

SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL SCREENING
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Abstract: Synthesis of a proline-rich cyclic hexapeptide – diandrine A [VI] was accomplished by coupling of tetrapeptide unit Boc-Gly-Pro-Trp-Pro-OH with dipeptide unit Tyr-Phe-OMe followed by cyclization of linear peptide unit [V] under alkaline condition. Structure of newly synthesized cyclopolypeptide was elucidated by means of spectral techniques including FTIR, ¹H NMR, ¹³C NMR, MS analyses. VI was subjected to pharmacological screening and found to exhibit good antifungal activity against dermatophytes. Further, VI possessed potent antihelmintic activity against earthworms *M. konkanensis*, *P. corethruses* and *Eudrilus sp.*

Keywords: diandrine A, cyclic hexapeptide, peptide synthesis, antifungal activity, antihelmintic activity

Abbreviations: str – stretching, bend – bending (deformation), oop – out-of-plane, s – strong, m – medium, w – weak, m/br – medium/broad (FT-IR spectra); s – singlet, d – doublet, t – triplet, m – multiplet, br. s – broad singlet, dd – doublet overlapped over doublet (NMR spectra)

Plant cyclooligopeptides obtained from parts like roots, rhizomes, stems, seeds, branches, leaves, twigs, latex and fruits (1-7) have received attention nowadays, due to their complex structures and wide biological spectrum including anti-inflammatory activity (8), cytotoxic activity (9, 10), antimicrobial activity (11), immunosuppressive and antimalarial activity (12), and tyrosinase inhibitory activity (13). Further, plant-derived cyclic peptides play a vital role in drug design and have provided significant promise for future endeavors (14). A natural cyclopolypeptide, diandrine A, was isolated from whole plants of *Drymaria diandra* (Caryophyllaceae) having selective inhibitory effect on collagen-induced platelet aggregation. Structure of isolated cyclopeptide was confirmed using 2D-NMR and FAB-MS (15).

As part of our investigation on new biologically active cyclic peptides from plant resources, we focused our attention on the synthesis of a natural proline-rich peptide. In this report, we describe the solution-phase synthesis, structure elucidation and screening of diandrine A for antibacterial, antifungal and antihelmintic potential.

EXPERIMENTAL

Materials and methods

Melting point was determined by open capillary method and was uncorrected. L-Amino acids and other chemicals used were obtained from Spectrochem Limited (Mumbai, India). IR spectra were recorded on Shimadzu 8700 FTIR spectrophotometer (Shimadzu, Japan) and ¹H and ¹³C NMR spectra were recorded on Bruker AC NMR spectrometer (Bruker, USA) at 300 MHz. FAB-MS was recorded on JMS-DX 303 Mass spectrometer (Jeol, Tokyo, Japan) operating at 70 eV. Elemental analyses were performed on Vario EL III elemental analyzer (Elementar, Germany) and optical rotation was measured on automatic polarimeter (Optics Tech, Ghaziabad, India) in a 2 dm tube at 25°C using methanol as solvent. Purity of all compounds was checked by TLC on precoated silica gel G plates using mixture of chloroform and methanol in different ratios (9 : 1 intermediate linear peptides and 7 : 3 for cyclopeptide).

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General method for the synthesis of dipeptide fragments I-III

Triethylamine (TEA, 2.8 mL, 0.021 M) was added to amino acid methyl ester hydrochloride (0.01 M) previously suspended in chloroform (20 mL) at 0°C and resulting reaction mixture was stirred for 15 min. To this, another mixture of Boc-amino acid (0.01 M) in chloroform (20 mL) and *N,N*-diisopropylcarbodiimide (DIPC) (1.26 g, 0.01 M) was added with stirring. After 24 h, the final reaction mixture was filtered and 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.

t-Butyloxycarbonyl-glycyl-proline methyl ester [I]

Semisolid mass, Yield 79.4%, [α]_D: -26.2°, R_f - 0.79; IR (CHCl₃, cm⁻¹): 3125 (m, -NH str, amide), 2999, 2994 (m, -CH str, cyclic CH₂ and CH), 2929 (m, -CH str, asym, CH₂), 2845 (m, -CH str, sym, CH₂), 1754 (s, -C=O str, ester), 1672, 1635 (s, -C=O str, 3° and 2° amide), 1535 (m, -NH bend, 2° amide), 1392, 1360 (m, -CH bend, group), 1272 (s, C-O str, ester) ¹H NMR (300 MHz, CDCl₃, δ , ppm): 6.39 (d, 1H, br. s, -NH), 3.50-3.48 (2H, d, *J* = 4.75 Hz, CH₂, Gly), 4.29-4.26 (1H, t, α -H, Pro), 3.75-3.72 (2H, t, δ -H's, Pro), 3.62 (3H, s, OCH₃), 2.08-2.02 (2H, m, β -H's, Pro), 1.99-1.93 (2H, m, γ -H's, Pro), 1.55 (9H, s, *t*-butyl group); ¹³C NMR (CDCl₃, 300 MHz, δ , ppm): 172.7, 169.2 (C=O, Gly and Pro), 155.3 (C=O, Boc), 79.2 (α -C, Boc), 58.1 (α -C, Pro), 52.5 (OCH₃), 47.7 (δ -C, Pro), 44.5 (CH₂, Gly), 29.1 (β -C, Pro), 27.3 (3C, β -C's, Boc), 24.8 (γ -C, Pro). Analysis: calcd. for C₁₃H₂₂N₂O₅: C, 54.53; H, 7.74; N, 9.78; found: C, 54.52; H, 7.77; N, 9.76%.

