Journal of Research & Development, Vol. 13 (2013) *ISSN 0972-5407*

Plant Coumarins - Occurrence, Biosynthesis and Perspectives

Mudasir A. Tantry[1]*, Bashir A. Ganai[1],Azra N. Kamili[1], Md Niamat Ali[1] and Abdul S. Shaw[2]

[1]Centre of Research for Development, University of Kashmir Srinagar 190006
[2]Indian Institute of Integrative Medicine, Sanatnagar Srinagar 190005
*Email: mudaek@gmail.com

ABSTRACT

Natural products deal with the isolation, identification, structure elucidation, and study of the chemical characteristics of chemical substances produced by living organisms. Coumarins are one of important class of natural products. This review highlights their occurrence, biosynthesis and perspectives.

Keywords: Plant Coumarins, occurrence, biosynthesis, perspectives

INTRODUCTION

Coumarins are derived from 1,2-benzopyrones. These molecules are found in higher plants where they originate from the general phenylpropanoid pathway (Harborne 1999) and are subject to numerous modifications. Coumarins continue to receive attention for their diverse bioactivities. Some natural coumarins have been used as human therapeutics, while 4-hydroxycoumarins are prominent examples of microbial modification which gave rise to the first generation molecules developed along with aspirin and heparin as anticoagulants (Mueller, 2004). Other applications appear possible in the course of new developments in various therapeutic fields, like symptomatic treatment of multiple sclerosis, photochemotherapy of T cell lymphoma, chemotherapy of multidrug resistant tumors, organ transplants, or treatment of smokers for nicotine addiction. Despite the importance of coumarins for

plant life and human uses, major details of their biosynthesis have remained unresolved. This review will give an update of coumarin biogenesis in plants with emphasis on the cytochrome P450 enzymes involved.

Coumarin (Fig. 1) is a natural product well known for its pleasant vanilla-like odor. It was reported from many plants of a variety of families, including Fabaceae i.e., Tonka bean (*Coumarouna odorata*) or sweetclover (*Melilotus alba)*, Lamiaceae i.e., lavender (*Lavandula officinalis*), and Lauraceae i.e., cinnamon (*Cinnamonum verum*). More recent studies have revealed the presence of *o*-coumaric acid in *Arabidopsis thaliana* root exudates. As cis-o-coumaric acid is unstable under acidic or neutral conditions and lactonizes spontaneously to coumarin it is conceivable that coumarin is formed in *Arabidopsis thaliana*. There have been many reports on the effect of coumarin in plants, at the organ, tissue and cellular levels. These observations tend to demonstrate that coumarin acts as a plant hormone. However, until now neither solid evidence for a physiological function nor the molecular mode of action of coumarin has been provided in plant tissues.

2. Occurrence and functions of coumarins in plants

Coumarins may be sub classified as simple coumarins (benzo-α-pyrones syn. 1,2-benzopyrone), 7-oxygenated coumarins (furanocoumarins syn. furobenzo-α-pyrones or furocoumarins), pyranocoumarins (benzodipyran-2-ones), and phenylcoumarins (benzo-benzopyrones. Simple coumarins, furanocoumarins and pyranocoumarins derive from the same pathway, whereas the most common phenylcoumarins (i.e., coumestans) originate from isoflavone metabolism.

2.1. Simple Coumarins

These compounds are widespread in plants and more than 700 structures have already been described (Harborne, 1999). Coumarins formed by plants originate via shikimic and chorismic acids as well as phenylalanine and cinnamic acids with carbon dioxide being the ultimate source. The formation of the phenylpropanoid amino acids

phenylalanine and tyrosine via this pathway and the conversion of former to trans-cinnamic acid through the action of phenylalanine ammonia-lyase. The first step in the biosynthesis of the coumarin nuleus involves *ortho*-hydroxylation of cinnamic acid. Brown (Brown *et al.,* 1966) proposed the basis of tracer investigation that *trans*-cinnamic acid is the common precursor of all coumarins and that the *ortho* or *para* hydroxylation leads to the elaboration of coumarin or the 7-hydroxycoumarin.

