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Total Synthesis and Anticancer Evaluation of BZR-cotoxin IV

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Abstract

Anticancer peptides (ACPs) is a potential alternative for future cancer therapy. ACPs specifically inhibit cancer cells through a non-enzymatic membranolysis mechanism. One potential anticancer peptide worthy of investigation is BZR-cotoxin IV. Currently, research on BZR-cotoxin IV is limited to isolation, making it an interesting area for further development. One approach to developing BZR-cotoxin IV is through chemical synthesis techniques. This compound belongs to the group of cyclodepsipeptides that are naturally produced by the fungus *Bipolaris zeicola* Race 3. The synthesis of BZR cotoxin IV involves 3 steps: (1) Synthesis of the hydroxy acid precursor by conversion of L-valine amino acid to (2*R*)-hydroxyisovaleric acid, (2) synthesis of the linear depsipeptide using solid-phase peptide synthesis on 2-chlorotrityl chloride resin and (3) cyclization of the linear depsipeptide using solution phase synthesis to produce the BZR cotoxin IV compound with a purity of 11.7 %. BZR-cotoxin IV was characterized using HR-TOF-MS and ^{1}H and ^{13}C-NMR to validate the desired product. Anticancer activity testing was performed using the Resazurin assay on the HeLa cancer cell line, resulting in an IC_{50} of 187.50 μg/mL (214.014 μM) categorized as moderate.

Keywords: BZR-cotoxin IV, Anticancer peptides, Solid-phase peptide synthesis, Peptide cyclization

Introduction

Cancer is one of the deadliest diseases that causes millions of deaths worldwide [1]. This disease is characterized by the presence of abnormal cells that can grow uncontrollably and have the ability to attack and move between cells and tissues of the body. Haider *et al.* [2] stated that more than 50 % of cancer cells are resistant to commonly used anticancer drugs. This phenomenon is called multidrug resistance (MDR) [2]. Due to this, the development of new anticancer drugs is needed as an alternative to overcome the MDR problem and reduce cases of death caused by cancer.

Peptide-based anticancer drugs (anticancer peptides/ACPs) are a potential anticancer alternative because they have the advantage of being effective on target tissues and having no side effects on other normal tissues [3]. The activity of anticancer peptides is often associated with some mechanism that inhibits cancer cell growth, such as: Inhibits angiogenesis, gene expression, signal transduction and enzyme activity

[4].

ACPs are small peptides consisting of less than 50 amino acids [5] and the majority of this group consists of basic (cationic) and hydrophobic residues [6]. A large number of peptides have been shown to be effective against cancer cells, including peptides from the cyclodepsipeptide group. Several cyclodepsipeptides with anticancer properties have been reported, for example, zygosporamide was found to inhibit the growth of colon/intestinal cancer cells (HCT-16) with an IC_{50} value of 13.6 μM [7]. Another compound, geodiamolide, has shown activity against T47D Carcinoma (breast cancer) with an IC_{50} value of 6 μM [8]. Compared to linear peptides, cyclodepsipeptides have advantages, such as rigid structure, biochemical stability and the ability to penetrate cell membranes [8].

Peptide BZR-cotoxin IV (**Figure 1**) is a cyclodepsipeptide compound with an interesting structure includes 7 amino acid residues and 1 hydroxy acid residue. Therefore it has 7 peptide bonds, including an amide-N-methylated bond formed, and 1 ester bond. This compound was first isolated from the endophytic fungus *Bipolaris zeicola* Race 3, a fungus that damages corn leaves [9]. Ali *et al.* [10] also successfully isolated BZR-cotoxin IV along with 3 other similar compounds, BZR-cotoxin I, II and III, from different fungi, namely *Bipolaris sorokiniana* LK12, obtained from stem plants, herb *Rhazia stricta*.

Research on BZR-cotoxin IV still focused on its isolation techniques and characterization. However, to obtain BZR-cotoxin IV strains from the endophytic fungi *Bipolaris zeicola* Race 3 and *Bipolaris sorokiniana* LK12, complex steps were required and the number of isolates produced was very low. In order to explore the chemistry and biology of BZR-cotoxin IV, other alternatives are required to obtain this compound. One technique that can be used to obtain BZR-cotoxin IV is through chemical synthesis.

It has been demonstrated that cyclodepsipeptide synthesis can be successfully accomplished using a combination of the solid phase peptide synthesis (SPPS) and solution phase techniques [11,12]. The solid-phase approach is applied to synthesize the linear peptide, and the solution-phase approach is used for cyclization. A cyclodepsipeptide contrasin was effectively synthesized by Coin *et al.* [13] using a combination of solid- and solution-phase method where unprotected hydroxy was connected to the resin at the first stage. Cyclization was carried out in solution to get the desired contrasin. Rahim *et al.* [14] used a protected hydroxy acid (Fmoc-D-Hiv-OH) to be attached on resin during the preparation of the linear precursor on resin before it was cyclized in solution phase.

