The lipoxygenase pathway in tulip (*Tulipa gesneriana*): detection of the ketol route

Alexander N. GRECHKIN^{*1}, Lucia S. MUKHTAROVA^{*} and Mats HAMBERG[†]

^{*}Kazan Institute of Biochemistry and Biophysics, Russian Academy of Sciences, P. O. Box 30, Kazan, 420503, Russia, and [†]Department of Physiological Chemistry II, Karolinska Institutet, S-17177, Stockholm, Sweden

The *in vitro* metabolism of [1-¹⁴C]linoleate, [1-¹⁴C]linolenate and their 9(*S*)-hydroperoxides was studied in cell-free preparations from tulip (*Tulipa gesneriana*) bulbs, leaves and flowers. Linoleate and its 9-hydroperoxide were converted by bulb and leaf preparations into three ketols: (12*Z*)-9-hydroxy-10-oxo-12-octadecadienoic acid (α -ketol), (11*E*)-10-oxo-13-hydroxy-11-octadecadienoic acid (γ -ketol) and a novel compound, (12*Z*)-10-oxo-11-hydroxy-12-octadecadienoic acid (10,11-ketol), in the approximate molar proportions of 10:3:1. The corresponding 15,16-dehydro α - and γ -ketols were the main metabolites of [1-¹⁴C]linolenate and its 9-hydroperoxide. Thus bulbs and leaves possessed 9-lipoxygenase and allene oxide synthase activities. Incubations with flower preparations gave α -ketol hydro(pero)xides as predominant metabolites. Bulb and leaf preparations possessed a novel enzyme activity, γ -ketol reductase, which reduces γ -ketol to 10-oxo-13-hydroxyoctadecanoic acid (dihydro- γ -ketol) in the presence of NADH.

Exogenous linolenate 13(*S*)-hydroperoxide was converted mostly into chiral (9*S*,13*S*)-12-oxo-10-phytodienoate (99.5% optical purity) by bulb preparations, while [1-¹⁴C]linolenate was a precursor for ketols only. Thus tulip bulbs possess abundant allene oxide cyclase activity, the substrate for which is linolenate 13(*S*)-hydroperoxide, even though 13(*S*)-lipoxygenase products were not detectable in the bulbs. The majority of the cyclase activity was found in the microsomes (10⁵ g pellet). Cyclase activity was not found in the other tissues examined, but only in the bulbs. The ketol route of the lipoxygenase pathway, mediated by 9-lipoxygenase and allene oxide synthase activities, has not been detected previously in the vegetative organs of any plant species.

Key words: hydroperoxides, linoleic acid, linolenic acid, metabolism.

INTRODUCTION

Lipoxygenase (LOX; linoleate:oxygen oxidoreductase; EC 1.13.11.12) is a widespread enzyme in higher plant species. The direction of the LOX pathway in a particular plant tissue depends on the LOX regiospecificity and the presence of enzymes of fatty acid hydroperoxide metabolism [1]. The primary LOX products, fatty acid hydroperoxides, undergo further conversions, which are controlled by a few known plant enzymes. The main enzymes of hydroperoxide metabolism are hydroperoxide lyase and allene oxide synthase (AOS; formerly referred to as hydroperoxide dehydrase or dehydratase) [2]. Allene oxides, short-lived products of AOS, undergo spontaneous hydrolysis into ketols [2–4]. Some allene oxides that have a double bond in the β,γ -position toward oxirane can also undergo spontaneous [5,6] or enzymic [7,8] cyclization. For instance, 18:3 allene oxide, (9*Z*,13*S*,15*Z*)-12,13-oxido-9,11,15-octadecatrienoic acid (12,13-EOT), forms a cyclopentenone, (15*Z*)-12-oxo-10,15-phytodienoic acid (12-oxo-PDA), as the predominant product in the presence of allene oxide cyclase (AOC) [2]. On the other hand, allene oxides lacking a β,γ -double bond, such as (9*Z*,13*S*)-12,13-oxido-9,11-octadecadienoic acid (12,13-EOD), are not convertible into cyclopentenones either spontaneously [1,9] or enzymically [7,8].

The product of 12,13-EOT cyclization, 12-oxo-PDA, is a

precursor of the phytohormone 7-*iso*-jasmonic acid [10]. Jasmonate biosynthesis is often considered as the main, if not the sole, physiological destination of the plant LOX pathway. However, the plant LOX cascade includes multiple routes [1]. The present paper is concerned with detection of the ‘ketol route’ of the AOS pathway in tulip bulbs, leaves and flowers. The metabolism of linoleate and α -linolenate in these tissues, which possess 9-LOX and AOS activities, is directed entirely towards ketols and related products. Two of the detected oxylipins are new compounds.

EXPERIMENTAL

Materials

[1-¹⁴C]Linoleic acid and α -[1-¹⁴C]linolenic acid were obtained from DuPont NEN (Boston, MA, U.S.A.). Alternatively, [1-¹⁴C]linolenic acid purchased from the Institute of Applied Chemistry (St. Petersburg, Russia) was used in some of the experiments. Unlabelled linoleic acid and α -linolenic acid were obtained from Nu-Chek-Prep (Elysian, MN, U.S.A.) or Fluka Chemie (Buchs, Switzerland). 1-¹⁴C-labelled (9*S*,10*E*,12*Z*)-9-hydroperoxy-10,12-octadecadienoic acid (9-HPOT), (9*S*,10*E*,12*Z*,15*Z*)-9-hydroperoxy-10,12,15-octadecatrienoic acid (9-HPOT) and (9*Z*,11*E*,13*S*,15*Z*)-13-hydroperoxy-9,11,15-

