Inhibitors of Abscisic Acid 8′-Hydroxylase†

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ABSTRACT: Structural analogues of the phytohormone (+)-abscisic acid (ABA) have been synthesized and tested as inhibitors of the catabolic enzyme (+)-ABA 8′-hydroxylase. Assays employed microsomes from suspension-cultured corn cells. Four of the analogues [(+)-8′-acetylene-ABA, (+)-9′-propargyl-ABA, (−)-9′-propargyl-ABA, and (+)-9′-allyl-ABA] proved to be suicide substrates of ABA 8′-hydroxylase. For each suicide substrate, inactivation required NADPH, increased with time, and was blocked by addition of the natural substrate, (+)-ABA. The most effective suicide substrate was (+)-9′-propargyl-ABA ($K_I = 0.27$ μM). Several analogues were competitive inhibitors of ABA 8′-hydroxylase, of which the most effective was (+)-8′-propargyl-ABA ($K_i = 1.1$ μM). Enzymes in the microsomal extracts also hydroxylated (−)-ABA at the 7′-position at a low rate. This activity was not inhibited by the suicide substrates, showing that the 7′-hydroxylation of (−)-ABA was catalyzed by a different enzyme from that which catalyzed 8′-hydroxylation of (+)-ABA. Based on the results described, a simple model for the positioning of substrates in the active site of ABA 8′-hydroxylase is proposed. In a representative physiological assay, inhibition of *Arabidopsis thaliana* seed germination, (+)-9′-propargyl-ABA and (+)-8′-acetylene-ABA exhibited substantially stronger hormonal activity than (+)-ABA itself.

(+)-ABA[1] is a key phytohormone that controls or influences numerous aspects of plant growth and development including embryo maturation, seed dormancy, water use, and responses to environmental stress (*1*). The mechanisms by which plant cells perceive and respond to ABA signals are being intensively studied in many laboratories but are not yet well understood (*2*).

The ABA biosynthetic pathway has been established in the past few years (*3, 4*) and key genes have been cloned (*5, 6*). The major catabolic pathway is initiated by hydroxylation of S−(+)-ABA at the 8′-carbon atom (Figure 1) (*1, 4*) mediated by the enzyme (+)-ABA 8′-hydroxylase. The initial product, 8′-hydroxy-ABA, cyclizes to (−)-PA, the product of the enzyme generally obtained on isolation. There are also two minor catabolic pathways (*7, 8*) (Figure 1). ABA and its oxidized products can also be conjugated as esters, particularly glucose esters, and as glycosides (*1, 3*).

The gene encoding ABA 8′-hydroxylase has not been cloned and the enzyme has proved difficult to characterize. The first in vitro assay for ABA 8′-hydroxylase was reported in 1976 (*9*) and little additional information has been reported from subsequent in vitro experiments (*10, 11*). Recently, we developed a sensitive assay for ABA 8′-hydroxylase using microsomal extracts from suspension-cultured corn cells (*8*). This in vitro experimental system enabled us to confirm the suggestion of Gillard and Walton (*9*) that the enzyme is a cytochrome P450 monooxygenase.

In mature seeds, increased catabolism of ABA was associated with release from seed dormancy (*12, 13*), and catabolism in epidermal and mesophyll cells may be important for regulating stomatal aperture (*14, 15*). The importance of ABA catabolism in ABA-regulated processes has been investigated with application of ABA analogues resistant to metabolism at the 8′-carbon atom. Experiments comparing the effects of exogenous ABA and ABA-d_6 (trideuterated at the 8′-carbon atom and therefore more slowly metabolized) on germination of cress seed (*16*) and yellow cedar embryos[2] and studies of oil production in *Brassica napus* embryos comparing the effects of ABA and 8′-methylene-ABA (a slowly metabolized analogue of ABA) (*17*−*19*) confirm that ABA catabolism plays an important role in controlling ABA-mediated effects. However, little is known of how ABA catabolism is regulated (*4*).

The norbornanodiazetine reagent tetcyclasis inhibits ABA 8′-hydroxylase in vitro (*8*) and has been employed to inhibit ABA 8′-hydroxylase in vivo (*20*). However, tetcyclasis is a general inhibitor of cytochrome P450 enzymes and also blocks many other P450-mediated oxidations.

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[1] Abbreviations: ABA, abscisic acid; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography; PA, phaseic acid; DPA, dihydrophaseic acid; EDTA, ethylenediaminetetraacetate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; HREIMS, high-resolution electron ionization mass spectrometry.

[2] Personal communication from Dr. Allison Kermode, Department of Biological Sciences, Simon Fraser University, British Columbia, Canada.

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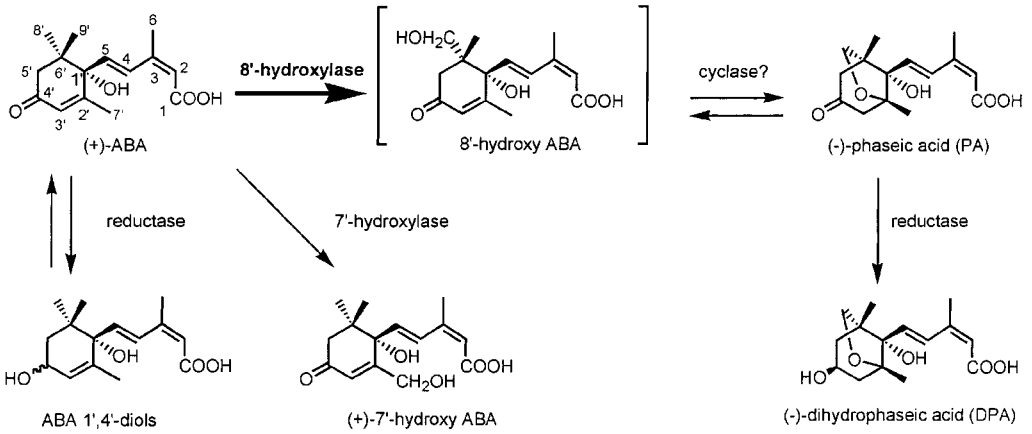

FIGURE 1: Oxidative catabolism of (+)-ABA in plants. This shows the pathways for oxidative catabolism and the reduction to form 1′, 4′ diols. Conjugate formation is not shown. The major catabolic pathway is via 8′-hydroxylation (*4*).

In view of the evident importance of ABA catabolism to plant growth and development, specific inhibitors of ABA 8′-hydroxylase would be exceedingly useful for future research in plant physiology, biochemistry, and genetics. Concerted efforts in enzyme characterization and assay development (*8*), as well as in method development for synthesis of 8′-altered ABA analogues (*17*, *21*), have recently made it possible to design, chemically synthesize, and test potential inhibitors.

A suicide substrate (or mechanism-based enzyme inactivator or inhibitor) is a substrate analogue that is converted by an enzyme, via its normal catalytic cycle, into a reactive species that can bind covalently to the enzyme without prior release from the active site (*22*). Substrate analogues with unsaturated carbon−carbon bonds (e.g., allene, propargyl, and acetylene groups) have proved very effective as suicide substrates for P450 enzymes (*23*), and the mechanism of activation of substrates and of the subsequent inactivation of the enzymes have been studied in depth (*24*, *25*).

