenoids on the basis of their characteristic UV absorptions (*23*) in the 281−313 or 308−339 nm range (**Figure 4**), as well as their APCI mass spectra.

The APCI mass spectrum of **7** (**Figure 3**) revealed a molecular mass of 280 Da, and a characteristic loss of 56 Da, corresponding to a loss of C_4H_8, as is often observed for prenylated stilbenes. A characteristic absorption in the 301−312 nm range suggested the absence of a conjugated double bond in the aliphatic side chain (*7*−*9*). Upon analysis of ^{1}H, ^{13}C NMR, and APCI-MS data, the structure of **7** was shown to match that of chiricanine A, a compound originally reported by Ioset et al. (*17*) from the root bark of the leguminous tree, *Lonchocarpus chiricanus*. Similarly, compound **9** (molecular mass 330 Da) (**Figure 3**) was reported as a constituent of the evergreen tree *Artocarpus dadah* (*18*). Neither of these compounds has been

Table 1. ^{1}H NMR Spectroscopic Data for Compounds **8**, **11**, **10**, and **12**

	δ_H (*J*)			
position	**8**[a]	**11**[b]	**10**[b]	**12**[a]
2, 6	6.63 s	6.56 s	6.52 s	6.57 d (1.4), 6.41 d (1.4)
7	6.93 d (16.0)[c−e]	6.97 d (16.2)[d]	6.76 d (16.8)[e]	6.75 d (16.3)
8	7.03 d (16.0)[c−e]	7.02 d (16.2)[d]	6.93 d (16.8)[e]	6.94 d (16.3)
2′, 6′	7.46 br d (8.0)	7.49 dd (1.2, 7.4)	7.34 d (8.5)	7.34 d (8.4)
3′, 5′	7.34 t (8.0)	7.32 t (7.4)	6.76 d (8.5)	6.79 d (8.4)
4′	7.24 t (8.0)	7.21 dt (1.2, 7.4)		
1″	6.27 dd (1.6, 16.4)	2.62 dd (10.0, 13.9)	2.60 dd (10.0, 14.0)	6.59 d (10.1)
		3.13 dd (2.3, 13.9)	3.12 dd (2.4, 14.0)	
2″	6.14 dd (7.2, 16.4)	3.56 dd (2.3, 10.0)	3.55 dd (2.4, 10.0)	5.58 d (10.1)
3″	2.55 doct (1.6, 7.2)			
4″, 5″	1.13 d (7.2)	1.25 s	1.24 s	1.42 s
OHs	5.10 br s			4.81 br s, 4.86 br s

[a] In $CDCl_3$. Spectra recorded at 400 MHz; *J* values given in hertz; chemical shift values presented in parts per million. [b] In CD_3OD. Spectra recorded at 400 MHz; *J* values given in hertz; chemical shift values presented in parts per million. [c−e] Assignments with identical superscripts are interchangeable. Assignments for compound **12** were verified by HMBC data.

Table 2. ^{13}C NMR Spectroscopic Data for Compounds **8**, **11**, **10**, and **12**

position	**8**[a]	**11**[b]	**10**[b]	**12**[a]
1	146.2 s	138.9 s	138.6 s	138.6 s
2	105.8 d	106.5 d	106.3 d	105.9 d
3	153.8 s	157.9 s	157.8 s	154.1 s
4	111.8 s	115.0 s	114.3 s	109.0 s
5	153.8 s	157.9 s	157.8 s	151.2 s
6	105.8 d	106.5 d	106.3 d	107.1 d
7	127.8 d[c−e]	128.4 d[d]	128.8 d[e]	126.1 d
8	128.0 d[c−e]	128.8 d[c]	130.5 d[e]	128.4 d
1′	137.2 s	138.0 s	127.0 s	130.2 s
2′	126.7 d	127.4 d	128.7 d	128.0 d
3′	128.8 d	129.7 d	116.5 d	115.6 d
4′	129.3 d	129.9 d	158.3 s	155.3 s
5′	128.8 d	129.7 d	116.5 d	115.6 d
6′	126.7 d	127.4 d	128.7 d	128.0 d
1″	117.2 d	26.8 t	26.7 t	116.3 d
2″	137.8 d	80.9 d	80.9 d	128.4 d
3″	32.3 d	74.0 s	74.0 s	76.1 s
4″	22.4 q	25.5 q	25.5 q	27.8 q
5″	22.4 q	25.3 q	25.3 q	27.8 q

