

Full Length Research Paper

# Synthetic and pharmacological studies on a natural cyclopeptide from *Gypsophila arabica*

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The present study deals with the synthesis of a novel proline-rich cyclopeptide - gypsin B 7 via coupling of tripeptide units Boc-L-Tyr-L-Phe-L-Pro-OH and Gly-L-Leu-L-Pro-OMe utilizing two different carbodiimides, followed by cyclization of linear polypeptide fragment. Structure elucidation of newly synthesized peptide was done on basis of FT-IR, <sup>1</sup>H-NMR, <sup>13</sup>C-NMR, ESI-MS/MS data. From biological evaluation, it was concluded that cyclic hexapeptide 7 exhibited potent antidermatophyte activity against pathogenic *Microsporum audouinii* and *Trichophyton mentagrophytes*. Also, good bioactivity against pathogenic *Candida albicans* and gram-negative bacteria, and moderate antihelmintic activity against three species of earthworms was observed for newly synthesized cyclohexapeptide.

**Key words:** *Gypsophila arabica*, cyclic hexapeptide, gypsin B, peptide coupling, antimicrobial activity, antihelmintic activity.

## INTRODUCTION

Diverse reports in past literature have proved the potential of roots of plants to produce a range of natural products with different pharmacological activities (Tan and Zhou, 2006). Among these, cyclic peptides have received special attention due to their wide pharmacological profile and may prove better candidates to overcome the problem of resistance towards conventional drugs. A novel cyclic hexapeptide, gypsin B has been isolated from roots of *Gypsophila arabica* (Caryophyllaceae) and its structure was elucidated from the spectroscopic and chemical evidence. The stereochemistry of all amino acid residues was found to be in the L-configuration by GC analysis (Bruzual De Abreu et al., 2008). Although, syntheses of natural products have remained a challenge for scientists since decades due to diverse complicated moieties present in their structures, yet syntheses of cyclopolypeptides employing solid as well as solution-phase techniques is carried out frequently by various research groups (Pettit

et al., 2000; Hamada and Shioiri, 2005; Stawikowski and Cudic, 2007; Morris and Phillips, 2010). As part of our continuing synthetic efforts on biologically active peptides (Dahiya et al., 2006; Dahiya and Kaur, 2007; Dahiya and Pathak, 2007; Dahiya and Sharma, 2008; Dahiya and Kumar, 2008; Dahiya et al., 2009; Dahiya and Gautam, 2010), the present investigation was aimed at the synthesis of a novel cyclic hexapeptide of plant origin. Keeping in view of significant bioactivities possessed by natural cyclopolypeptides (Dahiya and Pathak, 2006; Pathak and Dahiya, 2003), the above synthetic peptide was further subjected to antibacterial, antifungal and antihelmintic activity studies.

## MATERIALS AND METHODS

### General experimental part

Melting point was determined by open capillary method and is uncorrected. L-Amino acids, di-*tert*-butyldicarbonate (Boc<sub>2</sub>O), dicyclohexylcarbodiimide (DCC), diisopropylcarbodiimide (DIPC), trifluoroacetic acid (TFA), triethylamine (TEA), pyridine, *N,N*-dimethylformamide (DMF) and *N*-methylmorpholine (NMM) were purchased from Spectrochem Limited (Mumbai, India). The chemical structures of all newly synthesized compounds were

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elucidated by means of spectral as well as elemental analysis. The IR spectra were run on Shimadzu 8700 FTIR spectrophotometer using a thin film supported on KBr pellets or utilizing chloroform.  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra were recorded on Bruker AC NMR spectrometer using DMSO- $d_6$  as solvent. The mass spectra of the cyclopeptide was recorded on JMS-DX 303 Mass spectrometer (Jeol, Tokyo, Japan) operating at 70 eV by ESI-MS/MS. Optical rotation of synthesized peptide derivatives was measured on automatic polarimeter in a 2 dm tube at 25°C. Elemental analyses of all peptides were performed on Vario EL III elemental analyzer. Purity of all synthesized compounds was checked by TLC on precoated silica gel G plates utilizing chloroform / methanol as developing solvent system in different ratios (7:3 / 9:1 v/v).

#### General method for preparation of di/tripeptide intermediates

L-Amino acid methyl ester hydrochloride/dipeptide methyl ester (10 mmol) was dissolved in dichloromethane (DCM, 20 ml). To this, NMM (2.21 ml, 20 mmol) was added at 0°C and the reaction mixture was stirred for 15 min. Boc-L-amino acid (10 mmol) in DCM (20 ml) and DIPC/DCC (1.26 g/2.1 g, 10 mmol) were added with stirring. After 24 h, the reaction mixture was filtered and the residue was washed with DCM (30 ml) and added to the filtrate. The filtrate was washed with 5%  $\text{NaHCO}_3$  and saturated NaCl solutions. The organic layer was dried over anhydrous  $\text{Na}_2\text{SO}_4$ , filtered and evaporated in vacuum. The crude product was recrystallized from a mixture of chloroform and petroleum ether (b.p. 40 to 60°C) followed by cooling at 0°C.