t-Butyloxycarbonyl-tryptophanyl-proline methyl ester [II]

Semisolid mass, Yield 68.9%, [α]_D: -71.9°, R_f - 0.54; IR (CHCl₃, cm⁻¹): 3475 (m, -NH str, indole ring), 3073-3066 (w, -CH str, indole ring), 2849 (m, -CH str, sym, CH₂), 1752 (s, -C=O str, ester), 1670, 1637 (s, -C=O str, 3° and 2° amide), 1558, 1425 (m, skeletal bands, indole ring), 1535 (m, -NH bend, 2° amide), 1391, 1365 (m, -CH bend, *t*-butyl group), 1269 (s, C-O str, ester), 716, 692 (s, -CH bend, oop, aromatic ring); ¹H NMR (300 MHz, CDCl₃, δ , ppm): 8.95 (1H, br. s, -NH, indole ring), 7.12-7.03 (3H, m, δ - ζ -H's, indole ring), 7.55-7.53 (1H, d, *J* = 7.3 Hz, γ -H, indole ring), 6.35-6.33 (1H, d, *J* = 7.75 Hz, α -H, indole ring), 6.45 (1H, br. s, -NH, Trp), 5.08-5.03

(1H, q, *J* = 6.1 Hz, α -H, Trp), 4.28-4.25 (1H, t, α -H, Pro), 3.77-3.74 (2H, t, δ -H's, Pro), 3.64 (3H, s, OCH₃), 3.39-3.37 (2H, d, *J* = 5.65 Hz, β -H's, Trp), 2.07-2.01 (2H, m, β -H's, Pro), 1.99-1.92 (2H, m, γ -H's, Pro), 1.56 (9H, s, *t*-butyl group); ¹³C NMR (CDCl₃, 300 MHz, δ , ppm): 170.3, 166.7 (C=O, Trp and Pro), 154.5 (C=O, Boc), 138.6, 127.4 (α' -C and β' -C, indole ring), 125.3, 122.8 (α -C and δ -C, indole ring), 121.4, 119.6 (e-C and γ -C, indole ring), 113.3, 107.9 (ζ -C and β -C, indole ring), 79.9 (α -C, Boc), 66.4 (α -C, Trp), 56.1 (α -C, Pro), 54.3 (OCH₃), 47.0 (δ -C, Pro), 30.4 (β -C, Trp), 28.9 (β -C, Pro), 26.8 (3C, β -C's, Boc), 24.5 (γ -C, Pro). Anal. Calcd. for C₂₂H₂₉N₃O₅: C, 63.60; H, 7.03; N, 10.11; found: C, 63.59; H, 7.05; N, 10.14%.

t-Butyloxycarbonyl-tyrosinyl-phenylalanine methyl ester [III]

White solid, Yield 73.2%, [α]_D: +12.9°, R_f - 0.75; IR (KBr, cm⁻¹): 3128, 3123 (m, -NH str, amide), 3375 (m, -OH str, Tyr), 3069-3062 (w, -CH str, aromatic rings), 2926, 2923 (m, -CH str, asym, CH₂), 1746 (s, -C=O str, ester), 1645, 1637 (s, -C=O str, 2° amide), 1562, 1559, 1435, 1429 (m, skeletal bands, aromatic rings), 1535-1529 (m, -NH bend, 2° amide), 1388, 1369 (m, -CH bend, *t*-butyl group), 1273 (s, C-O str, ester), 714, 689 (s, -CH bend, oop, aromatic rings); ¹H NMR (300 MHz, CDCl₃): d 7.31-7.27 (2H, dd, *J* = 8.55, 4.9 Hz, m-H's, Tyr), 7.12-7.09 (1H, t, *J* = 6.1 Hz, p-H, Phe), 7.01-6.97 (2H, m, m-H's, Phe), 6.92-6.88 (2H, dd, *J* = 8.6, 5.25 Hz, o-H's, Tyr), 6.87-6.83 (2H, dd, *J* = 8.75, 4.1 Hz, o-H's, Phe), 6.67 (1H, br. s, -NH, Phe), 6.56 (1H, br. s, -NH, Tyr), 5.95 (1H, br. s, -OH, Tyr), 4.73-4.69 (1H, q, *J* = 7.9 Hz, α -H, Tyr), 4.61-4.56 (1H, q, *J* = 5.55 Hz, α -H, Phe), 3.53 (3H, s, OCH₃), 3.15-3.13 (2H, d, *J* = 5.5 Hz, β -H's, Tyr), 2.82-2.80 (2H, d, *J* = 5.9 Hz, β -H's, Phe), 1.54 (9H, s, *t*-butyl group); ¹³C NMR (CDCl₃, 300 MHz, δ , ppm): 170.9, 168.3 (C=O, Tyr and Phe), 156.3 (p-C, Tyr), 153.9 (C=O, Boc), 139.3 (γ -C, Phe), 136.1 (2C, m-C's, Tyr), 130.7 (2C, o-C's, Tyr), 128.5 (2C, o-C's, Phe), 127.9 (2C, m-C's, Phe), 127.2 (γ -C, Tyr), 125.7 (p-C, Phe), 79.5 (α -C, Boc), 55.8 (α -C, Tyr), 54.1 (α -C, Phe), 53.5 (OCH₃), 38.6 (β -C, Phe), 36.4 (β -C, Tyr), 27.6 (3C, β -C's, Boc). Analysis: calcd. for C₂₄H₃₀N₂O₆: C, 65.14; H, 6.83; N, 6.33; found: C, 65.11; H, 6.86; N, 6.35%.

