Conference paper

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Chemoecological studies on marine natural products: terpene chemistry from marine mollusks

Abstract: Some species of nudibranchs (Mollusca) protect themselves from predatory attacks by storing defensive terpene chemicals acquired from dietary sponges (Porifera) in specialized body parts called MDFs (mantle dermal formations), often advertising their unpalatability to potential predators by means of bright coloration patterns. Consequently, the survival of these trophic specialist species is closely related to the possibility of obtaining the defensive tools from sponges that live in their immediate vicinity; therefore, it is important to determine as precisely as possible the chemical composition of nudibranch extracts prior to any ecological studies addressing issues that involve their alimentary behavior and their defensive strategies, including the significance of their color patterns. Some of our recent studies on the chemical composition of terpene extracts from nudibranchs belonging to the genera *Chromodoris* and *Hypselodoris* are summarized. We also report the development of a method to assay extracts and purified metabolites for their feeding deterrent activity against co-occurring generalist predators. In a recent chemoecological study, showing that repugnant terpene chemicals are accumulated at extremely high concentrations in exposed parts of the nudibranchs' bodies, the feeding deterrence assays were carried out on the generalist marine shrimp *Palaemon elegans*, very common in the Mediterranean. We have modified this assay for use with the Australian shrimp species *P. serenus*, and confirmed the ecological validity of the assay by analysis of extracts from species of sponges and mollusks that live in the same habitat as *P. serenus*. The deterrent properties of haliclonacyclamine alkaloids isolated from the sponge *Haliclona* sp. were demonstrated, with the alkaloid mixture demonstrating palatability deterrence at concentrations as low as 0.05 mg/mL, and complete deterrence at 0.75 mg/mL. In contrast, the diterpene thuridillin metabolites from the sacoglossan mollusk *Thuridilla splendens* did not deter feeding by *P. serenus*.

Keywords: assay; deterrency; enantioselective synthesis; feeding; HPLC; IUPAC Congress-44, Life Chemistry; marine natural products; nudibranchs; sponges; terpenes.

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Marine natural products

Introduction

On our planet, it has been estimated that there are over 20 000 documented species of eukaryotic marine life, with more than 200 000 new species described in the last decade [1]. Life underwater is a competition for space (not too far different from the "competition" here in our conference city Istanbul for a seat on the trams or buses) in addition to competition for the essential nutrients required for survival and reproduction. Soft-bodied and sessile marine animals, such as marine sponges, use the strategy of chemical defense, commonly involving small organic molecules, to assist them in surviving in the complex underwater world. The defensive metabolites needed may be synthesized by the host animal or acquired from associated or symbiotic microbes. Specialist groups of marine bacteria, once considered unculturable, have been found to be rich sources of metabolites displaying unique chemical structures [2, 3]. The metagenomic mining of environmental DNA samples has provided detailed structural information on the extraordinary chemical diversity of bacterial strains [4].

Marine organisms are therefore a prolific source of natural products that potentially can be used as lead compounds in drug discovery or which have inspired organic chemists as synthetic targets [5]. In particular, the nudibranchs (Mollusca: Gastropoda: Opisthobranchia) are a group of marine animals that use small molecules for a variety of purposes, including communication, reproduction, and defense against predation [6, 7]. As of 2012, seven out of 20 natural products of marine origin (either directly or as a derivative) that have been approved for FDA use as a drug or in clinical trial were from mollusks or their prey foods [8]. This paper summarizes some of our recent research into the chemistry and chemical ecology of representatives from two families of nudibranchs commonly found in South East Queensland.

The chemistry of *Chromodoris* spp.

