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Phytochemistry 68 (2007) 1038–1045
PHYTOCHEMISTRY
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Biosynthesis of hyperforin and adhyperforin from amino acid precursors in shoot cultures of *Hypericum perforatum*

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Received 19 October 2006; received in revised form 13 December 2006
Available online 20 February 2007

Abstract

Hyperforin and adhyperforin contribute to the antidepressant effects of *Hypericum perforatum*. The involvement of branched-chain amino acids in the biosynthesis of hyperforin and adhyperforin was demonstrated in *H. perforatum* shoot cultures. L-[U-$^{13}C_5$]Valine and L-[U-$^{13}C_6$]isoleucine, upon administration to the shoot cultures, were incorporated into acyl side chain of hyperforin and adhyperforin, respectively. Feeding the shoot cultures with unlabelled L-isoleucine at a concentration of 2 mM induced a 3.7-fold increase in the production of adhyperforin. The addition of 3 mM L-threonine, a precursor of isoleucine, stimulated a 2.0-fold increase in the accumulation of adhyperforin. The administration of L-valine at concentrations of 0–5 mM had no stimulating effect on the hyperforin production in *H. perforatum* shoot cultures.

Keywords: *Hypericum perforatum*; Biosynthesis; Shoot culture; Adhyperforin; Hyperforin; Isoleucine; Threonine; Valine

1. Introduction

Extracts of *Hypericum perforatum* L., St. John's wort, are widely utilized for the treatment of mild to moderate depression (Müller, 2003). Hyperforin (Fig. 1a) and adhyperforin (Fig. 1b), which are polyprenylated acylphloroglucinols, have been shown to contribute to the antidepressant activity of *H. perforatum* by inhibiting the re-uptake of a number of neurotransmitters (Jensen et al., 2001; Müller, 2003; Medina et al., 2006). Hyperforin has also been found to exhibit antibiotic properties (Schempp et al., 1999) and to display antitumoral effects (Schempp et al., 2002; Medina et al., 2006).

The biosynthetic pathways for hyperforin and adhyperforin are not clear. However, polyketide-type pathways have been proposed (Adam et al., 2002; Klingauf et al., 2005). Quantitative NMR spectroscopy analysis of hyperforin after feeding *H. perforatum* cut sprouts with ^{13}C labelled glucose suggested that the phloroglucinol moiety of hyperforin is generated via a polyketide mechanism (Adam et al., 2002). The polyketide origin of hyperforin is also supported by the detection of type III polyketide synthase activity that catalyzes the condensation of one molecule of isobutyryl-CoA with three molecules of malonyl-CoA to form a hyperforin skeleton in cell-free extracts from *Hypericum calycinum* cell cultures (Klingauf et al., 2005). Adam et al. (2002) showed that isoprenoid moieties of hyperforin are derived predominantly via the deoxyxylulose phosphate pathway. A prenyltransferase activity, which is likely to catalyze the first prenylation step in the hyperforin biosynthesis, has recently been found in *H. calycinum* cell cultures (Boubakir et al., 2005).

Plant cell and tissue cultures have been recognized as promising alternatives to whole plants in the production of secondary metabolites. Numerous strategies have been employed to enhance the productivity of tissue cultures, including precursor feeding (Pras, 1992; Bourgaud et al.,

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doi:10.1016/j.phytochem.2007.01.001

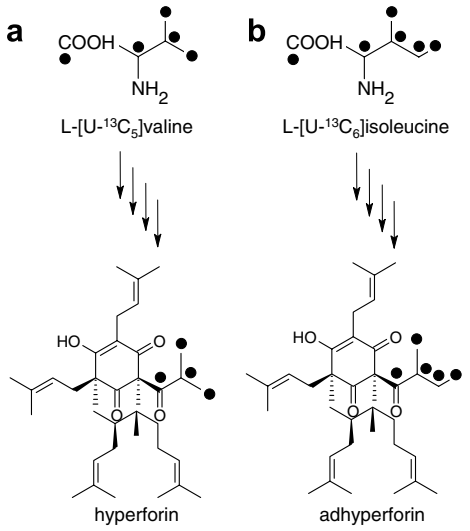

Fig. 1. Biosynthesis of hyperforins from amino acid precursors in shoot cultures of *H. perforatum*. (a) ^{13}C labelled hyperforin biosynthetically derived from L-[U-$^{13}C_5$]valine. (b) ^{13}C labelled adhyperforin biosynthetically derived from L-[U-$^{13}C_6$]isoleucine. '•' denotes position of ^{13}C labels.