*tert-Butyloxycarbonyl-L-tyrosinyl-L-phenylalanyl-L-prolinemethyl ester 4*: Yield 89%;  $[\alpha]_D -31.9^\circ$  (c, 0.25 in MeOH);  $R_f$  0.71; IR (CHCl<sub>3</sub>),  $\nu$  cm<sup>-1</sup>: 3372 (m/br, -OH str, Tyr), 3135-3129 (m, -NH str, amide), 3068-3062 (w, -CH str, ring), 2998-2995 (m, -CH str, Pro), 2875 (m, -CH str, sym, CH<sub>3</sub>), 2927 (m, -CH str, asym, CH<sub>2</sub>), 2852 (m, -CH str, sym, CH<sub>2</sub>), 1747 (s, -C=O str, ester), 1667, 1649, 1636 (s, -C=O str, 3° and 2° amide), 1555-1552, 1476-1469 (m, skeletal bands, rings), 1539-1535, 1528, 1523 (m, -NH bend, 2° amide), 1390, 1369 (s, -CH bend, *tert*-Butyl), 1266 (s, C-O str, ester), 1230 (s, C-O str, phenolic), 929 (w, CH<sub>3</sub> rocking, *tert*-Butyl), 827, 725, 689 (m, -CH def, oop, rings).  $^1\text{H-NMR}$  (DMSO- $d_6$ )  $\delta$ : 8.64 (1H, br. s, NH), 7.30 (2H, dd,  $J=8.6$ , 4.9 Hz, H-m, Tyr), 7.18 (2H, tt,  $J=6.75$ , 4.5 Hz, H-m, Phe), 6.98 (1H, t,  $J=6.25$  Hz, H-p, Phe), 6.92 (2H, dd,  $J=8.55$ , 5.3 Hz, H-o, Tyr), 6.81 (2H, dd,  $J=8.8$ , 4.15 Hz, H-o, Phe), 6.57 (1H, br. s, NH), 5.95 (1H, br. s, OH), 4.59 (1H, q,  $J=5.5$  Hz, H- $\alpha$ , Phe), 4.52 (1H, q,  $J=7.9$  Hz, H- $\alpha$ , Tyr), 3.94 (1H, t,  $J=6.9$  Hz, H- $\alpha$ , Pro), 3.62 (3H, s, OCH<sub>3</sub>), 3.40 (2H, t,  $J=7.25$  Hz, H- $\delta$ , Pro), 2.98-2.87 (4H, m, H- $\beta$ , Phe and Tyr), 2.05-1.99 (4H, m, H- $\beta$  and H- $\gamma$ , Pro), 1.53 (9H, s, *tert*-Butyl). *Anal.* Calcd for C<sub>29</sub>H<sub>37</sub>N<sub>3</sub>O<sub>7</sub>: C, 64.55; H, 6.91; N, 7.79. Found: C, 64.58; H, 6.89; N, 7.80.

*tert-Butyloxycarbonyl-glycyl-L-leucyl-L-proline methyl ester 5*: Yield 82%;  $[\alpha]_D -73.6^\circ$  (c, 0.25 in MeOH);  $R_f$  0.59; IR (CHCl<sub>3</sub>),  $\nu$  cm<sup>-1</sup>: 3137-3132 (m, -NH str, amide), 2997-2993 (m, -CH str, Pro), 2878 (m, -CH str, sym, CH<sub>3</sub>), 2929, 2926 (m, -CH str, asym, CH<sub>2</sub>), 2856 (m, -CH str, sym, CH<sub>2</sub>), 1746 (s, -C=O str, ester), 1668, 1645, 1637 (s, -C=O str, 3° and 2° amide), 1538, 1529-1522 (m, -NH bend, 2° amide), 1392, 1368 (s, -CH bend, *tert*-Butyl), 1385, 1363 (s, -CH bend, *iso*-propyl), 1268 (s, C-O str, ester), 926 (w, CH<sub>3</sub> rocking, *tert*-Butyl).  $^1\text{H-NMR}$  (DMSO- $d_6$ )  $\delta$ : 6.92 (1H, br. s, NH), 6.24 (1H, br. s, NH), 4.47 (1H, q,  $J=6.7$  Hz, H- $\alpha$ , Leu), 3.92 (1H, t,  $J=6.95$  Hz, H- $\alpha$ , Pro), 3.74 (2H, d,  $J=5.15$  Hz, H- $\alpha$ , Gly), 3.65 (3H, s, OCH<sub>3</sub>), 3.38 (2H, t,  $J=7.3$  Hz, H- $\delta$ , Pro), 2.04-1.96 (4H, m, H- $\beta$  and H- $\gamma$ , Pro), 1.76 (2H, t,  $J=7.95$  Hz, H- $\beta$ , Leu), 1.55 (9H, s, *tert*-Butyl), 1.49-1.42 (1H, m, H- $\gamma$ , Leu), 0.99-0.97 (6H, d,  $J=6.25$  Hz, H- $\delta$ , Leu). *Anal.* Calcd for C<sub>19</sub>H<sub>33</sub>N<sub>3</sub>O<sub>6</sub>: C, 57.13; H, 8.33; N, 10.52. Found: C, 57.15; H, 8.32; N, 10.55.

