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BIOLOGICAL ACTIVITY AND BIOSYNTHESIS OF PENTACYCLIC OXYLIPINS: THE LINOLEIC ACID PATHWAY*

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Key Word Index—12-oxo-phytodienoic acid (12-oxo-PDA); 15,16-dihydro-12-oxo-PDA; jasmonic acid; 9,10-dihydrojasmonic acid; 12-oxo-PDA reductase; biological activity; secondary metabolite induction; plant defense; plant cell cultures.

Abstract—The relevance of the postulated pathway from linoleic acid to dihydrojasmonic acid is analysed. Pentacyclic oxylipins having pentenyl or pentyl side chains were tested for their secondary metabolite inducing activity in seven different plant cell culture species which all responded well to 12-oxo-phytodienoic acid and jasmonic acid. The response towards the dihydro-derivatives 15,16-dihydro-12-oxo-phytodienoic acid and 9,10-dihydrojasmonic acid ranged from strong activity in *Eschscholzia californica* to no activity in *Lycopersicon esculentum*. 15,16-Dihydro-12-oxo-phytodienoic acid can be formed from linoleic acid (18:2) by a linseed acetone powder enzyme preparation. Application experiments with linoleic (18:2) and linolenic acid (18:3) showed that the bottleneck of the 18:2 pathway is most likely the cyclization of the intermediate allene oxide when compared to the ease by which 15,16-dihydro-12-oxo-phytodienoic acid is converted to dihydrojasmonic acid in plant systems. The metabolism of potential precursors of jasmonic and dihydrojasmonic acid was extensively studied in various cell cultures.

INTRODUCTION

Jasmonic acid (JA) was originally discovered as a plant hormone-like substance influencing developmental processes such as growth inhibition, induction of senescence, tuber formation and tendril coiling [1, 2]. Recently, the essential role of JA as a signal molecule in plant defense was recognized by brilliant experiments published in 1990 by Farmer and Ryan [3]. These authors showed for the first time that volatile methyljasmonate (MeJA) induces proteinase inhibitors, which function as high molecular defense compounds. Subsequently we demonstrated that also low molecular defense compounds, phytoalexins, are induced by jasmonates [4]. Since then, research on this topic produced a large body of further data confirming that pentacyclic oxylipins are essentially involved in the signal transduction process leading to defense gene activation producing in turn both high and low M_r compounds that are against plant predators and pests [5].

The biosynthetic route of JA has been elucidated by Vick and Zimmerman [6, 7] in an amazing depth without knowing at that time any further function of this compound mimicking the mammalian prostaglandins [8]. Later on, this pathway was modified with regard to the individual enzymes involved in the cyclization process [9, 10]. The biosynthesis starts from α-linolenic acid (18:3) that is oxidized to the 13(*S*)-hydroperoxide (HPOT). Dehydration by allene oxide synthase then forms an unstable epoxide (EOT) that is cyclized by a stereospecific allene oxide cyclase leading to 9(*S*),13(*S*)-12-oxo-phytodienoic acid (12-oxo-PDA), the first pentacyclic derivative in this pathway. The next step is a reduction of the pentacyclic ring, followed by a shortening of the side-chain by three cycles of β-oxidation yielding (+)-7-epi-JA.

In addition to JA, 9,10-dihydrojasmonic acid (DH-JA) was also found to occur in fungi and plants [11, 12]. Dihydro-oxylipins could arise by two different biosynthetic routes, either starting from linolenic acid (18:3) followed by a reduction of the double bond in the pentenyl side-chain at the cyclic oxylipin level, or directly from the linoleic acid (18:2). Such an alternative pathway beginning with linoleic acid was suggested by Blechert *et al.* [13] upon the finding that this 18:2 acid is converted to 15,16-dihydro-12-oxo-PDA (DH-12-oxo-PDA) by an enzyme extract from flax seedlings. This concept was supported by the ability of linoleic acid (18:2) to induce proteinase inhibitor [14], by the activity of DH-JA in phytoalexin induc-

* Dedicated to Prof. C. A. Ryan on the occasion of his 65th birthday.

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tion [13], and by the lack of reduction of the side-chain double bond when JA was fed to plant cell cultures and by the absence of dihydrojasmonate derivatives among the reisolated metabolites [15].

Compared to JA, DH-JA is biologically active only in a limited number of bioassays used, showing no activity with regard to tuber formation [16], induction of proteinase inhibitor in potato [17], tendril coiling in *Bryonia dioica* [18] and glucosinolate induction in *Brassica napus* [19]. Nevertheless, DH-JA is highly active in growth inhibition, induction of senescence [1], alkaloid accumulation in cell cultures of *Eschscholzia californica* [13] and moderately active in nicotine induction in *Nicotiana sylvestris* [20].

Literature reports on the cyclization of the 18:2 derived 12,13-(*S*)-epoxide are controversial. Some authors detected small amounts of DH-12-oxo-PDA [9, 13, 21, 22], others claim that no or only trace amounts of pentacyclic products are formed from 18:2 [23, 24, 25].

The aim of this work was to analyse the significance of the postulated dihydro-pathway. We checked the secondary metabolite inducing activity of the oxylipins and their dihydro-compounds in seven plant cell culture species and examined the *in vivo* formation and metabolism of the dihydro-oxylipins.

RESULTS

Enzymic synthesis of 12-oxo-PDA and DH-12-oxo-PDA

For 12-oxo-PDA synthesis, linolenic acid (18:3) was directly incubated with an enzyme extract from linseed acetone powder as described in the Experimental section. Since the linseed acetone powder contains 13-lipoxygenase and allene oxide synthase, but is free of cyclase activity [26], this method yields a racemic mixture of 12-oxo-PDA formed by spontaneous chemical cyclization of the epoxide. The main reaction products observed here were α-ketol, γ-ketol and 12-oxo-PDA [Fig. 1(A)]. If linoleic acid (18:2) was used as substrate for this enzyme preparation under the same conditions, DH-12-oxo-PDA is not formed in an amount detectable by HPLC, but addition of bovine serum albumin (BSA) to the incubation mixture enhanced the formation of DH-12-oxo-PDA [Fig. 1(B)]. BSA is known to promote the spontaneous, non-enzymic cyclization by stabilizing the epoxide [26]. This BSA effect is most likely shown also by specific enzymes present in higher plants under *in vivo* conditions. The mass spectrum of the enzymically formed DH-12-oxo-PDA is shown in Fig. 2.

Compared to linolenic acid (18:3), the above described conversion of the 18:2 acid yielded several additional products. One of these products could at first sight be mistaken for DH-12-oxo-PDA, because of its same mass m/z $[M]^+$ 294 and similar fragmentation pattern. But the UV-spectrum with a maximum at 278 nm is completely different from the 222 nm maximum of DH-12-oxo-PDA [Fig. 1(B)]. By comparison with published mass spectral data [27] the substance was identified as 13-keto-octadecadienoic acid (13-KOD). To unambiguously prove the identity of DH-12-oxo-PDA, the suspected metabolite was reduced with Pd/H_2 yielding 10,11-15,16-tetrahydro-12-oxo-phytodienoic acid (TH-12-oxo-PDA), which has a simple and characteristic fragmentation pattern EI mass spectrum 70 eV, m/z (rel. int.): 226 (25), 153 (25), 83 (100). The formation of DH-12-oxo-PDA from 13-hydroperoxy-octadecadienoic acid (HPOD) was independently confirmed by H. W. Gardener (personal communication) using a maize seed germ preparation and mass spectral analysis of the products formed.

