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IN VIVO FORMATION OF 1-MALONYLAMINOCYCLOPROPANE-1-CARBOXYLIC ACID AND ITS RELATIONSHIP TO ETHYLENE PRODUCTION IN COCKLEBUR SEED SEGMENTS: A TRACER STUDY WITH 1-AMINO-2-ETHYLCYCLOPROPANE-1-CARBOXYLIC ACID

SHIGERU SATOH and YOHJI ESASHI

Department of Biological Science, Tohoku University, Kawauchi, Sendai 980, Japan

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Key Word Index—*Xanthium pennsylvanicum*; Compositae; cocklebur; malonylation; ethylene production; D-amino acid; 1-aminocyclopropane-1-carboxylic acid; 1-amino-2-ethylcyclopropane-1-carboxylic acid.

Abstract—The *in vivo* formation of 1-malonylaminocyclopropane-1-carboxylic acid (malonyl-ACC) and its relationship to ethylene production in the axial tissue of cocklebur (*Xanthium pennsylvanicum*) seeds were investigated using the stereoisomers of the 2-ethyl derivative of ACC (AEC), as tracers of ACC. Of the four AEC isomers, the (1*R*, 2*S*)-isomer was converted most effectively to a malonyl conjugate as well as to 1-butene. Malonyl-AEC, once formed, was not decomposed, supporting the view that malonyl-ACC does not liberate free ACC for ethylene production in this tissue. D-Phenylalanine inhibited the formation of malonyl-AEC and, at the same time, promoted the evolution of 1-butene, whereas L-phenylalanine did not. Possibly, the D-amino-acid-stimulated ethylene production in cocklebur seed tissues is due to an increase in the amount of ACC available for ethylene production which results from the decrease of ACC malonylation in the tissues treated with D-amino acid. 2-Aminoisobutyric acid, a competitive inhibitor of ACC-ethylene conversion, did not affect the malonylation of AEC.

INTRODUCTION

ACC is a key intermediate of ethylene production in plants [1, 2]. Recent studies by Amrhein *et al.* [3] and Hoffman *et al.* [4, 5] have shown that ACC is present in both the free form and a conjugated form, malonyl-ACC, in plant tissues, and that exogenously applied ACC is converted to malonyl-ACC as well as to ethylene. Because the malonylation of D-amino acids commonly occurs in higher plant tissues [6, 7], ACC is considered to be recognized as one of the D-amino acids in the tissues and converted to malonyl-ACC in spite of its lack of an asymmetric α-carbon atom [3–5]. On the other hand, we have shown that some D-amino acids stimulate ethylene production in plant tissues [9]. More recently, the stimulation of ethylene production by D-amino acids has been interpreted as the consequence of their inhibitory action on the malonylation of ACC resulting in a higher ACC content [15, 16].

Isomers of AEC, the 2-ethyl derivative of ACC, are degraded to 1-butene in apple tissue and mung bean hypocotyls and the degradation is thought to be catalysed by the same enzyme as that responsible for the conversion of ACC to ethylene [8]. Of interest is the finding that the (1*R*,2*S*)-isomer is most preferentially converted to 1-butene in these tissues, suggesting the stereospecific conversion of ACC to ethylene [8].

On the basis of these preceding works, it was to be expected that AEC isomers would be converted to malonyl conjugates as well as to 1-butene in plant tissues by the respective enzymes responsible for the conversion of ACC to ethylene and malonyl-ACC. In this study, we found the formation of malonyl-AEC from exogenously applied AEC and the absence of decomposition of the conjugate in the axial segments of cocklebur seeds. Thereupon, we used AEC isomers as tracers of ACC in order to elucidate the properties of the malonylation of ACC and its relationship to ethylene production in seed tissues as follows: First, the possible stereospecificity in the malonylation of ACC was examined by analysis of the malonylation of AEC isomers. Second, the effects of D- and L-phenylalanine on the conversion of AEC to 1-butene and malonyl-AEC were examined in relation to D-amino acid-stimulated ethylene production [9, 10]. Finally, the effect of AIB, a competitive inhibitor of ACC-ethylene conversion [11, 12], on the malonylation of AEC was tested to characterize the malonylation of ACC.

