

Synthesis, Characterization, and Biological Activity Studies on Fanlizhicyclopeptide A

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Abstract

The synthesis of a proline-rich cyclic heptapeptide, fanlizhicyclopeptide A (8), previously isolated from the fruits of *Annona squamosa* (sugar-apples), is described via coupling of tetrapeptide L-prolyl-L-tyrosyl-L-leucyl-L-proline methyl ester with tripeptide Boc-glycyl-L-valyl-L-proline followed by cyclization of the linear fragment having seven amino acid units. Structure of the synthesized cyclooligopeptide was confirmed by the means of chemical and spectroscopic methods including FTIR, ¹H NMR, ¹³C NMR, FABMS and further, subjected to the anthelmintic, antibacterial and the antifungal activity studies. Bioactivity results indicated that the newly synthesized cyclic peptide displayed potent anthelmintic activity against the three earthworm species *Megascolex konkanensis*, *Pontoscotex corethruses* and *Eudrilus eugeniae* at 2 mg/mL and remarkable anti-dermatophytic activities against *Trichophyton mentagrophytes* and *Microsporum audouinii* at concentration of 6 µg/mL.

Keywords: Fanlizhicyclopeptide A; Cyclic heptapeptide; Solution-Phase synthesis; Macrocyclization; Pharmacological activity.

Introduction

Ever since ancient times, in search for rescue for their disease, the people looked for drugs in nature. Many of the powerful drugs were used in modern medicines originated in plants. Herbal medicines have a strong traditional or conceptual base and the potential to be useful as drugs in terms of safety and effectiveness leads to treating different diseases (1). Today's plant-based drugs treat a range of diseases, from headaches to cancer. Awareness of medicinal plants usage is a result of the many years of struggles against

illnesses due to which man learned to pursue drugs in barks, seeds, fruit bodies, and other parts of the plants (2). Nowadays, cyclooligopeptides from plant fruits have received more attention of researchers and scientists (3-6) because of their complex molecular structures and a wide spectrum of associated pharmacological activities like anti-inflammatory activity (7), vasorelaxant activity (8), cell growth inhibitory activity (9), and immunosuppressive activity (10). A natural cyclic heptapeptide, fanlizhicyclopeptide A was isolated from the fruits of *Annona squamosa* (sugar-apples) and its structure was elucidated by ESI MS/MS, 1D and 2D NMR data, and chemical degradation (11).

Keeping in view broad range of pharmacological activities possessed by

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cycloligopeptides (12-15) and to obtain a natural cyclic peptide in good yield in the laboratory, the present study aimed toward the solution-phase synthesis, structure elucidation and the screening of a proline-rich cycloheptapeptide, fanlizhicyclopeptide A for the anthelmintic, antibacterial and antifungal potential.

Experimental

Chemistry

Melting point was determined by the open capillary method and is uncorrected. IR spectra were recorded using an FTIR-8400S fourier transform spectrophotometer (Shimadzu, Kyoto, Japan). ¹H NMR and ¹³C NMR spectra were recorded on a Bruker AC 300 spectrometer at 300 MHz (Bruker, IL, USA). Mass spectra were recorded on a JMS-DX 303 spectrometer (Jeol, Tokyo, Japan). Elemental analysis was performed on a Vario EL III elemental analyzer (Elementar Vario EL III, Hanau, Germany) and optical rotation of the synthesized peptides was measured on an Optics Technology automatic polarimeter (OpticsTech, Delhi, India). Purity of the synthesized peptides was checked by TLC on precoated silica gel G plates (Kieselgel 0.25 mm, 60G F₂₅₄, Merck, Germany).

General method for the synthesis of linear tri/tetrapeptide segments (5, 6)

To the solution of the amino acid methyl ester hydrochloride or dipeptide methyl ester (0.01 mol) in dichloromethane (DCM, 20 mL), NMM (2.23 mL, 0.021 mol) was added at 0 °C, and the reaction mixture was stirred for 15 min. The Boc-dipeptide (0.01 mol) in DCM (20 mL), *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC.HCl, 1.92 g, 0.01 mol), and HOBt (1.34 g, 0.01 mol) were added with stirring to the above reaction mixture. Stirring of the resulting mixture was continued for 24 h at r.t. The reaction mixture was filtered and the residue was washed with DCM (20 mL) and added to the filtrate. The filtrate was washed with 5% NaHCO₃ and saturated NaCl solutions. The organic layer was dried over anhydrous Na₂SO₄, filtered, and evaporated in vacuum. The crude product was recrystallized from a mixture of chloroform and

petroleum ether (b.p. 40-60 °C) followed by cooling at 0 °C to get the title compounds.

tert-Butyloxycarbonyl-*L*-Prolyl-*L*-Tyrosyl-*L*-Leucyl-*L*-Proline methyl ester (5)

