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ARTICLE

Determination of Stereoselective Interaction between Enantiomers of Chiral γ-Aryl-1*H*-1,2,4-triazole Derivatives and *Penicillium digitatum*

Xiufang Cao,[†] Ming Hu,[†] Jie Zhang,[‡] Fei Li,[†] Yuhong Yang,[†] Deli Liu,*[,‡] and Sheng Hua Liu*[,†]

[†]Key Laboratory of Pesticide and Chemical Biology, Ministry of Education, College of Chemistry, Central China Normal University, Wuhan 430079, P. R. China, and [‡]College of Life Science, Central China Normal University, Wuhan 430079, P. R. China.

A series of chiral γ-aryl-1*H*-1,2,4-triazole derivatives has been synthesized and the respective analogues have been tested for their inhibitory activities against *Penicillium digitatum* (*P. digitatum*). In vitro experiments were indicative of a strong inhibitory effect of all of the compounds on *P. digitatum*, and seven of the compounds **5** exhibited better inhibition than the commercial fungicides triadimefon and triadimenol. The respective pairs of enantiomers showed significantly different inhibitory activities, most notably in the case of **5g-*R*** and **5g-*S***, for which a 230-fold difference was observed. These observations suggest that *P. digitatum* discriminates the enantiomers and that the *R* enantiomer better fits the active site of cytochrome P450.

KEYWORDS: Chirality; triazole; *P. digitatum*; CYP51; fungicide; binding constant

INTRODUCTION

Green mold, caused by *Penicillium digitatum*, is the most important postharvest disease of citrus fruits. This pathogen occurs in almost all regions of the world where citrus crops are grown and causes serious postharvest losses annually (*1*−*3*). Application of synthetic fungicides is the preferred method to control postharvest diseases caused by fungal phytopathogens in fruits and vegetables (*4*−*6*). For example, *o*-phenylphenol (OPP), sodium *o*-phenylphenate (SOPP), diphenyl (DP), thiabendazole (TBZ), imazalil (IMZ) (*7*), pyrimethanil (PYR), and prochloraz are widely used to control postharvest decay caused by various fungal pathogens during the storage and marketing of citrus fruit (**Figure 1**) (*8*, *9*). They may be used alone, combined in mixtures, or applied separately in sequence. Methods such as heating the fungicide solution or adding $NaHCO_3$ (*10*−*13*) have been introduced with the aim of improving the effectiveness of the fungicides so that they offer some control of resistant isolates. Resistance to SOPP among *P. digitatum* was first reported within a few years of its introduction, more than 50 years ago. Resistance to TBZ appeared about 2 years after its introduction in 1968. Resistance to IMZ developed more slowly and was first noted among *P. digitatum* isolates collected in California citrus packing houses in 1986, about 6 years after it was introduced. When resistance to a fungicide is detected, an alternative fungicide with a different mode of action should be developed and used (*8*, *14*). Recently, two new postharvest fungicides, pyrimethanil (PYR) (*15*−*18*) and fludioxonil, were introduced into the citrus industry, primarily for use in alternating strategies with the existing fungicides.

The agrochemical industry is continuously searching for new active pesticide compounds. The main goal of this research is to develop substances with lower application doses, increased selectivity, and reduced undesired ecological impact (*19*). Currently, chiral pesticides occupy about 25% of all pesticides used, and this proportion is continuously increasing as more complex structures are introduced. For chemical and economic reasons, chiral pesticides are primarily used as mixtures of enantiomers (*20*−*22*). However, the biological activity of chiral substances often depends upon their stereochemistry, since the living body is a highly chiral environment. The enantiomers usually differ in their biological properties as a result of their interaction with enzymes or other naturally occurring chiral molecules (*23*). Nowadays, both the public and the health authorities have become increasingly concerned about the presence of fungicides in food and the release of residues to the environment. As a direct result, research effort has been directed toward the development of alternative methods for the synthesis of chiral pesticides.

