Synthesis, Cytotoxic and Antimicrobial Screening of a Proline-Rich Cyclopolypeptide

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Present study describes the first total synthesis of a cyclic heptapeptide, stylisin 1 (**8**) *via* coupling of tetrapeptide Boc-L-tyrosinyl-L-prolyl-L-leucyl-L-proline-OH and tripeptide L-phenylalanyl-L-isoleucyl-L-proline-OMe followed by cyclization of linear heptapeptide segment. Structure elucidation of synthesized cyclopeptide was done on basis of detailed spectral as well as elemental analysis. From the results of pharmacological screening, it was concluded that cyclopeptide **8** possessed moderate cytotoxicity against Dalton's lymphoma ascites (DLA) and Ehrlich's ascites carcinoma (EAC) cell lines with IC_{50} (inhibitory concentration, 50%) values of 10.6 and 14.6 μM. Furthermore, cyclopeptide **8** exhibited moderate to good antimicrobial activity against Gram −ve (negative) bacteria *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, dermatophytes and *Candida albicans* with minimum inhibitory concentration (MIC) of 6 μg/ml.

Key words total synthesis; cyclopolypeptide; marine sponge; stylisin 1; cytotoxicity; antimicrobial activity

Marine environment especially sponges are well known to produce a wide variety of biomedically useful agents that may act as lead compounds for drug development.[1—5] Among them, cyclopeptides having unique structures and a wide pharmacological profile[6] are emerged as vital organic congeners which may prove better candidates to overcome the problem of widespread increase of resistance towards conventional drugs. Marine sponge-derived natural cyclopolypeptides possess numerous pharmacological activities including antimicrobial,[7—9] anti-inflammatory,[10] anti-human immunodeficiency virus (HIV),[11,12] nematocidal,[13] cytotoxic,[14,15] antifouling,[16] antitubercular,[17] histone deacetylase, tryptase, thrombin, protein phophatases 1, 2A and superoxide generation inhibitory properties.[18—22] A natural cyclic heptapeptide, stylisin 1 has been isolated from the Jamaican sponge *Stylissa caribica* and its structure was elucidated on basis of correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond coherence (HMBC) and nuclear overhauser enhancement spectroscopy (NOESY) experiments followed by determination of absolute configuration of amino acids by Marfey's analysis method.[23]

Prompted by the medicinal properties of proline-rich cyclic peptides[24—27] as well as to obtain a natural bioactive peptide in good yield, present investigation was aimed at first total synthesis of natural peptide, stylisin 1 (**8**) employing disconnection strategy. The potential cytotoxic, antibacterial and antifungal activities of the synthesized peptide were also evaluated.

