Total synthesis, structural and biological evaluation of stylissatin A and related analogues

Farzana Shaheen,[†,*] Almas Jabeen,[‡] Samreen Ashraf,[†] Muhammad Nadeem-ul Haq,[†] Zafar Ali Shah,[†] Muhammad Asad Ziaee,[†] Nida Dastagir,[‡] and A. Ganesan,[§]

[†]H. E. J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan

[‡]Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan

[§]School of Pharmacy, University of East Anglia, Norwich Research Park, Norwich NR4 7TJ, United Kingdom

Correspondence to: Prof. Dr. Farzana Shaheen, H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan,
Email: afnan.iccs@gmail.com

ABSTRACT:

The natural product cyclic peptide stylissatin A (**1a**), was reported to inhibit nitric oxide production in LPS-stimulated murine macrophage RAW 264.7 cells. In the current study, solid-phase total synthesis of stylissatin A was performed by using a safety-catch linker and yielded the peptide with a *trans*-Pro^6-Phe^7 linkage whereas the natural product is the trans rotamer at this position as evidenced by a marked difference in NMR chemical shifts. In order to preclude the possibility of **1b** beingan epimer of the natural product, we repeated the synthesis using D-*allo*-Ile in place of L-Ile and a different site formacrocyclization.The resulting product (D-*allo*-Ile^2)-stylissatin A (**1c**)was also found to have the *trans*-Pro^6-Phe^7 peptide conformations like rotamer **1b**. Applying the second routeto the synthesis of stylissatin A itself, we obtained stylissatin A natural rotamer **1a** accompanied by rotamer **1b** as the major product. Rotamers **1a**, **1b** and the epimer **1c** were separable by HPLC and **1a** was found to match the natural product in structure and biological activity. Six related analogues **2-7** of stylissatin A were synthesized on Wang resin and characterized by spectral analysis. The natural product (**1a**), the rotamer (**1b**), and (D-*allo*-Ile^2)-stylissatin A (**1c**) exhibited significant inhibition of NO·. Further investigations were focused on **1b**, which also inhibited proliferation of T-cells and inflammatory cytokine IL-2 production. The analogues **2-7** weakly inhibited NO· production, but strongly inhibited IL-2 cytokine production compared to synthetic peptide **1b**. All analogues inhibited the proliferation of T-cells, with analogue **7** having the strongest effect. In the analogues, the Pro^6 residue was replaced by Glu/Ala and the SAR indicates that the nature of this residue plays a role in the biological function of these peptides.

Keywords: solid-phase peptide synthesis, cyclic peptides, proline rotamers, inflammation, nitric oxide, interleukin 2, reactive oxygen species.

INTRODUCTION:

Recently, the cyclic peptide stylissatin A (**1**) has been isolated from the Papua New Guinean marine sponge *Stylissa massa*. The cyclic peptide **1** was reported to have an inhibitory effect on nitric oxide production in LPS-stimulated murine macrophage RAW264.7 cells with an IC_{50} value of 87 μM [1]. Inflammation is an immediate cellular response of the body against harmful foreign elements and tissue injury. Upon first exposure to pathogens or other stimuli phagocytes, mainly neutrophils and macrophages, release various mediators including reactive oxygen (ROS) and nitrogen species (RNS), chemokines and cytokines. The release of these mediators progresses the processes of inflammation and activation of cell mediated immune responses [2,3]. Reactive oxygen species (ROS) and nitric oxide (NO·**)** released by inflammatory cells are involved directly and indirectly in the advancement of oxidative damage [4]. Nitric oxide (NO·) has various physiological roles including maintenance of vessel homeostasis. However, the excessive production of NO· in inflammatory conditions has a damaging effect on cells and organs [5,6]. Meanwhile, T lymphocytes are the main cells involved in the generation of adaptive immune response. The activation and proliferation of various immune cells depends on the cytokines secreted by T- cells. IL-2 is an important immunomodulatory cytokine, secreted by T-cells. Along with the effect on production of other cytokines, it is known to activate T-cells in an autocrine manner. Activated T-cells have high affinity receptors for IL-2 on their surface [7]. Inhibition of IL-2 provides a strong immunosuppressive response by preventing the activation and proliferation of T-cells in transplantation rejection and other autoimmune diseases [8].

Given the interesting structure of stylissatin A (**1a**) with the inclusion of two proline residues that can exist as *cis* or *trans* rotamers and the biological activity in reducing NO· release, we embarked on investigations to complete the total synthesis of stylissatin A and a set of analogues to explore structure-activity relationships. During the course of our study, the synthesis of

stylissatin A was reported by a combination of solid and solution-phase synthesis and the synthetic sample also exhibited strong inhibition against NO· release (EC_{50} 73 µM) [9]. Although the proline rotatmers of the synthetic and natural stylissatin A were not assigned, analysis of the reported ^{13}CNMR data indicates that both are the *trans*, *cis* proline rotamers with a *trans*-peptide bond between Pro^4-Ile^5 and a *cis*-peptide bond between Pro^6-Phe^7.

