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Biosynthesis of the Active *Hypericum perforatum* Constituents

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ABSTRACT

Extracts from *Hypericum perforatum* (St. John's wort; Clusiaceae) are widely used for the treatment of mild to moderate depression. Four classes of constituents – hyperforins, hypericins, flavonoids, and xanthones – appear to contribute to the antidepressant activity. Interestingly, all four classes of secondary metabolites involve polyketide derivatives. Key reactions of their biosyntheses are catalysed by type III polyketide synthases. These enzymes differ in the starter substrates used, the number of extender units added, and the mode of intramolecular cyclization catalysed. Their products are metabolised by downstream enzymes, such as prenyltransferases and cytochrome P450 enzymes, to give the final active compounds. Despite the medicinal importance of *H. perforatum*, little is known about the metabolism generating the complex pattern of constituents.

Keywords: acylphloroglucinol, benzophenone, flavonoid, hyperforin, hypericin, polyketide, xanthone
Abbreviations: BPS, benzophenone synthase; **BUS**, isobutyrophenone synthase; **CHI**, chalcone-isomerase; **CHS**, chalcone synthase; **OKS**, oktaketide synthase; **PAL**, phenylalanine-ammonia lyase; **PKS**, polyketide synthase

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INTRODUCTION

Hypericum perforatum (St. John's wort; Clusiaceae) is a well-known medicinal plant which contains a complex mixture of secondary metabolites (Tatsis *et al.* 2007). Dried alcoholic extracts prepared from the flowering upper parts by highly sophisticated processes are widely used for the treatment of mild to moderate depression (Linde 2009). Their therapeutic efficacy was demonstrated in a number of placebo-controlled and comparative clinical trials *versus* standard antidepressants (Whiskey *et al.* 2001; Linde *et al.* 2008). The relatively low rate of adverse effects and the good tolerability lead to a high degree of compliance. *Hypericum* extracts are thus among the best characterised and top-selling herbal medicines worldwide (Percifield *et al.* 2007).

The antidepressant activity of St. John's wort preparations is attributed to several classes of constituents that exhibit additive, synergistic and partly antagonistic effects (Butterweck and Schmidt 2007; Hammer *et al.* 2007). Thus, the total extract is considered the active principle. The pharmacologically best studied constituent is hyperforin with multiple activities (**Fig. 1**; Beerhues 2006; Leuner *et al.* 2007). Flavonoids and hypericins are also among the well-characterised secondary metabolites and contribute to the antidepressant effect (Butterweck and Schmidt 2007). Appreciable quantities of xanthones which also exhibit antidepressant activity were found in the aerial parts of *H. perforatum* collected in India (Muruganandam *et al.* 2000).

Interestingly, all four classes of active constituents involve polyketide derivatives. Key reactions of their biosynthetic pathways are catalysed by type III polyketide synthases (PKSs) which sequentially condense acetyl units from the decarboxylation of malonyl-CoA with a specific starter molecule (Schröder 1999a; Austin and Noel 2003). This reaction sequence is reminiscent of fatty acid biosynthesis and PKSs are evolutionarily related to *ß*-ketoacyl synthases. Type III PKSs form an amazing array of natural products by varying the starter substrate (aliphatic or aromatic units), the number of acetyl additions (one to seven), and the mechanism of ring formation used to cyclise linear polyketide intermediates (Claisen condensation, aldol condensation, or heterocyclic lactone formation) (Austin and Noel 2003; Yu and Jez 2008; Flores-Sanchez and Verpoorte 2009). In *H. perforatum*, these variations in the principal reaction lead to the formation of the four classes of active polyketide derivatives.

FLAVONOIDS

Flavonoids are commonly involved in UV protection, flower pigmentation, and pathogen and herbivore resistance (Winkel-Shirley 2001). They also affect developmental processes, i.e. processes of primary metabolism, such as auxin transport, pollen germination, and signalling to microorganisms (Buer and Muday 2004; Taylor and Grotewold 2005). The flavonoid content in aerial parts of *H. perforatum* is 2-4% (Nahrstedt and Butterweck 1997).

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Invited Review

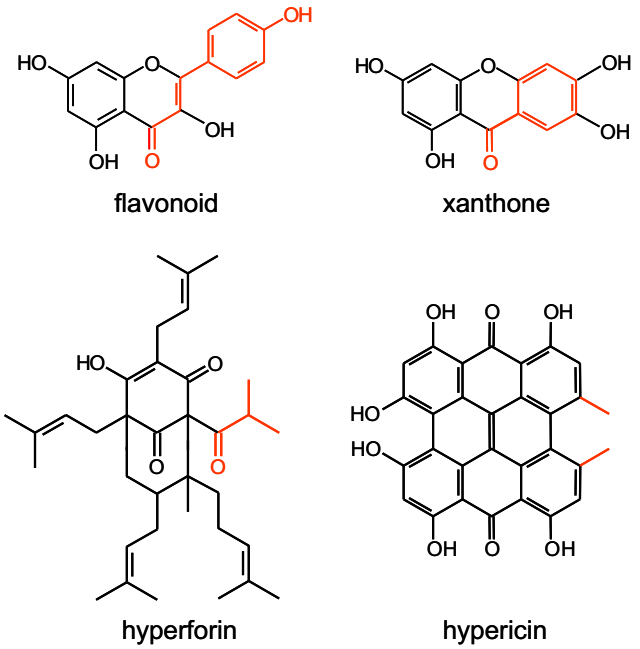

Fig. 1 Classes of active constituents. The biosynthetic starter units are highlighted in red.

