TECHNICAL

Fatty Acid Allene Oxides[1]

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The history of allene oxides in organic chemistry is short. It goes back to 1968, when Camp and Greene were able to isolate and characterize an allene oxide which they obtained by monoepoxidation of 1,3-di-*t*-butylallene with *m*-chloroperbenzoic acid (1). Since then a large number of allene oxides have been prepared by organic synthesis, mainly by monoepoxidation of allenes, but also by elimination reactions carried out with suitably substituted epoxide derivatives. Allene oxide is a very unusual molecule, containing the structural features of an epoxide, a double bond, and an enol ether. Because of the considerable ring strain, allene oxides are highly reactive. An interesting reaction is conversion into cyclopropanones, which may occur either directly or via the oxyallyl zwitterion. Another reaction which is typical for allene oxides is attack by nucleophiles, resulting in the formation of α-substituted ketones (2,3).

Formation of the allene oxide structure by an enzymatic reaction was discovered in 1987 (4). The present paper reviews our work on fatty acid allene oxides, including recent studies of the mechanism of biosynthesis of the plant cyclopentenone, 12-oxo-10,15(*Z*)-phytodienoic acid.

BIOSYNTHESIS OF FATTY ACID ALLENE OXIDES

In 1966 Zimmerman reported the presence in flaxseed of a "hydroperoxide isomerase" that catalyzed the conversion of 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (13(*S*)-HPOD) and 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid into α-ketol derivatives, i.e., 12-oxo-13-hydroxy-9(*Z*)-octadecenoic acid and 10-oxo-9-hydroxy-12(*Z*)-octadecenoic acid, respectively (5). Gardner described a similar type of transformation in corn germ, and also identified γ-ketol derivatives in the reaction product (6).

The mechanism of these transformations was clarified by a number of experimental approaches (4). In one set of experiments 13(*S*)-HPOD was stirred with a preparation of the corn enzyme at 0°C. Aliquots were removed 10, 30, 60 and 90 sec, and 5 min after addition of substrate and were then treated with 20 vol of methanol. Analysis by thin-layer chromatography revealed transient appearance of a trapping product, which was identified as 12-oxo-13-methoxy-9(*Z*)-octadecenoic acid. This finding indicated the existence of an unstable intermediate in the conversion of 13(*S*)-HPOD into α-ketol. Further experiments showed that the unstable compound: 1) retained one of the hydroperoxide oxygens and retained the hydrogens at C-9, C-10, and C-13, but lost the hydrogen at C-12 during its formation from 13(*S*)-HPOD; 2) had a half-life in aqueous medium of about 33 sec (0°C, pH 7.4); 3) reacted with water to produce 12-oxo-13-hydroxy-9(*Z*)-octadecenoic acid (α-ketol), and with methanol to produce 12-oxo-13-methoxy-9(*Z*)-octadecenoic acid; and 4) displayed an ultraviolet absorption band with λ_{max} = 236 nm. On the basis of those results an allene oxide structure was established for the unstable compound, i.e., 12,13(*S*)-epoxy-9(*Z*),11-octadecadienoic acid (12,13(*S*)-EOD) (4). As seen in Figure 1, two distinct steps are involved in the net isomerization of 13(*S*)-HPOD into α-ketol, i.e., an enzyme-catalyzed conversion of the hydroperoxide into fatty acid allene oxide, followed by spontaneous hydrolysis of the latter into α-ketol. Because the enzyme-catalyzed reaction is formally a dehydration, it was suggested that the enzyme earlier referred to as "hydroperoxide isomerase" be renamed "hydroperoxide dehydrase" (4). In a recent study, Brash and coworkers described isolation of the methyl ester of 12,13(*S*)-EOD (7). This work allowed NMR-spectrometric analysis of the allene oxide and thus confirmed our structural work.

