Synthesis of Some New (N^{α}-Dipicolinoyl)-*bis*-L-leucyl-DL-norvalyl Linear tetra and Cyclic octa Bridged Peptides as New Antiinflammatory Agents

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In continuation to our search for new amino acid and peptide based anti-inflammatory agents, the suggestion, synthesis, structure elucidation of some N^{α}-*bis*-dipicolinoyl amino acids, linear tetra and cyclic octa bridged peptides **1-9**, of which four are new compounds **6-9**, were herein realized. Accordingly, N^{α}-*bis*-dipicolinoyl-L-leucine methyl ester **1**, the corresponding acid **2**, its *bis*-DL-norvalyl methyl ester homologue **3**, the acid **4** and hydrazide **5** analogues were conventionally prepared.

The tetrachlorophthalic acid hydrazine conjugate **6**, anisaldehyde hydrazone **7**, the benzenetetracarboxylic acid and naphthalenetetracarboxylic acid *bis*-L-leucyl-DL-norvalyl cyclic octa bridged peptides **8** and **9** respectively, were newly synthesized *via* condensation of the hydrazide **5** with the corresponding aldehyde or anhydride.

The chromatographic, IR, NMR and mass spectral analysis confirmed the identities of the synthesized compounds.

Comparable to the two reference antiinflammatory drugs indomethacin® and voltaren® (100%), the determined antiinflammatory potency of the candidates (carrageenan® induced paw edema in rats) revealed a general significant activity (66 – 94%), except for the practically inactive **6** (∼1.5 % activity).

In particular, the potency of the (N^{α}-dipicolinoyl)-*bis*-L-leucyl-DL-norvalyl anisaldehyde hydrazone **7** was of 94 and 87%, comparable to the reference drugs. However, **7** also showed 58% protection against ulcer formation, comparable to null for indomethacin®. Additionally, an acceptable acute toxicity was observed (LD_{50}: 2833 mg/kg, comparable to 2700 and 2850 for indomethacin® and voltaren® respectively).

Key words: Dipicolinic Acid, Amino Acids, Peptides, Antiinflammatory Agents

Introduction

Inflammation could be generally defined as an abnormal 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 (prostaglandins), small or large peptides (bradykinin or interleukin-1, respectively). Inhibition of a released mediator is, consequently, of therapeutic impact [1].

Interestingly, some individual amino acids exemplified by valine, leucine, iso-leucine, glutamine, tryptophan, methionine and phenylalanine were reported to have antiinflammatory properties in different experimental models [2 – 12].

Similarly, some short peptides such as valyl-alanine, valyl-tryptophan and tyrosyl-valine [13] as well as longer peptides [14 – 16] showed antiinflammatory properties. Equally, the nonapeptides antiinflammatory, (antiflammins) having Lys-Val-Leu-Asp as a common amino acid sequence, [17 – 20], were found to posses a considerable antiinflammatory activity. The latter peptides are thought to be inhibitors of the inflammatory mediator phospholipdase A2 (PLA2, EC 3.1.1.4)].

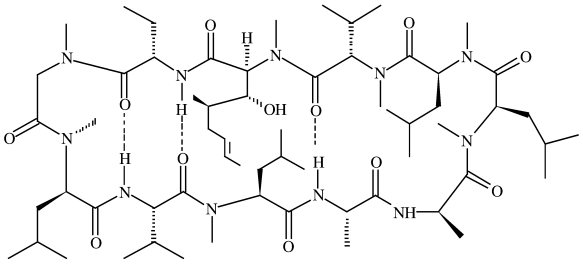
Met-Gln-Met-Lys-Lys-Val-Leu-Asp-Ser (1)

His-Asp-Met-Asn-Lys-Val-Leu-Asp-Leu (2)

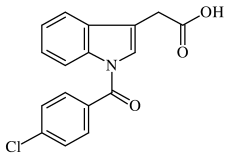
Met-Gln-Met-Asn-Lys-Val-Leu-Asp-Ser (3)

Antiinflammatory Nonapeptides (Antiflammins 1, 2 and 3)

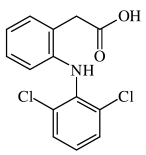
In particular, cyclic peptides related to cyclosporin A exhibit antiinflammatory characteristics [21 – 23].



