Development of Specific Inhibitors of CYP707A, a Key Enzyme in the Catabolism of Abscisic Acid

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Y. Todoroki*,[1] and K. Ueno*,[2]

[1]*Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, Shizuoka 422-8529, Japan*

[2] *Graduate School of Agricultural Science, Kobe University, Kobe 657-8501, Japan*

*Address correspondence to these authors at:

[1]Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, Shizuoka 422-8529, Japan

Tel: +81-54-238-4871; Fax: +81-54-238-4871

E-mail: aytodor@agr.shizuoka.ac.jp

[2] Graduate School of Agricultural Science, Kobe University, Kobe 657-8501, Japan

Tel: +81-78-803-5884; Fax: +81-78-803-5884

E-mail: ueno@crystal.kobe-u.ac.jp

Abstract: Abscisic acid (ABA) is a plant hormone involved in stress tolerance, stomatal closure, seed dormancy, and other physiological events. Although ABA is registered as a farm chemical (plant growth regulator), its practical use has been limited, mainly due to its weak effect in field trials, which is considered to be due to its rapid inactivation through biodegradation. Catabolic inactivation of ABA is mainly controlled by ABA 8'-hydroxylase (CYP707A), which is the cytochrome P450 catalyzing the C8'-hydroxylation of ABA into 8'-hydroxy-ABA and its more stable tautomer, phaseic acid, which has much lower hormonal activity than ABA. Thus, a specific inhibitor of CYP707A is promising not only as a chemical probe for the mechanism of ABA action, but also because of its potential use in agriculture and horticulture. This review article focuses on our recent research on the development of two types of specific inhibitors of CYP707A: the ABA analogue-type inhibitors that were designed on the basis of differences between the structural requirements for CYP707A and for ABA activity, and the azole-type P450 inhibitors that were developed by conformational restriction or enlargement of the plant growth retardant uniconazole.

Abbreviations: ABA, abscisic acid; PA, phaseic acid; UNI, uniconazole; GA, gibberellin; AHI, abscisic acid 8'-hydroxylase inhibitor; SRS, substrate recognition site; Abz, abscinazole.

Running header: Specific inhibitors of CYP707A

INTRODUCTION

Abscisic acid (ABA) is a plant hormone involved in stress tolerance, stomatal closure, seed dormancy, and other physiological events [1-4]. The endogenous levels of ABA in plants are cooperatively controlled by biosynthesis, transportation, and catabolic inactivation in response to environmental changes [1-4]. A natural or artificial chemical that perturbs this highly controlled system is promising not only as a chemical probe for the mechanism of ABA action [5], but also because of its potential use in agriculture and horticulture. Although ABA is registered as a farm chemical (plant growth regulator), its practical use has been limited, mainly due to its weak effect in field trials [6], which is considered to be due to its rapid inactivation through biodegradation.

Catabolism of ABA can be initiated by several reactions: hydroxylation at C7', C8'

or C9'; conjugation with glucose at C1 or C1'; and reduction at C4' (Fig. (**1**)) [1-4,7]. The major reactions are C8' hydroxylation and C1 conjugation; these are generally found in all plant species. The other catabolic pathways are minor or specific to certain plants. The C8' hydroxylation pathway, which produces dihydrophaseic acid via phaseic acid (PA), is considered to be as characteristic of ABA catabolism as the C1

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conjugation pathway, although it can also be a reversible inactivation reaction sequence to regenerate free ABA.

ABA 8'-hydroxylase is a microsomal cytochrome P450 monooxygenase. In vitro ABA 8'-hydroxylating activity has been reported for microsomal fractions from plant materials [8-11]. These reports revealed that ABA 8'-hydroxylation requires both NADPH and O_2, and is inhibited by CO, cytochrome c, and tetcyclacis, a P450 inhibitor. In 2004, the gene encoding ABA 8'-hydroxylase was identified by Kushiro $et\ al.$ [12] and Saito $et\ al.$ [13]. These groups independently demonstrated that members of the $Arabidopsis$ cytochrome P450 CYP707A family, $CYP707A1$ to $CYP707A4$, encode ABA 8'-hydroxylases, and since then, many CYP707A isozymes have been found in various plants [14]. CYP707A genes are upregulated under drought stress conditions and the mRNA level is significantly increased upon rehydration [12]. Mutants of $cyp707a2$ exhibit hyperdormancy in seeds and accumulate six-fold greater levels of ABA than wild type [12]. Gene knockdown and overexpression studies suggest that CYP707A is a key enzyme for controlling ABA concentration during water deficit stress, changes in humidity, and dormancy maintenance and breaking [15-17]. Microsomes from insect cells expressing CYP707A3 exhibit very active 8'-hydroxylation of (+)-ABA (K_M = 1.3 μM and k_{cat} = 15 min^{-1}), and solubilized CYP707A3 protein binds

(+)-ABA (K_s = 3.5 μM) [12]. The reaction product 8'-hydroxy-ABA is so unstable that it spontaneously isomerizes in vitro to a more stable tautomer, PA [18]. The kinetics of isomerization of 8'-hydroxy-ABA in the CYP707A3 reaction match those of 8'-hydroxy-ABA in buffered solutions, indicating that CYP707A3 catalyzes 8'-hydroxylation of (+)-ABA but not isomerization of 8'-hydroxy-ABA to PA [12]. These results demonstrate that genes in the CYP707A family play a key role in regulating the ABA level through the 8'-hydroxylation of (+)-ABA.

CYP707A is an attractive target for chemical regulation of environmental responses of plants. Recent research has revealed that some azole-containing P450 inhibitors, including tetcyclacis and uniconazole (UNI), function as inhibitors of CYP707A [12,19,20]. However, these compounds are plant growth retardants which inhibit gibberellin (GA) biosynthesis [21,22]. In addition, UNI also inhibits P450 enzymes in brassinosteroid biosynthesis [23, 24] and alters the level of other plant hormones, such as auxins, cytokinins, and ethylene [25]. This review article focuses on our recent research on the development of two types of specific inhibitors of CYP707A: ABA analogue-type inhibitors that were designed on the basis of differences between the structural requirements for CYP707A and for ABA activity, and azole-type P450 inhibitors that were developed by conformational restriction or enlargement of the plant

growth retardant UNI.

ABA ANALOGUE-TYPE INHIBITORS

A structural analogue of ABA designed on the basis of the substrate specificity of CYP707A can work as a specific and competitive inhibitor. However, ABA analogues can also have ABA activity, a serious drawback for in vivo experiments where the desired action is not as an agonist for ABA receptors but as an inhibitor of the enzyme. On this premise, the ideal enzyme inhibitor would have the ABA structural features required for substrate specificity, but not those involved in activating the ABA signal transduction pathway. Therefore, we must understand the differences between the structural requirements for CYP707A inhibition and ABA activity, as well as substrate specificity.

