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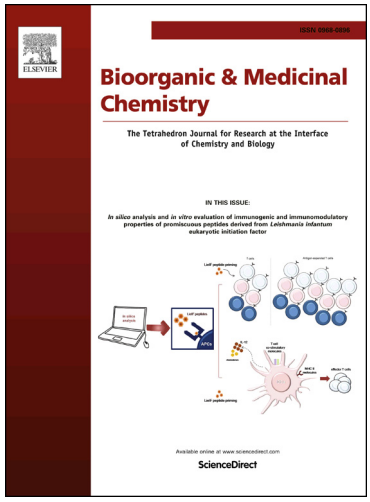

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Synthesis and biological evaluation of aminothiazoles against *Histoplasma capsulatum* and *Cryptococcus neoformans*

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Keywords

Aminothiazoles; Antifungal activity; Structure-Activity-Relationship*; Histoplasma capsulatum; Cryptococcus neoformans*

Abstract

The design and synthesis of a library of forty novel 2-aminoazole analogues as well as their evaluation as antifungal compounds against *Histoplasma capsulatum* and *Cryptococcus neoformans* is described. These structures were derived from *N*-[5-(1-naphthalenylmethyl)-2-thiazolyl]cyclohexanecarboxamide (41F5), a fungistatic agent previously identified through phenotypic screening (*Antimicrob Agents Chemother*. 2013; 57:4349). Modifications to improve potency and water-solubility of 41F5 focused primarily on the 5-naphthalenyl group, the thiazole core, and the methylene linker between these two structural elements. In general, compounds with lipophilic [5+6] bicyclics ring systems, such as the 7-benzothiophenyl- and 4-indanyl groups, at the 5-position were 2-3 times more active against both fungal species as compard to 41F5. Also, introduction of a carbonyl group at the methylene linker of 41F5 resulted in a 2-3 fold increase in potency. These highly active compounds also showed generally low toxicities against murine P388D1 macrophages resulting in selectivity indices ranging from 63 to >200. Compounds that were highly active against fluconazole-sensitive *C. neoformans* strains had almost identical activity against fluconazole-resistant variants of this fungus indicating that 14α-demethylase is not their molecular target. Highly active compounds also retained activity against *H. capsulatum* phagocytosed into P388D1 macrophages.

1. Introduction

Histoplasma capsulatum, the fungal pathogen causing histoplasmosis, is endemic to the Ohio- and Mississippi River valleys of North America as well as parts of Latin American and Africa, where up to 90% of the residents display signs of prior infection.[1] Up to 50 million people have had contracted *H. capsulatum* and an estimated 500,000 cases of new infections occur annually in the US.[2,3] Fortunately, most infections are subclinical but over 3,000 hospitalizations occur per year.[4] Histoplasmosis is the most common cause for hospitalization among endemic fungal disease.[4] The cost for each hospitalization is estimated to be $20,300 for children and $17,000 for adults.[4] *Cryptococcus neoformans*, the culprit of cryptococcosis, affects primarily the immunocompromised population in sub-Saharan Africa, where the human immunodeficiency virus (HIV) burden is the highest in the world. In sub-Saharan Africa, the approximate number of cases of cryptococcal meningitis is 720,000 and the 90-day case fatality is 70%.[5-8]

Major classes of current antifungal drugs include allylamines, azoles, pyrimidine analogues, polyenes, echinocandins, and oxaboroles.[9,10] These agents have different molecular targets found in the fungal cell membrane or in fungal biochemical processes.[11] All clinically utilized antifungals suffer from side effects and limited activity spectra.[9,10,12-20] In particular, echinocandins, which have the least host toxicity potential, are ineffective against *H. capsulatum* and *C. neoformans*.[12-14] Therefore, it is important to explore novel structural classes for the development of antifungal agents.

Edwards et al. performed a phenotypic screening of a purinome-focused library of 3600 commercially available compounds against *H. capsulatum* to find novel antifungal candidates and identified 41F5, which has a thiazole core structure, as the most active compound of this library (Table 1).[21] This agent displayed fungistatic activity with an IC_{50} value of 0.87 μM against *H. capsulatum* and a selectivity index of ~63 over macrophages. It was also found that 41F5 is active against *C. neoformans* with an IC_{50} of 1.25 μM but 41F5 was inactive against the fungi *Candida albicans*, *Aspergillus fumigatus* and *Blastomyces dermatitidis*.[21] 41F5 did not enhance sensitivity of *H. capsulatum* to fluconazole

suggesting the target of 41F5 differs from the cytochrome P450 sterol 14α-demethylase inhibited by azole-class antifungals.[22]

The thiazole core structure has been utilized as a pharmacophore in a number of biologically active agents.[23] Examples are the anticancer agents tiazofurin and dasatinib, the anti-HIV agent ritonavir, the antiparasitic drug nitazoxanide, the anti-inflammatory agents fanetizole, meloxicam, and fentiazac, the antiulcer agent nizatidine, and the insecticide thiamethoxam.[24-26] Abafungin is an antifungal drug that contains a thiazole ring systems but is otherwise structurally different from 41F5. This compound has inhibitory activity against sterol-C24-methyltransferase and can directly damage fungal cell membrane.[27] Abafungin is fungicidal rather than fungistatic, and it is active against *Candida albicans* and *Aspergillus fumigatus*.[24] Therefore, it was suggested that abafungin and 41F5 have different mechanisms of action.[21]

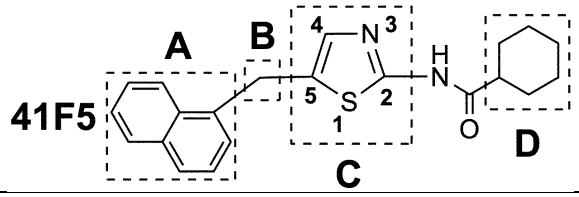
Khalil et al. synthesized 68 analogues of 41F5 to improve potency against *H. capsulatum* and *C. neoformans* and to develop a structure-activity relationship (SAR) for 41F5-derived antifungal compounds.[22] Unfortunately, compounds with higher potency than 41F5 were not identified. Therefore, we synthesized and evaluated further analogues of 41F5 with the intention to improve potency against *H. capsulatum* and *C. neoformans* and to enhance water solubility. Another objective was the synthesis of 41F5 analogues that have the potential to be used for molecular target identification by unbiased affinity chromatography techniques. The results of these studies are presentend in this paper.

2. Results

2.1. Design strategies

Table 1 summarizes the primary objectives of our studies and aligns these with areas of structural modifications of the 41F5 structure and the numbering system for all target compounds. Analogues of 41F5 were synthesized to (1) improve potency against *H. capsulatum* and *C. neoformans* yeasts, (2) enhance water solubility, and (3) identify the molecular target(s) of 41F5-derived antigfungal compounds, which is(are) currently unknown. Additional related objectives were the evaluation of compound

Table 1. Alignment of research objectives with areas of structural modifications at the 41F5 structure and the numbering system for all target compounds



Objective	Areas of chemical modifications	Target compounds
1 (Increased potency against *H. capsulatum* and *C. neoformans*)	A, B, C, and D	All the target compounds (**14**-**33**, **36**, **38**, **40**)
2 (Increased water solubility)	A and C	**28** (quinoline-substituted); **29**, **31**, **32** (pyridine-substituted); **40** (imidazole-substituted)
3 (Molecular target identification)	A, B and D	**14**, **15**, **33** (ester-modified at 2- or 5-substituents)

toxicities against fluconazole-resistant *C. neoformans* yeast, murine macrophages, and *H. capsulatum* phagocytosed into murine macrophages.

