1.24
Biosynthesis of Coumarins

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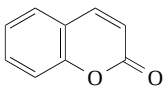
1.24.1 INTRODUCTION

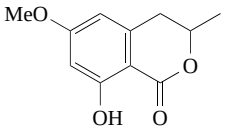
1.24.1.1 Definition and Survey

Coumarins are classified by their 2*H*-1-benzopyran-2-one core structure (**1**) and are distinguished from other benzopyranones such as the isocoumarins (**2**)[1] essentially for biosynthetic reasons, although coumarins and isocoumarins may accumulate coincidentally in the same tissues.[2] Coumarins are widely distributed in higher plants and a few examples showing noteworthy bioactivity, such as novobiocin,[3] have also been isolated from fungal and bacterial sources. Following the comprehensive treatise of Murray *et al*. in 1982,[1] a vast array of further reports has been published on the taxonomic distribution of familiar coumarins as well as on the synthesis and isolation of new derivatives. An update of chemical structures covering the period up to 1989 was published in 1991[4] and the ongoing interest in these compounds is best documented by the numerous reports that have appeared since then. This chapter is dedicated exclusively to coumarins from higher plants and focuses primarily on research concerning their biosynthesis and physiological regulation. Several constitutive plant coumarins have been isolated as glycosidic conjugates, for example, from *Ammi majus*.[5] Glycosidation generally appears to affect the subcellular distribution of phenolic metabolites rather than the biosynthesis of coumarins and will not be considered in detail. Among the dicotyledoneous plants, the *Apiaceae*, *Rutaceae*, and *Moraceae* are particularly rich sources of coumarins.[1,4] Several members of these plant families are used as spices and vegetables in human nutrition or for medicinal purposes and, accordingly, multiple studies have addressed the beneficial effects and potential hazards of the respective coumarin metabolites as well as of the related synthetic deriva-

tives. The toxic potential of linear furanocoumarins (psoralens), which is much less pronounced in the angular furanocoumarin series, has in fact been a major driving force behind research, and the capabilities of detoxification in herbivores feeding on plants such as celery have been intensively studied.[6]



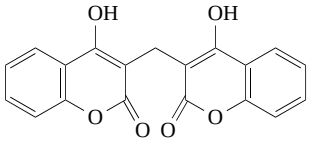
(1) Coumarin



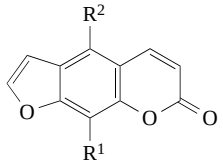
(2) 6-Methoxymellein
(Isocoumarin)

1.24.1.2 Medicinal and Biotechnological Applications

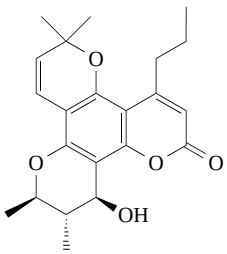
The bioactivities of dicoumarol derivatives (e.g. (**3**)) and of phototoxic psoralens (**4**–**7**) are common knowledge and several of these compounds are used in anticoagulant and antipsoriatic therapy, respectively. However, coumarins show further effects of medicinal value which justify and nourish the current committed research. In particular the platelet and lipoxygenase inhibitory activities[7,8] or the mechanistically related antiinflammatory activity[9] as well as the inhibition of DNA gyrase[10] or topoisomerase,[11] which may be linked to the anti-HIV activity,[12] the antitumor activity,[13] the inhibitory effect on T-cell activation,[14] and the inhibition of superoxide generation in activated neutrophiles[15] were significant in pharmacological testing and are under investigation. Among the active coumarins, the calanolides from the rainforest tree *Calophyllum lanigerum*, indigenous to Borneo, deserve particular mention; these compounds inhibited *in vitro* the replication and cytopathicity of HIV-1, but not HIV-2, and were active even against azidothymidine- (AZT) and pyridinone-resistant strains.[16] Calanolide A (**8**) was identified as an inhibitor of HIV-1-specific reverse transcriptase and represented a novel anti-HIV chemotype for drug development. Although the initially inappropriate taxonomic classification caused some confusion,[17] the active principle has meanwhile also been isolated from *Calophyllum teysmannii*[18] and *C. inophyllum*,[19] and the synthesis of calanolide A has been accomplished in several laboratories.[20] The fluorescence of substituted coumarins and the reactivity of 4-hydroxycoumarins have furthermore been exploited in diverse applications such as the labeling of transcription factors for the kinetic evaluation of dimer association[21] or the synthesis of L-proline conjugated chirality reagents.[22]



(3) Dicoumarol
(4-Hydroxycoumarin)



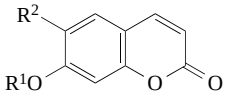
$R^1 = R^2 =$ H Psoralen **(4)**
$R^1 =$ H, $R^2 =$ OMe Bergapten **(5)**
$R^1 =$ OMe, $R^2 =$ H Xanthotoxin **(6)**
$R^1 = R^2 =$ OMe Isopimpinellin **(7)**



(8) Calanolide A

1.24.1.3 Ontogenetic Pattern and Physiological Significance

Healthy plants often accumulate considerable amounts of coumarins in oil tubes of the fruit and in the seed coats, as has been reported for wild parsnip, *Pastinaca sativa*,[23] but the genetic control of seed chemistry is barely understood.[23] A similar analysis of *Angelica archangelica* revealed high levels of coumarins in the seeds with low levels in the fruit tissues.[24] Nevertheless, coumarins were also found in the green tissues. For example, clear seasonal trends were reported for the petiole and leaf tissue of *Apium graveolens* where the bergapten (**5**) level increased during development and declined only at later stages of maturity.[25] This trend was basically supported by experiments with parsley plants, where older leaves appeared to be a richer source of coumarin-specific *O*-methyltransferases.[26] Many plants excrete their coumarins to the leaf surface, and this was monitored by a selective experimental approach which involved the extraction of leaf surface coumarins;[27] with this procedure, seasonal changes of surface levels of xanthotoxin (**6**), psoralen (**4**), and bergapten (**5**) were confirmed for *Heracleum lanatum*[27] and other umbelliferous plants,[28] which increased until mid-May and decreased thereafter until maturity. A similar analysis of mature *Ruta graveolens* plants revealed furthermore that the proportion of bergapten in comparison to psoralen and xanthotoxin increased during senescence.[28] Coumarins, and in particular furanocoumarins, are known to inhibit root tip growth and seem to induce membrane disturbances,[29] and their excretion on seed surfaces might be a means to delay germination. Coumarins are leached from the roots of some plants, such as wild *Avena*, into the soil,[30] where they provide a defense against hostile microorganisms. Alternatively, coumarins in the soil might play a specific role in bacterial root symbiosis as was reported for umbelliferone (**9**).[31] The excretion of coumarins to the leaf surface is likely to serve other allelopathic functions.[32]