Mollusks from the family Chromodorididae (order Nudibranchia) frequently contain oxygenated diterpene metabolites [9]. In an early study on a single specimen of a chromodorid mollusk (possibly *Chromodoris reticulata*, now known as *Goniobranchus reticulatus*, www.marinespecies.org), we identified the new dialdehyde metabolite **1** together with the ring-closed acetal **2** (Fig. 1) as well as four known diterpenes [10]. Terpenes **1** and **2**, both of which we found to show bioactivity against P388 mouse leukemia cells, were subsequently isolated from the Chinese nudibranch *Chromodoris sinensis* (now known as *Goniobranchus sinensis*) and have been implicated in feeding deterrency [11]. Chemical evaluation of two large specimens of *C. reticulata* provided six new oxygenated diterpenes (**3** – **8**) together with seventeen known diterpenes. The anatomical distribution of the diterpene compounds within the various tissue types of the mollusk was explored by dissection and analysis of one of the two specimens [12]. The known diterpene aplyroseol-2 (**9**) was the major compound in the mantle tissue along with dialdehydes, while the linear furan ambliofuran (**10**) was the only diterpene found solely in the internal (digestive) organs (viscera). As had been observed in *C. reticulata*, the presence of lactone-acetal-hemiacetal functionality was noted in many of the isolated compounds, and is considered to be the inevitable consequence of the sensitivity of reactive dialdehydes present in the mollusk extract to the isolation conditions employed. Two of the new diterpenes (**5**, **6**) contained imine functionality that also supported their likely origin from aldehyde precursors. We next investigated the nudibranch *Chromodoris albopunctata* (now known as *Goniobranchus albopunctatus*) which provided four new oxygenated diterpenes (**11** – **14**) in conjunction with three known diterpene metabolites. The known metabolite (+)-spongian-16-one (**15**) was isolated from both digestive tissue and mantle extracts. The known compounds 7α-acetoxyspongian-16-one (**16**) and (+)-isoagatholactone (**17**) were isolated from the digestive tissue extract in conjunction with the new metabolite 12α-acetoxyspongian-16-one (**11**), while additional new metabolites, 20-acetoxyspongian-16-one (**12**), 20-oxyspongian-16-one propionate (**13**) and 12α,20-dioxyspongian-16-one dipropionate (**14**) were isolated from the mantle extract [13]. In contrast to acetate esters, propionate esters are uncommon in marine natural products [14].

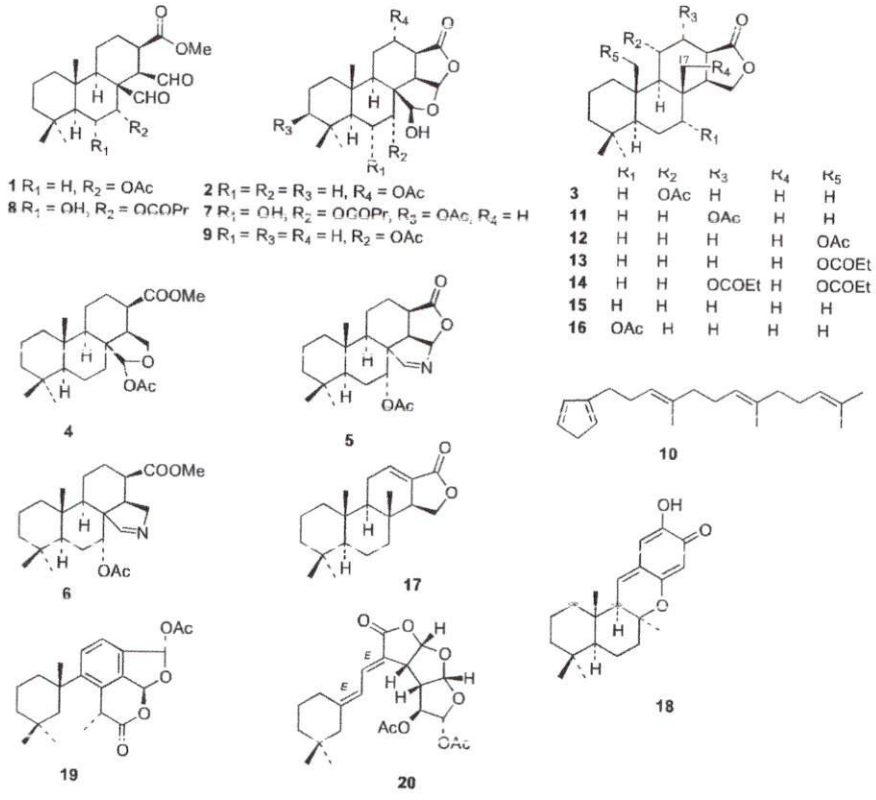


Fig. 1 Representative terpene metabolites isolated from *Chromodoris* mollusks.