Suicide substrates of other cytochrome P450 monooxygenases have been useful in several ways. For example, piperonylic acid is a specific suicide inhibitor of the key plant enzyme cinnamic acid 4-hydroxylase (*26*) and effectively blocks this enzyme in vivo without other effects on phenylpropanoid metabolism. In other studies, suicide substrates have been used to distinguish activities of closely related enzymes (e.g., ref *27*), for active site mapping (e.g., ref *28*), for mechanistic studies (e.g., ref *29*), and in the development of highly specific, nontoxic pharmaceuticals (*23*).

To develop novel ABA 8′-hydroxylase inhibitors, the targets (potential inhibitors) were chosen to resemble the natural substrate and to have unsaturated groups, especially acetylenic ones, located on or near the 8′-carbon atom of ABA. The first such ABA analogue produced was (+)-8′-methylene-ABA, a compound that proved to be a strong ABA agonist (*17*, *19*). The promising results with 8′-methylene-ABA encouraged us to synthesize other optically pure ABA analogues with carbon−carbon unsaturations at the 8′-position (Figure 2).

In this paper we analyze the kinetics of a number of 8′- and 9′-substituted ABA analogues as inhibitors of ABA 8′-hydroxylase. Further, we develop a model to explain the interaction of substrates and inhibitors with the activated oxygen in the active site. We also compare the biological activities of ABA and suicide substrates in a seed germination assay.

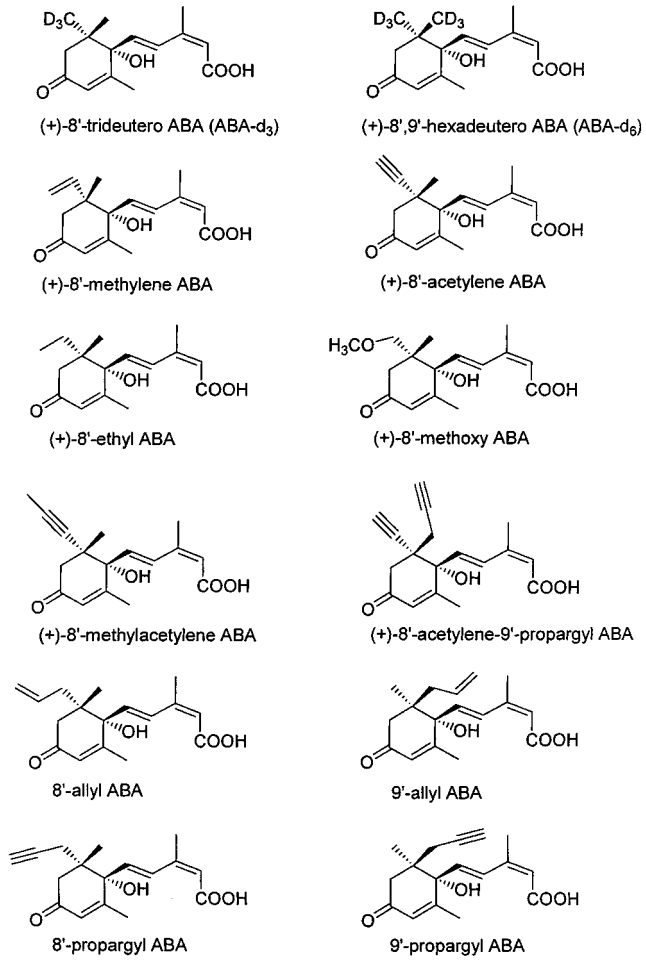

FIGURE 2: Structures of putative inhibitors of ABA 8′-hydroxylase.

EXPERIMENTAL PROCEDURES

Chemicals. Natural *S*-(+)-ABA was a gift from Dr. Yasuo Kamuro (BAL Planning Co., Japan). The chemical structures of the analogues employed in this study are shown in Figure 2. The compounds have the absolute configuration shown. For example, (+)-8′-methylene-ABA is (1′*S*, 6*R*) (*17*) and (+)-ABA is (1′*S*). Known compounds were obtained by methods previously described: ABA-d_6 [(+)- 8′,8′,8′,9′,9′,9′-

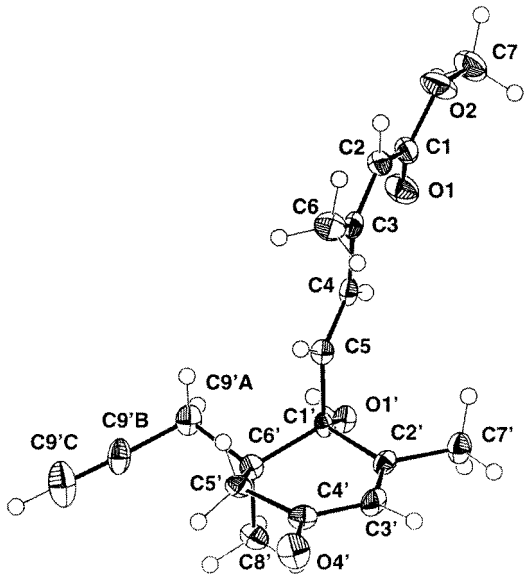

FIGURE 3: Three-dimensional structure of (−)-9′-propargyl-ABA determined by X-ray crystallography. Mirror image [i.e., (+)-isomer] is shown for better comparison with (+)-ABA. The crystal structure has been deposited at the Cambridge Crystallographic Data Centre.

hexadeutero-ABA] (*16*), (−)-ABA (*30*), (−)-PA (*31*), and (+)-7′-hydroxy-ABA (*32*).

The 8′-ethyl-, 8′-methylene-, 8′-methoxy-, 8′-methylacetylene-, and 8′-acetylene-ABA analogues were synthesized from the symmetrical cyclohexadienoate intermediate by reaction with the appropriate Grignard reagent, and the product was resolved as previously described (*17*, *21*).

The 8′- and 9′-allyl and propargyl analogues and the 8′-methylacetylene and 8′-acetylene-9′-propargyl analogues were prepared through a route that has previously been utilized to generate a number of ABA analogues (*16*, *33*). The 8′-acetylene-9′-propargyl analogue was prepared through a Grignard reaction on a substituted cyclohexadienoate. The preparation of these compounds will be reported in full elsewhere. The side chain was attached to the preformed ring bearing the 8′- and 9′-substituents, generating a mixture of diastereomers. The determination of which isomer was 8′- and which was 9′-substituted was established by X-ray crystallography of (−)-9′-allyl-ABA and (−)-9′-propargyl-ABA. Figure 3 shows the mirror image of the X-ray structure of (−)-9′-propargyl-ABA for better comparison with (+)-ABA.

2-*Trans*-8′-acetylene-ABA was prepared by photoisomerization of the corresponding 2-*cis* methyl ester, and the separated isomers were hydrolyzed.

Enzyme Activity and Inhibition. ABA 8′-hydroxylase was induced in suspension cultures of maize (*Zea mays* L. cv Black Mexican Sweet) by (+)-ABA treatment as described previously (*8*). Induced cells were harvested by vacuum infiltration and stored in aliquots at −80 °C until required. Microsomal membranes were prepared by ultracentrifugation as described previously (*8*) in an extraction buffer containing 0.33 M sucrose, 40 mM ascorbic acid, 0.1% BSA, 20 mM EDTA, and 200 mM potassium phosphate buffer, pH 7.6. Microsomal protein was resuspended in 0.1 M Hepes, pH = 7.6, and protein content was measured by the Bio-Rad protein assay (*34*).