1) Structural requirements for ABA activity

Fig. (2)

Structure-activity relationships for ABA have been investigated over the past forty years since it was first identified as a plant hormone. The consensus findings are

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summarized in Fig. (**2**) [26]. All the modifications except for those at C8' and C9'

decrease its activity. In particular, elimination of C6 and C7', reduction of the

pentadienoic acid side chain, a ring conformational change into the side chain equatorial,

and geometrical isomerization of the side chain into the *E*-form abolish activity. If any

of these modifications has no effect on the inhibitory activity of CYP707A, it would be

a significant target for designing CYP707A inhibitors.

2) Structural requirements for CYP707A inhibition

The detailed structural requirements for CYP707A inhibition have been investigated

using recombinant *Arabidopsis* CYP707A3 and structural analogues of ABA (Fig. (**2**))

[26,27]. The C1 carboxyl group of ABA has been believed to play a significant role in

the interaction of ABA with target molecules. Recently, in the case of ABA receptors,

this specific interaction was demonstrated by crystal structural determination of the

complexed form with ABA [28-32]. Also, in CYP707A inhibition, the C1 carboxylate is

an absolutely essential moiety. Esterification, reduction, or change in orientation by

2*E*-isomerization causes a remarkable decrease in the inhibitory potency. On the other

hand, elimination of the other two oxygen functional groups, the C1' hydroxy and the

C4' carbonyl groups, does not always abolish affinity. In particular, the C1' hydroxy group must have no involvement in binding to CYP707A3. Reduction of the α,β-unsaturated carbonyl group in the ring results in slight loss of affinity of the (S)-(+)-isomer for the enzyme and in acceptance of the (R)-(−)-isomer. (R)-(−)-ABA, the unnatural enantiomer of ABA, cannot bind to CYP707A3 at all [13], whereas (−)-4'-deoxo-ABA is a weak competitive inhibitor and (−)-2',3'-dihydro-4'-deoxo-ABA

is an effective competitive inhibitor (Table (1)) [27]. These observations suggest that the carbonyl moiety at C4' is related to asymmetrical binding of ABA, although it is not necessarily significant for substrate binding. Therefore, for oxygen functional groups, it appears that there is no significant difference in structural requirements between ABA activity and CYP707A inhibition.

Alteration at each of the four methyl groups of ABA has a different effect on affinity to CYP707A3. The C6'-methyl groups (C8' and C9'), not necessary for ABA activity, are strictly recognized by the enzyme, because their enlargement results in a considerable loss of affinity for CYP707A3, except for mono- and di-fluorine substitution, which yields a tolerable size that fits into the active site. Enlargement at C7' is tolerable up to additional one carbon. Removal of C7' decreases, but does not necessarily abolish, binding to CYP707A3. On the other hand, the elimination of the C6

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methyl has no effect on binding to CYP707A3, in contrast to the observation that the C6

methyl group is essential to ABA activity. Therefore, we can attenuate ABA activity by

elimination of C6 without lowering the inhibitory activity against CYP707A.

The ring conformation is also a significant factor in the structural requirements for

CYP707A inhibition. The ABA skeleton has enough flexibility to permit a variety of

conformations. The calculated energy minimum of the cyclohexadienone ring of ABA is

a half-chair with the side chain and C8' axial, and with the C1'-hydroxy and C9'

equatorial [33], the conformation reported for the crystal [34] and the most stable

structure in solutions [35]. The structure of ABA in the binding pocket of ABA receptors

is very similar to that of crystalline ABA [28-32]. A free energy difference between this

conformation and a half-chair with the side chain and C8' equatorial and with the

1'-hydroxy group and C9' axial is estimated to be 1.4 kcal mol^{-1}, while the free-energy

barrier to interconversion between these two conformations is calculated to be 11.2 kcal

mol^{-1} [33]. These relatively low values mean that the less stable half-chair could be the

binding conformation, because the binding energy would be sufficient to compensate

for the small loss of energy owing to adoption of the less stable conformation. However,

based on the behavior in enzyme reactions of bicyclic analogues with a variety of

conformational properties, the binding conformation to CYP707A3 was estimated to be

the more stable half-chair with the side chain and C8' axial. Thus, conformational

modification is not an effective strategy to develop CYP707A inhibitors with no ABA

activity.

3) AHI1

Based on the differences between the structural requirements for CYP707A

inhibition and ABA activity described above, we proposed AHI1 (abscisic acid

8'-hydroxylase inhibitor 1) as a candidate lead compound for the development of

CYP707A inhibitors (Fig. (3)) [36]. This compound was designed according to the

following criteria: (1) it has all the functional groups that are required for binding to the

enzyme; (2) it never substitutes for ABA as an activator of the signal transduction

pathway; and (3) it is easily prepared from a synthetic intermediate, which can be

modified to improve the properties of the final product. Racemic AHI1 acted as a

substrate and an effective competitive inhibitor of CYP707A3 (Table (1)) without

exhibiting ABA activity. In optically pure AHI1, (+)-AHI1, which corresponds to

(+)-ABA on C1' chirality, was apparently a poorer substrate than (+)-ABA, whereas

(−)-AHI1, which corresponds to the unnatural enantiomer (−)-ABA, yielded no enzyme

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reaction product, and acted as a more effective competitive inhibitor than (+)-AHI1. The

K_I value for (+)-AHI1 is slightly higher than the K_M value for (+)-ABA. Because the

elimination of C6 in (+)-ABA does not affect binding to the CYP707A active site, the

low affinity of (+)-AHI1 is likely a consequence of the absence of the enone moiety in

the ring. In contrast to (+)-enantiomers, removal of the ring enone allows

(−)-enantiomers to bind to the active site as described above. Although ABA has a chiral

center (C1'), the molecular shape of ABA is relatively symmetrical (Fig. (**4**)). This is

why (*R*)-(−)-ABA, which does not occur naturally, shows similar hormonal activity to

naturally occurring (*S*)-(+)-ABA in many bioassay systems. In the crystal structure of

the ABA-receptor complex [28-32], there is a space that can accommodate C8' of

(−)-ABA in the α axial direction at C2' of (+)-ABA, although not perfectly. On the other

hand, CYP707A cannot bind (−)-ABA, indicating that the CYP707A active site does not

have space for C8' of (−)-ABA. Nevertheless, (–)-AHI1 with the unnatural C1'

configuration can bind to the CYP707A active site. (–)-AHI1 may reinforce the affinity

by relatively nonspecific, hydrophobic interactions with the lipophilic ring, in a manner

Fig. (**4**)

not equivalent to that of (+)-ABA (Fig. (**4**)). Because AHI1 exhibits no significant ABA

bioactivity, it is a useful lead compound for the design and development of potent and

specific inhibitors of CYP707A.

