International Journal of Anatomy and Physiology ISSN: 2326-7275 Vol. 6 (11), pp. 001-007, November, 2017. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Synthesis, characterization and biological evaluation of cyclomontanin D

Rajiv Dahiya[1]* and Hemendra Gautam[2]

[1]Department of Pharmaceutical Chemistry, Globus College of Pharmacy, Bhojpur Road, Bhopal 462 045, Madhya Pradesh, India.

[2]Department of Pharmaceutical Chemistry, NRI Institute of Pharmacy, Raisen Road, Bhopal 462 021, Madhya Pradesh, India.

Accepted 25 March, 2017

In present study, it was of interest to synthesize a natural proline-rich cyclooligopeptide - cyclomontanin D 8 by coupling of tripeptide Boc-L-Asn(bzh)-L-Pro-Gly-OH and tetrapeptide L-Leu-L-Pro-L-Tyr-L-Ala-OMe followed by cyclization of linear polypeptide fragment. Structure of synthesized cyclopeptide was confirmed by detailed spectral analysis including FTIR, ^{1}H NMR, ^{13}C NMR, ESIMS/MS and elemental analysis. From the results of biological evaluation, it was concluded that newly synthesized cyclooligopeptide possessed good bioactivity against Gram-negative bacteria *Klebsiella pneumonia*, *Pseudomonas aeruginosa* as well as potent antidermatophyte activity against *Microsporum audouinii* and *Trichophyton mentagrophytes*, in comparison to reference drugs - ciprofloxacin and griseofulvin. Moreover, 8 exhibited moderate antifungal activity against pathogenic *Candida albicans* with minimum inhibitory concentration (MIC) value of 6 μg/ml.

Key words: *Annona montana*, cyclomontanin D, cyclic heptapeptide, peptide synthesis, antibacterial activity, antidermatophyte activity.

INTRODUCTION

Natural peptides especially cyclooligopeptides have attracted much attention in recent years owing to their intriguing chemical structures and wide biological profile. They have the property of greater resistance to *in vivo* enzymatic degradation and have greater bioavailability than non-cyclic congeners. Cyclopeptides especially those isolated from seeds, roots and latex of higher plants, are widely distributed in nature and are well known to exhibit broad spectrum of pharmacological activities such as immunosuppressive activity (Morita et al., 1999; Matsumoto et al., 2001), anticancer activity (Takeya et al., 1993; Wele et al., 2005; Mongkolvisut et al., 2006), antimalarial activity (Baraguey et al., 2000; Picur et al., 2006), vasorelaxant activity (Morita et al., 2006), estrogen-like activity (Itokawa et al., 1995) and tyrosinase, cyclooxygenase, plasmin and classic pathway activity of human complement inhibitory activity (Morita et al., 1994; Shin et al., 1996; Van Den Berg et al., 1995; Morita et al., 1997).

A natural cycloheptapeptide, cyclomontanin D has been isolated from the seeds of annonaceous plant *Annona montana* Macf. (mountain soursop) and its structure was elucidated on basis of ESIMS/MS fragment evidence, 2D NMR analysis and chemical means (Chuang et al., 2008).

Keeping in view the wide range of pharmacological activities exhibited by natural plant-originated cyclopeptides (Tan and Zhou, 2006; Morita and Takeya, 2010) and in continuation of our previous investigations on peptides (Dahiya and Pathak, 2006; Dahiya and Gautam, 2011), present investigation was directed toward the synthesis of a natural cyclic heptapeptide 8 employing solution-phase technique. Furthermore, synthesized product was also subjected to antibacterial and antifungal activity studies.

*Corresponding author. E-mail: drrajivdahiya@rediffmail.com. Tel: +91 9630229885, +91 755 4285308.

MATERIALS AND METHODS

General experimental part

Melting point of the synthesized product was determined in open glass capillary on melting point apparatus and was uncorrected. Laboratory chemicals were supplied by SpectroChem Ltd. The purity of the compounds was checked by thin layer chromatography (TLC) on precoated silica gel G plates utilizing $CHCl_3$/MeOH as developing solvent. IR spectra were recorded on Shimadzu 8700 FTIR spectrophotometer using KBr pellets for cyclopeptide and $CHCl_3$ as solvent for intermediate semisolids. ^1H NMR and ^{13}C NMR spectra were recorded on Bruker AC NMR spectrometer (300 MHz / 100 MHz). Chemical shifts are expressed in δ (ppm) relative to TMS as an internal standard using C_5D_5N as solvent. Mass spectra was recorded on JMS-DX 303 Mass spectrometer operating at 70 eV using ESIMS/MS technique. Elemental analyses of newly synthesized compounds were performed on Vario EL III elemental analyzer.

General procedure for synthesis of linear peptide segments (5-7)

Amino acid methyl ester hydrochloride/peptide methyl ester (0.01 mol) was dissolved in tetrahydrofuran (THF, 25 ml) . To this, pyridine (1.61 ml, 0.021 mol) was added at 0°C and the reaction mixture was stirred (25 min). Boc-amino acid/peptide (0.01 mol) dissolved in THF (25 ml) and DCC/DIPC (2.1 g/1.26 g, 0.01 mol) were added to above reaction mixture with stirring. After 24 h, the reaction mixture was filtered and the residue was washed with THF (30 ml) and added to the filtrate. The filtrate was washed with 5% sodium bicarbonate and saturated sodium chloride solutions. 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. 60 to 80°C) followed by cooling at 0°C.

