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Design and Optimization of Highly-Selective Fungal CYP51 Inhibitors

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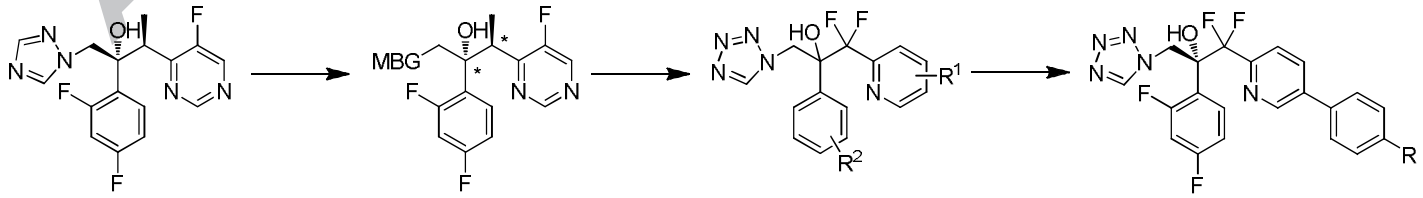
Viamet Pharmaceuticals Inc., Durham, NC 27703, USA

Abstract: While the orally-active azoles such as voriconazole and itraconazole are effective antifungal agents, they potently inhibit a broad range of off-target human cytochrome P450 enzymes (CYPs) leading to various safety issues (e.g., drug-drug interactions, liver toxicity). Herein, we describe rationally-designed, broad-spectrum antifungal agents that are more selective for the target fungal enzyme, CYP51, than related human CYP enzymes such as CYP3A4. Using proprietary methodology, the triazole metal-binding group found in current clinical agents was replaced with novel, less avid metal-binding groups in concert with potency-enhancing molecular scaffold modifications. This process produced a unique series of fungal CYP51-selective inhibitors that included the oral antifungal **7d** (VT-1161), now in Phase 2 clinical trials. This series exhibits excellent potency against key yeast and dermatophyte strains. The chemical methodology described is potentially applicable to the design of new and more effective metalloenzyme inhibitor treatments for a broad array of diseases.

Cytochrome P450 enzymes (CYPs), due to high homology inclusive of a common heme-iron motif, present a major challenge to the discovery of target-selective inhibitors. Many metalloenzyme inhibitors consist of two chemical components: the metal-binding group (MBG), the portion of the inhibitor designed to bind to the metal, and the scaffold, the portion of the inhibitor recognized by the amino acid residues that form the substrate-binding site of the metalloenzyme. The MBG is often a major contributor to the overall potency of the inhibitor, though metalloenzyme inhibitors have been reported that do not utilize a MBG. The attraction of the MBG to the metal ion is governed by electronic factors. The magnitude of the MBG's interaction with the metal, and therefore inhibitor potency, can be "tuned" by modulating its electronic character. If the metal/MBG interaction is strong, potent inhibition of the target enzyme may be achieved but unintended related metalloenzymes can be inhibited as well.

Scheme 1



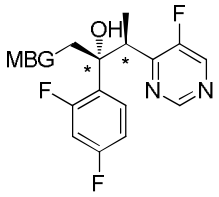
Voriconazole **1** **2** **7**

* Corresponding author. Tel: 919-467-8539, ext. 303; email: whoekstra@viamet.com

The azole class of antifungal drugs inhibits fungal CYP51 (lanosterol demethylase) activity through competitive, reversible binding to the heme cofactor located in the enzyme active site.[1] Historically, there has been relatively little variation in CYP51 inhibitor MBGs. First-generation antifungal drugs, such as miconazole and ketoconazole, utilized the 1-imidazole MBG, a high-affinity ligand for heme-iron (e.g., **1d**; Table 1). These drugs also inhibited off-target human hepatic CYP enzymes leading to severe and sometimes fatal liver problems.[2] Second-generation azole antifungal drugs (e.g. itraconazole, voriconazole) utilized a 1-(1,2,4-triazole) MBG. Compared to 1-imidazole, the triazole was a lower-affinity ligand for heme-iron and this MBG alternative led to improved tolerability, but liver toxicity and drug-drug interactions remained problematic.[3] Our strategy to discover new, more selective agents focused on alternative, low-affinity MBGs. Herein, we disclose a potent, selective series of inhibitors, including the Phase 2 antifungal clinical agent **7d** (VT-1161; Scheme 1, Table 3).[4]

We hypothesized that the MBG found in the azole class of drugs (e.g., voriconazole, itraconazole), the 1-(1,2,4-triazole), is too high in heme-iron affinity and may be the source of CYP non-selectivity. Our approach was to attenuate the magnitude of the MBG/metal interaction in order to improve target selectivity, likely at the initial expense of overall CYP51 affinity. Inhibitor potency was to be improved through modifications to the scaffold, thus increasing the magnitude of binding affinity within the substrate binding pocket rather than via the metal interaction.

