Pergamon

PII: S0031-9422(97)00299-9

Phytochemistry, Vol. 46, No. 2, pp. 215–222, 1997
© 1997 Elsevier Science Ltd. All rights reserved
Printed in Great Britain
0031-9422/97 $17.00+0.00

DEFINING STERIC, ELECTRONIC AND CONFORMATIONAL REQUIREMENTS OF CARRIER-MEDIATED UPTAKE OF ABSCISIC ACID IN BARLEY SUSPENSION CULTURE CELLS*

MICHEL PERRAS, PATRICIA A. ROSE,†‡ ERIC W. PASS,‡ K. BROCK CHATSON,‡ JOHN J. BALSEVICH‡ and SUZANNE R. ABRAMS‡

Department of Horticulture, University of Guelph, Guelph, ON N1G 2W1, Canada; ‡ Plant Biotechnology Institute, National Research Council of Canada, 110 Gymnasium Place, Saskatoon, SK S7N 0W9, Canada

(*Received* 26 *September* 1996; *in revised form* 13 *February* 1997)

Key Word Index—*Hordeum vulgare*; Gramineae; barley suspension culture cells; abscisic acid uptake; inhibitors; carrier; abscisic acid.

Abstract—The structural and conformational requirements of a carrier responsible for the saturable component of the uptake of the plant hormone (+)-abscisic acid in barley suspension culture cells have been probed through the use of a defined series of optically pure ABA analogues. Two analogues showed a tenfold increase over (+)-ABA in inhibiting the uptake of radiolabelled (+)-ABA. Results from different studies (molecular modelling, low temperature NMR spectroscopy and assays with sterically rigid analogues) have shown the likely conformation of ABA in the binding site of the carrier is that with the sidechain in an equatorial-like orientation. Structure/activity studies show that the C-1 carboxylic acid is essential for binding, the C-4 carbonyl is moderately important while the C-1′ hydroxyl group is not important for binding to the carrier.
© 1997 Elsevier Science Ltd

INTRODUCTION

It has been well documented that a saturable component in the uptake of the plant hormone (+)-abscisic acid (ABA, **1**) exists in many plant cell systems [1–8]. ABA can freely permeate membranes and cell walls, thus the role of the active carrier is unknown. One intriguing possibility is that the carrier could be involved in the ABA signal transduction pathway. Two recent reports [9, 10] suggesting that the initial perception site of the ABA signal is located on the outer face of the plasma membrane and may be an indication that the carrier is in fact an ABA receptor.

To date, strong inhibitors of ABA carrier-mediated uptake have not been found, yet they could prove to be useful tools for probing the role of the carrier. In the case of polar auxin transport (PAT), inhibitors have proven useful for defining how auxin functions in plants. For example, Okada *et al.* [11] have shown the requirement of the PAT system in the development of *Arabidopsis thaliana* floral bud formation and Avasarala *et al.* have phenocopied the *lanceolate* mutant in tomato using PAT inhibitors [12].

We have synthesized a series of optically pure ABA analogues and have tested them in barley suspension culture cells. We report for the first time analogues that compete ten times more strongly than (+)-ABA in the active uptake of radiolabelled (+)-ABA. Further, we have carried out a comprehensive structure–activity study using a series of optically pure ABA analogues, and have determined some of the structural requirements of the binding site within the putative carrier protein. This information will assist in the development of more effective inhibitors as well as to determine regions of the ABA molecule which can be used to attach a photoaffinity label, leading towards the isolation and identification of the carrier protein in future research.

RESULTS AND DISCUSSION

Inhibition experiments and K_i *determination*

The uptake of [^{3}H]-abscisic acid in barley (*Hordeum vulgare* L. cv. Heartland) cell cultures has been shown to be mediated through both non-saturable and saturable components [1]. The contribution of the saturable component of the uptake of ^{3}H-(+)-ABA can be measured by subtracting the diffusion component [measured in the presence of 100 times excess (50 μM) of non-radioactive (+)-ABA] from the total uptake

* NRCC No. 40713.

† Author to whom correspondence should be addressed.

[measured in the presence of labelled (+)-ABA (0.5 μM)]. Under these conditions, the saturable uptake, which accounts for 50–60% of the total uptake, can be gradually inhibited by increasing concentrations of unlabelled (+)-ABA or an ABA analogue.

The inhibition curves were plotted as 1/saturable uptake of (+)-^{3}H ABA against analogue concentration (Dixon plots). Dixon representations drawn on the values obtained from analogue concentrations ranging from 0 to 8 μM were found to be linear and could therefore be used to determine their respective K_i values, i.e. via the intercept on the abscissa. In that representation, (+)-ABA gave a measured K_i value of 3.1 and was used as a reference to assess the inhibitory activity of the different analogues. Analogues which show lower K_i values than 3.1 are more effective inhibitors than (+)-ABA, while analogues which show higher K_i values than 3.1 are either less effective or ineffective inhibitors of (+)-ABA uptake.

