# Cytotoxic and Other Metabolites of Aspergillus Inhabiting the Rhizosphere of Sonoran Desert Plants 1

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# Cytotoxic Constituents of *Aspergillus terreus* from the Rhizosphere of *Opuntia versicolor* of the Sonoran Desert

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A novel cyclopentenedione, asterredione (**1**), two new terrecyclic acid A derivatives, (+)-5(6)-dihydro-6-methoxyterrecyclic acid A (**2**) and (+)-5(6)-dihydro-6-hydroxyterrecyclic acid A (**3**), and five known compounds, (+)-terrecyclic acid A (**4**), (−)-quadrone (**5**), betulinan A (**6**), asterriquinone D (**7**), and asterriquinone C-1 (**8**), were isolated from *Aspergillus terreus* occurring in the rhizosphere of *Opuntia versicolor*, using bioassay-guided fractionation. Acid-catalyzed reaction of **2** under mild conditions afforded **4**, whereas under harsh conditions **2** yielded **5** and (−)-isoquadrone (**9**). Catalytic hydrogenation and methylation of **4** afforded 5(6)-dihydro-terrecyclic acid A (**10**) and (+)-terrecyclic acid A methyl ester (**11**), respectively. The structures of **1**−**11** were elucidated by spectroscopic methods. All compounds were evaluated for cytotoxicity in a panel of three sentinel cancer cell lines, NCI-H460 (non-small cell lung cancer), MCF-7 (breast cancer), and SF-268 (CNS glioma), and were found to be moderately active. Cell cycle analysis of **2**, **4**, and **5** using the NCI-H460 cell line indicated that **4** is capable of disrupting the cell cycle through an apparent arrest to progression at the $G_1$ and $G_2$/M phases in this p53 competent cell line. A pathway for the biosynthetic origin of asterredione (**1**) from asterriquinone D (**7**) is proposed.

Soil-derived microorganisms represent a valuable resource in the search for secondary metabolites with useful therapeutic applications.[1] Rhizosphere soil, defined as the volume of soil adjacent to and influenced by plant roots (generally less than 1 mm in thickness), constitutes a region of intense microbial activity.[2] Because of their proximity to plant root exudates, rhizosphere microbial communities are distinct from those of nonrhizosphere (bulk) soil, and the number of microorganisms occurring in the rhizosphere is normally 10−20 times greater than that in the nonrhizosphere soil.[3] This is not surprising given that each day plants release 10−40% of their photosynthate from their roots into the soil.[4] The amount released is influenced by a number of factors including the developmental stage of the plant, soil type, stress, plant nutrition, and microbial activities.[2a] As a consequence, the diversity of microbial species in the rhizosphere has been postulated to be influenced by different plant species,[5] their phylogeny, and environmental factors affecting plant growth.[6] Recent results have substantiated these hypotheses. Techniques based on the analysis of microbial DNA extracted from rhizosphere communities (such as 16s RNA sequence analysis and DNA melting hybridization) as well as analysis of fatty acid metabolic profiles indicate that the rhizosphere communities of plant species differ from each other and from nonrhizosphere communities in the surrounding soil.[7] This includes microorganisms found in the rhizospheres of desert plants.[8]

The demonstration that rhizosphere microbial diversity is strongly influenced by the diversity of plant species and environmental factors suggests a previously unexploited opportunity to harvest unique secondary metabolites from the rhizosphere of floristically diverse plant communities such as those in the Sonoran desert. We have recently initiated an extensive program to uncover anticancer agents in the rhizosphere microflora of Sonoran desert plants and thus far have collected over 20 000 rhizosphere bacteria and 3000 rhizosphere fungi. From this library of microorganisms, we have cultured over 1500 fungi, prepared extracts from these, and tested the extracts for cytotoxicity in a panel of three sentinel cancer cell lines [NCI-H460 (non-small cell lung cancer), MCF-7 (breast cancer), SF-268 (CNS glioma)] recently recommended by the NCI as a primary screen before submitting to the 60 cell-line assay.[9] Several fungal extracts exhibiting ≥90% inhibition of at least one of the three cancer cell lines at a dose of 10 $\mu$g/mL were selected for bioactivity-guided fractionation, and one such extract was derived from *Aspergillus terreus* Thom. (Moniliaceae), occurring in the rhizosphere of the staghorn cholla (*Opuntia versicolor* Engelm; Cactaceae). *Aspergillus* species have been extensively investigated before and have yielded anthraquinones,[10] asterriquinones,[11] aspochalasins,[12] aflatoxin analogues,[13] isoquinoline alkaloids,[14] *p*-terphenyls,[15] xanthones,[16] bioanthrones,[17] tryprostatin analogues,[18] and sesquiterpenes,[19] among other compounds. Some of these *Aspergillus* constituents exhibit interesting biological activities, such as the angioinhibitory effect of fumagillin from *A. fumigatus fresenius*.[20] In addition, reports have appeared that demonstrate in vitro and in situ inhibition of MAP2-dependent microtubule assembly by tryprostatin derivatives of *A. fumigatus* BM 939,[18] HIV-reverse transcriptase inhibitory activity of asterriquinones from *A. terreus*, [21] inhibition of binding of the Grb-2 adapter to phosphorylated EGF receptor tyrosine kinase by asterriquinones of *A. candidus*,[11b] and oral hypoglycemic activity of some asterriquinones from *Pseudomassaria*.[22]

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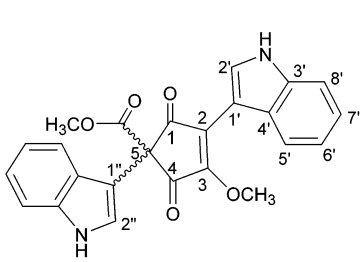
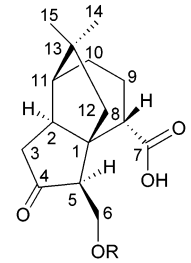
**Chart 1**

