ABSTRACT

Inhibition of cell expansion of excised embryonic axes of Phaseolus vulgaris was used to evaluate the growth-inhibiting activity of abscisic acid and related compounds. None of the 13 compounds tested was as active as abscisic acid. 4-Hydroxyisorphinone, a substance representative of the abscisic acid ring system was essentially inactive; cis, trans-3-methylsorbinic acid, a compound resembling the side chain of abscisic acid had low activity; and cis,trans-3-β-ionolidineacetic acid was one-sixth as active. Loss of the ring double bond results in a drastic decrease in biological activity. Comparison of our results with those reported previously leads to the suggestion that the double bond of the cyclohexyl moiety may have an important function in determining the degree of activity of cis,trans-ionolidineacetic acids. Two modes of action are discussed. It seems possible that the ring double bond is involved in covalent bonding in binding of the abscisic acid analogue to macromolecules. This may require formation of an intermediate epoxide. It can also be argued that stereochemical differences between cyclohexane derivatives are important factors in determining the degree of biological activity.

While noninjurious inhibition of plant growth is one of the well documented effects of (S)-abscisic acid (Structure I), fewer papers have dealt with structure-activity relations (1). Apparently, hydrolyzable substituents on the carboxylic acid group, such as esters (10) or glycosides (2), alter activity by their effect on transport (10). Both enantiomers of ABA are active, and an ABA analogue that does not have a tertiary hydroxyl group is, nevertheless, a strong growth inhibitor (12). Ionolidineacetic acids which do not have oxygenated substituents around the cyclohexene ring have been shown to be active (2), and this is also true of an epoxide (18). The activity of the epoxide isomer of ABA has been attributed to its reaction products, which under typical physiological conditions include about 20% ABA (17). While a number of authors have shown that the trans,trans analogue of ABA has much lower activity than ABA (1), only small differences were reported for growth inhibition of Lemna (14).

In the work reported here we employed the growth inhibition of excised embryonic axes of Phaseolus vulgaris. The method utilizes storible biological material and requires only a 12-hr incubation period. Growth, which is due primarily to cell elongation of existing cells, is followed by fresh weight increase (19). With this procedure we have evaluated the effectiveness of ABA and 12 other compounds and have observed more than 500-fold differences in potency. These test compounds were used to provide information on the role of the stereochernetry, functional groups, and carbon skeleton in determining the activity of ABA as a growth inhibitor.

EXPERIMENTAL PROCEDURE

Growth Assay Procedure. Axes are excised from the dry seed of P. vulgaris L. (var. White Marrowfat). Approximately 100 mg of axes, weighed to the nearest 0.1 mg, are incubated in 50-ml Erlenmeyer flasks in 2 ml of 0.01 m potassium phosphate buffers, pH 7.0, containing 20 μg m chloramphenicol and appropriate concentrations of test compound. The flasks are shaken at 26°C in a Dubnoff metabolic incubator, and triplicates are run for each control and test substance. Plating of the incubation medium at the end of the test period showed the bacterial contamination to be less than 1000 cells/ml. After a 12-hr incubation the axes are blotted and weighed to the nearest 0.1 mg. The parameter used for growth is the fresh weight increase above that due to imbibition (Fig. 1) (19). For those compounds for which a concentration could be found that gave more than 50% inhibition, the concentration necessary to give 50% inhibition was estimated from semiog plots of percentage inhibition versus concentration.

Test Compounds. (RS)-ABA and its trans,trans analogue were synthesized by the procedure of Cornforth et al. (5). The cis,trans- and trans,trans-3-β-ionolidineacetic acids [3-methyl-5-(2',6',6'-trimethyl-1'-cyclohexenyl)-2,4-pentadienoic acids] were prepared by the procedure of Schwiet er et al. (15). The cis,trans- and trans,trans-3-β-dehydroionolidineacetic acids [3-methyl-5-(2',6',6'-trimethyl-1',3'-cyclohexadienyl-2,4-pentadienoic acids] were also prepared by the above procedure (15). 4-Hydroxyisorphinone, (RS)-3,5,5-trimethyl-4-hydroxy-2-cyclohexene-l-one, was synthesized from isophorone (9).

cis,trans-2-dihydroionolidineacetic acid [3-methyl-5-(2',6',6'-trimethylcyclohexenyl)-2,4-pentadienoic acid] was prepared from dihydroyclocticral (4) and ethyl 4-bromo-3-methylcrotonate (8) by a Reformatsky reaction. The intermediate lactone on treatment with sodium ethoxide gives high yields of the cis,trans isomer. This procedure has been used previously for the preparation of cis,trans-3-β-ionolidineacetic acid (6).

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1 This study was supported by National Science Foundation Grant GB4262 and United States Department of Agriculture Forest Service Grant 3-4040.

