J. Iran. Chem. Soc., Vol. 5, No. 3, September 2008, pp. 445-452.

JOURNAL OF THE
Iranian
Chemical Society

Synthesis and *in vitro* Cytotoxic Activity of a Natural Peptide of Plant Origin

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(Received 20 October 2007, Accepted 10 November 2007)

Present work reports the first total synthesis of a phenylalanine and glycine-rich cyclopolypeptide podacycline B by coupling of tripeptide Boc-L-phe-gly-L-phe-OH and tetrapeptide L-ala-gly-L-thr-L-ile-OMe followed by cyclization of linear heptapeptide segment. The chemical structure of the compound was elucidated by FTIR, ^{1}H NMR, ^{13}C NMR, FAB MS spectral data and elemental analyses. The newly synthesized peptide was subjected to biological screening and found to possess high cytotoxicity against *Dalton's lymphoma ascites* (DLA) and *Ehrlich's ascites carcinoma* (EAC) cell lines with IC_{50} values of 13.2 and 15.5 μM, in addition to moderate anthelmintic activity against earthworms *M. konkanensis*, *P. corethruses* and *Eudrilus* sp. at a dose of 2 mg ml^{-1}.

Keywords: Podacycline B, Cycloheptapeptide, *Jatropha podagrica*, Cytotoxicity, Anthelmintic activity

INTRODUCTION

In past years, natural products have played a crucial role in the pharmaceutical research as biomedically useful agents or as lead compounds for drug development. Among these, cyclopolypeptides and related congeners have emerged as important organic compounds due to their unique structures and wide pharmacological profile [1-4] which may prove better candidates to overcome the problem of wide spread increase of resistance towards conventional drugs. Cyclic peptides isolated from different parts of plants, possess a wide range of pharmacological activities including cytotoxic activity [5], antimalarial activity [6], immunosuppressive activity [7], vasorelaxant activity [8] and angiotension converting enzyme and tyrosinase inhibitory activity [9,10]. A natural cyclic peptide podacycline B has been isolated from latex of *Jatropha podagrica* Hook which is well known for its immunomodulating properties [11]. Structure of isolated peptide was elucidated by a combination of amino acid analysis, FAB MS spectrometry and two-dimensional ^{1}H NMR spectroscopy (HOHAHA and ROESY).

In continuation of our synthetic work on natural cyclopolypeptides [12], the present investigation was aimed at synthesis of natural cyclic heptapeptide podacycline B. Keeping in view of significant bioactivities possessed by cyclopeptides [13], the above synthetic peptide was further subjected to cytotoxic, anthelmintic and antifungal screening.

EXPERIMENTAL

L-amino acids, di-*tert*-butylpyrocarbonate (Boc_2O), dicyclohexylcarbodiimide (DCC), trifluoroacetic acid (TFA), triethylamine (TEA), *N*-methylmorpholine (NMM), pyridine (C_5H_5N) were purchased from SpectroChem Pvt. Ltd., Mumbai (India). Melting points were determined by open capillary method and are uncorrected. IR spectra were recorded on Shimadzu 8700 FTIR spectrophotometer using a thin film supported on KBr or $CHCl_3$ as solvent. ^{1}H NMR and

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^{13}C NMR spectra were recorded on Bruker AC NMR spectrometer (300 MHz). Mass spectra were recorded on JMS-DX 303 Mass spectrometer operating at 70 eV using fast atom bombardment technique. Elemental analyses of all compounds were performed on Vario EL III elemental analyzer. Optical rotation was measured on automatic polarimeter in a 2 dm tube at 25 °C using sodium lamp. Purity of all compounds was checked by TLC on precoated silica gel G plates utilizing $CHCl_3$/MeOH as developing solvent in different ratios (9:1/7:3 v/v).

General Procedure for the Synthesis of Linear Peptide Fragments 1-7

Amino acid methyl ester hydrochloride/peptide methyl ester (0.01 mol) was dissolved in CH_2Cl_2 (20 ml). To this, TEA (2.8 ml, 0.021 mol) was added at 0 °C and the reaction mixture was stirred for 15 min. Boc-amino acid/peptide (0.01 mol) in $CHCl_3$ (20 ml) and DCC (2.1 g, 0.01 mol) were added with stirring. After 24 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 solutions. The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated in vacuum. The crude product was recrystallized from a mixture of chloroform and petroleum ether followed by cooling at 0 °C.

The carboxyl group of L-amino acids was protected by esterification with methanol using thionyl chloride and the free amino group of L-amino acids was protected by using Boc_2O. Furthermore, TFA (2.28 g, 0.02 mol) was used for the removal of Boc group and ester group was removed by alkaline hydrolysis with LiOH (0.36 g, 0.015 mol). Peptide units were synthesized by solution phase technique [14] using DCC as coupling agent and TEA as the base. Physical characterization data of all the synthesized compounds is summarized in Table 1.

