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BIOLOGICAL ACTIVITY OF AN AROMATIC ANALOGUE OF ABSCISIC ACID

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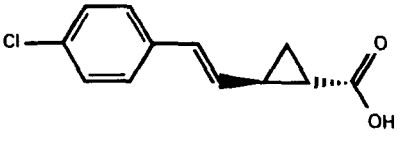
Key Word Index—Abscisic acid; 2-(2-*p*-chlorophenyl-*trans*-ethenyl)-cyclopropane carboxylic *trans*-acid; CPA; α-amylase activity; anti-transpirant; seed germination inhibitor; ethylene biosynthesis inhibitor.

Abstract—The activity of *trans*-2-(2-*p*-chlorophenyl-*trans*-ethenyl) cyclopropane carboxylic acid (CPA) as a mimic of abscisic acid (ABA) is reported. CPA had *ca.* the same inhibitory activity as ABA at 10^{-5} M and 10^{-6} M in an assay for gibberellic acid-induced α-amylase activity using embryoless barley seeds. At 10 μM, cress seed germination was inhibited by 62%; at 100 μM, lettuce seed germination was inhibited by 58%. ABA completely inhibited seed germination of both species at the respective concentrations. CPA was 1/5 to 1/3 as effective as ABA at equivalent concentrations in inhibiting water loss from detached leaves or intact barley seedlings. However, unlike ABA, at concentrations of 10^{-3} M and 10^{-4} M CPA inhibited ethylene biosynthesis by soybean hypocotyls. It was concluded that CPA mimics some of the biological functions of ABA at concentrations that are in general higher than ABA. When characterizing the activity of a xenobiotic suspected as being an analogue of a natural plant hormone it is important to complement assays designed to demonstrate activity of the hormone of interest with assays in which activity is unexpected.

INTRODUCTION

Abscisic acid (ABA) is a naturally occurring growth regulator (hormone) that is involved in regulating numerous vital processes and many events in plants, especially under conditions of environmental stress [1]. Spray applications of ABA to intact plants, or the addition of ABA to the incubation medium of cell cultures of wheat, potatoes and alfalfa increase their tolerance to freezing temperatures [2–4]. Observations of ABA effecting stomatal closure has led to the hypothesis that ABA acts as a natural anti-transpirant [1]. ABA is also a controlling element in seed storage protein synthesis [5] and seed dormancy [6, 7] which implies that manipulation of the levels of ABA during development may lead to an increase in the seed shelf life of some species. Another pragmatic suggestion is that ABA can substitute for the mutagenic compound, potassium bromate, in the brewing industry [8]. With these possibilities, it is apparent that ABA is a prime candidate for commercialization. However, the lability of the active molecule [9] and the extremely high synthetic costs make it impractical to pursue as a commercial product.

Most analogues of ABA that have been described have the cyclohexenone ring system of the ABA molecule, but two papers [10, 11] have reported that ABA activity is possessed by aromatic analogues. This study was undertaken in order to determine if other aromatic compounds could be found that would mimic the activity of ABA within the plant. It was theorized that compounds may be found that would selectively effect an ABA response in one organ or tissue and not in another, for example, affecting specific protein synthesis without affecting stomatal closure. Such a molecule that could elicit a specific affect of ABA independently of other responses may be of commercial interest and may add to information about the receptor sites involved in the recognition of the ABA molecule. An additional goal of the project was that these analogues be relatively inexpensive to synthesize. The aromatic molecules tested were styryl cyclopropane-carboxylates and this paper reports on the interesting biological activity of 2-*trans*-(2-*p*-chlorophenyl-*trans*-ethenyl) cyclopropane carboxylic acid (**1**).



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Scheme 1.

RESULTS AND DISCUSSION

Three bioassays were used initially to identify and confirm ABA-like activity. Gibberellic acid (GA_3)-induced α-amylase activity of embryoless barley seed [12]; water loss from detached primary leaves of barley [13]; and cress seed germination [14]. After ABA-like activity was identified, further experiments were carried out to confirm that CPA was truly ABA-like in character.