Very early in studies on coumarin biosynthesis the trans isomer of cinnamic acid, *trans*-2'-glucosyloxycinnamic acid and its aglycone were implicated. Kinetic studies after administration of $^{14}CO_2$ provided definite choice that trans-2'-glucosyloxycinnamic acid is an intermediate in the formation of coumarin. Glucosylation of trans-2'-hydroxycinnamic acid has been demonstrated in cell free extracts of *Morus alba*.

2.2. Hydroxylated and methoxylated coumarins

Prevalent hydroxylated coumarins are umbelliferone, herniarin and scoparone (2-methoxylated derivatives of umbelliferone), esculetin, fraxetin, isofraxidin, isoscopoletin, daphnetin and their corresponding glucosides (Fig. 1). As for scopoletin, these molecules are involved in plant responses to stressors like salicylic acid. Herniarin was demonstrated to be demethylated to umbelliferone by C4H from *Helianthus tuberosus* (CYP73A1) heterologously expressed in yeast; However, the *K*m was so high compared to cinnamate substrate that the implication of C4H for herniarin demethylation remains questionable.

Scopoletin and scopolin (7-β-$_D$-glucoside of scopoletin, Fig.1) were reported from many plants, e.g., rubber tree and cassava or carrot and cotton, but have been mainly studied in tobacco and sunflower. Scopoletin is a typical phytoalexin, its synthesis is post-infectionally activated in plants, but can also be triggered by various abiotic stresses. Scopoletin also displays radical scavenging properties toward reactive oxygen species and may be involved in the reduction of oxidative stress in plant cells. Until recently, there was no report of hydroxylated coumarins in *Arabidopsis*, however, recent metabolic studies have revealed that this plant can accumulate scopolin in

stems and roots. These findings demonstrate that stress induced hydroxylated coumarins are more common in higher plant species than previously assumed. As frequently described for other secondary metabolites (Harborne 1999), scopoletin is glucosylated to scopolin (Fig. 1) in the cytosol and then transferred to the vacuole.

Derivatives of daphnetin have attracted most attention recently. Cold acclimated rye expresses an *O*-methyltransferase with attenuated specificity for position 8. The product, 7-hydroxy-8- methoxycoumarin (hydrangetin) (Fig. 1), had been reported as a protein kinase inhibitor, and the modulating effect on protein kinases was proposed to function during exposure of rye to high photosystem II excitation pressure and cold acclimation. This might be the first example of a coumarin involved in hormone-like signaling. Polyhydroxylated coumarins, like 6,7,8-trihydroxycoumarin, have been described from *Pelargonium sinoides*, which demonstrates that plants are capable of multiple-step hydroxylations leading to more complex coumarin patterns.

