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Antifungal Activity of Artemisinin Derivatives

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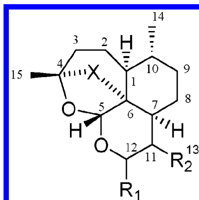
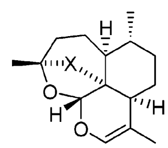
A series of 29 artemisinin derivatives (**2**–**30**), including four new compounds (**16**–**18**, **20**), together with artemisinin (**1**), artemisinic acid (**31**), and arteannuin B (**32**), were tested for antifungal activity against two opportunistic pathogens, *Candida albicans* and *Cryptoccocus neoformans*. Of all the compounds tested, anhydrodihydro-artemisinin (**3**) demonstrated more potent antifungal activity against *C. neoformans* than amphotericin B. Also, *β*-arteether (**7**) and α-arteether (**8**) showed marked activity against *C. neoformans*. Against *C. albicans,* the overall antifungal effect of these compounds was weak or negligible. Derivatives **2**–**30** were prepared according to literature procedures.

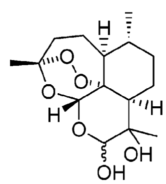
During the past two decades, the incidence of fungal infections, in particular those associated with immunocompromised patients, has increased dramatically.[1] *Cryptococcus neoformans* is the cause of the most common life-threatening opportunistic fungal infections in patients with HIV/AIDS.[2] Although the occurrence of *C. neoformans* among this group of patients has decreased in the past few years due to the introduction of triple HIV therapy, the incidence remains high, particularly in developing countries.[3] Cryptococcosis caused by *C. neoformans* involves infection of the central nervous system, which is often manifested as meningitis, and is now seen most often in patients with AIDS, of whom 2–20% develop this condition.[4] In addition, candidiasis caused by *Candida albicans* is also one of the most frequent (though uncommonly life-threatening) fungal infections attacking persons with HIV/AIDS.[5] On the other hand, development of resistance, particularly with suppressed immunity, is a challenging problem in the treatment of fungal infections.[6]As part of a continuing search for new, safer, and more effective antifungal drugs acting on the opportunistic pathogens *C. albicans* and *C. neoformans*, we herein report on the in vitro antifungal activity of a series of artemisinin derivatives against these organisms. A series of 29 artemisinin derivatives was prepared for this study. With the exception of compounds **16**, **17**, **18**, and **20**, which are new, the remainder of compounds **2**–**30** are known. The structures of the new compounds were determined on the basis of spectroscopic data interpretation, as shown in the Experimental Section. The relative stereochemistry at C-16 and C-19 of compounds **17** and **18** was established on the basis of NOESY data. In compound **17**, the α-oriented H-12 displayed a NOESY correlation with H-16, and the *β*-oriented H-15 correlated with H-19, suggesting similar stereo-orientations. In compound **18**, the *β*-oriented H-15 showed NOESY correlations with both H-16 and H-19; thus H-16 and H-19 were assigned as having *β*-orientation.The antifungal activity of compounds **1**–**32** was evaluated against two opportunistic pathogens, C. albicans and *C. neoformans*, with amphotericin B as a positive control, following standard procedures.[7] The results are shown in Table 1.

Chart 1





	X
3	O-O
22	O



4

	X	R_1	R_2
1	O-O	=O	β-CH_3
2	O-O	OH	β-CH_3
5	O-O	β-OMe	β-CH_3
6	O-O	α-OMe	β-CH_3
7	O-O	β-OEt	β-CH_3
8	O-O	α-OEt	β-CH_3
9	O-O	=O	=CH_2
12	O-O	β-OOH	=CH_2
21	O	=O	β-CH_3
23	O	α-OOH	=CH_2
24	O	α-OH	=CH_2
25	O	=O	=CH_2
26	O	β-OMe	=CH_2

Compounds **2**, **3**, **7**, and **8**, which all contain a peroxide group, were the most active derivatives, as shown in Table 1. Compound **4**, obtained as a mixture of two epimers,[8] was inactive, although it has a peroxide group.