Cyclosporin A



Indomethacin®



Voltaren®

Additionally, the specific inhibition of the inflammatory enzyme cyclo-oxygenase-2 (COX-2) by the natriuretic peptides has recently been reported [24].

However, adverse effects of non-steroidal antiinflammatory drugs (NSAIDs) exemplified by voltaren® and indomethacin® has become a problem. The most common toxic effects are gastric and/or duodenal ulcers, bleeding, perforation, or obstruction of the gastrointestinal tract and alterations in kidney function.

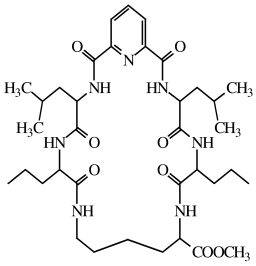
Consequently, a strong motivation for the search for more potent and less toxic new antiinflammatory agents becomes an up dated area of a multidisciplinary bio-organic research [25 – 27].

In this context, we have previously approached the synthesis and investigation of some new conjugates of non-proteinogenic and proteinogenic amino acid condensed with voltaren®. The synthesized candidates proved to be considerably potent and less ulcerogenic than the parent drug [28 – 29].

Results and Discussion

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* [30]. Structurally, the diacid, *via* its two 2,6-dicarboxylates, allows a *bis*-symmetrical derivatization with several nucleophiles like the N-terminal free amino acids and peptides.

Additionally, peptides, either cyclic or linear, constitute an excellent class of molecules for rapid drug



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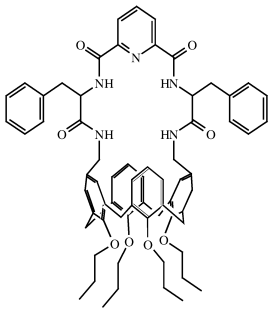


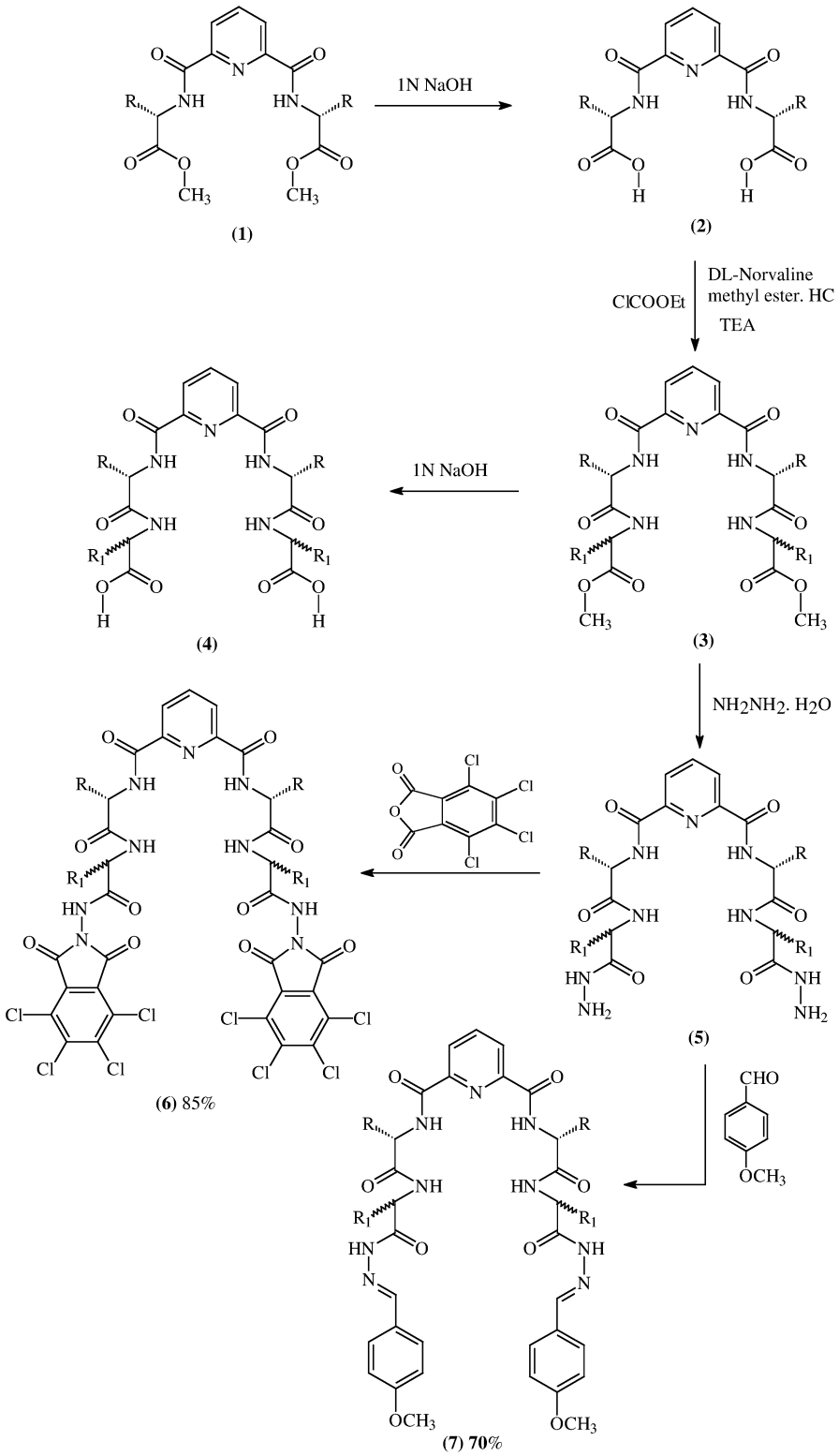
[B]