From 12-oxo-PDA, 3-oxo-2-(2′-pentenyl)cyclopentaneoctanoic acid (OPC 8:0) and TH-12-oxo-PDA were synthesized chemically, thus all four C_{18} pentacyclic oxylipins, 12-oxo-PDA, OPC 8:0, DH-12-oxo-PDA and TH-12-oxo-PDA were available for biological testing.

Biological activity of pentacyclic oxylipins and their fatty acid precursors

To determine the ability of the different oxylipins to induce secondary metabolites, a wide concentration range of these potential signal substances was tested. Examples of dose effect curves are given in Fig. 3. The secondary metabolites formed within the cultures were either quantitated by extinction measurement of the extract in case of coloured products or by HPLC analysis (e.g. Fig. 4).

Seven plant species in cell culture were tested with the above described set of pentacyclic oxylipins and their fatty acid precursors. The results are summarized in Fig. 5. As compared to *E. californica*, which produces benzo[c]phenanthridine alkaloids upon elicitation [28], two other species belonging to the Fabaceae, *Erythrina crista-galli* and *Crotalaria cobalticola* [Fig. 4(A)], which produce either isoflavonoids or isobavachalcone demonstrated a weaker response to the addition of dihydro-oxylipins. In the case of *Rauvolfia serpentina*, which produces mainly indole alkaloids, and *Nicotiana tabacum*, the activity of the dihydro-compounds is further reduced, but still significant. In *Tinospora cordifolia* and *Lycopersicon esculentum* [Fig. 4(B)], the dihydro-compounds are completely inactive. The biological activity of the dihydro-oxylipins studied varies, therefore, strongly between the individual plant species, but is more or less the same within the two biosynthetic groups 12-oxo-PDA/OPC 8:0/JA and DH-12-oxo-PDA/TH-12-oxo-PDA/DH-JA (Fig. 5). While the 12-oxo-PDA family of compounds in all seven species tested caused a strong response of low M_r secondary metabolite induction, the DH-12-oxo-PDA family derived from linoleic acid (9:1) seems to be very species selective in its ability of defense gene activation.

We now turn to the elicitation capacity of the free

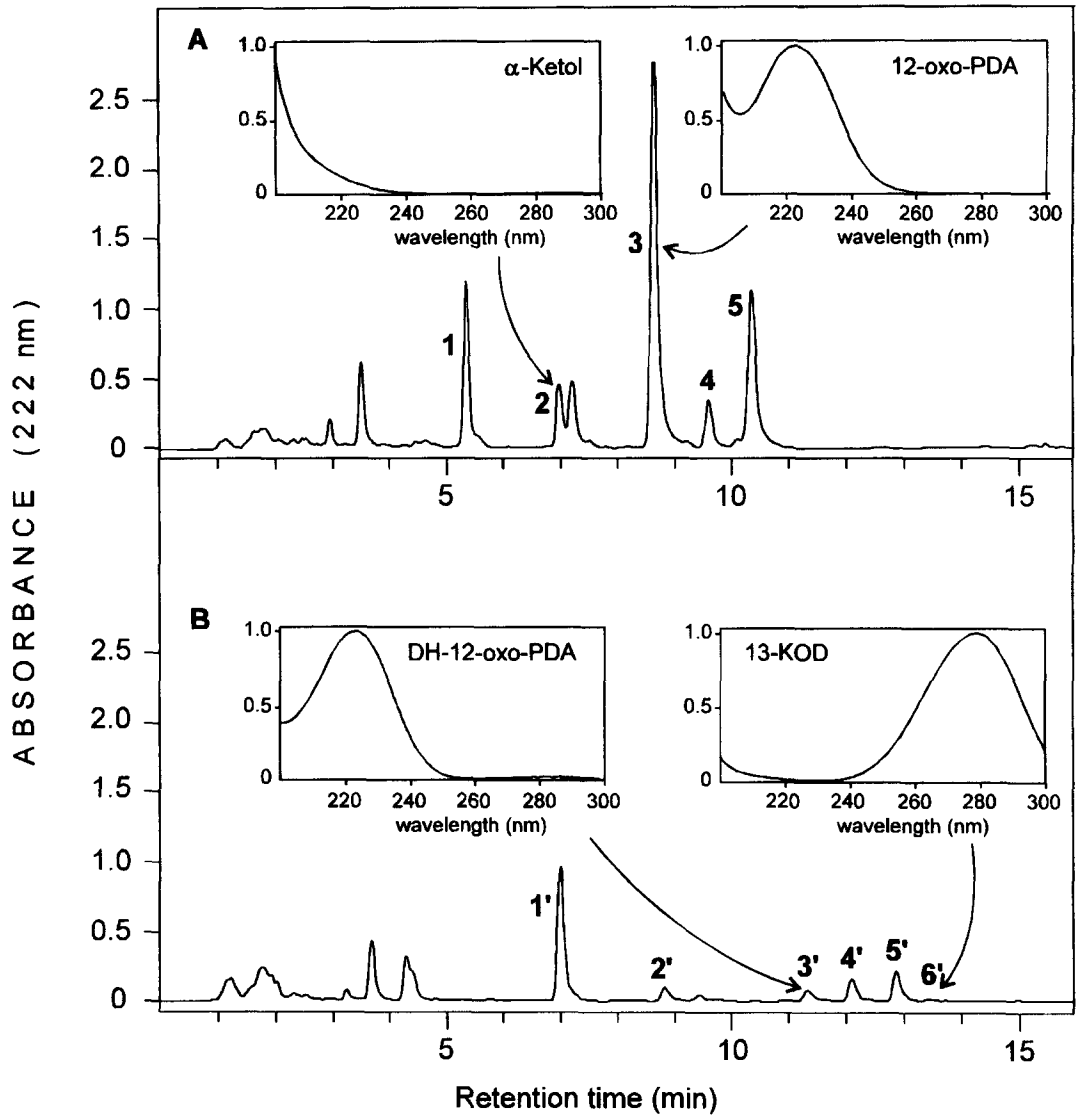

Fig. 1. HPLC separation of products formed from (A) linolenic acid (18:3) and (B) linoleic acid (18:2) after incubation with a linseed enzyme preparation. 18:3 derived products: (1) γ-ketol, (2) α-ketol, (3) 12-oxo-PDA, (4) 13-hydroxy-octadecatrienoic acid (HOT), (5) 13-hydroperoxy-octadecatrienoic acid. 18:2 derived products: (1′) γ-ketol, (2′) α-ketol, (3′) 15,16-dihydro-12-oxo-PDA (DH-12-oxo-PDA), (4′) 13-hydroxy-octadecadienoic acid (HOD), (5′) 13-hydroperoxy-octadecadienoic acid (HPOD), (6′) 13-keto-octadecadienoic acid (KOD). The inserts show the respective UV-spectra, the structures are depicted in Fig. 6.

