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Original research paper

First total synthesis and biological screening of hymenamide E

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A new potent bioactive, proline-rich cyclic heptapeptide hymenamide E (**13**) was synthesized using the solution phase technique by cyclization of the linear peptide Boc--Phe-Pro-Thr-Thr-Pro-Tyr-Phe-OMe (**12**) after proper deprotection at carboxyl and amino terminals. Linear peptide segment was prepared by coupling the tripeptide unit Boc-Phe-Pro-Thr-OH (**10a**) with the tetrapeptide unit Thr-Pro-Tyr-Phe-OMe (**11a**) using dicyclohexylcarbodiimide as the coupling agent and *N*-methylmorpholine as the base. Structures of all new compounds were characterized by IR, ^{1}H NMR spectral data as well as elemental analyses. In addition, the structure of compound **13** was verified by ^{13}C NMR, fast atom bombardment mass spectroscopy and differential scanning calorimetry. The newly synthesized cyclopeptide was screened for its antibacterial, antifungal and anthelmintic activities against eight pathogenic microbes and two earthworm species. Compound **13** showed potent antifungal activity against *Candida albicans* and *Ganoderma* species comparable to that of griseofulvin as a reference drug and potent anthelmintic activity against earthworms *Megascoplex konkanensis* and *Eudrilus* species in comparison to piperazine citrate.

Keywords: hymenamide E, cyclic heptapeptide, antibacterial activity, antifungal activity, anthelmintic activity

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The current literature indicates that natural peptides from marine environment possess diverse pharmacological activities, including antimicrobial (1–3), cytotoxic (4, 5), anti-HIV (6), anti-inflammatory (7, 8), nematocidal (9) and antimalarial (10). Among various marine sponges, sponges belonging to genus *Hymeniacidon* have received special attention in providing cyclic congeners with a wide array of bioactivities (11–14). In the past decades, hymenamides A-K, natural polypeptides isolated from the Okinawan marine sponge *Hymeniacidon* species bearing seven to eight amino acid residues, have emerged as novel organic cyclic structures exhibiting antifungal (15), cytotoxic and inhibitory activity against protein tyrosine kinase (16). Among these, hymenamide E is unique in

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having two adjacent threonine units in its structure. It was isolated from extracts of marine sponge of genus *Hymeniacidon* by Tsuda *et al.* (17). As per IUPAC rules, hymenamide E can be named 6,9-dibenzyl-12-(4-hydroxybenzyl)-20,23-di(1-hydroxyethyl)perhydrodipyrrolo[1,2-a:1,2-j][1,4,7,10,13,16,19]heptaaza cyclohenicosine-2,5,8,11,14,17,20-heptaone. Hymenamide E exhibited potent antifungal activity against pathogenic *Cryptococcus neoformans* (*MIC* 133 μg mL^{-1}). The minute quantities of this bioactive cyclopeptide obtained from natural sources (0.0006% yield from methanolic extracts of sponge) restricted scientists to investigate its biological profile in detail. Further, the wide-spread increase of fungal and helminth resistance towards conventional antifungal and anthelmintic agents encourage the development of novel moieties with unexploited mechanisms of action. Hence, keeping in mind the biological potential of extracts of marine sponge *Hymeniacidon* sp. and to obtain a bioactive peptide in good yield, the present investigation aims the first total synthesis of a natural cyclic heptapeptide hymenamide E using the solution phase technique in a simple and economical manner in the laboratory. The study also includes testing of the synthesized compound for its expected antifungal along with anthelmintic and antibacterial effects.

EXPERIMENTAL

Materials and equipment

All reactions requiring anhydrous conditions were conducted in a flame dried apparatus. Melting points were determined by the open capillary method and were uncorrected. Confirmation of melting points was done by differential scanning calorimetry thermograms recorded on a DSC Q10 Calorimeter (TA Instruments, USA). L-Amino acids, di-*tert*-butylpyrocarbonate (Boc_2O), dicyclohexylcarbodiimide (DCC), trifluoroacetic acid (TFA), *p*-nitrophenol (pnp) and *N*-methylmorpholine (NMM) were obtained from Spectrochem Limited (India). IR spectra were recorded on a Shimadzu 8700 FTIR spectrophotometer (Shimadzu, Japan) using a thin film supported on KBr pellets for the synthesized cyclic heptapeptide hymenamide E and $CHCl_3$ as solvent for intermediate semisolids. ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker AC NMR spectrometer (300 MHz), (Brucker, USA) using $CDCl_3$ as solvent and tetramethylsilane (TMS) as internal standard. Mass spectra were recorded on a JMS-DX 303 mass spectrometer (Jeol, Japan) operating at 70 eV using the fast atom bombardment technique (FAB MS). Elemental analyses of all compounds were performed on a Vario EL III elemental analyzer (Elementar, Germany). Purity of all compounds was checked by TLC on precoated silica gel G plates (Kieselgel 0.25 mm, 60G F254, Merck, Germany). Chloroform/methanol (9:1, *V/V*) was used as the developing solvent system and dark brown spots were detected on exposure to iodine vapours in a tightly closed chamber.

*Synthesis of Boc-amino acids (**1**–**3**)*

L-Phenylalanine (3.3 g, 0.02 mol) was dissolved in 1 mol L^{-1} NaOH (20 mL) and *i*-propanol (20 mL). Boc_2O (6 mL, 0.026 mol) in *i*-propanol (10 mL) was added followed by 1 mol L^{-1} NaOH (20 mL) to the resulting solution. The solution was stirred at r.t. for 2 h, washed with light petroleum ether (b.p. 40–60 °C) (20 mL), acidified to pH 3.0 with 1

Table I. Physical and analytical data of compounds ***1–13***

Compd. No.	Physical state	M.p. (°C)	Yield (%)	Mol. formula (M_r)	Elemental analysis Calcd./found (%)		
					C	H	N
1	White crystals	84–85	89	$C_{14}H_{19}NO_4$ (265.31)	63.38 63.25	7.22 7.28	5.28 5.25
2	White crystals	74–76	80	$C_9H_{17}NO_5$ (219.24)	49.31 49.22	7.82 7.85	6.39 6.45
3	White crystals	135–136	88	$C_{14}H_{19}NO_5$ (281.31)	59.78 59.77	6.81 6.68	4.98 4.89
4	Viscous liquid	–	91	$C_6H_{12}ClNO_2$ (165.62)	43.51 43.65	7.30 7.28	8.46 8.55
5	White crystals	160–162	78	$C_{10}H_{14}ClNO_2$ (215.68)	55.69 55.76	6.54 6.55	6.49 6.58
6	Viscous liquid	–	86	$C_5H_{12}ClNO_3$ (169.61)	35.41 35.32	7.13 7.17	8.26 8.22
7	Semisolid mass	–	77	$C_{20}H_{28}N_2O_5$ (376.45)	63.81 64.02	7.50 7.63	7.44 7.48
7a	White solid	155–157	71	$C_{19}H_{26}N_2O_5$ (362.42)	62.97 62.69	7.23 7.26	7.73 7.68
8	Semisolid mass	–	79	$C_{15}H_{26}N_2O_6$ (330.38)	54.53 54.55	7.93 7.90	8.48 8.39
8a	White solid	189–190	70	$C_{14}H_{24}N_2O_6$ (316.35)	53.15 52.98	7.65 7.82	8.86 8.84
9	Semisolid mass	–	82	$C_{24}H_{30}N_2O_6$ (442.51)	65.14 64.98	6.83 6.89	6.33 6.28
9a	Semisolid mass	–	73	$C_{19}H_{22}N_2O_4$ (342.39)	66.65 66.66	6.48 6.52	8.18 8.09
10	Semisolid mass	–	72	$C_{24}H_{35}N_3O_7$ (477.55)	60.36 59.89	7.39 7.67	8.80 8.69
10a	White solid	111–112	70	$C_{23}H_{33}N_3O_7$ (463.53)	59.60 59.54	7.18 7.09	9.07 8.98
11	Semisolid mass	–	76	$C_{33}H_{44}N_4O_9$ (640.73)	61.86 61.60	6.92 6.98	8.74 8.66
11a	Semisolid mass	–	79	$C_{28}H_{36}N_4O_7$ (540.61)	62.21 62.26	6.71 6.64	10.36 10.39
12	Semisolid mass	–	66	$C_{51}H_{67}N_7O_{13}$ (986.12)	62.12 62.10	6.85 6.86	9.94 10.01
12a	White solid	149–150	70	$C_{50}H_{65}N_7O_{13}$ (972.09)	61.78 61.83	6.74 6.69	10.09 9.98
12b	Semisolid mass	–	92	$C_{56}H_{68}N_8O_{15}$ (1093.19)	61.53 61.49	6.27 6.33	10.25 10.32
12c	Semisolid mass	–	89	$C_{51}H_{60}N_8O_{13}$ (993.08)	61.68 61.72	6.09 6.12	11.28 11.33
13	Light brown solid	175–176	72	$C_{45}H_{55}N_7O_{10}$ (853.97)	63.29 63.14	6.49 6.54	11.48 11.50

