CYP53A15 of *Cochliobolus lunatus*, a Target for Natural Antifungal Compounds†

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A novel cytochrome P450, CYP53A15, was identified in the pathogenic filamentous ascomycete *Cochliobolus lunatus*. The protein, classified into the CYP53 family, was capable of para hydroxylation of benzoate. Benzoate is a key intermediate in the metabolism of aromatic compounds in fungi and yet basically toxic to the organism. To guide functional analyses, protein structure was predicted by homology modeling. Since many naturally occurring antifungal phenolic compounds are structurally similar to CYP53A15 substrates, we tested their putative binding into the active site of CYP53A15. Some of these compounds inhibited CYP53A15. Increased antifungal activity was observed when tested in the presence of benzoate. Some results suggest that CYP53A15 O-demethylation activity is important in detoxification of other antifungal substances. With the design of potent inhibitors, CYP53 enzymes could serve as alternative antifungal drug targets.

Introduction

Benzoic acid derivatives and other phenolic compounds (e.g., eugenol, isoeugenol, vanillin, thymol) play a crucial role in plant resistance to fungal infection.[1] The inhibitory antifungal action of benzoate has been proposed to be due to membrane disruption, inhibition of essential metabolic reactions, stress on intracellular pH homeostasis, accumulation of toxic anions, or the induction of an energetically expensive stress response.[2]

Nevertheless, benzoate is one of the key intermediates in the metabolism of aromatic compounds in fungi.[3] It is detoxified via the β-ketoadipate pathway.[4] The β-ketoadipate pathway is a convergent pathway for the degradation of aromatic compounds and is present in many soil bacteria and fungi, especially those associated with plants. There are several reports that ascomycetes and basidomycetes degrade aromatic compounds such as phenylalanine, toluene, and cinnamic acid via benzoate.[3,5,6] In all fungi studied, the sole pathway of benzoate metabolism is through the hydroxylation of benzoic acid to 4-hydroxybenzoate, leading to protocatechuate as the ring fission substrate.[7]

Enzymes with benzoate para-hydroxylase activity are cytochromes P450 belonging to the CYP53 family. The ascomycete *Aspergillus niger* CYP53A1 was the first enzyme shown to catalyze para-hydroxylation of benzoic acid.[8] Notably, CYP53A1 also exhibits O-demethylation activity. With benzoate para-hydroxylase (bph[a]) gene deletion mutant studies in *A. nidulans*, it was confirmed that benzoate para-hydroxylase is an essential enzyme for benzoate detoxification.[9]

In the present study we describe cloning and characterization of the first cytochrome P450, CYP53A15, from the filamentous fungus *Cochliobolus lunatus*, a known plant and opportunistic human pathogen. To guide functional analyses, the three-dimensional structure of the protein was predicted by homology modeling. Since many naturally occurring antifungal phenolic compounds are similar in structure to CYP53 substrates, we tested their putative binding into the active site of CYP53A15.

Natural compounds could serve as effective alternatives to conventional antifungal or antimycotic agents, which present a hazard to human health and the environment. Conventional antifungal therapy is mainly based on inhibitors directed toward one enzyme, CYP51, or 14α-demethylase.[10] This cytochrome P450 is a key enzyme in the synthesis of ergosterol. Through structural modeling and in vivo inhibitor testing, we suggest a new cytochrome P450 drug target, conserved in many pathogenic fungi.

Results and Discussion

CYP53A15 Gene. A putative cytochrome P450 benzoate para-hydroxylase gene was identified in the filamentous fungus *Cochliobolus lunatus* and tentatively designated as *bph*. In the 1560 bp gene, the presence of a 54 bp intron was confirmed with the cDNA transcript sequence. The *C. lunatus* benzoate para-hydroxylase gene sequence was stored under Genbank accession number EU597483. The cytochrome P450 nomenclature committee classified the corresponding amino acid sequence into the CYP53 family and designated the protein as CYP53A15.

Putative fungal cytochrome P450 genes, encoding proteins with high similarity to the CYP53 family, are now available from genome sequencing projects. CYP53 proteins of filamentous ascomycetes (e.g., *Aspergillus* sp., *Fusarium* sp.) and basidiomycetes (e.g., *Ustilago maydis*, *P. chrysosporium*, *R. minuta*) share close to 50% amino acid identity, which is comparable to amino acid identity of well-known house-keeping enzymes, such as 14α-demethylase (CYP51), of the same fungal species. The benzoate para-hydroxylase activity has evidently been conserved in genera, having an important function in the life of fungi.[11] Since *C. lunatus* var. *Curvularia lunata* is a

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[a] Abbreviations: bp, base pair; bph, benzoate para-hydroxylase; CPR, NADPH-cytochrome P450 reductase; K_i, constant of inhibition; MIC, minimum inhibitory concentration; NADPH, nicotinamide adenine dinucleotide phosphate; SAR, structure−activity relationship; RACE, rapid amplification of cDNA ends; DIG, digoxigenin; RGR, radial growth rate; IGI, initial growth inhibition.

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pathogen, the benzoate para-hydroxylase found was tested as a putative antifungal target.

Functional Expression in *E. coli*, Purification, and Catalytic Properties. The full-length CYP53A15 cDNA encoding 501 amino acid residues with a calculated molecular weight of 60 kDa was cloned and expressed in *E. coli*. The CO difference spectra of the isolated and purified protein with a characteristic maximum at 450 nm confirmed expression of a functional enzyme.

To optimize the expression procedure, several parameters were tested. Selection of the *E. coli* strain was found to be the most significant variable. The use of C43 (DE3) strain increased the expression level of CYP53A15 from 300 nmol/L in the Bl21(DE3) strain to 800−900 nmol/L in C43 (DE3) under the same growth conditions, probably because of differences in plasmid stability.[12] Two modifications of the N-terminal part of the protein were designed in order to achieve optimal expression levels: a protein with the Barnes modification of eight N-terminal amino acids[13] and an N-terminally truncated form. Yet there were no significant differences in expression levels of the two N-terminally modified proteins compared to the wild type.