^{t}Butyloxycarbonyl-L-asparaginyl(bzh)-L-prolyl-glycine methyl ester (5)

Semisolid mass; yield, 88%; R_f 0.61; $[D]$ –55.7 (*c* 0.25 in MeOH); IR ($CHCl_3$) *v* cm^{-1}: 3135, 3122 (m, N–H str, 2° amide), 3075, 3049-3044 (w, C–H str, rings), 2999- 2992 (m, C–H str, Pro), 2877 (m, C–H str, sym, CH_3), 2929-2925, 2854 (m, C–H str, asym and sym, CH_2), 1754 (s, C=O str, ester), 1659, 1644, 1635 (s, C=O str, 3 and 2° amide), 1577, 1574, 1475, 1472 (m, skeletal bands, rings), 1538, 1522 (m, N–H bend, 2° amide), 1392, 1374 (s, C–H bend, butyl-*t*), 1268 (s, C−O str, ester), 726, 721, 689, 684 (s, C–H bend, oop, rings); ^1H NMR ($CDCl_3$) δ (ppm): 8.62 (br. s, 1H, NH, Gly), 8.39 (br. s, 1H, NH, Asn), 7.24-7.15 (m, 6H, H- *m* and H-*p*, rings, bzh), 7.10-7.04 (m, 4H, H-*o*, rings, bzh), 6.89 (d, 1H, *J* = 5.6 Hz, CONH, Asn), 5.94 (d, 1H, *J* = 5.4 Hz, H-α, bzh), 5.94 (t, 1H, *J* = 6.9 Hz, H-α, Pro), 4.34 (q, 1H, H-α, Asn), 4.02 (d, 2H, *J* = 8.2 Hz, H-α, Gly), 3.67 (t, 2H, *J* = 7.2 Hz, H-δ, Pro), 3.59 (s, 3H, OCH_3), 3.07 (d, 2H, *J* = 3.8 Hz, H-β , Asn), 2.65 (q, 2H, H-β, Pro), 1.96-1.89 (m, 2H, H-γ, Pro), 1.57 (s, 9H, butyl-*t*); *Anal*. Calc. for $C_{30}H_{38}N_4O_7$ (566): C, 63.59; H, 6.76; N, 9.89. Found: C, 63.56; H, 6.74; N, 9.90%.

^{t}Butyloxycarbonyl-L-leucyl-L-prolyl-L-tyrosinyl-L-alanine methyl ester (6)

Semisolid mass; yield, 84%; R_f 0.87; $[D]$ –103.5 (*c* 0.25 in MeOH); IR ($CHCl_3$) *v* cm^{-1}: 3369 (m, O–H str, Tyr), 3139, 3133 (m, N–H str, 2° amide), 3064 (w, C–H str, ring), 2998-2989 (m, C–H str, Pro), 2956, 2870 (m, C–H str, asym and sym, CH_3), 2922, 2856 (m, C–H str, asym and sym, CH_2), 1753 (s, C=O str, ester), 1658, 1642-1635 (s, C=O str, 3° and 2° amide), 1572, 1479 (m, skeletal bands, ring), 1536-1525 (m, N–H bend, 2° amide), 1395, 1373 (s, C–H bend, butyl- *t*), 1383, 1362 (s, C–H def, propyl- *i*), 1272 (s, C–O str, ester), 1234 (s, C–O str, phenolic), 933, 921 (w, CH_3 rock, butyl-*t* and propyl-*i*), 829 (s, C–H bend, oop, ring); ^1H NMR ($CDCl_3$) δ (ppm): 7.04-6.96 (m, 2H, H-*m*, Tyr), 6.94 (br. s, 1H, NH, Ala), 6.92 (dd, 2H, *J* = 7.3 Hz, H- *o*, Tyr), 6.54 (br. s, 1H, NH, Tyr), 5.97 (br. s, 1H, OH, Tyr), 5.89 (br. s, 1H, NH, Leu), 4.97 (q, 1H, H-α, Tyr), 4.47 (t, 1H, *J* = 6.8 Hz, H-α, Pro), 4.21 (q, 1H, H-α, Leu), 3.82-3.75 (m, 1H, H-α, Ala), 3.69 (t, 2H, *J* = 7.3 Hz, H-δ, Pro), 3.60 (s, 3H, OCH_3), 2.89 (d, 2H, *J* = 4.0 Hz, H-β, Tyr), 2.67 (q, 2H, H-β, Pro), 1.98-1.86 (m, 4H, H-γ, Pro and H-β, Leu), 1.60-1.55 (m, 1H, H-γ, Leu), 1.52 (s, 9H, butyl-*t*), 1.28 (d, 3H, *J* = 7.8 Hz, H-β, Ala), 1.18 (d, 6H, *J* = 6.2 Hz, H-δ, Leu); *Anal*. Calc. for $C_{29}H_{44}N_4O_8$ (576): C, 60.40; H, 7.69; N, 9.72. Found: C, 60.38; H, 7.72; N, 9.70%.

