Acta Poloniae Pharmaceutica – Drug Research, Vol. 64 No. 6 pp. 509–516, 2007 ISSN 0001-6837
Polish Pharmaceutical Society

DRUG SYNTHESIS

SYNTHESIS OF A PHENYLALANINE-RICH PEPTIDE AS POTENTIAL ANTHELMINTIC AND CYTOTOXIC AGENT

RAJIV DAHIYA*

Department of Pharmaceutical Chemistry, Rajiv Academy for Pharmacy,
Mathura – 281 001, UP, India

Abstract: A natural cyclic heptapeptide segetalin D [**VIII**] was synthesized by coupling of tripeptide unit Boc-Pro-Gly-Leu-OMe [**V**] with tetrapeptide unit Boc-Ser-Phe-Ala-Phe-OMe [**VI**] after proper deprotection at carboxyl and amino ends followed by cyclization of linear peptide segment. Structure of **VIII** was confirmed by spectral and elemental analyses. The newly synthesized cyclopeptide was tested for its antibacterial, antifungal, anthelmintic and cytotoxic activities. Compound **VIII** showed high cytotoxicity against *Dalton's lymphoma ascites* (DLA) and *Ehrlich's ascites carcinoma* (EAC) cell lines with CTC_{50} values of 7.54 and 13.56 μM. Moreover, **VIII** exhibited potent anthelmintic activity against earthworms *Eudrilus species*, *Megascoplex konkanensis* and *Pontoscotex corethruses* at a dose of 2 mg/mL.

Keywords: segetalin D, cyclic heptapeptide, antimicrobial activity, anthelmintic activity, cytotoxicity.
Abbreviations: str – stretching, bend – bending (deformation), oop – out-of-plane, s – strong, m – medium, w – weak, m/br – medium/broad (IR spectra); s – singlet, d – doublet, t – triplet, m – multiplet, br. s – broad singlet, tt – triplet overlapped over triplet (NMR spectra).

During last three decades, plants have well proved their potential to produce a wide array of natural products with interesting biological activities (1-6). Among these, cyclopolypeptides (7) and related congeners (8-10) have received special attention due to their unique structures and wide pharmacological profile which may prove better candidates to overcome the problem of wide spread increase of resistance towards conventional drugs. Diverse bioactivities exhibited by plant-derived cyclic peptides include cytotoxic activity (11,12), immunosuppressive and antimalarial activity (13), vasorelaxant activity (14,15), tyrosinase inhibitory activity (16) and estrogen-like activity (17). A natural cyclic polypeptide, segetalin D, was isolated from the seeds of higher plant *Vaccaria segetalis* (Caryophyllaceae) which have been used to activate blood flow and for treatment of amenorrhea and breast infections. Structure of the isolated peptide was elucidated by 2D-NMR and chemical degradation (18).

As part of ongoing efforts on synthetic aspects of bioactive cyclic peptides (19-22), the present investigation was aimed at the synthesis of novel cyclic heptapeptide segetalin D. Keeping in view significant bioactivities possessed by various cyclopeptides, the above synthetic peptide was further subjected to antibacterial, antifungal, anthelmintic and cytotoxic activity studies.

EXPERIMENTAL

Materials and methods

All the reactions requiring anhydrous conditions were conducted in flame dried apparatus. Melting point was determined by open capillary method and was uncorrected. L-Amino acids, dicyclohexylcarbodiimide (DCC), trifluoroacetic acid (TFA), *p*-nitrophenol (PNP), N-methylmorpholine (NMM), triethylamine (TEA), di-*tert*-butylpyrocarbonate (Boc_2O) and pyridine (C_5H_5N) were obtained from Spectrochem Limited (Mumbai, India). IR spectra were recorded on Shimadzu 8700 FTIR spectrophotometer (Shimadzu, Japan) using a thin film supported on KBr pellets for synthesized cyclopeptide and $CHCl_3$ as solvent for intermediate semisolids. ^1H NMR and ^{13}C NMR spectra were recorded on Bruker AC NMR spectrometer (300 MHz), (Bruker, USA) using $CDCl_3$ as solvent and tetramethylsilane (TMS) 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 all compounds were performed on Vario

* Correspondence: e-mail: rajivdahiya04@yahoo.co.in, rajivdahiya77@rediffmail.com

EL III elemental analyzer (Elementar, Germany). Optical rotation of the synthesized peptides was measured on automatic polarimeter (Optics Tech, Ghaziabad, India) in a 2 dm tube at 25°C using sodium lamp and methanol as solvent. Purity of synthesized cyclopeptide as well as intermediates was checked by TLC on precoated silica gel G plates utilizing $CHCl_3$/MeOH as developing solvent system in different ratios (9:1/8:2 v/v) and brown spots were detected on exposure to iodine vapors in a tightly closed chamber.

General method for the synthesis of linear peptide fragments **I-VII**

Amino acid methyl ester hydrochloride/peptide methyl ester (0.01 mol) was dissolved in $CHCl_3$ (20 mL). To this, NMM (2.23 mL, 0.021 mol) was added at 0°C and the reaction mixture was stirred for 15 min. Boc-amino acid/peptide (0.01 mol) in $CHCl_3$ (20 mL) and DCC (2.1 g, 0.01 mol) were added with stirring. After 24 h, the reaction mixture was filtered and the residue was washed with $CHCl_3$ (30 mL) and added to the filtrate. The filtrate was washed with 5% $NaHCO_3$ and saturated NaCl solutions. The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated in vacuum. The crude product was recrystallized from a mixture of chloroform and petroleum ether followed by cooling at 0°C.

