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Design and synthesis of a novel dipeptide mimetic of the 4th loop of neurotrophin-3 and its pharmacological effects

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1. CHEMISTRY

NMR spectra were recorded on the Bruker Fourier 300 HD (Bruker Corporation, Germany, 300 and 75 MHz for ^{1}H- and ^{13}C) at room temperature; chemical shifts (δ) were measured relative to tetramethylsilane (0 ppm), DMSO-d_6 (2.50 ppm for ^{1}H) and $CDCl_3$ (7.26 ppm for ^{1}H). Chemical shifts (δ) are given in ppm; *J* values are given in Hz. Signal symbols: s – singlet, d – doublet, t – triplet, m – multiplet, br – broadened signal. When necessary, assignments of signals in NMR spectra were made using 2D techniques. High-resolution mass spectra (HRMS) were performed on the Bruker microTOF II instrument using electrospray ionization (ESI). The measurements were made in a positive ion mode (interface capillary voltage 4500 V).

Thin-layer chromatography (TLS) was performed on the glass silica gel plates DC Kieselgel 60 G/F254 (Merck, Germany) in solvent systems: $CHCl_3$ - MeOH – H_2O- AcOH, 15: 10: 2: 3 (A); *n*-BuOH - AcOH - H_2O, 3: 1: 1 (B); hexane - EtOAc, 1: 1 (C); hexane - EtOAc, 1: 5 (D). Amino-containing compounds were detected by ninhydrin, compounds with amide groups - using chlorine and o-tolidine.

Final product was purified by reverse-phase (RP) HPLC. Preparative RP HPLC was carried out using the GILSON system (France) on a Reprosil 100 C18 column (15 × 250 mm, 10 μm) (Dr. Maisch, Germany). The volume of the loop was 2 ml. The mobile phase A consisted of 0.1% solution of TFA in water, the mobile phase B consisted of 0.1% solution of TFA in CH_3CN.

Gradient elution mode was used (from 0% to 27% phase B, 20 min). Flow rate 10 mL/min was used.

Analytical RP HPLC was performed using the KNAUER system (Germany). The detection was with the wavelength of 220 nm. The analysis was carried out at room temperature. The Luna C18 column (4.6 × 250 mm, 5 μm, Phenomenex, USA) was used. The volume of the loop was 20 μl. The mobile phase A (0.05% TFA in a solution of 50 ml of CH_3CN + 950 ml of water) and the mobile phase B (0.05% AcOH solution in CH_3CN) were used. Gradient elution mode was used (from 5% to 50% phase B, 20 min). Flow rate 1 mL/min was used.

Melting points were determined on the Optimelt MPA100 device (Stanford Research Systems, USA) in open capillaries without correction. Specific optical rotation was recorded on the automatic polarimeter ADP 440 Polarimeter (Bellingham+Stanley Ltd., Great Britain).

***N*-*tert*-Butoxycarbonyl-L-asparagine (Boc-L-Asn-OH) (1)** was obtained according to [S1] from asparagine monohydrate (56.75 g, 0.378 mol). Yield 79.5 g (0.343 mol, 91%); white crystalls. R_f 0.70 (B); mp 176-176.4°C (decomp); $[\alpha]_D^{21}$ – 9.6° (c 2, DMF). Lit. [1]: mp 176°C (decomp); $[\alpha]_D^{20}$ – 7.2° (c 2, DMF).

Pentafluorophenyl ester of *N*-*tert*-butoxycarbonyl-L-asparagine (Boc-L-Asn-OPfp) (2) was obtained according to [S2] from Boc-L-Asn-OH (10.0 g, 43 mmol). Yield 13.08 g (32.5 mmol, 76%); white powder. R_f 0.66 (D); mp. 120-123ºC (EtOAc/hexane); $[\alpha]_D^{23}$ – 17.9° (c 1, EtOAc). δ_H ($CDCl_3$): 1.47 (s, 9H, -OC(CH_3)$_3$), 2.91 (dd, ^2J 16.5 Hz; ^3J 4.1 Hz, 1H, C$^{\beta}$H$_2$ Asn), 3.15 (dd, ^2J 16.5 Hz, ^3J 4.5 Hz, 1H, C$^{\beta}$H$_2$ Asn), 4.91 (m, 1H, C$^{\alpha}$H Asn), 5.65 и 5.79 (two s, 2H, -C(O)NH_2 Asn), 5.87 (d, ^3J 6.15 Hz, 1H, NH Asn).

Lit. [2]: mp 124-126 °C (diethyl ether/hexan); $[\alpha]_D^{25}$ – 16.7° (c 1, EtOAc).

Bis(*N*-*tert*-butyloxycarbonyl-L-asparagine) hexamethylenediamide, (Boc-L-Asn-NH-)$_2$(CH_2)$_6$ (3). A solution of hexamethylenediamine (0.51 g, 4.40 mmol) in DMF (15 ml) was added to solution of Boc-L-Asn-OPfp (3.87 g, 9.73 mmol) in DMF (20 ml) with stirring. The reaction mass was mixed for 4 h at room temperature, then left overnight without mixing. DMF was evaporated (40°C), distilled water (200 ml, 43-45°C) was added to the residue and left at room temperature until precipitate formation. The precipitate was filtered off, washed with water, acetone (20 ml), petroleum ether (2x40 ml), dried *in vacuo* (15 Torr) over $CaCl_2$ and paraffin. The yield was 1.90 g (7.67 mmol, 79%), white solid. R_f 0.75 (B); mp 206-211ºC (decomp); $[\alpha]_D^{21}$ – 2.0° (c 1, DMSO). δ_H (DMSO-d_6): 1.21 (m, 4H, 2 C^{3}H$_2$ spacer), 1.28-1.45 (m, 4H, 2 C^{2}H$_2$ spacer), 1.37 (s, 18H, 2 –OC(CH_3)$_3$), 2.27-2.43 (m, 4H, 2 C$^{\beta}$H$_2$ Asn), 3.02 (m, 4H, 2

C^1H_2 spacer), 4.16 (m, 2H, $C^{\alpha}H$ Asn), 6.81 (d, ^3J 8.1 Hz, 2H, NH Asn) 6.86 and 7.25 (two s, 4H, 2 -C(O)NH_2 Asn), 7.65 (t, ^3J 5.3 Hz, 2H, -N<u>H</u>$(CH_2)_6$N<u>H</u>-).

