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Full Length Research Paper

Solid-phase total synthesis of cyclic pentapeptide Longicalycinin A, by using 2-chlorotrityl chloride resin

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Naturally isolated cyclic pentapeptide Longicalycinin A, which showed cytotoxicity to Hep G2 cancer cell line with an IC_{50} value 13.52 *μ*g/mL, has been successfully synthesized by solid-phase methodology with Fmoc/*t*-Bu protecting schemes *via* solution-phase macrocyclization. 2-chlorotrityl chloride resin was used as solid support. Solution phase macrocyclization of linear pentapeptide precursor was carried out by two different routes with mild and severe acidic conditions correspondingly and found that percentage yield of Longicalycinin A under mild acidic condition (33%) was better than severe acidic conditions (13.7%). In addition, the cytotoxicity against Dalton's lymphoma ascites (DLA) and Ehrlich's ascites carcinoma (EAC) cell lines with CTC_{50} values were found to be 2.62 and 6.17 *μ*M respectively.

Key words: Longicalycinin A, solid phase synthesis, 2-chlorotrityl chloride, solution-phase macrocyclization, cytotoxicity.

INTRODUCTION

The researchers have paid special attention to the cyclic peptides due to their unique structures and wide spread pharmacological profile, which may solve the problem of wide spread increase of confrontation towards conventional drugs (Poteau and Trinquier, 2005; Tan and Zhou, 2006; Morel et al., 2002; Jennings et al., 2001). Naturally isolated cyclopeptides show various pharmacological activities, such as antitumour (Salvatella et al., 2003; Takeya et al., 1993; Morita et al., 1995), antimalarial (Linington et al., 2007; Isaka et al., 2007), estrogen-like (Itokawa et al., 1995) and tyrosinase inhibitory (Morita et al., 1994).

Solid phase synthesis has many advantages over the classical peptide extraction like the automation of the reaction and the problems of purification and solubilization of the peptide no longer exist since it remains attached to the solid matrix. The most critical feature of solid-phase synthesis is the attachment of substrate to the polymeric resin (Scott, 2009), so the first anchoring unit should be loaded efficiently with polystyrene support and the linkage should be stable enough to tolerate the subsequent chemical transformations and chemical environment. Trityl linker is commonly used in solid-phase peptide synthesis which allows the "protected" compound to be subjected to various chemical manipulations and consequently to afford pure compounds without numerous purification steps (Park et al., 2004; Olsen et al., 2004; Lundquist et al., 2006; Crestey et al., 2008). Additionally, the trityl linker is readily cleaved under mild acidic condition (1% TFA in dichloromethane) owing to the high stability of trityl cations (Rothman et al., 2003).

Cyclic head-to-tail connected peptides can easily be synthesized in good purity using standard procedures

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and orthogonally protected amino acid residues. However, the cyclization step is critical and depends upon the sequence of peptide, structural constraints, and the resulting ring size. A number of studies have been developed to improve this crucial step and to obtain cyclic peptides in good yield with minimum side reactions (Yang and Morriello, 1999; Li et al., 2002).

A new cyclic pentapeptide Longicalycinin A [*cyclo*-(Gly^1–Phe^2–Pro^3–Tyr^4–Phe^5–)] was isolated from the plant *Dianthus superbus var. longicalycinus* which has been used for treating carcinoma, diuretic and inflammatory diseases showed cytotoxicity to Hep G2 cancer cell line with an IC_{50} value 13.52 μg/mL (Hsieh et al., 2005). By keeping in mind the significance of cytotoxic activity of Longicalycinin A and solid-phase peptide synthesis, this cyclic pentapeptide has been successfully synthesized by solid-phase methodology with Fmoc/*t*-Bu protecting scheme *via* 2-chlorotrityl chloride resin, while macrocyclization was carried out in solution-phase. Solution phase macrocyclization of linear pentapeptide precursor was carried out by two different routes with mild and severe acidic conditions simultaneously and found that percentage yield of Longicalycinin A under mild acidic condition (33%) was better than severe acidic conditions (13.7%). In addition, the cytotoxicity against Dalton's lymphoma ascites (DLA) and Ehrlich's ascites carcinoma (EAC) cell lines with CTC_{50} values were found to be 2.62 and 6.17 μM, respectively.

MATERIALS AND METHODS

All other commercially obtained reagents and solvents were used without further purification. Trifluoroacetic acid (TFA), triisopropylsilane (TIS), Fmoc amino acids and coupling reagents N-hydroxybenzotriazole (HOBt), O-(benzotriazol-1-yl)-*N,N,N′,N′*–tetramethyluranium hexafluoro phosphate (HBTU) were supplied by novabiochem. Solvents like acetonitrile (MeCN), pyridine, piperazine, *N,N*-diisopropylethylamine (DIPEA) and diethyl ether were purchased from Fisher Scientific. 2-chlorotrityl chloride resin (1% DVB, 75~150 µm, 100~200 mesh, sub. rate 1.22 mmol/g) was purchased from Wako. Dichloromethane (DCM), *N, N*-dimethyl formamide (DMF) and methanol (MeOH) were dried according to the Grubb's methods (supporting information).

1HNMR spectra were recorded on Bruker NMR spectrometers operating at 600 MHz. Proton chemical shifts (δ) are reported in ppm. Data are reported as follows: chemical shift (multiplicity [singlet (s), doublet (d), triplet (t), quartet (q) and multiplet (m)], and integration). 13CNMR spectra were recorded on Bruker NMR spectrometers operating at 150 MHz, with complete proton decoupling. NMR data were collected at 25°C. Analytical thin-layer chromatography (TLC) was performed using Silica Gel 60 Å F-254 pre-coated plates (0.25 mm thickness). Visualization was accomplished after spraying with cerric sulphate reagent. High-resolution mass spectra, mass measurements and fast atom bombardment (FAB) mass measurements were recorded on mass spectrometers JEOL JMS HX-110; FAB source using glycerol or thioglycerol as the matrix and cesium iodide (CsI) as internal standard. Optical rotations were measured with a JASCO DIP-360 digital polarimeter at the sodium D line (path length 50 mm). Low-resolution electron impact mass spectra were recorded on a Finnigan MAT-311 with MASPEC Data system. Peak matching, field desorption (FD) and field ionization (FI) were performed on the Finnigan MAT-312 mass spectrometer. Reverse phase LC-908W recycling preparative HPLC was performed on polyamine column with gradient elution using acetonitrile (MeCN) and HPLC grade water (H_2O) (1:2) respectively.

Loading condition for the first amino acid

To the 2-chlorotrityl chloride resin, preswollen in dichloromethane (DCM) (1h), Fmoc-Gly (713.5 mg, 2.4 mmol, 2 equivalents), and *N,N*-diisopropylethylamine (DIPEA) (1.07 mL, 6.10 mmol, 5 equivalents) were added under anhydrous condition. The reaction mixture was stirred under nitrogen atmosphere for 4 h. The reaction was terminated by addition of MeOH/DIPEA (9:1) (10 mL) as scavenger to remove side products. The Fmoc-peptidyl resin **1** was immediately filtered and washed sequentially with DMF (5 mL × 3), DCM (5 mL × 3), and MeOH (5 mL × 3) respectively. The loading of 2 chlorotrityle chloride resin-bound Fmoc glycine in good yields (65 % loading level) has been obtained. After washing, the resin was dried under vacuum for 24 h. The loading efficiency of 2-chlorotrityl chloride resin was determined by loading efficiency procedure (supporting information). The loading degree was found to be 65%, which was determined by UV spectrophotometric analysis (Qin et al., 2003). For the synthesis of the remaining linear pentapeptide, the standard Fmoc protocol was followed (Veber et al., 1984).

