Total synthesis of five proline-enriched cyclic heptapeptides from the marine sponge *Stylissa carteri*

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Total synthesis of five proline-enriched cyclic heptapeptides from the marine sponge *Stylissa carteri*

Yulei Li[a,b] , Qi Chang[a,b], Minghao Wu[a] , Xia Zhao [a,b] *

[a] *Key Laboratory of Marine Drugs, Ministry of Education, School of Medicine and Pharmacy, Ocean University of China, Qingdao 266003, China*
[b] *Laboratory for Marine Drugs and Bioproducts of Qingdao National Laboratory for Marine Science and Technology, Qingdao 266237, China*

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ABSTRACT

The total synthesis of five naturally occurring cyclic proline-enriched heptapeptides from the marine sponge *Stylissa carteri* was reported. The five cyclic heptapeptides were synthesized by applying a two-step solid-phase/solution synthesis strategy. The linear heptapeptides were assembled by standard Fmoc chemistry on 2-chlorotrityl chloride resin, cleaved off-resin with acetic acid/trifluoroethanol/dichloromethane to keep side-chain protecting groups intact, and subsequently cyclization was achieved by a solution method. The final products were purified by a preparative RP-HPLC system, and their structures were characterized by HR-QTOF-MS NMR. The spectral data of synthetic peptides were found to be identical to that reported for the natural products.

1. Introduction

Marine organisms are excellent sources of biologically active natural products, which have been considered as promising resources for lead compounds or drug candidates.[1] Peptides are important bioactive natural products which are present in many marine species and have attracted increasing attention for their broad spectra of bioactivities, low toxicity and high specificity.[2] These marine peptides have high potential medicinal values and show a hopeful and brilliant prospect for treatment or prevention on various diseases. In 2010, the global sales of four of 60 peptide drugs on the market reached in excess of US $ 1 billion, and more than 500 peptides are under clinical development.[3,4] Cyclic peptides are one of the underexplored classes of bioactive peptides with a marine origin that have great promise in pharmaceutical areas. Unlike linear peptides, they are more stable in vivo, more lipophilic and membrane permeable, and show higher specificity for target receptors. These bioactive cyclic peptides may be considered as attractive targets for drug discovery and biomedical research.[5,6] Especially for the poly-proline cyclic peptides, proline residue can reduce their backbone flexibility, thus enhancing the selectivity and affinity for protein binding.[7] Recently, an emerging class poly-proline cyclic peptides have been mainly isolated from lower sea organisms, however, their contents are so low that it was not enough for drug development. What is more, they have been associated with strong anticancer and antimicrobial activities.[8] For further research of biological activity and structure-activity relationships, many researchers have focused on the synthesis of cyclic peptides, especially by solid-phase peptide synthesis method.[9] The five cyclic peptides (**Fig. 1**) are poly-proline cyclic heptapeptides from the marine sponge *Stylissa carteri*. [10] Among them, *Phakellistatin* 13 (**3**) was first reported in 2003 by zhong-hua wang and his colleagues as a secondary metabolite from the sponge Phakellia fusca Thiele. This compound was significantly cytotoxic against the human hepatoma BEL-7404 cell line with an $ED_{50}<10^{-2}$ μg/mL. [11] And *Hymenamides* C (**4**) and D (**5**) were described in 1993 by Kobayashi and his team from the okinawan marine sponge hymeniacidon sp.[12] Carteritins A (**1**) is a new cyclic heptapeptide exhibited cytotoxic activity against Hela, HCT116 and RAW264 cells with IC_{50} values of 0.70, 1.30 and 1.50 μM, respectively. The five cyclic heptapeptides have been previously isolated in very low yields (<1%) from the marine

