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Biosynthesis of furanocoumarins: mevalonate-independent prenylation of umbelliferone in *Apium graveolens* (Apiaceae)

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Abstract

Preincubation of detached leaves of *Apium graveolens* with deuterium labelled [5,5-$^2H_2$]-deoxy-D-xylulose prior to the elicitation of furanocoumarin biosynthesis by jasmonic acid, resulted in high incorporation of a deuterated prenyl segment into all genuine furanocoumarins. Psoralen, xanthotoxin, bergapten and *iso*-pimpinellin consistently showed at C-3′ of the furan moiety the presence of a single deuterium atom (31% ^2H). With respect to previous studies, that indicated only a very low incorporation of mevalonate into furanocoumarins, our results clearly demonstrate that the prenylation of umbelliferone in *A. graveolens* is achieved via the novel mevalonate independent pathway. This is the first example of the participation of DOX-derived DMAPP in a prenylation reaction.

Keywords: Biosynthesis; Furanocoumarins; Xanthotoxin; Psoralen; Desoxy-D-xylulose; Mevalonate; Prenylation

1. Introduction

In higher plants the biosynthesis of isoprenoids proceeds either through the classical acetate/mevalonate pathway or via the novel nonmevalonate 1-deoxy-D-xylulose (DOX) pathway (Rohmer, Knani, Simonin, Sutter, & Sahm, 1993; Rohmer, Seemann, Horbach, Bringer-Meyer, & Sahm, 1996; Lichtenthaler, Rohmer, & Schwender, 1997; Arigoni et al., 1997). Both routes finally provide *iso*-pentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the two principal building blocks of all terpenoids (Scheme 1). The diphosphate of DOX is a novel, central intermediate that ultimately derives from glucose via pyruvate and glyceraldehyde-3-phosphate (Rohmer et al., 1996; Duvold, Cali, Bravo, & Rohmer, 1997). Following an intramolecular transketolase-type skeletal rearrangement, the methyl-branched isoprenoid building block *iso*-pentenyl diphosphate (IPP) is formed (Rohmer et al., 1993, 1996; Arigoni et al., 1997).

While the mevalonate-independent pathway appears to be typical for plastidic isoprenoids like for example, carotenoids, plastoquinone-9, phytol and isoprene (Zeidler, Lichtenthaler, May, & Lichtenthaler, 1997), sterols and vacuolar saponins are preferentially (Lichtenthaler, Schwender, Disch, & Rohmer, 1997), but not exclusively (Arigoni et al., 1997), synthesized via the classical acetate/mevalonate route. Since many procaryotes, such as the cyanobacterium *Synechocystis*, presumed ancestors of the plastids of higher plants, also synthesise their terpenoids through the mevalonate-independent pathway (Lichtenthaler, Rohmer, & Schwender, 1997), the novel pathway is most likely restricted to these cellular organelles. The hypothesis is supported further by the observation that a typical plastid transit signal peptide sequence of a nuclear gene, *CLA1*, of *Arabidopsis thaliana*, exhibits a high homology to the gene of DOX-synthase in *E. coli* and other bacteria, including *Synechocystis* (Mandel, Feldmann, Herrera-Estrella, Rocha-Sosa, & Leon, 1996; Sprenger et al., 1997). This localization of the mevalonate-independent pathway in the chloroplast raises the question whether or not the IPP/DMAPP, synthesized by this route, is utilized for the very common prenylation reactions of aromatic phenols and amines like, for example, umbelliferone, flavones, cytokinins and many others. Prenylation of umbelliferone en route to the linear and angular furano- and pyranocoumarins of the Apiaceae is particularly interesting, since this represents the first step in the biosynthesis of a large number of compounds serving as inducible defenses in plants (Matern, Lüer, & Kreusch, 1988; Somssich & Hahlbrock, 1998).

