Preservation of hypericin and related polycyclic quinone pigments in fossil crinoids

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The fringelite pigments, a group of phenanthroperylene quinones discovered in purple coloured specimens of the Upper Jurassic crinoid *Liliocrinus*, demonstrate exceptional preservation of organic compounds in macrofossils. Here we report the finding of hypericin and related phenanthroperylene quinones in *Liliocrinus munsterianus* from the original 'Fringeli' locality and in the Middle Triassic crinoid *Carnallicrinus carnalli*. Our results show that fringelites in fact consist of hypericin and closely related derivatives and that the stratigraphic range of phenanthroperylene quinones is much wider than previously known. The fossil occurrence of hypericin indicates a polyketide biosynthesis of hypericin-type pigments in Mesozoic crinoids analogous to similar polyketides, which occur in living crinoids. The common presence of a characteristic distribution pattern of the fossil pigments and related polycyclic aromatic hydrocarbons further suggests that this assemblage is the result of a stepwise degradation of hypericin via a general diagenetic pathway.

Keywords: fossil crinoids; molecular preservation; organic pigments; mass spectrometry; diagenesis

1. INTRODUCTION

Since the nineteenth century certain fossil crinoids (Echinodermata) from Jurassic (e.g. Parkinson 1808; Bather 1893; Taylor 1983; Salamon & Zatoń 2005) and Triassic (e.g. Jaekel 1894; Biese 1927; Hagdorn 1982, 1999) deposits have been reported to show an unusual purple coloration. Several attempts were undertaken to analyse the compounds that give such specimens their characteristic colour (Bather 1893; Biese 1927), but they failed to detect specific organic or inorganic pigments. However, Blumer (1951, 1960, 1962a, 1965) discovered a remarkable group of organic pigments (fringelites) in purple to violet coloured specimens of the Jurassic crinoid 'Millericrinus' (= *Liliocrinus*) from northern Switzerland. These pigments are also accompanied by several polycyclic aromatic hydrocarbons (PAHs), which were proposed as transformation products of the pigments (Blumer 1962b; Thomas & Blumer 1964; Blumer 1965). Based on recent colour measurements using diffuse reflectance spectroscopy (Falk *et al.* 1994; Falk & Mayr 1997) and description of purple coloured crinoids cited above, a wider occurrence of fringelite pigments in the fossil record can be assumed, but to date, none of these possible occurrences has been confirmed by chemical analysis.

The fringelite pigments have been described as hydroxylated phenanthroperylene quinones, which differ in the numbers of their hydroxyl groups (Blumer 1960, 1962a, 1965). However, due to the lack of complementary analytical techniques at the time of their discovery, these structures were described mainly on the basis of their

absorption spectra. Furthermore, two fringelite structures were tentatively revised (Blumer 1971), but were reported according to the original assignment in a later review (Blumer 1976). Although several fringelites have also been synthesized (fringelite D: Freeman *et al.* 1994, Falk & Mayr 1995; fringelite F: Cameron & Raverty 1976, Rodewald *et al.* 1977; fringelite H: Brockmann *et al.* 1950), the structures of the original fossil pigments have not been verified until now.

To obtain further insights into the occurrence, structure and diagenesis of the fringelite pigments, we analysed purple coloured crinoid specimens from two of the previously described localities in detail: (i) three specimens of the Middle Triassic *Carnallicrinus carnalli* (formerly *Chelocrinus carnalli*) (Lower Muschelkalk, Jena Formation, Freyburg/Unstrut, Germany; Hagdorn 1999), representing different types of preservation; and (ii) one specimen of the Upper Jurassic *Liliocrinus munsterianus* (Bärschwil Formation, Bärschwil, Switzerland; Blumer 1960) from the original 'Fringeli' locality as a reference. Specimens were examined by microscopic analysis of thin sections and by chemical analysis of the extractable organic matter using mass spectrometry and high-performance liquid chromatography (HPLC).

