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Biosynthesis of 14,15-dehydro-12-oxo-phytodienoic acid and related cyclopentenones via the phytoprostane D_1 pathway

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Dedicated to Meinhart H. Zenk on the occasion of his 70th birthday

Abstract

A novel group of cyclopentenone prostaglandin-like compounds, deoxy phytoprostanes J_1, together with their precursors, phytoprostanes D_1, were identified in tobacco, tomato and *Arabidopsis*. Previously, it was thought that 14,15-dehydro-12-oxo-phytodienoic acid, a member of the deoxy phytoprostanes J_1 family, is derived from either 12-oxo-phytodienoic acid or diketols via the allene oxide synthase pathway. Results suggest that 14,15-dehydro-12-oxo-phytodienoic acid as well as structurally related cyclopentenones of the chromomoric acid family are synthesized via the phytoprostane D_1 pathway in planta. Notably, 14,15-dehydro-12-oxo-phytodienoic acid is more abundant than 12-oxo-phytodienoic acid in all three species so far analyzed.

Keywords: Cross conjugated cyclopentenone; Isoprostane; Prostaglandin; Deoxy phytoprostane J_1; 12-Oxo-phytodienoic acid; 14,15-Dehydro-12-oxo-phytodienoic acid; Chromomoric acid; Jasmonate

1. Introduction

In 1981–1982 Bohlmann and co-workers discovered a novel group of prostaglandin-like octadecanoids in *Chromolaena* species (compounds **1**, **2** and **3**, Fig. 1) that are characterized by a cyclopentenone ring system (Bohlmann et al., 1981; Bohlmann et al., 1982a, b). Originally it was proposed that compound **1**, termed chomomoric acid, as well as compounds **2** and **3** are derived from prostaglandin G-like compounds since prostaglandin G is a common precursor for all prostanoids in animals (Bohlmann et al., 1982a). However, a cyclooxygenase enzyme activity which is essential for the formation of prostaglandin G in animals has not yet been identified in plants. In contrast to animals, plants utilize α-linolenic acid to synthesize prostaglandin-like compounds of the jasmonate family via the allene oxide synthase pathway (Mueller, 1997). Hydroxylation of the first cyclic fatty acid of this pathway, 12-oxo-phytodienoic acid (OPDA), at position 12 would directly yield chromomoric acid (Fig. 1). For this reason, it has been assumed that compound **1** (chromomoric acid), **2** and **3** are derived from OPDA (Grechkin, 1995). However, all attempts to convert OPDA into any of the compounds **1** to **3** were unsuccessful (Grechkin, 1995). Therefore, an alternative biosynthetic route to **1** has been proposed which starts from α-ketol (Fig. 1), another product of the allene oxide synthase pathway (Grechkin, 1995, 1998). α- as well as the γ-diketones have been implicated as intermediates in this pathway. However, these diketones have not been found in nature and enzymes catalyzing the proposed reactions downstream of allene oxide have also not yet been identified.

Recently, we have discovered that a series of octadecanoids with characteristic prostaglandin ring systems may be formed via the dinor isoprostanoid pathway in plants. Isoprostanoids are products of free radical catalyzed oxidation of polyunsaturated fatty acids and represent a family of prostaglandin isomers. They are classified according to a nomenclature system that conforms with prostaglandin convention (Rokach et al., 1997). Dinor isoprostanes derived from α-linolenic acid have been termed phytoprostanes in order to distinguish

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them from γ-linolenic acid derived dinor isoprostanes in animals (Imbusch and Mueller, 2000a, b). The phytoprostane pathway in plants [see (Mueller, 1998) for review] is initiated by free radical attack of α-linolenic acid yielding a linolenate radical that readily oxidizes and cyclizes to two regioisomeric, prostaglandin G-like compounds (Fig. 2) termed phytoprostanes G_1 type I and II (PPG_1-I and PPG_1-II) in vitro (O'Connor et al., 1981, 1984). G-ring prostanoids rapidly rearrange to D- and E-ring compounds in aqueous environment (Hamberg et al., 1974; Morrow et al., 1994). In vivo, PPG_1 may be either reduced to phytoprostanes F_1 (PPF_1) or converted to phytoprostanes E_1 (PPE_1). Non-enzymatically formed PPE_1 and PPF_1 were detected virtually in every plant species so far investigated (Parchmann and Mueller, 1998; Imbusch and Mueller, 2000a, b). Since PPE_1 occur apparently ubiquitously in plants, it is likely that nonenzymatic phytoprostane D_1 (PPD_1) formation takes also place in planta. PPD_1 have not been characterized yet, possibly because they are unstable molecules that readily dehydrate to deoxy phytoprostanes J_1 type I and II ($dPPJ_1$-I and $dPPJ_1$-II). Dehydration of D-ring prostanoids occurs apparently rapidly both in vitro (Maxey et al., 2000) and in mammals in vivo (Shibata et al., 2002). However, $dPPJ_1$-I is identical with 14,15-dehydro-OPDA or compound **2**, discovered 20 years ago by Bohlmanns group in *Chromolaena* species (Bohlmann et al., 1982a, b). We therefore hypothesized that the originally proposed biosynthesis of **2** and related compounds via bicyclic endoperoxide, prostaglandin G-like intermediates as proposed by Bohlmann (1981) is indeed correct. Our hypothesis predicts that compound **2** is an ubiquitously occurring compound common to all plants and therefore not limited to *Chromolaena* species. Moreover, if compound **2** ($dPPJ_1$-I) is formed via the phytoprostane pathway, an isomer of **2** must exist that has the structure of $dPPJ_1$-II. In addition, the precursors PPD_1-I and

