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ACTIVITY AND UTILITY OF ABSCISIC ACID HAVING A 3′ THIOETHER LINKER ARM*

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Key Word Index—*Lepidium sativum*; abscisic acid; linker arm; affinity chromatography; antibody; analogues; preparation; cress; germination.

Abstract—Abscisic acid (ABA) was functionalized at the 3′-position by reaction of its α-2′,3′-epoxide with 1,4-butanedithiol to afford the corresponding derivative having a six-atom linker arm containing a terminal sulphhydryl group. This derivative was coupled with epoxy activated Sepharose 6B affording a gel containing *ca* 1.4 mg of (+)-ABA ml^{-1}. This gel was shown to bind specifically an anti-ABA antibody from hybridoma culture broth, and its binding capacity was determined. The (+)-ABA analogue possessing the thiol linker arm was also derivatized by reaction with a fluorescent derivatizing agent and these compounds were shown to inhibit cress seed germination. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Affinity chromatography can be a valuable method for the isolation and purification of low abundance proteins [1–3]. (+)-Abscisic acid (ABA, **1**) is a plant hormone for which it is postulated that high affinity, low abundance proteins acting as receptors, exist [4]. The availability of affinity matrices containing ABA would thus be highly desirable for the isolation and purification of putative receptors; unfortunately, any chemical modification of ABA, necessary for its attachment to a suitable support, would have some impact on its geometry and physical properties and, consequently, its ability to bind the desired protein(s). Although this is a general problem in preparing affinity matrices, the situation with ABA is particularly relevant due to its small size (M_r 264). The position and type of linkage required to attach ABA to a gel is thus crucial. Perhaps the most obvious means of attachment is via the C-1 carboxylic acid (via ester formation) or C-4′ ketone (via hydrazone formation), and the corresponding ABA conjugates for preparation of antibodies have been made [5–7]; however, in the case of C-1, it would appear that not only is this position very important for ABA activity, but the presence of the carboxylic acid group is also necessary.‡ Linkage through the C-4′ carbonyl group has been based on the formation of hydrazones derived from aromatic aminohydrazides [5, 6], but the basicity associated with these linkers means that the derivatized ABAs would exist as charge separated zwitterionic species. The high charge polarization associated with them would almost certainly affect their ability to bind an ABA receptor. The hydrazone (through C-4′) and ester linkages (through C-1) are also susceptible to hydrolysis under certain conditions.

We therefore sought to prepare an analogue that would contain a chemically stable, neutral linker arm attached to ABA at a position that would minimally impact its binding to a receptor. The criterion for whether a substituent at a position would minimally impact binding was based on whether such an analogue would exhibit ABA activity in various bioassays. Todoroki *et al.* [8] prepared (+)-3′-fluoroABA (**2**) from 2′α,3′α-epoxyABA (**3**), which itself was obtained by alkaline hydrogen peroxide epoxidation of (+)-ABA (Figs 1 and 2), and found it to be better than (+)-ABA in inhibition of lettuce seed germination and almost as good as (+)-ABA in a rice seedling elongation assay. The high activity associated with this 3′-substituted analogue suggested this position as an attractive point of attachment for a linker arm, while previous studies, aimed at identifying gibberellin receptors [9, 10], had established the utility of linker arms containing a thioether bridge and a terminal sulphhydryl group. Accordingly, we describe here the preparation of an ABA analogue possessing a neutral linker at the 3′-position, its attachment to epoxy activated Sepharose as well as a fluorescent thiol derivatizing agent, its activity in cress seed germination inhibition, and the ability of

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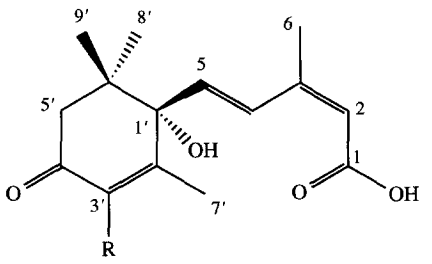
‡Results on the importance of the C-1 carboxylic acid were presented at the Plant Growth Regulator Meeting in Minneapolis, MN, 17–21 July, 1995, and will be published in the near future.