The carboxyl group of L-amino acids was protected by esterification with methanol using thionyl chloride. Furthermore, trifluoroacetic acid was used for the removal of Boc group and ester group was removed by alkaline hydrolysis with lithium hydroxide. Peptides units were synthesized by solution phase technique (23) using DCC as the coupling agent and NMM as the base.

tert-Butyloxycarbonyl-prolyl-glycine methyl ester [**I**]

White crystals, m.p. 79°C, yield 69.9% (2.0 g), $[\alpha]_D$ –11.6°, R_f – 0.79, IR (KBr, cm^{-1}): 3128 (m, -NH str, amide), 2997, 2994 (m, -CH str, cyclic CH_2 and CH), 2927 (m, -CH str, asym, CH_2), 2848 (m, -CH str, sym, CH_2), 1752 (s, -C=O str, ester), 1672, 1635 (s, -C=O str, 3° and 2° amide), 1532 (m, -NH bend, 2° amide), 1390, 1362 (m, -CH bend, *tert*-butyl group), 1270 (s, C-O str, ester); ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 5.76 (1H, br. s, -NH), 3.98-3.96 (2H, d, *J* = 4.8 Hz, CH_2, Gly), 3.86-3.82 (1H, t, α-H, Pro), 3.64 (3H, s, OCH_3), 3.25-3.20 (2H, t, δ-H's, Pro), 2.55-2.46 (2H, m, β-H's, Pro), 1.94-1.86 (2H, m, γ-H's, Pro), 1.48 (9H, s, *tert*-butyl group) ppm, Analysis: calcd. for $C_{13}H_{22}N_2O_5$: C, 54.53; H, 7.74; N, 9.78%. Found: C, 54.49; H, 7.75; N, 9.82%.

tert-Butyloxycarbonyl-seryl-phenylalanine methyl ester [**II**]

Semisolid mass, yield 73.5% (2.69 g), $[\alpha]_D$ +69.6°, R_f – 0.61, IR ($CHCl_3$, cm^{-1}): 3337 (m/br, OH str, Ser), 3135 (m, -NH str, amide), 3075 (w, -CH str, phenyl ring), 2848 (m, -CH str, sym, CH_2), 1750 (s, -C=O str, ester), 1644, 1632 (s, -C=O str, 2° amide), 1584, 1475 (m, skeletal bands, phenyl ring), 1534 (m, -NH bend, 2° amide), 1465 (m, CH bend (scissoring), CH_2), 1390, 1365 (m, -CH bend, *tert-b*utyl group), 1272 (s, C-O str, ester), 715, 694 (s, -CH bend, out of plane, aromatic ring), 666 (m/br, OH bend, out of plane, Ser); ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 7.12-7.07 (1H, t, *p*-H, Phe), 7.04 (1H, br. s, -NH), 7.00-6.96 (2H, m, *m*-H's, Phe), 6.93 (1H, br. s, -NH), 6.86-6.84 (2H, dd, *J* = 6.45 Hz, *o*-H's, Phe), 5.70 (1H, s, -OH, Ser), 4.59-4.52 (1H, m, α-H, Phe), 4.32-4.28 (1H, m, α-H, Ser), 4.06-4.01 (2H, t, β-H's, Ser), 3.55 (3H, s, OCH_3), 2.80-2.78 (2H, d, *J* = 5.9 Hz, β-H's, Phe), 1.54 (9H, s, *tert-b*utyl group). Analysis: calcd. for $C_{18}H_{26}N_2O_6$: C, 59.00; H, 7.15; N, 7.65%. Found: C, 58.97; H, 7.15; N, 7.66%.

tert-Butyloxycarbonyl-alanyl-phenylalanine methyl ester [**III**]

Semisolid mass; yield 78.3% (2.74 g); $[\alpha]_D$ +2.8°; R_f – 0.48; IR ($CHCl_3$, cm^{-1}): 3123 (m, -NH str, amide), 3052 (w, -CH str, arom. ring), 2956, 2926 (m, -CH str, asym, CH_3 and CH_2), 1750 (s, -C=O str, ester), 1643 (s, -C=O str, 2° amide), 1584, 1475 (m, skeletal bands, arom. ring), 1535 (m, -NH bend, 2° amide), 1390, 1367 (m, -CH bend, *tert-b*utyl group), 1269 (s, C-O str, ester), 710, 690 (s, -CH bend, oop, phenyl ring). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 7.13-7.09 (1H, t, *p*-H, Phe), 7.00-6.97 (2H, tt, *m*-H's, Phe), 6.89 (1H, br. s, -NH), 6.86-6.84 (2H, dd, *J* = 6.45 Hz, *o*-H's, Phe), 6.55 (1H, br. s, -NH), 4.60-4.48 (2H, m, α-H's, Phe and Ala), 3.54 (3H, s, OCH_3), 3.00-2.98 (2H, d, *J* = 5.9 Hz, β-H's, Phe), 1.59-1.57 (3H, d, *J* = 4.2 Hz, β-H's, Ala), 1.54 (9H, s, *tert-b*utyl group) ppm, Analysis: calcd. for $C_{18}H_{26}N_2O_5$: C, 61.70; H, 7.48; N, 7.99%. Found : C, 61.73; H, 7.48; N, 7.98%.

