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Original Article

Clathriamide, an hexapeptide isolated from the marine sponge *Clathria* (*Clathria*) *nicoleae*

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ABSTRACT

Chemical investigation of the aqueous fraction of the ethanol extract from the Brazilian endemic marine sponge *Clathria* (*Clathria*) *nicoleae* Vieira de Barros, Santos & Pinheiro, 2013, Microcionidae, sampled from a 55 m deep rhodolith bed at the Amazon River mouth, led to the isolation of a new hexapeptide, clathriamide (**1**). HP-20 resin was used to capture compound **1** from the aqueous fraction, which was purified by additional chromatographic steps. The absolute configuration of the amino acids of **1** was determined by advanced Marfey's analysis using 5-fluoro-2,4-dinitrophenyl-Nα-L-tryptophanamide. The amino acid derivatives analyzed by ultra-performance liquid chromatography coupled to a mass spectrometry using a C_8 column enabled a good chromatographic resolution of L-Ile and L-*allo*-Ile, previously unfeasible using C_{18} column.

Introduction

Marine sponges are a rich source of both ribosomal and non-ribosomal bioactive peptides that frequently present unusual aminoacids (Fusetani and Matsunaga, 1993; Carroll et al., 2019). Marine sponges belonging to the genus *Clathria* (>350 valid species) have yielded an array of modified peptides (Van Soest et al., 2019). These include eurypamide A, that contains (2*S*,3*S*,4*R*)-3,4-dihydroxyarginine, isolated from *Microciona eurypa* [currently *Clathria (Thalysias) eurypa*] (Reddy et al., 1998), microcionamides A–D, that contain a 2-phenylethylenamine residue, isolated from *Clathria* (*Thalysias*) *abietina* and from *Clathria* (*Clathria*) *basilana* (Davis et al., 2004; Mokhlesi et al., 2017), as well as gombamides A–D, that present a pyroglutamic acid residue, isolated from *Clathria* (*Clathria*) *gombawuiensis* and from *Clathria* (*Clathria*) *basilana* (Woo et al., 2013; Mokhlesi et al., 2017). These modified peptides clearly arise from non-ribosomal peptide synthetases (NRPS) biosynthetic machineries, of widespread occurrence in microorganisms, suggesting a microbial origin for *Clathria* spp. peptides.

Samples of *Clathria* (*Clathria*) *nicoleae* Barros et al., 2013, Microcionidae, were collected during an expedition to the Amazon River mouth, where an extensive reef system has been recently described (Moura et al., 2016), revealing large rhodolith beds and a rich sponge fauna, including endemic species (Collette and Rützler, 1977; Leal et al., 2017). We have recently reported the first investigation of bioactive metabolites from *Dictyonella* sp., a sponge collected at the same location that yielded bromopyrrole alkaloids inhibitors of the proteasome (Souza et al., 2018).

The EtOH extract of *C. nicoleae* was defatted with hexane. After evaporation, the polar residue was partitioned between H_2O and EtOAc. The H_2O fraction was adsorbed onto Diaion HP-20 resin, which was washed with H_2O and desorbed with MeOH, then with 1:1 MeOH/acetone. The MeOH and 1:1 acetone/MeOH fractions from the H_2O extract were pooled and subjected to a series of separations by reversed-phase chromatography and HPLC (see Materials and Methods), to give clathriamide (**1**) as an optically active glassy solid.