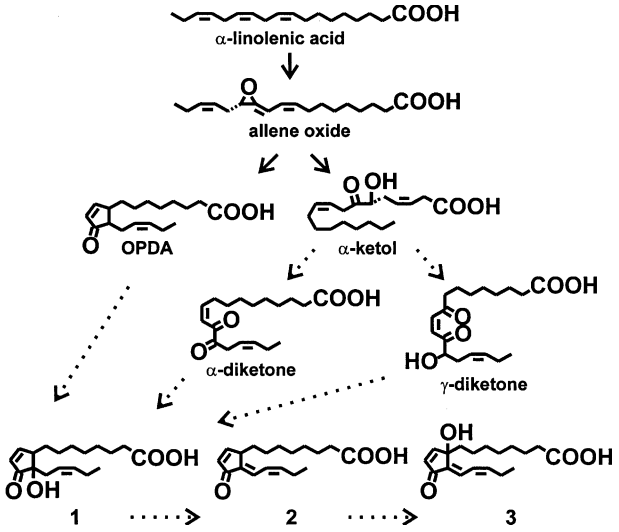

Fig. 1. Hypotheses for the formation of chromomoric acid **1** and the related compounds **2** and **3**. Biosynthesis of 12-oxo-phytodienoic acid (OPDA) and α-ketol from α-linolenate has been well established and occurs probably ubiquitously in plants (solid arrows) (Mueller, 1997). Three pathways starting from allene oxide metabolites have been suggested for the formation of **1**, **2** and **3** (broken arrows) in *Chromolaena* species. In one of these, OPDA is hydroxylated to chromomoric acid **1** which in turn is dehydrated to yield **2** which is further hydroxylated to form **3** (Bohlmann et al., 1981; Grechkin, 1995). Alternatively, α- or γ-diketones formed from α-ketol may be converted to **1**, **2** and **3** (Grechkin, 1998). However, neither one of the diketones nor enzymes catalyzing one of the postulated reactions (broken arrows) have been identified yet.

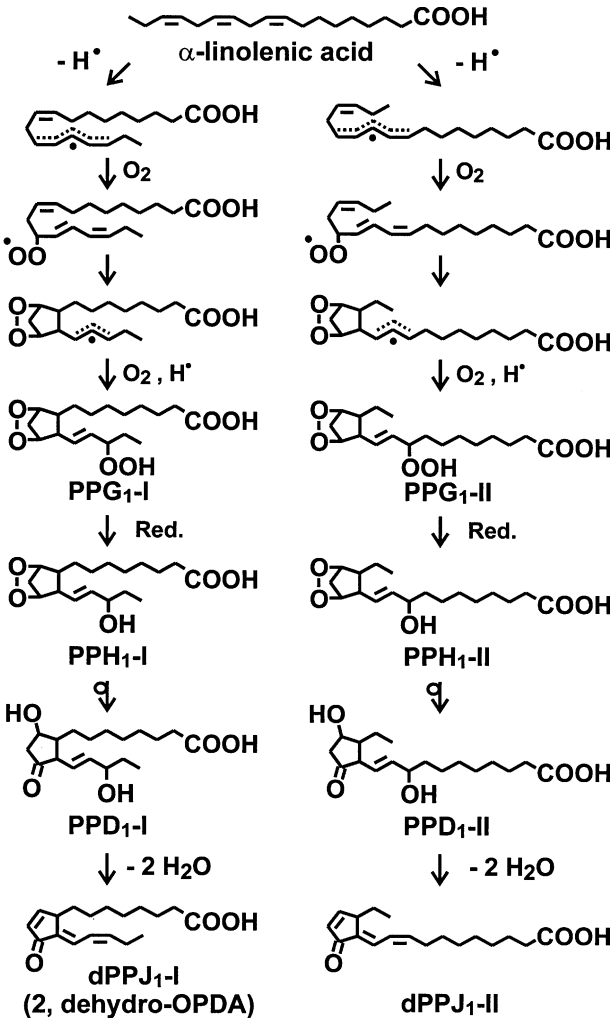

Fig. 2. Postulated phytoprostane D_1 pathway. Free radical catalyzed oxidation of linolenate yields two unstable, racemic phytoprostane G_1 regioisomers (PPG_1-I and PPG_1-II). The endoperoxide group of PPG_1 rapidly rearranges to a keto- and a hydroxy-function in aqueous environment thereby forming two regioisomeric phytoprostanes D_1 (PPD_1-I and PPD_1-II). D-ring prostanoids are prone to undergo nonenzymatic dehydration to yield deoxy J-ring compounds, both in vitro and in vivo in animals (Shibata et al., 2002). Similarly, PPD_1 may dehydrate to form two racemic, regioisomeric deoxy phytoprostanes J_1 ($dPPJ_1$-I and $dPPJ_1$-II) in plants. All geometric isomers of $dPPJ_1$ may be formed since $dPPJ_1$ are photolabile and isomerize rapidly in the daylight. One major isomer, $dPPJ_1$-I (as shown) is identical with 13,14-dehydro-12-oxo-phytodienoic acid or **2**.

