Phytochemistry, Vol. 32, No. 4, pp. 869–873, 1993
Printed in Great Britain.

0031-9422/93 $6.00 + 0.00

SYNTHESIS AND PROPERTIES OF C-1-AZIDO-ABA

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(*Received* 9 *June* 1992)

Key Word Index—Abscisic acid; indole acetic acid; gibberellin; azide; bioassay; photoaffinity labelling; receptor.

Abstract—The acyl azido derivative of ABA has been synthesized in high yield via a mixed anhydride. It is stable in most organic solvents but rearranges in water ($t_{1/2}$ of 30 min at 30°) to yield 1'-hydroxy-4'-oxo-α-ionone, presumably via an isocyanate intermediate. Photolysis of (±) *RS*-[G-^{3}H]azido-ABA in a 1% solution of bovine serum albumin with low intensity UV_{254nm} light gave labelled bovine serum albumin (BSA). Racemic 1-azido-ABA inhibited wheat embryo growth with about 10% the efficacy of racemic ABA. Racemic 1-azido-ABA was resolved into its enantiomers on a chiralcel HPLC column and both enantiomers were bioassayed. The (+)*S*-enantiomer caused stomatal closure and inhibited growth of *Lemna gibba* with about 10% of the potency of ABA but the (−) *R*-enantiomer was inactive in both the stomatal closure and *Lemna gibba* bioassays. It is suggested that in one of the two canonical forms of the azide the α-nitrogen atom carries the negative charge in the same position as the carboxyl group of ABA and so is able to substitute for it

INTRODUCTION

The identification and purification of plant hormone receptors using the traditional techniques used for animal hormone receptors have been ineffective or inconclusive. Purification of plant hormone binding proteins using affinity chromatography has been successful [1, 2], but it is not clear if these proteins are hormone receptors or if they have some other function in either the transport or metabolism of the hormones.

A promising technique for the identification of a plant hormone receptor has been to label the hormone receptor *in situ* with a photoaffinity probe and to purify the labelled protein(s) [3, 4]. The photoaffinity probe should satisfy the following five criteria: (1) it should be biologically active in the same way as the parent compound; (2) it should be stable under experimental conditions; (3) photolysis should be efficient and rapid; (4) highly reactive, short lived intermediates should be formed on photolysis; (5) photolytic conditions should not significantly damage cells or denature proteins [3]. These criteria have been met for a photoaffinity probe to the GA receptor in barley aleurone layers but the loss of biological activity that usually accompanies modifications to ABA have limited the possiblities for the synthesis of a photoaffinity probe to the ABA receptor [5]. We have synthesized and tested the acyl azido derivative of ABA (AB-Az) (**1**) against the criteria described above with a view to identifying one or more or the ABA receptors.

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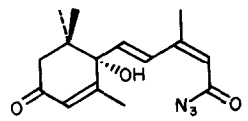
RESULTS

AB-Az was synthesized via a mixed anhydride [6] in high yield (95%). Other methods were also attempted [7, 8] however, the overall yields with these methods were low (< 20%) and the product required extensive purification. Tritiated and deuteriated samples of AB-Az were



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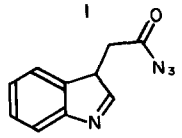

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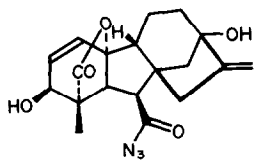

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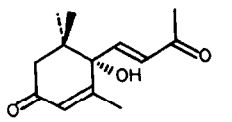

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synthesized using labelled ABA as the starting material. The tritiated AB-Az was obtained in only 30% yield, probably as a result of scaling down the synthesis to 26 ng. The mixed anhydride method was also used to convert IAA and GA_3 into their respective acyl-azido derivatives, **2** and **3**, in the same high yield (95%).

