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Solid-phase total synthesis of cyclic peptide brachystemin A and its immunomodulating activity

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Abstract: Brachystemin A (**1**) is a biologically active peptide from a Chinese traditional plant *Brachystemma calycinum* D. Don. (Caryophyllaceae). The current study describes the complete solid-phase total synthesis of peptide **1** by using Kenner's sulfonamide safety-catch linker strategy. It was identified by QTOF/MS data and NMR studies. Synthetic peptide **1** was tested for its immunomodulatory effect on different inflammatory parameters, including production of inflammatory cytokines, interleukin 2 (IL-2), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β), and generation of nitric oxide ($NO^{\cdot}$). The synthetic peptide **1** showed a moderate to low level of inhibition on the production of IL-2 (35.2%), TNF-α (19.3%), and IL-1β(7.5%) at a concentration of 25 μM. The effect of the compound on viability of cells was also evaluated, and it was found to be nontoxic on 3T3 cells.

Key words: *Brachystemma calycinum*, cyclic peptide, brachystemin A, safety-catch linker, immunomodulatory effect, cytokines

1. Introduction

Plants belonging to the family Caryophyllaceae produce cyclic peptides containing 5–9 proteinogenic amino acids.[1–3] Most of them are biologically active in mammalian systems. The medicinal plant *Brachystemma calycinum* D.Don. (Caryophyllaceae) is known to contain the cyclopeptides brachystemins A–I. *B. calycinum* is used in Chinese folk medicine for the treatment of rheumatoid arthritis, impotence, limb numbness, and gonorrhea. On the basis of the traditional uses of this herb in inflammatory diseases, the cyclic peptide constituents of this plant were tested in vitro for their inhibitory effects on the secretion of chemokine ligand-2, interleukin 6, and collagen IV by high-glucose-stimulated mesangial cells. Brachystemin A (**1**) was identified as the most active constituent as it significantly inhibited the secretion of interleukin 6, chemokine ligand-2, and collagen IV and exhibited no toxic effect in mesangial cells.[4,5]

Inflammation and oxidative stress are involved in many pathological conditions. The role of inflammatory mediators, including cytokines and free radicals, in the disease pathology is well established. Various diseases caused by deregulation of the immune system, including rheumatoid arthritis, atherosclerosis, inflammatory

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bowel disease, diabetes, and neurodegenerative disorders, pose serious health problems worldwide. New therapeutic approaches to target inflammatory parameters, mainly involved in disease pathogenesis, are continuously needed.[6]

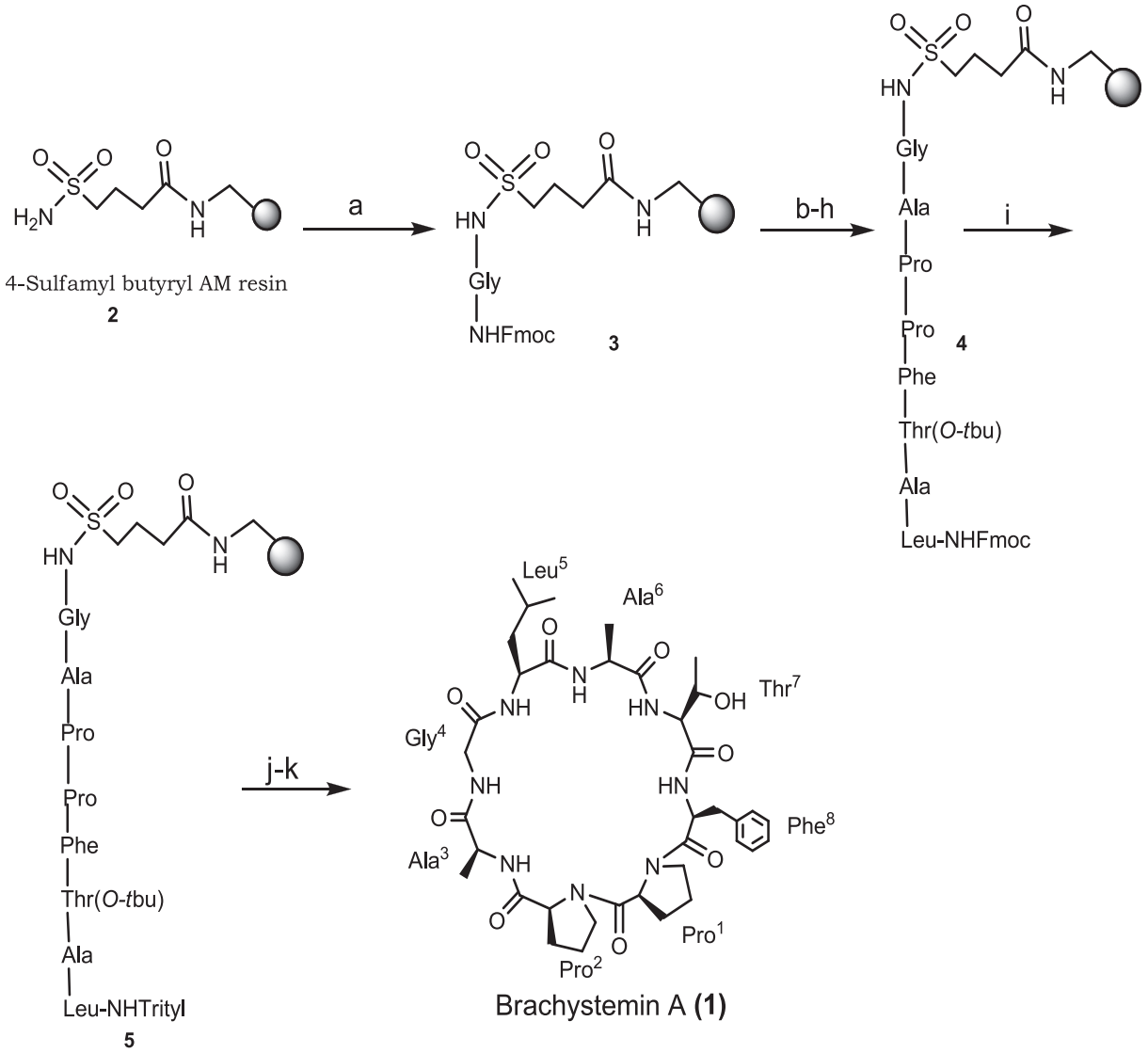
Biologically active cyclic peptides are reported from many natural sources.[7,8] However, they are usually obtained from natural sources in very low yields. Many investigators have reported the synthesis of cyclic peptides by different strategies[9−12] to obtain them in sufficient quantities for subsequent development as medicinal agents. The yield of cyclic peptides through various cyclization approaches are often low due to many reasons, including difficulties in the cyclization of peptides having three to eight amino acids on resin,[13−17] epimerization at the C-terminal amino acid, and the formation of cyclic dimers and oligomers.[18,19] Among the various synthetic methods, the on-resin cyclization approach has been successfully employed in the synthesis of many biologically active natural products.[20−23] The current study describes the solid-phase synthesis of brachystemin A (**1**) by using Kenner's sulfonamide safety-catch linker. The synthetic peptide was evaluated for its immunomodulatory activity by performing different cell-based assays.

