Aspirochlorine Class Compounds from *Aspergillus flavus* Inhibit Azole-Resistant *Candida albicans*

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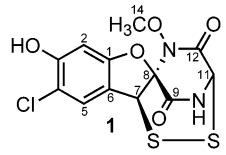
Dereplication of the antifungal extracts of *Aspergillus flavus* indicated that the primary antifungal compound present was the known aspirochlorine (**1**). Preparative isolation work resulted in the identification of the new compounds tetrathioaspirochlorine (**2**) and cyclo(D-*N*-methyl-Leu-L-Trp) (**3**).

A high-throughput screen to detect extracts that inhibited the growth of drug-resistant bacteria and fungi was used to analyze ~140 000 extracts from the National Cancer Institute's Natural Products Repository.[1] Among the antifungal hits identified in the screen was an organic extract from an *Aspergillus flavus* culture. This extract exhibited exceptional potency against an azole-resistant strain of *Candida albicans* and yet was rather nontoxic to human CEM-SS lymphocytes.

The rich chemical history of *A. flavus* portended a significant challenge to our rapid HPLC-ELSD-UV-MS-bioassay dereplication analysis.[2] Several peaks observed by ELSD and UV absorbance in the HPLC chromatogram were, upon bioassay, found to possess antifungal activity. The most potent of these contained a substance with an apparent molecular ion cluster at *m/z* 360/362 [MH^+], indicating the presence of chlorine; the UV absorption and molecular weight corresponded to those of aspirochlorine (**1**).[3−5] In the process of purifying small quantities of **1** for further evaluation of its antifungal activity, we also isolated the new tetrathio analogue **2** and diketopiperazine **3**. Additionally, we found UV and MS evidence for the presence of the trithio analogue (**4**) of aspirochlorine, but could not secure a sufficient quantity for NMR analysis and structure confirmation.

The identity of the primary antifungal isolate as aspirochlorine was confirmed by comparison of the NMR, MS, and UV data with those reported in the literature.[5] Compound **2** was likewise confirmed as the tetrathio analogue of **1**; the NMR spectra were virtually identical to those of **1**, while the mass spectrum indicated the presence of two additional sulfur atoms.

Compound **3** appeared unrelated to the aspirochlorines, analyzing for $C_{18}H_{23}N_3O_2$ by HRESIMS and exhibiting a UV spectrum characteristic of indoles. Examination of the ^1H and COSY NMR spectra revealed three spin systems: a 3-substituted indole (after consideration of the UV and ^{13}C NMR data), an isopentyl chain, and an ABX system comprised of a methylene/methine pair. The remaining structural elements were two carbonyl groups (δ 168.8 and 170.2), an N-methyl group (δ 2.67), an NH, and another



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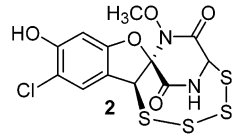

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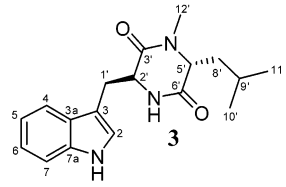

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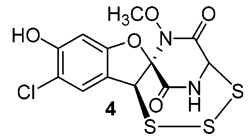

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site of unsaturation. The most likely structure to accommodate all these moieties was a diketopiperazine derived from tryptophan and leucine, one of which was N-methylated. This proposed assemblage was confirmed by HMBC and HSQC correlations; the placement of the methyl group on the leucine nitrogen was established by HMBC correlations from the tryptophan carbonyl and the leucine α-methine to the methyl protons.

The configuration of this dipeptide remained to be established. Cyclo(L-Leu-L-Trp) is a known compound and exhibits some unusual chemical shifts because of anisotropic shielding of the side chain of the leucine residue by the indole ring.[6,7] In particular, the terminal methyl groups and the adjacent methine are shielded to δ 0.45/0.55 and 1.22, respectively, in the L/L isomer. In **3**, the comparable chemical shifts were δ 0.86, 0.87, and 1.61, suggesting a D/L or L/D configuration. The configuration was defined by hydrolysis and analysis of Marfey's derivatives by HPLC.[8] Using L-*N*-methyl leucine and D/L-*N*-methyl leucine as standards, the configuration of the *N*-methyl leucine residue in **3** was demonstrated to be D; thus, **3** is cyclo(D-*N*-methyl-leucine-L-tryptophan).

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Table 1. Antimicrobial and Cytotoxicity Data for Compounds **1**–**3**

compound	*C. albicans*[a]	CEM[a]	TI[b]
1	0.028	0.58	20
2	0.083	4.2	50
3	>31	>31	none
amphotericin B	0.16	160	1000
gliotoxin	3.1	0.15	none
griseofulvin	>28	10	none

[a] IC_{50} values (μM) average of a minimum of three replicates. [b] Therapeutic index (CEM IC_{50}/*C.a.* IC_{50}).

