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 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, 1H NMR spectral data as well as elemental analyses. In addition, the structure of compound 13 was verified by 13C 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 gri-seofulvin as a reference drug and potent anthelmintic activity against earthworms *Megascopex konkanensis* and *Eudrilus* species in comparison to piperazine citrate.

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

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)perhydropyrrolo[1,2-a:1,2-j][1,4,7,10,13,16,19]heptaaza cyclohexacosine-2,5,8,11,14,17,20-heptakonone. 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 (Boc₂O), 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 CHCl₃ as solvent for intermediate semisolids. ¹H NMR and ¹³C NMR spectra were recorded on a Bruker AC NMR spectrometer (300 MHz), (Bruker, USA) using CDCl₃ 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). Boc₂O (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

<table>
<thead>
<tr>
<th>Compd. No.</th>
<th>Physical state</th>
<th>M.p. (°C)</th>
<th>Yield (%)</th>
<th>Mol. formula</th>
<th>Elemental analysis Calcd./found (%)</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>C</td>
</tr>
<tr>
<td>1</td>
<td>White crystals</td>
<td>84–85</td>
<td>89</td>
<td>C_{14}H_{19}NO_{4} (265.31)</td>
<td>63.38</td>
</tr>
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<td>2</td>
<td>White crystals</td>
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<td>80</td>
<td>C_{9}H_{17}NO_{5} (219.24)</td>
<td>49.31</td>
</tr>
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<td>3</td>
<td>White crystals</td>
<td>135–136</td>
<td>88</td>
<td>C_{14}H_{19}NO_{5} (281.31)</td>
<td>59.78</td>
</tr>
<tr>
<td>4</td>
<td>Viscous liquid</td>
<td>–</td>
<td>91</td>
<td>C_{6}H_{12}ClNO_{2} (165.62)</td>
<td>43.51</td>
</tr>
<tr>
<td>5</td>
<td>White crystals</td>
<td>160–162</td>
<td>78</td>
<td>C_{10}H_{14}ClNO_{2} (215.68)</td>
<td>55.69</td>
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<tr>
<td>6</td>
<td>Viscous liquid</td>
<td>–</td>
<td>86</td>
<td>C_{6}H_{12}ClNO_{3} (169.61)</td>
<td>35.41</td>
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<tr>
<td>7</td>
<td>Semisolid mass</td>
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<td>77</td>
<td>C_{20}H_{28}N_{2}O_{5} (376.45)</td>
<td>63.81</td>
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<tr>
<td>7a</td>
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<td>155–157</td>
<td>71</td>
<td>C_{19}H_{26}N_{2}O_{5} (362.42)</td>
<td>62.97</td>
</tr>
<tr>
<td>8</td>
<td>Semisolid mass</td>
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<td>79</td>
<td>C_{15}H_{26}N_{2}O_{6} (330.38)</td>
<td>54.53</td>
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<tr>
<td>8a</td>
<td>White solid</td>
<td>189–190</td>
<td>70</td>
<td>C_{14}H_{24}N_{2}O_{6} (316.35)</td>
<td>53.15</td>
</tr>
<tr>
<td>9</td>
<td>Semisolid mass</td>
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<td>82</td>
<td>C_{24}H_{30}N_{2}O_{6} (442.51)</td>
<td>65.14</td>
</tr>
<tr>
<td>9a</td>
<td>Semisolid mass</td>
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<td>73</td>
<td>C_{19}H_{28}N_{2}O_{4} (342.39)</td>
<td>66.65</td>
</tr>
<tr>
<td>10</td>
<td>Semisolid mass</td>
<td>–</td>
<td>72</td>
<td>C_{24}H_{32}N_{2}O_{7} (477.55)</td>
<td>60.36</td>
</tr>
<tr>
<td>10a</td>
<td>White solid</td>
<td>111–112</td>
<td>70</td>
<td>C_{23}H_{33}N_{2}O_{7} (463.53)</td>
<td>59.60</td>
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<td>11</td>
<td>Semisolid mass</td>
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<td>76</td>
<td>C_{33}H_{44}N_{2}O_{9} (640.73)</td>
<td>61.86</td>
</tr>
<tr>
<td>11a</td>
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<td>79</td>
<td>C_{26}H_{32}N_{2}O_{7} (540.61)</td>
<td>62.21</td>
</tr>
<tr>
<td>12</td>
<td>Semisolid mass</td>
<td>–</td>
<td>66</td>
<td>C_{51}H_{67}N_{2}O_{13} (986.12)</td>
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</tr>
<tr>
<td>12a</td>
<td>White solid</td>
<td>149–150</td>
<td>70</td>
<td>C_{50}H_{65}N_{2}O_{13} (972.09)</td>
<td>61.78</td>
</tr>
<tr>
<td>12b</td>
<td>Semisolid mass</td>
<td>–</td>
<td>92</td>
<td>C_{56}H_{68}N_{2}O_{15} (1093.19)</td>
<td>61.53</td>
</tr>
<tr>
<td>12c</td>
<td>Semisolid mass</td>
<td>–</td>
<td>89</td>
<td>C_{51}H_{60}N_{2}O_{13} (993.08)</td>
<td>61.68</td>
</tr>
<tr>
<td>13</td>
<td>Light brown solid</td>
<td>175–176</td>
<td>72</td>
<td>C_{45}H_{55}N_{2}O_{10} (853.97)</td>
<td>63.29</td>
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</tbody>
</table>
mol L\(^{-1}\) H\(_2\)SO\(_4\) 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\(_2\)O (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 CHCl\(_3\) (30 mL) and added to the filtrate. The filtrate was washed with 5% NaHCO\(_3\) and saturated NaCl solution (25 mL each). The organic layer was dried over anhydrous Na\(_2\)SO\(_4\), 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\(_2\)O (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\(_2\)SO\(_4\). The aqueous layer was extracted with Et\(_2\)O (3 × 25 mL). Combined organic extracts were dried over anhydrous Na\(_2\)SO\(_4\) 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