For measuring suicide inhibition, a preincubation mixture was made containing 0.5−2 mg of microsomal protein in 0.1 M HEPES buffer, pH = 7.6, an appropriate concentration of a potential suicide substrate, and 3 mM NADPH (added last) in a total volume of 1.55 mL. This mixture was maintained at 30 °C and agitated gently (Thermomixer, Eppendorf). Immediately (zero time) and subsequently at 5 min intervals, 300 μL aliquots were removed from the preincubation mixture and diluted into an assay mixture containing a final concentration of 400 μM (+)-ABA, 3 mM NADPH, and 0.1 M HEPES, pH = 7.6, in a total volume of 400 μL. Assays were incubated at 30 °C for 90 min in a temperature-controlled incubator (Thermomixer, Eppendorf) and then stopped by addition of 80 μL of 1 N HCl. Denatured protein was removed by centrifugation. Control preincubations lacked inhibitor and/or NADPH. Assay controls contained all ingredients but were terminated immediately by addition of HCl.

For measurements of K_M and in competitive inhibition experiments, assays were performed in duplicate in a total volume of 400 μL and contained 200−400 μg of microsomal protein, (+)-ABA (range of concentration 12−400 μM), and (for competitive inhibition experiments only) an appropriate concentration of inhibitor. Assays were started by addition of NADPH to a final concentration of 5 mM and were incubated for 60 min at 30 °C in a temperature-controlled incubator (Thermomixer, Eppendorf). Assays were stopped by addition of 80 μL of 1 N HCl, and precipitated protein was removed by centrifugation. Assay controls lacked NADPH or contained all ingredients but were terminated immediately by addition of HCl.

For measuring 7′-hydroxylation of (−)-ABA, the procedure described above was modified by using increased amounts of protein in each assay (400−800 μg) and extending the assay time to 6 h.

In assays of (+)-ABA 8′-hydroxylase, product accumulation was linear for at least 2 h and directly proportional to protein content over the range employed in these experiments. Product accumulation was not linear over the 6 h incubation times used in assays of (−)-ABA 7′-hydroxylase; therefore, results from these assays were not used for calculation of kinetic parameters.

ABA and products were isolated from the clarified assay mixture by use of Oasis HLB extraction cartridges (Waters) according to the manufacturer's instructions. The methanolic eluates (1 mL) were evaporated to dryness by centrifugation under vacuum in a Vacufuge (Eppendorf) and then redissolved in 500 μL of methanol/water (1:1) for HPLC analysis.

Chromatography and Data Analysis. HPLC analysis was performed on a Hewlett-Packard 1100 instrument. Injected sample size was 20 μL and metabolites were separated on a Supelcosil LC-18 column (length 3.3 cm, internal diameter 4.6 mm, and pore size 3 μm) with an isocratic flow of solvent (flow rate 1.5 mL/ min) containing a 70:30 (v/v) mixture of 0.1% aqueous trifluoroacetic acid and 30% methanol at 45 °C. The eluate was monitored at 262 nm. Concentrations of ABA, ABA catabolites (PA, 8′-hydroxy-ABA, and 7′-hydroxy-ABA), and inhibitors were calculated by comparison with standard curves generated by injecting various known amounts of pure compounds.

Kinetic parameters were calculated by fitting plots of rate (V) vs substrate concentration (S) (competitive inhibition)

or percentage inhibition of initial enzyme activity vs inhibitor concentration (irreversible inhibition) to the equation $y = ax/(b + x)$ with SigmaPlot software (SPSS Science, version 4.0). For suicide inhibition experiments, results are reported from experiments in which the $r^2 > 0.96$ and standard errors of the fitted parameters were <30% for both. For K_M and competitive inhibition experiments, results are reported from experiments with $r^2 > 0.99$ and standard errors <13% of both calculated parameters.

Extraction and Analysis of Metabolic Product of (+)-8′-Acetylene-ABA. Newly subcultured corn cells (approximately 4 g) were transferred into each of 5 × 500 mL flasks and mixed with 100 mL of fresh culture medium supplemented with 100 μM (+)-8′-acetylene-ABA. The flasks were incubated on a rotary shaker at room temperature for 4 days. At the end of the culture period, cells were removed by filtration and the filtrate was frozen at −80 °C until extracted.

The metabolite was purified from the culture medium through ethyl acetate extraction and preparative TLC. The culture medium was filtered through a double layer of Whatman no. 1 filter paper, and the pH was adjusted to 3.0 with 1 N HCl. The media was extracted with ethyl acetate (4 × 500 mL) and the combined organic extracts were washed with brine solution and dried over sodium sulfate. Concentration of the organic phase gave 40 mg of dry residue. To further purify this material, the extract was redissolved in ethyl acetate and partitioned between $NaHCO_3$ solution and ethyl acetate. The neutral compounds were removed with two washes of the bicarbonate layer with ethyl acetate. The aqueous layer was reacidified with 1N HCl and extracted with ethyl acetate (4 × 100 mL). The combined organic extracts were again washed with brine, dried over sodium sulfate, and concentrated to give 5.8 mg of crude acidic components. Preparative TLC (silica gel 60 GF_{254}, 20 cm × 20 cm × 0.25 mm, toluene−ethyl acetate−acetic acid 25:15:2) of the crude acid extract gave mainly recovered (+)-8′-acetylene-ABA; however, a minor component (<0.25 mg) was isolated and identified as 8′-acetic acid lactone on the basis of the following spectral properties:

HREIMS $[M]^+$ at *m/z* 290.1144 ($C_{16}H_{18}O_5$ requires 290.1154); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.72 (d, 1H, J = 16.4 Hz, H-4), 6.13 (s, 1H, −CH), 6.07 (d, 1H, J = 16.6 Hz, H-5), 5.84 (s, 1 H, −CH), 2.68 (d, 1H, J = 17.2 Hz), 2.54 (d, 1H, J = 17.6 Hz), 2.49 (d, 1H, J = 17.4 Hz), 2.33 (d, 1H, J = 17.0 Hz), 2.06 (s, 3H, $-CH_3$), 1.96 (d, 3H, J = 1.1 Hz, $-CCH_3$), 1.15 (s, 3H, H-9′).

Seed Germination. For each replicate assay, 50 seeds of *Arabidopsis thaliana* (cv. Columbia) were placed on two sheets of filter paper (Whatman no. 1, 70 mm) in a Petri plate (100 × 15 mm) wetted with 2 mL of sterile water containing the additives indicated. Plates were sealed with Parafilm and incubated at 24 °C, with a 16 h/8 h light/dark cycle, for the duration of the experiment (7 days). The number of seeds germinated in each plate was recorded twice each day. ABA and ABA analogues were added from ethanolic stock solutions, and control plates contained ethanol at the highest amount in the test plates. The results were quantified by a weighted germination index (*35*).

RESULTS

K_M and V_{max} for ABA and ABA-d_6. As a prerequisite for undertaking kinetic experiments with specific inhibitors of ABA 8′-hydroxylase, the K_M of ABA 8′-hydroxylase for (+)-ABA was calculated as 13.8 ± 1.1 μM, based on 14 separate experiments (two individual determinations are shown as part of data presented later). Use of ABA-d_6 as substrate revealed a kinetic isotope effect of approximately 2 in both the measured V_{max} and K_M values (Table 1).