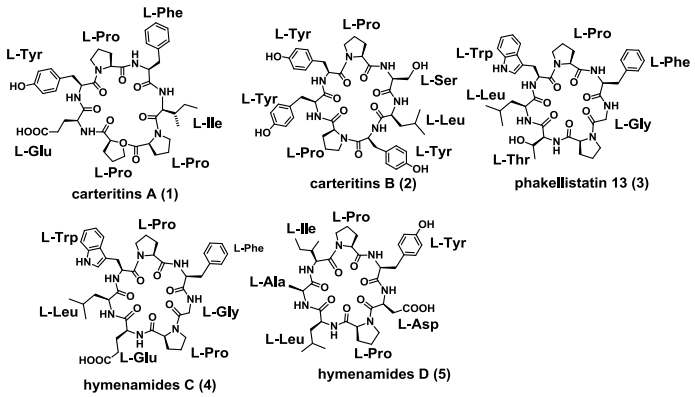
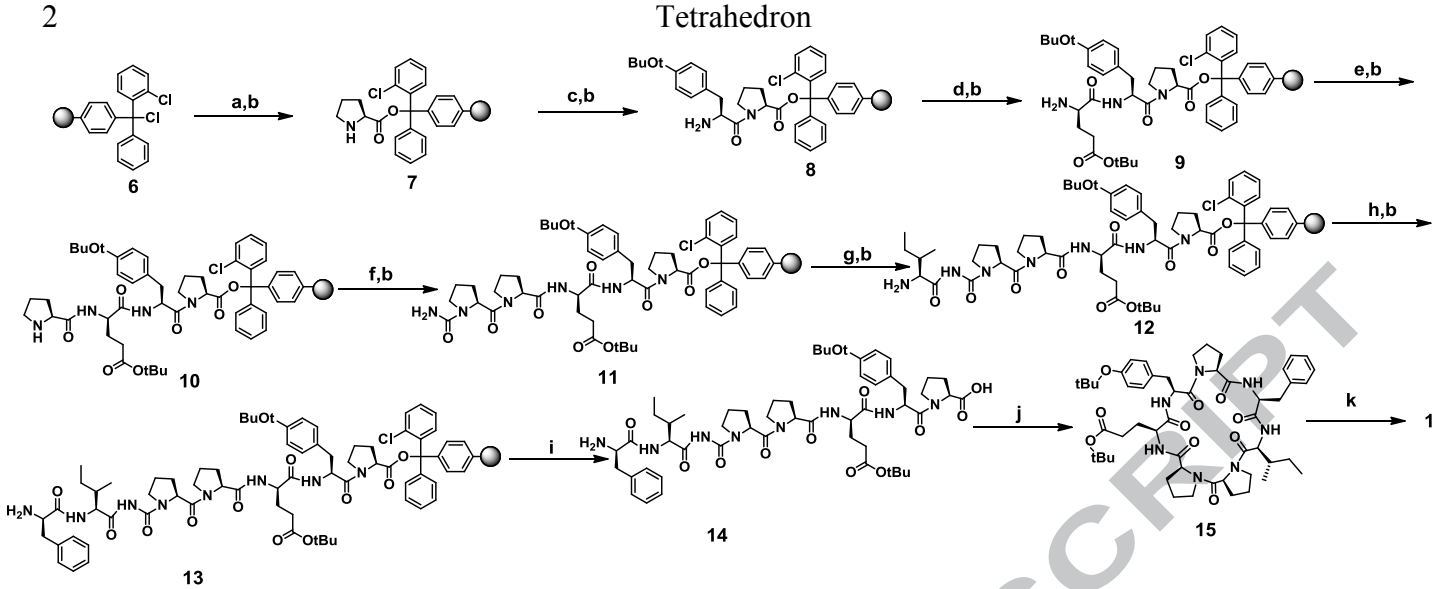


Fig 1. Structures of compound 1-5 (all the amino acids are L-configuration).

* Corresponding author: Tel & Fax: 86-532- 82031560. E-mail address: zhaoxia@ouc.edu.cn (X. Zhao).