2001). An exogenous supply of a biosynthetic precursor may improve the production of specific compounds in plant tissue cultures if the endogenous level of the precursor is a limiting factor of the biosynthesis (Bourgaud et al., 2001). The production of medicinally important secondary metabolites has been successfully enhanced in *in vitro* cultures by feeding cultures with biosynthetic precursors. For example, feeding with geraniol increased the content of the anticancer compound taxol in *Taxus baccata* suspension cultures (Hirasuna et al., 1996), and cinnamyl alcohol enhanced the production of cinnamyl glycosides in compact callus aggregate cultures of *Rhodiola rosea* (György et al., 2004).

In plants, branched-chain amino acids serve as precursors for secondary metabolism in addition to their role in the protein synthesis. In this study, we fed shoot cultures of *H. perforatum* with L-[U-$^{13}C_5$]valine, L-[U-$^{13}C_6$]isoleucine and L-[U-$^{13}C_6$]leucine to examine the involvement of branched-chain amino acids in the biosynthesis of hyperforin and adhyperforin. The incorporation of ^{13}C labels from amino acids into hyperforin and adhyperforin was subsequently monitored by high-performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS). The *H. perforatum* shoot cultures were also fed with unlabelled L-valine, L-isoleucine, L-leucine and L-threonine to explore the possibility to enhance the production of hyperforin and adhyperforin through biotransformation of amino acid precursors.

2. Results and discussion

2.1. Incorporation of ^{13}C labels from branched-chain amino acids into hyperforin and adhyperforin

After the addition of 2 mM L-[U-$^{13}C_5$]valine into shoot cultures of *H. perforatum*, the mass spectrum of hyperforin showed appearance of $[M-H+4]^-$ and $[M-H+5]^-$ molecular species (Fig. 2a) that were not detected in the natural spectrum of hyperforin (Fig. 2b). The abundances of $[M-H+4]^-$ and $[M-H+5]^-$ species of hyperforin in shoot cultures treated with L-[U-$^{13}C_5$]valine (analysed by

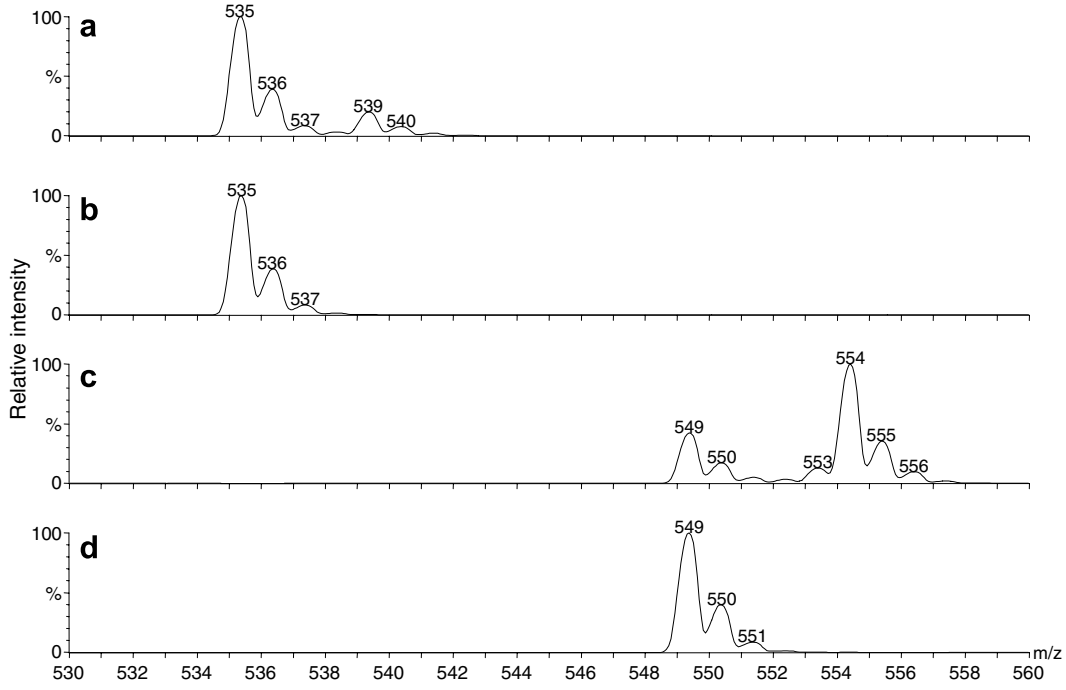

Fig. 2. Mass spectra of (a) hyperforin after treatment with L-[U-$^{13}C_5$]valine, (b) natural hyperforin, (c) adhyperforin after treatment with L-[U-$^{13}C_6$]isoleucine and (d) natural adhyperforin.