The synthesis of cyclodepsipeptide is known to be problematic [15]. It is well known that the production of ester bonds (depside) in solid phase is considerably more challenging than that of amide bonds [16]. Furthermore, Coin *et al.* [13] mentioned that diketopiperazine (DKP) can be readily formed when there is a depside bond in the backbone. The formation of DKP during the deprotection of the Fmoc can be overcome by the addition of a depside bond at the end of the coupling process [17]. Peptides are also susceptible to side reactions that may occur during the reaction process, purification and storage [18]. One example of a side reaction that is frequently observed is fragmentation/deletion. Fragmentation/deletion could selectively address peptide with characteristic sequences like N-terminal NAc-N-Alkyl, N-Alkyl-Xaa-bond, N-terminal- His-Pro-Xaa, etc [19].

In this study, we describe the strategy of synthesis of cyclodepsipeptide BZR-cotoxin IV and describe its anticancer evaluation towards HeLa cell line.

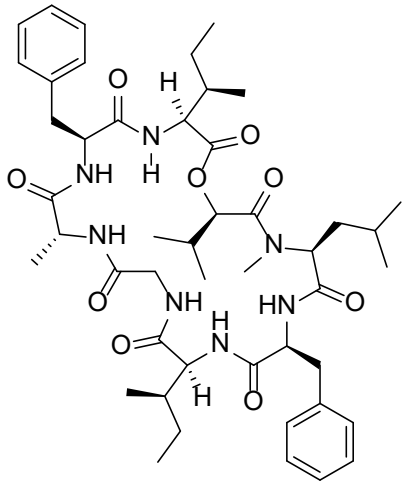


Figure 1 Chemical structure of BZR-cotoxin IV.

The anticancer activity of this compound will be evaluated using the Resazurin (7-hydroxy-10-oxidophenoxazin-10-ium-3-one) assay. The Resazurin assay is a toxicity test that employs a fluorometric approach to assess the viability of cells through the conversion of resazurin into highly fluorescent resorufin [20]. The final result can be quantified by measuring the absorbance of light at a specific wavelength using an enzyme-linked immunosorbent assay (ELISA) reader. The half-maximal inhibitory concentration (IC_{50}) value is employed to assess the toxicity of the substance in question.

Materials and methods

Materials

All the amino acid used in this study were purchased from GL-Biochem Ltd., Shanghai-China, including: L-Valin (L-Val), 9-fluorenylmetoxycarbonyl-Glycine-hydroxy (Fmoc-Gly-OH), 9-fluorenylmetoxycarbonyl-L-Isoleucine-hydroxy (Fmoc-L-Ile-OH), 9-fluorenylmetoxycarbonyl-L-Phenylalanin-hydroxy (Fmoc-L-Phe-OH), 9-fluorenylmetoxycarbonyl-N-Methyl-Leucine-hydroxy (Fmoc-N-MeLeu-OH), 9-fluorenylmetoxycarbonyl-D-Alanine-hydroxy (Fmoc-D-Ala-OH). Coupling reagents, resin and solvents were purchased from GL-Biochem Ltd., Shanghai-China, including: Hexafluorophosphate aza-benzotriazole tetramethyl uranium (HATU), 1-hydroxy-7-azabenzotriazole (HOAt), *N,N*-diisopropilcarbodiimida (DIC), *N,N*-dimethylaminopiridine (DMAP), 2-chlorotrytil chloride (CTC) resin, *N,N*-diisopropyletyleamine (DIPEA), dimethylformamide (DMF), dichloromethane (DCM), trifluoroacetic acid (TFA), chloranil reagent, piperidine, acetonitrile, methanol, propanol, acetic acid, sodium bicarbonate, sodium chloride, sodium nitrite, sodium sulfate anhydrous, sulfuric acid and ethyl acetate. All materials used in this synthesis are pro-analysis grade. With regard to the testing of cytotoxic activity, the following materials may be employed: Roswell Park Memorial Institute Medium (RPMI) (Gibco 11875-093), Fetal Bovine Serum (FBS) (Gibco 10270-106), Antibiotic (Sigma Aldrich P4333), Cisplatin (EDQM C2210000), Resazurin Sodium Salt-Powder BioReagent (Sigma Aldrich R7017), Phosphate buffered saline (PBS) (Gibco 18912-014), Trypsin-EDTA (Gibco 25200-056), trypan blue exclusion (Sigma Aldrich T-8154). While the tools used in this study are: Analytical RP-HPLC was performed on Waters Alliance 2998 using photo diode array (PDA) detector with Acquity BEH C-18 column 1.7 µm (2.1×50 mm^2), and also on semi-preparative RP-HPLC was performed on Waters Alliance e2695 using photo diode array (PDA) 2489 with RP-18e LichroCart column 5 µm (4.6×250 mm^2). UV/Vis with Tecan metro p200 device, FT-IR Perkin Elmer equipped with KBr plate, Mass spectrometry spectra

were recorded on Waters HR-TOF-MS Lockspray, ^{1}H and ^{13}C-NMR spectra were recorded on Agilent NMR 500 MHz (^{1}H) and 125 MHz (^{13}C) using CD_3OD.

