Absolute Configuration of *cis*-12-Oxophytodienoic Acid of Flaxseed: Implications for the Mechanism of Biosynthesis from the 13(*S*)-Hydroperoxide of Linolenic Acid†

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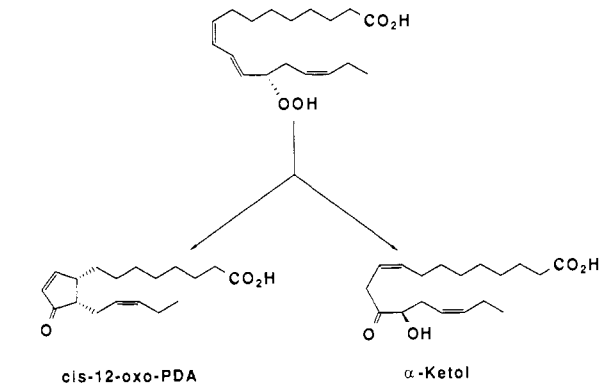
ABSTRACT: *cis*-12-Oxophytodienoic acid (*cis*-12-oxo-PDA) is a C_{18} cyclopentenone formed from the 13-(*S*)-hydroperoxide of linolenic acid in flaxseed and other plant tissues. Although the structure of *cis*-12-oxo-PDA is well established, the absolute configuration of the side chains has not been determined. We have now measured this important parameter by two independent approaches. The CD spectrum of freshly prepared *cis*-12-oxo-PDA showed no deviations from base line—implying that the product is racemic. This conclusion was checked by a high-pressure liquid chromatography (HPLC) method capable of resolving the enantiomers; *cis*-12-oxo-PDA was reduced to two saturated hydroxy analogues which were each converted to (−)-menthoxycarbonyl diastereomers and analyzed by HPLC. Each epimer was resolved as two peaks of equal area, thus confirming that their *cis*-12-oxo-PDA parent is a racemic mixture, enantiomeric at the ring junctures. We propose that the biosynthesis of racemic *cis*-12-oxo-PDA proceeds by dehydration of the 13(*S*)-hydroperoxide to an allene oxide. A major fate of the allene oxide is hydrolysis to an α-ketol, which is always formed together with *cis*-12-oxo-PDA. The allene oxide also opens to a zwitterion, which undergoes charge delocalization to form a planar intermediate; this structure is the achiral precursor of the stable end product of pericyclic ring closure, viz., racemic *cis*-12-oxo-PDA.

Flaxseed and other plant tissues convert the 13(*S*)-hydroperoxide of linolenic acid to an α-ketol (Zimmerman, 1966; Zimmerman & Vick, 1970; Gardner, 1970) and to a cyclopentenone named *cis*-12-oxophytodienoic acid (12-oxo-PDA)[1] (Zimmerman & Feng, 1978; Vick & Zimmerman, 1979), Scheme I. The α-ketol has a 12-oxo-13-hydroxy structure and was originally presumed to arise via the action of a "hydroperoxide isomerase". Subsequent work has shown that the transformation is not an isomerization of the hydroperoxide. The oxygen in the C-12 carbonyl arises from the original hydroperoxide group (Veldink et al., 1970), whereas the oxygen of the 13-hydroxyl comes from the solvent. Thus, "isomerase" is not an appropriate name for the enzyme. Additional work on the mechanism of biosynthesis of the α-ketol yielded two significant findings: (i) the 13-hydroxyl has the opposite absolute configuration to the hydroperoxide of the substrate [i.e., the α-ketol is 12-oxo-13(*R*)-hydroxy], and (ii) the presence of high concentrations of methanol or other nucleophiles during the biosynthesis gives rise to the corresponding substituent at C-13 (e.g., 13-methoxy) (Gardner, 1975). From these observations Gardner deduced that a 12,13-oxido structure is an intermediate in formation of the α-ketol and that S_N2 attack by water gives the C-13 hydroxyl with inversion to the 13*R* configuration (Gardner, 1979).

Formation of the α-ketol is always associated with biosynthesis of the cyclopentenone *cis*-12-oxo-PDA. A "hydroperoxide cyclase" is said to be responsible for this transformation. *cis*-12-Oxo-PDA bears a close structural resemblance to jasmonic acid, Figure 1, a 12-carbon cyclopentanone constituent of many plants (Meyer, 1984). Conversion of the 18-carbon cyclopentenone *cis*-12-oxo-PDA through to jasmonic acid was

Scheme I



demonstrated in tissue slices of *Vicia faba* L. pericarp (Vick & Zimmerman, 1983) and later in five other plant species (Vick & Zimmerman, 1984), thus providing strong evidence for the precursor/product relationship.

An important aspect of the biosynthesis of *cis*-12-oxo-PDA remains to be addressed. This concerns the absolute configuration of the molecule at the chiral centers in the ring juncture carbons 9 and 13. It is known from work in the 1960s that jasmonic acid is a chiral molecule (Demole et al., 1962; Hill & Edwards, 1965). The absolute configuration of the upper side chain is as presented in Figure 1; both *cis*- and *trans*-jasmonic acids have been isolated from natural sources, but it has been suggested that the *trans*-jasmonic acid arises from epimerization during isolation and purification procedures (Hill & Edwards, 1965; Vick & Zimmerman, 1984). We considered that determination of the absolute configuration of *cis*-

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[1] Abbreviations: PDA, phytodienoic acid; RP-HPLC, reversed-phase high-pressure liquid chromatography; SP-HPLC, straight-phase high-pressure liquid chromatography; DNBPG, [(dinitrobenzoyl)phenyl]-glycine; GC–MS, gas chromatography–mass spectrometry; COSY, correlation spectroscopy; PG, prostaglandin; TMS, trimethylsilyl; MC, menthoxycarbonyl; MeOH, methanol; HAc, glacial acetic acid.

