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A new non-azole inhibitor of ABA 8'-hydroxylase: Effect of the hydroxyl group substituted for geminal methyl groups in the six-membered ring

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Abstract—We designed and synthesized AHI4 that has an axial hydroxyl group instead of geminal methyl groups at C-6' of AHI1, previously reported as a lead compound for the development of non-azole inhibitors of ABA 8'-hydroxylase. (+)-AHI4 competitive-ly inhibited 8'-hydroxylation of ABA by recombinant CYP707A3. The K_I value was found to be 0.14 μ M, 10-fold less than that of (+)-AHI1, suggesting that enzyme affinity increased by a factor of 10 due to substitution of the hydroxyl group by the geminal methyls at C-6'. This finding should assist in the design of more effective, non-azole ABA 8'-hydroxylase inhibitors. © 2006 Elsevier Ltd. All rights reserved.

Abscisic acid (ABA) 8'-hydroxylase is a cytochrome P450 monooxygenase and a key catabolic enzyme controlling inactivation of ABA, a plant hormone involved in stress tolerance.^{1–4} In addition to ABA biosynthesis and transport, catabolism of ABA is crucial for controlling ABA concentration in vivo. Chemical regulation of ABA catabolism by use of enzyme inhibitors is a practical method to control concentration. Because ABA is largely catabolized through 8'-hydroxylation by the cytochrome P450 monooxygenase (Fig. 1), ABA 8'-hydroxylase (e.g., Arabidopsis CYP707A1-CYP707A4).5,6 specific inhibitors of this enzyme are likely to be very useful tools for probing cellular and molecular events involving ABA. Kitahata et al. reported that (+)-diniconazole, a triazole-containing fungicide, strongly inhibits ABA 8'-hydroxylase.⁷ However, azole-containing P450 inhibitors can function as inhibitors of other P450 enzymes; in fact, diniconazole and uniconazole-P act as GA biosynthesis inhibitors.⁷ This loose specificity for target enzymes derives from structural properties of the azole-containing inhibitors. The azole-type



Figure 1. Major catabolic pathway of ABA in plants. The key step is the 8'-hydroxylation catalyzed by ABA 8'-hydroxylase. 8'-Hydroxy-ABA is spontaneously isomerized to phaseic acid which is (probably enzymatically) reduced to give dihydrophaseic acid. The activity of phaseic acid is one-tenth to one-hundredth of that of ABA. Dihydrophaseic acid exhibits no ABA activity.

inhibitors bind to the P450 active site by both coordinating the heme-iron atom and interacting with the surrounding protein residues.⁸ The heme coordination results from the intrinsic affinity of the ligand nitrogen electron pair for the heme iron. Azole-type inhibitors tend to be non-specific owing to this intrinsic effect.

Keywords: Abscisic acid; ABA; Cytochrome P450; ABA 8'-hydroxy-lase; Inhibitor.

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Figure 2. Chemical structures of ABA 8'-hydroxylase inhibitors (AHI) and their isomers.

Recently, we proposed a lead compound, ABA 8'-hydroxylase inhibitor 1 (AHI1, Fig. 2), for the development of non-azole inhibitors.⁹ This compound has a simple ring structure which easily allows structural modifications of the ring methyl groups due to the low cost of synthesis. Verification of an effect of the modifications on ABA 8'-hydroxylase inhibition is done more easily than by using the full ABA structure. Methyl groups on the ABA ring are thought to stabilize the enzyme–ABA complex through van der Waals or CH– π interactions. If the interaction were a weak hydrogen bond such as the CH- π interaction, a more acidic hydrogen at these sites may reinforce affinity for ABA 8'-hydroxylase. However, substituents with additional carbons at these methyls substantially reduce affinity for the enzyme,¹⁰ suggesting that the substrate-binding cavity accommodates ABA with little or no tolerance for bulkier structures. It follows, therefore, that an acidic hydrogen should be introduced directly on the ring carbon, instead of methyl groups. Thus, AHI4 (Fig. 2) was designed which has an axial hydroxyl group instead of geminal methyl groups at C-6' of AHI1. In this paper, we describe the synthesis, optical resolution, and determination of the absolute configuration of AHI4 and evaluate its inhibitory activity against ABA 8'hydroxylase.