The substrate specificity of hydroperoxide dehydrase from corn was recently examined. In addition to 13(*S*)-HPOD, the following fatty acid hydroperoxides served as substrates and were converted into allene oxides: 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid (derived from linoleic acid), 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid (derived from α-linolenic acid), 9(*S*)-

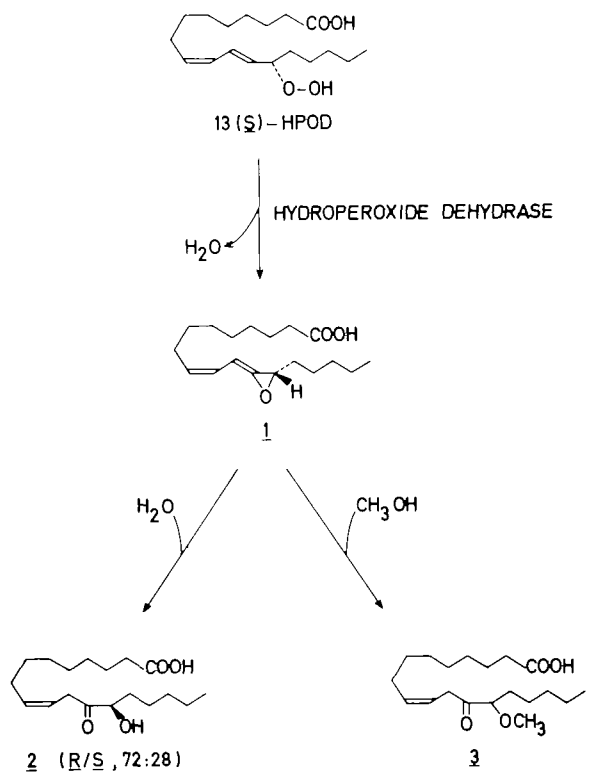

FIG. 1. Mechanism of formation of α-ketol derivatives from fatty acid hydroperoxides. 1, 12,13(*S*)-epoxy-9(Z),11-octadecadienoic acid (allene oxide); 2, 12-oxo-13-hydroxy-9(Z)-octadecenoic acid (α-ketol); 3, 12-oxo-13-methoxy-9(Z)-octadecenoic acid (trapping product formed from 1 in methanol).

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hydroperoxy-6(*Z*),10(*E*),12(*Z*)-octadecatrienoic acid (derived from γ-linolenic acid), 15(*S*)-hydroperoxy-11(*Z*),13(*E*),17(*Z*)-eicosatrienoic acid (derived from bis-homo-α-linolenic acid), 15(*S*)-hydroperoxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid (derived from arachidonic acid), and 15(*S*)-hydroperoxy-5(*Z*),8(*Z*),11(*Z*),13(*E*),17(*Z*)-eicosapentaenoic acid (derived from eicosapentaenoic acid).

NONENZYMATIC CONVERSIONS OF FATTY ACID ALLENE OXIDES

Nonenzymatic conversions of the allene oxide 12,13(*S*)-EOD are summarized in Figure 2. As mentioned above, 12,13(*S*)-EOD (1) underwent rapid hydrolysis in aqueous medium into the α-ketol derivative 12-oxo-13-hydroxy-9(*Z*)-octadecenoic acid (2). Steric analysis showed that the α-ketol had largely the *R* configuration at C-13 (13(*R*)/13(*S*), 72:28) (4). Thus, formation of α-ketols from allene oxides apparently occurred by an S_N2 type of displacement at the saturated epoxide carbon (resulting in inversion of the configuration), as well as by an S_N1 type reaction involving the oxypentadienyl zwitterion (resulting in racemization). In addition to the α-ketol derivative, small amounts of the isomeric γ-ketol (3; formed via the oxypentadienyl zwitterion) were also formed by hydrolysis of allene oxide.

Treatment of 12,13(*S*)-EOD with sodium borohydride in methanol yielded 12-hydroxy-9-octadecenoic acid (5 in Fig. 2). When reduction was accomplished with sodium borodeuteride, two atoms of deuterium were incorporated in the hydroxy acid (at C-12 and C-13), whereas treatment of 12,13(*S*)-EOD generated from 13(*S*)-[9,10,12,13-$^2H_4$]-HPOD with sodium borohydride led to the formation of 12-hydroxy-9-octadecenoic acid containing three atoms of deuterium (at C-9, C-10, and C-13). Thus, reduction of the allene oxide by sodium borohydride occurred by addition of hydride at C-13, opening and ketonization of the allene oxide structure, and reduction of the 12-oxo group by addition of hydride at C-12.