#### Procedure for synthesis of linear hexapeptide 6

5.25 g (10 mmol) of Boc-L-Tyr-L-Phe-L-Pro-OH was dissolved in DMF (25 ml) and solution was neutralized with 2.8 ml (21 mmol) of TEA at 0°C and the resulting mixture was stirred for 15 min. 3.0 g (10 mmol) of Gly-L-Leu-L-Pro-OMe was dissolved in DMF (25 ml) and resulting solution along with 2.1 g/1.26 g (0.01 mol) of DCC / DIPC were added to above mixture. Stirring was first done for 1 h at 0 to 5°C and then further for 35 h at room temperature (RT). 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 (b.p. 40-60°C) followed by cooling at 0°C to get Boc-L-Tyr-L-Phe-L-Pro-Gly-L-Leu-L-Pro-OMe 6 as pale-yellow semisolid mass.

*tert-Butyloxycarbonyl-L-tyrosinyl-L-phenylalanyl-L-prolyl-glycyl-L-leucyl-L-proline methyl ester 6*: Yield 76%;  $[\alpha]_D -55.8^\circ$  (c, 0.15 in MeOH);  $R_f$  0.88; IR (CHCl<sub>3</sub>),  $\nu$  cm<sup>-1</sup>: 3378 (m/br, -OH str, Tyr), 3137-3128 (m, -NH str, amide), 3067, 3059 (w, -CH str, ring), 2999-2992 (m, -CH str, Pro), 2962 (m, -CH str, asym, CH<sub>3</sub>), 2877 (m, -CH str, sym, CH<sub>3</sub>), 2928, 2925 (m, -CH str, asym, CH<sub>2</sub>), 2854 (m, -CH str, sym, CH<sub>2</sub>), 1749 (s, -C=O str, ester), 1666, 1644-1635 (s, -C=O str, 3° and 2° amide), 1556-1552, 1474-1467 (m, skeletal bands, rings), 1538-1533, 1529, 1523 (m, -NH bend, 2° amide), 1392, 1368 (s, -CH bend, *tert*-Butyl), 1386, 1362 (s, -CH bend, *iso*-propyl), 1268 (s, C-O str, ester), 1233 (s, C-O str, phenolic), 927 (w, CH<sub>3</sub> rocking, *tert*-Butyl), 829, 722, 687 (m, -CH def, oop, rings).  $^1\text{H-NMR}$  (DMSO- $d_6$ )  $\delta$ : 9.45 (1H, br. s, NH), 8.65 (1H, br. s, NH), 7.78 (1H, br. s, NH), 7.32 (2H, dd,  $J=8.65$ , 4.9 Hz, H-m, Tyr), 7.19 (2H, tt,  $J=6.8$ , 4.45 Hz, H-m, Phe), 7.02 (1H, t,  $J=6.3$  Hz, H-p, Phe), 6.94 (2H, dd,  $J=8.6$ , 5.25 Hz, H-o, Tyr), 6.83 (2H, dd,  $J=8.75$ , 4.2 Hz, H-o, Phe), 6.59 (1H, br. s, NH), 5.97 (1H, br. s, OH), 4.55 (1H, q,  $J=7.85$  Hz, H- $\alpha$ , Tyr), 4.42 (1H, q,  $J=5.5$  Hz, H- $\alpha$ , Phe), 4.12 (1H, q,  $J=6.65$  Hz, H- $\alpha$ , Leu), 4.05 (1H, t,  $J=6.9$  Hz, H- $\alpha$ , Pro-1), 4.01 (2H, d,  $J=5.2$  Hz, H- $\alpha$ , Gly), 3.89 (1H, t,  $J=6.85$  Hz, H- $\alpha$ , Pro-2), 3.66 (3H, s, OCH<sub>3</sub>), 3.42 (2H, t,  $J=7.3$  Hz, H- $\delta$ , Pro-2), 3.35 (2H, t,  $J=7.25$  Hz, H- $\delta$ , Pro-1), 3.04-2.89 (4H, m, H- $\beta$ , Phe and Tyr), 2.68-2.63 (2H, m, H- $\beta$ , Pro-1), 2.05-1.97 (4H, m, H- $\beta$  and H- $\gamma$ , Pro-2), 1.94-1.89 (2H, m, H- $\gamma$ , Pro-1), 1.78 (2H, t,  $J=7.9$  Hz, H- $\beta$ , Leu), 1.56 (9H, s, *tert*-Butyl), 1.50-1.44 (1H, m, H- $\gamma$ , Leu), 0.98-0.96 (6H, d,  $J=6.2$  Hz, H- $\delta$ , Leu). *Anal.* Calcd for C<sub>42</sub>H<sub>58</sub>N<sub>6</sub>O<sub>10</sub>: C, 62.51; H, 7.24; N, 10.41. Found: C, 62.49; H, 7.25; N, 10.44.

