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Potent In Vitro Antifungal Activities of Naturally Occurring Acetylenic Acids▽

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Our continuing effort in antifungal natural product discovery has led to the identification of five 6-acetylenic acids with chain lengths from C_{16} to C_{20}: 6-hexadecynoic acid (compound 1), 6-heptadecynoic acid (compound 2), 6-octadecynoic acid (compound 3), 6-nonadecynoic acid (compound 4), and 6-icosynoic acid (compound 5) from the plant *Sommera sabiceoides*. Compounds 2 and 5 represent newly isolated fatty acids. The five acetylenic acids were evaluated for their in vitro antifungal activities against *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida tropicalis*, *Candida parapsilosis*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Trichophyton mentagrophytes*, and *Trichophyton rubrum* by comparison with the positive control drugs amphotericin B, fluconazole, ketoconazole, caspofungin, terbinafine, and undecylenic acid. The compounds showed various degrees of antifungal activity against the 21 tested strains. Compound 4 was the most active, in particular against the dermatophytes *T. mentagrophytes* and *T. rubrum* and the opportunistic pathogens *C. albicans* and *A. fumigatus*, with MICs comparable to several control drugs. Inclusion of two commercially available acetylenic acids, 9-octadecynoic acid (compound 6) and 5,8,11,14-eicosatetraynoic acid (compound 7), in the in vitro antifungal testing further demonstrated that the antifungal activities of the acetylenic acids were associated with their chain lengths and positional triple bonds. In vitro toxicity testing against mammalian cell lines indicated that compounds 1 to 5 were not toxic at concentrations up to 32 μM. Furthermore, compounds 3 and 4 did not produce obvious toxic effects in mice at a dose of 34 μmol/kg of body weight when administered intraperitoneally. Taking into account the low in vitro and in vivo toxicities and significant antifungal potencies, these 6-acetylenic acids may be excellent leads for further preclinical studies.

The antifungal and antimicrobial properties of fatty acids have been known for centuries. Compared to saturated fatty acids, unsaturated fatty acids with double and/or triple bonds are, in general, more potent against fungal pathogens (16). The representative unsaturated fatty acid with a single double bond at C-10, undecylenic acid (UDA), is still on the market as a cost-effective antifungal agent and the active ingredient of many topical over-the-counter antifungal preparations (20). We have previously demonstrated that two acetylenic acids with a triple bond at C-6, 6-octadecynoic acid (compound 3) and 6-nonadecynoic acid (compound 4), isolated from the plant *Pentagonia gigantifolia*, show potent in vitro antifungal activities against *Candida albicans* and fluconazole-resistant *C. albicans* patient isolates (25). In our ongoing effort to search for prototype natural product antifungal leads (1), the plant *Sommera sabiceoides*, which, along with *P. gigantifolia*, is a member of the family Rubiaceae, was selected for bioassay-guided fractionation due to the potent antifungal activity of its crude methanol extract against *C. albicans* ATCC 90028 (50% inhibitory concentration [IC_{50}], <20 μg/ml). As a result, five antifungal acetylenic acids, 6-hexadecynoic acid (compound 1), 6-heptadecynoic acid (compound 2), compound 3, compound 4, and 6-icosynoic acid (compound 5), were isolated. Compounds 2 and 5 are newly isolated natural products. This paper describes the isolation and structure elucidation of the new natural products and the antifungal activities of all the isolated compounds along with two commercially available analogs, 9-octadecynoic acid (compound 6) and 5,8,11,14-eicosatetraynoic acid (compound 7). A particular focus will be on the potential of these antifungal compounds as leads for the development of new treatments for topical fungal infections.

MATERIALS AND METHODS

General experimental procedures. Melting points (mp) were measured with a Thomas Hoover capillary melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded on an ATI Mattson Genesis Series FTIR spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance DRX-400 NMR spectrometer for the ^{1}H- and ^{13}C-NMR spectra and calibrated by residual $CDCl_3$ for δ_C 77.4 and δ_H 7.24 ppm. Electrospray ionization (ESI)-Fourier transform mass spectrometry (FTMS) was measured on a Bruker-Magnex BioAPEX 30es ion cyclotron high-resolution high-performance liquid chromatography (HPLC)–FT spectrometer by direct injection into an electrospray interface. Liquid chromatography-mass spectrometry (LC-MS) was conducted on a ThermoFinnigan aQa LC/MS system employing ESI and atmospheric pressure chemical ionization, interfaced with a ThermoFinnigan HPLC system with photodiode array detector. Column chromatography was run using a reverse-phase silica gel (RP-18; 40 μm; J. T. Baker). Thin-layer chromatography was performed on silica gel sheets (Alugram Sil G/UV_{254}; Macherey-Nagel, Germany) and reverse-phase plates (RP-18 F_{254S}; Merck, Germany). The antifungal drugs amphotericin B (AMB; ICN Biomedicals, OH), fluconazole (FLU; Sequoia Research Products Ltd., United Kingdom), ketoconazole (KTC; Sigma-Aldrich, MO), UDA (Sigma-Aldrich, MO), terbinafine (TRB; Novartis, NJ), and caspofungin (CASPO; Merck, NJ) were used as positive controls for in vitro

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antifungal susceptibility testing. Also included in the assay were commercial products 9-octadecynoic acid (compound 6; Alfa Aesar, MD) and 5,8,11,14-eicosatetraynoic acid (compound 7; A. G. Scientific, Inc., CA).