HPLC-MS/MS in MRM mode) were 20% and 5.5%, respectively, relative to $[M-H]^-$ (Table 1). These results provide clear evidence for the incorporation of ^{13}C labels from L-[U-$^{13}C_5$]valine into hyperforin. Furthermore, the large abundance of $[M-H+4]^-$ species of hyperforin after L-[U-$^{13}C_5$]valine treatment suggests that four of the five ^{13}C labels of the L-[U-$^{13}C_5$]valine were incorporated into hyperforin. The $[M-H+5]^-$ ion is part of the isotopic cluster of the $[M-H+4]^-$ species of hyperforin. No incorporation of ^{13}C labels from L-[U-$^{13}C_5$]valine into adhyperforin was detected (Table 1), which indicates that L-valine is not an efficient precursor for adhyperforin.

After the treatment of *H. perforatum* shoot cultures with 2 mM L-[U-$^{13}C_6$]isoleucine, the mass spectrum of adhyperforin showed appearance of molecular species (Fig. 2c) that were not detected in the natural spectrum of adhyperforin (Fig. 2d). The abundances of $[M-H+4]^-$ and $[M-H+5]^-$ species of adhyperforin in shoot cultures treated with L-[U-$^{13}C_6$]isoleucine were 23% and 181%, respectively, relative to $[M-H]^-$ (Table 1), clearly indicating the incorporation of ^{13}C labels from L-[U-$^{13}C_6$]isoleucine into adhyperforin. The highest abundance of adhyperforin $[M-H+5]^-$ species after feeding indicates the incorporation of five of the six ^{13}C labels of the L-[U-$^{13}C_6$]isoleucine into adhyperforin. Interestingly, the incorporation rate of L-[U-$^{13}C_6$]isoleucine into adhyperforin was higher compared to the incorporation of L-[U-$^{13}C_5$]valine into hyperforin. This might be due to a low endogenous isoleucine pool in the *H. perforatum* shoots leading to a high rate of incorporation when exogenous L-isoleucine is administered. No incorporation of ^{13}C labels from L-[U-$^{13}C_6$]isoleucine into hyperforin was detected (Table 1), which shows that L-isoleucine is not utilized for the biosynthesis of hyperforin.

L-Leucine was not an efficient precursor for either hyperforin or adhyperforin (Table 1). However, a slight increase in the abundances of $[M-H+4]^-$ and $[M-H+5]^-$ species of adhyperforin were detected after feeding the shoot cultures with 2 mM L-[U-$^{13}C_6$]leucine. This increase is probably caused by impurities, i.e. traces of labelled L-isoleucine in the chemical used for the feeding experiments with labelled L-leucine.

The above results clearly demonstrate the incorporation of valine and isoleucine into hyperforin and adhyperforin, respectively. Thus, our results confirm earlier suggestions about the biosynthetic origin of hyperforin via valine metabolism (Adam et al., 2002). Previously, Adam et al. (2002) suggested a polyketide origin of hyperforin biosynthesis through condensation of three malonyl-CoA with isobutyryl-CoA which could be derived from valine through α-ketoisovalerate. To exactly locate the positions of ^{13}C labels in hyperforin and adhyperforin after feeding the shoot cultures of *H. perforatum* with L-[U-$^{13}C_5$]valine and L-[U-$^{13}C_6$]isoleucine, MS/MS fragmentation studies were performed.

The MS/MS fragmentation spectra and proposed fragmentation patterns of $[M-H]^-$ and $[M-H+4]^-$ ions of hyperforin after treatment with L-[U-$^{13}C_5$]valine, and $[M-H]^-$ and $[M-H+5]^-$ ions of adhyperforin after treatment with L-[U-$^{13}C_6$]isoleucine are shown in Fig. 3. By comparing the fragmentation spectra of $[M-H]^-$ (Fig. 3a) and $[M-H+4]^-$ (Fig. 3b) ions of hyperforin, it can be seen that the peaks at *m/z* 313 and *m/z* 395 are the same for both spectra. All other peaks in the spectra of $[M-H+4]^-$ ion of hyperforin appear four mass units higher compared with the spectra of $[M-H]^-$ ion. The peaks at *m/z* 313 and *m/z* 395 refer to a loss of acyl side chain from ions at *m/z* 383 and at *m/z* 466, respectively, in the MS/MS spectra of $[M-H]^-$ ion of hyperforin and at *m/z* 387 and at *m/z* 470, respectively, in the MS/MS spectra of $[M-H+4]^-$ ion of hyperforin. The analogous fragmentation behaviour can be seen in the spectra of $[M-H]^-$ (Fig. 3c) and $[M-H+5]^-$ (Fig. 3d) ions of adhyperforin. The peaks at *m/z* 313 and *m/z* 395 are the same for both spectra, and all other peaks appear five mass units higher in the spectra of $[M-H+5]^-$ ion of adhyperforin. The peaks at *m/z* 313 and *m/z* 395 refer to a loss of acyl side chain from ions at *m/z* 397 and at *m/z* 480, respectively, in the MS/MS spectra of $[M-H]^-$ ion of adhyperforin and the peaks at *m/z* 402 and at *m/z* 485, respectively, in the MS/MS spectra of $[M-H+5]^-$ ion of adhyperforin. Thus, our results clearly show that the ^{13}C labels from both L-[U-$^{13}C_5$]valine and L-[U-$^{13}C_6$]isoleucine were incorporated into acyl side chains of hyperforin and adhyperforin, respectively (Fig. 1).