[a] In $CDCl_3$. [b] In CD_3OD. Spectra recorded at 100 MHz; chemical shift values presented in parts per million. Carbon multiplicities are shown next to the shifts and were established by DEPT experiments. Position assignments were made on the basis of chemical shifts, multiplicities, and comparisons with similar analogues in the literature. A different numbering system was used in ref *24* for a close analogue of **12**, but the ^{13}C NMR shifts corresponding to C4 and C5 in the present paper were apparently accidentally reversed in that reference. [c−e] Assignments with identical superscripts are interchangeable. Assignments for compound **12** were verified by HMBC data.

reported previously as a peanut metabolite. All four of the new metabolites described below are related to these known compounds, and their structure assignments were facilitated by this resemblance. As no common name was proposed by the authors for compound **9** (*18*) and because this compound was isolated during the present research from peanuts together with structurally similar compounds **10** and **11** (**Figure 3**), we propose the name arahypin-2 for compound **9**.

The mass spectrometric data for **8** (**Figure 3**) matched very closely with those of chiricanine A (**7**), which is an isomer of **8**. However, a UV_{max} at 327 nm (**Figure 4**) suggested the presence of a conjugated double bond in the side chain in this instance. Analysis of ^{1}H, ^{13}C, and DEPT NMR data for **8** in comparison to those for **7** confirmed the very close structural similarities. Interpretation of these data was straightforward and led to recognition that the structure of **8** differs from that of **7** only in that the side-chain olefin unit is located between C1″ and C2″, rather than between C2″ and C3″. This difference is reflected in the replacement of the NMR signals for the vinylic methyls, the trisubstituted olefin, and the adjacent methylene unit in the data for **7** with signals for an isopropyl group linked to a *trans*-olefin unit in the spectra of **8** (**Tables 1** and **2**). Compound **8** has not been previously reported to our knowledge. Thus, a common name, arahypin-1, is proposed for **8**. A compound similar to **8**, but somewhat less polar, 3,5-dimethoxy-4′-deoxyarachidin-3, has been reported in peanut root mucilage, albeit only with a tentative structure assignment (*11*).

The UV (**Figure 4**) and mass spectra of compound **10** were similar to those of **9** (*18*), which again suggested a close structural similarity. The molecular formula of **10** was determined to be $C_{19}H_{22}O_5$ (nine degrees of unsaturation) on the basis of ^{1}H, ^{13}C NMR, DEPT, and HRMS data. Analysis of the ^{1}H, ^{13}C, and DEPT NMR data (**Tables 1** and **2**) revealed that **10** contains a dihydroxylated prenyl group analogous to that of **9** (*18*), which was also encountered in the extract as noted above. This conclusion was enabled by observation of NMR signals for a dimethylated OH bearing a quaternary carbon and an isolated CH_2-CHOH unit in place of those corresponding to the prenyl groups found in the spectra of **7** and **8**. The NMR spectra for **10** also clearly indicated the presence of an oxygenated para-disubstituted benzene ring, as well as the same type of symmetrical 1,2,3,5-tetrasubstituted ring found in **7** and **8**. These results indicate that the dihydroxylated prenyl group in **10**is located on the dihydroxylated ring, by analogy to the situation in **7** and **8**, rather than on the monooxygenated ring, as in **9**. Compound **10** (**Figure 3**) also appears to be a new natural product and was assigned the common name arahypin-3.

Compound **11** has a molecular formula of $C_{19}H_{22}O_4$ (nine degrees of unsaturation), as established by the analysis of NMR (**Tables 1** and **2**) and HRMS data. It differs from **10** only in the absence of the hydroxy group at C4′. The presence of one fewer oxygen atom in the molecular formula relative to that of **10**, together with replacement of the para-disubstituted patterns present in the ^{1}H and ^{13}C NMR spectra of **10** with diagnostic phenyl group NMR signals in the data for **11**, made the assignment particularly straightforward. This structure assignment for **11** is consistent with its significantly lower polarity, as reflected by a considerable difference in retention times for **10** and **11** (**Figure 6**). It should be noted that when the original seed extract was analyzed directly by HPLC without any prepurification procedure, **11** coeluted with arachidin-2 (**3**), but