General method for the synthesis of linear tetra/hexapeptide fragments IV-V

Boc-protected dipeptide/tetrapeptide (0.01 M) was dissolved in 25 mL of *N,N*-dimethylformamide (DMF) and solution was neutralized with 2.21 mL (0.021 M) of *N*-methylmorpholine (NMM) at 0°C

and the resulting mixture was stirred for 15 min. Dipeptide methyl ester (0.01 M) was dissolved in 25 mL of DMF and resulting solution with DIPC (1.26 g, 0.01 M) were added to the above mixture. Stirring was first carried out for 1 h at 0-5°C and then, further for 24 h at room temperature. After completion of the reaction, the reaction mixture was diluted with an equal amount of water and the semisolid mass obtained was washed with water and purified from a mixture of chloroform and petroleum ether (b.p. 40-60°C) followed by cooling at 0°C.

t-Butyloxycarbonyl-glycyl-prolyl-tryptophanyl-proline methyl ester [IV]

Semisolid mass, Yield 78.8%, $[\alpha]_D^{20}$: +92.7°, R_f – 0.48, IR (CHCl₃, cm⁻¹): 3479 (m, -NH str, indole ring), 3129 (m, -NH str, amide), 3077-3068 (w, -CH str, indole ring), 2997-2989 (m, -CH str, cyclic CH₂ and CH), 2927 (m, -CH str, asym, CH₂), 2840 (m, -CH str, sym, CH₂), 1751 (s, -C=O str, ester), 1675, 1639, 1635 (s, -C=O str, 3° and 2° amide), 1556, 1429 (m, skeletal bands, indole ring), 1539-1536 (m, -NH bend, 2° amide), 1390, 1362 (m, -CH bend, t-butyl group), 1269 (s, C-O str, ester) 715, 696 (s, -CH bend, oop, indole ring), ¹H NMR (300 MHz, CDCl₃, δ, ppm): 8.93 (1H, br. s, -NH, indole ring), 6.39-6.37 (1H, d, *J* = 7.8 Hz, α-H, indole ring), 7.24-7.22 (1H, d, *J* = 7.25 Hz, γ-H, indole ring), 7.15-7.06 (3H, m, δ-ζ-H's, indole ring), 6.88 (1H, br. s, -NH, Gly), 6.41 (1H, br. s, -NH, Trp), 5.35-5.31 (1H, q, *J* = 6.15 Hz, α-H, Trp), 4.46-4.43 (1H, t, *J* = 6.8 Hz, α-H, Pro-1), 3.91-3.87 (1H, t, *J* = 6.85 Hz, α-H, Pro-2), 3.62-3.59 (2H, t, δ-H's, Pro-1), 3.57 (3H, s, OCH₃), 3.54-3.52 (2H, d, *J* = 4.8 Hz, CH₂, Gly), 3.41-3.38 (2H, t, δ-H's, Pro-2), 3.20-3.18 (2H, d, *J* = 5.7 Hz, β-H's, Trp), 2.69-2.64 (2H, m, β-H's, Pro-1), 2.01-1.93 (4H, m, γ-H's, Pro-2), 1.92-1.87 (2H, m, γ-H's, Pro-1), 1.55 (9H, s, t-butyl group), ¹³C NMR (CDCl₃, 300 MHz, δ, ppm): 176.5, 169.9 (C=O, Pro-1 and Pro-2), 169.3, 167.6 (C=O, Gly and Trp), 155.0 (C=O, Boc), 136.9, 128.6 (α'-C and β'-C, indole ring), 125.3, 124.4 (α-C and ε-C, indole ring), 120.9, 119.3 (δ-C and γ-C, indole ring), 111.3, 109.6 (β-C and ζ-C, indole ring), 79.2 (α-C, Boc), 63.7 (α-C, Trp), 57.2 (α-C, Pro-2), 56.8 (α-C, Pro-1), 53.5 (OCH₃), 47.1 (CH₂, Gly), 46.0, 45.3 (δ-C's, Pro-1 and Pro-2), 30.8 (β-C, Trp), 28.9 (β-C, Pro-2), 28.0 (3C, β-C's, Boc), 27.4 (β-C, Pro-1), 24.6, 23.7 (2C, γ-C's, Pro-1 and Pro-2). Analysis: calcd. for C₂₉H₃₉N₅O₇: C, 61.15; H, 6.90; N, 12.29. found : C, 61.17; H, 6.89; N, 12.32%.

t-Butyloxycarbonyl-glycyl-prolyl-tryptophanyl-prolyl-tyrosinyl-phenylalanine methyl ester [V]