The inhibition of the saturable component of (+)-ABA uptake in barley cell suspension culture using ABA analogues is a rapid assay (10 min), which is an important criterion when carrying out structure–activity studies. Problems such as the metabolism or the stability of analogues, loss of label from ^{3}H-(+)-ABA through exchange, and longer term ABA effects of the analogues on the cells (such as cell growth inhibition) are reduced.

Milborrow has previously shown that a saturable uptake carrier for ABA in carrot suspension culture cells had a higher affinity for analogues having the same configuration as (+)-ABA [7] and we saw the same trend in our earlier work [1]. For this present structure–activity study, we chose to test analogues having the same relative stereochemistry at C-1′ as (S)-(+)-ABA (natural ABA).

The enantiomerically pure compounds were chosen to probe the importance of the polar functional groups on ABA (the C1-acid, C1′-hydroxyl and C4′-carbonyl), to assess the steric requirements of the ring for possible sites of attachment of photoaffinity labels and to identify the conformation adopted by ABA within the binding site (sidechain-axial or sidechain-equatorial). The results of the assay are shown in Table 1.

Electronic factors

C-1′ hydroxy group. One of the most active inhibitors for the carrier system was (+)-C-1′-methyl ether of ABA, **2**. This compound was nearly ten times more effective at inhibiting ABA uptake than ABA itself. The addition of the methyl group on the C-1′ oxygen not only increases the bulk at this centre, but also removes its hydrogen bonding ability. Previously, this compound was found to exhibit low activity in inhibiting germination of wheat embryos and reducing transpiration of wheat seedlings, yet showed a strong growth inhibition in maize cell culture [13]. The strong inhibition shown by compound **2** in this system shows that the hydroxyl group of ABA is not required for binding to the carrier, even though it is important in other physiological processes.

Windsor *et al.* [7] has previously shown that in bean root segments 1′-deoxyABA has a similar K_i value to ABA, and also suggests that the hydroxyl group is not involved in binding to the carrier. The strong inhibition shown by methyl ether **2** indicates other factors, such as conformational properties, may be involved in its strong binding to the carrier. These will be discussed later.

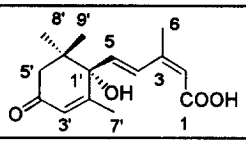
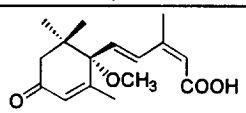
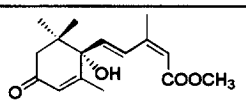
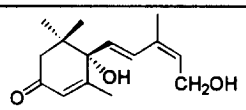
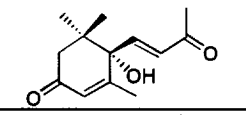
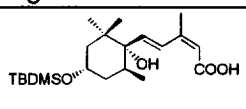
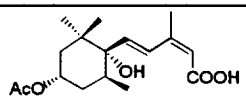
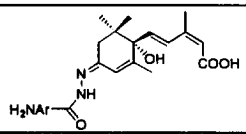
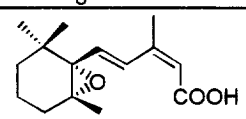
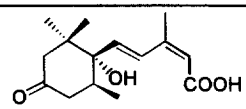
C-1 acid functionality. The acid group at the C-1 position is essential for the binding of ABA to the carrier, as shown by the lower K_i values of the analogues with different groups at C-1. The (+)-methyl ester of ABA **3** is a weak inhibitor with a K_i value of 15.3, while (+)-abscisyl alcohol **4** was completely ineffective with a K_i value greater than 100. Compound **5**, an analogue missing the last two carbons on the ABA sidechain, also was not an inhibitor for (+)-ABA uptake.

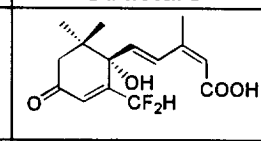
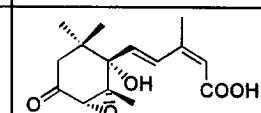
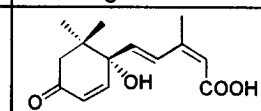
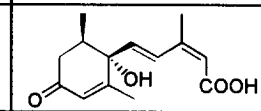
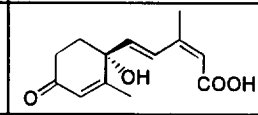
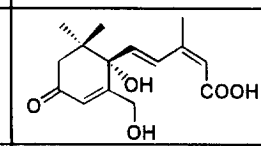
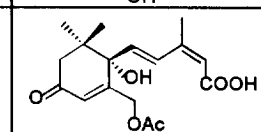
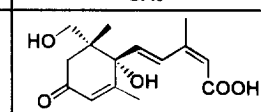
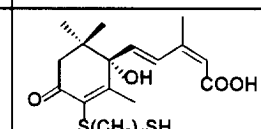
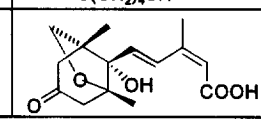
In related experiments, saturable uptake of the methyl ester of ^{3}H-(+)-ABA was not detected (results not shown), showing that the ester was not transported through the ABA carrier in barley cells. Similarly, Astle and Rubery [4] showed that in *Phaseolus coccineus* L. suspension culture cells that the methyl ester of ABA reversibly inhibited the carrier-mediated uptake of ABA, but was not itself a carrier substrate. These authors suggested that the methyl ester was not transported through the carrier as it did not have the potential to form a carboxylate anion required in the operation of an ABA^-/H^+ symport. Our results, along with those of Windsor *et al.* [7], who noted that all the analogues that could compete with ABA for saturable uptake sites in carrot suspension culture cells were capable of forming an acid at C-1, tend to support the idea that the carrier does indeed act as an ABA^-/H^+ co-transporter.

