Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry 11 (2003) 4463–4478

BIOORGANIC & MEDICINAL CHEMISTRY

# Synthesis and Biological Activities of Benzofuran Antifungal Agents Targeting Fungal *N*-Myristoyltransferase

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Received 19 February 2003; accepted 18 June 2003

**Abstract**—The C-4 side chain modification of lead compound **1** has resulted in the identification of a potent and selective *Candida albicans N*-myristoyltransferase (CaNmt) inhibitor RO-09-4609, which exhibits antifungal activity against *C. albicans* in vitro. Further modification of its C-2 substituent has led to the discovery of RO-09-4879, which exhibits antifungal activity in vivo. The drug design is based on X-ray crystal analysis of a CaNmt complex with benzofuran derivative **4a**. The optimization incorporates various biological investigations including a quasi in vivo assay and pharmacokinetic study. The computer aided drug design, synthesis, structure–activity relationships, and biological properties of RO-09-4879 are described in detail.


## Introduction

The incidence of fungal infection has increased significantly in the past 25 years. The growing number of immunocompromised patients as a result of cancer chemotherapy, organ transplantation, and HIV infection are the major factors contributing to this incidence. Since *Candida albicans* (*C. albicans*), and *Aspergillus fumigatus* (*A. fumigatus*) are the main causative fungi of the systemic mycosis, antifungal drugs for treating patients of deep mycosis should have a broad antifungal spectrum including at least these microorganisms. Currently only four classes of antifungal drugs, polyene macrolides (amphotericin B), azoles (fluconazole, miconazole, itraconazole and voriconazole), flucytosine, and candins (caspofungin acetate and micafungin), are available for treatment of systemic mycoses. Unfortunately, none of them is ideal in terms of efficacy, antifungal spectrum or safety. Although amphotericin B is efficacious against both candidiasis and aspergillosis, it shows severe renal toxicity. Azoles show drug–drug interactions with various drugs by inhibiting metabolic enzymes, the CYP450 isoenzymes.[1] The antifungal spectra of fluconazole and flucytosine are narrow (mainly against *C. albicans*) and they are prone to develop drug resistance. Although caspofungin and micafungin are fungicidal in *Candida,* they are fungistatic in *Aspergillus* and inactive against *Cryptococcus neoformans* (*C. neoformans*), a clinically emerging fungus. In addition, these drugs are given only by iv infusion due to poor oral bioavailability. To overcome the drawbacks of the current antifungal drugs and to obtain more efficacious drugs, an antifungal drug having a novel mode of action should be developed. Myristoyl CoA:protein *N*-myristoyltransferase (Nmt; EC 2.1.3.97) is a cytosolic monomeric enzyme that catalyzes the transfer of the myristoyl group from myristoyl CoA to the N-terminal glycine of a number of eukaryotic cellular proteins and viral proteins.[2–4] *N*-Myristoylproteins have diverse functions and intracellular destinations. *N*-Myristoylation is an irreversible protein modification that occurs co-translationally after the removal of the initiator methionine by cellular methionylaminopeptidases.[5,6] *N*-Myristoylation results in an increase of lipophilicity that triggers the promotion of reversible protein–membrane and protein–protein interactions.[7,8] *N*-Myristoylation relates to diverse biological processes including signal transduction cascades and apoptosis.[9,10] *N*-Myr-

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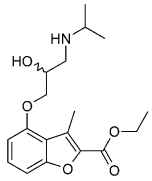
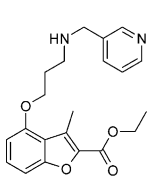
doi:10.1016/S0968-0896(03)00429-2

istoylation of several G-proteins, Gpa1, Arf1, Arf2, and Vps15, which are essential for fungal growth in vitro, has been reported to be indispensable for their function in *Saccharomyces cerevisiae*.[11–14] Nmt has also been proven to be essential for viability in vitro of fungi, including medically important pathogenic fungi such as *C. albicans* and *C. neoformans*, which cause systemic fungal infections in immunocompromised patients.[15,16] Furthermore, we have recently reported that a defect of Nmt causes the loss of the ability of *C. albicans* to infect in mice.[17] These data suggest that fungal Nmt is a potential target enzyme for the development of novel fungicidal drugs having a broad antifungal spectrum. In addition, since the mode of action is novel, the Nmt inhibitors might overcome the drawbacks of current antifungal drugs, such as resistance or drug–drug interactions. On the other hand, since Nmt is also distributed in mammalian cells, the inhibitors need to be fungal Nmt selective to avoid adverse events caused by inhibiting human Nmt. So far, peptidomimetic inhibitors,[18–26] myristic acid analogues,[27,28] benzothiazole inhibitors,[29,30] *p*-toluenesulfonamide inhibitors,[31] and benzofuran inhibitors[32–39] have been reported to be fungal Nmt inhibitors. Among them, the benzofuran inhibitors that we synthesized showed high selectivity over human Nmt and exhibited antifungal activity in vivo. We identified lead compound **1** that competitively inhibited CaNmt ($IC_{50}$: 0.98 μM) with high selectivity over human Nmt ($IC_{50}$: 194 μM) by a random screening of the Roche chemical libraries. Since antifungal activity in vitro of **1** was only marginal ($IC_{50}$ against *C. albicans* CY1002: 390 μM), our first aim was to improve the in vitro activity. The C-4 side-chain modification of **1** resulted in finding the potent and selective CaNmt inhibitor **10h** (RO-09-4609), which exhibited antifungal activity against *C. albicans* in vitro. Further modification of **10h** led to the discovery of **15g** (RO-09-4879), which exhibited antifungal activity in vivo. The drug design was based on X-ray crystallographic analysis of a CaNmt complex with a benzofuran derivative **4a**.[38] The optimization incorporated various biological investigations including a quasi in vivo assay[40,41] (an in vitro antifungal assay in 80% serum) and a cassette dosing pharmacokinetic (PK) study.[42–44] This paper describes in full the drug design, synthesis, structure–activity relationships (SARs), and lead optimization process.

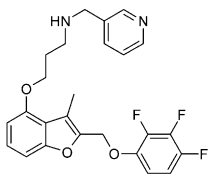
The biological properties of **15g** (RO-09-4879) are also discussed in detail.



**1**



**10h** (RO-09-4609)



**15g** (RO-09-4879)

## Chemistry

The general synthesis of the benzofuran derivatives is outlined in Scheme 1. 4-Oxiranylmethoxy derivatives **3a** and **3b** were prepared from 4-hydroxybenzofuran derivative **2** by treatment with excess (2*S*)- and (2*R*)-glycidyl tosylate in the presence of NaH in *N*,*N*-dimethylformamide (DMF), respectively. (*R*,*S*)-4-Oxiranylmethoxy derivative **3** was prepared from **2** by treatment with excess epichlorohydrin in the presence of $Cs_2CO_3$ in acetonitrile at 60 °C. Amination of epoxides **3**, **3a**, and **3b** with *tert*-butylamine in ethanol at 60 °C gave amino compounds **4**, **4a**, and **4b**, respectively. Treatment of **2** with excess alkylene dibromide in the presence of potassium carbonate in DMF gave *O*-bromoalkyl derivatives **5**, **6**, **7**, and **8**. Treatment of **5**, **6**, **7**, and **8** with various amines in ethanol at 50–70 °C gave the desired amino derivatives **9**, **10a–j**, **11**, and **12**, respectively. 3-(Pyridin-3-ylmethoxy)propoxy analogue **13** was also prepared from **6** by treatment with 3-pyridinemethanol in the presence of NaH in DMF. Ethers **15a–k**, **16**, and **17** were synthesized by Mitsunobu reaction of appropriate phenols and **14** that was obtained by $LiAlH_4$ reduction of **10h**. The modified Mitsunobu reaction using 1,1′-(azodicarbonyl)dipiperidine (ADDP) and tributylphosphine gave **15f** and **15g** in higher yields (40–80%) than the conventional Mitsunobu reaction using diethyl azodicarboxylate (DEAD) and triphenylphosphine (TPP).

## Results and Discussion

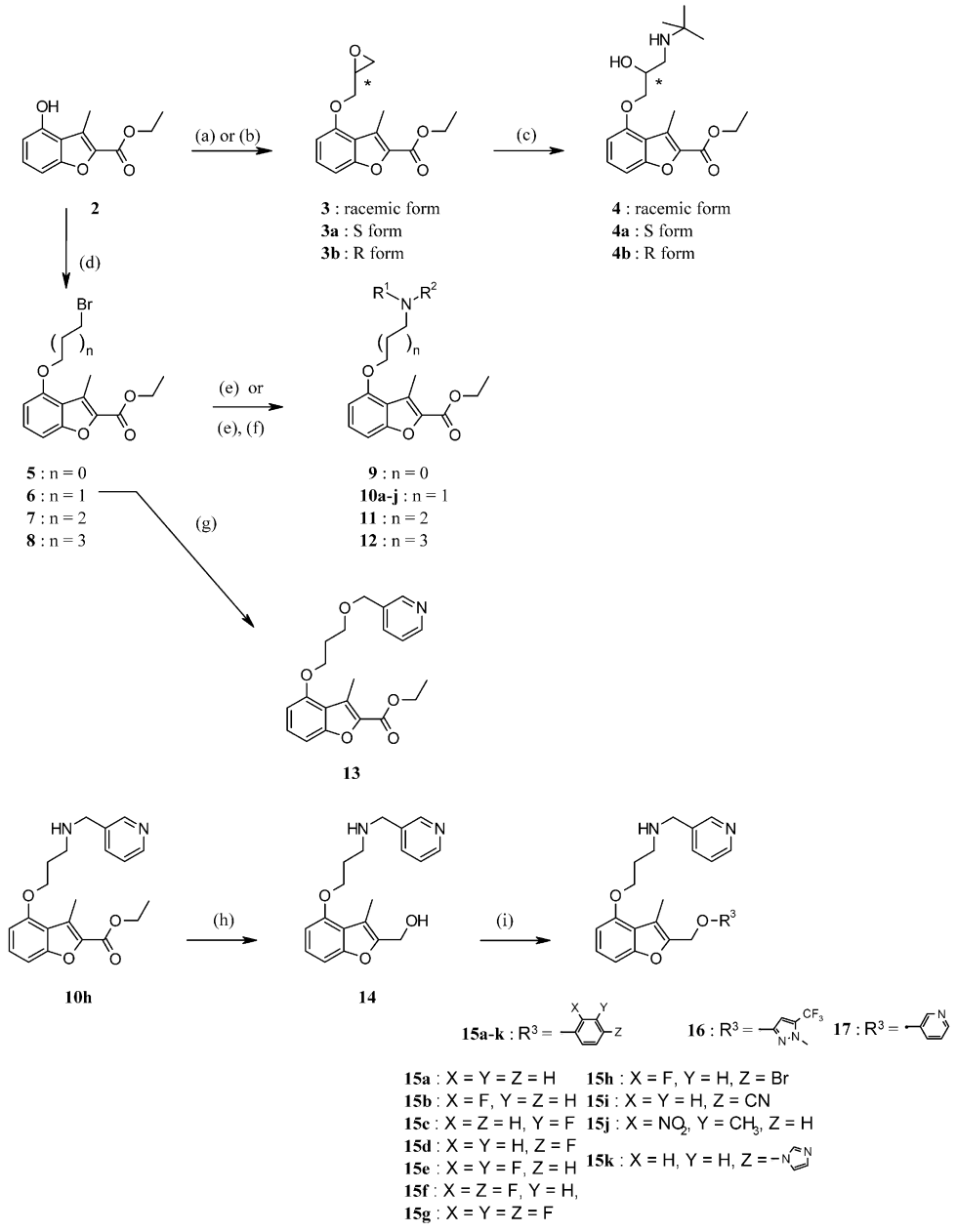
### Modification of the C-4 substituent of 1

Lead compound **1** was originally reported to have β-adrenoceptor blockade.[45] Since it is well known that an aromatic compound having a β-aminoalcohol moiety shows β-blocking action, we removed the hydroxy group of **1** to eliminate this activity. As expected, removing the hydroxy group lowered the β-adrenoceptor blockade significantly without affecting its CaNmt enzyme inhibitory activity (Table 1). The $IC_{50}$s of **10a** and **10b** in the β-adrenoceptor blockade assay were 38 and 25 μM, respectively, whereas those of the corresponding hydroxy derivatives **1** and **4** were 0.098 and 0.0043 μM, respectively. To find the optimal chain length of the C-4 position, ethylene **9**, tetramethylene **11**, and pentamethylene **12** derivatives were also prepared and their enzyme inhibitory activity was compared with that of the trimethylene derivative **10b**. The result indicated that the optimal chain length was C3 (trimethylene).