***tert*-Butyloxycarbonyl-phenylalanyl-glycine methyl ester (1).** IR ($CHCl_3$) ν_{max} (cm^{-1}): 3129 (m, -NH str, amide), 1745 (s, -C=O str, ester), 1644, 1639 (s, -C=O str, 2° amide), 1532, 1526 (m, -NH bend, 2° amide), 1273 (s, C-O str, ester); ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 7.50 (2H, t, J = 7.25 Hz, H-*m*), 7.09 (1H, br. s, NH), 6.91 (1H, t, J = 6.2 Hz, H-*p*), 6.83 (2H, d, J = 7.8 Hz, H-*o*), 6.15 (1H, br. s, NH), 4.96 (1H, td,

Table 1. Physical Characterization of the Synthesized Compounds

Compd.	Physical state	Mol. Formula (Mol. Wt.)	M.p. (ºC)	Yield (%)	R_f[a]	$\alpha_{[D]}$[b]	Elemental analysis Calcd./found (%)		
							C	H	N
1	Semisolid mass	$C_{17}H_{24}N_2O_5$ (336.39)	-	81.3	0.73	-11.3	60.00 59.97	7.19 7.20	8.33 8.35
2	Semisolid mass	$C_{11}H_{20}N_2O_5$ (260.29)	-	86.9	0.66	-62.7	50.76 50.79	7.74 7.72	10.76 10.75
3	Semisolid mass	$C_{16}H_{30}N_2O_6$ (346.42)	-	76.2	0.48	+8.3	55.47 55.48	8.73 8.72	8.09 8.12
4	White crystals	$C_{10}H_{14}ClNO_2$ (215.68)	160-162	78.0	0.29	+15.6	55.69 55.67	6.54 6.55	6.49 6.52
5	Semisolid mass	$C_{26}H_{33}N_3O_6$ (483.56)	-	69.8	0.54	-76.1[d]	64.58 64.60	6.88 6.88	8.69 8.70
6	Semisolid mass	$C_{21}H_{38}N_4O_8$ (474.55)	-	72.6	0.62	+19.4	53.15 54.16	8.07 8.05	11.81 11.84
7	Semisolid mass	$C_{41}H_{59}N_7O_{11}$ (825.95)	-	77.5	0.85[c]	-112.9[d]	59.62 59.62	7.20 7.24	11.87 11.85
8	White solid	$C_{35}H_{47}N_7O_8$ (693.79)	136-137	83.3	0.68[c]	-67.2[e]	60.59 61.62	6.83 7.82	14.13 14.15

[a]($CHCl_3$:MeOH/7:3). [b]*c*, 0.9 in MeOH. [c]($CHCl_3$:MeOH/9:1). [d]*c*, 0.5 in MeOH. [e]*c*, 0.25 in MeOH.

J = 4.6 Hz, J = 5.85 Hz, H-α, phe), 4.11 (2H, d, J = 5.5 Hz, H-α, gly), 3.65 (3H, s, OC$\underline{H}_3$), 2.97 (2H, d, J = 5.7 Hz, H-β, phe), 1.54 (9H, s, *tert*-Butyl).

***tert*-Butyloxycarbonyl-alanyl-glycine methyl ester (2).** IR ($CHCl_3$) ν_{max} (cm^{-1}): 3126 (m, -NH str, amide), 1747 (s, -C=O str, ester), 1641, 1638 (s, -C=O str, 2° amide), 1533 (m, -NH bend, 2° amide), 1269 (s, C-O str, ester); ^{1}H NMR ($CDCl_3$) δ (ppm): 7.05 (1H, br. s, N$\underline{H}$), 6.42 (1H, br. s, N$\underline{H}$), 4.59 (1H, q, J = 4.35 Hz, H-α, ala), 4.12 (2H, d, J = 5.45 Hz, H-α, gly), 3.64 (3H, s, OC$\underline{H}_3$), 1.55 (9H, s, *tert*-Butyl), 1.34 (3H, d, J = 4.2 Hz, H-β, ala).

***tert*-Butyloxycarbonyl-threonyl-isoleucine methyl ester (3).** IR ($CHCl_3$) ν_{max} (cm^{-1}): 3342 (m/br, -OH str), 3133 (m, -NH str, amide), 1742 (s, -C=O str, ester), 1643, 1639 (s, -C=O str, 2° amide), 1535 (m, -NH bend, 2° amide), 1274, 1093 (s, C-O str, ester, C-OH); ^{1}H NMR ($CDCl_3$) δ (ppm): 6.58 (1H, br. s, N$\underline{H}$), 6.45 (1H, br. s, N$\underline{H}$), 4.55 (1H, dd, J = 6.2 Hz, J = 4.9 Hz, H-α, thr), 4.42 (1H, br. s, O$\underline{H}$), 4.20 (1H, dd, J = 5.95 Hz, J = 4.3 Hz, H-α, ile), 3.68 (1H, m, H-β, thr), 3.52 (3H, s, OC$\underline{H}_3$), 2.02 (1H, m, H-β, ile), 1.65 (2H, q, J = 6.25 Hz, H-γ, ile), 1.54 (9H, s, *tert*-Butyl), 1.25 (3H, d, J = 5.8 Hz, H-γ, thr), 0.93 (3H, t, J = 7.75 Hz, H-δ, ile), 0.88 (3H, d, J = 5.9 Hz, H-γ', ile).

