Total Synthesis and Further Scrutiny of the *in vitro* Antifungal Activity of 6-Nonadecynoic Acid

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The antifungal, naturally occurring acetylenic fatty acid 6-nonadecynoic acid was synthesized in three steps (18% overall yield), for the first time starting with commercially available 1-tetradecyne. The synthesis developed herein will facilitate the further study of the antifungal properties of this naturally occurring acetylenic fatty acid. The 6-nonadecynoic acid exhibited the best antifungal activity (< 4.3 μM) against *Cryptococcus neoformans* ATCC 66031 in Sabouraud Dextrose Broth (SDB) media. In our hands, it was not active against *Candida albicans* ATCC 14053 and *Candida albicans* ATCC 60193.

Keywords: Synthesis; Fatty acids; Antifungal; Acetylenic acids; *C. neoformans*

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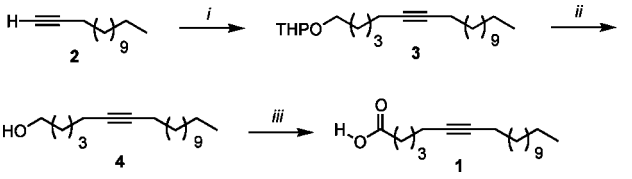
Introduction

The antimicrobial and antifungal properties of acetylenic fatty acids have been an active area of lipid research for some time [1–3]. Just recently, a novel acetylenic fatty acid, 6-nonadecynoic acid **1**, together with the known 6-octadecynoic acid, was isolated from an ethanol extract of the roots of *Pentagonia gigantifolia* [4]. The acetylenic acid **1** was reported to be fungistatic and specific to fluconazole-susceptible and resistant *Candida albicans* strains with MIC values (0.52 μg/mL) comparable to those of amphotericin B (0.52 μg/mL) and fluconazole (0.29 μg/mL) [4]. The antifungal mechanism of action of **1** has been suggested to be due to the interference of the compound with fungal sphingolipid biosynthesis [4]. Therefore, the nonadecynoic acid **1** is a potential antifungal lead substance for further studies. The synthesis of large quantities of **1** is needed for these studies. Herein, we report the first total synthesis of **1** and, in addition, we report that **1** is also fungistatic to a strain of *Cryptococcus neoformans* but not effective against other strains of *Candida albicans*. This finding is in contrast to what was previously reported for the 6-nonadecynoic acid (**1**), which was found to be specific towards *C. albicans* but not fungistatic towards *C. neoformans* [4].

Results and discussion

Our approach towards the total synthesis of 6-nonadecynoic acid (**1**) started with commercially available 1-tetradecyne (**2**) which was successfully alkylated with 2-(5-bromopentyloxy)tetrahydropyran in the presence of *n*-BuLi in THF-HMPA at −10 °C (better solubility at this temperature) which resulted in the tetrahydropyranyl protected alkyne **3** in 82% yield (Scheme 1). Deprotection of **3** was achieved with *p*-toluenesulfonic acid (PTSA) in methanol at 45 °C, which yielded the desired 6-nonadecyn-1-ol (**4**) in a 45% yield. Final oxidation of **4** with pyridinium dichromate (PDC) in DMF afforded the 6-nonadecynoic acid (**1**) in 49% isolated yield. The overall yield for this three-step synthesis was 18%. The synthetic acid **1** presented spectral data identical with the natural acetylenic fatty acid **1** as previously reported [4].



Scheme 1. Synthesis route for 6-nonadecynoic acid. Reagents and conditions: *i* Br-$(CH_2)_5$OTHP, *n*-BuLi, THF, HMPA, −10 °C; *ii* PTSA, MeOH, 45 °C; *iii* PDC, DMF, rt.

The antifungal activity of **1** against *Cryptococcus neoformans* ATCC 66031, *Candida albicans* ATCC 14053, *Candida albicans* ATCC 60193, and *Aspergillus niger* ATCC 16404 in SDB media were determined using a modified method of the National Committee for Clinical Laboratory Standards (NCCLS) as described by Galgiani and the more recent NCCLS M27-A microdilution methods as described previously [5-8]. As shown in Table 1 amphotericin B and

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Short Communication

Table 1. Antifungal activity (MIC values, μM)† against *Cryptococcus neoformans* (SDB), *Candida albicans* (SDB), and *Aspergillus niger* (SDB) at 35−37 °C after 24−48 h.

Compound	*C. neoformans* ATCC 66031	*C. albicans* ATCC 14053	*C. albicans* ATCC 60193	*A. niger* ATCC 16404
1	< 4.3	6672	8896	2224
Fluconazole	< 1.9	4003	> 4003	1001
Amphotericin B	< 0.2	< 0.2	< 0.2	< 0.2
DMSO	> 5000	> 5000	> 5000	> 5000

† The result is the average of three separate experiments.

fluconazole were used as positive controls. Acid **1** displayed antifungal activity against *C. neoformans* ATCC 66031 with a MIC of less than 4.3 μM but did not exhibit any activity against *A. niger* ATCC 16404 (MIC 2.224 μM) and, more surprisingly, neither against *Candida albicans* ATCC 14053 (MIC 6.672 μM) nor *Candida albicans* ATCC 60193 (MIC 8.896 μM). The two latter *C. albicans* strains are fluconazole resistant strains. It was previously reported that the natural acetylenic fatty acid **1** is specific towards *C. albicans* ATCC 90028 but not active against *C. neoformans*. This difference in fungitoxicity could be due to differences in the susceptibility of *C. neoformans* and *C. albicans* strains. Nevertheless, our results underline once again the importance of acid **1** as an antifungal compound.

