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Discovery of Novel Tetrazoles Featuring a Pyrazole Moiety as Potent and Highly Selective Antifungal Agents

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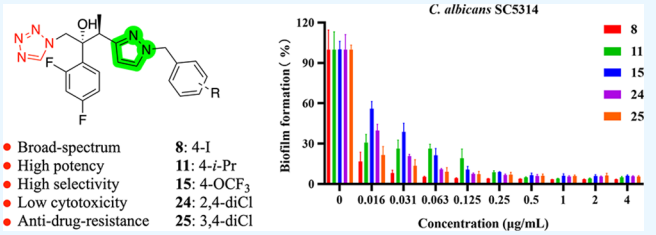
ABSTRACT: In pursuit of developing novel azole antifungals with potent activity and high selectivity, a series of (2*R*,3*S*)-3-(substituted-1*H*-pyrazol-3-yl)-2-(2,4-difluorophenyl)-1-(1*H*-tetrazol-1-yl)butan-2-ol derivatives were designed and synthesized based on our previous work. All compounds exhibited excellent *in vitro* antifungal activities against *Candida* spp. and *Cryptococcus neoformans* H99 with minimum inhibitory concentration values ranging from <0.008 to 4 μg/mL, with some even showing moderate activity against *Aspergillus fumigatus* 7544. The most active compounds (**8**, **11**, **15**, **24**, and **25**) displayed outstanding antifungal activity against six fluconazole-resistant *C. auris* clinical isolates and showed a potent inhibitory effect on biofilm formation of *C. albicans* SC5314 and *C. neoformans* H99. In addition, compounds **11** and **15** showed no inhibition of human CYP1A2 and low inhibitory activity against CYP3A4, indicating minimal risk of drug−drug interactions. Taken together, these promising tetrazoles with high *in vitro* potency and good safety profiles warrant further investigation.



1. INTRODUCTION

The incidence and severity of invasive fungal infections (IFIs) are more common than ever due to the increasing number of immunocompromised patients associated with cancer chemotherapy, AIDS, and organ transplantation.[1] Approximately, 1.6 million people die each year from IFIs, and the associated mortality rate is reported to exceed 50% due to drug resistance and the scarcity of available antifungal drugs.[2−4] Although two antifungal drugs, one tetrazole (oteseconazole/VT-1161)[5] and one triterpenoid (ibrexafungerp/SCY-078),[6] have been approved for marketing by the Food and Drug Administration in the last two years, the antifungal arsenal remains inadequate. Therefore, new antifungal drugs with high potency and selectivity are urgently needed.

Among the five classes of antifungals (flucytosine, polyenes, azoles, echinocandins, and triterpenoids), azoles are the most abundant and the most intensively studied in terms of structure−activity relationships. It is well known that the mode of action of azole antifungals is the inhibition of lanosterol 14α-demethylase (CYP51) to prevent the biosynthesis of ergosterol.[7,8] From imidazole to triazole and then to tetrazole, azole drugs have developed into a fourth generation. In general, the azole ring determines the antifungal activity, CYP selectivity, and hepatotoxicity, while side chains determine the physicochemical properties, bioavailability, and antifungal spectrum (Figure 1). For example, fluconazole (1990) and oteseconazole (2022) are mainly used for the treatment of candidiasis,[9,10] and voriconazole (2002), posaconazole (2006), and isavuconazole (2015) are mainly used for aspergillosis.[11−13] For the treatment of cryptococcal

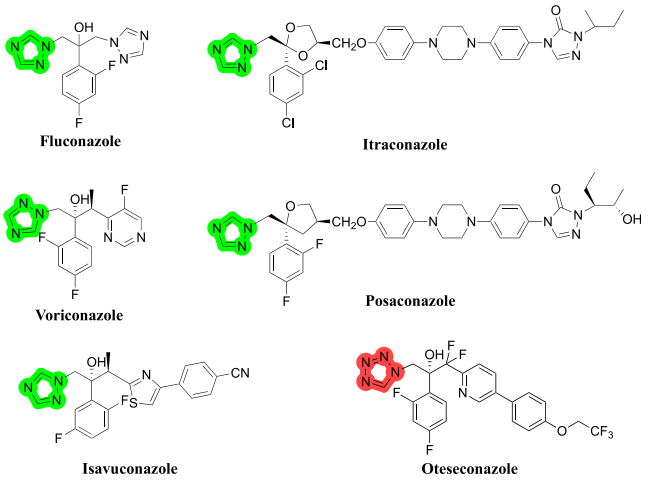


Figure 1. Azole drugs for IFIs.

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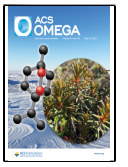


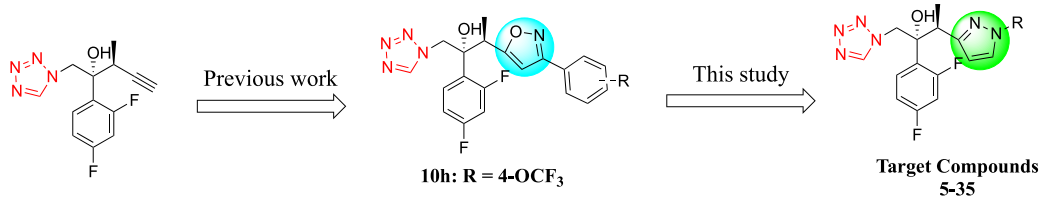
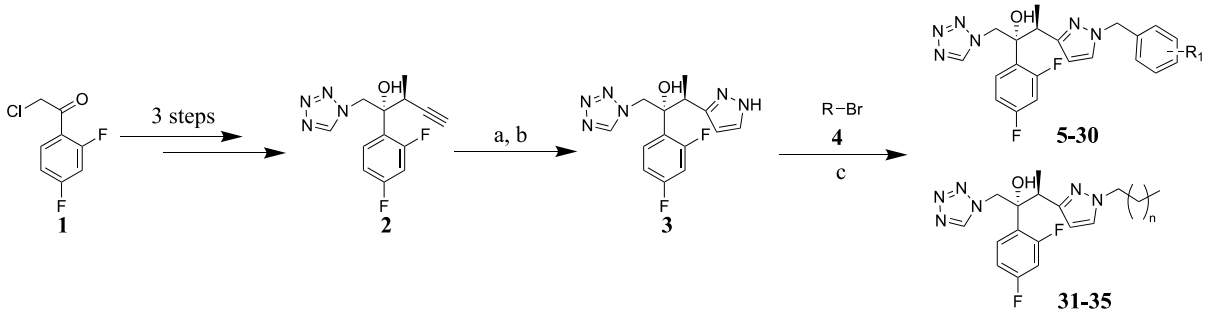


Figure 2. Previous work and this study.

Scheme 1. Reagents and Conditions[a]



5: R_1 = H **6**: R_1 = 4-F **7**: R_1 = 4-Cl **8**: R_1 = 4-I **9**: R_1 = 4-Me **10**: R_1 = 4-t-Bu **11**: R_1 = 4-i-Pr

12: R_1 = 4-CF_3 **13**: R_1 = 3-CF_3 **14**: R_1 = 4-CN **15**: R_1 = 4-OCF_3 **16**: R_1 = 2,3-diF **17**: R_1 = 2,4-diF **18**: R_1 = 2,5-diF

19: R_1 = 2,6-diF **20**: R_1 = 3,4-diF **21**: R_1 = 3,5-diF **22**: R_1 = 2,3,6-triF **23**: R_1 = 3,4,5-triF **24**: R_1 = 2,4-diCl **25**: R_1 = 3,4-diCl

26: R_1 = 3-Cl-2-F **27**: R_1 = 3-Cl-4-F **28**: R_1 = 4-Cl-2,6-diF **29**: R_1 = 3-CF_3-4-F **30**: R_1 = 3-OMe-4-F

31: n = 0 **32**: n = 1 **33**: n = 2 **34**: n = 3 **35**: n = 4

[a](a) $TMSCHN_2$, toluene, 110 °C, 24 h; (b) KF, MeOH, 1 h; (c) K_2CO_3, DMF, 50 °C, 6 h.

meningitis, fluconazole in combination with amphotericin B (AmB, 1958) and flucytosine (1971) still represents the gold standard therapy, but concerns over its safety lead to the need for close monitoring while patients are receiving treatment.[14] Moreover, none of the azoles have demonstrated broad-spectrum antifungal activity for the treatment of candidiasis, cryptococcosis, and aspergillosis in clinical practice. Overall, despite being a widely used class of antifungal drug, structure optimization of the azoles still represents a fundamental area of antifungal research.

Hoekstra et al. reported that the replacement of the triazole ring of voriconazole with a tetrazole ring resulted in a significant decrease in antifungal activity against *Candida albicans* with minimum inhibitory concentration (MIC) values increasing from 0.06 to 1 μg/mL. Even though the inhibition of CYP3A4 was slightly reduced, the selectivity deteriorated.[15] Then, by replacing the pyrimidine ring of voriconazole with a phenylpyridine moiety and replacing the chiral methyl with a difluoro substitution, highly selective and potent tetrazole compounds VT-1161 and VT-1129 were obtained, which were subsequently further optimized to obtain VT-1598 with a better broad spectrum.[16] In addition, other research teams have made attempts at tetrazole replacement strategies,[17,18] but it seems that a tetrazole replacement strategy is not universal, and only specific side chains, like the phenylpyridine moiety of oteseconazole, are required for tetrazole activity. Moreover, tetrazole−triazole hybrids had been reported earlier; however, these hybrids only showed moderate antifungal activity against pathogenic fungi.[19−21]

Inspired by the studies of Hoekstra et al., we also tried to design and synthesize a series of tetrazole compounds based on our previously reported triazole compound NT-a9.[22−24] Encouragingly, tetrazole compound **10h** proved to have no inhibitory effect on CYP1A2 and CYP3A4 and demonstrated high *in vivo* efficacy in a mouse model of *C. albicans* SC5314 infection, which prompted us to continue studying the structural optimization of tetrazole compound **10h** (Figure 2).[22] In this paper, we applied the concept of bioisosterism to design novel tetrazoles by replacing the isoxazole moiety with a pyrazole ring, a "biologically privileged" five-membered N-heteroaromatic ring. Herein, we report for the first time the rational design and synthesis of a series of tetrazole compounds containing a pyrazole moiety and evaluate their antifungal activity.

2. RESULTS AND DISCUSSION

2.1. Chemistry. The general synthetic route for the preparation of the target compounds (**5**−**35**) is depicted in Scheme 1. As a key intermediate of our designed compounds, alkyne **2** was synthesized according to the literature,[22,25,26] and the absolute configuration of **2** was confirmed by X-ray crystallographic analysis.[22] Intermediate **3** was prepared by cycloaddition of **2** with $TMSCHN_2$ at a high temperature. Subsequently, intermediate **3** was reacted with benzyl bromides or alkyl bromides in the presence of K_2CO_3 to form target compounds **5**−**35** in good yields.