Synthesis of (2*R*)-hydroxyisovaleric acid (Hiv).

A 2 g of amino acid L-Val (17 mmol) was dissolved in 30 mL sulfuric acid (1M). Then, 6.78 g of sodium nitrite solution in water (98.3 mmol) was added into the solution slowly at 0 °C for 2 h. The reaction mixture was stirred for 15 h at room temperature. The reaction was monitored using thin layer chromatography (Propanol: Methanol: Acetic acid - 7:2:1). 6 g Sodium bicarbonate (71.4 mmol) was then added into the reaction mixture until it reached pH 2. The acidity value must be controlled to pH 2 to remove unreacted remnants of starting material and precipitate unformed by-products. After which 40 mL of saturated sodium chloride solution (14.6 g, 249.8 mmol) was added to the solution. Then the reaction mixture was extracted with ethyl acetate (3×50 mL). The ethyl acetate phase was then dried with 10 g sodium sulfate anhydrous (68.9 mmol) and filtered. The filtrate was then concentrated with a rotary evaporator. Purity was analyzed by thin layer chromatography. The resulting product was then characterized using FT-IR, HR-TOF-MS, ^{1}H-NMR and ^{13}C-NMR.

Peptide synthesis and purification

Loading the resin

The 2-Chlorotrityl resin (400 mg) was placed in a clean SPPS reactor and swollen by dichloromethane for 10 min. The first amino acid Fmoc-L-Phe-OH (117 mg, 0.375 mmol) that was previously dissolved in dichloromethane (4 mL) and N, N-diisopropylethylamine (0.1275 mL, 0.75 mmol) was added to the resin. The mixture was shaken for 4 h. The loading value of the resin was determined by weighing 0.6 mg of resin in a vial. Then 2 mL of 20 % piperidine solution in DMF was added. The solution was shaken and allowed to stand for 40 min. The solution was measured for absorbance using a UV/Vis spectrophotometer at a wavelength of 290 nm. To make sure that there were no active Cl in the resin, the reaction was capped using 5 mL MeOH: DIPEA: DCM (2:1:7) for 5 min in 2 cycles. The reaction mixture was washed with dichloromethane 3 times and then drained to obtain dry Fmoc-L-Phe-resin.

Fmoc deprotection

Fmoc protecting group of amino acid (Fmoc-L-Phe-resin) was deprotected using 20 % piperidine in dimethylformamide (DMF) (v/v) (10 mL) for 10 min. The solid phase resin was then filtered and washed with dichloromethane, dimethylformamide and dichloromethane, successively. To indicate the Fmoc protecting group had been removed and the NH_2 was available, the chloranil test was applied to the resin bead for the appearance of a blue color.

Amino acid coupling

A solution of Fmoc-amino acid: HATU: HOAT: DIPEA (3:3:3:6 equivalents) in DMF (4 mL) was added to the peptidyl-resin. For the formation of ester/depsi bonds, Hiv is added with coupling reagents: DIC: DMAP: DIPEA (10:0.7:1.4 equivalents). The reaction was shaken for 4 h. The resin was then filtered and washed with dichloromethane, dimethylformamide and dichloromethane, successively. A small amount of drained resin beads was analysed by the chloranil test solution for the confirmation of the successfulness of the coupling reaction.

Fmoc deprotection

Deprotection/release of the Fmoc protecting group on Fmoc-Ile-O-Hiv-N-MeLeu-Phe-Ile-Gly-Ala-Phe-Resin (Fmoc-octadepsipeptidyl-resin) was performed using 20 % piperidine in DMF. The release of the Fmoc protecting group was monitored using the chloranil test.

Resin cleavage

The reagent of TFA 20 % in DCM (5 mL) was added to the Fmoc-octadepsipeptidyl-resin, and then shaken for 1 h in 2 cycles. The filtrate of the reaction was collected and the resin was then washed subsequently 5 mL of dichloromethane. The combined solutions were evaporated using rotatory evaporation to obtain the crude linear octadepsipeptide.

Peptide cyclization

The crude linear octadepsipetide (20 mg, 0.02 mmol) was dissolved in dichloromethane (50 mL) to give a concentration of 1.25×10^{-3} M solution of crude linear octadepsipeptide. Then, HATU (228.1 mg, 0.06 mmol) was added to the solution, followed by DIPEA in 1 % (26.15 mmol). The reaction mixture was stirred for 72 h. The reaction mixture was then evaporated using rotatory evaporation.

