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SEARCH FOR NEW DRUGS

SYNTHESIS AND *IN VITRO* NEUROPROTECTOR ACTIVITY OF DIASTEREOISOMERS OF A DIMERIC DIPEPTIDE MIMETIC OF NERVE GROWTH FACTOR GK-2

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Previously, a dimeric dipeptide mimetic *bis*-(*N*-monosuccinyl-L-glutamyl-L-lysine) hexamethylenediamide (GK-2) based on the β-turn of the fourth loop of nerve growth factor was prepared by us, activated TrkA, and exhibited neuroprotective activity *in vitro* (10^{-5} – 10^{-9} M) and *in vivo* (0.05 – 5 mg/kg i.p.). The present work reports the synthesis of two of its diastereomers, *bis*-(*N*-monosuccinyl-*L*-glutamyl-D-lysine) (GK-2LD) and *bis*-(*N*-monosuccinyl-*D*-glutamyl-*L*-lysine) hexamethylenediamides (GK-2DL). Studies of their neuroprotective activities using HT-22 neuronal culture under oxidative stress showed that switching L-lysine to D-lysine led to a significant activity decrease whereas switching L-glutamic acid to the D-stereoisomer caused it to disappear completely. Both dipeptide residues were concluded to be involved in interactions with the TrkA receptor.

Keywords: mimetic, NGF, neuroprotective activity, diastereomers, GK-2, TrkA.

Nerve growth factor (NGF) was the first representative of the neurotrophin family [1] and participates in the development and survival of central and peripheral neurons. NGF was discovered at the start of the 1950s [2] and immediately attracted attention as a possible agent for treating neurodegenerative diseases such as Alzheimer's, Parkinson's, Huntington's, and brain ischemia. Clinical use of NGF is limited by serious adverse effects, mainly hyperalgesia and weight loss, and unsatisfactory pharmacokinetic parameters associated with the proteinaceous nature of neurotrophins [3]. Therefore, the search for low-molecular-mass NGF mimetics without the drawbacks of the full-sized protein continues [4-6].

The beta-turn of the fourth loop of NGF was used as a platform at V. V. Zakusov SIP to design the dimeric dipeptide mimetic GK-2 or *bis*-(*N*-monosuccinyl-*L*-glutamyl-*L*-lysine) hexamethylenediamide [7]. Like NGF, GK-2 activated specific TrkA receptors and selectively the phosphatidylinositol-3 kinase (PI3K/Akt) post-receptor signaling pathway that promoted neuroprotector effects [8].

GK-2 in experiments *in vitro* at micro- and nanomolar concentrations exhibited high NGF-like neuroprotective activity [9]. GK-2 protected immortalized HT-22 mouse hippocampal neuron cells from death caused by H_2O_2 or glutamic acid and PC-12 rat pheochromocytoma cells from the effects of the neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) [10]. GK-2 was active in experiments *in vivo* on Alzheimer's and Parkinson's disease models and in acute and chronic rat brain ischemia models and did not cause hyperalgesia and mass loss [11 – 13]. Currently, GK-2 is in the final stage of preclinical studies as a potential neuroprotector.

The stereospecificity of the neuroprotective activity of GK-2 with TrkA receptors was studied to deepen our understanding of the mechanism of the interaction. For this, two of its diastereomers were synthesized, i.e., *bis*-(*N*-monosuccinyl-*L*-glutamyl-*D*-lysine) (GK-2LD) and *bis*-(*N*-monosuccinyl-

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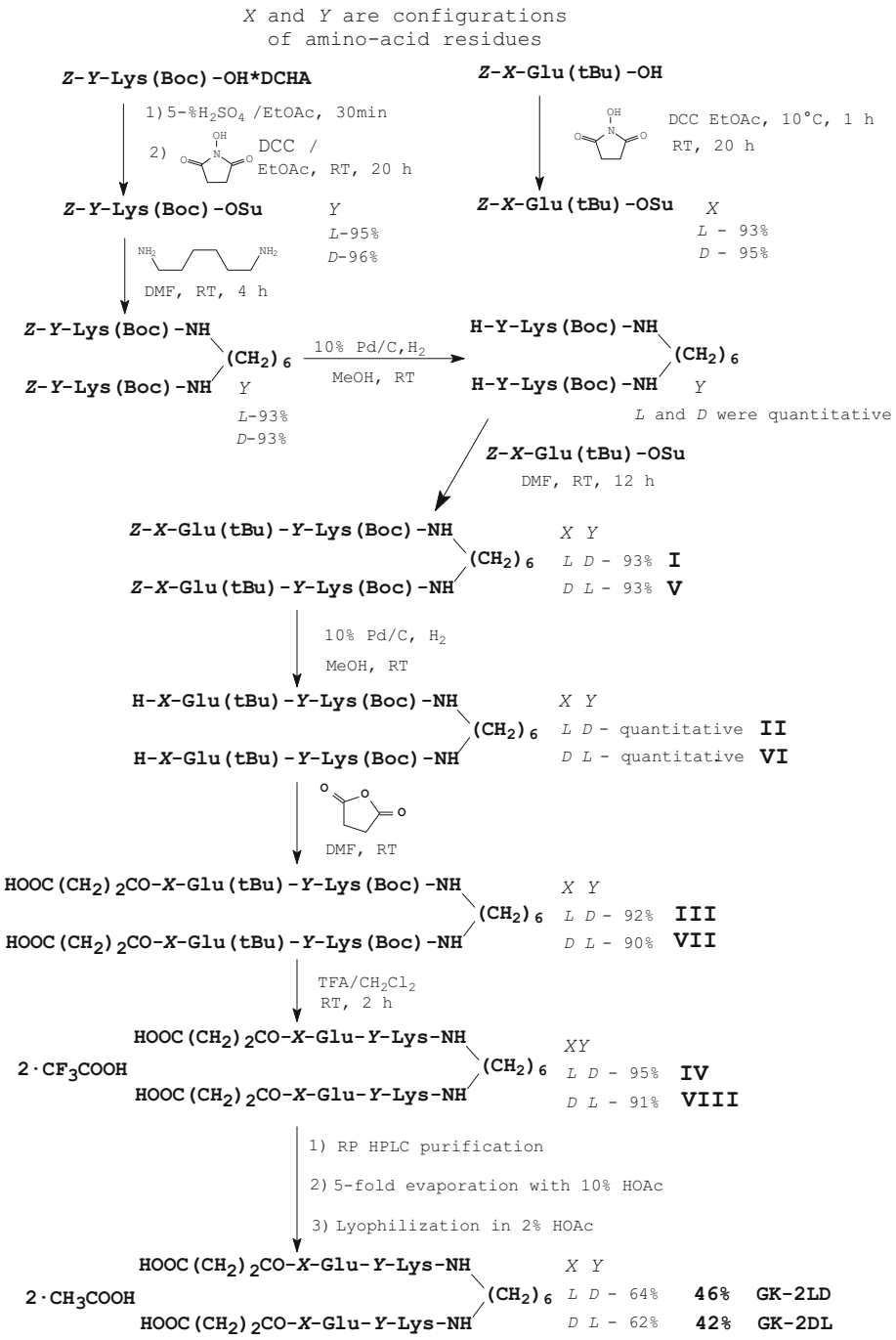

