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A lead compound for the development of ABA 8′-hydroxylase inhibitors

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Abstract—(1′*S**,2′*S**)-(±)-6-Nor-2′,3′-dihydro-4′-deoxo-ABA (**2**) was designed and synthesized as a candidate lead compound for developing a potent and specific inhibitor of ABA 8′-hydroxylase. This compound acted as an effective competitive inhibitor of the enzyme, with a K_I value of 0.40 μM, without exhibiting ABA activity. However, compound **2** also functioned as an enzyme substrate, making it a short-lived inhibitor. The 8′-difluorinated derivative of **2** (**4**) was synthesized as a long-lasting alternative. Compound **4** resisted 8′-hydroxylation, but inhibited ABA 8′-hydroxylation as effectively as **2**. These results suggest that compound **2** is a useful lead compound for the future design and development of an ideal ABA 8′-hydroxylase inhibitor.

The plant hormone (1′*S*)-(+)-abscisic acid (ABA, **1**) is an important regulator of plant growth, development, and responses to stresses such as desiccation and freezing.[1–4] In addition to ABA biosynthesis and transport, the metabolic inactivation of ABA is crucial for the control of ABA concentration in vivo. Since ABA is mainly metabolized through hydroxylation at C-8′ by the cytochrome P450 monooxygenase ABA 8′-hydroxylase (e.g., Arabidopsis CYP707A1-CYP707A4) (Fig. 1),[5–7] specific inhibitors of this enzyme would be potentially very useful tools for cellular and molecular investigations in the field of plant physiology. Tetcyclacis inhibited 8′-hydroxylation of ABA by CYP707A1,[6] and uniconazole-P acted as an inhibitor of ABA catabolism in cultured tobacco cells.[8] Diniconazole was a potent competitive inhibitor of CYP707A3.[8] However, these compounds also inhibit gibberellin biosynthesis, and, therefore, are not specific inhibitors of ABA 8′-hydroxylase. Since there are many metabolic pathways mediated by P450 enzymes in plants, there is a need for ABA 8′-hydroxylase inhibitors that block only the ABA metabolic inactivation pathway without affecting other pathways.

An ABA analogue designed on the basis of the substrate specificity of ABA 8′-hydroxylase has the potential to act as a specific inhibitor of the enzyme. 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. Additionally, the ideal inhibitor would bind with high affinity to the enzyme and have a relatively long-lasting activity. To develop an ABA 8′-hydroxylase inhibitor meeting these requirements, we need a lead compound, which should be designed according to the following criteria: (1) it has all the functional groups that are required for binding to the enzyme; (2) it never substitutes for ABA as an activator of the ABA signal transduction pathway; and (3) it is easily prepared from a synthetic intermediate which can be modified to improve the properties of the final product. In this study, we designed (1′*S**,2′*S**)-(±)-6-nor-2′,3′-dihydro-4′-deoxo-ABA (**2**, Fig. 2) as a candidate lead compound.

Keywords: Abscisic acid; ABA; Cytochrome P450; ABA 8′-hydroxylase, Inhibitor.
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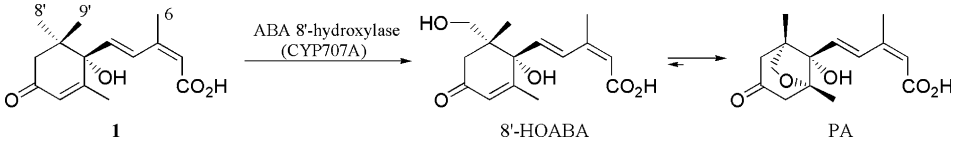

1 8'-HOABA PA

Figure 1. Initial step of metabolic inactivation of ABA in plants.

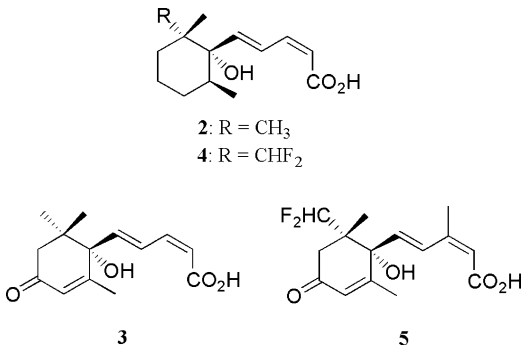

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Figure 2. Structures of ABA 8′-hydroxylase inhibitors. Compounds **2** and **4** are racemates, and compounds **3** and **5** are chiral compounds.