2 Abbreviation: ABA: abscisic acid.
To 5 g of dihydrocyclocitrinal in 10 ml of tetrahydrofuran were added 5 g of 30 mesh activated zinc. The mixture was heated to reflux in a nitrogen atmosphere and 8.6 g of ethyl 4-bromo-3-methylcrotonate in 10 ml of tetrahydrofuran were added slowly. After addition of the halide was completed, the mixture was refluxed for another 20 min. The cooled mixture was treated with excess 10% acetic acid in water, stirred vigorously for 1 h, extracted with ether, washed with water, and dried over anhydrous magnesium sulfate. Distillation at 3 mm yielded a fraction boiling at 140 to 147 °C which crystallized on standing at room temperature. Recrystallization from hexane and cooling in a Dry Ice-acetone bath yielded 1.76 g of lactone, with a melting point of 72 to 74 °C; R<sub>f</sub> 0.28 on 1-mm thin layer chromatographic plates coated with Silica Gel GF-254, benzene, ether, hexane (20:25:55, v/v/v); Calculated for C<sub>15</sub>H<sub>24</sub>O<sub>2</sub>: C, 76.23; H, 10.24. Found: C, 76.25; H, 10.30.

To 798 mg of the above lactone, 11 ml of methanol containing sodium methoxide, prepared by addition of 77.7 mg of sodium, were added. The solution was stored for 30 min at room temperature, the methanol was evaporated below 40 °C, and 10 ml of water was added. This solution was extracted with ether to remove unreacted lactone. The aqueous phase was acidified with 85% phosphoric acid and extracted with ether. The ether extract was washed with water, dried over anhydrous magnesium sulfate, and concentrated at reduced pressure. The remaining oil crystallized on scratching, yielding 210 mg of acid, with a melting point of 114 to 117 °C from acetonitrile; Calculated for C<sub>9</sub>H<sub>12</sub>O<sub>2</sub>: C, 76.23; H, 10.24. Found: C, 75.97; H, 10.28.

Cis-crotonic acid was prepared from the potassium salt shortly before use (13). The crude salt, 5.9 g, was dissolved in 10 ml of water and extracted with ether, and the extract was discarded. The aqueous phase was acidified with 2 N hydrochloric acid and extracted with ether. The extract was washed with small amounts of water and dried over anhydrous magnesium sulfate, and the solvent was removed under reduced pressure. The residue was sublimed at room temperature with good cooling. The sublimate was crystallized from 2 volumes of pentane, melting point 14 to 15 °C. Nuclear magnetic resonance spectra gave no evidence for the presence of any trans isomer. Monitoring of the ultraviolet spectrum of dilute aqueous solutions during 24-hr storage at 26 °C indicated no loss of light absorption attributable to polymerization.

Cis-trans-β-methylsorbic acid was synthesized according to Wiley and Smith (20).

RESULTS

Fresh weight increase of the embryonic axes is a stepwise process (Fig. 1). After a rapid imbibition phase a plateau is reached, followed by a linear increase and then a leveling off. Previous work has shown that the stage of linear increase in fresh weight is due to cell elongation and can be inhibited by antibiotics that interfere with RNA and protein synthesis (19). From Figure 1 it is noted that (RS)-ABA decreases the growth rate without affecting the imbibition or plateau stages. Those (RS)-ABA concentrations that are only partially inhibitory allow linear growth for a longer period than is observed with the controls. A 12-h incubation period was chosen for the determination of the growth-inhibitory activity of the test substances. During this time period linearity of the axes fresh weight increase is maintained, and the inhibitory effects of the various compounds are readily measured.

Table I summarizes the growth response data obtained with 12 compounds. The concentrations necessary to give 50% inhibition of the fresh weight increase between the 5th and 12th hr are reported and are compared to the response given by (RS)-ABA. While none of the tested compounds were more active than (RS)-ABA, all ionylidenecac acid derivatives were more effective than compounds that do not possess this carbon skeleton. Among the substances having this carbon skeleton, compounds A to G, the variation in potency is almost 70-fold. In the three cases in which the effect of the stereochemistry of the side chain can be compared—compounds A and C, B and F, and D and G—the cis, trans configuration leads to higher activity than the trans, trans form. The degree of unsaturation of the ring of the ionylidenecac acid has a strong effect. Maximum activity is observed with the cyclohexene derivative, compound B. An 8- to 10-fold decrease in activity results from introduction of a second double bond, compound D, or from the saturation of the ring, compound E.

Compounds H, I, and J are representative of the ABA side chain and have low but measurable activity. It seems noteworthy that cis-crotonic acid, which has only one double bond, is as effective as cis, trans-β-methylsorbic acid, and that trans, trans-sorbic acid is less active than cis, trans-β-methylsorbic acid. The ring substituents of (RS)-4-hydroxyisophorone, compound K, are very closely related to those of (RS)-ABA. Yet this compound is probably inactive in this system.