RESULTS AND DISCUSSION:

Among various synthetic methods to cyclic peptides, the on-resin cyclization approach has been successfully employed in the synthesis of several biologically active natural peptides. Several procedures have been developed for on-resin cyclization in good yields with minimum side products [10-12]. For the synthesis of peptide **1**, we selected Kenner's sulfonamide safety-catch linker strategy as employed previously for cyclic peptides by others [13-16] as well as ourselves [17-19]. In our first synthesis (route A), Fmoc-L-isoleucine was employed for loading on the sulphamylbutyryl Kenner safety-catch resin. Using Fmoc solid-phase peptide synthesis, the head-to-tail coupling of amino acids was carried out to produce the linear peptide precursor of **1**. The Fmoc group of the terminal amino acid Tyr was replaced by Boc before activation of the safety-catch linker through alkylation with iodoacetonitrile. The Boc group was then removed by using a TFA cocktail. Base treatment promoted on-resin macrocyclization between Ile and Tyr residues of the linear precursor to produce the cyclic peptide **1b** which was simultaneously cleaved from the resin (**Scheme 1**).

Reverse-phase recycling HPLC with an isocratic solvent system was used in the purification of the major product **1b**. A comparison of NMR chemical shifts of natural stylissatin **1a** and synthetic peptide **1b** in d_6-DMSO were performed (**Table 1**). Whereas, natural stylissatin A (**1a**) is the *trans, cis* rotamer at the Pro^4-Ile^5 and Pro^6-Phe^7 peptide bonds respectively as inferred from

analysis of ^{13}CNMR chemical shift differences of C_β–C_γ of Pro^4 and Pro^6 residues of natural product, he synthetic peptide **1b** from this study was found to be the *trans, trans* rotamer [19-22] as revealed by ^{13}CNMR chemical shift differences of Pro Δδ C_β–C_γ i.e., (Pro^4, Δδ C_β (29.0)–C_γ (24.5) = 4.5 and Pro^6, Δδ C_β (29.1) − C_γ (24.6) = 4.5) (**Figure 1**). In the NOESY spectrum clear cross-peaks were observed between Ile^5-Hα/Pro^4-Hδ_A and Pro^4-Hδ_B as well as between Phe^7-Hα/Pro^6-Hδ_A, and Pro^6-Hδ_B (**Figure S8**), that further supported the *trans* conformation of all proline peptidic linkages of **1b** which is in accordance with the literature [20,23,24]. This change in proline rotamer to *trans*-Pro^6-Pro^7 led to global changes in the NMR chemical shifts in synthetic **1b** compared to the natural **1a** as reported for other proline peptide conformers [22]. Our results combined with the previous synthesis [9] indicate the proline rotamer population can be significantly influenced by the macrocyclization method used.

In route A, since the isoleucine residue was activated and involved in the macrocyclization step, another possible explanation is that the differences in chemical shifts between **1a** and **1b** may be due to epimerization at Ile^2 during macrocyclization [9]. In order to preclude the possibility of **1b** as an epimer of the natural product, we carried out a second synthesis (route B) in which the macrocyclization was taking place at a different position between tyrosine and phenylalanine residues (**Scheme 2**). Furthermore, in this synthesis, we deliberately replaced L-Ile^2 by D-*allo*-Ile^2 to ensure full epimerization at this position. Afterh this synthesis, the peptide **1c** was purified and analyzed by reverse-phase high performance liquid chromatography. It showed a different retention time (**Figure 2**) and distinct ^{1}HNMR data compared to **1b** (**Table 1**). Detailed NMR studies revealed that **1c** also has *trans*-Pro^4-Ile^5 and *trans*-Pro^6-Phe^7 peptide conformations (^{13}CNMR chemical shift differences of Pro Δδ C_β–C_γ i.e., (Pro^4, Δδ C_β (28.9) − C_γ (24.3) = 4.6 and Pro^6, Δδ C_β (28.6) − C_γ (24.4)= 4.1) (**Figure 1**). The prominent cross peaks between Ile^5-

Hα / Pro^4-$H\delta_A$ and Pro^4-$H\delta_B$ as well as between Phe^7-Hα / Pro^6-$H\delta_A$, and Pro^6-$H\delta_B$ confirmed the *trans* linkage of both Pro^4 and Pro^6 residues (**Figure S9**).Although both **1b** and **1c** contain *trans* proline rotamers, they were distinct species and this confirms that **1b** is in fact a proline rotamer of natural stylissatin A (**1a**) rather than an epimer at the Ile^2 residue (Table-1).

The synthesis of stylissatin A was then performed using route B (**Scheme 3**) with L-Ile^2 to investigate whether the site of cyclization affects the rotamer populationDespite the different position of macrocyclization, the synthesis one again afforded the *trans*, *trans* proline rotamer **1b** as the major product as determined by HPLC and NMR comparison with the sample from route ANevertheless, a small amount of a second product was obtained that we were able to isolate and spectroscopically characterize as identical to the *trans, cis* natural product rotamer **1a**(Table-1)**.** Synthetic stylissatin A **1a** had ^{13}CNMR chemical shift differences of Pro $\Delta\delta$ C_β−C_γ i.e., (Pro^4, $\Delta\delta$ C_β (29.3) − C_γ (24.6) = 4.7 and Pro^6, $\Delta\delta$ C_β (29.5) − C_γ (21.6)= 7.9) (**Figure 1**) that matched the literature reports although asample of naturally isolated stylissatin A was not available for direct comparison. The cross-peak between Pro^6-Hα / Phe^7-Hα in ROESY spectrum established the *cis*-geometry for the Pro^6-Phe^7 peptide bond [22] (**Figure S10**). Stylissatin A (**1a**), its proline amide rotamer (**1b**) and its epimer (**1c**) have different retention time under similar HPLC conditions and can be distinguished from one another (**Figure 2**).