4) AHI derivatives

AHI2, the 8'-difluorinated derivative of AHI1, was developed to test the usefulness of AHI1 as a lead compound (Fig. (**3**)) [36]. This compound was expected to resist CYP707A, because 8',8'-difluoro-ABA yields no enzyme products and acts as an effective competitive inhibitor of CYP707A. In an in vitro experiment, racemic AHI2 competitively inhibited the reaction with recombinant CYP707A3 as effectively as AHI1 (Table (**1**)). In contrast to racemic AHI1, racemic AHI2 resisted hydroxylation at C8', a result consistent with the design concept. An optically active AHI2 that corresponds to unnatural (–)-ABA, (–)-AHI2, acted as the most effective of non-azole inhibitors, with a K_I value of 0.08 μM (unpublished data).

Because 7',8',9'-trinor-AHI1 does not inhibit CYP707A (unpublished data), in the case of 4'-deoxo analogues of ABA, including AHI1, methyl groups on the ring were thought to be one of the functional groups stabilizing the enzyme-ligand complex through van der Waals or CH-π interactions. If the interaction were a weak hydrogen bond such as a CH-π interaction, a more acidic hydrogen at these sites might reinforce affinity for ABA 8'-hydroxylase. However, substitutions with additional carbons at these

methyl groups substantially reduce affinity for the enzyme [26], suggesting that the substrate-binding cavity accommodates ABA with little or no tolerance for bulkier structures. It follows, therefore, that an acidic hydrogen should be introduced directly on the ring carbon, instead of the methyl groups. Thus, we designed and synthesized AHI4, which has an axial hydroxy group instead of geminal methyl groups at C6' of AHI1 [37]. (+)-AHI4, which has the same configuration at C1' as (+)-ABA, strongly inhibited recombinant CYP707A3. The inhibition mode was competitive, with a K_I value of 0.14 μM (Table (**1**)). This K_I value is about 10-fold less than that for (+)-AHI1, indicating a 10-fold increase in affinity for the active site due to the hydroxy group substitution for the geminal methyl groups at C6'. This result shows that the axial hydroxy group at C6' of (+)-AHI4 stabilizes the enzyme-ligand complex more strongly than the geminal methyls in (+)-AHI1. The acidic hydrogen of the hydroxy group is expected to provide a stronger interaction than the CH-π interaction. The inhibitory activity of (+)-AHI4 was more than 10 times that of (+)-AHI1, equal to that of (+)-6-nor-ABA. Because (+)-AHI1 was 10 times less effective than (+)-6-nor-ABA, owing to the absence of the ring enone, this finding should aid in the design of more effective non-azole CYP707A inhibitors. On the other hand, (–)-AHI4 and the epimer of AHI4 at C2', (±)-*epi*-AHI4, exhibited little inhibitory activity.

(+)-AHI4 did not exhibit ABA activity, but the compound enhanced the effect of (+)-ABA upon simultaneous application of both to rice seedlings. The activity of 1 μM ABA, at which the elongation inhibition ratio is 22%, was enhanced to 96% by addition of 30 μM (+)-AHI4 [37]. This suggests that (+)-AHI4 inhibits 8'-hydroxylation of (+)-ABA in vivo. Radish seedlings acquired drought-stress tolerance when treated with 400 μM (+)-AHI4 in the absence of (+)-ABA [34]. (+)-AHI4 is thought to induce drought-stress tolerance by slowing endogenous ABA catabolism in radish seedlings. (+)-AHI4 may be more effective when ABA is required (e.g., under stress conditions) than when it is not (e.g., germination and growth).

AZOLE-TYPE P450 INHIBITORS

1) UNI

Fig. (5)

S-(+)-UNI (Fig. (5)) is an azole-containing cytochrome P450 inhibitor developed as a plant growth retardant, uniconazole P, in the 1980s [38,39]. 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

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oxidation of *ent*-kaurene to *ent*-kaurenoic acid [22], biosynthetic precursors of the plant

hormone GA. This activity has prompted researchers to use UNI as a chemical tool

inhibiting GA biosynthesis. However, UNI also inhibits brassinosteroid biosynthesis

[23,24] and alters the level of other plant hormones, such as auxins, cytokinins, ethylene

and ABA [25]. Recently, Kitahata *et al.* [20] and Saito *et al.* [19] revealed that UNI

strongly inhibits CYP707A. The K_I value is 10 nM for recombinant *Arabidopsis*

CYP707A3 expressed by *E. coli*, whereas the K_M value of the native substrate, (+)-ABA,

is 3.4 μM for the same enzyme [40]. This shows that UNI fits well into the substrate

pocket of CYP707A3. Nevertheless, UNI is also an inhibitor of CYP701A and CYPs in

brassinosteroid biosynthesis [23-25], the native substrates of which are structurally quite

different: e.g., ABA (sesquiterpene) for CYP707A; *ent*-kaurene (diterpene) for

CYP701A; and steroids for brassinosteroid biosynthetic enzymes. Mammalian P450

enzymes metabolizing xenobiotics have a large conformational flexibility to adapt to

substrates of different sizes [41]. Because some plant P450 species possess xenobiotic

detoxification activity [42], they may be highly flexible in conformation. On the other

hand, CYP707A has relatively high substrate specificity [26]. If other UNI-inhibiting

P450 enzymes involved in biosynthesis and catabolism of plant hormones also have

high substrate specificity, even though their specificity has not yet been investigated in

detail, explanations other than enzyme flexibility may be required to explain the adaptation of UNI.

We cannot use UNI in expectation of only inhibiting ABA catabolism because of its relatively broad inhibition spectrum. Nevertheless, its very strong inhibitory effect on CYP707A is very attractive in helping to develop a selective and strong inhibitor of this enzyme. If some structural modification of UNI increases enzyme specificity with no effect on its strong affinity for CYP707A, then this would be a good strategy for developing a potent and specific inhibitor of CYP707A. To provide direction for the lead modification, we considered why UNI has a relatively broad inhibitory spectrum

Fig. (**6**)

(Fig. (**6**)).

