SUPPORTING INFORMATION

Complete NMR assignment of succinimide, its detection and quantification in peptides and intact proteins

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Supplementary Materials and Methods

Materials. All N^{α}-Fmoc amino acids, Fmoc-Rink amide MBHA, N,N,-dimethylformamide (DMF), 1-methyl-2-pyrrolidinone (NMP), dichloromethane (DCM), diethylether (Et_2O), N,N'-diisopropylethylamine (DIPEA) were purchased from Iris Biotech GmbH (Marktredwitz, Germany). 1-Hydroxybenzotriazole (HOBt), (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (HBTU), trifluoroacetic acid (TFA) and piperidine were obtained from Biosolve (Valkenswaard, The Netherlands). HPLC-grade acetonitrile (ACN), triisopropylsilane (TIS), thioanisole (TIA), acetic anhydride (Ac_2O) were from Sigma-Aldrich (Steinheim, Germany). HPLC-grade TFA was from Alfa-Aesar (Karlsruhe, Germany).

Fmoc-based SPPS. SPPS was automatically performed using a Syro-I (MultiSynTech) peptide synthesizer and Fmoc/tBu strategy. Syntheses were carried out on a 0.018 mmol scale by employing a Rink amide MBHA resin (loading: 0.45 mmol/g). Couplings were performed by using Fmoc-AA-OH/HOBt 1:1 (5 eq.), HBTU (4.9 eq.), and DIPEA (10 eq.), in DMF/NMP 7:3. Each coupling was performed twice for 45 min. N-terminal deprotection was carried out with a 3 min treatment with 30% piperidine in DMF, followed by a 12 min treatment with 15% piperidine in DMF. After the coupling of the last residue, the N-terminal residue was acetylated by treating the resin with Ac_2O (10 eq.) and DIPEA (11 eq.) in DMF for 15 min, followed by washing with DMF, DCM and Et_2O (3 times each) and vacuum drying overnight. The peptide was cleaved from the resin and simultaneously side-chain deprotected by using the mixture TFA/H_2O/TIS/TIA (90:4:3:3) for 2.5 h, followed by precipitation with ice-cold Et_2O and centrifugation at 4°C for 8 min. The crude peptide was washed repeatedly with ice-cold Et_2O and finally vacuum dried overnight. Analytical HPLC and MS data are shown in Table S-21 and Figure S-2.

For the characterization of the side product $\mathbf{2^{Pip}}$ the resin-bound peptide assembled for the preparation of **2** was treated with 30% piperidine in DMF for 20 min prior TFA cleavage (Figures S-3 and S-8). For the characterization of the side product $\mathbf{2^{DKP}}$ the sequence SDGQPEN was assembled with the same protocol used for peptide **2**. HPLC and MS analyses revealed the presence of two peaks with the same molecular mass corresponding to that of the linear sequence with water loss (Figure S-3). To distinguish the DKP-containing peptide $\mathbf{2^{DKP}}$ from its precursor, the Snn-containing species (**2Δ**), the resin-bound peptide was either acetylated or treated with piperidine as described above. The two treatments led to the N-

terminal acetylation (**Ac-2Δ**) or to the Asp(piperidide) (**2Δ^{Pip}**) formation of the Snn-containing peptide, respectively, whereas the DKP-containing peptide remained unchanged (Figure S-3).

Analytical HPLC and MALDI-TOF-MS of the synthetic peptides. Analytical RP-HPLC was performed using a Thermo Scientific™ Dionex™ UltiMate™ 3000 HPLC system (Thermo Fisher Scientific, Germering, Germany) and either a Syncronics C18 column (100 Å, 5 µm, 250 mm x 4.6 mm, at a flow rate of 1.5 mL/min, from Thermo Fisher Scientific) or a Nucleosil C18 column (100 Å, 5 µm, 250 mm x 4.6 mm at a flow rate of 1 mL·min^{-1}, from Macherey-Nagel). Unless specifically stated, the first one was used. The UV detection was set at 220 nm. A binary elution system was used, which consisted of the mobile phase A with 0.06% (v/v) TFA in water, and of the mobile phase B with 0.05% (v/v) TFA in ACN. The crude products were dissolved in ACN/H_2O 10:90 (v/v) containing 0.l % TFA. The analytical HPLC gradient applied for the decapeptides was: 10% B for 5 min, followed by 10-70% B in 40 min. The analytical HPLC gradient applied for the heptapeptides was: 5% B for 5 min, followed by 5-60% B in 45 min. MALDI-TOF-MS analysis of the synthetic peptides was performed both in negative and positive mode on an Autoflex mass spectrometer from Bruker Daltonics using α-cyano-4-hydroxycinnamic acid as matrix (Acros Organics, Germany).

Expression and purification of the recombinant Fc/2 fragment of human IgG1 in *E. coli*. For enhanced expression in bacteria, the DNA sequence of the CH2-CH3 domain (amino acid 241-450) of Rituximab (Fc/2) was altered by codon harmonization.[1] Therefore, the original codons of the DNA sequence corresponding to the CH2-CH3 domain of Rituximab were substituted with synonymous codons from *E. coli* having the same or similar usage frequencies as used in the original gene. The original codons of the first eleven amino acids were replaced by low frequency and thus rare codons. The harmonized sequence (Figure S13c) was synthesized and cloned into the pGH vector by ATG:biosynthetics GmbH (Merzhausen, Germany). The Fc/2 fragment was amplified by polymerase chain reaction (PCR) and digested with the restriction enzymes *Nde I* and *Xho I* (New England BioLabs, Ipswich, MA, USA). The digested Fc/2 fragment was cloned into the pET-based expression vector pHIS parallel 2.[2] The construct was transformed into *E. coli* Rosetta gamiB pLys S (Novagen, Gibbson, NJ, USA). Bacteria were plated on five LB-Amp plates and incubated overnight at 37°C. Two 5 L flasks containing 2.5 L LB-Amp medium were inoculated with