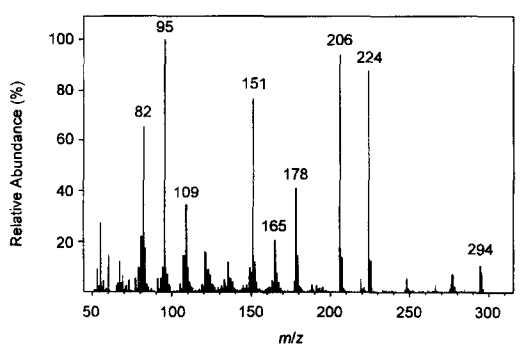

Fig. 2. Mass spectrum (EI mode) of 15,16-dihydro-12-oxo-PDA (DH-12-oxo-PDA) formed from linoleic acid (9:1) by a linseed enzyme preparation.

and exogenously applied C_{18}-fatty acids. Both linolenic (18:3) and linoleic (18:2) acid have previously been shown to be able to provoke the formation of proteinase inhibitor [14] and tendril coiling [18].

T. cordifolia was the only one of the species analysed in which the induction properties of 18:3 nearly equalled that of cyclic oxylipins [Fig. 3(B)]. In the other six cultures, 18:3 showed only a minimal activity with a 2- to 5-fold induction over the controls (Fig. 5). This demonstrates that exogenously applied free linolenic acid (18:3) is normally not sufficient to trigger the signal cascade leading to secondary defense metabolite induction.

Linoleic acid (18:2) generally displayed no activity except in *L. esculentum* in which the induction factor

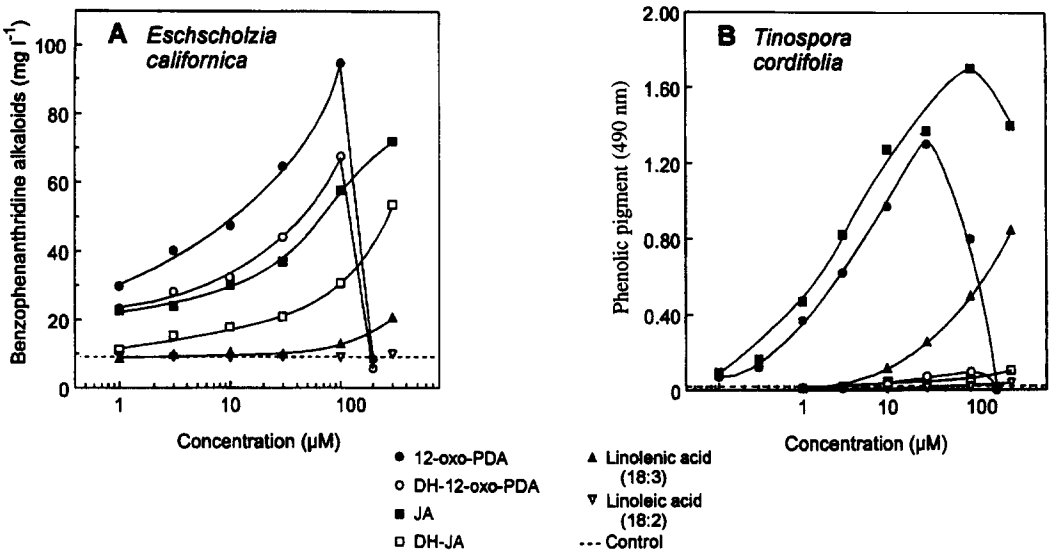

Fig. 3. Induction of low molecular weight defense compounds in cell suspension cultures after the addition of potential signal compounds for 4 days. Dose effect curves showing the differential reaction towards pentacyclic oxylipins and dihydro-oxylipins. Both, 12-oxo-PDA and DH-12-oxo-PDA induce cell death at 200 μM. (A) Total benzo[c]phenanthridine alkaloids produced by *Eschscholzia californica* cultures. (B) A phenol produced by *Tinospora cordifolia* cultures.

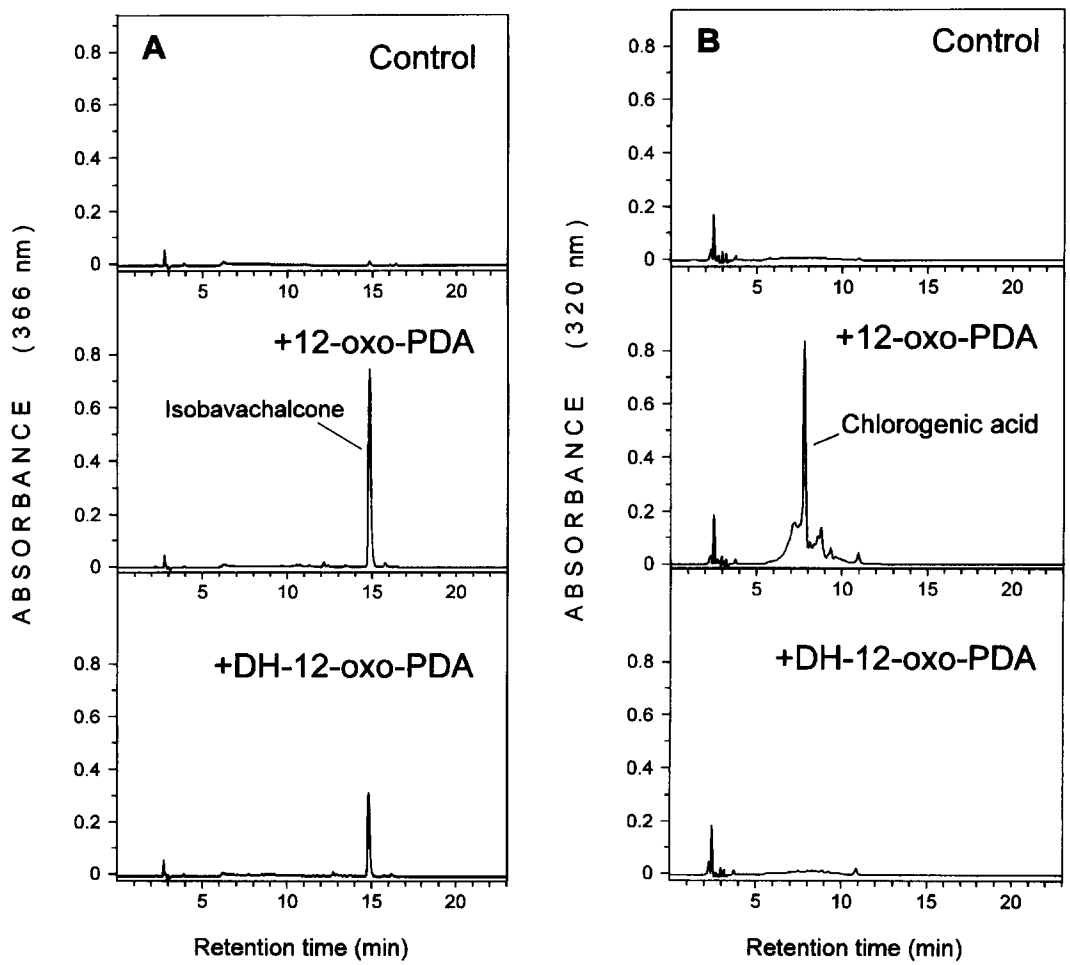

Fig. 4. HPLC analysis of secondary metabolites induced by the signal compounds 12-oxo-PDA and DH-12-oxo-PDA in cell suspension cultures of (A) *Crotalaria cobalticola* producing isobavachalcone [4] and (B) *Lycopersicon esculentum* producing as main metabolite chlorogenic acid.