#### Cyclization of linear hexapeptide fragment

To synthesize 7, linear hexapeptide unit 6 (4.0 g, 5 mmol) was deprotected at carboxyl end using lithium hydroxide (0.18 g, 7.5 mmol) to get Boc-L-Tyr-L-Phe-L-Pro-Gly-L-Leu-L-Pro-OH. The deprotected hexapeptide unit (3.97 g, 5 mmol) was now dissolved in chloroform (35 ml) at 0°C. To the above solution, DIPC (0.63 g, 5 mmol) and *p*-nitrophenol / pentafluorophenol (0.94 g/1.23 g, 6.7 mmol) were added and stirring was done at RT (12 h). The reaction mixture was filtered and the filtrate was washed with 10%  $\text{NaHCO}_3$  (3 × 15 ml) and 5% HCl (2 × 10 ml) to get the corresponding *p*-nitrophenyl/pentafluorophenyl ester Boc-L-Tyr-L-Phe-L-Pro-Gly-L-Leu-L-Pro-Opnp/pfp. To this compound (3.66 g/3.84 g, 4 mmol) dissolved in chloroform (25 ml), TFA (0.91 g, 8 mmol) was added, stirred at RT (1 h) and washed with 10%  $\text{NaHCO}_3$  solution (2 × 25 ml). The organic layer was dried over anhydrous  $\text{Na}_2\text{SO}_4$  to get L-Tyr-L-Phe-L-Pro-Gly-L-Leu-L-Pro-Opnp/pfp which was dissolved in chloroform (25 ml) and TEA/NMM/pyridine (2.8 ml/2.21 ml/1.61 ml, 21 mmol) was added. Then, whole contents were kept at 0°C (7 days). The reaction mixture was washed with sufficient quantity of 10%  $\text{NaHCO}_3$  solution until the by-product *p*-nitrophenol/

**Table 1.** Antibacterial activity data of 7.

Compound	Diameter of zone of inhibition (ZI) in mm			
	Bacterial strains			
	<i>B. subtilis</i>	<i>S. epidermidis</i>	<i>P. aeruginosa</i>	<i>K. pneumoniae</i>
7	9(25)	11(12.5)	27(6.25)	26(6.25)
Control <sup>b</sup>	—	—	—	—
Gatifloxacin	20(12.5)	28(6.25)	24(6.25)	25(6.25)

<sup>a</sup> Values in bracket are MIC values ( $\mu\text{g/ml}$ ); <sup>b</sup> DMF; '—' indicates 'no activity'.

pentafluorophenol was removed completely and finally washed with 5 % HCl (3  $\times$  25 ml). The organic layer was dried over anhydrous  $\text{Na}_2\text{SO}_4$ . Finally, chloroform was distilled off to get the crude cyclized product. Purification of crude compound was done by dissolving it in chloroform, filtering repeatedly and finally adding *n*-hexane dropwise in filtered cooled solution to get pure cyclic product 7 as white solid.

