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Chemical diversity in the scleractinian coral *Astroides calycularis*

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ABSTRACT

The detailed chemical inspection of the Mediterranean coral *Astroides calycularis* led to the isolation and structure characterization of two families of alkaloids. Derivatives of orthidine were for the first time isolated from this species. The second family of alkaloids includes the aplysinopsins among which a new derivative is described. The structure was identified on the basis of extensive NMR data interpretation. These results are of chemotaxonomic relevance in order to link this species to the Atlantic *Tubastrea aurea*.

1. Introduction

The monotypic genus *Astroides* (phylum Cnidaria, class Anthozoa, subclass Hexacorallia, order Scleractinia, family Dendrophylliidae) is composed of the sole species *Astroides calycularis* (Pallas, 1766), an azooxanthellate colonial coral strictly protected by the Convention on the conservation of European Wildlife and natural Habitats (Cairns et al., 2001). After a progressive extinction in the northern Mediterranean the species was only found in the south-western Mediterranean until the 90′ (Zibrowius, 1995). Since then, *A. calycularis* seems to have recovered the north-eastern Mediterranean shallow waters that may be due to climate change effects (Fig. 1) (Bianchi, 2007; Grubelic et al., 2004). As part of an ongoing research program to describe the chemodiversity of the emblematic benthic Mediterranean communities, *A. calycularis* was investigated in order to obtain a large chemical overview for this species. As for many invertebrates of the benthic Mediterranean community, few chemical studies have been reported on *A. calycularis*. Aplysinopsin (**1**) and three analogues **2**–**4** were previously isolated by the group of Fattorusso (Fattorusso et al., 1985), along with the pteridine derivative **5** (Fig. 2) (Aiello et al., 1987). We report herein the isolation and structure characterization of a new derivative of the aplysinopsin family **6** together with known compounds **1**–**2**. We were also able to identify, for the first time in this species, orthidine derivatives **7**–**9** which is of chemotaxonomic relevance for this group of organisms.

2. Results and discussion

2.1. Structure elucidation

The structures of the aplysinopsin derivatives **1** and **2** were determined by comparison with spectroscopic data of the literature (Aiello et al., 1987; Guella et al., 1988) and a new aplysinopsin derivative, named 6-bromo-*N*-methylaplysinopsin (**6**), was isolated and characterized as a 1:2 mixture of stereoisomers (*E*/*Z*).

A comparative analysis of the spectroscopic data of **6**, isolated as a bright yellow oil, with those of **2**, revealed a high level of similarity between both structures, especially for the indole part of the molecule. ^{1}H NMR (Table 1) and MS (*m*/*z* 347.0 (1 0 0), 349.0 (1 0 0) $[M+H]^+$) data suggested the presence of an additional methylene group in **6** instead of an exchangeable proton in **2**. The methylcreatinine moiety of **2** was clearly modified in **6** and the key C-3′/C$\underline{H_3}$N-3′ HMBC correlation indicated the presence of an additional methyl group at N-C3′ (Fig. 3). The further key HMBC correlations between C-3′, C$\underline{H_3}$N-2′ and C$\underline{H_3}$N-4′ and between C-1′ and C$\underline{H_3}$N-2′ led us to fully characterize the trimethyl-substituted creatinine of **6**. Comparison of the NMR data with those of a non brominated *N*-methylaplysinopsin, isolated from an Australian sponge, confirmed the structure of **6** (Taylor et al., 1981).

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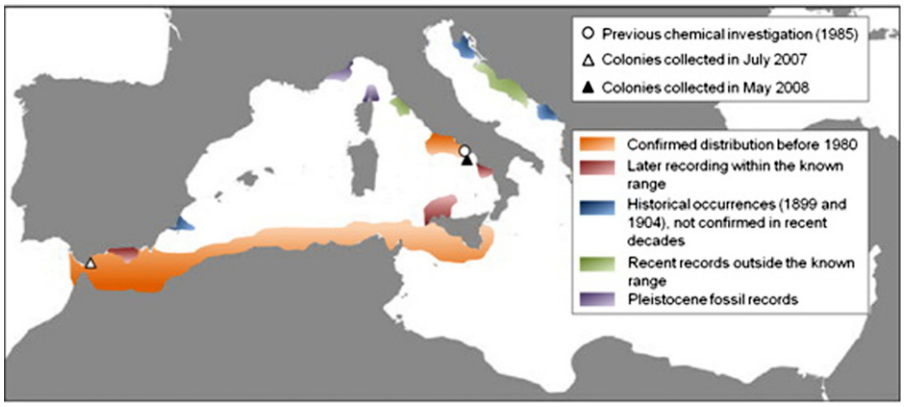