Table 1: Kinetic Parameters for (+)-ABA and (+)-ABA-d_6 as Substrates for ABA 8′-Hydroxylase[a]

substrate	K_M (μM)	V_{max} [nm h^{-1} (mg of protein)$^{-1}$]
(+)-ABA	16.0	13.7
(+)-ABA-d_6	8.3	6.4

[a] Kinetic parameters for both compounds were measured in the same experiment so that V_{max} values could be directly compared.

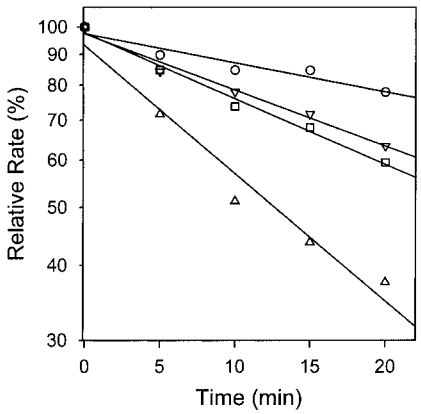

FIGURE 4: Time-dependent irreversible inhibition of ABA 8′-hydroxylase by 8′-acetylene-ABA and protection of the enzyme by its natural substrate (+)-ABA. Preincubations contained 50 μM (+)-8′-acetylene-ABA (○); NADPH (□); 50 μM (+)-8′-acetylene-ABA and NADPH (△); or 50 μM (+)-8′-acetylene-ABA, 50 μM (+)-ABA, and NADPH (▽). Enzyme activities were corrected for the production of PA during the preincubation by enzyme assays stopped with HCl immediately after addition of aliquots of the preincubation mixture.

Analogue Development. Potential inhibitors and substrates of ABA 8′-hydroxylase (Figure 2) were designed to resemble the natural hormone, with alterations made only at the 8′- or 9′-carbon atom. The side chain and cyclohexenone ring were unmodified. The analogues were all prepared in optically active form, as both mirror image forms of ABA can elicit hormonal responses (*36*, *37*). Both mirror image forms of ABA can be hydroxylated at the 8′-carbon atom in corn cell suspension cultures, although the pathway for (−)-ABA is a very minor one (*38*). We originally intended to test only 8′-derivatives because there had been no published evidence for ABA hydroxylation at the 9′-position. However, in preliminary experiments, it quickly became evident that 9′-derivatives were in some cases even more effective as inhibitors than the corresponding 8′-derivatives. Therefore, whenever possible, we tested corresponding pairs of 8′- and 9′-derivatives.

Inhibition Kinetics. Each potential suicide substrate was tested for its ability to cause time-dependent enzyme inactivation as shown for (+)-8′-acetylene-ABA in Figure 4. Microsomes from corn cells were preincubated with NADPH and (+)-8′-acetylene-ABA and aliquots of the preincubation mixture were removed at various time intervals to measure residual ABA 8′-hydroxylase activity. The data in Figure 4 show that both NADPH and (+)-8′-acetylene-

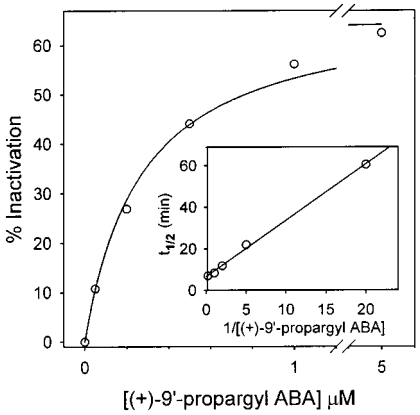

FIGURE 5: Concentration dependence of the inhibition of ABA 8′-hydroxylase by (+)-9′-propargyl-ABA. In the inset, the data are replotted on a Kitz−Wilson plot (*22*) with $t_{1/2}$ as the time required for 50% enzyme inactivation. The percent inactivation at each concentration was converted to a corresponding $t_{1/2}$ value by means of the linear relationship between percent activity remaining and the corresponding incubation time on a log plot such as Figure 4.

ABA were required for substantial inactivation to occur. As is commonly observed with suicide substrates (*22*), the rate of inactivation exhibited pseudo-first-order kinetics with respect to enzyme activity over the time course of these experiments. Deviations from linearity occurred after longer preincubations (generally more than 30 min, data not shown). A low rate of background inactivation was observed in the absence of NADPH and was unaffected by the presence (as shown in Figure 4) or absence of (+)-8′-acetylene-ABA. A somewhat greater rate of inactivation was observed when the enzyme was incubated with NADPH in the absence of the inhibitor. Such an NADPH-dependent background is often observed with monooxygenases in the absence of substrate (e.g., ref *27*).

In addition to (+)-8′-acetylene-ABA, three other compounds caused time-dependent inactivation of ABA 8′-hydroxylase analogous to the results shown in Figure 4. For all four compounds, kinetic parameters were calculated by measuring the dependence of the rate of enzyme inactivation on the concentration of inhibitor (*22*). A representative example of the experiments performed is shown in Figure 5 for (+)-9′-propargyl-ABA. The data in Figure 5 was corrected for inactivation caused by NADPH alone (not shown), as done with analogous experimental systems (e.g., ref *27*). The inset Kitz and Wilson plot verifies that the half-life for inactivation ($t_{1/2}$) is linearly related to the reciprocal of inhibitor concentration, as expected for an irreversible inhibitor (*22*).

Kinetic parameters for the four suicide substrates identified are listed in Table 2. The most effective suicide substrate (highest k_{inact}/K_I) was (+)-9′-propargyl-ABA, and three of the four compounds were substituted at the 9′-position. Both isomers of 9′-propargyl-ABA were inhibitors.

ABA analogues, modified at the 8′- and 9′-positions, that did not cause irreversible inhibition were tested for their ability to act as competitive, reversible inhibitors of ABA 8′-hydroxylase at concentrations ≤ 400 μM. The most effective competitive inhibitor was (+)-8′-propargyl-ABA, and a representative experiment demonstrating its ability to inhibit ABA 8′-hydroxylase is shown in Figure 6. The only (−)-isomer that was an effective competitive inhibitor was (−)-8′-propargyl-ABA.

Table 2: Kinetic Parameters for Suicide Inhibitors of ABA 8′-Hydroxylase[a]

compounds	K_I (μM)	k_{inact} (min^{-1})	k_{inact}/K_I
(+)-9′-propargyl-ABA	0.27	0.113	4.19×10^{-1}
(−)-9′-propargyl-ABA	13.5	0.028	2.07×10^{-3}
(+)-8′-acetylene-ABA	19.0	0.053	2.79×10^{-3}
(+)-9′-allyl-ABA	5.5	0.029	5.27×10^{-3}

[a] K_I is the concentration at which the rate of enzyme inactivation is half of the maximum rate, and k_{inact} is the pseudo-first-order rate constant for enzyme inactivation at saturation.

