

Tổng hợp và đánh giá độc tính tế bào của các dẫn xuất benzimidazole kết hợp amino acid

TÓM TẮT

Benzimidazole và các dẫn xuất của nó thể hiện hoạt tính sinh học đa dạng, gồm đặc tính kháng khuẩn, kháng viêm, kháng virus và đặc biệt là kháng ung thư. Các nghiên cứu gần đây cũng chỉ ra tiềm năng của các cấu trúc kết hợp giữa benzimidazole và amino acid/peptide trong nghiên cứu thiết kế các tác nhân kháng khuẩn và kháng ung thư mới. Trong báo cáo này, một quy trình tổng hợp hiệu quả gồm ba bước đã được phát triển thành công hướng đến các cấu trúc lai hóa bằng cách kết hợp các amino acid vào khung benzimidazole. Dựa trên quy trình này, sáu dẫn xuất benzimidazole kết hợp amino acid mới đã được tổng hợp thành công với hiệu suất 45–80%. Cấu trúc của các dẫn xuất này đã được xác định dựa trên các dữ liệu phổ nghiệm gồm 1D, 2D-NMR và HR-ESI-MS. Đánh giá độc tính tế bào chỉ ra rằng các dẫn xuất **7a** và **7c** có độc tính tế bào từ trung bình đến khá tốt đối với ba dòng tế bào ung thư bao gồm ung thư phổi, ung thư cổ tử cung và ung thư vú. Đáng chú ý, hợp chất **7c** ($IC_{50} = 18,88 \mu\text{M}$) mang nhóm L-phenylalanine ethyl ester thể hiện độc tính tốt trên dòng tế bào ung thư vú.

Từ khóa: *Amino acid, benzimidazole, độc tính tế bào, hợp chất dị vòng, lai hóa phân tử.*

Synthesis and cytotoxicity evaluation of benzimidazole conjugated amino acid derivatives

ABSTRACT

Benzimidazole and its derivatives displayed diverse biological activities, including antibacterial, anti-inflammatory, antiviral, and particularly anticancer properties. Recent studies also showed that the benzimidazole and amino acid/peptide conjugates displayed potential in designing novel antimicrobial and anticancer agents. In this report, an efficient three-step synthetic method has been successfully developed towards hybrid structures *via* conjugating amino acids to the benzimidazole framework. Based on this procedure, six novel benzimidazole-amino acid hybrid derivatives have been successfully synthesized in 45–80% yields. The structures of these derivatives were fully determined based on spectroscopic data including 1D, 2D-NMR, and HR-ESI-MS. Cytotoxicity assays indicated that compounds **7a** and **7c** possessed moderate to rather good cytotoxicities towards three cancer cell lines including lung cancer, cervical cancer, and breast cancer. Notedly, compound **7c** ($IC_{50} = 18.88 \mu\text{M}$) with the L-phenylalanine ethyl ester moiety displayed good cytotoxicity on the breast cancer cell line.

Keywords: *Amino acids, benzimidazole, cytotoxicity, heterocyclic compounds, molecular hybridization.*

1. INTRODUCTION

Nitrogen-containing heterocyclic compounds have exhibited diverse pharmacological activities, due to the ability of the nitrogen atom to easily form hydrogen bonding with biological receptors.¹ Among those, benzimidazole, an integral part of the structure of vitamin B₁₂, has exhibited a broad spectrum of biological activities including antimicrobial,^{2,3} antiviral,^{4,5} anti-inflammatory,⁶ antiulcer,⁷ and especially anticancer.⁸⁻¹⁴ Several anticancer drugs bearing the benzimidazole moiety's core structure have been approved by the FDA such as bendamustine for treating chronic lymphocytic leukemia, selumetinib for treating neurofibromatosis type 1, binimetinib for treating certain types of melanoma.

