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Synthesis, Characterization, and Evaluation of Antimicrobial Activity of Some 1,2,4-Triazole Derivatives Bearing an Antipyryl Moiety

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Summary. Some novel 4-[[2-[[5-(2-furanyl)-4-alkyl/aryl-4*H*-1,2,4-triazol-3-yl]thio]-acetyl/propionyl]-amino]-1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazoles were synthesized and evaluated for *in vitro* antibacterial activity against *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, and *Enterococcus faecalis* ATCC 29212 and antifungal activity against *Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6258, *Candida parapsilosis*, *Trichophyton mentagrophytes var. erinacei* NCPF 375, *Microsporum gypseum* NCPF 580, and *Trichophyton rubrum* using the microbroth dilution method. All of the compounds were found to be ineffective against the above bacteria within the applied MIC ranges. On the other hand, they were effective against fungi to different degrees. Three compounds showed high activity against *C. parapsilosis* and *T. mentagrophytes var. erinacei* NCPF 375 (MIC = $8\,\mu g\,cm^{-3}$). The *in vitro* antimycobacterial activity of the new compounds was also investigated against *Mycobacterium tuberculosis* $H_{37}RV$ (ATCC 27294) in BACTEC 12B medium using a broth microdilution assay, the Microplate Alamar Blue Assay (MABA). Compounds exhibiting fluorescence were tested in the BACTEC 460 radiometric system. The most active compound was found with 66% inhibition at $> 6.25\,\mu g\,cm^{-3}$.

Keywords. Antipyrines; 1,2,4-Triazoles; Antibacterial activity; Antifungal activity; Antimycobacterial activity.

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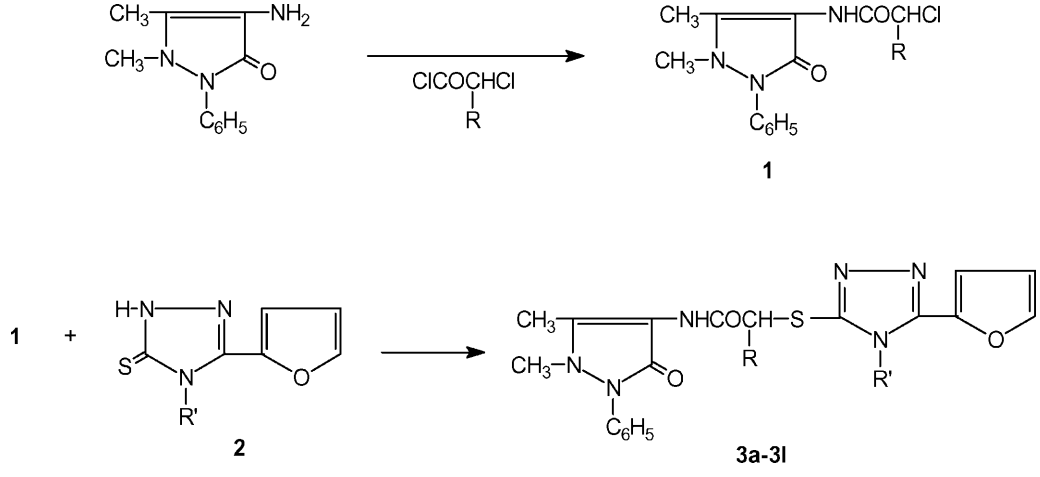
Introduction

The presence of the triazole ring in compounds with a wide range of biological activities has contributed over the years to enlarge the interest for the closely related 1,2,4-triazoles. Many 1,2,4-triazole derivatives have been shown to exhibit bactericidal [1–11], fungicidal [12–22] and antitubercular [23–25] properties. In this study, we have synthesized new compounds by combining 1,2,4-triazoles with antipyrine, 1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazol, and we have investigated the effect of this entering group on the antimicrobial activity of 1,2,4-triazoles. Therefore, as a continuation of our work on 1,2,4-triazoles [26–30] it appeared interesting to prepare and to evaluate the biological potentials of new compounds obtained by incorporating these moieties in a single molecule.