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FIGURE 1: Absolute configurations of *cis*- and *trans*-jasmonic acid.

12-oxo-PDA would provide fundamental insight into the mechanism of cyclization of linolenic acid hydroperoxide and also provide further information regarding the relationship of *cis*-12-oxo-PDA and jasmonic acid. We have made the surprising finding that *cis*-12-oxo-PDA is a racemate, and this has prompted additional experiments to explore the nature of the enzymatic transformation of the hydroperoxide to the α-ketol and the cyclopentenone.

Experimental Procedures

Materials. Unlabeled linolenic acid was purchased from Nu Chek Inc. and radiolabeled linolenic acid from Amersham Corp. (−)-Menthyl chloroformate was obtained from Aldrich and BSTFA from Supelco. All solvents were distilled-in-glass grade from Burdick & Jackson, Muskegon, MI.

Preparation of Hydroperoxide Substrates. Soybean lipoxygenase (type IV, Sigma) was used to prepare the 13-(*S*)-hydroperoxyoctadeca-9(*Z*),11(*E*),15(*Z*)-trienoic acid from α-linolenic acid. This material was purified by SP-HPLC and quantified by UV spectroscopy (ϵ = 28 000). The corresponding racemic (13*RS*) hydroperoxide was obtained by controlled autoxidation of linolenic acid in the presence of vitamin E (Peers & Coxon, 1983). The resulting mixture of four monohydroperoxides (plus the hydroxy derivatives which appear when the free acid is autoxidized) was initially chromatographed on SP-HPLC, with further resolution by RP-HPLC (on a 5S ODS Bio-Rad analytical column, 4 × 250 mm). The final preparation of racemic material was of comparable purity to the 13*S* enzymic product.

Preparation of Acetone Powder. Acetone powder of flaxseed was prepared by homogenization of 10 g of seeds in 500 mL of acetone at −10 °C in a Polytron homogenizer (Brinkmann) operated at full speed for 30 s. The cloudy supernatant was decanted, and the procedure was repeated on the remaining solids. The powder was dried under vacuum and stored at −20 °C.

Incubation and Extraction. The acetone powder was made up as a fresh stock solution of 50 mg/mL in 50 mM potassium phosphate buffer at pH 7.0, containing 3 mM Zwittergent 3-14 (Calbiochem). The enzyme solution was stirred 30 min on ice and then centrifuged for 5 min in a Beckman microfuge. The resulting supernatant was diluted 1:40 with 50 mM phosphate buffer; 5 μL of the original supernatant efficiently converted 10 μg of the substrate to products. Reactions were started by addition of the 13(*S*)-hydroperoxide of α-linolenic acid (10 or 50 μg/mL final concentration in a few microliters of ethanol). Reaction was stopped by addition of 20% by volume of 200 mM NaH_2PO_4 plus a predetermined volume of 1 N HCl to give pH 4 and immediate extraction with dichloromethane or ethyl acetate. The extract was then washed with water prior to concentration.

HPLC Methods. Products formed from 13(*S*)-hydroperoxylinolenic acid were purified by SP-HPLC on an Alltech 5-μm silica column (250 × 4.6 mm) and a solvent system of hexane/IPA/HAc (100/1.6/0.1 by volume); the retention volumes of the 13-hydroperoxide, *trans*-12-oxo-PDA, the α-ketol, and *cis*-12-oxo-PDA were 16, 16.5, 19, and 21.5 mL, respectively. An Alltech 10-μm silica column (250 × 10 mm) was used for preparative work. A Hewlett-Packard 1040A diode array detector was used to monitor UV absorbance at 205, 220, 235, and 270 nm and to record UV spectra.

RP-HPLC was run with an Altex 5-μm Ultrasphere column (250 × 4.6 mm). A solvent system of MeOH/H_2O/HAc (75/25/0.01 by volume) was used for free acids, MeOH/H_2O (90/10 v/v) for benzyl esters, and 100% acetonitrile for menthoxycarbonyl derivatives.

Chiral-phase HPLC separations used a Baker DNBPG column (250 × 4.6 mm) and a solvent of hexane/2-propanol (100/0.1) for the benzyl ester menthoxycarbonyl derivatives.

Incubations in D_2O. For experiments in D_2O, a pH 7 potassium phosphate buffer was lyophilized and reconstituted with 99+% D_2O (Aldrich). After addition of enzyme solution (acetone powder in D_2O), the 13(*S*)-hydroperoxylinolenic acid substrate was added in a few microliters of ethanol. Reaction was allowed to proceed for 3 min at room temperature and was then terminated by addition of 1 mg of $NaBH_4$/mL of buffer; 2 min later, the solution was acidified to pH 4 by addition of NaH_2PO_4 plus a predetermined volume of 1 N HCl, and the products were extracted with ethyl acetate. The diols resulting from reduction of the α-ketol were purified by RP-HPLC and analyzed by GC–MS as the methyl ester TMS ether derivatives.