Chemistry To carry out the synthesis of stylisin 1 (**8**), the cyclic heptapeptide molecule was split into three dipeptide units Boc-L-tyrosinyl-L-proline-OMe (**1**), Boc-L-leucyl-L-proline-OMe (**2**), Boc-L-phenylalanyl-L-isoleucine-OMe (**3**) and single amino acid unit L-proline-OMe·HCl (**4**). The required dipeptide units **1**—**3** were prepared by coupling of Boc-amino acids *viz.* Boc-L-tyrosine-OH, Boc-L-leucine-OH and Boc-L-phenylalanine-OH with corresponding amino acid methyl ester hydrochlorides such as L-proline-OMe·HCl and L-isoleucine-OMe·HCl using DCC as coupling agent.[28]
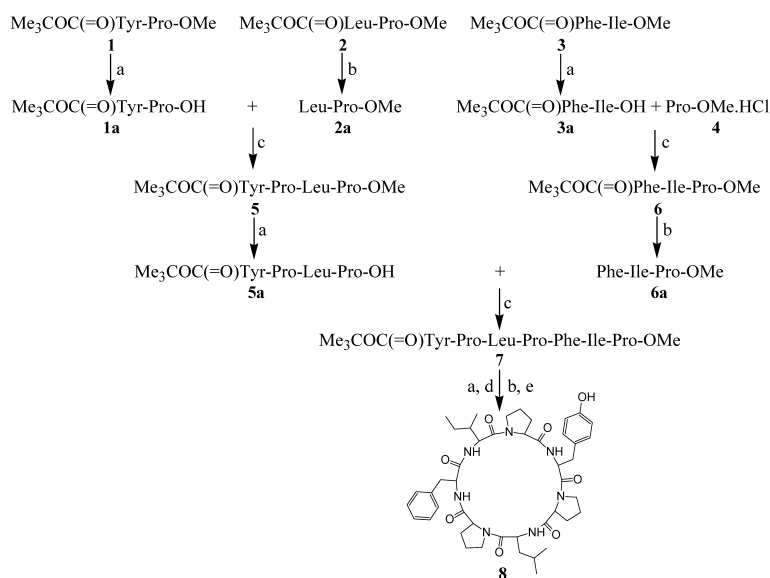
Dipeptide **1** after deprotection at carboxyl end, was coupled with dipeptide **2** deprotected at amino terminal, to get the tetrapeptide unit Boc-L-tyrosinyl-L-prolyl-L-leucyl-L-proline-OMe (**5**). Similarly, ester group of dipeptide **3** was removed by alkaline hydrolysis with LiOH and deprotected peptide was coupled with compound **4** to get the tripeptide unit Boc-L-phenylalanyl-L-isoleucyl-L-proline-OMe (**6**). After removal of ester and Boc groups of tetrapeptide **5** and tripeptide **6**, deprotected peptide units were coupled with each other to get linear heptapeptide unit Boc-L-tyrosinyl-L-prolyl-L-leucyl-L-prolyl-L-phenylalanyl-L-isoleucyl-L-proline-OMe (**7**). The ester group of linear fragment was removed using LiOH and *p*-nitrophenyl or pentafluorophenyl (pnp or pfp) ester groups were introduced. The Boc-group was removed using trifluoroacetic acid (CF_3COOH) and deprotected linear fragment was now cyclized by keeping the whole contents at 0 °C (7 d) in presence of catalytic amount of triethylamine (TEA)/*N*-methylmorpholine (NMM)/pyridine to get cyclic product **8** (Chart 1).

Structure of the newly synthesized cyclic heptapeptide as well as intermediates di/tri/tetrapeptides were confirmed by infrared (IR), nuclear magnetic resonance (^{1}H-NMR) and elemental analysis. In addition, ^{13}C-NMR and mass spectra were recorded for the linear as well as cycloheptapeptide.

Cytotoxicity Activity The synthesized linear and cyclic heptapeptides **7**, **8** were subjected to short term *in vitro* cytotoxicity study[29] against Dalton's lymphoma ascites (DLA) and Ehrlich's ascites carcinoma (EAC) cell lines at 62.5—3.91 μg/ml using 5-fluorouracil (5-FU) as reference compound. Activity was assessed by determining the percentage inhibition of DLA and EAC cells. IC_{50} values were determined by graphical extrapolation method. The cytotoxic activity data of synthesized compounds **7**, **8** is tabulated in Table 1.

Antibacterial and Antifungal Activity The synthesized linear and cycloheptapeptides **7**, **8** were also evaluated for their antimicrobial activity[30] against six bacterial strains *Corynebacterium pyogenes*, *Staphylococcus aureus*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*

Me₃COC(=O)Tyr-Pro-OMe   Me₃COC(=O)Leu-Pro-OMe   Me₃COC(=O)Phe-Ile-OMe
1   **2**   **3**

Me₃COC(=O)Tyr-Pro-OH   +   Leu-Pro-OMe   Me₃COC(=O)Phe-Ile-OH + Pro-OMe.HCl
1a   **2a**   **3a**   **4**