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Table 1. Effect of CPA and ABA on GA-induced α-amylase activity in embryoless barley

Treatment	O.D.	(s.d.)*	%Activity†
No hormones	0.409	(0.042)	100
$+GA_3$ (10^{-6}M)	0.052	(0.003)	0
$+GA_3$ (10^{-6}M)+ABA (10^{-5}M)	0.213	(0.013)	17
$+GA_3$ (10^{-6}M)+CPA (10^{-5}M)	0.280	(0.059)	24
$+GA_3$ (10^{-6}M)+ABA (5×10^{-6}M)	0.099	(0.024)	5
$+GA_3$ (10^{-6}M)+CPA (5×10^{-6}M)	0.116	(0.009)	7
$+GA_3$ (10^{-6}M)+ABA (10^{-6}M)	0.077	(0.023)	3
$+GA_3$ (10^{-6}M)+CPA (10^{-6}M)	0.090	(0.010)	4
$+GA_3$ (10^{-6}M)+ABA (5×10^{-6}M) +CPA (5×10^{-6}M)	0.194	(0.024)	19

*s.d. standard deviation of triplicates.

$$\dagger\ \%\text{Activity} = 100 - \left[\frac{A_{\text{no hormone}} - A_{\text{treatment}}}{A_{\text{no hormone}} - A_{GA^3}}\right] \times 100.$$

Amylase activity

CPA had activity equal to that of ABA in inhibiting GA_3-induced α-amylase activity (Table 1). The greater degree of inhibition demonstrated was not, in later similar experiments, statistically significant. When ABA and CPA were added to the assay system at equimolar concentrations the inhibitory effect on amylase activity was additive (Table 1). This lends support, but does not prove, that CPA acts at the same site as ABA. To confirm that CPA and ABA do not interfere with enzyme activity *per se* the compounds were incubated with the isolated enzyme from barley malt. Inhibition by direct interaction of ABA with amylase enzyme has been reported [15]. However, the activity of the isolated enzyme from barley malt was unaffected by the addition of either 10 μM CPA or 10 μM ABA (data not shown). The effect of CPA and ABA on the total activity of α-amylase rather than that which was secreted was also examined. The ratios of the activity of the secreted enzyme to that of the extracted enzyme from aleurone layers incubated in the presence of either CPA or ABA were similar (Table 2). Thus, the extent to which CPA affected α-amylase activity was similar to ABA at each concentration tested. Apparently high concentrations (0.1 mM) of both CPA and ABA tended to inhibit the secreted enzyme to a greater extent than the extractable enzyme.

Seed germination

CPA was less phytotoxic to plants than ABA. High concentrations of ABA, e.g. 0.1 mM, caused chlorosis after spraying or application through the transpiration stream to barley or cotton; CPA did not. Similarly CPA only reduced cress seed germination by 62% and lettuce seed by 58% at a concentration (10 μM or 0.1 mM respectively) at which ABA inhibited germination completely.

Anti-transpirant activity

CPA was less effective than ABA at inhibiting water loss from detached primary leaves and intact seedlings of barley although the anti-transpirant effect was reproducible and frequently statistically significant at the 5% probability level (Table 3). CPA was also incubated with isolated epidermal strips from *Vicia faba*. At the concentrations of the compounds tested the stomata did not close as tightly in response to CPA as they did in the presence of ABA. CPA caused the pores to close to less than 0.5 units on the recticule whereas ABA closed them completely. Open stomata from strips incubated on water (plus an amount of ethanol comparable to test solns) were

Table 2. Effect of ABA and CPA on the ratio of secreted to extracted activity of GA3-induced α-amylase activity in isolated aleurone layers

Treatment	Secreted α-amylase activity / extractable α-amylase activity
$+GA_3$(10^{-6}M)	1.54
No hormones	0.63
$+GA_3$ (10^{-6}M)+CPA (10^{-4}M)	0.60
$+GA_3$ (10^{-6}M)+ABA (10^{-4}M)	0.67
$+GA_3$ (10^{-6}M)+CPA (10^{-5}M)	1.75
$+GA_3$ (10^{-6}M)+ABA (10^{-5}M)	1.51
$+GA_3$ (10^{-6}M)+CPA (10^{-6}M)	1.75
$+GA_3$ (10^{-6}M)+ABA (10^{-6}M)	1.86