2. Results and discussion

Brachystemin A (**1**) was first isolated from *B. calycinum* by Cheng and coworkers. It was identified as cyclo-Pro_1-Phe_2-Leu_3-Ala_4-Thr_5-Pro_6-Ala_7-Gly_8. Later, this compound was re-isolated and its primary structure was revised as cyclo-Pro_1-Pro_2-Ala_3-Gly_4-Leu_5-Ala_6-Thr_7-Phe_8 by quadrupole-time-of-flight (QTOF) mass spectrometers and X-ray diffraction analysis. During the course of the current study, Lijing Fang et al. reported the combination of solid and solution-phase synthesis of brachystemin A in which triphosgene (BTC) was used as the coupling agent in the synthesis of the linear precursor of brachystemin A. The linear peptide was cleaved off from the Wang resin and finally subjected to cyclization.

Herein, we report another convenient route to the complete solid-phase synthesis of peptide **1** by using resin bound sulfonamide anchor (Scheme). The use of sulfonamide linker allows the cyclization and cleavage steps simultaneously from the resin, thus minimizing the formation of side products. The first amino acid is usually loaded twice on the solid-supported safety-catch linker to ensure maximum loading. In order to avoid the expected racemization during the loading step, as well as to increase the yield of target peptide **1**, the less sterically hindered amino acid residue of **1**, i.e. glycine, was selected as the first amino acid for coupling with the resin in the synthesis of peptide **1** (Scheme). It is also expected that the terminal free amino group of linear peptide precursor would more readily attack a less sterically hindered electrophilic carbonyl of glycine during the macrocyclization step. The two proline residues present in brachystemin A (**1**) also serve as turn-inducers.[24] It would further make the two ends close to each other, and can support macrocyclization.

On the basis of the above consideration, the first amino acid Fmoc-Gly was loaded on sulfonamide resin by using the coupling agent (benzotriazol-1-yloxy) tripyrrolidino- phosphoniumhexafluorophosphate (PyBOP) and base N,N-diisopropylethylamine (DIEA). The loading capacity of the first amino acid was analyzed by UV spectroscopy.[25] The linear peptidyl resin **4** was constructed by using the Fmoc protocol. The Fmoc group of terminal amino acid of peptidyl resin was replaced by the bulkier trityl group before the activation of the safety-catch linker by cyanomethylation. The terminal amino acid of the linear peptide was made free by using 5% triflouro acetic acid (TFA) in dichloro- methane. The cyclization and cleavage of peptide from resin were carried out in the presence of DIEA and tetrahydrofuran. The crude peptide was finally deprotected and then purified by recycling reversed-phase high performance liquid chromatography (RP-HPLC) by using a reverse-phase (C18) column to obtain cyclic peptide **1**. The structure of synthetic peptide **1** was fully characterized by



Reagents and reaction conditions: (a) PyBOP, DIEA, Fmoc-Gly-OH/DMF, 0 °C, 12 h, repeated twice; (b) (i) 20% 4-methylpiperidine/DMF, 20 min (ii) Fmoc-Ala-OH/DMF, PyBOP, DIEA, 4 h; (c) (i) 20% 4-methylpiperidine/DMF, 20 min (ii) Fmoc-Pro-OH/DMF, PyBOP, DIEA, 4 h; (d) (i) 20% 4-methylpiperidine/DMF, 20 min (ii) Fmoc-Pro-OH/DMF, PyBOP, DIEA, 4 h; (e) (i) 20% 4-methylpiperidine/DMF, 20 min (ii) Fmoc-Phe-OH/DMF, PyBOP, DIEA, 4 h; (f) (i) 20% 4-methylpiperidine/DMF, 20 min (ii) Fmoc-Thr ($O-t$bu)-OH/DMF, PyBOP, DIEA, 4 h; (g) (i) 20% 4-methylpiperidine/DMF, 20 min (ii) Fmoc-Ala-OH/DMF, PyBOP, DIEA, 4 h; (h) (i) 20% 4-methylpiperidine/DMF, 20 min (ii) Fmoc-Leu-OH/DMF, PyBOP, DIEA, 4 h; (i) (i) 20% 4-methylpiperidine/DMF, 20 min (ii) trityl chloride, DIEA; (j) ICH_2CN, DIEA, NMP, 24 h; (k) (i) 5% TFA/DCM, 0.5 h, (ii) DIEA, 20 h in THF, (iii) TFA/TIS/H_2O (9.5:0.25:0.25).

Scheme 1. Synthesis of brachystemin A (**1**).

1D and 2D-NMR, as well as QTOF data. Thus, the current study describes the complete solid-phase synthesis of brachystemin A via an on-resin cyclization approach. The overall yield of the finally pure product (7.4%) is higher than that of the earlier reported synthesis of cyclic peptides by using the safety-catch linker strategy (Figures S1–S8, supporting information; on the journal's website).

The molecular formula of cyclic peptide **1** was deduced as $C_{37}H_{54}N_8O_9$ by high resolution electron spray ionization (HRESI) mass spectrometry, which exhibited the molecular ion $[M+H]^+$ at m/z 755.4068 (calcd. 755.4092). NMR data were recorded in d_5-pyridine as it was used for natural brachystemin A previously. A comparative study of ^{1}H NMR data of natural and synthetic brachystemin A exhibited close resemblance (Table 1). ^{1}H NMR showed six amide protons, resonating at δ_H 10.56 (1H, b, Ala_3-NHCO), 9.60 (1H, d, Ala_6-NHCO), 8.96 (1H, t, Gly_4-NHCO), 7.70 (1H, d, Phe_8-NHCO), 7.51 (1H, d, Thr_7-NHCO), and 7.15 (1H, s, Leu_5-NHCO). These amide proton resonances were the same as those observed with natural brachystemin A. Furthermore, L-amino acid residues of cyclic peptide **1** showed alpha protons at δ_H 5.39 (1H, Leu_5-CH), 5.21 (1H, m, Ala_3-CH), 5.05 (1H, m, Phe_8-CH), 5.03 (1H, d (J = 6.0 Hz), Pro_1-CH), 4.79 (1H, d, J = 8.1 Hz, Thr_7- CH), 4.60 (1H, m, Pro_2-CH), and 4.29 (1H, m, Ala_6-CH) (Table 1). The ^{13}C NMR chemical shift differences of Pro_1 ($\Delta\delta$ C_β-C_γ = 3.1) and Pro_2($\Delta\delta$ C_β-C_γ= 3.6) indicated that the amide bonds in the two Pro residues are *trans*,[26] similar to the natural product (Table 1). The structure of cyclic peptide **1** was further confirmed by QTOF/MS data, which showed a series of b_n (+1) ion peaks at m/z 737, 608, 507, 436, and 266, corresponding to the successive loss of Phe, Thr, Ala, Leu-Gly, and the terminal tripeptide ion Pro-Pro-Ala (Table 2).

