Total Synthesis and Biological Activity of 13,14-Dehydro-12-Oxo-Phytodienoic Acids (Deoxy-J_1-Phytoprostanes)

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In plants, almost all abiotic and biotic stresses are associated with enhanced endogenous production of reactive oxygen species and free radicals. Free radicals in turn readily attack unsaturated fatty acids in membrane lipids that can be non-enzymatically oxidised to a variety of products in situ. Eventually oxidised lipids are released from membrane stores. It becomes increasingly clear that several of these oxidised lipids, such as malondialdehyde and the recently discovered phytoprostanes that occur ubiquitously in plants, can be induced by oxidative stress and display potent biological activities.[1,2] Consequently it has been postulated that oxidised lipids represent archetype mediators of oxidative stress, not only in plants but also in animals.[3] The major metabolites of the phytoprostane pathway are deoxy-J_1-phytoprostanes (dPPJ_1), which structurally resemble potent, enzymatically formed defence mediators in plants and animals such as 12-oxo-phytodienoic acid (OPDA) and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (dPGJ_2; Scheme 1). Notably, these cyclopentenone phytoprostanes are more abundant than OPDA even in young and unstressed plants.[4] However, it is not known whether the recently discovered dPPJ_1s are merely markers of oxidative stress or are biologically active stress metabolites. It was the aim of this study to systematically prepare and evaluate these cyclopentenone phytoprostanes.

In 1976, Pryor and co-workers discovered that α-linolenic acid is prone to undergoing autoxidation to yield a series of prostaglandin-like compounds by a free-radical-catalysed lipid-peroxidation process in vitro.[5] This finding remained a laboratory curiosity until 1990, when it was shown that arachidonic acid undergoes a similar radical-catalysed oxidation to yield

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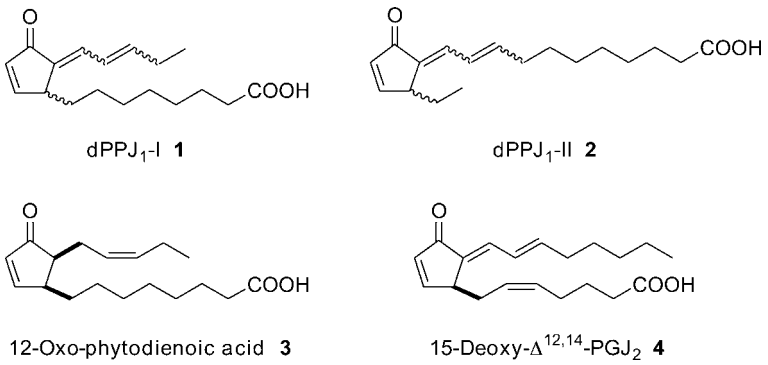


Scheme 1. *Structures of deoxy-J_1-phytoprostanes (dPPJ$_1$) and structurally related lipid mediators from plants (12-oxo-phytodienoic acid, OPDA) and mammals 15-deoxy-$\Delta^{12,14}$-PGJ$_2$ (dPGJ$_2$).*

isomeric prostaglandins, termed isoprostanes, both in vitro and in vivo.[6,7] Today, isoprostanes are regarded as reliable markers of oxidative stress.[8] Moreover, total synthesis of several isoprostanes was accomplished, and it has been shown, that many—but not all—isoprostanes bind to and activate prostaglandin receptors in the nanomolar concentration range and might therefore represent mediators of oxidative stress in animals and man.[9,10]