Besides, α -amino acids are essential for the human body's production of proteins and participation in vital metabolic processes. Both structural modification and drug synthesis have frequently used these structural characteristics. It has been observed that the conjugation of amino acids and pharmacophores results in novel hybrid compounds with enhanced pharmacological activity,^{15,16} improved water solubility,¹⁷ and reduced cytotoxicity.¹⁸

Several benzimidazole-conjugated amino acid derivatives have been known to demonstrate as lead anticancer agents. As shown in Figure 1, the poly (ADP-ribose) polymerase (PARP) inhibitor, veliparib¹⁹ (an anticancer drug) and the strong cytotoxic agent, compound **1**²⁰ share the common structural features of a benzimidazole core conjugated to an amino acid moiety at the C-2 position. Additionally, compound **2**²¹ bearing the tryptophan moiety at the C-5 position of the heterocyclic ring, acted as a potent sirtuin (Sirt) inhibitor. However, there have not been many reports on the synthesis and assessment of anticancer activity for benzimidazole derivatives that contain amino acids at the *N*-1 position, despite the fact that this was also a crucial position that frequently results in anticancer activity.^{13,22,23} In light of these facts, this paper reports the synthesis of novel benzimidazole-conjugated amino acid derivatives *via* *N*-1 position of the benzimidazole core and their *in vitro* cytotoxicity towards the three cancer cell lines, including HeLa (cervical cancer), MCF-7 (breast cancer), and A549 (lung cancer) cell lines.

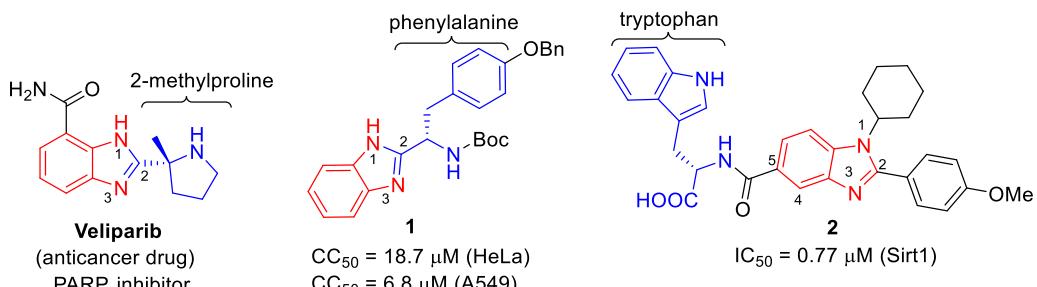


Figure 1. Several benzimidazole-amino acid hybrid derivatives as potent cytotoxic agents

2. MATERIALS AND METHODS

2.1. Chemistry

2.1.1. General information

Reactions were monitored by thin layer chromatography (TLC) on 0.2 mm pre-coated silica gel 60 F254 plates (Merck). 1D and 2D-NMR spectra were measured with a JEOL 400 MHz spectrometer using DMSO-d₆ as solvent. HR-ESI-MS (high resolution electrospray ionization) data were performed on a X500R qTOF spectrometer. Chemical shifts are displayed in ppm (parts per million) compared to Me₄Si ($\delta = 0$). For column chromatography, silica gel 60 (0.063–0.200 mm, Prolabo) was utilized. Melting points (Mp, °C) were determined on an INE-X-4 melting point apparatus with microscope. Chemicals and solvents used are sourced from Macklin (1-fluoro-4-methyl-2-nitrobenzene, 4-chloro-1-fluoro-2-nitrobenzene, H-L-Tle-OME-HCl, H-L-Phe-OEt-HCl, H-L-Val-OEt-HCl), Merck (4-methoxybenzaldehyde, DMSO, MeOH), China (LiOH·H₂O, NH₄Cl, NaOAc, NaHCO₃, Na₂SO₄, Na₂S₂O₄, ethyl acetate), Singapore (*n*-hexane) and Vietnam (NaCl, HCl, H₂O).

2.1.2. General procedure for the synthesis of benzimidazole-amino acid derivatives

Synthesis of derivatives 5a-c

To a solution of compound 3a-b (1 mmol), L-amino acid ethyl/methyl ester hydrochlorides 4a-c (1.5 mmol), NaHCO₃ (1.5 mmol), and NaOAc (10 mmol) in water (4 mL) was refluxed and stirred at 100°C for 8 hours. After completion, the temperature of reaction mixture was cooled down. The saturated aqueous solution of NH₄Cl was used to bring the mixture's pH to 7. Ethyl acetate (3×20 mL) was used to extract the combination, and washed with brine solution, dried on Na₂SO₄, and then solvent was then evaporated under reduced pressure to give compounds 5a-c, which was utilized without any additional purification for the following phase.

Ethyl (4-chloro-2-nitrophenyl)-L-valinate (5a): Yield 88%. Orange gel-like.

Methyl (S)-3,3-dimethyl-2-((4-methyl-2-nitrophenyl)amino)butanoate (5b): Yield 76%. Orange gel-like.

