Full Paper

Synthesis of New (N^{α}-Dipicolinoyl)-bis-L-valyl-L-phenylalanyl Linear and Macrocyclic Bridged Peptides as Anti-Inflammatory Agents

Abd El-Galil E. Amr[1], Mohamed H. Abo-Ghalia[2], and Mohamed M. Abdalah[3]

[1] Applied Organic Chemistry Department, National Research Center, Dokki, Cairo, Egypt
[2] Peptide Chemistry Department, National Research Center, Dokki, Cairo, Egypt
[3] Research Units, Hi-Care Pharmaceutical Co., Cairo, Egypt

In continuation to our search for new chiral macrocyclic peptide-based anti-inflammatories, the suggestion, synthesis, structure elucidation of some N^{α}-*bis*-dipicolinoyl amino acids, linear, tetra and cyclic (penta and octa)-bridged peptides **3 – 10**, were realized herein. The newly synthesized compounds showed potent anti-inflammatory activity with low toxicity (LD_{50}) comparable to indomethacin and diclofenac as reference anti-inflammatory drugs.

Keywords: Amino acids / Anti-inflammatory agents / Macrocyclic peptides

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Introduction

Generally, inflammation can be defined as an abnormal, but protective biological response to a tissue injury that is induced by physical trauma, chemicals, microbial agents, or even an autoimmune reaction. The inflammatory reaction is initially activated by a release of chemical mediators from the injured tissue and/or migrating cells. The acting mediators may include amines (histamine), lipids (prostaglandin), small or large peptides (bradykinin or interleukin-1). Inhibition of a released mediator is, consequently, of a therapeutic impact [1]. Interestingly, some individual amino acids exemplified by valine, leucine, isoleucine, some glutamine, tryptophane, methionine, and phenylalanine were early reported to have anti-inflammatory properties [2 – 7]. Similarly, some short peptides as valyl-alanine, valyl-typtophan, and tyrosyl-valine [8] as well as, some longer peptides [9, 10] showed anti-inflammatory properties. Additionally, the specific inhibition of the inflammatory enzyme cyclooxygenase-2 (COX-2) by the natriuretic peptides has been equally reported [11]. In this context, we have previously approached the synthesis and investigation of some new conjugates of non-proteinogenic and proteinogenic amino acid condensed with diclophenac. The synthesized candidates proved to be considerably potent and less toxic than the parent drugs [12 – 14].

Correspondence: Abd El-Galil E. Amr, Applied Organic Chemistry Department, National Research Center, Dokki, Cairo, Egypt.
E-mail: aamr1963@yahoo.com
Fax: +20 2337-0931

Results and discussions

Chemistry

Biochemically, dipicolinic acid (pyridine-2,6-dicarboxylic acid, DPA, CAS, 499-83-2) is a naturally occurring dicarboxylic acid that is found mainly in bacterial endospores, as recently indicated by spectrometric analysis of *Bacillus* spores [15]. We have previously explored the analytical and biological characteristics of some of rationalized *bis*-amino acid and peptide conjugates of dipicolinic acid [16]. Our studies of these compounds, exemplified by compound **A** (Fig. 1), revealed an interesting anti-cancer activity, probably via DNA intercalation, as well as outstanding metal sensors properties, particularly, for the serious pollutant, lead cations (Pb^{2+}) [17 – 19]. Cyclization of some of these *bis*-conjugates with the molecular baskets calix[4]arene as presented by compound **B** (Fig. 1)

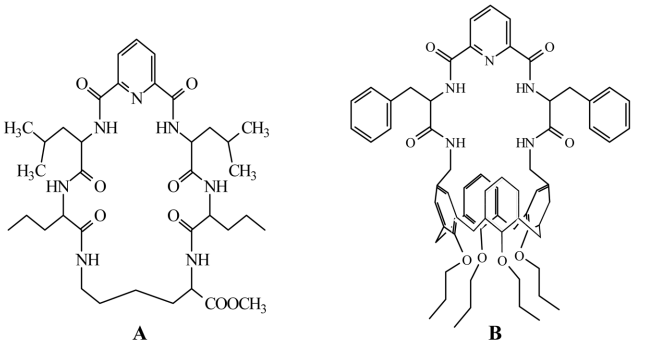


Figure 1. Chemical structures of cyclic and macrocyclic compounds **A** and **B**.