The effect of synthetic peptide **1** was observed on inflammatory cytokines TNF-α, on IL-1β produced from THP-1 cells, and on IL-2 produced from Jurkat cells at a concentration of 25 μM. All biological assays were performed in triplicate. Standard deviation values are presented in Table 3. The peptide moderately inhibited the production of TNF-α(19.3%) and IL-2 (35.2%), whereas a low level of inhibition was also observed on IL-1β(7.5%). The peptide was also evaluated for its effect on nitric oxide (NO$\cdot$) generation by using lipopolysaccharide activated macrophages from the J774.2 cell line. Similar to the natural product, the synthetic peptide showed a very weak inhibitory effect (7.5%) at a concentration of 30 μM. The peptide was further evaluated for cytotoxicity against fibroblast cell line 3T3, where it was found to be nontoxic (Table 3). The data for cytotoxicity are plotted as percent viability explaining the number of viable cells at different concentrations of peptide **1** and standard drug (Figure). The inactivity on nitric oxide (NO$\cdot$) and noncytotoxic effect of peptide **1** was in agreement with the previously described results of isolated peptide.

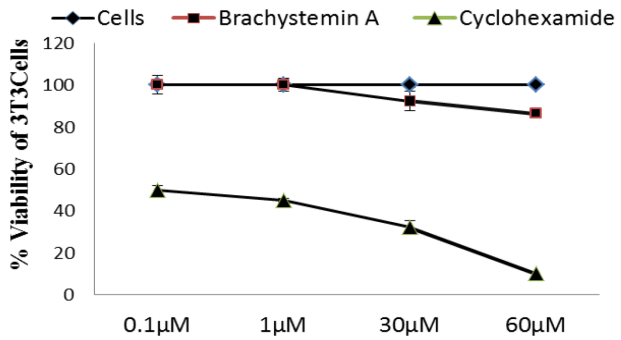


Figure. Effect of brachystemin A (**1**) on viability of 3T3 cells. Cyclohexamide was used as standard drug for cytotoxicity.

Table 1. ^{1}H and ^{13}C spectral data of cyclic peptide **1.**

Amino Acid	Position	Natural Peptide **1**		Synthetic Peptide **1**	
		δ^1 H (J in Hz)	δ^{13} C	δ^1 H (J in Hz)	δ^{13} C
Phe	α	5.01 m	55.2	5.05 m	55.67
	β	3.45 m	36.1	3.45 m	36.42
		3.34 m		3.34 m	
	γ		138.9		138.7
	σ	7.18–7.53	130.1 129.0 127.2	7.18–7.51*	129.9 128.8 127.0
	NH	7.73, d, (J = 5.6)		7.70, d, (J = 5.4)	
	C=O		169.8		169.6
Thr	α	4.79, d, (J = 8.0)	59.8	4.79, d, (J = 8.15)	59.5
	β	5.03 m	68.0	5.03 m	66.3
	$CH_3\gamma$	1.40, d, (J = 6.4)	21.8	1.39, d, (J = 6.3)	21.8
	NH	7.50, d, (J = 7.6)		7.51, d, (J = 7.6)	
	C=O		172.2		171.7
Ala^6	α	4.30 m	53.6	4.29 m	53.4
	CH_3	1.55, d, (J = 5.6)	16.8	1.55, d, (J = 5.2)	16.7
	NH	10.61, d, (J = 2.0)		10.56, bs	
	C=O		174.2		173.7
Leu	α	5.43 m	49.7	5.39 m	49.0
	β	1.31m	29.1	1.31 m	28.1
		1.25m		1.24 m	
	γ	2.03m	24.8	2.03m	24.5
	$2CH_3\sigma$	0.89, d, J (6.8), 0.99, d, J =(6.0)	21.3, 23.8	0.73, d, J (6.3), 0.97, d, J = (6.3)	20.9, 24.5
	NH	7.10(br. s)		7.15 (bs)	
	C=O		177.2		177.17
Gly	α	4.54, dd, (J = 16.8, 5.6) 3.87 dd (16.8, 5.6)	44.2	4.53, Overlap	44.1
	β	3.87, (J = 16.8,5.6)		3.86, Overlap	
	NH	8.96, t, (J = 6.4)		8.96, t, (J = 6.0)	
	C=O		169.39		169.3
Ala^3	α	5.20 (m)	48.8	5.21(m)	48.6
	CH_3	1.84, d, (J = 7.6)	18.8	1.85, d, (J = 7.2)	18.7
	NH	7.95, d, (J = 9.6)		7.95, d, (J = 9.6)	
	C=O		173.7		173.5
Pro^2	α	4.61 m	59.8	4.60 m	63.9
	β	2.02 m	30.0	2.01 m	28.9
	γ	1.55 m	26.4	1.71 m	26.2
		1.32 m		1.34 m	
	σ	3.16 m	47.2	3.15 m	47.0
		3.38 m		3.37 m	
	C=0		172.0		171.7
Pro^1	α	4.94 d	66.6	5.03 Overlap	64.5
	β	2.31 m	28.2	2.29 m	28.1
		1.80 m		1.79 m	
	γ	2.05 m	25.1	2.05 m	25.0
		1.96 m		1.98 m	
	σ	4.01 m	47.6	4.00 m	47.4
		3.40 m		3.41 m	
	C=0		172.4		172.2

Table 2. QTOF/MS sequence ions (m/z) of the protonated molecular ions of cyclic peptide **1.**

Proposed fragment structure	MS/MS fragmentation of $[M+H^+]$
Pro_1–Pro_2–Ala_3–Gly_4–Leu_5–Ala_6–Thr_7–Phe_8	775
$[M+H^+]$	737
$[H–Pro_1–Ala_3–Gly_4–Leu_5–Ala_6–Thr_7]^+$	608
$[(H–Pro_1–Pro_2–Ala_3–Gly_4–Leu_5–Ala_6–Thr_7]\text{-}H_2O]^+$	590
$[H–Pro_1–Pro_2–Ala_3–Gly_4–Leu_5–Ala_6]^+$	507
$[H–Pro_1–Pro_2–Ala_3–Gly_4–Leu_5]^+$	436
$[H–Pro_1–Pro_2–Ala_3]^+$	266

Table 3. Effect of brachystemin A (**1**) on production of inflammatory cytokines TNF-α, IL-1β, IL-2, and nitric oxide. Effect of peptide on viability of 3T3 cells was also evaluated using MTT assay. The results are presented as mean ± SD of triplicates.

Compound	TNF-α % Inh	IL-1β % Inh	IL-2 % Inh	NO % Inh	Cytotoxicity IC_{50} (μM)
Brachystemin A	19.3 ± 1.0	7.5 ± 1.8	35.2 ± 11.4	7.4 ± 0.1	> 60
N^G Methyl L-Arginine Acetate	-	-	-	65.65 ± 1.1	-
Cyclohexamide	-	-	-	-	0.1 ± 0.2

In conclusion, the total synthesis of natural peptide brachystemin A (**1**), involving a solid-phase route by using safety-catch linker, was carried out. The structure was identified by mass spectrometry and nuclear magnetic resonance spectroscopy. Furthermore, this peptide was found to be nontoxic on a normal cell line (3T3 fibroblast cells). In this study, brachystemin A was also found to be a moderate inhibitor of IL-2 and TNF-α. Thus, it can be an important lead for drug discovery against inflammatory diseases. However, in vivo studies are necessary to evaluate the effects of brachystemin A (**1**) in inflammatory diseases.

