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Differential accumulation of hyperforin and secohyperforin in *Hypericum perforatum* tissue cultures

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Abstract

Hyperforin is a pharmacologically active constituent of *Hypericum perforatum* (St. John's wort). *In vitro* cultures of this medicinal plant were found to contain hyperforin and three related polyprenylated acylphloroglucinol derivatives. The accumulation of these compounds was coupled to shoot regeneration, with secohyperforin being the major constituent in morphogenic cultures. The structure of secohyperforin was elucidated online by LC-DAD, -MS, and -NMR. In multiple shoot cultures, the ratio of hyperforin to secohyperforin was strongly influenced by the phytohormones N^6-benzylaminopurine (BAP) and naphthalene-1-acetic acid (NAA). While increasing concentrations of BAP stimulated the formation of hyperforin, increasing concentrations of NAA elevated the level of secohyperforin. No differential stimulation was observed after elicitor treatment. Hyperforin and secohyperforin are proposed to arise from a branch point in the biosynthetic pathway.

Keywords: *Hypericum perforatum*; Clusiaceae; *In vitro* cultures; Hyperforin; Secohyperforin; HPLC-NMR; Online analysis; Biosynthesis

1. Introduction

Hypericum perforatum (St. John's wort) is a well-known medicinal plant which contains a complex mixture of secondary metabolites (Tatsis et al., 2007). Preparations from the flowering upper parts are widely used for the treatment of mild to moderate depressions (Müller, 2003). Their therapeutic efficacy was demonstrated in a number of controlled clinical trials (Whiskey et al., 2001). The antidepressant activity is attributed to hyperforins, hypericins, and flavonoids (Butterweck, 2003). The best characterized constituent of *H. perforatum* is hyperforin (**1**, Fig. 1), a polyprenylated bicyclic acylphloroglucinol derivative (Beerhues, 2006).

Hyperforin (**1**) exhibits intriguing pharmacological activities, as reviewed by Beerhues (2006) and Medina et al. (2006). The antidepressant activity is due to the broad-band inhibition of the reuptake of neurotransmitters (Müller, 2003). In addition, compound **1** has antitumoral,

Abbreviations: BAP, N^6-benzylaminopurine; DAD, diode array detector; LC, liquid chromatography; MS, mass spectrometry; NAA, naphthalene-1-acetic acid; NMR, nuclear magnetic resonance.

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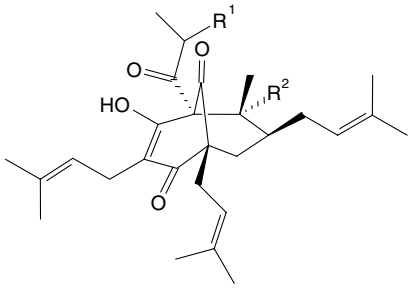

	R^1	R^2
hyperforin (**1**)	CH_3	
adhyperforin (**2**)	CH_2-CH_3	
secohyperforin (**3**)	CH_3	CH_3
adsecohyperforin (**4**)	CH_2-CH_3	CH_3

Fig. 1. Chemical structures of hyperforins **1**–**4**.

antibacterial, and antiinflammatory properties (Schempp et al., 1999, 2002; Albert et al., 2002). However, it also contributes to the adverse effects of St. John's wort preparations by affecting the expression of CYP3A4 and P-glycoprotein (Moore et al., 2000; Gutmann et al., 2006).

The biosynthesis of hyperforin (**1**) is divided into two sections, viz. formation of the nucleus and attachment of the prenyl side chains. The skeleton is formed by isobutyrophenone synthase from isobutyryl-CoA and three molecules of malonyl-CoA (Klingauf et al., 2005). Isobutyryl-CoA is derived from L-valine (Adam et al., 2002; Karppinen et al., 2007). The subsequent prenylation steps involve five isoprenoid units, which arise from the non-mevalonate (MEP) pathway (Adam et al., 2002). The enzyme catalyzing the first prenylation reaction was characterized as a soluble and Fe^{2+}-dependent dimethylallyltransferase (Boubakir et al., 2005). The replacement of isobutyryl-CoA with 2-methylbutyryl-CoA as a starter substrate results in the formation of the homologue adhyperforin (**2**), which is a minor component of St. John's wort extracts.

