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# Synthetic and biological studies on a cyclopolypeptide of plant origin

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**Abstract:** Objective: A natural cyclic peptide previously isolated from *Citrus medica* was synthesized by coupling of tetrapeptide units Boc-Leu-Pro-Trp-Leu-OMe and Boc-Ile-Ala-Ala-Gly-OMe after proper deprotection at carboxyl and amino terminals followed by cyclization of linear octapeptide segment. Methods: Solution phase technique was adopted for the synthesis of cyclooctapeptide—sarcodactylamide. Required tetrapeptide units were prepared by coupling of Boc-protected dipeptides viz. Boc-Leu-Pro-OH and Boc-Ile-Ala-OH with respective dipeptide methyl esters Trp-Leu-OMe and Ala-Gly-OMe. Cyclization of linear octapeptide unit was done by *p*-nitrophenyl ester method. The structure of synthesized cyclopolypeptide was elucidated by FTIR, $^{1}$H NMR, $^{13}$C NMR, FABMS spectral data and elemental analysis. The newly synthesized peptide was evaluated for different pharmacological activities including antimicrobial, anthelmintic and cytotoxic activities. Results: Synthesis of sarcodactylamide was accomplished with >78% yield utilizing dicyclohexylcarbodiimide (DCC) as coupling agent. Newly synthesized peptide possessed potent cytotoxic activity against Dalton's lymphoma ascites (DLA) and Ehrlich's ascites carcinoma (EAC) cell lines, in addition to moderate anthelmintic activity against earthworms *Megascoplex konkanensis*, *Pontoscotex corethruses* and *Eudrilus* sp. Moreover, cyclopolypeptide displayed good antimicrobial activity against pathogenic fungi *Candida albicans* and Gram-negative bacteria *Pseudomonas aeruginosa*, in comparison to standard drugs griseofulvin and ciprofloxacin. Conclusion: Solution phase technique employing DCC and triethylamine (TEA) as base proved to be effective for the synthesis of natural cyclooctapeptide. *N*-methyl morpholine (NMM) was found to be a better base for the cyclization of linear octapeptide unit in comparison to TEA and pyridine.

**Key words:** Cyclic octapeptide, *Citrus medica* var. *sarcodactylis*, Sarcodactylamide, Peptide synthesis, Anthelmintic activity, Antimicrobial activity, Cytotoxicity

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## INTRODUCTION

Literature is enriched with several reports indicating the potential of higher plants to produce a wide array of natural products with interesting pharmacological activities (Lago *et al*., 2007; Wele *et al*., 2005; Morel *et al*., 2005; Akendengue *et al*., 2005; Svangård *et al*., 2004; Kumarasamy *et al*., 2003; Nogueira *et al*., 1996; Marston *et al*., 1993). Among these, cyclopolypeptides and related congeners have received special attention due to their unique structures and wide biological profile, and may prove better candidates to overcome the problem of resistance towards conventional drugs. A novel cyclic peptide, cyclo(-Gly-Leu-Pro-Trp-Leu-Ile-Ala-Ala-), has been isolated from fruit peels of *Citrus medica* var. *sarcodactylis* SWINGLE (Rutaceae) and the structure was elucidated by intensive two-dimentional (2D) NMR analysis and chemical evidence. The absolute stereochemistry of each amino acid component of cyclooctapeptide was determined to be of an L-configuration by HPLC analysis of the derivatives of the acid hydrolysate by treatment with Marfey's reagent (Matsumoto *et al*., 2002).

In continuation of our synthetic efforts on natural cyclic polypeptides of biological interest—halolitoralins A~C (Dahiya and Pathak, 2006a; 2007a; 2007b), cherimolacyclopeptides E and G (Dahiya, 2007a; 2008), segetalins C~E (Dahiya and Kaur, 2007; 2008; Dahiya, 2007b), longicalycinin A (Dahiya, 2007c) and hymenamide E (Dahiya *et al.*, 2006), the present investigation was aimed at the synthesis of a novel cyclic octapeptide of plant origin. Keeping in view of significant biological activities possessed by

natural cyclopolypeptides (Dahiya and Pathak, 2006b; Pathak and Dahiya, 2003), the above synthetic peptide, sarcodactylamide, was further subjected to the studies of antibacterial, antifungal, anthelmintic and cytotoxic activities.

## MATERIALS AND METHODS

### Chemistry

All the reactions requiring anhydrous conditions were conducted in flame dried apparatus. Melting point was determined by open capillary method and uncorrected. L-amino acids, dicyclohexylcarbodiimide (DCC), trifluoroacetic acid (TFA), *p*-nitrophenol (pNP), *N*-methyl morpholine (NMM), triethylamine (TEA), di-*tert*-butylpyrocarbonate ($Boc_2O$) and pyridine ($C_5H_5N$) were obtained from Spectrochem. Ltd. (Mumbai, India). IR spectra were recorded on Shimadzu 8700 FTIR spectrophotometer (Shimadzu, Japan) by using a thin film supported on KBr pellets for synthesized cyclic octapeptide and $CHCl_3$ as solvent for intermediate semisolids. $^1H$ NMR and $^{13}C$ NMR spectra were recorded on Bruker AC NMR spectrometer (300 MHz, Brucker, USA) with $CDCl_3$ as solvent and tetramethylsilane (TMS) as internal standard. Mass spectra were recorded on JMS-DX 303 mass spectrometer (Jeol, Tokyo, Japan) at 70 eV by using fast atom bombardment technique. Elemental analyses of all compounds were performed on Vario EL III elemental analyzer (Elementar, Germany). Optical rotation of the synthesized peptides was measured on automatic polarimeter (Optics Tech., Ghaziabad, India) in a 20 cm tube at 25 °C with sodium lamp and methanol as solvent. Purity of synthesized cyclopolypeptide as well as intermediates was checked by thin-layer chromatography (TLC) on precoated silica gel G plates utilizing $CHCl_3$/MeOH as developing solvent in different ratios (9:1 or 7:3, v/v), and brown spots were detected on exposure to iodine vapours in a tightly closed chamber.