Many proline-rich synthetic cyclic peptides such as stylopeptides, axinastins and cherimolacyclopeptide E have not reproduced the biological activity reported forthe naturally isolated material[19, 24]. One reason for this discrepancy is *cis* / *trans* isomerization at proline linkages in such peptides having an effect on the bioactivity [24]. In the current study, despite the rotamer difference, our synthetic sample of stylissatin A **1a** and its rotamer **1b** show similar

nitric oxide inhibitory activity. This reveals that the *cis* / *trans* conformation of the Pro^6 residue in stylissatin A has no impact on biological activity of these peptides.

In order to better understand the structure-activity relationships of stylissatin A, we embarked on the synthesis of six analogues **2-7** (Table-2) (**Scheme 4**). Given the potential for multiple rotamers with the safety-catch strategy, we switched to an on-resin cyclization approach using the Wang resin [25-28]. Linear precursors of peptide analogues were anchored to the Wang resin through the side chain of glutamic acid. Fmoc-peptide coupling and Fmoc group deprotection followed standard procedures that were used in the natural product synthesis. After the synthesis of the linear chain, the allyl protecting group of the Glu residue was removed by using palladium (0) (Scheme 1). The Fmoc group of the terminal amino acid residue was removed by 4-methylpiperidine in DMF. On-resin cyclization (27-29) was carried out by using Oxymapure / DIC and a TFA cocktail cleaved the final product from the resin. The structures of analogues were confirmed by MALDI (Table S1) and NMR studies (Table 3,4). Purification of all peptides was achieved by using preparative HPLC.

The peptide **1b** and related six analogues **2-7** were evaluated for their anti-inflammatory potential by investigating different immune parameters including effects on production of $NO^{\bullet}$, intracellular ROS and interleukin 2 (IL-2) cytokine (Table 5). The natural product (**1a**), the synthetic proline amide rotamer of stylissatin A (**1b**), and (D-*allo*-Ile^2)-stylissatin A (**1c**) exhibited significant inhibition of $NO^{\bullet}$ produced from LPS activated J774.2 macrophages with an IC_{50} value in the range of 60-69 μM, while the standard nitric oxide inhibitor L-NMMA had an IC_{50} = 97.5 μM. Further investigations were focused on peptide **1b** and its related analogues **2-7**. No inhibition was observed when **1b** was tested for its effect on ROS produced from whole blood and isolated neutrophils from the blood of healthy human volunteers. The peptide showed strong inhibitory

effect against proliferation of T-cells whereas it moderately inhibited the production of cytokine interleukin 2 (IL-2) (Table-5).

The analogues **2-7** showed weak inhibitory activity of NO· and more potent inhibition of interleukin 2 production compared to parent peptide **1**. The common difference among the peptide sequence of natural peptide **1** and **2-7** is that Pro^6 is replaced by other amino acid residues in all analogues. That resulted in varying biological activity of natural peptide and its analogues (Table 5). It also appeared that by substitution of both proline residues (Pro^4 and Pro^6) of peptide **1**, with Ala and Glu respectively, the resulting peptide analogue **2** was found to be a strong inhibitor of ROS with no inhibition of NO·. It potently inhibited ROS from neutrophils as well as strongly inhibited the release of cytokine interleukin 2 (IC_{50} 13.4 ± 0.1 μM**)** (Table 5). This indicated that both proline residues are involved in the mechanism of NO· inhibition. The peptide analogue **7,** in which Ile^2 and Pro^6 were replaced by Ala and Glu, respectively, was found to be the most potent inhibitor of interleukin 2 (IC_{50} 6.0 ± 0.9 μM**)** release among the peptides **1-7**. The results obtained from current studies showed significant anti-inflammatory potential of synthetic peptide **1b** and its analogues **2-7**.

CONCLUSION

Stylissatin A (**1a**), proline rotamer of stylissatin A (**1b**), an epimer (**1c**) and six cyclic analogues **2-7** were successfully synthesized on solid-phase by employing different cyclization routes. All peptides were characterized by mass and NMR studies. The position of macrocyclization was found to influence the proline rotamer population of synthetic sytlissatin A. Synthetic stylissatin **1a** was found to have similar activity to that reported in the literature. The synthetic stylissatin A **1a** and its *trans, trans* rotamer **1b** potently inhibited the NO· production. Analogue **2** inhibited

ROS on whole blood, as well as neutrophils. Related analogues of stylissatin A were identified as more potent inhibitors of interleukin 2 release.