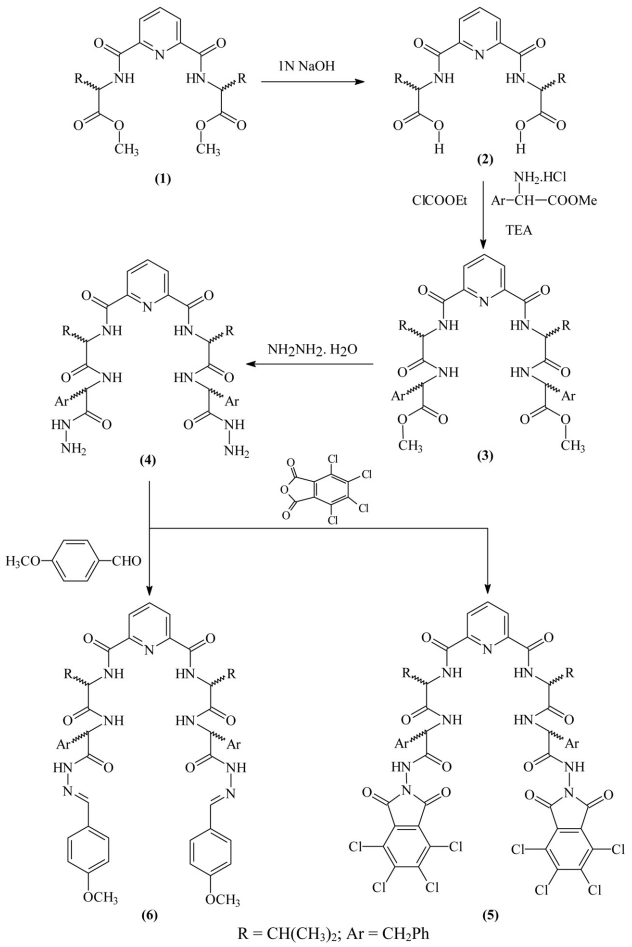
provided new biologically and chemically interesting molecular architectures [20].

In this work, the synthesis and investigation of anti-inflammatory characteristics of some *bis*-N^{α}-L-valine, and N^{α}-L-valyl-L-phenylalanine linear, tetra and cyclic (penta and octa) bridged peptides of dipicolinic acid were undertaken.

L-Valine methyl ester was initially coupled with dipicolinic acid via the conventional acid chloride method [20]. The peptide linkage was then formed with L-phenylalanine methyl ester. While the synthesis of compounds **1** and **2** were previously reported [20], coupling of diacid **2** with L-phenylalanine methyl ester by mixed anhydride method afforded the corresponding N^{α}-dipicolinoyl-*bis*-[L-valyl-L-phenylalanine methyl ester] **3**. In this context, the used mixed anhydride coupling method, low temperature, weak bases (TEA) proved generally more potent and less racemizing than the alternatives methods, e.g. the acid chloride or the azide coupling procedures. Hydrazonolysis of **3** with hydrazine hydrate afforded the corresponding *bis*-hydrazide **4** (Scheme 1).

Compounds **5**–**8** are newly suggested and synthesized candidates obtained via simple condensation of the hydrazide **4** with tetrachlorophthalic anhydride thus affording the 2,6-*bis*-imide pyridine derivative **5**, with anisaldehyde in methanol giving the corresponding hydrazone derivative **6** and with 1,2,4,5-benzenetetra-carboxylic dianhydride or 1,8,4,5-naphthaline-tetracarboxylic dianhydride affording the conjugates **7** and **8**, respectively (Scheme 2).

N^{α}-Dipicolinoyl-*bis*-[L-valyl-L-phenylalanine methyl ester] **3** was hydrolyzed with 1 N sodium hydroxide in methanol to give the corresponding *bis*-acid **9**, which was cyclized with L-lysine methyl ester in the presence of ethyl chloroformate/triethylamine (mixed anhydride method) to afford the cyclopentapeptide derivative **10** (45%), which was equally prepared by azide coupling with 35% yield (Scheme 3). Generally, peptide cyclization