Purification

The crude of cyclic product was purified using semi-preparative RP-HPLC using H_2O: Acetonitrile (70:30 %), continued by a linear gradient to 80 % acetonitrile within 35 min with a flow rate of 2.0 mL/min using a C-18 column 5 µm (4.6×250 mm^2). The product was analysed using analytical RP-HPLC H_2O: Acetonitrile (70:30 %), continued by a linear gradient to 80 % acetonitrile within 35 min with a flow rate of 1.0 mL/min using a C-18 column 1.7 µm (2.1×50 mm^2).

Cytotoxic activity

Resazurin assay procedure

The anticancer activity of BZR-cotoxin IV was tested *in vitro* using a cytotoxicity test with the Resazurin assay. The cytotoxic effect of BZR-Cotoxin IV on cancer cells was tested using HeLa cancer cells. The test procedure is as follows: The cells were maintained in the Roswel Park Memorial Institute (RPMI) medium supplemented with 10 % (v/v) Fetal Bovine Serum (FBS) and 50 µL/50 mL antibiotics, and then cultures were incubated at 37 °C in a humidified atmosphere of 5 % CO_2. Similarly, cisplatin was prepared as a standard/control positive, while a DMSO blank solution was used as a negative control. The cells were seeded in 96-well plates at a density of 2×10^4 cells (in 100 µL of medium) per well. After incubation (24 h), the BZR-cotoxin IV samples at 8 concentrations 3.91, 7.81, 15.63, 31.25, 62.50, 125.00, 250.00 and 500.00 in 2 % DMSO, were added to the wells. Following 96 h, viability was determined by measuring the metabolic conversion of Resazurin substrate into pink fluorescent Resofurin product, yielded from the reduction in viable cells. The absorbance was read using a multimode reader at 570 nm. Each concentration of the compounds was tested, then IC_{50} values were calculated by linear regression method using Microsoft Excel software.

The criteria used to categorize the cytotoxicity of BZR-cotoxin IV against HeLa cancer cells based on U.S. National Cancer Institute (NCI) was as follows: $IC_{50} \leq 20$ µg/mL = highly cytotoxic, IC_{50} ranged between 21 and 200 µg/mL = moderately cytotoxic, IC_{50} ranged between 201 and 500 µg/mL = weakly cytotoxic and $IC_{50} > 501$ µg/mL = no cytotoxicity [21].

Results and discussion

Synthesis of (2*R*)-hydroxyisovaleric acid (Hiv) precursor

The hydroxy acid of BZR-cotoxin IV, (2*R*)-hydroxyisovaleric acid (Hiv), was prepared through a diazotization reaction between L-valine and sodium nitrite in acid condition, resulting (2*R*)-hydroxyisovaleric acid (**Figure 2**). The amount of Hiv product was 818.2 mg or 40.6 % yield (white powder). The IR (KBr) λ_{max} (cm^{-1}) spectrum showed wavenumbers of 3,416 cm^{-1} (OH- hydroxyl), 2,965 cm^{-1} (CH- aliphatic), 1,704 cm^{-1} (C=O- carboxylic group), 1,389 cm^{-1} (gem- dimethyl) and 1,025 cm^{-1} (C-O) (**Figure 3**). The HR-TOF-ESI mass spectrometry showed $[M+H]^+$ ion peak at *m/z* of 117.0552, $C_5H_{10}O_3$ of the product. The structure of product was also confirmed using ^{1}H-NMR (500 MHz, CD_3OD) and ^{13}C-NMR (125 MHz, CD_3OD).

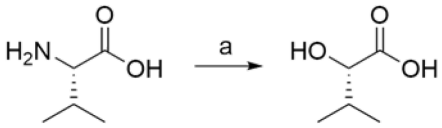


Figure 2 Synthesis of (2*R*)-hydroxyisovaleric acid (Hiv).

