Electronic Supplementary Information

A contamination-insensitive probe for imaging specific biomolecules by secondary ion mass spectrometry

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1. Materials and General Methods for Chemical Synthesis

Reagents. Starting materials and reagents were of the highest grade available from commercial sources and were used as supplied. ^{15}N-Glycine, ^{15}N-diethylamine hydrochloride and ^{15}N-biuret were purchased from Deutero (Kastellaun, Germany). Fmoc-protected amino acids, coupling reagents and Sieber amide resin were purchased from GL Biochem (Shanghai, China). Star635-NHS ester was obtained from Abberior (Göttingen, Germany). Flash column chromatography on silica was performed using Macherey-Nagel Silica Gel 60 with a particle size of 0.063–0.2 mm (Düren, Germany). Thin layer chromatography (TLC) was conducted on aluminum backed plates of silica gel 60 F254 (layer thickness: 0.20 mm) from Merck (Darmstadt, Germany) to detect UV-active spots by fluorescence quenching at 254 nm.

Solvents. Anhydrous solvents (DMF, EtOH) of extra dry or puriss. absolute grade (over molecular sieves) were obtained from Acros-Organics (Geel, Belgium) and Sigma Aldrich (Schnelldorf, Germany). Deuterated DMSO was purchased from Deutero (Kastellaun, Germany). Technical solvents were distilled prior to use in flash column chromatography. Acetonitrile and MeOH for HPLC (in respective grade and all other solvents of the grade puriss. p. a.) were purchased from commercially available sources and were used as supplied. Water for HPLC and buffer was purified using a Simplicity water purification device from Millipore (Bedford, UK).

Reactions. All air- and water-sensitive reactions were conducted under inert atmosphere. Therefore, glass equipment utilized for large scale reactions was flame-dried before use. For small reactions which were performed in non-heatable tubes a purge-and-refill technique was applied.

Instruments. ^{1}H- and ^{13}C-NMR spectra were performed using the Varian instruments Mercury VX 300 and INOVA 500 (Palo Alto, CA, USA). Chemical shifts are quoted in ppm (TMS = 0 ppm). The resonances of the rest protons of deuterated solvents were taken as internal standards. Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad, dd = doublet of doublets, dt = doublet of triplets, dq = doublet of quartets, ddd = doublet of doublet of doublets. Coupling constants $^{n}J_{x,x}$ are stated in Hz, n is the number of bonds mediating the coupling, x stands for the nuclei. ^{13}C-NMR spectra were recorded as broadband ^{1}H-decoupled spectra. Electrospray ionization (ESI) mass spectra were recorded with a Finnigan Ion Trap LCQ spectrometer (Thermo Finnigan, San Jose, CA, USA) and a Bruker micrOTOF spectrometer. The values are given as *m/z* relation. High-resolution mass spectra (HR-MS-ESI) were obtained with a Bruker FT-ICR-MS Apex IV instrument (Bremen, Germany). Infrared (IR) spectra were recorded with a Jasco FTIR-4100 spectrometer (Groß-Umstadt, Germany) from a range of 450–4000 cm^{-1}, whereas the given peaks are classified in vs (very strong), s (strong), m (medium) and w (weak). Microwave-mediated SPPS was realized on a CEM Discover microwave instrument (Kamp-Lintfort, Germany). High-performance

liquid chromatography (HPLC) was performed on a Pharmacia Äkta Basic system (GE Healthcare, London, UK) with a pump-type P-903, and a variable wavelength detector ultraviolet (UV)-900 using a Macherey-Nagel Nucleodur® RP C-18 analytical HPLC column (250 × 4.6 mm, 5 µm) and a semipreparative HPLC column (250 × 10 mm, 5 µm). The HPLC runs were carried out using a linear gradient of 0.1% aq. TFA (solvent A) and 80% aq. CH_3CN/0.1% TFA (solvent B) in 30 min. Flow rates were taken as 1 mL/min for the analytical, and 3 mL/min for the semi-preparative purpose. The UV absorbance of peptides could be detected at three different wavelengths simultaneously (215 nm, 254 nm and 280 nm) for peptides. For compounds containing Star635-NHS fluorophore the respective major absorption wavelength was used for detection (635 nm) instead of 280 nm. Freeze-drying of compounds from aqueous solutions containing minimal amounts of MeOH or acetonitrile was performed using a Christ-Alpha-2-4 lyophilizer attached to a high vacuum pump and a Christ RCV-2-18 ultracentrifuge (Osterode am Harz, Germany). UV-Spectra for determining the concentration of fluorophore-labelled peptides were recorded with a Thermo Scientific Nanodrop 2000c (Waltham, Massachusetts, USA). Estimation of the loading capacity of the Sieber amide resin was achieved by UV analysis with a JASCO V-550 UV/VIS spectrometer (Groß-Umstadt, Germany).

2. Materials and General Methods for Biochemical and Cellular Experiments

Materials. The compounds used for cell biology experiments were purchased mainly from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany) or from VWR (Hannover, Germany), if the producer is not indicated. Propargyl-L-lysine (PRK) was synthesized by Sirius Fine Chemicals SiChem GmbH (Bremen, Germany).

Cells. The three proteins investigated in this study, SNAP-25, syntaxin 1 and syntaxin 13, were expressed in baby hamster kidney (BHK) fibroblasts. The BHK cells were maintained in culture in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Darmstadt, Germany) to which a final concentration of 10% tryptose phosphate, 5% fetal calf serum, 2 mM L-glutamine, 60 U/mL penicillin, and 60 U/mL streptomycin were provided.

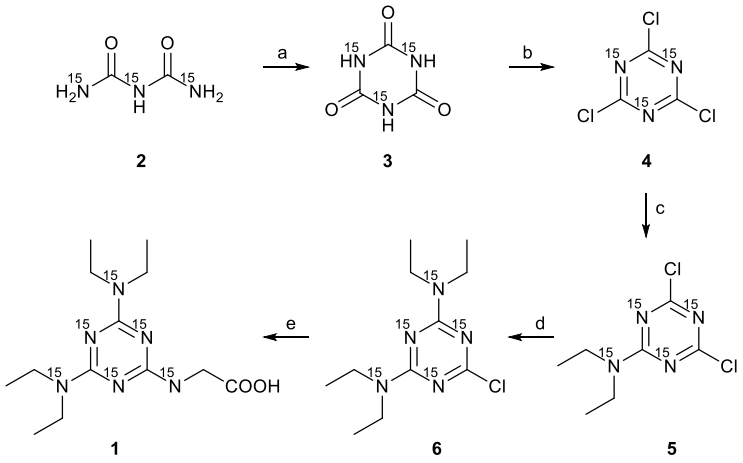
Constructs. The SNAP-25B (pEYFP-C1) and syntaxin 1A (pEYFP-N1) vectors were kindly given to us by Professor Thorsten Lang (LIMES Institute, University of Bonn, Germany). For the present experiments, SNAP-25 coding sequence was cloned into a pEGFP-N1 (Clontech) vector, and an Amber stop codon was introduced at amino acid position 84 (F84TAG) via site directed mutagenesis. Syntaxin 1 (in pEYFP-N1) was directly mutagenized to a V255TAG variant. Constructs lacking the *C*-terminal fluorescent protein (FP) domain were obtained by digestion with restriction enzymes, religation, and site-directed mutagenesis to introduce of Ochre stop codons after the protein coding sequence.[1]

Syntaxin 13 (pET28a) was a gift from Professor Reinhard Jahn (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany). It was subsequently cloned into vectors with the Amber stop codons pEGFP-N1 and pN1, from which the GFP was excised enzymatically and an Ochre stop codon was added.[1]

The pCMV tRNA-PylRS WT vector was a gift from Dr. Edward Lemke (EMBL, Heidelberg, Germany) and here we employ it according to previous protocols.[2]

3. Synthesis of ^{15}N-Triazine Peptides

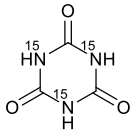
3.1 Synthetic Scheme and Procedures for $^{15}N_6$-TriazGly-OH (1)



Supplementary Scheme S1: Synthesis of ($^{15}N_2$-4,6-bis(diethylamino)-$^{15}N_3$-1,3,5-triazin-2-yl-1,3,5)-^{15}N-glycine ($^{15}N_6$-triazGly-OH, **1**). Reagents and conditions (a) $(EtO)_2CO$, NaOEt, EtOH, 16 h, reflux; 84%. (b) PCl_5, $POCl_3$, 11 h reflux. (c) $Et_2NH{\cdot}HCl$, K_2CO_3, 18-crown-6, DCM, 1 h, 0 °C; 18%. (d) $Et_2NH{\cdot}HCl$, K_2CO_3, 18-crown-6, toluene, 2 d, r.t.; 95%. (e) Glycine, K_2CO_3, DMSO, 16 h, 88–94 °C; 65%.

$^{15}N_3$-1,3,5-Triazinane-2,4,6-trione ($^{15}N_3$-cyanuric acid) (3)

Literature-known compound (patent).[3] The natural analogue, 1,3,5-triazinane-2,4,6-trione (cyanuric acid), was synthesized similarly to the described procedure below.



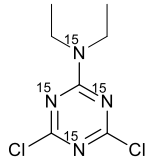
$C_3H_3{}^{15}N_3O_3$ [132.05]

Sodium metal (640 mg, 28.0 mmol, 3.30 eq) was added in small portions to dry ethanol (35 mL) under argon. To the resulting NaOEt/EtOH solution $^{15}N_3$-biuret (**2**) (900 mg, 8.49 mmol, 1.00 eq) was added followed by diethyl carbonate (2.21 mL, 2.16 g, 18.3 mmol, 2.15 eq) and the mixture was heated under argon to reflux for 16 h. Then the reaction mixture was allowed to cool down to ambient temperature, the solvent was removed *in vacuo* and the crude product was diluted with H_2O (5 mL). After cooling down to 0 °C, the solution was acidified with conc. HCl to pH 1. The precipitate was filtered off, washed with ice-cold H_2O (3 × 5 mL) and pre-dried under vacuum followed by lyophilisation. Compound **3** (940 mg, 7.14 mmol, 84%) was obtained as a white solid.