C-4′-carbonyl group. Increasing the steric bulk around the 4′-carbonyl of ABA is barely tolerated by the carrier. The addition of a bulky non-polar t-butyl dimethylsiloxy group on the same face of the ring as the C-1′ hydroxyl group gives an analogue which is not an inhibitor of (+)-ABA uptake (**6**, $K_i > 100$). Compound **7**, which has the smaller acetoxy substituent at the same position, is a poor inhibitor ($K_i = 16.0$), whereas 4′-aminobenzoyl hydrazone of (+)-ABA (**8**, $K_i = 8.8$), which has a large 4′-substituent in the plane of the ring, is a slightly stronger inhibitor than **7**, but is still weak in comparison to (+)-ABA.

Interestingly, complete removal of the carbonyl (**9**, $K_i = 0.3$) produces a strong competitive inhibitor. This compound was the strongest inhibitor found in the study, showing a tenfold increase over (+)-ABA in inhibiting the uptake of radiolabelled (+)-ABA. This may be due to the conformational properties of compound **9**, which will be discussed later.

Table 1. Effect of ABA analogues as competitive inhibitors of the saturable uptake of [^{3}H]-(+)-ABA by barley suspension culture cells

Entry	Structure	*Ki*
1		3.1
2		0.4
3		15.3
4		>100
5		>100
6		>100
7		16.0
8		8.8
9		0.3
10		0.5

Entry	Structure	*Ki*
11		0.9
12		2.7
13		4.3
14		4.7
15		11.8
16		4.0
17		6.9
18		7.9
19		2.1
20		>100

(+)-2′,3′-Dihydro-ABA **10** was a very effective inhibitor of the uptake of ^{3}H-(+)-ABA with a calculated K_i value of 0.5, much lower than that of the natural substrate. At high concentrations, the uptake of **10** is similar to that of ABA, as reported in our initial work [1]. Measuring uptake at lower concentrations differentiates the activities of the analogues, and in this case shows **10** to be much more active than ABA. Windsor *et al.* [7], who also tested the dihydro compound, found **10** to show a similar K_i to that of ABA in carrot suspension culture cells. Possibly there are differences between the steric or electronic requirements of the carrier binding site in different systems.

The 4′-carbonyl of **10** is no longer conjugated to a 2′,3′ double bond, therefore the oxygen of the carbonyl has a higher electron density than in the 4′-carbonyl of ABA. The more polarized carbonyl may be capable of forming a stronger hydrogen bond within the carrier, thus increasing the binding strength of the analogue. Similarly, activating the carbonyl of placing fluorines on the 7′-methyl group (**11**, $K_i = 0.9$), removing the double bond through epoxidation at the 2′,3′-position (**12**, $K_i = 2.7$) or addition of electron

rich groups at the 3′ position (**19**, $K_i = 2.1$) also produces stronger inhibitors. This correlation suggests that one can design effective competitive inhibitors of the carrier by increasing the hydrogen bonding capability at the C-4′-carbonyl.

Steric factors

Typically, photoreactive groups which are used for crosslinking ligands to proteins are aromatic azides. ABA is a small molecule, and the addition of a large aromatic group can greatly decrease the biological activity of the analogue. An ABA-photoaffinity probe must show high activity in order for it to be useful. In the carrier assay, using the C-1 acid or the C-4′-carbonyl as attachments sites for photoaffinity probes is not possible, as the C-1 is critical for binding to the carrier, and increased steric bulk around the C-4′ carbonyl produces very weak inhibitors. The strong inhibitor, C-1′-methyl ether of ABA, suggests the C-1′ group would be an excellent position to exploit. Unfortunately, the steric hinderance around this centre makes it synthetically very difficult to incorporate anything larger than a methyl group. We looked, therefore, at analogues with changes in steric bulk at the ring methyl groups and at the C-3′-position on the ring.