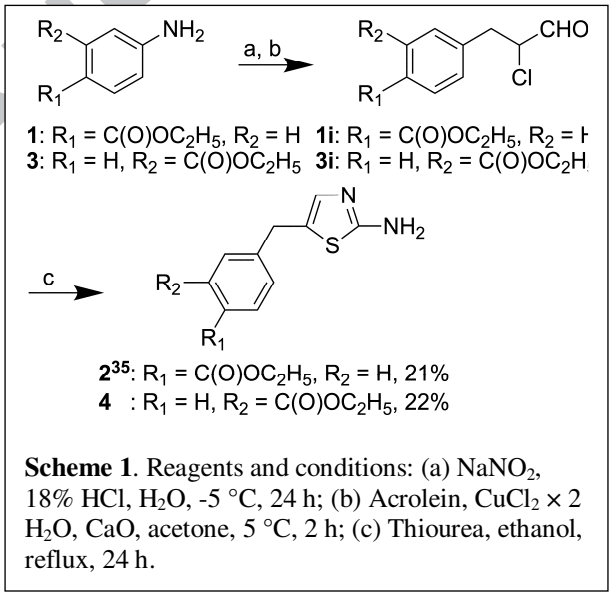
The "A" group was extensively modified to achieve all the three objectives (Table 1). In order to investigate the effect of the size on antifungal activity, the naphthalenyl group at the 5-position was replaced with tricyclic rings (fluorenyl), bicyclic rings (benzothiophenyl, benzofuranyl, 2,1,3-benzothiadiazolyl, indanyl, quinolinyl and tetralinyl), monocyclic rings (pyridinyl, cyclopentyl, and ester-modified phenyl) or the methyl group. Bioisosteric considerations played an important role in modifying this group (objective 1).[28-30] To potentially improve water solubility (objective 2), pyridine- (pKa = 5.2) (**29a/b**, **31**, **32a/b**)[31] and quinoline substituents (pKa = 4.85) (**28a/b**)[32] were introduced at the 5-position. The phenyl ring at the 5-position of the thiazole core was substituted with an ester group either at the 3- or 4-position (objective 3). Using standard chemical methodology, such an ester function could be converted to an alkyne-containing amide group, which could be coupled to an azide-containing solid support matrix via click chemistry. A stationary phase modified in this way could potentially be used in an unbiased affinity chromatography approach for molecular target identification,[33] which may have the potential to facilitate the structure-based design of 41F5-derived antifungal compounds. A crucial milestone in pursuing this methodololgy would be to initially evaluate the toxicity of ester containing compounds against *H. capsulatum* and *C. neoformans*. If such compounds would have significantly reduced activity as compared to 41F5, the fairly bulky ester group must interfere with binding to the target protein. In such a case, it would be reasonable to assume

that further increased steric hindrance by subsequent chemical modifications would be even more detrimental to binding, thus, rendering this affinity chromatography approach futile. In the "B" area, the methylene linker at the 5-position was replaced with a carbonyl group in order to explore the nature of the linker on potency (objective 1). In the "C" area, other heteroaromatic ring systems, such as oxazole (**38a** and **38b**) and imidazole (**40**) were introduced to address objective 1. In addition, imidazole (pKa = 6.95)[34] was also chosen as a core replacement to explore objective 2 . Similar to the attachment of an ester group to a phenyl group at the 5-position ("A" area), modifications of the "D" area encompassed introduction of ester groups to a cyclohexane ring at the 2-position (**33a**, **33b** and **33c**) to potentially explore affinity chromatography approaches for molecular target identification (objective 3).

2.2. Chemistry

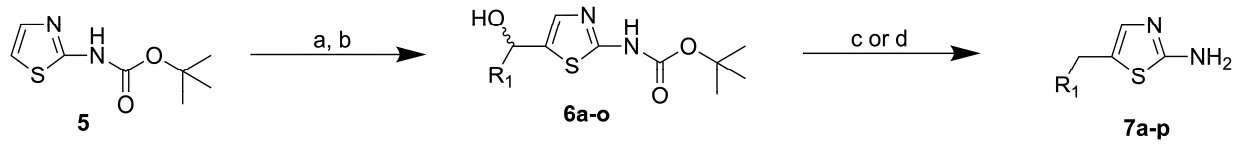
2.2.1. Precursor synthesis

Compound **2** (Scheme 1) was synthesized from commercially-available compound **1** as a precursor molecule according to a published procedure.[35] This method was then adapted for the synthesis of **4** from commercially available compound **3**. Briefly, the reactions of ethyl 4-aminobenzoate (**1**) and ethyl 3-aminobenzoate (**3**) with sodium nitrite resulted in the formations of corresponding diazonium salts, which were subsequently exposed to acrolein in the presence of copper(II) chloride dihydrate to yield the α-chloro aldehyde intermediates **1i** and **3i**. Cyclization of these intermediates with thiourea afforded products **2** and **4** in 21% and 22% over all yield, respectively. Intermediates **1i** and **3i** were not isolated and converted *in situ* to their respective end products **2** and **4**.



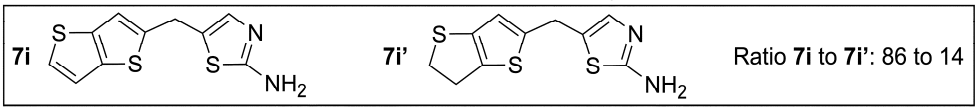
Scheme 1. Reagents and conditions: (a) $NaNO_2$, 18% HCl, H_2O, -5 °C, 24 h; (b) Acrolein, $CuCl_2 \times 2$ H_2O, CaO, acetone, 5 °C, 2 h; (c) Thiourea, ethanol, reflux, 24 h.

Commercially available compound **5** was lithiated using lithium diisopropyl amide (LDA) followed by reaction with aldehydes to afford **6a**-**o** in yields ranging from 9% to 81% (Scheme 2).[36]



6a (26%): R_1 = 2-Fluorenyl
6b (29%): R_1 = 7-Benzothiophenyl
6c (13%): R_1 = 1-Fluorenyl
6d (37%): R_1 = 4-Fluorenyl
6e (53%): R_1 = 2,1,3-Benzothiadiazol-4-yl
6f (81%): R_1 = 7-Benzofuranyl
6g (57%): R_1 = 4-Benzothiophenyl
6h (54%): R_1 = 3-Benzothiophenyl
6i (9%): R_1 = 2-Thieno[3,2-b]thiophenyl
6j (59%): R_1 = Cyclopentyl
6k (53%): R_1 = 4-Indanyl
6l (53%): R_1 = 5-Tetralinyl
6m (27%): R_1 = 4-Quinolinyl
6n (32%): R_1 = 2-Pyridinyl
6o[22] (52%): R_1 = 1-Naphthalenyl

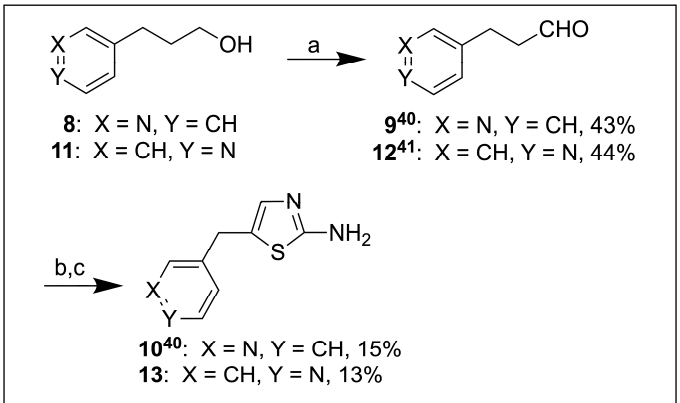
7a (76%): R_1 = 2-Fluorenyl (rxn c)
7b (74%): R_1 = 7-Benzothiophenyl (rxn c)
7c (77%): R_1 = 1-Fluorenyl (rxn c)
7d (73%): R_1 = 4-Fluorenyl (rxn c)
7e (66%): R_1 = 2,1,3-benzothiadiazol-4-yl (rxn c)
7f (71%): R_1 = 7-Benzofuranyl (rxn c)
7g (52%): R_1 = 4-Benzothiophenyl (rxn c)
7h (52%): R_1 = 3-Benzothiophenyl (rxn c)
7i (14%): R_1 = 2-Thieno[3,2-b]thiophenyl (rxn c)
7j (88%): R_1 = Cyclopentyl (rxn c)
7k (45%): R_1 = 4-Indanyl (rxn c)
7l (85%): R_1 = 5-Tetralinyl (rxn c)
7m (10%): R_1 = 4-Quinolinyl (rxn d)
7n (43%): R_1 = 2-Pyridinyl (rxn d)
7o[22] (61%): R_1 = 1-Naphthalenyl (rxn c)
7p*: R_1 = Methyl



