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8′-Methylene Abscisic Acid[1]

An Effective and Persistent Analog of Abscisic Acid

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We report here the synthesis and biological activity of a new persistent abscisic acid (ABA) analog, 8′-methylene ABA. This ABA analog has one additional carbon atom attached through a double bond to the 8′-carbon of the ABA molecule. (+)-8′-Methylene ABA is more active than the natural hormone (+)-ABA in inhibiting germination of cress seed and excised wheat embryos, in reducing growth of suspension-cultured corn cells, and in reducing transpiration in wheat seedlings. The (+)-8′-methylene analog is slightly weaker than (+)-ABA in increasing expression of ABA-inducible genes in transgenic tobacco, but is equally active in stimulating a transient elevation of the pH of the medium of corn cell cultures. In corn cells, both (+)-ABA and (+)-8′-methylene ABA are oxidized at the 8′ position. ABA is oxidized to phaseic acid and (+)-8′-methylene ABA is converted more slowly to two isomeric epoxides. The alteration in the ABA structure causes the analog to be metabolized more slowly than ABA, resulting in longer-lasting and more effective biological activity relative to ABA.

The plant hormone (+)-ABA (Fig. 1) regulates diverse aspects of plant growth, including development and germination of seeds, transpiration, and adaptive responses to environmental stresses (Zeevaart and Creelman, 1988; Davies and Jones, 1991). Considerable progress has been made in the identification of ABA-responsive genes, mutant characterization, and signaling (Bray, 1993; Chandler and Robertson, 1994; Giraudat et al., 1994). However, examination of the mechanisms of ABA action, identification of receptor proteins, and cellular localization of the hormone have been restricted by the rapid turnover of ABA in plants. Similarly, agricultural uses of applied ABA have been limited by its rapid metabolism in plants. Thus, the aim of the current research was to develop potent biologically stable ABA analogs that can be used to prolong ABA-like effects in plants for agricultural and basic research applications.

Biologically stable analogs of other plant hormones, especially of the auxins, have proven to be useful tools for investigating hormone action and to have widespread agricultural uses. The synthetic auxins, such as the chlorophenoxy acetic acids, are not metabolized as readily by IAA oxidases, and thus persist in plants longer than the natural auxins (for review, see Moore [1979]).

In plants, the predominant pathway of metabolism of (+)-ABA involves hydroxylation at the 8′ position, affording 8′-hydroxyABA, which undergoes cyclization by attack of the 8′-hydroxyl group onto the enone system, producing PA (Fig. 1; Gillard and Walton, 1976; Loveys and Milborrow, 1984; Zeevaart and Creelman, 1988; Balsevich et al., 1994). 8′-HydroxyABA has rarely been found in plant extracts, most probably because it readily cyclizes during manipulation (Milborrow, 1969). Recently, in the first report of the activity of 8′-hydroxyABA, it was found to be as active as ABA in increasing very-long-chain fatty acid production and oleosin transcript accumulation in *Brassica napus* embryos (Zou et al., 1995). PA, the more readily isolable ABA catabolite, has little or no activity in most assays (for example, see Loveys [1991]; Gusta et al. [1992]; Robertson et al. [1994]; Hill et al. [1995]; Todoroki et al. [1995]; Zou et al. [1995]).

In earlier work we examined the importance of the 8′-methyl group of ABA. We synthesized and studied the activity of an analog with three ^{2}H atoms at the 8′-C atom (Lamb et al., 1996). This compound is sterically and electronically almost identical to (+)-ABA, but is more active in the inhibition of germination of cress seeds and is correspondingly more persistent in suspension-cultured corn cells. The latter observation is consistent with a slower metabolism due to the greater strength of the 8′-C—^{2}H bond relative to the C—H bond (breakage of the C—H bond is the presumed rate-limiting step in the enzymatic hydroxylation of ABA). We have also synthesized an ABA analog lacking the 8′-methyl group and showed that this group is not strictly required for inhibiting germination of excised wheat embryos (Walker-Simmons et al., 1994).

Our research is directed at altering the 8′-C atom of ABA to develop analogs that are resistant to enzymatic oxidation

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Abbreviations: c, grams per 100 mL; *d*, doublet; *dd*, doublet of doublets; δ, chemical shifts; H, proton; HRMS, high resolution mass spectrum; *J*, coupling constant; PA, phaseic acid; ppm, parts per million; *s*, singlet; THF, tetrahydrofuran.

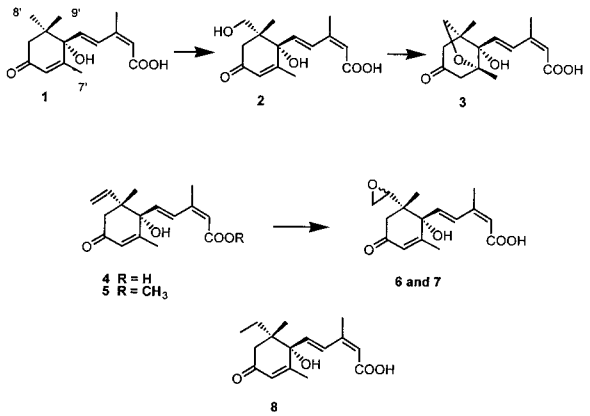

Figure 1. Chemical structures of (+)-ABA (**1**), its 8′-oxidized metabolites 8′-hydroxyABA (**2**) and PA (**3**), (+)-8′-methylene ABA (**4**), (+)-methyl 8′-methylene ABA (**5**), metabolites of **4** (**6** and **7**), and (+)-8′-methyl ABA (**8**).