Results and Discussion

4-(Chloroacetyl/α-chloropropionyl)-amino-1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazoles (**1**) [31] were reacted with the anion generated from 5-(2-furanyl)-4-alkyl/aryl-2,4-dihydro-3*H*-1,2,4-triazole-3-thiones (**2**) [32] in the presence of K_2CO_3, to afford 4-[[2-[[5-(2-furanyl)-4-alkyl/aryl-4*H*-1,2,4-triazol-3-yl]-thio]-acetyl/propionyl]-amino]-1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazoles (**3a**–**3l**) (Scheme 1). Analytical (CHN) and spectral data (IR, ^{1}H NMR, EIMS) supported the structures.

The stretching vibrations of =C–H (3116–2957 cm^{-1}), C=N/C=C (1617–1431 cm^{-1}), amide C=O (1698–1667 cm^{-1}), and pyrazolone C=O (1660–1622 cm^{-1}) observed in the IR spectra provided substantial proof for the formation of the desired products **3a**–**3l**. The ^{1}H NMR spectral data of **3** were also consistent with the assigned structures. The C–CH_3, N–CH_3 protons of the pyrazolone moiety, and the NH–CO proton appeared at 2.14–2.08, 3.08–3.03, and 9.39–9.37 ppm [31]. The mass spectra of the compounds **3d** and **3j** were recorded by EIMS. The compounds showed molecular ions (M^+) which confirmed their molecular weights. Fragmentation followed the route in accordance with literature [31, 33].



Scheme 1

Compounds **3a–3l** were evaluated for *in vitro* antibacterial and antifungal activity against representative bacteria: *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, and

Table 1. Antibacterial activity of compounds **3a–3l**

Comp./Microorg.[a]	MIC/$\mu g\,cm^{-3}$			
	A	B	C	D
3a	> 32	> 32	> 32	> 32
3b	> 32	> 32	> 32	> 32
3c	> 32	> 32	> 32	> 32
3d	> 32	> 32	> 32	> 32
3e	> 32	> 32	> 32	> 32
3f	> 32	> 32	> 32	> 32
3g	> 32	> 32	> 32	> 32
3h	> 32	> 32	> 32	> 32
3i	> 32	> 32	> 32	> 32
3j	> 32	> 32	> 32	> 32
3k	> 32	> 32	> 32	> 32
3l	> 32	> 32	> 32	> 32
Cefotaxime	2	8	0.06	n.t.
Tetracycline	n.t.[b]	n.t.[b]	n.t.[b]	16

[a] A = *S. aureus* ATCC 29213, B = *P. aeruginosa* ATCC 27853, C = *E. coli* ATCC 25922, D = *E. faecalis* ATCC 29212; [b] n.t. = not tested

Table 2. Antifungal activity of compounds **3a–3l**

Comp./Microorg.[a]	MIC/$\mu g\,cm^{-3}$						
	A	B	C	D	E	F	G
3a	16	16	16	8	8	> 32	> 32
3b	16	16	16	8	8	> 32	> 32
3c	16	16	16	8	8	> 32	> 32
3d	16	16	16	16	32	> 32	> 32
3e	16	16	16	16	32	> 32	> 32
3f	16	16	16	8	16	> 32	> 32
3g	16	16	16	8	16	> 32	> 32
3h	16	16	16	8	16	> 32	> 32
3i	16	16	16	8	> 32	> 32	> 32
3j	16	16	16	8	> 32	> 32	32
3k	32	32	32	16	16	> 32	32
3l	16	16	32	8	16	> 32	32
Ketoconazole	n.t.[c]	0.25	0.5	0.5	n.t.[c]	n.t.[c]	n.t.[c]
Itraconazole[b]	n.t.[c]	0.25	0.5	0.25	0.5	0.5	1

[a] A = *C. albicans* ATCC 10231, B = *C. parapsilosis* ATCC 22019, C = *C. krusei* ATCC 6258, D = *C. parapsilosis*, E = *T. mentagrophytes var. erinacei* NCPF 375, F = *M. gypseum* NCPF 580, G = *T. rubrum*; [b] Quality control strain *C. parapsilosis* ATCC 22019 MIC value 0.25 $\mu g\,cm^{-3}$; [c] n.t. = not tested

Table 3. Antimycobacterial activity of **3a–l**[a]