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Materials and methods

General experimental procedures

The ultraviolet spectrum was recorded on a UV-3600 Shimadzu UV spectrophotometer. The optical rotation was measured with a Polartronic H Schmidt+Haensch polarimeter. The sample was diluted in MeOH in a concentration of 0.01 mg ml^{-1}. The infrared (IR) analysis was performed on a Si plate using a Shimadzu model IRAffinity instrument. NMR spectra were obtained at 25 °C on a Bruker Avance III spectrometer (9.4 T) operating at either 600 MHz (^{1}H) or 150 MHz (^{13}C), using TMS as standard. HRESIMS measurements were recorded on a Waters Acquity UPLC H-class liquid chromatograph coupled to a Waters Xevo G2-XS QToF mass spectrometer with an electrospray interface (ESI). A UPLC column Acquity BEH C_{18} (2.1 × 50.0 mm, 1.7 μm; Waters) was used for the HRMS and HRMS/MS analyses, with an elution gradient from 95:5 to 0:100 H_2O/CH_3CN (with 0.1% of formic acid) during 3.5 min, then from 0:100 to 95:5 of H_2O/CH_3CN (with 0.1% of formic acid) during 3 min, then maintained in 95:5 of H_2O/CH_3CN (0.1% of formic acid) during 1.4 min. The flow rate was 0.500 ml min^{-1}. The volume of injection was 0.1 μl of a 0.1 mg ml^{-1} solution (in MeOH). The column and samples were maintained at 40 °C and at 15 °C, respectively. Acquisition of mass spectra was performed in MS and MS/MS mode, with acquisition time of 5 min. The ESI source was set up for a mass range between *m/z* 50 and 900, for a scan time of 0.200 s^{-1} and collision energy (for MS/MS) of 25 eV. The positive mode ESI conditions were: 1.2 kV capillary voltage, 30 V cone voltage, 100 °C source temperature, desolvation temperature of 450 °C, 50 l h^{-1} cone gas flow and 750 l h^{-1} desolvation gas flow. A solution of leucine enkephalin at 200 pg ml^{-1}, infused by the lock-mass probe with a flow rate of 10 μl min^{-1}, was used for internal calibration. HPLC-UV-ELSD-MS analyses were performed with a chromatography system consisting of a Waters 2695 Alliance control system coupled to a Waters 2696 UV-visible spectrophotometric detector with photodiode array detector, connected sequentially with a splitter that divides the flow between a Waters Micromass ZQ 2000 mass spectrometry detector and a Waters ELSD 2424 detector, both of which are operated using Empower platform. Analyses were performed using a Waters C_{18} X-Terra reversed phase column (4.6 × 250 mm, 5 μm). The mass spectrometer detector was used under the following conditions: capillary voltage: 3 kV; temperature of the source: 100 °C; desolvation temperature: 350 °C; ESI mode, acquisition range *m/z* 100–1000; gas flow without cone: 50 l h^{-1} and desolvation gas flow: 350 l h^{-1}. Samples were diluted in MeOH to a concentration of 1.00 mg ml^{-1}.

Sponge material

The sponge *Clathria* (*Clathria*) *nicoleae* Barros et al., 2013, Microcionidae (Barros et al., 2013) was collected on September 27th, 2014, at the coordinates 1.3 °N 46.78 °W, by bottom trawl net, at a 55 m deep rhodolith bed in Pará State, Brazil. The collection was during an oceanographic expedition on the continental shelf of the Equatorial margin at the Amazon River mouth, aboard the Brazilian Navy ship *NHo Cruzeiro do Sul*. The species was identified by F. C. Moraes based on morphological and anatomical comparison to literature descriptions (Muricy et al., 2008; Barros et al., 2013) and comparative material. Voucher specimens have been deposited at Museu Nacional, Universidade Federal do Rio de Janeiro, under registry numbers MNRJ 18754, MNRJ 18813 and MNRJ 18814 (Moura et al., 2016).

