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Anticancer Activity of Thiazole-Incorporated Synthetic Wainunuamide

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A cyclic heptapeptide wainunuamide isolated from Fijian marine sponge was synthesized by using solution phase peptide synthesis technique. The synthesized wainunuamide compound had shown moderate activity towards HeLa Cancer cell lines. To increase the anticancer activity of the synthesized compound a thiazole moiety is incorporated in the wainunuamide compound. The synthesized compounds were characterized by FTIR, ^{1}H NMR and mass spectral analysis. The thiazole incorporated synthetic wainunuamide was tested for its anticancer activity (cell line-MCF7 cells). The compound exhibited potent anticancer activity.

Key Words: Wainunuamide, Cyclic heptapeptide, Solution phase synthesis, Anticancer activity.

INTRODUCTION

A cyclic heptapeptide wainunuamide, Cyclo-L-[Phe-Pro-His-Pro-Pro-Gly-Leu] isolated from a Fijian marine sponge of *Stylotella aurantium*, showed moderate cytotoxic activity[1,2]. The role of proline in these molecules has been linked to the control of conformation of the molecule in solution because of the restricted rotation of proline[3,4].

A review of the structures of cyclic peptides exhibiting anticancer activity showed presence of proline units in the molecule. Various cytotoxic cyclic peptides were found to contain heterocyclic moieties as a part of the structure[5-7]. Hence an attempt has been made to synthesize thiazole-incorporated wainunuamide, cyclic heptapeptide. The molecule cyclo-L-[4-amino-5-methylthiazol-2-carboxyl-Phe-Pro-His-Pro-Pro-Gly-Leu] was synthesized by solution phase technique of peptide synthesis using dicyclohexylcarbodimide (DCC) as the coupling agent and triethylamine (Et_3N) as a base.

EXPERIMENTAL

Anticancer activity: All culture manipulations were preformed under aseptic conditions employing sterile culture techniques. MCF7 cell line were routinely

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maintained in RPMI 1640 growth medium (pH 7.4) supplemenated with 10 % heat maintained fetal calf serum (FCS) at 37 ºC in 10 cm^2 cell culture dishes in a humidified atmosphere with 5 % CO_2.

Cytototoxicity was determined by using a modified MTT assay previously described by Carmicheal *et al.*[8] and Masmaan *et al.*[9]. Cells in exponential growth were trypsinized, [0.25 % trypsin] counted and diluted to 30,000 cells/mL. Aliquots of 200 µL of the cell suspensions were added to each well of a 96-well culture plate and incubated at 37 ºC for 24 h prior to the addition of the drug solutions. Cisplatin (positive control) was intially solubilized in 0.5 % DMSO and stock concentrations of 100 µM were prepared in growth medium. Aliquots of 200 µL of the drug solutions were added to the wells such that the final drug concentration per well was 100, 75 and 50 µM and the cells were incubated at 37 ºC (5 % CO_2 humidified atmosphere) for 48 h. Drug solutions were then aspirated and replaced with 200 µL of 0.5 mg/mL MTT growth medium and incubated further 3 h at 37 ºC. The MTT solution was aspirated and replaced with 200 µL of DMSO to dissolve the MTT formazan precepitate. The plates were then agitated on a shaker for 5 min and the absorbance was read at 540 nm on a multiscan MS multiwell scanning spectrophotometer.

All the reactions required anhydrous conditions were conducted in flame dried apparatus. Solvents and reagents were purified by standard methods. Organic extracts were dried over anhydrous sodium sulphate. All amino acids and other chemicals were obtained from spectrochem Ltd. (Mumbai, India). Melting points were determined in open capillary tubes and are uncorrected. Purity of the compounds was checked by pre-coated TLC plate. IR spectra were recorded on Thermo Nicolet FTIR 330 spectrometer using a thin film supported on KBr pellets. ^1H NMR spectra were recorded on Bruker AC NMR spectrometer using $CDCl_3$ as solvent. FAB mass spectra were recorded on a Joel Sx 102/DA-6000.

