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A coumarin as a fluorescent compound in scorpion cuticle

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Summary

A new compound fluorescent under UV light is identified in cuticular extracts from 11 scorpion species: 4-methyl-7-hydroxycoumarin (4-methylumbelliferone, hymecromone). This is the first record of a coumarin in arthropods.

Introduction

All scorpions (Arachnida, Scorpiones) fluoresce in the visible spectrum (*c.* 500 nm) when irradiated by UV-light. This unique phenomenon was first reported by the Italian zoologist M. Pavan (Pavan, 1954; Pavan & Vachon, 1954), and, later that same year, by R. F. Lawrence (1954) in South Africa. Scorpion fluorescence (photoluminescence; Fasel *et al.*, 1997) has been since widely used to locate animals in the field, effectively revolutionizing the study of scorpion ecology and biology (Stahnke, 1972; Sissom *et al.*, 1990). The adaptive function (if any) of this phenomenon is still hypothetical, and its chemical nature until recently remained unknown.

The fluorescence is contained in a very thin ($<4\mu$m; Hjelle, 1990), outermost layer of the exoskeleton, the so-called hyaline exocuticle, which mainly consists of mucopolysaccharides and lipoproteins; the amount of chitin in this layer is debatable (Kennaugh, 1959; Malek, 1963). A few attempts to isolate the fluorescent compound (Gain, 1973; Anglade *et al.*, 1990) have not been effective. Stachel *et al.* (1999) were the first to identify a fluorescent organic compound from scorpion cuticle as a beta-carboline (a tricyclic nitrogen heterocycle). Here, we report another fluorescent compound, which belongs to the entirely different class of organic chemicals—a coumarin, detected in the ethanol extracts from scorpion cuticle.

Material and methods

Specimens. We used extracts from 11 scorpion species, which belong to nine families spanning the entire taxonomical, geographic, and ecological diversity of this order. The taxa used were: *Bothriurus araguaye* (Bothriuridae); *Centruroides exilicauda*, *Apistobuthus pterygocerus* (Buthidae); *Broteas gervaisii* (Chactidae); *Didymocentrus leseurii* (Diplocentridae); *Euscorpius mingrelicus*, *Megacormus gertschi* (Euscorpiidae); *Hadogenes bicolor* (Ischnuridae); *Hadrurus arizonensis* (Iuridae); *Heterometrus longimanus* (Scorpionidae); and *Paruroctonus gracilior* (Vaejovidae).

Extraction. Specimens had been kept in a mixture of 70% ethanol and 30% water for between one and ten years. Anecdotal evidence shows that the preservative after some time normally acquires fluorescence under UV light, therefore a passive extraction of a fluorescent compound has taken place.

Reverse Phase High Performance Liquid Chromatography (HPLC) separation. The extracted solution was acidified with acetic acid and injected onto a narrow bore reverse phase column (RP-18 Spheri-5, 2.1X3 mm). Buffer A was 0.1% trifluoroacetic acid (TFA) in water, and buffer B was 0.085% TFA in 80% acetonitrile. The gradient consisted of 5% acetonitrile/min. Fractions were collected every 2 minutes throughout the gradient.

Fluorescence detection. The fluorescence of each HPLC fraction was measured using a fluorimeter (ISA Fluorolog tau3). The excitation wavelength used was 409.5 nm.

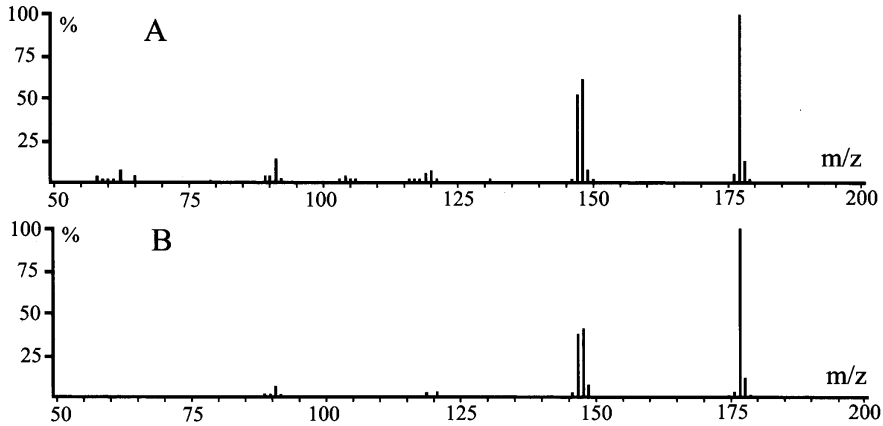

Fig. 1: **A** EI mass spectrum of the compound at m/z 176 from the scorpion extract. **B** EI mass spectrum of pure 4-methyl-7-hydroxycoumarin.

Mass spectrometry. The HPLC fractions were lyophilized to dryness and reconstituted in methanol. The samples were then injected into a Gas Chromatography Mass Spectrometer (GCMS) (Saturn 2000, Varian) equipped with a quadrupole ion trap mass analyser. The compounds were ionized by both electron impact and chemical ionization.