NMR. Spectra were recorded on a Bruker AM-400 or an IBM/Bruker NR-300 at 400 and 300 MHz, respectively, with $CDCl_3$ as solvent. Chemical shifts are reported in relation to tetramethylsilane (δ 0.0), and residual $CHCl_3$ was observed at δ 7.26. Typical parameters for 1-D spectra were 16K sampling and data points, 60° pulse, and 2.5-s relaxation delay. Generally, 16 scans provided good signal-to-noise after transformation. For measurement of coupling constants, gaussian multiplication (providing resolution enhancement) was employed before transformation. For measurement of NOE difference spectra, 1024 scans of each irradiation spot were acquired before subtraction, and 0.5–1.0 Hz of exponential line broadening was used. COSY spectra were obtained with the standard Bruker microprogram. A total of 256 1K spectra were recorded with a 90–τ–45 pulse sequence. Sine-bell apodization, magnitude calculation, and symmetrization were employed.

Gas Chromatography–Mass Spectrometry. The instrument was a Nermag R10-10C coupled to a Varian Vista 6000 gas chromatograph. Samples were run on a 10-m DB-1 capillary column with temperature programming from 150 °C at 10 deg/min. Spectra were recorded in the electron impact mode with an electron energy of 70 eV.

Ultraviolet Spectroscopy. A Beckman DU-7 scanning spectrophotometer was used. The calibration was checked with a holmium oxide filter by use of the absorbance peak at 279 nm (λ_{max} observed at 279.2 nm).

Circular Dichroism. A Jasco J-500A recording spectropolarimeter was used to obtain CD measurements. Samples in acetonitrile were scanned from 400 to 200 nm in a 0.8-mL sample holder with 1-cm path length. The spectrum was recorded 4 times and averaged before plotting.

HPLC Method for Measurement of Enantiomers of cis-12-Oxo-PDA. Approximately 100 μg of *cis*-12-oxo-PDA (and a separate sample of the trans isomer) was treated with a few milligrams of $NaBH_4$ in 0.5 mL of methanol; reaction was allowed to proceed for 1 h at 0 °C and for a further 30 min at room temperature. The reaction mixture was then quenched with aqueous NaH_2PO_4, further acidified with 1 N HCl, and extracted with ethyl acetate. The organic phase was washed with water and taken to dryness.

The benzyl ester derivative of the hydroxy epimers was prepared by dissolving the sample in 80 μL of dimethylacet-

Table I: ^{1}H NMR (400 MHz) of *cis*- and *trans*-12-Oxophytodienoic Acid

proton	multiplicity	δ	coupling constants (Hz)
	(A) *cis*-12-Oxophytodienoic Acid		
H2a,b	t	2.35	$J_{2ab,3ab}$ = 7.4
H3a,b	m	1.63	
H4a,b	m	1.30–1.35	
H5a,b	m	1.30–1.35	
H6a,b	m	1.30–1.35	
H7a,b	m	1.30–1.35	
H8a	m	1.15	
H8b	m	1.73	
H9[a]	m	2.98	$J_{9,10}$ = 2.8 $J_{9,11}$ = 1.8
H10	dd	7.74	$J_{10,11}$ = 5.8
H11	dd	6.19	
H13[a]	m	≈2.43	$J_{13,14a}$ = 6.2 $J_{13,14b}$ = 4.5
H14a	m	≈2.13	$J_{14a,14b}$ = 14.4
H14b	m	≈2.51	
H15	m	≈5.38	$J_{15,16}$ = 11.1
H16	m	≈5.44	$J_{16,17}$ = 7.4
H17a,b	p	2.07	$J_{17ab,18abc}$ = 7.4
H18a,b,c	t	0.98	
	(B) *trans*-12-Oxophytodienoic Acid		
H2a,b	t	2.365	
H3a,b	m	≈1.63	
H4a,b	m	1.30–1.35	
H5a,b	m	1.30–1.35	
H6a,b	m	1.30–1.35	
H7a,b	m	1.30–1.35	
H8a	≈q	≈1.49	$J_{8a,9}$ = ≈7
H8b	≈q	≈1.49	$J_{8b,9}$ = ≈7
H9[a]	m	≈2.58	$J_{9,10}$ = 2.4 $J_{9,11}$ = 1.9; $J_{9,13}$ = ≈2
H10	dd	7.60	$J_{10,11}$ = 5.7
H11	dd	6.13	
H13[a]	m	≈2.02	$J_{13,14a}$ = ≈8.1 $J_{13,14b}$ = ≈6.0
H14a	m	≈2.28	$J_{14a,14b}$ = 14.5 $J_{14a,15}$ = 8.1
H14b	m	≈2.465	$J_{14b,15}$ = ≈6.0
H15	m	≈5.27	
H16	m	≈5.45	$J_{16,17ab}$ = 7.3
H17a,b	m	≈2.055	$J_{17ab,18abc}$ = ≈7.3
H18a,b,c	t	0.965	

[a] Assignment in the reference Vick et al. (1979) is incorrect.

Table II: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) of the α-Ketol Product

(A) ^{1}H NMR

proton	multiplicity	δ	coupling constants (Hz)
H2a,b	t	2.35	
H3a,b	p	1.63	
H4a,b	m	1.25–1.45	
H5a,b	m	1.25–1.45	
H6a,b	m	1.25–1.45	
H7a,b	m	1.25–1.45	
H8a,b	q	2.02	
H9	m	5.57–5.68	$J_{9,10}$ = 10.7
H10	m	5.50–5.68	
H11a	dd	3.22	
H11b	dd	3.28	
H13	t	4.30	
H14a	m	2.45	
H14b	m	2.60	
H15	m	5.26–5.37	$J_{15,16}$ = 10.7
H16	m	5.47–5.60	
H17a,b	p	2.07	
H18a,b,c	t	0.965	