tert-Butyloxycarbonyl-prolyl-glycyl-leucine methyl ester [**V**]

Semisolid mass, yield 78.2% (3.12 g), $[\alpha]_D$ –86.0°, R_f – 0.83, IR ($CHCl_3$, cm^{-1}): 3135 (m, -NH str, amide), 2995, 2992 (m, -CH str, cyclic CH_2 and CH), 2929, 2926 (m, -CH str, asym, CH_2), 2852 (m, -CH str, sym, CH_2), 1750 (s, -C=O str, ester), 1668, 1636, 1633 (s, -C=O str, 3° and 2° amide), 1532, 1524 (m, -NH bend, 2° amide), 1468 (m, CH bend

(scissoring), CH_2), 1389, 1360 (m, -CH bend, *tert-b*utyl group), 1365, 1339 (s, -CH bend, isopropyl group), 1270 (s, C-O str, ester), 916 (w, CH_3 rocking, isopropyl group). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 7.52 (1H, br. s, -NH), 6.03 (1H, br. s, -NH), 3.89-3.85 (1H, t, α-H, Pro), 3.77-3.75 (2H, d, *J* = 4.75 Hz, CH_2, Gly), 3.60 (3H, s, OCH_3), 3.50-3.47 (1H, m, α-H, Leu), 3.23-3.19 (2H, t, δ-H's, Pro), 2.52-2.48 (2H, t, β-H's, Pro), 1.95-1.88 (2H, m, γ-H's, Pro), 1.49 (9H, s, *tert-b*utyl group), 1.46-1.41 (1H, m, γ-H, Leu), 1.30-1.27 (2H, t, β-H's, Leu), 0.97-0.95 (6H, d, *J* = 6.0 Hz, δ-H's, Leu) ppm, Anal. Calcd. for $C_{19}H_{33}N_3O_6$: C, 57.13; H, 8.33; N, 10.52. Found : C, 57.09; H, 8.35; N, 10.52%.

tert-Butyloxycarbonyl -seryl-phenylalanyl-alanyl-phenylalanine methyl ester [**VI**]

Semisolid mass, yield 73.1% (4.27 g), $[\alpha]_D$ –94.2°, R_f – 0.39, IR ($CHCl_3$, cm^{-1}): 3334 (m/br, OH str, Ser), 3132 (m, -NH str, amide), 3078, 3073 (w, -CH str, phenyl rings), 2952, 2925, 2922 (m, -CH str, asym, CH_3 and CH_2), 2872, 2869 (m, -CH str, sym, CH_3), 2849, 2845 (m, -CH str, sym, CH_2), 1752 (s, -C=O str, ester), 1642, 1640, 1635 (s, -C=O str, 2° amide), 1583, 1472, 1468 (m, skeletal bands, phenyl rings), 1537, 1532 (m, -NH bend, 2° amide), 1392, 1366 (m, -CH bend, *tert-b*utyl group), 1268 (s, C-O str, ester), 715, 712, 694, 689 (s, -CH bend, oop, aromatic ring), 665 (m/br, OH bend, oop, Ser). ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 8.88 (1H, br. s, -NH), 8.15 (1H, br. s, -NH), 7.19-7.15 (2H, tt, *m*-H's, Phe[1]), 7.12-7.08 (1H, t, *p*-H, Phe[2]), 7.02-6.96 (3H, m, *p*-H, Phe[1] and *m*-H's, Phe[2]), 6.92 (1H, br. s, -NH), 6.87-6.81 (4H, m, *o*-H's, Phe[1] and Phe[2]), 6.78 (1H, br. s, -NH), 5.71 (1H, s, -OH, Ser), 4.33-4.17 (3H, m, α-H's, Ala, Phe[1] and Ser), 4.08-4.03 (2H, t, β-H's, Ser), 3.95-3.89 (1H, m, α-H, Phe[2]), 3.54 (3H, s, OCH_3), 3.01-2.60 (4H, m, β-H's, Phe[1] and Phe[2]), 1.55 (9H, s, *tert-b*utyl group), 1.50-1.48 (3H, d, *J* = 4.15 Hz, β-H's, Ala) ppm, Analysis: calcd. for $C_{30}H_{40}N_4O_8$: C, 61.63; H, 6.90; N, 9.58%. Found: C, 61.64; H, 6.86; N, 9.60%.

tert-Butyloxycarbonyl-prolyl-glycyl-leucyl-seryl-phenylalanyl-alanyl-phenylalanine methyl ester [**VII**]