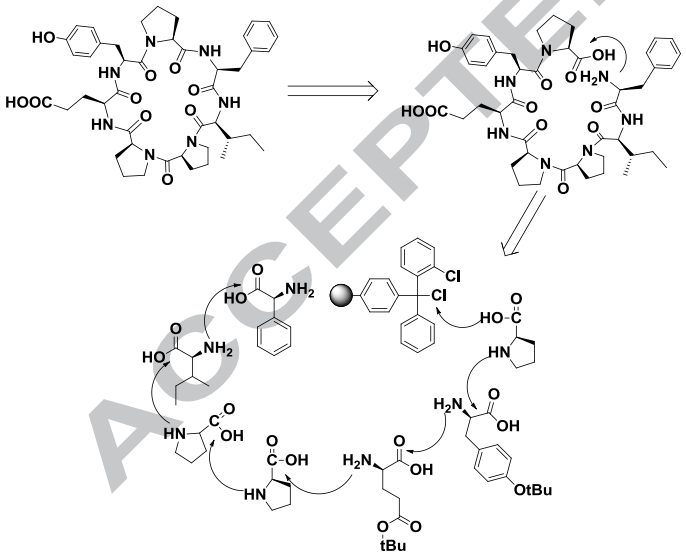


Scheme 2. Total synthesis of Carteritins A (**a**)Fmoc-Pro-OH, DMF, DCM, DIPEA,1h; (**b**) 20% piperidine in DMF, (2×5min); (**c**)Fmoc-Tyr(tBu)-OH, HCTU, DIPEA, DMF, 1h; (**d**) Fmoc-Glu(Trt)-OH, HCTU, DIPEA, DMF, 1h; (**e**)Fmoc-Pro-OH, HCTU, DIPEA, DMF, 1h; (**f**) Fmoc-Pro-OH, HCTU, DIPEA, DMF, 1h; (**g**)Fmoc-Ile-OH, HCTU, DIPEA, DMF, 1h; (**h**)Fmoc-Phe-OH, HCTU, DIPEA, DMF, 1h; (**i**) AcOH/TFE/DCM (1:2:16), 3 h; (**j**) PyBop, HOBt, DIPEA, NMP, DCM, 0 ^{o}C-r.t. overnight; (**k**) TFA/TIPS/ phenol/H_2O(88/5/5/2, v/v) for 1h with shaking

sponge Stylissa carteri.[10] It is obvious that the extraction procedure is very difficult and has an extremely low yield, which greatly hinders further biological activity and development, so chemical synthesis becomes of great importance. As a result, these cyclic heptapeptides were obtained by combining solid-phase method with conventional solution method for its further biological studies in this paper.

Results and discussion

Retrosynthetic strategy



Scheme1. Retrosynthetic analysis of Carteritins A

Scheme 1 describes a retrosynthetic method to obtain Carteritins A, and the method can be applied to the retrosynthetic analysis of Carteritins B, Phakellistatin 13, hymenamides C and D. It is well known that the head-to-tail connected cyclic peptides can be easily synthesized in good purity using standard procedures and orthogonally protected amino acid residues. However, the cyclization step is critical and essentially depended on the peptide sequence, structural constraint, and the resulting ring size.[13,14,15] These peptides are poly-proline cyclic heptapeptides. According to our previous experience in synthesizing natural cyclic heptapeptides stylissatin A,[16] we found that the residues of Pro and the beta location of carbon which is not replaced are the favourable points of cyclization to get the target compounds. So the point of cyclization Carteritins A , Carteritins B, Phakellistatin 13, hymenamides C and D is Pro - Phe, Pro - Tyr, Pro - Phe, Pro - phe, and Pro – Leu, respectively, to obtain the linear precursor with Pro as the C terminal amino acid, and another residue as the N-terminal amino acid. The linear precursors were synthesized directly by standard solid-phase peptide synthesis procedures, using a 9-fluorenylmethyloxycarbonyl/tert-butoxycarbonylation(Fmoc/Boc) protecting scheme and 2-chlorotrityl chloride resin (CLTR-Cl) as a solid support. The cyclization of the linear precursors was prepared in solution. The final crude cyclic heptapeptides were purified by a preparative RP-HPLC system to give the target compounds.

