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Synthesis and Properties of Amino-Acid Analogues in Ethylene Biosynthesis

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C. ABSTRACT

The present research was undertaken in order to check whether ACC analogues can be used to manipulate ethylene biosynthesis in plants. Most of the analogues which were synthesized only slightly inhibited ethylene biosynthesis in etiolated mung bean hypocotyl sections, citrus leaf discs and apple pericarp discs. Part of this inhibitory effect was due to reduction of the uptake of 1-aminocyclopropane-1-carboxylic acid (ACC) which was applied in the experimental medium. Two of the analogues tested, 2-methyl-1-aminocyclopropane carboxylic acid (Me-ACC) and 1-amino-2-cyclopropylcyclopropane carboxylic acid (cyclopropyl-ACC) were relatively more active. Me-ACC, which was found to compete with ACC for the same site, was further used to study the role of cyanide in the regulation of ethylene biosynthesis. The data suggest that cyanide is not involved in the regulation of this process. The involvement of lipoxygenase in ethylene biosynthesis was also discounted. Phenoxy acids were found to effectively inhibit ethylene forming enzyme (EFE), thus offering a new approach for controlling ethylene biosynthesis.

D. OBJECTIVES

D1. OBJECTIVES OF THE ORIGINAL RESEARCH PROPOSAL

- (a) The development of new, efficient, and practical syntheses of analogues of ACC and AVG.
- (b) To use these analogues to learn more of the biochemistry of ethylene biosynthesis and particularly of the enzymatic system (EFE) catalyzing the conversion of ACC to ethylene.
- (c) To use these analogues to bind to the EFE to permit its isolation.
- (d) To test the physiological and practical potential of the analogues to be used as a tool for controlling processes which are mediated by ethylene like fruit ripening, abscission and senescence.

D2. DEVIATION FROM THE ORIGINAL OBJECTIVES

- a. None of the analogues synthesized was highly active as an inhibitor of EFE activity. Therefore, objective (c) was not dealt with.
- b. During the course of this study, it has been found that phenoxy acids inhibit EFE activity. Studies of this phenomenon were included in this research.

E. SYNTHESIS AND BIOLOGICAL ACTIVITY OF ACC ANALOGUES

E1. EXPERIMENTAL

(a) Synthesis of analogues

Detailed description of the synthesis of ACC analogues is presented in the following separately for each compound.

(b) Physiological studies

Plant material

The plant species tested were mung bean (*Vigna radiata* L), apple (*Malus silvestris* Mill, var. Golden Delicious), citrus (*Citrus sinensis* [L.] Osbeck, var. Shamouti Orange), and avocado (*Persea americana*, var. Hass).

Seeds of mung bean were germinated and grown in Vermiculite for 4 days in darkness at 25°C. Ten 1 cm long hypocotyl segments were cut 1 cm below the hook, weighed, and incubated in 3 ml of 50 mM MES buffer (pH 6.1) or test solutions in a 25 ml Erlenmeyer flask.

Apples were obtained from commercial storage houses and stored in our laboratory at 4°C. Plugs (9 mm in diameter and 2 cm long) were excised from the pericarp with a cork borer and further cut into discs, about 3 mm thick. Six discs were weighed and incubated in 2.5 ml of 50 mM MES buffer (pH 6.1) or test solutions in a 25 ml Erlenmeyer flask.

Mature citrus leaves were harvested from trees grown in Rehovot. Discs, 9 mm in diameter, were excised with a cork borer and kept in a moistened Petri dish until treatment. Ten discs were floated on 3 ml of 50 mM MES buffer (pH 6.1) or test solutions with the adaxial surface down. After treatment, the discs were blotted dry on a filter paper and then transferred to a test tube for measuring ethylene production rates.

Mature preclimacteric avocado fruits were harvested from trees grown in Rehovot. Test solutions were applied to the fruits through the cut pedicels under pressure. After treatment, fruits were transferred to 2.2 liter jars for measuring ethylene. In some experiments plugs (9 mm in diameter) were excised from the pericarp with a cork borer and further cut into discs, about 3 mm thick. Six discs were incubated in 3 ml of MES buffer (pH 6.1) or test solutions in a 25 ml Erlenmeyer flask.

Determination of Ethylene and Propene (Propylene)

For monitoring ethylene and propene production, the vessels containing the plant samples were sealed with a rubber serum cup and incubated at 25°C. Apple, mung bean and avocado tissues were shaken during incubation. At various periods during incubation, a 2-ml air sample was withdrawn from the vessels with a hypodermic syringe. After each sampling, the vessels were flushed with ethylene-free air and resealed for an additional determination. The gases were assayed using a gas chromatograph equipped with an alumina column and a flame ionization detector.

Extraction and Determination of ACC

Tissue was ground with a mortar and pestle in 2 ml 80% ethanol. The mortar and pestle were washed twice with 2 ml 80% ethanol each time. The extract and washings were combined and centrifuged for 10 min at 10,000g. The supernatant was concentrated under reduced pressure at 45°C. Residues were brought to a volume of 2 ml with distilled water and clarified by centrifugation as above. ACC in the aqueous extract was assayed by the method of Lizada and Yang (Anal. Biochem. 100:140-145. 1979).

EFE Activity

EFE activity was determined by measuring conversion of applied ACC to ethylene *in vivo*. Different concentrations of ACC in MES buffer were added to the tissues and ethylene evolution was measured as described above.

E2. RESULTS

a) 1-Amino-2-cyclopropylcyclopropane carboxylic acid

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Cyclopropyl-Substituted Aminocyclopropane Carboxylic Acid (Cyclopropyl-ACC) —an Investigation of the Mechanism of Ethylene Biosynthesis**

By Michael Pirrung* and Gerard M. McGeehan

We report here the synthesis of 1-amino-2-cyclopropylcyclopropane carboxylic acid (cyclopropyl-ACC) 3 and its use in investigating the mechanism of ethylene biosynthesis in plants. The application of the *Schöllkopf*^[1] procedure to 1,2-dibromides has proven the method of choice for synthesis of ACC analogues.^[2] In this case, ethyl isocyanoacetate reacts with 1,2'-dibromoethylecyclopropane 1^[3] in the presence of NaH in ether/dimethylsulfoxide (DMSO) to give the protected bis-cyclopropane 2 in 20-27% yield. A two-step hydrolysis procedure and ion-exchange chromatography gives cyclopropyl-ACC 3^[4] as a mixture of stereoisomers (> 7:1) in 85-95% yield. The structure shown was assigned to the major isomer by comparison of its ¹³C-NMR spectra with those of the known methyl-ACC^[2] and by bioassay.

Yang et al. have established that in alkyl-ACC derivatives the alkyl and carboxyl groups must be in *trans* positions to be metabolized by plant tissue.^[5] The *trans*-methyl-ACC serves as a good inhibitor of ethylene production ($K_i = 0.5$ mM) and is a substrate for propylene production in mungbean hypocotyl segments.^[2] A Dixon analysis of the inhibition of ethylene biosynthesis in mungbean by cyclopropyl-ACC 3 shows a K_i of 1.5 mM.^[6] The bulkier cyclopropyl group probably makes it more difficult for 3 to penetrate to the active center of ethylene biosynthesis.