The stereoisomers **17** and **18** exhibited almost identical antifungal activity with IC_{50} values of 1.0 and 0.9 μg mL^{-1}, respectively, against *Cryptococcus neoformans*. However, both compounds were inactive against *Candida albicans.* Among the deoxy-artemisinin derivatives studied, four compounds, **21**, **22**, **25**, and **27**, exhibited marginal activity.

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Chart 2

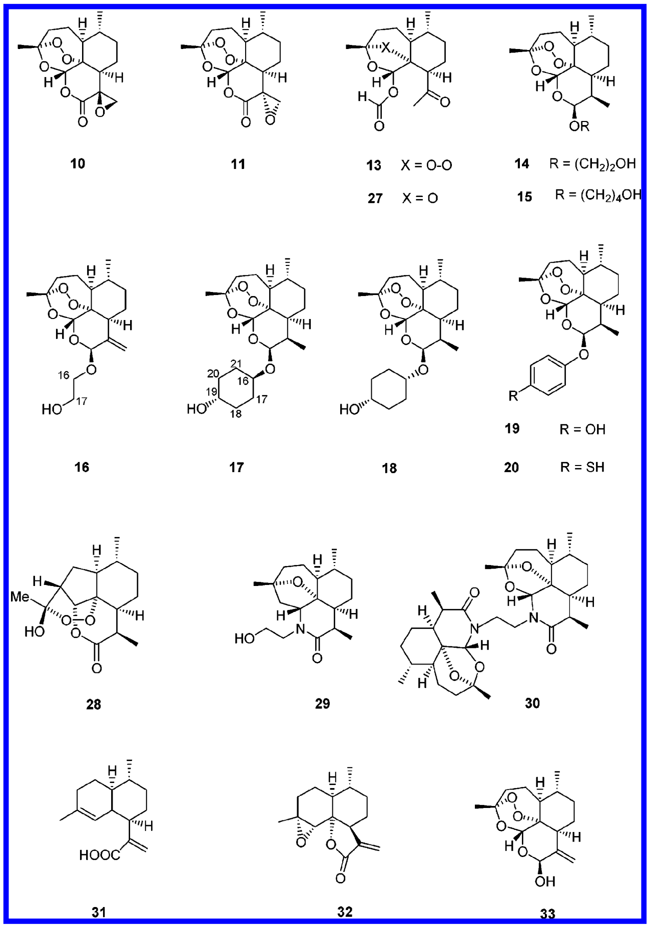

Table 1. Antifungal Activity of Artemisinin Derivatives

compound	IC$_{50}$/MIC (μg mL^{-1}) *C. albicans*	IC$_{50}$/MIC (μg mL^{-1}) *C. neoformans*
1	8.0/50	2.0/50
2	9.0/–[a]	0.09/12.5
3	7.0/50	0.045/0.195
5	–	0.6/25
6	–	1.5/–
7	15/50	0.085/3.13
8	3.0/–	0.045/1.56
9	–/–	0.045/–
15	–/–	0.6/–
16	–/–	3.0/–
17	–/–	1.0/–
18	–/–	0.9/–
19	–/–	0.087/–
20	–/–	0.31/–
22	30.0/–	6.0/–
amphotericin B	0.1/0.15	0.35/0.625

[a] – = inactive at highest test concentration of 50 μg mL^{-1}.

The rest of the compounds were totally inactive. In addition, the two aza-deoxyartemisinin derivatives **29** and **30** were also inactive. Compound **28**, which carries a peroxide function, was weakly active, which suggests that the presence of the typical tetracyclic trioxane skeleton of artemisinin is crucial for activity. The requirement of the peroxide function as part of the typical tetracyclic trioxane skeleton of artemisinin and its derivatives for the exhibition of antifungal activity was also observed as an essential functionality for their antimalarial and cytotoxic activities.[9,10]

