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# Differences between the structural requirements for ABA 8′-hydroxylase inhibition and for ABA activity

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**Abstract**—A major catabolic enzyme of the plant hormone abscisic acid (ABA) is the cytochrome P450 monooxygenase ABA 8′-hydroxylase. For designing a specific inhibitor of this enzyme, the substrate specificity and inhibition of CYP707A3, an ABA 8′-hydroxylase from *Arabidopsis thaliana*, was investigated using 45 structural analogues of ABA and compared to the structural requirements for ABA activity. Substrate recognition by the enzyme strictly required the 6′-methyl groups (C-8′ and C-9′), which were unnecessary for ABA activity, whereas elimination of the 3-methyl (C-6) and 1′-hydroxyl groups, which significantly affected ABA activity, had little effect on the ability of analogues to competitively inhibit the enzyme. Fluorination at C-8′ and C-9′ resulted in resistance to 8′-hydroxylation and competitive inhibition of the enzyme. In particular, 8′,8′-difluoro-ABA and 9′,9′-difluoro-ABA yielded no enzyme reaction products and strongly inhibited the enzyme ($K_I$ = 0.16 and 0.25 μM, respectively).


## 1. Introduction

The plant hormone (1′*S*)-(+)-abscisic acid (ABA) is an essential mediator of plant responses to environmental stresses such as desiccation and freezing.[1–4] The biosynthesis, transportation, and catabolism of ABA have to be appropriately controlled to produce adaptive responses to changes in temperature and moisture. Catabolism of ABA can be initiated by several reactions: hydroxylation at C-7′, C-8′, or C-9′; conjugation with glucose at C-1 or C-1′; and reduction at C-4′ (Fig. 1).[1–5] The major reactions are C-8′ hydroxylation and C-1 conjugation; these are generally found in all plant species. The other catabolic pathways are minor or specific to certain plants. The C-8′ hydroxylation pathway, which produces dihydrophaseic acid via phaseic acid (PA), is considered to be as characteristic of ABA catabolism as the C-1 conjugation pathway, although which can be a reversible inactivation reaction sequence used 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.[6–9] 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.[10] and Saito et al.[11] These groups independently demonstrated that members of the Arabidopsis cytochrome P450 CYP707A family, *CYP707A1–CYP707A4*, encode ABA 8′-hydroxylases. CYP707A genes were upregulated during drought stress conditions and the mRNA level significantly increased upon rehydration.[10] Mutants of *cyp707a2* exhibited hyperdormancy in seeds and accumulated sixfold greater levels of ABA than wild type.[10] Microsomes from insect cells expressing CYP707A3 exhibited very active 8′-hydroxylation of (+)-ABA ($K_M$ = 1.3 μM and $k_{cat}$ = 15 $min^{-1}$) and solubilized CYP707A3 protein bound (+)-ABA ($K_s$ = 3.5 μM).[11] The reaction product 8′-hydroxy-ABA (8′-HOABA) is so unstable that it spontaneously isomerizes in vitro to a more stable tautomer, PA.[12] The kinetics of isomerization of 8′-HOABA in the CYP707A3 reaction matched those of 8′-HOABA in buffered solutions, indicating that CYP707A3 catalyzed

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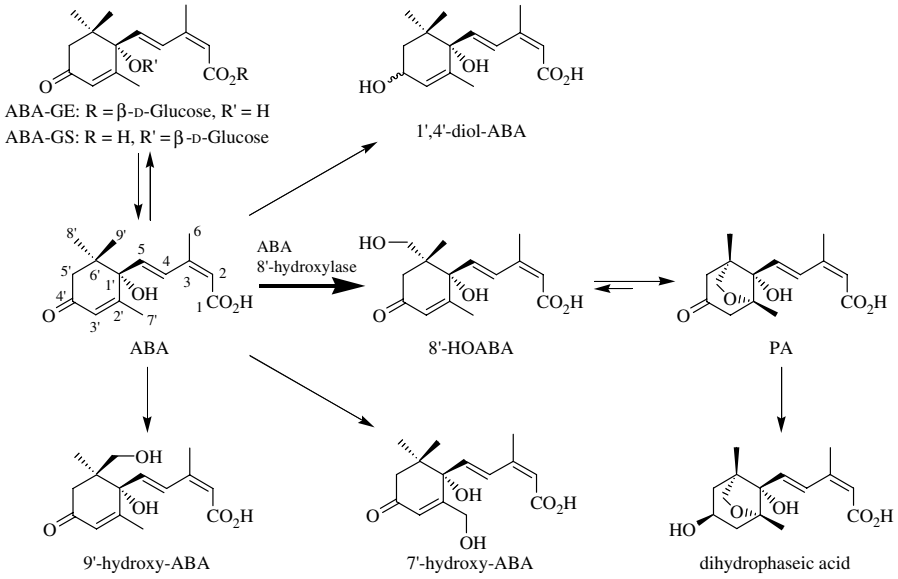


**Figure 1.** Catabolism of ABA in plants. The major catabolic pathway is via 8′-hydroxylation.

8′-hydroxylation of (+)-ABA but not isomerization of 8′-HOABA to PA.[11] These results demonstrate that genes in the CYP707A family play a key role in regulating the ABA level through the 8′-hydroxylation of (+)-ABA.

Specific inhibitors of ABA 8′-hydroxylase are potentially very useful tools for cellular and molecular investigations in the field of plant physiology. Kushiro et al. reported that tetcyclacis inhibited 8′-hydroxylation of ABA by CYP707A1.[10] However, tetcyclacis, which is also an inhibitor of gibberellin biosynthesis,[13] is not a specific inhibitor of ABA 8′-hydroxylase. An ABA analogue designed on the basis of the substrate specificity of the enzyme can be a specific inhibitor of ABA 8′-hydroxylase. 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 ABA 8′-hydroxylase. 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. Cutler et al. demonstrated that structural analogues of ABA, that were modified mainly at C-8′ or C-9′, functioned as suicide or competitive inhibitors of ABA 8′-hydoxylase in the microsomal fraction from suspension-cultured maize cells.[14] These results gave us significant information about the substrate specificity of ABA 8′-hydroxylase, even though the substrate affinity of the maize enzymes is 1/10 that of CYP707A3 and the experiments were limited to the space around C-8′ and C-9′ in ABA. The substrate specificity of the CYP707As has never been investigated in detail, except for the determination that (−)-ABA does not bind to CYP707A3.[11]

In our previous work,[15] we designed and synthesized many structural analogues of ABA, as potent regulators of plant growth, and for use as probes to investigate the molecular mechanism of ABA action and catabolism. These analogues, which have already been good tools for investigating structure–activity relationships, are mainly modified on the cyclohexenone-ring moiety of ABA. Since the CYP707A enzymes oxidize the methyl group on the ring of ABA and should strictly recognize this part of the substrate, the ring-modified analogues should be good probes for investigating the substrate specificity of these enzymes. In fact, a ring-modified analogue with strong ABA activity was inactivated more slowly than ABA in radish seedlings,[16] indicating that it interacts imperfectly with the active site of ABA 8′-hydroxylase. In addition, we have prepared new or known analogues modified on the side-chain of ABA for probing the structural requirements of this part of the molecule for enzyme activity. For this paper, we have tested 45 ABA analogues in CYP707A3 reactions and analyzed their kinetics to provide a detailed substrate specificity and inhibition of this enzyme. We also discuss the differences between the structural requirements for ABA 8′-hydroxylase inhibition and for ABA activity, and exploit these to design a specific inhibitor of ABA 8′-hydroxylase.