Azole-type P450 inhibitors bind to the target P450 active site both by coordinating to the heme-iron atom and interacting with surrounding protein residues. Because heme coordination 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. Thus, one reason for the broad inhibitory spectrum of UNI must be that UNI is small enough to embed itself into various substrate-binding pockets. If UNI just invades the pocket, even if its geometry does not completely coincide with that of the pocket, the structural plasticity of a P450 active site can allow it to fit well enough to

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make the aza-nitrogen interact with the heme iron. In addition, because UNI is structurally flexible as described later, the structural fit between UNI and P450 enzymes may depend on the conformational flexibility of UNI rather than that of P450 active sites. Based on these possibilities, we developed two types of UNI analogues, enlarged and conformationally restricted types, as candidates for a more specific CYP707A inhibitor.

2) Enlarged-type analogues of UNI

Although the crystal structures of P450s indicate an overall similarity in structural folding in spite of limited sequence identity, regions responsible for substrate binding differ substantially. These regions correspond to the substrate recognition sites, SRS1 to SRS6 [43]. In particular, SRS1-3 and 6, which are far from the heme in the active site, are structurally quite variable and are involved significantly in substrate specificity. In the case of UNI, the portion of the molecule far from the triazole interacting with the heme is the 4-chlorine of the phenyl ring, which might interact with SRS1-3 and 6. Thus, we selected C4' in UNI as a scaffold for enlarging the UNI molecule.

A set of 11 kinds of enlarged UNI analogues (UT) was synthesized using click chemistry [44] (Fig. (7)), by which a variety of compounds is easily prepared [45]. The alkyl-type UT, except for the longest-chain UT15, exhibited high inhibitory activity (greater than 90%) against recombinant *Arabidopsis* CYP707A3 coexpressed with *Arabidopsis* P450 reductase (ATR2) in *E. coli*; the inhibitory activity was equivalent to

that of UNI (Table (2)). The mode of inhibition of UT4 was determined to be competitive; the inhibition constant (K_I) was 195 nM. Although this value is larger than that for UNI (10 nM), UT4 has strong affinity to the active site of CYP707A, considering that the K_M value for (+)-ABA was 3.4 μM under the same experimental conditions. The alcohol-types UT1H, UT2H, and UT4H were also effective (75-85% inhibition) but were less potent than the alkyl-types. The acid-type UT4A was much less effective than the alcohol-types. These results suggest the following: (1) linear elongation at C4' in UNI is generally not crucial to binding to the active site of CYP707A; (2) the protic group in the elongated portion of the molecule may have a negative effect on binding to the enzyme, and (3) there is an upper limit to the length and hydrophobicity of the elongated portion that allows binding to the enzyme.

The alkyl-types of UT are highly hydrophobic; UT7-15 had an especially high estimated log *P* value (> 5) (Table (2)). Compounds with very high log *P* value are too

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poorly water soluble. In fact, UT7-15 did not dissolve in water, although it dissolved in

the buffer used in the enzyme assay. These compounds are not practical to use as plant

regulators, although they may be useful as chemical probes for in vitro enzymatic

studies. Thus, only the UT compounds with a log P value < 4 were assayed for rice

seedling arrest. The acid- and alcohol-types of UT were not effective even at 100 μM,

whereas the alkyl-types showed a significant effect at a concentration greater than 30

μM (Fig. (**8**)) [45]. These results suggest that elongation at C4' in UNI depresses the

affinity to enzymes involved in seedling growth; the hydrophilic substitution is

especially crucial. Considering its potency in CYP707A inhibition, its small effect on

seedling growth, and its ease of application, UT was the good candidate for a highly

selective inhibitor of CYP707A. Recently, we have developed a more potent and higher

selective UT analogue, abscinazole-E1 (Abz-E1). This inhibitor has almost same K_I and

log P values as those of UNI, whereas it does not inhibit rice seedling growth even at

100 μM (unpublished data). Further evaluation as the specific CYP707A inhibitor is

now in progress.

3) Conformationally restricted analogues of UNI

The conformation of UNI depends on two hydrophilic functional groups, the aza-nitrogen (N2") and the hydroxy group at C3. First of all, we synthesized

C3-modified UNI analogues (UNI-OMe, UNI-F, UNI-H, IMI, IMI-OMe, IMI-F, and IMI-H) (Fig. (**9**)) as probes to explore the conformational preference of UNI in binding to CYP707A [46]. The C3-hydroxy group was replaced by methoxy, fluorine, or hydrogen moieties, whereas the aza-nitrogen (N2") was substituted by carbon. The hydroxy group can act as both a donor and an acceptor in a hydrogen bond, whereas the methoxy group can act only as an acceptor. Fluorine can act only as a weaker acceptor than hydroxy and methoxy groups. The aliphatic hydrogen can be neither donor nor acceptor in a conventional hydrogen bond. The introduction of a carbon at the 2"-position of a triazole ring can eliminate the intramolecular hydrogen bond. These UNI and IMI analogues were expected to have different conformational profiles and different interactions with the CYP707A active site. Although it is not necessary for the ligand conformation in the protein-ligand complex to be similar to the preferred conformation in the uncomplexed situation, conformational consistency between the uncomplexed and complexed molecules should be advantageous energetically. Thus, an analogue with a conformational preference different from that of UNI may have a different inhibition spectrum, even if it is a flexible molecule.

We constructed four initial geometries for UNI and IMI analogues, conformers **A**–**D** (Fig. (**10**)), on the basis of the C2-N1" and C2-C3 bond rotations [46]. The most stable geometries of each conformer and their energies were obtained by theoretical calculations. Because conformers **A** and **B** in UNI were much more stable than conformers **C** and **D**, the populations of which were less than 5% in both the gas and aqueous phases, we focused on only the **A/B** population ratio in all the analogues (Table

(**3**)). UNI prefers conformer **A** in gas phase and **B** in aqueous phase. UNI-OMe and UNI-F prefer conformer **B** in both phases, whereas UNI-H and all the IMI analogues do not show a distinct conformational preference in either phase. These observations agreed with results from conformational studies using NMR and X-ray crystallographic analyses.