1 R = H (+)-Abscisic acid
2 R = F

Fig. 1. Structure and numbering system of ABA (**1**) and 3′-fluoroABA (**2**).

the Sepharose-linked ABA to bind selectively an anti-ABA MAb as demonstration of the potential utility of the gel for isolation/purification of putative ABA receptors.

RESULTS AND DISCUSSION

(+)-ABA can be converted into the 2′3′-α-epoxide **3** in *ca* 50% yield by treatment with hydrogen peroxide under basic conditions [8]. Although ring cleavage of epoxides by nucleophiles generally requires stringent conditions, the activating presence of the keto group adjacent to the 3′-position of epoxide **3** and the use of a strongly nucleophilic thiolate reagent resulted in rapid reaction under relatively mild conditions. For the linker arm 1,4-butanedithiol was chosen because it was symmetrical and of sufficient length that further cyclization of the free sulphhydryl group on to the enone of ABA would not be a problem (it would result in the unfavourble formation of an eight-membered dithiane ring). The desired product **6** would have a six-atom linker arm connected to ABA at the 3′-position by a stable thioether bond; the terminus of the linker arm would have a free sulphhydryl group available for further attachment (Fig. 2).

Initially, reaction of **3** with a 50% excess of the butanedithiol in tetrahydrofuran and two equivalents of sodium hydride resulted in a very rapid reaction which gave two main products, of which the minor (30%) was the desired **6**. The major product (58%) was the dimer **7** formed by reaction of the free sulphhydryl of **6** with the starting epoxide **3**; apparently, under these conditions, the rate of reaction of **6** with **3** was much greater than that of butanedithiol with **3**. Neither of the putative 3′-substituted-2′-hydroxy intermediates **4** or **5** was observed in the reaction mixture, which indicated that β elimination (**5** → **6**) was occurring *in situ* very rapidly. Doubling or tripling the amount of butanedithiol in the reaction did not drastically improve the proportion of **6** in the mixture. Use of an excess of butanedithiol coupled with changing the solvent to methanol–aqueous NaOH, however, did result in a more selective reaction in which the desired 3′-substituted derivative **6** was obtained in 78% yield. The reaction in methanol was slower, requiring 20 hr for completion, and was performed under a nitrogen atmosphere to minimize oxidative coupling of the sulphhydryl groups.

Attachment of **6** (in this case using racemic material*) to epoxy activated Sepharose 6B was achieved in aqueous 20% methanol under basic conditions. The amount of ABA attached to the Sepharose was determined by two means: firstly, by measuring the amount of ABA derivatives recovered after washing the Sepharose, and secondly, by hydrolysing an aliquot of the Sepharose-linked ABA **8** and quantifying the amount of ABA present by UV spectrophotometry. Both methods were consistent, indicating that the gel contained *ca* 2.8 mg of racemic ABA ml^{-1} or 1.4 mg of (+)-ABA ml^{-1}. Since the epoxy activated Sepharose 6B contained a hydrophilic linker arm 12 atoms long, and the ABA derivative **6**, a lipophilic linker arm six atoms long, the Sepharose-linked ABA **8** thus consisted of a sugar-backboned polymer to which ABA was attached via an 18 atom neutral, chemically stable, linker arm. Proximate to the sugar end the linker arm was hydrophilic, which was desirable for enhancing the stability of the bed material (as a consequence of hydrogen bonding to the carbohydrate chains of the gel matrix), while proximate to the ABA end, the linker arm was lipophilic, in character with an expected membrane bound receptor protein. The length of the linker arm was appropriate for binding even large proteins [3].