It is interesting to note the inactivity of most of these compounds for *C. albicans*. While both *C. neoformans* and *C. albicans* are yeasts, the disparity in activity could be explained by the artemisinin derivatives inhibiting molecular targets or processes only present in *C. neoformans*. Moreover, all of these compounds are only fungistatic, not fungicidal, at the highest test concentration of 50 μg mL^{-1} used, suggesting a mechanism of action other than fungicidal drugs such as amphotericin B and caspofungin. On the basis of the observation that polymorphonuclear leukocytes kill *C. neoformans*, at least in part via generation of fungicidal oxidants,[11] one might speculate on the mode of action of this class of compounds as the liberation of free radicals that interfere with the growth of these organisms.[12,13] It is hoped that these preliminary results will stimulate the further study of the potential of artemisinin derivatives as antifungal agents.

Experimental Section

General Experimental Procedures. Melting points were recorded on an Electrothermal 9100 instrument. Optical rotations were recorded at ambient temperature using a JASCO DIP 370 digital polarimeter. IR spectra were obtained using an ATI Mattson Genesis Series FTIR spectrometer. The NMR spectra were recorded on Bruker Avance DRX 400 and 500 spectrometers using the solvent peak as reference. 2D NMR data were measured with standard pulse programs and acquisition parameters. HRESIMS were obtained on a Bruker BioAPEX 30es ion cyclotron high-resolution HPLC-FT spectrometer by direct injection onto an electrospray interface.

Test Compounds. Compounds **2**–**30** were prepared following literature procedures,[14–20] while artemisinin (**1**), artemisinic acid (**31**), and arteanuine B (**32**) were isolated from *Artemisia annua,* as previously reported.[21] Compound **33** was prepared according to a literature procedure.[16] Reactions were run in oven-dried round-bottomed flasks. Diethyl ether was distilled from sodium benzophenone ketyl and stored prior to use under an atmosphere of argon. All dihydroxy alcohols were dried by Al_2O_3-grade I prior to use. All other compounds were purchased from Aldrich Chemical Co. and used without further purification. Column chromatography was performed using flash silica gel (Merck, particle size 230–400 mesh). Analytical thin-layer chromatography (TLC) was performed with silica gel 60 F_{254} plates (Merck, 250 μm thickness). Visualization was accomplished by spraying with *p*-anisaldehyde spray reagent followed by heating using a hot-air gun.

Compound Preparation. Preparation of Compound 16. To a stirred solution of **33** (75 mg, 0.27 mmol) in dry ether (15 mL) and ethylene glycol (52 mg) was added $BF_3{\cdot}OEt_2$ (18 μL). The reaction mixture was left for 24 h, after which it was quenched with 25 mL of 2% aqueous $NaHCO_3$, and extracted with ether (50 mL × 3). The combined ether extract was washed with water, dried over Na_2SO_4, and concentrated under reduced pressure. Column chromatography of the oily crude product using a gradient of EtOAc in hexane (20% → 50%) afforded **16** (10 mg, gum): $[\alpha]_D$ +160.7° (*c* 0.028, MeOH); IR (film) ν_{max} 3436 (br, OH), 2923, 2873, 1454, 1378, 1103, 1006, 986 cm^{-1}; ^{1}H NMR (C_5D_5N, 400 MHz) δ 5.93 (1H, s, H-5), 5.54 (1H, s, H-12), 5.12 (1H, s, H-13a), 4.94 (1H, s, H-13b), 4.12 (1H, t, J = 5.2 Hz, H-16a), 4.00 (2H, q, J = 4.9 Hz, H-17a, H-17b), 3.79 (1H, q, J = 5.1 Hz, H-16b), 2.32 (1H, m, H-7), 1.42 (3H, s, Me-15), 1.20 (2H, m, H-1, H-10), 0.76 (3H, d, J = 5.4 Hz, Me-14); ^{13}C NMR (C_5D_5N, 100 MHz) δ 143.8 (C, C-11), 114.5 (CH_2, C-13), 104.0 (C, C-4), 101.6 (CH, C-12), 88.3 (CH, C-5), 81.5 (C, C-6), 66.6 (CH, C-16), 61.6 (CH_2, C-17), 52.2 (CH, C-1), 48.5 (CH, C-7), 37.3 (CH, C-10), 36.7 (CH_2, C-3), 34.4 (CH_2, C-9), 31.7 (CH_2, C-8), 24.7 (CH_2, C-2), 26.1 (CH_3, C-15), 20.2 (CH_3, C-14); HRESIFTMS *m/z* 349.1637 [M + Na]$^+$ (calcd for $C_{17}H_{26}O_6Na$, 349.1621); R_f 0.17 (hexane–EtOAc, 1:1).