Scheme. Syntheses of GK-2DL and GK-2LD.

D-glutamyl-*L*-lysine) hexamethylenediamides (GK-2DL). Their neuroprotective activity was studied *in vitro* using HT-22 neuronal culture under oxidative stress induced by H_2O_2.

Diastereomers of GK-2 were synthesized using classical solution peptide-synthesis methods for growing the peptide chain from the C-terminus, Z/Boc protecting groups, and activated *N*-hydroxysuccinimide esters according to the general scheme given below.

Compounds GK-2DL and GK-2LD were synthesized from commercially available *Z*-*L*-Lys(Boc)-OH, *Z*-*D*-Lys-(Boc)-OH, *Z*-*L*-Glu(tBu)-OH, and *Z*-*D*-Glu(tBu)-OH. Activated *N*-hydroxysuccinimidyl esters of protected amino acids were prepared by standard methods [15]. The activated esters of protected L- and D-lysine were condensed with hexamethylenediamine in DMF at room temperature (RT) to give in identical yields (93%) [*Z*-*L*-Lys(Boc)-NH-]$_2$(CH$_2$)$_6$ and [*Z*-*D*-Lys(Boc)-NH-]$_2$(CH$_2$)$_6$ hexamethylenediamides

that then were *Z*-deblocked using hydrogenolysis. The obtained products were condensed in DMF with the corresponding activated esters of protected glutamic acid *Z*-*D*-Glu(tBu)-OSu and *Z*-*L*-Glu(tBu)-OSu to produce in 93% yield condensation products [*Z*-*L*-Glu(tBu)-*D*-Lys(Boc)-NH-]$_2$(CH$_2$)$_6$ (**I**) and [*Z*-*D*-Glu(tBu)-*L*-Lys(Boc)-NH-]$_2$(CH$_2$)$_6$ (V). Hydrogenolysis produced [H-*L*-Glu(tBu)-*D*-Lys(Boc)-NH-]$_2$(CH$_2$)$_6$ (**II**) and [H-*D*-Glu(tBu)-*L*-Lys(Boc)-NH-]$_2$(CH$_2$)$_6$ (**VI**) that then were acylated by succinic anhydride in DMF to afford in 90 – 92% yields *N*-monosuccinyl derivatives [HOOC-CH$_2$CH$_2$-CO-*L*-Glu(tBu)-*D*-Lys(Boc)-NH-]$_2$(CH$_2$)$_6$ (**III**) and [HOOC-CH$_2$CH$_2$-CO-*D*-Glu(tBu)-*L*-Lys(Boc)-NH-]$_2$(CH$_2$)$_6$ (**VII**). Subsequent removal of the Boc-protection by TFA in CH$_2$Cl$_2$ gave the ditriflates of *bis*-(*N*-monosuccinyl-*L*-glutamyl-*D*-lysine) (**IV**) and *bis*-(*N*-monosuccinyl-*D*-glutamyl-*L*-lysine) hexamethylenediamides (**VIII**). Preparative RP HPLC purification gave the ditriflates (97 – 98%), which were converted to the acetates by multiple evaporations with HOAc (10%) followed by lyophilization.

The target products were homogeneous according to TLC and RP HPLC. The structures and diastereomeric purities of the products were confirmed using PMR spectroscopy.

Neuroprotective activity of the synthesized diastereomers was studied under oxidative stress with H_2O_2 using a culture of immortalized HT-22 mouse hippocampal cell line at concentrations of 10^{-5} – 10^{-8} M. The peptides were added 24 h before adding the stressor.

Table 1 shows that the neuroprotective effect decreased by about three times on going from the L,L- to the L,D-diastereomer. However, the neuroprotective effect disappeared completely on going from the L,L- to the D, L-isomer. Apparently, both amino-acid residues of the NGF dipeptide mimetic were involved in the interaction with the receptor. The configuration of the lysine residue may have been less influential because its side chain was rather long and flexible. This could allow the terminal positive charge on the D-lysine side chain to occupy almost the same position in the complex with the receptor as that of L-lysine.

TABLE 1. Influence of *bis*-(*N*-Monosuccinylglutamyllysine) Hexamethylenediamide Diastereomers on Neuron Survival Under Oxidative Stress

Compound	Concentration, M	Optical absorption		Activity, %
		with H_2O_2	without H_2O_2	
GK-2 [9], (L, L-isomer)	10^{-5}	0.422 ± 0.063*		94
	10^{-8}	0.416 ± 0.059*		96
	10^{-9}	0.378 ± 0.042*		36
	10^{-10}	0.358 ± 0.038		10
	0 (control)	0.350 ± 0.032#	0.427 ± 0.071	
GK-2DL (D, L-isomer)	10^{-5}	0.160 ± 0.009		10
	10^{-6}	0.170 ± 0.013		19
	10^{-7}	0.166 ± 0.005		16
	10^{-8}	0.158 ± 0.006		8
	0 (control)	0.149 ± 0.005#	0.259 ± 0.011	
GS-2LD (L, D-isomer)	10^{-5}	0.176 ± 0.011*		27
	10^{-6}	0.175 ± 0.011*		27
	10^{-7}	0.179 ± 0.007*		31
	10^{-8}	0.184 ± 0.013*		36
	0 (control)	0.152 ± 0.008#	0.240 ± 0.012	
NGF [9]	100 ng/mL (~10^{-9})	0.425 ± 0.047*		97
	0 (control)	0.350 ± 0.032#	0.427 ± 0.071	

Note: Tests used HT-22 mouse hippocampal neuronal cell line. Compounds were injected 24 h before insult. Cell survival was assayed using the MTT test:

* $p < 0.05$ by the Mann-Whitney criterion vs. a control with H_2O_2;

\# $p < 0.05$ by the Mann-Whitney criterion vs. a control without H_2O_2.