Me₃COC(=O)Tyr-Pro-Leu-Pro-OMe   Me₃COC(=O)Phe-Ile-Pro-OMe
5   **6**

Me₃COC(=O)Tyr-Pro-Leu-Pro-OH   +   Phe-Ile-Pro-OMe
5a   **6a**

Me₃COC(=O)Tyr-Pro-Leu-Pro-Phe-Ile-Pro-OMe
7

8

Reaction conditions:
a = LiOH:H₂O (1:1), rt, 1 h; b = CF₃COOH, CHCl₃, RT, 1 h;
c = EDC.HCl/DIPC, TEA/NMM, THF/DMF, rt, 24—36 h;
d = DIPC, pnp/pfp, rt, 12 h; e = TEA/NMM/pyridine, 7 d, 0 °C

Chart 1.   Synthetic Route to Stylisin 1 (**8**)

Table 1.   Cytotoxic Activity Data against Pathogenic Cell Lines

Compd.	Cell lines	
	DLA cells	EAC cells
7	14.8[a]	20.3
8	10.6	14.6
Control	—	—
5-Fluorouracil	37.4	90.6

a) IC₅₀ values in μM.

and *Escherichia coli*, and five fungal strains *Candida albicans*, *Aspergillus niger*, *Ganoderma* sp., *Microsporum audouinii* and *Trichophyton mentagrophytes* at 25—6 μg/ml. Minimum inhibitory concentration (MIC) values of test compounds were determined by tube dilution technique. The solvents dimethylformamide (DMF) and dimethylsulphoxide (DMSO) were used as negative controls, and gatifloxacin and griseofulvin were used as antibacterial and antifungal standards. The antimicrobial activity shown by synthesized compounds **7**, **8** towards various microbes is recorded in Tables 2 and 3.

Results and Discussion

Synthesis of stylisin 1 (**8**) was carried out successfully with good yield (>85%) utilizing three different bases for cyclization of linear heptapeptide fragment. Presence of $M^+ + 1$ ion peak at *m/z* 828 corresponding to the molecular formula $C_{45}H_{61}N_7O_8$ in mass spectra of **8**, along with other fragment ion peaks resulting from cleavage at 'Pro–Leu' amide bond level, showed exact sequence of attachment of all the seven amino acid moieties in a chain. Presence of peaks at 618, 590 and 261 mu in mass spectrum of **8** further indicated fragmentation at 'Pro–Phe' and 'Pro–Tyr' amide bond levels. This fact was further confirmed by presence of additional fragment ion peaks at 521, 148 and 374, 164 mu

which are formed only if peptide bond between 'Pro–Phe' and 'Pro–Tyr', breaks. In addition, elemental analysis of **8** afforded average values (± 0.03) strictly in accordance to the molecular composition.

Synthesized cyclopeptide **8** possessed moderate cytotoxic activity against DLA and EAC cell lines with IC₅₀ values of 10.6 and 14.6 μM respectively, in comparison to 5-FU (IC₅₀ values, 37.4 and 90.6 μM). Comparison of antimicrobial screening data suggested that **8** exhibited good antibacterial activity against pathogenic microbes *K. pneumoniae* and *P. aeruginosa* with MIC value of 6 μg/ml, in comparison to standard drug, gatifloxacin. The mechanism of antibacterial action of **8** may be interference with the cell membrane functions by penetrating the outer membrane of bacteria through porin channels. Moreover, **8** displayed moderate level of activity against dermatophytes *M. audouinii* and *T. mentagrophytes*, and pathogenic *Candida albicans* with MIC values of 6 μg/ml. However, **8** displayed no significant activity against neither Gram +ve (positive) bacteria nor pathogenic *Ganoderma* sp. and *A. niger*.

In addition, analysis of pharmacological activity data revealed that cycloheptapeptide **8** displayed more bioactivity against pathogenic microbes and cell lines when compared to its linear form **7**. This is possibly because, cyclization of peptides reduces the degree of freedom for each constituent within the ring and thus substantially leads to reduced flexibility, increased potency and selectivity of cyclic peptides. Further, inherent flexibility of linear peptides leads to different conformations which can bind to more than one receptor molecules, resulting in undesirable adverse effects.[31,32]

Conclusions

First total synthesis of natural peptide, stylisin 1 (**8**) was accomplished with good yield *via* coupling reactions utilizing different carbodiimides. Diisopropylcarbodiimide (DIPC)/TEA coupling method proved to be yield-effective,

Table 2. Antibacterial Activity Data against Pathogenic Bacteria

Compd.	Bacterial strains					
	C. pyogenes	S. aureus	B. subtilis	K. pneumoniae	P. aeruginosa	E. coli
7	25[a]	12.5	25	6	6	12.5
8	25	12.5	25	6	6	12.5
Control	—	—	—	—	—	—
Gatifloxacin	12.5	6	12.5	6	6	6

a) MIC values in μg/ml.