Semisolid mass; Yield 89%; $[\alpha]_D = -76.3^\circ$ ($c = 0.25$, MeOH); $R_f = 0.69$ (CHCl₃:MeOH - 8:2); IR (CHCl₃): $\nu = 3373$ (O-H_{str}, aromatic ring), 3127, 3124 (N-H_{str}, amide), 3069-3062 (Ar-H_{str}, aromatic ring), 2999-2992 (C-H_{str}, cyclic CH₂), 2968, 2924-2921 (C-H_{str}, asym, CH₃ and CH₂), 2843, 2839 (C-H_{str}, sym, CH₂), 1743 (C=O_{str}, ester), 1669-1666, 1643, 1639 (C=O_{str}, 3° and 2° amide), 1565, 1437 (skeletal bands), 1534, 1530 (N-H_{def}, amide), 1388, 1369 (C-H_{def}, tert-butyl), 1383, 1362 (C-H_{def}, iso-propyl), 1271 (C-O_{str}, ester), 716, 689 (C-H_{def}, oop, aromatic ring) cm⁻¹; ¹H NMR (CDCl₃): $\delta = 7.52$ (br. s, 1 H, NH, Leu), 6.99, 6.96 (dd, $J = 8.55, 5.25$ Hz, 2 H, *m*-H's, Tyr), 6.92 (br. s, 1 H, NH, Tyr), 6.89, 6.86 (dd, $J = 8.6, 4.9$ Hz, 2 H, *o*-H's, Tyr), 5.95 (br. s, 1 H, OH, Tyr), 4.56-4.52 (q, $J = 7.9$ Hz, 1 H, α -H, Tyr), 4.15-4.11 (m, $J = 6.7$ Hz, 1 H, α -H, Leu), 4.10 (t, $J = 6.9$ Hz, 1 H, α -H, Pro-1), 3.89 (t, $J = 6.85$ Hz, 1 H, α -H, Pro-2), 3.62 (s, 3 H, OCH₃), 3.39 (t, $J = 7.15$ Hz, 2 H, δ -H, Pro-2), 3.23 (t, $J = 7.2$ Hz, 2 H, δ -H, Pro-1), 2.89 (d, $J = 5.5$ Hz, 2 H, β -H's, Tyr), 2.57-2.54 (m, 2 H, β -H's, Pro-1), 2.03-1.98 (m, 4 H, β -H's, γ -H's, Pro-2), 1.93-1.89 (m, 2 H, γ -H's, Pro-1), 1.76 (t, 2 H, $J = 5.9$ Hz, β -H's, Leu), 1.49 (s, 9 H, tert-butyl), 1.47-1.42 (m, 1 H, γ -H, Leu), 0.98 (d, 6 H, $J = 6.25$ Hz, δ -H's, Leu); C₃₁H₄₆N₄O₈ (602): calcd. C 61.78, H 7.69, N 9.30; found C 61.75, H 7.68, N 9.32.

tert-Butyloxycarbonyl-*Glycyl-L-Valyl-L-Proline methyl ester (6)*

Semisolid mass; Yield 79%; $[\alpha]_D = +49.2^\circ$ ($c = 0.25$, MeOH); $R_f = 0.56$ (CHCl₃:MeOH - 8:2); IR (CHCl₃): $\nu = 3128, 3123$ (N-H_{str}, amide), 2999-2993 (C-H_{str}, cyclic CH₂), 2925-2921 (C-H_{str}, asym, CH₂), 2853, 2849 (C-H_{str}, sym, CH₂), 1743 (C=O_{str}, ester), 1667, 1645 (C=O_{str}, 3° and 2° amide), 1537 (N-H_{def}, 2° amide), 1389, 1368 (C-H_{def}, tert-butyl), 1382, 1363 (C-H_{def}, iso-propyl), 1268 (C-O_{str}, ester) cm⁻¹; ¹H NMR (CDCl₃): $\delta = 7.83$ (br. s, 1 H, NH, Val), 6.82 (br. s, 1 H, NH, Gly), 4.45 (t, $J = 5.85$ Hz, 1 H, α -H, Val), 3.75 (d, $J = 5.5$ Hz, 2 H, α -H's, Gly),

3.65 (t, 1 H, $J = 6.9$ Hz, α -H, Pro), 3.63 (s, 3 H, OCH_3), 3.12 (t, 2 H, $J = 7.15$ Hz, δ -H, Pro), 2.06-1.97 (m, 5 H, β -H's, γ -H's, Pro and β -H, Val), 1.54 (s, 9 H, tert-butyl), 1.07 (d, 6 H, $J = 4.6$ Hz, γ -H's, Val); $\text{C}_{18}\text{H}_{31}\text{N}_3\text{O}_6$ (385): calcd. C 56.09, H 8.11, N 10.90; found C 56.11, H 8.14, N 10.89.

Deprotection of the tetrapeptide unit at the amino terminal

Boc-protected tetrapeptide 5 (6.02 g, 0.01 mol) was dissolved in CHCl_3 (15 mL) and treated with CF_3COOH (2.28 g, 0.02 mol). The resulting solution was stirred at room temperature for 1 h and washed with a saturated NaHCO_3 solution (25 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by crystallization from CHCl_3 and petroleum ether (b.p. 40-60 °C) to get the pure deprotected compound 5a.

L-Prolyl-L-Tyrosyl-L-Leucyl-L-Proline methyl ester (5a)

