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Qualitative variation of alkaloids in color morphs of *Cystodytes* (Ascidiacea)

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Abstract

Despite their great variability, particularly of color, Mediterranean specimens of the genus *Cystodytes* are usually attributed to the cosmopolitan species *Cystodytes dellechiajei*. In this study, we used MALDI-TOF and HPLC techniques to assess alkaloid distribution in the four most abundant color morphs of *Cystodytes* in the western Mediterranean (green, purple, brown and blue). The intraspecimen location of these compounds (either in tunic or zooids) was also analyzed. Two major chemotypes were found: (1) that of the purple morph was based on the sulfur-containing pyridoacridines, shermilamine B, kuanoniamine D and their deacetylated forms; (2) the chemotype of the blue and green morphs was based on the C_9-unsubstituted pyridoacridines, ascididemin and 11-hydroxyascididemin. In the brown morph, ascididemin was only detected by MALDI-TOF. All of these alkaloids were present in both the

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tunic and zooids, with the exception of the purple morph, in which shermilamine B and kuanoniamine D were found in the tunic, whilst their deacetylated forms were found in both tunic and zooids. Whereas a clear link between these pigments (pyridoacridines) and color was found in the purple morph, color in the other morphs may depend on other unknown molecules. MALDI-TOF proved to be a rapid and reliable tool with which to detect targeted compounds of low molecular mass at both colony and intraspecimen levels, making it effective for the rapid assessment of chemotypes. The chemical differences found raised questions about the taxonomic status of the color morphs attributed to the nominal species *C. dellechiajei* in the Mediterranean. Our results stress the importance of a detailed morphotype description when working with marine natural products, especially for taxa whose taxonomy is not well resolved, in order to understand fully the variation in secondary chemistry within and between species.

Keywords: *Cystodytes*; Chemotype; Morphotype; MALDI-TOF; HPLC; Ascidian; Color variation; Pyridoacridine alkaloid

1. Introduction

Marine invertebrates are a rich source of bioactive secondary metabolites with cytotoxic, antimicrobial, antifungal, antiviral and antifouling activities (Ireland et al., 1988; Bhakuni and Jain, 1990; Uriz et al., 1991; Becerro et al., 1997; McClintock and Baker, 2001; Faulkner, 2002). These secondary metabolites are assumed to have ecological functions as chemical defenses against predators, pathogens, spatial competitors, ultraviolet radiation and fouling organisms (Bakus, 1981; Coll et al., 1982; Paul, 1992; Pawlik, 1993; Hay, 1996; Becerro et al., 1997; McClintock, 1987; Lindquist, 2002).

Investigations into the qualitative variation of secondary metabolites in invertebrate taxa are crucial to studies of chemical ecology and its potential biotechnological applications, especially when species boundaries are ill-defined due to lack of reliable morphological characters. Groups such as sponges and ascidians are prominent in this respect (Solé-Cava and Boury-Esnault, 1999; Knowlton, 2000). The role of microsymbionts in the production of bioactive substances may also lead to distinct qualitative profiles within and across taxa (Faulkner et al., 1994; Hentschel et al., 2003; Schmidt et al., 2004). Studies on variation in profiles of secondary metabolites are scarce, and are sometimes believed to reflect previously un-noticed taxonomic diversity (Miller et al., 2001; Loukaci et al., 2004) and/or variability in symbiont populations (Bewley et al., 1996; McGovern and Hellberg, 2003). Even less is known about the intraspecimen location and production of chemical defenses (e.g. Uriz et al., 1996; Becerro et al., 1998; Tarjuelo et al., 2002). Underpinning studies of intraspecimen distribution is the optimal defense theory, which assumes that chemical and morphological defenses in living organisms are costly and that natural selection will favor an allocation of resources to defenses that optimize their benefit/cost ratio in terms of fitness (Rhoades, 1979; Fagerström et al., 1987). Therefore, reproductive parts (e.g. Steinberg, 1984; Schupp et al., 1999),

young, actively growing tissues (e.g. Cronin and Hay, 1996; Van Alstyne et al., 1999) or parts in contact with competitors (e.g. Uriz et al., 1996; Turon et al., 1996) should be preferentially defended. However, demonstration and quantification of costs is elusive (Hay and Steinberg, 1992), and some exceptions to the postulates of optimal defense theory exist (Pavia et al., 2002).