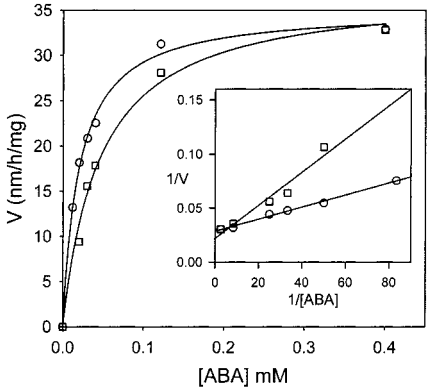

FIGURE 6: Competitive inhibition of ABA 8′-hydroxylase by (+)-8′-propargyl-ABA. Assays contained (+)-ABA (○) or (+)-ABA and 1 μM (+)-8′-propargyl-ABA (□). The inset is a double-reciprocal plot of the same data.

Table 3: Inhibition Constants for Competitive Inhibitors of ABA 8′-Hydroxylase

compounds	K_I (μM)
(+)-8′-propargyl-ABA	1.1
(+)-1′-methoxy-ABA	11.1
(+)-8′-ethyl-ABA	12.9
(−)-8′-propargyl-ABA	56
(+)-8′-methylene-ABA	122
(+)-8′-cyano-ABA	187
(+)-8′-methylacetylene-ABA	284
(+)-ABA methyl ester	NI[a]
(−)-ABA	NI
(+)-8′-methoxy-ABA	NI
(+)-8′-acetylene-9′-allyl-ABA	NI
(−)-8′-acetylene-ABA	NI

[a] No measurable inhibition.

Among the compounds tested as competitive inhibitors was 8′-methylene-ABA, previously shown to be a powerful ABA agonist (*17*). In corn cell cultures, the vinyl group of (+)-8′-methylene-ABA was oxidized to the corresponding epoxide by ABA 8′-hydroxylase (*17*). Enhanced hormonal activity relative to ABA was at least partly due to the fact that (+)-8′-methylene-ABA was a relatively poor substrate for ABA 8′-hydroxylase and was therefore more persistent in plant tissues. At that time, it was not possible to show whether 8′-methylene-ABA was a suicide substrate for ABA 8′-hydroxylase. We found that it was ineffective as a suicide substrate (data not shown) and was only a relatively weak competitive inhibitor (Table 3). This is a manifestation of the fact that 8′-methylene-ABA has a relatively low affinity for ABA 8′-hydroxylase, which also results in the low rate of oxidation of 8′-methylene-ABA by the enzyme (*17*, *19*).

Metabolism of 8′-Acetylene-ABA. Suicide inhibitors can either bind to the enzyme, causing inhibition, or react with

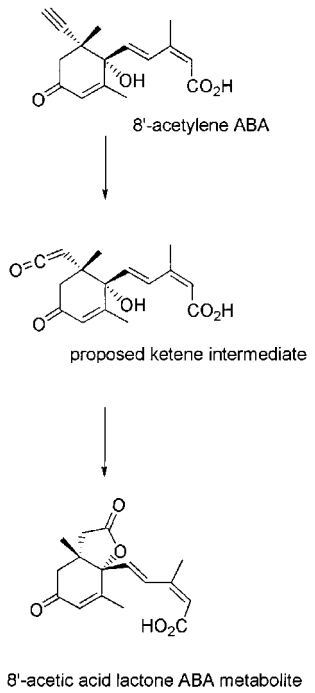

FIGURE 7: Product formed by enzymatic oxidation of (+)-8′-acetylene-ABA.

water, releasing a product and leaving the enzyme unaffected. Indeed, only a small proportion of catalytic turnovers result in enzyme inhibition (*22*). We examined one of the suicide substrates, (+)-8′-acetylene-ABA, in detail to verify that it was converted to an oxidized product consistent with the mode of action of the enzyme and the reactivity of the acetylene group (Figure 7). The identity of this product was established following an in vivo incubation, since insufficient quantities of product were produced by microsomal in vitro assays. Suspension-cultured corn cells were incubated with (+)-8′-acetylene-ABA for 4 days and one metabolite was isolated from the medium in very small quantity. HPLC analysis of the crude acid extract showed there was an approximate 20:1 ratio of starting 8′-acetylene-ABA to metabolite. High-resolution mass spectroscopy showed that the metabolite contained one oxygen atom more than the starting compound. Proton NMR of the isolated metabolite clearly showed that the side chain and enone moiety in the ring had remained intact. However, two new protons were present, possessing shifts and coupling constants consistent with a methylene adjacent to a carbonyl. On the basis of the known mode of oxidation of acetylenic substrates by cytochrome P450 enzymes (*24*, *25*) and the above spectral data, the metabolite was assigned the structure of 8′-acetic acid lactone (Figure 7).

7′- versus 8′-Hydroxylation. Both enantiomers of ABA are enzymatically oxidized in vivo and the predominant

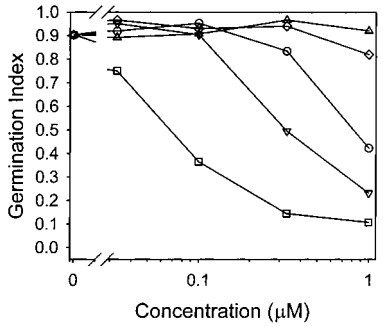

FIGURE 8: Inhibition of *Arabidopsis thaliana* var. Columbia seed germination by inhibitors of ABA 8′-hydroxylase: (+)-ABA (○); (+)-9′-propargyl-ABA (□); (+)-8′-acetylene-ABA (▽); (+)-9′-allyl-ABA (◇); (+)-8′-propargyl-ABA (△).

products are (−)-PA from (+)-ABA and (−)-7′-hydroxy-ABA from (−)-ABA (*39*). It is unclear whether both these oxidations are carried out by ABA 8′-hydroxylase or another enzyme(s) is involved. Therefore, we performed an experiment to determine whether two representative suicide substrates of ABA 8′-hydroxylase also inhibited 7′-hydroxylation. The results in Table 4 show that neither (+)-8′-acetylene-ABA nor (+)-9′-propargyl-ABA increased the amount of 7′-hydroxylase inactivation above that seen with NADPH alone. The failure of these compounds to inhibit 7′-hydroxylation of (−)-ABA showed that this oxidation is carried out by a separate enzyme from that responsible for 8′-hydroxylation.

Germination Assays of Suicide Substrates. As a demonstration of the potential of ABA 8′-hydroxylase inhibitors to act as powerful ABA agonists, three of the four suicide substrates (Table 2) and (+)-8′-propargyl-ABA (the most effective competitive inhibitor of ABA 8′-hydroxylase, Table 3) were compared with ABA as germination inhibitors (Figure 8). Both (+)-9′-propargyl-ABA and (+)-8′-acetylene-ABA are stronger inhibitors than (+)-ABA, showing that at least some ABA 8′-hydroxylase inhibitors are very powerful hormone agonists. For example, approximately 0.07 μM (+)-9′-propargyl-ABA reduces the germination index to 0.5, whereas about 0.9 μM (+)-ABA is required to produce the same effect. However, (+)-9′-allyl-ABA and (+)-8′-propargyl-ABA are relatively weak germination inhibitors at the concentrations tested.

