# A radio-iodinated abscisic acid photoaffinity probe

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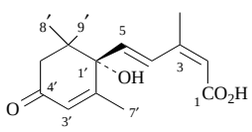
**The construction of a photoaffinity probe based on abscisic acid is described. A strategy based on C-4′-tethering of aromatic hydrazides led to abscisic acid 4′-(3″-azido-4″-hydroxybenzoylhydrazide). This compound is of moderate biological activity in cereal aleurone assays and was readily iodinated with chloramine T and sodium iodide, conditions that were used for the preparation of the [$^{125}$I]-labelled compound in high radiochemical yield.**

Plant hormones, in common with their counterparts in insects and animals, are potent compounds which control fundamental growth and developmental processes. The identification of receptors and other molecules involved in transduction of plant hormone signals is an important area of research in plant science. We have been employing[1–3] the technique of photoaffinity labelling to identify gibberellin (GA) binding proteins (potential receptors) in plant tissue derived from cereal seed aleurone. The aleurone is a layer of specialist cells that, on seed germination, react to endogenous GA by producing hydrolytic enzymes such as α-amylase. The effect of GA is counteracted by abscisic acid (ABA) **1**. This is another well known terpenoid plant hormone that is involved[4] in a number of important growth responses in plants, including seed dormancy. The possibility that the cereal aleurone response is coordinated by the activation of GA and ABA receptors led us to complement our efforts with GA photoaffinity labelling with a similar strategy for ABA receptors. The provision of bioactive ABA photoaffinity probes would not only be useful for the investigation of ABA action in aleurone but also in other ABA-responsive plant tissues such as stomatal guard cells where it has an important regulatory function in controlling water loss. Experiments on the photo-cross-linking of ABA, by photoactivation of the enone function, to guard cell protoplasts, have been described.[5] However, although specific labelling was observed the ABA-binding proteins so identified have not been further investigated.

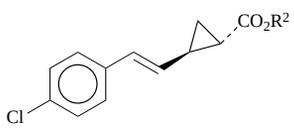
One of the problems with using enones as photo-activatable cross linking groups is the long and intense UV exposure needed to achieve efficient labelling. These conditions can lead to damage of the proteins being labelled.[6] We have sought to prepare biologically active ABA-photoaffinity labels with more suitable photochemistry. In this paper we describe the successful preparation of a bioactive arylazido derivative of ABA that can be radio-iodinated. We also describe the preparation of an ABA-biotin conjugate suitable for use in avidin affinity chromatography.

## Results and discussion

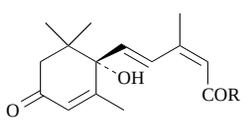
In a preliminary study we examined the potential of the aromatic analogue[7] **2** and the 4-azidoanilide **3**, prepared by mixed anhydride coupling of ABA with 4-azidoaniline. Both compounds were of low biological activity in aleurone and therefore were not further developed into photoaffinity reagents. ABA-acyl azide **4**, was prepared as described by Willows and Milborrow.[8] We found this compound to be unstable in solution



