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# 3′-Azidoabscisic Acid as a Photoaffinity Reagent for Abscisic Acid Binding Proteins

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**Abstract**—3′-Azidoabscisic acid was synthesized as a potential photoaffinity reagent for abscisic acid binding proteins. This compound was stable in organic and aqueous solutions in the dark, but was decomposed by UV irradiation. Its biological activity was equivalent to that of abscisic acid, suggesting that it may be an effective photoaffinity reagent. 

Abscisic acid [(1′*S*)-(+)-ABA (**1**)] is the primary hormone that induces adaptive reactions to protect plants from environmental stresses such as desiccation and freezing.[1–4] A thorough understanding of the mechanism of ABA action is important not only as a point of academic interest, but also to promote the agricultural application of ABA. The initial perception and metabolic inactivation of ABA by target cells remain obscure at the molecular level. ABA receptors are believed to be membrane proteins,[5,6] and the catabolic enzyme that catalyzes the first step of ABA catabolism is likely to be the microsomal cytochrome P-450 monooxygenase.[7] However, the corresponding proteins and genes have not yet been isolated.

A powerful technique for identifying hormone-binding proteins is photoaffinity labeling. ABA can naturally form covalent bonds under UV irradiation, since ABA contains a UV-sensitive α,β-unsaturated carbonyl group, an enone structure, in its six-membered ring. Indeed, Hornberg and Weiler used this characteristic of ABA in their study.[8] More recently, Cornelussen et al. tried to optimize the conditions for UV-induced cross-linking of ABA in a model experiment.[9] ABA analogues with a more photosensitive functional group, azide, have also been synthesized.[10] In 1993, Willows and Milborrow synthesized 1-azido-ABA (**2**), which was 10% as effective as ABA in stomatal closure and *Lemna gibba* growth assays.[10] Photolysis of tritiated **2** in a solution of bovine serum albumin (BSA) gave labeled BSA. However, compound **2** was so unstable and rapidly broken down that it may not be useful as a photoaffinity label for the ABA receptor. The 4′-carbonyl group is a good site for tethering functional groups for photoaffinity, since most ABA derivatives modified at C-4′ exhibit moderate biological activities. Kohler et al. synthesized [$^{125}$I]-labeled ABA that tethered an aromatic hydrazide at C-4′ (**3**) as the first radioiodinated ABA photoaffinity probe.[11] This compound was about one-tenth as active as ABA in the inhibition of GA-induced α-amylase. An anthracenone ABA analogue (**4**) was reported by Irvine et al.[12] This analogue was designed based on the fact that benzophenone-containing substrate analogues are advantageous with regard to chemical stability, a long activation wavelength (350–360 nm), and many excitation–relaxation cycles. The activity of **4** was one-third that of ABA in the inhibition of corn cell growth. There is some doubt whether these analogues function as a specific photoaffinity reagents for the ABA receptor because these modifications of the parent compound involved the loss of a significant group for bioactivity, alteration of the ABA skeleton, or the addition of a large substituent which may interfere with ABA–receptor interactions, although the investigation of ABA-binding proteins using these probes has not yet been reported.

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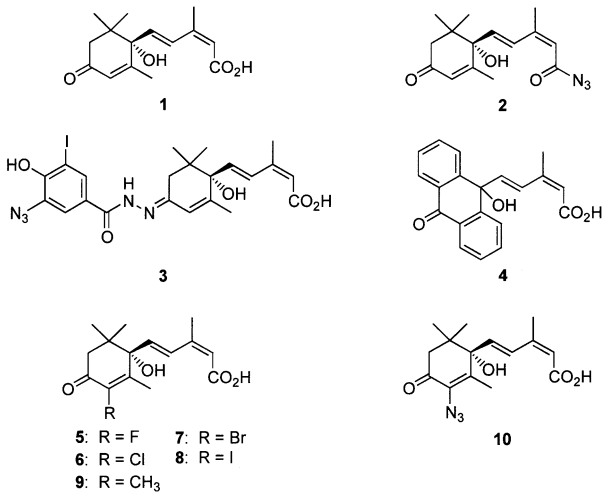


The 3′-position of ABA can also be modified while maintaining bioactivity. In our previous paper,[13] we reported that the bioactivities of 3′-halogenated ABA (**5**–**8**) and 3′-methyl-ABA (**9**) were slightly stronger than that of ABA. Introducing a hydrophobic substituent at C-3′ may cause a hydrophobic interaction to reinforce binding with the ABA receptor. This means that activation of a photolabile group at C-3′ may result in effectively labeling the ABA-binding proteins. We selected an azide as the photolabile group because of its size and easy introduction at C-3′. By introducing it at C-3′, we can build an aryl azide, which is a popular and useful group for photoaffinity labeling studies.[14–16] Its relatively small size would result in high affinity and specificity for ABA-binding proteins. In this paper, we describe the synthesis and biological activity of 3′-azido-ABA (**10**). We also describe the stability and photolysis of **10** in various solutions to estimate its potential as a photoaffinity reagent.