For further optimization, we used the crystal structure of a binary complex of CaNmt and **4a** that was obtained at 3.5 Å resolution by a soaking experiment (Fig. 1).[38] The benzofuran moiety is located at the center of a deep pocket, surrounded by hydrophobic residues. The C-4 substituent on the benzofuran ring extends to a C-terminal leucine (Leu 451) of the polypeptide chain and the *tert*-butyl group is surrounded by a hydorophobic pocket consisting of aromatic amino acid residues, Tyr 107, Tyr 119, and Phe 176. The amino group in the C-4 side chain makes a salt bridge with the carboxyl group of Leu 451. This interaction should be essential for the enzyme inhibitory activity because the

C-terminal carboxylate plays an important role in the catalytic mechanism.[48] This structural analysis suggested that the replacement of the *tert*-butyl amino group by another hydrophobic amino group or an alkyl amino group with an aromatic ring would increase the enzyme inhibitory activity. The weak inhibitory activity of primary amine **10c** supported this analysis (Table 2). Therefore, a number of benzofurans having a hydrophobic group or an alkyl group with an aromatic ring on the amino group were synthesized.

The aniline derivative **10e** and pyridin-3-ylmethoxy derivative **13** were devoid of the inhibitory activity, suggesting that the strong basicity of an aliphatic amino group is essential for the inhibitory activity. The (pyridin-3-ylmethyl)amino derivative **10h** (RO-09-4609) showed the most potent enzyme inhibitory activity against CaNmt with extremely high selectivity (> 5000-fold) over human Nmt. Its increased enzyme inhibitory activity over benzyl derivative **10f** indicated the presence of additional favorable hydrogen bonding between the pyridine nitrogen and an amino acid residue of CaNmt (*vide infra*). Since other heteroaromatic derivatives such as (pyridin-2-ylmethyl)amino **10g**, (pyridin-4-ylmethyl)-amino **10i**, and (pyridin-3-ylethyl)amino **10j** derivatives showed weaker activity than did **10h**, the position of the




**Scheme 1.** Preparation of benzofuran derivatives. Reagents and conditions: (a) (2*S*)- or (2*R*)-glycidyl tosylate, NaH, DMF, rt, 76–83%; (b) epichlorohydrin. $Cs_2CO_3$, $CH_3CN$, 60 °C, 86%; (c) *tert*-$BuNH_2$, EtOH, 60 °C, 74–95%; (d) alkylene dibromide, $K_2CO_3$, DMF, rt or 100 °C, 1–4 h, 43–88%; (e) $R^1R^2NH$, EtOH, 50–70 °C, 34–98%; (f) $H_2$, 10% Pd/C, EtOH, rt, 45% (g) 3-pyridinemethanol, NaH, DMF, rt, 11%; (h) $LiAlH_4$, THF, 0 °C, 91%; (i) Mitsunobu reaction.

pyridine nitrogen was very important to gain stronger binding affinity.

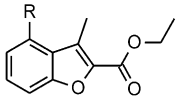
To find the optimal substituent of the C-3 position, the C-3 methyl group of **10h** was replaced by hydrogen, ethyl, cyclopropyl, and isopropyl; however, the methyl was still the best among them.[32]

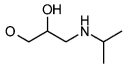
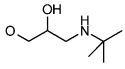
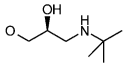
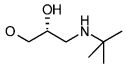
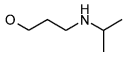
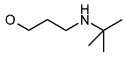
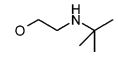
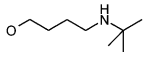
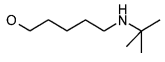
Although **10h** was about 10 times stronger than **1** in terms of CaNmt inhibitory activity and showed clear antifungal activity against *C. albicans*, it did not show antifungal activity in vivo in a murine systemic candidiasis model. Its antifungal activity might be weak and its metabolic stability (elimination half-life $t_{1/2}$: 0.42 h in mice) insufficient. The ester group of **10h** was easily hydrolyzed by esterases in mice to give an inactive carboxylic acid metabolite. To overcome these drawbacks, we modified the C-2 substituent of **10h**.

## Modification of the C-2 substituent of 10h

The crystal structure of a binary complex of CaNmt and **4a** (Fig. 1) revealed that the C-2 substituent is surrounded by three phenylalanine residues, Phe 115, Phe 240, and Phe 339. To strengthen the binding between the C-2 substituent and these phenylalanine residues through aromatic–aromatic interaction, we introduced a phenyl group to the C-2 position via various linkers: –CONH–, $–CH_2S–$, $–CH_2CH_2–$, and $–CH_2O–$ (**15a**) which are expected to be much more metabolically stable than an ethyl ester group. Among them, compound **15a** having an ether linker showed the most potent CaNmt inhibitory activity and antifungal activity. Next, we introduced various substituents on the phenyl ring of **15a**.[33] To increase the binding affinity, electron-withdrawing groups, such as fluoro, bromo, cyano, and nitro, were introduced to the phenyl ring of **15a** to

**Table 1.** Enzyme inhibitory activity, antifungal activity, and β-adrenoceptor blockade of benzofuran derivatives ($IC_{50}$: μM)



| Compd | R | Enzyme inhibition | | Antifungal activity[c] | β-Adrenoceptor blockade[d] |
|---|---|---|---|---|---|
| | | CaNmt[a] | HsNmt[b] | *C. albicans* CY1002 | |
| **1** |  | 0.98 | 194 | 390 | 0.098 |
| **4** |  | 1.2 | 470 | 210 | 0.0043 |
| **4a** |  | 1.1 | 190 | 39 | 0.079 |
| **4b** |  | 150 | >290 | 40 | 0.38 |
| **10a** |  | 4.4 | 380 | 470 | 38 |
| **10b** |  | 1.7 | 200 | 300 | 25 |
| **9** |  | 50 | >630 | 590 | NT[e] |
| **11** |  | 4.4 | >580 | 190 | NT |
| **12** |  | 15 | >550 | 53 | NT |
| Alprenolol | | NT | NT | NT | 0.0023 |

[a]Inhibitory activity against *C. albicans* Nmt (CaNmt) as assessed by $IC_{50}$ using substrate peptide GLTISKLFRR-$NH_2$ (0.5 μM) and myristoyl-CoA at 0.5 μM.
[b]Inhibitory activity against human Nmt (HsNmt) as assessed by $IC_{50}$ using substrate peptide GNAASARR-$NH_2$ (0.5 μM) and myristoyl-CoA at 0.5 μM.
[c]Antifungal activity against *C. albicans* CY1002 as assessed by $IC_{50}$ in YNBPB medium (1% glucose, 0.25% $K_2HPO_4$, pH 7).
[d]Adrenergic, beta, non-selective binding assay, performed by NOVA SCREEN according to a literature method with modifications.[46,47]
[e]NT, Not tested.

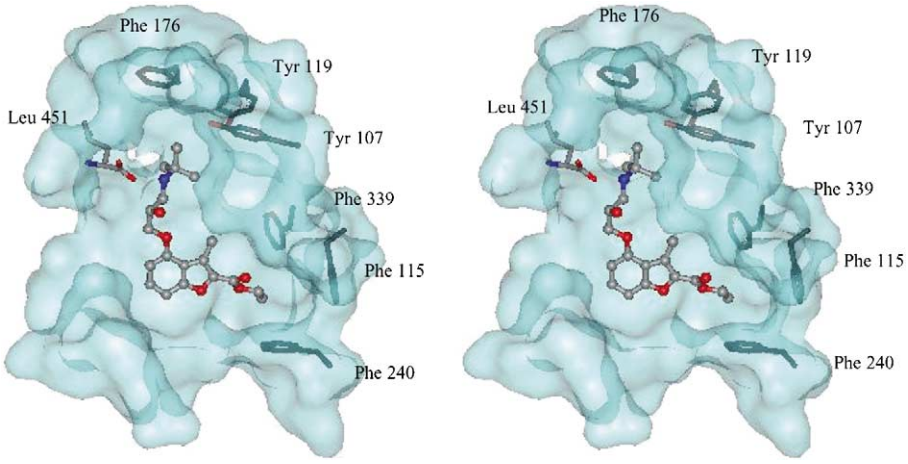



**Figure 1.** Crystal structure of a binary complex of CaNmt and **4a**. Only the binding site is shown.

interact with the aromatic residues of CaNmt more strongly.[49] As expected, most of the phenyl ether derivatives having electron-withdrawing group(s) showed stronger inhibitory activity than the un-substituted phenyl ether **15a** (Table 3). Among them, the 2-fluoro **15b**, 4-fluoro **15d**, 2,3-difluoro **15e**, 2,4-difluoro **15f** (RO-09-4746), 2,3,4-trifluoro **15g** (RO-09-4879), 4-cyano **15i**, and 2-nitro-3-methyl **15j** derivatives showed very strong inhibitory activity against CaNmt ($IC_{50}$: 3.7–9.4 nM). In contrast to this, the 3-fluoro derivative **15c** was 10–30 times less active. This variation in inhibitory activity among the ether derivatives could be explained by the differences in the electron density on the ether oxygen calculated by the electrostatic potentials around the aromatic rings of the compounds using a molecular orbital method.[37] We also introduced heteroaromatics, 1-methyl-5-trifluoromethyl-1*H*-pyrazole **16** and 3-pyridyl **17**, to the C-2 position via $–CH_2O–$. The enzyme inhibitory activity of **16** was as strong as the fluorophenyl derivatives. The binding mode of phenyl ether derivatives was examined by solving a crystal structure of a binary complex of CaNmt and **15e** that was obtained at 3.5 Å resolution by a soaking experiment.[38] The positions of the fluorine atoms were not well defined because of their poor electron density. A schematic representation of the hydrogen-bonding interactions between **15e** and CaNmt are shown in Figure 2. **15e** is located at a hydrophobic pocket composed of hydrophobic amino acid residues including Tyr 107, Phe 115, Phe 117, Tyr 119, Phe 176, His 227, Phe 240, Tyr 225, Tyr 354, and Phe 339. The benzofuran ring of **15e** is stacked parallel to Tyr 225 and interacts with Tyr 354 in a perpendicular orientation in the proximity of Phe 117 and Phe 339. The benzene ring of the benzofuran occupies the hydrophobic pocket with Leu 394, Tyr 225, and Cys 393 of the polypeptide chain and little space is left for an extra substituent at the C-6 and C-7 positions of the benzofuran. This observation is consistent with the SARs that show mono-methylation at the C-7 position lowered CaNmt inhibitory activity by a factor of 250. The ring oxygen of the benzofuran is located in the proximity of the His 227 imidazole ring and makes a hydrogen bond to a ring nitrogen of His 227. Since {3-[2-(2,4-difluoro-phenoxymethyl)-3-methylbenzothiophene-4-yloxy]propyl}pyridin-3-ylmethylamine, a benzothiophene analogue of compound **15f**, showed about 1000-fold lower inhibitory activity, the hydrogen bond interaction of the benzofuran ring with the imidazole ring of His 227 might greatly contribute to the inhibitor binding. The phenyl ring of the C-2 substituent of **15e** is surrounded by three phenylalanine residues, Phe 115, Phe 240, and Phe 339, that make hydrophobic packing interactions, and is situated exactly between Phe 240 and Phe 339. The crystal structure also suggested the important hydrogen bond between the amido-$NH_2$ of Asn 392 and the ether oxygen of the C-2 substituent. The 3-pyridyl group in the C-4 side chain is surrounded by hydrophobic aromatic amino acid residues, Tyr 107, Tyr 119, and Phe 176. In addition, hydrogen bonding between the pyridine nitrogen and the Tyr 119 hydroxy group was observed. The increased enzyme inhibitory activity of **10h** over the benzyl derivative **10f** can be explained by this additional hydrogen bonding, supported by the finding that the inhibitory activity of **10h** against a mutant CaNmt having alanine for Tyr 119 was 14 times weaker than that against the wild CaNmt.[32]

## Quasi in vivo assay and cassette dosing PK study for evaluating benzofuran inhibitors

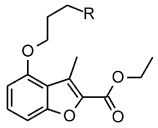
The results of enzyme inhibitory activity, antifungal activity in vitro, quasi in vivo activity, pharmacokinetic (PK) properties, and in vivo antifungal activity are shown in Table 3. To predict in vivo efficacy, we introduced an antifungal assay of 80% calf serum supplemented with 10 μM $FeCl_3 \cdot 6H_2O$, 10 μM deferoxamine and 2% dextrose (quasi in vivo assay). The quasi in vivo assay was reported to be more predictive for in vivo efficacy than the conventional in vitro antifungal assay.[40,41] In addition, a PK study would also be important for predicting in vivo efficacy. To carry out the PK study efficiently and to reduce the number of test animals, we introduced cassette dosing PK.[42–44] Five compounds (2 mg/kg each) were intravenously administered to one rat and the plasma concentration of each compound was measured by LC–MS. This method allowed efficient selection of compounds for in vivo tests. Compounds **15b**, **15d**, **15e**, **15f**, **15g**, **15j**, and **16**

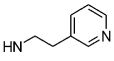
were as potent as fluconazole ($IC_{50}$: 0.5 μM in this assay protocol) in the quasi in vivo assay, although the addition of calf serum lowered their antifungal activity. All the ether derivatives in Table 3 with the exception of **17** showed longer $t_{1/2}$ than did **10h**, as we had expected. Thus, most of the compounds selected for further in vivo evaluation showed reasonable PK profiles and strong quasi in vivo activity. Among them the fluoro derivatives **15f** and **15g**, which showed the strongest quasi in vivo antifungal activity, exhibited the most potent antifungal activity in the in vivo model with $ED_{50}$s of 7.1 mg/kg (Fig. 3). The in vivo activity of **15h** was not as strong ($ED_{50}$: >30 mg/kg) in spite of its reasonable PK profile. Its weak activity in the quasi in vivo assay may explain its weak in vivo activity. The in vivo activity of the ether derivatives was well predicted by the results of the quasi in vivo assay and PK properties.

