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Synthesis and Biological Activity of 4′-Methoxy Derivatives of Abscisic Acid

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Abstract—Replacing the 4′-carbonyl group of abscisic acid with a methoxy group does not affect the abscisic acid (ABA)-like activities of the product in barley aleurone protoplasts, although the reduction of ABA to 4′-hydroxyl derivatives significantly reduces the ABA-like activity of the products. This suggests that methoxy derivatives of abscisic acid might be used to produce probes for ABA binding proteins. © 2000 Elsevier Science Ltd. All rights reserved.

Abscisic acid (ABA) is involved in the control of many processes in plants, including the acceleration of abscission, induction of dormancy, inhibition of rooting, and stimulation of stomatal closure.[1] In addition, ABA has attracted considerable attention because it is thought to play an important role in the response to environmental stresses. In spite of understanding the roles of ABA in plant physiology, there is no information about the actions of ABA on its receptors. To develop probes for ABA-binding proteins, as well as to develop plant growth regulators targeted towards specific biological activities, it is important to identify regions of the ABA molecule that can be modified without affecting its biological activity.

To determine the structural features of the ABA molecule that are needed for initiating ABA responses in many specific physiologic processes, the modification and derivatization of ABA molecules and biological assays have been carried out since the discovery of ABA.[2] In a review, Milborrow[3] concluded that almost any change of the ABA molecule destroys or attenuates its activity in stomatal bioassays, including replacement of the 4′ carbonyl with a group that is not rapidly metabolized. However, Hornberg and Weiler[4] reported later that 0.1 μM ABA-4′-tyrosylhydrazone had ABA-like activity in a stomatal bioassay. A fluorescence-labeled abscisic acid (**1**), in which a fluorescent functional group was tagged to ABA-4′-aminophenylcarbohydrazone, showed ABA-like activity in a gibberellin-induced α-amylase synthesis test in barley aleurone protoplasts,[5] which suggests that ABA should retain its activity with the substitution of other functional groups at its 4′-position.

Rose et al. synthesized ABA analogues with a methyl ether at C-1′ for probing the role of the hydroxyl group of ABA.[6] (+)-*C*-1′-*O*-Methyl-ABA (**2**) is rapidly metabolized by suspension-cultured maize cells to (+)-*C*-1′-*O*-methyl-phaseic acid (**3**) in a process seen as analogous to ABA.[7] The presence of a methyl ether on the 1′-position of ABA does not interfere with enzymatic oxidation at the 8′-carbon. Although both enantiomers were less active than ABA in a wheat embryo germination inhibition assay, in maize cell growth inhibition, (+)-*C*-1′-*O*-methyl ABA exhibited stronger activity than (+)-ABA, suggesting that a free C-1′-hydroxyl group is not essential for the biological activity of ABA in maize (*Zea mays*). Todoroki et al. reported the potent activity of 8′- and 9′-methoxyabscisic acids (**4** and **5**, respectively) as anti-metabolic analogues of abscisic acid.[8] These results suggest that if a methoxy group is properly introduced into the ABA molecule, derivatives likely keep the intrinsic activity of ABA. In addition, if methoxy derivatives of abscisic acid possess ABA-like activity, we might synthesize a molecular probe for ABA binding protein by replacing the methoxy group with a functionalized alkoxy group.

In this context, we substituted a methoxy group for the 4′ carbonyl of ABA to develop new compounds to use as molecular probes to investigate ABA-binding proteins in barley aleurone cells.