## Synthesis

Racemic ABA, purchased from Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan, was converted to the methyl ester of 2′α,3′α-deoxy-2′α,3′α-epoxy-ABA (**11**) in two steps as reported elsewhere (Fig. 1).[17] The epoxide **11** was treated with 2 equivalents of $NaN_3$ in DMF in the presence of $NH_4Cl$ at 80 °C to give the methyl ester of 3′-azido-ABA (**10a**) in 33% yield. The racemate **10a** was optically resolved by HPLC with a chiral column, Chiralcel OD (250×4.6 mm, Daicel; solvent, 5% *i*-PrOH in hexane; flow rate, 1 mL $min^{-1}$; detection, 254 nm) to give (+)- and (−)-**10a**. Each enantiomer was hydrolyzed with porcine liver esterase (EC 3.1.1.1, Sigma E3019) in MeOH (3 mL) and potassium phosphate buffer (0.1 M, pH 8.0, 16 mL). The obtained free acids (+)- and (−)-**10** were purified by chromatography on silica gel with toluene–EtOAc (7:3) containing 5% AcOH. These enantiomers were identified by spectral data.[18] Since the Cotton effects in the CD spectra of (+)- and (−)-**10** were similar to those of (1′*S*)-(+)- and (1′*R*)-(−)-**1**,[19] respectively, the absolute configuration at C-1′ was determined to be *S* for (+)-**10** and *R* for (−)-**10**.

## Biological Activity

The biological activities of optically active **1** and **10** were evaluated in the two bioassays (Fig. 2).[20] In the inhibition of lettuce seed germination, the activity of (+)-**10** was equivalent to that of (+)-**1**, whereas (−)-**10** was not effective at the concentrations tested. In the inhibition of rice seedling elongation, (+)-**10** showed 1/3 the activity of (+)-**1**, whereas (−)-**10** showed no activity. The high bioactivities of (+)-**10** mean that (+)-**10** may be an effective photoaffinity reagent. The ABA receptors should interact with the azide (+)-**10** in a manner similar to (+)-**1**. On the other hand, the lack of an effect of (−)-**10** in the bioassays can be explained by destruction of the pseudo-symmetrical structure of ABA. The high bioactivity of unnatural (−)-**1** has been observed in most bioassays.[1–4] ABA receptors can recognize unnatural (−)-**1** to induce ABA-activity, probably due to its pseudo-symmetrical structure,[21] which is derived from the pseudo-symmetrical cyclohexenone ring that has a chiral center at C-1′ and methyl groups at both C-2′ and C-6′. Therefore, the introduction of a substituent on the ring of (−)-**1** tends to eliminate the pseudo-symmetry and thus the bioactivity. Indeed, most of the unnatural enantiomers of ring-modified analogues are biologically inactive.[13,22–27]