Ethyl (4-chloro-2-nitrophenyl)-L-phenylalaninate (5c): Yield 95%. Orange gel-like.

Synthesis of derivatives 7a-c

A mixture of compound 5a-c (1 mmol), aldehyde 6 (1.1 mmol), Na₂S₂O₄ (4 mmol) in dimethylsulfoxide (DMSO, 4 mL) was stirred at 90°C for 2-4 hours. After completion, the temperature of reaction mixture was cooled down. The saturated aqueous solution of NaHCO₃ was used to bring the mixture's pH to 7. Ethyl acetate (3×20 mL) was used to extract the combination, then washed with brine solution, dried on Na₂SO₄, and solvent was evaporated under reduced pressure. Purification of the crude products using silica gel column chromatography (*n*-hexane/ethyl acetate as eluent) afforded derivatives 7a-c.

Ethyl (S)-2-(5-chloro-2-(4-methoxyphenyl)-1H-benzo[d]imidazol-1-yl)-3-methylbutanoate (7a): Orange-yellow gel-like. Yield 48%. $R_f = 0.61$ (*n*-hexane:ethyl acetate=3:1). ¹H-NMR (400 MHz, δ ppm) (J in Hertz): 7.77 – 7.74 (m, 2H), 7.68 – 7.64 (m, 2H), 7.32 (dd, $J = 2.2, 8.7$ Hz, 1H), 7.19 – 7.15 (m, 2H), 4.66 (d, $J = 11.0$ Hz, 1H), 4.24 (q, $J = 7.1$ Hz, 2H), 3.87 (s, 3H), 2.84 – 2.75 (m, 1H), 1.2 (t, $J = 7.1$ Hz, 3H), 0.96 (d, $J = 6.5$ Hz, 3H), 0.29 (d, $J = 6.7$ Hz, 3H). ¹³C-NMR (100 MHz, δ ppm): 168.5, 160.6, 155.9, 143.8, 132.5, 131.1, 126.8, 122.6, 121.3, 118.7, 114.3, 114.1, 65.1, 61.7, 55.3, 28.0, 19.5, 18.0, 13.8. HRMS (*m/z*): [M+H]⁺ found 387.1474 (calcd. for C₂₁H₂₄ClN₂O₃⁺, 387.1470).

Methyl (S)-2-(2-(4-methoxyphenyl)-5-methyl-1H-benzo[d]imidazol-1-yl)-3,3-dimethylbutanoate (7b): White solid. Mp 128–130°C. Yield 45%. $R_f = 0.40$ (*n*-hexane:ethyl acetate=3:1). ¹H-NMR (400 MHz, δ ppm) (J in

Hertz): 7.58 (d, J = 8.7 Hz, 2H), 7.45 (s, 1H), 7.39 (d, J = 8.4 Hz, 1H), 7.15 (d, J = 8.7 Hz, 2H), 7.06 (dd, J = 1.2, 8.5 Hz, 1H), 5.13 (s, 1H), 3.86 (s, 3H), 3.75 (s, 3H), 2.41 (s, 3H), 0.82 (s, 9H). ^{13}C -NMR (100 MHz, δ ppm): 168.5, 160.1, 154.6, 143.0, 132.3, 131.0, 123.6, 122.7, 118.9, 114.3, 113.2, 65.4, 55.2, 52.5, 37.2, 27.6, 20.9. HRMS (m/z): $[\text{M}+\text{H}]^+$ found 367.2017 (calcd. for $\text{C}_{22}\text{H}_{27}\text{N}_2\text{O}_3^+$, 367.2016).

Ethyl (S)-2-(5-chloro-2-(4-methoxyphenyl)-1H-benzo[d]imidazol-1-yl)-3-phenylpropanoate (7c): Orange-yellow gel-like. Yield 53%. R_f = 0.49 (*n*-hexane:ethyl acetate=3:1). ^1H -NMR (400 MHz, δ ppm) (J in Hertz): 7.71 (d, J = 2.0 Hz, 1H), 7.62 (d, J = 8.7 Hz, 1H), 7.35 (dd, J = 2.1, 8.7 Hz, 1H), 7.10 (t, J = 7.4 Hz, 1H), 7.03 – 6.96 (m, 6H), 6.54 (d, J = 7.1 Hz, 2H), 5.43 (t, J = 8.0 Hz, 1H), 4.26 – 4.20 (m, 2H), 3.82 (s, 3H), 3.44 (d, J = 8.1 Hz, 2H), 1.17 (t, J = 7.1 Hz, 3H). ^{13}C -NMR (100 MHz, δ ppm): 168.6, 160.3, 155.6, 143.7, 135.8, 132.4, 130.5, 128.4, 128.1, 126.7, 126.6, 122.6, 121.1, 118.8, 113.8, 113.2, 61.8, 59.6, 55.2, 34.3, 13.8. HRMS (m/z): $[\text{M}+\text{H}]^+$ found 435.1473 (calcd. for $\text{C}_{25}\text{H}_{24}\text{ClN}_2\text{O}_3^+$, 435.1470).