Based on the previously proposed mechanism of ethylene biosynthesis,^[7] it was anticipated that the radical 5, in

Scheme 1.

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which the radical center is adjacent to a cyclopropane ring, would be generated via 4 during the metabolism of 3 by plants, and that this species might undergo a second ring opening. Incubation of apple tissue with 3 results in slow evolution of 1,4-pentadiene 10 (identified by GC/MS). In contrast, treatment of 3 with NaOCl, a reagent known to convert ACC into ethylene by a concerted mechanism,^[1] provides only vinylcyclopropane.

Because 3 is a good inhibitor of ethylene biosynthesis but 1,4-pentadiene is generated only slowly, it seemed likely that an intermediate radical was being diverted by another reaction, perhaps with the ethylene-forming system. An assay for time-dependent inactivation was conducted by incubating mungbean hypocotyl segments with various concentrations of 3. Samples were periodically removed and incubated with excess ACC. A decrease in the rate of ethylene production^[6] with time was observed (Fig. 1). While not strictly first order, the rate constant for the inactivation was estimated to be $1.7 \times 10^{-2} \text{ s}^{-1}$. Excess ACC protects against inactivation and oxygen is required but it was impossible to determine an inactivation/turnover ratio for 3 since the yield of 1,4-pentadiene was too low. Mechanism-based inactivation of ethylene biosynthesis in plants is thus shown for 3.

Mechanistically, this study provides further evidence for the presence of a "half-opened" radical and the sequential

Fig. 1. Plot of the negative logarithm of the activity remaining ($-\lg A_t$) versus the incubation time. Top left: concentrations of 3. See text.

single electron transfer pathway in ethylene biosynthesis. The formation of 1,4-pentadiene 10 is best accommodated by a mechanism involving a 1,2-hydrogen migration in a radical intermediate such as 9 (Scheme 1). It seems likely that the divergent reactivity of the "half-opened" radical formed from ACC and from 3 is responsible for the low conversion as well as the "suicide" inactivation.

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[1] U. Schöllkopf, R. Harms, D. Hoppe, *Justus Liebigs Ann. Chem.* 1973, 611.

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[3] Prepared in 59% overall yield from methyl cyclopropyl ketone by a procedure involving bromination (1. Br_2 , MeOH , 0°C , 40 min; 2. H_2O , 20 min, room temperature), reduction (NaBH_4 , MeOH , 0°C , 20 min) (89.6%,

two steps), and OH-Br exchange (Ph_3P , Br_2 , CH_3CN , 2 h). $^1\text{H-NMR}$ (CDCl_3): δ = 0.41 (1H, m), 0.68 (1H, m), 0.77 (1H, m), 0.96 (1H, m), 1.30 (1H, m), 3.64 (1H, ddd, J = 12.3, 8.1, 5.4 Hz), 3.83 (1H, dd, J = 10.4, 8.1 Hz), 3.89 (1H, dd, J = 10.4, 5.4 Hz); IR (film): ν = 3090, 3000, 1430, 1140, 1020, 935, 830 cm^{-1} ; correct elemental analysis.

[4] M.p. = 211 (decomp.), $^1\text{H-NMR}$ (D_2O): δ = 0.14 (2H, m), 0.44 (2H, m), 0.55 (1H, m), 0.79 (1H, t, J = 6.5 Hz), 1.22 (1H, dd, J = 9.8, 6.5 Hz), 1.38 (1H, ddd, J = 9.8, 6.5, 5.7 Hz); $^{13}\text{C-NMR}$ (D_2O): δ = 3.55, 3.98, 7.35, 16.82, 27.17, 39.83, 175.68; correct elemental analysis.

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b) 1-Hydroxycyclopropane carboxylic acid (ACC-OH)

1. Synthesis

- (a) *1,2-Bis (trimethylsilyloxy)-cyclobutene*. A 3-neck, 1 liter round bottom flask was fitted with a condenser, a constant rate addition funnel, and a mechanical stirrer with a water jacketed condensing sleeve. The flask was charged with 275 ml toluene and 9.90 g (0.43 mol) of sodium. The solvent was refluxed and the stirrer operated vigorously until the sodium was dispersed. A solution of 17.76 g (0.10 mol) of diethylsuccinate and 48.48 g (0.45 mol) chlorotrimethylsilane in 125 mL toluene was added dropwise over 2 h. After refluxing for 42 h, the deep brownish-purple mixture was cooled to room temperature and filtered through a 150 mm coarse funnel using three 20 mL portions of ether for washing. The solvents were removed by rotary evaporation. The remaining yellow liquid was vacuum distilled through a 10 cm vacuum jacketed column affording 23.3359 g (100%) of a colourless liquid, bp 84-86° (10 mm); ^1H NMR (CDCl_3) δ 0.21 (18, s), 2.8 (4, s).
- (b) *1-Hydroxycyclopropane carboxylic acid*. A 3-neck, 100 mL round bottom flask containing a stir bar was fitted with a pressure equalizing addition funnel, a thermometer, and a condenser. A solution of 5.7477 (24.9 mmol) of 1,2-bis(trimethylsilyloxy)-cyclobutene in 6 mL of n-pentane was added to the flask. Exposure to light was minimized. The reaction flask was cooled to and maintained at -60°C while adding a solution of 3.9883 g (25.0 mmol) of Br_2 in 6 mL of n-pentane over 0.5 h. The mixture containing a yellow precipitate was heated to reflux for 2 h. It was then cooled to 0° and the pH adjusted to 9 by adding 62.5 mL of 1 N NaOH cooled to 0° over 0.5 h. The pH was then adjusted to 1 with 1 N

HCl. The pentane and H_2O were removed by rotary evaporator (15 mm, 25° and 0.05 mm, 35°). The yellow solid was dissolved in anhydrous acetone and the liquid was decanted from the salts which were washed with three 5 mL portions of acetone. The acetone was removed by rotary evaporation and the remaining yellow liquid was Kugelrohr distilled (110-120°C, 0.02 mm) affording 1.8914 g (74%) of a white crystalline solid: mp 102-103°C; IR (KBr): 3380, 1700, 1180 cm^{-1} ; 1H NMR ($(CD_3)_2CO$): δ 1.06 (2, m), 1.21 (2, m), 4.98 var (2, broad s).

2. Biological activity

Buffer-treated hypocotyl sections of mung bean produced almost no ethylene during the incubation period (data not shown). Treatment with ACC resulted in increased ethylene evolution (Fig. 1). ACC-OH reduced ethylene formation from ACC (EFE activity). The inhibitory effect of ACC-OH was related to its concentration in the medium.

Figure 1: Effect of ACC-OH concentration on EFE activity in mung bean hypocotyl sections. Sections were pretreated with different concentrations of ACC-OH as indicated for 1.5 h and then 0.1 mM ACC was added.