2.2. *In Vitro* Antifungal Activities of Target Compounds. The *in vitro* antifungal activities of compounds **5**−**35** were evaluated against seven common pathogenic fungi,

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Table 1. *In Vitro* Antifungal Activity of the Selected Compounds[a]

Compd	R_1 or n	MIC (μg/mL)						
		C. alb SC5314	*C. gla* 537	*C. par* 22019	*C. kru* 4946	*C. tro* 2718	*C. neo* H99	*A. fum* 7544
5	H	<0.008	<0.008	0.0625	1	<0.008	0.25	>64
6	4-F	<0.008	<0.008	0.0625	1	<0.008	0.125	>64
7	4-Cl	<0.008	<0.008	0.0313	0.125	<0.008	<0.008	64
8	4-I	<0.008	<0.008	<0.008	<0.008	<0.008	<0.008	>64
9	4-Me	<0.008	<0.008	0.0625	0.25	<0.008	<0.008	>64
10	4-t-Bu	2	<0.008	0.125	1	<0.008	1	>64
11	4-i-Pr	<0.008	<0.008	<0.008	<0.008	<0.008	<0.008	>64
12	4-CF_3	<0.008	<0.008	0.0313	0.25	<0.008	<0.008	64
13	3- CF_3	<0.008	<0.008	0.125	0.0625	<0.008	<0.008	>64
14	4-CN	0.016	<0.008	0.0625	0.25	<0.008	1	>64
15	4-OCF_3	<0.008	<0.008	0.0625	<0.008	<0.008	<0.008	>64
16	2,3-diF	<0.008	<0.008	0.125	0.0313	<0.008	0.25	>64
17	2,4-diF	<0.008	<0.008	0.125	<0.008	<0.008	0.125	32
18	2,5-diF	0.032	<0.008	0.0625	0.5	<0.008	0.25	>64
19	2,6-diF	<0.008	<0.008	0.0625	0.25	<0.008	0.0625	>64
20	3,4-diF	<0.008	<0.008	0.0625	<0.008	<0.008	0.0625	64
21	3,5-diF	<0.008	<0.008	0.125	<0.008	<0.008	0.25	16
22	2,3,6-triF	<0.008	<0.008	0.0625	<0.008	<0.008	0.125	>64
23	3,4,5-triF	0.016	<0.008	0.0625	2	<0.008	0.0625	>64
24	2,4-diCl	<0.008	<0.008	<0.008	<0.008	<0.008	<0.008	64
25	3,4-diCl	<0.008	<0.008	<0.008	<0.008	0.016	<0.008	32
26	3-Cl-2-F	<0.008	<0.008	0.0625	<0.008	<0.008	<0.008	>64
27	3-Cl-4-F	<0.008	<0.008	0.0625	0.5	<0.008	<0.008	>64
28	4-Cl-2,6-diF	<0.008	<0.008	0.125	0.0625	<0.008	<0.008	>64
29	3-CF_3-4-F	0.0625	<0.008	0.5	4	<0.008	0.5	>64
30	3-OMe-4-F	<0.008	<0.008	0.125	0.125	<0.008	<0.008	>64
31	n = 0	0.0625	0.0313	0.5	>4	1	4	>64
32	n = 1	0.0156	0.0313	0.125	2	0.5	2	>64
33	n = 2	<0.008	0.0156	0.0313	0.5	0.0313	0.25	>64
34	n = 3	<0.008	<0.008	0.0625	0.5	>4	0.25	>64
35	n = 4	<0.008	<0.008	<0.008	1	0.0625	0.5	>64
OTC	-	<0.008	<0.008	<0.008	<0.008	<0.008	<0.008	>64
POS	-	<0.008	<0.008	0.0313	0.016	<0.008	<0.008	0.125
VOC	-	<0.008	<0.008	0.016	0.25	<0.008	0.0625	0.125
FLC	-	0.25	0.5	1	32	<0.125	8	>64

[a]Abbreviations: MIC, minimum inhibitory concentration of drugs to inhibit ≥50% growth of fungal cells compared to that of a drug-free control at 30 °C for 24 h for *Candida* spp., 72 h incubation for *Cryptococcus neoformans*, and at 35 °C for 48 h for *Aspergillus fumigatus*; *C. alb*, *Candida albicans*; *C. par*, *Candida parapsilosis*; *C. tro*, *Candida tropicalis*; *C. gla*, *Candida glabrata*; *C. kru*, *Candida krusei*; *C. neo*, *Cryptococcus neoformans*; *A. fum*, *Aspergillus fumigatus*; OTC, oteseconazole; POS, posaconazole; VOC, voriconazole; FLC, fluconazole.

Candida albicans SC5314, *Candida glabrata* 537, *Candida parapsilosis* 22019, *Candida krusei* 4946, *Candida tropicalis* 2718, *Cryptococcus neoformans* H99, and *Aspergillus fumigatus* 7544. The MIC was measured according to the standard micro-broth dilution method of the Clinical and Laboratory Standards Institute (CLSI, M27-A3 and M60).[27,28] The MIC

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was defined as the first well with ≥50% reduction in growth compared to the growth of the drug-free well. Compounds were dissolved in dimethyl sulfoxide (DMSO), serially diluted in growth medium, inoculated, and incubated at 30 or 35 °C. The MIC was determined at 24 h for *C. albicans*, at 48 h for *C. glabrata*, at 72 h for *C. neoformans*, and at 48 h for *A. fumigatus*. Oteseconazole (OTC, VT-1161), posaconazole (POS), voriconazole (VOC), and fluconazole (FLC) were used as reference drugs. The results are summarized in Table 1.

Initially, we synthesized unsubstituted benzyl analogue **5**, which showed potent broad-spectrum antifungal activity with the exception of *A. fumigatus*. These results highlight that pyrazole substitution is feasible and encouraged us to investigate the contribution of substituents on the benzyl group to the antifungal activity. Therefore, subsequently synthesized compounds will all use compound **5** as a reference. The introduction of 4-F, 4-Cl, and 4-I into the benzyl ring gave compounds **6**, **7**, and **8**, respectively. Compared to **5**, compounds **7** (4-Cl) and **8** (4-I) showed 8-fold and 125-fold improvement in activity against *C. krusei* 4946, respectively, and both showed 32-fold improvement in activity against *C. neoformans* H99. The introduction of 4-Me, 4-t-Bu, and 4-i-Pr into the benzyl ring gave compounds **9**, **10**, and **11**, respectively. The *para*-position of the benzyl group substituted with 4-Me was well tolerated, and substitution of 4-i-Pr was beneficial for a broad spectrum and showed excellent antifungal activity with a MIC value of <0.008 μg/mL against all test fungi with the exception of *A. fumigatus* 7544. The substitution of 4-t-Bu was unfavored and showed a 250-fold, 2-fold, and 4-fold decrease in activity against *C. albicans* SC5314, *C. parapsilosis* 22019, and *C. neoformans* H99, respectively, when compared to compound **5**. Compounds **12** (4-CF_3) and **13** (3-CF_3) showed 4-fold and 16-fold improvement in activity against *C. krusei* 4946, respectively, while compound **13** showed relatively low potency against *C. parapsilosis* 22019, suggesting that an electron-withdrawing group at the *para*-position is more favorable. Disappointingly, compound **14** with 4-CN substitution proved to be less potent than compound **5**. Compound **15** (4-OCF_3) showed 125-fold and 32-fold improvement in activity against *C. krusei* 4946 and *C. neoformans* H99, respectively. Encouraged by the promising results obtained with compounds **6**, **12**, and **15**, two or more fluorine atoms were introduced into the benzyl ring, giving compounds **16** (2,3-diF), **17** (2,4-diF), **18** (2,5-diF), **19** (2,6-diF), **20** (3,4-diF), **21** (3,5-diF), **22** (2,3,6-triF), and **23** (3,4,5-triF). The MIC values of these compounds indicated that fluorine substitution was well tolerated, and compounds **17** (2,4-diF), **20** (3,4-diF), and **21** (3,5-diF) even showed moderate activity against *A. fumigatus* with MIC values of 32, 64, and 16 μg/mL, respectively. We also examined the effect of introducing a second chlorine atom into compound **7**. Surprisingly, compounds **24** (2,4-diCl) and **25** (3,4-diCl) displayed moderate to excellent antifungal activity against all test fungi. Finally, we combined −Cl, −CF_3, and −OMe with −F to obtain compounds **26** (3-Cl-2-F), **27** (3-Cl-4-F), **28** (4-Cl-2,6-diF), **29** (3-CF_3-4-F), and **30** (3-OMe-4-F). Although these compounds showed excellent antifungal activity against *C. albicans*, *C. glabrata*, *C. tropicalis*, *and C. neoformans*, there was no improvement in activity against *C. krusei*, *C. parapsilosis*, and *A. fumigatus* when compared to compounds **24** and **25**.

To further verify the importance of the benzyl group, we replaced the benzyl group with alkyl groups to obtain compounds **31** (ethyl), **32** (*n*-propyl), **33** (*n*-butyl), **34** (*n*-pentyl), and **35** (*n*-hexyl). The results showed that while all the alkyl-substituted compounds exhibited excellent activity against *C. albicans* SC5314, they were less active against *C. krusei*, *C. tropicalis*, and *C. neoformans*. Clearly, the length of the alkyl chain was critical for antifungal activity, with compound **33** (*n*-butyl) having the optimal antifungal activity. Overall, the broad spectrum of the alkyl-substituted compounds was inferior to that of the benzyl-substituted compounds.

In recent years, *Candida auris*, a multidrug-resistant yeast that exhibits resistance to azoles, AmB, and echinocandins, has globally emerged as a nosocomial pathogen.[29] Five outstanding compounds **8**, **11**, **15**, **24**, and **25** were therefore selected for evaluation of *in vitro* activity against six drug-resistant *C. auris* clinical isolates. FLC showed MIC values greater than 64 μg/mL against all six drug-resistant *C. auris* isolates, while compounds **8**, **11**, **15**, **24**, and **25** showed MIC values in the range of <0.0625−64 μg/mL, significantly better than FLC (Table 2). Compounds **8** and **15** showed the best potency against *C. auris* 789 (0.125 and 0.25 μg/mL), *C. auris* D12 (0.125 and 0.5 μg/mL), and *C. auris* BS12372 (<0.0625 and <0.0625 μg/mL), respectively, and were even superior to OTC. These encouraging results highlight the anti-resistance advantage of the pyrazole moiety and warrant further study.

Table 2. *In Vitro* Antifungal Activity of Selected Compounds[a]

	MIC (μg/mL)					
Compd	*C. aur* 789	*C. aur* 791	*C. aur* 887	*C. aur* 888	*C. aur* D12	*C. aur* BS12372
8	0.125	1	2	4	0.125	<0.0625
11	2	4	4	8	1	0.125
15	0.25	1	2	1	0.5	<0.0625
24	16	16	64	64	16	0.5
25	4	8	8	16	2	<0.0625
OTC	2	8	4	8	0.25	<0.0625
FLC	>64	>64	>64	>64	>64	>64

[a]Abbreviations: MIC, minimum inhibitory concentration of drugs to inhibit ≥50% growth of fungal cells compared to that of a drug-free control at 30 °C for 24 h for *Candida auris*; *C. aur*, *Candida auris*; OTC, oteseconazole; FLC, fluconazole.