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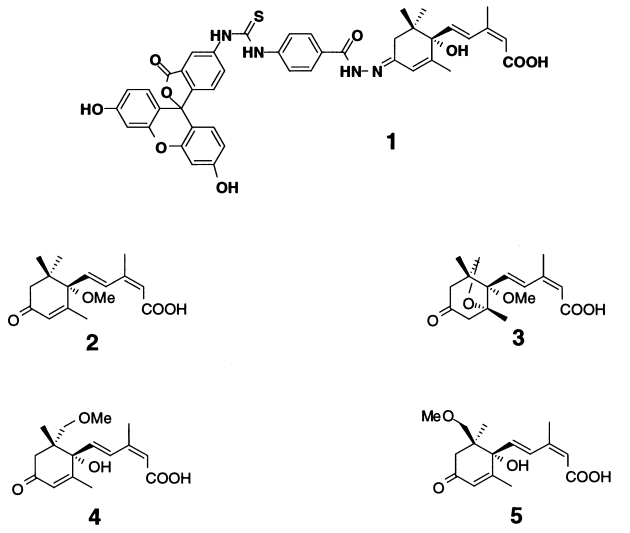


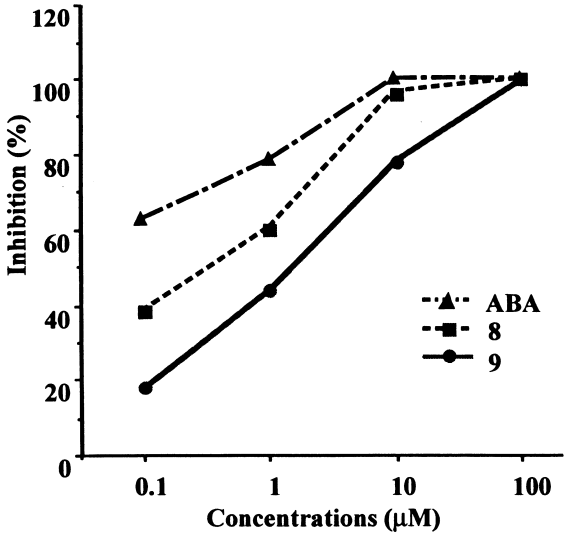


Figure 1. Inhibition of cress germination by ABA and ABA derivatives.

Synthesis

(*S*)-(+)-ABA, a kind gift from Toray Co. Ltd. Tokyo, was modified to methyl abscisate by diazomethane treatment and reduced successively with $NaBH_4$ to give 4′-hydroxy derivatives of ABA, **6** and **7**, respectively. The epimers of the diols were easily separated by silica gel column chromatography. Both diols were then methylated with methyl iodide after being deprotonated with 1.1 equivalents of NaH in DMF. Finally, hydrolysis of the methyl esters with KOH–EtOH gave the corresponding target compounds, **8** and **9**, respectively (Scheme 1). The structures of **6** and **7** were elucidated according to the report by Hirai et al.[9,10]

Activities

One of the typical activities of ABA is the inhibition of seed germination.[11] The inhibition of GA_3-induced α-amylase induction and induction of the accumulation of dehydrin in unstressed barley seeds are also well-demonstrated effects of ABA that have been used to determine whether chemicals have ABA-like activity.[12,13] Therefore, the activities of chemicals were evaluated using cress seeds and barley aleurone protoplasts, as reported by Asami et al.[14] The results of the bioassays are shown in Figures 1–3. In the cress germination test (Fig. 1), 4′-methoxy derivative **8** shows inhibitory activity at the same level as ABA, whereas 4′-methoxy derivative **9**, in which the methoxy group has a different configuration from **8**, is less active than **8**. In the α-amylase induction test using barley aleurone protoplasts (Fig. 2), **8** and ABA possess similar potencies, but **9** is not as active as **8** and ABA. The effects of **8**, **9**, and ABA on the induction of dehydrin gene expression were also tested in aleurone protoplasts by measuring dehydrin promoter activity in transient assays (Fig. 3).[20] (±)-ABA (10 μM) increased

