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Novel antifungal agents: Triazolopyridines as inhibitors of β-1,6-glucan synthesis

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ABSTRACT

Preparations and in vitro antifungal activities of triazolopyridines, imidazopyridines, and a pyrazolopyridine were reported. Among those scaffolds, triazolopyridine was found to be the specific inhibitor of the synthesis of β-1,6-glucan, an essential component of the fungal cell wall, and to show potent antifungal activities against several *Candida* species.

1. Introduction

Fungal infection has dramatically increased in immunosuppressed patients such as those with AIDS or who have had organ or marrow transplants over the past decades.[1–3] Currently, some antifungal agents of major drug classes: azoles, polyenes, and candins, have been launched and are demonstrating remarkable success for the treatment of fungal systemic infections.[2–7] However, these usages are often limited mainly due to their unsatisfactory antifungal activity, narrow spectrum, and side effects, causing rapid development of drug resistance and a high rate of mortality.[7–10] In consideration of these facts, alternative agents for the treatment and prevention of fungal infections are urgently required, preferably with a novel mode of action.

Pyridobenzimidazole derivatives **1**, **2**, and **3** have been reported as antifungal agents inhibiting the synthesis of β-1,6-glucan, an essential component of the fungal cell wall (Fig. 1).[11,12] It was also reported that their primary target was one of the β-1,6-glucan syntheses encoded by *KRE6* gene, which was conserved in various fungi, and that no homolog was found in mammalian cells.[13–15] These facts suggest that Kre6p could be a novel target for the treatment of fungal infections. According to the previous structure–activity relationships study (SARs) by Takeshita et al., substituents such as cyano, methyl, phenyl, and (3*S*)-(dimethylamino)pyrrolidine on the pyridine core of **2** were required for potent antifungal activity against *Candida* species (*Candida* spp.).[12] However, the role of the benzene core in the tricyclic scaffold has not been elucidated so far. Although compound **2** showed potent antifungal activity, its physicochemical properties such as water solubility and metabolic stability were not satisfactory for drug therapies. It is known that water solubility and metabolic stability of compounds are often improved by reducing their lipophilicity. If certain bicyclic compounds in which the benzene core was removed from tricyclic **2** showed antifungal activity in the same manner as compound **2**, we could reduce lipophilicity and improve their physicochemical properties. Herein, we would like to report the design, synthesis, and evaluation of antifungal activity of novel bicyclic derivatives such as triazolopyridines, imidazopyridines, and a pyrazolopyridine.

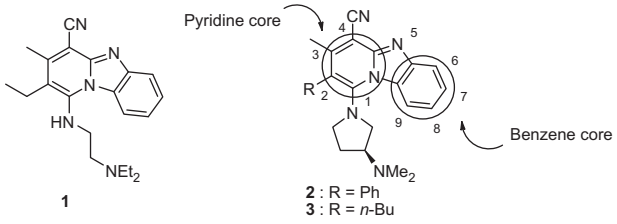

Figure 1. The structure of compounds **1**–**3**.

2. Design

As shown in Figure 2, three new heteroaromatic bicyclic scaffolds: triazolopyridines, imidazopyridines, and pyrazolopyridines,

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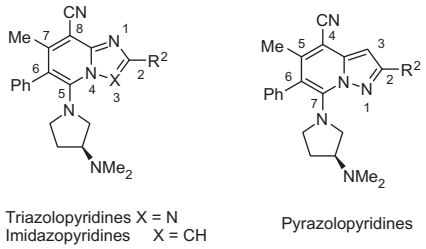


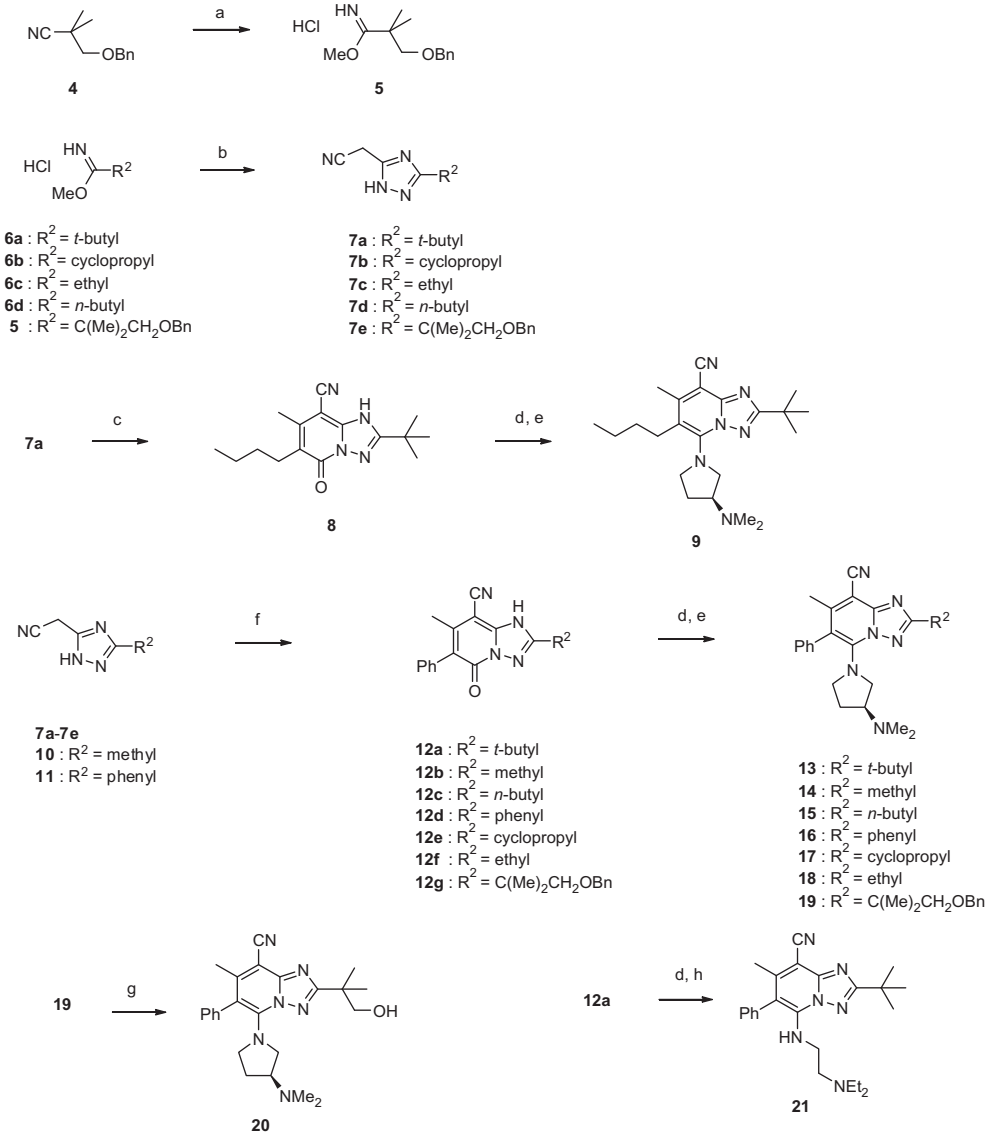
Figure 2. Design of novel fused-pyridine scaffolds.

were designed. Based on the results reported by Takeshita, substituents such as cyano, methyl, phenyl, and (3*S*)-(dimethylamino)pyrrolidine on the rings were considered to be essential to show antifungal activity against *Candida* spp. Therefore, we decided to fix these substituents and to focus on finding efficient substituents on R^2.

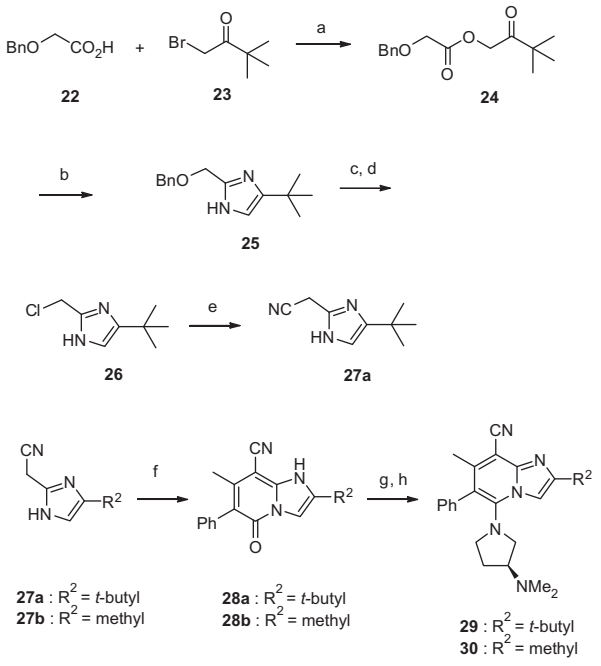
3. Chemistry

Triazolopyridines **9** and **13**–**21** were synthesized as depicted in Scheme 1. Imidoate **5** was synthesized from cyanide **4**.[16] Compounds **6a**–**6d**[17,18] and **5** were treated with cyanoacetohydrazide to give triazoles **7a**–**7e**. Compound **7a** was then condensed with ethyl 2-acetylhexanoate in the presence of ammonium acetate to afford **8**, which was chlorinated with $POCl_3$ followed by the introduction of (3*S*)-3-(dimethylamino)pyrrolidine to give triazolopyridine **9**. Triazolopyridines **13**–**19** and **21** were obtained from triazoles **7a**–**7e**, **10,** and **11** following a similar manner as described above. Compound **20** was obtained by hydrogenolysis of **19**.

Imidazopyridines **29** and **30** were synthesized as shown in Scheme 2. Imidazole **27a** was prepared in five steps from (benzyloxy)acetic acid (**22**) and 1-bromo-3,3-dimethylbutan-2-one (**23**). Compounds **27a** and **27b**[19] were converted to corresponding imidazopyridines **29** and **30** by the same manner as described in Scheme 1.