PPD_1-II should also occur in nature. However, $dPPJ_1$-II as well as the PPD_1 precursors have not yet been identified in any natural source.

In order to test the phytoprostane hypothesis, a novel method for analysis of prostaglandin J_1-like compounds was developed which allowed simultanous quantification of $dPPJ_1$ and OPDA in plants. Analysis of three plant species revealed that both regioisomeric $dPPJ_1$ occur in planta at even higher levels than OPDA that is biosynthesized via the allene oxide synthase pathway. Moreover, the predicted unstable precursors of $dPPJ_1$, PPD_1-I and PPD_1-II, were also identified in all three plant species.

2. Results and discussion

2.1. Synthesis and analysis of deoxy phytoprostanes J_1

For the development of appropriate methods for PPD_1 and $dPPJ_1$ analysis, synthesis of authentic reference compounds was required. A mixture of all theoretical isomers of PPF_1, that have been isolated from autoxidized linolenate (Imbusch and Mueller, 2000a), was used as starting material. For conversion of PPF_1 into PPD_1, a chemical reaction sequence (Fig. 3) that has originally been developed for prostaglandin D_2 synthesis was employed (Nishizawa et al., 1975). The method relies on selective oxidation of one of the two ring hydroxyls of the F-ring system. However, we observed oxidation of either one of the ring hydroxyls yielding a mixture of PPD_1 and PPE_1 with an overall yield of 15 and 5%, respectively. Since PPD_1 and PPE_1 are each comprised of theoretically 32 isomers which are difficult to separate, no attempt was made to isolate single isomers. The PPD_1/PPE_1 mixture was subjected to acid catalyzed dehydration to obtain $dPPJ_1$ and deoxy phytoprostanes A_1 ($dPPA_1$). $dPPJ_1$ and $dPPA_1$ each consist of two regioisomers (type I and II) which could be separated by HPLC (Fig. 4). The UV spectra of peak I and II display absorption maxima at 227 and 309 nm. They are identical to the UV spectrum of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 indicating the presence of a cross conjugated cyclopentenone triene system (Fig. 4). In contrast, HPLC peaks III and IV have absorption maxima similar to 15-deoxy-prostaglandin A_1 (data not shown). HPLC peaks I and II ($dPPJ_1$) were analyzed as methyl ester derivatives by gas chromatography-electron impact ionization mass spectroscopy (GC-EIMS). Cross conjugated cyclopentenones such as $dPPJ_1$ or 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 ($dPGJ_2$) isomerized already in the injector port of the GC yielding a series of peaks differing with respect to their double bond geometry. However, GC-EIMS analysis of HPLC peak I and II revealed that all isomers comprised in each peak exhibit a characteristic fragmentation pattern. Fragmentation

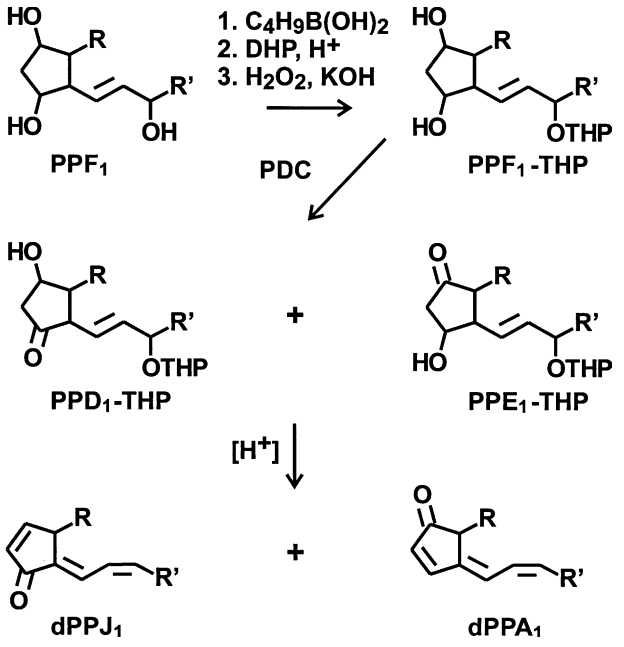

Fig. 3. Synthesis of deoxy phytoprostanes J_1. Deoxy phytoprostanes J_1 were synthesized from a racemic mixture of phytoprostanes F_1 comprising the type I ($R = C_7H_{14}COOH$, $R' = C_2H_5$) and type II ($R = C_2H_5$, $R' = C_7H_{14}COOH$) regioisomers. The hydroxy group of the side chain was selectively derivatized with dihydropyran (DHP) after protection of the ring hydroxyls with butylboronic acid. The butylboronate ester was cleaved with alkaline H_2O_2. Pyridinium dichromat (PDC) oxidation of tetrahydropyranyl (THP) ether protected PPF_1 afforded a mixture of PPD_1- and PPE_1-derivatives in a ratio of 3:1. D_1/E_1-ring phytoprostanes were deprotected and dehydrated with CF_3COOH to give a mixture of $dPPJ_1$ and $dPPA_1$. $dPPJ_1$-I and $dPPJ_1$-II were separated and purified by preparative HPLC.