Ascidians, like other marine taxa, show some cases of great intraspecies variability (e.g. *Botryllus schlosseri* and *Ciona intestinalis*: Hoshino and Nishikawa, 1985; Monniot and Monniot, 2001; Stoner et al., 2002). Color variation has been frequently described, but its significance for delimiting species boundaries seems to be highly variable in this group (e.g. Sabbadin, 1982; Aron and Solé-Cava, 1991; Dalby, 1997; Tarjuelo et al., 2004). Ascidians also show one of the strongest cytotoxic, antibacterial and antifungal bioactivities found among benthic invertebrates (Uriz et al., 1991), although the chemical compounds responsible for these activities have rarely been identified (Paul et al., 1990; Davis, 1991; Vervoort et al., 1998).

Cystodytes (Della Valle, 1877) (Aplousobranchiata, Polycitoridae) is a colonial ascidian genus widely distributed in both tropical and temperate waters. Despite its marked variability, previous reports of *Cystodytes* in the Mediterranean waters have been attributed to the widespread species *Cystodytes dellechiajei*. Color variation in *C. dellechiajei* has been extensively reported (reviewed by Kott, 1990), which led some authors to suggest that it might in fact be several species (Turon, 1987; Brunetti, 1994). Recently, in a study of this genus in the western Mediterranean, López-Legentil and Turon (2005) found 15 different color morphs and 3 kinds of spicules. However, on the basis of phylogenetic studies using mtDNA data, the authors concluded that these morphological traits were not consistent enough to differentiate between *Cystodytes* species. In addition, several pyridoacridines, a group of highly colored polycyclic aromatic alkaloids, have been reported in *C. dellechiajei* (Bonnard et al., 1995; Delfourne et al., 2000; Rottmayr et al., 2001). Among these are: ascididemin (**1**, Fig. 1), first reported in the genus *Didemnum* (Kobayashi et al., 1988a) and *Eudistoma* (He and Faulkner, 1991); 11-hydroxyascididemin (**2**, Fig. 1), first isolated from *Leptoclinides* sp. (Schmitz et al., 1991); cystodytins A–I (Kobayashi et al., 1988b; Kobayashi et al., 1991); shermilamine B (**3**, Fig. 1), first isolated from a *Tridemnum* species (Carroll et al., 1989); kuanoniamine D (**4**, Fig. 1), first found in an unidentified ascidian (Caroll and Scheuer, 1990); and finally, sebastianines A and B (Torres et al., 2002). Some of these substances exhibit strong bioactivities, including antileukemic properties (Rottmayr et al., 2001) and high cytotoxicity (see Bowden, 2000). Ascididemin also has antibacterial, antifungal (Lindsay et al., 1995) and antifouling activities (Debard et al., 1998).

Most studies of secondary chemistry do not provide detailed information about morphology or spicular types of the source organism. Therefore, it is at present impossible to match chemical and morphological variation in this polymorphic genus. This problem hinders studies of potential biotechnological and pharmaceutical applications of these compounds, or of the systematics and taxonomy of *Cystodytes*.

Nowadays, new techniques have been developed for the detection of substances in biological samples, such as secondary ion mass spectrometry (SIMS) and