Activity was calculated using the formula $A(\%) = (D_{exp} - D_{H_2O_2})/(D_{contr} - D_{H_2O_2})\cdot 100\%$, where D_{exp} is the optical absorption of the test solution; D_{H_2O}, optical absorption of a control solution (with H_2O_2); and D_{contr}, optical absorption of a passive control (without H_2O_2).

The results could be useful for designing new NGF mimetics.

EXPERIMENTAL CHEMICAL PART

Commercial amino acids and their derivatives (Sigma, Alfa Aesar) were used in the work. DMF was purified by distillation over ninhydrin. Et_2O was stored over NaOH and used without further purification. EtOAc, CH_2Cl_2, $CHCl_3$, C_6H_6, Me_2CO, hexane, MeOH, and EtOH (all chemically pure) were used without further purification. Melting points were measured on an Optimelt MPA100 apparatus (Stanford Research Systems, Great Britain) in open capillaries and were uncorrected. Specific optical rotation was recorded on an automated ADP 440 Polarimeter (Bellingham+Stanley Ltd., Great Britain). PMR spectra were recorded in DMSO-d_6 at a concentration of 30 mg/mL with TMS internal standard on the δ-scale in ppm on a Bruker Fourier 300 HD spectrometer (Germany). Resonances were assigned by analyzing one-dimensional PMR spectra and two-dimensional COSY spectra. TLC used Kieselgel 60 G/F_{254} glass plates (Merck, Germany) and solvent systems $CHCl_3$—MeOH (6:1, A); C_6H_6—MeOH (1:4, B); $CHCl_3$—MeOH— H_2O—HOAc (15:10:2:3, C); hexane—EtOAc (1:5, D); *n*-BuOH—HOAc—H_2O (3:1:1, E); $CHCl_3$—MeOH—HOAc (80:10:1, F); hexane—EtOAc (1:1, G); *i*-PrOH—HOAc—H_2O (7:3:1, H); C_6H_6—MeOH (2:1, I); and dioxane—H_2O (9:1, J). Compounds containing amines were detected by ninhydrin; amides, Cl—toluidine; carboxylic acids, bromocresol green; aromatic rings, UV light.

HPLC of the dipeptides used a Wellchrom 2001 chromatography system (Knauer, Germany) and a Diasorb-130-C16T analytical column (4.0 × 150 mm, 7 μm) and a Diasorb 130-C16T preparative column (15 × 250 mm, C_{16}, 9 μm). The loop volumes were 20 μL (analytical) and 2 mL (preparative). Mobile phases A (0.05% TFA in H_2O) and B (0.05% TFA in MeCN) were used at 0-100% B in 0-30 min. The flow rate was 0.6 mL/min for the analytical and 6 mL/min for the preparative column. Detection was made at 220 nm. The analyses were made at room temperature.

[*Z*-*L*-Lys(Boc)-NH-]$_2$(CH$_2$)$_6$, **[*Z*-*D*-Lys(Boc)-NH-]$_2$(CH$_2$)$_6$**, **[H-*L*-Lys(Boc)-NH-]$_2$(CH$_2$)$_6$**, and **[H-*D*-Lys(Boc)-NH-]$_2$(CH$_2$)$_6$** were prepared before in our laboratory [17 – 19].

***N*-Hydroxysuccinimide ester of *N*-benzyloxycarbonyl-*N*$^{\varepsilon}$-*tert*-butyloxycarbonyl-*L*-lysine [*Z*-*L*-Lys(Boc)-OSu]** was prepared as before [18] in 95% yield as white crystals. R_f 0.73 (A), R_f 0.73 (D). mp 95 – 99°C. $[\alpha]_D^{22}$ – 16.1° (s 2, dioxane). Lit. [18]: mp 97 – 99°C. $[\alpha]_D^{22}$ – 16.0° (s 2, dioxane).

N-Hydroxysuccinimide ester of *N*-benzyloxycarbonyl-*N*$^{\varepsilon}$-*tert*-butyloxycarbonyl-*D*-lysine [*Z*-*D*-Lys(Boc)-OSu] was prepared as before [19] in 96% yield as white crystals. R_f 0.73 (A), R_f 0.73 (D). mp 91 – 97°C (Et_2O). $[\alpha]_D^{27}$ + 16.2° (s, 2; dioxane). Lit. [19]: mp 97 – 99°C. $[\alpha]_D^{22}$ + 16.0° (s, 2; dioxane).

***N*-Hydroxysuccinimide ester of *N*-benzyloxycarbonyl-γ-*tert*-butyl-*L*-glutamic acid [*Z*-*L*-Glu(tBu)-OSu]** was prepared according to the literature [14] in 93% yield as white crystals. R_f 0.87 (EtOAc), R_f 0.83 (D), R_f 0.52 (G). mp 98 – 101°C. $[\alpha]_D^{21}$ – 29.7 (s, 2.4, EtOH), $[\alpha]_D^{26}$ – 21.3° (s, 1, EtOAc), $[\alpha]_D^{25}$ – 31.4° (s, 1, MeOH). Lit. [14]: mp 86 – 89°C. $[\alpha]_D^{25}$ – 31.4 (s, 1, MeOH); [20]: mp 105 °C.

