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SYNTHESIS AND *IN VITRO* NEUROPROTECTIVE ACTIVITY OF GLYCINE ANALOGS OF GK-2 DIMERIC DIPEPTIDE MIMETIC OF NERVE GROWTH FACTOR 4TH LOOP

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A dimeric dipeptide mimetic of nerve growth factor (NGF), *bis*-(*N*-monosuccinyl-L-glutamyl-L-lysine) hexamethylenediamide (GK-2), was previously developed at V. V. Zakusov State Institute of Pharmacology, activated specific TrkA receptors, and exhibited neuroprotective activity *in vitro* (10^{-5} – 10^{-9} M) and *in vivo* (0.1 – 10 mg/kg i.p. and p.o.). GK-2 was designed based on the beta-turn (-Asp^{94}-Glu^{95}-Lys^{96}-Gln^{97}-) of the NGF 4th loop and preserved the central dipeptide fragment (-Glu^{95}-Lys^{96}-). The Asp^{94} residue was replaced by its monosuccinyl bioisostere. The dimeric structure of NGF was reproduced using a bivalent hexamethylenediamine spacer. The structure—activity (neuroprotective) relationship for GK-2 was studied in the present work using a glycine scan, i.e., successive replacement of the peptide side groups by H. The *bis*-(*N*-acetyl-L-glutamyl-L-lysine) (GK-2Ac), *bis*-(*N*-monosuccinylglycyl-L-lysine) (GK-2-Gly1), and *bis*-(*N*-monosuccinyl-L-glutamylglycine) hexamethylenediamides (GK-2-Gly2) were less active with neuroprotective activity *in vitro* under oxidative stress for HT22 cells at concentrations 10 – 100 times greater than GK-2. The conclusion was drawn that each side radical of GK-2 was important for manifestation of the full neuroprotective activity of dimeric dipeptide GK-2, a mimetic of the NGF 4th loop. However, removal of any of the side radicals would probably not change the active structure of the beta-turn so that the two remaining side radicals should retain the ability to bind to their TrkA subsites. This could explain the retention of neuroprotective activity in the GK-2 glycine analogs.

Keywords: mimetic, dipeptide, GK-2, NGF, neuroprotective activity, glycine scan, structure—activity relationship.

The dimeric dipeptide *bis*-(*N*-monosuccinyl-L-glutamyl-L-lysine) (GK-2) (RU Pat. No. 2,410,392, 2011; US Pat. No. 9,683,014 B2, 2017; CN Pat. No. 102365294 B, 2016; EP 2397488, 2019) was previously designed at V. V. Zakusov State Institute of Pharmacology as a mimetic of the NGF 4th loop β-turn [1]. The design preserved the central dipeptide fragment of the β-turn (Glu^{95}-Lys^{96}) and replaced the preceding Asp^{94} with a bioisosteric monosuccinyl radical. GK-2 reproduced the homodimeric NGF structure and was a TrkA receptor agonist, like neurotrophin itself [1]. GK-2 in *in vitro* experiments at micro- and nanomolar concentrations exhibited neuroprotective activity typical of full-sized NGF [2]. It prevented death caused by H_2O_2 or glutamic acid of HT-22 immortalized murine hippocampal neurons and protected PC-12 rat pheochromocytoma cell line from the neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). The neuroprotective effect of GK-2 was confirmed in *in vivo* experiments using models of Alzheimer's and Parkinson's diseases and ischemic cerebral stroke in rodents [3]. In contrast with full-sized NGF, GK-2 did not cause hyperalgesia and mass loss [4]. GK-2 has now passed a full cycle of preclinical studies as a potential neuroprotective drug for treating cerebral circulatory disorders.

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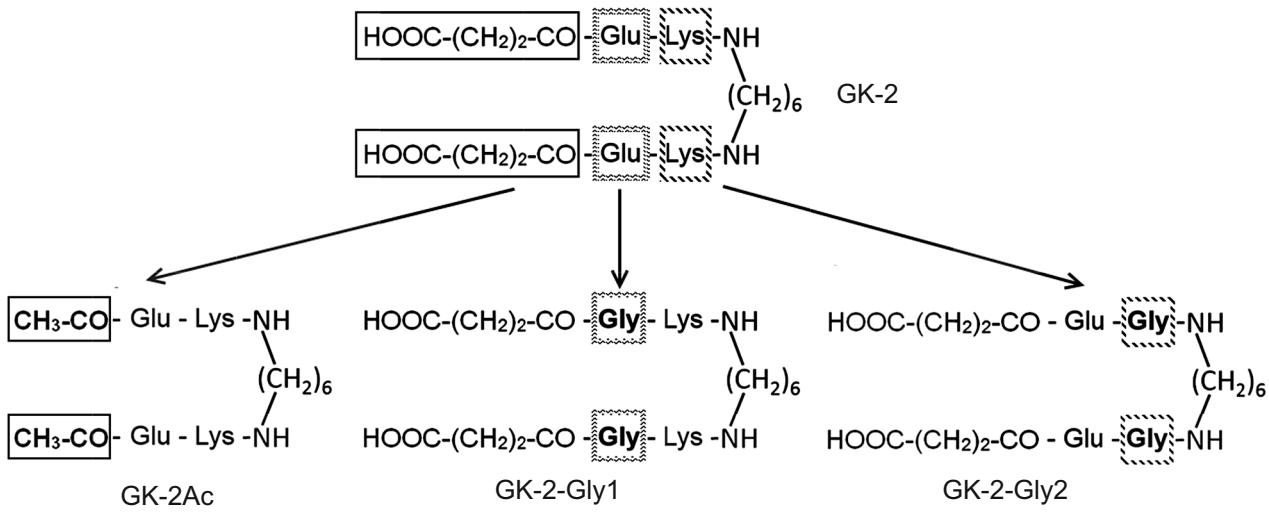