Semisolid mass, yield 79.0% (6.68 g), $[\alpha]_D$ +61.5°, R_f – 0.43, IR ($CHCl_3$, cm^{-1}): 3338 (m/br, OH str, Ser), 3138, 3120 (m, -NH str, amide), 3076, 3072 (w, -CH str, phenyl rings), 2998, 2993 (m, -CH str, cyclic CH_2 and CH), 2950, 2928, 2924 (m, -CH str, asym, CH_3 and CH_2), 2872, 2848, 2842 (m, -CH str, sym, CH_3 and CH_2), 1754 (s, -C=O str, ester), 1669, 1640 (s, -C=O str, 3° and 2° amide), 1585, 1470, 1469 (m, skeletal bands, phenyl rings), 1534, 1526 (m, -NH bend, 2° amide), 1388, 1369 (m, -CH bend, *tert*-butyl group), 1362, 1340 (s, -CH bend, isopropyl group), 1269 (s, C-O str, ester), 933, 914 (w, CH_3 rocking, isopropyl and *tert-b*utyl groups), 717, 698, 687 (s, -CH bend, oop, aromatic rings), 667 (m/br, OH bend, oop, Ser). ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 8.19, 8.15, 7.98, 7.84, 7.47 (5H, broad singlets, -NH), 7.21-7.16 (2H, tt, *m*-H's, Phe[1]), 7.13-7.09 (1H, t, *p*-H, Phe[2]), 7.02-6.97 (3H, m, *m*-H's, Phe[2] and *p*-H, Phe[1]), 6.87-6.81 (4H, m, *o*-H's, Phe[1] and Phe[2]), 6.78 (1H, br. s, -NH), 6.72 (1H, s, -OH, Ser), 4.32-4.25 (1H, m, α-H, Ala), 4.25-4.21 (1H, m, a-H, Phe[1]), 4.15-4.11 (1H, m, α-H, Ser), 4.10-4.07 (1H, t, α-H, Pro), 4.95-4.88 (1H, m, α-H, Phe[2]), 4.87-4.84 (1H, m, α-H, Leu), 3.82-3.80 (2H, d, *J* = 4.75 Hz, CH_2, Gly), 3.72-3.63 (2H, t, β-H's, Ser), 3.56 (3H, s, OCH_3), 3.24-3.20 (2H, t, δ-H's, Pro), 2.98-2.82 (4H, m, β-H's, Phe[1] and Phe[2]), 2.58-2.53 (2H, m, β-H's, Pro), 1.95-1.72 (4H, m, β-H's, Leu and γ-H's, Pro), 1.51-1.49 (3H, d, *J* = 4.25 Hz, β-H's, Ala), 1.48 (9H, s, *tert-b*utyl group), 1.46-1.38 (1H, m, γ-H, Leu), 1.01-0.99 (6H, d, *J* = 6.15 Hz, δ-H's, Leu) ppm, Analysis: calcd. for $C_{43}H_{61}N_7O_{11}$: C, 60.62; H, 7.22; N, 11.51%. Found: C, 60.59; H, 7.25; N, 11.50%.

Synthesis of cyclic heptapeptide, segetalin D [**VIII**]

To synthesize compound **VIII**, linear octapeptide unit **VII** (0.005 mol) was deprotected at carboxyl end using LiOH (0.18 g, 0.0075 mol) to get Boc-Pro-Gly-Leu-Ser-Phe-Ala-Phe-OH. The deprotected heptapeptide unit (0.005 mol) was now dissolved in $CHCl_3$ (50 mL) at 0°C. To the above solution, *p*-nitrophenol (0.94 g, 0.0067 mol) was added and stirred at RT for 12 h. The reaction mixture was filtered and the filtrate was washed with 10% $NaHCO_3$ solution (3 × 15 mL) until excess of *p*-nitrophenol was removed and finally washed with 5% HCl (2 × 10 mL) to get the corresponding *p*-nitrophenyl ester Boc-Pro-Gly-Leu-Ser-Phe-Ala-Phe-O-PNP. To this compound (0.004 mol) dissolved in $CHCl_3$ (35 mL), CF_3COOH (0.91 g, 0.008 mol) was added, stirred at RT for 1 h and washed with 10% $NaHCO_3$ solution (2 × 25 mL). The organic layer was dried over anhydrous Na_2SO_4 to get Pro-Gly-Leu-Ser-Phe-Ala-Phe-O-PNP which was dissolved in $CHCl_3$ (25 mL) and TEA/NMM/C_5H_5N (2.8 mL/2.21 mL/1.61 mL, 0.02 mol) was added. Then, the whole content was kept at 0°C for 7 days. The reaction mixture was washed with 10% $NaHCO_3$ solution until the byproduct *p*-nitrophenol was removed completely and finally washed with 5% HCl (3 × 15 mL). The organic layer was dried

over anhydrous Na_2SO_4. Finally, chloroform was distilled off and crude cyclized product was crystallized from $CHCl_3$ and n-hexane to get pure cyclo (prolyl-glycyl-leucyl-seryl-phenylalanyl-alanyl-phenylalanyl) [**VIII**].

