DOI: 10.1002/cmdc.201000040

Synthesis and in vitro and in vivo Antifungal Activity of the Hydroxy Metabolites of Saperconazole

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Herein we describe the scalable diastereoselective and enantioselective syntheses of eight enantiomers of hydroxy metabolites of saperconazole. The in vitro antifungal activity of the eight stereoisomers (compounds **1**–**8**) was compared against a broad panel of *Candida* spp. ($n=93$), *Aspergillus* spp. ($n=10$), *Cryptococcus* spp. ($n=19$), and dermatophytes ($n=27$). The four 2*S* isomers **1**–**4** of the new agent were generally slightly more active than the four 2*R* isomers **5**–**8**. All eight isomers were tested in a model of experimental *A. fumigatus* infection in guinea pigs by intravenous inoculation of the fungal conidia. Treatment doses were 1.25 mg kg^{-1} and 2.5 mg kg^{-1} per day. Infection severity was measured in terms of mean survival time (MST) after infection and mean tissue burdens in brain, liver, spleen, and kidney at postmortem examination. Among the eight isomers, the 2*S* diastereomers **1**–**4** showed a generally higher level of activity than the 2*R* diastereomers **5**–**8**, revealing compounds **1** and **4** as the most potent overall in eradicating tissue burden and MST. Compared with reference compounds itraconazole and saperconazole, the hydroxy isomers **1**–**8** are less potent inhibitors of the growth of *A. fumigatus* in vitro and of ergosterol biosynthesis in both *A. fumigatus* and *C. albicans*.

Introduction

Systemic fungal infections (SFI) are life-threatening conditions that most commonly affect patients with decreased immunity, which often results from therapeutic interventions to treat malignant diseases.[1–10] The number of various fungi that have been involved in SFI is large and still growing. Despite many cases of invasive candidiasis and aspergillosis, there has been an increased incidence of infections due to other molds such as *Scedosporium apiospermum*, *Fusarium* spp., and *Zygomycetes*, *Rhizopus* and *Mucor* spp.[9–11] Therefore, effective therapeutic agents to treat all these infections must have a very broad spectrum of activity. Over the past few decades itraconazole,[12–14] fluconazole,[15] ketoconazole,[16] and intravenous or liposomal amphotericin B[17] have been used to treat SFI, and all these agents have their limitations with regard to spectrum, safety, or ease of administration.

More recently, a third generation of azoles has been investigated and introduced to the market (Figure 1), improving the treatment options in intensive care units. Voriconazole (Vfend™)[18,19] and posaconazole (Noxofil™)[20–23] have shown much improvement in the treatment of life-threatening invasive SFI such as candidiasis, aspergillosis, and infections due to *Fusarium* spp. at clinically relevant dosages. Moreover, posaconazole shows efficacy against infections caused by emerging species of *Zygomycota*.[24] Echinocandins such as anidulafungin, caspofungin, and micafungin, which are noncompetitive inhibitors of 1,3-β-glucan synthesis in fungal cell walls, display high efficacy against *Candida* and *Aspergillus* spp., but have no activity against *Cryptococcus*, *Fusarium*, or *Zygomycetes* spp.[25,26] Of all antimycotic agents, azoles still represent a unique class of compounds that display the broadest antifungal spectrum via inhibition of 14-α-demethylase, an enzyme essential for ergosterol biosynthesis in fungi.[27]

Taking itraconazole as a starting point for further structural optimization, we have from previous work that replacement of the 2,4-dichlorophenyl moiety with a 2,4-difluorophenyl moiety (see saperconazole, Figure 1) results in significant improvement of potency and spectrum, especially against *Aspergillus* spp.[28,29] Moreover, strategies to enhance the aqueous solubility of poorly soluble drugs often rely on prodrug design.[30] The prodrug design we envisaged started from the identification of hydroxyitraconazole as a major antifungal-

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.201000040: Individual dose–response curves for the in vitro antifungal activity of compounds **1**–**8** against a broad range of yeasts and moulds.

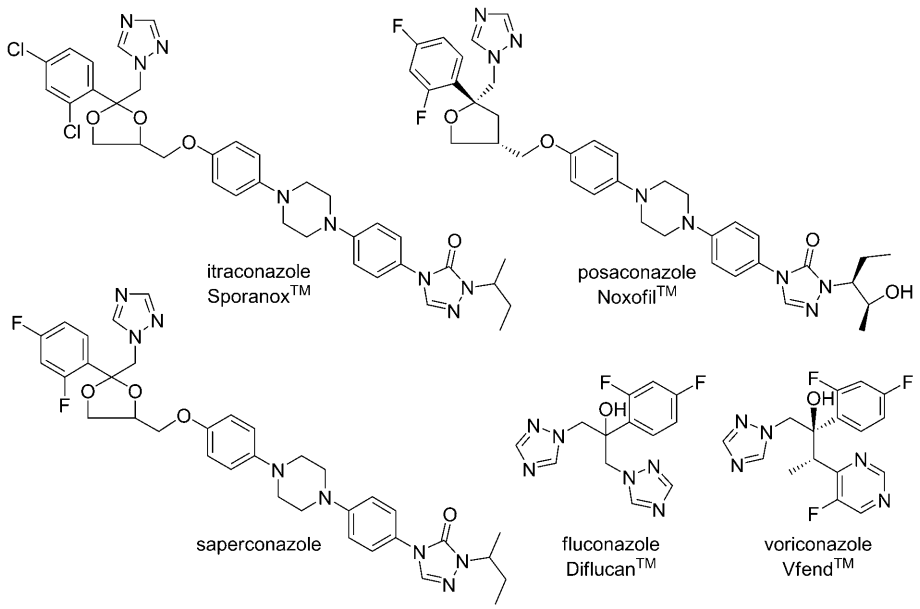


Figure 1. Antifungal azoles for the treatment of systemic fungal infections (SFI).

active metabolite of itraconazole.[31–35] A similar metabolite is generated in vivo from saperconazole. The hydroxy metabolites **1**–**8** (Figure 2) have four chiral centers each, resulting in 16 possible enantiomers. Fortunately the *trans*-dioxolane isomers[36,37] proved to be far less active than the *cis* isomers, which left us with only eight isomers to be evaluated for their antifungal spectrum and potency.

The antifungal activity of racemic hydroxyitraconazole[33] has been published, but there are no reports that describe the activity of the individual isomers, or that of hydroxysaperconazole metabolites. Moreover, posaconazole is a single enantiomeric hydroxy metabolite of the investigational drug Sch-51048.[38,39] The enantioselective synthesis and antifungal activity of the various enantiomers have been reviewed.[40] In addition, others have described an enantioselective synthesis of (2*R*,4*S*,2′*S*,3′*R*)-hydroxyitraconazole without discussing its antifungal spectrum.[41]

Herein we describe the diastereoselective and enantioselective synthesis of eight hydroxy metabolites of saperconazole (compounds **1**–**8**; Figure 2), their antifungal spectrum against a broad panel of *Candida* spp. ($n=93$), *Cryptococcus* spp. ($n=19$), *Aspergillus* spp. ($n=10$), and dermatophytes ($n=27$). We also discuss the results of a treatment of an experimentally disseminated *A. fumigatus*-induced infection in guinea pigs with the various hydroxy isomers **1**–**8**, and the mode of action of these compounds against *A. fumigatus* and *C. albicans*.

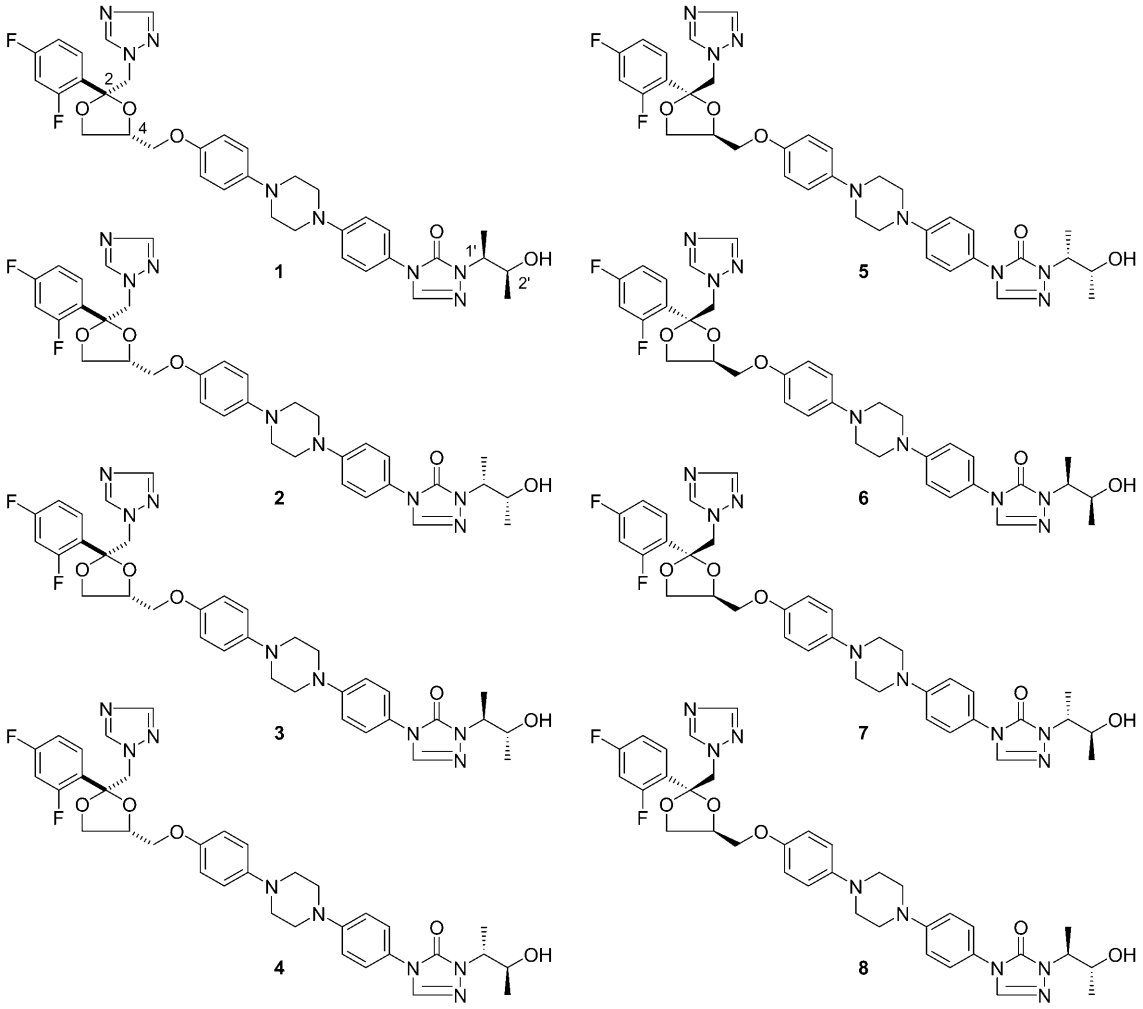
Results and Discussion

Chemistry

A convergent synthesis was used to prepare target compounds **1**–**8** (Figure 2). The eight diastereomers **1**–**8** were obtained in moderate to good yields starting from the optically pure *cis*-dioxolanes **17a** (2*S*) and **18** (2*R*), which were previously described,[42] and the phenols **12a**–**d** in dry DMF in the presence of sodium hydroxide pellets (see Scheme 3 in the Experimental Section below). An improved yield (91%) and a scalable method for compound **1** was worked out by changing the solvent to DMA and by optimizing the workup method without the need for chromatographic purification of target product **1**, by starting from dioxolane **17b** and phenol **12a**.