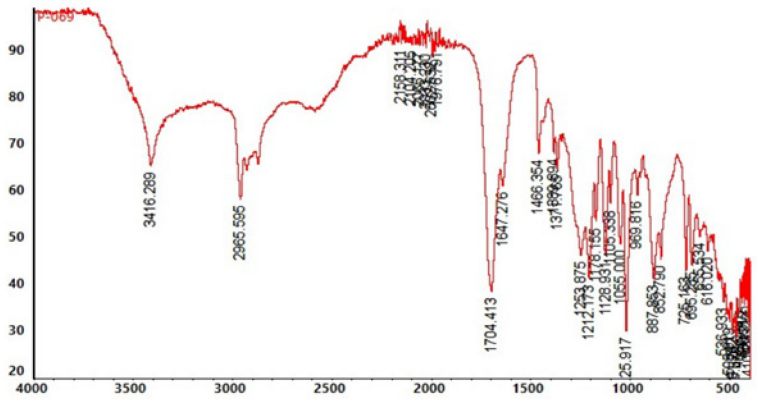
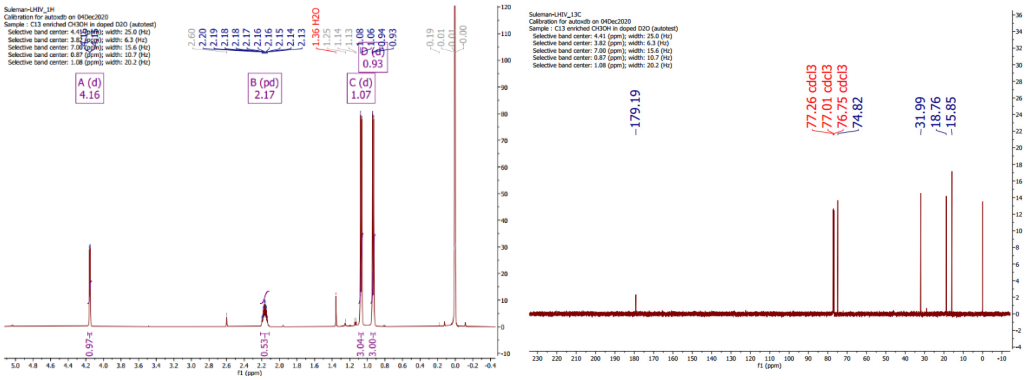


Figure 3 IR spectrum of Precursor (2*R*)-hydroxyisovaleric acid.

^{1}H-NMR δ_H (ppm) 4.16 (d, J = 5 Hz, 1H, H-2), 2.13 - 2.20 (m, 1H, H-3), 1.07 (d, J = 10 Hz, 3H, H-4) and 0.93 (d, J = 5 Hz, 3H, H-4') (**Figure 4(a)**). ^{13}C-NMR δ_C (ppm) 179.19 (C-1), 74.82 (C-2), 31.95 (C-3), 18.76 (C-4) and 15.85 (C-4') (**Figure 4(b)**).



(a) **(b)**

Figure 4 (a) ^{1}H-NMR and (b) ^{13}C-NMR spectrum of Precursor (2*R*)-hydroxyisovaleric acid.

Total synthesis of BZR-cotoxin IV

The synthesis of BZR-cotoxin IV was accomplished through a combination of solid-phase peptide synthesis (SPPS) and solution-phase methods (**Figure 3**). The linear precursor (linear octadepsipeptide) of BZR-cotoxin IV was synthesized on CTC resin and HATU/HOAt coupling reagent was employed for amide bond formation. The formation of ester (depside) bond took advantage of DIC/DMAP as coupling reagent.