EXPERIMENTAL SECTION

General Experimental Procedures. Bruker 600 MHz NMR spectrometer were used for Proton and Carbon-13 NMR spectra. NMR data were collected at 25°C. ESI mass spectra were recorded on QSTAR XL MS/MS SYSTEMS (AB SCIEX, USA). Matrix-assisted laser desorption/ionization was carried out on Ultraflex III TOF/TOF (Bruker Daltonics, Bremen, Germany) mass spectrometer. Preparative RPHPLC separation was performed by using a Jaigel ODS-MAT 80 (C18) column using elution with acetonitrile / water (60:40) in 0.08 % TFA. Optical rotation was measured with a JASCO DIP 360 polarimeter at the sodium D line (path length 50 mm).

Stylissatin A Synthesis. 4-Sulfamylbutyryl-aminomethyl resin (0.7 mmol/g) from Novabiochem and was used in the synthesis. Solid-phase synthesis was accomplished manually by using a step-wise Fmoc solid-phase peptide procedure. Agitations were performed with an orbital shaker or magnetic stirrer. Synthesis of linear peptidyl chain was achieved by applying same procedure as reported previously [16, 18, 19].

Cleavage of Peptides from Resin after Cyclization. After activation of linker and removal of Boc group from terminal amino acid as shown in scheme **1-3**, the peptide resin was soaked in THF. It was then treated with 20 % DIEA / THF for 24 h in nitrogen atmosphere. The resin was filtered and washed with THF and DCM (3 × 25 mL each) and the filtrate was collected. After evaporation of solvents under reduced pressure, the crude cyclized product was precipitated with

cold diethyl ether, and dried in *vacuum.* The crude peptides were precipitated from cold diethyl ether and further purified on RPHPLC.

Synthesis of Stylissatin A Analogues: Solid phase peptide synthesis of all analogues was accomplished by using a step-wise Fmoc solid-phase peptide procedure; all reagents were from Chem-Impex USA and Novabiochem. Wang resin (loading capacity; 0.9 mmol/g) was used as solid support. Fmoc-Glu(OAllyl)–OH was coupled to resin first in all six analogues by using oxymapure and diisopropylcarbodiimide (DIC) in dichloromethane (DCM) / dimethylformamide (DMF). After loading the first amino acid (AA), the resin was treated with acetic anhydride (2 equiv.) and Pyridine (2 equiv.) in DCM for 30 min to cap un-reacted hydroxyl groups. Linear peptide resins were synthesized using Fmoc AAs with oxymapure / DIC as coupling reagents and 20 % 4-methyl piperidine in DMF for Fmoc group deprotection. After completing the linear peptide sequences, the allyl group of the glutamic acid was removed using Pd-catalyst (tetrakis (triphenylphosphine) palladium (0) (0.51 equiv.) in an inert environment in mixture of DCM / acetic acid / NMM of 18.5:1:0.5. After washing, the Fmoc of terminal AA was deprotected by treatment with 20 % 4-methylpiperidene in DMF. The macrocyclization was performed as previously reported [29]. A cocktail of trifluoroacetic acid / DCM / triisopropylsilane of 19:0.8:0.2 v/v was used to cleave the peptide from resin. Filtrates containing crude peptide were concentrated and precipitated using cold diethyl ether and further purified on RPHPLC.

Chemiluminescence assay: The luminol-enhanced chemiluminescence assay was performed, as described by Jantan *et al* [30].

Nitric oxide Assay: The nitric oxide assay was performed on mouse macrophage cell line J774.2 (European Collection of Cell Cultures, UK) using three different concentrations (2, 10 and 50 μM) of peptide and with method as described in Shaheen et al [31].

IL-2 Production and Quantification:

Jurkat (human T lymphocyte leukemia) cells were maintained in RPMI-1640 (BioM Laboratories, Chemical Division, Malaysia) supplemented with 5% FBS and 1% penicillin / streptomycin (GIBCO New York U.S). Upon 70% confluency cells were plated in 96-well flat bottom plates (Costar, NY, USA) at a concentration of 2×10^6 cells/mL. The cells were activated by using 20 ng/mL of phorbol myristate acetate (PMA) and, 7.5 µg/mL of phytoheamagglutinin (PHA) (SERVA, Heidelberg, Germany). Cells were then treated with three different concentrations (2, 10, 50 µM) of compound and plate was incubated for 18 hours at 37 ºC in 5% CO_2. Supernatants were collected and IL-2 quantification was performed using the human IL-2 Kits Duo Set (R&D systems, Minneapolis, USA) and according to manufacturer's instructions [32].

T-cell Proliferation Assay.

Cell proliferation assay performed by standard thymidine, incorporation assay [20]. Briefly, T-cells were isolated from peripheral blood of healthy volunteers using Ficoll paque method. The cells were plated at a concentration of 2×10^6 cells/mL in a round bottom 96-well tissue culture plates IWAKI (Chiba, Japan). Peptides were added in three different concentrations (2, 10 and 50 µM) in triplicates. The plates were then incubated for 48hrs at 37 °C in 5% CO_2 incubator. After 48 hrs, cultures were pulsed with 0.5 μCi / well (methyl-^{3}H) thymidine Amersham Pharmacia (Biotech, UK), and further incubated for 18 hrs. Thymidine incorporation into the cells was measured by a LS65000 liquid scintillation counter Beckman coulter (Fullerton, CA, USA). Results were expressed as mean count per minute (CPM).