The possibility that the inhibitory effect of ACC-OH resulted from interfering with ACC uptake was further investigated. Similar to the data presented in Figure 1, ACC-OH inhibited EFE activity (Fig. 2). However, the insert shows that ACC-OH-

treated sections contained lower levels of ACC, indicating that the inhibitor reduced ACC uptake. Endogenous ACC levels in mung bean tissues are negligible, so that measurement of endogenous ACC levels reflects only ACC taken up by the tissues. A similar effect on EFE activity was also detected in citrus leaf discs (Fig. 3).

Figure 2: Effect of ACC-OH concentration on EFE activity and ACC content (insert) in hypocotyl sections of mung bean. Details as in Fig. 1. ACC was determined after 8 h of incubation.

In the two previous plant systems EFE activity was dependent on uptake of ACC from the medium. Therefore, we studied the effect of ACC-OH on ethylene production in apple pericarp discs which contain natural endogenous ACC. ACC-OH somewhat reduced ethylene production during the first 2 h of incubation (Fig. 4). This inhibition was paralleled by a slight increase in endogenous ACC levels (Fig. 5).

Figure 3: Effect of ACC-OH concentration on EFE activity in citrus leaf discs. Discs were floated on ACC-OH for 3 h and then 0.2 mM ACC was added for additional 20 min. Ethylene was determined at different periods after termination of the ACC treatment.

Figure 4. Effect of ACC-OH concentration on ethylene production of apple pericarp discs. ACC-OH was pre-sent during the entire incubation period.

Figure 5: Effect of ACC-OH concentration on endogenous ACC levels in apple pericarp discs. ACC was measured after 6 h of incubation with the inhibitor. The data are of two different experiments.

The plant tissues used in this study differ from each other in their endogenous ACC levels. Mung bean and citrus tissues contain very low levels of endogenous ACC and analysis of EFE in these tissues requires addition of exogenous ACC. Apple tissues contain significant levels of ACC. However, a direct analysis of EFE activity in this tissue is almost impossible because we measure ethylene, the end product, whose level is determined by both ACC synthesis and the conversion of ACC to ethylene. Specific inhibition of EFE activity can be determined only by accumulation of ACC. The above data from mung bean and citrus tissues suggest that ACC-OH somewhat inhibited EFE activity. However, we conclude that this in fact was not the case, since ACC levels decreased rather than increased in ACC-OH-treated tissues (Fig. 2). Results with apple tissues further corroborate this conclusion. In apple tissues, ACC-OH caused a very slight inhibition of ethylene production (Fig. 4) which was

paralleled by a slight accumulation of ACC (Fig. 5). These data indicate that ACC-OH is a very weak inhibitor of EFE activity.

c) *N,N*-Dimethyl 1-aminocyclopropane carboxylic acid (ACC-dimethyl)

1. Synthesis

(a) *Ethyl N,N*-Dimethyl-1-aminocyclopropane carboxylate. To a solution of ACC-ethyl ester hydrochloride in 3.5 mL HCO_2H was added 0.47 g KOAc and 1 g of 37% formalin solution. The reaction mixture was warmed at 60° for 4 h, cooled and evaporated. The solids were suspended in 5 mL brine and 5 mL CH_2Cl_2 , then basified to pH 10 with 10% NaOH. Extraction with CH_2Cl_2 , drying over K_2CO_3 , filtration and evaporation provided 0.216 g (94%) of the title compound. Kugelrohr distillation provided an analytical sample. *Anal.* Calcd for $\text{C}_8\text{H}_{15}\text{O}_2\text{N}$: C, 61.10; H, 9.61; N, 8.94. Found: C, 61.11; H, 9.35; N, 8.74. ^1H NMR (CDCl_3): δ 0.93 (2 H, m), 1.24 (2 H, m), 1.25 (3 H, t, $J=7$), 2.54 (6 H, s), 4.12 (2 H, q, $J=7$). IR: 3100, 2960, 2920, 2860, 2780, 1725, 1470, 1440, 1360, 1335, 1270, 1210, 1220, 1025, 930, 910, 725 cm^{-1} .

(b) *N,N*-Dimethyl 1-aminocyclopropane carboxylic acid. To 0.30 g (1.91 mmol) of the above ester was added 4 mL of 4.5 M KOH. After heating at reflux for 2 h, the

solution was cooled, neutralized, and loaded onto a 100 g Dowex 50 (H^+) column. The column was washed with water and the amino acid was eluted with 20% EtOH in 1 M NH_3 . Evaporation provided 170 mg (69%) of **3** mp 197-199°C (d). 1H NMR (D_2O): δ 1.27 (2H, m), 1.53 (2 H, m), 2.96 (6 H, m), 2.96 (6 H, m). HRMS Calcd for $C_6H_{11}NO_2$: 129.0790. Found: 129.0790.

2. Biological activity

ACC-dimethyl showed a significant inhibitory effect of EFE activity in mung bean hypocotyl sections (Fig. 6). However, in apple tissues (Fig. 6) as well as in citrus leaf discs (Fig. 7) it was almost ineffective. The data suggest that, similar to the former analog, the inhibitory effect in mung bean tissues probably resulted from reduction in ACC uptake.

Figure 6: Effect of ACC-dimethyl concentration on EFE activity in mung bean hypocotyl sections and apple pericarp discs. Hypocotyl sections were pre-treated with ACC-dimethyl for 2 h and then 0.1 mM ACC was added for 20 min. EFE activity was determined between 6 and 8 h after the termination of treatment. Apple pericarp plugs were treated with ACC-dimethyl for 4 h. EFE activity was determined during the 4-h incubation period.

Figure 7: Effect of ACC-dimethyl concentration on EFE activity in citrus leaf discs. Discs were pretreated with ACC-dimethyl for 2 h and then ACC at different concentrations was added for 20 min. EFE activity was determined in a 4-h period after the termination of ACC treatment.

d. N-Cyclopropyl Glycine

1. Synthesis

(a) *Ethyl N-cyclopropyl glycinate*. To a solution of 2.84 g cyclopropylamine (49.8 mmol) in 50 mL CH₂Cl₂ was added ethyl bromoacetate (2.8 mL, 25.3 mmol) over 10 min dropwise. TLC monitoring (silica gel, 1:1 EtOAc/hexane) showed a single spot at 0.28 R_f. After stirring for 1 h, 30 mL 5% HCl was added, the layers were separated, and the aqueous phase was extracted with CH₂Cl₂. Basification and extraction with EtOAc gave an organic phase which was dried with MgSO₄ and evaporated to provide 1.408 g (39%).

IR: 3340, 3080, 2980, 1730, 1440, 1420, 1370, 1340, 1200, 1100, 1020, 920, 810, 770, 740 cm^{-1} .

NMR (CDCl_3) δ 4.18 (2H, q, $J=7$), 3.43 (2H, br s), 2.22 (1H, m), 1.27 (3H, t, $J=7$), 0.3-0.5 (2H, m).