Semisolid mass, Yield 82.4%, $[\alpha]_D^{20}$: -48.1°, R_f – 0.68, IR (CHCl₃, cm⁻¹): 3475 (m, -NH str, indole ring), 3372 (m/br, -OH str, Tyr), 3127-3123 (m, -NH str, amide), 3079-3064 (w, -CH str, aromatic rings), 2999-2993 (m, -CH str, cyclic CH₂ and CH), 2928, 2923-2919 (m, -CH str, asym, CH₂), 2843, 2839 (m, -CH str, sym, CH₂), 1749 (s, -C=O str, ester), 1678, 1672, 1638-1632 (s, -C=O str, 3° and 2° amide), 1562, 1559, 1435-1429 (m, skeletal bands, aromatic rings), 1538, 1533 (m, -NH bend, 2° amide), 1389, 1365 (m, -CH bend, t-butyl group), 1271 (s, C-O str, ester), 715, 695-689 (s, -CH bend, oop, aromatic rings), ¹H NMR (300 MHz, CDCl₃, δ, ppm): 9.96 (1H, br. s, -NH, Tyr), 9.79 (1H, br. s, -NH, Trp), 7.45 (2H, br. s, -NH, indole ring and -OH, Tyr), 7.41-7.39 (1H, d, *J* = 7.8 Hz, α-H, indole ring), 7.23-7.21 (1H, d, *J* = 7.25 Hz, γ-H, indole ring), 7.16-7.07 (3H, m, δ-ζ-H's, indole ring), 7.05-7.02 (1H, t, *J* = 6.2 Hz, p-H, Phe), 7.01-6.94 (4H, m, m-H's, Tyr and Phe), 6.92-6.90 (2H, dd, *J* = 8.6, 5.3 Hz, o-H's, Tyr), 6.87-6.83 (2H, dd, *J* = 8.75, 4.15 Hz, o-H's, Phe), 6.81 (1H, br. s, -NH, Gly), 6.05 (1H, br. s, -NH, Phe), 5.40-4.36 (1H, q, *J* = 6.15 Hz, α-H, Trp), 5.05-5.01 (1H, q, *J* = 7.9 Hz, α-H, Tyr), 4.45-4.42 (1H, t, *J* = 6.8 Hz, α-H, Pro-1), 3.95-3.91 (1H, q, *J* = 5.55 Hz, α-H, Phe), 3.87-3.84 (1H, t, *J* = 6.85 Hz, α-H, Pro-2), 3.65-3.62 (2H, t, δ-H's, Pro-1), 3.54 (3H, s, OCH₃), 3.51-3.49 (2H, d, *J* = 4.8 Hz, CH₂, Gly), 3.28-3.25 (2H, t, δ-H's, Pro-2), 3.21-3.19 (2H, d, *J* = 5.7 Hz, β-H's, Trp), 2.98-2.96 (2H, d, *J* = 5.9 Hz, β-H's, Phe), 2.89-2.87 (2H, d, *J* = 5.5 Hz, β-H's, Tyr), 2.76-2.67 (4H, m, β-H's, Pro-1 and Pro-2), 1.96-1.89 (4H, m, γ-H's, Pro-1 and Pro-2), 1.53 (9H, s, t-butyl group), ¹³C NMR (CDCl₃, 300 MHz, δ, ppm): 175.1, 171.0 (C=O, Pro-1 and Pro-2), 170.3, 169.1 (C=O, Tyr and Phe), 168.9, 167.8 (C=O, Trp and Trp), 156.6 (C=O, Boc), 153.8 (p-C, Tyr), 136.7 (γ-C, Phe), 135.2 (α'-C, indole ring), 130.2 (2C, o-C's, Tyr), 128.8 (γ-C, Tyr), 128.2 (2C, o-C's, Phe), 127.7 (2C, m-C's, Tyr), 127.0 (2C, m-C's, Phe), 126.5 (β'-C, indole ring), 126.1 (p-C, Phe), 123.9, 123.0 (α-C and ε-C, indole ring), 120.5, 119.0 (δ-C and γ-C, indole ring), 110.7, 109.9 (β-C and ζ-C, indole ring), 79.5 (α-C, Boc), 62.3 (α-C, Trp), 55.8 (α-C, Pro-2), 53.6 (α-C, Phe), 52.7 (α-C, Pro-1), 51.9 (OCH₃), 49.1 (α-C, Tyr), 47.3, 46.7 (δ-C's, Pro-1 and Pro-2), 46.0 (CH₂, Gly), 38.3 (β-C, Phe), 37.0 (β-C, Tyr), 30.3 (β-C, Trp), 28.6 (3C, β-C's, Boc), 27.3 (β-C, Pro-2), 26.5 (β-C, Pro-1), 24.4, 23.0 (2C, γ-C's, Pro-1 and Pro-2). Analysis: calcd. for C₄₇H₅₇N₇O₁₀: C, 64.15; H, 6.53; N, 11.14; found: C, 64.18; H, 6.50; N, 11.15%.

Synthesis of cyclic hexapeptide, diandrine A [VI]

To synthesize compound **VI**, linear hexapeptide unit **V** (0.005 M) was deprotected at carboxyl end using LiOH (0.18 g, 0.0075 M) in THF : H₂O (1 : 1) to get Boc-Gly-Pro-Trp-Pro-Tyr-Phe-OH. The deprotected hexapeptide unit (0.005 M) was now dissolved in CHCl₃ (50 mL) at 0°C. To the above solution, pentafluorophenol (1.23 g, 0.0067 M) and DIPC (0.63 g, 0.005 M) was added and stirred at room temp. for 12 h. The reaction mixture was filtered and the filtrate was washed with 10% NaHCO₃ solution (2 × 25 mL) and 5% HCl (3 × 15 mL) to get the corresponding pentafluorophenyl ester Boc-Gly-Pro-Trp-Pro-Tyr-Phe-O-*pf*p. To this compound (0.004 M) dissolved in chloroform (25 mL), trifluoroacetic acid (0.91 g, 0.008 M) was added, stirred at room temp. for 1 h and washed with 10% NaHCO₃ solution (3 × 20 mL). The organic layer was dried over anhydrous Na₂SO₄ to get Gly-Pro-Trp-Pro-Tyr-Phe-O-*pf*p which was dissolved in CHCl₃ (25 mL) and TEA/NMM/pyridine (2.8 mL/2.21 mL/1.61 mL, 0.02 M) was added. Then, whole content was kept for 1 week time at 0°C. The reaction mixture was washed with 10% NaHCO₃ and 5% HCl solutions (3 × 25 mL). The organic layer was dried over anhydrous Na₂SO₄. Finally, chloroform was distilled off and crude cyclized product was crystallized from CHCl₃/*n*-hexane to get pure cyclo (glycyl-prolyl-tryptophanyl-prolyl-tyrosinyl-phenylalanyl) [**VI**].