Deprotection of Fmoc group

The Fmoc deprotected peptidyl resin was obtained by the addition of 20% piperidine / DMF solution (15 mL) in such a way that the solution just covered the surface of dried Fmoc-peptidyl resin in a two necked round bottom flask (100 mL), with a nitrogen balloon fitted syringe in one neck while the other neck was tightened with a rubber septum and shaked for 30 min, the reaction flask was then placed on a shaker (IKA® -AS130.1) (80 cycles/min). The peptidyl resin was filtered under vacuum, and sequentially washed with 20% piperidine / DMF (10 mL), DMF (5 mL × 5), and finally by DCM (5 mL × 5). The resulting compound was left to dry for 12 h. The Kaiser ninhydrin colorimetric test (supporting information) was performed to confirm the Fmoc deprotection.

Coupling of further amino acids

After loading the first amino acid on polystyrene resin, the resulting peptidyl resin was soaked in DCM (10 mL) for 1 h and then in 2 equivalent concentration of Fmoc-amino acid and treated with coupling reagents [HOBt (324.2 mg, 2.4 mmol, 2 equivalents) and HBTU (910.2 mg, 2.4 mmol, 2 equivalents)] in DIPEA (820 μL, 4 equivalents) for 2 min to form a solution, which was then added to the presoaked (DCM) peptidyl resin and agitated for 4 h at room temperature under nitrogen atmosphere. The reaction was terminated by performing the Kaiser test. After coupling and Fmoc deprotected (20% piperidine/DMF), the anchored resin was filtered and washed with DMF (5 mL × 3) and DCM (5 mL × 3), respectively.

Cytotoxicity

Synthesized Longicalycinin A was subjected to short term *in vitro* cytotoxicity study against Dalton's lymphoma ascites (DLA) (NCRC 101) and Ehrlich's ascites carcinoma (EAC) (NCRC 69) cell lines at 65.5, 32.75, 16.37, 8.18 and 4.09 μg/mL using 5-fluorouracil (5-FU) as reference compound (Kuttan et al., 1985) and three experiments were carried out with each concentration. Activity was assessed by

determining the percentage inhibition of DLA and EAC cells. CTC values were determined by graphical extrapolation method.

The 2-chlorotrityl–Gly^1–Fmoc **1** was subjected to alternate five coupling and five Fmoc deprotection processes, according to general Fmoc deprotection method and general amino acid coupling method as described above, to get compound 2-chlorotrityl–Gly^1–Phe^2–Pro^3–Tyr^4(O*t*-Bu)–Phe^5 **10** (Scheme 1). The progress of each step was confirmed by Kaiser test (Kaiser et al., 1970); the negative test (red color of granular resin) confirmed the coupling, while the positive test (blue color of resin) confirmed the deprotection of Fmoc.

The resulted linear pentapeptide anchored resin **10** was divided in two equal masses and subjected to solution phase macrocyclization through two different routes **A** and **B** (Scheme 2): in one route, first the cleavage step was completed then solution phase macrocyclization was performed and at the end, deprotection of the side chain (O*t*-Bu) was achieved to produce the target molecule Longicalycinin A, while in the other route the cleavage from the solid support and side chain deprotection of O*t*-Bu occurred in a single step followed by solution phase macrocyclization to obtain the target compound (Scheme 2). After cleavage of pentapeptidyl anchored resin, each step was purified by reverse-phase recycling preparative HPLC by using the solvent system of CH_3CN: H_2O, 1:2. A graphical representation of Schemes 1 and 2 is shown in Figure 1.

Macrocyclization Route A

***Synthesis of compound* Gly^1–Phe^2–Pro^3–Tyr^4(O*t*-Bu)–Phe^5 11**

A mixture of 1% trifluoroacetic acid/dichloromethane TFA/DCM was agitated with linear pentapeptide anchored resin **10** for 2 min under nitrogen atmosphere and then filtered under vacuum. The filtrate was treated with a mixture of 10% pyridine/MeOH (50 mL). The same procedure was repeated 10 times and then the filtrate was concentrated in a rotavapor and the crude linear side chain protected pentapeptide Gly^1–Phe^2–Pro^3–Tyr^4(O*t*-Bu)–Phe^5 **11** was purified by HPLC (CH_3CN : H_2O, 1:2) to give one major peak identified on the bases of FAB-MS and ^1H spectrum data as linear side chain protected pentapeptide **11**.

Synthesis of compound [cyclo-(Gly^1-Ph^2-Pro^3-Tyr^4(Ot-Bu)-Phe^5-)] 12

Side chain protected linear pentapeptide **11** (250.7 mg, 0.364 mmol) was dissolved in CH_3CN (360 mL) and treated with HOBT (50.0 mg, 0.364 mmol), HBTU (138.1 mg, 0.364 mmol) and DIPEA (0.38 mL, 2.184 mmol). The solution was maintained at 23°C for 4 days by continuous slow stirring under nitrogen atmosphere and after 4 days, the solution was concentrated in a rotavapor resulting in a yellowish powder which was purified by HPLC (CH_3CN : H_2O, 1:2) to give one major peak identified on the bases of FAB-MS data as a side chain protected cyclic pentapeptide [*cyclo*-(Gly^1-Ph^2-Pro^3-Tyr^4(O*t*-Bu)-Phe^5-)] **12**, (248.3 mg, 99.04 % with respect to compound **11**) (Scheme 2).

Deprotection of compound [cyclo-(Gly^1–Phe^2–Pro^3–Tyr^4(Ot-Bu)–Phe^5)] 12

The side chain deprotection of [*cyclo*-(Gly^1–Phe^2–Pro^3–Tyr^4(O*t*-Bu)–Phe^5)] **12** was achieved by a 2 mL mixture of TFA/H_2O/TIS (95:2.5:2.5). The reaction mixture was kept under stirring for 1 h under nitrogen atmosphere, the solution was concentrated in a rotavapor and the crude cyclic pentapeptide was purified by HPLC (CH_3CN : H_2O 1:2) to give a pure target cyclic pentapeptide Longicalycinin A (81.93 mg, 32.9 % with respect to compound **12**). Furthermore, the recycling of carboxylic acid (e.g. preferably trifluoroacetic acid) trityl ester resins was done by treating the resin with HC1 in CH_2C1_2.

Macrocyclization by Route B

Synthesis of compound Gly^1-Phe^2-Pro^3-Tyr^4-Phe^5 13

A mixture of trifluoroacetic acid/dichloromethane/triisopropylsilane (TFA/DCM/TIPS) (10:9:1) was added to the linear pentapeptide anchored resin 2-chlorotrityl–Gly^1–Phe^2–Pro^3–Tyr^4(O*t*-Bu)–Phe^5 **10** and stirred for 30 min. Under such strong acidic condition cleavage of linear pentapeptide (Gly^1-Phe^2-Pro^3-Tyr^4(O*t*-Bu)-Phe^5) from the 2-chlorotrityl resin as well as side chain deprotection (O*t*-Bu) were carried out in one step. Then this mixture was filtered and washed with a solution of TFA/DCM/TIPS (10:9:1, 20 mL), and DCM (30 mL) after which the filtrate was concentrated in a rotavapor under vacuum and the crude linear pentapeptide was purified by HPLC (CH_3CN : H_2O, 1:2); the HPLC profile showed one major peak which was identified on the bases of FABMS data as linear pentapeptide (Gly^1-Phe^2-Pro^3-Tyr^4-Phe^5) **13** (245.1 mg) (Scheme 2).