The common mollusk *Chromodoris elisabethina*, which we frequently find associated with sponges in the field, shows a very different chemical profile. In contrast to all the other species that we have investigated, extracts prepared from *C. elisabethina* always contain the same oxygenated diterpene metabolite puupehenone (**18**) [15]. Although puupehenone is known as a, cytotoxin [16], and shows antitubercular activity [17], its ecological effects are not yet well understood.

Chromodoris splendida is a brilliantly colored mollusk that, in the field at Gneerings Reef, Mooloolaba, is often observed feeding on the bright yellow-encrusting sponge *Darwinella tango* (Order Dendroceratida). Our isolation studies revealed aplysulfurin (**19**) [18] as the major metabolite in both sponge and nudibranch, a finding that establishes a specific predator-prey link for this mollusk. A detailed investigation of the minor metabolites in *C. splendida* led to the isolation of gracilin metabolites (e.g., gracilin C **20**) and so revealed that the mollusk does not prey exclusively on *D. tango*.

The chemistry of *Hypselodoris* spp.

Members of the genus *Hypselodoris*, among the most brightly colored of nudibranchs, are frequently characterized by the presence of sesquiterpene metabolites containing a furan moiety [9]. Specimens of three different *Hypselodoris* species were collected for a comparative biogeographical study on their chemistry prior to understanding the deterrency or palatability associated with these mollusks.

The three known metabolites dendrolasin (**21**), (−)-euryfuran (**22**), and (+)-pallescensin A (**23**) (Fig. 2) were identified from the diethyl ether extract obtained from two individuals of *H. obscura* collected at the Gold Coast Seaway. Dendrolasin (**21**) has been isolated from both terrestrial and marine sources, including from the nudibranchs *Chromodoris lochi* and from the marine sponges *Spongia microfijiensis* and *Dictyodendrilla* sp. [19] It has also been isolated from Californian and Mediterranean species of *Hypselodoris* [20, 21].

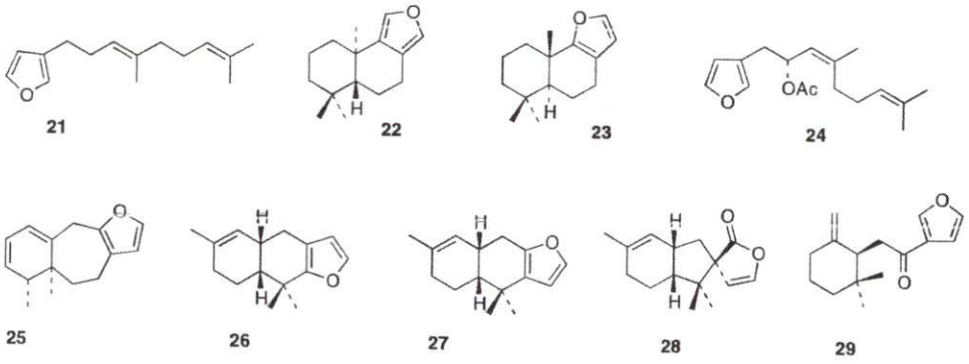
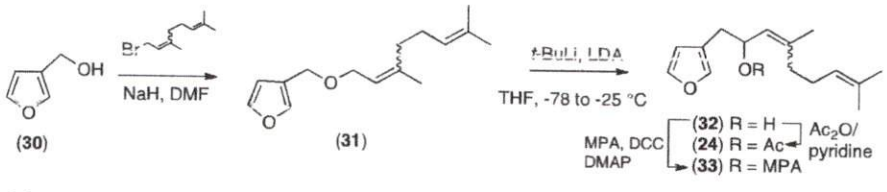


Fig. 2 Representative sesquiterpene metabolites isolated from *Hypselodoris* mollusks.

