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Design and Synthesis of Novel Benzofurans as a New Class of Antifungal Agents Targeting Fungal *N*-Myristoyltransferase. Part 2

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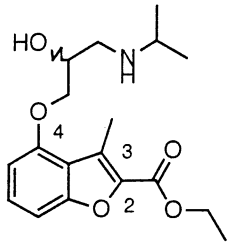
Abstract—Modification of the C-2 position of a benzofuran derivative **6** (RO-09-4609), an *N*-myristoyltransferase (Nmt) inhibitor, has led us to discover antifungal agents that are active in a murine systemic candidiasis model. The drug design is based on the analysis of a crystal structure of a *Candida* Nmt complex with **2**. The optimization has been guided by various biological evaluations including a quasi in vivo assay and pharmacokinetic analysis.

N-Myristoyltransferase (Nmt) has been proven to be essential for the viability of fungi, including medically important pathogenic fungi, *Candida albicans*[1] and *Cryptococcus neoformance*,[2] making it a possible target for the development of fungicidal drugs with a novel mode of action. An Nmt inhibitor could therefore be a new type of antifungal agent. Because of its novel mechanism of action, an Nmt inhibitor is expected to have advantages over azole antifungal agents in terms of activity against azole resistant fungal strains and lack of the drug–drug interactions that are a drawback of the azole agents.[3] In our previous paper,[4] we reported our optimization of the C-3 and C-4 substituents of a novel Nmt inhibitor, a benzofuran derivative **1**, discovered by random screening. The modification resulted in identification of **6** (RO-09-4609) that showed improved activity in both *C. albicans* Nmt (CaNmt) inhibitory activity (IC_{50}: 0.1 μM) and in vitro antifungal activity (IC_{50} against *C. albicans* CY1002: 1.6 μM) as compared with **1**. This compound, however, did not show in vivo efficacy in a murine systemic candidiasis model, probably because of its weak antifungal activity and its short elimination half-life ($t_{1/2}$: 0.42 h) in mice. The ester group of **6** was easily hydrolyzed by esterases in the mice to give an inactive carboxylic acid metabolite. To overcome these problems, we modified the C-2 substituent of compound **6**. The inhibitors were designed and optimized based on the analysis of the X-ray structure of a CaNmt complex with a benzofuran inhibitor **2**[4] and the structure–activity relationship (SAR) analysis. The modification was guided by the enzyme inhibitory activity, the antifungal activity in YNBPB medium, the antifungal activity in 80% calf serum (quasi in vivo assay), and the pharmacokinetic (PK) properties. The quasi in vivo assay could be more predictive for in vivo efficacy than the conventional in vitro antifungal assay, since the former assay system includes a factor of drug–protein binding.



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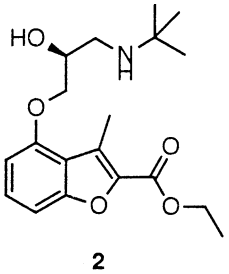

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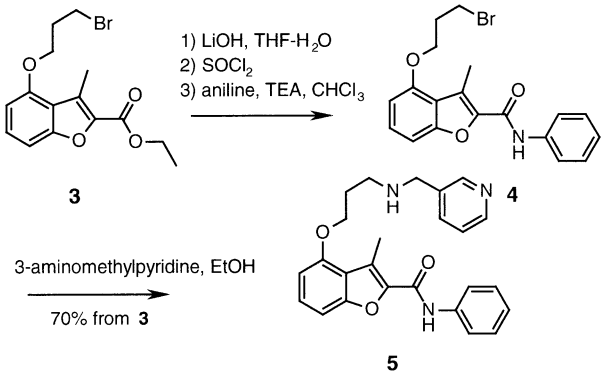
In this paper, we wish to report the design and synthesis of metabolically more stable derivatives at the C-2 position of compound **6** and their biological activities including antifungal activity in vivo.

Synthesis

N-Phenylcarbamoyl derivative **5** was prepared from ester **3** in a good yield as outlined in Scheme 1. Ester **3** was hydrolyzed by lithium hydroxide, and the resulting acid was converted into its acid chloride by treatment with thionyl chloride, which was immediately subjected to the next anilide formation reaction. The resulting *N*-phenylcarbamoyl compound **4** was heated to 70 °C with 3-aminomethylpyridine in EtOH to give **5**.