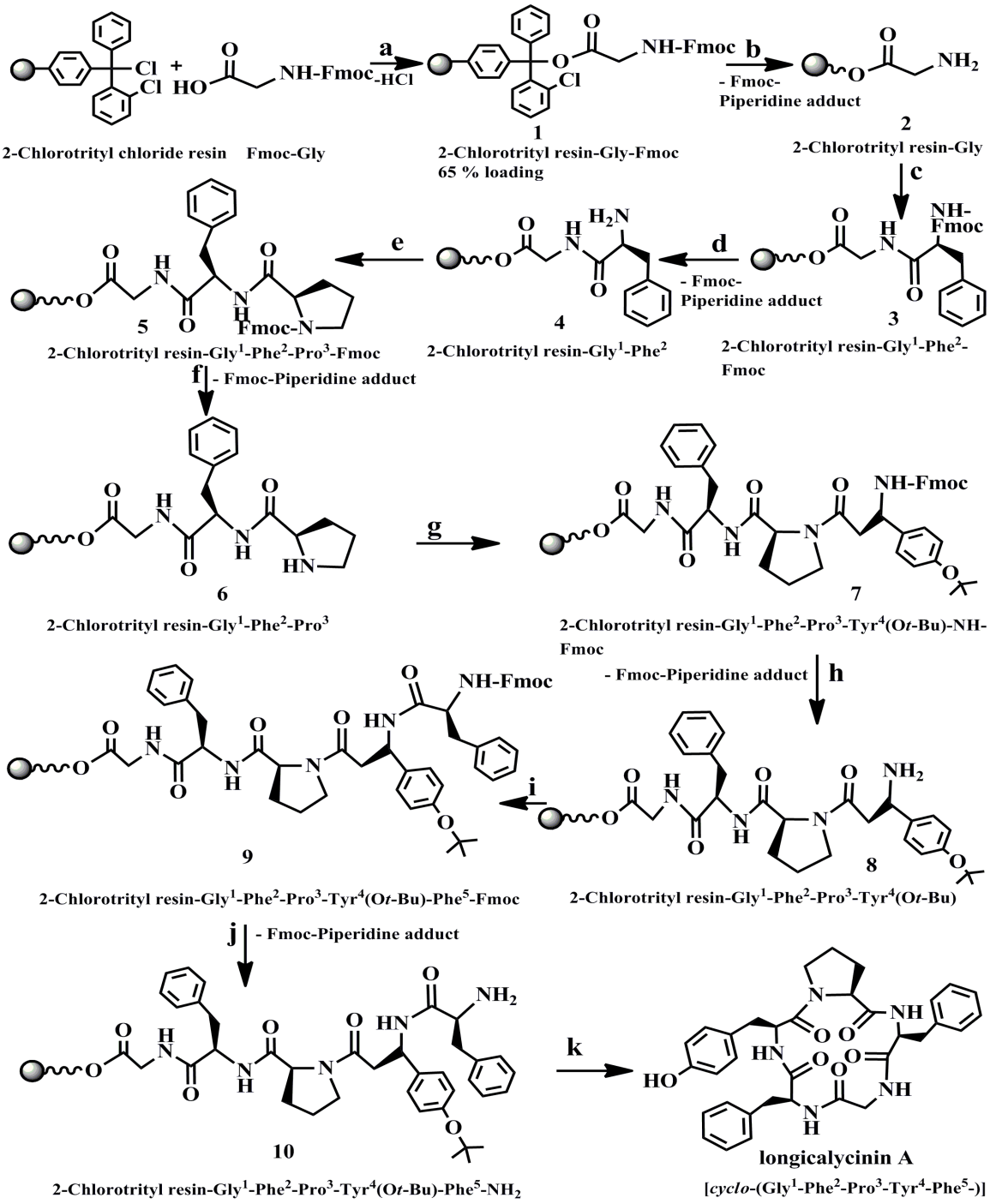
Solution-phase macrocyclization of linear pentapeptide Gly^1-Phe^2-Pro^3-Tyr^4 -Phe^5 13

Solution-phase macrocyclization of linear pentapeptide Gly^1-Phe^2-Pro^3-Tyr^4 -Phe^5 **13** was carried out by dissolving 20 mg, 0.0317 mmol of compound **13** in CH_3CN (31.79 mL) and treated with HOBT (4.4 mg, 0.0317 mmol), HBTU (12.0 mg, 0.0317 mmol) and N,N-diisopropylethylamine (DIPEA) (0.038 mL, 0.2184 mmol). The solution was kept at 23°C for 4 days by continuous slow stirring under nitrogen atmosphere and then the solution was concentrated *in vacuo*, resulting in a white yellowish powder and was purified by HPLC (CH_3CN:H_2O, 1:2). The major peak was identified on the bases of FAB-MS, 1H spectrum, 13C spectrum (Table 1), HMBC and HMQC co-relation data as cyclic pentapeptide [*cyclo*-(Gly^1-Phe^2-Pro^3-Tyr^4-Phe^5-)] Longicalycinin A (33.5 mg, 13.7 % with respect to compound **13**).