2.3. Determination of the Sterol Composition in *C. albicans*. Azole drugs inhibit lanosterol 14α-demethylase (CYP51), a key enzyme in the ergosterol biosynthesis pathway, and as a result cause the accumulation of methylated sterols, which cause a fungistatic effect in yeast.[30] To verify the mechanism of action of our target compounds **8**, **11**, **15**, **24**, and **25**, sterol composition analysis in fungal cells was performed with gas chromatography−mass spectrometry (GC−MS). *C. albicans* SC5314 was treated with selected compounds **8**, **11**, **15**, **24**, and **25** at 0.008 μg/mL and FLC at 8 μg/mL. The sterol composition analysis results are shown in Figure 3. In the control group, ergosterol was the major sterol, accounting for 75.33%, in contrast to lanosterol of 9.15% and zymosterol of 15.53%. When fungal cells were treated with compounds **8**, **11**, **15**, **24**, and **25** at 0.008 μg/mL and FLC at 8 μg/mL, ergosterol was almost completely depleted with the exception of the compound **8**-treated group. The contents of lanosterol, eburicol, and obtusifoliol increased to a similar extent as the FLC-treated group, indicating that our target tetrazole compounds have the same mechanism of action as

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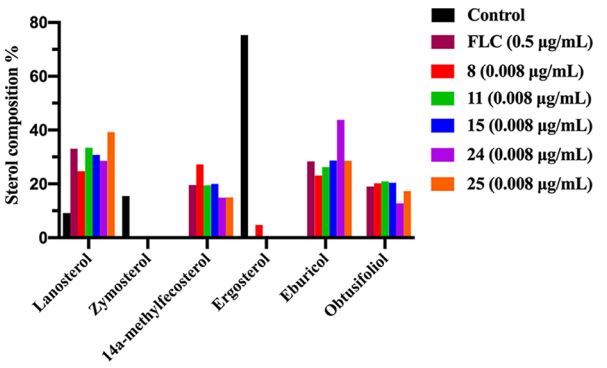

Figure 3. Sterol analysis in *C. albicans* SC5314 cells by GC−MS. The fungal strain was treated with DMSO (control), FLC at 0.5 μg/mL, compounds **8**, **11**, **15**, **24**, and **25** at 0.008 μg/mL for 8 h. FLC, fluconazole.

FLC, that is to block the biosynthesis of ergosterol by inhibiting CYP51.

2.4. Compounds Inhibit the Biofilm Formation of *C. albicans* and *C. neoformans*. Biofilm formation is an important factor for the pathogenesis of *C. albicans*, which leads to high levels of resistance to a wide range of antifungals.[31] Herein, we used cell counting kit-8 (CCK-8) reduction assays, which examined the inhibitory effect of compounds **8**, **11**, **15**, **24**, and **25** on biofilm formation of *C. albicans* SC5314 and *C. neoformans* H99. As shown in Figure 4A,B, all compounds showed potent inhibitory effects on biofilm formation of *C. albicans* SC5314 in a dose-dependent manner and were significantly superior to FLC at the same concentrations. The inhibitory activity on biofilm was enhanced at higher concentrations of test compounds, and over 90% of the biofilms were inhibited in the presence of 0.25 μg/mL of all tested compounds. However, the biofilms were not completely inhibited even when the drug concentration was increased to 4 μg/mL. On the other hand, although all compounds showed a potent inhibitory effect on biofilm formation of *C. neoformans* H99 in a dose-dependent manner and were significantly superior to FLC at the same concentrations, the effect of these compounds on biofilm formation of *C. neoformans* H99 was slightly less effective than that on *C. albicans* SC5314. In comparison, compound **8** manifestly exhibited the best inhibitory potency on the biofilm formation of both *C. albicans* SC5314 and *C. neoformans* H99.

2.5. Compounds Inhibit the Growth of *C. albicans* and *C. neoformans*. To further confirm the antifungal activity of compounds **8**, **11**, **15**, **24**, and **25**, growth curve assays were performed for *C. albicans* SC5314, *C. neoformans* H99, *C. glabrata* 537, *C. krusei* 4946, *C. parapsilosis* 22019, and *C. tropicalis* 2718 in yeast extract−peptone−dextrose (YPD) liquid medium. FLC was used as a reference drug. The results are shown in Figure 5. In general, all compounds showed stronger growth inhibition than FLC at low concentrations against six pathogenic fungi, but the inhibitory activity varied among compounds. First, except for compound **24**, the other four compounds completely inhibited the growth of *C. albicans* SC5314 at an extremely low concentration of 0.002 μg/mL (Figure 5A). While all compounds could not completely inhibit the growth of *C. glabrata* 537 at 0.125 μg/mL, their inhibitory potency was slightly better than that of FLC at 2 μg/mL (Figure 5B). Second, compounds **8**, **11**, and **15** could significantly inhibit the growth of *C. parapsilosis* 22019 at a concentration of 0.008 μg/mL, whereas compounds **24** and **25** were relatively less active (Figure 5C). Frustratingly, all compounds showed poor inhibition against *C. tropicalis* 2718

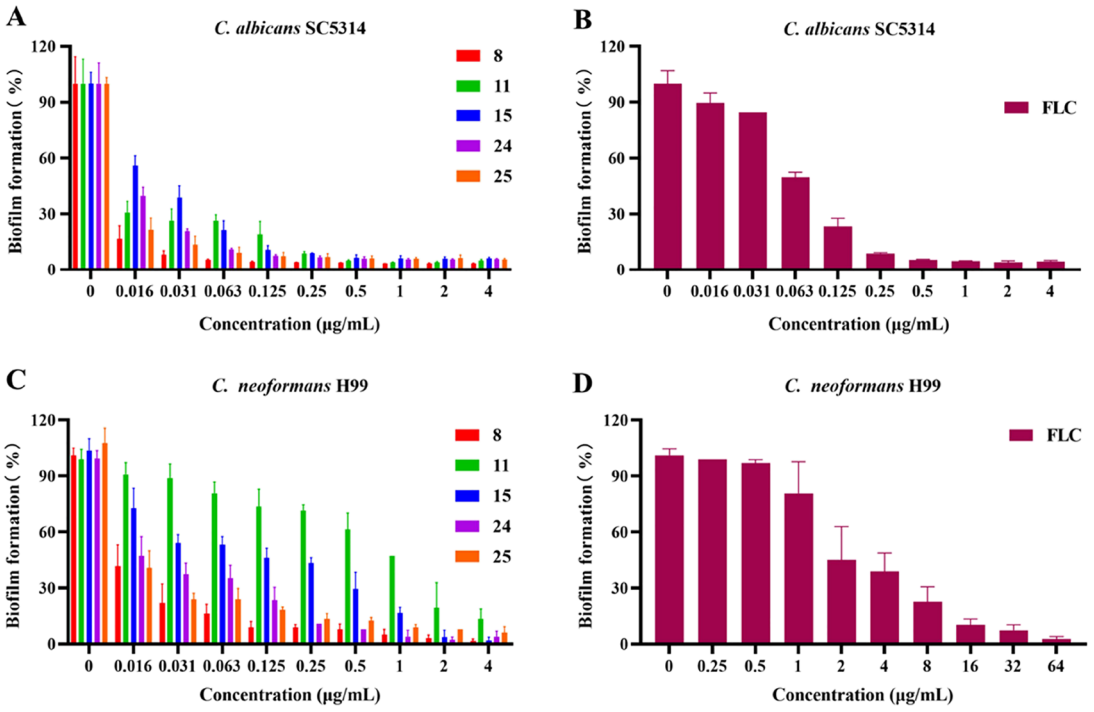

Figure 4. (A) Compounds **8**, **11**, **15**, **24**, and **25** inhibit *C. albicans* SC5314 biofilm formation *in vitro*. (B) Fluconazole inhibits *C. albicans* biofilm formation *in vitro*. (C) Compounds **8**, **11**, **15**, **24**, and **25** inhibit *C. neoformans* H99 biofilm formation *in vitro*. (D) Fluconazole inhibits *C. neoformans* H99 biofilm formation *in vitro*. Biofilm formation was evaluated by the CCK-8 assay, and the results were repeated three times. FLC, fluconazole.

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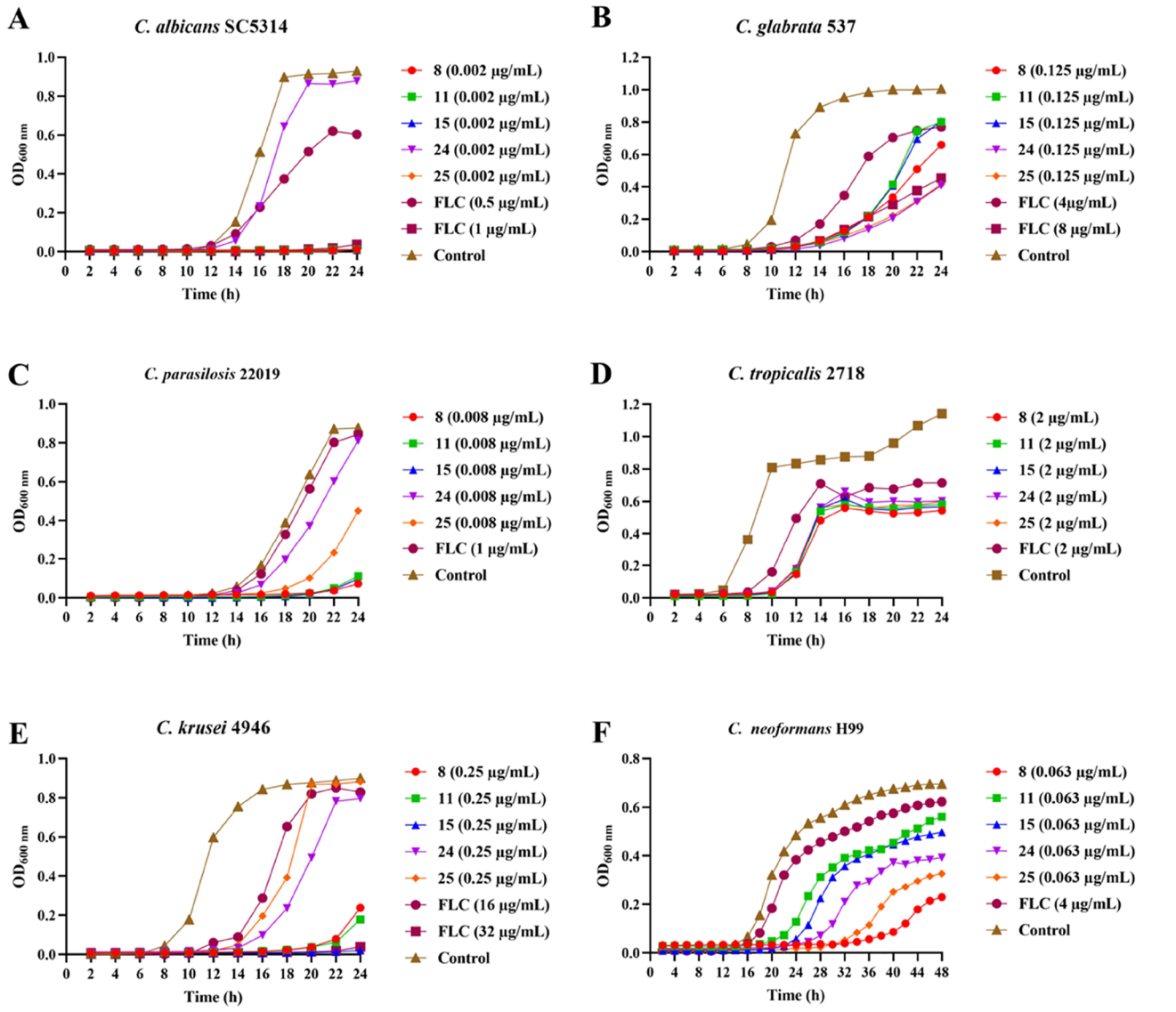

Figure 5. Growth curves of six pathogenic fungi in the presence of test compounds. (A) *C. albicans* SC5314, (B) *C. glabrata* 537, (C) *C. parapsilosis* 22019, (D) *C. tropicalis* 2718C, (E) *C. krusei* 4946, and (F) *C. neoformans* H99 were treated with different concentrations of test compounds as shown in the figure. Strains were grown in YPD medium with a starting inoculum of 10^3 CFU/mL. The strain without treatment was used as the control. Optical density (OD_{600nm}) was measured at a time interval of 2 h for 24 or 48 h.

at a concentration of 2 μg/mL but were comparable to FLC (Figure 5D). On the other hand, compound **15** was able to completely inhibit the growth of *C. krusei* 4946 at 0.25 μg/mL, whereas compounds **8** and **11** also showed strong inhibition and were significantly better than compounds **24** and **25** (Figure 5E). Finally, for *C. neoformans* H99, all compounds showed stronger inhibitory activity at 0.0625 μg/mL concentration than FLC at 4 μg/mL (Figure 5F), and the rank order of inhibitory activity of the five compounds was **8** > **25** > **24** > **15** ≈ **11**. In conclusion, compounds **8**, **11**, and **15** deserve further investigation based on growth curve assays.

