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Research Article

BIOACTIVE TERPENOID FROM THE BALINESE NUDIBRANCH *Hypselodoris infucata*

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ABSTRACT

Marine organisms, in particular nudibranchs (Mollusca: nudibranchia), are known as a rich source of chemically diverse secondary metabolites exhibiting potential as antimalarial, anti-inflammatory, antiviral and anticancer activity. We presented the chemical investigation of an extract of nudibranch *Hypselodoris infucata* collected from Bali, an unexplored water but rich in nudibranch diversity. The extract contained the known (−)-furodysinin (**1**), a furanosesquiterpene that for the first time isolated from this species. Metabolite **1** was purified by chromatography and the structure was characterised by comparison of ^{1}H NMR data with that of the reported data. The absolute configuration was determined by comparing the optical rotation values with the known enantiomer. *In vitro* cytotoxic activity of compound **1** against HeLa cell line displayed an IC_{50} at 102.7µg/mL. We also report for the first time the development of a method to assay nudibranch extracts for their feeding deterrence activity against local shrimps *Penaeus vannamei*. The extract show food rejection with highly significant difference in respect to the control (P = 0.0061) at natural concentration of 3.0mg/mL.

Keywords: nudibranch, natural product, feeding deterrence, *Hypselodoris*

INTRODUCTION

Secondary metabolites play an important role as a starting point in the drug discovery process. Marine organisms offer an abundant source of chemically diverse and biologically active secondary metabolites. Among marine organisms, sponges and nudibranchs are prolific sources of diverse natural products, and the study of their chemical properties has led to the discovery of many biologically potent chemicals with antimalarial, anti-inflammatory, antiviral and anticancer activity (Blunt *et al*., 2015).

Nudibranchs (Mollusca: nudibranchia) are slow-moving marine molluscs with soft bodies and often present bright and attractive coloration. However, despite slow movements and the absence of physical attributes, only few predators have been documented. Chemoecological studies showed that nudibranchs may employ secondary metabolites as chemical defence mechanism against predators (Cimino and Ghiselin, 2009; Mudianta *et al*., 2014). The metabolites are either derived from their diets mainly sponges or biosynthesized in *de novo* fashion (Fontana *et al*., 2012). This sponge-prey relationship has resulted an array of metabolites with intriguing framework, which are not found in their terrestrial counterparts and showed the potential as new pharmaceutical agents (Gerwick and Moore, 2012).

Furanosesquiterpenoids are the most dominant metabolites characterised from nudibranchs of the genus *Hypselodoris* (Gastropoda: nudibranchia) and they seem to be a specific chemotaxonomic marker in these organisms (Fontana *et al*., 2001). Additionally, some species within this genus have also been reported to produce diterpenoids (*Hochlowski et al.,* 1982) as well as sesterpenoids (Cimino *et al*., 1993). There have been 17 different species of *Hypselodoris* chemically investigated and reported from 1982 until 2012. The animals were collected from disparate geographical regions including California (USA), Brazil (Latin America), the Mediterranean (Europe), South Africa, and India.

Here we presented an initial chemical investigation of nudibranch *Hypselodoris infucata* collected from Tulamben-Bali, a less-explored waters but rich in species diversity. Our research group have pioneered the chemical investigation of marine invertebrate mainly sponges and nudibranchs from this prolific site (Mándi *et al*., 2015; Mudianta *et al*., 2014). We also reported for the first time the development of a method to assay nudibranch extracts for their feeding deterrent activity against local shrimps *Penaeus vannamei*.

MATERIAL AND METHIODS

Materials

Two specimens of *Hypselodoris infucata* (crawling length 2cm) were collected during fieldwork at Tulamben Bay, Bali in November 2014. The specimens were identified by comparing the surface pictures with that in the encyclopaedia of nudibranch as well as with the online database such as nudipixel (www.nudipixel.net) and WoRM (www.marine-species.org). The first specimen (coded by the authors as 28-11-14-12) was extracted for chemical study, while the second specimen (coded as 28-11-14-13) was employed in the feeding assay.