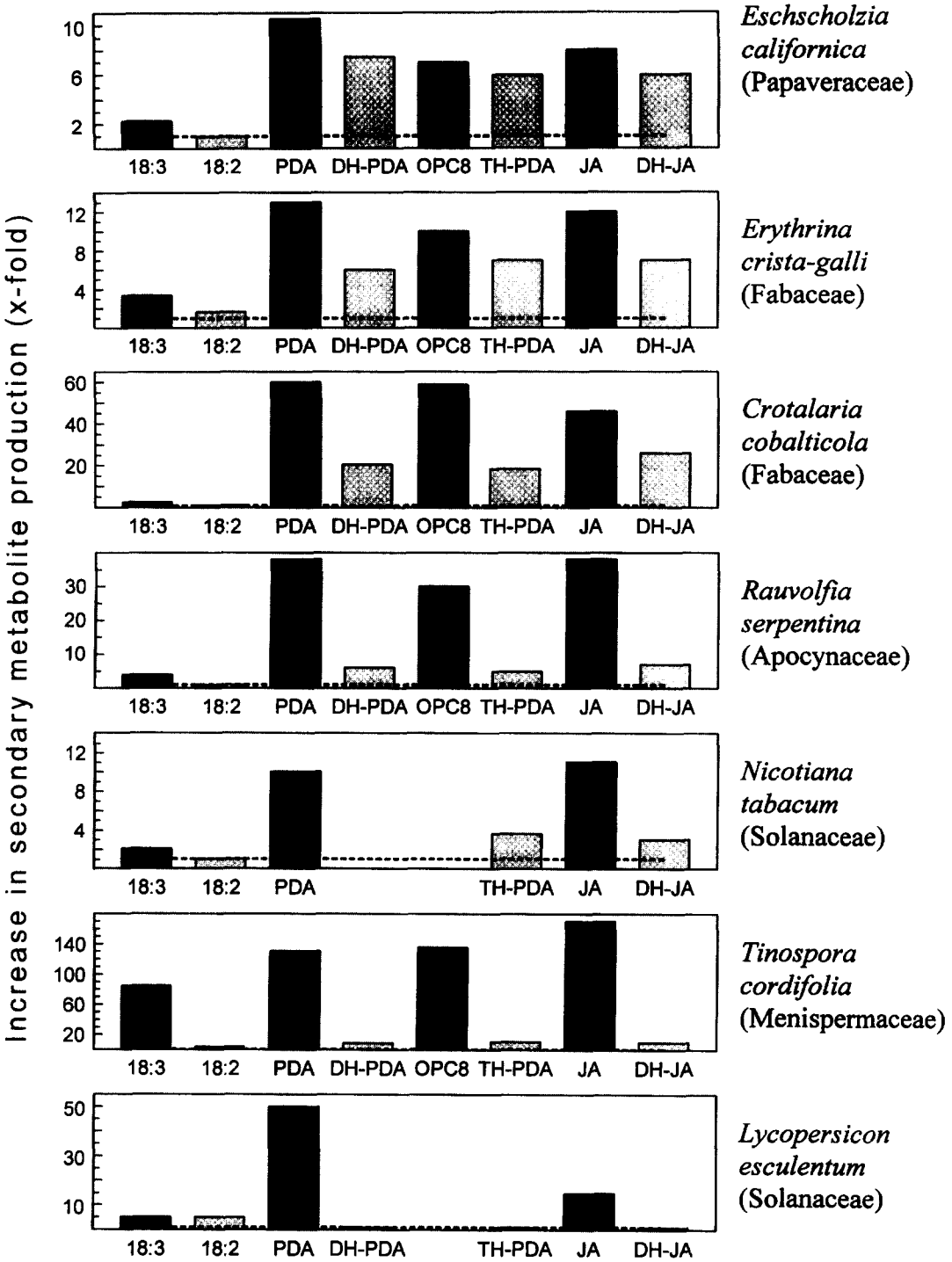

Fig. 5. Secondary product inducing-potential of various naturally occurring signal compounds at optimal concentrations in different plant suspension cultures. The values are depicted as *x*-fold increase in secondary metabolite content compared to untreated control cultures. 18:3, linolenic acid; 18:2, linoleic acid; PDA, 12-oxo-phytodienoic acid; DH-PDA, 15,16-dihydro-12-oxo-phytodienoic acid; OPC8, 3-oxo-2-(2′-pentenyl)cyclopentaneoctanoic acid; TH-PDA, 10,11–15,16-tetrahydro-12-oxo-phytodienoic acid; JA, jasmonic acid; DH-JA, 9,10-dihydrojasmonic acid; – – –, control level.

of 5 was the same as for linolenic acid (18:3). The biological activity of the exogenously applied 18:2 fatty acid could possible be explained by a conversion to 18:3. Application experiments with [1-^{14}C]-labelled 18:2 showed that such a desaturation really occurs. After an incubation period of 24 hr, carrier-free [^{14}C]-labelled 18:2 was converted to 18:3 to about 4% of the recovered radioactivity in the fatty acid phase of the *L. esculentum* cell culture and to 6% in tomato leaves.

From the results presented, it is evident that DH-12-oxo-PDA can be formed from linoleic acid (18:2) *in vitro* and most likely also *in vivo* in the plant species investigated up to now and that dihydro-oxylipins display biological activity in several plant species in provoking the synthesis of low M_r defense compounds [13]. To elucidate the pathway from 12-oxo-PDA and DH-12-oxo-PDA to the respective jasmonates, 12-oxo-PDA reductase was partially purified and its substrate specificity analysed.

12-*oxo-PDA reductase*

The activity of the enzyme reducing the double bond in the pentacyclic ring system of 12-oxo-PDA has been previously detected in corn kernels [29]. We introduce here a new and simple assay for this enzyme based on the HPLC separation of the substrate 12-oxo-PDA and the product OPC 8:0. Using this assay system, this enzyme activity was detected in all nine cell cultures analysed for 12-oxo-PDA reductase: *Agrostis tenuis*, *Arabidopsis thaliana*, *E. californica*, *Glycine max*, *L. esculentum*, *Phaseolus vulgaris*, *R. serpentina*, *Rubia tinctorum* and *Ruta montana*. In crude enzyme extracts of these cell cultures the activity ranged from 0.2–1.7 pkat mg^{-1} protein. The enzyme was also found in acetone powder from linseeds and spinach leaf.