As a further check of the utility of the linker arm as well as for further characterization, **6** [derived from (+)-ABA] was reacted with the fluorescent thiol-derivatizing agent, tetramethylrhodamine-5-(and 6)-iodoacetamide, in aqueous methanol under basic conditions to yield a mixture of the fluorescent ABA derivatives **9** and **10**. The mixture was characterized by ES mass spectrometry, ^{1}H NMR and UV–VIS spectrophotometry.

Having the 3′-substituted derivatives **6**, and **9**/**10** in hand, their activity in cress (*Lepidium sativum* L.) seed germination inhibition [11] was examined and the results are presented in Figs 3 and 4. Both analogues showed weak activity at 10 μM, delaying germination by 3–6 hr, which was analogous to the activity of 1 μM (+)-ABA (data not shown). At 50–100 μM they caused significant inhibition of germination and were almost as potent as 10 μM (+)-ABA, which essentially inhibited germination completely over 50 hr at 23°. Since these derivatives possessed a chemically stable linkage at the 3′-position, the activity observed could not be due to transformation to ABA *in vivo*. Thus, although exhibiting reduced activity, these analogues nonetheless were active, supporting the premise that the

*Epoxy activated Sepharose 6B contains a high titre of reactive epoxide groups (10–20 μequiv ml^{-1}) and the use of racemic **6** for the coupling was not expected to alter significantly the binding or availability of (+)-ABA to a potential ligand.