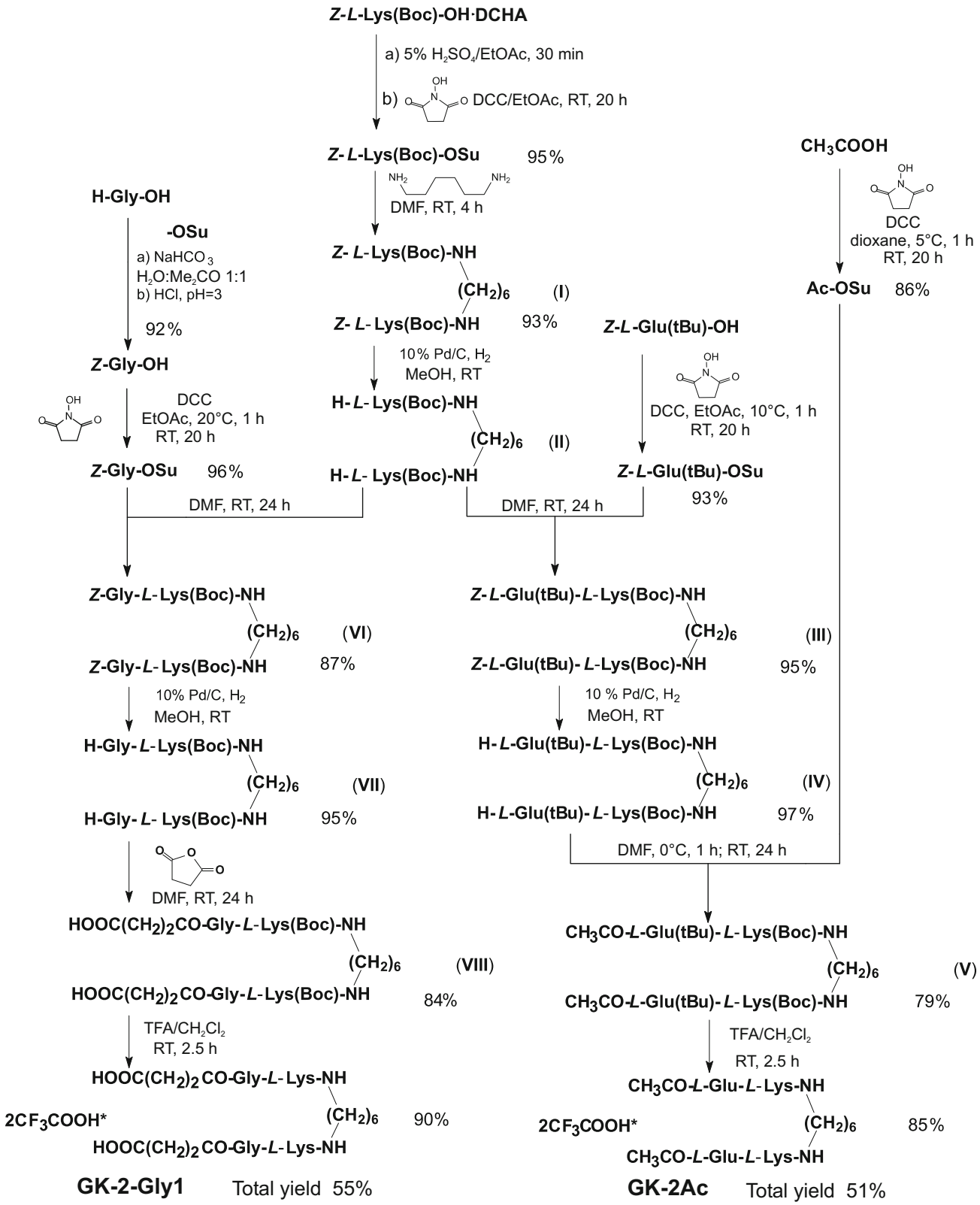
Fig. 1. Design of GK-2 glycine analogs (GK-2 glycine scan).

Two diastereomers of GK-2 were previously synthesized to study the stereospecificity of their interactions with the TrkA receptor [5]. These were *bis*-(*N*-monosuccinyl-L-glutamyl-D-lysine) (GK-2LD) and *bis*-(*N*-monosuccinyl-D-glutamyl-L-lysine) hexamethylenediamides (GK-2DL). Studies of their neuroprotective effects on HT-22 neuronal culture with oxidative stress showed that replacing L- by D-lysine decreased significantly the activity whereas replacing L- by D-glutamic acid destroyed it completely. It was concluded that the L-configuration of both dipeptide residues was important for interaction with the TrkA receptor. In continuation of this research, we studied the effects of the amino-acid side radicals on GK-2 activity. For this, a glycine scan was conducted by designing three GK-2 analogs (Fig. 1). These were *bis*-(*N*-acetyl-L-glutamyl-L-lysine) (GK-2Ac), *bis*-(*N*-monosuccinylglycyl-L-lysine) (GK-2-Gly1), and *bis*-(*N*-monosuccinyl-L-glutamylglycine) hexamethylenediamides (GK-2-Gly2). The compounds were synthesized so their neuroprotective effects on HT-22 neuronal culture could be studied *in vitro* with oxidative stress induced by H_2O_2.

Glycine-containing GK-2 analogs. The glycine GK-2 analogs were synthesized using classical wet methods for synthesizing peptides from the *C*-terminus using a Z/Boc-strategy of protecting groups and activated *N*-hydroxysuccinimide esters (Schemes 1 and 2).