(B) ^{13}C NMR

chemical shift (ppm)	assignment
209.9	C-12
178.2	C-1
135.6 134.4 122.2 119.7	olefinic carbons—C-9, C-10, C-15, C-16
75.76	C-13 (–OH)
37.06 33.69 31.55 29.12 28.99 (2 signals) 28.92 27.54 24.63 20.79	aliphatic CH_2's—C-2–C-8, C-11, C-14, C-17
14.07	C-18

amide and 20 μL of 2.4% methanolic tetramethylammonium hydroxide, followed by addition of 2μL of benzyl bromide. After 30 min at room temperature, 5 mL of water was added, and the sample was passed through a 500-mg C_{18} Bond-Elut extraction cartridge; the column was then washed with water, and the benzyl esters were recovered with ethyl acetate. The hydroxy products were purified by RP-HPLC on a 5-μm Ultrasphere column (25 × 0.46 cm) in a solvent of MeOH/H_2O (85:15 v/v) with UV detection at 210 nm. The reduced cis isomer eluted as minor and major hydroxy epimers at 19.5 and 23.2 mL, respectively. The trans isomer gave major and minor products at 20.2 and 22.4 mL, respectively.

Each hydroxy epimer was treated with 90 μL of dry toluene/10 μL of dry pyridine/5 μL of (–)-menthyl chloroformate for 2 h at 100 °C. The samples were then evaporated, and the MC derivative was dissolved in acetonitrile and purified by RP-HPLC with 100% acetonitrile as solvent (retention volumes ≈ 15 mL); care was taken to collect the whole chromatographic peak. The MC derivative of each hydroxy epimer was then chromatographed on SP-HPLC systems using either a Baker DNBPG chiral-phase column or a 5-μm Alltech silica column, a solvent of 0.1% 2-propanol in hexane, and UV detection at 210 nm. Peak areas were computed by a Hitachi D-2000 integrator.

RESULTS

Metabolism of the 13-Hydroperoxide of C18.3ω3. Acetone powders of flaxseed rapidly metabolize the 13(*S*)-hydroperoxide of α-linolenic acid to an α-ketol and a cyclopentenone. In our experiments the products were formed in the relative proportions of ca. 5:1 in favor of the α-ketol. We established the structures of the compounds by UV spectroscopy, GC–MS, and NMR. Key NMR data are given in Tables I and II. These initial experiments proved the compounds were 12-oxo-13-hydroxyoctadeca-9(*Z*),15(*Z*)-dienoic acid and *cis*-12-oxo-PDA (as in Scheme I), thus confirming the structures reported in the literature (Zimmerman & Feng, 1978; Vick & Zimmerman, 1979). The spectral assignments were aided by the use of the two-dimensional correlated spectroscopy (COSY) experiment (Figure 2). This method established the scalar coupling of all protons in *cis*-12-oxo-PDA (and subsequently in the base-isomerized *trans*-12-oxo-PDA) and showed that assignments for the ring-juncture protons H9 and H13 are the reverse of the chemical shifts given in the literature (Vick et al., 1979).

A complete structural analysis of the cyclopentenone involved the ability to distinguish the *cis*- and the *trans*-12-oxo-PDA. The distinction was based on several criteria. As described in the literature (Vick et al., 1979), the natural product is converted by base treatment to an isomer with different chromatographic mobility and with identical UV and

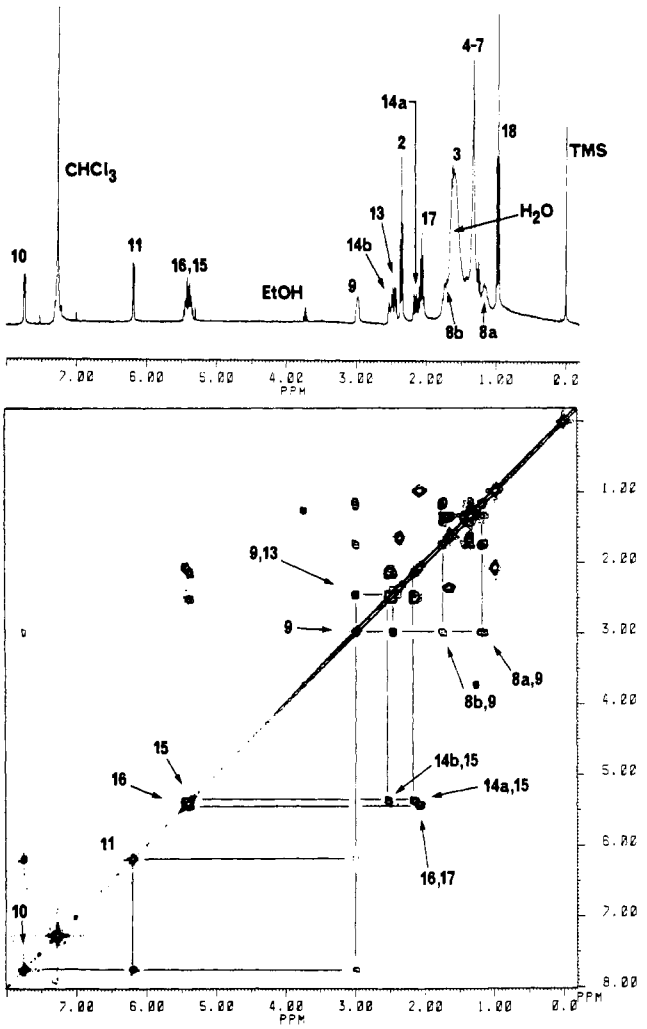


FIGURE 2: ^{1}H NMR spectrum (400 MHz) of *cis*-12-oxo-PDA (top) and the two-dimensional COSY spectrum (bottom).