Results

The fluorescent compound was extracted using an ethanol/water mixture. The extract was then fractionated by reverse phase HPLC, and the fluorescence of each fraction was measured. Fraction 1 was found to fluoresce at 441 nm using an excitation wavelength of 409.5 nm. Fraction 1 was analysed by GCMS along with fraction 2 as a negative control. A compound of m/z (mass over chagre) 176 was found in fraction 1 but was absent from fraction 2. The Electron Impact (EI) mass spectrum of this compound (Fig. 1A) was searched in the NIST database. The search identified a matching spectrum belonging to the compound 4-methyl-7-hydroxycoumarin.

When pure 4-methyl-7-hydroxycoumarin (Aldrich Chemical Co.; structure shown in Fig. 2) was analysed by GCMS, the EI mass spectrum was a perfect match with that of the unknown compound (Fig. 1B). The fluorescence of pure 4-methyl-7-hydroxycoumarin was measured using the same experimental conditions as the sample. The compound was found to fluoresce at 441 nm when excited with a wavelength of 409.5 nm. The pure 4-methyl-7-hydroxycoumarin was also found to coelute with the sample compound on the gas chromatograph.

Discussion

All specimens of scorpions analysed contained the same fluorescent substance identified as 4-methyl-7-hydroxycoumarin (Fig. 2). This compound is also known as 4-methylumbelliferone (4-MU), or hymecromone. Its synthetic form is widely used as a fluorogenic marker in enzyme assays (e.g. Miller *et al.*, 1998; Gee *et al.*, 1999; Trubetskoy & Shaw, 1999), and has a medical effect as an antispasmodic and choleretic (increasing bile flow) (e.g. Krawzak *et al.*, 1995; Calabuig *et al.*, 1996).

The remarkable aspect of this novel scorpion fluorochrome is its being a coumarin. Coumarins are almost exclusively plant compounds (O'Kennedy & Thornes, 1997), with some representation in fungi and bacteria. In animals, there are only two exceptional records of coumarins, in a prosobranch mollusc and in the scent glands of beavers (Murray *et al.*, 1982). No coumarins have been reported from arthropods.

Scorpions exhibit fluorescence only when their cuticle is hardened (freshly molted scorpions and first instar juveniles do not fluoresce (Stahnke, 1972; Hjelle, 1990). Presence of extractable 4-MU, therefore, might have something to do with cuticular sclerotization, a process in which cuticular

proteins are cross-linked, usually by aromatic molecules.

Stachel *et al.* (1999) were the first to identify a soluble chemical compound associated with scorpion fluorescence from two species, *Centruroides vittatus* (Buthidae) and *Pandinus imperator* (Scorpionidae). They identified a completely different compound, beta-carboline, in their ethanol extract from the scorpion cuticle. We could find no evidence of this compound in our fluorescent HPLC fraction. However, we used an excitation wavelength of 409.5 nm for all of our fractions, while they used an excitation wavelength of 350 nm. Therefore, we were not selecting for this compound when we tested our HPLC fractions for fluorescence. Stachel *et al.* (1999) do state that more than one fluorogenic species was present in their crude extract.

It is not known whether fluorescence in scorpions has any biological function (Lourenço & Cloudsley-Thompson, 1996). It has been suggested that it can be used by scorpions for communication (Anglade *et al.*, 1990; Hjelle, 1990; P. H. Brownell, pers. comm.); indeed, high photosensitivity of scorpions is well known (Hjelle, 1990).

We would like to propose an additional hypothesis: that fluorescent compounds in scorpion hyaline exocuticle are a relict feature, which could have served as a sunblock, shielding scorpions from the UV component in sunlight. Such adaptation might seem strange in modern scorpions, which are exclusively nocturnal animals. However, at the time of their terrestrialization (possibly Devonian; Selden & Jeram, 1989; Jeram, 1998) the early scorpions could have been daytime active. Hyaline exocuticle appears to be present already in the earliest fossil scorpions; in fact, due to its unusual stability, it is the only cuticular layer preserved in, and a very common component of, fossil assemblages (Bartram *et al.*, 1987). During this transition to land, protection from cell- and DNA-damaging UV light would be an extremely highly valuable selective trait, as it is in modern marine animals (see e.g. Dunlap & Shick, 1998). It is known that coumarins play the role of sunblock in young plants (O'Kennedy & Thornes, 1997).

In addition, another interesting, if maybe far–fetched, observation should be made here. The discovery of extractable, biologically active compounds in scorpion cuticle (beta-carboline, an alkaloid; 4-MU, a choleretic/antispasmodic) could perhaps give some credibility to the traditional folk remedies such as "scorpion oil" concoctions (Cloudsley-Thompson, 1990; Braunwalder & Cameron, 2001).

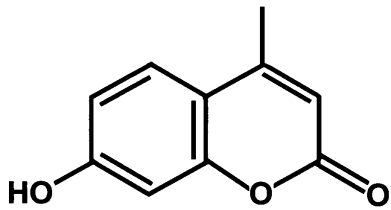

Fig. 2: Chemical structure of 4-methyl-7-hydroxy-coumarin (hymecromone).

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