ORIGINAL ARTICLE

SYNTHETIC AND PHARMACOLOGICAL STUDIES ON LONGICALYCININ A

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ABSTRACT
Present investigation describes the synthesis of a natural phenylalanine-rich cyclopolypeptide longicalycinin A (**10**) by coupling of dipeptide unit Boc-L-phe-L-tyr-OH with tripeptide unit L-pro-L-phe-gly-OMe followed by cyclization of linear segment. Synthesized cyclic pentapeptide was characterized by spectral techniques including FTIR, $^{1}H/^{13}C$ NMR, FAB MS and elemental analysis and screened for different pharmacological activities. It was found that it has good anthelmintic activity against *Megascoplex konkanensis*, *Pontoscotex corethruses* and *Eudrilus* sp. at 2 mg/ml concentration, in addition to high cytotoxicity against *Dalton's lymphoma ascites* (DLA) and *Ehrlich's ascites carcinoma* (EAC) cell lines with CTC_{50} values of 2.62 and 6.37 μM. Dermatophytes were found to be moderately sensitive towards newly synthesized peptide.

Keywords: *Dianthus superbus*, cyclic peptide, longicalycinin A, anthelmintic screening, antimicrobial activity, cytotoxicity.

INTRODUCTION

In past decades, higher plants are well recognized for their ability to produce a wide spectrum of natural products with interesting bioactivities (Liu *et al.,* 2007; Morita *et al.,* 2006; Pepeljnjak *et al.,* 2005; Tiew *et al.,* 2003; Min *et al.,* 2000). Among these, peptides especially cyclopolypeptides and related congeners (Tan and Zhou, 2006; Morel *et al.,* 2002; Jennings *et al.,* 2001) have received special attention due to their unique structures and wide pharmacological profile which may solve the problem of wide spread increase of resistance towards conventional drugs. Diverse pharmacological activities exhibited by plant cyclopeptides include antitumour activity (Takeya *et al.,* 1993; Morita *et al.,* 1995), antimalarial activity (Picur *et al.,* 2006), vasorelaxant activity (Morita *et al.,* 2006a), estrogen-like activity (Itokawa *et al.,* 1995) and tyrosinase inhibitory activity (Morita *et al.,* 1994). A natural cyclic pentapeptide, longicalycinin A, was isolated from plant *Dianthus superbus* var. *longicalycinus* which has been used for treating carcinoma, diuretic and inflammatory conditions. Structure of isolated cyclopeptide was elucidated on the basis of ESI tandem mass fragmentation analysis, chemical evidence and extensive 2D NMR methods (Hsieh *et al.,* 2005).

As part of our continuing efforts on synthesizing bioactive cyclic peptides (Dahiya and Pathak, 2007; Dahiya and Pathak, 2007a; Dahiya and Pathak, 2006; Dahiya *et al.,* 2006), present work aims at synthesis of novel cyclic pentapeptide, longicalycinin A. Keeping in view of significant biological activities possessed by various cyclopeptides (Dahiya and Pathak, 2006a; Pathak and Dahiya, 2003), above synthetic peptide was further subjected to antimicrobial, anthelmintic and cytotoxic activity studies.

MATERIALS AND METHODS

General experimental part

Melting points were determined by open capillary method and are uncorrected. IR spectra were recorded on FTIR-8400S fourier transform infrared spectrophotometer using a thin film supported on KBr pellets for solids and $CHCl_3$ as solvent for intermediate semisolids (cm^{-1}). ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker AC 300 spectrometer at 300 MHz using $CDCl_3$ as solvent and TMS as internal reference (chemical shifts in δ ppm). Mass spectra was recorded on JMS-DX 303 Mass spectrometer operating at 70 eV using fast atom bombardment technique. Elemental analyses of all compounds were performed on Vario EL III elemental analyzer. Optical rotation of the synthesized peptides was measured on Optics Technology automatic polarimeter 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 in different ratios (8:2/7:3 v/v). Bacterial and fungal cultures were obtained from the Manipal University Mycological Center (MUMC, Manipal, India) and earthworm species were arranged from Indian Council of Agricultural Research Breeding Center (ICARBC, Kasaragod, India).

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Preparation of Boc-amino acids (1, 2)

L-amino acid (20 mmol) was dissolved in 1N NaOH (20 ml) and isopropanol (20 ml). Di-*tert*-butylpyrocarbonate (6 ml, 26 mmol) in isopropanol (10 ml) was added followed by 1N NaOH (20 ml) to the resulting solution. The solution was stirred at RT for 2 h, washed with light petroleum ether (bp 40-60 °C) (20 ml), acidified to pH 3.0 with 2N H_2SO_4 and finally extracted with chloroform (3 × 20 ml). The organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to give the crude product which was finally crystallized from chloroform and petroleum ether (bp 40-60°C).