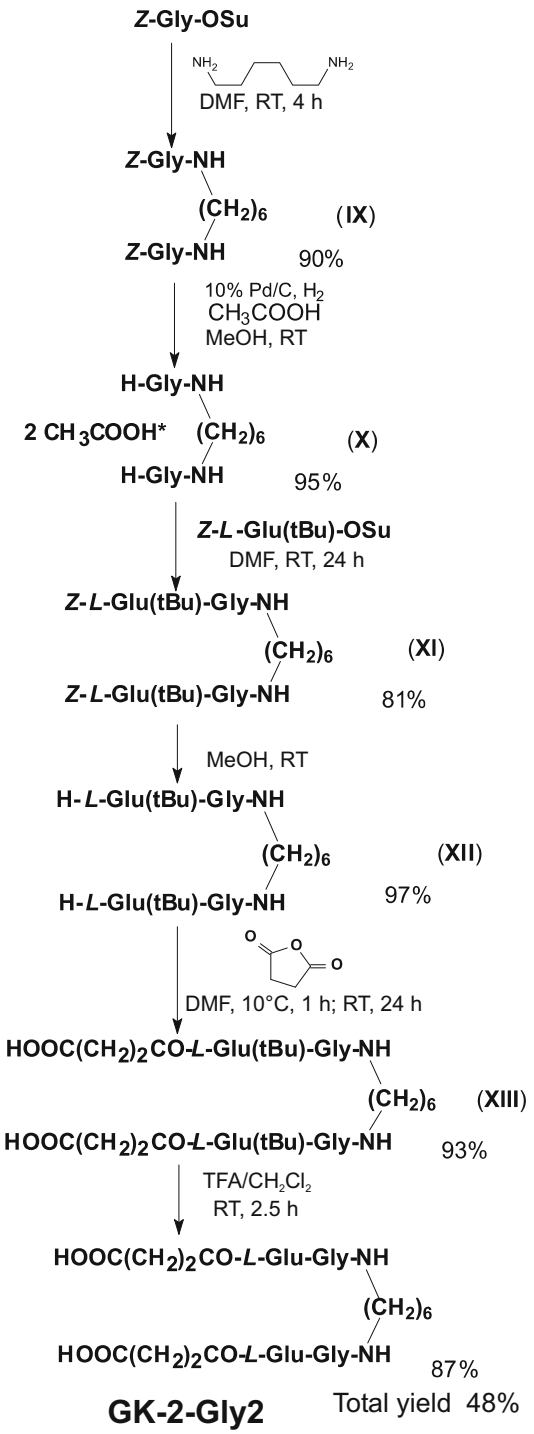
Peptides GK-2Ac, GK-2-Gly1, and GK-2-Gly2 were synthesized starting from commercially available Z-L-Lys(Boc)-OH, Z-L-Glu(tBu)-OH, and H-Gly-OH. Z-substituted glycine was prepared in 92% yield by reacting the sodium salt of glycine with benzyloxycarbonyloxysuccinimide (Z-OSu) in aqueous Me_2CO. Activated *N*-hydroxysuccinimide esters of acetic acid, Z/Boc-protected lysine, Z/tBu-protected glutamic acid, and Z-glycine were synthesized using *N*-hydroxysuccinimide and dicyclohexylcarbodiimide (DCC) at 5 – 10°C. Activated esters Z-L-Lys(Boc)-OSu and Z-Gly-OSu were condensed with hexamethylenediamine in DMF at room temperature (RT) to give in 93 and 90% yields $[Z\text{-L-Lys(Boc)-NH}]_2(CH_2)_6$ (**I**) and $(Z\text{-Gly-NH})_2(CH_2)_6$ (**IX**) that were then Z-deblocked via catalytic hydrogenolysis in the presence of Pd/C (10%). The *bis*-products $[H\text{-L-Lys(Boc)-NH}]_2(CH_2)_6$ (**II**) and $(H\text{-Gly-NH})_2(CH_2)_6$ (**X**) were condensed in DMF with Z-L-Glu(tBu)-OSu; *bis*-product **II** also with Z-Gly-OSu, to produce in 81 – 95% yields the corresponding *bis*-dipeptides $[Z\text{-L-Glu(tBu)-L-Lys(Boc)-NH}]_2(CH_2)_6$ (**III**), $[Z\text{-Gly-L-Lys(Boc)-NH}]_2(CH_2)_6$ (**VI**), and $[Z\text{-L-Glu(tBu)-Gly-NH}]_2(CH_2)_6$ (**XI**). Catalytic hydrogenolysis in the presence of Pd/C (10%) produced free α-amines on *bis*-dipeptides $[H\text{-L-Glu(tBu)-L-Lys(Boc)-NH}]_2(CH_2)_6$ (**IV**), $[H\text{-Gly-L-Lys(Boc)-NH}]_2(CH_2)_6$ (**VII**), and $[H\text{-L-Glu(tBu)-Gly-NH}]_2(CH_2)_6$ (**XII**). Reaction of **IV** with Ac-OSu in DMF gave the *N*-acetyl product $[Ac\text{-L-Glu(tBu)-L-Lys(Boc)-NH}]_2(CH_2)_6$ (**V**), which was deprotected by trifluoroacetic acid (TFA) in CH_2Cl_2 to give GK-2Ac as the trifluoroacetate in 51% total yield calculated for the starting protected lysine. *bis*-Dipeptides **VII** and **XII** were acylated by succinic anhydride in DMF in 84 and 93% yields to give *N*-monosuccinyl derivatives $[HOOC\text{-}CH_2CH_2\text{-CO-Gly-L-Lys(Boc)-NH}]_2(CH_2)_6$ (**VIII**) and $[HOOC\text{-}CH_2CH_2\text{-CO-L-Glu(tBu)-Gly-NH}]_2(CH_2)_6$ (**XIII**). Finally, removal of tBu- and Boc-protecting groups by TFA in CH_2Cl_2 produced the target products GK-2-Gly1 (as the trifluoroacetate) and GK-2-Gly2 in total yields of 55 and 48% calculated for starting protected lysine and glycine, respectively. The target peptides were homogeneous by TLC and reversed-phase HPLC. The structures and diastereomeric purity (>98%) of the obtained compounds were confirmed by PMR and ^{13}C NMR spectroscopy.

Neuroprotective activity of the synthesized peptides in the concentration range $10^{-5} - 10^{-8}$ M was studied using an



Scheme 1. Syntheses of GK-2Ac and GK-2-Gly1

H_2O_2-induced oxidative stress model and HT-22 immortalized murine hippocampal cell line [6]. Peptides were administered 24 h before the damaging agent. Table 1 presents the results.



Scheme 2. Synthesis of GK-2-Gly2.

Table 1 shows that replacing an *N*-monosuccinyl radical by *N*-acetyl (GK-2Ac) and also Glu by Gly (GK-2-Gly1) decreased by 100 times the neuroprotective activity according to the minimum active concentration (from 10^{-9} to 10^{-7} M) and by ~3 times according to the effects.

The GK-2 analog with Lys replaced by Gly (GK-2-Gly2) exhibited neuroprotective effects at concentrations down to 10^{-8} M, i.e., 10 times more active than the two previous GK-2 analogs but 10 times less than GK-2 itself according to active concentration and ~5 times by manifestation of effects.

Thus, both amino-acid residues and the *N*-terminus substituent acting as Asp^{94} were important for manifestation of neuroprotective effects by dimeric dipeptide GK-2, a mimetic of the NGF 4^{th} loop. The activity, although decreased by 10 – 100 times, was retained if any two side groups of the mimetic were preserved. This suggested that the β-turn active structure could be retained after removing one of the side radicals. Apparently, the two remaining side radicals were still capable of binding to their TrkA receptor subsites.

EXPERIMENTAL CHEMICAL PART

Commercially available enantiomerically pure L-amino acids and their derivatives (Sigma and Fluka) were used in the work. Melting points were determined in open capillaries on an Optimelt MPA100 apparatus (Stanford Research Systems, USA) and are uncorrected. PMR and ^{13}C NMR spectra were recorded in DMSO-d_6 solutions with TMS internal standard (0 ppm) on the δ-scale on a Bruker Fourier 300 HD spectrometer (300 and 75 MHz, respectively). Resonances were assigned by analyzing 1D and 2D homonuclear $^{1}H–^{1}H$ COSY and heteronuclear $^{1}H–^{13}C$ COSY spectra (HSQC and HMBC).

Specific optical rotation was recorded on an ADP 440 automated polarimeter (Bellingham+Stanley Ltd., Great Britain). TLC used DC 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 (4:1:1, E); $CHCl_3$–MeOH– HOAc (80:10:1, F); hexane–EtOAc (1:1, G); C_6H_6–MeOH (2:1, I); dioxane–H_2O (9:1, J); $CHCl_3$–MeOH (9:1, K).

Amine-containing compounds were detected by ninhydrin; amides, a chlorine-tolidine test; compounds with a free carboxylic acid, bromocresol green; aromatics, UV light.

HPLC of the dipeptides used a Wellchrom 2001 chromatography system (Knauer, Germany) with a Diasorb-110-C16 column (4.0 × 250 mm, 5 μm). The loop volume was 20 μL. Mobile phase A (0.05% aqueous TFA) and mobile phase B (0.05% TFA in MeCN) were used in gradient mode (0 – 100% B over 30 min). The flow rate was 0.6 mL/min. Detection was made at 220 nm. Analyses were made at room temperature.