DISCUSSION

The objective of this study was to develop novel substrates and inhibitors of ABA 8′-hydroxylase, the key enzyme of ABA catabolism in plants. The strategy was to develop specific, irreversible inhibitors based on precedents in which suicide substrates had been successfully developed for other eucaryotic cytochrome P450 enzymes (*24*, *40*). As a result, we produced an array of novel ABA derivatives with a range

Table 4: Effect on 7′-Hydroxylation of (−)-ABA of Addition of Suicide Inhibitors of 8′-Hydroxylation of (+)-ABA

additions	initial activity[a] [nm h^{-1} (mg of protein)$^{-1}$]	final activity[b] [nm h^{-1} (mg of protein)$^{-1}$]	loss of activity over 30 min (%)
NADPH	1.02	0.78	23.5
(+)-8′-acetylene-ABA, NADPH	1.06	0.88	17.0
(+)-9′-propargyl-ABA, NADPH	0.95	0.75	21.1

[a] Initial rate of formation of (−)-7′-hydroxy-ABA from (−)-ABA. [b] Rate of formation of (−)-7′-hydroxy-ABA from (−)-ABA after 30 min preincubation in a mixture containing 3 mM NADPH and either no inhibitor, 50 μM (+)-8′-acetylene-ABA, or 5 μM (+)-9′-propargyl-ABA.

of inhibitory properties, of which four are shown to be suicide substrates. We used the inhibitors to investigate the binding specificity of the enzyme and develop a model for the interaction of substrate with the activated heme-bound oxygen (or iron-oxo group) in the active site. Our approach to investigating ABA hydroxylation is necessarily empirical, as a crystal structure for only one eucaryotic membrane-bound cytochrome P450 is available (*41*), and a gene encoding ABA 8′-hydroxylase has not yet been reported. However, current evidence suggests that "..the active sites and catalytic mechanisms of P450 enzymes are remarkably similar" (*42*).

Inhibition Kinetics. Silverman (*22*) suggested experimental criteria for establishing whether an enzyme inhibitor acts as a suicide substrate. For the compounds listed in Table 2, we showed that inhibition of ABA 8′-hydroxylase is time-dependent (Figure 4) and saturable (Figure 5). In addition, in each case the enzyme is protected from inactivation by the presence of the natural substrate, (+)-ABA (Figure 4), and the requirement for NADPH (Figure 4) shows that enzyme catalysis is required.

Surprisingly, of the four synthesized compounds that that we identified as suicide substrates, three were 9′-derivatives. We have not yet developed a synthesis for 9′-acetylene-ABA, the remaining compound in the set of 8′- and 9′-acetylenic ABA analogues, to ascertain whether it is also a suicide inhibitor. Of the (−)-isomers, only (−)-9′-propargyl-ABA produced measurable irreversible inhibition. Inhibitors were compared by calculating the ratio k_{inact}/K_I (*23*), which is a measure of the effectiveness of each compound as an enzyme inhibitor. By this criterion, (+)-9′-propargyl-ABA was about 100−200-fold more effective than any of the other inhibitors (Table 2).

The calculated k_{inact} values are comparable to those previously reported for suicide substrates, such as 0.064 min^{-1} calculated for inhibition of cinnamic acid hydroxylase by piperonylic acid (*26*), 0.0432 min^{-1} for the inactivation of plant oleic acid ω-hydroxylase by (*Z*)-9-octadecen-17-ynoic acid, and 0.0360 min^{-1} for the inactivation of the same enzyme by (*Z*)-9,10-epoxyoctadecan-17-ynoic acid (*27*). Generally k_{inact} values fall in the range 0.01−1 min^{-1} (*22*). These rates are typically low relative to rates of catalysis (k_{cat}; the catalytic constant or turnover number) for normal substrates. For example, the cytochrome P450 enzymes cinnamic acid hydroxylase and berbamunine synthase (the former heterologously expressed in yeast and the latter purified from *Berberis stolonifera*) have k_{cat} values of 100−400 min^{-1} (*26*) and 50 min^{-1} (*43*), respectively. Enzymes generally have k_{cat} values in the range 100−10,000 min^{-1} (*22*).

The K_M of ABA 8′-hydroxylase for (+)-ABA is comparable with those determined for other plant cytochrome P450 enzymes. For example, cinnamic acid hydroxylase has a K_M of 4 μM for cinnamic acid (*43*; p321) and lauric acid ω-hydroxylase has a K_M of 55 μM for lauric acid (*29*). We observed a kinetic isotope effect for both V_{max} and K_M of approximately 2 (Table 1), a value similar to that seen in vivo (*16*). With the standard assumption that the enzyme (with saturating NADPH) acts in accordance with the Briggs−Haldane (steady-state) mechanism, the isotope effect on K_M indicates that enzyme-bound ABA is more likely to be oxidized than released unchanged[3] (*44*).

Inhibition Mechanism and Metabolic Products of 8′-Acetylene-ABA. We had previously utilized an in vivo protocol with intact cultured corn cells to establish qualitatively that (+)-8′-acetylene-ABA caused irreversible inhibition (*21*). The availability of an in vitro assay for ABA 8′-hydroxylase enabled us to repeat the experiment quantitatively and to prove that (+)-8′-acetylene-ABA is a suicide substrate (Table 1, Figure 4). Since (+)-8′-acetylene-ABA can be synthesized efficiently and in quantity, it is likely to be the most widely used inhibitor in future studies. Therefore, we undertook a more detailed analysis of enzyme inhibition and metabolism of this compound.

In the case of suicide substrates in which terminal acetylenes are oxidized, the mechanism is thought to be initiated by the attack of activated oxygen (attached to the iron of the active-site heme) on the triple bond. Inactivation of the enzyme can occur by either of two ways: either the oxygen atom attaches to the internal carbon of the acetylene while the terminal carbon binds covalently to the heme or the oxygen binds to the terminal carbon atom with the acetylenic hydrogen migrating to the internal carbon, leading to the formation of a ketene. The highly reactive ketene can bind covalently to the protein or heme (*24*, *25*). Either event leads to inactivation of the enzyme. Alkylated heme compounds have been identified as products of P450 enzymatic inactivations. Alkylated proteins have been observed, and active sites have been labeled in the case of arylacetylene suicide substrates (*45*). In the case of ketene formation, reaction with water rather than the enzyme results in formation of the acetic acid derivative of the substrate.

The 8′-acetic acid lactone was isolated after (+)-8′-acetylene-ABA was supplied to cells. Its formation was likely mediated by the ABA 8′-hydroxylase. The lactone was probably formed by initial oxidation of the acetylene to the oxirene, followed by rearrangement of the highly unstable oxirene to the ketene. Subsequent intramolecular attack of the ketene by the C1′-hydroxyl group or water, followed by lactone formation, affords the putative metabolite (Figure 7). The oxidation of 8′-acetylene-ABA indicates that the active site of the enzyme can accommodate the extra bulk of the additional carbon atom of the acetylene group.

We performed an additional experiment with (+)-8′-acetylene-ABA to clarify its rate of reaction in the enzyme active site. Inactivation by suicide substrates should be mediated through a reactive species that does not leave the active site (otherwise leading to nonspecific inactivation; *22*). Consistent with this, there was no evidence for delayed inactivation or for an increase of the rate of inactivation with time (Figure 4) as would be expected if the concentration of a reactive species built up in solution. In addition, adding the reducing agents dithiothreitol or glutathione failed to quench inactivation (data not shown), consistent with the reactive species not leaving the active site (these compounds

[3] The Briggs−Haldane (steady-state) assumption gives $K_M = (k_2 + k_{-1})/k_1$, where k_{-1} and k_1 are the rate constants for the dissociation and association of free enzyme and substrate to form the enzyme−substrate complex, respectively, and k_2 is the rate constant for breakdown of the complex to yield product and free enzyme. Since only k_2 (and hence V_{max}) is altered by the slower rate of C−D versus C−H bond breakage, the fact that a measurable isotope effect is observed on K_M implies that $k_2 > k_{-1}$. The magnitude of the isotope effect is discussed in ref *16*.

do not usually enter the active site and can therefore only quench reactive electrophiles that leave the active site).