Scheme 1. Reagents: (a) HCl, MeOH, 59%; (b) cyanoacetohydrazide, NaOH, MeOH, 53–88%; (c) ethyl 2-acetylhexanoate, $AcONH_4$, 63%; (d) $POCl_3$; (e) (3*S*)-3-(dimethylamino)pyrrolidine, Et_3N, DMF; (f) ethyl 3-oxo-2-phenylbutanoate, $AcONH_4$, 12–50%; (g) H_2, 10% Pd–C, MeOH, 87%; (h) 2-(diethylamino)ethylamine, Et_3N, DMF, 64% (two steps).

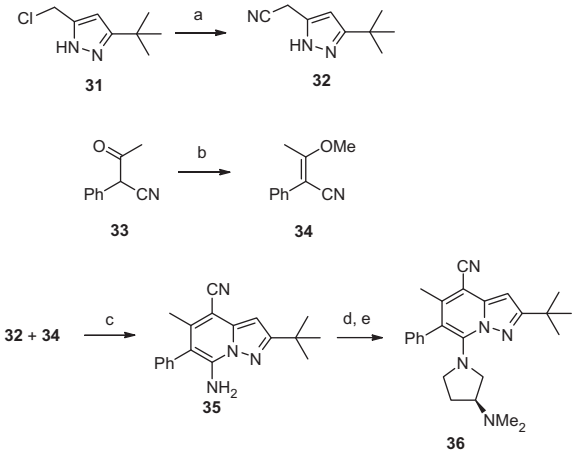


Scheme 2. Reagents: (a) K_2CO_3, DMF, 80%; (b) $AcONH_4$, 76%; (c) H_2, 10% Pd–C, EtOH; (d) $SOCl_2$, 80% (two steps); (e) KCN, EtOH–H_2O, 67%; (f) ethyl 3-oxo-2-phenylbutanoate, $AcONH_4$, 33–61%; (g) $POCl_3$; (h) (3*S*)-3-(dimethylamino)pyrrolidine, Et_3N, DMF; 24–96% (two steps).

Synthesis of pyrazolopyridine **36** was accomplished from pyrazole **31**[20] as shown in Scheme 3. Pyrazole **31** was converted to cyanide **32**, which on treatment with but-2-enenitrile **34** to give pyrazolopyridine **35**. Sandmeyer reaction[21] of **35**, followed by the introduction of (3*S*)-3-(dimethylamino)pyrrolidine gave pyrazolopyridine **36**.

4. Results and discussion

Synthesized compounds **9**, **13**–**21**, **29**, **30**, and **36** were evaluated for their in vitro antifungal activity against *Saccharomyces cerevisiae* (*S. cerevisiae*), *Candida albicans* (*C. albicans*), *Candida glabrata* (*C. glabrata*), *Candida tropicalis* (*C. tropicalis*), and *Candida krusei* (*C. krusei*). Pyridobenzimidazoles **2**, **3**, and Fluconazole (FLCZ) were used as positive control agents.



Scheme 3. Reagents: (a) KCN, EtOH–H_2O, 85%; (b) $MeC(OMe)_3$, 57%; (c) lithium diisopropylamide, THF, 42%; (d) *t*-BuONO, $CuCl_2$; (e) (3*S*)-3-(dimethylamino)pyrrolidine, Et_3N, DMF, 12% (two steps).

The MIC-0 (the lowest drug concentration producing an optically clear well) of the triazolopyridine derivatives **9**, **13**–**16**, and **21** were shown in Table 1. All the derivatives showed no inhibitory activity against *C. albicans*. Although pyridobenzimidazole **3** with *n*-butyl group exhibited potent activity against *C. glabrata* (⩽0.125 μg/mL), the corresponding triazolopyridine **9** showed weak antifungal activity against *C. glabrata* (32 μg/mL). However, the 6-phenyl analog **13** exhibited potent activity against *C. glabrata* (⩽0.125 μg/mL). The substituents at the 2-position of compounds **13**–**16** strongly affected their antifungal activities. Among 2-substituted analogs **13**–**16**, 2-*tert*-butyl derivative **13** was proved to exhibit the most potent inhibitory activity against *Candida* spp. The methyl and *n*-butyl derivatives **14** and **15** showed weak antifungal activity against *C. glabrata* (2 and 1 μg/mL, respectively). No activity was observed for 2-phenyl derivative **16**. The 5-[(3*S*)-3-(dimethylamino)pyrrolidin-1-yl] (cyclic amine) derivative **13** showed more potent antifungal activity than the 5-{[2-(diethylamio)ethyl]amino} (acyclic amine) derivative **21**, which was in agreement with the study of pyridobenzimidazole derivatives.[12] Among these derivatives, **14** showed significantly improved water solubility and metabolic stability.

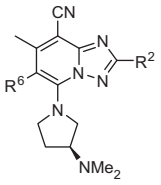
Since our product-treated cells often grew well but were severely clumped and could not be uniformly mixed, it was difficult to visually define the endpoint. To measure a reproducible and accurate MIC, an oxidation–reduction indicator, alamar blue was added to the medium.[11] Our compounds exhibited trailing growth phenomenon, which lead to significant differences in the MIC values among *C. albicans* and *C. tropicalis*.[25] For that reason, MIC-2 (the lowest drug concentration showing 50% growth inhibition compared to the control without drug) was measured as an endpoint for the experiments with *Candida* spp. (Table 2). Compared to the results of the MIC-0s for compound **2**, **3**, **13**, and FLCZ, the MIC-2s showed similar tendency of SARs and thus seemed not to have any problem to evaluate our derivatives in detail (Table 2). The MIC-2s of various triazolopyridines **13**, **17**-**20**, imidazopyridines **29** and **30**, and a pyrazolopyridine **36** were shown in Table 2. Almost all the bicyclic compounds showed no inhibitory activity against *C. albicans*. Among several 2-aliphatic triazolopyridine analogs, 2-*tert*-butyl derivative **13** exhibited the most potent growth inhibition against *Candida* spp., followed by cyclopropyl and ethyl derivatives **17** and **18** (0.25, 1, and 1 μg/mL against *C. tropicalis*, respectively). Results obtained above suggested that the preferable number of carbon atoms for potent antifungal activity was two to four for a substituent at the 2-position. Moreover, a sterically bulky substituent, such as *tert*-butyl and cyclopropyl, was required for potent antifungal activity. Interestingly, 2-(benzyloxy)-1,1-dimethylethyl derivative **19**, possessing a rather bulky substituent, also gave potent activity. In addition, the hydroxyl group, attached to the bulky substituent as in compound **20**, retained moderate activity against *C. glabrata* and *C. tropicalis* (0.25 and 0.25 μg/mL, respectively). These results indicated that the hydrophilic functional group, such as hydroxyl or alkoxy group, at the 2-position was tolerated for showing potent growth inhibition.

Similar results were observed in the SARs of imidazopyridine analogs **29** and **30**. The 2-*tert*-butyl analog **29** was more potent compared to the corresponding 2-methyl derivative **30** (0.25 and 0.5 μg/mL against *C. glabrata*, respectively), as in Table 2. On the other hand, pyrazolopyridine **36** with preferable substituents observed in **13** and **29** exhibited weak antifungal activities against several *Candida* spp.

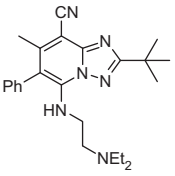
These results suggested that the nitrogen atoms at the 1-position as in triazolopyridine **13** and imidazopyridine **29** were indispensable for potent antifungal activity in comparison with the non-nitrogen pyrazolopyridine **36** at the 3-position (Fig. 2).

Pyrazolopyridine **36** showed high lipophilicity and poor solubility. Triazolopyridine **13** and imidazopyridine **29** demonstrated

Table 1
In vitro activity and physicochemical property of triazolopyridine derivatives



9, 13-16



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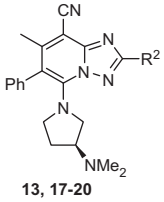
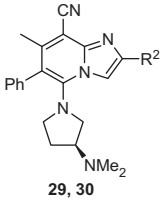
Compd	R^2	R^6	MIC-0 (μg/mL)[a]					Log D[c]	Sol.[d]	MS[e]
			S. cerevisiae AY-14	*C. albicans* ATCC 24433	*C. glabrata* IFO 0622	*C. tropicalis* TIMM 0313	*C. krusei* TIMM 0269	pH 6.8	pH 6.8 (μg/mL)	Human (%)
9	*t*-Bu	*n*-Bu	64	>128	32	>128	>128	3.2	146	11
13	*t*-Bu	Ph	⩽0.125	>128	⩽0.125	2	16	3.1	16	9
14	Me	Ph	8	>128	2	>128	>128	1.7	376	84
15	*n*-Bu	Ph	2	>128	1	>64	>64	3.2	33	5
16	Ph	Ph	>128	>128	>128	>128	>128	NT[f]	NT	NT
21	—	—	0.5	>128	0.5	>128	>128	3.3	2	10
2	—	—	⩽0.125	>128	⩽0.125	0.5	⩽0.125	3.1	5.3	29
3	—	—	0.25	>128	⩽0.125	0.5	0.5	3.2	26	57
FLCZ[b]	—	—	4	0.25	2	0.25	16	0.4[g]	>2000	96

[a] MIC-0s (in micrograms per milliliter) were determined by using the microdilution method.
[b] The lowest drug concentration producing a prominent reduction in turbidity was employed as an endpoint.
[c] Log D values were determined from the partition coefficient for 1-octanol/phosphate buffer saline (BPS) at pH 6.8.
[d] Water solubility was measured at pH 6.8.
[e] Metabolic stability for human liver microsome.
[f] Not tested.
[g] Log D value at pH 7.4.