Solvent purification. DMA was purified by distillation from ninhydrin. Et_2O was stored over solid NaOH. EtOAc, CH_2Cl_2, $CHCl_3$, C_6H_6, Me_2CO, hexane, MeOH, and EtOH (all chemically pure) were used without further purification.

Synthesis of starting compounds

***N*-Benzyloxycarbonylglycine (Z-Gly-OH)** was prepared as before [7] in 92% yield from H-Gly-OH (10.0 g, 0.13 mol). R_f 0.15 (EtOAc), R_f 0.71 (C); mp 116 – 120°C. Lit. [8] mp 120°C.

TABLE 1. Effect of NGF Mimetic GK-2 and Its Analogs GK-2Ac, GK-2-Gly1, and GK-2-Gly2 on Neuron Viability Under Oxidative Stress. Peptides Were Administered 24 h Before H_2O_2

Group	Concentration, M	MTT assay, D_{600}, mean ± RSD	Activity A, %
Control#	0	0.427 ± 0.071	100
H_2O_2#	1.5×10^{-3}	0.350 ± 0.032	0
NGF#	$\sim 10^{-9}$	0.425 ± 0.047*	97*
GK-2#	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
Control	0	0.184 ± 0.005	100
H_2O_2	1.5×10^{-3}	0.096 ± 0.011^	0
GK-2Ac	10^{-5}	0.125 ± 0.009***	33 ± 10***
	10^{-6}	0.124 ± 0.007***	31 ± 8***
	10^{-7}	0.118 ± 0.004*	25 ± 4*
	10^{-8}	0.115 ± 0.008	23 ± 8
Control	0	0.200 ± 0.014	100
H_2O_2	1.5×10^{-3}	0.118 ± 0.010^	0
GK-2-Gly1	10^{-5}	0.139 ± 0.015*	24 ± 19*
	10^{-6}	0.135 ± 0.012*	22 ± 15*
	10^{-7}	0.136 ± 0.005*	22 ± 5*
	10^{-8}	0.134 ± 0.014	20 ± 17
Control	0	0.217 ± 0.009	100
H_2O_2	1.5×10^{-3}	0.087 ± 0.004^	0
GK-2-Gly2	10^{-5}	0.104 ± 0.006*	13 ± 5*
	10^{-6}	0.107 ± 0.005***	15 ± 3***
	10^{-7}	0.106 ± 0.007**	15 ± 5**
	10^{-8}	0.104 ± 0.007*	13 ± 5*

Compounds administered 24 h before damage. Statistical significance of differences: ^, from control ($p \le 0.001$); *from H_2O_2 (*$p \le 0.05$; **$p \le 0.01$; ***$p \le 0.001$) Kruskal–Wallis criterion followed by Dunn test. $n = 12$ (GK-2); $n = 11$ (GK-2Ac); $n = 12$ (GK-2-Gly1); $n = 10$ (GK-2-Gly2). Activity was calculated using the formula: $A(\%) = \frac{(D_{exp} - D_{H_2O_2})}{(D_{contr} - D_{H_2O_2})} \cdot 100\%$, where D_{exp} is the experimental solution optical absorption; $D_{H_2O_2}$, optical absorption of active control (with H_2O_2); D_{contr}, optical absorption of passive control (without H_2O_2). #Literature data [1].

***N*-Benzyloxycarbonylglycine *N*-hydroxysuccinimide (Z-Gly-OSu)** was prepared as before [9] in 96% yield from Z-Gly-OH (50.0 g, 0.24 mol) in EtOAc. R_f 0.83 (EtOAc), R_f 0.43 (G); mp 108 – 110°C. PMR spectrum (DMSO-d_6), δ, ppm: 2.81 (s, 4H, -CH_2CH_2- -OSu), 4.21 (d, J 7.9 Hz, 2H, CH_2 Gly), 5.08 (s, 2H, -OCH_2- *Z*), 7.36 (m, 5H, C_6H_5 Z), 7.96 (t, J 7.9 Hz, 1H, NH Gly). Lit. [9]: mp 111 – 115°C.

***N*-Hydroxysuccinimide ester of acetic acid (Ac-OSu)** was prepared by the literature method [10] in 86% yield from HOAc (12.0 g, 0.2 mol). R_f 0.85 (A), R_f 0.56 (EtOAc), R_f 0.70 (J); mp 130 – 134°C. PMR spectrum (DMSO-d_6), δ, ppm: 2.34 (s, 3H, CH_3CO-), 2.80 (m, 4H, -CH_2CH_2- OSu). Lit. [10]: mp 130°C.

Z-L-Glu(tBu)-OSu and **Z-L-Lys(Boc)-OSu** activated esters and **[Z-L-Lys(Boc)-NH]$_2$(CH_2)$_6$ (I)**; **[H-L-Lys(Boc)-NH]$_2$(CH_2)$_6$ (II)**; **[Z-L-Glu(tBu)-L-Lys(Boc)-NH]$_2$(CH_2)$_6$ (III)**, and **[H-L-Glu(tBu)-L-Lys(Boc)-NH]$_2$(CH_2)$_6$ (IV)** were previously prepared by us according to the literature [11].

Synthesis of [CH_3CO-L-Glu-L-Lys-NH]$_2$(CH_2)$_6$, GK-2Ac

***bis*-(*N*-Acetyl-(-*tert*-butyl-L-glutamyl-N^{ε}-*tert*-butyloxycarbonyl-L-lysine) hexamethylenediamide, [CH_3CO-L-Glu(tBu)-L-Lys(Boc)-NH]$_2$(CH_2)$_6$ (V).** A solution of **IV** (6.0 g, 6.36 mmol) in DMF (40 mL) at RT was treated with Ac-OSu (2.2 g, 14 mmol) and stirred at RT. When the reaction was finished (TLC monitoring), the DMF was evaporated. The still fluid residue was treated with Me_2CO (50 mL) and stored at RT for 2 h. The resulting precipitate was filtered off and rinsed with Me_2CO (2 × 25 mL). The white crystals were dried under vacuum (15 mm Hg) in a desiccator over $CaCl_2$ to afford **V** (5.2 g, 79%). R_f (main spot) 0.60 (A), R_f 0.83 (E), R_f 0.81 (J); mp. 194 – 198°C; $[\alpha]_D^{27}$ – 21.8° (s, 1.04; MeOH). PMR spectrum (DMSO-d_6), δ, ppm: 1.15 – 1.48 (m, 16H, 2 $C^{\gamma}H_2C^{\delta}H_2$ Lys, -$NHCH_2(C\underline{H}_2)_4CH_2NH$-), 1.36 (s, 18H, 2-$OC(CH_3)_3$ Boc), 1.38 (s, 18H, 2-$OC(CH_3)_3$ tBu), 1.48 and 1.59 (two m, 4H, 2 $C^{\beta}H_2$ Lys), 1.67 and 1.86 (two m, 4H, 2 $C^{\beta}H_2$ Glu), 1.84 (s, 6H, CH_3-CO), 2.20 (t, J 8.0 Hz, 4H, 2 $C^{\gamma}H_2$ Glu), 2.86 (m, 4H, 2 $C^{\varepsilon}H_2$ Lys), 3.01 (m, 4H, -$NHC\underline{H}_2(CH_2)_4C\underline{H}_2NH$-), 4.09 – 4.16 (m, 2H, 2 $C^{\alpha}H$ Lys), 4.18 – 4.25 (m, 2H, 2 $C^{\alpha}H$ Glu), 6.75 (t, J 5.5 Hz, 2H, 2 NH Lys), 7.78 (t, J 5.5 Hz, 2H, -$N\underline{H}(CH_2)_6N\underline{H}$-), 7.85 (d, J 7.9 Hz, 2H, 2 NH Lys), 8.03 (d, J 7.7 Hz, 2H, 2 NH Glu).