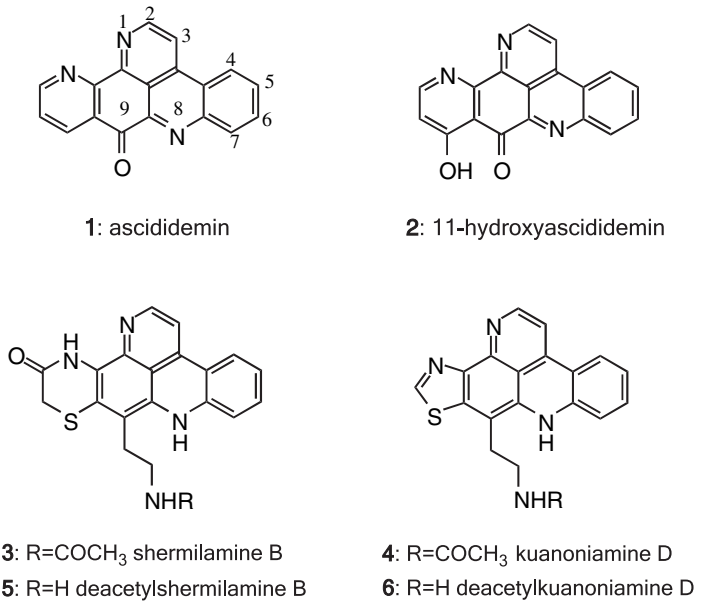

Fig. 1. Structures of the six main alkaloids isolated from the studied specimens of *Cystodytes*. (**1**) Ascididemin, (**2**) 11-hydroxyascididemin, (**3**) shermilamine B, (**4**) kuanoniamine D, (**5**) deacetylshermilamine B, (**6**) deacetylkuanoniamine D.

matrix-assisted laser desorption/ionization coupled with time-of-flight mass spectrometry (MALDI-TOF MS) (e.g. Pacholski and Winograd, 1999; Todd et al., 2001; Stoeckli et al., 2001). These methods are highly sensitive, require only a few milligrams of sample material, and allow rapid detection of even small amounts of targeted substances. In particular, MALDI-TOF MS has become a technique of choice for the location and characterization of peptides and proteins (Caprioli et al., 1997; Uttenweiler-Joseph et al., 1998; Stoeckli et al., 2001).

The main aim of this study was to ascertain the alkaloid composition of the most abundant color morphs of *Cystodytes* from the western Mediterranean (green, purple, brown, and blue). In addition, we analyzed the intraspecimen location of these alkaloids (tunic vs. zooids), and assessed the usefulness of MALDI-TOF MS techniques as a rapid and reliable tool in the detection of targeted substances, intraspecimen location and chemotype assignment.

2. Material and methods

2.1. Sample collection

Specimens of the ascidian *Cystodytes* were sampled in shallow water habitats (usually <7 m deep) by scuba diving in three zones of the western Mediterranean in

Fig. 2. Map showing the zones sampled (northern Catalonia, Ibiza-Formentera and Cabo de Gata) and the color morphs present at each locality.

2002 (Fig. 2): Catalonia (NE Spain), Cabo de Gata (SE Spain) and Ibiza-Formentera (Balearic Islands). Four main color morphs were found: blue, purple, green and brown. The specimens were identified by the authors as belonging to the nominal species *C. dellechiajei*, based on the descriptions of Turon (1987) and Kott (1990). To determine the intraspecimen location of the alkaloids, we separated the zooids from the tunic of living colonies using forceps under a binocular microscope. All samples were frozen and stored at −30 °C prior to lyophilization and further analyses.

For MALDI-TOF MS, three colonies of each color morph were analyzed separately for each locality. We also separated tunic from zooids of the purple and blue Catalonia morphs, and the green Cabo de Gata morph and performed with MALDI-TOF MS, three separate replicate analyses for each. For HPLC analysis, the extracts of several colonies of the same color morph were pooled together to isolate the main pyridoacridines.

2.2. MALDI-TOF analysis

We followed a standard micro-extraction protocol to detect a broad pattern of metabolites, including peptides. For each colony, between 0.5 and 5 mg of freeze-dried material were mixed with solvent A [acetonitrile:methanol:water, 1:1:1, supplemented with 0.3% TFA at a ratio of 1:5 (w:v)]. In a solvent mixture of dichloromethane:methanol (used for HPLC extraction), the alkaloids appear as free bases, whereas in solvent A they are extracted as TFA salts. Both methods allowed complete extraction of pyridoacridines. A slightly more hydrophobic solvent (acetonitrile) is commonly used in peptide detection, and does not interfere with pyridoacridine extraction. The supernatant (0.5 μl) was spotted onto a target well of a 100-position stainless steel sample plate and immediately mixed with 0.5 μl of the matrix solution (50 mg/ml 2,5-dihydroxybenzoic acid dissolved in solvent A).