General method for the synthesis of linear peptide fragments (compounds **1**~**6**): Amino acid methyl ester hydrochloride/peptide methyl ester (0.01 mol) was dissolved in 20 ml chloroform. To this, TEA (0.021 mol, 2.21 ml) was added at 0 °C and the reaction mixture was stirred for 15 min. Boc-amino acid/peptide (0.01 mol) dissolved in 20 ml chloroform and DCC (0.01 mol, 2.1 g) were added to above reaction mixture, with stirring. After 24 h, the reaction mixture was filtered, and the residue was washed with 30 ml chloroform and added to the filtrate. The filtrate was washed with 5% (w/v) sodium bicarbonate and saturated sodium chloride solutions. The organic layer was dried over anhydrous sodium sulphate, filtered and evaporated in vacuum. The crude product was recrystallized from a mixture of chloroform and petroleum ether (b.p. 60~80 °C) followed by cooling at 0 °C.

Peptides units were prepared by solution phase technique (Bodanszky and Bodanszky, 1984) employing DCC as coupling agent and TEA as base. The carboxyl group of L-amino acids was protected by esterification with methanol by using thionyl chloride. Moreover, Boc group was removed by TFA and ester group was removed by alkaline hydrolysis with LiOH. Physical characterization of the synthesized compounds is given in Table 1.

**Table 1 Physical characterization of the synthesized compounds**

| Compd. | Physical state | Formula | $M_w$ | m.p. (°C) | Yield (%) | $R_f$[a] | $\alpha_{[D]}$[b] | Elemental content (%)[#] | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | C | H | N |
| **1** | Semisolid mass | $C_{17}H_{30}N_2O_5$ | 342.43 | – | 84.3 | 0.82 | –19.2 | 59.63/59.65 | 8.83/8.79 | 8.18/8.17 |
| **2** | Semisolid mass | $C_{15}H_{28}N_2O_5$ | 316.39 | – | 66.4 | 0.65 | –112.5 | 56.94/56.95 | 8.92/8.90 | 8.85/8.85 |
| **3** | Semisolid mass | $C_{23}H_{33}N_3O_5$ | 431.53 | – | 72.1 | 0.54 | +11.3 | 64.02/64.00 | 7.71/7.69 | 9.74/9.75 |
| **4** | Semisolid mass | $C_{11}H_{20}N_2O_5$ | 260.29 | – | 75.8 | 0.49[*] | –17.6 | 50.76/50.75 | 7.74/7.70 | 10.76/10.79 |
| **5** | Semisolid mass | $C_{34}H_{51}N_5O_7$ | 641.80 | – | 79.7 | 0.58 | –110.6 | 63.63/63.00 | 8.01/8.04 | 10.91/10.90 |
| **6** | Semisolid mass | $C_{20}H_{36}N_4O_7$ | 444.52 | – | 75.0 | 0.63 | +98.1 | 54.04/54.05 | 8.16/8.15 | 12.60/12.62 |
| **7** | Semisolid mass | $C_{48}H_{75}N_9O_{11}$ | 954.17 | – | 83.5 | 0.81[*] | –70.9[†] | 60.42/60.45 | 7.92/7.90 | 13.21/13.22 |
| **8** | White solid | $C_{42}H_{63}N_9O_8$ | 822.01 | 245~246 (dec.)[c] | 78.3 | 0.70[*] | –81.2[‡] (–81.1)[d] | 61.37/61.39 | 7.72/7.72 | 15.34/15.35 |

Solvent systems used for determination of $R_f$ values: [a]$CHCl_3$:MeOH (7:3, v/v), [*]$CHCl_3$:MeOH (9:1, v/v); Concentrations of compound solutions for determination of specific rotation ($\alpha_{[D]}$) values: [b]0.5 mol/L in MeOH, [†]0.35 mol/L in MeOH, [‡]0.15 mol/L in MeOH; [c]dec.: Decomposed; [d]–81.1 is a specific rotation value for naturally isolated cyclic octapeptide which was synthesized; [#] Data expressed as calculated/found

Cyclization of linear octapeptide fragment (compound **7**): To synthesize sarcodactylamide (compound **8**), linear peptide unit (compound **7**) (0.005 mol, 4.77 g) was deprotected at carboxyl end with lithium hydroxide (0.0075 mol, 0.18 g) to get Boc-Leu-Pro-Trp-Leu-Ile-Ala-Ala-Gly-OH. The deprotected octapeptide unit (0.005 mol, 4.7 g) was now dissolved in 50 ml chloroform at 0 °C. To the above solution, *p*-nitrophenol (0.0067 mol, 0.94 g) was added and stirred at room temperature for 12 h. The reaction mixture was filtered, and the filtrate was washed with 10% (w/v) sodium bicarbonate solution (15 ml, thrice) until excess of *p*-nitrophenol was removed and finally washed with 5% (v/v) HCl (10 ml, twice) to get the corresponding *p*-nitrophenyl ester Boc-Leu-Pro-Trp-Leu-Ile-Ala-Ala-Gly-O-pNP. To this compound (0.004 mol, 4.24 g) dissolved in 35 ml chloroform, trifluoroacetic acid (0.008 mol, 0.91 g) was added, stirred at room temperature for 1 h, and washed with 10% sodium bicarbonate solution (25 ml, twice). The organic layer was dried over anhydrous sodium sulphate to get Leu-Pro-Trp-Leu-Ile-Ala-Ala-Gly-O-pNP that was dissolved in 25 ml of chloroform, and TEA/NMM/pyridine (0.021 mol, 2.21 ml/2.8 ml/1.61 ml) was added. Then, whole contents were kept at 0 °C for 7 d. The reaction mixture was washed with 10% sodium carbonate solution until the by-product *p*-nitrophenol was removed completely, and finally washed with 5% HCl (15 ml, thrice). The organic layer was dried over anhydrous sodium sulphate. Finally, chloroform was distilled off and crude cyclized product was crystallized from chloroform/*n*-hexane to get pure cyclo(-Leu-Pro-Trp-Leu-Ile-Ala-Ala-Gly-) (compound **8**, sarcodactylamide).

