Synthesis and Biological Activities of 8'-Methylene- and 8'-Methylidyneabscisic Acids

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8'-Methylene- and 8'-methylidyneabscisic acids which might act as suicide inhibitors of the 8'-hydroxylase of abscisic acid, were designed, synthesized, and optically resolved. The (+)-isomers showed stronger inhibitory activity in rice elongation and in lettuce seed germination than (+)-abscisic acid. The activity of (+)-8'-methylidyneabscisic acid was the strongest of the analogues synthesized to date, 40-fold stronger than abscisic acid.

Key words: abscisic acid; 8'-methyleabscisic acid; 8'-methylidyneabscisic acid; cytochrome P-450 monoxygenase; suicide inhibitor

(5S)-(+-)-Abscisic acid (ABA, 1) is a plant hormone that can transduce environmental stresses such as desiccation, freezing, and wounding into defensive responses at the molecular, cellular, and whole plant levels.1-3 The major metabolic inactivation of ABA in plants is initiated by hydroxylase at C-8' to produce 8'-hydroxy-ABA (2), which is cyclized spontaneously or enzymatically to biologically inactive (--)-phasic acid (3) (Fig. 1).4 Several reports suggested that hydroxylation at C-8' is catalyzed by a microsomal cytochrome P-450 monoxygenase.5-6 However, the 8'-hydroxylase of ABA has not been isolated or characterized.

The general strategy for isolation of cytochrome P-450 is tracing the specific absorption spectra, but in plants other heme proteins such as chlorophyll disturb the trace.7,8 Following the activity is also difficult due to spontaneous inactivation and decomposition under the purification conditions. The alternative strategy is affinity labeling using suicide inhibitor with a functional group that is activated by reaction with enzymes. Suicide inhibitors bind covalently to the residue in the neighborhood or sometimes to the catalytic group in the enzyme, resulting in its inactivation. In some instances, exo methylene and acetylene groups react with cytochrome P-450 monoxygenase, and bind to a prosthetic heme or to an amino acid residue of the binding site.9 These groups are small, so introducing them into C-8' of ABA might result in its becoming a suicide inhibitor without interfering with binding to 8'-hydroxylase. Thus, 8'-methylene- and 8'-methylidyne-ABA, 4 and 5, respectively, have been designed as candidates of suicide inhibitors for 8'-hydroxylase of ABA which can be used for affinity-labeling of 8'-hydroxylase in combination with radioisotopes. We describe here the synthesis and biological activities of 4 and 5.

Both analogues 4 and 5 were synthesized diastereoselectively from methyl (2Z,4E)-5-(2,6-dimethyl-1-hydroxy-4-oxocyclohexa-2,5-dienyl)-3-methylpent-2,4-dienoate (6) (Fig. 2).10 The copper(I) iodide catalyzed conjugate addition of vinylmagnesium bromide to 6 diastereoselectively yielding (+)-4a, which was hydrolyzed with alkali to afford the free acid (+)-4. The same reaction with ethynylmagnesium bromide gave (+)-5a, which was hydrolyzed with alkali to give the free acid (+)-5. These racemic compounds were optically resolved by HPLC on a chiral column. In the CD spectra, (+)-enantiomers showed a positive Cotton curve similar to that of (+)-ABA,11 showing that the (+)-enantiomers had an S configuration at C-1'. The absolute configuration of at C-1' of the (-)-enantiomers was confirmed as being R by the CD spectra.

The biological activities of optically active 4 and 5 were tested in four bioassays12: elongation of the second leaf sheath of rice seedlings; lettuce seed germination; α-amylase induction by gibberellic A3 in barley half-seeds without embryos; and stomatal opening of epidermal strips of spiderwort. Values for the concentration causing 50% inhibition (IC50) from the assays are summarized in the Table. (+)-Isomers of 4 and 5 showed stronger activities than (+)-ABA in the rice and lettuce assays. Particularly, (+)-5 showed the strongest activity among the analogues synthesized to date in the rice assay, a long-term assay. The activities of the (+)-isomers in the α-amylase and stomatal assays were similar to that of (+)-ABA. The activity in the stomatal assay suggested that the affinity of

[Diagram of metabolic pathway of ABA in plants]

Fig. 1. Major Metabolic Pathway of ABA in Plants.