mol L^{-1} H_2SO_4 and finally extracted with chloroform (3 × 20 mL). The organic layer was dried over anhydrous sodium sulphate and evaporated under reduced pressure to give the crude product, which was crystallized from chloroform and petroleum ether (b.p. 40–60 °C) to get pure Boc-phenylalanine (**1**). Similarly, Boc-threonine (**2**) and Boc-tyrosine (**3**) were prepared by stirring Boc_2O (6 mL, 0.026 mol) with L-threonine (2.38 g, 0.02 mol) and L-tyrosine (3.62 g, 0.02 mol), respectively (Tables I and II).

*Synthesis of L-amino acid methyl ester hydrochlorides (**4**–**6**)*

Thionyl chloride (1.4 mL, 0.02 mol) was slowly added to methanol (100 mL) at 0 °C and L-proline (2.3 g, 0.02 mol) was added to the above solution. The resulting mixture was refluxed for 8-10 h at ambient temperature. Methanol was evaporated and the residue was triturated with ether at 0 °C until excess dimethyl sulphite was removed. The crude solid was crystallized from methanol and ether at 0 °C to get L-proline methyl ester hydrochloride (**4**). Similarly, L-phenylalanine methyl ester hydrochloride (**5**) and L--threonine methyl ester hydrochloride (**6**) were prepared by refluxing L-phenylalanine (3.3 g, 0.02 mol)/L-threonine (2.38 g, 0.02 mol) with methanol (100 mL) in the presence of thionyl chloride (1.4 mL, 0.02 mol).

*Synthesis of Boc-dipeptide methyl esters (**7**–**9**)*

To a mixture of compound **4** (1.66 g, 0.01 mol) in $CHCl_3$ (20 mL), NMM (2.3 mL, 0.021 mol) was added at 0 °C. The reaction mixture was stirred for 15 min. Compound **1** (2.65 g, 0.01 mol) in $CHCl_3$ (20 mL) and DCC (2.1 g, 0.01 mol) were added under stirring to the above mixture. After 36 h, the reaction mixture was filtered and the residue was washed with $CHCI_3$ (30 mL) and added to the filtrate. The filtrate was washed with 5% $NaHCO_3$ and saturated NaCI solution (25 mL each). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated in vacuum. The crude product was crystallized from a mixture of chloroform and petroleum ether (b.p. 40–60 °C), followed by cooling at 0 °C to get Boc-Phe-Pro-OMe (**7**). Similarly, Boc-Thr-Pro-OMe (**8**) and Boc--Tyr-Phe-OMe (**9**) were prepared by stirring compounds **2** and **3** with amino acid methyl ester hydrochlorides **4** and **5,** respectively, in the presence of DCC and NMM.

*Deprotection of dipeptides at carboxyl end (**7a**, **8a**)*

To a solution of compound **7** (3.76 g, 0.01 mol) in THF/H_2O (1:1, 36 mL), LiOH (0.36 g, 0.015 mol) was added at 0 °C. The mixture was stirred at r.t. for 1 h and then acidified to pH 3.5 with 0.5 mol L^{-1} H_2SO_4. The aqueous layer was extracted with Et_2O (3 × 25 mL). 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 (**7a**). Similarly, compound **8** was hydrolyzed under alkaline conditions to obtain Boc-Thr-Pro-OH (**8a**).

*Deprotection of dipeptide at amino end (**9a**)*

Compound **9** (4.42 g, 0.01 mol) was dissolved in $CHCl_3$ (15 mL) and treated with trifluoroacetic acid (2.28 g, 0.02 mol). The resulting solution was stirred at r.t. for 1 h and washed with saturated $NaHCO_3$ solution (25 mL). The organic layer was dried over an-