***tert*-Butyloxycarbonyl-phenylalanyl-glycyl-phenyl-alanine methyl ester (5).** IR ($CHCl_3$) ν_{max} (cm^{-1}): 3133-3128 (m, -NH str, amide), 1752 (s, -C=O str, ester), 1640, 1637-1632 (s, -C=O str, 2° amide), 1538, 1525 (m, -NH bend, 2° amide), 1271 (s, C-O str, ester) 738-730, 696-689 (s, CH bend, out-of-plane, rings); ^{1}H NMR ($CDCl_3$) δ (ppm): 7.90 (1H, br. s, N$\underline{H}$), 7.50 (2H, t, J = 7.3 Hz, H-*m*, phe-1), 7.12 (1H, t, J = 6.2 Hz, H-*p*, phe-2), 6.99 (2H, t, H-*m*, J = 7.25 Hz, phe-2), 6.90 (1H, t, J = 6.25 Hz, H-*p*, phe-1), 6.85 (4H, m, H-*o*, phe-1, phe-2), 6.67 (1H, br. s, N$\underline{H}$), 6.32 (1H, br. s, N$\underline{H}$), 4.54 (1H, td, J = 4.55 Hz, J = 5.85 Hz, H-α, phe-1), 3.95 (1H, td, J = 4.55 Hz, J = 5.9 Hz, H-α, phe-2), 3.91 (2H, d, J = 5.45 Hz, H-α, gly), 3.54 (3H, s, OC$\underline{H}_3$), 3.10 (4H, m, phe-1, phe-2), 1.55 (9H, s, *tert*-Butyl).

***tert*-Butyloxycarbonyl-alanyl-glycyl-threonyl-isoleucine methyl ester (6).** IR ($CHCl_3$) ν_{max} (cm^{-1}): 3345 (m/br, -OH str), 3132-3125 (m, -NH str, amide), 1751 (s, -C=O str, ester), 1643-1637 (s, -C=O str, 2° amide), 1538, 1532 (m, -NH bend, 2° amide), 1273, 1092 (s, C-O str, ester, C-OH); ^{1}H NMR ($CDCl_3$) δ (ppm): 10.36 (1H, br. s, N$\underline{H}$), 7.60 (1H, br. s, N$\underline{H}$), 6.85 (1H, br. s, N$\underline{H}$), 6.52 (1H, br. s, N$\underline{H}$), 4.53 (1H, br. s, O$\underline{H}$), 4.38 (1H, q, J = 4.4 Hz, H-α, ala), 4.25 (1H, dd, J = 6.2 Hz, J = 4.95 Hz, H-α, thr), 4.11 (2H, d, J = 5.5 Hz, H-α, gly), 3.89 (1H, m, H-β, thr), 3.81 (1H, dd, J = 6.0 Hz, J = 4.25 Hz, H-α, ile), 3.51 (3H, s, OC$\underline{H}_3$), 2.03 (1H, m, H-β, ile), 1.68 (2H, q, J = 6.25 Hz, H-γ, ile), 1.58 (3H, d, J = 4.15 Hz, H-β, ala), 1.54 (9H, s, *tert*-Butyl), 1.24 (3H, d, J = 5.75 Hz, H-γ, thr), 0.93 (3H, t, J = 7.8 Hz, H-δ, ile), 0.89 (3H, d, J = 5.85 Hz, H-γ', ile).