Abbreviations used: LOX, lipoxygenase; AOS, allene oxide synthase; AOC, allene oxide cyclase; RP-HPLC, reverse-phase HPLC; TMS, trimethylsilyl; 9-HPOT, (9*S*,10*E*,12*Z*)-9-hydroperoxy-10,12-octadecadienoic acid; 9-HPOT, (9*S*,10*E*,12*Z*,15*Z*)-9-hydroperoxy-10,12,15-octadecatrienoic acid; 13-HPOT, (9*Z*,11*E*,13*S*,15*Z*)-13-hydroperoxy-9,11,15-octadecatrienoic acid; 9,10-EOD, (9*S*,12*Z*)-9,10-oxido-10,12-octadecadienoic acid; 9,10-EOT, (9*S*,12*Z*,15*Z*)-9,10-oxido-10,12,15-octadecatrienoic acid; 12,13-EOD, (9*Z*,13*S*)-12,13-oxido-9,11-octadecadienoic acid; 12,13-EOT, (9*Z*,13*S*,15*Z*)-12,13-oxido-9,11,15-octadecatrienoic acid; 12-oxo-PDA, (15*Z*)-12-oxo-10,15-phytodienoic acid.

¹ To whom correspondence should be addressed (e-mail grechkin@sci.kcn.ru).

octadecatrienoic acid (13-HPOT) were synthesized as described previously [3,11]. Preparations of [$1\text{-}^{14}\text{C}$] γ -ketol and [$1\text{-}^{14}\text{C}$]-hydro(pero)xy- α -ketol were collected during the reverse-phase HPLC (RP-HPLC) separation of products of [$1\text{-}^{14}\text{C}$]9-HPOD with a tulip leaf extract (as described below). [$1\text{-}^{14}\text{C}$]12-Oxo-PDA was obtained by incubation of [$1\text{-}^{14}\text{C}$]13-HPOT with a fresh extract of tulip bulbs (as described below) and purified by RP-HPLC. Tulip (*Tulipa gesneriana* L.) bulbs grown in gardens near Kazan were harvested at the end of summer. Otherwise, bulbs were purchased from vendors in The Netherlands.

Preparation of cell-free preparations, and their incubation with [$1\text{-}^{14}\text{C}$]linoleic acid and α -[$1\text{-}^{14}\text{C}$]linolenic acid and their 9-hydroperoxides

Tulip bulbs (10 g) were homogenized in 25 ml of Tris/HCl buffer (pH 7.4) after removing the scales at 0–4 °C. The homogenate was filtered through two layers of cheesecloth. Incubations with the filtrate were performed within 30 min at 23 °C, starting with the addition of 42 kBq of [$1\text{-}^{14}\text{C}$]linoleate or α -[$1\text{-}^{14}\text{C}$]linoleate (specific radioactivity 2.8 MBq/mmol) dissolved in 50 μ l of ethanol. Preparations from leaves and flower petals were prepared and incubations with them were performed under the same conditions. The same preparation methods and the same reaction conditions were used for incubations with [$1\text{-}^{14}\text{C}$]9-HPOD and [$1\text{-}^{14}\text{C}$]9-HPOT (both with a specific radioactivity of 5.7 MBq/mmol).

Preparation of subcellular fractions and their incubation with [$1\text{-}^{14}\text{C}$]13-HPOT

Tulip bulbs (30 g) were homogenized in 15 ml of 50 mM Tris/HCl buffer, pH 7.4. The filtrate of the homogenate was centrifuged at 800 *g* for 5 min and the supernatant was centrifuged again at 9300 *g* for 16 min. The resulting supernatant was centrifuged at 105000 *g* for 60 min. The pellets from the 800 *g*, 9300 *g* and 105000 *g* centrifugations were resuspended in 9 ml of the same buffer. These suspensions (3 ml each) and 3 ml of the 105000 *g* supernatant were incubated with 1.8 kBq of [$1\text{-}^{14}\text{C}$]13-HPOT (5.7 MBq/mmol) for 15 min.

Preparation of γ -ketol reductase and studies of its substrate specificity

Tulip leaves (10 g) were homogenized with an Ultra-Turrax homogenizer in 30 ml of 50 mM Tris/HCl buffer, pH 7.4, and filtered through two layers of cheesecloth. The filtrate was centrifuged at 9300 *g* for 16 min, and the resulting supernatant was centrifuged at 105000 *g* for 1 h. The supernatant was decanted and $(\text{NH}_4)_2\text{SO}_4$ was added to 70% saturation. After centrifugation at 9300 *g* for 16 min, the resulting pellet was stored in a freezer until the time of the experiment. The frozen pellet was then dissolved in 15 ml of 50 mM Tris/HCl buffer, pH 7.4. Equal parts of enzyme solution (3 ml) were incubated at 23 °C for 30 min with 150 nmol of one of the following five substrates: [$1\text{-}^{14}\text{C}$] γ -ketol [(11*E*)-10-oxo-13-hydroxy-11-octadecenoic acid] (compound II in Figure 1), [$1\text{-}^{14}\text{C}$]hydro(pero)xy- α -ketol (III), [$1\text{-}^{14}\text{C}$]12-oxo-PDA (VII), (10*E*,12*Z*)-9-oxo-10,12-[$1\text{-}^{14}\text{C}$]octadecadienoic acid or (9*Z*,11*E*)-13-oxo-9,11-[$1\text{-}^{14}\text{C}$]octadecadienoic acid. All substrates had a specific radioactivity of 2.89 MBq/mmol. Incubations were stopped by the addition of 3 ml of ethanol and products were extracted as described below. Products were analysed by HPLC (with radio-detection or UV detection at 210 nm) and by GC-MS.

