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Alkylated Piperazines and Piperazine-Azole Hybrids as Antifungal Agents

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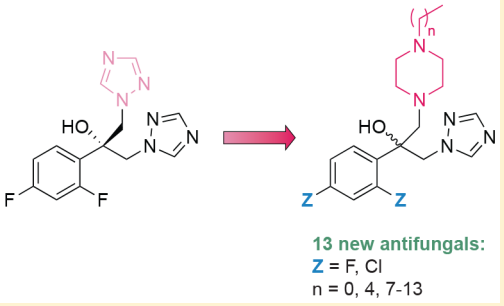
Supporting Information

ABSTRACT: The extensive use of fluconazole (FLC) and other azole drugs has caused the emergence and rise of azole-resistant fungi. The fungistatic nature of FLC in combination with toxicity concerns have resulted in an increased demand for new azole antifungal agents. Herein, we report the synthesis and antifungal activity of novel alkylated piperazines and alkylated piperazine-azole hybrids, their time-kill studies, their hemolytic activity against murine erythrocytes, as well as their cytotoxicity against mammalian cells. Many of these molecules exhibited broad-spectrum activity against all tested fungal strains, with excellent minimum inhibitory concentration (MIC) values against non-*albicans Candida* and *Aspergillus* strains. The most promising compounds were found to be less hemolytic than the FDA-approved antifungal agent voriconazole (VOR). Finally, we demonstrate that the synthetic alkylated piperazine-azole hybrids do not function by fungal membrane disruption, but instead by disruption of the ergosterol biosynthetic pathway via inhibition of the 14α-demethylase enzyme present in fungal cells.



INTRODUCTION

For the past decade, reported cases of diseases caused by fungi are on the rise, which presents a great threat to human health.[1] Fungal infections are especially problematic for patients with immune systems compromised either by HIV infections or the administration of immunosuppressive drugs.[2−9] The overreliance on conventional antifungal agents such as azoles (e.g., fluconazole (FLC), voriconazole (VOR), itraconazole (ITC), and posaconazole (POS)), polyenes (e.g., amphotericin B (AmB)), echinocandins (e.g., caspofungin (CAS) and micafungin), and allylamines (e.g., terbinafine and naftifine) has caused the development of drug-resistant fungal strains.[10−15] Therefore, the identification and development of novel antifungal agents capable of eliminating these drug-resistant fungi are a priority.[7,16,17] Fungal infections vary in pathogenicity; some are merely superficial and benign, whereas others are associated with significant morbidity and mortality. Serious fungal infections are caused by *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*.[18−20] Azole antifungal agents have emerged as front-line drugs for the treatment of fungal infections because of their favorable pharmacokinetics and better safety profiles than that of AmB, albeit they are still toxic at high concentrations.[21] Azoles disrupt the formation of ergosterol in fungi by inhibiting sterol 14α-demethylase, the enzyme that converts lanosterol into ergosterol in the ergosterol biosynthetic pathway.[22,23] FLC is one of the most important members of the azole family. FLC is well absorbed and exhibits high oral bioavailability. However, its fungistatic nature renders FLC inefficient against invasive fungal diseases. Consequently, second-generation analogues such as POS, VOR, and ITC were developed to help overcome some of these deficiencies. However, the extensive use of azoles has caused an increase in the number of azole-resistant fungi, which in combination with toxicity concerns created a need to improve on the existing azole scaffolds.

We and other groups previously reported that alkylation of various drug scaffolds can result in derivatives with promising antifungal properties.[24−30] For example, kanamycin B (KANB) and tobramycin (TOB) analogues with linear alkyl chains comprised of 12 and 14 carbons (C_{12} and C_{14}),[24,25] an *n*-alkylated ebsulfur derivative with a linear C_5 alkyl chain,[27] as well as 2,4-difluoro-2-(1*H*-1,2,4-triazo-1-yl)acetophenone analogues with linear C_5−C_8 alkyl chains,[28] displayed strong antifungal activity against *C. albicans* and *Aspergillus* spp. It is also well-known that tridemorph, a morpholine-based compound containing a linear C_{13} alkyl chain attached to the nitrogen atom, is a useful fungicide against plant fungal pathogens.[31] Inspired by the promise shown by KANB and TOB (C_{12} and C_{14}), ebsulfur (C_5), and 2,4-difluoro-2-(1*H*-1,2,4-triazo-1-yl)acetophenone (C_5−C_8) as antifungals, as well as the proven efficacy of tridemorph against plant pathogenic fungi, we decided to generate two new families of antifungal compounds: (1) alkylated piperazine analogues and (2) alkylated piperazine-azole hybrids. We opted for the piperazine moiety containing a nitrogen atom in place of the oxygen of the morpholine of tridemorph, as a morpholine-based linear alkyl chain could not be attached to the azole scaffold via its

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oxygen atom. In an effort to develop novel azole analogues, we decided to replace the triazole on the carbon α to the dihalophenyl ring of FLC by a linear alkylated piperazine.

Herein, we report the synthesis of nine piperazine derivatives (**1**−**9**) as well as that of 11 alkylated piperazine-azole hybrids (**16**−**26**) (Figure 1). We also report the synthesis of one

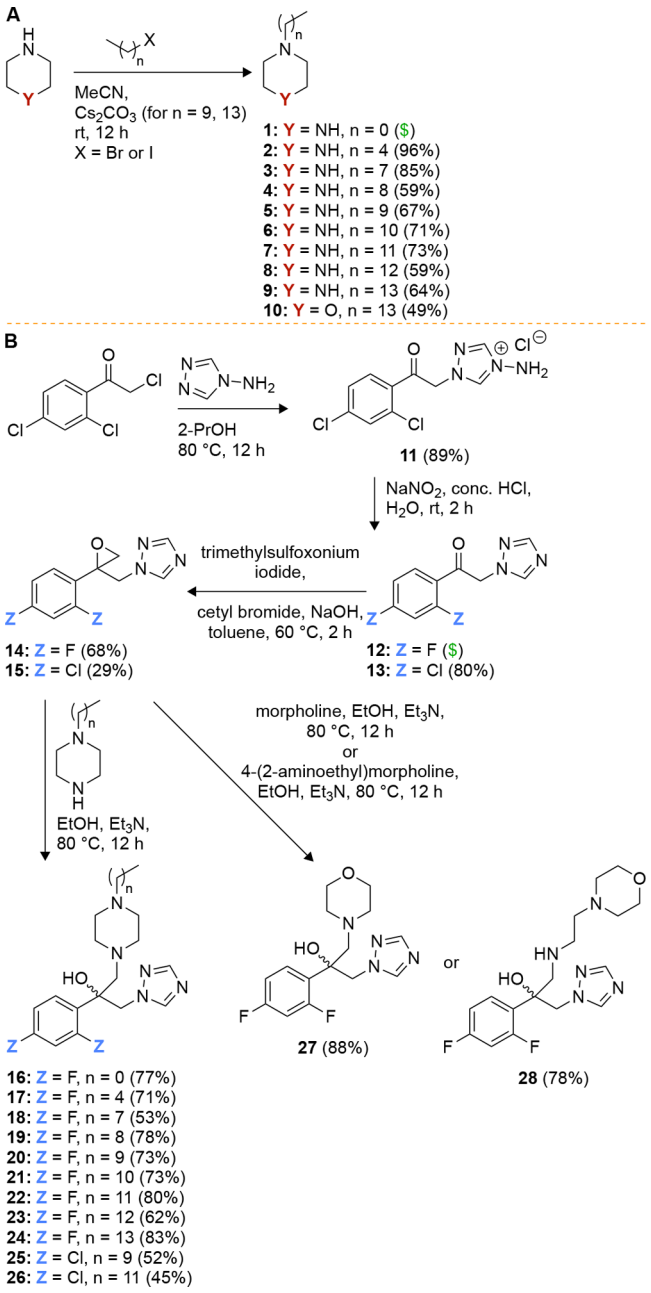

Figure 1. Synthetic scheme for the preparation of (A) piperazine derivatives **1**−**9** and morpholine derivative **10** and (B) novel azole analogues **16**−**28**.

alkylated morpholine derivative (**10**) and two morpholine-azole hybrids (**27** and **28**). We evaluate the antifungal activity of these compounds against a variety of *C. albicans*, non-*albicans Candida*, and *Aspergillus* strains by in vitro MIC determination as well as by time-kill studies. We also explore the hemolytic activity as well as cytotoxicity of these compounds against mouse erythrocytes and three mammalian cell lines, respectively. Finally, we investigate the potential mechanism of action of selected compounds by probing their ability to disrupt fungal membrane and/or the ergosterol biosynthetic pathway.

RESULTS AND DISCUSSION

Design and Synthesis of Two Families of Antifungal Compounds. We synthesized the alkylated piperazine derivatives **1**−**9** and morpholine derivative **10** in a single step. The nucleophilic substitution reactions of piperazine and morpholine with linear alkyl chain halides generated derivatives **1**−**10** (Figure 1A). The alkylated piperazines **1**−**9** were used in the preparation of alkylated piperazine-azole hybrids **16**−**26**, which proceeded in two steps from either the commercially available fluorinated compound **12** or its synthesized chlorinated counterpart **13**[32] (Figure 1B). The carbonyl group of compounds **12** and **13** was first converted to an epoxide by using trimethylsulfoxonium iodide in the presence of a strong base and a surfactant to yield the oxirane intermediates **14** and **15**, respectively, which were then reacted under mild basic conditions with piperazines **1**−**9** to afford hybrids **16**−**26**. Two additional hybrids, **27** and **28**, were also synthesized by reacting intermediate **14** with morpholine and 4-(2-aminoethyl)-morpholine, respectively.

Antifungal Activity in the Absence or Presence of Serum. The antifungal activity of the newly prepared alkylated piperazine/morpholine-azole hybrids **16**−**28**, of intermediate **14** that we used to verify that only final compounds exert antifungal activity, as well as that of alkylated piperazine/morpholine derivatives **1**−**10** were first evaluated against a panel of seven *C. albicans* (ATCC 10231 (**A**), ATCC 64124(R) (**B**), ATCC MYA-2876(S) (**C**), ATCC 90819(R) (**D**), ATCC MYA-2310(S) (**E**), ATCC MYA-1237(R) (**F**), and ATCC MYA-1003(R) (**G**)), three non-*albicans Candida* (*C. glabrata* ATCC 2001 (**H**), *C. krusei* ATCC 6258 (**I**), and *C. parapsilosis* ATCC 22019 (**J**)), and three *Aspergillus* (*A. flavus* ATCC MYA-3631 (**K**), *A. nidulans* ATCC 38163 (**L**), and *A. terreus* ATCC MYA-3633 (**M**)) strains using a concentration range of 0.03−31.3 μg/mL (Table 1). Commercially available antifungal agents such as AmB, CAS, FLC, and VOR were used as positive controls for comparison. For final compounds **1**−**10** and **16**−**28**, intermediate **14**, as well as the reference drugs AmB and CAS, we reported MIC-0 values, which correspond to no visible growth of the 13 fungal strains tested. We reported MIC-2 values (i.e., 50% growth inhibition) for FLC and VOR against all fungal strains tested with the exception of strain **A** by VOR. Out of the seven *C. albicans* strains, two were classified as sensitive (strains **C** and **E**), one as intermediate (strain **A**), and four as resistant (strains **B**, **D**, **F**, and **G**) against FLC and ITC as defined by American Type Culture Collection (ATCC). Henceforth, we define antifungal activity as excellent (0.015−1.95 μg/mL), good (3.9 μg/mL), moderate (7.8−15.6 μg/mL), or poor ($\geq$31.3 μg/mL) based on MIC values. It is important to note that all comparisons to follow will be made by using the MIC values reported in μg/mL as the data were collected that way. However, we also present in parentheses in Table 1 the corresponding values in micromolar units.

From a quick glance at the MIC data reported in Table 1, we could make the following observations. The inactivity of **14** confirmed that the intermediates generated during the synthesis of our target compounds did not exert any antifungal activity. In the case of the alkylated piperazines, the longer chains displayed better antifungal activity. Compounds **1**−**4** displayed poor activity against all fungal strains tested. Compounds **5** and **6** were found to be moderately active against all

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Table 1. MIC Values[a] (in μg/mL) (*Note*: MIC Values in Micromolar Are Presented in Parentheses) Determined for Compounds 1−10, 14, and 16−28, As Well As for Four Control Antifungal Agents (AmB, CAS, FLC, and VOR) against various Yeast Strains and Filamentous Fungi