Extraction and isolation

A sample (650 g) of the marine sponge *C. nicoleae* was exhaustively extracted with EtOH and this extract was evaporated to dryness. The EtOH extract (9 g) was dissolved in MeOH and partitioned with hexane. The MeOH fraction was evaporated, resuspended and partitioned between EtOAc and H_2O. Diaion HP-20 resin (50 g) was added to the water fraction with overnight shaking. The HP-20 resin was then collected by filtration and washed with H_2O. The organic adsorbed material was recovered by extraction from the resin with 100 ml of MeOH, then with 100 ml of 1:1 MeOH/acetone and 100 ml of acetone. The HP-20 organic fractions were combined and evaporated to dryness, resulting in a fraction (W7B; 390.3 mg), which was subjected to a separation by gel permeation chromatography on Sephadex LH-20 (dimensions: 140 × 2.5 cm) eluted with MeOH. Fourteen fractions (W7BA - W7BN) were obtained. After analysis by HPLC-UV-ELSD-MS and UPLC-HRMS, the fraction W7BH (104 mg) was selected for separation by HPLC-ELSD using semi-preparative C_{18} Inertsustain (10 × 250 mm, 5 μm, GL Science Inc). A gradient of elution of MeCN/MeOH/H_2O (4.0 ml min^{-1}) was used, starting with 12:13:75 during 15 min, then changing to 20:20:60 in 5 min, maintaining in this condition during 15 min, then changing to 50:50:0 in 5 min, maintaining in this condition during 10 min. Fraction W7BHI (18.6 mg), collected in the retention time between 26 and 28 min, was further separated by HPLC-ELSD using a X-Terra C_{18} analytical column (4.6 × 250 mm, 5 μm, Waters), with an isocratic elution of 10:15:75 MeCN:MeOH:H_2O (flow rate: 0.8 ml min^{-1}), to give 2.6 mg of pure clathriamide (**1**).

Clathriamide (**1**). White amorphous solid; $[\alpha]_D$ -2.97° (*c* 0.27, MeOH); UV (MeOH) λ_{max} (log ε) 202 (4.78) nm; IR (film) ν_{max} 3304, 2958, 2931, 2875, 2547, 1645, 1533, 1452, 1392, 1201, 1083, 700, 609 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 1; HRESIMS $[M+H]^+$ *m/z* 703.4047 (calcd. for $C_{35}H_{55}N_6O_9^+$ *m/z* 703.4026).

Acid hydrolysis of clathriamide

A 0.7 mg sample of **1** was dissolved in 500 μl of HCl 6 mol l^{-1} and heated at 115 °C, with stirring, for 12 h. The solution was cooled to 23 °C, dried, resuspended with water and evaporated to remove the residual HCl.

Advanced Marfey's analysis with 5-fluoro-2,4-dinitrophenyl-Nα-L-tryptophanamide (FDTA)

Clathriamide (**1**) acid hydrolysate was resuspended in 1 ml of H_2O. Two 50 μl aliquots of this solution were separately treated with 142 μl solutions of L- and D-FDTA (1% in acetone) containing 20 μl of $NaHCO_3$ (1 mol l^{-1}) each. Both derivatization reactions with L- and D-FDTA were heated at 40 °C, with stirring, during 1 h. The reaction was then cooled to room temperature and neutralized with 10 μl of 2 mol l^{-1} HCl and evaporated to dryness. The evaporation residue was resuspended in 500 μl of MeOH. UPLC-MS analyses of derivatized hydrolysates were performed with an Acquity BEH C_{18} column (2.1 × 50.0 mm, 1.7 μm; Waters) using a linear gradient elution from 8:2 to 5:5 of H_2O/MeCN (with 0.1% formic acid) during 8 min, with a flow rate of 0.500 ml min^{-1}. The same derivatization conditions and UPLC-MS analysis were used with the following amino acid standards: L-Leu, L-Ile, L-*allo*-Ile, L-Thr, L-Pro, L-Hyp, L-Phe. In the case of L-Leu, L-Ile and L-*allo*-Ile, the L-DTA derivatives were analyzed with an Acquity BEH C_8 column (2.1 × 100 mm, 1.7 μm, Waters), eluting with isocratic 73:27

Table 1
^{1}H, and ^{13}C NMR data for **1** in MeOH-d_4 (^{1}H: 600 MHz, ^{13}C: 150 MHz).