N-Hydroxysuccinimide ester of *N*-benzyloxycarbonyl-γ-*tert*-butyl-*D*-glutamic acid [*Z*-*D*-Glu(tBu)-OSu] was prepared analogously to *Z*-*L*-Glu(tBu)-OSu in 95% yield as white crystals. R_f 0.88 (EtOAc), R_f 0.84 (A). R_f 0.49 (G). mp 100 – 104°C. $[\alpha]_D^{24}$ + 18.3° (s, 1, EtOAc). PMR spectrum: 1.40 (s, 9H, OC(C$\underline{H}_3$)$_3$), 1.97 – 2.10 (m, 2H, C$^{\beta}$H$_2$ Lys), 2.42 (m, 2H, C$^{\gamma}$H$_2$ Lys), 2.82 (4H, s, -CH$_2$-CH$_2$-, OSu), 4.53 (m, 1H, C$^{\alpha}$H Lys), 5.07 (s, 2H, -OC$\underline{H}_2$C$_6$H$_5$), 7.36 (m, 5H, C$_6$H$_5$), 8.08 (d, 1H, J 7.8 Hz, NH).

bis-(*N*-Benzyloxycarbonyl-*N*$^{\varepsilon}$-*tert*-butyloxycarbonyl-*L*-lysine) hexamethylenediamide, [*Z*-*L*-Lys(Boc)-NH-]$_2$(CH$_2$)$_6$, was prepared according to the literature [14] in 93% yield as white crystals. R_f 0.63 (EtOAc), R_f 0.21 (A), R_f 0.86 (B), R_f 0.35 (D). mp 150 – 154°C, $[\alpha]_D^{25}$ – 9.3° (s, 0.29, EtOH), $[\alpha]_D^{25}$ – 9.1° (s, 1, MeOH). Lit. [14]: mp 150 – 154°C, $[\alpha]_D^{25}$ – 9.3° (s, 0.29, EtOH).

bis-(*N*-Benzyloxycarbonyl-*N*$^{\varepsilon}$-*tert*-butyloxycarbonyl-*D*-lysine) hexamethylenediamide, [*Z*-*D*-Lys(Boc)-NH-]$_2$(CH$_2$)$_6$, was prepared according to the literature [19] in 93% yield as white crystals with mp 154 – 157°C. R_f 0.57 (EtOAc), $[\alpha]_D^{24}$ + 7.6° (s, 1, MeOH). Lit. [19]: mp 151 – 154°C, $[\alpha]_D^{25}$ + 9.0° (s, 0.3, EtOH).

bis-(*N*$^{\varepsilon}$-*tert*-Butyloxycarbonyl-*L*-lysine) hexamethylenediamide, [H-*L*-Lys(Boc)-NH-]$_2$(CH$_2$)$_6$, was prepared as before [19] in quantitative yield as white crystals. R_f 0.33 (A), R_f 0.68 (D), R_f 0.39 (D), R_f 0.48 (G), R_f 0.21 (H). mp 81 – 84°C (Et_2O), $[\alpha]_D^{25}$ + 8.3 (s, 1, EtOH). PMR spectrum: 1.24 – 1.51 (m, 20H, -NHCH$_2$(C$\underline{H}_2$)$_4$CH$_2$NH-, 2C$^{\beta}$H$_2$C$^{\gamma}$H$_2$C$^{\delta}$H$_2$ Lys), 1.37 (s, 18H, 2-OC(CH$_3$)$_3$ Boc), 2.88 (m, 4H, C$^{\varepsilon}$H$_2$ Lys), 3.03 (m, 2H, 2 C$^{\alpha}$H Lys), 3.06 (m, 4H, -NHC$\underline{H}_2$(CH$_2$)$_4$C$\underline{H}_2$NH-), 6.71 (t, J 5.2 Hz, 2H, 2 *N*$^{\varepsilon}$H Lys), 7.76 (t, J 5.6 Hz, 2H, -N$\underline{H}$(CH$_2$)$_6$N$\underline{H}$-).

bis-(*N*$^{\varepsilon}$-*tert*-butyloxycarbonyl-*D*-lysine) hexamethylenediamide, [H-*D*-Lys(Boc)-NH-]$_2$(CH$_2$)$_6$, was prepared analogously [19] in quantitative yield as white crystals. R_f 0.33 (A), R_f 0.47 (B). mp 81 – 85°C, $[\alpha]_D^{24}$ – 12.6° (s, 1, MeOH). PMR spectrum: 1.24 – 1.55 (m, 20H, -NHCH$_2$(C$\underline{H}_2$)$_4$CH$_2$NH-, 2C$^{\beta}$H$_2$C$^{\gamma}$H$_2$C$^{\delta}$H$_2$ Lys), 1.37 (s, 18H, 2 -OC(CH$_3$)$_3$ Boc), 2.87 (m, 4H, C$^{\varepsilon}$H$_2$ Lys), 3.03 (m, 6H, 2 C$^{\alpha}$H Lys, 4H, -NHC$\underline{H}_2$(CH$_2$)$_4$C$\underline{H}_2$NH-), 6.75 (t, J 5.4 Hz, 2H, 2 *N*$^{\varepsilon}$H Lys), 7.78 (t, J 5.6 Hz, 2H, -N$\underline{H}$(CH$_2$)$_6$N$\underline{H}$-).

Synthesis of [HOOC(CH$_2$)$_2$CO-*L*-Glu(tBu)-*D*-Lys(Boc)-NH-]$_2$(CH$_2$)$_6$, GK-2LD