Although compound **2** lacks the α,β-unsaturated ketone in the cyclohexenone ring of ABA, modification of this functional group only slightly affects binding to ABA 8′-hydroxylase;[9] therefore, compound **2** was expected to bind to ABA 8′-hydroxylase as well as ABA. Furthermore, compound **2** was not expected to exhibit ABA activity, because (+)-6-nor-ABA (**3**) has no ABA activity.[9]

The methyl groups (C-7′, -8′, and -9′ of ABA) involved in binding to ABA 8′-hydroxylase are significant substituents for developing an ABA 8′-hydroxylase inhibitor that has a unique function, for example, long-lasting effects, suicide function, coordination to the heme iron atom, and protein modification. Compound **3** acted as the most ideal competitive inhibitor of ABA 8′-hydroxylase (K_I = 0.16 μM) among the ABA analogues we tested previously.[9] However, alternation of C-7′, -8′, and -9′ in compound **3** required more complicated synthetic procedures than in the case of compound **2**, which enables us to synthesize from a cyclohexanone that is easily modified at the corresponding sites to C-7′, -8′, and -9′ of **2**. Thus, compound **2** should be more advantageous for the future design of a portent and useful ABA 8′-hydroxylase inhibitor than compound **3**.

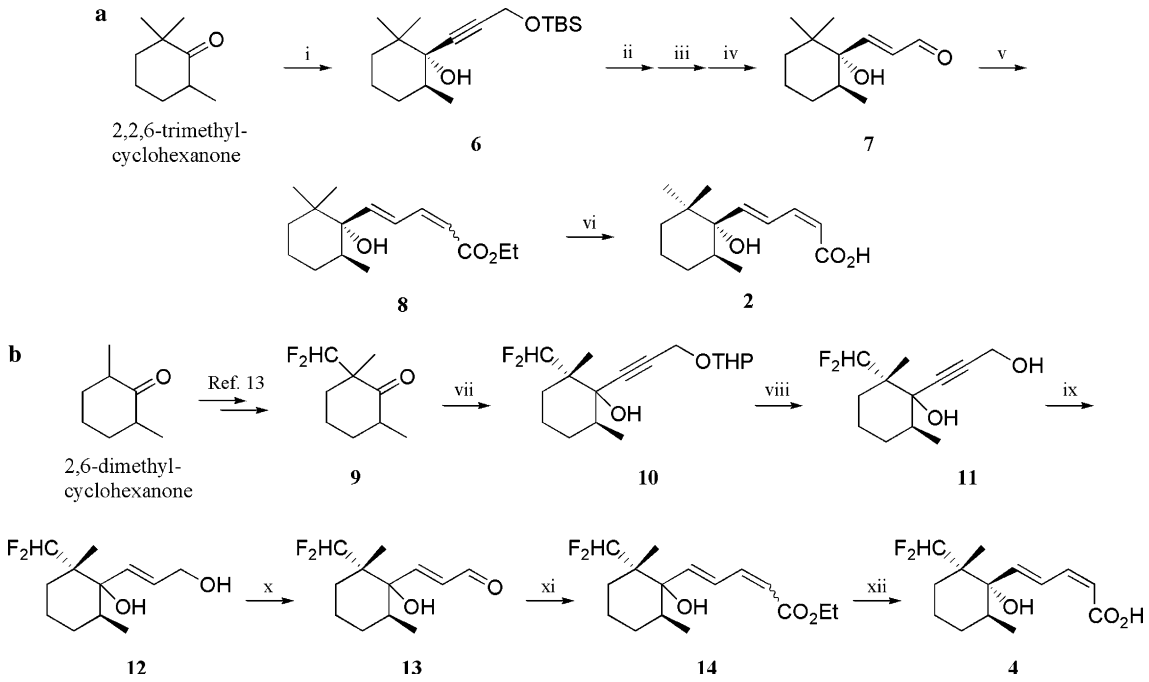
To test the usefulness of **2** as a lead compound, we designed its 8′-difluorinated derivative, compound **4**. This compound is expected to resist 8′-hydroxylation, because (+)-8′,8′-difluoro-ABA (**5**) yielded no enzyme products and acted as an effective competitive inhibitor of ABA 8′-hydroxylase (K_I = 0.17 μM).[9] In contrast, compound **2** may be converted into the 8′-hydroxylated product, because compound **2** has the methyl group corresponding to C-8′ of ABA and the similar conformation to ABA. This means that compound **2** is a short-lived inhibitor in in vivo experiments. Compound **4** is expected to be longer-lasting than **2**, making it a more useful in vivo inhibitor. In this paper, we describe the synthesis of compounds **2** and **4**, and assess their ability to inhibit ABA 8′-hydroxylase. We also discuss the usefulness of **2** as a lead compound for developing ABA 8′-hydroxylase inhibitors.