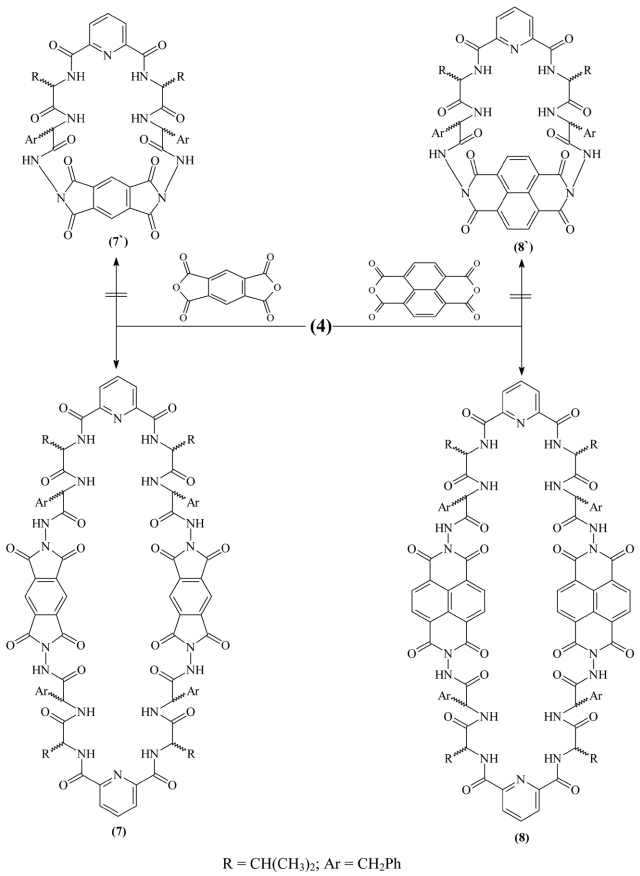


Scheme 1. Synthesis of new (N^{α}-dipicolinoyl)-*bis*-L-valyl-L-phenylalanyl linear tetra peptides **5** and **6**.

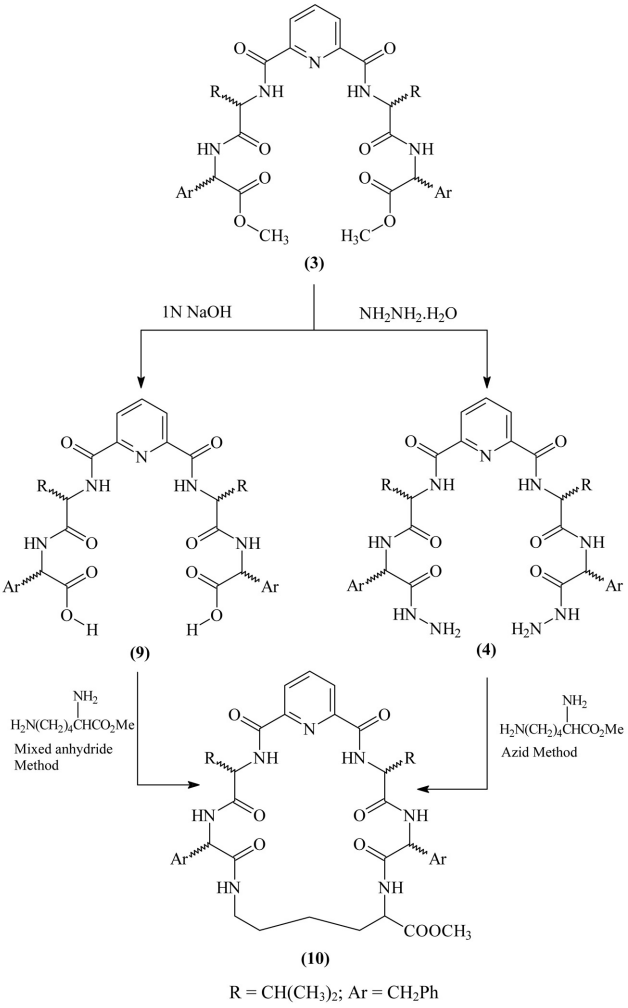
is associated with moderate to low reaction yields. In addition, the possibility of dimerization of compound **10** is chromatographically (TLC) not supported.

The possible modulation of the amino acid and peptide C-terminal ends as esters **1** and **3**, acids **2** and **9**, hydrazide **4**, tetrachlorophthalic hydrazine conjugate **5**, and finally hydrazone **6** permit the explorations of the corresponding activity of these biologically significant functional groups.

In addition, bridging with benzenetetracarboxylic acid exemplified by **7** and naphthalene tetracarboxylic acid affording compound **8** infer a planar molecular geometry for the obtained octa peptides. These conformationally restricted structures offer the perspectives of investigating the biological responses of these planar macrocycles. Structural resemblance to the biologically attractive crown ethers may also be significantly considered.



Scheme 2. Synthesis of new (N^{α}-dipicolinoyl)-*bis*-L-valyl-L-phenylalanyl cyclic octa-bridged peptides **7** and **8.**



Scheme 3. Synthesis of new (N^{α}-dipicolinoyl)-*bis*-L-valyl-L-phenylalanyl cyclic penta-bridged peptides **10.**

As expected, the mass spectral data (Scheme 2) confirmed that the bridged cyclic tetra peptides **7′**and **8′** were not formed. This may indicate that such cyclic molecules, contrary to the corresponding octapeptides **7** and **8**, are sterically hindered and consequently structurally unfavorable.