The key step for this synthesis, however, was the diastereoselective and enantioselective syntheses of compounds **12a**–**d**. The diastereoselective synthesis started from a selective reduction of ketone **11**, easily obtainable from alkylation of triazolone **9** and O-demethylation (Scheme 1). According to Cram's rule,[43–45] chelating conditions would result in a selective formation of the *SR*/*RS* **12c**,**d** isomer, and nonchelating conditions would give more *SS*/*RR* **12a**,**b**. Ideally this would require nonpolar solvents such as ethers; however, the poor solubility of ketone **11** in THF and Et_2O required a co-solvent such as DMF. Thus reduction of ketone **11** with K-selectride in a mixture of THF/DMF (1.5:5) at $-10\,^{\circ}C$ gave a 90:10 diastereomeric mixture of **12a**,**b**/**12c**,**d**, which was purified by chiral HPLC to give **12a** and **12b** in 31 and 34% yields, respectively. Despite chelating conditions using $Zn(BH_4)_2$ in a Et_2O/DMF (1.6:1) mixture at $0\,^{\circ}C$, the erythro diastereomeric selectivity was poor **12a**,**b**/**12c**,**d** (70:30). The fact that we had to use DMF as a co-solvent, which probably hampered the chelating properties of the zinc ion, explains this disappointing selectivity. Chiral separation of the diastereomeric mixture gave **12c** and **12d** in 14 and 13% yields, respectively (Scheme 1).

The need for a scalable method that would give access to all four enantiomers prompted us to look into enantioselective syntheses for alcohols **12a**–**d** (Scheme 2). Nucleophilic ring opening of *meso*-(4*R*,5*R*)-4,5-dimethyl-1,2,3-dioxathiolane-2,2-dioxide with triazolone **9** gave enantiomer **13c** in good yield (84%). This method, although independently discovered, was previously reported for the synthesis of itraconazole hydroxy metabolites.[41] Demethylation using a 1:1 mixture of 48% aqueous HBr in acetic acid saturated with HBr gas at $80\,^{\circ}C$ gave **12c** in moderate yield (72%). The optical rotation of the purified and re-crystallized sample of **12c** corresponds very well to the analytical data obtained for **12c** after diastereoselective reduction of **11** and chiral separation: for example, $[\alpha]_{20}^{D}=-7.66$ ($c=0.430$ *w*/*v*% in DMF; from Scheme 2) and $[\alpha]_{20}^{D}=-7.07$ ($c=0.976$ *w*/*v*% in DMF; from Scheme 1). Inversion of the alcohol of **13c** under Mitsunobu conditions gave



Compound	Stereochemistry	Starting Material	Yield [%][a]	mp [°C][b]	$[\alpha]_D^{20}$ [c]
1	2*S*, 4*R*, 1'*S*, 2'*S*	**17b**, **12a**	56 (91)*	189.4	–20.41
2	2*S*, 4*R*, 1'*R*, 2'*R*	**17a**, **12b**	70	ND	–7.71
3	2*S*, 4*R*, 1'*R*, 2'*S*	**17b**, **12c**	59	210.1	–17.79
4	2*S*, 4*R*, 1'*S*, 2'*R*	**17b**, **12d**	79	199.9	–9.36
5	2*R*, 4*S*, 1'*R*, 2'*R*	**18**, **12b**	64	190.3	19.49
6	2*R*, 4*S*, 1'*S*, 2'*S*	**18**, **12a**	57	ND	7.13
7	2*R*, 4*S*, 1'*S*, 2'*R*	**18**, **12d**	84	211.1	17.79
8	2*R*, 4*S*, 1'*R*, 2'*S*	**18**, **12c**	88	199.3	9.22

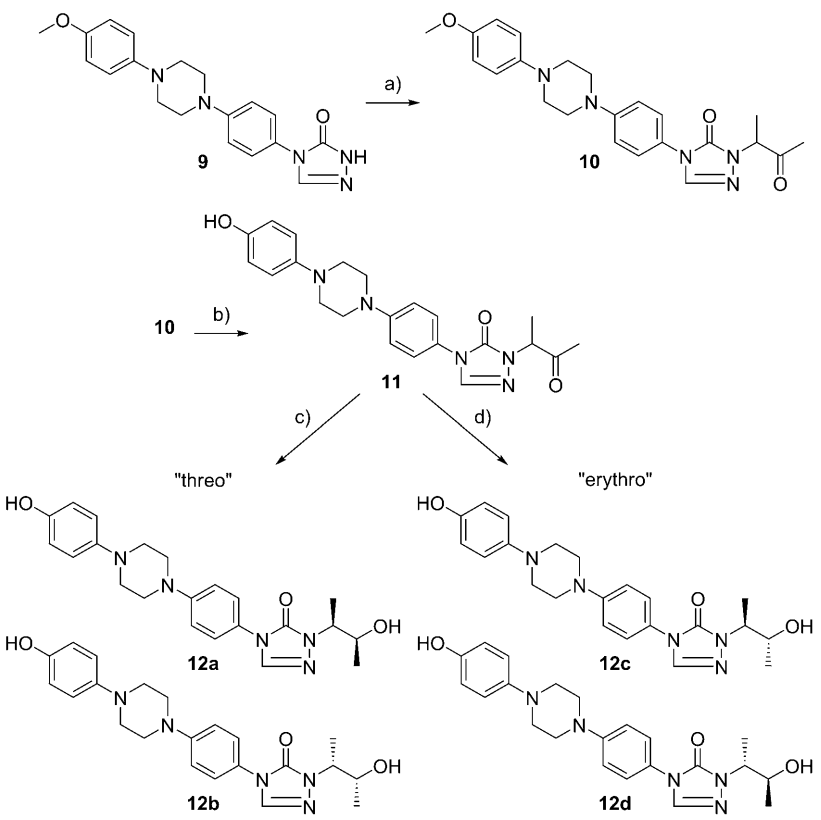
[a] Yields not optimized; *optimized scale-up yield. [b] ND: not determined. [c] All optical rotation values were determined in DMF solution; see Experimental Section for details.

Figure 2. The eight hydroxy metabolites of saperconazole and a summary of their syntheses.

13a in 71% yield, and subsequent demethylation with saturated hydrobromic acid in acetic acid gave **12a** in 63% yield. The optical rotation of **12a**, obtained via this enantioselective method, was $[\alpha]_{20}^{D} = -10.43$ ($c = 0.987$ *w*/*v*% in DMF) and corresponded very well to the previously obtained results for **12a** by selective reduction and chiral chromatography (Scheme 1) $[\alpha]_{20}^{D} = -10.81$ ($c = 1.008$ *w*/*v*% in DMF). The moderate yield of the Mitsunobu reaction of **13c** was caused mainly by elimination of the 4-nitrobenzoate to give compound **16**. The demethylation reaction could be further optimized during scale-up, as formation of acetate **15** was observed as the main side product, which could be easily hydrolyzed upon treatment with aqueous sodium hydroxide.

Biological activity

The in vitro activity of the various antifungal triazoles **1**–**8** was tested against several pathogenic fungi. The test strains included 20 isolates each of *Candida albicans*, *C. glabrata*, and *C. krusei*, 12 each of *C. lusitaniae* and *C. parapsilosis*, 9 of *C. tropicalis*, 19 of *Cr. neoformans*, 10 isolates of *A. fumigatus*, and 9 isolates each of *Microsporum canis*, *Trichophyton mentagrophytes*, and *T. rubrum*. The activity of the reference compound saperconazole and the test results for compounds **1**–**4** (2*S* isomers) and **5**–**8** (2*R* isomers) are summarized in Table 1. Against *A. fumigatus* and dermatophyte species, saperconazole showed a more potent in vitro activity than itraconazole; the opposite situation applied for *Candida* spp.[28,29]



Scheme 1. Reagents and conditions: a) 3-chloro-2-butanone, K_2CO_3, DMF/toluene (1:1), 110 °C, 16 h, 97%; b) $HBr_{(aq)}$ (48%), $NaHSO_3$, reflux, 5 h, 84%; c) K-selectride (1 M), THF/DMF (1.5:5), −10 °C→RT, 2 h, (**12a,b**/**12c,d** 90:10) Chiralpac AD: 31% **12a**, 34% **12b**; d) $Zn(BH_4)_2$, Et_2O/DMF (1.6:1), 0 °C→RT, 2 h, (**12a,b**/**12c,d** 70:30) Chiralpac AD: 14% **12c**, 13% **12d**.

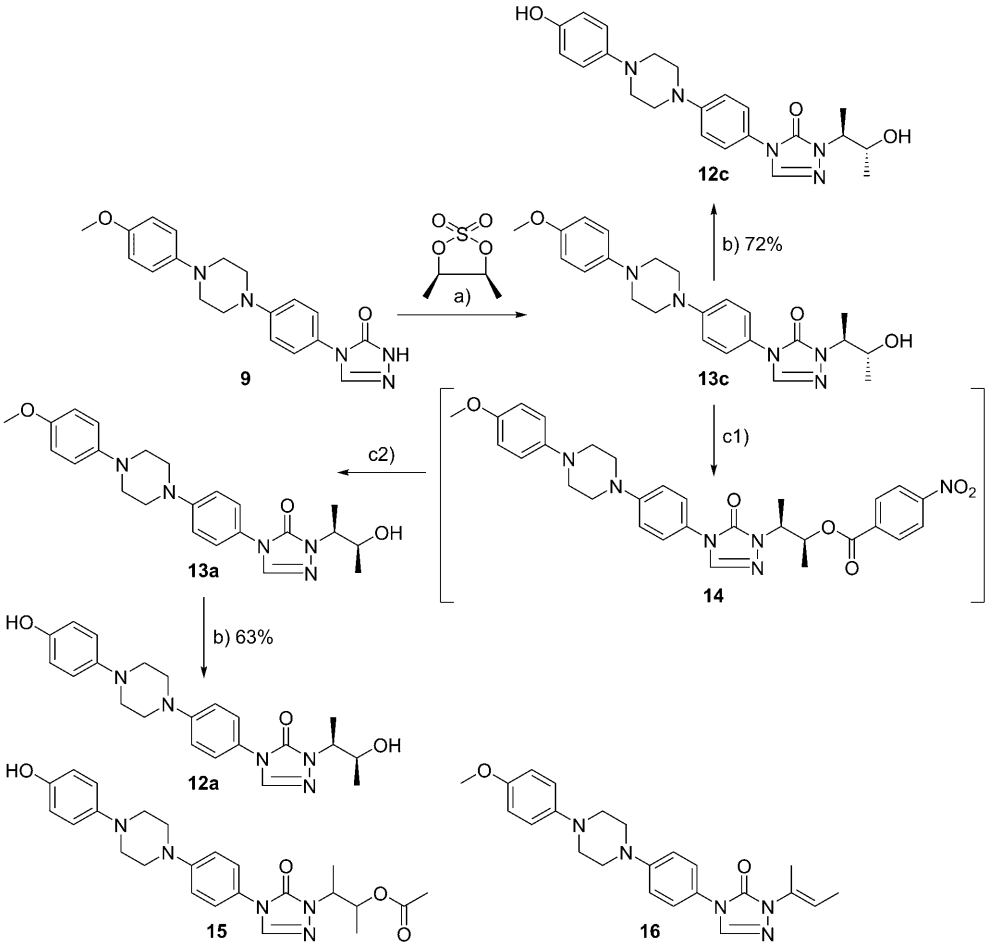
The 2*S* isomers **1**–**4** showed broadly equivalent inhibitory potency against most of the fungal species tested (Table 1), although compounds **3** and **4** were less potent than compounds **1** and **2** against *C. tropicalis* isolates. Compounds **1** and **2** were conspicuously more potent inhibitors than the two other isomers **3** and **4**, and saperconazole against *C. glabrata*.