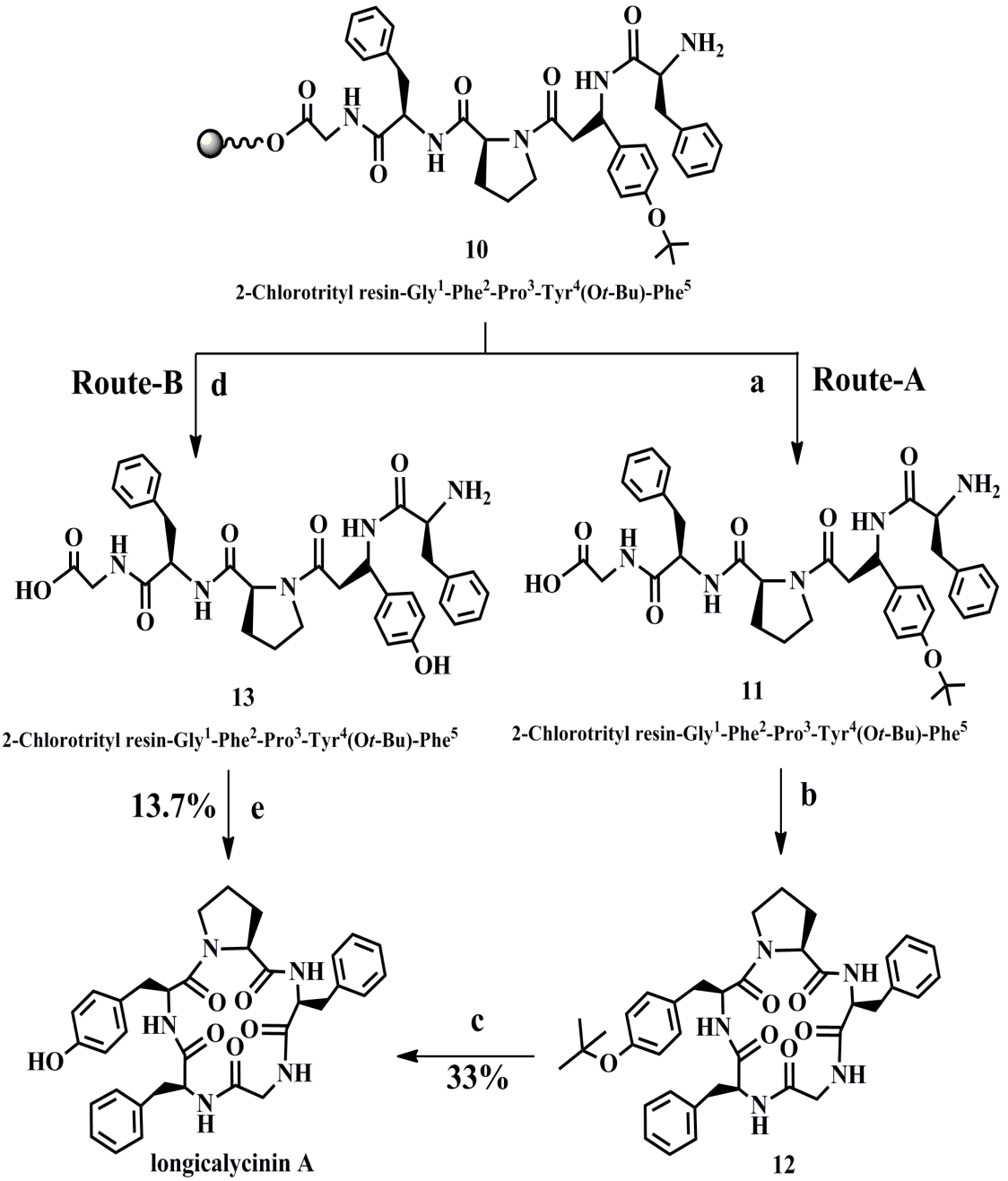
RESULTS

Characterization of Gly^1–Phe^2–Pro^3–Tyr^4(O*t*-Bu)–Phe^5 11

The physical appearance as an amorphous solid (250.7 mg, 54.05 % with respect to loaded glycine) (scheme 2), R*f* = 0.22 (2:8 MeOH/$CHCl_3$) ; FABMS (+ve) 686.81, 1HNMR (300 MHZ, C_5D_5N, 298 K, major conformation) δ 10.70 (s, OH), δ 8.03 (m, 3H (NH)), δ 7.49 (m, 4H), δ 7.18-7.29 (m, 8H), δ 6.94 (d, 2H), δ 5.15 (t, 1H), δ 5.11 (m, 2H(NH_2)), δ 4.40 (m, 1H), δ 4.14 (s, 2H), δ 3.97 (m, 5H), δ 3.44-3.51 (m, 3H), δ 2.86 (m, 1H), δ 2.32 (m, 2H), δ 2.02 (m, 2H), δ 1.41(s, 9H) (Figure 1B). 13CNMR (C_5D_5N, 125 MHz) δ 174.7, δ 170.5, δ 171.5, δ 170.2, δ 170.1, δ 157.2, δ 137.7, δ 136.4, δ 131.1, δ 128.6, δ 128.3, δ 128.1, δ127.6, δ 125.8, δ 124.8, δ 123.5, δ 116.2, δ 61.1, δ 56.2, δ 55.4, δ 55.7, δ 46.3, δ 44.4, δ 37.6, δ 37.3, δ 37.2, δ 31.7, δ 22.8, δ 11.5 (Figure 2).



Scheme 1. The Solid-Phase Synthesis of Longicalycinin A *via* 2-chlorotrityl Chloride Linker Reagents.
(**a**) (1) Fmoc-Gly-OH (713.5 mg, 2.4 mmol) (2 equivalents) , (2) DCM (10 mL), (3) DIPEA (1.07 ml, 6.10 mmol) (5 equivalents), Stirring 4h (under nitrogen), (4) MeOH/DIPEA (9:1); (**b**) 20% piperidine/DMF; (**c**) (1) Fmoc-Phe-OH (929.9 mg,2.4mmol) (2 equivalents.), (2) HBTU (910.2 mg, 2.4 mmol) (2 equivalents), (3) HOBT (324.2 mg, 2.4 mmol) (2 equivalents), (4) DIPEA 820 μL (4 equivalents); (**d**) 20% piperidine/DMF; (**e**) (1) Fmoc-Pro-OH (809.7 mg, 2.4 mmol) (2 equivalents), (2) HBTU (910.1 mg, 2.4 mmol) (2 equivalents), (3) HOBT (324.2 mg, 2.4 mmol) (2 equivalents), (4) DIPEA (820 μL) (4 equivalents); (**f**) 20% piperidine/DMF; (**g**) (1) Fmoc-Tyr (*O*t-Bu)-OH (1103.1 mg, 2.4 mmol) (2 equivalents), (2) HBTU (910.2 mg, 2.4 mmol) (2 equivalents), (3) HOBT (324.2 mg, 2.4 mmol) (2 equivalents), (4) DIPEA (820 μL) (4 equivalents); (**h**) 20% piperidine/DMF; (**i**) (1) Fmoc-Phe-OH (929.9 mg, 2.4 mmol) (2 equivalents), (2) HBTU (910.2 mg, 2.4 mmol) (2 equivalents), (3) HOBT (324.2 mg, 2.4 mmol) (2 equivalents), (4) DIPEA (820 μL) (4 equivalents); (**j**) 20% piperidine/DMF; (**k**) (1) TFA/DCM/TIPS (10:9:1) (20 mL), (2) CH_3CN (31.79 mL), (3) HOBT (4.4 mg, 0.0317 mmol), (4) HBTU (12.0 mg, 0.0317 mmol), (5) DIPEA (0.038 mL, 0.2184 mmol).



Scheme 2. The Solution-Phase Macrocyclization.
(a) (1) 1% TFA/DCM, (2) 10% pyridine/MeOH ((50 mL); (b) CH_3CN (360 mL), HOBT (50.0 mg, 0.364 mmol), HBTU (138.1 mg, 0.364 mmol), DIPEA (0.38 mL, 2.184 mmol); (c) TFA/H_2O/TIS (95:2.5:2.5); (d) TFA/DCM/TIPS (10:9:1), (e) CH_3CN (31.79 mL), HOBT (4.4 mg, 0.0317 mmol), HBTU (12.0 mg, 0.0317 mmol), (DIPEA) (0.038 mL, 0.2184 mmol).

Characterization of [*cyclo*-(Gly^1-Ph^2-Pro^3-Tyr^4(O*t*-Bu)-Phe^5-)] 12

R*f* = 0.21 (2:8 MeOH/$CHCl_3$) ; FABMS (+ve) 668.79, 1HNMR (300 MHZ, C_5D_5N, 298 K, major conformation) δ 8.0 (m, 2H(NH)), δ 7.40 (m, 4H), δ 7.27-7.29 (m, 6H), δ 7.18 (m, 2H), δ 6.94 (dd, 2H), δ 5.10 (m, 1H), δ 4.92 (m, 1H), δ 4.40 (m, 1H), δ 4.15 (d, 1H), δ 3.95 (m, 1H), δ 3.19-3.44 (m, 4H), δ 2.61-2.86 (m, 2H), δ 2.34 (m, 2H), δ 2.09 (m, 2H), δ 1.41 (s, 9H) (Figure 3). 13CNMR (C_5D_5N, 125 MHz) δ 172.7, δ 172.5, δ 172.3, δ 171.5, δ 170.4, δ 158.0, δ 138.5, δ 137.5, δ 130.0, δ 129.9, δ 129.8, δ 128.7, δ128.6, δ 126.8, δ 123.8, δ 123.8, δ 116.3, δ 61.7, δ 56.4, δ 55.7, δ 55.1, δ 46.9, δ 45.5, δ 38.2, δ 37.6, δ