(−)-Euryfuran (**22**) and (+)-pallescensin A (**23**) have also been reported from both nudibranchs and sponges [20, 22, 23]. For *H. jacksoni*, eight animals collected from two separate locations (Mudjimba Island, Mooloolaba; Shag Rock, Moreton Bay) were combined prior to extraction owing to their small size, yielding the new metabolite (−)-(5*R*,6*Z*)-dendrolasin-5-acetate (**24**) along with the known sesquiterpenes (+)-agassizin (**25**) [20], (−)-furodysin (**26**), (−)-furodysinin (**27**) [24], (−)-euryfuran, (−)-dehydroherbadysidolide (**28**) [25], and (+)-pallescensone (**29**) [26]. Finally eight specimens of *H. whitei* (collection site Shag Rock) provided (−)-euryfuran, (−)-furodysin, (−)-furosydinin, and dendrolasin. In common with their counterparts in Mediterranean, Indo-Pacific and Caribbean regions, *Hypselodoris* molluscs from South East Queensland may express a dietary preference for furanosesquiterpene-rich sponges. In our field work, we have rarely encountered *Hypselodoris* feeding on its sponge prey, and so a direct dietary link has not been firmly established. A small piece of a grey encrusting sponge, collected from a site near Mooloolaba (Sunshine Coast) and provisionally identified as a *Dysidea* species, contained traces of furodysin, furodysinin and dehydroherbadysolide, thereby confirming the availability of suitable sponge foods in SE Queensland habitats.

(+)-HRESIMS measurements showed that the new metabolite (−)-(5*R*,6*Z*)-dendrolasin-5-acetate (**24**) had the molecular formula $C_{17}H_{24}O_3$. The ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) showed diagnostic signals for a furan [δ_H 7.33 (1H), 7.23 (1H), 6.28 (1H); δ_C 142.7, 140.1, 111.8], two alkene signals [δ_H 5.15 (1H), 5.07 (1H); δ_C 123.7, 123.9], three methyl groups [δ_H 1.72, 1.67, and 1.59] and an acetate group [δ_H 2.01 (3H, s)]. These partial NMR assignments were comparable to those of dendrolasin (**21**) [19], except that the signal corresponding to H-5 in dendrolasin at δ_H 2.21 (2H) was shifted to δ_H 5.64 (1H, br dt, J = 6.4, 9.3 Hz) in **24** owing to the hydroxy substituent. Furthermore, the C-6/C-7 bond was deduced as *Z* from a 1D nOe experiment in which the signal intensity of CH_3-14 increased when H-6 was irradiated using a mixing time of 60 ms; in contrast, dendrolasin has a *E*-configured C-6/C-7 double bond. The ^{1}H NMR chemical shifts of H-6 and H_3-14 fitted this configurational assignment (**24**: δ_H 5.15 and 1.72 vs. **21**: δ_H 5.17 and 1.59).

A synthetic study, adapted from synthetic work by Tsubuki et al. [27], was undertaken (Scheme 1) to provide the absolute configuration at C-5 of **24** [28]. Furan-3-methanol (**30**), prepared in 78 % yield by reduction of 2-furoic acid with $LiAlH_4$ in dry Et_2O, was treated with NaH and a mixture of geranyl and neryl bromides to furnish the 3-furylmethyl ether **31** (65 % yield) as an *E*/*Z* mixture (3:1). The H-5 signal for the *E* and *Z* isomers of **31** resonated at δ_H 4.00 and δ_H 3.90, respectively, while irradiation of the H-5 signal for the *Z* isomer



Scheme 1 Synthetic route to dendrolasin-5-acetate (**24**).