Procedure

Extraction and isolation of metabolite

A single specimen of *Hypselodoris infucata* (Figure 1) was diced, extracted in acetone (3x10mL), and sonicated for 2min. The combined extracts were partitioned between water and dichloromethane (3x5mL) and subsequently the organic layer was dried with Na_2SO_4. Dried organic layer was evaporated to dryness to give a crude extract (5mg). The extract was passed through a normal-phase Sep-Pak cartridge eluting with 100% hexane (10mL) to provide compound **1** (1.3mg).

General

^{1}H NMR data were recorded using Bruker Advance 500MHz spectrometers (5mm inverse probe, gradient selection). Measurements were made in deuterochloroform ($CDCl_3$, referenced at: δ_H 7.26ppm, δ_C 77.16ppm). Chemical shifts (δ) were recorded in parts per million (ppm) and coupling constants (J values) were measured in Hertz (Hz). Positive ion electrospray mass spectra were determined using a Bruker Esquire HCT instrument (LRESIMS) with MeOH as solvent. Infra-red spectrum was recorded on a PerkinElmer FT-IR/FIR spectrometer. Specific optical rotations were measured at the sodium D line (589nm) at ambient temperature using a 1mL quartz cell with a 10cm path length, using a Jasco P-2000 polarimeter. TLC investigations were performed on TLC silica gel 60G F_{254} (Merck). Solvents were distilled prior to use. GC/MS analyses were performed on a Shimadzu GCMS QP-2010 Plus gas chromatograph mass spectrometer, operating at 70eV, fitted with a DB-5 column (30m, internal diameter 0.25mm, J&W Scientific). Standard GCMS programme: split mode; column flow 1.5mL/min; initial oven temperature 100°C (isothermal for 3min), ramp 16°C/min to 250°C held for 10min; injection temperature 250°C (total programme time 30min). Samples for GC/MS were prepared in HPLC grade *n*-hexane (1mg/mL).

Anticancer assay

In vitro anticancer assay of compound **1** against HeLa tumor cell line was performed by colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) (Hughes and Mehmet, 2003). The HeLa cervical cancer cell lines were cultured in RPMI 1640 medium containing 10% Fetal Bovine Serum (FBS), 0.5% fungizone and 2% Penicillin-Streptomycin. The HeLa cells were plated at 10,000 cells per well and incubated at 37°C with 5% of CO_2 flow for 24h. After that, tested compound and doxorubicin as positive control were tested in a single concentration of 30µg/mL (dissolved in RPMI medium) for 24h. Each sample was tested in three replicates. The cell-growth medium was removed from each well before the extracts were plated into the wells containing HeLa cells attached. Three kinds of controls were made, i.e. control of tumor cells, control of medium (medium without tumor cells) and control of samples (samples without tumor cells). After 24h treatment of the tested compound, the solution was removed from each well. After that, 100µL of MTT reagent (500µg/mL) was added into each wells and incubated for 4h in CO_2 incubator until purple precipitate was visible.

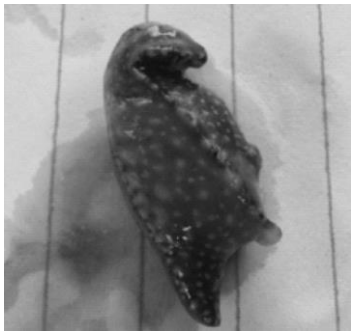


Figure 1. Surface picture of *H.infucata* (Ruppell and Leuckart, 1830) taken shortly after collection