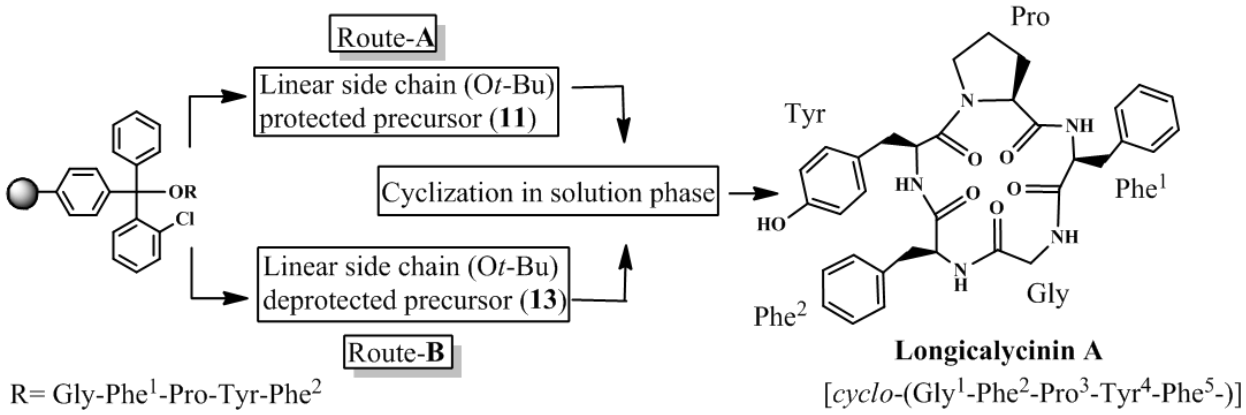


Figure 1A. Graphical abstract of Scheme 1 (The Solid-Phase Synthesis of Longicalycinin A *via* 2-chlorotrityl Chloride Linker) and Scheme 2 (The Solution-Phase Macrocyclization).

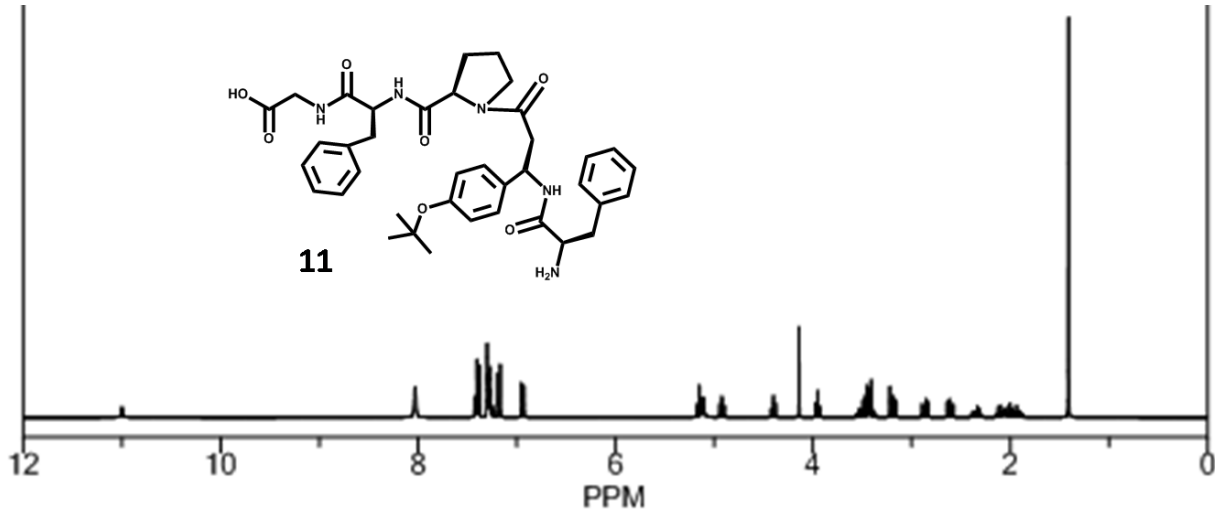


Figure 1B. 1HNMR of compound.

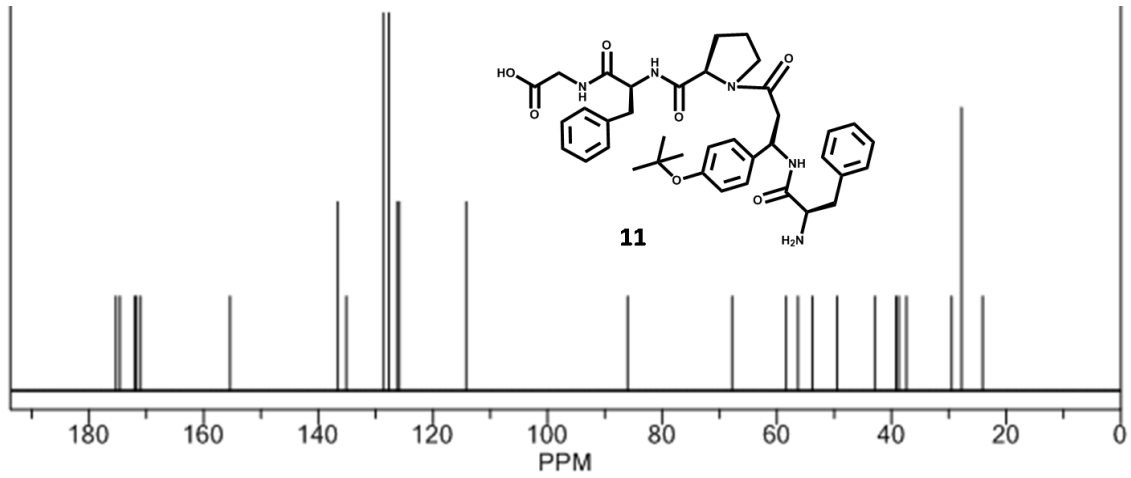


Figure 2. 13CNMR of compound 11

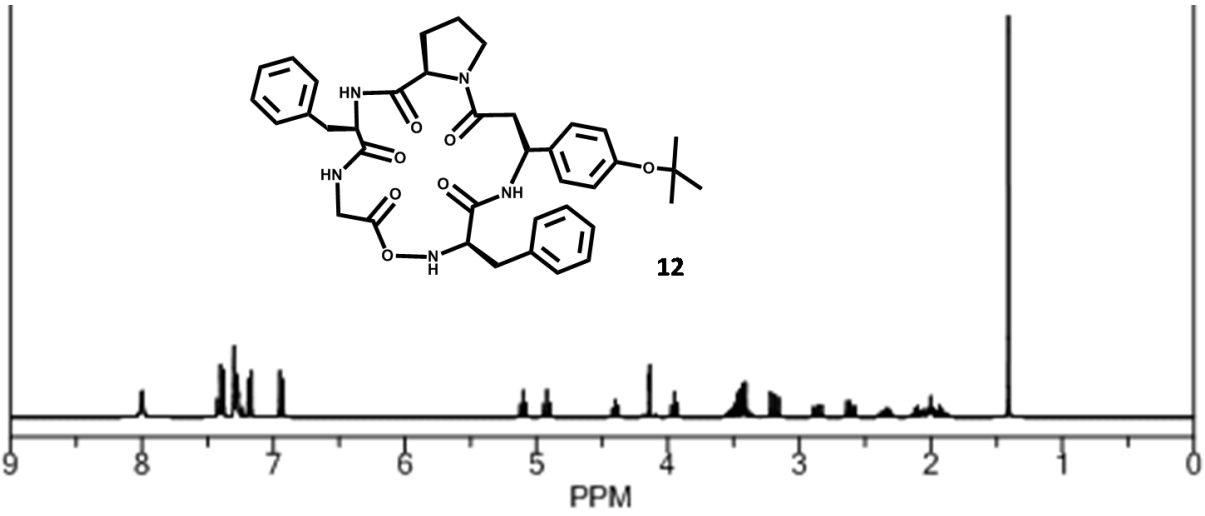


Figure 3. 1HNMR of compound.