Table 1. Antifungal Activity and CYP3A4 Potency of Pyrimidines.



1

Compound	MBG	*C. albicans* MIC[a]	CYP3A4 IC_{50} [b]
1a	1-(1,2,3-triazole)	>16	8.0
1b	1-tetrazole	1	32
1c	4-(1,2,4-triazole)	>16	51
1d	1-imidazole	0.5	0.8
1e	2-tetrazole	8	46
Voriconazole	1-(1,2,4-triazole)	0.06	13

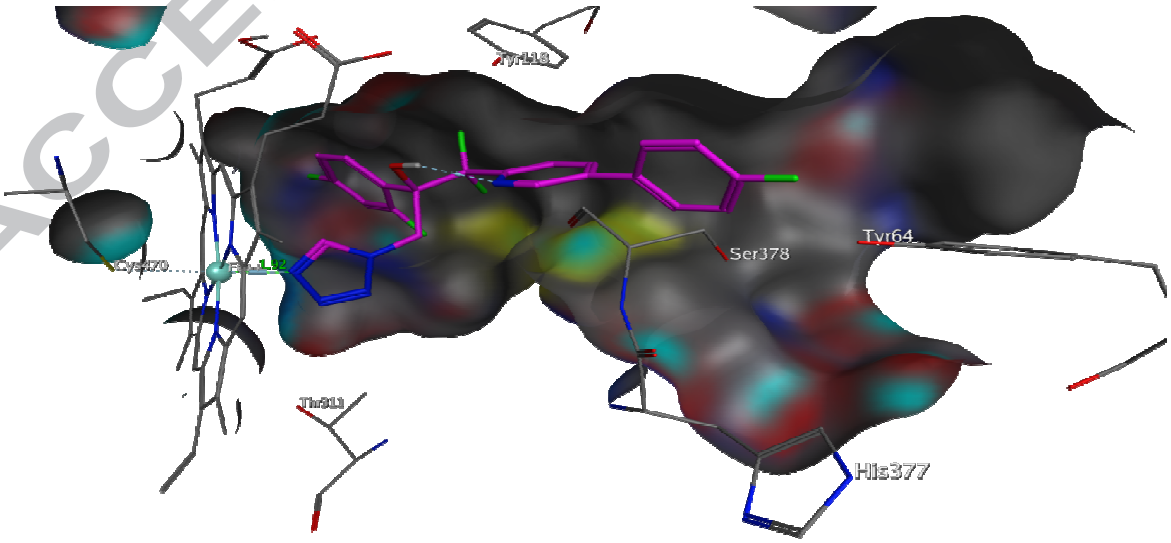
a. Minimum concentration that achieved 50% inhibition of fungal growth; MIC units in μg/mL.[5] b. Inhibition of CYP3A4 measured in microsomes obtained from pooled human hepatocytes; IC_{50} units in μM.[8]

In order to investigate our hypothesis, we used a process which combined the application of inorganic chemistry with classical medicinal chemistry. MBGs were initially selected using an *in silico* method that rank-ordered them by their predicted affinity for heme-iron. The approach is exemplified in Scheme 1 wherein voriconazole (**1a**) was chosen as a starting point, given its good potency (C. albicans MIC = 0.06 ug/mL)[5] and

physicochemical properties,[6] as well as its lower affinity for the anti-target, CYP3A4, than itraconazole (CYP3A4 IC_{50} = 0.07 uM) or posaconazole (CYP3A4 IC_{50} = 0.05 uM). A survey of potential alternative lower affinity MBGs, screened *in silico*,[7] led to discovery of the active, low basicity 1-tetrazole **1b** (Table 1).