Representative total synthesis of Carteritins A

Scheme 2 shows the total synthetic process of Carteritins A, including synthesis of the linear heptapeptide, cyclization and side-chain deprotection. The synthesis of the linear heptapeptide **13** was performed in a peptide synthesizer under anhydrous condition. The first amino acid Fmoc-Pro-OH was loaded on the 2-Cl-trityl-Cl resin (1 mmol/g) after treatment by N, N-Diisopropylethylamine (DIPEA) (540.0 μL) in a mixed solution of N, N-Dimethylformamide (DMF) (5 mL) and dichloromethane (DCM) (5 mL) for 1 h, then the solvent was drained and the resin was washed sequentially with DMF, DCM and MeOH (3-5mL). The Pro-linked resin was then used for construction of the full length peptide. The protocols employed were as follows: deprotection of Fmoc with 20% piperidine in DMF (15 min) and peptide coupling using 5 eq. of amino acid, 4.5 eq. of O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium-hexafluor-ophosphate (HCTU) and 10 eq. of DIPEA. All coupling reactions were performed 1 h at room temperature. In the synthesis of **8-13**, amino acids Fmoc-Tyr(tBu)-OH, Fmoc-Glu(tBu)-OH, Fmoc-Pro-OH, Fmoc-Pro-OH, Fmoc-Ile-OH, Fmoc-Phe-OH were sequentially installed to construct the linear peptide. To cleave the linear peptide from the solid phase with intact side chain protecting groups, the resin was treated with AcOH/TFE/DCM (1:2:16, v/v) at 35 ^{o}C with shaking for 3 h. The synthesis of the

linear heptapeptide **14** yielded a side-chain protected linear precursor in 70% of overall yield. By HPLC and ESI-MS analysis, the linear hexapeptide **14** was found to be obtained in high purity and used directly for subsequent cyclization without further purification. Cyclization was performed in dry DCM with the liner peptide concentration of 0.5 mg/mL at -5°C. Benzotriazol-1-yl-oxytripyrrolidinophosphonium-hexafluorophosphate(PyBOP) and 1-hyd-roxy-benzotriazole (HOBT) were chosen as the coupling reagents. The crude peptide was initially purified by a Sephadex LH-20 column. The deprotection of **15** was performed by using TFA/TIPS/Phenol/H_2O (88/5/5/2, v/v) with shaking for 3h, and then the crude cyclic peptide was precipitated at 0 °C with cool diethyl ether. The final crude cyclic peptide was purified by a preparative reversed-phase HPLC to give the target compound as a white solid powder, with a total yield of 21% and HPLC purity over 98%. The HR-QTOF-MS, ^{1}H and ^{13}C NMR spectroscopic data (Supporting Information) of synthetic **1** were in agreement with those of the natural product.

Total synthesis of Carteritins B, Phakellistatin 13, hymenamides C and D

As a result, the naturally occurring cyclic peptide Carteritins A (**1**) was successfully synthesized by a two-step solid-phase/solution synthesis strategy. Then the method was also applied to the synthesis of its close structural analogues, Carteritins B(**2**), Phakellistatin 13(**3**), hymenamides C(**4**) and D(**5**). All the peptides were obtained about total yields of 20%, and their HR-QTOF-MS, ^{1}H and ^{13}C NMR spectroscopic data , (Supporting Information) were in agreement with those of the natural products. The optical rotation value of the final products： Carteritins A (1): $[\alpha]^{20}_D$ -120° (c 0.33, MeOH); Carteritins B (2): $[\alpha]^{20}_D$ -98° (c 0.30, MeOH); Phakellistatin 13(3): $[\alpha]^{20}_D$ -126° (c 0.09, MeOH); Hymenamides C (4): $[\alpha]^{20}_D$ -138° (c 0.40, MeOH); Hymenamides D (5): $[\alpha]^{20}_D$ -87° (c 0.15, MeOH);