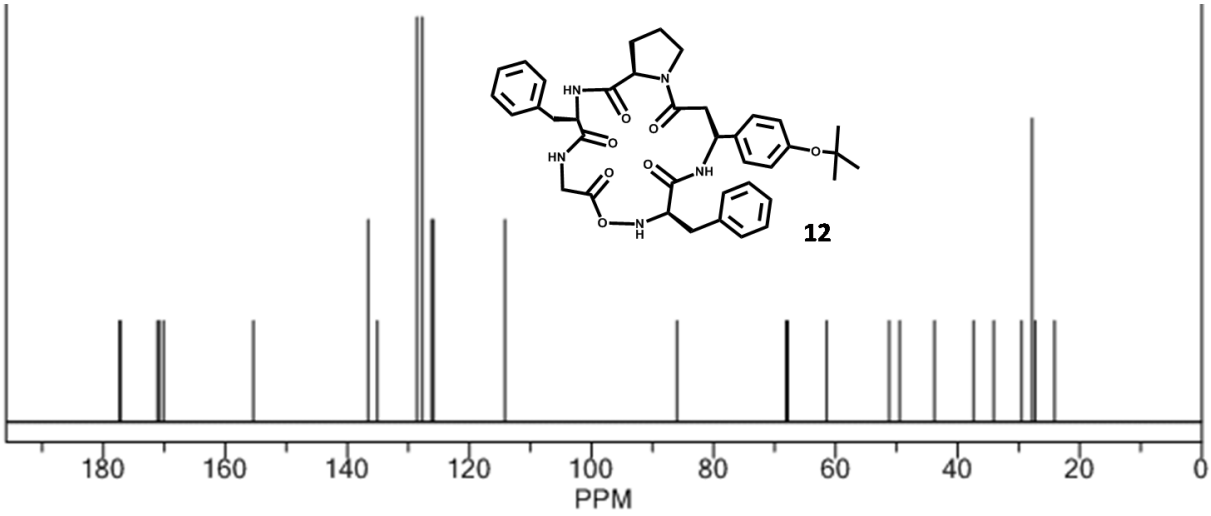


Figure 4. 13CNMR of compound 12.

37.5, δ 31.7, δ 22.9, δ 11.5 (Figure 4).

Characterization of compound Gly[1]-Phe[2]-Pro[3]-Tyr[4]-Phe[5] 13

R*f* = 0.24 (2:8 MeOH/$CHCl_3$) ; FABMS (+ve) 630.7, 1HNMR (300 MHZ, C_5D_5N, 298 K, major conformation) δ 10.86 (s, 1H(OH)), δ 8.03 (s, 3H), δ 7.40 (dd, 4H), δ 7.29 (dd, 4H), δ 7.12 (d, 2H), δ 6.74 (d, 2H), δ 5.35 (s, 1H(OH)), δ 5.17 (m, 2H(NH_2)), δ 5.15 (m, 1H), δ 4.92 (m, 1H), δ 4.40 (m, 1H), δ 4.14 (s, 2H), δ 3.95 (m, 1H), δ 3.44 (m, 4H), δ 2.95 (m, 2H), δ 2.34 (m, 2H), δ 2.02-2.09 (m, 4H) (Figure 5). 13CNMR (C_5D_5N, 125 MHz) δ 175.7, δ 172.5, δ 171.5, δ 170.6, δ 170.5, δ 157.6, δ 137.4, δ 136.7, δ 131.8, δ 129.6, δ 128.5, δ 128.1, δ 127.4, δ 125.6, δ 124.7, δ 123.5, δ 116.3, δ 61.2, δ 56.1, δ 55.6, δ 55.2, δ 46.1, δ 44.6, δ 37.5, δ 37.3, δ 37.2, δ 31.7, δ 22.8, δ 11.5 (Figure 6).

Characterization of [*cyclo*-(Gly[1]-Phe[2]-Pro[3]-Tyr[4]-Phe[5]-)] Longicalycinin A

Longicalycinin A [*cyclo*-(Gly[1]-Phe[2]-Pro[3]-Tyr[4]-Phe[5]-)] physical appeared as a white yellowish amorphous substance. The specific rotation $[\alpha]_D$ was -12° in MeCN solvent. The FAB-MS exhibited $[M+H]^+$ at m/*z* 612. The 1HNMR spectrum (Table 1) of compound [*cyclo*-(Gly[1]-Phe[2]-Pro[3]-Tyr[4]-Phe[5]-)] showed a broad singlet for NH of Phe[5] at δ 9.77. A broad doublet at δ 8.02 (*J* = 9.9 Hz) was due to (NH) proton of Tyr[4], a doublet of one proton at

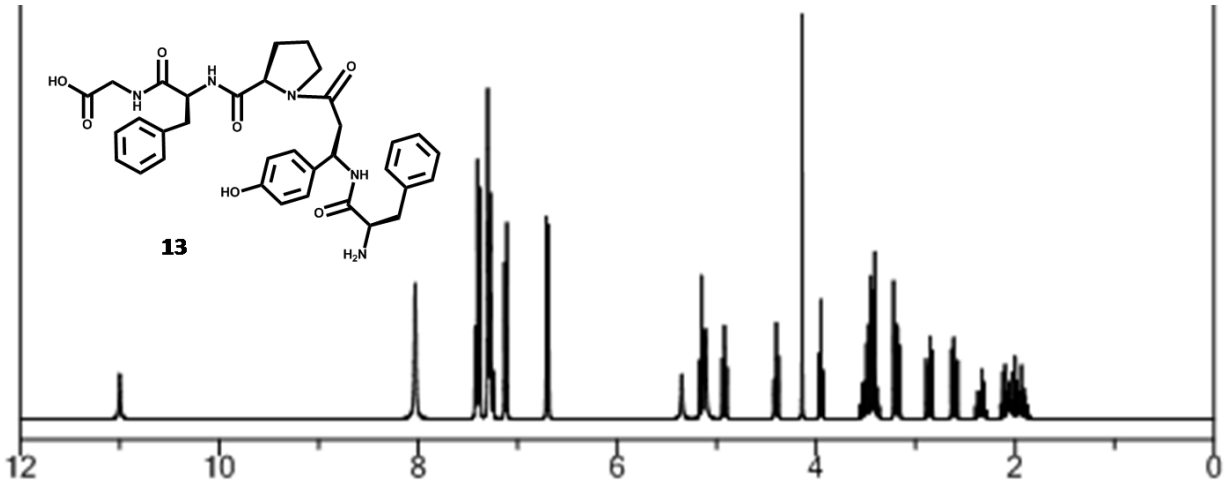


Figure 5. 1HNMR of compound.