(probably involving Cyt P450 monooxygenases) but that retain the bioactivity of the natural hormone. Adding a methylene group to the 8′-C of ABA, as in the analog 8′-methylene ABA, provides two possible mechanisms to increase resistance to oxidation. First, the methylene derivative should be a poorer substrate for the 8′ hydroxylase enzyme. Increasing the steric bulk with additional C atoms on the 8′-C atom should hinder binding in the active site of the hydroxylase enzyme, reducing the rate of oxidation, and thus prolonging the lifetime of the hormone analog. Second, terminal olefins analogous to 8′-methylene ABA have been shown to inactivate Cyt P450 monooxygenases (Ortiz de Montellano, 1991; He et al., 1996). The mechanism is thought to involve initial oxidation of the olefin, forming a reactive intermediate that may be radical or cationic (Newcomb et al., 1995) and may be internally quenched to produce an epoxide or alkylated by the protein, leading to irreversible enzyme inhibition. If the latter process occurs to a significant extent, increased persistence of the analog could result from a reduced level of active enzyme available for degrading the analog. In addition, 8′-methylene ABA may have enhanced activity because the cyclization to molecules such as PA may be reduced.

To be useful, ABA analogs must maintain the ability to act as hormone agonists. The steric hindrance of the additional C must not prevent binding to the hormone receptor protein. There is encouraging evidence that the binding of ABA to the receptor and to the hydroxylase are primarily dependent upon different structural features of the ABA molecule. Comparison of the relative importance of the 7′, 8′, and 9′ methyl groups of ABA in inhibiting wheat embryo germination indicates that the presence of the 7′ methyl group is absolutely essential, but that the others are less crucial (Walker-Simmons et al., 1994). This would suggest that there might be some latitude in altering the substrate binding site (8′ C) without affecting the part of the molecule that is critical for receptor binding (7′ C). ABA analogs bearing a methoxy or alkyl group on either the 8′- or 9′-C atom (Todoroki et al., 1994; Nakano et al., 1995) or fluorines on the 8′-C atom (Kim et al., 1995; Todoroki et al., 1995) are strong ABA agonists, suggesting that modifying the geminal dimethyl groups in some instances does not hinder recognition of the molecule as ABA-like. Similarly, alkyl substitutions at the 7′, 8′, and 9′ positions revealed that high hormonal activity was generally maintained for molecules with the same absolute stereochemistry as (+)-ABA (Nakano et al., 1995). In the course of these studies Nakano et al. (1995) showed that an analog with a methyl group added to the 8′-C atom, (+)-8′-methyl ABA (Fig. 1), was nearly as active as (+)-ABA in blocking GA_3-stimulated α-amylase activity, and was more active than (+)-ABA in inhibiting germination of lettuce seed, stomatal opening of spiderwort, and elongation of rice seedling leaves. The high activity of (+)-8′-methyl ABA suggested that the methylene analog (of similar steric bulk) ought also to be recognized as ABA-like. This also means that comparison of the biological activities of 8′-methyl ABA and 8′-methylene ABA would be of considerable interest because, although they are similar molecules, only 8′-methylene ABA has the potential to inactivate the 8′ hydroxylase irreversibly.

An important criterion in our chemical studies is that the analogs can be synthesized in short, efficient sequences to provide sufficient quantities of material for extensive testing of physiological activity and for further modification at the 8′-position. We report here an efficient chemical synthesis of a new ABA analog, 8′-methylene ABA, which is more slowly metabolized than ABA. We show that (+)-8′-methylene ABA is more effective than ABA in several biological assays, including inhibiting germination in cress and wheat, inhibiting growth of suspension-cultured corn cells, and reducing transpiration in wheat seedlings.

MATERIALS AND METHODS

Synthesis of 8′-Altered ABA Analogs

General Experimental Conditions

Melting points are uncorrected and were recorded on a hot stage melting point apparatus (Ernst Leitz Wetzlar, Germany). ^1H NMR spectra were recorded on a spectrometer at 500 MHz (model AMX-500, Bruker, Billerica, MA). ^{13}C NMR spectra were recorded on the same spectrometer at 125 MHz. $CDCl_3$ was used as a solvent in all NMR experiments with $CHCl_3$ as a reference. δ and *J* are reported as if they were first order. The conventional numbering system for ABA is used for proton assignments. HRMS were recorded in the electron impact mode using a double-focusing hybrid spectrometer (VG 70–250SEQ, Micromass, Manchester, UK) with a digital data system (PDP 11/73, Micromass). IR spectra were recorded on an Fourier transform IR spectrometer (Paragon 1000, Perkin-Elmer). Optical rotations were measured on a polarimeter (model 1000, Perkin-Elmer). Flash-column chromatography was performed using silica gel 60 (230–400 mesh, Merck). The solvent THF was dried by distillation from sodium and benzophenone. Unless otherwise indicated, all reactions were conducted under an atmosphere of dry argon.

