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Selectivity improvement of an azole inhibitor of CYP707A by replacing the monosubstituted azole with a disubstituted azole

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ABSTRACT

The plant growth-retardant uniconazole (UNI), a triazole inhibitor of gibberellin biosynthetic enzyme (CYP701A), inhibits multiple P450 enzymes including ABA 8′-hydroxylase (CYP707A), a key enzyme in ABA catabolism. Azole P450 inhibitors bind to a P450 active site by both coordinating to the heme-iron atom via sp^2 nitrogen and interacting with surrounding protein residues through a lipophilic region. We hypothesized that poor selectivity of UNI may result from adopting a distinct conformation and orientation for different active sites. Based on this hypothesis, we designed and synthesized novel UNI analogs with a disubstituted azole ring (DSI). These analogs were expected to have higher selectivity than UNI because the added functional group may interact with the active site to restrict orientation of the molecule in the active site. DSI-505ME and DSI-505MZ, which have an imidazolyl group with a methyl 5-acrylate, strongly inhibited recombinant CYP707A3, with no growth-retardant effect.

Uniconazole is an azole-containing cytochrome P450 inhibitor developed as a plant growth retardant in the 1980s.[1,2] UNI has since been used as a plant growth regulator in agriculture and horticulture. The main site of action of UNI is suggested to be *ent*-kaurene oxidase (CYP701A), which catalyzes the three-step oxidation of *ent*-kaurene to *ent*-kaurenoic acid,[3] a biosynthetic precursor of the plant hormone gibberellin (GA). This has prompted researchers to use UNI as a chemical tool inhibiting GA biosynthesis. However, UNI also inhibits brassinosteroid biosynthesis[4,5] and alters the level of other plant hormones, such as auxins, cytokinins, ethylene, and abscisic acid (ABA).[6] Recently, Kitahata et al.[7] and Saito et al.[8] revealed that UNI strongly inhibits ABA 8′-hydroxylase (CYP707A[9,10]), a key enzyme in ABA catabolism (Fig. 1).

Azole-type P450 inhibitors including UNI bind to the target P450 active site by both coordinating to the heme-iron atom and interacting with surrounding protein residues. Because heme co-ordination is a common property of azole-containing inhibitors, their affinity, and specificity for individual P450 enzymes depend on structural properties other than the azole group. UNI may be small and flexible enough to embed itself into various substrate-binding pockets, where UNI may adopt a distinct conformation and orientation for different active sites. Ketoconazole, a well-known antifungal drug, binds to various P450 active sites in different conformations and orientations (Fig. 2). In homology models for CYP707A3 and CYP701A3, the active site void spaces that are close to the heme differ in size and shape (Fig. 3). Although these models have low reliability because of the low sequence similarity (~25%) to

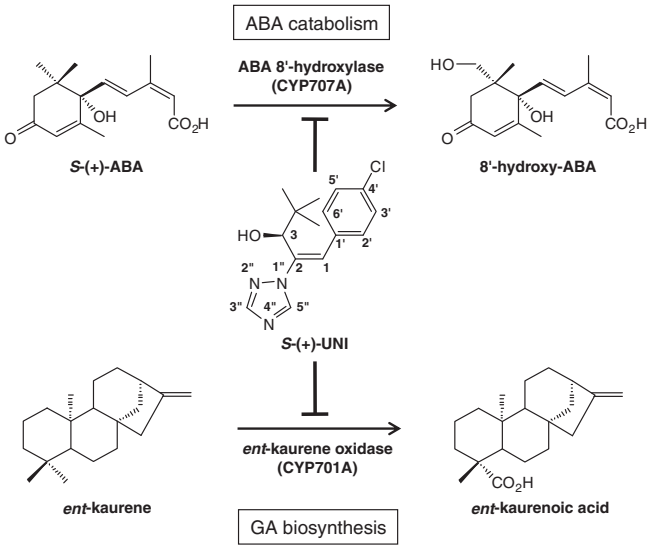

Figure 1. UNI is a potent inhibitor of ABA 8′-hydroxylase (CYP707A) and *ent*-kaurene oxidase (CYP701A).

