Callyptide A, a new cytotoxic peptide from the Red Sea marine sponge *Callyspongia* species

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1. Introduction

Marine organisms have been classified as the largest remaining reservoir of natural molecules to be evaluated for drug activity (Gerwick 1987; De Vries & Beart 1995). Recently,
marine peptides have opened a new perspective for pharmaceutical developments (Aneiros & Garateix 2004). Most peptides isolated from sponges have unique unprecedented structures in comparison to those from other sources. They are often cyclic or linear peptides containing unusual amino acids, which are either rare in terrestrial and microbial systems or even totally novel, and also frequently containing uncommon condensation between amino acids (Aneiros & Garateix 2004; de Silva et al. 1990). The genus *Callyspongia* (order Haplosclerida, family Callyspongiidae) includes about 182 species; about 15 of them have been chemically investigated (Ibrahim et al. 2010). The sponges of this species are known as rich sources of structurally unique compounds such as macrolides (Pham et al. 2014), polyacetylenes (Umeyama et al. 1997; Youssef et al. 2000, 2003), peptides (Berer et al. 2004; Ibrahim et al. 2008, 2010; Yang et al. 2009), diketopiperazines (Huang et al. 2010; Chen et al. 2014), terpenoids (Gray et al. 2006), alkaloids (Davies-Coleman et al. 1993; Yang et al. 2013; Plisson et al. 2014), fatty acids (Carballeria & Pagán 2001), polyketides (Kobayashi et al. 1997), sterols (Theobald et al. 1978), peroxides (Toth & Schmitz 1994), butenolides (Layne & Tinto 2006), nucleosides (Huang et al. 2011) and diazepine derivatives (Huang et al. 2010). These compounds showed interesting biological properties including cytotoxic (Kobayashi et al. 1997; Murakami et al. 2000; Ibrahim et al. 2008, 2010; Huang et al. 2010; Pham et al. 2014), antileishmanial (Gray et al. 2006), antifouling (Qian et al. 2006), α-glucosidase inhibition (Nakao et al. 2002) and antimicrobial (Qian et al. 2006; Ibrahim et al. 2010) activities. During the course of our searches into biologically active agents from marine sources, a new cytotoxic cyclic peptide named callyptide A (1) had been isolated from marine sponges *Callyspongia* sp. collected from the Red Sea. Details of the isolation, structure elucidation and biological activity of the new cyclic peptide are described in the present work.