^{t}Butyloxycarbonyl-L-asparaginyl(bzh)-L-prolyl-glycyl-L-leucyl-L-prolyl-L-tyrosinyl-L-alanine methyl ester (7)

Semisolid mass; yield, 77%; R_f 0.79; $[D]$ –19.8 (*c* 0.35 in MeOH); IR ($CHCl_3$) *v* cm^{-1}: 3369 (m, O–H str, Tyr), 3138- 3132 (m, N–H str, 2° amide), 3071-3064, 3047 (w, C–H str, rings), 2999-2989 (m, C–H str, Pro), 2959, 2877- 2873 (m, C–H str, asym and sym, CH_3), 2927-2924, 2858-2852 (m, C–H str, asym and sym, CH_2), 1756 (s, C=O str, ester), 1662-1659, 1646-1635 (s, C=O str, 3 and 2° amide), 1578-1573, 1476- 1471 (m, skeletal bands, rings), 1539, 1533, 1529 (m, N–H bend, 2° amide), 1390, 1376 (s, C–H bend, butyl- *t*), 1381, 1366 (s, C–H def, propyl-*i*), 1271 (s, C−O str, ester), 1236 (s, C–O str, phenolic), 932, 925 (w, CH_3 rock, butyl- *t* and propyl-*i*), 827, 725-721, 688-683 (s, C–H bend, oop, rings). ^1H NMR (pyridine-d_6) δ (ppm): 10.24 (br. s, 1H, NH, Gly), 8.39 (br. s, 1H, NH, Tyr), 8.34 (br. s, 1H, NH, Asn), 8.09 (br. s, 1H, NH, Leu), 7.28-7.17 (m, 6H, H-*m* and H-*p*, rings, bzh), 7.12- 7.05 (m, 4H, H-*o*, rings, bzh), 7.02-6.96 (m, 2H, H-*m*, Tyr), 6.92 (d, 1H, *J* = 5.7 Hz, CONH, Asn), 6.88 (br. s, 1H, NH, Ala), 6.83 (dd, 2H, *J* = 7.3 Hz, H-*o*, Tyr), 5.99 (d, 1H, *J* = 5.3 Hz, H-α, bzh), 5.94 (br. s, 1H, OH, Tyr), 5.17 (q, 1H, H-α, Leu), 5.05 (q, 1H, H- α, Tyr), 4.47 (t, 1H, *J* = 6.8 Hz, H-α, Pro- 1), 4.30 (q, 1H, H-α, Asn), 4.14 (t, 1H, *J* = 6.9 Hz, H-α, Pro-2), 3.98 (d, 2H, *J* = 8.2 Hz, H-α, Gly), 3.80-3.73 (m, 1H, H-α, Ala), 3.73 (t, 2H, *J* = 7.3 Hz, H-δ, Pro-1), 3.61 (s, 3H, OCH_3), 3.39 (t, 2H, *J* = 7.2 Hz, H-δ, Pro-2), 2.96 (d, 2H, *J* = 4.2 Hz, H-β, Tyr), 2.80 (d, 2H, *J* = 4.0 Hz, H-β, Asn), 3.54-3.47 (m, 4H, H-δ, Pro-1 and Pro-2), 2.92-2.85 (m, 2H, H-β, Leu), 2.68-2.59 (m, 1H, H-γ, Leu), 2.13-2.03 (m, 4H, H- β, Pro-1 and Pro- 2), 1.56 (d, 3H, *J* = 7.8 Hz, H-β, Ala), 1.51 (s, 9H, butyl-*t*), 1.13 (d, 6H, *J* = 6.1 Hz, H- δ, leu); *Anal*. Calc. for C_{53} $H_{70}N_8O_{12}$ (1011): C, 62.95; H, 6.98; N, 11.08. Found: C, 62.92; H, 6.99; N, 11.10%.

Procedure for cyclization of linear heptapeptide fragment

To synthesize 8, linear heptapeptide unit 7 (5.06 g, 0.005 mol) was deprotected at carboxyl end using lithium hydroxide (0.18 g, 0.0075 mol) to get Boc- L-Asn(bzh)-L-Pro-Gly-L-Leu-L-Pro-L-Tyr-L-Ala-OH. The deprotected linear peptide unit (4.98 g, 0.005 mmol) was now dissolved in chloroform (50 ml) at 0°C. To the above solution, pentafluorophenol (1.23 g, 0.0067 mol) was added and stirred at room temperature (12 h). The reaction mixture was filtered and the

Table 1. Antibacterial profile of newly synthesized linear/cyclic heptapeptide.

Compounds	Zone of inhibition† (MIC in μg/ml)			
	Tested bacteria			
	Gram-negative		Gram-positive	
	K. pneumoniae	*P. aeruginosa*	*B. subtilis*	*S. aureus*
7	17 (12.5)	24 (6)	–	9 (12.5)
8	22 (12.5)	28 (6)	9 (6)	10 (12.5)
Control‡	–	–	–	–
Ciprofloxacin	19 (12.5)	25 (6)	20 (6)	20 (12.5)

† in mm; ‡ DMF.

filtrate was washed with 10% sodium bicarbonate solution (3 x 25 ml) until excess of pentafluorophenol was removed and finally washed with 5% HCl (2 x 20 ml) to get the corresponding pentafluorophenyl ester Boc-L-Asn(bzh)-L-Pro-Gly-L- Leu-L-Pro- L-Tyr-L-Ala-Opfp. To this compound (4.65 g, 0.004 mol) dissolved in chloroform (35 ml), TFA (0.91 g, 0.008 mol) was added, stirred at room temperature (1 h) and washed with 10% sodium bicarbonate solution (2 x 25 ml). The organic layer was dried over anhydrous Na_2SO_4 to get L-Asn(bzh)-L-Pro-Gly-L-Leu-L-Pro-L-Tyr-L-Ala-Opfp which was dissolved in chloroform (25 ml) and TEA/NMM (2.21 ml/2.8 ml, 0.021 mol) was added. Then, whole contents were kept at 0°C (7 days) . The reaction mixture was washed with 10% sodium carbonate and 5% HCl (4 x 25 ml) solutions. The organic layer was dried over anhydrous Na_2SO_4. Finally, chloroform was distilled off and crude product was crystallized from chloroform/*n*-hexane to get pure cyclic product 8.

