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Discovery of novel indazole-linked triazoles as antifungal agents

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Abstract—The in vitro and in vivo activities of a series of (2*R*,3*R*)-2-(2,4-difluorophenyl)-3-(substituted indazol-1-yl)-1-(1*H*-1,2,4-triazol-1-yl)butan-2-ol as potential antifungal agents are described. In particular, the analog **12j** having 5-bromo substitution on the indazole ring exhibited significant antifungal activity against a variety of fungal cultures (*Candida* spp. and *Aspergillus* spp.). In addition, oral administration of **12j** showed its excellent efficacy against *Candida albicans* in a murine infection model and the significantly improved survival rates of the infected mice.

The incidence of systemic fungal infections such as candidosis, cryptococcosis, and aspergillosis has rapidly been increasing due to an increase of the immunocompromised hosts. Thus, the development of new and clinically useful antifungal azoles has been considered as one of the most attractive approaches for the treatment of these infections. In particular, attention has been paid to the triazole derivatives because of their broad antifungal spectrum and low toxicity.[1] Triazoles are known to displace lanosterol from lanosterol 14-demethylase (14 DM), a cytochrome P-450-dependent enzyme and block the biosynthesis of ergosterol, an essential component of fungal cell membrane.[2]

Fluconazole (**1**) is an antifungal agent of choice for the treatment of infections by *Candida albicans* and *Cryptococcus neoformans* due to its potent activity, excellent safety profile, and favorable pharmacokinetic characteristics.[3,4] However, its poor efficacy against *Aspergillus* infections[5] prompted the developments of new triazoles such as voriconazole (**2**),[6] albaconazole (**3**),[7] and ravuconazole (**4**),[8] which are therapeutically useful for the treatment of *Aspergillus* infection or under clinical trials (Fig. 1).

Considering necessity of the C3-methyl, which functions as an equivalent of the 13β-methyl group of lanosterol,[9,10] and the fluorobenzene moieties for high antifungal activity,[11,12] our structural modification focused on the variation of the arylalkyl moiety. Thus, our preliminary work on the development of new antifungal triazoles included an introduction of a variety of linker moiety such as hydrazine, semicarbazide, amide, urea, and amine. Among these, the hydrazine-linked triazole analogs, which have not been reported yet, were found to have the potent antifungal activity and the substituted benzyl-hydrazine analog (**5**) turned out to be most active. However, we extended the structural modification to a series of the indazole analogs depicted as **11** and **12**, which are the cyclic hydrazine fused with benzyl groups, because the hydrazine moiety was known as an inappropriate drug moiety in terms of stability.

In this paper, we describe synthesis and antifungal activity of the novel antifungal agents **11** and **12**.

The title compounds **11a**–**j**, **12b**, **12d**, and **12h**–**j** were prepared from the optically active oxirane intermediate (**10**),[13] which was conveniently derived from methyl (*R*)-lactate in eight steps as shown in Scheme 1. The amide intermediate **6** was prepared from methyl (*R*)-lactate by amidation with morpholine followed by THP protection. The amide **6** was then reacted with phenylmagnesium bromide, followed by epoxidation (Corey's reagent, DMSO) to afford the oxirane intermediate **7** as a diastereomeric mixture in favor of the desired diastereomer (1′*R*,2*R*)-**7** (4:1). The diastereomeric mixture was reacted with 1*H*-1,2,4-triazole in the presence of so-