Preparation of dipeptides: Amino acid methyl ester hydrochloride (10 mmol) was dissolved in chloroform (20 mL). To this, triethylamine (4 mL, 28.7 mmol) was added at 0 ºC and the reaction mixture was stirred for 15 min. Boc-amino acid (10 mmol) in $CHCl_3$ (20 mL) and DCC (10 mmol) were added with stirring. After 12 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 % $NaHCO_3$ (20 mL) and saturated NaCl (20 mL) solutions. The organic layer was filtered and evaporated in vacuum. To remove the traces of the dicyclohexylurea (DCU), the product was dissolved in minimum amount of chloroform and cooled to 0 ºC. The crystallized DCU was removed by filtration. Petroleum ether was added to the filtrate at 0 ºC to recrystallize the pure product. Boc-Phe-Pro-OMe (1), Boc-His-Pro-OMe (2) and Boc-Pro-Gly-OMe (3) were prepared in this manner.

Preparation of the tetrapeptide Boc-Phe-Pro-His-Pro-OMe: The tetrapeptide was prepared from the dipeptides Boc-Phe-Pro-OMe (1) and Boc-His-Pro-OMe (2) units after appropriate deprotection at the required functional groups. The

deprotected dipeptide units[10] were coupled using DCC/Et_3N to get the protected tetrapeptide by the procedure similar to that of the dipeptides.

Preparation of the tripeptide Boc-Pro-Gly-Leu-OMe: The tripeptide was prepared from the dipeptide Boc-Pro-Gly-OMe (3) and Leu-OMe (4) units after appropriate deprotection at the required functional groups using DCC/Et_3N to get the protected tripeptide.

Preparation of ethyl-4-amino-5-methylthiazol-2-carboxylate: Ethyl-2-chloroacetoacetate (0.1 mmol) was refluxed with thiourea (0.1 mmol) in dry acetone (20 mL) for 2 h. The completion of the reaction was monitored by TLC. Solvent system used was hexane: ethyl acetate (8:2). The product obtained was recrystallized with ethyl acetate. The ester group of carboxylic acid was deprotected by LiOH.

Preparation of the pentapeptides Boc-4-amino-5-methylthiazol-2-carboxyl-Phe-Pro-His-Pro-OMe: The pentapeptides were prepared from the tetrapeptide Boc-Phe-Pro-His-Pro-OMe (5) and Boc-4-amino-5-methylthiazol-2-carboxylic acid (6) units after appropriate deprotection at the required functional groups. The deprotected tetrapeptides and Boc-4-amino-5-methylthiazol-2-carboxylic acid units were coupled using DCC/Et_3N to get the protected pentapeptide by the procedure similar to that of the dipeptides.

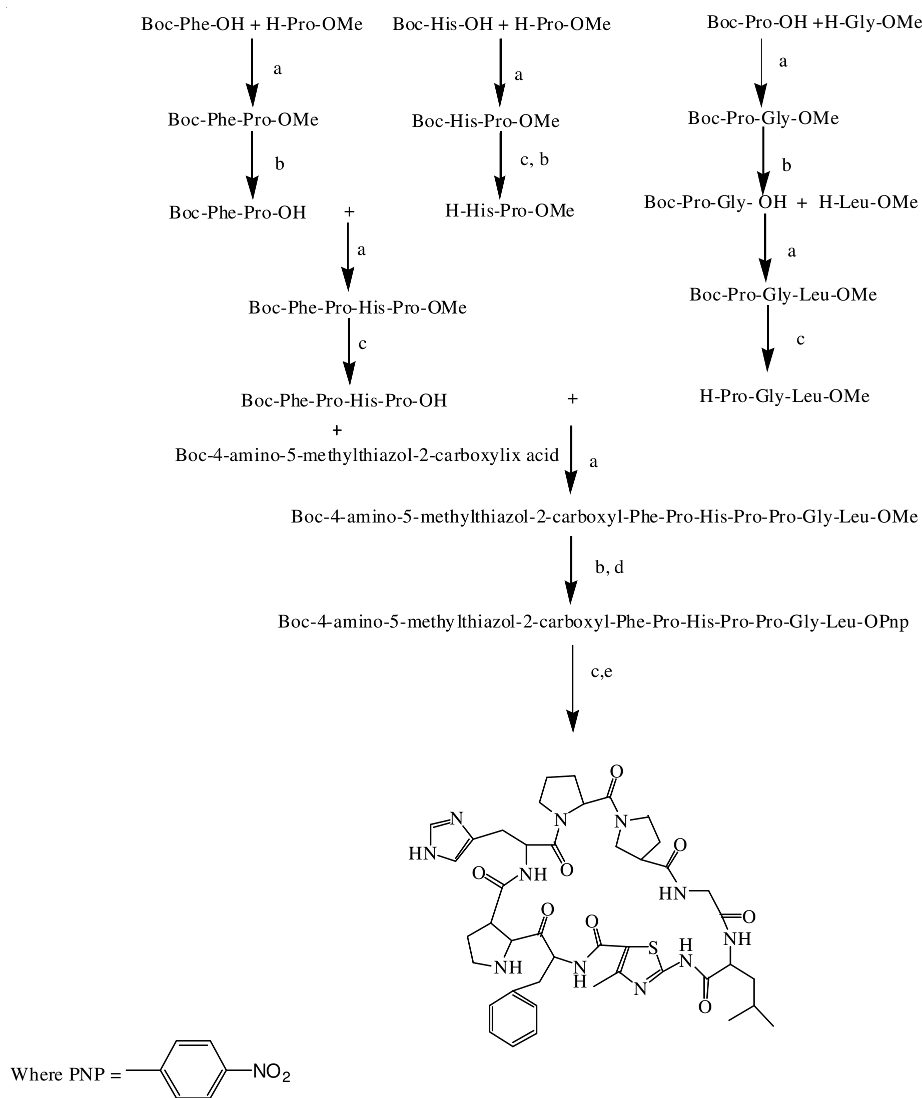
Preparation of linear heptapeptide: The Boc-group of the tripeptide (Boc-Pro-Gly-Leu-OMe) was removed and the ester group of the pentapeptide (Boc-4-amino-5-methylthiazol-2-carboxyl-Phe-Pro-His-Pro-OMe) was deprotected. Both the deprotected units were coupled to get the linear heptapeptide.