*^{t}Butyloxycarbonyl-phenylalanine (**1**)*

Yield 88%, $[\alpha]_D$ –8.0°, R_f - 0.52; ^{13}C NMR: 173.8 (COOH); 155.2 (C=O, boc); 138.0 (C-*γ*); 129.3 (2C, C-*o*); 128.2 (2C, C-*m*); 126.5 (C-*p*); 79.5 (C-*α*, butyl-*t*); 55.1 (C-*α*); 37.7 (C-*β*); 27.8 (3C, C-*β*, butyl-*t*). Anal. Calcd. for $C_{14}H_{19}NO_4$: C, 63.38; H, 7.22; N, 5.28. Found: C, 63.35; H, 7.23; N, 5.32 (Dahiya *et al.*, 2006).

*^{t}Butyloxycarbonyl-proline (**2**)*

White crystals, mp 136 °C, yield 79%, $[\alpha]_D$ +12.6°, R_f - 0.71; IR: 3302-2515 (m/br, OH str, COOH); 2998, 2986 (m, CH str, CH_2, pro); 1715 (s, C=O str, COOH); 1669 (s, C=O str, amide); 1386, 1363 (m, CH band, butyl-*t*); 925 (w, CH_3 rocking, butyl-*t*). ^{1}H NMR: 12.02 (br. s, 1H, OH); 4.31-4.26 (t, 1H, H-*α*); 3.40-3.36 (t, 2H, H-*δ*); 2.02-1.95 (m, 2H, H-*γ*); 1.90-1.85 (m, 2H, H-*β*); 1.49 (s, 9H, butyl-*t*). ^{13}C NMR: 178.2 (COOH); 154.9 (C=O, boc); 80.1 (C-*α*, butyl-*t*); 57.6 (C-*α*, pro); 46.6 (C-*δ*, pro); 31.7 (C-*β*, pro); 29.3 (3C, C-*β*, butyl-*t*); 22.5 (C-*γ*, pro). Anal. Calcd. for $C_{10}H_{17}NO_4$: C, 55.80; H, 7.96; N, 6.51. Found: C, 55.77; H, 8.01; N, 6.50.

Preparation of L-amino acid methyl ester hydrochlorides (3-5)

Thionyl chloride (1.4 ml, 20 mmol) was added to methanol (100 ml) slowly at 0°C and L-amino acid (20 mmol) was added to above solution. The resulting mixture was refluxed for 8-10 h at ambient temperature. Solvent was evaporated and the residue was triturated with ether at 0°C until excess dimethyl sulphite was removed. The crude product was crystallized from methanol and ether at 0°C to obtain the pure amino acid methyl ester hydrochloride.

*L-tyrosine methyl ester hydrochloride (**3**)*

White crystals, yield 84%, mp 190 °C, $[\alpha]_D$ –4.3°, R_f - 0.67; IR: 3372 (m/br, OH str); 3011-2863 (s/br, NH_3^+); 2928, 2848 (m, CH_2); 1750 (s, C=O, ester); 1588, 1475 (m, skeletal bands, ring); 1227 (s, C–O str, phenolic); 1272 (s, C–O str, ester); 825 (s, CH def, oop, ring). ^{1}H NMR: 7.80-7.78 (dd, 2H, H-*m*, J=7.4 Hz, J=5.0 Hz); 7.56-7.54 (dd, 2H, H-*o*, J=7.5 Hz, J=4.6 Hz), 5.40 (br. s, OH and NH_3^+), 4.13-4.09 (m, 1H, H-*α*); 4.12 (s, 3H, OCH_3); 2.20-2.18 (d, 2H, H-*β*, J=7.15 Hz). ^{13}C NMR: 168.8 (C=O, ester); 156.5 (C-*p*); 130.2 (2C, C-*o*); 122.2 (C-*γ*); 117.5 (2C, C-*m*); 53.1 (OCH_3); 50.5 (C-*α*); 32.9 (C-*β*). Anal. Calcd. for $C_{10}H_{14}ClNO_3$: C, 51.84; H, 6.09; N, 6.05. Found: C, 51.82; H, 6.10; N, 6.06.