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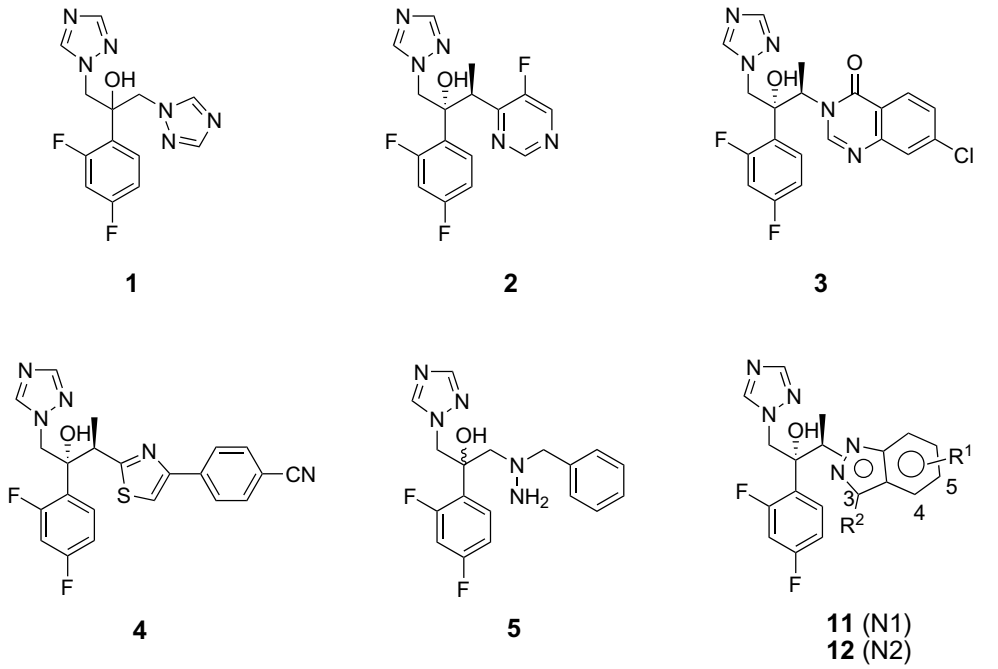
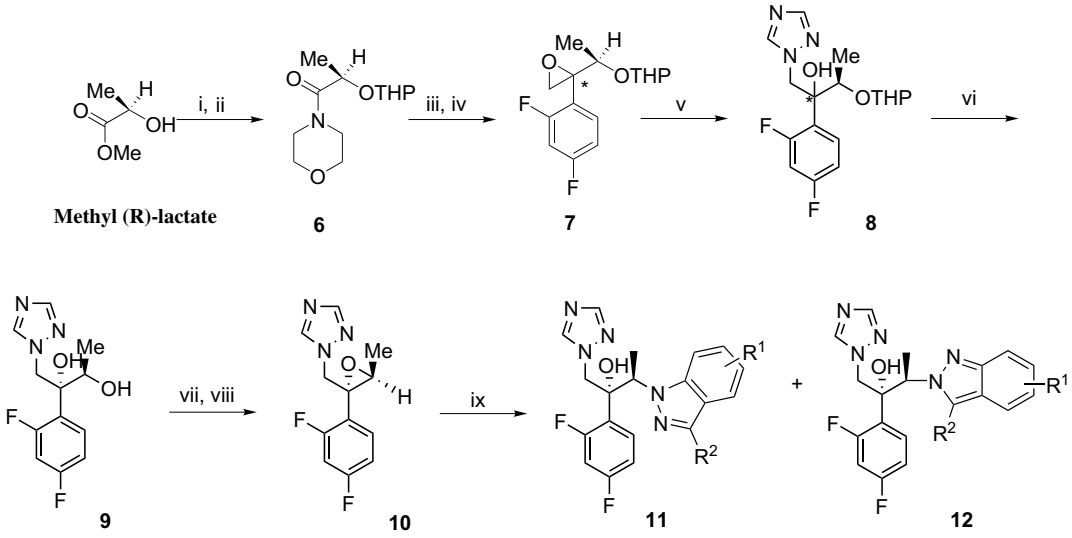


Figure 1. Structures of fluconazole (**1**), voriconazole (**2**), albaconazole (**3**), ravuconazole (**4**), and the novel triazole analogs (**5**, **11**, and **12**).