Yellowish needles, m.p. 135-137°C, Yield 79.8% (NMM), 70.3% (TEA), 66.8% (C₃H₅N), [α]_D: -67.8° (-67.6°), R_f - 0.59; IR (KBr, cm⁻¹): 3472 (m, -NH str, indole ring), 3375 (m/br, -OH str, Tyr), 3129-3125, 3121 (m, -NH str, amide), 3078, 3069-3062 (w, -CH str, aromatic rings), 2998-2992 (m, -CH str, cyclic CH₂ and CH), 2926, 2922, 2917 (m, -CH str, asym, CH₂), 2846, 2837-2833 (m, -CH str, sym, CH₂), 1679-1675, 1640, 1634-1629 (s, -C=O str, 3° and 2° amide), 1560, 1557, 1436-1427 (m, skeletal bands, aromatic rings), 1539, 1535-1532 (m, -NH bend, 2° amide), 718, 698-692 (s, -CH bend, oop, aromatic rings). ¹H NMR (300 MHz, CDCl₃, δ, ppm): 9.89 (1H, br. s, -NH, Tyr), 9.72 (1H, br. s, -NH, Trp), 7.75 (1H, br. s, -NH, Phe), 7.47 (2H, br. s, -NH, indole ring and -OH, Tyr), 7.38-7.36 (1H, d, *J* = 7.75 Hz, α-H, indole ring), 7.25-7.23 (1H, d, *J* = 7.3 Hz, γ-H, indole ring), 7.21-7.15 (4H, m, m-H's, Tyr and Phe), 7.14-7.05 (3H, m, δ-ζ-H's, indole ring), 7.02-6.99 (1H, t, *J* = 6.15 Hz, p-H, Phe), 6.91 (1H, br. s, -NH, Gly), 6.88-6.84 (2H, dd, *J* = 8.55, 5.3 Hz, o-H's, Tyr), 6.83-6.79 (2H, dd, *J* = 8.8, 4.15 Hz, o-H's, Phe), 5.65-5.61 (1H, q, *J* = 5.6 Hz, α-H, Phe), 5.30-5.28 (2H, d, *J* = 4.75 Hz, CH₂, Gly), 4.62-4.58 (1H, q, *J* = 6.2 Hz, α-

H, Trp), 4.25-4.21 (1H, q, *J* = 7.85 Hz, α-H, Tyr), 3.92-3.89 (1H, t, *J* = 6.9 Hz, α-H, Pro-2), 3.87-3.84 (1H, t, *J* = 6.75 Hz, α-H, Pro-1), 3.26-3.23 (2H, t, δ-H's, Pro-2), 3.21-3.18 (2H, t, δ-H's, Pro-1), 2.89-2.87 (2H, d, *J* = 5.65 Hz, β-H's, Trp), 2.72-2.63 (4H, m, β-H's, Pro-1 and Pro-2), 2.59-2.57 (2H, d, *J* = 5.45 Hz, β-H's, Tyr), 2.43-2.41 (2H, d, *J* = 5.85 Hz, β-H's, Phe), 1.89-1.82 (4H, m, γ-H's, Pro-1 and Pro-2). ¹³C NMR (CDCl₃, 300 MHz): 173.5, 172.0 (C=O, Pro-1 and Pro-2), 169.9, 169.3 (C=O, Tyr and Trp), 163.1, 162.3 (C=O, Gly and Phe), 154.4 (p-C, Tyr), 137.3 (γ-C, Phe), 135.9 (α'-C, indole ring), 132.2 (γ-C, Tyr), 129.8 (2C, o-C's, Tyr), 128.9 (2C, o-C's, Phe), 128.1 (2C, m-C's, Tyr), 127.4 (2C, m-C's, Phe), 127.0 (β'-C, indole ring), 126.1 (p-C, Phe), 123.3, 121.7 (α-C and ε-C, indole ring), 119.8, 118.5 (δ-C and γ-C, indole ring), 111.2, 109.6 (β-C and ζ-C, indole ring), 59.2 (α-C, Pro-2), 57.4 (α-C, Pro-1), 57.8 (α-C, Trp), 54.5 (α-C, Tyr), 53.0 (α-C, Phe), 49.7 (CH₂, Gly), 48.0, 46.9 (δ-C's, Pro-2 and Pro-1), 41.5 (β-C, Phe), 39.6 (β-C, Tyr), 30.3 (β-C, Pro-2), 27.8 (β-C, Pro-1), 27.0 (β-C, Trp), 25.2, 22.7 (2C, γ-C's, Pro-1 and Pro-2). FAB-MS: *m/z* 748.8 (M + H)⁺, 720.8 (748.8-CO)⁺, 691.8 (Pro-Trp-Pro-Tyr-Phe)⁺, 663.8 (691.8-CO)⁺, 651.7 (Tyr-Phe-Gly-Pro-Trp)⁺, 623.7 (651.7-CO)⁺, 601.6 (Gly-Pro-Trp-Pro-Tyr)⁺, 594.6 (Trp-Pro-Tyr-Phe)⁺, 585.6 (Phe-Gly-Pro-Trp-Pro)⁺, 573.6 (651.7-CO)⁺, 566.6 (594.6-CO)⁺, 557.6 (585.6-CO)⁺, 544.6 (Pro-Trp-Pro-Tyr)⁺, 516.6 (488.5-CO)⁺, 488.5 (Phe-Gly-Pro-Trp)⁺, 465.5 (Tyr-Phe-Gly-Pro)⁺, 460.5 (488.5-CO)⁺, 447.5 (Trp-Pro-Tyr)⁺, 438.5 (Gly-Pro-Trp-Pro)⁺, 437.5 (465.5-CO)⁺, 419.5 (447.5-CO)⁺, 410.5 (438.5-CO)⁺, 381.4 (Pro-Trp-Pro)⁺, 368.4 (Tyr-Phe-Gly)⁺, 341.4 (Gly-Pro-Trp)⁺, 340.4 (368.4-CO)⁺, 313.4 (341.4-CO)⁺, 311.3 (Tyr-Phe)⁺, 302.3 (Phe-Gly-Pro)⁺, 284.3 (Trp-Pro)⁺, 283.3 (311.3-CO)⁺, 256.3 (284.3-CO)⁺, 187.2 (Trp)⁺, 164.2 (Tyr)⁺, 159.2 (C₁₀H₁₁N₂)⁺, 155.2 (Gly-Pro)⁺, 136.2 (C₈H₁₀NO)⁺, 130.1 (C₉H₈N)⁺, 127.2 (155.2-CO)⁺, 120.2 (C₈H₁₀N)⁺, 116.1 (C₈H₈N)⁺, 107.1 (C₇H₇O)⁺, 93.1 (C₆H₅O)⁺, 91.1 (C₇H₇)⁺, 77.1 (C₆H₅)⁺, 70.1 (C₄H₈N)⁺, 58.0 (Gly)⁺, 30.0 (CH₄N)⁺. Analysis: calcd. for C₄₁H₄₅N₇O₇: C, 65.85; H, 6.06; N, 13.11; found: C, 65.88; H, 6.05; N, 13.09%.