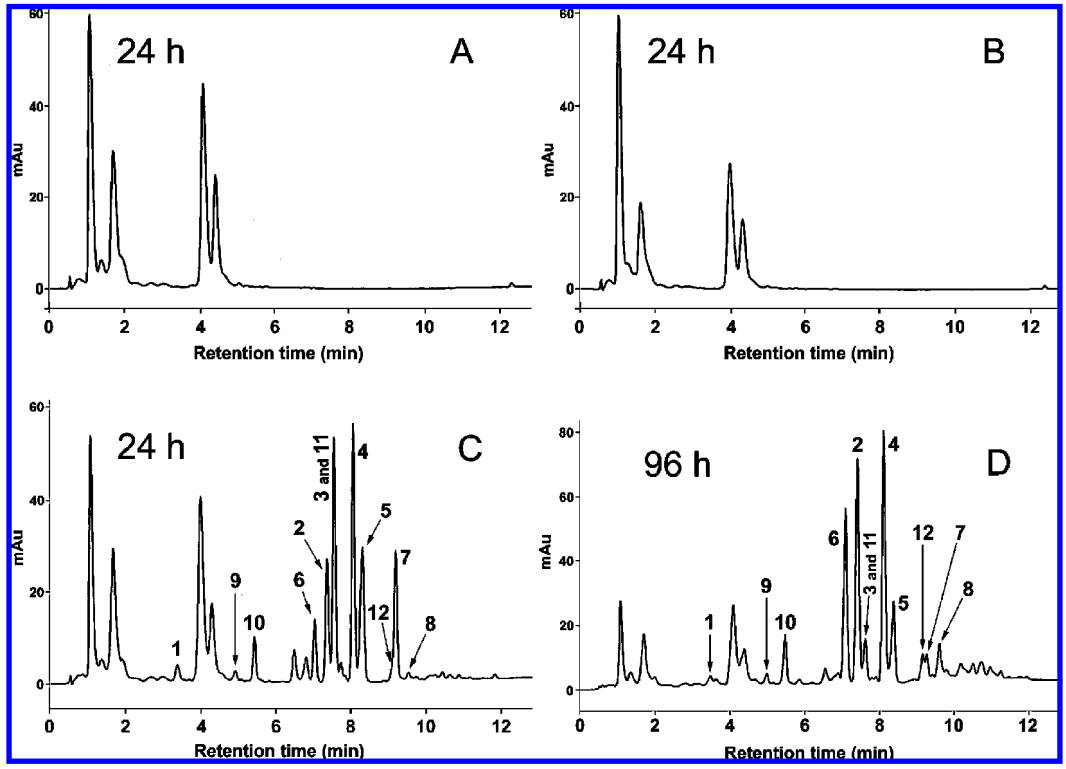

Figure 6. HPLC results for peanut seed extracts: (**A**) nonviable uninoculated seeds after 24 h of incubation; (**B**) viable uninoculated seeds after 24 h of incubation; (**C**) viable seeds after 24 h of incubation with *A. caelatus*; (**D**) viable seeds after 96 h of incubation with *A. caelatus*. Peaks: **1**, resveratrol; **2**, arachidin-1; **3**, arachidin-2; **4**, arachidin-3; **5**, *trans*-3′-isopentadienyl-3,5,4′-trihydroxystilbene; **6**, SB-1; **7**, chiricanine A; **8**, arahypin-1; **9**, arahypin-2; **10**, arahypin-3; **11**, arahypin-4; **12**, arahypin-5. Stilbenoids **1**−**12** were all detected in the *trans*-configuration. The chromatograms were recorded at 315 nm.

11 could be isolated by reversed phase HPLC after fractionation of the extract on silica gel. Compound **11** (**Figure 3**) was named arahypin-4.