The chemical characteristics of compounds **1**~**8** are shown as following:

1. *tert*-Butyloxycarbonyl-leucyl-proline methyl ester (compound **1**)

IR ($CHCl_3$), $v$ ($cm^{-1}$): 3133 (m, -NH str, amide), 2999, 2992 (m, -CH str, cyclic $CH_2$), 2926, 2851 (m, -CH str, asym & sym, $CH_2$), 2826 (m, -CH str, $OCH_3$), 1748 (s, -C=O str, ester), 1666, 1642, 1638 (s, -C=O str, 3° & 2° amide), 1536 (m, -NH bend, 2° amide), 1462 (m, -CH bend (scissoring), $CH_2$), 1396, 1372 (s, -CH bend, *tert*-butyl group), 1383, 1365 (s, -CH bend, isopropyl group), 1278 (s, C-O str, ester), 930, 923 (w, $CH_3$ rocking, *tert*-butyl and isopropyl groups); $^1$H NMR (300 MHz, $CDCl_3$), $\delta$ ($10^{-6}$): 5.89 (1H, br. s, -NH, Leu), 4.39~4.28 (2H, m, α-H's, Pro & Leu), 3.81~3.75 (2H, t, δ-H's, Pro), 3.62 (3H, s, $OCH_3$), 2.07~1.98 (4H, m, β- & γ-H's, Pro), 1.90~1.87 (2H, t, β-H's, Leu), 1.63~1.56 (1H, m, γ-H's, Leu), 1.55 (9H, s, *tert*-butyl group), 1.03~1.01 (6H, d, $J$=6.2 Hz, δ-H's, Leu).

2. *tert*-Butyloxycarbonyl-isoleucyl-alanine methyl ester (compound **2**)

IR ($CHCl_3$), $v$ ($cm^{-1}$): 3129, 3125 (m, -NH str, amide), 2962, 2959, 2874 (m, -CH str, asym & sym, $CH_3$), 2848 (m, -CH str, sym, $CH_2$), 2824 (m, -CH str, $OCH_3$), 1752 (s, -C=O str, ester), 1643, 1640 (s, -C=O str, 2° amide), 1536, 1529 (m, -NH bend, 2° amide), 1468 (m, -CH bend (scissoring), $CH_2$), 1394, 1370 (s, -CH bend, *tert*-butyl group), 1275 (s, C-O str, ester), 932 (w, $CH_3$ rocking, *tert*-butyl group); $^1$H NMR (300 MHz, $CDCl_3$), $\delta$ ($10^{-6}$): 6.74 (1H, br. s, -NH, Ala), 5.68 (1H, br. s, -NH, Ile), 4.46~4.39 (1H, q, α-H, Ala), 4.35~4.31 (1H, t, α-H, Ile), 3.59 (3H, s, $OCH_3$), 1.85~1.74 (1H, m, β-H, Ile), 1.63~1.57 (2H, q, γ-H's, Ile), 1.54 (9H, s, *tert*-butyl group), 1.30~1.28 (3H, d, $J$=5.25 Hz, β-H's, Ala), 1.05~1.03 (3H, d, $J$=5.85 Hz, γ′-H's, Ile), 0.99~0.96 (3H, t, δ-H's, Ile).

3. *tert*-Butyloxycarbonyl-tryptophanyl-leucine methyl ester (compound **3**)

IR ($CHCl_3$), $v$ ($cm^{-1}$): 3485 (m, -NH str, arom. ring), 3135, 3122 (m, -NH str, amide), 3072 (w, -CH str, arom. ring), 2927, 2850, 2847 (m, -CH str, asym & sym, $CH_2$), 2820 (m, -CH str, $OCH_3$), 1755 (s, -C=O str, ester), 1644, 1639 (s, -C=O str, 2° amide), 1572, 1433 (m, skeletal bands, arom. ring), 1535, 1526 (m, -NH bend, 2° amide), 1463 (m, -CH bend (scissoring), $CH_2$), 1395, 1370 (s, -CH bend, *tert*-butyl group), 1383, 1364 (s, -CH bend, isopropyl group), 1272 (s, C-O str, ester), 929, 920 (w, $CH_3$ rocking, *tert*-butyl and isopropyl groups), 735, 675 (s, -CH bend, oop, arom. ring); $^1$H NMR (300 MHz, $CDCl_3$), $\delta$ ($10^{-6}$): 8.94 (1H, br. s, -NH, Indole ring), 7.54~7.52 (1H, d, $J$=7.6 Hz, H at $C_4$, Trp), 7.13~7.05 (3H, m, H's at $C_{5\sim7}$, Trp), 6.98, 6.52 (2H, br. s, -NH, Leu & Trp), 6.23~6.21 (1H, d, $J$=7.85 Hz, H at $C_2$, Trp), 5.02~4.97 (1H, q, α-H, Trp), 4.16~4.11 (1H, q, α-H, Leu), 3.62 (3H, s, $OCH_3$), 3.16~3.14 (2H, d, $J$=5.7 Hz, β-H's, Trp), 1.55 (9H, s, *tert*-butyl group), 1.49~1.33 (1H, m, γ-H's, Leu), 1.28~1.25 (2H, t, β-H's, Leu), 0.96~0.94 (6H, d, $J$=6.15 Hz, δ-H's, Leu).

4. *tert*-Butyloxycarbonyl-alanyl-glycine methyl ester (compound **4**)

IR ($CHCl_3$), $v$ ($cm^{-1}$): 3126 (m, -NH str, amide), 2960, 2877 (m, -CH str, asym & sym, $CH_3$), 2928, 2852 (m, -CH str, asym & sym, $CH_2$), 2822 (m, -CH str, $OCH_3$), 1747 (s, -C=O str, ester), 1641, 1638 (s, -C=O str, 2° amide), 1533 (m, -NH bend, 2° amide), 1392, 1373 (s, -CH bend, *tert*-butyl group), 1269 (s, C-O str, ester), 928 (w, $CH_3$ rocking, *tert*-butyl group); $^1$H NMR (300 MHz, $CDCl_3$), $\delta$ ($10^{-6}$): 7.06, 6.54 (2H, br. s, -NH, Gly & Ala), 4.56~4.49 (1H, q, α-H, Ala), 4.15~4.13 (2H, d, $J$=5.15 Hz, α-H's, Gly), 3.64 (3H, s, $OCH_3$), 1.61~1.59 (3H, d, $J$=5.3 Hz, β-H's, Ala), 1.54 (9H, s, *tert*-butyl group).