Here, we report on the structural elucidation of a new hyperforin homologue, secohyperforin (**3**), by online analysis (LC-DAD, -MS, and -NMR) of the crude extract. In addition, the effect of phytohormones and elicitors on the ratio of hyperforin to secohyperforin in *H. perforatum* shoot cultures was studied. Finally, we propose a new branch point in the biosynthetic pathway of hyperforins.

2. Results and discussion

2.1. Identification of hyperforins in multiple shoot cultures of *H. perforatum*

LC-DAD analysis of methanolic extracts from *H. perforatum* shoot cultures revealed the presence of four constituents, **1**–**4**, with hyperforin-like UV spectra, i.e. a single maximum at ~275 nm (Fig. 2). Compound **1** ($R_t = 21.0$) was identified as hyperforin (Fig. 1) by co-chromatography with an authentic reference sample. Studies using LC-ESI MS confirmed this finding ($[M+H]^+$ at $m/z = 537$) and also permitted the assignment of the metabolites **2** ($R_t = 21.5$) and **4** ($R_t = 18.8$) as adhyperforin ($[M+H]^+$ at $m/z = 551$) and adsecohyperforin ($[M+H]^+$ at $m/z = 483$), respectively (Fig. 1). The LC mass spectrum of constituent **3** ($R_t = 18.1$; $[M+H]^+$ at $m/z = 469$) suggested that this compound bears a C_5 unit fewer than hyperforin (**1**). The position of this lacking substituent, however, could not be determined by LC-ESI MS alone. Thus, compound **3** was analyzed directly from the peak by LC-NMR using fully deuterated HPLC solvents to facilitate the measurement of 2D NMR spectra even without any solvent suppression procedure (Table 1).

Analysis of the online ^{1}H NMR spectrum of constituent **3** verified the absence of a C_5 side chain as only three signals appeared in the region around 5 ppm, typical of the protons attached to the double bond of prenyl residues. Furthermore, the ^{1}H NMR spectrum of **3** showed two singlet signals in the highfield region, each corresponding to a methyl group attached to a quaternary carbon atom, as compared to just one such signal in hyperforin (**1**). This already led to the assumption that C-15 in **3** may be a methyl substituent attached to C-3 instead of a further prenylated CH_2 unit as in **1**. The occurrence of a COSY cross signal between CH_3-14 and CH_3-15 clearly evidenced their proximity. Analysis of an online NOESY spectrum revealed cross couplings of CH_3-14 and CH_3-15 to both diastereomeric protons at C-21 and to CH_3-25, i.e. to the CH_2 group and one methyl substituent of the neighboring prenyl residue, respectively. An additional interaction was observed between CH_3-14 and CH-4. Taken together, these findings indicated the structure shown in Fig. 3. The chemical shifts of all proton-bearing carbon atoms were assigned using online-HSQC spectra. To further corroborate the molecular structure of metabolite **3**, online-HMBC spectra were recorded. Interactions between CH_3-14 and C-3, C-4 and C-15 as well as between CH_3-15 and C-3, C-4, and C-14 unambiguously established the structure **3** (Fig. 3).

The structural assignment was further confirmed by a complete attribution of the positions of the two other alkyl-substituted methyl groups, CH_3-12 and CH_3-13. In the online-NOESY spectrum, these groups interacted with each other and furthermore with CH-11. Finally, HMBC interactions clearly evidenced the location of these methyl substituents at the butyrate side chain, particularly due to the strong cross peaks to the carbonyl carbon atom, C-10.

The compound described thus corresponded to secohyperforin (**3**), which was previously reported as a minor constituent of *H. perforatum* by Erdelmeier et al. (1999), but as yet without providing any spectral evidence. Very recently, another hyperforin homologue lacking a C_5 unit has been detected in Greek *H. perforatum* and named hyperfirin (Tatsis et al., 2007). Interestingly, while secohyperforin