bacteria from plates and incubated at 200 rpm and 37°C until the OD_{600} reached a value of 0.6. The expression of the CH2-CH3 fragment was induced by the addition of 0.4 mM IPTG. The culture was harvested by centrifugation (20 min at 5000 g, 4°C) after expression for 20 h at 16°C. Cells were re-suspended in 1/10 culture volume of 20 mM NaH_2PO_4/Na_2HPO_4 pH 7.0 and disrupted by three repeated cycles of freezing and thawing, homogenization using a tissue homogenizer (ultra-turrax, 5 min on ice) and sonication (5 min on ice). The soluble protein fraction was obtained after a centrifugation step for 20 min at 13000 g and 4°C. The Fc/2 fragment was purified by a two-step process. The first step was an affinity chromatography using a 1 mL Protein G column (GE Healthcare, Chalfont St. Giles, UK) at a flow rate of 1 mL/min. Fractions of 1 mL were collected during 20 mL gradient elution to 0.1 M glycine adjusted to pH 2.7 with HCl. Immediately afterwards, 200 µL 1 M Tris HCl pH 9.0 were added to the fractions to neutralize the pH. Fractions containing the Fc/2 fragment were pooled and concentrated using 3 kDa cut-off Amicon Ultra Centrifugal Filters (Millipore, Billerica, MA). Concentrated samples were applied to size exclusion chromatography using a Sephadex 75 10/300 39 GL column at a flow rate of 0.6 mL/min (Äkta FPLC, GE Healthcare). The Fc/2 fragment was eluted using 5 mM NH_4HCO_3 pH 7.6. Fractions containing the purified Fc/2 fragment were pooled and concentrated using 3 kDa cut-off Amicon Ultra Centrifugal Filters (Millipore). Protein aliquots were stored at -20°C until further use.

Gel electrophoresis of Fc/2. Protein fractions and the purified protein were monitored by reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 15% gels. Proteins were visualized by staining with Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, USA).

Amino acid analysis of Fc/2. The amino acid analysis was performed in triplicates following the Pico-Tag™ method (Waters, Milford, MA). Reversed phase high-performance liquid chromatography (UltiMate 3000, Thermo Fischer, Waltham, USA) using a 3.0x150 mm XSELECT™ HSS T3 3.5 µm column (Waters) was used to analyze the phenylthiocarbamyl amino acid derivatives. Hydrolyzed amino acid peaks were quantified at 254 nm by peak area comparison to amino acid standard H (Pierce, Rockford, IL, USA).

Circular dichroism spectroscopy of Fc/2. The secondary structure of the recombinant Fc/2 fragment of Rituximab was investigated by circular dichroism spectroscopy. Circular

dichroism and thermal denaturation were recorded in 10 mM sodium phosphate pH 7.3 using a JASCO J-815 spectropolarimeter (Jasco, Tokyo, Japan) at a concentration of 0.1 mg/mL. Far-UV CD spectra (190 – 260 nm) spectra were recorded at 20°C, after heating up to 95°C and after cooling down to 20°C. Results are presented as the mean residue molar ellipticity.

Preparation of filgrastim for NMR measurements. For measurements of expired, but untreated filgrastim 14 vials of Neupogen® (each 0.48 mg filgrastim in 1.6 mL) from Amgen (expired 2014) were pooled; concentration and buffer exchange (10 mM ammonium acetate pH 4.0) were simultaneously accomplished with an Amicon ultrafiltration device (3 kDa cutoff). The final volume of 0.72 mL with the initial buffer diluted to 1/32 was lyophilized and afterwards dissolved in 500 µL 7 M urea-d_4 in D_2O (pH* 7.0) and 40 mM DTT-d_{10}. Despite the solution got gel-like, it was incubated for 30 min to allow reduction of disulfide bonds. After adjusting the pH* to 2.5 (uncorrected) with diluted DCl the solution cleared up and all precipitate dissolved. This solution was measured in a standard 5 mm NMR tube. To obtain Snn-containing filgrastim 14 vials of Neupogen® from Amgen (expired 2014) were pooled and mixed 1:1 with a 0.5 M ammonium acetate pH 4.0 in the presence of 2.4 mM TCEP. After incubation at 40°C for 65 h, the solution was concentrated in an Amicon ultrafiltration device and the starting buffer was diluted 36 times with a 10 mM ammonium acetate pH 4.0 buffer, resulting in a final volume of 1.8 mL. After lyophilization the sample was immediately dissolved in 300 uL 7 M urea-d_4 in D_2O (pH* 2.3, uncorrected), which was transferred into a 5 mm Shigemi tube for NMR measurements.

Preparation of Fc/2 for NMR measurements. For the detection of Snn in Fc/2, 15.5 mL of 7.8 µM recombinant Fc/2 protein solution in 5 mM NH_4HCO_3 buffer pH 7.4 were mixed 1:1 with 0.5 M ammonium acetate pH 4.0 and incubated at 40°C for 138 h (no TCEP). The entire volume was lyophilized and afterwards dissolved in 500 uL 7 M urea-d_4 in D_2O (pH* 2.3, uncorrected), containing 5 mM TCEP. After 1 h incubation at 37°C the sample was transferred to a standard 5 mm NMR tube.