5. *tert*-Butyloxycarbonyl-leucyl-prolyl-tryptophanyl-leucine methyl ester (compound **5**)

IR ($CHCl_3$), $v$ ($cm^{-1}$): 3488 (m, -NH str, arom. ring), 3135, 3129 (m, -NH str, amide), 3069 (w, -CH str, arom. ring), 2998~2993 (m, -CH str, cyclic $CH_2$), 2927, 2919, 2852, 2849 (m, -CH str, asym & sym, $CH_2$), 2825 (m, -CH str, $OCH_3$), 1747 (s, -C=O str, ester), 1669, 1644~1637 (s, -C=O str, 3° & 2° amide), 1569, 1443, 1437 (m, skeletal bands, arom. ring), 1536, 1524 (m, -NH bend, 2° amide), 1465 (m, -CH bend (scissoring), $CH_2$), 1392, 1370 (s, -CH bend, *tert*-butyl group), 1385, 1361 (s, -CH bend, isopropyl group), 933, 921 (w, $CH_3$ rocking, *tert*-butyl and isopropyl groups), 739, 677 (s, -CH bend, oop, arom. ring); $^1$H NMR (300 MHz, $CDCl_3$), $\delta$ ($10^{-6}$): 8.95 (1H, br. s, -NH, Indole ring), 7.28~7.26 (1H, d, $J$=7.9 Hz, H at $C_2$, Trp), 7.22~7.20 (1H, d, $J$=7.55 Hz, H at $C_4$, Trp), 7.16~7.08 (3H, m, H's at $C_{5\sim7}$, Trp), 6.77, 6.56, 5.90 (3H, br. s, -NH, $Leu^2$, Trp & $Leu^1$), 5.30~5.26 (1H, q, α-H, Trp), 4.48~4.45 (1H, t, α-H, Pro), 4.23~4.18 (1H, q, α-H, $Leu^1$), 3.74~3.69 (2H, t, δ-H's, Pro), 3.60 (3H, s, $OCH_3$), 3.53~3.49 (1H, q, α-H, $Leu^2$), 3.18~3.16 (2H, d, $J$=5.75 Hz, β-H's, Trp), 2.68~2.65 (2H, q, β-H's, Pro), 1.99~1.91 (4H, m, β-H's, $Leu^1$ & γ-H's, Pro), 1.66~1.57 (1H, m, γ-H's, $Leu^1$), 1.54 (9H, s, *tert*-butyl group), 1.49~1.40 (3H, m, β- & γ-H's, $Leu^2$), 1.01~0.99 (6H, d, $J$=6.15 Hz, δ-H's, $Leu^1$), 0.95~0.93 (6H, d, $J$ =6.2 Hz, δ-H's, $Leu^2$).

6. *tert*-Butyloxycarbonyl-isoleucyl-alanyl-alanyl-glycine methyl ester (compound **6**)

IR ($CHCl_3$), $v$ ($cm^{-1}$): 3132~3126 (m, -NH str, amide), 2965~2958, 2874~2869 (m, -CH str, asym & sym, $CH_3$), 2846 (m, -CH str & sym, $CH_2$), 2825 (m, -CH str, $OCH_3$), 1750 (s, -C=O str, ester), 1644~1640 (s, -C=O str, 2° amide), 1532, 1526 (m, -NH bend, 2° amide), 1466 (m, -CH bend (scissoring), $CH_2$), 1392, 1373 (s, -CH bend, *tert*-butyl group), 1268 (s, -C-O-str, ester), 930 (w, $CH_3$ rocking, *tert*-butyl group); $^1$H NMR (300 MHz, $CDCl_3$), $\delta$ ($10^{-6}$): 9.43, 9.35 (2H, br. s, -NH, $Ala^1$ & $Ala^2$), 7.25 (1H, br. s, -NH, Gly), 5.68 (1H, br. s, -NH, Ile), 4.47~4.41 (1H, q, α-H, $Ala^1$), 4.31~4.25 (1H, q, α-H, $Ala^2$), 4.22~4.19 (1H, t, α-H, Ile), 4.05~4.03 (2H, d, $J$=5.2 Hz, α-H's, Gly), 3.65 (3H, s, $OCH_3$), 1.84~1.75 (1H, m, β-H, Ile), 1.65~1.51 (2H, q, γ-H's, Ile), 1.55 (9H, s, *tert*-butyl group), 1.50~1.48 (3H, d, $J$=5.3 Hz, β-H's, $Ala^2$), 1.46~1.44 (3H, d, $J$=5.25 Hz, β-H's, $Ala^1$), 1.04~1.02 (3H, d, $J$=5.9 Hz, γ′-H's, Ile), 0.98~0.95 (3H, t, δ-H's, Ile).

7. *tert*-Butyloxycarbonyl-leucyl-prolyl-tryptophanyl-leucyl-isoleucyl-alanyl-alanyl-glycine methyl ester (compound **7**)

IR ($CHCl_3$), $v$ ($cm^{-1}$): 3485 (m, -NH str, arom. ring), 3128~3125 (m, -NH str, amide), 3066, 3052 (w, -CH str, arom. ring), 2999~2992 (m, -CH str, cyclic $CH_2$), 2957, 2953 (m, -CH str & asym, $CH_3$), 2927~2924, 2852 (m, -CH str, asym & sym, $CH_2$), 2827 (m, -CH str, $OCH_3$), 1746 (s, -C=O str, ester), 1666, 1646~1637 (s, -C=O str, 3° & 2° amide), 1573, 1433~1428 (m, skeletal bands, arom. ring), 1537~1525 (m, -NH bend, 2° amide), 1390, 1369 (s, -CH bend, *tert*-butyl group), 1382, 1368 (s, -CH bend, isopropyl group), 1271 (s, C-O str, ester), 933, 919 (w, $CH_3$ rocking, *tert*-butyl and isopropyl groups), 736, 672 (s, -CH bend, oop, arom. ring); $^1$H NMR (300 MHz, $CDCl_3$), $\delta$ ($10^{-6}$): 9.42 (1H, br. s, -NH, $Ala^2$), 8.94 (1H, br. s, -NH, indole ring), 7.96 (1H, br. s, -NH, $Ala^1$), 7.62, 7.56 (2H, br. s, -NH, Ile & $Leu^2$), 7.29~7.27 (1H, d, $J$=7.85 Hz, H at $C_2$, Trp), 7.25 (1H, br. s, -NH, Gly), 7.22~7.11 (4H, m, H's at $C_{4\sim7}$, Trp), 6.55, 5.91 (2H, br. s, -NH, Trp & $Leu^1$), 5.11~5.07 (1H, q, α-H, Trp), 4.48~4.45 (1H, t, α-H, Pro), 4.30~4.25 (1H, q, α-H, $Ala^2$), 4.24~4.21 (1H, q, α-H, $Leu^1$), 4.20~4.16 (1H, q, α-H, $Ala^1$), 4.08~4.05 (1H, t, α-H, Ile), 4.04~4.02 (2H, d, $J$=5.2 Hz, α-H's, Gly), 3.89~3.85 (1H, q, α-H, $Leu^2$), 3.71~3.68 (2H, t, δ-H's, Pro), 3.64 (3H, s, $OCH_3$), 3.14~3.12 (2H, d, $J$=5.7 Hz, β-H's, Trp), 2.70~2.65 (2H, q, β-H's, Pro), 2.09~2.02 (1H, m, β-H, Ile), 1.97~1.80 (6H, m, β-H's, $Leu^1$, $Leu^2$ & γ-H's, Pro), 1.67~1.61 (2H, q, γ-H's, Ile), 1.55 (9H, s, *tert*-butyl group), 1.49~1.34 (8H, m, γ-H's, $Leu^1$, $Leu^2$ & β-H's, $Ala^1$, $Ala^2$), 1.05~1.03 (3H, d, $J$=5.85 Hz, γ′-H's, Ile), 1.02~0.98 (12H, m, δ-H's, $Leu^1$ & $Leu^2$), 0.96~0.93 (3H, t, δ-H's, Ile).