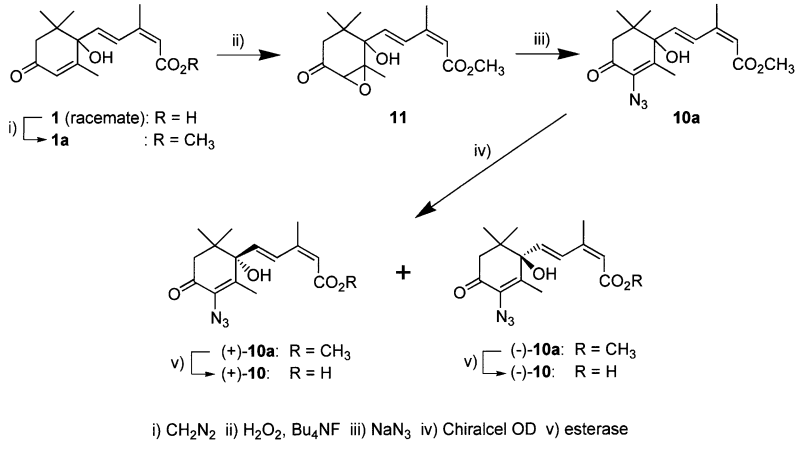


**Figure 1.** Synthesis and optical resolution of **10**.

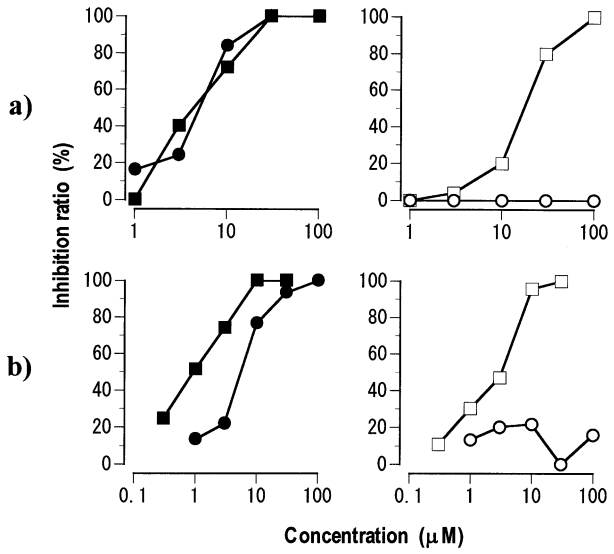


**Figure 2.** Inhibitory activities of optically active **1** and **10** in (a) lettuce seed germination and (b) rice seedling elongation. ■, (+)-**1**; ●, (+)-**10**; □, (−)-**1**; ○, (−)-**10**.

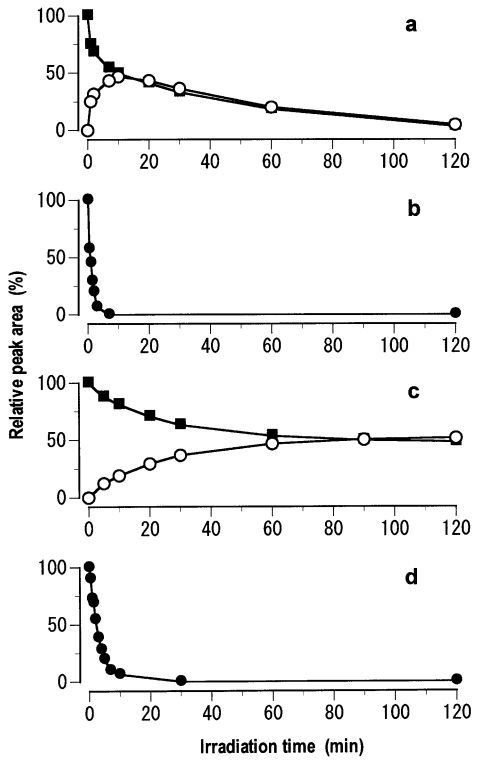


**Figure 3.** Photolysis of (+)-**1** and (+)-**10** by UV irradiation. The samples were analyzed by HPLC. a: ■ (+)-**1**, ○ (+)-2*E*-**1**, 254 nm; b: ● (+)-**10**, 254 nm; c: ■ (+)-**1**, ○ (+)-2*E*-**1**, 365 nm; d: ● (+)-**10**, 365 nm.

## Stability and Photolysis

The stability and photolysis of (+)-**10** were tested in MeOH and Tris–Mes (10 mM, pH 6.5)[28] and citrate–KOH (10 mM, pH 4) buffers, which are often used in photoaffinity labeling for a plasma membrane fraction.[29,30] Compound (+)-**10** was as stable as (+)-**1** in these three solvents in the dark at ambient temperature, and was relatively stable even under the fluorescent lamps (~500 lux) in the laboratory, and so was easily handled. UV irradiation at 254 and 365 nm was performed using compact UV lamps.[31] Sample solutions (0.15 mg per 1.5 mL solution) were prepared in quartz cells, and 10-μL aliquots were analyzed by HPLC using an ODS column (solvent: 50% MeOH in 0.1% aqueous AcOH at 1.0 mL $min^{-1}$; detection: 254 nm) at appropriate intervals after UV irradiation. Since the photolysis profiles of (+)-**1** and (+)-**10** were scarcely influenced by changing the solvent, those in Tris–Mes buffer are shown in Figure 3. A 10-min irradiation at 254 nm converted (+)-**1** to an equilibrium mixture of 2*Z*- and 2*E*-isomers (ca. 1:1). Decomposition of both isomers was observed upon further irradiation. Irradiation at 365 nm caused only *Z*/*E* isomerization, which proceeded more slowly than with irradiation at 254 nm. These results were similar to those reported by Cornelussen et al.[9] Compound (+)-**10** was completely decomposed to many compounds by 10 min of irradiation at both 254 and 365 nm. Most of the degradation products were more polar than the initial compound. The time-course of UV absorption for the photolysis (365 nm) of (+)-**10** is shown in Figure 4. Absorption maxima of (+)-**10** before irradiation were observed at 250 and 280 nm; the former is due to the dienoic acid side-chain, and the latter is due to the enone moiety with an azide group in the ring. The decrease in absorption after irradiation at 280 nm was greater than that at 250 nm, suggesting decomposition of the 3′-azide. This was supported by the fact that the absorption of an azide in the IR spectrum disappeared after irradiation. The present results suggest that (+)-**10** is a potential photoaffinity reagent for ABA-binding proteins.