White solid, m.p. 164-165°C (165-167°C), yield 82.6% (5.95 g, NMM), 64.5% (TEA), 58.2% (C_5H_5N), $[\alpha]_D$ +14.0° (+13.7°), R_f – 0.72, IR (KBr, cm^{-1}): 3332 (m/br, OH str, Ser), 3155, 3136, 3125 (m, -NH str, amide), 3084, 3076 (w, -CH str, phenyl rings), 2995, 2987 (m, -CH str, cyclic CH_2 and CH), 2955, 2932 (m, -CH str, asym, CH_3 and CH_2), 2873, 2846 (m, -CH str, sym, CH_3 and CH_2), 1668, 1637, 1630 (s, -C=O str, 3° and 2° amide), 1589, 1464, 1462 (m, skeletal band, phenyl rings), 1542, 1535, 1522 (m, -NH bend, 2° amide), 1466 (m, CH bend (scissoring), CH_2), 1359, 1342 (s, -CH bend, isopropyl group), 916 (w, CH_3 rocking, isopropyl group), 748-698 (s, -CH bend, oop, aromatic rings), 670 (m/br, OH bend, oop, Ser). ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 9.14, 8.37, 8.20, 7.91, 7.70, 7.54 (6H, broad singlets, -NH), 7.41 (1H, s, -OH, Ser), 7.21-7.15 (4H, m, *m*-H's, Phe^1 and Phe^2), 7.02-6.98 (2H, tt, *p*-H's, Phe^1 and Phe^2), 6.86-6.82 (4H, m, *o*-H's, Phe^1 and Phe^2), 6.15-6.10 (1H, m, α-H, Leu), 5.99-5.92 (1H, m, α-H, Ala), 5.83-5.75 (1H, m, α-H, Ser), 5.73-5.69 (1H, m, α-H, Phe^2), 5.57-5.50 (1H, m, α-H, Phe^1), 4.05-4.02 (2H, t, β-H's, Ser), 3.97-3.95 (2H, d, J = 4.8 Hz, CH_2, Gly), 3.90-3.87 (1H, t, α-H, Pro), 3.26-3.22 (2H, t, δ-H's, Pro), 2.70-2.65 (2H, m, β-H's, Pro), 2.60-2.52 (4H, m, β-H's, Phe^1 and Phe^2), 1.90-1.79 (4H, m, α-H's, Leu and γ-H's, Pro), 1.44-1.42 (3H, d, J = 4.2 Hz, β-H's, Ala), 1.00-0.98 (6H, d, J = 6.1 Hz, d-H's, Leu), 0.88-0.76 (1H, m, γ-H, Leu). ^{13}C NMR ($CDCl_3$, 300 MHz) δ (ppm): 172.8, 172.5 (C=O, Ala and Leu), 171.3, 171.0 (C=O, Phe^1 and Pro), 170.9, 170.2 (C=O, Ser and Phe^2), 169.9 (C=O, Gly), 138.0, 137.4 (γ-C's, Phe^1 and Phe^2), 130.1, 129.2 (*o*-C's, Phe^1 and Phe^2), 128.9, 128.5 (*m*-C's, Phe^1 and Phe^2), 127.2, 126.9 (*p*-C's, Phe^1 and Phe^2), 63.9 (β-C, Ser), 62.3 (α-C, Pro), 57.8 (α-C, Ser), 55.6 (α-C, Phe^1), 53.8 (α-C, Leu), 53.5 (α-C, Phe^2), 50.1 (α-C, Ala), 48.1 (δ-C, Pro), 44.2 (CH_2, Gly), 43.9 (β-C, Leu), 40.2, 36.6 (β-C's, Phe^1 and Phe^2), 29.6 (γ-C, Leu), 28.8, 24.9 (α- and δ-C's, Pro), 22.4 (δ-C's, Leu), 18.9 (β-C, Ala). FAB MS, m/z: 720.8 $(M + H)^+$, 692.8 $(720.8 - CO)^+$, 607.7 (Ser-Phe-Ala-Phe-Pro-Gly)$^+$, 579.6 $(607.7 - CO)^+$, 550.6 (Ser-Phe-Ala-Phe-Pro)$^+$, 522.6 $(550.6 - CO)^+$, 453.5 (Ser-Phe-Ala-Phe)$^+$, 425.5 $(453.5 - CO)^+$, 306.3 (Ser-Phe-Ala)$^+$, 278.3 $(306.3 - CO)^+$, 235.3 (Ser-Phe)$^+$, 207.2 $(235.3 - CO)^+$, 88.1 (Ser)$^+$, 60.1 $(88.1 - CO)^+$, 91.0 $(C_7H_7)^+$, 57.1 $(C_4H_9)^+$, 43.1 $(C_3H_7)^+$, 29.1 $(C_2H_5)^+$, 15.0 $(CH_3)^+$, Analysis: calcd. for $C_{37}H_{49}N_7O_8$: C, 61.74; H, 6.86; N, 13.62%. Found: C, 61.75; H, 6.84; N, 13.60%.

BIOLOGICAL ACTIVITY

Antimicrobial screening

The synthesized cyclopeptide was screened for its antimicrobial activity (24) against four bacterial strains *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* and four fungal strains *Microsporum audouinii*, *Trichophyton mentagrophytes*, *Candida albicans* and *Aspergillus niger* at 10 μg mL^{-1} concentration. MIC values 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^{-1}). 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 drug ciprofloxacin/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^{-1}. 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 screening

Anthelmintic activity studies (25) were carried out against three different species of earthworms *Megascoplex konkanensis*, *Pontoscotex corethruses* and *Eudrilus species* at 2 mg mL^{-1} 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