***bis*-(*N*-Acetyl-L-glutamyl-L-lysine) hexamethylenediamide ditrifluoroacetate, {[CH_3CO-L-Glu-L-Lys-NH]$_2$(CH_2)$_6$·2CF_3COOH}, GK-2Ac.** A suspension of **I** (1.00 g, 0.97 mmol) in CH_2Cl_2 (15 mL) was treated with TFA (5 mL) and stirred at RT. When the starting compound disappeared (TLC monitoring, ~2 h), the mixture was evaporated. The still fluid residue was triturated with Et_2O and decanted (3 × 20 mL). The precipitate was filtered off using an apparatus for filtering hygroscopic compounds to afford **GK-2Ac** (0.79 g, 85%) as a white crystalline compound. R_f 0.41 (K); τ = 9.0 min; mp 124 – 143°C (dec., did not have a

sharp melting point, hydroscopic); $[\alpha]_D^{25}$ – 28.8° (s, 1; MeOH). PMR spectrum (DMSO-d_6), δ, ppm: 1.23, 1.37, 1.51 and 1.68 (four m, 20H, 2 $C^{\beta}H_2C^{\gamma}H_2C^{\delta}H_2$ Lys, -NHCH_2(C$\underline{H}_2$)$_4$CH_2NH-), 1.71 – 1.93 (m, 4H, 2 $C^{\beta}H_2$ Glu), 1.85 (s, 6H, 2 CH_3CO-), 2.24 (t, J 2.26 Hz, 4H, 2 $C^{\gamma}H_2$ Glu), 2.75 (m, 4H, 2 $C^{\varepsilon}H_2$ Lys), 3.02 (m, 4H, -NHC$\underline{H}_2$(CH$_2$)$_4$C$\underline{H}_2$NH-), 4.12 – 4.23 (m, 4H, 2 C^{α}H Lys, 2 C^{α}H Glu), 7.76 (6H, br.s, 2 $N^{+}H_3$ Lys), 7.78 (t, 2H, -N$\underline{H}$(CH$_2$)$_6$N$\underline{H}$-), 7.92 (d, J 8.0 Hz, 2H, 2 NH Lys), 8.07 (d, J 8.0 Hz, 2H, 2 NH Glu).

Synthesis of [HOOC(CH$_2$)$_2$CO-Gly-L-Lys-NH]$_2$(CH$_2$)$_6$, GK-2-Gly1

***bis*-(*N*$^{\alpha}$-Benzyloxycarbonylglycyl-*N*$^{\varepsilon}$-*tert*-butyloxycarbonyl-L-lysine) hexamethylenediamide, [Z-Gly-L-Lys(Boc)-NH]$_2$(CH$_2$)$_6$ (VI).** A solution of **II** (10.00 g, 17.46 mmol) in DMF (40 mL) was treated with a solution of Z-Gly-OSu (12.83 g, 41.9 mmol) in DMF (45 mL), stirred at RT for 12 h, treated with *N*,*N*-dimethyl-1-aminopropane (DMAPA, 0.87 mL), stirred for 30 min, and poured slowly into distilled H_2O (800 mL) to produce a precipitate. The mixture was left overnight. The well-formed precipitate was filtered off; rinsed successively with distilled H_2O to pH 7, hexane (100 mL), and Et_2O (50 mL); and dried in a vacuum (15 mm Hg) desiccator over $CaCl_2$ to afford chromatographically homogeneous **VI** (15.11 g, 87%) as white crystals with a light-gray tint. R_f 0.63 (A), R_f 0.74 (F); mp 111 – 117°C (dec); $[\alpha]_D^{27}$ – 14.8° (s, 1; MeOH). PMR spectrum (DMSO-d_6), δ, ppm: 1.22 – 1.36 (m, 16H, 2 $C^{\gamma}H_2C^{\delta}H_2$ Lys, -NHCH_2(C$\underline{H}_2$)$_4$CH_2NH-), 1.36 (s, 18H, 2 -OC(CH_3)$_3$ Boc), 1.48 and 1.59 (two m, 4H, 2 $C^{\beta}H_2$ Lys), 2.86 (m, 4H, 2 $C^{\varepsilon}H_2$ Lys), 3.01 (m, 4H, -NHC$\underline{H}_2$(CH$_2$)$_4$C$\underline{H}_2$NH-), 3.65 (d, J 6.0 Hz, 2H, 2 CH_2 Gly), 4.14 – 4.21 (m, 2H, 2 C^{α}H Lys), 5.03 (s, 4H, 2 -OCH_2- *Z*), 6.74 (t, J 5.2 Hz, 2H, 2 N^{ε}H Lys), 7.35 (m, 10H, 2 -C_6H_5 *Z*), 7.46 (t, J 6.0 Hz, 2H, 2 NH Gly), 7.87 (t, J 5.3 Hz, 2H, -N$\underline{H}$(CH$_2$)$_6$N$\underline{H}$-), 7.91 (d, J 8.2 Hz, 2H, 2 NH Lys).

