Supporting Information

Modifications at Arg and Ile Give Neurotensin(8-13) Derivatives with High Stability and Retained NTS_1 Receptor Affinity

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1. General experimental conditions

If not otherwise stated, solvents and buffer components, purchased from commercial suppliers, were of analytical grade. Gradient grade MeOH for HPLC was obtained from Merck (Darmstadt, Germany) and gradient grade acetonitrile for HPLC was from Sigma-Aldrich (Taufkirchen, Germany). *N*,*N*-Diisopropylethylamine (DIPEA, 99%) was from ABCR (Karlsruhe, Germany). Anhydrous DMF (99.8%), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and 1-Methyl-D-Trp (**14**) were purchased from Sigma-Aldrich. DMF (for peptide synthesis, packed under nitrogen, code D/3848/PB17), NMP (for peptide synthesis, nitrogen flushed), anhydrous NMP (99,5%) and HOBt hydrate were obtained from Acros Organics/Fisher Scientific (Nidderau, Germany). Trifluoroacetic acid and absolute EtOH were purchased from Honeywell (Seelze, Germany). Piperidine and HBTU were from Iris Biotech (Marktredwitz, Germany). Bovine serum albumin (BSA) was purchased from Serva (Heidelberg, Germany), and ammonium acetate (98%) was from Merck. Peptide **1** (tris(hydrotrifluoroacetate)) was purchased from SynPeptide (Shanghai, China). Succinimidyl propionate (**10**) was prepared according to a described procedure.[1] Millipore water was used throughout for the preparation of buffers, stock solutions and HPLC eluents. 1.5- and 2-mL polypropylene reaction vessels with screw cap (in the following referred to as "reaction vessel with screw cap") from Süd-Laborbedarf (Gauting, Germany) were used for the preparation and storage of stock solutions, and for small-scale reactions. 1.5- or 2-mL polypropylene reaction vessels (in the following referred to as "reaction vessel") from Sarstedt (Nümbrecht, Germany) were used for the preparation of dilute solutions, and for the investigation of stabilities in plasma. Reactions under anhydrous conditions were performed under argon atmosphere using anhydrous solvents. For the evaporation of solvents in 1.5- or 2-mL reaction vessels a Savant Speed-Vac Plus SC110A vacuum concentrator (Thermo Fischer Scientific, Waltham, MA) was used. NMR spectra were recorded on a Bruker Avance 600 instrument (^{1}H: 600 MHz, T = 300 K) (Bruker, Karlsruhe, Germany). NMR spectra were calibrated based on the solvent residual peaks (^{1}H-NMR, DMSO-d_6: δ = 2.50 ppm) and data (^{1}H-NMR) are reported as follows: chemical shift δ in ppm (multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br s = broad singlet), integral, coupling constant *J* in Hz). High resolution mass spectra (HRMS) were acquired with an Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS system coupled to an Agilent 1290 HPLC system (Agilent Technologies, Santa Clara, CA), using an ESI source. Analyses were either performed with LC method A or B. Method A: column: Luna Omega C18, 1.6 µm, 50 × 2.1 mm (Phenomenex, Aschaffenburg, Germany), column temperature: 40 °C, flow: 0.6 mL/min,

solvent/linear gradient: 0–4 min: 0.1% aq HCOOH/0.1% HCOOH in MeCN 95:5–2:98, 4–5 min: 2:98. Method B: column: YMC Triart C18, 1.9 µm, 75 × 2 mm (YMC Europe, Dinslaken, Germany), column temperature: 40 °C, flow: 0.6 mL/min, solvent/linear gradient: 0–10 min: 0.1% aq HCOOH/0.1% HCOOH in MeCN 100:0–70:30, 10–11 min: 70:30–2:98, 11–12 min: 2:98. Preparative HPLC was performed with a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps and a K-2001 detector. A Kinetex-XB C18, 5 µm, 250 mm × 21 mm (Phenomenex) or a YMC-Actus Triart C8, 5 µm, 250 mm × 20 mm (YMC) served as RP-columns at flow rates of 20 mL/min and 18 mL/min, respectively. Mixtures of 0.2% aq TFA (A1) and acetonitrile (B), or 10 mM ammonium acetate buffer (pH 5) (A2) and B were used as mobile phase. A detection wavelength of 220 nm was used throughout. Collected fractions were lyophilized using an Alpha 2-4 LD apparatus (Martin Christ, Osterode am Harz, Germany) equipped with a vacuubrand RZ 6 rotary vane vacuum pump. Analytical HPLC analysis of compounds **2**-**9**, **11** and **12** was performed with a system from Agilent Technologies consisting of a 1290 Infinity binary pump equipped with a degasser, a 1290 Infinity Autosampler, a 1290 Infinity Thermostated Column Compartment, a 1260 Infinity Diode Array Detector and a 1260 Infinity Fluorescence Detector. A Kinetex-XB C18, 2.6 µm, 100 × 3 mm (Phenomenex) or a YMC Triart C8, S-5µm, 250 × 4.6 mm (YMC) were used as stationary phase at a flow rate of 0.6 mL/min and 1 mL/min, respectively. The oven temperature was set to 25 °C. Mixtures of 0.04% aq TFA (A3) and B, or A2 and B served as mobile phase. The following linear gradients were applied: compounds **2**-**4**, **6**-**9**, **11** and **12** (Kinetex-XB): 0–12 min: A3/B 90:10–70:30, 12–16 min: 70:30–5:95, 16–20 min: 5:95; compound **5** (YMC Triart): 0–25 min: A2/B 90:10–65:35, 25–27 min: 65:35–5:95, 27–35 min: 5:95. The injection volume was 20 µL. UV detection was performed at 220 nm and fluorescence detection at 275/305 nm. Retention (capacity) factors k were calculated from the retention times t_R according to $k = (t_R - t_0)/t_0$ (t_0 = dead time).

2. Experimental synthetic protocols and analytical data of compounds 1-9, 11 and 12

Compound Characterization. Synthesized peptides were characterized by ^{1}H-NMR spectroscopy, HRMS, and RP-HPLC analysis. Additionally, ^{1}H-COSY NMR spectra were acquired of peptides **2**-**6**, **9** and **11**. The purity of all final compounds, determined by RP-HPLC (220 nm), was ≥ 98%.

Annotation concerning the ^{1}H-NMR spectra (solvent: DMSO-d_6) of **4**, **5**, **7**-**9**, **11** and **12**: in order to allow an integration of the signals interfering with the broad water signal at ca 3.5 ppm, ^{1}H-NMR spectra were additionally recorded in DMSO-d_6/D_2O (11:1 or 4:1 v/v).

General Procedure for SPPS. Peptides were synthesized by manual SPPS according to the Fmoc strategy. 5-mL polypropylene/polyethylene syringes from B. Braun Melsungen (Melsungen, Germany) or 5-mL BD Discardit II syringes (Becton Dickinson, Heidelberg, Germany) equipped with a polyethylene frit (pore size 35 μm) served as reaction vessels. Protected standard L-amino acids and protected N-methylated L-amino acids were purchased from Merck Biosciences (Schwalbach am Taunus, Germany) (Fmoc-N-Me-Arg(Pbf)-OH, Fmoc-Ile-OH, Fmoc-N-Me-Ile-OH, Fmoc-N-Me-Leu-OH, Fmoc-Pro-OH, Fmoc-Tle-OH (Fmoc-α-*tert*-butylglycine)) or from Iris Biotech (Fmoc-Arg(Pbf)-OH, Fmoc-Tyr(*t*Bu)-OH, Fmoc-N-Me-Tyr(*t*Bu)-OH). The H-Leu-2-ClTrt resin and the 2-ClTrt-Cl resin were purchased from Merck Biosciences and Iris Biotech, respectively. A mixture of DMF and NMP (80:20 v/v) was used as solvent (ca. 2.2 mL per 1 mmol Fmoc-aa) for the coupling reactions and for Fmoc deprotection. In case of using the H-Leu-2-ClTrt solid support (synthesis of **2**-**8** and **11**), the resin was allowed to swell in the solvent for 45 min before the beginning of the synthesis. Protected standard amino acids (Fmoc-Arg(Pbf)-OH, Fmoc-Ile-OH, Fmoc-Pro-OH, Fmoc-Tyr(*t*Bu)-OH) as well as Fmoc-Tle-OH were used in 5-fold excess, and the protected N-methylated amino acids Fmoc-N-Me-Arg(Pbf)-OH, Fmoc-N-Me-Ile-OH and Fmoc-N-Me-Tyr(*t*Bu)-OH were used in 3.5-fold excess. Amino acid coupling was performed with HBTU/HOBt/DIPEA (standard amino acids and Fmoc-Tle-OH: 4.9/5/10 equiv., N-methylated amino acids: 3.45/3.5/7 equiv.). For the coupling of N-methylated amino acids anhydrous solvents (DMF, NMP) were used. All amino acids were pre-incubated with HBTU/HOBt/DIPEA at rt for 5 min prior to addition to the resin. The coupling reactions were performed under shaking at 35 °C (standard amino acids: 2 × 1 h ("double" coupling), N-methylated amino acids: 2 × 2 h). After coupling, the resin was washed with solvent (4 ×). Fmoc deprotection was performed with piperidine (20% in DMF/NMP (80:20 v/v)) at rt for

2 × 8-10 min. After Fmoc deprotection the resin was washed with solvent (6 ×). The last coupling and Fmoc deprotection step was followed by washing of the resin with solvent (4 ×) and CH_2Cl_2 (treated with K_2CO_3) (6 ×). Peptides were cleaved off the resin with CH_2Cl_2/HFIP (3:1 v/v) (rt, 2 × 20 min). The solutions, containing the crude peptide, were separated from the resin by filtration and the resin was washed with CH_2Cl_2/HFIP (3:1 v/v). The filtrates were combined, the volatiles were evaporated, TFA/water (95:5 v/v) (1-3 mL) was added and the mixture was stirred at rt for 3 h (after 2 h additional TFA (70-300 µL) was added). The mixture was transferred to a 100-mL flask containing water (33-100 mL) followed by lyophilization to obtain the crude peptides, which were purified by preparative HPLC.

Chemistry: synthesis protocols and analytical data of compounds 2-9, 11 and 12.

Arg-Arg-Pro-Tyr-2-*tert*-butyl-Gly-Leu tris(hydrotrifluoroacetate) (**2**). Peptide **2** was synthesized according to the general procedure using a H-Leu-2-ClTrt resin (loading 0.79 mmol/g) (100 mg, 0.079 mmol). Purification by preparative RP-HPLC (column: Kinetex-XB C18, gradient: 0–35 min: A1/B 92:8–57:43, t_R = 18 min) afforded **2** as white fluffy solid (70.6 mg, 77%). ^{1}H-NMR (600 MHz, DMSO-d_6): δ 0.79-0.95 (m, 15H), 1.43-1.74 (m, 11H), 1.74-1.92 (m, 3H), 1.92-2.06 (m, 1H), 2.63-2.76 (m, 1H), 2.80-2.95 (m, 1H), 3.03-3.17 (m, 4H), 3.48-3.69 (m, 2H), 3.73-3.95 (m, 1H), 4.17-4.25 (m, 1H), 4.25-4.31 (m, 1H), 4.31-4.39 (m, 1H), 4.40-4.57 (m, 2H), 6.56-6.68 (m, 2H), 6.68-7.13 (br s, 4H, interfering with the next listed signal), 6.98-7.01 (m, 2H), 7.13-7.55 (br s, 4H), 7.55-7.73 (m, 3H), 7.98 (d, 1H, *J* 7.9 Hz), 8.02-8.57 (m, 4H), 8.65 (d, 1H, *J* 6.7 Hz), 9.19 (s, 1H), 12.48 (br s, 1H). HRMS (LC method A): *m/z* $[M+2H]^{2+}$ calcd. for $[C_{38}H_{66}N_{12}O_8]^{2+}$ 409.2558, found 409.2563. RP-HPLC (220 nm): 99% (t_R = 6.3 min, k = 7.3). $C_{38}H_{64}N_{12}O_8 \cdot C_6H_3F_9O_6$ (817.01 + 342.07).

***N*$^{\alpha}$-Methyl-Arg-Arg-Pro-Tyr-Ile-Leu tris(hydrotrifluoroacetate)**[2] (**3**). Peptide **3** was synthesized according to the general procedure using a H-Leu-2-ClTrt resin (loading 0.79 mmol/g) (50 mg, 0.0395 mmol). Purification by preparative RP-HPLC (column: Kinetex-XB C18, gradient: 0–35 min: A1/B 92:8–57:43, t_R = 19 min) afforded **3** as white fluffy solid (30.8 mg, 67%). ^{1}H-NMR (600 MHz, DMSO-d_6): δ 0.78-0.92 (m, 12H), 0.99-1.09 (m, 1H), 1.38-1.87 (m, 16H), 1.92-2.06 (m, 1H), 2.41-2.48 (m, 3H), 2.67 (dd, 1H, *J* 8.3, 14.0 Hz), 2.83-2.91 (m, 1H), 3.02-3.21 (m, 4H), 3.48-3.67 (m, 2H), 3.69-3.88 (m, 1H), 4.14-4.29 (m, 2H), 4.29-4.38 (m, 1H), 4.38-4.59 (m, 2H), 6.58-6.64 (m, 2H), 6.64-7.15 (br s, 4H,

interfering with the next listed signal), 6.98-7.01 (m, 2H), 7.15-7.59 (br s, 4H), 7.59-7.85 (m, 3H), 7.90 (d, 1H, *J* 7.9 Hz), 8.20 (d, 1H, *J* 7.9 Hz), 8.64-9.29 (m, 4H), 12.39 (br s, 1H). HRMS (LC method A): *m/z* $[M+2H]^{2+}$ calcd. for $[C_{39}H_{68}N_{12}O_8]^{2+}$ 416.2636, found 416.2645. RP-HPLC (220 nm): > 99% (t_R = 6.6 min, *k* = 7.7). $C_{39}H_{66}N_{12}O_8 \cdot C_6H_3F_9O_6$ (831.03 + 342.07).

