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Original Article

Amphidinolide P from the Brazilian octocoral *Stragulum bicolor*

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ABSTRACT

Dinoflagellates are an important source of unique bioactive secondary metabolites. Symbiotic species, commonly named zooxanthellae, transfer most of their photosynthetically fixed carbon to their host. The mutualistic relationship provides the organic metabolites used for energy production but there are very few reports of the role of the dinoflagellates in the production of secondary metabolites in the symbiotic association. Corals and other related cnidarians are the most well-known animals containing symbiotic dinoflagellates. In the present paper we describe the isolation of amphidinolide P (**1**) from the octocoral *Stragulum bicolor* and its prey, the nudibranch *Marionia limceana*, collected off the coasts of Fortaleza (Ceará, Brazil). The coral extracts also contained 3-*O*-methyl derivative (**2**) of amphidinolide P, together with minor compounds still under investigation. Amphidinolides have been so far reported only in laboratory cultures of *Amphidinium* sp., thus compounds **1** and **2** represents the first identification of these polyketides in invertebrates. The finding proves the possibility to isolate amphidinolides from a natural symbiosis, enabling further biological and biotechnological studies.

Introduction

Many marine invertebrates host symbiotic dinoflagellates, commonly referred to as zooxanthellae, which provide nutritional requirements in the form of photosynthetic products and also facilitate the assimilation of dissolved inorganic nitrogen (Hallock, 2001; Kita et al., 2010). Dinoflagellates are unicellular eukaryotes which exhibit a great diversity of form and lifestyle. Free living species are an important source of natural products, including marine toxins and bioactive compounds (Wang, 2008; Blunt et al., 2012), whereas symbiotic microalgae have been often suggested to be responsible for the production of secondary metabolites in marine invertebrates, especially cnidarians and porifera (Kobayashi and Ishibashi, 1993; Kobayashi and Kubota, 2010; Mydlarz et al., 2003). Symbiotic community of dinoflagellates comprises a heterogeneous group of many strains and species that in part can be isolated (Andersen and Kawachi, 2005). Although some of these isolates have proven to be able to synthesize secondary metabolites in lab culture (Keller et al., 1987), the symbiotic association imposes a bilateral exchange between host and symbionts, which leads to production of metabolites that are not generally formed by either organism separately (Trench, 1993). Thus, synthesis of secondary metabolites by zooxanthellae *in hospite* remains an open question.

Amphidinium is a widespread genus of dinoflagellates, found in temperate and tropical marine waters, in both free-living benthic and endosymbiotic states. *Amphidinium* species are often amongst the most abundant dinoflagellates in benthic ecosystems, and a few species are known as a prolific source of secondary metabolites. Symbiotic strains of the genus have been isolated from the Okinawan flatworm *Amphiscolops* sp. (Kobayashi and Tsuda, 2004). Along several generations, laboratory cultures of this dinoflagellate have produced a number of bioactive macrolactones designated as amphidinolides (Kobayashi, 2008). The family – to date almost 40 members – shows a variety of backbone skeletons and ring size from 12 to 29 atoms. Furthermore, due to their

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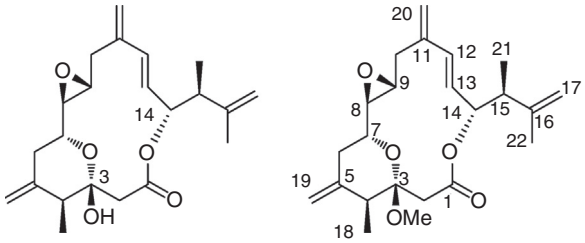
diverse functionality, stereochemical complexity and promising cytotoxicity activity, amphidinolides have been also subject of many chemical syntheses (Kobayashi and Kubota, 2010).

As part of a Bilateral Program on identification and biosynthesis of bioactive metabolites from Brazilian marine organisms, we have recently studied the chemical composition and cytotoxic activity of the extract of the octocoral *Stragulum bicolor* (Octocorallia: Clavulariidae) (Bumbeer and da Rocha, 2012). The aim of this work is to describe the identification of amphidinolide P (**1**) (Ishibashi et al., 1995) and its derivative 3-*O*-methyl amphidinolide P (**2**) from the invertebrate and from its predator, the nudibranch *Marionia limceana*.