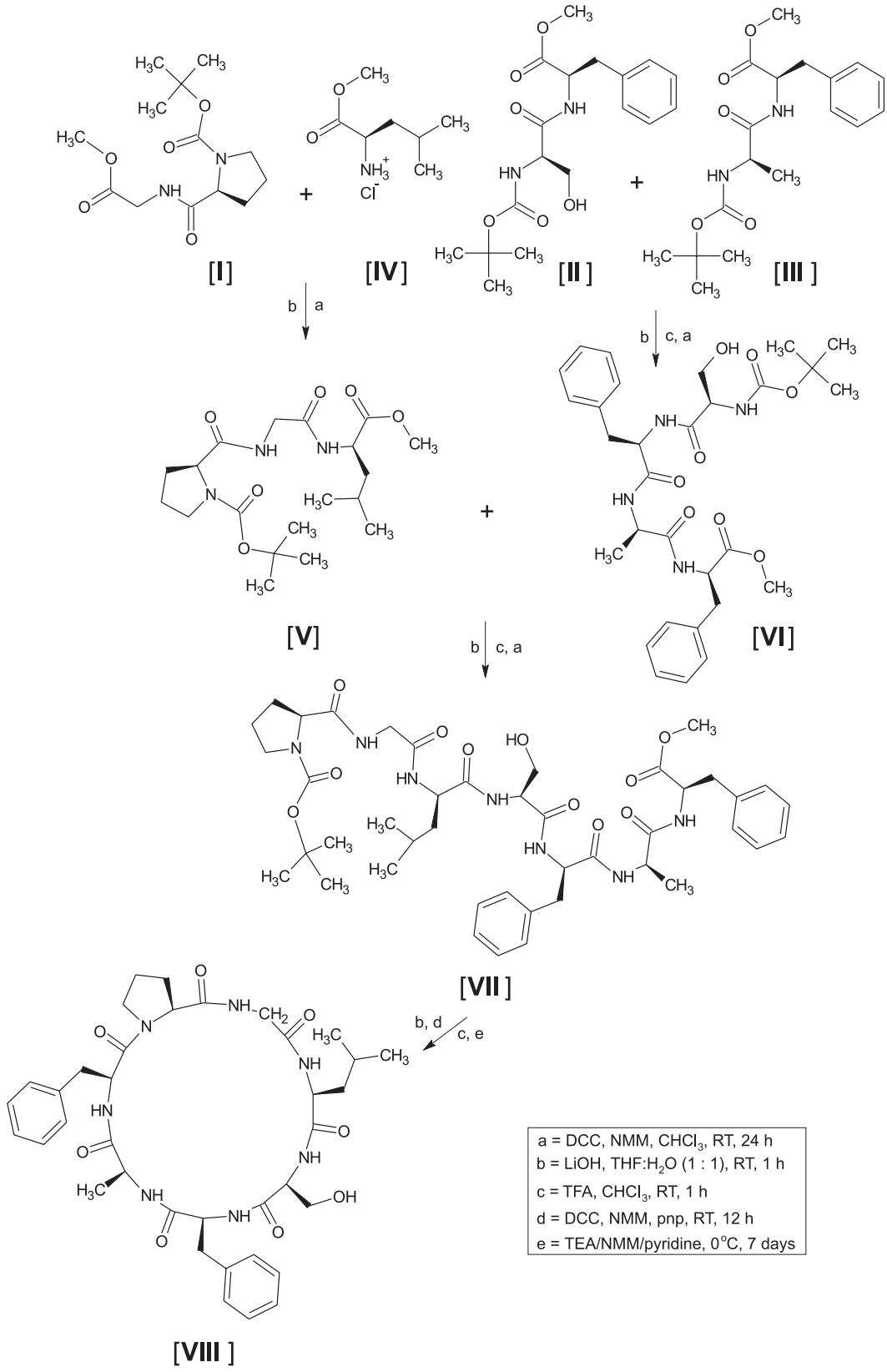

Figure 1. Synthetic pathway for segetalin D (**VIII**).

Table 1. Antimicrobial activity data for compound **VIII**.

Compd.	Diameter of zone of inhibition (mm)							
	Bacterial strains				Fungal strains			
	B. subtilis	*S. aureus*	*P. aeruginosa*	*E. coli*	*C. albicans*	*M. audouinii*	*A. niger*	*T. mentagrophytes*
VIII	8 (25)[a]	–	21(6)	16 (12.5)	17 (6)	–	9 (12.5)	11(12.5)
Control	–	–	–	–	–	–	–	–
Ciprofloxacin	20 (6)	20 (12.5)	25 (6)	19 (12.5)	–	–	–	–
Griseofulvin	–	–	–	–	20 (6)	18 (6)	18 (12.5)	20 (6)

[a] Values in brackets are MIC values (μg mL^{-1}).

Table 2. Anthelmintic activity data for compound **VIII**.

Compd.	Earthworm species					
	M. konkanensis		*P. corethruses*		*Eudrilus sp.*	
	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)
VIII[b]	08.25 ± 0.53	10.52 ± 0.42	12.27 ± 0.31	16.34 ± 0.59	07.46 ± 0.27	10.02 ± 0.19
Control[c]	–	–	–	–	–	–
Mebendazole[b]	10.55 ± 0.64	12.59 ± 0.53	17.58 ± 1.03	19.42 ± 1.20	11.35 ± 0.45	13.46 ± 0.62
Piperazine citrate[b]	12.39 ± 0.36	13.52 ± 0.49	19.06 ± 0.57	22.23 ± 0.78	12.46 ± 0.15	13.58 ± 0.47

[a] Data are given as the mean ± S.D. (n = 3); [b] c = 2 mg mL^{-1}; [c] 0.5% Tween 80 in distilled water.

Table 3. Cytotoxic activity data for compound **VIII**.