*Ratio of amylase units:

$$\alpha\text{-amylase units} = \frac{(A_{620\text{mm}}) \times \text{total volume}}{\text{time of incubation} \times \text{sample volume}}.$$

Table 3. Effect of CPA and ABA on water loss from (a) detached leaves or (b) intact seedlings of barley

	Water loss (% inhibition of control) Detached leaves		Intact seedling	
Treatment	10^{-4}M	5×10^{-5}M	10^{-3}M	10^{-4}M
ABA	51*	50*	68*	45*
CPA	12*	11*	23*	0

*Significant at 5% probability level.

2.5 units on the recticule (Table 4). The extent to which ABA closes stomata of *Commelina communis* is dose dependent [16] and therefore, it is correct to describe CPA as having weak ABA-like activity in its direct effect on stomata.

On this evidence it may be more correct to hypothesize that CPA acts as a phaseic acid (PA) analogue. Ho [17] has shown that PA was more active than ABA in the α-amylase assay and Sharkey and Raschke [18] have demonstrated CPA has anti-transpirant activity in some species. PA was weakly active in reducing stomatal conductance of leaves of detached barley and was also a weak inducer of stomatal closure when incubated with isolated epidermal strips of *V. faba.*

Table 4. Effect of ABA and CPA on opening of stomata in isolated epidermal strips of *Vicia faba*

Treatment	% Average open*
Control	100
ABA (10^{-4}M)	26
ABA (10^{-5}M)	49
CPA (10^{-4}M)	40
CPA (10^{-5}M)	55

*Average = average number of 100 stomata on each of 4 isolated strips.

Open = those pores open wider than 0.5 units on the recticule.

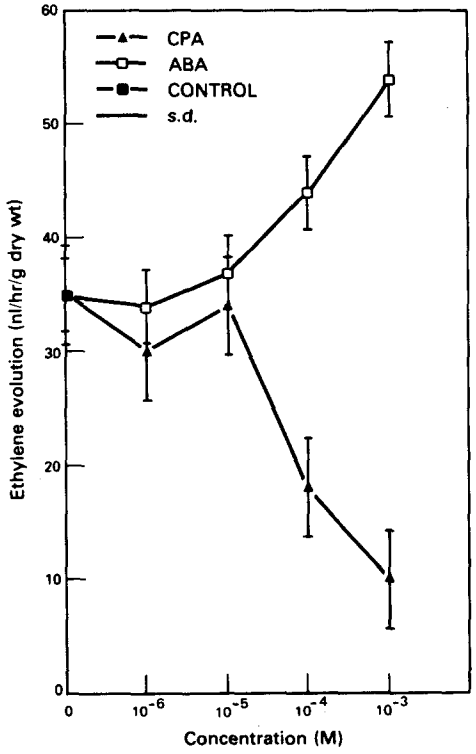

Fig. 1. Effect of CPA (▲) and ABA (□) on ethylene evolution in a soybean hypocotyl bioassay.

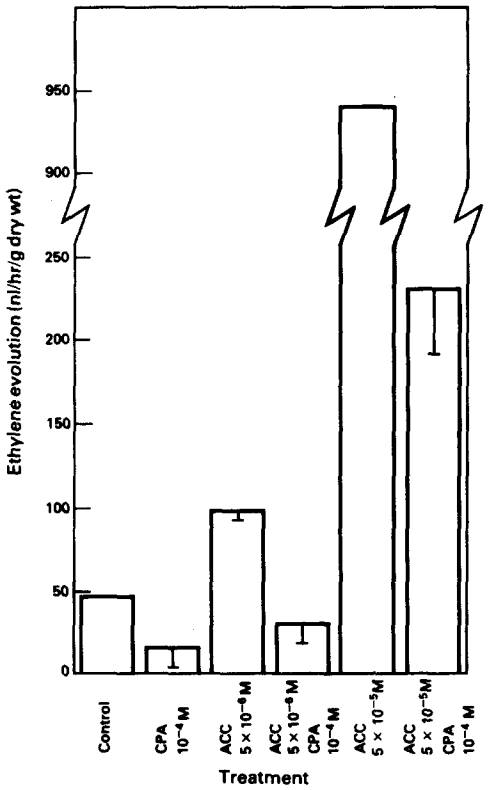

Fig. 2. Effect of CPA on the evolution of ethylene in the presence and absence of α-aminocyclopropane carboxylic acid (ACC) in a soybean hypocotyl bioassay.