*Cyclo(-L-tyrosinyl-L-phenylalanyl-L-prolyl-glycyl-L-leucyl-L-prolyl-)* 7: Yield 86% (NMM), 71% (TEA), 63% ( $\text{C}_5\text{H}_5\text{N}$ );  $[\alpha]_D^{25} -10.1^\circ$  (c, 0.1 in MeOH);  $R_f$  0.62; m.p.  $202^\circ\text{C}$  (d); IR (KBr),  $\nu$   $\text{cm}^{-1}$ : 3377 (m/br, -OH str, Tyr), 3139, 3135, 3128 (m, -NH str, amide), 3069, 3052 (w, -CH str, rings), 2998-2993 (m, -CH str, Pro), 2964, 2875 (m, -CH str, asym and sym,  $\text{CH}_3$ ), 2929, 2922 (m, -CH str, asym,  $\text{CH}_2$ ), 2857 (m, -CH str, sym,  $\text{CH}_2$ ), 1668, 1649-1636 (s, -C=O str, 3 and 2 $^\circ$  amide), 1558-1553, 1477-1469 (m, skeletal bands, rings), 1539, 1534, 1528-1522 (m, -NH bend, 2 $^\circ$  amide), 1384, 1359 (s, -CH bend, *iso*-propyl), 1236 (s, C-O str, phenolic), 825, 727, 684 (m, -CH def, oop, rings).  $^1\text{H-NMR}$  ( $\text{DMSO-}d_6$ )  $\delta$ : 9.89 (1H, br. s, NH), 9.15 (1H, br. s, NH), 7.59 (1H, br. s, NH), 7.38 (1H, br. s, NH), 7.17 (2H, tt,  $J=6.85, 4.5$  Hz, H-m, Phe), 7.04 (1H, t,  $J=6.25$  Hz, H-p, Phe), 6.97 (2H, dd,  $J=8.7, 4.9$  Hz, H-m, Tyr), 6.91 (2H, dd,  $J=8.65, 5.3$  Hz, H-o, Tyr), 6.84 (2H, dd,  $J=8.8, 4.2$  Hz, H-o, Phe), 6.30 (1H, q,  $J=6.7$  Hz, H- $\alpha$ , Leu), 5.95 (1H, br. s, OH), 5.74 (1H, q,  $J=5.45$  Hz, H- $\alpha$ , Phe), 4.22 (1H, q,  $J=7.9$  Hz, H- $\alpha$ , Tyr), 3.95 (2H, d,  $J=5.15$  Hz, H- $\alpha$ , Gly), 3.92 (1H, t,  $J=6.9$  Hz, H- $\alpha$ , Pro-2), 3.88 (1H, t,  $J=6.85$  Hz, H- $\alpha$ , Pro-1), 3.29-3.23 (4H, m, H- $\delta$ , Pro-2 and Pro-1), 2.69-2.62 (4H, m, H- $\beta$ , Pro-1 and Pro-2), 2.60-2.55 (4H, m, H- $\beta$ , Phe and Tyr), 1.88 (2H, t,  $J=7.85$  Hz, H- $\beta$ , Leu), 1.86-1.81 (4H, m, H- $\gamma$ , Pro-1 and Pro-2), 0.99-0.97 (6H, d,  $J=6.15$  Hz, H- $\delta$ , Leu), 0.88-0.79 (1H, m, H- $\gamma$ , Leu);  $^{13}\text{C NMR}$  ( $\text{DMSO-}d_6$ )  $\delta$ : 174.2 (C=O, Leu), 172.2 (C=O, Pro-1), 171.5 (C=O, Pro-2), 170.2 (C=O, Tyr), 169.9 (C=O, Phe), 169.1 (C=O, Gly), 153.3 (C-p, Tyr), 137.2, 133.8 (2C, C- $\gamma$ , Phe and Tyr), 130.9 (2C, C-o, Phe), 129.6 (2C, C-o, Tyr), 128.5 (2C, C-m, Phe), 128.1 (2C, C-m, Tyr), 127.7 (C-p, phe-1), 62.2, 58.3 (2C, C- $\alpha$ , Pro-1 and Pro-2), 57.1, 53.9 (2C, C- $\alpha$ , Tyr and Phe), 49.7 (C- $\alpha$ , leu), 48.2, 47.7 (2C, C- $\delta$ , Pro-1 and Pro-2), 44.5 (C- $\beta$ , Leu), 41.9 (C- $\alpha$ , Gly), 39.7, 36.2 (2C, C- $\beta$ , Tyr and Phe), 32.4, 29.6 (2C, C- $\beta$ , Pro-2 and Pro-1), 27.3 (C- $\gamma$ , Leu), 24.8 (C- $\gamma$ , Pro-1), 22.5 (2C, C- $\delta$ , Leu), 20.3 (C- $\gamma$ , Pro-2). ESI-MS/MS: ( $m/z$ , rel. int.) [Leu-Gly amide bond level]: 675.7 [(M + H)<sup>+</sup>, 100], 647.7 [(675.7-CO)<sup>+</sup>, 17], 618.7 [(Leu-Pro-Tyr-Phe-Pro)<sup>+</sup>, 59], 590.7 [(618.7-CO)<sup>+</sup>, 11], 521.6 [(Leu-Pro-Tyr-Phe)<sup>+</sup>, 73], 493.6 [(521.6-CO)<sup>+</sup>, 19], 374.4 [(Leu-Pro-Tyr)<sup>+</sup>, 29], 346.4 [(374.4-CO)<sup>+</sup>, 10], 211.3 [(Leu-Pro)<sup>+</sup>, 17], 183.3 [(211.3-CO)<sup>+</sup>, 9], 114.2 [(Leu)<sup>+</sup>, 11]; [Breakage at Pro-Phe amide bond level]: 528.6 [(Pro-Gly-Leu-Pro-Tyr)<sup>+</sup>, 36], 500.6 [(528.6-CO)<sup>+</sup>, 9], 268.3 [(Pro-Gly-Leu)<sup>+</sup>, 54], 240.3 [(268.3-CO)<sup>+</sup>, 11]; [Breakage at Pro-Leu amide bond level]: 562.6 [(Pro-Tyr-Phe-Pro-Gly)<sup>+</sup>, 22], 534.6 [(562.6-CO)<sup>+</sup>, 17], 505.5 [(Pro-Tyr-Phe-Pro)<sup>+</sup>, 14]; [Immonium ions]: Tyr: 136.2 [( $\text{C}_8\text{H}_{10}\text{NO}$ )<sup>+</sup>, 11]; Phe: 120.2 [( $\text{C}_8\text{H}_{10}\text{N}$ )<sup>+</sup>, 13]; Pro: 70.1 [( $\text{C}_4\text{H}_8\text{N}$ )<sup>+</sup>, 9]; Leu: 86.1

[( $\text{C}_5\text{H}_{12}\text{N}$ )<sup>+</sup>, 16]; Gly: 30.1 [( $\text{CH}_4\text{N}$ )<sup>+</sup>, 10]. *Anal.* Calcd for  $\text{C}_{36}\text{H}_{46}\text{N}_6\text{O}_7$ : C, 64.08; H, 6.87; N, 12.45. Found: C, 64.06; H, 6.89; N, 12.42.