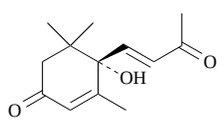

**1**




**2**

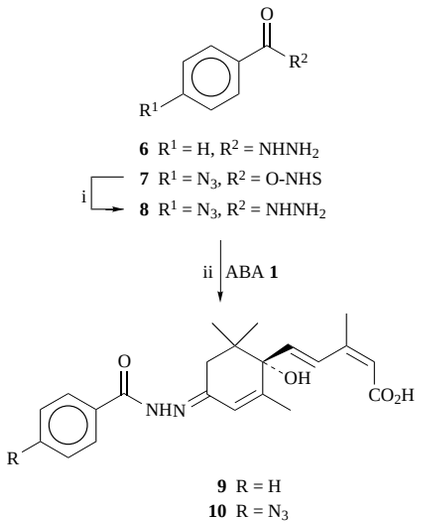



**3** R = $NHC_6H_4N_3$
**4** R = $N_3$




**5**

at room temperature in the dark. In chloroform the compound had a half-life of 18 h, decomposing to the isocyanate. When **4** was dissolved in alcoholic or aqueous solvents, the methyl ketone **5**, observed by Willows and Milborrow,[8] was formed over 22 h. The ketone **5** was found to have some activity in aleurone assays (see later). This, taken together with its inherent instability suggests that the acyl azide **4** is of limited utility for the identification of ABA receptors but may be used in the future for the labelling of partially characterised ABA binding proteins. It was clear from the above results that an effective photoaffinity probe would have to retain the C–1 carboxy group. In addition, there are literature reports indicating the importance of the side-chain double bonds[9] and the C–1′ tertiary hydroxy group[10] for retention of high biological activity. Thus, options for modification of the C–(1)–C–(1′) area of the structure appear limited. Therefore, we have examined the 4′-ketone group as a possible site for the tethering of photoactive groups. Alcohols derived from sodium borohydride reduction of the ketone are very active in some ABA bioassays,[9] although it has been suggested that the activity may be due to oxidation to ABA *in vivo*. It is also of interest that several analogues of ABA with ethylene ketal groups at C–4′ have good bioactivity.[11] The starting point for this work was a report[5] that ABA 4′-tyrosylhydrazone has moderate biological activity. This derivative has been of use in the preparation of hapten-protein conjugates for anti-ABA antibody product.[12,13] Following these methods, ABA was treated with benzoic acid hydrazide **6** in a 1 M solution of acetic acid in methanol. After 3 days at room temperature, protected from light to avoid isomerisation of the ABA side-chain, the hydrazone **9** was formed in quantitative yield (Scheme 1). In the $^1$H NMR spectrum the aromatic signals were clearly visible and the 3′-H signal was downfield from its




**Scheme 1** *Reagents:* i, $NH_2NH_2 \cdot H_2O$, dioxane, ii, AcOH, MeOH

position in ABA. The two doublets from the 5′-$H_2$ AB system were also affected and had converged relative to their position in ABA. The absence of doubling of any of the NMR signals indicated that a single geometric isomer had been produced. This was observed in all ABA 4′-arylhydrazones prepared in this work.

The azido analogue **10** was prepared as follows: diazotisation of 4-aminobenzoic acid followed by treatment with sodium azide yielded 4-azidobenzoic acid which was esterified with *N*-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide. The ester **7** was then converted into the hydrazide **8** by the method of Wetzel and Soll[14] and purified by flash chromatography, before being condensed with ABA to form the hydrazone **10** as above (Scheme 1). The hydrazones **9** and **10** were found to have moderate biological activity (see later) and therefore this synthetic strategy was adopted for the preparation of a radio-iodinatable affinity probe.

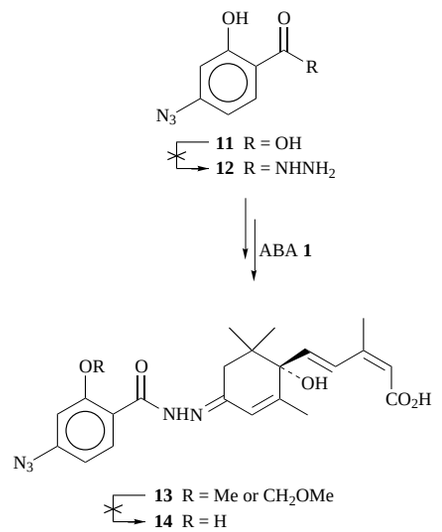
Previous work in this laboratory has used the 4-azidosalicylate group function to prepare a photoaffinity probe for gibberellin binding proteins.[3] The aryl azido moiety is commonly used as a photolabelling reagent[6] and the presence of the hydroxy group in salicylate allows the aromatic ring to be radioiodinated to high specific activity.[3] However, attempts to form 4-azidosalicylic acid hydrazide **12** by converting 4-azidosalicylic acid **11** into an ester or activated ester and treating that with hydrazine hydrate were unsuccessful. It was possible to form the hydrazide if the hydroxy group of 4-azidosalicylic acid was protected as a methyl or methoxymethyl ether but, after linking the hydrazide to ABA, to give **13** it was found that the hydrazone bond was unstable to the hydroxy deprotection conditions required and the required azidosalicyloyl hydrazone **14** could not be obtained (Scheme 2).

Having failed to prepare an ABA 4′-hydrazone using the salicyclic acid moiety, we turned to a commercially available compound, 3-amino-4-hydroxybenzoic acid hydrazide **15** (Scheme 3).This compound was successfully coupled to ABA to form the hydrazone **16**. The amino group was then converted into the azide by diazotisation and reaction of the resultant diazonium salt with sodium azide using the method of Pinney and Katzenellenbogen.[15] The aromatic azide **17** was formed, albeit in a disappointing yield of 25%. As a model reaction for radio-iodination and to provide a sample for biological assessment, the aromatic ring was then iodinated using chloramine-T and sodium iodide.[3] The mono iodide **18** was recovered in 71% yield. The hydrazone method was also used to link ABA to biotin hydrazide to yield the conjugate **19**. The availability of this conjugate will enable the isolation of ABA-binding proteins by monomeric avidin affinity chromatography.