Analytical Data: IR (diamond-ATR): $\tilde{\nu}$ [cm^{-1}] = 3197m and 3054m (NH), 1756w, 1735w and 1689vs (CO), 1441s, 1408s, 1389s, 1042m, 1031m, 782s, 756s, 742s, 689m. ^{1}H-NMR (300 MHz, DMSO-d_6, 308 K): δ = 11.10 (s, 3 H, 3 × ^{15}NH) ppm. ^{13}C-NMR (75 MHz, DMSO-d_6, 308 K): δ = 149.82 (dt, $^1J_{13C,15N}$ = 17.9 Hz, $^3J_{13C,15N}$ = 1.91 Hz, 3 × CO) ppm. *m/z* (ESI) = 131.0 $[M - H]^-$. *m/z* (HR-ESI-MS) = calculated: 131.0013 $[M - H]^-$, found: 131.0013 $[M - H]^-$.

$^{15}N_4$-4,6-Dichloro-*N*,*N*-diethyl-1,3,5-triazine-2-amine (5)

The natural analogue, 4,6-dichloro-*N*,*N*-diethyl-1,3,5-triazine-2-amine, was synthesized similarly to the described procedure below.



$C_7H_{10}Cl_2{}^{15}N_4$ [225.06]

A mixture of $^{15}N_3$-cyanuric acid (**3**) (300 mg, 2.27 mmol, 1.00 eq) and PCl_5 (1.49 g, 7.16 mmol, 3.15 eq) in $POCl_3$ (5 mL) were heated under argon to reflux for 11 h. After cooling down, the excess of $POCl_3$ was concentrated *in vacuo*. The residue was dissolved in DCM (10 mL), washed with ice-cold H_2O (10 mL) and dried over $MgSO_4$. After filtration, the isolated $^{15}N_3$-2,4,6-trichloro-1,3,5-triazine ($^{15}N_3$-cyanuric chloride) (**4**) in DCM was used without further purification.

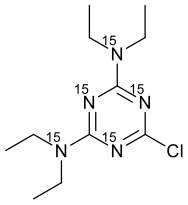
^{15}N-Diethylamine-hydrochloride (260 mg, 2.35 mmol, 1.04 eq), K_2CO_3 (640 mg, 4.63 mmol, 2.04 eq), 18-crown-6 (6.13 mg, 23.2 µmol, 0.01 eq) were suspended under argon in dry DCM (5 mL) and stirred for 2 h at ambient temperature. Then, $^{15}N_3$-cyanuric chloride (**4**) in DCM was added at –10 °C. The solution was stirred for 1 h at –10 °C and for 1 h at 0 °C. After dilution with H_2O (50 mL), the layers were separated. The water layer was extracted with EtOAc (2 × 50 mL) and the combined organic layers were dried over $MgSO_4$ followed by adsorbtion on silica gel. The crude product was

purified by flash column chromatography (pentane/EtOAc, 95:5, *v/v*). Compound **5** (92.8 mg, 410 µmol, 18%) was obtained as white crystals.

Analytical Data: TLC (pentane/EtOAc, 95:5, *v/v*): $R_f = 0.41$. *m/z* (ESI) = 225.0 $[M + H]^+$, 247.0 $[M + Na]^+$. *m/z* (HR-ESI-MS) = calculated: 225.0237 $[M + H]^+$, 247.0056 $[M + Na]^+$, found: 225.0238 $[M + H]^+$, 247.0051 $[M + Na]^+$.

$^{15}N_5$-6-Chloro-N^2,N^2,N^4,N^4-tetraethyl-1,3,5-triazine-2,4-diamine (6)

The natural analogue, 6-chloro-N^2,N^2,N^4,N^4-tetraethyl-1,3,5-triazine-2,4-diamine, was synthesized similarly to the described procedure below.



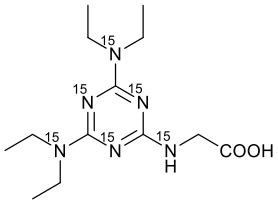
$C_{11}H_{20}Cl^{15}N_5$ [262.73]

To a mixture of $^{15}N_4$-4,6-dichloro-*N*,*N*-diethyl-1,3,5-triazine-2-amine (**5**) (92.8 mg, 412 µmol, 1.00 eq), K_2CO_3 (120 mg, 868 µmol, 2.11 eq) und 18-crown-6 (2.19 mg, 8.28 µmol, 0.02 eq) in dry toluene (5 mL) ^{15}N-diethylamine-hydrochloride (50.4 mg, 456 µmol, 1.11 eq) was added at –15 °C and stirred for 1 h at –15 °C. After return to ambient temperature, the reaction mixture was additionally stirred for 40 h. Then the solvent was removed *in vacuo* and the crude was diluted with H_2O (50 mL) and EtOAc (50 mL). After separation of the layers, the aqueous layer was extracted with EtOAc (2 × 50 mL). The combined organic layers were dried over $MgSO_4$ and the crude product was purified by flash column chromatography (pentane/EtOAc, 95:5, *v/v*). Compound **6** (103 mg, 390 µmol, 95%) was obtained as a facile melting white solid.

Analytical Data: TLC (pentane/EtOAc, 95:5, *v/v*): $R_f = 0.55$. ^{1}H-NMR (300 MHz, DMSO-d_6, 308 K): $\delta = 1.06–1.14$ (m, 12 H, 4 × CH_3), 3.49 (dq, $^3J_{1H,1H} = 6.84$ Hz, $^2J_{1H,15N} = 2.55$ Hz, 8 H, 4 × CH_2) ppm. ^{13}C-NMR (75 MHz, DMSO-d_6, 308 K): $\delta = 12.79$ (d, $^2J_{13C,15N} = 64$ Hz, 4 × CH_3), 41.00 (ddd, $^1J_{13C,15N} = 41$ Hz, $^3J_{13C,15N} = 11$ Hz, $^5J_{13C,15N} = 2.8$ Hz, 4 × CH_2), 163.28 (dd, $^1J_{13C,15N} = 0.2$ Hz, $^3J_{13C,15N} = 0.04$ Hz, C_{aromat}), 168.10–168.27 (m, 2 × C_{aromat}) ppm. *m/z* (ESI) = 263.2 $[M + H]^+$, 285.1 $[M + Na]^+$. *m/z* (HR-ESI-MS) = calculated: 263.1332 $[M + H]^+$, found: 263.1334 $[M + H]^+$.

$^{15}N_6$-((4,6-Bis(diethylamino)-1,3,5-triazine-2-yl)amino)glycine, $^{15}N_6$-TriazGly-OH (1)

The natural analogue, ((4,6-bis(diethylamino)-1,3,5-triazine-2-yl)amino)glycine, was synthesized similarly to the described procedure below.



$C_{13}H_{24}{}^{15}N_6O_2$ [302.33]

A mixture of $^{15}N_5$-6-chloro-N^2,N^2,N^4,N^4-tetraethyl-1,3,5-triazine-2,4-diamine (**6**) (42.0 mg, 160 µmol, 1.00 eq), ^{15}N-glycine (42.0 mg, 552 µmol, 3.57 eq) and K_2CO_3 (88.4 mg, 640 µmol, 4.00 eq) in DMSO (2 mL) was heated to 88 – 94 °C for 2 d. Then the solvent was removed *in vacuo* and the crude was diluted with H_2O (10 mL). After cooling down to 0 °C, the solution was acidified with aqueous 1 M HCl to pH 2. The aqueous solution was extracted with DCM (5 × 20 mL) and the combined organic layers were dried over $MgSO_4$. The crude product was purified by flash column chromatography (acetone/H_2O, 20:1 → 10:1, *v/v*). Compound **1** (31.2 mg, 103 µmol, 65%) was obtained as a white solid.

Analytical Data: TLC (acetone/H_2O, 95:5, *v/v*): R_f = 0.38. ^{1}H-NMR (300 MHz, DMSO-d_6, 308 K): δ = 1.07 (dt, $^3J_{1H,1H}$ = 6.9 Hz, $^3J_{1H,15N}$ = 2.5 Hz, 12 H, 4 × CH_3), 3.45 (q, $^3J_{1H,1H}$ = 7.0 Hz, 8 H, 4 × CH_2), 3.78 (d, $^3J_{1H,1H}$ = 6.6 Hz, 2 H, α-CH_2), 6.53 (d, $^1J_{1H,15N}$ = 93 Hz, 1 H, ^{15}NH) ppm. ^{13}C-NMR (75 MHz, DMSO-d_6, 308 K): δ = 13.37 (s, 4 × CH_3), 40.43 (s, 4 × CH_2), 42.52–42.70 (m, α-CH_2), 163.57–164.21 (m, C_{aromat}), 165.57 (d, $^1J_{13C,15N}$ = 26 Hz, 2 × C_{aromat}), 172.51 (s, COOH) ppm. *m/z* (ESI) = 303.2 $[M + H]^+$, 325.2 $[M + Na]^+$, 301.2 $[M - H]^-$. *m/z* (HR-ESI-MS) = calculated: 303.1856 $[M + H]^+$, 301.1710 $[M - H]^-$, found: 303.1855 $[M + H]^+$, 301.1713 $[M - H]^-$.

3.2 Artificial Amino Acid Synthesis

^{15}N-(((9*H*-Fluoren-9-yl)methoxy)carbonyl)glycine (Fmoc-^{15}N -Gly-OH, **7**)[4], the azide transfer reagent 1*H*-imidazole-1-sulfonyl azide hydrochloride (**8**)[5,6] and the azido amino acid (*S*)-2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-6-azidohexanoic acid (Fmoc-Lys(N_3)-OH, **9**)[6,7] were prepared as described in literature.