12-oxo-PDA reductase is constitutively present in all systems analysed so far by us and cannot be induced by treating cell cultures with a yeast elicitor preparation. The enzyme was enriched more than 400-fold from cell cultures of *R. serpentina*, the purification scheme is given in Table 1. The enzyme is not yet homogenous but was used in a highly purified stage. 12-oxo-PDA reductase from *R. serpentina* has a broad pH optimum ranging from pH 7 to 9 and a temp optimum at 45°. The enzyme is highly specific for NADPH. Using NADH (up to 4 mM) as cofactor no OPC 8:0 formation was observed. The K_M values are 140 μM for 12-oxo-PDA and 35 μM for NADPH. K_M values and pH optimum are similar to the values described for corn 12-oxo-PDA reductase [29]. The reverse reaction, the oxidation of OPC 8:0 to 12-oxo-PDA with NADP was not catalysed by the enriched enzyme preparation.

It turned out that 12-oxo-PDA reductase is not strictly substrate specific. It converts, in addition, 4,5-dehydro-JA, having a double bond in the pentacyclic ring, to JA as well as DH-12-oxo-PDA to TH-12-oxo-PDA. DH-12-oxo-PDA can, therefore, at that stage be metabolized by the same pathway as 12-oxo-PDA.

In vivo *transformation of TH-12-oxo-PDA, 12-oxo-PDA, linolenic and linoleic acid*

To elucidate the metabolism of the oxylipins after the reduction of the pentacyclic ring, TH-12-oxo-PDA and 12-oxo-PDA were applied to cell suspension cultures. In all four cultures analysed (*A. tenuis*, *N. tabacum*, *R. serpentina* and *T. cordifolia*). TH-12-oxo-PDA was converted to DH-JA and 12-oxo-PDA was converted to JA (Table 2). The corresponding C_{16} and C_{14} intermediates were also detected. Approximately 10-fold more OPC 4:0 and DH-OPC 4:0 accumulated than OPC 6:0 and DH-OPC 6:0. When linolenic acid (18:3) was exogenously applied to cell cultures, a distinct accumulation of 12-oxo-PDA and JA was observed. Feeding of linoleic acid (18:2) in parallel experiments did not result in the formation of DH-12-oxo-PDA or DH-JA. The concentration of products formed were most likely below the detection limit. If [U-^{13}C]linolenic acid (18:3, 97% ^{13}C atom access) was supplied exogenously to cell cultures of *A. tenuis*, the 12-oxo-PDA and JA formed exhibited negative ion mass peaks of the pentafluorobenzyl (PFB) derivatives of *m/z* [M-PFB]$^-$ 309 and 221, respectively, clearly proving that they both were derived from the [^{13}C]-labelled fatty acid. When [U-^{13}C]TH-12-oxo-PDA was synthesized from [U-^{13}C] 12-oxo-PDA by Pd/H_2 reduction and was subsequently supplied to *N. tabacum* cell cultures, [^{13}C]-labelled DH-JA (*m/z* [M-PFB]$^-$ 223) was detected. The [^{13}C]-labelled substances were fed in high excess, keeping the dilution of [^{13}-C]-labelled metabolites by the corresponding endogenous [^{12}C] compounds below the detection limit.

Biological activity of 12-*oxo-PDA compared to JA*

In a screening programme, the secondary metabolite inducing activity of 12-oxo-PDA and MeJA was up to now analysed in 165 different plant cell culture species. As a preliminary result, we found that 84%

Table 1. Scheme for partial purification of 12-oxo-PDA reductase from cell cultures of *Rauvolfia serpentina*

Purification step	Total protein (mg)	Specific activity (pkat mg^{-1})	Recovery (%)	Purification ion (*x*-fold)
Crude extract	1900	1.7	100	1
0–50% $(NH_4)_2SO_4$	437	7.1	96	4.2
Hydrophobic interaction (Phenyl-Sepharose)	20	56	35	33
Colour affinity (Orange 2)	0.9	363	10	213
Gel chromatography (Superose 12)	0.04	827	1	486

Table 2. *In vivo* formation of pentacyclic oxylipins after application of precursors to cell cultures of *Agrostis tenuis*, *Nicotiana tabacum*, *Rauvolfia serpentina* and *Tinospora cordifolia*

		Pentacyclic oxilipins detected after application of precursors									
Species	applied substance	12-oxo-PDA	OPC 8:0	OPC 6:0	OPC 4:0	JA	DH-12-oxo-PDA	TH-12-oxo-PDA	DH-OPC 6:0	DH-OPC 4:0	DH JA
Agrostis tenuis	18:3	+++	+	0	0	++	0	0	0	0	0
	18:2	0	0	0	0	0	0	0	0	0	0
	12-oxo-PDA		+	+	++	+++	0	0	0	0	0
	TH-12-oxo-PDA	0	0	0	0	0	0		+	++	+++
Nicotiana tabacum	18:3	++	0	+	+	+	0	0	0	0	0
	18:2	0	0	0	0	0	0	0	0	0	0
	12-oxo-PDA		+	++	+++	+++	0	0	0	0	0
	TH-12-oxo-PDA	0	0	0	0	0	0		+	+++	+++
Rauvolfia serpentina	18:3	++	0	0	0	++	0	0	0	0	0
	18:2	0	0	0	0	0	0	0	0	0	0
	TH-12-oxo-PDA	0	0	0	0	0	0		+	+++	+++
Tinospora cordifolia	18:3	0	0	0	0	+++	0	0	0	0	0
	18:2	0	0	0	0	0	0	0	0	0	0
	TH-12-oxo-PDA	0	0	0	0	0	0		0	0	+

Linolenic (18:3) and linoleic (18:2) acid were applied at a concentration of 300 μM, 12-oxo-PDA and TH-12-oxo-PDA at 50 μM. Endogenous pentacyclic oxilipins were below the detection limit (*ca* 0.5 pmol ml^{-1} cell culture). 0 = not detected, + = $\approx$ 10 pmol ml^{-1}, ++ = $\approx$ 100 pmol ml^{-1}, +++(*m*) = $\approx$ 1000 pmol ml.

of the cultures could be induced with 12-oxo-PDA and with MeJA. No difference was observed in the qualitative spectrum of 12-oxo-PDA and MeJA induced secondary metabolites, implicating that both signal compounds control the same biosynthetic pathways. This fact disproves clearly the possibility raised by Boland *et al.* [30] that 12-oxo-PDA and JA induce different pathways thus giving rise to different metabolic patterns in plants. It was observed in our study again that 12-oxo-PDA often shows considerably higher biological activity than JA (in 60% of 139 inducible cultures). Applied at the same concentration as JA, 12-oxo-PDA leads almost always to a higher increase in secondary metabolite concentration in cell cultures. Another typical effect of 12-oxo-PDA is its toxicity leading to cell death in concentrations of more than 100–200 μM (see Fig. 3). MeJA rarely caused such toxic effects and only in concentrations of more than 500 μM.

The equal but often higher biological activity of 12-oxo-PDA as compared to JA further corroborates that 12-oxo-PDA displays biological activity on its own. It was shown previously that the conversion to JA is not required for the biological activity of 12-oxo-PDA [13]. We, therefore, assume that 12-oxo-PDA is the effective molecule in the jasmonate cascade but jasmonate itself does of course possess biological activity. This is in accordance with previous assumptions [18]. Molecular genetic suppression of the 12-oxo-PDA reductase gene in a plant would serve to test this hypothesis.