NMR spectroscopy. Sample volumes of 500 µL of solutions in either 100% D_2O (Armar, Germany) or H_2O/D_2O (93:7 v/v) in 5 mm standard NMR tubes were used unless indicated otherwise. For samples in H_2O/D_2O we used a 3-9-19 Watergate composite pulse for suppression of the water resonance. For the long-range H-CO correlation experiment we adopted an ^{1}H-^{13}C

HSQC by applying all 90° pulses in the ^{13}C channel as selective Q5[3] and time-reverse Q5 pulses and all 180° pulses as REBURP pulses[4] and used several INEPT delays (Figure S-15). Due to the modulation of the signal by various homonuclear ^{1}H-^{1}H scalar couplings, the sign of cross-peaks sometimes changed, bearing the risk that some signals might undergo a zero-crossing and disappear. Therefore, we recorded three spectra with different INEPT delays, always a multiple of 13 ms to suppress transfer due to $^{1}J_{CH}$.

Mass spectrometry. Analysis was carried out on an HPLC system (UltiMate 3000, Thermo Fisher Scientific, Germering, Germany). Heptapeptides were separated at a flow rate of 100 µL min^{-1} on a Hypersil Gold aQ column (100 × 1.0 mm i.d., 1.9 µm particle size, 175 Å pore size, Thermo Fisher Scientific, Sunnyvale, CA, USA), operated at a temperature of 50°C. Mobile phase A was H_2O + 0.10% TFA, mobile phase B was composed of acetonitrile + 0.10% TFA. The gradient applied was: 1.0% B for 5 min, 1.0−15.0% B in 2.5 min, 15.0−60.0% B in 2.5 min, column regeneration at 100.0% B for 5 min and re-equilibration at 1.0% B for 20 min. Five microliters of sample were injected in in-line split-loop mode. UV-detection was carried out with a 2.5 µL flow-cell at 214 nm. Mass spectrometry was conducted on a Thermo Scientific™ Q Exactive™ Hybrid-Quadrupol-Orbitrap mass spectrometer equipped with an Ion Max source with a heated electrospray ionization (HESI) probe (Thermo Fisher Scientific, Bremen, Germany). Mass calibration of the instrument was conducted with Pierce™ LTQ Velos ESI Positive Ion Calibration Solution from Life Technologies (Vienna, Austria). The instrument settings were as follows: source heater temperature of 250°C, spray voltage of 3.5 kV, sheath gas flow of 20 arbitrary units, auxiliary gas flow of 5 arbitrary units, capillary temperature of 250°C, S-lens RF level of 70.0, AGC target of 1e6 and a maximum injection time of 200 ms. Peptides were analyzed with a full scan at a scan range of *m/z* 250-1,000 and a resolution of 17,500 at *m/z* 200, followed by three data-dependent scans (dd-MS^2) upon higher-energy induced collisional dissociation (HCD) at 25% normalized collision energy.

Lysozyme samples were diluted in 0.10% (v/v) aqueous formic acid (FA) to a final concentration of 0.50 mg mL^{-1}. Intramolecular disulfide bonds were reduced with 5 m mol·L^{-1} tris (2-carboxyethyl) phosphine hydrochloride at 60°C for 30 minutes. The analysis was carried out utilizing the same HPLC-MS system as described above operated at a flow rate of 200 µL min^{-1}. A Waters XBridge Protein BEH C4 column (150 × 2.1 mm i.d., 3.5 µm particle size, 300 Å pore size, Waters, Milford, MA, USA) was operated at a column

temperature of 60 °C. Two microliters of sample were injected in in-line split-loop mode. Separation was carried out with an initial equilibration at 5.0% (v/v) acetonitrile (ACN) in 0.10% (v/v) formic acid (FA) for 5 min, followed by a linear gradient of 5.0−50.0% ACN + 0.10% FA in 20 min, column regeneration at 99.99% ACN in 0.10% FA for 10 min and re-equilibration at 5.0% in 0.10% FA for 15 min. For lysozyme the instrument settings were as follows: source heater temperature of 200°C, spray voltage of 3.5 kV, sheath gas flow of 20 arbitrary units, auxiliary gas flow of 5 arbitrary units, capillary temperature of 250°C, S-lens RF level of 70.0, in-source CID of 20.0 eV, AGC target of 1e6 and a maximum injection time of 200 ms. The intact protein measurements were carried out in full scan mode with a range of *m/z* 500 – 2,500 at a resolution setting of 140,000. Top-down sequencing experiments were carried out in all-ion fragmentation mode (AIF) in the higher-energy collisional dissociation cell (HCD) with a normalized collision energy (NCE) of 16 % (corresponding to 48 eV collision energy at *m/z* 1,500) with a scan range of *m/z* 500 - 2,500 at a resolution of setting 140,000. Filgrastim and Fc/2 samples were diluted in 0.10% (v/v) aqueous formic acid (FA) to a final concentration of 0.10 mg mL^{-1}. The analysis was carried out utilizing the same HPLC-MS system as described above operated at a flow rate of 300 µL min^{-1}. A Waters XBridge Protein BEH C4 column (150 × 2.1 mm i.d., 3.5 µm particle size, 300 Å pore size, Waters, Milford, MA, USA) was operated at a column temperature of 60 °C. Five microliters of sample were injected in in-line split-loop mode. Separation was carried out with an initial equilibration at 20.0% (v/v) ACN in 0.10% FA for 2.5 min, followed by a linear gradient of 20.0−70.0% ACN + 0.10% FA in 5 min, column regeneration at 99.99% ACN in 0.10% FA for 2.5 min and re-equilibration at 20.0% in 0.10% FA for 10 min. For MS the instrument settings were as follows: source heater temperature of 250°C, spray voltage of 3.5 kV, sheath gas flow of 20 arbitrary units, auxiliary gas flow of 5 arbitrary units, capillary temperature of 250°C, S-lens RF level of 70.0, in-source CID of 20.0 eV, AGC target of 1e6 and a maximum injection time of 100 ms. The intact protein measurements were carried out in full scan mode with a range of *m/z* 1500 – 3,000 at a resolution setting of 140,000. Top-down sequencing experiments for filgrastim were carried out for the MH11+ charge state with 15% NCE (4.7 eV) in the HCD-cell. The C_H2-C_H3 domain was sequenced in AIF-mode at 10% NCE (30 eV) in the HCD-cell with a scan range of *m/z* 500 - 2,500. Deconvolution of the ion spectra was carried out with the sliding window option and the Xtract™ algorithm integrated into BioPharma Finder™ version 1.0.76.10 (Thermo Fisher Scientific). The fragment ion spectra were deconvoluted utilizing the Xtract algorithm in the software Xcalibur™ 3.0.63 (Thermo Fisher Scientific). Sequence coverage of fragmentation experiments was determined with