As shown in Scheme 2, phenylthioether **8**, a phenethyl derivative **11**, and phenylethers **12a**–**12k** were synthesized from alcohol **7** that was prepared by lithium aluminum hydride reduction of **6**. The treatment of alcohol **7** with thiophenol and trifluoroacetic acid (TFA) in dichloromethane gave the thioether **8**. *N*-Protection of **7** with Boc followed by oxidation of the resulting alcohol **9** with manganese dioxide gave the aldehyde **10**. Wittig reaction of **10**, hydrogenation of the resulting olefin, and subsequent deprotection gave the phenethyl derivative **11**. The phenylether derivatives **12a**–**12k** were synthesized from alcohol **7** and an appropriate phenol by Mitsunobu reaction. In most cases, a modified Mitsunobu reaction with 1,1′-(azodicarbonyl)dipiperidine (ADDP) and tributylphosphine in toluene[5] gave better yields (ca. 60%) than the standard Mitsunobu reaction (DEAD-TPP/THF).

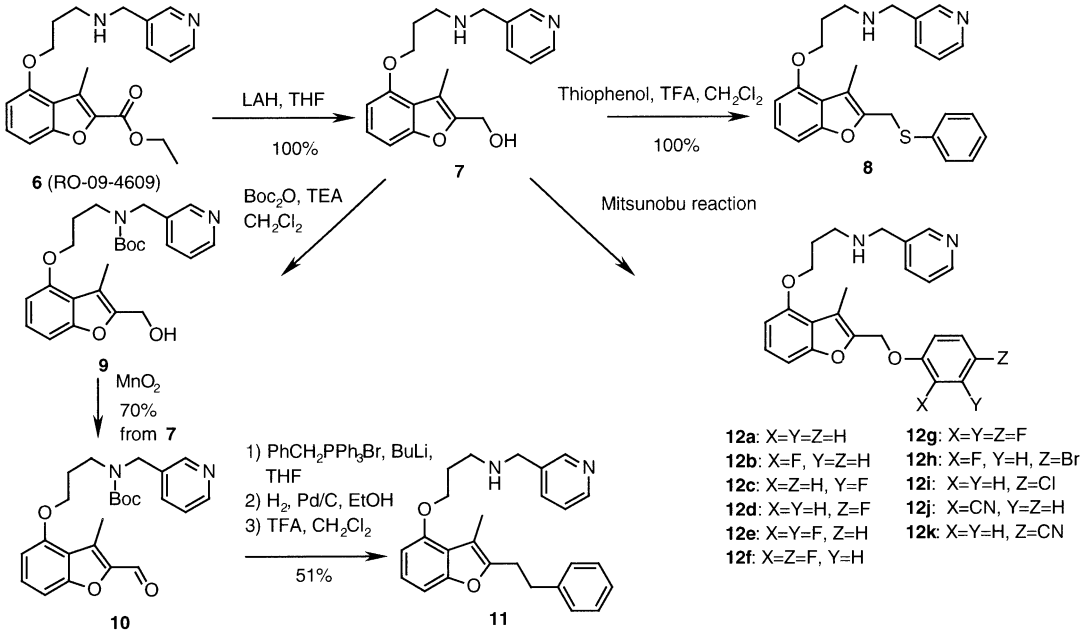


Scheme 1. Preparation of *N*-phenylcarbamoyl derivative **5**.

Results and Discussion

The binding mode of the compound 6 derivatives to CaNmt and their SARs

The crystal structure of a CaNmt complex with **2** revealed that the C-2 substituent of **2** was surrounded by three phenylalanine residues, Phe 115, Phe 240, and Phe 339.[4] To strengthen the binding between the C-2 substituent and these phenylalanine residues by aromatic–aromatic interaction, we introduced a phenyl group to the C-2 position via various linkers: –CONH– (**5**), $-CH_2S-$ (**8**), $-CH_2CH_2-$ (**11**), and $-CH_2O-$ (**12a**). We also expected that the replacement of the C-2 ester with the above-mentioned linkers would increase the metabolic stability of the inhibitors. Both the CaNmt inhibitory activity and the antifungal activity of these compounds were strongly affected by the linker structure. Namely, the compound **12a** having an ether linker was the most potent among the C-2 derivatives **5**, **8**, **11**, and **12a** (Table 1). This result suggested that the ether oxygen atom in **12a** and one of the ester oxygens in **6** were interacting with CaNmt through hydrogen bonding. To further increase the binding affinity, we introduced electron-withdrawing groups to the phenyl group of **12a**, because an aromatic ring having electron-with-



Scheme 2. Preparation of benzofuran derivatives.

