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Short communication

Pyrimidine alkaloids from *Eudistoma vannamei*

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ABSTRACT

Methanolic extracts of the Brazilian endemic ascidian *Eudistoma vannamei* have been extensively studied for their cytotoxic activity against several human cancer cell lines. Previous work reported the occurrence of purine derivatives and staurosporine alkaloids as the major nitrogen-containing compounds. In this study, we report the occurrence of three pyrimidine alkaloids in addition to cholesterol, sitosterol and stigmasterol.

Introductory remarks

The colonial ascidian *Eudistoma vannamei* Millar, 1977, an endemic species to the Brazilian Northeast coast, has long been a subject of our marine natural product research group. This, such as many tropical ascidian species, has shown to be a rich and prolific source of biologically active compounds, and publications have been spanning through the specialized literature for over 10 years.

We have previously reported cytotoxicity in the crude extract (Jimenez et al., 2003), induction of cellular apoptosis by derived fractions (Jimenez et al., 2008), and the isolation of novel highly cytotoxic staurosporine derivatives – 2-hydroxy-7-oxostaurosporine (**9**) and 3-hydroxy-7-oxostaurosporine (**10**) (Jimenez et al., 2012). Applying directly crude extracts analysis in electrospray ionization tandem mass spectrometry (ESI-MS/MS) method allowed the identification of four purine derivatives – adenine (**4**), 2′-deoxyadenosine (**5**), 2′-deoxynosine (**6**), and deoxyguanosine (**7**) (Takeara et al., 2007a) and the continuous chemical investigation of this extract afforded other new and unusual amino acid bearing molecules – 9-[*N*-(leucyl)-isoleucyl]-adenine (**1**), 8-hydroxy-8-isopentyl-7,8-dihydroadenine (**2**) and *N*-[*N*-(leucyl)-isoleucyl]phenethylamine (**3**) (Pimenta et al., 2014). Moreover, we have prospected the cultivable microorganisms associated to *E. vannamei* for producers of biologically active compounds and described the recovery of cytotoxic fungi strains, among which an *Aspergillus* sp. yielding mellein derivatives and penicillic acid (Montenegro et al., 2012). Regarding the bacteria, we have reported some bioactive strains of actinomycetes (Jimenez et al., 2013), a staurosporine-producing *Streptomyces* sp. (**8**) which can support the structure block for the final products **9** and **10** isolated in *E. vannamei* (Andréo et al., 2012), the isolation of novel anthracyclinones from a strain of *Micromonospora* sp. (Sousa et al., 2012), and the isolation of an anti-cytokinesis dithiolpyrrolone from *Streptomyces* sp. (Abreu et al., 2014). In the present short communication we are reporting a continuation to the chemical characterization of active extracts of *E. vannamei* collected on the coast of Ceará State, Brazil, where this species is highly abundant.

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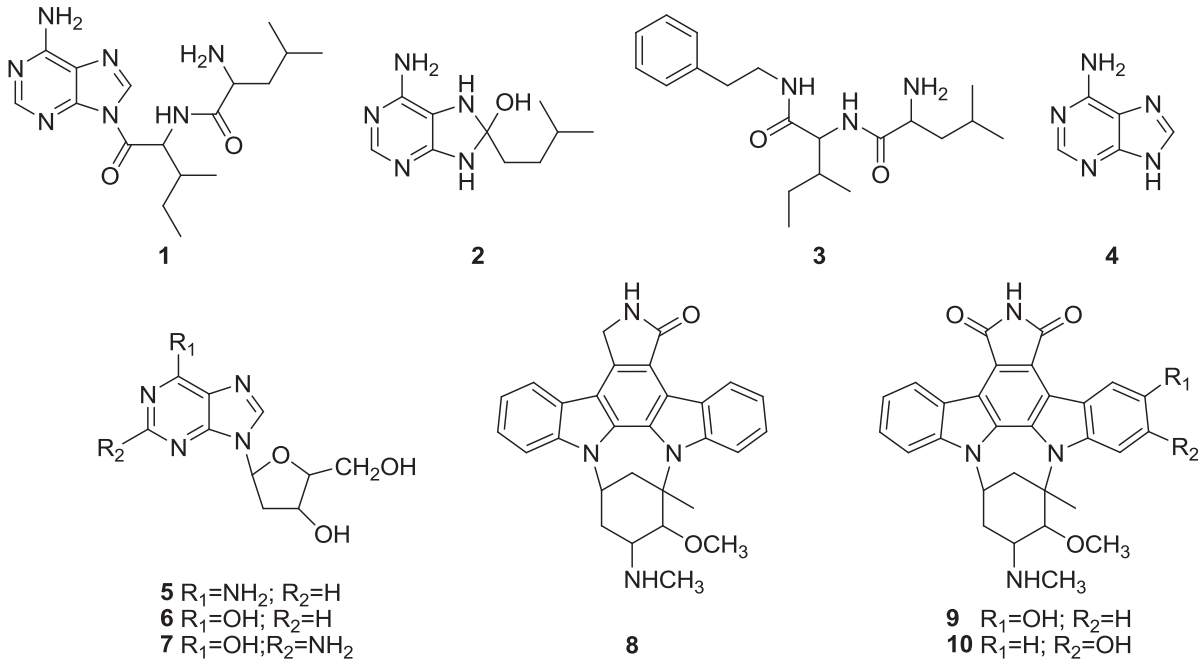


