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Phytochemistry 66 (2005) 139–145
PHYTOCHEMISTRY
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Biosynthesis of the hyperforin skeleton in *Hypericum calycinum* cell cultures

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Received 3 September 2004; received in revised form 10 November 2004
Available online 18 December 2004

Abstract

Hyperforin is an important antidepressant constituent of *Hypericum perforatum* (St. John's wort). Cell cultures of the related species *H. calycinum* were found to contain the homologue adhyperforin and to a low extent hyperforin, when grown in BDS medium in the dark. Adhyperforin formation paralleled cell culture growth. Cell-free extracts from the cell cultures contained isobutyrophenone synthase activity catalyzing the condensation of isobutyryl-CoA with three molecules of malonyl-CoA to give phlorisobutyrophenone, i.e. the hyperforin skeleton. The formation of the hyperforins during cell culture growth was preceded by an increase in isobutyrophenone synthase activity. The cell cultures also contained benzophenone synthase and chalcone synthase activities which are involved in xanthone and flavonoid biosyntheses, respectively. The three type III polyketide synthases were separated by anion exchange chromatography.

Keywords: *Hypericum calycinum*; Hyperforins; Isobutyrophenone synthase; Benzophenone synthase; Chalcone synthase; Cell cultures

1. Introduction

Alcoholic extracts from the flowering upper parts of *Hypericum perforatum* L. (St. John's wort) are widely used for the treatment of mild to moderate depression (Butterweck, 2003; Müller, 2003). Their antidepressant activity is attributed to hyperforins, flavonoids and hypericins (Butterweck, 2003). Indian *H. perforatum* plants contain appreciable quantities of xanthones which also exhibit antidepressant properties (Muruganandam et al., 2000).

Hyperforin (Fig. 1) is a polyprenylated acylphloroglucinol derivative and inhibits non-selectively the reuptake of a number of neurotransmitters (Müller, 2003). The homologue adhyperforin is a minor constituent of *H. perforatum* and exhibits similar pharmacological properties (Maisenbacher and Kovar, 1992; Jensen et al., 2001). In this study, we report the occurrence of hyperforin and adhyperforin in cell cultures of *H. calycinum* and the detection of three type III polyketide synthases (PKS), one of which participates in hyperforin biosynthesis.

Type III PKS generate a diverse array of secondary metabolites by condensing multiple acetyl units derived from malonyl-CoA to specific starter molecules (Jez

Abbreviations: BPS, benzophenone synthase; BUS, isobutyrophenone synthase; CHS, chalcone synthase; DEAE, diethylaminoethyl; DTT, dithiothreitol; PKS, polyketide synthase.

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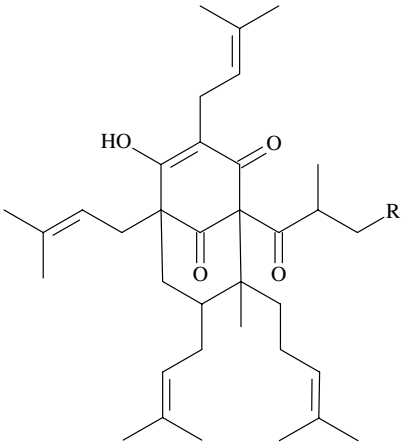
doi:10.1016/j.phytochem.2004.11.003



hyperforin R = H
adhyperforin R = CH_3

Fig. 1. Chemical structures of hyperforins.

et al., 2002). The homodimeric enzymes orchestrate a series of acyltransferase, decarboxylation, condensation, cyclization, and aromatization reactions at two functionally independent active sites. The structure of the active site dictates starter substrate preference, polyketide chain-length, and regio-specificity of polyketide cyclization. These strategies have increased considerably the functional diversity of type III PKS.