Semisolid mass; Yield 76%; $[\alpha]_D = -43.8^\circ$ ($c = 0.25$, MeOH). $R_f = 0.75$ ($\text{CHCl}_3 \cdot \text{MeOH} - 8:2$); IR (CHCl_3): $\nu = 3369$ (N-H_{str} , amine), 3371 (O-H_{str} , aromatic ring), 3129, 3126 (N-H_{str} , amide), 3067-3062 (Ar-H_{str} , aromatic ring), 2997-2991 (C-H_{str} , cyclic CH_2), 2969, 2926, 2920 (C-H_{str} , asym, CH_3 and CH_2), 2845, 2841 (C-H_{str} , sym, CH_2), 1741 (C=O_{str} , ester), 1667, 1642-1638 (C=O_{str} , 3° and 2° amide), 1617 (N-H_{def} , amine), 1565, 1436 (skeletal bands), 1536, 1529 (N-H_{def} , amide), 1382, 1361 (C-H_{def} , iso-propyl), 1273 (C-O_{str} , ester), 715, 687 (C-H_{def} , oop, aromatic ring) cm^{-1} ; $^1\text{H NMR}$ (CDCl_3): $\delta = 7.95$ (br. s, 1 H, NH, Tyr), 7.49 (br. s, 1 H, NH, Leu), 6.98, 6.95 (dd, $J = 8.6, 5.25$ Hz, 2 H, m -H's, Tyr), 6.91, 6.87 (dd, $J = 8.55, 4.85$ Hz, 2 H, o -H's, Tyr), 6.44 (br. s, 2 H, OH, Tyr and NH, Pro-1), 4.36-4.31 (q, $J = 7.85$ Hz, 1 H, α -H, Tyr), 4.14-4.09 (m, $J = 6.65$ Hz, 1 H, α -H, Leu), 3.92 (t, $J = 6.9$ Hz, 1 H, α -H, Pro-2), 3.64 (s, 3 H, OCH_3), 3.50 (t, $J = 6.85$ Hz, 1 H, α -H, Pro-1), 3.42 (t, $J = 7.2$ Hz, 2 H, δ -H, Pro-2), 2.95 (d, $J = 5.45$ Hz, 2 H, β -H's, Tyr), 2.76 (t, $J = 7.15$ Hz, 2 H, δ -H, Pro-1), 2.06-1.99 (m, 4 H, β -H's, -H's, Pro-2), 1.89-1.85 (m, 2 H, β -H's, Pro-1), 1.78-1.73 (m, 2 H, γ -H's, Pro-1), 1.67 (t, 2 H, $J = 5.85$ Hz, β -H's, Leu), 1.52-1.47 (m, 1 H, γ -H, Leu), 0.99 (d, 6

H, $J = 6.3$ Hz, δ -H's, Leu); $\text{C}_{26}\text{H}_{38}\text{N}_4\text{O}_6$ (502): calcd. C 62.13, H 7.62, N 11.15; found C 62.15, H 7.65, N 11.12.

Deprotection of the tripeptide unit at the carboxyl terminal

To a solution of the tripeptide 6 (3.85 g, 0.01 mol) in $\text{THF} \cdot \text{H}_2\text{O}$ (1:1, 36 mL), LiOH (0.36 g, 0.015 mol) 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_2SO_4 . The aqueous layer was extracted with Et_2O (3×25 mL). The combined organic extracts were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was finally crystallized from methanol and ether to get the pure deprotected compound 6a.

tert-Butyloxycarbonyl-Glycyl-L-Valyl-L-Proline (6a)

Semisolid mass; Yield 88%; $[\alpha]_D = +114.7^\circ$ ($c = 0.25$, MeOH); $R_f = 0.79$ ($\text{CHCl}_3 \cdot \text{MeOH} - 8:2$); IR (CHCl_3): $\nu = 3295$ -2517 (O-H_{str} , COOH), 3129, 3122 (N-H_{str} , amide), 2997-2991 (C-H_{str} , cyclic CH_2), 2927, 2922 (C-H_{str} , asym, CH_2), 2852, 2849 (C-H_{str} , sym, CH_2), 1715 (C=O_{str} , COOH), 1665, 1642 (C=O_{str} , 3° and 2° amide), 1535 (N-H_{def} , 2° amide), 1387, 1369 (C-H_{def} , tert-butyl), 1384, 1362 (C-H_{def} , iso-propyl) cm^{-1} ; $^1\text{H NMR}$ (CDCl_3): $\delta = 10.46$ (br. s, 1 H, OH, COOH), 7.82 (br. s, 1 H, NH, Val), 6.85 (br. s, 1 H, NH, Gly), 5.25 (t, $J = 5.9$ Hz, 1 H, α -H, Val), 3.82 (t, 1 H, $J = 6.85$ Hz, α -H, Pro), 3.74 (d, $J = 5.45$ Hz, 2 H, α -H's, Gly), 3.13 (t, 2 H, $J = 7.2$ Hz, δ -H, Pro), 2.07-1.98 (m, 5 H, β -H's, γ -H's, Pro and β -H, Val), 1.55 (s, 9 H, tert-butyl), 1.05 (d, 6 H, $J = 4.55$ Hz, γ -H's, Val); $\text{C}_{17}\text{H}_{29}\text{N}_3\text{O}_6$ (371): calcd. C 54.97, H 7.87, N 11.31; found C 54.95, H 7.88, N 11.33.

Procedure for preparation of linear heptapeptide unit and its cyclized form (7, 8)

Tetrapeptide methyl ester, L-Pro-L-Tyr-L-Leu-L-Pro-OMe (5a, 5.02 g, 0.01 mol) was dissolved in 30 mL of THF and 2.8 mL (0.021 mol) of TEA was added at 0 °C and the resulting mixture was stirred for 15 min. Boc-protected tripeptide, Boc-Gly-L-Val-L-Pro-OH (6a, 3.71 g, 0.01 mol) was dissolved in 30 mL of THF and DIPC/EDC.HCl (1.26 g/1.92 g, 0.01 mol) and