Table II. Spectral data of compounds ***1–13***

Compd. No.	IR ($CHCl_3$, cm^{-1}), ^1H NMR ($CDCl_3$, δ ppm), FAB MS (*m/z*)
1	3298–2485 (m/br, OH str, COOH), 3089, 3034 (w, CH str, ring), 2928 (m, CH str, asym, aliph. CH_2), 2897 (m, CH str, >CH–), 1715 (s, C=O str, COOH), 1582, 1482 (m, skeletal bands, ring), 1542 (m, NH bend, amide), 1387, 1365 (m, CH bend, ^{t}butyl), 933 (w, CH_3 rocking, ^{t}butyl), 730, 693 (s, CH bend, out-of-plane, monosub. ring) 10.48 (s, 1H, OH, COOH), 7.30–7.26 (tt, 2H, *m*-H's, Phe), 7.09–7.07 (dd, 2H, *o*-H's, Phe, *J* = 6.45 Hz), 7.00–6.96 (t, 1H, *p*-H, Phe), 5.89 (br. s, 1H, NH), 5.12–5.08 (m, 1H, α-H, Phe), 3.25–3.23 (d, 2H, β-H's, Phe, *J* = 4.5 Hz), 1.54 (s, 9H, ^{t}butyl)
2	3341–2528 (m/br, OH str, Thr and OH str, COOH), 2959 (m, CH str, asym, aliph. CH_3), 2898, 2892 (m, CH str, >CH–), 2873 (m, CH str, sym, aliph. CH_3), 1712 (s, C=O str, COOH), 1544 (m, NH bend, amide), 1389, 1365 (m, CH bend, ^{t}butyl), 929 (w, CH_3 rock, ^{t}butyl), 665 (m/br, OH bend, oop, Thr) 6.49 (ss, 2H, β-OH, Thr and OH, COOH), 5.88 (br. s, 1H, NH), 4.49–4.42 (m, 1H, β-H, Thr), 4.19–4.15 (t, 1H, α-H, Thr), 1.54 (s, 9H, ^{t}butyl), 1.40–1.38 (d, 3H, β-CH_3, Thr, *J* = 5.9 Hz)
3	3369 (m/br, OH str, phenolic), 3292–2472 (m/br, OH str, COOH), 3085, 3022 (w, CH str, ring), 2927 (m, CH str, asym, aliph. CH_2), 2855 (m, CH str, sym, aliph. CH_2), 2898 (m, CH str, >CH–), 1709 (s, C=O str, COOH), 1587, 1484 (m, skeletal bands, ring), 1543 (m, NH bend, amide), 1387, 1366 (m, CH bend, ^{t}butyl), 1225 (s, C–O str, phenolic), 930 (w, CH_3 rocking, ^{t}butyl), 822 (s, CH bend, oop, 1,4-disub. ring) 8.23 (ss, 2H, *p*-OH, ring and OH, COOH), 7.20–7.18 (dd, 2H, *o*-H's, Tyr, *J* = 7.15 Hz), 7.08–7.06 (dd, 2H, *m*-H's, Tyr, *J* = 7.1 Hz), 5.90 (br. s, 1H, NH), 5.13–5.08 (m, 1H, α-H, Tyr), 3.24–3.22 (d, 2H, β-H's, Tyr, *J* = 6.2 Hz), 1.55 (s, 9H, ^{t}butyl)
4	3050–2840 (s/br, $>NH_2^+$ str, asym and sym), 2995, 2987 (m, CH str, cyclic CH_2 and CH), 2822 (m, CH str, OCH_3), 1745 (s, C=O str, ester), 1465 (m, CH bend, OCH_3), 1440 (s/br, $>NH_2^+$ bend), 1206 (s, C–O str, ester) 4.73–4.70 (t, 1H, δ-H of Pro), 4.02–3.97 (t, 2H, α-H's, Pro), 3.85 (s, 3H, OCH_3), 2.48–2.41 (m, 2H, γ-H's, Pro), 2.40–2.31 (m, 2H, β-H's, Pro), 2.35 (br. s, 2H, NH_2^+)
5	3010–2855 (s/br, NH_3^+ str, asym and sym), 3076, 3030 (w, CH str, ring), 2926 (m, CH str, asym, aliph. CH_2), 2894 (m, CH str, >CH–), 2828 (m, CH str, OCH_3), 1742 (s, C=O str, ester), 1605, 1503 (s/br, NH_3^+ bend, asym and sym), 1582, 1485 (m, skeletal bands, ring), 1205 (s, C–O str, ester), 732, 695 (s, CH bend, out-of-plane, monosub. ring) 7.66–7.61 (tt, 2H, *m*-H's, Phe), 7.53–7.49 (t, 1H, *p*-H, Phe), 7.47–7.45 (dd, 2H, *o*-H's, Phe, *J* = 6.5 Hz), 5.17 (br. s, 3H, NH_3^+), 4.15–4.11 (m, 1H, α-H, Phe), 4.09 (s, 3H, OCH_3), 2.31–2.29 (d, 2H, β-H's, Phe, *J* = 4.45 Hz)
6	3339 (m/br, OH str, Thr), 3008–2859 (s/br, NH_3^+ str, asym and sym), 2957 (m, CH str, asym, aliph. CH_3), 2896 (m, CH str, >CH–), 1746 (s, C=O str, ester), 1602, 1498 (s/br, NH_3^+ bend, asym and sym), 1208 (s, C–O str, ester), 667 (m/br, OH bend, oop, Thr) 4.11 (s, 3H, OCH_3), 3.84 (ss, 4H, NH_3^+ and OH), 3.81–3.79 (t, 1H, α-H, Thr), 3.65–3.57 (m, 1H, β-H, Thr), 1.89–1.87 (d, 3H, β-CH_3, Thr, *J* = 6.0 Hz)

Table II. contd.

Compd. No.	IR ($CHCl_3$, cm^{-1}), ^1H NMR ($CDCl_3$, δ ppm)
7	3325 (s, NH str, amide), 3087, 3033 (w, CH str, ring), 2992, 2986 (m, CH str, cyclic CH_2 and CH), 2927 (m, CH str, asym, aliph. CH_2), 2895 (m, CH str, >CH–), 2823 (m, CH str, OCH_3), 1752 (s, C=O str, ester), 1669, 1643 (s, C=O str, 3° and 2° amide), 1589, 1486 (m, skeletal bands, ring), 1545 (m, NH bend, 2° amide), 1386, 1367 (m, CH bend, ^{t}butyl), 1206 (s, C–O str, ester), 732, 693 (s, CH bend, oop, ring) 7.53–7.46 (m, 2H, *m*-H's, Phe), 6.95–6.90 (t, 1H, *p*-H, Phe), 6.86–6.83 (dd, 2H, *o*-H's, Phe, *J* = 6.5 Hz), 6.44 (br. s, 1H, NH), 4.80–4.73 (m, 1H, α-H, Phe), 4.34–4.29 (t, 1H, δ-H of Pro), 3.75–3.72 (t, 2H, α-H's, Pro), 3.64 (s, 3H, OCH_3), 3.15–3.13 (d, 2H, β-H's, Phe, *J* = 4.5 Hz), 2.08–1.95 (m, 4H, β- and γ-H's, Pro), 1.55 (s, 9H, ^{t}butyl)
8	3345 (m/br, OH str, Thr), 3315 (s, NH str, amide), 2997, 2990 (m, CH str, cyclic CH_2 and CH), 2957 (m, CH str, asym, aliph. CH_3), 2870 (m, CH str, sym, aliph. CH_3), 2812 (m, CH str, OCH_3), 1749 (s, C=O str, ester), 1672, 1639 (s, C=O str, 3° and 2° amide), 1544 (m, NH bend, 2° amide), 1462 (m, CH bend, OCH_3), 1390, 1365 (m, CH bend, ^{t}butyl), 1205 (s, C–O str, ester), 928 (w, CH_3 rock, ^{t}butyl), 667 (m/br, OH bend, oop, Thr) 6.44 (br. s, 1H, NH), 4.47–4.43 (m, 2H, α-H and β-OH, Thr), 4.30–4.22 (t, 1H, δ-H, Pro), 3.78–3.69 (t, 2H, α-H's, Pro), 3.67–3.59 (m, 1H, β-H, Thr), 3.62 (s, 3H, OCH_3), 2.09–1.95 (m, 4H, β- and γ-H's, Pro), 1.54 (s, 9H, ^{t}butyl), 1.27–1.25 (d, 3H, β-CH_3, Thr, *J* = 6.0 Hz)
9	7.34–7.28 (dd, 2H, *m*-H's, Tyr, *J* = 7.0 Hz), 7.14–7.08 (t, 1H, *p*-H, Phe), 7.02–6.96 (tt, 2H, m-H's, Phe), 6.93–6.91 (dd, 2H, o-H's, Tyr, *J* = 7.2 Hz), 6.87–6.83 (dd, 2H, o-H's, Phe, *J* = 7.1 Hz), 6.68 (br. s, 1H, NH), 6.57 (br. s, 1H, NH), 5.95 (1H, s, *p*-OH, Tyr), 4.75–4.67 (m, 1H, α-H, Tyr), 4.62–4.57 (m, 1H, α-H, Phe), 3.55 (s, 3H, OCH_3), 3.15–2.74 (m, 4H, β-H's, Tyr and Phe), 1.54 (s, 9H, ^{t}butyl)
10	3340, 3332 (m/br, OH str, Thr), 3320, 3244 (s, NH str, amide), 3095, 3030 (w, CH str, ring), 2993, 2989 (m, CH str, cyclic CH_2 and CH), 2961, 2924 (m, CH str, asym, aliph. CH_3 and CH_2), 2898, 2894 (m, CH str, >CH–), 2820 (m, CH str, OCH_3), 1748 (s, C=O str, ester), 1675, 1639 (s, C=O str, 3° and 2° amide), 1588, 1488 (m, skeletal bands, ring), 1544, 1535 (m, NH bend, 2° amide), 1388, 1369 (m, CH bend, ^{t}butyl), 1204, 1093 (s, C–O str, ester and C–OH), 730, 690 (s, CH bend, oop, ring) 8.60 (br. s, 1H, NH), 7.55–7.45 (m, 2H, m-H's, Phe), 6.95 (br. s, 1H, NH), 6.93–6.84 (m, 3H, *o*- and *p*-H's, Phe), 4.84 (q, 1H, α-H, Phe), 4.65 (t, 1H, α-H, Thr), 4.62–4.54 (m, 1H, β-H, Thr), 4.22 (t, 1H, δ-H, Pro), 3.64 (t, 2H, α-H's, Pro), 3.62 (s, 3H, OCH_3), 3.27 (s, 1H, β-OH, Thr), 3.16 (d, 2H, β-H's, Phe, *J* = 4.5 Hz), 2.77–2.70 (q, 2H, γ-H's, Pro), 1.97–1.88 (m, 2H, β-H's, Pro), 1.54 (s, 9H, ^{t}butyl), 1.32 (d, 3H, β-CH_3 of Thr, *J* = 6.0 Hz)
10a	3345–2505 (m/br, OH str, Thr and OH str, COOH), 3315, 3257 (s, NH str, amide), 3079, 3026 (w, CH str, ring), 2995, 2990 (m, CH str, cyclic CH_2 and CH), 2962, 2922 (m, CH str, asym, aliph. CH_3 and CH_2), 1710 (s, C=O str, COOH), 1678, 1642 (s, C=O str, 3° and 2° amide), 1589, 1485 (m, skeletal bands, ring), 1548, 1537 (m, NH bend, 2° amide), 1387, 1365 (m, CH bend, ^{t}butyl), 1093 (s, C–O str, OH, Thr), 730, 692 (s, CH bend, oop, mono-sub. ring)