Scheme 2. Reagents and conditions: (a) LDA, THF, -78 °C, 30 min; (b) R_1CHO, r.t., 16 h; (c) Et_3SiH, TFA, DCM, r.t., 16 h; (d) 57% aq. HI, acetic acid, 100 °C, 4 h; *compound was purchased.

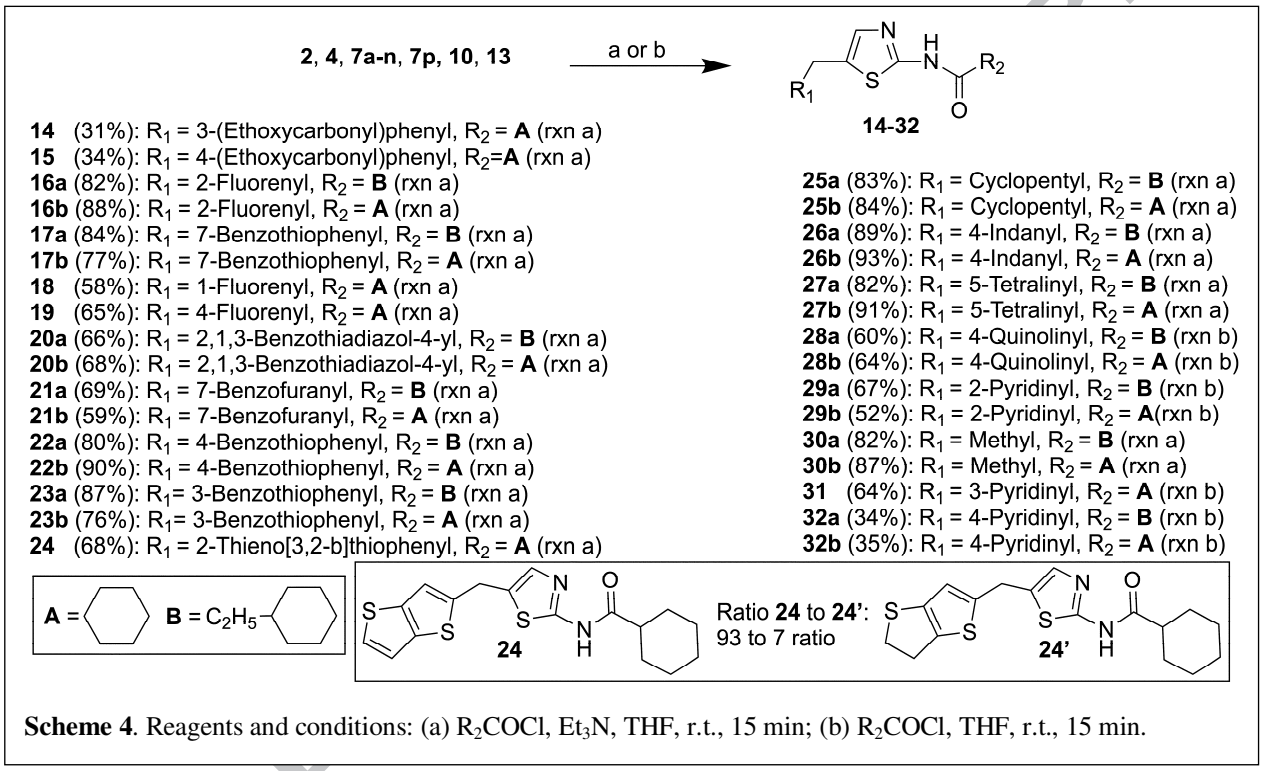
Compounds **6a-l** and **6o** were converted to **7a-l** and **7o** by reduction of secondary alcohols and deprotection of *t*-butoxycarbonyl (Boc) with triethylsilane and trifluoroacetic acid (TFA), respectively, in yields ranging from 14% to 88%.[22] Compounds **7m** and **7n** were prepared by simultaneous reduction of the hydroxyl group and removal of the Boc protective group of **6m** and **6n** using hydriodic acid in 10% and 43% yields, respectively.[37] Boc-deprotection and deoxygenation of **6i** afforded not only **7i** but also **7i'** in a molar ratio of 84:16 as determined by ^{1}H NMR and HR-ESI-MS (Scheme 2). It was not possible to separate this mixture by column chromatography. The deoxygenation of thiophene derivatives with triethylsilane in the presence of strong acids has been reported previously.[38,39]

Aldehyde **9** was synthesized from



Scheme 3. Reagents and conditions: (a) Dess-Martin periodinane, 4Å molecular sieves, DCM, r.t., 16 h; (b) *t*-BuBr, DMSO, acetonitrile, 65 °C, 16 h; (c) Thiourea, ethanol, reflux, 16 h.

commercially available compound **8** according to a published procedure (Scheme 3),[40] which was adapted for the synthesis of **12**[41] from starting material **11**. Briefly, 3- (**8**) and 4-pyridinepropanol (**11**) were oxidized with Dess-Martin periodinane to afford **9** and **12** in 43% and 44% yield, respectively. Compounds **9** and **12** were then α-brominated with *t*-butyl bromide and dimethyl sulfoxide (DMSO) followed by cyclization with thiourea to afford **10**[40] and **13** in 15% and 13% yield, respectively.

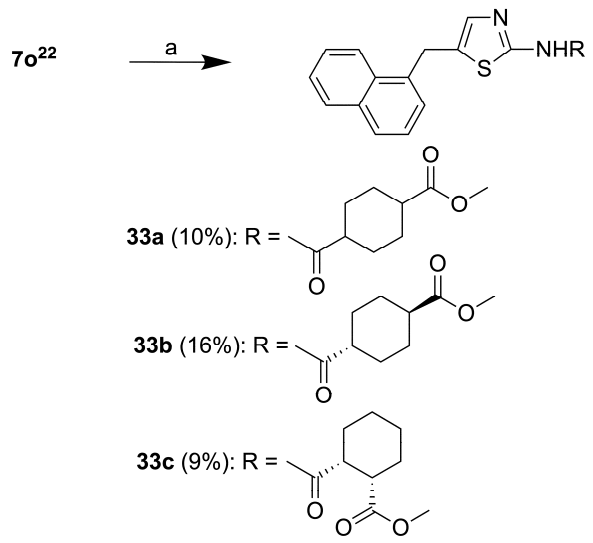


Scheme 4. Reagents and conditions: (a) R_2COCl, Et_3N, THF, r.t., 15 min; (b) R_2COCl, THF, r.t., 15 min.