increased the intensity of the signal associated with the adjacent methyl (CH_3-14). The isomeric mixture of **31** was immediately subjected to [1,2]-Wittig rearrangement with *t*-BuLi (4 equiv) and LDA at –78 °C to afford racemic 5-hydroxy dendrolasin (**32**) in 39 % yield. A 3:1 ratio of *E*/*Z* isomers in **32** was confirmed via analytical HPLC, and by comparison of ^{1}H NMR data with those of individual stereoisomers obtained by preparative HPLC (hexanes:EtOAc, 90:10). Assignment of the signals corresponding to the groups attached to the double bonds was made using HMBC and nOe data. The signal for CH_3-14 in the *Z* isomer resonated downfield (δ_H 1.74) compared to that in the *E* isomer (δ_H 1.65) while the signal for H-5 appeared slightly upfield (δ_H 4.48) in the *Z* isomer compared to that in its *E* counterpart (δ_H 4.51). 1D-NOESY irradiation of the CH_3-14 signal for the *Z* isomer resulted in enhancement of the alkene signal at δ_H 5.24 (H-6), fully consistent with the *Z* configuration; in contrast, there was no corresponding nOe between CH_3-14 and H-6 in the *E* isomer.

The racemic sample of (*Z*)-5-hydroxy dendrolasin (**32**) was separated by preparative enantioselective HPLC and each individual enantiomer then converted to an acetate derivative on treatment with Ac_2O/pyr. Surprisingly, (+)-(*Z*)-**32** ($[\alpha]_D$ +9.3) gave an acetate derivative with $[\alpha]_D$ –8.7 and vice versa ($[\alpha]_D$ –12 for (–)-(*Z*)-**32**, but $[\alpha]_D$ +13 for its acetate derivative). The ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of the synthetic (–)-(6*Z*) acetate was fully superimposable with that of the natural sample isolated from *H. jacksoni*. The absolute configuration of C-5 in the natural acetate derivative was determined as follows: first, (±)-**32** was esterified with (*R*)-MPA acid in the presence of DMAP and DCC at room temperature to give a mixture of the diastereomers (*R,R*)-**33** and (*R,S*)-**33**, which could be separated by reverse phase (RP) HPLC (MeCN:H_2O; 63:35). The H-6 protons in (*R,R*)-**33** and (*R,S*)-**33** resonated at δ_H 5.04 and at δ_H 5.13, respectively, in agreement with H-6 of the (*R,R*)-isomer sitting in the shielding cone of the MPA aryl ring. Conversely, the diastereotopic H-4 signals in (*R,S*)-**33** at δ_H 2.51 and 2.58 were shielded compared to those in (*R,R*)-**33** (δ_H 2.64, 2.74). MPA esters were next prepared from the individually purified enantiomers of synthetic (6*Z*)-5-hydroxy dendrolasin. The ^{1}H NMR spectra of the MPA esters from (+)-**32** and (–)-**32** were identical to those of synthetic (*R,R*)-**33** and (*R,S*)-**33**, respectively. Thus, (+)-5-hydroxy-dendrolasin (**32**) had an *R* configuration. Given the change in sign of $[\alpha]_D$ between the alcohol and its acetate derivative, these data verified that the naturally-occurring (–)-acetate **24** had a 5*R* configuration [28].

Feeding deterrency assays with *Palaemon serenus*

Ecological studies initiated by one of us (E.M.) have led to the development of a convenient feeding deterrency assay using the marine shrimp *Palaemon elegans*, a generalist feeder that includes small mollusks in its diet, as a model predator [29]. Given that field experiments prevent assessing the state of conservation and the purity of the compounds immediately prior to the assays in remote locations, the choice of the model predator fell on easy available shrimps with broad adaptability, allowing their survival for long time in a small volume of seawater in the chemical laboratory. *P. elegans* is commonly found throughout the Mediterranean region, but some assays conducted with it have been carried out on compounds isolated from nudibranchs collected in other parts of the world [11]. Even though the use of a non-local model predator can have the advantage of excluding confounding effects due to avoidance-learning [11], the general ecological validity of the assay is not yet well established, given that different consumers can vary in their response to particular extracts or purified metabolites. The availability of the model organisms near to the chemical laboratory where the bioassays have to be carried out is desirable. Palaemonids live worldwide, thus we decided to use the Australian shrimp species *Palaemon serenus*, the almost transparent exoskeleton of which enabled us to easily detect the occurrence of the red food in the viscera.