Compound **12** was assigned a molecular formula of $C_{19}H_{18}O_3$ (11 degrees of unsaturation) by analysis of NMR and HRMS data and clearly contained a unit not found in metabolites **7**−**11**. Unlike compounds **7** and **8**, which displayed a characteristic loss of 56 Da (C_4H_8), and compounds **9**, **11**, and **12**, which showed a major neutral loss of H_2O (18 Da), compound **12** produced a highly fragmented spectrum (**Figure 5**) that included a distinctive loss of 28 Da (CO or C_2H_4). The ^{1}H spectrum included signals representative of an isolated *cis*-olefin unit that was suggestive of the cyclization of a prenyl unit with an ortho position of the aromatic ring to form a dimethyl coumarin unit. The position of the prenyl group cyclization was based on comparison of NMR data with those of similar compounds in the literature (*24*), together with the fact that all of the other stilbenoids in the mixture, described here and in earlier papers (*12*), that have a prenyl group linked to the dihydroxylated ring, place the group in a position analogous to that shown for **12**. To verify this regiochemical assignment, and to enable unambiguous NMR shift assignments for all positions, an HMBC experiment was performed. As expected, the signal for H-1″ showed correlations to both of the oxygenated carbons of the adjoining aromatic ring (C-3 and C-5), whereas both H-2 and H-6 showed correlations to C-7. These results confirmed the location of the new ring as shown in structure **12**. Although a compound containing a methoxy group in place of the 3-OH group in **12** has been reported from Cubé resin (*24*), to our knowledge, compound **12** is also a previously unreported compound and was assigned the name arahypin-5.

Given the propensity for stilbenoids to undergo olefin photoisomerization under certain conditions, it should be noted that, in all of the experiments described here, compounds **1**−**12** (**Figures 2** and **3**) were detected strictly in the *trans*-configuration on the basis of the large *J*value (16−17 Hz) for the corresponding olefinic ^{1}H signals in each case. For compounds **9**−**11**, another stereochemical issue is present, that is, the absolute configuration at the stereocenter in each side chain. Studies reported for known compound **9** afforded the intriguing result that **9** was originally isolated (from *A. dadah*) in a 5:3 enantiomeric ratio favoring the *R*-isomer (*18*). Comparison of the specific rotation of the sample of **9** obtained in the current study (+8.1) with that of the sample described in the literature (+4.0) indicated that the sample of **9** obtained from peanuts is also not a pure enantiomer, but is instead present in approximately a 2:1 *R*-to-*S* ratio, rather than a 5:3 ratio. Compounds **10** and **11** also showed positive specific rotations and are presumed to favor the *R*-form over the *S*-form by analogy with the case for compound **9**, but their enantiomeric identities and ratios were not rigorously determined.

Dynamics of Stilbenoid Production and Suggested Role of New Stilbenoids. An *A. caelatus* strain was chosen as a biotic phytoalexin elicitor because it demonstrated a slightly higher growth rate and a faster phytoalexin production response in our previous experiments (*25*). Panels **A** and **B** of **Figure 1** show sliced nonviable and viable peanut seeds, respectively, after 96 h of incubation with *A. caelatus*. Nonviable peanuts were completely colonized with the fungus, whereas only a few colonies were counted on viable peanuts. Apparently, under experimental conditions, viable peanuts were able to suppress fungal growth compared to nonviable peanuts. Viable peanuts produced an array of stilbenoids at significant concentrations (**Figure 6C** and **D**), whereas no stilbenoids were detected in nonviable or viable uninoculated control samples (**Figure 6A**,**B**). Apparently, slicing alone did not serve as the eliciting factor. Chromatograms shown

in **Figure 6A**,**B** were virtually indistinguishable from chromatograms that were obtained by analysis of the extract of frozen peanut samples at day 0. Most likely, the yellowish color of viable seeds (**Figure 1B**) can be attributed to the pigmented phytoalexin SB-1 (*12*), which was determined in the seeds at high concentrations; no other pigments were detected in infected peanut seeds at early times of incubation. **Figure 6C** illustrates that, just after 24 h of incubation, all of the known and new stilbenoids described here were simultaneously accumulated at considerable levels. At the same time, there was no microscopically visible evidence of spore germination, which allows the conclusion that peanuts promptly responded to dormant spores or germinating spores at very early stages of germination, even before germination could be visibly detected. Among these stilbenoids, the arachdins **2**, **3**, **4**, and **5** as well as chiricanine A (**7**) were the major components (**Figure 6C**). From 24 to 96 h (intermediate chromatograms are not shown), the profile gradually changed, so that the concentrations of arachdin-1 (**2**), arachidin-3 (**4**), and SB-1 (**6**) became significantly higher compared with the concentrations of other stilbenoids. The most noticeable difference occurred in the levels of arachidin-2 (**3**) and chiricanine A (**7**), which became significantly lower after 96 h (**Figure 6D**). It should be noted that **3** and **7** were the only stilbenoids in the present study that incorporated the prenyl side chain as a 3-methyl-2-butenyl group, rather than the 3-methyl-1-butenyl moiety found in **2**, **4**, **6**, and **8** (**Figures 2** and **3**).