Measurements were made in a VOYAGER DE-PRO time-of-flight mass spectrometer from Applied Biosystems (Foster City, USA) equipped with a reflectron.

No high-resolution imaging device was available. The acceleration voltage was set to 20 kV. Mass spectra obtained in delayed extraction mode allowed the determination of monoisotopic mass values (m/z; mass-to-charge ratio). For desorption of the components, a nitrogen laser beam ($\lambda = 337$ nm) was focused on the template. Analyses were performed in positive reflector mode. A spectrum was compiled by averaging results from at least 150 shots taken across the width of the sample. After determination of monoisotopic mass values, post-source decay (PSD) measurements for recording fragment ions were taken.

2.3. Pyridoacridine alkaloid isolation and identification (HPLC and NMR)

We followed the optimized protocol for extraction, isolation and identification of pyridoacridine alkaloids of *C. dellechiajei*, described by Bontemps (1996). The freeze-dried colonies were extracted three times in a 1:1 (v:v) mixture of dichloromethane and methanol and then the combined extracts were concentrated under vacuum leaving a powdery organic residue. The different crude extracts were compared by HPLC analysis (Alliance, Waters) with a photodiode array detector (PDA), and the resulting UV spectra of the different peaks were compared with the UV database available in our laboratory. To isolate and identify the major pyridoacridines, the organic extracts were submitted to successive chromatographic separations. Each residue was mixed with RP-8 material (LiChroprep®, 40–63 μm, Merck) at a ratio of 1:6 (w:w), evaporated to dryness, and then subjected to RP-8 column chromatography with water and increasing proportions of methanol. Fractions showing an orange coloration on TLC with Dragendorff reagent were purified by HPLC using Jasco 880-PU pumps under the following conditions: solvent, methanol:water 7:3 (v:v) with a flow rate of 2.5 ml/min; column, Uptisphere UP5ODB-25M; UV (Jasco 875 UV–vis detector) and light diffusion detector (Polymer Laboratories). Pyridoacridine alkaloids were characterized by proton and carbon nuclear magnetic resonance spectroscopy (^{1}H and ^{13}C NMR; Jeol EX 400 spectrometer) and UV spectroscopy (Hewlett Packard diode array spectrophotometer). Spectral values of the purified metabolites were compared with reference compounds available in our laboratory and with published values from the literature.

3. Results

3.1. MALDI-TOF MS results

Peak clusters at m/z 286 and m/z 301, corresponding to ascididemin (**1**) and 11-hydroxyascididemin (**2**), respectively, were found in the MS spectrum of the blue morph from Catalonia and Ibiza-Formentera and the green morph from Cabo de Gata and Ibiza-Formentera (Table 1). Ascididemin was also detected in the brown morph from Catalonia and Ibiza-Formentera. In the mass spectra obtained from the purple morph, we detected peaks at m/z 361 and 391, corresponding to protonated molecular ions of shermilamine B (**3**) and kuanoniamine D (**4**) $[M + H]^+$,

Table 1
Main pyridoacridine alkaloids present in the four color morphs of *Cystodytes* studied

Alkaloid	Mass observed (*m*/*z*)	Purple	Green	Blue	Brown
Ascididemin	286	–	HPLC/MS	HPLC/MS	MS
11-Hydroxyascididemin	301	–	HPLC/MS	HPLC/MS	–
Deacetylkuanoniamine D	319	HPLC/MS	–	–	–
Deacetylshermilamine B	349	HPLC/MS	–	–	–
Kuanoniamine D	361	HPLC/MS	–	–	–
Shermilamine B	391	HPLC/MS	–	–	–

HPLC: compounds isolated and identified after HPLC/PDA analyses and characterized by NMR and UV spectroscopy. MS: compounds detected by MALDI-TOF spectrometry, with indication of their mass signal.

respectively. The PSD-fragmentation spectra of the peaks at *m*/*z* 319 and 349 were very similar to those of shermilamine B and kuanoniamine D, but were shifted to a lower mass by 42 absolute mass units. Therefore, they were attributed to their deacetylated forms: deacetylshermilamine B (**5**) and deacetylkuanoniamine D (**6**), respectively. Independent analyses of the tunic and zooids revealed no major differences between body components in the green and blue morphs. In the tunic of the purple morph the four major compounds (**3**, **4**, **5**, **6**) were found together, whilst in the zooids only compounds **5** and **6** were present (Fig. 3). No differences in alkaloid composition were found between the replicate analyses of the same color morph, locality or intra-organism body component ($n = 3$).

