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THE SEARCH FOR NEW DRUGS

DESIGN AND SYNTHESIS OF DIPEPTIDE NERVE GROWTH FACTOR LOOP 1 MIMETICS AND *IN VITRO* STUDIES OF THEIR NEUROPROTECTIVE AND DIFFERENTIATION-INDUCING ACTIVITIES[#]

A. V. Tarasyuk,[1] N. M. Sazonova,[1,*] A. G. Rebeko,[1] I. O. Logvinov,[1] S. V. Nikolaev,[1] T. A. Antipova,[1] T. A. Gudasheva,[1] and S. B. Seredenin[1]

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Previous studies at the V. V. Zakusov Science Research Institute of Pharmacology created a dimeric dipeptide mimetic of the most exposed fourth loop of nerve growth factor (NGF), bis-(N-monosuccinyl-L-glutamyl-L-lysine) hexamethylenediamide (GK-2), which activates specific TrkA receptors and has neuroprotective activity *in vitro* (at 10^{-5} to 10^{-9} M) and *in vivo* (0.1 – 10 mg/kg, i.p., or p.o.). The present report describes the construction and synthesis of a mimetic of the first loop of NGF based on the β-turn (-Lys^{32}-Gly^{33}-Lys^{34}-Glu^{35}-), bis-(N-aminocaproyl-glycyl-L-lysine) hexamethylenediamide (GK-6). The structure of GK-6 preserves the central dipeptide fragment of the β-turn -Gly^{33}-Lys^{34}-, the Lys^{32} residue being substituted by its bioisostere - a 6-aminocaproic acid residue - and the dimeric structure of NGF being reproduced by dimerization at the C-terminal using a bivalent hexamethylenediamine spacer. Structure-activity relationships of GK-6 were studied by sequential substitution of the side groups of the peptide by hydrogen to produce bis-(N-acetyl-glycyl-L-lysine) hexamethylenediamide (GTS-611) and bis-(N-aminocaproyl-glycyl-glycine) hexamethylenediamine (GTS-613). Mimetic GL-6 and its analogs GTS-613, which contains the N-aminocaproyl radical, had neuroprotective effects at concentrations of 10^{-6} and 10^{-5} M in conditions of oxidative stress in HT-22 neuron cultures. Dipeptide GTS-611, in which the aminocaproyl fragment was replaced by an acetyl residue, had no neuroprotective activity in these conditions, pointing to the importance of Lys^{32} in NGF for this property. In contrast to the loop 4 mimetic GK-2, the loop 1 mimetics GK-6 and GTS-611 showed differentiation-inducing activity on PC12 cells.

Keywords: GK-6, GTS-611, GTS-613, NGF, neuroprotective activity, differentiation-inducing activity, structure-activity relationships.

Previous studies at the V. V. Zakusov Science Research Institute of Pharmacology created a dimeric dipeptide mimetic based on the structure of the β-turn of the most exposed loop 4 of nerve growth factor (NGF), i.e., bis-(N-monosuccinate-L-glutamyl-L-lysine) hexamethylenediamide (GK-2) [1 – 5]. GK-2 was shown to be able to activate TrkA receptors and the PI3/Akt signal pathway [6, 7]. In vitro experiments showed that micronanomolar concentrations of GK-2 displayed the neuroprotective activity intrinsic to NGF [8]. In *in vivo* experiments, the neuroprotective properties of GK-2 were demonstrated in experimental models of Alzheimer's disease, Parkinson's disease, and ischemic cerebral stroke [7]. In contrast to full-size NGF, GK-2 had no differentiation-inducing activity [1].

We report here the preparation of three mimetics of another loop of NGF, loop 1. Their neuroprotective and differentiation-inducing activities were studied *in vitro*. Construc-

[1] V. V. Zakusov Science Research Institute of Pharmacology, 8 Baltiiskaya Street, 125315 Moscow.

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[*] Corresponding author: Tel.: 8(916)803-33-35; e-mail: tata-sosnovka@mail.ru

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tion of the novel mimetics was based on published data on the crystal structure of the NGF homodimer (pdb ID: 1btg) [9]. Loop 1 can be seen, like loop 4, to be exposed, which may make a contribution to the binding of the neurotrophin to TrkA receptors. The basis for modeling was the sequence of the most exposed part of NGF loop 1, i.e., its β-turn (-Lys^{32}-Gly^{33}-Lys^{34}-Glu^{35}-). The structure of the mimetic preserved the central dipeptide fragment of the β-turn (Gly^{33}-Lys^{34}) which, on the basis of geometrical considerations, may penetrate more deeply into the receptor binding zone and be most completely recognized by it. The preceding amino acid residue, Lys^{32}, was substituted by its bioisostere - a 6-aminocaproic acid residue. Based on data on the interactions of neurotrophins with Trk receptors in the dimeric form, the two mimetics of the β-turn were dimerized head-to-head with a hexamethylenediamine spacer. This yielded the dimeric dipeptide NGF loop 1 mimetic GK-6, i.e., bis-(N-aminocaproyl-glycyl-L-lysine) hexamethylenediamide. The effects of the nature of the N-acyl and side radical of lysine on the activity of GK-6 were studied by constructing two analogs of GK-6 (Fig. 1): bis-(N-acetyl-glycyl-L-lysine) hexamethylenediamide (GTS-611) and bis-(N-aminocaproyl-glycyl-glycine) hexamethylenediamine (GTS-613). The neuroprotective activities of these compounds were studied *in vitro* on HT-22 neuronal cultures in conditions of oxidative stress induced by H_2O_2. In addition, the differentiation-inducing effects of mimetics GK-6 and GTS-611 were studied in PC12 cells.

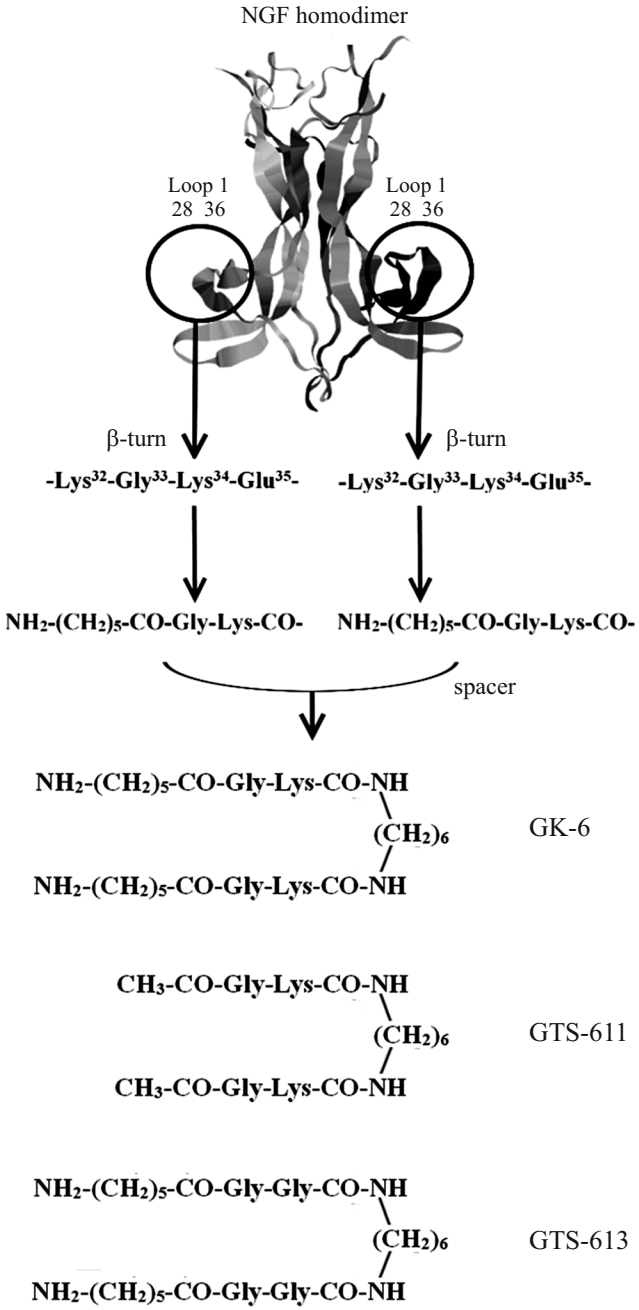


Fig. 1. Construction of NGF mimetics GK-6, GTS-611, and GTS-613.