## 2. Results

### 2.1. Preparation of ABA analogues

To investigate the substrate specificity of ABA 8′-hydroxylase and differentiate it from the structural requirements for ABA activity, ABA analogues modified at various positions (Fig. 2) were prepared. Most of the ABA analogues had been synthesized previously by us,[17–26] but some known analogues were synthesized for this study using the methods reported by other groups.[27–29] The analogues (±)-7′-nor-ABA and the methyl ester of (±)-6-nor-ABA, which had been previously described, attracted our attention because they have no ABA activity.[30,31] These compounds were potential starting points in the development of a specific

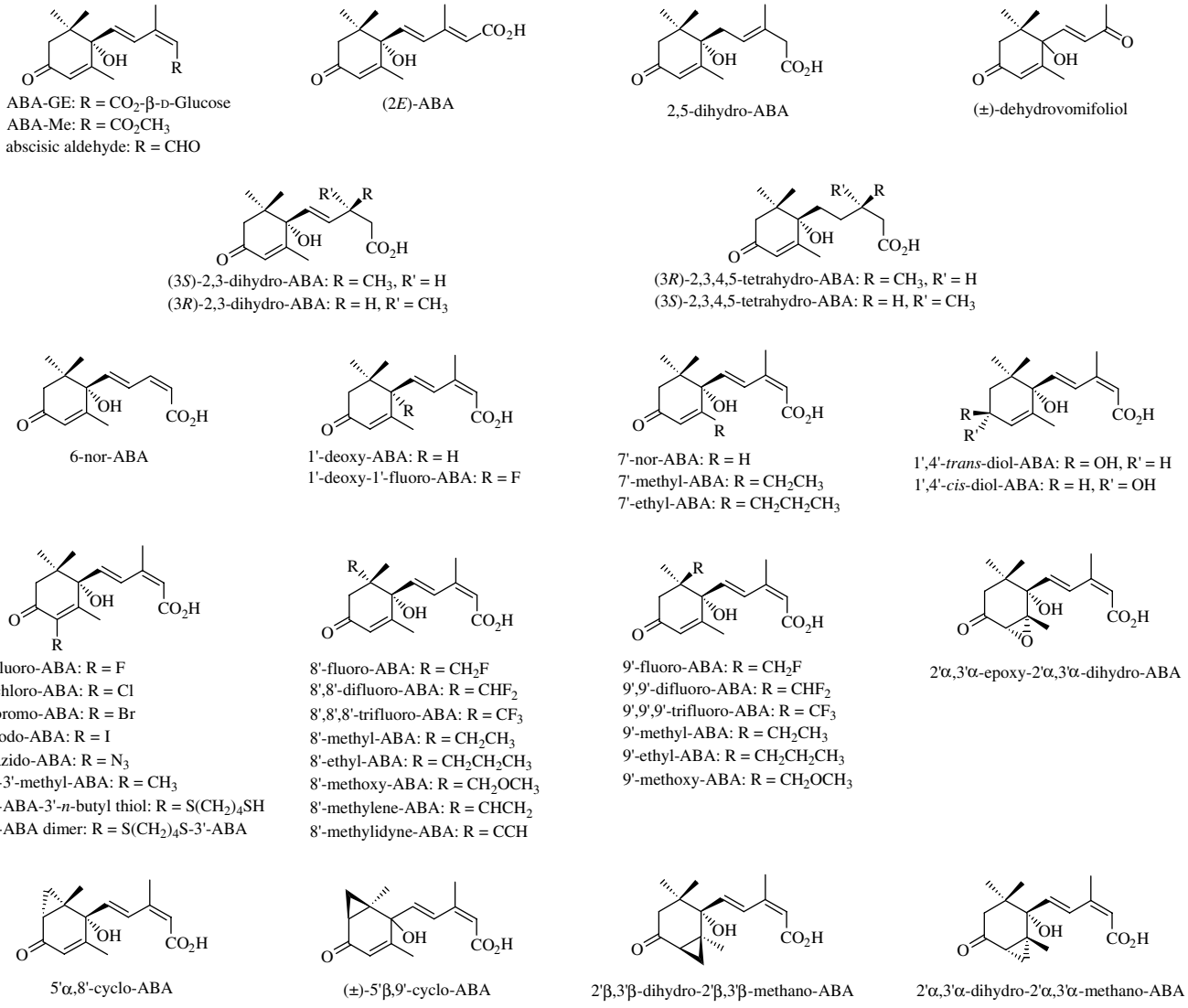


**Figure 2.** Structures of ABA analogues.

inhibitor of ABA 8′-hydroxylase that has no affinity for the ABA receptors. (±)-7′-Nor-ABA was synthesized by the method reported by Nanzyo et al.,[30] whereas (±)-6-nor-ABA was synthesized by a route different from that reported by Yamashita et al.,[31] as shown in Figure 3. Briefly, the monoacetal of 4-oxoisophorone was treated with 2-propynyl *tert*-butyldiphenylsilyl (TBDPS) ether and *n*-butyllithium (*n*-BuLi) to give the ether **1**. De-protection, reduction, and oxidation of **1** afforded the aldehyde **2**. Horner–Emmons reaction of **2** with ethyl di-*o*-tolylphosphonoacetate yielded ethyl 6-norabscisate, followed by basic hydrolysis of the ester to give (±)-6-nor-ABA. (±)-7′-Nor-ABA and (±)-6-nor-ABA were optically resolved by HPLC on a chiral column to obtain the corresponding (+)-isomers with the same exciton chirality as that of (*S*)-(+)-ABA.[32] Since the unnatural type of ABA, (*R*)-(−)-ABA, is not a substrate for ABA 8′-hydroxylase,[11] we generally used optically active analogues with the same exciton chirality as that of the natural type of ABA, (*S*)-(+)-ABA, for the assays using CYP707A3 microsomes (to avoid confusion, we have not shown the sign of chirality for these analogues in this paper). A few ABA analogues that were prepared as the racemate were tested in the enzyme reaction without optical resolution unless they showed interesting properties.

## 2.2. Hydroxylation assay

Initially, all the ABA analogues were incubated with CYP707A3 microsomes and NADPH for 10 min and 1 h before HPLC analysis, to determine which are substrates for ABA 8′-hydroxylase. In the HPLC analysis, we regarded the new peaks, which were absent when NADPH was omitted from the reaction, as the enzyme products. By this definition, analogues that yielded enzyme products were classified as substrates of ABA 8′-hydroxylase, whereas analogues that yielded no enzyme products were classified as nonsubstrates. As shown in Table 1, the best substrates were 1′-deoxy-1′-fluoro-ABA, 1′-deoxy-ABA, 3′-fluoro-ABA, and 6-nor-ABA; these yielded as much enzyme products as ABA. (±)-3′-Methyl-ABA, 7′-nor-ABA, and 9′-fluoro-ABA functioned as enzyme substrates, although more poorly than

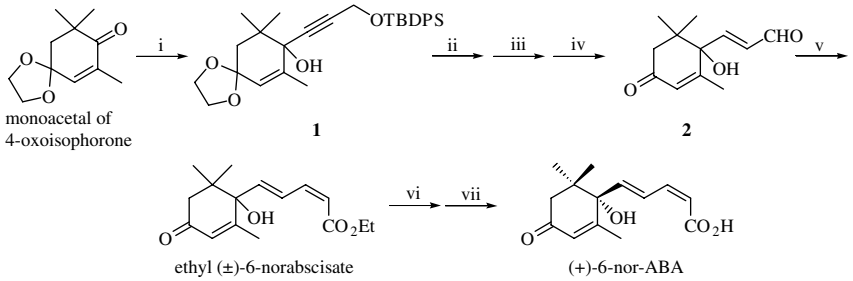


**Figure 3.** Synthesis of (+)-6-nor-ABA. Reagents: (i) 2-propynyl-TBDPS ether, *n*-BuLi; (ii) tetrabutylammonium fluoride; (iii) lithium aluminum hydride, HCl; (iv) pyridinium dichromate, Celite; (v) ethyl di-*o*-tolylphosphonoacetate, NaH; (vi) NaOH; and (vii) optical resolution with Chiralcel OD.