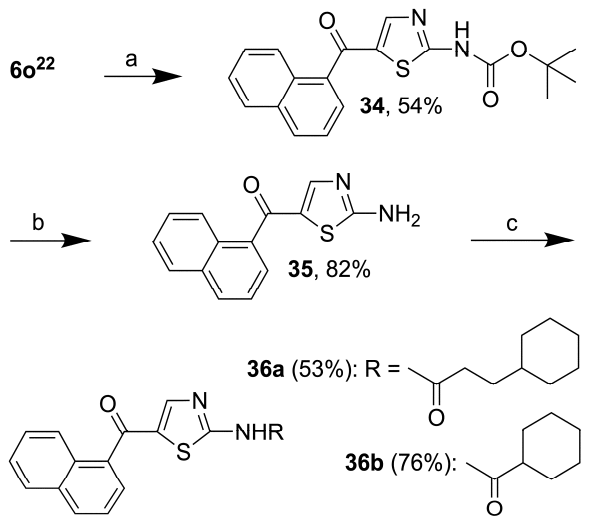
2.2.1. Target compound synthesis

Reactions of the precursor molecules **2**, **4**, **7a**-**n**, **7p**, **10** and **13** containing free amino groups with acid chlorides in the presence/absence of triethylamine (Scheme 4) yielded target compounds **14**-**32**. As **7i** was contaminated with **7i'** (*vide supra*), two target compounds **24** and **24'** were produced in molar ratio of 93:7 as determined by ^{1}H NMR (SM-S.6.17.1) and HR-ESI-MS (SM-S.6.17.3). As in the case of the precursor molecules, it was not possible to separate this mixture by column chromatography. Precursor molecule **7o** was coupled with commercially-available ester-substituted cyclohexane-1-carboxylic acids

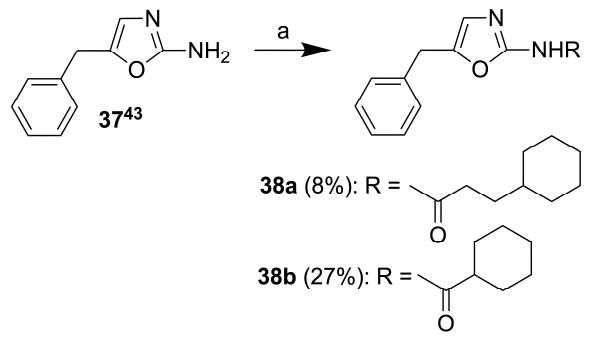
in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI)[22] to afford **33a**, **33b** and **33c** in 10%, 16% and 9% yield, respectively (Scheme 5). Carboxylic acids were used in these experiments because the corresponding acid chlorides were not commercially available.



Scheme 5. Reagents and conditions: (a) (1*R/S*,4*R/S*)-4-(methoxycarbonyl)cyclohexane-1-carboxylic acid or (1*R*,4*R*)-4-(methoxycarbonyl)cyclohexane-1-carboxylic acid or (1*R*,2*S*)-2-(methoxycarbonyl)cyclohexane-1-carboxylic acid, EDCI, DMAP, Et_3N, DCM/DMF (3:1, v/v), r.t., 16 h.



Scheme 6. Reagents and conditions: (a) Dess-Martin periodinane, DCM, r.t., 16 h; (b) TFA, DCM, r.t., 16 h; (c) 3-Cyclohexylpropanoyl chloride or cyclohexanecarbonyl chloride, Et_3N, THF, r.t., 15 min.

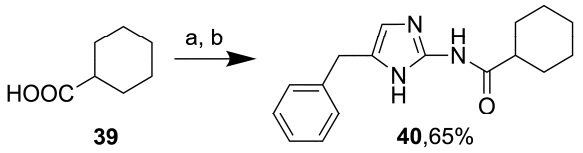


Scheme 7. Reagents and conditions: (a) 3-Cyclohexylpropanoyl chloride or cyclohexanecarbonyl chloride, Et_3N, THF, r.t., 15 min.

The secondary alcohol of compound **6o** was oxidized with Dess-Martin periodinane to a carbonyl group to afford **34** in 54% yield (Scheme 6)[42] followed by the removal of the Boc protective group with trifluoroacetic acid (TFA) to yield **35** in 82% yield. Compounds **36a** and **36b** were synthesized from **35** in 53% and 76% yields, respectively, using the method described for the synthesis of **14**-**27**, and **30a/b**.

The oxazole derivatives **38a** and **38b** (Scheme 7) were prepared from a reported compound **37**[43] in 8% and 27% yields, respectively, using the method described for synthesis of **14**-**27**, **30a/b**, and **36a/b**. Imidazole derivative **40** was synthesized in 65% yield from commercially available

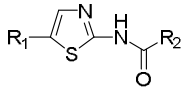
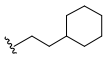
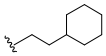
cyclohexanecarboxylic acid **39** by reaction with hydroxybenzotriazole (HOBt) in the presence of EDCI hydrochloride followed by reaction with commercially available 5-benzyl-1*H*-imidazol-2-amine hydrochloride (Scheme 8). Acylation occurred exclusively at the amino group under the applied reaction conditions.



39 **40**,65%

Scheme 8. Reagents and conditions: (a) HOBt, Et_3N, EDCI hydrochloride, acetonitrile, r.t., 10 min; (b) 5-Benzyl-1*H*-imidazol-2-amine □ HCl, 80 °C, 16 h.

Table 2. Highly active thiazole antigfungal compounds synthesized and evaluated by Khalil et al.[22]

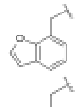
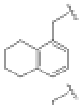
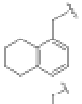
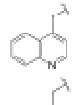
Cmpd			*H. capsulatum*	*C. neoformans* (H99 strain)
	R_1	R_2	IC_{50} (μM)	IC_{50} (μM)
41F5			0.4	0.4
A			0.4	> 10
B			1.6	1.3
C			0.7	> 10

2.3. Biology

Compounds **A**-**C** and 41F5, previously reported by Khalil et. al,[22] were used in our biological studies as general reference compounds because they showed significant antifungal activity and are structurally similar to the newly synthesized compounds (Table 2). The cytoxicities of target compounds (**14**-**33**, **36**, **38** and **40**) were evaluated *in vitro* against *H. capsulatum* and *C. neoformans* yeasts as well as in murine P388D1 macrophages and the obtained data are summarized in the Tables 3-5.

Table 3 shows data of target compounds having a bicyclic ring system at the 5-position. Amongst the group of compounds with a cyclohexylamide substituent at the 2-position, compounds **17b**, **22b** and **26b**, having a 7-benzothiophenyl-, 4-benzothiophenyl-, and indanyl group, respectively, at the 5-position, were ~2 times more active than 41F5 both against *H. capsulatum* and *C. neoformans*. Compound **36b**, substituted at the 2-position with cyclohexylamide and having a carbonyl linked naphthalenyl group at the 5-position, was also ~ 2 times more active than 41F5 against *H. capsulatum* but appears to be slightly less active than 41F5 against *C. neoformans.* The activities of benzofuran-substituted **21a/b** (Table 3) against *H. capsulatum* and *C. neoformans* were similar to that of 41F5 and compound **A** (Table 2).