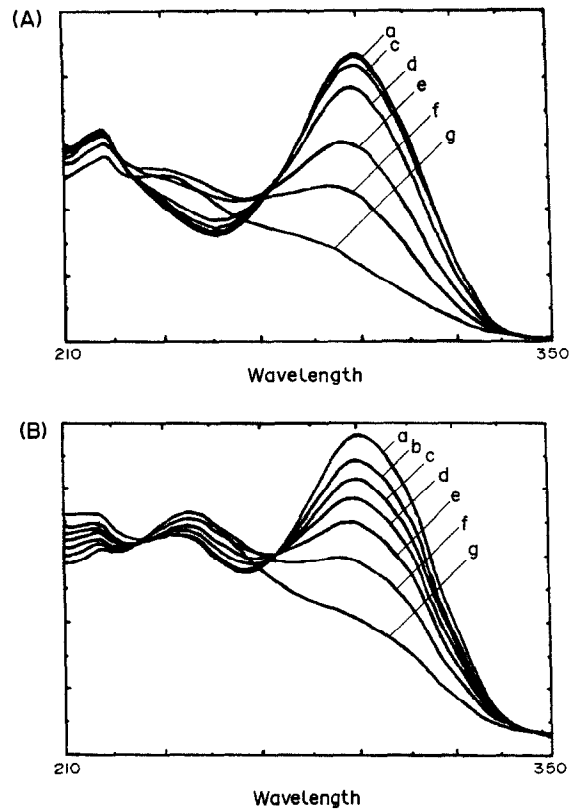

Fig. 1. Time course for the photolysis of AB-Az in (A) hexane: IPA(24: v/v) at; (a) 0 sec, (b) 5 sec, (c) 10 sec, (d) 30 sec, (e) 60 sec, (f) 120 sec and (g) 240 sec. (B) Potassium phosphate (10 mn) pH 7 at; (a) 0 sec, (b) 5 sec, (c) 10 sec, (d) 20 sec, (e) 40 sec, (f) 80 sec and (g) 160 sec.

The stability of acyl azides varies considerably and they often hydrolyse in water to yield the free acid as they usually have similar properties to the corresponding acid chlorides [9]. Breakdown of AB-Az was monitored by UV spectroscopy and by HPLC and the compound was found to be stable when kept in solution in hexane or in methanol at 30° for two days in darkness. However, it rearranged to **4** with a $t_{1/2}$ of 30 min at 30° in 10 mM potassium phosphate buffer at pH 7. No hydrolysis of AB-Az to ABA was detected over the time period of these experiments (limits *ca* 0.01%).

The photolysis of AB-Az was complete within 160 sec when an aqueous solution was irradiated 150 mm from a 15 Watt UV_{254nm} lamp. Figure 1.1 and 1.2 shows the effect on the UV spectrum during photolysis in hexane: IPA (24:1, v/v) and water, respectively.

The ability of the photolytic intermediates to react with a protein was tested by photolysing (±)-[G-^{3}H]AB-Az or (±)-[G-^{3}H]ABA in the presence of BSA (Table 1). TCA was used to precipitate the BSA, however a high background binding was observed for ABA and un-irradiated controls, probably due to hydrophobic interactions. The rearrangement of organic azides to reactive isocyanates is catalysed by acid as well as by light and is probably the cause of the binding of a high proportion of AB-Az in both irradiated and non-irradiated samples. Precipitation of BSA with acetone minimizes both the non-specific binding and the conversion into isocyanate, and it was found that 25% of the added (±)-[G-^{3}H]AB-Az was bound irreversibly to the acetone-precipitated BSA. This compares with 5% bound in the dark control and 0.2–0.3% in the ABA controls. This suggests that AB-Az will be useful for photoaffinity labelling of the ABA receptor.