ABA. Other ABA analogues had little or no activity as enzyme substrates, including the C-1 conjugated forms, all of the side-chain modified analogues except for 6-nor-ABA, the 3′-halogenated analogues except for 3′-fluoro-ABA, the alkylated and fluorinated analogues at C-7′, 8′, and 9′ except for 9′-fluoro-ABA, and the cyclopropane analogues.

### 2.3. Inhibition assay

We examined the ability of each ABA analogue (at 50 μM) to inhibit the 8′-hydroxylase reaction for ABA (at 5 μM) (Table 1). As expected, the good substrates for ABA 8′-hydroxylase, including 6-nor-ABA, 1′-deoxy-ABA, 1′-deoxy-1′-fluoro-ABA, and 3′-fluoro-ABA, were effective inhibitors. The other three substrates, (±)-3′-methyl-ABA, 7′-nor-ABA, and 9′-fluoro-ABA, also showed inhibitory activity. In addition, we found inhibitors among the nonsubstrates: all the 3′-modified analogues except for ABA dimer; 7′-methyl- and ethyl-ABA; all the analogues fluorinated or methylated at C-8′ and C-9′; 8′-methylene-ABA; 5′α,8′-cyclo-ABA; 2′α,3′α-dihydro-2′α,3′α-epoxy-ABA; and abscisic aldehyde. The analogues mono- and di-fluorinated at C-8′ and C-9′ exhibited especially strong inhibition (ca. 80%). Analogues that were not inhibitors of ABA 8′-hydroxylase included the C-1 conjugated forms, all the side-chain modified analogues except for 6-nor-ABA, the analogues ethylated and methoxylated at C-8′ and C-9′, all the cyclopropane analogues except for 5′α,8′-cyclo-ABA, and the 4′-reduced analogues. The C-8′ unsaturated analogue, 8′-methylidyne-ABA, was also a noninhibitor. Since Cutler et al. reported that 8′-methylidyne-ABA irreversibly inhibited ABA 8′-hydroxylase from corn cells, we attempted to repeat this result using CYP707A3 microsomes. However, no similar inhibition was observed (data not shown).

### 2.4. Inhibitor kinetics

To gain insight into the mode of inhibition, we examined the kinetics of the strong inhibitors: all the analogues fluorinated at C-8′ and C-9′; the analogues methylated at C-8′ and C-9′; and 6-nor-ABA. All the compounds tested showed competitive inhibition analogous to the representative results shown in Figure 4. The best inhibitors were 6-nor-ABA and 8′,8′-difluoro-ABA ($K_I$ = 0.16 and 0.17 μM, respectively) (Table 2). The 9′-modified analogues were a little less effective than the corresponding 8′-modified analogues.

### 2.5. Bioactivity of 6-nor-ABA

6-Nor-ABA was the best substrate and competitive inhibitor of ABA 8′-hydroxylase. In contrast, the biological activity of 6-nor-ABA was expected to be lower than that of ABA, according to the reported activity of the methyl ester of (±)-6-nor-ABA.[31,33] This characteristic suggested that 6-nor-ABA would be a good starting compound for development of a specific inhibitor of ABA 8′-hydroxylase that has no ABA activity. Therefore, we verified the biological activity of optically active 6-nor-ABA with the same chirality as that of the natural type of ABA. In the rice seedling elongation assay, the $IC_{50}$ value of 6-nor-ABA was 38 μM, whereas that of ABA was 2.3 μM (Table 3). In the lettuce seed germination assay, the $IC_{50}$ value of 6-nor-ABA was 110 μM, whereas that of ABA was 4.4 μM. These results suggest that 6-nor-ABA is a much poorer ligand than ABA for the ABA receptors, in contrast to the situation for ABA 8′-hydroxylase.

## 3. Discussion

### 3.1. The 8′-modified ABA analogues as competitive inhibitors of ABA 8′-hydroxylase

Since ABA 8′-hydroxylase recognizes and inserts an oxygen atom into the 8′-C–H in ABA, modification at C-8′ could interfere with substrate recognition to decrease affinity to the enzyme, or it could inhibit only the catalytic reaction without affecting binding. In fact, our present data show that the steric size of the substituent at C-8′ has a great effect on substrate recognition and the catalytic reaction of ABA 8′-hydroxylase. All the 8′-fluorinated analogues functioned as competitive inhibitors, whereas they yielded little or no enzyme products, meaning that the fluorine atom at C-8′ inhibits not the binding to but rather the catalytic action of ABA 8′-hydroxylase. This effect seems to depend on the physicochemical properties of fluorine: the smallest steric size (next to hydrogen), and the stronger C–F bond energy compared to the C–H bond.[34] The former property of fluorine results in a good affinity to the enzyme, and the latter causes resistance to hydrogen abstraction, a

**Table 1.** Comparison among 45 ABA analogues of biological activity on plants and inhibitory activity for ABA 8′-hydroxylase

| Compounds | Bioactivity[a] | Products[b] | Inhibition[c] (%) |
|---|---|---|---|
| ABA (assay control) | +++ | 100 | |
| (±)-ABA-GE | – | ND[d] | NI[e] |
| ABA-Me | +++ | ND | NI |
| Abscisic aldehyde | ++ | ND | 31 |
| (2*E*)-ABA | – | ND | NI |
| 2,3,4,5-Tetrahydro-3,4-dehydro-ABA | – | ND | NI |
| (3*S*)-2,3-Dihydro-ABA | – | ND | NI |
| (3*R*)-2,3-Dihydro-ABA | – | ND | NI |
| (3*R*)-2,3,4,5-Tetrahydro-ABA | – | ND | NI |
| (3*S*)-2,3,4,5-Tetrahydro-ABA | – | ND | NI |
| (±)-Dehydrovomifoliol | – | ND | NI |
| 6-Nor-ABA | + | 60 | 88 |
| 1′-Deoxy-ABA | ++ | 99 | 73 |
| 1′-Deoxy-1′-fluoro-ABA | ++ | 94 | 100 |
| 1′,4′-*trans*-Diol-ABA | ++ | ND | NI |
| 1′,4′-*cis*-Diol-ABA | ++ | ND | NI |
| 7′-Nor-ABA | – | 15 | 28 |
| 7′-Methyl-ABA | +++ | 15 | 83 |
| 7′-Ethyl-ABA | ++ | ND | 26 |
| 3′-Fluoro-ABA | +++ | 68 | 84 |
| 3′-Chloro-ABA | +++ | 19 | 70 |
| 3′-Bromo-ABA | +++ | 5 | 65 |
| 3′-Iodo-ABA | ++ | ND | 54 |
| 3′-Azido-ABA | +++ | ND | 38 |
| (±)-3′-Methyl-ABA | +++ | 32 | 64 |
| (±)-ABA-3′-thio-*n*-butyl thiol | ++ | 2 | 51 |
| (±)-ABA dimer | – | ND | NI |
| 8′-Fluoro-ABA | +++ | 11 | 83 |
| 8′,8′-Difluoro-ABA | ++++ | ND | 83 |
| 8′,8′,8′-Trifluoro-ABA | +++++ | ND | 38 |
| 8′-Methyl-ABA | +++ | ND | 35 |
| 8′-Ethyl-ABA | ++ | ND | NI |
| 8′-Methoxy-ABA | ++++ | ND | NI |
| 8′-Methylene-ABA | ++++ | 4 | 33 |
| 8′-Methylidyne-ABA | +++++ | ND | NI |
| 9′-Fluoro-ABA | +++ | 33 | 83 |
| 9′,9′-Difluoro-ABA | +++ | 3 | 76 |
| 9′,9′,9′-Trifluoro-ABA | +++ | ND | 55 |
| 9′-Methyl-ABA | +++ | 3 | 26 |
| 9′-Ethyl-ABA | ++ | ND | NI |
| 9′-Methoxy-ABA | +++ | ND | NI |
| 5′α,8′-Cyclo-ABA | +++++ | ND | 28 |
| (±)-5′β,9′-Cyclo-ABA | – | ND | NI |
| 2′α,3′α-Dihydro-2′α,3′α-methano-ABA | – | ND | NI |
| 2′β,3′β-Dihydro-2′β,3′β-methano-ABA | ++ | 3 | NI |
| 2′α,3′α-Dihydro-2′α,3′α-epoxy-ABA | – | 19 | 56 |