Because UNI-H and IMI-H prefer **A** and **B** almost equally in both gas and aqueous phases, the difference in activity between UNI-H and IMI-H was expected to be a straightforward reflection of the difference in the affinity for the P450 active site of the triazole and imidazole rings, independent of their conformational preferences. In the theoretical analysis, imidazole bound to the heme more strongly than triazole by a factor of ca. 30. This calculation agreed with the ratio of K_I (UNI-H)/K_I (IMI-H), 32, in the CYP707A3 inhibition assay. However, the ratio of K_I (UNI-X)/K_I (IMI-X) decreases in

the order -H (32) > -F (23) > -OMe (7) > -OH (5). The smaller influence of -F, -OMe, and -OH compared to that of -H may indicate that the preference for conformer **B** in a polar environment contributes energetically to the affinity for the CYP707A active site. On the other hand, the inhibitory activity on growth of rice seedlings did not differ for the triazole and imidazole rings on the basis of the ratio of IC_{50} (UNI-H)/IC_{50} (IMI-H), 0.8. All the IMI analogues had weaker activity than UNI and similar activity to UNI-H, independent of the 3-substituent, whereas UNI analogues with the polar 3-substituent showed stronger activity than UNI-H. This result may indicate that the P450 active sites involved in rice seedling growth, e.g., GA biosynthetic enzymes CYP701A or CYP88A, also prefer conformer **B**. These findings suggest that the conformational manipulation of UNI may not be effective for increasing the enzyme selectivity of UNI. Nevertheless, we focused on the conformationally restricted analogues of UNI [40], expecting that their rigid properties would increase selectivity, because restriction of conformation is a strategy that has been widely used in drug design to develop potent and selective enzyme inhibitors [47].

Fig. (**11**)

We searched for and found a known conformationally restricted UNI derivative [48], which we named UFAP1 (Fig. (**11**)). UFAP1 has a structure bridged with a carbonyl group between C5" in the triazole ring and the oxygen of the 3-hydroxy in UNI;

therefore, it is expected to be restricted in a similar conformation to the **B**-type form of UNI (Fig. (**10**)). UFAP1, the C3 stereochemistry of which is not shown in the literature, has been described as a fungicide and plant growth retardant [48]. However, its conformational structure-activity relationship and enzyme selectivity have never been investigated. Thus, we first synthesized UFAP1 and examined its inhibitory effect on CYP707A and its activity as a plant growth retardant. UFAP1 functioned as a plant growth retardant as effectively as UNI, whereas it did not inhibit CYP707A3 at all, unlike UNI. This suggested that a novel, highly specific P450 inhibitor can be produced by conformational restriction. Restriction in a different manner from that of UFAP1 might produce a selective inhibitor of CYP707A that does not inhibit other UNI-inhibited P450s, and thus will never cause plant growth inhibition, in contrast to UFAP1. We designed and synthesized various UNI analogues with conformations (**A** or **B**) restricted by different bridge moieties to identify a selective inhibitor of CYP707A (Fig. (**11**)).

In the CYP707A3 inhibition assay, among the **A**-type analogues, the cyclic ether-bridged analogue IFAP2 exhibited significant inhibition, whereas among the **B**-type analogues, only the lactol-bridged analogues UFAP3 and IFSP3 exhibited significant inhibition; IFSP3 was an especially strong inhibitor, as was IFAP2 (Table

Table (**4**)

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(**4**)). Other analogues of both types were poor inhibitors. The cyclic ether-bridged compounds (UFAP2, IFSP2, and IFAP2), which are the most simple conformationally restricted UNI analogues, have no additional functional group compared to UNI-OMe which is as effective as UNI [46]. This means that the effect of conformational restriction is dominant in the interaction with the active site. Therefore, the difference in CYP707A3 inhibition between the **A**-type (IFAP2) and **B**-type (UFAP2 and IFSP2) suggests that the CYP707A3 active site prefers the **A**-type conformation of UNI, contrary to the speculation based on the preferred conformation and activity relationships of C3-modified UNI analogues. On the other hand, for the lactol- and lactone-bridged analogues, this conformational preference did not hold, probably because the additional hydroxy and carbonyl groups were advantageous or disadvantageous according to their orientation in the active site. The potent activity of **B**-type analogues UFAP3 and IFSP3 indicates that the lactol moiety of the **B**-type analogue has a significant role in binding to compensate for the conformational disadvantage.

The most potent analogues IFSP3 and IFAP2 were examined in greater detail for each stereoisomer at C3. Kinetic analysis revealed that these analogues function as competitive inhibitors; however, their affinity for the enzyme active site was estimated

to be less than one-tenth that of *S*-UNI on the basis of the K_I values. Interestingly, 3*R*-IFSP3 was slightly more effective than the 3*S*-enantiomer (K_I = 420 and 970 nM, respectively), unlike the case of UNI. Analogue IFSP3 may fit into the active site of CYP707A in a quite different manner from UNI, because of its **B**-type conformational rigidity and lactol moiety.

In an inhibition assay of rice seedling growth, comparing the cyclic ether-bridged analogues, the **A**-type analogue (IFAP2) was more potent than the **B**-type analogues (UFAP2 and IFSP2), suggesting that the active site of P450 enzymes involved in rice seedling growth (CYP701A or CYP88A) prefers the **A** conformation, as in the case of CYP707A. However, the addition of carbonyl or hydroxy groups to the bridge of the **B**-type analogues had an opposite effect on the activity, depending on the type of azole ring. The lactone and lactol-bridged **B**-type analogues with a triazole (UFAP1 and UFAP3) were stronger than the **A**-type analogues, whereas those with an imidazole (IFSP1 and IFSP3) were not active at all. This result suggests that the polar moiety on the bridge acts in cooperation with the 2"-aza nitrogen in the triazole in binding to CYP701A or CYP88A.

Using the conformational restriction strategy, we found three azole-type P450 inhibitors that were more selective than UNI, although we cannot discuss the reason in

detail due to the lack of structural characterization of CYP707A. UFAP1 and UFAP3 act

as effective plant growth retardants without inhibiting ABA catabolism, whereas IFSP3,

renamed abscinazole-F1 (Abz-F1), acts as an effective inhibitor of ABA catabolism

without inhibiting plant growth.

DROUGHT TOLERANCE INDUCED BY AHI1, UT4, AND ABZ-F1

To determine whether in vivo inhibition of AHI1, UT4, and Abz-F1 against

CYP707A is enough to intensify ABA-induced physiological responses, we tested the

effect of these CYP707A inhibitors on drought tolerance of apple seedlings. All the

inhibitors induced significant drought tolerance by a one-time spray treatment (Fig.

Fig. (**12**)

(**12**)) [40,45]. In the *S*-UNI treatment at high concentration, leaf browning was observed.

This side effect, which may have been caused by inhibition of other enzymes including

P450s, was not observed in treatments using our selective CYP707A inhibitors. This

result suggests that AHI1, UT4, and Abz-F1 function as more selective inhibitors of

CYP707A than UNI.

CONCLUSIONS AND PERSPECTIVE

We developed two types of specific inhibitors of CYP707A, a key enzyme of ABA

catabolism. The ABA analogue-type inhibitors, AHI1 and its derivatives, were

generated on the basis of differences between the structural requirements for CYP707A

inhibition and for ABA activity. Although AHIs are a successful example in the context

of developing a CYP707A inhibitor, they do not have any ripple effect in the context of

a general and effective method of developing selective inhibitors of plant P450 enzymes.