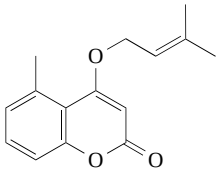
$R^1 = R^2 = H$ Umbelliferone **(9)**

$R^1 = H$, $R^2 = OH$ Aesculetin **(10)**

$R^1 = H$, $R^2 = OMe$ Scopoletin **(11)**

1.24.1.4 Biosynthetic Classification

Coumarins and isocoumarins differ greatly in terms of biogenesis. Whereas isocoumarins are synthesized by polyketide synthases,[1,33,34] coumarins result from the cyclization of cinnamic acids.[1] Furthermore, plant phenylcoumarins and 4-hydroxycoumarins appear to fall into the same category as the isocoumarins, e.g., the formation of 4-hydroxy-5-methylcoumarin in the course of gerberacoumarin (**12**) biosynthesis has been shown to proceed via the polyketide pathway in *Gerbera jamesoni* similar to the fungal synthesis of a 5-methylcoumarin.[35] It is likely that bacteria also use the polyketide route for the synthesis of 4-hydroxycoumarins, accounting for the lack of the general phenylpropanoid pathway. This aspect is particularly relevant in the context of ^{18}O-labeling studies on novobiocin biosynthesis,[1] which revealed that the ring-oxygen of the 4-hydroxycoumarin nucleus was derived from a carboxyl group and suggested the lactonization of a suitable hydroxylated precursor acid. It is obvious, therefore, that this result cannot be considered as a precedent for the cyclization reaction of plant coumarins. Analogous to the isocoumarins, phenyl- and 4-hydroxycoumarins are beyond the scope of this review.

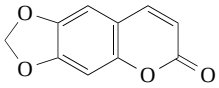


(12) Gerberacoumarin

1.24.2 MODULATION OF COUMARIN ACCUMULATION

Several environmental factors have been found to influence the coumarin content of plants. Treatment with heavy metals or irradiation with short wavelength UV light induced the accumu-

lation of scopoletin (**11**) and ayapin (**13**) in sunflower.[36] Experimental exposure of celery plants to acid fog (pH 2.0) for 4 h, which was initiated to simulate the conditions near urban centers in California, increased the coumarin content of leaves and petioles approximately fivefold as measured at 120 h post treatment.[37] Spraying *Ruta graveolens* plants with sulfuric acid at pH 2.4 or saturated sodium chloride solutions decreased the total concentration of furanocoumarins but increased the relative percentage on the surface of the leaves.[38] A single ozone treatment (200 nL L^{-1}) for 10 h acted as a cross-inducer of flavonoid glucoside and furanocoumarin biosynthesis in parsley plants,[39] and in other instances the accumulation of furanocoumarins was induced by mechanical wounding and herbivore attack of plants,[40] infection by pathogens,[1,41] or by treatment with airborne methyl jasmonate, which is a "broad spectrum plant activator".[42] Furthermore, scopoletin was reported as a phytoalexin from taxonomically diverse plants such as sweet potato,[43] tobacco,[44] carrot,[2] sunflower,[45] *Citrus*,[46] *Hevea*,[47] and cotton[48] and the accumulation of coumarins may thus be regarded as a very general defense response. Nevertheless, the induction of celery leaves with jasmonic acid or analogues of amino acid conjugates of jasmonate revealed a peculiar effect on the proportion of furanocoumarins sequestered to the surface.[32] Whereas the ratio of bergapten (**5**) to xanthotoxin (**6**) within the leaf remained at 1.3 : 1.0, xanthotoxin dominated in the surface lipids at a ratio of 0.8 : 1.0 and indicated that the export is not a simple diffusive translocation.[32] These results are reminiscent of the data reported for *Ruta graveolens*[28] and fit the observation that jasmonate conceivably induces rapid senescence.



(13) Ayapin

Cell cultures of umbelliferous plants proved particularly suitable for regulatory studies and the induction of liner furanocoumarin phytoalexins was first demonstrated in parsley cultures treated with fungal elicitors.[49] Most of the coumarins were secreted into the culture fluid under the conditions of elicitation. Numerous studies have followed on the regulation and molecular biology of the phenylpropanoid pathways in such cell cultures[50,51] and these aspects will be elaborated in more detail below. In case of *Ammi majus* cell cultures, the effects of growth media nutrients or of known intermediates in the biosynthetic pathway on coumarin accumulation were investigated and 3% sucrose or the addition of fairly high concentrations of L-phenylalanine were found most effective.[52] Besides, preincubations with methyl jasmonate[53] or 2,6-dichloroisonicotinic and 5-chlorosalicyclic acid,[54] respectively, conditioned the cells for a greatly enhanced accumulation of coumarins on subsequent challenge with fungal elicitor. 2,6-Dichloroisonicotinic acid is available commercially as an activator of the plant systemic acquired resistance response; its mode of action appears to be more subtle than that of methyl jasmonate, generating much less active oxygen species and causing no phenotypic change of the cells.[54] Data suggest that plant activators like 2,6-dichloroisonicotinic acid induce particularly the late enzymes of the phenylpropanoid pathways, catalyzing reactions beyond that of phenylalanine ammonia-lyase (PAL), while PAL is not induced in the plant cells.[55] This raises the possibility that the enzyme system mediating the cyclization of the benzopyranone moiety might also be selectively induced by plant activator chemicals. Another interesting facet of the fungal-induced furanocoumarin synthesis is the concomitant *in vivo* inhibition of phytosterol biosynthesis, which was observed in *Ammi majus*[56] as well as in *Petroselinum crispum* cells[57] and suggested the specific inhibition of one of the enzymes on the mevalonic acid to dimethylallyl diphosphate pathway span of the cytosolic microsomal pathway of the terpenoid biosynthesis.[56] This effect was indirectly supported by the observation that various furanocoumarins inhibited the production of trichothecenes in *Fusarium culmorum*.[58] However, the induction of *Petroselinum crispum* with fungal elicitor was shown to cause pleiotropic effects on gene expression and included the repression of cell cycle-related genes.[59]