<table>
<thead>
<tr>
<th>Compd. No.</th>
<th>IR (CHCl₃, cm⁻¹), ¹H NMR (CDCl₃, δ ppm), FAB MS (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>3298–2485 (m/br, OH str, COOH), 3089, 3034 (w, CH str, ring), 2928 (m, CH str, asym, aliph. CH₂), 2897 (m, CH str, &gt;CH–), 1715 (s, C=O str, COOH), 1582, 1482 (m, skeletal bands, ring), 1542 (m, NH bend, amide), 1387, 1365 (m, CH bend, ³butyl), 1393 (w, CH₃ rocking, ³butyl), 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, ³butyl)</td>
</tr>
<tr>
<td>2</td>
<td>3341–2528 (m/br, OH str, Thr and OH str, COOH), 2959 (m, CH str, asym, aliph. CH₃), 2898, 2892 (m, CH str, &gt;CH–), 2873 (m, CH str, sym, aliph. CH₃), 1712 (s, C=O str, COOH), 1544 (m, NH bend, amide), 1389, 1365 (m, CH bend, ³butyl), 1225 (s, C–O str, phenolic), 930 (w, CH₃ rocking, ³butyl), 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, ³butyl), 1.40–1.38 (d, 3H, β-CH₃, Thr, J = 5.9 Hz)</td>
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<td>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. CH₂), 2855 (m, CH str, sym, aliph. CH₂), 2898 (m, CH str, &gt;CH–), 1709 (s, C=O str, COOH), 1587, 1484 (m, skeletal bands, ring), 1543 (m, NH bend, amide), 1387, 1366 (m, CH bend, ³butyl), 1225 (s, C–O str, phenolic), 930 (w, CH₃ rocking, ³butyl), 822 (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, ³butyl)</td>
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<td>4</td>
<td>3050–2840 (s/br, &gt;NH₃⁺ str, asym and sym), 2995, 2987 (m, CH str, cyclic CH₂ and CH, 2822 (m, CH str, OCH₃), 1745 (s, C=O str, ester), 1465 (m, CH bend, OCH₃), 1440 (s/br, &gt;NH₂⁺ 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, OCH₃), 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, NH₂⁺)</td>
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<td>5</td>
<td>3010–2855 (s/br, NH₄⁺ str, asym and sym), 3076, 3030 (w, CH str, ring), 2926 (m, CH str, asym, aliph. CH₂), 2894 (m, CH str, &gt;CH–), 2828 (m, CH str, OCH₃), 1742 (s, C=O str, ester), 1605, 1503 (s/br, NH₂⁺ 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, NH₃⁺), 4.15–4.11 (m, 1H, α-H, Phe), 4.09 (s, 3H, OCH₃), 2.31–2.29 (d, 2H, β-H’s, Phe, J = 4.45 Hz)</td>
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<td>6</td>
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</tr>
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<td>Compd. No.</td>
<td>IR (CHCl₃, cm⁻¹), ¹H NMR (CDCl₃, δ ppm)</td>
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<td>--------------------------------------</td>
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</tr>
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</tr>
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Table II. contd.