The results obtained from the assays designed to identify ABA-like activity indicated that CPA behaved as an ABA, or PA, analogue. In addition, it appeared that the dose at which CPA elicited the ABA-like response was dependent upon the tissue. Thus, it might be possible that responses typical to ABA could be elicited independently of one another by CPA. However, when the two compounds were tested for their effect on ethylene biosynthesis their activity differed (Fig. 1). In the soybean hypocotyl bioassay, ABA was not considered to significantly affect ethylene biosynthesis and the stimulation observed at high levels of ABA was ascribed to a non-physiological, toxic response. CPA had significant activity inhibiting ethylene biosynthesis at concentrations of 0.1mM and higher. The site of inhibition was probably between α-aminocyclopropanecarboxylic acid (ACC) and ethylene (Fig. 2). Ethylene has been implicated in affecting α-amylase secretion from barley aleurone layers [19, 20], and so another inhibitor of ethylene biosynthesis, aminoethoxyvinyl glycine (AVG), was assayed in the half-seed bioassay. AVG had no effect on the activity of secreted α-amylase (Table 5). It is unlikely that the ABA-like biological activity of CPA is related entirely to its effect on ethylene biosynthesis because CPA elicited ABA-like responses at concentrations (1–10 μM) which did not affect the former, and another inhibitor of ethylene biosynthesis, AVG, had no similar effects on α-amylase activity. Of course, the inactivity of AVG may be due to poor penetration or specificity of tissue type. Also AVG,

Table 5. Effect of AVG, CPA and ABA on GA_3-induced α-amylase activity in embryoless half-seeds of barley

Treatment	A620 (s.d.)	% Inhibition†
No hormones	0.780 (0.023)	100
GA_3 (10^{-6}M)	0.033 (0.005)	0
GA_3 (10^{-6}M)+ABA (10^{-4}M)	0.761 (0.009)	97
GA_3 (10^{-6}M)+ABA (10^{-5}M)	0.405 (0.032)	50
GA_3 (10^{-6}M)+ABA (10^{-6}M)	0.091 (0.017)	8
GA_3 (10^{-6}M)+CPA (10^{-4}M)	0.818 (0.038)	105
GA_3 (10^{-6}M)+CPA (10^{-5}M)	0.162 (0.009)	17
GA_3 (10^{-6}M)+CPA (10^{-6}M)	0.083 (0.010)	7
No hormones	0.679 (0.022)	100
GA_3 (10^{-6}M)	0.029 (0.002)	0
GA_3 (10^{-6}M)+ABA (10^{-5}M)	0.515 (0.034)	75
GA_3 (10^{-6}M)+ABA (10^{-6}M)	0.191 (0.018)	25
GA_3 (10^{-6}M)+AVG (10^{-4}M)	0.031 (0.004)	1
GA_3 (10^{-6}M)+AVG (10^{-5}M)	0.042 (0.003)	2
GA_3 (10^{-6}M)+AVG (10^{-6}M)	0.035 (0.035)	1

*s.d. standard deviation.

$$\dagger\ \%\text{Inhibition} = 100 - \left[\frac{A_{\text{no hormone}} - A_{\text{treatment}}}{A_{\text{no hormone}} - A_{GA_3}} \times 100\right].$$

unlike CPA, apparently inhibits ACC formation [21] whereas CPA blocked the conversion of ACC to ethylene. Therefore, one argument may be that CPA acts through its effect on the accumulation of ACC rather than inhibition of ethylene biosynthesis *per se*.

The data presented here indicate that aromatic structures do possess ABA-like activity but in view of the inhibitory activity on ethylene biosynthesis CPA may have other activity as well. Speculation on any structure–activity implications must be cautious. However, it is noteworthy that for biological activity it is essential the dienoic side chain of ABA be in the 2-*cis*,4-*trans* configuration [22]. The side chain of CPA is held by the cyclopropane ring in the *trans* configuration.