discovery and lead optimization. This is promised by their high degree of structural diversity and conformational states as well as the expected reduced toxicity of both the candidates and metabolites [31 and references cited therein].

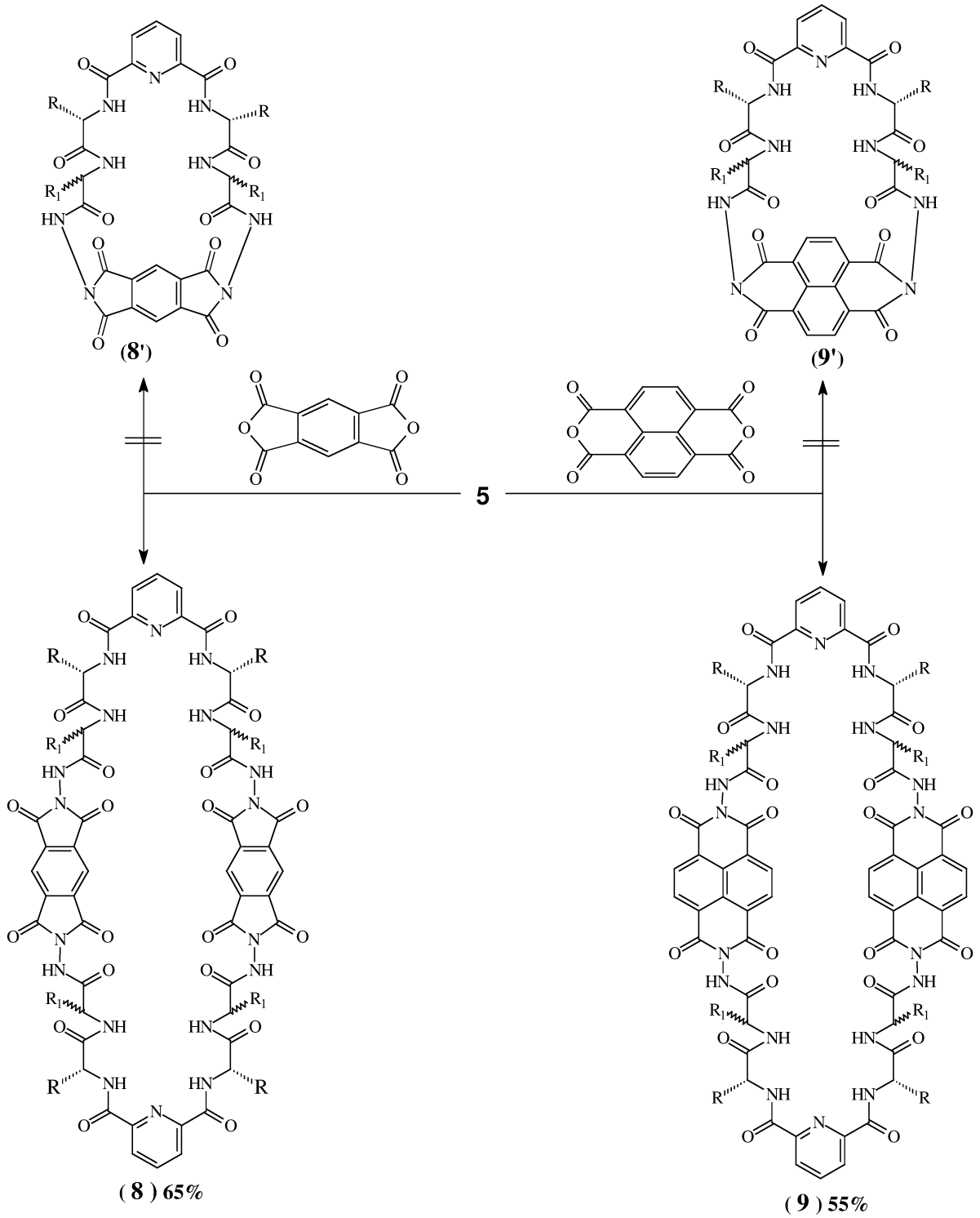
We have previously explored the analytical and biological characteristics of some *bis*-amino acid and peptide conjugates of dipicolinic acid [32]. Our studies of these compounds exemplified by compound [**A**] revealed an interesting anticancer activity, probably *via* DNA intercalation, as well as an outstanding metal sensor property, particularly, for pollutant lead (Pb^{2+}) cations [32]. Cyclization of some of these *bis* conjugates with molecular baskets based on calix[4]arene as represented by compound [B] provided new biologically and chemically interesting molecular architectures [33].