2. Results

2.1. Detection of hyperforins

Cell cultures of *H. calycinum* formed adhyperforin and to a low extent hyperforin, when grown in BDS medium in the dark. Light repressed the formation of hyperforins, as previously observed with xanthone production in cultured cells of *H. androsaemum* (Schmidt et al., 2000). The identity of the two hyperforins was shown by co-chromatography (HPLC) with samples of authentic reference compounds and HPLC–MS. The mass spectra of the constituents were consistent with those of the authentic reference compounds and agreed with published data (Maisenbacher and Kovar, 1992; Erdelmeier, 1998). The level of hyperforins in cultured cells was not affected by addition of methyl jasmonate and jasmonoyl-isoleucine. Both elicitors also failed to induce hyperforin formation in cell cultures of *H. androsaemum*, *H. gnidioides*, and *H. perforatum*.

2.2. Characterization of H. calycinum cell cultures

After a 2-day-lag phase, the dry weight of cultured cells increased from day 2 to day 4 before the cell cultures entered the deceleration and stationary phases

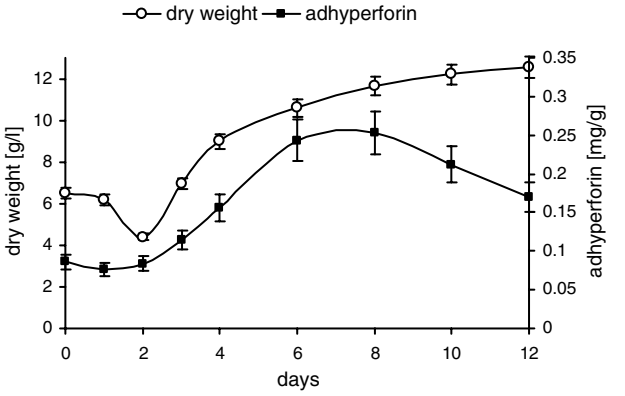


Fig. 2. Growth and adhyperforin formation of *H. calycinum* cell cultures. Bars represent means and standard deviations from three determinations.

(Fig. 2). The formation of adhyperforin paralleled cell culture growth, i.e. accumulation increased linearly from day 2 to day 6, with the maximum yield being reached at day 8. The adhyperforin content was 0.03% of dry weight. The hyperforin content was approx. one tenth of the adhyperforin concentration. No hyperforins were detected in the cell culture medium.

2.3. Detection of isobutyrophenone synthase (BUS) activity

Incubation of desalted cell-free extracts from *H. calycinum* cell cultures with isobutyryl-CoA and malonyl-CoA resulted in the formation of phlorisobutyrophenone (Fig. 3). The enzymatic product was identified by HPLC and GC–MS in comparison with a sample of chemically synthesized reference compound (Fig. 4). Besides co-chromatography, the mass spectrum of the acetylated enzymatic product was identical with that of the acetylated synthetic product. No formation of the enzymatic product occurred when incubation assays contained heat-denatured protein extract.

2.4. Detection of benzophenone synthase (BPS) and chalcone synthase (CHS) activities

Enzymatic conversions were also observed when isobutyryl-CoA as starter substrate was replaced by benzoyl-CoA and 4-coumaroyl-CoA (Fig. 3). The products formed were identified as phlorbenzophenone and naringenin, respectively, by co-chromatography with samples of authentic reference compounds.

2.5. Separation of three PKS by anion exchange chromatography

Cell-free extracts were subjected to ammonium sulphate precipitation. The protein fraction precipitating between 55% and 70% saturation was chromatographed