*L-phenylalanine methyl ester hydrochloride (**4**)*

Yield 78%, $[\alpha]_D$ +15.6°, R_f - 0.29; ^{13}C NMR: 168.9 (C=O, ester); 137.2 (C-*γ*); 131.0 (2C, C-*o*); 129.2 (2C, C-*m*); 125.8 (C-*p*); 53.3 (OCH_3); 52.5 (C-*α*); 36.3 (C-*β*). Anal. Calcd. for $C_{10}H_{14}ClNO_2$: C, 55.69; H, 6.54; N, 6.49. Found: C, 55.67; H, 6.55; N, 6.52 (Dahiya *et al.*, 2006).

*Glycine methyl ester hydrochloride (**5**)*

White solid, yield 90%, mp 174-175 °C, $[\alpha]_D$ –88.2°, R_f - 0.45; IR: 3015-2853 (s/br, NH_3^+ str, asym and sym); 2925, 2847 (m, CH str, asym and sym, CH_2); 1744 (s, C=O str, ester); 1599, 1502 (s/br, NH_3^+ bend, asym and sym); 1386, 1370 (m, CH def, butyl-*t*); 1268 (s, C–O str, ester), 932 (w, CH_3 rock, butyl-*t*). ^{1}H NMR: 4.75 (br. s, NH_3^+); 4.19 (s, 3H, OCH_3); 3.87-3.85 (d, 2H, H-*α*, J=5.5 Hz, gly); ^{13}C NMR: 170.2 (C=O, ester); 52.3 (OCH_3); 40.8 (C-*α*). Anal. Calcd. for $C_3H_8ClNO_2$: C, 28.70; H, 6.42; N, 11.16. Found: C, 28.69; H, 6.42; N, 11.20.

Preparation of linear peptide fragments (6-10)

L-Amino acid methyl ester hydrochloride/peptide methyl ester (0.01 mol) was dissolved in $CHCl_3$ (20 ml). To this, triethylamine (TEA) (2.8 ml, 0.02 mol) was added at 0 °C and the reaction mixture was stirred for 15 min. Boc-L-amino acid/peptide (0.01 mol) in $CHCl_3$ (20 ml) and dicyclohexylcarbodiimide (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 (b.p. 40-60°C) followed by cooling at 0°C.

Furthermore, trifluoroacetic acid (TFA) was used for the removal of Boc group and ester group was removed by alkaline hydrolysis with lithium hydroxide (LiOH). Peptides units were synthesized by solution phase technique (Bodanszky and Bodanszky, 1984) using DCC as coupling agent and TEA as the base.

*^{t}Butyloxycarbonyl-L-phenylalanyl-L-tyrosine methyl ester (**6**)*

Semisolid mass, yield 78%, $[\alpha]_D$ –106.2°, R_f - 0.42; IR: 3078, 3022 (w, CH str, rings); 2929, 2925, 2847 (m, CH str, asym and sym, CH_2); 1752 (s, C=O str, ester); 1633, 1629 (s, C=O str, amide); 1586, 1482, 1472 (m, skeletal bands, ring); 1538 (m, NH bend, amide); 1385, 1365 (m, CH bend, butyl-*t*); 929 (w, CH_3 rock, butyl-*t*); 1269 (s, C–O str, ester); 1229 (s, C–O str, phenolic); 729, 822, 690

(s, CH bend, oop, rings). ^{1}H NMR: 7.50-7.46 (tt, 2H, H-*m*, phe); 6.95-6.92 (t, 1H, H-*p*, phe); 6.91-6.89 (dd, 2H, H-*o*, J=7.55 Hz, J=4.6 Hz); 6.84-6.82 (dd, 2H, H-*o*, J=7.85 Hz, J=4.5 Hz, phe), 6.78-6.76 (dd, 2H, H-*m*, J=7.5 Hz, J=4.95 Hz); 6.69 (br. s, 1H, NH); 6.56 (br. s, 1H, NH); 5.98 (br. s, 1H, OH); 4.75-4.69 (m, 1H, H-α, phe); 4.60-4.55 (q, 1H, H-α); 3.56 (s, 3H, OCH_3); 3.10-2.83 (m, 4H, H-β, phe and tyr); 1.54 (s, 9H, butyl-*t*). ^{13}C NMR: 172.3 (C=O, amide); 169.8 (C=O, ester); 154.0 (C-*p*); 150.2 (C=O, boc); 133.3 (C-γ, phe); 130.8 (2C, C-*m*, phe); 129.7 (2C, C-*o*); 129.2 (2C, C-*o*, phe); 128.9 (C-*p*, phe); 125.5 (2C, C-*m*); 117.4 (C-γ); 79.8 (C-α, butyl-*t*); 57.1, 54.9 (C-α, phe and tyr); 50.8 (OCH_3); 38.5, 37.3 (C-β, tyr and phe); 27.5 (3C, C-β, butyl-*t*). Anal. Calcd. for $C_{24}H_{30}N_2O_6$: C, 65.14; H, 6.83; N, 6.33. Found: C, 65.15; H, 6.85; N, 6.30.