3.2. Pyridoacridine alkaloid isolation and identification (HPLC and NMR)

The major pyridoacridine alkaloids isolated from the different color morphs of *Cystodytes* are listed in Table 1. They belonged to two classes: C_9-unsubstitued pyridoacridines and sulfur-containing pyridoacridine ethylamine alkaloids. Although **1** and **2** were detected in all the blue and green samples, **1** was isolated from the blue morph found in Catalonia and **2** from the green morph found in southern Spain. In the purple specimens collected in Catalonia four significant peaks were observed. The first two were identified as **3** and **4**. The UV spectra of the other two peaks were identical to **3** and **4**, and after their structure was clarified by NMR, were identified as their deacetylated forms: **5** (Fig. 1) and **6** (Fig. 1; Eder et al., 1998). No alkaloid was observed in the brown morph from Ibiza-Formentera, examined with the same extraction and analytical procedures.

4. Discussion

Our study revealed two major chemotypes within the four color morphs of *Cystodytes* studied. The first had sulfur-containing pyridoacridines and corresponded to the purple morph, whilst the second had C_9-unsubstituted pyridoacridine alkaloids and was found in the blue and green morphs. The purple

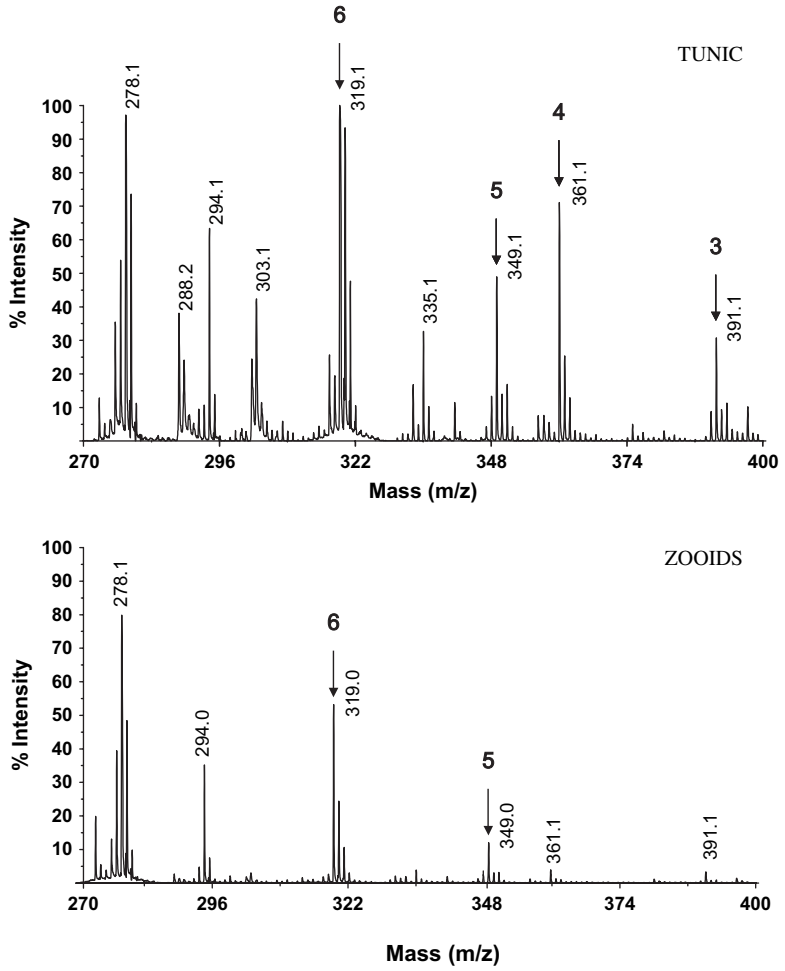

Fig. 3. Purple morph MALDI-TOF spectra of the intraspecimen location (tunic and zooids) of the major compounds. (**3**) shermilamine B, (**4**) kuanoniamine D, (**5**) deacetylshermilamine B, and (**6**) deacetylkuanoniamine D. In the tunic spectra, peak **6** (*m*/*z* 319.1) comes to saturation.