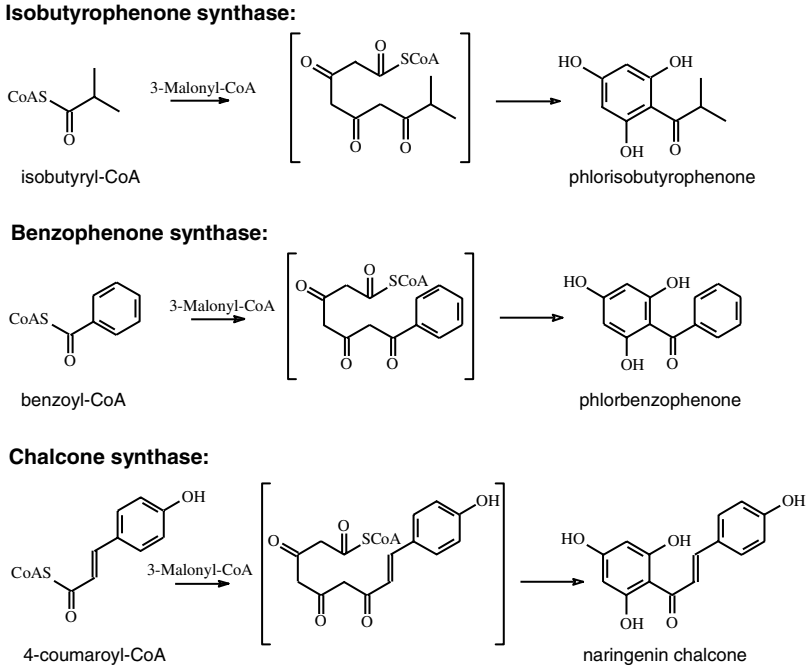


Fig. 3. Reactions catalyzed by three type III PKS from *H. calycinum* cell cultures.

on a DEAE-anion exchange column, resulting in the separation of three PKS activities (Fig. 5). In the order of elution, the enzymes preferred benzoyl-CoA, isobutyryl-CoA and 4-coumaroyl-CoA as starter substrates and converted the respective two other starter substrates less efficiently. Thus, the three PKS resolved were BPS, BUS and CHS, respectively.

2.6. Changes in the PKS activities during cell culture growth

BUS activity rapidly increased after cell culture transfer into new growth medium (Fig. 6). Thereafter, the enzyme activity was high between day 1 and day 4. In contrast, CHS activity increased slowly and reached its maximum at days 4–5. BPS activity appeared to be high at day 3. Its starter substrate benzoyl-CoA was also efficiently converted by BUS but this enzyme activity exhibited the plateau between day 1 and day 4. The maximum specific activities of BUS and CHS were 0.85 and 1.4 μkat/kg. The specific activity with benzoyl-CoA as starter molecule increased to 2.5 μkat/kg.

3. Discussion

Cell cultures of *H. calycinum* were found to be a valuable in vitro system for studying hyperforin biosynthesis. In contrast to intact plants of *H. perforatum* which accumulate hyperforin, cell cultures of *H. calycinum* contained mainly the homologue adhyperforin. So far, hyperforins have not been detected in differentiated plants of *H. calycinum*, although several dearomatized polyprenylated acylphloroglucinols have been isolated from this species (Decosterd et al., 1998; Gronquist et al., 2001). Where the lipophilic compounds accumulate in differentiated plants and cultured cells of *Hypericum* species is still open.

Three type III PKS were detected in cell-free extracts from *H. calycinum* cell cultures and separated by anion exchange chromatography. One of them, BUS, is responsible for the formation of the hyperforin skeleton. It catalyzes the condensation of one molecule of isobutyryl-CoA with three molecules of malonyl-CoA to give phlorisobutyrophenone. As expected, an increase in BUS activity preceded formation of hyperforins. While isobutyryl-CoA is the starter substrate for hyperforin, adhyperforin is derived from 2-methylbutyryl-CoA. The latter substrate appears to be the preferred starter molecule in cell cultures of *H. calycinum* which contain mainly adhyperforin.

An enzyme which functionally resembles BUS participates in the biosynthesis of bitter acids in glandular hairs of hop cones. This enzyme was purified (Paniego et al., 1999) and its gene was isolated (Okada and Ito, 2001). In both species, *Humulus lupulus* and *H. calycinum*, the PKS products undergo stepwise prenylation to give bitter acids and hyperforins, respectively.

