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Detection of hypericins in the "red glands" of *Hypericum elodes* by ESI–MS/MS

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Abstract

The biologically active naphthodianthrones hypericin and pseudohypericin were detected by electrospray ionization mass spectrometry (ESI-MS/MS) in microsamples from the sepals of *Hypericum elodes* (Hypericaceae) containing the so-called "red glands", i.e. stipitate glands with red-coloured heads. The occurrence of hypericins in the red glands of *H. elodes* supports the taxonomic position of the section *Elodes* within the genus *Hypericum* and provides evidence that the ability of carrying out the biosynthetic pathway leading to the naphthodianthrone compounds, rather than the absolute amounts produced, should be regarded as a chemical marker of the phylogenetically more advanced sections of genus *Hypericum*. The biologically active phloroglucinol derivatives hyperforin and adhyperforin, so far found only in *H. perforatum*, were also detected and evidence for their localization in the sepal secretory canals with large lumen, is given.

Keywords: Hypericum elodes; Hypericaceae; Red glands; ESI–MS/MS; Naphthodianthrones; Hypericin; Phloroglucinols; Hyperforin

1. Introduction

The red pigments hypericin and pseudohypericin are naturally occurring naphthodianthrones exhibiting important biological activities, namely photodynamic, antidepressive and antiviral activities (Roth, 1990; Weiss, 1991; Bombardelli and Morazzoni, 1995; Gulick et al., 1999).

Hypericin and pseudohypericin have been found only in *Hypericum* species (Brockmann and Sanne, 1957; Mathis and Ourisson, 1963; Kitanov, 2001) and have an important taxonomic value for the infrageneric classification of *Hypericum* (Robson, 1977). These natural products are absent in species from the primitive sections and seem to be specific only for the taxa of phylogenetically more advanced sections (Kitanov, 2001).

There is convincing evidence and general agreement about localization of hypericin and pseudohypericin in the "black glands", sometimes referred to as black nodules. The occurrence of black glands in an organ is regarded as an accurate index of the presence of hypericin and probably pseudohypericin (Robson, 1981).

The occurrence of hypericins in *Hypericum elodes* has long been a debated topic. *H. elodes*, the only taxon of the monotypic section *Elodes* (Robson, 1981, 1996), lacks black glands, but "red glands", i.e. stipitate glands with a peduncle and a red-coloured head, are present at the sepal and bract margins. No hypericin was detected in *H. elodes* by Mathis and Ourisson (1963); on the other hand, as pointed out by Robson (1981), if the glands are red, hypericins may occur in low amounts, and may not be detectable by the methods adopted. In the red glands of *H. elodes* the presence of anthocyanins was suggested by Bottega et al. (1999). Recently (Koch, 2001), several anthraquinone and naphthodianthrone derivatives have been detected in a number of *Hypericum* species including *H. elodes*.

The present paper deals with hypericin and pseudohypericin detection in the red glands from the sepals of *Hypericum elodes* by electrospray ionization mass spectrometry (ESI–MS/MS) approach, which in previous research had proven to be a suitable analitycal approach

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when detecting natural products occurring in low amounts in complex mixtures (Piovan et al., 1998; Favretto et al., 1998). The biologically active phloroglucinol derivatives (Muller et al., 1998; Singer et al., 1999; Schempp et al., 1999), hyperforin and adhyperforin, were also detected in sepals and their localization discussed.

2. Results and conclusion

Different secretory structures are known to occur in the sepals of *H. elodes* (Bottega et al., 1999), namely:

- glandular emergences or stipitate glands with colourless to red-coloured heads, typically located on the margin of the sepals (Fig. 1); and
- canals with large lumen distributed sparsely within the thickness of the sepals (type-B canals according to Bottega et al., 1999).

Only type-B canals were present in the sample from the middle of sepals (Sample 2); both the secretory structures occurred in the sample from the sepal margin (Sample 1).