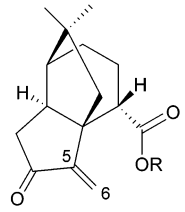


**1**



**2** R = $CH_3$

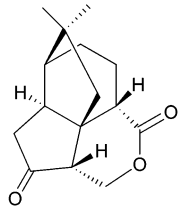
**3** R = H



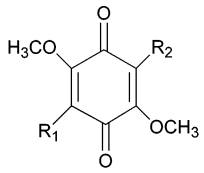
**4** R = H

**10** R = H ; 5(6)-dihydro

**11** R = $CH_3$



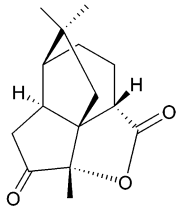
**5**



**6** $R_1 = R_2$ = Ph

**7** $R_1 = R_2$ =

**8** $R_1$ = $R_2$ =



**9**

Using a bioassay-guided approach, we now report that the cytotoxic EtOAc extract of *A. terreus* occurring in the rhizosphere of staghorn cholla contains a novel type of cyclopentenedione, asterredione (**1**), two new sesquiterpenes, (+)-5(6)-dihydro-6-methoxyterrecyclic acid A (**2**) and (+)-5(6)-dihydro-6-hydroxyterrecyclic acid A (**3**), and the known compounds (+)-terrecyclic acid A (**4**),[23] quadrone (**5**),[19,24] betulinan A (**6**),[25] asterriquinone D (**7**),[26] and asterriquinone C-1 (**8**).[26] The structures of **2**−**5** were supported by chemical interconversions, which also resulted in the formation of (−)-isoquadrone (**9**).[27,28] Catalytic hydrogenation and methylation of **4** afforded the corresponding derivatives **10** and **11**. Herein we report the isolation of **1**−**8**, structure elucidation of **1**−**3**, cytotoxicities of **1**−**11** toward three sentinel cancer cell lines, and the effect of **2**, **4**, and **5** on the cell cycle progression of the non-small cell lung cancer cell line NCI-H460.

## Results and Discussion

The bioactive $CHCl_3$ fraction obtained from an EtOAc extract of the rhizosphere fungus *A. terreus* on gel permeation and silica gel chromatography afforded compounds **1**−**8**. Asterredione (**1**) was obtained as a yellow solid that analyzed for $C_{24}H_{18}N_2O_5$ by a combination of HRMS and $^{13}C$ NMR spectroscopy and indicated 17 degrees of unsaturation. Its IR spectrum had absorption bands at 3392, 1751, and 1718 $cm^{-1}$, suggesting the presence of NH/OH, ester carbonyl, and $\alpha,\beta$-unsaturated ketone carbonyl. The $^1H$ NMR spectrum of **1** indicated the presence of two $OCH_3$ singlets at $\delta$ 4.45 and 3.78 and two 3-substituted indole residues. The $^{13}C$ NMR spectrum, while confirming the above functionalities in **1**, suggested the presence of two ketone carbonyls ($\delta$ 192.9 and 191.2), an ester carbonyl ($\delta$ 167.3), two olefinic carbons ($\delta$ 160.6 and 132.5), of which one is oxygenated, and a quaternary carbon ($\delta$ 64.3). In the HMBC spectrum, in addition to the expected correlations within the two indole moieties, the $OCH_3$ signal at $\delta_H$ 4.45 showed a correlation to the CO at $\delta_C$ 167.3, indicating the presence of a $CO_2CH_3$ group. The second $OCH_3$ signal at $\delta_H$ 3.78 had a strong correlation to the olefinic carbon at $\delta_C$ 160.6. The two indolyl moieties, $CO_2CH_3$ and $OCH_3$ groups account for $C_{19}H_{18}N_2O_3$ and 13 units of unsaturation. Thus, the remaining fragment of **1** has the partial formula $C_5O_2$ with 4 units of unsaturation and two carbonyl functions, which suggested a fully substituted cyclopentenone. The close $^{13}C$ chemical shifts of the carbonyls ($\delta$ 192.9 and 191.2) supported a cyclopent-2-ene-1,4-dione and not a cyclopent-4-ene-1,2-dione structure. On the basis of the differences in chemical shifts of 2-H of the two indole groups, the one having 2-H at $\delta$ 8.21 was placed at the C-2/C-3 olefinic carbon of the cyclopent-2-ene-1,4-dione and the other with 2-H at $\delta$ 7.27 at the quaternary carbon, C-5. Since the $OCH_3$ group is attached to an olefinic carbon (C-2/C-3; see above), the $CO_2CH_3$ group was placed at C-5. The $^{13}C$ NMR data for the cyclopentenedione moiety of **1** showed very close resemblance to those of the spirodione moiety of the recently reported methyl mutadione A.[29] Therefore, the structure of asterredione was elucidated as 2,5-di(indol-3′-yl)-3-methoxy-5-methoxycarbonylcyclopent-2-ene-1,4-dione (**1**). With the available data it was not possible to determine the stereochemical disposition of the indole and methoxy-