Triazole compounds constitute some well-known fungicides that are used in agriculture to control a wide range of fungi on fruit and vegetables. However, often because of chemical reasons, triazoles are produced and applied as racemates. In fact, only one enantiomer/diastereomer in many chiral triazoles is highly fungicidally active (*20*). Recently, we obtained a novel series of chiral γ-aryl-1*H*-1,2,4-triazole derivatives (**Figure 2**) by using a chiral auxiliary as a controlling reagent, and in our previous paper (*24*) we reported that this class of compounds displayed comparably high inhibitory rates to commercial fungicides and a broad spectrum of activity. To gain further insight into the biological

*To whom correspondence should be addressed. For S.H.L.: e-mail, chshliu@mail.ccnu.edu.cn. For D.L.: e-mail, deliliu2002@yahoo.com.cn.

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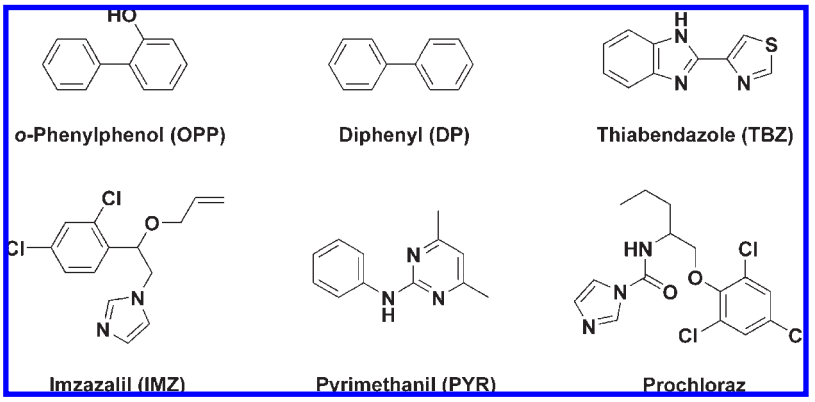


Figure 1. Some representative structures of fungicides for citrus fruits.

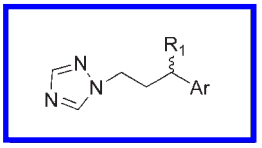


Figure 2. Chiral γ-aryl-1*H*-1,2,4-triazole derivatives.

activity of these compounds, we have now tested their activity against *P. digitatum*.

As is well-known, triazole fungicides are ergosterol-biosynthesis-inhibiting (EBI) fungicides, which interfere with the cellular permeability of pathogenic fungi. Like other azole derivatives, they belong to the demethylation inhibitor (DMI) group of fungicides, which inhibit a cytochrome P450 dependent enzyme that is involved in fungi cell-wall synthesis (*25*, *26*). DMI fungicides are the main group of fungicides for agricultural and medical use (*27*−*29*). For exploratory investigations aimed at finding novel DMIs, assessment of the binding ability of chemical compounds to the target enzyme CYP51 offers a quick screening method (*30*). Cytochrome P450 isoenzyme 51 (CYP51/lanosterol-14α-demethylase) belongs to a family of phylogenetic highly conserved monooxygenases found in mycobacteria, fungi, plants, animals, and humans (*31*, *32*). To date, many different methods have been reported for measurement of the interaction between DMI fungicides and CYP51, such as GC/MS (*33*), HPLC (*34*), isotope tracer labeling, TLC scanning (*35*), and binding spectrum measurements (*36*), and so on. Among these methods, the binding spectrum method is simple and quick. The affinity of a drug to the target enzyme CYP51 can be quantitatively measured through identifying the shift in the binding spectrum, thereby allowing assessment of the potential effect of the compound as a DMI fungicide.

In this study, we have evaluated the inhibitory activities of chiral γ-aryl-1*H*-1,2,4-triazole derivatives against *P. digitatum* using the binding spectrum method and have compared the effects of the respective enantiomers on growth inhibition activity against *P. digitatum*.