Bis(hydrotrifluoroacetate) of bis(L-asparagine) hexamethylenediamide, (CF_3COOH*H-L-Asn-NH-)$_2(CH_2)_6$ (4). A solution of (Boc-L-Asn-NH-)$_2(CH_2)_6$ (1.20 g, 46.45 mmol) in CH_2Cl_2 and trifluoroacetic acid (TFA) (30 ml, 1:1) was mixed at room temperature for 2 h, concentrated *in vacuo* with CH_2Cl_2 (2x15 ml). The residue was triturated under dry diethyl ether with decantation (2x20 ml) and left under diethyl ether (20 ml) for 2 h to form solid precipitate. The precipitate was filtered off and dried *in vacuo* (15 Torr) over $CaCl_2$. The yield was 2.00 g (36.45 mmol, 87%), white solid, R_f 0.36 (A), R_f 0.17 (B); mp 160-178ºC (Et_2O, decomp); $[\alpha]_D^{25}$ +4.7° (c, 1; MeOH), $[\alpha]_D^{25}$ +1.0° (c 1, DMSO). δ_H (DMSO-d_6): 1.26 (m, 4H, 2 C^3H_2 spacer), 1.40 (m, 4H, 2 C^2H_2 spacer), 2.53-2.69 (m, 4H, 2 $C^{\beta}H_2$ Asn), 3.09 (m, 4 H, 2 C^1H_2 spacer), 3.98 (m, 2H, 2 $C^{\alpha}H$ Asn), 7.24 and 7.66 (two s, 4H, 2 -C(O)NH_2 Asn), 8.08 (br s, 6H, 2 N^+H_3 Asn), 8.33 (t, ^3J 5.3 Hz, 2H, -N<u>H</u>$(CH_2)_6$N<u>H</u>-).

***N*-Benzyloxycarbonyl-γ-*tert*-butyl-L-glutamic acid *N*-hydroxysuccinimide ester (Cbz-L-Glu(Bu^t)-OSu)** was prepared according to [S3] with yield of 93% in the form of white crystals. R_f 0.52 (C); mp 98-101°C (ethyl acetate-n-hexane); $[\alpha]_D^{25}$ – 29.88° (c 2.4, EtOH). Lit. [S3]: mp 101-103°C. $[\alpha]_D^{24}$ -29.9 (c 2.43, EtOH).

Bis(*N*-benzyloxycarbonyl-γ-*tert*-butyl-L-glutamyl-L-asparagine) hexamethylenediamide, (Cbz-L-Glu(tBu)-L-Asn-NH-)$_2(CH_2)_6$ (5). DIPEA (0.74 ml, 4.26 mmol) and Cbz-L-Glu(tBu)-OSu (1.94 g, 4.46 mmol) were added to a solution of (CF_3COOH*H-*L*-Asn-NH-)$_2(CH_2)_6$ (1.16 g, 2.03 mmol) in DMF (20 ml) with stirring. The reaction mixture was stirred for 4 h at room temperature, left overnight without stirring. DMF was evaporated *in vacuo* (53°C), the residue was co-evaporated with water (2x20 ml). Warm distilled water (45°C, 50 ml) was added to the residue and left until the precipitate formed. The precipitate was filtered off, washed with water to neutral pH, acetone (20 ml), petroleum ether (20 ml), diethyl ether (2x20 ml) and dried in air. The yield was 1.10 g (1.12 mmol, 55%), cream-shade solid. R_f 0.83 (B); mp 211-218°C (decomp); $[\alpha]_D^{25}$ -5.2º (c 1, DMSO). δ_H (DMSO-d_6): 1.19 (m, 4H, 2 C^3H_2 spacer), 1.25-1.45 (m, 4H, 2 C^2H_2 spacer), 1.38 (s, 18H, 2 –OC$(CH_3)_3$)), 1.72 and 1.85 (two m, 4H, 2 $C^{\beta}H_2$ Glu), 2.24 (t, ^3J 7.6 Hz, 4H, 2 $C^{\gamma}H_2$ Glu), 2.46-2.50 (m, 4H, 2 $C^{\beta}H_2$ Asn), 2.99 (m, 4H, 2 C^1H_2 spacer), 3.98 (m, 2H, 2 $C^{\alpha}H$ Clu), 4.45 (m, 2H, 2 $C^{\alpha}H$ Asn), 5.00 and 5.06 (two d, ^2J 12.7 Hz, 4H, 2 -OCH_2- Z), 6.90 (s, 2H, H -C(O)NH_2 Asn),

7.31-7.37 (m, 12H, 2 -C_6H_5 Z и 2H, -C(O)NH_2 Asn), 7.54-7.62 (d, 2H, 2 NH Glu, t, 2H, -N<u>H</u>-$(CH_2)_6$-N<u>H</u>-), 8.13 (d, ^3J 7.9 Hz, 2H, NH Asn).

Bis(γ-*tert*-butyl-L-glutamyl-L-asparagine) hexamethylenediamide, (H-L-Glu(Bu^t)-L-Asn-NH-)$_2$(CH_2)$_6$ (6). Through the suspension of (Z-L-Glu(tBu)-L-Asn-NH-)$_2$(CH_2)$_6$ (5.0 g, 5.08 mmol) in MeOH (180 ml), hydrogen gas was passed under the conditions of catalytic hydrogenolysis (1.0 g 10% Pd/C, 50% wet, at room temperature, TLC control). The catalyst was filtered off and washed with MeOH (100 ml). The solvent was evaporated, then the residue was co-evaporated with benzene (30 ml), the resulting oil was crystallized under the petroleum ether (30 ml). The precipitate was filtered off, dried *in vacuo* (15 Torr) over paraffin. The yield was 3.06 g (4.33 mmol, 85%), amorphous white substance. R_f 0.81 (A), R_f 0.48 (B); mp 174-187°C (decomp); $[\alpha]_D^{25}$ –5.7° (c 1, DMSO). δ_H (DMSO-d_6): 1.20 (m, 4H, -2 C^3H_2 spacer), 1.25-1.45 (m, 4H, 2 C^2H_2 spacer), 1.38 (s, 18H, 2 –OC(CH_3)$_3$)), 1.56 and 1.79 (two m, 4H, 2 $C^{\beta}H_2$ Glu), 1.91 (br s, 4H, 2 NH_2 Glu), 2.24 (t, ^3J 7.6 Hz, 4H, 2 $C^{\gamma}H_2$ Glu), 2.37-2.50 (m, 4H, 2 $C^{\beta}H_2$ Asn), 3.00 (m, 4 H, 2 C^1H_2 spacer), 3.16 (m, 2H, 2 C^{α}H Clu), 4.45 (m, 2H, 2 C^{α}H Asn), 6.86 and 7.38 (two s, 4H, 2 -C(O)NH_2 Asn), 7.74 (t, ^3J 7.2 Hz, 2H, -N<u>H</u>(CH_2)$_6$N<u>H</u>-), 8.21 (br d, 2H, NH Asn).