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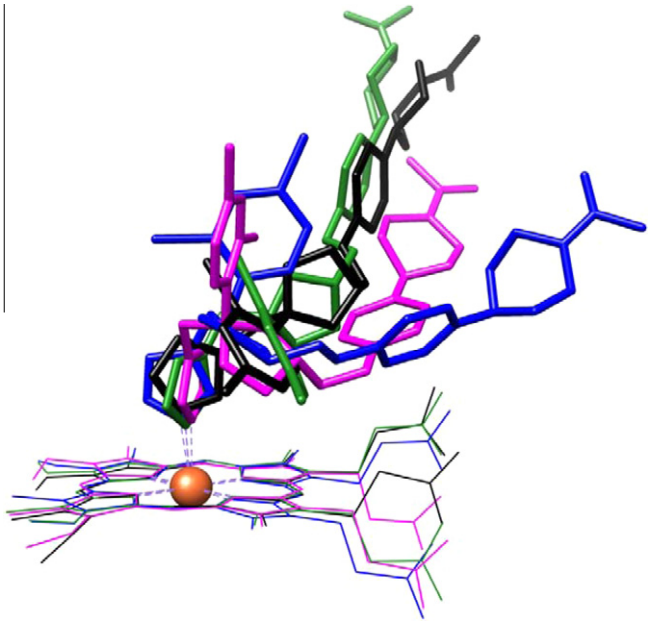
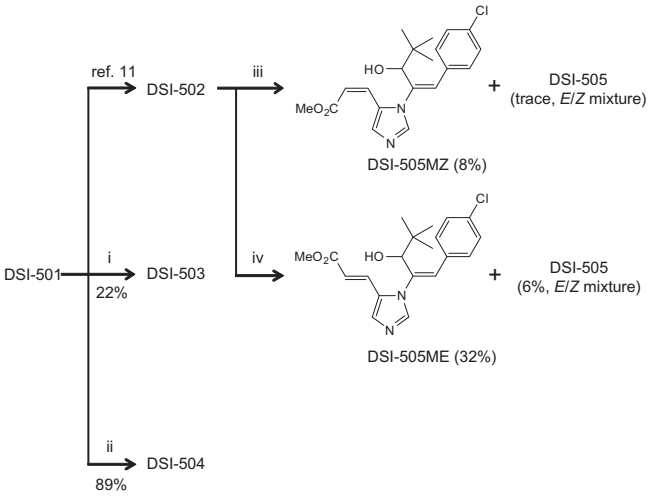


Figure 2. Overlaid structures of ketoconazole-binding P450 active sites: black, 3LD6 (human CYP51); pink, 2V0M (human CYP3A4); blue, 1JIN (bacterial CYP107A1); green, 2JJP (bacterial CYP113A1). The figures were manually drawn using Chimera after automatic overlay.

template proteins, we speculated that UNI may inhibit various P450 enzymes in a manner similar to the case of ketoconazole. Based on this speculation, in previous works, we generated selective inhibitors of CYP707A by elongating or conformationally freezing the UNI molecule.[11,12] In this study, we focused on restricting azole orientation by using a disubstituted azole ring for the monosubstituted ring of UNI. A functional group introduced into the azole of UNI can have a new interaction with the active site residues, causing advantageous or disadvantageous reorientation in binding to the active site. This reorientation depends on the active site profile, which will be different for different P450 enzymes. We designed and synthesized seven UNI analogs with a disubstituted imidazole (DSI) (Fig. 4), which has an oxidized functional group at C2″ or C5″, in hopes of electrostatic or hydrogen-bonding interactions in addi-



Scheme 1. Synthesis of DSI. Reagents and conditions: (i) NaH, MeI, DMF, 0 °C, 22%; (ii) NaH, EtI, DMF, 0 °C, 89%; (iii) NaH, $(CF_3CH_2O)_2P(O)CH_2CO_2Me$, THF, 0–75 °C, 8% (DSI-505MZ) and trace (DSI-505); (iv) NaH, $(CF_3CH_2O)_2P(O)CH_2CO_2Me$, THF, 0 °C to rt, 32% (DSI-505ME) and 6% (DSI-505).