Table II. contd.

Compd. No	IR ($CHCl_3$, cm^{-1}), ^1H NMR ($CDCl_3$, δ ppm)
11	3372, 3343 (m/br, OH str, phenolic and Thr), 3315–3244 (s, NH str, amide), 3088–3020 (w, CH str, rings), 2998, 2992 (m, CH str, cyclic CH_2 and CH), 2959, 2927 (m, CH str, asym, aliph. CH_3 and CH_2), 2872, 2853 (m, CH str, sym, aliph. CH_3 and CH_2), 2897 (m, CH str, >CH–), 2816 (m, CH str, OCH_3), 1748 (s, C=O str, ester), 1675, 1639 (s, C=O str, 3° and 2° amide), 1590, 1588 (m, skeletal bands, rings), 1540–1520 (m, NH bend, 2° amide), 1460 (m, CH bend, OCH_3), 1388, 1369 (m, CH bend, ^{t}butyl), 1223, 1206 (s, C–O str, phenolic and ester), 930 (w, CH_3 rock, ^{t}butyl), 825 (s, CH bend, oop, 1,4-disub. ring) 727, 689 (s, CH bend, oop, monosub. ring), 665 (m/br, OH bend, oop, Thr) 9.94 (br. s, 1H, NH), 7.14–7.11 (t, 1H, *p*-H, Phe), 7.02–6.83 (m, 8H, *o*- and *m*-H's, Phe and Tyr), 6.50 (br. s, 1H, NH), 6.06 (br. s, 1H, NH), 5.26–5.21 (m, 1H, α-H, Tyr), 5.20 (2H, s, p-OH, Tyr and β-OH, Thr), 4.41 (t, 1H, α-H, Thr), 4.28–4.23 (t, 1H, δ-H, Pro), 3.96–3.89 (m, 1H, α-H, Phe), 3.67–3.58 (m, 3H, α-H's, Pro and β-H, Thr), 3.55 (s, 3H, OCH_3), 3.03–2.70 (m, 6H, β-H's, Tyr, Phe and γ-H's, Pro), 1.97-1.88 (q, 2H, β-H's, Pro), 1.54 (s, 9H, ^{t}butyl), 1.26 (d, 3H, β-CH_3, Thr, J = 6.0 Hz)
11a	3495, 3398 (w, free NH str, NH_2, asym and sym), 3370, 3345 (m/br, OH str, phenolic and Thr), 3310–3245 (s, NH str, amide), 3090–3015 (w, CH str, rings), 2997, 2992 (m, CH str, cyclic CH_2 and CH), 2962, 2925 (m, CH str, asym, aliph. CH_3 and CH_2), 2817 (m, CH str, OCH_3), 1751 (s, C=O str, ester), 1676, 1640 (s, C=O str, 3° and 2° amide), 1590, 1589 (m, skeletal bands, rings), 1538–1522 (m, NH bend, 2° amide), 1225, 1208 (s, C–O str, phenolic and ester), 1055 (m, C–N str, NH_2, Thr), 828 (s, CH bend, oop, 1,4-disub. ring) 726, 689 (s, CH bend, oop, monosub. ring), 667 (m/br, OH bend, oop, Thr)
12	3370–3329 (m/br, OH str, phenolic and Thr), 3322–3258 (s, NH str, amide), 3055, 3032 (w, CH str, arom. rings), 3010–2990 (m, CH str, cyclic CH_2 and CH), 2960–2922 (m, CH str, asym, aliph. CH_3 and CH_2), 2870–2848 (m, –CH str, sym, aliph. CH_3 and CH_2), 2898, 2891 (m, –CH str, >CH–), 2814 (m, CH str, OCH_3), 1950–1780 (w, overtone bands), 1750 (s, C=O str, ester), 1682–1633 (s, –C=O str, 3° and 2° amide), 1589, 1582 (m, skeletal bands, arom. rings), 1542–1533 (m, NH bend, 2° amide), 1466 (m, scissor, aliph. CH_2), 1387, 1370 (m, CH bend, ^{t}butyl), 1224, 1207 (s, C–O str, phenolic and ester), 1125–1080 (m, CH bend, in-plane, rings), 930 (w, CH_3 rock, ^{t}butyl), 823 (s, CH bend, oop, 1,4-disub. ring), 800–755 (m/br, NH wagg, 2° amide), 729–687 (s, CH bend, oop, monosub. rings) 9.91 (br. s, 1H, NH), 8.49 (br. s, 1H, NH), 7.52–7.48 (m, 2H, *m*-H's, Phe^1), 7.13–7.08 (t, 1H, *p*-H, Phe^2), 7.02–6.83 (m, 11H, *o*-, *m*- and *p*-H's, Phe^2, Tyr, Phe^1), 6.94 (br. s, 1H, NH), 6.53 (br. s, 1H, NH), 6.45 (br. s, 1H, NH), 5.71 (ss, 3H, singlet of p-OH of Tyr overlapped over singlets of β-OHs, Thr^1 and Thr^2), 5.26–5.20 (m, 1H, α-H, Tyr), 4.92–4.88 (t, 1H, α-H, Thr^1), 4.62–4.56 (m, 1H, α-H, Phe^1), 4.47–4.45 (t, 1H, δ-H, Pro^1), 4.21–4.17 (t, 1H, α-H, Thr^2), 4.11–4.08 (t, 1H, δ-H, Pro^2), 3.96–3.67 (m, 5H, α-H, Phe^2, β-H's, Thr^1 and Thr^2, α-H's, Pro^1), 3.55 (s, 3H, OCH_3), 3.30–3.24 (t, 2H, α-H's, Pro^2), 3.14–2.71 (m, 6H, β-H's, Phe^1, Tyr and Phe^2), 2.70–2.64 (tt, 4H, triplet overlapped over triplet, γ-H's, Pro^1 and Pro^2), 1.97–1.88 (m, 4H, β-H's, Pro^1 and Pro^2), 1.54 (s, 9H, ^{t}butyl), 1.30 (d, 3H, β-CH_3, Thr^1, J = 6.0 Hz), 1.25 (d, 3H, β-CH_3, Thr^2, J = 6.0 Hz)

Table II. contd.