Bis(*N*-γ-hydroxybutyryl-γ-*tert*-butyl-L-glutamyl-L-asparagine) hexamethylenediamide, (GHB-L-Glu(Bu^t)-L-Asn-NH-)$_2$(CH_2)$_6$ (7). A suspension of (H-L-Glu(Bu^t)-L-Asn-NH-)$_2$(CH_2)$_6$ **6** (2.5 g, 3.49 mmol) in 20 ml (265 mmol, 38-x excess) γ-butyrolactone was stirred at 60-70°C until complete dissolution and then stirred at room temperature for another 12 h. Then diethyl ether (15 ml) was added, the precipitate was thoroughly triturated, diethyl ether was decanted, this procedure was repeated 4 times. The solid residue was suspended in acetone (50°C, 30 ml), filtered using device for hygroscopic substances, and dried *in vacuo* (15 Torr) over $CaCl_2$. The yield was 2.07 g (2.33 mmol, 67%), white crystalline substance. R_f 0.79 (A), R_f 0.64 (B); mp 162-173°C (diethyl ether); $[\alpha]_D^{25}$ –4.8° (c 1, DMSO). δ_H (DMSO-d_6): 1.21 (m, 4H, 2 C^3H_2 spacer), 1.25-1.45 (m, 4H, 2 C^2H_2 spacer), 1.39 (s, 18H, 2 –OC(CH_3)$_3$)), 1.65 and 1.84 (two m, 8H, 2 $C^{\beta}H_2$ Glu and 2 C^3H_2 GHB), 2.17-2.27 (two t, 8H, 2 C^4H_2 GHB, 2 $C^{\gamma}H_2$ Glu), 2.40-2.50 (m, 4H, 2 $C^{\beta}H_2$ Asn), 3.00 (m, 4 H, 2 C^1H_2 spacer), 3.38 (t, ^3J 6.3 Hz, 2 C^2H_2 GHB), 4.11 (m, 2H, 2 C^{α}H Glu), 4.41 (m, 2H, 2 C^{α}H Asn), 6.90 and 7.39 (two s, 4H, 2 -C(O)-NH_2 Asn), 7.53 (br t, 2 H, -N<u>H</u>(CH_2)$_6$N<u>H</u>-), 8.16 (d, 2H, 2 NH Asn), 8.18 (d, 2H, 2 NH Glu).

Bis(*N*-γ-hydroxybutyryl-L-glutamyl-L-asparagine) hexamethylenediamide, (GHB-L-Glu-L-Asn-NH-)$_2$(CH_2)$_6$, (GTS-302). To a suspension of (GHB-*L*-Glu(Bu^t)-*L*-Asn-NH-)$_2$(CH_2)$_6$ **7** (1.50 g, 1.69 mmol) in 45 ml CH_2Cl_2, TFA (15 ml) was added, and this was stirred for 2 h at room

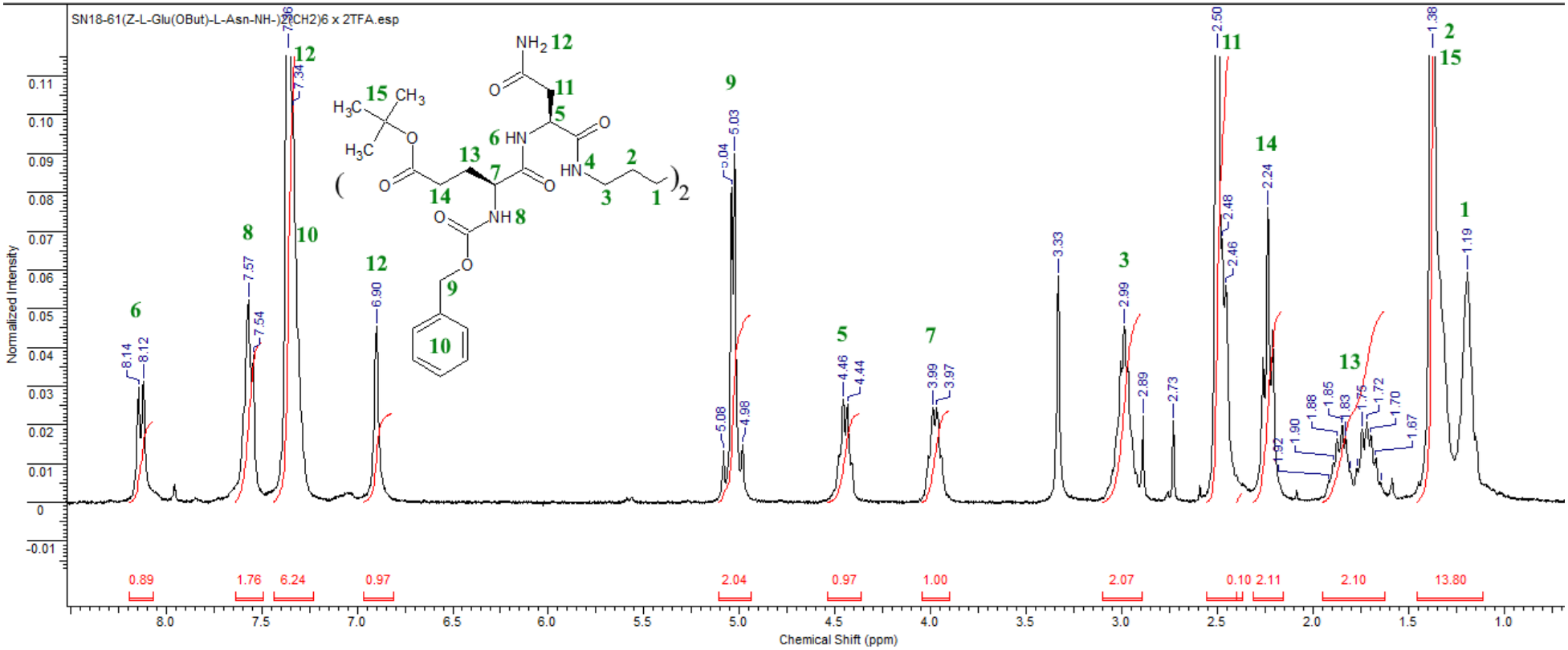
temperature. The reaction mass was concentrated *in vacuo*, then it was co-evaporated with CH_2Cl_2 (2x15 ml), the residue was triturated with dry diethyl ether and decanted (2x20 ml), left under the diethyl ether (20 ml) for 2 h for the formation of precipitate. The solid precipitate was filtered off. The product was purified with HPLC and lyophilized. The yield was 1.0 g (1.12 mmol, 76 %), white powder. R_f 0.75 (A), R_f 0.25 (B); τ = 11.0 min; mp 173-178°C (decomp); $[\alpha]_D^{23}$ –7.76° (c 1, DMSO). HRMS $[M + Na]^+$: calcd. for $C_{31}H_{54}N_8O_{14}$ 797.36, found 797.3647.