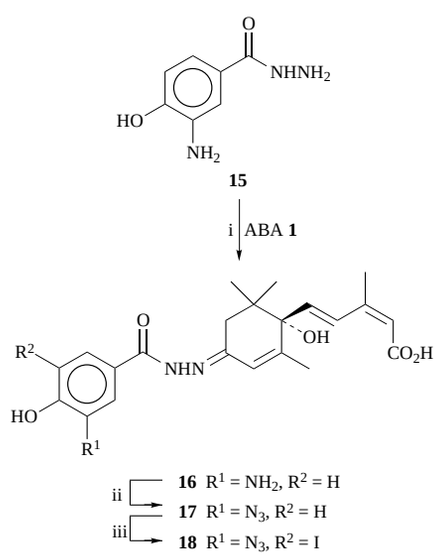
**Table 1** Biological activity of ABA derivatives

| Compound | Inhibition of GA-induced α-amylase $IC_{50}$ (μM)[a] | RAB gene expression $C_{50}$ (μM)[b] |
|---|---|---|
| ABA **1** | 0.7 | 1.0 |
| **2** | ND * | >1000 |
| **5** | 580 | 40 |
| **9** | 8.3 | ND * |
| **10** | 8.3 | 40 |
| **17** | 5.5 | ND * |
| **18** | 4.0 | 600 |
| **19** | 7.4 | 100 |

[a] Concentration of compound required to inhibit, by 50%, the amount of α-amylase secreted by 8 half-seeds in $10^{-8}$ M $GA_3$ ($IC_{50}$ for ABA = 0.7 μM). [b] Concentration of compound required to induce half-maximal expression of RAB gene (ABA = 1 μM). * ND = not determined.




**Scheme 2**

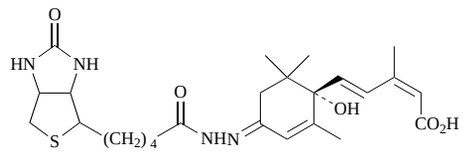



**Scheme 3** *Reagents:* i, AcOH, MeOH, ii, $NaNO_2$, $NaN_3$, $HCL_{(aq)}$, acetone, iii, Chloramine-T, NaI, MeCN, $H_2O$

## Biological activity

The derivatives were tested for ABA-like activity in an assay based on the inhibition of α-amylase production in GA treated, de-embryolated half-seeds of barley. The results are shown in Table 1. Selected compounds were also tested for the induction of RAB-16 gene expression in isolated barley aleurone protoplasts. The 4′-hydrazones **9**, **10**, **17–19** all showed consistent moderate inhibition of the α-amylase production with $IC_{50}$ in the range 4-9 μM compared with ABA which showed an $IC_{50}$ of

0.7 μM. In the RAB gene expression system hydrazones **10**, **18** and **19** showed less consistency in their induction, but this may reflect the more complex nature of this protoplast-based assay.



**19**

### Stability and purity of ABA-4′-hydrazones

While this work was in progress, Hite *et al.* reported[16] on the 4-aminobenzoic acid hydrazone of ABA. They suggested that ABA-4′-hydrazones are unstable and that any observed biological activity was due to ABA formed by hydrolysis of hydrazone *in vivo*. We were concerned, therefore, that our encouraging assay results may be due to ABA being regenerated under the assay conditions. NMR samples of the benzoylhydrazide **9** were made in $CD_3OD$ or in mixtures of $CD_3OD$ and $D_2O$. These were analysed by $^1H$ NMR spectroscopy and stored at room temperature. After 1 and 2 days no change could be seen in the NMR spectra of the samples. To account for the observed biological activity the sample would have to contain 10% ABA, a level that would certainly be visible in the NMR spectrum. In another experiment hydrazone **18** was stored in ethanol for several days and the solution monitored by thin layer chromatography. No ABA could be observed. Similar analysis of standard solutions containing a range of concentrations of ABA revealed that a 1% level of contamination of the hydrazone with ABA would be detectable by this assay. Thus, it appears that the hydrazones prepared in this work are stable enough in aqueous and alcoholic solvents for accurate biological assessment. The azido derivatives are to be used in photoaffinity labelling experiments which will only require a brief exposure to aqueous environments. Indeed, the intended use will be to label proteins in isolated plasma membrane fractions, where the incubation time with **18**, prior to photocrosslinking, will be short (minutes).

### Radio-iodination

For radio-iodination, methods developed[3] for GA-photoaffinity probes, including the facile isolation of the product by adsorption of the reaction mixture onto an ion-exchange chromatography cartridge, were applied effectively to **17**. This led to high yields of [$^{125}$I]-**18** which was radiochemically pure by TLC-autoradiography.