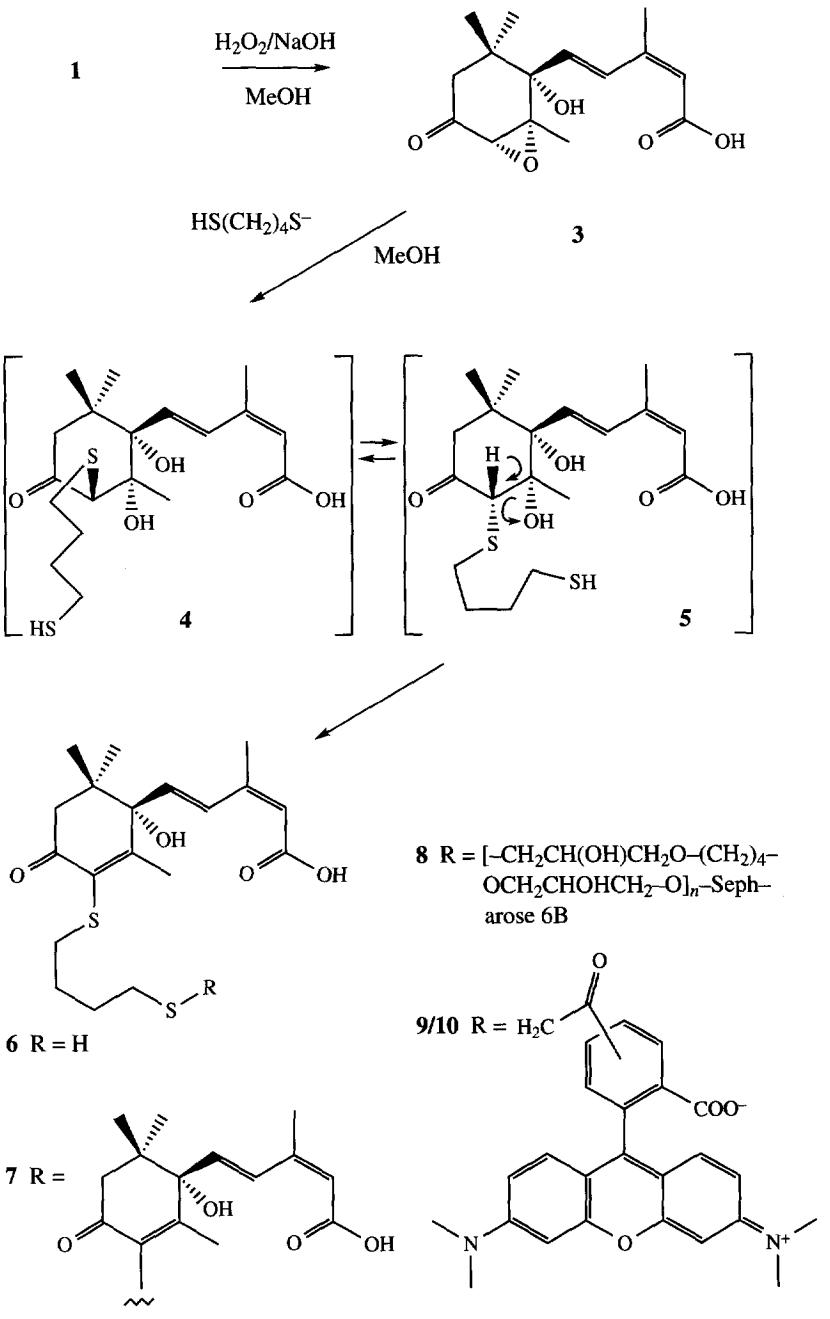


Fig. 2. Preparation and structures of various 3′-substituted ABA derivatives.

3′-position was appropriate for preparation of an affinity matrix.

The utility of the Sepharose-linked ABA was tested by its ability to bind a high specificity anti-ABA antibody, which had previously been prepared against a 4′-hydrazone conjugate [7]. Anti-ABA antibody A2, an IgG_1, had high specificity for natural (+)-ABA and exhibited no cross-reactivity with its enantiomer, (−)-ABA or its C-1 conjugate, (+)-ABA glucose ester. Even a relatively minor change in the ABA structure [i.e. (+)-ABA methyl ester] eliminated reactivity of the antibody with the ligand. This antibody thus represented a plausible model of a receptor binding site, and its isolation from hybridoma culture fluid, which contains several hundred proteins, would provide evidence of the gel's utility.

As anticipated, the gel (**8**) selectively afforded anti-ABA antibodies directly from the hybridoma culture fluid. Estimates of immunoglobulin and protein content in eluted fractions both indicated a binding capacity of the ABA–Sepharose gel of *ca* 1.79±0.31 mg of anti-ABA immunoglobulin ml^{-1} of matrix. The antibody represented 1% (0.035 mg ml^{-1}) of the total protein in the culture fluid (3.17±0.52 mg ml^{-1}). A Brilliant

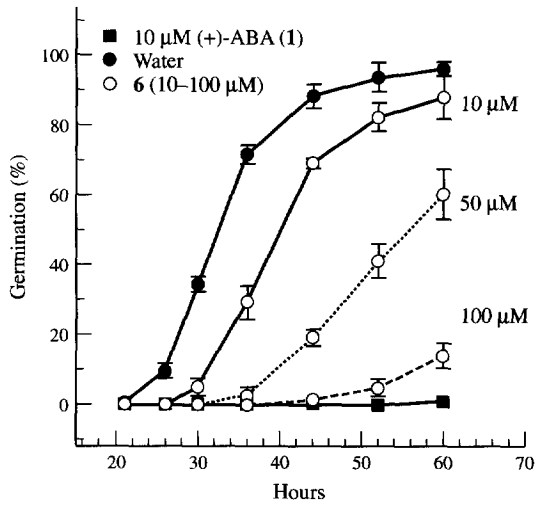


Fig. 3. Germination of cress seed in presence of (+)-ABA (10 μM) and 3′-substituted ABA **6** (10–100 μM). Water was used as the control.

