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

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 β-ketoacyl syntheses. 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).
PKSs 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 tetra-ketide (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 (Ferré 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 cinnamyl-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 acid residues that define catalytic and structural motifs and might provide a framework for designing new catalysts.

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 2008). Furthermore, rutin was found to be essential for the activity of extracts (Nöldner and Schötz, 2002). Flavonoids efficient inhibited benzodiazepine binding to the $\gamma$-aminobutyric acid (GABA) receptor (Nahrstedt and Butterweck 1997; Baureithel et al. 1997; Holzl 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 tetra-ketide (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 (Ferré 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.

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

**Fig. 2** Reaction mechanism catalysed by chalcone synthase (CHS). 1,2,3: decarboxylative condensations with malonyl-CoA; CHI: chalcone isomerase. Adapted from Schröder 1999b.
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; Höhlz 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 in vitro with *Hypericum* cell cultures, suggesting that xan-thones function in *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-β-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-
zophenone, is regiospecifically hydroxylated by benzophenone 3'-hydroxylase, a cytochrome P450 monoxygenase, 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 by cytochrome P450 enzymes named xanthone synthases. The isomeric products appear to be the precursors of all

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 prenylation. Concomitant intramolecular cyclizations of the attached C5 and C10 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 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 Mártoniť 1997; Tekélová 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 keratinoctye differentiation (Müller 2003). Furthermore, hyperforin is a promising novel anticancer agent which induces apoptosis, inhibits angiogenesis, and suppresses metastasis formation and lymphangiogenesis (Schempp et al. 2002; Doná 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 pregnant 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 isobutyrophene none synthase (BUS). In contrast to CHS and BPS which

| Fig. 4 | Prenylation and cyclization of benzophenones, yielding polyprenylated derivatives and xanthones, respectively. |
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 tetraketidintermediate which undergoes intramolecular Claisen condensation to yield phlorisobutyrophene. This reaction was detected in cell-free extracts from *H. calycinum* cell cultures which contain mainly the homologue adhyperforin (Klingaat 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 (Karpinnen 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; Karpinnen et al. 2007). Feeding *H. perforatum* shoot cultures with isoleucine and its biosynthetic precursors, 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; Zauber 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 N²-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, hyperforin, 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 pseudo-hypericin 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 and are effective against 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 destruc-
tive 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; Karpinnen 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 subsequently 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 OKSs from H. perforatum, as determined by real-time PCR, correlated with the contents of hypericins in various tissues (Karpinnen and Hohlotla 2008). Furthermore, transcripts encoding OKSs from H. perforatum were localised in dark nodules of leaf margins, flower petals and stamens using in situ RNA hybridization (Karpinnen et al. 2008). Dark nodules consist of a core of large interior cells surrounded by a biseriate sheath of flattened cells (Curris and Lerssen 1990; Onelli et al. 2002). OKS transcripts were detected in the large cells and in some of the innermost flat cells (Karpinnen 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 enzyme 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 (Kosuth 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 (Kosuth et al. 2007).

**PERSPECTIVES**

The active constituents of H. perforatum exhibit multiple pharmacological activities. Beside antidepressant activity, they possess antimutual, 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.

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