Full Paper

Total Synthesis and Biological Potential of Psammosilenin A

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The present work reports the synthesis of a plant-originated cyclooctapeptide – psammosilenin A **8** by cyclization of linear peptide fragment phe-pro-phe-phe-ala-pro-leu-pro-Opfp which was prepared by coupling of tetrapeptide units Boc-phe-pro-phe-phe-OH and ala-pro-leu-pro-OMe followed by proper deprotection of terminal groups and activation of the acid functionality. During synthesis, dicyclohexylcarbodiimide (DCC) and *N*,*N*-diisopropylcarbodiimide (DIPC) were used as the coupling agents and *N*-methylmorpholine (NMM)/triethylamine (TEA) were used as bases. Structure of the synthesized peptide was elucidated by spectral as well as elemental data. The newly synthesized peptide was subjected to biological screening and found to possess potent cytotoxic activity against DLA and EAC cell lines with IC_{50} value of 7.93 and 17.06 μM, respectively. Furthermore, good anthelmintic activity against earthworms *M. konkanensis* and *Eudrilus* species at 1 and 2 mg/mL was also observed for the synthesized cyclic peptide. Studies indicated that *N*-methylmorpholine was a more useful base for cyclization of linear peptide unit in comparison to pyridine.

Keywords: Cyclic octapeptide / Macrocyclization / Psammosilenin A / Pharmacological activities / *Psammosilene tunicoides*

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Introduction

The literature is enriched with several reports proving the significant role of natural products in the pharmaceutical research as biomedically useful agents or as lead compounds for drug development. Among these, cyclopolypeptides and related congeners having unique structures and a wide pharmacological profile emerged as vital organic congeners which may prove better candidates to overcome the problem of the widespread increase of resistance towards conventional agents [1–3]. Plant-derived natural cyclic peptides exhibit a wide range of pharmacological activities such as cytotoxic activity [4, 5], vasorelaxant activity [6, 7], estrogen-like activity [8], antimalarial activity [9] and tyrosinase inhibitory activity [10]. A natural cyclic octapeptide – psammosilenin A has been isolated from the roots of higher plant *Psammosilene tunicoides* which is used as anodyne and haemastatic agent. The structure of isolated cyclopeptide was elucidated using FABMS (Fast Atom Bombardment Mass Spectroscopy) and 2D NMR techniques including ^{1}H-^{1}H COSY (Correlated SpectroscopY), HMQC (Heteronuclear Multiple Quantum Coherence) and HMBC (Heteronuclear Multiple Bond Coherence) [11]. In continuation of our synthetic work on natural cyclopolypeptides [12–15], the present investigation was aimed at synthesis of natural cyclopeptide – psammosilenin A. Keeping in view of significant bioactivities possessed by cyclopeptides [16, 17], the above synthetic peptide was further subjected to cytotoxic and anthelmintic screening.

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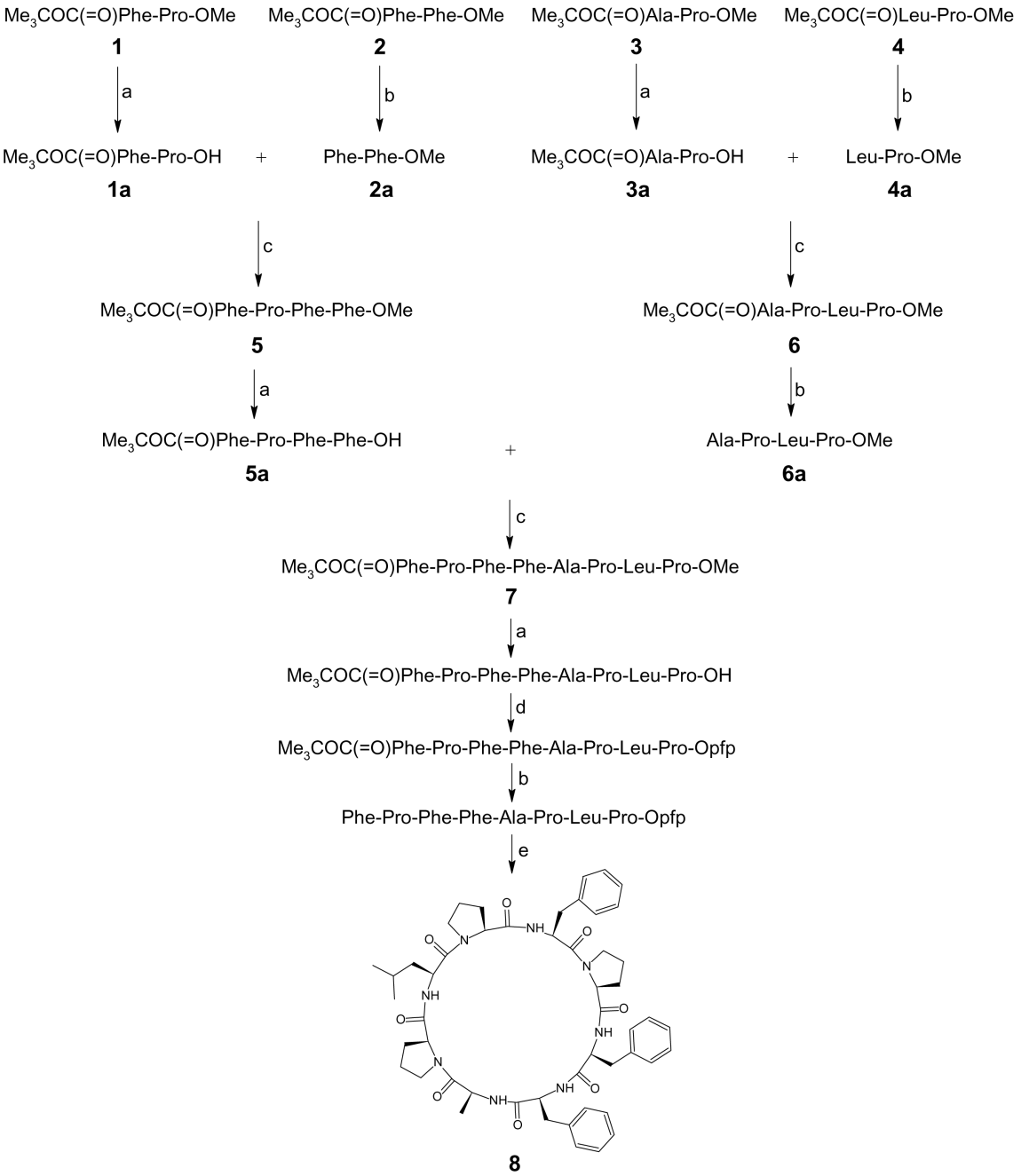
Abbreviations: dicyclohexylcarbodiimide (DCC); *N*,*N*-diisopropylcarbodiimide (DIPC); Dalton's lymphoma ascites (DLA); Ehrlich's ascites carcinoma (EAC); *N*-methylmorpholine (NMM); pentafluorophenyl (pfp); triethylamine (TEA)