HOBt (1.34 g, 0.01 mol) were added to above mixture with stirring. Stirring was continued for 24 h, after which the reaction mixture was filtered and the filtrate was washed with 25 mL each of 5% NaHCO₃ and saturated NaCl solutions. The organic layer was dried over anhydrous Na₂SO₄, filtered and evaporated in vacuum. The crude product was recrystallized from a mixture of chloroform and petroleum ether (b.p. 40-60 °C) followed by cooling at 0 °C to get the Boc-Gly-L-Val-L-Pro-L-Pro-L-Tyr-L-Leu-L-Pro-OMe (7) as yellowish semisolid mass. Linear heptapeptide unit (7, 4.28 g, 0.005 mol) was then, deprotected at the carboxyl terminal using lithium hydroxide (LiOH, 0.18 g, 0.0075 mol) to obtain the Boc-Gly-L-Val-L-Pro-L-Pro-L-Tyr-L-Leu-L-Pro-OH. To a solution of the deprotected heptapeptide (4.21 g, 0.005 mol) in CHCl₃ (50 mL), pentafluorophenol (*ppf*, 1.23 g, 0.0067 mol) and DCC (1.06 g, 0.005 mol) were added followed by stirring at r.t. for 12 h. Filtrate of the above reaction mixture was washed with 10% NaHCO₃ (3 × 20 mL) and 5% HCl (2 × 20 mL) solutions to obtain the corresponding pentafluorophenyl ester Boc-Gly-L-Val-L-Pro-L-Pro-L-Tyr-L-Leu-L-Pro-*Opfp*. Boc-group of the resulting unit (4.03 g, 0.004 mol) was removed using CF₃COOH (0.91 g, 0.008 mol) to get the deprotected product Gly-L-Val-L-Pro-L-Pro-L-Tyr-L-Leu-L-Pro-*Opfp* which was dissolved in CHCl₃ (25 mL) and TEA/NMM/pyridine (2.8 mL/2.21 mL/1.61 mL, 0.021 mol) was added. Then, the whole contents were kept at 0 °C for 7 days. The reaction mixture was washed with 10% NaHCO₃ (3 × 25 mL) and 5% HCl (2 × 25 mL) solutions. The organic layer was dried over anhydrous Na₂SO₄ and crude cyclized compound was recrystallized from CH₂Cl₂/*n*-hexane to obtain the pure product *cyclo* (Glycyl-L-Valyl-L-Prolyl-L-Prolyl-L-Tyrosyl-L-Leucyl-L-Proyl) (8).

tert-Butyloxycarbonyl-Glycyl-L-Valyl-L-Prolyl-L-Prolyl-L-Tyrosyl-L-Leucyl-L-Proline (7)

Semisolid mass; Yield 69%; $[\alpha]_D = -63.4^\circ$ ($c = 0.25$, MeOH); $R_f = 0.49$ (CHCl₃:MeOH - 9:1); IR (CHCl₃): $\nu = 3374$ (O-H_{str}, aromatic ring), 3129-3125, 3122 (N-H_{str}, amide), 3064-3059 (Ar-H_{str}, aromatic ring), 2999-2992 (C-H_{str}, cyclic CH₂), 2969, 2926-2918 (C-H_{str}, asym,

CH₃ and CH₂), 2851-2843 (C-H_{str}, sym, CH₂), 1742 (C=O_{str}, ester), 1669-1665, 1644, 1639 (C=O_{str}, 3° and 2° amide), 1568, 1435 (skeletal bands), 1537, 1533-1528 (N-H_{def}, 2° amide), 1389, 1372 (C-H_{def}, tert-butyl), 1382, 1363 (C-H_{def}, iso-propyl), 1271 (C-O_{str}, ester), 712, 689 (C-H_{def}, oop, aromatic ring) cm⁻¹; ¹H NMR (CDCl₃): $\delta = 7.84$ (br. s, 1 H, NH, Val), 7.52 (br. s, 1 H, NH, Leu), 6.99, 6.96 (dd, $J = 8.55, 5.3$ Hz, 2 H, *m*-H's, Tyr), 6.90, 6.86 (dd, $J = 8.6, 4.9$ Hz, 2 H, *o*-H's, Tyr), 6.79 (br. s, 1 H, NH, Gly), 6.51 (br. s, 1 H, NH, Tyr), 5.95 (br. s, 1 H, OH, Tyr), 4.83-4.79 (q, $J = 7.9$ Hz, 1 H, α -H, Tyr), 4.48 (t, $J = 5.85$ Hz, 1 H, α -H, Val), 4.42 (t, 1 H, $J = 6.9$ Hz, α -H, Pro-2), 4.13-4.08 (m, $J = 6.7$ Hz, 1 H, α -H, Leu), 3.93 (t, 1 H, $J = 6.85$ Hz, α -H, Pro-3), 3.88 (t, $J = 6.9$ Hz, 1 H, α -H, Pro-1), 3.72 (d, $J = 5.5$ Hz, 2 H, α -H's, Gly), 3.68 (t, 2 H, $J = 7.15$ Hz, δ -H, Pro-2), 3.62 (s, 3 H, OCH₃), 3.41 (t, $J = 7.2$ Hz, 2 H, δ -H, Pro-3), 3.08 (t, $J = 7.15$ Hz, 2 H, δ -H, Pro-1), 2.89 (d, $J = 5.5$ Hz, 2 H, β -H's, Tyr), 2.71-2.67 (m, 2 H, β -H's, Pro-1), 2.65-2.61 (m, 2 H, β -H's, Pro-2), 2.07-1.99 (m, 5 H, β -H's, γ -H's, Pro-3 and β -H, Val), 1.95-1.89 (m, 4 H, γ -H's, Pro-2 and Pro-1), 1.82 (t, 2 H, $J = 5.9$ Hz, β -H's, Leu), 1.53 (s, 9 H, tert-butyl), 1.50-1.45 (m, 1 H, γ -H, Leu), 1.08 (d, 6 H, $J = 4.6$ Hz, γ -H's, Val), 0.98 (d, 6 H, $J = 6.3$ Hz, δ -H's, Leu); ¹³C NMR (CDCl₃): $\delta = 174.2$ (C=O, Tyr), 172.5, 169.8, 168.4 (3 C, C=O, Pro-2, Pro-1 and Pro-3), 167.9, 167.1 (2 C, C=O, Leu and Val), 165.6 (C=O, Gly), 157.7 (C=O, Boc), 152.2 (*p*-C, Tyr), 130.7 (2 C, *o*-C's, Tyr), 129.3 (γ -C, Tyr), 128.0 (2 C, *m*-C's, Tyr), 79.3 (α -C, Boc), 60.5, 58.6, 55.9 (3 C, α -C's, Pro-1, Pro-3 and Pro-2), 52.9 (OCH₃), 49.4 (α -C, Val), 48.6, 47.8 (2 C, α -C's, Leu and Tyr), 47.5, 46.0, 44.7 (3 C, -C's, Pro-1, Pro-2 and Pro-3), 42.0 (α -C, Gly), 39.6, 37.0 (2 C, β -C's, Leu and Tyr), 31.5, 29.2 (2 C, β -C's, Pro-1 and Val), 28.9 (β -C, Pro-3), 26.7 (3 C, β -C's, Boc), 25.5 (β -C, Pro-2), 24.9, 24.5, 23.8 (3 C, γ -C's, Pro-3, Pro-1 and Pro-2), 22.0 (γ -C, Leu), 21.4 (2 C, δ -C's, Leu), 18.7 (2 C, γ -C's, Val); C₄₃H₆₅N₇O₁₁ (856): calcd. C 60.33, H 7.65, N 11.45; found C 60.35, H 7.66, N 11.47.