The resolution of the two enantiomers of AB-Az was achieved on a cellulose-based chiral column that was used to resolve the methyl ester of ABA [10]. Baseline resolution was achieved using IPA: hexane (2:23, v/v) and the peak eluting at 10.8 min was identified as the (+) *S*-enantiomer by synthesizing the (+) *S*-enantiomer

Table 1. Photolabelling of BSA with AB-Az

Treatment	TCA precipitate		Acetone precipitate	
	dpm	% bound	dpm	% bound
Irradiated				
ABA	17126	10	168	0.3
AB-Az	5523	48	2510	22
Dark controls				
ABA	13347	10	88	0.2
AB-Az	6860	60	678	6

(−) *R*-[G-^{3}H] AB-Az (30 Ci mmol^{-1}) or (±) *R,S*-[G-^{3}H] ABA (30 Ci mmol^{-1}) was added to an aqueous solution of BSA (1%; 0.5 ml). The solution, after 2 min preincubation at 25°, was either irradiated for 30 sec under UV_{254nm} light or kept in darkness. The protein was then precipitated with either aqueous TCA (5%; 2 ml) or acetone (2 ml). After 15 min the precipitate was sedimented by centrifugation and washed twice with the precipitating agent. The pellet was resuspended in 0.5 ml of water and counted, after the addition of a scintillation cocktail, in a scintillation counter.

Table 2. Effects of azido derivatives of ABA on growth of excised wheat embryos (three days) and *Lemna* (seven days)

Bioassay	$I_{50}(\mu M)$
Wheat embryo	
(±)-ABA	1.1–4.9
(±)-AB-Az	56.9
(+)-AB-Az	10.4
(−)-AB-Az	25
Lemna gibba	
(+)-ABA	2.7
(+)-AB-Az	30
(−)-ABA	83
(−)-AB-Az	no inhibition
(+)-*trans*-ABA	3.8
(−)-*trans*-ABA	42
(+)-*trans*-AB-Az	161
(−)-*trans*-AB-Az	97
(±)-1′-Hydroxy-4′-oxo-α-ionone	no inhibition*

*Growth promotion.

The increases in growth as a percentage of control growth (initial values subtracted) was plotted as a probit against log concentration. The concentrations causing a 50% inhibition in growth were obtained by interpolation (I_{50}).

Table 3. Effects of mixtures of (+)-*S*-ABA and (±)-1′-hydroxy-4′-oxo-α-ionone on the growth of *Lemna*

(+) S-ABA	(±)-1′-hydroxy-4′-oxo-α-ionone (mg l^{-1})			
(mg l^{-1})	30	10	3	0
3	14	13	20	19
0.9	73	80	89	76
0.3	101	105	75	94
0	125	109	86	100

Sixteen fronds per tube were used as an inoculum and were counted at intervals. The figures are percentages of the control, after subtraction of the initial frond number, at eight days. The fronds in the solutions without ABA were of normal size and appearance. Figures are the mean of two tubes, except the control (3).

of AB-Az from (+) *S*-ABA. The (−) *R*-enantiomer eluted at 12.8 min.

The AB-Az was tested in growth inhibition bioassays with *Lemna gibba* [11] and with excised wheat embryos [12]. The I_{50} values of the AB-Az in each of these bioassays with ABA are shown in Table 2, with the 2-*trans* isomers and **4** as a comparison.

Compound **4** was found to be growth promoting at high concentration ($\geq 30\ \mu g\ ml^{-1}$) in the *Lemna gibba* bioassay and it was tested with ABA, using a latin square design, to determine if the growth promoting activity could overcome growth inhibition by ABA. Compound **4** had no effect on the growth inhibition of *Lemna gibba* by ABA, although on its own, it promotes growth (Table 3).

The two enantiomers of AB-Az were also tested in two stomatal closure bioassays. Neither the (+) *S*- or the (−) *R*-enantiomers were active in a stomatal conductance bioassay, using *Helianthus annuus* leaves (B. R. Loveys, unpublished data), in which the AB-Az was supplied via the transpiration stream. However, the stomatal conductance bioassay is performed under relatively high light intensities and it is likely that the AB-Az will be photolysed before it can reach the stomata. In contrast, (+) *S*-AB-Az but not the (−) *R*-enantiomer, caused stomatal closure when applied directly to epidermal strips of *Valeriana officinalis*, as shown in Table 4.