Precursor studies with *Pimpinella magna* and *Pas-*

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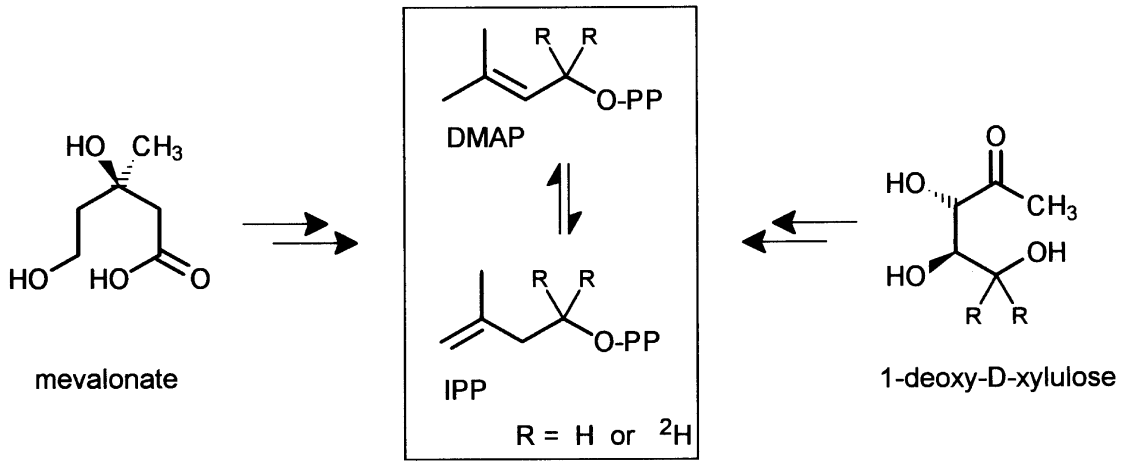


Scheme 1. Dual origin of dimethylallyl diphosphate and isopentenyl diphosphate from 1-deoxy-[5,5-$^2H_2$]-D-xylulose (DOX-d_2) and mevalonic acid. The C-5 labelled DOX-d_2 is converted into [1,1-$^2H_2$]-DMAPP with retention of both deuterium isotopes.

tinacia sativa (Floss & Mothes, 1966) plants or *Thamnosma montana* cell cultures (Brown, 1970) have shown that these compounds are derived from umbelliferone by prenylation in the 6- (demethylsuberosin, cf. Scheme 2) and 8-position (osthenol). However, the extremely low incorporation rates of labelled mevalonate, together with the recent finding that an elicitor-inducible flavonoid, dimethylallyl transferase, was found to be associated with the chloroplast (Biggs, Welle, & Griesebach, 1990), encouraged us to reinvestigate the biosynthesis of linear furanocoumarins with particular emphasis on the prenylation of umbelliferone. Although the mechanistic details of the biosynthesis of IPP from 1-deoxy-D-xylulose are not yet known in detail, it has been shown that 1-deoxy-[5,5-$^2H_2$]-D-xylulose is converted in *E. coli*, with high efficiency and without loss of deuterium atoms, from C-5 into [1,1-$^2H_2$]-*iso*-pentenyl diphosphate (Scheme 2) (Broers, 1994). We report here, using leaves of *Apium graveolens* as a model system and synthetic [5,5-$^2H_2$ $^2H_2$]-DOX (Piel & Boland, 1997), that the prenyl unit of non-mevalonate origin is, indeed, efficiently incorporated into the furane moiety of psoralen.