An analogous scenario apparently exists in plants, which, generally devoid of arachidonic acid (C20:4), use α-linolenic acid (C18:3) as a precursor for non-enzymatic (C18-) isoprostane formation. Several classes of plant isoprostanes, termed phytoprostanes, have been shown to be present in virtually all plants so far investigated.[1,11] Recently, it has been shown that the most abundant phytoprostanes in several plant species are apparently dPPJ$_1$s.[4] Two regioisomers, dPPJ$_1$-I (13,14-dehydro-12-oxo-phytodienoic acid) and dPPJ$_1$-II can be formed in plants. dPPJ$_1$-I, was first isolated as the methyl ester by Bohlmann et al. in 1981–1982 from dried material of *Chromolaena* species.[12–14] dPPJ$_1$-I methyl esters comprised four geometric double-bond isomers (with *trans*,*trans*, *cis*,*cis*, *trans*,*cis*, and *cis*,*trans* configurations).[13,14] The stereochemistry of the C9 asymmetric centre has not yet been unambiguously determined; however, if the compounds are derived via the phytoprostane pathway they should be racemic. In contrast, Bohlmann et al. report an optical rotation of +20 for the isolated dPPJ$_1$-I methyl ester.[13] However, the possibility that these compounds may be rapidly metabolised in an enantiomer-selective way and undergo isomerisation in the dried plant material or after extraction in the presence of light should not be discounted. Both epimerisation and double-bond isomerisation of dPGJ$_2$, the mammalian congener of dPPJ$_1$-I, have been shown to occur rapidly in the presence of light.[15]

Several 13,15-geometric isomers of dPPJ$_1$-I have been synthesised previously in racemic form by both the Bohlmann[16] and Liu[17] groups. Since it is to be expected that different isomers also display different biological activities, we first prepared racemic dPPJ$_1$ by autoxidation of linolenic acid in vitro to obtain a racemic mixture of dPPJ$_1$-I and dPPJ$_1$-II isomers as they most likely occur in nature.[4] Proton NMR spectroscopic analysis of both dPPJ$_1$ regioisomers revealed that the predominant isomers obtained by this route possess the *trans*,*trans*-dienone configuration (more than 80% as judged by proton NMR spectroscopy) and not the *cis*,*trans* configuration as has been suggested previously.[4]

Naturally occurring cyclopentenone A$_1$- and B$_1$-phytoprostanes are not only induced by oxidative stress but also display powerful biological activities including induction of secondary metabolism as well as glutathione-*S*-transferase and activation of mitogen-activated protein kinases.[1,2] It has been proposed that the biological activity of these compounds is at least in part due to the electrophilic properties of the cyclopentenone ring system. Deoxy-J-ring compounds are by far the most reactive electrophiles within the prostanoid series and readily bind by a Michael-type addition reaction to free thiol groups in proteins, a mechanism that is believed to be important in signal transduction. When racemic synthetic dPPJ$_1$-I and dPPJ$_1$-II were applied to tobacco cell cultures (10 μM each), a transient increase of scopoletin in the medium of the cell culture was observed (Figure 1a). The highest scopoletin accumulations (5–6-fold increases) were found 3–4 h after addition of the phytoprostanes. A similar time course of scopoletin accumulation (18-fold increase after 3 h) was found after application of racemic OPDA (10 μM). Albeit less active than OPDA, dPPJ$_1$ was revealed to be as active as the established phytohormone jasmonic acid and other phytoprostanes in this bioassay.[1] Scopoletin is known to accumulate in solanaceous plants upon oxidative stress and infection, and is generally considered to be an antimicrobial, antiviral and antioxidative plant-defence compound.[18,19] Hence, dPPJ$_1$s are non-enzymatic lipid-oxidation products that can induce antioxidative secondary metabolites that might help plants to cope better with oxidative stress.