EXPERIMENTAL CHEMICAL SECTION

These studies used commercially available *L*-amino acids and there derivatives from Sigma and Fluka. Melting temperatures were determined on an Optimelt MPA100 instrument (Stanford Research Systems, USA) in open capillaries without correction. ^{1}H and ^{13}C NMR spectra were recorded on the ppm scale using a Bruker Fourier 300 HD spectrometer (300 and 75 MHz respectively) in DMSO-d_6 solution with tetramethylsilane (0 ppm) as the internal standard. Signal assignments were made on the basis of analysis of one-dimensional and two-dimensional homonuclear ^{1}H-^{1}H COSY spectra and heteronuclear ^{1}H-^{13}C COSY spectra (HSQC and HMBC).

Specific optical rotation was recorded on an ADP 440 automatic polarimeter (Bellingham + Stanley Ltd., UK). TLC was run on glass DC Kieselgel 60 G/F_{254} plates (Merck, Germany) in the following solvent systems: chloroform and methanol, 6:1 (A), chloroform and acetone, 2:1 (B); chloroform, methanol, water, and acetic acid, 15:10:2:3 (C), chloroform, methanol, water, and acetic acid, 8:10:2:3 (D), *n*-butanol, acetic acid, water, 3:1:1 (E), chloroform, methanol, water, acetic acid, 10:15:2:3 (F), benzene and methanol (I), and dioxane and water, 9:1 (K).

Amine-containing compounds were detected with ninhydrin, compounds containing amide groups were detected with the chlorotoluidine test, compounds with open carboxyl groups were detected with bromocresol green, and compounds containing aromatic groups were detected in UV light.

DMF was purified by redistillation over ninhydrin. Diethyl ether was stored over solid NaOH. Ethyl acetate, dichloromethane, chloroform, benzene, acetone, hexane, petro-

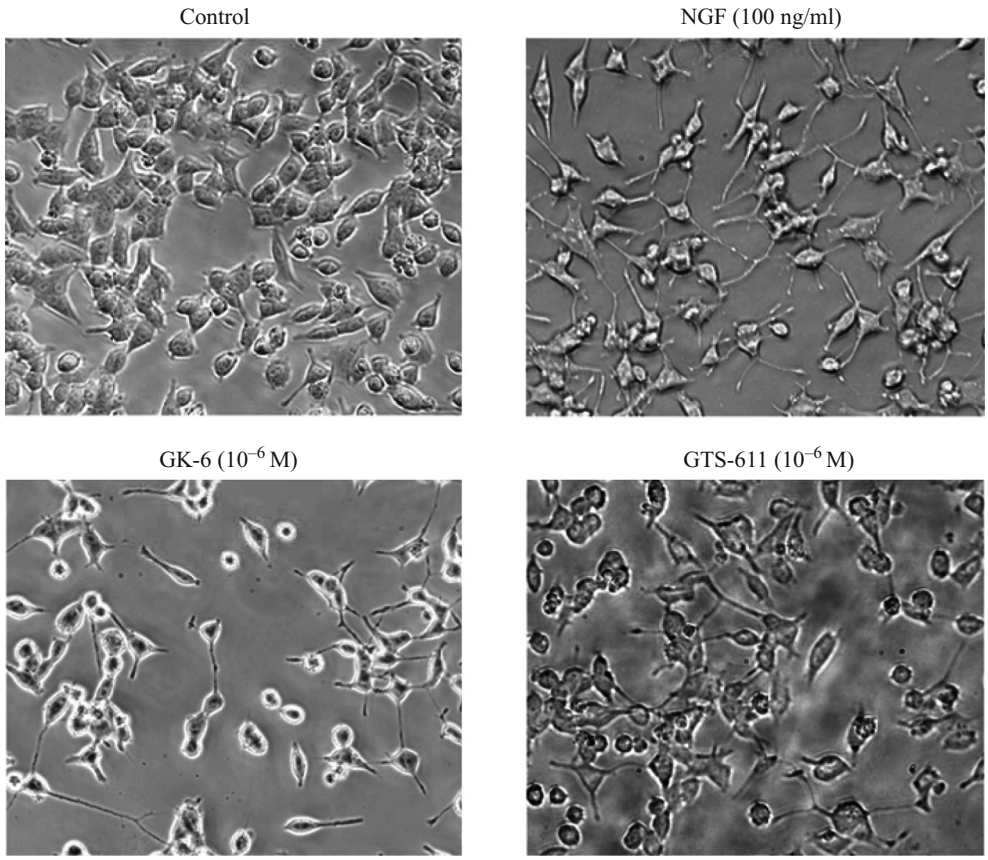


Fig. 2. Differentiation-inducing activity of peptides GK-6 and GTS-611 in rat PC-12 pheochromocytoma cells. Phase contrast, magnification ×100.

leum ether, methanol, and ethanol (all reagent grade) were used without additional purification.

Compounds obtained previously in our laboratory were: Z-Gly-OH (compound 1), Z-Gly-OSu (2), (Z-Gly-L-Lys(Boc)-NH-)$_2$(CH$_2$)$_6$ (8), (H-Gly-L-Lys-(Boc)-NH-)$_2$-(CH$_2$)$_6$ (9) [17]; Z-L-Lys(Boc)-OSu (5), (Z-L-Lys(Boc)-NH-)$_2$(CH$_2$)$_6$ (6), (H-L-Lys(Boc)-NH-)$_2$(CH$_2$)$_6$ (7) [18].

Synthesis of Starting Compounds

N-*tert*-Butyloxycarbonyl-6-aminohexanoic acid, (Boc-NH-(CH$_2$)$_5$-COOH) (3), was prepared as described in [11]. A solution of 5.72 g of $NaHCO_3$ in 57.2 ml of water and 115 ml of isopropyl alcohol were added sequentially to a solution of 15.00 g (114.4 mmol) of 6-aminocaproic acid in 115 ml of 1 M NaOH with mixing. Di-*tert*-butylpyrocarbonate (35 ml 139.5 mmol) was then added to the reaction mix in portions over 20 min at room temperature and the resulting reaction was mixed for 18 h. The reaction was treated with 150 ml of water and extracted with 2 x 110 ml of petroleum ether (to remove excess di-*tert*-butylpyrocarbonate). The aqueous solution was acidified with 1 M HCl at 0 – 5°C to pH ~3.0 and product was extracted with 3 × 200 ml of ethyl acetate. The ethyl acetate solution was washed with 200 ml of water and 190 ml of saturated NaCl solution, dried over Na_2SO_4, and evaporated. This yielded 21.82 g (~82%) of product as a yellow material, which was dried in vacuo and used without further purification. R_f 0.74 (A), R_f 0.65 (B), R_f 0.79 (ethyl acetate). The ^{1}H NMR spectrum, DMSO-d$_6$, δ, ppm, was: 1.19 – 1.26 (m, 2H, C$^{\gamma}$H$_2$ Aca), 1.30 – 1.36 (m, 11H, C$^{\delta}$H$_2$ Aca, -O(CH$_3$)$_3$), 1.42 – 1.52 (m, 2H, C$^{\beta}$H$_2$ Asa), 2.17 (t, J 7.4 Hz, 2H, C$^{\alpha}$H$_2$ Aca), 2.87 (m, J 5.4 Hz, 2H, C$^{\varepsilon}$H$_2$ Aca), 6.78 (t, ^{3}J 5.40 Hz, 1H, HN-(CH$_2$)$_5$-CO Aca), 12.00 (s, 1H, COOH).