In this work, the synthesis and investigation of antiinflammatory characteristics of some *bis*-N^{α}-L-leucine, and N^{α}-L-leucyl-DL-norvaline linear, tetra and cyclic octa bridged peptides of dipicolinc acid, were undertaken. Schemes 1 and 2 outline the adopted synthetic routes for the candidates.

L-leucine methyl ester was initially coupled with dipicolinic acid *via* the conventional acid chloride method [34]. The peptide linkage was then formed with DL-norvaline methyl ester. The mixed anhydride (ethyl chloroformate/triethyl amine) proved practically more convenient than the azide or carbodiimide methodologies [32]. While the synthesis of compounds **1-5** were previously reported [32, 34]. Compounds **6-9** were obtained *via* simple condensation of the hydrazide **5** with anisaldehyde in methanol affording the hydrazone **7**. Similarly, tetrachlorophthalic anhydride, 1,2,4,5-benzenetetracarboxylic dianhydride and 1,8,4,5-naphthalene-tetracarboxylic dianhydride were condensed with **5** in glacial acetic acid giving the hydrazine conjugates **6**, **8** and **9** respectively.



Scheme 1. Synthesis of new (N^{α}-dipicolinoyl)-*bis*-L-leucyl-DL-norvalyl linear tetrapeptides **6** and **7**. R = $CH_2CH(CH_3)_2$; R_1 = $CH_2CH_2CH_3$.



Scheme 2. Synthesis of new (N^{α}-dipicolinoyl)-*bis*-L-leucyl-DL-norvalyl cyclic bridged octapeptides **8** and **9**. R = $CH_2CH(CH_3)_2$; R_1 = $CH_2CH_2CH_3$.

The modification of the amino acid and peptide C-terminal ends as esters **1** and **3** acids **2** and **4**, hydrazide **5** hydrazone **7** and finally as tetrachlorophthalic hydrazine conjugate **6** permits the exploration of the influence of these functional groups on biological activity.

Bridging with benzenetetracarboxylic acid and naphthalenetetracarboxylic acid to afford octapeptides **8** and **9** infers for these compounds a planar geometry. These conformationally restricted structures offer the perspective of investigating the biological responses of planar macrocycles up to the hypothetical inflammatory receptor. It is noteworthy to mention that the compounds could be enzymatically resistant due to the encountered non-peptidic (DPA-Leu) and racemized peptidic L-Leu-DL-Nval linkages.