Fig. 1. Distribution of *A. calycularis* within the Mediterranean Sea and sites of collection for chemical studies.

A second family of natural products was evidenced in the methanolic fraction as suggested by the presence of compounds displaying distinct UV profiles in the HPLC-DAD spectra. In the 1H NMR spectrum of **8**, a catechol moiety was evidenced by the presence of characteristic aromatic signals at δ_H 6.91 (1H, d, J = 8.5 Hz, H-5), 6.97 (1H, dd, J = 2.0, 8.5 Hz, H-6) and 7.06 (1H, d, J = 2.0 Hz, H-8) ppm. On the basis of HRESIMS, MS and NMR data, **7**–**9** were identified as isomers with all the molecular formula $C_{18}H_{20}N_6O_4$. Interpretation of the 1H and the 13C NMR spectra of **8**, as well as HSQC and HMBC data, established the presence of a tubastrine-like substructure accounting for $C_9H_9N_3O_2$ of the required molecular formula. Remaining NMR signals indicated the presence of a partial fragment made of a 3,4-dihydroxysubstituted phenyl group, a guanidine group (δ_C 158.7 ppm) and two oxymethines at δ_C 77.6 (C-2), δ_H 4.90 (d, J = 5.5 Hz, H-2) and δ_C 79.9 (C-3), δ_H 5.62 (d, J = 5.5 Hz, H-3) ppm. Interpretation of the key C-4a/H-3 and C-8a/H-2 HMBC correlations and MS/MS fragments, allowed the binding of these two units through the oxygens of the catechol moiety. Orthidine C was recently isolated from the New Zealand ascidian Aplidium orthium (Pearce et al., 2008) and comparison with the NMR data of this compound revealed that **8** has the same structure. Compounds **7** and **9**, isolated as a 1:1 mixture, shared the same basic structure. Comparison with spectroscopic data of other analogues isolated in the same ascidian allowed us to identify orthidine A (**7**) and D (**9**). All these orthidine derivatives are isolated for the first time from *A. calycularis*. The measure of specific rotatory powers indicated very weak values which suggested the presence of racemic mixtures for all the orthidines as previously described for the ascidian (Pearce et al., 2008). This result is still in accordance with the putative biosynthetic pathway proposed by the authors for these com-