***tert*-Butyloxycarbonyl-6-aminohexanoic acid N-oxysuccinimide ester, (Boc-NH-(CH$_2$)$_5$-COOSu) (4).** N-hydroxysuccinimide (11.94 g, 0.1037 mol) and 80 ml of ethyl acetate were sequentially added to a solution of 21.82 g (0.0943 mol) of Boc-Aca-OH in 150 ml of ethyl acetate with mixing. The reaction mix was cooled to 0 – 5°C and 22.38 g (0.1084 mol) of DCHC in 120 ml of ethyl acetate was added. The reaction was mixed with cooling for 1.5 h and at room temperature for 20 h. Oxalic acid (1.26 g) was then added (to remove excess DCHC) and after 1 h the precipitate of DCHM was collected by filtration and washed on the filter with 100 ml of ethyl acetate. The filtrate was evaporated in vacuo at 45°C. The resulting oil was triturated under diethyl ether and held overnight in a refrigerator; the resulting precipitate was collected by filtration, thoroughly squeezed, and

dried over $CaCl_2$ in a desiccator. The yield was 27.7 g (~90%) as a white crystalline substance. R_f 0.88 (A), R_f 0.82 (ethyl acetate); T_m 81 – 85°C. The ^{1}H NMR spectrum, DMSO-d_6, δ, ppm was: 1.31 – 1.37 (m, 13H, $C^{\gamma}H_2$ $C^{\delta}H_2$ Aca, -O$(CH_3)_3$), 1.56 – 1.65 (m, 2H, $C^{\beta}H_2$ Aca), 2.65 (t, J 7.4 Hz, 2H, $C^{\alpha}H_2$ Aca), 2.80 (s, 4H, -CH_2-CH_2-OSu), 2.89 (m, J 5.4 Hz, 2H, C^{ε}H 2 Aca), 6.79 (t, J 5.4 Hz, 1H, NH Aca).

Acetic acid N-oxysuccinimide ester (Ac-OSu) was prepared as described in [19] from 13.4 g (0.1164 mol) of N-hydroxysuccinimide with a yield of 96%. R_f 0.85 (A), R_f 0.56 (ethyl acetate), R_f 0.70 (K); T_m was 130 – 134°C. The ^{1}H NMR spectrum, DMSO-d_6, δ, ppm was: 2.34 (s, 3H, CH_3CO-), 2.80 (m, 4H, -CH_2CH_2-OSu). Published data [19]: T_m 133 – 134°C.

N-*tert*-Butyloxycarbonyl-glycine (Boc-Gly-OH) (12) was prepared as described in [11] with a yield of 81%. R_f 0.75 (C), R_f 0.91 (E), R_f 0.25 (B); T_m 88 – 91°C. Published data [20]: T_m 87 – 88°C.

***Tert*-Butyloxycarbonyl-glycine acid N-oxysuccinimide ester (Boc-Gly-OSu) (13)** was prepared as described in [18] with a yield of 81%. R_f 0.76 (C), R_f 0.75 (B), R_f 0.81 (ethyl acetate); T_m 131 – 134°C. Published data [21]: T_m 155°C.

Synthesis of Bis-(6-aminocaproyl-glycyl-L-lysine) Hexamethylenediamide, GK-6

Bis-(N-*tert*-butyloxycarbonyl-6-aminocaproyl-glycyl-N$^{\varepsilon}$-*tert*-butyloxycarbonyl-lysine hexamethylenediamide (Boc-HN-$(CH_2)_5$-CO-Gly-L-Lys(Boc)-NH-)$_2(CH_2)_6$ (10). A solution of 4.73 g (14.4 mol) of Boc-Aca-OSu in 45 ml of DMF was added to a solution of 4.50 g (6.6 mmol) of (H-Gly-L-Lys-(Boc)-NH-)$_2(CH_2)_6$ (9) in 40 ml of DMF and the reaction was mixed for 12 h at room temperature, after which 0.16 ml of N,N-dimethyl-1-aminopropane (DMAPA) was added and mixed for 30 min. The reaction mix was diluted with 200 ml of ethyl acetate and 150 ml of water and the aqueous layer was extracted with 200 ml of ethyl acetate. The ethyl acetate solution was washed with 100 ml of water and evaporated, and the residue was taken up in 200 ml of diethyl ether and held overnight in a refrigerator; the precipitate was collected by filtration and washed with diethyl ether (30 ml). The material was dried in a desiccator over $CaCl_2$ and the yield was 6.2 g (~85%) of chromatographically homogeneous product as a white crystalline substance. R_f 0.90 (C), R_f 0.81 (E), R_f 0.6 (A); T_m was 148 – 155°C. The ^{1}H NMR spectrum, DMSO-d_6, δ, ppm was: 1.23, 1.37, 1.45 – 1.61 (four m, 64H, 2 $C^{\beta}H_2C^{\gamma}H_2C^{\alpha}H_2$ Lys, -HN-CH_2-$(CH_2)_4$-CH_2-NH-, 2 $C^{\alpha}H_2C^{\beta}H_2C^{\gamma}H_2$ Aca, 4 -O$(CH_3)_3$, Boc), 2.11 (t, J 6.8 Hz, 4H, 2 $C^{\alpha}H_2$ Aca), 2.87 (m, 8H, 2 $C^{\varepsilon}H_2$ Lys, 2 $C^{\varepsilon}H_2$ Aca), 3.02 (m, 4H, HN-C<u>H</u>$_2$-$(CH_2)_4$-C<u>H</u>$_2$-NH-), 3.68 (broad s, 4H, 2 CH_2 Gly), 4.15 (m, 2H, 2 C^{α}H Lys), 6.75 (broad s, 4H, 2 N$^{\varepsilon}$H Lys and 2 NH Aca), 7.87 – 7.90 (m, 2H, 2 NH Lys, -<u>H</u>N-$(CH_2)_6$-N<u>H</u>-), 8.05 (broad s, 2H, 2 NH Gly).