The lead compound **2** was synthesized from 2,2,6-trimethylcyclohexanone as shown in Scheme 1a. The reaction of 2,2,6-trimethylcyclohexanone with 2-propynyl *tert*-butyldimethylsilyl (TBS) ether and *n*-butyllithium (*n*-BuLi) gave the ether **6**, whose relative configuration is (1′*S**,2′*S**), and its diastereomer, whose relative configuration is (1′*S**,2′*R**), in a ratio of 12:1. The 1′-hydroxyl group of **6** was in the equatorial orientation, which was most likely formed by the preferential axial attack on the 2,2,6-trimethylcyclohexanone by the acetylide anion.[10,11] Reduction with sodium bis(methoxyethoxy)aluminum hydride (SMEAH), de-protection with AcOH and H_2O in tetrahydrofuran (THF), and oxidation with pyridinium dichromate (PDC) yielded the aldehyde **7**. The Horner–Emmons reaction of **7** with ethyl di-*o*-tolylphosphonoacetate yielded the ethyl ester **8**; the ratio of 2*Z*/2*E* isomers was 4:1. Basic hydrolysis of **8** and separation of the isomers by silica gel column chromatography with 8–15% acetone in CH_2Cl_2 containing 0.5% AcOH, and by octadecylsilyl (ODS) column chromatography using Sep-Pak Plus C18 cartridges (Waters) with 75% MeOH in H_2O containing 0.1% AcOH, produced compound **2** as a colorless oil (29% yield from 2,2,6-trimethylcyclohexanone).[12]

In difference NOE and 2D NOE (NOESY) experiments in a $CDCl_3$ solution, the 5-proton of **2** exhibited NOEs to the 3′-, 5′-, 7′-, and 9′-protons, and the 8′-protons showed NOE to the 2′-proton (Fig. 3). The result suggests that the ring conformation of **2** is a chair with an axial side chain and that the relative configuration of **2** is (1′*S**,2′*S**); that is, the 2′-methyl group is *cis* to the side chain.

The 8′-difluorinated derivative, (1′*S**,2′*S**,6′*S**)-(±)-6-nor-2′,3′-dihydro-4′-deoxo-8′,8′-difluoro-ABA (**4**), was synthesized from compound **9** (prepared as reported previously)[13] by almost the same method used for **2** (Scheme 1b) and was identified by spectral data.[14] The conformation of **4** is a chair with an axial side chain and the relative configuration of **4** is (1′*S**,2′*S**,6′*S**), which was confirmed by difference NOE and NOESY experiments (Fig. 3).

The biological activities of **2** and **4** were evaluated in two bioassays.[15] In the rice seedling elongation assay, the IC_{50} values of both compounds were more than 300 μM, whereas that of (±)-ABA was 2 μM. On the other hand, the IC_{50} value of **4** in the lettuce seed germination assay was 150 μM, whereas that of (±)-ABA was 12 μM. The IC_{50} value of **2** in the same assay was more



Scheme 1. Synthetic routes of compound **2** (a) and compound **4** (b). Reagents and conditions: (i) 2-propynyl TBS ether (3.1 equiv), *n*-BuLi (2.0 equiv), dry THF, −78 °C, 50 min, and then addition of 2,2,6-trimethylcyclohexanone, −78 to −11 °C, 2.5 h, 87%; (ii) SMEAH (2.9 equiv), dry THF, rt, 1.5 h; (iii) THF/AcOH/H_2O = 1.5:3:1 (v/v), rt, 3d, 71% from **6**; (iv) PDC (1.3 equiv), Celite, dry CH_2Cl_2, rt, 4 h, 85%; (v) ethyl di-*o*-tolylphosphonoacetate (3.3 equiv), NaH (4.6 equiv), dry THF, 0 °C, 30 min, then addition of **7** at −78 °C, −78 to −24 °C, 70 min, 87%; (vi) NaOH, MeOH, rt, 4 h, and purification with Sep-Pak Plus C18 cartridges, 63%; (vii) 2-propynyl THP ether (2.7 equiv), *n*-BuLi (2.1 equiv), dry THF, −75 °C, 1 h, and then addition of **9**, −30 to −15 °C, 2.5 h, 46%; (viii) pyridinium *p*-toluenesulfonate (0.1 equiv), EtOH, 55 °C, 3.5 h, 83%; (ix) SMEAH (10.5 equiv), dry THF, rt, 1.5 h, 85%; (x) MnO_2 (19.3 equiv), CH_2Cl_2, rt, 2.5 h, 74%; (xi) ethyl di-*o*-tolylphosphonoacetate (1.7 equiv), NaH (3.0 equiv), dry THF, 0 °C, 30 min, then addition of **13** at −75 °C, −75 to −40 °C, 1.5 h, quantitative; and (xii) separation with HPLC ODS column, and NaOH, MeOH, rt, 13.5 h, 32%. Compound **9** was prepared from 2,6-dimethylcyclohexanone as reported previously.[13] Compounds **2**, **4**, and **6**–**8** are a racemic mixture, and compounds **9**–**14** are a diastereomeric mixture.