MATERIALS AND METHODS

Chemistry. Unless otherwise noted, all materials were commercially available and were used directly without further purification. All solvents were redistilled before use. *P. digitatum* was provided through the courtesy of the Center for Bioassay, Central China Normal University. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Mercury-Plus 400 spectrometer at 400 or 600 MHz, respectively. The chemical shifts are reported relative to $CDCl_3$ with TMS as the internal reference. MS spectra were determined using a TraceMS 2000 organic mass spectrometer. Elemental analyses were performed on a Vario EL III elemental analysis instrument. Optical rotations were measured on a JASCO P-1010 polarimeter. Melting points were taken on a Buchi B-545 melting point apparatus and uncorrected.

General Synthesis for Target Compounds. The chemical and physical data of compounds **5a**−**j** were reported in the literature (*24*), and these data of newly synthesized derivatives **5k**, **5l** are reported in Supporting Information together with the intermediates **2k**−**4k** and **2l**−**4l**, which were synthesized as reported procedures (*24*, *37*).

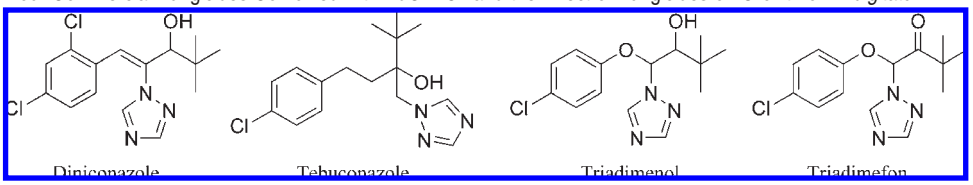
Antifungal Activity Determination. (Growth inhibition test) The in vitro fungicidal activities against *P. digitatum* were tested according to the reported method (*24*, *38*). The medium was amended with aliquots of each tested compounds solution to provide concentration of 50 mg/L. The tested compounds were dissolved in 0.3 mL of DMSO (dimethyl sulfoxide) and added aseptically to molten agar after autoclaving, when the agar had cooled to approximately 45−50 °C. The concentration of solvent never exceeded 0.1 mg/L. The mixed medium without sample was used as the blank control. The inocula, 5 mm in diameter, were removed from the margins of actively growing colonies of mycelium, placed in the centers of the above plates. Three replicates were done for each concentration, and the control plates were sealed with parafilm and incubated at 26 °C in darkness. The diameter of the mycelium was measured for 48 h. The inhibition percent was used to describe the control efficiency of the compounds. Inhibition percent (%) = (hyphal diameter in the control − hyphal diameter in the treatment)/hyphal diameter in the control.

Preparation of the Heterogeneous Expressed CYP51 Protein of *P. digitatum*. Cloning and expression of PdCYP51 was carried out by using reverse transcription PCR (RT-PCR) with the purified mRNA as a template isolated from *P. digitatum* (*39*). The amplified PdCYP51 cDNA fragment was cloned into pMD-T vecter for sequencing and then subcloned into pET-28 for expression. The recombinant plasmid pET-PdCYP51 was transformed into *Escherichia coli* BL21 (DE3), and the positive colonies were selected. The expression of recombinant protein in *E. coli* BL21 (DE3) was induced by 0.5 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) for 6 h at 25 °C before harvesting at 5000 g for 10 min. After the being harvested, cells were washed twice with 100 mM phosphate-buffered saline (PBS) and suspended in buffer A (50 mM KH_2PO_4, 50 mM K_2HPO_4, 20% glycerol, 1 mM EDTA, 0.5% Triton X-100) with 1 mM dithiothreitol (DTT) and 1 mM PMSF. The cells were lysed by sonication on ice, in a UP200S cell disruptor at an intensity of 60 W for 5 min, with 30 s rest period between each burst. The lysate was centrifuged at 15000*g* (30 min, 4 °C), and the supernatant containing soluble cellular material was purified using the Ni-NTA affinity resin according to the manufacturer's protocol with certain modifications. All the buffers were adjusted to pH 7.2. The purified protein was dialyzed into 10 mM PBS (pH 7.2) to desalt, concentrated, and stored at −80 °C until use (*40*).