δ_H (DMSO-d_6): 1.20 (m, 4H, 2 C^3H_2 spacer), 1.35 (m, 4H, 2 C^2H_2 spacer), 1.65 (m, 4H, 2 C^3H_2 GHB), 1.73 and 1.88 (two m, 4H, 2 $C^{\beta}H_2$ Glu), 2.18 и 2.20 (two d, ^3J 7.2 Hz, ^3J 7.8 Hz, 4H, 2 C^4H_2 GHB), 2.26 (t, ^3J 7.7 Hz, 4H, 2 $C^{\gamma}H_2$ Glu), 2.42-2.51 (m, 4H, 2 $C^{\beta}H_2$ Asn), 3.00 (m, 4 H, 2 C^1H_2 spacer), 3.38 (t, ^3J 6.4 Hz, 4H, 2 C^2H_2 GHB), 4.12 (m, 2H, 2 $C^{\alpha}H$ Clu), 4.41 (m, 2H, 2 $C^{\alpha}H$ Asn), 6.91 and 7.34 (two s, 4H, 2 -$CONH_2$ Asn), 7.52 (t, ^3J 5.5 Hz, 2 H, -N<u>H</u>$(CH_2)_6$N<u>H</u>-), 8.09 (d, ^3J 8.3 Hz, 2H, 2 NH Asn), 8.12 (d, ^3J 7.0 Hz, 2 H, 2-NH Glu), 12.07 (br s, 2H, 2 COOH Glu).

δ_C (DMSO-d_6): 26.38 (s, 2C, 2 C^3 spacer), 27.25 (s, 2C, 2 C^{β} Glu), 28.87 (s, 2C, 2 C^3 GHB), 29.30 (s, 2C, 2 C^2 spacer), 30.57 (s, 2C, 2C^{γ} Glu), 32.39 (s, 2C, 2 C^2 GHB), 37.07 (s, 2C, 2 C^{β}Asn), 39.11 (s, 2C, 2 C^1 spacer), 50.26 (s, 2C, 2 C^{α} Asn), 53.04 (s, 2C, 2 C^{α} Glu), 60.80 (s, 2C, 2 C^4 GHB), 170.81 (s, 2C, 2CO (Asn)spacer), 171.51 (s, 2C, 2CO, Glu), 172.31 (s, 2C, 2 $CONH_2$ Asn), 173.71 (s, 2C, 2 CO GHB), 174.44 (s, 2C, 2COOH).

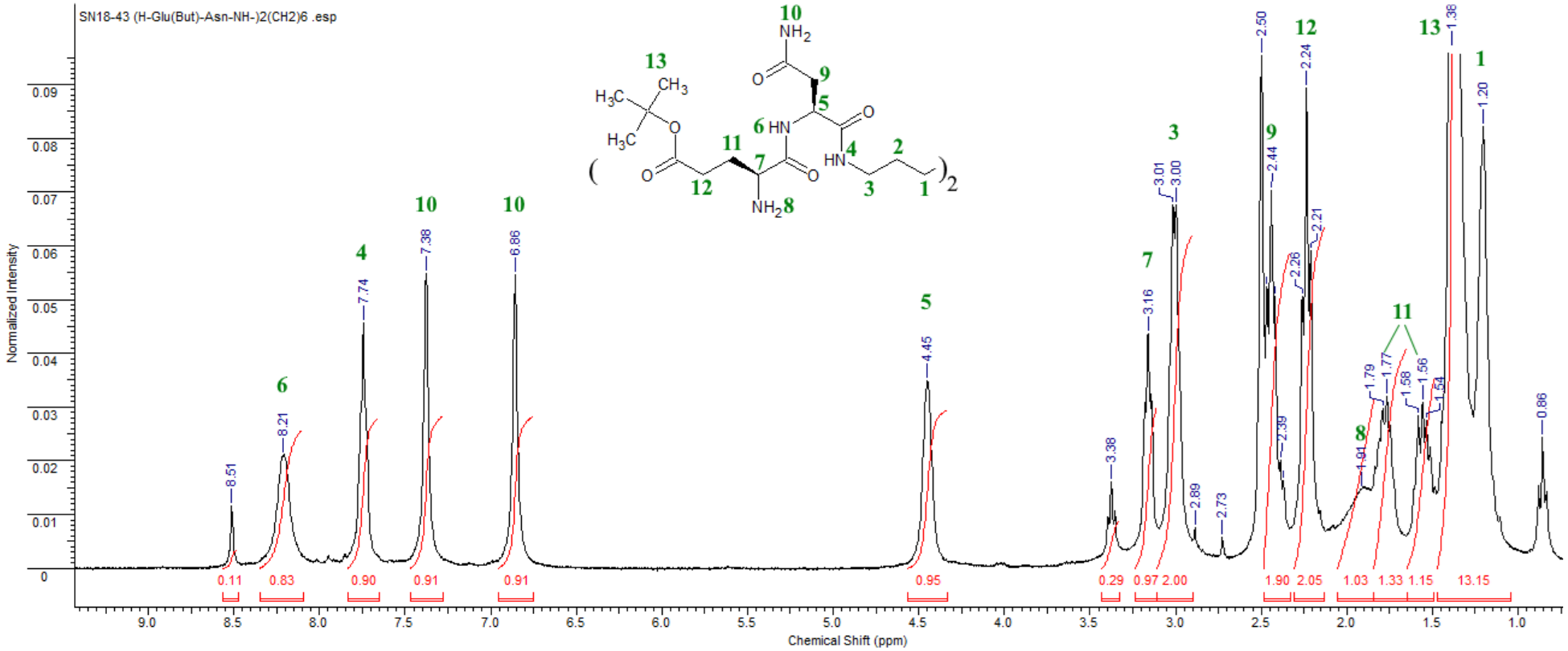
2. NMR SPECTRA

^{1}H-NMR (Cbz-L-Glu(Bu^{t})-L-Asn-NH-)$_2$(CH$_2$)$_6$ (**5**).