*^{t}Butyloxycarbonyl-L-prolyl-L-phenylalanine methyl ester (**7**)*

Yield 83%, $[\alpha]_D$ –9.6°, R_f - 0.33; ^{13}C NMR: 170.2 (C=O, amide); 167.9 (C=O, ester); 158.3 (C=O, boc); 137.0 (C-γ, phe); 129.3 (2C, C-*o*); 127.5 (2C, C-*m*); 126.5 (C-*p*); 80.2 (C-α, butyl-*t*); 62.6, 55.6 (C-α, pro and phe); 52.8 (OCH_3); 46.8 (C-δ, pro); 38.8, 29.6 (C-β, phe and pro); 27.7 (3C, C-β, butyl-*t*); 24.1 (C-γ, pro). Anal. Calcd. for $C_{20}H_{28}N_2O_5$: C, 63.81; H, 7.50; N, 7.44. Found: C, 63.82; H, 7.50; N, 7.42 (Poojary *et al.*, 2001).

*^{t}Butyloxycarbonyl-L-prolyl-L-phenylalanyl-glycine methyl ester (**8**)*

Semisolid mass, yield 69%, $[\alpha]_D$ –66.3°, R_f - 0.82; IR: 3072 (w, CH str, ring); 2995, 2988 (m, CH str, CH_2, pro); 2928, 2924, 2842 (m, CH str, asym and sym, CH_2); 1745 (s, C=O str, ester); 1667, 1639 (s, C=O str, amide); 1587, 1486 (m, skeletal bands, ring); 1537 (m, NH bend, amide); 1386, 1361 (m, CH bend, butyl-*t*); 733, 694 (s, CH bend, oop, ring). ^{1}H NMR: 7.20-7.15 (tt, 2H, H-*m*); 7.01-6.98 (t, 1H, H-*p*); 6.93 (br. s, 1H, NH); 6.85-6.83 (dd, 2H, H-*o*, J=7.75 Hz, J=4.5 Hz); 6.50 (br. s, 1H, NH); 4.98-4.93 (m, 1H, H-α); 4.10-4.07 (t, 1H, H-α, pro); 4.05-4.03 (d, 2H, H-α, J=5.6 Hz, gly); 3.65 (s, 3H, OCH_3); 3.23-3.19 (t, 2H, H-δ, pro); 2.98-2.96 (d, 2H, H-β, J=5.75 Hz); 2.57-2.52 (m, 2H, H-β, pro); 1.96-1.87 (m, 2H, H-γ, pro); 1.49 (s, 9H, butyl-*t*). ^{13}C NMR: 171.1, 169.8 (C=O, amide); 168.2 (C=O, ester); 156.2 (C=O, boc); 138.6 (C-γ); 129.8 (2C, C-*o*); 127.2 (2C, C-*m*); 127.2 (C-*p*); 79.9 (C-α, butyl-*t*); 61.2, 53.9 (C-α, pro and phe); 51.7 (OCH_3); 46.7 (C-δ, pro); 40.8 (C-α, gly); 36.4, 29.2 (C-β, phe and pro); 28.3 (3C, C-β, butyl-*t*); 23.9 (C-γ, pro). Anal. Calcd. for $C_{22}H_{31}N_3O_6$: C, 60.96; H, 7.21; N, 9.69. Found: C, 61.00; H, 7.22; N, 9.66.

*^{t}Butyloxycarbonyl-L-phenylalanyl-L-tyrosinyl-L-prolyl-L-phenylalanyl-glycine methyl ester (**9**)*

Semisolid mass, yield 72%, $[\alpha]_D$ –15.7°, R_f - 0.58; IR: 3085, 3025-3019 (w, CH str, rings); 2999, 2986 (m, CH str, CH_2, pro); 2928, 2925, 2922 (m, CH str, asym, CH_2);

Table 1: Antimicrobial activity data for compound **10**

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*
10	–	–	10(12.5)	9(12.5)	–	15(6)	8(12.5)	16(6)
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 bracket are MIC values (μg/ml).