Determination of Proteins Content. The protein concentration was determined by the Bradford method using bovine serum albumin (BSA) as the protein standard (*41*).

Determination of CYP51 Activity and Binding Spectrum Analysis. According to the methods described in the literature (*42*), the CYP51 content and the activity were determined. The solutions of CYP51 were prepared with 100 mM PBS (pH 7.2), and UV−visible absorption spectra

Table 1. K_d Values of Four Commercial Fungicides Combined with PdCYP51 and the Effect of Fungicides on Growth of *P. digitatum*



fungicide	*Kd* (μM)	EC_{50} (mg/L)	inhibitory rate (%) (50 mg/L)
diniconazole	0.12	0.09	100
tebuconazole	0.32	0.37	100
triadimenol	0.64	1.44	100
triadimefon	0.99	2.27	100

were recorded for the oxidized species on a UV-240 IPC spectrophotometer at 25 °C. CYP51 solutions were reduced using a small amount of sodium dithionite and bubbled briefly with carbon monoxide to form the P450−CO complex and measure the precise P450 concentration at 25 °C (*43*). The purified soluble PdCYP51 was diluted to appropriate concentration and fill into a cuvette. After its baseline was scanned at 350−500 nm on an S-3100 spectrophotometer, the drug was added to the cuvette. After staying for 1 min under room temperature the absorption spectrum of the mixture was recorded (concentration of DMSO in mixture was maintained $<0.5\%$). The binding constant K_d was calculated by the following equation and Hanes−Woolf (*44*) chart [I] versus [I]/ΔA:

$$A = A_{max}[I]/(K_d+[I])$$

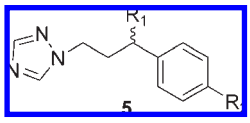
In the above equation, A is the value of the maximum, subtracting the minimum observed shift in absorption at different drug concentrations, A_{max} is the value of the maximum subtracting the minimum observed shift in absorption at saturation, [I] is drug concentration, and K_d is the binding constant.

RESULTS AND DISCUSSION

According to the previously described method, we evaluated the K_d values of four commercial fungicides toward PdCYP51 as well as their EC_{50} values, and the results are shown in **Table 1**. It was found that all four of these fungicides totally inhibited the growth of *P. digitatum* at a concentration of 50 mg/L. The 48 h EC_{50} values of the four fungicides on the growth of *P. digitatum* are shown in **Table 1**. Among the four fungicides tested, diniconazole showed the strongest antifungal effect on *P. digitatum*, with an EC_{50} value of 0.09 mg/L, while triadimefon was the least effective. As can also be seen from **Table 1**, the binding constants, K_d, of diniconazole, tebuconazole, triadimenol, and triadimefon were 0.12, 0.32, 0.64, and 0.99 μM, respectively. In other words, the affinities of the four fungicides to CYP51 of *P. digitatum* decreased in the order diniconazole > tebuconazole > triadimenol > triadimefon, consistent with the results of the growth inhibition experiments. These results indicated that the binding constants, K_d, determined by the established binding spectrum method did indeed reflect the antifungal activities of the fungicides.

The binding spectra analysis was based on the spectral characteristics of cytochrome P450 and the shift thereof upon ligand binding. There are different types of binding spectra, designated as type I and type II. Type I is characterized by the appearance of a trough at 420 nm and an absorption peak at 385−390 nm; however, type II presents a trough at 390−400 nm and an absorption peak at 430 nm (*45*). The spectra obtained with diniconazole, triadimenol, and triadimefon when binding to heterologously expressed CYP51 of *P. digitatum* (**Figure 3**) showed a maximum absorption at 420−430 nm and a minimum absorption at 390−410 nm, conforming to type II, whereas that obtained with tebuconazole showed an absorption peak at 390 nm and a trough at 420 nm, thus conforming to type I. The shifts of the spectra were possibly due to interference from other components on the membrane, and moreover, there are certain shifts for cytochrome P450 binding with different compounds.