2. Results and discussion

Compound 1 (Figure 1) was obtained as a white amorphous powder. It gave negative ninhydrin reaction, suggesting the N-terminus was a blocked or a part of a cyclic peptide. The ESI-MS spectrum of 1 showed pseudo-molecular ion peak at \( m/z \) 1001.5345 \([M + H]^+\) (Calcd for 1001.5348). Its molecular formula was established as \( \text{C}_{52}\text{H}_{72}\text{N}_8\text{O}_{12} \) by HRESIMS, requiring
21 degrees of unsaturation. Considering the molecular formula and degrees of unsaturation, 1 is a cyclic peptide. The IR spectrum showed absorption bands due to hydroxy (3445 cm\(^{-1}\)) and amide carbonyl (1660 cm\(^{-1}\)) functionalities. In the \(^1\)H NMR spectrum (Supplementary Figure S1), eight amide NH groups at 8.43–7.19 ppm coupled to the signals in the region of \(\alpha\)-protons of amino acids at 4.58–3.85 ppm, suggesting the peptide nature of 1. This was confirmed by the presence of eight carbonyl carbons at \(\delta_C\) 176.3–166.4 and 8 \(\alpha\)-amino acid carbon atom resonances at \(\delta_C\) 55.6–47.8. The \(^1\)C NMR (Supplementary Figure S2), HSQC (Supplementary Figure S4) spectra revealed the presence of 52 carbon resonances: 8 methyls, 5 methylenes, 26 methines, 2 of them are oxygen-bonded, and 13 quaternary carbons including 8 amide carbonyls. The \(^1\)H–\(^1\)H COSY experiment (Supplementary Figures S5 and S6) was used to characterise the major features of the constituent amino acids and the resulting assignments were confirmed by HSQC and HMBC (Supplementary Figures S3 and S6) spectra. Detailed analysis of the 1D and 2D NMR data indicated the presence of nine amino acid residues, seven of which were common amino acids; two tyrosines (Try), alanine (Ala), leucine (Leu), isoleucine (Ile), phenyl alanine (Phe) and threonine (Thr). The remaining non-standard amino acid residue was assigned as 3-hydroxy leucine (3-OHLeu) on the basis of 1D and 2D NMR spectral data. The observed \(^1\)H and \(^1\)C NMR signals at \(\delta_H/\delta_C\) 4.50 (dd, \(J = 10.4, 7.8\) Hz, H-39)/55.6 (C-39), 4.75 (dd, \(J = 10.4, 7.1\) Hz, H-40)/74.5 (C-40), 1.72 (m, H-41)/28.8 (C-41), 0.84 (d, \(J = 6.9\) Hz, H-42, 43)/23.2 (C-42, 43) and 8.41 (d, \(J = 7.8\) Hz, 39-NH), in association with the HMBC correlations from H-39 to C-40 and C-41, H-40 to C-38, C-41, C-42 and C-43, and H-42 and H-43 to C-40 and C-41, as well as the COSY correlations from H-39 to 39-NH and H-40 and H-41 to H-40, H-42 and H-43 established the 3-OHLeu residue (Lu et al. 2011). The amino acids sequence was established by the HMBC correlations of NH and \(H_\alpha\) of each amino acid to the amide carbonyl carbons through \(^3\)J\(_{\text{CH}}\) and \(^2\)J\(_{\text{CH}}\). The HMBC correlations of 2-NH and H-2 to C-5 and 2-NH to C-6 established the connectivity between Thr-1 and Ala-2 [substructure A]. Leu-3 and Tyr-4 were linked to give di-peptide substructure B on the basis of HMBC cross-peaks of 9-NH and H-9 to C-14 and 9-NH to C-15. The connectivity of substructures A and B to give tetra-peptide substructure was secured by the \(^3\)J HMBC cross-peak H-6 to C-8 and the \(^2\)J HMBC cross-peak 6-NH to the same carbon. The HMBC (Supplementary Figures S5 and S6) correlations of 24-NH to C-32 and C-33 and H-24 to C-32 assigned the amide linkage of Phe-5 to Ile-6 to give substructure C. 3-OHLeu-7 and Tyr-8 were linked to give a di-peptide substructure D. Substructures C and D were connected through the observed HMBC correlations of 33-NH to C-38 and C-39 and H-33 to C-38. HMBC (Supplementary Figures S5 and S6) correlations of 15-NH and H-15 to C-23, 15-NH to C-24, H-45 to C-1, and 45-NH to C-1 and C-2 established the cyclisation via the amide bond formed between Phe-5/Tyr-4 and Tyr-8/Thr-1. The \(R\)-configuration of C-3 in 3-OHLeu was determined on the basis of comparison of the \(^1\)H and \(^1\)C chemical shift as well as coupling constant value (\(\mathcal{J}_{H_2H_3} = 10.4\) Hz) with the literature (Lu et al. 2011). Also, a large \(J\) value (\(\mathcal{J}_{H_2H_3} = 10.1\) Hz) in Thr determined the absolute configuration of C-3 to be \(R\) (Lu et al. 2011). The stereochemical assignments of the individual amino acid residues of 1 were determined using Marfey’s method coupled with LC-MS analysis of the acid hydrolysate of 1 derivatised with N-\(\alpha\)-(5-fluoro-2,4-dinitrophenyl)-L-leucinamide. The results indicated that all amino acids were \(L\)-configured. Based on the data obtained from 1D and 2D NMR experiments and by comparison with the literature, the structure of 1 was unambiguously elucidated and named as callyptide A.