Compd.	Conc. (μg/mL)	DLA cells				EAC cells			
		Live cells counted	No. of dead cells	% growth inhibition[a]	CTC_{50}[b] ((μM)	Live cells counted	No. of dead cells	% growth inhibition	CTC_{50} (μM)
VIII	62.5	0	38	100.0		0	28	100.0	
	31.25	2	36	94.74		2	24	92.86	
	15.63	4	34	89.47	7.54	11	17	60.71	13.56
	7.81	16	22	57.89		15	13	46.43	
	3.91	21	17	44.74		24	4	14.29	
Control	62.5	38	0	–		28	0	–	
	31.25	38	0	–		28	0	–	
	15.63	38	0	–	–	28	0	–	–
	7.81	38	0	–		28	0	–	
	3.91	38	0	–		28	0	–	
Standard (5-FU)	62.5	0	38	100.0		0	28	100.0	
	31.25	0	38	100.0		0	28	100.0	
	15.63	10	28	73.68	37.36	11	17	60.71	90.55
	7.81	13	25	65.79		19	9	32.14	
	3.91	22	16	42.11		23 5	17.86		

[a] % growth inhibition = $100 - [\{(Cell_{total} - Cell_{dead}) \times 100\} / Cell_{total}]$; [b] CTC_{50} = cytotoxic conc. inhibiting 50% of percentage growth.

inch in length) were placed in Petri plates of 4 inch diameter containing 50 mL of suspension of test sample and reference drug at RT. 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 means were calculated for triplicate sets. The death time was ascertained by placing the earthworms in warm water (50°C) which stimulated the movement, if the worm was alive.

Cytotoxicity screening

Synthesized cyclopeptide **VIII** was subjected to short term *in vitro* cytotoxicity study (26) at 62.5–3.91 μg mL^{-1} using 5-fluorouracil (5-FU) as reference compound. Activity was assessed by determining the percentage inhibition of DLA and EAC cells. Both cells were cultured in the peritoneal cavity of healthy albino mice by injecting the suspension of cells (1×10^6 cells/mL) intraperitoneally. After 15-20 days, cells were withdrawn from the peritoneal cavity of the mice with help of a sterile syringe and counted using hemocytometer and adjusted to 1×10^6 cells/mL. Different dilutions of synthesized compound **VIII** ranging from 62.5–3.91 μg mL^{-1} were prepared in Dulbecco's minimum essential medium and 0.1 mL of each diluted test compound was added to 0.1 mL of DLA cells (1×10^6 cells/mL) and EAC cells (1×10^6 cells/mL). The resulted suspensions were incubated at 37°C for 3 h. After 3 h, tryphan blue dye exclusion test was performed and the percentage growth inhibition was calculated. CTC_{50} values were determined by graphical extrapolation method. Controls were also tested at 62.5–3.91 μg mL^{-1} against both cell lines.

RESULTS

Chemistry

In the present work, disconnection strategy was employed to carry out the first total synthesis of segetalin D. The cyclic heptapeptide molecule was split into three dipeptide units Boc-Pro-Gly-OMe [**I**], Boc-Ser-Phe-OMe [**II**], Boc-Ala-Phe-OMe [**III**] and single amino acid unit Leu-OMe.HCl [**IV**]. The required dipeptide units **I-III** were prepared by coupling of Boc-amino acids viz. Boc-Pro, Boc-Ser and Boc-Ala with corresponding amino acid methyl ester hydrochlorides such as Gly-OMe·HCl and Phe-OMe·HCl employing dicyclohexylcarbodiimide (DCC) as coupling agent. Ester group of dipeptide **I** was removed by alkaline hydrolysis with LiOH and deprotected peptide was coupled with amino acid methyl ester hydrochloride **IV** using DCC and N-methylmorpholine (NMM) as the base, to get the tripeptide unit Boc-Pro-Gly-Leu-OMe [**V**]. Similarly, dipeptide **II** after deprotection at the carboxyl end, was coupled with dipeptide **III** deprotected at the amino terminal, to get the tetrapeptide unit Boc-Ser-Phe-Ala-Phe-OMe [**VI**]. After removal of ester and Boc group of tripeptide **V** / tetrapeptide **VI**, deprotected units were coupled with each other to get the linear heptapeptide unit Boc-Pro-Gly-Leu-Ser-Phe-Ala-Phe-OMe [**VII**]. The ester group of the linear fragment was removed using LiOH and *p*-nitrophenyl (PNP) ester group was introduced. The Boc-group was removed using CF_3COOH and deprotected linear fragment was now cyclized by keeping the whole contents at 0°C for 7 days in a presence of catalytic amount of TEA/NMM/pyridine to get cyclic compound **VIII** (Figure 1).

The structure of the newly synthesized cyclic heptapeptide as well as intermediates linear di/tri/tetra/heptapeptides were confirmed by IR, ^1H NMR as well as elemental analysis. In addition, ^{13}C NMR and mass spectra were recorded for the cyclopeptide.

Pharmacology

Synthesized compound **VIII** 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* by Kirby-Bauer disk diffusion method (24) and for anthelmintic activity against earthworms *Megascoplex konkanensis, Pontoscotex corethruses* and *Eudrilus species* by Garg method (25).The synthesized cyclopeptide was also subjected to short term *in vitro* cytotoxicity study against DLA and EAC cell lines by Kuttan method (26). The results of biological activity studies are presented in Tables 1-3.

DISCUSSION AND CONCLUSION

The synthesis of segetalin D was carried out successfully with good yield and NMM was proved to be a yield effective base for cyclization of linear heptapeptide fragment. The structure of the synthesized peptide was confirmed by spectral as well as elemental analyses. Cyclization of linear peptide fragment was indicated by disappearance of absorption bands at 1754 cm^{-1} and 1388, 1369 cm^{-1} (-C=O stretching of ester and -CH bending of *tert*-butyl group) and presence of additional amide I and amide II bands of the -CO-NH- moiety at 1630 cm^{-1} and

1545-1542 cm^{-1} in the IR spectra of compound **VIII**. The formation of cyclopeptide was further confirmed by disappearance of a singlet at 1.48 ppm corresponding to nine protons of *tert-b*utyl group of Boc, in ^{1}H NMR spectrum of compound **VIII**. Furthermore, ^{1}H NMR and ^{13}C NMR spectra of the synthesized cyclic heptapeptide showed characteristic peaks confirming the presence of all 49 protons and 37 carbon atoms. The presence of $(M + 1)^{+}$ ion peak at m/z 720.8 corresponding to the molecular formula $C_{37}H_{49}N_7O_8$ in mass spectra of compound **VIII**, along with other fragment ion peaks resulting from the cleavage at Leu-Ser amide bond level, showed an exact sequence of attachment of all the eight amino acid moieties in a chain. In addition, elemental analysis of compound **VIII** afforded values (± 0.02) strictly in accordance with the molecular composition.