Besides BUS, *H. calycinum* cell cultures contained BPS and CHS which form the carbon skeletons of xan- thones and flavonoids, respectively. Both classes of compounds have previously been found in intact *H. calycinum* plants (Decosterd et al., 1998; Gronquist et al., 2001). While CHS is the best-known member of the superfamily of

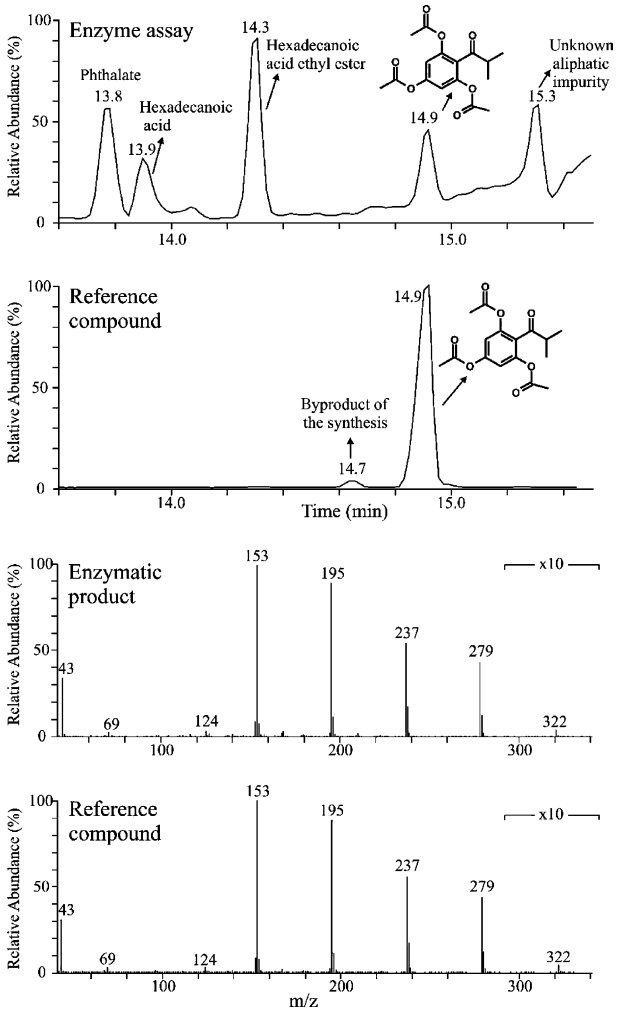


Fig. 4. GC–MS analysis of BUS assays. The enzymatic product and the reference compound were acetylated. Phthalate, hexadecanoic acid, hexadecanoic acid ethyl ester and an unknown aliphatic compound were also detected in control assays containing heat-denatured cell-free extract.

type III PKS (Schröder, 1999), BPS has been cloned only recently from cell cultures of *H. androsaemum* (Liu et al., 2003). BPS preferred benzoyl-CoA as starter substrate and discriminated 4-coumaroyl-CoA which, however, was the most efficient starter molecule for CHS. The products resulting from these two PKS reactions are phlorbenzophenone and naringenin chalcone. These intermediates can undergo intramolecular cyclization to give xanthones and flavonoids, respectively.

Another class of active *Hypericum* constituents are hypericins. These naphthodianthrones result from dimerization of emodin anthrone (Bais et al., 2003). The biosynthesis of these octaketide-derived monomers might be catalyzed by a type III PKS. Recently, a novel CHS-like protein from *Rheum palmatum* was found to form an aromatic heptaketide (Abe et al., 2004). In conclusion, polyketide metabolism plays a central role in the biosynthesis of the active *Hypericum* constituents.