2. MATERIAL AND METHODS

(a) Fossil samples and reference compounds

Crinoid specimens were selected from collection material of the following institutions: Institut für Geologische Wissenschaften und Geiseltalmuseum, Universität Halle-Wittenberg: MLU. WOL 2005.2 (sample Ch 8) and MLU. WOL 2005.3 (sample Ch 10); Museum für Naturkunde Berlin: MB. E. 533 (sample Ch 9); Naturhistorisches

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Museum Basel: NMB M 8908 (sample Li 1). For reference we used an authentic sample of hypericin from *Hypericum perforatum* and synthetic samples of fringelite F and 1,2,3,4,5,6-hexahydrophenanthro[1,10,9,8-*opqra*]perylene (HHPP) prepared according to Rodewald *et al.* (1977) and Wolkenstein *et al.* (2002), respectively.

(b) Sample preparation and extraction

Small stalk parts (0.5–3.2 g) of the specimens were isolated from the surrounding sediment and cleaned extensively with acetone to remove any contaminants. After dissolution of the carbonate with 10 M HCl, the residues were separated by centrifugation, washed free of acid with distilled water and dried overnight at room temperature under vacuum (10 Torr). Residues were then sequentially extracted (with protection from light) by sonication (1 h, 40 °C) and centrifugation in toluene (3×), methanol (3×) and dimethyl sulphoxide (1×). To obtain additional information on the nature of the pigment species in the fossil material, a sample of *Liliocrinus* was ground to a fine powder; one part of the sample was extracted as described previously, while an equal part of the sample was extracted directly, without prior dissolution of the carbonate.

(c) HPLC analysis

Aliquots of all extracts were subjected to HPLC (toluene aliquots were diluted 1 : 1 with acetonitrile) using a Kontron system equipped with a dual wavelength detector with a spectral range of 190–400 nm. Separation was achieved at 25 °C on a Purospher STAR RP-18 endcapped column (55×4 mm i.d., 3 µm, Merck) protected with a Purospher STAR RP-18 endcapped guard column (4×4 mm i.d., 5 µm, Merck). For the toluene extracts, the HPLC program consisted of a linear gradient of acetonitrile/water (70 : 30) to 100% acetonitrile in 20 min, followed by isocratic elution at 100% acetonitrile; the flow rate was 1 ml min⁻¹ and absorbance was detected at 300 nm. For the methanol and dimethyl sulphoxide extracts, the HPLC program consisted of a linear gradient of methanol/50 mM sodium acetate solution adjusted to pH 7.0 with acetic acid (60 : 40) to 100% methanol in 20 min, followed by isocratic elution at 100% methanol; the flow rate was 1 ml min⁻¹ and absorbance was detected at 254 nm.

(d) Mass spectrometry

Low- and high-resolution 70 eV electron ionization (EI) mass spectra were obtained from a JEOL JMS-700 magnetic sector instrument. Samples were introduced via the direct probe. For accurate mass measurements, the resolution was set to $R=5000$ and perfluorokerosene was used for internal mass calibration. Low-resolution electrospray ionization (ESI) mass spectrometry and ESI tandem mass spectrometry (MS/MS) measurements were performed on a Finnigan TSQ 700 triple quadrupole mass spectrometer. Samples were sprayed using a MasCom nanoESI interface (1.0 kV spray voltage, desolvation capillary heated to 100 °C). Spectra are the averages of 10–20 scans at 10 s per scan over the m/z 50–1000 range. For ESI-MS/MS experiments, argon at 0.9 mTorr was used as collision gas and a collision offset of 59 V was applied. Empirical formulae of the pigments were determined by ESI Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) on a Bruker Apex III instrument equipped with a 7 T magnet. Mass calibration was established externally using perfluorocarboxylate ions.

Figure 1. Crown of the Middle Triassic crinoid *Carnallicrinus carnalli* (MB. E. 13), with exceptional preservation of fossil pigments. Freyburg/Unstrut, Germany. Scale bar, 1 cm.

3. RESULTS AND DISCUSSION

(a) Preservation of fossil material

Both localities are known for the occurrence of well-preserved articulated crinoid specimens (Blumer 1960; Hagdorn 1999). In addition, almost all crinoid specimens display a distinct purple to violet colour, although the intensity of colour varies between the individual specimens and parts of them. This characteristic coloration of the crinoids is in strong contrast to the surrounding sediment (figure 1) and other fossils, which are completely devoid of pigments, indicating a preservation of endogenous compounds in the fossil material.