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<th>Compd. No</th>
<th>IR (CHCl₃, cm⁻¹), ¹H NMR (CDCl₃, δ ppm)</th>
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</tr>
<tr>
<td>11a</td>
<td>3495, 3398 (w, free NH str, NH₂, 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 CH₂ and CH), 2962, 2925 (m, CH str, asym, aliph. CH₃ and CH₂), 2817 (m, CH str, OCH₃), 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, NH₂, Thr) 9.91 (br. s, 1H, NH), 8.49 (br. s, 1H, NH), 7.52–7.48 (m, 2H, m-H’s, Phe₁), 7.13–7.08 (t, 1H, p-H, Phe₂), 7.02–6.83 (m, 11H, o-, m- and p-H’s, Phe₂, Tyr, Phe₁), 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, Thr¹ and Thr²), 5.26–5.20 (m, 1H, α-H, Tyr), 4.92–4.88 (t, 1H, α-H, Thr¹), 4.62–4.56 (m, 1H, α-H, Phe₁), 4.47–4.45 (t, 1H, δ-H, Pro¹), 4.21–4.17 (t, 1H, α-H, Thr²), 4.11–4.08 (t, 1H, δ-H, Pro²), 3.96–3.67 (m, 5H, α-H, Phe₂, β-H’s, Thr¹ and Thr², α-H’s, Pro¹), 3.55 (s, 3H, OCH₃), 3.30–3.24 (t, 2H, α-H’s, Pro²), 3.14–2.71 (m, 6H, β-H’s, Phe₁, Tyr and Phe₂), 2.70–2.64 (tt, 4H, triplet overlapped over triplet, γ-H’s, Pro¹ and Pro²), 1.97–1.88 (m, 4H, β-H’s, Pro¹ and Pro²), 1.54 (s, 9H, tert-butyl), 1.30 (d, 3H, CH₃, Thr¹, J = 6.0 Hz), 1.25 (d, 3H, β-CH₃, Thr², J = 6.0 Hz)</td>
</tr>
<tr>
<td>12</td>
<td>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 CH₂ and CH), 2960–2922 (m, CH str, asym, aliph. CH₃ and CH₂), 2870–2848 (m, –CH str, sym, aliph. CH₃ and CH₂), 2898, 2891 (m, –CH str, &gt;CH–), 2814 (m, CH str, OCH₃), 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. CH₂), 1387, 1370 (m, CH bend, tert-butyl), 1224, 1207 (s, C–O str, phenolic and ester), 1125–1080 (m, CH bend, in-plane, rings), 930 (w, CH₃ rock, tert-butyl), 823 (s, CH bend, oop, 1,4-disub. ring) 726, 689 (s, CH bend, oop, monosub. ring), 667 (m/br, OH bend, oop, Thr) 9.91 (br. s, 1H, NH), 8.49 (br. s, 1H, NH), 7.52–7.48 (m, 2H, m-H’s, Phe₁), 7.13–7.08 (t, 1H, p-H, Phe₂), 7.02–6.83 (m, 11H, o-, m- and p-H’s, Phe₂, Tyr, Phe₁), 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, Thr¹ and Thr²), 5.26–5.20 (m, 1H, α-H, Tyr), 4.92–4.88 (t, 1H, α-H, Thr¹), 4.62–4.56 (m, 1H, α-H, Phe₁), 4.47–4.45 (t, 1H, δ-H, Pro¹), 4.21–4.17 (t, 1H, α-H, Thr²), 4.11–4.08 (t, 1H, δ-H, Pro²), 3.96–3.67 (m, 5H, α-H, Phe₂, β-H’s, Thr¹ and Thr², α-H’s, Pro¹), 3.55 (s, 3H, OCH₃), 3.30–3.24 (t, 2H, α-H’s, Pro²), 3.14–2.71 (m, 6H, β-H’s, Phe₁, Tyr and Phe₂), 2.70–2.64 (tt, 4H, triplet overlapped over triplet, γ-H’s, Pro¹ and Pro²), 1.97–1.88 (m, 4H, β-H’s, Pro¹ and Pro²), 1.54 (s, 9H, tert-butyl), 1.30 (d, 3H, CH₃, Thr¹, J = 6.0 Hz), 1.25 (d, 3H, β-CH₃, Thr², J = 6.0 Hz)</td>
</tr>
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</table>
Table II. contd.