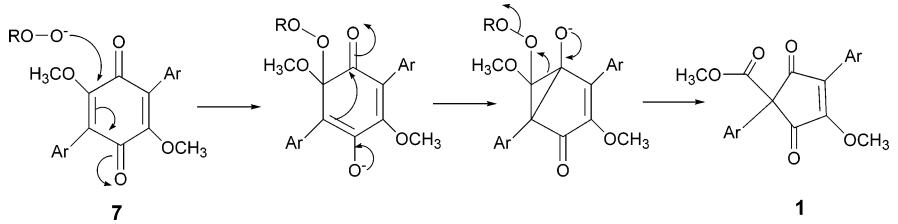



**Figure 1.** Proposed biosynthetic pathway to asterredione (**1**) from asterriquinone D (**7**).

**Table 1.** $^1$H (500 MHz) and $^{13}$C (125 MHz) NMR Data for **2** and **3** in $CDCl_3$

| | **2** | | | **3** | |
|---|---|---|---|---|---|
| position | $\delta_H$[a] | $\delta_C$[b] | HMBC | $\delta_H$[a] | $\delta_C$[b] |
| 1 | | 53.8 s | H-6, H-9 | | 53.3 s |
| 2 | 2.61 dd (12.0, 6.8) | 45.3 d | | 2.76 dd (11.9, 7.5) | 45.2 d |
| 3 | 2.52 ddd (19.9, 12.0, 1.0) | 40.7 t | | 2.57 dd (20.3, 11.9) | 40.9 t |
| | 2.34 dd (19.9, 6.8) | | | 2.39 dd (20.3, 7.5) | |
| 4 | | 213.8 s | H-2, H-6 | | 218.3 s |
| 5 | 2.94 dd (9.8, 3.6) | 56.6 d | H-3 | 2.85 t (6.7) | 57.9 d |
| 6 | 3.44 t (9.8) | 71.1 t | $OCH_3$-6 | 3.71 dd (11.0, 7.0) | 60.6 t |
| | 3.93 dd (9.8, 3.6) | | | 4.01 dd (11.0, 6.7) | |
| 7 | | 175.5 s | H-9 | | 178.3 s |
| 8 | 2.90 d (7.3) | 50.5 d | H-12 | 2.88 t (3.0) | 48.7 d |
| 9 | 1.85 m | 22.2 t | | 1.76−1.92 m | 22.0 t |
| | 1.97 t (6.3) | | | | |
| 10 | 1.76 m | 28.5 t | H-8 | 1.76−1.92 m | 28.6 t |
| | 1.92 m | | | | |
| 11 | 1.83 m | 49.3 d | H-3 | 1.76−1.92 m | 48.8 d |
| 12 | 1.46 d (14.6) | 47.7 t | | 1.51 s | 47.6 t |
| | 1.55 d (14.6) | | | | |
| 13 | | 39.9 s | H-10, H-11 | | 40.0 s |
| 14 | 1.10 s | 34.1 q | | 1.12 s | 34.2 q |
| 15 | 1.15 s | 27.1 q | | 1.15 s | 27.1 q |
| $OCH_3$ | 3.41 s | 59.5 q | | | |

[a] Multiplicities deduced from HSQC; coupling constants (*J* values in Hz) are in parentheses. [b] Multiplicities deduced from DEPT.

carbonyl groups at C-5. Furthermore, **1** resisted crystallization, precluding its X-ray crystal structure determination. Biogenetically, **1** may arise by a hitherto unknown hydroperoxy-mediated ring contraction of asterriquinone D (**7**) (Figure 1), which was found to co-occur in the same extract (see below).

Compound **2** crystallized as white needles. Its molecular formula, $C_{16}H_{24}O_4$, determined by a combination of HRMS and $^{13}$C NMR spectroscopy, indicated 5 degrees of unsaturation. It showed IR absorption bands at 3438, 1732, and 1706 $cm^{-1}$ and had characteristic signals in its $^{13}$C NMR spectrum at $\delta$ 213.8 and 175.5, suggesting the presence of a ketone carbonyl and a carboxylic acid group. The $^1$H NMR spectrum (Table 1) consisted of three 3H singlets and four independent spin systems. On the basis of their chemical shifts the 3H singlets were assigned to a $OCH_3$ ($\delta$ 3.41) and two $CH_3$ groups ($\delta$ 1.15 and 1.10) on quaternary carbons. One of the spin systems consisted of two 1H doublets ($J$ = 14.6 Hz) at $\delta$ 1.46 and 1.55 due to a $CH_2$ sandwiched by quaternary carbons in a cyclic system. Two signals centered at $\delta$ 3.44 (t, $J$ = 9.8 Hz) and 3.93 (dd, $J$ = 9.8 and 3.6 Hz) together with a signal at $\delta$ 2.94 (dd, $J$ = 9.8 and 3.6 Hz) suggested the presence of an ABX spin system. The chemical shifts and coupling constants of A and B were indicative of nonequivalent methylene protons adjacent to an oxygen atom. The presence of another ABX spin system in **2** was apparent from the two signals centered at $\delta$ 2.34 (dd, $J$ = 19.9 and 6.8 Hz) and 2.52 (ddd, $J$ = 19.9, 12.0, and 1.0 Hz) together with a signal at $\delta$ 2.61 (dd, $J$ = 12.0 and 6.8 Hz). The remaining spin system contained six protons in a complex pattern assignable to a $CHCH_2CH_2CH$ moiety. The $^{13}$C NMR spectrum of **1** (Table 1) when analyzed with the help of HSQC showed the presence of two methyl, one methoxy, five methylene (one of which is attached to an oxygen atom), four methine, and four quaternary carbons (two of which were due to a ketone and a carboxylic acid carbonyl). On the basis of the HMBC correlations (Table 1) the carbon skeleton of **2** was determined to be that of a tricyclic sesquiterpene related to quadrone (**5**).[19,24] The $^2J$ and $^3J$ correlations in the HMBC spectrum were also useful in locating the carbonyl, $CH_2$-$OCH_3$, and $CO_2H$ groups in the tricyclic skeleton. On the basis of the foregoing evidence, the structure of **2** was determined to be 5(6)-dihydro-6-methoxyterreyclic acid A. The presence of (+)-terrecyclic acid A (**4**) and (−)-quadrone (**5**) in the same extract and the use of MeOH for extraction of the fungus suggested possible artifactual origin of **2** from **4**. However, both **4** and **5** failed to react with MeOH under a variety of conditions including such harsh conditions as refluxing MeOH in the presence of *p*-toluenesulfonic acid (*p*-TSA). Furthermore, when MeOH was replaced with *n*-BuOH in the extraction process, **2** was found to be present in the resulting extract, suggesting that this compound is a genuine natural product.