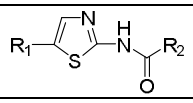
Table 3. Activity of 41F5-derived antifungals with bicyclic ring systems at the 5-position

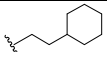
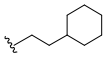
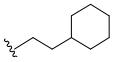
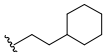
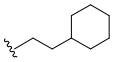
			*H. capsulatum*		*C. neoformans* (H99 strain)		P388D1	SI	SI
Cmpd	R_1	R_2	IC_{50} (∝M) [±SD]	MIC (∝M)	IC_{50} (∝M) [±SD]	MIC (∝M)	IC_{50} (∝M) [±SD]	(Hc)	(Cn)
A			0.60 [±0.03]	1.25	> 20	> 40	27.59 [±4.12]	46	nd
41F5			0.48 [±0.01]	1.25	0.67 [±0.06]	1.25	> 40	> 83	> 60
17a			0.92 [±0.38]	5.00	> 20	> 40	18.87 [±3.05]	21	nd
17b			0.20 [±0.01]	0.31	0.34 [±0.04]	0.63	> 40	> 200	> 118
20a			> 20	> 40	> 20	> 40	nd	nd	nd
20b			1.54 [±0.42]	2.50	1.52 [±0.22]	5.00	> 40	> 26	> 26
21a			0.40 [±0.01]	0.63	> 20	> 40	17.75 [±5.00]	44	nd
21b			0.42 [±0.01]	1.25	0.62 [±0.12]	2.50	> 40	> 95	> 65
22a			0.20 [±0.01]	0.31	> 20	> 40	> 40	> 200	nd
22b			0.20 [±0.01]	0.31	0.37 [±0.09]	1.25	> 40	> 200	> 108
23a			0.27 [±0.02]	0.63	> 20	> 40	16.90 [±1.75]	63	nd
23b			> 20	> 40	> 20	> 40	nd	nd	nd
24			> 20	> 40	> 20	> 40	nd	nd	nd
26a			0.38 [±0.02]	0.63	> 20	> 40	> 40	> 105	nd
26b			0.25 [±0.02]	0.63	0.41 [±0.07]	1.25	> 40	> 160	> 98
27a			0.90 [±0.29]	2.50	> 20	> 40	24.27	27	nd
27b			> 20	> 40	> 20	> 40	nd	nd	nd
28a			> 20	> 40	> 20	> 40	nd	nd	nd
28b			3.05 [±0.25]	10.00	3.24 [±0.42]	10.00	13.92 [±1.19]	5	5
36a			0.64 [±0.03]	1.25	> 20	> 40	> 40	> 63	nd
36b			0.19 [±0.01]	0.31	0.89 [±0.32]	2.50	> 40	> 211	> 45

SD: Standard deviation; SI: Selectivity index, nd: not determined

Amongst the group of compounds with a cyclohexylethylamide substituent at the 2-position, compounds **22a** and **23a** with a 4- and 3-benzothiophenyl group, respectively, were ~ 2 times more active against *H. capsulatum* than 41F5. Compounds **21a** and **26a**, substituted with a 7-benzofuranyl- and an indanyl group, respectively, had similar activity against *H. capsulatum* as 41F5 whereas compound **36a**, with a carboranyl linked naphthalenyl group, was slightly less active against this fungal species. A limited set of compounds with relatively small alicyclic (**25a/b**) and aliphatic (**30a/b**) substituents at the 5-position was evaluated in assays with *H. capsulatum* and *C. neoformans* (Table 4). Compound **25b**, with a cyclohexylamide subsituents at the 2-position and a cyclopentylmethyl group at the 5-position, was active against *H. capsulatum* in the same range as reference compounds **B** and **C**.

Table 4. Activity of 41F5-derived antifungals with monocyclic ring systems or an acyclic ethyl group at the 5-position



Comp	R_1	R_2	*H. capsulatum* IC_{50} (μM) [±SD]	*H. capsulatum* MIC (μM)	*C. neoformans* (H99 strain) IC_{50} (μM) [±SD]	*C. neoformans* (H99 strain) MIC (μM)	P388D1 IC_{50} (μM) [±SD]	SI (Hc)	SI (Cn)
C			0.80 [±0.04]	1.25	> 20	> 40	> 40	> 50	nd
B			1.06 [±0.06]	2.50	1.36 [±0.10]	2.50	> 40	> 38	> 29
25a			> 20	> 40	> 20	> 40	nd	nd	nd
25b			0.76 [±0.05]	1.25	> 20	> 40	> 40	> 53	nd
29a			8.09 [±0.70]	20.00	> 20	> 40	> 40	> 5	nd
29b			18.30 [±1.06]	40.00	7.17 [±0.84]	20.00	> 40	> 2	> 6
30a	C_2H_5		> 20	> 40	> 20	> 40	nd	nd	nd
30b	C_2H_5		> 20	> 40	> 20	> 40	nd	nd	nd
31			> 20	> 40	> 20	> 40	nd	nd	nd
32a			7.67 [±0.25]	20.00	> 20	> 40	> 40	> 5	nd
32b			> 20	> 40	> 20	> 40	nd	nd	nd

SD: Standard deviation; SI: Selectivity index, nd: not determined

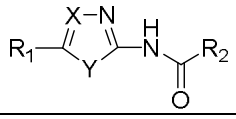
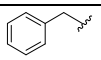
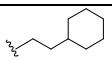
Khalil et. al.[22] reported that 41F5-derived thiazole antifungal compounds with a cyclohexylethylamide group at the 2-position (e.g. **A** and **C**, Table 2) were highly active only against *H. capsulatum* whereas those having a cyclohexylamide group at the 2-position (e.g. 41F5 and **B**, Table 2)

were highly active against both *H. capsulatum* and *C. neoformans*. Surprisingly, this SAR pattern was not observed for cyclohexylethylamide substituted compounds **20a** and **28a**, which were inactive against both *H. capsulatum* and *C. neoformans*, whereas their cyclohexylamide counterparts **20b** and **28b** were moderately active against both fungal species in accordance with the previously observed SAR pattern (Table 3). In contrast, the cyclohexylamide-substituted compounds **23b** and **27b** were inactive against both *H. capsulatum* and *C. neoformans* whereas their cyclohexylethylamide counterparts **23a** and **27a** adhered to the previously established SAR trend and were active/moderately active against *H. capsulatum* and inactive against *C. neoformans* (Table 3). Yet another SAR profile was observed for the **25a/b** pair (Table 4). In this case, activity was observed only for cyclohexylamide-substituted compound **25b** in *H. capsulatum*. Studies with compounds **20a/b**, **23a/b**, **25a/b**, **27a/b**, and **28a/b** were repeated several times to exclude the possibility of experimental error. The obtained set of data indicates that differences in the SAR pattern between both fungal species are more complex than originally proposed by Khalil et al.[22]

Compounds **28a/b, 29a/b**, **31** and **32a/b** were moderately active or inactive against *H. capsulatum* and *C. neoformans* indicating that the presence of a nitrogen in the ring system at the 5-position negatively affects activity (Tables 3 and 4). No significant differences were observed when the activities of **20a/b**, **28a/b, 29a/b**, **31** and **32a/b** against both fungal species were evaluated comparatively at pH 5 and and pH 7 (Supplementary Material [SM]-S.2). On the other hand, compounds with oxygen in the ring system at the 5-position (**21a/b**) had approximately similar activities as 41F5. These results are consistent with those reported by Khalil et al.,[22] which indicates that polar atoms within the ring systems at the 2- and 5-positions generally have no positive effect on the activities of 41F5-derived antifungals. In addition, calculations with ACD/Percepta software (Version 14.1.0, Advanced Chemistry Development, Inc., Toronto, ON, Canada) indicated increased aqueous solubilities of compounds **28b**, **29a/b**, **31**, **32a/b** by factors ranging from 15 to 298 at pH 5 and from 10 to 88 at pH 7 as compared to that of 41F5 (SM-S.3), which suggests that improved water solubility may also have no marked effect on the activities of 41F5-derived antifungals.

The compounds shown in Table 5, having oxazole (**38a/b**) or imidazole (**40**) core ring structures, were evaluated in assays with *H. capsulatum* and *C. neoformans* to explore the effect of modifications to the thiazole core structure on activity. Imidazole **40** was weakly active against *H. capsulatum* and inactive against *C. neoformans* whereas **38a** and **38b** were inactive against both fungal species. These results are consistent with those previously reported by Khalil et al.,[22] which strongly suggests that the thiazole core structure is essential for activity.