Table 3. Antifungal Activity Data against Pathogenic Fungi

Compd.	Fungal strains				
	C. albicans	A. niger	Ganoderma sp.	M. audouinii	T. mentagrophytes
7	6[a]	25	—	6	6
8	6	25	—	6	6
Control	—	—	—	—	—
Griseofulvin	6	12.5	6	6	6

a) MIC values in μg/ml.

in comparison to methods utilizing 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide hydrochloride (EDC·HCl) and TEA or NMM, providing 10—12% additional yield. Pentafluorophenyl ester was proved to be better for the activation of acid functionality of linear heptapeptide unit when compared to *p*-nitrophenol. NMM was found to be a good base for intramolecular cyclization of linear peptide fragment in comparison to TEA and pyridine. Synthesized cycloheptapeptide displayed moderate cytotoxicity as well as antimicrobial activity against *K. pneumoniae* and *P. aeruginosa*, dermatophytes and *C. albicans*. In comparison, Gram −ve bacteria were found to be more sensitive than Gram +ve bacteria towards the newly synthesized peptide. Cyclopolypeptide **8** showed sufficient promise as antimicrobial and cytotoxic agent and should be subjected for further toxicity tests.

Experimental

Materials and Methods All the reactions requiring anhydrous conditions were conducted in flame dried apparatus. Melting points were determined using a Jindal Scientific melting point apparatus (Jindal, Delhi, India) by open capillary method and are uncorrected. IR spectra were recorded on FTIR-8400S fourier transform infrared spectrophotometer (Shimadzu, Kyoto, Japan) using a thin film supported on KBr pellets for solids and CHCl$_3$ as solvent for intermediate semisolids. ^{1}H- and ^{13}C-NMR spectra were recorded on Bruker AC 300 spectrometer at 300 MHz (Brucker, IL, U.S.A.) using CDCl$_3$ or acetone-d_6 as solvent and tetramethylsilane as internal standard. Mass spectra were recorded on JMS-DX 303 Mass spectrometer (Jeol, Tokyo, Japan) operating at 70 eV using fast atom bombardment technique. Elemental analyses of cyclopeptide as well as intermediates were performed on Vario EL III elemental analyzer (Elementar Vario EL III, Hanau, Germany). Optical rotation of the synthesized peptides was measured on Optics Technology automatic polarimeter (OpticsTech, Delhi, India) in a 2 dm tube at 25 °C using sodium lamp and methanol as solvent. Purity of synthesized cyclopeptide was checked by TLC on precoated silica gel G plates (Kieselgel 0.25 mm, 60G F$_{254}$, Merck, Germany) utilizing CHCl$_3$: AcOH : H$_2$O as developing solvent and brown spots were detected on exposure to iodine vapours in a tightly closed chamber.

General Procedure for the Preparation of Linear Tri/Tetrapeptide Segments L-Amino acid methyl ester hydrochloride or dipeptide methyl ester (0.01 mol) was dissolved in DMF (25 ml). To this, TEA or NMM (0.021 mol) was added at 0 °C and the reaction mixture was stirred for 15 min. Boc-dipeptide (0.01 mol) in DMF (25 ml) and EDC·HCl or DIPC (0.01 mol) were added with stirring. Stirring was first done for 1 h at 0—

5 °C and then further for 24 h at room temperature. After the completion of reaction, the reaction mixture was diluted with equal amount of water. The precipitated solid was filtered, washed with water and recrystallized from a mixture of chloroform and petroleum ether (bp 40—60 °C) followed by cooling at 0 °C to get the title compounds.