On the other hand, the azole-type P450 inhibitors, UT4 and Abz-F1, were developed by

conformational restriction and enlargement, respectively, of the plant growth retardant

UNI, which has a broad inhibition spectrum. These UNI derivatives acted as effective

inhibitors of CYP707A both in vitro and in vivo with no growth-retardant effect (Fig.

(**13**)).

Fig. (**13**)

Selectivity is an important aspect of the drug discovery process. Substrate analogues

have an advantage in P450 selectivity, but their cost of development is comparatively

high, whereas azole-containing compounds, which are easier to develop by modifying

the structure, have an unfavorable position in terms of selectivity because of their

common mechanism of inhibition. Some azole-type P450 inhibitors cause serious side

effects because of their inhibition of non-target P450 enzymes. Well-known azole

inhibitors such as fluconazole and ketoconazole, which are used as anti-fungal compounds in mammals, are toxic to the liver of the host [49,50]. Triadimenol and tebuconazole, which are azole fungicides, also inhibit plant sterol 14α-demethylase (CYP51) [51]. UNI and paclobutrazol inhibit at least two P450 enzymes, CYP701A and CYP707A [19]. An enlargement or conformational restriction strategy was successful in increasing the selectivity of UNI. However, we cannot exclude the possibility that UT4 and Abz-F1 inhibit P450 enzymes other than CYP707A without examining their inhibition spectrum on all P450s, which are present in all organisms. Nevertheless, the azole-type P450 inhibitors will be attractive in developing novel inhibitors of plant P450 enzymes owing to their low developmental cost, easy structural evolution, and strong inhibitory potency. Our modification strategy has been useful for improving the P450 enzyme selectivity of a lead compound with a broad inhibition spectrum.

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Figure legends

Fig. (1). Catabolism of ABA in plants [1-4]. The major catabolic pathway is via 8'-hydroxylation catalyzed by CYP707A. PA, phaseic acid; DPA, dihydrophaseic acid; ABA-GE, β-D-glucosyl ester of ABA; ABA-GS, β-D-glucoside of ABA; HMG, 3-hydroxy-3-methylglutaryl.

Fig. (2). Structural requirements for ABA activity and for CYP707A inhibition [26]. The relative activities of structural analogues are shown in numbers. ABA activity was evaluated in rice seedling elongation assay. CYP707A inhibition was measured using a described method that uses recombinant Arabidopsis CYP707A3 microsomes [19,26]. The significant functional groups were shown outlined in bold line.

Fig. (3). AHI1 and its derivatives [36,37].

Fig. (4). Putative mechanism of asymmetrical ligand binding at the CYP707A active site [27].

Fig. (5). UNI, a potent inhibitor of CYP707A and CYP701A [19,20,22].

Fig. (6). Hypothetical mechanism of multiple P450 enzyme inhibition by UNI [40,45,46].

Fig. (7). UT, enlarged analogues of UNI [45].

Fig. (8). Inhibitory effect of UT and UNI on rice seedling growth [45].

Fig. (9). Structures of UNI-X and IMI-X [46].

Fig. (10). Four conformers, **A-D**, of UNI-X and IMI-X [46].

Fig. (11). Conformationally restricted analogues of UNI [40].

Fig. (12). Drought tolerance of apple seedlings treated with AHI1 (unpublished data), UT4 [45], and Abz-F1 [40].

Fig. (13). Conformational restriction and enlargement yield highly selective P450 inhibitors.

Table (1). Inhibition constants for representative ABA analogues, AHI1 and its derivatives.[a]

compounds	K_I [μM]
(+)-ABA	0.71 (K_M)
(+)-6-nor-ABA	0.16
(+)-8',8'-difluoro-ABA	0.17
(+)-8'-methylene-ABA	5.4
(+)-8'-methylidyne-ABA	NI[b]
(−)-ABA	NI
(−)-4'-deoxo-ABA	27
(−)-2',3'-dihydro-4'-deoxo-ABA	0.45
(±)-AHI1	0.40
(+)-AHI1	1.3
(−)-AHI1	0.30
(±)-AHI2	0.41
(−)-AHI2	0.08
(+)-AHI4	0.14
(−)-AHI4	NI
(±)-*epi*-AHI4	NI
(+)-*epi*-AHI1	2.50
(−)-*epi*-AHI1	1.63

[a] Ref. 27, 36, and 37.

[b] No significant inhibition

Table (2). Inhibitory activities of UT against CYP707A3 and rice seedling elongation.[a]

compound	structural properties	CYP707A3	rice
	$\log P^b$	inhibition^c (%)	IC$_{50}$ (μM)
S-UNI	3.13	100 (K_I = 10 nM)	0.18
UT4A	2.64	63	NI^d
UT1H	1.55	83	NI
UT2H	2.12	77	NI
UT4H	3.08	92	NI
UT3	3.51	92	21
UT4	3.96	100 (K_I = 195 nM)	42
UT7	5.29	96	NT^e
UT9	6.18	100	NT
UT11	7.07	100	NT
UT13	7.96	95	NT
UT15	8.85	54	NT

^{a} Ref. 45.

^{b} Calculated partition coefficient.

^{c} Inhibition ratio of compounds (10 μM) in the 8'-hydroxylation of ABA (5 μM) (average of two sets of experiments).

^{d} No significant inhibition even at 100 μM (max concentration tested).

^{e} Not tested because the compound was not dissolved in water.

Table (3). Conformational properties and CYP707A3 inhibitory activities of UNI-X and IMI-X.[a]

| compound | calculated **A/B** ratio[b] | | CYP707A3 inhibition | rice growth inhibition |
	gas[c]	aq.[d]	K_I (nM)	IC_{50} (μM)
UNI	99/1	19/81	2.7	0.18
UNI-OMe	1/99	11/89	8.6	0.90
UNI-F	1/99	17/83	34	0.65
UNI-H	70/30	64/36	450	2.7
IMI	58/42	52/48	0.6	2.3
IMI-OMe	54/46	49/51	1.2	2.6
IMI-F	62/38	54/46	1.5	3.0
IMI-H	57/43	58/42	14	3.4

[a] Ref. 46.

[b] Calculated based on the energy difference between the optimized **A** and **B** conformations.

[c] B3LYP/6-311++G(2df,2p)//B3LYP/6-31G(d).

[d] B3LYP/6-311++G(2df,2p)//B3LYP/6-31G(d). Geometry optimization and frequency calculations were performed using the Onsager method, and the single point energy calculations were performed using the IEF-PCM method.