Isolation of acetylenic acids 1 to 5 from *Sommera sabiceoides*. The roots of *Sommera sabiceoides* Schuman were collected by Manuel Rimachi in Loreto, Peru, in April 1996 and identified by M. Rimachi and Sidney McDaniel. A voucher specimen is deposited at the Herbarium of Mississippi State University (voucher IBE-MR 11586). Ground dried roots (210 g) of *S. sabiceoides* were percolated with 95% ethanol (EtOH; 2.5 liters, three times). Removal of the solvent under vacuum at 40°C yielded an EtOH extract (8.3 g). An 8.1-g sample of the EtOH extract was chromatographed on a reverse-phase silica gel (C_{18}; 200 g) using a gradient solvent system of aqueous CH_3CN (80% CH_3CN, 3 liters; 85% CH_3CN, 2 liters; and finally 100% CH_3CN, 2 liters). Each fraction was collected in about 20 ml and examined on a reverse-phase silica gel plate (RP-18). The fractions showing a single spot on thin-layer chromatography were combined. Five pure compounds (1 to 5) were obtained.

6-Hexadecynoic acid (compound 1). White powder; mp, 40°C; IR (KBr) ν_{max}, 3,200 (br, weak absorption), 2,917, 2,850, 1,707, 1,460, 1,409, 1,312, 1,263, 1,206, 1,138, 1,076, 899, 717 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 0.87 (3H, t, J = 6.9 Hz, H-16), 1.25 to 1.39 (12H, m, H-10-15), 1.45 (2H, br quint, J = 6.9 Hz, H-9), 1.51 (2H, br quint, J = 7.1 Hz, H-4), 1.71 (2H, br quint, J = 7.2 Hz, H-3), 2.11 (2H, br t, J = 6.8 Hz, H-8), 2.16 (2H, br t, J = 6.8 Hz, H-5), 2.34 (2H, t, J = 7.4 Hz, H-2), 10.20 (1H, br s, COOH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.5 (C-16), 18.9 (C-5), 19.1 (C-8), 23.1 (C-15), 24.3 (C-3), 28.9 (C-4). 29.3, 29.5, 29.6, 29.7 and 29.9 (C-9 to C-13), 32.3 (C-14), 34.2 (C-2), 79.7 (C-6), 81.2 (C-7), 180.3 (C-1); ESI-MS m/z 253.2181 {calculated for $[M(C_{16}H_{28}O_2) + H]^+$, 253.2162}. To our knowledge, no NMR spectroscopic data for this compound are available in the literature (34).

6-Heptadecynoic acid (compound 2). White powder; mp, 39°C; IR (KBr) ν_{max}, 3,200 (br, weak absorption), 2,918, 2,850, 1,711, 1,470, 1,409, 1,261, 1,205, 1,075, 868, 716 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.87 (3H, t, J = 6.8 Hz, H-17), 1.20 to 1.40 (14H, m, H-10-16), 1.45 (2H, br quint, J = 6.8 Hz, H-9), 1.51 (2H, br quint, J = 7.0 Hz, H-4), 1.71 (2H, br quint, J = 7.2 Hz, H-3), 2.11 (2H, br t, J = 6.9 Hz, H-8), 2.16 (2H, br t, J = 6.8 Hz, H-5), 2.33 (2H, t, J = 7.4 Hz, H-2), 10.30 (1H, br s, COOH); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 14.6 (C-17), 18.9 (C-5), 19.2 (C-8), 23.1 (C-16), 24.4 (C-3), 28.9 (C-4), 29.4, 29.59, 29.63, 29.8, 30.02, and 30.06 (C-9 to C-14), 32.4 (C-15), 34.4 (C-2), 79.8 (C-6), 81.2 (C-7), 180.3 (C-1); ESI-MS m/z 267.2299 {calculated for $[M(C_{17}H_{30}O_2) + H]^+$, 267.2319}.

6-Octadecynoic acid (compound 3). Reference 25 includes a report of the physical and spectral data for compound 3.

6-Nonadecynoic acid (compound 4). Reference 25 includes a report of the physical and spectral data for compound 4.

6-Icosynoic acid (compound 5). White powder; mp, 53°C; IR (KBr) ν_{max}, 3,200 (br, weak absorption), 2,916, 2,849, 1,714, 1,539, 1,471, 1,409, 1,312, 1,250, 1,206, 1,076, 964, 899, 868, 717 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.87 (3H, t, J = 6.8 Hz, H-20), 1.20 to 1.40 (20H, m, H-10-19), 1.46 (2H, br quint, J = 7.2 Hz, H-9), 1.52 (2H, br quint, J = 7.2 Hz, H-4), 1.72 (2H, br quint, J = 7.2 Hz, H-3), 2.12 (2H, br t, J = 6.8 Hz, H-8), 2.17 (2H, br t, J = 6.8 Hz, H-5), 2.35 (2H, t, J = 7.4 Hz, H-2), 10.50 (1H, br s, COOH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.5 (C-20), 18.9 (C-5), 19.1 (C-8), 23.1 (C-19), 24.3 (C-3), 28.8 (C-4), 29.3, 29.5, 29.6, 29.8, 29.98, 30.07 (×4), and 30.12 (C-9 to C-18), 32.3 (C-19), 34.2 (C-2), 79.7 (C-6), 81.2 (C-7), 180.4 (C-1); ESI-MS m/z 309.2773 {calculated for $[M(C_{20}H_{36}O_2) + H]^+$, 309.2788}.

