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著者	Todoroki Yasushi, Naiki Kumi, Muramatsu Taku, Ohnishi Toshiyuki, Ueno Kotomi, Mizutani Masaharu, Hirai Nobuhiro
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A conformationally restricted uniconazole analogue as a specific inhibitor of rice *ent*-kaurene oxidase, CYP701A6

Yasushi Todoroki^{a,*}, Kumi Naiki^a, Taku Muramatsu^a, Toshiyuki Ohnishi^b, Kotomi Ueno^c, Masaharu Mizutani^c, Nobuhiro Hirai^d

^a *Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, Shizuoka 422-8529, Japan*

^b *Division of Global Research Leaders, Shizuoka University, Shizuoka 422-8529, Japan*

^c *Graduate School of Agricultural Science, Kobe University, Kobe 657-8501, Japan*

^d *Graduate School of Agriculture, Kyoto University, Kyoto 606-8501, Japan*

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* Corresponding author. Tel./fax: +81 54 238 4871.

E-mail address: aytodor@ipc.shizuoka.ac.jp (Y. Todoroki)

ABSTRACT

The plant growth retardant uniconazole (UNI), which has been used as an effective inhibitor of *ent*-kaurene oxidase (CYP701A) involved in gibberellin biosynthesis, also strongly inhibits ABA 8'-hydroxylase (CYP707A), a key enzyme in abscisic acid catabolism. Azole P450 inhibitors bind to the P450 active site by both coordinating to the heme-iron atom via an sp² nitrogen and interacting with surrounding protein residues through a lipophilic region. We hypothesized that poor selectivity of UNI may result from its small molecular size and flexible conformation that allows it to fit into active sites differing in size and shape. To find a selective inhibitor of CYP701A based on this hypothesis, we examined inhibitory activities of three types of UNI analogues, which were conformationally constrained, enlarged in width, and enlarged in length, against recombinant rice CYP701A6 and *Arabidopsis* CYP707A3. Conformationally restricted analogues, UFAP2 and UFAP2N, inhibited CYP701A6 as strongly as UNI, whereas it inhibited CYP707A3 less than UNI.

S-uniconazole (UNI) is an azole inhibitor of cytochrome P450.^{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 (KA),³ a biosynthetic precursor of the plant hormone gibberellin (GA). However, UNI also inhibits brassinosteroid (BR) biosynthesis^{4,5} and alters the level of other plant hormones, such as auxins, cytokinins, ethylene and abscisic acid (ABA).⁶ In particular, UNI strongly inhibits ABA 8'-hydroxylase (CYP707A), a key enzyme in ABA catabolism (Figure 1).^{7,8} *S*-Diniconazole and paclobutrazol, which have a structure very similar to *S*-UNI, also inhibits both CYP701A and CYP707A.⁷⁻⁹ Because the selectivity for target proteins is very important to chemical tools for biological research, these azole compounds are not ideal inhibitors of a specific enzyme.

Azole inhibitors of P450 bind to the target P450 active site by both coordinating to the heme-iron atom and interacting with surrounding protein residues. Because heme coordination is a common property of azole inhibitors, their affinity and specificity for individual P450 enzymes depends on structural properties other than those of the azole group. UNI may be small and flexible enough to embed itself into various substrate-binding pockets, where it may adopt a distinct conformation and orientation for different active sites. Based on this speculation, in previous work, we generated selective inhibitors of CYP707A by enlarging or conformationally freezing the UNI molecule (Figure 2).¹⁰⁻¹² Here we focused on the inhibitory activity of these UNI analogues against CYP701A. Although our previous studies revealed an inhibitory effect on rice seedling growth,¹⁰⁻¹² it did not completely parallel the inhibitory potency against CYP701A enzymes because the dwarf phenotype is caused by loss-of-function of various enzymes involved in biosynthesis or catabolism of plant hormones and other bioactive molecules. Some genes encoding members of the plant *ent*-kaurene oxidase CYP701 family have been reported.¹³ Among them, rice CYP701A6 (OsKO2)¹⁴ and *Arabidopsis* CYP701A3^{15,16} were heterologously expressed and their three-step oxidation activity was verified by in vitro assays. However, no direct evidence for CYP701A inhibition of UNI and related compounds has been reported. In this study, we examined the inhibitory effect of UNI

and structural analogues against recombinant rice CYP701A6 produced in a baculovirus-insect cell expression system to find a CYP701A inhibitor with higher enzyme specificity than UNI.