[a] The activity in rice seedling elongation assay: +++++, 100×; ++++, 10×; +++, 1×; ++, 1/10; +, 1/100; –, <1/100.[17–30]
[b] The ratio of the enzyme products of analogues to those of ABA when the reaction mixture was incubated for 10 min in the hydroxylation assay.
[c] The ability of ABA analogues (50 μM) to inhibit the 8′-hydroxylase reaction for ABA (5 μM).
[d] Not detected.
[e] No significant inhibition.

step in the P450 hydroxylation reaction.[35] Based on steric size (Fig. 5), we can deduce why 8′,8′,8′-trifluoro-ABA has a larger $K_I$ value than the mono- and di-fluorinated analogues. The mono- and di-fluoromethyl groups will have the tolerable size, which fits into the active site, whereas the trifluoromethyl group will be just a little too large to fit. On the other hand, 8′-fluoro-ABA and 8′,8′-difluoro-ABA retain an 8′-C–H but still resisted the hydroxylation reaction. Considering that the C–H bond energy should be weakened by the geminal fluorine(s),[34] this resistance may indicate that the steric or electronic properties of fluorine itself interfered with the catalytic action.

The analogues alkylated at C-8′ varied in their properties; although neither analogue gave enzyme products, 8′-methyl-ABA functioned as a competitive inhibitor, whereas 8′-ethyl-ABA did not. This difference can be explained by the steric size of the substituent at C-8′. The methyl group at C-8′ may be the maximum tolerable size for fitting into the catalytic site. This interpretation is supported by the fact that 8′-methoxy-ABA, whose

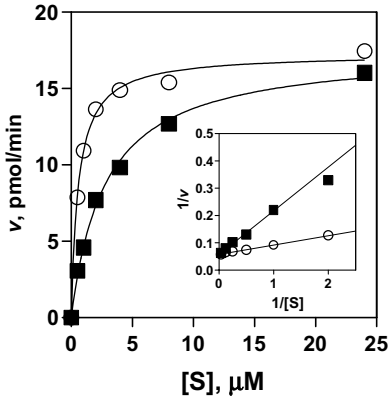


**Figure 4.** Competitive inhibition of ABA 8′-hydroxylase by 8′,8′-difluoro-ABA. Assays contained ABA (○) or ABA and 0.5 μM 8′,8′-difluoro-ABA (■). The inset is a double-reciprocal plot for the same data.

**Table 2.** Inhibition constants for competitive inhibitors of ABA 8′-hydroxylase

| Compounds | $K_I$ (μM) |
|---|---|
| 6-Nor-ABA | 0.16 ± 0.01 |
| 8′,8′-Difluoro-ABA | 0.17 ± 0.03 |
| 9′,9′-Difluoro-ABA | 0.25 ± 0.05 |
| 8′-Fluoro-ABA | 0.27 ± 0.17 |
| 8′,8′,8′-Trifluoro-ABA | 0.71 ± 0.21 |
| 9′-Fluoro-ABA | 0.78 ± 0.27 |
| 8′-Methyl-ABA | 0.94 ± 0.11 |
| 9′,9′,9′-Trifluoro-ABA | 1.06 ± 0.04 |
| 9′-Methyl-ABA | 4.29 ± 0.34 |
| 8′-Methylene-ABA | 5.43 ± 1.45 |

**Table 3.** The $IC_{50}$ values of ABA and 6-nor-ABA in bioassays

| | $IC_{50}$ in assay (μM) | |
|---|---|---|
| | Lettuce seed germination | Rice seedling elongation |
| (+)-ABA | 4.4 | 2.3 |
| (+)-6-Nor-ABA | 110 | 38 |

methoxy group is similar in steric size to the ethyl group (Fig. 5), gave the same results as 8′-ethyl-ABA. The analogues allylated at C-8′ also showed varied properties; 8′-methylene-ABA weakly inhibited ABA 8′-hydroxylation, whereas 8′-methylidyne-ABA had no affinity for the enzyme. The steric size of the methylidyne moiety in the axial direction to the cyclohexenone-ring of the ABA skeleton is similar to that of the ethyl and methoxy groups (Fig. 5), suggesting that the steric constraints in this direction are very strict. On the other hand, the steric size of the methylene moiety differs little from the methyl group. If, for the C-8′ substituent, the steric size is the only factor affecting affinity to the enzyme, 8′-methylene-ABA and 8′-methyl-ABA should show similar competitive inhibition of ABA 8′-hydroxylase. However, the $K_I$ value of 8′-methylene-ABA was 5.43 μM, while that of 8′-methyl-ABA was 0.94 μM. This difference in $K_I$ values suggests another possibility: that 8′-unsaturated groups inhibit binding to the catalytic site of ABA 8′-hydroxylase. Terminal methylene and acetylene can function as suicide inhibitors of P450 enzymes,[36] by reacting with the P450 heme. Therefore, if these moieties have a negative effect on binding to ABA 8′-hydroxylase, it should be from their interaction with the apoprotein rather than the heme.

We synthesized 8′-methylene-ABA and 8′-methylidyne-ABA as suicide inhibitors of ABA 8′-hydroxylase.[21] In fact, Cutler et al. also developed these analogues for the same purpose, and demonstrated that 8′-methylidyne-ABA (referred to as 8′-acetylene-ABA) showed suicide inhibition of the 8′-hydroxylating activity of the microsomal fraction from corn cells.[14] However, our present results show that 8′-methylidyne-ABA did not act as any type of substrate for Arabidopsis CYP707A3 microsomes. We cannot explain this contradiction.

All the 8′-modified ABA analogues tested in this paper have bioactivity equivalent to or stronger than ABA;[17–21] that is, the structural requirements at C-8′ for ABA receptors are much less stringent than those for ABA 8′-hydroxylase. This result was expected, because C-8′ is the site acted on by ABA 8′-hydroxylase but is not necessary to induce ABA responses.[37] Among the 8′-modified analogues, 8′,8′,8′-trifluoro-ABA and 8′-methylidyne-ABA are the most biologically active; this was especially true for inhibition of rice seedling elongation, where these analogues were more active than ABA by a factor of 30–40.[17,21] In previous papers, we interpreted these results not in terms of the affinity of these analogues to the ABA receptor but rather their

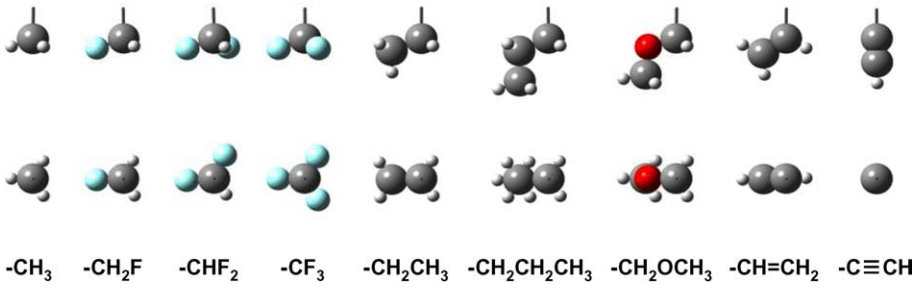


**Figure 5.** Relative steric size of the substituents at C-8′. The top view is from the equatorial direction and the bottom view is from the axial direction. The atoms are illustrated at the equivalent scale of the van der Waals covalent radii. Gray atoms are carbons, white atoms are hydrogen, red atoms are oxygens, and light blue atoms are fluorines.

resistance to 8′-hydroxylation.[17,21] However, the present results show that all the 8′-modified analogues are almost completely resistant to 8′-hydroxylation, suggesting that another factor is involved in determining bioactivity. In future, we may have to take into consideration that the 8′-substituents may make some contribution to binding of ABA and its analogues to the ABA receptor.