As described above, fatty acid allene oxides were unstable in hydroxylic solvents such as water and methanol. As would be expected, stability was greatly enhanced in non-hydroxylic, water-miscible solvents such as acetonitrile and tetrahydrofuran. Thus, the half-life of 12,13(*S*)-EOD in acetonitrile/potassium phosphate buffer pH 7.4 (20:1, v/v) at 0°C was 7.4 min, as compared to 33 sec in buffer (8). Interestingly, degradation of allene oxide in aqueous acetonitrile did not result in the formation of α-ketol but in two unique macrolactones, 12-oxo-9(*Z*)-octadecen-11-olide and 12-oxo-9(*Z*)-octadecen-13-olide (8; **6** and **7** in Fig. 2). The latter lactone was subjected to steric analysis and found to be essentially racemic (ratio between *R* and *S* enantiomers, 53:47). Therefore, it seemed likely that macrolactone formation from 12,13(*S*)-EOD occurred by way of the oxypentadienyl zwitterion, in which there is no asymmetry. Formation of macrolactones required that the carboxyl group of the allene oxide existed in the form of the nucleophilic carboxylate anion. Thus, when allene oxide was allowed to decompose in acidified acetonitrile solution, formation of macrolactones was inhibited. Instead, slow formation of α-ketol (2) and γ-ketol (3), as well as a number of unidentified products, was observed.

The fatty acid allene oxides were stabilized in the presence of vertebrate serum albumins (9). Thus, the aqueous half-lives at 0°C of 12,13(*S*)-EOD in the presence of 15 mg/ml of bovine, human, and equine serum albumins were 14.1 ± 1.8, 11.6 ± 1.2, and 4.8 ± 0.5 min, respectively, as compared to 33 sec in the absence of albumin. This stabilization was probably due to binding of allene oxide to hydrophobic sites of the albumin molecule, resulting in protection from hydrolysis in the aqueous phase. In the course of these studies we found that degradation of 12,13(*S*)-EOD in the presence of bovine serum albumin led to the formation of a novel cyclopentenone derivative, i.e., 3-oxo-2-pentyl-cyclopent-4-en-1-octanoic acid (9; **8a** and **8b** in Fig. 2). Steric analysis demonstrated that the relative configuration of the side

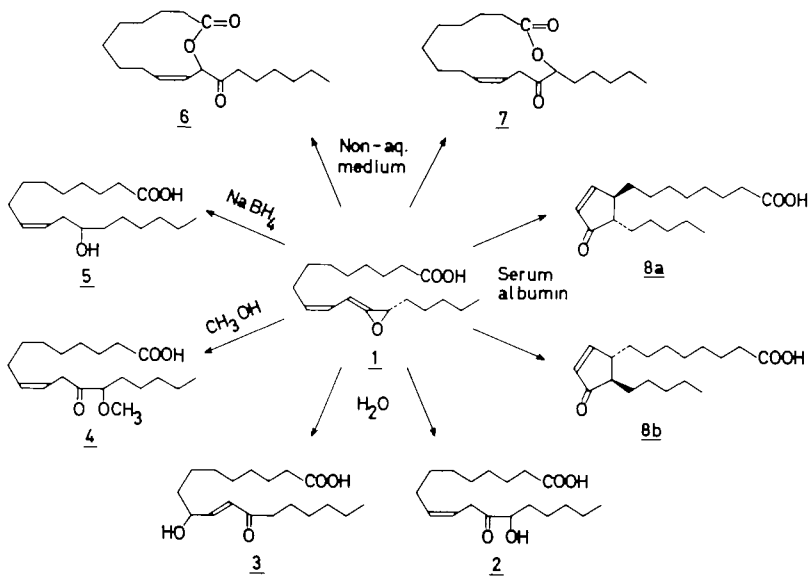


FIG. 2. Nonenzymatic reactions of the fatty acid allene oxide 12,13(*S*)-epoxy-9(*Z*),11-octadecadienoic acid (1).

chains attached to the cyclopentenone ring was *trans*, and that the compound was largely racemic (ratio between **8a** [9(*R*),13(*S*)] and **8b** [9(*S*),13(*R*)], 58:42).

MECHANISM OF THE BIOSYNTHESIS OF 12-OXOPHYTODIENOIC ACID

Conversion of α-linolenic acid into a cyclopentenone derivative, 12-oxo-10,15(*Z*)-phytodienoic acid (12-oxo-PDA), was reported by Zimmerman and Feng in 1978 (10). The initial step of the conversion consisted of lipoxygenase-catalyzed oxygenation of α-linolenic acid into 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid (13(*S*)-HPOT). A "hydroperoxide cyclase" enzyme was postulated for the conversion of the hydroperoxide into cyclopentenone. Vick and Zimmerman (11) later reported that 12-oxo-PDA serves as the precursor of 7-iso-jasmonic acid, a C-12 cyclopentanone derivative which has been isolated from a large number of plants and has growth-regulating effects (12).