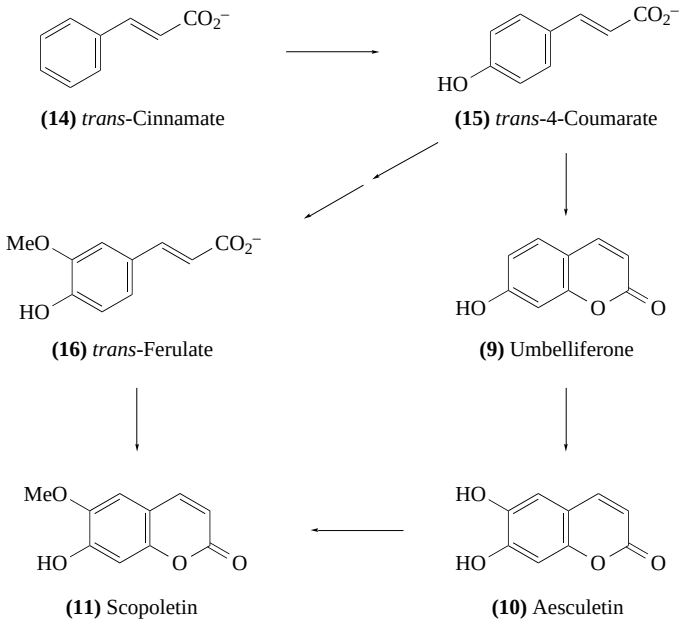
1.24.3 BIOSYNTHESIS

1.24.3.1 Coumarin and Umbelliferone

Most intermediates in the formation of coumarins in higher plants were identified by classical precursor feeding studies,[1,52] and the overall pattern of biosynthesis was proposed from such

experiments. The biosynthesis of coumarins proceeds from *trans*-cinnamic acid,[1] which is generated from primary metabolites through the shikimate and general phenylpropanoid pathways. It has become clear, however, that an in depth mechanistic description of the pathway can only be elaborated from supplementary, thorough *in vitro* studies. The enzymology of the shikimate pathway has been reviewed in detail,[60,61] (see also Chapter 1.22), and the general phenylpropanoid pathway is outlined in Chapter 1.26. Precise knowledge of the subcellular topology of these pathways is particularly desirable with respect to the subsequent formation of the benzopyranone moiety. Several reports published since the late 1980s seem to support the view that the entire shikimate pathway has to be assigned exclusively to the plastid compartment, which would also favor the plastids for the synthesis of coumarins. Subsequent data,[60] however, have called this idea into question, and it appears reasonable at present to leave the case of compartmentation of the coumarin-committed pathway open, pending further experimental evidence.

Cinnamate (**14**) may be hydroxylated to 4-coumarate (**15**) by a cytochome P450-dependent monooxygenase (Scheme 1), which was initially cloned from *Helianthus tuberosus* and later functionally characterized after expression in an optimized yeast system.[62] This enzyme has meanwhile been cloned also from *Phaseolus aureus*, *Medicago sativa*, and *Pisum sativum*.[63] Formally, cinnamate and 4-coumarate were considered the immediate precursors of coumarin (**1**) and umbelliferone (**9**), respectively, biosyntheses which require the 2-hydroxylation of the aromatic ring and/or the cyclization reaction (Scheme 1).[1,50,52] Both the lactonization of 2-hydroxycinnamic acids and the direct cyclization of coumaric acid via a spirodiene intermediate appeared feasible and have been outlined.[50,64] In the case of cinnamate the 2′-hydroxylation is likely to precede the cyclization to coumarin, since in *Melilotus* the 2′-*O*-glucoside of *trans*-2′-hydroxycinnamic acid is delivered through the tonoplast and stored in the vacuole predominantly in the *cis*-configuration.[65] Nevertheless, early reports on the enzymatic *ortho*-hydroxylation of cinnamic and 4-coumaric acids *in vitro* could not be repeated and the discrepancies have been discussed elsewhere.[50,64] However, in an entirely different context the *ortho*-hydroxylation of benzoate to salicylate has been reported and this enzyme was identified as a soluble cytochrome P450-dependent monooxygenase.[66] The benzoate 2-hydroxylase might be considered as a precedent enzyme for cinnamate 2′-hydroxylation, but this assumption definitely requires further experimental verification. The gap in our knowledge of coumarin biosynthesis therefore concerns both the mechanism of cyclization, including the prerequisite *cis*- to *trans*-isomerization of cinnamic acids, as well as the exact chemical nature of the substrates, since the respective CoA-esters cannot be ruled out at the present time. Provided that the formation of the benzoypyranone moiety proceeds in two steps (i.e., hydroxylation and subsequent cyclization) the CoA-activation of the acids might increase the rate of lactonization which can also proceed spontaneously.[1]



(14) *trans*-Cinnamate **(15)** *trans*-4-Coumarate **(16)** *trans*-Ferulate **(9)** Umbelliferone **(11)** Scopoletin **(10)** Aesculetin

Scheme 1

1.24.3.2 Aesculetin and Scopoletin

Coumarin itself and the hydroxylated, alkoxylated, or alkylated derivatives were regarded as simple coumarins in contrast to, for example, the furanocoumarins.[52] The biosynthetic path of simple coumarins, e.g., aesculetin (**10**) and scopoletin (**11**), had to be traced on the basis of *in vivo* precursor feeding studies,[1,52,61,64] owing to the fact that the benzopyranone cyclization had not been reliably accomplished *in vitro*. Most of these experiments were carried out on plant tissues which constitutively produce coumarins, yielding fairly low rates of precursor incorporation and the results were summarized previously.[50,52,64] The studies revealed inconsistent results concerning the sequence of cyclization and hydroxylation in different plants. In *Cichorium intybus* aesculetin was proposed to be formed by hydroxylation of umbelliferone (**9**),[67] whereas enzyme activities from other plants readily catalyzed the synthesis from caffeic acid *in vitro*.[68,69] Scopoletin (**11**) was proposed to be formed directly from ferulic acid (**16**) in tobacco mosaic virus (TMV) infected tobacco, although tobacco expresses an *O*-methyltransferase activity methylating aesculetin to scopoletin.[1,44,50] However, feeding studies in *Daphne mezereum*[70] and *Agathosma puberula*[71] corroborated the role of aesculetin as the intermediate between umbelliferone and scopoletin, and the pathway in tobacco was considered exceptional.[52,71] An investigation of scopoletin biosynthesis in elicited sunflower plants again yielded ambiguous results, and here also the methylation of aesculetin to scopoletin was catalyzed *in vitro*.[36]