Cyclo (-asparaginyl-prolyl-glycyl-leucyl-prolyl-tyrosinyl-alanyl-) (8)

White solid; M.p. 202°C (d); yield, (89%, NMM), (68%, TEA); R_f 0.66; $[D]$ –41.3 (–41.2 for natural peptide, *c* 0.001 in MeOH); Yield IR (KBr) *v* cm^{-1}: 3372 (m, O–H str, Tyr), 3349, 3175 (m, N–H str, asym and sym, 1° amide), 3138-3132, 3128-3125 (m, N–H str, 2° amide), 3069 (w, C–H str, ring), 2997-2989 (m, C–H str, Pro), 2959-2954, 2872 (m, C–H str, asym and sym, CH_3), 2928, 2922, 2858 (m, C–H str, asym and sym, CH_2), 1660, 1655 (s, C=O str, 3° and 1° amide), 1647- 1639, 1636, 1632 (s, C=O str, 2° amide), 1623 (m, -NH_2 bend, 1° amide), 1575, 1478 (m, skeletal bands, ring), 1539-1528, 1523 (m, N–H bend, 2° amide), 1468 (m, C–H bend(scissoring), CH_2), 1409 (m, C–N str, 1° amide), 1381, 1364 (s, C–H def, propyl-*i*), 1231 (s, C–O str, phenolic), 923 (w, CH_3 rocking, propyl- *i*), 827 (s, C–H bend, oop, ring); ^{1}H NMR (pyridine-d_6) δ (ppm): 9.34 (br. s, 1H, NH, Gly), 9.27 (br. s, 1H, NH, Tyr), 8.91 (br. s, 1H, NH, Ala), 8.63 (br. s, 1H, NH, Asn), 8.05 (br. s, 1H, NH, Leu), 7.99-7.92 (m, 2H, H-*m*, Tyr), 7.14 (dd, 2H, *J* = 7.4 Hz, H-*o*, Tyr), 6.73 (s, 2H, CONH_2, Asn), 5.94 (br. s, 1H, OH, Tyr), 5.41 (q, 1H, H-α, Tyr), 5.29 (q, 1H, H-α, Leu), 4.94 (q, 1H, H-α, Asn), 4.85-4.79 (m, 1H, H-α, Ala), 4.04 (d, 2H, *J* = 8.3 Hz, H-α, Gly), 4.53-4.45 (m, 2H, H-α, Pro-1 and Pro-2), 3.56-3.47 (m, 4H, H-δ, Pro- 1 and Pro-2), 3.36 (d, 2H, *J* = 4.1 Hz, H-β, Tyr), 3.07 (d, 2H, *J* = 3.9 Hz, H-β, Asn), 2.97-2.92 (m, 2H, H-β, Leu), 2.55-2.48 (m, 1H, H-γ, Leu), 2.03-1.96 (m, 4H, H-β, Pro-1 and Pro-2), 1.85 (d, 3H, *J* = 7.9 Hz, H-β, Ala), 1.69-1.57 (m, 4H, H-γ, Pro-1 and Pro-2), 1.18 (d, 6H, *J* = 6.1 Hz, H-δ, leu); ^{13}C NMR (pyridine-d_6) δ (ppm): 175.9 (C=O, C-γ, Asn), 173.6 (C=O, Asn), 173.1 (C=O, Tyr), 172.7, 171.9 (2C, C=O, Pro-1 and Pro-2), 171.7 (C=O, Ala), 171.5 (C=O, Leu), 171.1 (C=O, Gly), 157.6 (C-*p*, Tyr), 131.2 (2C, C-*o*, Tyr), 130.5 (C-γ, Tyr), 116.0 (2C, C-*m*, Tyr), 65.2, 63.2 (2C, C-α, Pro-1 and Pro-2), 59.3 (C-α, Tyr), 56.8, 54.4, 52.2 (3C, C-α, Asn, Leu and Ala), 48.9, 48.0 (2C, C-δ, Pro-1 and Pro-2), 43.9 (C-α, Gly), 39.7, 36.8 (2C, C-β, Tyr and Asn), 33.5 (C-γ, Leu), 31.6 (C-β, Leu), 30.6, 29.9 (2C, C-β, Pro-2 and Pro-1), 26.8, 25.1 (2C, C-γ, Pro-1 and Pro-2), 19.8 (2C, C-δ, Leu), 17.7 (C-β, Ala); ESIMS/MS (*m/z*, rel. int.): 713 [$(M + H)^+$, 100], 642 [$(Asn\text{-}Pro\text{-}Gly\text{-}Leu\text{-}Pro\text{-}Tyr)^+$, 29], 614 [$(642–CO)^+$, 16], 685 [$(713–CO)^+$, 13], 599 [$(Pro\text{-}Gly\text{-}Leu\text{-}Pro\text{-}Tyr\text{-}Ala)^+$, 21], 571 [$(599–CO)^+$, 10], 528 [$(Pro\text{-}Gly\text{-}Leu\text{-}Pro\text{-}Tyr)^+$, 39], 500 [$(528–CO)^+$, 17], 446 [$(Pro\text{-}Tyr\text{-}Ala\text{-}Asn)^+$, 55], 365 [$(Pro\text{-}Gly\text{-}Leu\text{-}Pro)^+$, 51], 332 [$(Pro\text{-}Tyr\text{-}Ala)^+$, 18], 304 [$(332–CO)^+$, 11], 283 [$(Ala\text{-}Asn\text{-}Pro)^+$, 34], 269 [$(Asn\text{-}Pro\text{-}Gly)^+$, 18], 268 [$(Pro\text{-}Gly\text{-}Leu)^+$, 72], 240 [$(268–CO)^+$, 15], 212 [$(Asn\text{-}Pro)^+$, 10], 186 [$(Ala\text{-}Asn)^+$, 25], 184 [$(212–CO)^+$, 21], 158 [$(186–CO)^+$, 9], 155 [$(Pro\text{-}Gly)^+$, 42], 136 [Tyr immonium ion $(C_8H_{10}NO)^+$, 13], 127 [$(155–CO)^+$, 14], 116 [$(Asn)^+$, 16], 107 [$(C_7H_7O)^+$, 10], 87 [Asn immonium ion $(C_3H_7N_2O)^+$, 15], 86 [Leu immonium ion $(C_5H_{12}N)^+$, 19], 70 [Pro immonium ion $(C_4H_8N)^+$, 23], 58 [$(C_2H_4NO)^+$, 9], 57 [$(C_4H_9)^+$, 11], 44 [Ala immonium ion $(C_2H_6N)^+$, 9], 43 [$(C_3H_7)^+$, 8], 30 [Gly immonium ion $(CH_4N)^+$, 11], 15 [$(CH_3)^+$, 10]; *Anal*. Calc. for $C_{34}H_{48}N_8O_9$ (712): C, 57.29; H, 6.79; N, 15.72. Found: C, 57.32; H, 6.80; N, 15.69%.