The azido derivative of IAA (IA-Az) (**2**) was bioassayed as an aqueous solution applied to tomato shoots and the azido derivative of GA_3 (GA_3-Az) (**3**) was bioassayed on barley aleurone layers. The IA-Az-induced epinasty of tomato leaves typical of IAA and GA_3-Az stimulated the synthesis of α-amylase by the aleurone cells, so these compounds also may be capable of photolytically labelling their receptors. However, it must be noted that the azido group in these derivatives is not allylic and so may not be as stable as that of ABA and no attempt was made to detect if the respective free acids were formed from them by hydrolysis.

Excised barley embryos (40) were supplied with either (−) *R*- or (+) *S*-[G-^{3}H]AB-Az (0.36 mCi $mmol^{-1}$; 69 μM) in water and incubated for two days at 25°. In two experiments with (+) *S*-AB-Az, traces of (+) *S*-[G-^{3}H] ABA were extracted, which was 0.46% and 0.07% of the added [G-^{3}H]AB-Az. In an experiment with (−) *R*-[G-

Table 4. Stomatal closure bioassay with *Valeriana officinalis*

	Treatment time (min)	Stomata Closed	Stomata Total	Closed (%)
Hexane (control)	20	28	85	33%
(±)*RS*-ABA	23	142	179	80%
(±)*S*-AB-Az	26	111	142	78%
(−)*R*-AB-Az	29	38	112	34%

Solutions of the compounds (100 $\mu g\ ml^{-1}$) in hexane (3 μl) were applied to a 5 mm × 5 mm section on the undersurface of a single leaf. The hexane evaporated immediately. The treated section is then excised and the stomata examined under a microscope at × 200 magnification after 20–30 min.

^{3}H] AB-Az, traces of (−) *R*-[G-^{3}H] ABA were detected which was 0.16% of the added (−) *R*-[G-^{3}H] AB-Az. The ABA formed from the AB-Az in these experiments was between 26 and 184 ng of ABA per gram of embryo (fresh weight). The maximum value (184 ng g^{-1} embryo fr. wt) was compared with the amount of ABA present in embryos supplied with (+) *S*-ABA (B. V. Milborrow and H. S. Lee, unpublished results). The growth inhibition caused by this concentration was interpolated to be 12% and so the ABA formed from AB-Az cannot account for the 90% growth inhibition induced by AB-Az. Such an effect requires greater than 3500 ng of (+) *S*-ABA per gram fresh weight (B. V. Milborrow and H. S. Lee, unpublished results).

Two tomato shoots (*ca* 2 g each) were supplied with (+) *S*-[G-^{3}H]AB-Az (77 500 dpm and 67 000 dpm, respectively; 30 Ci mmol^{-1}) via the transpiration stream. 3560 dpm and 2860 dpm were recovered, respectively, as ABA after 2 hr. This is 4.6 and 4.3% of the AB-Az fed.

DISCUSSION

The azide of ABA was synthesized to test whether it could react with the receptor of the hormone and so could be used as a photoaffinity label. The results show clearly that it has the potential to be used for this purpose because it acts as a growth inhibitor and causes stomata to close. Although traces of labelled ABA were detected these were equivalent to less than 5% of the AB-Az and this concentration cannot increase with time because the azide breaks down rapidly in water to **4** and this C_{13} degradation product cannot give rise to ABA.

The growth inhibitory effect of the AB-Az is unlikely to be caused by traces of ABA formed from it because the quantities are too small and the (+) *S*- and (−) *R*-ABA are potent inhibitors of growth whereas the (+) *S*-AB-Az is active and the (−) *R*-AB-Az is considerably less so. If the growth inhibition were caused by the release of ABA then the (−)-azide would be expected to show a high degree of activity.

The breakdown product **4** stimulates the increase in frond number of *Lemna* and it was thought possible that the weaker effect of AB-Az than ABA could be attributed, in part, to competition between the potent growth inhibitory effect of the azide and the counteracting, stimulatory effect of **4** on growth. This was disproved by the data of a competition experiment (Table 3), where **4** had no effect on growth in the presence of exogenous concentrations of ABA whereas on its own it stimulated frond production. It can be hypothesized that, like the β-ionone [13] it causes the membranes to be more permeable to ABA. So, in the absence of ABA, the endogenous hormone leaks out and so the fronds divide more rapidly while in the presence of relatively high ABA concentrations, compound **4** could facilitate entry of the exogenous ABA and so aid the growth inhibition.