Compd. No.	IR ($CHCl_3$, cm^{-1}), ^1H NMR ($CDCl_3$, δ ppm), ^{13}C NMR ($CDCl_3$, δ ppm)
12b	3355–3323 (m/br, OH str, phenolic and Thr), 3277, 3258 (s, NH str, amide), 3052–3045 (w, CH str, arom. rings), 3012–2992 (m, CH str, cyclic CH_2 and CH), 2959–2922 (m, CH str, asym, aliph. CH_3 and CH_2), 2870–2849 (m, –CH str, sym, aliph. CH_3 and CH_2), 2894 (m, –CH str, >CH–), 1763 (s, C=O str, aromatic ester), 1683–1632 (s, –C=O str, 3° and 2° amide), 1589, 1584, 1580 (m, skeletal bands, arom. rings), 1542–1532 (m, NH bend, 2° amide), 1518 (s, asym NO stretch, arom. NO_2 grp), 1392, 1365 (m, CH bend, ^{t}butyl), 1349 (s, sym NO stretch, arom. NO_2 grp), 1224, 1215 (s, C–O str, phenolic and arom. ester), 929 (w, CH_3 rock, ^{t}butyl), 822 (s, CH bend, oop, 1,4-disub. ring), 731–688 (s, CH bend, oop, monosub. rings)
12c	3497, 3392 (w, free NH stretch, asymm and symm, NH_2 grp), 3352–3326 (m/br, OH str, phenolic and Thr), 3255, 3251 (s, NH str, amide), 3050–3043 (w, CH str, arom. rings), 3010–2994 (m, CH str, cyclic CH_2 and CH), 2962–2928 (m, CH str, asym, aliph. CH_3 and CH_2), 2872–2846 (m, –CH str, sym, aliph. CH_3 and CH_2), 2897, 2894 (m, –CH str, >CH–), 1760 (s, C=O str, aromatic ester), 1683–1630 (s, –C=O str, 3° and 2° amide), 1590, 1584, 1580 (m, skeletal bands, arom. rings), 1540–1532 (m, NH bend, 2° amide), 1520 (s, asym NO stretch, arom. NO_2), 1350 (s, sym NO stretch, arom. NO_2 grp), 1225, 1215 (s, C–O str, phenolic and arom. ester), 1057 (m, C–N stretch, NH_2), 819 (s, CH bend, oop, 1,4-disub. ring), 730–689 (s, CH bend, oop, monosub. rings)
13[a]	3400, 3335 (m/br, OH str, phenolic and Thr), 3327–3180 (s, NH str, amide), 3055, 3032 (w, CH str, arom. rings), 3010–2990 (m, CH str, cyclic CH_2 and CH), 2952–2924 (m, CH str, asym, aliph. CH_3 and CH_2), 2853–2847 (m, CH str, sym, aliph. CH_2), 1682–1633 (s, C=O str, 3° and 2° amide), 1593–1554 (m, skeletal bands, rings), 1535, 1512 (m, NH bend, 2° amide), 1449 (m, CH bend, CH_2 scissoring), 1224 (s, C–O str, phenolic), 1125–1025 (m, CH bend, in-plane, rings), 825 (s, CH bend, oop, 1,4-disub. ring), 727–674 (s, –CH bend, oop, monosub. rings), 664 (m/br, OH bend, oop, Thr) 9.88 (br. s, 1H, NH), 9.84 (br. s, 1H, NH), 7.75 (br. s, 1H, NH), 7.70 (br. s, 1H, NH), 7.61 (br. s, 1H, NH), 7.20–6.82 (m, 14H, *o*-, *m*- and *p*-H's, Phe^1, Tyr and Phe^2), 7.05 (s, 3H, β-OH, Thr^1, Thr^2 and p-OH, Tyr), 5.80–5.61 (m, 3H, α-H, Thr^2 and α-H's, Phe^1, Phe^2), 4.41–4.36 (t, 1H, α-H, Thr^1), 4.23–4.16 (m, 1H, α-H, Tyr), 3.93–3.87 (tt, 2H, triplet overlapped over triplet, δ-H's, Pro^1 and Pro^2), 3.84–3.74 (m, 2H, β-H's, Thr^1 and Thr^2), 3.28–3.22 (tt, 4H, α-H's, Pro^1 and Pro^2), 2.70–2.64 (m, 4H, γ-H's, Pro^1 and Pro^2), 2.63–2.35 (m, 6H, β-H's, Phe^2, Tyr and Phe^1), 1.88–1.79 (m, 4H, β-H's, Pro^1 and Pro^2), 1.42–1.40 (dd, 6H, β-H's, Thr^1 and Thr^2, J = 6.2 Hz) 173.0 (s, C=O), 171.2 (d, C=O), 171.1 (s, C=O), 170.0 (s, C=O), 169.7 (s, C=O), 153.8 (s, ζ-C, Tyr), 137.9 (s, γ-C, Phe^2), 137.4 (s, γ-C, Phe^1), 133.9 (s, γ-C, Tyr), 130.2 (s, δ-C and θ-C, Phe^1), 129.6 (s, δ-C and θ-C, Tyr), 129.3 (s, δ-C and θ-C, Phe^2), 128.9 (s, ε-C, η-C, Phe^1 and Phe^2), 128.1 (s, ε-C and η-C, Tyr), 127.9 (d, ζ-C, Phe^1 and Phe^2), 67.1 (s, β-C, Thr^2), 66.1 (s, β-C, Thr^1), 58.3 (s, α-C, Thr^1), 58.2 (s, α-C, Pro^1 and Pro^2), 58.1 (s, α-C, Thr^2), 53.8 (s, α-C, Phe^1), 53.5 (s, α-C, Phe^2), 52.8 (s, α-C, Tyr), 46.6 (s, δ-C, Pro^2 and Pro^1), 36.5 (s, β-C, Tyr and Phe^2), 35.8 (s, β-C, Phe^1), 29.6 (s, β-C, Pro^2 and Pro^1), 21.9 (s, β-CH_3, Thr^1), 21.4 (s, γ-C, Pro^2 and Pro^1), 21.0 (s, β-CH_3, Thr^2). 854.9 $(M + 1)^+$, 826.8 $(854–CO)^+$, 707.7 $(Phe\text{-}Pro\text{-}Thr\text{-}Thr\text{-}Pro\text{-}Tyr)^+$, 679.7 $(707–CO)^+$, 544.5 $(Phe\text{-}Pro\text{-}Thr\text{-}Thr\text{-}Pro)^+$, 516.6 $(544–CO)^+$, 447.5 $(Phe\text{-}Pro\text{-}Thr\text{-}Thr)^+$, 419.5 $(447–CO)^+$, 346.6 $(Phe\text{-}Pro\text{-}Thr)^+$, 318.4 $(346–CO)^+$, 245.4 $(Phe\text{-}Pro)^+$, 217.2 $(245–CO)^+$, 148.2 $(Phe)^+$, 120.1 $(148–CO)^+$.

[a] IR spectrum was taken using KBr.

hydrous 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 give pure Tyr-Phe-OMe (**9a**).