Removal of the 7′ or 8′ methyl groups from the ring didn't affect the K_i's of the analogues greatly (**13** and **14**, $K_i = 4.3$ and 4.7 respectively), though removal of both the 8′ and 9′ methyl groups gave much weaker inhibitors (**15**, $K_i = 11.8$). Interestingly, in a wheat embryo germination inhibition assay, all activity was lost by removal of the 7′-methyl group [14], showing a clear difference in the structural requirements for ABA-like activity in different processes.

Increasing the size of substituents on the 7′-methyl group from methyl (ABA, $K_i = 3.1$) to hydroxy (**16**, $K_i = 4.0$) to acetoxy (**17**, $K_i = 7.0$) gave less effective inhibitors, but shows that bulk can be tolerated in this position. Similarly, increasing the size and hydrophilicity at the 8′-methyl causes a slight decrease in competitive binding to the carrier as seen with 8′-hydroxyABA (**18**, $K_i = 8.0$).

One of the more interesting analogues tested had a butane dithiol group linked at the 3′-carbon, **19**, and was found to compete more strongly than ABA in the uptake of radiolabelled ABA with a K_i of 2.1. This may be a good position for attaching labelled probes in future research. The 3′-substituted ABA has been conjugated to Sepharose and found to be useful in purifying monoclonal antibodies to (+)-ABA [15].

ABA metabolites

7′-HydroxyABA **16** and PA **20** have previously been tested by Windsor *et al.* [7] as inhibitors of the saturable uptake of ABA in carrot cell suspension cultures, who found, as in the barley system, that 7′-hydroxyABA is as effective an inhibitor as ABA, while phaseic acid (PA) does not inhibit the uptake of (+)-ABA. Interestingly, we have found that the immediate precursor to PA, 8′-hydroxyABA **18**, is moderately active. Both 7′-hydroxyABA and 8′-hydroxyABA have shown some activity in ABA assays. 7′-HydroxyABA inhibits GA-stimulated α-amylase in barley aleurone layers [16] and 8′-hydroxyABA acts like ABA in *B. napus* embryos in fatty acid biosynthesis [17]. PA, on the other hand, is typically inactive in ABA bioassays. The biological activity of the ABA metabolites correlates to their measured K_i values in the barley carrier assay.

Conformational studies

In the barley carrier assay, the inability of natural PA (**20**, $K_i > 100$) to inhibit ABA uptake (PA is sterically held in a chair conformation with the sidechain axial) and the strong inhibition shown by epoxy β-ionylidene acetic acid (**9**, $K_i = 0.3$) (a compound where the sidechain is in a more equatorial-like conformation due to the constraints of the ring epoxide) suggested to us that the conformation of ABA in the carrier is likely a pseudo-chair with an equatorial sidechain. To test this theory, we carried out conformational analyses on ABA and some of the stronger inhibitors of (+)-ABA uptake through NMR spectroscopic experiments and computational chemistry. Similar to X-ray crystallography, these methods help define the lowest energy conformations a compound may adopt, which only gives an indication of the conformation within a binding site.

The crystal structure of ABA was reported in 1977 by two groups [18, 19], with both showing that the ABA molecule in the crystalline state adopts a pseudo-chair conformation with the sidechain axial. Willows and Milborrow [20] carried out molecular dynamic studies on ABA, and were unable to find direct evidence of low energy conformations of ABA having a pseudo-chair conformation with the sidechain in an equatorial orientation. NMR spectroscopic experiments in solution measuring nuclear Overhauser effects (NOE) [21] did show the presence of the axial conformation through an NOE signal between the C-5′ and C-5 protons. However, the experimental evidence did not eliminate the possibility of there being a significant population of ABA in an equatorial conformation in solution. In a non-rigid molecule such as ABA, the NOE signals which are observed are an average signal derived from any contributing conformations in solution. It would be unlikely that one could observe the equatorial conformation of ABA by NOE, as it would not be expected to have any signals distinctive from the signals of the axial conformation, such as the very distinctive C-5′H–C5H signal in the axial conformation.

To investigate the available low energy conformations of ABA, a molecular modelling experiment was carried out using RANDOMSEARCH software by TRIPOS. We used an energy minimized start-

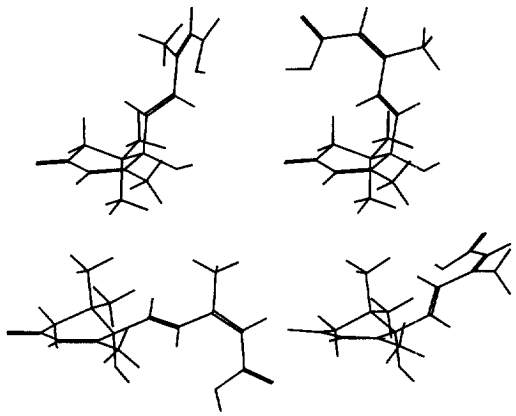


Fig. 1. Minimum energy conformations for ABA found through RANDOMSEARCH calculations. Axial sidechain with C-5H oriented towards the ring (top left, $E = 0.95$ kcal mol^{-1}) and away from the ring (top right, $E = 0.85$ kcal mol^{-1}). Equatorial sidechain with C-5H oriented towards the ring (lower left, $E = 0.67$ kcal mol^{-1}) and away from the ring (lower right, $E = 2.40$ kcal mol^{-1}).