Table 2. K_d Values of Compounds **5** Combined with PdCYP51 and the in Vitro Fungicidal Activities (50 mg L^{-1}, Inhibitory Rate Percent)



entry	compd	R_1	R_2	configuration	ee (%)[a]	K_d (μM)	inhibitory rate (%)[b]
1	**5a-*R***	Et	H	*R*	98	0.034	84 ± 2
2	**5a-*S***	Et	H	*S*	98	1.655	20 ± 3
3	**5b-*R***	Et	F	*R*	82	0.081	88 ± 1
4	**5b-*S***	Et	F	*S*	85	0.23	78 ± 2
5	**5c-*R***	Et	Cl	*R*	84	0.15	92 ± 1
6	**5c-*S***	Et	Cl	*S*	80	1.14	23 ± 3
7	**5d-*R***	Et	OMe	*R*	>99	0.063	89 ± 2
8	**5d-*S***	Et	OMe	*S*	70	0.505	77 ± 2
9	**5e-*R***	*n*-Pr	H	*R*	92	0.14	90 ± 1
10	**5e-*S***	*n*-Pr	H	*S*	95	0.21	79 ± 2
11	**5f-*R***	*n*-Pr	F	*R*	82	0.25	89 ± 1
12	**5f-*S***	*n*-Pr	F	*S*	84	no binding	78 ± 2
13	**5g-*R***	*n*-Pr	OMe	*R*	72	0.15	90 ± 1
14	**5g-*S***	*n*-Pr	OMe	*S*	86	no binding	<5
15	**5h-*R***	*n*-Bu	H	*R*	80	0.76	73 ± 2
16	**5h-*S***	*n*-Bu	H	*S*	88	no binding	65 ± 3
17	**5i-R**	*n*-Bu	F	*R*	86	0.09	100 ± 1
18	**5i-*S***	*n*-Bu	F	*S*	84	0.15	95 ± 1
19	**5j-*R***	*n*-Bu	Cl	*R*	96	0.35	88 ± 1
20	**5j-*S***	*n*-Bu	Cl	*S*	97	0.34	83 ± 2
21	**5k-*R***	*n*-Bu	OMe	*R*	88	0.049	100 ± 1
22	**5k-*S***	*n*-Bu	OMe	*S*	96	0.18	97 ± 1
23	**5l-R**	*n*-Bu	Me	*R*	88	0.046	93 ± 1
24	**5l-*S***	*n*-Bu	Me	*S*	92	1.22	87 ± 2

[a] Excesses of enantiomers, which were determined by HPLC analysis (Chiralcel OJ-H or OD-H). [b] Mean value for relative inhibition calculated from at least three determinations.

The compounds of the synthesized series of chiral γ-aryl-1*H*-1,2,4-triazole derivatives were then tested for their inhibitory activity against *P. digitatum*. The results are presented in **Table 2** and can be compared with those for the four commercial antifungals (diniconazole, tebuconazole, triadimenol, and triadimefon). Most of the compounds **5** displayed a good inhibitory effect against *P. digitatum* (inhibition rates of 65−100% at 50 mg/L) except for **5a-*S***, **5c-*S***, and **5g-*S***. For example, compounds **5c-*R***, **5e-*R***, **5g-*R***, **5i-*S***, **5k-*S***, and **5l-*R*** displayed inhibition rates of 92%, 90%, 90%, 95%, 97%, and 93%, respectively, at a dosage of 50 mg/L, and most notably, compounds **5i-*R*** and