RESULTS AND DISCUSSION

Formation of AEC conjugate and its identification

Each of the four isomers of AEC was converted to a malonyl conjugate as well as to 1-butene, when administered exogenously to the axial segments of cocklebur seeds (Table 1). To characterize the conjugate, it was prepared from 20 g of segments which had been fed with (1*R*,2*S*)-AEC. The conjugate was extracted with 70%

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AEC, 1-amino-2-ethylcyclopropane-1-carboxylic acid; AIB, 2-aminoisobutyric acid; malonyl-ACC, 1-malonylaminocyclopropane-1-carboxylic acid; malonyl-AEC, 1-malonylamino-2-ethylcyclopropane-1-carboxylic acid; Na-PIPES, sodium piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

Table 1. Conversion of AEC isomers to 1-butene and malonyl-AEC in axial segments

AEC-isomers (0.5 mM)	1-Butene [nmol/g (8 hr)]	Malonyl-AEC (nmol/g)	AEC (nmol/g)
(1*R*,2*S*)	50.2 ± 1.0	236 ± 16	656 ± 45
(1*R*,2*R*)	1.1 ± 0.6	42.0 ± 2.5	202 ± 16
(1*S*,2*R*)	8.8 ± 1.0	37.0 ± 1.0	805 ± 18
(1*S*,2*S*)	0.4 ± 0.6	34.5 ± 2.0	915 ± 16

Axial segments were incubated in the solns of AEC isomers for 8 hr. At the end of the incubation period, the gases evolved were analysed for 1-butene and the segments for the contents of free and conjugated AEC.

EtOH and recovered in an organic acid fraction. It chromatographed as one compound on cellulose TLC with solvents 1 and 2. The relationship between the R_f values of the free and conjugated forms of AEC were almost the same as those for the corresponding forms of ACC (Solvent 1: free and conjugated-AEC, 0.7 and 0.9; free and malonyl-ACC, 0.45 and 0.73. Solvent 2: 0.75 and 0.43; 0.5 and 0.25).

After further separation of the AEC conjugate by cellulose TLC with solvent 2 followed by purification by HPLC (× 2), the conjugate yielded one compound (R_t 10.07 min). Its amount was estimated as *ca* 0.2 μmoles by measuring AEC after hydrolyis. The purified AEC conjugate was hydrolysed and separated into amino acid and organic acid fractions by ion exchange chromatography. HPLC of the organic acid fraction gave only one compound, the R_t (7.83 min) of which was coincident with that of authentic malonic acid. The amino acid fraction gave 1-butene after degradation with NaOCl according to Lizada and Yang. The conjugated AEC, therefore, is in all probability malonyl-AEC.

Metabolic stability of pre-formed malonyl-AEC.

A pulse experiment with (1*R*,2*S*)-AEC was conducted to see whether malonyl-AEC could be further metabolized (Fig. 1). The AEC content of the AEC-fed segments began to decrease just after the pulse period of three hours and fell to almost zero at 24 hours. The change in 1-butene production was similar to that in the AEC content, but with a delay of three hours. In contrast, the malonyl-AEC content continued to increase for a further three hours after the pulse period, and slightly decreased thereafter. But, this decrease was superficial due to the increase in fresh weight during the incubation period. Thus, malonyl-AEC, once formed, was not metabolized and did not liberate free AEC in imbibed axial segments. The present findings provide further support for the view [13] that malonyl-ACC does not act as a source of free ACC for ethylene production in germinating cocklebur seeds.

Comparison among AEC-isomers in 1-butene and malonyl-AEC formation

Table 1 shows the amounts of 1-butene evolved from each of the four isomers over an eight hour incubation period with axial segments and the amounts of AEC and malonyl-AEC remaining after the incubation. The