(Glycyl-L-Valyl-L-Prolyl-L-Prolyl-L-Tyrosyl-L-Leucyl-L-Proyl) (8)

Pale yellow solid; m.p. 137-139 °C (d); Yield

85 % (C₅H₅N), 78 % (NMM), 68 % (TEA); $[\alpha]_D = -74.2^\circ$ ($c = 0.54$, MeOH) (-74.1° for natural fanlizhicyclopeptide A [11]); $R_f = 0.77$ (CHCl₃:MeOH - 9:1); IR (KBr): $\nu = 3372$ (O–H_{str}, aromatic ring), 3128-3125, 3123-3119 (N–H_{str}, amide), 3067-3061 (ArH_{str}, aromatic ring), 2999, 2996-2991 (C–H_{str}, cyclic CH₂), 2967, 2925-2919 (C–H_{str}, asym, CH₃ and CH₂), 2853, 2949-2843 (C–H_{str}, sym, CH₂), 1668-1664, 1642, 1639 (C=O_{str}, 3° and 2° amide), 1566, 1439 (skeletal bands), 1538, 1532-1529 (N–H_{def}, 2 amide), 1380, 1362 (C–H_{def}, iso-propyl), 716, 687 (C–H_{def}, oop, aromatic ring) cm⁻¹; ¹H NMR (CDCl₃): $\delta = 9.88$ (br. s, 1 H, NH, Tyr), 9.69 (br. s, 1 H, NH, Leu), 9.18 (br. s, 1 H, NH, Gly), 7.85 (br. s, 1 H, NH, Val), 6.99, 6.95 (dd, $J = 8.6, 5.25$ Hz, 2 H, *m*-H's, Tyr), 6.89, 6.86 (dd, $J = 8.55, 4.9$ Hz, 2 H, *o*-H's, Tyr), 6.55 (t, $J = 5.9$ Hz, 1 H, α -H, Val), 6.33-6.28 (m, $J = 6.7$ Hz, 1 H, α -H, Leu), 5.97 (br. s, 1 H, OH, Tyr), 4.25 (t, 1 H, $J = 6.85$ Hz, α -H, Pro-2), 4.21-4.37 (q, $J = 7.85$ Hz, 1 H, α -H, Tyr), 4.02 (d, $J = 5.45$ Hz, 2 H, α -H's, Gly), 3.89 (t, 1 H, $J = 6.9$ Hz, α -H, Pro-3), 3.75 (t, $J = 6.85$ Hz, 1 H, α -H, Pro-1), 3.51 (t, 2 H, $J = 7.2$ Hz, δ -H, Pro-2), 3.23 (t, $J = 7.15$ Hz, 2 H, δ -H, Pro-3), 2.95 (t, $J = 7.2$ Hz, 2 H, δ -H, Pro-1), 2.71-2.65 (m, 4 H, β -H's, Pro-1 and Pro-3), 2.64-2.60 (m, 2 H, β -H's, Pro-2), 2.57 (d, $J = 5.45$ Hz, 2 H, β -H's, Tyr), 1.89 (t, 2 H, $J = 5.85$ Hz, β -H's, Leu), 1.87-1.78 (m, 6 H, γ -H's, Pro-2, Pro-3 and Pro-1), 1.67-1.62 (m, 1 H, β -H, Val), 1.15 (d, 6 H, $J = 4.55$ Hz, γ -H's, Val), 0.99 (d, 6 H, $J = 6.25$ Hz, δ -H's, Leu), 0.86-0.79 (m, 1 H, γ -H, Leu); ¹³C NMR (CDCl₃): $\delta = 173.9$ (C=O, Leu), 172.0, 171.3 (2 C, C=O, Pro-3 and Tyr), 170.8, 170.3, (2 C, C=O, Pro-2 and Val), 169.7, 169.1 (2 C, C=O, Pro-1 and Gly), 153.8 (*p*-C, Tyr), 135.6 (γ -C, Tyr), 131.3 (2 C, *m*-C's, Tyr), 129.1 (2 C, *o*-C's, Tyr), 62.2, 58.4, 56.1 (3 C, α -C's, Pro-3, Pro-2 and Pro-1), 55.8 (α -C, Val), 54.9, 54.5 (2 C, α -C's, Leu and Tyr), 49.3, 46.5, 45.1 (3 C, δ -C's, Pro-3, Pro-2 and Pro-1), 43.6, 42.0 (2 C, β -C's, Leu and Tyr), 40.9 (α -C, Gly), 34.6, 33.7, 30.1 (3 C, β -C's, Pro-3, Pro-1 and Pro-2), 29.9 (β -C, Val), 29.0 (γ -C, Leu), 24.1, 23.8 (2 C, γ -C's, Pro-3 and Pro-1), 23.1 (2 C, δ -C's, Leu), 20.7 (γ -C, Pro-2), 18.9 (2 C, γ -C's, Val); MS (FAB, 70 eV): m/z (%) = 724 (100) [M + 1]⁺, 696 (11) [724-CO]⁺, 667 (39) [Val-Pro-Pro-Tyr-Leu-Pro]⁺, 639 (17) [667-CO]⁺, 627