3.3 Solid Phase Peptide Synthesis (SPPS)

Loading of the first amino acid. Fmoc-protected Sieber amide resin (113 µmol) was pre-swollen in a BD syringe (Becton Dickinson, Fraga, Spain) in DMF for 1 h at room temperature and was thoroughly washed with DMF, DCM, DMF (6 × 8 mL each) followed by Fmoc-deprotection applying 20% piperidine in DMF (2 × 4 mL for 20 min). After subsequent washing of the resin with DMF, DCM, DMF (6 × 8 mL each), a prior activated solution of Fmoc-^{15}N-Gly-OH (**7**) (5.0 eq), *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) (4.5 eq), 1-hydroxy-7-azabenzotriazole (HOAt) (5 eq), DIEA (10 eq) in DMF (4 mL) was added and the coupling was carried out by microwave irradiation (10 min, 40°C, 20 W). Double coupling was performed and the resin was subsequently washed with DCM, DMF, DCM, MeOH (6 x 8 mL each) and dried *in vacuo*. After resin-loading, the loading density (0.75 mmol/g) was estimated via UV analysis of the Fmoc-dibenzofulven deprotection product.[8]

Microwave assisted SPPS. Manual SPPS of peptides was performed under microwave irradiation starting with a 10 µmol scale on the Fmoc-^{15}N-Gly-preloaded Sieber amide resin (0.75 mmol/g). The resin was swollen in a BD syringe for 2 h in DMF followed by washing with DMF (3×), DCM (3×) and NMP (3×). The coupling cycle was started by microwave supported Fmoc-deprotection, applying 20% piperidine in DMF (2×, 30 sec, 50 W, 50 °C and 3 min, 50 °C, 50 W) followed by successive washing with DMF (3×), DCM (3×) and NMP (3×). For Asp containing sequences 0.1 M 1-hydroxybenzotriazole (HOBt) was added to the piperidine solution. For pre-activation and coupling, the respective amino acid (5.0 eq), *O*-(benzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) (4.5 eq) and HOBt (5.0 eq) were dissolved in NMP (0.4 mL). Directly before transferring the coupling cocktail to the resin, DIEA (10 eq) was added to activate the mixture. Double coupling of each amino acid (3.0 eq) was performed with HBTU (2.7 eq), HOBt (3.0 eq) and DIEA (10 eq). $^{15}N_6$-TriazGly-OH (**1**) (3.0 eq) was introduced under standard coupling conditions. However, to obtain better coupling yields, HATU and HOAt were used as activation reagents followed by double coupling. Each coupling was carried out by microwave irradiation (10 min, 50 °C, 25 W) followed by washing with DMF (3×), DCM (3×), NMP (3×). After the final coupling cycle, the resin was washed with DMF, DCM, NMP, DCM, MeOH, DCM (5× each) and was dried *in vacuo*.

The natural analogues were synthesized applying the same procedure described above on a Fmoc-Gly-preloaded Sieber amide resin (0.76 mmol/g). Special care had to be taken for Cys coupling to avoid racemization. In this case, either a mixture of HBTU (4.5 eq/ 2.7 eq), HOBt (5.0 eq/ 3.0 eq) and DIEA (10 eq) in NMP or *N*,*N*′-diisopropylcarbodiimide (DIC) (4.5 eq/ 2.7 eq) and HOBt (5.0 eq/ 2.7 eq) in NMP were used, followed by milder microwave irradiation conditions (40 °C, 20 W, 10 min).

Cleavage. Cleavage of the peptide from the Sieber amide resin and simultaneous deprotection of protecting groups was obtained after 1–2 h at ambient temperature by using

- 10% of cleavage cocktail (95% trifluoroacetic acid (TFA)/ 2.5% H_2O/ 1% triisopropylsilane (TIS), *v*/*v*/*v*) in DCM for peptide **10**,
- 10% of cleavage cocktail (94% TFA/ 2.5% H_2O/ 2.5% 1,2-ethanedithiol (EDT)/ 1% TIS, *v*/*v*/*v*/*v*) in DCM for peptide **11**, and
- 10% cleavage cocktail (94% TFA/ 2.5% H_2O/ 2.5% 2-propanethiol/ 1% TIS, *v*/*v*/*v*/*v*) in DCM for peptide **12**.

Post-cleavage work-up. After cleavage, the resulting solution was concentrated under a nitrogen stream, followed by precipitation using cold Et_2O. The resulting suspension was centrifuged at –5 °C. The supernatant was discarded and the peptide pellet was washed with cold Et_2O (3×) and dried *in vacuo*. The crude peptide was purified by HPLC unless otherwise specified.

$^{15}N_6$-TriazGly-Lys($^{15}N_6$-triazGly)-Asp-Glu-Lys(N_3)-^{15}N-Gly-Asp-Glu-Lys-^{15}N-Gly-NH_2 (10) as a negatively charged peptide scaffold

The natural analogue, triazGly-Lys(triazGly)-Asp-Glu-Lys(N_3)-Gly-Asp-Glu-Lys-Gly-NH_2, was synthesized similarly to the described procedure below.

The following amino acid building blocks were used in the microwave-mediated SPPS: Fmoc-Lys(Boc)-OH, Fmoc-Glu(O^{t}Bu)-OH, Fmoc-^{15}N-Gly-OH (**7**), Fmoc-Lys(N_3)-OH (**9**), Fmoc-Asp(O^{t}Bu)-OH, Fmoc-Lys(Fmoc)-OH and $^{15}N_6$-triazGly-OH (**1**). Peptide **10** was obtained as a white solid after HPLC purification and lyophilisation.

Analytical data: HPLC (analytical, gradient 40 → 80% B in 30 min): t_R = 13.80 min. *m/z* (ESI) = 534.3 $[M + 3H]^{3+}$, 800.9 $[M + 2H]^{2+}$, 1600.8 $[M + H]^{+}$, 798.9 $[M - 2H]^{2-}$, 1598.8 $[M - H]^{-}$. *m/z* (HR-ESI-MS) = calculated: 534.2784 $[M + 3H]^{3+}$, 800.9140 $[M + 2H]^{2+}$, 1600.8207 $[M + H]^{+}$, found: 534.2781 $[M + 3H]^{3+}$, 800.9134 $[M + 2H]^{2+}$, 1600.8219 $[M + H]^{+}$.

TriazGly-Lys(triazGly)-Cys-Gly-Lys(N_3)-Gly-NH_2 (11) as an uncharged peptide scaffold

The following amino acid building blocks were used in the microwave-mediated SPPS: Fmoc-Gly-OH, Fmoc-Lys(N_3)-OH (**9**), Fmoc-Cys(Trt)-OH, Fmoc-Lys(Fmoc)-OH and triazGly-OH (**13**). Peptide **11** was obtained as a white solid after HPLC purification and lyophilisation.

Analytical data: HPLC (analytical, gradient 40 → 90% B in 30 min): t_R = 13.54 min. *m/z* (ESI) = 537.3 $[M + 2H]^{2+}$, 1073.7 $[M + H]^{+}$. *m/z* (HR-ESI-MS) = calculated: 537.3223 $[M + 2H]^{2+}$, 1073.6374 $[M + H]^{+}$, found: 537.3224 $[M + 2H]^{2+}$, 1073.6373 $[M + H]^{+}$.

TriazGly-Lys(triazGly)-Cys-Gly-Lys(N_3)-Lys-Lys-Lys-Gly-NH_2 (12) as a positively charged peptide scaffold

The following amino acid building blocks were used in the microwave-mediated SPPS: Fmoc-Gly-OH, Fmoc-Lys(N_3)-OH (**9**), Fmoc-Lys(Boc)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Lys(Fmoc)-OH and triazGly-OH (**1**). Peptide **12** was obtained as a white solid after post-cleavage workup and drying *in vacuo*.*[1]

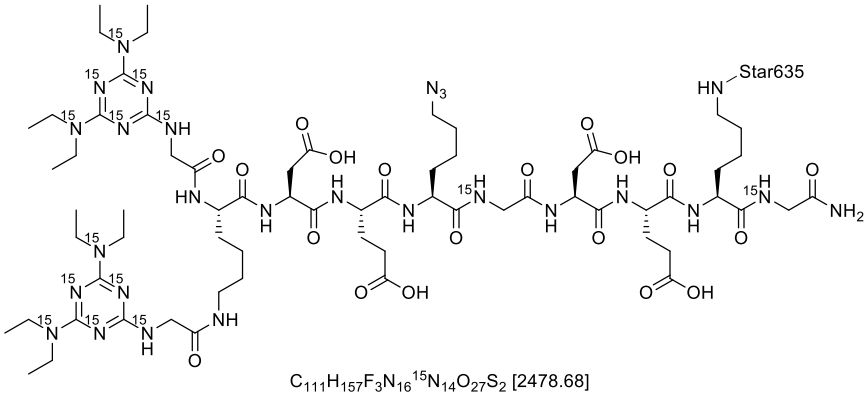
Analytical data: HPLC (analytical, gradient 40 → 90% B in 30 min): t_R = 22.17 min. *m/z* (ESI) = 486.6 $[M + 3H]^{3+}$, 729.5 $[M + 2H]^{2+}$, 1457.9 $[M + H]^{+}$. *m/z* (HR-ESI-MS) = calculated: 486.6456 $[M + 3H]^{3+}$, 729.4648 $[M + 2H]^{2+}$, 1457.9223 $[M + H]^{+}$, found: 486.6467 $[M + 3H]^{3+}$, 729.4648 $[M + 2H]^{2+}$, 1457.9226 $[M + H]^{+}$.

*[1] The product **12** was used without further purification, due to the fast proceeding oxidation of the thiol moiety forming an intermolecular disulfide bridge after HPLC purification. Therefore, a volatile thiol-containing scavenger in the cleavage cocktail was used (2-propanethiol). TCEP as an *in situ* reducing reagent prior fluorophore attachment was avoided to protect azide functionality from reduction.

3.4 Synthesis of Star635-Labelled Peptide

$^{15}N_6$-TriazGly-Lys($^{15}N_6$-triazGly)-Asp-Glu-Lys(N_3)-^{15}N-Gly-Asp-Glu-Lys(Star635)-^{15}N-Gly-NH_2 (TriazNF1) as a negatively peptide

The natural analogue, triazGly-Lys(triazGly)-Asp-Glu-Lys(N_3)- Gly-Asp-Glu-Lys(Star635)- Gly-NH_2, was synthesized similarly to the described procedure below.