(±)-Methyl 8′-Methylene Abscisate

Methyl (2Z, 4E)-5-(2,6-dimethyl-1-hydroxy-4-oxocyclohexa-2,5-dienyl)- 3-methylpent-2,4-dienoate (3.60 g, 13.7 mmol; Lei et al., 1994) was dissolved in dry THF (300 mL) and cooled to −78°C with an external dry ice/acetone bath. Methyllithium (Aldrich; 1.4 M in THF, 9.7 mL, 13.7 mmol) was added and the solution was stirred for 15 min. To this a solution of vinylmagnesium bromide (Aldrich; 1.0 M in THF, 41 mL), which had been stirred with anhydrous cuprous iodide (Aldrich; 440 mg, 2.3 mmol) for 0.5 h at 0°C, was transferred via cannula. The resultant dark solution was stirred at −78°C for 20 min, diluted with ether (200 mL), and then quenched with 200 mL of 10:1 saturated NH_4Cl/20% NH_4OH solution. The organic layer separated off and was washed with saturated sodium chloride solution (100 mL). The combined aqueous layers were extracted with ether (2× 100 mL). The organic layers were combined, dried over anhydrous $MgSO_4$, and concentrated. The residue was purified by column chromatography 25% ethyl acetate/hexane) to yield 2.74 g (69%) of product as a solid. Recrystallization from ether/hexane yielded white crystals with a melting point of 116 to 117°C; ^{1}H NMR: δ 7.85 (*d*, 1 H, *J* = 16.0 Hz, H-4), 6.06 (*dd*, 1 H, *J* = 10.8, 17.6 Hz, H-8′), 6.06 (*d*, 1 H, *J* = 16.0 Hz, H-5), 5.96 (*s*, 1 H, H-3′), 5.73 (*s*, 1 H, H-2), 5.29 (*d*, 1 H, *J* = 10.8 Hz, H-10′, *cis* to H-8′), 5.23 (*d*, 1 H, *J* = 17.6 Hz, H-10′, *trans* to H-8′), 3.68 (*s*, 3 H, OMe), 2.49 (*d*, 1 H, *J* = 17.2 Hz, H-5′), 2.41 (*d*, 1 H, *J* = 17.2 Hz, H-5′), 2.00 (*s*, 3 H, C-6, Me), 1.89 (*d*, 3 H, *J* = 0.9 Hz, C-7′, Me), and 1.12 (s, 3 H, C-9′, Me) ppm. ^{13}C NMR: δ 197.0, 166.3, 163.0, 149.2, 140.7, 134.9, 129.1, 127.3, 118.5, 118.0, 78.2, 51.2, 47.7, 47.5, 21.1, 20.6, and 19.2 ppm. HRMS, calculated for $C_{17}H_{22}O_4$ (M^+) 290.1518, found 290.1522.

Resolution of (±)-Methyl 8′-Methylene Abscisate

Resolution was effected by preparative chiral HPLC. A solution of racemic methyl ester in 1:4 2-propanol/hexane (20 mg mL^{-1}) was injected onto an OD column (Diacel Chemical Industries, Ltd., Chiral Technologies, Exton, PA; 250- × 10-mm i.d., preceded by a guard column, CSK1 HC Pellosil, Whatman) and eluted with 1:4 2-propanol/hexane at 2 mL min^{-1}, with UV detection at 262 nm. Five 1-mL injections (20 mg per injection) afforded (+)-methyl 8′-methylene abscisate (55 mg), eluting at 14 min, and (−)-methyl 8′-methylene abscisate (44 mg), eluting at 23 min. Each isomer was >99% optically pure, as determined by analytical HPLC (Diacel Chemical Industries, Ltd.), and gave ^{1}H and ^{13}C NMR spectra identical to those of the racemic mixture: (+)-methyl 8′-methylene abscisate $[\alpha]_D^{24}$ +386.6° (c = 1.3 in $CHCl_3$); melting point (ether/hexane) 124 to 125°C; (−)-methyl 8′-methylene abscisate, $[\alpha]_D^{24}$ −386.5°(c = 1.7 in $CHCl_3$); melting point (ether/hexane) 124 to 125°C.

Proof of structure was established by conversion of (−)-methyl 8′-methylene abscisate to methyl 8′-hydroxyABA and methyl phaseate using standard functional group manipulations. Cleavage of the vinyl group was accomplished by ozonolysis, and the resultant aldehyde reduced to the alcohol with sodium borohydride in ethanol at 0°C. Both methyl 8′-hydroxyABA and methyl phaseate were obtained in pure form after chromatography. The methyl phaseate obtained was identical to natural methyl phaseate as shown by NMR (Abrams et al., 1990), and gave the same retention time on a chiral GC column as unnatural (+)-methyl phaseate (Balsevich et al., 1994). These results confirmed the configuration of (−)-methyl 8′-methylene abscisate to be (1R, 6S).

(+)-8′-Methylene ABA

(+)-Methyl 8′-methylene abscisate (23 mg) was dissolved in methanol (7 mL) and 7 mL of 2 N NaOH solution was added dropwise. The solution was stirred at room temperature for 4 h and concentrated to remove most of the methanol. The residue was dissolved in 2 N NaOH (5 mL) and washed with ether (5 mL). The aqueous layer was acidified with 10% HCl solution and then extracted with ethyl acetate (3× 10 mL). The combined ethyl acetate layers were dried over anhydrous Na_2SO_4 and evaporated to afford (+)-8′-methylene ABA (21.3 mg, 97%) as a solid. $[\alpha]_D^{24}$ +351.3° (c = 2.3 in $CHCl_3$), ^{1}H NMR: δ 7.76 (*d*, 1 H, *J* = 16.1 Hz, H-4), 6.06 (*d*, 1 H, *J* = 16.1 Hz, H-5), 6.03 (*dd*, 1 H, *J* = 11.0, 17.5 Hz, H-8′), 5.94 (*s*, 1 H, H-3′), 5.71 (*s*, 1 H, H-2), 5.23 (*d*, 1 H, *J* = 11.0 Hz, H-10′, *cis* to H-8′), 5.17 (*d*, 1 H, *J* = 17.5 Hz, H-10′, *trans* to H-8′), 2.51 (*d*, 1 H, *J* = 17.3 Hz, H-5′), 2.42 (*d*, 1 H, *J* = 17.3 Hz, H-5′), 2.00 (s, 3 H, C-6, Me), 1.85 (s, 3 H, C-7′, Me), and 1.09 (s, 3 H, C-9′, Me) ppm. ^{13}C NMR: δ 197.3, 170.9, 163.2, 151.5, 140.8, 135.7, 129.2, 127.3, 118.2, 117.8, 78.5, 47.7, 47.3, 21.4, 20.7, and 19.3 ppm. HRMS calculated for $C_{16}H_{22}O_4$ (M^+) was 276.1362, found 276.1354; the melting point (ether/hexane) was 128 to 131°C.