(78) [Pro-Tyr-Leu-Pro-Gly-Val]⁺, 625 (49) [Pro-Pro-Tyr-Leu-Pro-Gly]⁺, 611 (64) [Pro-Gly-Val-Pro-Pro-Tyr]⁺, 599 (19) [627-CO]⁺, 597 (16) [625-CO]⁺, 583 (16) [611-CO]⁺, 570 (48) [Val-Pro-Pro-Tyr-Leu]⁺, 568 (37) [Pro-Pro-Tyr-Leu-Pro]⁺, 542 (11) [570-CO]⁺, 540 (13) [568-CO]⁺, 530 (76) [Tyr-Leu-Pro-Gly-Val]⁺, 528 (41) [Pro-Tyr-Leu-Pro-Gly]⁺, 502 (11) [530-CO]⁺, 500 (15) [528-CO]⁺, 471 (76) [Pro-Pro-Tyr-Leu]⁺, 457 (23) [Val-Pro-Pro-Tyr]⁺, 448 (52) [Pro-Gly-Val-Pro-Pro]⁺, 443 (29) [471-CO]⁺, 431 (23) [Tyr-Leu-Pro-Gly]⁺, 429 (16) [457-CO]⁺, 420 (11) [448-CO]⁺, 403 (14) [431-CO]⁺, 374 (48) [Pro-Tyr-Leu]⁺, 358 (61) [Pro-Pro-Tyr]⁺, 351 (72) [Pro-Gly-Val-Pro]⁺, 346 (17) [374-CO]⁺, 330 (14) [358-CO]⁺, 323 (16) [351-CO]⁺, 294 (38) [Val-Pro-Pro]⁺, 277 (41) [Tyr-Leu]⁺, 261 (33) [Pro-Tyr]⁺, 254 (33) [Pro-Gly-Val]⁺, 233 (10) [261-CO]⁺, 226 (14) [254-CO]⁺, 195 (27) [Pro-Pro]⁺, 167 (11) [195-CO]⁺, 155 (29) [Pro-Gly]⁺, 136 (19) [Tyr immonium ion, C₈H₁₀NO]⁺, 127 (21) [155-CO]⁺, 107 (10) [C₇H₇O]⁺, 98 (22) [Pro]⁺, 93 (13) [C₆H₅O]⁺, 86 (21) [Leu immonium ion, C₅H₁₂N]⁺, 72 (26) [Val immonium ion, C₄H₁₀N]⁺, 70 (34) [Pro immonium ion, C₄H₈N]⁺, 57 (14) [C₄H₉]⁺, 43 (28) [C₃H₇]⁺, 30 (16) [Gly immonium ion, CH₄N]⁺, 17 (10) [OH]⁺, 15 (21) [CH₃]⁺; C₃₇H₅₃N₇O₈ (723): calcd. C 61.39, H 7.38, N 13.54; found C 61.41, H 7.36, N 13.55.

Anthelmintic evaluation

Newly synthesized linear heptapeptide and heptacyclopeptide 7, 8 were subjected to anthelmintic activity studies against three earthworm species *Megascolex konkanensis*, *Pontoscotex corethruses* and *Eudrilus eugeniae* at 2 mg/mL concentration using Garg's method (16). Tween 80 (0.5%) in distilled water was used as control and mebendazole was used as standard drug. The results of anthelmintic screening are compiled in Table 1.

Antibacterial and antifungal evaluation

The synthesized linear and heptacyclopeptide 7, 8 were evaluated for their antimicrobial activity against Gram-positive bacteria *Bacillus subtilis*, *Staphylococcus aureus*, Gram-negative bacteria *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, dermatophytes *Microsporium audouinii*, *Trichophyton mentagrophytes*,

Table 1. Anthelmintic evaluation data for the linear and cycloheptapeptide 7, 8.

| Compound | Earthworm species | | | | | |
|-------------|-----------------------------|------------------------|----------------------------|-----------------------|----------------------------|-----------------------|
| | M. konk. | | P. core. | | E. euge. | |
| | Mean paralyzing time (min)‡ | Mean death time (min)‡ | Mean paralyzing time (min) | Mean death time (min) | Mean paralyzing time (min) | Mean death time (min) |
| 7 | 14.35 ± 0.13 | 23.09 ± 0.31 | 18.04 ± 0.40 | 28.49 ± 0.17 | 13.22 ± 0.11 | 24.55 ± 0.47 |
| 8 | 10.08 ± 0.42 | 17.13 ± 0.49 | 13.66 ± 0.27 | 22.08 ± 0.21 | 11.28 ± 0.34 | 20.18 ± 0.22 |
| Control# | – | – | – | – | – | – |
| Mebendazole | 13.63 ± 0.33 | 22.43 ± 0.27 | 17.56 ± 0.49 | 29.49 ± 0.15 | 13.50 ± 0.39 | 24.07 ± 0.44 |

M. konk.: Megascoplex konkanensis, P. core.: Pontoscotex corethruses, E. euge.: Eudrilus eugeniae

‡ Data are given as mean ± S.D. (n = 3) # Tween 80 (0.5%) in distilled water

diamorphic fungi *Candida albicans*, and other fungal strains, including *Aspergillus niger* at 25–6 µg/mL concentration by using modified Kirby-Bauer disc diffusion method (17). MIC values of test compounds were determined by tube dilution technique. The Petri plates inoculated with bacterial cultures were incubated at 37 °C for 18 h and those inoculated with fungal cultures were incubated at 37 °C for 48 h. Gatifloxacin and griseofulvin were used as reference drugs and DMF/DMSO were used as control. The results of antibacterial and antifungal studies are presented in Table 2.