In the forced swimming test, the quercetin glycosides isoquercitrin, hyperoside, and miquelianin exhibited antidepressant activity (Butterweck *et al.* 2000; Paulke *et al.* 2008). Furthermore, rutin was found to be essential for the activity of extracts (Nöldner and Schötz, 2002). Flavonoids play an important role in the modulation of the function of the hypothalamic-pituitary-adrenal (HPA) axis (Butterweck *et al.* 2004). The biflavonoid amentoflavone which is a minor constituent of buds and blossoms (0.01-0.05%) efficiently inhibited benzodiazepine binding to the γ-aminobutyric acid (GABA) receptor (Nahrstedt and Butterweck 1997; Baureithel *et al.* 1997; Hölzl and Petersen 2003). Flavonoids are able to penetrate the blood-brain-barrier and to reach the central nervous system (Gutmann *et al.* 2002; Jürgenliemk *et al.* 2003; Paulke *et al.* 2008).

Biosynthesis of flavonoids is initiated by chalcone synthase (CHS). This enzyme is the most common type III PKS in higher plants and the prototype enzyme of the superfamily (Schröder 1999a, 1999b; Austin and Noel 2003). CHS uses 4-coumaroyl-CoA as a starter substrate and catalyses the sequential addition of three acetyl units from the decarboxylation of malonyl-CoA to yield a linear tetraketide (**Fig. 2**). In the same active site cavity, this intermediate is subjected to intramolecular cyclization *via* C6→C1 Claisen condensation to give naringenin chalcone which is released. This product then undergoes either chalcone-isomerase (CHI)-catalysed stereospecific or spontaneous random isomerization to yield naringenin (Jez *et al.* 2000). Downstream enzymes metabolise this flavanone to the diverse subgroups of flavonoids, such as flavonols and flavones (Ferrer *et al.* 2008). The latter group was induced in *H. perforatum* cell cultures by methyl jasmonate treatment (Conceição *et al.* 2006). Similarly, jasmonic acid stimulated flavonoid accumulation and upregulated phenylalanine-ammonia lyase (PAL) and CHI activities (Gadzovska *et al.* 2007). So far, downstream enzymes involved in flavonoid metabolism of *Hypericum* species have not yet been studied.

cDNAs encoding CHSs from *H. perforatum*, *H. androsaemum*, and *H. calycinum* were cloned and the enzymes were functionally expressed in *Escherichia coli* (Liu *et al.* 2003; Klingauf and Beerhues, unpublished). The preferred starter substrates for the PKSs were 4-coumaroyl-CoA and cinnamoyl-CoA. The enzymes were active as homodimers consisting of 41-44 kDa subunits, which is true for all type III PKSs so far studied (Schröder 1999b). CHS from *H. androsaemum* was subjected to site-directed mutagenesis of amino acids shaping the active site cavity (Liu *et al.* 2003). A triple mutant (L263M/F265Y/S338G) preferred benzoyl-CoA over 4-coumaroyl-CoA.

The first crystal structure of a type III PKS, CHS2 from *Medicago sativa*, was determined by Ferrer *et al.* (1999) and provided a framework for understanding the substrate and product specificities and facilitated rational engineering of new enzyme activities. Three catalytic residues are highly conserved in type III PKSs. Cys 164 (numbering in *M. sativa* CHS2) serves as the nucleophile in the loading reaction and as the attachment site of the polyketide during the elongation reactions. The thiolate anion is stabilised by an ionic interaction with His 303 as an imidazolium cation (Jez and Noel 2000). His 303 and Asn 336 catalyse the decarboxylation of malonyl-CoA and stabilise the transition state during the condensation steps. The crystal structure defined, beside this catalytic triad, a number of conserved amino