### 3.2. The 9′-modified ABA analogues as competitive inhibitors

Contrary to our expectations, none of the 9′-modified ABA analogues tested, except for 9′-fluoro-ABA, were converted to products by ABA 8′-hydroxylase, similar to the 8′-modified analogues. 9′-Ethyl-ABA and 9′-methoxy-ABA are considered to have lost affinity to the enzyme owing to steric bulk at C-9′, as in the case of 8′-ethyl-ABA and 8′-methoxy-ABA. Like the corresponding 8′-substituted ABAs, three 9′-fluorinated ABAs and 9′-methyl-ABA functioned as competitive inhibitors. This result is striking in view of the fact that C-9′ is not a site involved in the hydroxylation reaction of ABA 8′-hydroxylase. We have no clear interpretation for competitive inhibition by the 9′-fluorinated and 9′-methylated analogues, none of which are substrates. One possibility is that the fluorines or methyl moiety at C-9′ interfere indirectly with the catalytic reaction. Recent studies of the catalytic mechanism of bacterial P450cam suggest that the amino acid residues and water molecules in the active site contribute to lowering the free-energy barrier of the substrate hydrogen atom abstraction step.[38] The 9′-substituents may sterically or electronically disrupt the protein environment necessary for the hydroxylation reaction.

The biological activity of all the 9′-modified ABA analogues tested is almost equal to that of ABA. This finding means that for C-9′, as for C-8′, there is a difference between the requirements for affinity to the ABA receptor and the ABA 8′-hydroxylase.

### 3.3. Groups necessary and unnecessary for binding to ABA 8′-hydroxylase

For designing a specific inhibitor, it is important to know whether a functional group is necessary for binding to the enzyme. In general, the oxygen functional groups of ABA are believed to play a significant role in interacting with its target molecules. In the induction of ABA responses, the 1-carboxyl group is an absolutely essential moiety, whereas elimination of the other two oxygen functional groups, the 1′-hydroxyl and 4′-ketone, is not always fatal.[4] Our present results suggest that this is also the case for binding to ABA 8′-hydroxylase. The conjugated analogues, the glucosyl ester of (±)-ABA (ABA-GE) and the methyl ester of ABA (ABA-Me), and (2*E*)-ABA did not bind to the enzyme, suggesting that not only the electrostatic properties but also the directivity of the free carboxylic acid is significant. The 1-carboxyl group must have an electronic and specific interaction with the enzyme protein as a free acid. Shortening or reduction of the side-chain of ABA seriously diminished both ABA activity and affinity to the enzyme. This result also highlights the importance of the physicochemical properties and orientation of the 1-carboxyl group. Abscisic aldehyde, which does not have the carboxyl group, may have weak affinity to the enzyme owing to the 1-carbonyl moiety. The conjugated analogues also have a carbonyl group; however, the steric bulk of these molecules should interfere with specific interaction. The 1′-hydroxyl group, the elimination of which slightly decreases ABA activity, has no involvement in binding to the enzyme. Therefore, we do not have to consider the 1′-hydroxyl in the design of a specific inhibitor. Reduction of the 4′-carbonyl group to the alcohol resulted in loss of affinity to the enzyme. Since this conversion has a large effect on the conformation of the cyclohexenone ring of ABA, the loss of affinity may depend on a steric rather than an electronic effect. This possibility limits our ability to assess the role of the 4′-carbonyl group.

The methyl groups at positions other than C-8′ and C-9′, the 3-methyl (C-6) and 2′-methyl (C-7′) groups, are believed to play a significant role in ABA activity, because 6-nor-ABA and 7′-nor-ABA have little ABA activity. These methyl groups seem somewhat less important for binding to ABA 8′-hydroxylase. Removal or enlargement of the C-7′ group decreased, but did not necessarily abolish, binding to ABA 8′-hydroxylase; the C-6 group was not involved in binding to the enzyme at all.

### 3.4. Steric limitation in the equatorial orientation at C-3′

ABA 8′-hydroxylase was sensitive to modifications at C-3′ in ABA. The yield of reaction products decreased significantly as the steric size of the 3′-substituent ($H < F < CH_3 < Cl < Br < I < N_3$) was increased. The inhibitory activity decreased in a similar manner to product yield, although more gradually. This finding suggests that steric hindrance in the equatorial orientation around C-3′ moves C-8′ away from the appropriate catalytic site by causing binding to the active site in a distorted manner. Thio-*n*-butyl-thiol linker at C-3′ caused unexpectedly strong inhibition (51%) of the enzyme, despite being the largest among the 3′-substituents tested. The thiol group, which is a known inhibitor of P450,[36] may have caused the strong inhibition. The ABA dimer formed using this linker at C-3′ completely lost affinity to the enzyme. These results suggest that modifying C-3′ with a functional group, such as a fluorophore or radioactive moiety, will not produce ABA analogues that can be used for probing the enzyme.

### 3.5. The putative conformation of ABA in the active site

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 C-8′ axial, and with the 1′-OH and C-9′ equatorial[39] (Fig. 6, left). This conformation was reported for the crystal[40] and is the most stable structure in solutions.[41] In a previous paper, we

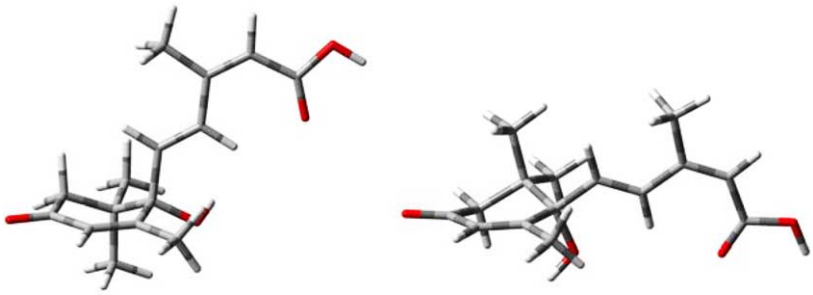

**Figure 6.** Two conformers of ABA. The dominant form is on the left.

estimated a free-energy difference of 1.4 kcal $mol^{-1}$ between this conformation and another energy minimum, a half-chair with the side-chain and C-8′ equatorial and with 1′-OH and C-9′ axial[39] (Fig. 6, right). The free-energy barrier to interconversion between these two conformations was calculated to be 11.2 kcal $mol^{-1}$. This relatively low value means 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. Ideally, the exact binding conformation of the substrate should be determined from the structure of a complex between the substrate and the enzyme. Nevertheless, we should be able to infer a putative binding conformation from the behavior in the reaction of bicyclic analogues with a variety of conformational properties (Table 4). Using this same approach, we inferred that the binding conformation to the ABA receptors is the half-chair with the side-chain and C-8′ axial.[39] All the bicyclic analogues fused with cyclopropane at C2′–C3′ and C5′–C6′ were poor substrates, whereas significant enzyme inhibition was observed only for 5′α,8′-cyclo-ABA. This result suggests that the binding conformation may be the half-chair with the side-chain and C-8′ axial, and that 5′α,8′-cyclo-ABA, which adopts the boat conformation with the side-chain and C-8′ axial, was a poor substrate for ABA 8′-hydroxylase owing to C-8′ incorporation into the cyclopropyl ring.[16] However, 2′α,3′α-dihydro-2′α,3′α-epoxy-ABA, which has a similar conformation to that of 2′α,3′α-dihydro-2′α,3′α-methano-ABA, showed significant enzyme inhibition. An ether oxygen can potentially interact electronically with the iron cation of P450 heme.[42] Therefore, the epoxide in 2′α,3′α-dihydro-2′α,3′α-epoxy-ABA might interact with the heme of ABA 8′-hydroxylase to prevent the hydroxylation of ABA.