3. Experimental

3.1. General experimental procedures

Protected amino acids, resin, and all other chemicals and reagents were purchased from Sigma Aldrich, Chemimpex, and Novabiochem. The peptide **1** was purified by RP-HPLC (LC-900 Japan). C18 Column Jaigel ODS-MAT 80 was used in the purification of the peptide at a flow rate of 4 mL/min, and H_2O/CH_3OH (50:50) was used a mobile phase. A Bruker 500 MHz was used for recording ^1H and ^{13}C nuclear magnetic resonance spectra, and chemical shifts were reported in parts per million. Electron spray ionization mass spectra were recorded on a QSTAR XL (Applied Biosystems).

3.2. Peptide synthesis

4-Sulfamylbutyryl AM resin was soaked in dimethylformamide (DMF) for 1 h. Fmoc-amino acid (4 equiv.) was synthesized manually in a 10-mL polypropylene syringe fitted with a filter disc and agitation was performed on an orbital shaker. PyBOP (2.28 g, 4 equiv.), and DIEA (1.5 mL, 8 equiv.) in DMF were added to the resin. The reaction mixture was stirred for 24 h. This coupling step was repeated to achieve maximum loading.

3.3. Peptide coupling

The swollen peptidyl resin **3** was deprotected by 20% 4-methylpiperidine in DMF for 20 min. After the deprotection step, the resin was washed with DMF. The next Fmoc-amino acid (3 equiv.) was activated by PyBOP (4 equiv.), and DIEA (4 equiv.) in 5 mL of DMF.

3.4. Activation of sulfonamide linker

The Fmoc protecting group of linear peptidyl resin **4** was removed by 20% 4-methylpiperidine in DMF for 20 min, and the amino group of the terminal amino acid was protected by trityl chloride (1.227 g, 4 equiv.) in the presence of DIEA (1.5 mL, 8 equiv.) for 2 h. The sulfonamide linker was activated by reaction with iodoacetonitrile (0.8 mL, 10 equiv.) in the presence of DIEA (2.25 mL, 12 equiv.) and *N*-methylpyrrolidinone (NMP) for 12 h under N_2. The reaction was protected from light by covering the reaction vessel with aluminum foil. The resin was washed, and the trityl group was removed by 5% trifluoroacetic acid/dichloromethane for 2 h.

3.5. Cyclization and release of peptide from the resin

The activated *N*-acylsulfonamide linker was soaked in tetrahydrofuran and treated with base DIEA (565 μL, 3 equiv.) for 24 h under N_2. The resin was filtered and washed with tetrahydrofuran and dichloromethane (3 $\times$ 25 mL each). The filtrate was concentrated to remove solvents and the crude residue was precipitated with cold diethyl ether. Finally, the side chain protecting groups were removed by treatment with TFA/TIS/H_2O (9.5:0.25:0.25). The peptide residue was precipitated by cold ether, lyophilized, and then purified by RP-HPLC.

Brachystemin A (**1**) Cyclo-(Pro$_1$-Pro$_2$-Ala$_3$-Gly$_4$-Leu$_5$-Ala$_6$-Thr$_7$-Phe$_8$); 59.5 mg (7.4%); $[\alpha]_D^{25}$ –23 (c0.0005, MeOH); ^{1}H NMR (500 MHz) and ^{13}C NMR (125-MHz) (Table 1); ESI-MS m/z 755 $[M + H]^+$; HR-TOF-ESI-MS m/z755.4068 $[M + H]^+$ (calcd. for $C_{37}H_{45}N_8O_9$, 755.4092).

3.6. Nitrite concentration in mouse macrophage culture medium

The mouse macrophage cell line J774.2 (European Collection of Cell Cultures, UK) was cultured in 75-cc flasks (IWAKI Asahi Techno Glass, Tokyo, Japan) in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, Steinheim, Germany) that contained 10% fetal bovine serum (GIBCO, New York, NY, USA) supplemented with 1% streptomycin/penicillin. The flasks were kept at 37 °C in humidified air containing 5% CO_2. Cells (10^6 cells/mL) were then transferred to a 24-well plate. The nitric oxide synthase (NOS-2) in macrophages was induced by the addition of 30 μg/mL *E. coli* lipopolysaccharide (LPS) (Difco Laboratories, Detroit, MI, USA). The test compounds were added at 30-μM concentration and cells were further incubated at 37 °C in 5% CO_2. The supernatant was collected after 48 h for analysis. Nitrite accumulation in cell culture supernatant was measured using the Griess method described.[27]

3.7. Cytokine production and quantification

THP-1 (human monocytic leukemia cells) was obtained from the European Collection of Cell Cultures (UK). The cells were maintained in endotoxin-free RPMI-1640 containing 5.5 mmol/L glucose (BioM Laboratories, Chemical Division, Malaysia), 50 μmol/L mercaptoethanol (Merck, Darmstadt, Germany), 10% fetal bovine

serum (FBS), 2 mmol/L L-glutamine (PAA Laboratories, GmbH, Pasching, Austria), 1 mmol/L sodium pyruvate (GIBCO, Grand Island, NY, USA), and 10 mmol/L (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (MP Biomedicals, Illkirch, France). Cells were grown in 75-cc flasks until they attained 70% confluence, and then were plated in 24-well tissue culture plates at a concentration of 2.5×10^5 cells/mL. The cells were differentiated into macrophage-like cells by using phorbol myristate acetate (PMA) (SERVA, Heidelberg, Germany) at a final concentration of 20 ng/mL and incubated for 24 h at 37 °C in 5% CO_2. The cells were then stimulated with *E. coli* Lipopolysacchride B (Difco Laboratories) at a final concentration of 50 ng/mL and treated with peptide **1** at a concentration of 25 μM. The cells were then incubated for 4 h at 37 °C in 5% CO_2. The supernatants collected were analyzed for the level of TNF-α and IL-1β. Jurkat (human T lymphocyte leukemia) cells were kindly provided by Prof Daniel Hoessli (University of Geneva, Switzerland). The cells were maintained in RPMI-1640 supplemented with 5% FBS and 1% penicillin/streptomycin. Upon 70% confluence the cells were plated in 96-well flat bottom plates at a concentration of 2×10^6 cells/mL. The cells were activated by using 20 ng/mL phorbol myristate acetate (PMA) and 7.5 μg/mL phytohemagglutinin (PHA) (SERVA, Heidelberg, Germany). The cells were then treated with peptide **1** at a concentration of 25 μM and plate was incubated for 18 h at 37 °C in 5% CO_2. Supernatants were collected and analyzed for interleukin-2 cytokine. Cytokine quantification in supernatants was performed using the human TNF-α, IL-1β, and IL-2 Kits Duo Set (R&D Systems, Minneapolis, MN, USA), and according to the manufacturer's instructions.