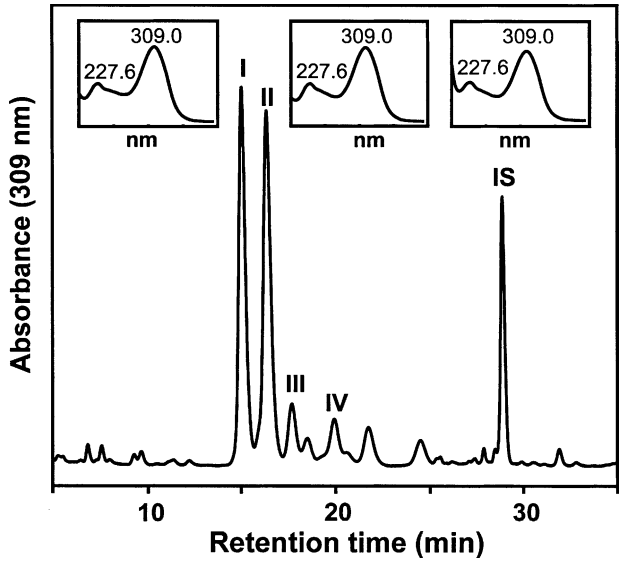

Fig. 4. HPLC separation of deoxy phytoprostanes J_1 and A_1. Regioisomers of deoxy phytoprostanes J_1 and A_1 could be separated by reversed phase HPLC. The order of elution was: $dPPJ_1$-I (peak I), $dPPJ_1$-II (peak II), $dPPA_1$-I (III) and $dPPA_1$-II (IV). Deoxy-prostaglandin J_2 was used as internal standard for quantification (IS). Insets show UV spectra of $dPPJ_1$-I, $dPPJ_1$-II and $dPGJ_2$, respectively.

patterns of methyl esters of HPLC peak I and II compounds (EIMS) are: Peak I *m/z* (rel. int.): 304 $[M]^{\cdot+}$ (100), 289 (2), 275 (67), 273 (23), 257 (7), 244 (9), 215 (11), 203 (10), 189 (10), 175 (86), 162 (41), 147 (72), 133 (76), 119 (46). Peak II *m/z* (rel. int.): 304 $[M]^{\cdot+}$ (86), 289 (2), 272 (31), 254 (11), 243 (17), 229 (6), 215 (20), 201 (10), 189 (21), 175 (61), 161 (18), 149 (74), 147 (56), 133 (49), 121 (100).

High resolution GC-EIMS analysis revealed that all GC peaks from HPLC compounds I–IV (Fig. 4) delivered molecular ions at *m/z* 304.2039 ± 5 ppm consistent with the elemental composition $C_{19}H_{28}O_3$ and the structures of $dPPJ_1$ and $dPPA_1$ methyl esters. After catalytic hydrogenation, molecular ions of HPLC compounds I–IV were observed at *m/z* 310, indicating the presence of three double bonds in each of the underivatized molecules. The orientation of the side chains relative to the cyclopentanone ring system of the derivatives could be determined unequivocally by EIMS analysis of the hydrogenated compounds. Analysis revealed that peaks I, II, III and IV isolated by HPLC (Fig. 4) represent $dPPJ_1$-I, $dPPJ_1$-II, $dPPA_1$-I and $dPPA_1$-II, respectively. Fragmentation patterns of hydrogenated methyl esters of I–IV (EI-MS) are: Peak I *m/z* (rel. int.): 310 $[M]^{\cdot+}$ (1.5), 279 $[M\text{-}OCH_3]^+$ (14), 241 (16), 240 $[M\text{-}C_5H_{10}]^+$ (99), 211 (8),180 (6), 159 (15), 158 (39),153 $[M\text{-}C_9H_{17}O_2]^+$ (100), 115 (9), 112 (10). The same fragmentation pattern and GC retention time was obtained from hydrogenated OPDA methyl ester which confirms the correct assignment of the side chains of peak I. Peak II *m/z* (rel. int.): 310 $[M]^{\cdot+}$ (1.1), 281 $[M\text{-}C_2H_5)^+$ (8), 279 $[M\text{-}OCH_3]^+$ (3), 249 (15), 149 (6), 113 (9), 112 $[M\text{-}C_{12}H_{22}O_2]^+$ (100), 105 (6). Peak III *m/z* (rel. int.): 310 $[M]^{\cdot+}$ (7), 279 $[M\text{-}OCH_3]^+$ (11), 239 $[M\text{-}C_5H_{11})^+$ (14), 155 (13), 154 $[M\text{-}C_9H_{16}O_2]^+$ (100), 125 (20), 124 (11), 111 (11), 109 (11). Peak IV *m/z* (rel. int.): 310 $[M]^{\cdot+}$ (3), 282 $[M\text{-}C_2H_4]^+$ (24), 279 $[M\text{-}OCH_3)^+$ (8), 200 (6), 147 (6), 129 (18), 112 (17), 111 $[M\text{-}C_{12}H_{23}O_2]^+$ (100), 109 (9).

Cross conjugated cyclopentenones such as $dPGJ_2$ (or in analogy $dPPJ_1$) are photolabile compounds that rapidly isomerize at room temperature in normal daylight to form mixtures of all theoretically possible geometric isomers that have similar spectral characteristics (Maxey et al., 2000). The major compound obtained from chemical dehydration of PGD_2 was found to have the *trans-cis*-$\Delta^{12,14}$ diene component (Maxey et al., 2000). In analogy, we assume that the major dehydration products of PPD_1 have the *trans,cis*-diene structure as shown in Figs. 1 and 2. NMR analysis of $dPPJ_1$-I and $dPPJ_1$-II revealed that they indeed consist of mixtures of (theoretical four) geometrical isomers (data not shown). All four geometric isomers of compound **1** ($dPPJ_1$-I) have been isolated from *Chromolaena* species (Bohlmann et al., 1982a, b). The configuration of carbon 9 of **1** has not been determined. However, if **1** is derived from PPD_1 it is a racemic compound.