(b) *N*-Cyclopropyl glycine. To 1.32 g (9.23 mmol) of the above ester was added 2.05 g KOH (31.1 mmol) in 20 mL water and 3 mL methanol. The reaction mixture was stirred overnight, heated at reflux for 1 h, cooled, and neutralized. Ion-exchange chromatography (Dowex 50, NH_3 elution) and evaporation gave 0.93 g of the title compound.

NMR (D_2O): 3.66 (2H, br s), 2.71 (1H, m), 0.7-0.9 (4H, m).

2. Biological activity

N-Cyclopropyl glycine had almost no inhibitory effect on ethylene production in apple pericarp discs (Fig. 8). It showed, however, an increasing inhibition of EFE activity up to 20 mM concentration in mung bean tissue, but again no parallel increase in ACC could be observed. In fact, there was a slight decrease in ACC content.

e) 1-Aminocyclobutyl-1-carboxylic acid

1. Synthesis

The cyclobutane analog was prepared by the substitution of dibromopropane for dibromoethane in the synthesis of ACC (Schöllkopf et al. (1973). *Liebigs Ann. Chem.* 611).

Figure 8: Effect of N-cyclopropyl glycine on ethylene production in apple pericarp discs and on EFE activity and ACC levels in mung bean hypocotyl sections. Mung bean sections incubated for 20 min in 0.1 mM ACC and ethylene was determined between 4 and 6 h after the termination of ACC treatment. The same tissue was used for analysis of endogenous ACC. EFE activity in apple pericarp discs was determined during a 4-h incubation period.

Figure 9: Effect of 1-aminocyclobutyl-1-carboxylic acid on EFE activity and ACC levels in mung bean hypocotyl sections. ACC (0.2 mM) was present during the 8 h incubation period. Levels of ACC in the tissues, were determined at the end of the incubation period.

2. Biological activity

1-Aminocyclobutyl-1-carboxylic acid inhibited EFE activity in mung bean tissue between 4 to 8 h after the initiation of treatment (Fig. 9). The inhibition was concentration dependent. ACC, measured after 8 h, decreased in a parallel pattern.

In apples, however, the inhibition of ethylene production by the analog was accompanied by an accumulation of ACC (Fig. 10). The inhibition of EFE activity decreased with time and did not show a clear relationship with the analog concentration.

Figure 10: Effect of 1-aminocyclobutyl-1-carboxylic acid on EFE activity and ACC levels in apple pericarp discs. ACC (0.2 mM) was present during the 6 h incubation period. Levels of ACC in the tissues, were determined at the end of the incubation period.

f) 1-Aminomethyl-1-cyclopropane carboxylic acid (Homo-ACC)

1. Synthesis

- (a) *1-Aminomethyl-1-cyclopropane methanol*. To a 1 M solution of LiAlH_4 in ether (40 mL) was added dropwise under nitrogen a solution of methyl 1-cyano-1-cyclopropanecarboxylate (1.0 g, 8 mmol) in 20 mM ether. After stirring under reflux for 5 h, the reaction was cooled. Water (1.9 mL), 15% NaOH (1.9 mL), and water (5.7 mL) were added sequentially. The solid was filtered and the cake was washed with ethyl acetate. Concentration provided 0.75 g (93%) of the title compound. $^1\text{H-NMR}$ (CDCl_3): δ 3.59 (s, 2H), 2.82 (s, 2H), 0.48 (t, 2H), 0.40 (t, 2H).
- (b) *1-Aminomethyl-1-cyclopropane carboxylic acid*. 1-Aminomethyl-1-cyclopropane methanol (2.2g) was dissolved in ethyl trifluoroacetate (5 mL) and heated at reflux for 1 h. Concentration provided 4.2 g (98%) of crude trifluoroacetamide. This compound was dissolved in 500 mL acetone and a 1.67 M solution of Jones' reagent was added until the orange colour persists. After 30 min at 20° , isopropanol was added. The reaction mixture was partitioned between ethyl acetate and water. The organic phase was extracted with NaHCO_3 and the aqueous phase acidified. Extraction with ethyl acetate, drying, and concentration provided 2.71 g (60%) of acid trifluoroacetamide. This was stirred for 15 h at 20° with 10 mL 20% NaOH. Neutralization and ion-exchange chromatography gave 1.1 g (73%) of the title compound. $^1\text{H-NMR}$ (D_2O): δ 2.67 (2H, s), 0.76 (2H, m), 0.46 (2H, m).

2. Biological activity

Homo-ACC did not show significant effect on EFE activity in mung bean hypocotyl sections or on ethylene production in apple pericarp discs (Fig. 11).

Figure 11: Effect of Homo-ACC on EFE activity in mung bean hypocotyl sections and on ethylene production in apple pericarp discs. Mung bean sections were incubated with 0.2 mM ACC and EFE activity was determined between 4 and 8 h of the incubation period. Ethylene production in apple pericarp discs was determined as above.

g) 2-Methyl-1-aminocyclopropane carboxylic acid (Me-ACC)

1. Synthesis

The synthesis and some preliminary studies concerning the competitive nature of the analog are described in the following paper.

2. Biological activity

As shown above Me-ACC competes with ACC on the same site of EFE. Further experiments were designed to test its inhibitory effect on EFE activity. In both mung bean hypocotyls (Fig. 12) and citrus leaf discs (Fig. 13), Me-ACC showed an increasing inhibition of ethylene production from ACC with the rise in Me-ACC concentrations. No ethylene was formed in the absence of ACC. However, the tissues were able to produce propene from the analog. In the presence of ACC, the propene evolution was somewhat lower than that without ACC. This was most evident in mung bean tissue treated with 5 mM Me-ACC (Fig. 12).

Figure 12: Effect of Me-ACC concentration on the release of ethylene from ACC in mung bean hypocotyl sections. Propene released from the analog was also recorded. Sections were pretreated with Me-ACC for 20 min. Ethylene and propene were determined between 0 and 6 h after the termination of the ACC treatment.

Figure 13: Effect of Me-ACC concentration on the release of ethylene from ACC in citrus leaf discs. Propene released from the analog was also recorded. Sections were pretreated with Me-ACC for 4.5 h and then with 0.5 mM ACC for 20 min. Ethylene and propene were determined between 0 and 4 h after the termination of the ACC treatment.

h) Aminoethoxyvinyl glycine (AVG).

Although AVG is not an ACC analog an attempt was made to develop a chemical synthesis of aminoethoxyvinyl glycine (AVG, 1), an inhibitor of ACC synthase and therefore of ethylene biosynthesis (1). The method which was adopted was based on a literature procedure for the preparation of β , γ -unsaturated amino acids.(2) (Eq. 1).

For the synthesis of AVG, a β -alkoxy-substituted α,β -unsaturated aldehyde was

needed. Three different compounds were prepared by the procedure of Quang.(3)

Each was submitted to reduction/oxidation (50% overall yield) to obtain the α,β -unsaturated aldehyde. (Eq. 2). Extensive attempts have been made to convert these compounds to the amino acids. These have included condensation with benzylamine and subsequent treatment with TMSCN by the Greenlee procedure, or conversion to

the N-trimethylsilyl imine by the method of Hart (4) and condensation with TMSCN. In the former case, the nitrile is obtained in impure form, but attempts to hydrolyze to the amino acid in base were unsuccessful. In the latter case, no characterizable product could be isolated at all. Our final effort is completing the synthesis, currently underway, is shown in Eq. 3.