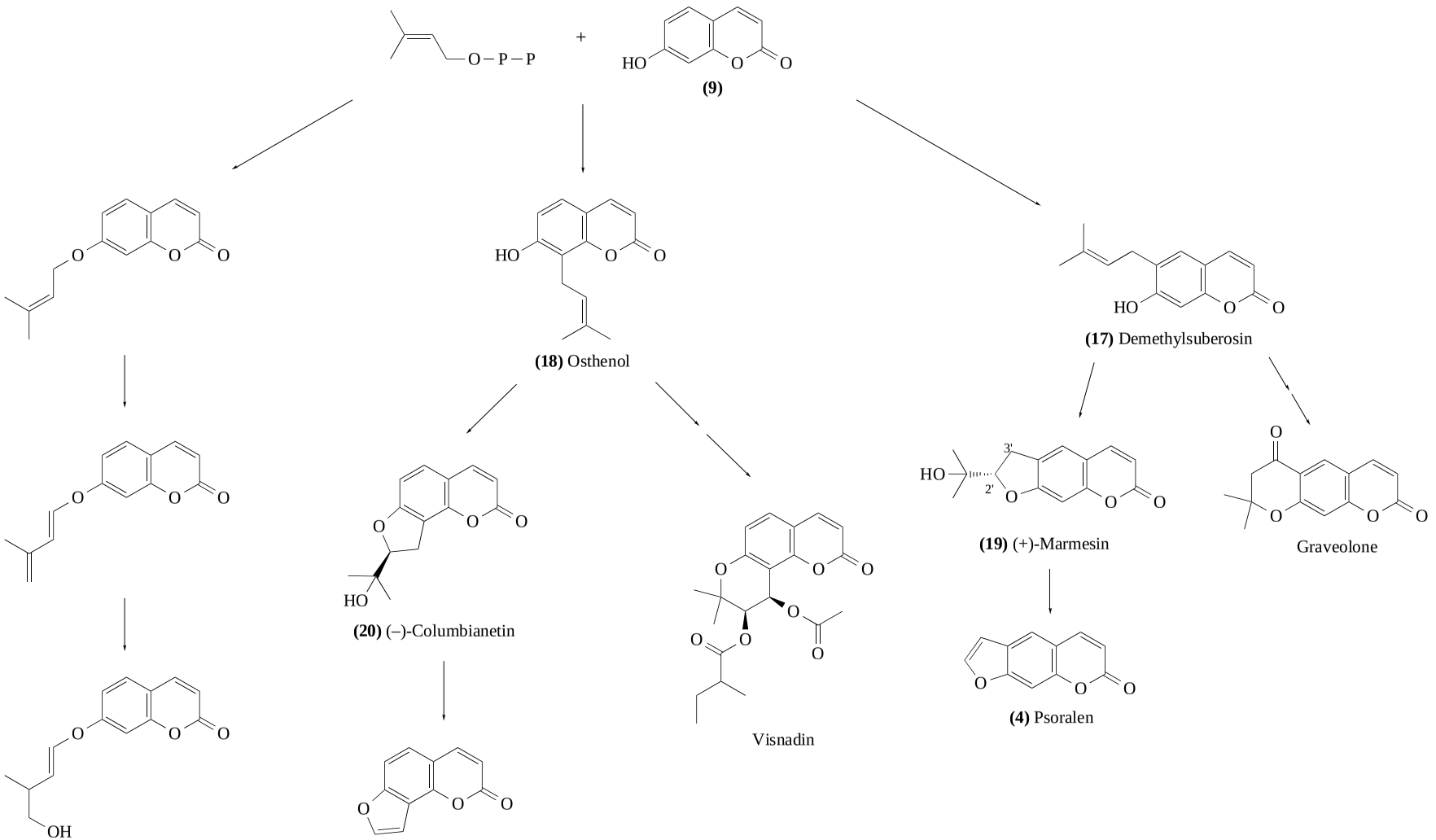
1.24.3.3 Prenylcoumarins

The prenylations of umbelliferone (**9**) in the 6- or 8-position yield demethylsuberosin (**17**) and osthenol (**18**), respectively, and give access to the branch pathways to linear or angular furano- and pyranocoumarins, which are predominantly found in the *Umbelliferae* (Scheme 2). Pyranocoumarins have been reported as major metabolites of, for example, *Petroselinum crispum*[72] and *Ammi visnaga*.[73] Prenylated coumarins have furthermore been isolated from various plants and, in particular, from *Rutaceae* species.[74] Following the first report by Dhillon and Brown on the *in vitro* prenylation of umbelliferone with fractions from *Ruta graveolens* plastids in the presence of Mn^{2+} ions,[1,52] several dimethylallyl transferase activities prenylating umbelliferone[50,64,75] and other aromatic substrates such as flavonoids,[76,77] acridones,[78] and 4-hydroxybenzoate[79] or forming geranyl and farnesyl diphosphate for lower terpenoid biosynthesis[80,81] have been described from plants. The enzyme activities were always associated with the microsomal fraction and assigned in soybean and French bean to the envelope membrane of plastids.[76] Although only the 6-*C*- and 7-*O*-prenylation of umbelliferone have been described *in vitro*,[64,75] it is to be expected that the prenylation at the 8-position (Scheme 2) in the course of angular furano- and pyranocoumarin biosyntheses is catalyzed by a homologous membrane-bound transferase. This 8-*C*-prenyltransferase must clearly be a separate enzyme entity.

Despite the numerous prenylated phenolic plant metabolites that have been isolated particularly in the field of flavonoids,[76] neither one of the corresponding prenyltransferases has been purified and thoroughly studied. This is in sharp contrast to fungal and mammalian farnesyl- and geranylgeranyltransferases,[82–84] which fulfil important regulatory functions in cellular homoeostasis, or to the polyprenyl diphosphate synthases from plant[85,86] and bacterial sources.[87] The stereochemical mode of action of bacterial polyprenyl diphosphate synthases has been investigated and several such enzymes have been cloned;[87] highly conserved sequence domains of these genes might be helpful in the cloning of plant dimethylallyl transferases.

1.24.3.4 Dihydrofuranocoumarins and Cytochrome P450 Catalysis

The oxidative cyclization reactions of 6- and 8-prenylated umbelliferone were proposed to yield the dihydrofuranocoumarins (+)-marmesin (**19**) and (−)-columbianetin (**20**), respectively, which are the immediate precursors of linear and angular furanocoumarins[1] (Scheme 2). The cyclization of demethylsuberosin to (+)-marmesin (Scheme 2) was accomplished *in vitro* in the presence of NADPH using microsomal fractions of umbelliferous plant cells (*Petroselinum crispum*, *Ammi majus*, *Arracacia xanthorhiza*) that had been elicited with fungal elicitor.[50,64] Inhibition studies identified the ''marmesin synthase'' as a cytochrome P450-dependent monooxygenase,[88] which did not release any intermediates during the reaction.[50,64] Following an earlier proposal,[1,52] it was nevertheless suggested that the epoxidation of the side-chain double bond as a hypothetical intermediate initiated the reaction.[50] The transfer of oxygen to alkenes by P450 enzymes typically results


O – P – P
+
HO
O
O
(9)
HO
O
O
(18) Osthenol
HO
O
O
(17) Demethylsuberosin
O
O
O
OH
HO
O
O
O
(20) (–)-Columbianetin
HO
3'
2'
O
O
O
(19) (+)-Marmesin
O
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O
Graveolone
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O
O
O
O
O
Visnadin
O
O
O
(4) Psoralen
O
O
O
(21) Angelicin

Scheme 2

in epoxidation rather than hydroxylation reactions[89] and the reaction is formally considered as an insertion of an "oxen".[90] Furthermore, the product often deactivates the enzyme by concomitant alkylation of the prosthetic heme group.[91] In the case of marmesin synthase, the proper spatial orientation of the 7-hydroxy group of demethylsuberosin (**17**) during catalysis is probably responsible for delocalization of the electron density from the double bond and favors the cyclization reaction. Conceivably, the formation of (−)-columbianetin from osthenol (Scheme 2) follows an analogous route and is also catalyzed by a P450 monooxygenase, but the experimental support for this assumption is still lacking.