Scheme 1. Reagents and conditions: (i) Morpholine, 85 °C, 88%; (ii) 3,4-dihydro-2*H*-pyran, *p*-TsOH, CH_2Cl_2, 0 °C, 85%; (iii) 2,4-$F_2C_6H_3MgBr$, THF, 88%; (iv) $(CH_3)_3S^+OI^-$, NaH, DMSO; (v) 1,2,4-triazole, NaH, DMF, 80 °C, 61% for two steps; (vi) PPTS, EtOH, 55 °C, then recrystallization (IPE), 54%; (vii) MsCl, EtOAc–CH_2Cl_2, 0 °C; (viii) NaOMe, MeOH, 0 °C, 80% for two steps; (ix) indazoles, K_2CO_3, DMF, 120 °C, 25–47%.

dium hydride to give **8**. The THP group of **8** was removed with pyridinium *p*-toluenesulfonate and the resulting diol was recrystallized to give the diastereomerically pure diol (2*R*,3*R*)-**9**. The optically active diol **9** was converted to the oxirane (2*R*,3*S*)-**10** by selective mesylation and sodium methoxide-assisted epoxidation. Finally, the oxirane **10** was reacted with various indazoles in the presence of K_2CO_3 in DMF to give the condensation products[14] as a single product or a mixture of **11** and **12**, which were separated by flash column chromatography or preparative HPLC. The ^{1}H NMR spectra showed the characteristic resonance for C3-CH_3 of **11** in more upfield compared to those of **12**.[15,16] The ratio of isolated yields of the above two isomers suggested that the **11b**, **11d**, and **11h–j** isomers are the predominated one. The steric and electronic factors of substituents seem to affect the ratio of the produced isomers as reported earlier.[17]

The antifungal activities of **11a–j**, **12b**, **12d**, and **12h–j** were evaluated by in vitro broth microdilution assay and their MIC_{80}s are summarized in Table 1. Generally, the indazole analogs **11** and **12** were more potent than the hydrazine analog **5**. The C_5-nitro indazole analog (**11c**) exhibited the most potent activity as well as the broad spectrum compared to other analogs, which have

Table 1. In vitro antifungal activity of R^1-substituted analogs

Compound	R^1	R^2	MIC_{80} (μg/mL) of compounds and standard drugs against fungal culture[a]										
			CA	CG	CK	CN	CL	CT	CP	AF	Af	AT	AN
5			1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
11a	H	H	⩽0.015	2	0.5	0.063	0.063	0.125	0.063	2	1	2	4
11b	4-NO_2	H	⩽0.015	2	1	0.031	⩽0.015	⩽0.015	0.031	1	2	1	2
11c	5-NO_2	H	⩽0.015	4	0.25	0.063	⩽0.015	0.031	0.031	1	1	2	2
11d	6-NO_2	H	⩽0.015	8	0.5	0.25	⩽0.015	⩽0.015	⩽0.015	2	2	2	4
11e	7-NO_2	H	⩽0.015	4	0.5	0.125	⩽0.015	⩽0.015	⩽0.015	2	1	2	4
11f	5-NH_2	H	0.25	>8	8	1	0.25	1	2	nd	nd	nd	nd
11g	5-$N(CH_3)_2$	H	0.063	8	1	0.25	0.063	0.5	0.5	1	1	2	2
11h	5-F	H	⩽0.015	4	1	0.063	⩽0.015	0.25	0.125	4	4	4	8
11i	5-C1	H	⩽0.015	4	2	0.031	⩽0.015	0.5	0.125	2	4	4	8
11j	5-Br	H	⩽0.015	4	2	⩽0.015	⩽0.015	1	0.125	2	4	4	4
12b	4-NO_2	H	⩽0.015	>8	4	0.063	0.125	1	0.25	1	2	1	4
12d	6-NO_2	H	0.063	>8	>8	0.5	0.5	8	0.25	32	32	128	64
12h	5-F	H	⩽0.015	4	0.5	0.063	⩽0.015	0.125	0.031	0.25	0.5	0.5	1
12i	5-C1	H	⩽0.015	4	0.125	0.015	⩽0.015	0.031	⩽0.015	0.25	0.5	0.5	1
12j	5-Br	H	⩽0.015	2	0.125	⩽0.015	⩽0.015	⩽0.015	⩽0.015	0.25	0.5	0.5	1
1 (Fluconazole)			2	>8	>8	8	8	>8	>8	>128	>128	>128	>128

nd, not determined.