Thus, using the 4′-hydrazone route we have succeeded in preparing the first radio-iodinated ABA photoaffinity probe. This compound is now being employed alongside the radio-iodinated GA-photoaffinity probe[3] to investigate plant hormone binding proteins in cereal aleurone and other plant tissues.

## Experimental

Abscisic acid used in this work was racemic and purchased from Fluka (Gillingham, Dorset, UK) or Maybridge Chemical Co. (Tintagel, Cornwall, UK). General experimental details are as previously described.[3] 4-Azidobenzoic acid hydrazide **8** was prepared from 4-azidobenzoic acid *N*-hydroxysuccinimidyl ester **7** by the method of Wetzel and Soll.[14] Purity of compounds prior to bioassay was assessed by $^{13}$C NMR and TLC analysis.

### Abscisic acid 4′-benzoylhydrazide 9

Abscisic acid (70 mg, 270 μmol) and benzoic acid hydrazide **6** (72 mg, 500 μmol) were dissolved in methanol (5 cm$^3$). Acetic acid (290 mm$^3$, 5.0 mmol) was added to the solution which was then stirred at room temperature, protected from light, for 3 days. After removal of the solvent from the mixture *in vacuo*, the residue was dissolved in ethyl acetate (20 cm$^3$) and the solution was extracted with sodium borate buffer (0.1 M, pH 9; 2 × 10 cm$^3$). The aqueous layer was then acidified with hydrochloric acid (2 mol dm$^{-3}$) and extracted with ethyl acetate (3 × 10 cm$^3$); the combined extracts were washed with brine (10 cm$^3$), dried ($MgSO_4$) and concentrated to an oil. Flash chromatography of this on silica gel eluted with ethyl acetate–hexane–acetic acid (70:30:0.5 to 80:20:0.5) yielded the hydrazone **9** as a solid (101 mg, 270 μmol, 100%), mp 140–143 °C; $\delta_H$(400 MHz; $CDCl_3$) [7.9–7.6 (4 H, m), 7.5–7.4 (2 H, m) ArH, NH], 6.30 (1 H, s, 3′-H), 6.09 (1 H, d, *J* 15, 5-H), 5.70 (1 H, s, 2-H), 2.52 (1 H, d, *J* 15, 5′-H), 2.39 (1 H, d, *J* 15, 5′-H), 2.00 (3 H, s, 7′-$H_3$), 1.81 (3 H, s, 6-$H_3$) and 1.00 (6 H, s, 8′-$H_3$ + 9′-$H_3$); $\delta_C$(100 MHz; $CDCl_3$) 176.7, 170.5, 164.7, 154.9, 151.4, 149.6 (6 s, C-1, C-3, C-2′, C-4′, C-1″, CONH), 137.7 (d, C-5), 132.1, 128.8, 127.9, 127.5, 124.5, 118.0 (6 d, C-2, C-4, C-3′, [C-2″ + C-6″], [C-3″ + C-5″], C-4″), 79.6 (s, C-1′), 39.3 (s, C-6′), 37.0 (t, C-5′), 24.6, 23.7, 21.5 and 20.9 (4 q, C-6, C-7′, C-8′, C-9′); *m/z* (FAB; negative ion, thioglycerol-glycerol, 1:1) 381 ($M^-$ − H, 25%), 119 (55) and 102 (100).

### Abscisic acid 4′-(4″-azidobenzoyl)hydrazide 10

Abscisic acid (72 mg, 270 μmol) and *p*-azidobenzoyl hydrazide **8** (160 mg, 820 μmol) were dissolved in methanol (10 cm$^3$) and acetic acid (78 mm$^3$, 1.4 mmol) was added to the solution. This mixture was heated under reflux for 24 h after which it was cooled and evaporated *in vacuo*. The residue was dissolved in ethyl acetate (40 cm$^3$) and the solution then washed with 10% aqueous citric acid (3 × 10 cm$^3$) and then brine (10 cm$^3$), dried ($MgSO_4$) and concentrated. The product **10** was purified by flash chromatography on silica gel, eluting with ethanol–ethyl acetate–acetic acid (10:90:0.25), to give a pale yellow gum (103 mg, 245 μmol, 90%); $\delta_H$(400 Mz; $CD_3OD$) 7.91 (2 H, d, *J* 9, 2″-H + 6″-H), 7.72 (1 H, d, *J* 16, 5-H), 7.18 (2 H, d, *J* 9, 3″-H + 5″-H), 6.25 (1 H, s, 3′-H), 6.18 (1 H, d, *J* 16, 4-H), 5.71 (1 H, s, 2-H), 2.67 (1 H, d, *J* 17, 5′-H), 2.48 (1 H, d, *J* 17, 5′-H), 2.03 (3 H, s, 7′-$H_3$) and 1.87 (3 H, s, 6-$H_3$) [1.07 (3 H, s) and 1.05 (3 H, s) 8′-$H_3$, 9′-$H_3$]; $\delta_C$(100 MHz, $CD_3OD$) 169.7, 166.7, 158.7, 152.1, 151.5, 151.3, 145.5 (7 s, C-1, C-3, C-2′, C-4′, C-1″, C-4″, CONH), 139.2 (d, C-5), 131.2, 129.2, 125.4, 120.2, 119.4 (5 d, C-2, C-4, C-3′, [C-2″ + C-6″], [C-3″ + C-5″]), 80.6 (s, C-1′), 41.5 (s, C-6′), 38.5 (t, C-5′), 24.9, 23.9, 21.5, 19.2 (4 q, C-6, C-7′, C-8′, C-9′); *m/z* (FAB; negative ion, thioglycerol-glycerol, 1:1) 422 ($M^-$ − H, 30%) and 102 (100).