The fact that a cyclopentenone derivative could be formed from the allene oxide 12,13(*S*)-EOD in a nonenzymatic reaction (bovine serum albumin) (9), coupled with the fact that incubation of 13(*S*)-HPOT with corn hydroperoxide dehydrase afforded 12-oxo-PDA (9,13), suggested that 12,13(*S*)-epoxy-9(*Z*),11,15(*Z*)-octadecatrienoic acid (12,13(*S*)-EOT; the allene oxide generated from 13(*S*)-HPOT) served as the immediate precursor of 12-oxo-PDA. Recent experiments, e.g., showing transient appearance of 12,13(*S*)-EOT parallelling the synthesis of 12-oxo-PDA, have verified the correctness of this hypothesis (14). In that work, allene oxide 12,13(*S*)-EOT was generated by incubation of 13(*S*)-HPOT with the 105,000 × *g* particle fraction of homogenate of corn (the subcellular fraction containing the major part of the hydroperoxide dehydrase activity in corn). Interestingly, incubation with the particle fraction resuspended in buffer resulted in formation of only modest amounts of 12-oxo-PDA, (ratio 12-oxo-PDA/α-ketol, 0.14:1, irrespective of the concentration of 13(*S*)-HPOT used for generation of allene oxide), whereas the corresponding incubation carried out in the presence of 105,000 × *g* supernatant led to pronounced stimulation of 12-oxo-PDA formation. This enhancement of 12-oxo-PDA formation was highest at low substrate concentrations and declined when higher concentrations of substrate were used (e.g., ratios 12-oxo-PDA/α-ketol at 3 and 150 μM 13(*S*)-HPOT, 2.88:1 and 0.46:1, respectively). These results suggested that, in the absence of 105,000 × *g* supernatant, formation of 12-oxo-PDA occurred by nonenzymatic, chemical cyclization of the allene oxide, whereas 12-oxo-PDA formation observed in the presence of 105,000 × *g* supernatant occurred mainly by action of a saturable activity that selectively catalyzed formation of 12-oxo-PDA at the expense of α-ketol. Further evidence for formation of 12-oxo-PDA by both enzymatic and nonenzymatic routes was provided by steric analysis using a recently developed method (13). It was found that 12-oxo-PDA formed in the absence of 105,000 × *g* supernatant was racemic, irrespective of the substrate concentration used (14,15). On the other hand, 12-oxo-PDA produced in the presence of supernatant was enriched with respect to the 9(*S*),13(*S*) enantiomer. This enrichment was dependent upon the substrate concentration. For example, 12-oxo-PDA was 96% and 76% optically pure when formed from allene oxide generated from 3 and 150 μM 13(*S*)-HPOT, respectively.

The soluble enzyme activity catalyzing the formation of 9(*S*),13(*S*)-12-oxo-PDA from allene oxide was named "allene oxide cyclase" (14, Fig. 3). As expected, the enzyme activity was heat labile, destroyed by proteolytic digestion, and saturable with respect to substrate. The activity was precipitated by ammonium sulfate at 30–55% saturation and migrated as a protein with a molecular weight of about 45,000 upon gel filtration (14). Further support for allene oxide cyclase being a specific enzyme came from experiments with (±)-*cis*-12,13-epoxy-9(*Z*)-octadecenoic acid and (±)-*cis*-12,13-epoxy-9(*Z*),15(*Z*)-octadecadienoic acid. These epoxy acids, in which the epoxide group is located in the same position as in the allene oxide substrate, inhibited formation of 12-oxo-