(−)-8′-Methylene ABA

Hydrolysis of (−)-methyl 8′-methylene abscisate (26 mg) with the method described above yielded (−)-8′-methylene ABA (21.5 mg, 87%) as a solid. $[\alpha]_D^{24}$ −368.1° (c = 2.3 in $CHCl_3$). The ^{1}H and ^{13}C NMR spectra were identical to those of the (+) enantiomer. The melting point (ether/hexane) was 130 to 133°C.

Synthesis of (+)-8′-Methyl ABA

(±)-Methyl 8′-methylabscisate was synthesized using the same procedure as for (±) methyl 8′-methylene abscisate by substituting ethylmagnesium bromide for the vinylmagnesium bromide. The two enantiomers of (±)-methyl 8′-methyl abscisate were separated by chiral HPLC and the esters were hydrolyzed to the corresponding acids in the same manner as for (+)-8′-methylene ABA. Spectral properties of both acids and esters agreed with those previously reported (Nakano et al., 1995).

Metabolites of (+)-8′-Methylene ABA from Suspension-Cultured Corn Cells

Eighteen grams of cv Black Mexican Sweet corn cells were subcultured into a 1-L flask containing 500 mL of sterile medium, as described previously (Balsevich et al., 1994). The following day, 13.5 mg of (+)-8′-methylene

ABA, dissolved in 0.5 mL of ethanol, was introduced to the medium at a final concentration of 100 μM. The culture was incubated at room temperature on a rotary shaker at 150 rpm for 90 H, at which time HPLC analysis showed a high concentration of metabolites. At the end of the culture period, the cells were removed by filtration and the filtrate was frozen until processed for isolation.

The metabolites were extracted from the culture filtrate by a chromatographic procedure (Amberlite XAD-2 resin, Supelco, Bellefonte, PA; Balsevich et al., 1994). The crude product isolated from the resin was partially purified by preparative TLC (silica gel 60 GF_{254}, 20 cm × 20 cm × 1 mm, toluene-EtOAc-HOAc 25:15:2 as eluent), yielding two isomeric acids. The acids were separately reacted with diazomethane, and then further purified by HPLC as their methyl esters, yielding approximately 0.5 mg of each metabolite. Spectral data indicated that the metabolites were two isomeric epoxides.

Analysis of metabolite 1, the methyl ester of 8′-methyleneoxide ABA: Fourier transform IR spectroscopy (neat) υ_{max} cm^{-1}: 3446 (O—H), 1717 (C═O, ester), 1654 (C═O, enone), electron impact HRMS: $[M+1]^+$ at m/z 307.1573 ($C_{17}H_{23}O_5$ requires 307.1545); ^1H NMR: δ 7.89 (*d*, *J* = 16 Hz, 1 H-4), 6.01 (*d*, *J* = 15.9 Hz, 1 H-5), 6.00 (*s*, 1 H-3′), 5.74 (*s*, 1 H-2), 3.69 (*s*, 3 H, CO_2CH_3), 3.30 (*t*, *J* = 3.0 Hz, 1 h-8′), 3.04 (*s*, 1 H, OH), 2.67 (*t*, *J* = 4.2 Hz, 1 H-10′), 2.61 (*dd*, *J* = 3.0, 4.1 Hz, 1 H-10′), 2.48 (*d*, *J* = 17.9 Hz, 1 H-5′), 2.33 (*d*, *J* = 17.8 Hz, 1 H-5′), 1.99 (*s*, 6 H-6, 7′), and 0.91 (*s*, 3 H-9′).

Analysis of metabolite 2, the methyl ester of 8′-methyleneoxide ABA: Fourier transform IR spectroscopy (neat) υ_{max} cm^{-1}: 3447 (O—H), 1717 (C═O, ester), 1654 (C═O, enone), electron impact HRMS: $[M+1]^+$ at m/z 307.1560 ($C_{17}H_{23}O_5$ requires 307.1545); ^1H NMR: δ 7.87 (*d*, *J* = 16.1 Hz, 1 H-4), 6.01 (*d*, *J* = 16.1 Hz, 1 H-5), 5.99 (*d*, *J* = 1.2 Hz, 1 H-5), 5.76 (*s*, 1 H-2), 3.69 (*s*, 3 H, CO_2CH_3), 3.23 (*dd*, *J* = 4.0, 3.1 Hz, 1 H-8′), 2.77 (*s*, 1 H, OH), 2.74 (*t*, *J* = 4.1 Hz, 1 H-10′), 2.69 (*t*, *J* = 3.0 Hz, 1 H-10′), 2.34 (*s*, 2 H-5′), 1.99 (*d*, *J* = 1.0 Hz, 3 H-6/7′), and 1.94 (*d*, *J* = 1.1 Hz, 3 H-6/7′), 1.02 (*s*, 3 H-9′).

Comparison of Depletion of (+)-8-Methylene ABA, (+)-8′-Methyl ABA, and (+)-ABA from Corn Cell Culture Media

Ethanolic stock solutions of (+)-ABA, (+)-8′-methylene ABA, and (+)-8′-methyl ABA were added to flasks containing 0.2 g of corn cells and 10 mL of culture medium to obtain a final concentration of 100 μM of hormone or analog. Aliquots of the medium (100 μL) were removed at intervals and analyzed for ABA or ABA analog content by HPLC, as described previously (Balsevich et al., 1994). Each compound was inoculated into three flasks and duplicate samples were removed at the indicated time points. The corn cells were used 1 or 2 d after subculturing.