[a] CA, *C. albicans* ATCC 36082, CG, *C. glabrata*, ATCC 34138. CK, *C. krusei* ATCC 6528, CN, *Cryptococcus neoformans* ATCC 24065, CL, *Candida lusitanea* ATCC 42720, CT, *Candida tropicalis* ATCC 750, CP, *Candida parapsilosis* ATCC 21019, AF, *Aspergillus fumigatus* ATCC 16424, Af, *Aspergillus flavus* ATCC MYA-1004, AT, *Aspergillus terreus* ATCC 28301, AN, *Aspergillus niger* ATCC 9042.

Table 2. In vitro antifungal activity of R^1 and R^2-substituted compounds

Compound	R^1	R^2	MIC_{80} (μg/mL) of compounds and standard drugs against fungal culture										
			CA	CG	CK	CN	CL	CT	CP	AF	Af	AT	AN
11c	5-NO_2	H	⩽0.015	4	0.25	0.063	⩽0.015	0.031	0.031	1	1	2	2
11k	5-NO_2	Me	0.015	8	4	0.25	0.063	4	0.125	4	16	8	16
111	5-NO_2	Ph	2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
11h	5-F	H	⩽0.015	4	1	0.063	⩽0.015	0.25	0.125	4	4	4	8
11m	5-F	Me	0.031	8	4	0.5	0.125	4	0.5	4	8	4	16
11n	5-F	Et	⩽0.015	4	2	0.125	0.063	2	0.125	16	>16	>16	>16
11o	4-F	Me	⩽0.015	8	1	0.125	0.125	1	0.25	16	>16	>16	>16
11p	6-F	Me	0.031	>8	8	0.5	0.25	>8	0.5	16	>16	>16	>16
11q	5-C1	Me	⩽0.015	4	2	0.25	⩽0.015	1	0.125	>16	>16	>16	>16
11r	5-CF_3	Me	⩽0.015	8	4	1	0.25	>8	1	>16	>16	>16	>16

nd, not determined.

nitro-substituent at other position. In particular, the utmost of **11c** in antifungal activity was shown against *Aspergillus* spp.

Thus, other substituents (**11f–11j**) were introduced at the C_5-position of the indazole ring and the antifungal activities of the corresponding analogs were evaluated. The analogs with halogens at C_5-position (**11h–j**) showed remarkable antifungal activities against *Aspergillus* spp. Generally, the potency decreased especially against *Candida glabrata* and *Candida krusei*, known as the fluconazole-resistant species, as the size of halogen increases. Introduction of free amines at C_5-position (**11f**) almost eliminated the antifungal activity. However, the *N*,*N*-dimethyl amine-substituted analog (**11g**) showed an antifungal activity although it was less potent against *Candida* spp. than the analogs, which possess the electron-withdrawing substituent such as the nitro group. The *N*-2 regioisomers **12h–j** having halogen groups were generally more active than the *N*-1 isomers

Table 3. In vitro antifungal activity of **12j**

Compound	MIC_{80} (μg/mL) of **12j** and standard drugs against fungal culture										
	CA	CG	CK	CN	CL	CT	CP	AF	Af	AT	AN
12j	<0.015	2	0.125	⩽0.015	⩽0.015	⩽0.015	⩽0.015	0.25	0.5	0.5	1
Fluconazole	2	>8	>8	8	8	>8	>8	>128	>128	>128	>128
Voriconazole	⩽0.015	4	1	0.125	0.063	0.25	0.25	0.25	0.5	1	1
Albaconazole	⩽0.015	8	0.25	⩽0.015	⩽0.015	0.031	⩽0.015	0.5	0.5	1	2
Ravuconazole	⩽0.015	2	0.125	⩽0.015	⩽0.015	⩽0.015	⩽0.015	0.125	0.5	0.5	1
Amphotericin B	nd	nd	nd	nd	nd	nd	nd	1	4	2	0.5

nd, not determined.