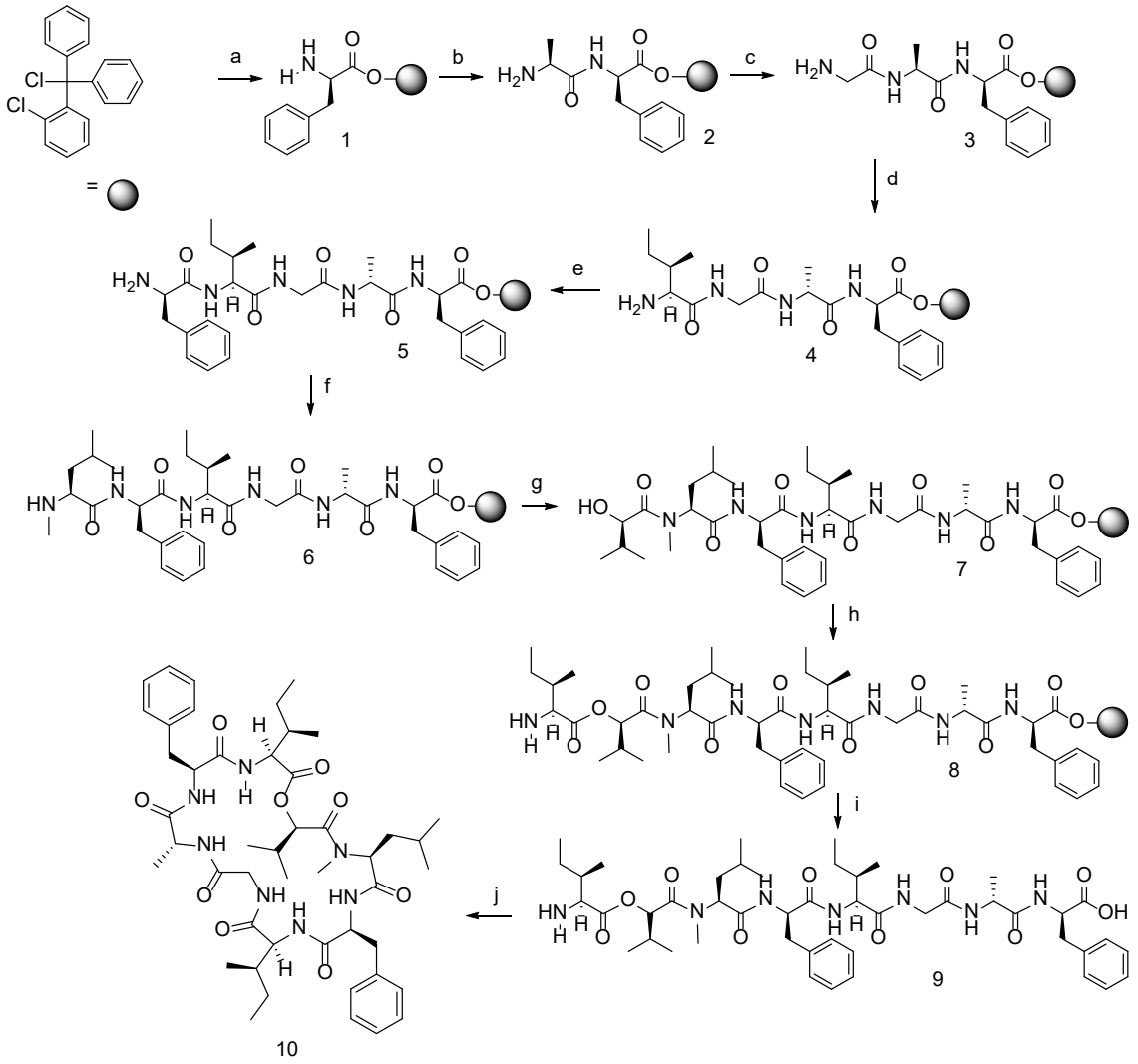


Figure 5 SPPS and cyclization of BZR-cotoxin IV (a) (1) Fmoc-L-Phe-OH: DIPEA (1:2 equivalents), 4 mL CH_2Cl_2, 4 h; (2) MeOH: DIPEA: CH_2Cl_2 (15:5:80 equivalents), 2×5 min; (3) 20 % piperidine, 10 min., (Dep.); (b). (1) Fmoc-L-Ala-OH: HATU: HOAt: DIPEA (3:3:3:6 equivalents), (2) Dep., (c) (1) Fmoc-Gly-OH: HATU: HOAt: DIPEA (3:3:3:6 equivalents), (2) Dep.; (d) (1) Fmoc-L-Ile-OH: HATU: HOAt: DIPEA (3:3:3:6 equivalents), (2) Dep.; (e) (1) Fmoc-L-Phe-OH: HATU: HOAt: DIPEA (3:3:3:6 equivalents), (2) Dep., (f) (1) Fmoc-L-NMe-Leu-OH: HATU: HOAt: DIPEA (3:3:3:6 equivalents), (2) Dep.; (g) (1) Hiv: HATU: HOAt: DIPEA (3:3:3:6 equivalents), (2) Dep.; (h) (1) Fmoc-L-Ile-OH: DIC: DMAP: DIPEA (7:10:0.7:1.4 equivalents) (b-g in 4 mL DMF, 4 h), (2) Dep.; (i) (TFA) 20 % 2×1 h. (j) HATU (3 equivalents), 1 % DIPEA, 72 h.

Fmoc chemistry was applied to synthesize the linear octadepsipeptide **9**. The first amino acid, Fmoc-L-Phe-OH, was attached onto 2-chlorotrityl chloride resin in DCM and in the presence of basic DIPEA to provide 0.45 mmol L-phenilalanin per g resin. The unreacted sites on the resin were then capped by reacting the resin with MeOH: DIPEA: DCM (2:1:7).

The Fmoc group is removed using 20 % piperidine in DMF to create a free amino group, which is then ready to react with the next amino acid. The second amino acid, Fmoc-D-Ala-OH, was coupled to resin-Phe-NH_2 **1** using a combination of coupling reagents HATU and HOAt in the presence of DIPEA base. The 3 to 6 equivalents of HATU/HOAt and DIPEA were found to provide effective coupling reactions for different types of peptides. The success of the coupling reaction was monitored using a chloranil test. The peptide chain was extended by linking the next amino acids, namely Gly, L-Ile, L-Phe, N-MeLeu, Hiv and L-Ile, to form the desired linear octadepsipeptide-resin **8**.

An interesting point in this synthesis is the formation of an ester bond (depside) at the last amino acid coupling (Fmoc-L-Ile-OH). The procedure applied in this synthesis uses the steglich esterification method following the protocol for the synthesis of exumolides A and B [17] with temperature modification (room temperature). The coupling of Fmoc-L-Ile-OH to the hepta-peptidyl resin **7** showed perfect incorporation after coupling twice. Furthermore, the utilisation of (2R)-hydroxyisovaleric acid (Hiv) precursors in the absence of protective groups and the formation of ester bonds in the solid phase led to more efficient reactions due to the shorter reaction pathways, in comparison to the formation of ester bonds in the solution phase.