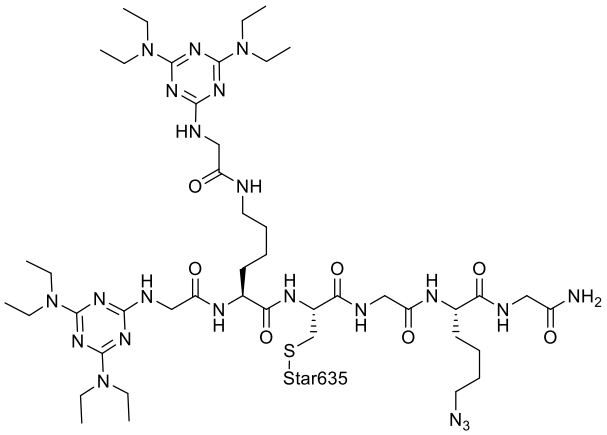


$C_{111}H_{157}F_3N_{16}{}^{15}N_{14}O_{27}S_2$ [2478.68]

Peptide **10** (1.10 mg, 0.69 µmol, 2.7 eq) was transferred to an Eppendorf tube. Under argon atmosphere, a solution of DIEA (2.72 µL, 15.6 µmol, 60 eq) in dry DMF (500 µL) was added, followed by the addition of Star635-NHS ester (0.25 mg, 0.26 µmol, 1.0 eq) in dry DMF (25 µL) under light exclusion. After stirring for 19 h at ambient temperature, the solvent was removed *in vacuo* and the crude product was purified by HPLC. Product **TriazNF1** (0.30 mg, 0.12 µmol, 47%) was obtained as a blue solid after lyophilisation.

Analytical data: HPLC (analytical, gradient 40 → 90% B in 30 min): t_R = 19.04–20.72 min. *m/z* (ESI) = 1240.1 $[M + 2H]^{2+}$, 1262.1 $[M + 2Na]^{2+}$, 825.0 $[M - 3H]^{3-}$, 1238.0 $[M - 2H]^{2-}$. *m/z* (HR-ESI-MS) = calculated: 848.6830 $[M + 3Na]^{3+}$, 1239.5479 $[M + 2H]^{2+}$, 1261.5299 $[M + 2Na]^{2+}$, 1237.5334 $[M - 2H]^{2-}$, found: 848.6826 $[M + 3Na]^{3+}$, 1239.5489 $[M + 2H]^{2+}$, 1261.5302 $[M + 2Na]^{2+}$, 1237.5332 $[M - 2H]^{2-}$.

TriazGly-Lys(triazGly)-Cys(Star635)-Gly-Lys(N_3)-Gly-NH_2 (TriazNF2) as an uncharged peptide

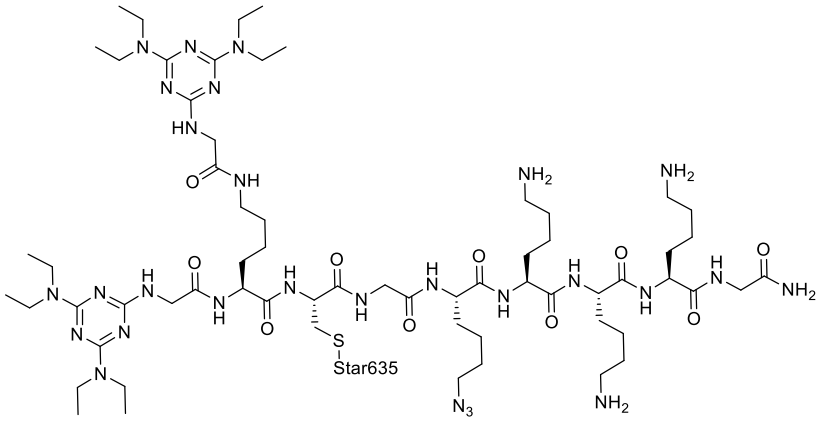


$C_{96}H_{134}F_3N_{27}O_{17}S_3$ [2091.48]

Peptide **11** (1.05 mg, 0.98 µmol, 2.50 eq) was transferred in an Eppendorf-tube under argon atmosphere followed by the addition of degassed PBS buffer (pH 7.26, 20 mM phosphate buffer + 100 mM NaCl, 950 µL) and dry DMF (100 µL). Afterwards Star635-maleimide (40 µL, 0.40 mg, 0.39 µmol, 1.0 eq) in dry DMF was added to the solution. After stirring for 3 h at ambient temperature under light exclusion the reaction mixture was stored over night at +4 °C and the crude was purified by HPLC. Compound **TriazNF2** (0.34 mg, 0.16 µmol, 42%) was obtained as a blue solid after lyophilisation.

Analytical data: HPLC (analytical, gradient 30 → 90% B in 30 min): t_R = 20.85–22.93 min. *m/z* (ESI) = 1046.5 $[M + 2H]^{2+}$, 2092.0 $[M + H]^{+}$. *m/z* (HR-ESI-MS) = calculated: 697.6595 $[M + 3H]^{3+}$, 1045.9855 $[M + 2H]^{2+}$, 2090.9638 $[M + H]^{+}$, found: 697.6610 $[M + 3H]^{3+}$, 1045.9854 $[M + 2H]^{2+}$, 2090.9638 $[M + H]^{+}$.

TriazGly-Lys(triazGly)-Cys(Star635)-Gly-Lys(N_3)-Lys-Lys-Lys-Gly-NH_2 (TriazNF3) as a positively charged peptide



$C_{114}H_{170}F_3N_{33}O_{20}S_3$ [2476.00]

Peptide **12** (0.60 mg, 0.42 μmol, 1.0 eq) was transferred to an Eppendorf-tube under argon atmosphere followed by the addition of degassed PBS buffer (pH 7.26, 20 mM phosphate buffer + 100 mM NaCl, 600 μL) and dry DMF (100 μL). Subsequently, Star635-maleimide (43 μL, 0.43 mg, 0.42 μmol, 1.0 eq) in dry DMF was added to the solution. After stirring for 2 h at ambient temperature under light exclusion the reaction mixture was stored over night at +4 °C and the crude was purified by HPLC. Upon lyophilization, compound **TriazNF3** (0.24 mg, 0.10 μmol, 24%) was obtained as a blue solid.

Analytical data: HPLC (analytical, gradient 40 → 90% B in 30 min): t_R = 15.04–16.25 min. *m/z* (ESI) = 619.8 $[M + 4H]^{4+}$, 826.1 $[M + 3H]^{3+}$, 1238.7 $[M + 2H]^{2+}$. *m/z* (HR-ESI-MS) = calculated: 619.5688 $[M + 4H]^{4+}$, 825.7583 $[M + 3H]^{3+}$, 1238.1286 $[M + 2H]^{2+}$, found: 619.5676 $[M + 4H]^{4+}$, 825.7544 $[M + 3H]^{3+}$, 1238.1280 $[M + 2H]^{2+}$.

4. Biochemical and Cellular Experiments

Site-directed mutagenesis. The Amber and Ochre stop codons were introduced at the desired positions in the constructs via site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit II (Stratagene). The primers and the protocols were designed according to the instructions provided with this kit.

Treatment with L-leucine-2-^{13}C. One day before fixation, the BHK cell medium was exchanged to ^{13}C-medium: leucine-free DMEM supplemented with 5% dialysed fetal calf serum, 2 mM glutamine and 200 mg/L L-leucine-2-^{13}C (20 mg/mL stock in PBS). After 6 h the medium was replaced to fresh ^{13}C-medium to which 250 μM PRK has been added and transfection was performed (see below). Overall, BHK cells were allowed to incorporate ^{13}C-labelled leucine for 24 h and PRK for 18 h before fixation and further labelling reactions.

Co-transfection. The cells were co-transfected following the instructions of the manufacturer using Lipofectamine 2000 (Life Technologies, Darmstadt, Germany) and two vectors: pCMV tRNA-Pyl RS WT that encodes for the aminoacyl-tRNA-synthetase/tRNA pair,[2] and another vector for the protein of interest, SNAP-25, syntaxin 1, and, respectively, syntaxin 13. The transfection was carried out by separately incubating the plasmids and the Lipofectamine® 2000 for 5 min in Opti-MEM Gibco® (Life Technologies GmbH, Darmstadt, Germany), then mixing the two solutions and waiting for further 20 min before applying them to the cells. The cells were allowed to express the proteins of interest for 18 h at 37 °C. Then the medium was exchanged to DMEM without antibiotic and the unnatural amino acid PRK was washed off for 2 h before proceeding to fixation and further sample processing.

Click reaction. Fixation was performed using 0.2% glutaraldehyde and 4% PFA in phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.3. This was followed by quenching for 20 min in 100 mM NH_4Cl and 100 mM glycine in PBS and a brief PBS wash before the samples were permeabilised with 0.1% Triton X-100 in PBS. A 15-minute blocking step (with 2× blocking solution exchange) using 5% BSA and 5% peptone in 0.1% Triton-X 100-PBS was employed to reduce the background. The cells were shortly incubated with 3% BSA in PBS before the 30-minute click reaction (at RT, in a dark humidified chamber). In agreement with the protocol of the manufacturer, the click solution was freshly prepared and contained: 1× component A (cell reaction buffer), 2 mM component B ($CuSO_4$), a 10× dilution of component C (Click-iT reaction buffer additive; Click-iT® Cell Reaction Buffer Kit, Life Technologies, cat. no. C10269), and 7 μM of clickable peptide **TriazNF1**, **TriazNF2**, and **TriazNF3**, respectively (from 1 M stocks in DMSO). The samples were washed after the labelling step for 15 min (2× solution exchange) with 5% BSA and 5% peptone in PBS and with PBS, respectively.