Anti-inflammatory activity

Table 1 resumes the anti-inflammatory activity and LD_{50} of the candidates. Comparable to the two reference anti-inflammatory drugs namely, indomethacin and diclophenac (100%), the determined anti-inflammatory activity of the candidates (carrageenan induced paw edema in rats) revealed a general significant activity (64 – 84%). In particular, the potency of the (N^{α}-dipicolinoyl)-*bis*-L-valyl-L-phenylalanyl-*p*-methoxybenzaldehyde hydrazone **6** was of 72% and 84% relative to the reference drugs, respectively. Additionally, an acceptable acute toxicity was observed (LD_{50}: 2980 mg/kg comparable to 2700 and 2850 for indomethacin and diclophenac, respectively).

Other significant conclusions can be outlined in the following: The cyclic compound **7** proved to be more potent than the simple parent hydrazide **4**. In all cases, elongation with L-phenylalanine had a potentiating effect. The general pronounced anti-inflammatory activity of the compounds may accept the hypothesis that the cyclooxygenase (COX) may be the working inflammatory receptor.

Experimental

Chemistry

Melting points were determined on open glass capillaries using an Electrothermal IA 9000 SERIES digital melting point apparatus (Electrothermal, Essex, UK) and are uncorrected. Elemental analyses were performed in the Microanalytical Unit, National Research Centre, Cairo, Egypt, and were found within ± 0.4% of the theoretical values. Analytical data were obtained from the Microanalytical Unit, Cairo University, Egypt. The IR spectra

Table 1. Anti-inflammatory potency and toxicity (LD_{50}) of the synthesized compounds.

Compound	% Increase in the weight of rat paw edema (g)	% Protection			
		A[a)]	B[b)]	C[c)]	LD_{50} (mg/Kg)
Diclofenac	10.318 ± 0.321	82.70	107.24	100	2850
Indomethacin	13.865 ± 0.828	77.11	100	93.24	2700
1	17.286 ± 0.726	71.47	80.20	59.68	2090
2	20.666 ± 0.823	64.24	70.90	49.92	2500
3	27.649 ± 0.768	54.36	50.14	37.31	2433
4	23.539 ± 0.468	61.06	58.90	43.83	2520
5	41.718 ± 1.178	31.15	33.23	24.73	2315
6	16.351 ± 1.108	72.20	84.79	63.10	2980
7	16.847 ± 0.891	72.19	82.29	61.24	2741
8	27.814 ± 0.763	54.09	49.84	37.09	2713
9	21.613 ± 0.591	64.33	64.15	47.73	2870

a) A: absolute figure.
b) B: % relative to Indomethacin.
c) C: % relative to Diclophenac.

Table 2. Physicochemical data of newly synthesized compounds.

Comp. N°	Yield (%)	M.p. (°C)	$[\alpha]_D^{25}$ (c 1, MeOH)	Color and solvent for cryst.	Mol. Formula (Mol. wt)
3	76	165 – 168	+ 82	White MeOH	$C_{37}H_{45}N_5O_8$ (687.79)
4	70	120 – 122	+ 71	White EtOAc	$C_{35}H_{45}N_9O_6$ (687.79)
5	80	>250	+ 95	White EtOH	$C_{51}H_{41}Cl_8N_9O_{10}$ (1223.56)
6	82	217 – 219	+ 74	Orange MeOH	$C_{51}H_{57}N_9O_8$ (924.06)
7	68	160 – 162	+ 84	Yellow MeOH	$C_{90}H_{86}N_{18}O_{20}$ (1739.78)
8	64	>250	+ 92	Brown MeOH	$C_{98}H_{90}N_{18}O_{20}$ (1839.90)
9	72	115 – 117	+ 78	White MeOH	$C_{35}H_{41}N_5O_8$ (659.73)
10	45 [A] 35 [B]	Oil	+ 68	Pale yellow EtOAc	$C_{42}H_{53}N_7O_8$ (783.92)

(KBr) were recorded on a FT IR-8201 PC Spectrophotometer (Shimadzu, Tokyo, Japan). The ^{1}H NMR spectra were measured with JEOL 270 MHz (JEOL, Tokyo, Japan) in DMSO-d_6. The chemical shifts were recorded (δ, ppm) relative to TMS. The mass spectra were run at 70 eV with a Finnigan SSQ 7000 spectrometer (Thermo-Instrument system incorporation, Santa Fe, NM, USA) using EI and the values of m/z are indicated in Dalton. The starting materials **1** and **2** were prepared according to published procedure [17]. The reactions were followed by TLC (Silica gel, aluminum sheets 60 F_{254}, Merck, Darmstadt, Germany).