Results and discussion

Chemistry

In the present work, disconnection strategy was employed to carry out the first total synthesis of psammo-



Reagents and conditions: a) LiOH, THF : H_2O (1 : 1), r.t., 1 h; b) CF_3COOH, $CHCl_3$, r.t. 1 h; c) DCC/DIPC, TEA/NMM, $CHCl_3$/THF/DMF, r.t., 24 – 36 h; d) DCC, pfp, r.t. 12 h; e) NMM/pyridine, 7 days, 0°C.

Scheme 1. Synthetic route to psammosilenin **8**.

silenin A. The cyclic octapeptide molecule was split into four dipeptide units Boc-phe-pro-OMe **1**, Boc-phe-phe-OMe **2**, Boc-ala-pro-OMe **3** and Boc-leu-pro-OMe **4**. The required dipeptide units **1** – **4** were prepared by coupling of Boc-amino acids viz. Boc-phe-OH, Boc-ala-OH, and Boc-leu-OH with corresponding amino acid methyl ester hydrochlorides such as pro-OMe.HCl and phe-OMe.HCl employing DCC/DIPC as coupling agent according to the Bodanszky-and-Bodanszky method [18] with certain modifications. The ester group of dipeptide **1** was removed by alkaline hydrolysis with LiOH, and the Boc-group of another dipeptide **2** was removed using CF_3COOH. Both deprotected units were coupled with each other using DCC and triethylamine (TEA) as base, to get the first tetra-

peptide unit Boc-phe-pro-phe-phe-OMe **5**. Similarly, dipeptide **3**, after deprotection at carboxyl terminal, was coupled with dipeptide **4**, after deprotection at amino end, to get another tetrapeptide Boc-ala-pro-leu-pro-OMe **6**. After removal of the ester and Boc-groups of the tetrapeptides **5** and **6**, those deprotected units were coupled to get the linear octapeptide Boc-phe-pro-phe-phe-ala-pro-leu-pro-OMe **7**. The ester group of the linear fragment was removed using LiOH, and pentafluorophenyl (pfp) ester group was introduced. The Boc-group was removed using CF_3COOH and the deprotected linear fragment was now cyclized by keeping the whole contents at 0°C to get cyclo (phe-pro-phe-phe-ala-pro-leu-pro) **8** (Scheme 1).

Synthesis of psammosilenin A **8** was carried out successfully with good yield (>85%). Cyclization of the linear octapeptide unit was indicated by the disappearance of absorption bands at 1747, 1270 cm^{-1} and 1389, 1366 cm^{-1} (corresponding to C-O stretching of the ester and C-H deformation of the *tert*-Butyl group), and the presence of additional Amide-I and Amide-II bands of the -CO-NH- moiety at 1638–1635 cm^{-1} and 1531–1528 cm^{-1} in the IR spectra of compound **8**. Formation of a cyclopeptide was further confirmed by the disappearance of singlets at 79.8 and 29.0 ppm corresponding to alpha and beta carbons of the *tert*-Butyl group of Boc and one singlet at 53.4 ppm corresponding to the carbon of the methyl ester n the ^{13}C-NMR spectrum. Also, a singlet at 1.54 ppm disappeared, corresponding to nine protons of the Boc-*tert*-Butyl group and a singlet at 3.62 ppm, corresponding to three protons of the methyl ester in ^{1}H-NMR spectrum of compound **8**. Furthermore, the ^{1}H-NMR and ^{13}C-NMR spectra of the synthesized cyclooctapeptide showed characteristic peaks confirming the presence all the 64 protons and 51 carbon atoms. Presence of the $[M+1]^+$ ion peak at m/z 917.5, corresponding to the molecular formula $C_{51}H_{64}N_8O_8$ in the mass spectrum of compound **8**, along with other fragment-ion peaks resulting from cleavage at 'leu-pro', 'phe-pro', and 'ala-pro' amide bond levels, showed the exact sequence of the attachment of all the eight amino acid moieties in a chain. The presence of the immonium-ion peaks at m/z 120.2 (phe), 86.2 (leu), 70.1 (pro), and 44.1 (ala) further confirmed the presence of these amino acid moieties in the cyclopeptide structure.

Mild acid hydrolysis (1.2 N HCl, 105°C, 1 h) of the cyclic peptide followed by C18-HPLC led to the recovery of the full-length cyclic peptide, which resulted from cleavage of the 'leu–pro-3' peptide bond and four smaller fragments ('pro-phe' and 'pro-phe-phe-ala-pro-leu') and ('pro-phe-pro-phe-phe-ala' and 'pro-leu') formed by a subsequent cut at 'phe-1–pro-1' and 'ala–pro-2' linkages, respectively, as evidenced by LC-MS analysis. In addition, elemental analysis of compound **8** afforded values (± 0.03) strictly in accordance to the molecular composition.

The absolute configuration of the amino acid residues was determined by acid hydrolysis of **8** followed by derivatization with Marfey's reagent [*N*-(3-fluoro-4,6-dinitrophenyl)-L-alaninamide, FDAA] and subsequent HPLC analysis. By comparing the chromatograms with those of derivatives of commercially available amino acids, it was found that cyclopeptide **8** contained L-ala, L-leu, L-phe, and L-pro in the ratio 1.1/1/3.1/3.2, showing the presence of one L-ala, one L-leu, three L-phe, and three L-pro in the structure.