Bis-(N-6-aminocaproyl-glycyl-L-lysine) hexamethylenediamide acetate, 4CH_3COOH·(Aca-Gly-L-Lys-NH-)$_2$ (GK-6). Compound 10 (6.2 g, 5.6 mmol) was treated with 100 ml of 100% TFA and after 1 h the reaction mix was evaporated; the residue was triturated with 200 ml of diethyl ether. Solvent was decanted and the resulting product was dissolved in 500 ml of water and purified on a column containing 100 ml of SP-Sephadex in a 0.1 > 0.6 M gradient of pyridine acetate buffer supplemented with ammonia. The corresponding fractions (monitored by TLC) were collected and evaporated, redistilled with isopropanol, and the resulting precipitate was dried in a vacuum desiccator over $CaCl_2$ to yield 3.6 g (90%) of final product as a solid white substance. R_f 0.02 (C), R_f 0.08 (E), R_f 0.50 (F); $[\alpha]_D^{20}$ was -23.34° (c, 1; water). The ^{1}H NMR spectrum, DMSO-d_6, δ, ppm was: 1.23, 1.27, 1.37, 1.48, and 1.61 (five m, 32H, 2 $C^{\beta}H_2C^{\gamma}H_2C^{\alpha}H_2$ Lys, -HN-CH_2-(C<u>H</u>$_2$)$_4$-CH_2-NH-, 2 $C^{\alpha}H_2C^{\beta}H_2C^{\gamma}H_2$ Aca), 1.73 (s, 12H, 4CH_3 Ac), 2.12 (t, J 7.2 Hz, 4H, 2 $C^{\alpha}H_2$ Aca), 2.64 (m, 8H, 2 $C^{\varepsilon}H_2$ 2 Lys, 2 $C^{\varepsilon}H_2$ Aca), 3.01 (m, 4H, HN-CH_2-$(CH_2)_4$-C<u>H</u>$_2$-NH-), 3.67 (broad s, 4H, 2 CH_2 Gly), 4.16 (m, 2H, 2 C^{α}H Lys), 8.08 (t, J 5.3 Hz, 2H, -<u>H</u>N-$(CH_2)_6$-N<u>H</u>-), 8.18 (d, J 8.0 Hz, 2H, 2 NH Lys), 8.46 (t, J 5.6 Hz, 2H, 2 NH Gly). 2N$^+$H$_3$ Lys and 2N$^+$H$_3$ Aca exchanged with solvent H_2O.

Synthesis of Bis-(N-acetyl-glycine-L-lysine), GTS-611

Bis-(N-acetyl-glycyl-N$^{\varepsilon}$-*tert*-butyloxycarbonyl-L-lysine), (Ac-Gly-L-Lys-(Boc)-NH-)$_2(CH_2)_6$ (11). AcOSu (1.51 g, 9.59 mmol) was added as one batch to a solution of 3.0 g (4.36 mmol) of (H-Gly-L-Lys-(Boc)-NH-)$_2(CH_2)_6$ (9) in 40 ml of DMF with mixing at 5°C on a magnetic stirrer. The reaction mix was held at this temperature for 1.5 h and then at room temperature for 12 h. DMF was evaporated in vacuo at 40°C and the "gel" residue was supplemented with 40 ml of acetone and held for 1 h to formation of a crystalline precipitate. The acetone solution was decanted and the precipitate was washed with hot acetone 2 × 30 ml with filtration using an attachment for hygroscopic substances, dried using a water flow vacuum pump for 2 h and then in a desiccator over $CaCl_2$, and this procedure yielded 2.3 g (81%) of chromatographically homogeneous product (11) as white crystals with a mild beige tinge. R_f 0.91 (D), R_f 0.82 (I), R_f 0.78 (ethyl acetate); T_m 125 – 139°C. The ^{1}H NMR spectrum, DMSO-d_6, δ, ppm was: 1.21, 1.36, and 1.59 (three m, 38H, 2 $C^{\gamma}H_2$ $C^{\alpha}H_2$ $C^{\beta}H_2$ Lys, -NH-CH_2-(C<u>H</u>$_2$)$_4$-CH_2-NH-, 2 -OC$(CH_3)_3$ Boc), 1.84 (s, 6H, 2 CH_3 Ac), 2.86 (m, 4H, 2 $C^{\varepsilon}H_2$ Lys), 3.00 (m, 4H, -NH-C<u>H</u>$_2$-$(CH_2)_4$-C<u>H</u>$_2$-NH-), 3.69 (broad s, 4H, 2 CH_2 Gly), 4.14 (m, 2H, 2 C^{α}H Lys), 6.76 (t, J 5.4 Hz, 2H, 2 N$^{\varepsilon}$H Lys), 7.84 (t, J 5.4 Hz 2H, -N<u>H</u>-$(CH_2)_6$-N<u>H</u>-), 7.93 (d, J 7.7 Hz, 2H, 2 NH Lys), 8.10 (t, J 5.3 Hz, 2H, 2 NH Gly).

Bis-(N-acetyl-glycyl-L-lysine) hexamethylenediamide ditrifluoroacetate, 2CF_3COOH·(Ac-Gly-L-Lys-NH-)$_2$-$(CH_2)_6$ (GTS-611). A solution of 0.3 g (0.46 mmol) of compound 11 in 10 ml of a mixture of 100% TFA and CH_2Cl_2

(1:1) was mixed the room temperature for 2 h, after which the reaction mix was evaporated and redistilled with methylene chloride 2 x 15 ml); the residue was triturated under dry diethyl ether with decantation (3 × 20 ml) and was left under diethyl ether (20 ml) for 2 h to form a precipitate. The precipitate was collected by filtration and dried on an attachment for hygroscopic substances, and dried in a desiccator in vacuo over $CaCl_2$ (15) mmHg). This yielded 0.26 g (87%) of chromatographically homogeneous product as a white crystalline substance (T_m not measured, hygroscopic). R_f 0.27 (D). The ^{1}H NMR spectrum, DMSO-d_6, δ, ppm was: 1.21, 1.36, 1.49, and 1.64 (four m, 20H, 2 $C^{\gamma}H_2$ $C^{\alpha}H_2$ $C^{\beta}H_2$ Lys, -NH-CH_2-(C<u>H</u>$_2$)$_4$-CH_2-NH-), 1.84 (s, 6H, 2 CH_3 Ac), 2.74 (m, 4H, 2 $C^{\varepsilon}H_2$ Lys), 3.01 (m, 4H, -NH-C<u>H</u>$_2$-$(CH_2)_4$-C<u>H</u>$_2$-NH-), 3.69 (d, J 4.0 Hz, 4H, 2 CH_2 Gly), 4.16 (m, 2H, 2 $C^{\alpha}H$ Lys), 7.74 (broad s, 6H, 2 N^+H_3 Lys), 7.88 (t, J 7.9 Hz, 2H, -N<u>H</u>-$(CH_2)_6$-N<u>H</u>-), 8.01 (d, J 8.0 Hz, 2H, 2 NH Lys), 8.18 (t, J 8.2 Hz, 2H, 2 NH Gly).