Extraction of products

The incubation mixtures were diluted with 1 vol. of distilled water, acidified to pH 4 and extracted twice with diethyl ether. The combined ether phases were washed with water, concentrated in a rotary evaporator and then taken to dryness under a stream of argon. The dry residue was dissolved in 3 ml of chloroform/propan-2-ol (2:1, v/v), then passed through a Supelclean NH_2 cartridge. The cartridge was washed with 7 ml of the same solvent, and the acidic fraction was eluted with 10 ml of ethyl acetate/acetic acid (98:2, v/v). The acidic fraction was taken to dryness under a stream of argon. The dry residue was dissolved in the mobile phase for subsequent HPLC analyses.

Separation of products

Products were separated as free acids either by isocratic RP-radio-HPLC on a Macherey-Nagel Nucleosil 5 ODS column (250 mm $\times$ 4.6 mm; Macherey-Nagel, Duren, Germany), with a solvent mixture of acetonitrile/water/acetic acid (50:50:0.01, by vol.) at a flow rate of 1 ml/min, with detection with BetaRam liquid scintillation detector (IN/US Systems, Tampa, FL, U.S.A.) and a Spectromonitor III ultraviolet detector (LDC, Riviera Beach, FL, U.S.A.). Alternatively, free acids were separated by gradient RP-HPLC on an Octadecyl-Daltosil 100 column (5 μ m; 4.6 mm $\times$ 250 mm; Serva Feinbiochemica, Heidelberg, Germany), using an elution solvent mixture of methanol/water/acetic acid (linear gradient from 60:40:0.1 to 96:4:0.1, by vol., for 55 min, then a further 25 min under isocratic conditions), at a flow rate of 0.4 ml/min. Radioactivity in the eluate was detected with an HPLC radiomonitor model 171 (Beckman Instruments, Fullerton, CA, U.S.A.) with a solid scintillator cell (125 μ l). Fractions collected during the RP-HPLC were finally purified on a normal-phase Separon SIX column (5 μ m; 3.2 mm $\times$ 150 mm; Tessek, Prague, Czech Republic) with isocratic elution using the solvent mixture hexane/propan-2-ol/acetic acid (98.5:1.5:0.1, by vol.), at a flow rate of 0.4 ml/min.

Derivatization methods

Portions of total samples or separate fractions after HPLC analyses were methylated with ethereal diazomethane. For GC-MS analyses, methylated samples were treated with a mixture of trimethylchlorosilane/hexamethyldisilazane/pyridine (1:1:1, by vol.) at 23 °C for 30 min. After evaporation of the silylating reagents and pyridine *in vacuo*, the dry residue was dissolved in hexane.

Elucidation of the chirality of 12-oxo-PDA

12-Oxo-PDA was treated as described previously [12], in several stages: (1) alkaline isomerization to the *trans* isomer; (2) reduction with sodium borohydride; (3) methylation with ethereal diazomethane; and (4) catalytic hydrogenation. The products of these procedures, a mixture of the *erythro* and *threo* isomers of *trans*-12-hydroxyphytonic acid (methyl esters), were separated by TLC, using as the mobile phase hexane/ethyl acetate (3:1, v/v). The zone of the *erythro* isomer (R_f 0.51) was scraped from the plate and eluted from the silica gel with ethyl acetate. After evaporation of solvent, the substance was converted into its (–)-menthoxycarbonyl derivative by treatment with (–)-menthyl-

Figure 1 Structural formulae of tulip oxylipins and their derivatives

Compounds: **(I)** α -ketol (18:2), (12Z)-9-hydroxy-10-oxo-12-octadecenoic acid; **(Ia)** α -ketol (18:3), (12Z,15Z)-9-hydroxy-10-oxo-12,15-octadecadienoic acid; **(II)** γ -ketol (18:2), (11E)-10-oxo-13-hydroxy-11-octadecenoic acid; **(IIa)** γ -ketol (18:3), (11E,15Z)-10-oxo-13-hydroxy-11,15-octadecadienoic acid; **(III)** hydro(peroxy)- α -ketol (18:2), (11E)-9-hydroxy-10-oxo-13-hydro(peroxy)-11-octadecenoic acid; **(IIIa)** hydro(peroxy)- α -ketol (18:3), (11E,15Z)-9-hydroxy-10-oxo-13-hydro(peroxy)-11,15-octadecadienoic acid; **(IV)** α -ketol (18:2) methyl ester methoxime; **(V)** 10,11-ketol, (12Z)-10-oxo-11-hydroxy-12-octadecenoic acid; **(VI)** 10,11-ketol methyl ester methoxime; **(VII)** (9S,13S,15Z)-12-oxo-10,15-phytodienoic acid (12-oxo-PDA); **(VIII)** dihydro- γ -ketol, 10-oxo-13-hydroxyoctadecanoic acid; **(IX)** dihydro- γ -ketol, (15Z)-9-hydroxy-12-oxo-15-octadecenoic acid.

chloroformate/toluene/pyridine (10:10:3, by vol.) at 85 °C for 3 h. Steric analyses of the (–)-menthoxycarbonyl derivative were performed by GC with a Hewlett-Packard (Avondale, PA, U.S.A.) chromatograph equipped with a DB-210 capillary column (25 m; film thickness 0.33 μ m) at 190 °C, with a helium flow rate of 36 cm/s.