Antimicrobial activity

Antibacterial activity of synthesized cyclooligopeptide 8 were carried out against four bacterial strains *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, according to modified disc diffusion method at 12.5-6 g/ml (Bauer et al., 1966). Antifungal screening was performed against four fungal strains *Candida albicans*, *Microsporum audouinii*, *Trichophyton mentagrophytes*, *Aspergillus niger*) according to serial plate dilution method at 12.5 to 6 g/ml (Khan, 1997). Ciprofloxacin and griseofulvin were used as standard drugs against bacterial and fungal strains. Minimum inhibitory concentration (MIC) values of test compounds were determined by tube dilution technique using sterile DMF. The Petri plates inoculated with bacterial and fungal cultures were incubated at 37°C for 18 and 48 h, respectively. The average diameters of the zones of inhibition (in mm) of test compounds were calculated for triplicate sets and compared with that produced by the standard drugs (Tables 1 and 2). Experimental details of antibacterial and antifungal activity procedures are given in previously published reports by Dahiya and Pathak (2007).

Table 2. Antifungal profile of newly synthesized linear/cyclic heptapeptide

Compounds	Zone of inhibition† (MIC in μg/ml)			
	Tested fungi			
	Dermatophytes		Yeast	
	M. audouinii	*T. mentagrophytes*	*C. albicans*	*A. niger*
7	13 (6)	19 (6)	12 (6)	–
8	18 (6)	22 (6)	15 (6)	–
Control‡	–	–	–	–
Griseofulvin	17 (6)	20 (6)	20 (6)	18 (12.5)

†in mm; ‡ DMSO.