2.6. Cytotoxicity and Hemolysis Assays. We then evaluated the cytotoxicity and hemolysis of compounds **8**, **11**, **15**, **24**, and **25**. FLC and AmB were used as reference drugs. The cytotoxicity of test compounds was determined by the CCK-8 assay: human umbilical vein endothelial cells (HUVECs) were treated with various concentrations (1, 2, 4, 8, 16, 32, and 64 μg/mL) of FLC, AmB, **8**, **11**, **15**, **24**, and **25** for 24 h. As shown in Figure 6, we found that compound **24**

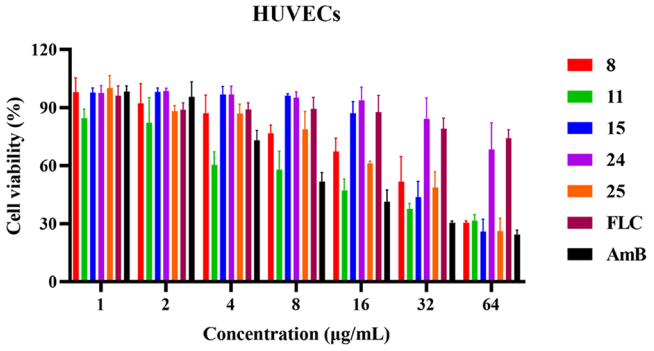

Figure 6. *In vitro* toxicity evaluation of **8**, **11**, **15**, **24**, and **25**, and fluconazole and amphotericin B. The cytotoxic effects of compounds **8**, **11**, **15**, **24**, and **25**, compared to that of FLC, on HUVEC viability was assessed by the CCK-8 test following a 2 h treatment; FLC: fluconazole; AmB: amphotericin B.

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showed no cytotoxicity, and no significant difference was observed between the tested compounds and FLC. However, compounds **8**, **11**, **15**, and **25** displayed mild cytotoxicity at high concentrations of 32−64 μg/mL, which was comparable to AMB.

The average hemolysis data for the test compounds against rabbit red blood cells are shown in Figure 7. AmB showed

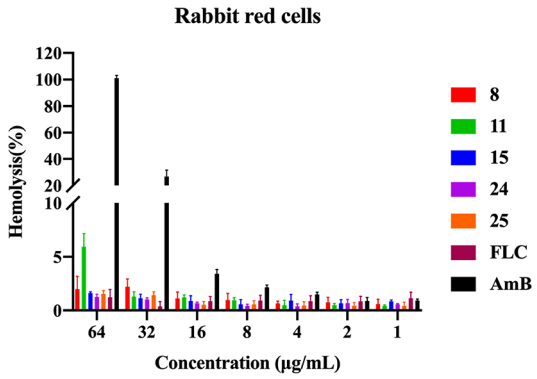

Figure 7. Hemolytic effect of **8**, **11**, **15**, **24**, and **25**, and fluconazole and amphotericin B against rabbit red blood cells at different indicated concentrations.

significant hemolysis of rabbit erythrocytes at 32 and 64 μg/mL with 26.8 and 100% hemolysis, respectively. In contrast, compounds **8**, **11**, **15**, **24**, and **25** and FLC did not show significant hemolysis at 64 μg/mL.

The cytotoxicity and hemolysis assays indicated that the designed tetrazole compounds have good safety profiles.

2.7. Inhibition of Human CYP450 Enzymes. Drug−drug interactions are the main side effect of azole drugs, so it is necessary to test the newly synthesized compounds for their CYP450 enzyme inhibitory activity. The activity against human CYP450 enzymes of compounds **8**, **11**, **15**, **24**, and **25** was investigated in cocktail-probe incubation systems, and the results are presented in Table 3. The IC_{50} value of

Table 3. Inhibition of Human CYP450 Enzyme Activity by Representative Compounds

	IC_{50} (μM)				
Compd	CYP1A2	CYP2C9	CYP2C19	CYP2D6	CYP3A4-M
8	77.4	2.37	0.262	0.619	8.04
11	>100	4.22	5.33	6.24	15.2
15	>100	4.08	1.39	0.549	19.8
24	>100	3.07	0.634	0.150	2.90
25	48.5	4.30	0.728	5.49	5.18
ketoconazole					0.0145

ketoconazole against CYP3A4 was 0.0145 μM. Compared with ketoconazole, compounds **8**, **11**, **15**, **24**, and **25** showed relatively lower inhibitory effects on CYP3A4 with IC_{50} values of 8.04, 15.2, 19.8, 2.90, and 5.18 μM, respectively. We determined that the halogen-containing compounds **8**, **24**, and **25** showed relatively strong inhibitory activities against the two enzymes CYP2C19 and CYP2D6, which is consistent with the data from our previous study.[22] Notably, compounds **11**, **15**, and **24** showed no inhibition of CYP1A2 with IC_{50} values of >100 μM. Among the five compounds, compound **11** exhibited the lowest CYP450 enzyme inhibition; nevertheless, it showed moderate inhibitory activity against CYP2C9, CYP2C9, and CYP2D6 with IC_{50} values of 4.22, 5.33, and 6.24 μM, respectively. Considering that CYP3A4 plays a prominent role in liver metabolism, we focused on comparing the IC_{50} values of compound **11** against CYP3A4 and the MIC_{50} values against *C. albicans* SC5314, which had a selectivity index (IC_{50}/MIC_{50}) greater than 1900.

2.8. Theoretical Evaluation of ADME/T Properties. Since compounds **8**, **11**, **15**, **24**, and **25** exhibited excellent antifungal activity, the ADMET (absorption, distribution, metabolism, excretion, toxicity) properties of these five compounds were theoretically evaluated by the DS-ADMET and DS-TOPKAT module.

As shown in Figure 8, compounds **24** and **25** are isomers of each other, so their prediction points overlap. All compounds

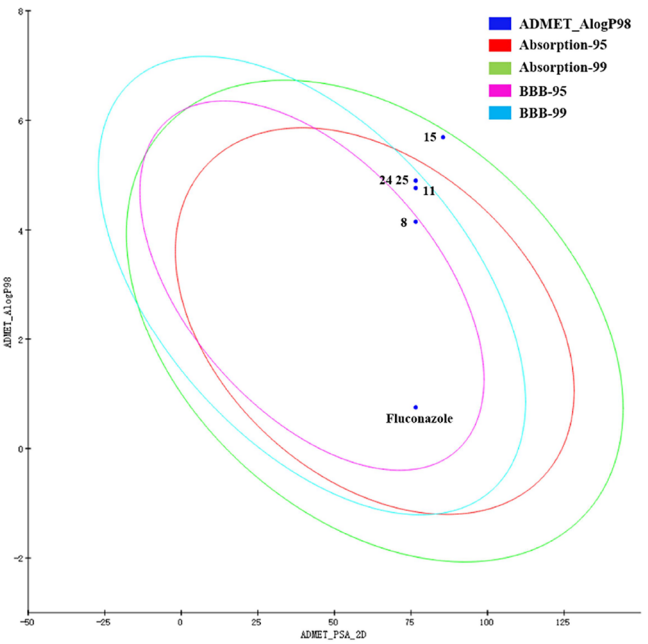

Figure 8. Evaluation chart of A log *P* and PSA of target compounds. The 95 and 99% confidence limit ellipses of BBB and human intestinal absorption (Absorption) models.

positioned in the 99% confidence ellipses for human intestinal absorption. Compounds **8**, **11**, **24**, and **25** positioned in the 99% confidence ellipses for human intestinal absorption and blood−brain barrier (BBB) penetration models. Only compound **8** positioned in the 95 and 99% confidence ellipses for both human intestinal absorption and BBB penetration models as FLC. These results indicated that with proper aqueous solubility, these compounds might enter blood circulation through intestinal absorption to exert their antifungal effect *in vivo*.

We also analyzed the A log *P* and polar surface area (PSA) of compounds **8**, **11**, **15**, **24**, and **25**. Their values were distributed within a reasonable absorption range relative to that of FLC, suggesting that these compounds can achieve therapeutic drug concentrations *in vivo* (Table 4). In addition, the toxicological properties of **8**, **11**, **15**, **24**, and **25** were further predicted according to the TOPKAT calculation module. We found these compounds to have no safety concerns with the exception of skin irritation, which significantly reduces the drug risk.

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Table 4. In Silico ADME/T Prediction of Target Compounds with Fluconazole[a]

ADME/T parameters	8	11	15	24	25	FLC[g]
aqueous solubility[a]	2	2	1	2	2	4
BBB penetration[b]	2	1	4	1	1	3
A log P98[c]	4.15	4.77	5.69	4.90	4.90	0.80
PSA[d]	76.6	76.6	85.5	76.6	76.6	76.6
PPB[e]	13.6	13.3	13.6	13.7	14.6	10.3
Ames mutagenicity	none	none	none	none	none	none
FDA[f] rodent carcinogenicity	none	none	none	none	none	none
Skin_Irritancy	irritant	irritant	irritant	irritant	irritant	none
Skin_sensitization	irritant	irritant	irritant	irritant	irritant	irritant
Mouse_Female_FDA[f]	none	none	none	none	none	none

[a]Abbreviations: a. Level of aqueous solubility predicted: 0 (extremely low), 1 (very low, but possible), 2 (low), 3 (good), 4 (optimal), 5 (too soluble), and 6 (warning: molecules with one or more unknown A log *P* calculations). b. BBB (blood−brain barrier), level of BBB penetration prediction: 0 (very high penetration), 1 (high), 2 (medium), 3 (low), and 4 (undefined). c. A log P98 (atom-based log P) (≤2.0 or ≥7.0: very low absorption). d. PSA (polar surface area) (>150: very low absorption). e. PPB, plasma protein binding. f. FDA, Food and Drug Administration. g. FLC, fluconazole.

3. CONCLUSIONS

In summary, 31 novel tetrazoles containing a pyrazole moiety were designed and synthesized based on our previous work and well characterized by HRMS, ^{1}H NMR, and ^{13}C NMR. All compounds exhibited moderate to excellent *in vitro* antifungal activities against seven human pathogenic fungi, including *C. albicans* SC5314, *C. glabrata* 537, *C. parapsilosis* 22019, *C. krusei* 4946, *C. tropicalis* 2718, *C. neoformans* H99, and *A. fumigatus* 7544. Among them, the most active compounds (**8**, **11**, **15**, **24**, and **25**) exhibited high potency against six drug-resistant *C. auris* clinical isolates. In addition, compared to fluconazole, compounds **8**, **11**, **15**, **24**, and **25** showed stronger anti-biofilm formation activity in *C. albicans* SC5314 and *C. neoformans* H99. Furthermore, the high potency of compounds **8**, **11**, **15**, **24**, and **25** was further confirmed by growth curve assays in YPD liquid medium. Notably, compounds **11** and **15** showed no inhibition of human CYP1A2 with IC_{50} values of >100 μM and low inhibitory activity against CYP3A4 with IC_{50} values of 15.2 and 19.8 μM, respectively, suggesting a low risk of drug−drug interactions. With high-potency *in vitro* and good safety profiles, these tetrazoles will be further investigated.