Table 2: Anthelmintic activity data for compound **10**

Compd.	Earthworm species					
	M. konkanensis		*P. corethruses*		*Eudrilus* sp.	
	Mean paralyzing time (min)[a]	Mean death time (min)[a]	Mean paralyzing time (min)	Mean death time (min)	Mean paralyzing time (min)	Mean death time (min)
10[b]	13.43 ± 0.34	14.55 ± 0.42	22.27 ± 0.49	24.53 ± 0.36	13.28 ± 0.23	14.37 ± 0.11
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 mean ± S.D. (n = 3); [b] *c* = 2 mg/ml ; [c] 0.5% Tween 80 in distilled water.

Table 3: Cytotoxic activity data for compound **10**

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)
10	62.5	0	38	100.0		0	28	100.0	
	31.25	1	37	97.37		2	26	92.86	
	15.63	7	31	81.58	2.62	10	18	64.29	6.37
	7.81	10	28	73.68		16	12	42.86	
	3.91	20	18	47.37		21	7	25.00	
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	62.5	0	38	100.0		0	28	100.0	
(5-FU)	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.

2848, 2843 (m, CH str, sym, CH_2); 1748 (s, C=O str, ester); 1665, 1644-1635 (s, C=O str, amide); 1589, 1485-1478 (m, skeletal bands, rings); 1539, 1532 (m, NH bend, amide); 1385, 1363 (m, CH bend, butyl-*t*); 1270 (s, C–O str, ester); 1232 (s, C–O str, phenolic); 933 (w, CH_3 rock, butyl-*t*); 732-686 (s, CH bend, oop, rings). ^{1}H NMR: 8.65 (br. s, 1H, NH); 7.53-7.50 (tt, 2H, H-*m*, phe-1); 7.22-7.19 (tt, 2H, H-*m*, phe-2); 7.05-7.02 (t, 1H, H-*p*, phe-2); 7.00-6.98 (dd, 2H, H-*m*, J=7.45 Hz, J=4.9 Hz, tyr); 6.95-6.92 (t, 1H, H-*p*, phe-1); 6.91-6.89 (dd, 2H, H-*o*, J=7.55 Hz, J=4.6 Hz, tyr); 6.86-6.81 (m, 4H, H-*o*, phe-1 and phe-2); 6.59 (br. s, 1H, NH); 6.55 (br. s, 1H, NH); 6.50 (br. s, 1H, NH); 5.96 (br. s, 1H, OH); 5.29-5.23 (m, 1H, H-*α*, phe-2); 4.55-4.49 (m, 1H, H-*α*, phe-1); 4.44-4.39 (m, 1H, H-*α*, tyr); 4.12-4.09 (t, 1H, H-*α*, pro); 4.04-4.02 (d, 2H, H-*α*, J=5.5 Hz, gly); 3.64 (s, 3H, OCH_3); 3.35-3.31 (t, 2H, H-*δ*, pro); 2.98-2.83 (m, 6H, H-*β*, phe-1, phe-2 and tyr); 2.70-2.65 (m, 2H, H-*β*, pro); 1.95-1.88 (m, 2H, H-*γ*, pro); 1.55 (s, 9H, butyl-*t*). ^{13}C NMR: 172.3, 170.7, 169.4, 167.5 (C=O, amide); 168.2 (C=O, ester); 154.2 (C-*p*, tyr); 152.0 (C=O, boc); 141.3, 133.2 (C-*γ*, phe-2 and phe-1); 132.0 (C-*γ*, tyr); 130.9 (2C, C-*m*, phe-1); 129.6, 129.3 (4C, C-*m*, tyr and phe-2); 129.0 (2C, C-*o*, phe-1); 128.6 (C-*p*, phe-1); 128.3 (2C, C-*o*, phe-2); 128.0 (2C, C-*m*, tyr); 126.4 (C-*p*, phe-2); 79.8 (C-*α*, butyl-*t*); 55.5, 53.6, 51.8 (C-*α*, pro, phe-1 and phe-2); 50.5 (OCH_3); 48.8 (C-*α*, tyr); 47.2 (C-*δ*, pro); 41.6 (C-*α*, gly); 37.7, 37.4, 36.1 (C-*β*, phe-1, tyr and phe-2); 29.1 (3C, C-*β*, butyl-*t*); 26.5 (C-*β*, pro); 23.2 (C-*γ*, pro). Anal. Calcd. for $C_{40}H_{49}N_5O_9$: C, 64.59; H, 6.64; N, 9.41. Found: C, 64.56; H, 6.65; N, 9.43.