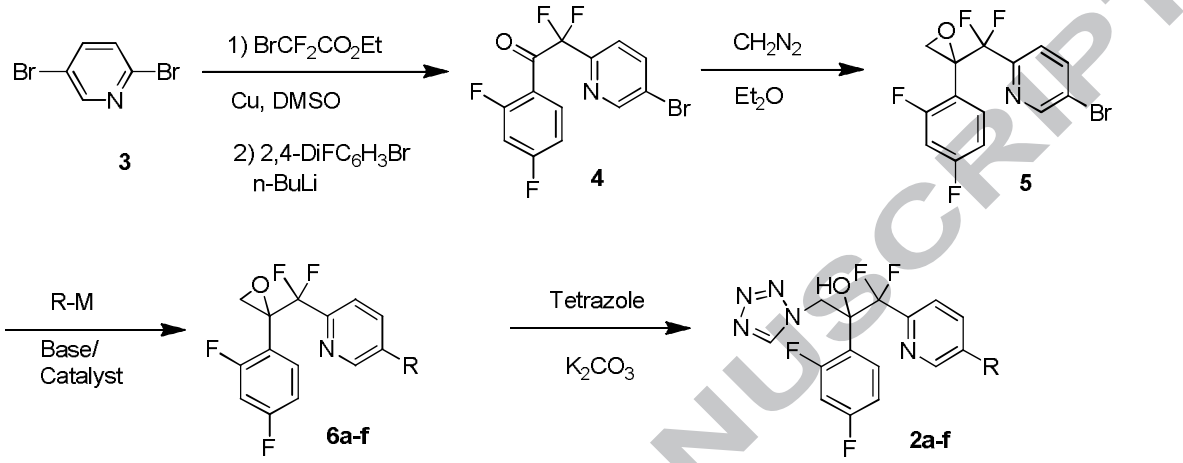
Compounds **1a**-**1e** are representatives from an array that was synthesized and then tested to assess *in vitro* potency in fungal growth[5] and CYP3A4 enzyme[8] assays (Table 1). The yeast, *C. albicans*, was chosen for initial antifungal testing because it is the species that most frequently causes invasive human infections.[9] CYP3A4 was selected as the representative anti-target due to its prominent role in liver metabolism, and its inhibition by the azole antifungals is a recognized source of numerous drug-drug interactions.[10] The target molecules were synthesized in modest yields (10-40%) as mixtures of the indicated enantiomeric pairs by coupling the requisite azole-methyl 2,4-difluorophenyl ketone intermediate with LDA-treated 4-ethyl-5-fluoro-pyrimidine.[6] Heteroaromatic MBGs were selected to capture a range of basicities and the focus was placed on low-affinity moieties that could be further optimized with scaffold modifications to realize both antifungal potency (*C. albicans* MIC ≤1 μg/mL initially) and CYP selectivity targets (CYP3A4 IC_{50} > 50 μM). Analogues with promising *C. albicans* activity in the 0.5-1 μg/mL range from the array were 1-tetrazole **1b** and 1-imidazole **1d**. The 1-imidazole was not pursued further given its marked basicity (pKa = 6.8) and pronounced CYP3A4 inhibition (IC_{50} = 0.8 μM). Though **1a**-**1e** did not attain the initial 50-fold selectivity goal, analogue **1b**, encouragingly inhibited CYP3A4 activity weakly (IC_{50} = 32 μM). It was selected for further exploration due to the low-basicity (and predicted iron affinity) of the 1-tetrazole MBG [basic pKa = -1.1 vs. 2.3 for the potent standard 1-(1,2,4-triazole)].[7]

Figure 1. CYP51 active site view of (*R*)-(+)-**2d** docked into a *C. albicans*-CYP51 homology model.



Homology model dockings of **1b** utilizing a *C. albicans*-CYP51 construct indicated that a potential interaction could be facilitated with the conserved tyrosine-118 residue by incorporation of an aromatic group vicinal to the *N*-1 position of the pyrimidine moiety (Fig. 1).[11] A π-stacking interaction between a lipophilic, aromatic replacement for the pyrimidine and the Tyr-118 was proposed.