Mechanism of Inhibition by 9′-Propargyl Derivatives of ABA. The crystal structure of (−)-9′-propargyl-ABA is shown in Figure 3. Both ABA and the 9′-propargyl analogue have similar conformations (*46*). The acetylene group is 2.6 Å longer than the C−H of the 9-methyl group, which the active site must be able to accommodate. This compound [and also (+)-9′-propargyl-ABA] must adopt a conformation in the active site such that oxidation by the iron-oxo group occurs, creating a radical species that itself, or on rearrangement, inactivates the enzyme. At this stage we can only speculate on possible mechanisms of inactivation. These include either abstraction of a hydrogen from the 8′-methyl group, with a hydrogen shift from the 9′-carbon affording the enzyme inactivator, or abstraction of a hydrogen directly from the 9′-propargyl system, or attack of the reactive oxygen on the triple bond directly. In future studies, identification of the products of metabolism of propargyl derivatives of ABA could clarify whether primary attack is on the 8′- or 9′-methyl group.

Active Site of ABA 8′-Hydroxylase. For cytochrome P450 monooxygenases, hydrophobicity and steric interactions determine binding and orientation of substrates. The positioning of the substrate with respect to the reactive iron-oxo species of the heme group active site is the critical factor determining the site of oxidation (*42*). For the natural substrate (+)-ABA, hydroxylation is only observed at the 8′-methyl group. The ABA molecule must be oriented so that only the 8′-methyl group is close to the reactive iron-bound oxygen, particularly as primary C−H bonds of the methyl group are the least reactive type of C−H bond in such enzymatic processes (*47*). There is no published evidence for, nor have we ever observed, 9′-hydroxylation of (+)-ABA, even as a minor reaction. In solution the ABA molecule is highly flexible, and extreme conformations with the side chain axial and equatorial are readily interconverted (*48*). In the active site of the enzyme, the mobility of the ABA molecule must be restricted. In a previous structure/activity study on the conformational requirements of carrier-mediated uptake of ABA, we proposed that the conformation of ABA in the carrier binding site was that with the side chain equatorial (*48*), based upon activity of compounds with side chains restricted to an equatorial-like conformation. In this study, we have no evidence for ABA adopting either extreme or some intermediate conformation. Whatever the conformation of ABA in the active site, the critical feature is that only the 8′-methyl group is oxidized.

The ability of ABA analogues to act as substrates and inhibitors of ABA 8′-hydroxylase provides some clues about steric and electronic requirements for ligand binding in the active site, including geometrical constraints as well as activation of the ferryl system. Substitution of the 8′- and 9′-carbon atoms by acetylene, propargyl, or allyl groups afforded compounds with the greatest biological activity either as suicide substrates or competitive inhibitors. Overall, the results summarized in Tables 2 and 3 showed that inhibitors contain hydrophobic substituents, with not more than two additional atoms, at either the 8′- or 9′-positions of ABA. The ABA analogue 8′-acetylene-9′-allyl-ABA was made with the intention of producing a more potent inhibitor by combining the functional groups of two known suicide substrates. However, this compound exhibited no irreversible or reversible inhibition of ABA 8′-hydroxylase (Table 3), suggesting that the additional substituent limited effective entry into the active site.

Interaction between the apoprotein and the carboxyl group is likely important for ligand binding, since (unlike the corresponding *cis*-isomer) (+)-2-*trans*-8′-acetylene-ABA is not a suicide substrate for the enzyme (data not shown) and ABA methyl ester does not act as a competitive inhibitor (Table 3). On the other hand, 1′-methoxy-ABA is an effective competitive inhibitor (Table 3) and also a substrate for ABA 8′-hydroxylase, being oxidized at the 8′-methyl group by corn cells in vivo (*49*). This implies that the 1′-hydroxyl group is not essential for effective binding of ABA in the active site.

The ABA analogue (+)-8′-methoxy-ABA failed to inhibit ABA 8′-hydroxylase (Table 3), although the 8′-methoxy substituent is of similar size to allyl and propargyl substituents and is more flexible. Also, this compound is an analogue of 8′-hydroxy-ABA, the normal enzyme product. Since steric bulk is not limiting, the result implies that only hydrophobic groups around the site of oxidation are compatible with tight inhibitor binding. Interestingly, 8′-hydroxy-ABA and both 8′- and 9′-methoxy-ABA retain high hormonal activity (*18*, *50*).

Some compounds were weak or ineffective inhibitors of ABA oxidation at concentrations <400 μM even though they are structurally similar to effective suicide or competitive inhibitors. Notable examples include 8′-cyano-ABA and 8′-methylacetylene-ABA (Table 3). Foroozesh et al. (*51*) found that methylacetylenes were suicide substrates for one class of P450 enzymes and were more active than the corresponding acetylene. In the present case the 8′-methylacetylene was not a suicide substrate and was only a weak competitive inhibitor. It is difficult to make specific deductions from these negative results except that they are consistent with spatial constraints in the active site.

These results taken together allow us to propose a simple model for oxidation of ABA by ABA 8′-hydroxylase (Figure 9). The ABA molecule must be constrained by steric factors in a specific conformation. Here we arbitrarily show ABA with the 8′-carbon equatorial, as proposed for protein-mediated uptake (*48*). Hydrogen bonding or other electronic interactions between the apoprotein and the carboxyl group is probably important, as discussed above. Since neither (+)-2-*trans*-8′-acetylene-ABA or 2-*trans*-ABA is an enzyme substrate (data not shown), we propose that there is a region of steric hindrance that prevents docking of trans isomers. There must be flexibility within the active site so as to accommodate the steric bulk of the alkyl substituents of the active analogues. More detailed understanding of the active site and the factors that control oxidation will be forthcoming when purified, recombinant enzyme is available.