SDS-PAGE analysis of the microsomal fraction prepared from insect cells infected with baculoviruses encoding full-length CYP701A6 showed a major band at around 50 kDa, which agrees with the molecular mass (56 kDa) deduced from the amino acid sequence ([Supplementary Figure S1](#)). The CO difference spectrum of the reduced microsomes exhibited a prominent peak at 450 nm ([Supplementary Figure S2](#)). Incubation of the microsomal fraction with *ent*-kaurene in the presence of the *Arabidopsis* P450 reductase ATR2 and NADPH supplementation yielded KA, detected as the methyl ester (Me KA), which has a molecular ion of m/z 316 in GC-MS analysis ([Figure 3a](#)). When 0.1 μ M UNI was added in the presence of 10 μ M *ent*-kaurene in the same assay system, Me KA was not detected ([Figure 3b](#)). Dose-response analysis revealed that the IC_{50} value of UNI was 58 nM (Table 1). This is the first direct evidence that UNI functions as a potent inhibitor of CYP701A activity.

The 14 structural UNI analogues, that were synthesized in our previous work¹⁰⁻¹² except for UFAP2N which is a new compound, were tested in the CYP701A6 inhibition assay. UFAP2N was prepared in a similar manner¹⁰ to UFAP2 ([Supplementary Scheme S1](#)). Activity was evaluated based on the decrease in the amount of the enzymatic product, KA, caused by addition of a test compound at a concentration one-tenth (1 μ M) that of the substrate *ent*-kaurene (10 μ M). Four compounds, IFAP2, UFAP2, UFAP2N and DSI-501, showed 100% activity, whereas the activity of other analogues was less than 50% ([Figure 4](#)). The IC_{50} values of UFAP2 and UFAP2N in the CYP701A6 inhibition assay were 231 and 153 nM, respectively, which are equivalent to that of UNI (58 nM) ([Table 1](#)). IFAP2 and DSI-501 are relatively potent inhibitors of CYP707A3, although their activity is much less than that of UNI, whereas UFAP2 and UFAP2N are very poor inhibitors of CYP707A3; especially, UFAP2N is less effective than UNI by a factor of 4000 on the basis of the K_i values. These results suggest that UFAP2 and UFAP2N are more specific CYP701A inhibitors than UNI. Abz-F1 and Abz-E1, which were developed as specific inhibitors of ABA 8'-hydroxylase CYP707A, were much less effective than UNI ([Figure 4](#)), as expected from their poor activity in a rice growth assay.^{10,11}

UFAP2 and UFAP2N were designed as a conformationally restricted analogue of UNI, based on the speculation that introduction of rigidity to the structure of UNI may cause an increase in enzyme specificity if the low enzyme specificity of UNI depends on a flexible structure.¹⁰ Although UFAP2 and UFAP2N inhibited the elongation of rice seedlings, its activity was much lower than that of UNI. The IC₅₀ values were *ca.* 20 μ M for UFAP2 and UFAP2N, whereas it was 0.16 μ M for UNI (Table 1), meaning that UFAP2 and UFAP2N are 100-fold less effective than UNI. It is not clear why there is a discrepancy in the activity in vitro and in vivo. The very strong retardant effect of UNI might be caused from combined inhibition of many P450 enzymes involved in the biosynthesis or catabolism of GA, ABA, and BR.

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Supplementary data

The procedures for preparation of a new compound UFAP2N, enzyme expression, enzyme assay, and biological assays are given. Supplementary data associated with this article can be found in the online version at doi:xx.xxxx/j.bmcl.2012.xx.xxx.