Cytochrome P450 monooxygenases appear to play an outstanding role in the overall biosynthesis of furanocoumarins and some basic mechanistic parameters will be recalled briefly for a better understanding of the coumarin-specific pathway. P450 monooxygenases catalyze the reductive activation of molecular oxygen to yield "active oxygen" species, capable of supporting the free radical homolytic cleavage of C—H or C—C bonds. Several steps of the activation process comprise one-electron transfers, that is, movement of either an electron or a hydrogen atom.[90,91] The enzymes contain Fe–protoporphyrin IX and, in the resting state, the ferric prosthetic group is held in a hexacoordinated position by a cysteinyl thiolate as a fifth ligand and coordination of a water molecule provides a sixth ligand (Figure 1). Binding of substrate displaces the water molecule and the iron spin state changes from a low spin to a high spin, pentacoordinated state. Furthermore, the reduction of the prosthetic group to a high spin, ferrous state by a single-electron transfer from cytochrome P450–NADPH reductase is facilitated in the substrate-bound P450, which then readily binds molecular oxygen to form a ferrous dioxygen complex. The structural information on the short-lived radicals beyond the ferrous dioxygen complex is rather limited and must be inferred from model systems.[90,91] Two steps lead to the "reactive oxygen species" as the eventual catalysts. A redox reaction in the complex produces a ferrisuperoxide ion, $[Fe^{III}—O—O]^{\bullet}$, which is most probably converted to the ferrihydroperoxy derivative, $[Fe^{III}—O—OH]$ (Figure 1), by transfer of another single electron (from NADPH–cytochrome P450 reductase or ferrous cytochrome b_5) and a proton or abstraction of a free radical hydrogen atom (from the substrate). The stoichiometric generation of H_2O_2 from NADPH and O_2 by liver microsomes in the absence of substrate was taken as evidence for the existence of the ferric-hydroperoxy species, which cannot be observed directly. $[Fe^{III}—O—OH]$ thus formed is itself a strong nucleophile and was proposed to act as the reactive intermediate in various P450 reactions, e.g., nitric oxide synthase or acyl-carbon cleavage reaction in the conversion of pregnenolone to the 16-en steroid products.[90–92] The instantaneous heterolytic cleavage of the oxygen–oxygen bond in the ferric-hydroperoxy species, which is probably assisted by the cysteinyl thiolate ligand, yields the reactive oxo-derivative as well as water. The oxo-derivative (oxoferryl or iron-oxo radical) may be represented in several resonance forms, for example, $Fe^{V}{=}O$, $[Fe^{IV}{=}O]^{+\bullet}$ or $[Fe^{IV}—O]^{\bullet}$. $[Fe^{IV}{=}O]^{+\bullet}$ and the oxo-Fe^{IV} porphyrin radical $[Fe^{IV}—O]^{\bullet}$ are electrophilic and behave like an alkoxy radical, respectively; these radicals are capable of abstracting hydrogen from a C—H bond, e.g., in the hydroxylation cycle, to produce Fe^{IV}—OH and a substrate carbon radical,[90,91] which immediately recombine ("oxygen rebound" in the hydroxylation pathway) to yield an alcohol. This recombination event must take place very rapidly in a reaction cage to account for the stereospecificity of the hydroxylation. Both the nucleophilic ferri-hydroperoxy species and the electrophilic oxo-Fe^{IV} radical species might participate in the reactions catalyzed by P450 monooxygenases during the course of furanocoumarin biosynthesis.

Besides the hydroxylation and C—C bond cleavage reactions P450 enzymes are capable of desaturating aliphatic substrates. It is noteworthy in the context of dihydrofuranocoumarin formation that P450 enzyme activities of the microsomes isolated from elicited *Ammi majus* cells metabolized 7-*O*-prenylumbelliferone (Scheme 2), which cannot cyclize to a dihydrofurane, by $\Delta^{1'}$-desaturation to yield butenyl ethers rather than epoxidation of the prenyl residue.[93] Desaturation reactions brought about by mammalian P450 monooxygenases[90,91] or by the mechanistically analogous non-heme-iron oxygenases of plants[94] can proceed without the intermediacy of an alcohol and instead require the successive abstraction of two hydrogen atoms. Accordingly, the iron-bound hydroxy radical formed initially by abstraction of the first hydrogen atom from the substrate functions here as an oxidant and abstracts the second hydrogen atom to give rise to a water molecule rather than to recombine with the substrate radical as suggested for the P450 hydroxylations.[90,91,94] The underlying mechanism has been outlined in detail elsewhere.[91,94]

The reaction mechanism of epoxidation of alkenic double bonds by P450 monooxygenases has not yet been solved, although a concerted process has been ruled out already.[90] Nevertheless, the model mechanisms proposed for the primary event in the interaction of the P450 oxo-derivative with aliphatic double bonds[91] provide a reasonable basis for the cyclization of demethylsuberosin to (+)-marmesin without the formation of an intermediate epoxide. The double-bond π system is