Activation of mitogen-activated protein kinases (MAPK) is a common reaction of plant cells in oxidative-stress and defence-related signal-transduction pathways. Recently, it has been shown that some members of the phytoprostanes can rapidly activate MAPK, while other phytoprostanes and jasmonic acid do not activate it.[1] The effect of dPPJ$_1$-I and II purified from autoxidised linolenate was tested in tomato cell cultures. Tyrosin-phosphorylated proteins were immunoprecipitated with a phospho-Tyr-specific MAPK antibody and analysed by an in-gel kinase assay with the model substrate, myelin basic protein (MBP).[1] As shown in Figure 1c, both dPPJ$_1$ types resulted in fast and strong activation of a MBP phosphorylating kinase activity, and thus a putative MAPK. Additionally, it has been shown previously that a variety of cyclopentenones, including OPDA and A$_1$- and B$_1$-phytoprostanes, induce glutathione-*S*-transferase 1 (*GST1*) expression in *Arabidopsis*.[1,20] When a racemic isomeric mixture of dPPJ$_1$-I and dPPJ$_1$-II (4 nmol) was infiltrated into leaves of a transgenic *Arabidopsis* line expressing a *GST1* promoter:β-glucuronidase reporter gene construct, we observed a dramatic increased glucuronidase activity after 3, 6 and 24 h post infiltration (Figure 1d).