DCC was found to be a yield-effective coupling agent giving a highly insoluble by-product dicyclohexylurea (DCU), in comparison to DIPC. Pentafluorophenyl ester was proved to be better for the activation of the acid functionality of the linear octapeptide unit and NMM was found to be a good base for intramolecular cyclization of the linear peptide fragment, in comparison to pyridine.

Pharmacology

Results of pharmacological activity studies are summarized in Tables 1–3. Comparison of cytotoxic activity data suggested that synthesized cyclooctapeptide **8** exhibited a high level of cytotoxic activity against Dalton's lymphoma ascites (DLA) and Ehrlich's ascites carcinoma (EAC) cell lines with IC_{50} values of 7.93 and 17.06 μM (7.26 and 15.63 μg/mL respectively) in comparison to the standard drug – 5-fluorouracil (IC_{50} values – 37.36 and 90.55 μM), whereas **8** showed no toxic effect against normal human hepatoma cells (HeP_2). A possible mechanism of cytotoxic action may be through apoptosis via induction of early cell death, nuclear fragmentation, and internucleosomal DNA scission. Anthelmintic activity data revealed that compound **8** showed higher activity against *M. konkanensis* and *Eudrilus* species at 1 and 2 mg/mL concentration, in comparison to standard drugs albendazole and mebendazole. However, anthelmintic activity possessed by compound **8** against *P. corethruses* was greater than that possessed by mebendazole but lower than that showed by albendazole. Antimicrobial activity data indicated that compound **8** exhibited higher antibacterial activity than reference drug – gatifloxacin, against pathogenic Gram-negative bacteria *P. aeruginosa* and *K. pneumoniae* at 6 μg/mL concentration. The mechanism of antibacterial action may involve cell lysis, breakdown of the cytoplasmic membrane barrier or interaction with a cytoplasmic target such as DNA. Furthermore, synthesized cyclopeptide showed moderate antifungal activity against dermatophytes, in comparison to the standard drug – griseofulvin. However, compound **8** displayed no

Table 1. *In-vitro cytotoxicity of compound* **8** *against DLA, EAC, and HeP_2 cell lines.*

Compound	Conc. (μg mL^{-1})	DLA cells			EAC cells			HeP_2 cells		
		No. of dead cells	% growth Inhibition[a)]	IC_{50}[b)] (μM)	No. of dead cells	% growth Inhibition	IC_{50} (μM)	No. of dead cells	% growth inhibition	IC_{50} (μM)
8	62.5	38	100.0		28	100.0		0	–	
	31.25	36	94.74		25	89.29		0	–	
	15.63	29	76.32	7.93	14	50.00	17.06	0	–	–
	7.81	20	52.63		8	28.57		0	–	
	3.91	13	34.21		4	31.58		0	–	
Control	62.5	0	–		0	–		0	–	
	31.25	0	–		0	–		0	–	
	15.63	0	–	–	0	–	–	0	–	–
	7.81	0	–		0	–		0	–	
	3.91	0	–		0	–		0	–	
Standard (5-FU)	62.5	38	100.0		28	100.0		0	–	
	31.25	38	100.0		28	100.0		0	–	
	15.63	28	73.68	37.36	17	60.71	90.55	0	–	–
	7.81	25	65.79		9	32.14		0	–	
	3.91	16	42.11		5	17.86		0	–	

a) % growth inhibition = 100 − [{($Cell_{total}$ − $Cell_{dead}$) × 100}/$Cell_{total}$].

b) IC_{50} = cytotoxic concentration inhibiting 50% of percentage growth.

Table 2. Anthelmintic activity of compound **8** against three earthworm species.

Compound	Concentration (mg)	Earthworm species					
		M. konkanensis		*P. corethruses*		*Eudrilus species*	
		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)
8	100	08.26 ± 0.40	14.28 ± 0.11	15.05 ± 0.41	26.09 ± 0.17	10.29 ± 0.48	21.05 ± 0.39
	200	05.08 ± 0.22	10.14 ± 0.34	10.34 ± 0.36	18.53 ± 0.25	06.44 ± 0.30	14.59 ± 0.17
Albendazole	100	11.25 ± 0.18	19.44 ± 0.40	13.20 ± 0.54	24.22 ± 0.11	12.32 ± 0.33	23.35 ± 0.42
	200	06.40 ± 0.37	13.12 ± 0.83	08.60 ± 0.50	18.04 ± 0.19	07.37 ± 0.28	16.11 ± 0.21
Mebendazole	100	13.85 ± 0.64	22.90 ± 0.53	17.85 ± 0.48	29.64 ± 0.73	13.54 ± 0.45	24.05 ± 0.62
	200	08.21 ± 0.48	15.11 ± 0.28	12.44 ± 0.21	21.05 ± 0.39	10.18 ± 0.77	18.52 ± 0.30
Control	–	–	–	–	–	–	–

a) Data are given as mean ± S.D. (n = 3).

Table 3. Antimicrobial activity of compound **8** against eight pathogenic bacteria and fungi.

Compound	Diameter of zone of inhibition (mm)							
	Bacterial strains				Fungal strains			
	B. subtilis	*S. epiderm.*	*P. aeruginosa*	*K. pneumoniae*	*C. albicans*	*M. audouinii*	*A. niger*	*T. mentagro.*
8	10 (25)[a)]	9 (12.5)	28 (6)	27 (6)	8 (12.5)	13 (6)	10(25)	17 (6)
Control	–	–	–	–	–	–	–	–
Gatifloxacin	20 (12.5)	28 (6)	24 (6)	25 (6)	–	–	–	–
Griseofulvin	–	–	–	–	20 (6)	17 (6)	18 (12.5)	22 (6)

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

significant activity against Gram positive bacteria and other pathogenic fungi tested. On passing toxicity tests, cyclic octapeptide **8** may prove a good candidate for clinical studies and could be a novel cytotoxic, anthelmintic, and antibacterial drug of the future.