The branched-chain amino acids have also been shown to play a crucial role in the biosynthesis of *Humulus lupulus* acylphloroglucinols, which are produced via a polyketide

Table 1
Incorporation of ^{13}C labels from branched-chain amino acids into hyperforin and adhyperforin of *H. perforatum* shoot cultures

Treatment	*n*	Hyperforin		Adhyperforin	
		$[M-H+4]^-$ (%)[a]	$[M-H+5]^-$ (%)[a]	$[M-H+4]^-$ (%)[a]	$[M-H+5]^-$ (%)[a]
Calculated spectrum[b]		0.15	0.015	0.17	0.017
Untreated	6	–	–	–	–
L-[U-$^{13}C_5$]Valine	6	20 ± 7	5.5 ± 2.2	–	–
L-[U-$^{13}C_6$]Isoleucine	4	–	–	23 ± 10	181 ± 74
L-[U-$^{13}C_6$]Leucine	4	–	–	0.50 ± 0.03	2.0 ± 0.2

'–' denotes abundance of <0.3%.

[a] Results are presented as means ± SD percent of peak integrals in HPLC-MRM-chromatograms relative to the non-labelled molecular species $[M-H]^-$.

[b] Produced with MassLynx version 3.5.

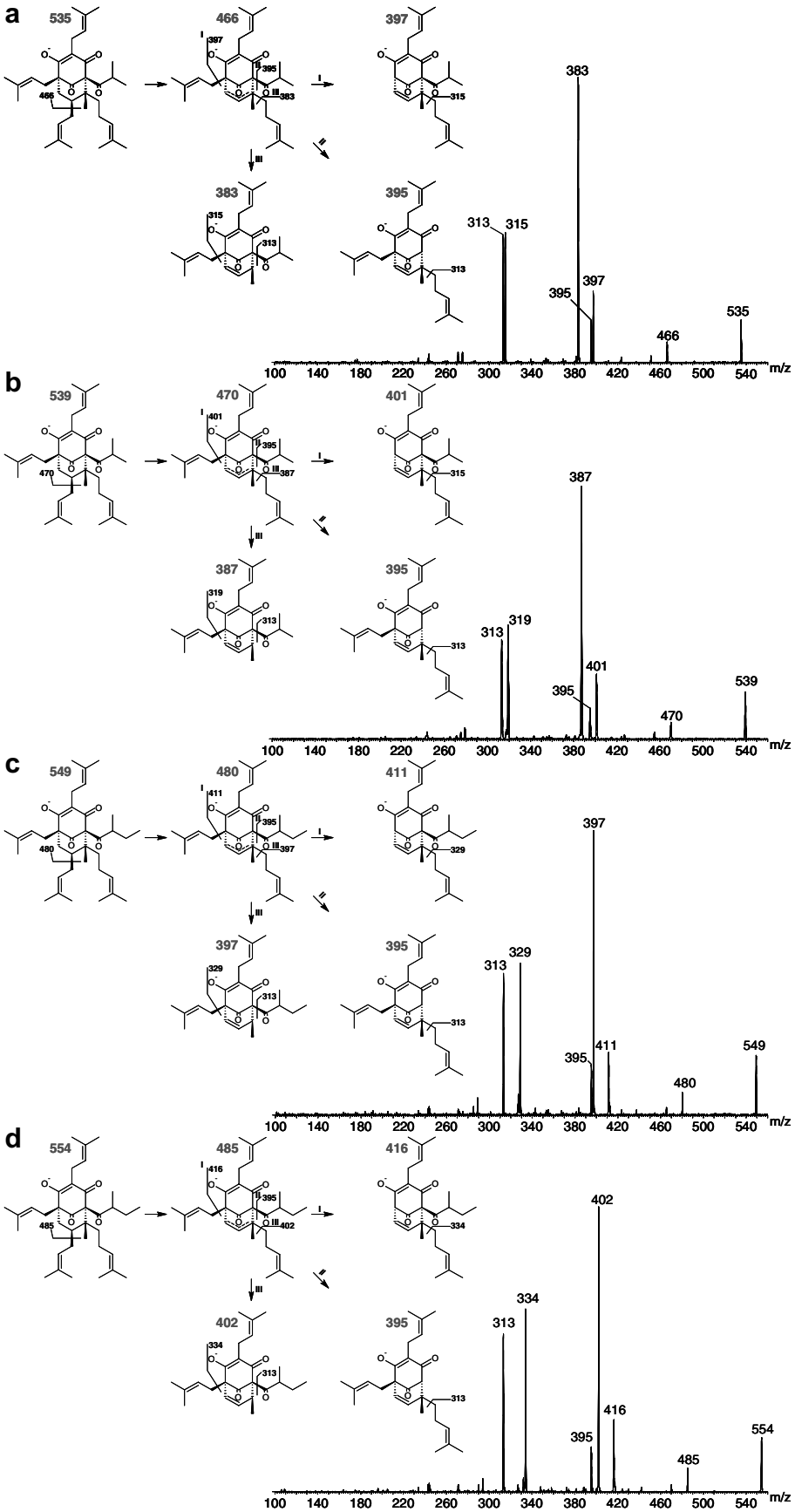

Fig. 3. MS/MS fragmentation spectra and proposed fragmentation patterns of (a) non-labelled $[M-H]^-$ and (b) labelled $[M-H+4]^-$ parent ion of hyperforin after treatment with L-[U-$^{13}C_5$]valine and (c) non-labelled $[M-H]^-$ and (d) labelled $[M-H+5]^-$ parent ion of adhyperforin after treatment with L-[U-$^{13}C_6$]isoleucine.