Table (4). Inhibitory activities of conformationally restricted analogues of UNI on CYP707A3 and rice seedling elongation.[a]

compound		structural properties[b]			CYP707A3		rice
		azole	bridge	conf.	inhibition[c] (%)	K_I (nM)	IC$_{50}$ (μM)
UNI	S	Trz		**A/B**	100	10	0.18
	R	Trz		**A/B**	79	1450	7.8
UFAP1	±	Trz	Lon	**B**	14	NM[d]	2.3
	S	Trz	Lon	**B**	10	NM	1.3
	R	Trz	Lon	**B**	27	NM	46
UFAP2	±	Trz	Ether	**B**	31	NM	78
UFAP3	±	Trz	Lol	**B**	69	NM	3.2
IFSP1	±	Imz	Lon	**B**	35	NM	NI[e]
IFSP2	±	Imz	Ether	**B**	13	NM	28
IFSP3 (Abz-F1)	±	Imz	Lol	**B**	91	NM	NI
	3S	Imz	Lol	**B**	96	970	NI
	3R	Imz	Lol	**B**	95	420	NI
IFAP1	±	Imz	Lon	**A**	52	NM	58
IFAP2	±	Imz	Ether	**A**	100	NM	9.0
	S	Imz	Ether	**A**	100	160	8.2
	R	Imz	Ether	**A**	87	520	12

[a] Ref. 40.

[b] azole, type of azole (Trz, triazole; Imz, imidazole); bridge, bridging moiety (Lon, lactone; Ether, cyclic ether; Lol, lactol); conf., type of conformation.

[c] Inhibition ratio of compounds (10 μM) in the 8'-hydroxylation of ABA (5 μM) (average of at least three sets of experiments).

[d] Not measured.

[e] No significant inhibition even at 100 μM (max concentration tested).

Fig. (1)

For ABA activity

demethyl: ~1
didemethyl: ~0.1
hydroxymethyl: ~0.1
ethyl: ~1
mono (di, tri)
    fluoromethyl: 1~

propyl: ~0.1
methoxymethyl: 1~
acetylene: 1~
ethylene: 1~

axial   *equatorial*: ~0

dimethyl   methyl   **demethyl: ~0**

S-configuration

R-configuration: 0.1~1

2*Z*, 4*E*-diene   2*E*: ~0
   dihydro: ~0

CO_2H free acid   ester: 0~1
   aldehyde: ~0.1
   alcohol: ~0.01

deoxo: ~0.1   oxo

OH

hydrogen

hydroxy   deoxy: ~0.1
   fluoro: ~0.1

halogen: ~1
methyl: ~1
azide: ~1

methyl   demethyl: ~0
   ethyl: ~1
   propyl: ~0.1

ene   dihydro: ~0.1

For CYP707A inhibition

ethyl: <0.1
propyl: ~0
methoxymethyl: ~0

acetylene: ~0
methylene: ~0.1
mono, difluoromethyl: ~4
trifluoromethyl: ~1

axial   *equatorial*: ~0

dimethyl   methyl   **demethyl: ~4**

S-configuration

R-configuration: ~0

2*Z*,4*E*-diene   2*E*: ~0
   dihydro: ~0

CO_2H free acid   ester: ~0
   aldehyde: <0.1

oxo

(*S*)-deoxo: ~0.3
(*R*)-deoxo: ~0.03

OH

hydrogen

hydroxy   deoxy: ~1
   fluoro: ~1

halogen: 0.1~1
azide: <0.1

methyl   demethyl: <0.1
   ethyl: ~1
   propyl: <0.1

enone   ene

(*S*)-dihydro-deoxo: ~0.1
(*R*)-dihydro-deoxo: ~1

(*S*)-dihydro: ~0.1
(*R*)-dihydro: ~0

Fig. (**2**)

Fig. (3)

(+)-ABA

(−)-ABA

not accommodated

not accommodated

(+)-AHI1

(−)-AHI1

(+)-ABA

(−)-ABA

(+)-AHI1

(−)-AHI1

Fig. (**4**)

ABA catabolism

CYP707A

ABA

8'-hydroxy-ABA

S-(+)-UNI

ent-kaurene

ent-kaurene oxidase
(CYP701A)

GA biosynthesis

ent-kaurenoic acid

Fig. (5)

Fig. (**6**)

UT4A •~~~CO₂H

UT1H •~OH

UT2H •(OH)

UT4H •(OH)~~~

UT3 •~~

UT4 •~~~

UT7 •~~~~~

UT9 •~~~~~~

UT11 •~~~~~~~

UT13 •~~~~~~~~

UT15 •~~~~~~~~~

Fig. (**7**)

UT4

UNI

0   0.03   0.1   0.3   1   3   10   30   100 µM

Fig. (**8**)

	R	X
UNI	N	OH
UNI-OMe	N	OMe
UNI-F	N	F
UNI-H	N	H
IMI	CH	OH
IMI-OMe	CH	OMe
IMI-F	CH	F
IMI-H	CH	H

Fig. (**9**)
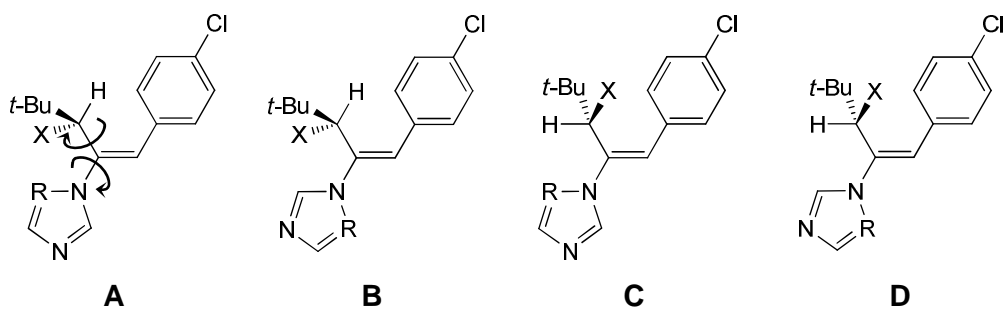
Fig. (**10**)