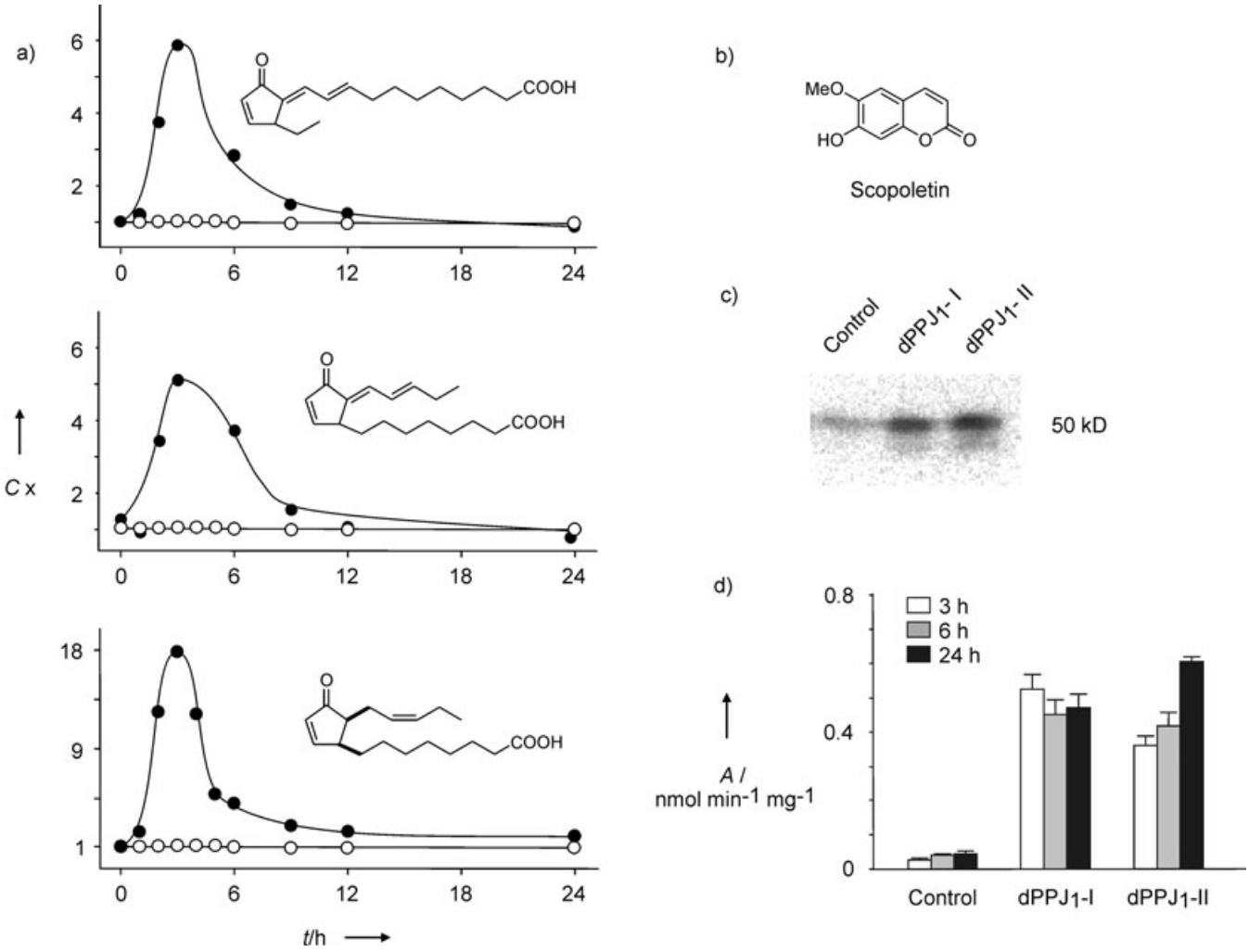


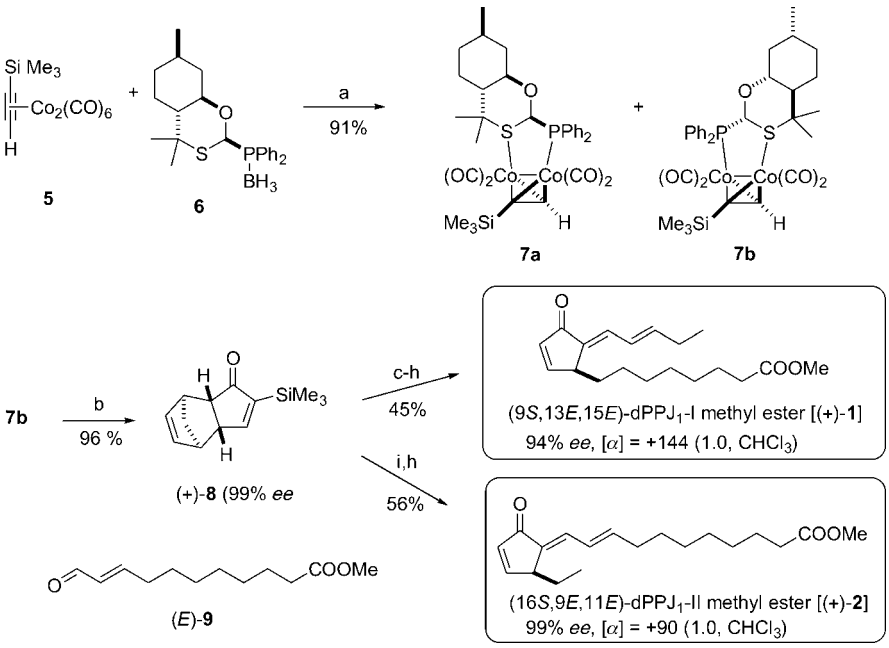
Figure 1. *Biological activities of racemic dPPJ$_1$ regioisomers. a) Time course of scopoletin levels (x-fold increase) in the medium of tobacco cell cultures when treated with different cyclopentenones (10 μM each, ●) or water (○). Each data point is the average of measurements from two independent experiments. b) Structure of scopoletin. c) Activation of mitogen-activated protein kinases. Tomato suspension cell cultures were treated with dPPJ$_1$-I (75 μM), dPPJ$_1$-II (75 μM) or water (control). Samples were taken after 5 min and analysed by in-gel kinase assay. d) Activation of the glutathione-S-transferase (GST1) promoter in transgenic* Arabidopsis thaliana *(Col-0) leaves containing the β-glucuronidase (GUS)-coding region driven by the GST1 promoter after infiltration of dPPJ$_1$ (4 nmol) or water (control) through stomata into the leaves. GUS activity was measured after 3, 6 and 24 h. Values are the mean ($\pm$ SD) of three independent experiments.*

Both regioisomers were almost equally active in inducing *GST1*, which is not only a marker gene of oxidative stress in *Arabidopsis* but also an enzyme that is thought to be involved in the detoxification of certain lipid-peroxidation products, such as electrophilic α,β-unsaturated aldehydes and ketones including cyclopentenones themselves.[1]