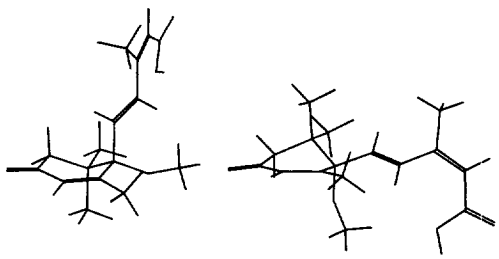


Fig. 2. Minimum energy conformations for **2** (C-1′-methyl ether of ABA) found through RANDOMSEARCH calculations. Axial sidechain (left, $E = 9.47$ kcal mol^{-1}). Equatorial sidechain (right, $E = 2.93$ kcal mol^{-1}).

ing structure generated from the coordinates given for the crystal structure of ABA [18]. From the search four families of conformations were found, all showing the ring in a pseudo-chair conformation. Two families have axial sidechains; one with the C5 hydrogen oriented towards the ring and one away from the ring. Similarly, the remaining two families of conformations have equatorial sidechains, with the C5 hydrogen either pointing towards the ring or away from the ring. Each family of structures contain various orientations of the rotatable bonds within the sidechain. The lowest energy conformation of each family is shown in Fig. 1. The lowest energy structure found was ABA with the sidechain in an equatorial conformation, and the C5 hydrogen oriented away from the ring (Fig. 1, lower left hand structure). The corresponding conformation with the C5 hydrogen oriented away from the ring (Fig. 1, lower right hand structure) is 1.8 kcal per mole higher in energy due to steric interactions between the hydrogens on the sidechain and the methyl groups on the ring. The difference between the lowest energy sidechain-axial (the structure adopted in the crystalline state, upper left hand structure in Fig. 1) and sidechain-equatorial conformations was less than 0.5 kcal per mole, suggesting that the two conformations are equally attainable.

One of the more active analogues tested in the series was the C-1′-methyl ether of ABA **2**. Analysis of a conformational search on this molecule showed that the sidechain-equatorial conformation was 7 kcal per mole lower in energy than the sidechain-axial conformation (Fig. 2). This large difference in energy is due to the bulky methoxy group on the C-1′-carbon, in which steric interactions with the ring methyl groups are reduced when the sidechain is in an equatorial orientation.

To confirm the modelling information experimentally, low temperature NMR spectroscopy was carried out on ABA, the methyl ester of ABA (MeABA) and the methyl ester of C-1′-methyl ether of ABA. Low temperature NMR spectroscopy (in $CDCl_3$) the methyl ether showed the presence of both sidechain-equatorial and -axial conformers in solution. At 18° below the coalescence temperature of 257 K, the two conformers were present in a 3:1 ratio (Fig. 3), with presumably the major component being the lower energy sidechain-equatorial conformation. The added steric bulk of the methyl group at the C-1′-hydroxy group increases the energy required for the molecule to interconvert between conformations (calculated to be *ca* 12 kcal mol^{-1} from the coalescence temperature), slowing the rate of interconversion enough at low temperature that both conformations can be observed. An attempt was made to observe the conformers of MeABA (in $CDCl_3$ and CD_3CD_2OD) and ABA (in CD_3CD_2OD) by low temperature NMR spectroscopy. However, we were unable to reach a temperature low enough to see two conformers in any case. This suggests (for example in the case of ABA) that there is either one conformation of ABA present in solution, or the interconversion between conformers is so facile that even at 175 K the frequency of the ring inversion is still faster than the rate of the nuclear transition observed. The latter is the more likely possibility as the C-1′-methyl ether of ABA (which would have a much higher energy for the ring inversion than ABA) did show both conformations.

If the preferred conformation within the binding site of the carrier is one having an equatorial sidechain, then the strong inhibition of ABA uptake shown by C-1′-methyl ether of ABA **2** may be due to its higher probability of being in the preferred conformation. Similarly, the strong inhibition shown by epoxy β-ionylidene acetic acid **9** (which is held in a more equatorial-like conformation) supports this idea.