Spectral studies

During HPLC analyses, UV topograms were recorded on-line with an RSD 2140 diode array detector (LKB, Bromma, Sweden). Acquisition of spectral data was performed using Wavescan EG software (LKB). Alternatively, UV spectra of purified products were recorded with a Hitachi U2000 spectrophotometer. The chemical-ionization mass spectra of purified compounds were recorded with a Finnigan MAT 212 instrument (isobutane as reagent gas; direct sample insertion). The products, as methyl esters of trimethylsilyl (TMS) ether derivatives, were analysed by GC-MS with a Hewlett-Packard model 5970B mass-selective detector connected to a Hewlett-Packard model 5890 gas chromatograph equipped with a methyl silicone fused capillary column (length 12 m; film thickness 0.33 μ m). Helium at a flow rate of 32 cm/s was used as the carrier gas. Injections were made in the split mode using an initial column temperature of 120 °C, which was raised to 240 °C at 10 °C/min.

RESULTS

Metabolism of [1-¹⁴C]linoleic acid and [1-¹⁴C]9-HPOD in tulip bulbs, leaves and flowers

As shown in Figure 2(A), two main products (**I** and **II**; see Figure 1) were formed after a 15 min incubation of [1-¹⁴C]linoleate with a tulip bulb extract. Compounds (**I**) and (**II**) have $\lambda_{\max}$ values of 205 nm and 225 nm respectively. Prolonged (60 min) incubations were accompanied by a decline of radioactivity in product (**I**) and an increase of label incorporation into metabolite (**III**) ($\lambda_{\max}$ at 229 nm). Compounds (**I**)–(**III**) were separated by RP-HPLC. Final purification of products (**I**)–(**III**) was performed by normal-phase HPLC as described below. Incubation of bulbs with [1-¹⁴C]9-HPOD led to the formation of the same products (**I**)–(**III**) in similar proportions (results not illustrated).

Similar patterns of the same metabolites (**I**)–(**III**) were found after incubation of [1-¹⁴C]linoleate with a leaf extract (Figure 2B). These same products were also formed after *in vitro* incubations with a flower extract (Figure 2C), but in this case the label was incorporated mainly into metabolite (**III**) (Figure 2C).

Compound (**I**), identified as (12Z)-9-hydroxy-10-oxo-12-octadecenoic acid (α -ketol), is known as a major product of AOS [1]. The following prominent ions were detected in the electron-impact mass spectrum (Figure 3A) of the methyl ester, TMS derivative of compound **I**: m/z [ion attribution] (relative intensity,

Figure 2 Separation of *in vitro* biosynthesized tulip oxylipins by RP-radio-HPLC

Products of [$1\text{-}^{14}\text{C}$]linoleate metabolism by tulip bulb (A), leaf (B) and flower (C) extracts are shown; (D) shows products of α -[$1\text{-}^{14}\text{C}$]linolenate metabolism by tulip leaf extract. Details of experimental conditions are described in the Experimental section. CPM = c.p.m.

%): 383 [$M\text{-CH}_3$] $^+$ (3); 367 [$M\text{-CH}_3\text{O}$] $^+$ (1); 259 [$M\text{-C10/C18}$] $^+$ (100); 227 [$259\text{-CH}_3\text{OH}$] $^+$ (2); 155 [$259\text{-CH}_3\text{O-Si(CH}_3)_3$] $^+$ (34); 109 [$M\text{-259-CH}_2\text{O}$] $^+$ (19); 73 [$\text{Si(CH}_3)_3$] $^+$ (53). After reaction of the methyl ester, TMS derivative of compound (I) with methoxyamine, the resulting methoxime (compound IV) possessed the following main fragmentation patterns on GC-MS analysis: m/z [ion attribution] (relative intensity, %): 427 [M] $^+$ (2); 412 [$M\text{-CH}_3$] $^+$ (19); 396 [$M\text{-CH}_3\text{O}$] $^+$ (14); 306 [396-TMSOH] $^+$ (14); 271 [$M\text{-C1/C8+H}$] $^+$ (66); 270 [$M\text{-C1/C8}$] $^+$ (41); 259 [$M\text{-C10/C18}$] $^+$ (23); 240 [$271\text{-CH}_3\text{O}$] $^+$ (39); 155 [$259\text{-CH}_3\text{O-Si(CH}_3)_3$] $^+$ (40); 73 [$\text{Si(CH}_3)_3$] $^+$ (100). Reduction of compound (I) with sodium borohydride, followed by treatment of the product with lead tetra-acetate, gave a single labelled product. This compound was identified as 9-oxononanoic acid by chemical-ionization MS (isobutane as the reagent gas); m/z (element composition; relative intensity, %): 173 ($\text{C}_9\text{H}_{17}\text{O}_3$) [$M\text{+H}$] $^+$ (100). This result indicated the presence of a 9-hydroxy-10-oxo function in product (I). Thus the obtained spectral data for compound (I) and its methoxime derivative (IV) allow us to assign the structure (12*Z*)-9-hydroxy-10-oxo-12-octadecenoic acid to product (I).

GC-MS analysis of the α -ketol fraction (I) allowed us to detect, along with the major component (I), a minor product (compound V). The electron-impact mass spectrum of its TMS ether, methyl ester derivative (Figure 3C) possessed the following most prominent ions: m/z [ion attribution] (relative intensity, %): 383 [$M\text{-CH}_3$] $^+$ (4); 367 [$M\text{-CH}_3\text{O}$] $^+$ (2); 277 [$M\text{-CH}_3\text{O-TMSOH}$] $^+$ (1); 199 [$M\text{-C1/C10}$] $^+$ (100); 129 [$M\text{-C14/C18+H}$] $^+$ (44); 73 [$\text{Si(CH}_3)_3$] $^+$ (54). Upon further treatment with methoxyamine, the compound was converted

into its methoxime, TMS ether, methyl ester derivative (VI). The following ions were the most prominent in the electron-impact mass spectrum of compound (VI): m/z [ion attribution] (relative intensity, %): 427 [M] $^+$ (5); 412 [$M\text{-CH}_3$] $^+$ (9); 396 [$M\text{-CH}_3\text{O}$] $^+$ (8); 339 [$M\text{-TMSOH}$] $^+$ (4); 306 [396-TMSOH] $^+$ (9); 271 [$M\text{-C1/C8+H}$] $^+$ (66); 270 [$M\text{-C1/C8}$] $^+$ (41); 259 [$M\text{-C10/C18}$] $^+$ (23); 240 [$271\text{-CH}_3\text{O}$] $^+$ (39); 155 [$259\text{-CH}_3\text{O-Si(CH}_3)_3$] $^+$ (40); 73 [$\text{Si(CH}_3)_3$] $^+$ (100).