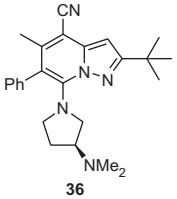
Table 2
In vitro activity and physicochemical property of various bicyclic derivatives



13, 17-20



29, 30



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Compd	R^2	MIC-2 (μg/mL)[a]					Log D[b]	Sol.[c]	MS[d]
		S. cerevisiae AY-14	*C. albicans* ATCC 24433	*C. glabrata* ATCC 48435	*C. tropicalis* IAM 4965	*C. krusei* ATCC 44507	pH 6.8	pH 6.8 (μg/mL)	Human (%)
13	*t*-Bu	0.063	>4	0.063	0.25	>4	3.1	16	9
17	Cyclopropyl	0.032	>4	0.063	1	1	2.4	57	48
18	Ethyl	0.063	>4	0.125	1	>4	2.1	228	54
19	$C(Me)_2CH_2OBn$	0.063	>4	0.125	0.125	>4	3.8	<3	9
20	$C(Me)_2CH_2OH$	0.125	>4	0.25	0.25	>4	1.8	140	64
29	*t*-Bu	0.125	4	0.25	4	>4	3.0	27	16
30	Me	1	>4	0.5	>4	>4	1.9	356	71
36	—	1	4	1	1	4	4.8	<2	12
2	—	0.004	>4	0.063	0.25	0.125	3.1	5.3	29
3	—	0.125	>4	0.125	0.5	1	3.2	26	57
FLCZ	—	4	0.25	2	0.25	16	0.4[e]	>2000	96

[a] MIC-2 was determined by using the microdilution method with alamar blue.
[b] Log D values were determined from the partition coefficient for 1-octanol/phosphate buffer saline (BPS) at pH 6.8.
[c] Water solubility was measured at pH 6.8.
[d] Metabolic stability for human liver microsome.
[e] Log D value at pH 7.4.

similar physicochemical properties. Potent antifungal activity and improved physicochemical properties were observed for triazolopyridine derivatives such as **17**, **18**, and **20** compared to **2** and the other bicyclic compounds. These results suggested that triazolopyridine was considered to be the most promising scaffold with potent antifungal activities and improved physicochemical properties.

Incorporation studies of pyridobenzimidazole analogs **1** and **2** with growing cells of *S. cerevisiae* or *C. albicans* using [^{14}C]-glucose revealed that they were specific inhibitors of β-1,6-glucan synthesis. To determine the mode of action of our bicyclic compounds obtained above, we selected two potent antifungal analogs, triazolopyridine **13** and imidazopyridine **29**, and adopted the biochemical approach based on the methods described by Kitamura

et al.[11] Triazolopyridine **13** and imidazopyridine **29** were evaluated by incorporation studies with growing cells of *C. albicans* using [^{14}C]-glucose. As shown in Figure 3, these bicyclic analogs markedly reduced the radioactivity in the fraction of β-1,6-glucan in a dose-dependent manner. No reductions in the fractions of β-1,3-glucan and chitin were observed in each case. Results obtained above suggested that bicyclic analogs **13** and **29** were also specific inhibitors of β-1,6-glucan synthesis.

5. Conclusion

We prepared triazolopyridines **9** and **13**–**21**, imidazopyridines **29** and **30**, and pyrazolopyridine **36** and evaluated their antifungal activities against *Candida* spp. Among them, triazolopyridine was found to be the most promising scaffold for specific inhibitors of β-1,6-glucan synthesis with potent antifungal growth inhibition and improved physicochemical properties.

6. Experimental section

6.1. Chemistry

Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. Melting points were taken on a Yanako MP-500D melting point apparatus and are uncorrected. Optical rotations were measured in a 0.5-dm cell at 25 °C at 589 nm with a HORIBA SEPA-300 polarimeter. ^{1}H NMR spectra were determined on a JEOL JNM-EX400 spectrometer. ^{13}C NMR spectra were determined on a JEOL JNM-ECP500 spectrometer. Chemical shifts are reported in parts per million relative to tetramethylsilane as an internal standard. Significant ^{1}H NMR data are tabulated in the order: number of protons, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad), coupling constant(s) in hertz. Infrared (IR) spectra were obtained on a HORIBA FT-720 spectrometer or a JASCO FT/IR-6100 type A. High-resolution mass spectra were obtained on a JEOL JMS-700 mass spectrometer under electron impact ionization conditions (EI), electron spray ionization conditions (ESI), or fast atom bombardment ionization conditions (FAB). Elemental analyzes are indicated only by the symbols of the elements; analytical results were within 0.4% of the theoretical values unless otherwise noted, which indicates ⩾95% purity of the tested compounds. Column chromatography refers to flash column chromatography conducted on Merck silica gel 60, 230–400 mesh ASTM. Thin-layer chromatography (TLC) was performed with Merck silica gel 60 F_{254} TLC plates, and compound visualization was effected with a 5% solution of molybdophosphoric acid in ethanol, UV-lamp, iodine, or Wako Ninhydrin Spray.

6.1.1. Methyl 3-(benzyloxy)-2,2-dimethylpropanimidoate hydrochloride (5)

Gaseous hydrogen chloride was bubbled through a stirred solution of 2,2-dimethyl-3-(benzyloxy)propionitrile (**4**)[16] (18.9 g, 99.9 mmol) and MeOH (8.1 mL, 200 mmol) in CH_2Cl_2 (200 mL) at −5 °C for 1 h. After being stirred at 0 °C for 15 h, the mixture was concentrated to dryness. Et_2O was added to the residue and the resulting precipitate was collected by filtration and washed with Et_2O to afford the title compound (15.2 g, 59%) as a pale yellow solid. ^{1}H NMR ($CDCl_3$) δ: 1.38 (6H, s), 3.63 (2H, s), 4.33 (3H, s), 4.55 (2H, s), 7.27–7.37 (5H, m).

6.1.2. General procedure for preparation of (1*H*-1,2,4-triazol-5-yl)acetonitrile 7a–7e

A mixture of sodium hydroxide (51.0 mmol), imidoate hydrochloride (50.0 mmol), and cyanoacetohydrazide (51.5 mmol) in dry MeOH (100 mL) was refluxed for 2.5 h. The solvent was evaporated, and the residue was chromatographed on a silica gel column ($CHCl_3$/MeOH = 50/1, v/v) to afford compounds **7a**–**7e**.

6.1.2.1. (3-*tert*-Butyl-1*H*-1,2,4-triazol-5-yl)acetonitrile (7a). Yield, 63% from methyl 2,2-dimethylpropanimidoate hydrochloride (**6a**),[17] colorless solid. Mp: 110–112 °C. MS (FAB) *m*/*z*: 165 $(M+1)^+$. ^{1}H NMR ($CDCl_3$) δ: 1.42 (9H, s), 3.87 (2H, s), 11.0 (1H, br s). ^{13}C NMR ($CDCl_3$) δ: 18.1, 29.0 (3C), 32.3, 115.8, 154.2, 166.6. IR (KBr): 2973, 2255, 1565, 1414, 1255, 1049, 1021, 940 cm^{-1}. Anal. Calcd for $C_8H_{12}N_4$: C, 58.51; H, 7.37; N, 34.12. Found: C, 58.40; H, 7.30; N, 33.76.

6.1.2.2. (3-Cyclopropyl-1*H*-1,2,4-triazol-5-yl)acetonitrile (7b). Yield, 53% from methyl cyclopropanecarboximidoate hydrochloride (**6b**), colorless solid. Mp: 138–140 °C. HRMS (ESI) Calcd for $C_7H_8N_4$+H: 149.0827. Found: 149.0849. ^{1}H NMR ($CDCl_3$) δ: 1.06–1.14 (4H, m), 1.93–2.00 (1H, m), 3.79 (2H, s), 10.44 (1H, br s). ^{13}C NMR ($CDCl_3$) δ: 7.1, 8.4 (2C), 17.7, 115.6, 153.8, 161.0. IR (KBr): 3024, 2903, 2751, 2658, 2255, 1582, 1519, 1439, 1353, 1101 cm^{-1}.

6.1.2.3. (3-Ethyl-1*H*-1,2,4-triazol-5-yl)acetonitrile (7c). Yield, 88% from **6c**,[18] colorless solid. MS (FAB) *m*/*z*: 137 $(M+1)^+$. ^{1}H NMR ($CDCl_3$) δ: 1.37 (3H, t, J = 7.5 Hz), 2.86 (2H, q, J = 7.5 Hz), 3.88 (2H, s), 11.61 (1H, br s).

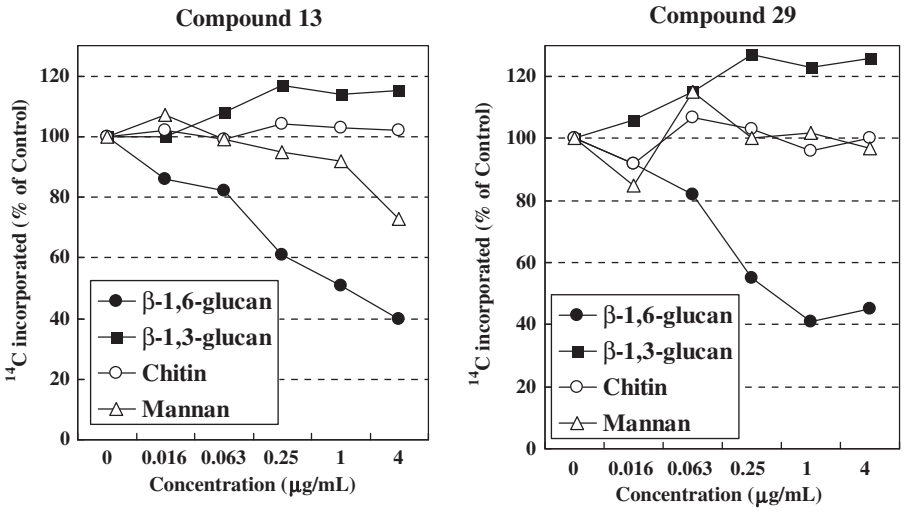

Figure 3. Incorporation studies of the bicyclic analogs **13** and **29** with growing cells of *C. albicans* ATCC MYA-573 using [^{14}C]-glucose.

6.1.2.4. (3-Butyl-1*H*-1,2,4-triazol-5-yl)acetonitrile (7d). Yield, 76% from methyl pentanimidoate hydrochloride **6d**, colorless solid. Mp: 70–71 °C. HRMS (ESI) Calcd for $C_8H_{12}N_4$+H: 165.1140. Found: 165.1148. ^{1}H NMR ($CDCl_3$) δ: 0.95 (3H, t, J = 7.4 Hz), 1.36–1.45 (2H, m), 1.70–1.79 (2H, m), 2.80 (2H, t, J = 7.7 Hz), 3.85 (2H, s), 10.79 (1H, br s). ^{13}C NMR ($CDCl_3$) δ: 13.6, 18.1, 22.2, 26.2, 29.8, 115.8, 154.2, 159.1. IR (KBr): 2934, 2260, 1665, 1566, 1508, 1417, 1068 cm^{-1}.