## 4. Conclusions

In this paper, we found several differences in the structural requirements for ABA activity and ABA 8′-hydroxylase inhibition (Table 5). The 3-methyl (C-6) and 1′-hydroxyl groups, which are essential for ABA activity, were not necessary for binding to ABA 8′-hydroxylase, whereas the 6′-methyls (C-8′ and C-9′), not necessary for ABA activity, were strictly recognized by the enzyme. These differences are useful for designing specific inhibitors of ABA 8′-hydroxylase.

## 5. Experimental

### 5.1. Nomenclature of chemicals

The ABA analogues used in this paper were named exactly according to IUPAC substitutive and subtractive nomenclature. Therefore, several analogues were given different names from those used in other papers, specifically: 8′-methylidyne-ABA for 8′-acetylene-ABA; 6-nor-ABA for 3-demethyl-ABA; and 7′-nor-ABA for 2′-demethyl-ABA.

### 5.2. Chemicals

(+)-ABA was a gift from Toray Industries Inc., Tokyo, Japan. (±)-ABA-GE was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. PA was

**Table 4.** Ring conformational properties of ABA and bicyclic ABA analogues

| Compounds | Ring | Ratio of the axial/equatorial side-chain[a] |
|---|---|---|
| ABA | Half-chair | 98.7/1.3 (97.8/2.2)[b] |
| 5′α,8′-Cyclo-ABA | Boat | Axial only[c] |
| 5′β,9′-Cyclo-ABA | Boat | Equatorial only[c] |
| 2′α,3′α-Dihydro-2′α,3′α-methano-ABA | Half-chair | 0.1/99.9 |
| 2′β,3′β-Dihydro-2′β,3′β-methano-ABA | Half-chair | 24.8/75.2 |
| 2′α,3′α-Dihydro-2′α,3′α-epoxy-ABA | Boat/half-chair[d] | 3.8/96.2 |

[a] Based on the B3LYP/6-311++G(2df,2p)//B3LYP/6-31G(d) calculations.
[b] Measured by low-temperature NMR.[39,46]
[c] Only one energy-minimum because of the similar type of ring to cyclohexadienone.[39]
[d] Boat with the side-chain axial and half-chair with the side-chain equatorial.

**Table 5.** Differences in structural requirements for competitive inhibition of ABA 8′-hydroxylase and for ABA activity

| C (functional group) | For inhibiting ABA 8′-hydroxylase | For exhibiting ABA activity |
|---|---|---|
| 1 (Carboxylic acid) | Required free acid | Required free acid |
| 2 (Double bond) | *Z*-Configuration | *Z*-Configuration |
| 3 (Methyl: C-6) | Not required | Required |
| 1′ (Hydroxyl) | Not required | Moderately required |
| 2′ (Methyl: C-7′) | Required | Required |
| 3′ (Hydrogen) | Replaceable with halogens | Replaceable with halogens |
| 6′ (Methyl: C-8′ and C-9′) | Sterically strict | Not required |

prepared as reported previously.[43] Syntheses of most of the ABA analogues were reported previously.[17–25] Dehydrovomifoliol was synthesized from the monoacetal of 4-oxoisophorone, which was coupled with the lithium salt of 1-methyl-2-propynyl *tert*-butyldimethylsilyl ether before being reduced and de-protected to obtain vomifoliol as a diastereomeric mixture. Oxidation of vomifoliol with pyridinium dichromate gave dehydrovomifoliol. The spectral data were consistent with those reported.[28] (+)-1′,4′-*cis*- and *trans*-diol-ABA, (±)-ABA-3′-*n*-butyl thiol, and abscisic aldehyde were prepared by the same method as reported.[26,27,29] (±)-7′-Nor-ABA was synthesized by the reported method,[30] and was optically resolved by HPLC: column, Chiralcel OD (250 × 4.6 mm, Dicel); solvent, 15% 2-propanol in hexane containing 0.1% trifluoroacetic acid (TFA); flow rate, 1.0 mL $min^{-1}$; detection, 254 nm. The materials at $t_R$ 9.3 and 15.4 min yielded (−)-7′-nor-ABA (2.0 mg) and (+)-enantiomer (2.0 mg) with an optical purity of 95.6% and 99.8%, respectively. (+)-7′-Nor-ABA: $[\alpha]_D^{27}$ +373 (MeOH; *c* 0.10); CD $\lambda_{ext}$ (MeOH) nm ($\Delta\varepsilon$): 334 (−2.2), 261 (20.8), 221 (−16.6). (−)-7′-Nor-ABA: $[\alpha]_D^{27}$ −373 (MeOH; *c* 0.10); CD $\lambda_{ext}$ (MeOH) nm ($\Delta\varepsilon$): 333 (2.4), 260 (−22.6), 221 (18.2).

### 5.3. Synthesis of (+)-6-nor-ABA

**5.3.1. (±)-3-(1′-Hydroxy-4′,4′-ethylenedioxy-2′,6′,6′-trimethyl-2′-cyclohexen-1′-yl)-2-butynol-TBDPS ether (1).** A 1.6 M hexane solution of *n*-butyllithium (3.2 mL, 5.1 mmol) was added dropwise to a stirred solution of 2-propynyl TBDPS ether (2.3 g, 7.8 mmol) in dry tetrahydrofuran (THF) (25 mL) over 5 min at −78 °C under Ar. After being stirred for 1 h, a solution of the monoacetal of 4-oxoisophorone (0.52 g, 2.7 mmol) in dry THF (10 mL) was added dropwise to the stirred mixture over 5 min. The mixture was stirred for 2.5 h at −78 to −11 °C. $H_2O$ was added to the mixture, which was then extracted with EtOAc (25 mL × 4). The organic layer was washed with $H_2O$, dried over $Na_2SO_4$, and concentrated. The residual oil was purified by column chromatography on silica gel (100 g) with hexane–EtOAc (4:1) to obtain **1** (1.2 g, 92% yield) as a colorless oil. $^1H$ NMR (270 MHz, $CDCl_3$): $\delta$ 1.04 (12H, s, Me-6′ and *tert*-butyl–Si), 1.08 (3H, s, Me-6′), 1.81 (3H, d, $J$ = 1.7 Hz, $H_3$-7′), 1.85 and 1.87 (2H, s, $H_2$-5′), 3.88–3.97 (4H, m, $OCH_2CH_2O$), 4.38 (2H, s, $H_2$-1), 5.33 (1H, s, H-3′), 7.35–7.47 (6H, m, Ph–Si), 7.69–7.72 (4H, m, Ph–Si); HRFABMS (3-nitrobenzyl alcohol): $[M+H]^+$ at *m/z* 491.2604 ($C_{30}H_{39}O_4Si$ requires 491.2618).