Compound **3** was obtained as a colorless semisolid. Its molecular formula, $C_{15}H_{22}O_4$, was determined by a combination of HRMS and $^{13}$C NMR data. Comparison of the $^1$H and $^{13}$C NMR data of **3** with **2** indicated that they are structurally related, the major difference being the presence of an OH in **3** in place of the $OCH_3$ in **2**. This was further supported by the HRMS data, which showed that the molecular formula of **3** had 14 mass units less than that of **2**, identifying **3** as 5(6)-dihydro-6-hydroxyterrecyclic acid A. Compound **3** has previously been obtained as an intermediate during the total synthesis of quadrone (**5**).[28,30] The stereochemistry at C-5 in **2** and **3** remains undetermined, though the failure to observe any spontaneous lactonization of these to quadrone (**5**) suggests that the

**Table 2.** Cytotoxicities of the Compounds **1**−**11** against a Panel of Tumor Cell Lines[a]

| compound | cell line[b] | | |
|---|---|---|---|
| | NCI-H460 | MCF-7 | SF-268 |
| **1** | 17.4 | 25.2 | 20.7 |
| **2** | 19.4 | 20.6 | 21.4 |
| **3** | 14.5 | 16.5 | 7.8 |
| **4** | 10.6 | 24.1 | 14.7 |
| **5** | 12.4 | 14.3 | 16.9 |
| **6** | 58.4 | 8.7 | 28.0 |
| **7** | 19.9 | 17.7 | 15.8 |
| **8** | 24.2 | 4.1 | 25.7 |
| **9** | NA[c] | NA[c] | NA[c] |
| **10** | NA[c] | NA[c] | NA[c] |
| **11** | 9.4 | 16.7 | 10.2 |
| Taxol | 9.5 | 11.2 | 21.7 |

[a] Results are expressed as $IC_{50}$ values in $\mu$M except for Taxol, which is in nM. [b] Key: NCI-H460 = human non-small cell lung cancer; MCF-7 = human breast cancer; SF-268 = human CNS cancer (glioma). [c] NA= not active at 10.0 $\mu$g/mL.

−$CH_2$OR groups to be of $\beta$-orientation as shown. This is further supported by the absence of NOEs between H-5 and H-8 in both **2** and **3**. A comparison of spectral and optical rotation data of compounds **4** and **5** with those reported in the literature allowed these to be identified as (+)-terrecyclic acid A[23,31] and (−)-quadrone,[19] respectively. Treatment of both **2** and **3** with *p*-TSA in toluene at 55 °C for 30 min afforded (+)-terrecyclic acid A (**4**) identical with the natural sample. However, prolonged treatment of **2**, **3**, or **4** with *p*-TSA in toluene under reflux afforded a mixture of (−)-quadrone (**5**) and (+)-isoquadrone (**9**). The former was identified by comparison with the natural sample obtained above and the latter by comparison of the spectral data with those reported in the literature for (+)-isoquadrone (**9**).[27,28] This is the first report of the occurrence of betulinan A (**6**) in an *Aspergillus* species.

Compounds **1**−**11** were evaluated for in vitro cytotoxicity against a panel of three sentinel cancer cell lines, NCI-H460 (non-small cell lung), MCF-7 (breast), and SF-268 (CNS glioma). Cells were exposed to serial dilutions of test compounds for 48 h in RPMI 1640 media supplemented with 10% fetal bovine serum, and cell viability was evaluated by the MTT assay.[31] As shown in Table 2, all except (+)-isoquadrone (**9**) and 5(6)-dihydroterrecyclic acid A (**10**) were found to be cytotoxic. The concentrations resulting in 50% inhibition of cell proliferation/survival as measured by MTT assay ($IC_{50}$) were found to range between 4.1 and 58.4 $\mu$M. Compound **3** showed selective activity toward the SF-268 cell line, whereas **6** and **8** were found to be more toxic to the MCF-7 cell line. The cytotoxic activities of (+)-terrecyclic acid A (**4**),[32] (−)-quadrone (**5**),[19,33] asterriquinone D (**7**),[34] and asterriquinone C-1 (**8**)[34] have previously been reported, and a mechanism for the cytotoxic action of asterriquinones has been reported.[35] In the sesquiterpene series (**2**−**5** and **9**−**11**) our results suggest that the cytotoxic activity is mainly dependent on the presence of an electrophilic α-methylene carbonyl moiety, which can covalently bind to DNA or to a protein target in a Michael-type reaction. Thus, 5(6)-dihydroterrecyclic acid A (**10**), which lacks this reactive moiety, was found to be completely devoid of cytotoxicity. The cytotoxic activities of **2**, **3**, and **5** may be due to their ready conversion into **4** under the culture conditions of the bioassay. The inability of (+)-isoquadrone (**9**) to generate an α-methylene carbonyl moiety due to the absence of a proton at C-5 provides a reasonable explanation as to why this compound is inactive in the cytotoxicity assay. As a first step in elucidating the mechanism(s) of cytotoxicity of this class of compounds, **2**, **4**, and **5** were subjected to flow cytometric cell cycle analysis using the non-small cell lung cancer cell line (NCI-H460), which was found to be the most sensitive to these compounds (Table 3). This cell line expresses wild-type p53 and displays an 18 h doubling time under exponential growth conditions.[36] Of the three compounds tested, terrecyclic acid A (**4**) and quadrone (**5**) induced the most marked alterations in the distribution of asynchronous NCI-H460 cells within the cell cycle (Figure 2). These compounds resulted in the accumulation of cells with 2n and 4n DNA content and associated depletion of S phase cells. This pattern is consistent with blocks to progression at the $G_1$ and $G_2$/M phases of the cell cycle and may underlie the cytotoxic activity observed for these compounds.[37] Studies to elucidate the molecular mechanism(s) of action of these compounds and animal studies to evaluate their antitumor potential are currently in progress.

**Table 3.** Effect of **2**, **4**, and **5** Treatment on NCI-H460 Cell Cycle Progression

| compound | $G_1$ (%) | S (%) | $G_2$/M (%) |
|---|---|---|---|
| **2** | 44.3 | 21.4 | 34.3 |
| **4** | 40.7 | 16.0 | 43.7 |
| **5** | 38.8 | 6.3 | 54.8 |
| DMSO[a] | 33.1 | 35.5 | 31.4 |
| Taxol[b] | 15.5 | 14.0 | 70.5 |
| nocodazole[b] | 13.0 | 6.9 | 80.1 |

[a] Negative control. [b] Positive controls.