Enantiomeric Selectivity and Possible 8′-Hydroxylation of (−)-ABA. The finding that (−)-ABA did not competitively inhibit (+)-ABA 8′-hydroxylase (Table 3) and that (−)-isomers of ABA analogues were generally inactive relative to their (+)-enantiomers suggested that the enzyme active site is selective for (+)-isomers. However, results obtained with propargyl derivatives indicates that enantiomeric selectivity is not absolute. (+)-ABA compounds substituted with propargyl groups at either the 8′- or 9′-positions were strong inhibitors with high affinities for the ABA 8′-

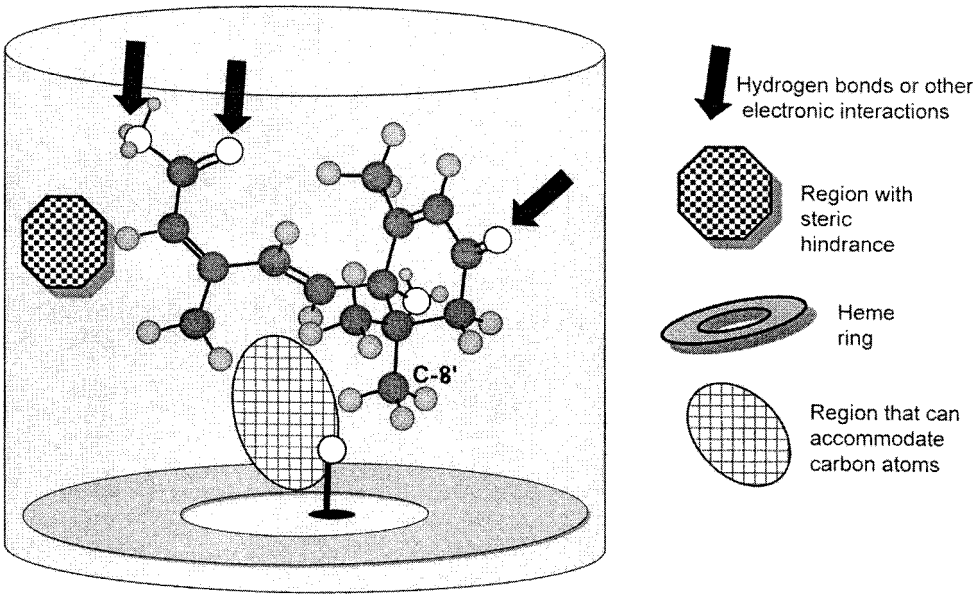

FIGURE 9: Model of (+)-ABA in the active site of ABA 8′-hydroxylase. The tubular shape represents the open access channel by which the substrate reaches the heme-containing active site in the interior of the protein. Orientation of the substrate is stabilized by steric and electronic interactions involving the carboxyl group. Hydrogen bonding with the 4′-carbonyl group may also be significant. The volume around the 8′-methyl group is sufficient to accommodate substituents with an additional two carbon atoms at either the 8′- or 9′-methyl group but not both. In the ABA ball-and-stick structure, dark gray atoms are carbons, light gray are hydrogens, and white atoms are oxygens. Small light gray spheres on hydroxyl oxygens are lone pairs of electrons.

hydroxylase active site, significantly higher than for the corresponding isomers substituted with acetylene or allyl groups. The enhanced affinity was sufficient to reveal that the enzyme is not entirely specific for (+)-isomers since significant inhibition of ABA 8′-hydroxylase by (−)-isomers of 8′- and 9′-propargyl-ABA was detected (Table 2 and 3). Furthermore, as noted above, the fact that (−)-9′-propargyl-ABA was a suicide substrate also revealed that its conformation in the active site is such that the 8′/9′ side of the cyclohexenone ring is presented to the iron-oxo group.

The conversion of (−)-ABA to (+)-PA has been observed after prolonged in vivo incubations in corn cells (*38*) and other systems (*52*). Because ABA 8′-hydroxylase is not absolutely specific for (+)-enantiomers, it is at least possible that 8′-hydroxylation of (−)-ABA is also carried out by this enzyme. However, the failure of (−)-ABA to act as a competitive inhibitor (Table 3) shows that the affinity of the enzyme for the potential substrate, (−)-ABA, is undetectable in the in vitro assay. Nevertheless, as discussed above, the observation that (−)-9′-propargyl-ABA is a suicide substrate (Table 2) reveals that (−)-isomers may enter the active site and are oriented so that 8′-oxidation can occur. Therefore, 8′-hydroxylation of (−)-ABA may be a minor activity of ABA 8′-hydroxylase, although further experiments will be required to establish whether this occurs or not.

7′-Hydroxylation of (−)-ABA Is Not Catalyzed by ABA 8′-Hydroxylase. The unnatural enantiomer (−)-ABA is oxidized at the 7′-position in corn cells and other plants. It has not been clear whether ABA 8′-hydroxylase is also responsible for this conversion. We previously observed that in corn cells in liquid culture (in vivo experiments) (−)-ABA was predominantly converted to (−)-7′-hydroxy-ABA (*31*, *53*). In corn microsomes (−)-ABA was metabolized to (−)-7′-hydroxy-ABA at approximately 5% of the rate with which (+)-ABA was converted to (−)-PA (Tables 1 and 4).

The ABA molecule is pseudosymmetrical about the cyclohexenone ring with the either the 8′- or 9′-methyl groups almost equivalent to the 7′-methyl group, depending on the conformation of the ABA molecule. In the active site, (−)-ABA might assume a conformation in which the carboxyl, 4′-ketone, and 1′-hydroxyl groups occupy the same relative positions as (+)-ABA. In this conformation the 7′-methyl group of (−)-ABA could be presented to the iron-oxo group for oxidation instead of the 8′-methyl group of (+)-ABA. Therefore, both 8′- and 7′-hydroxylations might be catalyzed by a single enzyme as suggested by Boyer and Zeevaart (*39*). However, results from in vivo labeling experiments were difficult to reconcile with a single hydroxylase enzyme (*31*). The results in Table 4 confirmed that a different enzyme, insensitive to suicide substrates, catalyzes 7′-hydroxylation of (−)-ABA and therefore that (−)-ABA cannot adopt a conformation such that the 7′-methyl group is presented to the iron-oxo group in the active site of ABA 8′-hydroxylase.

Germination Inhibition. The fact that (+)-9′-propargyl-ABA and (+)-8′-acetylene-ABA were significantly stronger than ABA itself in inhibiting *Arabidopsis* seed germination (Figure 8) is consistent with catabolism limiting the hormonal effects of ABA. Clearly one route to developing highly active ABA analogues is to design them to autoinhibit normal catabolic processes. However, the relatively weak activity of (+)-9′-allyl-ABA and (+)-8′-propargyl-ABA (relative to ABA) suggests that blocking ABA catabolism is not alone sufficient for strong ABA-like hormonal activity. It seems likely that analogues must not only reduce catabolism of endogenous ABA but also induce hormonal effects themselves. Therefore (+)-9′-allyl-ABA and (+)-8′-propargyl-ABA may bind only weakly to ABA receptors relative to (+)-ABA, (+)-9′-propargyl-ABA, and (+)-8′-acetylene-ABA.

[4] K. Walker-Simmons (USDA, Washington) and S. R. Abrams, experiments in progress.

Conclusions. In addition to potential field and greenhouse applications as antitranspirants and for blocking germination, the inhibitors described here should be useful for a variety of research purposes. The analogues that are more potent than the natural hormone can be employed to investigate the role of ABA in physiological processes. For example, 8′-acetylene-ABA is being employed to examine the role of ABA in dormancy in cereal seeds.[4] The 8′-acetylene-ABA analogue can be prepared efficiently and in quantity (*21*) and may therefore be the most convenient inhibitor to synthesize and use. In addition to strongly inhibiting *Arabidopsis* seed germination, it has high hormonal activity in inhibiting corn cell growth (*21*) and wheat embryo germination (*54*).

Another potential application for the suicide substrates described here is for mapping the amino acids of the ABA 8′-hydroxylase active site. Also, they will be useful reagents for distinguishing the relative roles of 7′- and 8′-hydroxylation in modulating the effects of ABA. In general, we anticipate that the inhibitors that we have described here will provide a repertoire of reagents that will help elucidate the role of 8′-hydroxylation in controlling ABA flux and resultant hormonal effects.

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