In order to elucidate the chemical nature of the pigments occurring in the red heads of the capitate glands from *H. elodes* sepals, at first we used a simple hystochemical approach to verify the possible occurrence of anthocyanins in the red glands of *H. elodes*, suggested by Bottega et al. (1999). It is well known that the colour of anthocyanin solutions depends on pH values (Brouillard, 1982); therefore, we treated the peltate glands with aqueous solutions at different pH values, but no colour change in the red heads was observed.

To ascertain the occurrence of the red pigments hypericins in the red-coloured heads of stipitate glands, the methanolic extracts from both samples were analysed by direct ESI–MS under negative ESI conditions. These conditions were chosen since hypericin and related compounds show very little tendency to form $[M+H]^+$ quasi molecular ions by addiction of protons but, on the other hand, deprotonation with production of $[M-H]^-$ ions is more likely to occur (Piperopoulos et al., 1997).

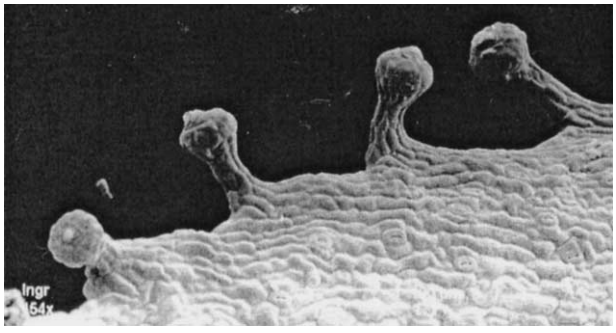

Fig. 1. Glandular emergences or stipitate glands on the margin of the sepals.

The negative ion mass spectrum of the extract from the sepal margins (Sample 1) showed three major ionic species at m/z 503, 519 and 535 corresponding to the $[M-H]^-$ molecular ions of hypericin, pseudohypericin and hyperforin, respectively. We isolated the ionic species at m/z 503 and 535 and obtained their collisionally induced dissociation (CID) product ion spectra (MS/MS and MS^n). The comparison with CID MS/MS and MS^n spectra obtained under the same ESI conditions for the deprotonated molecules of pure standards of hypericin and hyperforin, fully confirmed the original identification.

Furthermore, the ionic species at m/z 519 reasonably corresponds to pseudohypericin on the basis of its molecular weight. Unfortunately, the pure standard of pseudohypericin was not available, but we were able to compare the CID MS/MS spectrum of the species at m/z 407 corresponding at $[M–CH_4CH_3OH]^-$ with that of $[M–CH_4]^-$ ions produced by CID of deprotonated hypericin at m/z 503. These spectra are perfectly superimposable and the data are consistent with the structure of pseudohypericin.

The minor ionic species at m/z 549 corresponds to $[M-H]^-$ of adhyperforin. The MS/MS spectrum indicates the formation of ionic species at m/z 480, 397 and 313 corresponding to losses of $(CH_3)_2CCHCH_2$, $(CH_3)_2CCH(CH_2)_2$ and $CH_3(CH_2)CO(CH_3)CHCO$, respectively: all these data are consistent with the structure of adhyperforin. The ESI–MS/MS data are reported in Table 1.

The negative ion mass spectrum of the extract from the innermost sepal blade (Sample 2) showed a major ionic species corresponding to $[M-H]^-$ of hyperforin at m/z 535 and the ionic species corresponding to $[M-H]^-$ of adhyperforin at m/z 549. Sample 2 was completely devoid of the red naphthodianthrone pigments hypericin and pseudohypericin.

Our results provide additional evidence of versatility and usefulness of this analytical procedure, which might be regarded as the preferential option whenever only plant microsamples are available and low concentrations of non-volatile known natural compounds occur in complex mixtures.

A contribution is given to hyperforin and adhyperforin distribution within the genus *Hypericum*. In fact, at our knowledge these phloroglucinol derivatives had never been previously recorded in *H. elodes*; so far hyperforin seemed to be restricted to the species *H. perforatum*. Hypericin and pseudohypericin detection in *H. elodes* supports the rather advanced position given to section Elodes (Robson, 1996). We can assume that the occurrence of red or black glands is a good index of the occurrence of hypericins in different concentrations.