This involves the formation of a cyanohydrin tosylate and the introduction of both amino groups simultaneously via the azide.

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4. Examining the role of cyanide in ethylene biosynthesis by the use of Me-ACC

Me-ACC may be used as a tool to examine the hypothesis that cyanide plays a role in the regulation of ethylene biosynthesis; Me-ACC serves as a substrate for EFE, thus releasing cyanide as a by-product to propene. The latter has a much lower physiological activity than ethylene. Therefore, it is reasonable to assume that any physiological response caused by the application of Me-ACC might be attributed to cyanide. This hypothesis was tested in preclimacteric avocado fruits which contain negligible amounts of endogenous ACC. Avocado fruits were treated with either H₂O or 10 mM Me-ACC for 24 h and then pericarp discs were excised and treated with ACC. It was expected that if cyanide plays a role in inducing EFE activity, addition of ACC to Me-ACC pretreated fruits would result in relatively high levels of ethylene. The data show that there was no difference between the two treatments, indicating that cyanide did not affect EFE activity (Fig. 14). It may be argued that discs excised from fruits pretreated with Me-ACC did not respond fully to ACC because of the competition with Me-ACC. However, this was not the case since discs excised from fruits pretreated with Me-ACC produced almost no propene between 2 to 8 h after excision (data not shown).

When Me-ACC was applied to whole preclimacteric fruits, significant amounts of propene were produced (Fig. 15). Propene production reached a peak 24 h after the initiation of Me-ACC treatment and declined after 72 h almost to zero values. We assume that this decline resulted both from a decrease in Me-ACC uptake and conjugation. It is evident that Me-ACC did not induce any significant ethylene production. As expected, ACC induced increased ethylene production. Me-ACC-treated fruits did not show any advanced ripening compared to untreated fruits (data not shown). Again, these data do not indicate any involvement of cyanide in the regulation of ethylene biosynthesis.

Figure 14: Effect of Me-ACC pretreatment of intact fruits on the release of ethylene from ACC in avocado pericarp discs. Discs were treated with 1.5 mM ACC for measuring EFE activity.

Figure 15: Time course of ethylene and propene evolution in avocado fruits treated with either 10 mM Me-ACC or 5 mM ACC. The treatment solutions were introduced through the pedicel during the entire incubation period.

Even when fruits were pretreated for 21 h with Me-ACC and ACC was then added, the results were quite similar (Fig. 16). No ethylene or propene were detected when fruits were treated in water only (H₂O + H₂O). Addition of ACC to H₂O-

pretreated fruits ($\text{H}_2\text{O} + \text{ACC}$) increased ethylene production rates with time. A similar pattern was observed when ACC was added after pretreatment with Me-ACC (Me-ACC + ACC). The latter treatment showed a sharp decrease in propene production during the first 24 h which leveled off thereafter. Addition of ACC to ACC-pretreated fruits resulted in high levels of ethylene.

Figure 16. Time course of ethylene and propene production in avocado fruits pretreated with 10 mM Me-ACC or 5 mM ACC for 21 h and then with 5 mM ACC.

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Inhibition of Activity of the Ethylene-Forming Enzyme by α (*p*-Chlorophenoxy)Isobutyric Acid

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Abstract. α (*p*-Chlorophenoxy)isobutyric acid (PCIB) inhibited indole-3-acetic acid (IAA)-induced ethylene production in etiolated mung bean hypocotyl sections. The endogenous level of 1-aminocyclopropane-1-carboxylic acid (ACC) was not significantly affected by PCIB, indicating that PCIB exerted its effect primarily by inhibiting the activity of the ethylene-forming enzyme (EFE). This conclusion was supported by the observations that PCIB inhibited the conversion of exogenously applied ACC to ethylene. The inhibitory effect of PCIB was already evident with 0.05 mM PCIB, and it increased with time after application of the inhibitor. PCIB also significantly inhibited ethylene production in apple fruit tissues, but it only slightly reduced the level of endogenous ACC. Similar to mung bean, EFE activity in apple tissue was significantly inhibited by PCIB. The possibility that PCIB also inhibits auxin-induced ACC synthase activity is discussed.

α (*p*-Chlorophenoxy)isobutyric acid (PCIB) was classified as a substance possessing antiauxin properties (Burstrom 1950). Later its antiauxin properties were studied in detail, particularly in regard to auxin-binding sites in subcellular fractions from maize coleoptiles (Dohrmann et al. 1978) and from *Cucurbita* hypocotyls (Jacobs and Hertel 1978). In all these experiments, it has been shown that PCIB efficiently and specifically competes with indole-3-acetic acid (IAA) for auxin-binding sites in vitro. PCIB has been widely employed to demonstrate the role of endogenous auxin in plant growth processes. Thus, PCIB was first employed by Burstrom (1950) to demonstrate the role of endogenous auxin in the two phases of growth of wheat roots. Subsequently, it was utilized for studying growth of *Avena* coleoptiles (Fransson and Ingestad 1955), elongation of *Avena* leaf sections (Cleland 1964), and curvature of citrus

flower petals (Goldschmidt 1968). Recently, PCIB was found to abolish the geotropic response of roots of cress (Katekar and Geissler 1980). Also, it was used to examine the primary action of IAA in crown gall tumors in potato tuber tissue (Rausch et al. 1984).

Frenkel and Haard (1973) were the first to show that PCIB inhibited ethylene production in pears and attributed it to the antiauxin activity of PCIB. In a recent study, Tsai and Arteca (1984) observed that PCIB inhibited IAA-induced ethylene production in mung bean hypocotyl segments. They suggested that the inhibitory effect of PCIB resulted from the blocking of auxin transport and action. Other investigators also utilized PCIB to examine the involvement of auxin in the regulation of ethylene production (Imaseki et al. 1975) and to study the mechanism of the stimulatory effect of thidiazuron on ethylene production (Suttle 1984, Yip and Yang, 1986).

During studies of ethylene biosynthesis in cucumber growth tips and hypocotyls, we have observed that PCIB also inhibits ethylene-forming enzyme (EFE) activity, a step in the ethylene biosynthetic pathway, which is thought to be independent of auxin control (Yu and Yang 1979). The results of a study on the effect of PCIB on EFE activity are presented in this paper.

Materials and Methods

Plant Material and Treatments

The plant species tested were mung bean (*Vigna radiata* L.) and apple (*Malus silvestris* Mill., var. Golden Delicious). Seeds of mung bean were germinated and grown in Vermiculite for 4 days in darkness at 25°C. Ten 1-cm long hypocotyl segments, 1–3 cm below the hook, were weighed and incubated in 1 ml 50 mM Mes buffer (pH 6.1) in a 22-ml vial.

