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Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 16 (2006) 3302–3305

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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Received 14 February 2006; revised 7 March 2006; accepted 9 March 2006
Available online 24 March 2006

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 competitively inhibited 8′-hydroxylation of ABA by recombinant CYP707A3. The K_I value was found to be 0.14 μM, 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.

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 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.

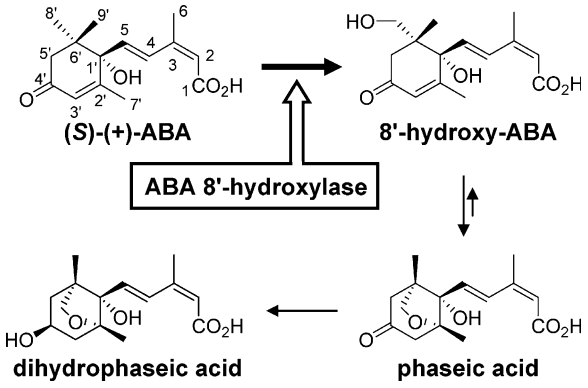

Figure 1. Major catabolic pathway of ABA in plants. The key step is the 8′-hydroxylation catalyzed by ABA 8′-hydroxylase. 8′-Hydroxy-ABA is spontaneously isomerized to phaseic acid which is (probably enzymatically) reduced to give dihydrophaseic acid. The activity of phaseic acid is one-tenth to one-hundredth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

Keywords: Abscisic acid; ABA; Cytochrome P450; ABA 8′-hydroxylase; Inhibitor.
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doi:10.1016/j.bmcl.2006.03.024

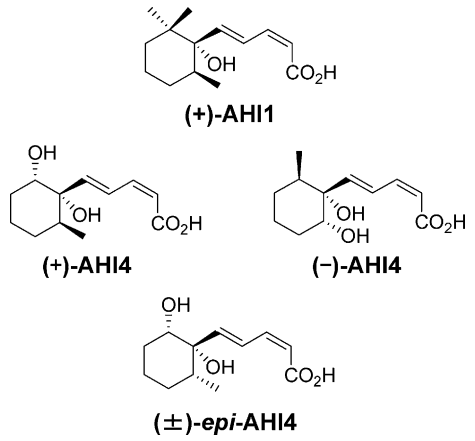
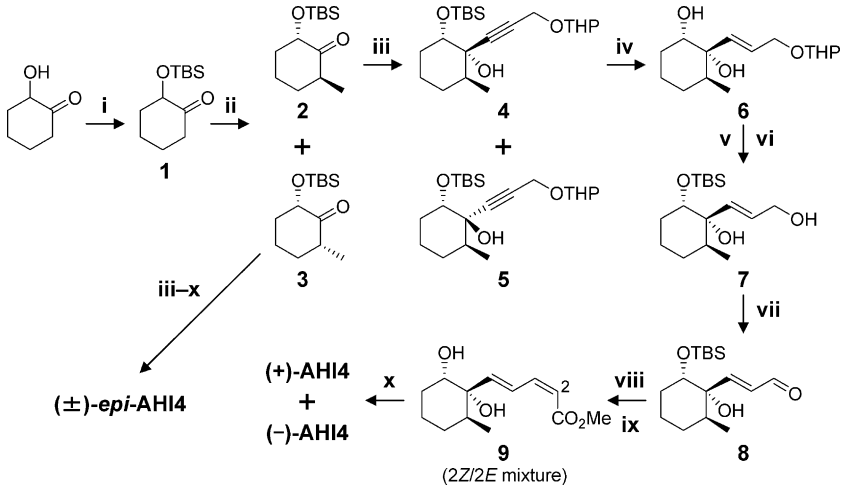


Figure 2. Chemical structures of ABA 8′-hydroxylase inhibitors (AHI) and their isomers.

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 (±)-2-hydroxycyclohexanone (Scheme 1). Protection of the 2-hydroxyl of (±)-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 2*Z*/2*E* mixture (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 (±)-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) MnO_2 (15 equiv), CH_2Cl_2, 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.