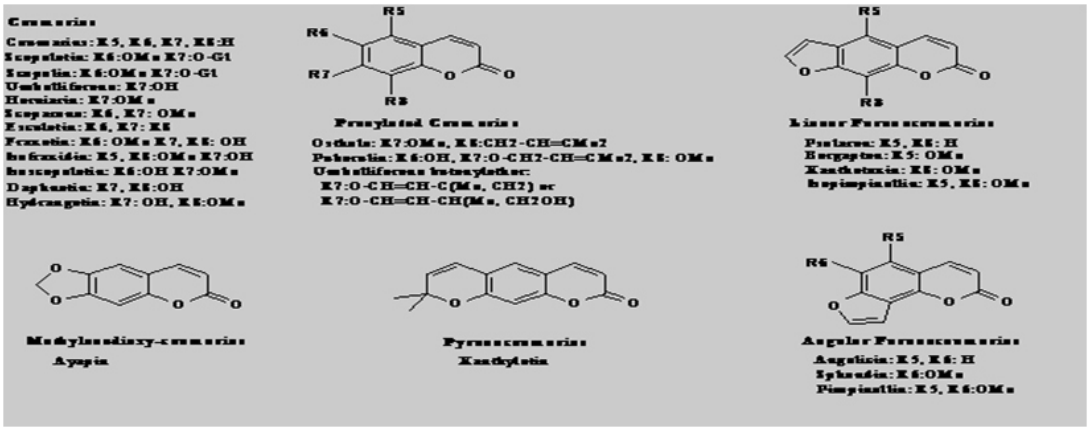


Fig. 1 Types of coumarins found in higher plants

2.3. Minor coumarins

There have been reports on many other minor coumarins in the phytochemical literature, which are beyond the scope of this review. Amongst this vast chemical diversity, methylenedioxy-substituted coumarins, i.e., ayapin (Fig. 1), and prenylated coumarins, like osthole and puberulin (Fig. 1), deserve mentioning. Ayapin has been described from Asteraceae only and was characterized as a phytoalexin. Methylenedioxy bridge-formation commonly occurs through cyclization of an ortho-methoxyphenol and is catalyzed by cytochrome P450-dependent activities. Such compounds are difficult to detoxify by phytopathogenic fungi, and it is noteworthy that the methylenedioxy moiety is known as a potent P450 inhibitor group requiring bioactivation. Osthole and puberulin have been frequently reported from Rutaceae and Apiaceae. *O*-Prenylated coumarins may be desaturated further to the corresponding butenyl ethers (Fig. 1) as shown in *Ammi majus*, and these reactions are likely also catalyzed through P450 enzymes. The butenylethers are labile and release a potentially toxic aldehyde moiety, which contributes to their role as phytoalexins. Thus, the aliphatic substitution of umbelliferone may provide new substrates for further cytochrome P450 modifications, but neither of these enzymes has so far been identified. As in case of ayapin in Asteraceae, the P450 monooxygenases must be considered as essential ecological factors.

2.4. Furanocoumarins

Furanocoumarins can be grouped into the linear type, where the (dihydro)furan ring is attached at C(6) and C(7), and the angular type, carrying the substitution at C(7) and C(8). Linear furocoumarins (syn. psoralens) are principally distributed in four angiosperm families: Apiaceae, Moraceae, Rutaceae and Leguminosae (restricted to Psoralea and Coronilla generae). The angular (dihydro)furanocoumarins are less widely distributed and primarily confined to the Apiaceae and Leguminosae. The most

abundant linear furanocoumarins are psoralen, xanthotoxin, bergapten and isopimpinellin, whereas the angular type is mostly represented by angelicin, sphondin, and pimpinellin (Fig 1). As was mentioned for the simple coumarins, numerous minor furocoumarins have been described in the literature, like bergamottin (5- geranoxy-psoralen) which has received attention recently as a major grapefruit component interfering with drug metabolism by intestinal CYP3A4. Furanocoumarins are recognized as potent phytoalexins and allele-chemical compounds. An outstanding feature of linear furanocoumarins is their ability to intercalate into dsDNA and create covalent cross-links primarily with thymidine residues. Cross linking proceeds readily under photoactivation and potentially blocks DNA replication and transcription. Accordingly, psoralens exhibit strong genotoxicity toward all living organisms, whereas the angular furanocoumarins are just capable of forming mono-adducts with DNA creating much less damage. Another remarkable property of furanocoumarins is their reactivity to inactivate P450 enzymes as mentioned above for bergamottin. This kind of enzyme inhibition has been demonstrated for P450s from vertebrate, insect and plant sources (Gravot et al. 2004). Psoralens inactivate by a mechanism-based inhibition (also referred as suicide inhibition) which requires their conversion to reactive intermediates by the enzyme itself. These intermediates form covalent links to the apoprotein and permanently inactivate the enzyme. The reactivity of furanocoumarins bears considerable ecotoxicological consequences, i.e., attributing these compounds an important role as allelochemicals during plant-insect interactions. Only herbivores able to tolerate furanocoumarins can feed on psoralen-rich plants, and xanthotoxin-insensitive P450 forms have been described from *Papilio polyxenes*, a papilionid butterfly adapted to furanocoumarin- accumulating host plants. This insensitivity was supposed to be the result of coevolution of insect detoxifying enzymes and the particular phytochemical defense since *Papilio glaucus*-whose host-plants do not contain furanocoumarins- exhibits sensitive P450s. The race of coevolution of butterflies on Apiaceae host plants has been studied in detail. The capacity of *Papilio*