*N$^{\alpha}$-Dipicolinoyl-bis-[L-valyl-L-phenylalanine methyl ester] **3***

To a cold and stirred dry dichloromethane solution (25 mL, −20°C) of diacid **2** (0.36 g, 1 mmol), ethyl chloroformate (0.2 g, 2 mmol) and triethylamine (0.2 g, 2 mmol) were successively added. Ten minutes later, a cold methylene chloride solution (10 mL, −20°C) of L-phenylalanine methyl ester (0.35 g, 2 mmol) was added. Stirring of the cold reaction mixture (−20°C) was continued for 3 h and then at room temperature overnight. The solution was washed with water, 1 N hydrochloric acid, 1 N sodium bicarbonate, and finally with water (2 × 3 mL). The dried solution (anhydrous $CaCl_2$) was evaporated and the obtained oily residue was solidified by dry ether trituration, filtered off, dried under vacuum, and crystallized from methanol / ether to afford **3** (Table 2).

IR (KBr, cm^{-1}): 3392 (NH), 1739 (CO, ester), 1660 (CO, amide). ^{1}H-NMR (DMSO-d_6) δ: 0.90 – 1.00 (m, 12H, 4 × *CH*$_3$), 2.28 – 2.34 (m, 2H, 2 × *CH*), 3.35 (d, 4H, 2 × *CH*$_2$Ph), 3.58 (s, 6H, 2 × O*CH*$_3$), 4.18 – 4.25 (m, 2H, 2 × *CH*-NH), 4.55 – 4.65 (m, 2H, 2 × *CH*-NH), 6.92 – 7.24 (m, 10H, 2 × Ph-*H*), 8.15 – 8.35 (m, 3H, pyr-*H*) and 8.45, 8.55 (2s, 4H, 4 × *NH*, exchangeable with D_2O). ^{13}C-NMR (DMSO-d_6) δ: 17.35, 18.66 (*CH*$_3$), 33.76 (*CH*), 42.12 (*CH*$_2$-Ph), 52.57, 52.80 (2 × *CH*-NH), 55.66 (O*CH*$_3$-ester), 124.12, 128.34, 129.46, 138.64 (Ar-*C*), 125.42, 140.00, 148.43 (pyr-*C*), 163.73, 168.96 (*CO*-amide), 172.44 (*CO*-ester). MS m/z (%): 687 [M$^+$] (25), corresponding to the molecular formula $C_{37}H_{45}N_5O_8$ and at 331 (100), base peak.

*N$^{\alpha}$-dipicolinoyl-bis-[L-valyl-L-phenylalanine hydrazide] **4***

Hydrazine hydrate (90%, 0.5 g, 10 mmol) was added to a methanolic solution (10 mL) of 3 (0.68 g, 1 mmol). The reaction mixture was refluxed for 5 h after which the solvent was evaporated. The obtained residue was triturated with ether, filtered off, and crystallized from methanol/ether to afford **4** (Table 2).

IR (KBr, cm^{-1}): 3460 – 3350 (br, NH_2, NH), 1666 (CO, amide). ^{1}H-NMR (DMSO-d_6) δ: 0.86 – 0.98 (m, 12H, 4 × *CH*$_3$), 2.18 – 2.28 (m, 2H, 2 × *CH*), 3.42 (d, 4H, 2 × *CH*$_2$Ph), 3.48 (brs, 4H, 2 × *NH*$_2$, exchangeable with D_2O), 4.22 – 4.28 (m, 2H, 2 × *CH*-NH), 4.54 – 4.68 (m, 2H, 2 × *CH*-NH), 6.95 – 7.15 (m, 10H, 2 × Ph-*H*), 8.18 – 8.25 (m, 3H, pyr-*H*), 8.60, 8.74, 9.10 (3s, 6H, 6 × *NH*, exchangeable with D_2O). ^{13}C-NMR (DMSO-d_6): δ 17.32, 18.58 (*CH*$_3$), 33.72 (*CH*), 42.22 (*CH*$_2$-Ph), 52.44, 53.00 (2 × *CH*-NH), 124.10, 128.22, 129.38, 138.68 (Ar-*C*),

125.34, 139.92, 148.36 (pyr-*C*), 163.73, 169.05 (*CO*-amide), 170.44 (*CO*-hydrazide). MS m/z (%):687 [M$^+$] (15), corresponding to the molecular formula $C_{35}H_{45}N_9O_6$ and at 331 (100), base peak.

N$^{\alpha}$-dipicolonoyl-bis-[L-valyl-L-phenylalanyl]-tetrachlorophthalic-1,2-hydrazine conjugate **5**

A stirred glacial acetic acid suspension (50 mL) of *N$^{\alpha}$*-dipicolonyl-*bis*-[L-valyl-L-phenylalanine hydrazide] (**4**, 0.68 g, 1 mmol) and tetrachlorophthalic anhydride (0.57 g, 2 mmol) was heated (80°C) for 6 hrs. The reaction mixture was concentrated under reduced pressure, cooled and the separated solid was collected by filtration, dried and crystallized from acetic acid/ether to yield the corresponding compound **5** (Table 2).