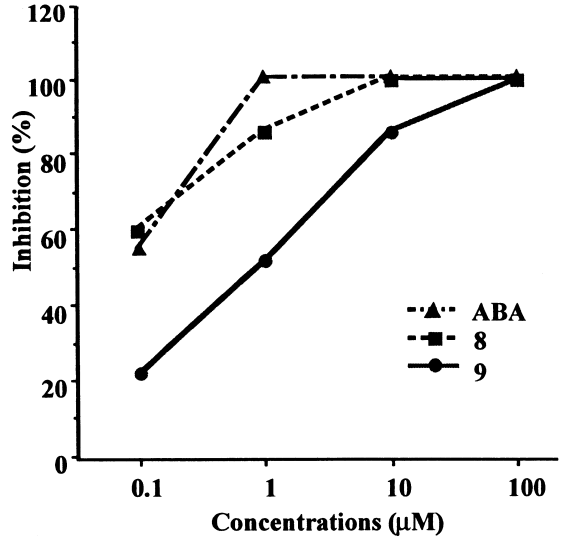
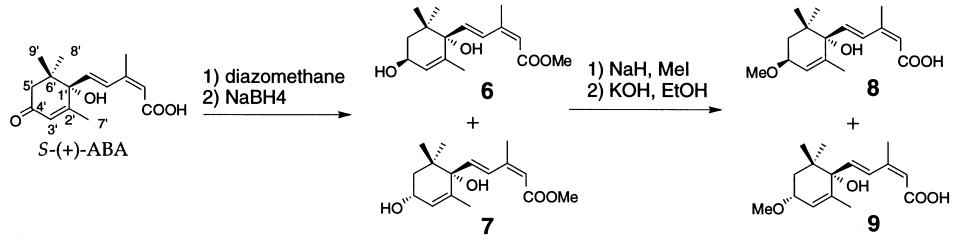


Figure 2. Inhibition of GA-inducible α-amylase induction by ABA and ABA derivatives in barley aleurone protoplasts.



Scheme 1.

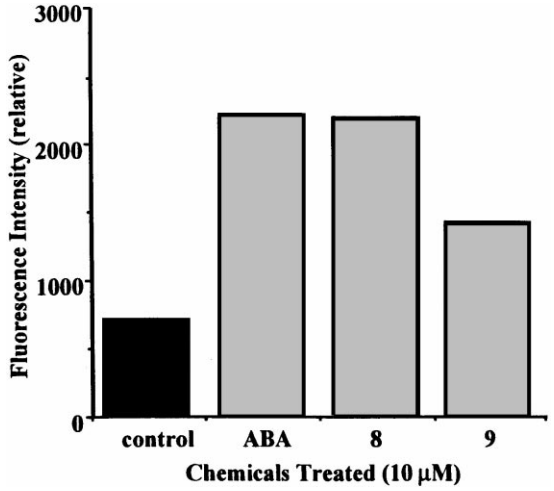


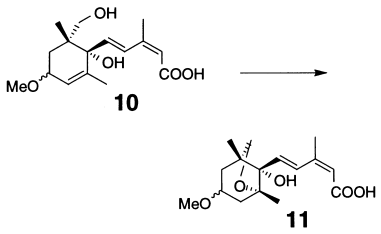
Figure 3. Dehydrin induction activity of ABA and ABA derivatives in barley aleurone protoplasts.

GUS activity about 3-fold over control levels. **8** and **9** also increased the GUS activity significantly, but were less active than (±)-ABA. The order of effectiveness of these compounds is the same as that in the α-amylase induction inhibition bioassay: (±)-ABA >**8** >**9**.

Discussion

4′-Methoxy ABA derivatives (**8** and **9**) showed ABA-like activity in inhibiting cress germination and GA-inducible α-amylase. However, these results alone are not sufficient to demonstrate that **8** and **9** have the same mechanism of action as ABA and interact with an ABA receptor. **8** and **9** may be general inhibitors of metabolic processes. Some phenyl compounds structurally mimicking ABA are reported to inhibit plant growth, transpiration, or GA-induced α-amylase synthesis, but they do not mimic ABA in all the assays for ABA.[15–17] Alternatively, **8** and **9** may inhibit steps in signal transduction between GA perception and the expression of α-amylase gene by a mechanism that differs from that of ABA. For example, okadaic acid, a protein phosphatase inhibitor, blocked GA-inducible α-amylase production and greatly reduced the accumulation of α-amylase mRNA, but did not lead to the accumulation of ABA-inducible products.[18] Therefore, to determine whether **8** and **9** mimic ABA, we examined the activity of **8** and **9** on ABA up-regulated expression of the dehydrin gene. The promoter activity indicated that **8** and **9** have dehydrin-induction activity, and the combined results show that **8** and **9** act as ABA agonists, not as so-called general toxins. The ABA-like activity of **8** and **9** suggests that the analogue may have the same mechanism of action as ABA and may be recognized as active ABA in in vitro assay systems.