Compounds **10** and **11** are likely the products of peanut seeds rather than the products of their detoxification by the fungus because a similar compound **9** was isolated from unchallenged tree bark (*18*). Compound **9** was also isolated from peanut seeds in the present research. Indirect support for this suggestion comes from the fact that detoxification of the isoflavanone kievitone, which bears the same type of prenyl side chain as **3** and **7**, occurs by hydration of the chain. This hydration is performed by a fungal extracellular enzyme (*26*). The pterocarpan phaseollidin is also detoxified by hydration of its prenyl side chain by an intracellular oxygenase enzyme (*26*). In these instances, only one hydroxy group is added to the side chain. The mechanism of formation of **9**−**11** seems to be different and not related to fungal activity. The fact that the concentrations of compounds **3** and **7** were considerably higher after 24 h compared with the concentrations of **10** and **11**, but were about equal after 96 h, indicates that compounds **3** and **7** may be precursors of **10** and **11**, respectively (**Figures 2** and **3**). Also, one of the most abundant stilbenoids, arachidin-2 (**3**), is a likely precursor of compound **12**, which could arise from either an enzyme- or acid-catalyzed cyclization to form the dimethylpyran unit.

Overall, the production patterns of major phytoalexins elicited by the *A. caelatus* strain were in agreement with the production patterns elicited by other *Aspergillus* species (*25*).

The following calculations allow estimation of the ratio of phytoalexin accumulation by the peanuts to spore weight that triggered this accumulation. Sliced peanut seeds were evenly sprayed with a suspension of spores, so that a 20 cm^2 area on the tray with peanuts that represented 6 g of seeds was covered with about 1×10^7 spores. Assuming that average spore weight is 0.1 pg/spore (*27*), the total concentration of spores can be estimated at 0.17 μg/g of peanuts. Overall production of stilbenoids after 24 h of incubation was estimated at 2.1 mg/g of seed wet weight on the basis of the assumption, for simplicity, that the new stilbenoids have molar extinction coefficients similar to that of chiricanine A (**7**) (*17*). Taking into account that the highest concentrations of phytoalexins are produced by peanut tissues in very close proximity to the site of infection (*25*), one may assume that the major part of the 2.1 mg of stilbenoids was concentrated close to the surfaces of the slices around the fungal spores, but not close to the surfaces that were free from the spores. The yellow coloration of the sliced seeds on the infected surfaces indirectly supports this assumption. Accordingly, the ratio of the spore weight to phytoalexin weight (0.17 μg vs 2.1 mg) is roughly equal to 1:12000, which indicates that the weight balance is shifted significantly toward the phytoalexins. Nevertheless, under the laboratory conditions, the fungus eventually wins the "battle." High concentrations of fungal spores that were applied on heavily wounded peanuts may be responsible for the outcome of the plant−fungus interaction. Further experiments with low spore concentrations and/or even individual spores may clarify the mechanism of this interaction.

Stilbene derivatives in general are known for their biological activity (*4*, *28*−*30*). Chiricanine A (**7**) demonstrated antifungal effects against *Cladosporium cucumerinum* and also showed toxicity toward larvae of the yellow fever-transmitting mosquito *Aedes aegypti* (*17*). It was demonstrated that the presence of the prenyl chain in **7** was required for the antifungal properties of this compound (*17*). It is reasonable to suggest that prenylated compound **8** may possess antifungal properties as well, on the basis of the fact that arachidin-2 (**3**) and arachidin-3 (**4**), which differ from each other only by the position of the double bond, like **7** and **8**, both possess antifungal properties and inhibit spore germination and hyphal extension of *A. flavus* (*28*). A similar suggestion may be valid for compounds **10** and **11** that bear the same dihydroxydimethylpropyl group as known compound **9** (**Figure 3**). Compound **9** demonstrated inhibitory effects against cyclooxygenases and particularly against cyclooxygenase-1 (*18*).