Table 5. Activity of antifungals with oxazole- and imidazole core structures

					*H. capsulatum*		*C. neoformans* (H99 strain)		P388D1	SI (Hc)	SI (Cn)
Cmpd	X	Y	R_1	R_2	IC_{50} (μM) [±SD]	MIC (μM)	IC_{50} (μM)	MIC (μM)	IC_{50} (μM)		
38a	CH	O			> 20	> 40	> 20	> 40	nd	nd	nd
38b	CH	O			> 20	> 40	> 20	> 40	nd	nd	nd
40	CH	NH			8.11 [±0.33]	20.00	> 20	> 40	> 40	> 5	> 2

SD: Standard deviation; SI: Selectivity index, nd: not determined

Linking tricyclic fluorene rings via methylene spacer either through the 2- (**16a/b**), 1- (**18**), or 4-position (**19**) (Scheme 4) to the 5-position of the thiazole core structure resulted in loss of activity both in *H. capsulatum* and *C. neoformans* (IC_{50}s = >20 μM/MIC = >40 μM) indicating that substituents bulkier than bicyclic naphthalene are not tolerated at this position. Compounds **14**, **15**, and **33a/b/c** (Schemes 4 and 5) were designed and synthesized with the intention of molecular target identification (*vide supra*). Unfortunatley, these compounds were inactive (IC_{50}s = >20 μM/MIC = >40 μM) both against *H. capsulatum* and *C. neoformans*, which indicates that the identification of the molecular target of 41F5-derived antifungals via unbiased affinity chromatography may be difficult to accomplish.

A small libray of highly active compounds, composed of **17b**, **21b**, **22b**, **26b**, and **36b**, was evaluated in a comparative assay against *C. neoformans* using both fluconazole-sensitive (H99 and B3501) and -resistant (TES9 and MRL862) strains (Table 6).[44] Fluconazole and 41F5 were used as

Table 6. Activity of 41F5-derived antifungals against fluconazole-resistant *Cryptococcus* isolates

Cmpd	H99 (serotype A-Flu^S)		B3501 (serotype D-Flu^S)		TES9[a] (serotype A-Flu^R)		MRL862[a] (serotype A-Flu^R)	
	IC_{50} (μM) [±SD][b]	MIC (μM)	IC_{50} (μM) [±SD]	MIC (μM)	IC_{50} (μM) [±SD]	MIC (μM)	IC_{50} (μM) [±SD]	MIC (μM)
41F5	0.67 [±0.06]	1.25	0.18 [±0.17]	0.63	0.20 [±0.03]	0.63	0.23 [±0.04]	0.63
Fluconazole	0.95 [±0.63]	4.08	0.13 [±0.22]	2.04	27.38 [±2.17]	65.36	14.61 [±0.65]	32.68
17b	0.34 [±0.04]	0.63	0.27 [±0.02]	0.63	0.23 [±0.01]	0.63	0.20 [±0.02]	0.63
21b	0.62 [±0.12]	2.50	0.32 [±0.02]	0.63	0.41 [±0.02]	0.63	0.15 [±0.09]	0.63
22b	0.21 [±0.09]	0.63	0.16 [±0.03]	0.63	0.19 [±0.01]	0.31	0.13 [±0.01]	0.31
26b	0.41 [±0.07]	1.25	0.42 [±0.06]	1.25	0.45 [±0.07]	1.25	0.37 [±0.58]	1.25
36b	0.89 [±0.32]	2.50	0.38 [±0.06]	1.25	0.47 [±0.05]	1.25	0.41 [±0.05]	1.25

[a]TES9 and MRL862 are fluconazole-resistant (Flu^R) strains due to mutation of the azole target Erg11.

[b]SD: Standard deviation

reference compounds in this assay. The results indicate that **17b**, **21b**, **22b**, **26b**, **36b**, and 41F5 were active to approximately the same extent against both fluconazole-sensitive and -resistant strains. In contrast, fluconazole was notably less active in the resistant strains as compared to the sensitive strains. These observations suggest that 14α-demethylase is not the molecular target of **17b**, **21b**, **22b**, **26b**, **36b**, and 41F5. It should be noted that clinically-used fluconazole, which has fungistatic or funcicidal dose-dependent activity in *Cryptococcus*,[45] has approximately the same activity levels against the fluconazole-sensitive H99- and B3501 *C. neoformans* strains as the experimental compounds **17b**, **21b**, **22b**, **26b**, **36b**, and 41F5.

The activities of compounds 41F5, **17b**, **22b** and **36b** were also evaluated against *H. capsulatum* yeasts phagocytozed into murine P388D1 macrophages. Compound **36b** displayed slightly improved IC_{50}/MIC values as compared to those of 41F5 (0.71±0.15 μM/1.25 μM v.s. 1.26±0.17 μM/2.5 μM) whereas **17b** (1.38±0.10 μM/2.5 μM) and **22b** (1.21±0.0.7 μM/2.5 μM) had similar inhibitory activities. As already discussed (*vide supra*), compounds **17b**, **22b** and **36b** showed ~2-fold increase in activity against *H. capsulatum* yeast *in vitro* as compared to 41F5. However, the same trend was only observed in the case of **36b** for intramacrophage *H. capsulatum*. In addition, **17b**, **22b**, **36b**, and 41F5 showed

moderately reduced activity against intramacrophage *H. capsulatum* as compared to *H. capsulatum* yeast *in vitro* (Tables), which might be due to the fact that the compounds have to permeate into the phagosome to inhibit the intracellular yeasts.

3. Summary and Conclusions

The highest activities against both *C. neoformans* and *H. capsulatum* were found for compounds containing [5,6]- and [6,5]-bicyclic ring systems, such as benzothiophene or indane groups, at the 5-position (**17b**, **22a**, **22b**, **23a**, **26a**, **26b**) and a [6,6]-bicyclic ring system linked via carbonyl spacer (**36b**) to the same position of the thiazole core structure. These compounds also showed low toxicities against murine P388D1 macrophages resulting in excellent selectivity indices (SIs). Highly active compounds, such as **17b**, **21b**, **22b**, **26b**, and **36b**, remained active against fluconazole-resistent *C. neoformans* TES9 and MRL862 strains and *H. capsulatum* yeasts phagocytozed into murine P388D1 macrophages. Compounds with reduced activity had ester functions in cyclic systems either at the 2- or the 5-position (**14**, **15**, **33**), a tricyclic ring system at the 5-position (**16**, **18**, **19**), an ionizable nitrogen atom in aromatic systems at the 5-position (**28**, **29**, **31**, **32**), a small ethyl substituent at the 5-position (**30**), or a modified core structure (**38, 40**). Several other modifications did also not lead to improved activity (**17a**, **20**, **21**, **23b**, **24**, **25**, **27**, **36a**). Based on the obtained results we were able to extend our understaning of the *H. capsulatum* specific SAR for 41F5-derived antifungals, which is illustrated in Figure 1.

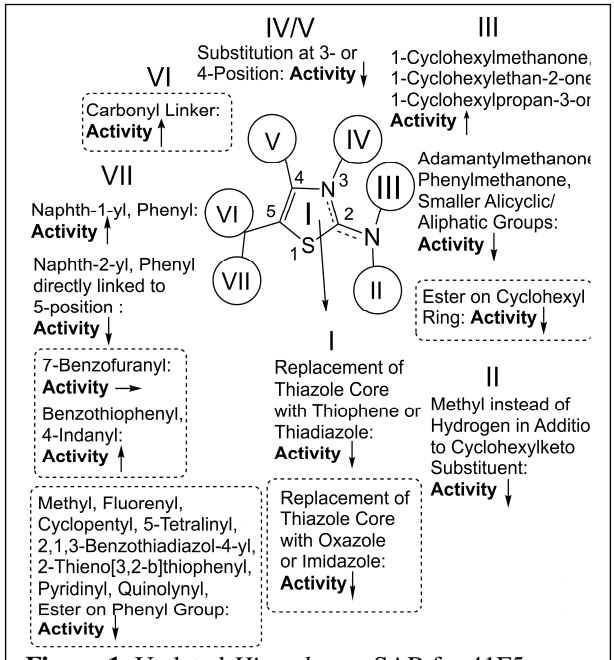


Figure 1. Updated *Histoplasma* SAR for 41F5-derived antifungal compounds. Areas circled with dotted lines indicate findings described in this paper. All other findings were reported previously by Khalil et al.