Compd.	R	R	Assay	MIC ($\mu g\,cm^{-3}$)	Inhibition %
3a	H	CH_3	Alamar	> 6.25	n.a.
3b	H	C_4H_9	Alamar	> 6.25	n.a.
3c	H	C_6H_{11}	Alamar	> 6.25	n.a.
3d	H	C_6H_5	Alamar	> 6.25	5
3e	H	$C_6H_4CH_3(4-)$	Alamar	> 6.25	n.a.
3f	H	$C_6H_4F(4-)$	Alamar	> 6.25	n.a.
3g	H	$C_6H_4Cl(4-)$	Alamar	> 6.25	n.a.
3h	H	$C_6H_4Br(4-)$	Alamar	> 6.25	3
3i	CH_3	C_6H_{11}	Alamar	> 6.25	66
3j	CH_3	C_6H_5	Alamar	> 6.25	3
3k	CH_3	$C_6H_4CH_3(4-)$	Alamar	> 6.25	n.a.
3l	CH_3	$C_6H_4Cl(4-)$	Alamar	> 6.25	1

[a] MIC RMP = 0.25 $\mu g\,cm^{-3}$, 97–99% inhibition *vs. M. tuberculosis* $H_{37}Rv$; n.a. = not active

Enterococcus faecalis ATCC 29212, and fungi: *Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6258, *Candida parapsilosis, Trichophyton mentagrophytes var. erinacei* NCPF 375, *Microsporum gypseum* NCPF 580, and *Trichophyton rubrum* using the microbroth dilution method [34–38]. All of the compounds were found to be ineffective against the bacteria at least within the applied MIC ranges (Table 1) and they were found to be effective against the fungi to different degrees (Table 2). Particularly R = H and R′ = alkyl (methyl, butyl and cyclohexyl) substituted derivatives **3a**, **3b**, and **3c** showed high activities against *C. parapsilosis* and *T. mentagrophytes var. erinacei* NCPF 375 (MIC = 8 $\mu g\,cm^{-3}$) (Table 2). Compounds **3a–3l** were also evaluated for *in vitro* antimycobacterial activity against *Mycobacterium tuberculosis* $H_{37}Rv$ (ATCC 27294) in BACTEC 12B medium using a broth microdilution assay, the Microplate Alamar Blue Assay (MABA) [39]. Compounds exhibiting fluorescence were tested in the BACTEC 460 radiometric system [39]. As can be seen in Table 3, the most active compound was compound **3i** (66% inhibition) which had $R = CH_3$ and a cyclohexyl group at the 4-position of the triazole ring.

Experimental

Melting points were determined using a Büchi 530 melting point apparatus in open capillary tubes (uncorrected). Elemental analyses were performed on a Carlo Erba 1106 elemental analyzer; the results were in good agreement with the calculated values. IR spectra were recorded on KBr discs, using a Perkin Elmer 1600 FTIR spectrophotometer. ^1H NMR (*DMSO*-d_6/*TMS*) spectra were measured on a Bruker AC 200 (200 MHz) spectrometer. EI mass spectra were recorded on a VG Zab Spec (70 eV) instrument. The starting materials were either commercially available or synthesized according to the references cited.

4-[[2-[[5-(2-Furanyl)-4-alkyl/aryl-4H-1,2,4-triazol-3-yl]thio]acetyl/propionyl]-amino]-1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazoles (**3a–3l**) – General Procedure

To a solution/suspension of 0.005 mol **2** in 30 cm^3 CH_3COCH_3, 0.005 mol of **1** and 0.02 mol of K_2CO_3 were added. The reaction mixture was refluxed for 21 h, cooled and poured into ice water.

The precipitate was collected by filtration. The residue was washed with water and recrystallized from C_2H_5OH.

4-[[2-[[5-(2-Furanyl)-4-methyl-4H-1,2,4-triazol-3-yl]-thio]-acetyl]-amino]-1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazoles (**3a**, $C_{20}H_{20}N_6O_3S \cdot \frac{1}{2}H_2O$)

Yield 79%; mp 226–227°C; IR (KBr): $\bar{\nu} = 3466$ (H_2O, O–H), 3128 (=C–H), 1595, 1490, 1456 (C=N/C=C), 1692 (amide C=O), 1660 (pyrazolone C=O) cm^{-1}.