Residue	Position	δ_C, type	δ_H (*J* in Hz)
L-Leu	CO	170.9, C	
	α	51.7, CH	4.31, dd (9.8, 3.9)
	β	40.9, CH_2	1.80, m
	γ	25.6, CH	1.91, m
	δ	24.0, CH_3	1.12, d (6.4)
	δ'	21.8, CH_3	1.14, d (6.4)
L-Pro	CO	173.6, C	
	α	61.1, CH	4.89*
	β	29.9, CH_2	1.83, m; 2.45, m
	γ	26.9, CH_2	2.14, m; 2.21, m
	δ	49.5, CH_2	3.58, m; 3.91, ddd (3.2, 7.8, 9.8)
L-Hyp	CO	173.1, C	
	α	61.1, CH	4.57, dd (4.7, 7.9)
	β	40.5, CH_2	2.13, m
	γ	68.4, CH	3.58, m
	δ	54.4, CH_2	3.13, dd (6.1, 11.9); 3.43, dd (5.1, 11.9)
L-Phe	CO	174.1, C	
	α	59.1, CH	4.44, dd (4.1, 12.6)
	β	38.9, CH_2	3.27, dd (12.6, 13.7); 3.39, dd (3.9, 13.7)
	aromatic	1: 139.1, C	
		2: 130.4, CH	7.41, m
		3: 128.5, CH	7.32, m
		4: 130.1, CH	7.40, m
		5: 128.5, CH	7.32, m
		6: 130.4, CH	7.41, m
L-Thr	CO	172.2, C	
	α	58.7, CH	4.59, d (2.7)
	β	71.4, CH	4.49, dd (2.7, 6.4)
	γ	19.3, CH_3	1.22, d (6.4)
L-Ile	CO	179.0, C	
	α	62.5, CH	4.09, d (4.8)
	β	37.9, CH	1.99, m
	γ	26.1, CH_2	1.29, m; 1.57, m
	γ'	17.0, CH_3	1.02, d (6.4)
	δ	12.6, CH_3	0.98, d (7.3)

* Superimposed by H_2O signal.

Table 2
UPLC Retention Times (t_R, min) and order of elution for L-DTA and D-DTA derivatives of clathriamide (**1**) amino acids.

Amino acid DTA derivative	Order of elution	t_R, min		Δt
		L	D	
4-*trans*-L-Hyp	D → L	2.50[a]	2.41[a]	−0.09
L-Thr	L → D	3.03[a]	3.99[a]	0.96
L-Pro	L → D	4.08[a]	4.63[a]	0.55
L-Phe	L → D	5.79[a]	6.88[a]	1.09
L-Ile	L → D	5.73[a]	7.11[a]	1.38
L-*allo*-Ile	L → D	5.77[a]	7.15[a]	1.38
L-Leu	L → D	5.90[a]	7.27[a]	1.37
L-Ile	L → D	17.05[b]		
L-*allo*-Ile	L → D	17.49[b]		
L-Leu	L → D	19.28[b]		

[a] Separation conditions: column: Waters ACQUITY BEH C_{18} (2.1 × 50.0 mm, 1.7 μm); eluent: linear gradient 8:2 to 5:5 H_2O/MeCN (+0.1% formic acid) during 8 min.; flow rate: 0.50 ml min^{-1}.

[b] Separation conditions: Waters ACQUITY BEH C_8 column (2.1 × 100 mm, 1.7 μm, Waters); eluent: 73:27 H_2O/MeCN (+0.1% formic acid) during 20 min.; flow rate: 0.500 ml min^{-1}.

H_2O/MeCN (with 0.1% formic acid) during 20 min, with a 0.500 ml min^{-1} flow rate. The retention time (t_R) of standard amino acid L-DTA and D-DTA derivatives is indicated in Table 2.

Results and discussion

HRESIMS analysis of clathriamide (**1**) displayed a $[M+H]^+$ at *m/z* 703.4047, corresponding to the molecular formula $C_{35}H_{54}N_6O_9$.