1 **2** **3** **4**

5 R_1=NH_2; R_2=H
6 R_1=OH; R_2=H
7 R_1=OH;R_2=NH_2

8

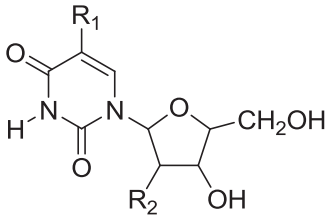
9 R_1=OH; R_2=H
10 R_1=H; R_2=OH

Materials and methods

The NMR spectra were obtained in a Bruker Avance DRX 400. Samples were dissolved in deuterated DMSO and transferred to 5 mm tubes. ESI-MS analysis was carried out in Quatro LC machine (Micromass – Waters). Solutions were infused into the ESI source at 5 μl min^{-1} using a Harvard Apparatus model 1746 (Holliston, MA) syringe pump. CID fragmentation was performed using argon as collision gas at 25 eV. For steroids and triterpene analysis, a gas chromatograph (GC-MS-QP-2010, Shimadzu) coupled to a quadruple mass spectrometer with electron impact (EI) ionization at 70 eV was applied. Samples were infused to EI machine through the gas chromatograph equipment furnished with an auto sampler AOC-20i and 1 μl split injections 1/10 were performed at 250 °C. Separations were carried out on an HP1 column (30 m × 0.25 mm × 0.25 μm film thickness). The oven temperature program for separation conditions was 200 °C for 5 min followed by temperature increases to 280 °C at 5 °C min^{-1} and 280 °C for 30 min. The carrier gas was ultrapure helium (grade 5.5) at constant flow rates of 1.10 ml min^{-1}. The spectra of all metabolites were compared with Wiley Mass Spectral Database and with authentic samples from NPPNS (Núcleo de Pesquisas em Produtos Naturais e Sintéticos) standards. For classical CC (column chromatography) and TLC (thin layer chromatography), silica gel 60 (70–230 mesh ASTM, Sigma) and silica gel GF254 (Merck) were used, respectively. Finally, for pyrimidine semi-preparative purification in HPLC (Shimadzu LC-6A, with UV-detector SPD-6A) was applied Shimpack ODS-C column (20 mm × 250 mm) and the solvent system flow was 9.7 min/ml in MeOH/H_2O gradiente.