LC-MS analysis of the crude ethanol extract. Analysis was done on an ODS PhenoSphere-*Next* column (C_{18}; 125 by 4.0 mm) using a mobile phase of CH_3CN-H_2O (80:20) at a flow rate of 0.8 ml/min. Five major peaks at t_R of 7.17, 9.97, 14.82, 19.81, and 26.84 min were identified from the total ion current chromatogram and corresponded to the mass units of 251, 265, 279, 293, and 307, respectively, in the negative ion ESI-MS mode. The peaks for t_R of 14.82 and 19.81 min were identified as compounds 3 and 4, respectively, by using authentic compounds isolated from *P. gigantifolia* (25).

In vitro antifungal susceptibility testing. CLSI (formerly NCCLS) methods were used for susceptibility testing (29, 30). Organisms were obtained from the American Type Culture Collection (Manassas, VA) and included *Candida albicans* ATCC 90028, *C. albicans* ATCC 14053, *C. albicans* ATCC 60193, *C. albicans* ATCC 32354, *C. albicans* ATCC 200955, *Candida glabrata* ATCC 90030, *Candida krusei* ATCC 6258, *Candida parapsilosis* ATCC 22019, *Candida tropicalis* ATCC 750, *Cryptococcus neoformans* ATCC 90113, *Cryptococcus neoformans* ATCC 66031, *Aspergillus fumigatus* ATCC 90906, *A. fumigatus* ATCC 204305, *Aspergillus flavus* ATCC 204304, *Aspergillus niger* ATCC 16404, *Trichophyton mentagrophytes* ATCC 9533, *T. mentagrophytes* ATCC MYA-4439, *Trichophyton rubrum* ATCC MYA-4438, and *T. rubrum* ATCC 10218. *C. albicans* patient isolates 1 (first isolate) and 17 (fluconazole resistant) were kindly provided by T. C. White and S. W. Redding (25, 35).

On the day of the assay, suspensions of test organisms were prepared either by suspending three to five colonies from 24- to 72-h Sabouraud dextrose (SD) agar plates in sterile normal saline (*Candida* spp. and *Cryptococcus* spp.) or by scraping the growth from 1- to 6-week-old potato dextrose (PD) slants and filtering through sterile Miracloth (Calbiochem, San Diego, CA) (*Trichophyton* spp. and *Aspergillus* spp.). Standard curves of the optical density (OD) at 630 nm versus CFU/ml of saline suspensions of test organisms were generated using linear regression in order to calculate target assay inocula. Since no significant differences were observed in OD readings between 630 nm and 530 nm (CLSI recommended wavelength), OD measurements for inoculum preparation were performed at 630 nm. Assay inocula were prepared by using the standard curves to appropriately dilute microbe suspensions in incubation broth (RPMI 1640; 0.2% dextrose, 0.03% glutamine, buffered with 0.165 M morpholinepropanesulfonic acid [MOPS] at pH 7.0; Cellgro, Herndon, VA) for *Candida* spp., SD broth (BD Diagnostic Systems, Sparks, MD) for *C. neoformans*, and 5% Alamar blue (Biosource International, Camarillo, CA)–RPMI 1640 broth (0.2% dextrose, 0.03% glutamine, buffered with 0.165 M MOPS at pH 7.0) for *Aspergillus* spp. and *Trichophyton* spp. to afford final target inocula of 1.5×10^3 CFU/ml for *Candida* spp. and *Cryptococcus* spp. or 2.7×10^4 CFU/ml for *Aspergillus* spp. and *Trichophyton* spp. Inocula were verified for each assay by plating onto SD or PD agar for colony enumeration.

For *Cryptococcus neoformans*, SD broth was used instead of RPMI broth due to low growth. Two separate experiments using RPMI broth with both *C. neoformans* strains afforded poor assay quality and poor *Z* prime analysis, as the OD difference at 530 nm between medium blanks and solvent controls was very small. Therefore, the test medium only for *C. neoformans* was changed to our standard natural product-screening medium of SD broth. However, all other CLSI conditions were used (inoculum size, incubation temperature, and incubation time). Therefore, this should be taken into consideration when comparing data.

Test samples were serially diluted in 20% dimethyl sulfoxide–saline and transferred in duplicate to 96-well flat-bottom microplates. The highest test concentrations of the compounds and control drugs are indicated in Tables 1 and 2, respectively. All compounds and control drugs were assayed using twofold serial dilutions with a total of 11 test concentrations. Test organisms were added to assay plates to afford a final volume of 200 μl. *Candida* spp. and *C. neoformans* were read at 530 nm using the Biotek Powerwave XS plate reader (Bio-Tek Instruments, VT) prior to and after incubation (*Candida* spp. at 35°C for 48 h; *C. neoformans* at 35°C for 72 h). *A. fumigatus* and *T. mentagrophytes* were read at 544 nm excitation and 590 nm emission using the Polarstar Galaxy plate reader (BMG LabTechnologies, Germany) prior to and after incubation (*Aspergillus* spp. at 35°C for 48 h; *Trichophyton* spp. at 35°C for 5 days). Percent growth was calculated, and IC_{50}s were generated using the XLFit software (dose-response model 201; IDBS, Alameda, CA). The MIC is defined as the lowest test concentration that allows no detectable growth (or no more than 20% growth for the azoles and caspofungin). Minimum fungicidal concentrations (MFCs) were determined by removing 100 μl from each clear (or blue) well, transferring to PD (filamentous fungi) or SD (*Candida* spp. and *Cryptococcus* spp.) agar, and incubating as described above. The MFC is defined as the lowest test concentration that allows no growth of the organism on agar.