Apples were obtained from commercial storage houses and stored in our laboratory at 4°C. Peel strips were cut from the equatorial region of apples using a domestic potato peeler and were further cut into 2-cm long sections. Each section was placed with the adhering cortical tissue downward on 2 ml 50 mM Mes buffer (pH 6.1) in a 25-ml Erlenmeyer flask. Plugs (1 cm in diameter and 2 cm long) were excised from the pericarp with a cork borer and further cut into disks, about 2 mm thick. Seven disks were weighed and incubated in 3 ml 50 mM Mes buffer (pH 6.1) in a 25-ml Erlenmeyer flask. Where indicated, PCIB, IAA, 1-aminocyclopropane-1-carboxylic acid (ACC), or aminoethoxyvinylglycine (AVG) was added to the medium.

Determination of Ethylene

For monitoring ethylene production, the vessels containing the plant samples were sealed with a rubber serum cap and shaken in the dark at 27°C. At various periods during incubation, a 2-ml air sample was withdrawn from the vessels with a hypodermic syringe. After each sampling, the vessels were flushed with ethylene-free air and resealed for an additional determination of

ethylene. Ethylene was assayed using a gas chromatograph equipped with an alumina column and a flame ionization detector.

Extraction and Determination of ACC

Mung bean hypocotyls were ground with a mortar and pestle in 2 ml 80% ethanol. The mortar and pestle were washed twice with 2 ml 80% ethanol each time. The extract and washings were combined and centrifuged for 10 min at 10,000g. The supernatant was concentrated under reduced pressure at 45°C. Residues were brought to a volume of 2 ml with distilled water and clarified by centrifugation as above.

Apple tissues were homogenized in 4 ml 80% ethanol by means of a Polytron homogenizer. The shaft was washed with additional 4 ml 80% ethanol. The extract and washing were combined and filtered through Miracloth. The ensuing procedure was as for mung bean hypocotyls. ACC in the aqueous extract was assayed by the method of Lizada and Yang (1979).

Assay of EFE Activity

EFE activity was determined by measuring conversion of applied ACC to ethylene *in vivo*. Tissue was placed in a 25-ml Erlenmeyer flask containing different concentrations of ACC in 1 or 2 ml Mes buffer (pH 6.1). In one experiment using apple peel tissues, AVG was also added to ensure measurement of EFE activity only. Ethylene evolution was measured as described above.

Results

Effect of PCIB on IAA-Induced Ethylene

Consistent with previous data (Yu and Yang 1979), application of 50 μ M IAA to mung bean hypocotyls increased ethylene evolution and ACC production (Fig. 1). Treatment with 1 mM PCIB inhibited IAA-induced ethylene. This inhibition was evident after 4 h of incubation, at a time when IAA-induced ethylene started to increase, and it increased gradually with time. After 8 h of incubation, PCIB inhibited IAA-induced ethylene production by 65% (Fig. 1A). Surprisingly, the endogenous level of ACC in IAA-treated hypocotyls was not significantly affected by PCIB (Fig. 1B). Data from similar experiments showed a consistency in the effect of PCIB on IAA-induced ethylene. However, the content of ACC in hypocotyls treated with IAA plus PCIB was either higher or slightly lower than in hypocotyls treated with IAA only.

Effect of PCIB on EFE Activity in Mung Bean Hypocotyls

To examine EFE activity in mung bean hypocotyls, ACC was directly utilized as an ethylene precursor. EFE activity, measured by application of 0.05 and

0.2 mM ACC to hypocotyl sections, was significantly inhibited by PCIB (Fig. 2). In both ACC concentrations, the inhibition was apparent after 2 h of incubation, but it was much higher in the lower concentration of ACC. The inhibition of EFE activity by PCIB increased with time, reaching 95 and 80% of control with 0.05 and 0.2 mM ACC, respectively, after 8 h of incubation.

The effect of different concentrations of PCIB on EFE activity was also examined (Fig. 3). Over a range of 0.05–0.5 mM, the inhibition by PCIB increased progressively from 20 to 75%. The concentration of PCIB required for 50% inhibition of ethylene production was estimated to be about 0.2 mM (Fig. 3).

Attempts to analyze the kinetics of inhibition by a Lineweaver-Burk plot failed to give a clear-cut conclusion about the nature of the inhibition.

Effect of PCIB on Ethylene Production in Apple Tissues

The rate of ethylene production in apple pericarp disks was initially high, but it decreased rapidly during an incubation period of 8 h (Fig. 4A). PCIB significantly inhibited ethylene production, the magnitude of the inhibition reaching ~70% of the control after 8 h of incubation (Fig. 4A). PCIB also reduced ACC level in apple tissues after 8 h of incubation (Figs. 4B, 5B). However, ACC level was only about 20% lower than that in the control, while inhibition of ethylene production was 70% (Fig. 4).

Similar results were obtained in apple peel tissue, where ethylene production was inhibited by 70% and ACC level was reduced by ~20% (Fig. 5). Additional experiments showed that, as in mung bean hypocotyls, there was a consistency in the effect of PCIB on ethylene production (~70% inhibition), but its effect on ACC level varied, so that in most experiments there was a slight reduction whereas in others PCIB somewhat enhanced ACC content (data not shown).

Effect of PCIB on EFE Activity in Apple Peel

The initial ethylene production in freshly excised apple tissues was high (Figs. 4, 5). For measuring EFE activity, AVG was used to accelerate the deprivation of endogenous ACC from apple peel tissue. Measurements of EFE activity started 4 h after excision of the tissues. Under these conditions EFE activity in control tissues was almost constant throughout the experimental period. PCIB reduced EFE activity by ~70% (Fig. 6).

Discussion

The properties of PCIB as an antiauxin agent are well documented (Burstrom 1950, Dohrmann et al. 1978, Jacobs and Hertel 1978). Among its various effects, PCIB was found to inhibit ethylene evolution in Bartlett pears (Frenkel and Haard 1973) and IAA-induced ethylene production in mung bean hypo-

Fig. 1. Effect of PCIB on ethylene production rate (A) and ACC level (B) in mung bean hypocotyls treated with IAA. Ten hypocotyl segments were incubated in Mes buffer (pH 6.1) containing 50 μ M IAA with or without 1 mM PCIB. Ethylene production was monitored during an 8-h incubation period. ACC levels were determined at the end of incubation. Bars indicate 1 SE.

Fig. 2. Time course of the inhibition of EFE activity in mung bean hypocotyl segments by PCIB. Ten hypocotyl segments were incubated in Mes buffer (pH 6.1) containing 1 mM PCIB and 0.05 (●) or 0.2 (○) mM ACC.

Fig. 3. Dose-response curve for the inhibition of EFE activity by PCIB in mung bean hypocotyls. Ten hypocotyl segments were incubated in Mes buffer (pH 6.1) containing 0.1 mM ACC and various concentrations of PCIB. Ethylene production was measured after an 8-h incubation.

Fig. 4. Effect of PCIB on ethylene production rate (A) and ACC level (B) in apple pericarp discs. Seven disks were incubated in Mes buffer (pH 6.1) containing 1 mM PCIB. Ethylene production was monitored during an 8-h incubation period. ACC level was determined at the end of incubation. Bars indicate 1 SE.

cotyls (Tsai and Arteca 1984). This effect was attributed by the above investigators to the antiauxin properties of PCIB.