Results and discussion

Chromatography fractionation of the methanolic extracts afforded several of the secondary metabolites previously isolated and structures were confirmed by TLC or ESI-MS data in negative mode. Fraction 16 (from a total of twenty polled fractions) showed signals relative to $[M-H]^-$ of **11** (*m*/*z* 241), **12** (*m*/*z* 227) and **13** (*m*/*z* 243) in ESI-MS. Our previous investigation with the ascidian *Didemnum psammatodes* resulted in the isolation and identification of same three nucleosides in addition to 2′-deoxyinosine and 2′-deoxyguanosine (Takeara et al., 2007b). Seeing confirmation of such structures, fraction 16 (8 mg) was submitted to purification by HPLC and pure samples were submitted to NMR analysis. ^{1}H NMR and ^{13}C NMR (DMSO, 400 MHz) spectra of **11** exhibited the signals: δ_H 7.7 (brd, H-6), 6.16 (s, H-1′), 4.20 (s, H-3′), 3.76 (m, H-4′), 3.56 (m, H-5′), 2.07 (m, H-2′), 1.77 (s, CH_3) and δ_C 164.0 (C-4), 151.2 (C-2), 136.4 (C-6), 109.4 (C-5), 88.5 (C-4′), 84.4 (C-1′), 71,3 (C-3′), 62.1 (C-5′), 40.0 (C-2′), 12.7 (s, CH_3). This data, in addition with the deprotonated ion, are in agreement with thymidine (Pouchert and Behnke, 1993). ^{1}H NMR and ^{13}C NMR (DMSO, 400 MHz) spectra of **12** exhibited the signals: δ_H 7.86 (d, *J* = 8.0, H-6), 6.16 (brd, H-1′), 5.63 (d, *J* = 8.0, H-5), 4.21 (s, H-3′), 3.77 (m, H-4′), 3.55 (m, H-5′), 2.07 (m, H-2′) and δ_C 163.0 (C-4), 150.0 (C-2), 139.0 (C-6), 101.4 (C-5), 86.5 (C-4′), 83.0 (C-1′), 70,3 (C-3′), 61.0 (C-5′), 40.0 (C-2′). This data, in addition with the deprotonated ion are, in agreement with NMR data from Pouchert and Behnke (1993) for 2′-deoxyuridine. Compound **13**, uridine, also has similar ^{1}H NMR (DMSO, 400 MHz) spectra to our previous work with *D. psammatodes* (Takeara et al., 2007a) and previously published data (Kitajima et al., 1999): δ_H 7.88 (d, *J* = 8.1, H-6), 5.77 (d, *J* = 5.2, H-1′), 5.64 (d, *J* = 8.1, H-5), 3.96 (m, H-3′), 3.84 (m, H-4′), 3.58 (m, H-5′), 4.02 (m, H-2′). Finally, CG-MS analysis of apolar fractions allowed the identification of a mixture of steroids containing cholesterol, sitosterol and stigmasterol. The occurrence of plant steroids in the extract of this filter-feeding invertebrate can be related with the presence of plankton in the ascidian's diet (Takeara et al., 2007a).



11 R_1=CH_3; R_2=H
12 R_1=R_2=H
13 R_1=H; R_2=OH

In this work, pyrimidine compounds were reported for the first time in *E. vannamei*, however they have been previously reported in the following ascidians: *Aplidium pantherinum* (Kim et al., 1993);

Atriolum robustum (Kehraus et al., 2004); an unidentified *Eudistoma* sp. (Schupp et al., 2003); *Didemnun* spp. (Takeara et al., 2007a; Mitchell et al., 1996); *Tridemnum cereum* (Demattè et al., 1985). The occurrence of thymidine, 2′-deoxyuridine and uridine are in agreement with observed bioactivity for the extract of *E. vannamei*. As a matter of fact, marine nucleosides are well known for their cytotoxic activity and have been engineered into drugs to treat viral and parasitical infections, as well as cancer (Huang et al., 2014). Notably, sponge pyrimidine arabinonucleosides isolated from *Crytothetya crypta* (Bergmann and Feeney, 1951) were developed into cytarabine (ARA-C) (Evans et al., 1961) and vidarabine (ARA-A) (Privat de Garilhe and De Rudder, 1964) in the early 1970s while transforming the existing logic applied to rational design of unnatural nucleosides. Cytotoxicity of such molecules are mostly a consequence of their capacity of misleading DNA-polymerase in to inserting a false, although similar, substrate in the newly synthetized DNA chain.

Naturally occurring nucleosides remain of great interest to scientists and industry alike, as these compounds have served as a prototype to a considerable number of pharmaceuticals, while enlightening the mechanisms of fundamental cellular processes. Marine organisms, including the ascidian *E. vannamei* chemically addressed herein, may be a promising source of lead molecules to new bioactive compounds of this class.

Author's contributions

RT, TOB and PCJ conducted extraction, isolation and structures identification of the secondary metabolites, respectively and the interpretation of all these data. LVCL, NPL, JLCL wrote and revised the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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