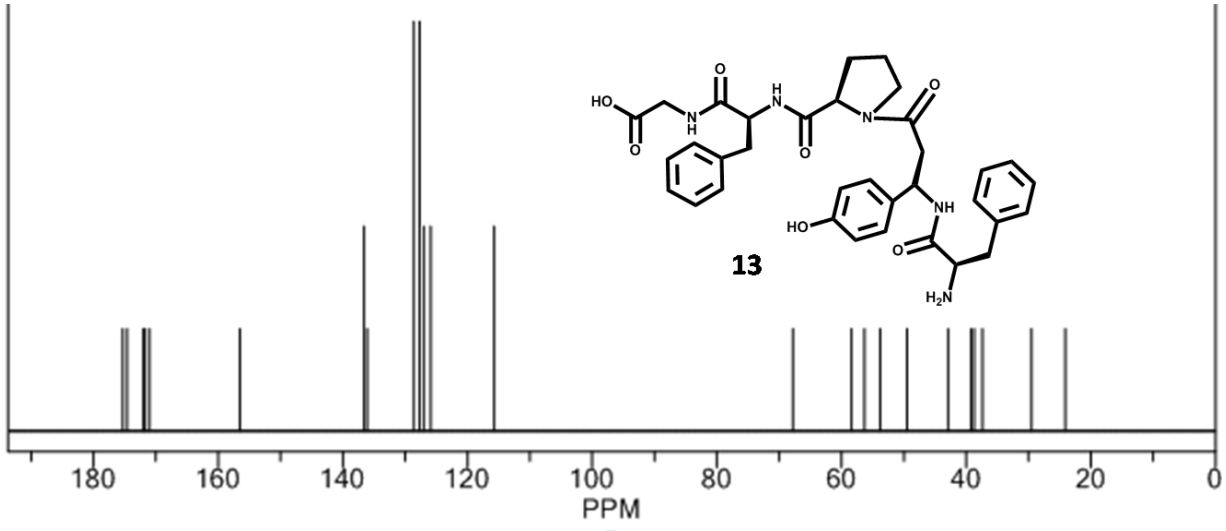


Figure 6. 13CNMR of compound 13.

δ 9.05 (J = 8.2 Hz) was due to NH of Gly[1], a doublet of one proton at δ 7.92 (J = 8.6 Hz) was due to NH of Phe[2] two doublets at δ 7.45 (J = 8.4 Hz) and δ 7.01 (J = 8.4 Hz) were due to aromatic protons of Tyr[4], a triplet at δ 3.70 (J = 9.9 Hz) was due to α proton of Pro[3]. A negative ninhydrin test indicated its cyclic nature. The difference of 13CNMR chemical shifts of Pro[4] ($\Delta\delta$ C_β-C_γ 8.8 ppm) provided evidence that the amide bond in the Pro[3] residue is *cis* (Mergler et al., 1988), (Figures 7, 8).

Cytotoxicity

The results of mean percentage inhibition (mean) and standard deviation (SD) are shown in Tables 2 and 3. Synthesized cyclopeptide exhibited high cytotoxic activity against DLA and EAC cell lines with CTC values of 2.62 and 6.17 μM respectively which in comparison to standard drug 5-fluorouracil (5-FU) (CTC) values were 37.36 and 90.25 μM.

DISCUSSION

To the best of our knowledge the solid-phase syntheses of Longicalycinin A was for the first time carried out using Fmoc/*t*-Bu (Góngora-Benítez et al., 2013) protecting schemes *via* solution-phase Macrocyclization (Jain et al., 2009) Figure 1 A. All the Fmoc-protected amino acids were activated by the HOBt/HBTU in the presence of diisopropylethylamine (DIPEA) before coupling. Immobilization was carried out on 2-chlorotritylchloride resin

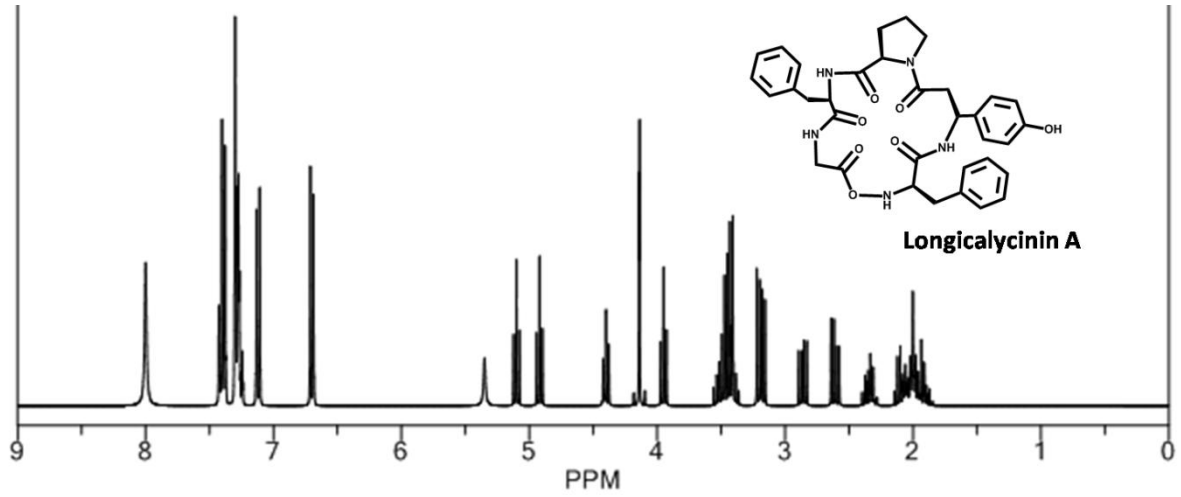


Figure 7. 1HNMR of compound Longicalycinin.

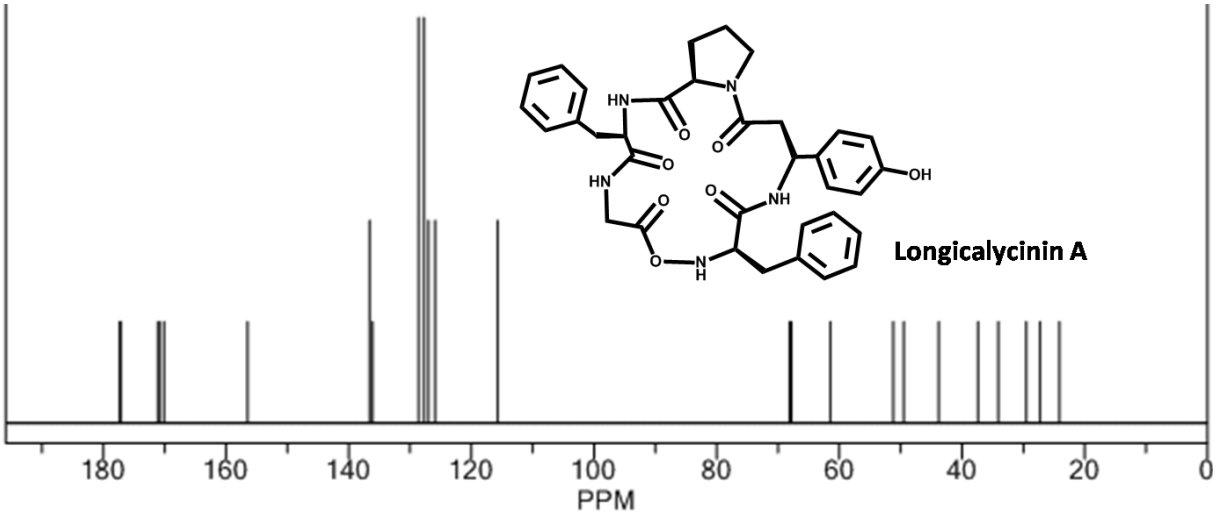


Figure 8. 13HNMR of compound Longicalycinin A.

through the carboxylic acid group of the first Fmoc-Gly *via* 2-chlorotritylchloride linker (Tegge et al., 2010) by DIPEA treatment under anhydrous conditions. The loading step was repeated twice to achieve best loading level. The resulting loading degree (65%) was determined by UV spectrophotometeric analysis (Qin et al., 2003). The resin was subjected to various coupling-deprotection steps to build the linear pentapeptide as the precursor for the cyclic pentapeptide Longicalycinin A. The progress of amino acid coupling was checked through Kaiser's ninhydrin colorimetric test (Kaiser et al., 1970). Fmoc deprotection before each coupling step was achieved by treatment of peptidyl resin with 20% solution of piperidine in DMF.