AHI4 was synthesized from (\pm) -2-hydroxycyclohexanone (Scheme 1). Protection of the 2-hydroxyl of (\pm) -2-hydroxycyclohexanone with tert-butyldimethylsilyl chloride (TBSCl) yielded the TBS ether 1, which was converted into a diastereomeric mixture of 2 and 3 in a ratio of 1:4, by 6-methylation using methyl iodide. The minor diastereomer 2 gave a diastereomeric mixture of 4 and 5 in a ratio of 19:1 by introduction of the side chain with alkynyl lithium. Reduction of the triple bond of 4 coupled with de-protection of the secondary alcohol gave the diol 6, which was converted to 7 by re-protection of the secondary alcohol and de-protection of the primary alcohol. Oxidation of 7 gave the aldehyde 8, which was converted to the methyl ester 9 as a 2Z/2Emixture (3:1). The methyl ester 9 was purified and separated into (+)-AHI4 and the enantiomer by HPLC using ODS and chiral columns.¹¹ (±)-epi-AHI4 was synthesized from 3 in a similar manner to AHI4.¹²

In the NOESY spectrum of (\pm) -AHI4, an NOE was observed between the 5-hydrogen and the 3'- and 5'-hydrogens (Fig. 3), indicating that AHI4 adopts a chair form with the side-chain axial. In this conformation, observed NOEs between the 5-hydrogen and the 6'- and 7'-hydrogens indicate that the 6'-hydrogens and 2'-methyl (C-7') are *cis* to the side chain. Thus, we determined the rela-



Scheme 1. Synthesis of AHI4 and *epi*-AHI4. Reagents and conditions: (i) TBSCl (1.2 equiv), imidazole (2.4 equiv), DMF, 0 °C, 15 h, 98%; (ii) *n*-BuLi (1.5 equiv), diisopropylamine (1.5 equiv), THF, -78 to -40 °C, 50 min, followed by addition of 1, -40 to -28 °C, 1.5 h, followed by addition of iodomethane (1.5 equiv), -50 °C, 4 h, 8%; (iii) 2-propynyl THP ether (2.1 equiv), *n*-BuLi (2.0 equiv), THF, -78 °C, 1 h, followed by addition of 2, -78 to -62 °C, 1 h, 79%; (iv) sodium bis(methoxyethoxy)aluminum hydride (4.5 equiv), THF, 1.5 h, 78%; (v) TBSCl (2.0 equiv), imidazole (4.0 equiv), DMF, 0 °C to room temperature, 15 h, 82%; (vi) pyridinium *p*-TsOH (0.35 equiv), EtOH, 60 °C, 1.5 h, 94%; (vii) MnO₂ (15 equiv), CH₂Cl₂, 22 h, 79%; (viii) bis(2,2,2-trifluoroethyl)(methoxycarbonylmethyl)phosphonate (3.0 equiv), KN(TMS)₂ (2.2 equiv), toluene, 0 °C, 1 h, followed by addition of 8, 1 h, 87%; (ix) *p*-TsOH (0.5 equiv), THF–EtOH (2:1), 60 °C, 4 h, 44%; and (x) NaOH, MeOH, 1 h, 98%, followed by HPLC separation using ODS and chiral columns. Compounds 2–9 are a racemic mixture.