Table 3. In Vitro fungicidal Activities of Compounds **5** Comparison with Four Commercial Fungicides Expressed as 50% Effective Concentration (EC_{50}, mg/L)

compd	R_1	R_2	configuration	ee (%)[a]	K_d (μM)	EC_{50} (mg/L)[b]	*R*/*S* (EC_{50})
5a-*R*	Et	H	*R*	98	0.034	0.577	1:155
5a-*S*	Et	H	*S*	98	1.655	89.58	
5c-*R*	Et	Cl	*R*	84	0.15	0.587	1:104
5c-S	Et	Cl	*S*	80	1.14	61.15	
5g-*R*	*n*-Pr	OMe	*R*	72	0.15	0.603	1:230
5g-*S*	*n*-Pr	OMe	*S*	86	no binding	138.4	
5i-*R*	*n*-Bu	F	*R*	86	0.09	0.88	1:1.3
5i-*S*	*n*-Bu	F	*S*	84	0.15	1.15	
5k-*R*	*n*-Bu	OMe	*R*	88	0.049	0.62	1:1.6
5k-*S*	*n*-Bu	OMe	*S*	96	0.18	0.97	
diniconazole					0.12	0.09	
tebuconazole					0.32	0.37	
triadimenol					0.64	1.44	
triadimef on					0.99	2.27	

[a] Excesses of enantiomers, which were determined by HPLC analysis (Chiralcel OJ-H or OD-H). [b] Mean value for relative inhibition calculated from at least three determinations.

5k-*R* displayed 100% inhibition rates. These compounds showed tight binding of PdCYP51, giving low K_d values of 0.046−0.18 μM. The results have indicated that recording the binding spectra of the fungicides with lanosterol 14α-demethylase can serve as a reliable and fast method for the screening of novel fungicides, which further implies that the binding constants, K_d, adequately reflect the antifungal activity of the fungicides.

It is well-known that the substitution of hydrogen by a halogen atom (such as fluorine) or an electron-donating substituent (methyl or methoxy) at the para-position of a benzene ring can alter biological activity. As can be seen from entries 1−8 and 15−24 in **Table 2**, the compounds **5** with an unsubstituted benzene ring showed lower inhibitory activities against *P. digitatum*. On the other hand, entries 9−14 in **Table 2** suggest that the substituents of the benzene ring have less influence on the biological activity with R_1 as *n*-Pr. It is also worthy of note that the compounds **5** with R_1 as *n*-Bu generally showed higher antifungal activities.

Interestingly, the results shown in **Table 2** are indicative of different biological activities of the enantiomers; the (*R*)-enantiomers of **5** displayed strong inhibitory activity against *P. digitatum*, whereas the (*S*)-enantiomers were weaker inhibitors against this