The results of these experiments emphasize the importance of using assays other than those designed to specifically identify mimics of the plant hormone of interest when characterizing xenobiotics or pursuing structure–activity relationships. To stop at recognized assays for any one plant growth regulator can easily lead to incomplete conclusions as to the physiological acitvity of the chemical.

Although CPA may not be true analogue (mimic) of ABA these results do confirm that plant processes under the control of ABA can be specifically manipulated with aromatic xenobiotics. Future interesting experiments with CPA will include further characterization of its mode of action in the aleurone system and also testing it in assays to determine its effect on enhancing the freezing tolerance of plants and cell cultures.

EXPERIMENTAL

Synthesis of ethyl cis,trans-2-(2-p-*chlorophenyl*-cis, trans-*ethenyl*) *cyclopropane carboxylate*

In 30 ml absolute EtOH were placed 1 g (0.04 M) of Na. This soln of NaOEt was added to 100 ml of EtOH containing 17 g (0.04 M) *p*-chlorobenzyl triphenylphosphonium chloride and 5.6 g (0.04 M) ethyl 2-formyl cyclopropane carboxylate. The reactants were stirred overnight at room temp. Removal of solvent and NaCl followed by Kugel Rohr dist yielded 4 g (40%) of a nearly colourless oil, bp 105–110° (0.05 mm). 1H NMR. (300 MHz, $CDCl_3$): δ 1.05 (1H, *m*, cyclopropyl H), 1.28 (3H, *t* Me), 1.50 (1H, *m*, cyclopropyl H), 1.72 (1H, *m* cyclopropyl H), 2.15 (1H, *m*, cyclopropyl H) 2.38 (1H, *m* cyclopropyl H), 4.15 (2H, OCH_2), 5.12 (1H, vinyl H), 5.70 (1H, vinyl H), 6.41 (1H, vinyl H), 7.30 (4H, aromatic) GC/MS. (EIMS, 70 eV) 61:38 *cis/trans m/z* (rel. int.): 250 $[M]^+$ (20), 176 $[M - HCO_2C_2H_5]^+$ (95), 142 [M −C], $CO_2C_2H_5]^+$(100). IR $\nu_{max}^{CH_2Cl_2}$ cm^{-1}: 3000–2800 CH; 1721 C=O; 1651 C=C; 1593, 1491, 1447 aromatic; 1244–1182 C–OC; 1092 aromatic-Cl. Analytical results, found: C, 66.85; H, 5.94 $C_{14}H_{15}ClO_2$ requires C, 67.20; H, 6.00

Synthesis of trans-2-(2-p-*chlorophenyl*-trans-*ethenyl*)*cyclopropane caroxylic acid*. The dist. residue from the previously described ester prep was used as it was determined by NMR to be > 90% *trans* isomer about the double bond. This ester 1.3 g (0.0005 M) was dissolved in 45 ml EtOH and THF–H_2O (1:1:1) and 1 g (0.025 M) NaOH added. The soln was stirred at room temp overnight. The solvents were removed and the solid was taken up in 10% NaOH and washed with Et_2O. The basic soln was neutralized and extd with Et_2O and dried (Na_2SO_4). Removal of Et_2O and crystallization of the solid from hexane–EtOAc yielded 0.6 g (51%) of a white solid, mp 132–135°. ^{1}H NMR. (300 MHz, $CDCl_3$): δ 1.15 (1H, *m*, cyclopropyl H), 1.55 (1H, *m*, cyclopropyl H), 1.75 (1H, cyclopropyl H), 2.25 (1H, cyclopropyl H) 5.70 (1H, *m*, olefin H), 6.50 (1, *d*, olefin H), 7.25 (4H, aromatic H). GC/MS. (EIMS, 70 eV), *m/z* (rel. int.): 222 $[M]^+$[(25), 176 $[M - COOH]^+$(50), 141[M-81]$^+$)100). IR $\nu_{max}^{CH_2Cl_2}$ cm^{-1}: 3500–2400 OH; 1736, 1695 C–O, 1493–1431 aromatic. Analytical results: Found: C, 64.71, H, 5.02 $C_{12}H_{11}ClO_2$ requires C, 64.86, H, 4.95.