***bis*-(Glycyl-*N*$^{\varepsilon}$-*tert*-butyloxycarbonyl-L-lysine) hexamethylenediamide, [H-Gly-L-Lys(Boc)-NH]$_2$(CH$_2$)$_6$ (VII).** A solution of **VI** (7.00 g, 0.0078 mol) in MeOH (190 mL) was treated with Pd/C (10%, 0.80 g, 50% moisture) and stirred under a H_2 atmosphere at RT. When the starting compound disappeared (TLC monitoring), the catalyst was filtered off and rinsed with MeOH (100 mL). The MeOH solution was evaporated. The residue was treated with Et_2O (60 mL) and left overnight in a refrigerator. The Et_2O was decanted. The residue was dried under vacuum (15 mm Hg, 40°C, ~1 h) to afford **VII** (4.79 g, 95%) as a white amorphous compound. R_f (main spot) 0.69 (C), R_f 0.21 (B); mp 71 – 79°C; $[\alpha]_D^{24}$ – 10.4° (s, 1; MeOH). PMR spectrum (DMSO-d_6), δ, ppm: 1.15 – 1.36 (m, 16H, 2 $C^{\gamma}H_2C^{\delta}H_2$ Lys, -NHCH_2(C$\underline{H}_2$)$_4$CH_2NH-), 1.36 (s, 18H, 2 -OC(CH_3)$_3$ Boc), 1.49 and 1.58 (two m, 4H, 2 $C^{\beta}H_2$ Lys), 2.31 (br.s. 2H, NH_2 Gly), 2.86 (m, 4H, 2 $C^{\varepsilon}H_2$ Lys), 3.02 (m, 4H, -NHC$\underline{H}_2$(CH$_2$)$_4$C$\underline{H}_2$NH-), 3.09 (br.s, 4H, 2 CH_2 Gly), 4.21 (m, 2H, 2 C^{α}H Lys), 6.74 (t, J 5.0 Hz, 2H, 2 N^{α}H Lys), 7.92 (d, 2H, 2 NH Lys), 7.95 (t, 2H, -N$\underline{H}$(CH$_2$)$_6$N$\underline{H}$-).

***bis*-(*N*-Monosuccinylglycyl-*N*$^{\varepsilon}$-*tert*-butyloxycarbonyl-L-lysine) hexamethylenediamide, [HOOC(CH$_2$)$_2$CO-Gly-L-Lys(Boc)-NH]$_2$(CH$_2$)$_6$ (VIII).** A solution of **VII** (4.26 g, 6.2 mmol) in DMF (60 mL) at 0°C was stirred, treated at once with succinic anhydride (1.40 g, 14 mmol), and stirred with cooling for 30 min and at RT for 12 h. When the reaction was finished (TLC monitoring), the DMF was evaporated. The residue was treated with MeCN (100 mL) and left for 40 min. The well-formed precipitate was filtered off and rinsed with MeCN (2 × 25 mL) and Et_2O (25 mL). The resulting white powder was dried in a vacuum (15 mm Hg) desiccator over $CaCl_2$ to afford **VIII** (4.40 g, 84%). R_f (main sport) 0.71 (B), R_f 0.64 (E); mp 131 – 149°C (dec); $[\alpha]_D^{24}$ – 10.7° (c, 1.05; MeOH). PMR spectrum (DMSO-d_6), δ, ppm: 1.15 – 1.42 (m, 16H, 2 $C^{\gamma}H_2C^{\delta}H_2$ Lys, -NHCH_2(C$\underline{H}_2$)$_4$CH_2NH-), 1.36 (s, 18H, 2 -OC(CH_3)$_3$ Boc), 1.48 and 1.61 (two m, 4H, 2 $C^{\beta}H_2$ Lys), 2.37 – 2.43 (m, 8H, 2 -CH_2CH_2- Suc), 2.86 (m, 4H, 2 $C^{\varepsilon}H_2$ Lys), 3.01 (m, 4H, -NHC$\underline{H}_2$(CH$_2$)$_4$C$\underline{H}_2$NH-), 3.70 (d, 4H, 2 CH_2 Gly), 4.10 – 4.17 (m, 2H, 2 C^{α}H Lys), 6.75 (t, J 5.4 Hz, 2H, 2 N^{ε}H Lys), 7.79 (t, J 5.4 Hz, 2H, -N$\underline{H}$(CH$_2$)$_6$N$\underline{H}$-), 7.85 (d, J 8.2 Hz, 2H, 2 NH Lys), 8.17 (t, J 5.7 Hz, 2H, 2 NH Gly), 12.11 (br.s, 2H, 2 -COOH).

***bis*-(*N*-Monosuccinylglycyl-L-lysine) hexamethylenediamide ditrifluoroacetate, [HOOC(CH$_2$)$_2$CO-Gly-L-Lys-NH]$_2$(CH$_2$)$_6$·2CF$_3$COOH, GK-2-Gly1.** A suspension of **VIII** (1.00 g, 1.1 mmol) in CH_2Cl_2 (15 mL) was treated with TFA (5 mL) and stirred at RT. When the starting material disappeared (TLC monitoring, ~1 h), the mixture was evaporated. The still fluid residue was triturated with Et_2O and decanted (3 × 20 mL). The precipitate was filtered using a head for filtering hygroscopic compounds to afford **GK-2-Gly1** (0.79 g, 90%) as a white crystalline compound. R_f (main spot) 0.21 (C); τ = 9.1 min, mp 141 – 151°C (dec, did not have a sharp melting point, hygroscopic); $[\alpha]_D^{26}$ – 12.0° (c, 1.1; MeOH). PMR spectrum (DMSO-d_6), δ, ppm: 1.23, 1.38, 1.51 and 1.66 (four m, 20H, 2 $C^{\beta}H_2C^{\gamma}H_2C^{\delta}H_2$ Lys, -NHCH_2(C$\underline{H}_2$)$_4$CH_2NH-), 2.44 (m, 8H, 2 -CH_2CH_2- Suc), 2.75 (m, 4H, 2 $C^{\varepsilon}H_2$ Lys), 3.02 (m, 4H, -NHCH_2(C$\underline{H}_2$)$_4$CH_2NH-), 3.70 (d, J 5.7 Hz, 4H, 2 CH_2 Gly), 4.13 – 4.19 (m, 2H, 2 C^{α}H Lys), 7.71 (br.s, 6H, 2 $N^{+}H_3$ Lys), 7.80 (t, J 5.5 Hz, 2H, -N$\underline{H}$(CH$_2$)$_6$N$\underline{H}$-), 7.87 (d, J 8.0 Hz, 2H, 2 NH Lys), 8.20 (t, J 5.7 Hz, 2H, 2 NH Gly).

Synthesis of [HOOC(CH$_2$)$_2$CO-L-Glu-Gly-NH]$_2$(CH$_2$)$_6$, GK-2-Gly2

***bis*-(*N*-Benzyloxycarbonylglycine) hexamethylenediamide, (Z-Gly-NH)$_2$(CH$_2$)$_6$ (IX).** A solution of Z-Gly-OSu (10.00 g, 32.6 mmol) in DMF (45 mL) was treated with a solution of hexamethylenediamine (1.60 g, 13.6 mmol) in DMF (10 mL), stirred at RT for 4 h, left overnight without

stirring, stirred, treated slowly with distilled H_2O (850 mL) heated beforehand to 43 – 45°C, stirred until the temperature reached 20 – 22°C by cooling naturally, and left for 12 h at 12 – 17°C. The well-formed precipitate was filtered off; rinsed with H_2O (200 mL) to pH ~7, hexane (40 mL), and Et_2O (40 mL); and dried in air to afford chromatographically homogeneous **IX** (7.36 g, 90%) as white crystals. R_f 0.56 (A), R_f 0.32 (K); mp 175 – 177°C. PMR spectrum (DMSO-d_6), δ, ppm: 1.23 (m, 4H, -NH$(CH_2)_2(C\underline{H}_2)_2(CH_2)_2$NH-), 1.37 (m, 4H, NHCH$_2C\underline{H}_2(CH_2)_2C\underline{H}_2CH_2$NH), 3.04 (m, 4H, -NHC$\underline{H}_2(CH_2)_4C\underline{H}_2$NH-), 3.57 (d, J 7.4 Hz, 4H, 2 CH_2 Gly), 5.02 (c, 4H, 2 -OCH_2- *Z*), 7.35 (s, 10H, 2 C_6H_5 *Z*), 7.41 (t, J 7.4 Hz, 2H, 2 NH Gly), 7.80 (t, J 7.8 Hz, 2H, -N$\underline{H}(CH_2)_6N\underline{H}$-).