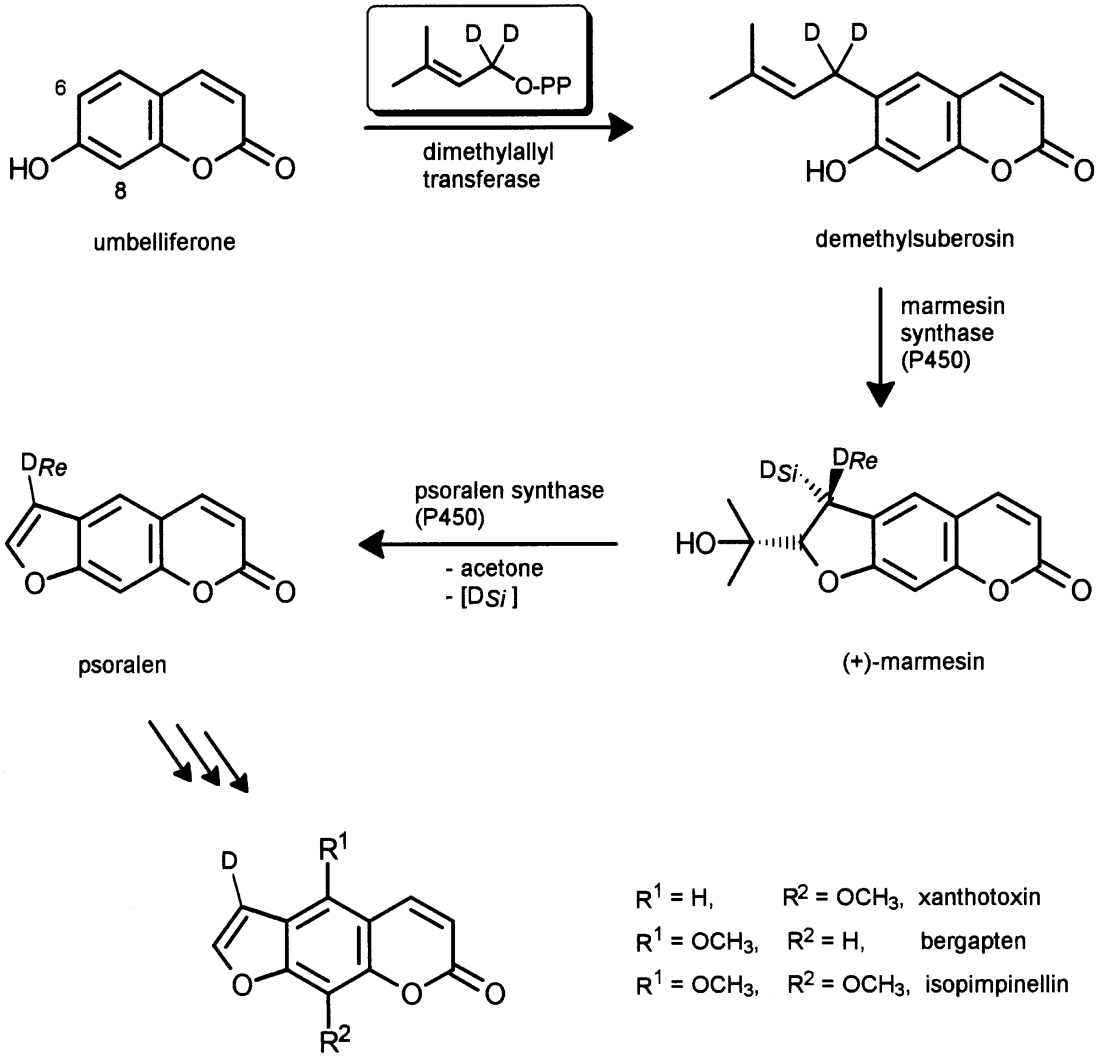
2. Results and discussion

The biosynthesis of the linear furanocoumarins in leaves of *A. graveolens* is efficiently stimulated by treatment of freshly cut leaves through the petiole with a solution of free jasmonic acid as described previously (Miksch & Boland, 1996). Approximately 48 h after the onset of the JA treatment, psoralen and its methoxylated products xanthotoxin, bergapten and iso-pimpinellin began to accumulate in the leaf tissue. Of total amount of the newly synthesized furanocoumarins, ca. 30% were exported to and deposited on the leaf surface (Stanjek, Herhaus, Ritgen, Boland, & Städler, 1997). In order to determine the origin of the DMAPP used for the prenylation of umbelliferone (cf. Scheme 1), leaves were preincubated for two days with a solution of [5,5-$^2H_2$]-DOX (1.0 mmol) to give a high level of the deoxysugar within the leaf tissue prior to the stimulation of the biosynthesis of furanocoumarins by addition of jasmonic acid. Four days after JA-treatment, the leaves were dried, extracted and the furanocoumarins were analyzed by GC–MS. Alternatively surface extracts were used without further sample pretreatment after concentration for GC–MS analysis.