***tert*-Butyloxycarbonyl-phenylalanyl-glycyl-phenyl-alanyl-alanyl-glycyl-threonyl-isoleucine methyl ester (7).** IR ($CHCl_3$) ν_{max} (cm^{-1}): 3342 (m/br, -OH str), 3132-3123 (m, -NH str, amide), 1748 (s, -C=O str, ester), 1648-1635 (s, -C=O str, 2° amide); 1269, 1088 (s, C-O str, ester, C-OH) 737-733, 698-689 (s, -CH bend, out-of-plane, rings); ^{1}H NMR ($CDCl_3$) δ (ppm): 10.35, 9.45, 8.89, 8.25, 8.12 (5H, br. s, N$\underline{H}$), 7.50 (2H, t, J = 7.3 Hz, H-*m*, phe-1), 7.18 (2H, t, J = 7.3 Hz, H-*m*, phe-2), 7.01 (1H, t, J = 6.2 Hz, H-*p*, phe-2), 6.90 (1H, t, J = 6.25 Hz, H-*p*, phe-1), 6.84 (4H, m, H-*o*, phe-1, phe-2), 6.79, 6.65 (2H, br. s, N$\underline{H}$), 4.79 (1H, td, J = 4.6 Hz, J = 5.9 Hz, H-α, phe-1), 4.55 (1H, br. s, O$\underline{H}$), 4.27 (1H, dd, J = 6.15 Hz, J = 4.9 Hz, H-α, thr), 4.17 (1H, q, J = 4.35 Hz, H-α, ala), 4.12 (2H, d, J = 5.5 Hz, H-α, gly-1), 4.02 (2H, d, J = 5.45 Hz, H-α, gly-2), 3.94 (1H, td, J = 4.55 Hz, J = 5.9 Hz, H-α, phe-2), 3.87 (1H, m, H-β, thr), 3.82 (1H, dd, J = 5.9 Hz, J = 4.25 Hz, H-α, ile), 3.52 (3H, s, OC$\underline{H}_3$), 3.07 (4H, m, H-β, phe-1, phe-2), 2.02 (1H, m, H-β, ile), 1.68 (2H, q, J = 6.3 Hz, H-γ, ile), 1.54 (9H, s, *tert*-Butyl), 1.49 (3H, d, J = 4.2 Hz, H-β, ala), 1.25 (3H, d, J = 5.8 Hz, H-γ, thr), 0.93 (3H, t, J = 7.8 Hz, H-δ, ile), 0.88 (3H, d, J = 5.85 Hz, H-γ', ile); ^{13}C NMR (300 MHz, DMSO-d_6) δ (ppm): 173.9, 170.4, 169.6, 168.0, 167.1, 166.7, 165.2, (7C, $\underline{C}$=O, amide), 156.8 ($\underline{C}$=O, Boc), 139.2, 137.5 (2C, C-γ, phe-1, phe-2), 130.9 (2C, C-*m*, phe-1), 130.2 (2C, C-*o*, phe-2), 129.4 (2C, C-*o*, phe-1), 128.9 (C-*p*, phe-1), 126.6 (2C, C-*m*, phe-2), 125.9 (C-*p*, phe-2), 79.9 (C-α, *tert*-Butyl), 67.5 (C-β, thr), 56.4, 55.5 (2C, C-α, thr, ile), 54.6 (O$\underline{C}H_3$), 54.3, 52.1, 51.6 (3C, C-α, ala, phe-1, phe-2), 45.2, 40.6 (2C, C-α, gly-1, gly-2), 38.1, 37.0, 35.9 (3C, C-β, ile, phe-1, phe-2), 29.6 (3C, C-β, *tert*-Butyl), 24.2, 20.5 (2C, C-γ, ile, thr), 18.6 (C-β, ala), 15.2 (C-γ', ile), 10.3 (C-δ, ile).

Synthesis of Cyclic Heptapeptide, Podacycline B (8)

To synthesize compound **8**, linear heptapeptide unit **7** (4.13

g, 0.005 mol) was deprotected at carboxyl end using LiOH (0.18 g, 0.0075 mol) to get Boc-L-phe-gly-L-phe-L-ala-gly-L-thr-L-ile-OH. The deprotected heptapeptide unit (4.06 g, 0.005 mol) was now dissolved in $CHCl_3$ (50 ml) at 0 °C. To the above solution, *p*-nitrophenol (0.94 g, 0.0067 mol) and DCC (1.06 g, 0.005 mol) were added and stirred at RT for 12 h. The reaction mixture was filtered and the filtrate was washed with 10% $NaHCO_3$ solution (3 × 15 ml) until excess of *p*-nitrophenol was removed and finally washed with 5% HCl (2 × 10 ml) to get the corresponding *p*-nitrophenyl ester Boc-L-phe-gly-L-phe-L-ala-gly-L-thr-L-ile-O-pnp. To this compound (3.73 g, 0.004 mol) dissolved in $CHCl_3$ (35 ml), TFA (0.91 g, 0.008 mol) was added, stirred at RT 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 L-phe-gly-L-phe-L-ala-gly-L-thr-L-ile-O-pnp which was dissolved in $CHCl_3$ (25 ml) and TEA/NMM/C_5H_5N (2.8 ml/2.21 ml/1.61 ml, 0.021 mol) was added. Then, whole contents were kept at 0 °C for 7 days. The reaction mixture was washed with 10% $NaHCO_3$ (3 × 25 ml) and 5% HCl (3 × 15 ml) solutions. The organic layer was dried over anhydrous Na_2SO_4 and crude cyclized product was crystallized from $CHCl_3$/n-hexane to get pure cyclo(-L-phenylalanyl-glycyl-L-phenylalanyl-L-alanyl-glycyl-L-threonyl-L-isoleucyl-) (**8**).