Microscopic analysis revealed the presence of numerous micrometre-sized inclusions, which are dispersed along a meshwork-like pattern (most obvious in *Liliocrinus*) within the calcite matrix of the fossils (figure 2). Thus, due to the presence of a highly porous stereom microstructure as a common feature of the echinoderm endoskeleton (Donovan 1991), we suggest that original organic matter was trapped within the former pores of the stereom by the precipitation of carbonate cement. Accordingly, early cementation of the stereom seems to be an important factor for the preservation of organic material in fossil crinoids.

(b) Characterization of organic compounds

HPLC analysis of the (purple coloured) methanol extracts revealed the presence of three major compounds, which could be further characterized by ESI-MS. The ESI spectra (negative-ion mode) of the methanol extracts show a very similar pattern for both crinoids, *Liliocrinus* and *Carnallicrinus*, with a base peak at a mass-to-charge

Figure 2. Photomicrograph of a longitudinal section from a column of *Liliocrinus munsterianus* (NMB M 8908), showing organic matter trapped within the carbonate cement that fills the pores of the crinoid skeleton (stereom). Scale bar, 200 µm.

Figure 3. Negative-ion ESI mass spectra of methanol extracts. (a) Sample of *Liliocrinus munsterianus*, Upper Jurassic, Switzerland. (b) Samples of *Carnallicrinus carnalli*, Middle Triassic, Germany.

ratio (m/z) of 475 and minor peaks at m/z 489 and 503 (figure 3). Characteristic fragmentation patterns of the corresponding ions were obtained by ESI-MS/MS (figure 4; for comparison, see Piperopoulos *et al.* 1997), and empirical formulae were determined by ESI-FT-ICR-MS. By direct comparison with authentic reference compounds, the fossil pigments could be identified as a series of hexahydroxyphenanthroperylene quinones: fringelite F ($C_{28}H_{12}O_8$, $[M-H]^-$ at m/z 475), demethylhypericin ($C_{29}H_{14}O_8$, $[M-H]^-$ at m/z 489) and hypericin ($C_{30}H_{16}O_8$, $[M-H]^-$ at m/z 503). These results show the presence of one of the previously described fringelite pigments, but provide the first evidence of hypericin and demethylhypericin in the fossil record.

HPLC analysis of the toluene extracts revealed the presence of numerous additional compounds of low

Figure 4. Negative-ion ESI collision-induced dissociation mass spectra of reference compounds and fossil pigments. (a) Fringelite F. (c) Hypericin. (b, d) Corresponding signals from a sample of *Carnallicrinus carnalli* (Ch 9), Middle Triassic, Germany.

abundance. The main compound was isolated by HPLC and subjected to low- and high-resolution EI mass spectrometry. By comparison with a synthetic reference (Wolkenstein *et al.* 2002), the fossil compound could be identified as HHPP, which is consistent with the structural assignment of previous studies (Blumer 1962b, Thomas & Blumer 1964). Additional related PAHs could be characterized by their empirical formulae. Further extraction of the residues with dimethyl sulphoxide yielded additional amounts of extractable organic matter, including the pigments mentioned above, but could not provide evidence for the presence of the previously described fringelite D, fringelite E and the very refractory fringelite H (soluble in dimethyl sulphoxide according to Falk *et al.* (1992)), which was suggested as the main pigment in *Liliocrinus* (Thomas & Blumer 1964; Blumer 1965). Due to the lack of all these compounds in the extracts, we conclude that the originally described fringelite pigments in fact consist of hypericin and closely related derivatives. As a consequence, the term 'fringelites' would be no longer valid for this group of fossil pigments.