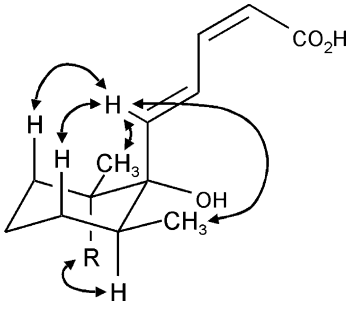


Figure 3. Favored conformations of **2** and **4**. **2**: R = CH_3; **4**: R = CHF_2. Arrows represent the observed NOEs.

Table 1. Inhibitory activity of **2** and **4** against CYP707A3

Compound	Inhibition[a] (%)	IC_{50}[b] (μM)	K_I (μM)	Products[c] (%)
2	90.9 ± 1.8	0.91	0.40 ± 0.03	17
4	94.0 ± 0.1	0.63	0.41 ± 0.17	ND[d]

[a] Inhibition ratio in the 8′-hydroxylation for ABA (5 μM). The concentration of **2** and **4** was 50 μM.
[b] The concentrations for 50% inhibition in the 8′-hydroxylase reaction for ABA (1 μM).
[c] The percent ratio of the enzyme products from inhibitors to those from ABA when the reaction mixture was incubated for 10 min at 30 °C.
[d] Not detected.

than 300 μM. These results suggest that compounds **2** and **4** are much poorer agonists than ABA of the ABA receptors.

To examine whether compounds **2** and **4** can inhibit the ABA 8′-hydroxylase reaction, we carried out in vitro ABA 8′-hydroxylase assays using recombinant CYP707A3.[16] The inhibition constants (K_I) and inhibitor types were determined from a Dixon plot. As shown in Table 1, compounds **2** and **4** inhibited the CYP707A3 reaction (IC_{50} = 0.91 and 0.63 μM, respectively). The kinetic analysis revealed that both compounds acted as competitive inhibitors of the enzyme; the K_I values were 0.40 μM for **2** and 0.41 μM for **4**. These values are lower than the K_M value for ABA (1.3 μM),[7] suggesting that compounds **2** and **4** are effective inhibitors of CYP707A3. Compound **2** functioned as an enzyme substrate, although the yield of enzyme products from **2** relative to those from ABA was estimated to be 17% (Table 1). In contrast, compound **4** resisted hydroxylation at C-8′, which is consistent with the design concept.

Compound **2** is an effective inhibitor of ABA 8′-hydroxylase and rarely activates the ABA signal transduction pathway. Additionally, this compound is easily prepared and modified, such as by fluorination to prevent 8′-hydroxylation. The present findings show that compound

2 is a useful lead compound for the future design and development of potent and specific inhibitors of ABA 8′-hydroxylase.