The feasibility of a "red spot" palatability assay using the Australian shrimp *Palaemon serenus* was first explored using a crude alkaloid extract prepared from the Great Barrier Reef sponge *Haliclona* sp. #628, and was based on the experimental method published by Mollo et al. [11, 29]. A crude alkaloid fraction (CAF) prepared from sponge tissue using an acid-base extraction protocol, and containing a mixture of 3-alkylpiperidine alkaloids containing haliclonacyclamine A (**34**) as the major component, was dissolved in acetone (0.5 mL), and combined with freeze dried squid (50 mg), alginic acid (30 mg) and purified sea sand

(30 mg). Following solvent evaporation, the mixture was homogenized in water (1 mL), red food dye (1 drop) was added, and the resulting thick paste loaded into a syringe and extruded into calcium chloride solution (0.25 M) to make spaghetti-like strands. After a 2 min interval for strand hardening, individual pieces were rinsed in water and then cut into pellets of size 4 mm. Shrimp were familiarized with a daily feeding regime of squid mantle, and starved for 3 days prior to assay. Seventy randomly selected shrimp were separated into individual containers, and acclimatized for 30 min before being fed either control or treatment pellets (n=10 for each treatment). After 20 min the shrimp were analyzed for the presence of a red spot in the digestive tract, indicating acceptance (i.e., palatability) of the pellet. Concentrations of CAF equal to or >0.05 mg/mL show a significant feeding deterrence on generalist marine shrimp, supporting earlier palatability studies by Garson et al. that had tested CAF extracts at ecologically-relevant concentrations against natural populations of reef fish [30]. The current result further indicates the role of the haliclonacyclamines as a deterrent to general marine predators. The dose response curve (Fig. 3) of the CAF extract confirmed the reproducibility of the assay method developed by Mollo et al. [11, 29]. However mollusk extracts are not necessarily deterrent. A second set of assay experiments using *P. serenus* (n=70) explored the deterrent effects of crude extracts from the sacoglossan *Thuridilla splendens* (Family Elysioidea). This mollusk species is common through South East Queensland, but in this case extracts, containing thuridillin A (**35**) [31] as the major metabolite, were found to be fully palatable (>90 %) at all concentrations tested up to 9 mg/mL.

The 44th World Chemistry Congress took place in Istanbul in Turkey, the country in which is also situated the ancient city of Troy. Consequently, it is highly appropriate to finish this overview article by mentioning the "Trojan horse" defensive stratagem presented by Carbone et al. [11] as an analogy to nudibranch chemical defenses. In the *Aeneid* of Virgil, following a ten year siege, the Greek army hid soldiers inside a giant wooden horse, which they left outside the gates of Troy when they sailed away. The horse was pulled into Troy as a victory trophy, but during cover of darkness, the Greek force crept out of the horse and opened the gates for the rest of the Greek army, which had sailed back under cover of night. Destruction of the city of Troy led to the end of the war. Consequently, the term "Trojan Horse" has come to mean any trick or stratagem that causes a target to invite a foe into a securely protected bastion or space. Carbone et al. have proposed that nudibranchs store sequestered deterrent chemicals in specialized glands called mantle dermal formations (MDFs) that are located around the mantle borders of the animal. When under attack, the nudibranchs retract their vital chemosensory organs (gills and rhinophores) and instead offer chemically-protected mantle tissue to the predator. When damaged following an attack, the MDFs release high concentrations of the deterrent compound directly into the mouth of the predator and it is then repelled. The "Trojan horse" study explored the role of several chromodorid terpenes, including a mixture of aplysiasecol-2 (**9**) and dialdehyde (**1**), in preventing predation by shrimp and showing deterrency at concentrations lower than those encountered in the MDFs [11].