As discussed (*vide supra*), the *C. neoformans* specific SAR deviates from this pattern to some degree.

The results obtained for the **36a/b** tandem (carbonyl linker at the 5-position) indicate that the exploration of e.g. ether-, thioether-, thiocarbonyl-, thionyl- and sulfonamide linkers at the 2- and 5 position have the potential to further improve activity of 41F5-derived antifungals. Overall, however, the chemical space specific for this compound class has been explored fairly exhaustively by our group in the present and a previous study.[22] Although compounds such as **17b**, **21b**, **22b**, **26b**, and **36b** appeared to have a ~2 fold increased activity both against *H. capsulatum* and *C. neoformans* as compared to 41F5, compounds with markedly improved potency could not be indentified. Therefore, it is doubtful if additional SAR studies using a phenotypic screening approach with a further extended compound library can produce any dramatic advancement.

As discussed (*vide supra*), it is conceivable that structural information for the molecular target(s) of 41F5-derived antifungal compounds could potentially facilitate a more rational structure-based drug design approach. However, low antifungal activities obtained for ester-modified compounds make it questionable if an unbiased affinity chromatography approach for molecular target indentification is a viable strategy.

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Footnotes

Abbreviations: Boc, *tert*-butyloxycarbonyl; DMAP, 4-dimethylaminopyridine; DCM, dichloromethane; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; EDCI, 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide; HIV, human immunodeficiency virus; HOBt, hydroxybenzotriazole; HR-ESI, high resolution–electrospray ionization; LDA lithium diisopropyl amide; MIC, minimal inhibitory concentration; rxn, reaction; SAR, structure-activity-relationship; SD, standard deviation; SM, supplementary material; SI, selectivity index; TFA, trifluoroacedic acid; THF, tetrahydrofurane.

A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at…… This material includes data on antifungal activities and calculated aqueous solubilities of 41F5-derived antifungal compounds at pH5 and pH7, calculated ionization energy levels of 41F5-derived antifungal compounds, a full experimental section, and NMR/MS spectra for target compound

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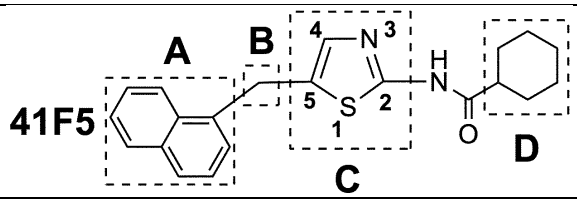
Table 1. Alignment of research objectives with areas of structural modifications at the 41F5 structure and the numbering system for all target compounds



Objective	Areas of chemical modifications	Target compounds
1 (Increased potency against *H. capsulatum* and *C. neoformans*)	A, B, C, and D	All the target compounds (**14**-**33**, **36**, **38**, **40**)
2 (Increased water solubility)	A and C	**28** (quinoline-substituted); **29**, **31**, **32** (pyridine-substituted); **40** (imidazole-substituted)
3 (Molecular target identification)	A, B and D	**14**, **15**, **33** (ester-modified at 2- or 5-substituents)

Table 2. Highly active thiazole antigfungal compounds synthesized and evaluated by Khalil et al.[22]

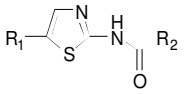
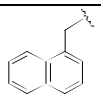
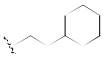
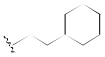
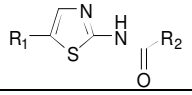
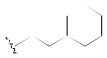
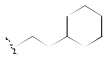
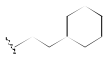
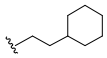
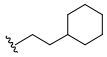
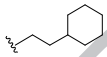
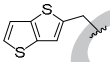
Cmpd			*H. capsulatum*	*C. neoformans* (H99 strain)
	R_1	R_2	IC_{50} (μM)	IC_{50} (μM)
41F5			0.4	0.4
A			0.4	> 10
B			1.6	1.3
C			0.7	> 10

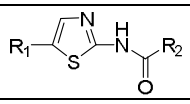
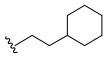
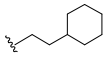
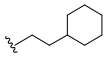
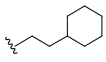
Table 3. Activity of 41F5-derived antifungals with bicyclic ring systems at the 5-position

			*H. capsulatum*		*C. neoformans* (H99 strain)		P388D1	SI (Hc)	SI (Cn)
Cmpd	R_1	R_2	IC_{50} (μM) [±SD]	MIC (μM)	IC_{50} (μM) [±SD]	MIC (μM)	IC_{50} (μM) [±SD]		
A			0.60 [±0.03]	1.25	> 20	> 40	27.59 [±4.12]	46	nd
41F5			0.48 [±0.01]	1.25	0.67 [±0.06]	1.25	> 40	> 83	> 60
17a			0.92 [±0.38]	5.00	> 20	> 40	18.87 [±3.05]	21	nd
17b			0.20 [±0.01]	0.31	0.34 [±0.04]	0.63	> 40	> 200	> 118
20a			> 20	> 40	> 20	> 40	nd	nd	nd
20b			1.54 [±0.42]	2.50	1.52 [±0.22]	5.00	> 40	> 26	> 26
21a			0.40 [±0.01]	0.63	> 20	> 40	17.75 [±5.00]	44	nd
21b			0.42 [±0.01]	1.25	0.62 [±0.12]	2.50	> 40	> 95	> 65
22a			0.20 [±0.01]	0.31	> 20	> 40	> 40	> 200	nd
22b			0.20 [±0.01]	0.31	0.37 [±0.09]	1.25	> 40	> 200	> 108
23a			0.27 [±0.02]	0.63	> 20	> 40	16.90 [±1.75]	63	nd
23b			> 20	> 40	> 20	> 40	nd	nd	nd
24			> 20	> 40	> 20	> 40	nd	nd	nd

SD: Standard deviation; SI: Selectivity index, nd: not determined

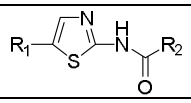
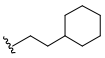
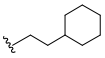
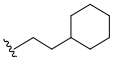
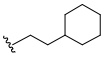
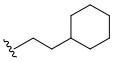
continued

Table 3. continued

			*H. capsulatum*		*C. neoformans* (H99 strain)		P388D1	SI (Hc)	SI (Cn)
Cmpd	R_1	R_2	IC_{50} (μM) [±SD]	MIC (μM)	IC_{50} (μM) [±SD]	MIC (μM)	IC_{50} (μM) [±SD]		
26a			0.38 [±0.02]	0.63	> 20	> 40	> 40	> 105	nd
26b			0.25 [±0.02]	0.63	0.41 [±0.07]	1.25	> 40	> 160	> 98
27a			0.90 [±0.29]	2.50	> 20	> 40	24.27	27	nd
27b			> 20	> 40	> 20	> 40	nd	nd	nd
28a			> 20	> 40	> 20	> 40	nd	nd	nd
28b			3.05 [±0.25]	10.00	3.24 [±0.42]	10.00	13.92 [±1.19]	5	5
36a			0.64 [±0.03]	1.25	> 20	> 40	> 40	> 63	nd
36b			0.19 [±0.01]	0.31	0.89 [±0.32]	2.50	> 40	> 211	> 45