polyxenes to detoxify furanocoumarins through CYP6B1 follows the order xanthotoxin > psoralen > angelicin (Wen et al. 2003), but a synergistic effect has been described between angular furanocoumarins and psoralen or xanthotoxin in response to insect attack. Considering the minor direct toxicity of angular furanocoumarins, the synergism is conceivably based on the inhibition of psoralen detoxifying CYP by angelicin. Furthermore, the accumulation of angular furanocoumarins is confined to a few taxons only. It was hypothesized, therefore, that the capacity for angular furanocoumarin biosynthesis has evolved later and presumably as a consequence to compensate for the success of herbivores in the detoxification of psoralens. It remains to be established, whether the enzymes for angular furanocoumarin biosynthesis have evolved from the biosynthesis of linear furanocoumarins. Most plants accumulating furanocoumarins possess a highly inducible biosynthetic pathway, which can be triggered by various biotic and abiotic stresses. *Ruta graveolens*, and possibly other Rutaceae, are exceptional because they do not respond to stressors and synthesize constitutively furanocoumarins in all tissues. However, the elicitation is still possible in *Ruta graveolens* dedifferentiated cells. The tissue-specific distribution of furanocoumarins has been studied in Apiaceae and Rutaceae. Obviously, these compounds accumulate in cells as well on the surface of plants. The pronounced accumulation on seeds and reproductive organs matches the optimal defense theory which predicts that defense compounds are principally allocated to the organs that play a key-role in plant fitness. The sub cellular localization of furanocoumarins is still unknown, but glucosylated forms have been frequently reported, suggesting a probable vacuolar compartmentation.

2.5. Pyranocoumarins

Pyranocoumarins, like xanthyletin (Fig. 1), have been mainly described from. As for furanocoumarins, linear and angular forms can be distinguished. To our knowledge, there is no proposal on their functions in plants, however, due to the structural

relationship with furanocoumarins, a role as phytoalexins may be assumed. The biosynthesis of pyranocoumarins has not yet been investigated.

3. **Biosynthesis of coumarins in plan**ts

Main enzymes and genes implicated in coumarins biosynthesis and that have been sufficiently documented.

3.1. Cinnamic acid to coumarin

The pathway of coumarin biosynthesis has been largely outlined during the '60s and '70s, with the help of tracer feeding experiments. Radiolabeled cinnamic acid was incorporated into coumarin and 7-hydroxycoumarins. Other tracer experiments conducted with *Lavandula officinalis*, a plant that produces coumarin as well as 7-hydroxylated coumarins, revealed that in the latter instance *para*-hydroxylation preceded the *ortho*-hydroxylation required for lactonization. This indicated that umbelliferone (Fig. 1) is derived from *cis*-*p*-coumaric acid, whereas coumarin originates from *cis*-cinnamic acid (Fig. 2), and may imply different enzymes for the *ortho*-hydroxylation/lactonization of coumarin versus umbelliferone.

The *ortho*-hydroxylation is a key step of coumarin biosynthesis, that has received insufficient attention. In initial experiments, double-labeled (*ortho*-^{3}H, *ring*-1-^{14}C) cinnamic acid was fed to *Melilotus alba* shoots or *Gaultheria procumbens* leaves, and the retention of label was monitored upon conversion to *o*-coumaric acid. An NIH shift was proposed because of insignificant decrease of the ^{3}H:^{14}C ratio, which is an indication of a cytochrome P450 monooxygenase reaction mechanism. A following report addressed the formation of coumarin with extracts from *Melilotus alba*, a plant that produces high levels of coumarin. This study allocated the *ortho*-hydroxylation of cinnamic acid to the chloroplast and again suggested a P450-dependent hydroxylation mechanism. Unfortunately, the in vitro results could not be reproduced, and the class of the enzyme involved as well as its subcellular site remain to be established. As revealed later, the early experiments may have suffered from fundamental analytical problems,

since the chromatography and recrystallization techniques employed were likely insufficient to separate the various cinnamic acids. Nevertheless, the proposed conversion of cinnamic to *o*-coumaric acid received some support by precursor feeding studies done with *Petunia* chloroplasts, which ascribed cinnamate 2-hydroxylase, including the formation of coumarin, and lack of cinnamate 4-hydroxylase to these organelles. In light of the studies done since with *Ammi majus* microsomes on the biosynthesis of furanocoumarins it appears possible that the 'ortho-hydroxylase' is an exceptionally labile CYP enzyme, in contrast to the CYPs hydroxylating cinnamic acids in *para* or *meta*- position (Fig. 2). Overall, the *ortho*-hydroxylation of cinnamic (or 4-coumaric) acid, being of pivotal importance for all coumarins, remains a missing link in the network of phenylpropanoid biosynthesis.