Biological activity**Antibacterial and antifungal activity studies**

The synthesized cyclic hexapeptide was subjected to antibacterial and antifungal activity studies using Modified Kirby-Bauer's method (16) against strains *B. subtilis*, *S. aureus*, *P. aeruginosa*, *E. coli* and *M. audouinii*, *T. mentagrophytes*, *C. albicans* and *A. niger* at 10 mg/mL concentration. MIC values

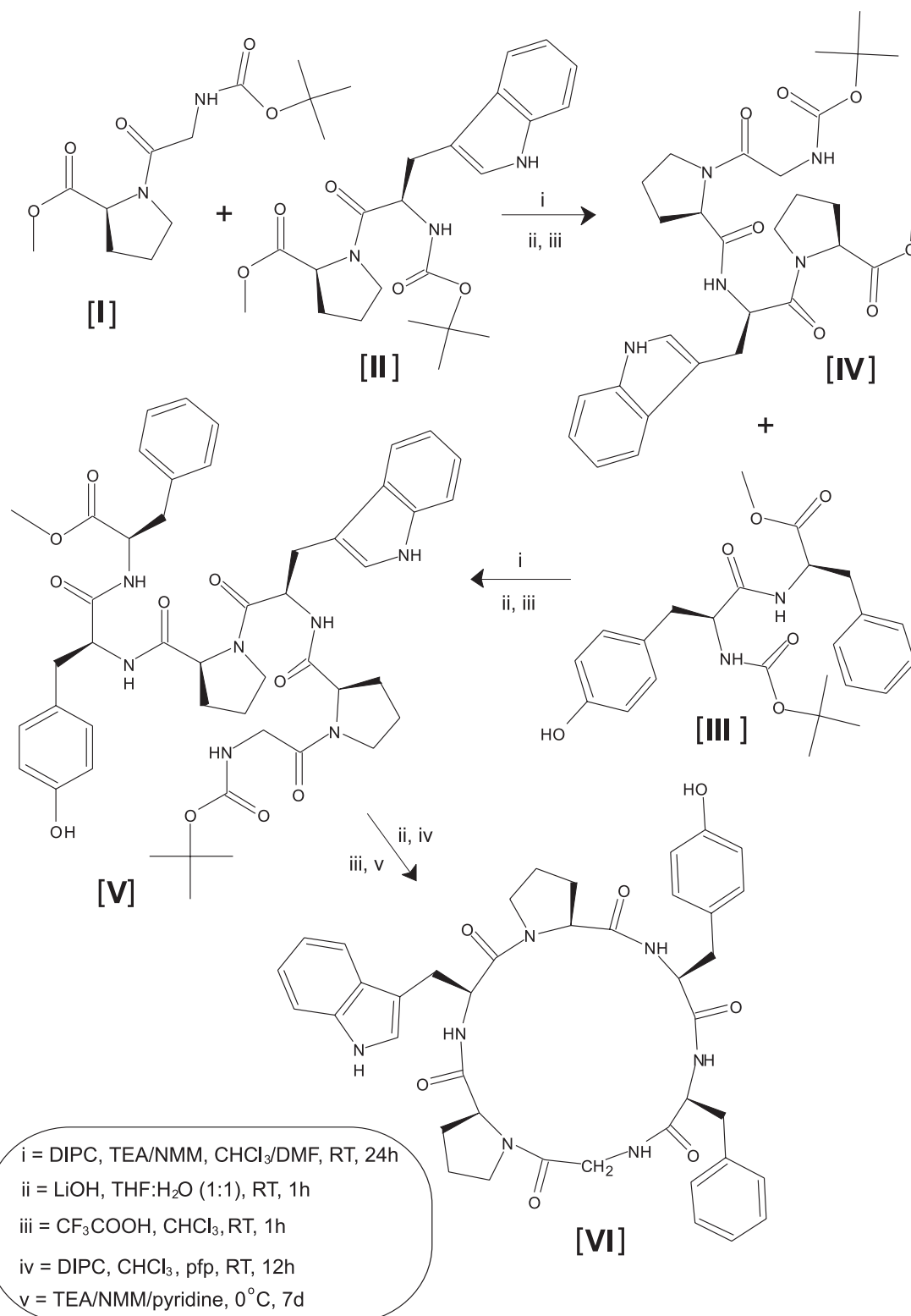


Figure 1. Synthesis of cyclic hexapeptide – diandrine A [VI]

of test compound were determined by tube dilution technique using DMF and DMSO. A spore suspension in sterile distilled water was prepared from 5 days old culture of the test bacteria/fungi growing on nutrient broth media/Sabouraud's broth media. About 20 mL of the growth medium was transferred into sterilized Petri plates and inoculated with 1.5 mL of the spore suspension (spore concentration – 6×10^4 spores/mL). Filter paper disks of 6 mm diameter and 2 mm thickness were sterilized by autoclaving at 121°C for 15 min. Each Petri plate was divided into five equal portions along the diameter to place one disc. Three discs of test sample were placed on three portions together with one disc with reference drugs ciprofloxacin and griseofulvin and a disk impregnated with the solvent (DMF/DMSO) as negative control. Reference drugs were also tested at the same concentration of 10 mg/mL. The Petri plates inoculated with bacterial/fungal cultures were incubated at 37°C for 18 h and 48 h, respectively. Diameters of the zones of inhibition (in mm) were measured and the average diameters for test sample were calculated of triplicate sets. The diameters obtained for the test sample were compared with that produced by the standard drug.

Anthelmintic activity studies

Anthelmintic activity studies were carried out using Garg's method (17) against different species of earthworms like *M. konkanensis*, *P. corethruses* and *Eudrilus sp.* at 2 mg/mL concentration. Suspensions of samples were prepared by triturating synthesized compounds (100 mg) with Tween 80 (0.5%) and distilled water and the resulting mixtures were stirred using a mechanical stirrer for 30 min. The suspensions were diluted to contain 0.2% w/v of the test samples. Suspension of reference drugs, mebendazole and piperazine citrate were prepared with the same concentration in a similar way. Three sets of five earthworms of almost similar sizes (2