N^{α}-Argininyl-N^{α}-methyl-Arg-Pro-Tyr-Ile-Leu tris(hydrotrifluoroacetate)[3] (**4**). Peptide **4** was synthesized according to the general procedure using a H-Leu-2-ClTrt resin (loading 0.79 mmol/g) (50 mg, 0.0395 mmol). Purification by preparative RP-HPLC (column: Kinetex-XB C18, gradient: 0–35 min: A1/B 92:8–57:43, t_R = 19 min) afforded **4** as white fluffy solid (26.0 mg, 56%). ^{1}H-NMR (600 MHz, DMSO-d_6): δ 0.78-0.86 (m, 9H), 0.90 (d, 3H, *J* 6.6 Hz), 1.02-1.10 (m, 1H), 1.37-1.46 (m, 3H), 1.49-1.80 (m, 13H), 1.94-2.02 (m, 1H), 2.68 (dd, 1H, *J* 8.7, 14.1 Hz), 2.85-2.90 (m, 1H), 2.92 (s, 3H), 3.06-3.15 (m, 4H), 3.27-3.29 (m, 1H), 3.42-3.55 (m, 1H), 4.15-4.25 (m, 2H), 4.25-4.33 (m, 1H), 4.33-4.51 (m, 2H), 5.08-5.19 (m, 1H), 6.58-6.67 (m, 2H), 6.67-7.13 (br s, 4H, interfering with the next listed signal), 6.99-7.02 (m, 2H), 7.13-7.61 (br s, 4H, interfering with the next listed signal), 7.52-7.56 (m, 1H), 7.63-7.77 (m, 2H), 7.91 (d, 1H, *J* 7.8 Hz), 7.99-8.36 (m, 4H), 9.17 (s, 1H), 12.49 (br s, 1H). HRMS (LC method A): *m/z* $[M+H]^+$ calcd. for $[C_{39}H_{67}N_{12}O_8]^+$ 831.5199, found 831.5202. RP-HPLC (220 nm): > 99% (t_R = 6.7 min, *k* = 7.8). $C_{39}H_{66}N_{12}O_8 \cdot C_6H_3F_9O_6$ (831.03 + 342.07).

Arg-Arg-Pro-N^{α}-methyl-Tyr-Ile-Leu tris(hydroacetate) (**5**).[3-4] Peptide **5** was synthesized according to the general procedure using a H-Leu-2-ClTrt resin (loading 0.79 mmol/g) (100 mg, 0.079 mmol). Purification by preparative RP-HPLC (column: YMC-Actus Triart C8, gradient: 0–30 min: A2/B 90:10–65:35, t_R = 17 min) afforded **5** as white solid (14.6 mg, 18%). ^{1}H-NMR (600 MHz, DMSO-d_6): δ 0.74-0.89 (m, 13H), 0.97-1.08 (m, 1H), 1.11-1.19 (m, 1H), 1.24-1.32 (m, 1H), 1.33-1.66 (m, 12H), 1.79-1.85 (m, 11H), 2.63-2.72 (m, 3H), 2.74-2.79 (m, 1H), 2.88-2.92 (m, 1H), 2.99-3.05 (m, 2H), 3.11-3.15 (m, 3H), 3.44-3.46 (m, 2H), 3.97-4.00 (m, 1H), 4.21-4.27 (m, 1H), 4.40-4.47 (m, 1H), 4.53-4.60 (m, 1H), 4.95-5.04 (m, 1H), 6.60-6.67 (m, 2H), 6.99-7.08 (m, 2H), 7.27-7.81 (m, 9H), 8.41-8.52 (m, 1H), 8.58-8.74 (m, 2H), 9.19 (s, 1H). Note: five exchangeable protons (NH, OH) of the 3-fold protonated molecule were not apparent. HRMS (LC method A): *m/z* $[M+H]^+$ calcd. for $[C_{39}H_{67}N_{12}O_8]^+$ 831.5199, found 831.5194. RP-HPLC (220 nm): 99% (t_R = 7.6 min, *k* = 9.0). $C_{39}H_{66}N_{12}O_8 \cdot C_6H_{12}O_6$ (831.03 + 180.16).

Arg-Arg-Pro-Tyr-*N*$^{\alpha}$-methyl-Ile-Leu tris(hydrotrifluoroacetate) (**6**).[3] Peptide **6** was synthesized according to the general procedure using a H-Leu-2-ClTrt resin (loading 0.79 mmol/g) (50 mg, 0.0395 mmol). Purification by preparative RP-HPLC (column: Kinetex-XB C18, gradient: 0–35 min: A1/B 92:8–57:43, t_R = 20 min) afforded **6** as white fluffy solid (6.7 mg, 15%). ^{1}H-NMR (600 MHz, DMSO-d_6): δ 0.72-0.93 (m, 13H), 1.17-1.25 (m, 1H), 1.40-1.76 (m, 12H), 1.78-1.92 (m, 3H), 1.96-2.12 (m, 1H), 2.61-2.98 (m, 5H), 2.98-3.27 (m, 4H), 3.52-3.73 (m, 2H), 3.74-3.89 (m, 1H), 4.04-4.22 (m, 1H), 4.31-4.39 (m, 1H), 4.43-4.52 (m, 1H), 4.68-5.07 (m, 2H), 6.57-6.68 (m, 2H), 6.68-7.13 (br s, 4H, interfering with the next listed signal), 6.94-6.99 (m, 2H), 7.13-7.56 (br s, 4H), 7.56-7.78 (m, 2H), 7.86-8.90 (m, 6H), 9.24 (s, 1H), 12.38 (br s, 1H). HRMS (LC method A): *m/z* $[M+2H]^{2+}$ calcd. for $[C_{39}H_{68}N_{12}O_8]^{2+}$ 416.2636, found 416.2643. RP-HPLC (220 nm): > 99% (t_R = 7.0 min, k = 8.2). $C_{39}H_{66}N_{12}O_8 \cdot C_6H_3F_9O_6$ (831.03 + 342.07).

***N*$^{\alpha}$-Methyl-Arg-Arg-Pro-Tyr-2-*tert*-butyl-Gly-Leu tris(hydrotrifluoroacetate)** (**7**). Peptide **7** was synthesized according to the general procedure using a H-Leu-2-ClTrt resin (loading 0.79 mmol/g) (45 mg, 0.0356 mmol). Purification by preparative RP-HPLC (column: Kinetex-XB C18, gradient: 0–35 min: A1/B 92:8–57:43, t_R = 17 min) afforded **7** as white fluffy solid (17.7 mg, 42%). ^{1}H-NMR (600 MHz, DMSO-d_6): δ 0.83 (d, 3H, *J* 6.5 Hz), 0.85-0.94 (m, 12H), 1.41-1.87 (m, 14H), 1.93-2.05 (m, 1H), 2.46-2.48 (m, 3H), 2.68 (dd, 1H, *J* 8.4, 14.1 Hz), 2.85-2.91 (m, 1H), 3.05-3.14 (m, 4H), 3.54-3.57 (m, 1H), 3.59-3.61 (m, 1H), 3.75-3.80 (m, 1H), 4.18-4.24 (m, 1H), 4.26-4.30 (m, 1H), 4.32-4.38 (m, 1H), 4.43-4.60 (m, 2H), 6.61 (d, 2H, *J* 8.5 Hz), 6.64-7.12 (br s, 4H, interfering with the next listed signal), 6.98-7.00 (m, 2H), 7.12-7.55 (br s, 4H), 7.55-7.67 (m, 3H), 7.89-8.09 (m, 1H), 8.15-8.30 (m, 1H), 8.72-9.07 (m, 3H), 9.10-9.27 (m, 1H), 12.48 (br s, 1H). HRMS (LC method A): *m/z* $[M+2H]^{2+}$ calcd. for $[C_{39}H_{68}N_{12}O_8]^{2+}$ 416.2636, found 416.2644. RP-HPLC (220 nm): > 99% (t_R = 6.3 min, k = 7.3). $C_{39}H_{66}N_{12}O_8 \cdot C_6H_3F_9O_6$ (831.03 + 342.07).

***N*$^{\alpha}$-Argininyl-*N*$^{\alpha}$-methyl-Arg-Pro-Tyr-2-*tert*-butyl-Gly-Leu tris(hydrotrifluoroacetate)** (**8**). Peptide **8** was synthesized according to the general procedure using a H-Leu-2-ClTrt resin (loading 0.79 mmol/g) (45 mg, 0.0356 mmol). Purification by preparative RP-HPLC (column: Kinetex-XB C18, gradient: 0–35 min: A1/B 92:8–57:43, t_R = 18 min) afforded **8** as white fluffy solid (15.9 mg, 38%). ^{1}H-NMR (600 MHz, DMSO-d_6): δ 0.81-0.85 (m, 3H), 0.87-0.93 (m, 12H), 1.40-1.79 (m, 14H), 1.93-2.02 (m, 1H), 2.64-2.72 (m, 1H), 2.85-2.96 (m, 4H), 3.08-3.15 (m, 4H), 3.30-3.32 (m, 1H), 3.50-3.52 (m, 1H), 4.18-4.24 (m, 1H), 4.25-4.32 (m, 2H),

4.32-4.39 (m, 1H), 4.41-4.49 (m, 1H), 5.09-5.16 (m, 1H), 6.59-6.64 (m, 2H), 6.64-7.12 (br s, 4H, interfering with the next listed signal), 6.99-7.02 (m, 2H), 7.12-7.52 (br s, 4H), 7.52-7.61 (m, 2H), 7.69-7.73 (m, 1H), 7.95-8.01 (m, 1H), 8.06-8.29 (m, 4H), 9.17 (s, 1H), 12.47 (br s, 1H). HRMS (LC method A): *m/z* $[M+2H]^{2+}$ calcd. for $[C_{39}H_{68}N_{12}O_8]^{2+}$ 416.2636, found 416.2646. RP-HPLC (220 nm): > 99% (t_R = 6.3 min, k = 7.3). $C_{39}H_{66}N_{12}O_8 \cdot C_6H_3F_9O_6$ (831.03 + 342.07).

Arg-Arg-Pro-Tyr-Ile-N^{α}-methyl-Leu tris(hydrotrifluoroacetate) (9).[3] Peptide **9** was synthesized on a 2-ClTrt-Cl resin (loading 1.6 mmol/g) (50 mg, 0.08 mmol). The resin was treated with a solution of Fmoc-N-Me-Leu-OH (29.4 mg, 0.08 mmol) and DIPEA (34.8 µL, 0.2 mmol) in CH_2Cl_2 (0.5 mL) at 35 °C for 14 h. MeOH (50 µL) and CH_2Cl_2 (100 µL) were added and shaking was continued at rt for 15 min. The liquid was removed by filtration and the resin was washed with CH_2Cl_2 (3 ×), MeOH (3 ×) and DMF/NMP (80:20 v/v) (4 ×). The loading of the resin with Fmoc-N-Me-Leu was estimated to amount to 0.8 mmol/g (50 mg, 0.04 mmol) (basis for the calculation of the yield). Fmoc-deprotection of N-Me-Leu and further SPPS was performed according to the general procedure. Purification by preparative RP-HPLC (column: Kinetex-XB C18, gradient: 0–20 min: A1/B 92:8–72:28, 20–42 min: A1/B 72:28–35:65, t_R = 22 min) afforded **9** as white fluffy solid (9.1 mg, 20%). ^{1}H-NMR (600 MHz, DMSO-d_6): δ 0.73-0.90 (m, 12H), 1.01-1.12 (m, 1H), 1.30-1.38 (m, 1H), 1.44-1.60 (m, 7H), 1.62-1.73 (m, 4H), 1.75-1.87 (m, 4H), 1.95-2.03 (m, 1H), 2.56-2.62 (m, 1H), 2.75-2.80 (m, 1H), 2.95 (s, 3H), 3.05-3.12 (m, 4H), 3.54-3.56 (m, 1H), 3.58-3.60 (m, 1H), 3.78-3.82 (m, 1H), 4.29-4.34 (m, 1H), 4.38-4.52 (m, 2H), 4.54-4.64 (m, 1H), 5.05-5.17 (m, 1H), 6.59-6.66 (m, 2H), 6.68-7.13 (br s, 4H, interfering with the next listed signal), 7.00-7.03 (m, 2H), 7.13-7.56 (br s, 4H), 7.56-7.67 (m, 2H), 7.81-8.04 (m, 1H), 8.06-8.30 (m, 4H), 8.48-8.70 (m, 1H), 9.20 (s, 1H), 12.71 (br s, 1H). HRMS (LC method A): *m/z* $[M+2H]^{2+}$ calcd. for $[C_{39}H_{68}N_{12}O_8]^{2+}$ 416.2636, found 416.2644. RP-HPLC (220 nm): > 99% (t_R = 8.2 min, k = 9.8). $C_{39}H_{66}N_{12}O_8 \cdot C_6H_3F_9O_6$ (831.03 + 342.07).

N^{α}-Propanoyl-Arg-Arg-Pro-Tyr-Ile-Leu bis(hydrotrifluoroacetate) (11). Peptide **11** was synthesized according to the general procedure using a H-Leu-2-ClTrt resin (loading 0.79 mmol/g) (50 mg, 0.0395 mmol). After the last amino acid coupling and Fmoc deprotection step, the resin was treated with a solution of **10** (20.3 mg, 0.119 mmol, 3 equiv.) and DIPEA (20.6 µL, 0.119 mmol, 3 equiv.) in DMF/NMP (80:20 v/v) (2 mL) under shaking at rt for 1 h. The resin was washed with DMF/NMP (80:20 v/v) (6 ×) and the peptide was

cleaved off the resin as described in the general procedure. Purification by preparative RP-HPLC (column: Kinetex-XB C18, gradient: 0–35 min: A1/B 92:8–57:43, t_R = 21 min) afforded **11** as white fluffy solid (24.5 mg, 56%). ^{1}H-NMR (600 MHz, DMSO-d_6): δ 0.76-0.81 (m, 3H), 0.84 (d, 6H, *J* 6.4 Hz), 0.90 (d, 3H, *J* 6.6 Hz), 0.94-1.01 (m, 3H), 1.01-1.10 (m, 1H), 1.39-1.57 (m, 9H), 1.59-1.72 (m, 4H), 1.72-1.87 (m, 3H), 1.95-2.02 (m, 1H), 2.08-2.18 (m, 2H), 2.68 (dd, 1H, *J* 8.2, 14.0 Hz), 2.86 (dd, 1H, *J* 5.1, 14.0 Hz), 3.03-3.12 (m, 4H), 3.57-3.58 (m, 1H), 3.60-3.61 (m, 1H), 4.17-4.24 (m, 2H), 4.24-4.30 (m, 1H), 4.30-4.36 (m, 1H), 4.36-4.43 (m, 1H), 4.43-4.50 (m, 1H), 6.52-6.62 (m, 2H), 6.62-7.06 (br s, 4H, interfering with the next listed signal), 6.98-7.01 (m, 2H), 7.06-7.62 (br s, 4H, interfering with the next listed signal), 7.43-7.50 (m, 2H), 7.69-7.79 (m, 1H), 7.79-8.02 (m, 2H), 8.02-8.16 (m, 1H), 8.16-8.30 (m, 1H), 9.01-9.29 (m, 1H), 12.49 (br s, 1H). HRMS (LC method A): *m/z* $[M+2H]^{2+}$ calcd. for $[C_{41}H_{70}N_{12}O_9]^{2+}$ 437.2689, found 437.2697. RP-HPLC (220 nm): > 98% (t_R = 8.1 min, *k* = 9.7). $C_{41}H_{68}N_{12}O_9 \cdot C_4H_2F_6O_4$ (873.07 + 228.05).

