Occurrence of symmetrical diacylguanidines triophamine and limaciamine in three Polyceridae species from Canary Islands: are they chemical markers of these nudibranchs?

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Abstract

Two symmetrical diacylguanidines, triophamine (**1**) and limaciamine (**2**), have been found to occur in three Polyceridae nudibranchs from Canary Islands. These compounds were previously reported from taxonomically related species collected from distinct geographical areas. Due to the peculiar occurrence of **1** and **2** in Polyceridae nudibranchs and, in particular, exclusively in members of Polycerinae and Triophinae subfamilies, it should be suggested that these diacylguanidines are distinctive chemical markers of this group of nudibranchs.

1. Subject and source

Nudibranchs are naked and apparently vulnerable gastropods constituting the largest order among the marine heterobranchs (Phylum Mollusca) (WoRMS database, 2018; Bouchet et al., 2017). They are important consumers in benthic communities, feeding mostly upon sessile invertebrates (e.g. sponges, cnidarians, tunicates, bryozoans) although some species hunt either other nudibranchs, echinoderms, fish eggs or mollusk eggs (Farmer, 1978; McDonald and Nybakken, 1991; Calado and Urgorri, 2001, 2002; Megina and Cervera, 2003; Behrens, 2005; Nakano et al., 2011; Nakano, 2017). Having lost the physical protection of a shell, nudibranchs often utilize chemicals to deter predators. These chemicals showing an extraordinary variety of molecular architectures are typically sequestered from prey species even though some nudibranchs have been demonstrated to be able of producing their own defensive molecules by *de novo* biosynthesis. Based on their general morphology and digestive gland, nudibranchs can be separated into two distinct clades: Doridina (= Anthobranchia) including the vast group of dorids and Cladobranchia embracing aeolids and other non-dorid nudibranchs (Wägele and Willan, 2000). These two clades have been confirmed recently by molecular phylogenetic analyses (Mahguib and Valdés, 2015). All dorids have a compact digestive gland and most are distinguished by a feather-like plume of gills on their dorsal side (except in the families Phyllidiidae and Corambidae) whereas most of cladobranchs have a branched digestive gland and lack gills (Wägele and Willan, 2000).

Among dorids, the family Polyceridae includes a group of nudibranchs that have elongate and limaciform bodies and a reduced mantle skirt. The family comprises three main subfamilies: Polycerinae, Triophinae, and Nembrothinae along with two minor subfamilies, Kalinginae and Kankelibranchinae, both including only one genus (Burn, 1967; Rudman, 1998; Ortea et al., 2005; Bouchet et al., 2017). All species belonging to Polycerinae and Triophinae subfamilies feed on encrusting or erect bryozoans whereas the subfamily

Nembrothinae embraces species exhibiting a variety of feeding habits, i.e. arborescent bryozoans (*Tambja*), ascidians (*Nembrotha*) and nudibranchs (*Roboastra*).

Three species have been chemically investigated for the first time in this work, *Thecacera pennigera* Montagu, 1813 and *Polycera elegans* Bergh, 1894, both belonging to subfamily Polycerinae, and *Plocamopherus maderae* R. T. Lowe, 1842, a member of subfamily Triophinae. The nudibranchs were collected in the marine micro-area of El Cabrón, at the East of Gran Canaria (Canary Islands). This site is one of the most biodiverse areas of Gran Canaria, being characterized by good conservation status, variety of habitats, and high biodiversity in both fish communities and invertebrates, in shallow waters (less than 30 m depth) (Aristegui et al., 2009).

2. Previous work

In comparison with other taxa of the clade Doridina, the chemistry of Polyceridae nudibranchs has been scarcely investigated and a few number of papers reporting their chemical components have been appeared in the literature to date (Cimino et al., 2001; Dean and Prinsep, 2017). Most of them concerns with a group of dietary antimicrobial and cytotoxic alkaloids, the tambjamines, that have been found in diverse species (*Tambja*, *Nembrotha*, *Roboastra*) of the subfamily Nembrothinae as well as in their bryozoan preys (Cartè and Faulkner, 1983, 1986; Paul et al. 1990; Lindquist and Fenical, 1991; Karuso and Scheuer, 2003; Granato et al., 2005; Carbone et al. 2010; Pereira et al., 2012).