8. Cyclo(-leucyl-prolyl-tryptophanyl-leucyl-isoleucyl-alanyl-alanyl-glycyl-) (compound **8**, sarcodactylamide)

Yield: 78.3% (3.22 g, NMM), 62% (2.55 g, TEA), 55.5% (2.28 g, $C_5H_5N$); IR (KBr), $\nu$ ($cm^{-1}$): 3489 (m, -NH str, arom. ring), 3132~3125 (m, -NH str, amide), 3068 (w, -CH str, arom. ring), 2998~2993 (m, -CH str, cyclic $CH_2$), 2959, 2956, 2870 (m, -CH str, asym & sym, $CH_3$), 2924, 2853, 2849 (m, -CH str, asym & sym, $CH_2$), 1668, 1649~1638 (s, -C=O str, 3° & 2° amide), 1576, 1429 (m, skeletal bands, arom. ring), 1538~ 1524 (m, -NH bend, 2° amide), 1464 (m, -CH bend (scissoring), $CH_2$), 1385, 1366 (s, -CH bend, isopropyl group), 921 (w, $CH_3$ rocking, isopropyl group), 732, 676 (s, -CH bend, oop, arom. ring); $^1$H NMR (300 MHz, $CDCl_3$), $\delta$ ($10^{-6}$): 9.86, 9.23 (2H, br. s, -NH, Trp & Ile), 8.95 (1H, br. s, -NH, indole ring), 8.30, 7.99 (2H, br. s, -NH, Ala$^1$ & Ala$^2$), 7.40, 7.35 (2H, br. s, -NH, Leu$^1$ & Gly), 7.27~7.22 (2H, m, H's at $C_2$ & $C_4$, Trp), 7.19 (1H, br. s, -NH, Leu$^2$), 7.15~7.07 (3H, m, H's at $C_{5\sim7}$, Trp), 6.33~6.24 (2H, m, α-H's, Leu$^1$ & Leu$^2$), 6.00~5.92 (2H, m, α-H's, Ala$^1$ & Ala$^2$), 5.28~5.23 (3H, m, α-H's, Ile & Gly), 4.27~4.22 (1H, q, α-H, Trp), 3.92~3.89 (1H, t, α-H, Pro), 3.27~3.23 (2H, t, δ-H's, Pro), 2.88~2.86 (2H, d, *J*=5.65 Hz, β-H's, Trp), 2.69~2.62 (2H, q, β-H's, Pro), 1.89~1.48 (9H, m, β-H's, Leu$^1$, Leu$^2$, Ile & γ-H's, Ile, Pro), 1.45~1.43 (3H, d, *J*=5.3 Hz, β-H's, Ala$^1$), 1.40~1.38 (3H, d, *J*=5.25 Hz, β-H's, Ala$^2$), 1.04~1.02 (3H, d, *J*=5.9 Hz, γ′-H's, Ile), 1.00~0.96 (12H, m, δ-H's, Leu$^1$ & Leu$^2$), 0.94~0.91 (3H, t, δ-H's, Ile), 0.86~0.72 (2H, m, γ-H's, Leu$^1$ & Leu$^2$); $^{13}$C NMR (300 MHz, $CDCl_3$), $\delta$ ($10^{-6}$): 173.6, 172.9, 172.1 (C=O, Ala$^1$, Pro & Trp), 171.7, 171.3, 170.7 (C=O, Ile, Ala$^2$ & Leu$^2$), 170.1, 168.2 (C=O, Leu$^1$ & Gly), 135.2 ($C_2$′, Trp), 129.0 ($C_3$′, Trp), 124.3 ($C_5$, Trp), 121.0 ($C_2$, Trp), 119.5 ($C_4$, Trp), 114.8 ($C_6$, Trp), 111.5 ($C_7$, Trp), 109.7 ($C_3$, Trp), 60.2, 58.8 (2C, α-C's, Ile & Pro), 56.3, 53.7 (2C, α-C's, Leu$^2$ & Trp), 50.2, 49.4 (2C, α-C's, Leu$^1$ & Ala$^1$), 48.9 (α-C, Ala$^2$), 46.1 (δ-C, Pro), 44.5, 44.0 (2C, β-C's, Leu$^1$ & Leu$^2$), 41.6 (α-C, Gly), 37.9, 33.6 (2C, β-C's, Ile & Pro), 26.8 (2C, γ-C's, Leu$^1$ & Leu$^2$), 26.2 (γ-C, Ile), 25.5 (β-C, Trp), 24.8 (γ-C, Pro), 23.5 (2C, δ-C's, Leu$^2$), 21.3 (2C, δ-C's, Leu$^1$), 18.2, 17.3 (2C, β-C's, Ala$^1$ & Ala$^2$), 15.1 (γ′-C, Ile), 10.3 (δ-C, Ile); FAB MS (*m*/*z*, relative intensity): 823 [$(M+H)^+$, 100], 795 [$(823\text{-}CO)^+$, 17.6], 709 [$(Ala\text{-}Ala\text{-}Gly\text{-}Leu\text{-}Pro\text{-}Trp\text{-}Leu)^+$, 10.8], 681 [$(709\text{-}CO)^+$, 10.3], 652 [$(Pro\text{-}Trp\text{-}Leu\text{-}Ile\text{-}Ala\text{-}Ala)^+$, 22.3], 612 [$(Trp\text{-}Leu\text{-}Ile\text{-}Ala\text{-}Ala\text{-}Gly)^+$, 32.9], 596 [$(Ala\text{-}Ala\text{-}Gly\text{-}Leu\text{-}Pro\text{-}Trp)^+$, 51.2], 581 [$(Pro\text{-}Trp\text{-}Leu\text{-}Ile\text{-}Ala)^+$, 48.1], 553 [$(581\text{-}CO)^+$, 19.8], 484 [$(Trp\text{-}Leu\text{-}Ile\text{-}Ala)^+$, 32.9], 456 [$(484\text{-}CO)^+$, 9.3], 426 [$(Ile\text{-}Ala\text{-}Ala\text{-}Gly\text{-}Leu)^+$, 60.2], 413 [$(Trp\text{-}Leu\text{-}Ile)^+$, 12.6], 410 [$(Ala\text{-}Ala\text{-}Gly\text{-}Leu\text{-}Pro)^+$, 28.7], 398 [$(426\text{-}CO)^+$, 7.7], 397 [$(Pro\text{-}Trp\text{-}Leu)^+$, 24.4], 313 [$(Ala\text{-}Ala\text{-}Gly\text{-}Leu)^+$, 18.3], 285 [$(313\text{-}CO)^+$, 10.8], 284 [$(Pro\text{-}Trp)^+$, 17.9], 256 [$(Ile\text{-}Ala\text{-}Ala)^+$, 16.4], 187 [$(Trp)^+$, 11.3], 185 [$(Ile\text{-}Ala)^+$, 14.8], 143 [$(Ala\text{-}Ala)^+$, 9.1], 130 [$(C_9H_8N)^+$, 7.5], 116 [$(C_8H_6N)^+$, 6.8], 115 [$(143\text{-}CO)^+$, 4.6], 72 [$(Ala)^+$, 7.8], 57 [$(C_4H_9)^+$, 15.3], 43 [$(C_3H_7)^+$, 13.8], 42 [$(C_3H_6)^+$, 8.9], 30 [$(CH_4N)^+$, 1.6], 29 [$(C_2H_5)^+$, 2.4], 15 [$(CH_3)^+$, 5.6].