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Table 1: NMR data of stylissatin A (**1a**) rotamer (**1b)** and epimer (**1c**) in DMSO-d_6

Residue	**Positions**	**Synthetic Rotamer (1b)**	**Epimer (1c)**	**Stylissatin A Reported data**	**Natural Product 1a from Route b**

						[1]		
		δ_C[a]	δ_H[b]	δ_C[a]	δ_H[b]	δ_H	δ_C[a]	δ_H[b]
Tyr[1]	NH		7.69(1H, d, 8.9)		8.32 (d, 8.3)	7.20 (1H, d, 4.7)		7.20
	α	56.6	4.10(1H, m)	54.9	4.23, m	4.07(1H, ddd, 9.8, 4.7, 2.6)	55.5	4.05 (1H, m)
	β	37.5	2.59 (1H, dd, 9.0, 13.8)	35.9	2.34 (m)	3.09(1H, dd, 13.1, 2.6)	36.4	3.07
			2.69 (1H, dd, 5.0, 13.8)		2.89 (m)	2.67(1H, dd)		2.65 (1H,dd, 13.3, 3.0)
	1	128.9		129.9			128.2	
	2,6	130.1	6.98 (2H, d, 8.4)	129.0	6.93 (8.3)	7.19 (2H, d)	129.6	7.12 (2H, 7.8)
	3,5	115.8	6.66 (2H, d, 8.4)	114.7	6.55 (8.4)	6.70 (2H, d)	114.7	6.68 (2H, 8.0)
	4	155.8		155.6			156.0	
	CO	169.4		170.8			170.0	
	OH		9.3 (1H, bs)		9.01	8.54 (1H, s)		
Ile[2]	NH		7.90(1H, bs)		8.15 (d, 6.4)	7.34(1H, d)		7.33
	α	53.2	3.89 (1H, m)	57.9	3.80 (t, 7.3)	4.19(1H, dd)	59.5	4.19
	β	36.3	1.72 (1H, m)	34.9	1.32	1.46 (1H, m)	36.8	1.43
	γ	24.3	1.52 (1H, m)	24.1	0.90 (m)	1.45 (1H, m)	24.1	1.47
			1.34 (1H, m)		0.65 (m)	1.10 (1H, m)		1.10
	γ'	15.1	0.81 (3H, d, 6.6)	14.6	0.27 (d, 6.6)	0.85 (3H, d, 6.7)	14.4	0.83 t (6.6)
	δ	11.2	0.78 (3H, m)	10.4	0.55 (t, 7.0)	0.78 (3H, t, 7.1)	9.9	0.78 (7.3)
	CO	170.2		171.2			171.5	
Phe[3]	NH		8.00 (1H,m)		7.82 (d, 7.4)	8.45(1H, d, 6.7)		8.44 d (6.6)
	α	54.0	4.47(1H, dd, 4.8, 8.0)	53.6	4.63 (t, 7.5. 14.6)	3.71(1H, ddd, 11.4, 6.7, 6.6)	57.7	3.69 ddd(4.2, 6.5, 11.3)
	β	36.9	2.99(1H, m)	38.7	2.80 5.7, 13.5	3.42 (1H)	34.0	3.42 (1H, m)
			2.82(1H,		2.92 m	3.25 (1H)		3.22 (1H, m)
	1	137.9		137.6			139.1	
	2,6	128.0	7.22 (m)[c]	128.2	7.24 (2H)[c]	7.19 (2H, brd)	129.6	7.19(2H)[c]
	3,5	126.1	7.18 (m)[c]	126.0	7.12 (2H) [c]	7.27 (2H, br, t)	128.2	7.25(2H)[c]
	4	129.2	7.24 (bs)\[c]	129.9	7.16 (1H)[c]	7.31 (1H, br)	129.1	7.28(1H)[c]
	CO	170.9		171.0			171.1	
Pro[4]	α	59.2	4.43(1H,	59.3	4.43	3.88 (1H, m)	60.5	3.86 (t)