On the other side, with regards to the subfamilies Polycerinae and Triophinae, only three species, *Polycera tricolor*, *Triopha catalinae* and *Limacia clavigera*, have been chemically investigated until now (Cimino et al., 2001; Dean and Prinsep, 2017). These studies have resulted in the isolation of two unique closely related alkaloids, triophamine (**1**) and limaciamine (**2**) (Fig. 1), both exhibiting a guanidine moiety, which is symmetrically bis-acylated by two polyketide acyl residues. Compound **1** was first reported as main metabolite of the lipophilic extract of *T. catalinae* collected from the western coasts of North America (British Columbia) (Gustafson and Andersen, 1982). Subsequently, compound **1** was also isolated as main component from the lipophilic extract of specimens of *P. tricolor*, that were sampled in the same geographical area (Gustafson and Andersen, 1985). The structure of triophamine (**1**) has been confirmed by synthesis (Piers et al., 1984). Due to the absence of **1** in the bryozoan preys of the mollusk as well as the occurrence in *T. catalinae* specimens collected from different sites, a probable *de novo* biosynthesis of **1** in *T. catalinae* was suggested (Graziani and Andersen, 1996). This hypothesis was rigorously proven by *in vivo*

feeding experiments using suitable precursors labeled with stable isotopes that led to significant incorporation of labeled acetate (Graziani and Andersen, 1996) and butyrate (Kubanek and Andersen, 1997) units in the alkyl parts of **1**. Finally, limaciamine (**2**), the structure of which is closely related to compound **1**, was later isolated from the skin of *L. clavigera*, collected in Norway (Graziani and Andersen, 1998).

3. Present study

In this communication we report the results of the chemical investigation of three Polyceridae species collected at "El Cabrón" (27°52'26.3"N 15°22'56.7"W), at the East of Gran Canaria Island, during February 2018. *T. pennigera* (40 individuals, size ~1.7 cm), *P. elegans* (40 individuals, size ~2.1 cm) and *P. maderae* (25 individuals, size ~3.5 cm) were collected on rocks at a depth 14 ±2 m during night SCUBA diving and immediately frozen at -20 °C. The species were identified by one of us (A.H.-B.). Frozen biological material was then transferred to ICB laboratory where it was stored at -20°C until the extraction.

In order to highlight differences in the secondary metabolites content of the external and internal tissues, *T. pennigera*, *P. elegans*, and *P. maderae* were separately extracted with acetone by using the same procedure as following described. Frozen individuals of each species were first immersed in acetone (30 mL x 3) and sonicated with ultrasounds for 1 min to get metabolites present in the external part of the mollusks. The organic solvent was evaporated under reduced pressure and the residual water was lyophilized to obtain the corresponding crude acetone extracts of the external parts (36.7 mg for *T. pennigera*; 112.0 mg for *P. elegans*; 36.0 mg for *P. maderae*). These extracts were directly analyzed without further partition. After the first extraction, the whole animal residues were transferred into a mortar, crumbled with a pestle and extracted again with acetone (30 mL x 3). The extracts were concentrated under reduced pressure until the evaporation of the organic solvent, and the resulting aqueous residues were partitioned in Et_2O (40 mL x 3) and, subsequently, in *n*-BuOH (30 mL x 3) affording for *T. pennigera*, *P. elegans*, and *P. maderae* the corresponding Et_2O (36.7 mg, 55.3 mg, and 24.7 mg, respectively) and *n*-BuOH (21.1 mg, 16.5 mg, and 18.8 mg, respectively) extracts of the internal parts. All extracts were analyzed by TLC chromatography (Merck Silica Gel 60 F254 plates) in different eluent systems. An intense UV–visible band (R_f 0.75–0.85, petroleum ether/ Et_2O, 1:1) was detected, along with usual lipid components (i.e. sterols and fatty acids), in the acetone extracts of external parts as well as in the Et_2O extracts of internal parts of all three species. All distinct extracts obtained for each species were separately fractionated by SiO_2 column chromatography (Merck Kieselgel