*Synthesis of Boc-tri/tetrapeptide methyl esters (**10**, **11**)*

To synthesize Boc-Phe-Pro-Thr-OMe (**10**), dipeptide unit **7a** (3.62 g, 0.01 mol) was coupled with amino acid methyl ester hydrochloride **6** (1.7 g, 0.01 mol) in the presence of DCC and NMM the following the same procedure as adopted for the synthesis of Boc-dipeptide methyl esters **7**–**9**. Similarly, Boc-Thr-Pro-Tyr-Phe-OMe (**11**) was prepared by coupling deprotected dipeptide units **8a** (3.16 g, 0.01 mol) and **9a** (3.42 g, 0.01 mol) using DCC as the coupling agent and NMM as the base.

*Synthesis of Boc-heptapeptide methyl ester (**12**)*

To synthesize Boc-Phe-Pro-Thr-Thr-Pro-Tyr-Phe-OMe (**12**), tripeptide unit **10** (4.77 g, 0.01 mol) was deprotected at carboxyl end to get Boc-Phe-Pro-Thr-OH (**10a**) following the same procedure as adopted for the synthesis of compounds **7a** and **8a** from compounds **7** and **8**, respectively. Tetrapeptide unit **11** (6.4 g, 0.01 mol) was deprotected at amino end to get Thr-Pro-Tyr-Phe-OMe (**11a**) following the same procedure as adopted for the synthesis of compound **9a** from compound **9**. The deprotected tripeptide unit **10a** (4.63 g, 0.01 mol) and tetrapeptide unit **11a** (5.4 g, 0.01 mol) were coupled in the presence of DCC and NMM to get linear heptapeptide unit **12** under same the experimental conditions as adopted for the synthesis of Boc-dipeptide methyl esters **7**–**9**.

*Synthesis of cyclic heptapeptide, hymenamide E (**13**)*

To synthesize cyclo(Phe-Pro-Thr-Thr-Pro-Tyr-Phe) (**13**), linear heptapeptide unit **12** (4.93 g, 0.005 mol) was deprotected at carboxyl end using LiOH (0.18 g, 0.0075 mol) to get Boc-Phe-Pro-Thr-Thr-Pro-Tyr-Phe-OH (**12a**) following the same procedure as adopted for the synthesis of compounds **7a** and **8a** from compounds **7** and **8** respectively.

The deprotected heptapeptide unit **12a** (4.86 g, 0.005 mol) was dissolved in $CHCl_3$ (50 mL) at 0 °C. To the above solution, pnp (0.94 g, 0.0067 mol) was added and stirred at r.t. for 12 h. The reaction mixture was filtered and the filtrate was washed with 10% $NaHCO_3$ solution (3 × 15 mL) until excess of pnp was removed and finally washed with 5% HCI (2 × 10 mL) to get the corresponding *p*-nitrophenyl ester Boc-Phe-Pro-Thr-Thr-Pro-Tyr-Phe-O-pnp (**12b**).

To compound **12b** (4.37 g, 0.004 mol) dissolved in $CHCI_3$ (35 mL), TFA (0.91 g, 0.008 mol) was added, stirred at r.t. 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 Phe-Pro-Thr-Thr-Pro-Tyr-Phe-O-pnp (**12c**), which was dissolved in $CHCI_3$ (25 mL) and NMM (2.3 mL, 0.021 mol) was added. Then, all 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% HCI (3 × 15 mL). The organic layer was dried over anhydrous Na_2SO_4. Finally, chloroform was distilled off and the crude cyclized product was crystallized from $CHCl_3$ and hexane to get the pure compound **13**. Synthe-

tic pathway for compounds **7–13** is given in Scheme 1 and various steric and lipophilicity parameters were calculated for synthesized cyclopeptide **13**, which are needed to describe the intermolecular forces of drug-receptor interaction as well as transport and distribution of the drug. The physicochemical and spectral data for compound **13** are given in Tables I and II.

Microbes and earthworms

The newly synthesized cyclic heptapeptide **13** was screened for *in vitro* antibacterial and antifungal activity against Gram positive bacteria *Bacillus subtilis* (MUMC 408) and *Staphylococcus aureus* (MUMC 377), Gram negative bacteria *Pseudomonas aeruginosa* (MUMC 266), *Escherichia coli* (MUMC 106) and cutaneous fungi *Microsporum audouinii* (MUMC 545) and *Trichophyton mentagrophytes* (MUMC 665), diamorphic fungi *Candida albicans* (MUMC 29) and plant pathogenic fungi *Ganoderma* sp. (MUMC 196) and for anthelmintic activity against two different species of earthworms, *Eudrilus* sp. (ICARBC 042) and *Megascoplex konkanensis* (ICARBC 211). Bacterial and fungal cultures were obtained from the Manipal University, Mycological Center (MUMC, Manipal, India), and earthworm species were obtained from the Indian Council of Agricultural Research Breeding Center (ICARBC, Kasaragod, India).

Antibacterial and antifungal activity

Antimicrobial activity studies were carried out against eight pathogenic microorganisms for compound **13** according to the modified Kirby-Bauer disc diffusion method (18). A spore suspension in sterile distilled water was prepared from the 5 days old culture of test bacteria growing on nutrient broth media and test fungi on Sabouraud's broth/ganoderma selective broth media. About 20 mL of the growth medium was transferred into sterilized Petri plates and inoculated with 1–2 mL of the spore suspension (spore concentration 6×10^4 spores mL^{-1}). Filter paper disks of 6 mm diameter and 1 mm thickness were sterilized by autoclaving at 121 °C (1.05×10^5 Pa) for 15 min.

Each Petri plate was divided into five equal portions along the diameter. Each portion was used to place one disk. Three disks of test sample **13** were placed on three portions together with one disk with the reference drug ciprofloxacin/griseofulvin and a disk impregnated with the solvent (sterile DMF) as negative control. Test sample and reference drugs were tested at 10 mg mL^{-1} concentration the propanol. 5 μL solution was transferred onto the disk using a micropipette (50 mg of the supstance applied). The Petri plates inoculated with bacterial cultures were incubated at 37 °C for 18 h and those with fungal cultures were incubated at 25 °C for 48 h. Diameters of the inhibition zones (in mm) were measured and the average diameters of the test sample were calculated for triplicate sets. The diameters obtained for the test sample were compared with those produced the standard drugs, ciprofloxacin for antibacterial activity and griseofulvin for antifungal activity. The results of antimicrobial activity are listed in Table IV.

Table III. Various steric and lipophilicity parameters for compound ***13***

Parameter	Calculated value
Molar refractivity (MR^{20})	226.31 ± 0.4 cm^3
Molar volume (MV^{20})	611.8 ± 5.0 cm^3
Parachor (P_r)	1805.8 ± 6.0 cm^3
Refractive index (n^{20})	1.661 ± 0.03
Surface tension (γ^{20})	0.0758 ± 0.005 N m^{-1}
Density (d^{20})	1.39 ± 0.1 g cm^{-3}
Polarizability (α)	89.71 ± 0.5 10^{-24} cm^3
Logarithm of partition coefficient (log *P*) (*n*-octanol/water)	–4.63 ± 0.91