1 **2**

Materials and methods

General

Optical rotations were measured on a Jasco P-2000 Polarimeter. UV spectra were acquired on a Jasco V-650 spectrophotometer. High resolution electrospray ionization mass spectra (HRESIMS) were acquired on a Q-Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific) or AmaZon SL (Bruker® – Billa Rica, USA) instrument. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectra were performed on a Bruker Avance DRX 600 equipped with a cryoprobe. Chemical shifts values are reported in ppm (δ) and referenced to internal signals of residual protons (C_6D_6 ^{1}H δ 7.15, ^{13}C 128.0 ppm). Solid phase extraction was carried out with polystyrene–divinyl benzene resin (CHROMABOND® HR-X). Silica gel chromatography was performed using precoated Merck F254 plates and Merck Kieselgel 60 powder.

Sample collection

Stragulum bicolor was collected on the underside of beach rocks in the intertidal zone along Caponga beach (04°02′ S 38°11′ W) located 61 km east from Fortaleza, Ceará, Brazil. Specimen identification was carried out by Prof. Tito Monteiro da Cruz Lotufo at University of São Paulo, and a voucher sample (ColBIO TL 1364 e ColBIO TL 1365) were deposited at the "Prof. Edmundo F. Nonato" collection in the Oceanographic Institute of the University of São Paulo (Brazil). First sampling was carried in August 2013, and the octocoral was collected along with its predator, the nudibranch *Marionia limceana*. The mollusc was identifed by the biologist Felipe de Vasconcelos Silva of the Federal University of Ceará (Fortaleza, Brasil). Colonies were washed with sterile seawater and kept in ice until MeOH extraction in the laboratory. In April 2014, new samples were collected aimed to isolation of associated microalgae by using the protocol described by Andersen and Kawachi (2005). The colonies were transported to the laboratory in sterile seawater and kept in ice. After a vigorously agitation, the supernatant was collected in a Petri dish, and dinoflagellates were isolated with a micropipette under inverted microscopy (200×). Each sample was transferred to a 24 multiwell plate containing K medium (Keller et al., 1987) and maintained at 23 °C under a photoperiod of 12 h (light):12 h (dark).

Cytotoxic activity

Cytotoxicity was evaluated against the adenocarcinoma colon cancer cell line HCT-116 (ATCC CCL-247™). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (v/v), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin at 37 °C under a 5% CO_2 atmosphere. Extracts and fractions were tested at concentrations ranging from 0.001 to 50 μg/ml, while compounds (**1** and **2**) were tested at concentrations ranging from 0.001 to 25 μM during 72 h. The effect on cell proliferation was evaluated *in vitro* using the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromide] assay, as described by Mosmann (1983). Doxorubicin was used as positive control. IC_{50} (the concentration that inhibits growth in 50%) values were calculated, along with the respective 95% CI (confidence interval), by non-linear regression using GraphPad Prism 5.0.

Extraction and isolation of metabolites

The octocoral colonies were successively extracted with MeOH (3× 100 ml). This dry extract (250 mg) was resuspended in 1.5 ml water and fractionated on 2.5 g HR-X resin by elution with 90 ml H_2O, 60 ml CH_3CN/H_2O 7:3, 45 ml CH_3CN and 45 ml CH_2Cl_2/CH_3OH 9:1 (Cutignano et al., 2015). The active CH_3CN fraction (11.2 mg) was chromatographed on silica gel with a gradient of diethyl ether in petroleum ether to give twelve fractions (A–L). Fraction F and H contained compound **2** (0.1 mg) and **1** (0.5 mg) respectively. LC–MS and NMR analyses of fraction J, K and L revealed minor analogs whose investigation is currently in progress.

Amphidinolide P (**1**): colorless oil; $[\alpha]^{25}_{D}$ +12.8 (c 0.04, MeOH); UV (MeOH) λ_{max} (ε) 225 (26,452), 231 (28,101), 239 (18,095) nm; HRESIMS $[M+Na]^+$ *m/z* 397.1982 (calcd for $C_{22}H_{30}O_5Na$ 397.1991).