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Experimental

Melting point was determined by open capillary method and is uncorrected. Dicyclohexylcarbodiimide (DCC), trifluoroacetic acid (TFA), triethylamine (TEA), *N*-methylmorpholine (NMM), pyridine (C_5H_5N) were purchased from SpectroChem Pvt. Ltd., Mumbai (India). Boc-amino acids, amino acid methyl ester hydrochlorides, *N*,*N*-diisopropylcarbodiimide (DIPC), and pentafluorophenol (pfp) were procured from RANKEM RFCL Ltd., New Delhi (India). IR spectra were recorded on Shimadzu 8700 FTIR spectrophotometer (Shimadzu, Japan) using a thin film supported on KBr or $CHCl_3$ as solvent. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on Bruker AC NMR spectrometer (300 MHz) (Brucker, USA) using $CDCl_3$/DMSO-d_6 as solvent and tetramethylsilane (TMS) as internal standard. FABMS was recorded on JMS-DX 303 Mass spectrometer (Jeol, Tokyo, Japan) operating at 70 eV using fast atom bombardment technique and electron spray ionization mass spectra were obtained on a Hewlett-Packard HP 1100 integrated LC-MS system (Hewlett Packard, USA). Elemental analyses of all compounds were performed on Vario EL III elemental analyzer (Elementar, Germany). Optical rotation was measured on automatic polarimeter (Optics Tech, Ghaziabad, India) in a 2 dm tube at 25°C using sodium-D light. Purity of all compounds was checked by TLC on precoated silica gel G plates (Kieselgel 0.25 mm, 60G F254, Merck, Germany) utilizing $CHCl_3$/MeOH (9 : 1/7 : 3) and $CHCl_3$/AcOH/H_2O (3 : 2 : 5) as developing solvents.

General procedure for the preparation of dipeptide fragments 1–4

1.66 g (0.01 mol) of L-proline methyl ester hydrochloride was dissolved in 20 mL of $CHCl_3$. To this solution, 2.8 mL (0.021 mol) of TEA was added at 0°C and the resulting mixture was stirred for 15 min. Then 2.65 g/1.89 g/2.31 g (0.01 mol) of Boc-L-phe-OH/Boc-L-ala-OH/Boc-L-leu-OH dissolved in 20 mL of $CHCl_3$ and 2.1 g/1.26 g (0.01 mol) of DCC/DIPC were added to above mixture with stirring. Stirring was continued for 24 h, after which the reaction mixture was filtered and the residue was washed with 25 mL of $CHCl_3$ and added to the filtrate. The filtrate was washed with 25 mL each of 5% $NaHCO_3$ and saturated NaCl solutions. The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated *in vacuo*. The crude product was recrystallized from a mixture of chloroform and petroleum ether (b.p. 40–60°C) followed by cooling at 0°C to get compounds **1**, **3**, and **4** as pale yellow semisolid masses (**1**: Yield 79%, $[\alpha]_D = -39.4^\circ$ (c = 0.25 M, MeOH), R_f = 0.71 ($CHCl_3$: MeOH/7 : 3), Anal. calcd. for $C_{20}H_{28}N_2O_5$: C, 63.81; H, 7.50; N, 7.44. Found: C, 63.79; H, 7.54; N, 7.45. **3**: Yield 83%, $[\alpha]_D = -113.6^\circ$ (c 0.25, MeOH), R_f = 0.68 ($CHCl_3$: MeOH/7 : 3). Anal. calcd. for $C_{14}H_{24}N_2O_5$: C, 55.99; H, 8.05; N, 9.33. Found: C, 56.02; H, 8.05; N, 9.35. **4**: Yield 87%, $[\alpha]_D$ = -2.8° (c = 0.25 M, MeOH), R_f = 0.77 ($CHCl_3$: MeOH/7 : 3), Anal. calcd. for $C_{17}H_{30}N_2O_5$: C, 59.63; H, 8.83; N, 8.18. Found: C, 59.61; H, 8.85; N, 8.22). Similarly, 2.16 g (0.01 mol) of L-phenylalanine methyl ester hydrochloride was coupled with 2.65 g (0.01 mol) of Boc-L-phe-OH using DCC/DIPC as coupling agents, to afford the desired compound **2** (Yield 76%; $[\alpha]_D = +63.5^\circ$ (c = 0.25 M, MeOH); R_f = 0.59 ($CHCl_3$: MeOH/7 : 3), Anal. calcd. for $C_{24}H_{30}N_2O_5$: C, 67.59; H, 7.09; N, 6.57. Found: C, 67.60; H, 7.12; N, 6.58).

Deprotection of dipeptide units at carboxyl terminal

To a solution of the compound **1** (3.76 g, 0.01 mol) in THF : H_2O (1 : 1, 36 mL), 0.36 g (0.015 mol) of LiOH was added at 0°C. The mixture was stirred at r.t. for 1 h and then acidified to pH = 3.5 with 1 N H_2SO_4. The aqueous layer was extracted with three equal proportions of 25 mL of Et_2O. The combined organic extracts were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was crystallized from methanol and ether to get Boc-phe-pro-OH **1a** as viscous liquid (Yield 86%, R_f = 0.57 ($CHCl_3$: MeOH/7 : 3), Anal. calcd. for $C_{19}H_{26}N_2O_5$: C, 62.97; H, 7.23; N, 7.73. Found: C, 62.98; H, 7.22; N, 7.75). Similarly, compound **3** (3.0 g, 0.01 mol) was hydrolyzed under alkaline conditions to obtain Boc-ala-pro-OH **3a** (Yield 89%, R_f = 0.79 ($CHCl_3$: MeOH/7 : 3), Anal. calcd. for $C_{13}H_{22}N_2O_5$: C, 54.53; H, 7.74; N, 9.78. Found: C, 54.56; H, 7.75; N, 9.76).

Deprotection of dipeptide units at amino terminal

Compound **2**/compound **4** (4.27 g/3.42 g, 0.01 mol) was dissolved in 15 mL of $CHCl_3$ and treated with 2.28 g (0.02 mol) of trifluoroacetic acid. The resulting solution was stirred at r.t. for 1 h, washed with 25 mL of saturated $NaHCO_3$ solution. The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by crystallization from $CHCl_3$ and petroleum ether (b.p. 40–60°C) to get pure phe-phe-OMe **2a**/leu-pro-OMe **4a** as semisolid masses (**2a**: Yield 74%, R_f = 0.73 ($CHCl_3$: MeOH/7 : 3), Anal. calcd. for $C_{19}H_{22}N_2O_3$: C, 69.92; H, 6.79; N, 8.58. Found: C, 69.93; H, 6.77; N, 8.60; **4a**: Yield 77%, R_f = 0.55 ($CHCl_3$: MeOH / 7 : 3), Anal. calcd. for $C_{12}H_{22}N_2O_3$: C, 59.48; H, 9.15; N, 11.56. Found: C, 59.45; H, 9.17; N, 11.56).