4-[[2-[[5-(2-Furanyl)-4-butyl-4H-1,2,4-triazol-3-yl]-thio]-acetyl]-amino]-1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazoles (**3b**, $C_{23}H_{26}N_6O_3S$)

Yield 63%; mp 164–165°C; IR (KBr): $\bar{\nu} = 3169$ (=C–H), 1603, 1587, 1540, 1489, 1457 (C=N/C=C), 1681 (amide C=O), 1640 (pyrazolone C=O) cm^{-1}.

4-[[2-[[5-(2-Furanyl)-4-cyclohexyl-4H-1,2,4-triazol-3-yl]-thio]-acetyl]-amino]-1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazoles (**3c**, $C_{25}H_{28}N_6O_3S$)

Yield 50%; mp 187–188°C; IR (KBr): $\bar{\nu} = 3060$ (=C–H), 1610, 1589, 1550, 1496, 1474, 1457 (C=N/C=C), 1698 (amide C=O), 1639 (pyrazolone C=O) cm^{-1}.

4-[[2-[[5-(2-Furanyl)-4-phenyl-4H-1,2,4-triazol-3-yl]-thio]-acetyl]-amino]-1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazoles (**3d**, $C_{25}H_{22}N_6O_3S \cdot H_2O$)

Yield 88%; mp 243–244°C; IR (KBr): $\bar{\nu} = 3446$ (H_2O, O–H), 3042 (=C–H), 1588, 1540, 1496, 1443 (C=N/C=C), 1679 (amide C=O), 1638 (pyrazolone C=O) cm^{-1}; ^1H NMR (*DMSO*-d_6, 200 MHz): $\delta = 9.37$ (s, 1H, NH), 7.69–7.27 (m, 11H, phenyl and furan C_5–H), 6.98 (dd, $J = 3.9$, 0.9 Hz, 1H, furan C_4–H), 6.74 (d, $J = 3.2$ Hz, 1H, furan C_3–H), 4.08 (s, 2H, CH_2–S), 3.03 (s, 3H, N–CH_3), 2.08 (s, 3H, C–CH_3) ppm; EIMS: m/z (%) = 486 (M^+, 0.3), 300 (100), 276 (1), 259 (72), 258 (42), 244 (6), 243 (8), 242 (4), 230 (23), 187 (5), 203 (48), 202 (13), 84 (19).

4-[[2-[[5-(2-Furanyl)-4-(4-methylphenyl)-4H-1,2,4-triazol-3-yl]-thio]-acetyl]-amino]-1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazoles (**3e**, $C_{26}H_{24}N_6O_3S$)

Yield 89%; mp 242–243°C; IR (KBr): $\bar{\nu} = 3037$ (=C–H), 1587, 1534, 1516, 1484, 1452 (C=N/C=C), 1685 (amide C=O), 1642 (pyrazolone C=O) cm^{-1}.

4-[[2-[[5-(2-Furanyl)-4-(4-fluorophenyl)-4H-1,2,4-triazol-3-yl]-thio]-acetyl]-amino]-1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazoles (**3f**, $C_{25}H_{21}FN_6O_3S$)

Yield 79%; mp 218–219°C; IR (KBr): $\bar{\nu} = 3041$ (=C–H), 1540, 1512, 1486, 1453 (C=N/C=C), 1691 (amide C=O), 1647 (pyrazolone C=O) cm^{-1}.

4-[[2-[[5-(2-Furanyl)-4-(4-chlorophenyl)-4H-1,2,4-triazol-3-yl]-thio]-acetyl]-amino]-1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazoles (**3g**, $C_{25}H_{21}ClN_6O_3S$)

Yield 85%; mp 214–215°C; IR (KBr): $\bar{\nu} = 2983$ (=C–H), 1587, 1531, 1495, 1452 (C=N/C=C), 1689 (amide C=O), 1643 (pyrazolone C=O) cm^{-1}.

4-[[2-[[5-(2-Furanyl)-4-(4-bromophenyl)-4H-1,2,4-triazol-3-yl]-thio]-acetyl]-amino]-1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazoles (**3h**, $C_{25}H_{21}BrN_6O_3S \cdot 1\frac{1}{2}H_2O$)

Yield 77%; mp 209–210°C; IR (KBr): $\bar{\nu} = 3466$ (H_2O, O–H), 2981 (=C–H), 1601, 1586, 1526, 1492, 1453 (C=N/C=C), 1688 (amide C=O), 1642 (pyrazolone C=O) cm^{-1}.