In vitro cytotoxicity assay. The mammalian cell lines SK-MEL (melanoma), KB (epidermal carcinoma, oral), BT-549 (ductal carcinoma, breast), SK-OV-3 (ovary carcinoma), Vero (African green monkey kidney fibroblast), and LLC-PK-1 (pig kidney epithelial) used in this study were obtained from ATCC (Manassas, VA). Cytotoxicity was determined by the neutral red method (4) up to a highest concentration of 32 μM. The detailed procedure was described in a previous paper (39).

In vivo cytotoxicity assay. Mice (CD-1; Harlan, Indianapolis, IN) were weighed and randomly distributed into seven groups (n = 3/group). They were kept in filter top cages and housed in environmentally controlled rooms (temperature, 22.2 ± 0.8°C; relative humidity, 30 to 50%) with a 12-h day and night light cycle. Food and water were provided ad libitum. The test compounds (3 and 4) were dissolved in peanut oil to the desired concentration. Six groups of mice were given intraperitoneal injections of 100 μl, affording doses of 3.4, 17, or 34 μmol/kg of body weight of compound 3 or 4. One group of mice was given 100 μl of peanut oil. Mice were observed for any discomfort at 2 h, 5 h, and 8 h postinjection. The body weights were recorded daily. At the end of 3 days, necropsy was performed and gross observations were recorded.

RESULTS AND DISCUSSION

Initial dereplication analysis of the antifungal methanol extract of *S. sabiceoides* by HPLC–ESI-MS revealed the presence of five acetylenic acid derivatives, including 6-octadecynoic acid (compound 3) and 6-nonadecynoic acid (compound 4), which were previously isolated from the plant *P. gigantifolia* (25). Although compounds 3 and 4 had been previously demonstrated to be active against *C. albicans* (25), the remaining three natural products were of great interest from the perspective of structure-activity relationships. Subsequent reverse-phase silica gel column chromatography of the extract using aqueous acetonitrile led to the isolation of five acetylenic acids (compounds 1 to 5), which correspond to the five peaks in the HPLC–ESI-MS (negative mode) analysis with retention times/pseudo-molecular ions of 7.17 min/251, 9.97 min/265, 14.82 min/279, 19.81 min/293, and 26.84 min/307, respectively.

The mass data from the HPLC–ESI-MS analysis indicated that compounds 1 to 5 are analogs with an increase of a methylene group (14 mass units) starting from 1 to 5. The molecular formulae of compounds 1 to 5 were further confirmed by high-resolution ESI-MS as $C_{16}H_{28}O_2$, $C_{17}H_{30}O_2$, $C_{18}H_{32}O_2$, $C_{19}H_{34}O_2$, and $C_{20}H_{36}O_2$, respectively. The structural identification of the three acetylenic acid analogs (compounds 1, 2, and 5) were readily achieved by careful comparison of their NMR spectra with those of compounds 3 and 4, whose structures were established by detailed chemical and spectroscopic methods (25). As indicated in the ^{13}C-NMR spectra for compounds 1 to 5, the chemical shifts of these compounds are very similar, particularly for C-1 to C-8 and the last three carbons starting from the terminal methyl group. The only difference is the number of the carbon signals attributable to the multimethylenes in the center of their straight carbon chains that resonate in the range of δ 29 to 31 ppm, showing the presence of 6, 7, 8, 9, and 10 carbon signals for compounds 1 to 5, respectively. This finding is also supported by the ^{1}H-NMR spectra, which showed close resemblance, with the exception of the integral protons in the range of δ 1.2 to 1.4 ppm from 12H, 14H, 16H, 18H, and 20H for compounds 1 to 5, respectively. Therefore, the structures of compounds 1, 2, and 5 are 6-hexadecynoic acid, 6-heptadecynoic acid, and 6-icosynoic acid, respectively. The last two represent new additions to the class of acetylenic acids. It is worthwhile to note that the aqueous acetonitrile system is an excellent mobile phase for column chromatography on reverse-phase silica gel (RP-18) to separate these fatty acids with minor structural modifications.

Compounds 1 to 5 and two commercially available acetylenic acid analogs, 9-octadecynoic acid (compound 6, a DNA binding agent [2]) and 5,8,11,14-eicosatetraynoic acid (compound 7, an inhibitor of arachidonic acid metabolism [31]), were tested for in vitro antifungal activity against 21 fungal pathogens: seven strains of *Candida albicans*, including a fluconazole-resistant isolate (35); four species of non-albicans *Candida*, including *C. glabrata*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis*; two strains of *Cryptococcus neoformans*; four strains of *Aspergillus*, including *A. fumigatus*, *A. flavus*, and *A. niger*; two strains of *Trichophyton mentagrophytes*; and two strains of *T. rubrum*. Six antifungal drugs with different mechanisms of action were used as positive controls: the polyene AMB, which interacts with fungal ergosterol, thereby disrupting the cytoplasmic membrane (15); the azoles FLU and KTC, which inhibit 14α-lanosterol demethylase in the ergosterol biosynthesis pathway (15); the lipopeptide CASPO, which inhibits the synthesis of 1,3-β-glucan (15); the fatty acid UDA, inhibiting morphogenesis of *C. albicans* (27); and the allyamine TRB, which inhibits squalene epoxidase in the ergosterol synthesis pathway (15). The results are shown in Tables 1 and 2.