60 powder) using a petroleum ether/ Et_2O gradient as eluent. All fractions from each column purifications were carefully analyzed by ^1H NMR revealing the presence in those fractions which contained the UV–visible band of a same product in *T. pennigera* and *P. maderae* whereas a different but related molecule was detected in *P. elegans*. The subsequent purification of these selected fractions was carried out by reversed-phase HPLC [C5 Phenomenex column (5 μm; 250 x 4.6 mm); MeOH/H_2O, 8:2; flow 1 mL/min; UV 254 nm] to obtain triophamine (**1**) from *P. elegans* and limaciamine (**2**) from *T. pennigera* and *P. maderae*. No further related minor metabolites were detected in the extracts of the animals. Compounds **1** and **2** were identified by spectroscopic data (1D and 2D NMR, and MS, see Supplementary material) and comparison with the literature (Gustafson and Andersen, 1982, 1985; Piers et al., 1984; Graziani and Andersen, 1998).

4. Chemotaxonomic significance

The finding of unique diacylguanidines triophamine (**1**) and limaciamine (**2**) in three Polyceridae species, *Thecacera pennigera*, *Polycera elegans,* and *Plocamopherus maderae,* from Canary Islands, is in agreement with the chemistry reported in the literature for taxonomically close nudibranchs, *Triopha catalinae*, *Polycera tricolor*, and *Limacia clavigera,* that were collected in distinct geographical areas including British Columbia and Norway coasts (Cimino et al., 2001; Dean and Princeps, 2017). All these nudibranchs belong to either Polycerinae or Triophinae subfamily and each species has been found to contain only one of the two compounds (Fig. 1). Even though the number of chemically investigated species is limited, the peculiar occurrence of either triophamine (**1**) or limaciamine (**2**) seems to be surprisingly distinctive for the members of both Polycerinae and Triophinae subfamilies. It should be noted that these compounds, the structures of which are strictly related, have been found in nature exclusively in these organisms. In addition, having *de novo* biosynthesis of **1** been demonstrated in *T. catalinae*, it is reasonable to suggest the same biosynthetic origin in all other investigated species.

With the exception of those included in peptide architectures, diacylguanidines are not frequently encountered in marine organisms (Berlinck et al., 2017, and previous in this series). In particular, with regards to marine mollusks, only two diacylguanidines, dotofide (Putz et al., 2011) and actinofide (Carbone et al., 2017), have been reported to date, in addition to compounds **1** and **2** here reported. From a structural point of view, dotofide and actinofide are markedly different from **1** and **2** being both characterized by terpenoid rather than polyketide acyl residues linked to the guanidine moiety. These compounds occur in two

nudibranch species belonging to two different clades: dotofide was isolated from nudibranch *Doto pinnatifida* (clade Cladobranchia) (Putz et al., 2011) whereas actinofide was found in dorid *Actinocyclus papillatus* (clade Doridina) (Carbone et al., 2017).