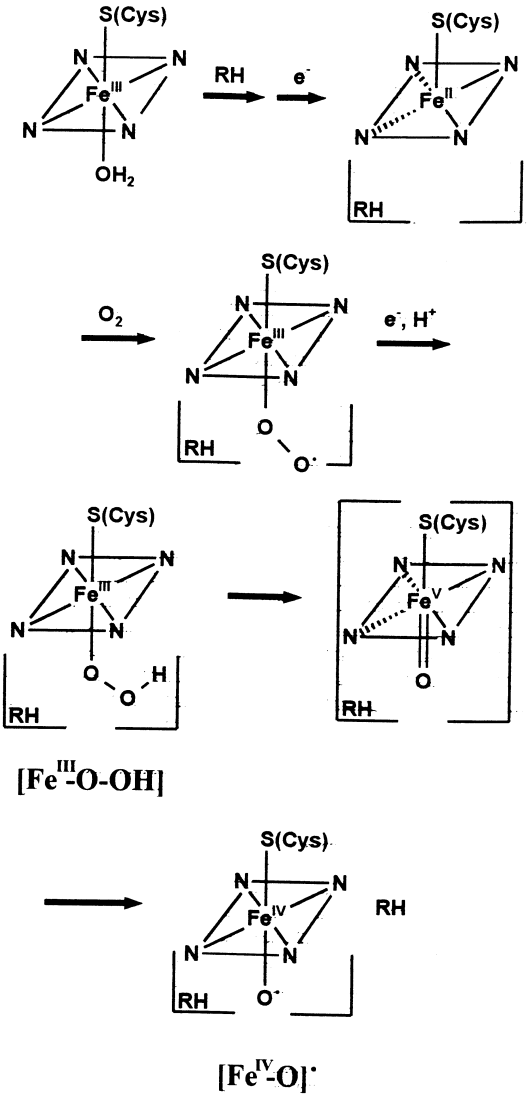


Figure 1 The sequence of events involved in the activation of cytochrome P450 leading to the ferric-hydroperoxy species and to the iron-oxo radical. The enzyme contains a low-spin iron in the resting state, which is converted to high-spin iron upon substrate binding.

predominantly suited to undergo electrophilic additions and the electrophilic rather than the radical character of the oxo-derivative might be emphasized. Under these premises, the addition in 3′ of demethylsuberosin (**17**) produces a 2′-cationic intermediate which would support the spontaneous cyclization to the dihydrofuran coincidentally with the formation of the 3′-tertiary alcohol (Scheme 2). It is experimentally difficult to distinguish this type of reaction mechanism from the pathway involving an intermediate epoxide, since in either case the dihydrofuran-ring oxygen must stem entirely from the umbelliferone substrate and the tertiary marmesin alcohol originates from molecular oxygen. Provided that the substrate specificity of marmesin synthase resides entirely in the prenyl chain, the same enzyme species might also catalyze the cyclization of osthenol to (−)-columbianetin (Scheme 2). However, this reaction has not yet been studied *in vitro*.

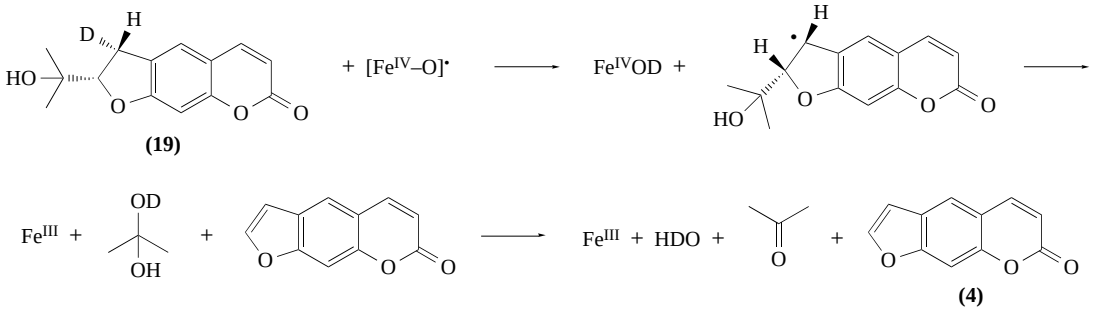
1.24.3.5 Psoralen

The conversion of (+)-marmesin (**19**) to psoralen (**4**) involves a C—C bond cleavage, which formally releases a C_3-fragment in the form of acetone and the concomitant 1′-desaturation of the dihydrofuran moiety[1] (one-step process). The system of carbon numbering (Scheme 2) was chosen

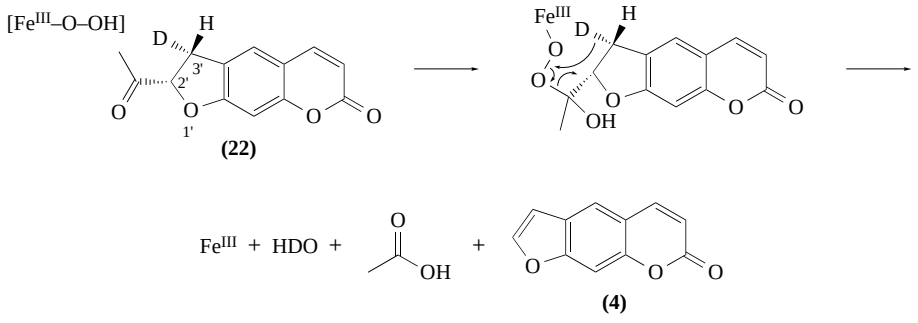
for convenience, as in most of the relevant literature, and does not conform to the standard nomenclature. The release of acetone in a plant biosynthetic pathway is rather unusual and the scheme dates back to an earlier proposal by Birch *et al.*[95] which included the generation of a 3′-carbenium ion with subsequent 1,3-elimination. However, this proposal has not received experimental confirmation. An alternative mechanism might involve the stepwise oxidation and removal of, for example, one carbon of the isopropyloxy side chain in the form of an aldehyde or acid followed by removal of the residual acetyl group (two-step process). Precedent enzymes for the two-step process and C—C bond cleavage can be commonly found in the P450-type steroid metabolism.[90,91] The psoralen synthase was identified as a cytochrome P450 monooxygenase.[50,64] Furthermore, inhibitor studies clearly revealed that the psoralen synthase reaction was catalyzed by a P450 entity different from marmesin synthase.[64] These results are in accordance with the pertinent literature suggesting that plant P450s involved in biosynthetic pathways show narrow substrate specificities in contrast to the detoxifying P450 enzymes.[63,89] The high regio- and stereoselectivity of such enzymes is thought to be imposed by structural constraints within the active site rather than by the nature of the catalytic mechanism.[90]