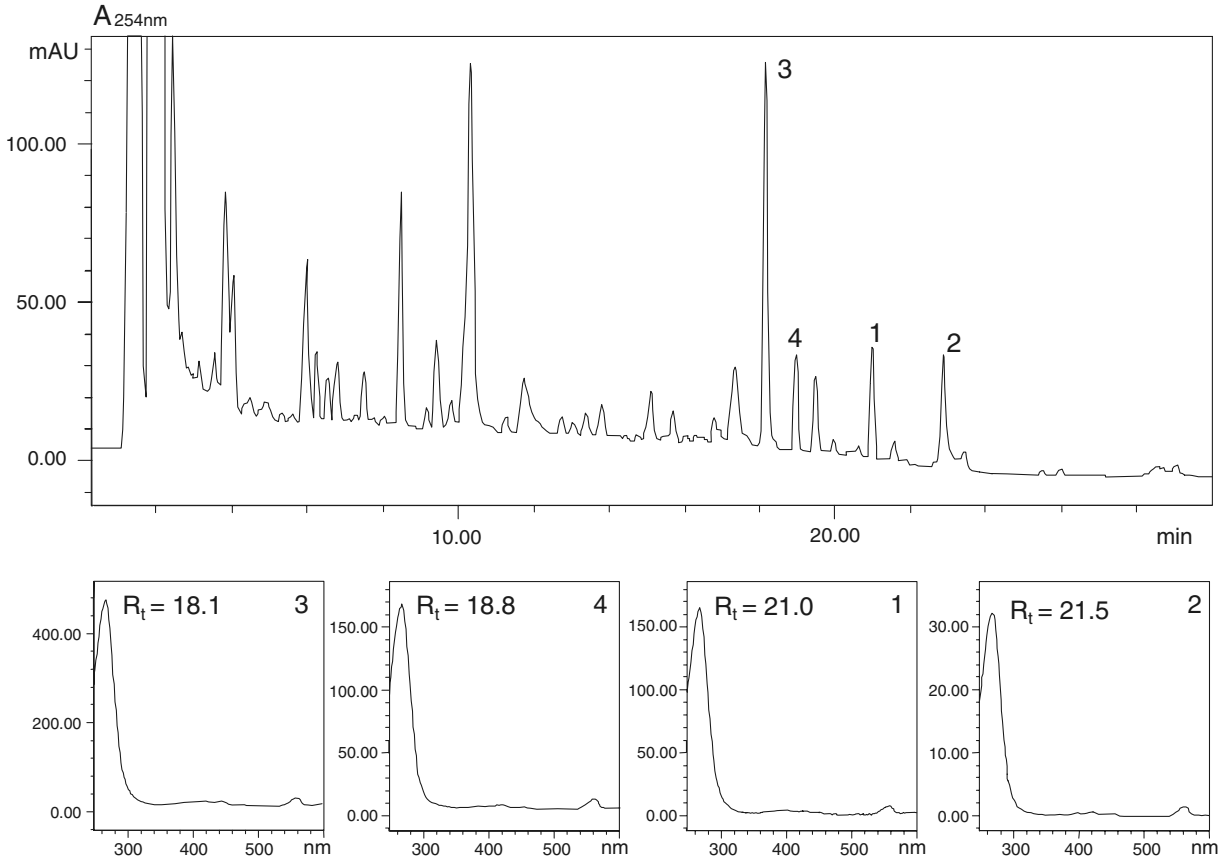

Fig. 2. LC-DAD detection of hyperforins in methanolic extracts from multiple shoot cultures of *H. perforatum*. R_t 18.10: secohyperforin (**3**); R_t 18.85: adsecohyperforin (**4**); R_t 21.04: hyperforin (**1**); R_t 21.53: adhyperforin (**2**).

(**3**) lacks the prenyl group at C-15, hyperfirin lacks the prenyl group at C-4.

Unlike secohyperforin (**3**) and adsecohyperforin (**4**), hyperforin (**1**) and adhyperforin (**2**) were also detected in extracts from flowering plants collected in Armenia. These extracts contained in addition an oxygenated hyperforin derivative (R_t 19.75; $[M+H]^+$ at m/z 553.1), which was previously reported by Brolis et al. (1998). This constituent, however, was not detectable in multiple shoot cultures.