Among the 2*R* isomers of the new triazoles **5**–**8** no major differences in potency were apparent against *A. fumigatus* and *Cr. neoformans* (Table 1). Compounds **5** and **6** were more potent inhibitors of *C. glabrata* than the two other 2*R* isomers **7** and **8** and saperconazole racemate. These two compounds were also the most potent inhibitors of the dermatophyte isolates tested (Table 1). Further interspecies variations in susceptibility were apparent at the level of 2*S* isomers **1**–**4** versus 2*R* isomers **5**–**8**. Against *Candida* spp. and *A. fumigatus* (Table 1) the 2*S* isomers showed a slightly higher level of activity than the 2*R* isomers in terms of IC_{50} values. However, against *Cr. neoformans* the 2*R* isomers showed a trend toward lower IC_{50} and IC_{90} values than the 2*S* isomers. Compounds **1** and **2** both showed a good in vitro activity against *Candida* spp. and *A. fumigatus*, although they were less active than saperconazole against *Cr. neoformans* and dermatophyte fungi.

Among the eight stereoisomers of the novel triazole antifungal agents studied in vitro, there was an overall modest trend toward higher activity among the 2*S* diastereomers **1**–**4** than the 2*R* diastereomers **5**–**8**, but within each species tested, differences in activity of the eight compounds were minor.

To evaluate possible activity differentials in vivo, a disseminated aspergillosis model in guinea pigs was chosen as a suitable test system, as such infections are not easy to treat and are therefore likely to reveal clearer differences in activity among the test compounds **1**–**8**. The test compounds were formulated in 20% hydroxypropy-11-β-cyclodextrin and were administered into guinea pigs intravenously by a permanently implanted catheter connected to an infusion pump via a proprietary swivel apparatus devised to allow the animal full mobility within the cage. The catheter was kept patent with a continuous flush of physiological saline when no treatment was administered. The activities of the placebo solution (20% hydroxypropy-11-β-cyclodextrin) and the test compounds **1**–**8** were compared those of itraconazole and saperconazole. To minimize the number of animals involved, experimentation to evaluate the method was held to a minimum (the model without the indwelling intravenous catheters has been described before[29]). For similar reasons only two doses of the test agents **1**–**8** and itraconazole and saperconazole, 1.25 and 2.5 mg kg^{-1} per day, were found necessary to evaluate their activities (Tables 2 and 3). These doses were selected on the basis of previous experience obtained with itraconazole and saperconazole as reference substances.

The indwelling catheter as a means of intravenous administration of test agents was the only difference from previous work with this model;[29] nevertheless, the evaluation of stereoisomers of antifungal agents on the basis of the relatively few results presented in this report requires a realistic appreciation of the limitations of the data. The highly complex nature of the surgical preparation for catheter insertion, the specially designed apparatus, and the requirement for a continuous infusion pump for each animal tested precluded repetition of the experiments with greater numbers of animals. Despite this caveat, the experiments described did show a number of consistent features that suggest superior efficacy among some of the isomers tested. The infection parameters in placebo-treated guinea pigs showed that a reasonably consistent degree of infection was achieved in terms of mean survival time and in tissue burdens of *A. fumigatus* measured in the liver and



Scheme 2. Reagents and conditions: a) 1. *t*BuOK, THF/DMA, 60 °C, 2 h, 2. H_2SO_4, 60 °C, 16 h, 84%; b) 1. $HBr_{(aq)}$ (48%), CH_3COOH (HBr-satd.), 80 °C, 24 h, 2. NaOH; c1) PPh_3, *p*-$NO_2(C_6H_4)COOH$, THF/DMA, DEAD, RT→50 °C, 2.5 h; c2) NaOH (1 N), 50 °C, 71%.

spleen (see the Experimental Section below and Tables 2 and 3).

From these data the superior anti-*A. fumigatus* potency of saperconazole over itraconazole was apparent in the data for mean survival times and CFU g^{-1} in the organs examined.[28,29] The data for itraconazole at both doses showed no efficacy at all in terms of prolongation of survival of the infected animals over controls and only modest decreases in the fungal tissue burdens. Neither compound was particularly effective in eliminating *A. fumigatus* from the liver, spleen, or kidney of infected animals, however a higher proportion of fungus-negative organs was observed in saperconazole-treated animals as compared with itraconazole-treated animals (Table 2 and Table 3).

Among the test compounds **1**–**8**, the activity of the 2*S* compounds **1**–**4** in vivo was once again generally superior to that of the 2*R* compounds **5**–**8** as previously noted in vitro (Table 1). However, differences in antifungal efficacy under the test conditions used were evident within these groups of isomers (Tables 2 and 3). Among the 2*S* series **1**–**4**, isomer **2** was markedly less active than the other three by all parameters measured. Compound **1** was the most active of the four 2*S* isomers in terms of all parameters measured and was clearly more potent than saperconazole. Compounds **3** and **4** showed an in vivo efficacy profile that similar to that of saperconazole. Among the 2*R* series **5**–**8**, isomer **5** was notably the least effective and compound **6** the most potent, showing a prolonged mean survival time (MST) to eight days at 2.5 mg kg^{-1} and a tissue burden decrease similar to or slightly better than that of saperconazole. Compounds **1** and **4** prolonged the MST to seven or more days at at least one of the concentrations tested, and both eradicated *A. fumigatus* by 70% or more in liver, brain, kidney, and/or spleen samples at at least one of the concentrations tested (Tables 2 and 3).

The mode of action of azole antifungal agents has been well established as inhibition of the P450 14-α-demethylase (CYP51, *Erg11*) of the ergosterol biosynthetic pathway.[46,47] Itraconazole and saperconazole have been reported as potent inhibitors of ergosterol biosynthesis in *A. fumigatus* and *C. albicans*.[14,46,48,49] In order to identify the mode of action of the novel triazoles, a study was designed to examine the interaction of compounds **1**–**8** with ergosterol biosynthesis in fungal cells. The results obtained with these compounds were compared with itraconazole and saperconazole as internal references in the same experiment. The effects of the compounds tested on the growth of *A. fumigatus*, and the incorporation of [^{14}C]acetate into ergosterol by *C. albicans* and *A. fumigatus* were measured as de-

Table 1. Activity of saperconazole and eight stereoisomers against various fungal pathogens in vitro.

Test species	*n*	Compound	MIC data [μg mL^{-1}] Range	IC_{50}[a]	IC_{90}[a]
All *Candida* spp.	93	saperconazole	≤0.05–>25	0.10	>25
		1	≤0.05–>25	0.20	>25
		2	≤0.05–>25	0.40	>25
		3	≤0.05–>25	0.20	>25
		4	≤0.05–>25	0.40	>25
		5	≤0.05–>25	0.80	>25
		6	≤0.05–>25	0.40	>25
		7	≤0.05–>25	0.80	>25
		8	≤0.05–>25	0.40	>25
C. glabrata	20	saperconazole	0.8–>25	>25	>25
		1	0.4–>25	6.3	>25
		2	0.4–>25	25	>25
		3	0.4–>25	>25	>25
		4	0.8–>25	>25	>25
		5	0.4–>25	6.3	>25
		6	0.4–>25	>25	>25
		7	3.2–>25	>25	>25
		8	0.8–>25	>25	>25
C. tropicalis	9	saperconazole	0.05–>25	13	>25
		1	≤0.05–>25	0.40	>25
		2	≤0.05–>25	0.20	>25
		3	≤0.05–>25	6.3	>25
		4	≤0.05–>25	13	>25
		5	0.05–>25	13	>25
		6	0.05–>25	0.80	>25
		7	0.05–>25	0.80	>25
		8	0.05–>25	1.6	>25
Cr. neoformans	19	saperconazole	≤0.05–0.40	0.20	0.40
		1	≤0.05–0.80	0.20	0.80
		2	≤0.05–0.80	0.20	0.80
		3	≤0.05–1.6	0.20	1.60
		4	≤0.05–0.80	0.20	0.80
		5	≤0.05–0.40	0.20	0.40
		6	0.05–0.80	0.10	0.80
		7	≤0.05–0.80	0.20	0.40
		8	≤0.05–0.80	0.20	0.40
A. fumigatus	10	saperconazole	≤0.05–0.20	0.10	0.20
		1	0.05–0.20	0.10	0.20
		2	≤0.05–0.40	0.20	0.40
		3	≤0.05–0.20	0.10	0.20
		4	≤0.05–0.20	0.10	0.20
		5	0.10–0.80	0.40	0.80
		6	≤0.05–0.20	0.40	0.80
		7	≤0.05–0.05	0.20	0.40
		8	≤0.05–0.40	0.20	0.40
All dermatophytes	27	saperconazole	≤0.05–0.80	0.05	0.05
		1	≤0.05–0.40	≤0.05	0.10
		2	≤0.05–0.40	≤0.05	0.10
		3	≤0.05–0.10	≤0.05	0.05
		4	≤0.05–0.40	≤0.05	0.10
		5	≤0.05–0.40	0.05	0.20
		6	≤0.05–0.40	0.10	0.10
		7	≤0.05–0.40	≤0.05	0.10
		8	≤0.05–0.10	≤0.05	0.10

[a] IC_{50} and IC_{90} values indicate the concentrations required to inhibit 50 and 90% of the test isolates, respectively.

scribed previously.[48] The effects of azole antifungal agents on the growth of *A. fumigatus*, listed in Table 4, confirm those obtained previously with itraconazole and saperconazole.[48,49] Both triazole derivatives are potent inhibitors of the growth of *A. fumigatus*. The hydroxy isomers **1**–**8** were generally 4- to 19-fold less active than the parent compound saperconazole. The most potent enantiomers were compounds **3**, **8**, **7**, **4**, and **1**. These results are based on 50% growth inhibition of a single *A. fumigatus* strain, whereas the results in Table 1 are based on growth inhibition of a panel of 10 *A. fumigatus* isolates, and therefore give a more comprehensive picture of breadth of spectrum, which is clinically more relevant. Despite this caveat, the experimental data listed in Table 4 do confirm a trend for higher potency with the 2*S* isomers **1**–**4** on growth inhibition in *A. fumigatus* relative to the 2*R* isomers **5**–**8**: for example, **1** and **3** were better than their 2*R* enantiomers **5** and **7**, and **2** and **4** were similar to **6** and **8**. The effects on ergosterol biosynthesis in *A. fumigatus* and *C. albicans* were measured after 16 h incubation of the fungi in the presence of itraconazole, saperconazole, or the hydroxy isomers **1**–**8**, according to experimental procedures previously described.[48] Inhibition (IC_{50} values) of ergosterol biosynthesis from [^{14}C]acetate was reached at 0.02 and 0.04 μM for saperconazole and itraconazole, respectively. The hydroxy isomer showed a consistently lower potency in this assay relative to saperconazole and itraconazole, with compounds **2** and **4** being the most potent. Surprisingly, compound **1** (IC_{50} = 0.20 μM) appeared to be the least potent of all 2*S* isomers. Head-to-head comparison of the enantiomeric pairs, however, again revealed a higher potency for the 2*S* diastereomers **1**–**4** in comparison with their 2*R* counterparts **5**–**8**. For the inhibition of ergosterol biosynthesis in *C. albicans*, however, we observed a consistently higher potency for the 2*R* isomers: for example, **1** < **5**, **2** < **6**, **3** ~ **7**, and **4** < **8**.

The weak trend for 2*S*-dioxolane isomers **1**–**4** to be more active than the 2*R* isomers **5**–**8** is in contrast with the much more significant difference between the 5*S* dystomer and 5*R* eutomer[50,51] of the tetrahydrofuran series related to posaconazole.[40] Given the strong similarity between the posaconazole series and compounds **1**–**8**, it is not surprising that compound **1**, one of the most potent among the series tested in vivo, has the same absolute configuration as posaconazole (Figure 1).