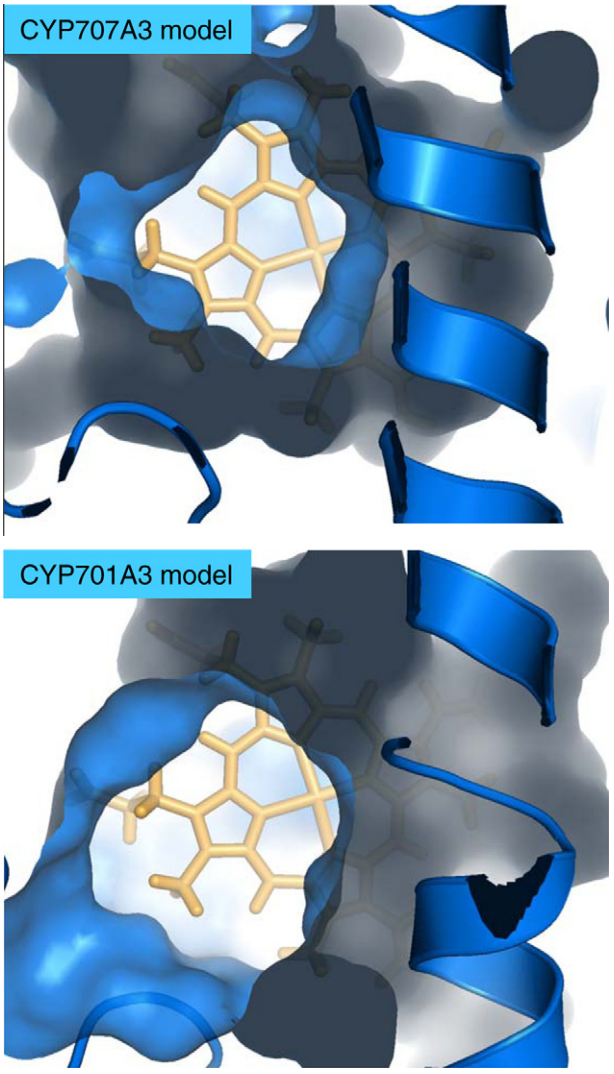


Figure 3. Ligand binding sites in the homology models of CYP707A3 (top) and CYP701A3 (bottom). Molecular surfaces were calculated and displayed with PyMOL.

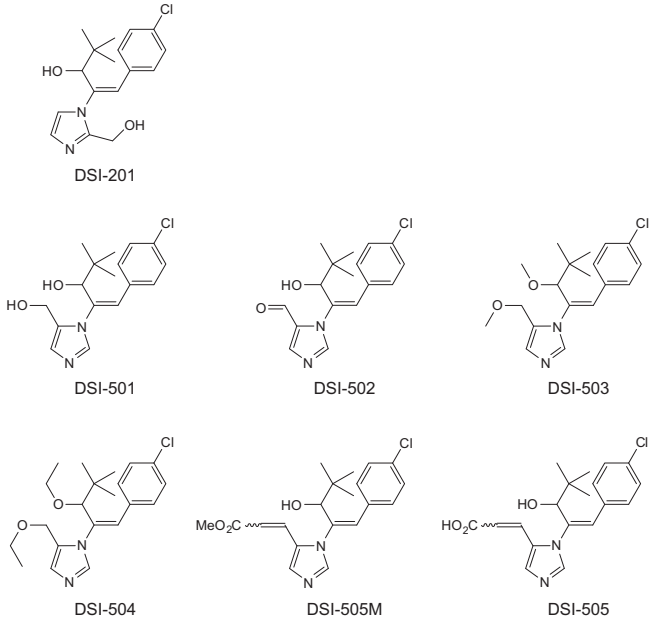
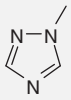
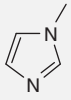
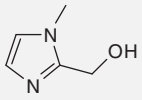
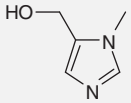
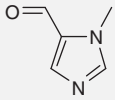
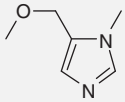
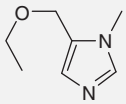
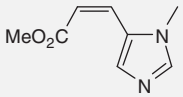
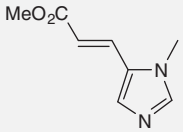
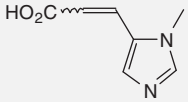