In our hands, aspirochlorine was a potent antifungal, superior to three standard compounds against azole-resistant *C. albicans* (Table 1). Tetrathioaspirochlorine (**2**) was only slightly less potent in the antifungal assay. Aspirochlorine has recently been shown to exert its antifungal activity by inhibition of fungal, but not bacterial or mammalian, protein synthesis.[9] In the NCI's 60 cell line anticancer panel, **1** gave a mean GI_{50} value near 1 μM (log GI_{50} −6.06); the leukemia panel was particularly sensitive to **1** at the GI_{50} level (log Leu panel GI_{50} −7.04), but there was no other discernible pattern of differential cytotoxicity.[10]

Experimental Section

General Experimental Procedures. IR spectra were acquired using a Perkin-Elmer Spectrum 2000 FT-IR spectrometer. Optical rotation was determined with a Perkin-Elmer 241 polarimeter. UV spectra were acquired on a Lambda 20 UV/vis spectrometer (Perkin-Elmer). NMR data were recorded at 27 °C in CD_3OD or CD_2Cl_2, with TMS as internal standard, on a Varian 500 MHz INOVA at 500 and 125 MHz for ^1H and ^{13}C experiments, respectively. Positive ion ESIMS was carried out on a Thermo Finnigan TSQ Quantum AM triple quadrupole mass spectrometer using a PEG mixture as an internal calibration standard. HPLC-MS hardware consisted of a Waters 600 pump, a Micromass ZMD electrospray mass spectrometer (cone voltage = 30), a Waters 996 photodiodearray spectrometer, and a Sedex 75 evaporative light scattering detector. Preparative separations were accomplished using Dynamax HPLC columns including C_8, C_{18}, or cyano (21 × 250 mm, 8 μm). All fractions were analyzed with a Waters XTerra C_8 or C_{18} column (4.6 × 250 mm, 5 μm).

Biological Material. *Aspergillus* spores were obtained from the Michigan Department of Health (MDH-1420). A plated culture of the spores was visually identified as *A. flavus* by S. M. Shipley and confirmed as *A. flavus* Link by 16S ribosomal RNA sequencing by the National Fungal Identification Service, Agriculture and Agri-food Canada (http://res2.agr.gc.ca/ecorc/daom/nfis-snif_e.htm). A frozen culture is stored at the DTP Natural Products Repository in Frederick, MD, as 0G0S0151.

Extraction and Isolation. Spores of *A. flavus* were cultured on a potato dextrose broth agar plate for 14 days. Mycelia from the plate were used to seed 10 × 6 L shake flasks containing 1500 mL of potato dextrose broth media (agitation speed = 150 rpm, T = 25 °C). The broth and mycelia were harvested after 21 days, resulting in 15 L of material that was subjected to liquid−liquid partitioning in CH_2Cl_2−10% aqueous MeOH. The CH_2Cl_2 phase was evaporated in vacuo, yielding 5.6 g of crude organic extract. Removal of nonpolar lipid material from the extract was accomplished by trituration with 80 mL of hexane. The hexane-insoluble fraction was dissolved in 80 mL of MeCN−H_2O (1:1) and centrifuged at 2500 rpm to precipitate insoluble material. The supernatant was loaded onto several C_{18} solid phase extraction cartridges (Varian) and eluted with MeCN−H_2O (1:1), MeOH and with CH_2Cl_2. The MeCN−H_2O (1:1) and MeOH fractions were combined, filtered, and chromatographed on a C_{18} Dynamax HPLC column. Elution with a MeCN−H_2O gradient resulted in 16 fractions after pooling, several of which showed toxicity to *C. albicans*. Fraction 4 was purified by cyano bonded phase HPLC (MeCN−H_2O), yielding 8 mg of **3**, a novel compound that exhibited no *C. albicans* toxicity. Fractions 5 and 6 were combined and chromatographed successively on C_{18} (MeCN−MeOH−H_2O) and cyano (MeCN−H_2O) bonded phase columns, yielding 1 mg of compound **1**. Further purification of fractions 5 and 6 by C_8 HPLC (MeCN−H_2O) led to the isolation of 0.1 mg of compound **2**.

Aspirochlorine (1): pale yellow powder; UV (MeOH) λ_{max} (log ϵ) 203 (4.4), 297 (3.6) nm; ^1H NMR (CD_2Cl_2, 500 MHz) δ 7.17 (1H, d, J = 1.3 Hz, H-5), 6.73 (1H, s, H-2), 6.57 (1H, br, NH), 5.87 (1H, br, OH), 5.18 (1H, d, J = 5.5 Hz, H-11), 4.91 (1H, d, J = 1.1, H-7), 3.94 (3H, s, OCH_3).

Tetrathioaspirochlorine (2): pale yellow powder; UV (MeOH) λ_{max} (log ϵ) 205 (4.0), 300 (3.3) nm; ^1H NMR (CD_2Cl_2, 500 MHz) δ 7.30 (1H, d, J = 1.2 Hz, H-5), 6.67 (1H, s, H-2), 6.57 (1H, br, NH), 5.86 (1H, br, OH), 5.41 (1H, d, J = 4.3 Hz, H-11), 4.96 (1H, d, J = 1.0 Hz, H-7), 3.94 (3H, s, OCH3).