The protein was purified to apparent homogeneity in a single chromatographic step. The deletion of the N-terminus was expected to increase solubility and purification yields; however, the opposite was true. A large portion of the truncated enzyme remained in the membrane fraction as well. The truncated form was less stable and more prone to proteolyzis. In addition, a higher portion of the inactive P420 form (increased absorption at 420 nm in CO difference spectra) was found during the purification procedure. An elevated amount of the dimeric form of the truncated enzyme was observed by SDS−PAGE analysis of the membrane fractions, probably due to aggregation.

Catalytic properties of the purified CYP53A15 enzyme were investigated in a reconstitution system with mammalian NADPH-cytochrome P450 reductase (CPR) in vitro. The results of HPLC analyses of the reaction mixture confirmed conversion of benzoic acid to para-hydroxybenzoic acid. K_M and k_{cat} values were 0.4 ± 0.2 mM and 1.4 ± 0.5 μmol min^{-1}, respectively. The benzoate hydroxylation turnover number was 1.4 ± 0.5 min^{-1}, which is rather low in comparison to 240 min^{-1} obtained in *A. niger* microsomes containing CYP53A1.[8] In this study, however, only 1% of activity of the microsomal fraction was preserved when the purified CYP53A1 was reconstituted with its natural redox partner, CPR from *A. niger*. The low turnover could be due to the instability of the enzyme and suboptimal reconstitution with its redox partner.

3D Structural Homology Model. To guide and facilitate functional analyses, a 3D structural homology model was constructed on the basis of structural alignment of amino acid residues lining the active site cavity of two human drug metabolizing cytochromes P450 2C8 (PDB code 1PQ2) and 2A6 (PDB code 1Z10). Despite the low amino acid identity (21%) between template candidates and CYP53A15, extensive refinement and dynamic simulations performed on the homology model resulted in a very stable and, according to PROCHECK, a high stereochemical quality protein structure. The active site predicted by the model is well suited for accommodation of small planar aromatic compounds (Figure 1). A favorable interaction is evident between the π-electron system of Phe-80 and the aromatic ring of substrates such as benzoic acid. Phe-451, which lies perpendicularly to Phe-80, is also involved in a hydrophobic reaction with substrates. Ile-274 and Leu-452 close the entrance to the iron in the active site and do not allow bulkier side substituents on the aromatic ring. The slightly asymmetric shape of the cavity could, however, be suitable for monosubstituted substrates.

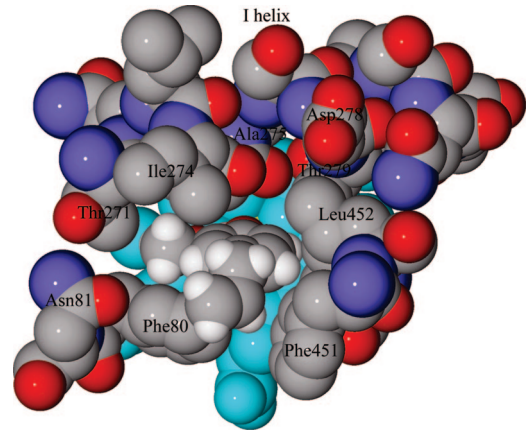


Figure 1. Space-fill model of CYP53A15 active site with docked isoeugenol. Hydrogen atoms are omitted from protein residues and from the heme moiety (cyan). The hydroxyl group of isoeugenol is situated just above the iron (small yellow spot), and the methoxy group is sandwiched between Phe-80 and Ile-274. Leu-452 is in close contact with the phenol ring, thus preventing the entrance of ligands with bulky substituents at both meta positions (PMDB ID: PM0075149).

Although not used as a template in homology modeling, the crystal structure of another human cytochrome P450, 2D6 (PDB code 2F9Q),[14] is strikingly similar to the predicted active site of CYP53A15. 2D6 generally recognizes substrates with a flat hydrophobic region, a negative molecular electrostatic potential, and a basic nitrogen.[15] Typical reactions include hydroxylation and O-demethylation. As is evident from a comparison of the active sites of both enzymes (Figure 2), several amino acids reported as key residues in 2D6 such as Phe-120 and Phe-483 are similarly positioned in CYP53A15 (Phe-80 and Phe-451, respectively). Thr-279 of CYP53A15 could be aligned with Thr-309 of 2D6 reported to be crucial in hydrogen-bonding to the water molecule formed from the cleavage of the dioxygen bond during the P450 catalytic cycle.[15]

Structure−Activity Relationship (SAR) of CYP53A15. Thirty putative substrates were tested for their ability to induce substrate binding spectra in CYP53A15 (Table 1A, Supporting Information). Besides benzoic acid, some derivatives of benzoic acid were also shown to induce type I substrate binding spectra in CYP53A15, among others, 2-hydroxy-, 2-chloro-, 2-methylbenzoate, but not 2-methoxybenzoate, 3-hydroxybenzoate, 3-methoxybenzoate, or 4-hydroxybenzoate.

Although putative substrates such as isoeugenol, eugenol, vanillin, vanillic acid, and 3-methoxybenzoic acid did not show substrate binding spectra, they were nevertheless tested with the activity assay. Among them, only 3-methoxybenzoic acid was converted. In this case, O-demethylation activity was observed and 3-hydroxybenzoic acid was detected as a product by HPLC.

Structures of vanillin, eugenol, and isoeugenol possess a common structural motif, the 3-methoxy and 4-hydroxy groups on the phenol ring. We tested whether they could fit into the active site in spite of their side substituents. Subsequent docking of isoeugenol clearly revealed its good accommodation into the cavity of the enzyme. The 3-methoxy group of isoeugenol is sandwiched between two hydrophobic residues, Ile-274 and Phe-80. Leu-452 is in close contact with the phenol ring. This could also provide an explanation of why no substrate binding spectra were obtained for 3-methoxybenzoic acid. A slight disorientation