### Abscisic acid 4′-(3″-amino-4″-hydroxybenzoyl)hydrazide 16

Abscisic acid (122 mg, 462 μmol) and 3-amino-4-hydroxybenzoyl hydrazide (230 mg, 138 μmol) in a round-bottomed flask were dissolved in methanol (10 cm$^3$). Acetic acid (260 mm$^3$, 4.6 mmol) was added to the solution which was then stirred at room temperature, protected from light, for 5 days. After this the mixture was evaporated *in vacuo*. The product was purified by flash chromatography on silica gel, eluting with ethanol–ethyl acetate–acetic acid (0:100:0.25 to 10:90:0.25) yielded the hydrazone **16** as a pale yellow oil (89 mg, 215 μmol, 47%); $\delta_H$(400 MHz; $CD_3OD$) 7.68 (1 H, d, *J* 15, 5-H), 7.24 (1 H, s, 2″-H), 7.13 (1 H, d, *J* 9, 6″-H), 6.72 (1 H, d, *J* 9, 5″-H), 6.29 (1 H, s, 3′-H), 6.13 (1 H, d, *J* 15, 4-H), 5.68 (1 H, s, 2-H), 2.62 (1 H, d, *J* 15, 5′-H), 2.44 (1 H, d, *J* 15, 5′-H), 1.97 (3 H, s, 7′-$H_3$), 1.81 (3 H, s, 6-$H_3$), [1.03 (3 H, s) and 1.00 (3 H, s) 8′-$H_3$, 9′-$H_3$]; $\delta_C$(100 MHz; $CD_3OD$) 175.5, 169.9, 169.0 (3 s, C-1, C-4′, CONH), 158.0, 151.2, 150.5 (3 s, C-3, C-2′, C-4″), 139.2 (d, C-5), 136.8, 125.8 (2 s, C-3′, C-3″), 128.8, 125.5, 120.3, 119.5, 116.4, 115.0 (6 d, C-2, C-4, C-3′, C-2″, C-5″, C-6″), 80.5 (s, C-1′), 49.8 (t, C-5′), 40.5 (s, C-6′), 24.9, 24.0, 21.5, 19.2 (4 q, C-6, C-7′, C-8′, C-9′); *m/z* (FAB; negative ion, triethanolamine) 412 ($M^-$ − H, 25%), 297 (70) and 148 (100).

### Abscisic acid 4′-(3″-azido-4″-hydroxybenzoyl)hydrazide 17

A solution of the amine **16** (92 mg, 220 μmol) in acetone (5 cm$^3$)