**Antimicrobial activity**

The synthesized cyclopolypeptide, sarcodactylamide (12.5~6 μg/ml), was screened for its antimicrobial activity (Bauer *et al*., 1966) against four bacterial strains: *Bacillus subtilis* (NCIM 2063), *Staphylococcus aureus* (NCIM 2079), *Pseudomonas aeruginosa* (NCIM 2034) and *Escherichia coli* (NCIM 2065), and four fungal strains: *Microsporum audouinii* (MUCC 545), *Trichophyton mentagrophytes* (MUCC 665), *Candida albicans* (MUCC 29) and *Aspergillus niger* (MUCC 177). MIC (minimum inhibitory concentration) values of test compounds were determined by tube dilution technique with dimethyl formamide (DMF) and dimethyl sulphoxide (DMSO). A spore suspension in sterile distilled water was prepared from 5 d 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 $-6\times10^4$ spores/ml). Filter paper disks of 6 mm diameter and 1 mm thickness were sterilized by autoclaving at 121 °C for 15 min. Each Petri dish was divided into five equal portions along the diameter, and one disk was placed in each portion. The five disks placed inside the Petri dish included three containing test samples, one with ciprofloxacin/griseofulvin (reference drug), and the remaining one with solvent (DMF/DMSO) as negative control. Reference drugs were also tested at the same concentration of 12.5~6 μg/ml. The Petri plates inoculated with bacterial/fungal cultures were incu-

bated at 37 °C for 18 and 48 h, respectively. Diameters (in mm) of the zones of inhibition were measured and the average diameters for test sample were calculated in triplicate sets. The diameters obtained for the test sample were compared with that produced by the standard drug.

**Anthelmintic activity**

Anthelmintic activity studies (Garg and Atal, 1963) were carried out against three different species of earthworms: *Megascoplex konkanensis* (ICARBC 211), *Pontoscotex corethruses* (ICARBC 408) and *Eudrilus* sp. (ICARBC 042) at 2 mg/ml concentration. Suspensions of sarcodactylamide were prepared by triturating synthesized compound (100 mg) with Tween 80 (0.5%, w/v) in distilled water and the resulting mixtures were stirred with a mechanical stirrer for 30 min. The suspensions were diluted to contain 0.2% (w/v) of the test samples. Suspensions of reference drugs, mebendazole and piperazine citrate were prepared at the same concentration in a similar way. Three sets of five earthworms of almost similar sizes (5 cm in length) were placed in Petri plates of 10 cm diameter containing 50 ml of suspensions of test sample and reference drug at room temperature (RT). Another set of five earthworms was kept as control in 50 ml suspension of Tween 80 (0.5%, w/v) in distilled water. 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 (60 °C) which stimulated the movement, if the worm was alive.

**Cytotoxicity activity**

Synthesized cyclopolypeptide, sarcodactylamide (compound **8**), was subjected to short term in vitro cytotoxicity study (Kuttan *et al*., 1985) at 62.5~3.91 μg/ml with 5-fluorouracil (5-FU) as reference compound. Activity was assessed by determining the inhibition percentage of Dalton's lymphoma ascites (DLA) and Ehrlich's ascites carcinoma (EAC) cells. Both cells were cultured in the peritoneal cavities of healthy albino mice by injecting the suspension of cells ($1\times10^6$ cells/ml) intraperitoneally. After 15~20 d, cells were withdrawn from the peritoneal cavities of the mice with help of sterile syringe, counted by using haemocytometer and adjusted to $1\times10^6$ cells/ml. Different dilutions of synthesized compound **8** ranging from 62.5 to 3.91 μg/ml were prepared in dulbecoccs minimum essential medium, and 0.1 ml of each diluted test compound was added to 0.1 ml of DLA cells ($1\times10^6$ cells/ml) and EAC cells ($1\times10^6$ cells/ml). Resulted suspensions were incubated at 37 °C for 3 h. After 3 h, tryphan blue dye exclusion test was performed and growth inhibition percentage was calculated. $CTC_{50}$ (cytotoxic concentration inhibiting 50% of growth percentage) values were determined by graphical extrapolation method. Controls were also tested at 62.5~3.91 μg/ml against both cell lines.