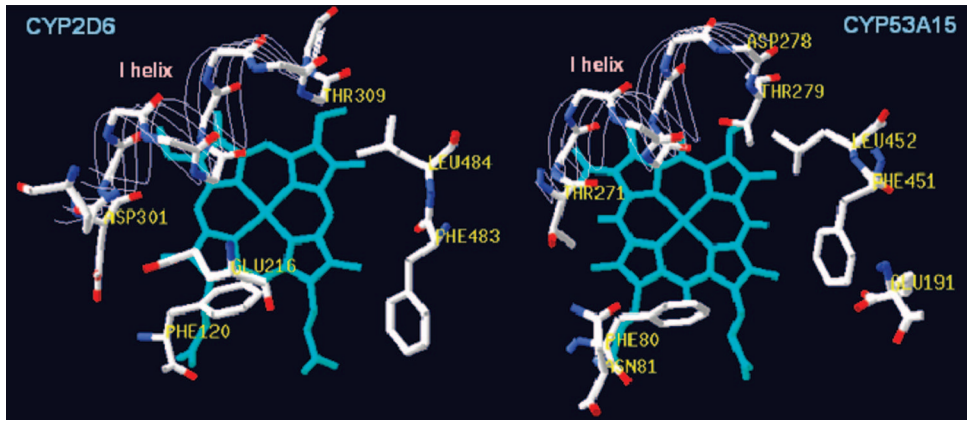


Figure 2. Comparison of key residues in CYP 2D6 and CYP53A15 active sites. Heme is marked in cyan. Key residues involved in positioning of aromatic substrates in CYP 2D6, Phe-120 and Phe-483, are similarly positioned in CYP53A15 (Phe-80 and Phe-451, respectively). Thr-309 of CYP 2D6, crucial in hydrogen-bonding to the water molecule formed during the P450 catalytic cycle, corresponds to Thr-279 of CYP53A15. Basic substrates bind to CYP 2D6 through Asp-301 and Glu-216, whereas in CYP53A15 Asn-81 could form a hydrogen bond with the carboxyl group of substrates.

of the substrate due to the bulkier 3-methoxy group prevents closer contact with the heme prosthetic group. As a consequence, no spin change is induced. Nevertheless, the positioning of substrates still allows for O-demethylation of the 3-methoxy group. It is possible that conformational changes caused by the binding of the redox partner enable proper positioning of 3-methoxybenzoic acid and induce spin state change.

The active site of CYP2A6 used as a template for homology modeling is one of the smallest compared to other human drug metabolizing CYPs. Tighter packing interactions in the molecule result in a more compact structure and less pliant active site cavity.[16] This could also be the case in CYP53A15, since structural requirements for substrates able to occupy the active site and induce spectral shifts were rather strict. Only monosubstituted derivatives of benzoic acid were substrates, whereas 2,4-dichlorobenzoic acid or 3,5-dimethylbenzoic acid were not. Substitutions at position 2 were only allowed for smaller hydroxy, chloro, or methyl but not for the larger methoxy group. On the other hand, the hydroxy group at the meta position was not allowed, but chloro and methyl groups were.

The carboxyl group seems essential for tight binding into the active site, since no substrate binding spectra were obtained with substrates lacking the carboxyl group or substrates with the carboxyl group further away from the aromatic ring, such as L or D-phenylglycine. As is evident from the 3D model, the Asn-81 is available for hydrogen bonding with the carboxyl group of substrates. Asp-278, also located in the active site of CYP53A15, could be involved in proper positioning of substrates possessing the carboxyl group by charge repulsion. Deduced CYP53A15 substrate structure requirements are presented in Table 1B of Supporting Information.

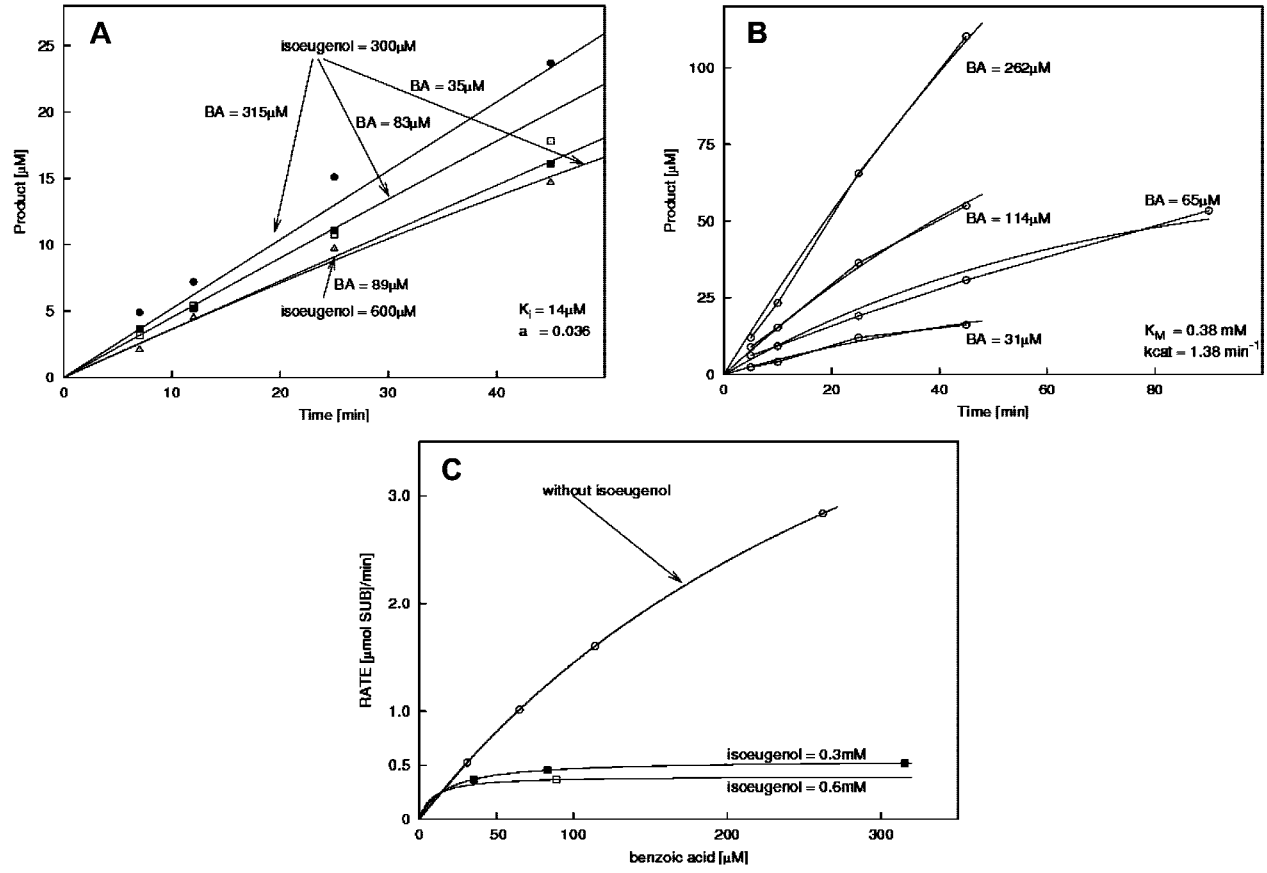
Inhibition of CYP53A15 by Naturally Occurring Antimicrobial Compounds in Vitro. Eugenol, isoeugenol, vanillin, and thymol are naturally occurring phenolic compounds with antifungal properties. Their structures resemble the structures of CYP53A15 substrates (Table 1A, Supporting Information). We tested their effects on benzoic acid hydroxylation by CYP53A15. Substrate binding experiments and activity assays were performed in the presence of different concentrations of these compounds. All substances tested acted as inhibitors of CYP53A15. In Figure 3, progress curves in the absence and presence of isoeugenol are shown. Among several tested kinetic models, the partial noncompetitive inhibition model was chosen for which the best agreement between theoretical curves and the data was obtained. The calculated constants of inhibition (K_i) for eugenol, isoeugenol, vanillin, and thymol and the mode of inhibition were similar for all four compounds, which was expected, given the similarity of their structures.