and hydrochloric acid (0.5 mol $dm^{-3}$; 4 $cm^3$) was cooled with an ice–salt bath and a solution of sodium nitrite (15 mg, 220 μmol) in water (0.5 $cm^3$) added dropwise to it over 1 min. After 20 min a solution of sodium azide (43 mg, 670 μmol) in water (0.5 $cm^3$) was added dropwise to the mixture over 1 min. The mixture was then stirred at 0–5 °C for 30 min after which it was extracted with ethyl acetate (3 × 20 $cm^3$). The combined extracts were washed with brine (10 $cm^3$), dried ($MgSO_4$) and concentrated to give a red–brown oil. The azide **17** was purified by flash chromatography on silica gel eluting with ethyl acetate–hexane–acetic acid (70:30:0.25 to 100:0:0.25), followed by ethanol–ethyl acetate–acetic acid (10:90:0.25) to give a pale yellow oil (24 mg, 50 μmol, 25%); $\delta_H$(400 MHz, $CD_3OD$) 7.68 (1 H, d, *J* 16, 5-H), 7.53 (1 H, d, *J* 9, 6″-H), 7.49 (1 H, s, 2″-H), 6.86 (1 H, d, *J* 9, 5″-H), 6.19 (1 H, s, 3′-H), 6.14 (1 H, d, *J* 16, 4-H), 5.68 (1 H, s, 2-H), 2.62 (1 H, d, *J* 17, 5′-H), 2.44 (1 H, d, *J* 17, 5′-H), 1.98 (3 H, s, 7′-$H_3$) and 1.82 (3 H, s, 6-$H_3$) [1.02 (3 H, s), 1.00 (3 H, s) 8′-$H_3$, 9′-$H_3$]; $\delta_C$(100 MHz; $CD_3OD$) 175.0, 169.8, 166.6, 158.5, 151.8, 151.4, 126.4 (7 s, C-1, C-3, C-2′, C-4′, C-1″, C-3″, C-4″, CONH), 139.2 (d, C-5), 128.9, 127.1, 125.5, 121.8, 119.4, 117.1 (6 d, C-2, C-4, C-3′, C-2″, C-5″, C-6″), 80.6 (s, C-1′), 40.4 (s, C-6′), 24.9, 23.9, 21.5 and 19.2 (4 q, C-6, C-7′, C-8′, C-9′); *m/z* (FAB; positive ion, glycerol) 440 ($M^+$ + H, 50%), 185 (55) and 136 (100).

### Abscisic acid 4′-(3″-azido-4″-hydroxy-5″-iodobenzoyl)hydrazide 18

A solution of the azide **17** (24 mg, 50 μmol) in acetonitrile (4 $cm^3$) and water (2 $cm^3$) was cooled with an ice-bath and sodium iodide (10 mg, 70 μmol) was added to it followed by chloramine-T (14 mg, 60 μmol); this caused a red colour to develop. After 30 min the solution was partitioned between water (10 $cm^3$) and ethyl acetate (10 $cm^3$). The organic layer was separated, washed with brine (5 $cm^3$) and dried ($MgSO_4$). Flash chromatography on silica gel, eluting with ethanol–ethyl acetate–acetic acid (10:90:0.25), yielded a sample of the iodide contaminated with chloramine-T derived material. The sample was dissolved in aqueous sodium hydrogen carbonate (10 $cm^3$) and washed with ethyl acetate (10 $cm^3$). The aqueous layer was then acidified with hydrochloric acid and extracted with ethyl acetate (10 $cm^3$). The extract washed with brine (10 $cm^3$), dried ($MgSO_4$) and concentrated to yield the pure iodide **18** as a pale oil (20 mg, 40 μmol, 71%); $\delta_H$(400 MHz; $(CD_3)_2CO$) 8.16 (1 H, br m, 6″-H), 7.84 (1 H, d, *J* 14, 5-H), 7.79 (1 H, d, *J* 6, 2″-H), 6.25 (1 H, d, *J* 14, 4-H), 6.12 (1 H, br s, 3′-H), 5.73 (1 H, s, 2-H), 2.80 (1 H, d, *J* 17, 5′-H), 2.52 ( 1 H, d, *J* 17, 5′-H), 2.00 (3 H, s, 7′-$H_3$), 1.82 (3 H, s, 6-$H_3$) and 1.04 (6 H, s, 8′-$H_3$ × 9′-$H_3$); $\delta_C$ (100 MHz; $CD_3OD$) 169.7, 165.2, 159.0, 153.2, 152.0, 151.5, 129.2 (7 s, C-1, C-3, C-2′, C-1″, C-3″, C-4″, CONH), 139.2 (d, C-5), 136.5, 128.9, 127.3, 120.7, 119.2 (5 d, C-2, C-4, C-3′, C-2″, C-6″), 85.7 (s, C-5″), 80.6 (C-1′), 50.0 (t, C-5′), 40.6 (s, C-6′), 25.0, 23.9, 21.5 and 19.3 (4 q, C-6, C-7′, C-8′, C-9′); *m/z* (FAB; positive ion, thioglycerol) 566 ($M^+$ + H, 100%), 440 (30), 391 (60), 327 (45) and 302 (55).