3(*S*)-(1*H*-Indol-3-ylmethyl)-6(*R*)-isobutyl-1-methylpiperazine-2,5-dione (3) [Cyclo(D-*N*-methylLeu-L-Trp)]: pale yellow powder; $[\alpha]^{25}_D$ −13.4° (*c* 2.87, MeOH); UV (MeOH) λ_{max} (log ϵ) 219 (4.52), 270 (3.75), 280 (3.77), 288 (3.68) nm; IR (NaCl) ν_{max} 3287, 2958, 1682, 1457, 1100, 736 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) δ 7.55 (1H, dt, J = 8.0, 1.0 Hz, H-4), 7.32 (1H, dt, J = 8.0, 0.9 Hz, H-7), 7.08 (1H, ddd, J = 8.0, 1.0 Hz, H-6), 7.06 (1H, s, H-2), 7.00 (1H, ddd, J = 8.0, 1.0 Hz, H-5), 4.34 (1H, t, J = 4.5 Hz, H-2′), 3.40 (1H, dd, J = 14.7, 4.5 Hz, H-1′), 3.24 (1H, dd, J = 14.7, 4.5 Hz, H-1′), 3.21 (1H, t, J = 6.0, H-5′), 2.67 (3H, s, H-12′), 1.67 (2H, m, H-8′), 1.61 (1H, m, H-9′), 0.87 (3H, d, J = 6.4, H-11′), 0.86 (3H, d, J = 6.4, H-10′); ^{13}C NMR, (CD_3OD, 125 MHz) δ 170.2 (C, C-6′), 168.8 (C, C-3′), 138.0 (C, C-7a), 128.7 (C, C-3a), 125.8 (CH, C-2), 122.5 (CH, C-6), 119.9 (CH, C-5), 119.8 (CH, C-4), 112.2 (CH, C-7), 109.1 (C, C-3), 61.5 (CH, C-5′), 58.3 (CH, C-2′), 40.4 (CH_2, C-8′), 32.9 (CH_3, C-12′), 30.7 (CH_2, C-1′), 25.5 (CH, C-9′), 23.6 (CH_3, C-11′), 22.8 (CH_3, C-10′); HRESIMS *m/z* 314.183 $[MH]^+$ (calcd for $C_{18}H_{24}N_3O_2$, 314.187); LRESIMS *m/z* 314 $[MH]^+$ (91), 130 [M − $C_9H_{15}N_2O_2$] (100).

Acid Hydrolysis and Determination of the Configuration of 3.[8] Compound **3** (100 μg) was dissolved in MeOH (100 μL) and transferred to a hydrolysis tube. The MeOH was removed under vacuum, and the tube was filled with 6 N HCl. After degassing, the solution was heated in an oil bath at 105 °C for 18 h, at which time the hydrolysis reaction was stopped. The HCl was evaporated under a nitrogen stream, and the hydrolysis products were rinsed twice with MeOH followed by overnight drying under vacuum.

Both the hydrolyzed compound **3** and the amino acid standards *N*-methyl-L-leucine and *N*-methyl-D/L leucine (Sigma) were dissolved in MeOH−H_2O (4:1, 1 mg/mL) and transferred (25 μL) into separate HPLC autosampler vials. All traces of solvent were removed under vacuum. To each sample was added 15 μL of 6% TEA and 7.5 μL of FDAA (Marfey's reagent, Pierce). The samples were heated to 40 °C for 1 h, then cooled prior to C_{18} HPLC analysis with MeCN−5% aqueous HOAc (1:9 to 1:1).

Biological Assays. The *C. albicans* bioassay was performed as described previously.[1] This *C. albicans* strain, 99-788, is resistant to the azole antibiotics miconazole and fluconazole with minimum inhibitory concentrations of 8 and >100 μg/mL, respectively. Additionally, the toxicity of all samples was assessed using CEM cells (human leukemia cells) that were grown at 37 °C with 5% CO_2 in RPMI-1640 (without phenol red) supplemented with 10% (v/v) fetal bovine serum. Cells were diluted to 50 000 cells per mL in RPMI-1640 (without phenol red) supplemented with 5% (v/v) fetal bovine serum; 0.2 mL of the diluted cells was added to each well of a 96-well plate containing the test compound, and 2.5 μM adriamycin was used as a kill control. The cells were then incubated at 37 °C with 5% CO_2 for 96 h. "Staining media" was prepared by dissolving 1 mg/mL of 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide in RPMI-1640 (without phenol red) and then adding 0.6% (w/v) *N*-methylphenazonium methosulfate.[10] A 50 μL portion of the

staining media was added to each well of the 96-well plate and was incubated for 4 h at 37 °C. The plates were read at 650 nm, and the results were reported as the % T/C.

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Supporting Information Available: ^{1}H NMR spectra of compounds **1**−**3**, ^{13}C NMR spectra of compound **3**, HPLC/LRESIMS of compounds **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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