3-O-Methyl-amphidinolide P (**2**): colorless oil; UV (MeOH) λ_{max} (ε) 225 (16,452), 231 (18,101), 239 (12,095) nm; ^{1}H and ^{13}C NMR data see Table 2; HRESIMS $[M+Na]^+$ *m/z* 411.2149 (calcd for $C_{23}H_{32}O_5Na$ 411.2147).

Mass spectrometry analysis (ESI-MS/MS)

Comparative analysis of nudibranch extracts and pure **1** was performed on AmaZon SL (Bruker® – Billa Rica, USA) instruments. Nitrogen was used as a nebulizing (10 psi) and drying gas (5 l/min, 180 °C) while the capillary high voltage was set to 3.5 kV and the end plate at 500 V. Samples were infused (5 μl/min) by syringe pump (Bruker® – Billa Rica, USA).

Results and discussion

Stragulum bicolor belongs to a recently described genus and species of octocoral that commonly occurs along the shallow waters of the Brazilian coasts (Bumbeer and da Rocha, 2012). The species has attracted much attention for its suspected invasive nature but so far there is no report of secondary metabolites from this organism. The octocoral is also the main component of the diet of the new species of nudibranch *Marionia limceana* (Nudibranchia: Tritoniidae) (Silva et al., 2013). Both invertebrates were collected in the intertidal zone of the beach rocks on the coast of Caponga Beach (Cascavel, Ceará, Brazil) in August 2013. The cnidarian and two specimens of nudibranchs were extracted by MeOH. The organic extract of *S. bicolor* was further fractionated using a novel protocol of solid phase extraction (SPE) based on polystyrene-divinyl benzene columns (CHROMABOND® HR-X) (Cutignano et al., 2015).

Table 1
Cytotoxic activity of extracts, SPE-HRX fractions (#1–#4) and pure compounds **1** and **2** from the octocoral *Stragulum bicolor* and of the methanol extract of the nudibranch *Marionia limceana*. Values are expressed as IC_{50} in μg/ml against the colon adenocarcinoma cell line HCT-116.

Sample	IC_{50} CI 95% (μg/ml)
S. bicolor (methanol extract)	0.505 0.24–1.07
S. bicolor (Fraction #1)	>50
S. bicolor (Fraction #2)	>50
S. bicolor (Fraction #3)	12.44
S. bicolor (Fraction #4)	>50
Amphidinolide P (1)	>25[a]
3-*O*-Methyl-amphidinolide P (2)	12.15[a] 6.98–21.17
M. limceana (methanol extract)	47.20 3.46–643.8
Doxorubicin	0.02[a] 0.02–0.03

[a] These values are expressed in μM concentration.

Extracts and fractions of *S. bicolor* were screened for cytotoxicity using the MTT assay on adenocarcinoma cell line HCT-116 and relevant cytotoxicity (>80% inhibition) was found only in the fraction eluted by CH_3CN (Table 1). This material was further fractionated on silica gel column with an elution gradient of diethyl ether in petroleum ether to give amphidinolide P (**1**, 0.5 mg), its 3-*O*-methyl derivative (**2**, 0.1 mg) and a number of minor compounds that are still under investigation.

Compound **1** was identified by NMR analysis and comparison of the resulting NMR and MS data with those reported in the literature (Ishibashi et al., 1995; Trost et al., 2005) whereas, despite the clear structural relationship, the assignment of compound **2** required an accurate spectroscopic study. In fact, the ^1H NMR spectrum of compound **2** was rather different from that of **1** mainly for what concerned the presence of a methyl singlet at 3.20 ppm and the distribution of the allylic protons around 2.5 ppm and methyl signals between 0.84 and 0.93 ppm (Fig. 1). However, 2D-NMR experiments allowed an unambiguous characterization of all spin systems according to the depicted structure (Table 2).

Recollection of *S. bicolor* from different sites off Ceará coast in April 2014 confirmed the presence of amphidinolide P (**1**) in the tissue of the cnidarian. Furthermore, full scan ESI-MS analysis carried out by direct infusion of the methanol extract of the nudibranch *M. limceana* collected upon the octocoral showed a major ion species at *m/z* 397.2 ($C_{22}H_{30}NaO_5^+$) (Fig. 2). The MS/MS fragmentation pattern of this ion was superimposable to sodiated amphidinolide P (**1**) that gave two series of fragments arising by either neutral loss of water (*m/z* 379.2) or sequential loss of CO_2, water and ketene (*m/z* 353.2, 335.2 and 311.2, respectively) (see Supporting Information) according to the mechanism previously described for the polyketides tetronasin and mycolactone (Fonseca et al., 2004; Hong et al., 2003).