General procedure for the preparation of tetrapeptide fragments 5, 6

3.62 g (0.01 mol) of compound **1a** was dissolved in 25 mL of DMF and solution was neutralized with 2.21 mL (0.021 mol) of NMM at 0°C and the resulting mixture was stirred for 15 min. 3.26 g (0.01 mol) of compound **2a** were dissolved in 25 mL of DMF and resulting solution with 2.1 g/1.26 g (0.01 mol) of DCC/DIPC were added to the above mixture. Stirring was first done for 1 h at 0–5°C and then further for 24 h at r.t. After completion of the reaction, the reaction mixture was diluted with an 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 compound

5 as yellowish semisolid mass (Yield 71%, $[\alpha]_D$ = +22.7° (c = 0.5 M, MeOH), R_f = 0.81 ($CHCl_3$: MeOH/9 : 1), Anal. calcd. for $C_{38}H_{46}N_4O_7$: C, 68.04; H, 6.91; N, 8.35. Found: C, 68.05; H, 6.89; N, 8.38). Similarly, compounds **3a** and **4a** (2.86 g/2.42 g, 0.01 mol) were coupled using DCC/DIPC to yield compound **6** (Yield 82%, $[\alpha]_D$ = −77.3° (c = 0.5 M, MeOH), R_f = 0.67 ($CHCl_3$: MeOH/9 : 1), Anal. calcd. for $C_{25}H_{42}N_4O_7$: C, 58.81 H, 8.29; N, 10.97. Found: C, 58.85; H, 8.26; N, 10.99).

Deprotection of tetrapeptide units at the carboxyl and amino terminals

Hydrolysis of compound **5** (6.71 g, 0.01 mol) was carried out using 0.36 g (0.015 mol) of lithium hydroxide and the boc-group of compound **6** (5.11 g, 0.01 mol) was removed using 2.28 g (0.02 mol) of trifluoroacetic acid according to the procedures which were adopted for preparation of compound **1a** and **3a** from compound **1** and **3** respectively, to afford compound **5a** and **6a** as pale yellow semisolid masses (**5a**: Yield 79%, $[\alpha]_D$ = +52.6° (c = 0.5 M, MeOH), R_f = 0.60 ($CHCl_3$: MeOH/9 : 1), Anal. calcd. for $C_{37}H_{44}N_4O_7$: C, 67.67; H, 6.75; N, 8.53. Found: C, 67.65; H, 6.79; N, 8.52; **6a**: Yield 76%, $[\alpha]_D$ = -37.2° (c = 0.5 M, MeOH), R_f = 0.78 ($CHCl_3$: MeOH / 9 : 1), Anal. calcd. for $C_{20}H_{34}N_4O_5$: C, 58.52 H, 8.35; N, 13.65. Found: C, 58.53; H, 8.35; N, 13.69).

Procedure for the preparation of linear octapeptide unit 7

4.11 g (0.01 mol) of compound **6a** was dissolved in 35 mL of THF. To this solution, 2.8 mL (0.021 mol) of TEA was added at 0°C and the resulting mixture was stirred for 15 min. 6.57 g (0.01 mol) of compound **5a** was dissolved in 35 mL of THF and 2.1 g/1.26 g (0.01 mol) of DCC/DIPC were added to above mixture with stirring. Stirring was continued for 36 h, after which the reaction mixture was filtered and the filtrate was washed with 30 mL each of 5% $NaHCO_3$ and saturated NaCl solutions. The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated *in vacuo*. The crude product was recrystallized from a mixture of chloroform and petroleum ether (b.p. 40 – 60°C) followed by cooling at 0°C to get compound **7** as yellowish semisolid mass.

tert*-Butyloxycarbonyl-L-phenylalanyl-L-prolyl-L-phenylalanyl-L-phenylalanyl-L-alanyl-L-prolyl-L-leucyl-L-proline methyl ester *7