Significant incorporation of deuterium from [5,5-$^2H_2$]-DOX was found in all four furanocoumarins produced during induction experiments. (Fig. 1)

Fig. 1 shows the mass spectrum of [3′-$^2H_2$]-xanthotoxin, the major furanocoumarin obtained from leaves of *A. graveolens*. After correction for the natural abundance of ^{13}C-isotopomers, present in the $[M]^+$ cluster (i.e. *m*/*z* 216 (unlabelled), 217 [+1 ^2H]) of the labelled xanthotoxin, the degree of deuterium labelling at C-3′ of the furanocoumarin amounted to 31% ^2H. As expected, control experiments applying only jasmonic acid without [5,5-$^2H_2$]-DOX preincubation did not result in the production of labelled furanocoumarins (cf. inset of Fig. 1).

Since all methoxylated furanocoumarins are produced from psoralen, the extend of deuterium labelling was found to be identical in all four furanocoumarins present in the leaf tissue, namely psoralen, xanthotoxin, bergapten and iso-pimpinellin, respectively. The position of the deuterium atom within the furan moiety of xanthotoxin and the other furanocoumarins, was determined from the MS fragmentation pattern. Due to the characteristic and constant distribution of the $^2H_2$-isotopomers within the peak cluster of the molecular ions, at *m*/*z* 216/217 Da, and subsequently within the peak clusters of the next lower masses, which resulted from the loss of a methyl group and the extrusion of CO from the lactone substructure (Tang et al., 1979), the deuterium atom



Scheme 2. Biosynthetic sequence leading from umbelliferone and DMAPP-d_2 to the linear furanocoumarins poralen, xanthotoxin, bergapten and isopimpinellin. The psoralen synthase removes the C-3′-H_{Si} together with the *syn* oriented *i*-propyloxy substituent of (−)-marmesin yielding psoralen, acetone and water as the sole products (Stanjek, Miksch, & Boland, 1997; Stanjek et al., 1998). A sequence of oxidation and alkylation generates the methoxylated furanocoumarins as metabolites from psoralen.

clearly had to reside either on the aromatic nucleus or within the furan moiety. A further loss of CO from the central nucleus furnished a fragment ion at *m/z* 146, still containing the isotopic label. Moreover, since the bismethoxylated furanocoumarin iso-pimpinellin, that possesses no hydrogen atoms on the central aromatic nucleus, also exhibited this typical pattern of $^1H/^2H$-isotopomers, the deuterium atom must have been located on the furan moiety, excluding any isotopic scrambling during the biosynthetic sequence.

3. Conclusions

Considering the previous report on the rather low incorporation (0.5%) of labelled mevalonate into the furanocoumarins skeleton (Floss & Mothes, 1966), together with the high level of deuterium present in all four furanocoumarins, after preincubation with [5,5-$^2H_2$]-DOX, it is clear that prenylation of umbelliferone is, indeed, achieved with DMAPP largely derived from the nonmevalonate pathway. Following prenylation, the resulting demethylsuberosin (alkylation at C-6, cf. Scheme 2) is oxidatively attacked at the double bond of the prenyl substituent by a P450-type enzyme, yielding (−)-marmesin as the only product (Hamerski & Matern, 1988). Then, a second P450-type enzyme, psoralen synthase (Wendorff & Matern, 1986), stereospecifically attacks the C-3′-H_{Si} hydrogen atom of the furane moiety creating a benzylic radical (Stanjek, Miksch, Lüer, Matern, & Boland, 1998). The latter undergoes *β*-cleavage yielding acetone, water and psoralen as end products. The overall process is a *syn*-elimination of the single C-3′-H_{Si} hydrogen atom and the vicinal *i*-pro-