## Biological properties of 15g

**Antifungal activity in a rat systemic candidiasis model.** Fisher rats ($n=5$) were infected intravenously with a

**Table 2.** Optimization of the C-4 substituent ($IC_{50}$:μM)



| Compound | R | Enzyme inhibition | | Antifungal activity[c] |
|---|---|---|---|---|
| | | CaNmt[a] | HsNmt[b] | *C. albicans* CY1002 |
| **10a** |  | 4.4 | 380 | 470 |
| **10b** |  | 1.7 | 200 | 300 |
| **10c** |  | 11 | >720 | 260 |
| **10d** |  | 4.1 | 38 | 84 |
| **10e** |  | >570 | >570 | >280 |
| **10f** |  | 3.3 | 530 | 10 |
| **10g** |  | 11 | 270 | 200 |
| **10h** (RO-09-4609) |  | 0.1 | >540 | 1.6 |
| **10i** |  | 1.9 | >540 | 180 |
| **10j** |  | 0.39 | >520 | 7.8 |
| **13** |  | >540 | >540 | NT[d] |
| SC-58272[e] | | 0.83 | >140 | 200 |
| Fluconazole | | NT | NT | 0.72 |

[a]Inhibitory activity against *C. albicans* Nmt (CaNmt) as assessed by $IC_{50}$ using substrate peptide GLTISKLFRR-$NH_2$ (0.5 μM) and myristoyl-CoA at 0.5 μM.
[b]Inhibitory activity against human Nmt (HsNmt) as assessed by $IC_{50}$ using substrate peptide GNAASARR-$NH_2$ (0.5 M) and myristoyl-CoA at 0.5 μM.
[c]Antifungal activity against *C. albicans* CY1002 as assessed by $IC_{50}$ in YNBPB medium (1% glucose, 0.25% $K_2HPO_4$, pH 7).
[d]Not tested.
[e]A dipeptide CaNmt inhibitor.[22,23,25]

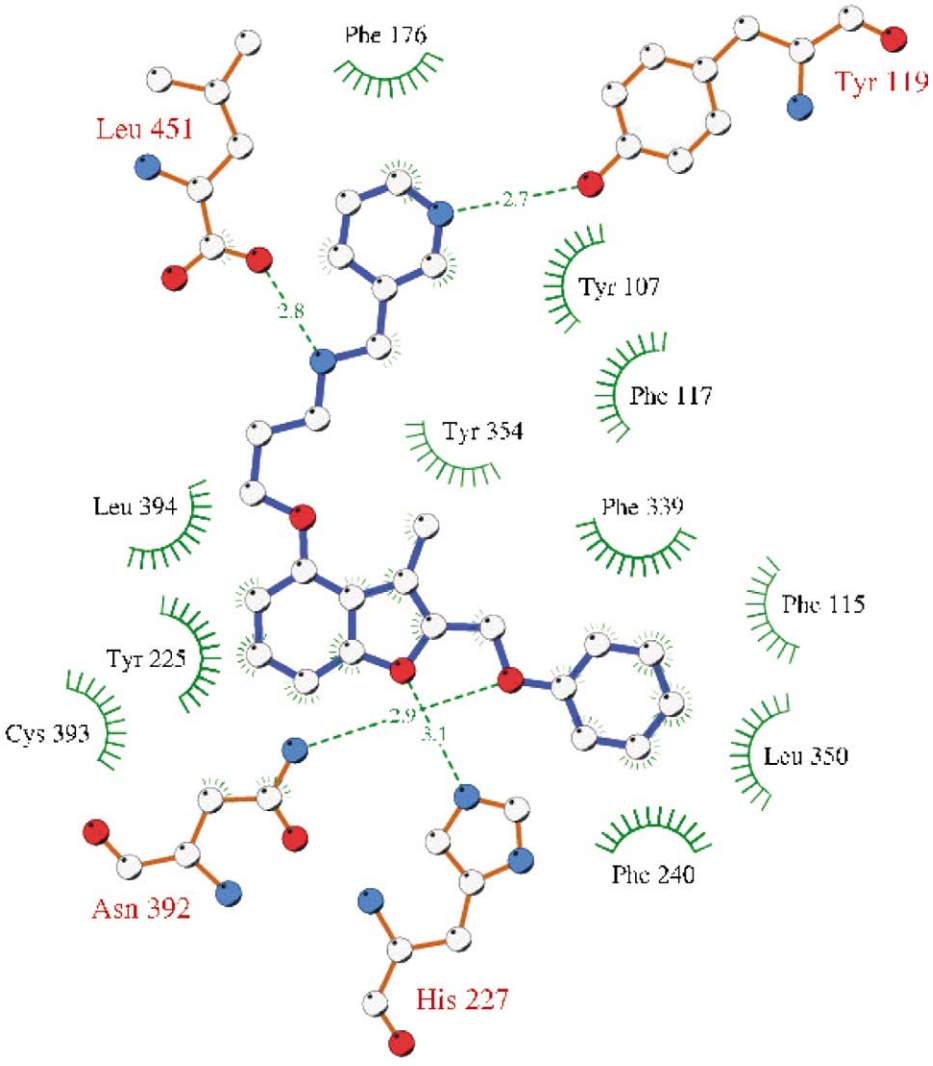



**Figure 2.** Schematic drawing of the interactions between CaNmt and **15e**, generated by LIGPLOT.[50] Residues forming van der Waals interactions are indicated; those participating in hydrogen bonds are represented by a ball-and-stick figure. The inhibitor is also represented by a ball-and-stick figure. Hydrogen bonds are depicted as dotted lines with the donor-acceptor distance given in Å.

lethal dose of *C. albicans* (CY1002) and treated intravenously (iv) with multiple doses (2 tid) of the test compound. Efficacy of the compounds was calculated as the effective dose (mg/kg) for 50% survival ($ED_{50}$) on day 7. The antifungal activity in vivo of the representative compound **15g** is shown in Figure 3. All the animals in the control group died within 2 days. The benzofuran derivative produced a statistically significant increase in survival compared with the control in a dose-dependent manner, although its efficacy was fairly weaker than fluconazole ($ED_{50}$: 0.5 mg/kg, iv).

**Efficacy in the disseminated candidiasis target organ assay of 15g.** The results of the target organ assay of **15g** in a rat systemic candidiasis model are shown in Figure 4. The benzofuran derivative was tested for its activity in reducing the numbers of recoverable yeast cells from the brain and kidney of Fisher rats ($n=3$) challenged intravenously with *C. albicans* CY1002 ($3\times10^3$ cells/g, 0.5 mL). Compound **15g** was administered intravenously in the disseminated candidiasis models, three rats per therapy group. Fluconazole (FCZ) was administered intraperitoneally (ip). Figure 4 shows the mean log10 colony forming unit (CFU) per brain or kidney at day 2 after infection in the groups treated with antifungal agents and that of sham-treated control groups. Compound **15g** reduced the counts of yeast cells recovered from the brain and kidney significantly. The results suggest that the survival of the rats tested was prolonged by inhibiting fungal growth in the rats.

**Time–kill kinetics of 15g.** To confirm the antifungal activity of **15g**, the time–kill kinetics of **15g** for *C. albicans* CY3003 in 80% calf serum was investigated at 2, 4, 15, and 30 times concentrations of the $IC_{50}$ (Fig. 5). Compound **15g** reduced CFU in a dose- and time-dependent manner at concentrations of $15\times IC_{50}$ and $30\times IC_{50}$ showing that it had fungicidal activity. In contrast, fluconazole did not reduce the CFU at a concentration of $200\times IC_{50}$ in the same experiment.

**Antifungal spectrum of 15g.** The antifungal spectra of **15g** and fluconazole are shown in Table 4. Antifungal susceptibility assays were performed using the broth

**Table 3.** Enzyme inhibitory activity, antifungal activity in vitro, quasi in vivo activity, pharmacokinetic (PK) properties, and in vivo antifungal activity

| Compd | Enzyme inhibition $IC_{50}$ (μM) | | Antifungal activity $IC_{50}$ (μM) | | PK parameters in rats[e] | | In vivo antifungal activity[f] $ED_{50}$ (mg/kg) |
|---|---|---|---|---|---|---|---|
| | CaNmt[a] | HsNmt[b] | Serum (−)[c] | Serum (+)[d] | AUC (ng·h/mL) | $t_{1/2}$ (h) | |
| **10h** (RO-09-4609) | 0.10 | >450 | 1.6 | NT[g] | 330 | 0.42 | NT |
| **15a** | 0.072 | 77 | 0.37 | NT | 670 | 1.9 | NT |
| **15b** | 0.0083 | >470 | 0.040 | 0.60 | 560 | 1.6 | NT |
| **15c** | 0.11 | >110 | 0.11 | 3.3 | 370 | 1.5 | NT |
| **15d** | 0.0052 | 67 | 0.021 | 0.70 | 340 | 1.9 | NT |
| **15e** | 0.0037 | 62 | 0.080 | 0.57 | 500 | 1.9 | NT |
| **15f** (RO-09-4746) | 0.0075 | >450 | 0.030 | 0.34 | 420 | 1.6 | 7.1 |
| **15g** (RO-09-4879) | 0.0057 | >430 | 0.035 | 0.33 | 330 | 2.0 | 7.1 |
| **15h** | 0.028 | >400 | 0.074 | 1.3 | 360 | 3.6 | >30 |
| **15i** | 0.0094 | >460 | 0.16 | 1.4 | 340 | 1.2 | 20 |
| **15j** | 0.0039 | >280 | 0.12 | 0.65 | 320 | 1.4 | 22 |
| **15k** | 0.19 | 430 | 0.21 | 5.5 | 400 | 1.6 | >15 |
| **16** | 0.0071 | >420 | 0.12 | 0.86 | 260 | 1.7 | 16 |
| **17** | 0.057 | 120 | 0.20 | 5.2 | 420 | 0.37 | >30 |

[a]Inhibitory activity against *C. albicans* Nmt (CaNmt) as assessed by $IC_{50}$ using substrate peptide GLTISKLFRR-$NH_2$ (0.5 μM) and myristoyl-CoA at 0.5 μM.
[b]Inhibitory activity against human Nmt (HsNmt) as assessed by $IC_{50}$ using substrate peptide GNAASARR-$NH_2$ (0.5 μM) and myristoyl-CoA at 0.5 μM.
[c]Antifungal activity against *C. albicans* CY1002 as assessed by $IC_{50}$ in YNBPB medium (1% glucose, 0.25% $K_2HPO_4$, pH 7).
[d]Antifungal activity against *C. albicans* CY1002 as assessed by $IC_{50}$ in 80% calf serum (80% calf serum supplemented with 10 μM $FeCl_3 \cdot 6H_2O$, 10 μM deferoxamine, 2% glucose).
[e]Cassette dosing: Five compounds were intravenously administered to a rat and the plasma concentration of each compound was measured by LC-MS.
[f]Fisher rats ($n = 5$) were infected intravenously with a lethal dose of *C. albicans* (CY1002) and treated intravenously (iv) with multiple doses (2 tid) of the test compound. Efficacy of the compounds was calculated as the effective dose (mg/kg) for 50% survival (ED50) on day 7.
[g]NT, Not tested.