mass spectral characteristics. This is interpreted as isomerization of the *cis*-12-oxo-PDA to the thermodynamically more stable trans arrangement of the side chains. More tangible evidence for the side-chain configuration was provided by NMR analyses, particularly the nuclear Overhauser effect (NOE) difference spectra involving the ring-juncture protons on carbons 9 and 13. Thus, gated irradiation of H9 showed marked NOE to H13 in the non-base-treated compound, giving evidence of the spatial proximity of the two protons (Figure 3). The same NOE difference experiment performed on the base-isomerized 12-oxo-PDA showed no detectable NOE from H9 to H13 (experiment not shown), indicating that H9 and H13 are much further apart spatially, consistent with a trans arrangement of the side chains. The complementary experiment (gated irradiation of H13) was also performed (Figure 3), and this confirmed the cis and trans assignment. The conclusion is further supported by a comparison of the chemical shifts of H9 and H13 in the two isomers of 12-oxo-PDA. The chemical shifts of H9 and H13 in the base-treated compound are upfield of the values in the natural compound by ≈0.4 ppm. This upfield shift is induced by electronic shielding from the alkyl side chain, which is syn to the vicinal juncture protons in the trans compound ["syn upfield" rule (Anteunis & Danneels, 1975)]. Thus, the upfield shifts of the ring-juncture protons are indicative of the trans side chain configuration in the base-treated compound.

The specificity of the enzymic transformation of hydroperoxide was investigated by comparing 13(*S*)- and 13(*RS*)-hydroperoxides as substrates. Under conditions in which the 13(*S*)-hydroperoxide was completely metabolized, approximately half of the racemic substrate remained unreacted. This unreacted portion of substrate was recovered by extraction and HPLC. The hydroperoxide was reduced with triphenylphosphine, repurified by HPLC, and analyzed by chiral-phase HPLC in comparison with 13(*S*)- and 13(*RS*)-hydroxy standards. The results showed that the unreacted substrate from the incubation with 13(*RS*)-hydroperoxide was nearly pure 13*R* enantiomer, Figure 4. Thus, the transformation to the α-ketol and *cis*-12-oxo-PDA is highly specific for the hydroperoxide of the 13*S* configuration.

Absolute Configuration of cis-12-Oxo-PDA. *cis*-12-Oxo-PDA and prostaglandin A_2 were prepared in acetonitrile solution at approximately 30 μg/mL. The UV spectra of the *cis*-12-oxo-PDA and the PGA_2 are shown in Figure 5A and the CD spectra in Figure 5B,C. The positive Cotton effect in the CD spectrum of the prostaglandin A_2 reflects the chirality at C-12 (Schneider et al., 1977). The CD spectrum of *cis*-12-oxo-PDA is a flat line. The implication of this result is that *cis*-12-oxo-PDA is a racemate.

A potential artifact in the CD analysis is a significant contamination of *cis*-12-oxo-PDA with the trans side chain analogue. Isomerization of cis to trans is associated with racemization at the ring junctures; the keto–enol tautomerism allows epimerization at both C-9 and C-13, leading to racemization. The *cis*-12-oxo-PDA sample was analyzed by SP-HPLC and was found to contain ≤2% of the trans isomer, thus effectively eliminating the possibility of contamination with racemic *trans*-12-oxo-PDA in the CD analysis.

An independent procedure was developed to check the chirality of *cis*-12-oxo-PDA: (a) The compound was reduced with sodium borohydride, giving major and minor hydroxy cyclopentanes in ca. 3:1 proportion (this step fixes the configuration at the ring juncture). The hydroxy epimers were each subjected to the sequence (b) hydrogenation (to eliminate the possibility of cis/trans isomers at the 15,16 double bond), (c) preparation of the benzyl ester (to allow UV detection on HPLC), and (d) conversion of the free hydroxyl to the (−)-menthoxycarbonyl (MC) derivative (this yields MC diastereomers which are amenable to chromatographic resolution).

The benzyl ester MC derivatives from the major and minor hydroxy epimers were each examined on RP-HPLC, SP-HPLC, and chiral-phase HPLC. Each epimer was resolved into two peaks of equal area in one or more of these systems. Figure 6 shows these separations. As noted earlier, *trans*-12-oxo-PDA is known to be racemic, and each hydroxy epimer would be expected to resolve as two MC diastereomers. This is indeed observed. These analyses show that the epimers of the cis isomer are also resolved. The results provide an independent line of evidence, which verifies the inference from the CD analysis. The conclusion is that *cis*-12-oxo-PDA is totally racemic.

Mechanism of Biosynthesis from the 13(S)-Hydroperoxide. In the introduction we referred to Gardner's proposal that the α-ketol is formed via a 12,13-oxido intermediate (Gardner, 1979). Vick, Feng, and Zimmerman later suggested that *cis*-12-oxo-PDA arises by cyclization of the same intermediate (Vick et al., 1980). In our own appraisal of the mechanism we also contemplated that both products would arise through a common pathway, but we differ from Gardner in details of the scheme. We consider the most plausible mechanism involves enzymatic conversion of the 13(*S*)-hydroperoxide to an allene oxide. Hydrolysis of allene oxides and cyclization to cyclopentenones have strong precedence in the chemical literature (Bordwell & Carlson, 1969a,b; Roumestant et al., 1976; Malacria & Roumestant, 1977; Chan & Ong, 1980).