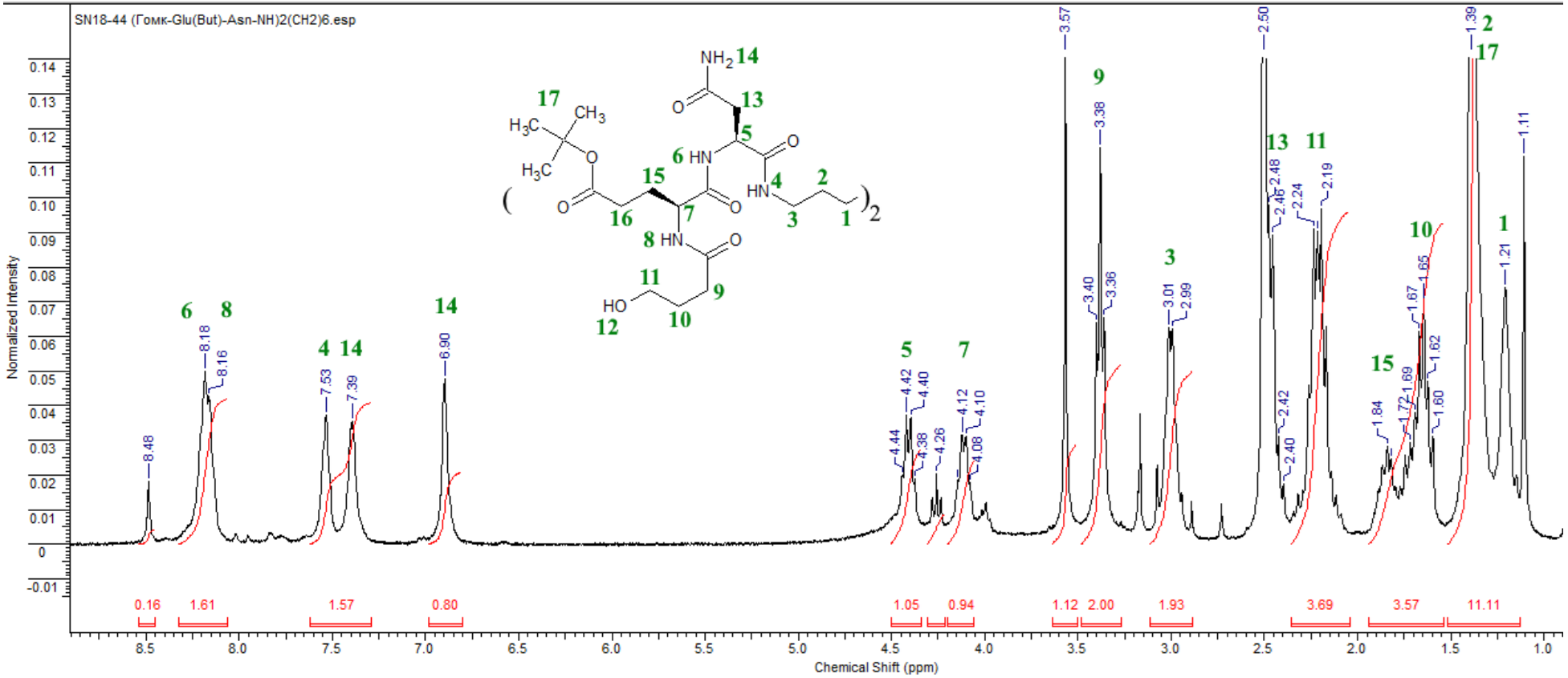
δ_H (DMSO-d$_6$): 1.19 (m, 4H, 2 C^{3}H$_2$ spacer), 1.25-1.45 (m, 4H, 2 C^{2}H$_2$ spacer), 1.38 (s, 18H, 2 –OC(CH$_3$)$_3$)), 1.72 and 1.85 (two m, 4H, 2 C$^{\beta}$H$_2$ Glu), 2.24 (t, ^3J 7.6 Hz, 4H, 2 C$^{\gamma}$H$_2$ Glu), 2.46-2.50 (m, 4H, 2 C$^{\beta}$H$_2$ Asn), 2.99 (m, 4H, 2 C^{1}H$_2$ spacer), 3.98 (m, 2H, 2 C$^{\alpha}$H Clu), 4.45 (m, 2H, 2 C$^{\alpha}$H Asn), 5.00 and 5.06 (two d, ^2J 12.7 Hz, 4H, 2 -OCH$_2$- Z), 6.90 (s, 2H, H -C(O)NH$_2$ Asn), 7.31-7.37 (m, 12H, 2 -C$_6$H$_5$ Z и 2H, -C(O)NH$_2$ Asn), 7.54-7.62 (d, 2H, 2 NH Glu, t, 2H, -N<u>H</u>-(CH$_2$)$_6$-N<u>H</u>-), 8.13 (d, ^3J 7.9 Hz, 2H, NH Asn).

^{1}H-NMR (H-L-Glu(Bu^{t})-L-Asn-NH-)$_2$(CH$_2$)$_6$ (**6**).



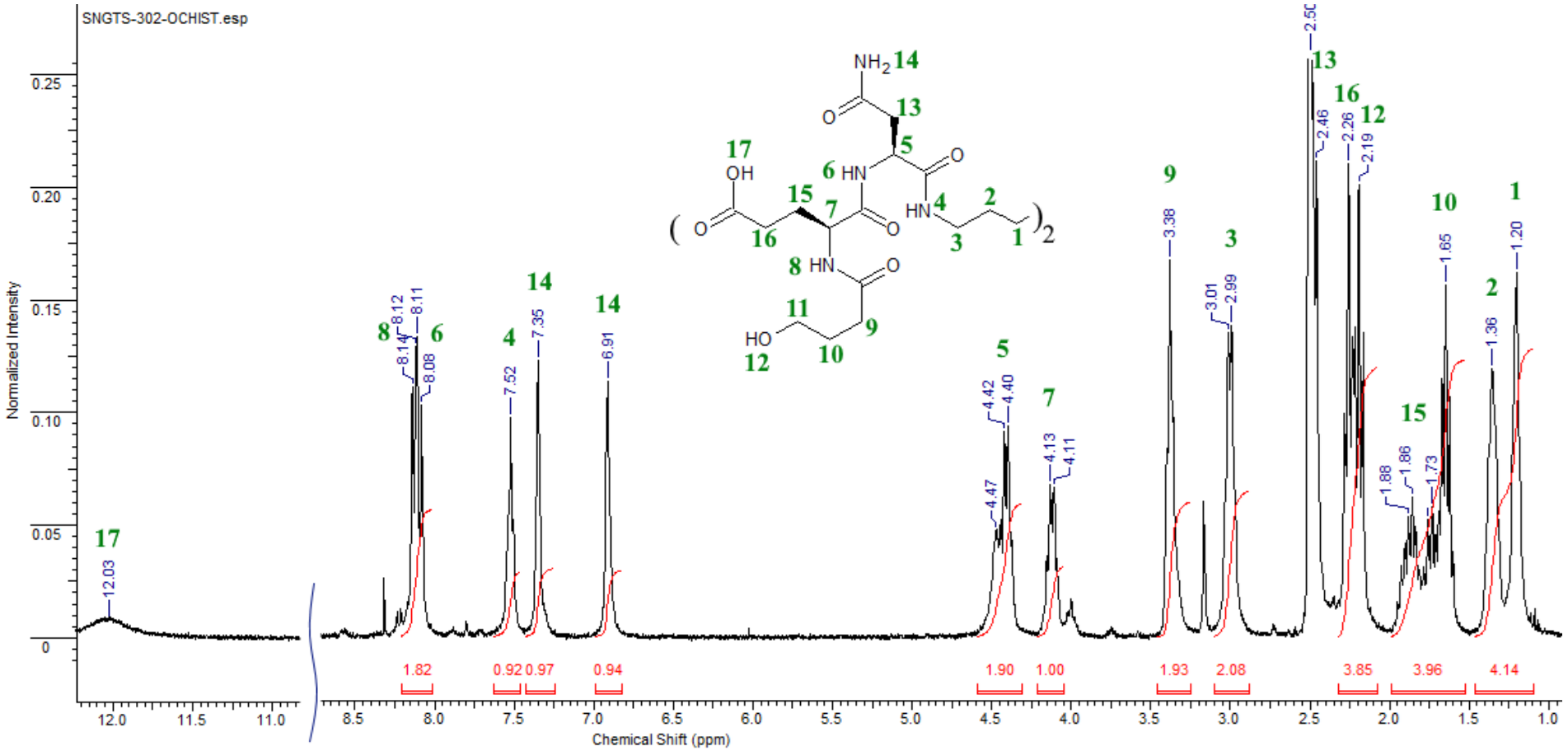
δ_H (DMSO-d$_6$): 1.20 (m, 4H, -2 C^{3}H$_2$ spacer), 1.25-1.45 (m, 4H, 2 C^{2}H$_2$ spacer), 1.38 (s, 18H, 2 –OC(CH$_3$)$_3$)), 1.56 and 1.79 (two m, 4H, 2 C$^\beta$H$_2$ Glu), 1.91 (br s, 4H, 2 NH$_2$ Glu), 2.24 (t, ^3J 7.6 Hz, 4H, 2 C$^\gamma$H$_2$ Glu), 2.37-2.50 (m, 4H, 2 C$^\beta$H$_2$ Asn), 3.00 (m, 4 H, 2 C^{1}H$_2$ spacer), 3.16 (m, 2H, 2 C$^\alpha$H Clu), 4.45 (m, 2H, 2 C$^\alpha$H Asn), 6.86 and 7.38 (two s, 4H, 2 -C(O)NH$_2$ Asn), 7.74 (t, ^3J 7.2 Hz, 2H, -N<u>H</u>(CH$_2$)$_6$N<u>H</u>-), 8.21 (br d, 2H, NH Asn).