Table 1. Enzyme inhibitory activity, antifungal activity, and pharmacokinetic (PK) properties

Compd	Enzyme inhibition (μM)		Antifungal activity (μM)		PK parameters[e]	
	CaNmt[a]	HsNmt[b]	Serum(−)[c]	Serum(+)[d]	AUC (ng∗h/ml)	$t_{1/2}$ (h)
5	2.2	>480	12	NT[f]	240	0.9
6	0.1	>450	1.6	NT	330	0.4
8	0.62	>470	11	180	390	1.4
11	1.2	12	13	>240	290	1.1
12a	0.072	77	0.37	NT	670	1.9
12b	0.0083	>470	0.04	0.6	560	1.6
12c	0.11	>110	0.11	3.3	370	1.5
12d	0.0052	67	0.021	0.7	340	1.9
12e	0.0037	62	0.08	0.57	500	1.9
12f	0.0075	>450	0.03	0.34	420	1.6
12g	0.0057	>430	0.035	0.33	330	2.0
12 h	0.028	>400	0.074	1.3	360	3.6
12i	0.073	>110	0.92	6.6	330	2.4
12j	0.017	19	1.2	3.3	280	0.5
12k	0.0094	>460	0.16	1.4	340	1.2
Fluconazole	NT	NT	0.72	0.5	19,600	4.0

[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% dextrose).
[e]Cassette dosing: five compounds were intravenously administered to a rat and the plasma concentration of each compound was measured by LC–MS.[9]
[f]NT, not tested.

drawing groups is known to interact with another aromatic ring more strongly than does an unsubstituted aromatic ring.[6] We thus introduced fluoro, chloro, bromo, and cyano groups to the phenyl ring of the phenylether **12a**. As we expected, most of the phenylether derivatives having electron-withdrawing groups showed stronger inhibitory activity against CaNmt than the unsubstituted phenylether **12a** (Table 1). Among them, the 2-fluoro **12b**, 4-fluoro **12d**, 2,3-difluoro **12e**, 2,4-difluoro **12f**, 2,3,4-trifluoro **12g**, and 4-cyano **12k** derivatives showed very strong inhibitory activity against CaNmt (IC_{50}: 3.7–9.4 nM). In contrast, the 3-fluoro derivative **12c** was 10–30 times less active than those derivatives. The variation of the inhibitory activity among the ether derivatives could be explained by the electron density on the ether oxygen that was calculated by the electrostatic potentials around aromatic rings of the compounds using a molecular orbital method. The theoretical calculation and the interpretation of the SARs will be reported in detail separately.

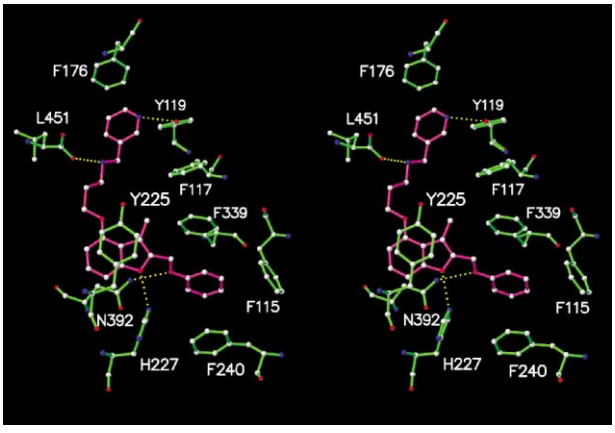


Figure 1. The crystal structure of a CaNmt complex with **12e**.