Deprotection of Tetrapeptide Unit at Carboxyl Terminal To a solution of the tetrapeptide **5** (6.0 g, 0.01 mol) in tetrahydrofuran (THF) : H$_2$O (1 : 1, 36 ml), 0.36 g (0.015 mol) of LiOH was added at 0 °C. The mixture was stirred at room temperature for 1 h and then acidified to pH 3.5 with 1 N H$_2$SO$_4$. The aqueous layer was extracted with Et$_2$O (3×25 ml). The combined organic extracts were dried over anhydrous Na$_2$SO$_4$ and concentrated under reduced pressure. The crude product was finally crystallized from methanol and ether to get pure deprotected compound.

Deprotection of Tripeptide Unit at Amino Terminal Tripeptide **6** (4.9 g, 0.01 mol) was dissolved in CHCl$_3$ (15 ml) and treated with 2.28 g (0.02 mol) of CF$_3$COOH. The resulting solution was stirred at room temperature for 1 h, washed with saturated NaHCO$_3$ solution (25 ml). The organic layer was dried over anhydrous Na$_2$SO$_4$ and concentrated under reduced pressure. The crude product was purified by crystallization from CHCl$_3$ and petroleum ether (bp 40—60 °C) to get pure deprotected compound.

Procedure for the Synthesis of Linear Heptapeptide Unit Compound **6a** (3.9 g, 0.01 mol) was dissolved in THF (35 ml). To this solution, TEA (2.8 ml, 0.021 mol) was added at 0 °C and the resulting mixture was stirred for 15 min. 5.9 g (0.01 mol) of compound **5a** was dissolved in THF (35 ml) and DIPC (1.26 g, 0.01 mol) was added to above mixture with stirring. Stirring was continued for 36 h, after which the reaction mixture was filtered and the filtrate was washed with 5% NaHCO$_3$ and saturated NaCl solutions (30 ml each). The organic layer was dried over anhydrous Na$_2$SO$_4$, filtered and evaporated in vacuum. The crude product was recrystallized from a mixture of chloroform and petroleum ether (bp 40—60 °C) followed by cooling at 0 °C.

Synthesis of the Cyclic Heptapeptide (8) To synthesize stylisin 1 (**8**), linear heptapeptide unit **7** (4.8 g, 0.005 mol) was deprotected at carboxyl end using LiOH (0.18 g, 0.0075 mol) to get Boc-L-tyrosinyl-L-prolyl-L-leucyl-L-prolyl-L-phenylalanyl-L-isoleucyl-L-proline-OH. The deprotected heptapeptide unit (4.73 g, 0.005 mol) was now dissolved in CHCl$_3$ (50 ml) at 0 °C. To this solution, 0.0067 mol of *p*-nitrophenol or pentafluorophenol (0.94 g or 1.23 g) and DIPC (0.63 g, 0.005 mol) were added and stirring was done at room temperature for 12 h. The reaction mixture was filtered and the filtrate was washed with 10% NaHCO$_3$ solution (3×25 ml) and finally washed with 5% HCl (2×30 ml) to get the corresponding *p*-nitrophenyl or pentafluorophenyl ester Boc-L-tyrosinyl-L-prolyl-L-leucyl-L-prolyl-L-phenylalanyl-L-isoleucyl-L-proline-Opnp or Boc-L-tyrosinyl-L-prolyl-L-leucyl-L-prolyl-L-phenylalanyl-L-isoleucyl-L-proline-Opfp. To this compound (4.27 g or 4.45 g, 0.004 mol) dissolved in CHCl$_3$ (35 ml), CF$_3$COOH (0.91 g, 0.008 mol) was added, stirred at room temperature for 1 h and washed with 10% NaHCO$_3$ solution (2×25 ml). The organic layer was dried over anhydrous

Na$_2$SO$_4$ to get L-tyrosinyl-L-prolyl-L-leucyl-L-prolyl-L-phenylalanyl-L-isoleucyl-L-proline-Opnp or L-tyrosinyl-L-prolyl-L-leucyl-L-prolyl-L-phenylalanyl-L-isoleucyl-L-proline-Opfp which was dissolved in CHCl$_3$ (25 ml) and TEA/NMM/pyridine (2.8 ml or 2.21 ml or 1.61 ml, 0.021 mol) was added. Then, whole contents were kept at 0 °C for 7 d. The reaction mixture was washed with 10% NaHCO$_3$ (3×25 ml) and 5% HCl (2×30 ml) solutions. The organic layer was dried over anhydrous Na$_2$SO$_4$ and crude cyclized product was crystallized from CHCl$_3$/n-hexane to get pure cyclic product.