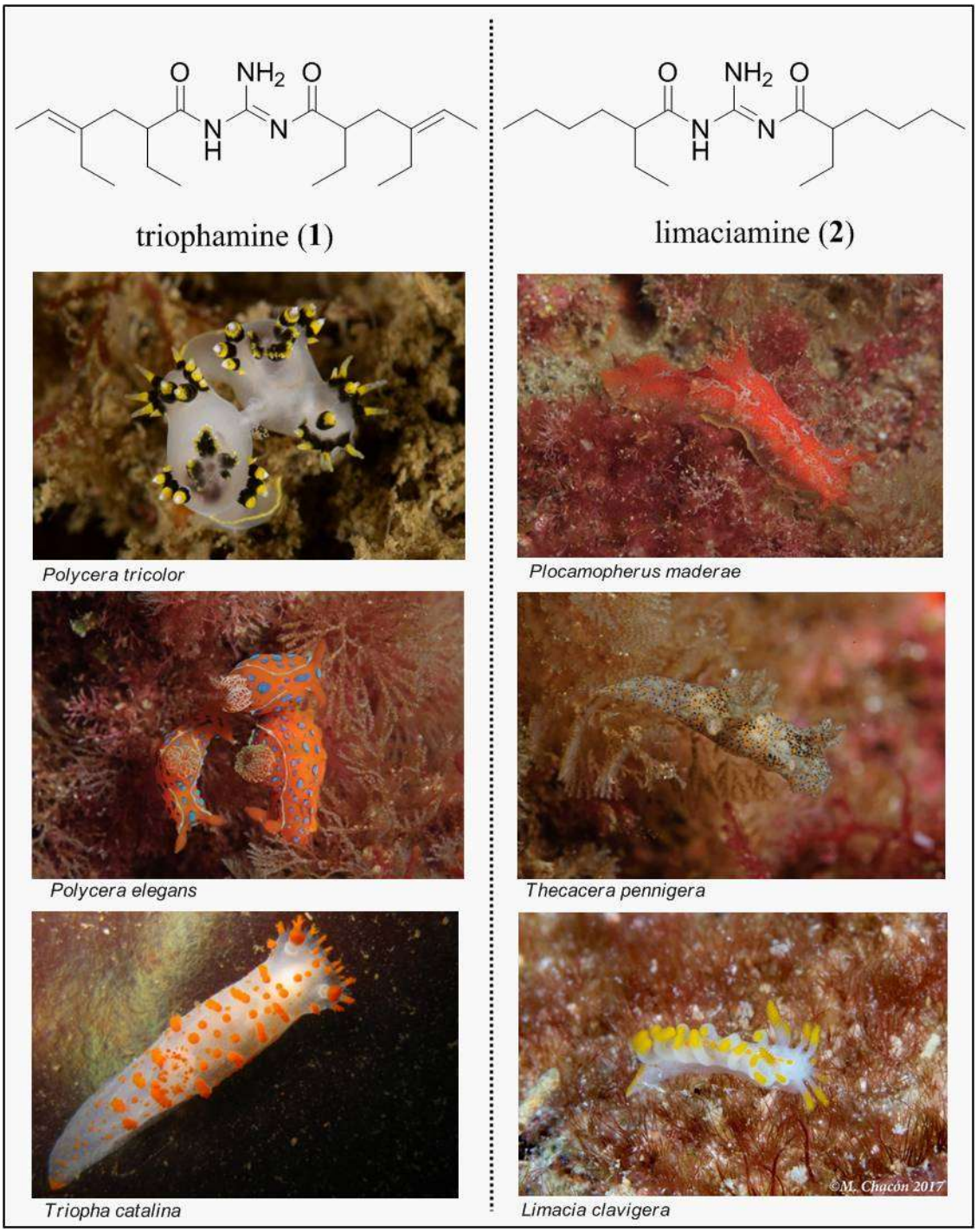

Figure 1. Distribution of triophamine (**1**) and limaciamine (**2**) in Polycerinae and Triophinae nudibranchs studied previously and in this work.

On the other side, the occurrence of diacylguanidines **1** and **2** is highly specific in all Polycerinae and Triophinae species that have been chemically investigated to date. In contrast with the members of Nembrothinae subfamily that are characterized by different metabolite patterns (Cimino et al., 2001; Dean and Prinsep, 2017), the chemistry of Polycerinae and Triophinae nudibranchs is substantially similar and, in addition, triophamine (**1**) and limaciamine (**2**) appear to be 'interchangeable' in the chemical pattern of these two groups of Polyceridae (Fig. 1). Thus, based on these data, it could be suggested that symmetrical diacylguanidines **1** and **2** are chemical markers of these selected nudibranchs.

However, it should be considered that i) a complete molecular phylogenetic analysis is lacking for Polyceridae family and ii) only few species belonging to this family have been chemically investigated. This implies that the chemotaxonomic scenario of Polyceridae is almost incomplete and needs further molecular phylogenetic and chemical studies to be rigorously confirmed.

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