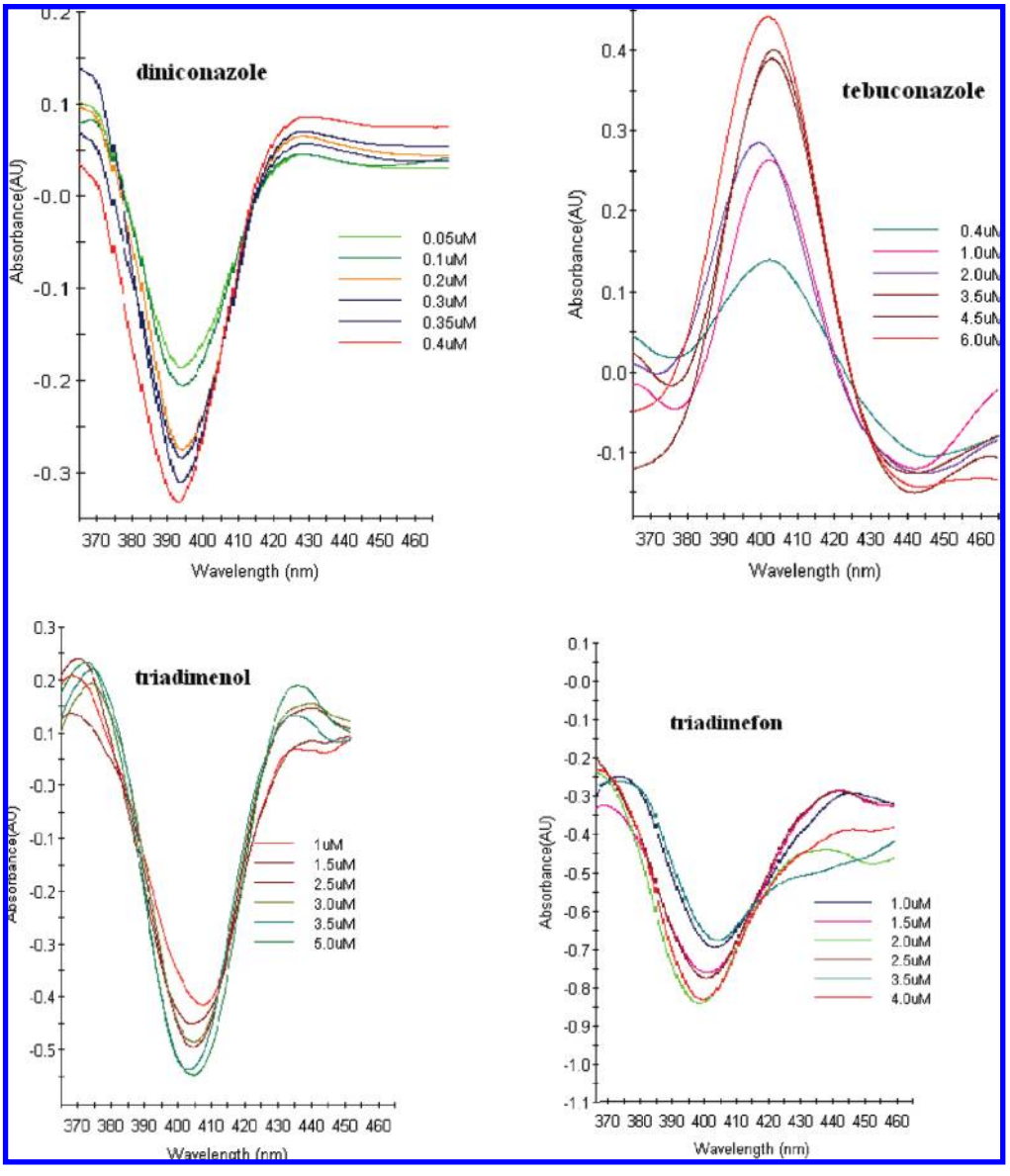


Figure 3. Difference spectra of diniconazole, tebuconazole, triadimenol, and triadimefon binding to CYP51 of *P. digitatum*, respectively.

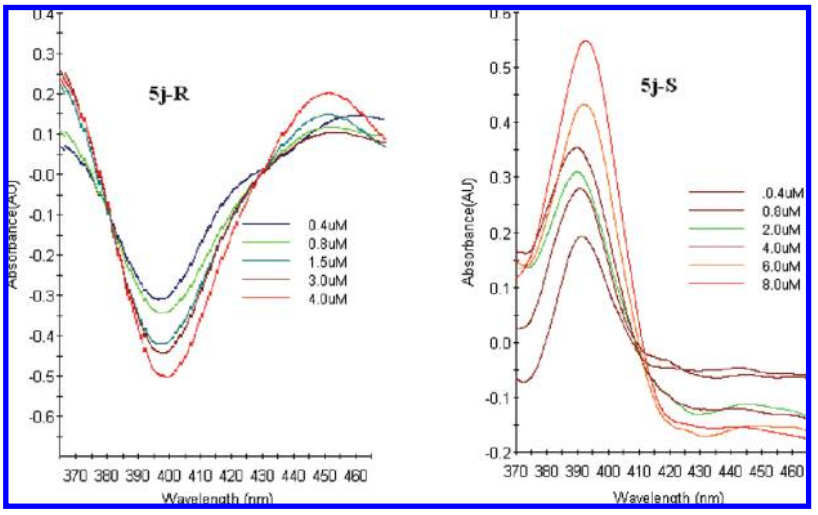


Figure 4. Difference spectra of **5j-*R* and 5j-*S* binding to CYP51 of *P. digitatum*, respectively.**