	yeast strains[b]										filamentous fungi[c]		
compd	A	B	C	D	E	F	G	H	I	J	K	L	M
1	>31.3 (>312.5)	>31.3 (>312.5)	>31.3 (>312.5)	>31.3 (>312.5)	>31.3 (>312.5)	>31.3 (>312.5)	>31.3 (>312.5)	>31.3 (>312.5)	>31.3 (>312.5)	>31.3 (>312.5)	>31.3 (>312.5)	>31.3 (>312.5)	>31.3 (>312.5)
2	>31.3 (>200.3)	>31.3 (>200.3)	>31.3 (>200.3)	>31.3 (>200.3)	>31.3 (>200.3)	>31.3 (>200.3)	>31.3 (>200.3)	>31.3 (>200.3)	>31.3 (>200.3)	>31.3 (>200.3)	>31.3 (>200.3)	>31.3 (>200.3)	>31.3 (>200.3)
3	>31.3 (>157.8)	>31.3 (>157.8)	>31.3 (>157.8)	>31.3 (>157.8)	>31.3 (>157.8)	>31.3 (>157.8)	>31.3 (>157.8)	>31.3 (>157.8)	>31.3 (>157.8)	>31.3 (>157.8)	>31.3 (>157.8)	>31.3 (>157.8)	>31.3 (>157.8)
4	31.3 (147.4)	>31.3 (>147.4)	>31.3 (>147.4)	31.3 (147.4)	31.3 (147.4)	31.3 (147.4)	>31.3 (>147.4)	31.3 (147.4)	31.3 (147.4)	>31.3 (>147.4)	31.3 (147.4)	>31.3 (>147.4)	>31.3 (>147.4)
5	15.6 (68.9)	15.6 (68.9)	15.6 (68.9)	15.6 (68.9)	15.6 (68.9)	15.6 (68.9)	15.6 (68.9)	7.8 (34.5)	7.8 (34.5)	15.6 (68.9)	31.3 (138.2)	15.6 (68.9)	15.6 (68.9)
6	7.8 (32.4)	7.8 (32.4)	15.6 (64.8)	7.8 (32.4)	7.8 (32.4)	7.8 (32.4)	15.6 (64.8)	7.8 (32.4)	7.8 (32.4)	7.8 (32.4)	31.3 (129.6)	15.6 (64.8)	15.6 (64.8)
7	1.95 (7.7)	7.8 (30.7)	3.9 (15.3)	7.8 (30.7)	3.9 (15.3)	3.9 (15.3)	3.9 (15.3)	1.95 (7.7)	1.95 (7.7)	1.95 (7.7)	3.9 (15.3)	1.95 (7.7)	7.8 (30.7)
8	0.975 (3.6)	0.975 (3.6)	1.95 (7.2)	0.975 (3.6)	1.95 (7.2)	1.95 (7.2)	1.95 (7.2)	0.48 (1.8)	0.975 (3.6)	1.95 (7.2)	1.95 (7.2)	0.975 (3.6)	1.95 (7.2)
9	0.975 (3.5)	0.975 (3.5)	0.975 (3.5)	1.95 (6.9)	0.975 (3.5)	1.95 (6.9)	1.95 (6.9)	0.975 (3.5)	0.975 (3.5)	0.975 (3.5)	0.975 (3.5)	0.975 (3.5)	0.975 (3.5)
10	>31.3 (>110.4)	>31.3 (>110.4)	>31.3 (>110.4)	>31.3 (>110.4)	>31.3 (>110.4)	>31.3 (>110.4)	>31.3 (>110.4)	>31.3 (>110.4)	>31.3 (>110.4)	>31.3 (>110.4)	>31.3 (>110.4)	>31.3 (>110.4)	>31.3 (>110.4)
14	>31.3 (>132.0)	>31.3 (>132.0)	>31.3 (>132.0)	>31.3 (>132.0)	>31.3 (>132.0)	>31.3 (>132.0)	>31.3 (>132.0)	>31.3 (>132.0)	>31.3 (>132.0)	>31.3 (>132.0)	>31.3 (>132.0)	>31.3 (>132.0)	>31.3 (>132.0)
16	7.8 (23.1)	31.3 (92.8)	7.8 (23.1)	31.3 (92.8)	7.8 (23.1)	31.3 (92.8)	31.3 (92.8)	31.3 (92.8)	15.6 (46.2)	0.975 (2.9)	31.3 (92.8)	0.975 (2.9)	31.3 (92.8)
17	>31.3 (>79.5)	>31.3 (>79.5)	>31.3 (>79.5)	31.3 (79.5)	>31.3 (>79.5)	>31.3 (>79.5)	>31.3 (>79.5)	>31.3 (>79.5)	>31.3 (>79.5)	15.6 (39.6)	>31.3 (>79.5)	>31.3 (>79.5)	>31.3 (>79.5)
18	3.9 (9.0)	31.3 (71.9)	31.3 (71.9)	15.6 (35.8)	>31.3 (>71.9)	31.3 (71.9)	0.975−31.3 (2.2−71.9)	15.6 (35.8)	7.8 (17.9)	0.03 (0.1)	1.95 (4.5)	3.9 (9.0)	3.9 (9.0)
19	0.975 (2.2)	7.8 (17.6)	3.9 (8.8)	7.8 (17.6)	15.6 (35.2)	15.6 (35.2)	15.6 (35.2)	3.9 (8.8)	0.975 (2.2)	0.24 (0.55)	0.48 (1.1)	0.975 (2.2)	1.95 (4.4)
20	0.975 (2.1)	3.9 (8.4)	7.8 (16.8)	3.9 (8.4)	7.8 (16.8)	7.8 (16.8)	3.9 (8.4)	>31.3 (>67.5)	1.95 (4.2)	0.015 (0.03)	0.24 (0.5)	0.975 (2.1)	0.975 (2.1)
21	1.95 (4.1)	15.6 (32.8)	7.8 (16.4)	15.6 (32.8)	15.6 (32.8)	15.6 (32.8)	15.6 (32.8)	3.9 (8.2)	1.95 (4.1)	0.24 (0.50)	1.95 (4.1)	1.95 (4.1)	1.95 (4.1)
22	1.95 (3.9)	7.8 (15.9)	3.9 (7.9)	3.9 (7.9)	7.8 (15.9)	7.8 (15.9)	15.6 (31.7)	0.975 (2.0)	0.48 (0.98)	0.015 (0.03)	1.95 (3.9)	3.9 (7.9)	3.9 (7.9)
23	1.95 (3.9)	7.8 (15.4)	1.95 (3.9)	1.95 (3.9)	3.9 (7.7)	3.9 (7.7)	3.9 (7.7)	0.975 (1.9)	0.975 (1.9)	<0.24 (<0.47)	0.975 (1.9)	0.975 (1.9)	0.975 (1.9)
24	1.95 (3.8)	31.3 (60.2)	7.8 (15.0)	1.95 (3.8)	3.9 (7.5)	3.9 (7.5)	1.95 (3.8)	>31.3 (>60.2)	0.975 (1.9)	>31.3 (>60.2)	0.48 (0.9)	1.95 (3.8)	0.975 (1.9)
25	1.95 (3.9)	15.6 (31.4)	7.8 (15.7)	3.9 (7.9)	7.8 (15.7)	7.8 (15.7)	7.8 (15.7)	0.975 (2.0)	1.95 (3.9)	0.24 (0.48)	3.9 (7.9)	3.9 (7.9)	3.9 (7.9)
26	1.95 (3.7)	7.8 (14.9)	3.9 (7.4)	3.9 (7.4)	3.9 (7.4)	3.9 (7.4)	1.95 (3.7)	0.975 (1.9)	1.95 (3.7)	0.24 (0.46)	3.9 (7.4)	1.95 (3.7)	1.95 (3.7)
27	>31.3 (>96.5)	>31.3 (>96.5)	>31.3 (>96.5)	3.9−31.3 (12.0−96.5)	7.8−31.3 (24.0−96.5)	>31.3 (>96.5)	31.3 (96.5)	31.3 (96.5)	>31.3 (>96.5)	31.3 (>96.5)	7.8 (24.0)	1.95 (6.0)	15.6 (48.1)
28	31.3 (85.2)	31.3 (85.2)	31.3 (85.2)	>31.3 (>85.2)	>31.3 (>85.2)	31.3 (85.2)	>31.3 (>85.2)	31.3 (85.2)	>31.3 (>85.2)	31.3 (85.2)	>31.3 (>85.2)	>31.3 (>85.2)	>31.3 (>85.2)
AmB[d]	3.9 (4.2)	3.9 (4.2)	1.95 (2.1)	0.975 (1.1)	1.95 (2.1)	3.9 (4.2)	3.9 (4.2)	1.95 (2.1)	3.9 (4.2)	1.95 (2.1)	15.6 (15.6)	3.9 (4.2)	3.9 (4.2)
CAS[d]	0.975 (0.8)	0.24 (0.2)	0.06 (0.05)	0.12 (0.1)	0.12 (0.1)	0.24 (0.2)	0.48 (0.4)	0.06 (0.05)	0.48 (0.4)	1.95 (1.6)	>31.3 (>25.8)	>31.3 (>25.8)	>31.3 (>25.8)
FLC[d]	62.5 (204.1)	>125 (>408.1)	15.6 (50.9)	>125 (>408.1)	>125 (>408.1)	62.5 (204.1)	62.5 (204.1)	>31.3 (>102.2)	>31.3 (>102.2)	1.95 (6.4)	62.5 (204.1)	62.5 (204.1)	62.5 (204.1)
VOR[d]	0.24 (0.69)	3.9 (11.2)	1.95 (5.6)	1.95 (5.6)	0.975 (2.8)	7.8 (22.3)	1.95 (5.6)	0.06 (0.17)	0.12 (0.34)	<0.03 (<0.06)	0.24 (0.69)	0.12 (0.34)	0.12 (0.34)

[a]For yeast strains: MIC-0 values are reported for FLC analogues **1−10**, **14**, **16−28**, AmB, and CAS, whereas MIC-2 values are reported for FLC and VOR. For filamentous fungi (strains **K−M**), MIC-0 values are reported for all compounds. [b]Yeast strains: **A** = *Candida albicans* ATCC 10231, **B** = *C. albicans* ATCC 64124(R), **C** = *C. albicans* ATCC MYA-2876(S), **D** = *C. albicans* ATCC 90819(R), **E** = *C. albicans* ATCC MYA-2310(S), **F** = *C. albicans* ATCC MYA-1237(R), **G** = *C. albicans* ATCC MYA-1003(R), **H** = *Candida glabrata* ATCC 2001, **I** = *Candida krusei* ATCC 6258, **J** = *Candida parapsilosis* ATCC 22019. NOTE: Here, the (S) and (R) indicate that ATCC reports these strains to be susceptible (S) and resistant (R) to ITC and FLC. [c]Filamentous fungi: **K** = *Aspergillus flavus* ATCC MYA-3631, **L** = *Aspergillus nidulans* ATCC 38163, **M** = *Aspergillus terreus* ATCC MYA-3633. [d]Known antifungal agents: AmB = amphotericin B, CAS = caspofungin, FLC = fluconazole, VOR = voriconazole.

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strains tested, with the exception of being inactive against *Aspergillus flavus* (strain **K**). Compound **7** was found to display good activity against five (strains **A**, **C**, and **E**-**G**) of the seven *C. albicans* strains tested, all three non-*albicans Candida* (strains **H**−**J**), and two (strains **K** and **L**) of the three *Aspergillus* strains tested. Among the alkylated piperazines, compounds **8** and **9** were found to be the best. They exhibited an excellent and broad spectrum of activity. Surprisingly, the corresponding morpholine analogue of **9**, compound **10**, displayed poor activity against all of the tested strains.

Overall, we found the morpholine-azole hybrids **27** and **28** to be inactive as antifungal agents, with the exception of compound **27** against strains **K** (7.8 μg/mL) and **L** (1.95 μg/mL). In general, for the alkylated piperazine-azole hybrids **16**−**26**, we observed better activity against non-*albicans Candida* and *Aspergillus* strains than against *C. albicans*. Overall, compounds **20**−**26** displayed good to excellent antifungal activity against most strains tested. Compounds **16** and **18**, although generally not as potent as **20**−**26**, also displayed good to excellent activity against a few fungal strains. However, compound **17** was found to be basically inactive against all fungal strains tested.

When focusing on *Aspergillus* strains, we observed that compounds **19**−**21**, **23**, and **24** displayed excellent activity against strains **K**−**M**. Compounds **18**, **22**, **25**, and **26** were good against strains **K**−**M**, whereas compounds **16** and **17** were completely inactive, with the exception of compound **16** against strain **L** (0.975 μg/mL). When looking at the non-*albicans Candida*, we found that the three strains tested were susceptible to the majority of the alkylated piperazine-azole hybrids, with strain **J** being the most susceptible. Indeed, compounds **16**, **18**−**23**, **25**, and **26** showed excellent activity against strain **J**. In most cases, compounds **18**−**26** displayed moderate to excellent activity against strains **H** and **I**. When examining the MIC values against *C. albicans* strains **A**−**G**, we observed that compounds **19**−**26** exhibited good to moderate activity, whereas compounds **16**−**18** generally displayed poor activity. From all of these observations made on compounds **1**−**28**, we can conclude that compounds **7**−**9**, **20**, **22**, **23**, **25**, and **26** displayed better overall activity. It is important to point out that these compounds maintained activity against the azole-resistant *C. albicans* strains.

As it is known that some of the azole antifungals on the market tend to bind to proteins and be less efficient in intercellular matrices, we tested the best compounds **7**, **9**, **20**, **22**, **25**, and **26** against strains **A**, **J**, and **L** in the presence and absence of fetal bovine serum (FBS) (Table 2) in order to test the potential effect of protein binding to our compounds. We found that the alkylated piperazine-azole hybrids **20**, **22**, **25**, and **26** retained their full antifungal activity in the presence of FBS, whereas the alkylated piperazine derivatives **7** and **9** exhibited a 2−8-fold decrease in activity in the presence of this agent. It is important to note that even with this small loss in activity, compounds **7** and **9** remained good antifungals.

Structure−Activity Relationship (SAR) Summary. From a deeper analysis of the antifungal MIC data (Table 1), we could answer the five following questions: (i) Which chain length confers better activity to the alkylated piperazine derivatives **1**−**9**? (ii) Which chain length confers superior activity to the alkylated piperazine-azole hybrids **16**−**26**? (iii) Which scaffold (alkylated piperazine versus alkylated piperazine-azole hybrid) produces the best antifungal activity? (iv) Does the identity of the halogen atoms on the phenyl ring play a role in antifungal activity? (v) How do the compounds generated compare to antifungals currently used in the clinic?

For the alkylated piperazine derivatives **1**−**9**, the optimal chain lengths for maximal activity were found to be C_{14}, C_{13}, and C_{12}, and the general trend for activity versus chain length was $C_{14} = C_{13} > C_{12} > C_{10} > C_9 = C_8 = C_5 = C_1$. For the alkylated piperazine-azole hybrids **16**−**26**, the optimal chain lengths were found to be C_{13}, C_{12}, and C_{10}, and the general trend for activity versus chain length was $C_{13} > C_{12} > C_{10} > C_{14} > C_{11} > C_9 > C_8 > C_1 > C_5$. By performing pairwise comparisons of the activity of alkylated piperazine derivatives **1**−**9** to that of their direct azole hybrid counterparts **16**−**24** (e.g., **1** versus **16**, **2** versus **17**, etc.), we determined that the hybrids conferred superior antifungal activity, with the exception of compounds **8** and **9**, which displayed overall superior antifungal activity than hybrids **23** and **24**. By investigating the activity of the dichlorinated hybrids **25** and **26** and comparing them to those of the difluorinated counterparts **20** and **22**, respectively, we observed that the identity of the halogen atoms on the phenyl ring did not greatly influence the antifungal activity of the compounds. For the C_{10}-containing hybrids (**20** and **25**), a slightly superior activity was observed with the difluorinated compound **20**, whereas for C_{12}-containing hybrids (**22** and **26**), comparable antifungal activity was observed with both compounds against all strains tested. In general, we found that the most potent antifungal compounds synthesized (**7**−**9**, **20**, **22**, **23**, **25**, and **26**) displayed better activity than FLC and similar or better activity than AmB against all fungal strains tested, as well as better activity than CAS against the three *Aspergillus* strains tested. For the remainder of the study, we

Table 2. MIC Values (in μg/mL) Determined for Compounds 7, 9, 20, 22, 25, and 26 As Well As for Two Control Antifungal Agents (AmB and VOR) against Various Yeast Strains and Filamentous Fungi

	yeast strains				filamentous fungi	
compd	*Candida albicans* ATCC 10231 (**A**) (no FBS)	*Candida albicans* ATCC 10231 (**A**) (+10% FBS)	*Candida parapsilosis* ATCC 22019 (**J**) (no FBS)	*Candida parapsilosis* ATCC 22019 (**J**) (+10% FBS)	*Aspergillus nidulans* ATCC 38163 (**L**) (no FBS)	*Aspergillus nidulans* ATCC 38163 (**L**) (+10% FBS)
7	1.95	3.9	1.95	7.8	1.95	7.8
9	0.975	7.8	0.975	7.8	0.975	7.8
20	0.975	0.975	0.015	0.06	0.975	0.975
22	1.95	1.95	0.015	0.06	3.9	3.9
25	1.95	1.95	0.24	0.24	3.9	3.9
26	1.95	1.95	0.24	0.24	1.95	3.9
AmB	3.9	7.8	1.95	15.6	3.9	15.6
VOR	0.48	0.48	0.015	0.015	0.12	0.12

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decided to use active compounds with alkyl chains containing an even number carbons as representatives.

Antibiofilm Activity of Synthetic Compounds. Biofilms are complex functional communities of one or more species of microorganisms that are encased in extracellular polymeric substances and attached to both a solid surface and to each other. Being complex matrices, antifungal agents have difficulty reaching the pathogens embedded in these difficult-to-treat networks. The antibiofilm activity of compounds **7**, **9**, **20**, **22**, **25**, and **26**, as well as that of VOR were evaluated against biofilms of *C. albicans* ATCC 64124 (strain **B**) by XTT reduction assay (Table 3). The $SMIC_{50}$ and $SMIC_{80}$ values

Table 3. Antibiofilm Activity of Compounds 7, 9, 20, 22, 25, and 26, As Well As VOR against *C. albicans* ATCC 64124 (Strain B) Biofilms

compd	$SMIC_{50}$[a] (μg/mL)	$SMIC_{80}$[b] (μg/mL)
7	7.8	31.3
9	15.6	62.5
20	15.6	62.5
22	7.8	15.6
25	15.6	31.3
26	15.6	31.3
VOR	62.5	62.5

[a]$SMIC_{50}$ = sessile minimum inhibitory concentration that reduced the metabolic activity of biofilms by 50%. [b]$SMIC_{80}$ = sessile minimum inhibitory concentration that reduced the metabolic activity of biofilms by 80%.

($SMIC_{50}$ and $SMIC_{80}$ are defined as the drug concentration required to inhibit the metabolic activity of the biofilm by 50% and 80%, respectively) for all tested compounds ranged between 7.8 and 15.6 μg/mL and 31.3−62.5 μg/mL, respectively, and were both 62.5 μg/mL for VOR, indicating the superiority of the alkylated piperazine-azole hybrids as *C. albicans* antibiofilm agents. Although it has been reported that *Candida* biofilms can exhibit higher degree of resistance (with 30 to 2000 times higher MIC values than their corresponding planktonic MIC values) to current antifungals such as AmB and azoles,[33] we found the MIC values of alkylated piperazine-azole hybrids not to be greatly affected in biofilms. By comparing the $SMIC_{50}$ and $SMIC_{80}$ values to the MIC values in liquid cultures for each compound, we found that compounds worked best in biofilms in the following order: **22** and **25** (1−2-fold increase in MIC in biofilms) > **7** (1−4-fold increase) > **26** (2−4-fold increase) > **20** (4−16-fold increase) > **9** (16−64-fold increase). Our results suggested that all hybrids tested with the exception of **9** could eliminate preformed biofilms of *C. albicans* ATCC 64124 (strain **B**), at least in vitro.

Time-Kill Assays. To determine if the antifungal activity of the alkylated piperazine-azole hybrids is fungistatic or fungicidal, we performed time-kill assays over 24 h periods by using compounds **20** and **22**, each against two fungal strains (strains **A** and **J** for **20**; **H** and **J** for **22**) (Figure 2). We found compounds **20** and **22** to be fungicidal at 4× their respective MIC values against strains **A** and **H**, respectively. With their fungicidal activity, compounds **20** and **22** appear to be better antifungals than the control FDA-approved drug VOR, which displayed fungistatic activity against these two fungal strains. However, when tested against strain **J**, both compounds displayed fungistatic activity at up to 8× their MIC values. Although fungistatic against strain **J**, at 1× their respective MIC values, compounds **20** and **22** displayed a larger reduction in fungal growth than VOR, confirming their superiority as antifungals.