4. Experimental

4.1. Chemicals

Hyperforin and adhyperforin were a kind gift from Dr. Erdelmeier (Dr. Willmar Schwabe, Karlsruhe, Germany). In addition, hyperforin was purchased from HWI Analytik (Rheinzabern, Germany). Methyl jasmonate was obtained from Serva (Heidelberg, Germany). Jasmonoyl-isoleucine was kindly provided by Prof. W. Boland (MPI for Chemical Ecology, Jena, Germany). Benzoyl-CoA, isobutyryl-CoA and malonyl-CoA were obtained from Sigma (Deisenhofen, Germany). 4-Coumaroyl-CoA was synthesized as reported earlier (Abd El-Mawla and Beerhues, 2002). Phlorbenzophenone and naringenin were purchased from ICN (Mecken-

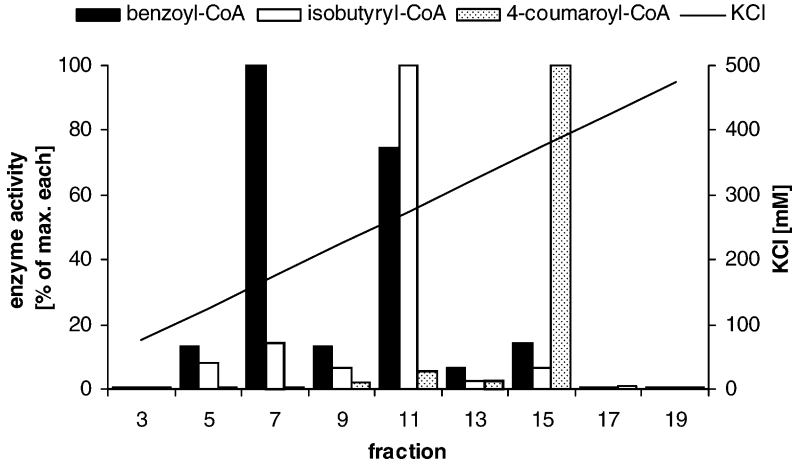


Fig. 5. Separation by DEAE-anion exchange chromatography of BPS, BUS and CHS from *H. calycinum* cell cultures. Data are mean values of two independent experiments.

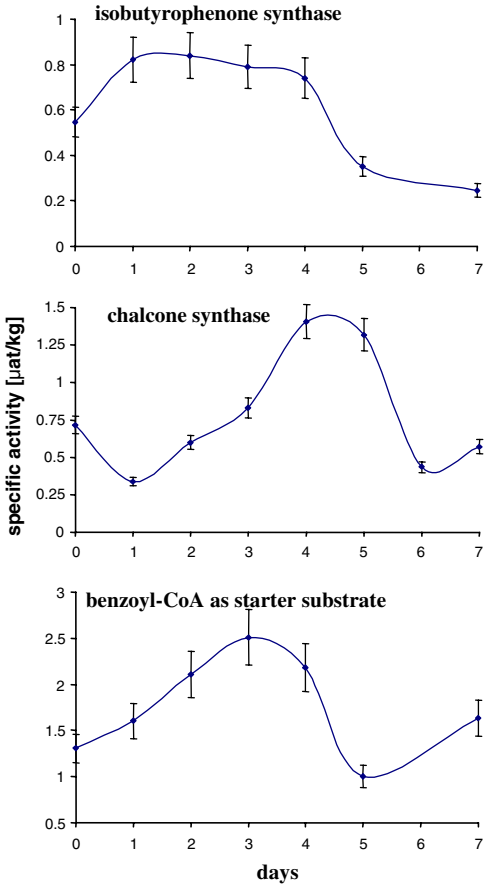


Fig. 6. Changes in the specific activities of BUS and CHS during cell culture growth. Benzoyl-CoA as starter substrate was efficiently converted by both BPS and BUS. Bars represent means and standard deviations from three determinations.

heim, Germany) and Sigma (Taufkirchen, Germany), respectively.

4.2. Cell cultures and elicitation

Callus of *H. calycinum* was initiated from young shoots collected in the Botanical Garden of the University of Bonn, Germany. Surface-sterilized stem segments were placed on LS (Linsmaier and Skoog, 1965), MS (Murashige and Skoog, 1962), 4× (Peters et al., 1998) and BDS media (Gamborg and Eveleigh, 1968). 4× and BDS are modified B5 media. Callus aliquots were transferred to the respective liquid media and the resulting cell cultures (50 ml) were shaken in Erlenmeyer flasks (300 ml) at 100 rpm and 25 °C either in the dark or under continuous light. Cell cultures were propagated by weekly transfer of aliquots (4 g) of the fine suspensions into fresh growth medium.