Experimental details of the biological activity studies are described in our previously published

reports (18-22).

Results and Discussion

Chemistry

The cycloheptapeptide molecule was split into three dipeptide units Boc-L-Pro-L-Tyr-OMe (1), Boc-L-Leu-L-Pro-OMe (2), and Boc-Gly-L-Val-OMe (3) and also a single amino acid unit L-Pro-OMe·HCl (4). Dipeptide units 1-3 were prepared by coupling of Boc-amino acids such as Boc-L-Pro, Boc-L-Leu, and Boc-Gly with corresponding amino acid methyl ester hydrochlorides such as L-Tyr-OMe·HCl, L-Pro-OMe·HCl, and L-Val-OMe·HCl by following

Table 2. Antimicrobial evaluation data for linear and cycloheptapeptide 7, 8.

| Compound | Diameter of zone of inhibition (mm) | | | | | | | |
|--------------|-------------------------------------|---------|----------|----------|----------------|----------|----------|----------|
| | Bacterial strains | | | | Fungal strains | | | |
| | B. sub. | S. aur. | P. aeru. | K. pneu. | C. alb. | M. audo. | A. niger | T. menta |
| 7 | – | – | 14(12.5) | – | 10(25) | 17(6) | – | 18(6) |
| 8 | 11(25) | – | 17(12.5) | 14(25) | 13(25) | 21(6) | 10(25) | 22(6) |
| Control# | – | – | – | – | – | – | – | – |
| Gatifloxacin | 18(12.5)† | 27(6) | 23(6) | 25(6) | – | – | – | – |
| Griseofulvin | – | – | – | – | 20(6) | 18(6) | 20(12.5) | 19(6) |

B. sub.: Bacillus subtilis, S. aur.: Staphylococcus aureus, P. aeru.: Pseudomonas aeruginosa,

K. pneu.: Klebsiella pneumoniae, C. alb.: Candida albicans, M. audo.: Microsporium audouinii,

A. niger: Aspergillus niger, T. menta.: Trichophyton mentagrophytes

† Values in bracket are MIC values (µg/mL) # DMF / DMSO

the modified Bodanzsky and Bodanzsky method (23). After deprotection at the carboxy terminus, dipeptide 1 was coupled with dipeptide 2 deprotected at the amino terminus, to get the tetrapeptide unit Boc-L-Pro-L-Tyr-L-Leu-L-Pro-OMe (5). The carboxyl group of dipeptide 3 was removed by alkaline hydrolysis using lithium hydroxide (LiOH) and the deprotected peptide was coupled with amino acid unit 4, utilizing three different carbodiimides to get the tripeptide unit Boc-Gly-L-Val-L-Pro-OMe (6). After removal of the ester group of tripeptide 6 and the Boc group of tetrapeptide 5, the deprotected units were coupled to get the linear heptapeptide unit Boc-L-Pro-L-Tyr-L-Leu-L-Pro-Gly-L-Val-L-Pro-OMe (7). The methyl ester group of the linear peptide fragment was replaced by pentafluorophenyl (*pfp*) ester group. The Boc-group of resulting compound was removed using trifluoroacetic acid (CF₃COOH), and the deprotected linear fragment was now cyclized by keeping the whole contents at 0 °C for 7 days in the presence of catalytic amounts of TEA or NMM or pyridine to get the cyclic product 8 (Scheme 1).

The structure of the newly synthesized cyclooligopeptide as well as that of the intermediate tri/tetra/heptapeptides was confirmed by FT-IR, ¹H NMR spectroscopy and elemental analysis. In addition, mass spectra and ¹³C NMR spectroscopy were recorded for the linear and cyclic heptapeptide.

The synthesis of cyclooligopeptide 8 was accomplished with 85% yield, and pyridine proved to be an effective base for cyclization of the linear heptapeptide unit. Cyclization of the linear peptide fragment was supported by the disappearance of absorption bands at 1742, 1271 and 1387, 1373 cm⁻¹ (C=O_{str}, C-O_{str}, ester and C-H_{def} tert-butyl groups) in IR spectra of 8. The formation of the cyclopeptide was further confirmed by the disappearance of singlets at 3.62 and 1.53 ppm corresponding to three protons of the methyl ester group and nine protons of the tert-butyl group of Boc in the ¹H NMR spectrum and the disappearance of the singlets at 157.7, 79.3 and 52.9, 26.7 ppm corresponding to carbon atoms of ester and tert-butyl groups in the ¹³C NMR spectrum of 8. Furthermore, the ¹H NMR and ¹³C NMR spectra