4-[[2-[[5-(2-Furanyl)-4-cyclohexyl-4H-1,2,4-triazol-3-yl]-thio]-propionyl]-amino]-1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazoles (**3i**, $C_{26}H_{30}N_6O_3S$)

Yield 28%; mp 166–167°C; IR (KBr): $\bar{\nu} = 3116$ (=C–H), 1617, 1539, 1491, 1466 (C=N/C=C), 1684 (amide C=O), 1654 (pyrazolone C=O) cm^{-1}; ^{1}H NMR (*DMSO*-d_6, 200 MHz): $\delta = 9.39$ (s, 1H, NH), 7.88–7.30 (m, 6H, phenyl and furan C_5–H), 6.98 (d, $J = 3.3$ Hz, 1H, furan C_3–H), 6.68 (dd, $J = 3.4$, 1.5 Hz, 1H, furan C_4–H), 3.72 (q, $J = 6.8$ Hz, 1H, C*H*–CH_3), 3.41–3.38 (m, 1H, cyclohexyl C_1–H), 3.08 (s, 3H, N–CH_3), 2.14 (s, 3H, C–CH_3), 2.08–1.02 (m, 13H, cyclohexyl C_{2-6}H and CH–C*H*$_3$).

4-[[2-[[5-(2-Furanyl)-4-phenyl-4H-1,2,4-triazol-3-yl]-thio]-propionyl]-amino]-1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazoles (**3j**, $C_{26}H_{24}N_6O_3S$)

Yield 72%; mp 204–205°C; IR (KBr): $\bar{\nu} = 2996$ (=C–H), 1588, 1533, 1497, 1455 (C=N/C=C), 1674 (amide C=O), 1638 (pyrazolone C=O) cm^{-1}; ^{1}H NMR (*DMSO*-d_6, 200 MHz): $\delta = 9.39$ (s, 1H, NH), 7.82–7.27 (m, 11H, phenyl and furan C_5–H), 6.50 (dd, $J = 3.3$, 1.8 Hz, 1H, furan C_4–H), 6.16 (d, $J = 3.4$ Hz, 1H, furan C_3–H), 4.56 (q, $J = 6.8$ Hz, 1H, C*H*–CH_3), 3.04 (s, 3H, N–CH_3), 2.09 (s, 3H, C–CH_3), 1.54 (d, $J = 6.8$ Hz, 3H, CH–C*H*$_3$); EIMS: m/z (%) = 500 (M^+, 23), 298 (100), 270 (22), 258 (5), 243 (45), 242 (15), 230 (20), 203 (7), 202 (7), 84 (6).

4-[[2-[[5-(2-Furanyl)-4-(4-methylphenyl)-4H-1,2,4-triazol-3-yl]-thio]-propionyl]-amino]-1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazoles (**3k**, $C_{27}H_{26}N_6O_3S \cdot \frac{1}{2}H_2O$)

Yield 46%; mp 233–234°C; IR (KBr): $\bar{\nu} = 3395$ (H_2O, O–H), 2989 (=C–H), 1590, 1533, 1512, 1487, 1455, 1431 (C=N/C=C), 1670 (amide C=O), 1638 (pyrazolone C=O) cm^{-1}.

4-[[2-[[5-(2-Furanyl)-4-(4-chlorophenyl)-4H-1,2,4-triazol-3-yl]-thio]-propionyl]-amino]-1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazoles (**3l**, $C_{26}H_{23}ClN_6O_3S \cdot 1\frac{1}{2}H_2O$)

Yield 87%; mp 133–134°C; IR (KBr): $\bar{\nu} = 3488$ (H_2O, O–H), 2989 (=C–H), 1580, 1511, 1492, 1455 (C=N/C=C), 1667 (amide C=O), 1622 (pyrazolone C=O) cm^{-1}.

Microbiology

All the compounds to be tested were dissolved in dimethyl sulfoxide (*DMSO*) at a stock concentration of 3200 $\mu g\,cm^{-3}$. The final desired concentrations were prepared with RPMI 1640 medium for *Candida* species and dermatophytes and with *Mueller-Hinton* broth for bacteria. The final *DMSO* concentration was adjusted to 1%.