Hemolysis Assay. Because our synthetic hybrids exhibited promising antifungal activity, we wanted to confirm that they would display none or reasonable (<50% hemolysis at 10 × MIC) hemolytic activity against murine red blood cells (mRBCs). We first investigated the alkylated piperazine derivatives **7** and **9** and found them to exhibit 52% and 93% hemolysis of mRBCs at 7.8 μg/mL (1−8-fold higher concentration than their overall antifungal MIC values), potentially indicating that very long alkyl chains might not be optimal for the development of these compounds as antifungals (Figure 3). We next tested all of the alkylated piperazine-azole hybrids generated, with the exception of compound **17**, that we had found to be inactive. Compounds **16** (C_1) and **18** (C_8) displayed <10% hemolysis at a concentration 62.5 μg/mL, which is 2−2000-fold higher than their overall MIC values. Similarly, compound **20** induced only 23% lysis at 31.3 μg/mL, which is 1−2000-fold higher than the MIC values reported for this compound. Because compound **20** is one of the best molecules in terms of MIC values, the low hemolytic activity for this analogue is highly encouraging. However, similarly to their counterparts **7** and **9**, hybrids **22** and **24** with C_{12} and C_{14} alkyl chains were found to display >50% hemolysis at 7.8 μg/mL, confirming that very long alkyl chains are not optimal. Subsequently, we analyzed the hemolytic activity of the dichlorinated compounds **25** and **26** and compared them with their difluorinated counterparts **20** and **22**. Similarly to compounds **20** and **22**, hybrids **25** and **26** showed chain-dependent hemolytic activity in the order of **26** (C_{12}) > **25** (C_{10}). Compound **25** showed only 22% hemolysis at 7.8 μg/mL (1−32.5-fold higher concentration than its antifungal MIC values). However, at that same concentration of 7.8 μg/mL, compound **26** displayed 85% hemolysis of mRBCs. Overall, the difluorinated analogues **20** and **22** displayed lower or equal hemolytic activity when compared to their dichlorinated counterparts **25** and **26**. Importantly, the newly synthesized compound **20** displayed less hemolytic effect than the FDA-approved control drug VOR.

In Vitro Cytotoxicity Assay. Because fungi are eukaryotes with similar biological properties, the drugs that are designed to target fungal cells may cause unwanted toxicity to mammalian cells. Therefore, a crucial parameter to consider when developing antifungal drugs is their selectivity for fungal over mammalian cells. To determine the selectivity of the synthetic hybrids toward fungi, we tested the most active compounds **7**, **9**, **18**, **20**, **22**, **25**, and **26** against three different cell lines, HEK-293, A549, and BEAS-2B, along with the FDA-approved antifungal agent VOR as a control (Figure 4). We observed that the alkylated piperazine derivatives **7** and **9** were toxic to all three cell lines tested, with the longer alkyl chains resulting in higher toxicity to mammalian cells. Compound **18** (C_8) was nontoxic to A549 at up to 15.5 μg/mL, but exhibited some toxicity against HEK-293 and BEAS-2B at 7.8 μg/mL (1−260-fold higher concentration than its overall antifungal MIC values). Similarly, hybrid **20** displayed no toxicity at up to 7.8 μg/mL against A549 and also exhibited some toxicity against HEK-293 and BEAS-2B at 7.8 μg/mL, which is 1−520-fold higher than the MIC values reported for this compound. As we postulated, for compound **22** with the longer alkyl chain, less than 40% cell survival was reported against all

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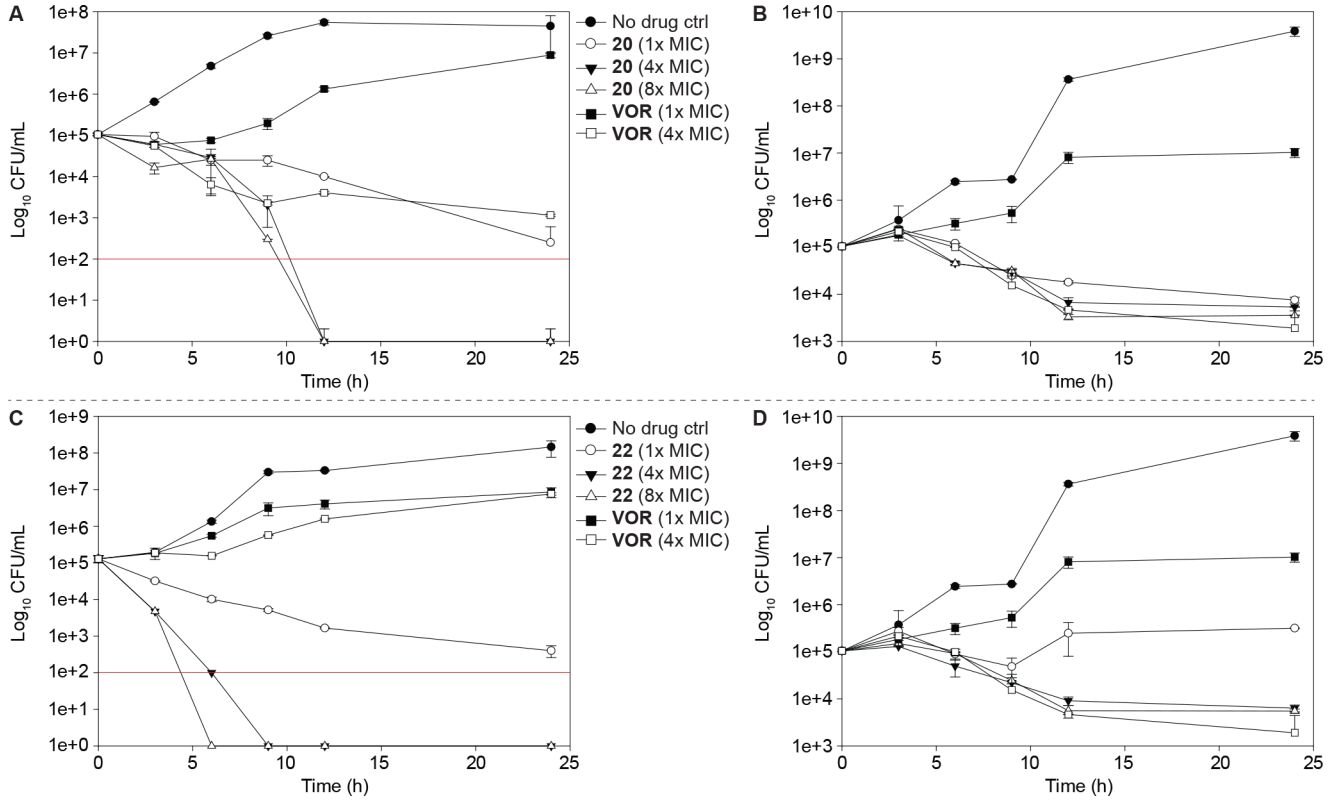

Figure 2. Representative time-kill curves for compound **20** against (A) *C. albicans* ATCC 10231 (strain **A**) and (B) *C. parapsilosis* ATCC 22019 (strain **J**), as well as for compound **22** against (C). *C. glabrata* ATCC 2001 (strain **H**), and (D) strain **J**. Fungal strains were treated with no drug (black circles), 1 × MIC (white circles), 4 × MIC (inverted black triangles), or 8 × MIC (white triangles) of compounds **20** or **22**, or with 1 × MIC (black squares) or 4 × MIC (white squares) of VOR. The detection limit of this assay was 100 CFU/mL (represented by the red line in (A) and (C)).

three cell lines when tested at a concentration of 7.8 μg/mL (1−520-fold higher concentration than its overall antifungal MIC values). The dichlorinated compounds **25** and **26** exhibited similar trends, and the cytotoxicity values were 1−32.5-fold higher concentration than the overall antifungal MIC values reported for these compounds. Overall, when considering the very low MIC values for hybrids **18**, **20**, **22**, **25**, and **26** against specific fungal strains, these cytotoxicity data are encouraging.

Membrane Permeabilization Assay. Previous studies from our group have demonstrated that amphiphilic molecules can cause membrane disruption to result in fungal cell death. On the basis of these findings, we assumed that the hybrids generated in this study could also potentially cause fungal death by disrupting the fungal membrane. To investigate this possible mechanism of action, we evaluated the effect of compounds **20** and **26** on fungal cell membrane integrity by using propidium iodide (PI) dye as a probe (Figure 5). The PI dye can only enter the cells with compromised membrane. Afterward, the dye binds to nucleic acid and fluoresces, which can be observed under a fluorescence microscope. We used compounds **20** and **26** with C_{10} and C_{12} linear alkyl chains to determine the impact of chain length on membrane disruption. The KANB (C_{14}) derivative with a 14-carbon linear alkyl chain and FLC were used as positive and negative controls, respectively. At 2 × MIC, the positive control KANB (C_{14}) significantly increased PI dye uptake by *C. albicans* ATCC 10231 (strain **A**). As expected, at 7.8 μg/mL, the highest concentration achievable in this assay, the negative control FLC did not allow for PI uptake by the fungal cells. Regardless of their chain length, neither compound **20** nor **26** (at 4 × MIC) induced cellular uptake of PI dye into *C. albicans* ATCC 10231 (strain **A**). From this study, we concluded that the possible mechanism of action of our compounds is not membrane disruption.

Determination of Sterol Composition in *C. albicans* in the Presence and Absence of Drugs. Because our synthetic hybrids did not cause membrane disruption of *C. albicans* ATCC 10231 (strain **A**) in the membrane permeabilization assay, we predicted they could potentially act like the other conventional azoles, and decided to explore their effect on sterol composition. By using gas chromatography−mass spectrometry (GC-MS), we investigated the potential of the synthetic compounds to exert their antifungal activity by inhibiting the sterol 14α-demethylase enzyme of the ergosterol biosynthetic pathway (Figure 6). We selected two of the best compounds, **20** and **26**, and evaluated their effects on sterol composition in *C. albicans* ATCC 10231 (strain **A**) at sub-MIC levels of 0.48 μg/mL (Figure 6E) and 0.975 μg/mL (Figure 6F), respectively. We also used FLC and VOR at 1.95 and 0.12 μg/mL, respectively, for comparison studies (Figure 6C,D). A no drug control was also performed (Figure 6A). The sterol profile results are summarized in Figure 6G. The sterol profile in the absence of drug indicated that strain **A** accumulated 100% ergosterol (**2**), suggesting that the sterol biosynthesis was fully functional in this fungal strain. When treated with FLC, we found predominance of ergosterol (**2**, 95.90%) in strain **A**, which indicated that FLC had no effect on ergosterol biosynthesis in this specific fungal strain. This observation could be easily explained by the fact that we have used FLC concentration that corresponds to 32-fold lower than the

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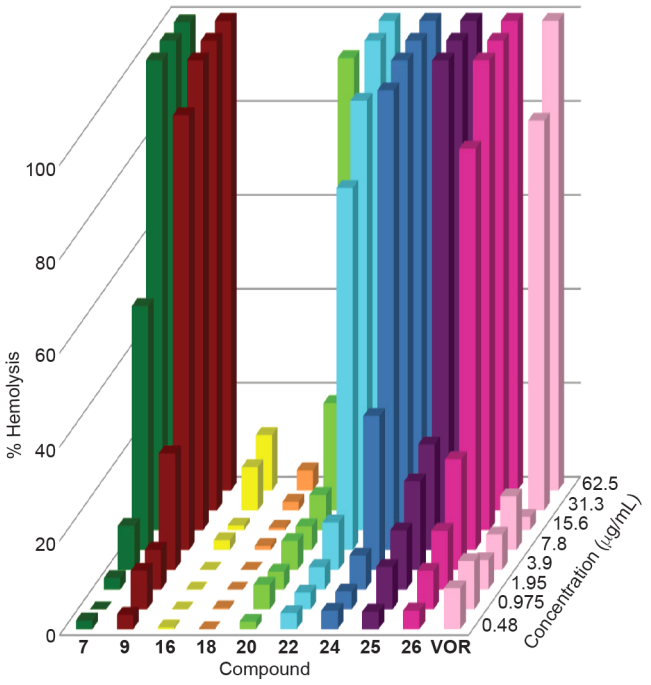

Figure 3. 3D bar graph depicting the dose-dependent hemolytic activity of azole derivatives against mouse erythrocytes. Mouse erythrocytes were treated and incubated for 1 h at 37 °C with compounds **7**, **9**, **16**, **18**, **20**, **22**, and **24**–**26**, and VOR at concentrations ranging from 0.48 to 62.5 μg/mL. Triton X-100 (1% v/v) was used as a positive control (100% hemolysis, not shown).

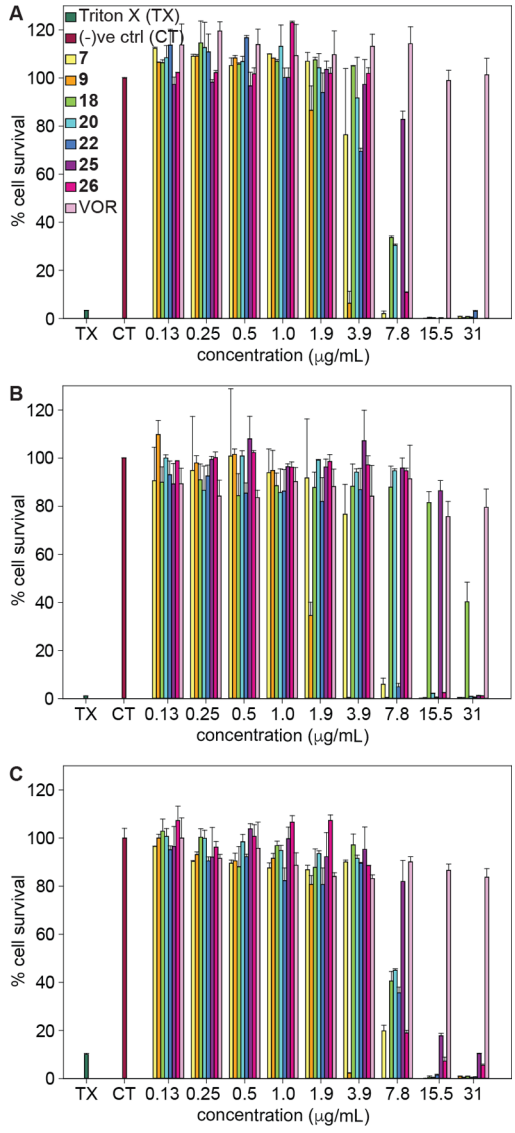

Figure 4. Representative cytotoxicity assays of piperazine and azole analogues against three mammalian cell lines: (A) HEK-293, (B) A549, and (C) BEAS-2B. Cells were treated with various concentrations of compounds **7** (yellow bars), **9** (orange bars), **18** (green bars), **20** (turquoise bars), **22** (blue bars), **25** (purple bars), **26** (hot-pink bars), and VOR (light-pink bars). The positive control consisted of cells treated with Triton X-100 (TX, 12.5% v/v). The negative control consisted of cells treated with DMSO (no drug).

antifungal MIC value (62.5 μg/mL) for FLC against this strain. When treating strain **A** with VOR, we detected a lower amount of ergosterol (**2**, 64.93%) and an increased amount of lanosterol (**1**, 18.49%) and eburicol (**3**, 2.14%) in comparison to what we observed with FLC. However, when strain **A** was treated with compound **26**, we observed a relatively low amount of ergosterol (**2**, 50.64%), as well as a higher quantity of lanosterol (**1**, 29.49%), eburicol (**3**, 1.15%), and the fungistatic metabolite 14α-methyl ergosta-8,24(28)-diene-3β,6α-diol (**4**, 11.05%). Interestingly, when strain **A** was treated with compound **20**, we detected a greater reduction in the amount of ergosterol (**2**, 30.15%) along with a related increase in lanosterol (**1**, 33.37%), eburicol (**3**, 1.25%), and the fungistatic metabolite 14α-methyl ergosta-8,24(28)-diene-3β,6α-diol (**4**, 15.40%). Finally, both compounds inhibited the ergosterol biosynthesis better than the azole drug controls FLC and VOR. These results indicated that the synthetic analogues **20** and **26** act by inhibiting the 14α-demethylase enzyme present in fungal cells, thereby affecting the ergosterol biosynthetic pathway.

Molecular Docking Study. To provide further confirmation of the inhibition of sterol 14α-demethylase with the new FLC derivatives, we performed docking studies of analogue **20** with the *C. albicans* CYP51. The synthetic analogue **20** was docked based on the crystal structure of VT-1161 with *C. albicans* CYP51 (PDB 3P99[34]) (Figure 7). The pharmacophore of **20** was unambiguously positioned based on VT-1161, and the rest of the molecule was positioned isosterically with the features of the inhibitor VT-1161. The difluorophenyl ring, triazole, and piperazine ring of the synthetic analogue **20** are isosteric with the difluorophenyl ring, tetrazole, and pyridine ring of VT-1161. The alkyl chain of **20** is isosteric with the 4-(2,2,2-trifluoroethoxy)phenyl ring of VT-1161. Even though the alkyl chain is longer, it fits perfectly into the hydrophobic region. The triazole ring is coordinated to the iron (Fe) of the heme, and the difluorophenyl ring is largely occupying the hydrophobic environment of Phe126, Ile131, Phe228, and, on the other side, Tyr132. The hydroxyl group of **20** makes water molecule mediated hydrogen bonding with the hydroxyl of Tyr132 and carboxylate group of the heme. The piperazine ring is surrounded by Leu121, Met508, and Leu376, which is favored hydrophobically. Additional hydrophobic interactions with the phenyl ring of Tyr118 was observed for the piperazine ring. Similarly to the 4-(2,2,2-trifluoroethoxy)phenyl group of VT-1161, the alkyl chain of compound **20** occupies a large solvent accessible predominantly hydrophobic pocket surrounded by Tyr64, Leu87, Leu88, Pro230, Val234, and His377. In sum, the synthetic analogue **20** exhibited many favorable interactions with the CYP51 enzyme, which further

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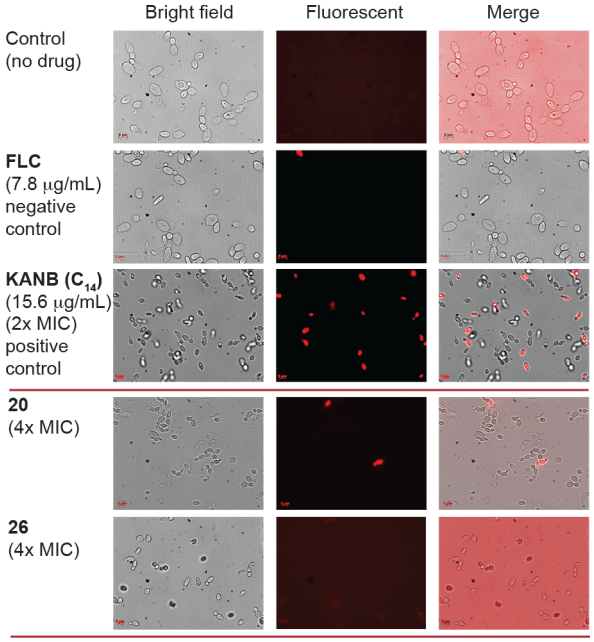

Figure 5. Effect of FLC and compounds **20** and **26** on the cell membrane integrity of *C. albicans* ATCC 10231 (strain **A**). From the top to bottom: Propidium iodide (PI) dye uptake by yeast cells without drug, with FLC (7.8 μg/mL), KANB (C_{14}) (15.6 μg/mL), compound **20** (4 × MIC), and compound **26** (4 × MIC).

support the results observed by the determination of sterol composition experiments.