<table>
<thead>
<tr>
<th>Compd.</th>
<th>IR (CHCl₃, cm⁻¹), ¹H NMR (CDCl₃, δ ppm), ¹³C NMR (CDCl₃, δ ppm)</th>
</tr>
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<tr>
<td>12b</td>
<td>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, asym, aliph. CH₃ and CH₂), 2870–2849 (m, –CH str, sym, aliph. CH₃ and CH₂), 2894 (m, –CH str, &gt;CH–), 1638–1632 (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. NO₂ grp), 1392, 1365 (m, CH bend, 3-butyl), 1349 (s, sym NO stretch, arom. NO₂ grp), 1224, 1215 (s, C–O str, phenolic and arom. ester), 929 (w, CH₃ rock, 3-butyl), 822 (s, CH bend, oop, 1,4-disub. ring), 731–688 (s, CH bend, oop, monosub. rings)</td>
</tr>
<tr>
<td>12c</td>
<td>3497, 3392 (w, free NH stretch, asymm and symm, NH₂ 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 CH₂ and CH), 2962–2928 (m, CH str, asym, aliph. CH₃ and CH₂), 2872–2846 (m, CH str, sym, aliph. CH₃ and CH₂), 1638–1630 (s, –C=O str, 3° and 2° amide), 1590, 1584, 1580 (m, skeletal bands, arom. rings), 1540–1532 (m, NH bend, 2° amide), 1518 (s, asym NO stretch, arom. NO₂), 1350 (s, sym NO stretch, arom. NO₂ grp), 1225, 1215 (s, C–O str, phenolic and arom. ester), 1057 (m, C–N stretch, NH₂), 819 (s, CH bend, oop, 1,4-disub. ring), 730–689 (s, CH bend, oop, monosub. rings)</td>
</tr>
<tr>
<td>13a</td>
<td>3400, 3335 (m/br, OH str, phenolic and Thr), 3207–3180 (s, NH str, amide), 3055, 3032 (w, CH str, arom. rings), 3010–2994 (m, CH str, cyclic CH₂ and CH), 2925–2924 (m, CH str, asym, aliph. CH₃ and CH₂), 2853–2847 (m, CH str, sym, aliph. CH₂), 1682–1633 (s, C=O str, 3° and 2° amide), 1593–1554 (m, skeletal bands, rings), 1535, 1512 (m, CH bend, 2° amide), 1449 (m, CH bend, CH₂ scissoring), 1125–1025 (m, C–N stretch, NH₂), 825 (s, CH bend, oop, 1,4-disub. ring), 727–674 (s, CH bend, oop, monosub. rings)</td>
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854.9 (M + 1)+, 826.8 (854–CO)+, 707.7 (Phe-Pro-Thr-Thr-Pro-Thr)+, 679.7 (707–CO)+, 544.5 (Phe-Pro-Thr-3-butyl)+, 447.5 (Phe-Pro-Thr-Thr)+, 419.5 (447–CO)+, 346.6 (Phe-Pro-Thr)+, 318.4 (346–CO)+, 245.4 (Phe-Pro)+, 217.2 (245–CO)+, 148.2 (Phe)+, 120.1 (148–CO)+.