3.8. Cytotoxicity assay

An in vitro cytotoxicity assay was performed as described previously.[28] Briefly 3T3 cells were harvested and suspended in Dulbecco's Modified Eagle's Medium supplemented with 5% FBS. Then 100 μL of 6×10^4 cells/mL were plated in 96-well flat bottomed plates and the plates were incubated for 24 h at 37 °C in 5% CO_2. After incubation, media was carefully removed and the cells were charged with different concentrations (10–100 μM) of cyclopeptide in triplicate; the final volume of 200 μL in each well was adjusted with complete Dulbecco's Modified Eagle's Medium. Plates were then further incubated for 48 h at 37 °C in a CO_2 incubator. After 48 h, the supernatant was carefully removed and 50 μL of 0.5 mg/mL (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) from 5 mg/mL stock was added to each well and the plates were then incubated for a further 4 h. After incubation, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was aspirated and formazan crystals were dissolved by addition of 100 μL of dimethyl sulfoxide with gentle agitation for 10–15 min in an orbital shaker (MTS 2/4 Digital Microtiter Shaker, IKS, Staufen, Germany). The extent of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction to formazan within cells was calculated by measuring the absorbance at 540 nm, using a spectrophotometer. The cytotoxicity was recorded as concentration causing 50% growth inhibition (IC_{50}).

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Supporting Information

NMR, ESI-MS/MS spectra, analytical high performance liquid chromatography profile of peptide brachystemin A.

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Solid-phase total synthesis of cyclic peptide brachystemin A and its immunomodulating activity

Supporting Information

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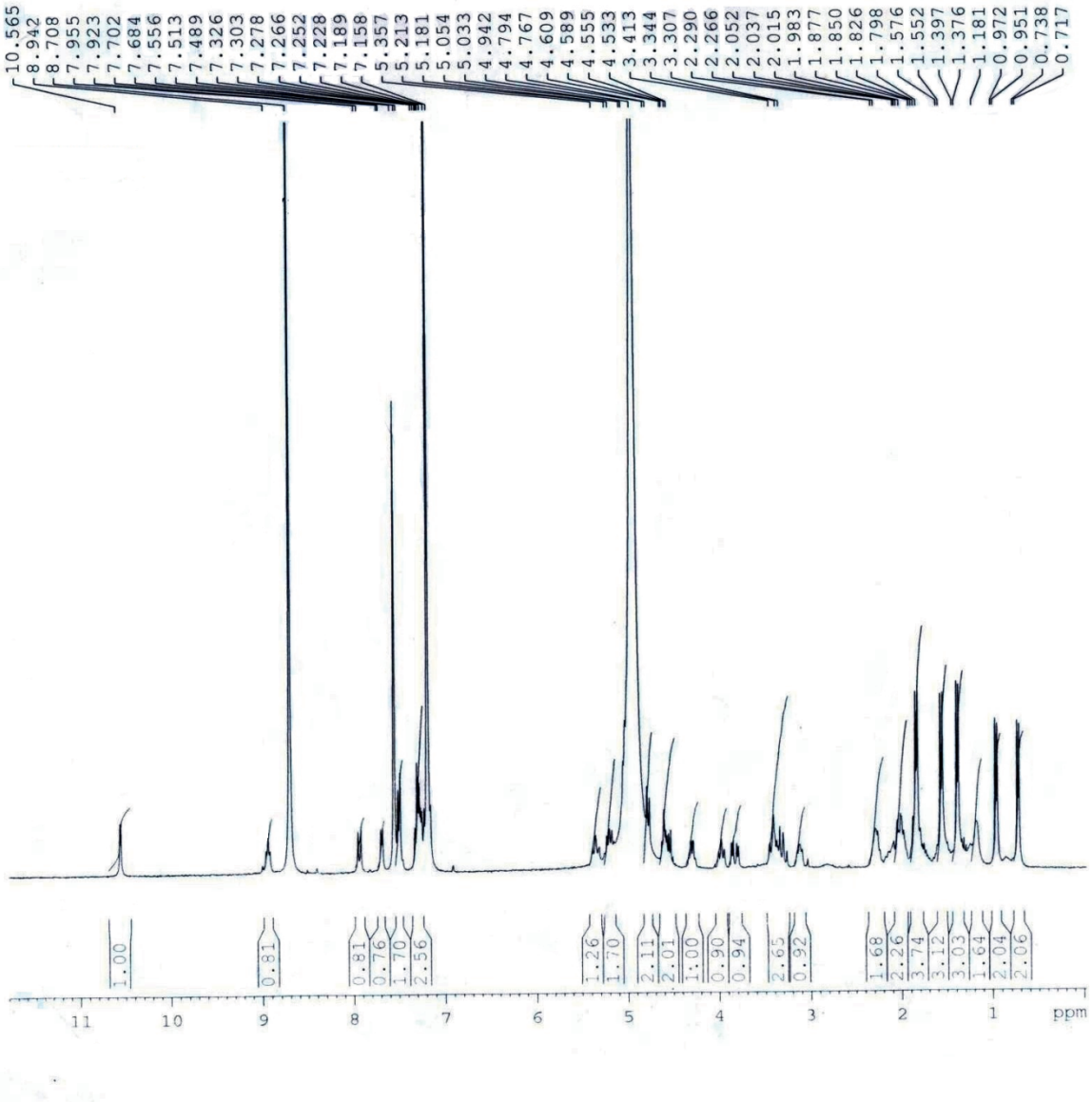


Figure S1. ^{1}H NMR spectrum of peptide (**1**) in d_5-pyridine (300 MHz).