Chemical and Physical Properties. FLC is known for its bioavailability, but in bulk form, it appears crystalline and is only slightly soluble in water compared to organic solvents such as DMSO where it is highly soluble. Our best compounds, **7**, **8**, **9**, **20**, **22**, **23**, **25**, and **26**, displayed similar solubility behaviors to that of FLC. The Lipinski's rule of five is often used as a starting point to evaluate drug likeness or determine if a compound with a certain pharmacological or biological activity has properties that would make it a likely active drug in humans. We calculated the Log*P* values of all the compounds (Supporting Information, Table S2). In general, an orally active drug is allowed no more than one violations of the rule of five. We investigated compounds **7**, **8**, **9**, **20**, **22**, **23**, **25**, and **26** for potential violations of the rule of five. In the case of the alkylated piperazines **7**, **8**, and **9**, we observed only one violation (their Log*P* values were greater than 5), but their molecular weights <500, not more than five hydrogen bond donors, and 10 hydrogen bond acceptors were in line with the rule of five. Similar results were observed for the alkylated piperazine-azole hybrids **20**, **22**, **23**, **25**, and **26**. Compounds **20**, **22**, **23**, and **25** exhibited <500, <5 hydrogen bond donors, and <10 hydrogen bond acceptors with Log*P* values higher than 5. Only compound **26** displayed two violations with greater than 500 molecular weight and Log*P* value greater than 5. From these observations, we can conclude that these compounds should be, in the future (outside the scope of this study), investigated in animal studies.

CONCLUSION

In summary, we have synthesized nine novel alkylated piperazine derivatives (**1**–**9**) as well as 11 alkylated piperazine-azole hybrids (**16**–**26**) along with an alkylated morpholine derivative (**10**) and two morpholine-azole hybrids (**27**–**28**) with alkyl chains of various lengths (C_1, C_5, C_8–C_{14}). We did not detect any antifungal activity with the alkylated morpholine derivative and the two morpholine-azole hybrids. We observed that the antifungal activity of alkylated piperazines and alkylated piperazine-azole hybrids depended on the length of the alkyl chains. We identified compounds **7**, **9**, **20**, **22**, **25**, and **26** as promising antifungal agents with low hemolytic activity, low cytotoxicity, and great activity against *C. albicans*, non-*albicans Candida*, and *Aspergillus* strains. In most cases, compounds **7**–**9**, **20**, **22**, **23**, **25**, and **26** displayed enhanced or comparable antifungal activity against fungal strains when compared to the commercial antifungal drugs AmB, CAS, and FLC. These compounds also exhibited superior activity compared to the control drug VOR against *Candida* biofilms. Contrary to the mechanism of action (i.e., membrane disruption) previously reported for molecules containing long alkyl chains, the compounds in this study did not disrupt the fungal membrane. Instead, the molecules studied killed the fungal cells by disrupting ergosterol biosynthesis by targeting the sterol 14α-demethylase enzyme of the ergosterol biosynthetic pathway. In the future, it will be interesting to see how these promising molecules will fare in in vivo studies.

EXPERIMENTAL SECTION

Chemistry. *Materials and Instrumentation.* All the chemicals used in this study (including compounds **1** and **12**) were purchased from Sigma-Aldrich (St. Louis, MO) or AK Scientific (Union City, CA) and used without any further purification. DMF and THF were freshly distilled prior to use. Chemical reactions were monitored by TLC (Merck, Silica gel 60 F254). Visualization was achieved using one of the following methods: iodine stain (I_2 in SiO_2 gel) or UV light. Compounds were purified by SiO_2 flash chromatography (Dynamic Adsorbents Inc., Flash SiO_2 gel 32–63 μ). ^{1}H and ^{13}C NMR spectra were recorded on a Varian 400 MHz spectrometer. Mass spectra were recorded using an Agilent 1200 series quaternary LC system equipped with a diode array detector, an Eclipse XDB-C_{18} column (250 mm × 4.6 mm, 5 μm), and an Agilent 6120 quadrupole MSD mass spectrometer. Further confirmation of purity for the final azole molecules was obtained by RP-HPLC, which was performed on an Agilent Technologies 1260 Infinity HPLC system by using the following general method 1: flow rate = 1 mL/min; λ = 254 nm; column = Vydac 201SP C18, 250 mm × 4.6 mm, 90 Å, 5 μm. Eluents: A = H_2O + 0.1% TFA, B = MeCN; gradient profile, starting from 1% B, increasing from 1% B to 100% B over 20 min, holding at 100% B from 20 to 25 min, and decreasing from 100% B to 1% B from 25 to 30 min. Prior to each injection, the HPLC column was equilibrated for 15 min with 1% B. All compounds synthesized were determined to be ≥96% pure. For compounds **19**, **21**, and **23**, the following general method 2 was used: flow rate = 0.4 mL/min; λ = 254 nm; column = Apollo C18, 250 mm × 4.6 mm, 5 μm. Eluents: A = H_2O + 0.1% formic acid, B = MeCN 0.1% formic acid; gradient profile, starting from 5% B, increasing from 5% B to 100% B over 16 min, holding at 100% B from 16 to 20 min, and decreasing from 100% B to 5% B from 20 to 30 min. All reactions were carried out under nitrogen atmosphere, and all yields reported represent isolated yields. Known compounds were characterized by ^{1}H NMR and are in complete agreement with samples reported in the literature. Compounds **2** and **7** were synthesized as previously reported.[26]

Experimental Protocols for the Preparation of Compounds 3, 4, 6–8, and 10–22. *1-Octyl Piperazine (3).* To a solution of piperazine (669 mg, 7.77 mmol) in MeCN (15 mL), 1-bromooctane (300 mg, 1.55 mmol) was added. The reaction mixture was stirred at room temperature for 12 h, and progress of the reaction was monitored by TLC (1:1/MeOH:CH_2Cl_2, R_f 0.20). The organic layer was removed under reduced pressure, and the residue was purified by flash column chromatography (SiO_2, 1:4/MeOH:CH_2Cl_2) to afford compound **3** (260 mg, 85%) as a white solid. ^{1}H NMR

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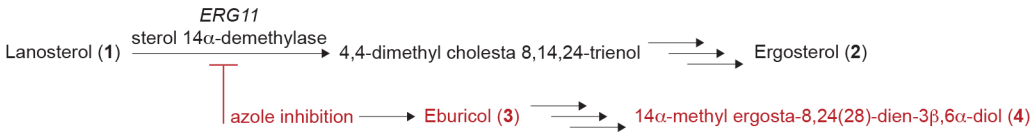
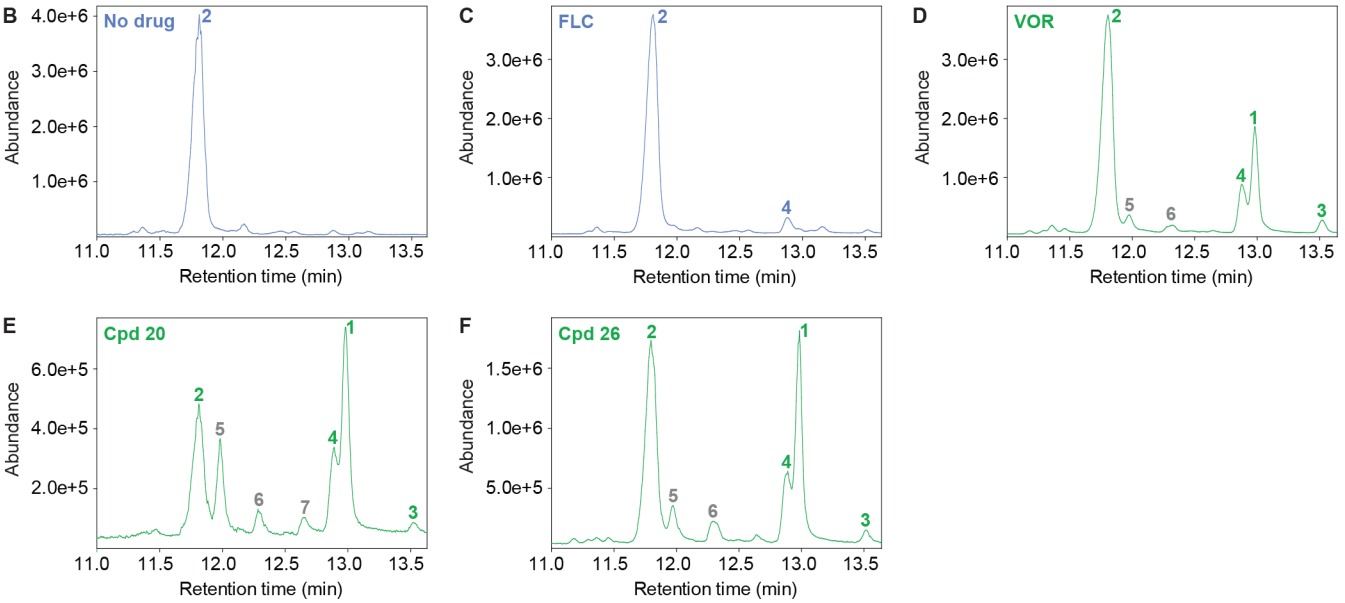
A. Simplified ergosterol biosynthetic pathway and products resulting from inhibition of *ERG11*:





G. Sterol composition of untreated and FLC-, VOR-, cpd 20-, and cpd 26-treated *C. albicans* ATCC 10231:

	Sterol composition (%)				
Sterol	**No drug**	**FLC**	**VOR**	**Cpd 20**	**Cpd 26**
Lanosterol (**1**)	ND	ND	18.49	33.37	29.49
Ergosterol (**2**)	100.00	95.90	64.93	30.15	50.64
Eburicol (**3**)	ND	ND	2.14	1.25	1.15
14α-methyl ergosta-8,24(28)-dien-3β,6α-diol (**4**)	ND	4.10	10.05	15.40	11.05
Unknown sterol (**5**)	ND	ND	2.79	14.42	3.56
Unknown sterol (**6**)	ND	ND	1.56	2.80	4.08
Unknown sterol (**7**)	ND	ND	ND	2.55	ND

ND = not detected

Figure 6. (A) A simplified ergosterol biosynthetic pathway and products resulting from inhibition of ERG11. (B–F) GC-MS chromatograms of the sterols extracted from untreated and antifungal-treated *C. albicans* ATCC 10231 (strain **A**). The fungal strain was treated with DMSO (no drug control (B)), FLC at 1.95 μg/mL (C), VOR at 0.12 μg/mL (D), compound **20** at 0.48 μg/mL (E), and compound **26** at 0.975 μg/mL (F). The peaks in these chromatograms are for lanosterol (**1**), ergosterol (**2**), eburicol (**3**), 14α-methyl ergosta-8,24(28)-dien-3β,6α-diol (**4**), and three unknown sterols (**5**–**7**). (G) Table summarizing the percentage of each sterol from B–F.

(400 MHz, CD_3OD, Supporting Information, Figure S1) δ 2.96 (t, *J* = 5.1 Hz, 4H), 2.53 (very br s, 4H), 2.37 (m, 2H), 1.53–1.47 (p, *J* = 7.2 Hz, 2H), 1.32–1.26 (m, 10H), 0.88 (t, *J* = 6.6 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD, Supporting Information, Figure S2) δ 58.4, 51.8, 44.0, 31.6, 29.1, 29.0, 27.1, 25.8, 22.3, 13.0. LRMS *m*/*z* calcd for $C_{12}H_{26}N_2$ 198.2, found 199.2 $[M + H]^+$.

*1-Nonyl Piperazine (**4**).* To a solution of piperazine (1.01 g, 12.1 mmol) in MeCN (10 mL), 1-bromononane (500 mg, 2.41 mmol) was added. The reaction mixture was stirred at room temperature for 12 h, and progress of the reaction was monitored by TLC (1:1/MeOH:CH_2Cl_2, R_f 0.21). The organic layer was removed under reduced pressure, and the residue was purified by flash column chromatography (SiO_2, 1:4/MeOH:CH_2Cl_2) to afford compound **4** (301 mg, 59%) as a white solid. ^1H NMR (400 MHz, CD_3OD, Supporting Information, Figure S3) δ 2.83 (t, *J* = 5.0 Hz, 4H), 2.43 (very br s, 4H), 2.35–2.27 (m, 2H), 1.55–1.45 (m, 2H), 1.34–1.23 (m, 12H), 0.88 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD, Supporting Information, Figure S4) δ 59.0, 53.3, 44.5, 31.6, 29.3, 29.2, 29.0, 27.3, 25.8, 22.3, 13.0. LRMS *m*/*z* calcd for $C_{13}H_{28}N_2$ 212.2, found 213.2 $[M + H]^+$.

*1-Decyl Piperazine (**5**).* To a solution of piperazine (1.95 g, 22.6 mmol) in MeCN (15 mL), 1-bromodecane (1.00 g, 4.52 mmol) and Cs_2CO_3 (736 mg, 2.26 mmol) were added. The reaction mixture was stirred at room temperature for 12 h, and progress of the reaction was monitored by TLC (1:1/MeOH:CH_2Cl_2, R_f 0.10). The organic layer was removed under reduced pressure, and the residue was purified by flash column chromatography (SiO_2, 1:4/MeOH:CH_2Cl_2) to afford compound **5** (690 mg, 67%) as a white solid. ^1H NMR (400 MHz, CD_3OD, Supporting Information, Figure S5) δ 2.91 (t, *J* = 5.1 Hz, 4H), 2.50 (very br s, 4H), 2.36 (m, 2H), 1.51 (p, *J* = 6.4 Hz, 2H), 1.32–1.26 (m, 14H), 0.90 (t, *J* = 6.7 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD, Supporting Information, Figure S6) δ 58.8, 52.9, 44.4, 31.6, 29.28, 29.26, 29.2, 29.0, 27.2, 25.8, 22.3, 13.0. LRMS *m*/*z* calcd for $C_{14}H_{30}N_2$ 226.2, found 227.2 $[M + H]^+$.

*1-Undecyl Piperazine (**6**).* To a solution of piperazine (883 mg, 10.6 mmol) in MeCN (10 mL), 1-bromoundecane (500 mg, 2.13 mmol) was added. The reaction mixture was stirred at room temperature for 12 h, and progress of the reaction was monitored by TLC (1:1/MeOH:CH_2Cl_2, R_f 0.24). The organic layer was removed under reduced pressure, and the residue was purified by flash column chromatography (SiO_2, 1:4/MeOH:CH_2Cl_2) to afford compound **6** (363 mg, 71%) as a white solid. ^1H NMR (400 MHz, CD_3OD, Supporting Information, Figure S7) δ 2.89 (t, *J* = 5.0 Hz, 4H),

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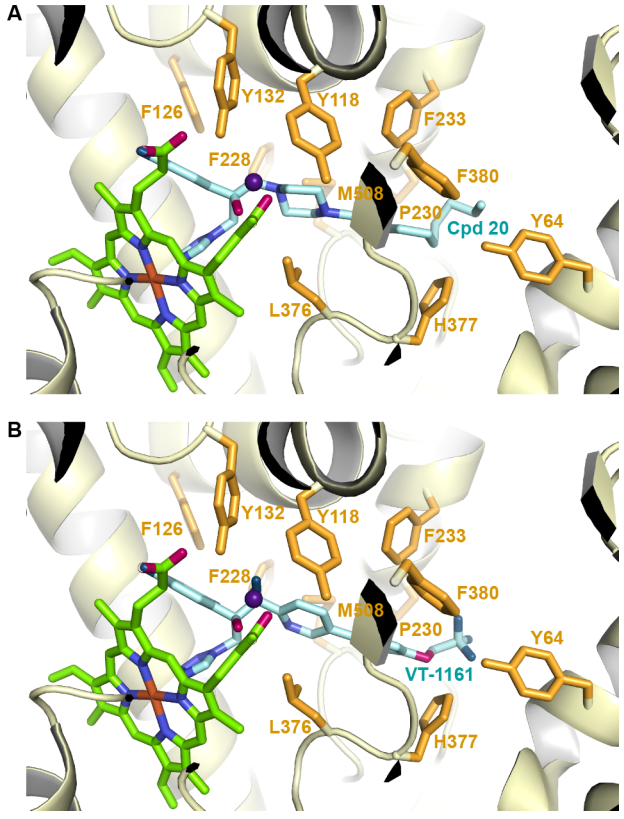

Figure 7. (A) Docking of compound **20** to the *C. albicans* sterol 14α-demethylase CYP51 (PDB 3P99[34]). (B) Co-crystal structure of VT-1161 bound to the *C. albicans* sterol 14α-demethylase CYP51 (PDB 3P99[34]). In both panels, the residues interacting with the bound molecules are shown as orange sticks. A water molecule is depicted as a purple sphere. The heme is shown as green sticks with its iron in dark orange. Compounds **20** and VT-1161 are depicted as turquoise sticks, with their oxygen, nitrogen, and fluorine atoms in red, blue, and dark turquoise, respectively.