pathogen, especially in the cases of compounds **5a**, **5c**, and **5g**. The (*R*)-enantiomers combined with PdCYP51 more tightly than the (*S*)-enantiomers. These observations suggest that PdCYP51 discriminates the enantiomers of compounds **5** and that the (*R*)-enantiomers more favorably fit the active site of the PdCYP51.

In order to further investigate the activity differences of the enantiomers, EC_{50} values were evaluated and compared to those of the four standard commercial antifungals (diniconazole, tebuconazole, triadimenol, and triadimefon). The EC_{50} values of the target compounds **5a**, **5c**, **5g**, **5i**, and **5k** against *P. digitatum* are presented in **Table 3**. The results further testify that most of the synthesized triazole derivatives **5** exhibited higher inhibition activity than the commercial triadimefon and triadimenol under the same conditions. More importantly, various activity differences are evident between the respective enantiomers. For example, the enantiomers of compound **5a** exhibit significant activity differences against *P. digitatum*, with the EC_{50} value of the (*R*)-enantiomer **5a-*R*** being 0.577 mg/L and that of the (*S*)-enantiomer **5a-*S*** being 89.58 mg/L. Similarly, the enantiomers of compound **5c** also show distinctly different levels of inhibition, with the (*R*)-enantiomer **5c-*R*** showing better inhibition activity than the (*S*)-enantiomer **5c-*S***, the respective EC_{50} values against *P. digitatum* being 0.59 and 61.1 mg/L. The most remarkable result is the huge difference in the biological activities of the enantiomers of compound **5g**, the activity of **5g-*R*** being about 230-fold greater than that of **5g-*S***. Indeed, **5g-*S*** showed no binding to the CYP51 of *P. digitatum*, as shown in **Table 3**. Conversely, the enantiomers of compounds **5i** and **5k** display little difference in terms of their activities against *P. digitatum*, the EC_{50} values of both enantiomers being low at about 1 mg/L, implying that they might be further developed as fungicides.

The spectra obtained for the chiral γ-aryl-1*H*-1,2,4-triazole derivatives (compounds **5** derivatives) upon binding to heterologously expressed CYP51 of *P. digitatum* conformed to type II, showing a maximum absorption at 420−430 nm and a minimum absorption at 385−400 nm except for that obtained with compound **5j-*S***, which showed a maximum absorption at 390 nm and a minimum absorption at 420 nm, conforming to type I. Interestingly, the two enantiomers of **5j** gave rise to different types of spectra (**Figure 4**), possibly reflecting different binding modes to PdCYP51, although the binding constants, K_d, of **5j-*R*** and **5j-*S*** were of the same order. In the cases of compounds **5f**, **5g**, and **5h**, the (*R*)-enantiomer bound to the PdCYP51, but the (*S*)-enantiomer did not.

The antifungal tests reported here indicated that most of the chiral γ-aryl-1*H*-1,2,4-triazole derivatives (compounds **5** derivatives) have excellent antifungal activities against *P. digitatum*, better than those of some commercial antifungal agents (triadimefon and triadimenol), suggesting that they have potential to be developed as highly potent fungicides.

Supporting Information Available: The chemical and physical data of newly synthesized derivatives **5k**, **5l**, and the intermediates **2k**−**4k** and **2l**−**4l**. This material is available free of charge via the Internet at http://pubs.acs.org.

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