Product (II) collected from the RP-HPLC column was subjected to methylation and trimethylsilylation, and the resulting derivative was analysed by GC-MS. The electron-impact mass spectrum of compound (II) methyl ester TMS derivative (Figure 3B) possessed the following most prominent and characteristic ions: m/z [ion attribution] (relative intensity, %): 398 [M] $^+$ (6); 383 [$M\text{-CH}_3$] $^+$ (5); 367 [$M\text{-CH}_3\text{O}$] $^+$ (2); 351 [$M\text{-CH}_3\text{-CH}_3\text{OH}$] $^+$ (3); 327 [$M\text{-C14/C18}$] $^+$ (58); 295 [$327\text{-CH}_3\text{OH}$] $^+$ (16); 227 [$M\text{-C1/C9}$] $^+$ (3); 199 [$M\text{-C1/C10}$] $^+$ (31); 184 [199-CH_3] $^+$ (20); 73 [$\text{Si(CH}_3)_3$] $^+$ (100). The physical data obtained allowed us to identify product (II) as a γ -ketol, (11*E*)-10-oxo-13-hydroxy-11-octadecenoic acid, which was originally described by Gardner [13].

After collection from the RP-HPLC column, compound (III) was reduced with NaBH_4 . The reduction product (λ_{max} at 203 nm) was methylated with diazomethane and silylated. The resulting methyl ester TMS derivative was analysed by GC-MS. The total ion current chromatogram possessed three unequal peaks having identical mass spectra (Figure 4A): m/z [ion attribution] (relative intensity, %): 545 [$M\text{-CH}_3$] $^+$ (0.1); 460 [$545\text{-C}_5\text{H}_{11}\text{-CH}_3\text{+H}$] $^+$ (6); 439 [$M\text{-TMSOH-CH}_3\text{O}$] $^+$ (0.6); 381 [$M\text{-TMSO-TMSOH}$] $^+$ (3); 301 [$M\text{-C1/C9}$] $^+$ (1); 259

Figure 3 Electron-impact mass spectra of products (I), (II) and (V)

Shown are the spectra of the methyl ester TMS derivatives of (A) compound (I), (B) compound (II) and (C) compound (V). Abbreviation: R, relative intensity.

$[M-301]^+$ (100); 211 $[301-\text{TMSOH}]^+$ (7); 155 $[259-\text{CH}_3\text{O}-\text{Si}(\text{CH}_3)_3]^+$ (37); 73 $[\text{Si}(\text{CH}_3)_3]^+$ (86). Alternatively, metabolite (III) was reduced with NaB^2H_4 (sodium borodeuteride). The reduction product was converted into its methyl ester TMS derivative and analysed by GC-MS. Mass spectral data (Figure 4B) demonstrated incorporation of one ^2H atom at C-10: m/z [ion attribution] (relative intensity, %): 546 $[M-\text{CH}_3]^+$ (0.1); 461 $[546-\text{C}_5\text{H}_{11}-\text{CH}_3+\text{H}]^+$ (5); 440 $[M-\text{TMSOH}-\text{CH}_3\text{O}]^+$ (0.6); 382 $[M-\text{TMSO}-\text{TMSOH}]^+$ (2); 302 $[M-\text{C1/C9}]^+$ (2); 259 $[M-301]^+$ (100); 212 $[302-\text{TMSOH}]^+$ (5); 155 $[259-\text{CH}_3\text{O}-\text{Si}(\text{CH}_3)_3]^+$ (30); 73 $[\text{Si}(\text{CH}_3)_3]^+$ (71). A shift of the UV absorbance maximum after NaBH_4 reduction of compound (III) from 229 to 203 nm, as well as the incorporation of deuterium from NaB^2H_4 at C-10, proved that product (III) is a conjugated oxoene having a carbonyl group at C-10. Thus the obtained data allowed

Figure 4 Electron-impact mass spectra of derivatives of product (III)

A Spectrum of methyl ester TMS derivative obtained through (1) NaBH_4 reduction of compound (III), (2) diazomethane methylation, and (3) trimethylsilylation. **B** Spectrum of an analogous derivative of compound (III) obtained through (1) sodium borodeuteride (NaB^2H_4) reduction of compound (III), (2) diazomethane methylation, and (3) trimethylsilylation. Abbreviation: R, relative intensity. Peaks of ions containing ^2H are marked with asterisks (*).

us to identify compound (III) as (11*E*)-9-hydroxy-10-oxo-13-hydro(peroxy)-11-octadecenoic acid.

Subcellular fractions obtained by differential centrifugation of a leaf extract were incubated with $[1-^{14}\text{C}]$ linoleate. The results revealed that the main part of AOS activity is localized in the microsomes ($10^5 g$ pellet). Similar experiments were performed with a bulb extract; however, the exceptionally high level of AOS activity in bulbs did not allow us to determine the enzyme localization. The substrate was completely converted into products (mostly α -ketol) in all particle fractions and in the $10^5 g$ supernatant.