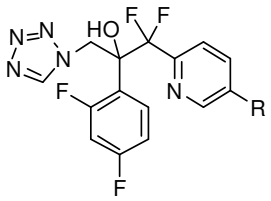
Scheme 2



Thus, a representative series of 1-tetrazoles **2a**-**2f** containing an electron-deficient 2,5-disubstituted pyridine capable of interacting with Tyr-118, connected through a metabolically-resistant, electron-withdrawing difluoromethyl linker,[12] were synthesized and tested (Scheme 2, Table 2). Small substituents at the 5-position of the pyridine (e.g., **2a-b**; prepared from 5-H or 5-Cl analogues of **3**) gave modest yeast potency improvement compared to pyrimidine **1b** while relatively large substituents provided significant yeast potency increases (**2d-f**); substitutions at the 5-position of the pyridine had only a small effect on CYP3A4 potency (Table 2). Several molecules in this chemical series exhibited robust oral efficacy in a mouse model[13] of invasive candidiasis (**2a**, **2b**, **2d**, **2e**), indicative of both adequate plasma exposure and sustained metabolic stability. Monocyclic and bicyclic heteroaromatic alternatives to pyridine were not pursued due to poor *in vivo* efficacy (data not shown).[14]

Further examination of the homology model around the meta/para position of biaryls **2d**-**2f** indicated that potency could be further improved through an additional H-bonding interaction with Ser-378 in this binding pocket region (e.g., with an ether moiety).[15] The trifluoromethyl ether **7c** and trifluoroethyl ether **7d** were synthesized as racemates, and following resolution of the single isomers,[15-17] furnished highly-potent growth inhibitors of representative yeast (*C. albicans*) and dermatophyte (*T. rubrum*) isolates (Table 3).

Table 2. *In Vitro* Potency and Oral Antifungal Activity of Difluoromethyl-Pyridines.



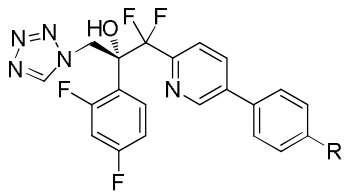
2

Compound	R	*C. albicans* MIC[a]	CYP3A4 IC_{50} [b]	Mouse Efficacy[c]
1b	-	1	32	93%
2a	H	0.25	136	98%
2b	Cl	0.25	74	100%
2c	OCH_2CF_3	0.06	72	81%
2d	4-F-Ph	0.016	53	99% [d]
2e	4-CN-Ph	≤0.016	16	100%
2f	4-CF_3-Ph	≤0.016	>60	94%[e]

a. Minimum concentration that achieved 50% inhibition of fungal growth; MIC units in μg/mL.[5] b. Inhibition of CYP3A4 measured in microsomes obtained from pooled human hepatocytes; IC_{50} units in μM.[8] c. Percent reduction in kidney fungal burden[13] following an oral dose of 50 mg/kg of **2a-c, 2e**. All values were significantly different than vehicle-treated controls ($P < 0.01$), except for **2c**. d. Oral dose of 10 mg/kg. e. Oral dose of 20 mg/kg.

While other variably-substituted phenyl and pyridine analogues at the carbinol center were prepared and tested (e.g., the racemic 2-F-4-Cl-Ph analogue of **7c** had a *C. albicans* MIC = 0.016 μg/mL), the 2,4-difluorophenyl moiety consistently furnished superior potency. Small meta/para-disubstituents at the R position of **2** provided inferior potency compared to **7c** as well (e.g., the racemic 3,4-diF-Ph analogue of **7c** had a *C. albicans* MIC = 0.016 μg/mL). Ether **7d** exhibited marked affinity for *C. albicans*-CYP51 with $K_d \leq$ 0.039 μM (>2200-fold yeast selectivity v. human CYP51, manuscript in preparation). The *in vitro* safety profiles of **7c** and **7d** (CYP3A4 IC_{50} = 79 μM and 65 μM, respectively; Table 3) were superior to marketed azoles such as itraconazole (CYP3A4 IC_{50} = 0.07 μM). Additionally, ether **7d** exhibited weak activity for CYP2C9 (IC_{50} = 99 μM) and CYP2C19 (IC_{50} = 72 μM). These highly-selective CYP51 inhibitors (e.g., **7a**-**d**) are, in general, extremely resistant to liver CYP metabolism. For example, ether **7d** was recovered in >99% yield following 2 hour incubations in animal and human liver microsome preparations, and has a long oral half-life in humans (>> 24 hours; manuscript in preparation) desired for an infectious disease therapeutic. Orally-administered **7d** was highly-active in *in vivo* models of yeast and dermatophyte infection. In a mouse invasive candidiasis model, a single oral dose of 10 mg/kg **7d** reduced kidney fungal burden by 99%.[14] In a guinea pig dermatophytosis model (*T. mentagrophytes*),[18] **7d** provided 86% mycological efficacy following 9 daily, oral doses of 10 mg/kg.