Synthesis of cyclic pentapeptide, longicalycinin A (10)

To synthesize compound **10**, linear pentapeptide unit **9** (0.005 mol) was deprotected at carboxyl end using LiOH (0.18 g, 0.0075 mol) to get Boc-L-phe-L-tyr-L-pro-L-phe-gly-OH. The deprotected pentapeptide unit (0.005 mol) was now dissolved in $CHCl_3$ (50 ml) at 0 °C. To the above solution, p-nitrophenol (pnp) (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-L-phe-L-tyr-L-pro-L-phe-gly-Opnp. To this compound (0.004 mol) dissolved in $CHCl_3$ (35 ml), TFA (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 L-phe-L-tyr-L-pro-L-phe-gly-Opnp which was dissolved in $CHCl_3$ (25 ml) and NMM/C_5H_5N (2.21 ml/1.61 ml, 0.02 mol) was added. Then, whole contents were 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 (L-phe-L-tyr-L-pro-L-phe-gly) (**10**).

Light yellow solid; m.p. 153 °C; Yield 81% (2.5 g, NMM), 62% (C_5H_5N); $[\alpha]_D$ –11.9° (–12°); R_f - 0.76; IR: 3079, 3025-3018 (w, CH str, rings); 2998, 2987-2985 (m, CH str, CH_2, pro); 2926, 2925-2921 (m, CH str, asym, CH_2); 2850-2847, 2842 (m, CH str, sym, CH_2); 1669, 1649-1634 (s, C=O str, amide); 1588, 1487-1479 (m, skeletal bands, rings); 1538, 1533-1529 (m, NH bend, amide); 1229 (s, C–O str, phenolic); 730-685 (s, CH bend, oop, rings). ^{1}H NMR: 9.89 (br. s, 1H, NH); 8.05 (br. s,

1H, NH); 7.64 (br. s, 1H, NH); 7.22-7.12 (m, 4H, H-*m*, phe-1 and phe-2); 7.08 (br. s, 1H, NH); 7.03-6.99 (m, 2H, H-*p*, phe-1 and phe-2); 6.98-6.96 (dd, 2H, H-*m*, J=7.5 Hz, J=4.95 Hz, tyr); 6.92-6.90 (dd, 2H, H-*o*, J=7.5 Hz, J=4.65 Hz, tyr); 6.85-6.81 (m, 4H, H-*o*, phe-1 and phe-2); 5.95 (br. s, 1H, OH); 5.76-5.71 (m, 1H, H-α, tyr); 5.65-5.61 (m, 1H, H-α, phe-1); 5.25-5.23 (d, 2H, H-α, J=5.6 Hz, gly); 4.35-4.29 (m, 1H, H-α, phe-2); 3.92-3.89 (t, 1H, H-α, pro); 3.29-3.25 (t, 2H, H-δ, pro); 2.71-2.67 (m, 2H, H-β, pro); 2.64-2.57 (m, 6H, H-β, phe-1, phe-2 and tyr); 1.89-1.78 (m, 2H, H-γ, pro). ^{13}C NMR: 172.3, 171.8, 171.0, 170.2, 169.3 (C=O, amide); 154.0 (C-*p*, tyr); 140.2, 137.8 (2C, C-γ, phe-1 and phe-2); 132.7 (C-γ, tyr); 130.4 (4C, C-*o*, phe-1 and phe-2); 128.9 (4C, C-*m*, phe-1 and phe-2); 128.4 (2C, C-*o*, tyr); 127.7 (2C, C-*m*, tyr); 126.9 (2C, C-*p*, phe-1 and phe-2); 58.3, 53.9, 51.9 (3C, C-α, pro, tyr and phe-1); 51.3 (C-α, phe-2); 48.1 (C-δ, pro); 42.9 (C-α, gly); 38.7, 37.8 (3C, C-β, phe-1, phe-2 and tyr); 31.3 (C-β, pro); 24.9 (C-γ, pro). FAB MS: m/z [%] 612.4 [$(M + H)^+$, 100]; 584.4 [$(612.4–CO)^+$, 29]; 465.3 [$(tyr\text{-}pro\text{-}phe\text{-}gly)^+$, 23]; 449.3 [$(pro\text{-}phe\text{-}gly\text{-}phe)^+$, 14]; 437.3 [$(465.3–CO)^+$, 14]; 421.3 [$(449.3–CO)^+$, 23]; 408.3 [$(tyr\text{-}pro\text{-}phe)^+$, 10]; 380.3 [$(408.3–CO)^+$, 12]; 311.3 [$(phe\text{-}tyr)^+$, 9]; 302.3 [$(pro\text{-}phe\text{-}gly)^+$, 18]; 261.2 [$(tyr\text{-}pro)^+$, 54]; 245.2 [$(pro\text{-}phe)^+$, 21]; 233.2 [$(261.2–CO)^+$, 43]; 164.2 [$(tyr)^+$, 7]; 148.2 [$(phe)^+$, 13]; 136.2 [$(164.2–CO)^+$, 11]; 120.2 [$(148.2–CO)^+$, 12]; 107.1 [$(C_7H_7O)^+$, 16]; 91.0 [$(C_7H_7)^+$, 18]; 65.0 [$(C_5H_5)^+$, 7]. Anal. Calcd. for $C_{34}H_{37}N_5O_6$: C, 66.76; H, 6.10; N, 11.45. Found: C, 66.75; H, 6.13; N, 11.46.