(c) Chemical stability of pigments

It has been shown that hydroxyphenanthroperylene quinones, such as hypericin, form salts due to the strong acidity of their bay region hydroxyl groups (Falk 1999). Thus, the exceptional chemical stability of hypericin and its derivatives in fossils can be understood from their ability to form extremely insoluble salts with bivalent ions, such as Ca^{2+} , as observed in a previous study of fringelite D (Falk & Mayr 1997). Furthermore, chelation involving

Figure 5. Proposed diagenetic pathway for the degradation of hypericin in fossil crinoids.

the *peri* hydroxyl and carbonyl groups with transition metal ions could lead to a further stabilization of the pigments (Falk & Mayr 1997).

Quantitative HPLC measurements of solvent fractions (toluene, methanol, dimethyl sulphoxide) from *Liliocrinus* obtained by extraction following treatment with hydrochloric acid in comparison to those obtained by extraction without prior dissolution of the carbonate showed that HHPP and the other PAHs could be extracted in equal amounts from both parts of the sample. In contrast, only traces of the pigments could be removed directly from the carbonate with methanol as the solvent, and even with dimethyl sulphoxide only about 10% of the pigments, which were extractable after dissolution of the carbonate, could be dissolved. This behaviour strongly suggests that hypericin and its derivatives occur in the fossil material as salts, providing an effective mechanism for chemical stabilization.

(d) Biosynthesis

Hypericin is usually known by its occurrence in the common medicinal plant St John's wort (*H. perforatum*) as a constituent with important pharmacological activities (Falk 1999). However, related anthraquinone, bianthrone and phenanthroperylene quinone pigments are also present in living comatulid crinoids (Rideout & Sutherland 1985), and, moreover, a group of brominated hypericin derivatives was reported from the living stalked crinoid *Gymnocrinus richeri* (De Riccardis *et al.* 1991). All these polyketide compounds are thought to be biosynthesized via the acetate-malonate pathway (for a review on the biosynthesis of aromatic polyketides, see Shen 2000). Thus, the finding of hypericin in two crinoids of Triassic and Jurassic age also implicates a polyketide biosynthesis of hypericin-type compounds in Mesozoic crinoids. Due to the widespread occurrence of present-day crinoidal polyketides in the form of their sulphate esters (Rideout *et al.* 1979; De Riccardis *et al.* 1991; Takahashi *et al.* 2002), which have been reported to show antifeedant activities against predatory fish (Rideout *et al.* 1979; Takahashi *et al.* 2002), the precursors of the fossil pigments may have acted in a similar way.

(e) Diagenesis

The characteristic assemblage of closely related phenanthroperylene quinones and related PAHs in *Liliocrinus* and *Carnallicrinus* would not be expected in a living organism for biogenetic reasons (e.g. since the pigments cannot be formed via the same biosynthetic route). Indeed, all these compounds, with the exception of hypericin, are unique to these fossil crinoids. We therefore suppose a diagenetic

formation of demethylhypericin, fringelite F and the accompanying PAHs. The presence of demethylhypericin as an intermediate between hypericin and fringelite F indicates a stepwise elimination of methyl groups, leading to fringelite F. In addition, numerous PAHs have been interpreted as diagenetic products derived from natural precursor compounds (Simoneit 1998; Neilson & Hynning 1998) and, in particular, perylene has been suggested to be derived from perylene quinone precursors (Jiang *et al.* 2000). Thus, an analogous transformation of fringelite F to HHPP would be plausible as well. Based on the *in situ* preservation of hypericin and its presumed degradation products, we propose the diagenetic pathway shown in figure 5. This pathway is also supported by quantitative results from HPLC analysis (table 1), as described in the following paragraphs.

The overall pigment concentration of the Jurassic *Liliocrinus* is in reasonable agreement with previous results obtained by semiquantitative determination on kilogram quantities of fossil material (Blumer 1960). However, these concentrations are about one order of magnitude higher than those of the Triassic *Carnallicrinus*, suggesting a degradation of organic matter on a geological time scale. In addition, there are significant differences in the pigment concentrations among the Triassic samples, which are related to different degrees of alteration, evident from the type of sediment. The distinctly coloured *Carnallicrinus* samples (Ch 8, Ch 9) from a micritic limestone microfacies show a more than fourfold higher pigment concentration than the faintly coloured sample (Ch 10) from a highly porous limestone microfacies, which is altered by dissolution processes. This is also in agreement with the general observation that specimens with distinct colour from the Freyburg area (such as the specimen shown in figure 1) are mainly preserved in micritic limestones, whereas specimens with only faint colour occur in porous limestones.