In Vivo Inhibitor Testing. Eugenol, isoeugenol, vanillin, and thymol were tested in vivo for their ability to inhibit fungal growth. Other naturally occurring phenolic antifungals, such as *trans*-cinnamic acid, *trans*-4-hydroxycinnamic acid, and carvacrol, as well as potassium sorbate, a weak acid, and a potent antifungal compound tropolon β-thujaplicin were taken for comparison. All compounds listed above were tested alone and in combination with benzoic acid. Radial growth rate (RGR, mm/h) and initial growth inhibition (IGI, h) were calculated for every inhibitor and combination (Figure 4). The minimum inhibitory concentration (MIC), which is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation was determined for benzoic acid.[17]

The MIC for benzoic acid was 5 mM. Lower concentrations of benzoic acid did not inhibit fungal growth. When tested at 5 mM, isoeugenol, thymol, *trans*-cinnamic acid, carvacrol, potassium sorbate, and β-thujaplicin all showed growth inhibiton of the *C. lunatus* to a different extent. The most potent inhibitor was β-thujaplicin with an initial growth inhibition time of 88 h. On the other hand, eugenol, vanillin, and *trans*-4-hydroxycinnamic acid effected no growth inhibition at 5 mM. However, when tested in combination with 0.1 mM benzoic acid, which alone had no growth inhibitory effects, vanillin and eugenol did cause growth inhibition. When isoeugenol was combined with 0.1 mM benzoic acid, the inhibition of fungal growth was even more pronounced than for β-thujaplicin (initial growth inhibition time of 145 h).

These observations are in correlation with in vitro experiments in which vanillin, eugenol, and isoeugenol inhibited CYP53A15. An increased toxic effect could be explained by higher intracellular levels of benzoic acid, a consequence of parallel inhibition of CYP53A15. Although inhibition of CYP53A15 in substrate binding experiments was also observed with thymol, the synergistic inhibitory effect on growth in combination with 0.1 mM benzoic acid could not be observed in in vivo experiments.

The effect of benzoic acid and other inhibitors was also tested in vivo in the Δbph strain of *C. lunatus*. Since benzoate para-



K_i values and type of inhibition of CYP53A15 for natural antifungal phenolic compounds.

Constituent	Isoeugenol	Eugenol	Vanillin	Thymol
Ki (μM)	14.2±8.2	26.2±6.4	29.7±7.7	8.2±3.5
Type of inhibition	Partial uncompetitive	Partial uncompetitive	Partial uncompetitive	Partial uncompetitive

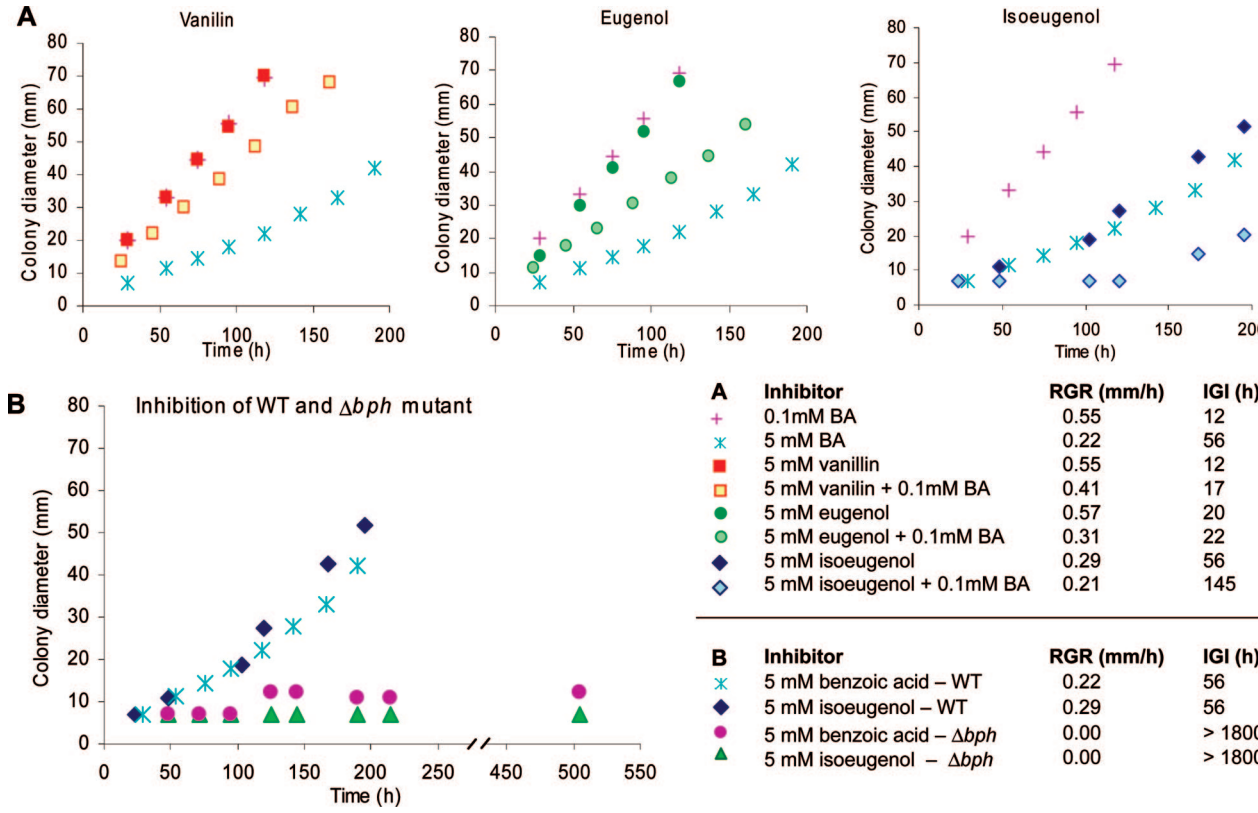
Figure 3. Progress curves for the reaction between CYP53A15 and benzoic acid without inhibitor (A) and in the presence of the inhibitor isoeugenol (B). Results were obtained by quantitative HPLC product analysis of the quenched reaction after different incubation times. A simulation of the direct plot vs [isoeugenol] is shown in part C. Theoretical initial rates and curves were calculated using values of kinetic constants as estimated from the simultaneous progress curves analysis. Inhibition experiments were performed at inhibitor concentrations of 300 and 600 μM; CYP53A15 concentration was 5 μM. The table below shows K_i values and type of inhibition of CYP53A15 for natural antifungal phenolic compounds (benzoic acid, BA).