2.2. Ratio of hyperforin (*1*) to secohyperforin (*3*) in different in vitro cultures

Callus cultures of *H. perforatum* contained only low amounts of hyperforin (**1**) and secohyperforin (**3**, Fig. 4). However, high levels of these compounds occurred in morphogenic and shoot cultures, indicating that the formation of hyperforins depends on the regeneration level of the *in vitro* cultures, as found earlier (Dias and Ferreira, 2003). Similar observations were made with hypericins, which accumulate in red-colored secretory cavities (Kirakosyan et al., 2000). Like intact plants, regenerated shoots contain both red-colored and translucent glands. The morphological differentiation of these secretory structures is accompanied by the accumulation of specific secondary metabolites (Čellárová et al., 1995).

In morphogenic cultures, the level of secohyperforin (**3**) was three times that of hyperforin (**1**, Fig. 4). By contrast, similar concentrations of the two compounds were observed in multiple shoot cultures, indicating a developmental regulation of the hyperforin to secohyperforin ratio.

2.3. Effect of elicitors on the formation of hyperforins in in vitro cultures

Callus cultures did not accumulate hyperforins in response to elicitors (jasmonic acid, methyl jasmonate and mannan), which is most likely due to the lack of glandular structures as accumulation site. Translucent glands have recently been found to accumulate hyperforin (Soelberg et al., 2007). Unlike callus, elicitor-treated morphogenic cultures (2-week-old) and multiple shoot cultures contained elevated levels of hyperforins (Fig. 5). A differential stimulation of hyperforin (**1**) and secohyperforin (**3**) formation was not observed. Compared to control cultures, the content of hyperforins was increased twofold after treatment with jasmonic acid or methyl jasmonate and threefold after addition of mannan. Unexpectedly, yeast extract, from which mannan is isolated, inhibited both the formation of hyperforins and the growth of shoot cultures. None of the elicitors used induced morphological changes.

Table 1
NMR (^{1}H 600 MHz, ^{13}C 150 MHz) data of compound **3** in CD_3CN with traces of D_2O (online HPLC-NMR measurements), buffered with 0.01% TFA

Position	^{1}H	*J*	^{13}C	HMBC	NOESY
1			[b]		
2			[b]		
3			46.8		
4	1.49	*m*	43.8		14, 15
5	1.39	*m*	[b]		15
	1.85[a]				
6			[b]		
7			[b]		
8			[b]		
9			[b]		
10			210.6[c]		
11	2.15	br	41.5[c]		12, 13
12	0.94	*d* (6.7)	21.6	10, 11, 13	11, 13
13	1.03	*d* (6.5)	20.9	10, 11, 12	11, 12
14	1.16	*s*	23.6	3, 4, 15	4, 15, 21, 25
15	0.93	*s*	16.0	3, 4, 14	4, 5, 14, 21, 25
21	2.07[a]		27.5[c]		14, 15
22	4.96	*m*	123.6		24
23			133.8[c]		
24	1.64	*s*	25.7	22, 23, 25	22
25	1.53	*s*	17.7	22, 23, 24	14, 15
26	3.06	*d* (6.8)	22.2	32	27
27	5.04	*m*	121.8		27, 29
28			133.9[c]		
29	1.63	*s*	25.6	27, 28, 30	27
30	1.67	*s*	17.9	27, 28, 29	
31	2.40	*dd* (7.1, 14.9)	30.1		
	2.47	br	30.1		
32	4.93	*m*	120.6		32
33			134.4[c]		
34	1.61	*s*	25.8	32, 33, 35	34
35	1.63	*s*	18.0	32, 33, 34	

[a] Chemical shifts taken from NOESY spectra due to signal overlap with solvent signals in ^{1}H NMR.
[b] Signals not detected.
[c] Chemical shifts taken from HMBC cross peaks.