### Antibacterial screening

The newly synthesized cyclopeptide 7 was evaluated for its antibacterial potential against two Gram-positive bacteria *Bacillus subtilis*, *Staphylococcus epidermidis* and two Gram-negative bacteria *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* at 25-6.25  $\mu\text{g/ml}$  concentration by using modified Kirby-Bauer disc diffusion method (Bauer et al., 1966). MIC values of test compound was determined by Tube Dilution Technique. Synthesized cyclopeptide 7 was dissolved to prepare a stock solution of 1 mg/ml using DMF. Stock solution was aseptically transferred and suitably diluted with sterile broth medium to contain seven different concentrations of test compound in different test tubes. All the tubes were inoculated with one loopful of one of the test bacterium. The process was repeated with different test bacteria. Tubes inoculated with bacterial cultures were incubated at  $37^\circ\text{C}$  for 18 h and the presence/absence of growth of the bacteria was observed. From these results, minimum inhibitory concentration (MIC) of each test compound was determined against each test bacterium. A spore suspension in sterile distilled water was prepared from 5 days old culture of the test bacteria growing on nutrient 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 -  $6 \times 10^4$  spores/ml). Filter paper disks of 6 mm diameter and 2 mm thickness were sterilized by autoclaving at  $121^\circ\text{C}$  (15 psig) 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 - gatifloxacin and a disk impregnated with the solvent (DMF) as negative control. The petri plates inoculated with bacterial cultures were incubated at  $37^\circ\text{C}$  for 18 h. Diameters of the zones of inhibition (in mm) were measured and the average diameters for test sample were calculated for triplicate sets. The diameters obtained for the test sample were compared with that produced by the standard drug. The results of antibacterial studies are presented in Table 1.

### Antifungal screening

Serial plate dilution method was employed for the evaluation of antifungal activity against diamorphic fungal strain *C. albicans* and three other fungal strains, including *Aspergillus niger* and two cutaneous fungal strains *M. audouinii* and *T. mentagrophytes* at 25-.25  $\mu\text{g/ml}$  concentration (Khan, 1997). MIC values of synthesized 6 cyclopeptide 7 was determined by employing the same technique as used for antibacterial studies using DMSO instead of DMF and

**Table 2.** Antifungal activity data of 7.

Compound	Diameter of zone of inhibition (ZI) in mm			
	Fungal strains			
	<i>C. albicans</i>	<i>M. audouinii</i>	<i>A. niger</i>	<i>T. mentagrophytes</i>
7	19(6.25)	24(6.25)	9(25)	28(6.25)
Control <sup>b</sup>	—	—	—	—
Griseofulvin	20(6.25)	17(6.25)	18(12.5)	22(6.25)

<sup>a</sup> Values in bracket are MIC values ( $\mu\text{g/ml}$ ); <sup>b</sup> DMSO; '—' indicates 'no activity'.

**Table 3.** Antihelmintic activity data of 7.

Compound	Earthworm species					
	<i>M. konkanensis</i>		<i>M. konkanensis</i>		<i>M. konkanensis</i>	
	Mean paralyzing time (min) <sup>a</sup>	Mean death time (min)	Mean paralyzing time (min)	Mean death time (min)	Mean paralyzing time (min)	Mean death time (min)
7 <sup>b</sup>	14.22 $\pm$ 0.21	16.47 $\pm$ 0.53	20.24 $\pm$ 0.41	22.46 $\pm$ 0.20	15.02 $\pm$ 0.38	17.09 $\pm$ 0.11
Control <sup>c</sup>	—	—	—	—	—	—
Mebendazole <sup>b</sup>	10.55 $\pm$ 0.64	12.59 $\pm$ 0.53	17.58 $\pm$ 1.03	19.42 $\pm$ 1.20	11.35 $\pm$ 0.45	13.46 $\pm$ 0.62

<sup>a</sup> Data are given as mean  $\pm$  S.D. (n = 3); <sup>b</sup> c = 2 mg/ml; <sup>c</sup> 0.5% tween 80 in distilled water.

tubes inoculated with fungal cultures were incubated at 37 °C for 48 h. After incubation, the presence/absence of growth of the fungi was observed and MIC of test compound was determined against each test fungus. A spore suspension in normal saline was prepared from culture of the test fungi on sabouraud's broth media. After transferring growth medium, petri plates were inoculated with spore suspension. After drying, wells were made using an agar punch and test sample, reference drug - griseofulvin and negative control (DMSO) were placed in labeled wells in each petri plate. The petri plates inoculated with fungal cultures were incubated at 37°C for 48 h. Antifungal activity was determined by measuring the diameter of the inhibition zone for triplicate sets. Activity of test compound was compared with reference standard. The results of antifungal studies are given in the Table 2.