Bioassays of (+)-8′-Methylene ABA

Growth Inhibition of Corn Cells and pH Effects

Cell growth was measured by the change in fresh weight over a 4-d period, as previously described (Balsevich et al., 1994). The medium pH changes associated with addition of (+)-ABA and (+)-8′-methylene ABA were also measured as described by Balsevich et al. (1994).

Transpiration in Wheat Seedlings

The transpiration rate of 6- to 10-d-old wheat seedlings (*Triticum aestivum* L. cv Katepwa) was measured as previously described (Rose et al., 1996). Antitranspirant activity of the analogs (prepared as a 1% ethanol solution in water) was measured over a range of concentrations to determine the concentration at which the transpiration rate of the wheat seedling was reduced by 50%. The transpiration rate (μmol H_2O cm^{-2} s^{-1}) was calculated and then given as a percentage of the initial transpiration rate of the plant (typically 0.28–0.35 μmol H_2O cm^{-2} s^{-1}), corrected for the effect of the control (1% ethanol).

Cress Seed Germination

Cress seed germination inhibition studies were performed as described by Gusta et al. (1992), except that the experiments were carried out at 23°C instead of 25°C, and ethanol was used in place of acetone to dissolve the analogs. The final concentration of the ethanol in the assay solutions was <0.05%. Assays were conducted with 100 seeds in each Petri dish and performed in triplicate. The germination measurements were based on the growth of the primary root. A seed was considered germinated when the radicle was approximately the same length as the seed.

Wheat Embryo Germination

Grains of soft white wheat (*T. aestivum* L. cv Clark's Cream) were used. For bioassay, embryos with some adhering endosperm and with the pericarp attached were cut from the grains with a razor blade. Solutions of analogs were prepared by dissolving the compounds in a minimal amount of DMSO to prepare a 0.1 M solution, and then the stock solution was diluted with 10 mM Mes, pH 5.8, to 0.01, 0.1, 1, 10, and 100 μM. For each analog concentration, 6 replicate germination assays were conducted on 10 embryos each at 30°C. Embryos were incubated in Petri dishes (100× 15 mm) containing 6 mL of solution. The number of germinated embryos was counted daily for 4 d and a weighted germination index was calculated (Walker-Simmons et al., 1992).

Gene Expression in Transgenic Tobacco

GUS-specific activity in homogenates of cotyledons from homozygous tobacco seedlings containing the transgene P*cor6.6*-GUS were measured as described by H. Wang et al. (1995). The results shown are an average of duplicate samples containing cotyledons from 34 seedlings.

RESULTS AND DISCUSSION

Chemical Synthesis of (+)-8′-Methylene ABA

We report here the first synthesis of 8′-methylene ABA (Fig. 1). The full experimental details of the new synthetic

chemistry and structure proof are given in "Materials and Methods." The preparation of the compound involves six steps from commercially available starting materials, and can be carried out to produce gram quantities of racemic 8′-methylene ABA. The racemic material can readily be resolved into the two optical isomeric forms. We determined that the (+)-form of 8′-methylene ABA had the same absolute configuration as natural (+)-ABA. Compound **4** in Figure 1 shows the absolute stereochemistry for (+)-8′-methylene ABA. In the biological assays the activity of (+)-8′-methylene ABA was compared with natural (+)-ABA. In the wheat embryo growth inhibition test, in which both enantiomers of ABA were equally effective, both enantiomers of (+)-8′-methylene ABA were compared with (+)-ABA. The (+)-8′-methyl ABA homolog was prepared so that its biological activity could be compared with the previously reported 8′-methyl ABA (Todoroki et al., 1994).

Metabolism and Persistence of 8′-Methylene ABA in Suspension Culture of Corn Cells

The rates of disappearance of (+)-ABA and (+)-8′-methylene ABA from the medium were compared to provide a simple estimate of the relative rates of metabolism. As can be seen in Figure 2, 50% of the (+)-ABA had disappeared from the medium by 24 H, whereas the (+)-8′-methylene ABA was only approximately 60% consumed by 100 h. 8′-Methyl ABA is consumed more slowly than the methylene derivative. These experiments show that the 8′-methylene ABA is considerably more persistent than the natural hormone.

To assess the stability and pathway of metabolism of (+)-8′-methylene ABA, its metabolites were isolated and identified using the suspension culture in which ABA metabolism has been previously described (Balsevich et al., 1994). (+)-8′-Methylene ABA was fed to the cells and two metabolites were isolated from the culture medium and purified as their methyl esters. Mass spectral analysis showed that the metabolites each contained one O more than the methyl ester of 8′-methylene ABA. The ^{1}H NMR of the two metabolites were very similar, each having lost the signals for the three vinylic protons and each gaining three proton signals, with δ consistent with epoxide formation. The two isomeric epoxides appear to have been formed by oxidation from both faces of the double bond. It is possible that the epoxide oxidation products that cannot cyclize to PA-like molecules are also active. Further studies are under way to synthesize sufficient quantities of these epoxides to test their biological activities.