ProSight Lite v1.3 Build 1.3.5744.16422 with an allowed mass tolerance of 10 ppm (http://prosightlite.northwestern.edu) provided by the Kelleher Research Group (Northwestern University, Evanston, IL, USA). Ultrapure water was produced in-house by a Millipore Integral 3 from Merck/Millipore (Billerica, MA, USA). Unless stated otherwise, chemicals were obtained from Sigma-Aldrich (Vienna, Austria).

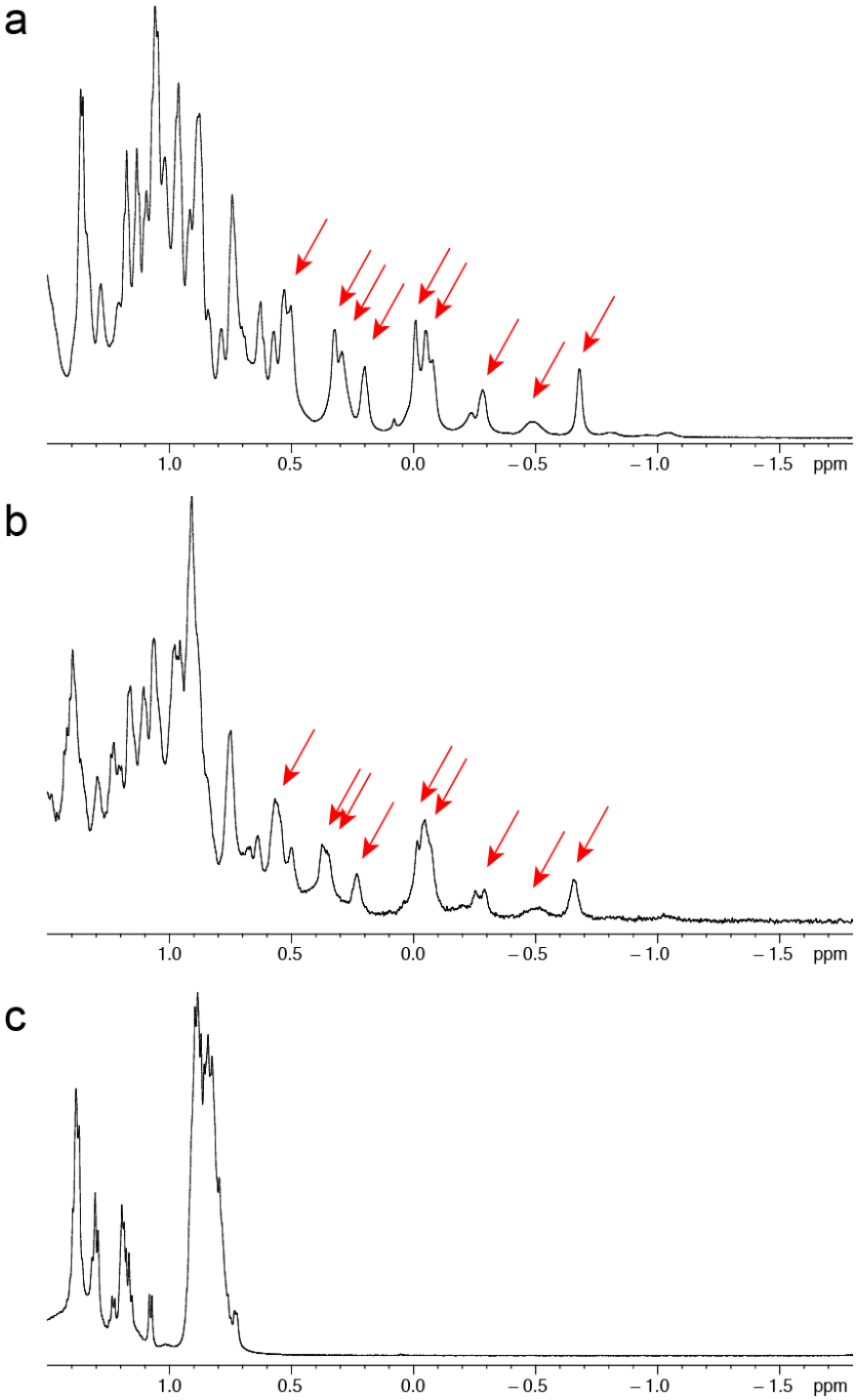


Figure S-1. 1D ^{1}H NMR spectra of lysozyme illustrate the degree of folding. (a) ^{1}H spectrum of fresh lysozyme dissolved in D_2O recorded at 298 K, 600 MHz, a protein concentration of 2 mmol·L^{-1} and ns=16. Shown is the region of methyl signals. Upfield shifted signals highlighted with an