Typically, different isomers of isoprostanes in animals and phytoprostanes display different biological activities. In order to explore possible structure–activity relationships, we also engaged in a total synthesis of racemic dPPJ$_1$ isomers using our recently disclosed conjugate addition–olefination approach.[21,22] Following these successful racemic syntheses (not shown), the corresponding asymmetric version was approached in an analogous manner with optically active **8**, available by following an asymmetric Pauson–Khand cycloaddition and employing the bidentate chiral ligand **6**.[23] By using this ligand, readily available from pulegone in multigram scale, both diastereomeric cobalt complexes **7a** and **7b** can be easily separated by crystallisation or chromatography (Scheme 2). The subsequent Pauson–Khand cyclisation of each diastereomer affords either enantiomer of **8**. In our case, based on the structural similarity of dPPJ$_1$-I **1** to OPDA **3**, the 9*S* configuration was targeted. Starting from the crystalline complex **7b**, the Pauson–Khand reaction afforded (+)-**8** in high yield and enantiomeric excess. From this material, 1,4-conjugate addition with the functionalised organometallic and in situ Peterson olefination with *trans*-pent-2-enal gave the adduct in which only the *trans*,*trans* isomer was detected. Subsequently, a standard deprotection–oxidation sequence furnished the corresponding carboxylic acid. At this stage, it was possible to remove cyclopentadiene directly; however, better yields were observed if this was performed by using the corresponding methyl ester. Thus, treatment of the cyclopentadienyl-protected methyl ester with $MeAlCl_2$ and maleic anhydride under microwave conditions afforded (+)-**1.** HPLC analysis indicated an enantiomeric excess of 94%. Following a more convergent

route, (+)-**2** was similarly accessed from the functionalised α,β-unsaturated aldehyde **9** in 99% enantiomeric excess. Both compounds recorded strongly dextrorotary readings; however, the value obtained for (+)-**1** differed significantly from the +20 value reported under similar conditions previously.

Next we analysed whether different isomers of the $dPPJ_1$ obtained by total synthesis differ in their scopoletin-inducing potency. Scopoletin accumulation in the cell culture medium was determined 4 h after application of the isomers (10 μM each) to tobacco cell cultures. As shown in Figure 2, all $dPPJ_1$s induced a four- to sixfold accumulation of scopoletin; this was similar to the induction evoked by methyl jasmonate. Thus, biological activity did not significantly differ between the *cis*,*trans* and *trans*,*trans* geometric isomers of $dPPJ_1$-I and $dPPJ_1$-II. In addition, the configuration also appears not to have a significant impact on the activity, at least in this bioassay. Methyl esters of $dPPJ_1$ were all almost inactive, while OPDA methyl ester and methyl jasmonate were somewhat less active than the corresponding free acids.



Scheme 2. *Total synthesis of* ***1*** *and* ***2****. Conditions: a) 1,4-diazabicyclo[2.2.2]octane, PhMe, 80°C; b) norbornadiene,* N*-methylmorpholine* N*-oxide, dichloromethane (DCM), 45°C; then recrystallisation from hexane; c) [TBSO(CH₂)₈]₂CuLi (TBS =* tert*-butyldimethylsilanyl), Et₂O/pentane (2:1), −78→−5°C; then* trans*-pent-2-enal, −78°C→RT; d) AcOH/H₂O/THF (6:3:1), RT; e) Dess–Martin periodinane, DCM, RT; f) NaClO₂, tBuOH/H₂O (1:1), RT; g) Me₃SiCHN₂, PhH/MeOH (3:1), RT; h) MeAlCl₂, maleic anhydride, DCM, microwave, 100°C; i) Et₂CuLi, Et₂O, −78→−5°C; then* trans*-**9**, −78°C→RT.*