IR spectrum was taken using KBr.
hydrous Na$_2$SO$_4$ 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-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-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% HCl (3 × 15 mL). The organic layer was dried over anhydrous Na$_2$SO$_4$, filtered, and the filtrate was distilled off to get the corresponding p-nitrophenyl ester Boc-Phe-Pro-Thr-Pro-Tyr-Phe-O-pnp (12b).

To compound 12b (4.37 g, 0.004 mol) dissolved in CHCl$_3$ (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$_2$SO$_4$ to get Phe-Pro-Thr-Pro-Tyr-Phe-O-pnp (12c), which was dissolved in CHCl$_3$ (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% HCl (3 × 15 mL). The organic layer was dried over anhydrous Na$_2$SO$_4$. 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 $6 \times 10^4$ spores mL$^{-1}$). Filter paper disks of 6 mm diameter and 1 mm thickness were sterilized by autoclaving at 121 °C (1.05 $\times 10^5$ 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.
An anthelmintic activity study was performed 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, respectively.

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 earthworms 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 409.

Scheme 1

\[
\begin{align*}
\text{1} & \quad \text{2} & \quad \text{3} & \quad \text{4} & \quad \text{5} \\
\text{7} & \quad \text{6} & \quad \text{8} & \quad \text{9} & \quad \text{9a} \\
\text{10} & \quad \text{11} & \quad \text{12} & \quad \text{13}
\end{align*}
\]

\( \text{a} = \text{DCC, NMM, CHCl}_3, \text{r.t., 36 h}; \quad \text{b} = \text{LiOH, THF/H}_2\text{O (1:1), r.t., 1 h} \)
\( \text{c} = \text{TFA, CHCl}_3, \text{r.t., 1 h}; \quad \text{d} = \text{pnp, CHCl}_3, \text{r.t., 12 h} \)
\( \text{e} = \text{NMM, CHCl}_3, \text{7 days, 0 }^\circ\text{C} \)
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). Boc₂O 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 SOCl₂. 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, ¹H NMR, ¹³C NMR and FAB MS spectral data (Table II). All characterization data of synthesized 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 ¹H 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 OCH₃ moiety in ¹H 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

\[
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Compd.} & \textbf{Bacteria} & \textbf{Fungi} \\
\hline
 & \textit{Bacillus subtilis} & \textit{Escherichia coli} & \textit{Staphylococcus aureus} & \textit{Pseudomonas aeruginosa} \\
\hline
DMF & – & – & – & – \\
Ciprofloxacin & 18 & 20 & 22 & 18 \\
\textbf{13} & – & – & 13 & 12 \\
\hline
\textbf{Compd.} & \textit{Candida albicans} & \textit{Ganoderma species} & \textit{Microsporum audouinii} & \textit{Trichophyton mentagrophytes} \\
\hline
DMF & – & – & – & – \\
Griseofulvin & 16 & 12 & 15 & 14 \\
\textbf{13} & 22 & 19 & – & – \\
\hline
\end{tabular}
\]

\[\text{a} \gamma = 10 \text{ mg mL}^{-1} (50 \mu\text{g per test}).\]
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, tbutyl) and a weak band at 929 cm⁻¹ (CH₃ rocking of tbutyl), along with the presence of a medium absorption band at 1057 cm⁻¹ (C–N stretch, NH₂ group) and weak bands at 3497 cm⁻¹ and 3392 cm⁻¹ corresponding to asymmetric and symmetric free NH stretching of NH₂ group in the IR spectra. The structure of compound 13 was confirmed by disappearance 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 C₄₅H₅₅N₇O₁₀ in mass spectra, along with other fragment ion peaks indicating the exact sequence of attachment of seven amino acid moieties in a chain. ¹H NMR and ¹³C 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.
antibacterial and antifungal screening. Also, great thanks are due to C.P.C.R.I., Kasaragod, Kerala (India), for providing earthworms for anthelmintic activity testing.

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Prva potpuna sinteza i biološko vrednovanje himenamida E

RAJIV Dahiya, DEVENDER PATHAK, MALIPEDI HIMAJA I SUNIKA BHATT

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 1H NMR spektroskopijom i elementarnom analizom, a struktura spoja 13 i 13C 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 piperazincitrat.

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

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