Cyclo (L-Tyrosinyl-L-prolyl-L-leucyl-L-prolyl-L-phenylalanyl-L-isoleucyl-L-prolyl) (**8**): Yield: 3.64 g (88%, NMM), 3.1 g (75%, TEA), 2.86 g (69%, C$_5$H$_5$N). mp 225 °C (dec). R_f 0.68 (CHCl$_3$: AcOH : H$_2$O, 3 : 2 : 5). ^{1}H-NMR (acetone-d_6) δ: 0.81 (1H, m), 0.94 (3H, t, $J=7.8$ Hz), 0.99 (6H, d, $J=6.3$ Hz), 1.02 (3H, d, $J=5.85$ Hz), 1.37 (3H, m), 1.75 (4H, m), 1.83 (2H, m), 1.89 (2H, t, $J=7.9$ Hz), 2.59 (4H, m), 2.67 (6H, m), 3.23 (2H, t, $J=7.3$ Hz), 3.27 (2H, t, $J=7.25$ Hz), 3.32 (2H, t, $J=7.3$ Hz), 3.87 (1H, t, $J=6.85$ Hz), 3.92 (1H, t, $J=6.9$ Hz), 3.96 (1H, t, $J=6.9$ Hz), 4.20 (1H, m), 4.40 (1H, m), 5.13 (1H, m), 5.25 (1H, dd, $J=5.9$, 4.25 Hz), 5.97 (1H, br s), 6.80 (2H, dd, $J=8.75$, 4.2 Hz), 6.92 (2H, dd, $J=8.6$, 5.25 Hz), 6.96 (2H, dd, $J=8.55$, 4.9 Hz), 7.02 (1H, t, $J=6.3$ Hz), 7.25 (2H, tt, $J=6.8$, 4.45 Hz), 9.05 (1H, br s), 9.22 (1H, br s), 9.72 (1H, br s), 9.88 (1H, br s). ^{13}C-NMR δ: 10.2, 17.7, 22.1, 21.5, 22.7, 23.1 (2C), 24.4, 26.2, 33.0, 31.5, 34.7, 39.0, 36.8, 43.5, 42.2, 46.4, 48.3, 49.1, 51.7, 52.1, 55.8, 55.2, 56.9, 58.1, 58.5, 128.0, 128.9 (2C), 129.6 (2C), 130.3 (2C), 132.6 (2C), 134.7, 139.1, 154.7, 168.5, 169.3, 169.8, 170.1, 172.5, 173.2, 174.8. IR (KBr) cm^{-1}: 3377, 3128, 3125, 3120, 3059, 3055, 2999, 2995, 2989, 2964, 2927, 2921, 2869, 2847, 2842, 1675, 1671, 1666, 1663, 1646, 1640, 1586, 1582, 1477, 1474, 1538, 1532, 1529, 1382, 1365, 1225, 864, 821, 713, 698. MS m/z: 829, 828 (M$^+$+1), 801, 731, 618, 590, 568, 540, 521, 471, 443, 374, 358, 330, 261, 211, 183, 164, 148, 136, 114, 120, 107, 93, 91, 86, 70, 65, 57, 56, 43, 42, 29, 17, 15. $[\alpha]_D$ −44.7 ($c=0.2$, MeOH) (−44.9 for natural stylisin 1). *Anal.* Calcd for C$_{45}$H$_{61}$N$_7$O$_8$: C, 65.28; H, 7.43; N, 11.84. Found: C, 65.25; H, 7.43; N, 11.85.

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