### Radio-iodination of the azide 17

**CAUTION:** All work with radioiodine was carried out in a well-ventilated hood and with appropriate lead or lead-impregnated acrylic shielding. To [$^{125}$I]sodium iodide (2 mCi, NEN-Dupont) was added the azide **17** [4.5 μg in 9 $mm^3$ of phosphate buffer (100 mmol $dm^{-3}$ pH 8.5], followed by chloramine-T (2.5 μg in 10 $mm^3$ of buffer). After 10 min aqueous sodium metabisulfite (5%; 10 $mm^3$) was added to the mixture. For purification, the reaction mixture was transferred to a Bond Elut 3cc SAX cartridge (Varian) which had been pre-eluted with water at pH 8 (5 $cm^3$). The reaction mixture was washed onto the column with 100 $mm^3$ (× 3) of water (pH 8). The cartridge was eluted in 1 $cm^3$ fractions with the following solvents: water [(pH 8; 2 $cm^3$ (frs. 1,2)], hexane [containing 2 mmol $dm^{-3}$ acetic acid; 2 $cm^3$ (frs. 3-,4], ethyl acetate [2 mmol $dm^{-3}$ acetic acid; 4 $cm^3$ (frs. 5-8)] and ethanol [2 mmol $dm^{-3}$ acetic acid; 2 $cm^3$ (frs. 9,10)]. Fraction 5 contained [$^{125}$I]-**18** (1.9 mCi). Fraction 6 contained further pure [$^{125}$I]-**18** (0.2 mCi).

TLC-autoradiographic analysis of radio-iodination products was carried out as follows. Unlabelled **17** and **18** were spotted onto the origin of each track before diluted aliquots representing 1/20 000 of each fraction were applied. The plate was developed with ethyl acetate–hexane–acetic acid (80:20:2.5). After drying, the carrier spots were identified under a UV lamp (**17** $R_f$ 0.05 **18** $R_f$ 0.2). Direct autoradiography was carried out with X-ray film and exposure times 4–20 h as necessary. Both fractions 5 and 6 contained single radioactive spots coincident with marker spots of **18**, demonstrating that the product obtained was of high radiochemical purity.

### Abscisic acid 4′-biotinylhydrazide 19

Biotin hydrazide (46 mg, 180 μmol) and abscisic acid (95 mg, 360 μmol) were dissolved in methanol (10 $cm^3$). Acetic acid (51 $mm^3$, 900 μmol) was added to the mixture which was then stirred at room temperature, protected from light, for 3 days. The cloudy solution was then filtered and the filtrate concentrated *in vacuo*. Flash chromatography of the residue from silica gel, eluting with ethanol–ethyl acetate–acetic acid (10:90:0.25) yielded the hydrazone **19** as an oil (88 mg, 170 μmol, 98% based on biotin hydrazide); $\delta_C$ (400 MHz; $CD_3OD$) 7.60 (1 H, d, *J* 16, 5-H), 6.15 (1 H, s, 3′-H), 5.97 (1 H, d, *J* 16, 4-H), 5.71 (1 H, s, 2-H), [4.46 (1 H, m), 4.28 (1 H, m) 1″-H, 3″-H], 3.19 (1 H, m, 4″-H), 2.88 (1 H, dd, *J* 15, 5, 6″-H), 2.63 (1 H, d, *J* 15, 6″-H), 2.46 (1 H, d, *J* 17, 5′-H), 2.4–2.2 (3 H, m, 5′-H, 10″-$H_2$), [1.90 (3 H, s), 1.78 (3 H, s) 6-$H_3$, 7′-$H_3$], 1.81–1.5 (4 H, m, 7″-$H_2$, 9″-$H_2$) and 1.5–1.4 (2 H, m, 8″-$H_2$) [1.03 (3 H, s), 0.99 (3 H, s) 8′-$H_3$, 9′-$H_3$]; $\delta_C$ (100 MHz; $CD_3OD$) 178.2, 173.4, 166.2, 156.7, 152.2, 146.2 (6 s, C-1, C-3, C-2′, C-4′, C-2″, C-11″), 136.8 (d, C-5), 129.5, 125.1, 124.1 (3 d, C-2, C-4, C-3′), 80.5 (s, C-1′), 63.5, 61.8, 57.1 (3 d, C-3a″, C-6a″, C-4″), 41.2 (s, C-6′), 40.4, 40.3, 38.5, 35.0, 26.8 (5 t, C-6″, C-7″, C-8″, C-9″, C-10″), 29.9, 25.8, 25.0 and 21.1 (4 q, C-6, C-7′, C-8′, C-9′); *m/z* (FAB; negative ion, thioglycerol–glycerol matrix 1:1) 503 ($M^-$ − H, 20%) and 102 (100).

### Biological assays

Barley seed (*Hordeum vulgare* L. cv. Himalaya), harvest 1985 (RAB-16 gene expression) and harvest 1991 (α-amylase inhibition) were obtained from Dept. of Agronomy, Washington State University, Pullman, USA.