Compound 1 was evaluated for its growth inhibition activity against three cancer cell lines including breast adenocarcinoma (MDA-MB-231, ATCC: HTB 38), human lung carcinoma
(A549 (ATCC: CCL-185) and human colorectal carcinoma (HT-29 (ATCC: HTB 38). It showed inhibition activity with GI\textsubscript{50} values of 29, 18.5 and 30 μM, respectively. The positive control doxorubicin showed IG\textsubscript{50} with the values 0.30, 0.35 and 0.40 μM, respectively.

3. Experimental

3.1. General experimental procedures
Optical rotations were measured on a JASCO DIP-370 digital polarimeter (Jasco Co., Tokyo, Japan) at 25 °C at the sodium D line (589 nm). UV spectra were measured on a Hitachi 300 spectrophotometer (Hitachi High-Technologies Corporation, Kyoto, Japan). IR spectra were measured on a Shimadzu Infrared-400 spectrophotometer (Shimadzu, Kyoto, Japan). Positive mode HRESIMS data were obtained on a Finnigan MAT-312 spectrometer (ThermoFinnigan GmbH, Tokyo, Japan). NMR spectra were obtained in DMSO-\textsubscript{d6} on Varian Inova 500 spectrometer (Varian Inc., Palo Alto, CA, USA) at 500 MHz for \textsuperscript{1}H NMR and 125 MHz for \textsuperscript{13}C NMR. NMR chemical shifts are expressed in parts per million (ppm) referenced to residual DMSO-\textsubscript{d6} solvent signals (δ\textsubscript{H} 2.49 for \textsuperscript{1}H and δ\textsubscript{C} 39.54 for \textsuperscript{13}C). For column chromatography, silica gel (70–230 mesh, Merck, Darmstadt, Germany) was used. Pre-coated SiO\textsubscript{2} 60 F\textsubscript{254} plates (Merck, Darmstadt, Germany) were used for TLC.

3.2. Biological material
The sponge was collected in November 2013 by hands using SCUBA at depths between 9 and 15 m near Obhur, Saudi Arabia. The sponge materials were frozen immediately after collection and kept frozen at −20 °C until processed. The sponge is funnel-shaped, pink-coloured and up to 20 cm high, with a terminal aperture of 3–4 cm diameter, and a 1–2-mm-thick tube wall. The surface is optically smooth, but irregularly undulated. The sponge shows compressible consistency and was easy to tear. The skeleton of spongin fibres is cored by thin strongyles, 60–85 × 1–2 μm. The ectosomal skeleton is double-meshed, with larger triangular meshes 300–1500 μm in diameter enclosed by primary fibres of 30–55 μm diameter cored by 3–5 spicules, enclosing smaller polygonal meshes of 60–120 μm diameter formed by secondary fibres of 10–15 μm diameter cored by a single spicule. The choanosomal skeleton is a basically rectangular but rather irregular system of primary fibres similar in size and coring to those of the ectosomal skeleton and similar secondary fibres recognisable only by their position and coring with a single spicule, forming large meshes of 250–700 μm in diameter. The sponge was classified as Callyspongia species (class Demospongiae, order Haplosclerida, family Callyspongiidae). A voucher specimen (No. ZMA POR. 17,064) was deposited at the Zoological Museum of the University of Amsterdam.

3.3. Extraction and purification of compound 1
The sponge material (0.56 kg) was extracted with a mixture of CH\textsubscript{2}Cl\textsubscript{2}/MeOH (1:1). The crude extract was partitioned between 60% MeOH in H\textsubscript{2}O (500 mL) and CH\textsubscript{2}Cl\textsubscript{2} (3 × 500 mL). The cytotoxic CH\textsubscript{2}Cl\textsubscript{2} extract (1.4 g) was subjected to a flash silica gel column eluted with n-hexane-CH\textsubscript{2}Cl\textsubscript{2}/acetone gradients to give 10 fractions. The collected fractions were subjected to the evaluation of their cytotoxic activity. Fractions 7 and 8 were cytotoxic to the cancer cell
Therefore, fractions 7 and 8 were combined and the residue (71 mg) was subjected to HPLC purification on a semi-preparative HPLC column (Cosmosil, 250 × 10 mm) using 75% CH₃CN in H₂O as an eluting solvent with a flow rate of 2 ml/min and a detection at 220 nm to give compound 1 (5.5 mg).