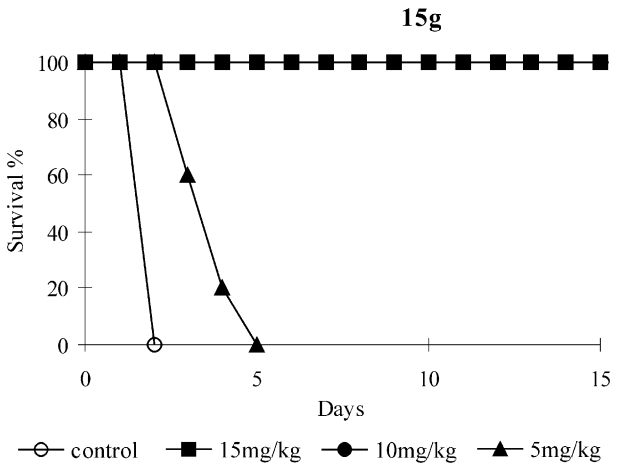



**Figure 3.** Antifungal activity of **15g** in a rat systemic candidiasis model. Animals: normal male Fischer rats, mean B.W. 56.9 g on day 0. Infection: *C. albicans* CY1002, $2.5 \times 10^4$ cells/g, 0.5 mL iv on day 0. Treatment: **15g**, 5% DMSO, 5% PEG and 10% HPCD, 0.5 mL, iv 2 tid.

dilution method according to M27-A guidelines recommended by the National Committee for Clinical Laboratory Standards (NCCLS). Compound **15g** displayed a broad spectrum and potent inhibitory activity against a variety of fungal species. This compound exhibited stronger antifungal activity in vitro against all the strains tested than did fluconazole. It exhibited excellent antifungal activity against a wide range of *Candida* species including clinically isolated *C. albicans* and *non-albicans Candida* such as *C. tropicalis* and *C. parapsilosis*. It was also active against mucorales including *Absidia corymbifera* and dermatophytes such as *Trichophyton mentagrophytes*. It was, however, only marginally active against *Cryptococcus neoformans* and *Aspergillus fumigatus*.

**Antifungal activity of 15g against *C. albicans* mutants in efflux transporter.** Compound **15g**, fluconazole, and itraconazole were tested for their antifungal activity against *C. albicans* mutants with deletions in several multidrug efflux transporter genes including *CDR1*, *CDR2*, *CaMDR1*, and *FLU1* (Table 5). The assays of the two azoles resulted in greatly enhanced activity against all deleted mutants. Especially, of all the transporter mutants tested, the quadruple deletion mutants (Δ *cdr1* Δ *cdr2* Δ *flu1* Δ *camdr1*) were most susceptible to both azoles. In contrast, the antifungal activity of **15g** was not affected by the deletion of the transporters. This clearly indicates that **15g** is not a substrate either for ABC (ATP binding cassette, *CDR1* and *CDR2*) transporters or for MFS (major facilitators superfamily, *CaMDR1* and *FLU1*) transporters, suggesting that **15g** would show antifungal activity against azole resistant *C. albicans* strains expressing multidrug efflux transporters.

## Summary and Conclusion

The modification work was carried out by the combination of rational drug design based on the crystal structures of CaNmt bound with benzofuran inhibitors, and SAR analysis guided by various biological assays including a quasi in vivo assay and cassette dosing PK studies in rats. This 'multidimensional optimization'

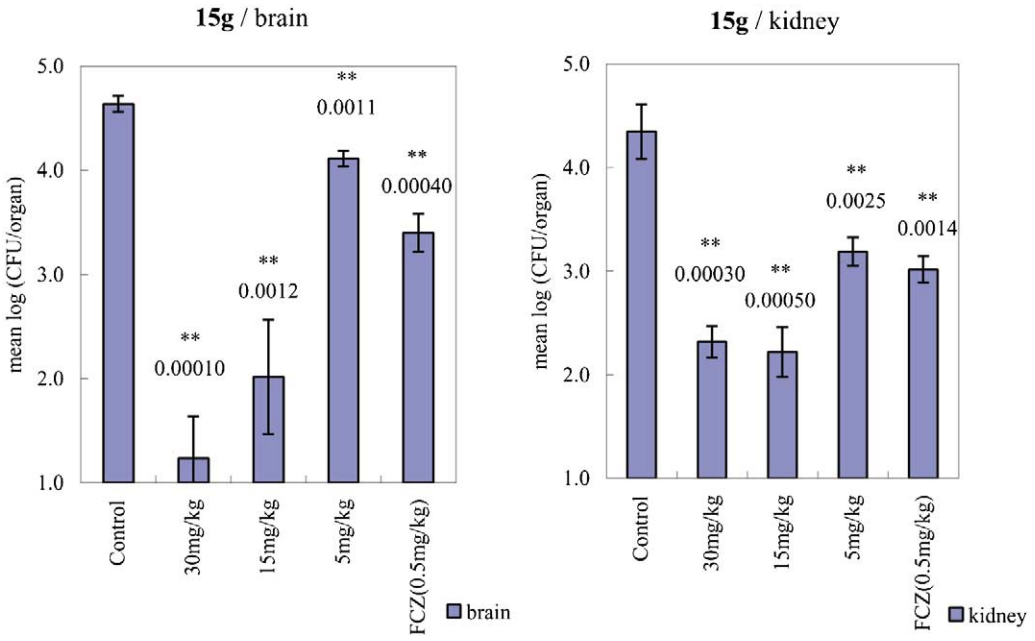



**Figure 4.** Efficacy of **15g** in a candidiasis target organ assay. Animals: Normal male Fischer rats, mean B.W. 62.5 g on day 0. Infection: *C. albicans* CY1002, $3\times10^3$ cells/g, 0.5 mL iv on day 0. Treatment: **15g**: 5% DMSO, 5% PEG, 10% HPCD, 0.5 mL, iv tid (30 mg/kg) or 2 tid (5 and 15 mg/kg). Fluconazole (FCZ): saline, 0.5 mg/kg, 0.5 mL, ip 2 tid. Control: 5% DMSO, 5% PEG, 10% HPCD and distilled water, 0.5 mL, iv 2 tid. $*p<0.05$, $**<0.01$

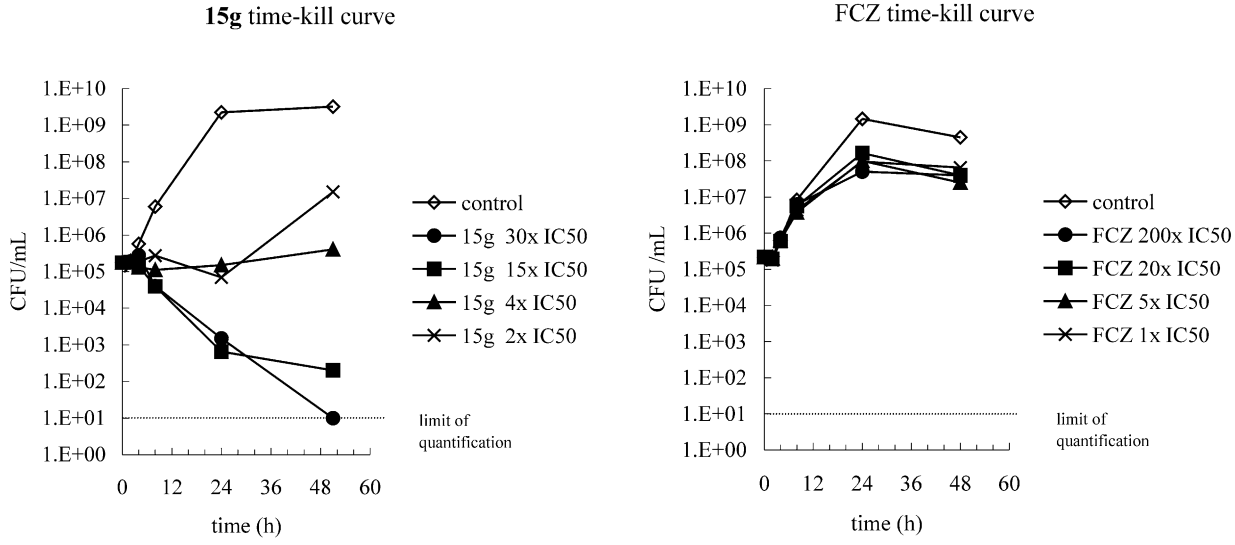



**Figure 5.** Time–kill curve of **15g** for *C. albicans* CY3003 in 80% calf serum. Medium: 80% calf serum (80% calf serum supplemented with 10 μM $FeCl_3{\cdot}6H_2O$, 10 μM deferoxamine, 2% glucose). Strain: *C. albicans* CY3003. Incubation: 35 °C. Inoculum size: $1\times10^5$ (CFU/mL). $IC_{50}$s of **15g** and fluconazole against *C. albicans* CY3003 were 0.5 and 1.6 μM, respectively, under the following conditions: medium: 80% calf serum (80% calf serum supplemented with 10 μM $FeCl_3{\cdot}6H_2O$, 10 μM deferoxamine, 2% glucose). Inoculum size: $1\times10^4$ (CFU/mL).

enabled us to rapidly identify compounds **15f** and **15g** that were active in vivo, starting from the enzyme inhibitor **1** with only marginal antifungal activity even in vitro. The candidiasis target organ assay suggested that the survival of the rats tested was prolonged by inhibiting fungal growth. Further biological investigations on **15g** revealed that (1) it had stronger and broader antifungal activity in vitro than did fluconazole, (2) it had fungicidal activity, and (3) it could not be a substrate of efflux transporters that cause azole resistance. In addition, since the oral bio-availability (*F* value) of **15g** in dogs was determined to be 33% and its stability in human liver microsome was better than in dog, oral administrations of this agent might be clinically useful. The novel mode of action, fungicidal activity, and potent antifungal activity in vivo make the benzofurans attractive for the development of antifungal drugs for systemic mycoses.

**Table 4.** Antifungal spectrum of **15g**

| Strains | | **15g** (μM) | | | Fluconazole (μM) | | |
|---|---|---|---|---|---|---|---|
| | | $IC_{50}$ | $IC_{80}$ | MIC | $IC_{50}$ | $IC_{80}$ | MIC |
| *Candida albicans* | Clinical Isolate | 0.026 | 0.044 | 0.11 | 0.82 | 1.2 | >650 |
| *Candida glabrata* | IFO0005 | 2.1 | 7.5 | 14 | 11 | 22 | 82 |
| *Candida guilliermondii* | ATCC9058 | 0.081 | 0.13 | 0.44 | 2 | 2.4 | 10 |
| *Candida tropicalis* | ATCC13803 | 0.033 | 0.046 | 0.11 | >650 | >650 | >650 |
| *Candida parapsilosis* | ATCC22019 | 0.0054 | 0.01 | 0.055 | 1.6 | 2.30 | 20 |
| *Cryptococcus neoformans* | MTU13001 | 4.6 | 5.9 | 6.9 | 4.6 | 9.5 | 41 |
| *Aspergillus fumigatus* | MTU06002 | 7.2 | 12 | 55 | 313 | 555 | >650 |
| *Aspergillus niger* | IFO6341 | 7.2 | 19 | 55 | >650 | >650 | >650 |
| *Absidia corymbifera* | IFO8084 | 0.64 | 2.9 | 14 | >650 | >650 | >650 |
| *Fusarium solani* | IFO8509 | 13 | 22 | 55 | >650 | >650 | >650 |
| *Trichophyton tonsurans* | IFO5928 | 1.4 | 2.1 | 14 | 30 | 144 | 330 |
| *Trichophyton mentagrophytes* | IFO5974 | 0.44 | 0.7 | 1.7 | 0.98 | 1.2 | 5.1 |
| *Trichophyton rubrum* | IFO5807 | 0.64 | 0.83 | 3.4 | 2.22 | 3.6 | 10 |

Medium: YNBPB (=YNB+1% glucose+0.25% $K_2HPO_4$) (pH 7.0) for yeasts. Medium: YNBPA (=YNB+1% glucose+0.25% $K_2HPO_4$+LMPA) (pH 7.0) for filamentous fungi. Incubation: 35 °C. Inoculum size: $1\times10^4$ (CFU/mL).