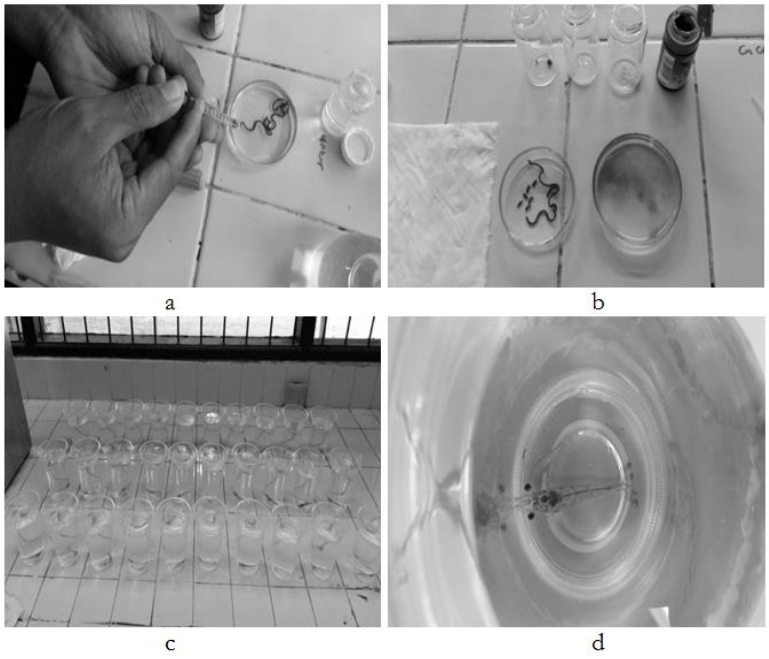


Figure 2. spaghetti-strand like making (a); red-colored shrimp pellet (b); shrimp assayin progress (c); and red spot in the digestive tube of the shrimp (d)

As much as 100µL of sodium dodecyl sulphate (SDS) 10% was added into each well, and incubated at room temperature (±27°C) in the dark for 12h. After incubation, the absorbance of each well was measured by DYNEX micro-plate reader at wavelength of 570nm. The percentage of cell death was calculated using the formula:

$$\%\ mortality = \frac{(A-D)-(B-C)}{(A-D)} \times 100\%$$

Description: *A* = absorbance of tumor cell control, *B* = absorbance of the sample, *C* = absorbance of sample control, *D* = absorbance of medium control.

The anticancer activity against HeLa was then tested in serial concentration of 12.5, 25, 50, and 75µg/mL. The Inhibition Concentration 50 (IC_{50}) value was then calculated using MINITAB 16.0 probit analysis.

Feeding Deterrence Assay

The second specimen of *Hypselodoris infucata* (coded as 28-11-14-13) was extracted in the same manner as the first one to give 6 mg crude extract. The feeding assay was carried out according to protocol reported by Mollo *et al.* (2008) (Mollo *et al.*, 2008). In this assay we employed local shrimps *Penaeus vannamei* instead of the marine generalist shrimp

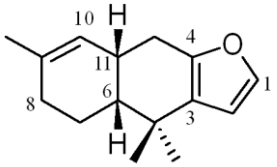
Palaemon elegans

The replacement of the shrimp species in Mollo's protocol was due to the availability of the shrimps in the local area in Bali. The rest of the protocol was performed exactly as described by Mollo *et al.* (2008). The crude nudibranch extract (1mg) was dissolved in 0.5mL of acetone was added to a mixture composed of alginic acid (30mg), ground freeze-dried squid mantle (50mg), and purified sea sand (3 mg; granular size 0.1-0.3mm). Sand was included in the mixture to prevent the floating of the pellets on the surface of the water during the experiments. After evaporation of the solvent, one drop of food coloring and distilled water was added to 1-mL volume. Food coloring was added for an easy detection of the ingested food in the digestive tube of the shrimps. The mixture was stirred, loaded into a 5mL syringe, and extruded into a 0.25M calcium chloride solution for 2min to harden (Figure 2a). The resulting spaghetti-like red strand was cut into 10-mm-long pellets (Figure 2b). Control foods were made in the same manner, with the addition of 0.5mL of acetone but without the purified metabolites. Shrimps (average size 30mm), obtained from shrimp breeding site in North Bali, and were kept in an aquarium for 1 week to get them accustomed to the daily proposed artificial food. After 3 days of total fasting, they were individually placed in 500mL beakers filled with 300mL of sea water (Figure 2c). Control or treated pellets were presented to shrimps in series of 10 independent replicates. After 30min, the presence of an evident red spot in the digestive tube of the shrimps was assumed as proof of acceptance and, conversely, its absence was the sign of a rejection response (Figure 2d). The significance of differences in the consumption of treated vs. control pellets were evaluated by two-tailed Fisher's exact test. *P* values <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The chemistry of *Hypselodoris infucata*