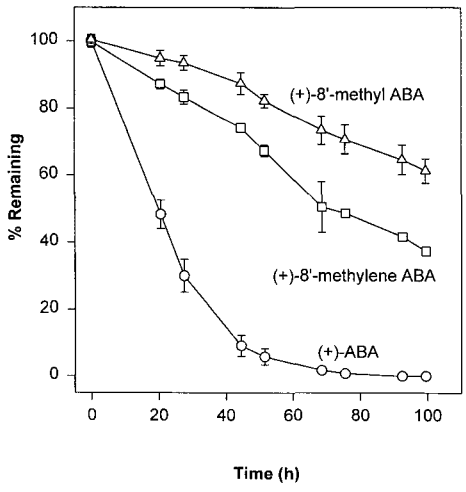

Figure 2. Time course of the persistence of (+)-ABA, (+)-8′-methylene ABA, and (+)-8′-methyl ABA in the medium of suspension cultures of corn cells. Each datum point represents the mean (± SD) of six concentration determinations (three replicate cultures, concentration determinations performed in duplicate for each culture). Average values at each concentration were normalized to a control (untreated) value of 100%.

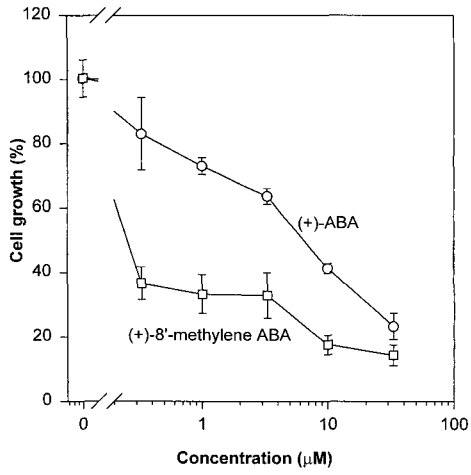

Figure 3. Effect of (+)-ABA and (+)-8-methylene ABA on corn cell growth. The effect of these compounds was determined at each concentration by calculating the percentage increase in fresh weight after 4 d. Measurements at each concentration were performed in triplicate and the average values (± SD) were normalized to a control (untreated) value of 100%.

Biological Activity of (+)-8′-Methylene ABA in BMS Suspension Culture of Corn Cells

Suspension-cultured corn cells are a well-characterized experimental system that has been useful for comparing the biological activity and metabolism of ABA and ABA analogs (Balsevich et al., 1994; Rose et al., 1996). (+)-ABA inhibits the growth of suspension-cultured corn cells over a 4-d period (Balsevich et al., 1994). In this study, (+)-8′-methylene ABA exhibited stronger growth inhibition activity than (+)-ABA at all tested concentrations (Fig. 3). The increased potency of the derivative is more marked at low concentrations. At 0.33 μM, (+)-ABA inhibited growth by 17% relative to the control, whereas (+)-8′-methylene ABA produced a 64% reduction. The inhibition caused by 8′-methyl ABA was comparable to that produced by (+)-ABA (Fig. 4). The (+)-8′-methylene ABA is significantly stronger than either (+)-ABA or (+)-8′-methyl ABA. The enhanced activity must be due to more than the simple steric bulk of the extra C atom. Because the 8′-methylene ABA is depleted from the culture medium more rapidly than the 8′-methyl analog, 8′-methylene ABA must have some additional properties, per-

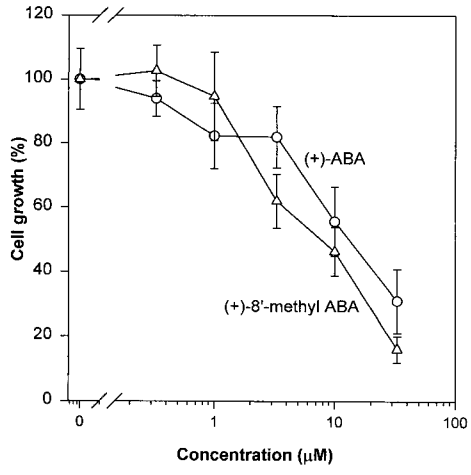

Figure 4. Effect of (+)-ABA and (+)-8-methyl ABA on corn cell growth. The effect of these compounds was determined at each concentration by calculating the percentage increase in fresh weight after 4 d. Measurements at each concentration were performed in triplicate and the average values (± SD) were normalized to a control (untreated) value of 100%.

haps higher affinity for the receptor. One cannot discount the possibility that the epoxides may possess biological activity. This question is currently being pursued.

Natural ABA causes a transient elevation of pH of the corn cell culture medium, reaching a maximum at about 6 h after ABA addition (Balsevich et al., 1994). Figure 5 shows the effect of a 10 μM solution of (+)-8′-methylene ABA, compared with the same concentration of natural ABA, on the pH of the medium of suspension-cultured corn cells. Although the significance of the pH change is not clear, it seems to be an early manifestation of ABA action, which is not produced by PA and is only weakly produced by the unnatural ABA (−)-isomer (Balsevich et al., 1994). The utility of this assay is that it is a relatively rapid test of the inherent hormonal activity of added compounds. The 8′-methylene compound causes a shift in pH comparable to that produced by ABA, suggesting that it acts in a similar way.

Biological Activity of 8′-Methylene ABA in Germination Inhibition

Inhibition of Germination of Embryos from Dormant Wheat Grains

Embryos isolated from dormant cereal grains are highly responsive to exogenous ABA as a germination inhibitor and dormancy can be restored by the application of ABA (Walker-Simmons, 1987; M. Wang et al., 1995). Both natural (+)-ABA and its enantiomer (−)-ABA are equally effective in inhibiting germination when supplied to excised embryos of wheat (Walker-Simmons et al., 1992). As shown in Figure 6, (+)-8′-methylene ABA is at least 10 times more effective than natural ABA, with 1.0 μM (+)-8′-methylene ABA giving equivalent inhibition results to 10 μM (+)-ABA. Although we have not measured the metabolism of the analog in the embryos, the increased activity over ABA is likely due in part to greater persistence. These results support the conclusion (M. Wang et al., 1995) that maintaining ABA levels in hydrated dormant grain embryos contributes to their growth arrest, and indicate that long-lasting ABA analogs such as 8′-methylene ABA will be useful tools to examine the role of ABA in both induction and maintenance of seed dormancy.