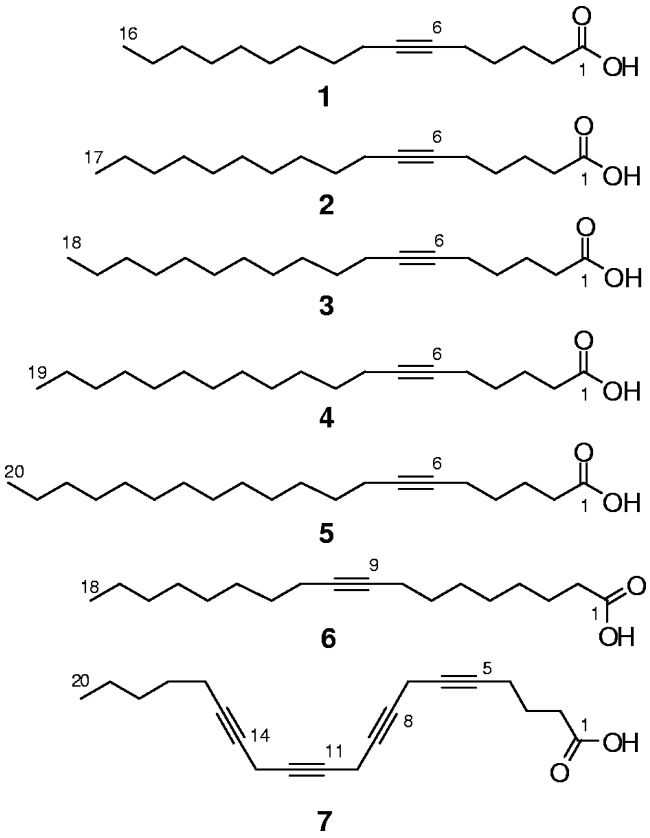

FIG. 1. Structures of acetylenic acids 1 to 7.

The seven acetylenic acids tested (Fig. 1), which differ only in their chain lengths or the position or number of triple bonds, showed various degrees of antifungal activity (Table 1). Compounds 3 and 4, which have a C-6 triple bond and carbon chain lengths of 18 and 19, respectively, demonstrated consistently good activity against the *C. albicans* strains. The remaining three 6-acetylenic acids, compounds 1, 2, and 5, which have chain lengths of 16, 17, and 20, respectively, were essentially inactive or marginally active, as was compound 6, which has a carbon chain length of 18 but a triple bond located at C-9 instead of C-6. These susceptibility patterns for compounds 1 to 6 seem to be similar to those against the *Trichophyton* spp. For example, compounds 3 and 4 showed potent fungicidal activities against *T. mentagrophytes* ATCC MYA-4439, *T. mentagrophytes* ATCC 9533, and *T. rubrum* ATCC 10218, with MFCs ranging from 0.7 to 11.1 μM, while compounds 1 and 2 exhibited decreased activities and compounds 5 and 6 were essentially inactive. These data suggest that both the chain length and the position of the triple bond are important for antifungal activity. Additionally, it appears that multiple triple bonds diminish or eliminate activity, as with compound 7,

TABLE 1. In vitro antifungal activities of acetylenic acids 1 to 7[a]