RESULTS AND DISCUSSION

In order to carry out the synthesis, the cyclic heptapeptide was disconnected into one tripeptides and pentapeptides unit. Boc-Pro-Gly-Leu-OMe and Boc-4-amino-5-methylthiazol-2-carboxyl-Phe-Pro-His-Pro-OMe. These units were properly deprotected and coupled together to get the linear heptapeptide and finally cyclized using *p*-nitrophenyl ester method (**Scheme-I**).

FTIR spectra of cyclic heptapeptide unit showed characteristic medium to strong bands corresponding to carbonyl stretching at 1698-1645 cm^{-1} (amide I band) and NH bending at 1542-1509 cm^{-1} (amide II band), confirming the coupling reaction. ^{1}H NMR spectra of the synthesized compound clearly indicates the coupling of amino acids and peptides. The mass spectral data indicates stable molecular ion peak for the synthesized compound. Therefore the cyclic heptapeptide structure was confirmed by FTIR, ^{1}H NMR and mass spectral data.

Cyclic heptapeptide (CHP) significantly reduced the growth of MCF7 cell as shown in the Table-1. Screening concentrations of 50 µM lead to a reduction in viable cell of 0.3-0.6 %, respectively, while the 100 and 75 µM screening concentrations of cyclic heptapeptide also reduced viable MCF7 cell numbers by 0.2-0.4 %. Cyclic heptaplptide at 50 µM produced moderate inhibitory effects against MCF7



a = $CHCl_3$, DCC, Et_3N, 12h, RT,
b = THF: H_2O (1:1), LiOH, 1h, RT,
c = $CF_3COOH/CHCl_3$, 1h, RT
d = p-nitrophenol, e = $CHCl_3$, pyridine, 3 days, 0^0C

Scheme-I

TABLE-1
EFFECT OF CYCLIC HEPTAPEPTIDE (CHP) ON CANCER CELL LINE

Concentration (μM)	MCF7 cell
100	0.285, 0.387, 0.489
75	0.299, 0.459, 0.488
50	0.477, 0.444, 0.598
Cisplatin (100)	0.8892

cancer cell line, except for CHP at 100 and 75 μM which showed little activity against MCF7 cells. The results of the study clearly indicate the positive control cisplatin to have the most significant inhibition of tumor cell growth for the cell line in comparison to the CHP. Cisplatin at 100 μM produced the most significant inhibitory effect on the MCF7 cell line resulting in a 0.8892 inhibition.