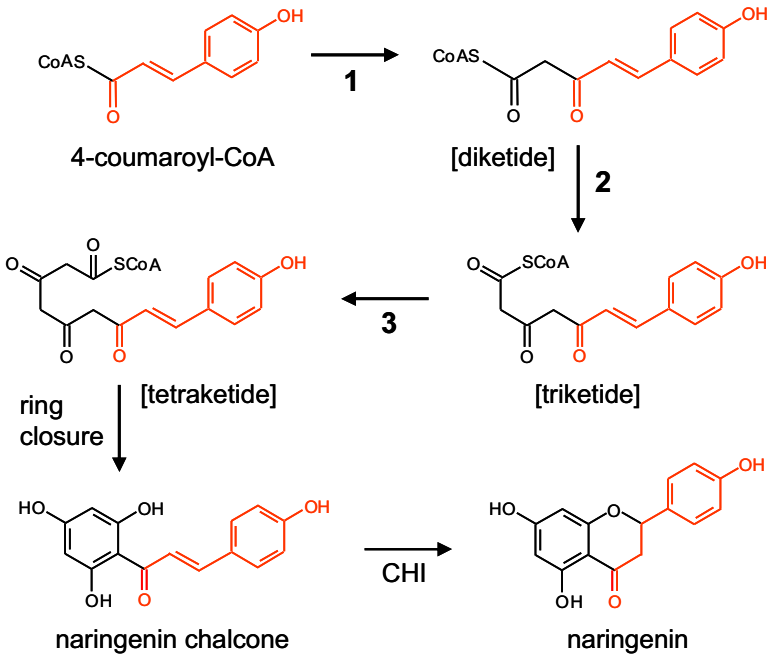

Fig. 2 Reaction mechanism catalysed by chalcone synthase (CHS). 1,2,3: decarboxylative condensations with malonyl-CoA; CHI: chalcone isomerase. Adapted from Schröder 1999b.

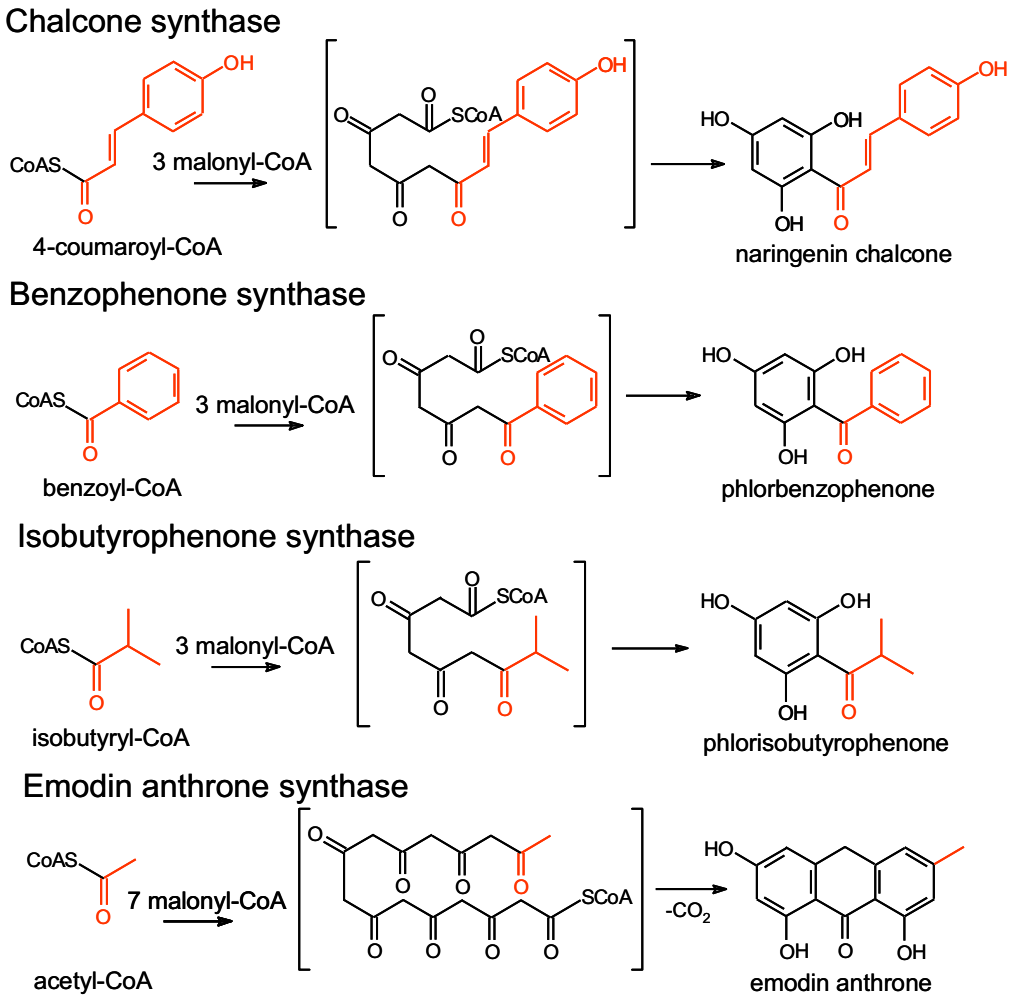

Fig. 3 Reactions catalysed by four type III polyketide synthases in *H. perforatum*.

acids that line the internal bi-lobed initiation/elongation cavity. One lobe of this catalytic center forms the starter unit binding pocket and the other accommodates the growing polyketide chain (Ferrer *et al.* 1999; Austin and Noel 2003).