3.2. Cinnamic acid to umbelliferone and other hydroxylated coumarins

The formation of umbelliferone proceeds from 4-coumaric acid or its ester derivatives (Fig. 2). The conversion of cinnamic acid to 4-coumaric acid is catalyzed by cinnamate 4-hydroxylase, a cytochrome P450 monooxygenase from the CYP73A family. This enzyme constitutes the P450 enzyme most studied to date and sets the stage for several branch pathways, such as the lignification (Anterola and Lewis, 2002) or flavonoid biosynthesis (Harborne, 1999).

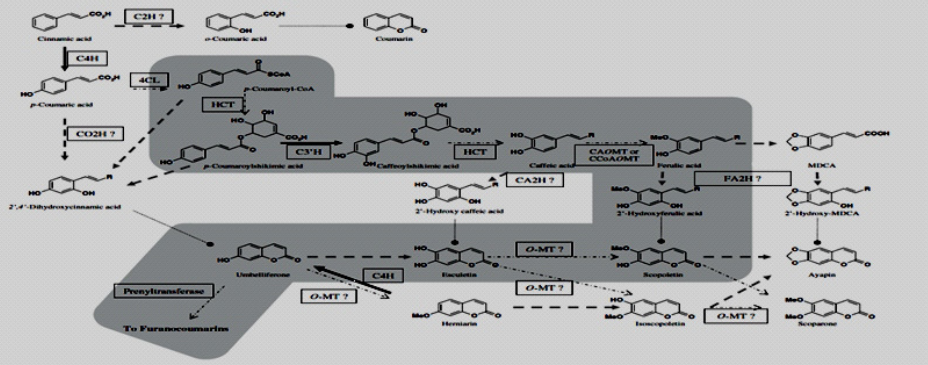


Fig. 2 Phenylpropanoid pathway leading to coumarins Pathways in gray have been unequivocally established. Glycosylated compounds are not shown for clarity. Enzymes assigned by a question mark are hypothetical. R = CO_2H or CO-SCoA C2H, cinnamic acid 2-hydroxylase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CO2H, 4-coumaric acid 2-hydroxylase; HCT, hydroxycinnamoyl-transferase; CA*O*MT, caffeic acid *O*-methyltransferase; CCoA*O*MT, caffeoyl CoA *O*-methyltransferase, CA2H, caffeic acid 2-hydroxylase; FA2H, ferulic acid 2-hydroxylase; MDCA2H, methylenedioxycinnamic acid 2-hydroxylase; *O*-MT, *O*-methyltransferase

Following the pertaining literature, 4-coumaric acid is *ortho*-hydroxylated to 2,4-dihydroxycinnamic acid. The respective enzyme activity was reported exclusively from *Hydrangea macrophylla* and assigned to the chloroplasts. This enzyme fraction was demonstrated to slowly convert cinnamic acid to *o*-coumaric acid but was more active to transform *p*-coumaric acid and ferulic acid respectively to umbelliferone and scopoletin. Although this report is unique in describing the *ortho*-hydroxylation of a hydroxylated coumarin in vitro and suggesting one plastidic fraction for the *o*-hydroxylation of both *p*-coumaric and ferulic acid as well as benzoic acid, the conversion of ferulic acid to scopoletin had been postulated before from precursor feeding studies in tobacco tissue cultures. This biosynthetic course of scopoletin/scopolin has been recently established in *Arabidopsis thaliana*. T-DNA insertion mutants within the gene encoding CYP98A3, which catalyzes 3´-hydroxylation of *p*-coumarate, revealed a dramatic decrease in both scopoletin and scopolin contents, confirming the origin from ferulic acid in *Arabidopsis*. This is in contrast to the results obtained for puberulin (Fig. 1) biosynthesis in *Agathosma puberula*. Here, as well as in *Daphne mezereum*, ferulic acid was not readily

incorporated as opposed to umbelliferone, therefore making esculetin (Fig. 1) a likely precursor for the synthesis of scopoletin. The formation of esculetin (Fig. 1; 6,7-dihydroxy coumarin) was examined in *Cichorium intybus*. These studies revealed that umbelliferone was an efficient precursor but not caffeic acid, suggesting 6-hydroxylation of umbelliferone, probably by the action of a P450 monooxygenase. This deserves mentioning, because the conversion of caffeic acid to esculetin is readily accomplished in vitro with various plant extracts containing phenol oxidase activity, but has not been confirmed in plants. Similar to esculetin, daphnetin (Fig. 1, 7,8-dihydroxycoumarin) in *Daphne mezereum*, was shown to be derived from umbelliferone rather than caffeic acid.