arrow are typical for well-folded proteins. (b) ^{1}H spectrum of lysozyme dissolved in a fully deuterated solution of 7 mol·L^{-1} urea-d_4 and 45 mmol·L^{-1} DTT-d_{10} pH* 7 recorded at 298 K, 600 MHz, a protein concentration of 2 mmol·L^{-1} and ns=1. (c) ^{1}H spectrum of lysozyme dissolved in 7 mol·L^{-1} urea-d_4 and 45 mmol·L^{-1} DTT-d_{10} pH adjusted to pH* 2.3, recorded at 298 K, 600 MHz, a protein concentration of 2 mmol·L^{-1} and ns=1.

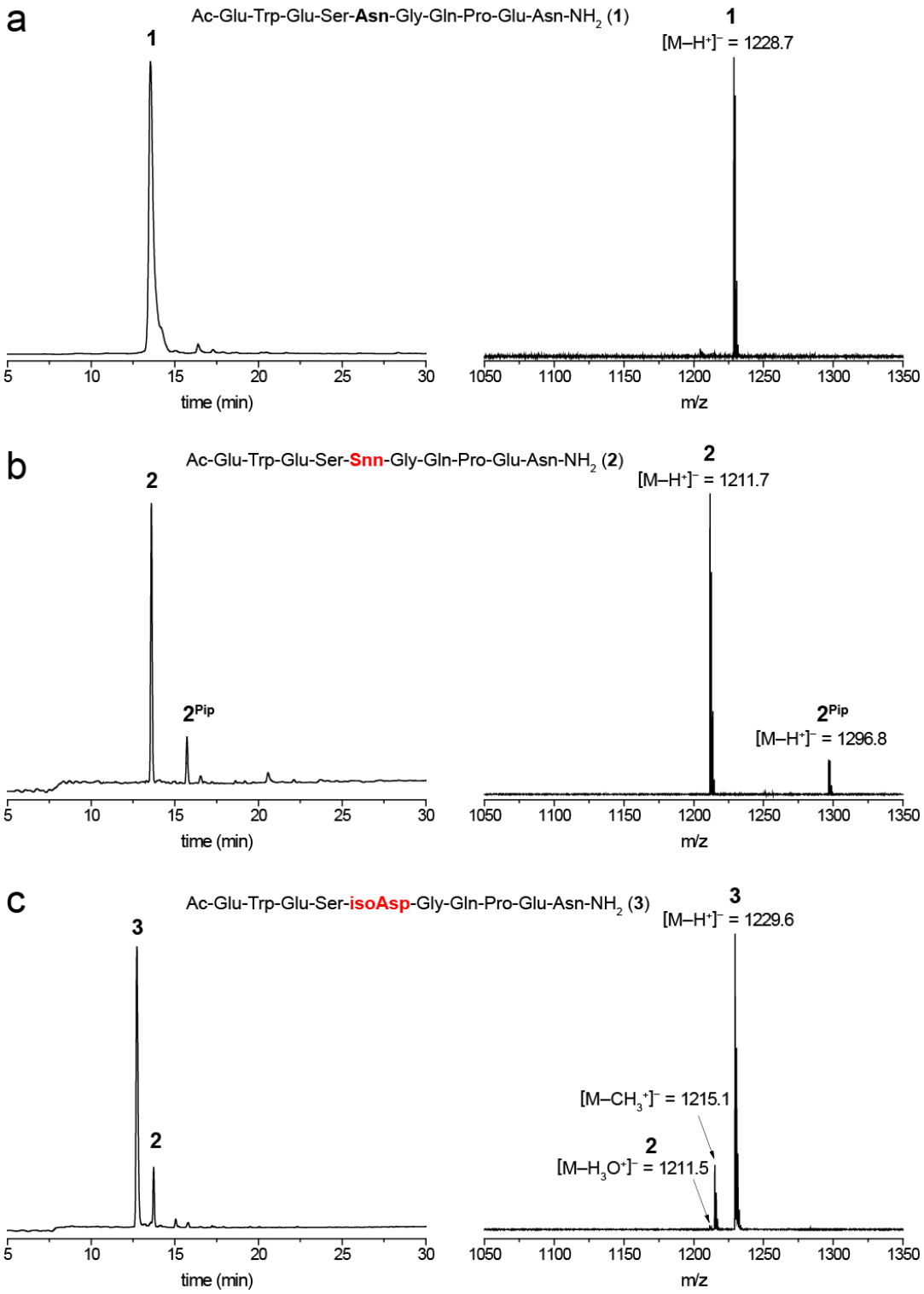


Figure S-2. Characterization of the synthesized peptides by analytical HPLC and MALDI-TOF-MS. (a) The purity of **1** was 95%. (b) The ratio between **2** and $\mathbf{2}^{\mathbf{Pip}}$ was 6:1. (c) The ratio between **3** and **2** was 6:1.