pathway (Paniego et al., 1999). Valine, isoleucine and leucine have been detected as precursors for isobutyryl, 2-methylbutyryl and isovaleryl residues, respectively, of bitter acid compounds (Drawert and Beier, 1976; Goese et al., 1999). Our results provide support for the previous suggestions that bitter acids and hyperforins might be biosynthesized via the same type of mechanism (Klingauf et al., 2005).

2.2. Biotransformation studies

The supplementation of L-valine or L-leucine at final concentrations of 0-5 mM had no significant stimulating effects on the production of adhyperforin (Fig. 4a) or hyperforin (Fig. 4b) in *H. perforatum* shoot cultures. This implies that although we showed that L-valine is a precursor for hyperforin, the concentration of valine seems not to be the limiting factor in the biosynthesis of hyperforin in *H. perforatum* shoot cultures. Similar observations have been made previously when feeding plant tissue cultures with an indirect precursor has been ineffective in stimulating the biosynthesis of specific secondary products (Robins et al., 1987). To explain this phenomenon, it has been proposed that the precursors of primary metabolism are usually not the factor limiting the secondary metabolite production in plant tissue cultures (Robins et al., 1987).

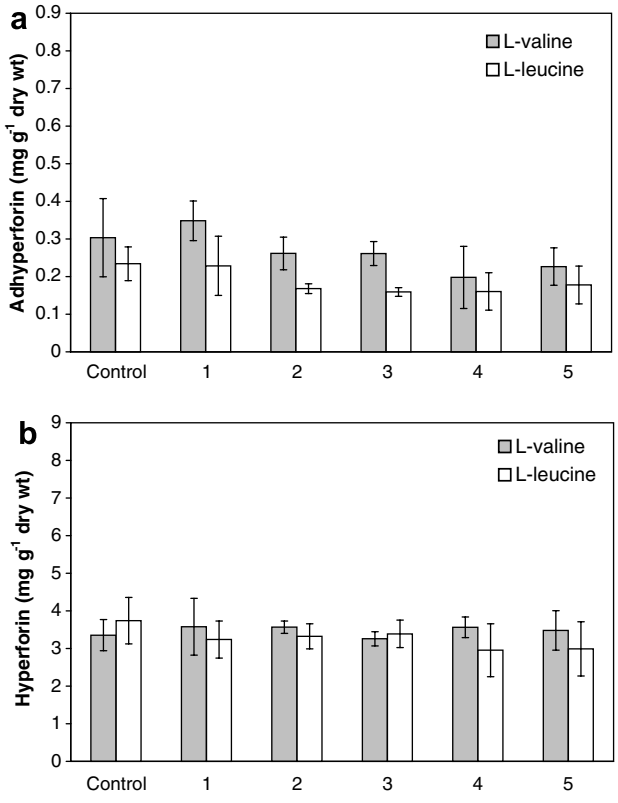

Fig. 4. Effect of different concentrations (mM) of L-valine and L-leucine on the production of (a) adhyperforin and (b) hyperforin in *H. perforatum* shoot cultures. Data represent means ± SD of three replicates.