DAPI labelling. For epifluorescence imaging purposes, the cell nuclei were labelled using a solution of 1 μg/mL DAPI in PBS for 5 minutes. This was followed by three PBS washes (5 min each) and mounting in Mowiol (24% w/v glycerol, 0.1 M Tris-HCl, pH 8.5, 9.6% w/v Mowiol 4-88; Carl Roth GmbH, Karlsruhe, Germany). An immunostaining procedure, for calnexin (Figure 1) was performed exactly as described.[1]

Plastic embedding and thin sectioning. For NanoSIMS measurements, the samples were subjected to post-fixation with 0.2% glutaraldehyde-4% PFA in PBS for 30 min, then briefly washed twice with PBS and quenched for 20 min in 100 mM NH_4Cl and 100 mM glycine in PBS. Another 15-minute washing step with PBS (2× buffer exchange) was performed prior to the embedding procedure. LR White resin (London Resin Company Ltd, Berkshire, England) was used using a previously described protocol.[9] The resulting samples were sectioned at a thickness of 200 nm using an EM UC6 ultramicrotome (Leica Microsystems). The thin sections were then placed on a silicone wafer.

5. Imaging

Confocal microscopy. Confocal imaging was conducted using a Leica TCS SP5 microscope (Leica Microsystems) equipped with a 100× 1.4 N.A. HCX PL APO CS oil objective (Leica). The Star635 and Cy2 fluorophores in the samples were visualised with a helium-neon laser at 633 nm and an argon laser at 488 nm, respectively. An AOTF filter (Leica) allowed us to select an appropriate emission interval and photomultiplier tubes enabled us to detect the signal. Image acquisition was performed at 7.5× zoom using line scanning at 1,000 Hz of a 20.68×20.68 μm area to render 1024×1024-pixel images with a pixel size of 20.2×20.2 nm.

Secondary Ion Mass Spectrometry (SIMS). The samples were analysed with a NanoSIMS 50L instrument (Cameca, Gennevilliers, France) using a Cs^+ primary ion beam at 16 keV energy and primary current of ~1-2 pA to release secondary ions. These ions were mass-separated with a reliable resolution for potential isobars and detected using electron multipliers. Prior to each measurement an implantation of Cs^+ ions was performed to increase ionization efficiency. Scanning was performed for 10×10 µm areas with a 512×512 pixel format and a 10 ms dwell-time per pixel. The following ions of interest were detected: $^{12}C^-$, $^{13}C^-$, $^{19}F^-$, $^{12}C^{14}N^-$, $^{12}C^{15}N^-$, $^{32}S^-$, mentioned in the rest of the manuscript as ^{12}C, ^{13}C, ^{19}F, ^{14}N, ^{15}N, and ^{32}S, respectively.

Epifluorescence microscopy. Samples were imaged using an inverted fluorescence microscope (Olympus IX 71, Olympus, Hamburg, Germany) with a 20× objective (Olympus, 0.50 N.A.). A 100 W mercury lamp (Olympus) provided the necessary illumination, while images were recorded by a charge-coupled device (CCD) camera (FView II, Olympus) operated through the interface of the CellF software (Olympus). Fluorescence in the different channels was detected using filter systems from AHF (Tübingen, Germany). For the DAPI channel, a 377/50 excitation filter, a 409 nm long pass beam splitter and a 447/60 emission filter were used. In the green channel (GFP or YFP/FITC), we employed a combination of the 480/40 HQ excitation filter, 505 LP Q beam splitter, and 527/30 HQ emission filter. Finally, the 620/60 HQ excitation filter, the 660 LP Q beam splitter, and the 700/75 HQ emission filter were used to detect the Star635 fluorescence.

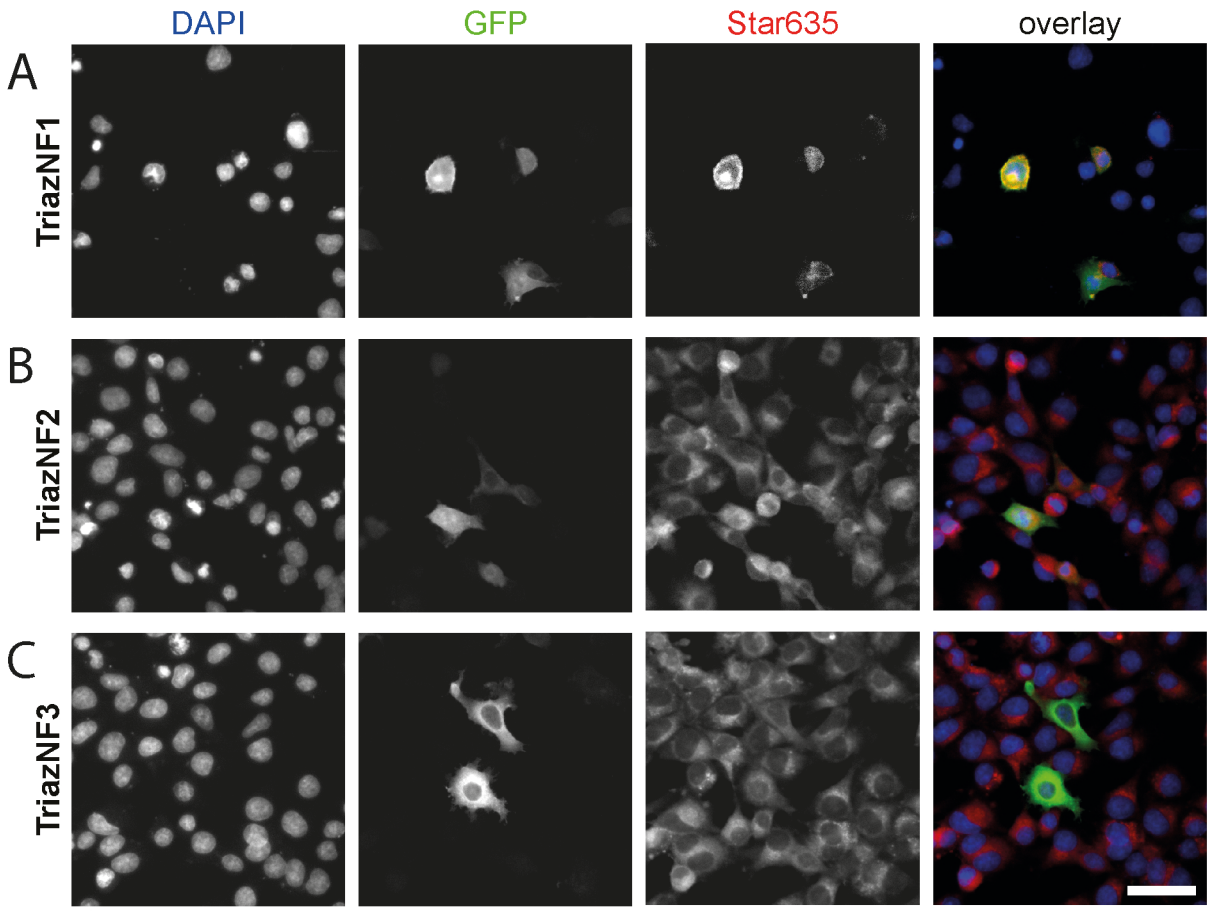
Data analysis. Matlab routines (the Mathworks Inc, Natick, MA) were used for all data analysis in this manuscript. Star635 confocal and SIMS images were overlaid and were corrected for rotational misalignments before analysis. Circular regions of 9 pixels in diameter were then collected manually in the different images. The average isotopic counts and their ratios were determined and shown in Figure 1.

Calculation of TriazNF1 copy numbers. The isotopic counts for ^{14}N and ^{15}N were determined as indicated under Data analysis, in regions of cells that were transfected with the different proteins of interest. To determine number of **TriazNF1** that were detected by isotopic imaging from each region, the following system of equations was considered:

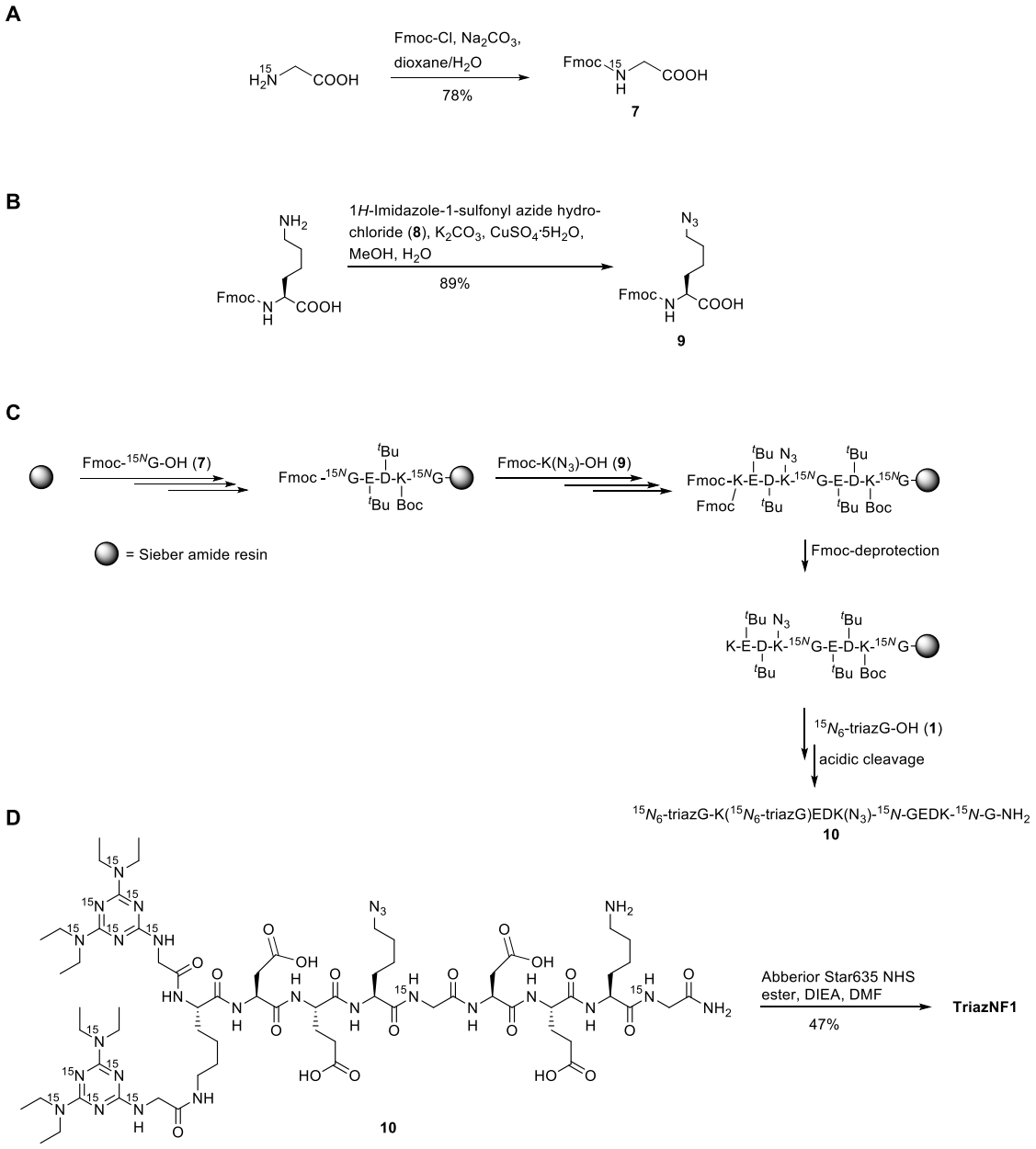
(1): $Number^{15}N$ + 14* TriazNF1 copy number = ^{15}N counts
(2): $Number^{14}N$ + 16* TriazNF1 copy number = ^{14}N counts
(3): $Number^{15}N$/ $Number^{14}N$ = 0.00367 (meaning 0.367%)
where $Number^{15}N$ and $Number^{14}N$ represent the numbers of ^{15}N and ^{14}N isotopes detected from the all the naturally occurring biomolecules in the respective region. The measured copy number of TriazNF1 is then equivalent to (^{15}N counts - ^{14}N counts*0.00367)/(14 - 0.00367*16).