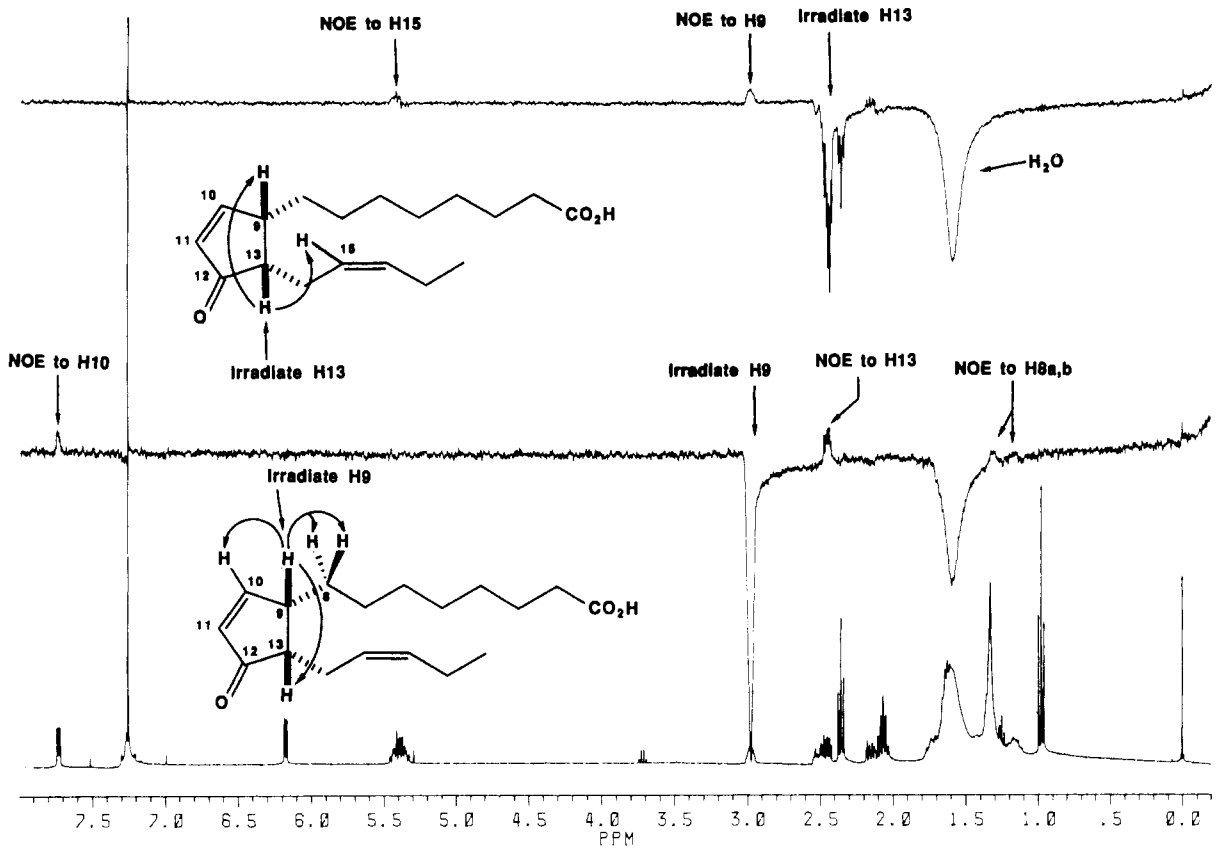


FIGURE 3: ^1H NMR nuclear Overhauser effect (NOE) difference spectra show that the side chains are cis. Irradiation of H13 (top trace) reveals a strong NOE to H9, indicating the spatial proximity of the two protons. Irradiation of H9 (lower trace) shows complementary NOE to H13.

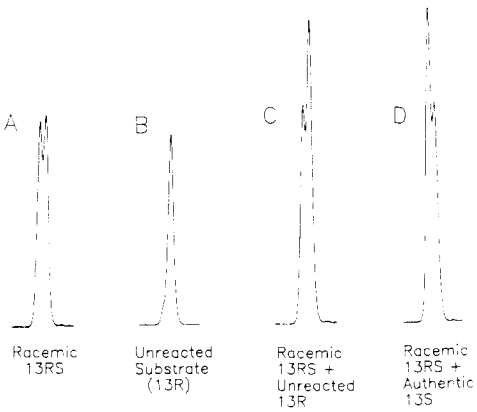
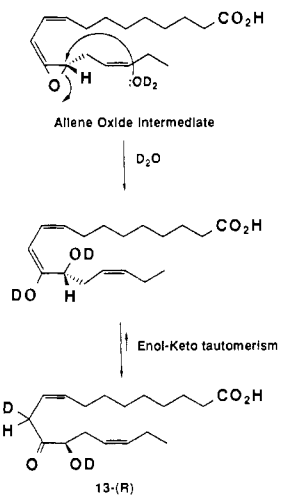


FIGURE 4: Chiral-phase HPLC analysis of 13(*RS*)-hydro(per)oxylinolenic acid before and after reaction with flaxseed enzymes. The 13-hydroperoxides were reduced with triphenylphosphine, repurified by SP-HPLC, methylated with diazomethane, and analyzed by chiral-phase HPLC. Conditions: Baker DNBPG column (25 × 0.4 cm) with a solvent of 0.5% 2-propanol in hexane, flow rate 0.5 mL/min, and UV detection at 235 nm. The 13*S* and 13*R* enantiomers were retained 22.3 and 22.8 mL, respectively; only the chromatographic peaks are shown. (A) Resolution of the two enantiomers of 13-(*RS*)-hydroxylinolenate. (B) Unreacted substrate from incubation of racemic 13-hydroperoxide with flaxseed. (C) Separation of a mixture of racemic 13-hydroxylinolenate and unreacted substrate from (B). (D) Racemic 13-hydroxylinolenate plus authentic 13*S* enantiomer. It can be seen that the unreacted substrate is nearly pure 13R enantiomer.