2.48 (very br s, 4H), 2.36−2.32 (m, 2H), 1.54−1.46 (m, 2H), 1.35−1.22 (m, 16H), 0.88 (t, J = 6.6 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD, Supporting Information, Figure S8) δ 58.7, 52.4, 44.2, 31.6, 29.30 (2C), 29.27, 29.2, 29.0, 27.2, 25.8, 22.3, 13.0. LRMS m/z calcd for $C_{15}H_{32}N_2$ 240.3, found 241.2 $[M + H]^+$.

*1-Tridecyl Piperazine (**8**).* To a solution of piperazine (788 mg, 9.49 mmol) in MeCN (10 mL), 1-bromotridecane (500 mg, 1.90 mmol) was added. The reaction mixture was stirred at room temperature for 12 h, and progress of the reaction was monitored by TLC (1:1/MeOH:CH_2Cl_2, R_f 0.28). The organic layer was removed under reduced pressure, and the residue was purified by flash column chromatography (SiO_2, 1:4/MeOH:CH_2Cl_2) to afford compound **8** (301 mg, 59%) as a white solid. ^{1}H NMR (400 MHz, CD_3OD, Supporting Information, Figure S9) δ 2.83 (t, J = 5.0 Hz, 4H), 2.44 (very br s, 4H), 2.34−2.29 (m, 2H), 1.55−1.45 (m, 2H), 1.35−1.22 (m, 20H), 0.88 (t, J = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD, Supporting Information, Figure S10) δ 59.0, 53.3, 44.5, 31.7, 29.4, 29.34 (2C), 29.29, 29.27, 29.2, 29.0, 27.3, 25.8, 22.3, 13.0. LRMS m/z calcd for $C_{17}H_{36}N_2$ 268.3, found 269.2 $[M + H]^+$.

*1-Tetradecyl Piperazine (**9**).* To a solution of piperazine (1.55 g, 18.0 mmol) in MeCN (15 mL), 1-bromotetradecane (1.00 g, 3.61 mmol) and Cs_2CO_3 (588 mg, 1.81 mmol) were added. The reaction mixture was stirred at room temperature for 12 h, and progress of the reaction was monitored by TLC (1:1/MeOH:CH_2Cl_2, R_f 0.20). The organic layer was removed under reduced pressure, and the residue was purified by flash column chromatography (SiO_2, 1:4/MeOH:CH_2Cl_2) to afford compound **9** (650 mg, 64%) as a white solid. ^{1}H NMR (400 MHz, CD_3OD, Supporting Information, Figure S11) δ 2.93 (t, J = 5.1 Hz, 4H), 2.52 (very br s, 4H), 2.37 (m, 2H), 1.52 (p, J = 7.4 Hz, 2H), 1.32−1.26 (m, 20H), 0.90 (t, J = 6.5 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD, Supporting Information, Figure S12) δ 58.7, 52.6, 44.3, 31.7, 29.37, 29.35, 29.33 (2C), 29.28, 29.27, 29.2, 29.1, 27.2, 25.8, 22.3, 13.0. LRMS m/z calcd for $C_{18}H_{38}N_2$ 282.3, found 283.3 $[M + H]^+$.

*4-Tetradecyl Morpholine (**10**).* To a solution of morpholine (200 mg, 2.30 mmol) in MeCN (8 mL), 1-bromotetradecane (1.3 mL, 4.59 mmol) was added. The reaction mixture was stirred at room temperature for 12 h, and progress of the reaction was monitored by TLC (1:1/MeOH:CH_2Cl_2, R_f 0.50). The organic layer was removed under reduced pressure, and the residue was purified by flash column chromatography (SiO_2, 1:9/MeOH:CH_2Cl_2) to afford compound **10** (320 mg, 49%) as a colorless liquid. ^{1}H NMR (400 MHz, CD_3OD, Supporting Information, Figure S13) δ 3.67 (t, J = 4.7 Hz, 4H), 2.43 (br s, 4H), 2.31 (m, 2H), 1.53−1.45 (m, 2H), 1.38−1.21 (m, 22H), 0.88 (t, J = 7.0 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD, Supporting Information, Figure S14) δ 66.1, 58.8, 53.4, 31.7, 29.40, 29.38, 29.37 (2C), 29.31, 29.30, 29.2, 29.1, 27.2, 25.8, 22.3, 13.1. LRMS m/z calcd for $C_{18}H_{37}NO$ 283.3, found 284.3 $[M + H]^+$.

*2,4-Dichloro-2-(1H-3-amino-1,2,4-triazo-1-yl)acetophenone (**11**).* To a solution of 2,2′,4′-trichloroacetophenone (2.60 g, 11.6 mmol) in 2-propanol (15 mL), 4-amino-1,2,4-triazole (783 mg, 9.31 mmol) was added and the resulting mixture was refluxed for 12 h. The solvent was evaporated, and to the residue CH_2Cl_2 (15 mL) was added, and the solution was stirred for 30 min at room temperature and filtered. The solid was washed with CH_2Cl_2 (2 × 10 mL) to afford compound **11** (3.2 g, 89%) as a white solid. ^{1}H NMR (400 MHz, CD_3OD, which matches the lit.,[28] Supporting Information, Figure S15) δ 9.04 (s, 1H), 8.77 (s, 1H), 7.92 (d, J = 8.5 Hz, 1H), 7.69 (d, J = 2.0 Hz, 1H), 7.53 (dd, J_1 = 7.8 Hz, J_2 = 2.0 Hz, 1H), 4.84 (s, 2H).

*2,4-Dichloro-2-(1H-1,2,4-triazo-1-yl)acetophenone (**13**).* A solution of compound **11** (1.50 g, 4.88 mmol) in H_2O (15 mL) was cooled to 0 °C followed by the addition of conc HCl (0.5 mL) and sodium nitrite (370 mg, 5.36 mmol). The reaction mixture was then stirred at room temperature for 2 h, and the completion of the reaction was observed by TLC (1:9/MeOH:CH_2Cl_2, R_f 0.70). The reaction mixture was neutralized with a saturated $NaHCO_3$ solution, and the solid formed was filtered. The residue was purified by flash column chromatography (SiO_2, 1:9/MeOH:CH_2Cl_2) to afford compound **13** (960 mg, 80%) as a yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$ which matches the lit.,[28] Supporting Information, Figure S16) δ 8.28 (s, 1H), 7.98 (s, 1H), 7.64 (d, J = 8.4 Hz, 1H), 7.49 (d, J = 2.0 Hz, 1H), 7.36 (dd, J_1 = 8.4 Hz, J_2 = 2.0 Hz, 1H), 5.62 (s, 2H).

*1-[[2-(2,4-Difluorophenyl)-2-oxiranyl]methyl]-1H-1,2,4-triazole (**14**).* To a solution of 2,4-difuoro-2-(1*H*-1,2,4-triazo-1-yl)acetophenone (1.00 g, 4.48 mmol) in toluene (10 mL), trimethylsulfoxonium iodide (1.08 g, 4.93 mmol), hexadecyltrimethylammonium bromide (163 mg, 0.45 mmol), and 1.5 mL of 20% (v/v) sodium hydroxide was added. The reaction mixture was stirred at 60 °C for 1 h, and progress of the reaction was monitored by TLC (3:2/EtOAc:hexanes, R_f 0.35). The reaction mixture was diluted with EtOAc (20 mL) and washed with H_2O (20 mL). The organic layer was removed under reduced pressure, and the residue was purified by flash column chromatography (SiO_2, 3:2/EtOAc:hexanes) to afford compound **14** (720 mg, 68%) as a yellow gummy liquid. ^{1}H NMR (400 MHz, $CDCl_3$, Supporting Information, Figure S17) δ 8.08 (s, 1H), 7.86 (s, 1H), 7.20−7.11 (m, 1H), 6.86−6.75 (m, 2H), 4.82 (d, J = 14.8 Hz, 1H), 4.49 (d, J = 14.8 Hz, 1H), 2.93 (d, J = 4.6 Hz, 1H), 2.87 (d, J = 4.6 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$, Supporting Information, Figure S18) δ 164.4, 164.3, 161.9, 161.8, 161.7, 159.4, 159.2, 151.8, 144.1, 129.64, 129.59, 129.55, 129.49, 111.90, 111.87, 111.69, 111.65, 104.3, 104.1, 103.8, 56.3, 53.59, 53.55, 52.2. LRMS m/z calcd for $C_{11}H_9F_2N_3O$ 237.1, found 238.1 $[M + H]^+$.

*1-[[2-(2,4-Dichlorophenyl)-2-oxiranyl]methyl]-1H-1,2,4-triazole (**15**).* To a solution of 2,4-dichloro-2-(1*H*-1,2,4-triazo-1-yl)acetophenone (2.00 g, 7.81 mmol) in toluene (20 mL), trimethylsulfoxonium iodide (1.89 g, 8.59 mmol), hexadecyltrimethylammonium bromide (94 mg, 0.26 mmol), and 20% (w/w) sodium hydroxide (344 mg) were added. The reaction mixture was stirred at 60 °C for 2 h, and progress of the

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reaction was monitored by TLC (3:2/EtOAc:hexanes, R_f 0.74). The reaction mixture was diluted with EtOAc (60 mL) and washed with H_2O (20 mL) and brine (30 mL) and then dried over $MgSO_4$. The organic layer was removed under reduced pressure, and the residue was purified by flash column chromatography (SiO_2, 1:1/EtOAc:hexanes) to afford compound **15** (620 mg, 29%) as a brown liquid. ^{1}H NMR (400 MHz, $CDCl_3$, Supporting Information, Figure S19) δ 8.10 (s, 1H), 7.88 (s, 1H), 7.38 (d, J = 1.8 Hz, 1H), 7.16 (dd, J_1 = 8.2 Hz, J_2 = 1.8 Hz, 1H), 7.10 (d, J = 8.2 Hz, 1H), 4.86 (d, J = 14.9 Hz, 1H), 4.48 (d, J = 14.9 Hz, 1H), 2.96 (d, J = 4.3 Hz, 1H), 2.88 (d, J = 4.3 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$, Supporting Information, Figure S20) δ 151.8, 144.1, 135.5, 133.2, 132.7, 130.2, 129.2, 127.5, 58.5, 53.0, 52.4. LRMS m/z calcd for $C_{11}H_9Cl_2N_3O$ 269.0, found 270.0 $[M + H]^+$.

*2-(2,4-Difluorophenyl)-1-(1H-1,2,4-triazo1-yl)-3-(4-methylpiperazine)propan-2-ol (**16**).* To a solution of compound **14** (50 mg, 0.21 mmol) in EtOH (3 mL), *N*-methylpiperazine (31 mg, 0.32 mmol) and Et_3N (0.04 mL, 0.32 mmol) were added. The reaction mixture was stirred at 80 °C for 12 h, and progress of the reaction was monitored by TLC (1:9/MeOH:CH_2Cl_2, R_f 0.30). The organic layer was removed under reduced pressure, and the residue was purified by flash column chromatography (SiO_2, 1:19/MeOH:CH_2Cl_2) to afford compound **16** (55 mg, 77%) as a colorless liquid. ^{1}H NMR (400 MHz, $CDCl_3$, Supporting Information, Figure S21) δ 8.08 (s, 1H), 7.77 (s, 1H), 7.55−7.46 (m, 1H), 6.82−6.75 (m, 2H), 4.53 (d, J = 15.0 Hz, 1H), 4.49 (d, J = 15.0 Hz, 1H), 3.06 (d, J = 13.6 Hz, 1H), 2.68 (d, J = 13.6 Hz, 1H), 2.48 (m, 8H), 2.32 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, Supporting Information, Figure S22) δ 164.0, 163.9, 161.5, 161.4, 160.1, 160.0, 157.7, 157.6, 151.0, 144.6, 129.33, 129.27, 129.23, 129.18, 126.2, 126.1, 126.03, 125.00, 111.64, 111.61, 111.43, 111.41, 104.5, 104.28, 104.26, 104.0, 72.01, 71.96, 62.2, 62.1, 56.3, 56.2, 54.9, 54.0, 45.7. LRMS m/z calcd for $C_{16}H_{21}F_2N_5O$ 337.2, found 338.1 $[M + H]^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: R_t = 3.39 min (99% pure; Supporting Information, Figure S23).

*2-(2,4-Difluorophenyl)-1-(1H-1,2,4-triazol-1-yl)-3-(4-pentylpiperazine)propan-2-ol (**17**).* To a solution of compound **14** (35 mg, 0.15 mmol) in EtOH (3 mL), *N*-pentylpiperazine (34 mg, 0.22 mmol) and Et_3N (0.03 mL, 0.22 mmol) were added. The reaction mixture was stirred at 80 °C for 12 h, and progress of the reaction was monitored by TLC (1:9/MeOH:CH_2Cl_2, R_f 0.40). The organic layer was removed under reduced pressure, and the residue was purified by flash column chromatography (SiO_2, 1:19/MeOH:CH_2Cl_2) to afford compound **17** (42 mg, 71%) as a colorless liquid. ^{1}H NMR (400 MHz, CD_3OD, Supporting Information, Figure S24) δ 8.30 (s, 1H), 7.78 (s, 1H), 7.50−7.44 (m, 1H), 6.97−6.84 (m, 2H), 4.70 (d, J = 14.3 Hz, 1H), 4.65 (d, J = 14.3 Hz, 1H), 3.06 (dd, J_1 = 13.9 Hz, J_2 = 1.5 Hz, 1H), 3.05−2.86 (m, 4H), 2.83 (d, J = 13.9 Hz, 1H), 2.80−2.62 (m, 4H), 1.63 (p, J = 7.6 Hz, 2H), 1.40−1.29 (m, 6H), 0.93 (t, J = 6.9 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD, Supporting Information, Figure S25) δ 164.0, 163.9, 161.5, 157.6, 149.7, 129.51, 129.45, 129.41, 129.35, 125.6, 125.5, 110.7, 110.5, 103.7, 103.5, 103.2, 73.84, 73.79, 62.92, 62.88, 57.9, 56.00, 55.95, 53.2, 52.6, 29.2, 25.2, 22.1, 12.8. LRMS m/z calcd for $C_{20}H_{29}F_2N_5O$ 393.2, found 394.2 $[M + H]^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: R_t = 3.46 min (96% pure; Supporting Information, Figure S26).

*2-(2,4-Difluorophenyl)-1-(1H-1,2,4-triazol-1-yl)-3-(4-octylpiperazine)propan-2-ol (**18**).* To a solution of compound **14** (50 mg, 0.21 mmol) in EtOH (3 mL), compound **3** (63 mg, 0.32 mmol) and Et_3N (0.04 mL, 0.32 mmol) were added. The reaction mixture was stirred at 80 °C for 12 h, and progress of the reaction was monitored by TLC (1:9/MeOH:CH_2Cl_2, R_f 0.45). The organic layer was removed under reduced pressure, and the residue was purified by flash column chromatography (SiO_2, 1:19/MeOH:CH_2Cl_2) to afford compound **18** (48 mg, 53%) as a colorless liquid. ^{1}H NMR (400 MHz, CD_3OD, Supporting Information, Figure S27) δ 8.30 (s, 1H), 7.74 (s, 1H), 7.48−7.41 (m, 1H), 6.94−6.81 (m, 2H), 4.67 (d, J = 14.3 Hz, 1H), 4.60 (d, J = 14.3 Hz, 1H), 2.99 (dd, J_1 = 13.9 Hz, J_2 = 1.5 Hz, 1H), 2.79 (d, J = 13.9 Hz, 1H), 2.70−2.42 (m, 10H), 1.51 (m, 2H), 1.30−1.20 (m, 10H), 0.87 (t, J = 6.7 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD, Figure S28) δ 164.0, 163.9, 161.5, 161.4, 160.5, 160.4, 158.1, 158.0, 149.7, 144.6, 129.54, 129.48, 129.44, 129.38, 125.60, 125.56, 125.47, 125.43, 110.72, 110.69, 110.51, 110.48, 103.7, 103.47, 103.45, 103.2, 74.0, 73.9, 62.9, 62.8, 57.7, 56.0. 55.92, 52.88, 52.5, 31.5, 29.0, 28.9, 26.9, 25.2, 22.3, 13.0. LRMS m/z calcd for $C_{23}H_{35}F_2N_5O$ 435.3, found 436.2 $[M + H]^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: R_t = 3.39 min (98% pure; Supporting Information, Figure S29).