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Abbreviations: ABA, abscisic acid; IC50, concentration causing 50% inhibition; THF, tetrahydrofuran.
the (+)-isomers to receptors of ABA did not change since the stomatal assay most reflects the affinity of analogues for the receptors, and is hardly affected by metabolism due to being a short-term assay. The differences between the activities of the (+)-isomers in the two assays resembled those of metabolism-resistant analogues which were modified at C-8 with functional groups resisting the hydroxylation. The metabolism-resistant analogues showed activities similar to those of ABA in the stomatal assays, and showed higher activities than ABA in the rice assay. This suggested some interactions between these (+)-isomers and 8'-hydroxylase. One of the interactions might be covalent binding to the enzyme since these (+)-isomers have different functional groups from the metabolism-resistant analogues. The high activities of (+)-4 and 5 may be explained by inactivation of 8'-hydroxylase by such binding: some molecules of (+)-4 and 5 inactivate 8'-hydroxylase through covalent binding, and the metabolism of the other intact molecules of (+)-4 and 5, and of endogenous ABA is suppressed. In that case, (+)-5 which was more active than (+)-4 can be a promising reagent for labeling the 8'-hydroxylase of ABA. The possibility that the inhibitors resist metabolism to the phaseic acid-like, inactive bicyclic compounds and show high activity could not be excluded. A part of metabolism of (+)-4 has very recently been reported as described below. Labeling and metabolic experiments with the (+)-isomers labeled with radioisotope are essential to know whether (+)-4 and 5 act as suicide inhibitors or not. The (-)-isomers of 4 and 5 were not active, which agrees with the observation that elongation of the carbon skeleton at C-8' caused a marked decrease in the activity of the (-)-isomer. The low activity of the (-)-isomers is probably due to a decrease in the affinity to receptors which is caused by loss of pseudosymmetry.

After our work was completed, synthesis, activity, and metabolism of (+)-4 were reported by Abrams et al. They found that (+)-4 showed higher inhibitory activities than ABA in assays of corn cell growth, cress seed germination, and others, and that the decrease of (+)-4 in the medium of corn cell cultures was slower than that of ABA. Metabolites of (+)-4 in the medium of corn cell culture were identified as two isomeric 8'-methyleneoxides. The slow metabolism of (+)-4 may be caused by its low affinity to 8'-hydroxylase as a substrate or by inactivation of 8'-hydroxylase by covalent binding although the formation of the 8'-methyleneoxides seems to deny the latter possibility. The high activity of (+)-4 was supposed to be caused not only by the slow metabolism but also by the activity of the 8'-methyleneoxides. Measurement of the activity of 8'-hydroxylase in the presence of (+)-4, and the activity of the 8'-methyleneoxides may reveal the basis of the slow metabolism and the high activity of (+)-4.

**Experimental**

*General procedures.* The 1H-NMR spectra were recorded with TMS as the internal standard using a Bruker ARX500 (500 MHz) and AC300 (300 MHz). For clarity, the names of all the compounds with the carbon skeleton of ABA were numbered as in ABA in the assignment of peaks. Mass spectra were recorded at 70eV with a JEOL JMS-DX300/DA5000 mass spectrometer. CD spectra were recorded with a JASCO J-720 spectropolarimeter.

(1S)-Methyl 8'-methyleneoxa(4)-bicyclic acid (4a). To a stirred solution of vinylmagnesium chloride (1.0 M solution in THF, 50 μl, 50 μmol) in 1 ml of THF was added manganese dioxide (0.5 mg, 2.6 μmol) at 0°C under nitrogen. The resulting mixture was stirred for 15 min at the same temperature. A solution of 6 (4.0 mg, 15.3 μmol) in 1 ml of THF was added at 0°C, and the resulting mixture was stirred at the same temperature for 30 min. After quenching with 10 mL of saturated aqueous NH4Cl, the mixture was extracted with 10 mL of EtOAc three times. The combined organic solutions were washed with H2O, dried over Na2SO4, and concentrated. The residue was purified by chromatography on silica gel with hexane-EtOAc (4:1) to afford 4a (5.6 mg, 81% yield) as a colorless oil. 4a. NMR δH (500 MHz, CDCl3): 1.15 (3H, s, H-9), 1.91 (3H, d, J=1.3 Hz, H-7), 2.02 (3H, d, J=1.2 Hz, H-6), 2.18 (1H, s, OH), 2.48