However, when *H. perforatum* shoot cultures were supplied with exogenous L-isoleucine, the production of adhyperforin was enhanced (Fig. 5a). The concentration of adhyperforin (0.67 ± 0.08 mg g^{-1} dry wt) in the shoots cultured in the presence of 2 mM of L-isoleucine was significantly higher (3.7-fold) in comparison with control cultures (0.18 ± 0.05 mg g^{-1} dry wt). Concentrations that were higher than 2 mM were not equally effective in the stimulation of adhyperforin production.

Treatment with L-threonine, a precursor of isoleucine, also stimulated the production of adhyperforin in *H. perforatum* shoot cultures (Fig. 5a). The adhyperforin concentration (0.68 ± 0.11 mg g^{-1} dry wt) was significantly higher than that of the control cultures (0.35 ± 0.04 mg g^{-1} dry wt) when 3 mM of L-threonine was supplied. Concentrations higher than 3 mM were not equally effective in the stimulation of adhyperforin production. The high concentrations of L-isoleucine and L-threonine could have caused inhibition in the adhyperforin biosynthetic pathway leading to inefficient biotransformation. The hyperforin concentration in shoot cultures of *H. perforatum* was not significantly affected by treatments with either L-isoleucine or L-threonine (Fig. 5b).

Our results show that adhyperforin concentration can be enhanced in *H. perforatum* shoot cultures through bio-

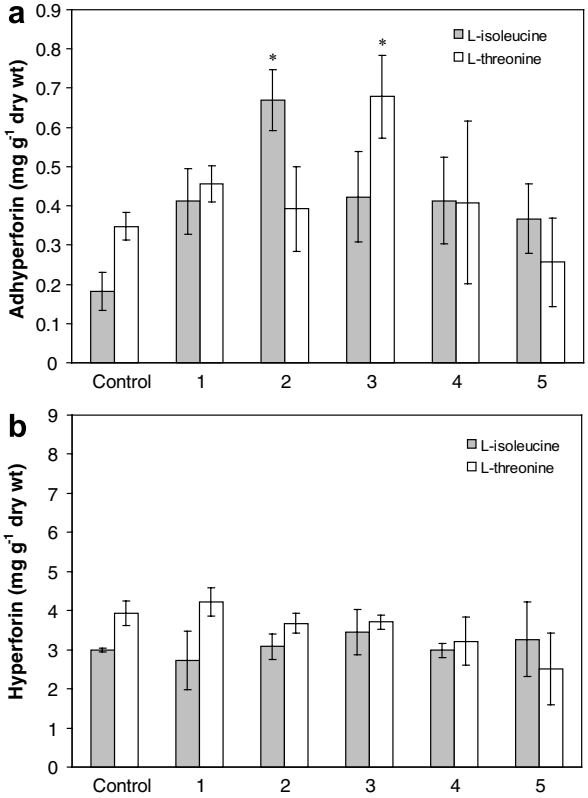

Fig. 5. Effect of different concentrations (mM) of L-isoleucine and L-threonine on the production of (a) adhyperforin and (b) hyperforin in *H. perforatum* shoot cultures. Data represent means ± SD of three replicates and * denotes values that are significantly different from corresponding control (Tukey, $P < 0.05$).

transformation of L-isoleucine and L-threonine while feeding with L-valine was found to be ineffective to elevate hyperforin concentration. The enhancement of adhyperforin concentration after administration of exogenous L-isoleucine indicates that the endogenous isoleucine pool in the *H. perforatum* shoots is low and that the intracellular amount of enzymes needed for the adhyperforin biosynthesis is not limiting. In contrast, hyperforin biosynthesis seems not to be limited by the endogenous valine level and thus hyperforin concentration could not be enhanced by biotransformation of L-valine.

In earlier studies, production of hyperforins has been enhanced in *in vitro* cultures of *H. perforatum* by means of optimization of culture conditions and by elicitation (Kirakosyan et al., 2004). Zobayed et al. (2003) reported an increased hyperforin content in *H. perforatum* plantlets grown at high sucrose concentrations. Hyperforin production was also stimulated by elicitation with salicylic acid and methyl jasmonate in meristem cultures of *H. perforatum* (Sirvent and Gibson, 2002). To our knowledge, no earlier studies on the effects of precursor feeding on the production of hyperforin or adhyperforin have been performed.