Figure 3. A favored conformation of AHI4 depicted in a stick model. Carbons, hydrogens, and oxygens are colored gray, white, and red, respectively. Arrows represent the observed NOEs.

tive configuration of (\pm) -AHI4 as $(1'S^*, 2'S^*, 6'S^*)$. The conformation of $(1'S^*, 2'S^*, 6'S^*)$ -AHI4 is consistent with theoretical calculations [B3LYP/6-311++G(2df,2p)//B3LYP/6-31G(d)];¹³ the chair with the equatorial methyl is more stable than that with the equatorial side chain by 1.3 kcal mol⁻¹. On the other hand, the relative configuration of (\pm) -*epi*-AHI4 was determined to be $(1'S^*, 2'R^*, 6'S^*)$ because it is the epimer of AHI4 at C-6'.

The absolute stereochemistry of both enantiomers of AHI4 was determined by Mosher's method¹⁴ using ¹H NMR spectra of (S)-(+)- α -methoxy- α -(trifluoromethyl)phenyl acetates (MTPA) of the methyl ester of both AHI4 enantiomers, which was prepared from compound 9 with (S)-(+)-MTPACl and subjected to subsequent HPLC. The side chain and ring protons of Me (+)-AHI4 shifted upfield and downfield, respectively, compared to those of Me (-)-AHI4 (Fig. 4), indicating that the absolute configuration at C-1' of (+)-AHI4 is S, whereas that of (-)-AHI4 is R. Finally we determined the absolute stereochemistry for (+)-AHI4 as (1'S,2'S,6'S) and for (-)-AHI4 as (1'R,2'R,6'R). Because (+)-AHI4 is the compound for which the C-8' of (+)-AHI1 is substituted with a hydroxyl group, the hydroxyl group of (+)-AHI4 is expected to mimic C-8' of (S)-(+)-ABA as the native hormone with respect to binding the active site.

(+)-AHI4 strongly inhibited 8'-hydroxylation of ABA by recombinant CYP707A3. The inhibition mode was competitive based on a Dixon plot. The $K_{\rm I}$ value was determined to be 0.14 μ M (Fig. 5), 10-fold less than that of (+)-AHI1 (1.4 μ M),¹⁵ indicating a 10-fold increase in affinity for the active site due to the hydroxyl group substitution for the geminal methyls at C-6'. On the other hand, (-)-AHI4 and (±)-*epi*-AHI4 exhibited little inhibitory activity. (+)-AHI4 did not exhibit ABA activity in either rice seedling elongation or lettuce seed germination assays even at a high concentration (400 μ M). This is because of the absence of the side-chain methyl (C-6), which has been shown to be essential for ABA bioactivity.¹⁰ However, (+)-AHI4 enhanced the effect of (+)-





Me (R)-MTPA-(1'S,2'S,6'S)-AHI4

Me (R)-MTPA-(1'R,2'R,6'R)-AHI4



Me (R)-MTPA-(+)-AHI4: $\Delta \delta = \delta$ (+) – δ (–)

Figure 4. Differences in chemical shifts between (R)-MTPA-esters of both enantiomers of Me AHI4.



Figure 5. A Dixon plot for the inhibition of recombinant CYP707A3 microsomes by (+)-AHI4.¹⁶

ABA upon simultaneous application of both compounds to rice seedlings (Fig. 6). The activity of 1 μ M ABA at which the inhibition ratio is 22% was enhanced to 96% by addition of 30 μ M (+)-AHI4. This suggests that (+)-AHI4 inhibits 8'-hydroxylation of (+)-ABA in vivo. Radish seedlings acquired drought-stress tolerance when treated with 400 μ M (+)-AHI4 in the absence of (+)-ABA (Fig. 7). (±)-*epi*-AHI4 was not found to induce tolerance at the same concentration. Thus, (+)-AHI4 is thought to induce drought-stress tolerance by slowing endogenous ABA catabolism in radish seedlings. (+)-AHI4 may be more effective when ABA is required (e.g., under stress conditions) than when it is not (e.g., germination and growth).