6.1.2.5. {3-[2-(Benzyloxy)-1,1-dimethylethyl]-1*H*-1,2,4-triazol-5-yl}acetonitrile (7e). Yield, 68% from **5**, colorless solid. MS (FAB) m/z: 271 (M+1)$^+$. ^{1}H NMR ($CDCl_3$) δ: 1.39 (6H, s), 3.53 (2H, s), 3.82 (2H, s), 4.59 (2H, s), 7.29–7.41 (5H, m), 11.23 (1H, br s).

6.1.3. 6-Butyl-2-*tert*-butyl-7-methyl-5-oxo-1,5-dihydro[1,2,4]-triazolo[1,5-a]pyridine-8-carbonitrile (8)

A mixture of **7a** (1.00 g, 6.09 mmol), ethyl 2-acetylhexanoate (1.19 g, 6.39 mmol), and ammonium acetate (986 mg, 12.8 mmol) was heated at 150 °C for 3.5 h. The mixture was cooled to room temperature and purified by silica gel chromatography eluting with $CHCl_3$/MeOH = 49/1, v/v to give the crude product. The crude product was recrystallized from MeCN to yield **8** (1.09 g, 63%) as a pale yellow solid. Mp: >280 °C. MS (FAB) m/z: 287 (M+1)$^+$. ^{1}H NMR ($CDCl_3$) δ: 0.93 (3H, t, J = 7.2 Hz), 1.35–1.52 (4H, m), 1.46 (9H, s), 2.42 (3H, s), 2.68 (2H, t, J = 7.7 Hz). ^{13}C NMR ($CDCl_3$) δ: 14.0, 18.2, 22.7, 26.5, 28.2 (3C), 30.9, 32.6, 71.0, 116.1, 118.5, 145.3, 146.3, 156.6, 161.9. IR (KBr): 2960, 2212, 1656, 1571, 1521, 1392, 1190, 990 cm^{-1}. Anal. Calcd for $C_{16}H_{22}N_4O$: C, 67.11; H, 7.74; N, 19.56. Found: C, 67.04; H, 7.72; N, 19.33.

6.1.4. 6-Butyl-2-*tert*-butyl-5-[(3*S*)-3-(dimethylamino)pyrrolidin-1-yl]-7-methyl[1,2,4]triazolo[1,5-*a*]pyridine-8-carbonitrile (9)

A mixture of **8** (900 mg, 3.14 mmol) in phosphorus oxychloride (5 mL) was refluxed for 1.5 h. The mixture was cooled to room temperature and concentrated in vacuo, and then $CHCl_3$ and water were added. The separated organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo to give 6-butyl-2-*tert*-butyl-5-chloro-7-methyl[1,2,4]triazolo[1,5-*a*]pyridine-8-carbonitrile (939 mg) as a pale yellow solid. The obtained compound was used without further purification. To a solution of the chloride (600 mg, 1.97 mmol) in DMF (6 mL) was added (3*S*)-3-(dimethylamino)pyrrolidine (275 μL, 2.17 mmol) and triethylamine (549 μL, 3.94 mmol) and the mixture was stirred at 90 °C for 4 h. The mixture was cooled to room temperature and concentrated in vacuo. The residue was dissolved with $CHCl_3$ and the mixture was washed with satd $NaHCO_3$ aq and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica gel column chromatography eluting with $CHCl_3$/MeOH = 99/1, v/v to afford **9** (723 mg, 97%) as a pale yellow solid. Mp: 49–53 °C. MS (FAB) m/z: 383 (M+1)$^+$. ^{1}H NMR ($CDCl_3$) δ: 0.97 (3H, t, J = 7.2 Hz), 1.38–1.50 (13H, m), 2.00–2.09 (1H, m), 2.22–2.29 (1H, m), 2.34 (6H, s), 2.63 (3H, s), 2.68–2.79 (2H, m), 3.09–3.19 (1H, m), 3.40 (1H, t, J = 8.3 Hz), 3.53 (1H, t d, J = 8.6, 2.9 Hz), 3.67–3.73 (2H, m). IR (KBr): 2956, 2869, 2772, 2216, 1607 cm^{-1}. Anal. Calcd for $C_{22}H_{34}N_6{\cdot}0.25H_2O$: C, 68.27; H, 8.98; N, 21.71. Found: C, 68.45; H, 8.89; N, 21.85. $[\alpha]_D^{25}$ −49.9 (c 1.00, $CHCl_3$).

6.1.5. General procedure for the synthesis of 7-methyl-5-oxo-6-phenyl-1,5-dihydro[1,2,4]triazolo[1,5-*a*]pyridine-8-carbonitriles 12a–12g

6.1.5.1. 2-tert-Butyl-7-methyl-5-oxo-6-phenyl-1,5-dihydro[1,2,4]-triazolo[1,5-a]pyridine-8-carbonitrile (12a). A mixture of **7a** (1.00 g, 6.09 mmol), ethyl 3-oxo-2-phenylbutanoate (1.32 g, 6.39 mmol), and ammonium acetate (986 mg, 12.8 mmol) was heated at 150 °C for 3.5 h. The mixture was cooled to room temperature and purified by silica gel chromatography eluting with $CHCl_3$/MeOH = 19/1, v/v to give the crude product. The crude product was recrystallized from MeCN to yield **12a** (621 mg, 33%) as a pale yellow solid. Mp: >280 °C. MS (ESI) m/z: 307 (M+1)$^+$. ^{1}H NMR ($CDCl_3$) δ: 1.43 (9H, s), 2.25 (3H, s), 7.26–7.44 (5H, m). ^{13}C NMR ($CDCl_3$) δ: 19.8, 28.3 (3C), 32.7, 71.8, 115.7, 119.4, 127.5, 128.4 (2C), 130.9 (2C), 135.4, 145.8, 148.2, 156.2, 162.2. IR (KBr): 2973, 2213, 1653, 1524, 1291, 989 cm^{-1}. Anal. Calcd for $C_{18}H_{18}N_4O$: C, 70.57; H, 5.92; N, 18.29. Found: C, 70.26; H, 5.77; N, 18.25.

6.1.5.2. 2,7-Dimethyl-5-oxo-6-phenyl-1,5-dihydro[1,2,4]triazolo[1,5-a]pyridine-8-carbonitrile (12b). Following the procedures as described for **12a**, the title compound (183 mg) was prepared in 12% yield from **10** (730 mg, 6.00 mmol) as a pale yellow solid. MS (FAB) m/z: 265 (M+1)$^+$. ^{1}H NMR (DMSO-d_6) δ: 2.16 (3H, s), 2.51 (3H, s), 7.23 (2H, m), 7.38 (3H, m).

6.1.5.3. 2-Butyl-7-methyl-5-oxo-6-phenyl-1,5-dihydro[1,2,4]triazolo[1,5-a]pyridine-8-carbonitrile (12c). Following the procedures as described for **12a**, the title compound (311 mg) was prepared in 17% yield from **7d** (1.00 g, 6.09 mmol) as a pale yellow solid. Mp: >280 °C. HRMS (ESI) Calcd for $C_{18}H_{18}N_4O$+H: 307.1559. Found: 307.1547. ^{1}H NMR (DMSO-d_6) δ: 0.92 (3H, t, J = 6.9 Hz), 1.29–1.40 (2H, m), 1.64–1.74 (2H, m), 2.11 (3H, s), 2.62–2.71 (2H, m), 6.67 (1H, br s), 7.17–7.40 (5H, m). IR (KBr): 3331, 2959, 2210, 1668, 1610, 1523, 1369, 1304, 727 cm^{-1}.

6.1.5.4. 7-Methyl-5-oxo-2,6-diphenyl-1,5-dihydro[1,2,4]triazolo[1,5-a]pyridine-8-carbonitrile (12d). Following the procedures as described for **12a**, the title compound (885 mg) was prepared in 50% yield from **11** (1.00 g, 5.43 mmol) as a colorless solid. MS (FAB) m/z: 327 (M+1)$^+$. ^{1}H NMR (DMSO-d_6) δ: 2.13 (3H, s), 7.13 (1H, br s), 7.24 (3H, m), 7.36 (2H, m), 7.46 (3H, m), 8.15 (2H, m).

6.1.5.5. 2-Cyclopropyl-7-methyl-5-oxo-6-phenyl-1,5-dihydro-[1,2,4]triazolo[1,5-a]pyridine-8-carbonitrile (12e). Following the procedures as described for **12a**, the title compound (366 mg) was prepared in 21% yield from **7b** (890 mg, 6.00 mmol) as a pale yellow solid. Mp: >280 °C. HRMS (ESI) Calcd for $C_{17}H_{14}N_4O$+H: 291.1246. Found: 291.1232. ^{1}H NMR (DMSO-d_6) δ: 0.80–0.96 (4H, m), 2.10 (3H, s), 2.14–2.23 (1H, m), 7.18–7.40 (5H, m). ^{13}C NMR (DMSO-d_6) δ: 8.1 (2C), 8.7, 19.3, 111.3, 118.7, 126.3, 127.8 (2C), 131.2 (2C), 137.3, 145.6, 151.9, 155.6, 166.5, 171.4. IR (KBr): 3469, 3396, 3204, 2205, 1662, 1611, 1530, 1363, 1297 cm^{-1}.

6.1.5.6. 2-Ethyl-7-methyl-5-oxo-6-phenyl-1,5-dihydro[1,2,4]-triazolo[1,5-a]pyridine-8-carbonitrile (12f). Following the procedures as described for **12a**, the title compound (350 mg) was prepared in 21% yield from **7c** (820 mg, 6.00 mmol) as a pale yellow solid. MS (FAB) m/z: 279 (M+1)$^+$. ^{1}H NMR (DMSO-d_6) δ: 1.26 (3H, t, J = 7.5 Hz), 2.13 (3H, s), 2.76 (2H, q, J = 7.5 Hz), 7.20 (2H, m), 7.29 (1H, m), 7.39 (2H, m).