Yield: (2.89 g, 83% (NMM), 71% (TEA), 59% (pyridine)); IR (KBr) ν_{max} (cm^{-1}): 3345 (m/br, -OH str), 3135-3124 (m, -NH str, amide), 1647-1631 (s, -C=O str, 2° amide), 1542, 1533, 1530-1525 (m, -NH bend, 2° amide), 1089 (s, C-O str, C-OH), 738-734, 696-692 (s, -CH bend, out-of-plane, rings); ^{1}H NMR ($CDCl_3$) δ (ppm): 9.30, 8.39, 8.08, 8.05, 7.72 (5H, br. s, NH, ile, phe-1, phe-2, thr, ala), 7.60 (1H, br. s, OH), 7.36 (1H, br. s, NH, gly-2), 7.18 (4H, tt, *J* = 7.3 Hz, *J* = 7.25 Hz, H-*m*, phe-1, phe-2), 7.05 (1H, br. s, NH, gly-1), 7.00 (2H, tt, *J* = 6.25 Hz, *J* = 6.2 Hz, H-*p*, phe-1, phe-2), 6.84 (4H, m, H-*o*, phe-1, phe-2), 5.95 (1H, q, *J* = 4.35 Hz, H-α, ala), 5.70 (1H, dd, *J* = 6.2 Hz, *J* = 4.95 Hz, H-α, thr), 5.63 (2H, m, H-α, phe-1, phe-2), 5.28 (1H, dd, *J* = 5.95 Hz, *J* = 4.25 Hz, H-α, ile), 5.23 (2H, d, *J* = 5.45 Hz, H-α, gly-1), 5.20 (2H, d, *J* = 5.5 Hz, H-α, gly-2), 3.80 (1H, m, H-β, thr), 2.61 (4H, m, H-β, phe-1, phe-2), 1.64 (3H, m, H-β, H-γ, ile), 1.42 (3H, d, *J* = 5.75 Hz, H-γ, thr), 1.44 (3H, d, *J* = 4.2 Hz, H-β, ala), 0.97 (6H, m, H-δ, H-γ′, ile); ^{13}C NMR (DMSO-d_6) δ (ppm): 173.4, 171.7, 170.8 (3C, C=O, ala, phe-2, ile), 169.8 (2C, C=O, gly-1, gly-2), 168.4, 161.8 (2C, C=O, thr, phe-1), 138.0 (2C, C-γ, phe-1, phe-2), 129.8, 129.5 (4C, C-*o*, phe-1, phe-2), 126.7, 126.3 (4C, C-*m*, phe-1, phe-2), 128.3 (2C, C-*p*, phe-1, phe-2), 66.9 (C-β, thr), 59.8, 58.2 (2C, C-α, ile, thr), 52.8 (2C, C-α, phe-1, phe-2), 48.9 (C-α, ala), 43.6 (2C, C-α, gly-1, gly-2), 38.8 (2C, C-β, phe-1, phe-2), 34.5 (C-β, ile), 23.8, 22.0 (2C, C-γ, ile, thr), 17.5 (C-β, ala), 15.9 (C-γ′, ile), 10.1 (C-δ, ile); FAB MS *m/z* [RI]: 694.8 [$(M+1)^+$, 100], 666.8 [$(694.8\text{-}CO)^+$, 17], 637.7 [$(phe\text{-}ala\text{-}gly\text{-}thr\text{-}ile\text{-}phe)^+$, 25], 593.7 [$(ile\text{-}phe\text{-}gly\text{-}phe\text{-}ala\text{-}gly)^+$, 38], 565.7 [$(593.7\text{-}CO)^+$, 19], 536.6 [$(ile\text{-}phe\text{-}gly\text{-}phe\text{-}ala)^+$, 26], 508.6 [$(536.6\text{-}CO)^+$, 10], 490.6 [$(phe\text{-}ala\text{-}gly\text{-}thr\text{-}ile)^+$, 56], 465.6 [$(ile\text{-}phe\text{-}gly\text{-}phe)^+$, 33], 462.2 [$(490.6\text{-}CO)^+$, 18], 377.4 [$(phe\text{-}ala\text{-}gly\text{-}thr)^+$, 39], 349.4 [$(377.4\text{-}CO)^+$, 15], 261.3 [$(ile\text{-}phe)^+$, 29], 233.3 [$(261.3\text{-}CO)^+$, 8], 219.3 [$(phe\text{-}ala)^+$, 22], 191.3 [$(219.3\text{-}CO)^+$, 9], 148.2 [$(phe)^+$, 14], 120.1 [$(C_8H_{10}N)^+$, 9], 114.2 [$(ile)^+$, 11], 91.1 [$(C_7H_7)^+$, 16], 86.1 [$(C_5H_{12}N)^+$, 6], 74.1 [$(C_3H_8NO)^+$, 7], 65.1 [$(C_5H_5)^+$, 11], 57.1 [$(C_4H_9)^+$, 6], 45.1 [$(C_2H_5O)^+$, 7], 44.1 [$(C_2H_6N)^+$, 5], 31.1 [$(CH_3O)^+$, 6], 30.0 [$(CH_4N)^+$, 5], 29.0 [$(C_2H_5)^+$, 9], 15.0 [$(CH_3)^+$, 12].

