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MODELING, SYNTHESIS AND BIOLOGICAL ACTIVITY OF NOVEL ANTIFUNGAL AGENTS (1)

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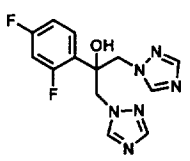
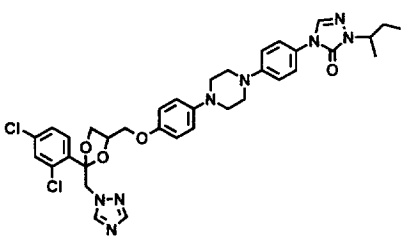
Abstract: Homology modeling of candida lanosterol C-14 demethylase, synthesis and *in vitro* antifungal activities of cyclohexyl analogs of restricticin are described.

Keyword: Antimicrobial; Chemotherapy; Fungi

The medicinal need for safe and effective systemic antifungal agents has been intensified due to rapid growth of the immunocompromised patient population.[1] Although azole antifungal agents, such as fluconazole, are most widely used today, they still have problems in efficacy (especially against *Asp. fumigatus*), resistance etc.[1] Thus, we tried to develop a new class of lanosterol C14-demethylase inhibitor which dose not have a (phenethyl)triazole moiety (***Fig.1***) found in all azole antifungals on the market, based on the structure of restricticin **1**.



Fluconazole



Itraconazole

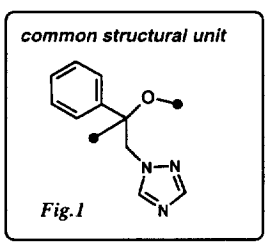


Fig.1

Restricticin **1** was discovered independently by Roche[2] and Merck[3] as a lanosterol C14-demethylase inhibitor. We have already reported the total synthesis of restrictinol **2**[4] and its corresponding carbacyclic analogs such as **Ro 09-2056.**[5] The initial modification study produced compounds whose chemical stabilities and *in vitro* antifungal activities were significantly imporoved over those of **1**.[5] **Ro 09-2056** however did not show *in vivo* efficacy in murine systemic candidiasis model due to strong protein binding despite its potent *in vitro* activity. To improve the pharmacokinetic profiles, we planed to introduce various hydrophilic functional groups at C-3 and C-4 position of **Ro 09-2056** without losing their strong hydrophobic interaction with the enzyme. In this paper, we describe the homology modeling of Candida C14-demethylase (P450DM) based on the crystal structure of Ps. putida's P450CAM, synthesis of **Ro09-2056** analogs and their biological results.

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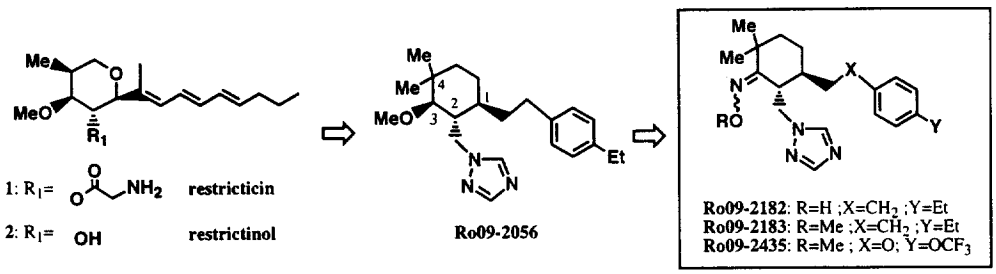


Our modification strategy of **Ro 09-2056** was to alter the pharmacokinetic parameters by increasing the hydrophilicity of the molecules. To do this we first needed to know which part of the molecule could accept hydrophilic functional groups without disturbing the strong affinity of the inhibitor to the enzyme. Scince the target enzyme Candida P450DM is a membrane bound enzyme, it is difficult to crystalize for X-ray analysis. Thus, we tried to build a putative 3D structure of Candida P450DM based on structure of the P450CAM of Ps. putida, which was the only P450 enzyme available with a known 3D structure.[6]