**5.3.2. (±)-3-(1′-Hydroxy-2′,6′,6′-trimethyl-4′-oxo-2′-cyclohexen-1′-yl)-propenal (2).** A 1.0 M solution of tetrabutylammonium fluoride in THF (2 mL, 2.0 mmol) was added to a solution of **1** (0.90 g, 1.8 mmol) in dry THF (60 mL) and stirred for 15 min at room temperature. The resulting mixture was quenched with $H_2O$ and extracted with EtOAc (40 mL × 5). The organic layer was washed with brine and $H_2O$, dried over $Na_2SO_4$, and concentrated. Column chromatography on silica gel (30 g) with hexane–EtOAc (2:3) gave the acetal alcohol (0.49 g) as a colorless oil. To a dry THF (50 mL) solution was added the acetal alcohol (0.43 g, 1.7 mmol) and lithium aluminum hydride (0.46 g, 12 mmol) at 0 °C under Ar. The mixture was stirred for 4 h at room temperature. After being cooled and added to $H_2O$, 3 M HCl (25 mL) was added to the mixture and stirred for 30 min at room temperature. The resulting mixture was extracted with EtOAc (25 mL × 9). The organic layer was washed with $H_2O$, dried over $Na_2SO_4$, and concentrated. The residual oil was purified by column chromatography on silica gel (51 g) with hexane–EtOAc (0.20 g) to give the keto alcohol as a colorless oil. Pyridinium dichromate and Celite in dry $CH_2Cl_2$ (1.5 mL) was added to a solution of the keto alcohol (0.18 g, 0.84 mmol) in dry $CH_2Cl_2$ (2 mL), and stirred for 4 h at room temperature. The mixture was filtered with Celite and the filtrate was concentrated. The residual oil was purified by column chromatography on silica gel (2.5 g) with hexane–EtOAc (9:1–3:2) to obtain **2** (0.13 g, 41% yield) as a colorless oil. $^1H$ NMR (270 MHz, $CDCl_3$): $\delta$ 1.06 (3H, s, $H_3$-9′), 1.14 (3H, s, $H_3$-8′), 1.91 (3H, d, $J$ = 1.3 Hz, $H_3$-7′), 2.35 (1H, s, HO-1′), 2.38 (1H, d, $J$ = 16.2 Hz, H-5′), 2.51 (1H, d, $J$ = 16.2 Hz, H-5′), 5.99 (1H, br s, H-3′), 6.50 (1H, dd, $J$ = 15.5 and 7.6 Hz, H-2), 6.89 (1H, d, $J$ = 15.5 Hz, H-3), 9.66 (1H, d, $J$ = 7.6 Hz, H-1); HREIMS: $[M]^+$ at *m/z* 208.1098 ($C_{12}H_{16}O_3$ requires 208.1099).

**5.3.3. (+)-6-Nor-ABA.** Ethyl di-*o*-tolylphosphonoacetate (0.20 g, 0.58 mmol) was added to a solution of NaH (44 mg, 1.1 mmol) in dry THF (1.5 mL) and stirred for 1 h at 0 °C under Ar. The resulting mixture was cooled at −78 °C and a solution of **2** (0.11 g, 0.51 mmol) in dry THF (0.5 mL) was added to the mixture. The mixture was stirred for 2 h at −78 to −15 °C and then poured into saturated $NH_4Cl$ at 0 °C. The resulting mixture was extracted with EtOAc (4 mL × 4). The organic layer was washed with $H_2O$, dried over $Na_2SO_4$, and concentrated. The residual oil was purified by column chromatography on silica gel (3.7 g) with hexane–EtOAc (7:3) and separated into isomers by

crystallization from hexane to give ethyl 6-norabscisate (83 mg) as colorless needles. A solution of 1 M NaOH (1 mL, 1 mmol) was added to a solution of 6-norabscisate (78 mg) in MeOH (2 mL), and the resulting mixture was stirred for 4 h at room temperature in the dark. $H_2O$ (60 mL) was added to the mixture. The solution was extracted with ether and the aqueous layer was extracted with EtOAc (15 mL × 5) after being acidified with 1 M HCl to pH 2. The organic layer was washed with $H_2O$, dried over $Na_2SO_4$, and concentrated. (±)-6-Nor-ABA was crystallized from toluene as colorless needles (60 mg, 50% yield). (±)-6-Nor-ABA. $^1$H NMR (270 MHz, $CD_3OD$): $\delta$ 1.01 (3H, s, $H_3$-9′), 1.05 (3H, s, $H_3$-8′), 1.92 (3H, d, $J$ = 1.3 Hz, $H_3$-7′), 2.19 (1H, d, $J$ = 17.2 Hz, H-5′ *proR*), 2.54 (1H, d, $J$ = 17.2 Hz, H-5′ *proS*), 5.68 (1H, d, $J$ = 11.6 Hz, H-2), 5.90 (1H, q, $J$ = 1.3 Hz, H-3′), 6.25 (1H, d, $J$ = 15.5 Hz, H-5), 6.72 (1H, dd, $J$ = 11.6 and 11.2 Hz, H-3), 7.63 (1H, dd, $J$ = 15.5 and 11.2 Hz, H-4); UV $\lambda_{max}$ (MeOH) nm ($\varepsilon$): 255.5 (22,500); HREIMS: $[M]^+$ at *m*/*z* 250.1208 ($C_{14}H_{18}O_4$ requires 250.1205).

A Chiralcel OD HPLC column (250 × 4.6 mm i.d., Daicel; solvent, 8% 2-propanol in hexane containing 0.1% TFA; flow rate, 1.8 mL min$^{-1}$; detection, 254 nm) was injected with (±)-6-nor-ABA. The materials at $t_R$ 7.3 and 9.3 min were collected to give (+)-6-nor-ABA (2.7 mg) and the (−)-enantiomer (2.6 mg) with an optical purity of 99.9% and 99.3%, respectively. (+)-6-Nor-ABA: $[\alpha]_D^{27}$ +471.2 (MeOH; *c* 0.110); CD $\lambda_{ext}$ (MeOH) nm ($\Delta\varepsilon$): 324.0 (−3.1), 262.0 (42.6), 227.8 (−34.6). (−)-6-Nor-ABA: $[\alpha]_D^{28}$ −479.8 (MeOH; *c* 0.125); CD $\lambda_{ext}$ (MeOH) nm ($\Delta\varepsilon$): 322.6 (3.4), 259.0 (−45.2), 227.4 (37.3).

### 5.4. Hydroxylation assay

The procedure was performed according to the reported method.[11] A reaction mixture (1 mL) containing 50 mM potassium phosphate (pH 7.25), 50 μg mL$^{-1}$ recombinant CYP707A3 microsomes, 50 μM NADPH, and 50 μM ABA analogue was incubated at 30 °C for 10 min or 1 h. Reactions were initiated by the addition of NADPH and stopped by acidification to pH 2 with 50 μL of 1 M HCl. The control experiments were performed in the same manner except for the absence of NADPH. To purify the reaction products, reaction mixtures were loaded onto Oasis HLB cartridges (1 mL, 30 mg; Waters) and washed with 1 mL of 10% MeOH in $H_2O$ containing 1% AcOH. The ABA analogue and metabolites were then eluted with 1 mL of MeOH containing 1% AcOH, and the eluate was concentrated in vacuo. The dried sample was redissolved in 100 μL of MeOH, and 10 μL of the sample was subjected to HPLC. The typical HPLC conditions were: octadecylsilyl (ODS) column, YMC AQ-311 (100 × 6.0 mm i.d.); solvent, 40% MeOH (0–5 min), 40–50% MeOH (linear gradient, 5–10 min), 50% MeOH (10–20 min), and 50–100% MeOH (linear gradient, 20–45 min) in $H_2O$ containing 0.1% AcOH; flow rate, 1.0 mL min$^{-1}$; detection, 254 nm. All the new peaks, which were absent in the control experiments, were regarded as enzyme reaction products; these were roughly quantified relative to the peak area of those produced from ABA under the same conditions.