*2-(2,4-Difluorophenyl)-1-(1H-1,2,4-triazol-1-yl)-3-(4-nonylpiperazine)propan-2-ol (**19**).* To a solution of compound **14** (100 mg, 0.42 mmol) in EtOH (5 mL), compound **4** (134 mg, 0.63 mmol) and Et_3N (0.09 mL, 0.63 mmol) were added. The reaction mixture was stirred at 80 °C for 12 h, and progress of the reaction was monitored by TLC (1:19/MeOH:CH_2Cl_2, R_f 0.35). The organic layer was removed under reduced pressure, and the residue was purified by flash column chromatography (SiO_2, 1:19/MeOH:CH_2Cl_2) to afford compound **19** (147 mg, 78%) as a yellow liquid. ^{1}H NMR (400 MHz, CD_3OD, Supporting Information, Figure S30) δ 8.31 (s, 1H), 7.72 (s, 1H), 7.48−7.39 (m, 1H), 6.93−6.78 (m, 2H), 4.66 (d, J = 14.2 Hz, 1H), 4.57 (d, J = 14.2 Hz, 1H), 2.97 (dd, J_1 = 13.8 Hz, J_2 = 1.6 Hz, 1H), 2.77 (d, J = 13.8 Hz, 1H), 2.50−2.42 (m, 4H), 2.42−2.32 (m, 4H), 2.29−2.24 (m, 2H), 1.48−1.41 (m, 2H), 1.33−1.22 (m, 12H), 0.87 (t, J = 6.7 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD, Supporting Information, Figure S31) δ 164.0, 163.9, 161.5, 161.4, 160.5, 160.4, 158.1, 158.0, 149.6, 144.6, 129.5, 129.41, 129.38, 129.3, 125.83, 125.80, 125.7, 110.7, 110.6, 110.5, 110.4, 103.7, 103.4, 103.2, 73.6, 73.5, 63.04, 63.0, 58.3, 56.1, 56.0, 53.8, 52.8, 31.6, 29.20, 29.18, 28.9, 27.2, 25.9, 22.3, 13.0. LRMS m/z calcd for $C_{26}H_{41}F_2N_5O$ 449.3, found 450.2 $[M + H]^+$. Purity of the compound was further confirmed by RP-HPLC by using method 2: R_t = 15.41 min (99% pure; Supporting Information, Figure S32).

*2-(2,4-Difluorophenyl)-1-(1H-1,2,4-triazol-1-yl)-3-(4-decylpiperazine)propan-2-ol (**20**).* To a solution of compound **14** (50 mg, 0.21 mmol) in EtOH (3 mL), compound **5** (72 mg, 0.32 mmol) and Et_3N (0.04 mL, 0.32 mmol) were added. The reaction mixture was stirred at 80 °C for 12 h, and progress of the reaction was monitored by TLC (1:9/MeOH:CH_2Cl_2, R_f 0.40). The organic layer was removed under reduced pressure, and the residue was purified by flash column chromatography (SiO_2, 1:19/MeOH:CH_2Cl_2) to afford compound **20** (71 mg, 73%) as a yellow liquid. ^{1}H NMR (400 MHz, CD_3OD, Supporting Information, Figure S33) δ 8.33 (s, 1H), 7.75 (s, 1H), 7.49−7.43 (m, 1H), 6.95−6.81 (m, 2H), 4.68 (d, J = 14.3 Hz, 1H), 4.59 (d, J = 14.3 Hz, 1H), 2.98 (dd, J_1 = 13.8 Hz, J_2 = 1.6 Hz, 1H), 2.79 (d, J = 14.3 Hz, 1H), 2.52−2.45 (m, 4H), 2.45−2.35 (m, 4H), 2.31−2.27 (m, 2H), 1.50−1.44 (m, 2H), 1.33−1.28 (m, 14H), 0.89 (t, J = 6.7 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD, Supporting Information, Figure S34) δ 164.0, 163.9, 161.5, 161.4, 160.6, 160.4, 158.1, 158.0, 149.6, 144.6, 129.5, 129.42, 129.39, 125.82, 125.79, 125.70, 125.66, 110.68, 110.65, 110.5, 110.4, 103.7, 103.4, 103.2, 73.6, 73.5, 63.04, 63.01, 58.3, 56.1, 56.0, 53.7, 52.8, 31.6, 29.26, 29.24, 29.18, 29.0, 27.2, 25.9, 22.3, 13.0. LRMS m/z calcd for $C_{25}H_{39}F_2N_5O$ 463.3, found 464.3 $[M + H]^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: R_t = 3.32 min (96% pure; Supporting Information, Figure S35).

*2-(2,4-Difluorophenyl)-1-(1H-1,2,4-triazol-1-yl)-3-(4-undecylpiperazine)propan-2-ol (**21**).* To a solution of compound **14** (100 mg, 0.42 mmol) in EtOH (5 mL), compound **6** (152 mg, 0.63 mmol) and Et_3N (0.09 mL, 0.63 mmol) were added. The reaction mixture was stirred at 80 °C for 12 h, and progress of the reaction was monitored by TLC (1:19/MeOH:CH_2Cl_2, R_f 0.44). The organic layer was removed under reduced pressure, and the residue was purified by flash column chromatography (SiO_2, 1:19/MeOH:CH_2Cl_2) to afford compound **21** (146 mg, 73%) as a yellow liquid. ^{1}H NMR (400 MHz, CD_3OD, Supporting Information, Figure S36) δ 8.31 (s, 1H), 7.72 (s, 1H), 7.47−7.41 (m, 1H), 6.93−6.78 (m, 2H), 4.66 (d, J = 14.2 Hz, 1H), 4.57 (d, J = 14.2 Hz, 1H), 2.97 (dd, J_1 = 13.8 Hz, J_2 = 1.6 Hz, 1H), 2.77 (d, J = 13.8 Hz, 1H), 2.50−2.43 (m, 4H), 2.42−2.32 (m, 4H), 2.29−2.24 (m, 2H), 1.48−1.40 (m, 2H), 1.33−1.22 (m, 16H), 0.87 (t, J = 6.7 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD,

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Supporting Information, Figure S37) δ 164.0, 163.9, 161.5, 161.4, 160.5, 160.4, 158.1, 158.0, 149.6, 144.6, 129.5, 1289.41, 128.38, 129.32, 125.8, 125.7, 110.7, 110.6, 110.4, 110.0, 103.7, 103.4, 103.2, 73.6, 73.5, 63.04, 62.99, 58.3, 56.1, 56.0, 53.7, 52.8, 31.6, 29.28, 29.27, 29.23, 29.17, 29.0, 27.2, 25.9, 22.3, 13.0. LRMS *m/z* calcd for $C_{26}H_{41}F_2N_5O$ 477.3, found 478.3 $[M + H]^+$. Purity of the compound was further confirmed by RP-HPLC by using method 2: R_t = 16.06 min (96% pure; Supporting Information, Figure S38).

2-(2,4-Difluorophenyl)-1-(1H-1,2,4-triazol-1-yl)-3-(4-dodecylpiperazine)propan-2-ol (**22**). To a solution of compound **14** (50 mg, 0.21 mmol) in EtOH (3 mL), compound **7** (81 mg, 0.32 mmol) and Et_3N (0.04 mL, 0.32 mmol) were added. The reaction mixture was stirred at 80 °C for 12 h, and progress of the reaction was monitored by TLC (1:9/MeOH:CH_2Cl_2, R_f 0.50). The organic layer was removed under reduced pressure, and the residue was purified by flash column chromatography (SiO_2, 1:19/MeOH:CH_2Cl_2) to afford compound **22** (80 mg, 80%) as a colorless liquid. ^{1}H NMR (400 MHz, $CDCl_3$, Supporting Information, Figure S39) δ 8.10 (s, 1H), 7.76 (s, 1H), 7.53−7.46 (m, 1H), 6.83−6.74 (m, 2H), 4.50 (s, 2H), 3.05 (d, *J* = 13.6 Hz, 1H), 2.65 (d, *J* = 13.6 Hz, 1H), 2.60−2.28 (m, 8H), 1.45−1.43 (m, 2H), 1.28−1.19 (m, 20H), 0.85 (t, *J* = 6.6 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, Supporting Information, Figure S40) δ 164.0, 163.9, 161.5, 161.4, 160.1, 160.0, 157.7, 157.6, 151.0, 144.6, 129.34, 129.28, 129.24, 129.19, 126.19, 126.16, 126.1, 126.0, 111.62, 111.59, 111.41, 111.38, 104.5, 104.3, 104.2, 104.0, 72.01, 71.95, 62.22, 62.18, 58.5, 56.31, 56.26, 54.0, 53.1, 31.9, 29.59, 29.57, 29.53, 29.49, 29.4, 29.3, 27.4, 26.5, 22.6, 14.1. LRMS *m/z* calcd for $C_{27}H_{43}F_2N_5O$ 491.3, found 492.3 $[M + H]^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: R_t = 3.12 min (97% pure; Supporting Information, Figure S41).

2-(2,4-Difluorophenyl)-1-(1H-1,2,4-triazol-1-yl)-3-(4-tridecylpiperazine)propan-2-ol (**23**). To a solution of compound **14** (100 mg, 0.42 mmol) in EtOH (5 mL), compound **8** (169 mg, 0.63 mmol) and Et_3N (0.09 mL, 0.63 mmol) were added. The reaction mixture was stirred at 80 °C for 12 h, and progress of the reaction was monitored by TLC (1:19/MeOH:CH_2Cl_2, R_f 0.48). The organic layer was removed under reduced pressure, and the residue was purified by flash column chromatography (SiO_2, 1:19/MeOH:CH_2Cl_2) to afford compound **23** (132 mg, 62%) as a yellow liquid. ^{1}H NMR (400 MHz, CD_3OD, Supporting Information, Figure S42) δ 8.31 (s, 1H), 7.72 (s, 1H), 7.47−7.40 (m, 1H), 6.93−6.79 (m, 2H), 4.66 (d, *J* = 14.2 Hz, 1H), 4.57 (d, *J* = 14.2 Hz, 1H), 2.97 (d, *J* = 13.8 Hz, 1H), 2.77 (d, *J* = 13.8 Hz, 1H), 2.50−2.45 (m, 4H), 2.45−2.32 (m, 4H), 2.30−2.24 (m, 2H), 1.48−1.39 (m, 2H), 1.33−1.20 (m, 20H), 0.87 (t, *J* = 6.6 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD, Supporting Information, Figure S43) δ 164.0, 163.9, 161.5, 161.4, 160.6, 160.4, 158.1, 158.0, 149.6, 144.6, 129.5, 129.42, 129.38, 129.32, 125.83, 125.79, 125.70, 125.67, 110.7, 110.6, 110.5, 110.4, 103.7, 103.44, 103.42, 103.2, 73.6, 73.5, 63.04, 63.00, 58.3, 56.1, 56.0, 53.8, 52.8, 31.6, 29.32, 29.30, 29.24, 29.21, 29.15, 29.0, 27.2, 25.9, 22.3, 13.0. LRMS *m/z* calcd for $C_{28}H_{45}F_2N_5O$ 505.4, found 506.3 $[M + H]^+$. Purity of the compound was further confirmed by RP-HPLC by using method 2: R_t = 16.82 min (97% pure; Supporting Information, Figure S44).

2-(2,4-Difluorophenyl)-1-(1H-1,2,4-triazol-1-yl)-3-(4-tetradecylpiperazine)propan-2-ol (**24**). To a solution of compound **14** (50 mg, 0.21 mmol) in EtOH (3 mL), compound **9** (89 mg, 0.32 mmol), and Et_3N (0.04 mL, 0.32 mmol) were added. The reaction mixture was stirred at 80 °C for 12 h, and progress of the reaction was monitored by TLC (1:9/MeOH:CH_2Cl_2, R_f 0.40). The organic layer was removed under reduced pressure, and the residue was purified by flash column chromatography (SiO_2, 1:19/MeOH:CH_2Cl_2) to afford compound **24** (91 mg, 83%) as a yellow solid. ^{1}H NMR (400 MHz, CD_3OD, Supporting Information, Figure S45) δ 8.33 (s, 1H), 7.75 (s, 1H), 7.49−7.43 (m, 1H), 6.95−6.81 (m, 2H), 4.68 (d, *J* = 14.3 Hz, 1H), 4.59 (d, *J* = 14.3 Hz, 1H), 2.98 (dd, J_1 = 13.8 Hz, J_2 = 1.6 Hz, 1H), 2.79 (d, *J* = 13.8 Hz, 1H), 2.55−2.45 (m, 4H), 2.45−2.32 (m, 4H), 2.31−2.27 (m, 2H), 1.46 (p, *J* = 7.4 Hz, 2H), 1.34−1.28 (m, 22H), 0.90 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD, Supporting Information, Figure S46) δ 164.0, 163.9, 161.5, 161.4, 160.6, 160.4, 158.1, 158.0, 150.7, 149.6, 144.6, 129.5, 129.42, 129.39, 129.3, 125.83, 125.80, 125.70, 125.67, 110.69, 110.65, 110.5, 110.4, 103.7, 103.5, 103.2, 73.6, 73.5, 63.1, 63.0, 58.3, 56.1, 56.0, 53.8, 52.8, 31.7, 29.36, 29.34, 29.32, 29.26, 29.24, 29.20, 29.18, 29.06, 27.2, 26.0, 22.3, 13.0. LRMS *m/z* calcd for $C_{29}H_{47}F_2N_5O$ 519.4, found 520.3 $[M + H]^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: R_t = 3.47 min (96% pure; Supporting Information, Figure S47).

2-(2,4-Dichlorophenyl)-1-(1H-1,2,4-triazol-1-yl)-3-(4-decylpiperazine)propan-2-ol (**25**). To a solution of compound **15** (50 mg, 0.19 mmol) in EtOH (3 mL), compound **5** (55 mg, 0.24 mmol) and Et_3N (0.03 mL, 0.24 mmol) were added. The reaction mixture was stirred at 80 °C for 12 h, and progress of the reaction was monitored by TLC (1:9/MeOH:CH_2Cl_2, R_f 0.40). The organic layer was removed under reduced pressure, and the residue was purified by flash column chromatography (SiO_2, 1:19/MeOH:CH_2Cl_2) to afford compound **25** (48 mg, 52%) as a yellow liquid. ^{1}H NMR (400 MHz, CD_3OD, Supporting Information, Figure S48) δ 8.31 (s, 1H), 7.74 (s, 1H), 7.61 (d, *J* = 8.6 Hz, 1H), 7.42 (d, *J* = 2.2 Hz, 1H), 7.20 (dd, J_1 = 8.6 Hz, J_2 = 2.2 Hz, 1H), 4.97 (d, *J* = 14.3 Hz, 1H), 4.67 (d, *J* = 14.3 Hz, 1H), 3.41 (d, *J* = 14.0 Hz, 1H), 2.83 (d, *J* = 14.0 Hz, 1H), 2.75−2.35 (m, 10H), 1.55−1.45 (m, 2H), 1.35−1.21 (m, 14H), 0.87 (t, *J* = 6.6 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD, Supporting Information, Figure S49) δ 149.7, 144.8, 138.4, 133.9, 131.5, 130.3, 130.0, 126.8, 74.9, 61.2, 57.5, 54.9, 52.3, 31.6, 29.3, 29.2, 29.1, 29.01, 28.97, 26.8, 24.9, 22.3, 13.0. LRMS *m/z* calcd for $C_{25}H_{39}Cl_2N_5O$ 495.3, found 496.3 $[M + H]^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: R_t = 3.45 min (97% pure; Supporting Information, Figure S50).

2-(2,4-Dichlorophenyl)-1-(1H-1,2,4-triazol-1-yl)-3-(4-dodecylpiperazine)propan-2-ol (**26**). To a solution of compound **15** (50 mg, 0.19 mmol) in EtOH (3 mL), compound **7** (61 mg, 0.24 mmol) and Et_3N (0.03 mL, 0.24 mmol) were added. The reaction mixture was stirred at 80 °C for 12 h, and progress of the reaction was monitored by TLC (1:9/MeOH:CH_2Cl_2, R_f 0.50). The organic layer was removed under reduced pressure, and the residue was purified by flash column chromatography (SiO_2, 1:19/MeOH:CH_2Cl_2) to afford compound **26** (44 mg, 45%) as a yellow liquid. ^{1}H NMR (400 MHz, CD_3OD, Supporting Information, Figure S51) δ 8.31 (s, 1H), 7.74 (s, 1H), 7.60 (d, *J* = 8.6 Hz, 1H), 7.42 (d, *J* = 2.1 Hz, 1H), 7.20 (dd, J_1 = 8.6 Hz, J_2 = 2.1 Hz, 1H), 4.98 (d, *J* = 14.3 Hz, 1H), 4.68 (d, *J* = 14.3 Hz, 1H), 3.43 (d, *J* = 14.0 Hz, 1H), 2.86 (d, *J* = 14.0 Hz, 1H), 2.78−2.43 (m, 10H), 1.58−1.51 (m, 2H), 1.35−1.18 (m, 18H), 0.87 (t, *J* = 6.4 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD, Supporting Information, Figure S52) δ 149.8, 144.7, 138.3, 133.9, 131.5, 130.3, 130.1, 126.8, 75.1, 61.2, 57.3, 54.9, 52.1, 52.0, 31.6, 29.3 (2C), 29.2, 29.1, 29.0, 28.9, 26.6, 24.6, 22.3, 13.0. LRMS *m/z* calcd for $C_{27}H_{43}Cl_2N_5O$ 523.3, found 524.3 $[M + H]^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: R_t = 3.46 min (98% pure; Supporting Information, Figure S53).