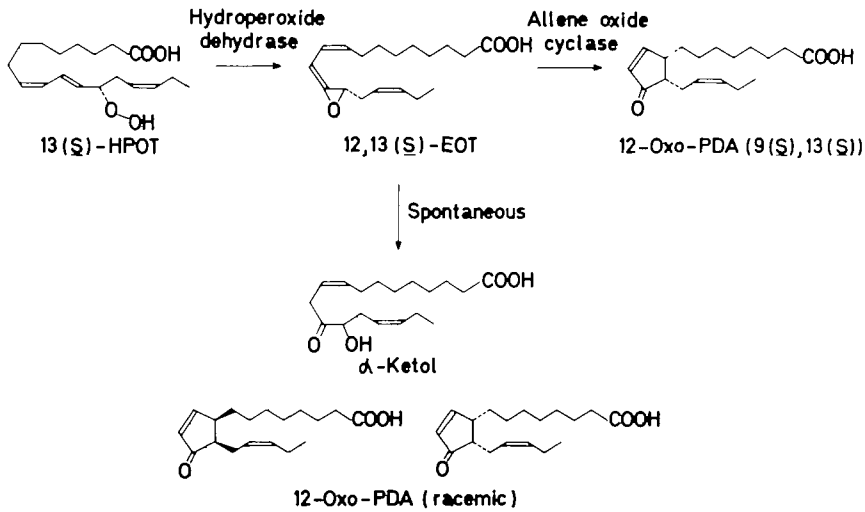


FIG. 3. Formation of 9(*S*),13(*S*)-12-oxo-PDA from 13(*S*-HPOT by sequential actions of hydroperoxide dehydrase and allene oxide cyclase. Also shown is the competing nonenzymatic conversion of allene oxide 12,13(*S*)-EOT into α-ketol and racemic 12-oxo-PDA.

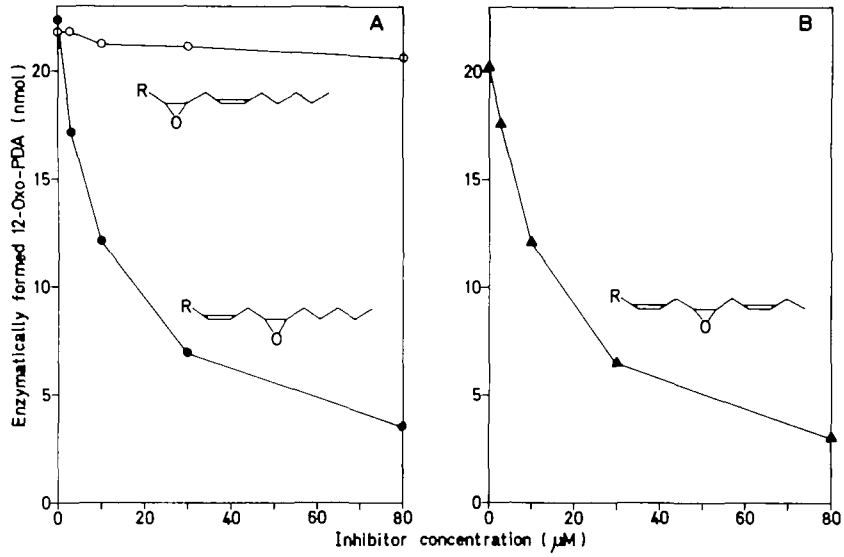


FIG. 4. Inhibition of allene oxide cyclase by epoxy acids. Suspensions of 105,000 × *g* particle fraction (source of hydroperoxide dehydrase) in 105,000 × *g* supernatant (source of allene oxide cyclase) of corn homogenate were stirred at 0°C with epoxy acids (0–80 μM) for 1 min. 13(*S*)-HPOT (11 μM) was added and the mixture stirred for an additional 10 min at 0°. The amounts of enzymatically formed 12-oxo-PDA were calculated following thin-layer radiochromatography as described by Hamberg (14). Inhibitors: -●-●-●-, (±)-*cis*-12,13-epoxy-9(*Z*)-octadecenoic acid; -○-○-○-, (±)-*cis*-9,10-epoxy-12(*Z*)-octadecenoic acid; -▲-▲—▲-, (±)-*cis*-12,13-epoxy-9(*Z*),15(*Z*)-octadecadienoic acid. R = $(CH_2)_7COOH$.