The linear pentapeptide part of peptidyl resin **10** was cleaved from resin *via* two routes as shown in Scheme 2. In route **A** the cleavage step was achieved by using mild acidic condition to get the side chain protected pentapeptide Gly^1–Phe^2–Pro^3–Tyr^4(O*t*-Bu)–Phe^5 **11** which was subjected to side chain deprotection followed by cyclization in solution phase to get the title compound, while in route **B** the severe acidic condition was used, by which both cleavage of linear pentapeptide from resin and side chain (O*t*-Bu) deprotection of tyrosine unit were done in a single step and then the cleaved side chain deprotected pentapeptide Gly^1-Phe^2-Pro^3-Tyr^4-Phe^5 **13** was subjected to solution phase cyclization to obtain Longicalycinin A. It was found that the percentage yield of Longicalycinin A under mild acidic condition (33%) was better than under severe acidic conditions (13.7%). In

Table 1. ^{1}HNMR and ^{13}CNMR data of Longicalycinin A in Pyridine (C_5D_5N).

Unit		δH, mult. (*J* in Hz)	δC
Gly[1]	C=O		172.3
	NH	7.92 (d, 8.2, 1H)	
	α	4.32 (dd, 2H)	45.5
Phe[2]	C=O		172.5
	NH	9.05 (d, 8.6, 1H)	
	α	5.18 (m, 1H)	55.1
	ß	3.32 (m, 1H)	38.2
		3.21 (m, 1H)	
	Ar	7.15-7.21 (m, 5H)	138.5
			128.6
			129.8
			123.8
Pro[3]	C=O		171.5
	NH		
	α	3.70 (t, 9.9, 1H)	61.7
	ß	1.45 (m, 2H)	31.7
	γ	2.02 (m, 1H)	22.9
		1.88 (m, 1H)	
	δ	3.82 (m, 1H)	46.9
		3.70 (m, H)	
Tyr[4]	C=O		170.4
	NH	8.02 (d, 9.9, 1H)	
	α	4.58 (m,1H)	55.7
	ß	3.08 (m, 1H)	37.6
		3.13 (m, 1H)	
	Ar	7.45 (d, 8.4, 2H)	130.0
		7.01 (d, 8.4, 2H)	126.8
			116.3
			158.0
Phe[5]	C=O		172.7
	NH	9.77 (br s, 1H)	
	α	5.18 (m, 1H)	56.4
	ß	3.40 (m, 2H)	37.5
	Ar	7.18-7.24 (m, 5H)	137.5
			128.7
			129.9
			123.8

route-**A** the tertiary butyl protecting group remained intact with the tyrosine unit which limits the chances of side products resulting in a higher yield in route-**A** as compared to route-**B**, in which deprotected side chain of the tyrosine unit may react with terminal carboxylic acid group of glycine to form an ester linkage. Also in route-B, severe acidic condition may cause the cleavage of amide bonds, and the carboxylic acid group of Gly[1] unit may also react with coupling reagents of HOBT and HBTU (Han et al., 2004).

Each resulting compound was purified by reverse phase recycling preparative HPLC and identified on the basis of Fast Atom Bombardment Mass Spectroscopy (FAB MS) data, 1HNMR spectrum, 13CNMR spectrum and Heteronuclear Multiple Bond Correlation (HMBC) and Heteronuclear Multiple-Quantum Correlation (HMQC). In addition, high cytotoxicity against Dalton's lymphoma ascites (DLA) and Ehrlich's ascites carcinoma (EAC) cell lines with CTC 50 values were found to be 2.62 and 6.17 μM. Recycling reaction may be carried out in organic solvents with high absorption capacity of HCl gas (such as alkylethers, DME, Diglyme, THF or dioxane). The recycling reaction is preferably carried out in the presence of THF or dioxane, most preferred in the presence of dioxane.

Conclusion

A novel, mild and rapid procedure for the loading of 2

Table 2. Cytotoxic activity data of Longicalycinin A for DLA cells.

Conc. (µg/ml)	Sample	Live cells counted	Dead cells counted	% growth inhibition	CTC_{50} (µm)	Mean % growth inhibition± SD	Standard % growth inhibition	Standard CTC_{50} (µm)
	LA1	0	40	100			100	
65.5	LA1	1	39	97.5		96.66±3.81	100	
	LA1	3	37	92.5			100	
	LA2	3	37	92.5			100	
32.75	LA2	3	37	92.5		91.66±1.44	100	
	LA2	4	36	90.0			100	
	LA3	8	32	80.0			75.0	
16.37	LA3	7	33	82.5	2.62	80.83±1.44	75.0	37.36
	LA3	8	32	80.0			75.0	
	LA4	12	28	70.0			65.0	
8.18	LA4	12	28	70.0		70.0±0.00	65.0	
	LA4	12	28	70.0			65.0	
	LA5	25	15	37.5			42.5	
4.09	LA5	26	14	40.0		39.16±1.44	42.5	
	LA5	26	14	40.0			42.5	

LA = Longicalycinine A, SD = Standard deviation, DLA = Dalton's lymphoma ascites, CTC = Common Toxicity Criteria.

Table 3. Cytotoxic activity data of Longicalycinin A for EAC cells.

Conc. (µg/ml)	Sample	Live cells counted	Dead cells counted	% growth inhibition	CTC_{50} (µm)	Mean % growth inhibition± SD	Standard % growth inhibition	Standard CTC_{50} (µm)
	LA6	0	30	100			100	
65.5	LA6	0	30	100		98.86±1.96	100	
	LA6	1	29	96.66			100	
	LA7	2	28	93.3			100	
32.75	LA7	2	28	93.3		93.3±0.00	100	
	LA7	2	28	93.3			100	
	LA8	10	20	66.6			63.3	
16.37	LA8	9	21	70.0	6.17	68.8±1.96	63.3	90.25
	LA8	9	21	70.0			63.3	
	LA9	16	14	46.6			36.6	
8.18	LA9	16	14	46.6		46.6±0.00	36.6	
	LA9	16	14	46.6			36.6	
	LA10	21	9	30.0			23.3	
4.09	LA10	22	9	30.0		31.0±1.73	23.3	
	LA10	20	10	33.3			23.3	

LA = Longicalycinine A, SD = Standard deviation, EAC = Ehrlich Ascites Carcinoma, CTC = Common Toxicity Criteria.

chlorotrityle chloride resin-bound Fmoc glycine in good yields (65 % loading level) has been developed, which will be useful for the solid-phase preparation of polypeptides containing glycine moiety in their structure. The resin recycling capability has been demonstrated using this protocol and the proposed reaction intermediates have been identified by performing the corresponding reactions in solution. The substitution level on the 2-chlorotrityl resin with Fmoc glycine was better and 0.73 mmol/g of amino acid was loaded resulting in a reasonable yield of the target peptide from route **A** (33%) by using mild acidic conditions, and route **B** (13.7%) with severe acidic condition, respectively. In route-**A** the tertiary butyl protecting group remained intact with tyrosine unit which limits the chances of side products thus resulting in a higher yield in route-**A** as compared to route-**B**. Synthesized cyclopeptide exhibited high cytotoxic activity against DLA and EAC cell lines with CTC values of 2.62 and 6.17 μM respectively, in comparison to standard drug 5-fluorouracil (5-FU) (CTC values – 37.36 and 90.25 μM). In future, Longicalycinin A will be prepared by using 4-Sulfamylbutyryl AM resin for comparative yield.

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