2.2. Analysis of phytoprostanes D_1 and deoxy J_1 in plant material

The most sensitive method reported for quantification of prostanoids in the literature is GC–MS in the negative chemical ionization mode as their corresponding methoxime, trimethylsilyl ether, pentafluorobenzyl ester derivatives. However, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 has not yet been identified by GC-MS in animals, possibly because of the high temperature induced isomerization of the compound. Chromatographic purification and detection of the compound by ELISA using a monoclonal antibody is currently state of the art (Shibata et al., 2002). When endogenous $dPPJ_1$ from plants were analyzed by GC–MS using standard methods (Chen et al., 1999), we obtained a series of overlapping, poorly resolved peaks (probably representing all geometric isomers) which match peaks of synthetic reference compounds. However, accurate GC–MS quantification of $dPPJ_1$ in complex plant matrices proved to be difficult.

Therefore, a novel analysis method was developed which makes use of the chemical reactivity of cyclopentenones. Cyclopentenone prostanoids are highly susceptible to nucleophilic Michael addition reactions with thiols (Chen et al., 1999) that occurs at the ß-carbon of the α,β-unsaturated ketone component of the cyclopentenone ring (Maxey et al., 2000). $dPPJ_1$, $dPGJ_2$ (used as internal standard) and OPDA were found to react rapidly with 7-mercapto-4-methylcoumarin in the presence of an acid catalyst yielding fluorescent products (Fig. 5). HPLC analysis of the resulting conjugated compounds revealed one distinct peak for each

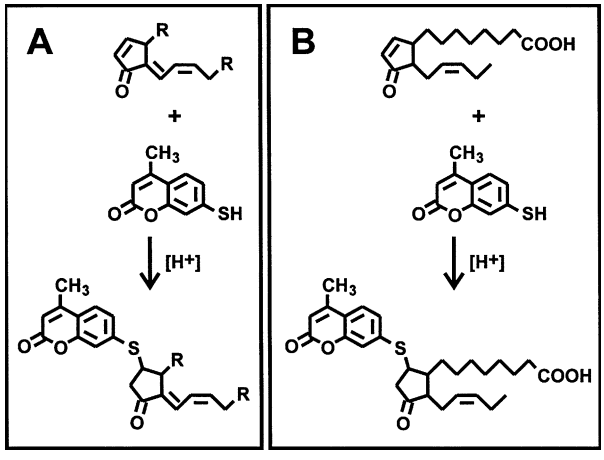

Fig. 5. Derivatization reactions of cyclopentenones with 7-mercapto-4-methycoumarin. Michael addition of thiol compounds to the α,β-unsaturated carbonyl group of $dPPJ_1$/deoxy prostaglandin J_2 (A) or OPDA (B) occurs rapidly in the presence of an acid catalyst (see Experimental section for details).

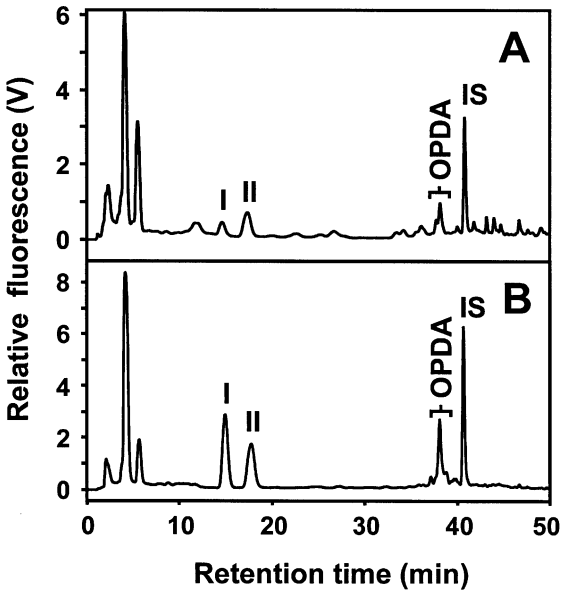

Fig. 6. HPLC chromatograms of endogenous cyclopentenones from *A. thaliana* (A) and reference compounds (B). Fresh leaves of *A. thaliana* cv. Columbia were treated with 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (internal standard, IS), extracted and processed as described in the Experimental section. Cyclopentenones were derivatized with 7-mercapto-4-methylcoumarin and analyzed by HPLC equipped with a fluorescence detector. Endogenous $dPPJ_1$-I (I), $dPPJ_1$-II (II) and 12-oxo-phytodienoic acid (OPDA) could be separated and quantified (A). Chromatogram B displays peaks derived from derivatisation of a standard reference mixture. *Cis*-configured OPDA, used as reference compound, partially racemizes during the derivatisation procedure yielding two peaks with somewhat varying ratios. Both peaks were integrated together.