3.3 The ortho-hydroxylation: a common route with salicylic acid

Analogous to C2H, another major *ortho*-hydroxylation step in phenolic metabolism is still controversial. Salicylic acid is a pivotal signal molecule in plant defense mechanisms but the biosynthesis pathway is still matter of debate. Two routes have been proposed. A pathway already shown to occur in bacteria has been proposed in tobacco through chorismate and isochorismate, via the general shikimic acid metabolism (Wildermuth *et al.,* 2001). Another route has been documented in tobacco and rice, via decarboxylation of transcinnamic acid to benzoic acid and subsequent 2-hydroxylation. This benzoic acid 2-hydroxylasewas characterized as a P450 enzyme but important biochemical characteristics are atypical for an eucaryotic P450 as it appears to be soluble and it exhibits an unusually high molecular weight. The corresponding P450 gene has not been reported so far. This benzoic acid 2-hydroxylase is unable to transform cinnamic acid into *o*-coumaric acid and consequently is unlikely to interfere with the coumarin pathway.

3.4. Biosynthesis of furanocoumarins in plants

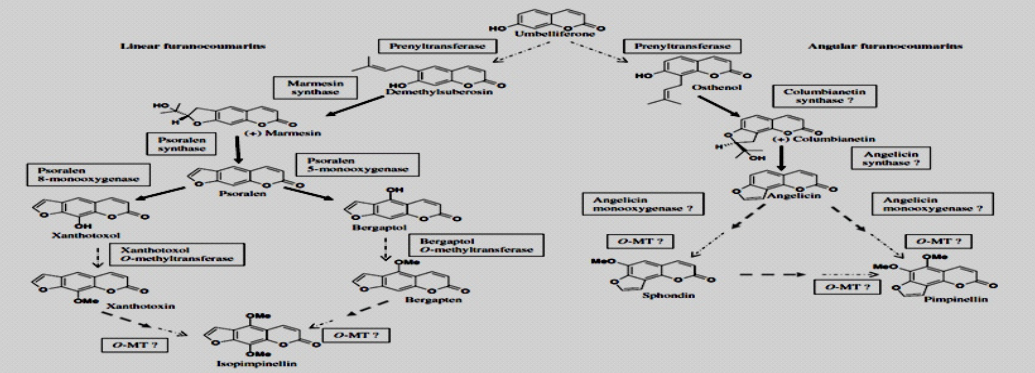
While coumarin biosynthesis remains a black box, several enzymes of the furanocoumarin pathway have been isolated and characterized (Fig. 3). Umbelliferone

rather than coumarin is the parent compound of furanocoumarins, as was reported a long time ago. It is first prenylated in 6- (for linear furanocoumarins) or 8-position (for angular furanocoumarins) to yield demethylsuberosin and osthenol, respectively (Fig. 3). Dimethylallyl diphosphate required for the 6-prenylation at least is provided in celery (*Apium graveolens*) by the deoxy-D-xylulose pathway and not through the mevalonate-dependent pathway. This is conceivably also the case in other plants, because the prenyltransferase has been identified in *Ruta graveolens* as a plastidic enzyme, and the activity was also documented in *Ammi majus*. The homologous enzyme for the angular furanocoumarins has not been isolated so far.