One aspect of hydrolysis of the allene oxide is open to straightforward experimental verification, viz., the implication that a proton from the solvent is incorporated in the α-ketol product (Scheme II). [This contrasts with the original proposal by Gardner in which an internal hydride shift was postulated (Gardner, 1979).] We measured this incorporation by conducting a biosynthesis in D_2O buffer, immediately

Scheme II



followed by $NaBH_4$ reduction in situ, and subsequent GC–MS analysis of the resulting 12,13-diols (Me ester TMS derivative). The prominent α-cleavage fragment representing carbons 12–18 (m/z 273) did not contain deuterium. The deuterium label was measured in the ion fragments normally present at m/z 299 (C-1–C-12) and m/z 270 (299 – 29); these ions were shifted 1 amu higher in the product formed in D_2O. The product contained one deuterium in this half of the molecule (C-1–C-12), and the probable location of the label is at C-11 (Scheme II). Control experiments indicated that there was approximately 12% incorporation of deuterium into the α-ketol when an unlabeled standard of α-ketol was incubated in D_2O and enzyme and reduced in situ with $NaBH_4$. Thus, the D_2O incubation supports hydrolysis of an allene oxide as shown in Scheme II.

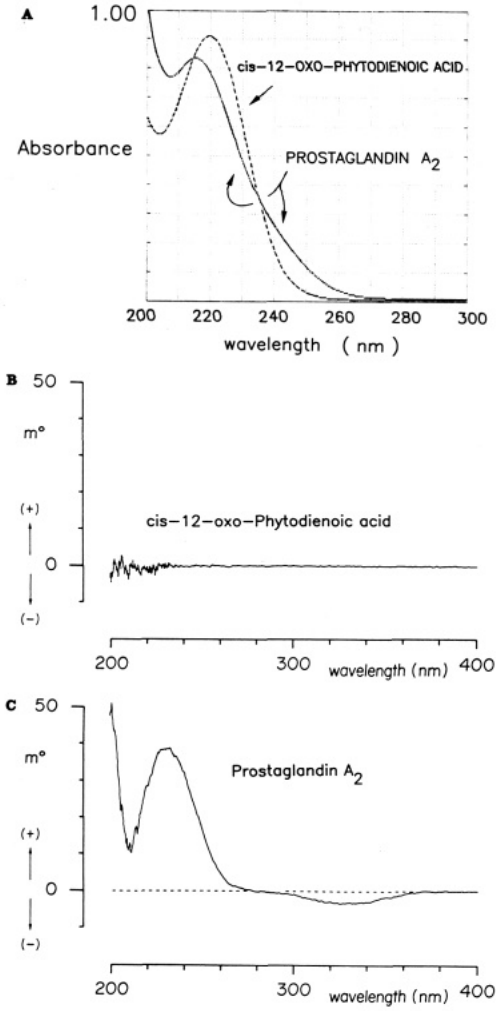
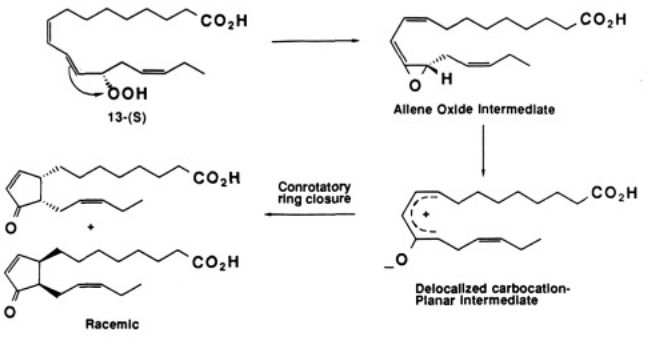


FIGURE 5: UV and CD spectra of *cis*-12-oxo-PDA and prostaglandin A_2. (A) UV spectra in acetonitrile. (B and C) The same solutions were used for CD measurements.

Scheme III



The biosynthesis of racemic *cis*-12-oxo-PDA follows in straightforward fashion from opening of the allene oxide, delocalization of the carbocation, and conrotatory ring closure as predicted by Woodward–Hoffman rules (Woodward & Hoffman, 1970), Scheme III. It follows a priori that racemic *cis*-12-oxo-PDA must be derived from an achiral noncyclic precursor. In Scheme III this is the delocalized zwitterion, a planar species that undergoes conrotatory ring closure, in both directions, to give *cis*-12-oxo-PDA.

Discussion

We decided to study the cyclization reactions in flaxseed because the biosynthesis of *cis*-12-oxo-PDA is the best documented example of a "hydroperoxide cyclase" reaction. Our interests in this type of transformation stem from a related