As expected, the mass spectral data (Scheme 2) confirmed that the bridged cyclic tetrapeptides **8'** and **9'** were not formed. This may indicate that such cyclic molecules are, contrary to the corresponding octapeptides **8** and **9**, sterically hindered and consequently structurally unfavorable.

The analytical and spectroscopic data namely IR, ^{1}H and ^{13}C NMR data (*cf.* Experimental Section) were concordant with the expected structures.

Antiinflammatory Activity

Table 1 resumes the antiinflammatory activity and LD_{50}, of the candidates and Table 2 demonstrates their antiulcerogenicity comparable to indomethacin ®

Comparable to the two reference antiinflammatory drugs namely, indomethacin ® and voltaren ® (100%), the determined antiinflammatory activity of the candidates (Carrageenan ® induced paw edema in rats) revealed a significant activity (66 – 94%), except for the practically inactive **6** (∼1.5%). In particular, the potency of the (N^{α}-dipicolinoyl)-*bis*-L-leucyl-DL-norvalyl-*p*-methoxybenzaldehyde hydrazone **7** was of 94 and 87 % relative to the two reference drugs respectively, however, with a favorable antiulcerogenicity namely, of 58% protection against ulcer formation, comparable to null for indomethacin ®. Additionally, an acceptable acute toxicity was observed (LD_{50}: 2833 mg/kg comparable to 2700 and 2850 for indomethacin ® and voltaren ® respectively).

Other significant structure / activity relationships include:

• The cyclic compounds **8** and **9** proved to be more potent than the parent simple hydrazide (**5**).

Table 1. Antiinflammatory potency of the synthesized candidates.

Compound	% Increase in the weight of rat paw edema (g)	% Protection			
		A*	B**	C***	LD_{50} (mg/kg)
1-Voltaren®	10.318 ± 0.321	82.7	107.24	(100)	
2-Indomethacin®	13.865 ± 0.828	77.11	100	(93.24)	
1	27.216 ± 0.938	55.08	71.43	(66.60)	2150
2	23.763 ± 0.963	60.78	78.82	(73.49)	2100
3	19.860 ± 0.394	67.21	87.17	(81.26)	
4	19.319 ± 1.213	68.11	88.32	(82.35)	2483
5	23.160 ± 1.114	61.77	80.10	(74.69)	
6	61.316 ± 0.818	1.19	1.54	(1.43)	
7	16.618 ± 0.613	72.57	94.11	(87.75)	2833
8	20.946 ± 0.721	65.43	84.85	(79.11)	
9	21.714 ± 0.591	64.16	83.20	(77.58)	

* Absolute figure; ** % relative to indomethacin ®; *** % relative to voltaren®.

Table 2. Comparative ulcerogenicity of some synthesized candidates.

Compound	Average number of ulcers X* ± S.E.	Average number of severity of ulcers Y** ± S.E.	% Incidence of ulcers/10	Ulcer Index	% Protection against ulcers
Indomethacin®	5.10 ± 0.10	2.138 ± 0.031	10	17	0
1	4.51 ± 0.634	1.218 ± 0.583	8.3	12	29
2	2.10 ± 0.281	1.416 ± 0.498	5.6	8	53
4	2.63 ± 0.318	1.819 ± 0.620	6.3	10	41
7	1.64 ± 0.391	1.010 ± 0.183	5.3	7	58

* Total number of ulcers in all animals; ** ulcer score severity.

• The acids **2** and **4** are more potent than the corresponding esters **3** and **1**.

• In all cases, elongation with DL-norvaline had a potentiated effect.

• The loss of activity of the hydrazide **5** (80.10%) upon condensation with tetrachlorophthalic acid (**6**, 1.54%) seems interesting, however, difficult to be interpreted.

• The generally pronounced antiinflammatory activity of the compounds with a concomitant low ulcerogenicity may support the hypothesis that the cyclooxygenase (COX) may be the working inflammatory receptor. A higher specificity of the candidates towards the isoenzymes COX-2 rather than COX-1 could be, assumed.