### Antihelmintic screening

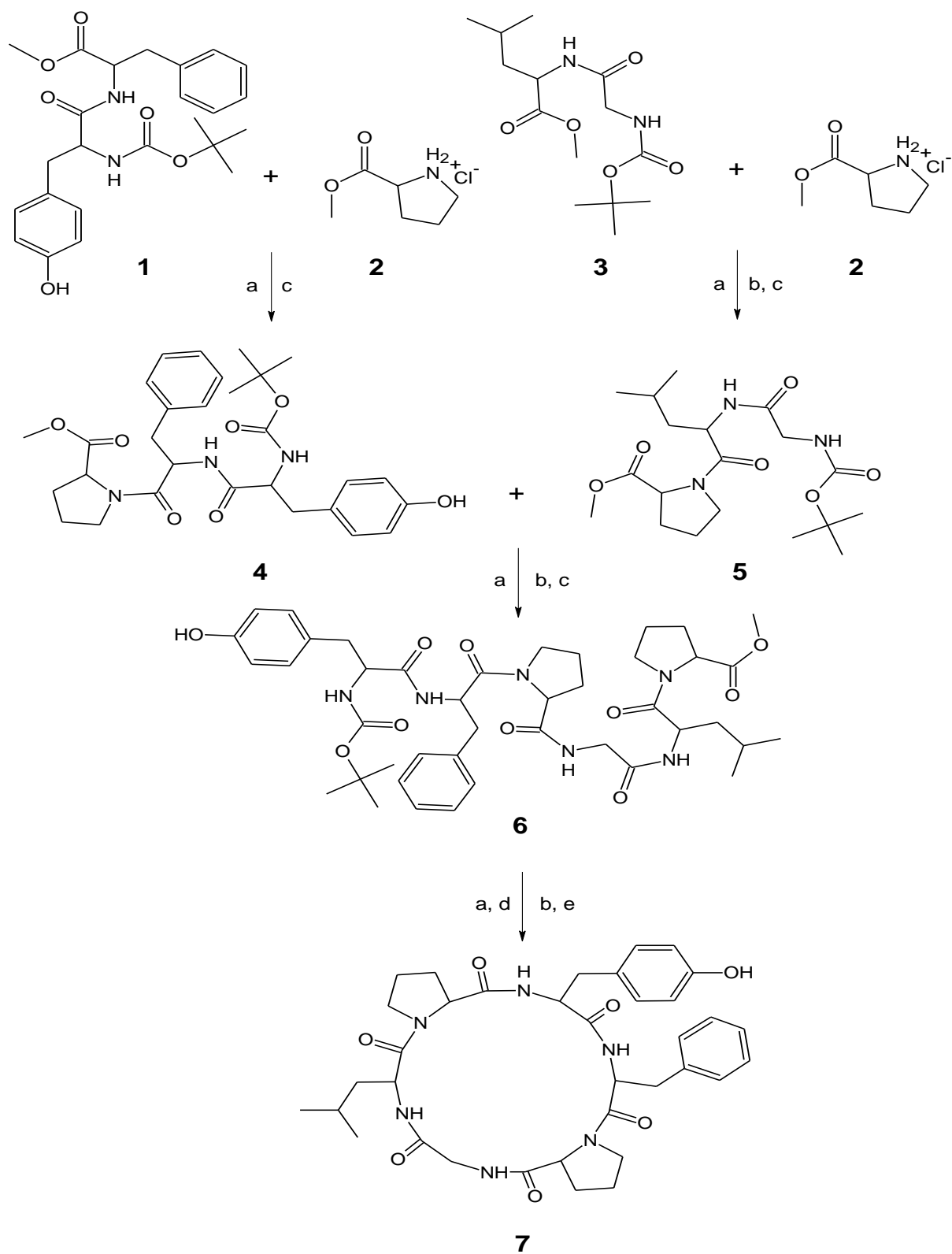
Antihelmintic activity studies were carried out against three different species of earthworms *Megascoplex konkanensis*, *Pontoscotex corethruses* and *Eudrilus species* at 2 mg/ml concentration (Garg and Atal, 1963). Suspension was prepared by triturating synthesized cyclopeptide 7 (100 mg) with tween 80 (0.5%) and distilled water and the resulting mixture was stirred using a mechanical stirrer for 30 min. The suspension was diluted to contain 0.2% w/v of the test sample. Suspension of reference drug - mebendazole was 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 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 mean was 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. The results of antihelmintic studies are tabulated in the Table 3.