XANTHONES

Xanthones are commonly minor constituents of the herb of *H. perforatum* (Nahrstedt and Butterweck 1997; Hölzl and Petersen 2003). No xanthones were found in *in vitro* regenerated shoots, in contrast to roots that contain the compounds (Pasqua *et al.* 2003). In cell cultures, xanthone formation was stimulated by addition of a fungal elicitor prepared from *Colletotrichum gloeosporioides* which causes St. John's wort wilt (Gärber and Schenk 2003; Conceição *et al.* 2006). The response was increased by preceding priming using either methyl jasmonate or salicylic acid. Induction of xanthone accumulation by elicitation was also observed with *H. androsaemum* cell cultures, suggesting that xanthones function in *Hypericum* species as phytoalexins against microbial pathogens (Abd El-Mawla *et al.* 2001; Franklin *et al.* 2009). In addition, their radical scavenging properties appear to protect the plant cells from oxidative damage by reactive oxygen species (ROS) (Franklin *et al.* 2009).

Xanthones, such as 1,3,5,8-tetrahydroxyxanthone, 1,5-dihydroxyxanthone, and 1,5,8-trihydroxy-3-methoxyxanthone, are selective and reversible inhibitors of the monoamine oxidase (MAO) isoenzyme A which is involved in oxidative deamination of neurotransmitters (Suzuki *et al.* 1981; Schaufelberger and Hostettmann 1988; Sparenberg *et al.* 1993; Rocha *et al.* 1994). Indian *H. perforatum* plants were found to contain appreciable quantities of xanthones (2-4%; Muruganandam *et al.* 2000). A xanthone-enriched fraction from these plants and some isolated xanthones exhibited significant antidepressant activity in the forced swimming test. They also caused changes in the regulation of some receptors.

The carbon skeleton of xanthones is formed by benzophenone synthase (BPS). cDNAs encoding BPS from cell cultures of *H. androsaemum* and *H. calycinum* were cloned and the enzymes were functionally expressed in *E. coli* (Liu *et al.* 2003; Klingauf and Beerhues, unpublished). BPS cDNAs were also isolated from *H. perforatum* but their products not yet characterised (Franklin *et al.* 2009; Klingauf and Beerhues, unpublished). BPS catalyses the iterative condensation of benzoyl-CoA with three molecules of malonyl-CoA to give a linear tetraketide intermediate which is cyclised into 2,4,6-trihydroxybenzophenone *via* intramolecular Claisen condensation (**Fig. 3**). Treatment of *H. perforatum* cell cultures with elicitor induced a transient accumulation of BPS transcripts, with a maximum mRNA level after 12 h (Franklin *et al.* 2009). In *H. androsaemum* cell cultures, the starter substrate for BPS, benzoyl-CoA, is derived from cinnamic acid by side-chain degradation *via* a CoA-dependent and non-*ß*-oxidative pathway (Abd El-Mawla and Beerhues 2002). Cinnamic acid is supplied by PAL-catalysed oxidative deamination of phenylalanine (Hanson and Havir 1981). Both PAL activity and transcripts were upregulated by elicitor treatment, indicating transcriptional regulation (Abd El-Mawla *et al.* 2001; Franklin *et al.* 2009). Recently, BPS from *H. androsaemum* was converted by a single amino acid substitution in the active site cavity into phenylpyrone synthase, a new type III PKS variant (Klundt *et al.* 2009). The changes in product and substrate specificities were rationalised by homology modeling.