2-(2,4-Difluorophenyl)-1-(1H-1,2,4-triazol-1-yl)-3-(mopholine)-propan-2-ol (**27**). To a solution of compound **14** (50 mg, 0.21 mmol) in EtOH (3 mL), morpholine (0.073 mL, 0.32 mmol) and Et_3N (0.04 mL, 0.32 mmol) were added. The reaction mixture was stirred at 80 °C for 12 h, and progress of the reaction was monitored by TLC (1:10/MeOH: CH_2Cl_2, R_f 0.02). The organic layer was removed under reduced pressure, and the residue was purified by column chromatography (SiO_2, 1:20/MeOH: CH_2Cl_2) to afford compound **27** (60 mg, 88%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$, Supporting Information, Figure S54) δ 8.12 (s, 1H), 7.77 (s, 1H), 7.55−7.53 (m, 1H), 6.82−6.76 (m, 2H), 5.11 (d, *J* = 8.2 Hz, 1H), 4.55 (d, *J* = 14.2 Hz, 1H), 4.48 (d, *J* = 14.2 Hz, 1H), 3.55 (m, 4H), 3.07 (d, *J* = 13.6 Hz, 1H), 2.61 (d, *J* = 12.8 Hz, 1H), 2.32 (m, 4H), 1.60 (br s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$, Supporting Information, Figure S55) δ 164.0, 163.9, 161.6, 161.4, 160.1, 160.0, 157.7, 157.6, 151.1, 144.7, 129.35, 129.29, 129.26, 129.20, 111.8, 111.7, 111.6, 111.5, 104.6, 104.3, 104.1, 72.2, 72.1, 66.8, 63.0, 62.9, 56.2, 56.1, 54.7. LRMS *m/z* calcd for $C_{15}H_{18}F_2N_4O_2$ 324.1, found 325.1 $[M + H]^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: R_t = 2.85 min (99% pure; Supporting Information, Figure S56).

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*2-(2,4-Difluorophenyl)-1-(1H-1,2,4-triazol-1-yl)-3-(4-(2-aminoethyl)morpholine)propan-2-ol (**28**).* To a solution of compound **14** (50 mg, 0.21 mmol) in EtOH (3 mL), 4-(2-aminoethyl)-morpholine (41 mg, 0.32 mmol) and Et_3N (0.04 mL, 0.32 mmol) were added. The reaction mixture was stirred at 80 °C for 12 h, and progress of the reaction was monitored by TLC (1:9/MeOH:CH_2Cl_2, R_f 0.20). The organic layer was removed under reduced pressure, and the residue was purified by flash column chromatography (SiO_2, 1:19/MeOH:CH_2Cl_2) to afford compound **28** (60 mg, 78%) as a colorless liquid. ^{1}H NMR (400 MHz, CD_3OD, Supporting Information, Figure S57) δ 8.32 (s, 1H), 7.75 (s, 1H), 7.51−7.44 (m, 1H), 6.96−6.81 (m, 2H), 4.66 (d, J = 14.3 Hz, 1H), 4.61 (d, J = 14.3 Hz, 1H), 3.62 (t, J = 4.7 Hz, 4H), 3.22 (d, J = 12.8 Hz, 1H), 2.97 (d, J = 12.8 Hz, 1H), 2.70 (td, J_1 = 12.5 Hz, J_2 = 6.1 Hz, 1H), 2.66 (td, J_1 = 12.5 Hz, J_2 = 6.1 Hz, 1H), 2.42−2.36 (m, 4H). ^{13}C NMR (100 MHz, CD_3OD, Supporting Information, Figure S58) δ 164.1, 163.9, 161.6, 161.5, 160.6, 160.5, 158.2, 158.1, 149.8, 144.6, 130.1, 130.04, 130.00, 129.9, 124.9, 124.84, 124.75, 124.7, 110.8, 110.7, 110.6, 110.5, 103.8, 103.57, 103.55, 103.3, 74.2, 74.1, 66.30, 66.25, 57.0, 55.9, 55.8, 54.53, 54.49, 53.2, 53.1, 45.3. LRMS m/z calcd for $C_{17}H_{23}F_2N_5O_2$ 367.2, found 368.1 $[M + H]^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: R_t = 3.14 min (96% pure; Supporting Information, Figure S59).

Biological Studies. *Antifungal Agents.* Azole derivatives **1**−**28** were purchased or chemically synthesized as described above. A 5 mg/mL stock solution of compounds **1**−**28** was prepared in DMSO and stored at −20 °C in the dark (foiled wrapped). The antifungal agents amphotericin B (AmB), fluconazole (FLC), and voriconazole (VOR) were obtained from AK Scientific Inc. (Mountain View, CA). The antifungal agent caspofungin (CAS) was purchased from Sigma-Aldrich (St. Louis, MO). AmB, FLC, VOR, and CAS were dissolved in DMSO at final concentrations of 5 mg/mL and were stored at −20 °C.

Organisms and Culture Conditions. *Candida albicans* ATCC 10231 (**A**), *C. albicans* ATCC 64124 (**B**), and *C. albicans* ATCC MYA-2876 (**C**) were kindly provided by Dr. Jon Y. Takemoto (Utah State University, Logan, UT, USA). *C. albicans* ATCC 90819 (**D**), *C. albicans* ATCC MYA-2310 (**E**), *C. albicans* ATCC MYA-1237 (**F**), *C. albicans* ATCC MYA-1003 (**G**), *Candida glabrata* ATCC 2001 (**H**), *Candida krusei* ATCC 6258 (**I**), *Candida parapsilosis* ATCC 22019 (**J**), *Aspergillus flavus* ATCC MYA-3631 (**K**), and *Aspergillus terreus* ATCC MYA-3633 (**M**) were obtained from the American Type Culture Collection (Manassas, VA, USA). *Aspergillus nidulans* ATCC 38163 (**L**) was received from Dr. Jon S. Thorson (University of Kentucky, Lexington, KY, USA). Filamentous fungi and yeasts were cultivated at 35 °C in RPMI 1640 medium (with L-glutamine, without sodium bicarbonate, Sigma-Aldrich, St. Louis, MO) buffered to a pH of 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma-Aldrich).

The human embryonic kidney cell line HEK-293 (ATCC CRL-1573), the human bronchus normal cell line BEAS-2B (ATCC CRL-9609), and the human lung carcinoma cell line A549 (ATCC CRL-185) were kind gifts from the laboratories of Dr. Matthew S. Gentry (University of Kentucky, Lexington, KY, USA) and Dr. David K. Orren (University of Kentucky, Lexington, KY, USA). The mammalian cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (ATCC, Manassas, VA, USA) with 10% fetal bovine serum (FBS) (ATCC, Manasas, VA, USA) and 1% Pen/Strep (ATCC, Manassas, VA, USA). Cell lines were cultured at 37 °C with 5% CO_2 and passaged by trypsinization with 0.05% trypsin−0.53 mM EDTA (ATCC, Manassas, VA, USA). Cell confluency was determined by using a Nikon Eclipse TS100 microscope (Minato, Tokyo, Japan).

MIC Value Determination by in Vitro Antifungal Assay. The MIC values of compounds **1**−**10**, **14**, and **16**−**28** against yeast cells were determined in 96-well plates as described in the CLSI document M27-A3 with minor modifications.[35] A single colony of freshly prepared yeast cells was used to inoculate 5 mL of yeast extract peptone dextrose broth (YPD) and incubated overnight with shaking at 200 rpm at 35 °C. Then 100 μL of actively growing yeast culture was transferred to 900 μL of sterile ddH_2O and readjusted to achieve OD_{600} of 0.12 (∼1 × 10^6 CFU/mL). The cell suspension was further diluted to achieve 2−4 × 10^3 CFU/mL in RPMI 1640 medium. Then 100 μL of cells (to achieve a final concentration of 1−2 × 10^3 CFU/mL) were added to the wells of a 96-well microtiter plates that contained 0.015−31.3 μg/mL of compounds **1**−**10**, **14**, and **16**−**28**, 0.975−62.5 μg/mL of FLC, 0.03−31.3 μg/mL of VOR, 0.48−31.3 μg/mL of AmB, or 0.03−31.3 μg/mL of CAS and incubated for 48 h at 35 °C. Each test was performed in triplicate. The final concentration of DMSO was ensured to be <1.25% in all experiments. The MIC values for compounds **1**−**10**, **14**, and **16**−**28**, AmB, and CAS were defined as the minimum drug concentration that yielded complete inhibition (also known as MIC-0). For FLC and VOR, the minimum drug concentration that yielded at least 50% growth inhibition (MIC-2) when compared with the growth control well was reported. One exception for the reporting of the MIC of the azoles was that of VOR against *C. albicans* ATCC 10231 (strain **A**), where the MIC-0 (indicating complete inhibition) was reported. These data are presented in Table 1.

Similarly, the MIC values of compounds **1**−**10**, **14**, and **16**−**28**, as well as that of all control drugs against filamentous fungi (strains **K**, **L**, and **M**) were determined as previously described in CLSI document M38-A2.[36] Spores were harvested from sporulating cultures growing on potato dextrose agar (PDA) by filtration through sterile glass wool and enumerated by using a hemocytometer to obtain the desired inoculum size. Serial dilutions of compounds **1**−**10**, **14**, and **16**−**28**, as well as VOR and CAS were made in sterile 96-well microplates in the ranges of 0.03−31.3 μg/mL in RPMI medium. The concentration ranges for FLC and AmB were 0.975−62.5 and 0.48−31.3 μg/mL, respectively. Spore suspensions were added to the wells to afford a final concentration of 5 × 10^5 CFU/mL. The plates were incubated at 35 °C for 48 h. The MIC values of compounds **1**−**10**, **14**, and **16**−**28**, azoles, AmB, and CAS against filamentous fungi were based on the complete inhibition of growth when compared to the growth control (MIC-0). Each test was performed in triplicate. These data are also presented in Table 1.

MIC Value Determination by in Vitro Antifungal Assay in the Presence of FBS. To investigate the effect of presence of serum on the MIC values of our novel azole derivatives, we selected compounds **7**, **9**, **20**, **22**, **25**, and **26**, with an even number of carbons in their side chain as representatives from here onward as they displayed strong activity in MIC assays and provide coverage of the range of chain lengths utilized. The procedures described in the Structure−Activity Relationship (SAR) Summary were utilized, and the RPMI 1640 medium or RPMI medium were supplemented with 10% fetal bovine serum (FBS) in parallel against *C. albicans* ATCC 10231 (strain **A**), *C. parapsilosis* ATCC 22019 (strain **J**), and *A. nidulans* ATCC 38163 (strain **L**). These data are presented in Table 2.

Time-Kill Assays. The time-kill curve analyses were performed using a previously published protocol.[37] The *Candida* strains, *C. albicans* ATCC 10231 (strain **A**), *C. glabrata* ATCC 2001 (strain **H**), and *C. parapsilosis* ATCC 22019 (strain **J**) were grown in culture tubes with YPD broth overnight. Afterward, from the culture tubes, volumes of 10−20 μL of cells were added to Eppendorf tubes and diluted with the appropriate amounts of RPMI 1640 medium (990−980 μL) to make up the working stocks with final volume of 1000 μL and OD_{600} of 0.12−0.13. The cell suspension working stocks were then thoroughly vortexed, and 300 μL of cells were added to new culture tubes, which were used for the time-kill experiments. The sterile controls consisted of culture tubes with 4000 μL of RPMI 1640 medium and no fungal cell. The untreated or growth controls were added to the aforementioned 300 μL of cell suspensions from the working stocks and 3700 μL of RPMI 1640 medium. Because of the low MIC values (0.015−0.975 μg/mL) of the tested compounds, the stock solutions of compounds **20** and **22** were prepared at 5000 or 1000 μg/mL. To perform the time-kill experiments with the drugs, tubes contained 300 μL of cells, the appropriate amounts of RPMI medium, and compounds **20** and **22** at concentrations of 1×, 4×, and 8× their respective MIC values to make up final volumes of 4000 μL. For the VOR controls, the tubes contained 300 μL of cells, the appropriate amount of RPMI medium, and VOR at concentrations of 1× and

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4× its MIC values. Once the culture tubes were prepared, they were incubated at 35 °C and agitated at 200 rpm for 24 h. At 0, 3, 6, 9, 12, and 24 h time points, 100 μL of cells from each tube were serially diluted with RPMI 1640 medium to the appropriate dilutions, which ranged from 10^2−10^7 dilutions depending on the turbidity and incubation time of each culture tube. After serial dilutions, the cells were plated onto PDA plates. The initial inoculum (at time 0 h) was determined to be between 1 and 4 × 10^5 CFU/mL. After 48 h incubation, fungal colonies on the plates were visible, counted, and reported. It is important to note that strain **A** was unique in the sense that the colonies grew very rapidly and thus, these plates were counted after 24 h incubation. The experiments were performed in duplicate. The detection limit of this assay was 100 CFU/mL. These data are presented in Figure 2.

Biofilm Assays. We investigated the biofilm reduction activity of our compounds against the azole-resistant *C. albicans* ATCC 64124 (strains **B**) by 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide [XTT] reduction assay with minor modification.[38] Briefly, *C. albicans* ATCC 64124 (strain **B**) was grown overnight in yeast peptone dextrose broth (YPDB) at 35 °C with agitation at 200 rpm. The culture was then diluted to an OD_{600} of 0.1 (~10^6 CFU/mL), and 100 μL was transferred into each well of a 96-well plate. The cells were grown statically at 37 °C for 24 h. The following day, all the spent medium containing planktonic cells were aspirated and each well was carefully washed three times with sterile PBS buffer (10 mM phosphate buffered saline, pH 7.2). Freshly prepared RPMI 1640 medium (100 μL) was added into each well containing serially diluted compounds **7**, **9**, **20**, **22**, **25**, and **26** and a positive control (VOR) at the final concentrations of 0.12−62.5 μg/mL, except for the wells that contains medium only (no drug and no biofilms) and biofilms alone (no drug). The plates were further incubated at 37 °C statically for an additional 24 h. After 24 h, the plates were carefully washed with sterile PBS. Then 100 μL of XTT (0.5 mg/mL)/menadione (1 μM) solution was added to each well of the plates, covered with aluminum foil, and incubated for 2 h at 37 °C. Then, 80 μL of the colored supernatant from each well was transferred to a new microtiter plate and the absorbance was read at 490 nm. The percent inhibition of metabolic activity of biofilms for each well containing drug was calculated by comparing the metabolic activity of biofilms formed in the absence of drug (growth control). The sessile minimum inhibitory concentrations, $SMIC_{50}$ and $SMIC_{80}$, were defined as the drug concentrations at which a 50% and 80% decrease in optical density was observed when compared to the growth control. The experiments were performed in duplicate. These data are presented in Table 3.

Hemolytic Activity Assays. The hemolytic activity of compounds **7**, **9**, **16**, **18**, **20**, **22**, and **25**−**26** was determined by using previously described methods with minor modifications.[25] Murine red blood cells (mRBCs) (1 mL) were suspended in 3 mL of PBS and centrifuged at 1000 rpm for 5 min to obtain the mRBCs. The mRBCs were washed four times in PBS and resuspended in the same buffer to a final concentration of 10^7 erythrocytes/mL. Serial dilution of compounds **7**, **9**, **16**, **18**, **20**, **22**, and **25**−**26** were prepared in Eppendorf tubes containing 100 μL of Milli-Q-H_2O, and 100 μL of mRBC suspension was added to achieve a final concentration of compounds ranging from 0.48 to 62.5 μg/mL and 5 × 10^6 erythrocytes/mL of RBC. The tubes were incubated at 37 °C for 60 min. Tubes with Milli-Q-H_2O (200 μL) and Triton X-100 (1% v/v, 2 μL) served as negative (blank) and positive controls, respectively. The percentage of hemolysis was calculated using the following equation: % hemolysis = [(absorbance of sample) − (absorbance of blank)] × 100/(absorbance of positive control). These data are presented in Supporting Information, Table S1, and Figure 3.