The common mode of action of P450 monooxygenases, in which the oxo-iron radical species promotes the initial abstraction of hydrogen atoms to yield a carbon radical, is difficult to reconcile with the direct generation of a carbenium ion as anticipated by Birch *et al.*[95] By analogy to the chemical elimination mechanism, the formation of psoralen by psoralen synthase was therefore assumed to involve the 3′-hydroxylation of (+)-marmesin as a typical P450-catalyzed oxygen-rebound process and followed by base-catalyzed *anti*-elimination.[50] A different point of view was put forward by Hakamatsuka *et al.*[96] in the context of rearrangement reactions, suggesting the initial homolytic abstraction of one of the 3′-hydrogens of (+)-marmesin, followed by disproportionation of the primary radical to psoralen concomitant with the release of the isopropyloxy side-chain radical which eventually recombines with the hydroxy radical of the P450-iron-hydroxy (oxygen-rebound process) to yield acetone and water. Neither of these two mechanisms had been sufficiently founded on experimental evidence. Therefore, the reaction mechanism of psoralen synthase was revisited in a fruitful collaboration with Boland and Stanjek.[97] A set of enzyme assays was conducted with microsomal fractions from induced *Ammi majus* cells and employing stereospecifically deuterated (±)-marmesin (**19**) or (±)-2′-acetyl-2′,3′-dihydropsoralen (**22**) (Schemes 3 and 4). The latter compound was included as a pseudosubstrate in an effort eventually to distinguish the one-step from the two-step process of catalysis. The microsomes converted both the substrate and pseudosubstrate to psoralen. Furthermore, the experiments clearly revealed that the reaction proceeds exclusively by *syn*-elimination. The side-chain release of acetate from (±)-2′-acetyl-2′,3′-dihydropsoralen (**22**) (Scheme 4) was reminiscent of the reaction catalyzed by 17α-hydroxylase-17,20-lyase involved in the formation of Δ^{16}-steroid from pregnenolone.[90,92] By analogy, psoralen synthase might therefore catalyze the initial addition of the nucleophilic ferric-hydroperoxy species to the side-chain carbonyl, forming a peroxyhemiketal, followed by decomposition of the adduct to yield acetate and a carbon radical, which then loses a hydrogen atom from the neighboring carbon with coincident desaturation (Scheme 4). In the course of this reaction, one atom of molecular oxygen must be incorporated into the acetate released.[90,92] In contrast to psoralen synthase, the authors reported a *trans*-scission process for the 17α-hydroxylase-17,20-lyase and claimed a radical rather than a concerted mechanism as a first example in C—C bond cleavage by P450 enzymes. This *anti*-elimination reaction, however, must be regarded as an exception to the rule, and the type of elimination is primarily governed by the spatial configuration of the active site rather than the catalytic mechanism. Additional model reactions nevertheless suggested that psoralen synthase operates via a different mode. Tetraphenyl-21*H*,23*H*-porphin-Fe^{III}-complex activated with iodosobenzene also converted deuterated (±)-marmesin or (±)-2′-acetyl-2′,3′-dihydropsoralen to psoralen, but under these conditions both *syn*- and *anti*-elimination was observed. More importantly, however, this activated porphin-Fe^{III} model complex does not deliver the ferrihydroperoxy species and the elimination reactions thus are more likely to have followed the common elimination mechanism which is initiated by the oxo-iron radical species.

In order to obtain unequivocal proof of the type of carbonyl compound released upon the psoralen synthase reaction *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine[98] was used as a trapping reagent in further assays conducted with appropriately deuterated (±)-marmesin. These experiments, in fact, revealed the stoichiometric release of acetone and psoralen from (+)-marmesin and provided the first solid evidence for the mechanistic mode of action of psoralen synthase (Scheme 3). The results predicted that the *syn*-elimination catalyzed by psoralen synthase is not necessarily the consequence of a concerted elimination mechanism. More likely, the oxo-Fe^{IV}-porphyrin radical $[Fe^{IV}—O]^{\bullet}$, acting like an alkoxy radical, abstracts the hydrogen atom from carbon-3′ of