^{1}H-NMR (GHB-L-Glu(Bu^{t})-L-Asn-NH-)$_2$(CH$_2$)$_6$ (**7**).



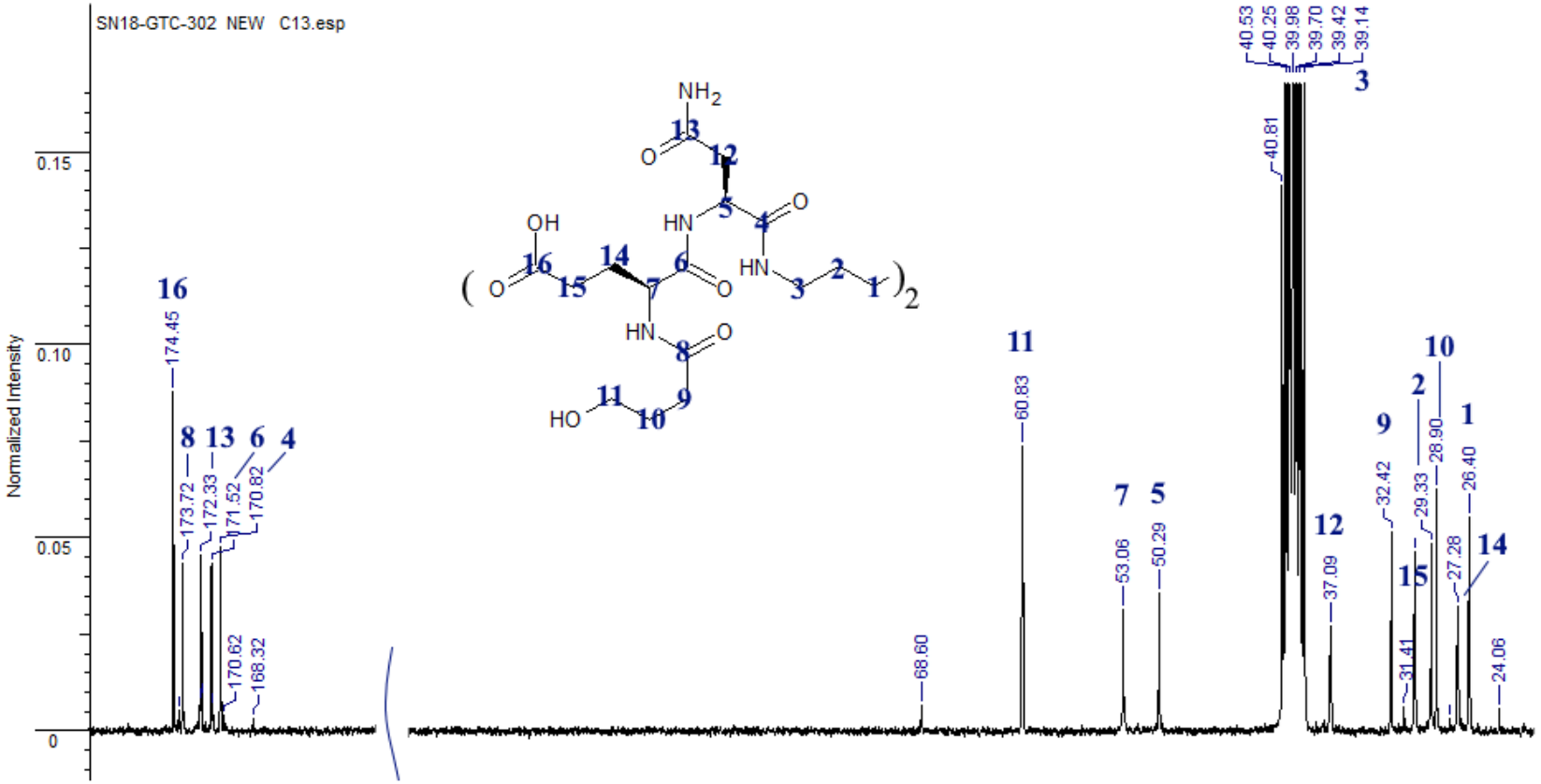
δ_H (DMSO-d$_6$): 1.21 (m, 4H, 2 C^{3}H$_2$ spacer), 1.25-1.45 (m, 4H, 2 C^{2}H$_2$ spacer), 1.39 (s, 18H, 2 –OC(CH$_3$)$_3$)), 1.65 and 1.84 (two m, 8H, 2 C$^{\beta}$H$_2$ Glu and 2 C^{3}H$_2$ GHB), 2.17-2.27 (two t, 8H, 2 C^{4}H$_2$ GHB, 2 C$^{\gamma}$H$_2$ Glu), 2.40-2.50 (m, 4H, 2 C$^{\beta}$H$_2$ Asn), 3.00 (m, 4 H, 2 C^{1}H$_2$ spacer), 3.38 (t, ^3J 6.3 Hz, 2 C^{2}H$_2$ GHB), 4.11 (m, 2H, 2 C$^{\alpha}$H Glu), 4.41 (m, 2H, 2 C$^{\alpha}$H Asn), 6.90 and 7.39 (two s, 4H, 2 -C(O)-NH$_2$ Asn), 7.53 (br t, 2 H, -NH(CH$_2$)$_6$NH-), 8.16 (d, 2H, 2 NH Asn), 8.18 (d, 2H, 2 NH Glu).

^{1}H-NMR (GHB-L-Glu-L-Asn-NH-)$_2$(CH_2)$_6$, (GTS-302).