4. EXPERIMENTAL SECTION

4.1. Strains and Agents. *C. albicans* SC5314, *C. glabrata* 537, *C. krusei* 4946, *C. parapsilosis* 22019, *C. tropicalis* 2718, *C. neoformans* H99, *C. auris* clinical isolates (789, 791, 887, 888, BS12372, and D12), and *A. fumigatus* 7544 were from our laboratory's fungi collection. Frozen stocks were stored at −80 °C in YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) supplemented with 40% (v/v) glycerol and were cultured twice on Sabouraud dextrose agar (1% peptone, 4% dextrose, and 1.8% agar) plates at 30 °C. Strains were grown in YPD overnight at 30 °C with shaking prior to use in each experiment.

4.2. Chemistry. For the chemical part, all the solvents and agents (including reference drugs: amphotericin B, voriconazole, fluconazole, itraconazole, and posaconazole) were purchased from Adamas-beta and used directly or dried prior to use if necessary. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were reported in DMSO-d_6 by a Bruker AC-300P spectrometer (^{1}H NMR, 300 MHz; ^{13}C NMR, 75 MHz). Chemical shifts (δ values) and coupling constants (J values) are given in ppm and Hz, respectively. HRMS analyses were performed on an Agilent Technologies 6500 UHD Accurate-Mass Q-TOF LC/MS. HPLC purity of the target compounds was determined by Agilent Technologies 1260 HPLC, and all the compounds are >95% pure by HPLC analysis.

4.2.1. Procedure for the Synthesis of Compound 2. The intermediate compound **2** was synthesized by a known procedure as previously reported,[22,25,26] and the specific experimental operation details can be found in the Supplementary Material.

4.2.2. Procedure for the Synthesis of Compound 3. $TMSCHN_2$ (50 mmol, 2 M in hexane) was added to a solution of compound **2** (50 mmol) in toluene (50 mL), and the resulting mixture was stirred at 110 °C for 24 h under a N_2 atmosphere. The reaction was monitored by thin-layer chromatography (TLC). Once the reaction had finished, the mixture was cooled to room temperature and concentrated in a vacuum. The residues were dissolved in MeOH (50 mL), and KF (50 mmol) was added. The resulting mixture was stirred at room temperature for 1 h. After the reaction was finished, the solid was filtered, and the filtrate was concentrated in a vacuum and directly purified by reverse phase (acetonitrile/H_2O) to give target compound **3** in a good yield.

3: ^{1}H NMR (300 MHz, DMSO) δ 12.78 (s, 1H), 9.03 (s, 1H), 7.69 (s, 1H), 7.27−7.17 (m, 2H), 6.90 (t, J = 7.4 Hz, 1H), 6.32 (s, 1H), 5.75 (s, 1H), 5.06 (d, J = 13.9 Hz, 1H), 4.27 (d, J = 14.3 Hz, 1H), 3.70 (q, J = 7.0 Hz, 1H), 0.96 (d, J = 7.1 Hz, 3H).^{13}C NMR (75 MHz, DMSO) δ 164.00, 163.84, 160.74, 160.68, 160.52, 157.41, 157.25, 144.73, 130.71, 130.63, 130.58, 130.50, 125.15, 125.03, 111.52, 111.48, 111.25, 104.88, 104.54, 104.20, 76.74, 76.67, 55.44, 16.15.

4.2.3. Procedure for the Synthesis of Target Compounds 5−35. Various benzyl bromides or alkyl bromides (1.1 mmol) were added to a solution of compound **3** (1 mmol) in dimethylformamide (DMF) (3 mL), and the resulting mixture was stirred at 50 °C for 5 h. The reaction was monitored by TLC. After the reaction had finished, the mixture was cooled to room temperature and was directly purified by reverse phase (acetonitrile/H_2O) to give target compounds **5**−**35** in good yields.

5: ^{1}H NMR (300 MHz, DMSO) δ 8.98 (s, 1H), 7.83 (s, 1H), 7.37−7.15 (m, 7H), 6.90 (t, J = 8.7 Hz, 1H), 6.34 (s, 1H), 5.75 (s, 1H), 5.36 (s, 2H), 5.01 (d, J = 14.5 Hz, 1H), 4.32 (d, J = 14.3 Hz, 1H), 3.63 (q, J = 6.9 Hz, 1H), 0.96 (d, J = 7.1 Hz, 3H).^{13}C NMR (75 MHz, DMSO) δ 163.99, 163.82,

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160.73, 160.56, 157.39, 157.23, 152.71, 144.69, 138.24, 131.71, 130.73, 130.66, 130.53, 128.98, 127.99, 127.83, 125.21, 125.10, 125.05, 111.48, 111.25, 105.86, 104.92, 104.55, 104.21, 76.75, 76.68, 55.46, 55.40, 55.11, 15.93. HRMS (ESI) *m/z* calcd for $C_{21}H_{20}F_2N_6O$ $[M + H]^+$: 411.1739, found: 411.1740.

6: ^{1}H NMR (300 MHz, DMSO) δ 8.99 (s, 1H), 7.83 (s, 1H), 7.33−7.14 (m, 6H), 6.90 (t, J = 8.5 Hz, 1H), 6.33 (s, 1H), 5.73 (s, 1H), 5.34 (s, 2H), 5.01 (d, J = 14.3 Hz, 1H), 4.31 (d, J = 14.3 Hz, 1H), 3.62 (q, J = 6.7 Hz, 1H), 0.95 (d, J = 7.1 Hz, 3H).^{13}C NMR (75 MHz, DMSO) δ 163.99, 163.82, 163.65, 160.73, 160.43, 157.39, 157.23, 152.79, 144.71, 134.46, 134.42, 131.62, 130.72, 130.60, 130.52, 130.06, 129.95, 125.23, 125.11, 125.06, 115.91, 115.63, 111.52, 111.24, 105.92, 104.92, 104.55, 104.20, 76.74, 76.67, 55.44, 55.39, 54.30, 15.92. HRMS (ESI) *m/z* calcd for $C_{21}H_{19}F_3N_6O$ $[M + H]^+$: 429.1645, found: 469.1648.

7: ^{1}H NMR (300 MHz, DMSO) δ 8.99 (s, 1H), 7.84 (s, 1H), 7.41 (d, J = 7.9 Hz, 2H), 7.28−7.16 (m, 4H), 6.90 (t, J = 8.4 Hz, 1H), 6.34 (s, 1H), 5.73 (s, 1H), 5.36 (s, 2H), 5.01 (d, J = 14.4 Hz, 1H), 4.32 (d, J = 14.3 Hz, 1H), 3.63 (q, J = 7.1 Hz, 1H), 0.96 (d, J = 7.1 Hz, 3H).^{13}C NMR (75 MHz, DMSO) δ 164.00, 163.83, 160.74, 160.57, 160.51, 157.40, 157.24, 152.92, 144.70, 137.26, 132.66, 131.78, 130.74, 130.66, 130.61, 130.53, 129.72, 128.96, 125.27, 125.22, 125.10, 125.05, 111.47, 111.24, 105.98, 104.91, 104.53, 104.19, 76.75, 76.68, 55.46, 55.40, 54.32, 15.92. HRMS (ESI) *m/z* calcd for $C_{21}H_{19}ClF_2N_6O$ $[M + H]^+$: 445.1350, found: 445.1349.

8: ^{1}H NMR (300 MHz, DMSO) δ 8.99 (s, 1H), 7.83 (s, 1H), 7.71 (d, J = 8.0 Hz, 2H),7.26−7.15 (m, 2H), 7.04 (d, J = 8.0 Hz, 2H),6.90 (t, J = 9.4 Hz, 1H), 6.33 (s, 1H), 5.73 (s, 1H), 5.32 (s, 2H), 5.00 (d, J = 14.4 Hz, 1H), 4.31 (d, J = 14.4 Hz, 1H), 3.61 (q, J = 7.0 Hz, 1H), 0.95 (d, J = 7.1 Hz, 3H).^{13}C NMR (75 MHz, DMSO) δ 163.99, 160.73, 160.56, 157.39, 152.88, 144.72, 138.08, 137.75, 131.81, 130.60, 130.16, 125.28, 125.11, 111.48, 111.21, 105.96, 104.92, 104.55, 104.20, 94.03, 76.74, 76.66, 55.44, 54.49, 15.92. HRMS (ESI) *m/z* calcd for $C_{21}H_{19}F_2IN_6O$ $[M + H]^+$: 537.0706, found: 537.0711.

9: ^{1}H NMR (300 MHz, DMSO) δ 8.97 (s, 1H), 7.79 (s, 1H), 7.24−7.15 (m, 6H), 6.89 (t, J = 8.4 Hz, 1H), 6.32 (s, 1H), 5.75 (s, 1H), 5.30 (s, 2H), 5.01 (d, J = 14.3 Hz, 1H), 4.31 (d, J = 14.3 Hz, 1H), 3.62 (q, J = 7.2 Hz, 1H), 2.25 (s, 3H), 0.96 (d, J = 7.1 Hz, 3H).^{13}C NMR (75 MHz, DMSO) δ 164.00, 163.83, 160.74, 160.67, 160.57, 160.51, 157.40, 157.24, 152.63, 144.67, 137.23, 135.14, 131.50, 130.75, 130.67, 130.63, 130.55, 129.52, 127.93, 125.25, 125.20, 125.08, 125.03, 111.47, 111.23, 111.19, 105.80, 104.90, 104.53, 104.18, 76.77, 76.70, 55.48, 55.42, 54.95, 21.10, 15.96. HRMS (ESI) *m/z* calcd for $C_{22}H_{22}F_2N_6O$ $[M + H]^+$: 425.1896, found: 425.1899.

10: ^{1}H NMR (300 MHz, DMSO) δ 8.96 (s, 1H), 7.81 (s, 1H), 7.35 (d, J = 8.0 Hz, 2H), 7.24−7.17 (m, 4H), 6.89 (t, J = 8.4 Hz, 1H), 6.32 (s, 1H), 5.75 (s, 1H), 5.31 (s, 2H), 5.01 (d, J = 14.3 Hz, 1H), 4.30 (d, J = 14.3 Hz, 1H), 3.62 (q, J = 6.9 Hz, 1H), 1.23 (s, 9H), 0.96 (d, J = 7.2 Hz, 3H).^{13}C NMR (75 MHz, DMSO) δ 163.99, 163.82, 160.73, 160.67, 160.56, 160.51, 157.40, 157.24, 152.63, 150.45, 144.66, 135.24, 131.57, 130.76, 130.67, 130.63, 130.55, 127.67, 125.70, 125.25, 125.21, 125.09, 125.04, 111.46, 111.19, 105.82, 104.90, 104.53, 104.18, 76.77, 76.70, 55.47, 55.41, 54.85, 34.66, 31.53, 15.92. HRMS (ESI) *m/z* calcd for $C_{25}H_{28}F_2N_6O$ $[M + H]^+$: 467.2365, found: 467.2369.