Blue-stained SDS–PAGE gel showed two major proteins present in the eluted antibody preparation. Their estimated molecular weights of 27 and 54 kDa corresponded closely to expected masses of immunoglobulin light and heavy chains, respectively. A corresponding Western blot prepared from a parallel gel and probed with anti-mouse heavy (IgG_1) and light (kappa) sera confirmed that both bands were immunoglobulin in nature. To confirm the specificity of the gel, hybridoma culture fluid containing anti-isopentenyladenosine antibodies [12] was passed over the column and found, unlike the anti-ABA antibody, to be removed by Tris-buffered saline (TBS) washes. These results showed that gel (**8**) specifically bound anti-ABA immunoglobulins and did not bind detectable amounts of non-

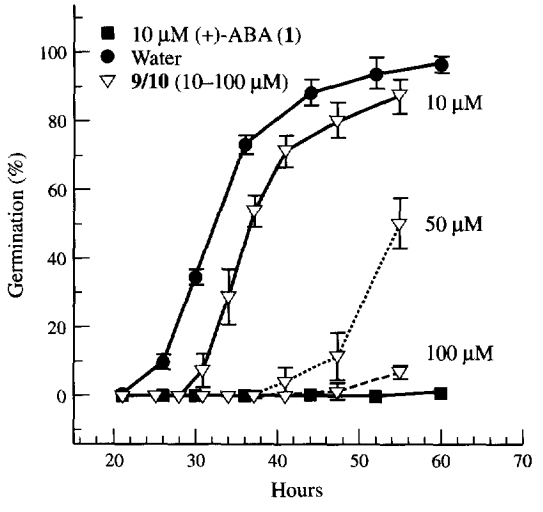


Fig. 4. Germination of cress seed in presence of (+)-ABA (10 μM) and 3′-substituted ABA **9/10** (10–100 μM). Water was used as the control.

immunoglobulin proteins present in the hybridoma culture supernatant.

In summary, a stable linker arm was attached to ABA at the 3′-position and this derivative, as well as one containing the fluorescent dye rhodamine, were shown to possess activity in cress seed germination inhibition. Attachment of ABA to Sepharose was achieved through this linker, and its ability to selectively bind a monoclonal anti-ABA antibody was shown. This ABA-linked-Sepharose should find use in the isolation and purification of other ABA binding proteins, while the analogue with the 3′ linker arm should prove useful for the preparation of biologically active photoaffinity labels.

EXPERIMENTAL

General. UV–VIS spectra were obtained (MeOH) with a Beckman DU-64 spectrophotometer. H NMR spectra were obtained with a Bruker AMX-500 spectrometer with samples in $CDCl_3$ unless otherwise noted. ESMS was performed on a Fision Trio 2000 instrument, and EIMS on a VG Analytical (Manchester, U.K.) 70-250 SEQ double focusing hybrid mass spectrometer. FABMS was performed on the same instrument, but equipped for continuous flow SIMS analysis according to ref. [13]. Optical rotations were obtained with a Perkin-Elmer 141 Polarimeter. IR spectra were obtained with a Perkin-Elmer Paragon 1000 FTIR.

Chemicals. Racemic ABA was from Aldrich (*ca* 98%, 86, 216-9). (+)-ABA was obtained by prep. HPLC resolution of (±)-Me abscisate followed by hydrolysis of the resolved esters, as previously described [14]. Solvents and reagents used were of reagent grade and used without further purification. Epoxy activated Sepharose 6B was from Pharmacia AB, tetramethylrhodamine 5-(and-6)-iodoacetamide was from Molecular Probes Inc. (Eugene, OR, U.S.A.), rabbit anti-mouse immunoglobulins were from Zymed Labs (South San Francisco, CA, U.S.A.) and alkaline-phosphatase labelled goat anti-rabbit IgG was from Sigma.