Detection of γ -ketol reductase and dihydro- γ -ketol

The minor metabolite (compound VIII) eluting after γ -ketol was detected during the RP-radio-HPLC analyses of extracts obtained after incubation of $[1-^{14}\text{C}]$ linoleate and $[1-^{14}\text{C}]$ 9-HPOD with bulbs and leaves. Incorporation of label into this peak was greatly increased after addition of NADH to the incubation mixtures. Compound (VIII), having a featureless UV spectrum, possessed the following main fragmentation patterns during its GC-MS analysis: m/z [ion attribution] (relative intensity, %): 385 $[M-\text{CH}_3]^+$ (33); 369 $[M-\text{CH}_3\text{O}]^+$ (5); 329 $[M-\text{C14/C18}]^+$ (69); 311 $[M-\text{TMSO}]^+$ (5); 310 $[M-\text{TMSOH}]^+$ (4); 279 $[310-\text{CH}_3\text{O}]^+$ (9); 239 $[311-\text{TMSOH}]^+$ (13); 207 $[239-\text{CH}_3\text{OH}]^+$

Table 1 ^1H -NMR spectrum of compound (VIII)NMR details were: 250 MHz; $[\text{D}_2]\text{chloroform}$; 298 K.

Proton (number of protons)	δ (p.p.m.)	Multiplicity	Coupling constant (Hz)
H2 (2)	2.32	t	7.4 (H3)
H3 (2)	1.52–1.73	m	
H4/H8 (10)	1.21–1.39	m	
H9 (2)	2.43	t	7.1 (H8)
H11 (2)	2.59	t	6.0 (H12)
H12 (2)	1.52–1.73	m	
H13 (1)	3.59	m	
H14 (2)	1.52–1.73	m	
H15/H17 (6)	1.21–1.39	m	
H18 (3)	0.89	t	6.4 (H17)

(24); 187 $[M-\text{CH}_2\text{CO}(\text{CH}_2)_8\text{COOCH}_3]^+$ (8); 186 $[M-\text{CH}_2\text{CO}(\text{CH}_2)_8\text{COOCH}_3-\text{H}]^+$ (11); 173 $[M-\text{CH}_2\text{CH}_2\text{CO}(\text{CH}_2)_8\text{COOCH}_3]^+$ (77); 144 $[329-(\text{CH}_2)_8\text{COOCH}_3+\text{H}-\text{CH}_3]^+$ (34); 143 $[M-(\text{CH}_2)_8\text{COOCH}_3-\text{CH}_3]^+$ (34); 130 $[329-\text{CO}(\text{CH}_2)_8\text{COOCH}_3]^+$ (51); 73 $[\text{Si}(\text{CH}_3)_3]^+$ (100). The ^1H -NMR spectrum of metabolite (VIII) is presented in Table 1. NMR data revealed that compound (VIII) has no double bonds. The spectral data allowed the identification of compound (VIII) as 10-oxo-13-hydroxyoctadecanoic acid, i.e. dihydro- γ -ketol.

The observed stimulation of dihydro- γ -ketol (compound VIII) formation by NADH allowed us to propose that this metabolite is formed through the NADH-dependent enzymic reduction of γ -ketol. To verify this proposal, we prepared an authentic γ -ketol, (11*E*)-10-oxo-13-hydroxy-11-octadecenoic acid (compound II), and incubated it in the presence of NADH with a 9300 g supernatant of tulip leaf extract (Figure 5A). This allowed us to detect a substantial yield of dihydro- γ -ketol. No reduction was observed during a similar incubation in the absence of NADH (Figure 5B).

To study the substrate specificity of the enzyme, we prepared a number of oxylipins having an α,β -unsaturated ketone function: γ -ketol $\{[1-^{14}\text{C}](11E)\text{-10-oxo-13-hydroxy-11-octadecenoic acid}\}$ (compound II); $[1-^{14}\text{C}]\text{hydro(pero)xy-}\alpha$ -ketol (compound III); $[1-^{14}\text{C}]\text{12-oxo-PDA}$ (compound VII), $[1-^{14}\text{C}](10E,12Z)\text{-9-oxo-10,12-octadecadienoic acid}$ and $[1-^{14}\text{C}](9Z,11E)\text{-13-oxo-9,11-octadecadienoic acid}$. These substrates were incubated in the presence of NADH with an enzyme preparation obtained from the 10^5 g supernatant fraction from tulip leaf extract. The results allowed us to establish that the last two named compounds, which are oxodienes, do not serve as substrates of the reductase. 12-Oxo-PDA (compound VII) was reduced to a small extent (11 % reduction). The greatest extent of reduction was observed with γ -ketol (100 % reduction) and (11*E*)-9,13-dihydroxy-10-oxo-11-octadecenoic acid (63 % reduction). Thus the reductase possesses a high degree of substrate specificity. The very small amount of 12-oxo-PDA reduction by the tulip enzyme allows one to propose that γ -ketol reductase is distinct from 12-oxo-PDA reductase.

Another dihydro- γ -ketol, compound (IX) (featureless UV spectrum), was detected during the incubation of the 10^5 g supernatant from bulbs with 13-HPOT. The methyl ester TMS derivative of compound (IX) possessed the following main fragmentation patterns on GC-MS analysis: m/z [ion attribution] (relative intensity, %): 383 $[M-\text{CH}_3]^+$ (27); 367 $[M-\text{CH}_3\text{O}]^+$ (4); 308 $[M-\text{TMSOH}]^+$ (61); 279 $[308-\text{CH}_2\text{CH}_3]^+$ (17); 277 $[308-\text{CH}_3\text{O}]^+$ (12); 273 $[M-\text{CH}_2\text{CO}(\text{CH}_2)_2\text{CHCHCH}_2\text{CH}_3]^+$

Figure 5 RP-radio-HPLC analyses of products of γ -ketol (compound II) incubation with an enzyme preparation from the 10^5 g supernatant from tulip leaves

Shown are the products of γ -ketol conversion in the presence (A) and in the absence (B) of NADH. Details of experimental conditions are described in the Experimental section. CPM = c.p.m.; II, peak of γ -ketol; VIII, peak of dihydro- γ -ketol.