6.1.5.7. 2-[2-(Benzyloxy)-1,1-dimethylethyl]-7-methyl-5-oxo-6-phenyl-1,5-dihydro[1,2,4]triazolo[1,5-a]pyridine-8-carbonitrile (12g). Following the procedures as described for **12a**, the title compound (2.00 g) was prepared in 26% yield from **7e** (5.00 g, 18.5 mmol) as a pale yellow solid. MS (FAB) m/z: 413 (M+1)$^+$. ^{1}H NMR ($CDCl_3$) δ: 1.35 (6H, s), 2.21 (3H, s), 3.56 (2H, s), 4.42 (2H, s), 7.18–7.42 (10H, m).

6.1.6. 2-*tert*-Butyl-5-[(3*S*)-3-(dimethylamino)pyrrolidin-1-yl]-7-methyl-6-phenyl[1,2,4]triazolo[1,5-*a*]pyridine-8-carbonitrile (13)

Compound **13** was prepared from **12a**, as described above for **9**. Yield, 99% as a pale yellow solid. Mp: 177–179 °C. MS (FAB) m/z: 403 (M+1)$^+$. ^{1}H NMR ($CDCl_3$) δ: 1.47 (9H, s), 1.56–1.69 (1H, m), 1.92–2.00 (1H, m), 2.16 (6H, s), 2.24 (3H, s), 2.57–2.67 (1H, m),

3.23–3.36 (2H, m), 3.50–3.59 (2H, m), 7.11–7.15 (1H, m), 7.22–7.26 (1H, m), 7.38–7.49 (3H, m). ^{13}C NMR ($CDCl_3$) δ: 20.4, 29.6 (3C), 29.7, 33.4, 43.9 (2C), 51.2, 55.9, 65.2, 89.5, 114.9, 116.1, 128.2, 128.8, 129.0, 131.2, 131.4, 136.5, 144.0, 148.3, 151.6, 174.2. IR (KBr): 2964, 2772, 2210, 1606, 1508 cm^{-1}. Anal. Calcd for $C_{24}H_{30}N_6$: C, 71.61; H, 7.51; N, 20.88. Found: C, 71.44; H, 7.49; N, 21.01. $[\alpha]_D^{25}$ +24.8 (*c* 0.839, $CHCl_3$).

6.1.7. 5-[(3*S*)-3-(Dimethylamino)pyrrolidin-1-yl]-2,7-dimethyl-6-phenyl[1,2,4]triazolo[1,5-*a*]pyridine-8-carbonitrile (14)

Compound **14** was prepared from **12b**, as described above for **9**. Yield, 41% as a pale yellow solid. Mp: 182–184 °C. MS (FAB) *m*/*z*: 361 (M+1)$^+$. ^{1}H NMR ($CDCl_3$) δ: 1.55–1.68 (1H, m), 1.93–2.02 (1H, m), 2.14 (6H, s), 2.26 (3H, s), 2.55–2.65 (1H, m), 2.60 (3H, s), 3.16 (1H, t, *J* = 9.5 Hz), 3.29–3.34 (1H, m), 3.42 (1H, dd, *J* = 10.0, 7.2 Hz), 3.58–3.65 (1H, m), 7.14 (1H, d, *J* = 7.4 Hz), 7.23–7.27 (1H, m), 7.40–7.49 (3H, m). ^{13}C NMR ($CDCl_3$) δ: 14.7, 20.4, 30.0, 44.1 (2C), 51.0, 56.0, 65.3, 89.6, 115.5, 115.7, 128.3, 128.9, 129.1, 131.0, 131.2, 136.2, 143.8, 148.6, 151.8, 163.5. IR (KBr): 2768, 2216, 1607, 1541, 1510, 1485, 1351 cm^{-1}. Anal. Calcd for $C_{21}H_{24}N_6$: C, 69.97; H, 6.71; N, 23.32. Found: C, 69.70; H, 6.73; N, 23.02. $[\alpha]_D^{25}$ +49.2 (*c* 0.438, $CHCl_3$).

6.1.8. 2-Butyl-5-[(3*S*)-3-(dimethylamino)pyrrolidin-1-yl]-7-methyl-6-phenyl[1,2,4]triazolo[1,5-*a*]pyridine-8-carbonitrile (15)

Compound **15** was prepared from **12c**, as described above for **9**. Yield, 89% as a pale yellow solid. Mp: 108–109 °C. MS (FAB) *m*/*z*: 403 (M+1)$^+$. ^{1}H NMR ($CDCl_3$) δ: 0.97 (3H, t, *J* = 7.4 Hz), 1.42–1.50 (2H, m), 1.58–1.67 (1H, m), 1.81–1.89 (2H, m), 1.94–2.01 (1H, m), 2.14 (6H, s), 2.26 (3H, s), 2.57–2.64 (1H, m), 2.92 (2H, t, *J* = 7.7 Hz), 3.20 (1H, t, *J* = 9.5 Hz), 3.29–3.34 (1H, m), 3.45 (1H, dd, *J* = 9.7, 6.9 Hz), 3.55–3.62 (1H, m), 7.12–7.15 (1H, m), 7.23–7.26 (1H, m), 7.39–7.48 (3H, m). ^{13}C NMR ($CDCl_3$) δ: 13.9, 20.4, 22.5, 28.6, 29.9, 30.3, 44.0 (2C), 51.1, 56.0, 65.3, 89.6, 115.5, 115.7, 128.3, 128.8, 129.1, 131.1, 131.3, 136.3, 143.9, 148.5, 151.6, 167.2. IR (KBr): 2956, 2771, 2209, 1610, 1508 cm^{-1}. Anal. Calcd for $C_{24}H_{30}N_6$: C, 71.61; H, 7.51; N, 20.88. Found: C, 71.49; H, 7.48; N, 20.70. $[\alpha]_D^{25}$ +42.3 (*c* 1.02, $CHCl_3$).

6.1.9. 5-[(3*S*)-3-(Dimethylamino)pyrrolidin-1-yl]-7-methyl-2,6-diphenyl[1,2,4]triazolo[1,5-*a*]pyridine-8-carbonitrile (16)

Compound **16** was prepared from **12d**, as described above for **9**. Yield, 91% as a pale yellow solid. Mp: 224–227 °C. MS (FAB) *m*/*z*: 423 (M+1)$^+$. ^{1}H NMR ($CDCl_3$) δ: 1.60–1.74 (1H, m), 1.95–2.08 (1H, m), 2.18 (6H, s), 2.23 (3H, s), 2.59–2.70 (1H, m), 3.31 (1H, t, *J* = 9.3 Hz), 3.40 (1H, m), 3.52 (1H, dd, *J* = 6.9, 9.9 Hz), 3.61–3.70 (1H, m), 7.14–7.19 (1H, m), 7.26–7.30 (1H, m), 7.40––7.53 (6H, m), 8.30–8.37 (2H, m). ^{13}C NMR ($CDCl_3$) δ: 20.4, 30.0, 44.1 (2C), 51.3, 56.2, 65.3, 89.8, 115.7, 115.8, 127.6 (2C), 128.3, 128.6 (2C), 128.9, 129.1, 130.2, 130.5, 131.1, 131.3, 136.3, 144.1, 148.9, 152.2, 163.5. IR (KBr): 2962, 2766, 2218, 1610, 1508, 1442 cm^{-1}. Anal. Calcd for $C_{26}H_{26}N_6{\cdot}0.25H_2O$: C, 73.13; H, 6.25; N, 19.68. Found: C, 72.99; H, 6.11; N, 19.74. $[\alpha]_D^{25}$ +47.2 (*c* 1.03, $CHCl_3$).

6.1.10. 2-Cyclopropyl-5-[(3*S*)-3-(dimethylamino)pyrrolidin-1-yl]-7-methyl-6-phenyl[1,2,4]triazolo[1,5-*a*]pyridine-8-carbonitrile (17)

Compound **17** was prepared from **12e**, as described above for **9**. Yield, 98% as a pale yellow solid. Mp: 167–169 °C. MS (FAB) *m*/*z*: 387 (M+1)$^+$. ^{1}H NMR ($CDCl_3$) δ: 1.03–1.09 (2H, m), 1.14–1.19 (2H, m), 1.58–1.68 (1H, m), 1.96 (1H, m), 2.14 (6H, s), 2.22 (1H, m), 2.24 (3H, s), 2.60 (1H, m), 3.17 (1H, dd, *J* = 8.7, 9.9 Hz), 3.29 (1H, m), 3.42 (1H, dd, *J* = 7.2, 9.9 Hz), 3.57 (1H, d t, *J* = 6.9, 10.2 Hz), 7.12 (1H, m), 7.24 (1H, m), 7.38–7.49 (3H, m). ^{13}C NMR ($CDCl_3$) δ: 9.0, 9.1, 9.6, 20.3, 29.9, 44.0 (2C), 51.0, 55.9, 65.3, 89.4, 115.5, 115.8, 128.2, 128.8, 129.1, 131.1, 131.3, 136.3, 143.7, 148.3, 151.7, 168.8. IR (KBr): 2771, 2210, 1606, 1542, 1508, 1477, 1360, 1270 cm^{-1}. Anal. Calcd for $C_{23}H_{26}N_6{\cdot}0.5H_2O$: C, 69.85; H, 6.88; N, 21.25. Found: C, 69.92; H, 6.68; N, 21.19. $[\alpha]_D^{25}$ +44.4 (*c* 1.01, $CHCl_3$).