In summary, we have accomplished the first total synthesis of the 6-nonadecynoic acid **1**, a novel potent antifungal acetylenic fatty acid. Our synthetic approach should provide access to considerable amounts of **1** for further biological evaluation. In addition, we have shown that **1** is indeed antifungal against *C. neoformans* at concentrations that are comparable with those of fluconazole, but in contrast to what was previously reported, it is active only against some strains of *C. albicans*.

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Experimental

IR spectra were recorded on a Nicolet 600 FT-IR spectrophotometer (Thermo-Nicolet, Madison, WI, USA). ^{1}H- and ^{13}C-NMR spectra were recorded on a General Electric QE-300, Bruker DPX-300 or on a Bruker DRX-500 spectrometer (General Electric Magnetic Resonance, Fremont, CA, USA or Bruker-Biospin, Billerica, MA, USA). ^{1}H-NMR chemical shifts are reported with respect to internal Me_4Si and ^{13}C chemical shifts are reported in parts per million (ppm) relative to $CDCl_3$ (77.0 ppm). Mass spectra data was acquired using a GC-MS (Hewlett-Packard 5972A MS ChemStation; Hewlett-Packard, Palo Alto, CA, USA) at 70 eV equipped with a 30 m × 0.25 mm special performance capillary column (HP-5MS) of polymethylsiloxane cross-linked with 5% phenyl methylpolysiloxane.

*1-[(*Tetrahydropyran-*2-yl)oxy]-6-nonadecyne (**3**)*

To a stirred solution of 1-tetradecyne (0.82 g, 4.2 mmol) in dry THF (5.0 mL), *n*-BuLi (2.5 M, 10.0 mmol) in dry hexane (4.0 mL) was added dropwise at room temperature. After 80 min, HMPA (10.0 mL) and 2-(5-bromopentyloxy)tetrahydropyran (1.06 g, 4.2 mmol) were added dropwise to the reaction mixture while maintaining the temperature approximately at − 60 °C. After 24 h, the reaction mixture was poured into a large volume of water and extracted with hexane (2 × 20 mL). The organic layer was washed with brine (1 × 20 mL) before drying over $MgSO_4$. Filtration, evaporation of the solvent and fractional distillation furnished 1.25 g of 1-[(tetrahydropyran-2-yl)oxy]-6-nonadecyne (**3**) in 82% yield. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 4.57 (1H, t, J = 2.3 Hz), 3.86−3.73 (2H, m), 3.49-3.38 (2H, m), 2.12 (4H, m, H-5, H-8), 1.81−1.68 (6H, m), 1.61−1.41 (8H, m), 1.24 (18H, m, -CH_2-), 0.87 (3H, t, J = 6.9 Hz, H-19); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 98.8 (d), 80.4 (s), 79.9 (s), 67.4 (t, C-1), 62.3 (t), 31.9 (t, C-17), 30.7 (t, C-2), 29.6 (t), 29.5 (t), 29.32 (t), 29.27 (t), 29.1 (t), 29.0 (t), 28.9 (t), 28.5 (t), 25.5 (t), 22.7 (t, C-18), 19.6 (t, C-3), 18.7 (t), 18.4 (t), 14.1 (q, C-19); GC-MS *m/z* (% rel. int.): $[M]^+$ 364 (1), 291 (4), 279 (2), 209 (2), 195 (8), 181 (1), 167 (1), 165 (1), 151 (1), 148 (1), 135 (2), 125 (3), 123 (3), 111 (4), 109 (9), 101 (10), 97 (5), 95 (14), 85 (100), 81 (14), 79 (12), 67 (22), 57 (12), 55 (19).

*6-Nonadecyn-1-ol (**4**)*

1-[(Tetrahydropyran-2-yl)oxy]-6-nonadecyne (1.4 g, 3.9 mmol) in methanol (10.0 mL), and catalytic amounts of *p*-toluene sulfonic acid (PTSA) were stirred at 45 °C for 3 h. The solvent was evaporated, hexane (5 mL) and diethyl ether (5 mL) were added to crystallize excess PTSA. Afterwards it was filtered and evaporated under high vacuum affording 0.05 g (45%) of 6-nonadecyn-1-ol (**4**). This product was used in the next step without further purification. IR (neat) ν_{max} 3314 (br), 2925, 2854, 2116, 1463, 1234, 1186, 1136, 1051 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 3.64 (2H, t, J = 6.5 Hz, H-1), 2.31 (1H, br, -OH), 2.14 (4H, m, H-5, H-8), 1.55 (2H, quintet, J = 6.7 Hz, H-2), 1.45 (4H, quintet, J = 6.9 Hz, H-4, H-9), 1.25 (20 H, m, -CH_2-), 0.87 (3H, t, J = 6.8 Hz, H-19); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 80.5 (s), 79.8 (s), 62.9 (t, C-1), 32.2 (t), 31.9 (t), 29.6 (t), 29.5 (t), 29.3 (t), 29.1 (t), 28.9 (t), 24.9 (t, C-3), 22.7 (t, C-18), 18.72 (t), 18.70 (t), 14.1 (q, C-19); GC-MS *m/z* (% rel. int.): $[M+1]^+$ 281 (1), 180 (2), 166 (2), 149 (3), 135 (8), 121 (10), 108 (34), 93 (66), 82 (100), 67 (83), 55 (76).