Species and strain or isolate	IC_{50}/MIC/MFC (μM) for compound no.[b]:						
	1	2	3	4	5	6	7
C. albicans							
ATCC 14053	81.8/−/−	>187.7[c]/−/−	1.0/2.8/5.6	0.8/1.3/2.7	>162.1/−/−	−/−/−	8.1/63.3/−
ATCC 60193	85.9/−/−	150.0/−/−	3.0/5.6/−	1.6/2.7/−	81.0/−/−	−/−/−	4.5/63.3/−
ATCC 32354	50.3/198.1/−	187.7/−/−	4.0/27.9/−	1.6/8.0/−	>162.1/−/−	−/−/−	4.0/31.7/−
ATCC 90028	56.8/99.1/−	95.4/−/−	2.0/2.8/11.1	1.3/2.7/−	67.3/−/−	−/−/−	5.0/42.2/−
ATCC 200955	47.8/198.1/−	66.7/−/−	1.9/11.1/−	0.9/1.3/−	53.4/−/−	−/−/−	2.0/5.3/21.1
Isolate 1[d]	40.5/99.1/−	82.2/−/−	4.2/13.9/−	1.7/2.7/−	86.6/−/−	−/−/−	1.7/42.2/−
Isolate 17[d]	23.3/99.1/198.1	18.8/70.4/−	3.6/16.7/−	1.5/8.0/−	55.0/−/−	−/−/−	3.5/42.2/−
Other *Candida* spp.							
C. glabrata ATCC 90030	>198.1/−/−	>187.7/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−
C. krusei ATCC 6258	2.9/6.2/49.5	1.9/4.4/−	3.4/5.6/−	4.1/10.6/−	106.1/−/−	>178.7/−/−	>168.9/−/−
C. parapsilosis ATCC 22019	>198.1/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	5.4/63.3/−
C. tropicalis ATCC 750	29.5/74.3/99.1	49.1/−/−	6.3/111.4/−	5.2/−/−	−/−/−	−/−/−	27.7/−/−
C. neoformans[e]							
ATCC 90113	52.4/−/−	>187.7/−/−	−/−/−	−/−/−	−/−/−	−/−/−	>168.9/−/−
ATCC 66031	1.2/49.5/99.1	1.2/187.7/−	1.4/−/−	1.1/−/−	−/−/−	>178.6/−/−	−/−/−
Aspergillus spp.							
A. fumigatus ATCC 204305	9.4/37.1/198.1	36.6/140.8/−	18.7/178.3/−	1.1/2.7/−	32.3/−/−	−/−/−	10.0/84.5/−
A. fumigatus ATCC 90906	165.6/−/−	−/−/−	−/−/−	1.4/6.6/−	81.0/−/−	−/−/−	13.3/84.5/−
A. flavus ATCC 204304	105.9/198.1/198.1	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	>168.9/−/−
A. niger ATCC 16404	100.9/−/−	>187.7/−/−	−/−/−	−/−/−	−/−/−	−/−/−	100.0/−/−
Trichophyton spp.							
T. mentagrophytes ATCC MYA-4439	3.9/9.3/12.4	3.0/5.9/5.9	1.2/2.8/2.8	0.4/0.7/0.7	>162.1/−/−	>178.6/−/−	6.7/31.7/−
T. mentagrophytes ATCC 9533	2.3/4.6/6.2	3.7/7.3/11.7	1.3/2.1/2.8	0.9/2.0/2.7	−/−/−	−/−/−	14.2/21.1/−
T. rubrum ATCC MYA-4438	3.8/31.0/198.1	4.1/29.3/−	1.8/5.6/−	0.6/3.3/−	−/−/−	80.2/−/−	2.9/31.7/−
T. rubrum ATCC 10218	8.7/24.8/24.8	6.5/17.6/23.5	2.7/5.6/11.1	1.0/2.0/5.3	−/−/−	>178.6/−/−	8.4/15.8/−

[a] Highest test concentrations for compounds 1 to 7 were 198.1, 187.7, 178.3, 169.8, 162.1, 178.6, and 168.9 μM, respectively.
[b] Values are means from two independent experiments, each with two replicates; the run-to-run variability was within one- to twofold. −, not active at the highest test concentration.
[c] Some growth inhibition (25 to 49% inhibition) was observed at that concentration.
[d] Patient isolates were obtained during fluconazole therapy; isolate 1 (first isolate) and isolate 17 (last isolate) were azole resistant (35).
[e] Values are means from one experiment using two replicates.

which has four triple bonds on a C_{20} carbon backbone. Interestingly, in the 6-acetylenic acid series compounds 1 to 4 are also active against *C. krusei* (MIC, 4.4 to 10.6 μM), which is inherently resistant to FLU (MIC, 163.3 μM). None of the other non-albicans *Candida* spp. nor the *C. neoformans*, *A. flavus*, or *A. niger* strains was significantly inhibited by any of the seven compounds tested. These data suggest that the antifungal activities of these acetylenic acids against respective fungal pathogens are strongly associated with their chain lengths and positional triple bonds.

Within the series of acetylenic acids tested, compound 4 showed the most consistent and potent activity across various strains within each species of fungal pathogen, with activity comparable to positive control drugs against *C. albicans* (MICs, 1.3 to 8.0 μM, compared to FLU MICs of 0.6 to >163.3 μM), *A. fumigatus* (MICs, 2.7 to 6.6 μM, compared to AMB MICs of 1.4 to 2.0 μM), and all four *Trichophyton* strains (MICs, 0.7 to 3.3 μM, compared with AMB MICs of 1.4 to 2.0 μM, KTZ MICs of 0.04 to 0.7 μM, and TRB MICs of 0.05 to 21.4 μM). Additionally, compound 4 has potent activity against the FLU-resistant *C. albicans* clinical isolate 17 (MIC, 8.0 μM; FLU MIC, >163.3 μM).

Our work reported here confirms the observations reported by others that the in vitro antifungal activity of an agent is largely dependent upon the susceptibility testing methods, and considerable variations for intra- and interlaboratory results may occur (19, 36), depending on the methods employed. For example, in a previous study, we isolated compound 4 from the plant *P. gigantifolia* and reported its activity against *C. albicans* ATCC 90028 (MIC, 1.8 μM [0.52 μg/ml]), as well as several FLU-resistant *C. albicans* strains (25). In that previous work, we used a slightly modified CLSI method, in which RPMI 1640 supplemented with 2% dextrose was used as the incubation medium, a larger inoculum size was employed, and plates were incubated at 37°C (25). In contrast, Carballeira et al. recently reported the synthesis and in vitro antifungal evaluation of compound 4 and reported it to be inactive against two strains of *C. albicans* (ATCC 14053, MIC of 6,672 μM; ATCC 60193, MIC of 8,896 μM) (6, 7) but active against *Cryptococcus neoformans* ATCC 66031 (MIC, <4.3 μM). Our results in the present study show compound 4 to be significantly active against a variety of *Candida* spp. strains (>800× more active than reported by Carballeira et al.) and inactive against *C. neoformans* (no MICs were observed). One explanation for these differences is that Carballeira et al. evaluated the antifungal activity of compound 4 by a modified CLSI method