Bioassays. All expts were repeated at least twice. *Bioassay* 1. Inhibition of GA_3-induced α-amylase activity. Measurements of secreted enzyme activity: barley, *Hordeum vulgare* L. cv, Himalaya seeds were cut in half transversely. α-Amylase production by the embryoless halves was measured according to ref. [23]. The buffer in which the halved seeds were incubated was assayed for starch degrading activity spectrophotometrically at 620 nM using I/KI soln. GA_3 was used at a concn of 1 μM. Measurements of secreted and extracted enzyme activity: the aleurone layers were sepd from the starchy endosperm before incubation with the compounds. Procedures were as described in ref. [12]. The only modification was that exts were made in 1 mM acetate buffer [24]. There were 10 half seeds or equivalent aleurone layers per replicate and each treatment was replicated × 3. The isolated enzyme from barley malt (Type VIII-A; 1–3 units per mg solid) was obtained from Sigma. The activity of the isolated enzyme was measured as for the secreted enzyme.

Bioassay 2. Antitranspirant activity. Detached leaves: Treatment solns (9 ml) were placed in 10-ml beakers and covered with parafilm. Four slits were made in the film. The excised end of a primary leaf of 10-day old barley, *Hordeum vulgare* L., cv 'Morex', cut 7 cm from the tip was inserted through each slit and submerged in the treatment soln. For each treatment, there were three replicates. The beakers with the test soln and leaves were weighed initially and after 4 days of continuous illumination. H_2O loss due to stomatal and cuticular transpiration was estimated by the difference in wt. Intact seedlings: barley, *H. vulgare* L., cv 'Himalaya', seeds were planted in Promix A potting mix (A. H. Hummert, St. Louis, MO) and grown in the greenhouse. They were thinned to three plants per pot. When the plants reached the 3-leaf stage, the pots, including the soil surface were wrapped in parafilm and Al foil to prevent H_2O loss from the soil and sprayed with the treatment soln. The compounds

were applied in an aq. Tween 20 soln (0.75%), 6 replicates per treatment. After spraying, the wts of the pots were recorded. The plants were placed in a growth chamber (17° day, 14° night, 70% rel humidity, 14 hr photoperiod, 1080 $\mu E/m^2/sec$). After 5 days the pots were reweighed and the H_2O lost through the plant could be estimated. The detached leaves and intact seedlings were visually rated at the end of the expt for toxicity. Isolated epidermal strips: strips were peeled from the underside of greenhouse grown plants of *Vicia faba* and floated in the light on aq. solns in the absence and presence of either ABA or CPA. Stomata were viewed under a microscope (magnification 512x) for scoring. For the antitranspirant tests all compounds were made up in 100% EtOH and dild accordingly. Controls had the same amount of EtOH in the treatment medium as the highest treatment dose.

Bioassay 3. Seed germination: garden cress, *Lepidium virginianum*, and lettuce, *Lactuca sativa* L., cv 'Grand Rapids', were imbibed 1 hr in the treatment soln. Fifty swelled seeds were placed on one sheet of Whatman No. 1 filter paper soaked with 1 ml treatment soln in a petri dish at 20–24°. After 4 days the number of seeds that had germinated was counted.

Bioassay 4. Inhibition of ethylene biosynthesis: soybean, *Glycine max* cv 'McCall', seeds were germinated in the dark for 4 days. Ten 2-cm hypocotyl segments were then incubated with 2 ml sodium hydrogen maleate buffer (5 mM; pH 5.3) in the presence or absence of the test compounds in a 25 ml flask. The flasks were left open for 2 hr in a shaking H_2O bath (28°) and then capped. After 4 hr a 1 ml sample was withdrawn from the head space and analysed by GC 4 ft glass column of 50/80 mesh activated alumina; air 280 ml/min, H_2 45 ml/min; carrier gas, He, 30 ml/min; detector temp and injection port 200°; oven temp 80°).

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