6.1.11. 5-[(3*S*)-3-(Dimethylamino)pyrrolidin-1-yl]-2-ethyl-7-methyl-6-phenyl[1,2,4]triazolo[1,5-*a*]pyridine-8-carbonitrile (18)

Compound **18** was prepared from **12f**, as described above for **9**. Yield, 98% as a pale yellow solid. Mp: 151–153 °C. MS (FAB) *m*/*z*: 375 (M+1)$^+$. ^{1}H NMR ($CDCl_3$) δ: 1.42 (3H, t, *J* = 7.5 Hz), 1.63 (1H, m), 1.98 (1H, m), 2.15 (6H, s), 2.26 (3H, s), 2.61 (1H, m), 2.95 (2H, q, *J* = 7.5 Hz), 3.21 (1H, dd, *J* = 8.7, 9.9 Hz), 3.32 (1H, m), 3.45 (1H, dd, *J* = 7.2, 9.6 Hz), 3.60 (1H, d t, *J* = 6.9, 10.2 Hz), 7.13 (1H, m), 7.24 (1H, m), 7.34–7.49 (3H, m). ^{13}C NMR ($CDCl_3$) δ: 12.5, 20.4, 22.4, 29.9, 44.1 (2C), 51.1, 56.0, 65.3, 89.5, 115.4, 115.7, 128.3, 128.8, 129.1, 131.1, 131.3, 136.3, 143.9, 148.5, 151.7, 168.1. IR (KBr): 2770, 2211, 1603, 1541, 1508, 1480, 1358, 1268 cm^{-1}. Anal. Calcd for $C_{22}H_{26}N_6{\cdot}0.2H_2O$: C, 69.89; H, 7.04; N, 22.23. Found: C, 70.15; H, 6.99; N, 21.97. $[\alpha]_D^{25}$ +44.7 (*c* 1.03, $CHCl_3$).

6.1.12. 2-[2-(Benzyloxy)-1,1-dimethylethyl]-5-[(3*S*)-3-(dimethylamino)pyrrolidin-1-yl]-7-methyl-6-phenyl[1,2,4]triazolo[1,5-*a*]pyridine-8-carbonitrile (19)

Compound **19** was prepared from **12g**, as described above for **9**. Yield, 95% as a pale yellow solid. Mp: 131–133 °C. MS (FAB) *m*/*z*: 509 (M+1)$^+$. ^{1}H NMR ($CDCl_3$) δ: 1.49 (6H, s), 1.61 (1H, m), 1.93 (1H, m), 2.14 (6H, s), 2.41 (3H, s), 2.60 (1H, m), 3.23 (1H, dd, *J* = 9.0, 9.9 Hz), 3.31 (1H, m), 3.48–3.59 (2H, m), 3.78 (2H, s), 4.55 (2H, m), 7.10–7.48 (10H, m). ^{13}C NMR ($CDCl_3$) δ: 20.4, 24.6, 24.6, 29.8, 38.2, 44.0 (2C), 51.3, 56.0, 65.2, 73.3, 78.4, 89.4, 114.8, 116.1, 127.3, 127.3 (2C), 128.2, 128.2 (2C), 128.8, 129.0, 131.2, 131.4, 136.5, 138.9, 144.0, 148.4, 151.6, 171.7. IR (KBr): 2869, 2207, 1604, 1537, 1504, 1468, 1349, 1091 cm^{-1}. Anal. Calcd for $C_{31}H_{36}N_6O$: C, 73.20; H, 7.13; N, 16.52. Found: C, 73.04; H, 7.11; N, 16.38. $[\alpha]_D^{25}$ +27.4 (*c* 1.04, $CHCl_3$).

6.1.13. 5-[(3*S*)-3-(Dimethylamino)pyrrolidin-1-yl]-2-(2-hydroxy-1,1-dimethylethyl)-7-methyl-6-phenyl[1,2,4]triazolo[1,5-*a*]-pyridine-8-carbonitrile (20)

To a solution of **19** (1.40 g, 2.80 mmol) in MeOH (14 mL) and THF (14 mL) was added 4 M HCl in 1,4-dioxane and the mixture was stirred for 10 min at room temperature. The mixture was concentrated in vacuo and the residue was dissolved with MeOH (30 mL). To the solution was added 10% Pd–C catalyst (560 mg, containing 55.6% of water), and the mixture was shaken vigorously under 4.5 kg/cm^2 of hydrogen for 30 min at ambient temperature. The mixture was filtered and concentrated in vacuo. Purification of the resulting residue by silica gel column chromatography ($CHCl_3$/MeOH = 50/1, v/v) gave **20** (1.0 g, 87%) as a pale yellow solid. Mp: 198–200 °C. MS (FAB) *m*/*z*: 419 (M+1)$^+$. ^{1}H NMR ($CDCl_3$) δ: 1.44 (6H, s), 1.63 (1H, m), 1.98 (1H, m), 2.15 (6H, s), 2.25 (3H, s), 2.61 (1H, m), 3.22 (1H, dd, *J* = 8.7, 10.2 Hz), 3.35 (1H, m), 3.44–3.60 (2H, m), 3.76 (1H, s), 3.86 (1H, br s), 7.11–7.14 (1H, m), 7.23–7.26 (1H, m), 7.39–7.50 (3H, m). ^{13}C NMR ($CDCl_3$) δ: 20.4, 24.6, 24.6, 29.9, 38.3, 44.0 (2C), 51.4, 56.2, 65.2, 71.3, 89.5, 115.5, 115.7, 128.4, 128.9, 129.1, 131.1, 131.3, 136.2, 144.0, 149.0, 151.3, 171.9. IR (KBr): 3155, 2965, 2214, 1603, 1536, 1503, 1469, 1347, 1065 cm^{-1}. Anal. Calcd for $C_{24}H_{30}N_6O{\cdot}0.25H_2O$: C, 68.14; H, 7.27; N, 19.87. Found: C, 68.32; H, 7.27; N, 19.73. $[\alpha]_D^{25}$ +36.5 (*c* 1.01, $CHCl_3$).

6.1.14. 2-*tert*-Butyl-5-{[2-(diethylamino)ethyl]amino}-7-methyl-6-phenyl[1,2,4]triazolo[1,5-*a*]pyridine-8-carbonitrile (21)

Compound **21** was prepared from **12a** and 2-(diethylamino)ethylamine, as described above for **9**. Yield, 64% as a pale yellow solid. Mp: 177–179 °C. MS (FAB) *m*/*z*: 405 (M+1)$^+$. ^{1}H NMR ($CDCl_3$) δ: 0.96 (6H, t, *J* = 7.2 Hz), 1.47 (9H, s), 2.22 (3H, s), 2.38–2.44 (6H,

m), 2.82–2.88 (2H, m), 7.19 (1H, br s), 7.24–7.27 (2H, m), 7.41–7.48 (3H, m). ^{13}C NMR ($CDCl_3$) δ: 12.2 (2C), 20.0, 29.6 (3C), 33.5, 41.5, 46.3 (2C), 51.3, 85.0, 107.5, 116.6, 128.5, 128.9 (2C), 131.7 (2C), 135.4, 143.2, 148.2, 150.5, 174.7. IR (KBr): 2964, 2816, 2215, 1614, 1556 cm^{-1}. Anal. Calcd for $C_{24}H_{32}N_6$: C, 71.25; H, 7.97; N, 20.77. Found: C, 71.07; H, 8.01; N, 20.68.

6.1.15. 3,3-Dimethyl-2-oxobutyl (benzyloxy)acetate (24)

(Benzyloxy)acetic acid (**22**) (1.00 g, 5.59 mmol) was dissolved in DMF (5 mL). To the solution was added K_2CO_3 and 1-bromo-3,3-dimethylbutan-2-one (**23**) (1.0 g, 5.59 mmol), and the mixture was stirred at room temperature for 24 h. The mixture was poured into 0.5 M HCl aq (40 mL) at 0 °C, and the resultant mixture was extracted with AcOEt. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The crude product was purified by silica gel column chromatography eluting with *n*-hexane/AcOEt = 95/5, v/v to yield **24** (1.17 g, 80%) as a colorless oil. HRMS (ESI) Calcd for $C_{15}H_{20}O_4$+H: 265.1440. Found: 265.1446. ^{1}H NMR ($CDCl_3$) δ: 1.20 (9H, s), 4.24 (2H, s), 4.67 (2H, s), 4.97 (2H, s), 7.28–7.40 (5H, m). IR (ATR): 1764, 1722, 1192, 1124, 1082, 987, 738, 698 cm^{-1}.

6.1.16. 2-[(Benzyloxy)methyl]-4-*tert*-butyl-1*H*-imidazole (25)

A mixture of **24** (6.46 g, 24.4 mmol) and ammonium acetate (18.8 g, 244 mmol) was heated at 140 °C for 12 h. The mixture was cooled to room temperature and then dissolved with $CHCl_3$ and 1 M NaOH aq. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The crude product was purified by silica gel column chromatography eluting with $CHCl_3$/MeOH = 49/1, v/v to yield **25** (4.52 g, 76%) as a pale yellow oil. HRMS (EI) Calcd for $C_{15}H_{20}N_2O$: 244.1576. Found: 244.1580. ^{1}H NMR ($CDCl_3$) δ: 1.27 (9H, s), 4.55 (2H, s), 4.61 (2H, s), 6.66 (1H, s), 7.28–7.37 (5H, m). IR (ATR): 2960, 1456, 1363, 1092, 1072, 735, 698 cm^{-1}.

6.1.17. 4-*tert*-Butyl-2-(chloromethyl)-1*H*-imidazole hydrochloride (26)

To a solution of **25** (4.48 g, 18.3 mmol) in EtOH (45 mL) was added 10% Pd–C catalyst (896 mg, containing 55.6% of water) and 1 M HCl aq (19.3 mL, 19.3 mmol), and the mixture was shaken vigorously under 4 kg/cm^2 of hydrogen atmosphere for 12.5 h at ambient temperature. The mixture was filtered and concentrated in vacuo to give a crude of (4-*tert*-butyl-1*H*-imidazol-2-yl)methanol (3.34 g). Thionyl chloride (6 mL) was added to the crude material and the mixture was stirred at ambient temperature for 9 h. The mixture was concentrated in vacuo and the residue was dissolved with Et_2O. The precipitate formed was collected by filtration, washed with Et_2O, and dried under a vacuum to afford the title compound (2.91 g, 80%) as a brown powder. HRMS (EI) Calcd for $C_8H_{13}ClN_2$: 172.0767. Found: 172.0755. ^{1}H NMR ($CDCl_3$) δ: 1.37 (9H, s), 4.91 (2H, s), 7.32 (1H, s). IR (ATR): 2686, 1682, 1630, 1282, 901, 823, 727 cm^{-1}.