Cytotoxic Activity Assay

Synthesized cyclopeptide **8** was subjected to short term *in vitro* cytotoxicity study [15] at 62.5-3.91 μg ml^{-1} using 5-fluorouracil (5-FU) as reference compound. Activity was assessed by determining the percentage inhibition of DLA and EAC cells. Both cells were cultured in the peritoneal cavity of healthy albino mice by injecting the suspension of cells (1 × 10^6 cells ml^{-1}) intraperitoneally. After 15-20 days, cells were withdrawn from the peritoneal cavity of the mice with the help of sterile syringe and counted using haemocytometer and adjusted to 1 × 10^6 cells ml^{-1}. Different dilutions of compound **8** ranging from 62.5-3.91 μg ml^{-1} were prepared in dulbecoccs minimum essential medium and 0.1 ml of each diluted test compound was added to 0.1 ml of DLA cells (1 × 10^6 cells ml^{-1}) and EAC cells (1 × 10^6 cells ml^{-1}). Resulted suspensions were incubated at 37 °C for 3 h. After 3 h, tryphan blue dye exclusion test was performed and percentage growth inhibition was calculated. IC_{50} values were determined by graphical extrapolation method. Controls were also tested at 62.5-3.91 μg ml^{-1} against both cell lines. The results of cytotoxic activity studies are summarized in Table 2.

Table 2. Cytotoxic Activity Data

Compd.	Conc. (μg ml^{-1})	DLA cells				EAC cells			
		Live cells counted	No. of dead cells	% Growth inhibition[a]	IC_{50}[b] (μM)	Live cells counted	No. of dead cells	% Growth inhibition	IC_{50} (μM)
8	62.5	0	38	100.0		0	28	100.0	
	31.25	2	36	94.74		3	25	89.29	
	15.63	14	24	63.16	13.2	9	19	67.85	15.5
	7.81	20	18	47.37		17	11	39.28	
	3.91	28	10	26.32		26	2	7.14	
Control	62.5	38	0	-		28	0	-	
	31.25	38	0	-		28	0	-	
	15.63	38	0	-	-	28	0	-	-
	7.81	38	0	-		28	0	-	
	3.91	38	0	-		28	0	-	
Standard	62.5	0	38	100.0		0	28	100.0	
(5-FU)	31.25	0	38	100.0		0	28	100.0	
	15.63	10	28	73.68	37.36	11	17	60.71	90.55
	7.81	13	25	65.79		19	9	32.14	
	3.91	22	16	42.11		23	5	17.86	

[a]%Growth inhibition = 100 - [{($Cell_{total}$ - $Cell_{dead}$) × 100}/$Cell_{total}$]; [b]IC_{50} = cytotoxic conc. inhibiting 50% of percentage growth.

Table 3. Anthelmintic Activity Data

Compd.	Earthworm species					
	M. konkanensis		*P. corethruses*		*Eudrilus* sp.	
	Mean paralyzing time (min)[a]	Mean death time (min)	Mean paralyzing time (min)	Mean death time (min)	Mean paralyzing time (min)	Mean death time (min)
8[b]	15.52 ± 0.23	18.07 ± 0.57	22.04 ± 0.31	25.16 ± 0.42	17.32 ± 0.44	19.25 ± 0.16
Control[c]	-	-	-	-	-	-
Mebendazole[b]	10.55 ± 0.64	12.59 ± 0.53	17.58 ± 1.03	19.42 ± 1.20	11.35 ± 0.45	13.46 ± 0.62
Piperazine citrate[b]	12.39 ± 0.36	13.52 ± 0.49	19.06 ± 0.57	22.23 ± 0.78	12.46 ± 0.15	13.58 ± 0.47

[a]Data are given as mean ± S.D. (n = 3); [b]*c* = 2 mg ml^{-1}; [c]0.5% tween 80 in distilled water.

Anthelmintic Activity Assay

Three earthworm species namely *Megascoplex konkanensis*, *Pontoscotex corethruses* and *Eudrilus* sp. were selected for anthelmintic testing of compound **8** at 2 mg ml^{-1} concentration [16]. Suspensions of sample were prepared by triturating synthesized compound (100 mg) with tween 80 (0.5%) and distilled water and the resulting mixtures were stirred using a mechanical stirrer for 30 min. The suspensions were diluted to contain 0.2% w/v of the test sample. Suspension of reference drugs, mebendazole and piperazine citrate were prepared with the same concentration in a similar way. Three sets of five earthworms of almost similar sizes (2 inch in length) were placed in petri plates of 4 inch diameter containing 50 ml of suspension of test sample and reference drug at RT. Another set of five earthworms was kept as control in 50 ml suspension of distilled water and tween 80 (0.5%). The paralyzing and death times were noted and their mean was calculated for triplicate sets. The death time was ascertained by placing the earthworms in warm water (50 °C) which stimulated the movement, if the worm was alive. The results of anthelmintic studies are given in Table 3.