Todomamic acid is a sesquiterpenic keto acid which has been
<table>
<thead>
<tr>
<th>Compound</th>
<th>Name</th>
<th>Formula</th>
<th>Concentration for 50% Inhibition μM</th>
<th>Relative Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. (RS)-ABA</td>
<td></td>
<td></td>
<td>5.5</td>
<td>100</td>
</tr>
<tr>
<td>B. Cis, trans-β-ionylideneacetic acid</td>
<td></td>
<td></td>
<td>30</td>
<td>18</td>
</tr>
<tr>
<td>C. (RS)-Trans, trans-ABA</td>
<td></td>
<td></td>
<td>95</td>
<td>6</td>
</tr>
<tr>
<td>D. Cis, trans-dehydroionylideneacetic acid</td>
<td></td>
<td></td>
<td>250</td>
<td>2</td>
</tr>
<tr>
<td>E. Cis, trans-dihydroionylideneacetic acid</td>
<td></td>
<td></td>
<td>300</td>
<td>2</td>
</tr>
<tr>
<td>F. Trans, trans-β-ionylideneacetic acid</td>
<td></td>
<td></td>
<td>350</td>
<td>1.5</td>
</tr>
<tr>
<td>G. Trans, trans-dehydroionylideneacetic acid</td>
<td></td>
<td></td>
<td>350</td>
<td>1.5</td>
</tr>
<tr>
<td>H. Cis-crotonic acid</td>
<td></td>
<td></td>
<td>650</td>
<td>1</td>
</tr>
<tr>
<td>I. Cis, trans-β-methylsorbic acid</td>
<td></td>
<td></td>
<td>750</td>
<td>0.5</td>
</tr>
<tr>
<td>J. Trans, trans-sorbic acid</td>
<td></td>
<td></td>
<td>1700</td>
<td>0.3</td>
</tr>
<tr>
<td>K. Isovaleric acid</td>
<td></td>
<td></td>
<td>1800</td>
<td>0.3</td>
</tr>
<tr>
<td>L. (RS)-4-Hydroxyisophorone</td>
<td></td>
<td></td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

1 Inhibition is low and not concentration-dependent.

isolated from bisulfite-treated pulpwood (18). Its methyl ester is isolated from balsam fir and has strong juvenile hormone activity in the hemipteran bug (3). Its structure (Structure II) is drawn here to stress its resemblance to ABA. Its assay as a growth inhibitor showed very low activity: 30% inhibition at a concentration of 800 μM.

*Cis, trans-β-ionylideneacetic acid*, compound B, had one-sixth the activity of (RS)-ABA in the bean embryonic axes system. Because of this relatively high activity we also tested this substance as a germination inhibitor of embryos excised from
STRUCTURE-ACTIVITY RELATIONS

Table II. Growth-inhibitory Activity of cis,trans-Ionylideneacetic Acid Derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>Activity</th>
<th>Assay Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td>High</td>
<td>Several</td>
</tr>
<tr>
<td>B.</td>
<td>High</td>
<td>Bean axes, rice seedlings</td>
</tr>
<tr>
<td>M.</td>
<td>High</td>
<td>Wheat embryo</td>
</tr>
<tr>
<td>N.</td>
<td>High</td>
<td>Several</td>
</tr>
<tr>
<td>O.</td>
<td>High</td>
<td>Rice seedlings</td>
</tr>
<tr>
<td>P.</td>
<td>High</td>
<td>Bean axes</td>
</tr>
<tr>
<td>Q.</td>
<td>Not detectable</td>
<td>Rice seedlings</td>
</tr>
<tr>
<td>E.</td>
<td>Low</td>
<td>Bean axes</td>
</tr>
<tr>
<td>D.</td>
<td>Low</td>
<td>Bean axes</td>
</tr>
</tbody>
</table>

The cyclohexyl moiety of all derivatives is attached to the side chain at carbon 1'.

The activity of (R)-ABA is almost equal to that of (S)-ABA (12).

The (RS)-methyl ester was tested and has about one-half the activity of (RS)-ABA (12).

This substance was tested as the racemate and appears to be as active as ABA in several assays (2).

This racemate was tested as the methyl ester and the free acid. Activity is in the same range as (RS)-ABA (18).

Two possible structures are presented for the major reaction product obtained from the epoxide isomer of ABA near pH 7 (17).

This compound was prepared from the epoxide, compound O (18).

Fraxinus ornus seeds. It has been shown previously that 10 μM (RS)-ABA inhibits the germination of these embryos about 90% during a 10-day period at 22 C (16). However, even at a concentration as high as 170 μM compound B was ineffective during the 10-day observation period.