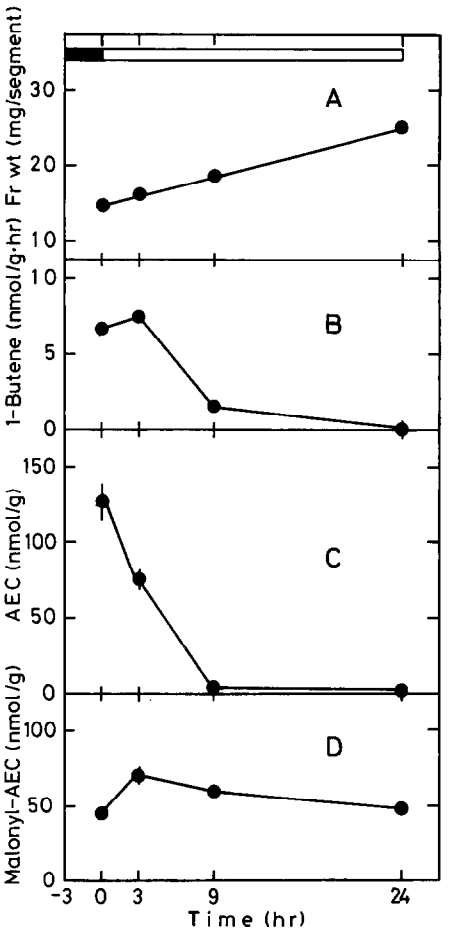

Fig. 1. Changes in fresh weight (A), 1-butene production (B), and the contents of AEC (C) and malonyl-AEC (D) in axial segments of cocklebur seeds. Segments were pulsed with 0.25 mM (1*R*, 2*S*)-AEC for 3 hr (solid bar), then incubated on wet filter paper (open bar). At given times of the incubation period the segments were again weighed and enclosed for 1 hr to determine 1-butene production, then assayed for AEC and malonyl-AEC contents. Bar indicates ± s.e. where larger than symbols.

(1*R*,2*S*)-isomer was the most preferentially converted to 1-butene, among the four isomers. Similarly the (1*S*,2*R*)-isomer was converted to 1-butene, although the efficiency was *ca* 25% of that with the (1*R*,2*S*)-isomer. These findings were different from the results with apple tissue and mung bean hypocotyls obtained by Hoffman *et al.* [8]. When fed at 0.25 mM, however, the amount of the (1*S*,2*R*)-isomer converted to 1-butene was only 6% of that of the (1*R*,2*S*)-one (data not shown). These suggest that the observed conversion capabilities are somewhat variable depending on experimental conditions. The conversions to 1-butene of the (1*S*,2*S*)- and (1*R*,2*R*)-isomers were negligible. Therefore, the present findings fundamentally agree with those of Hoffman *et al.* [8], who have described that a common enzyme metabolizes ACC and AEC-isomers to their respective alkenes by recognizing them as L-amino acids. This argument may be applicable to the results with cocklebur seed tissue.

The amount of malonyl-AEC in the segments fed with

the (1*R*,2*S*)-isomer was some five to seven times those with the other three isomers, indicating the preferred formation from the (1*R*,2*S*)-isomer. This difference in the malonylation of AEC-isomers, except for the (1*R*,2*R*)-one, seems to result from the different malonylation capability of each isomer, because their internal levels were still high as compared with those metabolized. With respect to the (1*R*,2*R*)-isomer, the low production of malonyl-AEC might be partly due to its low content, a quarter of the others. The low content of the (1*R*,2*R*)-isomer was thought to result from its low uptake, because more of it was found to remain in the incubation solution as compared to the others (data not shown).

It is presumed that the malonylation of AEC-isomers is mediated by an enzyme for the malonylation of ACC, as in the case of ACC-ethylene and AEC-butene conversion [8]. Amrhein *et al.* [15] and Liu *et al.* [16] have described that ACC and D-amino acids are malonylated by the same or separate enzymes having cross substrate-specificity. Therefore, AEC-isomers must be recognized as D-amino acids by the enzyme during malonylation. This recognition as D-amino acid possibly forms a part of the whole discrimination process of AEC-isomers during their malonylation. Evidence for the recognition of AEC-isomers as D-amino acids in the malonylation was shown next.

Inhibition of AEC malonylation and stimulation of 1-butene formation by D-phenylalanine.

We chose D-phenylalanine as a representative of those D-amino acids which are effective in stimulating ethylene procution [9, 10], and examined the effects of D- and L-phenylalanine on the formation of 1-butene and malonyl-AEC (Table 2). To avoid the interference of phenylalanine with AEC uptake, the segments were previously fed with the (1*R*,2*S*)-AEC for two hours, then treated for six hours with or without D- or L-phenylalanine at 10 mM. D-Phenylalanine completely prevented the formation of malonyl-AEC and increased the evolution of 1-butene by 50% over that of the buffer control. But L-phenylalanine had no effect. In D-phenylalanine-treated segments, the total amount of AECs including its metabolite (the sum of AEC, malonyl-AEC and 1-butene) remained almost constant during the six hour incubation period. But, the total amounts of AECs in the control and L-phenylalanine-treated segments at the end of the incubation were *ca* three-quarters of that at the start of the incubation. The reason for the loss in amount of AEC in the latter two segments was not further investigated. At all events, these findings suggest that the D-amino acid, when applied exogenously, causes a decrease of malonylation of endogenous ACC resulting in higher ACC content, and thereby a higher ethylene production in cocklebur seed tissue. This mechanism for the stimulation of ethylene production by D-amino acids has been shown for other plant tissues by Amrhein *et al.* [15] and Liu *et al.* [16].