morph contained **3** and **4**, as noted previously by Rottmayr et al. (2001), together with their deacetylated forms (**5** and **6**). Eder et al. (1998) described **6** in the sponge *Oceanapia* sp. Recently, Skyler and Heathcock (2002) proposed a pyridoacridine family tree in which they predicted the existence of natural pyridoacridines such as **5**, found here for the first time. To our knowledge, the coexistence of free ethylamine precursors and their acetamide forms has not been described before. Pyridoacridine alkaloids are pH-sensitive pigments. Compounds **3**, **4**, **5** and **6** are purple under acidic conditions. This helps to explain the purple color observed in the colonies, as the tunic of *Cystodytes* is highly acidic (pH < 1; Tarjuelo et al., 2002). Similarly, the violet color of another species, *Cystodytes violatinctus* Monniot, 1988, is probably

due to the presence of shermilamine analogs (Koren-Goldshlager et al., 1998, 2000). Both compounds **1** and **2** are yellow under acidic conditions. The link between color and pyridoacridine composition is less evident in these latter cases, and may depend on other unknown molecules.

In the tunic of the purple morph the four major compounds (**3**, **4**, **5**, **6**) were found together, whilst the apparent lack of **3** and **4** in the zooids indicates two possible hypotheses: (1) The pyridoacridines are produced as free amines (**5** and **6**) in the zooids, and then distributed and stored in the tunic as acetylated forms (**3** and **4**), with the tunic being the tissue most exposed to predators, or; (2) The most active metabolites are the free ethylamine compounds (**5** and **6**), present in both tunic and zooids. Particularly pertinent to this issue will be the identification and location of the type or types of cells responsible for the production and/or storage of the active compounds in the different forms and the assessment of their ecological role. Although at first glance it seems that redundancy of defense mechanisms violates the postulates of optimal defense theory, in fact these mechanisms may have evolved as a response to different predators and/or competitors, or they may work at different life history stages as suggested by Pisut and Pawlik (2002). Comparative studies of the bioactivity of the different forms, as well as of their potential metabolic costs, will be needed in order to adequately place our findings within the framework of optimal defense theory.

This study showed the usefulness of MALDI-TOF MS as a quick and reliable tool for the detection of targeted low molecular mass compounds at both colony and intraspecimen levels. In addition, and due to its high sensitivity, the MALDI-TOF technique provided clues to the presence of compound **1** in the brown morph, which was undetected under the HPLC conditions used. Although in this study we applied MALDI-TOF techniques qualitatively to assess chemotypes among morphotypes (i.e. the presence/absence of already known alkaloids), a further possible use involves the co-application of imaging techniques for fine intraspecimen location (at the cellular level) of targeted compounds. The full range of applications of this technique should improve our capacity for chemical detection, which represents an important aspect of all descriptive and experimental work in the field of marine chemical ecology.

Finally, the chemical differences between color morphs raised concerns about the taxonomic status of the specimens routinely attributed to *C. dellechiajei* in the western Mediterranean. Some authors favor the view that several species have been grouped under the name *C. dellechiajei* (e.g. Monniot, 1988), whilst others have found little basis for splitting this circumtropical species (e.g. Kott, 1990). Whether the chemical variability detected reflects the existence of 2 chemotypes within a single species or whether we are dealing with a group of sibling species is still an open question. Biological studies on their life cycles, as well as an assessment of population genetic structure, will be needed to clarify the taxonomic status of this taxon definitively. Our results highlight the importance of a detailed morphological description of the producer organism of marine natural products, especially for taxa whose taxonomy is not well resolved, in order to fully understand the variation in secondary chemistry within and between species.

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