hydroxylase functions in benzoate elimination, it was expected that the Δ*bph* mutant would be more susceptible to benzoate than the wild type.[9] Results showed great differences in initial growth inhibition time. Complete growth inhibition (more than 1800 h) of the Δ*bph* mutant was achieved, whereas growth inhibition time of the wild type was 56 h. No difference, however, was observed in MIC of benzoic acid (5 mM) for both strains. Growth recovery of the wild type strain as opposed to the Δ*bph* mutant demonstrates the essential nature of CYP53A15 in benzoate detoxification. The time needed for growth recovery of the wild type can be explained by the fact that the benzoate para-hydroxylase is inducible by its substrate.[18]

Surprisingly, the Δ*bph* mutant was completely inhibited with 5 mM isoeugenol alone, while the wild type recovered. The O-demethylation activity of CYP53A15 in the wild type could possibly be important for detoxification of other toxic phenolic compounds with the 3-methoxy moiety such as eugenol, isoeugenol, or vanillic acid. It was reported that vanillic acid is converted to protocatechuic acid via a demethylation reaction in white-rot and brown-rot basidiomycetes.[3,19] The reason that we were not able to detect an O-demethylated product of isoeugenol, eugenol, or vanillic acid could be that they are bad substrates. When tested in an activity assay with benzoic acid, they primarily behaved as inhibitors of CYP53A15. Interestingly, the two closest templates chosen for modeling CYP53A15, cytochromes P450 2C8 and 2A6, are involved in detoxification of small aromatic compounds via hydroxylation, as well as O-demethylation.[20] This is also true of 2D6 whose active site cavity architecture is similar to CYP53A15. A particularly important residue in the active site of 2D6 is Phe-120, considering that the substitution of this single amino acid allowed 2D6 to metabolize its classical inhibitor quinidine.[15] This residue forces quinidine to bind in an unproductive mode. In the case of CYP53A15, Phe-80 located in the active site could play a similar role in unproductive binding of isoeugenol, eugenol, or vanillic acid.

Conclusions

Recent studies on a number of fungal pathogens have demonstrated the effectiveness of natural phenolic compounds as antimicrobials or antimycotoxigenic agents.[21,22] None of these studies, however, clearly determined cellular targets for their activities. Possible modes of action of phenolic compounds have been reported in different reviews.[23] Lambert et al.[24] reported



A	Inhibitor	RGR (mm/h)	IGI (h)
+	0.1mM BA	0.55	12
✳	5 mM BA	0.22	56
■	5 mM vanillin	0.55	12
□	5 mM vanilin + 0.1mM BA	0.41	17
●	5 mM eugenol	0.57	20
○	5 mM eugenol + 0.1mM BA	0.31	22
◆	5 mM isoeugenol	0.29	56
◇	5 mM isoeugenol + 0.1mM BA	0.21	145

B	Inhibitor	RGR (mm/h)	IGI (h)
✳	5 mM benzoic acid – WT	0.22	56
◆	5 mM isoeugenol – WT	0.29	56
●	5 mM benzoic acid – Δbph	0.00	> 1800
▲	5 mM isoeugenol – Δbph	0.00	> 1800

Figure 4. In part A, growth curves of *C. lunatus* in the presence of different inhibitors and in combination of inhibitors with benzoic acid. Calculated values for radial growth rate (RGR, mm/h) and initial growth inhibition (IGI, h) of the *C. lunatus* wild type (WT) in the presence of inhibitors eugenol, isoeugenol, and vanillin are shown in the corresponding table below. In part B, growth curves for WT and Δ*bph* mutant strains in the presence of 5 mM benzoic acid and 5 mM isoeugenol, with calculated values for RGR and IGI, are given (benzoic acid, BA).

that thymol and carvacrol may inactivate essential enzymes, react with the cell membrane, or disturb the functionality of genetic material. Phenolic compounds also strongly influence cellular redox potentials.[25]

We have shown that isoeugenol, eugenol, thymol, and vanillin are inhibitors of benzoate para-hydroxylase, the enzyme responsible for benzoic acid detoxification. In in vivo experiments it was revealed that the antifungal potential of isoeugenol, eugenol, thymol, and vanillin could be amplified if applied in combination with benzoic acid. This observation is best explained by simultaneous inhibition of CYP53A15, which in turn increases intracellular levels of benzoic acid and finally results in increased growth inhibition of fungi.

Benzoate para-hydroxlase is a key enzyme in fungal primary (the ketodiapate pathway) and secondary (detoxification of phenolic compounds) metabolism.[9,11] There are homologues of CYP53 in several pathogenic fungi such as *Aspergillus fumigatus* and *Gibberella zeae*. Increased antifungal efficiency of combined naturally occurring phenolic compounds demonstrated in this study could potentially be used as an alternative to conventional fungicides or antifungal drugs.