IR (KBr, cm^{-1}): 3345, 3276 (NH), 1728 (C=O), 1656 (CO, amide). ^{1}H-NMR (DMSO-d_6) δ: 0.88 – 1.05 (m, 12H, 4 × CH_3), 2.20 – 2.27 (m, 2H, 2 × *CH*), 3.40 (d, 4H, 2 × CH_2Ph), 4.18 – 4.30 (m, 2H, 2 × *CH*N), 4.35 – 4.42 (m, 2H, 2 × *CH*-NH), 6.92 – 7.24 (m, 10H, 2 × Ph-*H*), 8.10 – 8.22 (m, 3H, py-*H*), 8.45 – 9.15 (brs, 6H, 6 × *NH*, exchangeable with D_2O). MS m/z (%): 1223 [M$^+$] (24), corresponding to the molecular formula $C_{51}H_{41}Cl_8N_9O_{10}$ and at 625 (100), base peak.

N$^{\alpha}$-dipicolonoyl-bis[L-valyl-L-phenylalanyl]-p-methoxybenzaldehyde hydrazone **6**

A stirred solution of *N$^{\alpha}$*-dipicolonyl-*bis*-[L-valyl-L-phenylalanine hydrazide] (**4**, 0.68 g, 1 mmol) and *p*-methoxy benzaldehyde (0.27 g, 2 mmol) in absolute methanol (50 mL) was refluxed reflux for 6 h. The reaction mixture was allowed to stand at room temperature overnight, then it was evaporated under reduced pressure. The obtained oily product was solidified by trituration with benzene/petrolether (40 – 60°C), the remaining solid was filtered off, dried, and crystallized from methanol to give the corresponding hydrazone **6** (Table 2).

IR (KBr, cm^{-1}): 3500 – 3250 (NH), 1660 (C=O, amid). ^{1}H-NMR (DMSO-d_6) δ 0.9 – 1.2 (m, 12H, 4 × CH_3), 2.22 – 2.28 (m, 2H, 2 × *CH*), 3.36 (d, 4H, 2 × CH_2Ph), 3.42 (s, 6H, 2 x OCH_3), 4.24 – 4.45 (m, 4H, 4 × *CH*-NH), 4.52 (s, 2H, 2 × *CH*=N), 6.95 – 7.44 (m, 18H, Ar-*H*), 8.12 – 8.32 (m, 3H, py-*H*), 8.60, 9.86, 10.00 (3 × s, 6H, 6 × *NH*, exchangeable with D_2O). ^{13}C-NMR (DMSO-d_6) δ: 17.49, 18.65 (CH_3), 33.86 (*CH*), 41.98 (CH_2-Ph), 52.09, 54.74 (*CH*-NH), 124.26, 127.26, 128.16, 128.68, 129.44, 130.25, 133,89, 138.66 (Ar-*C*), 147.84 (*CH*=N), 124.98, 140.00, 148.25 (pyr-*C*), 163.62, 169.40, 172.38 (C=O). MS m/z (%): 924 [M$^+$] (12), corresponding to the molecular formula $C_{51}H_{57}N_9O_8$ and at 331 (100), base peak.

Bis-[L-valyl-L-phenylalanyl] cyclic octa-bridged peptides **7** *and* **8**

A stirred suspension of a mixture of *N$^{\alpha}$*-dipicolonyl-*bis*-[L-valyl-L-phenylalanene hydrazide] (**4**; 0.68 g, 1 mmol) and 1,2,4,5-benzenetetracarboxylic acid dianhydride or 1,8,4,5-naphthaline-tetracarboxylic acid dianhydride (1 mmol) in glacial acetic acid (50 mL) was heated (80°C) for 7 h. The reaction mixture was concentrated under reduced pressure. The obtained solid was collected by filtration, dried, and crystallized from DMF/H_2O to give **7** and **8**, respectively (Table 2).

Benzene tetracarboxamide bis-[L-valyl-L-phenylalanyl]cyclic octa-bridged peptide **7**