regioisomer of $dPPJ_1$ as well as for $dPGJ_2$. However, OPDA isomerized during conjugation and eluted as two peaks from the HPLC column (see Fig. 6 B). For analysis of PPD_1 and cyclopentenones in fresh plants, a specific sample preparation method was developed. After addition of prostaglandin D_2 (PGD_2) and $dPGJ_2$ (used as internal standards), plant material was extracted and fractionated by solid phase extraction procedures into a fraction containing the hydroxylated oxylipins PGD_2/PPD_1 and a fraction containing the non-hydroxylated oxylipins $dPPJ_1$/OPDA/$dPGJ_2$. The first fraction was subjected to acid catalyzed dehydration to obtain the deoxy-J-ring compounds that were purified by solid phase extraction and coupled to 7-mercapto-4-methylcoumarin prior HPLC analysis. The later fraction was analyzed directly after coupling to the 7-mercapto-4-methylcoumarin. HPLC coupled to a fluorescence detector turned out to be a sensitive quantification method for conjugated cyclopentenones with a detection limit of 20 ng in plant material. The method is also highly selective since HPLC analysis of plant samples revealed that, besides peaks of the derivatization reagent, cyclopentenone conjugates were the major peaks observed (Fig. 6A). When 7-mercapto-4-methylcoumarin is omitted in the sample preparation procedure, no peaks could be detected in the region of interest of the chromatogram demonstrating selective formation and detection of cyclopentenone derivatives (data not shown).

After having established a specific analysis method for PPD_1, $dPPJ_1$ and OPDA, three plants species that often serve as model plants for biochemical studies were analyzed. Fresh leaves of *Arabidopsis thaliana* cv. Columbia, *Lycopersicon esculentum* cv. Moneymaker and *Nicotiana tabacum* cv. Samson were found to contain high levels (294 to 932 ng/g of dry weight) of compound **2** (13,14-dehydro-OPDA syn. $dPPJ_1$-I) which exceeded the levels of OPDA in all three plant species (Table 1). In addition, we also detected and quantitated $dPPJ_1$-II in all three plant species at levels ranging from 1074 to 2413 ng/g of dry weight. $dPPJ_1$-II cannot be formed from metabolites of the allene oxide synthase pathway or any other oxylipin pathway so far known. Moreover, we also detected the putative precursors of $dPPJ_1$, PPD_1-I and PPD_1-II, in all three plant species at even higher levels (Table 1). The amount of **2** (8 μg/g of dry wt.) isolated from air dried aerial parts of *Chromolaena chasleae* (Bohlmann et al., 1982b) is one order of magnitude higher than levels of **2** which we have detected in various fresh plants. Previously, we have shown that phytoprostane levels dramatically increase during drying of plants due to autoxidation of linolenate which may be the reason for the relatively high levels of **2** found in *C. chalseae*. We propose that compounds **2**, **3** and possibly also **1** (chromomoric acid) are derived from a common precursor, PPD_1-I. However, it cannot be excluded that compound **1** is derived from OPDA.

In order to clarify, whether or not PPD_1 and/or $dPPJ_1$ are formed by autoxidation of endogenous linolenate during the sample preparation procedure several control

Table 1
Levels of octadecanoids in selected plant species

	Oxylipins (mean±S.D.) (ng/g dry wt.)				
	PPD_1-I	PPD_1-II	$dPPJ_1$-I	$dPPJ_1$-II	OPDA
Lycopersicon esculentum cv. Moneymaker	459±130	698±161	294±20	1668±202	212±51
Nicotiana tabacum cv. Samson	348±83	492±139	576±181	1074±345	67±13
Arabidopsis thaliana cv. Columbia	2239±606	3995±258	932±202	2413±194	549±331

experiments were performed. Aliquots of plant samples were analyzed without addition of internal standards (prostaglandin D_2 or 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2). Instead 500 μg of purified arachidonic acid was added prior work up. Analyses revealed that isoprostanes D_2 or deoxy isoprostanes J_2 which could potentially be formed by autoxidation of arachidonate during sample preparation were below the limit of detection. Since arachidonate autoxidizes more readily than linolenate, these experiments suggest that phytoprostanes detected in plant leaves were entirely formed in plants in vivo prior harvest of the plants.

We have previously shown that the phytoprostane pathway is apparently present in all plant species due to the fact that the only requirements for phytoprostane formation (linolenic acid, molecular oxygen and reactive oxygen species) occur ubiquitously in plants (Mueller, 1998; Parchmann and Mueller, 1998). Phytoprostanes D_1 and $dPPJ_1$ are no exceptions of this rule. Beyond the fact, that the phytoprostane D_1 pathway has been identified as a novel oxylipin pathway in plants, our findings are also interesting with respect to other lines of research. It is obvious, that $dPPJ_1$ are structurally related to OPDA which is not only a precursor of jasmonic acid but also an established phytohormone that mediates plant defense responses in its own right (Stintzi et al., 2001). Since several structural congeners of OPDA and jasmonic acid display powerful biological activities (Haider et al., 2000) it remains to be shown if $dPPJ_1$ are biologically active molecules similar to the biologically highly active isoprostanes for which a role as mediators of oxidative stress in man has been proposed (Cracowski et al., 2001; Janssen, 2001). Notably, plant phytoprostanes as well as isoprostanes are dramatically induced by oxidative stress (Imbusch and Mueller, 2000b).

Moreover, $dPGJ_2$ and related cross conjugated cyclopentenones possess potent biological activities in animals [reviewed in (Straus and Glass, 2001)]. The spectrum of activities includes potent anti-inflammatory (Rossi et al., 2000), antiviral (Santoro, 1997) and apoptosis inducing activities (Kondo et al., 2002). Thus, it will also be interesting to investigate the pharmacological profile of these compounds in animals.