It is well accepted that IAA stimulates ethylene production by inducing the synthesis of ACC (Yang and Hoffman 1984). Several studies have shown that

Fig. 5. Effect of PCIB on ethylene production rate (A) and ACC level (B) in apple peel tissue. One 2-cm-long apple peel section was incubated in Mes buffer (pH 6.1) containing 1 mM PCIB. Ethylene production was monitored during an 8-h incubation. ACC content was determined at the end of incubation. Bars indicate 1 SE.

Fig. 6. Effect of PCIB on EFE activity in apple peel tissues. Peel sections were treated with 0.5 mM AVG with or without 1 mM PCIB. EFE activity was measured by application of 0.1 mM ACC. Hatched bars indicate EFE activity when tissue was treated with PCIB. Bars indicate 1 SE.

ethylene production rates induced by IAA closely paralleled the level of endogenous ACC and the activity of ACC synthase (Jones and Kende 1979, Yoshii and Imaseki 1982, Yu and Yang 1979). Accordingly, we expected that inhibition of IAA-induced ethylene production by PCIB (Fig. 1A) would be

accompanied by a parallel reduction in ACC level. Contrary to our expectations, this was not the case. ACC levels in IAA plus PCIB-treated hypocotyls were similar to levels in IAA-treated hypocotyls, and in some experiments even higher (Fig. 1B). Similarly, we observed that PCIB significantly inhibited ethylene production rates in apple tissues, whereas ACC levels were only slightly reduced (Figs. 4, 5). This discrepancy was clarified when the effect of PCIB on EFE activity was studied. PCIB strongly inhibited EFE activity in mung bean hypocotyls (Fig. 2). A significant inhibition was already observed with 0.05 and 0.1 mM PCIB (Fig. 3). Similar concentrations were used for negation of auxin action (Goldschmidt 1968, Rausch et al. 1984, Tsai and Arteca 1984). By using AVG to accelerate the deprivation of endogenous ACC from apple tissues, it could also be shown that PCIB inhibited EFE activity in apple tissues (Fig. 6). Because at present EFE activity can only be measured *in vivo*, we were unable to obtain reliable data on the nature of the inhibition. In a recent study on the stimulatory effect of thidiazuron on ethylene production, Yip and Yang (1986) also concluded that PCIB does not appear to be a specific inhibitor of IAA-mediated ethylene production.

It seems that the inhibition of ethylene production by PCIB does not result solely from the inhibition of EFE activity. If the conversion of ACC to ethylene were the only step in the ethylene biosynthetic pathway affected by PCIB, ACC levels in PCIB-treated tissues would have been well above those measured in untreated tissues. However, in both mung beans and apples, ACC levels in PCIB-treated tissues did not differ much from the levels of ACC in untreated tissues (Figs. 1B, 4B, 5B). The data suggest that PCIB also inhibits ACC synthase owing to its antiauxin properties. This assumption may be true also for apples, since there is evidence that auxin stimulates ethylene production in fruit tissues (Frenkel and Dyck 1973, Vendrell 1969).

The data obtained in this study may be interpreted to indicate that auxin also regulates EFE activity. It was recently hypothesized that auxins could be involved in the conversion of ACC to ethylene through their effects on membrane polarization (John 1983). Balague and Pech (1985) reported that EFE activity in pear cell cultures was increased by 2,4-D. They also suggested that auxin plays a role in the modulation of EFE activity. We, however, have found that various phenoxy acids, including some of those that possess auxin activity, also inhibited EFE activity in mung bean hypocotyls (data not shown). This indicates that the inhibition of EFE activity by PCIB is probably not related to its antiauxin properties.

Whatever the mechanism of the inhibition of EFE activity by PCIB may be, the use of PCIB to demonstrate the involvement of auxin in various physiological plant processes, and particularly in those in which ethylene is also involved, should be regarded with care.

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6. Effect of phenoxy acids on ethylene production in apple pericarp discs

Joseph Riov and Shang Fa Yang¹

In the previous study we observed that 2-(4-chlorophenoxy)isobutyric acid (PCIB) inhibited indole-3-acetic acid (IAA)-induced ethylene production (Trebitsh and Riov, 1987). The data obtained in the above study indicated that PCIB exerted its inhibitory effect primarily by blocking the activity of the ethylene-forming enzyme (EFE). Since PCIB is an antiauxin (Burstrom 1950), these observations may be interpreted to indicate that auxin regulates EFE activity in addition to 1-aminocyclopropane-1-carboxylic acid (ACC) synthase activity (Hoffman and Yang 1984). It was recently hypothesized that auxins could be involved in the conversion of ACC to ethylene through their effects on membrane polarization (John 1983). Balague and Pech (1985) reported that EFE activity in pear cell cultures was increased by 2,4-D. They also suggested that auxin plays a role in the modulation of EFE activity. There is a large number of phenoxy acids, most of which are potent auxins. We report here that various phenoxy acids inhibit EFE activity irrespective of their auxin activity.

Materials and Methods

Apples (*Malus silvestris* Mill., var. Golden Delicious) were obtained from local growers in California and stored at 4°C. Plugs, 8 mm in diameter and about 2 cm long, were excised from the pericarp with a cork borer and further cut into discs, about 4 mm thick. Four discs were weighed and placed on filter paper wetted with 0.45 ml of 50 mM Tris buffer (pH 6.1) containing 0.3 M mannitol and phenoxy acids at the desired concentration in a 25-ml glass vial. The vials were sealed and incubated in darkness at 25°C.

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For monitoring ethylene production, a 1-ml air sample was withdrawn from the vials with a hypodermic syringe. After each sampling, the vessels were flushed with ethylene-free air and resealed for an additional determination of ethylene. Ethylene was assayed using a gas chromatograph equipped with an alumina column and a flame ionization detector.

For determination of ACC and malonyl-ACC (MACC) levels, discs were extracted with 80% ethanol at 80°C. After removal of the ethanol, the extract was brought to a volume of 2.5 ml with water. An aliquot was assayed directly for ACC content by the method of Lizada and Yang (1979). For measuring MACC content, a 200- μ l aliquot was hydrolyzed with 2 N HCl for 3.5 h. After neutralization with NaOH, the ACC liberated was assayed as described above. MACC content was calculated as the difference in ACC content before and after hydrolysis.

The phenoxy acids used were: 4-chloro-2-methylphenoxyacetic acid (MCPA), 4-(4-chloro-2-methylphenoxy)butyric acid (MCPB), 2-(4-chloro-2-methylphenoxy)propionic acid (MCP), 4-chlorophenoxyacetic acid (4-CPA), 2-(4-chlorophenoxy)isobutyric acid (PCIB), 2-(4-chlorophenoxy)propionic acid (CPP), 2,4-dichlorophenoxy acetic acid (2,4-D), 4-(2,4-dichlorophenoxy)butyric acid (DCPB), phenoxyacetic acid (PA), phenoxybutyric acid (PB), 2-(2,4,5-trichlorophenoxy)propionic acid (2,4,5-TP).