3.4.1 *Linear furanocoumarins*

Demethylsuberosin is transformed to marmesin and further to psoralen by two separate cytochrome P450 enzymes. The enzymes were biochemically characterized, and evidence for their P450 nature was obtained from characteristic blue-light-reversible inhibition of the activities by carbon monoxide, and the use of specific inhibitors. The two enzymes formally catalyze very different reactions, the first forming the dihydrofuran-ring from the *ortho*-prenylated phenol (marmesin synthase) and the second catalyzing the oxidative carbon-carbon chain cleavage reaction (psoralen synthase). The mechanism of marmesin synthase has not been solved yet, but it might be speculated that some analogy exists to menthofuran synthase from *Mentha piperita* which belongs to the CYP71 family (Croteau et al., 2005). Psoralen synthase was found to operates by syn-elimination of acetone and one hydrogen from position 3´ (Fig. 3). This release of acetone is unique in plants. Psoralen synthase is very specific for (+)-marmesin and does not accept the ()-stereoisomer (nodakenetin) as a substrate. Neither of the two P450s has been characterized at the gene level.

3.4.1 *Linear furanocouumarins*



Psoralen 5-monooxygenase catalyzes the subsequent hydroxylation of psoralen to bergaptol and was also characterized as a cytochrome P450 enzyme from *Ammi majus* cell suspensions. Nevertheless, there is still the possibility that bergaptol could be formed from 5-hydroxymarmesin. Different plants might thus have developed a slightly different sequences. Bergaptol is then *O*-methylated to bergapten. The cDNA encoding the *O*-methyltransferase catalyzing this reaction was recently cloned and functionally characterized from *Ammi majus*. The enzyme was shown to be highly specific for bergaptol and does not accept xanthotoxol, the C(8) corresponding phenol. This corroborates previous reports on the separation of bergaptol and xanthotoxol methyltransferases from *Ruta graveolens* or *Petroselinum crispum*. The path for isopimpinellin formation (5, 8-dimethoxypsoralen) is uncertain. It was studied in *Heracleum lanatum*. In this plant xanthotoxin was the most efficient precursor. However, bergapten was found to be converted into isopimpinellin, although at a lower rate. Both 5- and 8-hydroxylation pathways can thus lead to final product, but 5,8-dihydroxypsoralen was also demonstrated to be a possible precursor in *Ruta graveolens*. Enzymatic turnover of the pathways could simply explain the prevalence of one of the three routes in a given plant.

3.4.2. Angular furanocoumarins

The transformation of columbianetin to angelicin is very similar from a mechanistic and stereochemical point of view to the conversion of marmesin to psoralen. As demonstrated by feeding studies using deuterium-labeled columbianetin with plants or leaf tissues. It is, thus, conceivable that the enzymes for angular furanocoumarin biosynthesis may have emerged by evolutionary adaptation from the linear pathway. This would be consistent with the fact that angular furanocoumarins are less abundant in plants than the linear type and that angular furanocoumarins are always found concomitantly with linear furanocoumarins. This hypothesis will be investigated once the genes for marmesin synthase and psoralen synthase, as well as those for umbelliferone 6- and 8-prenyltransferases, will be identified (Fig. 3). Unfortunately, no information is available yet at the genetic level.

3.4.3. Implication of P450s in furanocoumarin synthesis

Cytochrome P450 enzymes are pivotal enzymes of furanocoumarin biosynthesis, i.e., the formation of xanthotoxin relies, at least, on four sequential P450 reactions catalyzed by C4H, marmesin synthase, psoralen synthase and psoralen 8-monooxygenase. This was at a first glance puzzling because of the intrinsic capacity of furanocoumarins to inhibit very different cytochrome P450 enzymes, irrespective of the species, through a mechanism-based inactivation process. To understand how plants cope with this problem Gravot and co-workers compared inactivation by furanocoumarins (Gravot et al., 2004) of three different C4H: one from a plant that does not contain furanocoumarins (*Helianthus tuberosus*, CYP73 A1) and two from plants that synthesize furanocoumarins (*Ruta graveolens*, CYP73A32; *Petroselinum crispum* CYP73A10). This would suggest that plants producing furanocoumarins have adapted their P450 enzyme repertoire to the need for reduced inactivation while retaining the high catalytic efficiency. It is reasonable to expect a similar adaptation of all the P450 enzymes in the same pathway.

The evolution toward furanocoumarin accumulation must have occurred under strong selection pressure, since the biocidal and enzyme inactivation properties of furanocoumarins appear to be lethal to plants unless quick adaptation can be accomplished. This pressure might have built up by the exposure to herbivores and the need for efficient antifeedant metabolites. This would be fully compatible with the scheme of furanocoumarins as allele-chemicals in the warfare with insects only adapted to hatch on furanocoumarin producing plants (Schuler and Berenbaum, 2003). It will be interesting to compare the cytochrome P450 families recruited for the synthesis of furanocoumarins in the plant and their detoxification in insects.