inch in length) were placed in Petri plates of 4 inch diameter containing 50 mL of suspension of test sample and reference drug at room temp. Another set of five earthworms was kept as control in 50 mL suspension of distilled water and Tween 80 (0.5%). The paralyzing and death times were noted and their mean was calculated for triplicate sets.

Detailed experimental procedures of antimicrobial and antihelmintic activity studies are given in our previously published reports (18, 19).

RESULTS

Chemistry

In the present investigation, first total synthesis of diandrine A was accomplished using disconnection strategy (20). The cyclic hexapeptide molecule was split into three dipeptide units Boc-Gly-Pro-OMe [I], Boc-Trp-Pro-OMe [II] and Boc-Tyr-Phe-OMe [III]. The required dipeptide units I-III were prepared by coupling of Boc-amino acids viz. Boc-Gly, Boc-Trp and Boc-Tyr with corresponding amino acid methyl ester hydrochlorides such as Pro-OMe.HCl and Phe-OMe.HCl employing diisopropylcarbodiimide (DIPC) as coupling agent. Ester group of dipeptide I was removed by alkaline hydrolysis with LiOH and amino group of dipeptide II was removed by using trifluoroacetic acid. Both the deprotected dipeptides were coupled together using DIPC as coupling agent and *N*-methylmorpholine (NMM) as base, to get the tetrapeptide unit Boc-Gly-Pro-Trp-Pro-OMe [IV]. Similarly, dipeptide III after deprotection at amino end, was coupled with tetrapeptide IV deprotected at carboxyl terminal, to get the linear hexapeptide unit Boc-Gly-Pro-Trp-Pro-Tyr-Phe-OMe [V]. The ester group of linear fragment was removed using LiOH and pentafluorophenyl (pfp) ester group was introduced. The Boc-group was removed using CF₃COOH and deprotected linear fragment was now cyclized by

Table 1. Antimicrobial activity data for compound VI.

Compd.	Diameter of zone of inhibition (mm)							
	Bacterial strains				Fungal strains			
	<i>B. subtilis</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>E. coli</i>	<i>C. albicans</i>	<i>M. audouinii</i>	<i>A. niger</i>	<i>T. mentagrophytes</i>
VI	9(12.5) ^a	11(25)	18(6)	12(12.5)	13(6)	18(6)	-	20(6)
Control	-	-	-	-	-	-	-	-
Ciprofloxacin	20(6)	19(12.5)	25(6)	20(12.5)	-	-	-	-
Griseofulvin	-	-	-	-	20(6)	19(6)	18(12.5)	21(6)

^a Values in brackets are MIC values (g/mL).