3.4. Spectral data

Callyptide A (1): White amorphous powder; [α]D + 18° (c 0.4, MeOH); UV λmax (MeOH): 216, 284 nm; IR (KBr) νmax 3445, 2935, 1660, 1250, 1025 cm⁻¹; ¹H NMR (500 MHz, DMSO-d₆): Thr¹: δH 4.32 (1H, dd, J = 10.1, 6.9 Hz, H-2), 3.71 (1H, dd, J = 10.1, 6.8 Hz, H-3), 0.88 (3H, d, J = 6.8 Hz, H₃–4), 8.33 (1H, d, J = 6.9 Hz, NH); Ala²: 4.29 (1H, dq, J = 7.9, 7.1 Hz, H-6), 1.16 (3H, d, J = 7.1 Hz, H₃–7), 7.23 (1H, d, J = 7.9 Hz, NH); Leu³: 3.85 (1H, dd, J = 7.9, 6.9 Hz, H-9), 1.51 (2H, m, H-10), 1.47 (1H, m, H-11), 0.87 (3H, d, J = 6.8 Hz, H₃–12), 0.87 (3H, d, J = 6.7 Hz, H₃–13), 7.38 (1H, d, J = 7.9 Hz, NH); Tyr⁴: 4.58 (1H, dt, J = 8.6, 6.5 Hz, H-15), 2.86 (1H, d, J = 7.9 Hz, NH); 3-OHLeu⁷: 4.50 (1H, dd, J = 10.4, 7.8 Hz, H-39), 4.75 (1H, dd, J = 10.4, 7.1 Hz, H-40), 1.72 (1H, m, H-41), 0.84 (3H, d, J = 6.9 Hz, H₃–42), 0.84 (3H, d, J = 6.9 Hz, H₃–43), 8.41 (1H, d, J = 7.8 Hz, NH); Tyr⁹: 4.58 (1H, dt, J = 8.6, 6.5 Hz, H-45), 2.86 (1H, d, J = 13.5, 6.5 Hz, H-46B), 2.81 (1H, m, H-46B), 7.04 (2H, d, J = 7.9 Hz, H-48, 52), 6.60 (2H, d, J = 7.9 Hz, H-49, 51), 7.33 (1H, d, J = 8.6 Hz, NH); 3C NMR (125 MHz, DMSO-d₆); Ala²: δC 174.2 (C-1), 50.7 (C-2), 71.8 (C-3), 21.4 (C-4); Ala²: 172.5 (C-5), 47.8 (C-6), 18.0 (C-7); Leu³: 170.4 (C-14), 55.2 (C-15), 36.3 (C-16), 128.3 (C-17), 130.1 (C-18, 22), 114.8 (C-19, 21), 157.4 (C-20); Phe⁵: 166.4 (C-23), 55.5 (C-24), 36.5 (C-25), 135.4 (C-26), 129.1 (C-27, 31), 128.9 (C-28, 30), 127.9 (C-29); Ile⁶: 171.6 (C-32), 50.7 (C-33), 39.3 (C-34), 24.1 (C-35), 9.2 (C-36), 21.4 (C-37); 3-OHLeu⁷: 176.3 (C-38), 55.6 (C-39), 74.5 (C-40), 28.8 (C-41), 23.2 (C-42), 23.2 (C-43); Tyr⁹: 170.7 (C-44), 55.2 (C-45), 36.3 (C-46), 128.0 (C-47), 130.1 (C-48, 52), 114.8 (C-49, 51), 157.6 (C-50); HRESIMS m/z 1001.5345 [M + H]+ (Calcd for C₅₂H₇₃N₈O₁₂).

3.5. Determination of the absolute configuration of amino acids

3.5.1. Acid hydrolysis

About 1.0 mg of 1 was treated with 2 mL 6 N HCl (p.a.) and heated at 112 °C for 24 h in sealed ampoule. The resulting solution was concentrated under N₂ gas to dryness, with three consecutive addition of H₂O (5 mL each) to ensure complete elimination of HCl.