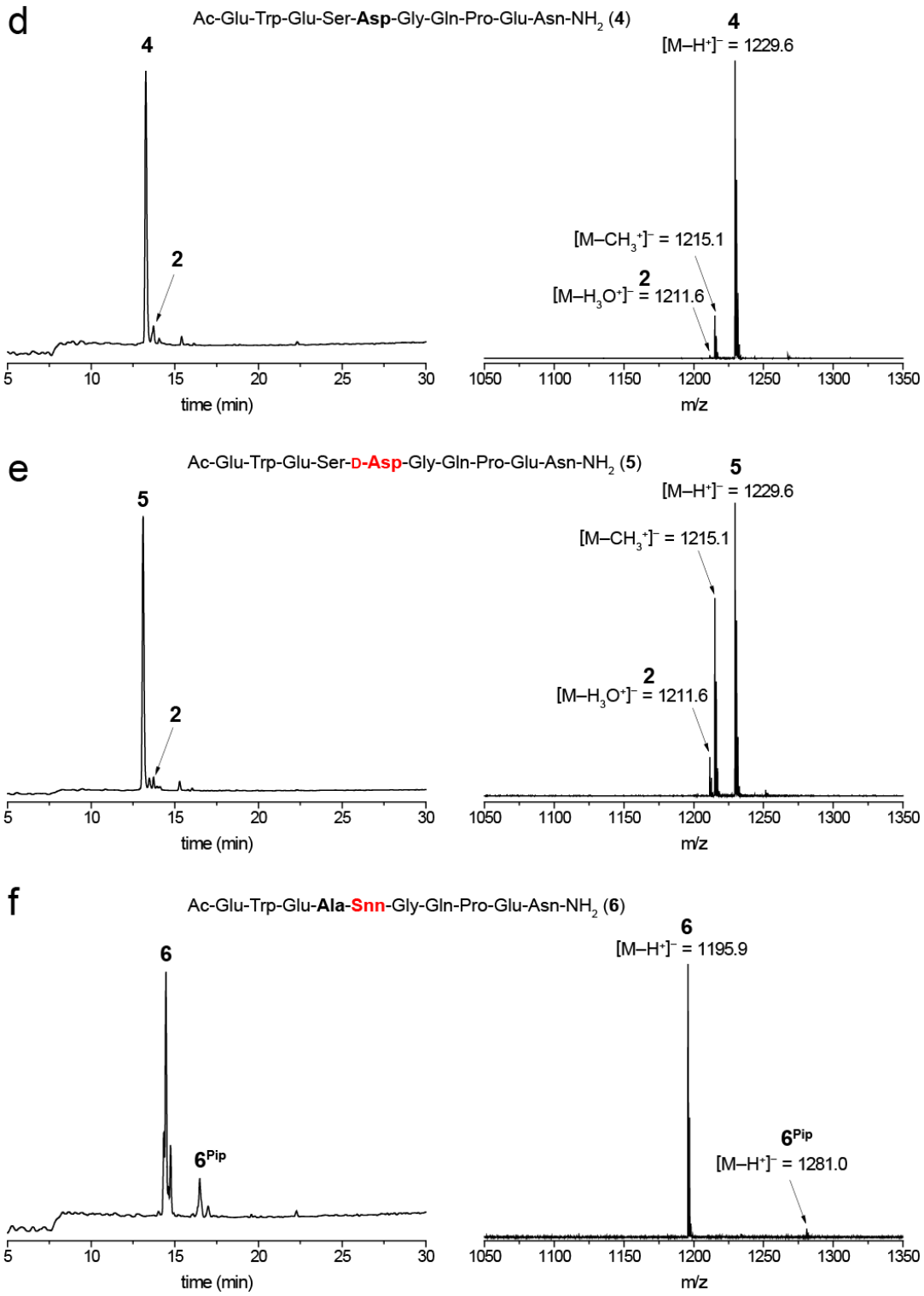


Figure S-2 (continued). (d) The purity of **4** was 91%. (e) The purity of **5** was 93%. (f) The ratio between **6** and $\mathbf{6}^{\mathbf{Pip}}$ was 1:4 (the splitting of the HPLC peaks is likely due to the presence of stereoisomers)