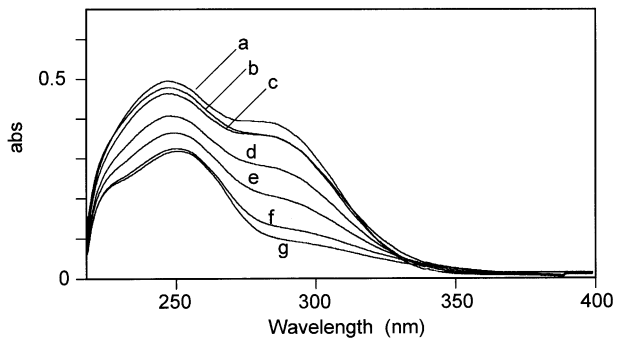


**Figure 4.** The time-course of UV absorption for the photolysis (365 nm) of (+)-**10** in Tris–Mes buffer at (a) 0 s, (b) 20 s, (c) 40 s, (d) 80 s, (e) 160 s, (f) 320 s and (g) 640 s. The initial concentration of (+)-**10** was 328 μM (100 μg $mL^{-1}$).

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18. (+)-**10**. $^1$H NMR (270 MHz, $CD_3OD$): δ 1.01 (3H, s, $H_3$-9′), 1.05 (3H, s, $H_3$-8′), 1.84 (3H, s, $H_3$-7′), 1.99 (3H, d, $J$ = 1.0 Hz, $H_3$-6), 2.30 (1H, d, $J$ = 16.5 Hz, H-5′), 2.61 (1H, d, $J$ = 16.5 Hz, H-5′), 5.77 (1H, s, H-2), 6.12 (1H, d, $J$ = 16.0 Hz, H-5), 7.67 (1H, d, $J$ = 16.0 Hz, H-4); IR $\nu_{max}$ ($CHCl_3$) cm$^{-1}$: 2122 ($N_3$); FAB-MS $m/z$ (rel. int.): 306 $[M+1]^+$ (5); UV $\lambda_{max}$ (MeOH) nm (ε): 251.6 (15,000), 281.4 (12,700); $[\alpha]_D^{25}$ +447.2° (MeOH; $c$ 0.197); CD $\lambda_{ext}$ (MeOH) nm (θ): 239 (−27,786), 284 (+93,953). (−)-**10**. $^1$H NMR (270 MHz, $CD_3OD$): δ 1.01 (3H, s, $H_3$-9′), 1.05 (3H, s, $H_3$-8′), 1.84 (3H, s, $H_3$-7′), 1.96 (3H, s, $H_3$-6), 2.29 (1H, d, $J$ = 16.5 Hz, H-5′), 2.62 (1H, d, $J$ = 16.5 Hz, H-5′), 5.79 (1H, s, H-2), 6.02 (1H, d, $J$ = 16.2 Hz, H-5), 7.58 (1H, d, $J$ = 16.2 Hz, H-4); UV $\lambda_{max}$ (MeOH) nm (ε): 251.0 (12,800), 281.4 (11,000); $[\alpha]_D^{25}$ −452.4° (MeOH; $c$ 0.116); CD $\lambda_{ext}$ (MeOH) nm (θ): 237 (+31,700), 283 (−94,900).
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31. 254 nm: UVG-11 (4 W), UVP, Inc. 365 nm: UVG-21 (4 W), UVP, Inc.