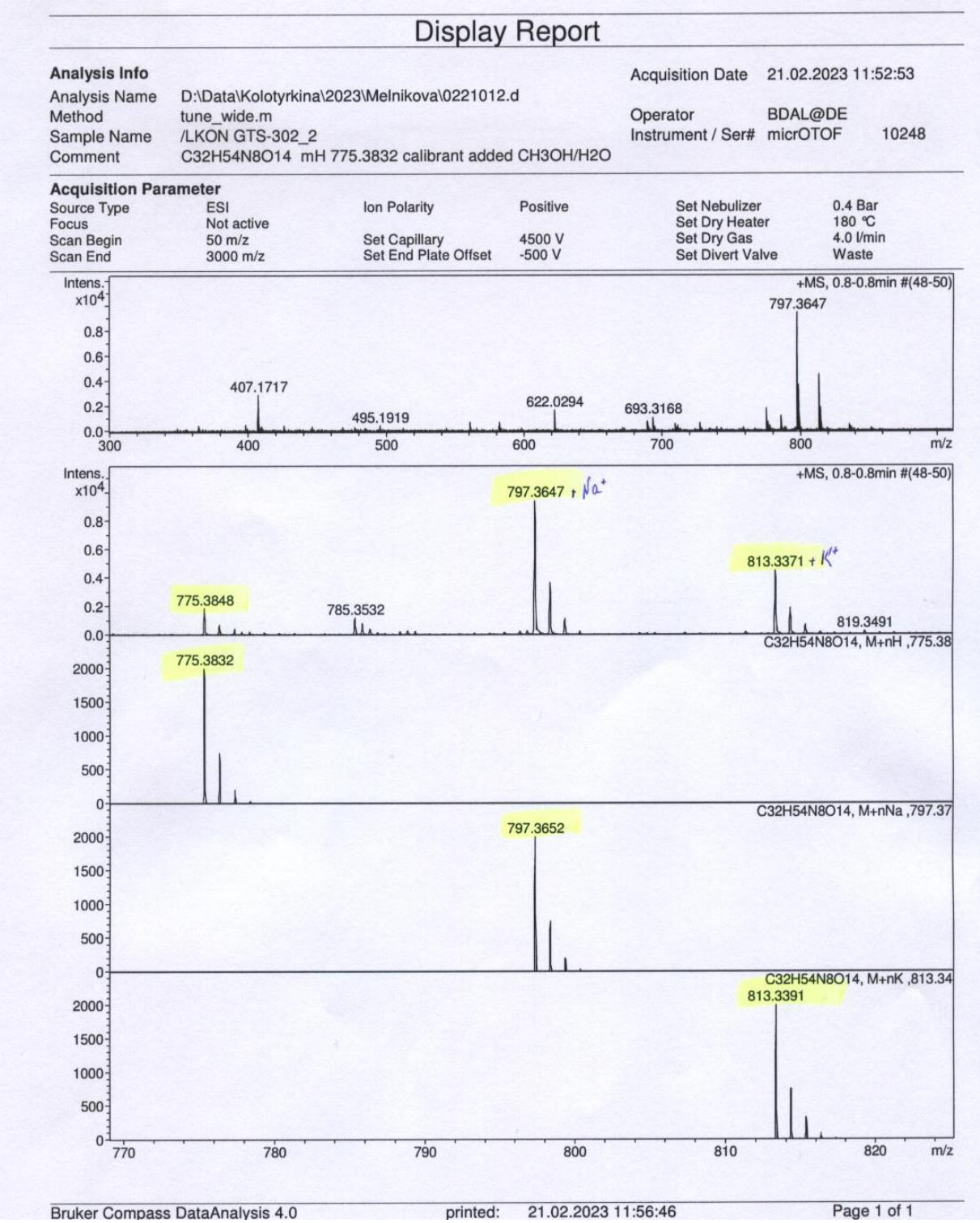
δH (DMSO-d6): 1.20 (m, 4H, 2 C^{3}H$_2$ spacer), 1.35 (m, 4H, 2 C^{2}H$_2$ spacer), 1.65 (m, 4H, 2 C^{3}H$_2$ GHB), 1.73 and 1.88 (two m, 4H, 2 C$^\beta$H$_2$ Glu), 2.18 и 2.20 (two d, ^3J 7.2 Hz, ^3J 7.8 Hz, 4H, 2 C^{4}H$_2$ GHB), 2.26 (t, ^3J 7.7 Hz, 4H, 2 C$^\gamma$H$_2$ Glu), 2.42-2.51 (m, 4H, 2 C$^\beta$H$_2$ Asn), 3.00 (m, 4 H, 2 C^{1}H$_2$ spacer), 3.38 (t, ^3J 6.4 Hz, 4H, 2 C^{2}H$_2$ GHB), 4.12 (m, 2H, 2 C$^\alpha$H Clu), 4.41 (m, 2H, 2 C$^\alpha$H Asn), 6.91 and 7.34 (two s, 4H, 2 -CONH$_2$ Asn), 7.52 (t, ^3J 5.5 Hz, 2 H, -NH(CH$_2$)$_6$NH-), 8.09 (d, ^3J 8.3 Hz, 2H, 2 NH Asn), 8.12 (d, ^3J 7.0 Hz, 2 H, 2-NH Glu), 12.07 (br s, 2H, 2 COOH Glu).

^{13}C-NMR (GHB-L-Glu-L-Asn-NH-)$_2$(CH$_2$)$_6$, (GTS-302).



δC (DMSO-d$_6$): 26.38 (s, 2C, 2 C^3 spacer), 27.25 (s, 2C, 2 C$^\beta$ Glu), 28.87 (s, 2C, 2 C^3 GHB), 29.30 (s, 2C, 2 C^2 spacer), 30.57 (s, 2C, 2C$^\gamma$ Glu), 32.39 (s, 2C, 2 C^2 GHB), 37.07 (s, 2C, 2 C$^\beta$Asn), 39.11 (s, 2C, 2 C^1 spacer), 50.26 (s, 2C, 2 C$^\alpha$ Asn), 53.04 (s, 2C, 2 C$^\alpha$ Glu), 60.80 (s, 2C, 2 C^4 GHB), 170.81 (s, 2C, 2CO (Asn)spacer), 171.51 (s, 2C, 2CO, Glu), 172.31 (s, 2C, 2 CONH$_2$ Asn), 173.71 (s, 2C, 2 CO GHB), 174.44 (s, 2C, 2COOH).

3. Mass spectra of GTS-302



HRMS $[M + Na]^+$: calcd. for $C_{31}H_{54}N_8O_{14}$ 797.36, found 797.3647

4. *IN VITRO* STUDIES

Materials and drugs. The following reagents were used: DMEM (HyClone, USA); FBS (Gibco, USA), L-glutamine (ICNPharmaceuticals, USA); poly-D-lysine (BD Biosciences, USA); H_2O_2 (Manufacturing pharmaceutical company «Renewal», Russia); DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (AppliChem, Panreac, Germany); Tris, glycine, TEMED, ammonium persulfate, SDS, NaCl, Tween-20, BSA (Bio-Rad, США); primary antibodies to phosphorylated forms of TrkA, TrkB and TrkC - anti-pY490 TrkA, anti-pY515 TrkB and anti-pY516 TrkC, primary antibodies to unphosphorylated TrkA, TrkB or TrkC (Invitrogen, Thermo Fisher Scientific, USA); secondary antibodies goat anti-rabbit IgG (Thermo Fisher Scientific, USA). The following equipment was used: culture flasks 75 cm^2, 96-well plates, pipettes 2 ml, 5 ml, 10 ml (TPP, Switzerland), spectrophotometer "Multiscan" (Thermo Fisher Scientific, USA).