## Experimental Section

**General Experimental Procedures.** Melting points were determined on a Gallenkamp micromelting point apparatus and are uncorrected. Optical rotations were measured with a Jasco Dip-1000 polarimeter using $CHCl_3$ as solvent. IR spectra for KBr disks were recorded on a Shimadzu FTIR-8300 spectrometer. 1D and 2D NMR spectra were recorded in $CDCl_3$ with a Bruker DRX-500 instrument at 500 MHz for $^1$H NMR and 125 MHz for $^{13}$C NMR using residual $CHCl_3$ as internal standard. The chemical shift values ($\delta$) are given in parts per million (ppm), and the coupling constants are in Hz. Low-resolution and high-resolution MS were recorded respectively on Shimadzu LCMS-8000QPα and JEOL HX110A spectrometers.

**Fungal Isolation, Identification, and Cultivation.** *Aspergillus terreus* Thom. was isolated from the rhizosphere of a staghorn cholla (*Opuntia versicolor* Engelm.) growing in the Tucson Mountains in southern Arizona. Excised roots of *O. versicolor* [1 cm long sections (ca. 5 g)] were placed in 5 mL of phosphate-buffered saline (PBS, 0.1 M, pH = 7.4), vortexed, and sonicated three times. A serial dilution of the suspension was plated onto potato dextrose agar (PDA, Difco, Plymouth, MN) amended with chloramphenicol and streptomycin. After 4 days of incubation at 25 °C, single colonies were transferred onto water agar containing the same antibiotics, and after 3 days a pure culture of *A. terreus* was obtained by hyphal tipping. The strain is deposited in the Department of Plant Pathology and the Southwest Center for Natural Products Research and Commercialization of the University of Arizona microbial culture collection under the code name AH-00-51-F7. The strain was subcultured using Petri dishes with PDA. For long-term storage isolates were subcultured on PDA slants, overlaid with 40% glycerol, and stored at −80 °C.

Flasks (125 mL) containing 100 mL of Sabouard medium (Difco Laboratories, Detroit, MI) were inoculated with a 5 mm mycelial plug from a pure culture and incubated with shaking (120 rpm) at 25 °C for one week. The mycelium was collected on filter paper by vacuum filtration, air-dried, frozen at −70 °C, and lyophilized for 24 h. The lyophilized mycelia were ground to a fine powder with liquid $N_2$ and stored at −20 °C. DNA extraction was performed using a plant DNA extraction kit (DNeasy, QIAGEN, Hilden, Germany) according to manu-

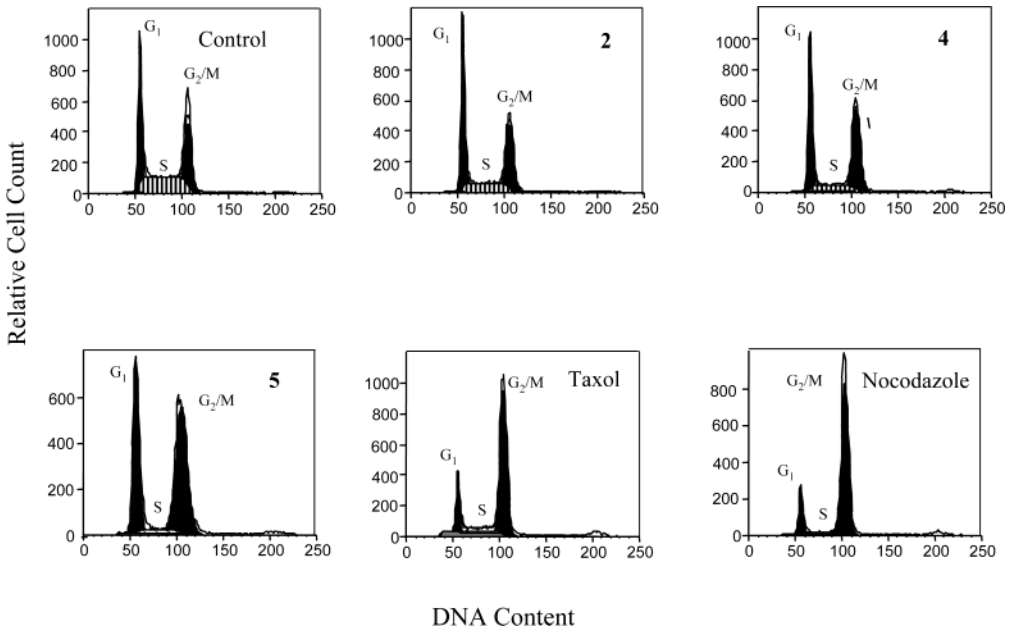



**Figure 2.** DNA content frequency histograms generated by flow cytometric analysis of NCI-H460 cells treated with control (DMSO), **2**, **4**, **5**, Taxol, and nocodazole.

facturer's instructions with the addition of a precipitation and wash step after the final DNA elution step. DNA was resuspended in 25 $\mu$L of TE (10 mM Tris pH 8.0, 1 mM EDTA). The intergenic spacer (ITS) regions of the ribosomal DNA were amplified by polymerase chain reaction (PCR) using primers ITS1 and ITS4 (ITS1: TCCGTAGGTGAACCTGCGG; ITS4: TCCTCCGCTTATTGATATGC).[38] Each 50 $\mu$L of reaction mixture contained 2 $\mu$L of DNA (conc $\geq$ 40 ng/$\mu$L), 5 $\mu$L of 10$\times$ reaction buffer, 2 $\mu$L each of 10 mM primer, 2 $\mu$L of 10 mM dNTP, 3 $\mu$L of 25 mM $MgCl_2$, 2 $\mu$L of Taq polymerase, and 32 $\mu$L of water. The reaction profile was 94 °C for 4 min, then (94 °C for 45 s, 52 °C for 45 s, and 72 °C for 4 min) for 36 cycles. The DNA product was purified using a PCR purification kit (Qiaquick, QIAGEN, Hilden, Germany) according to the manufacturer's instructions and sequenced at the University of Arizona Biotechnology Center. The DNA sequence was compared with the GenBank database (BLASTN search) and indicated 100% identity to *A. terreus*. Morphological characters consistent with known isolates of this fungus[39] are as follows: colonies pale grayish/brownish orange to camel in color; with a yellow to deep dirty brown reverse; yellowish soluble pigment sometimes present; conidial heads are compact, columnar pale orangish to tan with tightly packed metulae; aspergilla biseriate, metulae over the upper half to two-thirds of the vesicle, vesicles (7−) 12−20 $\mu$m wide, somewhat variable in shape, spherical or domelike; stipes smooth walled (70−) 100−250 (−300) $\mu$M long; phialides 5−7 (−9) $\times$ 1.5−2.5 $\mu$m; small smooth-walled conidia (2−2.5 $\mu$m) globose to ellipsoidal, aspergilla tightly packed, 5−7 $\times$ 2−3 $\mu$m (70−) 1000−250 (−300) $\mu$m long.