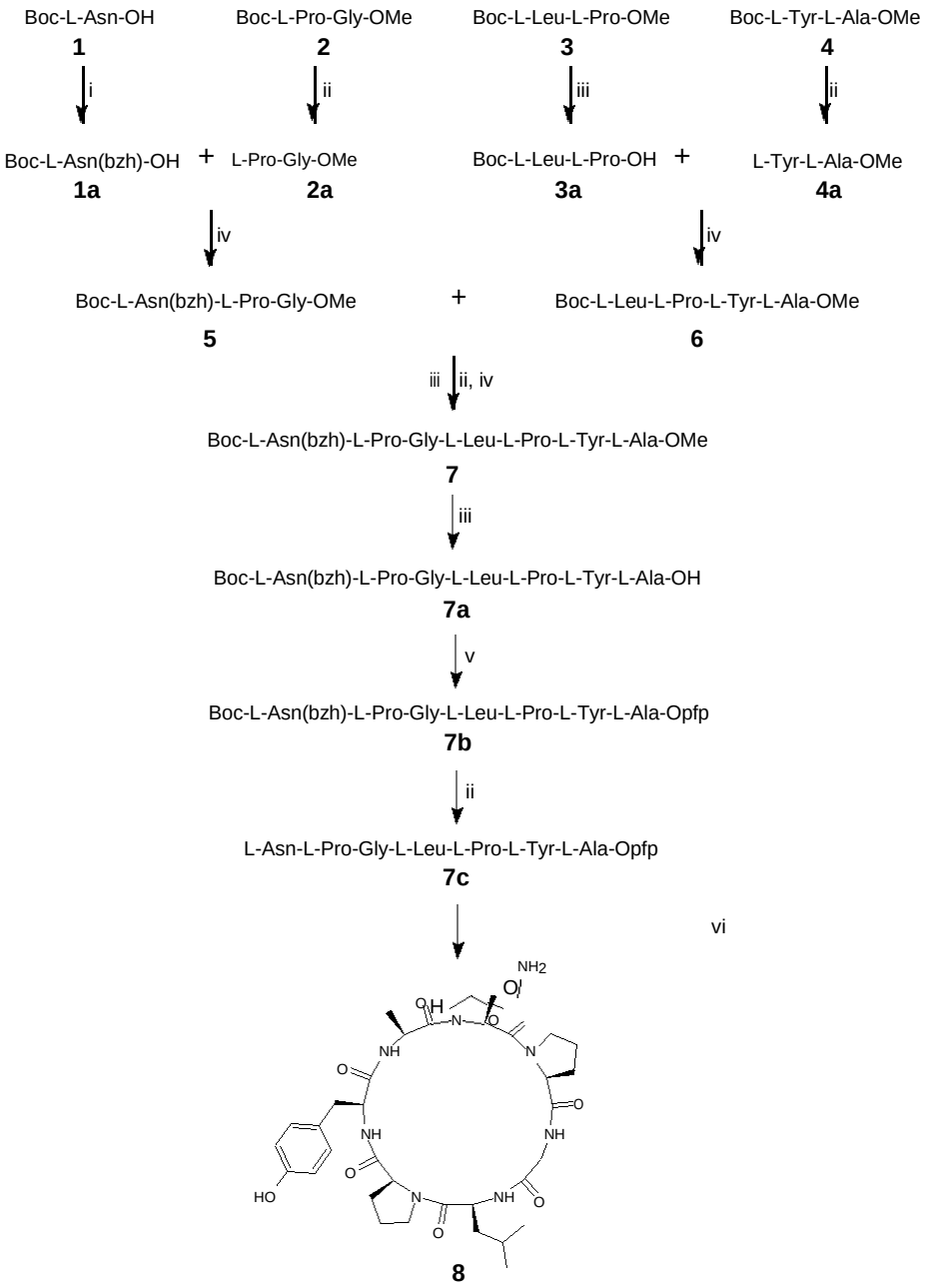
RESULTS AND DISCUSSION

Chemistry

In present investigation, disconnection strategy was utilized to carry out the first total synthesis of cyclomontanin D 8. The amino group of L-amino acids was protected by stirring with Boc_2O (di-*tert*-butylpyrocarbonate) and isopropanol. Carboxamide side chain of Boc-L-asparagine was protected using benzhydrol whereas Boc/benzhydrol groups were removed by treatment with trifluoroacetic acid (TFA). The carboxyl group of L-amino acids was protected by esterification with $MeOH/SOCl_2$ whereas methyl ester group was removed by alkaline hydrolysis with LiOH. All the above protections and deprotections were done according to the previously reported procedures (Dahiya and Kumar, 2007). All the coupling reactions were perfomed utilizing dicyclohexylcarbodiimide/diisopropylcarbodiimide (DCC/DIPC) as coupling agents and pyridine as base (Bodanzsky and Bodanzsky, 1984).

The cyclopeptide molecule was split into a single amino acid unit Boc-Asn-OH 1 and three dipeptide units Boc-L-Pro-Gly-OMe 2, Boc-L-Leu-L-Pro-OMe 3, Boc- L-Tyr-L-Ala-OMe 4. The carboxamide side chain of 1 was protected using benzhydrol to get Boc-L-Asn(bzh)-OH 1a. Dipeptide units 2-4 were prepared by coupling of Boc-amino acids viz. Boc-L-Pro-OH, Boc-L-Leu-OH and Boc-L-Tyr-OH with corresponding amino acid methyl ester hydrochlorides such as Gly-OMe.HCl, L-Pro-OMe.HCl and L-Ala-OMe.HCl employing DCC as coupling agent. Boc- groups of dipeptides 2 and 4 were removed using TFA to get deprotected units 2a and 4a, and dipeptide 3 was deprotected at carboxyl end by alkaline hydrolysis to obtain 3a. Deprotected dipeptide 2a was coupled with 1a to get the tripeptide unit Boc-L-Asn(bzh)-L-Pro- Gly-OMe 5 whereas deprotected dipeptde units 3a and 4a were coupled together to obtain tetrapeptide unit Boc-L-Leu-L-Pro-L-Tyr-L-Ala-OMe 6. Now, 5 was deprotected at carboxyl end and coupled with 6 deprotected at amino terminal using DCC/DIPC and pyridine as base, to get the linear heptapeptide unit Boc-L- Asn(bzh)-L-Pro-Gly-L-Leu-L-Pro-L-Tyr-L-Ala-OMe 7. The methyl ester group of linear peptide fragment was replaced by pentafluorophenyl (pfp) ester group. Boc and bzh groups of resulting unit were removed using TFA and deprotected linear fragment was now cyclized by keeping the whole contents at 0°C for 7 days in presence of catalytic amount of triethylamine (TEA) / *N*-methylmorpholine (NMM) to get final cyclized product 8 (Scheme 1). Structure of the newly synthesized cyclic heptapeptide as well as intermediates linear di/tri/tetra/heptapeptides were confirmed by spectral as well as elemental analysis.