**α-Amylase inhibition assay.** For this assay 8 sterilised, embryo-less barley half seeds were incubated in aqueous $CaCl_2$ [20 mmol $dm^{-3}$, containing $10^{-8}$ mol $dm^{-3}$ $GA_3$ plus ABA or ABA analogue; 500 $mm^3$] 25 °C, for 48 h. The activity of α-amylase secreted under these conditions was measured by modification of the literature method.[17] The incubation medium was removed. The half seeds were washed with water and the combined medium and washings made up to 10 $cm^3$ and frozen. The solutions were then thawed and centrifuged at 3000 rpm for 5 min to remove precipitated starch. Replicate aliquots (15–50 $mm^3$) from each sample were removed, made up to 100 $mm^3$ and treated with 100 $mm^3$ of a suspension made up from 1 Phadebas tablet (Pharmacia) in 1 $cm^3$ of buffer (100 mmol $dm^{-3}$ sodium acetate, 20 mmol $dm^{-3}$ $CaCl_2$). Samples were then incubated at 37 °C for 20 min and the reaction stopped by the addition of aqueous NaOH (0.2 mol $dm^{-3}$; 2.8 $cm^3$). The tubes were then centrifuged at 3000 rpm for 5 min after which the intensity of the blue chromophore released, by α-amylase action, from the Phadebas reagent was measured in a spectrophotometer set at 620 nm.

**RAB-16 gene expression assay.** A PIPES aqueous buffer containing mannitol (0.5 M), KCl (10 mmol $dm^{-3}$), $MgCl_2$ (1 mmol $dm^{-3}$), $CaCl_2$ (1 mmol $dm^{-3}$) and PIPES (pH 6.8; 10 mmol $dm^{-3}$), was used for both washing and incubation of the protoplasts. Barley aleurone protoplasts (4 × $10^5$ $cm^{-3}$) were pre-

pared essentially as described by Wang *et al*.[18] and incubated in buffer, with or without ABA or the analogue being tested, in the dark at 25 °C for 2 h. Total cellular RNA from $2 \times 10^6$ protoplasts was isolated and purified as described.[17] Northern blot analysis was carried out as previously described.[19] The amount of $^{32}$P-labelled probe hybridised to the RAB-16 mRNA was estimated by densitometry (Ultroscan KC densitometer LKB).

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## References

1 M. H. Beale, R. Hooley, S. J. Smith and R. P. Walker, *Phytochemistry*, 1992, **31**, 1459.
2 R. Hooley, S. J. Smith, M. H. Beale and R. P. Walker, *Aust. J. Plant. Physiol.*, 1993, **20**, 573.
3 M. H. Beale, R. Hooley, M. J. Lewis, S. J. Smith and J. Ward, *J. Chem. Soc., Perkin Trans. 1*, 1995, 657.
4 J. Giraudat, F. Parcy, N. Bertauche, F. Gosti, J. Leury, P. C. Morris, M. Bouvierdurand and N. Vartanian, *Plant Molecular Biology*, 1994, **26**, 1557.
5 C. Hornberg and E. W. Weiler, *Nature*, 1984, **310**, 321.
6 H. Bayley, *Photogenerated Reagents in Biochemistry and Molecular Biology*, Elsevier, Amsterdam, 1983.
7 J. A. R. Ladymann, J. R. Sanborn and E. E. Eelkema, *Phytochemistry*, 1988, **27**, 3751.
8 R. D. Willows and B. V. Milborrow, *Phytochemistry*, 1993, **32**, 869.
9 D. C. Walton, in *Abscisic Acid*, F. T. Addicott (ed.), Praeger, New York, 1983, 113.
10 R. M. Van der Meulen, F. Heidekamp, B. Jastorff, R. Horgan and M. Wang, *J. Plant Growth Regul.*, 1993, **12**, 13.
11 A. Flores and K. Dorffling, *J. Plant Growth Regul.*, 1990, **9**, 133.
12 E. W. Weiler, *Planta*, 1980, **148**, 262.
13 S. A. Quarrie and G. Galfre, *Biochemistry*, 1985, **151**, 389.
14 R. Wetzel and D. Soll, *Nucl. Acids Res.*, 1977, **4**, 1681.
15 K. G. Pinney and J. A. Katzenellenbogen, *J. Org. Chem.*, 1991, **56**, 3125.
16 D. R. C. Hite, W. H. Outlaw and M. A. Seavy, *Physiol. Plant.*, 1994, **92**, 79.
17 W. C. Barnes and A. B. Blakeney, *Die Starke*, 1974, **26**, 193.
18 M. Wang, B. Van Duijn and A. Schram, *FEBS Lett.*, 1991, **278**, 69.
19 M. Wang, B. Van Duijn, R. M. Van der Meulen and F. Heidekamp, in *Progress in Plant Growth Regulation*, Kluwer Academic Press, Dordrecht, The Netherlands, 1992, 635.

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