Synthesis of Bis-(N-6-aminocaproyl-glycyl-glycine) Hexamethylenediamide, GTS-613

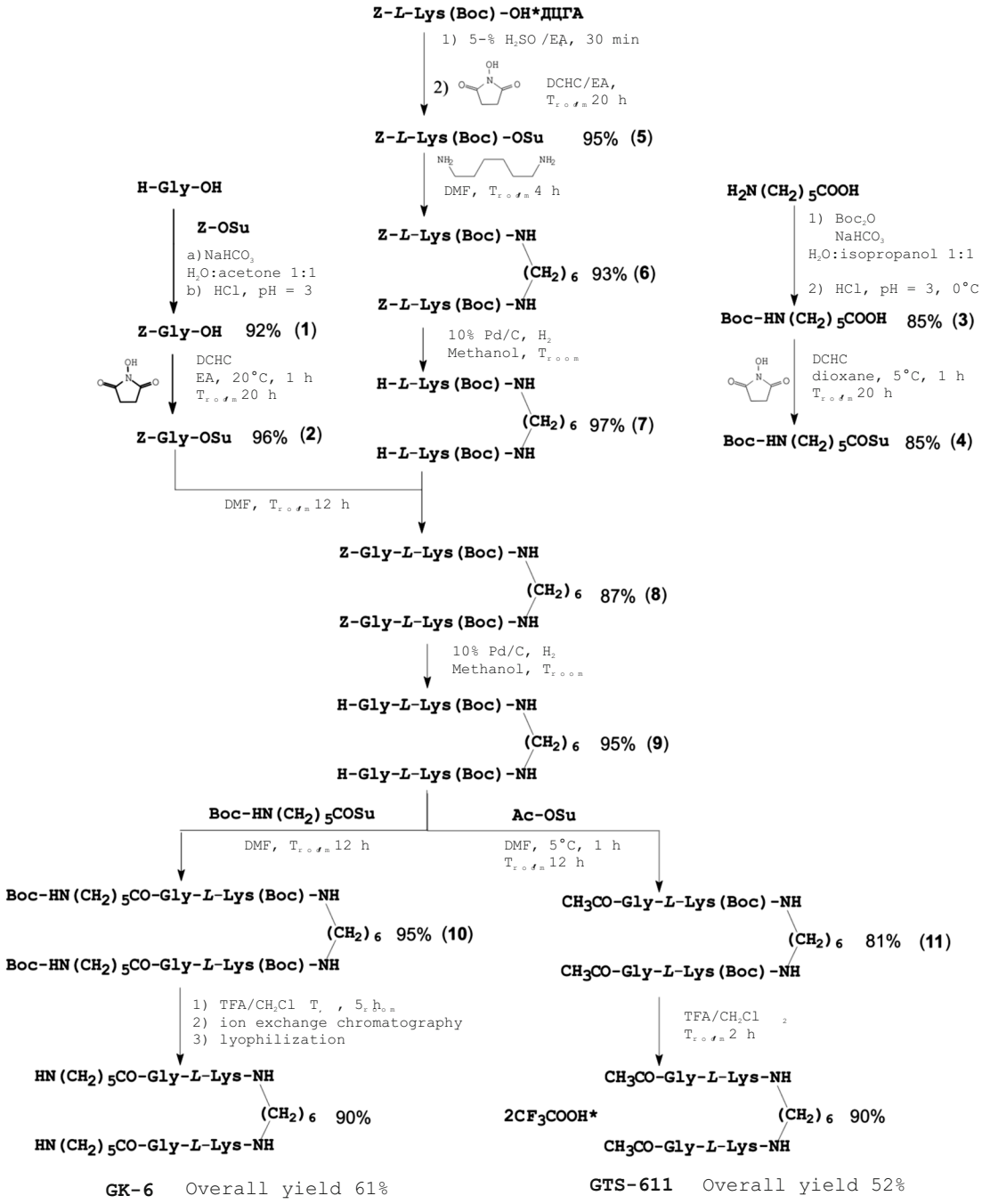
Bis-(*tert*-butyloxycarbonyl-glycine) hexamethylenediamide (Boc-Gly-NH-)$_2$(CH_2)$_6$ (14). A solution of 2.7 g (23.22 mmol) of hexamethylenediamine in 30 ml of DMF was poured into a solution of 13.9 g (51 mmol) of Boc-Gly-OSu (13) in 70 ml of DMF with mixing, resulting in precipitation of a small quantity of grayish material, which dissolved completely after 2.5 h. The reaction was mixed for 4 h at room temperature and was then left overnight without mixing. DMF was evaporated in vacuo at 40°C and 200 ml of distilled water previously warmed to 43 – 45°C was added to the residue, which was then left at room temperature until a precipitate formed. The fully formed precipitate was collected by filtration, washed with water to a neutral reaction followed by 50 ml of hexane, and dried in air. This yielded 9.0 g (90%) of chromatographically homogeneous compound 14 as a white crystalline substance. R_f 0.91 (C), R_f 0.88 (E), R_f 0.13 (B); T_m was 60 – 62°C. The ^{1}H NMR spectrum, DMSO-d_6, δ, ppm, was: 1.23 (m, 4H, -NH-$(CH_2)_2$-(C<u>H</u>$_2$)$_2$–$(CH_2)_2$-NH-), 1.37 (broad s, 22H, -NH-CH_2-C<u>H</u>$_2$-$(CH_2)_2$-C<u>H</u>$_2$-CH_2-NH-, 2 -OC$(CH_3)_3$), 3.03 (m, 4H, -NH-C<u>H</u>$_2$-$(CH_2)_4$-C<u>H</u>$_2$-NH-), 3.48 (d, J 6.0 Hz, 4H, 2 CH_2 Gly), 6.86 (t, J 6.0 Hz, 2H, 2NH Gly), 7.68 (t, J 5.1 Hz, 2H, -N<u>H</u>-$(CH_2)_6$-N<u>H</u>-).

Bis-glycine ditrifluoroacetate hexamethylenediamide, 2CF_3COOH·(H-Gly-NH)-2(CH_2)$_6$ (15). A solution of 2.0 g (46.45 mmol) of compound 14 in a mixture of 25 ml of CH_2Cl_2 and 10 ml of 100% TFA was mixed at room temperature for 2 h, after which the reaction mix was evaporated, redistilled with methylene chloride (2 × 15 ml), and the residue was triturated under dry diethyl ether with decantation (3 × 20 ml) and left under diethyl ether (20 ml) for 2 h to form a precipitate. The precipitate was collected by filtration and dried on an attachment for hygroscopic substances. This yielded 1.9 g (90%) of chromatographically homogeneous product 15 as a white crystalline substance (T_m not measured, hygroscopic). R_f 0.33 (D), R_f 0.28 (E); T_m was 165 – 167.5°C. The ^{1}H NMR spectrum, DMSO-d_6, δ, ppm, was: 1.27 (m, 4H, -NH-$(CH_2)_2$-(C<u>H</u>$_2$)$_2$-$(CH_2)_2$-NH-), 1.41 (m, 4H, -NH-CH_2-C<u>H</u>$_2$-$(CH_2)_2$-C<u>H</u>$_2$-CH_2-NH-), 3.10 (m, 4 H, -NH-C<u>H</u>$_2$-$(CH_2)_4$-C<u>H</u>$_2$-NH-), 3.52 (broad s, 4H, 2 CH_2 Gly), 8.10 (broad s, 6H, 2 N^+H_3 Gly), 8.39 (t, J 5.2 Hz, 2H, -N<u>H</u>-$(CH_2)_6$-N<u>H</u>-).

Bis-(*tert*-butyloxycarbonyl-glycyl-glycine) hexamethylenediamide, (Boc-Gly-Gly-NH-)$_2$(CH_2)$_6$ (16). DIPEA (0.83 ml, 4.8 mmol) was poured into a solution of 1.0 g (2.18 mmol) of compound 15 in 15 ml of DMF and 1.31 g (4.8 mmol) of Boc-Gly-OSu (13) was added with mixing. The reaction was mixed for 4 h at room temperature and then left overnight without mixing. DMF was evaporated in vacuo in a rotary evaporator at a temperature of 53°C and then redistilled with water (3 × 20 ml); 50 ml of water prewarned to 45°C was poured onto the residue and left to form a precipitate. The precipitate was collected by filtration, washed with water to a neutral reaction and then with hexane (20 ml) and acetone (20 ml), and dried in air. This produced 0.97 g (82%) of chromatographically homogeneous product 16 as a white crystalline substance. R_f 0.88 (C), R_f 0.73 (E), R_f 0.36 (A); T_m was 131 – 134°C. The ^{1}H NMR spectrum, DMSO-d_6, δ, ppm, was: 1.24 (m, 4H, -NH-$(CH_2)_2$-(C<u>H</u>$_2$)$_2$-$(CH_2)_2$-NH-), 1.39 (broad s, 22H, -NH-CH_2-C<u>H</u>$_2$-$(CH_2)_2$-C<u>H</u>$_2$-CH_2-NH-, 2 -OC$(CH_3)_3$), 3.03 (m, 4 H, -NH-C<u>H</u>$_2$-$(CH_2)_4$-C<u>H</u>$_2$-NH-), 3.55 and 3.65 (two d, J 5.8 and 5.6 Hz, 8H, 2 CH_2 ^{1}Gly and 2 CH_2 ^{2}Gly), 7.04 and 8.01 (two t, J 5.4, 5.1 Hz, 4H, 2 NH ^{1}Gly and 2 NH ^{2}Gly), 7.68 (t, J 5.1 Hz, 2H, -N<u>H</u>-$(CH_2)_6$-N<u>H</u>-).