11: ^{1}H NMR (300 MHz, DMSO) δ 8.97 (s, 1H), 7.81 (s, 1H), 7.25−7.16 (m, 6H), 6.89 (t, J = 8.8 Hz, 1H), 6.32 (s, 1H), 5.75 (s, 1H), 5.30 (s, 2H), 5.01 (d, J = 14.3 Hz, 1H), 4.30 (d, J = 14.3 Hz, 1H), 3.62 (q, J = 7.0 Hz, 1H), 2.84 (dt, J = 13.6, 6.8 Hz, 1H), 1.15 (d, J = 6.9 Hz, 6H), 0.96 (d, J = 7.1 Hz, 3H).^{13}C NMR (75 MHz, DMSO) δ 163.99, 163.82, 160.67, 160.56, 157.40, 157.23, 152.61, 148.21, 144.68, 135.61, 131.56, 130.74, 130.66, 130.53, 127.94, 126.87, 125.26, 125.21, 125.09, 125.04, 111.48, 111.23, 105.80, 104.91, 104.56, 104.20, 76.76, 76.69, 55.46, 55.40, 54.92, 33.55, 24.28, 15.92. HRMS (ESI) *m/z* calcd for $C_{24}H_{26}F_2N_6O$ $[M + H]^+$: 453.2209, found: 453.2209.

12: ^{1}H NMR (300 MHz, DMSO) δ 9.03 (s, 1H), 7.93 (s, 1H), 7.76 (d, J = 8.0 Hz, 2H), 7.46 (d, J = 8.0 Hz, 2H), 7.29−7.21 (m, 2H), 6.94 (t, J = 8.6 Hz, 1H), 6.41 (s, 1H), 5.78 (s, 1H), 5.53 (s, 2H), 5.06 (d, J = 14.3 Hz, 1H), 4.38 (d, J = 14.3 Hz, 1H), 3.68 (q, J = 7.2 Hz, 1H), 1.02 (d, J = 7.2 Hz, 3H).^{13}C NMR (75 MHz, DMSO) δ 164.00, 163.84, 160.74, 160.67, 160.57, 160.51, 157.40, 157.24, 153.11, 144.70, 143.07, 132.08, 130.74, 130.66, 130.61, 130.53, 128.81, 128.43, 127.97, 126.46, 125.97, 125.92, 125.87, 125.82, 125.27, 125.22, 125.10, 125.05, 122.86, 119.25, 111.50, 111.46, 111.23, 111.19, 106.11, 104.90, 104.53, 104.18, 76.75, 76.68, 55.44, 55.38, 54.47, 15.89. HRMS (ESI) *m/z* calcd for $C_{22}H_{19}F_5N_6O$ $[M + H]^+$: 479.1613, found: 479.1615.

13: ^{1}H NMR (300 MHz, DMSO) δ 8.98 (s, 1H), 7.90 (s, 1H), 7.65−7.53 (m, 4H), 7.20 (dd, J = 15.1, 8.8 Hz, 2H), 6.89 (t, J = 8.5 Hz, 1H), 6.36 (s, 1H), 5.73 (s, 1H), 5.48 (s, 2H), 5.02 (d, J = 14.3 Hz, 1H), 4.29 (d, J = 14.3 Hz, 1H), 3.64 (q, J = 6.9 Hz, 1H), 0.97 (d, J = 7.1 Hz, 3H).^{13}C NMR (75 MHz, DMSO) δ 164.00, 163.84, 160.74, 160.66, 160.57, 160.50, 157.39, 157.23, 153.14, 144.60, 139.84, 132.09, 131.90, 130.74, 130.66, 130.61, 130.53, 130.29, 130.10, 129.87, 129.45, 129.03, 126.34, 125.21, 125.16, 125.04, 124.99, 124.72, 124.67, 124.27, 124.22, 124.17, 124.12, 122.73, 119.12, 111.50, 111.23, 111.19, 106.04, 104.91, 104.56, 104.19, 76.72, 76.65, 55.44, 55.38, 54.31, 15.81. HRMS (ESI) *m/z* calcd for $C_{22}H_{19}F_5N_6O$ $[M + H]^+$: 479.1613, found: 479.1608.

14: ^{1}H NMR (300 MHz, DMSO) δ 8.99 (s, 1H), 7.88 (s, 1H), 7.82 (d, J = 7.7 Hz, 2H), 7.37 (d, J = 7.7 Hz, 2H), 7.24−7.16 (m, 2H), 6.90 (t, J = 8.3 Hz, 1H), 6.37 (s, 1H), 5.73 (s, 1H), 5.48 (s, 2H), 5.01 (d, J = 14.4 Hz, 1H), 4.33 (d, J = 14.4 Hz, 1H), 3.63 (q, J = 6.9 Hz, 1H), 0.97 (d, J = 7.1 Hz, 3H).^{13}C NMR (75 MHz, DMSO) δ 164.01, 163.84, 160.74, 160.67, 160.58, 160.51, 157.40, 157.24, 153.21, 144.72, 143.99, 132.97, 132.19, 130.73, 130.65, 130.61, 130.53, 128.50, 125.27, 125.22, 125.10, 125.05, 119.13, 111.51, 111.47, 111.24, 110.78, 106.16, 104.91, 104.53, 104.19, 76.74, 76.66, 55.44, 55.38, 54.51, 40.85, 15.89. HRMS (ESI) *m/z* calcd for $C_{22}H_{19}F_2N_7O$ $[M + H]^+$: 436.1692, found: 436.1701.

15: ^{1}H NMR (300 MHz, DMSO) δ 8.95 (s, 1H), 7.83 (s, 1H), 7.32 (s, 4H), 7.22−7.11 (m, 2H), 6.86 (t, J = 8.4 Hz, 1H), 6.31 (s, 1H), 5.70 (s, 1H), 5.37 (s, 2H), 4.98 (d, J = 14.2 Hz, 1H), 4.28 (d, J = 14.3 Hz, 1H), 3.59 (q, J = 7.4 Hz, 1H), 0.93 (d, J = 7.0 Hz, 3H).^{13}C NMR (75 MHz, DMSO) δ 163.82, 160.67, 157.39, 157.23, 152.94, 148.11, 144.70, 137.80, 131.86, 130.72, 130.64, 129.71, 125.28, 125.06, 122.20, 121.61, 118.80, 111.51, 111.20, 106.01, 104.92, 104.54, 104.20, 76.73, 76.66, 55.43, 54.20, 15.88. HRMS (ESI) *m/z* calcd for $C_{22}H_{19}F_5N_6O_2$ $[M + H]^+$: 495.1562, found: 495.1562.

16: ^{1}H NMR (300 MHz, DMSO) δ 8.98 (s, 1H), 7.86 (s, 1H), 7.39 (dd, J = 18.1, 8.4 Hz, 1H), 7.25−7.15 (m, 3H), 6.93 (dt, J = 17.2, 7.6 Hz, 2H), 6.35 (s, 1H), 5.74 (s, 1H), 5.48 (s, 2H), 5.00 (d, J = 14.3 Hz, 1H), 4.32 (d, J = 14.2 Hz, 1H), 3.61 (q, J = 7.0 Hz, 1H), 0.96 (d, J = 7.1 Hz, 3H).^{13}C NMR (75 MHz, DMSO) δ 164.00, 163.82, 160.73, 157.39, 157.23,

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153.08, 151.77, 151.61, 149.75, 149.57, 148.51, 148.35, 146.47, 146.30, 144.68, 132.03, 130.73, 130.65, 130.52, 127.70, 127.55, 125.51, 125.47, 125.43, 125.38, 125.25, 125.08, 125.03, 117.49, 117.27, 111.47, 111.20, 106.10, 104.91, 104.57, 104.19, 76.73, 76.66, 55.38, 55.32, 48.64, 15.84. HRMS (ESI) *m/z* calcd for $C_{21}H_{18}F_4N_6O$ $[M + H]^+$: 447.1551, found: 447.1551.

17: ^{1}H NMR (300 MHz, DMSO) δ 8.99 (s, 1H), 7.82 (s, 1H), 7.31−7.16 (m, 4H), 7.08 (t, J = 8.5 Hz, 1H), 6.89 (t, J = 8.4 Hz, 1H), 6.33 (d, J = 1.7 Hz, 1H), 5.75 (s, 1H), 5.39 (s, 2H), 5.00 (d, J = 14.4 Hz, 1H), 4.32 (d, J = 14.3 Hz, 1H), 3.61 (q, J = 6.9 Hz, 1H), 0.95 (d, J = 7.1 Hz, 3H).^{13}C NMR (75 MHz, DMSO) δ 164.14, 163.98, 163.83, 162.18, 162.01, 160.87, 160.71, 160.66, 160.57, 160.50, 158.89, 158.72, 157.39, 157.23, 152.95, 144.69, 131.95, 131.88, 131.82, 131.76, 130.73, 130.65, 130.60, 130.52, 125.25, 125.20, 125.08, 125.03, 121.49, 121.44, 121.29, 121.25, 112.32, 112.28, 112.04, 111.99, 111.50, 111.46, 111.23, 111.19, 106.00, 104.89, 104.78, 104.52, 104.44, 104.17, 104.09, 76.74, 76.67, 55.40, 55.33, 48.56, 48.52, 15.85. HRMS (ESI) *m/z* calcd for $C_{21}H_{18}F_4N_6O$ $[M + H]^+$: 447.1551, found: 447.1552.

18: ^{1}H NMR (300 MHz, DMSO) δ 8.98 (s, 1H), 7.85 (d, J = 2.1 Hz, 1H), 7.34−7.16 (m, 4H), 7.01−6.95 (m, 1H), 6.89 (td, J = 8.5, 2.3 Hz, 1H), 6.36 (d, J = 2.1 Hz, 1H), 5.75 (s, 1H), 5.42 (s, 2H), 5.02 (d, J = 14.4 Hz, 1H), 4.34 (d, J = 14.3 Hz, 1H), 3.62 (q, J = 7.2 Hz, 1H), 0.97 (d, J = 7.2 Hz, 3H).^{13}C NMR (75 MHz, DMSO) δ 164.01, 163.84, 160.74, 160.67, 160.57, 160.51, 160.17, 160.14, 158.02, 157.99, 157.40, 157.24, 156.99, 156.96, 154.82, 154.79, 153.13, 144.65, 132.05, 130.75, 130.67, 130.63, 130.55, 127.17, 127.06, 126.93, 126.83, 125.22, 125.17, 125.05, 125.00, 117.68, 117.56, 117.36, 117.24, 116.90, 116.82, 116.77, 116.58, 116.49, 116.43, 111.50, 111.46, 111.23, 111.19, 106.13, 104.89, 104.54, 104.51, 104.17, 76.72, 76.65, 55.38, 55.32, 48.79, 48.75, 15.81. HRMS (ESI) *m/z* calcd for $C_{21}H_{18}F_4N_6O$ $[M + H]^+$: 447.1551, found: 447.1551.

19: ^{1}H NMR (300 MHz, DMSO) δ 8.96 (s, 1H), 7.76 (d, J = 2.1 Hz, 1H), 7.52−7.42 (m, 1H), 7.24−7.12 (m, 4H), 6.89 (td, J = 8.5, 2.4 Hz, 1H), 6.30 (d, J = 2.2 Hz, 1H), 5.74 (s, 1H), 5.42 (s, 2H), 4.97 (d, J = 14.4 Hz, 1H), 4.28 (d, J = 14.4 Hz, 1H), 3.57 (q, J = 7.1 Hz, 1H), 0.94 (d, J = 7.2 Hz, 3H).^{13}C NMR (75 MHz, DMSO) δ 163.98, 163.81, 163.12, 163.01, 160.71, 160.65, 160.54, 160.49, 159.82, 159.72, 157.38, 157.22, 152.79, 144.62, 131.69, 131.56, 131.45, 130.72, 130.64, 130.60, 130.51, 125.21, 125.16, 125.04, 124.99, 113.21, 112.95, 112.70, 112.43, 112.33, 112.20, 112.10, 111.49, 111.44, 111.21, 111.17, 105.83, 104.87, 104.52, 104.49, 104.15, 76.74, 76.67, 55.31, 55.24, 43.07, 43.02, 15.76. HRMS (ESI) *m/z* calcd for $C_{21}H_{18}F_4N_6O$ $[M + H]^+$: 447.1551, found: 447.1552.