Effects of AIB on the formation of 1-butene and malonyl-AEC

AIB has been shown to inhibit ethylene production and to cause the accumulation of ACC in cocklebur seed tissue, by acting as a competitive inhibitor of the ACC-ethylene conversion [10–12]. In view of these findings, we examined the effects of AIB on the formation of 1-butene and malonyl-AEC from exogenously applied (1*R*,2*S*)-AEC. Also in this case, AEC was fed to axial segments prior to AIB treatment.

AIB at 10 mM inhibited the emanation of 1-butene by 44% as compared to the buffer control, but it did not affect the final amount of malonyl-AEC (Table 3). Under the same conditions, AIB inhibited endogenous ethylene production of the axial segments by almost the same degree (data not shown). Also, AIB has been shown not to affect the content of malonyl-ACC in cocklebur seed tissue [12]. These findings suggest that AIB inhibits the enzyme responsible for the ACC-ethylene and AEC-butene conversion, but not the enzyme for malonylation.

It has become apparent that ethylene production in cocklebur seed tissues depends upon their content of free ACC, which is partly regulated by the rate of the malonylation of ACC in addition to those of its synthesis and conversion to ethylene. An interesting problem for the future is to determine whether malonyl-ACC can act as a precursor for ethylene, and what conditions are necessary for it to occur, although this possibility is denied at the moment [5, 13].

EXPERIMENTAL

Materials and incubations. AEC isomers were generous gifts of Professors S. Sakamura and A. Ichihara, Faculty of Agriculture, Hokkaido University, Sapporo, Japan. Axial segments, 3 mm in length, of cocklebur (*Xanthium pennsylvanicum* Wallr.) seeds were prepared as described previously [9]. Prior to use, they were soaked in H_2O for 3 hr, during which period their seed coat fragments were removed, then incubated on a sheet of wet filter paper at 23° for another 17–20 hr in a dark room.

Table 2. Effects of D- and L-phenylalanine on the formation of 1-butene and malonyl-AEC from exogenously applied AEC

Treatment	1-Butene [nmol/g (6 hr)]	Malonyl-AEC (nmol/g)	AEC (nmol/g)
Control-1 (at the start of the 6 hr incubation)	—	$7.43^a \pm 0.56$	$34.9^a \pm 2.6$
Control-2 (at the end of the 6 hr incubation)	$11.5^a \pm 0.4$	$13.16^b \pm 1.00$	$4.70^b \pm 0.51$
10 mM D-phenylalanine	$17.5^b \pm 0.1$	$7.20^a \pm 0.35$	$15.5^c \pm 0.6$
10 mM L-phenylalanine	$12.3^a \pm 0.1$	$12.20^b \pm 0.82$	$4.58^b \pm 0.62$

Axial segments were initially treated with 0.1 mM (1*R*,2*S*)-AEC for 2 hr, and then incubated with or without D- or L-phenylalanine at 10 mM for 6 hr. Values with different letters in each column are significantly different by Student's *t*-test ($P < 0.01$).

Table 3. Effects of AIB on the formation of 1-butene and malonyl-AEC from exogenously applied AEC in axial segments

AIB (10 mM)	1-Butene [nmol/g (5 hr)]	Malonyl-AEC (nmol/g)	AEC (nmol/g)
−(Control)	32.3 ± 1.5	68.1 ± 7.8	46.7 ± 10.4
+	18.2* ± 0.7	69.2† ± 4.9	50.1† ± 10.7

Axial segments were treated with 0.25 mM (1*R*,2*S*)-AEC for 3 hr, then incubated with or without 10 mM AIB for 5 hr.

* Significantly different from the control by Student's *t*-test ($P < 0.001$).

† Not significantly different from the control.

In the experiments described in Table 1, batches of 25 segments were weighed and incubated in 1 ml solns containing 0.5 mM of the appropriate AEC isomer in a 30-ml vial. In the experiments described in Tables 2 and 3, segments (20 segments/ml) were incubated in (1*R*,2*S*)-AEC soln at the given concentrations for 2 or 3 hr, respectively, then rinsed with H_2O, blotted dry, and subdivided into batches of 25 segments. After weighing, each batch was transferred to 1 ml of test soln in a 30-ml vial. For the pulse experiment (Fig. 1), segments were similarly treated in 0.25 mM (1*R*,2*S*)-AEC soln for 3 hr, and subdivided into batches of 24 segments, then placed on filter paper wet with buffer soln in 9-cm Petri dishes for 0, 3, 9 and 24 hr, respectively, at 23° in the dark. After weighing, they were transferred into 30 ml vials containing 1 ml buffer soln.