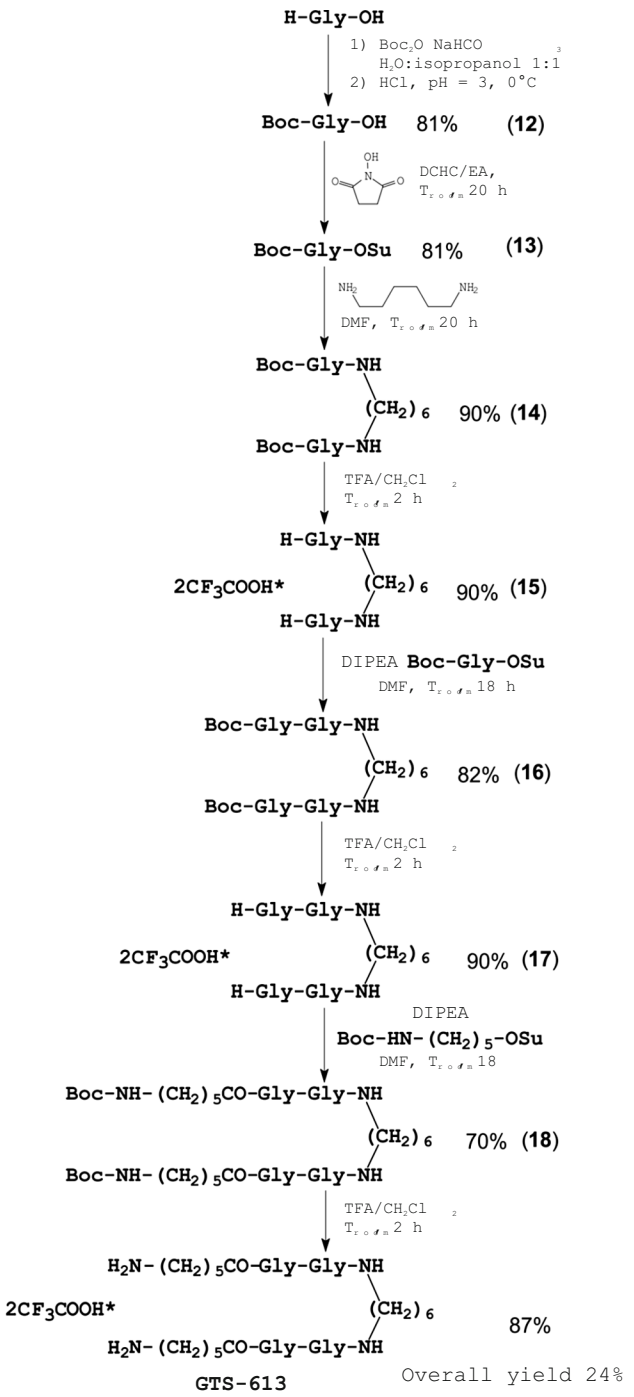
Bis-(glycyl-glycine) hexamethylenediamide ditrifluoroacetate, 2CF_3COOH·(H-Gly-Gly-NH-)$_2$(CH_2)$_6$ (17). A solution of 2.0 g (46.45 mmol) of compound 16 in a mixture of 30 ml of CH_2Cl_2 and 15 ml of 100% TFA was mixed for 2 h at room temperature, and the reaction mix was then evaporated, redistilled with methylene chloride (2 × 15 ml); the residue was washed (energetically shaken) with dry diethyl ether with decantation (2 × 15 ml); the diethyl ether residue was evaporated and the residue was supplemented with 35 ml of acetonitrile (which completely dissolved the oil) and held for 30 min; the resulting precipitate was collected by filtration using an attachment for hygroscopic substances. This yielded 1.89 g (90%) of chromatographically homogeneous product 17 as a white crystalline substance. R_f 0.18 (C), R_f 0.14 (E); T_m was 175 – 178°C. The ^{1}H NMR spectrum, DMSO-d_6, δ, ppm, was: 1.23 (m, 4H, -NH-$(CH_2)_2$-(C<u>H</u>$_2$)$_2$-$(CH_2)_2$-NH-), 1.37 (m, 4H, -NH-CH_2-C<u>H</u>$_2$-$(CH_2)_2$-C<u>H</u>$_2$-CH_2-NH-), 3.04 (m, 4 H, -NH-C<u>H</u>$_2$-$(CH_2)_4$-C<u>H</u>$_2$-NH-), 3.61 (m, 4H, 2CH_2 ^{2}Gly), 3.75 (d, J 5.7 Hz, 4H, 2 CH_2 ^{1}Gly), 7.96 (t, J 5.5 Hz, 2H, -N<u>H</u>-$(CH_2)_6$-N<u>H</u>-), 8.11 (broad s, 6H, 2 N^+H_3 ^{2}Gly), 8.64 (t, J 5.7 Hz 2H, NH ^{1}Gly).

Bis-(N-6-*tert*-butyloxycarbonyl-aminocaproyl-glycyl-glycine) hexamethylenediamide, Boc-Aca-Gly-Gly-NH-)$_2$(CH_2)$_6$ (18). Ditrifluoroacetate 17 (1.89 g, 3.3 mmol)



Scheme 1. Synthesis of NGF loop 1 dipeptide mimetics GK-6 and GTS-611.

was dissolved in 25 ml of DMF and 1.22 ml (7 mmol) of DIPEA was poured in; the reaction mix became gelatinous and a solution of 2.28 g (7.3 mmol) of Boc-Aca-OSu (4) in 15 ml of DMF was poured in and mixed for 18 h; DMF was evaporated on a rotary evaporator in vacuo using an oil pump at a temperature of 40°C. The residue, a mobile yellow gel, was supplemented with 40 ml of acetonitrile and left overnight to form a precipitate. The precipitate was collected by filtration and washed with 15 ml of acetonitrile and 15 ml of diethyl ether. The yield was 1.77 g (70%) as a white crystalline product. R_f 0.83 (C), R_f 0.11 (A); T_m was 220°C (with degradation). The ^{1}H NMR spectrum, DMSO-d_6, δ, ppm, was: 1.22 (m, 8H, -NH-$(CH_2)_2$-$(C\underline{H}_2)_2$-$(CH_2)_2$-NH-, 2 $C^{\gamma}H_2$ Aca), 1.36 (m, 8H, -NH-CH_2-$C\underline{H}_2$-$(CH_2)_2$-$C\underline{H}_2$-CH_2-NH-, 2 $C^{\alpha}H_2$ Aca), 1.47 (m, 4H 2 $C^{\beta}H_2$ Aca), 2.11 (t, J 5.7 Hz 2H, 2 $C^{\alpha}H_2$ Aca), 2.87 (m, 4H, 2 $C^{\varepsilon}H_2$ Aca), 3.03 (m, 4 H, -NH-$C\underline{H}_2$-$(CH_2)_4$-$C\underline{H}_2$-NH-), 3.63 (d, J 6.0 Hz 4H, 2CH_2 ^{2}Gly), 3.66 (d, J 6.0 Hz, 4H, 2 CH_2 ^{1}Gly), 6.78 (t, J 5.0 Hz



Scheme 2. Synthesis of NGF loop 1 dipeptide mimetic GTS-613.