			dd, 7.8, 13.8)		(1H, m)			(7.6)
	β	29.1	1.97 (1H, m)	28.9	1.97 (1H, m)	1.78 (1H, m)	29.3	1.73
			1.76 (1H, m)		1.82 (1H, m)	1.67(1H, m)		1.67
	γ	24.4	1.74 (1H, m)	24.3	1.83 (1H, m)	2.00 (1H, m)	24.6	1.97
			1.82 (1H, m)		1.81 (1H, m)	1.73(1H, m)		1.65
	δ	46.9	3.50 (1H, m)	47.1	3.48 (1H, m)	4.26(1H, m)	48.6	4.27 (1H, m)
			3.64 (1H, m)		3.65 (1H, m)	3.57(1H, m)		3.52 (1H, m)
	CO	169.4		169.8			169.8	
Ile[5]	NH		7.98 (1H, m)		7.91 (1H)	9.41 (1H, d)		9.4 d (8.4)
	α	54.5	4.34 (1H, m)	54.1	4.42 (1H, m)	4.22(1H, dd)	54.8	4.22
	β	36.7	1.76 (1H, m)	36.5	1.82 (1H, m)	2.02(1H, m)	34.8	1.98
	γ	24.0	1.05 (1H, m)	23.7	1.06 (1H, m)	1.45(1H, m)	23.9	1.42
			1.52 (1H, m)		1.50 (1H, m)	1.10(1H, m)		1.05
	γ'	15.4	0.86 (3H, d, 6.6)	10.9	0.89 (3H, d, 6.2)	0.86 (3H, d, 6.7)	14.7	0.89 (d, 6.3)
	δ	10.9	0.80 (3H, m)	15.3	0.75 (3H, t, 7.5)	0.79 (3H, t, 7.1)	9.5	0.75 (t, 7.2)
	CO	171.2		170.8				
Pro[6]	α	59.1	4.32 (1H, m)	59.6	4.28 (1H, m)	3.99(1H, m)	60.4	3.32(1H, m)
	β	29.0	1.98 (1H, m)	28.6	1.83	2.28(1H, m)	29.5	1.90
			1.74 (1H, m)		1.98	1.50(1H, m)		0.65
	γ	24.5	1.79 (1H, m)	24.5	1.82	1.95(1H, m)	21.6	1.95
			1.83 (1H, m)		1.79	1.59(1H, m)		1.65
	δ	47.1	3.49 (1H, m)	46.9	3.45	3.30 (2H, m)	45.7	3.19
			3.54 (1H, m)		3.63			3.24
	CO	171.2		170.8			169.1	
Phe[7]	NH		8.14(1H, 7.74)		7.92 (m)	8.77 (1H, br s)		
	α	52.3	4.68 (1H, m)	51.6	4.78 (8.5, 13.3)	4.01 (1H, brdd)	53.8	3.99 (4.3, 11.4)
	β	36.6	2.80(1H, m)	38.1	3.06 (3.9, 13.9)	2.89 (1H, dd)	37.1	2.88 (4.2, 12.1)

			3.01(1H, m)		2.95 (1H, m)	2.63 (1H, dd)		2.62 (m)
	1	137.3		137.6			135.2	
	2,6	128.3	7. 15 (2H)[c]	128.2	7.25 (2H)[c]	7.13 (2H, br d)	131.0	7.18
	3,5	126.5	7. 23 (2H)[c]	126.5	7.20(2H) [c]	7.27 (2H, br t)	126.9	7.17
	4	129.2	7.25 (1H)[c]	129.3	7.28 (1H)[c]	7.31 (1H, br t)	128.6	7.29
	CO	169.9		169.9			169.2	

[a]Recorded at 150 MHz. Multiplicity was derived from the DEPT and HSQC spectra.
[b]Recorded at 600 MHz. Coupling constants (Hz) are shown in parentheses.

[c]Signals overlapped**.**

Table 2. Characterization data of peptides 1-7

Peptide	substituted residue	$[M+H]^+$	Sequence of compound	$[\alpha]_D^{25}$	Overall Yield %
1a	none	878.4631 ($C_{49}H_{64}N_7O_8$)	cyclo (Pro^6-Phe^7-Tyr^1-Ile^2-Phe^3-Pro^4-Ile^5)	-29.76 (c 0.0672, MeOH)	2.3(route A
1b	none	900.4630 ($C_{49}H_{64}N_7NaO_8$)	cyclo (Pro^6-Phe^7-Tyr^1-Ile^2-Phe^3-Pro^4-Ile^5)	−40 (*c* 0.11, MeOH)	4.1(route B
1c	none	900.4644 ($C_{49}H_{64}N_7NaO_8$)	cyclo (Pro^6-Phe^7-Tyr^1-ile^2-Phe^3-Pro^4-Ile^5)	-24.7 (*c* 0.0932, MeOH)	5.9(route C)
2	Pro_6, Pro_4	883.4474 ($C_{47}H_{61}N_7O_{10}$) 906.4372 ($C_{47}H_{61}N_7O_{10}Na$)	cyclo (Glu^6-Phe^7-Tyr^1-Ile^2-Phe^3-Ala^4-Ile^5)	−21.8 (c 0.055 MeOH)	18.7
3	Pro_6, Pro_4	883.4474 ($C_{47}H_{61}N_7O_{10}$) 906.4372 ($C_{47}H_{61}N_7O_{10}Na$)	cyclo (Ala^6-Phe^7-Tyr^1-Ile^2-Phe^3-Glu^4-Ile^5)	−47.6 (c 0.05 MeOH)	16.1
4	Pro_6, Phe_7	833.4318 ($C_{43}H_{59}N_7O_{10}$) 856.4216 ($C_{43}H_{59}N_7O_{10}Na$)	cyclo (Glu^6-Ala^7-Tyr^1-Ile^2-Phe^3-Pro^4-Ile^5)	−77.3(c 0.052 MeOH)	16.5
5	Pro_6, Phe_3	833.4318 ($C_{43}H_{59}N_7O_{10}$) 856.4216 ($C_{43}H_{59}N_7O_{10}Na$)	cyclo (Glu^6-Phe^7-Tyr^1-Ile^2-Ala^3-Pro^4-Ile^5)	−49.5(c 0.047 MeOH)	13.1
6	Pro_6, Tyr_1	817.4368 ($C_{43}H_{59}N_7O_9$) 840.4266 ($C_{43}H_{59}N_7O_9Na$)	cyclo (Glu^6-Phe^7-Ala^1-Ile^2-Phe^3-Pro^4-Ile^5)	−71.1(c 0.06 MeOH)	15.1
7	Pro_6, Ile_2	867.4161 ($C_{46}H_{57}N_7O_{10}$) 890.4059 ($C_{46}H_{57}N_7O_{10}Na$)	cyclo (Glu^6-Phe^7-Tyr^1-Ala^2-Phe^3-Pro^4-Ile^5)	−60.3(c 0.055 MeOH)	19.5