The range of potent analogues of cytokinins, gibberellins and auxins has enabled photoaffinity probes with the characteristic biological activity to be synthesized for these plant hormones [1]. However, the failure to find a highly active analogue of ABA has limited the number of possibilities in the design of an ABA photoaffinity probe. Until now, the loss of an unmodified carboxyl group of ABA resulted in a total loss of biological activity, especially in stomatal closure bioassays, and other modifications also reduce the biological activity [5]. The ^{1}H NMR spectrum of AB-Az is virtually identical, down to the fine structure, with that of ABA indicating that the presence of the azido group at C-1 has not affected the conformation or eletronic structure of the rest of the molecule. The α-nitrogen atom of the azido group in one of its canonical forms, as shown in Fig. 2 [9], has a negative charge and this may occupy the same position as the $-O^-$ of the carboxyl group of ABA. Therefore, it is postulated that this charge on the α-nitrogen atom substitutes for the negative charge on the carboxyl group of ABA.

Fig. 2. The canonical forms of organic azides [8].

It is difficult to understand how such a short lived compound as AB-Az can have such a profound effect on growth if it does not react with the ABA receptor. The long lasting inhibition caused by AB-Az suggests that it binds to the ABA receptor molecules and makes them active for some time. All these observations and AB-Az's ability photolytically to label BSA point to labelled AB-Azs being useful as a photoaffinity label for the ABA receptor. An unexpected advantage is that just as (−) *R*-ABA was used as an inactive control for stomata [14] the (−) *R*-AB-Az, because it is so much less potent as a growth inhibitor, should be capable of the same function.

EXPERIMENTAL

General. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM 300 spectrometer operating at 300.165 MHz and 75.477 MHz, respectively. $CDCl_3$ was employed as the solvent with the residual $CHCl_3$ as ref. for ^{1}H. The new ABA numbering system [15] is used for all derivatives in the assignment of NMR data. IR spectra were recorded on a Pye Unicam SP2000 infrared spectrophotometer in $CHCl_3$ using a microcell. Methane PICI spectra were recorded on a Finnigan Model 3200 quadrupole GC-MS CI system [16] and were introduced into the ion source via the solids probe. UV spectra were recorded on a Beckman diode array UV/Vis spectrophotometer using a read average time of 0.5 sec.

Synthesis. AB-Az was synthesized by the mixed anhydride method [6]. ABA (10 mg; 38 μmol) and H_2O (10 μl) were stirred at 0° and sufficient Me_2CO (*ca* 100 μl) was added to dissolve the ABA. Triethyl amine (7 μl; 50 μmol) was added and stirred for 2 min when ethyl chloroformate (9 μl; 94 μmol) was added and stirring continued for 60 min at 0°. NaN_3 (80 μl of a satd soln in H_2O) was added and stirring continued for 30–60 min. The reaction

mixt. was then poured into 10 ml H_2O, extracted with Et_2O (2 × 20 ml) and the combined fractions dried over anhydrous $MgSO_4$. Further purification of the azide was by HPLC in a 8 mm × 250 mm silica column eluted with IPA:hexane (1:24, v/v) at 4 ml min^{-1}. ABZ eluted at 6.5 min. UV λ_{max} nm in hexane: 292 (26500 M^{-1} cm). IR $\nu_{max}^{CHCl_3}$ cm^{-1}:2140 (N_3), 1670 and 1595. ^1H NMR: δ H-2 5.67 (J = 0.7 Hz, H-5 and 0.8 Hz, H-4), H-4, 7.91 (*d*, J = 15.95 Hz, H-5, 1.2 Hz, H-6 and 0.8 Hz, H-2), H-5, 6.26 (*d*, J = 15.95 Hz, H-4 and 0.7 Hz, H-2), H-6, 2.04 (*s*, 1.2 Hz, H-4), H-3′ 5.95 (*s*, J = 1 Hz, H-5′$_{eq}$ and 1.4 Hz, H-7′), H-5′$_{ax}$ 2.46 (*d*, J = 17.25 Hz, H-5′$_{eq}$ and < 1 Hz, H-8′), H-5′$_{eq}$ 2.31 (*d*, J = 17.25 Hz, H-5′$_{ax}$ and < 1 Hz, H-9′), H-7′, 1.92 (*s*, J = 1.4 Hz, H-3′), H-8′ 1.11 (*s*, J < 1 Hz, H-5′$_{ax}$), H-9′, 1.01 (*s*, J < 1 Hz, H-5′$_{eq}$). ^{13}C NMR: δ 171.2 (C-1), 119.0 (C-2), 162.4 (C-3), 128.2 (C-4), 138.7 (C-5), 21.4 (C-6), 79.7 (C-1′), 153.1 (C-2′), 127.1 (C-3′), 197.8 (C-4′), 49.8 (C-5′), 18.9 (C-6′), 18.9 (C-7′), 23.1 (C-8′), 24.4 (C-9′). PICIMS *m/z* (rel. int.):153 (100), 181 (18), 202 (32), 219 (12), 229 (10), 234 (11), 244 (50), 261 (42), 262 (61), 290 (15), 302 (6).