Conclusions

We have found several analogues of ABA which are strong inhibitors of the carrier-mediated uptake of ABA in barley suspension cell cultures. These may prove to be useful tools for investigating the role of the

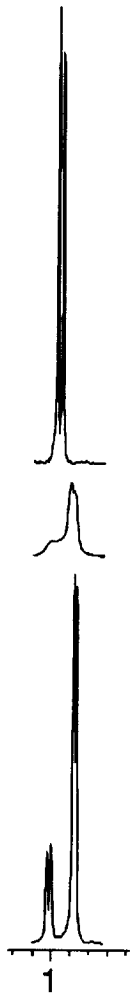


Fig. 3. Variable temperature NMR spectra of the methyl ester of **2** showing the 8′ and 9′ methyl groups at room temperature (top, 298° K), coalescence temperature (middle, 257° K) and showing both conformers (lower, 240° K).

ABA transport system in plants. Structure–activity, molecular modelling and low temperature NMR spectroscopic analysis results lead us to suggest that the ABA molecule and the analogues which are strong inhibitors adopted a conformation within the binding site of the carrier with the ring in a pseudo-chair conformation and an equatorial sidechain (see Fig. 4). The strong inhibition shown by the C-1′ methyl ether shows that the C-1′-hydroxyl group is not involved in binding to the carrier and may be facing away from the docking site. The carboxylic acid group is absolutely essential for inhibiting ABA uptake, strongly suggesting it is a key binding point to the carrier. The carbonyl at the C-4′ position is not essential, however increasing the hydrogen bonding capabilities of the 4′-carbonyl greatly increases the strength of the inhibition shown by the analogue, while increasing the steric bulk around the carbonyl has the opposite effect. This suggests that the carbonyl is weakly bound to the carrier. There is some flexibility for additional bulk around the 3′, 7′ area of the molecule, which may allow for the addition of a photoaffinity label in these positions.

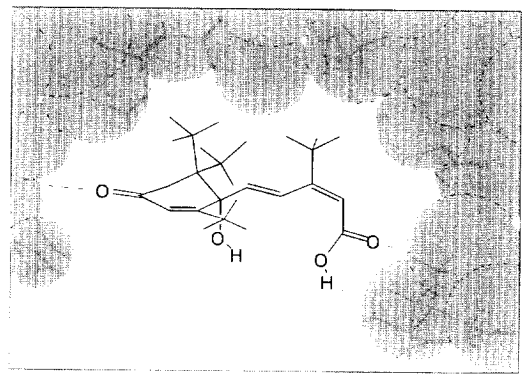


Fig. 4. ABA bound to the carrier.

EXPERIMENTAL

Plant material. Barley (*Hordeum vulgare* L. cv. Heartland) cell suspension cultures were provided by Dr K. Kartha, Plant Biotechnology Institute, Saskatoon. The suspensions originated from callus cultures derived from embryos as described by Kartha [22] and were maintained by subculturing every week into fresh Murashige and Skoog [23] medium supplemented with B5 vitamins and 5.0×10^{-6} M 2,4-D.

Uptake measurements. All uptake experiments were performed with 3- to 4-day-old barley cell suspension cultures (exponential growth phase). The cells were filtered through a 500 μm nylon membrane, collected on a piece of Miracloth paper and rinsed with sterile distilled H_2O. The cells were then resuspended in 0.2 M sodium phosphate–0.1 M citrate buffer adjusted to pH 4.5 supplemented with 2% sucrose at a density of 1 g fr. wt per 10 ml of buffered medium. The new cell suspension was put on a reciprocal shaker at 120 rpm and allowed to adjust to the new medium for at least 1 hr prior to uptake studies.

The uptake was initiated by adding 0.5 ml of the fresh cell suspension to the uptake medium consisting of 1 μl of 0.25 mM ^{3}H-(+)-ABA, 2 ml of phosphate–citrate buffer pH 4.5 containing 2% sucrose and, 1 μl of either EtOH (for the measure of total uptake) or 25 mM nonradioactive ABA (for the measure of the diffusion component). Both the total uptake and the diffusion component of uptake of ^{3}H-(+)-ABA was measured in the presence of 0, 1, 2, 8 and 20 μM of the different ABA analogues, in triplicate runs. The uptake was terminated after 10 min by adding 2 ml of distilled H_2O and immediately filtering the cells on a Whatman glass microfibre filter (GF/A) under vacuum. The filters (and cells) were then rinsed with 5 ml of phosphate–citrate buffer, transferred to plastic vials and 5 ml of scintillation liquid (ScintiVerse II, Fisher) was added. The vials were vigorously shaken and kept in the dark overnight before counting to ensure that all radioactivity had been extracted from the cells.

In all uptake experiments, the radioactivity which appeared to be associated with the free space as measured after a very short incubation period (less than 10 s) was routinely subtracted as indicated in the procedure followed by Bianco-Colomas *et al.* [5].