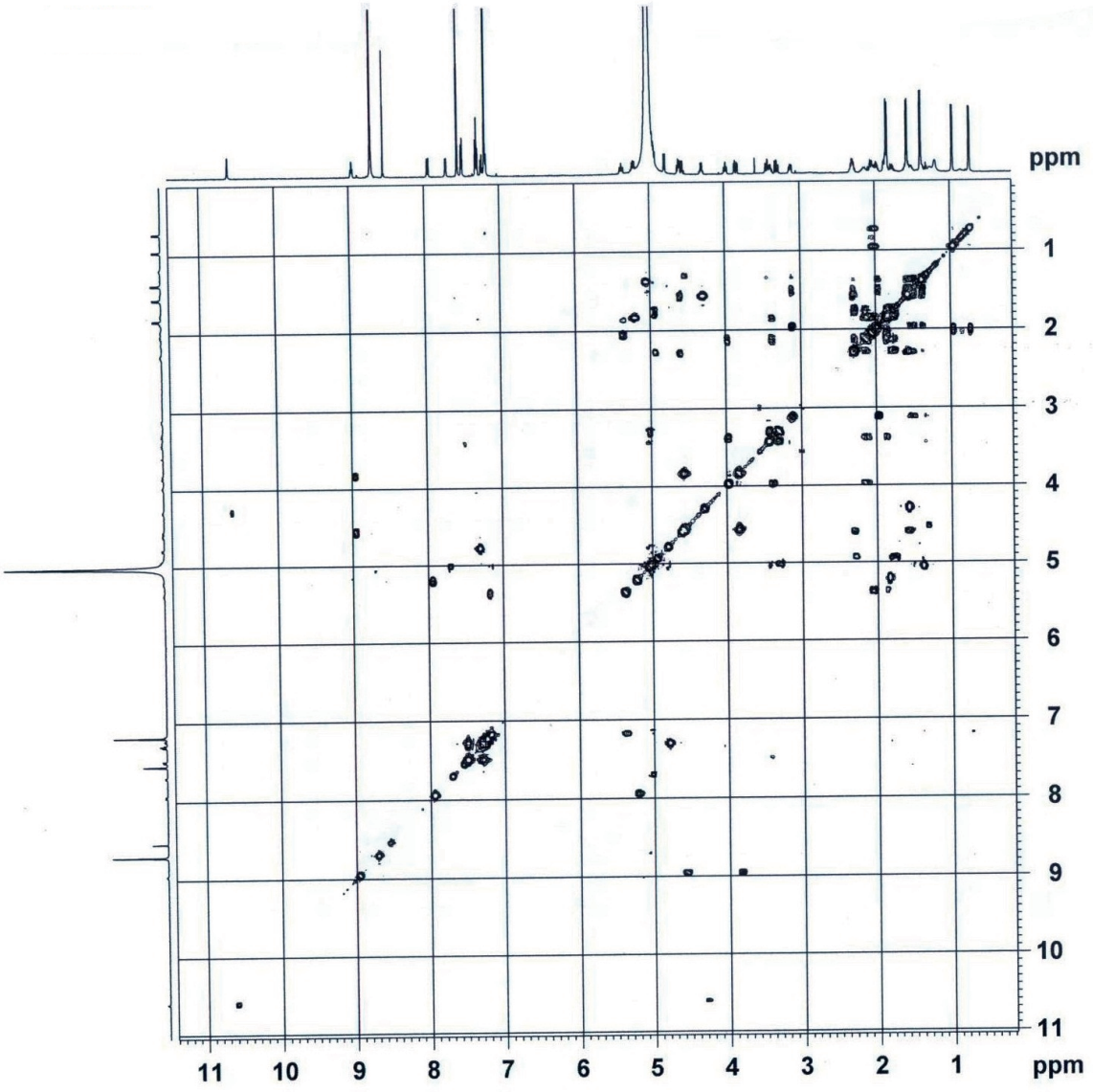


Figure S2. COSY NMR spectrum of peptide (**1**) in d_5-pyridine (500 MHz).

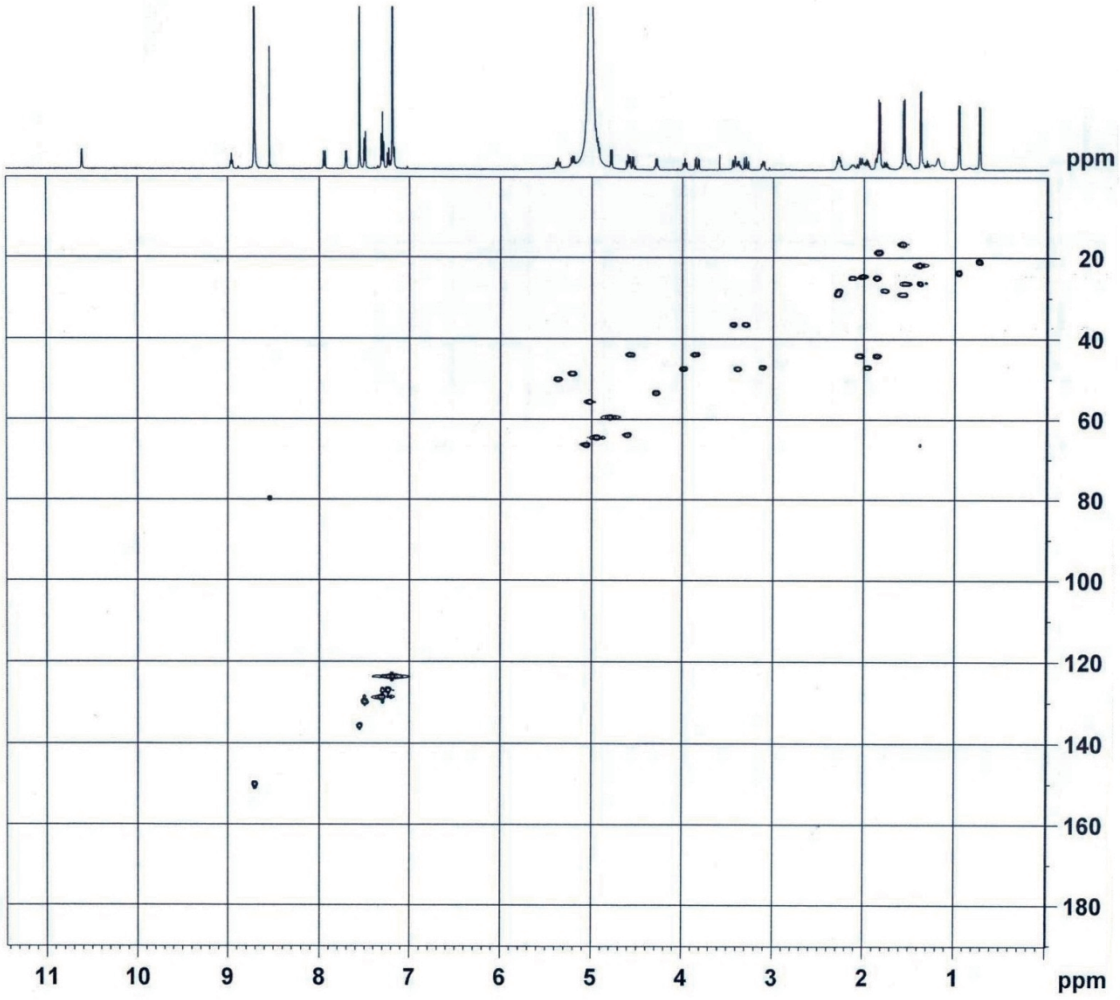


Figure S3. HSQC NMR spectrum of peptide (**1**) in d_5-pyridine (500 MHz).