In contrast to the shoot cultures, the compact callus aggregate cultures of *H. perforatum*, showing only a low level of tissue differentiation, could not biotransformate L-isoleucine into adhyperforin (data not shown). This indicates that the biosynthesis of adhyperforin requires a higher level of tissue differentiation than what is found in compact callus aggregates. In the case of many medicinal plants, callus cultures that lack cellular organisation and tissue differentiation have failed to produce the desired bioactive compounds that are found in intact plants. This has been claimed to be due to the connection of the biosynthesis of many plant secondary metabolites with morphological and biochemical differentiation processes (Dörnenburg and Knorr, 1995).

3. Conclusions

Due to the importance of *H. perforatum* as a therapeutic agent, the biosynthesis of the major bioactive compounds, hyperforins, has been examined during the recent years (Adam et al., 2002; Boubakir et al., 2005; Klingauf et al., 2005). The data obtained from the present study show that there is an active biosynthetic pathway from valine and isoleucine to hyperforin and adhyperforin, respectively, in *H. perforatum*, and that these amino acids are incorporated into the acyl side chains of these compounds. Furthermore, we showed that precursor feeding with L-isoleucine and L-threonine enhances the production of adhyperforin in shoot cultures of *H. perforatum*. In contrast, the production of hyperforin could not be enhanced by biotransformation of L-valine in this study indicating that the hyperforin biosynthesis is not limited by the amino acid precursor supply. Further research on the regulation of hyperforin and adhyperforin biosynthesis will aid in efficiently enhancing the production of hyperforins in *H. perforatum*.

4. Experimental

4.1. General experimental procedures

Hyperforin standard was purchased from Phytolab Labor Addipharma (Hamburg, Germany). L-[U-$^{13}C_5$]Valine (>98%), L-[U-$^{13}C_6$]isoleucine (>98%) and L-[U-$^{13}C_6$]leucine (>98%) were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Unlabelled amino acids (>98%) were purchased from Sigma (St. Louis, MO, USA). MeCN (LiChrosolv GG), HCO_2H (Suprapur), Me_2CO and MeOH (LiChrosolv GG) were purchased from Merck (Darmstadt, Germany). EtOH (99.5%, by weight) was obtained from Primalco Oy (Rajamäki, Finland). H_2O was purified with a Simplicity 185 water purifier (Millipore, Molsheim, France).

The HPLC-MS/MS analyses were performed with a Waters 2690 Alliance HPLC system (Waters, Milford, MA, USA). Multiple reaction monitoring (MRM) mass spectrometry experiments were performed using a Micromass Quattro II triple quadrupole mass spectrometer (Altrincham, UK) equipped with a Z-spray ionization source. For HPLC-DAD analysis, an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) fitted with Agilent 1100 autosampler, an Agilent 1100 binary pump and an Agilent 1100 photodiode-array detector was used in this study.

4.2. Plant material and initiation of shoot cultures

H. perforatum seeds were provided by the Botanical Garden, University of Helsinki (Helsinki, Finland). The seeds were collected from a wild population in Helsinki, Finland. The seeds were surface sterilized with 3.5% NaOCl for 10 min, followed by three washes with sterile distilled H_2O. For germination, the seeds were placed on a plant growth regulator-free half strength MS medium (Murashige and Skoog, 1962) supplemented with 30 g l^{-1} sucrose, 0.1 g l^{-1} *myo*-inositol and 3.5 g l^{-1} phytagel (Sigma Chemical Co., St. Louis, MO, USA) as a gelling agent. The seeds were incubated at 22 °C under a 16 h photoperiod at 18 μmol m^{-2} s^{-1} irradiance. After germination, the upper parts of the seedlings were excised and transferred onto an MS medium supplemented with 0.1 mg l^{-1} benzyladenine (BA), 30 g l^{-1} sucrose, 0.1 g l^{-1} *myo*-inositol and 3.5 g l^{-1} phytagel for shoot multiplication. The cultures were grown at 22 °C under a 16 h photoperiod at 18 μmol m^{-2} s^{-1} irradiance. A voucher specimen was deposited at the Botanical Garden, University of Oulu (Oulu, Finland).

For initiation of liquid shoot cultures, the shoots were cut into segments containing two leaves. The nodal seg-

ments were transferred into 50-ml Erlenmeyer flasks containing 20 ml MS-based culture media supplemented with 0.1 mg l^{-1} BA, 30 g l^{-1} sucrose, 0.1 g l^{-1} *myo*-inositol and 0.1 g l^{-1} ascorbic acid. This medium is later referred to as MS-S5. The flasks were incubated on a rotary shaker (Infors AG., CH-4103, Bottmingen, Switzerland) at 130 rpm under a 16 h photoperiod at 84 μmol m^{-2} s^{-1} irradiance at 22 °C. Subcultures were carried out in every 5–8 days by cutting the shoots into segments containing two leaves and placing the segments into a fresh MS-S5 medium.