Preparation of (+)-*ABA*-3′-*thio*-n-*butyl thiol* (**6**). A soln of **3** [8] (mg, 0.05 mmol) in MeOH (3 ml) under N_2 was treated with 1,4-butanedithiol (*ca* 20 mg, 0.17 mmol) and 1 M NaOH (aq., 0.25 ml). The reaction mixt. was stirred for 20 hr, diluted with H_2O (30 ml) and washed with Et_2O (2 × 15 ml). The aq. portion was acidified with 1 M citric acid (0.5 ml) and extracted with EtOAc (3 × 20 ml). After drying (Na_2SO_4), the EtOAc was removed *in vacuo* and the residue purified by TLC using C_6H_6–EtOAc–HOAc (50:35:1) as eluent to afford **6** (15 mg, 78%) as a film; $[\alpha]_D$ (T = 23°, $CHCl_3$) = +324° (c = 0.3); IR, ν_{max} cm^{-1}: 3438, 2932, 1670, 1602, 1570, 1246; UV, λ_{max} nm: 250 (ε = 18 140); ^{1}H NMR: δ 7.76 (1H, *t*, J = 16 Hz, C-5), 6.09 (1H, *t*, J = 16 Hz, C-4), 5.75 (1H, *s*, C-2), 3.66 (1H, *t*, J = 6 Hz, SH), 2.75 (1H, *t*, J = 7 Hz, ABA-S-C$\underline{H}_2$-), 2.53 (1H, AB, 17, C-5′β or α), 2.49 (2H, *q*,

$J = 7$ Hz, ABA-S-$(CH_2)_3\underline{CH_2}$-SH), 2.42 (1H, AB, 17, C-5′β or α), 2.19 (3H, *s*, C-7′), 1.99 (3H, *s*, C-6), 1.66 (2H, *q*, $J = 7$ Hz, ABA-S-$CH_2\underline{CH_2}(CH_2)_2$SH), 1.59 (2H, *q*, $J = 7$ Hz, ABA-S-$(CH_2)_2$ $\underline{CH_2}$ CH_2SH), 1.29 (1H, *t*, $J = 7$ Hz, SH), 1.06 (3H, *s*, C-8′ or -9′), 0.99 (3H, *s*, C-8′ or -9′). EIMS *m/z* (rel. int.): 384, 366 (2), 310 (4), 295 (4), 280 (4), 263 (12), 245 (31), 194 (19). FAB-HRMS: calc. for $C_{19}H_{29}O_4S_2$: 385.1506; found: 385.1507. On standing or in basic soln, **6** undergoes some oxidative coupling to give the disulphide dimer (ESMS *m/z* 789 $[M + H]^+$); **6** can be regenerated by treatment with dithiothreitol.

Preparation of Sepharose 6B-linked-ABA (**8**). Pharmacia freeze-dried epoxy activated Sepharose 6B (1 g) was treated with H_2O (*ca* 10 ml) and allowed to swell for 15 min at ambient temp. The gel was filtered and washed with several portions of H_2O (total *ca* 100 ml) and suspended in H_2O (12 ml). A soln of racemic **6** (23 mg) in MeOH (3 ml) in MeOH (3 ml) was treated with 0.1 M NaOH (1 ml) and added to the suspended gel with swirling. A further portion of 0.1 M NaOH (1 ml) was added and the mixt. was swirled, under N_2, at 150 rev min^{-1} at 30° for 20 hr. EtSH (*ca* 20 mg) was added and the mixt. swirled for a further 60 min, then filtered and washed H_2O (*ca* 50 ml), 0.1 M citric acid (30 ml), H_2O (50 ml) and 0.02% NaN_3 (20 ml). ABA-Sepharose gel (*ca* 3.3 ml) was stored in a refrigerator in 0.02% NaN_3 soln until needed.

Determination of amount of ABA linked to the Sepharose. Method A: The initial H_2O washings from above were treated with 1 M NaOH (1 ml) and washed with EtOAc (2 × 20 ml) to remove any neutrals. The EtOAc washings were discarded and the aq. portion acidified with citric acid and extracted with EtOAc (3 × 20 ml). The EtOAc extract was washed with H_2O (2 ×), dried (Na_2SO_4), and concd *in vacuo* to yield 9 mg acidic residue, implying that the Sepharose contained 14 mg racemic **6** or the equivalent of 9.6 mg (+/−)-ABA/3.3 ml (2.9 mg ml^{-1}). *Method B:* A portion of ABA-liked gel **8** (*ca* 0.3 ml) was suspended in 1 M HCl in 50% MeOH (*ca* 25 ml) and heated at 60° with vigorous stirring for 2 hr. The resultant soln was cooled to ambient temp., diluted to 100 ml with MeOH and used to obtain a UV spectrum. The spectrum yielded a max. absorbance of 250 nm of 0.56 which, using the extinction coefficient of 18 140 observed for **6**, led to an estimated titre of 1.19 mg/0.3 ml or 3.9 mg ml^{-1} of **6** (equivalent to 2.7 mg racemic ABA ml^{-1}).