Synthesis of derivatives 8a-c

To a round-bottom flask containing 0.5 mmol of ester **7a-c** and LiOH·H₂O (1.5 mmol) was added 4 mL of the solvent system (MeOH:H₂O=8:1), and stirred at room temperature for 24 hours. After completion, the mixture's pH was adjusted to 3 using 1N HCl solution. Ethyl acetate (3×10 mL) was used to extract the combination, then washed with brine solution, dried on Na₂SO₄, and solvent was evaporated under reduced pressure. Purification of the crude products using silica gel column chromatography (*n*-hexane/ethyl acetate as eluent) afforded derivatives **8a-c**.

(S)-2-(5-Chloro-2-(4-methoxyphenyl)-1H-benzo[d]imidazol-1-yl)-3-methylbutanoic acid (8a): White solid. Mp 201–204°C. Yield 80%. R_f = 0.20 (*n*-hexane:ethyl acetate=1:3). ^1H -NMR (400 MHz, δ ppm) (J in Hertz): 7.76 (d, J = 2.0 Hz, 1H), 7.74 (d, J = 8.8 Hz, 1H), 7.70 – 7.67 (m, 2H), 7.31 (dd, J = 2.1, 8.7 Hz, 1H), 7.19 – 7.16 (m, 2H), 4.61 (d, J = 11.0 Hz, 1H), 3.87 (s, 3H), 2.80 – 2.70 (m, 1H), 0.97 (d, J = 6.5 Hz, 3H), 0.26 (d, J = 6.7 Hz, 3H). ^{13}C -NMR (100 MHz, δ ppm): 170.1, 160.5, 155.9, 143.8, 132.5, 131.0, 126.7, 122.5, 121.5, 118.7, 114.3, 114.1, 65.2, 55.3, 27.7, 19.7, 17.9. HRMS (m/z): $[\text{M}+\text{H}]^+$ found 359.1159 (calcd. for $\text{C}_{19}\text{H}_{20}\text{ClN}_2\text{O}_3^+$, 359.1157).

(S)-2-(2-(4-Methoxyphenyl)-5-methyl-1H-benzo[d]imidazol-1-yl)-3,3-dimethylbutanoic acid (8b):

White solid. Mp 265–268°C. Yield 66%. R_f = 0.25 (*n*-hexane:ethyl acetate=1:3). ^1H -NMR (400 MHz, δ ppm) (J in Hertz): 7.56 (d, J = 8.7 Hz, 2H), 7.47 – 7.44 (m, 2H), 7.15 (d, J = 8.7 Hz, 2H), 7.05 (dd, J = 1.4, 8.5 Hz, 1H), 5.00 (s, 1H), 3.85 (s, 3H), 2.41 (s, 3H), 0.83 (s, 9H). ^{13}C -NMR (100 MHz, δ ppm): 169.6, 160.0, 154.7, 143.0, 132.3, 130.9, 130.8, 123.3, 122.9, 118.8, 114.2, 113.5, 65.6, 55.2, 36.8, 27.7, 20.9. HRMS (m/z): $[\text{M}+\text{H}]^+$ found 353.1860 (calcd. for $\text{C}_{21}\text{H}_{25}\text{N}_2\text{O}_3^+$, 353.1860).