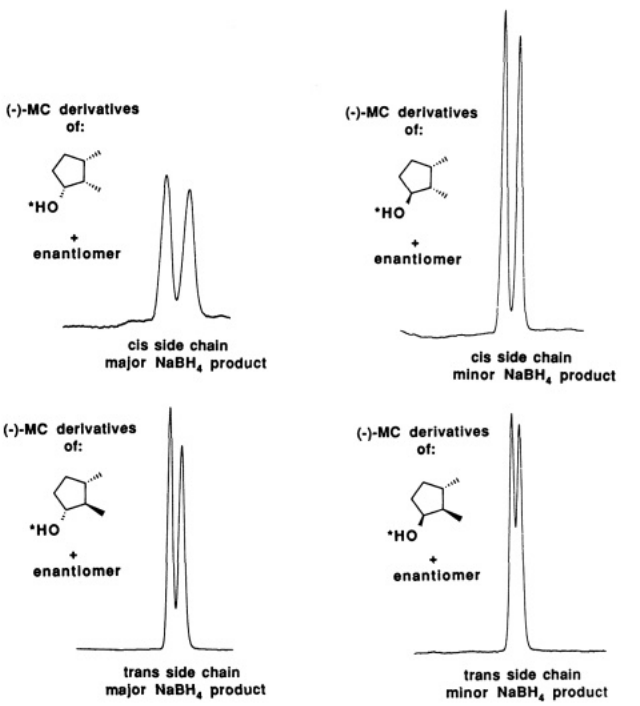


FIGURE 6: HPLC resolution of the side-chain enantiomers of *cis*- and *trans*-PDA. A method was developed to resolve the mirror image forms of *cis*- and *trans*-PDA (details are given under Experimental Procedures). Partial chromatograms are shown. The separation at top left was on SP-HPLC (silica column), while the other separations were on a chiral-phase DNBPG column. The two peaks of each pair represent equal amounts of the two enantiomers of opposite side-chain configuration. (*) Relative configuration of the hydroxyl was not determined.

project on the mechanism of prostaglandin A_2 biosynthesis in the coral *Plexaura homomalla*. This coral contains large amounts of prostaglandins, and there is evidence that the prostaglandin biosynthesis is not via a cyclooxygenase pathway (Corey et al., 1973). It has been suggested that the pathway involves an 8-lipoxygenase and a "hydroperoxide cyclase" (Corey et al., 1987). We have conducted extensive analyses of the products of arachidonic acid metabolism in *P. homomalla*, and we find that the enzyme system is very closely analogous to that of the flaxseed (Brash et al., 1987). Both tissues transform a chiral hydroperoxide to an α-ketol and a cyclopentenone. We determined the absolute configurations of the α-ketol and the cyclopentenone of the coral. We made the surprising finding that the cyclic product is racemic. In light of this result we considered it important to establish whether the same results would pertain to the flaxseed. The results indicate that *cis*-12-oxo-PDA is racemic, and thus the parallels in the two tissues extend to the chirality of the cyclic products.

The finding that *cis*-12-oxo-PDA is a racemic mixture leads to several new thoughts concerning the mechanism of biosynthesis from the 13(*S*)-hydroperoxide of linolenic acid. We deduced that conversion of the hydroperoxide to an allene oxide is the most likely route to the α-ketol and the cyclopentenone. On chemical grounds, the allene oxide is the most plausible intermediate; there is well-established precedent for the hydrolysis and cyclization reactions (Bordwell & Carlson, 1969a,b; Roumestant et al., 1976; Malacria & Roumestant, 1977; Chan & Ong, 1980). The existence of the allene oxide is strongly supported by the recent work of Hamberg, who used an analogous enzyme system in corn germ and linoleic acid as substrate; he isolated the intermediate of the corn germ "isomerase", recorded its UV spectrum, measured its chemical

half-life, and monitored its conversion to the α-ketol and novel breakdown products (Hamberg, 1987).

Formation of the allene oxide is a specific enzymatic transformation—our demonstration of the requirement for a hydroperoxide of the 13*S* configuration is one reflection of this specificity. Notably, however, the conversion of the allene oxide to the α-ketol and racemic cyclopentenone could well be nonenzymic in nature. The inherent instability of the allene oxide would allow the hydrolysis and cyclization reactions to proceed rapidly in aqueous solution at room temperature. Also, it seems distinctly unlikely that an enzyme would direct the synthesis of a racemic product (*cis*-12-oxo-PDA). Moreover, we know from our work in *P. homomalla* (Brash et al., 1987) and from the studies of Hamberg (1987) in corn germ that the α-ketol products are not optically pure; in fact, the chiralities are close to 70% of the opposite configuration of the hydroperoxide substrates. These results can readily be accounted for by S_N1 or S_N2 chemistry of the hydrolysis of the chiral allene oxide (Brash et al., 1987). The strong implication is that the "hydroperoxide cyclase" and "hydroperoxide isomerase" are not two distinct enzymes. It is relevant to point out that reported attempts to separate the "cyclase" and "isomerase" enzymes have not succeeded (Vick & Zimmerman, 1986), and this is compatible with the supposition that both activities are a reflection of one enzyme-directed step. An enzyme does initiate dehydration of the hydroperoxide, but subsequent breakdown of the common intermediate can easily be accounted for by nonenzymic processes.

The conversion of *cis*-12-oxo-PDA to *cis*-jasmonic acid in vivo has been demonstrated in several plant species (Vick & Zimmerman, 1983, 1984). Since it is known that jasmonic acid is chiral (Demole et al., 1962; Hill & Edwards, 1965), the finding that *cis*-12-oxo-PDA is racemic presents an intriguing biochemical problem. In general terms there are two solutions. Either there exists a mechanism to recover a chiral end product from the racemate, or a racemate is not formed under the natural conditions of biosynthesis. The first possibility entails that at some point in the pathway the enzymes must be selective for the correct side-chain enantiomer. This enantioselective step would then leave behind the "wrong" enantiomer—this could be epimerized to the correct configuration or have a separate metabolic fate. The second explanation involves a "missing" enzymic activity that can direct cyclization of the allene oxide. Notably, we prepared *cis*-12-oxo-PDA in very dilute solutions of protein, and with cofactors, lipids, and some enzymes lost in preparation of the acetone powder. Clearly a critical enzyme activity may be missing in this preparation, and the racemate may not be a natural product. Further study of the chiralities of the compounds in the jasmonic acid pathway are required to resolve these issues.

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