IA-Az was synthesized from IAA by the mixed anhydride method as described above for AB-Az. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 2140 (N_3), 1717 (C=O). PICIMS *m/z* (rel. int.): 89 (8), 130 (100), 158 (7), 172 (8), 173 (12), 201 (3), 213 (2). GA_3-Az was synthesized similarly from GA_3. IR $\nu_{max}^{CHCl_3}$ cm^{-1}:2140 (N_3), 1792 (C=CH_2), 1717 (C=O). PICI MS *m/z* (rel. int): 111 (90), 149 (50), 239 (46), 264 (25), 282 (44), 301 (24), 326 (100), 344 (15). Occasionally, the azides were contaminated with the isocyanate which is characterized by a strong band in the IR at *ca* 2210 cm^{-1}. This contamination can be reduced by performing all operations in dim light and ensuring everything is kept at <0° during the work up.

Bioassays. Growth inhibition bioassays were performed as described elsewhere [11, 12]. For the stomatal closure bioassay, a large leaf (90 mm × 25 mm) from *Valeriana officinalis* was cut with a razor blade under water, the cut end was placed in a 10 mM solution of KCl and the leaf was fanned under lights for 2–3 hr. The stomata on the undersurface were viewed under a microscope at × 200 magnification and when more than 60% of the stomata were open the leaf was used in the bioassay. Four 5 mm × 5 mm squares were marked on the intact leaf using a marker pen and these regions were treated with a hexane solution of (±) ABA (100 μg ml^{-1}; 3–5 μl), (+) *S*-AB-Az (100 μg ml^{-1}; 3–5 μl), (−) *R*-AB-Az(100 μg ml^{-1}; 3–5 μl) or hexane (3–5 μl) as a control. The leaf was then allowed to transpire as normal for 20–30 min and the treated sections were excised and the number of closed and open stomata were counted immediately under a microscope at × 200 magnification.

Stomatal conductance bioassays (B. R. Loveys, unpublished results) were performed with *Helianthus annuus* leaves.

IAA causes epinasty when sprayed on to the leaves of tomato plants and IA-Az was found to have a similar effect. GA_3 is known to induce the production of α-amylase by barley aleurone layers (17) and GA_3-Az was found to have a similar but weaker effect.

Resolution. Resolution of AB-Az was achieved on a chiralcel HPLC column [10]. IPA:hexane (2:23, v/v) at 1 ml min^{-1} resulted in baseline resolution of the (+)-enantiomer, 10.8 min, and the (−)-enantiomer, 12.8 min.

Acknowledgements—We thank Dr D. Bourne for help with mass spectrometry and Dr A. G. Netting for help with chromatography and much valuable discussion. We are grateful to Mrs J. LeClaire for the *Lemna* bioassays, Ms H. S. Lee for some of the wheat bioassays and Mr M. L. Windsor and Dr B. R. Loveys for the stomatal conductance bioassays. This work was supported by the Australian Research Committee.

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