Table 3. Antifungal Activity of Difluoromethyl-Pyridyl-Benzenes.



7

Compound	R	C. albicans MIC[a]	T. rubrum MIC[a]	CYP3A4 IC_{50}[b]	Selectivity Index[c]
7a	Cl	≤0.001	0.004	36	9,000
7b	CF_3	≤0.001	0.002	54	27,000
7c	OCF_3	≤0.001	≤0.001	79	>79,000
7d	OCH_2CF_3	≤0.001	≤0.001	65	>65,000
Itraconazole	-	0.016	0.062	0.07	1.1

a. Minimum concentration that achieved 50% inhibition of fungal growth; MIC units in μg/mL.[5] b. Inhibition of CYP3A4 measured in microsomes obtained from pooled human hepatocytes, IC_{50} units in μM.[8] c. *In vitro* selectivity calculated as CYP3A4 IC_{50} / *T. rubrum* MIC.

In summary, we have described the design and synthesis of novel, potent, and highly selective, 1-tetrazole based fungal CYP51 inhibitors.[19] A representative of this chemical series, VT-1161 (**7d**), has advanced into human clinical trials and exhibited linear pharmacokinetics coupled with excellent tolerability in an oral, multiple-ascending dose, Phase 1 study.[4] The MBG-based design process described herein is broadly applicable to the discovery of new, potent and selective inhibitors of the metalloenzyme target class. The same approach has produced highly-selective CYP17 inhibitors as potential treatments for prostate cancer[20,21] in addition to the fungal CYP51 inhibitors described herein.[4]

Acknowledgements. We thank Professors Thomas O'Halloran and Holden Thorp for their insightful guidance, and Mr. Sammy R. Shaver for assistance with the homology model.

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5. Minimum inhibitory concentrations (MIC) for standard ATCC isolates were determined under the CLSI guidelines M27-A3 (for yeasts such as *C. albicans*) and M38-A2 (for dermatophytes such as *T. rubrum*) and were read at 50% inhibition of fungal growth (Ricerca Biosciences, LLC).