***N*$^{\alpha}$-Propanoyl-Arg-Arg-Pro-Tyr-2-*tert*-butyl-Gly-Leu bis(hydrotrifluoroacetate) (12)**. Peptide **2** (22.2 mg, 0.0191 mmol) was dissolved in DMF/NMP (75:25 v/v) (180 µL). A solution of DIPEA (10 µL, 0.057 mmol) and **10** (9.8 mg, 0.057 mmol) in anhydrous DMF (23 µL) was added and the mixture was stirred at rt for 1 h followed by the addition of TFA/H_2O (1:9 v/v) (ca 0.06 mmol TFA). Purification by preparative RP-HPLC (column: Kinetex-XB C18, gradient: 0–35 min: A1/B 92:8–57:43, t_R = 20 min) afforded **12** as white fluffy solid (17.9 mg, 85%). ^{1}H-NMR (600 MHz, DMSO-d_6): δ 0.81-0.92 (m, 15H), 0.97-1.01 (m, 3H), 1.40-1.68 (m, 11H), 1.68-1.86 (m, 3H), 1.93-2.04 (m, 1H), 2.07-2.18 (m, 2H), 2.68 (dd, 1H, *J* 8.2, 13.9 Hz), 2.85-2.90 (m, 1H), 3.03-3.12 (m, 4H), 3.49-3.50 (m, 1H), 3.58-3.61 (m, 1H), 4.18-4.24 (m, 1H), 4.24-4.31 (m, 2H), 4.31-4.38 (m, 1H), 4.41-4.48 (m, 2H), 6.53-6.62 (m, 2H), 6.62-7.04 (br s, 4H, interfering with the next listed signal), 6.98-7.01 (m, 2H), 7.04-7.56 (br s, 4H, interfering with the next listed signal), 7.43-7.49 (m, 2H), 7.56-7.63 (m, 1H), 7.82-7.94 (m, 1H), 7.94-8.02 (m, 1H), 8.02-8.12 (m, 1H), 8.22 (d, 1H, *J* 7.5 Hz), 9.16 (s, 1H), 12.47 (br s, 1H). HRMS (LC method A): *m/z* $[M+2H]^{2+}$ calcd. for $[C_{41}H_{70}N_{12}O_9]^{2+}$ 437.2689, found 437.2706. RP-HPLC (220 nm): > 99% (t_R = 7.7 min, *k* = 9.1). $C_{41}H_{68}N_{12}O_9 \cdot C_4H_2F_6O_4$ (873.07 + 228.05).

3. Radioligand competition binding assay

Competition binding with [^{3}H]UR-MK300 ([^{3}H]**13**) at the hNTS$_1$R was performed at 23 ± 1 °C using intact human HT-29 colon carcinoma cells (grown in antibiotic-free McCoy's 5A medium supplemented with 5% FCS) as described previously.[5] Prior to the competition binding experiments, the K_d value of [^{3}H]**13** was determined by saturation binding at HT-29 cells as reported previously (data not shown).[5] The obtained K_d value amounted to 0.55 ± 0.03 nM (mean value ± SEM from two independent determinations performed in triplicate) being in excellent agreement with the reported K_d of [^{3}H]**13** (K_d = 0.51 nM).[5] Binding data were analyzed by plotting % specifically bound radioligand (100% = specifically bound radioligand in the absence of competitor) over log(concentration of competitor) followed by a four-parameter sigmoidal fit (SigmaPlot 11.0, Systat Software). Resulting pIC_{50} values were converted to IC_{50} values and K_i values were calculated from the IC_{50} values according to the Cheng-Prusoff equation[6] using a K_d value of 0.55 nM.

4. Experimental protocol for the investigation of the stability of **1**-**9**, **11** and **12** in human plasma

The metabolic stabilities of **1**-**9**, **11** and **12** were investigated in human blood plasma/PBS, pH 7.4 (1:2 v/v) (in the following referred to as plasma/PBS) at 37 °C using 1.5- or 2-mL polypropylene reaction vessels. The vessels were placed in a Thermocell mixing block from Bioer (Hangzhou, China). 1-Methyl-D-tryptophan (**14**) was used as internal standard. As the RP-HPLC purity of **14** was < 95% (data not shown), the compound was purified by preparative HPLC to give a purity of > 99%. Plasma was obtained by the collection of human blood from a healthy donor in 5.5-mL heparinized plasma-monovettes followed by centrifugation at 1,200 g at 4 °C for 10 min. The supernatants were pooled in two 50-mL Falcon tubes and centrifuged again at 1,200 g at 4 °C for 10 min. The plasma was aliquoted and stored at −80 °C.

For the addition of the peptides to plasma/PBS, 5 mM stock solutions in EtOH/HCl (20 mM) (50:50 v/v) (**1**) or MeCN/0.04% aq TFA (30:70 v/v) (**2**-**9**, **11** and **12**) were used. Recoveries were determined for peptide concentrations of 80 µM and 4 µM, and an internal standard concentration of 10 µM. For this purpose, **14** and the peptide were added to plasma/PBS (total volume: 70 µL), immediately followed by vortexing (ca 10 s) and precipitation of protein by the addition of ice-cold EtOH/MeCN (50:50 v/v) (140 µL). The mixture was vortexed for 5 min und centrifuged at 16,100 g at 4 °C for 10 min. Aliquots (180 µL) of the supernatant were transferred to 1.5-mL reaction vessels containing 10% aq TFA (5 µL). The volatiles were removed in a vacuum concentrator under reduced pressure at 40 °C (ca 60 min) and the residue was taken up in MeCN/0.04% aq TFA (10:90 v/v) (90 µL) by vortexing (2 min). The samples were filtrated through a 0.2 µm RC-membrane filter (Phenomenex, Aschaffenburg, Germany) and analyzed by RP-HPLC using the analytical HPLC system and the conditions as for the purity control of **2**-**4**, **6**-**9**, **11** and **12** (general experimental conditions), but applying the following gradient: 0–12 min: A3/B 90:10–73:27, 12–16 min: 73:27–5:95, 16–20 min: 5:95. On the day of an experiment four-point calibrations were performed for the respective peptides and the internal standard. Peak areas representing 100% recovery were obtained by analyzing 53.3 µM and 2.67 µM peptide solutions as well as a 6.67 µM solution of **14** (in duplicate each). All peak areas were transformed into concentrations (µM) and percent recoveries of the peptides and the internal standard were calculated based on the average values of the 100% reference samples (see **Table S1**). Recovery ratios were obtained by

dividing the recovery of the peptide by the recovery of **14** for each individual sample (n = 3-5, *cf*. **Table S1**).

For the investigation of the stabilities in plasma, the peptides and **14** were added to plasma/PBS at a concentration of 100 µM and 10 µM, respectively (in triplicate each). After 10 min, 1 h, 24 h and 48 h (**1** and **2**), 10 min, 30 min, 1 h and 2 h (**3**-**6**, **9** and **11**), or 1 h, 6 h, 24 h and 48 h (**7**, **8** and **12**) of incubation under shaking at 37 °C aliquots (70 µL) were taken and processed as described above for the determination of recoveries. On the day of an experiment four-point calibrations were performed for the respective peptides and **14**. Peak areas representing 100% recovery were obtained by analyzing 66.7 µM peptide solutions and a 6.67 µM solution of **14** (in duplicate).

Based on the calibration, the peak areas of the 100% references and of the samples were transformed into concentrations (µM). Recoveries of **1**-**9**, **11** and **12** were calculated by multiplying the recovery of **14**, obtained for each individual sample, with the recovery ratio obtained for the concentration of 80 µM (remaining peptide concentration in plasma/PBS > 20 µM) or 4 µM (remaining peptide concentration in plasma/PBS < 20 µM). The concentrations of the peptides in plasma/PBS were obtained by dividing the determined peptide concentration by the respective recovery. Degradation (%) of **1**-**9**, **11** and **12** was calculated based on the average values of the 100% reference samples.

Note: Data analysis was based on UV detection at 220 nm (**1**, **3**-**9**, **11**, **12** and **14**) or fluorescence detection at 275/305 nm (**2**).

The major degradation fragments of peptides **3**-**9**, **11** and **12** were identified by LC-HRMS analysis (LC-MS instrument and conditions see general experimental conditions; LC method A (**1**, **2**) or B (**3**-**9**, **11**, **12**)) using the same samples as used for HPLC analysis described above.

Table S1. Recoveries of peptides **1-9**, **11** and **12** from human plasma/PBS (1:2 v/v) for two different concentrations (80 μM and 4 μM), and ratios of peptide-recovery over recovery of internal standard (**14**).

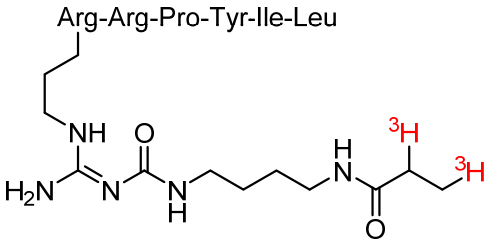
Compd.	Peptide concentration 80 μM			Peptide concentration 4 μM		
	recovery peptide (%)[a]	recovery **14** (%)[a]	ratio[b]	recovery peptide (%)[a]	recovery **14** (%)[a]	ratio[b]
1	78	91	0.85	79	92	0.86
	72	83	0.86	82	91	0.90
	80	89	0.90	76	93	0.82
	76	86	0.89	85	95	0.89
				80	95	0.85
			(0.88 ± 0.01)			(0.86 ± 0.01)
2	91	99	0.92	88	99	0.89
	90	96	0.93	86	90	0.95
	92	101	0.91	91	99	0.91
	92	103	0.90			
			(0.91 ± 0.01)			(0.92 ± 0.02)
3	101	95	1.06	92	94	0.98
	105	96	1.09	85	92	0.92
	100	97	1.03	91	91	1.01
	96	94	1.02	85	90	0.94
	96	93	1.02	93	90	1.04
			(1.04 ± 0.01)			(0.98 ± 0.02)
4	84	91	0.93	93	89	1.04
	77	82	0.94	97	93	1.04
	75	83	0.90	92	89	1.03
	92	96	0.96	87	84	1.03
	89	95	0.93	86	85	1.01
			(0.93 ± 0.01)			(1.03 ± 0.01)
5	88	95	0.92	96	92	1.05
	88	95	0.93	98	94	1.04
	87	93	0.93	98	94	1.05
	88	96	0.92	101	96	1.05
				97	92	1.06
			(0.92 ± 0.003)			(1.05 ± 0.003)
6	76	95	0.81	84	95	0.88
	89	104	0.86	83	98	0.85
	75	90	0.83	84	95	0.88
	82	98	0.84			
	78	99	0.80			
			(0.83 ± 0.01)			(0.87 ± 0.01)
7	89	94	0.95	105	98	1.07
	88	96	0.92	103	99	1.04
	93	99	0.94	108	105	1.03
	94	101	0.93	106	99	1.08
				107	106	1.01
			(0.93 ± 0.01)			(1.05 ± 0.01)
8	89	102	0.87	103	105	0.99
	87	98	0.89	103	101	1.02
	92	100	0.92	104	107	0.97
	93	103	0.90	100	100	0.99
	85	97	0.87	101	99	1.02
			(0.89 ± 0.01)			(1.00 ± 0.01)

Table S1 continued

9	90	104	0.87	85	92	0.92
	90	100	0.90	95	101	0.94
	88	98	0.90	90	99	0.91
	90	99	0.91			
			(0.89 ± 0.01)			(0.93 ± 0.01)
11	78	93	0.84	97	100	0.97
	89	101	0.88	85	88	0.96
	87	99	0.88	86	87	0.99
	81	93	0.87	101	104	0.97
	73	86	0.84			
			(0.86 ± 0.01)			(0.97 ± 0.005)
12	87	99	0.88	95	100	0.95
	86	99	0.87	100	102	0.97
	87	101	0.86	95	94	1.01
	92	101	0.91	103	103	1.00
	83	93	0.89	101	102	0.99
			(0.88 ± 0.01)			(0.98 ± 0.01)

[a]Recoveries of the peptides and of the internal standard (**14**) from human plasma/PBS (1:2 v/v) using a peptide concentration of 80 µM or 4 µM and an internal standard concentration of 10 µM (three, four or five independent experiments). [b]Ratios of peptide recovery over recovery of **14** calculated for individual experiments, as well as mean recovery ratios ± SEM (given in parenthesis).

5. Figures S1 and S2



[^{3}H]UR-MK300

Figure S1. Structure of the tritium-labeled NT(8-13) derivative [^{3}H]UR-MK300 ([^{3}H]**13**) used as radioligand for NTS_1R competition binding studies (reported K_d = 0.51 nM[5]).