Acknowledgments

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References and notes

1. Hirai, N. In Mori, K., Ed.; Comprehensive Natural Products Chemistry; Elsevier: Amsterdam, 1999; Vol. 8, pp 72–91.
2. *Abscisic Acid*; Davies, W. J., Jones, H. G., Eds.; BIOS Scientific Publishers: Oxford, 1991.
3. Zeevaart, J. A. D.; Creelman, R. A. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1998**, *39*, 439.
4. *Abscisic Acid*; Addicott, F. T., Ed.; Praeger: New York, 1983.
5. Krochko, J. E.; Abrams, G. D.; Loewen, M. K.; Abrams, S. R.; Cutler, A. J. *Plant Physiol.* **1998**, *118*, 849.
6. Kushiro, T.; Okamoto, M.; Nakabayashi, K.; Yamagishi, K.; Kitamura, S.; Asami, T.; Hirai, N.; Koshiba, T.; Kamiya, Y.; Nambara, E. *EMBO J.* **2004**, *23*, 1647.
7. Saito, S.; Hirai, N.; Matsumoto, C.; Ohigashi, H.; Ohta, D.; Sakata, K.; Mizutani, M. *Plant Physiol.* **2004**, *134*, 1439.
8. Kitahara, N.; Saito, S.; Miyazawa, Y.; Umezawa, T.; Shimada, Y.; Min, Y. K.; Mizutani, M.; Hirai, N.; Shinozaki, K.; Yoshida, S.; Asami, T. *Bioorg. Med. Chem.* **2005**, *13*, 4491.
9. Ueno, K.; Araki, Y.; Hirai, N.; Saito, S.; Mizutani, M.; Sakata, K.; Todoroki, Y. *Bioorg. Med. Chem.* **2005**, *13*, 3359.
10. Lamb, N.; Abrams, S. R. *Can. J. Chem.* **1990**, *68*, 1151.
11. Oritani, T.; Yamashita, K. *Agric. Biol. Chem.* **1982**, *46*, 817.
12. ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.78 (3H, d, J = 6.7 Hz, H_3-7′), 0.82 (3H, s, H_3-9′), 1.08 (3H, s, H_3-8′), 1.18 (1H, m, H-3′), 1.39 (1H, m, H-5′), 1.48 (1H, m, H-5′), 1.54 (1H, m, H-4′), 1.60 (1H, m, H-4′), 1.62 (1H, m, H-3′), 1.94 (1H, ddq, J = 13.0, 6.7, and 2.4 Hz, H-2′), 5.65 (1H, d, J = 11.3 Hz, H-2), 6.39 (1H, d, J = 15.3 Hz, H-5), 6.73 (1H, dd, J = 11.5 and 11.3 Hz, H-3), 7.55 (1H, dd, J = 15.3 and 11.5 Hz, H-4); UV λ_{max} (MeOH) nm (ε): 260.4 (21000); HREIMS: $[M]^+$ at m/z 238.1575 ($C_{14}H_{22}O_3$ requires 238.1569).
13. Todoroki, Y.; Hirai, N.; Koshimizu, K. *Phytochemistry* **1995**, *38*, 561.
14. ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.82 (3H, d, J = 6.7 Hz, H_3-7′), 0.88 (3H, s, H_3-9′), 1.24 (1H, m, H-3′), 1.33 (1H, m, H-5′), 1.56–1.72 (3H, m, H-3′ and H_2-4′), 2.00 (1H, m, H-5′), 2.11 (1H, m, H-2′), 5.71 (1H, d, J = 11.3 Hz, H-2), 6.14 (1H, t, $^2J_{HF}$ = 56.2 Hz, H-8′), 6.28 (1H, d, J = 15.3 Hz, H-5), 6.72 (1H, dd, J = 11.3 and 11.3 Hz, H-3), 7.59 (1H, dd, J = 15.3 and 11.3 Hz, H-4); UV λ_{max} (MeOH) nm (ε): 255.4 (21400); HREIMS: $[M]^+$ at m/z 274.1375 ($C_{14}H_{20}O_3F_2$ requires 274.1381).
15. 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 is the number of seeds that germinated when water was used, and B is the number of seeds that germinated when a test compound was used. 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 is the mean length of the second leaf sheath when water was used, and B is the mean length of the second leaf sheath when a test compound was used.
16. For description of assay protocols, see Ref. 9. Some procedures were modified described below. HPLC condition in inhibition assay: ODS column, YMC Hydrosphere C18 (150 × 6.0 mm i.d.); solvent, 40% MeOH in H_2O containing 0.1% AcOH; flow rate, 1.0 mL min^{-1}; detection, 254 nm. To estimate the IC_{50} values and the K_I of inhibitors, the 8′-hydroxylation activity of CYP707A3 was measured by the same manner for inhibition assay protocol, except for an appropriate concentration of inhibitor, and 0.5, 0.75, 1, and 2 μM of (+)-ABA in a reaction mixture. HPLC conditions in hydroxylation assay: ODS column, Hydrosphere C18 (150 × 6.0 mm i.d.); solvent, 45% MeOH (0–10 min), 45–100% MeOH (linear gradient, 10–37.5 min), and 100% MeOH (37.5–43 min) in H_2O containing 0.1% AcOH; flow rate, 1.0 mL min^{-1}; detection, 254 nm.