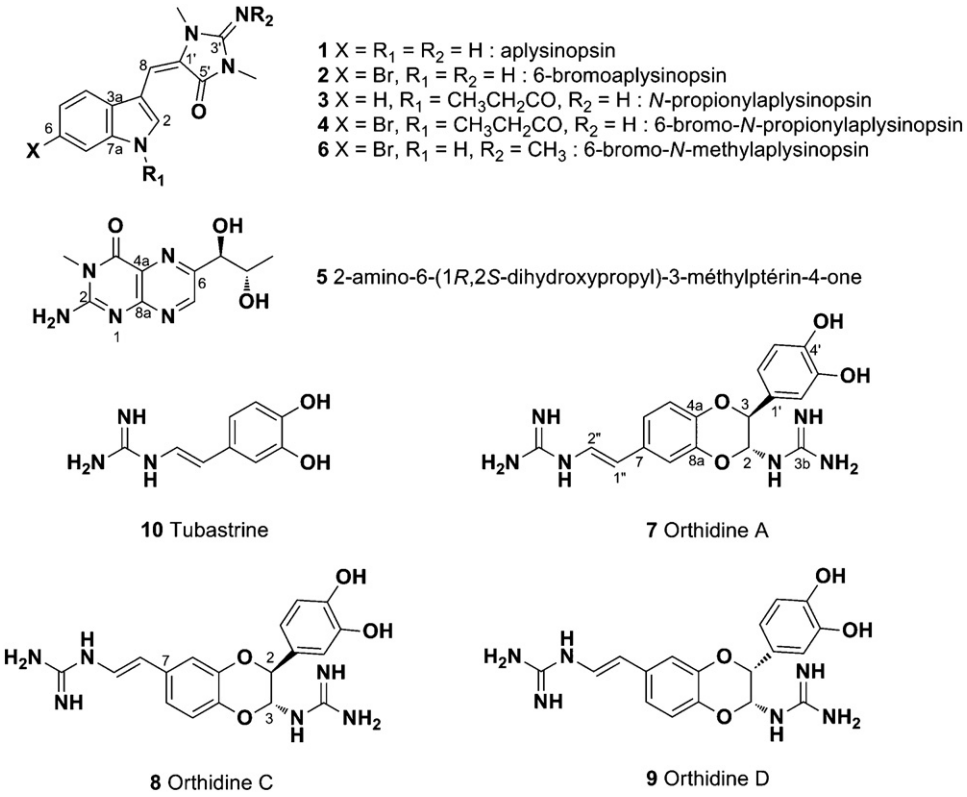

Fig. 2. Compounds **1**, **2**, **6**, **7** and **9** isolated from *A. calycularis* in this study and other compounds isolated previously from the same species and *Tubastrea aurea*.

Table 1
^{13}C (125 MHz) and ^{1}H (500 MHz) NMR data for **6** (CD_3OD).

Position	CH_n	(*Z*)-6		(*E*)-6	
		δ_C	δ_H (mult.)	δ_C	δ_H (mult.)
2	CH	121.3	7.67 (br s)	120.6	7.85 (br s)
3	C	108.8	–	110.1	–
3a	C	127.5	–	127.7	–
4	CH	132.0	7.80 (br s)	125.7	7.35 (m)
5	CH	115.5	7.50 (br s)	116.2	7.65 (m)
6	C	115.9	–	115.9	–
7	CH	131.1	7.72 (br s)	121.3	7.60 (s)
7a	C	138.9	–	138.2	–
8	CH	117.3	7.31 (s)	117.7	7.36 (s)
1′	C	123.3	–	124.2	–
3′	C	154.1	–	153.8	–
5′	C	161.2	–	161.8	–
Me-N2′	CH_3	29.1	3.55 (s)	31.0	3.65 (s)
Me-NC3′	CH_3	26.3	3.32 (s)	31.7	3.41 (s)
Me-N4′	CH_3	32.2	3.39 (s)	27.8	3.45 (s)

pounds. They can be formed by a non enzymatic and racemic radicalar dimerization of tubastrine (**10**).

2.2. Chemotaxonomic significance

The full chemical study of *A. calycularis* led to the isolation of two previously known aplysinopsin derivatives **1** and **2**, together with the new derivative **6**. We were not able to identify the propionyl-substituted analogues **3** and **4** in the extracts of our studied species whereas they were found by the group of Fattorusso in their original chemical study (Fattorusso et al., 1985). With more than 30 natural analogues, aplysinopsins represent a widely distributed family of indole alkaloids found in several groups of marine invertebrates (sponges, scleratinian corals, sea anemones, mollusks) and in various biota (Pacific, Indonesia, Caribbean, and Mediterranean areas) (Bialonska and Zjawiony, 2009). Aplysinopsin (**1**), the first member of this family, was isolated for the first time in 1977 as the major secondary metabolite of the sponge *Fascaplysinopsis reticulata* (=*Aplysinopsis reticulata*) (Kazlauskas et al., 1977). Compound **1** and other derivatives were later isolated from other species of *Aplysinopis* so that these compounds were initially proposed as chemotaxonomic markers of this genus. However, this proposition was not maintained after the isolation of close analogues in additional sponge species (Bialonska and Zjawiony, 2009; Djura et al., 1980). For the first time in 1985, the group of Fattorusso isolated aplysinopsin derivatives from the scleratinian coral *A. calycularis* (Fattorusso et al., 1985). Since then, aplysinopsins were described from many other species of sponges, corals, sea anemones and mollusks. The multiple sources of aplysinopsins suggest a common microbial origin, but, to date, no cellular localization study came to confirm this hypothesis. Weak antimicrobial activities were found to be associated to these compounds as well as cytotoxicity on some tumoral cell lines, antiplasmodial activity or, more significantly, properties related to neurotransmission modulation