***bis*-(*N*-Benzyloxycarbonyl-γ-*tert*-butyl-*L*-glutamyl-N^{ε}-*tert*-butyloxycarbonyl-*D*-lysine) hexamethylenediamide, [*Z*-*L*-Glu(tBu)-*D*-Lys(Boc)-NH-]$_2$(CH$_2$)$_6$, (I)**. A solution of [H-*D*-Lys(Boc)-NH-]$_2$(CH$_2$)$_6$ (5.94 g, 10.37 mmol) in DMF (30 mL) at room temperature was stirred on a magnetic stirrer, treated with *Z*-*L*-Glu(tBu)-OSu (9.50 g, 21.8 mmol, 5% excess) in DMF (25 mL), stirred for 4 h, and stored for another 12 h. The mixture was treated with *N*,*N*-dimethylpropylenediamine (DMPA, 0.13 mL), stirred for 30 min, and poured into H_2O (200 mL). The solution and resulting precipitate were left for 12 h at room temperature. The well-formed precipitate was filtered off, rinsed with H_2O (100 mL), pressed on the filter, rinsed successively with hexane (50 mL) and Et_2O (50 mL), and dried in a vacuum desiccator over $CaCl_2$ and paraffin to afford a cream-colored crystalline product (11.70 g, 93%). R_f 0.43 (EtOAc), R_f 0.77 (A), R_f 0.40 (F). mp 155 – 158°C, $[\alpha]_D^{25}$ + 5.0° (s, 1, MeOH). PMR spectrum: 1.21 – 1.50 (m, 20H, -NHCH$_2$(CH$_2$)$_4$CH$_2$NH-, 2 C$^{\beta}$H$_2$C$^{\gamma}$H$_2$C$^{\delta}$H$_2$ Lys), 1.36 (s, 18H, 2 -OC(CH$_3$)$_3$ Boc), 1.37 (c, 18H, 2 -OC(CH$_3$)$_3$ tBu), 1.59 – 1.84 (m, 4H, 2 C$^{\beta}$H$_2$ Glu), 2.20 (t, J 7.3 Hz, 4H, 2 C$^{\gamma}$H$_2$ Glu), 2.85 (m, 4H, C$^{\varepsilon}$H$_2$ Lys), 3.00 (m, 4H, -NHCH$_2$(CH$_2$)$_4$CH$_2$NH-), 4.03 (m, 2H, 2 C$^{\alpha}$H Glu), 4.15 (m, 2H, 2 C$^{\alpha}$H Lys), 5.02 (c, 4H, 2 -OCH$_2$C$_6$H$_5$), 6.72 (t, J 5.2 Hz, 2H, 2 N$^{\varepsilon}$H Lys), 7.34 (m, 10H, 2 -OCH$_2$C$_6$H$_5$), 7.51 (d, J 7.1 Hz, 2H, 2 NH Glu), 7.75 (t, J 6.6 Hz, 2H, -NH(CH$_2$)$_6$NH-), 8.05 (d, J 8.1 Hz, 2H, 2 NH Lys).

***bis*-(γ-*tert*-Butyl-*L*-glutamyl-N^{ε}-*tert*-butyloxycarbonyl-*D*-lysine) hexamethylenediamide, [H-*L*-Glu(tBu)-*D*-Lys(Boc)-NH-]$_2$(CH$_2$)$_6$, (II)**. Compound I (11.00 g, 9.1 mmol) was dissolved in MeOH (245 mL) and subjected to catalytic hydrogenolysis at room temperature in the presence of Pd/C (1.65 g, 10%, 50% moisture). When the starting material disappeared (TLC monitoring, EtOAc, A), the catalyst was filtered off and rinsed with MeOH (70 mL). The solvent was evaporated in vacuo. The solid was treated with C_6H_6 (30 mL) and evaporated in vacuo to remove traces of MeOH and H_2O. The foamy oil was triturated with Et_2O. The solvent was decanted. The amorphous white powder was dried in a vacuum desiccator over $CaCl_2$ and paraffin to afford the product (8.54 g, quantitative) as white crystals. R_f 0.02 (A). mp 89 – 112°C. $[\alpha]_D^{24}$ + 15.7° (s, 1, MeOH). PMR spectrum: 1.13 – 1.37 (m, 16H, -NHCH$_2$(CH$_2$)$_4$- CH$_2$NH-, 2 C$^{\gamma}$H$_2$C$^{\delta}$H$_2$ Lys), 1.35 (s, 18H, 2 -OC(CH$_3$)$_3$ Boc), 1.39 (c, 18H, 2 -OC(CH$_3$)$_3$ tBu), 1.40 and 1.55 (m, 4H, 2 C$^{\beta}$H$_2$ Lys), 1.57 – 1.80 (m, 4H, 2 C$^{\beta}$H$_2$ Glu), 2.22 (t, J 7.8 Hz, 4H, 2 C$^{\gamma}$H$_2$ Glu), 2.85 (m, 4H, C$^{\varepsilon}$H$_2$ Lys), 3.02 (m, 4H, -NHCH$_2$(CH$_2$)$_4$CH$_2$NH-), 3.17 (m, 2H, 2 C$^{\alpha}$H Glu), 4.16 (m, 2H, 2 C$^{\alpha}$H Lys), 6.72 (t, J 5.2 Hz, 2H, 2 N$^{\varepsilon}$H Lys), 7.89 (t, J 5.6 Hz, 2H, -NH(CH$_2$)$_6$NH-), 7.93 (d, J 8.8 Hz, 2H, 2 NH Lys).

bis-(*N*-Monosuccinyl-γ-*tert*-butyl-*L*-glutamyl-N^{ε}-*tert*-butyloxycarbonyl-*D*-lysine) hexamethylenediamide, [HOOC(CH$_2$)$_2$CO-*L*-Glu(tBu)-*D*-Lys(Boc)-NH-]$_2$(CH$_2$)$_6$, (III). A solution of II (8.14 g, 8.6 mmol) in DMF (35 mL) was cooled to +5°C, stirred, treated with succinic anhydride (2.08 g, 20.7 mmol, 20% excess), and stirred for 12 h at room temperature. When the reaction was finished (TLC monitoring), the mixture was treated with DMPA (0.437 mL), stirred for 30 min, treated slowly with distilled H_2O (200 mL), and stored overnight at room temperature. The resulting precipitate was filtered off, rinsed with H_2O (20 mL), pressed on the filter, rinsed successively with hexane (20 mL) and Et_2O (2 × 20 mL), and dried in a vacuum desiccator over $CaCl_2$ and paraffin to afford the product (9.07 g, 92%) as light cream-colored fine crystals. R_f 0.84 (B), R_f 0.23 (F). mp 164 – 168°C, $[\alpha]_D^{28}$ + 5.3° (s, 1, MeOH). PMR spectrum: 1.10 – 1.51 (m, 20H, -NHCH$_2$(CH$_2$)$_4$- CH$_2$NH-, 2 C$^{\beta}$H$_2$C$^{\gamma}$H$_2$C$^{\delta}$H$_2$ Lys), 1.35 (s, 18H, 2 -OC(CH$_3$)$_3$ Boc), 1.37 (c, 18H, 2-OC(CH$_3$)$_3$ tBu), 1.56 – 1.87 (m, 4H, 2 C$^{\beta}$H$_2$ Glu), 2.18 (m, 4H, 2 C$^{\gamma}$H$_2$ Glu), 2.45 (m, 8H, 2 -CH$_2$CH$_2$- Suc), 2.83 (m, 4H, 2 C$^{\varepsilon}$H$_2$ Lys), 3.01 (m, 4H, -NHCH$_2$(CH$_2$)$_4$CH$_2$NH-), 4.07 (m, 2H, 2 C$^{\alpha}$H Lys), 4.15 (m, 2H, 2 C$^{\alpha}$H Glu), 6.76 (t, J 5.4 Hz, 2H, 2 N$^{\varepsilon}$H Lys), 7.80 (t, J 5.1 Hz, 2H, -NH(CH$_2$)$_6$NH-), 8.11 (d, J 8.6 Hz, 2H, 2 NH Glu), 8.15 (d, J 8.1 Hz, 2H, 2 NH Lys).