**Table 5.** Effect of transporter disruption on antifungal activity of **15g**, fluconazole, and itraconazole

| Strains | $IC_{50}$ (μM) | | |
|---|---|---|---|
| | **15g** | Fluconazole | Itraconazole |
| *C. albicans* CAF2-1 parent (wild type) | 0.042 | >650 | >280 |
| *C. albicans* DSY448 Δ *cdr1* | 0.042 | 1.9 | 0.0068 |
| *C. albicans* DSY465 Δ *camdr1* | 0.059 | >650 | 0.78 |
| *C. albicans* DSY468 Δ *cdr1* Δ *camdr1* | 0.039 | 0.78 | 0.0032 |
| *C. albicans* DSY653 Δ *cdr2* | 0.046 | 7.8 | 0.028 |
| *C. albicans* DSY654 Δ *cdr1* Δ *cdr2* | 0.039 | 2 | 0.0025 |
| *C. albicans* DSY1024 Δ *cdr1* Δ *cdr2* Δ *flu1* Δ *camdr1* | 0.021 | 0.24 | 0.0017 |

Medium: YPD (1% Bacto Yeast extract+2% glucose+2% Bacto Peptone). Incubation: 35 °C. Inoculum size: $1\times10^4$ (CFU/mL).

## Experimental

### General methods

$^1$H NMR spectra were recorded on a Jeol JNM-EX270 spectrometer; chemical shifts are reported in parts per million (ppm) relative to TMS. Mass spectra were recorded on a Jeol DX-303 spectrometer. Optical rotation measurements were made using a JASCO DIP-140 polarimeter. All solvents and reagents were purchased from commercial sources and used without further purification. Wakogel® C-200 (Wako Pure Chemical Industries, Ltd., Japan) was used for chromatographic purification unless otherwise indicated. Thin layer chromatography (TLC) was performed on precoated silica gel $60F_{254}$ glass plates (Merck, Germany).

**(*S*)-3-Methyl-4-oxiranylmethoxybenzofuran-2-carboxylic acid ethyl ester (3a).** A mixture of 4-hydroxy-3-methylbenzofuran-2-carboxylic acid ethyl ester **2**[51] (100 mg, 0.45 mmol) and 60% NaH in mineral oil (22 mg, 0.55 mmol) in DMF (3 mL) was stirred at room temperature for 15 min. To the suspension was added (2*S*)-(+)-glycidyl tosylate (99 mg, 0.43 mmol) at room temperature. The mixture was stirred at room temperature for 23 h. The reaction mixture was diluted with ethyl acetate (50 mL) and washed with brine (50 mL). The organic layer was dried over anhydrous sodium sulfate and evaporated to dryness. The residue was purified by silica gel column chromatography (hexane/dichloromethane=5:1) to afford **3a** (104 mg, 83%) as a white powder. $[\alpha]_D^{22}$ (*c* 0.5, $CHCl_3$) +17.9. $^1$H NMR ($CDCl_3$) δ: 1.44 (3H, t, *J*=7 Hz), 2.77 (3H, s), 2.83 (1H, dd, *J*=5, 2.5 Hz), 2.96 (1H, dd, *J*=5, 4.5 Hz), 3.41-3.46 (1H, m), 4.08 (1H, dd, *J*=11, 5.5 Hz), 4.37 (1H, dd, *J*=11, 3 Hz), 4.44 (2H, q, *J*=7 Hz), 6.62 (1H, d, *J*=8 Hz), 7.15 (1H, d, *J*=8 Hz), 7.31 (1H, t, *J*=8 Hz). HRMS (*m*/*z*): calcd for $C_{15}H_{16}O_5$, 276.0998; found, 276.0996.

**(*R*,*S*)-3-Methyl-4-oxiranylmethoxybenzofuran-2-carboxylic acid ethyl ester (3).** To a mixture of 4-hydroxy-3-methylbenzofuran-2-carboxylic acid ethyl ester **2** (104 mg, 0.47 mmol) and cesium carbonate (240 mg, 0.74 mmol) in acetonitrile (5 mL) was added epichlorohydrin (440 mg, 4.7 mmol) at room temperature. The mixture was heated to 60 °C with stirring for 24 h. The reaction mixture was diluted with ethyl acetate (20 mL) and washed with water (20 mL). The organic layer was dried over anhydrous sodium sulfate and evaporated to dryness. The residue was purified by silica gel column chromatography (hexane/dichloromethane=4:1) to afford **3** (112 mg, 86%) as a white powder. $^1$H NMR ($CDCl_3$) δ: 1.44 (3H, t, *J*=7 Hz), 2.77 (3H, s), 2.82 (1H, dd, *J*=5, 2.5 Hz), 2.95 (1H, dd, *J*=5, 4.5 Hz), 3.40–3.46 (1H, m), 4.08 (1H, dd, *J*=11, 5.5 Hz), 4.37 (1H, dd, *J*=11, 3 Hz), 4.44 (2H, q, *J*=7 Hz), 6.62 (1H, d, *J*=8 Hz), 7.15 (1H, d, *J*=8 Hz), 7.31 (1H, t, *J*=8 Hz). EI–MS: *m*/*z* 276 ($M^+$). The $^1$H NMR data were practically identical with the reported values.[52]

**(*S*)-4-(3-*tert*-Butylamino-2-hydroxypropoxy)-3-methylbenzofuran-2-carboxylic acid ethyl ester (4a).** A mixture of **3a** (98 mg, 0.36 mmol) and *tert*-butylamine (0.4 mL) was stirred in ethanol (1 mL) at 60 °C for 13 h. The reaction mixture was diluted with ethyl acetate (10 mL) and washed with a saturated sodium hydrogen carbonate solution (5 mL) and water (5 mL). The organic phase was dried over anhydrous sodium sulfate and evaporated to dryness. The residue was purified by silica gel column chromatography (dichloromethane/methanol = 50:1→5:1) to afford **4a** (94 mg, 76%) as a colorless oil. $[\alpha]_D^{22}$ (*c* 0.9, $CHCl_3$) −23.7. $^1$H NMR ($CDCl_3$) δ: 1.15 (9H, s), 1.44 (3H, t, *J* = 7 Hz), 2.76 (3H, s), 2.75–2.80 (1H, m), 2.95 (1H, dd, *J* = 12, 3.5 Hz), 4.00–4.17 (3H, m), 4.44 (2H, q, *J* = 7 Hz), 6.64 (1H, d, *J* = 8 Hz), 7.13 (1H, d, *J* = 8 Hz), 7.31 (1H, t, *J* = 8 Hz). HRMS (*m*/*z*): calcd for $C_{19}H_{27}NO_5$, 349.1889; found, 349.1896.

**(*R*)-4-(3-*tert*-Butylamino-2-hydroxypropoxy)-3-methylbenzofuran-2-carboxylic acid ethyl ester (4b).** This compound was prepared by the same method as **4a**. Yield: 56% (two steps). Colorless oil. $[\alpha]_D^{22}$ (*c* 1.0, $CHCl_3$) +23.8. $^1$H NMR ($CDCl_3$) δ: 1.14 (9H, s), 1.43 (3H, t, *J* = 7 Hz), 2.75 (3H, s), 2.75–2.80 (1H, m), 2.94 (1H, dd, *J* = 12, 4 Hz), 3.98–4.07 (1H, m), 4.08–4.17 (2H, m), 4.44 (2H, q, *J* = 7 Hz), 6.64 (1H, d, *J* = 8 Hz), 7.13 (1H, d, *J* = 8 Hz), 7.31 (1H, t, *J* = 8 Hz). HRMS (*m*/*z*): calcd for $C_{19}H_{27}NO_5$, 349.1889; found, 349.1884.

**(*R,S*)-4-(3-*tert*-Butylamino-2-hydroxypropoxy)-3-methylbenzofuran-2-carboxylic acid ethyl ester (4).** Compound **4** was prepared from **3** by the same method as **4a**. Yield: 95%. Colorless oil. $^1$H NMR ($CDCl_3$) δ: 1.15 (9H, s), 1.43 (3H, t, *J* = 7 Hz), 2.76 (3H, s), 2.75–2.81 (1H, m), 2.96 (1H, dd, *J* = 12, 4 Hz), 4.01–4.18 (3H, m), 4.44 (2H, q, *J* = 7 Hz), 6.64 (1H, d, *J* = 8 Hz), 7.13 (1H, d, *J* = 8 Hz), 7.31 (1H, t, *J* = 8 Hz). HRMS (*m*/*z*): calcd for $C_{19}H_{27}NO_5$, 349.1889; found, 349.1886.

**4-(3-Bromopropoxy)-3-methylbenzofuran-2-carboxylic acid ethyl ester (6).** Compound **2** (500 mg, 2.27 mmol), potassium carbonate (1.64 g, 11.9 mmol), and 1,3-dibromopropane (1.15 mL, 11.9 mmol) were suspended in DMF (10 mL). The mixture was stirred at room temperature for 4 h. The reaction mixture was diluted with ethyl acetate (100 mL) and washed with water (100 mL). The organic layer was dried over anhydrous sodium sulfate and evaporated to dryness. The residue was purified by silica gel column chromatography (ethyl acetate/hexane = 50:1) to give a crystalline product, which was recrystallized from hexane to give **6** (620 mg, 80%) as colorless needles. $^1$H NMR ($CDCl_3$) δ: 1.43 (3H, t, *J* = 7 Hz), 2.37–2.46 (2H, m), 2.74 (3H, s), 3.65 (2H, t, *J* = 6.5 Hz), 4.23 (2H, t, *J* = 6.5 Hz), 4.45 (2H, q, *J* = 7 Hz), 6.65 (1H, d, *J* = 8 Hz), 7.13 (1H, d, *J* = 8 Hz), 7.31 (1H, t, *J* = 8 Hz). HRMS (*m*/*z*): calcd for $C_{15}H_{17}BrO_4$, 340.0310; found, 340.0306.

**4-(3-Isopropylaminopropoxy)-3-methylbenzofuran-2-carboxylic acid ethyl ester (10a).** Compound **6** (50 mg, 0.15 mmol) was stirred with isopropylamine (0.1 mL) in ethanol (1 mL) at 60 °C for 9 h. The reaction mixture was evaporated to dryness. The residue was purified by silica gel column chromatography (dichloromethane/methanol = 50:1→20:1) to afford **10a** (46 mg, 98%) as colorless crystals. $^1$H NMR ($CDCl_3$) δ: 1.30 (6H, d, *J* = 6.5 Hz), 1.43 (3H, t, *J* = 7 Hz), 2.26–2.36 (2H, m), 2.73 (3H, s), 3.04 (2H, t, *J* = 7.5 Hz), 3.07–3.17 (1H, m), 4.16 (2H, t, *J* = 6 Hz), 4.43 (2H, q, *J* = 7 Hz), 6.59 (1H, d, *J* = 8 Hz), 7.11 (1H, d, *J* = 8 Hz), 7.29 (1H, t, *J* = 8 Hz). HRMS (*m*/*z*): calcd for $C_{18}H_{25}NO_4$, 319.1784; found, 319.1789.

**4-(3-*tert*-Butylaminopropoxy)-3-methylbenzofuran-2-carboxylic acid ethyl ester (10b).** This compound was prepared by the same method as **10a**. Yield: 95%. Colorless crystals. $^1$H NMR ($CDCl_3$) δ: 1.41 (9H, s), 1.43 (3H, t, *J* = 7 Hz), 2.45–2.51 (2H, m), 2.73 (3H, s), 3.10 (2H, t, *J* = 7 Hz), 4.15 (2H, t, *J* = 6 Hz), 4.43 (2H, q, *J* = 7 Hz), 6.57 (1H, d, *J* = 8 Hz), 7.11 (1H, d, *J* = 8 Hz), 7.29 (1H, t, *J* = 8 Hz). HRMS (*m*/*z*): calcd for $C_{19}H_{27}NO_4$, 333.1940; found, 333.1935.

**3-Methyl-4-(3-piperidin-1-ylpropoxy)benzofuran-2-carboxylic acid ethyl ester (10d).** This compound was prepared by the same method as **10a**. Yield: 89%. Pale orange powder. $^1$H NMR ($CDCl_3$) δ: 1.43 (3H, t, *J* = 7 Hz), 1.43–1.50 (2H, m), 1.62 (4H, quintet, *J* = 6 Hz), 2.03-2.13 (2H, m), 2.43–2.45 (4H, m), 2.56 (2H, t, *J* = 7 Hz), 2.75 (3H, s), 4.12 (2H, t, *J* = 6 Hz), 4.44 (2H, q, *J* = 7 Hz), 6.62 (1H, d, *J* = 8 Hz), 7.11 (1H, d, *J* = 8 Hz), 7.30 (1H, t, *J* = 8 Hz). HRMS (*m*/*z*): calcd for $C_{20}H_{27}NO_4$, 345.1940; found, 345.1933.