Discussion

Among the advantages of our procedure for the evaluation of growth-inhibitory activity of ABA analogues are rapidity of response and the use of storable plant material. With the addition of chloramphenicol, bacterial contamination can be kept to an acceptably low value. As might be expected, the sensitivity of different batches of embryonic axes to the test compounds is somewhat variable. While this does not effect the relative values, the absolute activity detected may vary by 20 to 30%.

The degree of correlation between results obtained in this assay procedure and others is still uncertain. Comparison of our data with those of Tamura and Nagao (18), who used the second leaf sheath of rice seedlings, shows good agreement. However, while (RS)-ABA is both a good inhibitor of growth of embryonic bean axes and of germination of treated Fraxinus embryos, cis,trans-β-ionylideneacetic acid showed activity only with the bean axes. It may be that the structural requirements for growth inhibition in short time experiments are less stringent than those needed for long term inhibition of germination.

In Table II we have summarized the available data on the growth-inhibitory activity of cis,trans-ionylideneacetic acids. The compounds to which we attribute high activity are at least 10%; as active as (RS)-ABA in one or more assay systems. The effects of hydrolyzable substituents on the carboxylic acid group are ignored because, as stated previously, they seem to result from differences in uptake or transport (10). Since the epoxide isomer of ABA decomposes rapidly under typical physiological conditions (17), its major reaction products, compound Q and ABA, are considered here.

If it is assumed that the compounds discussed here act by a mechanism similar to that of ABA, a number of trends concerning structural requirements for ABA-like action can be seen.

1. With those substances that do not have the intact ionyldieneacetic acid structure, greatest effectiveness is found in substances that resemble the ABA side chain. Thus cis-crotonic acid and cis,trans-β-methylsorbic acid are more effective than iso-valeric acid, trans,trans,trans-sorbic acid, or 4-hydroxysolphorine. Since cis,trans-β-methylsorbic acid is no more effective than cis-crotonic acid, it seems likely that one of the requirements for activity is the presence of a cis double bond conjugated to a carboxylate or a group such as an ester or glycoside that can be readily converted to a carboxylate.

2. cis,trans-Ionylideneacetic acid derivatives have the highest activity. While compounds without keto or hydroxyl groups are active, substances in which all the ring carbons are tetrahedral, compounds E and Q, have low potency. However, this does not explain the low activity of cis,trans-dehydroionyldieneacetic acid, compound G. Possibly this is due to high chemical reactivity resulting from the extensive conjugation. Another factor that should not be ignored is that the presence of two asymmetric centers in the cyclohexane derivatives E and Q results in four stereomers. Since compound Q was prepared by hydrolysis of the epoxide (18), the preparation may be assumed to consist of the stereomer in which the hydroxyl groups are trans to each other. Compound E was prepared by catalytic hydrogenation of cyclocitral, and its stereochemistry is less predictable. What effects these stereochemical factors have on the activity of the test preparations is not known.

A number of mechanisms would account for the physiological behavior of these compounds. As has already been proposed for cis,trans-α-ionyldieneacetic acid, biological activity may require conversion in situ to ABA (2). While available data are insufficient
to evaluate the validity of this suggestion, it seems unlikely that the differences in growth-inhibitory activity of all the cis, trans-ionylideneacetic acids listed in Table II can be accounted for on this basis. Another explanation rests on the assumption that the epoxides are the active compounds and that the cyclohexene derivatives have to be converted to epoxides before growth-inhibitory activity is seen. It may be significant that an epoxide, compound O, had higher activity than the cyclohexene derivative, compound B, in the rice seedling assay (18). The surprising facility with which methyl β-ionylideneacetate is converted to the 1',2'-epoxide (shaking in air at room temperature) indicates that this process may not even require enzymes (18). Possibly the epoxides bind to a macromolecule, for example, a sulphydryl group of a protein, and elicit a physiological response. Inactivation of the epoxide could occur through hydrolysis.

Finally, the inhibitory activity of the cyclohexene derivatives and the epoxides can be explained on steric grounds. In compounds that have either a double bond or an epoxide ring at C-2', the C-2'-methyl group is coplanar with four-ring C atoms, while in the cyclohexane or the cyclohexanediol derivatives the C-2'-methyl group is either axial or equatorial. These differences may affect the fit between the ABA analogues and some required macromolecules and thereby play a dominant role in the determination of relative activities.

Note Added in Proof. S. Tamura and M. Nagoa (1969. Agr. Biol. Chem. 33: 1357–1360) have described an additional ABA analogue, ethyl 5-(1-hydroxy-2,6,6-trimethyl-1-cyclohexyl)-3-methyl-cis,trans-2,4-pentadienoate, which does not have a ring double bond and has very low biological activity.

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LITERATURE CITED