20: ^{1}H NMR (300 MHz, DMSO) δ 8.99 (s, 1H), 7.86 (s, 1H), 7.46−7.07 (m, 5H), 6.90 (t, J = 8.4 Hz, 1H), 6.34 (s, 1H), 5.72 (s, 1H), 5.36 (s, 2H), 5.01 (d, J = 14.4 Hz, 1H), 4.33 (d, J = 14.5 Hz, 1H), 3.62 (q, J = 7.6 Hz, 1H), 0.96 (d, J = 7.0 Hz, 3H).^{13}C NMR (75 MHz, DMSO) δ 164.00, 163.83, 160.50, 157.23, 153.00, 151.46, 151.29, 148.03, 147.59, 144.70, 136.03, 135.96, 131.83, 130.65, 125.22, 125.10, 124.85, 124.80, 124.76, 124.71, 118.19, 117.96, 117.14, 116.91, 111.48, 111.21, 106.07, 104.92, 104.54, 104.20, 76.72, 76.64, 55.35, 53.89, 15.87. HRMS (ESI) *m/z* calcd for $C_{21}H_{18}F_4N_6O$ $[M + H]^+$: 447.1551, found: 447.1553.

21: ^{1}H NMR (300 MHz, DMSO) δ 8.98 (s, 1H), 7.88 (s, 1H), 7.26−7.13 (m, 3H), 6.94−6.87 (m, 3H), 6.36 (s, 1H), 5.72 (s, 1H), 5.40 (s, 2H), 5.02 (d, J = 14.1 Hz, 1H), 4.34 (d, J = 14.3 Hz, 1H), 3.62 (q, J = 7.2 Hz, 1H), 0.97 (d, J = 7.1 Hz, 3H).^{13}C NMR (75 MHz, DMSO) δ 164.58, 164.41, 161.31, 161.14, 153.15, 144.67, 143.00, 142.88, 132.14, 130.66, 125.19, 125.03, 111.52, 111.25, 111.06, 110.95, 110.83, 110.72, 106.18, 104.92, 104.58, 104.20, 103.79, 103.45, 103.11, 76.69, 76.61, 55.39, 54.03, 15.82. HRMS (ESI) *m/z* calcd for $C_{21}H_{18}F_4N_6O$ $[M + H]^+$: 447.1551, found: 447.1548.

22: ^{1}H NMR (300 MHz, DMSO) δ 8.95 (s, 1H), 7.81 (d, J = 2.0 Hz, 1H), 7.59−7.48 (m, 1H), 7.24−7.14 (m, 3H), 6.88 (td, J = 8.5, 2.4 Hz, 1H), 6.30 (d, J = 2.2 Hz, 1H), 5.73 (s, 1H), 5.45 (s, 2H), 4.96 (d, J = 14.4 Hz, 1H), 4.28 (d, J = 14.4 Hz, 1H), 3.56 (q, J = 7.2 Hz, 1H), 0.94 (d, J = 7.2 Hz, 3H).^{13}C NMR (75 MHz, DMSO) δ 163.98, 163.81, 160.72, 160.65, 160.55, 160.49, 158.38, 157.38, 157.22, 155.08, 153.01, 150.53, 148.56, 147.13, 147.01, 145.30, 145.17, 144.62, 131.68, 130.73, 130.65, 130.61, 130.53, 125.21, 125.16, 125.04, 125.00, 118.45, 118.32, 118.20, 118.06, 115.30, 115.09, 115.01, 114.81, 112.39, 112.30, 112.24, 112.06, 112.01, 111.97, 111.92, 111.48, 111.20, 111.16, 105.95, 104.85, 104.48, 104.13, 76.72, 76.65, 55.27, 55.21, 43.20, 15.73. HRMS (ESI) *m/z* calcd for $C_{21}H_{17}F_5N_6O$ $[M + H]^+$: 465.1457, found: 465.1457.

23: ^{1}H NMR (300 MHz, DMSO) δ 8.98 (s, 1H), 7.87 (d, J = 2.2 Hz, 1H), 7.23−7.16 (m, 4H), 6.89 (td, J = 8.5, 2.5 Hz, 1H), 6.35 (d, J = 2.2 Hz, 1H), 5.71 (s, 1H), 5.36 (s, 2H), 5.01 (d, J = 14.5 Hz, 1H), 4.34 (d, J = 14.4 Hz, 1H), 3.62 (q, J = 7.1 Hz, 1H), 0.96 (d, J = 7.2 Hz, 3H).^{13}C NMR (75 MHz, DMSO) δ 164.00, 163.83, 160.74, 160.66, 160.57, 160.50, 157.39, 157.23, 153.24, 152.37, 152.32, 152.24, 152.19, 149.09, 149.04, 148.96, 148.91, 144.67, 140.22, 136.93, 136.73, 135.65, 135.59, 135.55, 135.49, 135.45, 135.39, 132.03, 130.74, 130.66, 130.61, 130.53, 125.24, 125.19, 125.07, 125.02, 112.69, 112.60, 112.49, 112.41, 111.50, 111.46, 111.23, 111.19, 106.23, 104.89, 104.55, 104.18, 76.68, 76.61, 55.39, 55.33, 53.66, 15.81. HRMS (ESI) *m/z* calcd for $C_{21}H_{17}F_5N_6O$ $[M + H]^+$: 465.1457, found: 465.1458.

24: ^{1}H NMR (300 MHz, DMSO) δ 9.00 (s, 1H), 7.86 (s, 1H), 7.68 (s, 1H), 7.43 (d, J = 8.3 Hz, 1H), 7.26−7.15 (m, 2H), 6.98−6.88 (m, 2H), 6.37 (s, 1H), 5.75 (s, 1H), 5.45 (s, 2H), 5.00 (d, J = 14.3 Hz, 1H), 4.34 (d, J = 14.3 Hz, 1H), 3.62 (q, J = 7.0 Hz, 1H), 0.97 (d, J = 7.1 Hz, 3H).^{13}C NMR (75 MHz, DMSO) δ 164.01, 163.84, 160.75, 160.67, 160.58, 160.51, 157.40, 157.24, 153.23, 144.71, 134.82, 133.60, 133.34, 132.33, 131.16, 130.74, 130.66, 130.62, 130.54, 129.29, 128.17, 125.24, 125.19, 125.07, 125.02, 111.48, 111.24, 106.13, 104.91, 104.56, 104.19, 76.76, 76.68, 55.43, 55.37, 52.28, 15.90. HRMS (ESI) *m/z* calcd for $C_{21}H_{18}Cl_2F_2N_6O$ $[M + H]^+$: 479.0960, found: 479.0956.

25: ^{1}H NMR (300 MHz, DMSO) δ 9.00 (s, 1H), 7.88 (s, 1H), 7.62 (d, J = 8.2 Hz, 1H), 7.51 (s, 1H), 7.26−7.15 (m, 3H), 6.90 (t, J = 8.3 Hz, 1H), 6.35 (s, 1H), 5.72 (s, 1H), 5.38 (s, 2H), 5.02 (d, J = 14.4 Hz, 1H), 4.31 (d, J = 14.3 Hz, 1H), 3.62 (q, J = 6.9 Hz, 1H), 0.96 (d, J = 7.0 Hz, 3H).^{13}C NMR (75 MHz, DMSO) δ 164.00, 163.83, 160.74, 160.67, 160.57, 157.40, 157.23, 153.14, 144.68, 139.41, 132.01, 131.54, 131.21, 130.73, 130.65, 130.53, 129.85, 128.19, 125.23, 125.19, 125.07, 125.02, 111.50, 111.46, 111.23, 111.19, 106.12, 104.90, 104.56, 104.19, 76.72, 76.65, 55.44, 55.37, 53.71, 15.82. HRMS (ESI) *m/z* calcd for $C_{21}H_{18}Cl_2F_2N_6O$ $[M + H]^+$: 479.0960, found: 479.0965.

26: ^{1}H NMR (300 MHz, DMSO) δ 8.98 (s, 1H), 7.85 (s, 1H), 7.52 (t, J = 7.5 Hz, 1H), 7.23−7.10 (m, 4H), 6.89 (t, J = 8.3 Hz, 1H), 6.35 (s, 1H), 5.74 (s, 1H), 5.47 (s, 2H), 5.00 (d, J = 14.3 Hz, 1H), 4.33 (d, J = 14.3 Hz, 1H), 3.62 (q, J = 7.0 Hz, 1H), 0.96 (d, J = 7.1 Hz, 3H).^{13}C NMR (75 MHz, DMSO) δ 164.00, 163.83, 160.74, 160.67, 160.57, 160.50, 157.40, 157.23,

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157.12, 153.83, 153.11, 144.67, 132.05, 130.74, 130.66, 130.56, 129.22, 129.18, 127.18, 126.99, 126.02, 125.96, 125.23, 125.18, 125.07, 125.02, 120.28, 120.05, 111.50, 111.46, 111.22, 111.18, 106.11, 104.89, 104.54, 104.17, 76.74, 76.67, 55.39, 55.33, 49.01, 48.96, 15.82. HRMS (ESI) *m/z* calcd for $C_{21}H_{18}ClF_3N_6O$ $[M + H]^+$: 463.1255, found: 463.1256.

27: ^{1}H NMR (300 MHz, DMSO) δ 8.99 (s, 1H), 7.87 (s, 1H), 7.48 (d, J = 7.1 Hz, 1H), 7.39 (t, J = 8.9 Hz, 1H), 7.28−7.15 (m, 3H), 6.89 (t, J = 8.0 Hz, 1H), 6.34 (s, 1H), 5.72 (s, 1H), 5.36 (s, 2H), 5.02 (d, J = 14.3 Hz, 1H), 4.31 (d, J = 14.3 Hz, 1H), 3.62 (q, J = 7.0 Hz, 1H), 0.96 (d, J = 7.2 Hz, 3H).^{13}C NMR (75 MHz, DMSO) δ 164.00, 163.83, 160.73, 160.56, 158.69, 157.39, 157.23, 155.43, 153.04, 144.69, 136.19, 136.14, 131.87, 130.72, 130.65, 130.52, 130.02, 128.73, 128.63, 125.24, 125.19, 125.07, 125.03, 119.97, 119.73, 117.62, 117.34, 111.47, 111.20, 106.07, 104.91, 104.57, 104.20, 76.71, 76.64, 55.42, 55.36, 53.70, 15.83. HRMS (ESI) *m/z* calcd for $C_{21}H_{18}ClF_3N_6O$ $[M + H]^+$: 463.1255, found: 463.1255.

28: ^{1}H NMR (300 MHz, DMSO) δ 8.95 (s, 1H), 7.78 (s, 1H), 7.44 (d, J = 7.5 Hz, 2H), 7.22−7.13 (m, 2H), 6.88 (t, J = 8.3 Hz, 1H), 6.29 (d, J = 1.9 Hz, 1H), 5.73 (s, 1H), 5.38 (s, 2H), 4.95 (d, J = 14.3 Hz, 1H), 4.27 (d, J = 14.3 Hz, 1H), 3.55 (q, J = 7.5 Hz, 1H), 0.92 (d, J = 7.2 Hz, 3H).^{13}C NMR (75 MHz, DMSO) δ 163.97, 163.07, 159.62, 157.21, 152.94, 144.64, 135.07, 134.89, 131.60, 130.71, 130.59, 125.21, 124.99, 122.57, 113.61, 113.22, 112.50, 111.49, 111.21, 105.89, 104.87, 104.49, 104.15, 76.73, 76.65, 55.27, 42.77, 15.75. HRMS (ESI) *m/z* calcd for $C_{21}H_{17}ClF_4N_6O$ $[M + H]^+$: 481.1161, found: 481.1163.