The product of the BPS reaction, 2,4,6-trihydroxyben-

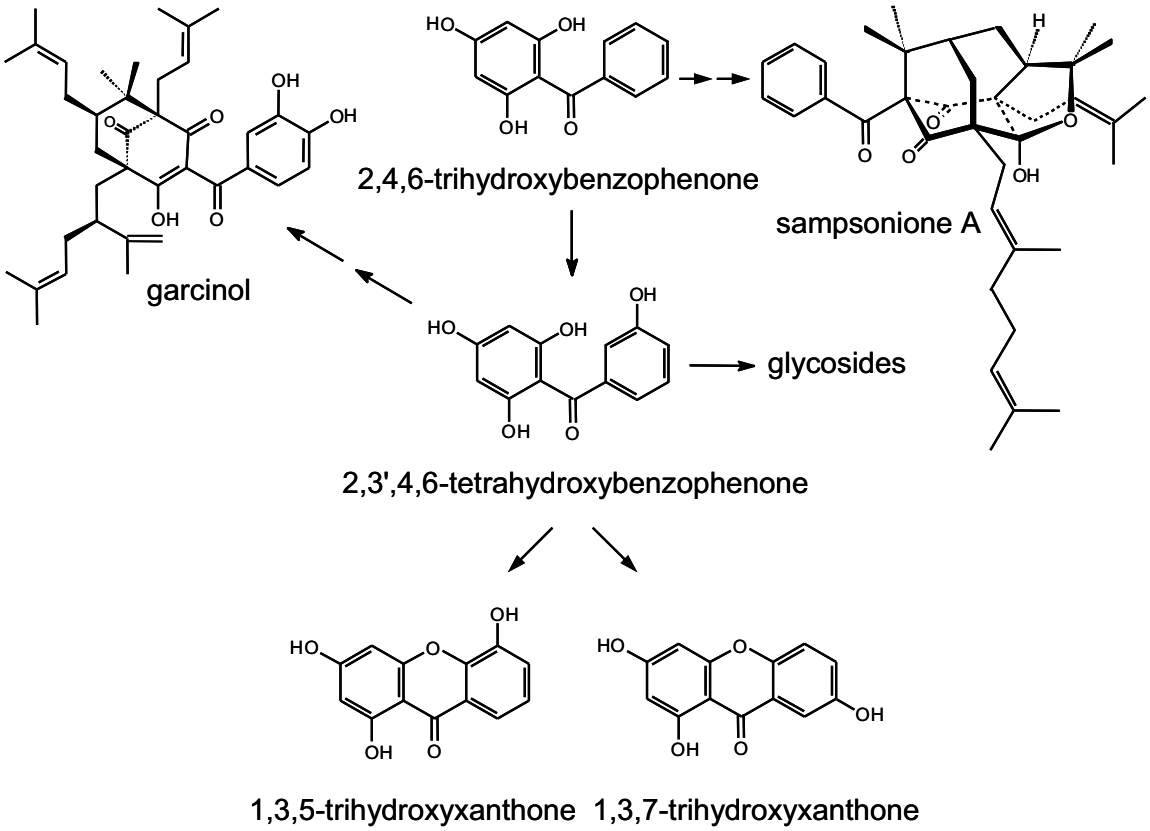

Fig. 4 Prenylation and cyclization of benzophenones, yielding polyprenylated derivatives and xanthones, respectively.

zophenone, is regiospecifically hydroxylated by benzophenone 3'-hydroxylase, a cytochrome P450 monooxygenase, detected in *H. androsaemum* cell cultures (**Fig. 4**; Schmidt and Beerhues 1997). 2,3',4,6-Tetrahydroxybenzophenone undergoes regioselective oxidative phenol coupling reactions either *ortho* or *para* to the 3'-hydroxy group, giving 1,3,5- and 1,3,7-trihydroxyxanthones, respectively (Peters *et al.* 1998). These intramolecular cyclizations are catalysed by cytochrome P450 enzymes named xanthone synthases. The isomeric products appear to be the precursors of all plant xanthones (Bennett and Lee 1989). They are subjected to 6-hydroxylation in cell cultures of *H. androsaemum* and *Centaurium erythraea* (Gentianaceae). The microsomal 6-hydroxylase activities preferred 1,3,7- and 1,3,5-trihydroxyxanthones, respectively (Schmidt *et al.* 2000).

The genus *Hypericum* encompasses about 450 species of trees, shrubs and herbs widely distributed in temperate regions across the globe (Robson 2003). Beside xanthones, polyprenylated polycyclic benzophenone derivatives are characteristic constituents. 2,4,6-Trihydroxybenzophenone and hydroxylated derivatives can undergo stepwise *C*-prenylation. Concomitant intramolecular cyclizations of the attached C_5 and C_{10} isoprenoid side chains result in the formation of bridged polycyclic compounds (**Fig. 4**; Hu and Sim 2000). These complex metabolites fulfill dual function as floral UV pigments and defence compounds (Gronquist *et al.* 2001). In the ovarian wall of *H. calycinum* flowers, polyprenylated benzoyl and acyl phloroglucinols amount to ~20% and protect the developing seeds against herbivores and microorganisms. A number of polyprenylated benzophenone derivatives with bi-, tri-, and tetracyclic skeletons possess interesting pharmacological properties, such as antitumoral and antibacterial activities (Yoshida *et al.* 2005; Hong *et al.* 2006).

HYPERFORINS

Hyperforins are bicyclic polyprenylated acylphloroglucinol derivatives with challenging structures and interesting activities (Beerhues 2006). They are abundant in reproductive organs, their content increasing from 2.5% in buds to 8.5% in capsules (Repčák and Mártonfi 1997; Tekel'ová *et al.* 2000). Relatively low levels are present in undifferentiated *in vitro* cultures, indicating that their accumulation is coupled to organ differentiation (Pasqua *et al.* 2003). Recently, the schizogenous translucent glands of *H. perforatum* were found to be the site of hyperforin accumulation (Soelberg *et al.* 2007; Hölscher *et al.* 2009). Dark nodules contained only minute amounts of hyperforins.

Hyperforin is a broad-band neurotransmitter reuptake inhibitor which does not directly interact with the transmitter transporters but elevates the intracellular sodium concentration, thereby inhibiting the gradient-driven neurotransmitter reuptake (Müller 2003). This is a novel mode of action. Recently, the molecular target of hyperforin was identified, the compound specifically activates TRPC6 channels (Treiber *et al.* 2005; Leuner *et al.* 2007). Transient receptor potential (TRP) channels constitute a group of non-selective cation channels, and hyperforin is the first selective TRPC activator. TRPC6 activation by hyperforin also stimulated keratinocyte differentiation (Müller *et al.* 2008). Furthermore, hyperforin is a promising novel anticancer agent which induces apoptosis, inhibits angiogenesis, and suppresses metastasis formation and lymphangiogenesis (Schempp *et al.* 2002; Donà *et al.* 2004; Martínez-Poveda *et al.* 2005; Rothley *et al.* 2009). Its antibacterial and anti-inflammatory activities may explain the traditional use of St. John's wort extracts for the local treatment of infected wounds and inflammatory skin disorders, respectively (Schempp *et al.* 1999; Albert *et al.* 2002; Hammer *et al.* 2007). Recently, hyperforin turned out to be a novel type of 5-lipoxygenase inhibitor (Feißt *et al.* 2009). However, hyperforin also contributes to drug-drug interactions by binding to the pregnane X receptor and affecting the expression of CYP3A4 and P-glycoprotein (Moore *et al.* 2000; Madabushi *et al.* 2006).