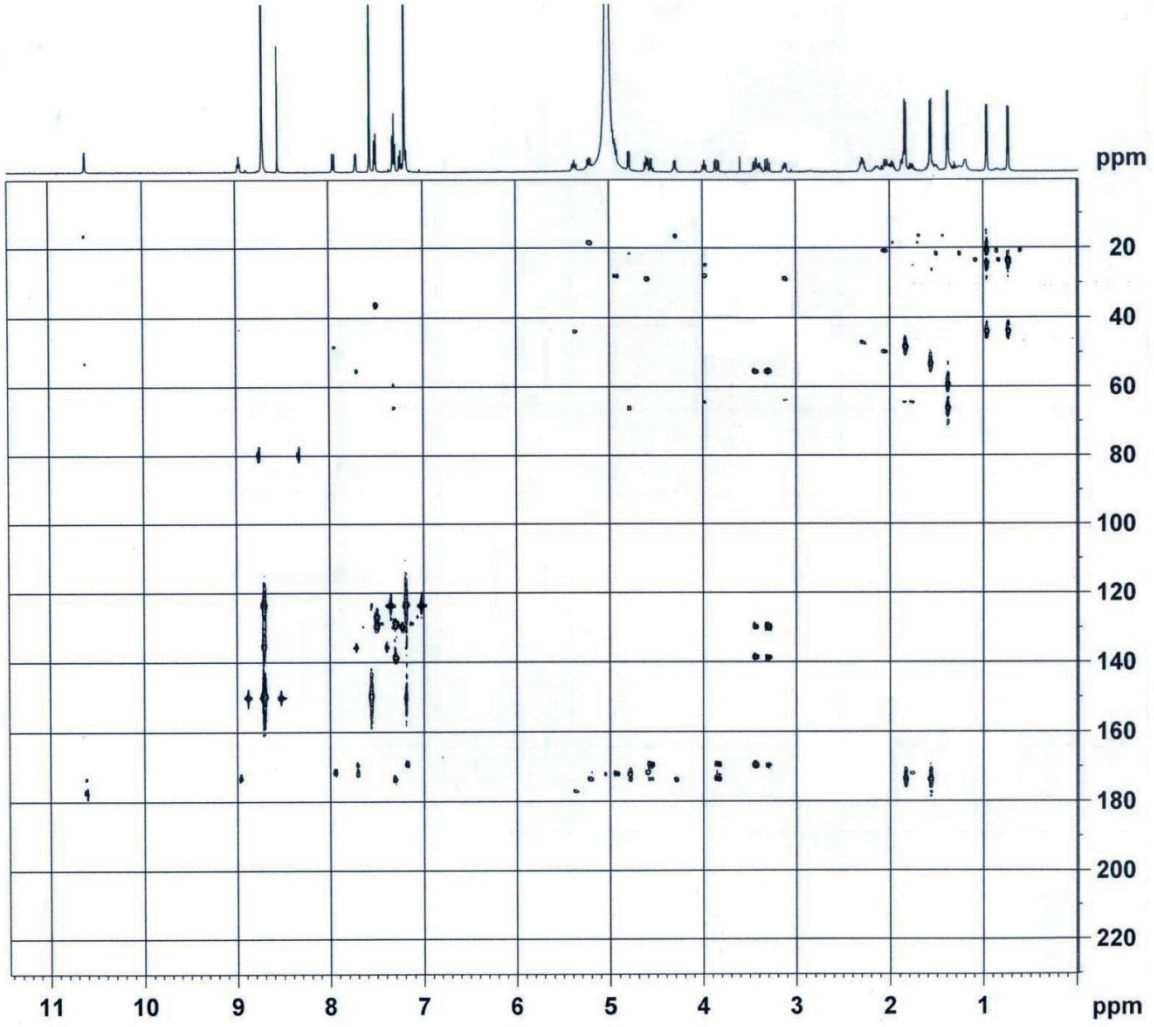


Figure S4. HMBC NMR spectrum of peptide (**1**) in d_5-pyridine (500 MHz).

Elemental composition calculator

Target m/z: +755.4068 amu
Tolerance: +10.0000 ppm
Result type: Elemental
Max num of results: 20
Min DBE: -0.5000 Max DBE: +50.0000
Electron state: OddAndEven
Num of charges: 0
Add water: N/A
Add proton: N/A
File Name: BA 19-8-14.wiff

	Elements	Min Number	Max Number
1	C	0	40
2	H	0	60
3	N	0	8
4	O	0	9

	Formula	Calculated m/z (amu)	mDa Error	PPM Error	DBE
1	C37 H55 N8 O9	755.4092	-2.4010	-3.1784	14.5

Figure S5. HR-ESI mass spectra of peptide (**1**).

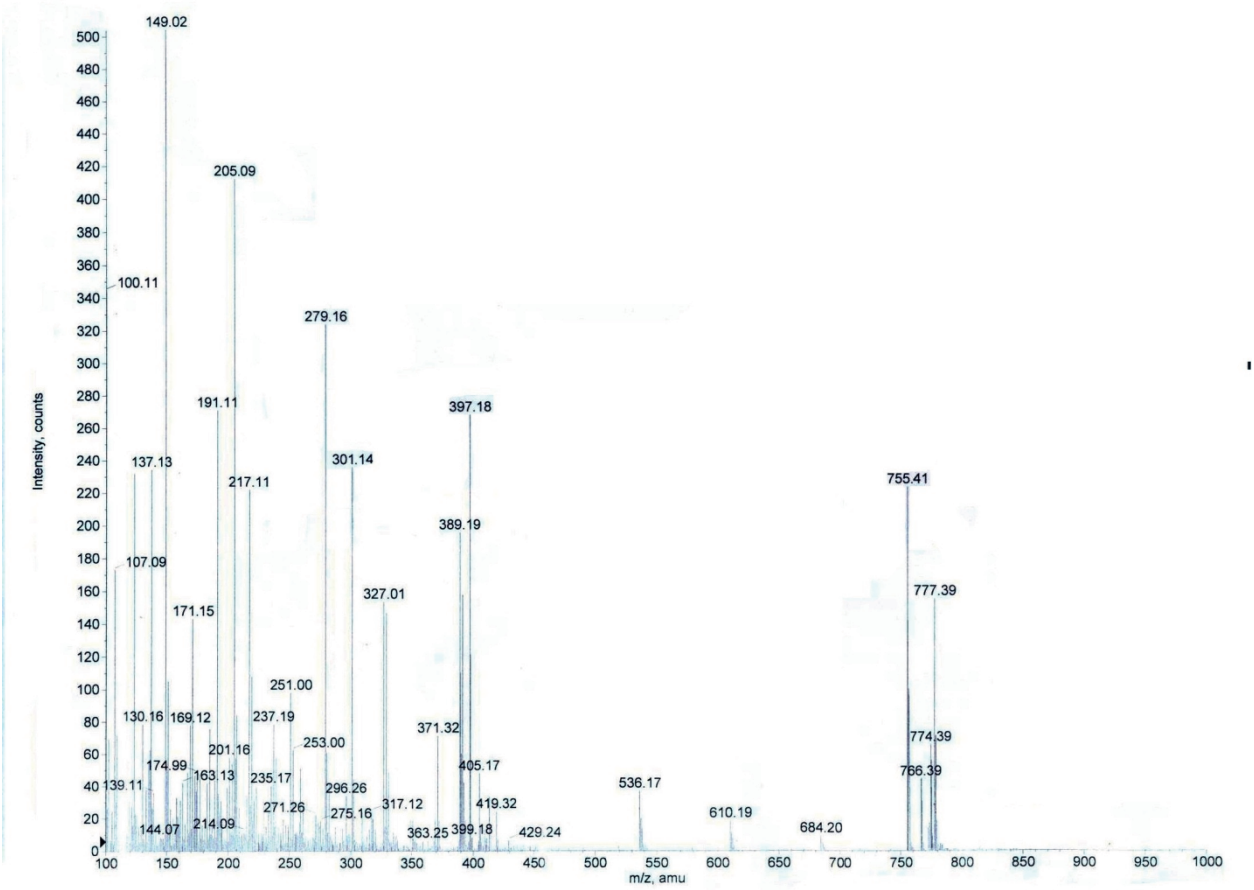


Figure S6. ESI mass spectra of peptide (**1**).