6. Compounds were synthesized as anti-racemates. For the synthetic method, see Dickinson, R.P.; Bell, A.S.; Hitchcock, C.A.; Narayanaswami, S.; Ray, S.J.; Richardson, K.; Troke, P.F. *Bioorg. Med. Chem. Lett*. **1996**, *6*(*16*), 2031-2036.
7. *In silico* enthalpies were also determined which generally aligned with *in silico* pKas. Briefly, using Spartan 2006 program package, Me-metal-binding group (Me-MBG) ligands were minimized using the MMFF-94 force field and optimized with the semi-empirical PM3 method. The CYP51 Fe-porphyrin construct[1] was minimized (MMFF-94) and then optimized using the PM3 semi-empirical method to obtain an unligated structure. Me-MBGs were introduced and the energy was determined by a single point calculation. The Fe-porphyrin and Me-MBG were complexed with only the Me-MBG ligand free to move during optimization. Next, the Me-MBG was submitted for geometry optimization and enthalpy measured. For the 1-imidazole and the 1-(1,2,4-triazole), the enthalpy values were determined to be -18.9 and -16.5 kcal/mol, respectively.
8. IC_{50} values for CYP3A4 enzyme were determined in human hepatocyte microsomes using 150 μM testosterone as substrate (a concentration equal to the K_m determined in the same lab). Reactions were analyzed for product using HPLC/MS/MS methods and IC_{50} values (in μM) were determined by fitting a 4-parameter logistical fit to the dose response data (OpAns, LLC).
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11. Homology modeling was conducting using Molecular Operating Environment (MOE; Chemical Computing Group, 1010 Sherbrooke St. W, Suite 910 Montreal, Quebec, Canada H3A 2R7). A series of homology models was constructed using the sequence information for *Candida albicans* and CYP51 crystal structures deposited in the Protein Data Bank. The model chosen for evaluation was developed from a *Trypanosoma cruzi* crystal structure with posaconazole bound (3K1O; Lepesheva, G.I.; Hargrove, T.Y.; Anderson, S.; Kleshchenko, Y.; Furtak, V.; Wawrzak, Z.; Villalta, F.; Waterman, M.R. *J. Biol. Chem.* **2010**, *285(33)*, 25582–25590). The homology model was then created using Amber99 as the protein modeling forcefield and PFROSST as the forcefield for energy minimizations, with a final RMS gradient of 0.5.
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13. Female CD-1 mice (7 weeks, 18-25 g) were made neutropenic with IP injections of cyclophosphamide (150 mg/kg) at 4 and 1 days before inoculation with *C. albicans* R303 via the tail vein. Compounds were administered orally 2 h after infection on day 1. Kidneys were collected from treated mice on day 2, PBS added, and then homogenized. Colony-forming units (CFU)/kidney were determined from number of colonies detected on SDA plates from serial dilutions of the homogenates. Fluconazole was used as a positive control and reduced kidney fungal burden 99% when orally dosed at 10 mg/kg (Ricerca Biosciences, LLC).
14. Hoekstra, W.J.; Schotzinger, R.J. PCT Int. Appl. **2012**, WO2012177725.
15. Compound **2f** was synthesized from **3** as follows[16]**:** To a suspension of Cu powder (2.68 g, 42.2 mmol) in DMSO (35 mL) was added ethyl bromodifluoroacetate (2.70 mL, 21.10 mmol), and the mixture was stirred for 1 h at RT. 2,5-Dibromopyridine (2.50 g, 10.55 mmol) was then added and stirring continued for 15 h at RT. The reaction was quenched with aqueous NH_4Cl and extracted with DCM (3 x 25 mL). The combined organic layers were washed with water and brine, dried over anhydrous (Na_2SO_4), and concentrated to afford a crude product mixture which upon column purification (EtOAc/hexane) afforded the ethyl ester intermediate (2.40 g, 8.57 mmol, 81%) as a pale yellow oil. To a stirred solution of 2,4-difluoro-bromobenzene (1.65 g, 8.57 mmol) in diethyl ether (10 mL) was added *n*-BuLi (3.70 mL, 8.57 mmol) at -70 ^oC followed by addition of ester (2.40 g, 8.57 mmol) in diethyl ether (5 mL) after 15 minutes. The reaction mixture was stirred for 1 h at -70 ^oC and warmed to RT and stirred 2 h. The reaction was quenched with aqueous NH_4Cl solution and extracted with EtOAc (3 x 20 mL). The combined organic layers were washed with water, washed with brine, dried (Na_2SO_4), and concentrated. The crude compound was purified by column chromatography to afford ketone **4** (1.30 g, 3.73 mmol, 43%) as a yellow liquid. To a stirred solution of ketone **4** (1.30 g, 3.73 mmol) in diethyl ether (300 mL) was added freshly prepared diazomethane at 0 ^oC followed by warming to RT. The reaction mixture was stirred for 2 h. The volatiles were removed to afford a crude product mixture which upon column