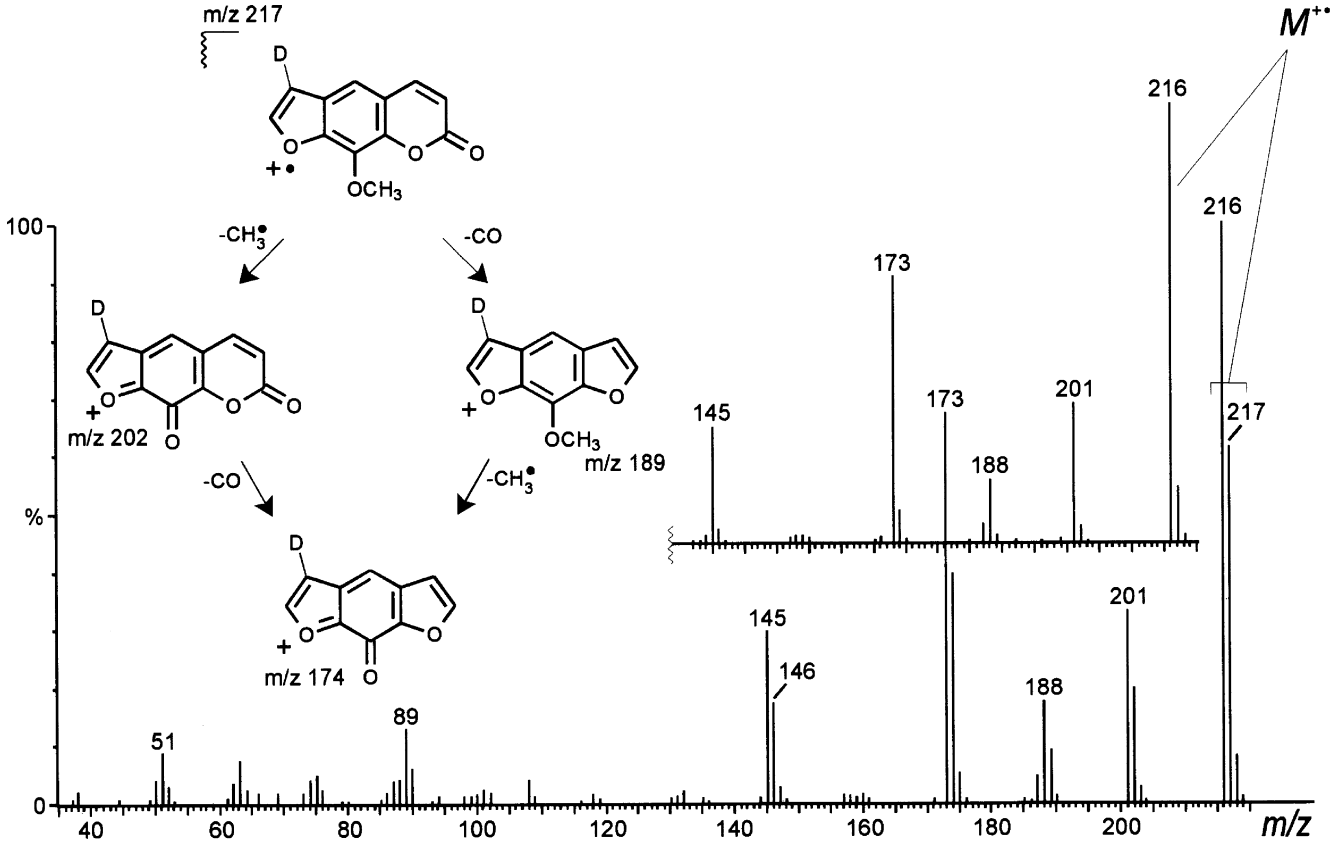


Fig. 1. Mass spectrum of partially deuterium labelled xanthotoxin resulting from the feeding experiment with DOX-d_2. Inset: molecular ion and first fragments of xanthotoxin produced in control experiments lacking DOX-d_2.

pyloxy substituent. In accord with this previously established mechanism, all four furanocoumarins show the presence of only a single deuterium atom in the molecule (cf. Fig. 1).