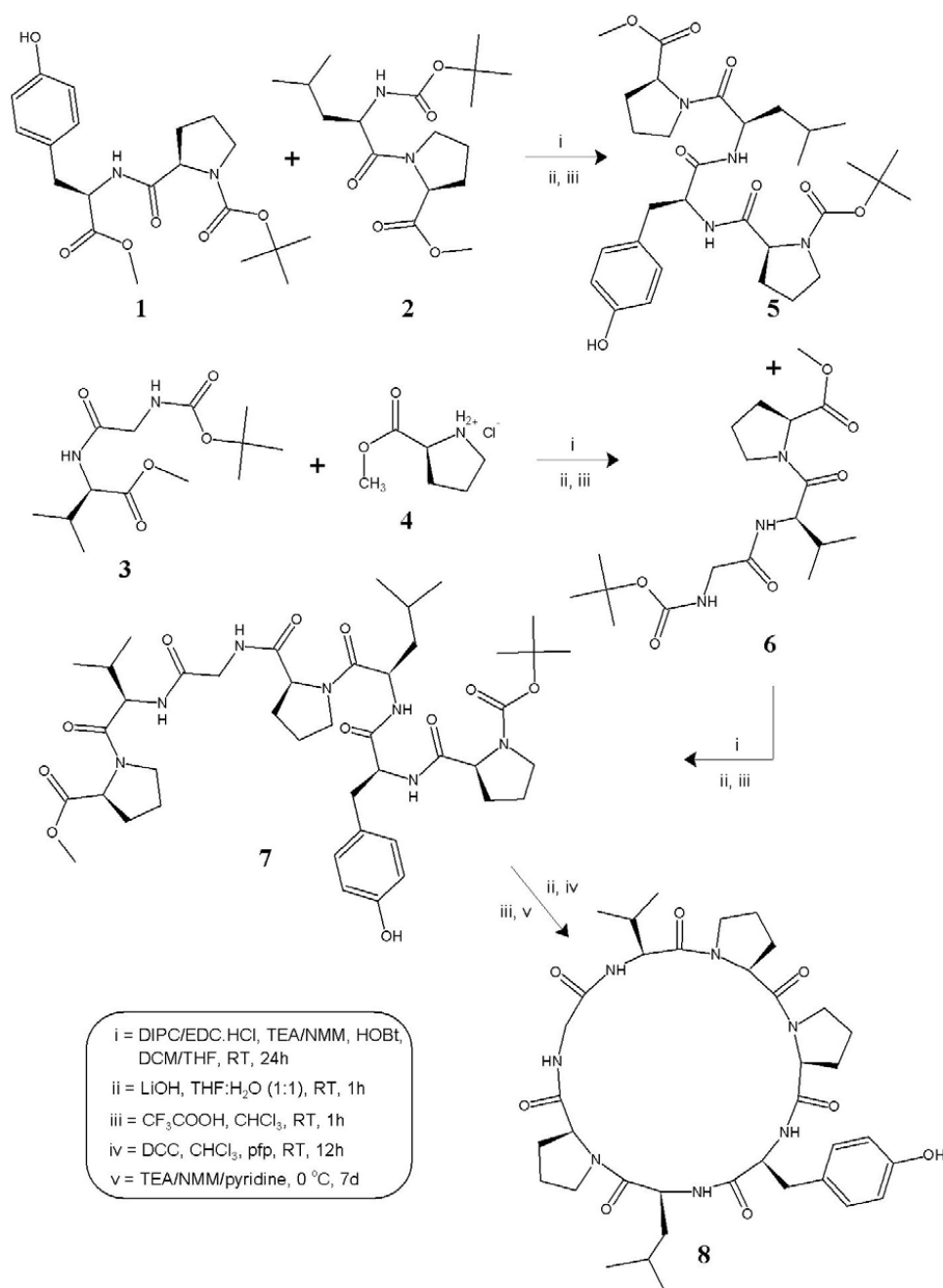
of the synthesized cyclic heptapeptide showed characteristic peaks confirming the presence of all the 53 protons and 37 carbon atoms. The appearance of the pseudomolecular ion peak (M + 1)⁺ at *m/z* = 724 corresponding to the molecular formula C₃₇H₅₃N₇O₈ in the mass spectrum of 8, along with other fragment ion peaks resulting from cleavage at 'Pro-Val', 'Pro-Pro', 'Pro-Leu', 'Tyr-Pro' and 'Val-Gly' amide bonds showed the exact sequence of the attachment of all the seven amino acid moieties in a chain. In addition, the presence of the immonium ion peaks at *m/z* = 136 (Tyr), 86 (Leu), 72 (Val), 70 (Pro), and 30 (Gly) further confirmed all the amino acid moieties in the cyclopeptide structure. Furthermore, the elemental analysis of 8 afforded values with tolerance of ± 0.02 strictly in accordance with the molecular composition.

Biological activity

Comparison of antifungal activity data suggested that 8 possessed potent bioactivity against dermatophytes *M. audouinii* and *T. mentagrophytes* with MIC values of 6 µg/mL when compared to the reference drug griseofulvin. From the analysis of anthelmintic activity data, it is observed that 8 displayed remarkable activity against all three earthworm species *M. konkanensis*, *P. corethruses* and *E. eugeniae*, in comparison to standard drug mebendazole. Moreover, a moderate level of activity was observed against Gram-negative bacteria *P. aeruginosa* for the newly synthesized cyclopeptide, in comparison to the standard drug gatifloxacin. However, 8 displayed no significant activity against neither Gram-positive and Gram-negative bacteria nor *Aspergillus niger*. In addition, the analysis of the pharmacological activity data revealed that heptacyclopeptide 8 displayed a higher bioactivity against pathogenic fungi and earthworms than its linear form 7, which is due to the fact that 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.

Conclusion

In conclusion, a first total successful synthesis



Scheme 1. Synthetic route to the cycloheptapeptide (fanlizhicyclopeptide A) 8.

of the natural peptide fanlizhicyclopeptide A (8) was accomplished in the present study, with good yield via coupling reactions utilizing different carbodiimides. The DIPC / TEA coupling method proved to be yield-effective, in comparison to methods utilizing EDC·HCl / DIPC and NMM, providing 10-12% additional

yield. The pentafluorophenyl ester was shown to be better for the activation of the acid functionality of the linear heptapeptide unit. Pyridine was found to be a good base for the intramolecular cyclization of the linear peptide fragment in comparison to TEA or NMM. The synthesized heptacyclopeptide displayed

potent anthelmintic activity and effectiveness against pathogenic dermatophytes. In addition, Gram-negative bacteria were found to be more sensitive than Gram-positive bacteria towards the newly synthesized peptide. On passing toxicity tests, heptacyclopeptide 8 may prove as a good candidate for clinical studies and can be a new anti-dermatophyte and anthelmintic drug of future.

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