In Vitro Cytotoxicity Assays. Mammalian cytotoxicity assays were performed as previously described with minor modifications.[27] HEK-293, BEAS-2B, and A549 mammalian cell lines were cultured as mentioned above. Once cell lines were about 80% confluent, cells were counted by a hemacytometer (Hausser Scientific, Horsham, PA, USA) and plated in 96-well microtiter plates at concentrations of 10000 cells per well for HEK-293 and 3000 cells per well for BEAS-2B and A549. The 96-well microtiter plates were allowed to incubate at 37 °C with 5% CO_2 for 16 h, after which the medium was replaced with 200 μL of fresh medium with 0.1% DMSO to make up the negative controls. For the piperazine analogues **7** and **9**, the DMSO concentrations used were 0.2% and 0.4% due to their low solubility, respectively. Please note that 0.1−0.4% DMSO was previously determined to be nontoxic to these cell lines. Additionally, the positive controls were prepared by adding 200 μL of medium containing Triton X-100 (12.5% v/v) to the cells. The sterile or background controls contained 200 μL of medium without any cells. Stock solutions of compounds **7**, **9**, **18**, **20**, **22**, **25**, **26**, and VOR were initially prepared at 31 mg/mL or 1000× the tested concentrations and serially diluted with biological DMSO in Eppendorf tubes to achieve 15.5, 7.8, 3.9, 1.9, 1.0, 0.5, 0.25, 0.13 mg/mL. For each concentration, 1 μL of each 1000× stock solution was added to 999 μL of fresh medium in new Eppendorf tubes to achieve final concentrations of 31, 15.5, 7.8, 3.9, 1.9, 1.0, 0.5, 0.25, 0.13 μg/mL. Afterward, each tested well was removed of old medium by vacuum suction and added 200 μL of the prepared medium containing the appropriate concentrations of compounds and 0.1−0.4% DMSO. The 96-well microtiter plates were then incubated for an additional 24 h. After the incubation period, cell survival was assessed via addition of resazurin (10 μL of 25 mg/L solution) for 6−10 h. Live cells produced the fluorescent pink compound resorufin, which was measured at λ_{560} absorption and λ_{590} emission by a SpectraMax M5 plate reader. Dead cells remained purple. The percentage survival rates were calculated by using the following formula: % cell survival = [(fluorescence of sample) − (fluorescence of background)] × 100/[(fluorescence of negative control) − (fluorescence of background)]. The experiments were performed in duplicate. These data are presented in Figure 4.

Membrane Permeabilization Assay Using Propidium Iodide Staining. A fresh culture of *C. albicans* ATCC 10231 (strain **A**) was prepared in 5 mL of YPD broth in a Falcon tube and was grown overnight at 35 °C at 200 rpm. An overnight culture (40 μL) was transferred to RPMI 1640 medium (1 mL) containing no drug (negative control) or compounds **20** at a concentration of 3.9 μg/mL (4 × MIC) or compound **26** at a concentration of 7.8 μg/mL (4 × MIC). FLC (7.8 μg/mL) and KANB derivative C_{14} (15.6 μg/mL)[25] were used as a negative and positive controls, respectively. The cell suspensions were then treated for 1 h at 35 °C with continuous agitation (200 rpm). The cells were then centrifuged and resuspended in 500 μL of PBS buffer (pH 7.2). Subsequently, cells were treated with propidium iodide (9 μM, final concentration) and incubated for 20 min at room temperature in the dark. Glass slides prepared with 10 μL of each mixture were observed in bright field and fluorescence modes (using Texas red filter set and excitation and emission wavelengths of 535 and 617 nm, respectively) using a Zeiss Axiovert 200 M fluorescence microscope. Data were obtained from at least two independent experiments. The images were also postprocessed utilizing automatic contrast and brightness setting in Microsoft PowerPoint 2013 to eliminate background noise. These data are presented in Figure 5.

Determination of Sterol Composition in C. albicans. A single colony of *C. albicans* ATCC 10231 (strain **A**) was picked from a fresh culture plate to inoculate 3 mL of yeast peptone dextrose broth (YPDB) and was incubated at 35 °C for ~18 h with continuous agitation (180 rpm). The overnight yeast culture was used to inoculate RPMI 1640 medium (15 mL), and the final inoculum concentration was adjusted to 1 × 10^6 CFU/mL (~OD_{600} = 0.12) by using spectrophotometric method. Afterward, the yeast cells were treated with compounds **20** (0.48 μg/mL), **26** (0.975 μg/mL), and VOR (0.12 μg/mL) at their sub-MIC values, except for FLC (1.95 μg/mL; *NOTE*: *C. albicans* ATCC 10231 (strain **A**) is resistant to FLC[39]). An equivalent amount of DMSO without drug (untreated control) was also prepared. Cells were harvested by centrifugation (5000 rpm) for 10 min at room temperature, and cell pellets were saponified at 80 °C for 2 h with 3 mL of MeOH, 2 mL of pyrogallol dissolved in MeOH (0.5%, w/v) (CAS 87-66-1, Sigma-Aldrich, St. Louis, MO), and 2 mL of KOH (60%, w/v). The nonsaponifiable sterols were then extracted three times with 5 mL of heptane. The extracts were evaporated under a stream of nitrogen to dryness and resuspended in 500 μL of heptane.

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The sterol suspension was then transferred to a GC-MS vial and derivatized with 250 μL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA, CAS 24589-78-4, Sigma-Aldrich, St. Louis, MO) at 70 °C for 20 min. GC-MS analyses were performed on an Agilent 7890A gas chromatograph with splitless injection, coupled to an Agilent 5970C inert XL mass spectrometer with a triple-axis detector and an Agilent 19091S-433 capillary column (30 m × 250 μm). The oven temperature was programmed to hold at 70 °C for 2 min and then ramped to 270 °C at a rate of 20 °C/min. Helium (10 psi) was used as the carrier gas, the electron ionization energy was 70 eV, and the inlet temperature 250 °C. Identification of sterols was achieved using the NIST (the National Institute of Standards and Technology) reference database. These data are presented in Figure 6.

Molecular Docking Study. The remarkable depletion of ergosterol content in *C. albicans* ATCC 10231 (strain **A**) by sub-MIC concentration of compounds **20** and **26** indicated that the potential target of these compounds could be the cytochrome P450 sterol 14α-demethylase. A molecular docking study was performed using Crystallographic Object Oriented Toolkit (COOT) software[40] in order to investigate the possible interactions and binding mode of compound **20** with the enzyme *C. albicans* CYP51 (PDB 3P99[34]). VT-1161, a known inhibitor of cytochrome P450 sterol 14α-demethylase sterol, served as a reference. The docking results are presented in Figure 7.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.7b01138.

^{1}H and ^{13}C NMR spectra and HPLC traces of final compounds tested; exact percentages with SDEV that were used to generate Figure 3; Log*P* values of compounds **1**−**28** (PDF)

Molecular formula strings (CSV)

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Author Contributions

N.T.C. and S.K.S. contributed equally to this work. N.T.C., S.K.S, and S.G.-T. design the study, analyzed the data, wrote the manuscript and Supporting Information and made figures. N.T.C. and K.C.H. synthesized all compounds used in this study. H.X.N. performed the cytotoxicity assays and time-kill studies and generated Figures 2 and 4. S.K.S. performed all other biochemical/biological experiments. O.V.T. performed the docking studies. All authors reviewed the manuscript and Supporting Information.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AmB, amphotericin B; ATCC, American Type Culture Collection; CAS, caspofungin; CLSI, Clinical and Laboratory Standards Institute; FLC, fluconazole; GC-MS, gas chromatography−mass spectrometry; ITC, itraconazole; KANB, kanamycin B; mRBCs, murine red blood cells; PI, propidium iodide; POS, posaconazole; SAR, structure−activity relationship; TOB, tobramycin; VOR, voriconazole; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide

■ REFERENCES

(1) Kathiravan, M. K.; Salake, A. B.; Chothe, A. S.; Dudhe, P. B.; Watode, R. P.; Mukta, M. S.; Gadhwe, S. The biology and chemistry of antifungal agents: a review. *Bioorg. Med. Chem.* **2012**, *20*, 5678−5698.

(2) Beck Sague, C.; Jarvis, W. R. Secular trends in the epidemiology of nosocomial fungal infections in the United States, 1980−1990. National Nosocomial Infections Surveillance System. *J. Infect. Dis.* **1993**, *167*, 1247−1251.

(3) Pfaller, M. A.; Diekema, D. J. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin. Microbiol. Rev.* **2007**, *20*, 133−163.

(4) Pannuti, C.; Gingrich, R.; Pfaller, M. A.; Kao, C.; Wenzel, R. P. Nosocomial pneumonia in patients having bone marrow transplant. Attributable mortality and risk factors. *Cancer* **1992**, *69*, 2653−2662.

(5) Carmona, E. M.; Limper, A. H. Overview of treatment approaches for fungal infections. *Clin. Chest Med.* **2017**, *38*, 393−402.

(6) Wiederhold, N. P. Antifungal resistance: current trends and future strategies to combat. *Infect. Drug Resist.* **2017**, *10*, 249−259.

(7) McCarthy, M. W.; Kontoyiannis, D. P.; Cornely, O. A.; Perfect, J. R.; Walsh, T. J. Novel agents and drug targets to meet the challenges of resistant fungi. *J. Infect. Dis.* **2017**, *216*, S474−S483.

(8) McCarthy, M. W.; Walsh, T. J. Drug development challenges and strategies to address emerging and resistant fungal pathogens. *Expert Rev. Anti-Infect. Ther.* **2017**, *15*, 577−584.

(9) Berkow, E. L.; Lockhart, S. R. Fluconazole resistance in *Candida* species: a current perspective. *Infect. Drug Resist.* **2017**, *10*, 237−245.

(10) Jiang, Z.; Wang, Y.; Wang, W.; Wang, S.; Xu, B.; Fan, G.; Dong, G.; Liu, Y.; Yao, J.; Miao, Z.; Zhang, W.; Sheng, C. Discovery of highly potent triazole antifungal derivatives by heterocycle-benzene bioisosteric replacement. *Eur. J. Med. Chem.* **2013**, *64*, 16−22.

(11) Morschhauser, J. Regulation of multidrug resistance in pathogenic fungi. *Fungal Genet. Biol.* **2010**, *47*, 94−106.

(12) Perlin, D. S.; Shor, E.; Zhao, Y. Update on antifungal drug resistance. *Curr. Clin. Microbiol. Rep.* **2015**, *2*, 84−95.

(13) Sanglard, D. Emerging threats in antifungal-resistant fungal pathogens. *Front. Med.* **2016**, *3*, 11.

(14) Srinivasan, A.; Lopez-Ribot, J. L.; Ramasubramanian, A. K. Overcoming antifungal resistance. *Drug Discovery Today: Technol.* **2014**, *11*, 65−71.

(15) Vandeputte, P.; Ferrari, S.; Coste, A. T. Antifungal resistance and new strategies to control fungal infections. *Int. J. Microbiol.* **2012**, *2012*, 713687.

(16) Zhou, C. H.; Wang, Y. Recent researches in triazole compounds as medicinal drugs. *Curr. Med. Chem.* **2012**, *19*, 239−280.

(17) Zhang, L.; Peng, X. M.; Damu, G. L.; Geng, R. X.; Zhou, C. H. Comprehensive review in current developments of imidazole-based medicinal chemistry. *Med. Res. Rev.* **2014**, *34*, 340−437.

(18) Garibotto, F. M.; Garro, A. D.; Masman, M. F.; Rodriguez, A. M.; Luiten, P. G.; Raimondi, M.; Zacchino, S. A.; Somlai, C.; Penke, B.; Enriz, R. D. New small-size peptides possessing antifungal activity. *Bioorg. Med. Chem.* **2010**, *18*, 158−167.

(19) Menzin, J.; Meyers, J. L.; Friedman, M.; Perfect, J. R.; Langston, A. A.; Danna, R. P.; Papadopoulos, G. Mortality, length of hospitalization, and costs associated with invasive fungal infections in high-risk patients. *Am. J. Health-Syst. Pharm.* **2009**, *66*, 1711−1717.

(20) Hahn-Ast, C.; Glasmacher, A.; Muckter, S.; Schmitz, A.; Kraemer, A.; Marklein, G.; Brossart, P.; von Lilienfeld-Toal, M. Overall survival and fungal infection-related mortality in patients with invasive fungal infection and neutropenia after myelosuppressive chemotherapy in a tertiary care centre from 1995 to 2006. *J. Antimicrob. Chemother.* **2010**, *65*, 761−768.

(21) Sheehan, D. J.; Hitchcock, C. A.; Sibley, C. M. Current and emerging azole antifungal agents. *Clin. Microbiol. Rev.* **1999**, *12*, 40−79.

DOI: 10.1021/acs.jmedchem.7b01138
J. Med. Chem. XXXX, XXX, XXX−XXX

(22) Vanden Bossche, H.; Koymans, L.; Moereels, H. P450 inhibitors of use in medical treatment: focus on mechanisms of action. *Pharmacol. Ther.* **1995**, *67*, 79−100.
(23) Emami, S.; Tavangar, P.; Keighobadi, M. An overview of azoles targeting sterol 14alpha-demethylase for antileishmanial therapy. *Eur. J. Med. Chem.* **2017**, *135*, 241−259.
(24) Shrestha, S. K.; Fosso, M. Y.; Green, K. D.; Garneau-Tsodikova, S. Amphiphilic tobramycin analogues as antibacterial and antifungal agents. *Antimicrob. Agents Chemother.* **2015**, *59*, 4861−4869.
(25) Fosso, M. Y.; Shrestha, S. K.; Green, K. D.; Garneau-Tsodikova, S. Synthesis and bioactivities of kanamycin B-derived cationic amphiphiles. *J. Med. Chem.* **2015**, *58*, 9124−9132.
(26) Chandrika, N. T.; Shrestha, S. K.; Ngo, H. X.; Garneau-Tsodikova, S. Synthesis and investigation of novel benzimidazole derivatives as antifungal agents. *Bioorg. Med. Chem.* **2016**, *24*, 3680−3686.
(27) Ngo, H. X.; Shrestha, S. K.; Garneau-Tsodikova, S. Identification of ebsulfur analogues with broad-spectrum antifungal activity. *ChemMedChem* **2016**, *11*, 1507−1516.
(28) Shrestha, S. K.; Garzan, A.; Garneau-Tsodikova, S. Novel alkylated azoles as potent antifungals. *Eur. J. Med. Chem.* **2017**, *133*, 309−318.
(29) Shrestha, S.; Grilley, M.; Fosso, M. Y.; Chang, C. W.; Takemoto, J. Y. Membrane lipid-modulated mechanism of action and non-cytotoxicity of novel fungicide aminoglycoside FG08. *PLoS One* **2013**, *8*, e73843.
(30) Chang, C. W.; Fosso, M.; Kawasaki, Y.; Shrestha, S.; Bensaci, M. F.; Wang, J.; Evans, C. K.; Takemoto, J. Y. Antibacterial to antifungal conversion of neamine aminoglycosides through alkyl modification. Strategy for reviving old drugs into agrofungicides. *J. Antibiot.* **2010**, *63*, 667−672.
(31) Bladocha, M.; Benveniste, P. Manipulation by tridemorph, a systemic fungicide, of the sterol composition of maize leaves and roots. *Plant Physiol.* **1983**, *71*, 756−762.
(32) Pore, V. S.; Agalave, S. G.; Singh, P.; Shukla, P. K.; Kumar, V.; Siddiqi, M. I. Design and synthesis of new fluconazole analogues. *Org. Biomol. Chem.* **2015**, *13*, 6551−6561.
(33) Hawser, S. P.; Douglas, L. J. Resistance of *Candida albicans* biofilms to antifungal agents *in vitro*. *Antimicrob. Agents Chemother.* **1995**, *39*, 2128−2131.
(34) Hargrove, T. Y.; Wawrzak, Z.; Lamb, D. C.; Guengerich, F. P.; Lepesheva, G. I. Structure-functional characterization of cytochrome P450 sterol 14alpha-demethylase (CYP51B) from *Aspergillus fumigatus* and molecular basis for the development of antifungal drugs. *J. Biol. Chem.* **2015**, *290*, 23916−23934.
(35) Clinical and Laboratory Standards Institute. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts—Approved Standard*; CLSI document M27-A3; Clinical and Laboratory Standards Institute: Wayne, PA, 2008.
(36) Clinical and Laboratory Standards Institute. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi*, 2nd ed.; CLSI document M38-A2; Clinical and Laboratory Standards Institute: Wayne, PA, 2008.
(37) Shrestha, S. K.; Fosso, M. Y.; Garneau-Tsodikova, S. A combination approach to treating fungal infections. *Sci. Rep.* **2015**, *5*, 17070.
(38) Pierce, C. G.; Uppuluri, P.; Tristan, A. R.; Wormley, F. L., Jr.; Mowat, E.; Ramage, G.; Lopez-Ribot, J. L. A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. *Nat. Protoc.* **2008**, *3*, 1494−1500.
(39) Oliveira, A. S.; Martinex-de-Oliveira, J.; Palmeira-de-Oliveira, R.; Palmeira-de-Oliveira, A. Antifungal activity of antidepressant sertraline against *Candida* species in vitro: A potential beneficial association with fluconazole. *Med. J. Obstet. Gynecol.* **2017**, *5*, 1095.
(40) Emsley, P.; Cowtan, K. Coot: model-building tools for molecular graphics. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2004**, *60*, 2126−2132.

DOI: 10.1021/acs.jmedchem.7b01138
J. Med. Chem. XXXX, XXX, XXX−XXX