4. **Perspectives**

Although no monooxygenase of the furanocoumarin pathway has been characterized at the gene level, techniques such as differential display and RT-PCR strategies have been developed for P450s, which should be readily applicable to furanocoumarin pathway. Such techniques already led to the characterization of the C4H and C3´H in the relevant plants. Inducible systems are needed to differentiate and correlate the individual transcript abundances with product accumulation Elicitor-treated *Ammi majus* cultures appear to qualify for this purpose. Numerous recent studies focused on the role of furanocoumarins as key allelochemicals, but the physiological relevance of coumarins reaches far beyond in the producing plants. This includes the potential role of simple coumarins as hormones and signaling molecules, which were shown in the past decade to be much more widespread in plant kingdom than previously assumed. More functional insight should be obtained once the mechanism, regulation of their biosynthesis and their subcellular localization will be known. Biosynthesis of L-phenylalanine proceeds in plastids while phenylalanine ammonia-lyase and C4H activities reside in the cytosol and endoplasmic reticulum. Subcellular localization of the pivotal *ortho*-hydroxylation of cinnamic or 4-coumaric acid, so far, remains unresolved. Investigation in *Ruta graveolens* assigned the subsequent 6-prenylation of

umbelliferone to plastidial membranes. Clarification of the localization of the 2-hydroxylation will be the further step to understand the physiological role of coumarins. It is probable that different routes to coumarins will be discovered to operate in plants, some of them might be confined to a taxonomic group. The formation of scopoletin is an example and derives either from esculetin or ferulic acid according to the plant species considered. It is currently unknown, whether the P450s involved in the furocoumarin pathway belong to a single family, as is the case with CYP71s in benzoxazine synthesis, or to multiple P450 families as shown for biosynthesis of cyanogenic glucosides (CYP71E1 and CYP79A1). In either case, the discovery of genes involved in coumarin synthesis will add another stage of complexity to the phenylpropanoid pathway. The recent detection of coumarin and hydroxylated coumarins in *Arabidopsis thaliana* have opened the way for new approaches. Metabolomics in conjunction with screening of mutant libraries is likely to reveal new players in the coumarin pathway.

REFERENCES

Anterola, A. M. and Lewis, G.N. 2004. Trends in lignin modifications - a comprehensive analysis of the effects of genetic manipulations/mutations on lignifications and vascular integrity. *Phytochemistry*, **72:** 176-181.

Brown, A. 1966. Coumarins of *Ferula penninervis*. *Chemistry of Natural Compounds*, **2:** 125- 127.

Croteau, S., Charron, M.C., Latham, K.E. and Naumova, A.K. 2005 Alternative splicing and imprinting control of the Meg3/Gtl2-Dlk1 locus in mouse embryos. *Mamm. Genome*, **14:** 231-241.

Gravot, A., Lietuad, A., Verret, F., Auroy, P., Vavasseur, A. and Richard, P. 2004. $AtHMA_3$ a plant P_{18}-ATPase functions as a Cd/Pb transporter in yeast. *FEBS Lett*., **561:** 22-28.

Harborne, J. B. 1999. Natural Coumarins chemistry and biochemistry, Verlag.

Mueller, B. N. 2004. Plant generation agents, aspirin, heparin and

coumarins. *Best Pract. Res. Clin Haematology*, **17:** 23-53.

Schuler, M. A. and Berenbaum, M.R. 2003. Molecular docking of substrates and inhibitors. *Proc. Natl. Acad*., **12**: 512-523.

Wildermuth, M. C., Dewdeny, J., Wu, G. and Ausubel, F. M. 2001. Isochorismate synthase is required to synthesise salicylic acid for plant defence. *Nature*, **414**: 562-565.

Wen, Z., Pan Z., Berenbaun M. R. and Schular, M. A. 2003. Metabolism of linear and angular furanocoumarins by *Papilio polyxenes* CYP6B1 coexpressed with NADPH cytochrome P450 reductase. *Insect Biochem. Mol. Biol.*, **33**: 937-947.