Figure 4. Chemical structures of DSI.

Table 1
Inhibitory activity of DSI against recombinant CYP707A3 and rice seedling elongation

Compound	Structural properties		CYP707A3		Rice growth
	2-**Az**	3-O**X**	Inhibition[c] (%)	K_I (nM)	IC_{50} (μM)
UNI[a]		H	**100**	10	0.18
IMI[b]		H	**100**	0.6	2.3
DSI-201		H	31	–[d]	**>100**
DSI-501		H	**95**	160	5.4
DSI-502		H	**91**	240	10
DSI-503		CH_3	**95**	190	9.6
DSI-504		C_2H_5	62	–	32
DSI-505MZ		H	**98**	220	**>100**
DSI-505ME		H	**100**	120	**>100**
DSI-505		H	31	–	**>100**

The values are averages of two independent experiments.
[a] Ref. 11.
[b] Ref. 13.
[c] Inhibition ratio of compounds (10 μM) in the 8′-hydroxylation of ABA (5 μM).
[d] Not measured.

tion to steric effects. We checked the enzyme selectivity of the DSI by examining both the inhibitory activity of DSI compounds against recombinant CYP707A and the plant growth-retardant effect caused mainly by inhibition of gibberellin biosynthetic enzymes including CYP701A and CYP88A.

DSI-201, DSI-501, and DSI-502 were prepared by a method reported earlier.[11] Oxidation of DSI-201 with MnO_2 gave not the aldehyde but the tautomeric isomer, the cyclic hemiacetal, which was previously reported as a conformationally restricted UNI analog.[11] Other disubstituted UNI analogs were synthesized from DSI-501 according to Scheme 1. Methylation and ethylation of DSI-501 with methyl iodide and ethyl iodide, respectively, gave DSI-503 and DSI-504 with 22% and 89% yield. DSI-505MZ and DSI-505ME were prepared by a Horner–Emmons reaction of DSI-502 with the phosphonoate. This reaction gave a mixture of *E* and *Z* isomers of DSI-505M, DSI-505ME, and DSI-505MZ. The major product was the *E*-isomer at room temperature, whereas it was converted into the *Z*-isomer by rising the reaction temperature to 75 °C. The free acid DSI-505 yielded a *E*/*Z* mixture (1:2) by partial alkaline hydrolysis during aqueous work-up of the reaction.

The inhibitory activity against ABA 8′-hydroxylase was examined using recombinant *Arabidopsis* CYP707A3 co-expressed with *Arabidopsis* P450 reductase (ATR2) in *Escherichia coli*.[12] The activity was evaluated based on the decrease in the enzymatic product, phaseic acid, caused by addition of a test compound at a concentration two times higher (10 μM) than the substrate *S*-(+)-ABA (5 μM).[12] For compounds showing >90% activity, the inhibition constant (K_I) was measured. The plant growth-retardant effect was examined using rice seedlings to estimate the concentration giving 50% inhibition (IC_{50}). The results are summarized in Table 1.