This formula determines the measured or perceived number of **TriazNF1** copies in the preparation. It is not an absolute number, since it makes no provision for the number of isotopes that did not ionize, and were not detected. However, this can be estimated for each NanoSIMS instrument by calibration with standard ^{14}N and ^{15}N-containing probes, which then enables the investigator to obtain absolute copy numbers for this probe.

6. Supporting Figures



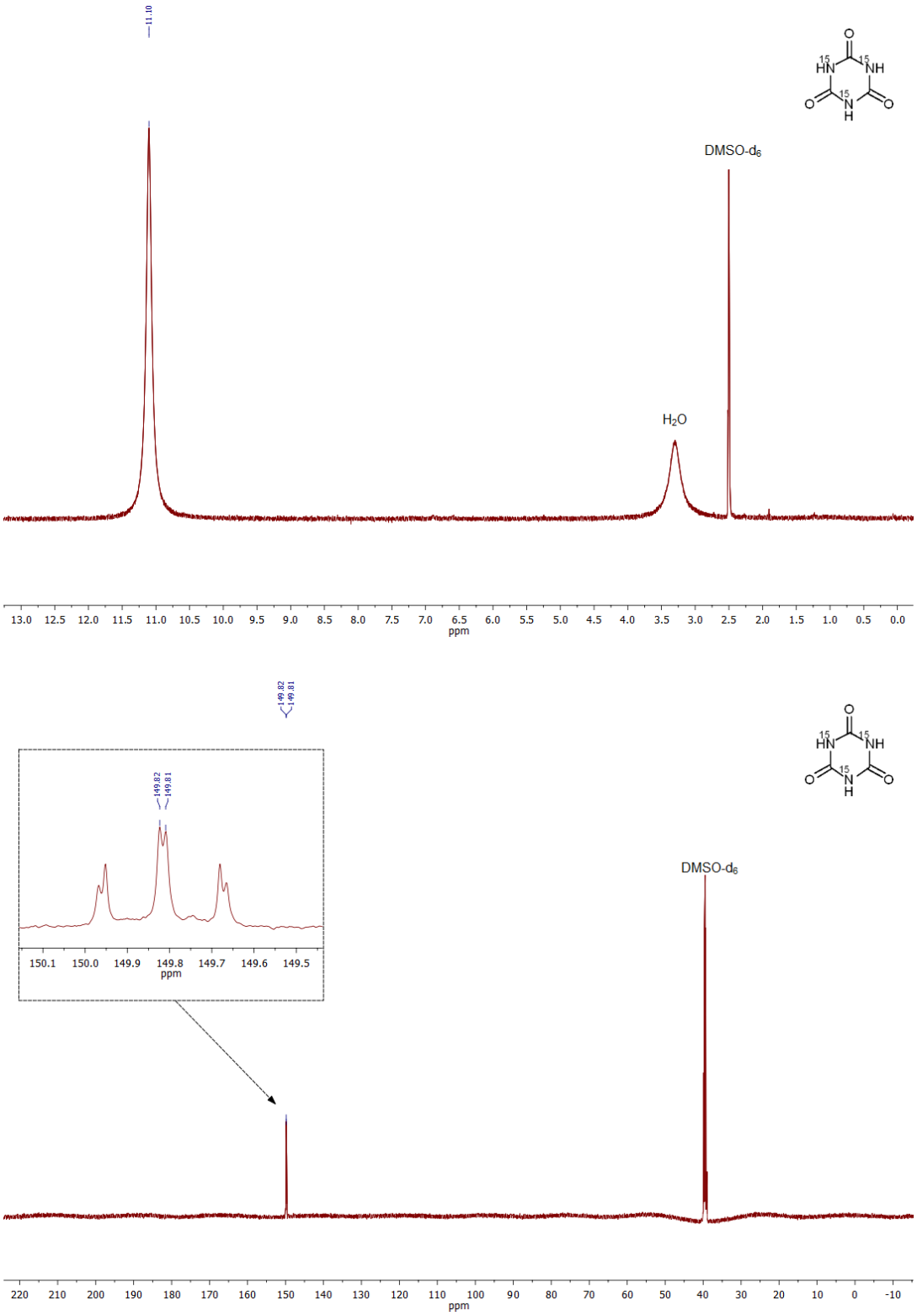
Supplementary Figure 1. Click reactions were performed with three differently charged peptides containing the natural analogue triazGly and coupled to Star635. Cells expressing SNAP-25-GFP in the presence of PRK were fixed and labelled with the following peptides: natural version of **TriazNF1** (**A**), **TriazNF2** (**B**), and **TriazNF3** (**C**). Additionally, the cell nuclei were revealed using DAPI staining. The DAPI and GFP images are identically scaled for all click conditions, whereas the Star635 images were adjusted separately for each peptide. Scale bar, 40 µm. Only the negatively charged peptide **TriazNF1** is specific. The two other peptides label both the transfected (green) and non-transfected cells. The non-specificity of uncharged **TriazNF2** is due to its hydrophobic character, which results in poor solubility under physiological conditions. Positively charged **TriazNF3** also shows non-specificity, despite good solubility under physiological conditions. This result could be due to electrostatic interactions with various cell compartments or with cytoplasmic polyanions, such as RNAs.



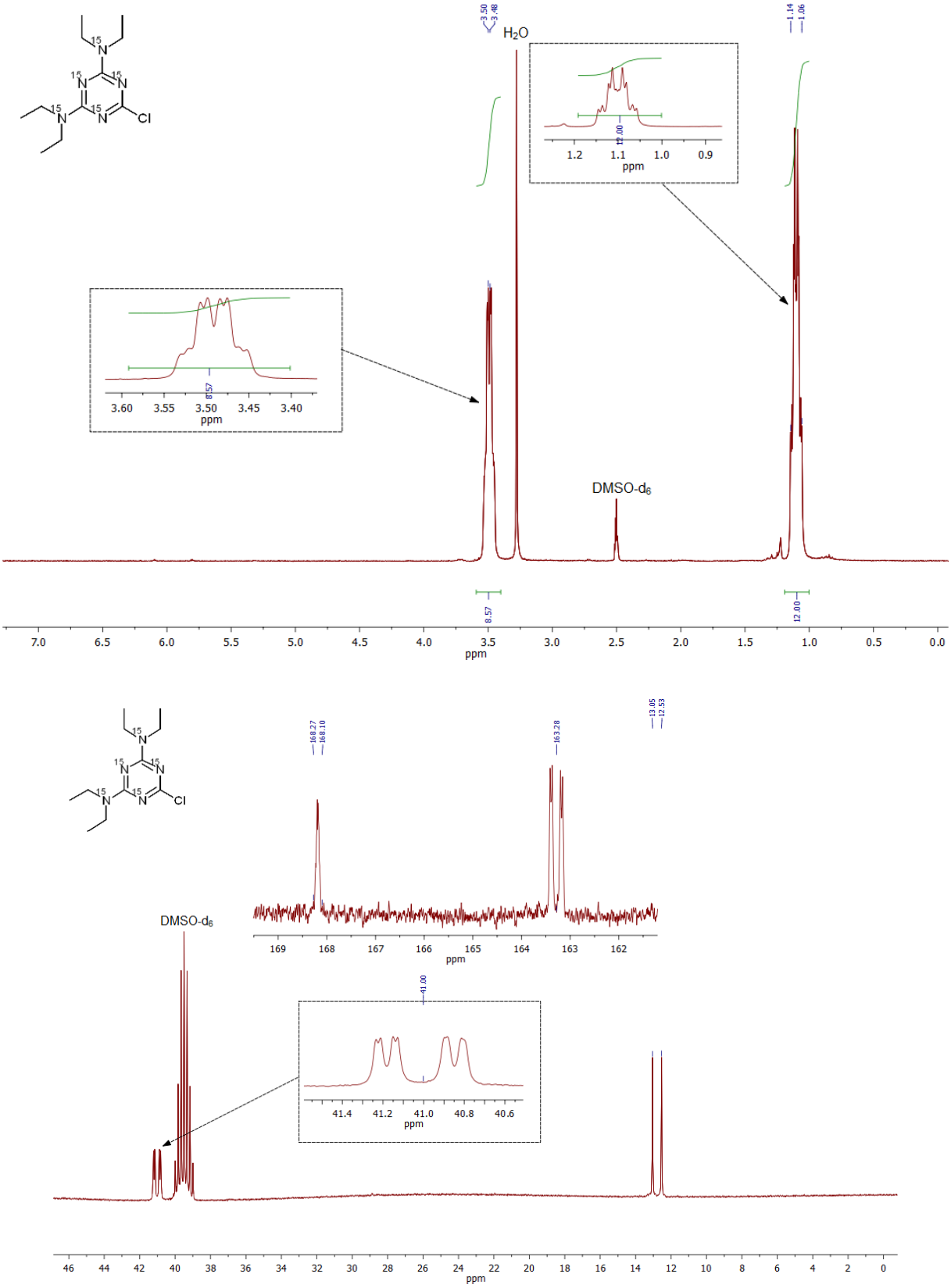
Supplementary Figure 2: Synthesis of **TriazNF1**. (**A**) ^{15}N-Gly-OH was protected gaining Fmoc-^{15}N-Gly-OH (**7**) prior to use in SPPS.[4] (**B**) Starting from commercially available Fmoc-Lys-OH, Fmoc-Lys(N_3)-OH (**9**)[6,7] was synthesized by a copper(II) catalysed diazo transfer reaction with previously prepared 1*H*-imidazole-1-sulfonyl azide hydrochloride (**8**)[5,6] as an azide transfer reagent. (**C**) Fmoc-Lys(N_3)-OH (**9**) was incorporated by manual solid phase peptide synthesis (SPPS) under microwave irradiation, following the Fmoc-protocol on Sieber amide resin. $^{15}N_6$-TriazGly-OH (**1**) was introduced following standard coupling conditions. Cleavage from the resin and simultaneous removal of protecting groups yielded crude peptide **10**, which was purified by HPLC and characterized by high-resolution mass spectrometry. (**D**) Labelling of peptide **10** with Star635 NHS ester was accomplished under light exclusion in solution to yield **TriazNF1**. This compound was purified by HPLC and characterised by high-resolution mass spectrometry.