Relative amounts of the individual compounds provide further information on the processes during diagenesis. Fringelite F, the final product of the proposed demethylation process, is the predominant compound in all analysed samples of both crinoids, whereas high amounts of demethylhypericin were detected only in *Liliocrinus*, and hypericin was found as a minor compound in *Liliocrinus* and *Carnallicrinus*. In contrast to the considerable differences in the pigment concentrations, HHPP was detected only in small amounts in both crinoids, suggesting that this compound does not represent a final product of the diagenetic degradation. However, HHPP/fringelite F ratios correlate with the degree of alteration of the corresponding samples, indicating a

Table 1. Pigment and hydrocarbon concentrations of *Liliocrinus* and *Carnallicrinus* samples ($\mu\text{g g}^{-1}$ of fossil). (Quantitative determination was performed by HPLC using external standards. The standard solutions of fringelite F were also used for the quantification of demethylhypericin. Reproducibility of the method was determined by analysing three replicates of a finely ground *Liliocrinus* sample. The relative standard deviation was in the range of 2–3% for all quantified compounds.)

sample	hypericin	demethyl-hypericin	fringelite F	HHPP
<i>Liliocrinus munsterianus</i> , Upper Jurassic				
Li 1	5.0	175.5	289.5	1.5
<i>Carnallicrinus carnalli</i> , Middle Triassic				
Ch 9	1.7	1.5	27.3	<0.8
Ch 8	<0.2	3.0	26.0	0.5
Ch 10	<0.1	<0.4	6.6	0.4

genetic relationship of these compounds. It should also be noted that in addition to HHPP ($\text{C}_{28}\text{H}_{20}$), the parent hydrocarbon of fringelite F, further PAHs ($\text{C}_{29}\text{H}_{22}$, $\text{C}_{30}\text{H}_{24}$) could be detected in the crinoid samples, which most probably represent the corresponding parent hydrocarbons of demethylhypericin and hypericin. Due to the presence of significant amounts of organic matter in the dimethyl sulphoxide fractions, which could not be characterized, and additional amounts of non-soluble organic matter, we assume that the final products of the diagenetic degradation occur as kerogen-like organic matter.

4. CONCLUSIONS

The discovery of hypericin and its derivatives in *Carnallicrinus* greatly extends the known range of phenanthroperylene quinones in the fossil record from the Upper Jurassic to the Middle Triassic, extending also the known geological range of polycyclic quinones in general. The only other fossil polycyclic quinones reported are the quincyte pigments, which were found in the sedimentary deposits of an Eocene lake (Prowse *et al.* 1991).

The common presence of hypericin, demethylhypericin and fringelite F in the Triassic *Carnallicrinus* (order Encrinida) and in the Jurassic *Liliocrinus* (order Millericrinida), two crinoids which belong to different periods, geographic locations and clades, suggests that the occurrence of these pigments in fossil crinoids is a widespread feature, not a local phenomenon. Moreover, the common presence of a characteristic distribution pattern of the closely related pigments and additional related PAHs suggests that this assemblage is the result of a general diagenetic pathway for the degradation of hypericin-type compounds in fossil crinoids. From a chemical point of view, such a pathway, including the demethylation of methyl aromatics, would be significant due to the high stability of the involved C–C bonds. Similar processes have been previously reported for methyl aromatics in petroleum (Behar *et al.* 1999). Therefore, we hypothesize a demethylation of hypericin via a natural thermal cracking process.

Considering the rare occurrence of fossil crinoids with preservation of organic pigments, available only in individual cases for (destructive) chemical analysis, our

study of phenanthroperylene quinones and their diagenetic products in fossil crinoids is far from complete. However, the results show that well-preserved fossil crinoids may serve as a unique source of information on the occurrence and fate of endogenous biomolecules in the fossil record. In particular, purple to violet coloured crinoids should be regarded as a potential source of molecular data. Based on the analysis of more diverse material, further significant discoveries can be expected.

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