A crystal structure (3.5 Å) of a CaNmt complex with **12e** was obtained by a soaking experiment and the structure of the binding site is shown in Figure 1. The positions of the fluorine atoms could not be well defined because of their poor electron density. The data strongly supported the supposition that the aromatic–aromatic interaction between the aryl group at the C-2 position and the hydrophobic pocket formed by Phe 115, Phe 240, and Phe 339 of CaNmt was very important in gaining binding affinity. The aryl group at C-2 position is exactly situated between two phenylalanine residues, Phe 240 and Phe 339. The crystal structure also suggested important hydrogen bonds between the amido-NH_2 of Asn 392 and the ether oxygen, and between one of the ring nitrogens of His 227 and the ring oxygen of the benzofuran. The importance of the latter hydrogen bond may be supported by very weak inhibitory activity (IC_{50}: 8.6 μM) of the benzothiophene analogue of compound **12f**, [3-[2-(2,4-difluoro-phenoxymethyl)-3-methyl-benzothiophene-4-yloxy]-propyl]-pyridin-3-ylmethyl-amine. The lack of activity of a benzothiophene analogue may be caused by the difference in hydrogen acceptor ability between the sulfur and oxygen atoms and/or the geometrical difference between the thiophene and furan rings. The phenylethers **12a**–**12k** showed extremely high selectivity to CaNmt over HsNmt (Table 1).

The quasi in vivo and in vivo antifungal activities

An in vitro antifungal assay containing serum was thought to be more predictive for in vivo efficacy than the conventional in vitro assay without serum. We

adopted an in vitro assay system in 80% calf serum supplemented with 10 μM $FeCl_3 \cdot 6H_2O$, 10 μM deferoxamine, and 2% dextrose in our assay cascade (quasi in vivo assay).[7,8] The compounds **12b**, **12d**, **12e**, **12f** and **12g** were as potent as fluconazole (IC_{50}: 0.5 μM in this assay protocol) in serum, although their antifungal activity was reduced by the addition of calf serum. Furthermore, all the phenylether derivatives in Table 1 showed longer $t_{1/2}$ than did compound **6**.

Since compounds **12f** and **12g** exhibited the highest quasi in vivo antifungal activity and compound **12h** showed the longest $t_{1/2}$ among the phenylether derivatives, they were further evaluated in a rat systemic candidiasis model. Fisher rats ($n=5$) were infected intravenously with a lethal dose of *C. albicans* (CY1002) and treated iv with multiple doses (three times per day for 2 days) 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 fluoro derivatives **12f** and **12g** were active in this model with ED_{50}s of 7.1 mg/kg. Although compound **12h** had a longer $t_{1/2}$ than did the other compounds, it showed much weaker in vivo efficacy in this model (ED_{50}: >30 mg/kg) than **12f** or **12g**. This in vivo result correlated with its weaker antifungal activity in the quasi in vivo assay. Thus, the in vivo efficacy was well predicted by the results of the quasi in vivo assay and the PK study. Although these compounds showed in vivo efficacy, they were still 10 times weaker than fluconazole in the rat systemic candidiasis model. Their weaker in vivo activity might be caused by their poorer PK profiles than those of fluconazole. Thus further modifications are required to develop a clinically useful fungicidal agent. Since the antifungal activity [serum (−)] against *C. albicans* of the benzofurans showed a good correlation with the enzyme inhibition activity against CaNmt, the growth inhibition of *C. albicans* was considered to be caused by the inhibition of the enzyme. The correlation coefficient (R) and F values of the correlation between log IC_{50} values of antifungal activity and those of enzyme inhibition activity were calculated to be 0.892 and 7.92×10^{-6}, respectively.

In summary, the extensive modification of the C-2 substituent of a novel CaNmt inhibitor, **6** (RO-09-4609), which showed moderate antifungal activity in vitro, led to identification of the novel derivatives, **12f** and **12g**, which were active in a rat systemic candidiasis model. The modification work was carried out by the combination of rational drug design based on the crystal structure of the binary complex of CaNmt with **2** and the SAR analysis guided by various biological investigations, including the quasi in vivo assay and the cassette dosing PK studies in rats.[9] This 'multi-dimensional optimization' enabled us to rapidly identify the compounds that are active in vivo, starting from the enzyme inhibitor **1** with only marginal antifungal activity in vitro.

In conclusion, we have discovered a new class of antifungal agents that selectively and strongly inhibited CaNmt and exhibited in vivo efficacy. Because of their unique mode of action, the Nmt inhibitors may overcome the drawbacks of current drugs such as resistant development and drug–drug interactions in azole antifungal agents as reported.[3] The identification of potent and selective CaNmt inhibitors, **12f** and **12g**, having enhanced in vivo efficacy warrants further modification studies to develop a new class of fungicidal agents.

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