To produce culture medium for isolation of secondary metabolites, the fungus was cultured in 80 T-flasks (800 mL), each containing 135 mL of PDA coated on five sides of the flasks (total surface area ca. 460 $cm^2$), for 30 days at 27 °C.

**Extraction and Isolation.** Methanol (200 mL/T-flask) was added to all 80 T-flasks, sonicated, and allowed to sit for 24 h at room temperature, and the resulting extract was filtered through Whatman No. 1 filter paper and a layer of Celite 545. The filtrate was concentrated to one-fourth of its original volume and extracted with EtOAc (5 $\times$ 600 mL). Combined EtOAc extracts were evaporated under reduced pressure to afford a dark brown semisolid (4.0 g), which was partitioned between hexane and 80% aqueous MeOH. The cytotoxic 80% aqueous MeOH fraction was diluted to 60% aqueous MeOH by the addition of $H_2O$ and extracted with $CHCl_3$. Evaporation of $CHCl_3$ under reduced pressure yielded a dark brown semisolid (2.75 g). A portion (2.60 g) of this was subjected to gel permeation chromatography on a column of Sephadex LH-20 (60.0 g) in hexane/$CH_2Cl_2$ (1:4) and eluted with hexane/$CH_2Cl_2$ (1:4) (450 mL) and $CH_2Cl_2$/acetone (3:2) (135 mL). Thirty-nine fractions (15 mL each) were collected and combined on the basis of their TLC patterns to yield seven cytotoxic fractions [A (48.5 mg), B (299.0 mg), C (584.0 mg), D (120.2 mg), E (298.5 mg), F (44.4 mg), and G (85.0 mg)]. Column chromatography of fraction A (48.5 mg) on silica gel (2.0 g) and elution with hexane/$CH_2Cl_2$ (1:1) afforded **6** as a yellow solid (6.1 mg). Chromatography of fraction B (100.0 mg) on silica gel (2.0 g) by elution with hexane/$CH_2Cl_2$ (1:1) and $CH_2Cl_2$ afforded two subfractions, B1 and B2. Subfraction B1 (1.2 mg) was a yellow solid identical with **6**, and subfraction B2 (52.9 mg) on crystallization with hexane/$CH_2Cl_2$ afforded **5** (37.9 mg). Fraction C (584.0 mg) was further fractionated on silica gel (20.0 g) by elution with hexane/$CH_2Cl_2$ (1:1), $CH_2Cl_2$, and increasing amounts of MeOH in $CH_2Cl_2$. Fractions eluted with 1% MeOH in $CH_2Cl_2$ were found to be cytotoxic, and these were combined and evaporated to yield a white solid (294.0 mg), which on recrystallization from hexane/$CH_2Cl_2$ afforded **2** (263.0 mg). Chromatography of the fraction D (120.2 mg) on silica gel (3.0 g) by elution with $CH_2Cl_2$ followed by increasing amounts of MeOH in $CH_2Cl_2$ afforded several fractions. Of these, the fraction (5.6 mg) eluted with 1% MeOH in $CH_2Cl_2$ was found to be cytotoxic, and this was further purified by preparative TLC on silica gel ($CH_2Cl_2$/MeOH, 99:1) to obtain **8** (2.1 mg). Fraction E (298.5 mg) was subfractionated on silica gel (7.0 g) using $CH_2Cl_2$ and a gradient of $CH_2Cl_2$ containing MeOH. The combined fractions (139.3 mg) eluted with 1% MeOH in $CH_2Cl_2$ was found to be cytotoxic, and this fraction was further fractionated on silica gel (3.0 g) using a gradient of hexane containing 2-propanol. The combined fractions (92.0 mg) eluted with 3% 2-propanol in hexane exhibited cytotoxicity. Purification of this fraction by reversed-phase preparative TLC on RP-18 (MeOH/$H_2O$, 90:10) furnished **4** (59.2 mg). Fraction F (44.4 mg) on column chromatography over silica gel (1.6 g) using a gradient of MeOH in $CH_2Cl_2$ afforded a cytotoxic fraction (4.3 mg) eluting with 0.5% MeOH in $CH_2Cl_2$. Further purification of this fraction on silica gel preparative TLC ($CH_2Cl_2$/MeOH, 96:4) furnished **7** as a dark brown powder (3.5 mg). The last cytotoxic fraction from the original Sephadex column, fraction G (85.0 mg), was separated by column chromatography on silica gel (2.0 g) using a gradient of MeOH in $CH_2Cl_2$. The cytotoxic fraction (5.2 mg) eluted with

0.5% MeOH in $CH_2Cl_2$ on further purification by preparative TLC (silica gel, $CH_2Cl_2$/MeOH, 96:4) yielded **1** (2.3 mg). The second cytotoxic fraction (33.7 mg) eluted with 1% MeOH in $CH_2Cl_2$ on further purification using silica gel preparative TLC ($CH_2Cl_2$/MeOH, 95:5) followed by RP-18 reversed-phase preparative TLC (MeOH/$H_2O$, 70:30) afforded **3** (11.3 mg).