The design and synthesis of the hydroxy derivatives **1**–**8** were based on the assumption of a similar metabolism of saperconazole compared with itraconazole, namely hydroxy metabolite formation at the *sec*-butyl substituent of the triazolone group.[31,32] However, equal formation and distribution of the individual hydroxy metabolites of saperconazole in vivo is unlikely to be the case. A more detailed study on the metabolism of itraconazole was recently published.[35] It shows a stereoselective metabolism of itraconazole in vitro and in vivo. Only the 2*R* isomers of itraconazole were metabolized by CYP3A4 into their corresponding hydroxy metabolites. The 2*S* isomers, independent of the stereochemistry of the *sec*-butyl group, appeared to be very stable upon incubation with CYP3A4. Similar observations were also made in healthy volunteers after single and multiple doses, in which a higher C_{max} value was measured

Table 2. Results of treatment of experimental disseminated *A. fumigatus* infection in guinea pigs.[a]

Treatment	No. animals	Dose [mg kg^{-1}]	MST [days][b]	Geometric mean log CFU (g tissue)$^{-1}$ ± SD in:				Negative tissues[c]	
				Brain	Liver	Spleen	Kidney	No. [*n*]	[%]
placebo	24	–	5.2	1.9 ± 1.7	4.0 ± 1.1	4.3 ± 0.8	3.3 ± 1.5	14/96	15
itraconazole	5	1.25	4.8	2.5 + 2.3	4.3 ± 0.2	4.4 ± 0.4	2.1 ± 2.0	4/20	20
	5	2.5	5.2	0.0 ± 0.0	3.7 ± 0.5	4.0 ± 0.6	2.7 ± 1.8	6/20	30
saperconazole	5	1.25	7.4	1.7 ± 2.3	2.6 ± 1.5	2.9 ± 1.7	1.1 ± 1.5	8/20	40
	5	2.5	8.4	0.7 ± 1.4	2.2 ± 1.4	2.8 ± 1.7	0.0 ± 0.0	11/20	55
1	5	1.25	9.0	0.5 ± 1.1	0.8 ± 1.8	0.7 ± 1.6	0.5 ± 1.0	16/20	80
	5	2.5	7.8	0.0 ± 0.0	1.6 ± 2.2	1.5 ± 2.0	0.4 ± 1.0	15/20	75
2	5	1.25	5.2	2.3 ± 2.1	3.9 ± 0.9	4.4 ± 1.3	3.6 ± 0.6	2/20	10
	4	2.5	4.8	1.6 ± 1.8	1.8 ± 2.2	2.6 ± 2.0	0.7 ± 1.4	4/16	25
3	4	1.25	7	0.7 ± 1.4	1.7 ± 2.0	2.8 ± 2.1	1.0 ± 2.0	9/16	56
	4	2.5	8.2	1.3 ± 1.5	1.8 ± 2.0	2.5 ± 1.8	0.6 ± 1.1	8/16	50
4	4	1.25	6.8	0.7 ± 1.3	0.9 ± 1.7	1.6 ± 1.9	0.7 ± 1.4	11/16	69
	4	2.5	7	0.0 ± 0.0	1.4 ± 1.7	1.0 ± 2.0	0.6 ± 1.1	12/16	75
5	5	1.25	5	1.3 ± 1.8	3.2 ± 1.8	3.3 ± 1.9	2.1 ± 1.2	6/20	30
	5	2,5	6.8	1.8 ± 1.7	4.0 ± 1.1	4.4 ± 0.7	2.7 ± 1.5	3/20	15
6	4	1.25	6.5	2.3 ± 2.7	3.2 ± 0.5	2.7 ± 2.0	2.8 ± 0.7	3/16	19
	3	2.5	8	0.0 ± 0.0	1.3 ± 2.3	1.9 ± 1.8	0.8 ± 1.3	8/12	67
7	4	1.25	5	1.7 ± 2.0	4.0 ± 0.8	4.2 ± 0.8	1.6 ± 1.8	4/16	25
	4	2.5	7.8	0.0 ± 0.0	1.8 ± 2.2	3.0 ± 2.2	1.0 ± 2.0	10/16	63
8	5	1.25	6.4	0.7 ± 1.5	2.1 ± 1.9	1.9 ± 1.8	0.9 ± 1.2	11/20	55
	5	2.5	7.6	1.3 ± 1.7	1.7 ± 2.3	3.3 ± 1.2	1.7 ± 1.7	12/20	60

[a] Animals were treated with two intravenous infusions (formulation in 20% hydroxypropy1-1-β-cyclodextrin) daily for a total of 9.5 days. [b] Mean survival time. [c] No fungus isolated by culture of tissue homogenates.

Table 3. Numbers and percentages of individual tissues that were culture-negative for *A. fumigatus*.

Treatment	No. animals	Dose [mg kg^{-1}]	Brain		Liver		Spleen		Kidney	
			No.	[%]	No.	[%]	No.	[%]	No.	[%]
placebo	24	–	10	42	1	4	0	0	3	13
itraconazole	5	1.25	2	40	0	0	0	0	2	40
	5	2.5	5	100	0	0	0	0	1	20
saperconazole	5	1.25	3	60	1	20	1	20	3	60
	5	2.5	4	80	1	20	1	20	5	100
1	5	1.25	4	80	4	80	4	80	4	80
	5	2.5	5	100	3	60	3	60	4	80
2	5	1.25	2	40	0	0	0	0	0	0
	4	2.5	2	50	1	25	0	0	1	25
3	4	1.25	4	100	2	50	1	25	3	75
	4	2.5	3	75	3	75	1	25	3	75
4	4	1.25	3	75	3	75	2	50	3	75
	4	2.5	4	100	2	50	3	75	3	75
5	5	1.25	3	60	1	20	1	20	1	20
	5	2.5	2	40	0	0	0	0	1	20
6	4	1.25	2	50	0	0	1	25	0	0
	3	2.5	3	100	2	67	1	33	2	67
7	4	1.25	2	50	0	0	0	0	2	50
	4	2.5	4	100	2	50	1	25	3	75
8	5	1.25	4	80	2	40	2	40	3	60
	5	2.5	3	60	3	60	0	0	2	40

for the 2*S* isomers relative to the 2*R* isomers.[35] Although a systematic in vivo pharmacokinetic evaluation of the new azole compounds **1**–**8** has not been done, one may assume a similar trend for the in vivo pharmacokinetics of the hydroxy derivatives **1**–**8**. Better bioavailability for the 2*S* isomers **1**–**4** over the 2*R* isomers **5**–**8** could explain the higher potency of the 2*S* isomers over the 2*R* isomers in the experimental *A. fumigatus* infection in guinea pigs. Compounds **1** and **4** appeared to be the most potent overall in eradicating tissue burden and in prolonging MST.

Conclusions

In summary, the goal of this study was to identify the most active hydroxy metabolite of saperconazole in experimental fungal infection as a potential starting point for prodrug design. Scaleable diastereoselective and enantioselective syntheses of hydroxy metabolites of saperconazole were described. The activities of eight diastereomers (compounds **1**–**8**) of a novel antifungal agent were evaluated against a panel of fungal isolates tested in vitro. The four 2*S* diastereomers **1**–**4** of the new agent were generally slightly more active than the four 2*R* diastereomers **5**–**8**, although none of the isomers matched the activity of the reference compounds itraconazole and saperconazole against dermatophytes and *Cr. neoformans* in vitro. Compounds **1** and **2** were, by a small margin, the two best inhibitors of fungal growth among the eight isomers, showing efficacy similar to that of itraconazole against a panel of 93 *Candida* isolates representing six pathogenic species, and activity similar to that of saperconazole against 10 isolates of *A. fumigatus*.

Compared with itraconazole and saperconazole, the hydroxy isomers **1**–**8** are less potent inhibitors of the growth of *A. fumigatus* and of ergosterol biosynthesis in both *A. fumigatus* and *C. albicans*. Among the enantiomers, compound **4** is the most potent inhibitor of ergosterol biosynthesis in *A. fumigatus*, whereas compound **8** is the best inhibitor of ergosterol biosynthesis in *C. albicans*.

Table 4. Effects on the growth of *A. fumigatus* and ergosterol biosynthesis in *A. fumigatus* and *C. albicans*.

Compounds	IC_{50} [μM]		
	A. fum. Growth	*A. fum.* Erg. Synth.	*C. alb.* Erg. Synth.
1	0.08	0.2	1.1
2	0.12	0.08	0.73
3	0.04	0.11	0.48
4	0.07	0.06	0.64
5	0.19	0.45	0.4
6	0.16	0.43	0.39
7	0.07	0.13	0.58
8	0.06	0.13	0.21
saperconazole	0.01	0.02	0.07
itraconazole	0.008	0.04	0.05

In vitro activity against a broad panel of *Candida* and *Aspergillus* spp., and good activity against disseminated aspergillosis in guinea pigs was the basis for selection of compound **1** as a suitable starting point for the design of derived prodrugs with improved solubility. Additional work on the design and antifungal activity of these prodrugs will be published in a forthcoming paper.

Experimental Section

Chemistry

General methods: Melting points were measured in open capillaries on a Büchi B545 instrument and are uncorrected. ^1H and ^{13}C NMR spectra were recorded on Bruker DPX 360 and Avance 600 spectrometers. $CDCl_3$ was used as solvent, unless otherwise mentioned. Chemical shifts (δ) are expressed in parts per million (ppm) with $(CH_3)_4Si$ as an internal standard. Elemental analyses were performed with a Carlo–Erba EA1110 analyzer. Mass spectra were obtained with a Waters-Micromass ZQ mass spectrometer with an electrospray ionization source operating in positive and negative ionization modes. Mass spectra were acquired by scanning in an area starting from 100 to 1000 mass units in 1 s using a dwell time of 0.1 s. The capillary needle voltage was 3 kV, and the source temperature was maintained at 140 °C; N_2 was used as the nebulizer gas. Cone voltage was 10 V for positive ionization mode and 20 V for negative ionization mode. Data acquisition was performed with a Waters-Micromass MassLynx-Openlynx data system. Silica gel thin-layer chromatography (TLC) was performed on precoated Kieselgel 60 F_{254} plates (E. Merck, AG Darmstadt, Germany). Silica gel column chromatography was performed with Kieselgel 60 (0.063–0.200 mm) (E. Merck, AG Darmstadt, Germany). Chiral HPLC purification was performed on a Daicel cellulose normal-phase Chiracel® OD™ HPLC column, purchased from Chiral Technologies Europe. Optical rotations were performed on a polarimeter (PerkinElmer). All commercial reagents and solvents were used without any pretreatment. All reactions were carried out under an atmosphere of dry N_2 gas. Brine is a saturated solution of NaCl in H_2O; 80% NaH (Sigma–Aldrich) was washed free of oil with hexanes prior to use. DEAD = diethylazodicarboxylate, DMA = *N*,*N*-dimethylacetamide, DMF = *N*,*N*-dimethylformamide, THF = tetrahydrofuran, MIK = 4-methyl-2-pentanone, DIPE = diisopropyl ether.