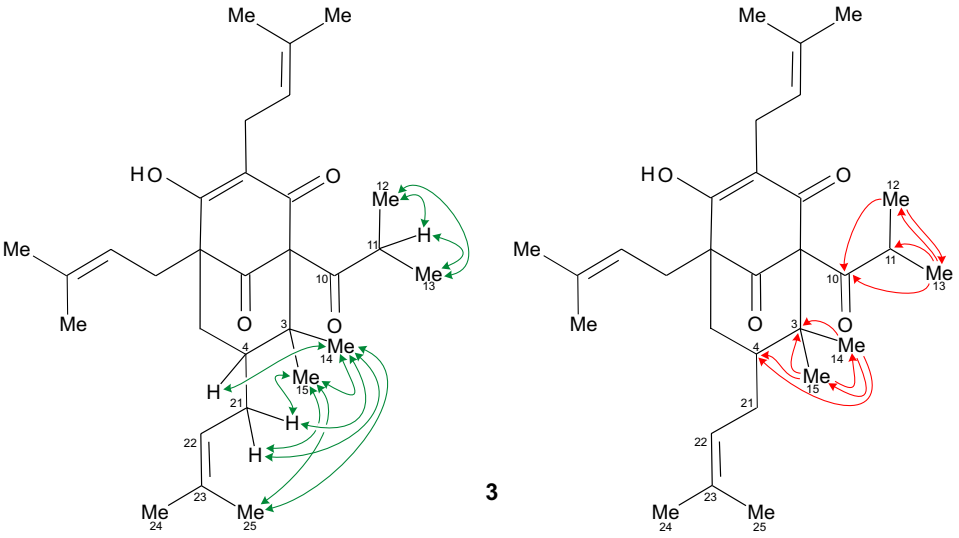

Fig. 3. Important online-NOESY (left) and online-HMBC (right) interactions used for the elucidation of the constitution of secohyperforin (**3**).

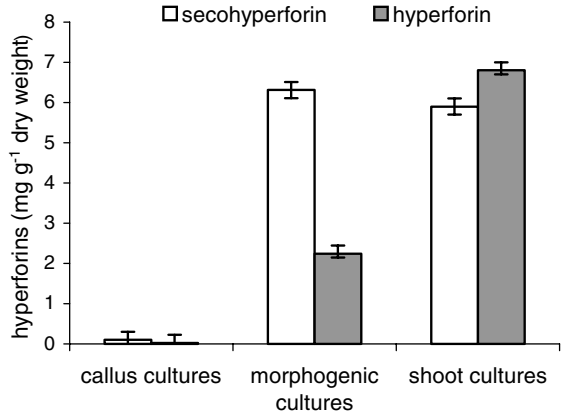

Fig. 4. Hyperforin (**1**) and secohyperforin (**3**) levels in various *H. perforatum in vitro* cultures. Data are means ± SD of three determinations.

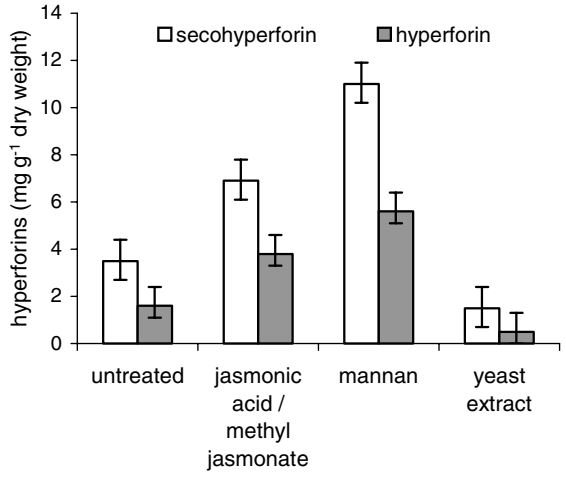

Fig. 5. Effect of elicitors on the formation of hyperforins in multiple shoot cultures of *H. perforatum*. Data are means ± SD of three determinations.

2.4. Effect of phytohormones on the ratio of hyperforin (*1*) to secohyperforin (*3*)

In multiple shoot cultures, varying concentrations of auxins and cytokinins led to morphological and metabolic changes. Increasing concentrations of BAP and kinetin stimulated the formation of dense and dark-green leaves. However, the density of shoots did not correlate with the cytokinin level. Increasing concentrations of NAA and 2,4-dichlorophenoxyacetic acid (2,4-D) led to the formation of yellow, light green or red callus tissue around the basis of developing shoots. In addition, some elongated stems were observed.

At the metabolite level, increasing concentrations of BAP strongly elevated the content of hyperforin (**1**), whereas that of secohyperforin (**3**) remained constant (Fig. 6). By contrast, increasing concentrations of NAA considerably stimulated the formation of secohyperforin (**3**), whereas that of hyperforin (**1**) was constant. This differential stimulation provides the opportunity for selective production of desired hyperforins in *H. perforatum* shoot cultures.