(18); 272 $[273-\text{H}]^+$ (17); 259 $[M-\text{CH}_2\text{CH}_2\text{CO}(\text{CH}_2)_2\text{CHCHCH}_2\text{CH}_3]^+$ (30); 241 $[M-(\text{CH}_2)_7\text{COOCH}_3]^+$ (70); 155 $[259-\text{TMS}-\text{CH}_3\text{O}]^+$ (27); 143 $[241-(\text{CH}_2)_2\text{CHCHCH}_2\text{CH}_3-\text{CH}_3]^+$ (29); 130 $[241-\text{CO}(\text{CH}_2)_2\text{CHCHCH}_2\text{CH}_3]^+$ (44); 73 $[\text{Si}(\text{CH}_3)_3]^+$ (100). The following most abundant ions were present in the electron-impact mass spectrum of compound (IX) methyl ester: m/z [ion attribution] (relative intensity, %): 308 $[M-\text{H}_2\text{O}]^+$ (100); 279 $[308-\text{C17/C18}]^+$ (16); 277 $[308-\text{CH}_3\text{O}]^+$ (27); 253 $[308-\text{C15/C18}]^+$ (10); 240 $[308-\text{C14/C18}+\text{H}]^+$ (26); 239 $[308-\text{C14/C18}]^+$ (6); 235 $[253-\text{H}_2\text{O}]^+$ (10); 222 $[253-\text{CH}_3\text{O}]^+$ (14); 208 $[240-\text{CH}_3\text{OH}]^+$ (26); 207 $[239-\text{CH}_3\text{OH}]^+$ (14); 183 $[308-\text{C11/C18}]^+$ (16); 182 $[183-\text{H}]^+$ (17); 165 $[308-\text{C12/C18}-\text{CH}_3\text{OH}]^+$ (36); 151 $[183-\text{CH}_3\text{OH}]^+$ (47); 150 $[182-\text{CH}_3\text{OH}]^+$ (57). The TMS ester derivative of compound (IX) possessed the following spectral data on GC-MS analysis: m/z [ion attribution] (relative intensity, %): 456 $[M]^+$ (13); 441 $[M-\text{CH}_3]^+$ (6); 366 $[M-\text{TMSOH}]^+$ (28); 351 $[441-\text{TMSOH}]^+$ (37); 331 $[M-\text{CH}_2\text{CO}(\text{CH}_2)_2\text{CHCHCH}_2\text{CH}_3]^+$ (28); 317 $[M-\text{CH}_2\text{CH}_2\text{CO}(\text{CH}_2)_2\text{CHCHCH}_2\text{CH}_3]^+$ (38); 298 $[M-\text{C13/C18}-\text{TMSOH}+\text{H}]^+$ (12); 241 $[M-(\text{CH}_2)_7\text{COOTMS}]^+$ (46); 229 $[331-\text{CH}_3-\text{TMSO}]^+$ (44); 73 $[\text{Si}(\text{CH}_3)_3]^+$ (100). The data allowed us to identify compound (IX) as (15*Z*)-9-hydroxy-12-oxo-15-octadecenoic acid.

Table 2 Substrate specificity of AOC from tulip bulbs: formation of cyclopentenones and α -ketols from fatty acid hydroperoxides via allene oxides

ND, not detected.

Substrate	Labelling of products (% of total radioactivity)	
	α -Ketol	Cyclopentenone
13-HPOT	6.45	77.6
13-HPOD	60.9	ND
9-HPOT	64.1	ND
9-HPOD	75.3	ND

Table 3 Distribution of AOC activity in fresh homogenate and subcellular fractions from tulip bulbs

The yield of 12-oxo-PDA related to enzymic synthesis; n.d., not determined.

Preparation used for incubation	Yield of 12-oxo-PDA (μ g/mg of protein)	12-Oxo-PDA/ α -ketol ratio
Fresh homogenate	n.d.	12.03
800 g pellet	1.14	0.44
9000 g pellet	1.59	0.62
105000 g pellet	9.64	1.85
105000 g supernatant	0.43	0.53

Metabolism of α -[1- 14 C]linolenic acid and its hydroperoxides

Exogenous α -[1- 14 C]linolenic acid was also converted rapidly into products during the incubation with tulip leaf (Figure 2D) and bulb extracts. Profiles of leaf (Figure 2D) and bulb (results not shown) products were similar to those observed in the case of linoleic acid conversion (Figure 2B). The spectral data (not shown) allowed us to identify linolenate metabolites (**Ia**)–(**IIIa**) as (12Z,15Z)-9-hydroxy-10-oxo-12,15-octadecadienoic acid (α -ketol), (11E,15Z)-10-oxo-13-hydroxy-10,15-octadecadienoic acid (γ -ketol) and (11E,15Z)-9-hydroxy-10-oxo-13-hydro(peroxy)-11,15-octadecadienoic acid respectively. The same metabolites were detected after the incubation of leaf extract with [1- 14 C]9-HPOT.

Incubations of [1- 14 C]13-HPOT with a filtrate of bulb homogenate led to two main products being formed, in the approximate molar proportions of 1:12. These compounds were identified as (9Z,15Z)-12-oxo-13-hydroxy-9,15-octadecadienoic acid (α -ketol) and 12-oxo-PDA (compound **VII**) respectively (Table 2). Physical data for these metabolites are not shown, since they were described previously. Thus, while incubations with linolenic acid and 9-HPOT afforded only ketols and no cyclopentenone, incubations with 13-HPOT resulted in the formation of 12-oxo-PDA as the predominant product (Table 2).