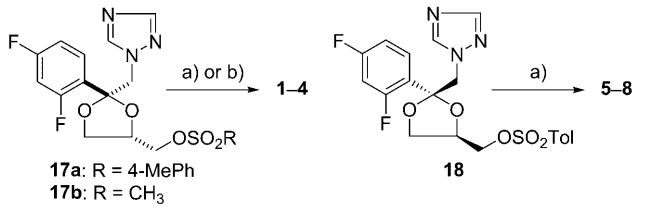
4-[4-[4-[4-[[(2*S*,4*R*)-2-(2,4-difluorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-1-piperazinyl]phenyl]-2,4-dihydro-2-[(1*S*,2*S*)-2-hydroxy-1-methylpropyl]-3*H*-1,2,4-triazol-3-one (1): According to the synthesis of compound **2** (see below) starting from dioxolane **17b** (5.4 g, 0.012 mol) and phenol **12a** (4.5 g, 0.0109 mol) to yield compound **1** as a white solid (4.7 g, 62%); mp = 189.4 °C (MeOH); $[\alpha]_{20}^{D} = -20.15$ ($c = 1.00$ *w*/*v*% in DMF); ^1H NMR (360 MHz, $CDCl_3$): $\delta = 1.25$ (d, $J = 6.4$ Hz, 3H), 1.46 (d, $J = 6.9$ Hz, 3H), 3.17 (d, $J = 8.1$ Hz, 1H), 3.21–3.25 (m, 4H), 3.34–3.39 (m, 4H), 3.49 (dd, $J = 9.7$, 6.3 Hz, 1H), 3.78 (dd, $J = 9.7$, 4.7 Hz, 1H), 3.82 (dd, $J = 8.4$, 5.2 Hz, 1H), 3.98 (dd, $J = 8.4$, 6.6 Hz, 1H), 3.97–4.05 (m, 1H), 4.25 (qd, $J = 6.9$, 5.6 Hz, 1H), 4.37–4.46 (m, $J = 6.4$, 6.4, 5.2, 4.7 Hz, 1H), 4.68 (d, $J = 14.7$ Hz, 1H), 4.73 (d, $J = 14.7$ Hz, 1H), 6.80 (d, $J = 9.0$ Hz, 2H), 6.85–6.92 (m, 2H), 6.94 (d, $J = 9.0$ Hz, 2H), 7.03 (d, $J = 8.9$ Hz, 2H), 7.41 (d, $J = 8.9$ Hz, 2H), 7.50 (td, $J = 8.9$, 6.4 Hz, 1H), 7.66 (s, 1H), 7.89 (s, 1H), 8.23 (s, 1H); Anal. calcd for $C_{35}H_{38}F_2N_8O_5$: C 61.04, H 5.56, N 16.27, found: C 61.04, H 5.47, N 16.33.

Scale-up optimized procedure for compound 1: Phenol **12a** (378.3 g, 0.93 mol), dioxolane **17b** (416.1 g, 1.108 mol), and NaOH pellets (44.3 g, 1.108 mol) were added to a 5 L round-bottomed flask, and after purging with N_2 gas, DMA (1390 mL) was added, followed by vigorous stirring. Methylisobutyl ketone (744 mL) was then added, whereupon the reaction mixture was heated to 80 °C under vigorous stirring. The initial viscous mixture became homogenous from 50 °C onward with the exception of the NaOH pellets. After stirring for 10 h at 80 °C, the reaction mixture was allowed to cool to room temperature, and stirring was continued for an additional 16 h. Ice-cooled H_2O (1.5 L) was added carefully over a period of 30 min (*exothermic!*), followed by stirring for 2 h at room temperature. The resulting precipitate was filtered off, washed with H_2O (1 L), MIK (1 L), and finally dried in vacuo at 40 °C to yield compound **1** (584 g, 91.2%). Compound **1** (435 g) was dissolved 1-methoxy-2-propanol (4.3 L) at reflux, filtered warm, and the filtrate was stirred for 16 h at room temperature. The precipitate was filtered off, washed with 2-propanol (200 mL), and dried in vacuo at 50 °C for 16 h to yield compound **1** (339 g). A second crop with the desired purity was obtained from the filtrate to yield 51 g of compound **1**, giving a total yield of 89% for the re-crystallization procedure.

4-[4-[4-[4-[[(2*S*,4*R*)-2-(2,4-difluorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-1-piperazinyl]phenyl]-2,4-dihydro-2-[(1*R*,2*R*)-2-hydroxy-1-methylpropyl]-3*H*-1,2,4-triazol-3-one (2): Compound **17a** (5.4 g, 0.012 mol), phenol **12b** (4.5 g, 0.0109 mol), and NaOH pellets (0.44 g, 0.011 mol) were added to a round-bottomed flask, and after purging with N_2 gas, DMF (150 mL) was added under vigorous stirring. The reaction mixture was stirred overnight at 70 °C, followed by cooling and subsequently poured out into an ice–H_2O mixture. The resulting precipitate was filtered off and dried to yield compound **2** (7 g). This fraction was purified by HPLC over Silica Motrex Amicon (20–45 μm; eluent: 1,1,2-trichloroethane/EtOH 90:10). The pure fractions were collected, and after evaporation of the solvent, the residue was triturated in warm MeOH, cooled, filtered off, and dried in vacuo at 40 °C to yield compound **2** as a white solid (5.3 g, 70%); $[\alpha]_{20}^{D} = -7.71$ ($c = 0.97$ *w*/*v*% in DMF); ^1H NMR (360 MHz, $CDCl_3$): $\delta = 1.25$ (d, $J = 6.4$ Hz, 3H), 1.47 (d, $J = 6.9$ Hz, 3H), 3.04 (d, $J = 8.5$ Hz, 1H), 3.20–3.27 (m, 4H), 3.34–3.40 (m, 4H), 3.48 (dd, $J = 9.7$, 6.3 Hz, 1H), 3.79 (dd, $J = 9.7$, 4.7 Hz, 1H), 3.82 (dd, $J = 8.5$, 5.2 Hz, 1H), 3.96–4.05 (m, 2H), 4.26 (qd, $J = 6.9$, 5.6 Hz, 1H), 4.38–4.45 (m, $J = 6.4$, 6.4, 5.2, 4.7 Hz, 1H), 4.68 (d, $J = 14.7$ Hz, 1H), 4.74 (d, $J = 14.7$ Hz, 1H), 6.80 (d, $J = 9.0$ Hz, 2H), 6.85–6.91 (m, 2H), 6.92–6.97 (m, 2H), 7.04 (d, $J = 8.9$ Hz, 2H), 7.42 (d, $J = 8.9$ Hz, 2H), 7.50 (td, $J = 8.9$, 6.4 Hz, 1H), 7.66 (s, 1H), 7.89 (s, 1H), 8.22 (s, 1H); Anal. calcd for $C_{35}H_{38}F_2N_8O_5$:

C 61.04, H 5.56, N 16.27, found: C 61.10, H 5.46, N 16.24; MS (ESI) *m/z* 689 ([*M*+H]$^+$).

The procedure for the preparation of compound **2** was used as a general method for the synthesis of compounds **1**–**8** (see Scheme 3).



Scheme 3. Reagents and conditions: a) **17a** or **18**: DMF, NaOH, 60–80 °C; b) **17b**, DMF or DMA, NaOH, 80 °C, 7–16 h.

4-[4-[4-[4-[[(2*S*,4*R*)-2-(2,4-difluorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-1-piperazinyl]phenyl]-2,4-dihydro-2-[(1*S*,2*R*)-2-hydroxy-1-methylpropyl]-3*H*-1,2,4-triazol-3-one (3): According to the synthesis of compound **2** starting from dioxolane **17b** (5.4 g, 0.012 mol) and phenol **12c** (4.5 g, 0.0109 mol) to yield compound **3** as a white solid (4.4 g, 59%); mp = 210.1 °C (MeOH); $[\alpha]_{20}^{D} = -17.79$ (589 nm, *c* = 0.99500 *w/v*%, DMF, 20 °C); ^{1}H NMR (360 MHz, $CDCl_3$): δ = 1.25 (d, *J* = 6.4 Hz, 3H), 1.43 (d, *J* = 6.9 Hz, 3H), 3.21–3.26 (m, 4H), 3.34–3.41 (m, 4H), 3.49 (dd, *J* = 9.7, 6.3 Hz, 1H), 3.69 (d, *J* = 2.7 Hz, 1H), 3.79 (dd, *J* = 9.7, 4.7 Hz, 1H), 3.82 (dd, *J* = 8.5, 5.2 Hz, 1H), 3.98 (dd, *J* = 8.4, 6.5 Hz, 1H), 4.17–4.25 (m, *J* = 6.4, 6.4, 6.4, 2.9, 2.7 Hz, 1H), 4.28 (qd, *J* = 6.9, 2.9 Hz, 1H), 4.36–4.46 (m, *J* = 6.4, 6.4, 5.2, 4.7 Hz, 1H), 4.68 (d, *J* = 14.7 Hz, 1H), 4.74 (d, *J* = 14.7 Hz, 1H), 6.80 (d, *J* = 8.9 Hz, 2H), 6.85–6.91 (m, 2H), 6.94 (d, *J* = 8.9 Hz, 2H), 7.04 (d, *J* = 8.9 Hz, 2H), 7.40 (d, *J* = 8.9 Hz, 2H), 7.50 (td, *J* = 8.9, 6.7 Hz, 1H), 7.64 (s, 1H), 7.89 (s, 1H), 8.22 (s, 1H); ^{13}C NMR (151 MHz): δ = 12.48 (s, 1C), 19.36 (s, 1C), 49.04 (s, 2C), 50.49 (s, 2C), 54.43 (d, *J* = 3.7 Hz, 1C), 57.28 (s, 1C), 67.54 (s, 1C), 67.59 (s, 1C), 69.83 (s, 1C), 74.90 (s, 1C), 105.12 (t, *J* = 25.9 Hz, 1C), 106.69 (d, *J* = 4.0 Hz, 1C), 111.18 (dd, *J* = 20.9, 3.4 Hz, 1C), 115.22 (s, 2C), 116.53 (s, 2C), 118.41 (s, 2C), 121.67 (dd, *J* = 13.0, 3.7 Hz, 1C), 123.70 (s, 2C), 125.30 (s, 1C), 129.19 (dd, *J* = 9.7, 4.6 Hz, 1C), 134.26 (s, 1C), 144.83 (s, 1C), 145.95 (s, 1C), 150.73 (s, 1C), 151.27 (s, 1C), 152.06 (s, 1C), 152.57 (s, 1C), 160.37 (dd, *J* = 252.7, 12.2 Hz, 1C), 163.64 (dd, *J* = 251.9, 10.9 Hz, 1C); Anal. calcd for $C_{35}H_{38}F_2N_8O_5$: C 61.04, H 5.56, N 16.27, found: C 60.68, H 5.44, N 16.17; MS (ESI) *m/z* 689 ([*M*+H]$^+$).

4-[4-[4-[4-[[(2*S*,4*R*)-2-(2,4-difluorophenyl)-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-1-piperazinyl]phenyl]-2,4-dihydro-2-[(1*R*,2*S*)-2-hydroxy-1-methylpropyl]-3H-1,2,4-triazol-3-one (4): According to the synthesis of compound **2** starting from dioxolane **17b** (5.4 g, 0.012 mol) and phenol **12d** (4.5 g, 0.0109 mol) to yield compound **4** as a white solid (5.9 g, 79%); mp = 199.9 °C (MeOH); $[\alpha]_{20}^{D} = -9.36$ (*c* = 1.01540 *w/v*% in DMF); ^{1}H NMR (360 MHz, [D$_6$]DMSO): δ = 0.98 (d, *J* = 6.2 Hz, 3H), 1.34 (d, *J* = 6.8 Hz, 3H), 3.11–3.22 (m, 4H), 3.26–3.36 (m, 4H), 3.65–3.83 (m, 4H), 3.91–4.00 (m, 2H), 4.35–4.43 (m, *J* = 5.7, 5.7, 5.7, 5.7 Hz, 1H), 4.66–4.76 (m, 2H), 4.94 (d, *J* = 5.9 Hz, 1H), 6.84 (d, *J* = 8.7 Hz, 2H), 6.96 (d, *J* = 8.7 Hz, 2H), 7.03–7.13 (m, 3H), 7.31 (ddd, *J* = 11.3, 9.1, 2.5 Hz, 1H), 7.44 (td, *J* = 8.9, 6.8 Hz, 1H), 7.50 (d, *J* = 8.6 Hz, 2H), 7.87 (s, 1H), 8.33 (s, 1H), 8.41 (s, 1H); ^{13}C NMR (151 MHz): δ = 12.49 (s, 1C), 19.35 (s, 1C), 49.03 (s, 2C), 50.47 (s, 2C), 54.41 (d, *J* = 3.4 Hz, 1C), 57.26 (s, 1C), 67.53 (s, 1C), 67.57 (s, 1C), 69.81 (s, 1C), 74.89 (s, 1C), 105.11 (t, *J* = 25.7 Hz, 1C), 106.68 (d, *J* = 4.2 Hz, 1C), 111.17 (dd, *J* = 21.2, 3.4 Hz, 1C), 115.2 (s, 2C), 116.51 (s, 2C), 118.39 (s, 2C), 121.66 (dd, *J* = 13.0, 3.7 Hz, 1C), 123.69 (s, 2C), 125.29 (s, 1C), 129.18 (dd, *J* = 9.7, 4.6 Hz, 1C), 134.25 (s, 1C), 144.81 (s, 1C), 145.93 (s, 1C), 150.71 (s, 1C), 151.26 (s, 1C), 152.05 (s, 1C), 152.56 (s, 1C), 160.36 (dd, *J* = 252.7, 12.2 Hz, 1C), 163.63 (dd, *J* = 251.6, 11.7 Hz, 1C); Anal. calcd for $C_{35}H_{38}F_2N_8O_5$: C 61.04, H 5.56, N 16.27, found: C 61.05, H 5.46, N 16.26; MS (ESI) *m/z* 689 ([*M*+H]$^+$).