Although it is reasonable to suggest that new compounds **8**, **10**, **11**, and **12** are likely to show biological activity on the basis of the comparisons above, experiments with appropriate test organisms or in vitro systems would be required to verify the presence of such activities. Considering the importance of knowledge of natural plant defense mechanisms, studies of the biological activity of these new stilbenoids are planned.

The present study revealed the production of four new stilbenoids, **8** and **10**−**12** (**Figure 3**), by fungus-challenged peanut seeds. In addition, two stilbenoids that have been reported previously from other sources (**7** and **9**) are reported in peanuts for the first time. Within 24 h, peanut seeds produced numerous stilbene phytoalexins, including the new stilbenoids, at significant concentrations before or at the very initial stage of fungal spore germination. Stilbenoids may play a protective role against fungi, as reflected in the literature (*2*, *6*, *10*, *17*, *28*−*30*).

ABBREVIATIONS USED

APCI-MS, atmospheric pressure chemical ionization mass spectrometry; DEPT, distortionless enhancement by polarization transfer.

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LITERATURE CITED

(1) Paxton, J. D. Biosynthesis and accumulation of legume phytoalexins. In *Mycotoxins and Phytoalexins*; Sharma, R. P.; Salunkhe, D. K., Eds.; CRC Press: Boca Raton, FL, 1991; pp 485−499.

(2) Subba Rao, P. V.; Strange, R. N. Chemistry, biology, and role of groundnut phytoalexins in resistance to fungal attack. In *Handbook of Phytoalexin Metabolism and Action*; Daniel, M.; Purkayastha, R. P., Eds.; Dekker: New York, 1995; pp 199−227.

(3) Cole, R. J.; Cox, R. H. *Handbook of Toxic Fungal Metabolites*; Academic Press: New York, 1981; pp 937.

(4) Cole, R. J.; Dorner, J. W. Peanut phytoalexins. In *Mycotoxins and Phytoalexins*; Sharma, R. P.; Salunkhe, D. K., Eds.; CRC Press: Boca Raton, FL, 1991; pp 501−509.

(5) Dorner, J. W.; Cole, R. J.; Sanders, T. H.; Blankenship, P. D. Interrelationship of kernel water activity, soil temperature, maturity, and phytoalexin production in preharvest aflatoxin contamination of drought-stressed peanuts. *Mycopathologia* **1989**, *105*, 117–128.

(6) Sobolev, V. S.; Guo, B. Z.; Holbrook, C. C.; Lynch, R. E. Interrelationship of phytoalexin production and disease resistance in selected peanut genotypes. *J. Agric. Food. Chem.* **2007**, *55*, 2195–2200.

(7) Ingham, J. L. 3,5,4′-Trihydroxystilbene as a phytoalexin from groundnuts (*Arachis hypogaea*). *Phytochemistry* **1976**, *15*, 1791–1793.

(8) Keen, N. T.; Ingham, J. L. New stilbene phytoalexins from American cultivars of *Arachis hypogaea*. *Phytochemistry* **1976**, *15*, 1794–1795.

(9) Aguamah, G. A.; Langcake, P.; Leworthy, D. P.; Page, J. A.; Pryce, R. J.; Strange, R. N. Two novel stilbene phytoalexins from *Arachis hypogaea*. *Phytochemistry* **1981**, *20*, 1381–1383.

(10) Cooksey, C. J.; Garratt, P. J.; Richards, S. E.; Strange, R. N. A dienyl stilbene phytoalexin from *Arachis hypogaea*. *Phytochemistry* **1988**, *27*, 1015–1016.

(11) Sobolev, V. S.; Potter, T. L.; Horn, B. W. Prenylated stilbenes from peanut root mucilage. *Phytochem. Anal.* **2006**, *17*, 312–322.

(12) Sobolev, V. S.; Deyrup, S. T.; Gloer, J. B. New peanut (*Arachis hypogaea*) phytoalexin with prenylated benzenoid and but-2-enolide moieties. *J. Agric. Food. Chem.* **2006**, *54*, 2111–2115.

(13) Hahlbrook, K.; Scheel, D. Physiology and molecular biology of phenylpropanoid metabolism. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1989**, *40*, 347–369.