3. Experimental

3.1. General experimental procedures

Arabidopsis thaliana ecotype Columbia were grown in soil in a growth chamber (9 h of light at 150 μmol m^{-2} s^{-1}) at 22 °C during the day and 20 °C at night. Leaves of 5-week-old *Arabidopsis* plants were collected, shock frozen in liquid nitrogen and analyzed for oxylipins directly. *Lycopersicon esculentum* cv. Moneymaker and *Nicotiana tabacum* cv. Samson plants were grown in soil in a green house with a 16/8 h light/dark photoperiod. Leaves of 6-week-old tomato plants and 10-week-old tobacco plants were collected, shock frozen in liquid nitrogen and analyzed for oxylipins directly.

α-Linolenic acid (natural, 70%), pyridinium dichromate (purum) and 7-mercapto-4-methylcoumarin (> 97%) were purchased from Fluka (Neu-Ulm, Germany). 3,4-Dihydro-2*H*-pyran (97%) was from Aldrich (Deisenhofen, Germany). *n*-Butaneboronic acid (96%) was from Lancaster (Frankfurt/Main, Germany). Solid phase extraction glass columns were obtained from Macherey and Nagel (Düren, Germany). All other solvents and chemicals used were of highest purity.

3.2. Synthesis of deoxy phytoprostanes J_1

Synthesis of PPD_1 from PPF_1 was performed according to the procedure of Nishizawa et al. (1975). A solution of 10 mg PPF_1 (30.5 μmol), isolated from autoxidized linolenate as described by Imbusch and Mueller (2000a), was treated with 50 mg of *n*-butaneboronic acid and heated to boiling under vigorous stirring. Evaporated solvent was replaced continuously. After 30 min, the reaction was completed as judged by thin layer chromatography. Thereafter, 2 ml of 3,4-dihydro-2*H*-pyran and 10 mg of *p*-toluenesulfonic acid were added and the reaction mixture was stirred at 40 °C for 20 h. Solvent was removed under reduced pressure and the residue was dissolved in 2 ml of methanol and 850 μl of 3 N aqueous KOH solution. 330 μl of 30% hydrogen peroxide and 2 ml of water were added and the mixture was allowed to stand for 2 h at room temperature. Methanol was removed under reduced pressure and the remaining liquid was diluted with 7 ml of water and washed twice with diethyl ether. After cooling to 0 °C, the aqueous solution was acidified with 2 N potassium bisulfate (pH 3) and extracted three times with ethyl acetate. The organic phases were combined, washed with brine and dried over anhydrous sodium sulfate.

The obtained PPF_1-tetrahydropyranylether was then oxidized with 1 ml of a solution of pyridinium dichromate in acetonitrile (0.5%, w/v) at room temperature for 10 min. The reaction mixture was diluted with 10 ml of water and extracted three times with 2 ml of ethyl acetate containing 1% acetic acid. The combined organic extracts were dried over anhydrous sodium sulfate, applied to a silica solid-phase extraction column and eluted with 3 ml of ethyl acetate containing 1% acetic acid.

After removal of the solvent, the PPD_1/E_1-tetrahydropyranyl ethers were treated with 2 ml of tetrahydrofurane:water:1 M citric acid (1:3:1,v/v) and allowed to stand for 1 h at 40 °C. The solution was then diluted with 5 ml of water and extracted three times

with 3 ml of diethyl ether. Organic phases were dried over anhydrous sodium sulfate and solvent was removed under vacuum to yield a residue, containing an isomeric mixture of PPD_1 and PPE_1 in a ratio of 3:1. Isolation of PPD_1 from the isomeric mixture proved to be difficult due to the large number of isomers. Therefore, PPD_1 in the mixture were converted to $dPPJ_1$ by acid catalyzed dehydration directly. Dehydration of PPD_1 was accomplished by incubation of the crude PPD_1 mixture in 500 μl of trifluoroacetic acid at 40 °C for 15 min. The sample was taken to dryness under a stream of nitrogen. The residue was reconstituted in chloroform and applied to a silica solid-phase extraction column (500 mg) and eluted with 9 ml of hexane:diethyl ether:acetic acid (50:50:1, v/v). A crude mixture of $dPPJ_1$ and $dPPA_1$ in a ratio of 5:1 was obtained. $dPPJ_1$ and $dPPA_1$ were quantitated by HPLC as described below. The overall yield of $dPPJ_1$ from the starting material was 1.18 mg (4.1 μmol, 13.4%). The mixture was evaporated to dryness, dissolved in ethyl acetate and stored under nitrogen at −80 °C.

3.3. Preparative purification of $dPPJ_1$ by HPLC

$dPPJ_1$-I and $dPPJ_1$-II were isolated from the crude $dPPJ_1$ mixture by HPLC (LiChrospher 100 RP18ec, 4 × 250mm, 5 μm particle size; Knauer, Berlin, Germany). The column was eluted at a flow rate of 1.5 ml/min using solvent A (acetonitrile:methanol:water:acetic acid; 29:29:42:0.1, v/v) and B (methanol:acetic acid; 100:0.1, v/v). Elution began with an isocratic flow of solvent A for 20 min, followed by a mixture of solvent A and B (55:45, v/v) for 10 min. Compounds were detected at 309 nm (Waters PDA 996, Milford, USA). Fractions of the eluat were taken, evaporated to dryness and characterized by GC/MS analysis (see below).