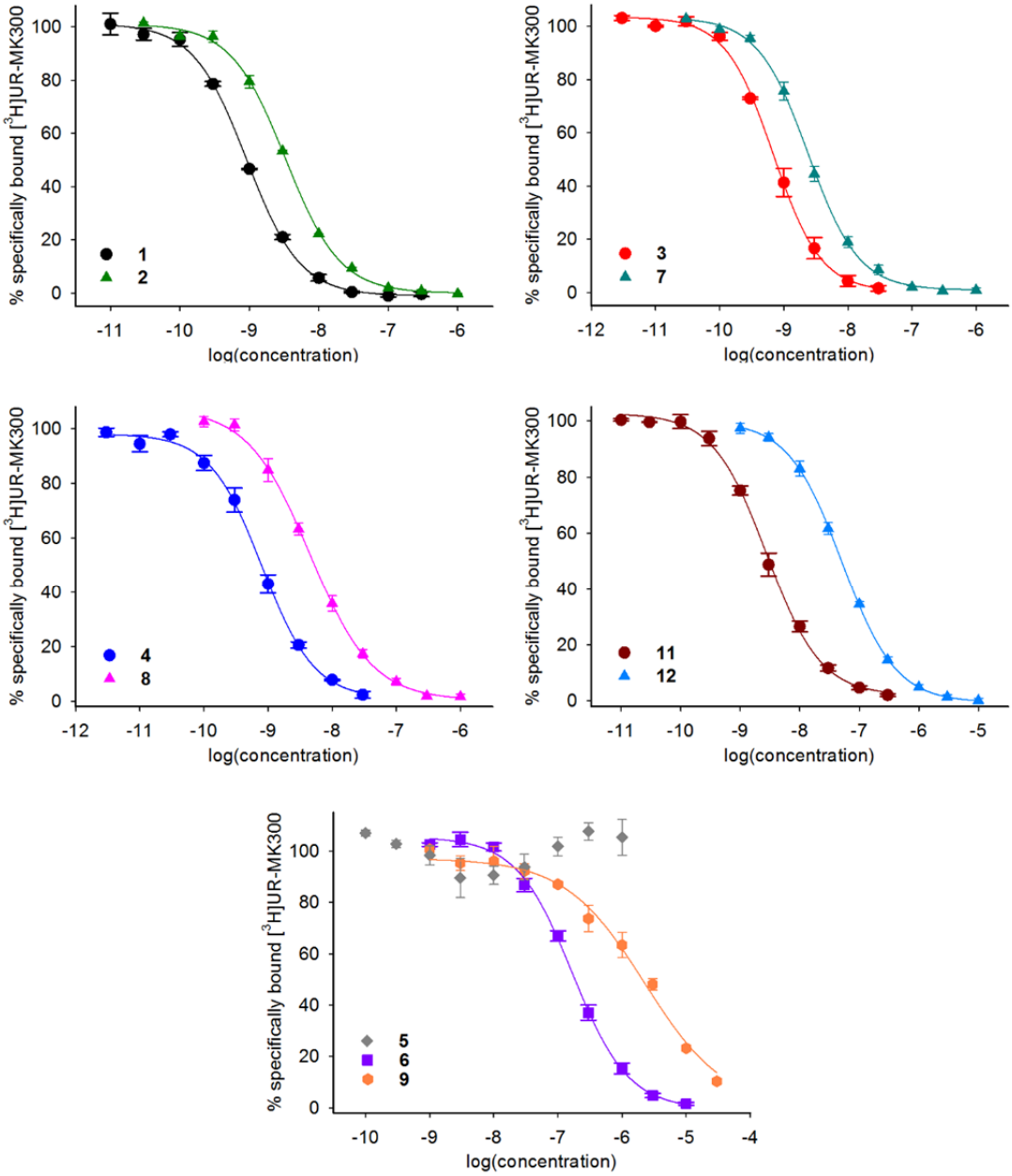
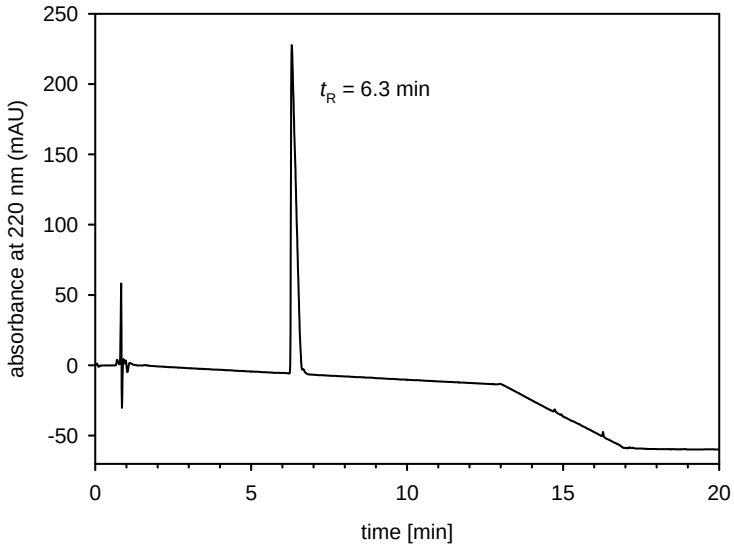
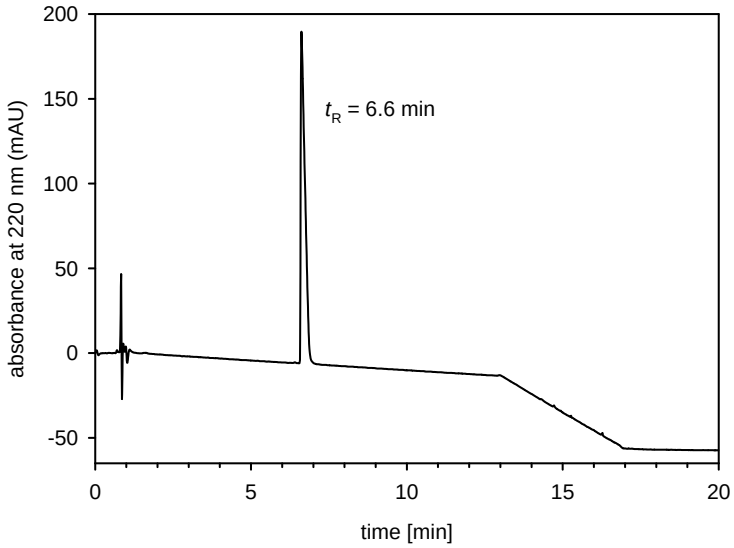


Figure S2. Displacement curves of [^{3}H]UR-MK300 ([^{3}H]**13**) (K_d = 0.55 nM, c = 1 nM) obtained from competition binding experiments with **1-9**, **11** and **12** at intact hNTS$_1$R expressing HT-29 cells. Compounds containing Ile^{12} are represented by circles, compounds containing Tle^{12} are represented by triangles. Data represent mean values ± SEM from at least two independent experiments (performed in triplicate).

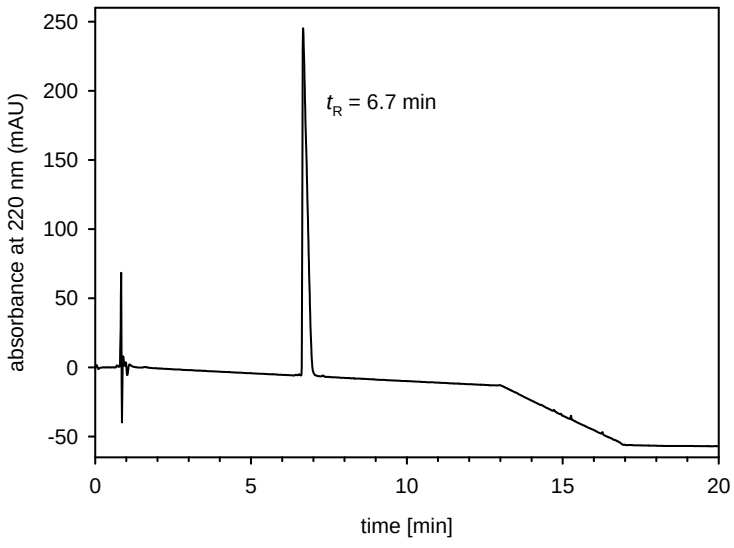
6. RP-HPLC chromatograms of compounds 2-9, 11 and 12



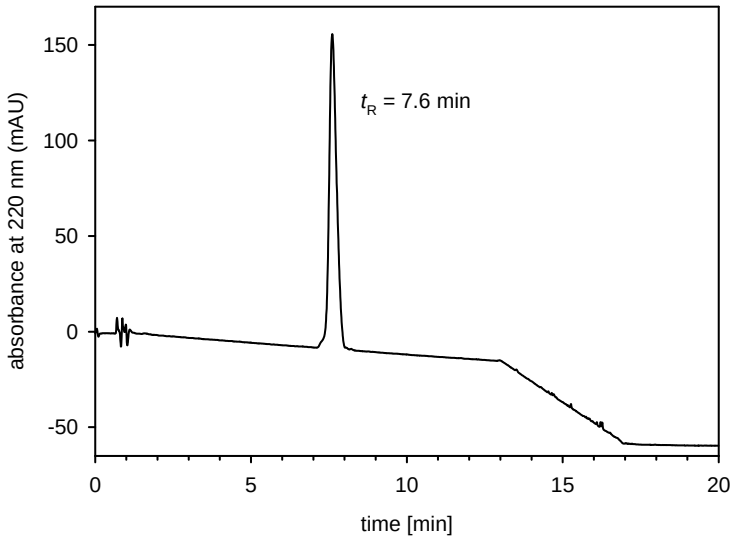
RP-HPLC analysis (purity control) of compound **2**



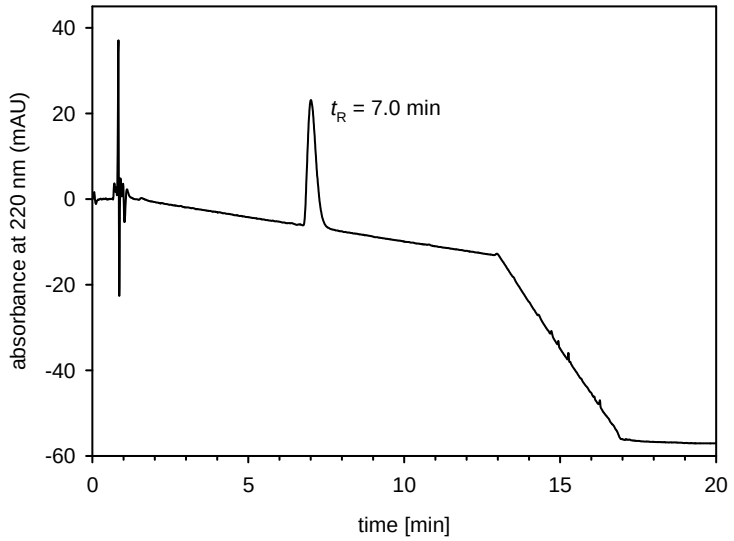
RP-HPLC analysis (purity control) of compound **3**



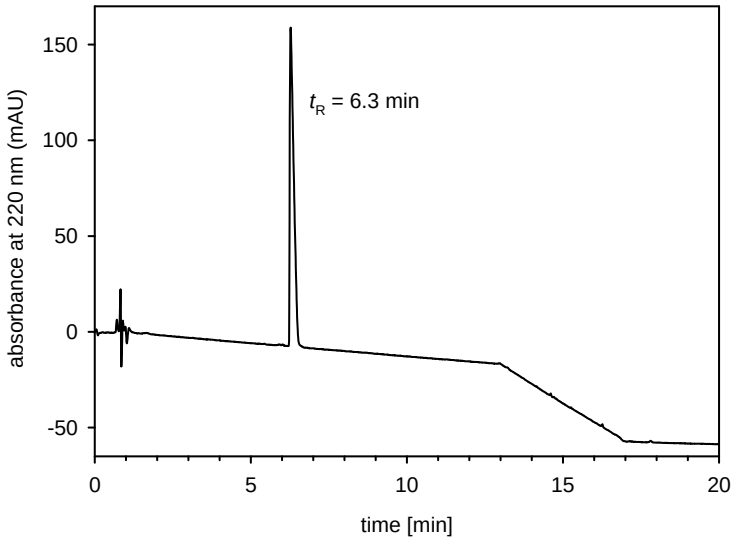
RP-HPLC analysis (purity control) of compound **4**



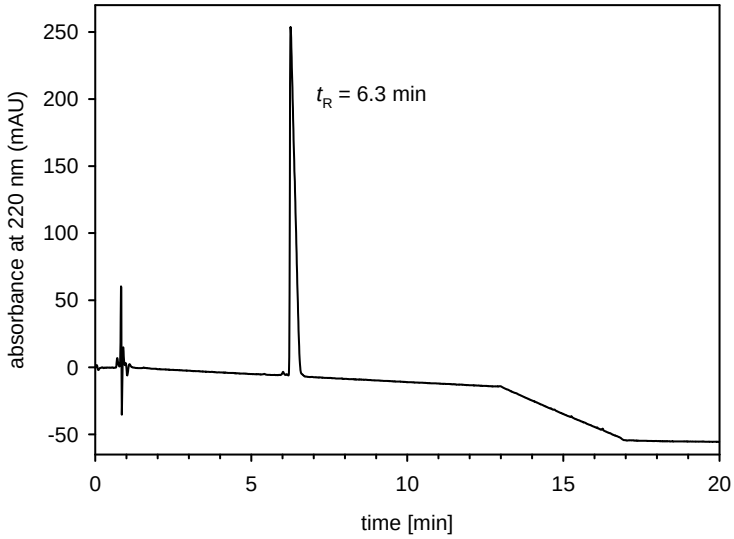
RP-HPLC analysis (purity control) of compound **5**



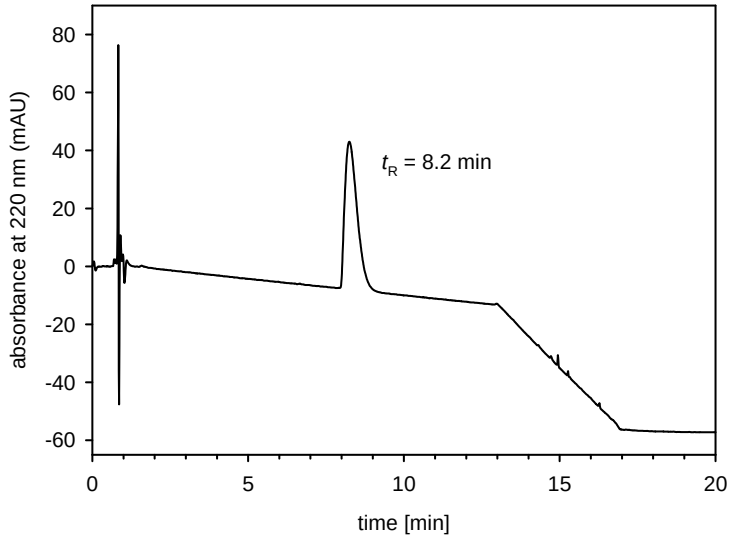
RP-HPLC analysis (purity control) of compound **6**