DISCUSSION

It was the aim of this contribution to investigate the occurrence and biological significance of the postulated linoleic acid (18:2) pathway in higher plants. Up to now, the description of dihydro-oxylipins in plants is limited. DH-JA has only been reported in several fungi and one plant species, *Vicia faba* [11, 12]. Other metabolites such as DH-12-oxo-PDA or TH-12-oxo-PDA have not been found to occur naturally. This could reflect a lack of analytical sensitivity, but it seems more likely that dihydro-oxylipins are not generally distributed as are 12-oxo-PDA and JA. In all nine plant cell culture species examined so far, dihydro-oxylipins could not be detected, at least not in concentrations comparable to JA (1–10 ng g^{-1} dry weight) [31].

Previous results of the alkaloid-inducing activity of TH-12-oxo-PDA and DH-JA in cell cultures of *E. californica* [13] were confirmed here also with DH-12-oxo-PDA [Fig. 3(A)]. While the jasmonate pathway for the activation of plant defense genes, as it was discovered by Ryan and associates [3], seems to be ubiquitously distributed in higher plants, the dihydro-oxylipin pathway seems to be more restricted. As shown in Fig. 5, there are plant species that clearly react to DH-12-oxo-PDA, TH-12-12-oxo-PDA and DH-JA such as *E. californica*, *E. crista-galli* and *C. cobalticola*, while others do not respond at all. Thus, the dihydro-oxylipin pathway may be restricted to certain species, which has to be taken into account in future research. Recently it has been clearly shown

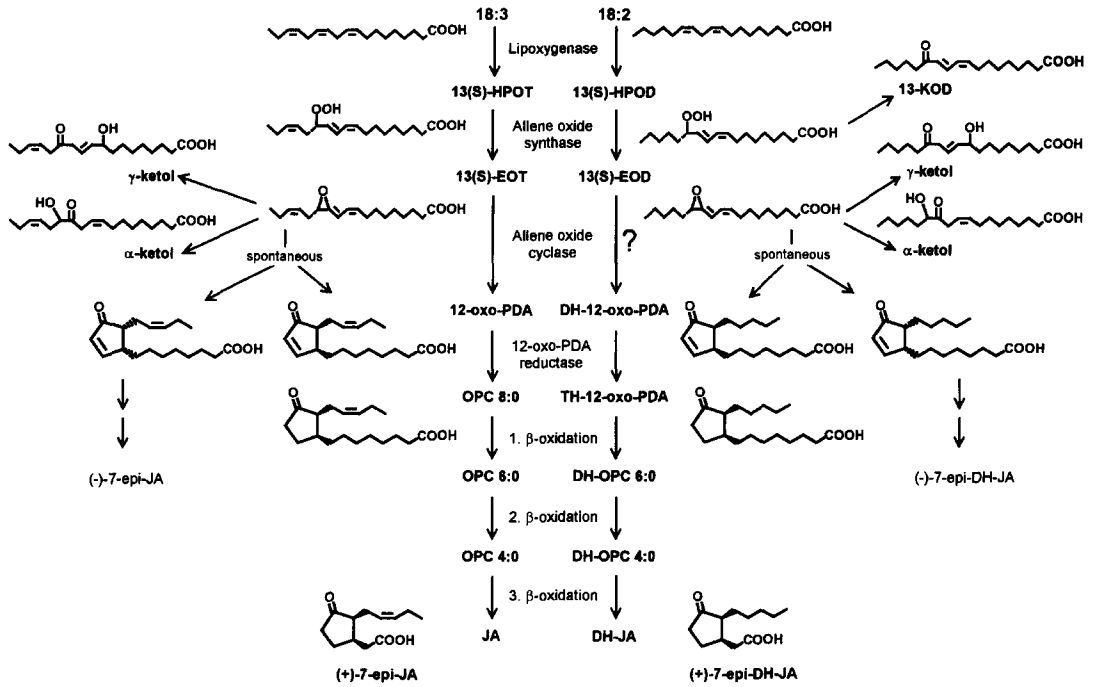

Fig. 6. Postulated biosynthetic pathways for the linolenic [6, 7, 9, 10] and linoleic route to jasmonate and dihydrojasmonate, respectively. 18:3, linolenic acid; 13(*S*)-HPOT, 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid; 13(*S*)-EOT, 12,13(*S*)-epoxy-9(*Z*),11,15(*Z*)-octadecatrienoic acid; α-ketol (from 18:3), 12-oxo-13-hydroxy-9(*Z*),15(*Z*)-octadecadienoic acid; γ-ketol (from 18:3), 12-oxo-9-hydroxy-10(*E*),15(*Z*)-octadecadienoic acid; 12-oxo-PDA, 12-oxo-phytodienoic acid; OPC 8:0, 3-oxo-2-(2′-pentenyl)cyclopentaneoctanoic acid; OPC 6:0, 3-oxo-2-(2′-pentenyl)cyclopentanehexanoic acid; OPC 4:0, 3-oxo-2-(2′-pentenyl)-cyclopentanebutanoic acid; JA, jasmonic acid; 18:2, linoleic acid; 13(*S*)-HPOD, 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid; 13(*S*)-EOD, 12,13(*S*)-epoxy-9(*Z*),11-octadecadienoic acid; α-ketol (from 18:2), 12-oxo-13-hydroxy-9(*Z*)-octadecenoic acid; γ-ketol (from 18:2), 12-oxo-9-hydroxy-10(*E*)-octadecenoic acid; 13-KOD, 13-keto-9(*Z*),11(*E*)-octadecadienoic acid; DH-12-oxo-PDA, 15,16-dihydro-12-oxo-phytodienoic acid; TH-12-oxo-PDA, 10,11–15,16-tetrahydro-12-oxo-phytodienoic acid; DH-OPC 8:0, 3-oxo-2-(2′-pentyl)cyclopentaneoctanoic acid; DH-OPC 6:0, 3-oxo-2-(2′-pentyl)cyclopentanehexanoic acid; DH-OPC 4:0, 3-oxo-2-(2′-pentyl)cyclopentanebutanoic acid; DH-JA, dihydrojasmonic acid.

that wounding of leaf tissue leads to a release of both linolenic (18:3) and linoleic (18:2) acid from cell membranes [32]. Both are precursors of 12-oxo-PDA and JA and potentially of DH-12-oxo-PDA and DH-JA as well. This fact strengthens strongly the possibility that both cascades leading to JA and DH-JA may be activated upon herbivore and pathogen attack.

The biosynthesis of DH-JA should proceed the following route starting from linoleic acid (Fig. 6): The first two enzymes of the Vick and Zimmerman pathway [7] have a broad substrate specificity. Lipoxygenase and allene oxide synthase convert 18:2 and 13-hydroperoxy-octadecadienoic acid (HPOD) and 18:3 and 13-hydroperoxy-octadecatrienoic acid (HPOT) equally well to the allene oxides 12,13-epoxy-octadecadienoic acid (EOD) and 12,13-epoxy-octadecatrienoic acid (EOT), respectively. Further down the pathway, after the cyclization reaction, 12-oxo-PDA reductase is also not substrate specific with regard to the pentyl or pentenyl side-chain. As demonstrated in this paper, in the next steps, through β-oxidation, TH-12-oxo-PDA is converted to DH-JA in the same way as OPC 8:0 is converted to JA. Even the corresponding intermediates DH-OPC 6:0 and DH-OPC 4:0 were detected.