Conclusion and Perspectives

Bis-dipicolinoyl linear and cyclic peptides appear as promising anti-inflammatory agents. Comparable to the known antiinflammatory drugs indomethacin ®

and voltaren®, eight of the nine prepared and tested compounds exhibited a significant antiinflammatory activity (66 – 94%), with a considerable protection against ulcer formation, and without compromising the acute toxicity (LD_{50}). The most potent candidate N^{α}-dipicolinoyl)-*bis*-L-leucyl-DL-norvalyl anisaldehyde hydrazone **7** showed of 94 and 87% antiinflammatory activity, comparable to indomethacin® and voltaren®, respectively with 58% protection against ulcer formation, comparable to null for indomethacin®.

Further chemical, pharmacological and biochemical studies, with regard to inhibition of the inflammatory enzymes cyclooxygenase, lipooxygenase and phospholipase A2 are intended.

Experimental Section

Syntheses were generally performed on 1 – 5 mmol scale and generally in ~5 ml solvent/mmol reactant. Melting points were recorded on an "Electrothermal IA 9000 SERIES" Digital Melting Point Apparatus (England) and are uncorrected. Analytical data were obtained from the Microanalytical Unit, National Research Center, Cairo, Egypt. The IR spectra were recorded on a FT IR-8201 PC Spectrophotometer (Shimadzu, Japan). ^{1}H- and ^{13}C-NMR spectra were recorded *via* (Jeol-GLM 270 MHz spectrometer and the chemical shifts were recorded relative to TMS as an internal standard. Mass spectra (EI-70 ev) were performed on a VG-2AB-3F Spectrometer. TLC was performed on silica gel, aluminum sheets 60 F_{254}, (Merck, Germany). Compounds **1-5** were prepared according to our published procedures [32, 34].

Synthesis of N^{α}-dipicolonyl-bis-[L-leucyl-DL-norvaline tetrachlorophthalic 1,2-hydrazine conjugate **6**

A stirred suspension (~50 ml) of N^{α}-dipicolonyl-*bis*-[L-leucyl-DL-norvaline hydrazide [34] (**5**, 0.62 g, ~1 mmol) in acetic acid and tetrachlorophthalic anhydride (0.57 g, ~2 mmol) was heated (80 °C) for 6 h. The reaction mixture was concentrated under reduced pressure and cooled. The separated solid was collected by filtration, dried and crystallized from acetic acid/ether to yield the corresponding compound **6**.

M. p. > 250 °C. – IR (film): ν = 3350, 3269 (NH), 1724 cm^{-1} (C=O). – ^{1}H NMR (270 MHz, DMSO-d_6,): δ = 0.95 – 0.80 (m, 18H, 6 × *CH*$_3$), 1.25 – 1.15 (m, 4H, 2 × *CH*$_2$), 1.60 – 1.45 (m, 4H, 2 × *CH*$_2$), 1.90 – 1.70 (m, 4H, 2 × *CH*$_2$), 2.35 (m, 2H, 2 × *CH*), 4.35 – 4.20 (m, 2H, 2 × *CH*N), 4.50 – 4.40 (m, 2H, 2 × *CH*N), 8.15 – 8.25 (m, 3H, py-*H*), 8.45, 8.80, 9.15 (3s, 6H, 6 × *NH*, exchangeable with D_2O). – MS (EI, 70 eV): *m/z* (%) = 1140 (11) [($M^+ + 2$)-H_2O], 557 (64) [M^+-$C_{16}H_2N_4O_4Cl_8$], 302 (100) [$C_{17}H_{24}N_3O_2$]. – $C_{45}H_{45}N_9O_{10}Cl_8$ (1155.53): calcd. C 46.77, H 3.92, N 10.90; found C 46.68, H 3.85, N 10.80.

Synthesis of N^{α}-dipicolonyl-bis-[L-leucyl-DL-norvaline p-methoxybenzaldehyde hydrazone **7**

A stirred solution of N^{α}-dipicolonyl-bis-[L-leucyl-DL-norvaline hydrazide (**5**, 0.62 g, 1 mmol) and *p*-methoxybenzaldehyde (0.27 g, 2 mmol) in absolute methanol (50 ml) was refluxed for 6 h. The reaction mixture was allowed to stand at room temperature overnight, then evaporated under reduced pressure. The obtained oily product was solidified by trituration with benzene/pet. ether (40 – 60 °C), the remaining solid was filtered off, dried and crystallized from methanol to give the corresponding hydrazone **7**.