Anthelmintic activity

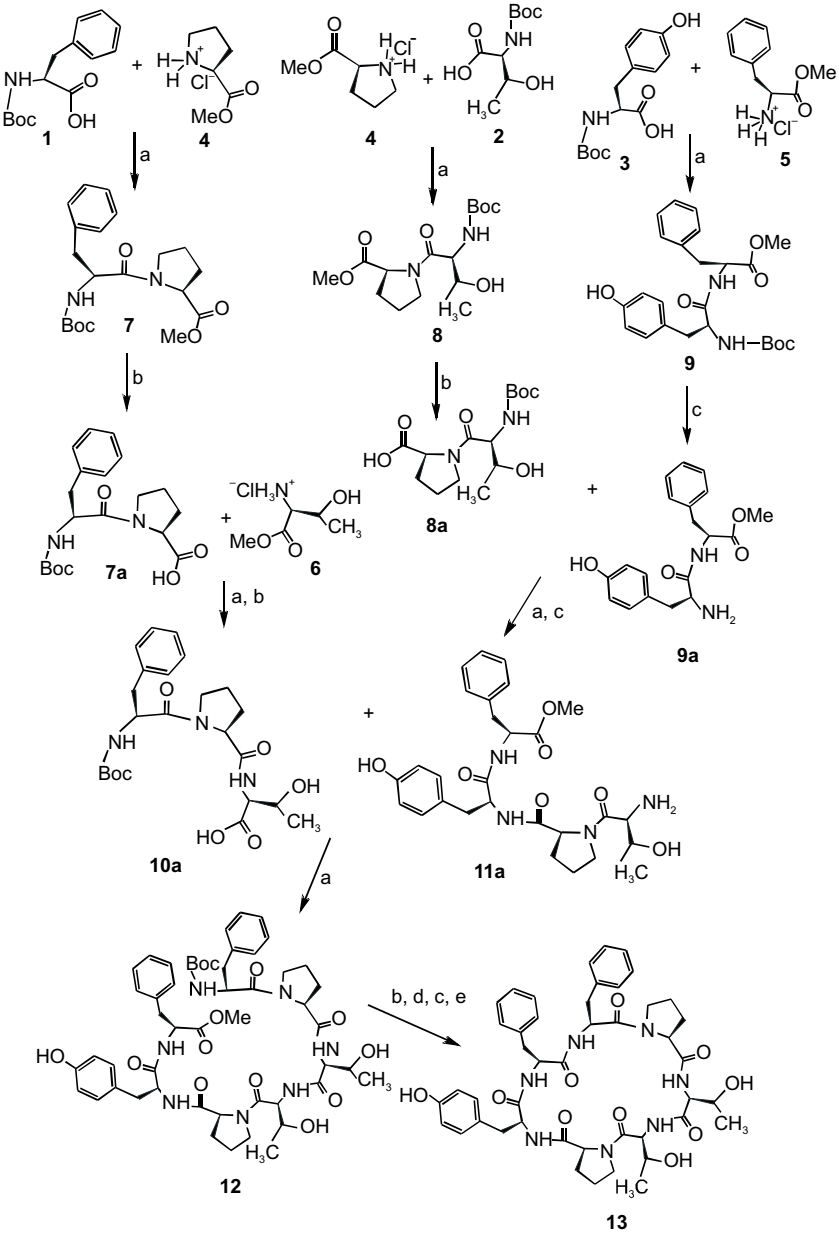
An anthelmintic activity study was perfomed for compound **13** against three different species of earthworms according to Garg's method (19). Suspension of the sample was prepared by triturating 200 mg of synthesized cyclopeptide **13** with 20 mL of Tween 80 (0.5%) and 20 mL of distilled water and the resulting mixture was stirred using a mechanical stirrer for 30 min. The suspension was diluted to contain 2 mg mL^{-1} of the test sample. Suspensions of reference drugs piperazine citrate and mebendazole were prepared in a similar way by triturating 100 mg of each drug with 10 mL of Tween 80 (0.5%) and 10 mL of distilled water separately and finally diluted to contain 2 mg mL^{-1} of piperazine citrate and mebendazole, repectively.

Two sets of five earthworms of similar sizes (5.1 cm in length) were placed in Petri plates of 10.2 cm diameter containing 50 mL of the suspension of the test sample and reference drugs (100 mg of the substance applied) at r.t. Another set of five earthworms was kept as control in a solution of 50 mL of Tween 80 (0.25%). The paralyzing and death times were noted and their mean was calculated for triplicate sets. The death time was ascertained by placing the earth worms in warm water (50 °C), which stimulated movement if the worm was alive. Results of the anthelmintic activity of the test compound and reference drugs are listed in Table V.

RESULTS AND DISCUSSION

Chemistry

The solution-phase technique was selected for peptide synthesis because it is simple and economic compared to solid phase peptide synthesis, which involves complicated chemistry utilizing costly linker resins (20, 21). In the present work, pnp and a novel base, NMM, are used for esterification and cyclization during the synthesis of cyclopeptide **13** from the linear peptide unit **12**, affording compound **13** in 72 % yield. There are previous literature reports on the synthesis of (nitro) hymenamide A and hymenamide



a = DCC, NMM, $CHCl_3$, r.t., 36 h; b = LiOH, THF/H_2O (1:1), r.t., 1 h
c = TFA, $CHCl_3$, r.t., 1 h; d = pnp, $CHCl_3$, r.t., 12 h
e = NMM, $CHCl_3$, 7 days, 0 °C

Scheme 1

Table IV. Antibacterial and antifungal activity of compound ***13***

Compd.	Zone of inhibition (mm)			
	Bacteria			
	Bacillus subtilis	*Escherichia coli*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
DMF	–	–	–	–
Ciprofloxacin[a]	18	20	22	18
13[a]	–	–	13	12
Compd.	Fungi			
	Candida albicans	*Ganoderma* species	*Microsporum audouinii*	*Trichophyton mentagrophytes*
DMF	–	–	–	–
Griseofulvin[a]	16	12	15	14
13[a]	22	19	–	–

[a] γ = 10 mg mL^{-1} (50 μg per test).

G by Belagali *et al*. (22, 23), who utilized pnp and pyridine for esterification and cyclization to get cyclopeptides in 56–61% yields. Peptide units were prepared by the Bodanszky method with certain modifications (24). Boc_2O was used to protect the amino group of L-amino acids. The carboxyl group of L-amino acids was protected by esterification with methanol utilizing $SOCl_2$. Furthermore, TFA was used to remove the Boc group and the ester group was removed by alkaline hydrolysis with lithium hydroxide.

To carry out the synthesis, hymenamide E was disconnected into a single amino acid unit **6** and three dipeptide units **7**, **8** and **9** (Scheme 1), which were coupled together after suitable deprotection at carboxyl or amino terminals to afford the linear heptapeptide **12** through tripeptide unit **10** and tetrapeptide unit **11**. Finally, linear fragment **12** was cyclized under basic conditions to yield cyclic heptapeptide **13**. Synthesis of compound **13** as well as linear segments **1**–**12** was carried out successfully with good yields and the structure of compound **13** was confirmed by IR, ^{1}H NMR, ^{13}C NMR and FAB MS spectral data (Table II). All characterization data of synthesied hymenamide E (spectral data and melting point) were found to be in agreement with characterization data of natural hymenamide E (17, 25).