Results and Discussion

In vegetative tissues, the rate of ethylene production is thought to be regulated by the internal level of auxin, and application of exogenous auxin stimulates ethylene production (Yang and Hoffman, 1984). Stimulation of ethylene biosynthesis by auxin is due to induction of ACC synthase activity (Yang and Hoffman 1984). Phenoxy acids possessing auxin activity may have two contrasting effects on ethylene biosynthesis: (a) induction of ACC synthase; and (b) inhibition of EFE activity. In order to avoid the possible induction of ACC synthase, we choose to work with apple tissues in which exogenous auxin does not affect ACC synthase activity.

Treatment of apple pericarp discs with 0.5 mM of different phenoxy acids resulted in various degrees of inhibition of ethylene production (Fig. 1). Phenoxy acids containing no chlorine were only slightly active regardless of the length of the side chain. Thus, PA and PB showed significant lower inhibition rates compared to the corresponding chloro-substituted phenoxy acids. This is in accordance with the higher biological activity of chloro-substituted phenoxy acids observed in previous studies (Audus 1972).

Figure 1: Inhibition of ethylene production in apple pericarp discs by various phenoxy acids (0.5 mM).

Inhibition of ethylene production by phenoxy acids was also dependent on the length of the side chain (Fig. 1). Chloro-substituted phenoxybutyric acids were

significantly more active than chloro-substituted phenoxyacetic acids (Fig. 1, A and C). Chloro-substituted phenoxy acids with a branched side chain showed a moderate activity (Fig. 1B). Fifty percent inhibition of ethylene production occurred at 0.12 mM of DCPB, whereas 0.48 mM of 2,4,5-TP was required to cause the same degree of inhibition (Fig. 2).

Figure 2: Effect of DCPB and 2,4,5-TP concentration on ethylene production in apple pericarp discs. ○, DCPB; ●, 2,4,5-TP.

ACC levels in apple pericarp discs increased following a .8-h treatment with various phenoxy acids (Table 1). These observations suggest that the inhibition of ethylene production by phenoxy acids is mainly due to inhibition of EFE activity. Similar results were previously reported for PCIB (Trebitsh and Riov 1987). MACC levels did not change significantly in the treated discs (data not shown).

The data obtained in the present study negate the hypothesis that auxin is responsible for the maintenance and modulation of membrane integrity and EFE activity (Balague and Pech 1985). The mechanism by which phenoxy acids inhibit EFE activity is not clear. The inhibition is not related to their auxin activity since both auxins and antiauxins are active. We only know that there is some structure-function relationship, and the longer the side-chain, the higher is the inhibitory effect.

Nevertheless, these compounds are widely utilized for both basic research and practical use and their inhibitory effect on EFE activity should be considered.

Table 1: Effect of various phenoxy acids (0.5 mM) on ethylene and ACC levels in apple pericarp discs. Pericarp discs were incubated for 7 h and then sealed for an additional h for measuring ethylene. Immediately after completion of the ethylene measurements, the same discs were employed for assaying ACC content

Phenoxy acid	Ethylene nl g ⁻¹ h ⁻¹	ACC nmol g ⁻¹
None	90.2 ± 16.4	6.9 ± 0.8
2,4,5-TP	27.5 ± 3.7	8.9 ± 0.7
DCPB	18.6 ± 2.4	7.8 ± 0.5
MCPB	15.8 ± 2.9	8.6 ± 0.7

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F. DESCRIPTION OF COOPERATION

During the entire research period a close cooperation was developed between the two groups. This consisted of a periodic exchange of information on each stage of the research. Prof. Pirrung synthesized the analogues and after preliminary tests the chemicals were sent to the Israeli laboratory for physiological tests. After performing the physiological tests, the results were sent to Prof. Pirrung as guide lines for synthesis of new analogues. At the beginning of the programme, Prof. Goren visited Stanford and during this visit a thorough planning of the research work and its details were discussed. Unfortunately, the planned visit of Prof. Pirrung to Rehovot during the second year was canceled for personnel reasons. Consequently, a second visit by Prof. Goren and Prof. Riov to Stanford was held in the fall of 1986 in order to assess the data obtained and discuss plans for the third year.

During the spring of 1987, a third meeting was held between the two parties in the U.S. In this meeting, we discussed the data obtained during the last year and set up guide lines for the final report.

The close contact between the two groups was very productive and insured a fruitful interaction.

G. EVALUATION OF THE RESEARCH ACHIEVEMENTS

G1. SYNTHESIS AND ACTIVITY OF ACC ANALOGUES

Much effort was devoted to the synthesis of ACC analogues and to determine their chemical and physical characteristics. In this respect the goals of the research were achieved. Unfortunately, only a few of the analogues synthesized can be considered for further research of ethylene biosynthesis. We assume that this is due to the strict specificity of ACC as a substrate for EFE. Although the nature of EFE has not been elucidated, the present research demonstrates that even slight structural changes in the ACC molecule cause the enzyme not to recognize the analogues.

Nevertheless, Me-ACC and 2-cyclopropylaminocyclopropane carboxylic acid showed relatively high specificity to the enzyme which was reflected by decreasing ethylene biosynthesis. These analogues can contribute to the studies of ethylene biosynthesis and indeed we used Me-ACC to assess the role of cyanide in ethylene biosynthesis (see below). Before being able to come to conclusive evaluation of the mode of action of these chemicals as analogues, further research is required. It is necessary to separate between their effects on ACC uptake and those on ethylene biosynthesis. The data presented might be a result, at least in part, from inhibition of ACC uptake. In this respect, the analogues can be useful for studying ACC uptake.

From a practical point of view, we suggest that further investigations towards ethylene biosynthesis inhibitors should involve other directions rather than the search for ACC analogues. Although the conclusion is of a negative nature, it is of both scientific and practical importance.

G2. INVOLVEMENT OF CYANIDE IN ETHYLENE BIOSYNTHESIS

Based on previous observations that HCN induced ethylene production, Pirrung has suggested that the cyanide released during the conversion of ACC to ethylene plays a regulatory role in ethylene biosynthesis. This hypothesis was tested in avocado tissues using Me-ACC, which proved to be useful for studying this question. The data obtained did not support the above hypothesis. Before arriving at final conclusions, other ethylene producing tissues, such as tomato tissue should be examined.

G3. INHIBITION OF ETHYLENE BIOSYNTHESIS BY PHENOXY ACIDS

During the course of this study we have observed that phenoxy acids inhibit ethylene biosynthesis, primarily by inhibiting EFE activity. To the best of our knowledge, this is the first report on the inhibition of EFE activity by phenoxy acids. Although the nature of this inhibition has not yet been elucidated, these observations may be utilized for studying the EFE. At present, it is clear that inhibition of EFE

activity is not related to the auxin activity of these compounds since phenoxy compounds which are auxins and antiauxins are active. It is premature to speculate whether phenoxy acids can be utilized for the practical control of ethylene biosynthesis.

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