The detailed procedure for modeling P450DM and docking Ro09-2056 with the model is described in reference 7. **Fig.2a** shows the sequence alignment of P450CAM and P450DM. Although the sequence identity between P450CAM and the P450DM is 10 % (similarity 26 %), some important residues are well conserved. The heme-binding residues of P450CAM, Gln108, Arg112, Arg299, His355 and Cys357 (red in Fig.2a), are replaced in P450DM with Lys178, His183, Lys358, His468 and Cys470, respectively. These residues except for Cys are conserved to maintain the electrostatic interaction with heme propionate groups. In the report of the P450DM model built by Boscott et al.[14], two of these heme-binding residues were not conserved. In order to check the accuracy of our model, a 3D profile score[15] was calculated. The score 74.3 indicates that our model (for 395 residues) was not largely misfolded, comparing with other experimentally determined structures.[16] **Fig.2b** shows water accessible surfaces of the active site pocket of the enzyme, which forms a putative inhibitor binding pocket. Ro09-2056 (yellow) was placed in the putative binding pocket. This model shows that C-1 side chain of the inhibitor is surrounded by lipophilic amino acid residues, whereas some amino acid residues with hydrogen bonding ability (e.g. Ser507, Glu354 and Tyr353) are located around the C-3 substituents. More interestingly, the C-4 substituent is located just below the entrance of the pocket (**Fig.2c**). This allows us to introduce hydrophilic functional groups without interfering the interaction between the enzyme and an inhibitor, because they can extend toward the outside of the pocket of the active site. Thus, we decided to introduce hydrophilic functional groups at C-3 and/or C-4 position.

The *in vitro* antifungal activity of C-3 derivatives summarized in ***Table 1*** in comparison with those of **Ro09-2056** and fluconazole.[12] **Ro09-2173** having hydroxyethoxy group showed extremely potent *in vitro* antifungal activity against *Candida. albicans*. **Ro09-2182** and **2183** both possessing an oxime group, were found to have extremely potent *in vitro* activity against *Aspergillus. fumigatus*. And **Ro09-2183** had an especially well-balanced activity against all three major systemic pathogens. However, these compounds were inactive in the murine systemic Candidiasis model. Thus, we investigated the metabolism of **Ro09-2182** using the rat liver microsome fraction. **Ro09-2182** was rapidly metabolized to two inactive metabolites **3** and **4**, in which the benzylic methylene group was oxidized(***Scheme 1***). In order to improve the metabolic stability, we investigated alternative C-1 side chains. ***Sheme 2*** shows the synthesis of representative compounds in this categoly.[17,18]

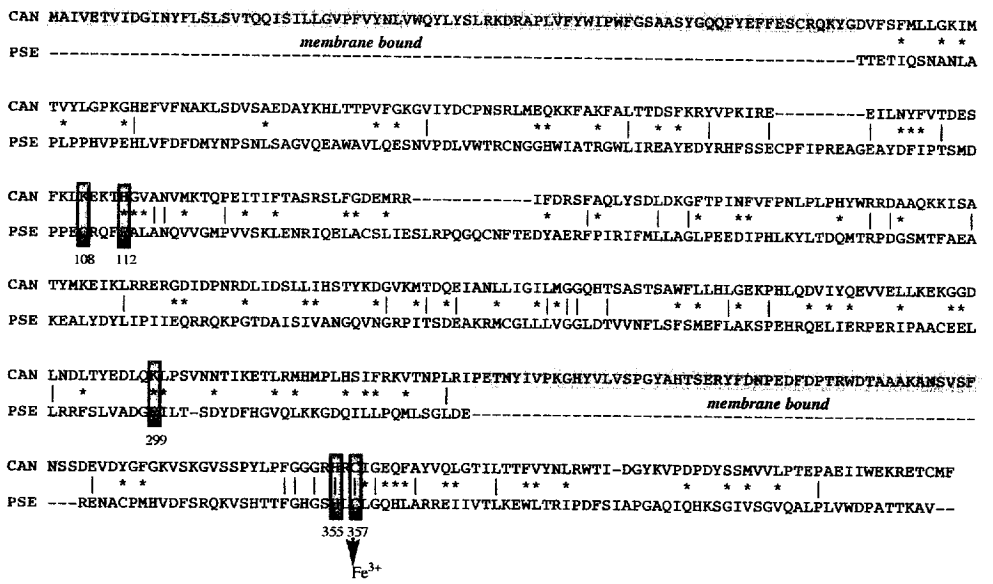


Fig.2a: Sequence alignment of P450CAM and P450DM

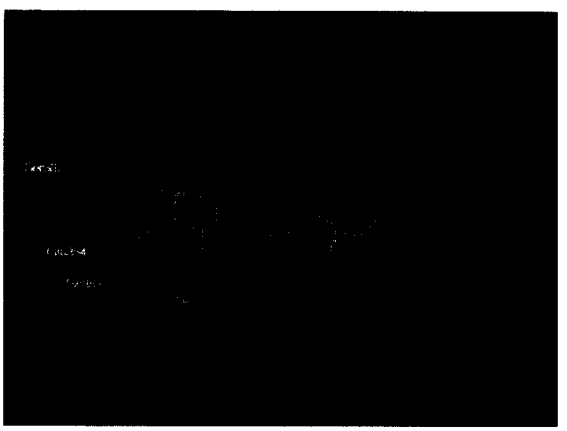


Fig.2b : Water accessible surfaces of P450DM active site: green dots / surface of carbon atom, red / oxygen atom / yellow: sulfur atom.