***bis*-(*N*-Monosuccinyl-*L*-glutamyl-*D*-lysine) hexamethylenediamide ditriflate, 2CF$_3$COOH·[HOOC(CH$_2$)$_2$CO-*L*-Glu-*D*-Lys-NH-]$_2$(CH$_2$)$_6$ (IV)**. Compound **III** (1.00 g, 0.87 mmol) was dissolved in a mixture of TFA (6 mL) and CH_2Cl_2 (18 mL), stirred at room temperature for 2 h, evaporated, and re-evaporated with CH_2Cl_2 (2 × 15 mL). The oily solid was triturated with anhydrous Et_2O (20 mL). The Et_2O was decanted. The operation was repeated. Then, more Et_2O (20 mL) was added. The mixture was left for 2 h to form a precipitate that was filtered off and dried in a vacuum desiccator over $CaCl_2$ and paraffin to afford the product (0.88 g, 95%) as white crystals. Analytical RP HPLC (τ = 8.6 min) showed the product was 83% pure. Next, compound IV was purified using preparative RP HPLC. A solution of IV (0.80 g) in deionized H_2O (6 mL) was filtered through a 0.22-μm polyestersulfone membrane filter (TPP, Switzerland) and injected (3 × 2 mL). Fractions corresponding to the peak maximum were collected and evaporated in a rotary evaporator to afford an oily product that was 97% pure (analytical RP HPLC). Then, it was dissolved in deionized H_2O (10 mL) and lyophilized to afford gray fine crystals (0.60 g, 75%) with mp 118 – 126°C (hygroscopic), $[\alpha]_D^{31}$ + 6.8° (s, 1, MeOH). PMR spectrum: 1.20 – 1.60 (m, 20H, -NHCH$_2$(CH$_2$)$_4$CH$_2$NH-, 2 C$^{\beta}$H$_2$C$^{\gamma}$H$_2$C$^{\delta}$H$_2$ Lys), 1.79 (m, 4H, 2 C$^{\beta}$H$_2$ Glu), 2.23 (m, 4H, 2 C$^{\gamma}$H$_2$ Glu), 2.40 (m, 8H, 2 -CH$_2$CH$_2$- Suc), 2.73 (m, 4H, 2 C$^{\varepsilon}$H$_2$ Lys), 3.02 (m, 4H, -NHCH$_2$(CH$_2$)$_4$CH$_2$NH-), 4.14 (m, 4H, 2 C$^{\alpha}$H Lys, 2 C$^{\alpha}$H Glu), 7.71 (br.s, 6H, 2 N^{+}H$_3$ Lys), 7.80 (t, J 5.6 Hz, 2H, -NH(CH$_2$)$_6$NH-), 8.09 (d, J 8.2 Hz, 2H, 2 NH Lys), 8.17 (d, J 6.7 Hz, 2H, 2 NH Glu), 12.14 (br.s, 4H, 2 -COOH Suc, 2 -COOH Glu).

bis-(*N*-Monosuccinyl-*L*-glutamyl-*D*-lysine) hexamethylenediamide diacetate, 2 CH_3COOH·[HOOC$(CH_2)_2$CO-*L*-Glu-*D*-Lys-NH-]$_2(CH_2)_6$ (GK-2LD). Compound **I**V (0.527 g, 0.5 mmol) in HOAc (10%, 10 mL) was evaporated in vacuo at 2°C, re-evaporated with HOAc (4 × 10 mL), and evaporated with C_6H_6 (3 × 10 mL) and Et_2O (2 × 5 mL) to afford an oily product that was 98% pure (analytical RP HPLC, $\tau = 8.6$ min). Then, it was dissolved in HOAc (20 mL, 2%) and lyophilized to afford a product (0.404 g, 85%) as hygroscopic crystals in overall yield 46%. The melting point was not determined (hygroscopic). $[\alpha]_D^{23}$ + 4.6° (s, 0.32 MeOH). PMR spectrum: 1.20 – 1.58 (m, 20H, 2-NHCH$_2$- (C$\underline{H}_2$)$_4$CH$_2$NH-, 2 C$^\beta$H$_2$C$^\gamma$H$_2$C$^\delta$H$_2$ Lys), 1.60 – 1.99 (m, 4H, 2 C$^\beta$H$_2$ Glu), 1.90 (s, 6H, 2 CH_3COOH), 2.21 (t, J 7.7 Hz, 4H, 2 C$^\gamma$H$_2$ Glu), 2.25 – 2.45 (m, 8H, 2-CH$_2$CH$_2$-Suc), 2.74 (t, J 7.4 Hz, 4H, 2 C$^\varepsilon$H$_2$ Lys), 3.01 (m, 4H, -NHC$\underline{H}_2$(CH$_2$)$_4$C$\underline{H}_2$NH-), 4.12 (m, 4H, 2 C$^\alpha$H Lys, 2 C$^\alpha$H Glu), 7.80 (t, J 5.2 Hz, 2H, -N$\underline{H}$(CH$_2$)$_6$N$\underline{H}$-), 8.12 (d, J 8.1 Hz, 2H, 2 NH Lys), 8.21 (d, J 6.6 Hz, 2H, 2 NH Glu).