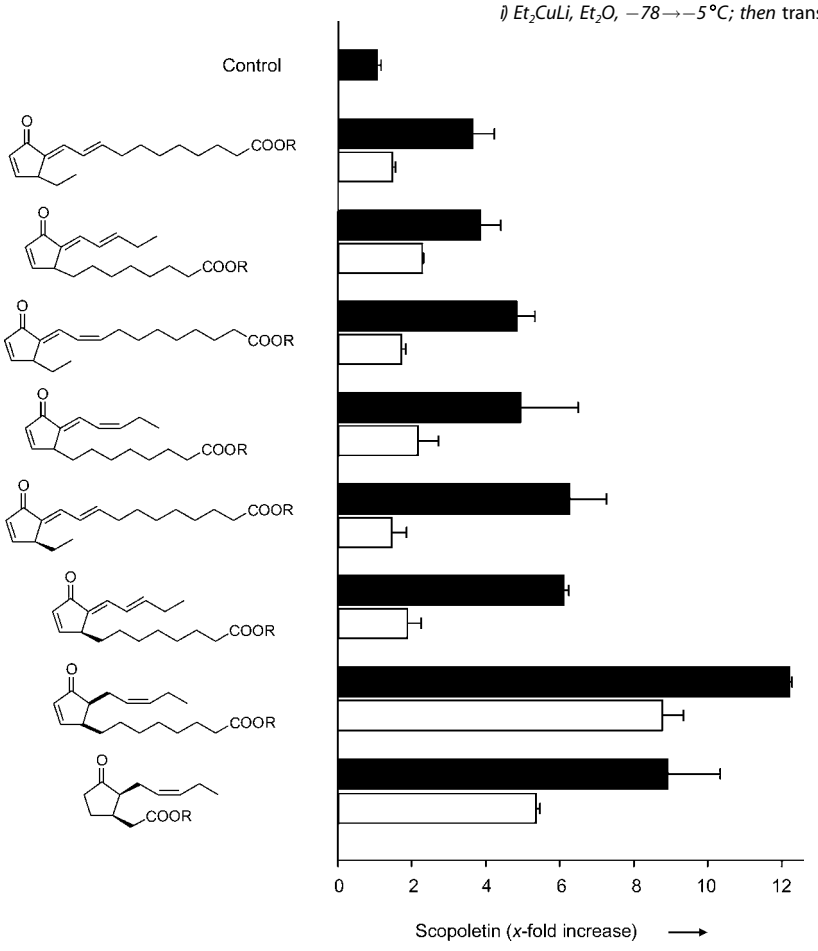


Figure 2. *Scopoletin increases in the medium of tobacco cell cultures 4 h after application of different oxylipins (10 μM each) applied as free acids (black bars) or as methyl esters (white bars). Values are the mean (± SD) of three independent experiments.*

In summary, several isomers of $dPPJ_1$ previously obtained by autoxidation of linolenate have been prepared by total synthesis thereby unambiguously confirming their structures and providing sufficient $dPPJ_1$ isomers for detailed analyses of their biological activity. Both naturally occurring regioisomers were equally active in three different bioassays; this indicates that $dPPJ_1$ might trigger plant defence responses. Moreover, all of the major isomers of autoxidised linolenate proved to be as active as jasmonates in inducing scopoletin in tobacco cells. These results strengthen a novel concept in which oxidative-stress metabolites, the phytoprostanes, function as mediators of oxidative stress in plants.[1,20,24] Since it has been shown that electrophilic cyclopentenones in general (i.e. $dPGJ_2$s, related prostaglandins, clavulones, punaglandins and cyclopentenone itself) display a common spectrum of pharmacological activities in man,[1,20,24] the current availability of chemically defined $dPPJ_1$ will allow their physiological function and mechanism of action in plants and mammals to be studied. The antiviral, anti-inflammatory and apoptosis-inducing activities in mammalian systems are currently being studied and will be reported in the future.

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Keywords: cyclization · isoprostane · jasmonate · phytoprostane · prostaglandins

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