***bis*-Glycine hexamethylenediamide diacetate, [H-Gly-NH]$_2$(CH$_2$)$_6$·2CH$_3$COOH (X).** A suspension of **IX** (7.00 g, 14 mmol) in MeOH (250 mL) was treated with Pd/C (10%, 0.79 g, 50% moisture) and HOAc (50%, 20 mL) and stirred under an H_2 atmosphere at RT. When the starting compound disappeared (TLC monitoring), the catalyst was filtered off and rinsed with MeOH (100 mL). The MeOH solution was evaporated and re-evaporated with toluene (3 × 50 mL). The oily residue was triturated with anhydrous Et_2O (50 mL) and decanted twice. Then Et_2O (50 mL) was added and the mixture was left for 2 h to form a precipitate, decanted, and dried in a rotary evaporator at 45°C for 30 min under vacuum (15 mm Hg, water aspirator) and then under high vacuum (diaphragm pump, 9 mm Hg, 1 h) to afford **X** (4.65 g, 95%) as a white crystalline compound. R_f 0.25 (C); mp 151 – 153°C. PMR spectrum (DMSO-d_6), δ, ppm: 1.25 (m, 4H, -NH$(CH_2)_2(C\underline{H}_2)_2(CH_2)_2$NH-), 1.39 (m, 4H, NHCH$_2C\underline{H}_2(CH_2)_2C\underline{H}_2CH_2$NH), 1.83 (s, 6H, 2 CH_3CO), 3.07 (m, 4H, -NHC$\underline{H}_2(CH_2)_4C\underline{H}_2$NH-), 3.22 (br.s, 4H, 2 CH_2 Gly), 7.09 (br.s, 6H, 2 N^+H_3 Gly), 8.06 (br.t, 2H, -N$\underline{H}(CH_2)_6N\underline{H}$-).

***bis*-(*N*-Benzyloxycarbonyl-(-*tert*-butyl-L-glutamylglycine) hexamethylenediamide, [Z-L-Glu(tBu)-Gly-NH]$_2$(CH$_2$)$_6$ (XI).** A solution of **X** (4.30 g, 104 mmol) in DMF (30 mL) at RT was stirred, treated with *N*-ethylmorpholine (3.9 mL, 24.5 mmol), and stirred for 20 min. The suspension was treated with a solution of Z-L-Glu(tBu)-OSu (12.45 g, 28.6 mmol) in DMF (50 mL), stirred for 20 h (during which the suspension turned to a solution), treated with DMAPA (0.5 mL, 4 mmol), stirred for 40 min, and poured into H_2O (400 mL). The oily product that separated was extracted with EtOAc (350 mL). The EtOAc extract was washed with H_2O (2 × 100 mL) and saturated NaCl solution (100 mL) and dried over anhydrous Na_2SO_4. The desiccant was filtered off and rinsed with EtOAc (30 mL). The EtOAc solution was evaporated. The residue was treated with a mixture (50 mL) of petroleum ether and Et_2O (3:1) and left for 3 h. The precipitate was filtered off, rinsed with petroleum ether (30 mL), and dried in air to afford **XI** (8.63 g, 81%) as a white crystalline compound. R_f 0.69 (I), R_f 0.75 (F); mp 103 – 106°C; $[\alpha]_D^{27}$ – 2.4° (s, 1.03; MeOH). PMR spectrum (DMSO-d_6), δ, ppm: 1.23 (m, 4H, -NH$(CH_2)_2(C\underline{H}_2)_2(CH_2)_2$NH-), 1.35 (s, 4H, -NHCH$_2C\underline{H}_2(CH_2)_2C\underline{H}_2CH_2$NH-), 1.38 (s, 18H, 2 -OC$(CH_3)_3$ tBu), 1.74 and 1.87 (two m, 4H, 2 C$^\beta H_2$ Glu), 2.25 (t, 4H, 2 C$^\gamma H_2$ Glu), 3.03 (m, 4H, -NHC$\underline{H}_2(CH_2)_4C\underline{H}_2$NH-), 3.65 (d, J 8.1 Hz, 4H, 2 CH_2 Gly), 3.99 (m, 2H, C$^\alpha$H Glu), 5.03 (s, 4H, 2 -OCH_2-*Z*), 7.35 (s, 10H, 2 C_6H_5 *Z*), 7.54 (d, J 7.6 Hz, 2H, 2 NH Glu), 7.65 (t, J 7.8 Hz, 2H, -N$\underline{H}(CH_2)_6N\underline{H}$-), 8.16 (t, J 8.1 Hz, 2H, 2 NH Gly).

***bis*-((-*tert*-Butyl-L-glutamylglycine) hexamethylenediamide, [H-L-Glu(tBu)-Gly-NH]$_2$(CH$_2$)$_6$ (XII).** A suspension of **XI** (8.63 g, 9.1 mmol) in MeOH (210 mL) was treated with Pd/C (10%, 0.90 g, 50% moisture) and stirred at RT under an H_2 atmosphere. When the starting compound disappeared (TLC monitoring), the catalyst was filtered off and rinsed with MeOH (70 mL). The solvent was removed under vacuum. The residue was re-evaporated with C_6H_6 (30 mL). The resulting oil was dried under vacuum (9 mm Hg, 40°C, ~1 h) to afford **XII** (5.78 g, 97%) as an amorphous white powder with a beige tint. R_f (main spot) 0.40 (B), R_f 0.71 (C), R_f 0.21 (E); mp (not determined, hygroscopic); $[\alpha]_D^{28}$ + 7.2° (s, 1.02; MeOH). PMR spectrum (DMSO-d_6), δ, ppm: 1.24 (m, 4H, -NH$(CH_2)_2(C\underline{H}_2)_2(CH_2)_2$NH-), 1.36 (s, 4H, -NHCH$_2C\underline{H}_2(CH_2)_2C\underline{H}_2CH_2$NH-), 1.39 (s, 18H, 2 -OC$(CH_3)_3$ tBu), 1.57 and 1.81 (two m, 4H, 2 C$^\beta H_2$ Glu), 1.91 (br.s, 4H, 2 NH_2 Glu), 2.26 (t, 4H, 2 C$^\gamma H_2$ Glu), 3.04 (m, 4H, -NHC$\underline{H}_2(CH_2)_4C\underline{H}_2$NH-), 3.15 (m, 2H, 2 C$^\alpha$H Glu), 3.66 (br.s, 4H, 2 CH_2 Gly), 7.77 (t, J 7.8 Hz, 2H, -N$\underline{H}(CH_2)_6N\underline{H}$-), 8.08 (br.t, 2H, 2 NH Gly).