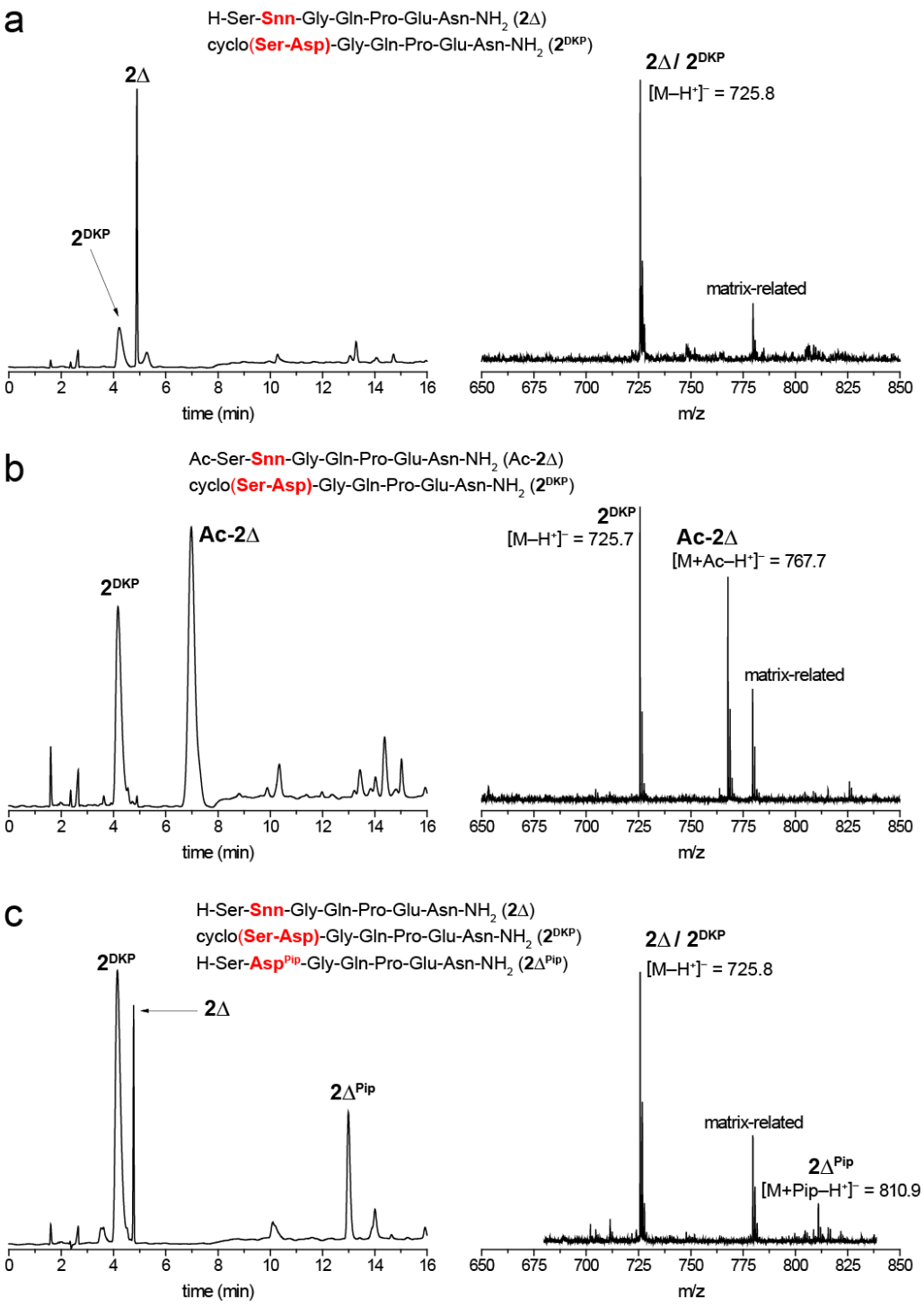


Figure S-3. Formation of Snn and DKP during SPPS with Fmoc-chemistry. (a) The assembly of the heptapeptide SDGQPEN using Fmoc-Asp(OAll)-OH led to the formation of Snn (**2Δ**). The latter partly rearranged to yield the Ser-Asp DKP ($\mathbf{2}^{\mathbf{DKP}}$). (b) Acetylation of the resin-bound mixture led to the acetylation of the Snn-containing peptide (**Ac-2Δ**), whereas the DKP-containing peptide $\mathbf{2}^{\mathbf{DKP}}$ remained unchanged. (c) Instead, treatment with 30% piperidine in DMF for 20 min. led to the aminolysis of Snn by nucleophilic attack of both piperidine ($\mathbf{2\Delta}^{\mathbf{Pip}}$) and the free N-terminus ($\mathbf{2}^{\mathbf{DKP}}$).