References and notes

1. Funaki, Y.; Ishiguri, T.; Kato, T.; Tanaka, S. *J. Pesticide Sci.* **1984**, *9*, 229.
2. Funaki, Y.; Oshita, H.; Yamamoto, S.; Tanaka, S.; Kato, T. (Sumitomo Chemical Co.). Ger. Offen. DE3010560, 1980.
3. Izumi, K.; Kamiya, Y.; Sakurai, A.; Oshio, H.; Takahashi, N. *Plant Cell Physiol.* **1985**, *26*, 821.
4. Iwasaki, T.; Shibaoka, H. *Plant Cell Physiol.* **1991**, *32*, 1007.
5. Asami, T.; Mizutani, M.; Fujioka, S.; Goda, H.; Min, Y. K.; Shimada, Y.; Nakano, T.; Takatsuto, S.; Matsuyama, T.; Nagata, N.; Sakata, K.; Yoshida, S. *J. Biol. Chem.* **2001**, *276*, 25687.
6. Izumi, K.; Nakagawa, S.; Kobayashi, M.; Oshio, H.; Sakurai, A.; Takahashi, N. *Plant Cell Physiol.* **1988**, *29*, 97.
7. Kitahata, N.; Saito, S.; Miyazawa, Y.; Umezawa, T.; Shimada, Y.; Min, Y. K.; Mizutani, M.; Hirai, N.; Shinozaki, K.; Yoshida, S.; Asami, T. *Bioorg. Med. Chem.* **2005**, *13*, 4491.
8. Saito, S.; Okamoto, M.; Shinoda, S.; Kushiro, T.; Koshiha, T.; Kamiya, Y.; Hirai, N.; Todoroki, Y.; Sakata, K.; Nambara, E.; Mizutani, M. *Biosci. Biotechnol. Biochem.* **2006**, *70*, 1731.
9. Fletcher, R. A.; Hofstra, G.; Gao, J.-G. *Plant Cell Physiol.* **1986**, *27*, 367.
10. Todoroki, Y.; Kobayashi, K.; Shirakura, M.; Aoyama, H.; Takatori, K.; Nimitkeatkai, H.; Jin, M.-H.; Hiramatsu, S.; Ueno, K.; Kondo, S.; Mizutani, M.; Hirai, N. *Bioorg. Med. Chem.* **2009**, *17*, 6620.
11. Todoroki, Y.; Aoyama, H.; Hiramatsu, S.; Shirakura, M.; Nimitkeatkai, H.; Kondo, S.; Ueno, K.; Mizutani, M.; Hirai, N. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 5782.
12. Okazaki, M.; Nimitkeatkai, H.; Muramatsu, T.; Aoyama, H.; Ueno, K.; Mizutani, M.; Hirai, N.; Kondo, S.; Ohnishi, T.; Todoroki, Y. *Bioorg. Med. Chem.* **2011**, *19*, 406.
13. Davidson, S. E.; Reid, J. B.; Helliwell, C. A. *Phytochem. Rev.* **2006**, *5*, 405.
14. Ko, K.-W.; Lin, F.; Katsumata, T.; Sugai, Y.; Miyazaki, S.; Kawaide, H.; Okada, K.; Nojiri, H.; Yamane, H. *Biosci. Biotechnol. Biochem.* **2008**, *72*, 3285.
15. Helliwell, C. A.; Poole, A.; Peacock, J.; Dennis, E. S. *Plant Physiol.* **1999**, *119*, 507.
16. Morrone, D.; Chen, X.; Coates, R. M.; Peters, R. J. *Biochem. J.* **2010**, *431*, 337.

Table and Figure legends

Table 1

Inhibitory activity of UNI and its analogues against recombinant CYP701A6 and CYP707A3. The values are averages of at least two independent experiments.

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

Figure 2. Chemical structures of UNI and the analogues used in this work.

Figure 3. Selected ion chromatograms (m/z 316) from GC-MS analysis of the recombinant CYP701A6 conversion of *ent*-kaurene into KA: (a) in the absence of inhibitors; (b) in the presence of 0.1 μ M UNI; (c) in the presence of 1 μ M UFAP2; and (d) in the absence of NADPH; (e) a standard sample of Me KA.

Figure 4. Activity of UNI analogues (1 μ M) in the CYP701A6 inhibition assay (10 μ M *ent*-kaurene). NI: no measurable inhibition at the tested concentration.

Table 1

Inhibitory activity of UNI and its analogues against recombinant CYP701A6 and CYP707A3. The values are averages of at least three independent experiments.

Compound	CYP701A6		CYP707A3		Rice growth
	Inhibition ^a (%)	IC ₅₀ (nM)	Inhibition ^b (%)	K _I (nM)	IC ₅₀ (μM)
S-UNI	100±0	58±9	100±0	9.9±0.4	0.16±0.03
UFAP2	100±0	231±56	31±10 ^c	— ^d	21±3
UFAP2N	100±0	153±45	8±7	40150±18300	22±3
IFAP2	100±0	264±26	100±0	161±42	9.0 ^c
DSI-501	100±0	68±27	95 ^e	156±80	5.4 ^e

^a Inhibition ratio of compounds (1 μM) in the presence of *ent*-kaurene (5 μM)

^b Inhibition ratio of compounds (10 μM) in the presence of *S*-ABA (5 μM)

^c See Ref. 10

^d Not measured

^e See Ref. 11

Figure 1

S-UNI

UFAP1

UFAP2: X = Cl
UFAP2N: X = NO₂

UFAP3

IFSP1

IFSP2

IFSP3 (Abz-F1)

IFAP1

IFAP2

DSI-201

DSI-501

DSI-504

DSI-505

Abz-E1

Figure 2

Figure 3

Figure 4