***bis*-(*N*-Monosuccinyl-(-*tert*-butyl-L-glutamylglycine)-hexamethylenediamide, [HOOC(CH$_2$)$_2$CO-L-Glu(tBu)-Gly-NH]$_2$(CH$_2$)$_6$ (XIII).** A solution of **XII** (5.64 g, 9.4 mmol) in DMF (20 mL) at 10°C was stirred, treated with succinic anhydride (2.06 g, 21 mmol, 20% excess), stirred for 30 min, warmed to RT, and stirred for another 12 h. When the reaction was finished (TLC monitoring), the solvent was evaporated (50°C). The resulting light-yellow oil was triturated with Et_2O (80 mL) and decanted. The precipitate on a filter was rinsed with Et_2O (2 × 25 mL) and MeCN (50 mL) and dried in a vacuum (15 mm Hg) desiccator over $CaCl_2$ to afford **XIII** (7.03 g, 93%) as a finely crystalline light-cream-colored powder. R_f (main spot) 0.88 (C), R_f 0.73 (B), R_f 0.69 (J); mp 170 – 176°C (dec); $[\alpha]_D^{26}$ – 2.8° (s 1; MeOH). PMR spectrum (DMSO-d_6), δ, ppm: 1.23 (m, 4H, -NH$(CH_2)_2(C\underline{H}_2)_2(CH_2)_2$ NH-), 1.36 (s, 4H, -NHCH$_2C\underline{H}_2(CH_2)_2C\underline{H}_2CH_2$NH-), 1.39 (s, 18H, 2 -OC$(CH_3)_3$ tBu), 1.73 and 1.87 (two m, 4H, 2 C$^\beta H_2$ Glu), 2.24(t, 4H, 2 C$^\gamma H_2$ Glu), 2.40 – 2.45 (m, 8H, 2 -CH_2CH_2-Suc), 3.03 (m, 4H, -NHC$\underline{H}_2(CH_2)_4C\underline{H}_2$NH-), 3.62 (d, 4H, 2 CH_2 Gly), 4.09 – 4.17 (m, 2H, 2 C$^\alpha$H Glu), 7.60 (t, J 7.75 Hz, 2H, -N$\underline{H}(CH_2)_6N\underline{H}$-), 8.10 (t, 2H, 2 NH Gly), 8.17 (d, 2H, 2 NH Glu), 12.11 (br.s, 2H, 2 -COOH).

***bis*-(*N*-Monosuccinyl-L-glutamylglycine) hexamethylenediamide, [HOOC(CH$_2$)$_2$CO-L-Glu-Gly-NH]$_2$(CH$_2$)$_6$,**

GK-2-Gly2. A suspension of **XIII** (2.0 g, 2.49 mmol) in CH_2Cl_2 (45 mL) at RT was treated with TFA (15 mL), stirred for 2 h, evaporated, and re-evaporated with CH_2Cl_2 (2 × 15 mL). The residue was triturated with anhydrous Et_2O and decanted (2 × 20 mL) and left under Et_2O (20 mL) for 2 h to form a precipitate that was filtered off and dried under vacuum (15 mm Hg) over $CaCl_2$ to afford **GK-2-Gly2** (1.5 g, 87%) as white crystals with a light-cream tint. Traces of TFA were removed by lyophilization (3×). R_f 0.49 (C), R_f 0.42 (E); τ = 9.8 min; mp 85 – 90°C (lyophilizate); –17.1° (*c*, 1; H_2O).

PMR spectrum (DMSO-d_6), δ, ppm: 1.23 (m, 4H, -NH$(CH_2)_2$($C\underline{H}_2$)$_2$$(CH_2)_2$NH-), 1.36 – 1.38 (m, 4H, -NHC$H_2$$C\underline{H}_2$$(CH_2)_2$$C\underline{H}_2$$CH_2$NH-), 1.75 and 1.89 (two m, 4H, 2 $C^{\beta}H_2$ Glu), 2.26 (t, 4H, 2 $C^{\gamma}H_2$ Glu), 2.40 – 2.45 (m, 8H, 2 -CH_2CH_2- Suc), 3.03 (m, 4H, -NHC$\underline{H}_2$$(CH_2)_4$$C\underline{H}_2$NH-), 3.63 (d, 4H, 2 CH_2 Gly), 4.09 – 4.17 (m, 2H, 2 C^{α}H Glu), 7.60 (t, J 7.75 Hz, 2H, -N$\underline{H}$$(CH_2)_6N\underline{H}$-), 8.10 (t, 2H, 2 NH Gly), 8.16 (d, 2H, 2 NH Glu).

^{13}C NMR spectrum (DMSO-d_6), δ, ppm: 174.40 and 174.38 (two s, 4C, 4 COOH, Suc, Glu), 172.2 and 172.0 (two s, 4C, 4 CO, Glu, Gly), 168.8 (s, 2C, 2 CO Gly), 52.97 (s, 2C, 2 C^{α} Glu), 42.54 (s, 2C, 2 C^{α} Gly), 39.99 (s, 2C, 2 C1 of spacer), 30.59 (c, 2C, C^{γ} Glu), 30.36 and 29.54 (two s, 4 C Suc), 29.44 (c, 2C, C^2 of spacer), 27.35 (c, 2C, 2 C^{β} Glu), 26.53 (c, 2C, 2 C^3 of spacer). Characteristic resonances (quartets) of CF_3 and CO groups of TFA were not observed in the ^{13}C NMR spectrum.

EXPERIMENTAL BIOLOGICAL PART

Neuroprotective activity was studied according to the literature [6] using HT-22 immortalized murine hippocampal cell line. Cells in DMEM medium (HyClon) were inoculated into 96-well plates at 3500 cells/well containing fetal bovine serum (5%, Invitrogen) and L-glutamine (2 mM; ICN) and incubated at 37°C in 5% CO_2 until a monolayer formed. Peptides at final concentrations from 10^{-5} to 10^{-8} M were added 24 h before the damaging effect.

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 [12]. Then, the medium was changed to the normal one. Cell viability was determined 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.

Activities in tests for counteraction to oxidative stress were calculated using the formula:

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

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

Statistical analysis used standard Statistica 6.0 programs (StatSoft Inc., USA). MTT assay results were analyzed using nonparametric statistics and qualitative analysis of the data by the Kruskal–Wallis criterion followed by a Dunn test (ANOVA). Results were considered statistically significant for $p \le 0.05$.

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