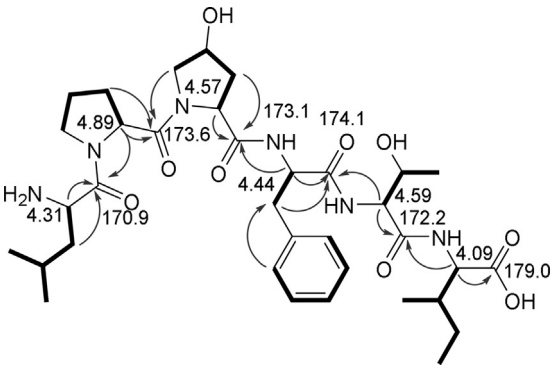
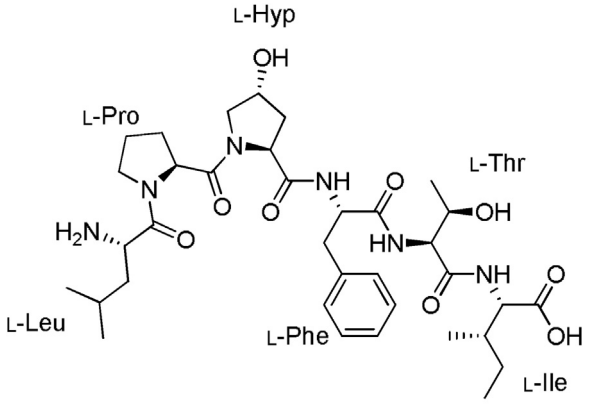


Fig. 1. ^{1}H NMR chemical shifts for α-hydrogens, ^{13}C NMR chemical shifts for amide and carboxylic acid carbonyl groups, key HMBC and COSY correlations observed for clathriamide (**1**) (600 MHz, MeOH-d_4).

Clathriamide ^{13}C NMR spectrum showed six signals for amide carbonyl groups and a carboxylic acid observed between δ_C 170.9 and 179.0, as well as six signals for amino acid α-carbons between δ_C 51.7 and 62.5. Its ^{1}H NMR spectrum also showed six α-hydrogens between δ_H 4.09 and 4.89, clearly indicating that **1** was an hexapeptide. Signals of ^{13}C and ^{1}H were also observed for an aromatic system at δ_C between 128.5 and 139.1 and at δ_H between 7.32 and 7.41, indicating the presence of a phenylalanine residue.



1

Inspection of clathriamide HSQC, COSY, HMBC and HSQC-TOCSY NMR data allowed the identification of six amino acids, leucine (Leu), proline (Pro), 4-hydroxyproline (Hyp), phenylalanine (Phe), threonine (Thr) and isoleucine (Ile) (Table 1). The connectivity between the amino acid residues was established by observation of key HMBC correlations between amide carbonyl groups and the corresponding α-hydrogens (Fig. 1). The confirmation of the amino acid sequence of clathriamide (**1**) could be established by HRESIMS/MS analysis. The fragmentation showed *b* and *y* series of fragments: $\boldsymbol{b_2}$ (*m/z* 211.1448, [M - Hyp - Phe - Thr - Ile]$^+$), $\boldsymbol{b_3}$ (*m/z* 324.1937, [M - Phe - Thr - Ile]$^+$), $\boldsymbol{b_4}$ (*m/z* 471.2613, [M - Thr - Ile]$^+$), $\boldsymbol{b_5}$ (*m/z* 572.3097, [M - Ile]$^+$); $\boldsymbol{y_2}$ (*m/z* 233.1356, [M+H - Leu - Pro - Hyp - Phe]$^+$), $\boldsymbol{y_3}$ (380.2187, [M+H - Leu - Pro - Hyp]$^+$), $\boldsymbol{y_4}$ (*m/z* 493.2670, [M+H - Leu - Pro]$^+$), $\boldsymbol{y_5}$ (*m/z* 590.3203, [M+H - Leu]$^+$) (Figure S2). The linear amino acid sequence of clathriamide (**1**) was thus defined as Leu-Pro-Hyp-Phe-Thr-Ile.