The above results suggest that the axial hydroxyl group at C-6' stabilizes the enzyme-ligand complex more strongly than the geminal methyls. The acidic hydrogen of the hydroxyl group is expected to provide a stronger



Figure 6. Enhanced effect of (+)-AHI4 on ABA activity in the rice seedling elongation assay.⁹



Figure 7. Drought tolerance of radish seedlings treated with (+)-AHI4.¹⁷

interaction relative to the CH– π interaction. The inhibitory activity of (+)-AHI4 is more than 10 times of that of (+)-AHI1, equal to that of (+)-6-nor-ABA,¹⁰ and less than one-tenth of that of uniconazole-P and (+)-diniconazole.⁷ In light of the fact that (+)-AHI1 is 10 times less effective than (+)-6-nor-ABA owing to the absence of the ring enone, the present findings should aid in the design of more effective, non-azole ABA 8'-hydroxylase inhibitors which are comparable to the azole-containing class.

Acknowledgment

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- [(2Z,4E)-5-((1S*,2S*,6S*)-1,2-dihydroxy-6-11. (±)-AHI4 methylcyclohexyl)penta-2,4-dienoic acid]: ¹H NMR $(500 \text{ MHz}, \text{CDCl}_3)$: $\delta 0.88 (3H, d, J = 7.0 \text{ Hz}, H_3-7')$, 1.21 (1H, m, H-3'proS), 1.51 (1H, m, H-4'), 1.62 (1H, m, H-3'proR), 1.68 (1H, m, H-4'), 1.70 (1H, m, H-5'proR), 1.83 (1H, m, H-5'proS), 2.00 (1H, m, H-2'), 3.73 (1H, dd, J = 5.2 and 2.7 Hz, H-6'), 4.81 (2H, br, HO-1' and HO-6'), 5.68 (1H, d, J = 11.3 Hz, H-2), 6.28 (1H, d, J = 15.3 Hz, H-5), 6.67 (1H, t, J = 11.3 Hz, H-3), 7.68 (1H, dd, J = 15.3 and 11.3 Hz, H-4); UV λ_{max} (MeOH) nm (e): 257.2 (20,200); HREIMS: $[M]^+$ at m/z 226.1209 (C₁₂H₁₈O₄ requires 226.1205). (+)-AHI4: $[\alpha]_D^{27}$ +32.5° (MeOH; *c* 0.0826). (-)-AHI4: $[\alpha]_{D}^{27}$ –32.3° (MeOH; c 0.0867). An optical purity was 99% and 98% for (+)- and (-)-enantiomers ($t_{\rm R}$ 11.8 and 9.5 min), respectively, on the basis of HPLC analysis (Chiralpak AD-H 250 × 4.6 mm i.d., Daicel; mobile phase, 15% 2-PrOH in hexane containing 0.1% TFA; flow rate, 1.0 mL min⁻¹; detection, 254 nm). ¹H NMR data of (+)and (-)-AHI4 agreed with that of (\pm) -AHI4.
- 12. (±)-*epi*-AHI4 [(2*Z*,4*E*)-5-((1*S**,2*R**,6*S**)-1,2-dihydroxy-6methylcyclohexyl)penta-2,4-dienoic acid]: ¹H NMR (500 MHz, CD₃OD): δ 0.84 (3H, d, *J* = 6.7 Hz, H₃-7'), 1.22 (1H, m, H-3'pro*S*), 1.44 (1H, m, H-4'pro*R*), 1.52 (2H, m, H-3'pro*R* and H-5'pro*S*), 1.60 (1H, m, H-2'), 1.76 (1H, m, H-4'pro*S*), 1.82 (1H, m, H-5'pro*R*), 3.49 (1H, dd, *J* = 11.3 and 4.6 Hz, H-6'), 5.65 (1H, d, *J* = 11.0 Hz, H-2), 6.28 (1H, d, *J* = 14.6 Hz, H-5), 6.70 (1H, t, *J* = 11.0 Hz, H-3), 7.64 (1H, dd, *J* = 14.6 and 11.0 Hz, H-4); UV λ_{max} (MeOH) nm (*e*): 256.8 (19,200); HREIMS: [M]⁺ at *m*/*z* 226.1211 (C₁₂H₁₈O₄ requires 226.1205).
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