Like other members of the family (Kobayashi and Kubota, 2010), amphidinolide P (**1**) has been never reported from other sources except for the laboratory cultures of the dinoflagellate *Amphidinium* sp. Thus, the presence of amphidinolide P (**1**) in the octocoral is the first report of this class of macrolides in invertebrates and, to the best of our knowledge, in a field sample. *S. bicolor* hosts a rich and apparently uncultivable community of dinoflagellates. Analysis of the *in hospite* group of zooxanthellae of *S. bicolor* collected in April 2014 revealed a major presence of a single type of dinoflagellate cell that was isolated and maintained for several weeks in multiwell plates by using different culture media (*k*, *f/2* and supplemented ES). Unfortunately the cells, whose identity is still under determination,

Table 2
^1H and ^{13}C NMR spectroscopic data of amphidinolide P (**1**) and 3-*O*-methyl-amphidinolide P (**2**).

	1[a,b]		**2**[a]	
No.	δ_H (multi, *J* in Hz)	δ_C	δ_H (multi, *J* in Hz)	δ_C
1		172.3		168.7
2	2.27, d (12.0) 2.36, d (12.0)	44.7	2.44, d (12.5) 2.57, d (12.5)	44.9
3		99.0		100.7
4	1.94, m	45.1	1.98, m	47.7
5	–	143.5	–	143.8
6	2.09, dd (12.0, 12.0) 2.51, dd (2.5, 12.0)	38.9	2.11, dd (12.0,12.6) 2.51, dd (2.5, 12.6)	39.1
7	3.46, m	73.3	3.13, ddd (2.5, 3.0, 8.0)	74.1
8	2.61, dd (2.0, 8.0)	62.3	2.71, dd (2.0, 8.0)	63.2
9	2.47, dd (2.0, 8.0)	57.4	2.57[c]	56.7
10	2.17, dd (8.0, 14,0) 2.64, d (14.0)	36.2	2.19, dd (9.0, 14,0) 2.74, d (14.0)	36.0
11		142.7		140.8
12	6.19, d (15.0)	133.4	6.33, d (15.0)	134.8
13	5.59, dd (8.0, 15.0)	128.8	5.57, dd (8.0, 15.0)	129.1
14	5.29, t (8.0)	78.1	5.33, t (8.0)	78.1
15	2.42, m	45.0	2.47, m	44.6
16	–	146.5	–	146.8
17	4.87, br s 4.88, br s	111.9	4.87, br s 4.90, br s	111.7
18	0.91, d (7.0)	11.4	0.84, d (7.0)	10.7
19	4.76, s 4.81, s	109.5	4.69, s 4.77, s	108.6
20	4.80, s 4.93, s	117.6	4.86, s 4.91, s	119.0
21	0.90, d (7.0)	16.0	0.93, d (7.0)	16.2
22	1.66, s	19.4	1.71, s	19.1
OMe	–	–	3.20, s	49.5

[a] Data were measured in C_6D_6 at 600 MHz.
[b] Ishibashi et al. J.O.C. 1995, 60, 6062–6066.
[c] Overlapped signals.