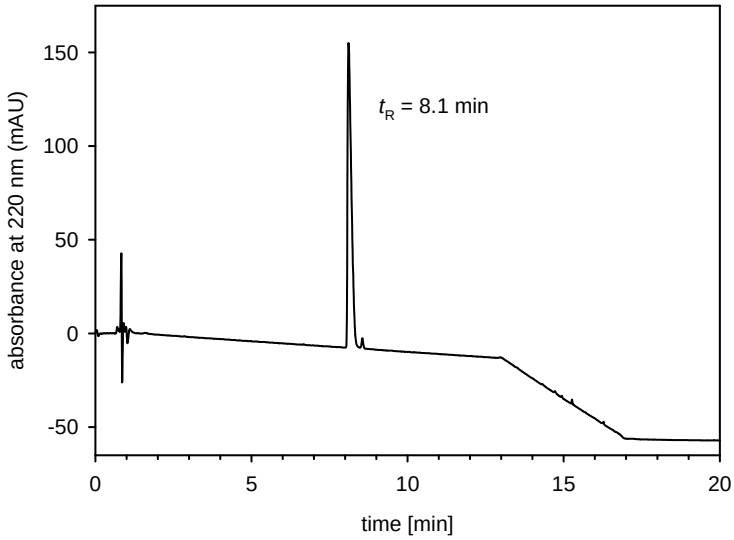
RP-HPLC analysis (purity control) of compound **7**



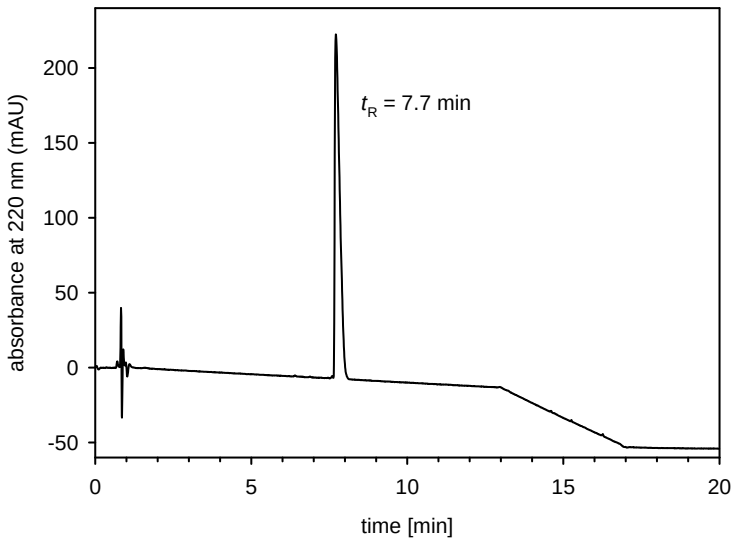
RP-HPLC analysis (purity control) of compound **8**



RP-HPLC analysis (purity control) of compound **9**

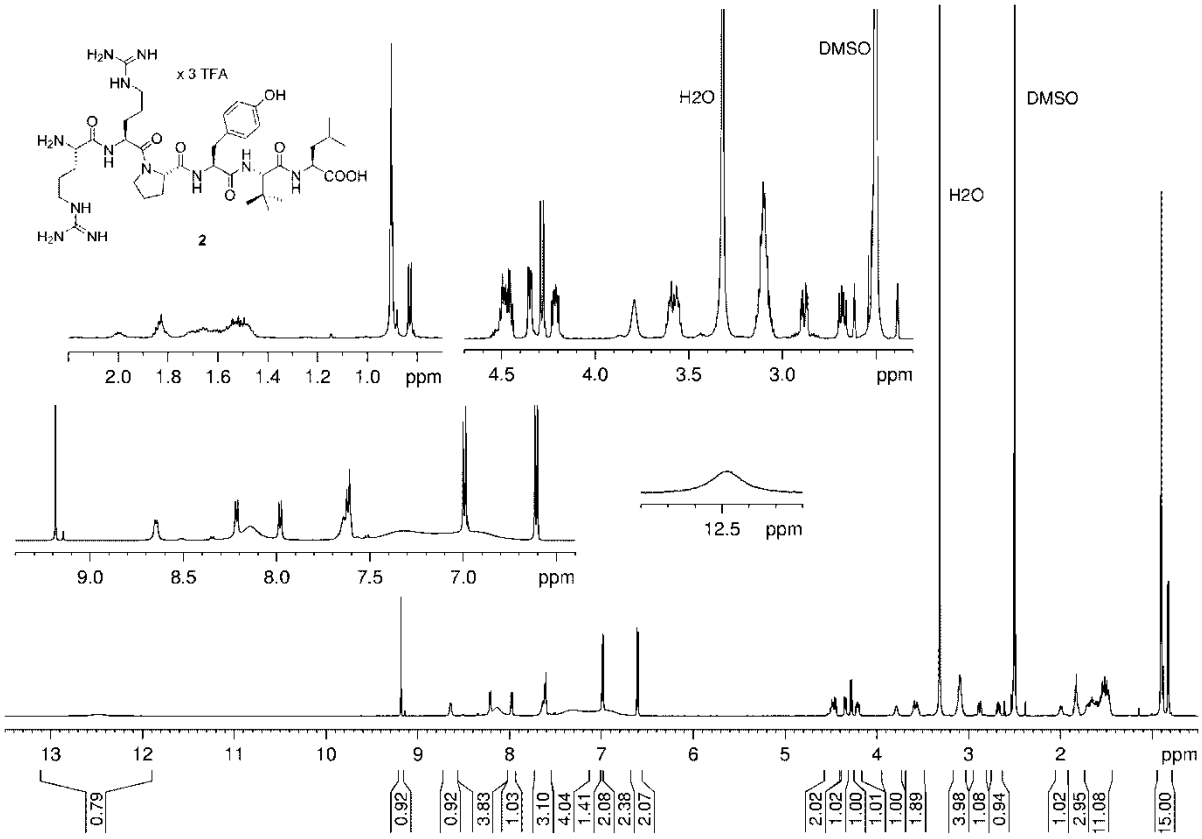


RP-HPLC analysis (purity control) of compound **11**

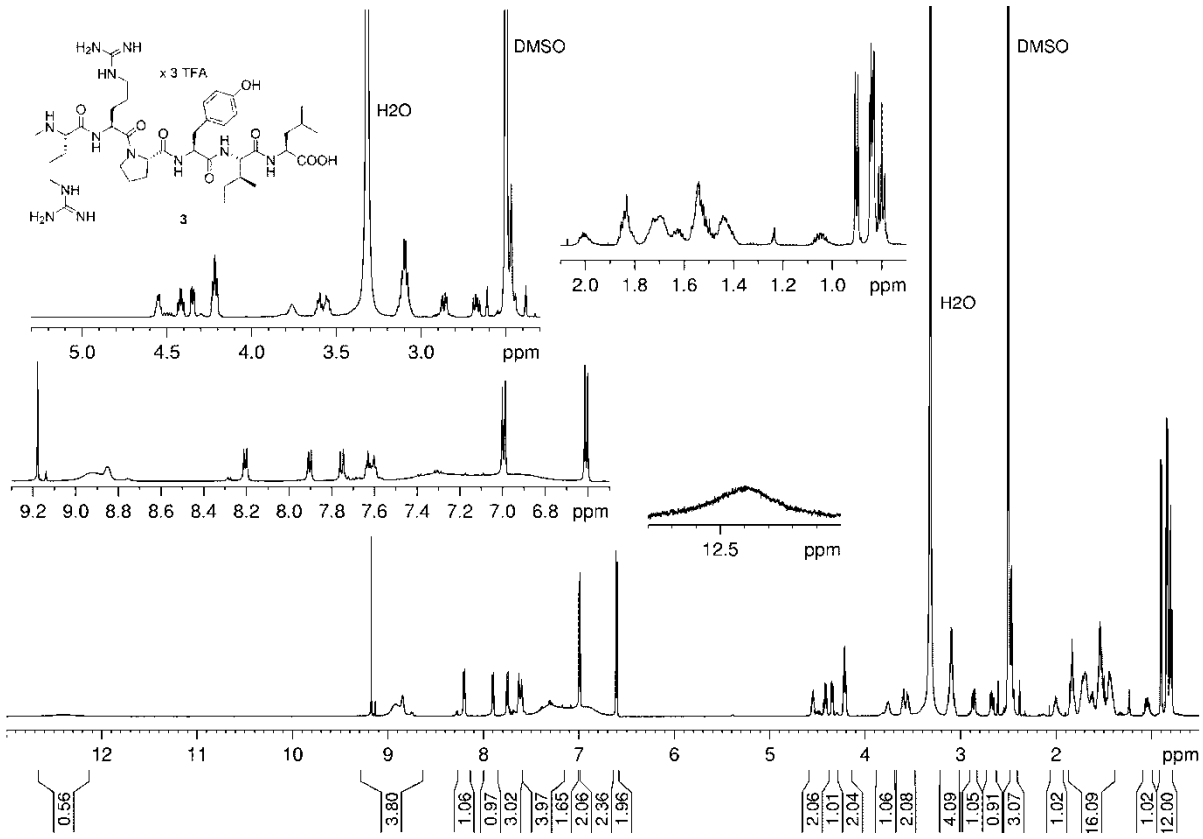


RP-HPLC analysis (purity control) of compound **12**

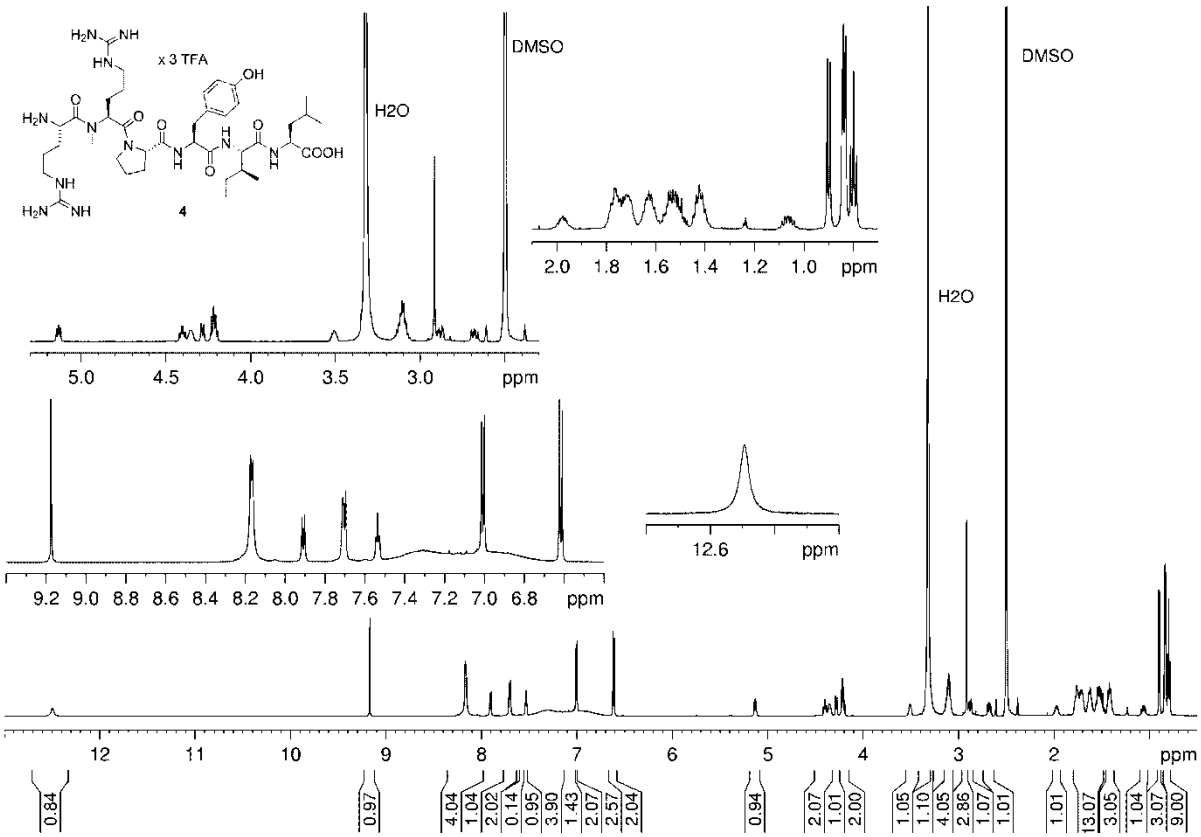
7. ^{1}H-NMR spectra of compounds 2-9, 11 and 12 in DMSO-d_6 and DMSO-d_6/D_2O



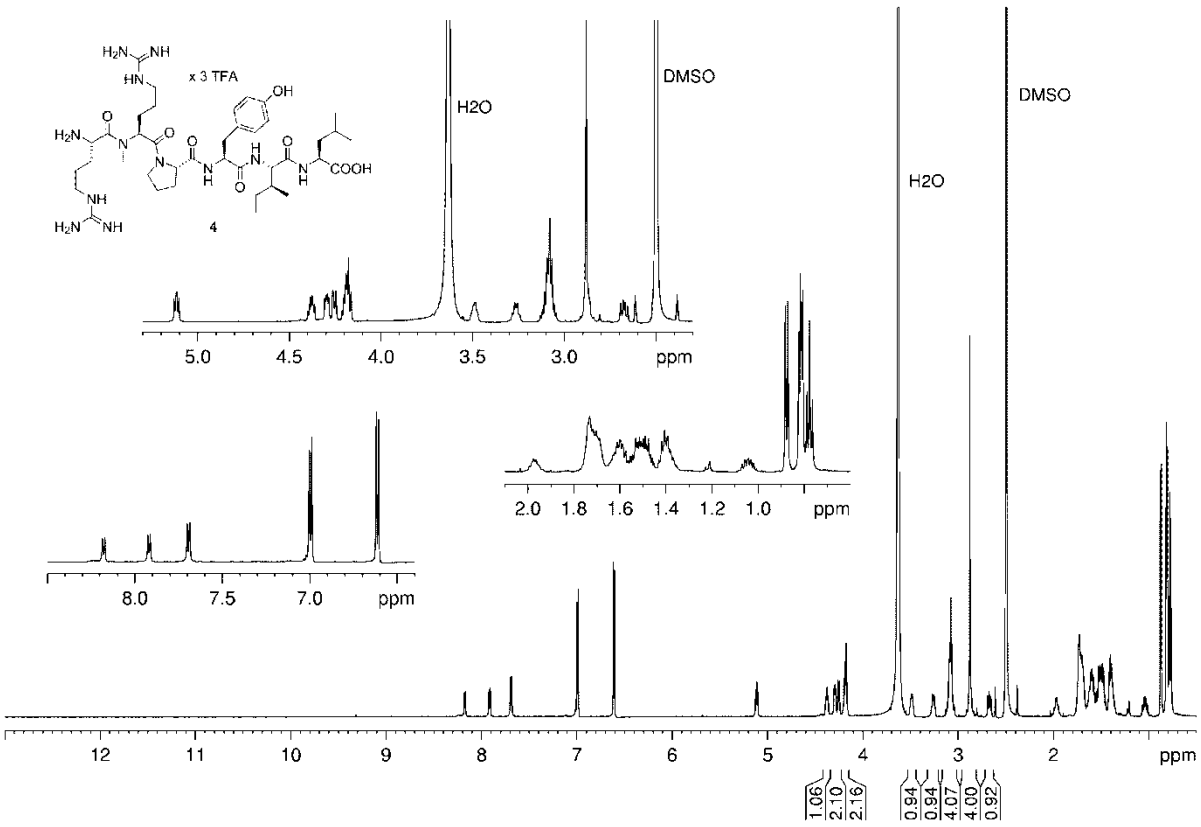
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **2**