Biosynthesis of hyperforins divides into two sections, i.e. formation of the nucleus and attachment of prenyl side chains. The hyperforin nucleus is formed by isobutyrophenone synthase (BUS). In contrast to CHS and BPS which

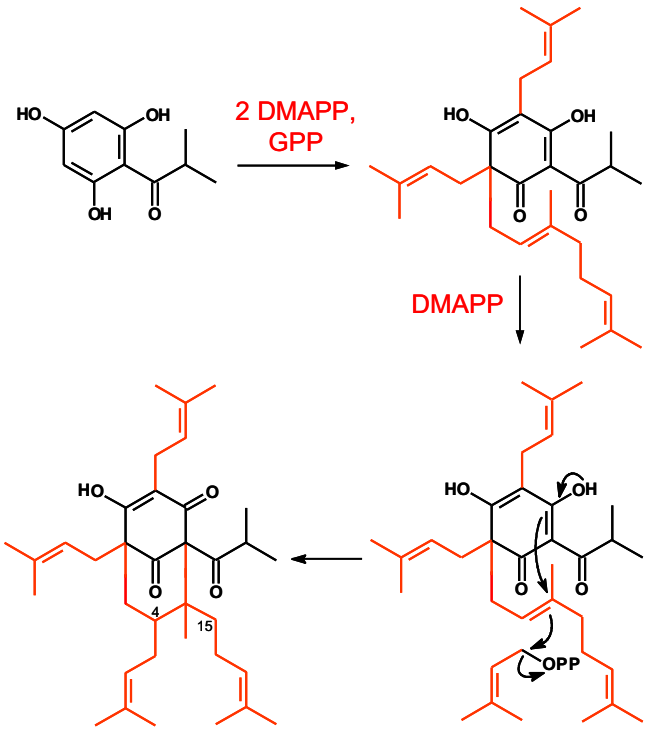

Fig. 5 Prenylation reactions in hyperforin biosynthesis. DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate. Adapted from Adam *et al.* 2002.

prefer aromatic starter substrates, BUS uses an aliphatic starter unit (**Fig. 3**). Isobutyryl-CoA is condensed with three molecules of malonyl-CoA to give a linear tetraketide intermediate which undergoes intramolecular Claisen condensation to yield phlorisobutyrophenone. This reaction was detected in cell-free extracts from *H. calycinum* cell cultures which contain mainly the homologue adhyperforin (Klingauf *et al.* 2005). Formation of hyperforins during cell culture growth was preceded by an increase in BUS activity. Adhyperforin originates from 2-methylbutyryl-CoA as a starter molecule. A putative BUS cDNA was cloned from *H. perforatum* but not yet functionally characterised (Karppinen and Hohtola 2008). However, the tissue-specific pattern of transcript expression correlated with that of hyperforin accumulation. A cDNA for a PKS preferring isovaleryl-CoA (3-methylbutyryl-CoA) was cloned from *Humulus lupulus* cones (Paniego *et al.* 1999; Okada and Ito 2001). Glands of this plant contain constituents that resemble hyperforins but lack the bicyclic structure. Isobutyryl-CoA, 2-methylbutyryl-CoA, and isovaleryl-CoA are derived from the amino acids valine, isoleucine, and leucine, respectively (Adam *et al.* 2002; Karppinen *et al.* 2007). Feeding *H. perforatum* shoot cultures with isoleucine and its biosynthetic precursor, threonine, significantly stimulated the formation of adhyperforin (Karppinen *et al.* 2007).

The hyperforin nucleus undergoes a series of *C*-prenylation reactions (**Fig. 5**). The isoprenoid residues used arise from the non-mevalonate (MEP) pathway (Adam *et al.* 2002). Triple electrophilic substitution of the unsubstituted hyperforin nucleus involves two dimethylallyl diphosphate (DMAPP) units and one geranyl diphosphate (GPP) molecule. The ring closure to give the bicyclic system is triggered by electrophilic attack of a third DMAPP on the 2′/3′ double bond of the preimplanted geranyl chain. The sequence of the steps is largely open. Since hyperforin accumulates together with essential oil in translucent glands, the chloroplast-containing cells surrounding the large cavity were proposed to be the biosynthetic site of both essential oil components and isoprenoid moieties of hyperforin (Soelberg *et al.* 2007). The first *C*-alkylation step is catalysed by a dimethylallyltransferase which was detected in *H. calycinum* cell cultures (Boubakir *et al.* 2005). Formation of hyperforins was preceded by an increase in prenyltransferase activity. The enzyme preferred dimethylallyldiphosphate (DMAPP) as prenyl donor and phlorisobutyrophenone as prenyl acceptor. The activity of the soluble enzyme was dependent on a divalent cation as cofactor, preferrably Fe^{2+}. The majority of so-called aromatic prenyltransferases are integral membrane proteins and contain a typical prenyl diphosphate binding site [(N/D)DXXD] (Pojer *et al.* 2003). Another class of transferases includes soluble enzymes that, however, lack the prenyl diphosphate binding site and thus the absolute requirement for a divalent cation. Prenyltransferases resembling the enzyme from *H. calycinum* cell cultures, i.e. soluble and ion-dependent, participate in the biosynthesis of bitter acids in hop (*Humulus lupulus*) and cannabinoids in hemp (*Cannabis sativa*) (Fellermeier and Zenk 1998; Zuurbier *et al.* 1998).