Yield 83%, $[\alpha]_D$ = −59.1° (c = 0.3 M, MeOH), R_f = 0.83 ($CHCl_3$: MeOH/9 : 1). FT-IR ($CHCl_3$) ν_{max} (cm^{-1}): 3125, 3122, 3117 (m, N-H str, amide), 2999, 2995, 2992 (m, C-H str, CH_2, pro), 2966, 2963, 2929-2924 (m, C-H str, asym, CH_3 and CH_2), 2875, 2844 – 2839 (m, C-H str, sym, CH_3 and CH_2), 1747 (s, C=O str, ester), 1666 – 1663, 1647, 1642, 1641, 1639 (s, C=O str, amide), 1589, 1582, 1478 – 1470 (m, skeletal bands, rings), 1538-1533, 1527 – 1523 (m, N-H def, amide), 1389, 1366 (s, C-H def, butyl-*t*), 1382, 1359 (s, C-H def, propyl-*i*), 1270 (s, C-O str, ester), 720 – 715, 698 – 694 (s, C-H bend, out-of-plane, rings). ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 8.85 (brs, 1H, NH, phe-1), 8.20 (brs, 1H, NH, phe-2), 7.53 (tt, *J* = 7.15, 4.35 Hz, 2H, H-*m*, phe-1), 7.29 (brs, 1H, NH, leu), 7.22-7.14 (m, 4H, H-*m*, phe-2 and phe-3), 7.08 – 7.01 (m, 2H, H-*p*, phe-2 and phe-3), 6.92 (t, *J* = 6.15 Hz, 1H, H-*p*, phe-1), 6.87 (dd, *J* = 8.75, 2H, 4.2 Hz, H-*o*, phe-1), 6.83 (dd, *J* = 8.8, 4.15 Hz, 2H, H-*o*, phe-2), 6.80 (dd, *J* = 8.75, 4.15 Hz, 2H, H-*o*, phe-3), 6.54 (brs, 1H, NH, phe-3), 6.44 (brs, 1H, NH, ala), 4.82 – 4.79 (td, *J* = 7.45, 3.8 Hz, 1H, H-α, phe-2), 4.68 – 4.63 (td, *J* = 6.2, 3.6 Hz, 1H, H-α, leu), 4.60-4.55 (td, *J* = 7.5, 3.75 Hz, 1H, H-α, phe-1), 4.46 (t, *J* = 6.85 Hz, 1H, H-α, pro-1), 4.25-4.18 (m, 2H, H-α, phe-3 and H-α, ala), 4.11 (t, *J* = 6.9 Hz, 1H, H-α, pro-2), 3.92 (t, *J* = 6.85 Hz, 1H, H-α, pro-3), 3.75 (t, *J* = 7.2 Hz, 2H, H-δ, pro-1), 3.62 (3H, s, OCH_3), 3.39 (t, *J* = 7.15 Hz, 2H, H-δ, pro-2), 3.32 (t, *J* = 7.15 Hz, 2H, H-δ, pro-3), 2.89 – 2.78 (m, 6H, H-β, phe-1, phe-2 and phe-3), 2.69 – 2.65 (m, 4H, H-β, pro-1 and pro-2), 2.04 – 1.99 (m, 4H, H-β and H-γ, pro-3), 1.95 – 1.89 (m, 4H, H-γ, pro-1 and pro-2), 1.78 (t, *J* = 4.6 Hz, 2H, H-β, leu), 1.54 (9H, s, butyl-*t*), 1.47 (d, *J* = 5.85 Hz, 3H, H-β, ala), 1.46 – 1.42 (m, 1H, H-β, leu), 0.98 (d, *J* = 6.2 Hz, 6H, H-δ, leu). ^{13}C-NMR (300 MHz, DMSO-d_6) δ (ppm): 177.2 (C=O, phe-3), 174.5 (C=O, pro-2), 173.4 (C=O, pro-1), 170.9 (C=O, phe-1), 169.6 (C=O, phe-2), 169.2 (C=O, ala), 168.6 (C=O, pro-3), 168.0 (C=O, leu), 153.2 (C=O, boc), 136.6 (C-γ, phe-2), 134.2 (C-γ, phe-3), 132.7 (C-γ, phe-1), 130.7 (2C, C-*m*, phe-1), 130.1 (2C, C-*o*, phe-3), 129.5 (2C, C-*o*, phe-2), 128.6 (2C, C-*m*, phe-2), 128.0 (2C, C-*m*, phe-3), 127.7 (2C, C-*o*, phe-1), 127.2 (C-*p*, phe-1), 126.9 (C-*p*, phe-2), 125.6 (C-*p*, phe-3), 79.8 (C-α, boc), 58.3 (C-α, pro-3), 56.1 (C-α, pro-2), 54.4 (C-α, pro-1), 53.4 (OCH_3), 51.8 (C-α, phe-1), 49.8 (C-α, ala), 49.2 (C-α, phe-3), 48.8 (C-δ, pro-1), 48.2 (C-α, phe-2), 47.5 (C-δ, pro-2), 46.9 (C-α, leu), 45.4 (C-δ, pro-3), 39.7 (C-β, leu), 38.3 (C-β, phe-1), 38.0 (C-β, phe-2), 37.6 (C-β, phe-3), 29.9 (C-β, pro-3), 29.0 (3C, C-β, boc), 26.8 (C-β, pro-2), 25.7 (C-β, pro-3), 25.1 (C-γ, pro-3), 24.8 (C-γ, pro-2), 24.2 (C-γ, pro-1), 22.9 (C-γ, leu), 22.2 (2C, C-δ, leu), 17.9 (C-β, ala). Anal. calcd. for $C_{57}H_{76}N_8O_{11}$: C, 65.25; H, 7.30; N, 10.68. Found: C, 65.26; H, 7.28; N, 10.65.

Synthesis of the cyclic octapeptide – psammosilenin A 8

To synthesize compound **8**, the linear octapeptide unit **7** (5.25 g, 0.005 mol) was deprotected at the carboxyl end using LiOH (0.18 g, 0.0075 mol) to get Boc-L-phe-L-pro-L-phe-L-phe-L-ala-L-pro-L-leu-L-pro-OH. The deprotected octapeptide unit (5.18 g, 0.005 mol) was now dissolved in 50 mL of $CHCl_3$ at 0°C. To this solution, 0.0067 mol of pentafluorophenol (1.23 g) and DCC (1.06 g, 0.005 mol) were added and stirring was done at r.t. for 12 h. The reaction mixture was filtered and the filtrate was washed with 10% $NaHCO_3$ solution (3 × 25 mL) and finally washed with 5% HCl (2 × 20 mL) to get the corresponding pentafluorophenyl ester Boc-L-phe-L-pro-L-phe-L-phe-L-ala-L-pro-L-leu-L-pro-Opfp. To this compound (4.8 g, 0.004 mol) dissolved in 35 mL of $CHCl_3$, 0.91 g (0.008 mol) of CF_3COOH was added, stirred at r.t. for 1 h and washed with two proportions each 25 mL of 10% $NaHCO_3$ solution. The organic layer was dried over anhydrous Na_2SO_4 to get L-phe-L-pro-L-phe-L-phe-L-ala-L-pro-L-leu-L-pro-Opfp which was dissolved in 25 mL of $CHCl_3$ and NMM/C_5H_5N (2.21 mL/1.61 mL, 0.021 mol) was added. Then, whole contents were kept at 0°C for seven days. The reaction mixture was washed with 10% $NaHCO_3$ (3 × 25 mL) and 5% HCl (2 × 20 mL) solutions. The organic layer was dried over anhydrous Na_2SO_4 and the crude cyclized product was crystallized from $CHCl_3$/n-hexane to get compound **8** as white crystals (m.p. 193 – 195°C).