Compound **1** (1.3mg) was isolated as a colorless oil ($[\alpha]_D$ –51) and showed a single GCMS peak at m/z 216 (M^+) with a strong diagnostic base peak at m/z 122 (C_8H_8O) arising from a retro Diels-Alder reaction of the carbocyclic ring adjacent to the furan ring (Dunlop *et al.,* 1982). The ^{1}H NMR ($CDCl_3$, 500MHz) spectrum of **1** revealed the presence of diagnostic furan protons at δ_H 7.21 (1H, d, J = 1.7Hz, H-1) and δ_H 6.23 (1H, d, J = 1.7Hz, H-2) together with an olefinic proton at δ_H 5.61 (1H, brs, H-10). There were also two geminal methyl groups resonated at δ_H 1.19 (3H, s, H-15) and δ_H 1.18 (3H, s, H-14) along with a methyl attached to an on olefinic carbon at δ_H 1.66 (3H, s, H-13). These ^{1}H NMR data of **1** were in close agreement with those of the synthetic sample of furodysinin reported by Vaillancourt *et al.* in 1991 (Figure 3) (Vaillancourt *et al.,* 1991).



1

Figure 3. ^{1}H NMR data comparison of compound **1**

The tricyclic furanosesquiterpene (+)-furodysinin (**2**), ($[\alpha]_D$ +64) was first reported by Wells *et al.* in 1978 from an Australian *Dysidea herbacea* (Kazlauskas *et al.*, 1978) without relative stereochemical assignment detail. Guella *et al.* subsequently characterised its enantiomer (–)-furodysinin ($[\alpha]_D$ –47) (Guella *et al.*, 1985) from a specimen of *D. tupha* collected from the Mediterranean. The absolute configuration of **2** has been established as as 6*R*,11*R* when optically pure (–)-furodysinin ($[\alpha]_D$ –54) was synthesised by Vaillancourt *et al.* from (+)-9-bromocamphor in four steps (Vaillancourt *et al.*, 1991). Recently, (–)-(6*R*,11*R*)-furodysinin (**1**) has been isolated from *D. herbacea* (Fiji) (Horton *et al.*, 1990), from *H. bayeri* collected from Cuba (Fontana *et al.*, 1994), and from *H. jacksoni* from South-East Queeensland, Australia. (–)-Furodysinin isolated from *H. infucata* in the current study has consistently been associated with a 6*R*, 11*R* configuration, whereas the positive counterpart shows a 6*S*, 11*S* configuration (Table I).

Table I. Summary of stereochemical variations and sources of furodysinin.

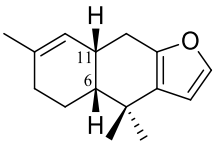
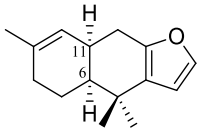
Compound	Configuration			Source	Collection sites
	$[\alpha]_D$	C-6	C-11		
 **1**	–47	*R*	*R*	*D.tupha* (Guella *et al,* 1985)	Mediterranean
	–54	*R*	*R*	Synthetic (Vaillancourt *et al,* 1991)	-
	–61	*R*	*R*	*D. herbacea* (Horton *et al,* 1990)	Fiji
	–55	*R*	*R*	*H. bayeri* (Fontana *et al,* 1994)	Cuba
	–51	*R*	*R*	*H. jacksoni*	Australia
 **2**	+64	-	-	*Dysidea sp.*(Kazlauskas *et al,* 1978)	Australia
	+30	*S*	*S*	*D. herbacea* (Searle *et al*, 1994)	Australia

Table II. 1H NMR data comparison of **1**

C	1 (experiment)	1 (literature)
	δ_H (ppm), mult., *J* (Hz)[a]	δ_H (ppm), mult., *J* (Hz) [b]
1	7.21, d, 1.7	7.21, d, 2.1
2	6.23, d, 1.7	6.24, d, 2.1
5	2.70, m	2.73, m
6	1.5, m	1.54, bd
7	1.20, m	1.32, m
8	2.03, m	2.03, m
10	5.61, brs	5.57, bs
13	1.66, s	1.66, s
14	1.18, s	1.18, s
15	1.19, s	1.19, s

[a]Chemical shifts (ppm) referenced to C*H*Cl_3 δ_H 7.24 ($CDCl_3$, 500 MHz);
[b]Chemical shifts (ppm) referenced to C*H*Cl_3 δ_H 7.24 ($CDCl_3$, 300 MHz);