3.5.2. Marfey analysis of the acid hydrolysate

100 μL FDNPL (1% N-(5-flouro-2,4-dinitrophenyl)-l-leucinamide in acetone) and 20 μL 1 M NaHCO₃ were added to 50 μL of acid hydrolysate (or authentic amino acid standard at comparable concentration, Youssef et al. 2014). The reaction mixture was heated at 40 °C for 1 h over a hot plate with frequent mixing. After cooling, 20 μL of 2 M HCl was added and the volume was completed to 1000 μL with MeOH. The amino acid standards (L and D) were
treated separately with FDNPL in the same manner. The FDNPL derivatives were analysed by LC-MS through the comparison of the retention time and molecular weight with those of standard amino acids FDNPL derivatives. The retention times of the D/L-FDNPL mixtures (with the FDNPL \( R \) underlined) were as follows: Thr \( \text{24.45, 28.64, } m/z 414 \ [\text{M} + \text{H}]^+ \); 3-OHLeu \( \text{30.26, 33.54, } m/z 442 \ [\text{M} + \text{H}]^+ \); Tyr \( \text{35.02, 39.85, } m/z 476 \ [\text{M} + \text{H}]^+ \); Phe \( \text{55.12, 68.25, } m/z 460 \ [\text{M} + \text{H}]^+ \); Ala \( \text{43.27, 48.53, } m/z 384 \ [\text{M} + \text{H}]^+ \); Leu \( \text{47.93, 53.56, } m/z 426 \ [\text{M} + \text{H}]^+ \); Ile \( \text{48.74, 55.02, } m/z 426 \ [\text{M} + \text{H}]^+ \). 

3.6. Cancer cell growth inhibition assay

Three cancer cell lines were used in this assay, namely lung carcinoma (A549, ATCC CCL-185), colorectal carcinoma (HT29, ATCC HTB-38) and breast adenocarcinoma cell (MDA-MB-231, ATCC HTB-26). The cancer cell lines were obtained from American Type Culture Collection (ATCC). The cell lines were maintained in RPMI medium supplemented with 10% foetal calf serum, 2 mM l-glutamine and 100 U/mL penicillin and streptomycin, at 37 °C and 5% CO\(_2\). Triplicate cultures were incubated for 72 h in the presence or absence of test compounds (at 10 concentrations ranging from 10 to 0.0026 μg/mL). For quantitative estimation of cancer cell growth inhibition, the colorimetric sulforhodamine B (SRB) method was used (Skehan et al. 1990). Briefly, the cells were washed twice with PBS, fixed for 15 min in 1% glutaraldehyde solution, rinsed twice in PBS and stained in 0.4% SRB solution for 30 min at room temperature. The cells were then rinsed several times with 1% acetic acid solution and air-dried. SRB was then extracted in 10 mM trizma base solution and the absorbance measured at 490 nm. Results are expressed as GI50, the concentration that causes 50% inhibition in cell growth after correction for cell count at the start of the experiment (NCI algorithm). Doxorubicin and DMSO (solvent) were used as the positive and negative controls in this assay. Prism 3.03 from GraphPad was used for the statistical analysis of the cell growth inhibition results.

4. Conclusion

In conclusion, investigation of the cytotoxic fraction of the organic extract of the Red Sea sponge *Callyspongia* species afforded the new peptide, callyptide A (1). Its structure was determined by extensive 1D and 2D NMR (COSY, HSQC and HMBC) studies and high-resolution mass spectral determination. The configuration of the amino acids was determined by Marfey’s analysis. Callyptide A was found to exhibit cytotoxic activity when tested against different cancer cell lines. It showed moderate cancer growth inhibition activities against these cancer cell lines.

Acknowledgments

This project was funded by the Deanship of Scientific Research (DSR), King Abdulaziz University, Jeddah, under grant no. (40 / 141 / 1434). The authors, therefore, acknowledge with thanks DSR technical and financial support. We would like to thank Dr. Rob van Soest for the taxonomic identification of the sponge material.

Disclosure statement

No potential conflict of interest was reported by the authors.
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