## RESULTS

**Chemistry**

In the present study, disconnection strategy was employed to carry out the first total synthesis of cyclic octapeptide (compound **8**, sarcodactylamide). The cyclopolypeptide molecule was split into four dipeptide units: Boc-Leu-Pro-OMe (compound **1**), Boc-Ile-Ala-OMe (compound **2**), Boc-Trp-Leu-OMe (compound **3**) and Boc-Ala-Gly-OMe (compound **4**). The required dipeptide units (compounds **1**~**4**) were prepared by coupling of Boc-amino acids viz. Boc-L-Leu, Boc-L-Ile, Boc-L-Trp and Boc-L-Ala with corresponding amino acid methyl ester hydrochlorides such as L-Pro-OMe·HCl, L-Ala-OMe·HCl, L-Leu-OMe·HCl and Gly-OMe·HCl employing DCC as coupling agent. Ester group of compound **1** was removed by alkaline hydrolysis with LiOH and Boc-group of compound **3** was removed by TFA. Both the deprotected units were coupled with DCC and TEA as base to get the first tetrapeptide unit Boc-Leu-Pro-Trp-Leu-OMe (compound **5**). Similarly, compound **2** after deprotection at carboxyl terminal was coupled with compound **4** after deprotection at amino end to get another tetrapeptide Boc-Ile-Ala-Ala-Gly-OMe (compound **6**). After removal of ester and Boc groups of compounds **5** and **6**, deprotected units were coupled to get linear octapeptide Boc-Leu-Pro-Trp-Leu-Ile-Ala-Ala-Gly-OMe (compound **7**). The ester group of linear fragment was removed with LiOH, and *p*-nitrophenyl (pNP) ester group was introduced. The Boc-group was removed with TFA, and deprotected linear fragment was now cyclized by keeping the whole contents at 0 °C for 7 d

in presence of catalytic amount of base to get cyclic compound **8** (Fig.1). Structures of the newly synthesized cyclic octapeptide, sarcodactylamide, as well as intermediates linear di/tetra/octapeptides were confirmed by IR, $^{1}$H NMR as well as elemental analysis. In addition, $^{13}$C NMR and mass spectra were recorded for the cyclopolypeptide.

## Pharmacology

Antimicrobial activity results for compound **8** against Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus*, Gram-negative bacteria *Pseudomonas aeruginosa* and *Escherichia coli*, cutaneous fungi *Microsporum audouinii* and *Trichophyton mentagrophytes*, and diamorphic fungi *Candida albicans* and *Aspergillus niger* by disk diffusion method, are tabulated in Table 2. Anthelmintic activity results against all three earthworm species are shown in Table 3 and in vitro cytotoxicity study results against DLA and EAC cell lines are tabulated in Table 4.

**Table 2 Antimicrobial activity data, diameter of inhibition zone (mm)**

| Compd. | Bacterial strains | | | | Fungal strains | | | |
|---|---|---|---|---|---|---|---|---|
| | *B. subtilis* | *S. aureus* | *P. aeruginosa* | *E. coli* | *C. albicans* | *M. audouinii* | *A. niger* | *T. mentagrophytes* |
| **8** | – | – | 28 (6) | 11 (12.5) | 22 (6) | 10 (12.5) | – | 11 (12.5) |
| Control | – | – | – | – | – | – | – | – |
| Ciprofloxacin | 20 (6) | 20 (12.5) | 25 (6) | 19 (12.5) | – | – | – | – |
| Griseofulvin | – | – | – | – | 20 (6) | 17 (6) | 18 (12.5) | 20 (6) |

Values in brackets are MIC values (μg/ml)

**Table 3 Anthelmintic activity data**

| Compd. | *M. konkanensis* | | *P. corethruses* | | *Eudrilus* sp. | |
|---|---|---|---|---|---|---|
| | Mean paralyzing time (min) | Mean death time (min) | Mean paralyzing time (min) | Mean death time (min) | Mean paralyzing time (min) | Mean death time (min) |
| **8**[a] | 14.29±0.35 | 17.47±0.22 | 20.42±0.41 | 24.08±0.58 | 16.44±0.48 | 21.28±0.12 |
| Control[b] | – | – | – | – | – | – |
| Mebendazole[a] | 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[a] | 12.39±0.36 | 13.52±0.49 | 19.06±0.57 | 22.23±0.78 | 12.46±0.15 | 13.58±0.47 |

All data are given as mean±*SD* (*n*=3); [a] Concentration at 2 mg/ml; [b] 0.5% (w/v) Tween 80 in distilled water

**Table 4 Cytotoxic activity data**

| Compd. | Conc. (μg/ml) | DLA cells | | | | EAC cells | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Live cells counted | No. of dead cells | Growth inhibition[a] (%) | $CTC_{50}$[b] (μmol/L) | Live cells counted | No. of dead cells | Growth inhibition (%) | $CTC_{50}$ (μmol/L) |
| **8** | 62.50 | 0 | 38 | 100.00 | 7.80 | 0 | 28 | 100.00 | 9.50 |
| | 31.25 | 2 | 36 | 94.74 | | 2 | 24 | 92.86 | |
| | 15.63 | 5 | 33 | 86.84 | | 9 | 19 | 67.85 | |
| | 7.81 | 14 | 24 | 63.16 | | 14 | 14 | 50.00 | |
| | 3.91 | 28 | 10 | 26.32 | | 26 | 2 | 7.14 | |
| Control | 62.50 | 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) | 62.50 | 0 | 38 | 100.00 | 37.36 | 0 | 28 | 100.00 | 90.55 |
| | 31.25 | 0 | 38 | 100.00 | | 0 | 28 | 100.00 | |
| | 15.63 | 10 | 28 | 73.68 | | 11 | 17 | 60.71 | |
| | 7.81 | 13 | 25 | 65.79 | | 19 | 9 | 32.14 | |
| | 3.91 | 22 | 16 | 42.11 | | 23 | 5 | 17.86 | |

[a] Growth inhibition (%)=100–live cell number×100/total cell number; [b] $CTC_{50}$: Cytotoxic concentration inhibiting 50% of growth percentage