The absolute configuration of clathriamide (**1**) amino acids was established by the advanced Marfey's analysis recently reported (Salib and Molinski, 2017), using 5-fluoro-2,4-dinitrophenyl-Nα-L-tryptophanamide (FDTA) instead of Nα-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (FDAA). Hydrolysis of clathriamide (**1**) followed by derivatization with L- and D-FDTA and UPLC-MS analysis using a C_{18} column enabled us to assign the absolute stereochemistry of four of clathriamide (**1**) amino acids as L-Pro,

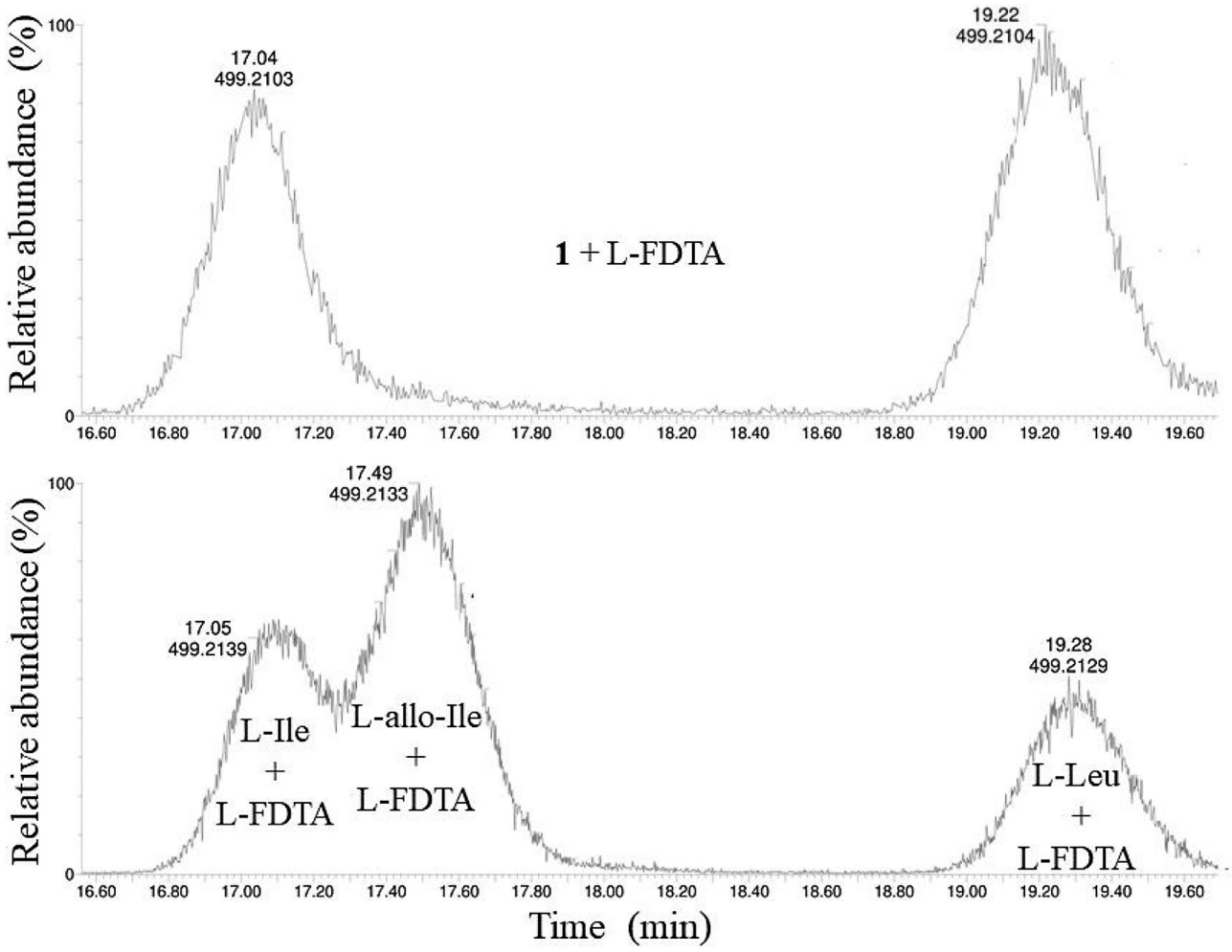


Fig. 2. UPLC trace for separation of L-Leu, L-Ile and L-*allo*-Ile standard DTA derivatives and comparison with clathriamide (**1**) L-FDTA derivative.