Synthesis of [HOOC$(CH_2)_2$CO-*D*-Glu(tBu)-*L*-Lys(Boc)-NH-]$_2(CH_2)_6$ (GK-2DL)

***bis*-(*N*$^\varepsilon$-Benzyloxycarbonyl-γ-*tert*-butyl-*D*-glutamyl-*N*$^\varepsilon$-*tert*-butyloxycarbonyl-*L*-lysine) hexamethylenediamide, [*Z*-*D*-Glu(tBu)-*L*-Lys(Boc)-NH-]$_2(CH_2)_6$**, (**V**) was prepared from [H-*L*-Lys(Boc)-NH-]$_2(CH_2)_6$ analogously to I in 93% yield. R_f 0.43 (EtOAc), R_f 0.77 (A), R_f 0.47 (F). mp 150 – 158°C, $[\alpha]_D^{25}$ – 7.4° (s, 1.05, MeOH). PMR spectrum: 1.21 – 1.50 (m, 20H, -NHCH$_2$(C$\underline{H}_2$)$_4$- CH$_2$NH-, 2 C$^\gamma$H$_2$C$^\gamma$H$_2$C$^\delta$H$_2$Lys), 1.36 (s, 18H, 2 -OC(CH$_3$)$_3$ Boc), 1.37 (c, 18H, 2 -OC(CH$_3$)$_3$ tBu), 1.59 – 1.84 (m, 4H, 2 C$^\beta$H$_2$ Glu), 2.20 (t, J 7.3 Hz, 4H, 2 C$^\beta$H$_2$ Glu), 2.85 (m, 4H, C$^\varepsilon$H$_2$ Lys), 3.00 (m, 4H, -NHC$\underline{H}_2$(CH$_2$)$_4$C$\underline{H}_2$NH-), 4.03 (m, 2H, 2 C$^\alpha$H Glu), 4.15 (m, 2H, 2 C$^\alpha$H Lys), 5.02 (c, 4H, 2 -OC$\underline{H}_2$C$_6$H$_5$), 6.72 (t, J 5.2 Hz, 2H, 2 N$^\varepsilon$H Lys), 7.34 (m, 10H, 2 -OCH$_2$C$_6\underline{H}_5$), 7.51 (d, J 7.1 Hz, 2H, 2 NH Glu), 7.75 (t, J 6.6 Hz, 2H, -N$\underline{H}$(CH$_2$)$_6$N$\underline{H}$-), 8.05 (d, J 8.1 Hz, 2H, 2 NH Lys).

bis-(γ-*tert*-Butyl-*D*-glutamyl-*N*$^\varepsilon$-*tert*-butyloxycarbonyl-*L*-lysine) hexamethylenediamide, [H-*D*-Glu(tBu)-*L*-Lys(Boc)-NH-]$_2(CH_2)_6$, (VI) was prepared from V analogously to **II** in quantitative yield. R_f 0.27 (A), R_f 0.84 (C), R_f 0.34 (F), R_f 0.31 (I), R_f 0.59 (J). mp 89 – 112°C, $[\alpha]_D^{24}$ – 13.8° (s, 1.1; MeOH). PMR spectrum: 1.13 – 1.37 (m, 16H, -NHCH$_2$(C$\underline{H}_2$)$_4$- CH$_2$NH-, 2 C$^\gamma$H$_2$C$^\delta$H$_2$ Lys), 1.35 (s, 18H, 2 -OC(CH$_3$)$_3$ Boc), 1.39 (c, 18H, 2 -OC(CH$_3$)$_3$ tBu), 1.40 and 1.55 (m, 4H, 2 C$^\beta$H$_2$ Lys), 1.57 – 1.80 (m, 4H, 2 C$^\beta$H$_2$ Glu), 2.22 (t, J 7.8 Hz, 4H, 2 C$^\gamma$H$_2$ Glu), 2.85 (m, 4H, C$^\varepsilon$H$_2$ Lys), 3.02 (m, 4H, -NHC$\underline{H}_2$(CH$_2$)$_4$C$\underline{H}_2$NH-), 3.17 (m, 2H, 2 C$^\alpha$H Glu), 4.16 (m, 2H, 2 C$^\alpha$H Lys), 6.72 (t, J 5.2 Hz, 2H, 2 N$^\varepsilon$H Lys), 7.89 (t, J 5.6 Hz, 2H, -N$\underline{H}$(CH$_2$)$_6$N$\underline{H}$-), 7.93 (d, J 8.8 Hz, 2H, 2 NH Lys).

bis-(*N*-Monosuccinyl-γ-*tert*-butyl-*D*-glutamyl-*N*$^\varepsilon$-*tert*-butyloxycarbonyl-*L*-lysine) hexamethylenediamide [HOOC$(CH_2)_2$CO-*D*-Glu(tBu)-*L*-Lys(Boc)-NH-]$_2(CH_2)_6$, (VII) was prepared from VI analogously to III in 90% yield. R_f 0.81 (B), R_f 0.36 (F). mp 164 – 168°C, $[\alpha]_{24}^{D}$ – 2.7° (s, 1.05 MeOH). PMR spectrum: 1.10 – 1.51 (m, 20H, -NHCH$_2$(C$\underline{H}_2$)$_4$- CH$_2$NH-, 2 C$^\beta$H$_2$C$^\gamma$H$_2$C$^\delta$H$_2$ Lys), 1.35 (s, 18H, 2 -OC(CH$_3$)$_3$ Boc), 1.37 (c, 18H, 2 -OC(CH$_3$)$_3$ tBu), 1.56 – 1.87 (m, 4H, 2 C$^\beta$H$_2$ Glu), 2.18 (m, 4H, 2 C$^\gamma$H$_2$ Glu), 2.45 (m, 8H, 2 -CH$_2$CH$_2$- Suc), 2.83 (m, 4H, 2 C$^\varepsilon$H$_2$ Lys), 3.01 (m, 4H, -NHC$\underline{H}_2$(CH$_2$)$_4$C$\underline{H}_2$NH-), 4.07 (m, 2H, 2 C$^\alpha$H Lys), 4.15 (m, 2H, 2 C$^\alpha$H Glu), 6.76 (t, J 5.4 Hz, 2H, 2 N$^\varepsilon$H Lys), 7.80 (t, J 5.1 Hz, 2H, -N$\underline{H}$(CH$_2$)$_6$N$\underline{H}$-), 8.11 (d, J 8.6 Hz, 2H, 2 NH Glu), 8.15 (d, J 8.1 Hz, 2H, 2 NH Lys).