Preparation of Compounds 17 and 18. In a 100 mL round-bottomed flask were introduced dihydroartemisinin (**2**) (850 mg, 3.0 mmol) and dry ether (25 mL). The mixture was stirred at room temperature, and cyclohexane-1,4-diol (mixture of *cis and trans*) (170 mg) was added. To the stirred solution,

$BF_3.OEt_2$ (570 μL) was then added by hypodermic syringe. The stirring was continued for 80 min, after which time the reaction was quenched and worked up as usual to leave a gummy residue (1.13 g). The residue was loaded onto a silica gel column (flash type, 270−400 mesh, 170 g) and eluted with increasing amounts of EtOAc in hexane (15 → 50%). Fractions of 5 mL were collected and similar fractions were pooled by guidance of TLC to afford two compounds, **17** and **18**. Compound **17** (184 mg, gum): $[\alpha]_D$ +180° (*c* 0.080, $CHCl_3$); IR (film) ν_{max} 3478 (br, OH), 2937, 2870, 1447, 1364, 1193, 1136, 1098, 1029, 993 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 5.42 (1H, s, H-5), 4.87 (1H, d, J = 3.4 Hz, H-12), 3.83 (1 H, m, H-16), 3.69 (1 H, m, H-19), 2.62 (1H, m, H-11), 1.45 (1H, m, H-7), 1.42 (3H, s, Me-15), 1.32 (1H, m, H-10), 1.23 (1H, m, H-1), 0.95 (3H, d, J = 6.2 Hz, Me-14), 0.90 (3H, d, J = 7.3 Hz, Me-13); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 104.4 (C, C-4), 100.3 (CH, C-12), 88.5 (CH, C-5), 81.6 (C, C-6), 71.6 (CH, C-16), 69.2 (CH, C-19), 53.0 (CH, C-1), 44.9 (CH, C-7), 37.9 (CH, C-10), 36.9 (CH_2, C-3), 35.1 (CH_2, C-9), 31.24 (CH, C-11), 31.2 (CH_2, C-20), 30.7 (CH_2, C-21), 30.1 (CH_2, C-18), 27.3 (CH_2, C-17), 26.6 (CH_3, C-15), 25.1 (CH_2, C-8), 24.9 (CH_2, C-2), 20.7 (CH_3, C-14), 13.5 (CH_3, C-13); HRESIFTMS m/z 405.2226 $[M + Na]^+$ (calcd for $C_{21}H_{34}O_6$ Na, 405.2253); R_f 0.17 (hexane−EtOAc, 6:4). Compound **18** (110.5 mg, gum): $[\alpha]_D$ +37.2° (*c* 0.086, $CHCl_3$); IR (film) ν_{max} 3478 (br, OH), 2935, 2870, 1450, 1375, 1100, 1024, 984 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 5.40 (1H, s, H-5), 4.88 (1H, d, J = 3.3 Hz, H-12), 3.70 (2H, m, H-16, H-19), 2.59 (1H, m, H-11), 1.45 (1H, m, H-7), 1.40 (3H, s, Me-15), 1.30 (1H, m, H-10), 1.25 (1H, m, H-1), 0.94 (3H, d, J = 6.3 Hz, Me-14), 0.86 (3H, d, J = 7.3 Hz, Me-13); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 104.5 (C, C-4), 100.4 (CH, C-12), 88.4 (CH, C-5), 81.5 (C, C-6), 74.4 (CH, C-16), 69.6 (CH, C-19), 53.0 (CH, C-1), 44.9 (CH, C-7), 37.9 (CH, C-10), 36.8 (CH_2, C-3), 35.1 (CH_2, C-9), 31.0 (CH_2, C-18, C-20), 28.5 (CH_2, C-17, C-21), 24.8 (CH_2, C-2), 25.0 (CH_2, C-8), 26.6 (CH_3, C-15), 20.7 (CH_3, C-14), 13.4 (CH_3, C-13); HRESIFTMS m/z 405.2223 $[M + Na]^+$ (calcd for $C_{21}H_{34}O_6$ Na, 405.2253); R_f 0.10 (hexane−EtOAc, 6:4).