29: ^{1}H NMR (300 MHz, DMSO) δ 8.93 (s, 1H), 7.83 (s, 1H), 7.62−7.55 (m, 2H), 7.44 (t, J = 9.6 Hz, 1H), 7.18−7.10 (m, 2H), 6.84 (t, J = 8.2 Hz, 1H), 6.29 (s, 1H), 5.67 (s, 1H), 5.39 (s, 2H), 4.96 (d, J = 14.7 Hz, 1H), 4.23 (d, J = 14.1 Hz, 1H), 3.57 (q, J = 6.4 Hz, 1H), 0.90 (d, J = 7.0 Hz, 3H).^{13}C NMR (75 MHz, DMSO) δ 164.00, 163.83, 160.65, 160.49, 160.32, 157.38, 156.99, 153.17, 144.63, 135.55, 135.50, 134.82, 134.70, 131.97, 130.72, 130.63, 130.51, 126.60, 126.55, 125.22, 125.05, 124.76, 121.15, 118.04, 117.77, 117.23, 117.07, 116.81, 116.64, 111.51, 111.20, 106.11, 104.91, 104.57, 104.19, 76.70, 76.62, 55.41, 55.34, 53.62, 15.80. HRMS (ESI) *m/z* calcd for $C_{22}H_{18}F_6N_6O$ $[M + H]^+$: 497.1519, found: 497.1521.

30: ^{1}H NMR (300 MHz, DMSO) δ 8.98 (s, 1H), 7.82 (d, J = 2.2 Hz, 1H), 7.25−7.10 (m, 4H), 6.89 (td, J = 8.6, 2.5 Hz, 1H), 6.80−6.75 (m, 1H), 6.33 (d, J = 2.2 Hz, 1H), 5.74 (s, 1H), 5.32 (s, 2H), 5.02 (d, J = 14.5 Hz, 1H), 4.31 (d, J = 14.4 Hz, 1H), 3.76 (s, 3H), 3.63 (q, J = 7.1 Hz, 1H), 0.96 (d, J = 7.2 Hz, 3H).^{13}C NMR (75 MHz, DMSO) δ 164.00, 163.83, 160.74, 160.66, 160.57, 160.49, 157.38, 157.22, 153.01, 152.75, 149.78, 147.53, 147.39, 144.65, 134.95, 134.90, 131.64, 130.74, 130.66, 130.61, 130.53, 125.25, 125.20, 125.08, 125.03, 120.27, 120.18, 116.38, 116.14, 113.68, 113.65, 111.52, 111.21, 105.94, 104.92, 104.57, 104.20, 76.75, 76.68, 56.33, 55.50, 55.44, 54.64, 15.94. HRMS (ESI) *m/z* calcd for $C_{22}H_{21}F_3N_6O_2$ $[M + H]^+$: 459.1751, found: 459.1750.

31: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.51 (s, 1H), 7.44−7.36 (m, 2H), 6.82−6.69 (m, 2H), 6.18 (d, J = 2.1 Hz, 1H), 5.88 (s, 1H), 5.03 (d, J = 14.2 Hz, 1H), 4.30 (d, J = 14.1 Hz, 1H), 4.17 (q, J = 7.3 Hz, 2H), 3.68 (q, J = 7.1 Hz, 1H), 1.50 (t, J = 7.3 Hz, 3H), 1.02 (d, J = 7.1 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.41, 164.24, 161.10, 160.93, 160.33, 160.18, 157.06, 156.90, 153.24, 142.96, 130.66, 130.58, 130.53, 130.46, 129.47, 123.10, 123.05, 122.94, 122.89, 111.57, 111.52, 111.30, 111.25, 105.38, 104.45, 104.11, 104.09, 103.75, 56.32, 56.24, 47.07, 38.68, 38.61, 17.13, 15.42. MS (ESI) *m/z*: 349.2 $[M + H]^+$.

32: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.50 (s, 1H), 7.43−7.34 (m, 2H), 6.82−6.68 (m, 2H), 6.17 (d, J = 2.1 Hz, 1H), 5.91 (s, 1H), 5.04 (d, J = 14.2 Hz, 1H), 4.28 (d, J = 14.1 Hz, 1H), 4.08 (t, J = 7.0 Hz, 2H), 3.67 (q, J = 7.1 Hz, 1H), 1.90 (q, J = 7.2 Hz, 2H), 1.02 (d, J = 7.1 Hz, 3H), 0.92 (t, J = 7.4 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.41, 164.24, 161.10, 160.93, 160.33, 160.17, 157.06, 156.97, 153.26, 142.96, 130.67, 130.60, 130.55, 130.47, 130.26, 123.06, 123.00, 122.90, 122.85, 111.56, 111.52, 111.29, 111.25, 105.30, 104.45, 104.11, 103.75, 56.27, 56.20, 53.89, 38.64, 38.58, 23.69, 17.09, 11.09. MS (ESI) *m/z*: 363.2 $[M + H]^+$.

33: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.49 (s, 1H), 7.42−7.34 (m, 2H), 6.81−6.68 (m, 2H), 6.17 (d, J = 1.9 Hz, 1H), 5.91 (s, 1H), 5.03 (d, J = 14.1 Hz, 1H), 4.27 (d, J = 14.1 Hz, 1H), 4.12 (t, J = 7.1 Hz, 2H), 3.67 (q, J = 7.1 Hz, 1H), 1.90−1.80 (m, 2H), 1.38−1.25 (m, 2H), 1.02 (d, J = 7.1 Hz, 3H), 0.94 (t, J = 7.4 Hz, 3H).^{13}C NMR (75 MHz, $CDCl_3$) δ 164.40, 164.23, 161.09, 160.93, 160.34, 160.18, 157.06, 156.90, 153.18, 142.96, 130.67, 130.59, 130.54, 130.46, 130.18, 123.06, 123.01, 122.90, 122.85, 111.55, 111.50, 111.27, 111.23, 105.32, 104.44, 104.10, 104.08, 103.74, 56.27, 56.19, 51.96, 38.63, 38.57, 32.29, 19.74, 17.08, 13.53. MS (ESI) *m/z*: 377.3 $[M + H]^+$.

34: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.50 (s, 1H), 7.43−7.34 (m, 2H), 6.81−6.69 (m, 2H), 6.17 (d, J = 2.0 Hz, 1H), 5.91 (s, 1H), 5.04 (d, J = 14.1 Hz, 1H), 4.27 (d, J = 14.1 Hz, 1H), 4.11 (t, J = 7.1 Hz, 2H), 3.67 (q, J = 7.1 Hz, 1H), 1.92−1.82 (m, 2H), 1.38−1.24 (m, 4H), 1.02 (d, J = 7.1 Hz, 3H), 0.88 (t, J = 7.0 Hz, 3H).^{13}C NMR (75 MHz, $CDCl_3$) δ 164.41, 164.25, 161.10, 160.93, 160.34, 160.18, 157.06, 156.91, 153.19, 142.93, 130.67, 130.59, 130.54, 130.47, 130.17, 123.04, 122.99, 122.88, 122.83, 111.56, 111.51, 111.29, 111.24, 105.31, 104.45, 104.11, 104.09, 103.75, 56.29, 56.21, 52.25, 38.65, 38.58, 29.94, 28.69, 22.11, 17.08, 13.91. MS (ESI) *m/z*: 391.3 $[M + H]^+$.

35: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.50 (s, 1H), 7.42−7.34 (m, 2H), 6.81−6.67 (m, 2H), 6.16 (d, J = 1.9 Hz, 1H), 5.90 (s, 1H), 5.04 (d, J = 14.1 Hz, 1H), 4.27 (d, J = 14.1 Hz, 1H), 4.10 (t, J = 7.1 Hz, 2H), 3.67 (q, J = 7.1 Hz, 1H), 1.88−1.83 (m, 2H), 1.28 (s, 6H), 1.01 (d, J = 7.1 Hz, 3H), 0.86 (d, J = 6.6 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.47, 164.31, 161.16, 161.00, 160.41, 160.26, 157.14, 156.98, 153.25, 143.02, 130.74, 130.67, 130.62, 130.54, 130.27, 123.13, 123.08, 122.97, 122.92, 111.62, 111.57, 111.34, 111.30, 105.38, 104.52, 104.17, 104.15, 103.81, 77.01, 76.95, 56.37, 56.30, 52.34, 38.73, 38.66, 31.27, 30.30, 26.28, 22.54, 17.16, 13.98. MS (ESI) *m/z*: 405.3 $[M + H]^+$.

4.3. *In Vitro* Antifungal Activity Assay. The MIC values of the target compounds were tested according to the broth microdilution protocol of the CLSI (M27-A3 and M60).[27,28] Specific experimental operation details can be found in the Supplementary Material.

4.4. Determination of the Fungal Sterol Compositions. The fungal sterol compositions were determined by a previously reported method.[32] Specific experimental operation details can be found in the Supplementary Material.

4.5. Biofilm Formation Assay. The *in vitro* biofilm formation assay was performed as described previously.[33] Specific experimental operation details can be found in the Supplementary Material.

4.6. Growth Curve Assay. Growth curve assays were performed as described previously with slight modifications.[34] Briefly, strains were grown in YPD medium with a starting

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inoculum of 10^3 CFU/mL. Different concentrations of tested compounds were added. The final DMSO concentration was less than <1% of the total test volume. The $OD_{600\ nm}$ was measured at a time interval of 2 h for 48 h using a Tecan plate reader (Infinite F200 PRO, Switzerland) at 30 °C with shaking.

4.7. Cell Viability Assay and Hemolysis. The cytotoxic effect of compounds on HUVEC viability was assessed using the CCK-8 (Target Molecule Corp. USA) assay.[32] Also, the hemolytic activity of the tested compounds on rabbit erythrocytes was assessed by a previously reported method.[35] The specific experimental operation details can be found in the Supplementary Material.

4.8. Human CYP450 Enzyme Inhibition Assay. This experiment was outsourced to 3D BioOptima Co. Ltd., and the specific experimental operation details can be found in the Supplementary Material.

4.9. ADME/T Prediction. The theoretic ADMET properties of compounds were predicted using the DS-ADMET and DS-TOPKAT module as described previously.[36] The specific experimental operation details can be found in the Supplementary Material.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c01421.

Experimental operation details (Section 4); NMR spectra of target compounds; HRMS spectra of representative compounds; and HPLC spectra of representative compounds (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AmB	amphotericin B
ADMET	absorption, distribution, metabolism, excretion, toxicity
A. fumigatus	*Aspergillus fumigatus*
ATCC	American Type Culture Collection
CLSI	Clinical and Laboratory Standards Institute
Caco2	intestinal epithelial cells
C. albicans	*Candida albicans*
C. auris	*Candida auris*
C. glabrata	*Candida glabrata*
C. krusei	*Candida krusei*
C. parapsilosis	*Candida parapsilosis*
C. tropicalis	*Candida tropicalis*
C. neoformans	*Cryptococcus neoformans*
Cl	clearance
CYP	cytochrome P450
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
FDA	Food and Drug Administration
FLC	fluconazole
HRMS	high-resolution mass spectrometer
HUVECs	human umbilical vein endothelial cells
IFIs	invasive fungal infections
MIC	minimum inhibitory concentration
NMR	nuclear magnetic resonance
OTC	oteseconazole
POS	posaconazole
TEA	triethylamine
VOC	voriconazole
YPD	yeast extract−peptone−dextrose

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