Steric analysis revealed that 12-oxo-PDA, resulting from incubation of 13-HPOT with fresh tulip bulb homogenate, was composed of 99.5% optically pure (9S,13S) epimer. Thus the results demonstrate that tulip bulbs exhibit high AOC activity, which possesses strong stereospecificity. The activity of AOS, which was unprecedentedly high in fresh homogenates, decreased progressively during the course of cell fractionation. Incubations of 13-HPOT with subcellular fractions revealed that the main part of the enzyme activity was associated with microsomes (Table 3).

DISCUSSION

The results of the present work demonstrate that the LOX pathway in tulip bulbs and leaves operates through the 9-LOX and AOS enzyme activities. Tulip is the second plant species that has been shown to possess predominantly 9-LOX activity in its leaves, along with the recently characterized potato leaf pathway [14]. All other plants that have been characterized previously exhibit exclusively or mainly 13-LOX in their leaves. 9-LOX products were detected in the air-dried leaves of *Glechoma hederacea* L. [15,16] and some other Labiatae [16]. However, the LOX activity in *G. hederacea* was not studied or characterized, and thus the ratio of 13-LOX and 9-LOX activities in *G. hederacea* leaves is unknown. One cannot exclude the possibility that 9-LOX activity is expressed specifically in *G. hederacea* leaves during the aging. Interestingly, 9-HOD, which is present in *G. hederacea* leaves, is a potent stimulator of platelet adenylate cyclase activity [15].

Jasmonic acid biosynthesis is often considered as the main, if not the sole, destination of the plant LOX pathway, particularly of the AOS route. Jasmonic acid is, undoubtedly, the best characterized of plant oxylipins with respect to its physiological activity. Vick [17] proposed that ketols are not formed in significant amounts (compared with 12-oxo-PDA) during normal *in vivo* metabolism in unstressed plants. However, some exceptions are known, such as maize germ [13,18], which possesses 9-LOX and AOS activities. Thus the LOX-dependent metabolism in maize germ is directed towards ketols. The results of the present study demonstrate that this pathway also operates in tulip bulbs, leaves and flowers.

Being under the control of 9-LOX and AOS activities, the metabolism of linoleate and linolenate in tulip leaves takes place with intermediate formation of the allene oxides (9S,12Z)-9,10-oxido-10,12-octadecadienoic acid (9,10-EOD) and (9S,12Z,15Z)-9,10-oxido-10,12,15-octadecatrienoic acid (9,10-EOT) respectively. Neither 9,10-EOD nor 9,10-EOT undergoes annulation into cyclopentenones, either spontaneously or enzymically [1]. 9,10-EOD (Scheme 1) and 9,10-EOT are only hydrolysed into the corresponding α - and γ -ketols. A third minor ketol, (12Z)-10-oxo-11-hydroxy-12-octadecenoic acid, was detected during the studies of [1- 14 C]linoleate conversion. Formation of a similar ketol, (9Z,15Z)-11-hydroxy-12-oxo-9,15-octadecadienoic acid, from 13-HPOT by flaxseed AOS has been detected previously [3,4].

As mentioned above, fresh bulb extracts possessed both AOC and AOS activities, converting exogenous 13-HPOT into (9S,13S,15Z)-12-oxo-PDA and α -ketol in a ratio of 12:1. Previously characterized AOCs from various species [7,8,19] yielded a ratio of 3:1 or less. Tulip AOC also exhibits the highest stereospecificity yet observed among AOCs from various species. Fractionation of a bulb homogenate by differential centrifugation revealed that the main part of the AOC activity is localized in the microsomes (Table 3). In this respect, tulip AOC appears not to be identical with previously described plant AOCs, which are soluble enzymes [7,8,19].

Tulip AOC possesses marked substrate specificity. Only 13-HPOT is converted into a cyclopentenone product (Table 2). In contrast, neither 13-HPOD nor 9-HPOD produces any cyclopentenones in the presence of tulip AOC (Table 2). This result is consistent with existing data [8,19,20].

The physiological properties of most plant oxylipins are much less well studied than those of the jasmonates. It seems logical to assume that ketols, being the predominant LOX products in such tissues as tulip bulbs and maize seeds, must have a physiological role. As shown previously, the allyl ketone function of α -ketols

Scheme 1 Linoleic acid metabolism through the LOX pathway in tulip (*Tulipa gesneriana*)

See the text for details.

is oxidized by LOXs, affording hydroperoxides [11]. The predominance of ketodiols over α -ketol hydroperoxides observed in the present work is, apparently, explained by the occurrence of hydroperoxidase activity in tulip bulbs. Abian et al. [18] detected and identified these products as ketodiols, similar to compound (III). The formation of ketodiols and γ -ketols in maize germ was dramatically stimulated by abscisic acid [18]. Thus it seems highly probable that ketols and their metabolites may be involved in cell signalling. Recent data [21] have demonstrated that α -ketol (compound Ia) is an endogenous flowering factor in *Lemna paucicostata*. γ -Ketol [(11*E*)-10-oxo-13-hydroxy-10-octadecenoic acid] [22] and the related macrolactone (11*E*)-10-oxo-11-octadecen-13-olide, isolated from maize seeds, exhibit strong cytostatic activity [23,24]. One can propose in this connection that reduction of γ -ketols and related oxylipins by γ -ketol reductase may have physiological importance as an intracellular mechanism for detoxification of these metabolites.

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