chromatography (EtOAc/hexane) afforded oxirane **5** (800 mg, 2.20 mmol, 59%) as light yellow solid. To a stirred solution of oxirane **5** (0.25 g, 0.69 mmol) in THF (20 mL) and water (7 mL) were added 4-(trifluoromethyl)phenylboronic acid (0.10 g, 0.55 mmol), Na_2CO_3 (0.16 g, 1.55 mmol) and $Pd(dppf)_2Cl_2$ (0.14 g, 0.17 mmol) at RT under argon. After purge with argon for a period of 30 min, the reaction mixture was heated to 75°C and stirring was continued for 4 h. The reaction mixture was cooled to RT and filtered through celite. The filtrate was concentrated to a residue and re-dissolved in EtOAc (30 mL). The organic layer was washed with water and brine, dried (Na_2SO_4), and concentrated. The crude compound was purified by column chromatography to afford **6f** (0.21 g, 0.49 mmol, 71%) as a white solid. To a stirred solution of **6f** (0.42 g, 0.98 mmol) in DMF (10 mL) was added K_2CO_3 (67 mg, 0.49 mmol) followed by 1*H*-tetrazole (68 mg, 0.98 mmol) at RT under argon. The reaction mixture was stirred for 5 h at 80 °C. The volatiles were removed *in vacuo* and the obtained residue was dissolved in EtOAc (30 mL). The organic layer was washed with water and brine, dried (Na_2SO_4), and concentrated. The crude compound was purified by column chromatography to afford **2f** (0.14 g, 0.28 mmol, 29 %) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$)**:** δ 8.76 (s, 1 H), 8.73 (s, 1 H), 8.01 (dd, *J* = 8.0, 2.0 Hz, 1 H), 7.78 (d, *J* = 8.5 Hz, 2 H), 7.72-7.67 (m, 3 H), 7.49 (s, 1 H), 7.44-7.37 (m, 1 H), 6.81-6.76 (m, 1 H), 6.71-6.65 (m, 1 H), 5.57 (d, *J* = 14.0 Hz, 1 H), 5.19 (d, *J* = 14.0 Hz, 1 H). HPLC: 97.3%. Mass: m/z 498 [M^++1]. (*R*)-(+)-Enantiomers (**7a**-**7d**) were isolated from racemates using chiral chromatography.

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17. A single-crystal x-ray diffraction method was employed to assign the (*R*)-absolute configuration of both **7c** and **7d** [all opposite, (-)-enantiomers of **7a**-**7d** exhibited immeasurable MICs].
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Graphical Abstract

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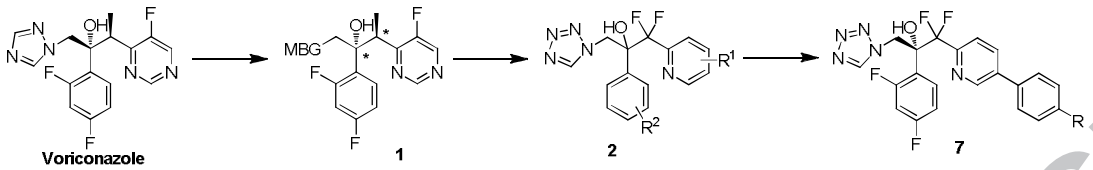
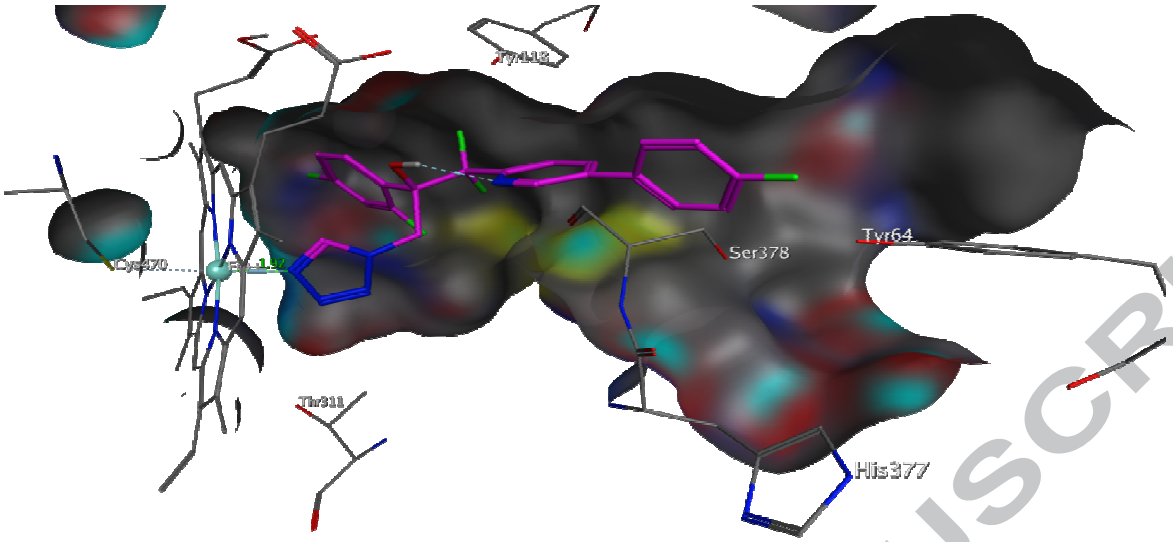


(MBG = metal-binding group)



Schemes