The vials were sealed with rubber stoppers and gently shaken at 23° in the dark. At the end of the incubation period, a 1-ml gas sample was taken from the sealed vials with a syringe and assayed by GC for 1-butene. Immediately after butene determination, the segments were submerged for 20 min in two changes of 80 ml ice-cold H_2O with occasional stirring to wash away exogenous AEC, and blotted dry on tissue paper. Then, they were weighed and stored at −20° until use.

All incubation solns were buffered at pH 6.8 with 10 mM Na-PIPES. The concentration of AEC in the incubation solns was determined by ninhydrin assay. Experiments were conducted with 3-replicated vials and the results are presented as means ± s.e. The fr. wt value of the segments used in the calculation were the average of the fr. wt measured before and after enclosure.

Extraction and determination of free and conjugated AEC. Segments were freeze-dried, defatted with Et_2O using a Soxhlet apparatus [10] and then homogenized in 5 ml ice-cold 70% aq. EtOH with a PHYSCOTRON NS-601 (NITI-ON, Chiba, Japan) at 20000 rpm (× 2) for 1 min with a cooling interval of 30 sec. After leaving it to stand for 1 hr at 0°, the homogenate was centrifuged at 10000 *g* for 15 min, then the supernatant collected. The extraction procedure was then repeated. The combined supernatants were taken to dryness under vacuum at 40°, taken up in 2 ml H_2O, then frozen. After thawing, the soln was again centrifuged to remove insoluble material. The resultant supernatant was used as the aq. EtOH extract for the determination of both free and conjugated AEC.

Free AEC was measured by degradation with NaOCl in the presence of Hg^{2+} according to Lizada and Yang [14]. In Table 2, however, an amino acid fraction was separated from the extract by ion exchange chromotography with a Dowex-50 W (H^+) column, and used for the determination.

Conjugated AEC was estimated from the amount of AEC determined after HCl-hydrolysis of an organic acid fraction (see below) of the extract, as described previously in the determination of conjugated ACC [12]. A fraction of the extract, which passed through a Dowex-50 W (H^+) column, was loaded onto a column of Dowex-1 ($HCOO^-$). The column was washed with H_2O, then eluted with 40% HCO_2H. The eluate was dried, taken up in H_2O and then subjected to HCl-hydrolysis. The free and conjugated AEC values were corrected for losses and for the presence of an inhibitor of AEC-butene conversion by adding a known amount of AEC as an internal standard to a replicate assay tube.

Large scale preparation and identification of conjugated AEC. Twenty gram segments were incubated in 70 ml 10 mM Na-PIPES, pH 6.8, containing *ca* 0.1 mM (1*R*,2*S*)-AEC at 23° for 25 hr in the dark with moderate shaking. An organic acid fraction was separated from an aq. EtOH extract of the segments by ion exchange chromatography. A part of the fraction was analysed for the AEC conjugate by cellulose TLC with solvent 1: 1-BuOH–HOAc–H_2O (12:3:5) and solvent 2: 1-PrOH–conc. NH_4OH (7:3), and the chromatographic behaviour of the AEC conjugate was compared with that of malonyl-ACC. The conjugates were located on the TLC plates as follows: 1-cm-width bands were scrapped off the plates and extracted with H_2O. The extracts were hydrolysed with HCl, the hydrolysates subjected to degradation with NaOCl and the gases evolved analysed by GC.

The AEC conjugate in the remainder of the fraction was purified by cellulose TLC with solvent 2, then by HPLC: An HPLC (Hitachi 635) with a refracto monitor (LDC SF-1107G) and a data processor (Hitachi 833) was equipped with a 300 × 8 mm i.d. column packed with Shodex Ionpak KC-811. The elution of the AEC conjugate was done at 40° with 0.1% phosphoric acid at a flow rate of 1 ml/min. Purification by HPLC was repeated twice.

The purified AEC conjugate was hydrolysed with 6 M HCl at 100° for 2 hr, and the hydrolysate taken up in H_2O after evaporation of the HCl. The hydrolysate was separated by ion exchange chromatography with a small column of Dowex-50 W (H^+) and, in turn, of Dowex-1 ($HCOO^-$) into amino acid and organic acid fractions. The organic acid fraction was analysed for the presence of malonic acid by HPLC (system described above), and the amino acid fraction was tested for AEC by degradation with NaOCl.

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