4-[4-[4-[4-[[(2*R*,4*S*)-2-(2,4-difluorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-1-piperazinyl]phenyl]-2,4-dihydro-2-[(1*R*,2*R*)-2-hydroxy-1-methylpropyl]-3*H*-1,2,4-triazol-3-one (5): According to the synthesis of compound **2** starting from dioxolane **18** (5.4 g, 0.012 mol) and phenol **12b** (4.5 g, 0.0109 mol) to yield compound **5** as a white solid (4.8 g, 64%); mp = 190.3 °C (MeOH); $[\alpha]_{20}^{D} = +19.49$ (589 nm, *c* = 1.03620 *w/v*%, DMF, 20 °C); ^{1}H NMR (360 MHz, $CDCl_3$): δ = 1.25 (d, *J* = 6.4 Hz, 3H), 1.46 (d, *J* = 6.9 Hz, 3H), 3.17 (d, *J* = 8.1 Hz, 1H), 3.21–3.25 (m, 4H), 3.34–3.39 (m, 4H), 3.49 (dd, *J* = 9.7, 6.3 Hz, 1H), 3.78 (dd, *J* = 9.7, 4.7 Hz, 1H), 3.82 (dd, *J* = 8.4, 5.2 Hz, 1H), 3.98 (dd, *J* = 8.4, 6.6 Hz, 1H), 3.97–4.05 (m, 1H), 4.25 (qd, *J* = 6.9, 5.6 Hz, 1H), 4.37–4.46 (m, *J* = 6.4, 6.4, 5.2, 4.7 Hz, 1H), 4.68 (d, *J* = 14.7 Hz, 1H), 4.73 (d, *J* = 14.7 Hz, 1H), 6.80 (d, *J* = 9.0 Hz, 2H), 6.85–6.92 (m, 2H), 6.94 (d, *J* = 9.0 Hz, 2H), 7.03 (d, *J* = 8.9 Hz, 2H), 7.41 (d, *J* = 8.9 Hz, 2H), 7.50 (td, *J* = 8.9, 6.4 Hz, 1H), 7.66 (s, 1H), 7.89 (s, 1H), 8.23 (s, 1H); Anal. calcd for $C_{35}H_{38}F_2N_8O_5$: C 61.04, H 5.56, N 16.27, found: C 61.11, H 5.51, N 16.27; MS (ESI) *m/z* 689 ([*M*+H]$^+$).

4-[4-[4-[4-[[(2*R*,4*S*)-2-(2,4-difluorophenyl)-2-(1i-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-1-piperazinyl]phenyl]-2,4-dihydro-2-[(1*S*,2*S*)-2-hydroxy-1-methylpropyl]-3*H*-1,2,4-triazol-3-one (6): According to the synthesis of compound **2** starting from dioxolane **18** (5.4 g, 0.012 mol) and phenol **12a** (4.5 g, 0.0109 mol) to yield compound **6** as a white solid (4.3 g, 57%); $[\alpha]_{20}^{D} = +7.13$ (*c* = 0.99540 *w/v*% in DMF); ^{1}H NMR (360 MHz, $CDCl_3$): δ = 1.25 (d, *J* = 6.4 Hz, 3H), 1.45 (d, *J* = 6.9 Hz, 3H), 3.20–3.27 (m, 4H), 3.28 (d, *J* = 7.8 Hz, 1H), 3.34–3.40 (m, 4H), 3.48 (dd, *J* = 9.7, 6.3 Hz, 1H), 3.79 (dd, *J* = 9.7, 4.7 Hz, 1H), 3.82 (dd, *J* = 8.5, 5.2 Hz, 1H), 3.96–4.05 (m, 2H), 4.26 (qd, *J* = 6.9, 5.6 Hz, 1H), 4.38–4.45 (m, *J* = 6.4, 6.4, 5.2, 4.7 Hz, 1H), 4.68 (d, *J* = 14.7 Hz, 1H), 4.74 (d, *J* = 14.7 Hz, 1H), 6.80 (d, *J* = 9.0 Hz, 2H), 6.85–6.91 (m, 2H), 6.92–6.97 (m, 2H), 7.04 (d, *J* = 8.9 Hz, 2H), 7.42 (d, *J* = 8.9 Hz, 2H), 7.50 (td, *J* = 8.9, 6.4 Hz, 1H), 7.66 (s, 1H), 7.89 (s, 1H), 8.22 (s, 1H); Anal. calcd for $C_{35}H_{38}F_2N_8O_5$: C 61.04, H 5.56, N 16.27, found: C 60.80, H 5.33, N 16.16; MS (ESI) *m/z* 689 ([*M*+H]$^+$).

4-[4-[4-[4-[[(2*R*,4*S*)-2-(2,4-difluorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-1-piperazinyl]phenyl]-2,4-dihydro-2-[(1*R*,2*S*)-2-hydroxy-1-methylpropyl]-3*H*-1,2,4-triazol-3-one (7): According to the synthesis of compound **2** starting from dioxolane **18** (5.4 g, 0.012 mol) and phenol **12d** (4.5 g, 0.0109 mol) to yield compound **7** as a white solid 6.3 g (84%); mp = 211.1 °C (MeOH); $[\alpha]_{20}^{D} = +17.79$ (*c* = 0.99520 *w/v*% in DMF); ^{1}H NMR (360 MHz, $CDCl_3$): δ = 1.25 (d, *J* = 6.4 Hz, 3H), 1.43 (d, *J* = 6.9 Hz, 3H), 3.21–3.26 (m, 4H), 3.34–3.41 (m, 4H), 3.49 (dd, *J* = 9.7, 6.3 Hz, 1H), 3.69 (d, *J* = 2.7 Hz, 1H), 3.79 (dd, *J* = 9.7, 4.7 Hz, 1H), 3.82 (dd, *J* = 8.5, 5.2 Hz, 1H), 3.98 (dd, *J* = 8.4, 6.5 Hz, 1H), 4.17–4.25 (m, *J* = 6.4, 6.4, 6.4, 2.9, 2.7 Hz, 1H), 4.28 (qd, *J* = 6.9, 2.9 Hz, 1H), 4.36–4.46 (m, *J* = 6.4, 6.4, 5.2, 4.7 Hz, 1H), 4.68 (d, *J* = 14.7 Hz, 1H), 4.74 (d, *J* = 14.7 Hz, 1H), 6.80 (d, *J* = 8.9 Hz, 2H), 6.85–6.91 (m, 2H), 6.94 (d, *J* = 8.9 Hz, 2H), 7.04 (d, *J* = 8.9 Hz, 2H), 7.40 (d, *J* = 8.9 Hz, 2H), 7.50 (td, *J* = 8.9, 6.7 Hz, 1H), 7.64 (s, 1H), 7.89 (s, 1H), 8.22 (s, 1H); Anal. calcd for $C_{35}H_{38}F_2N_8O_5$: C 61.04, H 5.56, N 16.27, found: C 61.23, H 5.49, N 16.28; MS (ESI) *m/z* 689 ([*M*+H]$^+$).

4-[4-[4-[4-[[(2*R*,4*S*)-2-(2,4-difluorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-1-piperazinyl]phenyl]-2,4-dihydro-2-[(1*S*,2*R*)-2-hydroxy-1-methylpropyl]-3*H*-1,2,4-triazol-3-one (8): According to the synthesis of compound **2** starting from dioxolane **18** (5.4 g, 0.012 mol) and phenol **12c** (4.5 g, 0.0109 mol) to yield compound **8** as a white solid (6.6 g, 88%); mp = 199.3 °C (MeOH); $[\alpha]_{20}^{D} = +9.22$ ($c = 1.03040$ *w/v*% in DMF); ^{1}H NMR (360 MHz, $CDCl_3$): $\delta = 1.24$ (d, $J = 6.4$ Hz, 3H), 1.43 (d, $J = 6.9$ Hz, 3H), 3.20–3.26 (m, 4H), 3.34–3.40 (m, 4H), 3.49 (dd, $J = 9.7$, 6.3 Hz, 1H), 3.75 (d, $J = 2.7$ Hz, 1H), 3.78 (dd, $J = 9.7$, 4.7 Hz, 1H), 3.82 (dd, $J = 8.4$, 5.2 Hz, 1H), 3.98 (dd, $J = 8.4$, 6.6 Hz, 1H), 4.17–4.24 (m, $J = 6.4$, 6.4, 6.4, 2.9, 2.7 Hz, 1H), 4.28 (qd, $J = 6.9$, 2.9 Hz, 1H), 4.38–4.46 (m, $J = 6.4$, 6.4, 5.2, 4.7 Hz, 1H), 4.68 (d, $J = 14.7$ Hz, 1H), 4.73 (d, $J = 14.7$ Hz, 1H), 6.80 (d, $J = 9.0$ Hz, 2H), 6.85–6.91 (m, 2H), 6.94 (d, $J = 9.0$ Hz, 2H), 7.03 (d, $J = 8.9$ Hz, 2H), 7.40 (d, $J = 8.9$ Hz, 2H), 7.50 (td, $J = 8.9$, 6.4 Hz, 1H), 7.64 (s, 1H), 7.89 (s, 1H), 8.23 (s, 1H); Anal. calcd for $C_{35}H_{38}F_2N_8O_5$: C 61.04, H 5.56, N 16.27, found: C 61.00, H 5.30, N 16.15; MS (ESI) *m/z* 689 ($[M+H]^+$).

2,4-dihydro-4-[4-[4-(4-methoxyphenyl)-1-piperazinyl]phenyl]-2-(1-methyl-2-oxopropyl)-3*H*-1,2,4-triazol-3-one (10): A mixture of triazolone **9** (61.5 g, 0.175 mol), 3-chlorobutan-2-one (23.6 g, 0.22 mol), and K_2CO_3 (20 g) in DMF (400 mL) and toluene (400 mL) was stirred overnight at 110 °C with azeotropic removal of H_2O. The reaction mixture was cooled, and H_2O (500 mL) and DIPE (500 mL) were added. The product was crystallized from the reaction mixture, filtered off, and washed with H_2O and a small amount of MeOH. Crystallizing the product from MIK, collecting and drying in vacuo at 60 °C gave pure compound **10** (71 g, 97%) as a white solid; mp = 172 °C (MIK); Anal. calcd for $C_{23}H_{27}N_5O_3$: C 65.54, H 6.46, N 16.62, found: C 65.20, H 6.24, N 16.65.