2H, 2 NH Aca), 7.72 (t, ^{3}J 5.5 Hz, 2H, -NH-$(CH_2)_6$-NH-), 8.09 (t, J 5.5 Hz, 2H, 2NH ^{2}Gly), 8.14 (t, J 5.0 Hz 2H, NH ^{1}Gly).

Bis-(N-6-aminocaproyl-glycyl-glycine) hexamethylenediamide ditrifluoroacetate, $2CF_3COOH\cdot$(Aca-Gly-Gly-NH-)$_2(CH_2)_6$ (GTS-613). A solution of 0.30 g (0.389 mmol) of compound 18 in 5 ml of a mixture of 100% TFA and 15 ml of CH_2Cl_2 was mixed at room temperature for 2 h; the reaction mix was evaporated and then redistilled with methylene chloride (2 × 15 ml); the residue was triturated under dry diethyl ether with decantation (3 × 20 ml) and left under diethyl ether (20 ml) for 2 h to form a precipitate. The precipitate was collected by filtration and dried on an attachment for hygroscopic substances, and then dried in a desiccator in vacuo over $CaCl_2$ (15 mmHg). The yield was 0.23 g (87%) of chromatographically homogeneous GTS-613 as a white crystalline substance. R_f 0.18 (C), R_f 0.14 (E); T_m was 112 – 120°C (with degradation). The ^{1}H NMR spectrum, DMSO-d_6, δ, ppm, was: 1.23 – 1.26 (m, 4H, -NH-$(CH_2)_2$-($C\underline{H}_2)_2$-$(CH_2)_2$-NH-), 1.29 – 1.31 (m, 4H, 2 $C^{\gamma}H_2$ Aca), 1.38 (m, 4H, -NH-CH_2-$C\underline{H}_2$-$(CH_2)_2$-$C\underline{H}_2$-CH_2-NH-), 1.46 – 1.57 (m, 8H, 2 $C^{\alpha}H_2$ and 2 $C^{\beta}H_2$ Aca), 2.11 (t, J 7.4 Hz, 4H, 2 C^{α}H Aca), 2.77 (m, 4H, 2 $C^{\varepsilon}H_2$ Aca), 3.03 (m, 4H, -NHC$\underline{H}_2(CH_2)_4CH_2$ NH-), 3.65 and 3.69 (two d, J 5.8, 5.6 Hz, 8H, 2 CH_2 ^{2}Gly and 2CH_2 ^{1}Gly), 7.75 (broad s, 8H, 2 N^+H_3 Aca, -N$\underline{H}$-$(CH_2)_6$-N$\underline{H}$-), 8.10 and 8.16 (two t, J 5.8, 5.6 Hz, 2 NH ^{2}Gly and 2 NH ^{1}Gly). The ^{13}C NMR spectrum (DMSO-d_6), δ, ppm, was: 173.18 (s, 2C, 2CO, Aca), 169.86 and 168.94 (two s, 4C, 2CO ^{2}Gly and 2CO ^{1}Gly), 158.79 (q, 2C, $^2J_{C\text{-}F}$ 34.27 Hz, CF_3COOH), 116.84 (q, 2C, $^1J_{C\text{-}F}$ 295.03 Hz, CF_3COOH), 42.74 and 42.47 (two s, 4C, 2C^{α} ^{2}Gly and 2C^{α} ^{1}Gly), 39.21 (s, 2C, 2C^{ε} Aca), 38.94 (s, 2C, 2 C^1 of spacer), 35.32 (s, 2C, 2C^{δ} Aca), 29.46 (s, 2C, C^2 of spacer), 27.29 (s, 2C, 2C^{δ} Aca), 26.51 (s, 2C, 2 C^3 of spacer), 25.95 (s, 2C, 2C^{γ} Aca), 25.01 (s, 2C, 2C^{β} Aca).

EXPERIMENTAL BIOLOGICAL SECTION

The neuroprotective activity of compounds was assessed [13] in cultures of immortalized mouse hippocampal cells, line HT-22. Cells were cultured in 96-well plates at a density of 3500 cells/well in DMEM medium (HyClone, USA) containing 5% fetal bovine serum (Gibco, USA) and 2 mM L-glutamate (ICN Pharmaceuticals, USA) and incubated at 37°C in 5% CO_2 to formation of a monolayer. Peptides were added 24 h before harmful actions in the concentration range 10^{-5} to 10^{-8} M.

Oxidative stress was modeled using H_2O_2 at a final concentration of 1.5 mM. Cells with H_2O_2 were incubated in a 5% CO_2 atmosphere at 37°C for 30 min [22]. Medium was then changed for normal medium and cell viability was assayed after 4 h using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (AppliChem, Panreac, Germany). Optical density was measured on a Multiscan EX spectrophotometer (Thermo) at a wavelength of 600 nm.

Activity in experiments against oxidative stress was computed as

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

where D_{exp} is the optical absorption in the experimental conditions, $D_{H_2O_2}$ is the optical absorption in the active control (with H_2O_2), and D_{contr} is the optical absorption in controls (without H_2O_2).

Statistical analysis was run using the standard program bundle Statistica 6.0 (StatSoft Inc. USA). MTT test results were analyzed using nonparametric statistics and qualitative data were analyzed using the Kruskal-Wallis test followed by the Dunn test (ANOVA). Results were regarded as significant at $p \le 0.05$.

Determination of the *differentiation-inducing activity of peptide mimetics of nerve growth factor NGF* was performed using pheochromocytoma cell line PC12. Cells were cultured in DMEM medium supplemented with 5% fetal bovine serum (FBS) in a CO_2 incubator at a temperature of 37°C and 5% CO_2 to monolayer formation. Undifferentiated PC12 cells were cultured at a density of 3500 per well in DMEM medium containing 1% FBS. At the moment of sowing, NGF (BD Biodiversity, UK) was added to the culture medium at a final concentration of 100 ng/ml ($\approx 10^{9}$ M) as positive control. This NGF concentration is used in experiments for detection of its neuroprotective and differentiation-inducing activities in PC12 cells [23] and is not cytotoxic [24]. Dipeptides GK-6 and GTS-611 were added to a concentration of 10^{6} M.

Study peptides and NGF were then added to the culture medium every 48 h for six days [25]. Cells were photographed on day 7 using a Nikon Eclipse TS1200-F microscope (Japan) at a magnification of ×100. Cells with processes longer than one cell diameter were regarded as differentiated.

Data were analyzed statistically using the Mann-Whitney *U* test. Data are presented as $m \pm s.d.$ Data were regarded as significant at $p \le 0.05$.

RESULTS AND DISCUSSION

Synthesis of Mimetics of NGF Loop 1 – GK-6, GTS-611, and GTS-613

FK-6 and GTS-611 were synthesized by classical peptide synthesis methods in solution using elongation of the peptide chain at the C terminus using the Z/Boc protective group strategy with N-hydroxysuccinimide-activated esters (scheme 1).