The synthesized cyclopeptide possessed high cytotoxic activity against DLA and EAC cell lines with CTC_{50} values of 7.54 and 13.56 μM, respectively, in comparison to standard drug 5-fluorouracil (5-FU) (CTC_{50} values – 37.36 and 90.55 μM) and potent anthelmintic activity against *M. konkanensis*, *P. corethruses* and *Eudrilus sp*. at 2 mg mL^{-1}, in comparison to the standard drugs, mebendazole and piperazine citrate. Moreover, compound **VIII** showed moderate level of activity against pathogenic microbes *P. aeruginosa*, *E. coli* and *C. albicans*. Gram positive bacteria were found to be resistant towards compound **VIII** in comparison to sensitive Gram negative bacteria. On passing toxicity tests, the synthesized cyclopeptide VIII may prove a good candidate for clinical studies and can be new anthelmintic and cytotoxic 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 analysis. Also, great thanks to Head, J.S.S. College of Pharmacy, Ooty for carrying out the cytotoxicity studies and Head, C.P.C.R.I., Kasaragod, Kerala for providing earthworms for testing anthelmintic activity.

REFERENCES

1. Morita H., Ishioka N., Takatsu H., Iizuka T., Kobayashi J.: J. Nat. Prod. 69, 418 (2006).
2. Hsieh P.W., Chang F.R., Wu C.C., Wu K.Y., Li C.M., Chen S.L., Wu Y.C.: J. Nat. Prod. 67, 1522 (2004).
3. Jayasinghe U.L., Puvanendran S., Hara N., Fujimoto Y.: Nat. Prod. Res. 18, 571 (2004).
4. Tiew P., Ioset J.R., Kokpol U., Chavasiri W., Hostettmann K.: Phytother. Res. 17, 190 (2003).
5. Bao G.H., Wang L.Q., Cheng K.F., Feng Y.H., Li X.Y., Qin G.W.: Planta Med. 69, 434 (2003).
6. Zamilpa A., Tortoriello J., Navarro V., Delgado G., Alvarez L.: Planta Med. 68, 281 (2002).
7. Tan N.H., Zhou J.: Chem. Rev. 106, 840 (2006).
8. Morel A.F., Maldaner G., Ilha V., Missau F., Silva U.F., Dalcol I.I.: Phytochemistry 66, 2571 (2005).
9. Gustafson K.R., McKee T.C., Bokesch H.R.: Curr. Protein Pept. Sci. 5, 331 (2004).
10. Jennings C., West J., Waine C., Craik D., Anderson M.: Proc. Natl. Acad. Sci. USA. 98, 10614 (2001).
11. Mongkolvisut W., Sutthivaiyakit S., Leutbecher H., et al.: J. Nat. Prod. 69, 1435 (2006).
12. Hsieh P.W., Chang F.R., Wu C.C., et al.: Chem. Pharm. Bull. (Tokyo) 53, 336 (2005).
13. Picur B., Cebrat M., Zabrocki J., Siemion I.Z.: J. Pept. Sci. 12, 569 (2006).
14. Morita H., Iizuka T., Choo C.Y., Chan K.L., Takeya K., Kobayashi J.: Bioorg. Med. Chem. Lett. 16, 4609 (2006).
15. Morita H., Iizuka T., Choo C.Y., Chan K.L., Itokawa H., Takeya K.: J. Nat. Prod. 68, 1686 (2005).
16. Morita H., Kayashita T., Kobata H., Gonda A., Takeya K.: Tetrahedron. 50, 9975 (1994).
17. Yun Y.S., Morita H., Takeya K., Itokawa H.: J. Nat. Prod. 60, 216 (1997).
18. Morita H., Yun Y.S., Takeya K., Itokawa H., Yamada K.: Tetrahedron 51, 6003 (1995).
19. Dahiya R., Pathak D.: Asian. J. Chem. 19, 1499 (2007).
20. Dahiya R., Pathak D.: J. Pharm. Res. 5, 69 (2006).
21. Dahiya R., Pathak D., Himaja M., Bhatt S.: Acta Pharm. 56, 399 (2006).
22. Dahiya R., Pathak D.: J. Serb. Chem. Soc. 72, 101 (2007).
23. Bodanszky M., Bodanszky A.: The Practice of Peptide Synthesis, p. 143, Springer-Verlag, New York 1984.
24. Bauer A.W., Kirby W.M., Sherris J.C., Turck M.: Am. J. Clin. Path. 45, 493 (1966).
25. Garg L.C., Atal C.K.: Ind. J. Pharm. Sci. 59, 240 (1963).
26. Kuttan R., Bhanumathy P., Nirmala K., George M.C.: Cancer Lett. 29, 197 (1985).

Received: 14.03.2007