Cytotoxicity assays

The synthesized cyclic heptapeptides **1–5** were evaluated for cytotoxicities against human cervical cancer cell line (HeLa), human colon cancer cell line (HCT116), and murine macrophage line (RAW264.7), human hepatoma(BEL-7402), human lung carcinoma cell (A-549). All the products showed cytotoxicities against the five cell lines with IC_{50} values of above 25μM, in despite of the HR-QTOF-MS, ^{1}H and ^{13}C NMR spectroscopic data (Supporting Information) of these targeted compounds were identical with those of the natural products. Several research groups have reported that although the synthetic proline-containing cyclic peptides are chemically equivalent to their natural counterparts, their biological activities are deviant in some cases.[1] It may be attributed to the conformational difference or the presence of biologically active impurity in the naturally isolated material, and/or the configuration was changed in the synthesis. The same reasons may explain the variation in biological activity of the synthetic and the natural compounds in our experiments.

Spectroscopic data of Carteritins A and B

^{1}H NMR, ^{13}C NMR, HR-QTOF-MS Data of Carteritins A:
Yield:21%. **^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm, J/Hz):**8.01(1H, d, *J* = 5.0Hz), 7.82(1H, d, *J* = 10.0Hz), 7.49(1H, d, *J* = 10.0Hz), 7.27(2H, t, *J* = 5.0Hz), 7.22(2H, t, *J* = 5.0Hz), 7.18(1H, t, *J* = 5.0Hz), 7.08(2H, t, *J* = 5.0Hz), 6.90(1H, t, *J* = 10.0Hz), 6.66(2H, t, *J* = 5.0Hz), 4.85(1H, t, *J* = 10.0Hz), 4.47(2H, td, *J* = 12.5,5.0Hz), 4.36(2H,q, *J* = 5.0Hz), 4.29(2H,d, *J* = 10.0Hz), 4.25(2H,t, *J* = 10.0Hz), 3.93(1H,t, *J* = 10.0Hz), 3.85(1H,m),3.76(1H, m), 3.47(1H, m), 3.35-3.25(4H, m), 2.93(1H, t, *J* = 15.0Hz), 2.45(1H, d, *J* = 15.0Hz), 2.11(3H, m), 2.03-1.97(4H, m), 1.88(2H, m), 1.84-1.71(4H, m), 1.60(2H, m), 1.49(2H, m), 1.23(1H, s), 1.03(1H, m), 0.90(3H, d, *J* = 5.0Hz), 0.76(3H, t, *J* = 5.0Hz)
^{13}C NMR (125MHz, DMSO-d_6, δ, ppm): 174.24, 172.31, 172.09, 171.91, 171.52, 170.77, 170.55, 168.20, 156.33, 138.99, 130.56, 129.08, 128.56, 127.58, 126.71, 115.42, 63.13, 60.76, 59.38, 56.18, 55.84, 54.40, 51.94, 47.32, 47.24, 47.22, 37.47, 36.53, 36.41, 32.32, 30.91, 28.89, 28.58, 27.32, 25.39, 24.69, 24.19, 22.29, 17.28, 12.33.
HR-QTOF-MS: $\mathbf{C_{44}H_{58}N_7O_{10}^+}$, Calcd. for, $(M+H)^+$, 844.4200, found, 844.4237, $\mathbf{C_{44}H_{57}N_7NaO_{10}^+}$ Calcd. for, $(M+Na)^+$, 866.4200, found, 866.4048.
^{1}H NMR, ^{13}C NMR, HR-QTOF-MS Data of Carteritins B:
Yield: 19.5%.**^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm, J/Hz):** 9.14(1H, s), 7.79(1H, d, *J* = 10.0Hz), 7.58(1H, d, *J* = 10.0Hz), 7.47(1H, d, *J* = 5.0Hz), 7.17(1H, d, *J* = 10.0Hz), 7.09(1H, d, *J* = 10.0Hz), 6.97(1H, d, *J* = 10.0Hz), 6.82(1H, d, *J* = 5.0Hz), 6.70(1H, d, *J* = 10.0Hz), 6.62(3H, m), 4.92(1H,m), 4.43(1H,m), 4.21(1H,m), 4.17(2H,m), 3.97(2H,m), 3.82(2H, m), 3.78(3H, m), 3.49(1H, d, *J* = 10.0Hz), 3.24(2H, m), 3.06(2H, m), 2.87(1H, dd, *J* = 10.0,15.0Hz), 2.81(1H, m), 2.74(1H, dd, *J* = 10.0,15.0Hz), 2.57(2H, m), 2.25(1H, m), 2.06(1H, m), 1.97(2H, m), 1.86(2H, m), 1.75(2H, m), 1.52(3H, m),1.34(2H, m), 1.26(1H, m), 1.03(1H, m), 0.94(3H, d, *J* = 5Hz), 0.90(3H, d, *J* = 5.0Hz),0.84(1H, d, *J* = 10.0Hz), 0.43(1H, m).
***^{13}C NMR* (125MHz, DMSO-d_6, δ, ppm):** 172.15, 171.59, 171.32, 170.72, 170.62, 170.41, 169.52, 156.59,156.50, 156.39, 131.09, 130.23, 129.78, 127.97, 127.92, 126.81, 115.89, 115.59, 115.29, 63.26, 61.46, 60.53, 57.53, 55.55, 54.15, 52.26, 52.01, 47.54, 46.45, 40.73, 37.65, 35.83, 30.86, 29.23, 25.61, 25.11, 23.97, 23.15, 22.49, 20.97.
HR-QTOF-MS:$\mathbf{C_{46}H_{58}N_7O_{10}^+}$, Calcd. for, $(M+H)^+$, 884.4100, found, 884.4171, $\mathbf{C_{46}H_{57}N_7NaO_{10}^+}$ Calcd. for, $(M+Na)^+$,906.4100, found, 906.3940.