(14) Morita, H.; Noguchi, H.; Schröder, J.; Abe, I. Novel polyketides synthesized with a higher plant stilbene synthase. *Eur. J. Biochem.* **2001**, *268*, 3759–3766.

(15) Dixon, R. A.; Pavia, N. L. Stress-induced phenylpropanoid metabolism. *Plant Cell* **1995**, *7*, 1085–1097.

(16) Hart, J. H. Role of phytostilbenes in decay and disease resistance. *Annu. Rev. Phytopathol.* **1981**, *19*, 437–458.

(17) Ioset, J.-R.; Marston, A.; Gupta, M. P.; Hostettman, K. Five new prenylated stilbenes from the root bark of *Lonchocarpus chiricanus*. *J. Nat. Prod.* **2001**, *64*, 710–715.

(18) Su, B.-N.; Cuendet, M.; Hawthorne, M. E.; Kardano, L. B. S.; Riswan, S.; Fong, H. H. S.; Mehta, R. G.; Pezzuto, J. M.; Kinghorn, A. D. Constituents of the bark and twigs of *Artocarpus dadah* with cyclooxygenase inhibitory activity. *J. Nat. Prod.* **2002**, *65*, 163–169.

(19) Langcake, P.; Pryce, R. J. A new class of phytoalexins from grapevines. *Experientia* **1977**, *33*, 151–152.

(20) Yoder, B. J.; Cao, S.; Norris, A.; Miller, J. S.; Ratovoson, F.; Razafitsalama, J.; Andriantsiferana, R.; Rasamison, V. E.; Kingston, D. G. I. Antiproliferative prenylated stilbenes and flavonoids from *Macaranga alnifolia* from the Madagascar rainforest. *J. Nat. Prod.* **2007**, *70*, 342–346.

(21) Huang, K.-S.; Wang, Y.-H.; Li, R.-L.; Lin, M. Stilbene dimers from the lianas of *Gnetum hainanense*. *Phytochemistry* **2000**, *54*, 875–881.

(22) Sobolev, V. S.; Cole, R. J.; Dorner, J. W.; Yagen, B. Isolation, purification, and liquid chromatographic determination of stilbene phytoalexins in peanuts. *J. AOAC Int.* **1995**, *78*, 1177–1182.

(23) Hillis, W. E.; Ishikura, N. The chromatographic and spectral properties of stilbene derivatives. *J. Chromatogr.* **1968**, *32*, 323–336.

(24) Fang, N.; Casida, J. E. New bioactive flavonoids and stilbenes in Cubé resin insecticide. *J. Nat. Prod.* **1999**, *62*, 205–210.

(25) Sobolev, V. S. Localized production of phytoalexins by peanut (*Arachis hypogaea*) kernels in response to invasion by *Aspergillus* species. *J. Agric. Food. Chem.* **2008**, *56*, 1949–1954.

(26) VanEtten, H. D.; Matthews, D. E.; Matthews, P. S. Phytoalexin detoxification: importance for pathogenicity and practical implications. *Annu. Rev. Phytopathol.* **1989**, *27*, 143–164.

(27) Smith, C. S.; Slade, S. J.; Nordheim, E. V.; Cascino, J. J.; Harris, R. F.; Andrews, J. H. Sources of variability in the measurement of fungal spore yields. *Appl. Environ. Microbiol.* **1988**, *54*, 1430–1435.

(28) Wotton, H. R.; Strange, R. N. Circumstantial evidence for phytoalexin involvement in the resistance of peanuts to *Aspergillus flavus*. *J. Gen. Microbiol.* **1985**, *131*, 487–494.

(29) Schultz, T. P.; Nicholas, D. D.; Fisher, T. H. Quantitative structure−activity relationships of stilbenes and related derivatives against wood-destroying fungi. *Recent Res. Dev. Agric. Food Chem* **1997**, *1*, 289–299.

(30) Schultz, T. P.; Boldin, W. D.; Fisher, T. H.; Nicholas, D. D.; McMurtrey, K. D.; Pobanz, K. Structure−fungicidal properties of some 3- and 4-hydroxylated stilbenes and bibenzyl analogues. *Phytochemistry* **1992**, *31*, 3801–3806.

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