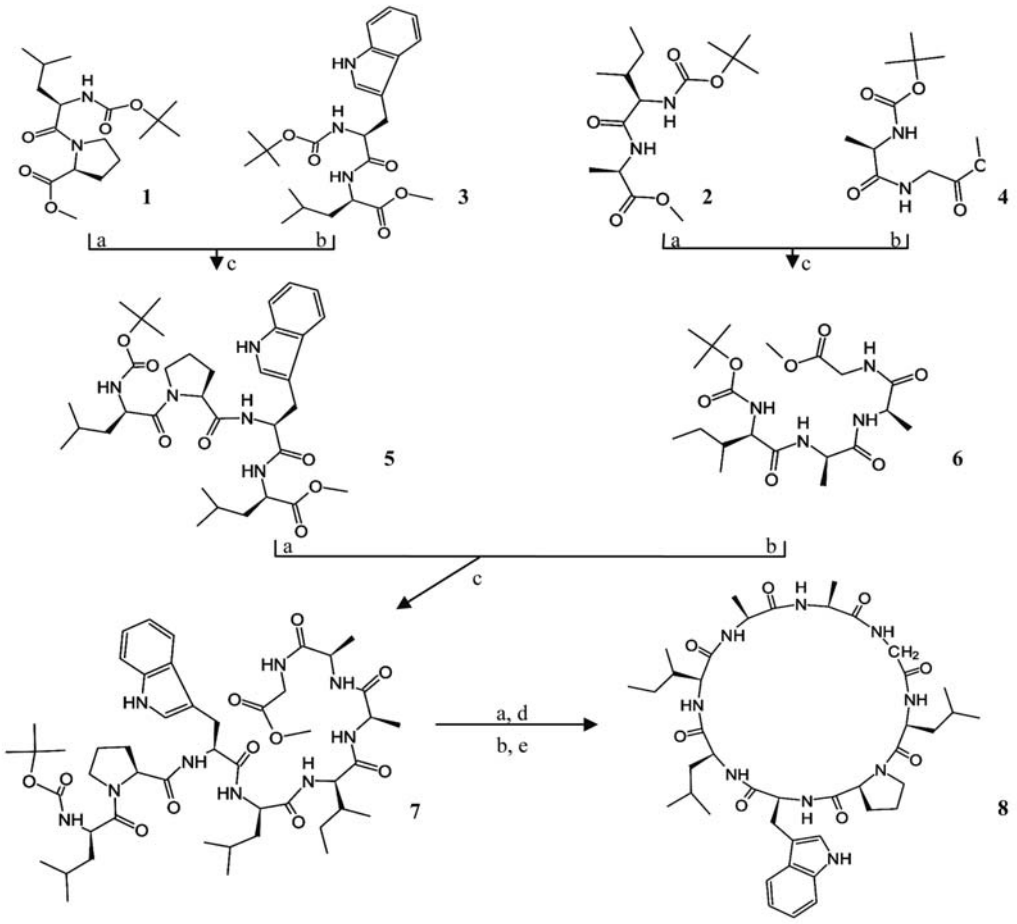


**Fig.1 Synthetic pathway for cyclic octapeptide, sarcodactylamide (compound 8)**

a: LiOH, THF:$H_2O$ (1:1, v/v), RT, 1 h; b: TFA, $CHCl_3$, RT, 1 h; c: DCC, TEA, $CHCl_3$, RT, 24 h; d: DCC, pNP, RT, 12 h; e: TEA/NMM/$C_5H_5N$, $CHCl_3$, 7 d, 0 °C

## DISCUSSION

Synthesis of cyclopolypeptide, sarcodactylamide, was carried out successfully with good yield, and NMM was proved to be an effective base for cyclization of linear octapeptide fragment. Structure of cyclic octapeptide was confirmed by spectral as well as elemental analysis. Cyclization of linear peptide fragment was indicated by disappearance of absorption bands at 1746 and 1390, 1369 $cm^{-1}$ (-C=O str of ester and -CH deformation of *tert*-butyl group) and presence of additional amide I bands of the -CO-NH- moiety at 1649~1647 $cm^{-1}$ in IR spectra of compound **8**. Formation of cyclopolypeptide was further confirmed by disappearance of singlet at $3.64\times10^{-6}$ and $1.55\times10^{-6}$ corresponding to three protons of methyl ester group and nine protons of *tert*-butyl group of Boc in $^1$H NMR spectra of compound **8**. Furthermore, $^1$H NMR and $^{13}$C NMR spectra of sarcodactylamide showed characteristic peaks confirming presence of all the 63 protons and 42 carbon atoms. Presence of $(M+1)^+$ ion peak at *m*/*z* 823 corresponding to the molecular formula $C_{42}H_{63}N_9O_8$ in mass spectra of compound **8**, along with other fragment ion peaks resulting from cleavage at 'Ile-Ala', 'Trp-Pro', 'Pro-Leu' and 'Leu-Ile' amide bond levels, which showed exact sequence of attachment of all the eight amino acid moieties in a chain. In addition, elemental analysis of compound **8** afforded values (±0.02) strictly in accordance to the molecular composition.

Synthesized cyclopolypeptide, sarcodactylamide (compound **8**), exhibited potent cytotoxic activity against DLA and EAC cell lines with $CTC_{50}$ values of 7.80 and 9.50 μmol/L respectively, in comparison to standard drug 5-FU ($CTC_{50}$ values −37.36 and 90.55

μmol/L), and moderate anthelmintic activity against *M. konkanensis*, *P. corethruses* and *Eudrilus* sp., in comparison to reference compounds mebendazole/piperazine citrate. Comparison of anthelmintic activity data suggested that *Eudrilus* sp. was less sensitive towards newly synthesized cyclopolypeptide, in comparison to *M. konkanensis* and *P. corethruses*. Moreover, compound **8** showed high level of activity against pathogenic microbes *C. albicans* and *P. aeruginosa*, in comparison to standard drugs griseofulvin/ciprofloxacin. On the other hand, synthesized cyclopolypeptide displayed only low level of antifungal activity against dermatophytes. Gram-positive bacteria were found to be resistant towards the compound **8** in comparison to sensitive Gram-negative bacteria. On passing toxicity tests, sarcodactylamide (compound **8**) may prove good candidate for clinical studies and can be a new cytotoxic and antimicrobial drug of future.

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