Table 3: H^1 and C^{13} NMR data of Peptides 2-4 in DMSO *d_6*

2			3			4		
Residue	**C^{13}**	**H^{1}**	**Residue**	**C^{13}**	**H1**	**Residue**	**C^{13}**	**H^{1}**
Tyr[1]		7.85	**Tyr[1]**			**Tyr[1]**		
NH			NH		7.89	NH		8.16
α	54.4	4.42	α	53.8	4.44	α	53.8	4.42
β	37.4	2.65	β	37.0	2.59	β	35.5	2.78
		2.82			2.79			2.94
1	136.0		1	137.5		1	137.6	
2,6	129.9	6.95	2,6	130.0	6.94	2,6	129.1	6.99
3,5	114.8	6.57	3,5	114.7	6.56	3,5	114.8	6.62
4	155.7		4	155.9		4	155.7	
CO	170.75		CO	170.1		CO	170.6	
OH		9.1	OH		9.1	OH		9.18
Ile[2]			**Ile[2]**			**Ile[2]**		
NH		7.71	NH		7.91	NH		7.88
α	56.5	4.12	α	56.78	4.14	α	56.6	4.10
β	36.1	1.60	β	36.8	1.58	β	36.3	1.62
γ	23.9	1.03	γ	24.0	0.95	γ	24.0	1.23
		1.35			1.28			0.95
γ’	11.0	0.65	γ’	14.60	0.66	γ’	10.82	0.70
δ	15.2	0.76	δ	14.88	0.78	δ	15.00	0.74
CO	170.45		CO	170.46		CO	172.84	
Phe[3]			**Phe[3]**			**Phe[3]**		
NH		8.06	NH		7.78	NH		8.01
α	53.5	4.51	α	56.0	4.18	α	53.9	4.33
β	36.5	2.74	β	36.54	2.99	β	28.5	2.81
		2.93			2.78			2.62
1	137.6		1	137.5		1	137.69	
2,6	129.1	7.16	2,6	128.2	7.21	2,6	129.98	7.19
3,5	127.9	7.15	3,5	127.8	7.22	3,5	127.98	7.18
4	126.8	7.15	4	127.5	7.23	4	126.12	7.19
CO	173.8		CO	170.92		CO	167.69	
Ala[4]			**Glu[4]**			**Pro[4]**		
NH		7.90	NH		7.85	α	51.3	4.26
α	47.9	4.25	α	52.5	4.4	β	29.2	1.96
β	18.2	1.13	β	29.8	2.6			1.86
CO	172.6				2.8	γ	23.7	1.03
Ile[5]			γ	27.7	1.98			1.46
NH		7.1			1.84	δ	46.6	3.69
α	58.9	3.84	CO	173				3.44
β	36.6	1.65	COOH	174		CO	170.61	
γ	24.3	1.26	**Ile5**			**Ile[5]**		
		1.30	NH		7.9	NH		7.72
γ’	11.0	0.75	A	56.78	4.16	α	54.3	4.26
δ	15.3	0.74	B	36.9	1.61	β	36.0	1.65
CO	170.45		Γ	24.2	1.27	γ	23.8	1.79
Glu[6]					1.32			1.93
NH		7.1	γ’	15.3	0.67	γ’	10.96	0.75
α	58.9	3.80	δ	12.2	0.75	δ	15.15	0.71
β	30.1	2.21	CO	170.51		CO	169.08	
		2.32	**Ala[6]**			**Glu[6]**		
γ	28.5	1.90	NH		8.15	NH		8.16
		1.70	α	48.08	4.34	α	59.20	4.32
CO	166.2		β	18.1	1.18	β	29.0	2.37
COOH	173.8		CO	172.5				2.38
Phe[7]			**Phe[7]**			γ	27.7	2.35
NH		7.99	NH		8.07			2.25
α	53.1	4.49	α	53.4	4.52	CO	167.69	
β	36.9	2.82	β	37.07	2.79	COOH	173.94	
		3.15			2.99	**Ala[7]**		
1	135.97		1	138.4		NH		8.14
2,6	130.33	7.13	2,6	129.00	7.22	α	47.2	3.85
3,5	130.29	6.95	3,5	129.18	7.23	β	18.9	1.25
4	130.00	7.12	4	128.29	7.21	CO	172.0	
CO	166.27		CO	170.12				

Table 4: H^1 and C^{13} NMR data of Peptides 5-7 in DMSO d_6.