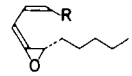
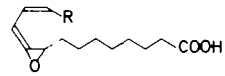
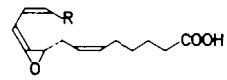
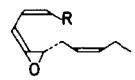
Fatty acid	Hydroperoxide	Allene oxide	Cyclization	
			Spontaneous	Enzymatic
Linoleic acid Arachidonic acid	13(S)-HPOD 15(S)-HPETE		–	–
Linoleic acid	9(S)-HPOD		–	–
γ-Linolenic acid	9(S)-HPOT ω6		+	–
α-Linolenic acid Bis-homo-α-linolenic acid Eicosapentaenoic acid	13(S)-HPOT ω3 15(S)-HPET ω3 15(S)-HPEP		+	+

FIG. 5. Spontaneous and enzymatic cyclization of allene oxides prepared from various fatty acid hydroperoxides. Allene oxides were generated by stirring the fatty acid hydroperoxides (10–150 μM) at 0°C with the 105,000 × *g* particle fraction of corn homogenate (source of hydroperoxide dehydrase), resuspended in either buffer or in 105,000 × *g* supernatant (source of allene oxide cyclase). Product composition and steric analysis of cyclopentenones were carried out as described by Hamberg (13,14).

PDA. On the other hand, (±)-*cis*-9,10-epoxy-12(Z)-octadecenoic acid, in which the epoxide group is not located in the correct position, gave only insignificant inhibition (Fig. 4). Additionally, allene oxide cyclase displayed substrate specificity. A number of allene oxides were generated by incubation of fatty acid hydroperoxides with dehydrase. As seen in Figure 5, spontaneous cyclization into cyclopentenones required the presence of a double bond β,γ to the epoxide (16). Enzyme-catalyzed cyclization was only observed with allene oxides having the epoxide group in the ω6,7 position and the β,γ double bond in the ω3 position. Enzymatic formation of cyclopentenones of the 12-oxo-PDA type thus seem to be restricted to allene oxides derived from the ω3 series of polyunsaturated fatty acids.

The configuration of enzymatically formed 12-oxo-PDA

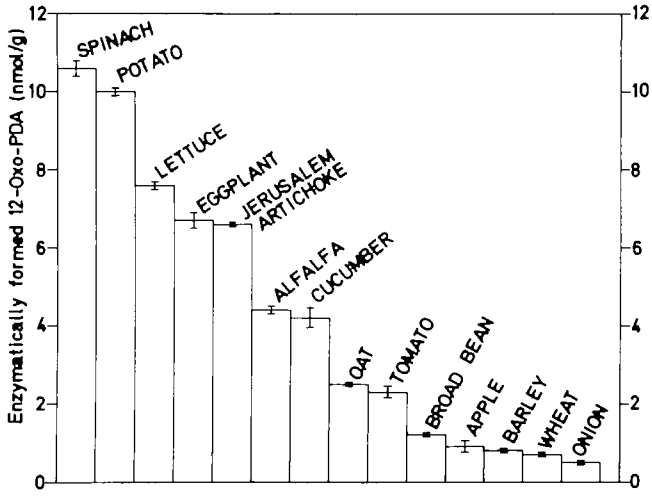


FIG. 6. Distribution of allene oxide cyclase activity in plant tissues. 13(*S*)-HPOT (27 μM) was incubated at 0°C for 15 sec with a suspension in buffer of the 105,000 × *g* particle fraction of corn homogenate. Three ml of the 9300 × *g* supernatant obtained from the different plant homogenates were added, and the mixture stirred at 0°C for 10 min. The amounts of enzymatically formed 12-oxo-PDA were calculated following thin layer radiochromatography (14).

(9(*S*),13(*S*)) corresponds to the configuration of its metabolite in plant tissue, 7-iso-jasmonic acid. Therefore, it seems likely that 7-iso-jasmonic acid is formed from α-linolenic acid by sequential actions of ω6-lipoxygenase, hydroperoxide dehydrase, and allene oxide cyclase, followed by Δ^{10} reductase and three steps of β-oxidation.

A number of plants have recently been screened for the presence of allene oxide cyclase. As shown in Figure 6, the highest activity (expressed in nmol of enzymatically formed 12-oxo-PDA per g of tissue) was found in spinach leaves and potato tubers. High activity was also noted in lettuce, eggplant fruit, jerusalem artichoke tubers, alfalfa seedlings and in cucumber. Low or insignificant activity was found in seedlings of barley and wheat and in yellow onion. We are presently investigating the possible existence of the hydroperoxide dehydrase-allene oxide cyclase pathway in mammalian tissue. The occurrence of such a pathway in mammals would be of great interest, because it would provide a means for biosynthesis of prostaglandin-like compounds by an aspirin-insensitive, non-cyclooxygenase route.

ACKNOWLEDGMENTS

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