M. p. 162 – 5 °C. – IR (film): $\tilde{\nu}$ = 3500 – 3250 (NH), 1660 cm^{-1} (C=O). – ^{1}H NMR (270 MHz, DMSO-d_6): δ = 0.9 – 1.2 (m, 20H, 6 × *CH*$_3$ + 2 × *CH*(CH$_3$)$_2$), 1.9 (t, 4H, 2 × *CH*$_2$), 2.15 – 2.20 (m, 4H, 2 × *CH*$_2$), 2.35 (m, 4H, 2 × *CH*$_2$), 3.46 (s, 6H, 2 × *OCH*$_3$), 3.95 – 4.00 (m, 4H, 4 × *CH*), 4.44 (s, 2H, 2 × *CH*=N), 7.08 – 7.37 (m, 8H, Ar-*H*), 8.22 – 8.42 (m, 3H, py-*H*), 8.63, 9.92, 10.12 (3s, 6H, 6 × *NH*, exchangeable with D_2O). – ^{13}C{^{1}H} NMR (270 MHz, DMSO-d_6): δ = 22.10, 27.80, 28.01, 30.09, 35.9, 37.00 (isobutyl and *n*-propyl carbons), 51.60 (OCH_3), 52.09, 54.74, 56.07 (CH), 148.93, 139.17, 125.67 (pyridyl-C), 127.02, 127.89, 128.76, 128.83, 136.24, 138.42 (Ar-C), 163.65, 169.42, 170.29, 172.28 (C=O). – MS (EI, 70 eV): *m/z* (%) = 856 (1.7) [M^+], 793 (5) [M^+-2OCH_3], 558 (14) [M^+-$C_{16}H_{18}N_4O_2$], 302 (100) [$C_{17}H_{24}N_3O_2$]. – $C_{45}H_{61}N_9O_8$ (856.03): calcd. C 63.13, H 7.18, N 14.72; found C 62.94, H 7.06, N 14.65.

Synthesis of bis[-L-leucyl-DL-norvalyl] cyclic octa bridged peptides **8** *and* **9** *(general procedure)*

A stirred suspension of mixture of N^{α}-dipicolonyl-*bis*-[L-leucyl-DL-norvaline hydrazide (**5**, 0.62 g, 1 mmol) and 1,2,4,5-benzenetetracarboxylic acid dianhydride or 1,8,4,5-naphthalenetetracarboxylic 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 **8** and **9**, respectively.

Benzene tetracarboxylic acid bis[-L-leucyl-DL-norvaly] cyclic octa bridged peptide **8**

M. p. 215 – 217 °C. – IR (film): $\tilde{\nu}$ = 3550 – 3300 (NH), 1742 cm^{-1} (C=O). – ^{1}H NMR (270 MHz, DMSO-d_6): δ = 0.83 – 0.92 (m, 36H, 12 × *CH*$_3$), 1.32 – 1.34 (m, 4H, 4 × *CH*(CH$_3$)$_2$), 1.55 – 1.58 (m, 8H, 4 × *CH*$_2$), 1.69 (t, 8H, 4 × *CH*$_2$), 1.83 (m, 8H, 4 × *CH*$_2$), 4.37 (t, 4H, 4 × *CH*N), 4.60 (t, 4H, 4 × *CH*N), 7.06 and 7.23 (2s, 4H, Ar-*H*), 8.01 – 8.03 (d, 4H, py-*H* – 3,5), 8.33 – 8.26 (t, 2H, py-*H* – 4), 8.91, 9.70, 9.77 (3s, 12H, 12 × *NH*, exchangeable with D_2O). – ^{13}C{^{1}H}NMR (270 MHz, DMSO-d_6):

δ = 14.19, 14.31, 19.07, 21.15, 22.46, 23.68, 25.23 (isobutyl and n-propyl carbons), 51.50, 52.46 (CHN), 125.42, 140.11, 149.57 (pyridine carbons), 135.60, 146.37, 150.9 (aromatic carbons), 163.34, 168.45, 171.05, 171.90, 172.14 (carbonyl carbons). – MS (EI, 70 eV): *m/z* (%) = 1603 (3) [M^+], 1502 (4) [M^+-$C_5H_{10}NO$], 1260 (3) [M^+ - $C_{15}H_{13}N_5O_5$], 1048 (6) [$C_{52}H_{61}N_{11}O_{13}$], 343 (10) [$C_{15}H_{13}N_5O_5$], 302 (16) [$C_{17}H_{24}N_3O_2$], 246 (14) [$C_{13}H_{14}N_2O_3$]. – $C_{78}H_{94}N_{18}O_{20}$ (1603.71): calcd. C 58.41, H 5.90, N 15.72; found C 58.34, H 5.78, N 15.66.