Preparation of rhodamine derivatives **9/10**. A soln of rhodamine 5/6-iodoacetamide (10 mg, 0.019 mmol) and (+)-ABA thiol **6** (6.5 mg, 0.017 mmol) in 80% aq. MeOH (6 ml) was treated with 10% NaOH soln (0.15 ml). The reaction mixt. was stirred for 18 hr under N_2, treated with tartaric acid (75 mg), and concd *in vacuo*. The residue was treated with EtOH (*ca* 0.5 ml), filtered and chromatographed on silica gel, using MeCN–H_2O (12:1) as eluent, to afford the rhodamine mixture **9/10** as a red film (6 mg, 42%); UV–VIS λ_{max}: (log ε). 538 (41 200), 354 (6 300), 296 (12 700), 250 (31 000), 234 (31 000), 218 (32 500); ^1H *NMR* (D_2O): δ 8.18, 7.94 (*s*, NH), 7.93 (overlapping peak, arom. H), 7.21 (*d*, $J = 16$ Hz, ABA C-5), 7.15–7.0 (overlapping peaks, arom. Hs), 6.66 (arom. H), 6.24 (arom. H), 5.83 (*d*, $J = 16$ Hz, ABA C-4H), 5.68 (*s*, ABA C-2H), 3.0 (*bs*, 4 × N-Me), 2.08 (*s*, ABA C-7′ Me), 1.69 (*s*, ABA C-6 Me), 0.84 (*bs*, ABA C-8′, 9′ Mes); ESMS: *m/z* 826 ($[M + H]^+$, 80%), 825 ($[M]^+$, 100%).

Cress seed germination inhibition studies. The studies were performed according to ref. [11], except the temp. was 23 instead of 25° and the initial stock solns of **6** and **9/10** were made up in EtOH rather than Me_2CO.

Determination of binding of anti-ABA Mab A-2 to **8**. The monoclonal anti-ABA antibody used in this study was developed against an (+)-ABA-KLH (keyhole limpet haemocyanin) C-4 conjugate as described in ref. [7]. Cross-reactivity of the antibody with ABA structural analogues was determined using a competitive ELISA [7]. Minimum binding capacity of the ABA–Sepharose was estimated by passing an excess of anti-ABA antibody-containing hybridoma culture supernatant over the matrix as follows. A 1-ml aliquot of ABA–Sepharose was placed into a polypropylene Econo-Column (BioRad Laboratories, Hercules, CA) and washed with 20 ml 0.1 M glycine–0.14 M NaCl (pH 2.3) followed by 20 ml 0.15 M TBS (pH 7.2). Anti-ABA hybridoma culture supernatant was mixed with an equal vol. TBS and 100 ml diluted culture supernatant passed over the column at *ca* 1.5 ml min^{-1}. The material that passed through the column was retained as the unbound fr. The column was washed with an additional 20 ml TBS, and anti-ABA immunoglobulins were eluted with 5 ml of the glycine-NaCl buffer (pH 2.3). Five 1-ml frs of the eluate were collected and analysed for anti-ABA antibody, mouse immunoglobulin content [15] and protein content [16]. Purity and identity of the eluted antibody prepns were assessed using SDS–PAGE [17] and Western blot [18]. The Western blot was developed by initial probing of the transferred proteins with rabbit anti-IgG_1 and rabbit anti-kappa chains followed by alkaline phosphatase labelled goat anti-rabbit IgG. Alkaline phosphatase substrate was prepd using a kit from Vector Labs (Burlingame, CA, U.S.A.).

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