Currently, the essential nature of CYP51 (sterol 14α-demethylase) in fungi is exploited for drug-targeted inhibition to combat clinical infections. Developed azole antifungal drugs, such as fluconazole, clotrimazole, and ketoconazole, possess the N-1 substituent groups of the azole molecule which selectively interacts with the fungal CYP51 protein over the human CYP51 protein.[26] Despite the in vitro efficacy of azole-based antifungal therapy, it frequently results in the recurrence of infection due to the development of fungal resistance through different mechanisms.[27]

The advantage of CYP53 over CYP51 as an antifungal drug target is that CYP53 does not have a homologue in higher eukaryotes. This would enable the design of more selective and potent inhibitors toward pathogenic fungi with less adverse side effects in higher eukaryotes.

Experimental Section

Fungal Species and Growth Conditions. The strain, obtained from the strain collection of the Friedrich Schiller University of Jena, Germany, was listed as the teleomorph *Cochliobolus lunatus* m118. It was also designated as noncompatible anamorph *Curvularia lunata* var. *lunata*, based on observation of induced conidial sporulation and genetic analyses.[28] The fungus was grown as described previously.[29] Mycelia were harvested by filtration and used for the preparation of protoplasts or were frozen in liquid N_2, ground, and used for DNA or RNA isolation or stored at −70 °C for further applications.

Construction and Characterization of *C. lunatus bph* Deletion Strain. The hygromycin resistance cassette (*hph*) of plasmid pAN7-1,[30] obtained after digestion with *Xba*I and *Bgl*II restriction endonucleases, was inserted into the polylinker site of pBlueScript, between *Xba*I and *Bam*HI sites. The 5′ of the *bph* gene was amplified in a PCR reaction using primers A and B. The PCR product was digested with *Not*I and *Xba*I and inserted next to the *hph* gene. The 3′ of the *bph* gene was also PCR amplified with primers C and D. After digestion with *Hind*III and *Kpn*I, the fragment was inserted on the other side of the *hph* cassette. Before transformation, the deletion cassette was released out of the plasmid with *Not*I and *Kpn*I. The protoplast based method was used for transformation of the strain, as described previously.[31] Transformants were selected on minimal medium or MEM agar plates, containing 1.2 M sorbitol and 100 μg of hygromycin/mL (Sigma, St. Louis, MO). Deletion transformants were discriminated from ectopic transformants in a duplex PCR reaction with primer E,

specific for the removed region of the *bph* gene, and primer F, specific for the *hph* gene, and the reverse primer D. Furthermore, deletion was confirmed with Southern blot analysis, using the same probe as for plaque lifting.

Expression of CYP53A15 in *E. coli.* *E. coli* C43 (DE3) cells, harboring the plasmid pCWori+ with gene constructs obtained from the following primer combinations of (1) P450N-B, P450C+, (2) P450N-B, P450C, (3) P450N-wt, P450C+, (4) P450N-wt, P450C, (5) P450N-RR, P450C+, and (6) P450N-RR, P450C, were grown in TB medium containing trace elements, 1 mM vitamin B1 (thiamin), and 100 μg of ampicillin per mL at 37 °C, with vigorous shaking until OD_{600} of 2.0 was reached. IPTG was then added to a final concentration of 0.8 mM to induce protein expression, as well as δ-aminolevulinic acid to a final concentration of 1 mM. Cells were grown for an additional 48 h at 30 °C.

Purification of CYP53A15. The cells were harvested by centrifugation (200 rpm for 15 min) and resuspended in 10 mL of lysozyme buffer (250 mM sucrose, 50 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, and 1 mg/mL lysozyme) per gram of biomass. After gentle stirring for 30 min, the suspension was centrifuged again at 200 rpm for 15 min. Cell pellets were sonicated in 5 mL of buffer A (50 mM potassium phosphate (pH 7.4), 20% glycerol, 0.1 mM DTT, 0.1 mM EDTA, 0.1% Triton-X-100, and 0.1 mM PMSF) per gram of pellet. After centrifugation (95 000 rpm for 1 h), supernatants were pooled, diluted with an equal amount of buffer B (50 mM potassium phosphate, 1 mM NaCl, pH 7.4, 20% glycerol, 0.1 mM DTT, 0.1 mM EDTA, 0.1% Triton-X-100, and 0.1 mM PMSF), and loaded onto a Ni-NTA column. The column was washed with 10 volumes of buffer B. CYP53A15 was subsequently eluted with buffer B containing 200 mM imidazole. Fractions were pooled on the basis of SDS−PAGE analysis and concentrated using ultrafiltration to approximately 1 mg/mL. The concentrate was dialyzed against buffer C (50 mM potassium phosphate (pH 7.4), 20% glycerol, 0.1 mM EDTA, 0.1% Triton-X-100) and stored at −70 °C.

Enzyme Assay. The concentration of CYP53A15 was determined from the absorbance at 420 nm (absolute spectrum) and from the CO difference spectrum. Samples were analyzed by SDS−PAGE, as well as on the Agilent Bioanalyzer 2000.

Reconstitution of CYP53A15 Activity. The 100 μL of NADPH cytochrome P450 reductase (CPR) (150 U/mL) from rabbit liver and 500 μL of dilauroylphosphatidylcholine (DLPC) (0.1% (w/v) in 10 mM phosphate buffer, pH 7.4) were added to 500 μL of CYP53A15 (21.2 μM). The reaction was started with the addition of 1 mM NADPH and 20 mM benzoate. CYP53A15 activity was measured by monitoring product formation by HPLC analysis, using an YMC-ODS chromatographic column with 10% acetonitrile, 0.1% trifluoroacetic acid as mobile phase, and multiple-wavelength UV and fluorescence detection, performed on the Waters Alliance system. Products were identified on the basis of their spectral and chromatographic properties. Compounds other than benzoate were tested spectrophotometrically for their ability to induce a type I spectral shift as described by ref 8.