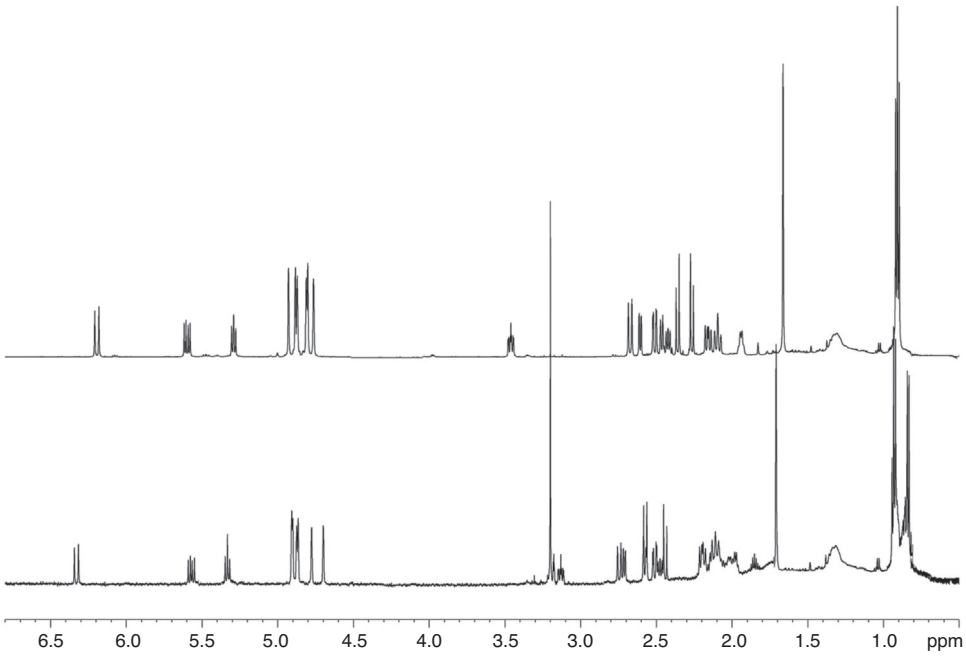


Fig. 1. ^{1}H NMR spectra (C_6D_6, 600 MHz) of compounds **1** (top) and **2** (bottom).

remained in their non-motile form and any attempt to grow them turned out to be unsuccessful so far. It is worth noting that despite the common belief that many secondary metabolites isolated from invertebrates may be produced by symbiotic zooxanthellae, only the origin of the terpene glycosides pseudopterosins from the soft coral *Pseudopterogorgia elisabethae* has been rigorously traced back to a symbiotic dinoflagellate of the genus *Symbiodinium* (Mydlarz et al., 2003; Boehnlein et al., 2005).

Amphidinolides are potent anticancer agents and some of them exhibited cytotoxicity to cancer cell lines in sub-nanomolar range (Kobayashi and Tsuda, 2004). Although previous studies indicated a moderate cytotoxicity for amphidinolide P (**1**) (Kobayashi and