4.3. Feeding with branched-chain amino acids

The feeding experiments with ^{13}C labelled and unlabelled branched-chain amino acids were performed in 50-ml Erlenmeyer flasks containing 20 ml MS-S5 media. The shoots grown in liquid cultures were cut into segments containing two leaves, and 0.14 g fresh weight of segments were inoculated into each flask. The shoot cultures were incubated as described above. For feeding experiments, the amino acids were dissolved in distilled H_2O and filter-sterilised using 0.22 μm membrane filters (Roth, Karlsruhe, Germany). L-[U-$^{13}C_5$]Valine, L-[U-$^{13}C_6$]isoleucine or L-[U-$^{13}C_6$]leucine were aseptically added to the culture media of 12-day-old shoot cultures in a final concentration of 2 mM and incubated for five days. Experiments were performed at least with four replicate flasks. For feeding experiments with unlabelled branched-chain amino acids, L-valine, L-isoleucine, L-leucine or L-threonine were aseptically supplied to the media of 12-day-old shoot cultures to give final concentrations of 0–5 mM in the medium. The cultures were incubated for five days, and experiments were performed with three replicate flasks. In both experiments the control cultures were left untreated. The shoot cultures from the labelling experiments were harvested for HPLC-MS/MS analyses. The shoot cultures from the biotransformation experiments were harvested for HPLC-DAD analyses.

4.4. HPLC-MS/MS analyses

To relatively quantify the effect of feeding with ^{13}C labelled amino acids on different molecular species of hyperforin and adhyperforin, the HPLC-MS/MS analyses from shoot cultures were performed. Sample extraction and chromatographic analysis were performed according to the method described by Tolonen et al. (2002) with small changes. The HPLC separation was performed using a Waters XTerra MS C_{18} 2.1 × 50 mm column with 3.5 μm particle size (Waters, Milford, MA, USA) at 30 °C. The HPLC eluents were aqueous 0.06% HCO_2H (A) and 0.06% HCO_2H in MeCN (B). The initial gradient condition was 40% A and 60% B, changing linearly to 100% B in 10 min. After analysis, the column was washed for 4 min with B and then equilibrated for 6 min with initial gradient conditions, giving a total analysis time of 20 min. The eluent flow rate was 0.3 ml min^{-1}.

The negative ion mode (ESI^-) was used in all experiments. The sample cone voltage of 35 V was used. The desolvation temperature was 300 °C. Nitrogen was used as both drying and nebulising gas with gas flow rates of 420 and 15 l h^{-1}, respectively. The collision gas was argon and the CID gas cell pressure was set to 1.6×10^{-3} mbar.

The dwell time for MRM monitoring each reaction was 0.2 s. Multiplier2 voltage was set to 730 V. The time windows for each reaction were 9–11.5 min for hyperforin species (m/z 535 > 383, m/z 539 > 387 and m/z 540 > 388 for non-labelled, 4-^{13}C and 5-^{13}C labelled, respectively) and 10–12.5 min for adhyperforin species (m/z 549 > 397, m/z 553 > 401 and m/z 554 > 402 for non-labelled, 4-^{13}C and 5-^{13}C labelled, respectively).

The MS/MS fragmentation spectra of $[M-H]^-$ and $[M-H+4]^-$ ions of hyperforin and $[M-H]^-$ and $[M-H+5]^-$ ions of adhyperforin were acquired using the same settings as in the MRM mode, but the last quadrupole (Q3) was set to scan the mass range m/z 50–600.

4.5. Analyses of hyperforins by HPLC-DAD

The HPLC-DAD analyses for the determination of contents of hyperforin and adhyperforin from shoot cultures treated with unlabelled amino acids were performed according to the method described by Tolonen et al. (2003) with small changes. The HPLC separation was performed with Waters XTerra RP_{18} 2.1 × 50 mm column (Waters, Milford, MA, USA) with 3.5 μm particle size together with a Security Guard cartridge C_{18} 4 mm × 3.0 mm precolumn (Phenomenex, Torrance, CA, USA). The temperature of the column oven was 50 °C, and the injection volume was 10 μl. Data were acquired and processed using Hewlett Packard Chemstation Software (Agilent Technologies, Palo Alto, CA, USA).

4.6. Statistical analyses

Statistical analyses were performed by a one-way analysis of variance (ANOVA) using the SPSS program, version 13.0 (SPSS Inc., Chicago, IL, USA).

Acknowledgements

This research was financially supported by grants from the Finnish Konkordia Fund and the Alfred Kordelin Foundation to K.K.

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