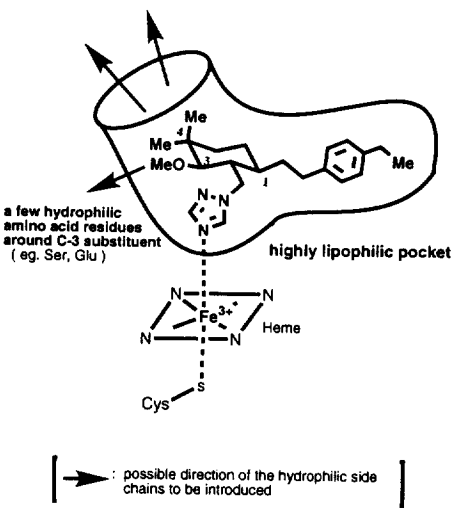
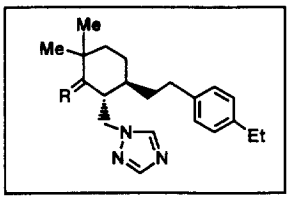


Fig.2c: Summary of the modeling

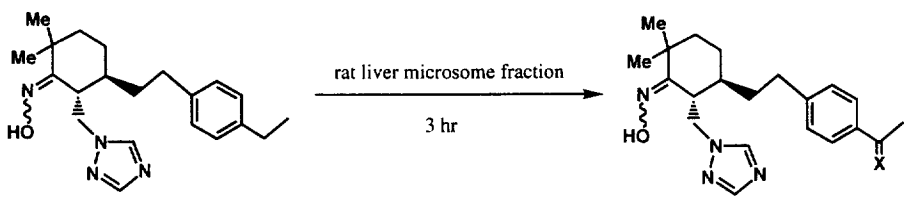
Table 1 : *in vitro* antifungal activity of C-3 derivatives



Compound		Antifungal activity IC_{80}(μg/ml)[a]			Enzyme inhibitory activity IC_{50}(μg/ml) [b]
Ro No.	R	*C. albicans* CY 3003	*C. neoformans* CY 1057	*A. fumigatus* CF 1004	
Ro09-2173	**•-O(CH$_2$)$_2$OH** (α orientation)	**0.00086**	0.36	>200	0.032
Ro09-2182	**•=N-OH**	**0.023**	130	**0.0011**	0.026
Ro09-2183	**•=N-OMe**	**0.024**	**0.48**	**0.064**	0.032
metabolite **3**	—	>200	>200	60	—
metabolite **4**	—	>200	>200	21	—
Ro09-2056	**•-OMe**	0.0042	0.74	>200	0.010
FCZ	—	1.3	6.2	150	0.042

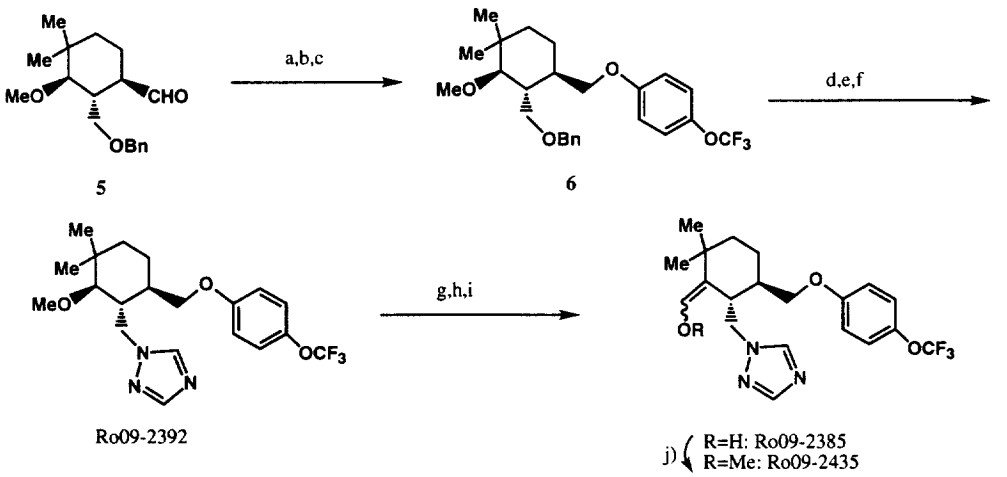
a) Broth dilution method; medium: YNBPB(=YNB+1% glucose+ 0.25% K_2HPO_4), pH 7.0, inoculum size; 1x10^4 cfu/ml, incubation: 1~2 days at 27°C. b) P450 lanosterol C14 demethylase (*C.albicans* CY1005)