**Asterredione (1):** yellow powder; mp dec >112 °C; $[\alpha]_D^{25}$ −2.26° (*c* 0.5, $CHCl_3$); UV (EtOH) $\lambda_{max}$ (log $\epsilon$) 411 (4.92), 269 (5.23), 218.5 (5.68) nm; IR (KBr) $\nu_{max}$ 3392, 1751, 1718, 1676, 1596, 1458, 1421, 1371, 1319, 1238, 1132, 1107, 744 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 500 MHz) $\delta$ 8.67 (1H, brs, NH), 8.21 (1H, d, $J$ = 2.9 Hz, H-2′), 8.18 (1H, brs, NH), 8.02 (1H, d, $J$ = 8.0 Hz, H-5′), 7.79 (1H, d, $J$ = 8.0 Hz, H-5″), 7.40 (1H, d, $J$ = 8.0 Hz, H-8′), 7.32 (1H, d, $J$ = 8.0 Hz, H-8″), 7.27 (1H, d, $J$ = 2.8 Hz, H-2″), 7.26 (1H, t, $J$ = 8 Hz, H-7′), 7.21 (1H, t, $J$ = 8.0 Hz, H-6′), 7.17 (1H, t, $J$ = 8.0 Hz, H-7″), 7.12 (1H, t, $J$ = 8.0 Hz, H-6″), 4.45 (3H, s, $-COCH_3$), 3.78 (3H, s, $-OCH_3$); $^{13}C$ NMR ($CDCl_3$, 125 MHz) $\delta$ 192.9 (C, C-1), 191.2 (C, C-4), 167.3 (C, $COCH_3$), 160.6 (C, C-3), 136.1 (C, C-9″), 132.5 (C, C-2), 129.7 (CH, C-2′), 125.9 (C, C-4″), 125.6 (C, C-4′), 124.2 (CH, C-2″), 123.2 (CH, C-5′), 123.2 (CH, C-7′), 122.6 (CH, C-7″), 121.2 (CH, C-6′), 121.1 (CH, C-5″), 120.4 (CH, C-6″), 111.4 (CH, C-8′), 111.3 (CH, C-8″), 108.4 (C, C-3″), 105.6 (C, C-3′), 64.3 (C, C-5), 60.2 ($CH_3$, $COCH_3$), 53.4 ($CH_3$, $OCH_3$); HRFABMS *m/z* 414.1216 $[M]^+$ (calcd for $C_{24}H_{18}N_2O_5$, 414.1216).

**(+)-5(6)-Dihydro-6-methoxyterrecyclic acid A (2):** white needles; mp 141−142 °C; $[\alpha]_D^{25}$ +7.70° (*c* 0.5, $CHCl_3$); IR (KBr) $\nu_{max}$ 2935,1732,1706, 1454, 1388,1263, 1209, 1170, 1107, 1091 $cm^{-1}$; $^1H$ and $^{13}C$ NMR data, see Table 1; HRFABMS *m/z* 281.3732 $[M + 1]^+$ (calcd for $C_{16}H_{25}O_4$, 281.3721).

**(+)-5(6)-Dihydro-6-hydroxyterrecyclic acid A (3):** white powder; mp 158−160 °C; $[\alpha]_D^{25}$ +27.30° (*c* 1.0 $CHCl_3$); IR (KBr) $\nu_{max}$ 3257, 2898, 1728, 1473, 1389, 1218, 1186, 1167, 1149, 1055, 1029 $cm^{-1}$; $^1H$ and $^{13}C$ NMR data, see Table 1; HRFABMS *m/z* 249.3310 $[M - H_2O + 1]^+$ (calcd for $C_{15}H_{21}O_3$, 249.3299).

**(+)-Terrecyclic acid A (4):** white solid; mp 122−124 °C; $[\alpha]_D^{25}$ +25.7° (*c* 0.5 $CHCl_3$); $^1H$ NMR and $^{13}C$ NMR data were consistent with literature values.[23]

**(−)-Quadrone (5):** white needles, mp 183−184 °C (lit.[24] 185−186 °C); $[\alpha]_D^{25}$ −60.53° (*c* 0.5, $CHCl_3$); $^1H$ NMR and $^{13}C$ NMR data were consistent with literature values.[24]

**Betulinan A (6):** yellow powder; mp 192−194 °C (lit.[25] 191−193 °C); UV, IR, and $^1H$ NMR data were consistent with those reported in the literature.[25]

**Asterriquinone D (7):** dark brown powder; mp 178−180 °C; UV (EtOH) $\lambda_{max}$ (log $\epsilon$) 480 (4.71), 282 (5.42), 222 (5.69) nm; IR (KBr) $\nu_{max}$ 3325, 1643, 1581, 1512, 1419, 1305, 1234, 1172, 1118, 1041, 948, 732 $cm^{-1}$; $^1H$ NMR ($CDCl_3$ + $CD_3OD$) $\delta$ 7.47 (2H, s, H-2′ and H-2″), 7.41 (2H, d, $J$ = 8.1 Hz, H-5′ and H-5″), 7.31 (2H, d, $J$ = 8.1 Hz, H-8′ and H-8″), 7.07 (2H, t, $J$ = 8.1 Hz, H-7′ and H-7″), 6.99 (2H, t, $J$ = 8.1 Hz, H-6′ and H-6″), 3.54 (6H, s, 2 × OMe); APCIMS (+) ve mode *m/z* 399$[M + 1]^+$; APCIMS (−) ve mode *m/z* 397 $[M - 1]^+$.