*Cyclo (L-phe-L-pro-L-phe-L-phe-L-ala-L-pro-L-leu-L-pro) **8***

Yield 3.94 g (86%, NMM), 3.25 g (71%, C_5H_5N), $[\alpha]_D$ = −108.2° (c = 0.4 M, MeOH), R_f = 0.63 ($CHCl_3$: AcOH : H_2O/3 : 2 : 5). FT-IR (KBr) ν_{max} (cm^{-1}): 3128, 3123, 3115 (m, N-H str, amide), 2998, 2996, 2992 (m, C-H str, CH_2 (cyclic)), 2967, 2964 (m, C-H str, asym, CH_3), 2928, 2925 (m, C-H str, asym, CH_2), 2877 (m, C-H str, sym, CH_3), 2845, 2836 (m, C-H str, sym, CH_2), 1668, 1662, 1645, 1638 – 1635 (s, C=O str, amide), 1588, 1585, 1475, 1469 (m, skeletal bands, rings), 1538, 1534, 1531-1528, 1525 (m, N-H def, amide), 1383,

1358 (s, C-H def, propyl-*i*), 722–717, 698–693 (s, C-H bend, out-of-plane, rings). ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 9.88 (brs, 1H, NH, phe-2), 9.75 (brs, 1H, NH, phe-1), 9.22 (brs, 1H, NH, leu), 7.75 (brs, 1H, NH, phe-3), 7.56 (brs, 1H, NH, ala), 7.23 (tt, *J* = 6.75, 4.45 Hz, 2H, H-*m*, phe-2), 7.19 (tt, *J* = 6.8, 4.5 Hz, 2H, H-*m*, phe-1), 7.16 (tt, *J* = 6.75, 4.5 Hz, 2H, H-*m*, phe-3), 7.04 (t, *J* = 6.15 Hz, 1H, H-*p*, phe-3), 7.01 (t, *J* = 6.2 Hz, 1H, H-*p*, phe-3), 6.98 (t, *J* = 6.1 Hz, 1H, H-*p*, phe-3), 6.86 (dd, *J* = 8.8, 4.15 Hz, 2H, H-*o*, phe-1), 6.84 (dd, *J* = 8.75, 4.15 Hz, 2H, H-*o*, phe-3), 6.81 (dd, *J* = 8.8, 4.2 Hz, 2H, H-*o*, phe-2), 6.04–5.98 (m, 1H, H-α, ala), 5.69–5.65 (td, *J* = 7.4, 3.75 Hz, 1H, H-α, phe-3), 5.17–5.12 (td, *J* = 6.15, 3.6 Hz, 1H, H-α, leu), 4.44–4.39 (td, *J* = 7.45, 3.8 Hz, 1H, H-α, phe-1), 4.23-4.19 (td, *J* = 7.5, 3.75 Hz, 1H, H-α, phe-2), 3.94 (t, *J* = 6.85 Hz, 1H, H-α, pro-2), 3.94 (t, *J* = 6.9 Hz, 1H, H-α, pro-1), 3.94 (t, *J* = 6.8 Hz, 1H, H-α, pro-3), 3.24 (t, *J* = 7.2 Hz, 2H, H-δ, pro-1), 3.21 (t, *J* = 7.15 Hz, 2H, H-δ, pro-3), 3.18 (t, *J* = 7.1 Hz, 2H, H-δ, pro-2), 2.72 (q, *J* = 5.9 Hz, 2H, H-β, pro-2), 2.69 (q, *J* = 5.85 Hz, 2H, H-β, pro-1), 2.65 (q, *J* = 5.9 Hz, 2H, H-β, pro-3), 2.63 (d, *J* = 5.6 Hz, 2H, H-β, phe-2), 2.59 (d, *J* = 5.55 Hz, 2H, H-β, phe-1), 2.56 (d, *J* = 5.6 Hz, 2H, H-β, phe-3), 1.89–1.85 (q, 2H, H-γ, pro-2), 1.84–1.78 (m, 4H, H-γ, pro-1 and pro-3), 1.72 (t, *J* = 4.55 Hz, 2H, H-β, leu), 1.43 (d, *J* = 5.9 Hz, 3H, H-β, ala), 0.99 (d, *J* = 6.15 Hz, 6H, H-δ, leu), 0.89–0.75 (m, 1H, H-γ, leu). ^{13}C-NMR (300 MHz, DMSO-d_6) δ (ppm): 173.0 (C=O, phe-3), 172.8 (C=O, leu), 171.6 (C=O, pro-3), 171.1 (C=O, pro-3), 170.7 (C=O, pro-1), 170.2 (C=O, phe-2), 169.6 (C=O, phe-1), 168.3 (C=O, ala), 140.1 (C-γ, phe-2), 139.8 (C-γ, phe-1), 137.5 (C-γ, phe-3), 131.5 (2C, C-*o*, phe-1), 130.3 (2C, C-*o*, phe-3), 129.1 (2C, C-*o*, phe-2), 128.9 (2C, C-*m*, phe-1), 128.4 (2C, C-*m*, phe-2), 127.8 (2C, C-*m*, phe-3), 127.3 (C-*p*, phe-1), 127.0 (C-*p*, phe-2), 126.7 (C-*p*, phe-3), 59.4 (C-α, pro-2), 58.9 (C-α, pro-3), 58.4 (C-α, pro-1), 57.2 (C-α, phe-2), 50.7 (C-α, phe-1), 49.6 (C-α, ala), 49.1 (C-α, phe-3), 48.3 (C-α, leu), 48.0 (C-δ, pro-1), 47.8 (C-δ, pro-2), 47.5 (C-δ, pro-3), 44.3 (C-β, leu), 39.1 (C-β, phe-2), 38.2 (C-β, phe-1), 37.5 (C-β, phe-3), 32.3 (C-β, pro-1), 31.9 (C-β, pro-2), 31.5 (C-β, pro-3), 28.2 (C-γ, leu), 22.7 (2C, C-δ, leu), 21.5 (C-γ, pro-1), 21.0 (C-γ, pro-2), 20.6 (C-γ, pro-3), 15.7 (C-β, ala). FAB MS *m/z* [rel. int.]: 917.5 $[M + 1]^+$, (100), 889.5 (21), 846.4 (42), 818.4 (17), 804.4 (33), 770.4 (67), 707.4 (56), 699.3 (23), 679.4 (19), 673.3 (41), 636.3 (32), 560.3 (27), 552.3 (69), 489.3 (27), 461.3 (17), 455.3 (48), 427.3 (22), 392.2 (31), 364.3 (11), 342.3 (23), 308.2 (19), 280.2 (12), 245.2 (28), 217.2 (10), 211.2 (15), 183.2 (21), 120.2 (29), 98.2 (17), 91.2 (28), 86.2 (14), 70.1 (21), 65.1 (21), 57.1 (19), 44.1 (9), 43.1 (6), 42.1 (16), 15.1 (7). Anal. calcd for $C_{51}H_{64}N_8O_8$: C, 66.79; H, 7.03; N, 12.22. Found: C, 66.80; H, 7.02; N, 12.25.