The linear octadepsipeptide were cleaved from the resin by using 20 % TFA in DCM for 2×1 h at room temperature. The resulting filtrate was collected and concentrated using a rotary evaporator, yielding 87.7 % based on the loading value of the resin. The crude linear octadepsipeptide **9** was purified using semi-preparative RP-HPLC equipped with a C18 column (5 µm, 4.6×250 mm^2) with 70 % H_2O as solvent A and 30 % acetonitrile as solvent B. Purification was carried out in a gradient from 30 to 80 % acetonitrile for 35 min with a flow rate of 2 mL/min, resulting in a yield of 8 % of the purified linear octadepsipeptide (3.2 mg, white powder). The analytical RP-HPLC analysis of the linear octadepsipeptide resulted in a single peak at a retention time of 8.490 min. The HR-TOF-MS analysis showed a measured molecular ion peak $[M+H]^+$ at 894.5334 m/z and a calculated mass of 894.5341 m/z with the molecular formula of $C_{47}H_{72}N_7O_{10}$.

Cyclization of linear octadepsipeptide was performed in solution phase. Cyclisation occurs through a head-to-tail reaction between phenylalanine as the C-terminal amino acid and isoleucine as the N-terminal amino acid. The cyclization reaction in this synthesis is achieved by adding HATU coupling reagent and DIPEA base in a DCM solvent mixture for 72 h at room temperature. The linear octadepsipeptide cyclization reaction was carried out under very dilute solution conditions (1.25×10^{-3} M) to minimize the formation of oligomers or cyclooligomers [22].

After the reaction was completed, the reaction mixture was concentrated and a yellow-brown cyclic crude was obtained. The cyclic crude was purified using semi-preparative RP-HPLC equipped with a C18 column (5 µm, 4.6×250 mm^2) using 70 % H_2O as solvent A and 30 % acetonitrile as solvent B. Purification was performed using a gradient from 30 to 80 % acetonitrile over 35 min at a flow rate of 2 mL, resulting in a 11.7 % yield of the cyclic product (3.5 mg, white powder). The analytical RP-HPLC analysis yielded a single peak with a retention time of 23.816 min, which is believed to be the compound BZR-cotoxin IV 10.

The molecular formula was established as $C_{47}H_{69}N_7O_9$ by HR-TOF-MS on the $[M+H]^+$ ion at 875.6954. The ^{1}H-NMR spectrum: δ_H (CD_3OD) (ppm) 0.75 - 0.83 (m, 3H), 0.89 - 0.95 (m, 18H), 0.97 - 1.18 (m, 3H), 1.44 - 1.62 (m, 3H), 1.47 (m, 3H), 2.08 - 2.27 (m, 3H), 2.52 (m, 1H), 3.00 (s, 3H), 3.05 - 3.15

(m, 2H), 3.15 - 3.26 (m, 2H), 3.99 (q, J = 7.8, 2H), 4.26 (m, 1H), 4.29 (m, 1H), 4.5 (m, 2H), 5.07 (m, 1H), 5.11 (m, 1H), 5.25 (m, 1H), 6.82 (t, J = 1.8, 1H), 7.1 - 7.3 (m, 1H), 7.48 (dd, J = 8.4, 1H), 8.44 (dd, J = 8.4, 1H) and 8.67 (dd, J = 4.5, 1H). The ^{13}C-NMR spectrum: δ_C (CD_3OD) (ppm) 174.61, 173.76, 172.83, 172.45, 172.25, 169.70, 169.66, 168.91, 135.01, 130.3, 129.40, 129.06, 128.54, 127.97, 121. 11, 66.28, 65.56, 64.42, 56.32, 56.19, 51.27, 41.70, 40.29, 38.82, 38.48, 36.78, 35.21, 31.67, 29.36, 24.15, 23.52, 22.63, 22.33, 20.77, 18.75, 15.98, 15.88, 15.77, 15.66 and 13.03.

In the ^{1}H-NMR spectrum (500 MHz, CD_3OD), a singlet proton signal at 3.00 ppm indicated the presence of a methyl group attached to an amide nitrogen. Four proton signals from the NH-amide proton were observed at 6.82 ppm (1H, d, J = 1.8 Hz), 7.48 ppm (1H, dd, J = 8.4), 8.44 ppm (1H, d, J = 1.3 Hz) and 8.67 ppm (1H, dd, J = 4.5 Hz). The proton signal from the other amide NH was obscured by the overlapped signals of 2 mono substituted phenyl rings at 7.04 - 7.33 ppm (11H, m). The signals between 0.75 - 5 ppm indicate α protons and some branches of amino acid residues. Signals between 3 and 5 ppm were considered to be α protons of amino acid residues and while the signals around 1 ppm indicated that branched amino acid residues [9].