IR spectra of all peptide units **7**–**13** showed characteristic medium to strong bands corresponding to carbonyl stretching at 1682–1633 cm^{-1} (amide I band) and NH bending at 1545–1512 cm^{-1} (amide II band), confirming the coupling reaction. The presence of the seven amino acid moieties was indicated by characteristic absorption bands in IR spectra and singlets/multiplets in the ^{1}H NMR spectra of the linear heptapeptide unit **12**. Alkaline hydrolysis of compound **12** afforded deprotected unit **12a**, whose structure was confirmed by disappearance of the singlet at δ 3.55 ppm corresponding to three protons of OCH_3 moiety in ^{1}H NMR spectra of compound **12a** and disappearance of absorption bands at 1750 cm^{-1} (carbonyl stretch, ester), 1207 cm^{-1} (C–O stretch, ester), along with

*Table V. Anthelmintic activity of compound **13***

Compd.	*Eudrilus* species		*Megascoplex konkanensis*	
	Mean paralyzing time ± SEM (min)	Mean death time ± SEM (min)	Mean paralyzing time ± SEM (min)	Mean death time ± SEM (min)
0.5% Tween 80 in distilled water	–	–	–	–
Piperazine citrate[a]	12.48 ± 0.18	13.73 ± 0.21	12.48 ± 0.37	13.58 ± 0.22
Mebendazole[a]	11.37 ± 0.39	13.53 ± 0.72	10.56 ± 0.93	12.73 ± 0.84
13[a]	11.41 ± 0.92	13.25 ± 0.96	11.24 ± 0.71	12.87 ± 0.38

[a] γ = 2 mg mL^{-1}[b] (100 mg per test) n = 3.

the presence of the strong absorption band at 1717 cm^{-1} (carbonyl stretch, COOH) and a broad band of medium intensity at 3332–2518 cm^{-1} (OH stretch, COOH) in the IR spectra. On treatment with *p*-nitrophenol, compound **12a** yielded the corresponding phenyl ester **12b**, which on further treatment with TFA afforded deprotected phenyl ester **12c**. Cyclization of compound **12c** at 0 °C in the presence of NMM yielded the title compound **13**. Structure of compound **12b** was confirmed by disappearance of absorption bands at 1717 cm^{-1} and 3332–2518 cm^{-1} (carbonyl and OH stretch, COOH), along with the presence of strong absorption bands at 1520 cm^{-1} and 1349 cm^{-1} (asymmetric and symmetric stretching of aromatic nitro group) and medium to strong bands at 1763 cm^{-1} and 1212 cm^{-1} corresponding to the carbonyl and C–O stretch of aromatic ester in the IR spectra, respectively. Further, the structure of compound **12c** was confirmed by disappearance of absorption bands at 1392 cm^{-1} and 1365 cm^{-1} (CH bending, ^{t}butyl) and a weak band at 929 cm^{-1} (CH_3 rocking of ^{t}butyl), along with the presence of a medium absorption band at 1057 cm^{-1} (C–N stretch, NH_2 group) and weak bands at 3497 cm^{-1} and 3392 cm^{-1} corresponding to asymmetric and symmetric free NH stretching of NH_2 group in the IR spectra. The structure of compound **13** was confirmed be disapearance of absorption bands at 1520 cm^{-1} and 1350 cm^{-1} (asymmetric and symmetric stretching of aromatic nitro group) and medium to strong bands at 1760 cm^{-1} and 1215 cm^{-1} corresponding to carbonyl and C–O stretch of aromatic ester in the IR spectra and the presence of $(M + 1)^+$ ion peak at *m/z* 854.9 corresponding to the molecular formula $C_{45}H_{55}N_7O_{10}$ in mass spectra, along with other fragment ion peaks indicating the exact sequence of attachment of seven amino acid moieties in a chain. ^{1}H NMR and ^{13}C NMR spectra of compound **13** showed characteristic peaks confirming the presence of all the 55 protons and 45 carbon atoms. Further, elemental analysis of compound **13** afforded values in accordance with the molecular composition (Table I).

Biological activity

Investigation of antimicrobial activity revealed that the synthesized cyclopeptide **13** possessed good antifungal activity against pathogenic fungi *Candida albicans* and *Ganoderma* species and moderate antibacterial activity against Gram negative bacteria *Pseudomonas aeruginosa* and Gram positive bacterium *Staphylococcus aureus*. Compound **13** was

almost 38% more active against *C. albicans* and 58–59% more active against pathogenic *Ganoderma sp*. compared to the reference drug griseofulvin and exhibited 59–67% antibacterial activity of the reference drug ciprofloxacin against bacteria *S. aureus* and *P. aeruginosa* at 10 mg mL^{-1} concentration (50 μg applied). Other bacteria and dermatophytes such as *Bacillus subtilis*, *Escherichia coli*, *Microsporum audouinii* and *Trichophyton mentagrophytes* were found to be resistant to the synthesized cyclopeptide **13**. The results of antibacterial and antifungal activity are presented in Table IV.

Comparison of anthelmintic data indicated that compound **13** exhibited 8–10% higher anthelmintic activity against both earthworms, *Eudrilus* species and *Megascoplex konkanensis*, in comparison to the standard drug piperazine citrate but showed up to 6% less activity compared to the reference drug mebendazole at 2 mg mL^{-1} concentration (100 mg applied). The results of anthelmintic activity are given in Table V.

Structure activity relationship

Detailed investigation of structures and biological potential of hymenamides A-K suggested that only hymenamides A, B, C and E showed potent antifungal activity against pathogenic *Candida* and *Cryptococcus* spp. All four hymenamides are cyclic heptapeptides and have two proline units and one phenylalanine unit in common, with one proline unit adjacent to the phenylalanine unit. The two proline units are either separated from each other by one/two amino acid units or may be adjacent to each other. Antifungal potential of hymenamides E may be attributed to this particular arrangement of two proline units and one phenylalanine unit in a cyclic structure bearing seven amino acid residues. Moreover, hymenamide E is unique among all hymenamides in having two threonine units adjacent to each other in a cyclic structure that may be responsible for its anthelmintic activity.

CONCLUSIONS

The solution phase technique employing catalytic amounts of the NMM base for cyclization and DCC as coupling agent provided yields effective for cyclopeptide synthesis. SAR studies revealed that the antifungal potential of compound **13** might be attributed to the presence of two proline and one phenylalanine units, with one proline unit adjacent to the phenylalanine unit. Likewise, anthelmintic potential may be due to the presence of adjacent threonine units in the cyclopeptide structure. On passing toxicity tests, compound **13** may be a good candidate for clinical studies and can be a new antifungal as well as anthelmintic agent in the future.

Abbreviations and acronyms – Boc – *tert*-butyloxycarbonyl, Boc_2O – di-*tert*-butylpyrocarbonate, DCC – dicyclohexylcarbodiimide, NMM – *N*-methylmorpholine, Phe – phenylalanine, pnp – *p*-nitrophenol, Pro – proline, TFA – trifluoroacetic acid, Thr – threonine, Tyr – tyrosine.

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S A Ž E T A K

Prva potpuna sinteza i biološko vrednovanje himenamida E

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Novi biološki aktivni ciklički heptapeptid himenamid E (**13**) sintetiziran je ciklizacijom linearnog peptida Boc-Phe-Pro-Thr-Thr-Pro-Tyr-Phe-OMe (**12**) nakon uklanjanja zaštitnih skupina sa C-terminalnih i N-terminalnih aminokiselina. Linearni peptidni segment pripravljen je spajanjem tripeptidne jedinice Boc-Phe-Pro-Thr-OH (**10a**) s tetrapeptidnom jedinicom Thr-Pro-Tyr-Phe-OMe (**11a**) u prisutnosti diciklohekSilkarbodiimida i *N*-metilmorfolina kao baze. Strukture novih spojeva potvrđene su IR i ^{1}H NMR spektroskopijom i elementarnom analizom, a struktura spoja **13** i ^{13}C NMR, spektroskopijom masa i diferencijalnom pretražnom kalorimetrijom. Novosintetizirani ciklopeptid testiran je na antibakterijsko, antifungalno i anthelmintičko djelovanje na osam patogenih mikroorganizama i dva parazita. Spoj **13** snažno djeluje antifungalno na gljivice *Candida albicans* i vrste *Ganoderma* i anthelmintički na nametnike *Megascoplex konkanensis* i vrste *Eudrilus*. Kao poredbene ljekovite tvari uporabljeni su grizeofulvin i piperazin citrat.

Ključne riječi: himenamid E, ciklički heptapeptid, antibakterijsko, antifungalno i anthelmintičko djelovanje

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