**3-Methyl-4-(3-phenylaminopropoxy)benzofuran-2-carboxylic acid ethyl ester (10e).** This compound was prepared by the same method as **10a**. Yield: 34%. Light brown powder. $^1$H NMR ($CDCl_3$) δ: 1.44 (3H, t, *J* = 7 Hz), 2.15–2.24 (2H, m), 2.79 (3H, s), 3.42 (2H, t, *J* = 7 Hz), 4.21 (2H, t, *J* = 6 Hz), 4.45 (2H, q, *J* = 7 Hz), 6.61-6.66 (3H, m), 6.71 (1H, t, *J* = 8 Hz), 7.13 (1H, d, *J* = 8 Hz), 7.15–7.21 (2H, m), 7.31 (1H, t, *J* = 8 Hz). HRMS (*m*/*z*): calcd for $C_{21}H_{23}NO_4$, 353.1627; found, 353.1612.

**4-(3-Benzylaminopropoxy)-3-methylbenzofuran-2-carboxylic acid ethyl ester (10f).** This compound was prepared by the same method as **10a**. Yield: 85%. Colorless oil. $^1$H NMR ($CDCl_3$) δ: 1.44 (3H, t, *J* = 7 Hz), 2.02–2.11 (2H, m), 2.67 (3H, s), 2.88 (2H, t, *J* = 7 Hz), 3.83 (2H, s), 4.17 (2H, t, *J* = 6 Hz), 4.43 (2H, q, *J* = 7 Hz), 6.62 (1H, d, *J* = 8 Hz), 7.11 (1H, d, *J* = 8 Hz), 7.27 (6H, m). HRMS (*m*/*z*): calcd for $C_{22}H_{25}NO_4$, 367.1784; found, 367.1791.

**3-Methyl-4-{3-[(pyridin-2-ylmethyl)amino]propoxy}benzofuran-2-carboxylic acid ethyl ester (10g).** This compound was prepared by the same method as **10a**. Yield: 89%. Colorless oil. $^1$H NMR ($CDCl_3$) δ: 1.44 (3H, t, *J* = 7 Hz), 2.06–2.13 (2H, m), 2.68 (3H, s), 2.91 (2H, t, *J* = 7 Hz), 3.95 (2H, s), 4.18 (2H, t, *J* = 6 Hz), 4.44 (2H, q, *J* = 7 Hz), 6.62 (1H, d, *J* = 8 Hz), 7.11 (1H, d, *J* = 8 Hz), 7.12–7.16 (1H, m), 7.30 (2H, t, *J* = 8 Hz), 7.60 (1H, dt, *J* = 8, 2 Hz), 8.53 (1H, d, *J* = 4 Hz). HRMS (*m*/*z*): calcd for $C_{21}H_{24}N_2O_4$, 368.1736; found, 368.1730.

**3-Methyl-4-{3-[(pyridin-3-ylmethyl)amino]propoxy}benzofuran-2-carboxylic acid ethyl ester (10h).** This compound

was prepared by the same method as **10a**. Yield: 77%. Colorless oil. $^1$H NMR ($CDCl_3$) δ: 1.44 (3H, t, $J$=7 Hz), 2.02–2.12 (2H, m), 2.67 (3H, s), 2.89 (2H, t, $J$=7 Hz), 3.84 (2H, s), 4.17 (2H, t, $J$=6 Hz), 4.44 (2H, q, $J$=7 Hz), 6.62 (1H, d, $J$=8 Hz), 7.12 (1H, d, $J$=8 Hz), 7.21 (1H, dd, $J$=8, 5 Hz), 7.30 (1H, t, $J$=8 Hz), 7.66 (1H, dd, $J$=8, 2 Hz), 8.49 (1H, dd, $J$=5, 2 Hz), 8.57 (1H, d, $J$=2 Hz). HRMS ($m/z$): calcd for $C_{21}H_{24}N_2O_4$, 368.1736; found, 368.1742.

**3-Methyl-4-{3-[(pyridin-4-ylmethyl)amino]propoxy}benzofuran-2-carboxylic acid ethyl ester (10i).** This compound was prepared by the same method as **10a**. Yield: 45%. Pale yellow oil. $^1$H NMR ($CDCl_3$) δ: 1.44 (3H, t, $J$=7 Hz), 2.03–2.13 (2H, m), 2.69 (3H, s), 2.89 (2H, t, $J$=7 Hz), 3.85 (2H, s), 4.19 (2H, t, $J$=6 Hz), 4.44 (2H, q, $J$=7 Hz), 6.63 (1H, d, $J$=8 Hz), 7.12 (1H, d, $J$=8 Hz), 7.24–7.27 (2H, m), 7.31 (1H, t, $J$=8 Hz), 8.52 (2H, dd, $J$=5, 2 Hz). HRMS ($m/z$): calcd for $C_{21}H_{24}N_2O_4$, 368.1736; found, 368.1742.

**3-Methyl-4-{3-[2-(pyridin-3-ylethyl)amino]propoxy}benzofuran-2-carboxylic acid ethyl ester (10j).** This compound was prepared by the same method as **10a**. Yield: 75%. Colorless oil. $^1$H NMR ($CDCl_3$) δ: 1.43 (3H, t, $J$=7 Hz), 2.00–2.09 (2H, m), 2.73 (3H, s), 2.87 (6H, m), 4.14 (2H, t, $J$=6 Hz), 4.44 (2H, q, $J$=7 Hz), 6.61 (1H, d, $J$=8 Hz), 7.12 (1H, d, $J$=8 Hz), 7.18 (1H, dd, $J$=8, 5 Hz ), 7.30 (1H, t, $J$=8 Hz), 7.52 (1H, dd, $J$=8, 2 Hz), 8.44 (1H, dd, $J$=5, 2 Hz), 8.48 (1H, d, $J$=2 Hz). HRMS ($m/z$): calcd for $C_{22}H_{26}N_2O_4$, 382.1893; found, 382.1899.

**4-(3-Aminopropoxy)-3-methylbenzofuran-2-carboxylic acid ethyl ester (10c).** Compound **10f** (80 mg, 0.22 mmol) was hydrogenated over 10% Pd/C (20 mg) in ethanol (4 mL) at room temperature for 14 h. Pd/C was filtered through a Celite pad. The filtrate was evaporated to dryness and the residue was purified by silica gel column chromatography (dichloromethane/methanol=50:1→5:1) to give **10c** (27 mg, 45%) as a colorless powder. $^1$H NMR ($CDCl_3$) δ: 1.44 (3H, t, $J$=7 Hz), 1.98–2.08 (2H, m), 2.75 (3H, s), 2.99 (2H, t, $J$=7 Hz), 4.17 (2H, t, $J$=6 Hz), 4.44 (2H, q, $J$=7 Hz), 6.63 (1H, d, $J$=8 Hz), 7.12 (1H, d, $J$=8 Hz), 7.31 (1H, t, $J$=8 Hz). HRMS ($m/z$): calcd for $C_{15}H_{19}NO_4$, 277.1314; found, 277.1316.

**4-(2-*tert*-Butylaminoethoxy)-3-methylbenzofuran-2-carboxylic acid ethyl ester (9).** Bromoethoxy derivative **5** was synthesized from **2** and 1,2-dibromoethane by practically the same preparation method for **6** except for the reaction temperature (100 °C). Compound **9** was obtained from **5** by the same preparation method as **10a**. Yield: 63% (two steps). Colorless oil. $^1$H NMR ($CDCl_3$) δ: 1.20 (9H, s), 1.44 (3H, t, $J$=7 Hz), 2.75 (3H, s), 3.08 (2H, t, $J$=5 Hz), 4.23 (2H, t, $J$=5 Hz), 4.44 (2H, q, $J$=7 Hz), 6.62 (1H, d, $J$=8 Hz), 7.11 (1H, d, $J$=8 Hz), 7.29 (1H, t, $J$=8 Hz). HRMS ($m/z$): calcd for $C_{18}H_{25}NO_4$, 319.1784; found, 319.1788.

**4-(4-*tert*-Butylaminobutoxy)-3-methylbenzofuran-2-carboxylic acid ethyl ester (11).** Bromobutoxy derivative **7** was synthesized from **2** and 1,4-dibromobutane by practically the same preparation method for **6**. Compound **11** was obtained from **7** by the same preparation method as **10a**. Yield: 43% (two steps). Colorless crystals. $^1$H NMR ($CDCl_3$) δ: 1.43 (3H, t, $J$=7 Hz), 1.46 (9H, s), 1.89–1.97 (2H, m), 2.23–2.34 (2H, m), 2.74 (3H, s), 3.02 (2H, br t, $J$=8 Hz), 4.02 (2H, t, $J$=6 Hz), 4.43 (2H, q, $J$=7 Hz), 6.51 (1H, d, $J$=8 Hz), 7.09 (1H, d, $J$=8 Hz), 7.25 (1H, t, $J$=8 Hz). HRMS ($m/z$): calcd for $C_{20}H_{29}NO_4$, 347.2097; found, 347.2095.

**4-(5-*tert*-Butylaminopentyloxy)-3-methylbenzofuran-2-carboxylic acid ethyl ester (12).** Bromopentyloxy derivative **8** was synthesized from **2** and 1,5-dibromopentane by practically the same preparation method for **6**. Compound **12** was obtained from **8** by the same preparation method as **10a**. Yield: 74% (2 steps). White powder. $^1$H NMR ($CDCl_3$) δ: 1.43 (3H, t, $J$=7 Hz), 1.50–1.65 (2H, m), 1.51 (9H, s), 1.83–1.93 (2H, m), 2.12–2.24 (2H, m), 2.71 (3H, s), 2.95 (2H, br t, $J$=8 Hz), 4.02 (2H, t, $J$=6 Hz), 4.42 (2H, q, $J$=7 Hz), 6.57 (1H, d, $J$=8 Hz), 7.09 (1H, d, $J$=8 Hz), 7.28 (1H, t, $J$=8 Hz). HRMS ($m/z$): calcd for $C_{21}H_{31}NO_4$, 361.2253; found, 361.2254.

**3-Methyl-4-[3-(pyridin-3-ylmethoxy)propoxy]benzofuran-2-carboxylic acid ethyl ester (13).** A suspension of 3-pyridinemethanol (43 μL, 0.44 mmol) and 60% NaH in mineral oil (19 mg, 0.44 mmol) in DMF (1 mL) was stirred at room temperature for 15 min. To the mixture was added **6** (100 mg, 0.29 mmol) and the reaction mixture was stirred at room temperature for 2 weeks. The mixture was diluted with ethyl acetate (30 mL) and washed with water (30 mL). The organic layer was dried over anhydrous sodium sulfate and evaporated to dryness. The residue was purified by preparative TLC (ethyl acetate/hexane=1:1) to give **13** (11.6 mg, 11%) as a colorless oil. $^1$H NMR ($CDCl_3$) δ: 1.44 (3H, t, $J$=7 Hz), 2.14–2.22 (2H, m), 2.68 (3H, s), 3.73 (2H, t, $J$=6 Hz), 4.20 (2H, t, $J$=6 Hz), 4.44 (2H, q, $J$=7 Hz), 4.55 (2H, s), 6.63 (1H, d, $J$=8 Hz), 7.12 (1H, d, $J$=8 Hz), 7.20 (1H, dd, $J$=8, 5 Hz), 7.31 (1H, t, $J$=8 Hz), 7.64 (1H, d, $J$=8 Hz), 8.51 (1H, dd, $J$=5, 1.5 Hz), 8.57 (1H, s). HRMS ($m/z$): calcd for $C_{21}H_{23}NO_5$, 369.1576; found, 369.1578.

**(3-Methyl-4-{3-[(pyridin-3-ylmethyl)amino]propoxy}benzofuran-2-yl)methanol (14).** To a solution of **10h** (7.9 g, 21 mmol) in dry tetrahydrofuran (THF) was added lithium aluminum hydride (3.2 g, 85 mmol) at 0 °C. The mixture was stirred at 0 °C for 30 min. To the reaction mixture were added a little water and then anhydrous sodium sulfate with stirring. Inorganic salt was removed by filtration and the filtrate was evaporated to dryness. The residue was purified by silica gel column chromatography (dichloromethane/methanol=50:1→10:1) to give **14** (6.31 g, 91%) as a colorless solid. $^1$H NMR ($CDCl_3$) δ: 1.97–2.07 (2H, m), 2.22 (3H, s), 2.83 (2H, t, $J$=7 Hz), 3.77 (2H, s), 4.10 (2H, t, $J$=6 Hz), 4.66 (2H, s), 6.55 (1H, d, $J$=8 Hz), 7.00 (1H, d, $J$=8 Hz), 7.12 (1H, t, $J$=8 Hz), 7.17 (1H, m), 7.63 (1H, br d, $J$=5 Hz), 8.42 (2H, m). HRMS ($m/z$): calcd for $C_{19}H_{22}N_2O_3$, 326.1630; found, 326.1636.