5			6			7		
Residue	**C^{13}**	**H^1**	**Residue**	**C^{13}**	**H^1**	**Residue**	**C^{13}**	**H^1**
Tyr[1]			**Ala[1]**			**Tyr[1]**		
NH		8.18	NH		7.99	NH		8.02
α	55.2	4.18	α	48.1	4.29	α	53.6	4.34
β	37.9	2.82	β	18.1	1.12	B	36.94	2.62
		3.10	CO	172.8				2.85
1	135.9		**Ile[2]**			1	135.9	
2,6	129.1	6.92	NH		8.18	2,6	130.0	6.92
3,5	114.7	6.56	α	56.6	4.12	3,5	114.7	6.58
4	155.7		β	36.1	1.65	4	155.7	
CO	167.0		γ	24.1	1.35	CO	170.4	
OH		9.10			1.47	OH		9.11
Ile[2]			γ’	14.9	0.81	**Ala[2]**		
NH		7.6	δ	10.8	0.72	NH		7.9
α	56.2	4.08	CO	170.6		α	48.6	4.17
β	36.5	1.59	**Phe[3]**			β	18.2	1.09
γ	24.0	1.79	NH		7.897	CO	172.8	
		1.82	α	53.5	4.48	**Phe[3]**		
γ’	10.8	0.70	β	37.2	2.77	NH		7.88
δ	15.1	0.62			2.83	α	54.0	4.38
CO	170.5		1	137.5		β	37.3	2.78
Ala[3]			2,6	130.3	7.12			2.95
NH		8.01	3,5	129.1	7.19	1	135.9	
α	48.1	4.18	4	126.1	7.14	2,6	130.2	7.10
β	17.5	1.13	CO	171.8		3,5	129.2	7.25
CO	172.5		**Pro[4]**			4	128.0	7.28
Pro[4]			α	59.2	4.34	CO	169.9	
α	59.1	4.32	β	28.5	1.77	**Pro[4]**		
β	28.6	1.70			1.79	α	59.2	4.32
		1.20	γ	24.2	1.76	β	29.0	1.73
								1.70
γ	24.3	1.22			1.13			
		1.05	δ	47.12	3.51	γ	24.1	1.48
								1.49
δ	47.1	3.52			3.69			
		3.72	CO	170.34		δ	47.1	3.49
CO	166.3		**Ile[5]**					3.68
Ile[5]			NH		8.18	CO	170.8	
NH		8.07	α	51.3	4.30	**Ile[5]**		
α	54.9	4.31	β	36.1	1.68	NH		8.07
β	36.1	1.70	γ	24.1	1.71	α	54.6	4.28
γ	24.2	1.50			1.79	β	37.9	1.70
		1.78	γ’	15.1	0.7	γ	24.3	1.02
γ’	11.0	0.81	δ	10.9	0.84			1.2
δ	14.9	0.88	CO	170.3		γ’	14.9	0.82
CO	170.0		**Glu[4]**			δ	10.8	0.78
Glu[6]			NH		8.21	CO	170.6	
NH		7.82	α	51.5	4.30	**Glu[6]**		
α	51.1	4.32	β	29.7	2.15	NH		8.07
β	30.2	2.22			2.25	α	51.5	4.29
		2.26	γ	28.6	1.79	β	29.8	2.26
								2.28
γ	28.5	1.79			1.80			
		1.98	CO	170.7		γ	28.2	1.70
CO	172.0		COOH	173.8				1.85
COOH	173.7		**Phe[7]**			CO	170.7	
Phe[7]			NH		7.89	COOH	174.0	
NH		8.2	α	53.8	4.48	**Phe[7]**		
α	52.9	4.51	β	37.2	2.78	NH		7.8
β	37.9	2.81			2.96	α	53.9	4.34
		3.15	1	137.5		β	36.0	2.83
1	135.9		2,6	127.9	7.14			2.92
2,6	130.4	7.12	3,5	129.2	7.19	1	135.9	

3,5	128.3	7.23	4	126.1	7.18	2,6	130.0	7.14
4	126.7	7.17	CO	171.3		3,5	128.5	7.07
CO	166.2					4	127.9	7.15
						CO	170.8	

Table 5: **Effect of peptides on oxidative burst, Nitric oxide (NO) and IL-2 production and T-cell proliferation.**

Compound No.	Oxidative Burst ($IC_{50}\mu M$)		$NO^{\cdot}$ ($IC_{50}\mu M$)	IL-2 (IC_{50} µM)	T-cell Proliferation (IC_{50} µM)
	Whole Blood	Neutrophils			
1a	nd	nd	63.0 ± 5.4	nd	nd
1b	>100	>100	60 ± 4.5	43.5 ± 5.6	8.76 ± 2.1
1c	nd	nd	69.7 ± 2.3	nd	nd
2	40.3 ± 7.1	15.0 ± 1.2	>200	13.4 ± 0.1	14.5 ± 2.1
3	>100	>100	154.6 ± 9	23.4 ± 0.2	40.6 ± 1.6
4	>100	>100	135.5 ± 19.2	18.2 ± 1.4	12.37 ± 2.1
5	>100	>100	128.6 ± 6	17.0 ± 2.0	56.7 ± 0.6
6	>100	>100	141.7 ± 20	14.9 ± 1.5	27.9 ± 0.4
7	>100	>100	137.8 ± 17.3	6.0 ± 0.9	10.9 ± 3.0
Ibuprofen	54.3 ± 9.2	12.1 ± 2.9	-	-	
L-NMMA	-	-	97.5 ± 3.2	-	
Cyclosporin	-	-	-	<0.06	1.5 ± 0.2

L-NMMA= N^G Monomethyl L-arginine acetate

Nd (not determined)