Spectral data

Tetrapeptide (I): Boc-Phe-Pro-His-Pro-OMe: Brown sticky semisolid mass, m.w.: 625, m.f.: $C_{32}H_{42}N_6O_7$ UV (100 % MeOH) λ_{max} 249; FITR (KBr, ν_{max}, cm^{-1}): 3227(-NH stretch), 2982 and 2964(-CH stretch), 1749 (CO of ester group), 1681 (CO stretch), 1570 (-NH bend), 1461 (-CH bend). ^1H NMR ($CDCl_3$): δ 8.0-7.5 (m, 7H, Ar-H), 7.2 (s, 1H, -NH of imidazole), 7.0-6.8 (s, 2H, -NH), 4.1-4.0 (m,-CH_2 of Phe), 3.4-3.3 (m, 2H, -CH_2 of Pro), 3.9-3.2 (m, 2H, -CH_2 of Pro). FAB mass m/z: 626 (M + 1).

Tripeptide (II): Boc-Pro-Gly-Leu-OMe: Pale yellowish sticky semisolid mass, m.w.: 399, m.f.: $C_{19}H_{33}N_3O_6$, UV (100 % MeOH) λ_{max} 232; FITR (KBr, ν_{max}, cm^{-1}): 3221 (-NH stretch), 2979 and 2960(-CH stretch), 1746 (CO of ester group), 1680 (CO stretch), 1576 (-NH bend), 1466 (-CH bend). ^1H NMR ($CDCl_3$): δ 7.0-6.7 (s, 2H, -NH), 3.4-3.2 (m, 2H, -CH_2 of Pro), 3.8-3.0 (m, 2H, -CH_2 of Pro). FAB mass m/z: 400 (M + 1).

Ethyl-4-amino-5-methylthiazol-2-carboxylate: White amphorous solid, m.w.: 186, m.f.: $C_7H_{10}N_2O_2S$, FTIR (KBr, ν_{max}, cm^{-1}): 3320 (-NH_2 stretch), 2989 and 2960 (-CH stretch), 1780 (CO stretch). ^1H NMR ($CDCl_3$): δ 9.2 (s, 1H, -NH_2), 4.3(q, 2H, -CH_2 of ester group), 2.8 (s, 3H, -CH_3), 1.5(t, 3H, -CH_3 of ester group). Mass m/z: 187 (M + 1).

Cyclic heptapeptide (III): Cyclo-L-[-4-amino-5-methylthiazol-2-carboxyl-Phe-Pro-His-Pro-Pro-Gly-Leu]: Pale yellowish semisolid mass, m.w.: 885, m.f.: $C_{43}H_{55}N_{11}O_8S$, $[\alpha]_D^{25}$-74.1^0 (c 0.011 MeOH); UV (100 % MeOH) λ_{max} 320; FTIR (KBr, ν_{max}, cm^{-1}): 3328 (-NH stretch), 2986 and 2967 (-CH stretch), 1721(CO stretch), 1575 (-NH bend), 1468 (-CH bend). ^1H NMR ($CDCl_3$): δ 7.6-7.4 (m, 7H, Ar-H), 7.2 (s, 1H, -NH of imidazole), 7.0-6.9 (s, 4H, -NH), 4.3-4.1 (m,-CH_2 of Phe), 3.5-3.4 (m, 2H, -CH_2 of Pro), 3.2-3.1 (m, 2H, -CH_2 of Pro), 2.8 (s, 3H, -CH_3), 2.0 (m, 2H, -CH_2 of Pro), 1.6 (m, 3H, -CH), 1.5 (s, 3H, -CH_3 group attached to the thiazole ring, 1.3-1.0 (m, 6H, 2(-CH_3) of Leu. FAB mass m/z: 886 (M + 1). ^{13}C NMR ($CDCl_3$): δ : 169.9-174.7 (CO of amide group), 53.3 (α-CH of Phe), α-CH_2 of Phe), 60.7-64.3 (Pro β-CH_2), 28.9-32.8 (Pro γ-CH_2), 22.7-25.8 (Pro δ-CH_2), 48-48.4 (Pro α-CH), 51(His α-CH), 29.3 (His β-CH_2), 20.8-23.1 (Leu α 2-CH_3), 44.7 (Gly α-CH_2).

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