**[3-(3-Methyl-2-phenoxymethylbenzofuran-4-yloxy)propyl]pyridin-3-ylmethylamine (15a).** To a suspension of **14** (33 mg, 0.1 mmol), phenol (10 mg, 0.11 mmol), and triphenylphosphine polymer bound (3 mmol/g resin, 50 mg, Fulka, Switzerland) in THF (1 mL) was added diethyl azodicarboxylate (40% toluene solution) at −45 °C under argon, and the mixture was slowly warmed to room temperature over a period of 18 h. The mixture was filtered off, and the filtrate was poured onto a saturated sodium hydrogen carbonate solution (10 mL). The mixture was extracted with ethyl acetate (20 mL). The organic layer was washed with brine (10 mL), dried over anhydrous sodium sulfate, and the solvent was evaporated under reduced pressure. The residue was purified on preparative TLC (dichloromethane/methanol = 10:1) to afford **15a** (8 mg, 20%) as a yellow oil. $^1$H NMR ($CDCl_3$) δ: 2.00–2.10 (2H, m), 2.34 (3H, s,), 2.88 (2H, t, $J$ = 7 Hz), 3.84 (2H, s), 4.15 (2H, t, $J$ = 6 Hz), 5.08 (2H, s), 6.60 (1H, d, $J$ = 8 Hz), 6.96–7.07 (2H, m), 7.15 (1H, d, $J$ = 8 Hz), 7.18–7.23 (1H, m), 7.28–7.34 (2H, m), 7.43–7.55 (2H, m), 7.66 (1H, d, $J$ = 8 Hz), 8.49 (1H, d, $J$ = 4 Hz), 8.57 (1H, s). HRMS ($m/z$): calcd for $C_{25}H_{26}N_2O_3$, 402.1943; found, 402.1939.

**{3-[2-(2-Fluorophenoxymethyl)-3-methylbenzofuran-4-yloxy]propyl}pyridin-3-ylmethylamine (15b).** This compound was prepared by the same method as **15a**. Yield: 21%. Pale yellow oil. $^1$H NMR ($CDCl_3$) δ: 2.00–2.09 (2H, m), 2.30 (3H, s), 2.87 (2H, t, $J$ = 7 Hz), 3.83 (2H, s), 4.13 (2H, t, $J$ = 6 Hz), 5.15 (2H, s), 6.59 (1H, d, $J$ = 8 Hz), 6.91–6.96 (1H, m), 7.03–7.10 (4H, m), 7.16 (1H, t, $J$ = 8 Hz), 7.20 (1H, dd, $J$ = 8, 7 Hz), 7.66 (1H, d, $J$ = 7 Hz), 8.47 (1H, d, $J$ = 4 Hz), 8.56 (1H, s). HRMS ($m/z$): calcd for $C_{25}H_{25}FN_2O_3$, 420.1849; found, 420.1855.

**{3-[2-(3-Fluorophenoxymethyl)-3-methylbenzofuran-4-yloxy]propyl}pyridin-3-ylmethylamine (15c).** This compound was prepared by the same method as **15a**. Yield: 18%. Pale yellow oil. $^1$H NMR ($CDCl_3$) δ: 2.00–2.10 (2H, m), 2.35 (3H, s), 2.88 (2H, t, $J$ = 7 Hz), 3.83 (2H, s), 4.15 (2H, t, $J$ = 6 Hz), 5.06 (2H, s), 6.60 (1H, d, $J$ = 8 Hz), 6.67-6.81 (3H, m), 7.05 (1H, d, $J$ = 8 Hz), 7.15–7.24 (3H, m), 7.66 (1H, d, $J$ = 7 Hz), 8.49 (1H, d, $J$ = 4 Hz,), 8.56 (1H, s). HRMS ($m/z$): calcd for $C_{25}H_{25}FN_2O_3$, 420.1849; found, 420.1844.

**{3-[2-(4-Fluorophenoxymethyl)-3-methylbenzofuran-4-yloxy]propyl}pyridin-3-ylmethylamine (15d).** This compound was prepared by the same method as **15a**. Yield: 25%. Yellow oil. $^1$H NMR ($CDCl_3$) δ: 2.01–2.11 (2H, m), 2.31 (3H, s), 2.88 (2H, t, $J$ = 6 Hz), 3.85 (2H, s), 4.14 (2H, t, $J$ = 7 Hz), 5.03 (2H, s), 6.58 (1H, d, $J$ = 8 Hz), 6.95–7.00 (4H, m), 7.04 (1H, d, $J$ = 9 Hz), 7.13 (1H, d, $J$ = 8 Hz), 7.19–7.22 (1H, m), 7.68 (1H, d, $J$ = 8 Hz), 8.48 (1H, d, $J$ = 4 Hz), 8.57 (1H, s). HRMS ($m/z$): calcd for $C_{25}H_{25}FN_2O_3$, 420.1849; found, 420.1841.

**{3-[2-(2,3-Difluorophenoxymethyl)-3-methylbenzofuran-4-yloxy]propyl}pyridin-3-ylmethylamine (15e).** This compound was prepared by the same method as **15a**. Yield: 35%. Pale yellow solid. $^1$H NMR ($CDCl_3$) δ: 2.00–2.09 (2H, m), 2.32 (3H, s), 2.87 (2H, t, $J$ = 7 Hz), 3.83 (2H, s), 4.14 (2H, t, $J$ = 6 Hz), 5.16 (2H, s), 6.59 (1H, d, $J$ = 7 Hz), 6.80 (1H, dq, $J$ = 8, 1.5 Hz), 6.88 (1H, dt, $J$ = 7, 1.5 Hz), 6.90–7.00 (1H, m), 7.04 (1H, d, $J$ = 8 Hz), 7.16 (1H, d, $J$ = 8 Hz), 7.19–7.22 (1H, m), 7.65 (1H, d, $J$ = 8 Hz), 8.47 (1H, d, $J$ = 4 Hz), 8.56 (1H, s). HRMS ($m/z$): calcd for $C_{25}H_{24}F_2N_2O_3$, 438.1755; found, 438.1751.

**{3-[2-(2,4-Difluorophenoxymethyl)-3-methylbenzofuran-4-yloxy]propyl}pyridin-3-ylmethylamine (15f).** To a solution of the compound **14** (65 mg, 0.20 mmol), tributylphosphine (74 μL, 0.30 mmol), and 2,4-difluorophenol (26 mg, 0.20 mmol) in THF (1 mL) was added 1,1′-(azodicarbonyl)dipiperidine (ADDP) (76 mg, 0.30 mmol) in THF (0.5 mL) at −45 °C under argon atmosphere, and the mixture was slowly warmed to room temperature over a period of 18 h. The mixture was poured onto a saturated sodium hydrogen carbonate solution (20 mL) and extracted with ethyl acetate (20 mL). The organic layer was washed with brine, dried over anhydrous sodium sulfate, and evaporated to dryness. The residue was purified by silica gel column chromatography (dichloromethane/methanol = 20:1) to afford **15f** (35 mg, 40%) as a yellow oil. $^1$H NMR ($CDCl_3$) δ: 2.00–2.09 (2H, m), 2.27 (3H, s), 2.89 (2H, t, $J$ = 7 Hz), 3.83 (2H, s), 4.13 (2H, t, $J$ = 6 Hz), 5.10 (2H, s), 6.59 (1H, d, $J$ = 8 Hz), 6.71–7.23 (6H, m), 7.66 (1H, dd, $J$ = 8, 2 Hz), 8.49 (1H, d, $J$ = 5 Hz), 8.56 (1H, s). HRMS ($m/z$): calcd for $C_{25}H_{24}F_2N_2O_3$, 438.1755; found, 438.1749.

**{3-[2-(2,3,4-Trifluorophenoxymethyl)-3-methylbenzofuran-4-yloxy]propyl}pyridin-3-ylmethylamine (15g).** This compound was synthesized from **14** by practically the same preparation method as **15f**. Toluene was used as a reaction solvent instead of THF. Yield: 80%. Yellow oil. $^1$H NMR ($CDCl_3$) δ: 2.00–2.10 (2H, m), 2.29 (3H, s), 2.88 (2H, t, $J$ = 7 Hz), 3.84 (2H, s), 4.14 (2H, t, $J$ = 6 Hz), 5.13 (2H, s), 6.60 (1H, d, $J$ = 8 Hz), 6.75–6.91 (2H, m), 7.04 (1H, d, $J$ = 8 Hz), 7.15–7.27 (2H, m,), 7.68 (1H, d, $J$ = 8 Hz), 8.49 (1H, d, $J$ = 4 Hz), 8.57 (1H, s). HRMS ($m/z$): calcd for $C_{25}H_{23}F_3N_2O_3$, 456.1661; found, 456.1653.

**{3-[2-(4-Bromo-2-fluorophenoxymethyl)-3-methylbenzofuran-4-yloxy]propyl}pyridin-3-ylmethylamine (15h).** To a solution of **14** (37 mg, 0.12 mmol), 1,1′-azobis(*N,N*-dimethylformamide) (41 mg, 0.24 mmol), and 4-bromo-2-fluorophenol (20 μL, 0.18 mmol) in THF (2 mL) was added tributylphosphine (89 μL, 0.36 mmol) at −30 °C under argon atmosphere, and the mixture was slowly warmed to room temperature over a period of 15 h. The precipitates were filtered off, and the filtrate was poured onto water (30 mL) and extracted with ethyl acetate (30 mL). The organic layer was washed with brine (30 mL), dried over anhydrous sodium sulfate, and evaporated to dryness. The residue was purified by silica gel column chromatography (dichloromethane/methanol = 20:1) to afford **15h** (24 mg, 40%) as a colorless oil. $^1$H NMR ($CDCl_3$) δ: 2.00–2.10 (2H, m), 2.30 (3H, s), 2.87 (2H, t, $J$ = 7 Hz), 3.84 (2H, s), 4.14 (2H, t, $J$ = 6 Hz), 5.13 (2H, s), 6.59 (1H, d, $J$ = 8 Hz), 6.95–7.09 (2H, m), 7.12–7.27 (4H, m), 7.66 (1H, dt, $J$ = 8, 2 Hz), 8.49 (1H, dd, $J$ = 5, 2 Hz), 8.56 (1H, d, $J$ = 2 Hz). HRMS ($m/z$): calcd for $C_{25}H_{24}BrFN_2O_3$, 498.0954; found, 498.0958.

**4-(3-Methyl-4-{3-[(pyridin-3-ylmethyl)amino]propoxy}-benzofuran-2-ylmethoxy)benzonitrile (15i).** This compound was prepared from **14** by the same method as **15h**. Yield: 46%. White powder. $^1$H NMR ($CDCl_3$) δ: 2.00–2.10 (2H, m), 2.35 (3H, s), 2.87 (2H, t, $J$=7 Hz), 3.84 (2H, s), 4.15 (2H, t, $J$=6 Hz), 5.13 (2H, s), 6.61 (1H, d, $J$=8 Hz), 7.05 (1H, d, $J$=8 Hz), 7.07 (2H, d, $J$=9 Hz), 7.19 (1H, t, $J$=8 Hz), 7.20–7.22 (1H, m), 7.61 (2H, d, $J$=9 Hz), 7.67 (1H, dd, $J$=8, 2 Hz), 8.49 (1H, dd, $J$=5, 2 Hz), 8.56 (1H, d, $J$=2 Hz). HRMS ($m/z$): calcd for $C_{26}H_{25}N_3O_3$, 427.1896; found, 427.1894.

**{3-[3-Methyl-2-(3-methyl-2-nitrophenoxymethyl)benzofuran-4-yloxy]propyl}pyridin-3-ylmethylamine (15j).** This compound was prepared from **14** by the same method as **15h**. Yield: 68%. Pale yellow powder. $^1$H NMR ($CDCl_3$) δ: 2.00–2.09 (2H, m), 2.29 (3H, s), 2.31 (3H, s), 2.87 (2H, t, $J$=7 Hz), 3.84 (2H, s), 4.14 (2H, t, $J$=6 Hz), 5.17 (2H, s), 6.59 (1H, d, $J$=8 Hz), 6.87 (1H, d, $J$=8 Hz), 7.03 (1H, d, $J$=8 Hz), 7.05 (1H, d, $J$=8 Hz), 7.16 (1H, d, $J$=8 Hz), 7.19–7.24 (1H, m), 7.30 (1H, d, $J$=8 Hz), 7.67 (1H, dt, $J$=8, 2 Hz), 8.48 (1H, dd, $J$=5, 2 Hz), 8.56 (1H, d, $J$=2 Hz). HRMS ($m/z$): calcd for $C_{26}H_{27}N_3O_5$, 461.1951; found, 461.1943.