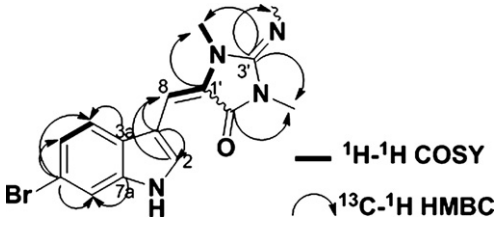

Fig. 3. Key COSY and HMBC correlations for the new 6-bromo-N-methylaplysinopsin (**6**).

(Bialonska and Zjawiony, 2009). In addition to the medicinal potential of aplysinopsins, some assumptions were made on their putative ecological roles. Some anti-feedant activity was deduced from the structure-activity studies performed on marine alkaloids (Lindel et al., 2000). Due to a non-destructive photoisomerization process, a possible function as UV protectors was also attributed to aplysinopsins (Guella et al., 1988, 1989). Even if this function could be useful for shallow-water tropical species, other aplysinopsin containing species, as *A. calycularis* or *Leptopsammia pruvoti*, are sometimes found in caves of temperate areas and do not seem to need protection from solar radiation. Another ecological study showed their significant role in eliminating potential space competitors by inhibiting larval growth of competitive sessile organisms (Koh and Sweatman, 2000). All these studies evidenced the clear ecological significance of these compounds in the marine ecosystems.

Our chemical study led to the identification of a second family of alkaloids from this species. The isolation of the orthidine derivatives **7**–**9** is of high interest for chemotaxonomic purposes. Indeed, tubastrine (**10**) is a bioactive monomer previously isolated from *Tubastraea aurea* (Sakai and Higa, 1987), a second Hexacorallia morphologically closely related to *A. calycularis*. *Tubastrea aurea* belongs to the same Dendrophilliidae family and is found in several oceans among them the Atlantic around the Macaronesian archipelagos. Previous chemical studies of this species revealed the presence of aplysinopsin derivatives (Iwagawa et al., 2008). Our report of orthidine derivatives in this species suggests that these two species are very closely chemotaxonomically related even if only the monomer has been identified in *T. aurea* so far. These morphological and chemical similarities may reflect a close evolutionary history between these two genera. While *Astroides* is mostly found in the western Mediterranean, *Tubastraea* can be found in the Caribbean, the Indo-Pacific Ocean, the Red Sea and the Atlantic Ocean. Then, an adaptation of *Tubastraea* to the Mediterranean conditions could have led to the *Astroides* genus, or *vice versa*, even if we lack clear genetic data to confirm this assumption. Such a migration was already observed for the Antipatharia *Antipathella wollastoni* (Anthozoa) (Ocaña, 2005; Ocaña et al., 2006). Because the presence of these compounds may be dependant from the collection site, we decided to investigate the chemical composition of a sample of *A. calycularis* geographically distant from the Strait of Gibraltar. In May 2008, a second sample of *A. calycularis* was collected off the coasts of Naples, site of the first chemically investigated sample of this species by the group of Fattorusso. Performing the same extraction and fractionation processes, the MeOH (100%) fraction was analyzed by LC/MS. The data analysis revealed the presence of aplysinopsins as well as orthidines in this fraction just like for our first collected sample. The MS data also underlined a pseudo-molecular ion at *m/z* 194.1 $[M + H]^+$ which could be assigned to tubastrine (**10**). Because no clear chemical differences were evidenced between two samples collected in distant Mediterranean areas, the metabolome of *A. calycularis* is mainly composed of two major secondary metabolites families, aplysinopsin and tubastrine derivatives. Recently, a genetic study on this species revealed a high level of connectivity and moderate level of differentiation among all the populations of the North Western Mediterranean (Casado-Amezu et al., 2012). Our chemical investigation clearly confirms these observations and consequently the progression of this species from the Atlantic to the Tyrrhenian Basin through the Alboran Sea. Of course, all these taxonomic considerations must take into account that a bacterial origin of these compounds has been proposed and genetic studies would give valuable information to infer the exact role of the associated microbiota into the production of these compounds.