Germination inhibitory activity was assessed for other 8′-ABA analogs, including (−)-8′-methylene ABA (the mirror image of (+)-8′ methylene ABA) and (+)-8′-methyl ABA (Fig. 7). The (−)-8′ methylene ABA is similar in potency to (+)-ABA. The (+)-8′-methyl ABA and (+)-8′-methylene ABA are both more active than (+)-ABA. The most potent compound over all concentrations and time points is (+)-8′-methylene ABA.

Cress Seed Germination

Figure 8 shows the effect of two concentrations of (+)-8′-methylene ABA and natural ABA on germination of

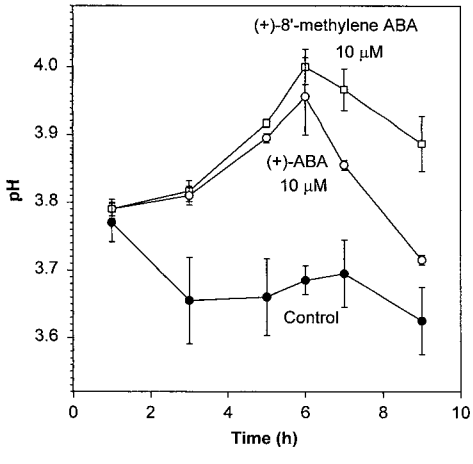

Figure 5. Changes in the culture medium pH of suspension cultures of corn cells supplied with 10 μM (+)-ABA and 10 μM 8′-methylene ABA. Each datum point represents the mean (± SD) of five measurements.

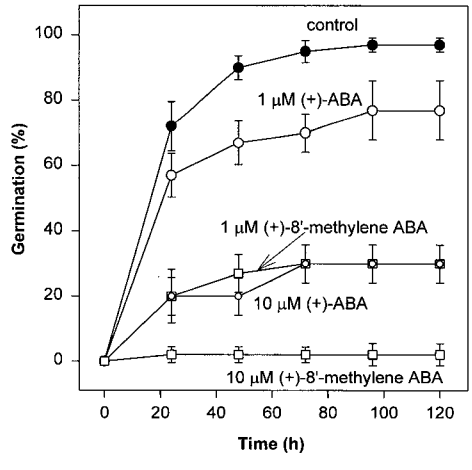

Figure 6. Biological activity of (+)-ABA and (+)-8′-methylene ABA as germination inhibitors of excised wheat embryos. The percentage of germinating embryos is reported as the mean ± SE of 6 replicate germination assays conducted on 10 embryos each.

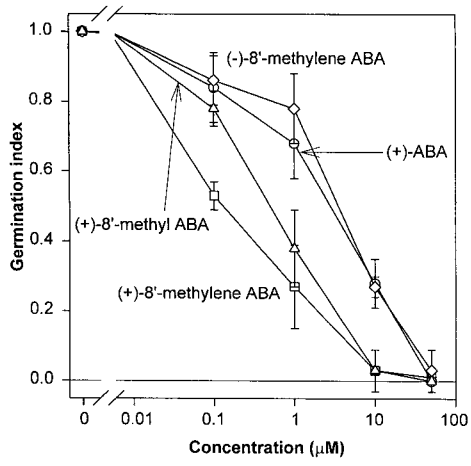

Figure 7. Biological activity of (+)-ABA, (+)-8′-methylene ABA, (−)-8′-methylene ABA, and (+)-8′-methyl ABA as germination inhibitors of excised wheat embryos. Effects of concentration on the embryonic germination index are shown and data are the mean ± SE of six replicates.

cress seed. At 1 μM the 8′-methylene analog is more potent than ABA, and at 5 μM the seeds supplied with ABA begin to germinate, whereas analog-imbibed seeds remain dormant. In summary, (+)-8′-methylene ABA is a more effective germination inhibitor in seeds of both monocots and dicots. It is anticipated that this analog will be more active than ABA in seed germination inhibition for species in which the hydroxylation of ABA to 8′-hydroxy ABA is the major pathway of degradation.

Biological Activity of 8′-Methylene ABA in Transpiration in Wheat Seedlings

ABA is a key signal molecule involved in regulating transpiration in plants (Davies and Mansfield, 1988). We previously determined that esters were as active as acids in this assay (data not shown), so the effect of the methyl esters of natural ABA, (+)-8′-methylene ABA, and 8′-methyl ABA on transpiration in intact wheat seedlings was compared 3 h after application. The most active analog is the 8′-methylene compound, which requires a concentration between 5 and 10 μM to reduce the transpiration rate of the wheat seedlings by 50%, with the ABA ester causing 50% inhibition at concentrations between 25 and 50 μM. The saturated 8′-side chain analog is relatively weak in this assay, with the 50% effective dose being 100 μM. This last result is very different from that reported by Nakano et al. (1995), who showed that the saturated 8′-methyl analog is only slightly weaker than ABA in a stomatal opening assay. The difference in responses may be due to reduced uptake of the 8′-methyl ABA in the intact wheat seedling.