**{3-[2-(4-Imidazol-1-ylphenoxymethyl)-3-methylbenzofuran-4-yloxy]propyl}pyridin-3-ylmethylamine (15k).** This compound was prepared by the same method as **15h**. Yield: 67%. Colorless oil. $^1$H NMR ($CDCl_3$) δ: 2.03–2.09 (2H, m), 2.35 (3H, s), 2.88 (2H, t, $J$=6.5 Hz), 3.84 (2H, s), 4.15 (2H, t, $J$=6 Hz), 5.12 (2H, s), 6.61 (1H, d, $J$=8 Hz), 7.06 (1H, d, $J$=8 Hz), 7.10 (2H, dd, $J$=6.5, 2 Hz), 7.16–7.22 (4H, m), 7.31 (2H, dd, $J$=6.5, 2 Hz), 7.67 (1H, d, $J$=7.5 Hz), 7.77 (1H, s), 8.49 (1H, dd, $J$=4.5, 2 Hz), 8.56 (1H, d, $J$=2 Hz). HRMS ($m/z$): calcd for $C_{28}H_{28}N_4O_3$, 468.2161; found, 468.2166.

**{3-[3-Methyl-2-(1-methyl-5-trifluoromethyl-1H-pyrazol-3-yloxymethyl)benzofuran-4-yloxy]propyl}pyridin-3-ylmethylamine (16).** This compound was prepared by the same method as **15h**. Yield: 86%. Colorless oil. $^1$H NMR ($CDCl_3$) δ: 2.01–2.11 (2H, m), 2.36 (3H, s), 2.88 (2H, t, $J$=7 Hz), 3.83 (2H, s), 3.84 (3H, s), 4.15 (2H, t, $J$=6 Hz), 5.22 (2H, s), 6.03 (1H, s), 6.59 (1H, d, $J$=8 Hz), 7.05 (1H, dd, $J$=8, 1 Hz), 7.17 (1H, t, $J$=8 Hz), 7.19–7.23 (1H, m), 7.67 (1H, dt, $J$=8, 2 Hz), 8.49 (1H, dd, $J$=5, 2 Hz), 8.57 (1H, d, $J$=2 Hz). HRMS ($m/z$): calcd for $C_{24}H_{25}F_3N_4O_3$, 474.1879; found, 474.1884.

**{3-[3-Methyl-2-(pyridin-3-yloxymethyl)benzofuran-4-yloxy]propyl}pyridin-3-ylmethylamine (17).** This compound was prepared by the same method as **15h**. Yield: 44%. Pale yellow oil. $^1$H NMR ($CDCl_3$) δ: 2.01–2.10 (2H, m), 2.35 (3H, s), 2.88 (2H, t, $J$=7 Hz), 3.84 (2H, s), 4.15 (2H, t, $J$=6 Hz), 5.13 (2H, s), 6.61 (1H, d, $J$=8 Hz), 7.05 (1H, d, $J$=8 Hz), 7.18 (1H, t, $J$=8 Hz), 7.25 (2H, m), 7.33 (1H, ddd, $J$=8.5, 3, 1.5 Hz), 7.67 (1H, dd, $J$=8, 2 Hz), 8.25 (1H, dd, $J$=5, 1.5 Hz), 8.42 (1H, d, $J$=3 Hz), 8.49 (1H, dd, $J$=5, 2 Hz), 8.56 (1H, d, $J$=2 Hz). HRMS ($m/z$): calcd for $C_{24}H_{25}N_3O_3$, 403.1896; found, 403.1891.

### Determination of enzyme inhibitory activity

Inhibitory activities against CaNmt and HsNmt were assessed by $IC_{50}$. Assays (100 μL) for CaNmt contained variable amounts of an inhibitor, 0.5 μM of [$^3$H]myristoyl-CoA (250 μCi, 62.0 Ci/mmol), 0.5 μM of substrate peptide GLTISKLFRR-$NH_2$, and 25 ng of purified *C. albicans* Nmt in a reaction buffer [30 mM Tris–HCl (Nacalai Tesque, Japan), pH 7.5, 0.45 mM EDTA (Dojindo, Japan), 0.5 mM EGTA (Dojindo, Japan), 4.5 mM 2-mercaptoethanol (Sigma, USA), 1.0% Triton-X-100 (Sigma, USA)]. Assays (100 μL) for HsNmt contained variable amounts of an inhibitor, 0.5 μM of [$^3$H]myristoyl-CoA (250 μCi, 62.0 Ci/mmol), 2.5 μM of substrate peptide peptide GNAASARR-$NH_2$, and 1 μg of purified human Nmt in a reaction buffer [30 mM Tris–HCl (Nacalai Tesque, Japan), pH 7.5, 0.45 mM EDTA, 0.5 mM EGTA, 4.5 mM 2-mercaptoethanol (Sigma, USA), 1.0% Triton-X-100 (Sigma, USA)]. Incubations were allowed to proceed for 60 min at 37 °C.

### Determination of antifungal activity in vitro

Antifungal activity in vitro of the benzofurans was determined using the *C. albicans* cells (strain CY1002) according to the broth microdilution procedure in the M27-A guidelines recommended by the National Committee for Clinical Laboratory Standards (NCCLS). Ten to the fourth power of cells in 100 μL of YNBPB medium [YNB (Difco, USA), 1% (w/v) glucose (Wako, Japan), 0.25% $K_2HPO_4$ (Wako, Japan), pH 7.0] containing various concentrations of compounds were dispensed in 96-well plates and incubated at 35 °C for 24 h. For quasi in vivo assay, the *C. albicans* CY1002 ($1\times10^4$ CFU/mL) was incubated in a 100 μL of 80% calf serum (Gibco, USA) containing 2% glucose, 10 μM $FeCl_3{\cdot}6H_2O$ (Wako, Japan), and 10 μM deferoxamine (Sigma, USA) with various concentrations of test compound using a 96-well plate. The plates were incubated at 35 °C for 24 h. The YNBPB medium was used for yeasts, while YNBPA medium [YNB (Difco, USA), 1% (w/v) glucose, 0.25% $K_2HPO_4$, 0.2% LMPA (Low Melting Point Agar, Gibco, USA), pH 7.0] was used for filamentous fungi. The turbidity of the cell suspension was measured using a microplate reader (WL320, Bio-Tek Instrument, USA) at 600 nm. Antifungal activity of each compound was indicated as 50% inhibition concentration ($IC_{50}$) values that were determined by calculation of the minimum concentration of the compound required for 50% reduction of the turbidity (OD600) of cells compared to untreated control cells. Minimum inhibitory concentrations (MICs), which were defined as the lowest concentrations of compound that completely inhibited visible growth (absence of detectable turbidity), were also determined by the microdilution format described above.

### Antifungal activity against disrupted mutants of *C. albicans*

*C. albicans* DSY448(Δ*cdr*1: *:hisG-URA3-his*G/Δ*cdr*1: *:hisG*), DSY465(Δ*camdr*1::*hisG-URA3-his*G/Δ*camdr*1::*hisG*), DSY468 (Δ*cdr*1::*his*G/Δ*cdr*1::*hisG* Δ*camdr*1::*hisG-URA3-his*G/Δ*camdr*1::*hisG*), DSY653 (Δ*cdr*2::*hisG-URA3-his*G/Δ*cdr*2::*hisG*), DSY654 (Δ*cdr*1::*his*G/Δ-

*cdr*1::*hisG* Δ*cdr*2::*hisG-URA3-his*G/Δ*cdr*2::*hisG*), and DSY1024 (Δ*cdr*1::*his*G/Δ*cdr*1::*hisG* Δ*cdr*2::*his*G/Δ*cdr*2::*hisG* Δ*camdr*1::*his*G/Δ*camdr*1::*hisG* Δ*flu*1::*his*G/Δ*flu*1::*hisG-URA3-his*G) were constructed by targeted gene deletions in the parent strain CAF2-1.[53–57] In 100 μL of YPD medium [1% (w/v) BactoYeast extract (Difco, USA), 2% (w/v) glucose, 2% (w/v) Bacto Peptone (Difco, USA)], the *C. albicans* strains ($1\times10^4$ CFU/mL) were incubated with various concentrations of test compound using a 96-well plate. The plates were incubated at 35 °C for 24 h and absorbance of wells measured with 600 nm.

### Time–kill curve procedure

The fungistatic or fungicidal effect of the compound was evaluated by time–kill curve studies. In a 100 μL of 80% calf serum containing 2% glucose, 10 μM $FeCl_3{\cdot}6H_2O$, and 10 μM deferoxamine, the *C. albicans* CY3003 ($1\times10^5$ CFU/mL) was incubated with various concentrations of a test compound using a 96-well plate. The plates were incubated at 35 °C under 10% $CO_2$ condition. At predetermined times, 30 μL samples were obtained from each well, serially diluted 10-fold, and then 100 μL of sample was plated onto YPG agar [2% Peptone (Becton Dickinson, USA), 1% Yeast extract (Becton Dickinson, USA), 2% glucose] for colony counting. Colony counts were performed following incubation at 35 °C for 24 h.

### Antifungal tests in vivo (rat systemic candidiasis model)

An in vivo rat candidiasis model was used to monitor the antifungal activity of the benzofuran derivatives. Groups of 5 male Fischer rats (mean body weight 56.9 g) were inoculated with 0.5 mL of a suspension containing $2.5\times10^4$ cells/g of *C. albican*s CY1002 by iv on day 0. The benzofuran derivatives were administered in 0.5 mL of solution of 5% DMSO (Wako, Japan), 5% PEG (Wako, Japan), and 10% HPCD (Nihon Shokuhin Kako, Japan) in distilled water by iv after infection (from day 0), three times daily for 2 days (2 tid). Fluconazole was administered in 0.5 mL of saline iv 2 tid. The control group received only the vehicle. The efficacies of the antifungal agents were assessed as the 50% effective dose ($ED_{50}$), calculated by probit analysis at day 7 after infection.

### Target organ study

Normal male Fischer rats (mean body weight 62.5 g, $n=3$) were inoculated with 0.5 mL of a suspension containing *C. albicans* CY1002 ($3\times10^3$ cells/g) iv on day 0. The benzofuran derivative was administered in 0.5 mL of solution of 5% DMSO, 5% PEG, and 10% HPCD in distilled water by iv after infection (day 0). The benzofuran derivative was administered three times daily for 2 days (2 tid) at the doses of 5 and 15 mg/kg, and for one day (1 tid) at the dose of 30 mg/kg. Fluconazole was administered in 0.5 mL of saline by ip 2 tid. The control group received only the vehicle iv 2 tid. The target organ assay for *C. albicans* monitored the number of CFU per brain or kidney homogenates at day 2 after infection (target organ brain assay or target organ kidney assay). The organs were put in sterile tubes containing 5 mL of sterile saline and then homogenized. The homogenates were serially diluted in saline, and aliquots were plated onto 1×GYPA agar [1% Peptone (Difco, USA), 0.5% Yeast extract, 1% glucose (Wako, Japan), 2% Agar (Difco, USA)] plates. The plates were incubated at room temperature for 48 h, and the colonies were counted. The mean number of CFU per organs of the treated groups was compared with those of the organs from sham-treated control animals.

### Cassette dosing pharmacokinetics

Male F344 rats ($n=6$), weighing 55–75 g were used in each cassette dosing PK experiment. Five compounds were dissolved individually in DMSO at a concentration of 20 mg/mL. The final concentration of each compound of the cocktail solution was 0.2 mg/mL in 5% DMSO, 5% PEG300 (Wako, Japan), and 10% HPCD (Nihon Shokuhin Kako, Japan). A 10 mL/kg cocktail solution was administered to rats intravenously at 2 mg/kg for each compound. Blood samples were obtained serially at selected time points after drug administration. After centrifugation, the resultant plasma was stored frozen at −20 °C until analysis. Concentrations of benzofurans in plasma were determined by the LC–MS/MS method following a protein precipitation. An aliquot of the extract was analyzed by LC–MS/MS in the positive ion mode using an API-300 triple quadrupole mass spectrometer (PE SCIEX, Concord, ON, Canada). The chromatographic column was a Develosil C8 column (4.6×50 mm, 5-μm particle size; Nomura Chemical Co. Ltd., Japan). The samples were centrifuged (3000 rpm×10 min), evaporated, redissolved in 65% aqueous acetonitrile and the supernatant was injected to the HPLC system. The HPLC mobile phase was 0.1% formic acid in methanol to 0.1% formic acid in water, 60:40 to 75:25 (v/v), or methanol to 0.1% formic acid and 5 mM ammonium formate in water (approximately pH 3), 60:40 to 75:25 (v/v). The flow rate was set at 1.0 mL/min.

## Acknowledgements

The authors thank Professor Dominique Sanglard (Institute of Microbiology, University Hospital Lausanne) for providing the disrupted mutants of *C. albicans*.

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