Table 1
Assignments of $[M-H]^-$ ions and their CID fragments

$[M-H]^-$	Fragments	Compound
503	487/459/443/431/415/405/388	Hypericin
519	503/487/459/443/431/415/405/388	Pseudohypericin
535	466/451/423/407/397/395/383/379/353/351/315/313/259	Hyperforin
549	480/397/313	Adhyperforin

The ability of carrying out the biosynthetic pathway leading to the naphthodianthrone compounds, rather than the absolute amounts produced, should be regarded as a chemical marker of the phylogenetically more advanced sections of genus *Hypericum*.

The present research provides new insight into localization of some biologically active phytoconstituents of *Hypericum*. In *H. elodes* the red naphthodianthrone pigments hypericin and pseudohypericin are obviously limited to the red heads of the stipitate glands.

Two hypotheses can be put forward to account for the fact that both hypericins and hyperforins can be found in the extract from the sepal margins (Sample 1):

- the stipitate glands on the margin contain both the naphthodianthrone red pigments and the phloroglucinol derivatives; and
- hyperforins are located in the canals with large lumen (referred to as type-B canals by Bottega et al., 1999), which are distributed sparsely within the thickness of the sepals.

Evidence supporting the latter possibility came from detection of only hyperforins in the extract from the middle sepal part where only type-B canals are present. Therefore, chemical diversity coupled with hystological diversity was shown to occur in the sepals of *H. elodes*. Similar chemical diversity was recorded for secrete from vittae and companion canals in the fruit of *Heracleum sphondylium* (Bicchi et al., 1990).

3. Experimental

3.1. Plant material and sample collection

Plants of *Hypericum elodes* L. cultivated on a peat-bog environment at the Botanical Garden of Padua, were used. These plants came from the only relic Italian population of *H. elodes* in Tuscany (biotope "Bosco del Palazzetto") and were transplanted at the Botanical Garden of Padua as a part of an ex situ conservation program for protection of this vulnerable species. Voucher specimen of the plant is deposited at the Botanical Garden of the University of Padua (HG 5168).

The capitate glands along the sepal margin show colourless heads before anthesis and accumulate the red pigments following fertilization. Due to the very reduced number of plants available, samples were collected only about 10 days after anthesis, when the heads of the sepal capitate glands had turned red.

Direct selective microsampling of the capitate glands being unfeasible, two different kinds of samples were collected, as follows:

- Sample 1: small fragments of the sepal margins (about 3 mm wide) containing intact red peltate glands, cut by a razor blade under a stereoscope; and
- Sample 2: small fragments of the sepals, taken from the middle of the sepal. Cautionary measures were taken in order to avoid sample contamination by the red glands from the sepal margins.

Sampling was carried out in triplicate.

3.2. SEM (scanning electronic microscopy)

Small pieces of sepals were fixed by 2.5% glutaraldehyde in phosphate buffer at pH 7.4, and postfixed in 0.2% OsO_4. The material was dehydrated with acetone, dried by critical point drying and gold coated. A Scanning Electron Microscope Philips 515 was utilised.

3.3. Chemicals

Authentic hypericin and hyperforin as reference compounds were purchased from Roth (Karlsruhe, Germany) and Addipharma GmbH (Wandalenweg, Germany), respectively.

3.4. Extraction procedure and chemical analysis

The samples were extracted in methanol (1 ml). The methanolic extracts were directly analysed by mass spectrometry.

All the mass spectrometric measurements were performed using a LCQ (Thermoquest, San José, CA, USA) ion trap mass spectrometer equipped with an electrospray ion source.

The samples were infused into the ESI source via a syringe pump at a flow rate of 10 μl/min. ESI parameters were: spray voltage 3 KV, capillary temperature 200 °C, N_2 sheath gas flow 60 a.u. (arbitrary units).

Multiple mass spectrometry (MS^n) experiments were performed by isolating either $[M-H]^-$ or fragment ions and applying a supplementary RF ('tickle voltage') of appropriate amplitude at the end caps of the ion trap. The hypericin and hyperforin detection was performed operating under negative ion ESI conditions.

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