Recently, a hyperforin homologue lacking a prenyl group at C-15 was detected in *H. perforatum* shoot cultures (Charchoglyan *et al.* 2007). The accumulation of hyperforin and its homologue, secohyperforin, was differentially stimulated by increasing concentrations of N^6-benzylaminopurine and naphthalene-1-acetic acid, respectively, which might be due to differentially regulated aromatic prenyltransferases exhibiting prenyl donor specificity for either DMAPP or GPP. Interestingly, Greek *H. perforatum* was found to contain another hyperforin homologue, hyperfirin, which lacks the prenyl group at C-4 (Tatsis *et al.* 2007).

HYPERICINS

Hypericins constitute the crimson pigments in flowers and leaves. They accumulate in multicellular nodules which appear as dark red to black dots or streaks (Curtis and Lersten 1990; Zobayed *et al.* 2006; Hölscher *et al.* 2009). The content of hypericins in the aerial parts varies between 0.08 and 0.44%, highest levels being observed in the reproductive organs (Hölzl and Petersen 2003). Stamens are particularly rich in dark nodules and thus hypericins (Zobayed *et al.* 2006). In leaves, the black dots are mostly arrayed around the margin. In callus and cell cultures, relatively high levels of hypericins correlated with the presence of globular cell aggregates (Kirakosyan *et al.* 2004). Recently, Kusari *et al.* (2008, 2009) detected an endophytic fungus from *H. perforatum*, identified as *Thielavia subthermophila*, that can accumulate emodin and hypericin in axenic cultures and may have industrial potential to meet the pharmaceutical demand for hypericin.

In the forced swimming test, hypericin and pseudohypericin exerted antidepressant activity when solubilised by addition of procyanidins (Butterweck *et al.* 1998). Evidence for the involvement of the dopaminergic system was also found. Hypericin downregulated the plasma levels of the adrenocorticotrophic hormone (ACTH) and corticosterone and affected the centers that control the activity of the HPA axis (Butterweck *et al.* 2001a, 2001b). A number of patients suffering from depression exhibit hypersecretion of ACTH and plasma cortisol. Hypericins also exhibit light-dependent antiviral activity against various enveloped but not non-enveloped viruses (Meruelo *et al.* 1988; Birt *et al.* 2009 and literature cited therein). Light-sensitised hypericin exhibits multiple modes of antiviral activity, such as inhibition of budding of new virions, cross-linking of capsids preventing viral uncoating, and inhibition of protein kinase activity required for replication of a number of viruses. Hypericins are also responsible for the photosensitizing effect of extracts, which is of limited clinical relevance in the dose ranges used in antidepressant therapy (Schulz 2006). However, due to its light-dependent tumor destructive properties hypericin may be applied as a potent photosensitizer in photodynamic therapy (PDT) of cancer (Kubin *et al.* 2005; Kiesslich *et al.* 2006; Olivo *et al.* 2006). In addition, it is a promising fluorescing agent for use in photodynamic diagnosis (PDD) of cancer (Kiesslich *et al.* 2006; Saw *et al.* 2006).