B-type restriction
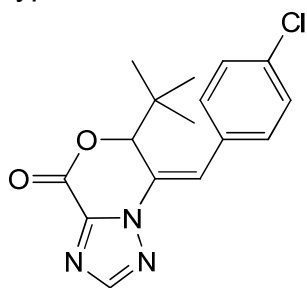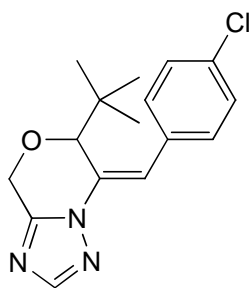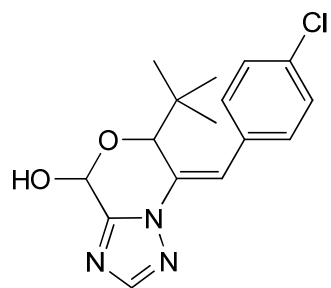
UFAP1   UFAP2   UFAP3
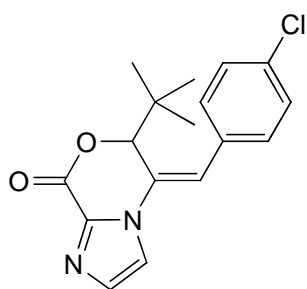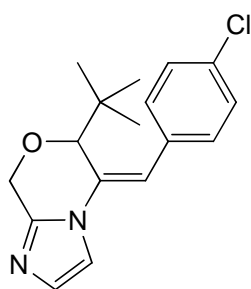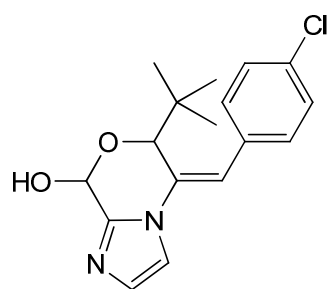
IFSP1   IFSP2   IFSP3 (**Abz-F1**)

A-type restriction
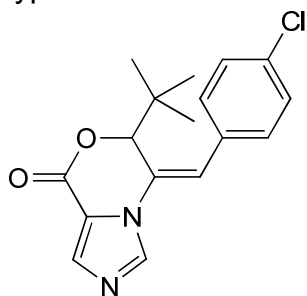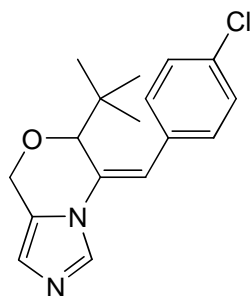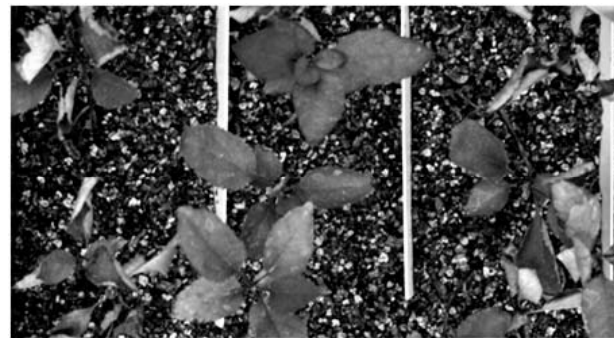
IFAP1   IFAP2

Fig. (**11**)

control

AHI1

control   UT4   UNI

control   Abz-F1   UNI

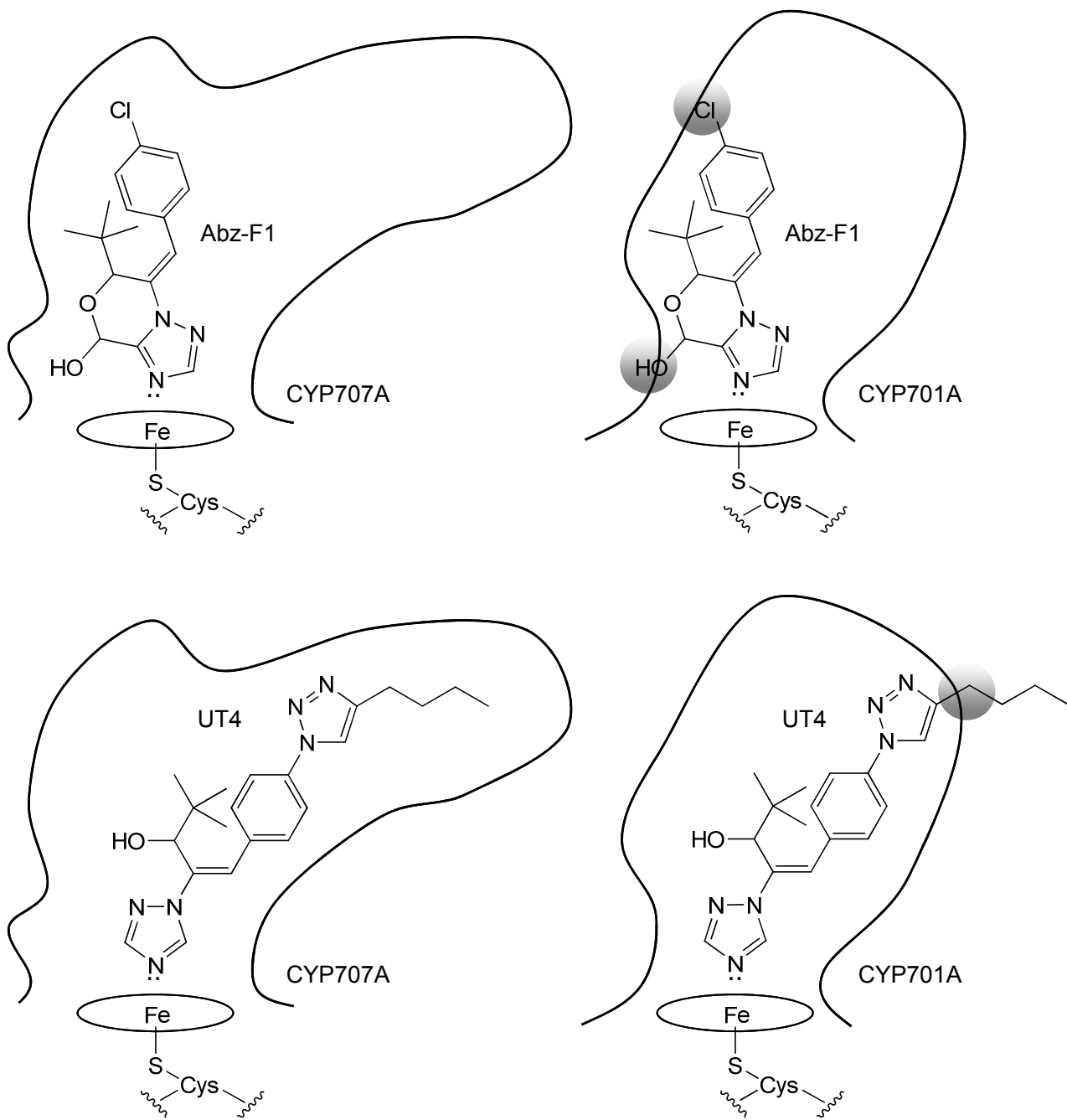Fig. (**12**)

Fig. (**13**)