2.5. Proposed branch point in the biosynthetic pathway

In conclusion, secohyperforin (**3**) is not simply a by-product of hyperforin biosynthesis. The formation of both, hyperforin (**1**) and secohyperforin (**3**), appears to be highly regulated by endogenous stimuli. The two compounds are proposed to result from a branch point in the biosynthetic pathway (Fig. 7). A route leading to the formation of hyperforin has been suggested by Adam et al. (2002). The diprenylated acylphloroglucinol intermediate is linked to either a dimethylallyl or a geranyl residue. Biochemical studies are in progress to reveal whether metabolic channeling at the branch point is due to differentially regulated aromatic prenyltransferases which exhibit prenyl donor specificity for either DMAPP or GPP.

Our findings contribute to the elucidation of the complexity of hyperforin metabolism. Formation of secohyperforin in shoot cultures and the recently detected accumulation of hyperfirin in Greek plants (Tatsis et al., 2007) are interesting branch pathways of hyperforin biosynthesis. In addition, high levels of adhyperforin were found in *H. perforatum* fruits (Maisenbacher and Kovar, 1992). Whether these hyperforin-like compounds serve different functions in the plant remains to be established.

3. Experimental

3.1. Chemicals

An authentic sample of hyperforin (**1**) was a kind gift from Prof. Panossian (Gulbenkian Drug Research Laboratory, Yerevan, Armenia). All other chemicals were purchased from Sigma (Taufkirchen, Germany). Solvents were of HPLC grade.

3.2. In vitro cultures

Callus and shoot cultures of *H. perforatum* were established as described previously (Kirakosyan et al., 2000). They were grown on a medium consisting of the basal salts of MS medium (Murashige and Skoog, 1962) and 30 g l^{-1} sucrose, 0.1 mg l^{-1} thiamine HCl, 0.1 mg l^{-1} nicotinic acid, and 0.1 mg l^{-1} pyridoxin HCl. The pH value was adjusted to 5.8. After autoclaving, the phytohormones were added from stock solutions prepared in DMSO and sterilized by filtration. The medium for callus cultures contained 0.05 mg l^{-1} NAA, that for shoot cultures in addition 0.5 mg l^{-1} BAP. All types of cultures were incubated in 50 ml medium in 500 ml Erlenmeyer flasks on a rotary shaker at 80 rpm, 25 °C, and 16 h photoperiod. Clusters of 7–10 shoots were subcultured at 15-day intervals, rare elongated shoots were eliminated.