Chemicals. Racemic ABA [(±)-*cis,trans*-abscisic acid] was purchased from Sigma. S-(+)-MeABA **3** was obtained through resolution of the racemic mixt. of the methyl esters on a chiral HPLC column [24], and was subsequently hydrolysed to give S-(+)-ABA **1**. (+)-ABA was tritiated by base mediated exchange with tritiated H_2O (2.5 Ci–0.5 ml) and obtained at a specific activity of 1.78 GBq $mmol^{-1}$ (or 54.0 Ci mol^{-1}) [25]. The optically pure enantiomers of the following compounds were synthesized as followed or as previously described: compound **2** [13]; **4** [26]; **5** [(+)-dehydrovomifoliol] was obtained in enantiomerically pure form after sepn of the racemic material, which was synthesized in a manner similar to that for ABA [27], and gave identical spectral data and equal and opposite optical rotation as that previously reported for (−)-dehydrovomifoliol [28]; compounds **6** and **7** were prepd through standard derivatization procedures of the sodium borohydride reduction product of (+)-dihydroABA **10**; compound **8** [29]; epoxy-β-ionylidene acetic acid **9** was synthesized racemically as previously published [30] and its enantiomers sepd as their methyl esters on a chiral HPLC column similarly to methyl ABA; **10** [31]; **11** [32]; **12** [33]; **13** [14]; **14** [35]; **16** [34]; compound **17** was prepd through the acetylation of 7′-hydroxyABA **16** by standard methods; **18** [17]; and **19** [15]. The synthesis of **15** will be reported elsewhere. The natural form of phaseic acid (PA) **20** was produced through the biotransformation of (+)-ABA using Black Mexican Sweet (BMS) maize suspension culture cells [25].

Molecular modelling. Structures for ABA and analogues were generated with SYBYL (version 6.1a) software by TRIPOS Associations Inc., employing the TRIPOS force field. Minimum energy conformations were found by employing the RANDOMSEARCH program, which randomly sets the torsion angle of three bonds within the molecule, minimizes the structure to the closest minimum, discards structures over a preset energy level, and then runs a comparison with conformations which have already been found. Over 240 unique structures of ABA were generated in the search, which through hierarchical cluster analysis showed four families of conformations. A similar procedure was carried out for the methyl ether of ABA and other analogues.

Low temperature NMR spectroscopy. Variable temp. NMR spectra were obtained on a Bruker AMX-500 spectrometer, or in the case of MeABA in CD_3CD_2OD on a Bruker AM-360-Wb spectrometer. The NMR spectra of ABA and MeABA were obtained in CD_3CD_2OD from 298 to 175 K for ABA and from 300 to 140 K for MeABA, with no sepn of the signals into separate conformers. MeABA was run in $CDCl_3$, to 223 K (just above the freezing point of $CDCl_3$), with the same result. Experiments with the methyl ether of methyl ABA were run in $CDCl_3$ at intervals from 298 to 240 K. At 240 K the spectra showed two complete sets of signals, in a 3:1 ratio: δ 7.76 (major, *d*, 1H, J = 16.1 Hz, H-4), 7.62 (minor, *d*, 1H, J = 16.3 Hz, H-4), 6.26 (minor, *d*, 1H, J = 16.4 Hz, H-5), 6.06 (major, *s*, 1H, H-3′). 6.00 (minor, 1H, *s*, H-3′), 5.91 (major, 1H, *d*, J = 16.2 Hz, H-4). 5.74 (major + minor, 1H each, *s*, H-2), 3.69 (major, 3H, *s*, $COOCH_3$), 3.65 (minor, 3H, *s*, $COOCH_3$), 3.47 (minor, 3H, *s*, OCH_3), 3.44 (major, 3H, *s*, OCH_3), 2.89 (major, 1H, *d*, J = 17.2 Hz, H-5′), 2.27 (minor, 2H, *d*, J = 16.6 Hz, H-5′), 2.04 (major + minor, 3H each, *s*, H-7′ or H-6 overlapping second H-5′), 2.00 (major + minor, 3H each, *s*, H-7′ or H-6 overlapping H-5′), 1.05 (minor, 3H, *s*, H-8′ or H-9′), 1.04 (minor, 3H, *s*, H-8′ or H-9′), 0.92 (major, 3H, *s*, H-8′ or H-9′), 0.90 (major, 3H, *s*, H-8′ or H-9′).

The coalescence temp. for the two conformers was 257 K, from which the energy barrier for the conversion between sidechain-axial and sidechain-equatorial conformations was calcd to be *ca* 12 kcal mol^{-1} [36].

Acknowledgements—The authors would like to thank Angela Shaw and Nancy Lamb for the synthesis of analogues, Greg Bishop for separating racemic methyl ABA into its enantiomers, Lawrence Hogge and Doug Olson for mass spectral analysis of new derivatives and Adrian Cutler and Nick Irvine for critical reviews of the manuscript.