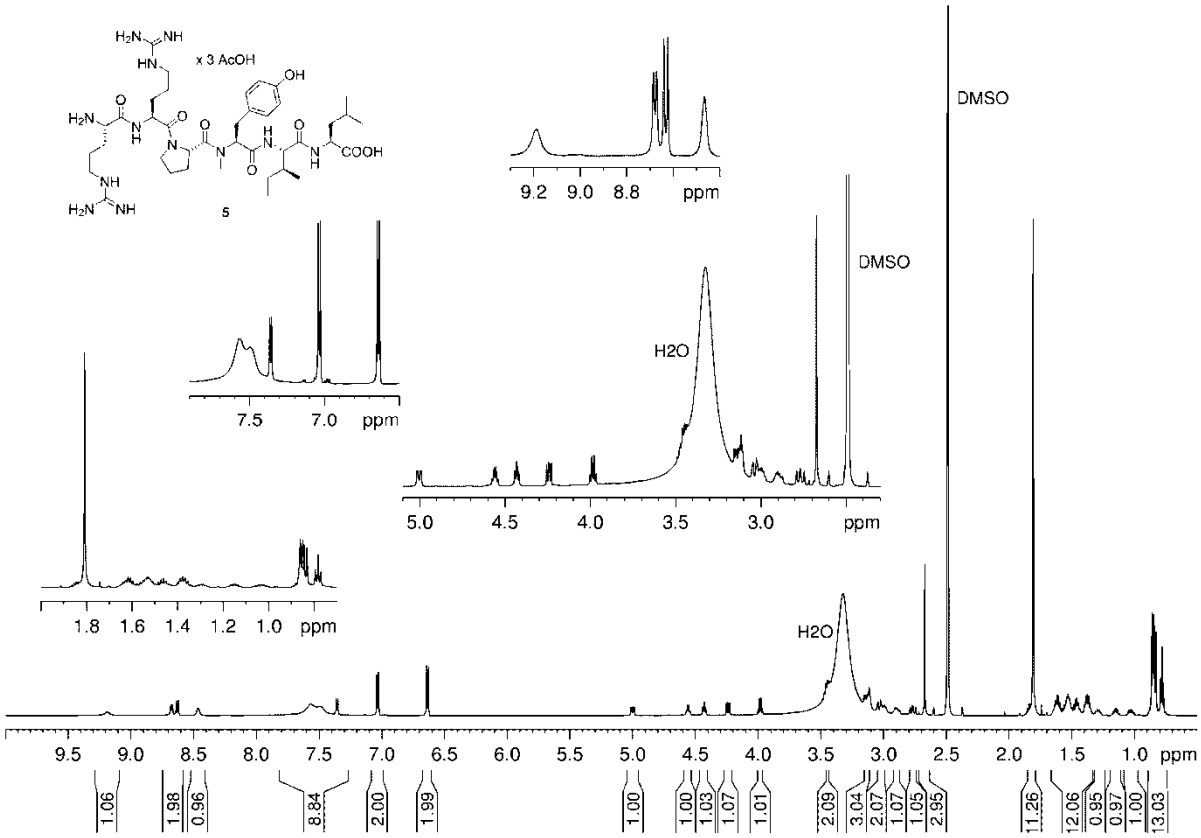
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **3**



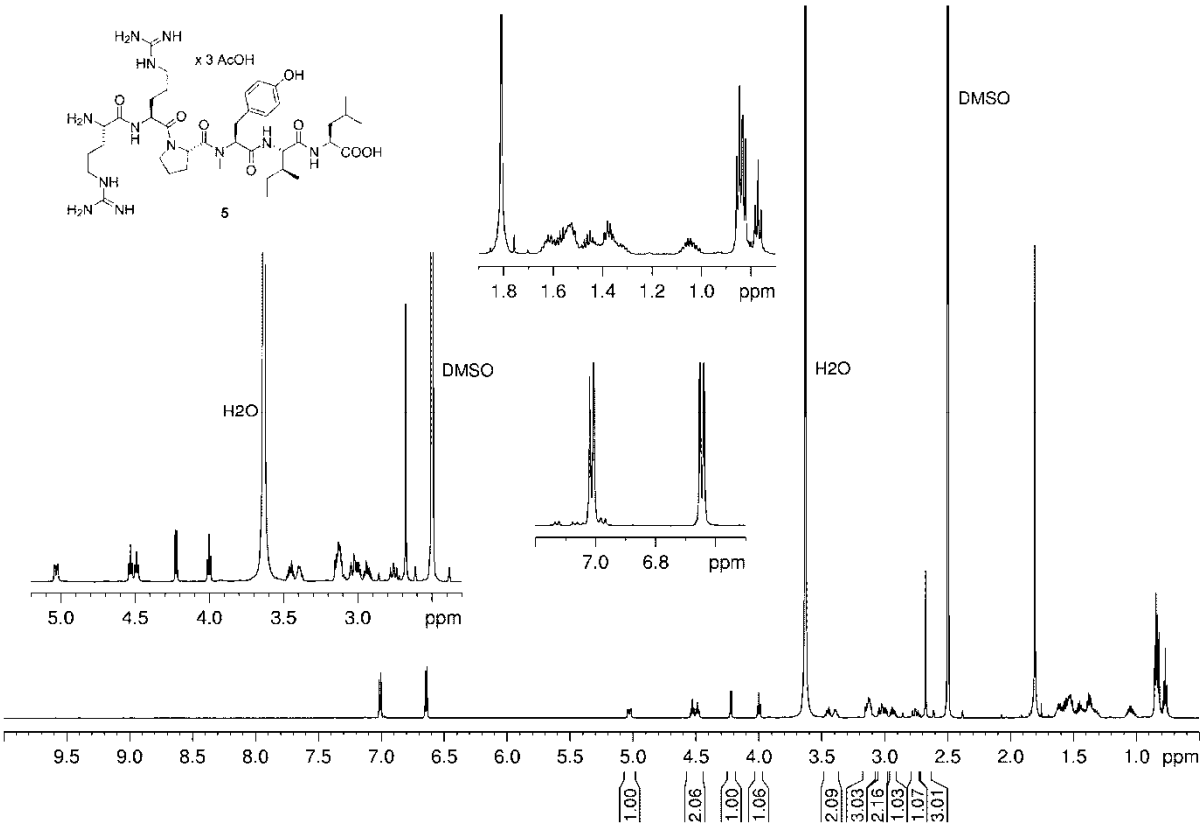
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **4**



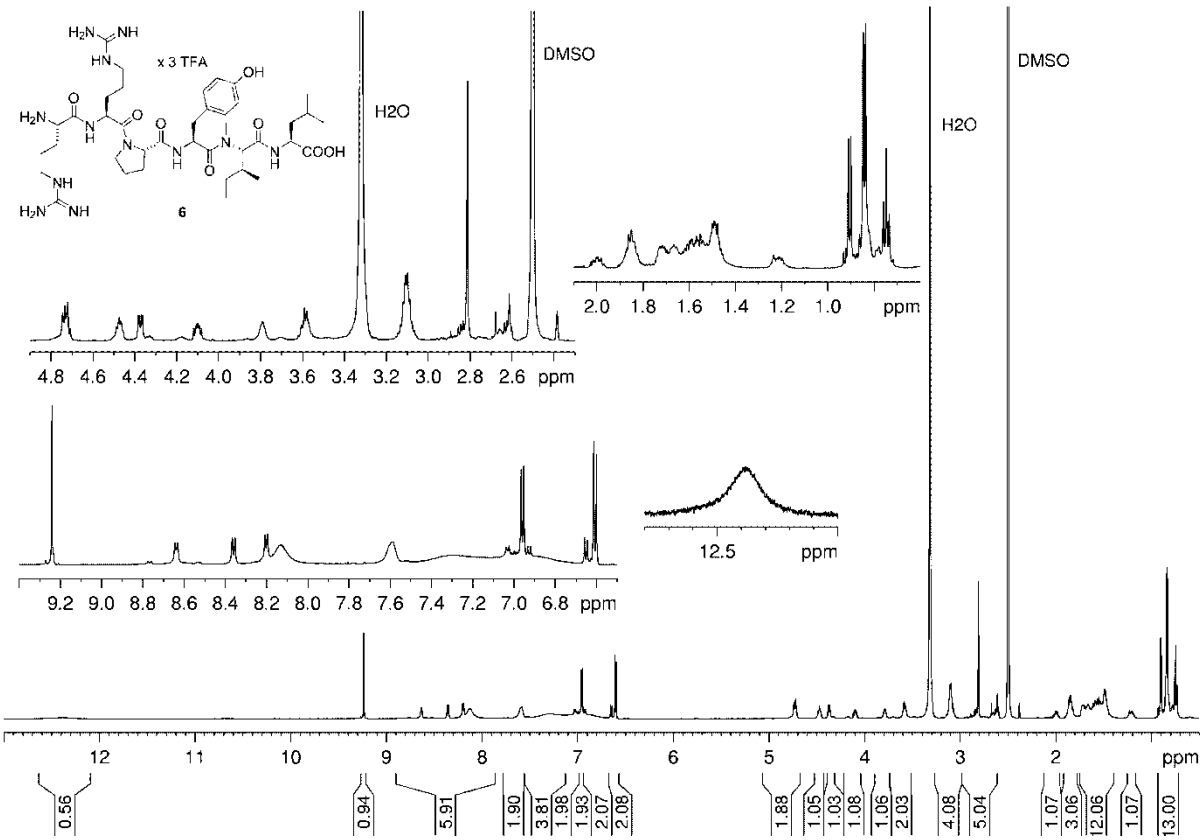
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 11:1 v/v) of compound **4**



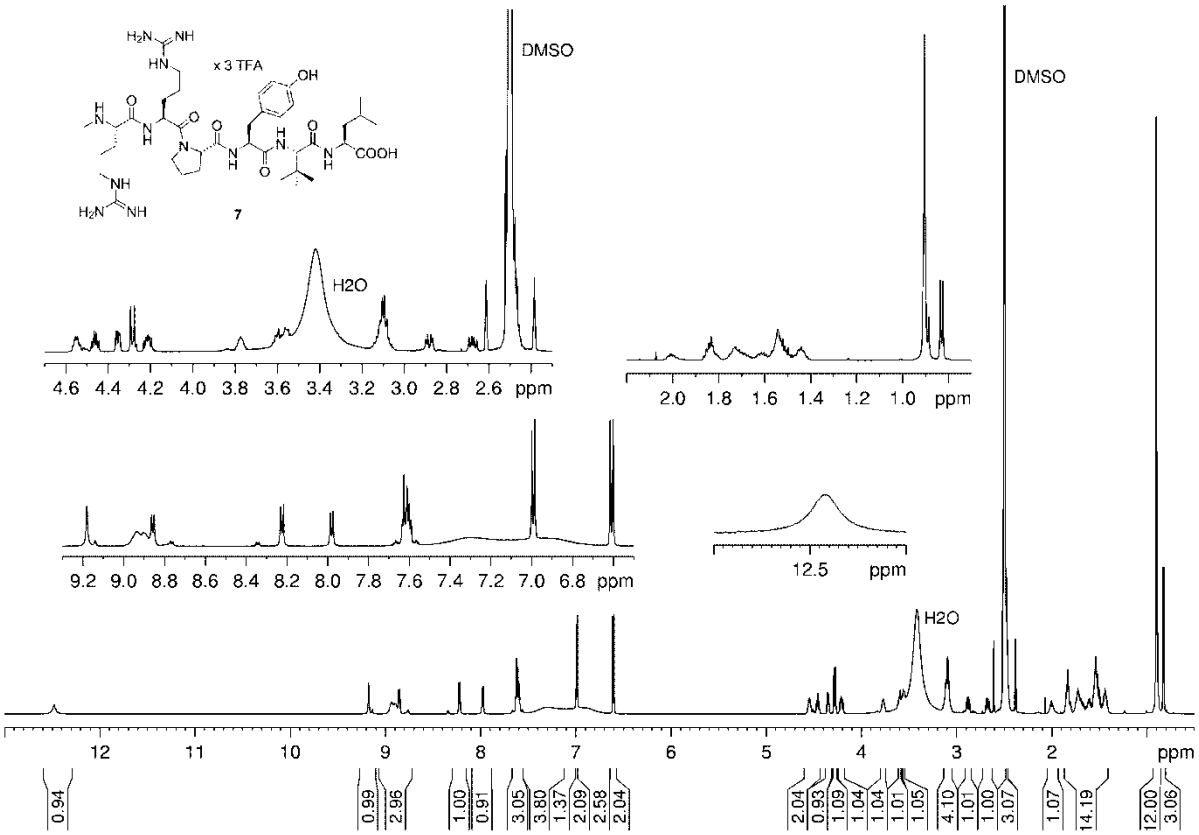
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **5**