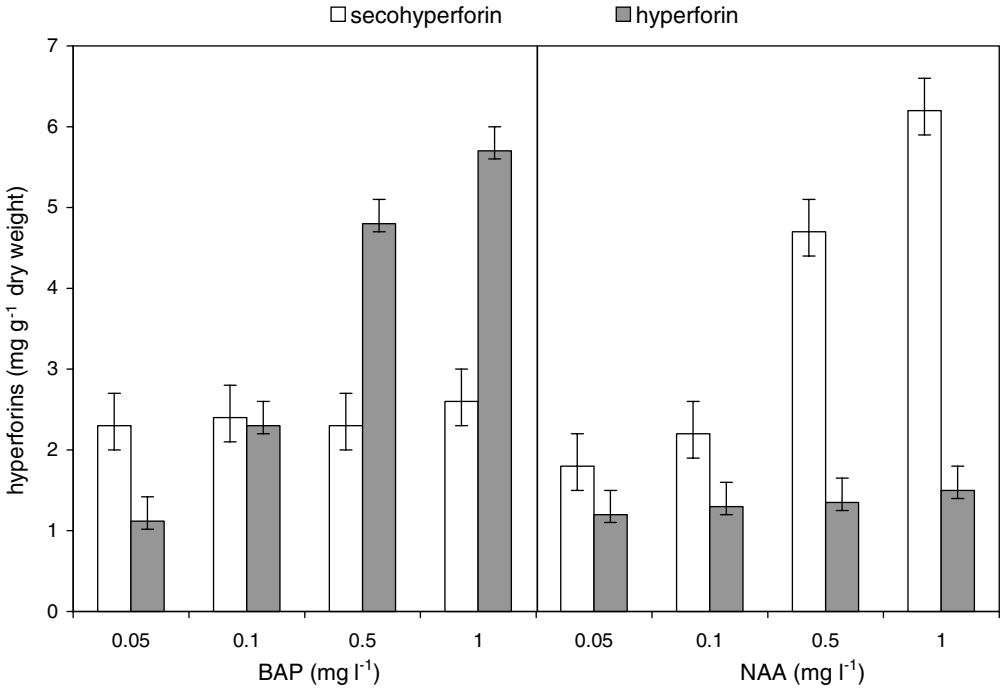

Fig. 6. Effect of increasing concentrations of BAP (in the presence of 0.05 mg l^{-1} NAA) and NAA (in the presence of 0.05 mg l^{-1} BAP) on the formation of hyperforin (**1**) and secohyperforin (**3**) in multiple shoot cultures of *H. perforatum*. Data are means ± SD of three determinations.

3.3. Intact plants

Flowering plants of *H. perforatum* were harvested in Yerevan, Armenia, and at the Institute of Plant Biochemistry (Halle/Saale, Germany) and freeze-dried.

3.4. Elicitation

Elicitors were used at the following final concentrations: jasmonic acid and methyl jasmonate (2.5 mg l^{-1}), mannan (2.0 mg l^{-1}) and yeast extract (0.5 mg l^{-1}). The elicitors were added to the medium before autoclaving. Flasks were then inoculated with approximately 3 g of biomass and shoots were harvested after 3 days of elicitor treatment. The analytical data presented are mean values of three replicates.

3.5. Extraction of hyperforins

Freshly harvested biomass (1 g) was freeze-dried and extracted overnight with 10 ml methanol containing 10 mM ascorbic acid. After filtration, the extract was evaporated to dryness in vacuo and the residue was dissolved in 2 ml methanol. An aliquot (20 μl) was analyzed by reversed-phase HPLC.

3.6. LC-DAD

A Tosoh 8020 system and a Hewlett-Packard 1100 system were used, both equipped with an autosampler and a photodiode array detector. Reversed-phase separations were carried out on an ODS-80TM column (4.6 × 150 mm; Tosoh) or a Eurospher-100 RP18 column (4 × 250 mm; Knauer), both maintained at 30 °C. The mobile phase consisted of a linear gradient from 50% CH_3CN in H_2O containing 0.01% phosphoric acid to 100% CH_3CN over 15 min, followed by isocratic elution at 100% CH_3CN for 15–30 min. The flow rate and the detection wavelength were 0.8 ml min^{-1} and 254 nm, respectively.

3.7. LC-MS

LC-ESI MS was performed on a ThermoQuest system equipped with a reversed-phase ODS-80TM column. The conditions were the same as described for HPLC. LC retention times of secohyperforin (**3**), adsecohyperforin (**4**), hyperforin (**1**), and adhyperforin (**2**) were 18.10, 18.85, 21.04 and 21.53 min, respectively. Multistage LC-ESI MS (positive): hyperforin (**1**) MS 537 $[M+H]^+$; MS/MS (precursor ion at *m/z* 537), 469, 413, 345. Multistage LC-ESI MS (negative): MS 535 $[M-H]^-$; MS/MS (precursor ion at *m/z* 535); 467, 383. Multistage LC-ESI MS (positive): adhyperforin (**2**) MS 551 $[M+H]^+$; MS/MS (precursor ion at *m/z* 551), 483, 427, 411, 355. Multistage LC-ESI MS (negative): MS 549 $[M-H]^-$; MS/MS (precursor ion at *m/z* 549); 481, 412, 397. Multistage LC-ESI MS (positive): secohyperforin (**3**) MS 469 $[M+H]^+$; MS/MS (precursor ion at *m/z* 469), 413, 401, 345. Multistage LC-ESI MS (negative): MS 467 $[M-H]^-$; MS/MS (precursor ion at *m/z* 467); 398. Multistage LC-ESI MS (positive): adsecohyperforin (**4**) MS 483 $[M+H]^+$; MS/MS (precursor ion at *m/z* 483), 415, 359. Multistage LC-ESI MS (negative): MS