4-*trans*-L-Hyp, L-Phe and L-Thr by comparison with the retention time of the corresponding L- and D-FDTA derivatized amino acid standards (Table 2). The absolute stereochemistry of the remaining amino acids was established as L-Leu, L-Ile and L-*allo*-Ile derivatized by the same procedure using a C_8 UPLC column. The elution order observed was L-L to L-D for all FDTA derivatives, except for Hyp, as previously described for FDLA (1-fluoro-2,4-dinitrophenyl-5-L- or D,L-leucinamide) derivatives (Bornancin et al., 2019). Derivatization with the FDTA enabled an excellent chromatographic resolution for the separation L-L and L-D amino acid diastereomeric mixtures by UPLC-MS. The use of a C_8 column enabled the separation of a mixture of L-Leu, L-Ile and L-*allo*-Ile (Fig. 2), previously not achieved for FDAA derivatives of these amino acids (Salib and Molinski, 2017). Thus, the use of the modified Marfey reagent FDTA, recently developed by Salib and Molinski (2017) proved to be the best solution for the chromatographic separation of Ile and *allo*-Ile stereoisomers.

Not only the structure of clathriamide is novel, the amino acid sequence of **1** is unique. A detailed search in SciFinder database did not provide any related penta-, tetra- or tripeptide, considering any possible variation with the same amino acid sequence of **1**. Only two dipeptides having the same sequence in clathriamide have been reported in the literature. The dipeptide L-4-Hyp-L-Phe showed an efficient catalyst for aldol reactions (Shi et al., 2004), while L-Pro-L-4-Hyp promoted the growth of mouse skin fibroblasts (Shigemura et al., 2009). After hydrolysis and derivatization of clathriamide (**1**), unfortunately not enough material was left for bioassay testing.

In conclusion, the present investigation reports the isolation of clathriamide (**1**), a novel peptide obtained from the aqueous fraction of the marine sponge *C. nicoleae*, collected at the recently described extensive reef system of the Amazon River mouth. The correct assignment of the absolute configuration of clathriamide (**1**) amino acids was possible by use of the newly developed derivatization reagent 5-fluoro-2,4-dinitrophenyl-Nα-L-tryptophanamide (FDTA) and of a UPLC C_8 column. This procedure enabled the separation of L-Leu, L-Ile and L-*allo*-Ile, demonstrating the usefulness of FDTA as a very suitable advanced Marfey derivatization agent.

Our previous investigation on the chemistry of a *Dictyonella* sp. sponge, collected at the same location where *Clathria* (*Clathria*) *nicoleae* was obtained, provided new bromopyrrole alkaloids (Sousa et al., 2018). The present investigation reports the isolation and identification of clathriamide, a novel peptide from *C. nicoleae*. We believe that future chemical investigations of sponge specimens collected at the Amazon river mouth should provide additional bioactive natural products, since the metabolism of these animals are possibly highly influenced by the unique environmental conditions at that location.

Authors' contributions

FCM, WCP, LTS, RCP, RLM and GMAF contributed in collecting and identifying the sponge material. VFF, JS, JIQB and RGSB contributed for the isolation and structural elucidation of the compound. AGF contributed in the NMR data acquisition. VFF and RGSB

elaborated the experimental design. RGSB supervised the laboratory work, chemicals purchase and instruments maintenance. RGSB and VFF wrote the largest portion of the manuscript. All authors have read the final manuscript and approved the submission.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflict of interest

All authors have none to declare

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bjp.2019.07.001.

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