Antibacterial Activity

Minimum inhibitory concentrations (MICs) were determined by the microbroth dilution method using the National Committee for Clinical Laboratory Standards (NCCLS) recommendations [34, 35]. *Mueller-Hinton* broth (Oxoid, England) was used as the test medium. An inoculum of approximately $5 \cdot 10^5$ CFU cm^{-3} was delivered per well. Serial twofold dilutions of the test compounds (32–0.25 $\mu g\,cm^{-3}$) and extra dilutions (0.12–0.015 $\mu g\,cm^{-3}$) for antibiotic standards were prepared.

Plates were incubated for 16–20 h at 35°C in an ambient air incubator. The lowest concentration of the test compounds inhibiting visible growth was taken as the MIC value.

Antifungal Activity for Candida Species

MICs were determined by the microbroth dilution method using the NCCLS recommendations [36]. RPMI broth was prepared from RPMI 1640 medium (Sigma, St. Louis-Mo-USA) supplemented with 0.3 g of glutamine per liter, buffered with 3-(*N*-morpholino)propanesulfonic acid (*MOPS*) and adjusted to *pH* 7.0. A working suspension of the inoculum was made by a 1:100 dilution of the 0.5 Mc Farland standard yeast suspension in 0.85% saline followed by a 1:20 dilution in RPMI broth. Two fold dilutions of test compounds from 32 to 0.25 $\mu g\,cm^{-3}$ were prepared with the working suspension of the inoculum. Extra dilutions (0.12–0.015 $\mu g\,cm^{-3}$) were added for itraconazole and ketoconazole. The plates were incubated at 35°C for 48 h in ambient air. The MIC is the lowest concentration of a compound that substantially inhibits growth of the organism as detected visually.

Antifungal Activity for Dermatophytes

MICs were determined by the microbroth dilution method according to the modification of previously described procedures [37, 38]. RPMI 1640 with glutamin and buffered at *pH* 7.0 with *MOPS* was the medium used for broth microdilution susceptibility testing. Test inocula of approximately $0.4 \cdot 10^3$ to $5 \cdot 10^3\,CFU\,cm^{-3}$ were evaluated. Dermatophytes were grown on potato dextrose agar slants. (Acumedia, Baltimore-Ma-USA) at 30°C for 7 days. Sterile normal saline 0.85% was added to the slant and the culture was gently swabbed with a cotton tip applicator to dislodge the conidia from the hyphal mat. The suspension was transferred to a sterile tube. After heavy particles were allowed to settle for 5 min the upper homogeneous suspensions were collected and vortexed. The densities of the conidial suspensions were read and adjusted to 70 to 82% transmittance at 530 nm. These suspensions were diluted 1:500 in RPMI 1640. This dilution yields $0.4 \cdot 10^3$ to $5 \cdot 10^3\,CFU\,cm^{-3}$ per well. Two fold dilutions of test compounds from (32–0.25 $\mu g\,cm^{-3}$) and additional dilutions for itraconazole (0.12–0.015 $\mu g\,cm^{-3}$) were prepared. For quality control checking *C. parapsilosis* ATCC 22019 was used. The microdilution plates were incubated at 35°C for 4 days. The minimum concentration at which no growth was observed was taken as the MIC value.

Antimycobacterial Activity

A primary screening was conducted at 6.25 $\mu g\,cm^{-3}$ (or molar equivalent of highest molecular weight compound in a series of congeners) against *Mycobacterium tuberculosis* $H_{37}Rv$ (ATCC 27294) in BACTEC 12B medium using a broth microdilution assay, the Microplate Alamar Blue Assay (MABA) [39]. Compounds exhibiting fluorescence were tested in the BACTEC 460 radiometric system. Compounds effecting $< 90\%$ inhibition in the primary screen (MIC $> 6.25\,\mu g\,cm^{-3}$) were not generally evaluated further. Compounds demonstrating at least 90% inhibition in the primary screen were retested at lower concentrations against *M. tuberculosis* $H_{37}Rv$ to determine the actual minimum inhibitory concentration (MIC) using MABA. The MIC was defined as the lowest concentration effecting a reduction in fluorescence of 90% relative to controls.