Synthesis of cyclopeptide 8 was accomplished with 89% yield and NMM was proved to be a yield effective base for cyclization of linear heptapeptide fragment. Cyclization of linear peptide fragment was indicated by disappearance of absorption bands at 1756, 1271 and 1390, 1376 cm^{-1} (C=O str / C–O str of ester and C–H bend of *tert*-Butyl group). Deprotection of asparagine was confirmed by presence of Amide I and II bands (1655, 1623 cm^{-1}) and bands at 3349, 3175 and 1409 cm^{-1} due to N–H and C– N str of the $-CONH_2$ moiety and disapppearance of strong out-of-plane deformation bands at 725- 721 cm^{-1} and 688- 683 cm^{-1} due to aromatic rings of bzh, in IR spectra and disapppearance of multiplet at 7.28 to 7.17 and 7.12 to 7.05 ppm due to 10 protons of phenyl rings of bzh group, in ^1H NMR spectra of 8. Formation of cyclopeptide was further confirmed by disappearance of singlets at 3.61 and 1.51 ppm corresponding to three protons of methyl ester group and nine protons of *tert*-Butyl group of Boc, in ^1H NMR spectrum of 8. Furthermore, ^1H NMR and ^{13}C NMR spectra of synthesized cyclic heptapeptide showed characteristic peaks confirming presence all the 48 protons and 34 carbon atoms. Appearance of pseudomolecular ion peak $(M + H)^+$ at *m/z* 713 corresponding to the molecular formula $C_{34}H_{48}N_8O_9$ in mass spectra of 8, along with other fragment ion peaks resulting from cleavage at 'Pro-Asn', 'Pro-Leu', 'Tyr-Ala'



Scheme 1. Synthetic pathway for cyclomontanin D (8).Reaction conditions: (i) bzh, AcOH, H_2SO_4, RT, 12 h; (ii) TFA, $CHCl_3$, RT, 12 h; (iii) LiOH, THF:H_2O (1:1), RT, 1 h; (iv) DCC/DIPC, pyridine, THF, RT, 24 h; v) DIPC, pfp, RT, 12 h; (vi) NMM/TEA, $CHCl_3$, 7 days, 0°C.

and 'Ala-Asn' amide bond levels, showed exact sequence of attachment of all the seven amino acid moieties in a chain. In addition, presence of immonium ion peaks at *m/z* 136, 70, 87, 86, 44 and 30 further confirmed all seven amino acid moieties in cyclopeptide structure. Furthermore, elemental analysis of 8 afforded values with tolerance of ± 0.03.

Pharmacology

The results of antibacterial and antifungal activity studies of cyclopeptide 8 against eight pathogenic microbes are summarized in Tables 1 and 2. Overall observation from the results of antimicrobial data revealed that 8 possesses potent antibacterial activity against *P.*

Table 3. ANOVA table for bacterial species.

Source of variation	Degree of freedom	Sum of squares	Mean square
Treatments (between columns)	3	702.92	234.31
Residuals (within columns)	8	137.88	17.167

Table 4. ANOVA table for fungal species.

Source of variation	Degree of freedom	Sum of squares	Mean square
Treatments (between columns)	3	343.02	114.34
Residuals (within columns)	7	247.17	35.310

aeruginosa and *K. pneumonia* and potent antifungal activity against *T. mentagrophytes* and *M. audouinii* with MIC values of 12.5 to 6 μg/ml, in comparison to standard drug - ciprofloxacin and griseofulvin. Moreover, 8 displayed moderate level of bioactivity against pathogenic *Candida albicans*. Newly synthesized cyclooligopeptide displayed no significant activity against neither *B. subtilis*, *S. aureus* nor pathogenic fungus *A. niger*. Analysis of antimicrobial activity data revealed that linear peptide 7 displayed less bioactivity against pathogenic bacteria and fungi when compared to corresponding cyclic form 8. This is because, cyclization of peptides reduces the degree of freedom for each constituent within the ring and thus substantially leads to reduced flexibility, increased potency and selectivity of cyclic peptides. Further, inherent flexibility of linear peptides lead to different conformations which can bind to more than one receptor molecules, resulting in undesirable adverse effects. Antibacterial and antifungal data was subjected to one way ANOVA with post test using GraphPad InStat software. The P value for antibacterial data was 0.0016 which was considered very significant and the variations among column means were significantly greater than expected by chance (Tables 3 and 4). The F_{calc} was found to be 13.649 and 3.238 whereas F_{tab} was 4.07 and 4.35 for antibacterial and antifungal data respectively. As the value of F_{calc} is greater than F_{tab} in case of bacterial species, the significant difference exists between the values of zone of inhibition of species tested. In case of fungal species, value of F_{calc} is less than F_{tab}, indicating that significant difference does not exist between the values of zone of inhibition of species tested.

Conclusion

First total synthesis of natural proline-rich cyclooligo-peptide 8 was accomplished with 89% yield utilizing different carbodiimides and bases. Pentafluorophenyl ester was found to be better for the activation of acid functionality of linear heptapeptide unit. NMM was found to be a good base for intramolecular cyclization of linear peptide fragment, in comparison to TEA. In all coupling reactions, DIPC was proved to be a yield-effective coupling agent, in comparison to DCC. Significant level of biopotential was observed for synthesized cycloheptapeptide against Gram-negative bacteria and dermatophytes. Gram-positive bacteria were found to be less sensitive toward newly synthesized peptide, in comparison to Gram-negative bacteria. On passing toxicity tests, proline-rich cyclopeptide 8 may prove good candidate for clinical studies and can be a novel antibacterial and antidermatophyte drug of the future.