(S)-2-(5-Chloro-2-(4-methoxyphenyl)-1H-benzo[d]imidazol-1-yl)-3-phenylpropanoic acid (8c):

White solid. Mp 122–124°C. Yield 68%. R_f = 0.13 (*n*-hexane:ethyl acetate=1:3). ^1H -NMR (400 MHz, δ ppm) (J in Hertz): 7.70 (d, J = 2.0 Hz, 1H), 7.64 (d, J = 8.7 Hz, 1H), 7.35 (dd, J = 2.0, 8.6 Hz, 1H), 7.09 (t, J = 7.3 Hz, 1H), 7.03 – 6.96 (m, 6H), 6.53 (d, J = 7.2 Hz, 2H), 5.36 – 5.32 (m, 1H), 3.81 (s, 3H), 3.43 – 3.41 (m, 2H). ^{13}C -NMR (100 MHz, δ ppm): 170.2, 160.2, 155.6, 143.7, 136.2, 132.5, 130.4, 128.3, 128.0, 126.6, 126.4, 122.5, 121.3, 118.7, 113.8, 113.3, 59.8, 55.2, 34.4. HRMS (m/z): $[\text{M}+\text{H}]^+$ found 407.1164 (calcd. for $\text{C}_{23}\text{H}_{20}\text{ClN}_2\text{O}_3^+$, 407.1157).

2.2. Cytotoxicity evaluation

Cytotoxicity of the synthesized derivatives against three cancer cell lines (MCF-7, HeLa, and A549) were evaluated base on the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay.²⁴ To create stock solutions (10 mM), the synthesized compounds were dissolved in DMSO and dilutions were prepared in the culture medium. 5-fluorouracil (5-FU), the positive control, was mixed in DMSO to create a 10 mM stock solution, which was then kept at –20°C until it was needed.

The human cancer cell lines were cultivated at 37°C with 5% CO₂ atmosphere in α-MEM (α-minimum essential medium), supplemented with 10% fetal bovine serum and 1% antibiotic antimycotic solution. Following their collection, cells at 80–90% confluence were centrifuged for three minutes at 3,000 rpm. In order to resuspend the cell pellet in new media, the supernatant was disposed of. The cells were seeded in 96-well plates (1×10⁴ cells/well) in aliquots (100 μL) and incubated for 24 hours. Five concentrations of the tested compounds (6.25, 12.5, 50, and 100 μM),

together with the 5-FU, were then introduced to the wells after the cells had been cleaned with (PBS) phosphate-buffered saline. Following a 72-hour incubation period, the cells were rinsed with PBS, and each well was filled with 100 μ L aliquots of media containing MTT solution (5 mg/mL) and incubated for three hours. A microplate reader was used to measure the absorbance at 570 nm. Percent proliferation inhibition was calculated using the following formula:

$$\% \text{ Proliferation cell inhibition} = [(A_t - A_b)/(A_c - A_b)] \times 100$$

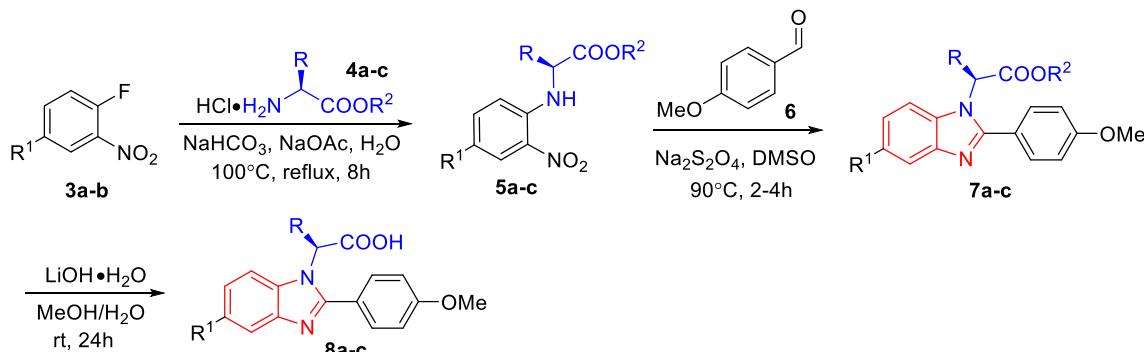
A_t : Absorbance of test compound, A_b : Absorbance of blank, A_c : Absorbance of control.

The IC_{50} values were determined by analyzing the correlation between the concentrations and the percentage of inhibition, using the GraphPad Prism 5.0 software. All data are shown as mean \pm standard deviation (S.D.), and each experiment was run three times.