## RESULTS AND DISCUSSION

### Chemistry

In order to carry out the first total synthesis of gypsin B 7, disconnection strategy was employed. The cyclic hexapeptide molecule was split into two dipeptide units Boc-L-Tyr-L-Phe-OMe (1), Boc-Gly-L-Leu-OMe (3) and a single amino acid unit L-Pro-OMe.HCl (2). The required dipeptide units 1 and 3 were prepared by coupling of Boc-amino acids viz. Boc-L-Tyr-OH and Boc-Gly-OH with corresponding amino acid methyl ester hydrochlorides such as L-Phe-OMe.HCl and L-Leu-OMe.HCl employing DCC as coupling agent (Bodanszky and Bodanszky, 1984). Ester group of dipeptide 1 was removed by alkaline hydrolysis with LiOH and deprotected peptide was coupled with amino acid methyl ester hydrochloride 2 using DCC/DIPC and TEA/NMM, to get the first tripeptide unit Boc-L-Tyr-L-Phe-L-Pro-OMe (4). Similarly, dipeptide 3 after deprotection at carboxyl end, was coupled with 2 to get the another tripeptide unit Boc-Gly-L-Leu-L-Pro-OMe (5). After removal of ester group of tripeptide 4 and Boc group of tripeptide 5, deprotected units were coupled to get linear hexapeptide unit Boc-L-Tyr-L-Phe-L-Pro-Gly-L-Leu-L-Pro-OMe (6). The methyl ester group of linear peptide fragment was replaced by



a = LiOH, THF:H<sub>2</sub>O (1:1), RT, 1 h; b = TFA, CHCl<sub>3</sub>, RT, 1 h; c = DIPC/DCC, NMM/TEA, DCM/DMF, RT, 24-36 h;  
 d = DIPC, pnp/pfp, RT, 12 h; e = TEA/NMM/pyridine, CHCl<sub>3</sub>, 7 days, 0 °C.

**Scheme 1.** Synthetic pathway for cyclohexapeptide - gypsin B 7.

*p*-nitrophenyl/pentafluorophenyl (pnp/pfp) ester group. The Boc-group of resulting compound was removed using TFA and deprotected linear fragment was now cyclized by keeping the whole contents at 0 °C for 7 days in presence of catalytic amount of TEA/NMM/pyridine to get cyclic product 7 (Scheme 1).

Synthesis of gypsin B 7 was carried out successfully with good yield. Cyclization of linear peptide was indicated by disappearance of absorption bands at 1749, 1268 cm<sup>-1</sup> and 1392, 1368 cm<sup>-1</sup> (CO stretching of ester and CH deformation of *tert*-Butyl group) in IR spectra of cyclopeptide 7. Formation of cyclopeptide was further confirmed by disappearance of singlet at 1.56 ppm corresponding to nine protons of *tert*-Butyl group of Boc and singlet at 3.66 ppm corresponding to three protons of methyl ester, in <sup>1</sup>H NMR spectrum of 7. Furthermore, <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra of synthesized cyclic hexapeptide showed characteristic peaks confirming presence all the 46 protons and 36 carbon atoms. Presence of (M + 1)<sup>+</sup> ion peak at *m/z* 675.7 corresponding to the molecular formula C<sub>36</sub>H<sub>46</sub>N<sub>6</sub>O<sub>7</sub> in mass spectra of 7, along with other fragment ion peaks resulting from cleavage at 'Leu-Gly', 'Pro-Phe' and 'Pro-Leu' amide bond levels, showed exact sequence of attachment of all the six amino acid moieties in a chain. In addition, elemental analysis of 7 afforded values (± 0.03) strictly in accordance to the molecular composition.

## Pharmacology

Results of antimicrobial and anthelmintic activity studies are summarized in Tables 1 to 3. Comparison of antimicrobial activity data suggested that synthesized cyclopeptide 7 exhibited high level of antidermatophyte activity against *M. audouinii* and *T. mentagrophytes* with MIC value of 6.25 µg/ml, in comparison to standard drug - griseofulvin. Antibacterial activity data further indicated that 7 possessed good level of bioactivity against pathogenic fungus *C. albicans* and bacteria *P. aeruginosa* and *K. pneumoniae* with MIC value of 6.25 µg/ml, in comparison to standard drug - gatifloxacin. Anthelmintic activity data revealed that 7 showed moderate anthelmintic activity against *M. konkanensis*, *P. corethruses* and *Eudrilus sp.* at 2 mg/ml concentration, in comparison to standard drug - mebendazole. However, 7 displayed no significant activity against Gram-positive bacteria and *A. niger*.

## Conclusion

First total synthesis of natural peptide, gypsin B 7 was accomplished with > 85% yield via coupling reactions utilizing different carbodiimides. DIPC was found to be a better coupling agent in comparison to DCC and

pentafluorophenyl ester was proved to be better for the activation of acid functionality of linear hexapeptide 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 cyclohexapeptide displayed significant antimicrobial activity against pathogenic *T. mentagrophytes*, *M. audouinii*, *C. albicans*, *K. pneumoniae* and *P. aeruginosa*. In comparison, gram-negative bacteria were found to be more sensitive than Gram-positive bacteria towards the newly synthesized peptide. On passing toxicity tests, newly synthesized cyclopeptide 7 may prove good candidate for clinical studies and can be a novel antimicrobial drug of the future.

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