We have demonstrated that ABA analogues of **8** and **9**, which have a methoxy group instead of a carbonyl group at the 4′-position of ABA, have ABA-like activity in various biological responses. This result suggests that modification of the 4′-position of ABA with an alkoxy group might be a way to design probes for ABA binding proteins. Furthermore, it is possible that they may tolerate catabolic inactivation to phaseic acid-type compounds (**11**), since **8** and **9** have a double bond in their ring systems that is not conjugated with the carbonyl group and makes it difficult to cyclize the hydroxylated intermediates (**10**). This may make them lead compounds as plant growth regulators because inactivation of ABA to phaseic acid is suggested as one of the reasons why ABA is not used in the field.[19]



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10. ^{1}H NMR (300 MHz, $CDCl_3$, δ): 0.91 (3H, s), 1.05 (3H, s), 1.68 (1H, dd, J = 13.4 and 9.8 Hz), 1.68 (3H, s), 1.80 (1H, dd, m), 2.02 (3H, d, J = 1.1 Hz), 3.39 (3H, s), 3.92 (1H, m), 5.66 (1H, s), 5.73 (1H, s), 6.21(1H, d, J = 16.2 Hz), 7.75 (1H, d, J = 16.2 Hz). **9**: ^{1}H NMR (300 MHz, $CDCl_3$, δ): 0.95 (3H, s), 1.04 (3H, s), 1.71 (3H, brs), 1.72 (1H, dd, J = 13.7 and 7.0 Hz),1.74 (1H, brs), 2.02 (3H, d, J = 1.1 Hz), 3.39 (3H, s), 3.80 (1H, m), 5.73 (1H, s), 5.74 (1H, m), 6.05 (1H, d, J = 16.2 Hz), 7.78 (1H, d, J = 16.2 Hz).
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20. Dehydrin promoter assay was conducted according to the procedure below. Protoplasts were resuspended in 1 mL of incubation medium (IM) consisting of 0.5 M mannitol, 0.11 M glucose, 0.055 M sucrose, 14 mM L-arginine, 10 mM MES, and 0.32% B5-Gamborg salt, pH 5.5. They were mixed gently

with 100 μg sheared salmon sperm DNA and 100 μg of Hv41 (-935)-IGN plasmid DNA, [21] and the suspension was left undisturbed for 1 min Protoplast transfection medium (PTM) consisting of 17.3% (w/v) polyethylene glycol 6000 (PEG), 10 mM Tris–HCl pH 9.0, 0.67 M mannitol, and 0.133 M $Ca(NO_3)_2$ was filter sterilized. Three volumes of PTM were added to the protoplasts, mixed, and left at room temperature for 20 min with occasional swirling. Then 40 mL of IM was added in 10 mL aliquots with 2 min between additions. The protoplasts were collected by centrifugation for 2 min at 50 *g* and washed twice in 30 mL of IM. Pelleted protoplasts were resuspended in an appropriate volume (generally 15 mL for protoplasts isolated from 150 grains) of IM containing 50 unit/mL nystatin, 150 μg/mL cefotaxime, 20 mM $CaCl_2$, 1.5 μg/mL aprotinin and 1.5 μg/mL leupeptin. One milliliter of the protoplasts was aliquoted into flasks, and samples were incubated in the dark at 25 °C for 24 h in the presence of 0, 10 μM (±) ABA, 10 μM **8**, or **9** in triplicate for each treatment. Fluorometric assays of GUS activity were performed in triplicate for each sample.

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