Biological activity studies

Antimicrobial screening

The synthesized cyclopeptide was evaluated for its antimicrobial activity (Bauer *et al.*, 1966) against four bacterial strains *Bacillus subtilis* (MUMC 408), *Staphylococcus aureus* (MUMC 377), *Pseudomonas aeruginosa* (MUMC 266) and *Escherichia coli* (MUMC 106) and four fungal strains *Microsporum audouinii* (MUMC 545), *Trichophyton mentagrophytes* (MUMC 665), *Candida albicans* (MUMC 29) and *Aspergillus niger* (MUMC 77) at 12.5–6 μg/ml concentration. MIC values of test compound were determined by tube dilution technique. The solvents DMF/DMSO were used as negative controls and ciprofloxacin/griseofulvin were used as standards.

Anthelmintic screening

Anthelmintic activity studies (Garg and Atal, 1963) were carried out against three different species of earthworms *Megascoplex konkanensis* (ICARBC 211), *Pontoscotex corethruses* (ICARBC 117) and *Eudrilus* sp. (ICARBC 042) at 2 mg/ml concentration. Tween 80 (0.5%) in distilled water was used as control and mebendazole/ piperazine citrate were used as standards.

Cytotoxicity screening

Synthesized cyclopeptide **10** was subjected to short term *in vitro* cytotoxicity study (Kuttan *et al.*, 1985) against *Dalton's lymphoma ascites* (NCRC 101) and *Ehrlich's ascites carcinoma* (NCRC 69) cell lines at 62.5–3.91 μg/ml using 5-fluorouracil (5-FU) as reference compound. Activity was assessed by determining the percentage inhibition of DLA and EAC cells. CTC_{50} values were determined by graphical extrapolation method.

RESULTS

Chemistry

In the present work, disconnection strategy was employed to carry out the first total synthesis of longicalycinin A (**10**). The cyclic pentapeptide molecule was split into single amino acid unit gly-OMe.HCl (**5**) and two dipeptide units Boc-L-phe-L-tyr-OMe (**6**), Boc-L-pro-L-phe-OMe (**7**). The required dipeptide units (**6**, **7**) were prepared by coupling of Boc-amino acids viz. Boc-L-phe-OH (**1**) and Boc-L-pro-OH (**2**) with corresponding amino acid methyl ester hydrochlorides such as L-tyr-OMe.HCl (**3**) and L-phe-OMe.HCl (**4**) employing DCC as coupling agent. Ester group of dipeptide **7** was removed by alkaline hydrolysis with LiOH and deprotected peptide was coupled with amino acid hydrochloride **5** using DCC and TEA as base, to get the tripeptide unit Boc-L-pro-L-phe-gly-OMe (**8**). Then, dipeptide **6** after deprotection at carboxyl end, was coupled with tripeptide **8** deprotected at amino terminal, to get the pentapeptide unit Boc-L-phe-L-tyr-L-pro-L-phe-gly-OMe (**9**). The ester group of linear fragment was removed using LiOH and p-nitrophenyl (pnp) ester group was introduced. The Boc-group 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 base to get cyclic product **10** (fig. 1).

Structure of the newly synthesized cyclic pentapeptide as well as intermediates di/tri/pentapeptides were confirmed by IR, ^{1}H / ^{13}C NMR as well as elemental analysis. In addition, mass spectra was recorded for the cyclopeptide.

Pharmacology

The results of *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* are presented in table 1. The results of anthelmintic activity against earthworms *M. konkanensis*, *P. corethruses* and *Eudrilus* sp. are given in table 2. Synthesized cyclopeptide was also subjected to short term *in vitro* cytotoxicity study against DLA and EAC cell lines and the results of such studies are tabulated in table 3.