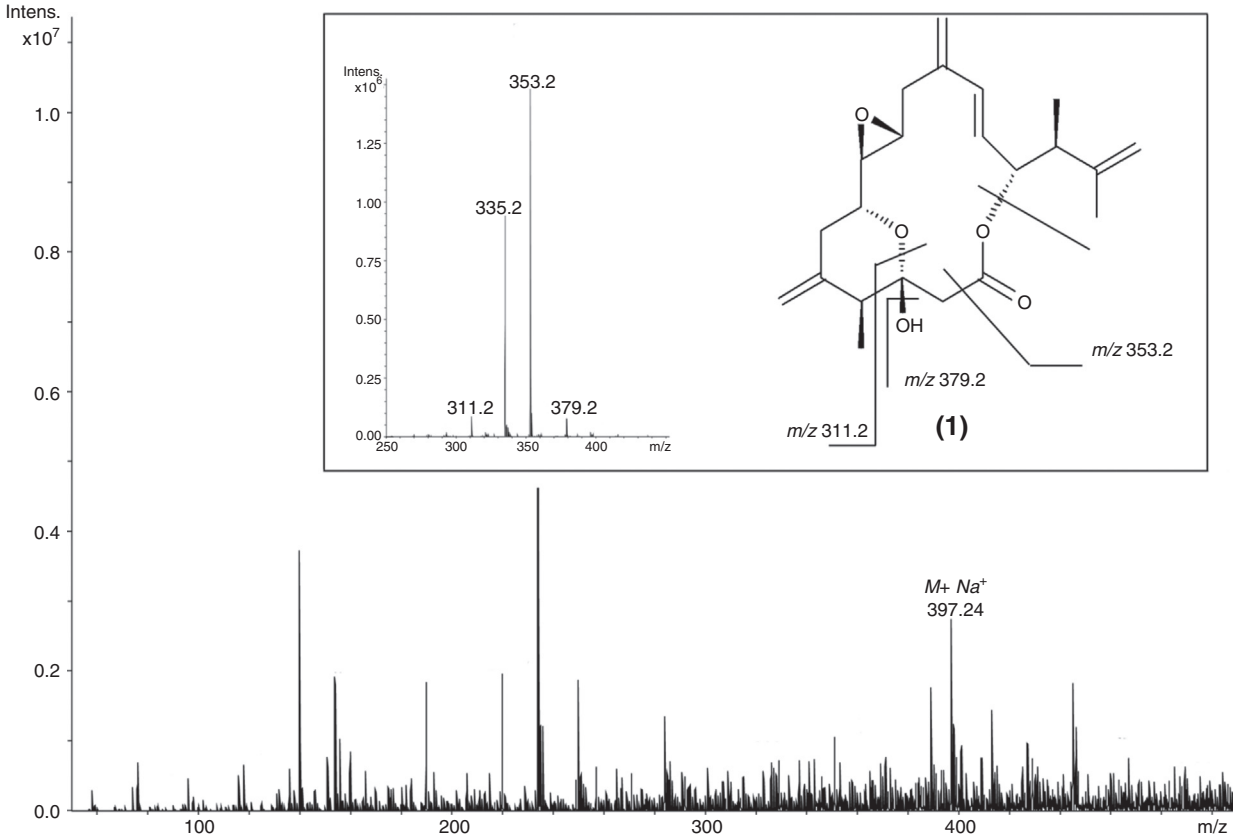


Fig. 2. Full-scan ESI-MS by direct infusion of the MeOH extract of the nudibranch *M. limceana*. The insert shows the diagnostic MS/MS fragmentation of the sodium adduct ion at *m*/*z* 397.2 corresponding to amphidinolide P (**1**) (see Supporting Material).

Tsuda, 2004; Kobayashi, 2008), the product of *S. bicolor* did not show any activity against adenocarninoma colon cancer HCT-116 cells, while compound **2** was only moderately active, presenting an IC_{50} of 12.2 μM.

In conclusion, we have identified amphidinolide P (**1**) and its derivative 3-*O*-methyl amphidinolide P (**2**) in the octocoral *S. bicolor* even if the latter product is probably an artifact that arises during extraction with MeOH. Amphidinolides have been recognized as potent anticancer agents (Kobayashi and Tsuda, 2004) but compounds **1** and **2** did not show significant cytotoxicity. Since the pure compounds were less active than raw fractions of the octocoral (Table 1), it is conceivable that other minor amphidinolides could be present in the tissue of the invertebrate. This brings into debate the synthesis of secondary metabolites by *S. bicolor* and the uncultivable strain of a putative *Amphidinium* symbiont. In fact, despite the primary ecological relevance of the interaction between cnidarians and dinoflagellate algae (Davy et al., 2012), the isolation of **1** is the first and so far only evidence about production of a polyketide by a symbiotic association. It is worth noting that amphidinolide P (**1**) has been recovered from the coral tissues in amount far higher than that reported from cultures of *Amphidinium* isolate (0.25% w/w in the octocoral extract for **1** and 0.005% w/w in the extract of the dinoflagellate pellet) (Ishibashi et al., 1995). This apparent increase in the production of the secondary metabolites is in agreement with the symbiotic origin of the product since it is well documented that the hosts plays an important role in regulating the type and the quantity of metabolites produced by the symbiont (Trench, 1993; Gordon and Leggat, 2010). Furthermore, occurrence of amphidinolides in a natural assemblage opens to the intriguing possibility to find compounds different from those so far reported from pure cultures of the dinoflagellate, as well as raises a question about the ecological or physiological role of these compounds. To this regards, the presence of amphidinolide P (**1**) in the extract of the nudibranch proves the dietary dependence of the two animals and provides the first evidence of transfer of this class of secondary metabolites along the trophic marine levels. This finding also suggests that these compounds may not have in the coral a defensive role against selective predators.

Authors contributions

GN, TSS and MCMT carried out purification and structure elucidation; EAS, BAG and ODLP contributed in collection and extraction of the animals; AC contributed in the NMR and MS investigations; NPL contributed in the mass spectrometry analysis and fragmentation studies on crude samples; AS isolated the symbiont; PCS, EAS and LVCS performed the biological assays. AF drafted the paper. AF and LVCL developed the project and interpreted the results. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2015.08.010.

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