bis-(*N*-Monosuccinyl-*D*-glutamyl-*L*-lysine) hexamethylenediamide ditriflate, 2CF_3COOH·[HOOC$(CH_2)_2$CO-*D*-Glu-*L*-Lys-NH-]$_2(CH_2)_6$, (VIII) was prepared and purified analogously to IV to afford a product that was 92% pure before purification (analytical RP HPLC, $\tau = 8.6$ min) and 98% after purification (RP HPLC) in 62% yield as gray fine crystals with mp 118 – 126°C (hygroscopic). $[\alpha]_D^{23}$ – 2.7° (s, 1.1, H_2O). PMR spectrum: 1.20 – 1.60 (m, 20H, -NHCH$_2$(C$\underline{H}_2$)$_4$CH$_2$NH-, 2 C$^\beta$H$_2$C$^\gamma$H$_2$C$^\delta$H$_2$ Lys), 1.79 (m, 4H, 2 C$^\beta$H$_2$ Glu), 2.23 (m, 4H, 2 C$^\gamma$H$_2$ Glu), 2.40 (m, 8H, 2 -CH$_2$CH$_2$- Suc), 2.73 (m, 4H, 2 C$^\varepsilon$H$_2$ Lys), 3.02 (m, 4H, -NHC$\underline{H}_2$(CH$_2$)$_4$C$\underline{H}_2$NH-), 4.14 (m, 4H, 2 C$^\alpha$H Lys, 2 C$^\alpha$H Glu), 7.71 (br.s, 6H, 2 N$^+$H$_3$ Lys), 7.80 (t, J 5.6 Hz, 2H, -N$\underline{H}$(CH$_2$)$_6$N$\underline{H}$-), 8.09 (d, J 8.2 Hz, 2H, 2 NH Lys), 8.17 (d, J 6.7 Hz, 2H, 2 NH Glu), 12.14 (br.s, 4H, 2 -COO$\underline{H}$ Suc, 2 -COO$\underline{H}$ Glu).

bis-(*N*-Monosuccinyl-*D*-glutamyl-*L*-lysine) hexamethylenediamide diacetate, 2CH_3COOH·[HOOC $(CH_2)_2$CO-*D*-Glu-*L*-Lys-NH-]$_2(CH_2)_6$, (GK-2DL) was prepared analogously to GK-2LD in overall yield (97% pure by RP HPLC) of 42%. R_f 0.19 (E). The melting point was not determined (hygroscopic). $[\alpha]_D^{23}$ – 3.1° (s, 0.32 MeOH). PMR spectrum: 1.20 – 1.58 (m, 20H, 2 -NHCH$_2$(C$\underline{H}_2$)$_4$CH$_2$NH-, 2 C$^\beta$H$_2$C$^\gamma$H$_2$C$^\delta$H$_2$ Lys), 1.60 – 1.99 (m, 4H, 2 C$^\beta$H$_2$ Glu), 1.90 (s, 6H, 2 CH_3COOH), 2.21 (t, J 7.7 Hz, 4H, 2 C$^\gamma$H$_2$ Glu), 2.25 – 2.45 (m, 8H, 2 -CH$_2$CH$_2$- Suc), 2.74 (t, J 7.4 Hz, 4H, 2 C$^\varepsilon$H$_2$ Lys), 3.01 (m, 4H, -NHC$\underline{H}_2$(CH$_2$)$_4$C$\underline{H}_2$NH-), 4.12 (m, 4H, 2 C$^\alpha$H Lys, 2 C$^\alpha$H Glu), 7.80 (t, J 5.2 Hz, 2H, -N$\underline{H}$(CH$_2$)$_6$N$\underline{H}$-), 8.12 (d, J 8.1 Hz, 2H, 2 NH Lys), 8.21 (d, J 6.6 Hz, 2H, 2 NH Glu).

EXPERIMENTAL BIOLOGICAL PART

Neuroprotective activity was studied as before [16] using a culture of immortalized HT-22 mouse hippocampal cell line. Cells were inoculated into 96-well plates with 3,500 cells per well in DMEM medium (HyClon) containing fetal bovine serum (5%, Invitrogen) and L-glutamine (2 mM, ICN) and incubated at 37°C with 5% CO_2 until a monolayer formed. Peptides at final concentrations of 10^{-5} to 10^{-8} M were added 24 h before the insult. The positive control was NGF at a concentration of 100 ng/mL in the medium.

Oxidative stress was modeled using H_2O_2 at a final concentration of 1.5 mM. Cells were incubated with H_2O_2 in 5% CO_2 at 37°C for 30 min [21]. Then, the medium was replaced by normal saline. Cell survival was assayed after 4 h using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma). Optical density was measured on a Multiscan EX spectrophotometer (Thermo) at 600 nm.

Activity in tests for counteraction to oxidative stress was calculated using the formula:

$$A(\%) = (D_{exp} - D_{H_2O_2})/(D_{contr} - D_{H_2O_2})\cdot 100\%,$$

where D_{exp} is the experimental solution optical absorption; $D_{H_2O_2}$, optical absorption of an active control solution (with H_2O_2); D_{contr}, optical absorption of a passive control (without H_2O_2).

Statistical analysis used standard Statistica 6.0 software (StatSoft Inc., USA). MTT assay results were analyzed using nonparametric statistics. Data quality was analyzed using the Mann-Whitney criterion. Results were considered statistically significant for $p \le 0.05$.

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