Data Analysis. The progress curve data of hydroxybenzoate formation by CYP53A15 at various starting benzoate concentrations in the presence and the absence of inhibitors were obtained by a quantitative HPLC product analysis of the quenched reaction after different incubation times. In order to include all collected data in the analysis, rather than just initial rates, we performed a comprehensive progress curve analysis by fitting differential equations for the Michaelis−Menten reaction mechanism in the absence and presence of an inhibitor, to the data. The kinetic parameters K_m and k_{cat}, and the corresponding inhibition constants and proportional factors were estimated by using a nonlinear least-squares equation-based program that evaluates parameters of a stiff system of differential equations from multiple data curves.[32] Among rival inhibition patterns for four inhibitors (eugenol, isoeugenol, thymol, and vanillin), the appropriate pattern was chosen according to Cleland's criteria of the goodness of fit.[33]

Homology Modeling. A homology model of CYP53A15 was built using cytochromes P450, 2C8 (PDB code 1PQ2), and 2A6 (PDB code 1Z10) as templates. An iterative multistep building procedure, using WHATIF,[34] a molecular modeling package, and CHARMM, a program for macromolecular simulations, was performed.[35] Isoeugenol was built and optimized quantum mechanically, using MOLDEN, a processing program of molecular and electronic structure, and Gaussian 03, an electronic structure program.[36] CHARMM force field parameters were assigned to the atoms of isoeugenol, which was docked into the active site cavity above the iron atom of the heme molecule by superimposing the corresponding atoms to the coumarin situated in the active site of 2A6. The complex structure was then subjected to 500 ps of constant pressure and temperature dynamic simulation (300 K, 1 bar, time step of 1 fs) using the EWALD summation for calculating the electrostatic interactions. The last frame was relaxed by 60 steps of QMMM refinement, assigning the heme and isoeugenol molecules quantum mechanically and assigning the protein and water molecules with H-bond contacts (112 of them) molecular mechanically.

Antifungal Activity Tests. In vivo antifungal activity of different inhibitors (benzoic acid, *trans*-cinnamic acid, *trans*-4-hydroxycinnamic acid, potassium sorbate, carvacrol, eugenol, isoeugenol, β-thujaplicin, thymol, and vanillin) was tested. Triplicate Petri dishes of every system for each inhibitor and a combination of individual inhibitors at 5 mM with 0.1 mM benzoic acid were prepared as described by Lopez-Malo et al.[37] Solidified agar was centrally inoculated with 7 mm diameter mycelia disks taken from a 48 h solid culture. To calculate initial growth inhibition time, the linear growth phase was extrapolated to a zero increase in diameter (7 mm diameter), and the intercept on the time axis was defined as growth inhibition time.[37] Minimum inhibitory concentration (MIC) was determined as described by Radford.[38]

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Supporting Information Available: Homology model of CYP53A15 (PMDB ID, PM0075149), basic experimental procedures for molecular manipulation and cloning, and tables listing oligonucleotide primers and substances tested. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Amborabe, B. E.; Fleurat-Lessard, P.; Chollet, J. F.; Roblin, G. Antifungal effects of salicylic acid and other benzoic acid derivatives towards Eutypa lata: structure−activity relationship. *Plant Physiol. Biochem.* **2002**, *40*, 1051–1060.

(2) Brul, S.; Coote, P. Preservative agents in foods. Mode of action and microbial resistance mechanisms. *Int. J. Food Microbiol.* **1999**, *50*, 1–17.

(3) Lapadatescu, C.; Ginies, C.; Le Quere, J. L.; Bonnarme, P. Novel scheme for biosynthesis of aryl metabolites from L-phenylalanine in the fungus *Bjerkandera adusta*. *Appl. Environ. Microbiol.* **2000**, *66*, 1517–1522.

(4) Harwood, C. S.; Parales, R. E. The beta-ketoadipate pathway and the biology of self-identity. *Annu. Rev. Microbiol.* **1996**, *50*, 553–590.

(5) Durham, D. R.; Mcnamee, C. G.; Stewart, D. B. Dissimilation of aromatic-compounds in Thodotorula-graminis. Biochemical-characterization of pleiotropically negative mutants. *J. Bacteriol.* **1984**, *160*, 771–777.

(6) Jensen, K. A.; Evans, K. M. C.; Kirk, T. K.; Hammel, K. E. Biosynthetic-pathway for veratryl alcohol in the ligninolytic fungus phanerochaete-chrysosporium. *Appl. Environ. Microbiol.* **1994**, *60*, 709–714.

(7) Wright, J. D. Fungal degradation of benzoic-acid and related-compounds. *World J. Microbiol. Biotechnol.* **1993**, *9*, 9–16.

(8) Faber, B. W.; van Gorcom, R. F. M.; Duine, J. A. Purification and characterization of benzoate-para-hydroxylase, a cytochrome P450 (CYP53A1), from *Aspergillus niger*. *Arch. Biochem. Biophys.* **2001**, *394*, 245–254.

(9) Fraser, J. A.; Davis, M. A.; Hynes, M. J. The genes gmdA, encoding an amidase, and bzuA, encoding a cytochrome P450, are required for benzamide utilization in *Aspergillus nidulans*. *Fungal Genet. Biol.* **2002**, *35*, 135–146.