Antifungal Activity Assay

Cultures of four fungal species namely *Candida albicans*, *Aspergillus niger*, *Microsporum audouinii* and *Trichophyton mentagrophytes* were used to investigate the antifungal activity of synthesized cyclopeptide **8** at 12.5-6 μg ml^{-1} concentration [17]. Griseofulvin, as a reference drug, and DMF, as the negative control, were also tested at the same concentration. MIC values of test compound **8** were determined by tube dilution technique. Diameters of the zones of inhibition (in mm) were measured for each fungal strain and the average diameters for test sample **8** were calculated for triplicate sets. The diameters obtained for the test sample **8** were compared with that produced by the reference drug.

RESULTS AND DISCUSSION

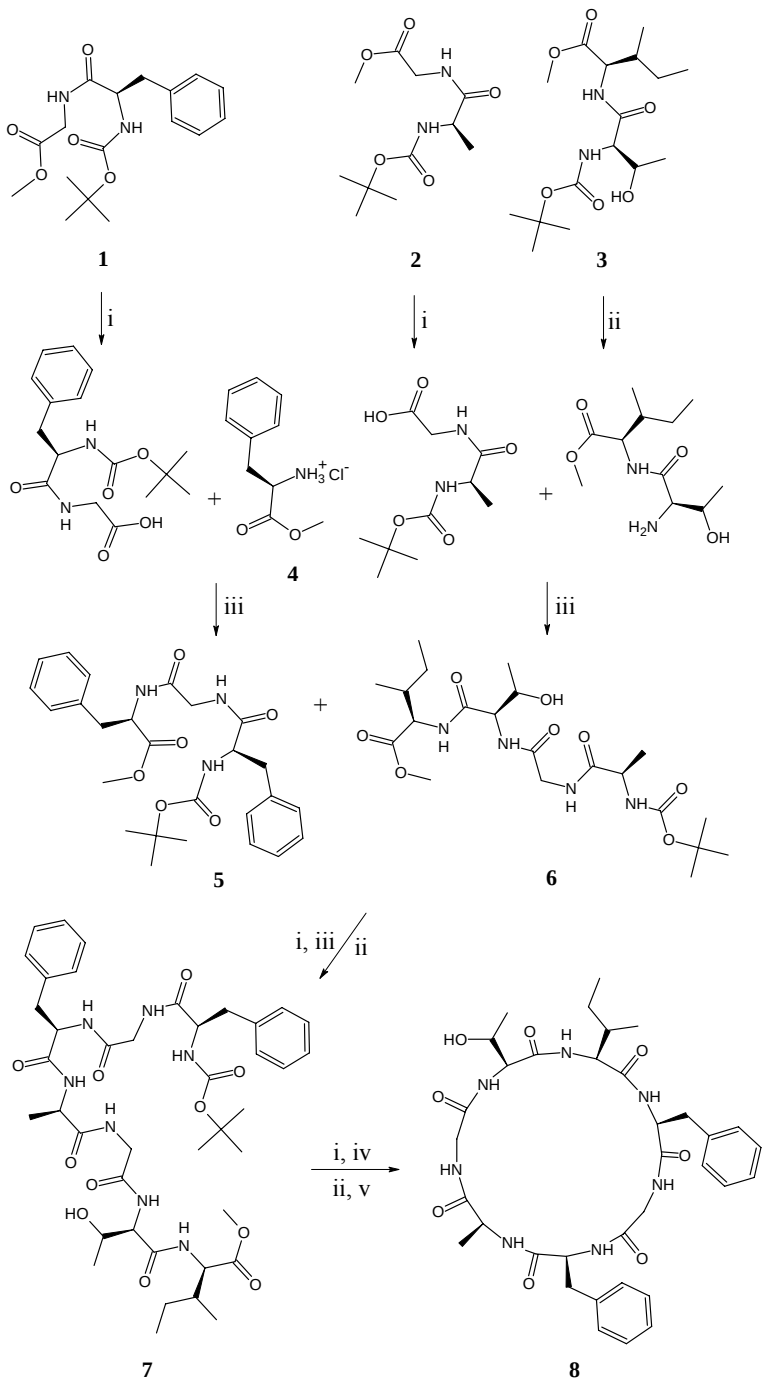
Chemistry

In the present work, disconnection strategy was employed to carry out the first total synthesis of podacycline B. The cyclic heptapeptide molecule was split into three dipeptide units Boc-L-phe-gly-OMe (**1**), Boc-L-ala-gly-OMe (**2**), Boc-L-thr-L-ile-OMe (**3**) and single amino acid unit L-phe-OMe.HCl (**4**). The required dipeptide units **1-3** were prepared by coupling of Boc-amino acids *viz*. Boc-L-phe, Boc-L-ala and Boc-L-thr with corresponding amino acid methyl ester hydrochlorides such as gly-OMe.HCl and L-ile-OMe.HCl employing DCC as coupling agent. Ester group of dipeptide **1** was removed by alkaline hydrolysis with LiOH and deprotected peptide was coupled with amino acid methyl ester hydrochloride **4** using DCC and TEA, to get the tripeptide unit Boc-L-phe-gly-L-phe-OMe (**5**).