Naphthalene tetracarboxylic acid bis-L-leucyl-DL-norvalyl cyclic octa bridged peptide ***9***

M.p. 218 – 220 °C. – IR (film): $\tilde{\nu}$ = 3560 – 3258 (NH), 1744 cm^{-1} (C=O). – ^{1}H NMR (270 MHz, DMSO-d_6): δ = 0.87 – 1.00 (m, 36H, 12 × *CH*$_3$), 1.34 (m, 4H, 4 × *CH*(CH$_3$)$_2$), 1.62 (m, 8H, 4 × *CH*$_2$), 2.70 (t, 8H, 4 × *CH*$_2$), 2.9 (m, 8H, 4 × *CH*$_2$), 3.85 (s, 4H, 4 × *CH*N), 4.22 (s, 4H, 4 × *CH*N), 7.35 – 7.60 (m, 8H, Ar-*H*), 8.15 – 8.20 (m, 6H, py-*H*), 9.00, 9.70, 10.20 (3s, 12H, 12 × *NH*, exchangeable with D_2O). – ^{13}C{^{1}H}NMR (270 MHz, DMSO-d_6): δ = 17.78, 18.47, 18.92, 20.00, 30.38, 30.58, 30.74 (isobutyl and *n*-propyl carbons), 54.30, 58.36 (CHN), 125.08, 140.08, 148.88 (pyridine carbons), 128.97, 129.24, 131.39, 134.77, 148.80 (aromatic carbons), 163.08, 163.21, 163.67, 167.64, 172.64 (carbonyl carbons). – MS (EI, 70 eV): *m/z* (%) = 1703 (7) [M^+], 987 (12) [M^+-$C_{36}H_{44}N_8O_8$], 718 (8) [$C_{36}H_{46}N_8O_8$], 302 (100) [$C_{17}H_{24}N_3O_2$]. – $C_{86}H_{98}N_{18}O_{20}$ (1703.83): calcd. C 60.62, H 5.80, N 14.80; found C 60.48, H 5.72, N 14.74.

Antiinflammatory Activity

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), Voltaren® (Novartis, Switzerland) were used as reference drugs. Carragenan® (BDH, England). Other used chemicals are of analytical grade (Sigma - Aldrich, USA).

Methodology

Three groups of rats were classified as followed: The first, of six rats received a vehicle of 0.2 ml of 7% aqueous solution of Tween 80® and served as control group. The second was divided into two subgroups of six rats, each received either indomethacin® or voltaren® (7 mg/kg). The third was divided in to nine subgroups of six rats each orally received a candidate compound (7 mg/kg). Identical conditions were followed for the second and third group as for the first one. The anti-inflammatory activity (Table 1) was principally determined according to Winter *et al.*, [35]. The rats 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 Carragenan in saline in to the sub plantar tissue of the rat hind paw which. Identical experimental conations, except for dosing, were followed for the reference group. Three hours after Carragenan® injection he average weight of edema in the rapidly excised paws of the decapitated rats was then 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 [36] A.0.05 level of probability was regarded as significant according to Sendecor and Cochran [37].

Antiulcerogenicity (Table 2)

The activity was principally determined according to Shing *et al.*, [38]. Animals were divided into three groups of eight rats, the first of which served as negative control group. Ulcerogenicity was induced in the second and third group by the subcutaneous injection of indomethacin (8 mg/kg rat body weight). The third group received the test candidates, 90 minutes later after ulcer induction by indomethacin. All the animals were decapitated sixty minutes following a candidate administration, The stomach was removed and examined for ulcer lessons. The ulcer index was calculated according to Meshali *et al.* [39].

LD_{50}

The LD_{50} determined by using rats and inject different increasing doses and calculate the dose that kill 50% of the animal, according to Austen *et al.* [40].

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