Table 4. In vitro hepatocyte toxicity of the selected analogs

	11a	12j	11o	11r	Ketoconazole
CC_{50} (μM)	599.0 ± 124.4	141.3 ± 4.3	8.1 ± 2.31[a]	2.9 ± 2.8[a]	51.0 ± 1.6

[a] % Inhibition at 50 μM. Values are means of two or three (for CC_{50}) experiments.

Table 5. In vivo efficacy (mean survival days) in *Candida albicans* infection model in mice

	Mean survival days	Control
11a	6.1	3.5
11c	12.4[a]	2.0
12j	13.5[a]	7.2

nd, not determined.
[a] $P < 0.05$ versus each vehicle control.

11h–j. However, The *N*-2 regioisomers **12b**, **d** having nitro groups were quite less active than the *N*-1 isomers **11b–e**.

Next, we investigated the C_3-substituent (R^2 group) effect of indazole ring on the antifungal activity, as shown in Table 2. The antifungal activity decreased as the size of the R^2 increases. Especially, the phenyl derivative **11l** was almost devoid of antifungal activity. In addition, the MICs of the analogs **11m**, **11o**, and **11p** possessing methyl as R^2 and fluorine as R^1 at various positions were compared. Again, the 5-fluoro analog **11m** turned out to be the most potent compound among the C3-methyl (R^2) series. The other C3-methyl analogs **11q** and **11r** were almost inactive against *Aspergillus* spp.

Table 3 shows the in vitro activity of **12j** against *Candida* spp. and *Aspergillus* spp. The analog **12j** exhibited an excellent antifungal activity and broad spectra against most of the fungal pathogens tested, which is superior or comparable to those of the currently available antifungal agents.

The hepatotoxic potential of the analogs **11a**, **12j**, **11o**, and **11r** was determined in human liver cancer cell HepG2 by the neutral red uptake assay according to the protocol described previously.[18,19] Ketoconazole was used as a reference antifungal agent. The CC_{50} (half concentration of cytotoxicity) value of ketoconazole (51 μM) was quite close to the CC_{50} which was previously reported. The hepatotoxic potentials of all the tested analogs were lower than that of ketoconazole (Table 4). In particular, the CC_{50}s of **11a** and **12j** were 599 μM and 141 μM, respectively.

In vivo efficacy of the selected analogs, which exhibited excellent in vitro antifungal activities, was evaluated in the systemic *C. albicans* infection model in mice.[20] The results are summarized in Table 5. Each analog was compared to the untreated control group. The analogs **11c** and **12j** showed good efficacies on oral administration and the significantly improved survival rates of the infected mice.

In conclusion, the indazole analogs with C_3-H and C_5-halogen substituent, respectively, were identified as the promising leads for antifungal therapy. These analogs showed potent antifungal activity, broad spectrum, significantly low hepatocyte toxicity, and good oral efficacy.

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15. Unambiguous assignments of the structures for *N*-1 and *N*-2 isomer were obtained by a 1D NOE experiment. Irradiation of the signal for the C-3 proton on indazole ring of one isomer resulted in an enhancement of the signals for the protons attached to C3-CH_3 and C3-H, consistent with the *N*-2 isomer. As required, the corresponding experiment with the *N*-1 isomer led to the quite low enhancement of the signal for the proton attached to C3-CH_3 only.
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20. SPF (specific pathogen-free) female ICR mice were used in these studies. Mice were infected systemically by intravenous injection of *C. albicans* ATCC 36082 (inoculum size about 5×10^5 per animal). Treatment of the selected analogs, which were orally administered, was started after 2 h post-infection and the antifungal agents were given once a day for 7 consecutive days. All the analogs were administered with 20 mg/kg of once daily doses. Efficacy was determined by mean of survival days for 21 or 28 days after infections.