Scheme 3



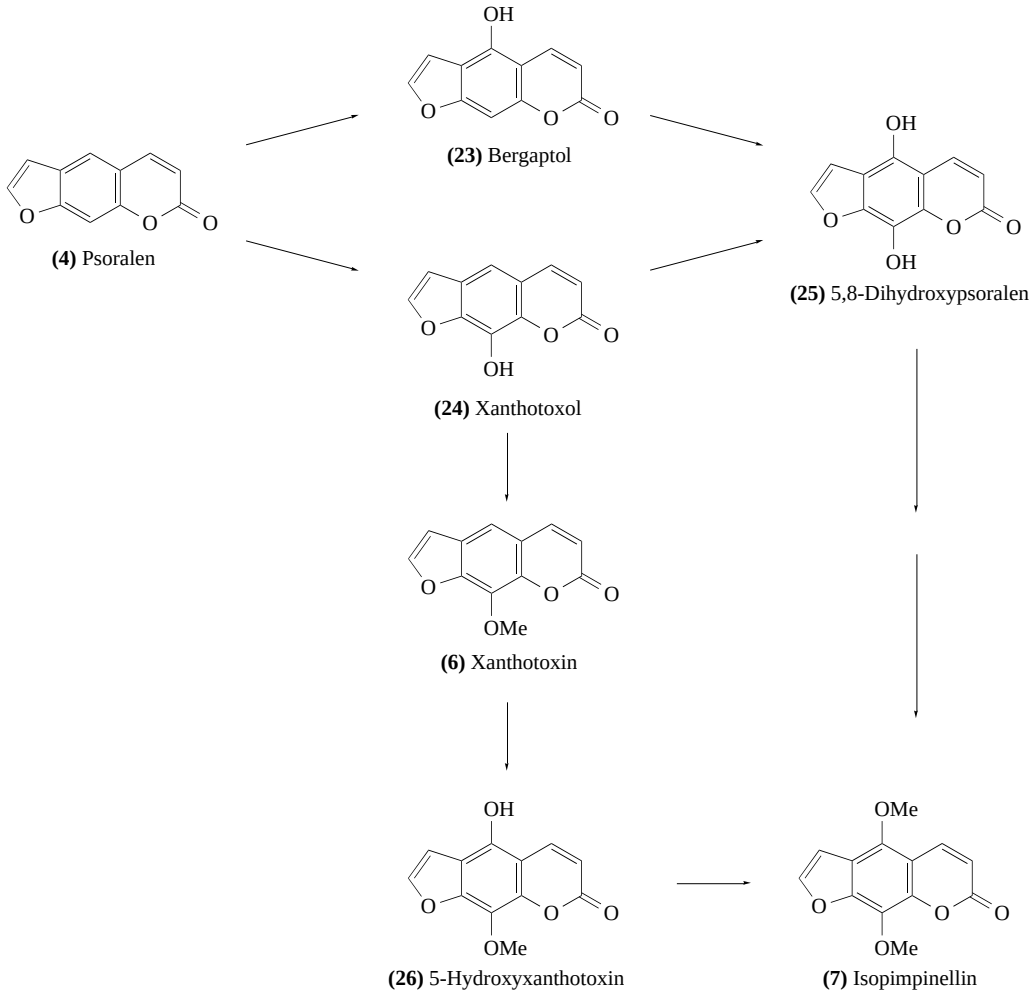
Scheme 4

(+)-marmesin in *syn*-orientation to the isopropyloxy side chain (Scheme 3) and instead of entering an oxygen-rebound process with the carbon radical, the reactive intermediate loses the side-chain isopropyl radical in a disproportionation, which coincidentally recombines with the hydroxy radical (oxygen-rebound) furnishing psoralen, acetone, and water. A primary kinetic isotopic effect ($k_{H\text{-}3'}/k_{D\text{-}3'}$) of about 4 was observed suggesting that the abstraction of the 3′-hydrogen atom is rate-limiting and supporting experimentally the proposed mechanistic sequence. The experiments *stricto sensu* do not rule out an intermediate (+)-3′-hydroxymarmesin. However, such an intermediate was not released during the enzyme assays, a hypothetical hydroxymarmesin would be likely to desaturate by *trans*-elimination, and precedent desaturation reactions by other P450 monooxygenases suggest the direct radical abstraction.[90,91] Overall, this again emphasizes the impact of the particular steric constraints of plant P450s on the course of the reaction, which is also the basis of their narrow substrate specificities. Nevertheless, as mentioned for marmesin synthase the eventual involvement of psoralen synthase in the formation of the angular series of furanocoumarins, i.e., the conversion of (−)-columbianetin (**20**) to angelicin (**21**), remains to be tested. Assays conducted with synthetic (±)-columbianetin and *Ammi majus* microsomes revealed that psoralen synthase does not catalyze the formation of angelicin.[99] Angelicin synthase, therefore, represents a separate enzyme entity which probably acts by a mechanism analogous to that of psoralen synthase.

1.24.3.6 Oxygenated Psoralens

The hydroxylation of psoralen in the 5- and/or 8-position formally yields bergaptol (**23**) or xanthoxol (**24**) and 5,8-dihydroxypsoralen (**25**), respectively. The latter compound is a fairly labile hydroquinone which has nevertheless been employed in precursor feeding studies concerned with the biosynthesis of isopimpinellin (**7**) (Scheme 5) in *Ruta graveolens* shoots,[1,52] although an alternative route via 5-hydroxyxanthotoxin (**26**) may be envisage.[52] These studies suggested that 5,8-dihydroxypsoralen is the major precursor of isopimpinellin,[52] which underlines the metabolic function of the hydroquinone. The sequence of hydroxylations leading to 5,8-dihydroxypsoralen via bergaptol or xanthotoxol has not yet been solved, but a psoralen 5-monooxygenase activity converting psoralen to bergaptol was identified in the microsomes from elicited *Ammi majus* cells.[100] Also this enzyme was identified as a P450 monooxygenase and inhibition studies again pointed to an enzyme different

from marmesin and psoralen synthases.[88] The reaction probably proceeds analogously to cinnamate and many other P450 hydroxylases by the oxygen-rebound process.[90,91] Bergaptol was the only product observed in these assays, suggesting that the formation of 8-hydroxypsoralen (xanthoxol) and 5,8-dihydroxypsoralen requires an additional one or two enzyme(s).



Scheme 5

The hydroxylated furanocoumarins may be further processed by *O*-alkylation and the methoxylated psoralens bergapten, xanthotoxin, and isopimpinellin (Scheme 5) accumulate in many plants as the final products of the pathway. Two *O*-methyltransferases catalyzing the methylation of bergaptol or xanthotoxol to bergapten (BMT) and xanthotoxin (XMT), respectively, were distinguished and extensively purified by affinity chromatography from *Ruta graveolens* and later from *Petroselinum crispum*.[1,52] The substrate specificities of the methyltransferases were considered helpful in clarifying the sequential order of biosynthetic reactions in the course of isopimpinellin biosynthesis. Precursor feeding studies with *Ruta graveolens* cultures had suggested that both xanthotoxin and bergapten were converted to isopimpinellin, although the route from xanthotoxin via 5-hydroxyxanthotoxin was preferred.[1,52] In *Petroselinum crispum* the XMT activity was shown to methylate exclusively xanthotoxol (8-hydroxyxanthotoxol), whereas the BMT catalyzed the 5-*O*-methylation of bergaptol (5-hydroxypsoralen) as well as the 5- and 8-*O*-methylations of 5,8-dihydroxypsoralen[101] (Scheme 5). Furthermore, 5-hydroxyxanthotoxin was methylated at a significantly higher rate than bergaptol and the authors considered another unidentified methyltransferase for the methylation of 8-hydroxybergapten in the course of isopimpinellin biosynthesis. Taken together, the methyltransferase activities did not shed much light on the late steps of isopimpinellin formation and the outcome is remeniscent of the difficulties encountered in studies on the formation of simple cou-

marins where the rather broad substrate specificities of *O*-methyltransferases caused ambiguous results.[36] The subcellular topology of *Petroselinum crispum* BMT in the epithelial cells of oil ducts[102] and the expression of the coding gene have been intensively studied in response to wounding or fungal infection.[103] The induction of BMT gene expression upon fungal elicitation was characterized as rather late in comparison to other genes of the inducible phenylpropanoid pathways,[103] which correlates with the catalytic function in the course of furanocoumarin biosynthesis.

1.24.4 CONCLUSIONS

Most of the biosynthetic pathway leading from cinnamic acid to alkylated furanocoumarins such as isopimpinellin has been unravelled by precursor feeding and *in vitro* studies. Psoralen synthase catalyzes a pivotal reaction in this pathway and the reaction mechanism has been elucidated and shown to differ from that previously proposed by Birch *et al.*[95] Cell culture systems inducible for the accumulation of coumarins provided a major breakthrough for the investigation of the enzymology, and these systems will certainly be helpful in tackling the still controversial course of *ortho*-hydroxylation and/or cyclization reactions of cinnamate and 4-coumarate as well as the classification of the enzyme introducing the 8-hydroxyl function in furanocoumarins. It is striking that 5–6 steps (cinnamate 4-hydroxylation, *ortho*-hydroxylation, formation of marmesin, psoralen, bergaptol and xanthotoxol/dihydroxypsoralen) of the total of 10 steps on the path to isopimpinellin in *Ammi majus* are catalyzed by cytochrome-P450-dependent monooxygenases, which carry out hydroxylation, desaturation, and cyclization reactions. Furthermore, the conversion of *O*-prenylumbelliferone to the corresponding butenyl ethers in *Ammi majus* is most likely to be brought about by P450 monooxygenases, which underlines the importance of this class of enzymes for overall coumarin accumulation. Coumarins accumulate in various plants in response to biotic or abiotic stressors, and it is conceivable that the expression of the committed enzymes must follow a coordinated pattern. One coumarin-specific cDNA (BMT) has been cloned[103] and the cloning of genes coding for the respective P450s will provide insight into their common regulatory sequences as well as into the basis of differential substrate specificities.

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