IR (KBr, cm^{-1}): 3550-3300 (NH), 1726 (C=O), 1655 (C=O, amid). ^{1}H-NMR (DMSO-d_6) δ: 0.82 – 0.90 (m, 24H, 8 × CH_3), 2.26 – 2.34 (m, 4H, 4 × *CH*), 3.36 – 3.40 (m, 8H, 4 × CH_2Ph), 4.28 – 4.37 (m, 4H, 4 × *CH*-NH), 4.55 – 4.60 (m, 4H, 4 × *CH*-NH), 6.95 – 7.30 (m, 20H, 4 × Ph-*H*), 7.40 (s, 4H, Ar-*H*), 8.10 – 8.28 (m, 6H, 2 × pyr-*H*), 8.88, 9.68, 9.72 (3s, 12H, 12 × *NH*, exchangeable with D_2O). ^{13}C-NMR (DMSO-d_6) δ: 17.69, 18.55 (CH_3), 33.86 (*CH*), 41.95, 42.12 (CH_2Ph), 51.50, 52.46 (*CH*-NH), 125.32, 140.15, 148.57 (pyr-*C*), 124.45, 128.43, 129.34, 134.50, 138.28, 152.46 (Ar-*C*), 164.34, 170.86, 172.10 (*CO*, amide), 174.18 (*CO*, imide). MS m/z (%): 1739 [M$^+$] (8), corresponding to the molecular formula $C_{90}H_{86}N_{18}O_{20}$ and at 331 (100), base peak.

Naphthalene tetracarboxamide bis-[L-valyl-L-phenylalanyl] cyclic octa-bridged peptide **8**

IR (KBr, cm^{-1}): 3560 – 3258 (NH), 1734 (CO), 1656 (CO, amide). ^{1}H-NMR (DMSO-d_6): δ = 0.85 – 1.00 (m, 24H, 8 × CH_3), 2.19 – 2.26 (m, 4H, 4 × *CH*), 3.38 – 3.44 (m, 8H, 4 × CH_2-Ph), 4.18 – 4.36 (m, 4H, 4 × *CH*-NH), 4.50 – 4.65 (m, 4H, 4 × *CH*-NH), 7.15 – 7.60 (m, 28H, Ar-*H*), 8.05 – 8.25 (m, 6H, 2 × pyr-*H*), 8.95, 9.64, 10.15 (3s, 12H, 12 × *NH*, exchangeable with D_2O). MS m/z (%): 1840 [M$^+$] (5), corresponding to the molecular formula $C_{98}H_{90}N_{18}O_{20}$ and at 625 (100), base peak.

N$^{\alpha}$-dipicolinoyl-bis-[L-valyl-L-phenylalanine] **9**

Sodium hydroxide (1 N, 25 mL) was added dropwise to a cold and stirred methanolic solution (0.68 g, 1 mmol, −5°C) of **3**. Stirring was continued at that temperature for 2 h and then for 12 h at room temperature followed by evaporation of the solvent. The cold reaction mixture was acidified with 1 N hydrochloric acid to pH ∼ 3, and the obtained solid was filtered off, washed with cold water, and crystallized from ethanol/ether mixture to afford **9** (Table 2).

IR (KBr, cm^{-1}): 3655 – 3540 (OH), 3325 (NH), 1725 (CO, acid), 1656 (C=O, amide). ^{1}H-NMR (DMSO-d_6) δ: 0.85 – 0.95 (m, 12H, 4 × CH_3), 2.16 – 2.33 (m, 2H, 2 × *CH*), 3.38 (d, 4H, 2 × CH_2Ph), 4.22 – 4.26 (m, 2H, 2 × *CH*-NH), 4.35 – 4.50 (m, 2H, 2 × *CH*-NH), 7.00 – 7.25 (m, 10H, 2 × Ph-*H*), 8.10 – 8.45 (m, 3H, pyr-*H*), 8.65, 8.82 (2s, 4H, 4 × *NH*, exchangeable with D_2O), 11.98 (s, 2H, 2 × *OH*, exchangeable with D_2O). ^{13}C-NMR (DMSO-d_6) δ: 17.98, 18.56 (CH_3), 33.68 (*CH*), 41.98 (CH_2-Ph), 52.54 (*CH*-NH), 124.18, 128.22, 129.40, 138.64 (Ar-*C*), 125.36, 139.89, 148.42 (pyr-*C*), 164.05, 169.10 (*CO*-amide), 171.40 (*CO*-acid). MS m/z (%): 659 [M$^+$] (65), corresponding to the molecular formula $C_{35}H_{41}N_5O_8$ and at 625 (100), base peak.

Cyclo-(N$^{\alpha}$-dipicolinoyl)-bis-[L-valyl-L-phenylalanyl]-L-lysine methyl ester **10**

Method A: Mixed anhydride method

Triethylamine (0.2 g, 2 mmol) was added to a cold and stirred dichloromethane (25 mL, −20°C) suspension of diacid **9** (0.65 g, 1 mmol), and ethyl chloroformate (0.22 g, 2 mmol). Stirring was continued for 20 min after which L-lysine methyl ester (0.16 g, 1 mmol) was added. The reaction mixture was stirred at (−20°C) for 3 h and then for 12 h at room temperature. The reaction mixture was washed with water, 1 N hydrochloric acid, 1 N sodium bicarbonate and water, then dried over anhydrous calcium chloride. The solvent was evaporated and the crude product was purified by preparative thin layer chromatography using methanol/benzene mixture (1 : 9 (*vol*/*vol*) as an eluent to give the corresponding cyclic peptide ester **10** (Table 2).