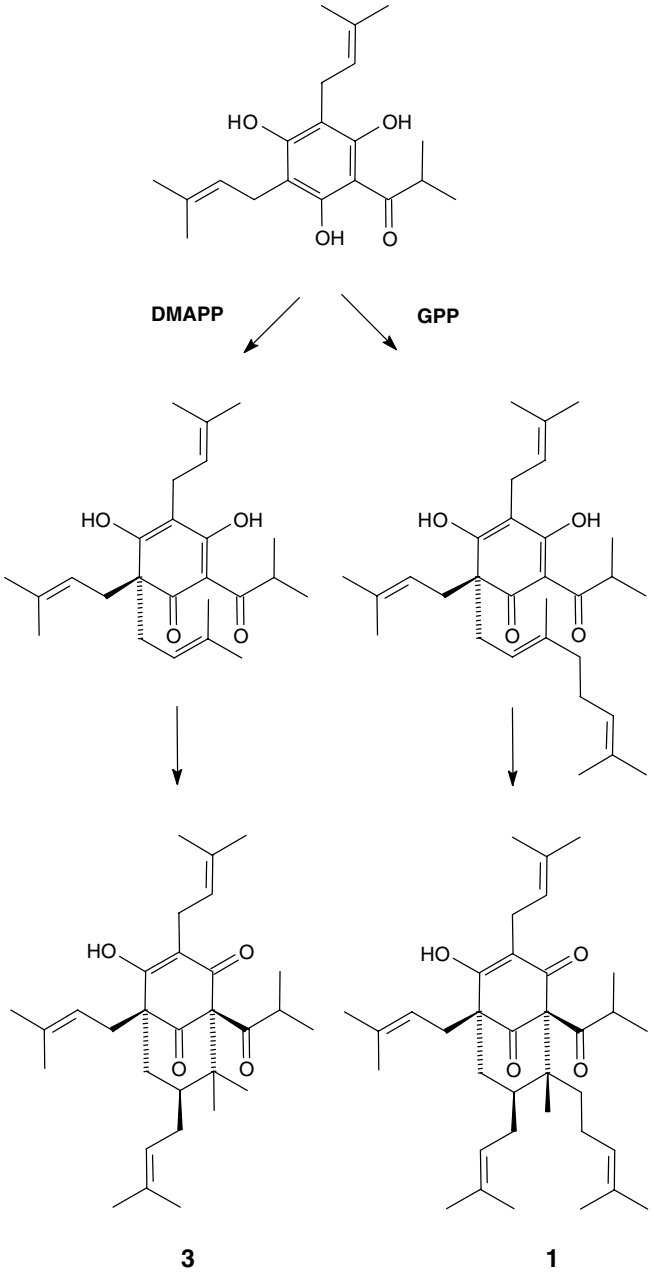

Fig. 7. Proposed branch point in the biosynthesis of hyperforins, which leads to the formation of secohyperforin (**3**) and hyperforin (**1**).

481 $[M-H]^-$; MS/MS (precursor ion at *m/z* 481); 441, 413, 383: Multistage LC-ESI MS (positive): an oxygenated hyperforin MS 553 $[M+H]^+$; MS/MS (precursor ion at *m/z* 553) 485, 427, 349. Multistage LC-ESI MS (negative): MS 567 $[M-H]^-$; MS/MS (precursor ion at *m/z 567*); 509, 439, 382. All experiments were performed in triplicate and standard errors were calculated by Microsoft® Excel.

3.8. LC-NMR

LC-NMR experiments were performed on a DMX-600 spectrometer (Bruker), equipped with a 5 mm DCH Cryo-Probe (Bruker), a 120 μl flow-insert (CryoFit, Bruker), and a BPSU-12 HP (Bruker Peak Sampling Unit) connecting the spectrometer and the chromatographic system (Agilent 1100 series). The latter consisted of a G1379A degasser, a G1311A quaternary pump, a G1314A VWD (variable wavelength detector), and a Waters Symmetry C_{18} column (4.6×250 mm) as the stationary phase. The linear gradient started from 50% CD_3CN in D_2O containing 0.01% TFA to 100% CD_3CN over 15 min, followed by isocratic elution at 100% CD_3CN for 20 min with a constant flow rate of 0.8 ml min^{-1} and a detection wavelength of 254 nm [R_t (**3**) = 19.6].

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