3.4. Derivatization and GC-EIMS analysis of $dPPJ_1$

For the identification of $dPPJ_1$ regioisomers, aliquots of HPLC fractions (peak I to IV in Fig. 4) were derivatized for GC-EIMS analysis. For the determination of the molecular weight of the compounds, aliquots of the samples were derivatized with diazomethane and analyzed. All HPLC peaks (I–IV) yielded a series of peaks with molecular ions at m/z 304. For analysis of the side chain lengths and their relative orientation with respect to the cyclopentenone ring system, aliquots of samples were hydrogenated using 20 mg of Adam catalyst. Hydrogen gas was bubbled through the sample for 10 min. Subsequently, the samples were filtered, taken to dryness, derivatized to their methyl ester using diazomethane and analyzed by GC-EIMS.

GC-EIMS-analysis was performed using a Varian 3400 gas chromatograph interfaced to a Finnigan MAT quadrupole SSQ 700 mass spectrometer. The MS source was set at 150 °C and the electron energy was 70eV. $dPPJ_1$ methyl esters were analyzed by using a 25 m, 0.25 μm film thickness Optima-5 column (Macherey-Nagel, Düren, Germany). The injector was set at 300 °C, the column temperature was programmed from 160 °C to 210 °C at 29 °C/min and from 210 °C to 300 °C at 5 °C/min. High resolution-EIMS was performed using an Agilent 6890 GC linked to a Jeol GC mate II high-resolution double-focusing magnetic sector mass spectrometer. The MS source was set at 200 °C and the electron energy was 70 eV. Samples were analyzed by using a 30 m, 0.25 μm film thickness Zebron ZB-5 column (Phenomenex, Aschaffenburg, Germany) at a linear He flow of 1 ml/min. The injector temperature was 300 °C and the column temperature was programmed as described above.

3.5. Analysis of $dPPJ_1$ and OPDA by HPLC

Leaves collected from single plants were mixed, ground at −80 °C and an aliquot was analyzed for oxylipins as described below. Three independent analyses from leaves of different plants were performed.

For analysis of $dPPJ_1$ in plants, frozen plant material (1–5 g of fresh wt.) was suspended in 10 ml of cold brine containing 100 μl of 1 M citric acid. After addition of 8 ml of cold ethyl acetate and 500 ng of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 as internal standard, the sample was homogenized for 3 min with an high-performance dispenser (Ultra-Turrax T25, IKA-Werk, Germany) at 24,000 rpm and centrifuged at 3500 rpm for 10 min. The ethyl acetate phase was passed through an aminopropyl solid-phase extraction column (500 mg). The column was washed with 9 ml of chloroform:isopropanol (2:1, v/v) and eluted with 6 ml of hexane:ethyl acetate:acetic acid (75:25:2, v/v). The eluate was taken to dryness under a stream of nitrogen, dissolved in 1 ml of chloroform and applied to a silica solid-phase extraction column (500 mg). The column was washed with 6 ml of chloroform and eluted with 9 ml of hexane:ethyl acetate:acetic acid (75:25:1, v/v). The sample was taken to dryness under a stream of nitrogen and reconstituted in 50 μl of ethyl acetate containing 0.1% (w/v) 7-mercapto-4-methyl-coumarin and 25 μl of trifluoroacetic acid as acidic catalyst. The mixture was allowed to stand at 0 °C for 60 min. The sample was taken to dryness under a stream of nitrogen and dissolved in 100 μl of acetonitrile. Ten–twenty microlitres were analyzed by HPLC on a reversed phase LiChrospher 100 column (RP18ec, 2 × 250 mm, 5 μm particle size; Knauer, Berlin, Germany) with a flow rate of 0.45 ml/min. Elution started with a linear gradient of 100% solvent A (water:acetonitrile:acetic acid 55:45:0,1, v/v) to 10% solvent B (acetonitrile:acetic acid 100:0,1, v/v) in 20 min, followed by an linear gradient to 100% solvent B within another 20 min. Thereafter, an isocratic flow of

100% solvent B was kept for 15 min. Fluorescent derivatives were detected with a Waters fluorescence detector 447 (Waters, Milford, USA) with an excitation wavelength set at 335 nm and an emission wavelength set at 420 nm. Retention times were determined with authentic standards. Response factors for $dPPJ_1$ and OPDA with respect to the internal standard were determined and used for quantification.

3.6. Analysis of PPD_1 by HPLC

Extraction of PPD_1 from plant material was performed as described above for $dPPJ_1$ using 500 ng of prostaglandin D_2 as internal standard. The ethyl acetate extract was taken to dryness, dissolved in chloroform and applied to a silica solid-phase extraction column (500 mg). The column was subsequently washed with 9 ml of chloroform:acetone:acetic acid (90:10:0,1, v/v) and 9 ml of hexane:ethyl acetate:acetic acid (70:30:0,1, v/v) to remove $dPPJ_1$, OPDA and contaminants. The column was then eluted with 9 ml of hexane:ethyl acetate:acetic acid (30:70:1,0, v/v) to recover PPD_1 and PGD_2. The solvent was removed and D-ring prostanoids were dehydrated into J-ring compounds by incubation of the sample with trifluoroacetic acid at 40 °C for 15 min. The sample was taken to dryness, derivatized with 7-mercapto-4-methylcoumarin and analyzed as described above.

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