6.1.18. (4-*tert*-Butyl-1*H*-imidazol-2-yl)acetonitrile (27a)

To a stirred solution of potassium cyanide (3.57 g, 54.9 mmol) in water (15 mL) was dropwised a solution of **26** (2.87 g, 13.7 mmol) in EtOH (61 mL) at 0 °C over 1 h. The mixture was stirred at room temperature for 2 h. The reaction mixture was filtered and concentrated in vacuo, and the residue was dissolved with AcOEt and 1 M NaOH aq. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The crude product was purified by silica gel column chromatography eluting with *n*-hexane/AcOEt = 1/2, v/v to yield **27a** (1.51 g, 67%) as an orange solid. Mp: 134–136 °C. MS (ESI) *m*/*z*: 164 (M+1)$^+$. ^{1}H NMR ($CDCl_3$) δ: 1.28 (9H, s), 3.90 (2H, s), 6.72 (1H, s). ^{13}C NMR (DMSO-d_6) δ: 17.6, 30.2 (3C), 30.9, 116.0, 117.2, 136.2, 146.5. IR (ATR): 2962, 2258, 1464, 1365, 1203, 754 cm^{-1}. Anal. Calcd for $C_9H_{13}N_3$: C, 66.23; H, 8.03; N, 25.74. Found: C, 66.28; H, 8.00; N, 25.81.

6.1.19. 2-*tert*-Butyl-7-methyl-5-oxo-6-phenyl-1,5-dihydroimidazo[1,2-*a*]pyridine-8-carbonitrile (28a)

Following the procedures as described for **12a**, the title compound (621 mg) was prepared in 33% yield from **27a** (300 mg, 1.84 mmol) as a pale brown solid. Mp: >280 °C. MS (ESI) *m*/*z*: 306 (M+1)$^+$. ^{1}H NMR ($CDCl_3$) δ: 1.38 (9H, s), 2.28 (3H, s), 7.26–7.40 (2H, m), 7.36–7.40 (2H, m), 7.44 (1H, s), 11.58 (1H, br s). ^{13}C NMR ($CDCl_3$) δ: 19.7, 29.1 (3C), 31.1, 70.0, 105.1, 116.3, 117.7, 127.2, 128.4 (2C), 131.0 (2C), 136.0, 142.2, 143.0, 147.3, 155.9. IR (ATR): 2208, 1645, 1518, 1317, 748, 717 cm^{-1}. Anal. Calcd for $C_{19}H_{19}N_3O$: C, 74.73; H, 6.27; N, 13.76. Found: C, 74.62; H, 6.27; N, 13.72.

6.1.20. 2,7-Dimethyl-5-oxo-6-phenyl-1,5-dihydroimidazo[1,2-*a*]pyridine-8-carbonitrile (28b)

Following the procedures as described for **12a**, the title compound (330 mg) was prepared in 61% yield from **27b** (250 mg, 2.06 mmol) as a brown solid. MS (FAB) *m*/*z*: 264 (M+1)$^+$. ^{1}H NMR (DMSO-d_6) δ: 2.15 (3H, s), 2.31 (3H, s), 7.21–7.23 (2H, m), 7.31 (1H, m), 7.38–7.41 (2H, m), 7.54 (1H, s). IR (ATR): 3156, 2204, 1650, 1616, 1523, 1317 cm^{-1}.

6.1.21. 2-*tert*-Butyl-5-[(3*S*)-3-(dimethylamino)pyrrolidin-1-yl]-7-methyl-6-phenylimidazo[1,2-*a*]pyridine-8-carbonitrile (29)

Compound **29** was prepared from **28a**, as described above for **9**. Yield, 96% as a pale red solid. Mp: 179–180 °C. MS (EI) *m*/*z*: 401 (M$^+$). ^{1}H NMR ($CDCl_3$) δ: 1.42 (9H, s), 1.60–1.72 (1H, m), 1.91–2.01 (1H, m), 2.14 (6H, s), 2.26 (3H, s), 2.51–2.59 (1H, m), 2.79–2.85 (1H, m), 2.95–3.05 (2H, m), 3.11 (1H, dd, *J* = 8.9, 7.2 Hz), 7.11–7.14 (1H, m), 7.16–7.20 (1H, m), 7.25 (1H, s), 7.40–7.49 (3H, m). ^{13}C NMR ($CDCl_3$) δ: 19.7, 30.1, 30.2 (3C), 32.6, 44.1 (2C), 49.0, 54.1, 65.6, 96.0, 105.5, 116.2, 120.3, 128.2, 128.9, 129.0, 130.4, 130.5, 135.9, 141.3, 143.5, 143.7, 158.0. IR (ATR): 2222, 1606, 1471, 1227, 1201, 1153, 912, 704 cm^{-1}. Anal. Calcd for $C_{25}H_{31}N_5$: C, 74.78; H, 7.78; N, 17.44. Found: C, 74.55; H, 7.79; N, 17.40. $[\alpha]_D^{25}$ −15.8 (*c* 1.01, $CHCl_3$).

6.1.22. 5-[(3*S*)-3-(Dimethylamino)pyrrolidin-1-yl]-2,7-dimethyl-6-phenylimidazo[1,2-*a*]pyridine-8-carbonitrile (30)

Compound **30** was prepared from **28b**, as described above for **9**. Yield, 24% as a pale red solid. Mp: 194–196 °C. MS (ESI) *m*/*z*: 360 (M+1)$^+$. ^{1}H NMR ($CDCl_3$) δ: 1.67 (1H, m), 1.95 (1H, m), 2.14 (6H, s), 2.27 (3H, s), 2.52 (3H, s), 2.56 (1H, m), 2.81 (1H, m), 2.94–3.03 (2H, m), 3.12 (1H, dd, *J* = 7.1, 9.1 Hz), 7.15 (1H, m), 7.19 (1H, m), 7.29 (1H, br s), 7.43–7.50 (3H, m). ^{13}C NMR ($CDCl_3$) δ: 14.7, 19.8, 30.1, 44.1 (2C), 49.0, 54.0, 65.6, 95.7, 108.6, 115.7, 120.6, 128.3, 129.0, 129.0, 130.3, 130.5, 135.7, 141.0, 143.7, 144.0, 144.2. IR (ATR): 2817, 2767, 2221, 1604, 1471 cm^{-1}. Anal. Calcd for $C_{22}H_{25}N_5$: C, 73.51; H, 7.01; N, 19.48. Found: C, 73.24; H, 6.93; N, 19.24. $[\alpha]_D^{25}$ −17.6 (*c* 1.01, $CHCl_3$).

6.1.23. (3-*tert*-Butyl-1*H*-pyrazol-5-yl)acetonitrile (32)

To a solution of potassium cyanide (19.2 g, 0.295 mol) in water (23 mL) was dropwised a solution of 3-*tert*-butyl-5-(chloromethyl)-1*H*-pyrazole hydrochloride (**31**)[20] (8.80 g, 42.1 mmol) in EtOH (80 mL) at 0 °C over 1 h. The mixture was stirred at room temperature for 4.5 h. The reaction mixture was filtered and concentrated in vacuo, and the residue was purified by silica gel column chromatography eluting with $CHCl_3$/MeOH = 100/1, v/v to yield **32** (5.87 g, 85%) as a yellow solid. MS (FAB) *m*/*z*: 164 (M+1)$^+$. ^{1}H NMR ($CDCl_3$) δ: 1.33 (9H, s), 3.74 (2H, s), 6.10 (1H, s).

6.1.24. (*Z*)-3-Methoxy-2-phenylbut-2-enenitrile (34)

A mixture of 3-oxo-2-phenylbutanenitrile (**33**) (25.0 g, 0.157 mol) and trimethyl orthoacetate (140 mL, 1.21 mol) was refluxed for 6 h. The reaction mixture was cooled to room temperature and concentrated in vacuo. The residue was purified by silica gel column chromatography eluting with *n*-hexane/AcOEt = 10/1, v/v to 5/1, v/v to give **34** (15.4 g, 57%) as a pale yellow oil. MS (FAB) *m*/*z*: 174 $(M+1)^+$. ^{1}H NMR ($CDCl_3$) δ: 2.45 (3H, s), 3.86 (3H, s), 7.18–7.26 (1H, m), 7.26–7.37 (2H, m), 7.58–7.64 (2H, m).

6.1.25. 7-Amino-2-*tert*-butyl-5-methyl-6-phenylpyrazolo[1,5-*a*]pyridine-4-carbonitrile (35)

Under a nitrogen atmosphere, lithium diisopropylamide solution (2.0 M in heptanes/THF/ethylbenzene, 2.02 mL, 4.05 mmol) was added dropwise to a solution of **32** (300 mg, 1.84 mmol) in THF (30 mL) at −30 °C. The mixture was stirred at the same temperature for 30 min and then a solution of **34** (318 mg, 1.84 mmol) in THF (5 mL) was added dropwise at −30 °C. After being stirred for 4 h at −30 °C, the mixture was stirred for 15 h at 0 °C. The reaction mixture was quenched with satd NH_4Cl aq and extracted with AcOEt. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica gel column chromatography eluting with *n*-hexane/AcOEt = 6/1, v/v to yield **35** (235 mg, 42%) as a pale yellow solid. MS (FAB) *m*/*z*: 305 $(M+1)^+$. ^{1}H NMR ($CDCl_3$) δ: 1.42 (9H, s), 2.25 (3H, s), 5.48 (2H, br s), 6.44 (1H, s), 7.23–7.30 (2H, m), 7.42–7.56 (3H, m).