The ease and the extent of the incorporation of deoxy-D-xylulose derived DMAPP into the prenyl side chain of furanocoumarins, as documented here, might be well representative for other prenylation reactions with DMAPP. By analogy with the above results, the prenyl side chain of the pyranocoumarins should be of non-mevalonate origin, since the biosynthesis of pyrano- and furanocoumarins diversifies only after the initial prenylation reaction (Grundon & McColl, 1975). Another example is the phytol side chain of chlorophyll, which is also of nonmevalonate origin (Lichtenthaler et al., 1997). The geranyl diphosphate, however, which condenses with *p*-hydroxybenzoic acid, en route to shikonin, is clearly derived form mevalonate (Li, Hennig, & Heide, 1998), suggesting that generalizations concerning the origin of prenylating species are not yet possible.

Given that the mevalonate-independent biosynthesis of IPP/DMAPP is restricted to the plastids, prenylation of plant secondary metabolites with DMAPP is only possible if either the acceptor, i.e. umbelliferone, is shuttled from the cytosol into the plastid or if IPP/DMAPP is exported from the plastid to the cytosol. Related observations strongly suggest a cooperative use of both the mevalonate-dependent and the mevalonate-independent pathways, in the biosynthesis of sesquiterpenoid volatiles, have been reported recently (Arigoni et al., 1997; Piel & Boland, 1998; Boland, Hopke, & Piel, 1998). Further, detailed studies on the involvement of the two pathways in terpenoid metabolism are needed to understand the significance of cellular compartimentation and the apparently 'combinatorial involvement' of the isoprenoid diphosphate building blocks in the production of different metabolites in different cellular locations.

4. Experimental

4.1. Plant material

Celery plants (*A. graveolens*, cv. *Secalinum*) were grown from seeds in 6-cm^2 diameter plastic pots filled with potting soil. The plants were kept at 23°C and were illuminated (ca. 270 $\mu E\ m^{-2}\ s^{-1}$, 14 h light and 10 h dark regime) using daylight fluorescence tubes. Plants used for induction and incubation experiments were 2–3-month old.

4.2. Administration of labelled DOX-d_2

1-Deoxy-[5,5-$^2H_2$]-D-xylulose was synthesized as described previously (Piel & Boland, 1997). Individual leaves were cut close to the stem and immediately placed into tap water (2.0 ml) containing 1.0 μmol ml^{-1} DOX-d_2. After 2 days jasmonic acid was added and adjusted to a final concentration of 1.0 μmol ml^{-1} to stimulate the biosynthesis of the furanocoumarins. The plants were kept in this solution for another four days prior to extraction. During the administration experiment the light–dark regime was maintained as described above. Control experiments were carried out, analogous to the incubation experiment, except that tap water without DOX-d_2 was used to rear the plants. Induction of furanocoumarin biosynthesis was achieved as above by addition of JA (1 μmol ml^{-1}).

4.3. Extraction of furanocoumarins from the leaf tissue

The incubated leaves were first dried for 24 h at 50°. Then, the dry leaves were ground in ethyl acetate (15.0 ml) using an ultraturrax (10 min at 20,000 rpm). The greenish suspension was stirred for 24 h at r.t. to complete the extraction. Solids were removed by filtration and the solvent was slowly removed in vacuo (ca. 120 Torr). The residue was redissolved in ethyl acetate (200 μl) and used without further purification for GC–MS analysis. Since ca. 30% of the furanocoumarins are exported to the leaf surface (Stanjek et al., 1997), the metabolites can be also obtained by brief dipping, avoiding damage, of the leaves (2×30 s) in CH_2Cl_2 (8.0 ml). The extract was filtered and the solvent was removed i.v. and the residue was redissolved in AcOEt (150 μl).

4.4. Mass spectroscopic analysis

The extracts (surface or tissue) were directly analyzed by GC–MS by injecting 1.0 μl of the solution (injection port: 250°C) onto a fused silica column, coated with *DB* 1 ($15\ m \times 0.25$ mm) using hydrogen as the carrier gas. Compounds were separated under programmed conditions (50°C for 2 min, then to 280°C at 20°C per min), and the eluting compounds were analyzed by mass spectrometry, Fisons MD 800 GC–MS system. Transfer line: 250°C. Scan range is 35–350 Da s^{-1}. Authentic references of the natural furanocoumarins as well as of the deuterium labelled compounds (Stanjek, Miksch, & Boland, 1997) were used for the unequivocal identification and quantification of the metabolites.

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