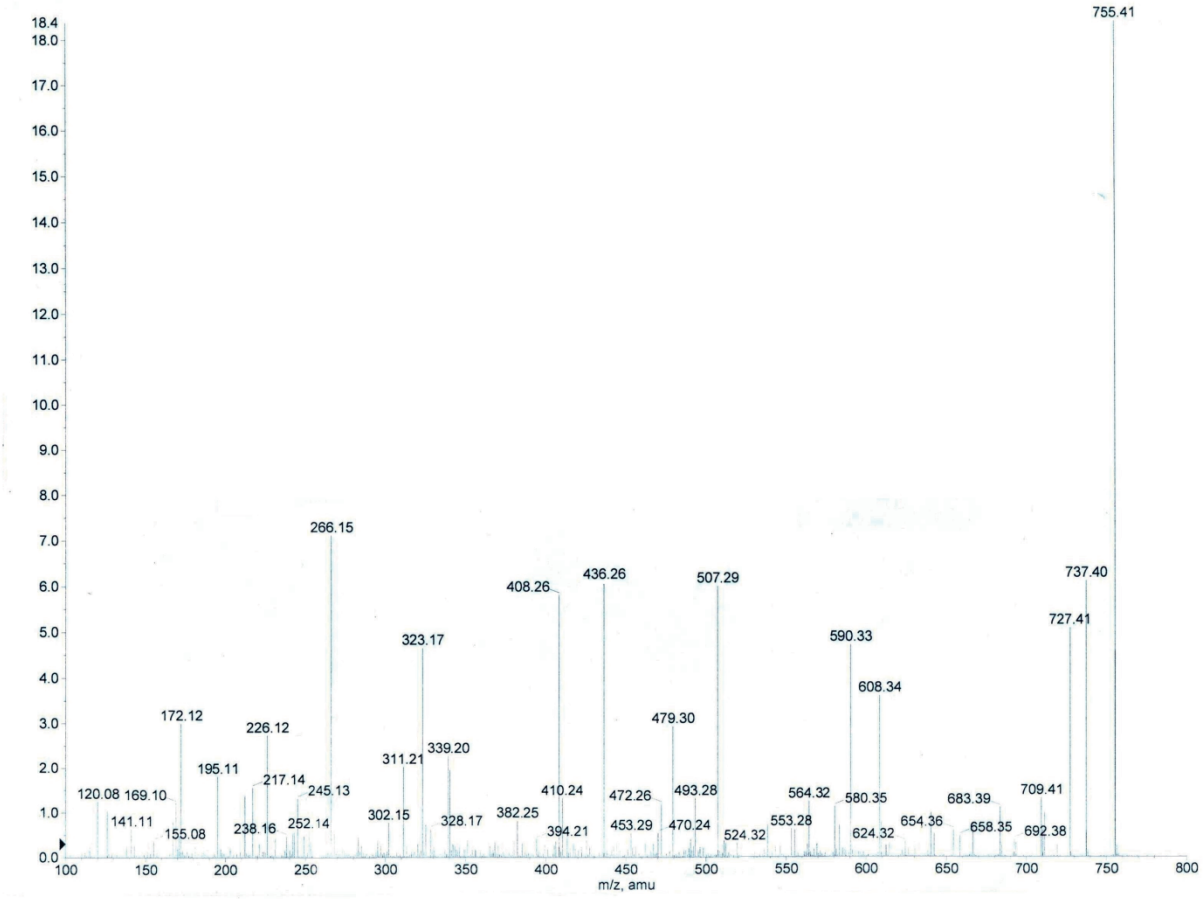
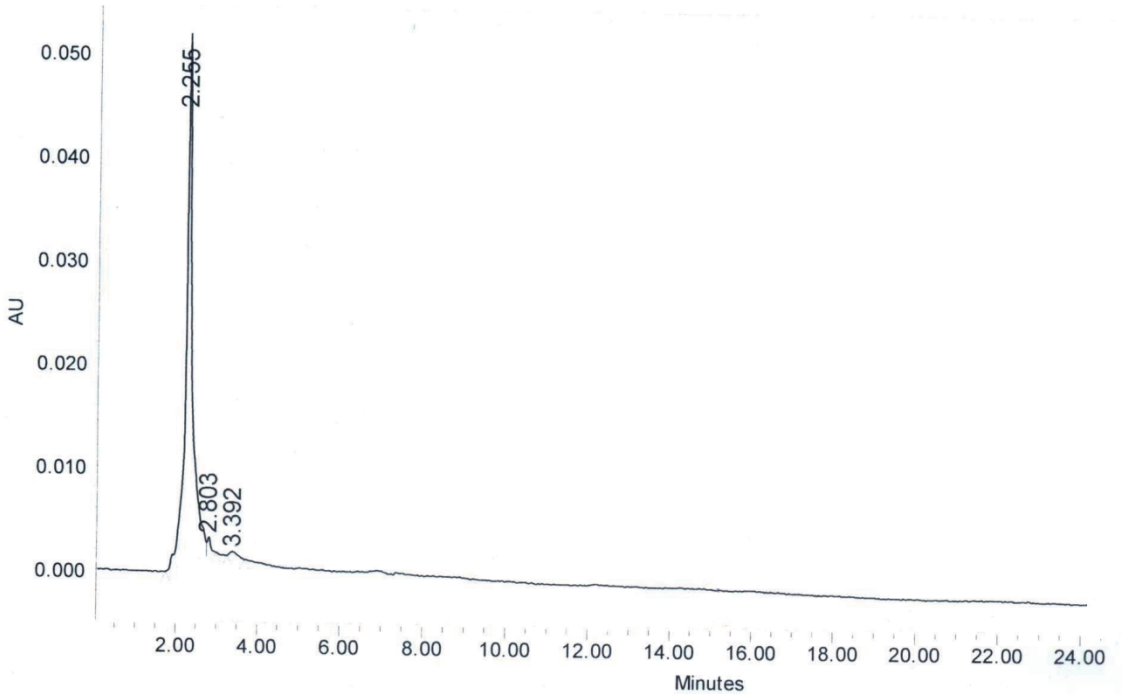


Figure S7. ESI MS/MS of peptide (**1**).



Peak Results

	RT	Area	% Area	Height (μV)	% Height
1	2.255	556531	97.05	50912	95.83
2	2.803	10717	1.87	1738	3.27
3	3.392	6172	1.08	480	0.90

Figure S8. Analytical HPLC profile of peptide (**1**).