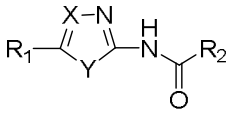
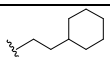
SD: Standard deviation; SI: Selectivity index. Not determined

Table 4. Activity of 41F5-derived antifungals with monocyclic ring systems or an acyclic ethyl group at the 5-position

			*H. capsulatum*		*C. neoformans* (H99 strain)		P388D1	SI (Hc)	SI (Cn)
Comp	R_1	R_2	IC_{50} (μM) [±SD]	MIC (μM)	IC_{50} (□M) [±SD]	MIC (μM)	IC_{50} (μM) [±SD]		
C			0.80 [±0.04]	1.25	> 20	> 40	> 40	> 50	nd
B			1.06 [±0.06]	2.50	1.36 [±0.10]	2.50	> 40	> 38	> 29
25a			> 20	> 40	> 20	> 40	nd	nd	nd
25b			0.76 [±0.05]	1.25	> 20	> 40	> 40	> 53	nd
29a			8.09 [±0.70]	20.00	> 20	> 40	> 40	> 5	nd
29b			18.30 [±1.06]	40.00	7.17 [±0.84]	20.00	> 40	> 2	> 6
30a	C_2H_5		> 20	> 40	> 20	> 40	nd	nd	nd
30b	C_2H_5		> 20	> 40	> 20	> 40	nd	nd	nd
31			> 20	> 40	> 20	> 40	nd	nd	nd
32a			7.67 [±0.25]	20.00	> 20	> 40	> 40	> 5	nd
32b			> 20	> 40	> 20	> 40	nd	nd	nd

SD: Standard deviation; SI: Selectivity index, nd: not determined

Table 5. Activity of antifungals with oxazole- and imidazole core structures

					*H. capsulatum*		*C. neoformans* (H99 strain)		P388D1	SI (Hc)	SI (Cn)
Cmpd	X	Y	R_1	R_2	IC_{50} (μM) [±SD]	MIC (μM)	IC_{50} (μM)	MIC (μM)	IC_{50} (μM)		
38a	CH	O			> 20	> 40	> 20	> 40	nd	nd	nd
38b	CH	O			> 20	> 40	> 20	> 40	nd	nd	nd
40	CH	NH			8.11 [±0.33]	20.00	> 20	> 40	> 40	> 5	> 2

SD: Standard deviation; SI: Selectivity index, nd: not determined

Table 6. Activity of 41F5-derived antifungals against fluconazole-resistant *Cryptococcus* isolates

Cmpd	H99 (serotype A-Flu^S)		B3501 (serotype D-Flu^S)		TES9[a] (serotype A-Flu^R)		MRL862[a] (serotype A-Flu^R)	
	IC_{50} (μM) [±SD][b]	MIC (μM)	IC_{50} (μM) [±SD]	MIC (μM)	IC_{50} (μM) [±SD]	MIC (μM)	IC_{50} (μM) [±SD]	MIC (μM)
41F5	0.67 [±0.06]	1.25	0.18 [±0.17]	0.63	0.20 [±0.03]	0.63	0.23 [±0.04]	0.63
Fluconazole	0.95 [±0.63]	4.08	0.13 [±0.22]	2.04	27.38 [±2.17]	65.36	14.61 [±0.65]	32.68
17b	0.34 [±0.04]	0.63	0.27 [±0.02]	0.63	0.23 [±0.01]	0.63	0.20 [±0.02]	0.63
21b	0.62 [±0.12]	2.50	0.32 [±0.02]	0.63	0.41 [±0.02]	0.63	0.15 [±0.09]	0.63
22b	0.21 [±0.09]	0.63	0.16 [±0.03]	0.63	0.19 [±0.01]	0.31	0.13 [±0.01]	0.31
26b	0.41 [±0.07]	1.25	0.42 [±0.06]	1.25	0.45 [±0.07]	1.25	0.37 [±0.58]	1.25
36b	0.89 [±0.32]	2.50	0.38 [±0.06]	1.25	0.47 [±0.05]	1.25	0.41 [±0.05]	1.25

[a]TES9 and MRL862 are fluconazole-resistant (Flu^R) strains due to mutation of the azole target Erg11.

[b]SD: Standard deviation

Captions

Figure 1. Updated *Histoplasma* SAR for 41F5-derived antifungal compounds. Areas circled with dotted lines indicate findings described in this paper. All other findings were reported previously by Khalil et al.

Scheme 1. Reagents and conditions: (a) $NaNO_2$, 18% HCl, H_2O, -5 °C, 24 h; (b) Acrolein, $CuCl_2 \times 2$ H_2O, CaO, acetone, 5 °C, 2 h; (c) Thiourea, ethanol, reflux, 24 h.

Scheme 2. Reagents and conditions: (a) LDA, THF, -78 °C, 30 min; (b) R_1CHO, r.t., 16 h; (c) Et_3SiH, TFA, DCM, r.t., 16 h; (d) 57% aq. HI, acetic acid, 100 °C, 4 h; *compound was purchased.

Scheme 3. Reagents and conditions: (a) Dess-Martin periodinane, 4Å molecular sieves, DCM, r.t., 16 h; (b) *t*-BuBr, DMSO, acetonitrile, 65 °C, 16 h; (c) Thiourea, ethanol, reflux, 16 h.

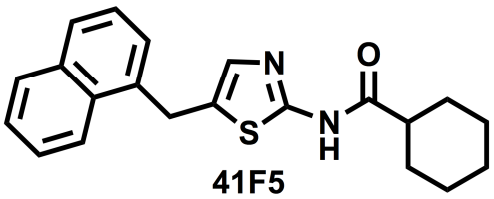
Scheme 4. Reagents and conditions: (a) R_2COCl, Et_3N, THF, r.t., 15 min; (b) R_2COCl, THF, r.t., 15 min.

Scheme 5. Reagents and conditions: (a) (1*R/S*,4*R/S*)-4-(methoxycarbonyl)cyclohexane-1-carboxylic acid or (1*R*,4*R*)-4-(methoxycarbonyl)cyclohexane-1-carboxylic acid or (1*R*,2*S*)-2-(methoxycarbonyl)cyclohexane-1-carboxylic acid, EDCI, DMAP, Et_3N, DCM/DMF (3:1, v/v), r.t., 16 h.

Scheme 6. Reagents and conditions: (a) Dess-Martin periodinane, DCM, r.t., 16 h; (b) TFA, DCM, r.t., 16 h; (c) 3-Cyclohexylpropanoyl chloride or cyclohexanecarbonyl chloride, Et_3N, THF, r.t., 15 min.

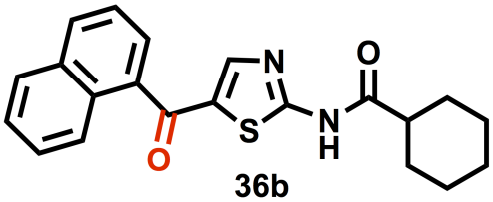
Scheme 7. Reagents and conditions: (a) 3-Cyclohexylpropanoyl chloride or cyclohexanecarbonyl chloride, Et_3N, THF, r.t., 15 min.

Scheme 8. Reagents and conditions: (a) HOBt, Et_3N, EDCI hydrochloride, acetonitrile, r.t., 10 min; (b) 5-Benzyl-1*H*-imidazol-2-amine □ HCl, 80 °C, 16 h.



41F5

$\underline{IC_{50}\text{ (Growth Inhibition)}}$
***Histoplasma capsulatum*: 0.48 μM**



36b

$\underline{IC_{50}\text{ (Growth Inhibition)}}$
***Histoplasma capsulatum*: 0.19 μM**