DSI-201 was much less effective in both assays. This compound is substituted at C2″ adjacent to N3″, which actually coordinates to the heme iron. The C2″ hydroxymethyl group may interfere with N–Fe co-ordination. DSI-501, DSI-502, and DSI-503 were less

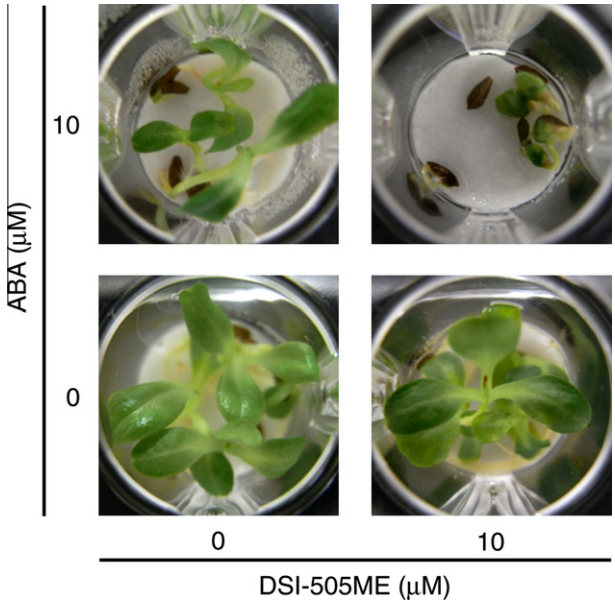


Figure 5. Enhanced effect of DSI-505ME on ABA activity in an early growth assay of lettuce.

effective than UNI by a factor of 20–50 in both assays. The hydroxymethyl, formyl, and methoxymethyl groups at C5″ may have a similar unfavorable effect on binding to the active sites of CYP707A and CYPs involved in plant growth. DSI-504, with the C5″ ethoxymethyl group, was less effective than DSI-501, DSI-502, and DSI-503 in both assays. This may be because of the larger steric effect by substituents at both C5″ and C3. DSI-505MZ and DSI-505ME exhibited CYP707A inhibitory activity equivalent to that of DSI-501, DSI-502, and DSI-503 in spite of its larger C5″ substituent, whereas it had little inhibitory activity on rice seedling growth. This suggests that the methyl acrylate moiety at C5″ is tolerated in the CYP707A active site, but not in those of CYPs involved in plant growth. Considering that DSI-505MZ and DSI-505ME were less effective CYP707A inhibitors than UNI and the inhibitory potency was largely independent of the acrylate *E*/*Z* geometry, the acrylate at C5″ may function as a steric obstacle, and neither an electrostatic nor a hydrogen-bonding donor or acceptor, for the amino acid residues in the active site; its sterically unfavorable effect may be too small for CYP707A to allow the inhibitor azole to orient to the heme iron, but too large for CYPs involved in plant growth to allow it. On the other hand, the free acid DSI-505 (*E*/*Z* mixture) exhibited little inhibitory activity in either assay. Because the P450 active site is generally hydrophobic, the carboxylate may not be accepted. We cannot discuss the structure–activity relationships in greater detail due to the lack of structural characterization of CYP707A and the CYPs involved in plant growth.

We tested the effect of DSI-505ME on early growth of lettuce in the presence and absence of ABA. DSI-505ME exhibited no significant effect at the tested concentrations of 0.1–100 μM, whereas it enhanced the effect of 10 μM ABA upon simultaneous application of 100 μM inhibitor (Fig. 5). This suggests that DSI-505ME acts as an inhibitor of CYP707A in vivo as well as in vitro to keep a high concentration of ABA.

Finally, we found a more selective azole inhibitor than UNI by replacing the monosubstituted azole with the disubstituted one. This design strategy may be useful for increasing the selectivity of azole CYP inhibitors.

Acknowledgments

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Supplementary data

Supplementary data (the synthetic procedure for new compounds, their ^{1}H NMR and high-resolution MS data, and the procedures for homology modeling, enzyme assay, and biological assays are given) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.07.067.

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