Four-day-old cell cultures were treated with either methyl jasmonate or jasmonoyl-isoleucine (100 µmol/l each). After 48 h, cultured cells were extracted and hyperforins quantitatively analyzed.

4.3. Extraction of hyperforins

Cultured cells (3 g) were collected by suction filtration and homogenized in a mortar with 5 ml $CHCl_3$ or CH_2Cl_2 for 5 min. After centrifugation at 10,000*g* for 10 min, the organic phase was evaporated to dryness in vacuo and the residue dissolved in 500 µl MeOH. The procedure was carried out at 0–4 °C.

4.4. HPLC

Hyperforins were separated on a Nucleosil C_{18} 100-5 column (25 × 0.4 cm; Macherey-Nagel, Düren, Germany) at a flow rate of 1 ml/min. The solvent was MeOH (85%) and the detection wavelength 275 nm. The R_t value of hyperforin was 6.0, that of adhyperforin 6.7. Standard solutions of authentic compounds were used for quantification.

Enzymatic products were analyzed using the solvents H_2O (A) and MeOH (B) and the following gradient: 40% B for 3 min, 40–70% B in 14 min, then isocratic at 70% B. The detection wavelengths were 285 nm for naringenin, 288 nm for phlorisobutyrophenone, and 306 nm for phlorbenzophenone.

4.5. Spectrometric methods

4.5.1. LC–MS

Analyses were performed on a Beckmann Gold Nouveau HPLC system with gradient pump 126 (Beckmann Coulter, Unterschleißheim, Germany) equipped with a Nucleosil C_{18} 100-5 column (25 × 0.4 cm). MeOH (85%) served as a solvent at a flow rate of 1 ml/min. Mass detection and fragmentation experiments were performed on a Finnigan MAT LCQ mass spectrometer (Thermo-Quest, Egelsbach, Germany) equipped with an ESI source and an ion trap mass analyzer which was controlled by the LCQ Navigator software. The LCQ was operated in the negative ion mode under the following conditions: source voltage 5 kV, capillary voltage −5 V, and capillary temperature 280 °C.

4.5.2. GC–MS

Acetylation of products and subsequent analysis were carried out as described previously (Liu et al., 2004).

4.6. Synthesis of phlorisobutyrophenone (2-methyl-1-(2,4,6-trihydroxyphenyl)-1-propanone)

The reaction was conducted as described by van Klink et al. (1999) starting with 2 g (12 mmol) of dry phloroglucinol (120 °C for 12 h). This was added to a solution of anhydrous $AlCl_3$ (4 g; 120 °C for 12 h) in phosphorous oxychloride (15 ml) under N_2 at 0 °C. Twelve mmol of 2-methylpropanoic acid (1.1 g) were added dropwise and the resulting solution was

stirred for 8 h at 0 °C under N_2, then for 48 h at ca. 6 °C. The reaction mixture was poured on crushed ice (ca. 100 ml) and the resulting mixture extracted three times with 100 ml of diethyl ether. The organic phases were combined and washed with saturated $NaHCO_3$ solution (2×250 ml) and dried over anhydrous Na_2SO_4. The organic solvent was removed and the crude reaction product chromatographed on a silica gel 60 column (2.5×40 cm) using the solvent diethyl ether:pentane (70:30). The NMR spectrum of the purified compound was in agreement with published data (Amer et al., 1983). For GC–MS, the compound was acetylated (Section 4.5).