Biosynthesis of hypericins was proposed to proceed *via*

emodin anthrone (Falk *et al.* 1993). The latter intermediate is likely to be formed by a type III PKS (**Fig. 3**). cDNAs encoding octaketide synthases (OKSs) were cloned from *Aloe arborescens* and *H. perforatum* and the enzymes were heterologously expressed in *E. coli* (Abe *et al.* 2005; Karppinen *et al.* 2008). Both enzymes catalysed the sequential condensation of acetyl-CoA with seven molecules of malonyl-CoA to give an intermediate octaketide which, however, was not cyclised into emodin anthrone *via* three intramolecular aldol condensations but instead converted to unnatural products called SEK 4 and SEK 4b. Since these products were not detected in the plants used as mRNA sources, the OKSs were speculated to be involved *in vivo* in anthrone biosynthesis. *In vitro*, the PKSs might lack cooperating enzymes or other factors that help to fold and cyclise the octaketide chain correctly. OKS from *A. arborescens* could use malonyl-CoA as sole substrate and generate its starter unit by decarboxylation (Abe *et al.* 2005). Recently, two additional OKS cDNAs were cloned from *A. arborescens*; however, the encoded enzymes did not differ functionally (Mizuuchi *et al.* 2009). A single amino acid in *A. arborescens* OKS was found to control polyketide chain length and substrate specificity by modulating the size and shape of the active site cavity (Abe *et al.* 2005). Interestingly, the spatial expression pattern of OKS from *H. perforatum*, as determined by real-time PCR, correlated with the contents of hypericins in various tissues (Karppinen and Hohtola 2008). Furthermore, transcripts encoding OKS from *H. perforatum* were localised in dark nodules of leaf margins, flower petals and stamens using *in situ* RNA hybridization (Karppinen *et al.* 2008). Dark nodules consist of a core of large interior cells surrounded by a biseriate sheath of flattened cells (Curtis and Lersten 1990; Onelli *et al.* 2002). OKS transcripts were detected in the large cells and in some of the innermost flat cells (Karppinen *et al.* 2008). The peripheral cells were previously proposed to be the site of hypericin biosynthesis (Kornfeld *et al.* 2007).

Conversion of emodin to hypericin is catalysed by Hyp-1 (Bais *et al.* 2003). The cDNA for this protein was isolated from a library derived from dark-grown *H. perforatum* cell cultures. The enzymic reaction was discussed to involve an aldol reaction between emodin and emodin anthrone to give dehydrodianthrone which then undergoes conversion to hypericin *via* protohypericin (**Fig. 6**; Bais *et al.* 2003; Dewick 2009). Southern blot analysis indicated that *hyp-1* is a single-copy gene. The endophytic fungus *Thielavia subthermophila* lacked a *hyp-1* gene (Kusari *et al.* 2009). Emodin as a precursor of hypericins was detected in dark nodules but not in surrounding tissues, suggesting that the site of hypericin biosynthesis is the dark nodule (Zobayed *et al.* 2006). However, the tissue-specific expression pattern of *hyp-1*, as determined by quantitative real-time PCR, did not correlate with the occurrence of dark nodules (Košuth *et al.* 2007). Nor was expression of *hyp-1* enhanced at early stages of leaf and concomitant nodule development. In contrast, relatively high expression levels were observed in roots that lack dark nodules and hypericins. These contradictory data raise questions as to the involvement of transport processes and the metabolic role of Hyp-1 (Košuth *et al.* 2007).

PERSPECTIVES

The active constituents of *H. perforatum* exhibit multiple pharmacological activities. Beside antidepressant activity, they possess antitumoral, antibacterial, antiviral, and anti-inflammatory properties. *H. perforatum* is among the best-studied medicinal plants with respect to phytochemistry and pharmacology, however, relatively little is known about the biochemistry and physiology of the active secondary metabolites. Understanding the biosynthetic pathways and the underlying regulatory processes may provide opportunities for metabolic engineering strategies. Manipulation of biosynthetic routes may enable tuning of production levels of secondary metabolites and formation of novel compounds with potentially improved or new properties. Generation of transgenic *H. perforatum* plants *via* either particle bombardment- or *Agrobacterium*-mediated transformation was reported (Di Guardo *et al.* 2003; Vinterhalter *et al.* 2006; Franklin *et al.* 2007, 2008). Alternatively, interesting biosynthetic pathways may be heterologously expressed in microbial hosts for yield improvement and structure modification, provided the genetic basis of these pathways is completely known.

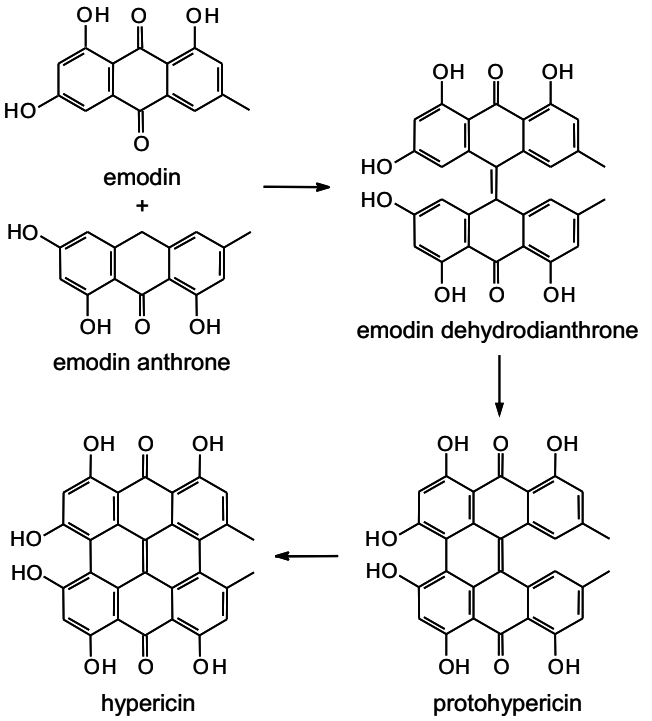

Fig. 6 Formation of hypericins from emodin anthrone and emodin. Adapted from Dewick 2009.

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