REFERENCES

1. Perras, M. R., Abrams, S. R. and Balsevich, J. J., *Journal of Experimental Botany*, 1994, **45**, 1565.
2. Astle, M. C. and Rubery, P. H., *Planta*, 1980, **150**, 312.
3. Astle, M. C. and Rubery, P. H., *Planta*, 1983, **157**, 53.
4. Astle, M. C. and Rubery, P. H., *Journal of Experimental Botany*, 1987, **38**, 150.
5. Bianco-Colomas, J., Bathe, P., Orlandi, M. and Le page-Degivry, M. T., *Plant Physiology*, 1991, **95**, 990.
6. Windsor, M. L., Milborrow, B. V. and McFarlane, I. J., *Plant Physiology*, 1992, **100**, 54.
7. Windsor, M. L., Milborrow, B. V. and Abrams, S. R., *Journal of Experimental Botany*, 1994, **45**, 227.
8. Daeter, W. and Hartung, W., *Planta*, 1993, **191**, 41.
9. Anderson, B. E., Ward, J. M. and Schroeder, J. I., *Plant Physiology*, 1994, **104**, 1177.
10. Gilroy, S. and Jones, R. L., *Plant Physiology*, 1994, **104**, 1185.
11. Okada, K., Ueda, J., Komaki, M. K., Bell, C. J. and Shimura, Y., *The Plant Cell*, 1991, **3**, 677.
12. Avasarala, S., Yang, J. and Caruso, J. L., *Journal of Experimental Botany*, 1996, **47**, 709.

13. Rose, P. A., Lei, B., Shaw, A. C., Barton, D. L., Walker-Simmons, M. K. and Abrams, S. R., *Phytochemistry*, 1996, **41**, 1251.
14. Walker-Simmons, M. K., Rose, P. A., Shaw, A. C. and Abrams, S. R., *Plant Physiology*, 1994, **106**, 1279.
15. Balsevich, J. J., Bishop, G. G. and Banowetz, G. M., *Phytochemistry*, 1997, **44**, 215. NRCC no. 40019.
16. Hill, R. D., Liu, J.-H., Durnin, D., Lamb, N., Shaw, A. and Abrams, S. R., *Plant Physiology*, 1995, **108**, 573.
17. Zou, J., Abrams, G. D., Barton, D. L., Taylor, D. C., Pomeroy, M. K. and Abrams, S. R., *Plant Physiology*, 1995, **108**, 563.
18. Ueda, H. and Tanaka, J., *Bulletin of the Chemical Society of Japan*, 1977, **50**, 1506.
19. Schmalle, V. H. W., Klaska, K. H. and Jarchow, O., *Acta Crystallography*, 1977, **B33**, 2218.
20. Willows, R. D. and Milborrow, B. V., *Phytochemistry*, 1993, **34**, 233.
21. Milborrow, B. V., *Biochemical Journal*, 1984, **220**, 325.
22. Kartha, K. K., Chibbar, R. N., Georges, F., Leung, N., Caswell, K., Kendall, E. and Qureshi, J., *Plant Cell Reports*, 1989, **8**, 429.
23. Murashige, T. and Skoog, F., *Physiologia Plantarum*, 1962, **15**, 473.
24. Dunstan, D. I., Bock, C. A., Abrams, G. D. and Abrams, S. R., *Phytochemistry*, 1991, **31**, 1451. NRCC no. 33520.
25. Balsevich, J. J., Cutler, A. J., Lamb, N., Friesen, L. J., Kurz, E. U., Perras, M. R. and Abrams, S. R., *Plant Physiology*, 1994, **106**, 135.
26. Rose, P. A., Abrams, S. R., and Shaw, A. C., *Tetrahedron: Asymmetry*, 1992, **3**, 443.
27. Mayer, H. J., Rigassi, N., Schwieter, U. and Weedon, B. C. L., *Helvetia Chimica Acta*, 1976, **59**, 1424.
28. Meyers, A. I. and Sturgess, M. A., *Tetrahedron Letters*, 1989, **30**, 1741.
29. Quarrie, S. A. and Galfre, G., *Analytical Biochemistry*, 1985, **151**, 389.
30. Tamura, S. and Nagao, M., *Agriculture and Biological Chemistry*, 1970, **34**, 1393.
31. Lamb, N. and Abrams, S. R., *Canadian Journal of Chemistry*, 1990, **68**, 1151.
32. Rose, P. A., Abrams, S. R. and Gusta, L. V., *Phytochemistry*, 1991, **31**, 1105.
33. Todoroki, Y., Hirai, N. and Ohigashi, H., *Tetrahedron*, 1995, **51**, 6911.
34. Nelson, L. A. K., Shaw, A. C. and Abrams, S. R., *Tetrahedron*, 1991, **47**, 3259. NRCC no. 32469.
35. Rose, P. A., Lei, B., Shaw, A. C., Walker-Simmons, M. K., Napper, S., Quail, J. W. and Abrams, S. R., *Canadian Journal of Chemistry*, 1996, **74**, 1836.
36. Eliel, E. J., Allinger, N. L., Angyal, A. J. and Morrison, G. A., *Conformational Analysis*. Interscience, New York, 1965, p. 152.