Radiometric Susceptibility Test

A total of 0.1 cm^3 of BACTEC 12B-passaged inoculum was delivered without prior dilution into 4 cm^3 of test medium. Subsequent determination of bacterial titers yielded average titers (three experiments) of $1 \cdot 10^5$, $2.5 \cdot 10^5$, and $3.25 \cdot 10^4\,CFU\,cm^{-3}$ of BACTEC 12B medium for *M. tuberculosis* $H_{37}Rv$. Frozen inocula were initially diluted 1:20 in BACTEC 12B medium, and then 0.1 cm^3 was delivered to test medium. This yielded $5.0 \cdot 10^5$ and $1.25 \cdot 10^5$ CFU per BACTEC vial for $H_{37}Rv$. Twofold drug

dilutions were prepared in either *DMSO* (Sigma) or distilled deionized H_2O and delivered *via* a 0.5 cm^3 insulin syringe in a 50 mm^3 volume. Drug-free control vials consisted of solvent with bacterial inoculum and solvent with a 1:100 dilution of bacterial inoculum (1:100 controls). Vials were incubated at 37°C, and the GI was determined in a BACTEC 460 instrument (Becton Dickinson) until the GI of the 1:100 controls reached at least 30. All vials were read the following day, and the GI and daily change in GI (ΔGI) were recorded for each drug dilution. The MIC was defined as the lowest concentration for which the ΔGI was less than the ΔGI of the 1:100 control. If the GI of the test sample was greater than 100, the sample was scored as resistant even if the ΔGI was less than the ΔGI of the 1:100 control.

Alamar Blue Susceptibility Test (MABA)

Antimicrobial susceptibility testing was performed in black, clear-bottomed, 96-well microplates (black view plates; Packard Instrument Company, Meriden, Conn.) in order to minimize background fluorescence. Outer perimeter wells were filled with sterile H_2O to prevent dehydration in experimental wells. Initial drug dilutions were prepared in either *DMSO* or distilled deionized H_2O, and subsequent twofold dilutions were performed in 0.1 cm^3 of 7H9GC (no Tween 80) in the microplates. BACTEC 12B-passaged inocula were initially diluted 1:2 in 7H9GC, and 0.1 cm^3 was added to wells. Subsequent determination of bacterial titers yielded $1 \cdot 10^6$, $2.5 \cdot 10^6$, and $3.25 \cdot 10^5$ CFU cm^{-3} in plate wells for *M. tuberculosis* H_{37}Rv. Frozen inocula were initially diluted 1:20 in BACTEC 12B medium followed by a 1:50 dilution in 7H9GC. Addition of 0.1 cm^3 to wells resulted in final bacterial titers of $2.0 \cdot 10^5$ and $5 \cdot 10^4$ CFU cm^{-3} for H_{37}Rv. Wells containing drug only were used to detect autofluorescence of compounds. Additional control wells consisted of bacteria only (B) and medium only (M). Plates were incubated at 37°C. Starting at day 4 of incubation, 20 mm^3 of 10X Alamar Blue solution (Alamar Biosciences/Accumed, Westlake, Ohio) and 12.5 mm^3 of 20% Tween 80 were added to one B well and one M well, and plates were reincubated at 37°C. Wells were observed at 12 and 24 h for a color change from blue to pink and for a reading of $\geq$ 50,000 fluorescence units (FU). Fluorescence was measured in a Cytofluor II microplate fluorometer (PerSeptive Biosystems, Framingham, Mass.) in bottom-reading mode with excitation at 530 nm and emission at 590 nm. If the B wells became pink by 24 h, reagent was added to the entire plate. If the well remained blue or $\leq$ 50,000 FU was measured, additional M and B wells were tested daily until a color change occured, at which time reagents were added to all remaining wells. Plates were then incubated at 37°C, and results were recorded at 24 h post-reagent addition. Visual MICs were defined as the lowest concentration of drug that prevented a color change. For fluorometric MICs, a background subtraction was performed on all wells with a mean of triplicate M wells. Percent inhibition was defined as 1 – (test well FU/mean FU of triplicate B wells) · 100. The lowest drug concentration effecting an inhibition of $\geq$ 90% was considered the MIC.

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