The bottleneck of a 18:2 pathway in the plants investigated seems to be the cyclization of EOD. Hamberg demonstrated that the 18:2 derived allene oxide is not converted by the allene oxide cyclase from corn [25]. Similar results were obtained here by *in vivo* application experiments with 18:2, which did not result in the formation of cyclic oxylipins. In contrast, the application of 18:3 yielded considerable quantities of 12-oxo-PDA and JA (Table 2). Nevertheless, DH-JA is existent in nature [11, 12] and it is conceivable that allene oxide cyclase may show different substrate and mechanistic specificities, or that spontaneous cyclization of EOD occurs *in vivo*.

It was shown *in vitro* using a cyclase activity-free enzyme preparation from linseed acetone powder [10] that small amounts (*ca* 1%) of DH-12-oxo-PDA can be formed from 18:2. This is in agreement with previous reports of *in vitro* DH-12-oxo-PDA formation [9, 13, 21, 22]. Other reports negating this conversion [24, 25] could be explained by a low detection sensitivity of the analytical procedure used. The question remains, though, whether the enzymic or spontaneous cyclization of EOD to DH-12-oxo-PDA is of significance *in vivo*. The fact that the 18:2 acid is rather selectively liberated upon wounding [32] is, however,

an indicator that this acid may be involved in a second defense signalling pathway parallel to the jasmonate pathway discovered by C. A. Ryan and his associates [8], which was a breakthrough in the mechanistic considerations of plant defense.

EXPERIMENTAL

Plant cell cultures and secondary metabolite quantitation. Plant cell cultures were provided by the departmental culture collection and were grown as previously described [4]. For induction experiments, cultures were inoculated with 12 g fr. wt cells, corresponding to 0.8 g dry wt per 75 ml medium, grown for 3 days and then 1 ml aliquots were transferred to wells of a 24-well Nunc plate. Test substances were added as a soln in 80% EtOH, max. 6 μl ml^{-1} cell culture, controls received only 80% EtOH. After incubation on a gyratory shaker (140 rpm) for 4 days, cells were harvested by centrifugation and extracted with MeOH for 2 hr at 60°. The extracts were subjected to HPLC analysis: LiChrospher RP C18 select B, 5 μm, 250 × 4 mm, A: H_2O–MeCN–H_3PO_4 (98:2:0.01), B: H_2O–MeCN–H_3PO_4, (2:98:0.01), 0–100% B in 15 min, 1 ml min^{-1}, diode array detection. Each substance was tested × 3 in concns from 1–300 μM. For the results shown in Fig. 5, the optimal concn from each dose effect curve was used, ranging from 30–300 μM and depending on the culture and test substance. Secondary metabolites were identified by MS and NMR spectroscopy.

Application experiments. Cell suspension cultures of *L. esculentum* were inoculated as described above. [1-^{14}C]Linoleic acid (18:2, 53 mCi mmol^{-1}, 1μCi) was added to 1 ml cell culture. After incubation on a gyratory shaker (140 rpm) for 24 hr, the cells were extracted with EtO_2–MeOH (19:1), the extract evapd and hydrolysed to liberate found fatty acids (1 M KOH in 95% EtOH, 1 hr, 80°), diluted with H_2O and acidified with conc. HCl. The fatty acids were extracted with Et_2O, sepd on RP C18 TLC (Merck), MeCN–H_2O–HAc (90:10:0.1) R_f 18:3 = 0.45, R_f 18:2 = 0.35 and quantitated by radio TLC scanning. For GC-MS analysis of metabolites, 18:3 and 18:2 fatty acids (unlabelled and [U-^{13}C]-labelled, 97% ^{13}C atom access) were applied to 1 ml cell culture at a concn of 300 μM, 12-oxo-PDA and TH-12-oxo-PDA at 50 μM and incubated for 2 hr.

GC-MS analysis. Pentacyclic oxylipins were extracted from the cells with hexane, purified and analysed as pentafluorobenzyl (PBF) derivatives (negative ion detection) under previously reported conditions [31] on a Varian 3400 gas chromatograph interfaced to a Finnigan MAT quadrupole SSQ700 mass spectrometer. The identity of DH-OPC 4:0 and DH-JA formed *in vivo* from applied TH-12-oxo-PDA was confirmed by GC-EIMS of the PFB derivatives: EIMS (3-oxo-2-(2′-pentyl)cyclopentanebutanoic acid) 70 eV, *m/z* (rel. int.): [M-PFB]$^+$ 239 (1), 169 (7), 153 (15), 83 (100); EIMS (9,10-dihydrojasmonic acid) 70 eV, *m/z* (rel. int.): [M-PFB]$^+$ 211 (6), 141 (100), 153 (14), 83 (60).

12-oxo-PDA synthesis and isolation. The previously described procedure [33] was modified for higher yield. Linseed Me_2CO powder (1.5 g) was suspended in 160 ml K-Pi buffer 100 mM, pH 7, stirred in an ice bath for 30 min and centrifuged 10 min at 2000 *g*. Linolenic acid (18:3) (100 mg) was dissolved in 2.6 g octane and mixed with 50 ml borate buffer, 100 mM, pH 9.6. The supernatant of the Me_2CO powder extraction and the 18:3 suspension were added to 800 ml K-Pi buffer, 25 mM, pH 7.0 and stirred vigorously for 1 hr at 30°. To recover the hydrophilic reaction products from the enzyme soln, 20 g of HP21 (Diainon HP21, Mitsubishi Kasei Corporation, Tokyo) pre-activated with MeOH, was added to the reaction mixt. and shaken for 1 hr. The HP21 material was recovered by filtration, washed with H_2O and extracted with MeOH (*ca* 200 ml). The 12-oxo-PDA containing MeOH extract of the HP21 material was evapd to dryness, dissolved in 1.5 ml MeOH and subjected to semi-prep. HPLC analysis: LiChrosorb RP C18, 7 μm, 250 × 25 mm, A: H_2O–MeCN–H_3PO_4 (98:2:0.01), B: H_2O–MeCN–H_3PO_4 (2:98:0.01), 0–30 min, linear gradient 50–55% B, 40 min 55% B, 45 min 100% B, 10 ml min^{-1}, detection at 222 nm, R_t PDA = 35 min. After evapn of MeCN, 12-oxo-PDA was extracted from H_2O phase with EtOAc yielding 15 mg, EIMS (12-oxo-phytodienoic acid) 70 eV, *m/z* (rel. int.): 292 [M]$^+$ (40), 224 (56), 206 (32), 178(23), 163 (45), 149 (36), 107 (60), 95 (100), 82 (51).