Conclusions

An efficient solid-phase/solution strategy was developed to the total synthesis of the natural products Carteritins A, Carteritins B, Phakellistatin 13, hymenamides C and D from the marine sponge *Stylissa carteri*. The spectral data of synthetic peptides were found to be identical to that reported for the natural products. However, a difference in biological activity was noted, the synthetic peptides were found to be weakly cytotoxic against five cell lines (A-549, Hela, BEL-7402, HCT116 and RAW264.7). It may be attributed to conformational difference or the presence of biologically active impurity in the naturally isolated material, or configuration was changed in the synthesis.

Acknowledgments

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Supplementary Material

Supplementary data associated with this article can be found

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Graphical Abstract

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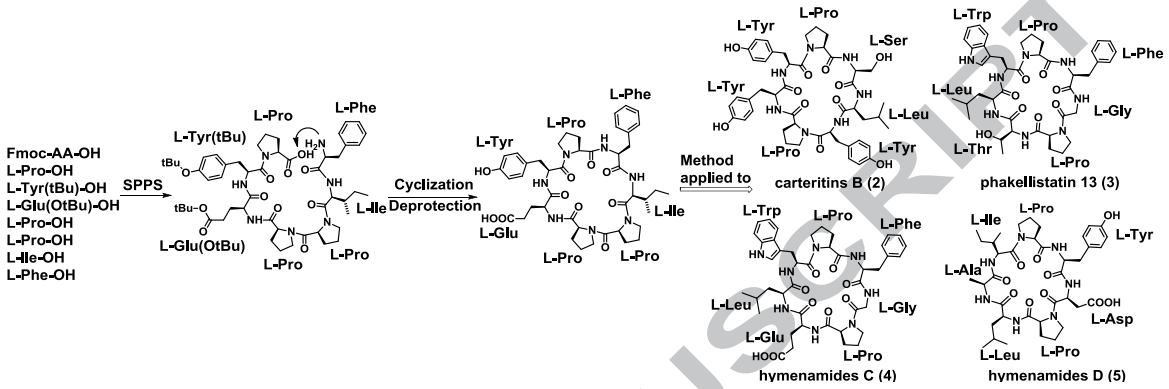

Highlights

• Five proline-enriched cyclic heptapeptides from marine sponge were synthesized totally.

• A two-step solid-phase/solution strategy was effective to cyclic peptides synthesis.

•Their structures were identical to those of natural products .