Cell culture. The experiments were carried out using HT-22 mouse hippocampal cells (Holland, Utrecht) and SH-SY5Y human neuroblastoma cells. All manipulations with cells were performed in strictly sterile conditions. Cells were cultivated at 37°C in the atmosphere of 5% CO_2 in DMEM (Dulbecco's modified Eagle's medium, HyClone, USA) containing of 5% FBS (fetal bovine serum) and 2mM L-glutamine. The culture medium was changed 24 hours after seeding and every subsequent 3 days. Seeding on culture flasks with total area of 75 cm^2 (TPP, Switzerland) was carried out every week.

Oxidative stress. Oxidative stress in the culture of HT-22 hippocampal neurons was induced as described [S4]. The cells were incubated in the presence of H_2O_2 at the final concentration of 1.5 mM in the atmosphere of 5% CO_2 for 30 min at 37°C in the DMEM medium containing of 5% FBS and 2 mM L-glutamine. Next, the culture medium containing H_2O_2 was replaced with a normal one, and cell viability was determined after 24 h.

6-OHDA - induced toxicity (cell model of Parkinson's disease). 6-OHDA at the final concentration of 100 μM was added to the SH-SY5Y cells and incubated for 24 h at 37°C in 5% CO_2 [S5], after that the medium was changed to normal and cell viability was determined after 24 h.

Cell viability assessment (MTT-test). At the end of the experiment, the culture medium was replaced with an MTT solution (0.5 mg ml^{-1}) and incubated for 30 min at 37°C. [S6]. Then the MTT solution was taken from the wells and DMSO was added to dissolve the formazan. After 15 min, light absorption was measured on the spectrophotometer "Multiscan" (Thermo) at the wavelength of 600 nm.

Treatment with GTS-302 dipeptide. Dipeptide GTS-302 was added 24 h before cell damage at the final concentrations from 10^{-5} to 10^{-11} M. NT-3 (100 ng ml^{-1}) was used as the positive control [S7].

Western blot analysis. Experiments were performed using HT-22 cells. Receptor activation was evaluated by phosphorylation of tyrosines in the cytoplasmic domain responsible for triggering the main postreceptor signaling pathways, PI3K/AKT and MAPK/ERK, p-TrkA (Tyr490), p-TrkC (Tyr516), and p-TrkB (Tyr515), respectively. Samples were lysed 5, 15, 30, 60, and 180 min after addition of NT-3 (100 ng ml^{-1}) or GTS-302 (10^{-6}M). Unphosphorylated TrkA, TrkB or TrkC were used as a loading control.

The protein concentration in the samples was measured by the Folin-Lowry method [S8]. Proteins were separated by electrophoresis in 10% polyacrylamide gel [S9]. Preincubation of Western blots with primary antibodies against TrkA or TrkB at the dilution of 1:1000 or TrkC at the dilution of 1:2000 was performed in TBS buffer with 1% Tween-20 and 3% (w/v) BSA overnight at +4 °C. Then, membranes were washed with TBS buffer with 1% Tween-20 and 3% (w/v) BSA, and were incubated at the presence of goat anti-rabbit IgG secondary antibodies conjugated with horseradish peroxidase (dilution 1: 1000) for 1 h. After washing off the second antibodies, protein detection was performed with ECL reagents a by the Alliance UVITEC gel-documentation system. Densitometry of the obtained images was performed using GIMP2 software.

Statistical analysis. To assess intergroup differences in the GTS-302 cytoprotective activity studying experiments, the Kruskal-Wallis test was used, followed by pairwise intergroup comparison according to Dunn's test; in the studying the activation of TrkA, TrkB and TrkC receptors experiments the Mann-Whitney U-test was used. Differences were considered statistically significant at $p \leq 0.05$.

5. *IN VIVO* STUDIES

Animals. The experiments were conducted using 100 male BALB/c mice (weight 19-22 g) obtained from the Stolbovaya Branch of the Scientific Center of Biomedical Technologies of the Federal Medical Biological Agency. The animals were kept in a vivarium with ad libitum feeding and access to water and natural light–dark cycle. The animal experiments were carried out in compliance with international regulations (Directive 2010/63/EU of the European Parliament and of the Council of the European Union of September 22, 2010, on the protection of animals used for scientific purposes). The experiments were approved by the

Biomedical Ethics Committee of the Zakusov Research Institute of Pharmacology (Protocol No. 5 dated April 19, 2021).

The forced swim test [S10] was carried out according to a modified method with two swimming sessions [S11]. The test setup consisted of 5 transparent plastic cylinders (10 cm diameter, 30 cm height). The cylinders were filled with water at the temperature of 22–23°C to 2/3 of the height, at this level the animals could not rest on the bottom of the cylinder with their paws or tail. First, the animals were placed in cylinders filled with water for 10 min. After 24 h, a test session was carried out - the animals were repeatedly placed under the same conditions for 5 min. The resulting video materials were processed in the Real-Timer software (Open Science, Russia) with the calculation of the total immobility time (refusal of active-defensive and research behavior).

Study design. The dipeptide GTS-302 was injected ip 1 h after the first swimming session. The classical antidepressant Amitriptyline was used as the reference drug (Moscow Endocrine Plant, Russia) and was injected according to the same scheme at the dose of 10 mg/kg, ip [S12].

There were two separate experiments with different doses of GTS-302. In Experiment 1, mice were randomly divided into the following groups:

1. Control (n=10)
2. GTS-302, 1 mg/kg (n=10)
3. GTS-302, 5 mg/kg (n=10)
4. GTS-302, 10 mg/kg (n=10)

Since GTS-302 was active at all these studied doses, we performed the additional study (Experiment 2) with lower and higher doses. In Experiment 2, mice were randomly divided into the following groups:

1. Control (n=11)
2. GTS-302, 0.1 mg/kg (n=10)
3. GTS-302, 0.5 mg/kg (n=10)
4. GTS-302, 10 mg/kg (n=10)
5. GTS-302, 20 mg/kg (n=10)
6. Amitriptyline, 10 mg/kg (n=9)

Statistical analysis was carried out using GraphPad Prism 7.0 software (GraphPad Software, Inc., San Diego, CA). The data were checked for normal distribution using the Shapiro-Wilk test. To identify intergroup differences, one-way analysis of variance (one-way ANOVA) was used, followed by multiple comparisons by Dunnett test. Differences were considered statistically significant at $p<0.05$.

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