(10) Lamb, D. C.; Waterman, M. R.; Kelly, S. L.; Guengerich, F. P. Cytochromes P450 and drug discovery. *Curr. Opin. Biotechnol.***2007**, *18*(6), 504–512.
(11) Fujii, T.; Nakamura, K.; Shibuya, K.; Tanase, S.; Gotoh, O.; Ogawa, T.; Fukuda, H. Structural characterization of the gene and corresponding cDNA for the cytochrome P450rm from *Rhodotorula minuta* which catalyzes formation of isobutene and 4-hydroxylation of benzoate. *Mol. Gen. Genet.* **1997**, *256*, 115–120.
(12) Dumon-Seignovert, L.; Cariot, G.; Vuillard, L. The toxicity of recombinant proteins in *Escherichia coli*: a comparison of overexpression in BL21(DE3), C41(DE3), and C43(DE3). *Protein Expression Purif.* **2004**, *37*, 203–206.
(13) Barnes, H. J. Maximizing expression of eukaryotic cytochrome P450s in *Escherichia coli*. *Methods Enzymol.* **1996**, *272*, 3–14.
(14) Rowland, P.; Blaney, F. E.; Smyth, M. G.; Jones, J. J.; Leydon, V. R.; Oxbrow, A. K.; Lewis, C. J.; Tennant, M. G.; Modi, S.; Eggleston, D. S.; Chenery, R. J.; Bridges, A. M. Crystal structure of human cytochrome P450 2D6. *J. Biol. Chem.* **2006**, *281*, 7614–7622.
(15) McLaughlin, L. A.; Paine, M. J. I.; Kemp, C. A.; Marechal, J. D.; Flanagan, J. U.; Ward, C. J.; Sutcliffe, M. J.; Roberts, G. C. K.; Wolf, C. R. Why is quinidine an inhibitor of cytochrome P450 2D6? The role of key active-site residues in quinidine binding. *J. Biol. Chem.* **2005**, *280*, 38617–38624.
(16) Yano, J. K.; Hsu, M. H.; Griffin, K. J.; Stout, C. D.; Johnson, E. F. Structures of human microsomal cytochrome P450 2A6 complexed with coumarin and methoxsalen. *Nat. Struct. Mol. Biol.* **2005**, *12*, 822–823.
(17) Andrews, J. M. Determination of minimum inhibitory concentrations. *J. Antimicrob. Chemother.* **2001**, *48*, 5–16.
(18) vandenBrink, J. M.; Vandenhondel, C. A. M. J.; vanGorcom, R. F. M. Optimization ef the benzoate inducible benzoate *p*-hydroxylase cytochrome P450 enzyme system in *Aspergillus niger*. *Appl. Microbiol. Biotechnol.* **1996**, *46*, 360–364.
(19) Kamada, F.; Abe, S.; Hiratsuka, N.; Wariishi, H.; Tanaka, H. Mineralization of aromatic compounds by brown-rot basidiomycetes—mechanisms involved in initial attack on the aromatic ring. *Microbiology* **2002**, *148*, 1939–1946.
(20) Yun, C. H.; Kim, K. H.; Calcutt, M. W.; Guengerich, F. P. Kinetic analysis of oxidation of coumarins by human cytochrome P450 2A6. *J. Biol. Chem.* **2005**, *280*, 12279–12291.
(21) Beekrum, S.; Govinden, R.; Padayachee, T.; Odhav, B. Naturally occurring phenols: a detoxification strategy for fumonisin B-1. *Food Addit. Contam.* **2003**, *20*, 490–493.
(22) Curir, P.; Dolci, M.; Dolci, P.; Lanzotti, V.; De Cooman, L. Fungitoxic phenols from carnation (*Dianthus caryophyllus*) effective against *Fusarium oxysporum* f. sp dianthi. *Phytochem. Anal.* **2003**, *14*, 8–12.
(23) Sofos, J. N.; Beuchat, L. R.; Davidson, P. M.; Johnson, E. A. Naturally occurring antimicrobials in food. *Regul. Toxicol. Pharmacol.* **1998**, *28*, 71–72.
(24) Lambert, R. J. W.; Skandamis, P. N.; Coote, P. J.; Nychas, G. J. E. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *J. Appl. Microbiol.* **2001**, *91*, 453–462.
(25) Kim, J. H.; Campbell, B. C.; Mahoney, N. E.; Chan, K. L.; Molyneux, R. J. Identification of phenolics for control of *Aspergillus flavus* using *Saccharomyces cerevisiae* in a model target-gene bioassay. *J. Agric. Food Chem.* **2004**, *52*, 7814–7821.
(26) Aoyama, Y. Recent progress in the CYP51 research focusing on its unique evolutionary and functional characteristics as a diversozyme P450. *Front. Biosci.* **2005**, *10*, 1546–1557.
(27) Sanglard, D. Resistance of human fungal pathogens to antifungal drugs. *Curr. Opin. Microbiol.* **2002**, *5*, 379–385.
(28) Rozman, D.; Komel, R. Isolation of genomic DNA from filamentous fungi with high glucan level. *BioTechniques* **1994**, *16*, 382–384.
(29) Plemenitas, A.; ZakeljMavric, M.; Komel, R. Hydroxysteroid dehydrogenase of *Cochliobolus lunatus*. *J. Steroid Biochem. Mol. Biol.* **1988**, *29*, 371–372.
(30) Punt, P. J.; Oliver, R. P.; Dingemanse, M. A.; Pouwels, P. H.; Vandenhondel, C. A. M. J. Transformation of Aspergillus based on the hygromycin-B resistance marker from *Escherichia coli*. *Gene* **1987**, *56*, 117–124.
(31) Punt, P. J.; Vandenhondel, C. A. M. J. Transformation of filamentous fungi based on hygromycin-B and phleomycin resistance markers. *Methods Enzymol.* **1992**, *216*, 447–457.
(32) Stojan, J. Analysis of progress curves in an acetylcholinesterase reaction: a numerical integration treatment. *J. Chem. Inf. Comput. Sci.* **1997**, *37*, 1025–1027.
(33) Cleland, W. W. The statistical-analysis of enzyme kinetic data. *Adv. Enzymol.* **1967**, 1–32.
(34) Vriend, G. WHATIF—a molecular modeling and drug design program. *J. Mol. Graphics* **1990**, *8*, 52.
(35) Brooks, B. R.; Bruccoleri, R. E.; Olafson, B. D.; States, D. J.; Swaminathan, S.; Karplus, M. Charmm—a program for macromolecular energy, minimization, and dynamics calculations. *J. Comput. Chem.* **1983**, *4*, 187–217.
(36) Schaftenaar, G.; Noordik, J. H. Molden: a pre- and post-processing program for molecular and electronic structures. *J. Comput.-Aided Mol. Des.* **2000**, *14*, 123–134.
(37) Lopez-Malo, A.; Alzamora, S. M.; Palou, E. *Aspergillus flavus* growth in the presence of chemical preservatives and naturally occurring antimicrobial compounds. *Int. J. Food Microbiol.* **2005**, *99*, 119–128.
(38) Radford, S. A.; Johnson, E. M.; Warnock, D. W. In vitro studies of activity of voriconazole (WK-109,496), a new triazole antifungal agent, against emerging and less-common mold pathogens. *Antimicrob. Agents Chemother.* **1997**, *41*, 841–843.

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