3. Experimental

3.1. General experimental procedures

NMR spectra were measured on a Bruker Avance 500 MHz spectrometer with pulsed field gradient and referenced to residual solvent signals (CD_3OD, at δ_H 3.31 and δ_C 49.0 ppm). HRESIMS data were measured with a LTQ Orbitrap mass spectrometer (Thermo Finnigan). HPLC–MS chromatograms were performed on a Waters Alliance 2695 connected to a Waters 2487 UV detector on-line with a Bruker Esquire 3000 mass spectrometer. HPLC purification was carried out on a Waters 600 system equipped with a Waters 717 Plus autosampler, a Waters 996 photodiode array detector, and a Sedex 55 evaporative light-scattering detector (Sedere, France).

3.2. Animal material

Approximately 10 medium-sized colonies of *A. calycularis* were collected by SCUBA at 20 m depth from three different sites near Ceuta (Spain-Strait of Gibraltar) in July 2007: "Callejones" (35°53′10″ N 5°17′30″ E), "Ciclon de Tierra" (35°53′50″ N 5°18′29″ E) and "Piedra Gorda" (35°53′8″ N 5°17′24″ E). A voucher sample (070702Ce5-04), identified by Oscar Ocaña, has been deposited in the Centre d'Océanologie de Marseille (Endoume, France). A second collection was made in May 2008 in a different location: the marine protected area of "Punta Campanella", Massa Lubrense, Italy (Fig. 1). Both collections were kept frozen until used.

3.3. Extraction, isolation and characterization of compounds

Colonies of *A. calycularis* from the first collection site were freeze-dried (250 g) and extracted three times with CH_2Cl_2/MeOH (3 × 1000 mL) at room temperature, yielding 15 g of brown oil after solvent evaporation. The crude extract was fractionated by RP-C_{18} flash chromatography with a step gradient from H_2O to MeOH and from MeOH to CH_2Cl_2 (500 mL per fraction). The MeOH (100%) fraction (288.1 mg) was then subjected to RP-semi-preparative HPLC (Phenomenex, Luna phenyl-hexyl, 250 mm × 10 mm, 5 μm) with a gradient of H_2O/CH_3CN/TFA (flow: 3.0 mL min^{-1} from 90:10:0.1 to 65:35:0.1–30 min). The subsequent mixtures were finally purified by RP-analytical HPLC (Phenomenex, Luna C_{18}, 150 mm × 4.6 mm, 5 μm) to afford pure compounds **1** (4.8 mg, $1.9.10^{-3}$% dry wt), **2** (2.4 mg, 10^{-3}% dry wt), **6** (1.2 mg, $0.5.10^{-3}$% dry wt) and **8** (5.4 mg, $2.2.10^{-3}$% dry wt), and compounds **7** and **9** as a 1:1 mixture (1.4 mg, $0.6.10^{-3}$% dry wt). The second collection of *A. calycularis* from Italy (50 g dry wt, 3.7 g crude extract) was processed in the same manner. Compounds **1–2** and **6–9** were also identified in the MeOH (100%) fraction by HPLC/MS.

3.3.1. 6-Bromo-N-methylaplysinopsin (**6**)

Yellow solid; UV/Vis (MeOH) λ_{max} 225 (log ε 4.2), 282 (3.5), and 398 (4.0) nm; IR (KBr) ν_{max} 3420, 1672 cm^{-1}; ^1H and ^{13}C NMR, see Table 1. HRESIMS (positive mode) *m/z* found 347.0512, 347.0502 calcd for $C_{15}H_{16}BrN_4O$ $[M + H]^+$.

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