**Asterriquinone C-1 (8):** dark brown powder; mp 162−164 °C; UV (EtOH) $\lambda_{max}$ (log $\epsilon$) 489 (4.19), 367 (3.97), 281 (5.12), 222 (5.49) nm; IR (KBr) $\nu_{max}$ 3394, 1720, 1637, 1596, 1460, 1301, 1263, 1060, 746 $cm^{-1}$; $^1H$ NMR ($CDCl_3$) $\delta$ 8.49 (1H, brs, NH), 8.09 (1H, brs, NH), 7.60 (1H, d, $J$ = 2.7 Hz, H-indole ring), 7.53 (1H, d, $J$ = 7.6 Hz, Ar-H), 7.42 (1H, d, $J$ = 8.0 Hz, Ar-H), 7.31 (1H, d, $J$ = 8.0 Hz, Ar-H), 7.27 (1H, d, $J$ = 7.6 Hz, Ar-H), 7.24 (1H, dt, $J$ = 7.6, 0.9 Hz, Ar-H), 7.17 (1H, dt, $J$ = 8.0, 0.8 Hz, Ar-H), 7.15 (1H, dt, $J$ = 7.6, 0.9 Hz, Ar-H), 7.07 (1H, dt, $J$ = 8.0, 0.8 Hz, Ar-H), 6.11 (1H, dd, $J$ = 17.4 Hz, 10.5 Hz, vinyl-H), 5.19 (1H, d, $J$ = 17.4 Hz, vinyl-H), 5.14 (1H, d, $J$ = 10.5 Hz, vinyl-H), 1.48 (6H, s, 2 × $CH_3$); APCIMS +ve mode *m/z* 467 $[M + 1]^+$.

**Conversion of 2 to 4.** A crystal of *p*-TSA was added to a stirred solution of **2** (5.0 mg) in dry toluene (0.5 mL), and the reaction mixture was heated at 55 °C for 30 min (TLC control). Toluene was removed under reduced pressure and the product purified by silica gel preparative TLC ($CH_2Cl_2$/MeOH, 92:8), giving a white solid (3.8 mg, 86%), which was identical with (+)-terrecyclic acid A (**4**) isolated above.

**Conversion of 2 to 5 and 9.** A crystal of *p*-TSA was added to a stirred solution of **2** (10.0 mg) in dry toluene (0.5 mL), and the reaction mixture was heated under reflux for 30 min. Toluene was removed under reduced pressure, and the crude mixture was subjected to column chromatography on silica gel with $CH_2Cl_2$ as eluant to obtain **5** (3.7 mg, 41%), identical with the above obtained natural sample, and (−)-isoquadrone (**9**) as a white solid (5.1 mg, 58%): mp 165−167 °C (lit.[28] 164−167 °C); $[\alpha]_D^{25}$ −1.40° (*c* 0.2, $CHCl_3$); IR and $^1H$ NMR data were consistent with literature values.[28]

**5(6)-Dihydroterrecyclic Acid A (10).** Pd on carbon (10%, 1.2 mg) was added to a solution of (+)-terrecyclic acid A (**4**; 5.0 mg) in ethanol (0.5 mL) and stirred under an atmosphere of $H_2$. The reaction was monitored by TLC, and after 30 min it was filtered through a cotton plug and ethanol was removed under reduced pressure to give **10** (5.0 mg, 99.2%). The product was a mixture of two diastereoisomers in the ratio 1:14 (by $^1H$ NMR): $^1H$ NMR (major isomer) $\delta$ 2.80 (1H, dd, $J$ = 10.6, 8.6 Hz, H-2), 2.60 (1H, d, $J$ = 6.1 Hz, H-8), 2.45−2.30 (3H, m), 1.90−1.55 (6H, m), 1.37 (1H, d, $J$ = 14.7 Hz, H-12a), 1.33 (1H, d, $J$ = 14.7 Hz, H-12b), 1.12 (3H, s, $CH_3$), 1.08 (3H, s, $CH_3$), 0.92 (3H, d, $J$ = 6.7 Hz, $CH_3$); APCIMS − ve mode *m/z* 249 $[M^+ - 1]$.

**Methyl Ester of (+)-Terrecyclic Acid A (11).** $CH_2N_2$ (in ether) was added dropwise to a stirred solution of (+)-terrecyclic acid A (**4**; 2.0 mg) in diethyl ether (0.4 mL) and methanol (2 drops) at −12 °C until the starting material disappeared (TLC control). Solvents were removed under reduced pressure, and the crude product was purified by preparative TLC (silica gel) using 5% methanol in $CH_2Cl_2$ as eluant to give **11** (2.1 mg, 99.0%): $^1H$ NMR ($CDCl_3$) $\delta$ 5.92 (1H, s, H-6a), 5.15 (1H, s, H-6b), 3.55 (3H, s, OMe), 3.01 (1H, dd, $J$ = 11.4, 9.6 Hz, H-2), 2.99 (1H, d, $J$ = 8.0 Hz, H-8), 2.61 (1H, dd, $J$ = 19.3, 11.4 Hz, H-3a), 2.50 (1H, dd, $J$ = 19.3, 9.6 Hz, H-3b), 2.09 (1H, m), 1.95 (1H, t, $J$ = 2.9 Hz, H-9a), 1.86−1.75 (3H, m), 1.74 (2H, s, H-12a and -12b), 1.23 (3H, s, $CH_3$) 1.17 (3H, s, $CH_3$); APCIMS +ve mode *m/z* 263 $[M^+ + 1]$.

**Cytotoxicity Bioassays.** The tetrazolium-based colorimetric assay (MTT assay)[31] was used for the in vitro assay of cytotoxicity to human non-small cell lung carcinoma (NCI-H460), human breast carcinoma (MCF-7), and human glioma (SF-268) cells.

**Cell Cycle Analysis.** NCI-H460 cells were harvested from flasks at 75−85% confluency, counted and plated into 60 mm dishes at a density of 2.5 × $10^5$ cells/dish, and allowed to reattach overnight. Test compounds, **2**, **4**, and **5** were prepared in stock solutions at 8 mg/mL in DMSO. Treatment solutions were prepared at twice the desired final concentration in complete RPMI 1640 medium such that the vehicle (DMSO) did not exceed a final concentration of 0.1 vol %. The final concentrations of the compounds were 10 $\mu$g/mL, and the final concentrations of the positive controls, Taxol and nocodozole, were 0.1 and 3 $\mu$M, respectively. A control dish was treated with the vehicle control (DMSO) alone. Cells were incubated overnight in the continuous presence of the various treatments, at which point they were rinsed with PBS, harvested by trypsination, resuspended in complete medium, pelleted by centrifugation, rinsed once with PBS, and resuspended in 1 mL of Krishan's buffer. Cells were analyzed by flow cytometric analysis on a Becton Dickinson FACScan instrument maintained by the Arizona Cancer Center as a core facility.

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