### 5.5. Inhibition assay

A reaction mixture (1 mL) containing 50 mM potassium phosphate (pH 7.25), 25 μg mL$^{-1}$ recombinant CYP707A3 microsomes, 50 μM ABA analogue, 5 μM (+)-ABA, and 50 μM NADPH was incubated for 10 min at 30 °C. The control experiments were performed simultaneously in the same manner except for the absence of an ABA analogue. Reactions were initiated by the addition of NADPH and stopped with 1 M HCl; the metabolites of ABA were isolated from the resulting mixture using Oasis HLB cartridges. 8′-HOABA and PA were eluted with 1 mL of 80% MeOH (MeOH–water–AcOH = 80:19:0.5), and the eluate was concentrated in vacuo. The dried sample was redissolved in 50 μL of MeOH, and 10 μL of the sample was subjected to HPLC under the following conditions: ODS column, YMC Hydrosphere C18 (250 × 4.6 mm i.d.); solvent, 40% MeOH in $H_2O$ containing 0.1% AcOH; flow rate, 1.0 mL min$^{-1}$; detection, 254 nm. The inhibition ratio was defined as $[(A - B)/A] \times 100$, where $A$ = the peak areas of products when an ABA analogue was absent from the reaction mixture (control), and $B$ = the peak areas of products when an ABA analogue was present. All tests were conducted at least twice. The enzyme activity was confirmed by determining the amounts of 8′-HOABA and PA in the control experiments before each set of measurements. The standard sample of 8′-HOABA was not prepared because 8′-HOABA is spontaneously converted to a tautomeric mixture of 8′-HOABA and PA. The amount of 8′-HOABA was estimated on the basis of a calibration curve for ABA (10, 25, 50, and 100 ng) because the oxygenation of C-8′ gives little effect on the molar absorbance coefficient of ABA.[20] A standard curve for ABA was produced using a solution of 0.01 mg mL$^{-1}$ of standard dissolved in MeOH. The amount of PA was determined on the basis of the relative ratio (1.18) of the molar absorption coefficient for 8′-HOABA/PA.[12] The total amount of 8′-HOABA and PA in the control experiments was estimated as 30.5 ± 4.9 ng, based on 20 sets of experiments; the error range was ±16%.

### 5.6. Inactivation of microsomal ABA 8′-hydroxylase by 8′-methylidyne-ABA

A reaction mixture (1 mL) containing 50 mM potassium phosphate (pH 7.25), 25 μg mL$^{-1}$ recombinant CYP707A3 microsomes, 50 μM NADPH, and 50 μM (+)-8′-methylidyne-ABA was incubated for 10 min at 30 °C. 50 μM (+)-ABA was added to the resulting mixture and then incubated for 10 min. After the reaction was stopped with 1 M HCl (50 μL), the catabolites were extracted and quantified as described above. To investigate whether 8′-methylidyne-ABA was a suicide substrate, CYP707A microsomes were preincubated with (+)-8′-methylidyne-ABA according to the method of Cutler et al.[14] A preincubation mixture (1.55 mL) was made containing 50 mM potassium phosphate (pH 7.25), 50 μg mL$^{-1}$ recombinant CYP707A3

microsomes, 50 μM NADPH, and 50 μM (+)-8′-methylidyne-ABA, and this mixture was maintained at 30 °C. Immediately after mixing (zero time), and subsequently at 10 min intervals, 500 μL of aliquots was removed from the preincubation mixture and diluted into an assay mixture containing a final concentration of 200 μM (+)-ABA, 200 μM NADPH, and 50 mM potassium phosphate (pH 7.25), in a total volume of 1 mL. Assays were incubated for 30 min at 30 °C and then stopped by addition of 50 μL of 1 M HCl. Control preincubations lacked 8′-methylidine-ABA.

### 5.7. Kinetic analysis

Reaction mixtures (1 mL) contained 50 mM potassium phosphate (pH 7.25), 25 μg $mL^{-1}$ recombinant CYP707A3 microsomes, (+)-ABA (0.5–24 μM), and an appropriate concentration of ABA analogue. Reactions were started by the addition of NADPH to a final concentration of 50 μM and were incubated at 30 °C for 10 min. Reactions were stopped by acidification to pH 2 with 50 μL of 1 M HCl and products were extracted as described above. The resulting materials were dissolved in 50 μL of MeOH, and 10 μL of the sample was subjected to HPLC under the same conditions described in '*Hydroxylation assay*'. 8′-HOABA and PA were quantified using the calibration curve for ABA described above. The reaction velocities in the presence and absence of the inhibitor were plotted on a double-reciprocal plot to determine the mode of inhibition. The kinetic constant was calculated from triplicate data sets plotted on a [S]/*v*-[S] plot ([S] is substrate concentration and *v* is initial velocity). For the noninhibited enzymatic reaction, the $K_M$ for (+)-ABA was calculated as 0.74 ± 0.29 μM, based on 10 separate experiments.

### 5.8. Bioassays

**5.8.1. Rice seedling elongation assay.** Seeds of rice (*Oryza sativa* L. cv. Nipponbare) were sterilized with EtOH for 5 min and washed with running tap water. The sterilized seeds were soaked in water to germinate for 3 days at 25 °C. The seeds were then placed in a glass tube (40 mm i.d.) containing 2 mL of a test solution and grown with the tube sealed with a plastic cap under continuous light (6000 lux) at 25 °C. When the seedlings were 7 days old, the length of the second leaf sheath was measured and the inhibition ratio was calculated. The inhibition ratio is defined as $[(A - B)/A] \times 100$, where $A$ = the mean length of the second leaf sheath when water was used, and $B$ = the mean length of the second leaf sheath when a test compound was used. All tests were conducted at least twice.

**5.8.2. Lettuce seed germination assay.** Twenty-five seeds of lettuce (*Lactuca sativa* L. cv. Grand Rapids) were placed on two sheets of Advantec No. 2 filter paper soaked in 2 mL of a test solution in a polystyrene dish (60 mm i.d.) and allowed to germinate under illumination (6000 lux) at 25 °C. After 48 h, the inhibition ratio was defined as $[(A - B)/A] \times 100$, where $A$ = the number of seeds that germinated when water was used and $B$ = the number of seeds that germinated when a test compound was used. All tests were conducted at least twice.

### 5.9. Conformational analysis

All of the minimum-energy conformers of ABA and bicyclic ABA analogues were generated using CAChe 3.11[44] or GaussView with Gaussian 03,[45] and minimized using MM3 or UFF built into the software. The six-membered ring of ABA, 2′α,3′α-dihydro-2′α,3′α-methano-ABA, 2′β,3′β-dihydro-2′β,3′β-methano-ABA, and 2′α,3′α-dihydro-2′α,3′α-epoxy-ABA was minimized from two initial conformers, half-chairs with the side-chain axial and equatorial. The minimum-energy rotamer, which was generated by C1′–C5 and C1′–O1′ bond rotations, was abstracted in each half-chair for further calculations. The MM3-minimized structures were fully optimized with density functional theory (DFT), using the Becke three parameter hybrid functional (B3LYP) method and the 6-31G(d) basis set [B3LYP/6-31G(d)] in Gaussian 03, followed by calculations of the harmonic vibrational frequencies at 298 K at the same level. The energy of optimized structures was calculated with B3LYP/6-311++G(2df,2p).

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