7. Supporting Spectra

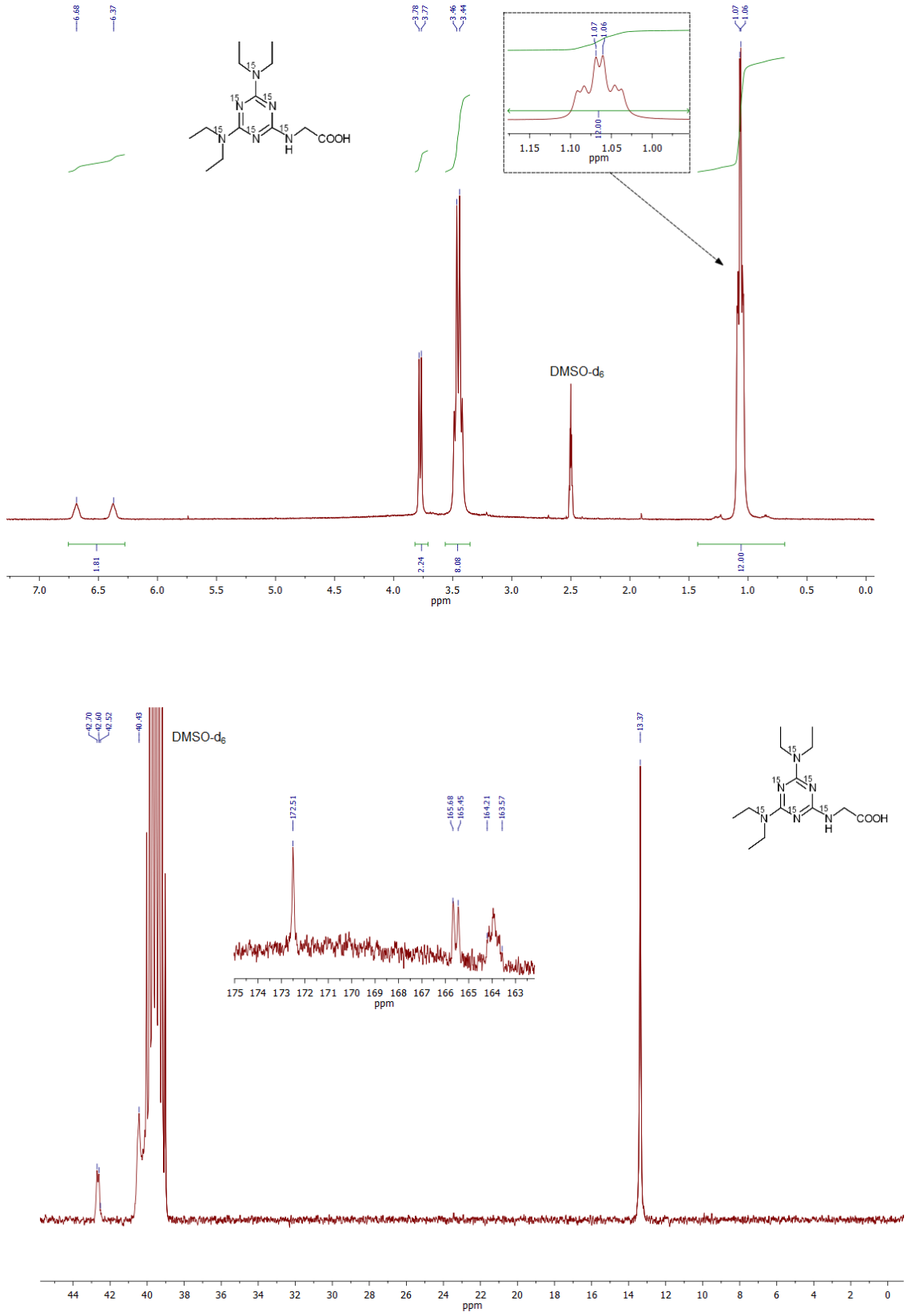
7.1 ^{1}H- and ^{13}C-NMR-Spectra of Compounds 1–6



Supplementary Spectra 1: ^{1}H-NMR- (above) and ^{13}C-NMR-spectra (below) of $^{15}N_3$-1,3,5-triazinane-2,4,6-trione ($^{15}N_3$-cyanuric acid) (**2**).

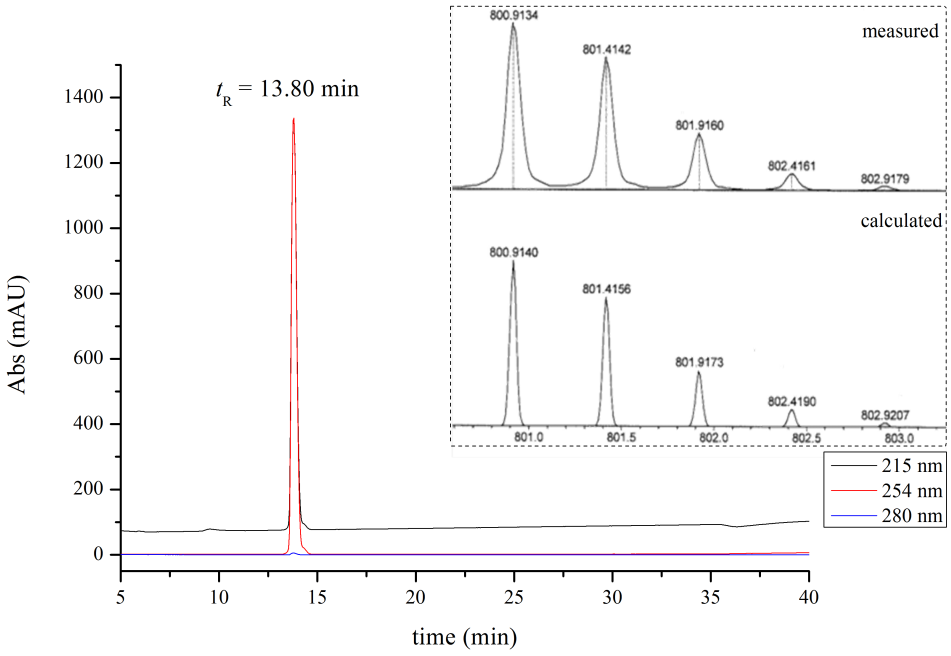


Supplementary Spectra 2: ^{1}H-NMR- (above) and ^{13}C-NMR-spectra (below) of $^{15}N_5$-6-chloro-N^2,N^2,N^4,N^4-tetraethyl-1,3,5-triazine-2,4-diamine (**6**).

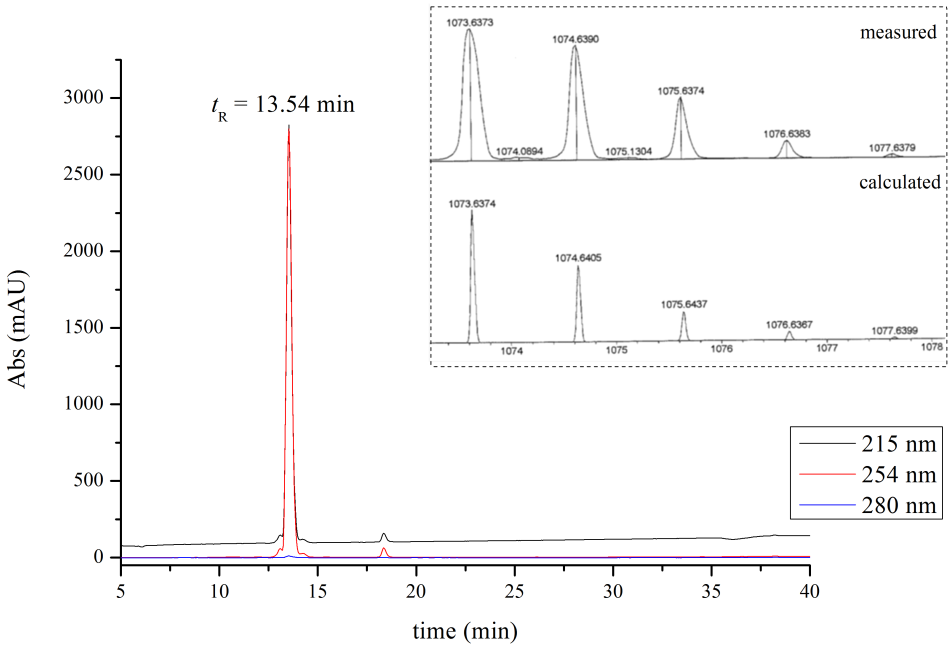


Supplementary Spectra 3: ^{1}H-NMR- (above) and ^{13}C-NMR-spectra (below) of $^{15}N_6$-((4,6-bis(diethylamino)-1,3,5-triazine-2-yl)amino)glycine, $^{15}N_6$-TriazGly-OH (**1**).