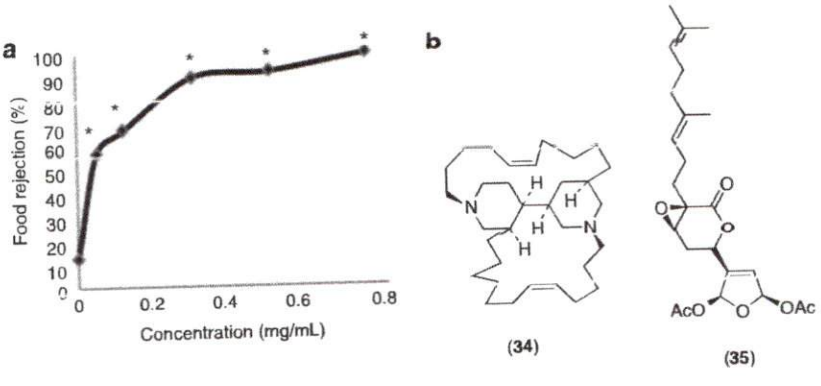


Fig. 3 (a) Dose-response curve for crude alkaloid fraction of *Haliclona* sp. 628 showing feeding deterrency against the shrimp *P. serenus*. A Tukey post-hoc test compared each concentration to the control. Statistical significance is indicated by (*) ($P < 0.001$); (b) Structures of haliclonacyclamine A (**34**) and of thuridillin A (**35**).

This defensive strategy is supported by field observations and reports in the literature, that nudibranchs can be deprived of part of their body but remain alive [11]. Interestingly, given that the shrimps are not big enough to eat their nudibranch prey whole, their ability to selectively attack exposed parts and protrusions of the nudibranch body makes them ideal models for this type of investigation. However, the ecological validity of the "Trojan horse" study can be questioned because of the involvement of a Mediterranean shrimp species to screen for the deterrency of extracts from mollusks collected in China. This is not necessarily a problem if an aim of the study is to document how repellant metabolites affect a generalist feeder in a dose-dependent manner. Numerous chemical studies have revealed that the same secondary metabolites can occur in nudibranchs from very different geographical areas [6, 7, 9]. However, chemoecological evaluations of deterrency should be carried out with predators from the same geographical region, as differing ecological conditions may influence an evolutionary arms race between predator and prey, and could drive divergence in predators' tolerance of unpalatable chemicals. Our own future work aims to validate better the ecological relevance of the shrimp "red spot" assay, and to compare these assay data against those from fish feeding assays using nudibranch and sponge extracts from South East Queensland.

Conclusions

This study revealed that extracts from nudibranchs of the genera *Chromodoris* and *Hypselodoris* collected in South East Queensland contain oxygenated diterpenes and sesquiterpenes, repectively. The new terpene (5*R*,6*Z*)-dendrolasin-5-acetate was isolated from *Hypselodoris jacksoni*, and its complete stereochemistry established by an enantiospecific synthesis. A palatability assay developed by Carbone et al. in 2013, and using the generalist Mediterranean-Atlantic marine shrimp *Palaemon elegans*, was modified for use with the Australian shrimp species *P. serenus*. The reproducibility of the assay has been established by analysis of extracts from sponges and mollusk species that live in the same location as *P. serenus*.

List of abbreviations

DCC	dicyclohexylcarbodiimide
DMAP	dimethylaminopyridine
FDA	food and drug administration (USA)
HPLC	high-performance liquid chromatography
HMBC	heteronuclear multiple bond correlation spectroscopy
HRESIMS	high-resolution electrospray mass spectrometry
MPA	methoxy phenylacetic acid
NMR	nuclear magnetic resonance
NOESY	nuclear Overhauser spectroscopy

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