(–)-Furodysinin (**1**): colorless oil (1.3mg); $[\alpha]_D = -51$ (*c* 0.25, $CHCl_3$); lit. $[\alpha]_D = -54$ (*c* 0.5, $CHCl_3$); (Vaillancourt *et al*., 1991) **^{1}H NMR ($CDCl_3$, 500 MHz)** δ_H 7.21 (1H, d, *J* = 1.7 Hz, H-1), 6.23 (1H, d, *J* = 1.7 Hz, H-2), 5.61 (1H, brs, H-10), 2.70 (2H, m, H-5), 2.03 (2H, m, H-8), 1.66 (3H, s, H-13), 1.50 (1H, overlap, H-6), 1.20 (1H, m, H-7), 1.19 (3H, s, H-15), 1.18 (3H, s, H-14);

GCMS *m/z* 216 $[M]^+$ (20), 201 (5), 145 95), 122 (100), 107 (20), 91 (15), 79 (25), 65 (5), 53 (5).

In vitro anticancer assay of (–)-furodysinin (**1**) determined by MTT method displayed inhibition at IC_{50} at 102.7μg/mL which was less potent compared to the positive control doxorubicin that showed a value of 2.3μg/mL. This result strongly suggested to explore other bioassay target for compound **1** including antibiotic as some furanosesquiterpene metabolites were reported to show promising bioactivity (Richou *et al*., 1989). The bioactivity of compound **1** has never been reported, however related derivative such as acetylthioxy-furodysinin lactone, a bioactive component of the marine sponge *Dysidea*, was reported to show as a potent LTB4 receptor partial agonist (Carte' *et al*., 1989).

Feeding deterrence profile of the extract of *Hypselodoris infucata*

The feeding deterrence assay of the extract of *H. infucata* was carried out by following a protocol that was first reported by Mollo *et al.* (2008) except for the use of shrimps *Penaeus vannamei* instead of *Palaemon elegans* (Mollo *et al*., 2008). Mollo employed the assay to assess palatability of brominated

tetrahydropyran isolated from *Hypselodoris cyanomarginata* against the generalist shrimp *P. elegans* collected at the Mediterranean seas. The brominated metabolites at its natural volumetric concentration found to show a 2.3 mg/mL food rejection with a highly significant difference in respect to the control (P = 0.0001).

da Cruz (2012) replicated the feeding deterrence assay assay toxicity in a nudibranch-sponge predator–prey association obtained at Portuguese coast (da Cruz *et al*., 2012). Palatability tests of the crude extract of the sponge *Dysidea fragilis* and the nudibranch *Hypselodoris cantabrica* revealed a more effective deterrence in the nudibranch extracts because significant rejection rates were observed at lower concentrations than those necessary for the sponge extracts to have the same effect.

The extract (Figure 4) show food rejection with highly significant difference in respect to the control (P = 0.0061) at natural concentration of 3.0mg/mL. This value is considered to be identical with that found by Mollo and presented additional palatability data of the *Hypselodoris* nudibranch.

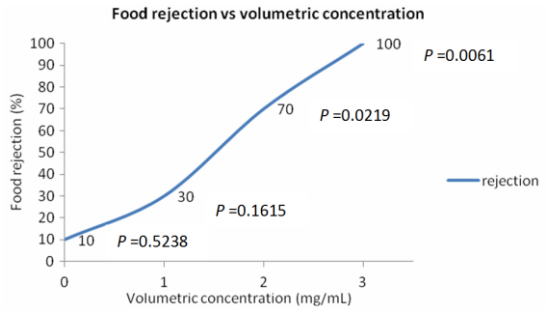


Figure 4. Dose-response curves for palatability tests of the extract of Hypselodoris infucata. Ten *Penaeus vannamei* specimens were randomly chosen for each test (n = 10). The significance of differences in the consumption of treated vs. control pellets were evaluated by two-tailed Fisher's exact test. P values <0.05 were considered statistically significant.

CONCLUSION

Chemical investigation of the extract of Balinese nudibranch *Hypselodoris infucata* yielded the known (–)-furodysinin (**1**). The metabolite was for the first time reported from this species and exemplified additional furanosesquiterpene metabolite isolated from the nudibranch. *In vitro* cytotoxic activity of compound **1** against HeLa cell line displayed an IC_{50} at 102.7 μg/mL. We also succeeded in replicating the feeding deterrence assay of the extract of *H. infucata* using local shrimps *Penaeus vannamei*.

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