2,4-dihydro-4-[4-[4-(4-hydroxyphenyl)-1-piperazinyl]phenyl]-2-(1-methyl-2-oxopropyl)-3*H*-1,2,4-triazol-3-one (11): A mixture of compound **10** (15 g, 0.035 mol) and $NaHSO_3$ (4 g) in 48% aqueous HBr (200 mL) was stirred and held at reflux for 5 h. The product was then crystallized from the cooled reaction mixture, and the solid was filtered off and washed with acetone. The resulting HBr salt was stirred in a mixture of an aqueous $NaHCO_3$ solution (~300 mL) and a solution of CH_2Cl_2/*n*-butanol (90:10, ~300 mL). The layers were separated, and the organic layer was washed with H_2O, brine, dried ($MgSO_4$), filtered, and the solvent was evaporated. The residue was triturated in MeOH, and the product was subsequently collected by suction and dried in vacuo at 50 °C to yield compound **11** (12 g, 84%) as white crystals; mp = 193.8 °C (MeOH); ^{1}H NMR (360 MHz, [D$_6$]DMSO): $\delta = 1.47$ (d, $J = 7.2$ Hz, 3H), 2.10 (s, 3H), 3.06–3.16 (m, 4H), 3.28–3.33 (m, 4H), 4.91 (q, $J = 7.2$ Hz, 1H), 6.68 (d, $J = 8.8$ Hz, 2H), 6.86 (d, $J = 8.8$ Hz, 2H), 7.11 (d, $J = 8.9$ Hz, 2H), 7.51 (d, $J = 8.9$ Hz, 2H), 8.43 (s, 1H), 8.88 (s, 1H); Anal. calcd for $C_{22}H_{25}N_5O_3$: C 64.85, H 6.18, N 17.19, found: C 64.78, H 6.06, N 17.35.

Synthesis of 12a and 12b: A stirred mixture of compound **11** (25 g, 0.06 mol) in dry DMF (500 mL) was purged with N_2 and cooled to −10 °C. Potassium tri(*sec*-butyl)borohydride (K-selectride; 150 mL of a 1 M solution in THF) was added dropwise. The mixture was allowed to warm to room temperature, followed by pouring out into ice–H_2O. The resulting precipitate was filtered off, washed with a small amount of MeOH, and crystallized from MeOH. The crystals were collected and dried in vacuo to yield 18.9 g of a mixture (90:10) of **12a,b** and **12c,d**. The mixture was purified by HPLC over Chiralpac AD (eluent: EtOH). Two pure fractions were collected (compound **12b** was eluted first followed by **12a**), and evaporated until dry. Each residue was triturated in MeOH, subsequently filtered off, and dried in vacuo at 40 °C to yield compound **12a** (7.3 g, 30%) and compound **12b** (8 g, 32.5%).

2,4-dihydro-2-[(1*S*,2*S*)-2-hydroxy-1-methylpropyl]-4-[4-[4-(4-hydroxyphenyl)-1-piperazinyl]phenyl]-3*H*-1,2,4-triazol-3-one (12a): mp = 201.2 °C (MeOH); $[\alpha]_{20}^{D} = -10.81$ ($c = 1.01$ *w/v*% in DMF); ^{1}H NMR (360 MHz, [D$_6$]DMSO): $\delta = 1.11$ (d, $J = 6.3$ Hz, 3H), 1.24 (d, $J = 7.0$ Hz, 3H), 3.04–3.13 (m, 4H), 3.26–3.33 (m, 4H), 3.75–3.84 (m, $J = 7.2$, 6.3, 6.3, 6.3, 5.2 Hz, 1H), 3.98–4.06 (m, $J = 7.2$, 6.9, 6.9, 6.9 Hz, 1H), 4.73 (d, $J = 5.2$ Hz, 1H), 6.68 (d, $J = 9.0$ Hz, 2H), 6.85 (d, $J = 9.0$ Hz, 2H), 7.09 (d, $J = 9.1$ Hz, 2H), 7.49 (d, $J = 9.1$ Hz, 2H), 8.30 (s, 1H), 8.89 (s, 1H); Anal. calcd for $C_{22}H_{27}N_5O_3$: C 64.53, H 6.65, N 17.10, found: C 64.77, H 6.51, N 17.09; MS (ESI) *m/z* 410 ($[M+H]^+$).

2,4-dihydro-2-[(1*R*,2*R*)-2-hydroxy-1-methylpropyl]-4-[4-[4-(4-hydroxyphenyl)-1-piperazinyl]phenyl]-3*H*-1,2,4-triazol-3-one (12b): mp = 200.4 °C (MeOH); $[\alpha]_{20}^{D} = +10.35$ ($c = 0.98$ *w/v*% in DMF); ^{1}H NMR (360 MHz, [D$_6$]DMSO): $\delta = 1.11$ (d, $J = 6.3$ Hz, 3H), 1.24 (d, $J = 7.0$ Hz, 3H), 3.04–3.13 (m, 4H), 3.26–3.33 (m, 4H), 3.75–3.84 (m, $J = 7.2$, 6.3, 6.3, 6.3, 5.2 Hz, 1H), 3.98–4.06 (m, $J = 7.2$, 6.9, 6.9, 6.9 Hz, 1H), 4.73 (d, $J = 5.2$ Hz, 1H), 6.68 (d, $J = 9.0$ Hz, 2H), 6.85 (d, $J = 9.0$ Hz, 2H), 7.09 (d, $J = 9.1$ Hz, 2H), 7.49 (d, $J = 9.1$ Hz, 2H), 8.30 (s, 1H), 8.89 (s, 1H); Anal. calcd for $C_{22}H_{27}N_5O_3$: C 64.53, H 6.65, N 17.10, found: C 63.26, H 6.66, N 16.70; MS (ESI) *m/z* 410 ($[M+H]^+$).

Synthesis of 12c and 12d: $Zn(BH_4)_2$ was prepared according to the following procedure: $ZnCl_2$ (40 g, 0.45 mol) was stirred in dry Et_2O (500 mL) at reflux until all $ZnCl_2$ was dissolved. This solution was added dropwise to a suspension of $NaBH_4$ (27 g, 0.17 mol) in dry Et_2O (300 mL), and the mixture was stirred overnight at room temperature. After filtration, the $Zn(BH_4)_2/Et_2O$ solution was added dropwise to an ice-cooled (0 °C) stirred solution of compound **11** (40.7 g, 0.1 mol) in dry DMF (500 mL). The reaction mixture was allowed to warm slowly to room temperature over a period of 3 h, and was poured into ice–H_2O. The resulting precipitate was filtered off and dried in vacuo to give **12a,b** and **12c,d** (40 g, 70:30 respective mixture). The reaction was repeated at the same amount and both crops were combined to give **12a,b/12c,d** (77 g), which was purified by HPLC over Amicon silica (eluent: CH_2Cl_2/EtOAc/MeOH 66:30:4). The desired erythro fractions were collected, and the solvent was evaporated to give **12c,d** 27.5 g (33%). This fraction was separated into its enantiomers over Chiralpac AD (eluent: 100% MeOH). Two pure fractions were collected and evaporated: first fraction yielding **12c** (11.5 g, 14%); second fraction **12d** (10.9 g, 13%). Both fractions were crystallized from MeOH, filtered, and dried in vacuo at 40 °C.

2-((1*S*,2*R*)-2-hydroxy-1-methylpropyl)-4-{4-[4-(4-hydroxyphenyl)-piperazin-1-yl]phenyl}-2,4-dihydro-3*H*-1,2,4-triazol-3-one (12c): mp = 221.9 °C (MeOH); $[\alpha]_{20}^{D} = -7.07$ ($c = 0.97600$ *w/v*% in DMF); ^{1}H NMR (360 MHz, [D$_6$]DMSO): $\delta = 0.97$ (d, $J = 6.2$ Hz, 3H), 1.34 (d, $J = 6.8$ Hz, 3H), 3.04–3.15 (m, 4H), 3.24–3.34 (m, 4H), 3.71–3.81 (m, $J = 7.7$, 6.2, 6.2, 6.2, 5.9 Hz, 1H), 3.89–3.99 (m, $J = 7.7$, 6.8, 6.8, 6.8 Hz, 1H), 4.92 (d, $J = 5.9$ Hz, 1H), 6.68 (d, $J = 8.6$ Hz, 2H), 6.85 (d, $J = 8.6$ Hz, 2H), 7.08 (d, $J = 8.7$ Hz, 2H), 7.48 (d, $J = 8.7$ Hz, 2H), 8.32 (s, 1H), 8.89 (s, 1H); Anal. calcd for $C_{22}H_{27}N_5O_3$: C 64.53, H 6.65, N 17.10, found: C 64.37, H 6.70, N 17.11; MS (ESI) *m/z* 410 ($[M+H]^+$).

2-((1*R*,2*S*)-2-hydroxy-1-methylpropyl)-4-{4-[4-(4-hydroxyphenyl)-piperazin-1-yl]phenyl}-2,4-dihydro-3*H*-1,2,4-triazol-3-one (12d): mp = 224.3 °C (MeOH); $[\alpha]_{20}^{D} = +6.86$ ($c = 0.99160$ *w/v*% in DMF); ^{1}H NMR (360 MHz, [D$_6$]DMSO): $\delta = 0.97$ (d, $J = 6.2$ Hz, 3H), 1.34 (d, $J = 6.8$ Hz, 3H), 3.04–3.15 (m, 4H), 3.24–3.34 (m, 4H), 3.71–3.81 (m, $J = 7.7$, 6.2, 6.2, 6.2, 5.9 Hz, 1H), 3.89–3.99 (m, $J = 7.7$, 6.8, 6.8, 6.8 Hz, 1H), 4.92 (d, $J = 5.9$ Hz, 1H), 6.68 (d, $J = 8.6$ Hz, 2H), 6.85 (d, $J = 8.6$ Hz, 2H), 7.08 (d, $J = 8.7$ Hz, 2H), 7.48 (d, $J = 8.7$ Hz, 2H), 8.32 (s, 1H), 8.89 (s, 1H); Anal. calcd for $C_{22}H_{27}N_5O_3$: C 64.53, H 6.65, N 17.10, found: C 64.41, H 6.67, N 17.09; MS (ESI) *m/z* 410 ($[M+H]^+$).

2,4-dihydro-2-[(1*S*,2*S*)-2-hydroxy-1-methylpropyl]-4-[4-[4-(4-methoxyphenyl)-1-piperazinyl]phenyl]-3*H*-1,2,4-triazol-3-one (13a): A mixture of intermediate **13c** (1.38 g, 0.00327 mol), PPh_3 (2.1 g, 0.00806 mol) and *p*-nitrobenzoic acid (1.2 g, 0.00717 mol) in a dry mixture of THF/DMA (3:2, 50 mL) was heated until complete dissolution, followed by cooling to room temperature, whereupon diethyl azodicarboxylate (1.4 g, 0.00806 mol) was added dropwise. The mixture was then stirred at room temperature for 90 min and for an additional 1 h at 50 °C, followed by the addition of NaOH (1 N, 10 mL) at 50 °C. The mixture was then poured out into a mixture of H_2O (100 mL) and 1 N NaOH (90 mL), followed by stirring. The resulting precipitate was filtered off and recrystallized from 2-propanol (60 mL). After 48 h, the precipitate was filtered off and dried in vacuo at 40 °C to yield compound **13a** (0.98 g, 71%), which was used in the next step without further purification (the preparation contained traces of compound **16**).

Synthesis of 12a from 13a: Compound **13a** (0.98 g, 0.0023 mol) was heated at 90 °C for 5 h in a mixture of HBr (48%, 25 mL) and HBr-saturated acetic acid (25 mL) in the presence of a small amount of $NaHSO_3$ (1 g). The mixture was stirred overnight at room temperature. The precipitate was filtered off, suspended in H_2O (200 mL), and after addition of 50% NaOH (20 mL) stirring was continued for another 2 h. After acidification to pH 4 with concentrated HCl and subsequent neutralization with an aqueous saturated solution of $NaHCO_3$, the precipitate was filtered off and purified by chromatography on silica gel (2% MeOH in CH_2Cl_2). The desired fraction was re-crystallized from MeOH to give (0.6 g, 63%): $[\alpha]_{20}^{D} = -10.2$ ($c = 0.995$ *w/v*% in DMF) corresponding to **12a**, confirmed by chiral HPLC Chiralpac AD (eluent: EtOH).