ACKNOWLEDGEMENTS

The authors wish to thank the Sophisticated Analytical Instrumentation Laboratory, School of Pharmaceutical Sciences, Rajiv Gandhi Proudyogiki Vishwavidyalaya, Bhopal, Madhya Pradesh for spectral analysis.

REFERENCES

Baraguey C, Blond A, Correia I, Pousset JT, Bodo B, Auvin-Guette C (2000). Mahafacyclin A, a cyclic heptapeptide from *Jatropha mahafalensis* exhibiting β-bulge conformation. Tetrahedron Lett., 41: 325-329.

Bauer AW, Kirby WM, Sherris JC, Turck M (1966). Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Path., 45: 493-496.

Bodanzsky M, Bodanzsky A (1984). The Practice of Peptide Synthesis. Springer- Verlag, New York, pp. 78-143.

Dahiya R, Gautam H (2011). Solution phase synthesis and bioevaluation of cordyheptapeptide B. Bull. Pharm. Res., 1: 1-10.

Dahiya R, Kumar A (2007). Synthesis and biological activity of a potent analog of natural cyclopeptide. Int. J. Nat. Appl. Sci., 3: 433-440.

Dahiya R, Pathak D (2006). Synthetic studies on a natural cyclic tetrapeptide - halolitoralin C. J. Pharm. Res., 5: 69-73.

Dahiya R, Pathak D (2007). Synthetic studies on novel benzimidazolopeptides with antimicrobial, cytotoxic and anthelmintic potential. Eur. J. Med. Chem., 42: 772-798.

Chuang PH, Hsieh PW, Yang YL, Hua KF, Chang FR, Shiea J, Wu SH,

Wu YC (2008). Cyclopeptides with anti-inflammatory activity from seeds of *Annona montana*. J. Nat. Prod., 71: 1365-1370.
Itokawa H, Yun Y, Morita H, Takeya K, Yamada K (1995). Estrogen-like activity of cyclic peptides from *Vaccaria segetalis* extracts. Planta Med., 61: 561-562.
Khan ZK (1997). *In vitro* and *vivo* screening techniques for bioactivity screening and evaluation. Proceeding Int. workshop UNIDO-CDRI, 210-211.
Matsumoto T, Shishido A, Morita H, Itokawa H, Takeya K (2001). Cyclolinopeptides F-I, cyclic peptides from linseed. Phytochemistry, 57: 251-260.
Mongkolvisut W, Sutthivaiyakit S, Leutbecher H, Mika S, Klaiber I, Moller W, Rosner H, Beifuss U, Conrad J (2006). Integerrimides A and B, cyclic heptapeptides from the latex of *Jatropha integerrima*. J. Nat. Prod., 69: 1435-1441.
Morita H, Kayashita T, Kobata H, Gonda A, Takeya K, Itokawa H (1994). Pseudostellarins A-C, new tyrosinase inhibitory cyclic peptides from *Pseudostellaria heterophylla*. Tetrahedron, 50: 6797-6804.
Morita H, Shishido A, Kayashita T, Takeya K, Itokawa H (1997). Cyclic peptides from higher plants. 39. Dichotomins F and G, cyclic peptides from *Stellaria dichotoma* var. lanceolata. J. Nat. Prod., 60: 404-407.
Morita H, Shishido A, Matsumoto T, Itokawa H, Takeya K (1999). Cyclolinopeptides B - E, new cyclic peptides from *Linum usitatissimum.* Tetrahedron, 55: 967-976.
Morita H, Iizuka T, Choo CY, Chan KL, Takeya K, Kobayashi J (2006). Vasorelaxant activity of cyclic peptide, cyclosquamosin B, from *Annona squamosa*. Bioorg. Med. Chem. Lett., 16: 4609-4611.
Morita H, Takeya K (2010). Bioactive cyclic peptides from higher plants. Heterocycles, 80: 739-764.
Picur B, Cebrat M, Zabrocki J, Siemion IZ (2006). Cyclopeptides of *Linum usitatissimum*. J. Pept. Sci., 12: 569-574.
Shin HJ, Matsuda H, Murakami M, Yamaguchi K (1996). Agardhipeptins A and B, two new cyclic hepta- and octapeptide, from the cyanobacterium Oscillatora agardhii (NIES-204). Tetrahedron, 52: 13129-13136.
Takeya K, Yamamiya T, Morita H, Itokawa H (1993). Two antitumour bicyclic hexapeptides from *Rubia cordifolia*. Phytochemistry, 33: 613-615.
Van Den Berg AJJ, Horsten SFAJ, Kettenes -Van Den Bosch JJ, Kroes BH, Beukelman CJ, Leeflang BR, Labadie RP (1995). Curcacycline A - a novel cyclic octapeptide isolated from the latex of *Jatropha curcas* L. FEBS Lett., 358: 215-218.
Wele A, Ndoye I, Zhang Y, Brouard J-P, Bodo B (2005). Cherimolacyclopeptide D, a novel cycloheptapeptide from the seeds of *Annona cherimola*. Phytochemistry, 66: 693-696.