Peptides GK-6 and GTS-611 were synthesized using commercially available Z-L-Lys(Boc)-OH, H-Gly-OH, and 6-aminocaproic acid. Z-protected glycine (1) was prepared by reaction of the sodium salt of glycine with benzyloxycarbonysuccinimide (Z-OSu) in aqueous-acetone solution [10]. Boc-protected aminocaproic acid Boc-Aca-OH was obtained using di-*tert*-butylpyrocarbonate using the Pozdnev method [11]. N-hydroxysuccinimide esters of Z/Boc-protected lysine (5), Boc-Aca-OH (4), and Z-glycine (2) were synthesized by the Andersen method [12] using N-hydroxysuccinimide and dicyclohexylcarbodiimide (DCHD) at temperatures from 0°C to +5°C. Condensation of the activated ester of protected L-lysine (5) with hexamethylenediamine in DMF at room temperature produced the corresponding (Z-L-Lys(Boc)-NH-)$_2$(CH$_2$)$_6$ hexamethylenediamide with a yield of 93%, which was then Z-deblocked by hydrogenolysis with hydrogen in the presence of 10% Pd/C. The resulting product 7 was condensed in DMF with Z-Gly-OSu (2) to produce (Z-Gly-L-Lys(Boc)-NH-)$_2$(CH$_2$)$_6$ (8) with a yield of 95%. Removal of the Z group by catalytic hydrogenolysis pro-

TABLE 1. Effects of NGF Mimetics GK-6, GTS-611, and GTS-613 on Neuron Viability in Oxidative Stress(MTT Test)

Group	Concentration, M	MTT test, D_{600}, mean ± SD	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*
Control	0	0.127 ± 0.005	100
H_2O_2	1.5×10^{-3}	0.097 ± 0.007^	0
GK-6	10^{-5}	0.117 ± 0.008*	66 ± 26*
	10^{-6}	0.117 ± 0.009*	66 ± 30*
	10^{-7}	0.100 ± 0.005	10 ± 17
	10^{-8}	0.112 ± 0.010	41 ± 33
Control	0	0.134 ± 0.008	100
H_2O_2	1.5×10^{-3}	0.093 ± 0.004^	0
GTS-611	10^{-5}	0.097 ± 0.008	11 ± 21
	10^{-6}	0.101 ± 0.007	22 ± 19
	10^{-7}	0.101 ± 0.005	22 ± 14
	10^{-8}	0.103 ± 0.006	16 ± 16
Control	0	0.176 ± 0.002	100
H_2O_2	1.5×10^{-3}	0.135 ± 0.004^	0
GTS-613	10^{-5}	0.155 ± 0.015*	49 ± 36**
	10^{-6}	0.153 ± 0.013*	44 ± 32*
	10^{-7}	0.150 ± 0.012	36 ± 29
	10^{-8}	0.149 ± 0.007	34 ± 17

Note. Substances were added 24 h before harmful treatments. Significant differences: ^ from control ($p \le 0.001$); * from H_2O_2 (* $p \le 0.05$; ** $p \le 0.01$), Kruskal-Wallis test with post hoc Dunn test, $n = 12$. Activity was calculated as $A(\%) = \frac{D_{exp} - D_{H_2O_2}}{D_{contr} - D_{H_2O_2}} \times 100\%$, where D_{exp} is the optical absorption in the experimental conditions, $D_{H_2O_2}$ is the optical absorption in the active control (with H_2O_2), and D_{contr} is the optical absorption in controls (without H_2O_2).

duced *bis*-dipeptide (H-Gly-L-Lys(Boc)-NH-)$_2$(CH$_2$)$_6$ (9), which was then acylated with Boc-Aca-OSu (4) and Ac-OSu in DMF to convert it to the N-aminocaproyl and N-acetyl derivatives (Boc-Aca-Gly-L-Lys(Boc)-NH-)$_2$(CH$_2$)$_6$ (10) and (Aca-Gly-L-Lys(Boc)-NH-)$_2$(CH$_2$)$_6$ (11) with yields of 95% and 81% respectively. Removal of Boc protection from *bis*-dipeptides 10 and 11 by acidolysis with TFA in dichloromethane produced trifluoroacetates GK-6 and GTS-611. Target peptide GK-6 was converted to the non-salt form by ion exchange chromatography (SP-Sephadex, pyridine-acetate buffer), purified by RP HPLC, and lyophilized. The overall yields of GK-16 and GTS-611 were 61% and 52% respectively. The structures and diastereomeric purities (>98%) of all compounds were confirmed by ^{1}H and ^{13}C NMR spectroscopy.

GTS-613 was synthesized using the Boc protective groups strategy (scheme 2). At the first stage, Boc-Gly-OH (12) was prepared from glycine, followed by preparation of its activated ester 13, which was condensed with hexamethylenediamine to produce *bis*-glycine 14 with a yield of 90%. Acidolysis with TFA in dichloromethane was then used to prepare *bis*-glycine trifluoroacetate 15, which was converted to free base using DIPEA and condensed with ester 13 to produce *bis*-dipeptide 16. Acidolysis of peptide 16 and further acylation with ester 4 produced the N-aminocaproyl derivative of *bis*-dipeptide 18, and final removal of Boc protection produced target compound GTS-611 as the ditrifluoroacetate with an overall yield in terms of glycine of 24%. Peptide GTS-613 was homogeneous by TLC. The structure and diastereomeric purity (98%) were confirmed by ^{1}H and ^{13}C NMR spectroscopy.

The neuroprotective activity of the peptides synthesized here was studied in a model of oxidative stress using hydrogen peroxide in cultures of immortalized mouse hippocampal cells line HT-22 [13] over the concentration range 10^{-5} to 10^{-8} M. Peptides were added 24 h before the harmful agent. The results are presented in Table 1.

GK-6 had neuroprotective activity at concentrations to 10^{-6} M, increasing cell viability by 66% of the maximum possible. In these conditions, NGF increased viability by 97%. Neuroprotective activity persisted when the lysine residue was replaced with a glycine residue (compound GTS-613). Dipeptide GTS-613 was active to a concentration of 10^{-6} M at a level of 44%. At the same time, substitution of the N-aminocaproyl radical with an acetyl radical (compound GTS-611) led to complete loss of activity.

Thus, the nature of the N-terminal substituent playing the role of Lys^{32} was critical for the neuroprotective activity of the NGF loop 1 mimetic, i.e., dimeric dipeptide GK-6. The C-terminal lysine residue corresponding to Lys^{34} in the NGF sequence does not play an important role in neuroprotective activity.

Differentiation-inducing activity on PC-12 cells of NGF mimetics GK-6 and GTS-611. The ability of peptide mimetics of nerve growth factor to induce differentiation was assessed in PC21 pheochromocytoma cells. These cells are known to contain TrkA and p75 receptors and addition of NGF is known to induce differentiation along the neuronal pathway [14]. Peptides GK-6 and GTC-611 were added to culture medium to a concentration of 10^{-6} M. As shown in Fig. 2, NGF induced differentiation of PC12 cells. The differentiating influence of GK-6 was weaker than that of NGF, while that of GTS-611 was even less marked. Mean process length in differentiated cells after addition of NGF was 59.6 ± 11.0 μm, compared with 44.5 ± 8.5 μm with GK-6 and 36.0 ± 6.2 μm after GTS-611 (Table 2).

Thus, substitution of the N-aminocaproyl radical of GK-6 with an acetyl radical led to loss of the neuroprotective but not the differentiation-inducing activity.

The causation of these relationships between the cellular effects of the compounds synthesized here and their structures could be linked with the specific features of the activation of the post-receptor transduction pathway, examples of which are given in our previous studies [15, 16].

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TABLE 2. Mena Process Length in Differentiated Cells after Addition of NGF, GK-6, and GTS-611 to PC-12 Rat Phaeochromocytoma Cells

Group	Mean process length of differentiated cells, μm
Control	0
NGF	59.6 ± 11.0*
GK-6	44.5 ± 8.5^
GTS-611	36.0 ± 6.2^#

Significant differences: * $p \le 0.05$ from control; ^$p \le 0.05$ from NGF; # $p \le 0.05$ from GK-6; Mann-Whitney U test.

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