4.6.1. 2-Methyl-1-(2,4,6-trihydroxyphenyl)-1-propanone

R_f (CH_2Cl_2:EtOAc:HOAc 79:20:1, silica gel) 0.38, (CH_2Cl_2:EtOAc:HOAc 69:30:1, silica gel) 0.84; RI (ZB1) 1930; ^{1}H NMR (400 MHz, acetone-d_6) δ 1.13 (6H, *d*, 2xCH_3, J = 7Hz), 3.97 (1H, sept., $(CH_3)_2CH$), 5.93 (2 H, *s*, $C_{arom.}$*H-3* and *H-5*), 9.14 (1H, *s*, O*H*), 11.7 (2H, *s*, O*H*). EIMS, 70eV, *m*/*z* (rel. int.): 196 (22, $[M]^+$), 153 (100, $[M - C_3H_7]^+$), 154 (7), 69 (6), 43 (4), 41 (4), 197 (2), 111 (2), 67 (2), 55 (2).

4.6.2. 2-Methyl-1-(2,4,6-tri-O-acetylphenyl)-1-propanone

RI (ZB1) 2031; EIMS, 70eV, *m*/*z* (rel. int.): 322 (0.4, $[M]^+$), 153 (100, $[M - C_3H_7 - 3(H_2C{=}C{=}O)]^+$), 195 (84, $[M - C_3H_7 - 2(H_2C{=}C{=}O)]^+$), 237 (59, $[M - C_3H_7 - (H_2C{=}C{=}O)]^+$), 279 (53, $[M - C_3H_7]^+$), 43 (40, H_3C_2O), 238 (19), 280 (15), 196 (11), 152 (10), 154 (7).

4.7. Enzyme extraction

Cultured cells (3 g) were collected by suction filtration, mixed with 0.3 g Polyclar AT (Serva, Heidelberg, Germany) and homogenized for 15 min in 3 ml of 0.1 M potassium phosphate buffer (pH 7.5) containing 10 μM DTT. After centrifugation at 12,000*g* for 10 min, the supernatant was passed through a PD-10 column (Amersham Biosciences, Freiburg, Germany) equilibrated with 0.1 M potassium phosphate buffer, pH 7.5. All steps were carried out at 0–4 °C. Protein was measured as described by Bradford (1976).

4.8. Enzyme assays

PKS assays (0.25 ml) contained 0.1 M potassium phosphate buffer, pH 7.5, 20–35 μg protein, 20 μM malonyl-CoA and 15 μM starter CoA ester. After incubation at 30 °C for 1 h, the reaction mixture was extracted twice with 0.25 ml ethyl acetate. The combined organic phases were evaporated to dryness. The residue was either taken up in 50 μl MeOH for HPLC or subjected to acetylation for GC–MS (Section 4.5).

4.9. Anion exchange chromatography

Crude extract from 2-day-old cultured cells (20 g) was subjected to ammonium sulphate precipitation. The protein fraction precipitating between 55% and 70% saturation was passed through a PD-10 column and loaded onto a DEAE-anion exchange column (HiTrap, 1 ml) connected to a BioLogic System (Bio-Rad, München, Germany). Both columns were purchased from Amersham Biosciences (Freiburg, Germany) and equilibrated with 20 mM Tris-HCL, pH 7.4 containing 1 mM DTT. After removal of unbound proteins by washing with three gel volumes of the same buffer, bound proteins were eluted with a linear gradient from 0 to 500 mM KCl at a flow rate of 2 ml/min. Fractions of 1 ml were collected.

Acknowledgements

We thank Dr. C. Erdelmeier (Dr. Willmar Schwabe GmbH & Co., Karlsruhe, Germany) and Prof. W. Boland (Max-Planck-Institut für Chemische Ökologie, Jena, Germany) for samples of hyperforin/adhyperforin and jasmonoyl-isoleucine, respectively. This work was supported by a Sokrates scholarship from the European Union (to P. Klingauf), a research fellowship from the Egyptian government (to S.A.M. El-Moghazy), a scholarship from the Libyan government (to Z. Boubakir), and a grant from the Deutsche Forschungsgemeinschaft (to L. Beerhues).

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