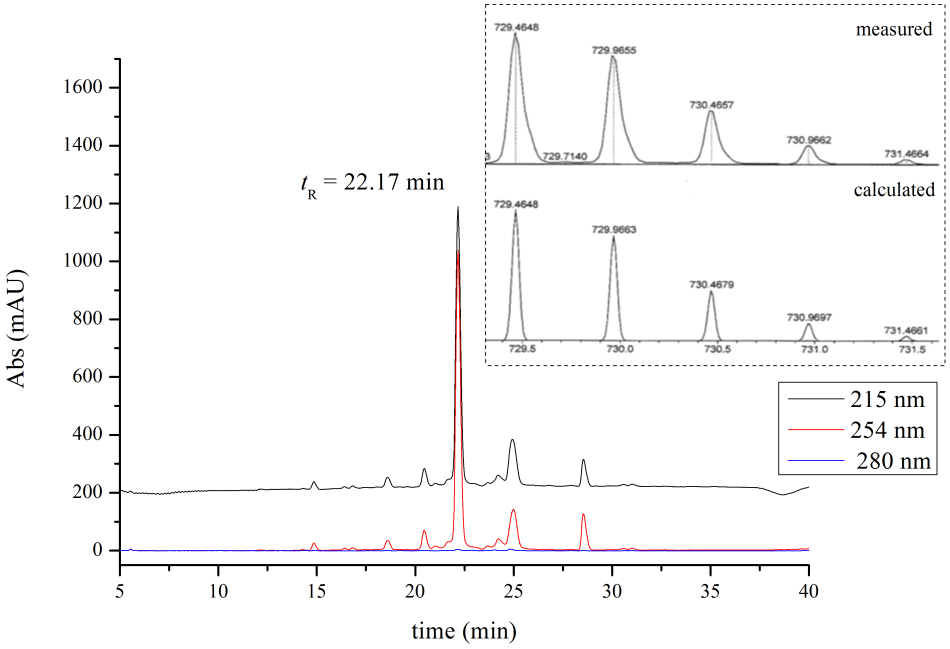
7.2 HPLC- and HR-MS-Spectra of Peptides 10–12, TriazNF1–3



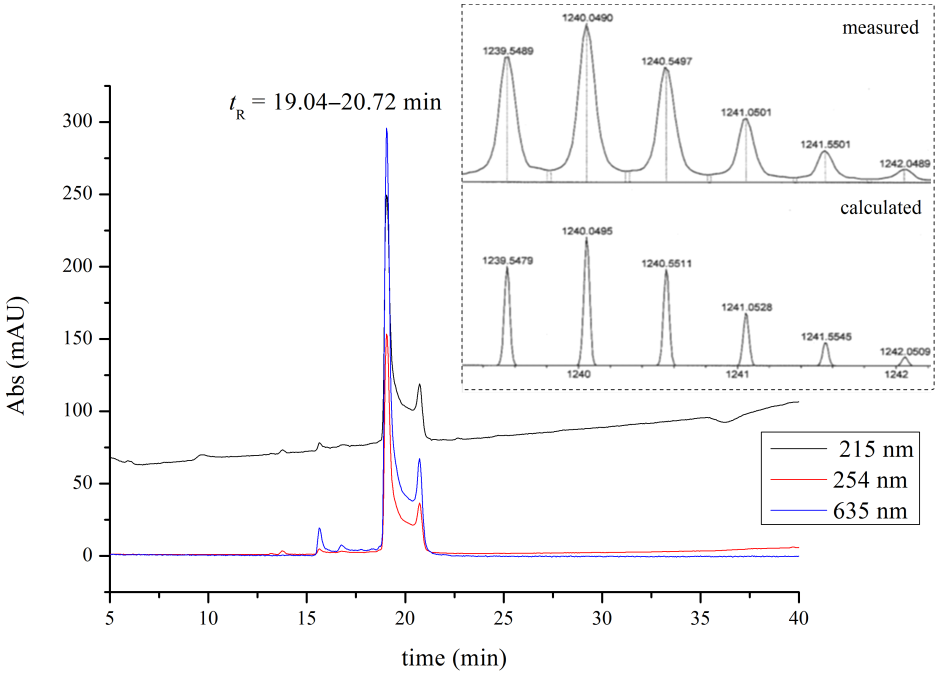
Supplementary Spectra 4: HPLC- and HR-MS-spectra of $^{15}N_6$-triazGly-Lys($^{15}N_6$-triazGly)-Asp-Glu-Lys(N_3)-^{15}N-Gly-Asp-Glu-Lys-^{15}N-Gly-NH_2 (**10**).



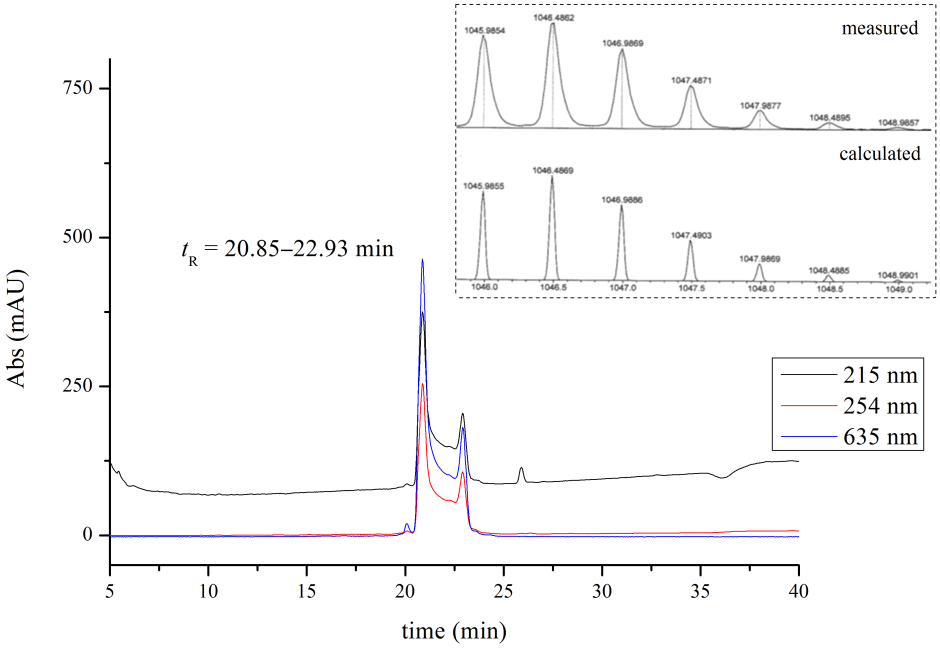
Supplementary Spectra 5: HPLC- and HR-MS-spectra of triazGly-Lys(triazGly)-Cys-Gly-Lys(N_3)-Gly-NH_2 (**11**).



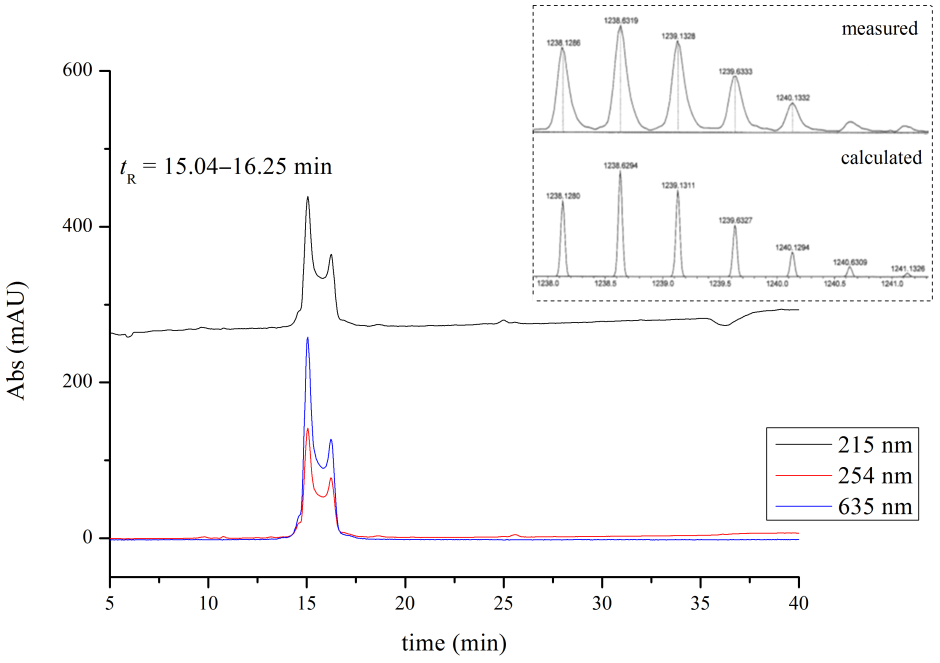
Supplementary Spectra 6: HPLC- and HR-MS-spectra of triazGly-Lys(triazGly)-Cys-Gly-Lys(N_3)-Lys-Lys-Lys-Gly-NH_2 (**12**).



Supplementary Spectra 7: HPLC- and HR-MS-spectra of $^{15}N_6$-triazGly-Lys($^{15}N_6$-triazGly)-Asp-Glu-Lys(N_3)-^{15}N-Gly-Asp-Glu-Lys(Star635)-^{15}N-Gly-NH_2 (**TriazNF1**).



Supplementary Spectra 8: HPLC- and HR-MS-spectra of triazGly-Lys(triazGly)-Cys(Star635)-Gly-Lys(N_3)-Gly-NH_2 (**TriazNF2**).



Supplementary Spectra 9: HPLC- and HR-MS-spectra of triazGly-Lys(triazGly)-Cys(Star635)-Gly-Lys(N_3)-Lys-Lys-Lys-Gly-NH_2 (**TriazNF3**).

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