*6-Nonadecynoic acid (**1**)*

To a stirred solution of 6-nonadecyn-1-ol (0.056 g, 0.20 mmol) in 3.0 mL DMF pyridinium dichromate (0.3 g, 0.80 mmol) was slowly added at room temperature. After 24 h, the reaction mixture was poured in 10 mL of water and extracted with hexane (3 × 10 mL). Once the solvent was evaporated, the mixture was dried under vacuum and impurities were vacuum distilled (Kugelrohr short-path distillation). Thereby, 0.029 g of 9-nonadecynoic acid (**1**) was obtained, resulting in a 49% yield. Acid **1** presented identical spectral data with the natural acetylenic acid **1** previously reported, and the purity of the compound was estimated to be >97% by ^{13}C-NMR [4].

Microorganisms

A. niger ATCC 16404, *C. neoformans* ATCC 66031, *C. albicans* ATCC 14053, and *C. albicans* ATCC 60193 were obtained from American Type Culture Collection, Manassas, VA, USA. Stock cultures were kept on Sabouraud Dextrose Agar (SDA; Becton-Dickinson and Co., Sparks, MD, USA). Subcultures were prepared on SDA at 35-37 °C. Suspension cultures were prepared by inoculation of single colonies in 7 ml of normal saline solution. Prior to preparation of susceptibility assays, yeast cells were resuspended in normal saline to yield a transmittance of 73-75% at 530 nm that equals a concentration of 10^6 cells/mL and spores of *A. niger* in saline medium produce a similar transmittance at 530 nm compared to the control tube. The media was Sabourad Dextrose Broth (SDB).

Chemicals and antifungal agents

Amphotericin B (AMB) was purchased from Acros, New Jersey, USA and was kept as a 5 mM stock in DMSO at 0 °C and used during one week of preparation. Fluconazole (FLC) was purchased from Medisa Inc., New York, NY, USA, or was provided from Vera Laboratories Ltd, Hyderabad, India, and was kept as a 20 mM stock solution at 0 °C. Working dilutions were made in SDB medium. Higher concentrations of compounds were used for those with weak antifungal activities. The final maximum concentration of DMSO in the assays was 5% (v/v). DMSO was not inhibitory to the organisms in the concentrations tested.

Susceptibility testing

Microdilutions for control experiments with *C. albicans*, *A. niger*, and *C. neoformans* were performed according to the modified method of National Committee for Clinical Laboratory Standards (NCCLS) method as described by Galgani [5] and according to the more recent NCCLS M27-A microdilution methods as described previously [7–8]. Dilutions were prepared in 0.1 mL of SDB; the inocula were either 10^4 *C. albicans* or *C. neoformans* cells or *A. niger* spores. The tubes were incubated for 24-48 h at 36 ± 1 °C, and turbidity was read visually. MICs were calculated in comparison to growth control as the lowest concentration that shows inhibition for AMB, FLC, and the test compounds.

References

[1] W. Konthikamee, J. R. Gilbertson, H. Langkamp, H. Gershon, *Antimicrob. Agents Chemother.* **1982**, *22*, 805–809.

[2] H. Gershon, L. Shanks, *Can. J. Microbiol.* **1978**, *24*, 593–597.

[3] N. McLain, R. Ascanio, C. Baker, R. A. Strohaver, J. W. Dolan, *Antimicrob. Agents Chemother.* **2000**, *44*, 2873–2875.

[4] X.-C. Li, M. R. Jacob, H. N. ElSohly, D. G. Nagle, T. J. Smillie, L. A.Walker, A. M. Clark, *J. Nat. Prod.* **2003**, *66*, 1132–1135.

[5] J. N. Galgiani, *Antimicrob. Agents Chemother.* **1993**, *37*, 2517–2521.

[6] National Committee for Clinical Laboratory Standards. Reference method for broth dilution of antifungal susceptibility testing for yeasts. Approval standard. Document M27-A. National Committee for Clinical laboratory Standards, Wayne, PA, **1997**, 17: no. 9.

[7] N. M. Carballeira, D. Ortiz, K. Parang, S. Sardari, *Arch.Pharm.-Pharm. Med. Chem. Pharm.* **2004**, *337*, 152–155.

[8] N. H. Nam, S. Sardari, M. Selecky, K. Parang, *Bioorg. Med. Chem.* **2004**, *12*, 6255–6269.