2-((1*S*,2*R*)-2-hydroxy-1-methylpropyl)-4-{4-[4-(4-methoxyphenyl)-piperazin-1-yl]phenyl}-2,4-dihydro-3*H*-1,2,4-triazol-3-one (13c): *t*BuOK (15.8 g, 0.165 mol) was added to a stirred mixture of compound **9**[37] (52.6 g, 0.15 mol) in dry DMA (500 mL) under a flow of N_2 gas. The mixture was stirred for 1 h at 100 °C and then cooled to 50 °C. *meso*-(4*R*,5*R*)-4,5-Dimethyl-1,2,3-dioxathiolane-2,2-dioxide[41] (25.1 g, 0.165 mol) was added dropwise. The mixture was stirred for 2 h at 50–60 °C, followed by dropwise addition of concentrated H_2SO_4 (20 mL), and stirring was continued at 60 °C for another 2 h. H_2O (20 mL) was then added, and stirring was continued for 20 h at 60 °C. Then the reaction mixture was cooled, poured out into ice–H_2O (1000 mL), and made alkaline (pH 8) with NaOH (50% *w/v*) under stirring. The resulting precipitate was filtered off, washed with H_2O, and dried in vacuo at 40 °C, followed by purification via column chromatography over silica gel (eluent: CH_2Cl_2/MeOH 99:1). The pure fractions were collected and evaporated to dryness. The residue was triturated in CH_2Cl_2 (100 mL) and DIPE (50 mL), filtered off, and dried in vacuo at 40 °C to yield compound **13c** (53.7 g, 84%) as a white solid; $[\alpha]_{20}^{D} = -5.44°$ ($c =$ 0.9735 *w/v*% DMF); Anal. calcd for $C_{23}H_{29}N_5O_3$: C 65.23, H 6.90, N 16.54, found: C 65.37, H 7.16, N 16.90.

Synthesis of 12c from 13c: Compound **13c** (5 g, 0.0118 mol) was heated at 90 °C for 5 h in a mixture of HBr (48%, 25 mL) and HBr-saturated acetic acid (25 mL) in the presence of $NaHSO_3$ (1 g). The mixture was stirred at room temperature overnight. The precipitate was filtered off, suspended in H_2O (200 mL), supplied with 50% NaOH (20 mL) and DMF (50 mL) and stirred for another 2 h. This mixture was acidified to pH 4 with concentrated HCl and subsequently neutralized with an aqueous saturated solution of $NaHCO_3$. The resulting precipitate was filtered off and purified by silica gel chromatography (2% MeOH in CH_2Cl_2). The desired fraction was re-crystallized from MeOH to give a solid (3 g, 62%) having an optical rotation of $[\alpha]_{20}^{D} = -6.59$ ($c = 0.500$ *w/v*% in DMF) corresponding to **12c**.

Biological assays

In vitro antifungal activities (results listed in Table 1): The compounds were dissolved in DMSO up to a concentration of 1.5 mg mL^{-1}. These stock solutions were stored at −20 °C for up to six months. For susceptibility determinations, doubling dilutions of the stock solutions were prepared in DMSO and these were further diluted in sterile H_2O to threefold the desired final test concentration. DMSO alone was diluted in H_2O for the preparation of drug-free controls. The antifungal dilutions and control solutions were dispensed in 50 μL volumes in microplate wells: the plates were stored at −20 °C for up to six months before use.

The test strains included 20 clinical isolates each of *C. albicans*, *C. glabrata* and *C. krusei*, 12 each of *C. lusitaniae* and *C. parapsilosis*, 9 of *C. tropicalis*, 19 of *Cr. neoformans*, 10 isolates of *A. fumigatus*, and 9 isolates each of *M. canis*, *T. mentagrophytes* and *T. rubrum*. The fungi, which were stored in suspensions at −70 °C with 10% glycerol as cryoprotectant were re-grown on Sabouraud glucose agar (Oxoid, UK) at appropriate incubation temperatures. Inocula of yeasts were prepared by growing the organisms to a concentration of 4×10^7 cells mL^{-1} at 30 °C with rotation at 20 rpm in CYGi broth.[52,53] Inocula of moulds were prepared on Sabouraud agar at 25–30 °C. The growth was flooded with sterile 0.05% SDS and agitated to provide a suspension with a turbidity OD value of ~1 at 530 nm. Mould inocula contained predominantly conidia or predominantly hyphal fragments, according to the level of sporulation achieved by the isolate concerned.

Susceptibility tests with all yeasts and moulds was done with the same medium: RPMI 1640 broth with extra glucose (final concentration 20 g L^{-1}) and buffered to pH 7.0 with 0.165 M MOPS. This medium was prepared at 1.5× final concentration. Fungal inoculum suspensions were diluted into the medium (20 μL per 10 mL) and 100 μL volumes of the inoculated broths were added to the 50 μL of drug dilution already present in the microdilution trays, thus bringing all components to the correct final concentration. The plates were sealed with adhesive stickers. Pathogenic yeasts were incubated at 37 °C; moulds at appropriate temperatures, usually 30 °C. The incubation time was 48 h for *Candida* spp. For all other isolates, the plates were inspected at intervals, and incubation was terminated when control wells contained richly turbid growth.

The turbidity of growth in the wells was estimated as $OD_{405\,nm}$ by means of a microplate spectrophotometer. Growth in the presence of test compounds was expressed as a percentage of control growth (with experimental and control readings corrected for background OD). The MIC of each compound for each isolate was defined as the IC_{50} (lowest concentration of test substance that decreased growth below 50% of control) and was estimated from dose–response curve data. Isolates for which control $OD_{405\,nm}$ readings were < 0.15 were excluded from analysis.

In vivo antifungal activity by intravenous infusion in experimental aspergillosis (results listed in Tables 2 and 3): For intravenous infusion the compounds were prepared as solutions in 20% hydroxypropy1-1-β-cyclodextrin.

Fungus and inoculum preparation: *A. fumigatus* strain B 19119, originally isolated from a pulmonary infection, was used throughout the experiments. The fungus was grown on potato glucose agar (Difco) for six days at 35 °C and conidia harvested in a solution

containing 20 μL Tween 80 per 100 mL physiological saline. The suspension was filtered through sterile gauze, centrifuged at 3000 *g* for 30 min, and re-suspended in sterile physiological saline/Tween 80. Sterile glycerol was then added to the suspension to a final concentration of 10% (*v*/*v*), and the suspension was distributed in 1 mL volumes in cryotubes. The tubes of inoculum suspension were stored at −70 °C, and a fresh tube was thawed and used for inoculation in each animal experiment. The number of viable *A. fumigatus* colony-forming units (CFU) per mL suspension was determined to be 1.75×10^9.

Animals and their inoculation: Animal experiments were approved by the Ethics Committee on Animal Experimentation at Johnson & Johnson Pharmaceutical Research & Development, a division of Janssen Pharmaceutica N.V. This animal research facility, located Turnhoutseweg 30, 2340 Beerse (Belgium) is permitted to perform animal experiments under national license number LA1100119.

Specific pathogen-free (SPF) guinea pigs with a weight of ~400–500 g were used in all experiments. A catheter was placed into the left jugular vein of each animal, the vein ligated, and the catheter connected (via a proprietary swivel device to provide free movement for the animal) to a microprocessor-controlled infusion pump (Greasby Medical) from which a continuous infusion of physiological saline was delivered. The animals were infected via the implanted catheter with a volume of inoculum calculated to deliver 4000 *A. fumigatus* CFU per gram body weight.

Treatment protocols: The first intravenous treatments began 1 h after infection of the animals. The test formulations were then administered on subsequent days as two 1 h infusions daily, separated by a period of 5 h, for a total of 9.5 days (19 infusions in total). On day 10, surviving animals were sacrificed. These, and animals that died before the end of treatment, were autopsied. The placement of the catheters and local reactions to catheter placement were assessed grossly at autopsy. Data from animals for which there was postmortem evidence of catheter misplacement or severe local reaction that might have interfered with intravenous administration of the test substances were excluded from analysis.

Samples of lung, brain, liver, spleen, and left kidney were removed with aseptic precautions, weighed, homogenized, and samples of homogenate were cultured on Sabouraud agar for enumeration of *A. fumigatus* CFU. Lung samples were so rarely positive for *A. fumigatus* that they were not included in the analysis of results.

Reproducibility of infection in placebo-treated animals: The intrajugular route of inoculation, continuous intravenous infusion of fluids, and the duration of treatments used in these experiments were all entirely novel and previously untried. Table 5 lists the data obtained for tissue burdens of *A. fumigatus* in the organs sampled postmortem to indicate the severity of infection achieved by intrajugular inoculation of the fungal conidia at the dose selected. The mean survival time (MST) for placebo-treated animals was ~5 days, with some variation between experiments (lowest MST was 4.8 days; highest was 5.8 days). The data in Table 3 show that only four of 24 animals suffered dissemination of the *A. fumigatus* to the lungs, whereas in 14 of 24, the infection disseminated to the brain. The liver, spleen, and kidney were the organs most severely affected, with few *A. fumigatus*-negative tissues among the placebo-treated guinea pigs. Quantitatively, the spleen was the most severely infected organ, with tissue burdens ranging from 2.4 to 5.8 log CFU g^{-1}.

Table 5. Tissue burdens *of A. fumigatus* in organs removed from placebo-treated guinea pigs.

$\log_{10}$ CFU g^{-1} in five organs of guinea pigs[a]				
Brain	Liver	Spleen	Kidney	Lung
0	4.6	4.5	4.3	0
0	3.7	3.7	4.4	0
4.2	3	3.9	0	0
3.4	4	3.7	2.7	0
0	0	2.4	3.2	0
3.3	5.2	4.6	2.9	0
3	3	3	0	0
3.9	4.6	4.3	4.5	0
2.4	5.5	4.4	2.9	0
3.7	5.4	5.8	4.2	2.6
0	4	3.9	4.3	0
0	4.6	3.9	4	3.4
3	4.8	5.1	4.2	0
3.8	3.8	4.1	3.5	0
2.2	4.3	4.7	3.6	0
0	3.6	3.5	3.3	0
4	3.9	3.7	2.2	0
3.2	3.5	3.8	2.2	0
2.9	4.3	4.6	4.6	2.3
0	4.1	5	0	0
3.1	4.2	4.6	3.4	2.7
0	4.4	5.2	5	0
0	4.1	5.1	3.9	0
0	4.1	4.2	4.5	0

[a] 0 indicates that no *A. fumigatus* was isolated.

Effects on the growth of *A. fumigatus* and ergosterol biosynthesis in *A. fumigatus* and *C. albicans*: The effects of the compounds tested on growth of *A. fumigatus*, and the incorporation of [^{14}C]acetate into ergosterol by *C. albicans* and *A. fumigatus* were measured as described previously.[48]

Azoles and [^{14}C]acetate were added to cultures of *A. fumigatus* immediately after inoculation. Cells were collected immediately after 16 h of growth, homogenized, protein content (a measure of growth; IC_{50} values: concentrations needed to achieve 50% decrease in protein content) determined, and lipids extracted. Sterols were separated by HPLC. *C. albicans* was first grown for 16 h (including 8 h in a reciprocal shaker) at 30 °C in polypeptone/yeast extract/glucose (10:10:40 g L^{-1}) medium, then washed and resuspended in a 0.1 M potassium phosphate buffer containing 56 mM glucose (pH 6.5). [^{14}C]Acetate, azole antifungal agents, and/or DMSO were added, and the cell suspensions were incubated for 2 h at 30 °C. Sterols were extracted and separated by TLC. IC_{50} values: concentrations needed to inhibit incorporation of the ^{14}C label into ergosterol by 50%.

Acknowledgements

The authors thank Ms. Ann Dierckx for technical assistance.

Keywords: antifungal agents · disseminated aspergillosis · metabolites · saperconazole · triazoles

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Received: January 30, 2010
Revised: March 4, 2010
Published online on April 8, 2010