TABLE 2. In vitro antifungal activities of antifungal drug controls[a]

Species and strain or isolate	IC_{50}/MIC/MFC (μM) of:[b]					
	AMB	FLU	KTC	CASPO	UDA	TRB
C. albicans						
ATCC 14053	0.3/1.0/1.4	0.5/1.3/−	0.01/0.02/−	0.05/0.10/0.26	>542.7[c]/−/−	13.7/−/−
ATCC 60193	0.2/1.0/1.4	0.4/0.6/−	0.01/0.02/−	0.03/0.10/0.52	>542.7/−/−	5.3/−/−
ATCC 32354	0.3/1.0/1.4	0.8/5.7/−	0.01/0.03/−	0.07/0.13/1.03	>542.7/−/−	2.6/−/−
ATCC 90028	0.3/1.0/1.4	0.8/6.4/−	<0.01/0.01/−	0.06/0.19/0.26	−/−/−	10.8/−/−
ATCC 200955	0.6/2.7/2.7	0.4/1.3/−	<0.01/0.01/−	0.05/0.13/0.13	>542.7/−/−	3.1/51.5/−
Isolate 1[d]	0.2/1.0/1.4	0.8/5.1/−	<0.01/0.10/−	0.05/0.10/0.26	>542.7/−/−	11.2/−/−
Isolate 17[d]	0.2/1.0/1.4	163.3/−/−	0.3/−/−	0.04/0.13/0.26	>542.7/−/−	>68.3/−/−
Other *Candida* spp.						
C. glabrata ATCC 90030	0.4/1.4/1.4	29.5/81.6/−	0.7/4.7/-	0.05/0.13/0.26	−/−/−	−/−/−
C. krusei ATCC 6258	0.7/2.0/2.7	125.7/163.3/−	0.4/1.1/−	0.1/0.4/1.0	>542.7/−/−	>68.3/−/−
C. parapsilosis ATCC 22019	0.3/2.0/5.4	3.4/10.2/−	0.04/0.13/−	0.2/0.5/−	>542.7/−/−	0.4/42.9/−
C. tropicalis ATCC 750	0.3/1.0/1.4	4.3/15.3/−	0.04/0.19/−	0.06/0.10/0.26	>542.7/−/−	45.4/−/−
C. neoformans[e]						
ATCC 90113	0.3/0.7/0.7	8.5/20.4/163.3	0.1/0.3/1.2	0.4/1.0/2.1	25.3/271.3/542.7	0.5/1.1/1.1
ATCC 66031	0.08/0.34/0.34	0.7/5.1/20.4	0.02/0.15/0.59	0.1/0.5/1.0	2.7/135.7/271.3	0.4/4.3/8.6
Aspergillus spp.						
A. fumigatus ATCC 204305	1.2/2.0/10.8	−/−/−	2.7/4.7/−	?[f]/−/−	−/−/−	1.1/4.3/−
A. fumigatus ATCC 90906	0.9/1.4/2.7	−/−/−	3.2/7.1/−	0.2/16.5/−	−/−/−	1.0/4.3/−
A. flavus ATCC 204304	1.5/2.7/−	−/−/−	2.8/4.7/−	?[f]/−/−	−/−/−	0.3/2.1/8.6
A. niger ATCC 16404	0.9/2.0/−	−/−/−	6.0/9.4/−	−/−/−	414.9/−/−	0.7/2.1/−
Trichophyton spp.						
T. mentagrophytes ATCC MYA-4439	0.9/1.4/1.4	24.2/81.6/−	0.1/0.7/−	0.2/−/−	378.9/542.7/−	<0.02/0.17/0.27
T. mentagrophytes ATCC 9533	0.6/2.0/2.7	29.5/61.2/−	0.2/0.4/−	0.3/−/−	279.0/542.7/−	<0.02/0.05/0.27
T. rubrum ATCC MYA-4438	0.6/1.4/5.4	2.7/6.4/−	0.01/0.08/−	0.06/0.10/−	111.3/542.7/−	4.2/21.4/−
T. rubrum ATCC 10218	0.6/1.4/1.4	1.8/1.9/163.3	0.02/0.04/−	−/−/−	270.0/542.7/−	<0.02/0.10/0.13

[a] The highest test concentrations of AMB, FLU, KTC, CASPO, UDA, and TRB were 10.8, 163.3, 9.4, 16.5, 542.7, and 68.3 μM, respectively.
[b] Values are means from two independent experiments, each with two replicates; the run-to-run variability was within one- or twofold. −, not active at the highest test concentration.
[c] Some growth inhibition (25 to 49% inhibition) was found at that concentration.
[d] Patient isolates were obtained during fluconazole therapy; isolate 1 (first isolate) and isolate 17 (last isolate) were azole resistant (35).
[e] Values are means from one experiment with two replicates.
[f] XLFit could not calculate an IC_{50}, as growth was present at high and low test concentrations but inhibition was observed at mid-concentrations, possibly due to a concentration effect.