3. RESULTS AND DISCUSSION

The three-step synthetic procedure towards the benzimidazole-amino acid hybrid derivatives was described in Scheme 1. The precursors *N*-substituted *o*-nitroanilines **5a-c**, were prepared *via* the nucleophilic aromatic

substitution reaction of *o*-fluoro-nitrobenzenes **3a-b** and the free amino moiety of **4a-c**.¹³ The aqueous solution of NaHCO_3 was used as the base to deliver the free amino group of **4a-c** and the excess of NaOAc to maintain the alkaline medium. The use of other base such as K_2CO_3 just led to lower yield, probably due to saponification of the ester moiety. Next, the condensation reaction between **5a-c** and aldehyde **6** using sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) in DMSO through a one-pot reductive cyclization route²⁵ afforded derivatives **7a-c** in moderate yields (Table 1). The reagent $\text{Na}_2\text{S}_2\text{O}_4$ allowed the synthesis of benzimidazoles in just one step from *N*-substituted *o*-nitroanilines, more efficiently than using the reagents $\text{Na}_2\text{S}_2\text{O}_5$ ²⁶ or NaHSO_3 ²⁷ (which required prior reduction of the nitro group). It seemed that the electron characteristic of the substituent at the C-4 position of the benzene ring of compounds **5a-c** did not affect the cyclization step although slightly higher yields were observed in the case of compound **5a** and **5c** with an electron withdrawing group Cl. Finally, the saponification reaction of **7a-c** took place readily using lithium hydroxide²¹ under mild conditions to afford the corresponding derivatives **8a-c** in reasonable to good yields (Table 1).



Scheme 1. Synthetic procedure towards the benzimidazole-conjugated amino acid derivatives

Table 1. Synthetic yield and cytotoxicity of the synthesized derivatives

Code	Structure	Yield ^a (%)	IC ₅₀ ^b (μ M)		
			HeLa	MCF-7	A549
7a		48	25.54 \pm 0.84	25.86 \pm 1.25	35.85 \pm 0.69
7b		45	>100	>100	>100

7c		53	21.57 ± 0.45	18.88 ± 0.13	25.97 ± 0.20
8a		80	>100	>100	>100
8b		66	>100	>100	>100
8c		68	>100	>100	>100
5-FU			6.36 ± 0.39	8.59 ± 0.72	3.92 ± 0.22

^aIsolated yields; ^bIC₅₀: 50% inhibitory concentration. Data are presented as mean ± standard deviation (S.D.) (n = 3).

The synthesized compounds **7a–c** and **8a–c** were tested for their cytotoxicities against the cancer cell lines. The results, summarized in Table 1, indicated that compounds **7b** and **8a–c** with the free carboxylic moiety showed no cytotoxicity towards the tested cancer cell lines at the tested concentration. In contrast, two compounds **7a** and **7c** (IC₅₀ = 18.88–35.85 μM) with the ester moiety being maintained exhibited rather good cytotoxicities compared with the positive control 5-FU (IC₅₀ = 3.92–8.59 μM). These results likely emphasized the role of the ester moiety compared with the corresponding free carboxylic moiety (compared the activity of **7a** and **7c** against **8a** and **8c**). The higher activity was probably due to the better penetration ability through the plasma membrane of the ester moiety than the carboxylic acid functionality as indicated in previous reports.^{28,29} Notedly, compound **7c** (IC₅₀ = 18.88 μM) with the L-phenylalanine ethyl ester moiety displayed the best cytotoxicity towards the MCF-7 cancer cell line as compared with 5-FU (IC₅₀ = 8.59 μM). The observed activity of **7c** could be related to the aromaticity and hydrophobicity of the phenylalanine moiety as indicated in the previous reports.^{16,20,30–32} The two structures **7a** and **7c** could be served as the starting point for further optimization towards the potent cytotoxicity agents. Studies have been actively continuing in our lab with two goals. The first is to validate the developed synthetic procedure to access the library of the benzimidazole and amino acid/peptide conjugates. The second is to clarify the structure-activity relationship (SAR) of the synthesized compounds for discovery of

hit structures targeting anticancer activity. These results will be reported in due course.

4. CONCLUSION

Six novel benzimidazole-conjugated amino acid derivatives were successfully designed and synthesized in reasonable to good yields based on a three-step synthetic procedure starting from commercially available *o*-fluoroneurobenzenes. Among those, compound **7c** displayed rather good cytotoxicity against the breast cancer cell line. This structure may be regarded as a lead molecule that merits more refinement and the creation of new anticancer drugs.

ACKNOWLEDGMENT

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