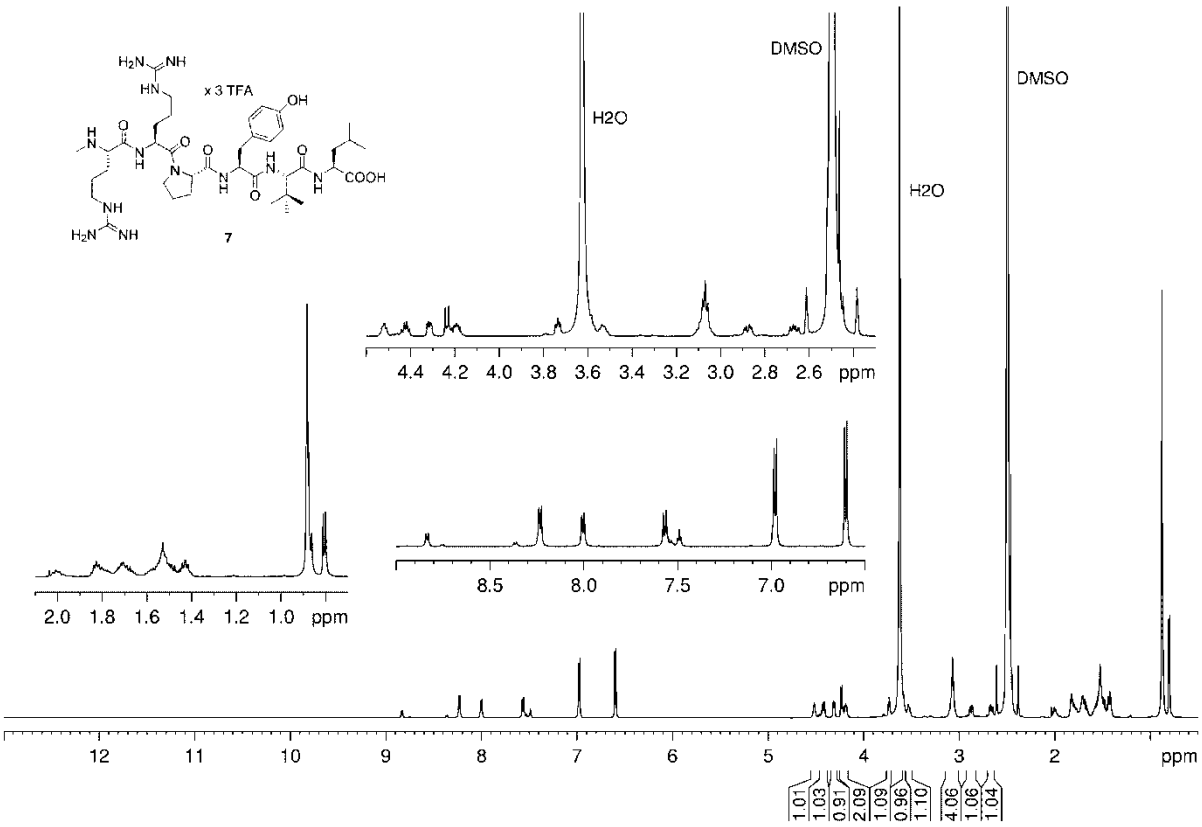
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 11:1 v/v) of compound **5**



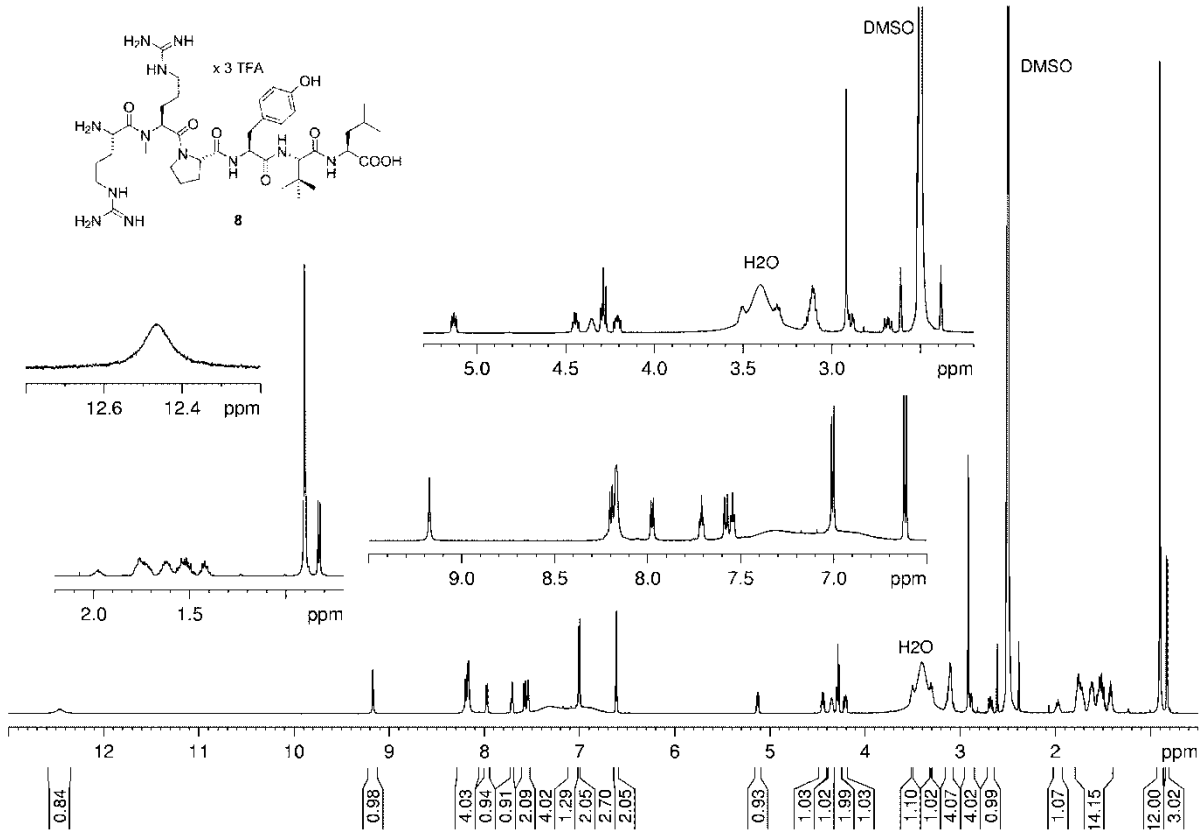
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **6**



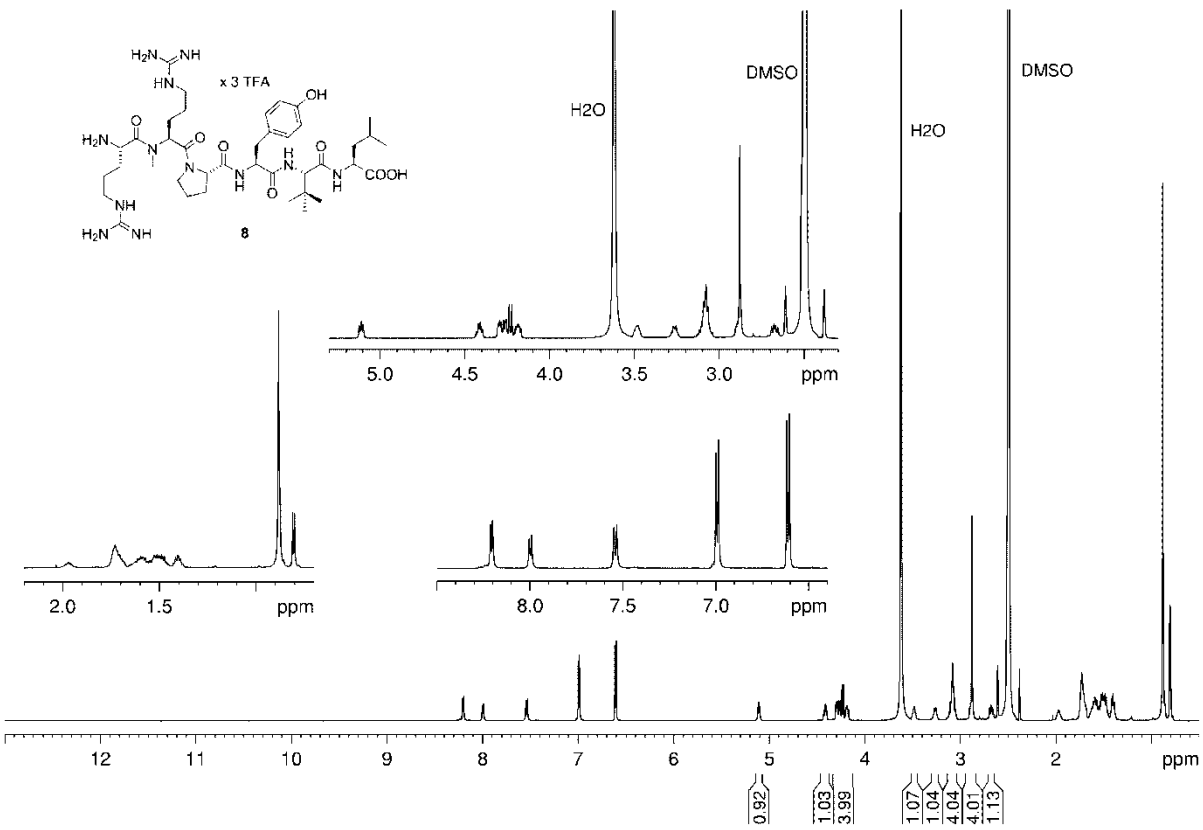
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **7**



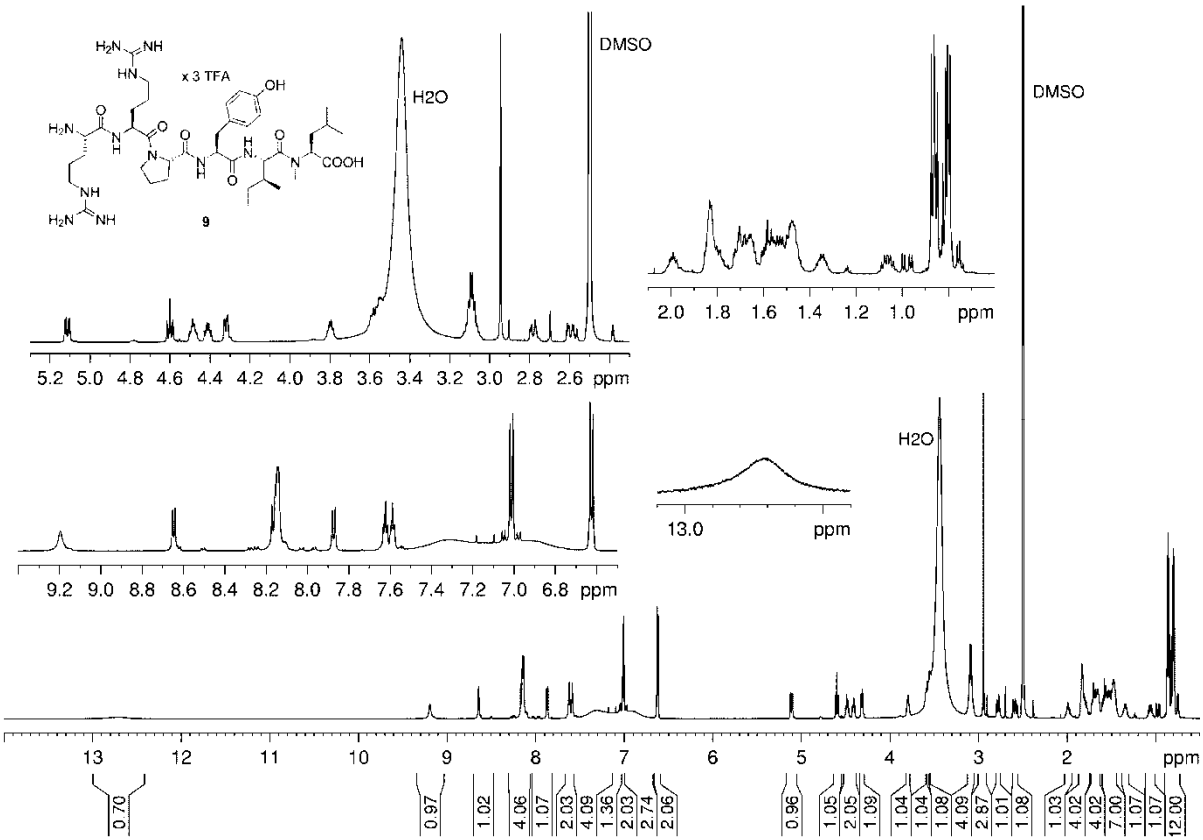
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 11:1 v/v) of compound **7**



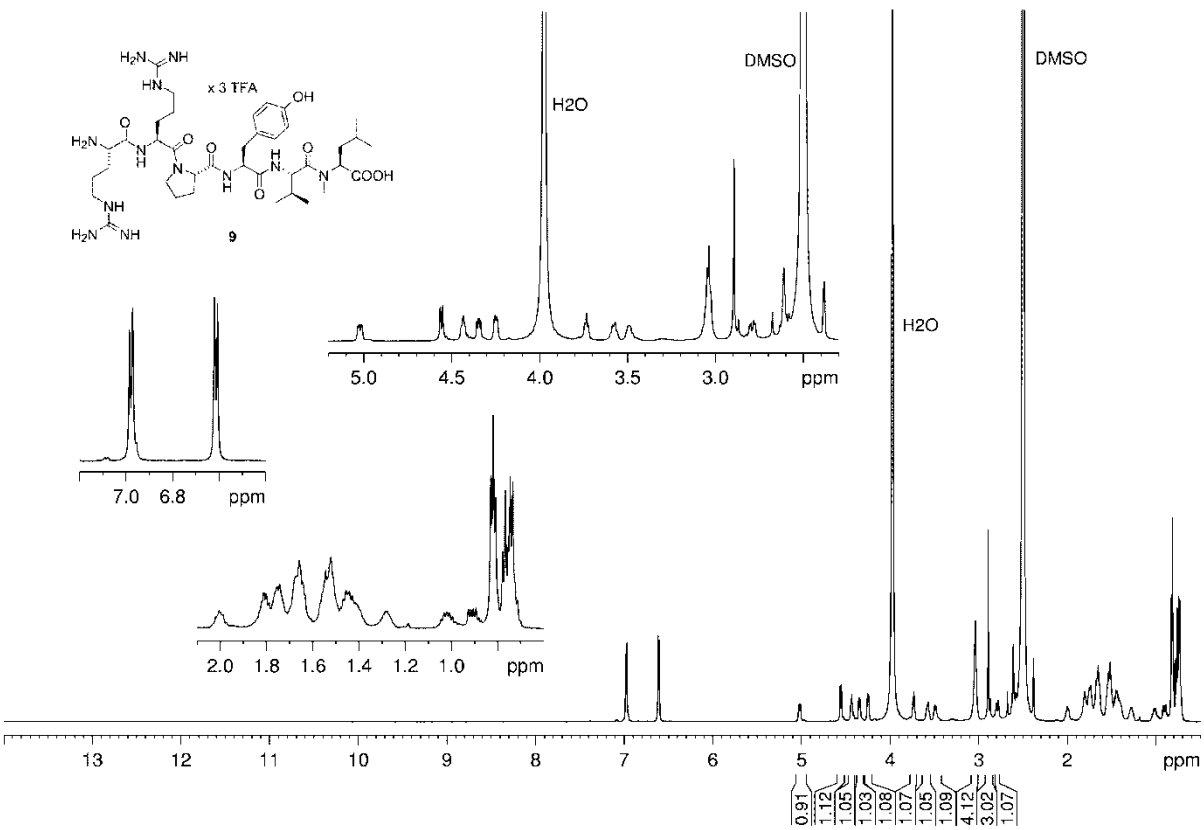
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **8**