using SD broth (rather than RPMI medium) and possibly a shorter incubation time (24 to 48 h, versus 48 h in our present study). In the present study, we followed the CLSI methods (29, 30), using the recommended inoculum size, temperature, incubation time, and medium conditions for all the *Candida* strains. Due to poor growth in RPMI, SD broth was substituted for analysis of activity against *C. neoformans* in order to afford susceptibility data. Our results indicate that compound 4, isolated from *S. sabiceoides*, showed potent antifungal activities against *C. albicans* ATCC 14053 (MIC, 1.3 μM), *C. albicans* ATCC 60193 (MIC, 2.7 μM), and *C. albicans* ATCC 90028 (MIC, 2.7 μM) (Table 1). In addition, this compound did not show strong activity against *C. neoformans* ATCC 66031, even though the same SD broth was used in both studies for this strain. While the reason for this discrepancy is unclear at this time, it is possible that other experimental conditions (such as inoculum size, incubation time, etc.) could account for these differences (6, 7).

To assess potential toxicity, the compounds were evaluated for in vitro cytotoxicity (39) in a panel of two noncancerous mammalian kidney cell lines (Vero and LLC-PK-1) and four human cancer cell lines (SK-MEL, KB, BT-549, and SK-OV-3). The results indicate that compounds 1 to 7 are not toxic up to the concentration of 32 μM. Follow-up evaluations of compounds 3 and 4 for in vivo toxicity in mice showed no obvious toxic effects at intraperitoneal doses up to 34 μmol/kg/day for 3 days.

Naturally occurring acetylenic acids are widely distributed in nature and are found in terrestrial plants, fungi, microorganisms, and marine organisms (3, 11). Biosynthetically, they are produced by further desaturation of olefinic systems in the fatty acid-derived molecules. The acetylenic acids containing multiple triple and/or double bonds are generally unstable, particularly for those with conjugated systems. For example, mycomycin (3,5,7,8-tridecatetraene-10,12-diynoic acid), isolated in 1950 and shown to be active against *Mycobacterium tuberculosis*, is highly unstable (8, 21). Compound 7 used in this study is also unstable. However, compounds 1 to 5, with a single triple bond, are stable enough for potential pharmaceutical development.

Extensive studies have been conducted regarding the chemistry and biological activities of acetylenic acids. A recent comprehensive review on acetylenic lipids, which also covered acetylenic acids, highlighted their anticancer activities (12).

The antibacterial, anti-human immunodeficiency virus, and pesticidal activities of some acetylenic acids have also been reported (5, 9, 13, 17, 18, 22). Several studies have also shown that natural and synthetic acetylenic acids have activity against human and agricultural fungal pathogens (5, 9, 14, 16, 17, 24). As with the antibacterial activity, their antifungal activity is generally associated with their chain length and positional triple bond (6, 16, 22, 28). For example, 4-, 5-, and 6-octynoic acids and 9-undecynoic acid are antifungal against *T. mentagrophytes*, with the last being the most active compound and slightly less potent than undecylenic acid (9). Our findings are consistent with those reported in the literature.

UDA is the only compound within the fatty acid class that is used as a topical antifungal drug for the treatment of dermatomycosis as well as oral thrush and denture stomatitis (20, 27). *T. mentagrophytes* and *T. rubrum* are two major causative fungal pathogens for dermatomycosis, while *C. albicans*, generally considered the most pathogenic *Candida* species, has been identified as the most prevalent yeast encountered in oral candidiasis (23). The antifungal mechanism of fatty acids may involve interference with fungal fatty acid biosynthesis (32, 33, 38). The advantage of UDA is its low toxicity and favorable safety profile; its shortcoming is its low cure rate due to its weak antifungal potency, as shown by the weak in vitro activity against the tested pathogens in Table 2, especially when compared to TRB, a powerful fungicidal drug for the treatment of dermatomycosis (with adverse events in 10.5% of the recipients) (10, 37). It is interesting that McLain et al. demonstrated that UDA inhibits morphogenesis of *C. albicans* at a 10 μM concentration; however, no inhibition of growth was observed up to 100 μM in vitro (27). Additionally, in that report, UDA was present at a very high concentration of approximately 70 mM in the denture liners used to study in vivo denture stomatitis. The discovery of these naturally occurring acetylenic acids with potent antifungal activities against *T. mentagrophytes*, *T. rubrum*, and *C. albicans* may represent an opportunity for the development of new topical antifungal agents derived from the fatty acid class.

In conclusion, the current study has demonstrated that acetylenic acids, e.g., compound 4, with low in vitro and in vivo toxicity profiles, are highly superior to UDA in terms of their in vitro antifungal potencies against *T. mentagrophytes*, *T. rubrum*, and *C. albicans* (Tables 1 and 2). Their strong fungicidal activities against *T. mentagrophytes* and *T. rubrum* indicates that they may quickly eradicate these pathogens and thus may reduce the chance of developing drug resistance in the treatment of topical infections. In addition, the synthetic methodologies for this class of compounds are available in the literature (6, 7, 26). Taking into account their antifungal potencies, low toxicities, synthetic accessibilities, and good pharmaceutical properties (similar to those of UDA), these 6-acetylenic acids may be excellent leads for further preclinical studies on their use as topical antifungal drug candidates to manage oral candidiasis and dermatomycosis.

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