Preparation and analysis of Marfey derivatives

0.5 mg of synthesized cyclooctapeptide **8** was hydrolyzed by heating in 1 mL of 6 M HCl at 110°C for 24 h. After cooling, the solution was evaporated to dryness and redissolved in 50 μL of water. 100 μL of a 1% w/v solution of FDAA (Marfey's reagent) in acetone was added to the acidic hydrolyzate solution (or to 50 μL of a 50 mM solution of the respective amino acid). After addition of 20 μL of 1 M $NaHCO_3$ solution, the mixture was incubated for 1 h at 40°C. The reaction was stopped by the addition of 10 μL of 2 M HCl. Finally, the solvents were evaporated to dryness and the residue was dissolved in 1 mL of MeOH. An aliquot of this solution (20 μL for cyclopeptide **8** and 10 μL for standards) was analyzed by HPLC (Phenomenex Luna C18, 4.6 × 250 mm, 5 μm, solvents: (A) water + 0.05% TFA, (B) MeCN, linear gradient: 0 min 35% B, 30 min 45% B, 1 mL min^{-1}, 25°C). Retention times (min) of the FDAA amino acid derivatives used as standards were as follows: L-ala (6.42), D-ala (7.67), L-leu (14.94), D-leu (20.58), L-phe (17.41), D-phe (22.32), L-pro (18.91), D-pro (20.22). Retention times (min) and relative peak area (%) of the observed peaks of the FDAA derivatized hydrolysis product of cyclopeptide **8** were as follows: L-ala (6.45, 2.62%), L-leu (14.96, 2.38%), L-phe (17.44, 7.39%), L-pro (18.93, 7.52%).

Partial hydrolysis of cyclopeptide 8 and LC-MS analysis

Compound **8** (0.1 mg) was dissolved in 200 μL of 1.2 N HCl and heated at 105°C for 1 h. The solution was then diluted with 600 μL of H_2O and stored frozed at −20°C prior to analysis. 50 μL of hydrolyzate was chromatographed in duplicate by linear ingredient C18-HPLC (Dynamax, 4.6 × 250 mm, 8 μm) employing 0–50% MeCN in H_2O (0.1% TFA) over 30 min (1.5 mL min^{-1}). Individual peaks were collected and chromatographed by LC-MS to identify the linear peptide fragments: $[M + H]^+$ *m/z* 691.8, appropriate for 'pro-phe-phe-ala-pro-leu', t_R = 12.8 min; $[M + H]^+$ m/z 725.8, for 'pro-phe-pro-phe-phe-ala', t_R = 14.1 min; $[M + H]^+$ m/z 263.3, for 'pro-phe', t_R = 15.6 min; $[M + H]^+$ m/z 229.3, for 'pro-leu', t_R = 17.2 min; $[M + H]^+$ m/z 936.2, for 'pro-phe-pro-phe-phe-ala-pro-leu', t_R = 18.4 min.

Biology

Cytotoxic activity assay

The synthesized cyclopeptide **8** was subjected to a short-term *in-vitro* cytotoxicity study [19] 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, EAC, and HeP_2 cells. Human hepatoma cells (HeP_2) were isolated from rat liver by perfusion method. Besides, DLA and EAC 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 sterile syringe and counted using haemocytometer and adjusted to $\times 10^6$ cells/mL. The stock solution of the tested compound **8** was prepared in DMSO and was used for serial dilutions ranging from 62.5–3.91 μg/mL in Dulbeccos minimum essential medium and 0.1 mL of 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). Resulted suspensions were incubated at 37°C for 3 h. After 3 h, tryphan blue dye exclusion test was performed and percentage growth inhibition was calculated. IC_{50} values were determined by graphical extrapolation method. Controls were also tested at 62.5–3.91 μg/mL against all three cell lines. The results of cytotoxic activity studies are summarized in Table 1.

Anthelmintic activity assay

Three earthworm species namely *Megascoplex konkanensis*, *Pontoscotex corethruses*, and *Eudrilus* sp. were selected for anthelmintic testing of compound **8** at 1 and 2 mg/mL concentration [20]. Suspensions of samples were prepared by triturating synthesized compound (100/200 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 0.1% and 0.2% w/v of the test sample. Suspension of reference drugs, albendazole, and mebendazole were prepared with the same concentration in a similar way. Three sets of five earthworms of almost similar sizes (2 inch in length) were placed in petri plates (4 inch in diameter) containing 50 mL of suspension of the test sample and reference drug at room temperature. 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 anthelmintic studies are given in Table 2.

Antibacterial and antifungal activity assay

The synthesized cyclopeptide **8** was screened against Gram-negative bacteria *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus epidermidis*, dermatophytes *Microsporum audouinii*, *Trichophyton mentagrophytes*, and other pathogenic fungi *Candida albicans* and *Aspergillus niger* using modified Kirby-Bauer disc diffusion method [21]. MIC values of test compound was determined by tube dilution technique. Cyclopeptide **8** was dissolved in sterile DMF to prepare a stock solution of 1 mg/mL which was suitably diluted with sterile nutrient broth media/Sabouraud's broth media to contain seven different concentrations of test compound ranging from 200-6 μg mL^{-1} in different test tubes. All the tubes were inoculated with one loopful of one of the test bacterium/fungus. The process was repeated with different test bacteria/fungi. Tubes inoculated with bacterial/fungal cultures were incubated at 37°C for 18 h/48 h and the presence/absence of growth of the bacteria/fungi was observed. From these results, MIC values of test compound were determined against each test bacterium/fungi. A spore suspension in sterile distilled water was prepared from five 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 $\times$ mL^{-1}). Filter paper disks of 6 mm diameter and 1 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 gatifloxacin/griseofulvin and a disk impregnated with the solvent (DMF) as negative control. Test sample and reference drugs were tested at the concentration of 25 – 6 μg mL^{-1}. The petri plates inoculated with bacterial/fungal cultures were incubated at 37°C for 18 h/48 h. 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 drugs. The results of antibacterial and antifungal studies are presented in Table 3.

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