The synthesis of BZR-cotoxin IV is not without difficulties. This compound contains several amino acids that present a challenge during the reaction. Due to the inherent attributes of certain peptide individuals, they could undergo various side reactions [18]. For example, fragmentation/deletion side reactions can occur during synthesis, purification and even storage. Peptide-containing N-alkylated amino acid (e.g. N-Me-amino acid) residues are inherited with decreased stabilities in acidic condition, compared with their counterparts with ordinary non-N-alkyllated amino acid. Therefore, peptides with these characteristics may undergo fragmentation/deletion reactions. BZR-cotoxin IV has an N-MeLeu residue in its chain, therefore it can undergo the peptide bond fragmentation/deletion, taken place at the site off N-MeLeu (Ile-O-Hiv-N-MeLeu) to form a pentapeptide (Phe-Ile-Gly-Ala-Phe). The deletion caused the reduction of the percentage of the targetted octadepsipetide (Ile-O-Hiv-N-MeLeu-Phe-Ile-Gly-Ala-Phe) in the final product.

Cytotoxic activity of BZR-cotoxin IV

The cytotoxic activities of BZR-cotoxin IV against HeLa cancer cells were determined at 570 nm by Resazurin assay. The cytotoxic data were obtained in the form of absorbance values. These data were then used to calculate the percentage of live cells. Furthermore, the IC_{50} value was analyzed using Excel (linear regression of log concentration vs. % cell viability). Cisplatin (16.24 µM) was used as a positive control. The result showed that BZR-cotoxin IV has IC_{50} of 187.50 µg/mL or 214.014 µM (**Figure 6**). According to a criteria based on US-NCI, BZR-cotoxin IV showed moderate cytotoxic activity against HeLa cancer cell.

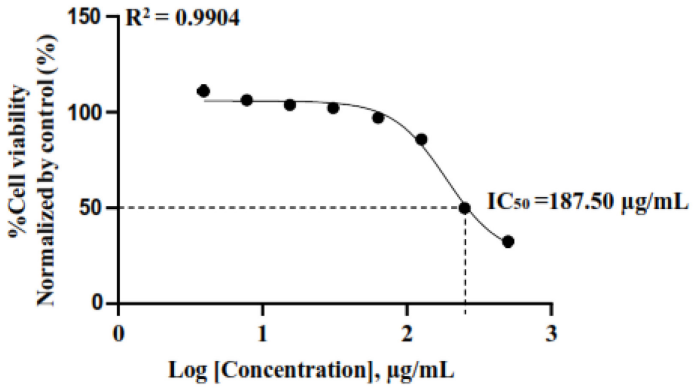


Figure 6 Graphic log concentration vs. % cell viability of BZR-cotoxin IV.

The toxicity of the BZR-cotoxin IV compound against HeLa cancer cells is attributed to its hydrophobicity. This property arises from the compound's composition, which includes hydrophobic amino acid residues, 2 phenylalanine residues, 2 isoleucine residues, 1 alanine residue and 1 N-methyl leucine residue and 1 hydrophobic hydroxy acid ((*2R*)-hydroxyisovaleric acid) [9]. The hydrophobicity facilitates the peptide penetration into the cancer cell membrane [23].

Research on the synthesis of BZR-cotoxin IV compounds is of great interest and importance. The synthesis of BZR-cotoxin IV, as one of the cyclodepsipeptides, presents unique challenges compared to cyclopeptides. The presence of an ester bond in its cyclic structure renders it susceptible to hydrolysis during the synthesis of its linear precursor, resulting in the formation of diketopiperazine (DKP) compounds as by-products. This discrepancy between the amino acid sequence arranged in the synthesis and the target compound can be circumvented by implementing a strategy that places the ester bond at the end of the coupling reaction. This strategy has not been employed to a significant extent, which represents one of the advantages of the synthesis of BZR-cotoxin IV compounds in this study.

To the best of our knowledge, exploration of BZR-cotoxin IV compounds is still limited to the isolation and structural characterization of these compounds from natural sources. While research on the chemical synthesis of BZR-cotoxin IV compounds and their anticancer activity tests has not been reported so far. Therefore, the synthesis and anticancer evaluation of BZR-cotoxin IV is firtsly reported in this paper.

Conclusions

We have successfully synthesized BZR-cotoxin IV using a combination of solid and solution phase peptide synthesis method. Cyclization strategy of L-phenilalanine residue on C-terminal and L-isoleusin residue on N-Terminal, which placed the ester bond formation at the end of the reaction, was successful to increase the product with overall yield of 11.7 %. BZR-cotoxin IV compound showed moderate cytotoxic activity (214.01 μM) against HeLa cancer cells.

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