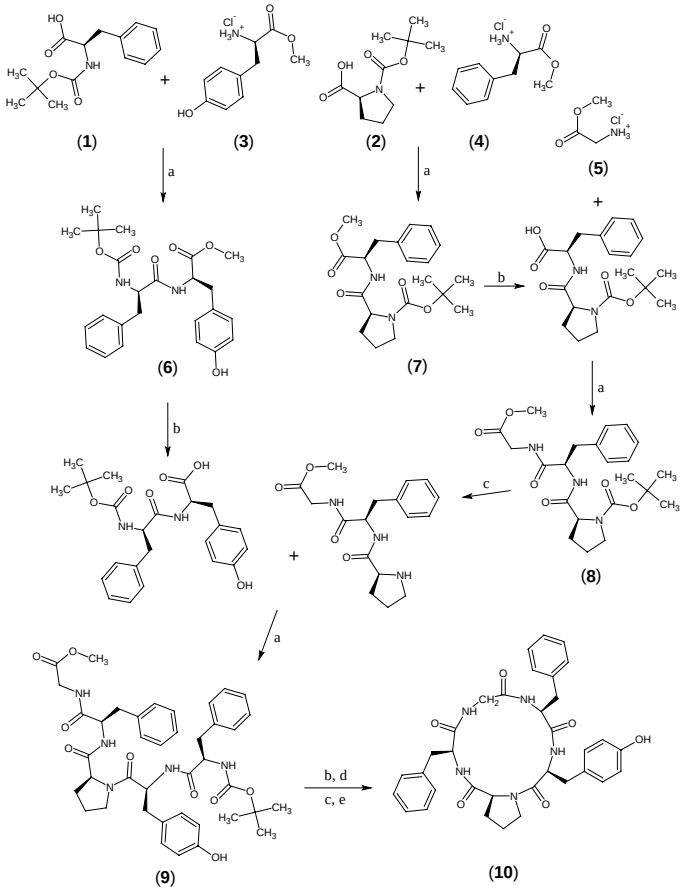

Fig. 1

DISCUSSION

Synthesis of longicalycinin A was carried out successfully with good yield and NMM was proved to be a yield effective base for cyclization of linear pentapeptide fragment. Cyclization of linear peptide fragment was indicated by disappearance of absorption bands at 1748 cm^{-1} and 1385, 1363 cm^{-1} (C=O stretching of ester and CH deformation of ^{t}Butyl group) and presence of additional Amide I and Amide II bands of the -CO-NH- moiety at 1649-1645 cm^{-1} and 1531-1529 cm^{-1} in IR spectra of the compound **10**. Formation of cyclopeptide was further confirmed by disappearance of singlets at 3.64 and 1.55 ppm corresponding to three protons of methyl ester group and nine protons of ^{t}Butyl group of Boc, in ^{1}H NMR spectrum of compound **10**. Furthermore, ^{1}H NMR and ^{13}C NMR spectra of synthesized cyclic pentapeptide showed characteristic peaks confirming presence all the 37 protons and 34 carbon atoms. Presence of $(M + 1)^{+}$ ion peak at m/z 612.4 corresponding to the molecular formula $C_{34}H_{37}N_5O_6$ in mass spectra of compound **10**, along with other fragment ion peaks resulting from cleavage at 'phe-tyr', 'tyr-pro' and 'gly-phe' amide bond levels, showed exact sequence of attachment of all the five amino acid moieties in a chain. In addition, elemental analysis of compound **10** afforded average values (± 0.02) strictly in accordance to the molecular composition.

Synthesized cyclopeptide exhibited high cytotoxic activity against DLA and EAC cell lines with CTC_{50} values of 2.62 and 6.37 µM respectively, in comparison to standard drug 5-fluorouracil (5-FU) (CTC_{50} values – 37.36 and 90.55 µM). Comparison of antimicrobial screening data suggested that compound **10** possessed moderate activity against dermatophytes *M. audouinii* and *T. mentagrophytes* with MIC values of 6 µg/ml, in comparison to standard drug griseofulvin. However, compound **10** displayed only little activity against gram negative bacteria and fungus *A. niger*. On the other hand, it exhibited no activity against gram positive bacteria and pathogenic *C. albicans*. Furthermore, compound **10**

showed good anthelmintic activity against earthworms *M. konkanensis*, *P. corethruses* and *Eudrilus* sp. at 2 mg/ml, as compared to reference drugs mebendazole and piperazine citrate. In comparison, *P. corethruses* was found to be least sensitive earthworm species towards the newly synthesized peptide. On passing toxicity tests, synthesized cyclopeptide **10** may prove good candidate for clinical studies and can be new cytotoxic and anthelmintic drug of future.

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