Biological Activity of 8′-Methylene ABA in ABA-Inducible Gene Expression

Figures 9 and 10 show the induction of ABA-responsive gene expression in transgenic tobacco seedlings that were imbibed with (+)-ABA, (+)-8′-methylene ABA, or (+)-8-methyl ABA for 24 h. The transgenic seedlings contained the ABA-responsive *cor6.6* promoter from *Arabidopsis thaliana* (H. Wang et al., 1995) fused to the protein coding sequence of GUS. The ABA inducibility and other properties of transgenic plants containing this construct have been described (H. Wang et al., 1995; Wang and Cutler, 1995). Although at 100 μM (+)-8′-methylene ABA was a slightly weaker inducer than ABA, the analog was highly effective in inducing ABA-responsive gene expression. 8′-Methyl ABA was about 3-fold weaker than ABA (Fig. 10).

CONCLUSIONS

8′-Methylene ABA is strongly active in all plant ABA responses investigated in this study. Based on these experiments with corn cells, it seems that the greater metabolic stability of 8′-methylene ABA is likely to be a significant

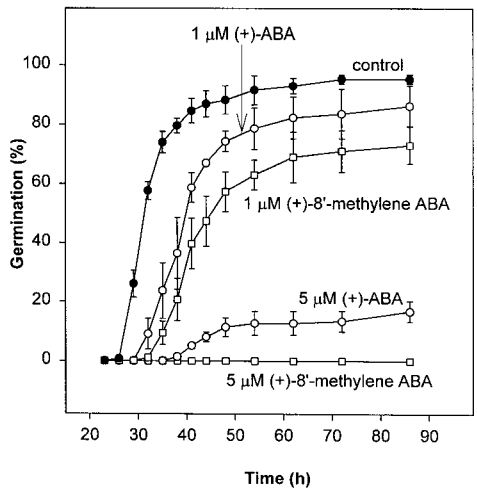

Figure 8. Biological activity of (+)-ABA and (+)-8′-methylene ABA as germination inhibitors of cress seed. The percentage of germinating embryos is reported as the mean ± SD of 3 replicate germination assays conducted on 100 seeds each.

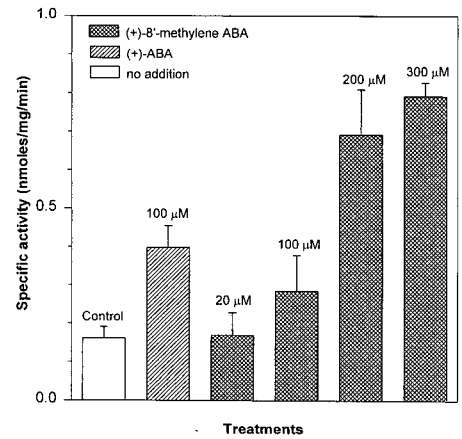

Figure 9. The effect of (+)-ABA and (+)-8′-methylene ABA on GUS-specific activity in homogenates of cotyledons from homozygous tobacco seedlings containing the transgene P*cor6.6*-GUS. The results shown are the average of duplicate samples containing cotyledons from 34 seedlings ± SD.

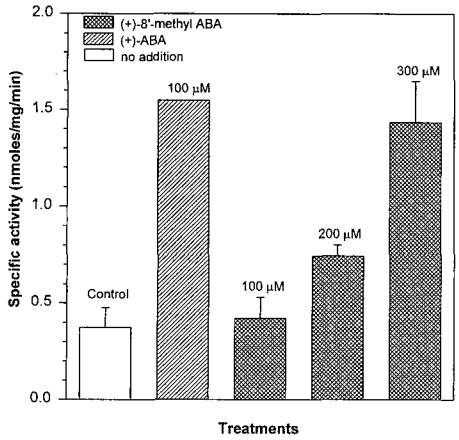

Figure 10. The effect of (+)-ABA and (+)-8′-methyl ABA on GUS specific activity in homogenates of cotyledons from homozygous tobacco seedlings containing the transgene P*cor6.6*-GUS. The results shown are the average of duplicate samples containing cotyledons from 34 seedlings ± SD.

factor in its high hormonal activity. We anticipate that this factor would be proportionately more important in longer-term biological assays. Indeed, the 8′ derivative is much more potent than ABA in the 4-d growth inhibition assay, in both germination assays and in transpiration reduction. In the short-term pH assays in corn both (+)-ABA and (+)-8′-methylene ABA gave comparable results. Only in the 1-d gene induction assay in tobacco (Fig. 9) is the 8′ methylene ABA slightly weaker than ABA. Therefore, this and other 8′ derivatives show promise for applications in which metabolic persistence is crucial, especially in systems in which 8′ hydroxylation is the most important first step in ABA metabolism. However, it should be noted that in some plants the predominant mode of hormone inactivation is by direct conjugation of ABA (Loveys and Milborrow, 1984). The susceptibility of 8′-methylene ABA to conjugation is not yet known.

The basis for the strong agonist activity of 8′-methylene ABA relative to both ABA and 8′-methyl ABA, especially in suspension-cultured corn cells, is unclear at present. The rate of metabolism is clearly slower than that of ABA (Fig. 2), and the results of the short-term pH assay suggest that ABA and 8′-methylene ABA have similar inherent hormonal activities. The finding that the rate of metabolism of 8′-methylene ABA is not slower than that of 8′-methyl ABA suggests that mechanism-based inhibition of the ABA 8′-hydroxylase is not the only factor. Further studies on the inhibitory properties of 8′-methylene ABA are in progress.

The utilization of auxin analogs in agriculture depended upon the production of compounds with high hormonal activity and improved stability (Moore, 1979). We suggest that similar developments for ABA analogs require a practical, efficient synthesis of analogs altered at the 8′-C atom, as reported here. The 8′-methylene analog of ABA is readily prepared and has activity that is stronger or comparable to ABA in a range of biological assays. This discovery of an ABA analog that is more persistent and more effective than ABA provides the means to maintain ABA responses ranging from the gene expression level to physiological processes including seed dormancy and stress tolerance.

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