Similarly, dipeptide **2** after deprotection at carboxyl end, was coupled with dipeptide **3** deprotected at amino terminal, to get the tetrapeptide unit Boc-L-ala-gly-L-thr-L-ile-OMe (**6**). After removal of ester group of tripeptide **5** and Boc group of tetrapeptide **6**, deprotected units were coupled to get linear heptapeptide unit Boc-L-phe-gly-L-phe-L-ala-gly-L-thr-L-ile-OMe (**7**). The methyl ester group of linear peptide fragment was replaced by *p*-nitrophenyl (pnp) ester group. The Boc-group of resulting compound was 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 TEA/NMM/pyridine to get cyclic compound **8** (Scheme 1).

Synthesis of podacycline B **8** was carried out successfully with good yield and NMM was proved to be a yield effective base for cyclization of linear heptapeptide fragment. Cyclization of linear peptide was indicated by disappearance of absorption bands at 1748, 1269 cm^{-1} and 1387, 1372 cm^{-1} (CO stretching of ester and CH deformation of *tert*-Butyl group) and presence of additional Amide I and Amide II bands of the -CO-NH- moiety at 1634-1631 cm^{-1} and 1542 cm^{-1} in IR spectra of the compound **8**. Formation of cyclopeptide was further confirmed by disappearance of singlets at 79.9, 29.6 ppm corresponding to alpha and beta carbons of *tert*-Butyl group of Boc and singlet at 54.6 ppm corresponding to carbon of methyl ester, in ^{13}C NMR spectrum and disappearance of singlet at 1.54 ppm corresponding to nine protons of *tert*-Butyl group of Boc and singlet at 3.52 ppm corresponding to three protons of methyl ester, in ^{1}H NMR spectrum of compound **8**.

Furthermore, ^{1}H NMR and ^{13}C NMR spectra of the synthesized cyclic heptapeptide showed characteristic peaks confirming presence all the 47 protons and 35 carbon atoms. Presence of $(M + 1)^{+}$ ion peak at *m/z* 694.8 corresponding to



i) LiOH, THF:H_2O (1:1), RT, 1 h
ii) TFA, $CHCl_3$, RT, 1 h
iii) DCC, TEA, $CHCl_3$, RT, 24 h
iv) DCC, pnp, RT, 12 h
v) TEA/NMM/C_5H_5N, $CHCl_3$, 7 days, 0 ^oC

Scheme 1. Synthetic pathway for podacycline B (**8**)

the molecular formula $C_{35}H_{47}N_7O_8$ in mass spectra of compound **8**, along with other fragment ion peaks resulting from cleavage at 'gly-phe' and 'thr-ile' amide bond levels, showed exact sequence of attachment of all the seven amino acid moieties in a chain. In addition, elemental analysis of compound **8** afforded values (±0.03) strictly in accordance to the molecular composition.

Pharmacology

Results of cytotoxic and anthelmintic activity studies are summarized in Tables 2 and 3. Comparison of cytotoxic activity data suggested that synthesized cyclopeptide **8** exhibited high level of cytotoxic activity against DLA and EAC cell lines with IC_{50} values of 13.2 and 15.5 μM (9.15 and 10.75 μg ml^{-1}, respectively) in comparison to standard drug 5-fluorouracil (IC_{50} values - 37.36 and 90.55 μM). Anthelmintic activity data revealed that compound **8** showed moderate anthelmintic activity against *M. konkanensis*, *P. corethruses* and *Eudrilus* sp. at 2 mg ml^{-1} concentration, in comparison to standard drugs mebendazole and piperazine citrate. However, compound **8** displayed no significant activity against any of the pathogenic fungal strains tested. On passing toxicity tests, compound **8** may prove good candidate for clinical studies and can be a novel cytotoxic drug of the future.

ACKNOWLEDGEMENTS

The author is thankful to U.S.I.C., DU, Delhi and Head, R.S.I.C., I.I.T., Delhi for spectral analysis. Also, great thanks to J.S.S. College of Pharmacy, Ooty for carrying out the cytotoxicity studies and C.P.C.R.I., Kasaragod, Kerala for providing earthworms for testing anthelmintic activity.

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