DH-12-oxo-PDA synthesis and isolation. Linoleic acid (18:2) (100 mg) was dissolved in 0.5 ml octane and added to 50 ml borate buffer 100 mM, pH 9.6, containing 6.1×10^5 U soybean 13-lipoxygenase (EC1.13.11.12, Sigma). O_2 was passed through the solution for 10 min, the reaction was stopped by the addition of 3 ml 1 M citric acid, 13-HPOD was extracted with EtOAc, evapd, dissolved in 200 ml MeOH and added to 50 ml enzyme extract from 1.5 g linseed Me_2CO powder, prepd as described above. After mixing for 30 sec, a soln of fatty acid-free BSA (Sigma; 900 mg 10 ml^{-1} K-Pi buffer, 100 mM pH 7.0) was added. The mixt. was stirred for 1 hr, solid citric acid was added until a pH of 3 was reached. Metabolites derived from linoleic acid were extracted with EtOAc, the organic solvent was evapd, the residue dissolved in 200 μl MeOH and sepd by semi-prep. HPLC: LiChrospher RP C18e, 5 μm, 250 × 10 mm A: H_2O–MeCN–HAc (98:2:0.1), B: MeCN–H_2O–HAc (98:2:0.1), 0–30 min, linear gradient 50–65% B, 20–35 min 60–100% B, 4.5 ml min^{-1}, detection at 222 nm, 45 min 100% B, 4.5 ml min^{-1}, detection at 222 nm, R_t DH-12-oxo-PDA = 22 min. The DH-12-oxo-PDA containing fr. was evapd, yielding 0.7 mg, EIMS (15,16-dihydro-12-oxo-phytodienoic acid) 70 eV, *m/z* (rel. int.): 294 (11), 224 (87), 206 (94), 178 (37), 165 (13), 151 (76), 109 (34), 95 (100), 82 (65).

13-KOD isolation. 13-KOD was isolated from the 13-HPOD prepn with the same column and solvents

as used for DH-12-oxo-PDA, 0–20 min 50–60% B, 20–35 min 60–100% B, detection 280 nm, R_t 13-KOD = 26 min, yield 2%, EIMS (13-keto-9(*Z*),11(*E*)-octadecadienoic acid) 70 eV, *m/z* (rel. int.): 294 (26), 238 (7), 223 (13), 177 (16), 151 (54), 99 (26), 95 (64), 81 (100).

TH-12-oxo-PDA and OPC 8:0 synthesis. Reduction of 12-oxo-PDA (soln 1 mg ml^{-1} MeOH) with 10% Pd on activated carbon gave TH-12-oxo-PDA, 97% yield, EIMS (10,11–15,16-tetrahydro-12-oxo-PDA) 70 eV, *m/z* (rel. int.): 226 (25), 153 (25), 83 (100). OPC 8:0 was synthesized by reduction of 12-oxo-PDA with $NaBH_4$ followed by reoxidation with Jones reagent (2.7 M CrO_3 in 4 M H_2SO_4) [29]. OPC 8:0 was purified by semi-prep. HPLC as described for DH-12-oxo-PDA, detection 204 nm, R_t OPC 8:0 = 21 min, yield 75%, EIMS (OPC 8:0) 70 eV, *m/z* (rel. int.): 294 (12), 226 (59), 151 (71), 109 (83), 95 (39), 83 (100).

[^{13}C]-12-oxo-PDA synthesis. 1 mg [U-^{13}C]linolenic acid (97% ^{13}C carbon access) was incubated for 30 min at 30° in 50 ml enzyme extract, prepd as described before from 250 mg linseed Me_2CO powder. The reaction was stopped by addition of 2 ml 1 M citric acid, the products were extracted ×2 with EtOAc, the organic solvent evapd, the residue dissolved in 200 μl MeOH and subjected to semi-prep. HPLC as described for DH-12-oxo-PDA: R_t 12-oxo-PDA = 16.5 min. The 12-oxo-PDA containing fr. was evapd, yielding 120 μg [U-^{13}C]-12-oxo-PDA, 97% ^{13}C carbon access, CIMS (isobutane) *m/z* $[M+H]^+$ 311.

Analytical HPLC. Linolenic and linoleic acid products formed by linseed enzyme prepn (Fig. 1) were analysed with the following system: LiChrospher RP C18, 5 μm, 250 × 4 mm, A: H_2O–MeCN–H_3PO_4 (98:2:0.01), B: MeCN–H_2O–H_3PO_4 (98:2:0.01). 0–15 min, 50–65% B, 20 min 100% B, 1.5 ml^{-1}, DAD detection.

Enzyme preparation and purification of 12-oxo-PDA reductase. Five day old suspension cultures of *R. serpentina* were frozen with liquid N_2, 800 g cell fr. wt (corresponding to 70 g dry wt) was thawed in 1.3 l 200 mM K-Pi buffer, pH 7.0 and stirred on ice for 30 min. The slurry was pressed through 4 layers of cheesecloth and the filtrate centrifuged at 22 000 *g* for 15 min. The supernatant was passed through an Amberlite XAD-2 column (8 × 30 cm). Finely ground solid $(NH_4)_2SO_4$) was added slowly to the eluate up to a satn of 50%, the pellet collected by centrifugation (22 000 *g*, 10 min) was dissolved in 300 ml 1 M KCl, 50 mM K-Pi buffer, pH 7.0, and applied to a Phenyl-Sepharose fast flow column (20 × 4 cm) pre-equilibrated with the same buffer. After washing with 500 ml, the enzyme was eluted with a linear gradient of 200 ml to 100% 1 mM K-Pi buffer, pH 7.0, followed by 400 ml of the same buffer, flow rate 8 ml min^{-1}. Frs (700–840 ml) containing 12-oxo-PDA reductase activity were pooled, dialysed against 10 mM K-Pi buffer, pH 7.0, and applied to a colour affinity column (Mimetic Orange 2, Promochem, 20 × 1.5 cm), pre-equilibrated with 10 mM K-Pi buffer, pH 7.0. The column was washed with 250 ml equilibration buffer, the adsorbed enzyme was eluted with a linear gradient of 150 ml to 250 mM KCl in 10 mM K-Pi buffer, followed by 50 ml 1 M KCl in 10 mM K-Pi buffer, flow rate 4 ml min^{-1}. The active frs (385–470 ml) were concd and desalted by membrane filtration (10 kD) and applied to a Superose 12 (Pharmacia LKB) column (1 × 30 cm), equilibrated and run with 100 mM K-Pi buffer, pH 7.0, 150 M KCl, 0.3 ml min^{-1}. The enzyme eluted at 14 ml. Protein was determined by the method of Bradford [34] using BSA as standard.

Enzyme assay for 12-oxo-PDA reductase. The assay mixt. contained in a total volume of 500 μl: 500 nmol NADPH, 200 nmol 12-oxo-PDA, 100 mM K-Pi buffer, pH 7.0, and protein up to 1 mg. After incubation at 30°, the reaction was terminated by the addition of 50 μl 1 M citric acid. The mixt. was extracted with 1.5 ml EtOAc, the EtOAc phase was evapd, the residue dissolved in 100 μl MeOH and 50 μl were analysed as described under analytical HPLC: R_t PDA = 8.8 min, R_t OPC 8:0 = 11.0 min (*cis*), 11.4 min (*trans*). OPC 8:0 was quantitated at 204 nm.

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