Preparation of Compound 20. To a stirred solution of dihydroartemisinin (60 mg, 0.216 mmol) in ether (10 mL) was added 4-thiophenol (29 mg) at room temperature, followed by addition of $BF_3{\cdot}OEt_2$ (311 μL). The stirring was continued for 30 min, after which time the reaction was quenched by the addition of 2 mL of 1% aqueous solution of $NaHCO_3$, diluted with ether, and washed twice with water. The organic portion was collected, dried over sodium sulfate, and concentrated to leave an oil. The crude reaction mixture was loaded on a silica gel column (10 g) and eluted with increasing amounts of EtOAc in hexane (10% → 50%) to yield impure **20**, which was rechromatographed using 6% MeCN in CH_2Cl_2. This column afforded **20** as a pure oil (11 mg, 13%): $[\alpha]_D$ +266.7° (*c* 0.032, CH_2Cl_2); IR (film) ν_{max} 3377 (SH), 2928, 2872, 1600, 1582, 1496, 1451, 1379, 1269, 1211, 1026, 932 cm^{-1}; ^1H NMR (C_6D_6, 400 MHz) δ 7.55 (2H, d, J = 8.4 Hz, H-18a, H-18b), 6.92 (2H, d, J = 8.4 Hz, H-17a, H-17b), 5.82 (1H, s, H-5), 5.32 (1H, d, J = 5.6 Hz, H-12), 3.08 (1H, m, H-11), 1.56 (2H, m, H-7, H-10), 1.41 (3H, s, Me-15), 1.39 (1H, m, H-1), 1.06 (1H, d, J = 7.3 Hz, Me-13), 0.99 (3H, d, J = 6.4 Hz, Me-14); ^{13}C NMR (C_6D_6, 100 MHz) δ 157.0 (C, C-16), 135.9 (CH_2, C-18, C-20), 125.3 (CH, C-19), 116.0 (CH, C-17, C-21), 105.3 (C, C-4), 92.7 (CH, C-12), 88.9 (CH, C-5), 81.6 (C, C-6), 53.0 (CH, C-1), 45.5 (CH, C-7), 37.7 (CH, C-10), 36.8 (CH_2, C-3), 34.8 (CH_2, C-9), 25.1 (CH_2, C-2), 24.7 (CH_2, C-8), 26.2 (CH_3, C-15), 20.7 (CH_3, C-14), 15.4 (CH_3, C-13); HRESIFTMS m/z 415.1545 $[M - H]^-$ (calcd for $C_{21}H_{27}O_5S$, 415.1549).

Antimicrobial Bioassay. Two opportunistic fungal strains (*C. albicans* ATCC 90028 and *C. neoformans* ATCC 90113) were used in the in vitro evaluation of antifungal activity. Susceptibility testing was performed using a modified version of the NCCLS methods. The microbial inocula were prepared by diluting the subcultured organism in its incubation broth. Test compounds were dissolved in DMSO, serially diluted using normal saline, and transferred in duplicate to 96-well microtiter plates. The microbial inoculum was added to achieve a final volume of 200 μL and final concentrations starting with 50 μg/mL for pure compounds. Amphotericin B (ICN Biomedicals, Aurora, OH) was used as a positive control, and blank (media only) controls were added to each test plate. The plates were read turbidimetrically at 630 nm using an EL-340 Biokinetics Reader (Biotek Instruments, Winooski, VT) prior to and after incubation. The percent growth was calculated and plotted versus concentration to afford the IC_{50}/MIC.

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