Scheme 1: Metabolism of Ro09-2182 in rat liver microsome fraction



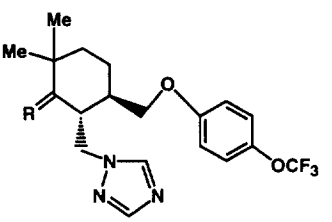
X=O (**3**)
X=H,OH (**4**)

The *in vitro* antifungal activity of the metabolically stable derivative is summarized in ***Table 2*** in comparison with those of **Ro09-2392** and fluconazole. **Ro09-2435** showed well-balanced antifungal activity. However, these compounds were still inactive in the murine systemic Candidiasis model in mouse. This was due to strong serum protein binding of the compound.[19] Thus, we tried to decrease the serum protein binding by introducing another hydrophilic functional group at C-4 position. Synthesis and antifungal activity of the new derivatives at C-4 position are reported in the following paper of this issue.



Scheme 2: (a) $LiAlH_4$, ether, rt; (b) MsCl, Et_3N, CH_2Cl_2, 0°C; (c) o-trifluomethoxyphenol,NaH, DMF, rt (76%, 3 steps); (d) H_2, 10% Pd/C, MeOH, rt; (e) MsCl, pyridine, CH_2Cl_2, 0°C; (f) 1,2,4-triazole sodium salt, DMF, rt (68%, 3 steps); (g) NaI, $AlCl_3$, MeCN, rt; (h) NCS, Me_2S then Et_3N; (i) $NH_2OH.HCl$, pyridine, EtOH; (j) NaH, MeI, DMF

Table 2 : *in vitro* antifungal activity of metabolically stable C-3 derivatives



Compound		Antifungal activity IC_{80}(μg/ml)[a]			Enzyme inhibitory activity IC_{50}(μg/ml) [b]
Ro No.	R	*C. albicans* CY 3003	*C. neoformans* CY 1057	*A. fumigatus* CF 1004	
Ro09-2364	=O(CH2)2OH (α orientation)	0.39	5.2	110	0.010
Ro09-2385	=N–OH	**0.35**	>200	**0.17**	0.054
Ro09-2435	=N–OMe	**0.12**	**7.6**	**0.4**	0.024
Ro09-2392	–OMe	0.18	0.51	56	0.049
FCZ	—	1.3	2.6	>200	0.039

a) Broth dilution method; medium: YNBPB(=YNB+1% glucose+ 0.25% K_2HPO_4), pH 7.0, inoculum size; $1x10^4$ cfu/ml, incubation: 1~2 days at 27°C. b) P450 lanosterol C14 demethylase (*C.albicans* CY1005)

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6) Candida C14-demethylase (P450DM) in ref. 8 is the only one fungal C14-demethylase whose amino acid sequence is known.
7) The amino acid sequence of P450DM was taken from that reported by Lai et al (ref.8). The sequence and coordinates of P450CAM were obtained from Brookhaven Protein Databank (ID-code: 3CPP, ref. 9). The sequence alignment was initially done by the GCG program package(ref. 10). Then it was manually optimized with the reference of the sequence alignment by Morris et al (ref. 11). Two large insertions (1-80 and 390-442 colored in blue) were speculated to be membrane bound regions due to the hydrophobicity so that they were omitted to model. The location is, however, predicted to be close to each other on the surface of the modeled P450DM molecule. The homology modeling of P450DM was performed with the program MOLOC (ref. 12). The inhibitor docking with the P450DM model was simulated by the following steps. Firstly, we investigated the geometry of heme coordinating His residues among 20 heme proteins of which structures were well clarified by X-ray crystallography. The average distance between Fe(III) and a coordinating N atom of imidazole and the average angle of N atom (heme)-Fe(III)-N atom (imidazole) were 2.1 Å and 90°, respectively. They were used for placing azole ring of Ro09-2056 in the C-2 position. Next the lowest energy conformation from a stochastic conformational analysis of the inhibitor itself was manually placed in the active site without any significant repulsion for the P450DM model as a initial position. 100 conformations were generated by molecular dynamics (MD) calculation at 1000 K and consequence energy minimization. The energetically stable conformation was selected as a hypothetical bound conformation. MD calculation and generation of water accessible surfaces were performed by the INSIGHT II and DISCOVER software package (ref. 13). All computational work was done on Silicon Graphics Indigo2 workstation.
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17) All new compounds gave spectroscopic data in agreement with the assigned structures.
18) Synthesis of compound **5**: see ref. 5)
19) Antifungal activity of Ro09-2435 (C.albicans CY 3003) with 10% mouse serum; IC_{80}=150μg/ml.