6.1.26. 2-*tert*-Butyl-7-[(3*S*)-3-(dimethylamino)pyrrolidin-1-yl]-5-methyl-6-phenylpyrazolo[1,5-*a*]pyridine-4-carbonitrile (36)

A mixture of *tert*-butyl nitrite (88 μL, 0.74 mmol) and copper (II) chloride (80 mg, 0.59 mmol) in MeCN (3 mL) was stirred at 75 °C for 5 min. To the reaction mixture was added **35** (150 mg, 0.49 mmol) at the same temperature. After being stirred for 1 h at 75 °C, the mixture was cooled to room temperature and diluted with $CHCl_3$. The resultant mixture was washed with 1 M HCl aq and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica gel column chromatography eluting with *n*-hexane/AcOEt = 8/1, v/v to afford a crude product of 2-*tert*-butyl-7-chloro-5-methyl-6-phenylpyrazolo[1,5-*a*]pyridine-4-carbonitrile. The obtained crude product was diluted with DMF (2 mL). (3*S*)-3-(Dimethylamino)pyrrolidine (21 mg, 0.185 mmol) and triethylamine (41 μL, 0.308 mmol) was added and the resulting mixture was stirred at 90 °C for 4 h. The mixture was cooled to room temperature and concentrated in vacuo. The residue was dissolved with AcOEt and the mixture was washed with satd $NaHCO_3$ aq and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica gel column chromatography eluting with $CHCl_3$/MeOH = 100/1, v/v to afford **36** (23 mg, 12%) as a yellow solid. Mp: 125–127 °C. MS (FAB) *m*/*z*: 402 $(M+1)^+$. ^{1}H NMR ($CDCl_3$) δ: 1.42 (9H, s), 1.50–1.70 (1H, m), 1.88–1.97 (1H, m), 2.17 (6H, s), 2.20 (3H, s), 2.63–2.75 (1H, m), 3.16–3.26 (2H, m), 3.38–3.59 (2H, m), 6.45 (1H, s), 7.09–7.23 (2H, m), 7.35–7.47 (3H, m). ^{13}C NMR ($CDCl_3$) δ: 20.0, 29.3, 30.6 (3C), 32.7, 43.6 (2C), 50.3, 54.7, 65.2, 91.8, 92.2, 115.0, 117.5, 127.8, 128.7, 128.8, 131.1, 131.2, 137.1, 140.9, 143.3, 143.9, 165.2. IR (KBr): 2960, 2865, 2817, 2768, 2209, 1603, 1515, 1468, 1443 cm^{-1}. Anal. Calcd for $C_{25}H_{31}N_5 \cdot 0.75H_2O$: C, 72.34; H, 7.89; N, 16.87. Found: C, 72.59; H, 7.63; N, 16.73. $[\alpha]_D^{25}$ −1.14 (*c* 1.02, $CHCl_3$).

6.2. In vitro antifungal activity

The MIC-0 against each fungal strain was measured by the microdilution method described by the Clinical and Laboratory Standard Institute (CLSI), except that the incubation temperature was 30 °C.[22] The MIC-0 was defined as the lowest drug concentration producing an optically clear well.

The MIC-2 (the lowest drug concentration showing 50% growth inhibition compared to the control without drug) was evaluated by the CLSI standard microdilution method with some modification using RPMI 1640 media with 0.165 M MOPS buffer as the medium. Briefly, in the experiments using RPMI 1640 media, alamar blue (Biosource, Camarillo, CA) was used to accurately define its MIC-2.[23–25] Test organisms were purchased from the American Type Culture Collection (Rockville, MD), the Institute for Fermentation Osaka (Osaka, Japan), or Teikyo Institute of Medical Mycology (Tokyo, Japan). Initial cell densities (1×10^3 to 1×10^4 cells/mL) and incubation time (18–72 h) were decided for each strain in consideration of its growth speed. The fungi were incubated at 30 °C. Following incubation, OD_{570} was measured with a Wallac 1420 ARVOsx multi-label counter (Wallac, Tokyo, Japan) and MIC-2s were calculated as a 50% reduction compared with the drug-free control in absorbance at 570 nm. Compounds were tested at different concentrations ranging from 0.004 to 128 μg/mL.

6.3. Incorporation study with growing cells using [^{14}C]-glucose

Incorporation study with growing cells was conducted based on the methods described by Kitamura et al.[11] Exponentially growing cells of *C. albicans* were suspended in an RPMI 1640 medium to give ~0.7 of absorbance at 595 nm. After the drug solution and radioactive [^{14}C]-glucose were added, the reaction tubes were incubated at 30 °C with occasional shaking. After 3 h incubation, samples were taken and crude fractions of β-1,3-glucan, β-1,6-glucan, chitin, and mannan were prepared as follows. The harvested cells were extracted with 3% NaOH at 80 °C in 1 h. Mannan fractions were prepared from the supernatant using Fehling's reaction. Insoluble material was washed and digested with Zymolyase 100T (Seikagaku-kougyou) overnight. After digestion, insoluble material was harvested as a chitin fraction. The supernatants were taken as glucan fractions (β-1,3-glucan + β-1,6-glucan fraction) and were dialyzed overnight. After dialysis, samples were taken as a β-1,6-glucan fraction. The radioactivity of each fraction was counted with a toluene scintillator. The radioactivities of the β-1,3-glucan fraction were calculated by subtracting that β-1,6-glucan fraction from the glucan fraction.

6.4. Distribution coefficient (Log *D*)

The distribution coefficients (Log *D*) were determined by the shake-flask method. Four hundred micromolar of compound solution of each compound in a 2 mL *n*-octanol–2 mL BPS solution was placed on a shaker for 30 min at pH 6.8. After centrifuging each solution separately at 3000 rpm for 10 min, an LC/MS method was used to assay each layer. The LC/MS system consisted of an 1100 Series LC/MSD (Agilent) and an X Terra® MSC18 3.5 μm, 3.0 × 30 mm column (Waters). The mobile phase was a 10 mM ammonium acetate buffer (pH 4.5)/0.05% (v/v) acetic acid mixture in acetonitrile; the gradient condition (95/5-10/90). Analyst software program (version 1.4, Applied BioSystems) was used to calculate the Log *D*.

6.5. Metabolic stability

Compounds (final 1 μM) were incubated with human liver microsome in sodium phosphate buffer (pH 7.4) for 20 min at 37 °C. The microsomal protein concentration in the assay was 0.5 mg/mL. Reaction was started by the addition of NADPH at 37 °C and stopped by addition of MeOH after 30 min. After centrifuging each solution separately at 3500 rpm for 10 min at 4 °C, the corresponding loss of parent compound was determined by LC/MS/MS.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.06.096. These data include MOL files and InChiKeys of the most important compounds described in this article.

References and notes

1. Rüping, M. J. G. T.; Vehreschild, J. J.; Cornely, O. A. *Drugs* **2008**, *68*, 1941.
2. Drew, R. *Int. J. Antimicrob. Agents* **2006**, *27S*, S36.3.
3. Pappas, P. G.; Rex, J. H.; Sobel, J. D.; Filler, S. G.; Dismukes, W. E.; Walsh, T. J.; Edwards, J. E. *Clin. Infect. Dis.* **2004**, *38*, 161.
4. Ostrosky-Zeichner, L.; Marr, K. A.; Rex, J. H.; Cohen, S. H. *Clin. Infect. Dis.* **2003**, *37*, 415.
5. Fridkin, S. K. *Clin. Infect. Dis.* **2005**, *41*, 1455.
6. Enoch, D. A.; Ludlam, H. A.; Brown, N. M. *J. Med. Microbiol.* **2006**, *55*, 809.
7. Georgopapadakou, N. H. *Curr. Opin. Microbiol.* **1998**, *1*, 547.
8. Pfaller, M. A.; Diekema, D. J. *J. Clin. Microbiol.* **2004**, *42*, 4419.
9. Singh, N. *Clin. Infect. Dis.* **2001**, *33*, 1692.
10. Fridkin, S. K.; Jarvis, W. R. *Clin. Microbiol. Rev.* **1996**, *9*, 499.
11. Kitamura, A.; Someya, K.; Hata, M.; Nakajima, R.; Takemura, M. *Antimicrob. Agents Chemother.* **2009**, *53*, 670.
12. Takeshita, H.; Watanabe, J.; Kimura, Y.; Kawakami, K.; Takahashi, H.; Takemura, M.; Kitamura, A.; Someya, K.; Nakajima, R. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 3893.
13. Kapteyn, J. C.; Hoyer, L. L.; Hecht, J. E.; Müller, W. H.; Andel, A.; Verkleij, A. J.; Makarow, M.; Ende, H. V. D.; Klis, F. M. *Mol. Microbiol.* **2000**, *35*, 601.
14. Roemer, T.; Paravicini, G.; Payton, M. A.; Bussey, H. *J. Cell. Biol.* **1994**, *127*, 567.
15. Umeyama, T.; Kaneko, A.; Watanabe, H.; Hirai, A.; Uehara, Y.; Niimi, M.; Azuma, M. *Infect. Immun.* **2006**, *74*, 2373.
16. Roth, G. A.; McClymont, E. L. *J. Agric. Food Chem.* **1991**, *39*, 612.
17. Connolly, D. J.; Guiry, P. J. *Synlett* **2001**, 1707.
18. Matsubara, R.; Berthiol, F.; Nguyen, H. V.; Kobayashi, S. *Bull. Chem. Soc. Jpn.* **2009**, *82*, 1083.
19. Tolmachova, N. A.; Gerus, I. I.; Vdovenko, S. I.; Haufe, G.; Kirzhner, Y. A. *Synthesis* **2007**, *24*, 3797.
20. Grotjahn, D. B.; Van, S.; Combs, D.; Lev, D. A.; Schneider, C.; Rideout, M.; Meyer, C.; Hernandez, G.; Mejorado, L. *J. Org. Chem.* **2002**, *67*, 9200.
21. Doyle, M. P.; Siegfried, B.; Dellaria, J. F., Jr. *J. Org. Chem.* **1977**, *42*, 2426.
22. Clinical and Laboratory Standard Institute. Reference method for broth dilution antifungal susceptibility testing of yeast—3rd ed: approved standard M27-A3. CLSI, Wayne, PA, USA, 2008.
23. Davey, K. G.; Szekely, A.; Johnson, E. M.; Warnock, D. W. *J. Antimicrob. Chemother.* **1998**, *42*, 439.
24. Tiballi, R. N.; He, X.; Zarins, L. T.; Revankar, S. G.; Kauffman, C. A. *J. Clin. Microbiol.* **1995**, *33*, 915.
25. Kitamura, A.; Someya, K.; Okumura, R.; Hata, M.; Takeshita, H.; Nakajima, R. *Biol. Pharm. Bull.* **2010**, *33*, 192.