Table 2. Anthelmintic activity data for compound VI.

Compd.	Earthworm species					
	<i>M. konkanensis</i>		<i>P. corethruses</i>		<i>Eudrilus sp.</i>	
	Mean paralyzing time (min) ^a	Mean death time (min)	Mean paralyzing time (min)	Mean death time (min)	Mean paralyzing time (min)	Mean death time (min)
VI ^b	07.32 ± 0.44	09.36 ± 0.29	15.09 ± 0.28	17.44 ± 0.39	08.22 ± 0.43	10.25 ± 0.37
Control ^c	–	–	–	–	–	–
Mebendazole ^b	10.52 ± 0.62	12.57 ± 0.49	18.02 ± 0.58	19.49 ± 0.37	11.29 ± 0.40	13.37 ± 0.42
Piperazine citrate ^b	12.38 ± 0.49	13.55 ± 0.27	19.17 ± 0.44	22.17 ± 0.26	12.45 ± 0.19	13.44 ± 0.36

^a Data are given as the mean ± S.D. (n = 3); bc = 2 mg/mL; c 0.5% Tween 80 in distilled water.

keeping the whole contents at 0°C for 7 days in presence of catalytic amount of TEA/NMM/pyridine to get cyclic compound VI (Fig. 1). Structure of the newly synthesized cyclic hexapeptide as well as intermediates linear di/tetra/hexapeptides were confirmed by IR, ¹H NMR, ¹³C NMR as well as elemental analysis. In addition, mass spectrum was recorded for the synthesized cyclohexapeptide.

Pharmacology

Synthesized cyclopeptide VI was screened for in vitro antimicrobial activity against Gram-positive bacteria *B. subtilis* and *S. aureus*, Gram-negative bacteria *P. aeruginosa* and *E. coli*, cutaneous fungi *M. audouinii* and *T. mentagrophytes*, diamorphic fungi *C. albicans* and *A. niger* and for anthelmintic activity against earthworms *M. konkanensis*, *P. corethruses* and *Eudrilus sp.* The results of pharmacological activity studies are tabulated in Tables 1 and 2.

DISCUSSION AND CONCLUSION

Synthesis of diandrine A was carried out successfully with good yield and NMM was proved to be a yield effective base for cyclization of linear hexapeptide fragment. Structure of synthesized peptide was confirmed by spectral as well as elemental analyses.

Disappearance of absorption bands at 1749, 1271 cm⁻¹ and 1389, 1365 cm⁻¹ (C=O_{str} and C-O_{str}, methyl ester group and C-H_{bend}, t-butyl group) in FT-IR spectrum of VI clearly indicated cyclization of linear hexapeptide unit. This fact was further supported by disappearance of two singlets at δ 1.53 and 3.54 ppm, corresponding to protons of t-butyl and methyl ester groups, in ¹H NMR spectrum and disappearance of singlets at δ 79.5, 28.6 and 51.9 ppm, corresponding to carbon atoms of t-butyl and methyl

ester groups, in ¹³C NMR spectrum of VI. Six signals between δ 5.65-3.84 ppm in the proton spectrum of VI suggested a peptidic structure for the synthesized product, with these signals being attributable to the α-protons of all amino acid units. The ¹H NMR spectrum of cyclized product showed presence of two broad singlets between δ 9.89-7.75, 6.91 ppm corresponding to the imino protons of the tyrosine, tryptophan, phenylalanine and glycine moieties, remaining amino acids being two proline units, indicating similarity of the structure of the newly synthesized cyclohexapeptide with the natural molecule. Moreover, ¹H/¹³C NMR spectra of the cyclized product VI showed characteristic peaks confirming presence all the 45 protons and 41 carbon atoms. The presence of pseudomolecular ion peak at m/z 748.8 corresponding to the molecular formula C₄₁H₄₅N₇O₇ in mass spectra of VI, along with other fragment ion peaks resulting from cleavage at 'Phe-Gly', 'Pro-Tyr', 'Pro-Trp', 'Gly-Pro' and 'Phe-Tyr' amide bond levels, showed exact sequence of attachment of all the six amino acid units in a chain. In addition, elemental analysis data of VI afforded values (± 0.03) strictly in accordance to the molecular composition.

Synthesized cyclopeptide exhibited potent anthelmintic activity against *M. konkanensis*, *P. corethruses* and *Eudrilus sp.* at 2 mg/mL, in comparison to standard drugs – mebendazole and piperazine citrate. Moreover, compound VI showed better activity against dermatophytes *M. audouinii*, *T. mentagrophytes* and moderate level of activity against pathogenic microbes *P. aeruginosa*, *E. coli* and *C. albicans*. Gram-negative bacteria were found to be more sensitive towards compound VI in comparison to Gram-positive bacteria. On passing toxicity tests, synthesized cyclopeptide VI may prove good candidate for clinical studies and can be new anthelmintic and antidermatophyte drug of the future.

Acknowledgments

The authors are thankful to Head, U.S.I.C., DU, Delhi and Head, R.S.I.C., I.I.T., Delhi for spectral analyses. Also, great thanks to Head, C.P.C.R.I., Kasaragod, Kerala for providing earthworms for testing antihelmintic activity.

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Received: 1. 07. 2016