![Fig. 2. Synthesis and Optical Resolution of 4 and 5.](image-url)
(1H, d, J = 17.2 Hz, H-Sp,nahl) 2.56 (1H, d, J = 17.2 Hz, H-Sp,nahl), 3.70 (3H, s, OMe), 5.25 (1H, d, J = 17.5 Hz, H-143, 5.32 (1H, d, J = 10.8 Hz, H-153, 5.76 (1H, br, s, H-2), 5.98 (1H, m, H-3), 6.08 (1H, dd, J = 17.5 and 10.8 Hz, H-8'), 6.08 (1H, dd, J = 16.1 Hz, H-5), 7.87 (1H, d, J = 16.1 Hz, H-4'); UV λmax (MeOH) nm (ε): 264 (21,000), IR νmax (Chrom. HCl) cm⁻¹: 3000, 1710, 1665, 1640, 1630; EIMS m/z (rel. int.): 290 [M⁺] (1), 272 [M – H₂O⁺] (6), 222 (6), 190 (100), 162 (22), 134 (24), 125 (6); HR-EIMS m/z [M⁺]: calc. for C₁₇H₁₂O₄; found: 290.1581.

Optical resolution. Racemic mixtures of 4 and 5 were separated into enantiomers on a Chiral OD HPLC column (4.6 d, × 250 mm, Daicel Chemical Industries, Ltd.) with isopropanol–hexane (1:1) containing 0.1% TFA as the eluent, at a flow rate of 1.0 ml min⁻¹. The materials at τ₄ 8.7 and 15.0 min of 4 (45 mg) were collected to give (+)-4 (21.7 mg) and (−)-4 (22.1 mg) with optical purity of 99.9%. The materials at τ₄ 11.9 and 15.0 min of 5 (22 mg) were collected to give (+)-5 (10.6 mg) and (−)-5 (10.6 mg) with optical purity of 99.9%. (+)-4 [α]D₂₀ +412.5° (c = 0.646, MeOH); CD (c = 0.0010, MeOH) nm (λ): 326.1 (–2.8), 265.2 (+4.65), 236.0 (–43.1), (−)-4 [α]D₂₀ +407.2° (c = 0.646, MeOH); CD (c = 0.0010, MeOH) nm (λ): 326.1 (+2.9), 265.9 (–43.7), 236.3 (+42.2), (+)-5 [α]D₂₀ +360.3° (c = 0.126, MeOH); CD (c = 0.0010, MeOH) nm (λ): 326.1 (–2.6), 263.2 (+37.2), 232.2 (–33.2), (−)-5 [α]D₂₀ –359.3° (c = 0.123, MeOH); CD (c = 0.0010, MeOH) nm (λ): 326.4 (±2.8), 264.6 (±37.4), 238.1 (±34.2).

Bioassays. Details have been reported previously. For lettuce germination assay, the number of germinated lettuce (Lactuca sativa L. cv. Grand Rapids) seeds was counted after incubation with the test solution at 25°C for 48 h. For rice seedling elongation assay, the length of the second leaf sheath of rice (Oryza sativa L. cv. Nihoenbare) seeds were measured after incubation with the test solution in continuous light at 30°C for 7 days. For x-ray amylase assay, after incubating barley (Hordeum vulgare L. cv. Himalaya) half-seeds without embryos in the test solution at 30°C for 48 h in the dark, the absorbance of the test solution at 660 nm was measured by the Somogyi-Nelson method. For stomata assay, the width of stomatal aperture on epidermal strips of spiderwort (Tradescantia reflexa Rafin) was measured after incubation with the test solution in continuous light at 25°C for 3 h.

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