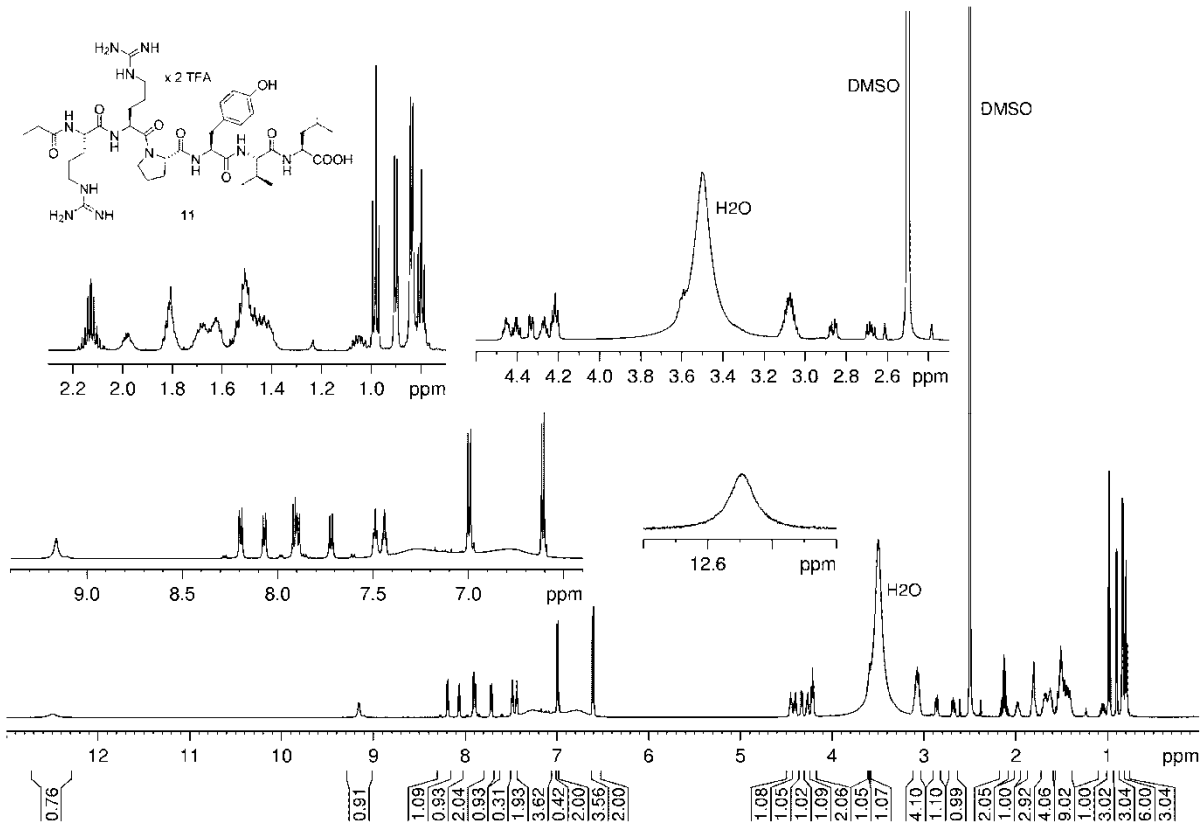
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 11:1 v/v) of compound **8**



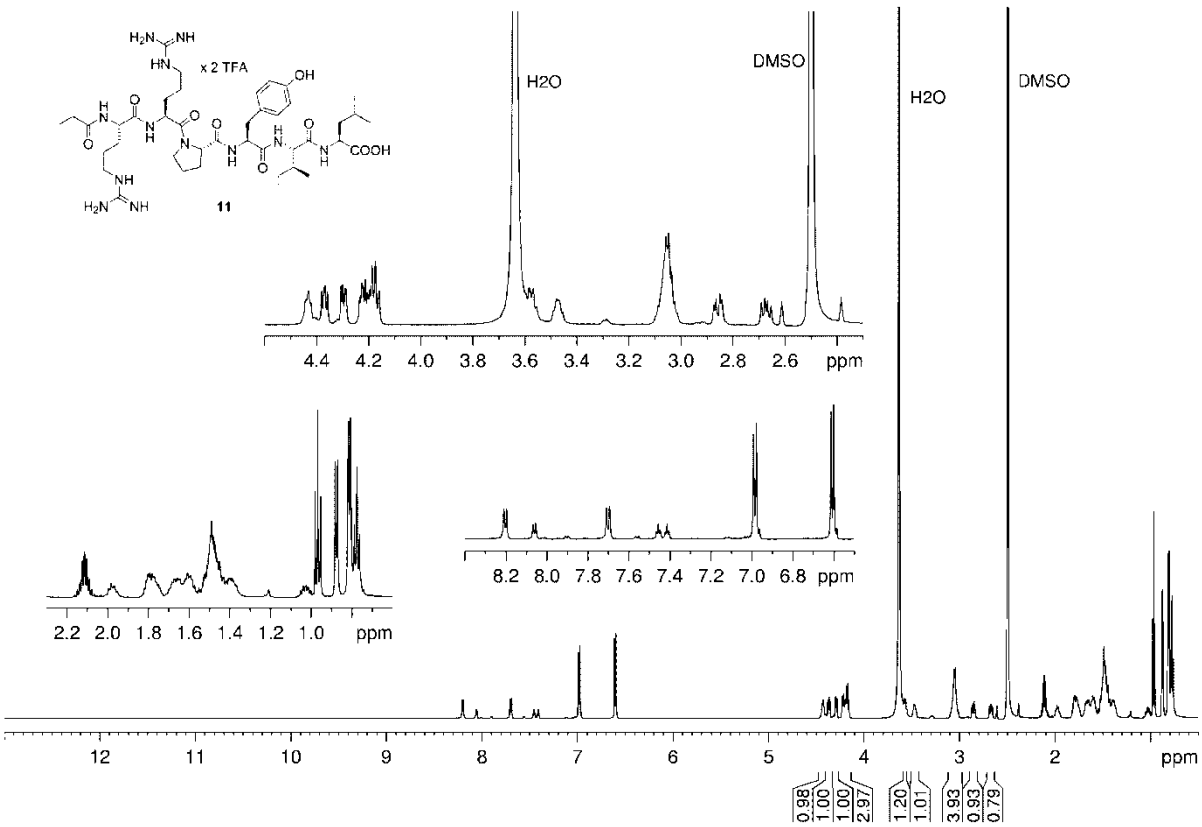
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **9**



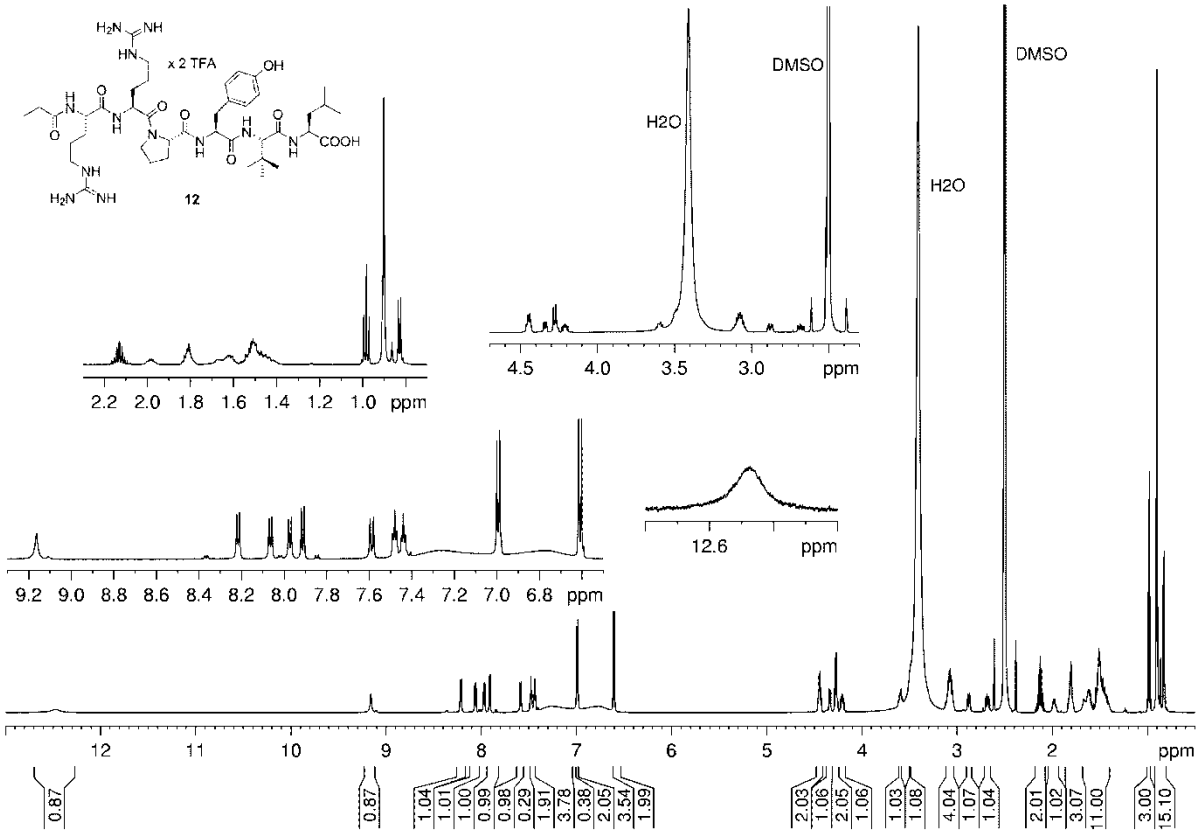
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **9**



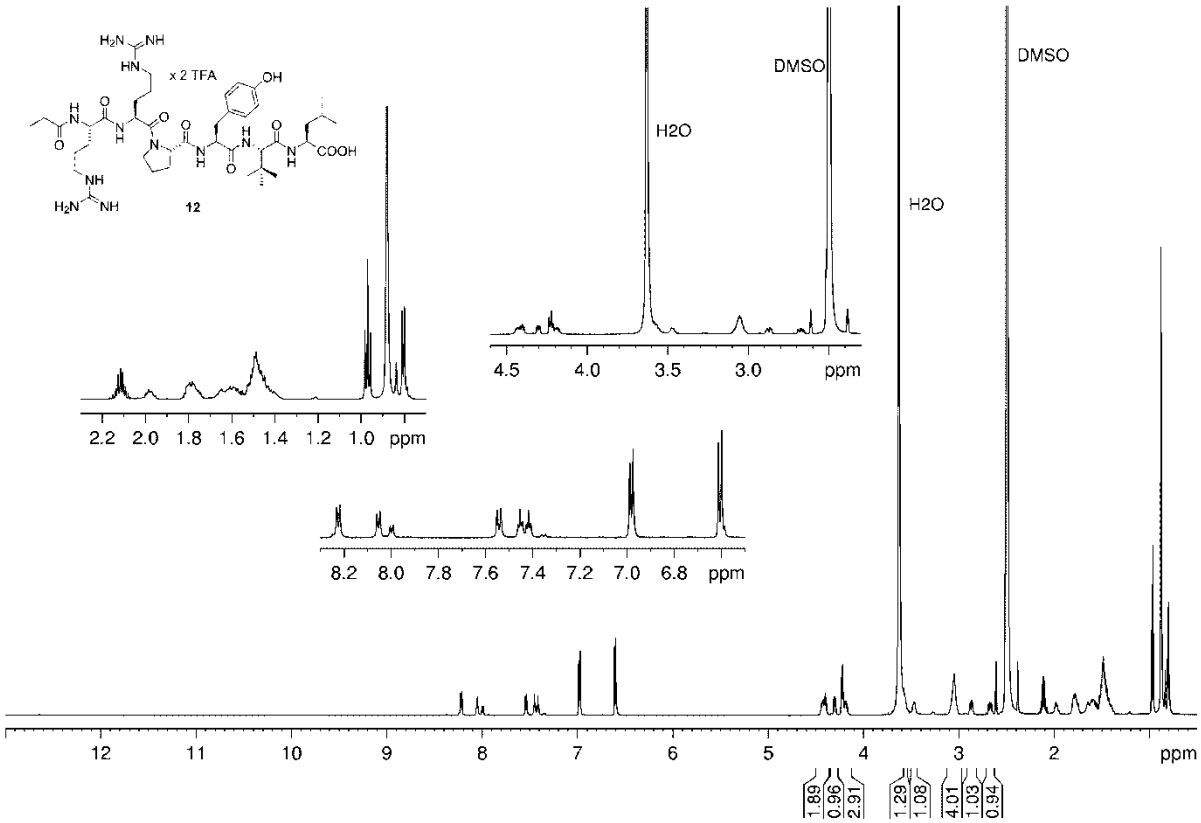
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **11**



^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 11:1 v/v) of compound **11**



^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **12**



^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 11:1 v/v) of compound **12**

8. References

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