IR (KBr, cm^{-1}): 3350 (NH), 1745 (C=O, ester), 1656 (C=O, amide). ^{1}H-NMR (DMSO-d_6) δ: 0.90 – 0.80 (m, 12H, 4 × CH_3), 1.24 – 1.46 (m, 4H, 2 × CH_2), 1.60 – 1.75 (m, 2H, CH_2), 2.30 – 2.35 (m, 2H, 2 × *CH*(CH_3)$_2$), 3.00 – 3.20 (m, 2H, CH_2), 3.32 (d, 4H, 2 × CH_2Ph), 3.55

(s, 3H, OCH_3), 3.90 – 4.05 (m, 4H, 4 × *CH*-NH), 4.38 – 4.44 (m, 1H, *CH*-NH), 7.12 – 7.30 (m, 10H, 2 × Ph-*H*), 8.05 – 8.18 (m, 3H, pyr-*H*), 8.88, 8.96, 9.15 (3s, 6H, 6 x *NH*, exchangeable with D_2O). ^{13}C-NMR (DMSO-d_6) δ: 17.65, 18.97 (*CH*$_3$), 22.56, 28.30, 30.35, 38.00 (4 × *CH*$_2$), 33.85 (*CH*), 41.99, 42.05 (2 *CH*$_2$-Ph), 52.36, 52.80 (4 × *CH* – NH), 54.48 (O*CH*$_3$-ester), 60.50 (*CH*-ester), 124.32, 128.30, 129.42, 138.74 (Ar-*C*), 125.23, 139.37, 149.57 (pyr-*C*), 163.81, 169.15, 170.64 (*CO*, amide), 173.89 (*CO*, ester). MS m/z (%): 784 [M$^+$] (16), corresponding to the molecular formula $C_{42}H_{53}N_7O_8$ and at 331 (100), base peak.

Method B: Azide method

An aqueous solution of sodium nitrite (10%, 0.13 g, 2 mmol) was added to a cold (– 5°C) and stirred solution of the dihydrazide **4** (0.68 g, 1 mmol) in 5 N HCl (3 mL) and acetic acid (3 mL). Stirring was continued for 30 min after which the reaction mixture was extracted with ether, washed with water, $NaHCO_3$, and water; then dried over anhydrous sodium sulfate. The cold ethereal solution (– 5°C) was then added to a cold (– 5°C) dichloromethane solution of L-lysine methyl ester (0.16 g, 1 mmol, 10 mL). Stirring was continued for 5 h and at room temperature for 2 h. The reaction mixture was washed with 1 N hydrochloric acid, water and then dried (anhydrous sodium sulfate). Evaporation of the solvent afforded crude **10** (Table 2), which upon preparative TLC was found identical with that obtained via the mixed anhydride method.

Pharmacological Screening

Anti-inflammatory assay

Animals: Adult male albino rats weighing ~120 – 200 g were obtained from the animal house colony, Research Institute for Ophthalmology, Cairo, Egypt and fed on standard laboratory diet. Drugs and chemicals: Indomethacin (Sigma, USA) and diclofenac (Novartis, Switzerland) were used as reference drugs. Carrageenan (BDH, England); other used chemicals are of analytical grade (Sigma-Aldrich, USA).

Methodology: Three groups of rats were classified as followed: The first group containing six rats received a vehicle of 0.2 mL of 7% aqueous solution of Tween 80® and served as control group. The second group was divided into two subgroups of six rats, each received either indomethacin or diclofenac (7 mg/kg). The third group was divided into nine subgroups of six rats each; the rats received a candidate compound (7 mg/kg) orally. As for the first group, identical conditions also were followed for the second and third group. The anti-inflammatory activity (Table 1) was determined principally according to Winter *et al.* [21]. The rates were dosed with a candidate or a reference drug. One hour later, a foot-paw edema was induced by sub-planter injection of 0.05 mL of 1% suspension of carrageenan in saline into the planter tissue of the rat hind paw. Identical experimental conditions, except for dosing, were followed for the reference group. Three hours after carrageenan injection the average weight of edema in the rapidly excised paws of the decapitated rats was determined. For each group, the mean value of the obtained results was then considered. Statistical analysis of data was computed via the Student`s t-test [22] A 0.05 level of probability was regarded as significant according to Sendecor and Cochran [23].

Toxicity

The LD_{50} was determined by using rats and inject different increasing doses and calculate the dose that kill 50% from the animal, according to Austen *et al.* [24].

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