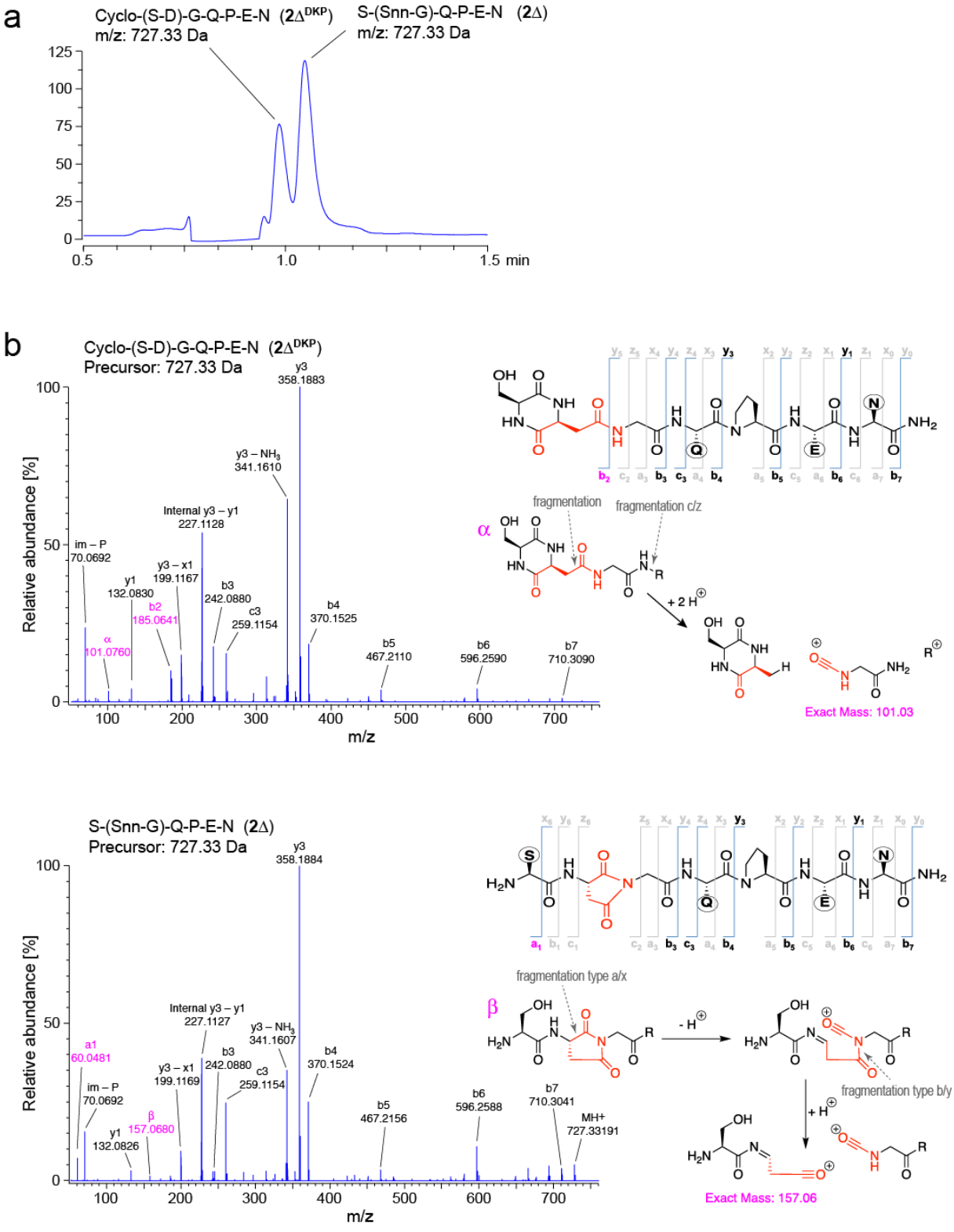


Figure S-4. HPLC-MS^2 analysis of the heptapeptides **2Δ** and **6Δ** confirm the presence of a mixture of Snn and diketopiperazine. (a) HPLC profile of the peptide **2Δ** revealing two signals with identical mass. (b) Corresponding MS^2 spectra of the two HPLC fractions of peptide **2Δ** (left) and proposed fragmentation patterns to explain the observed fragments (right).

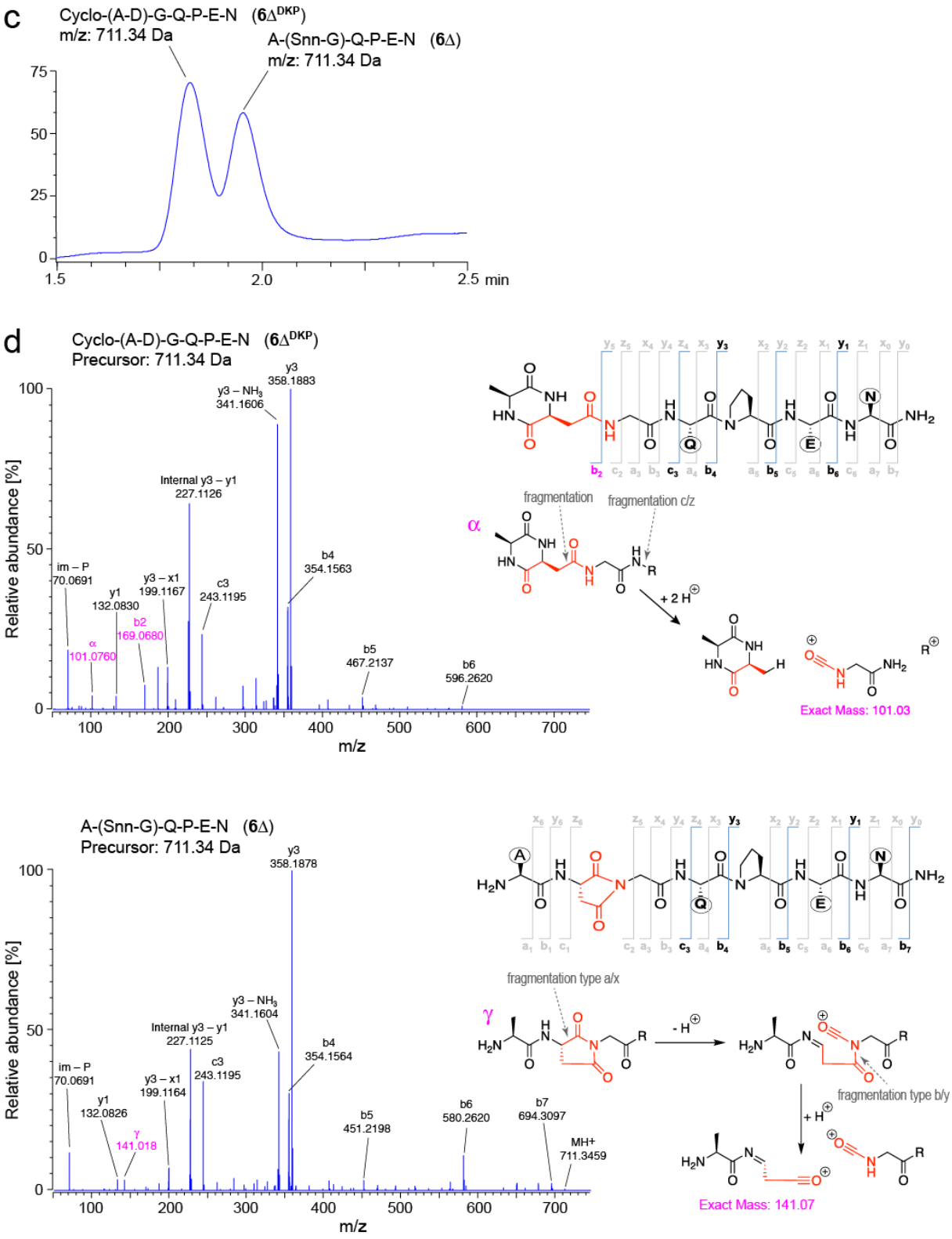


Figure S-4 (continued). (c) HPLC profile of the peptide **6Δ** revealing two signals with identical mass. (d) Corresponding MS^2 spectra of the two HPLC fractions of peptide **6Δ** (left) and proposed fragmentation patterns to explain the observed fragments (right).

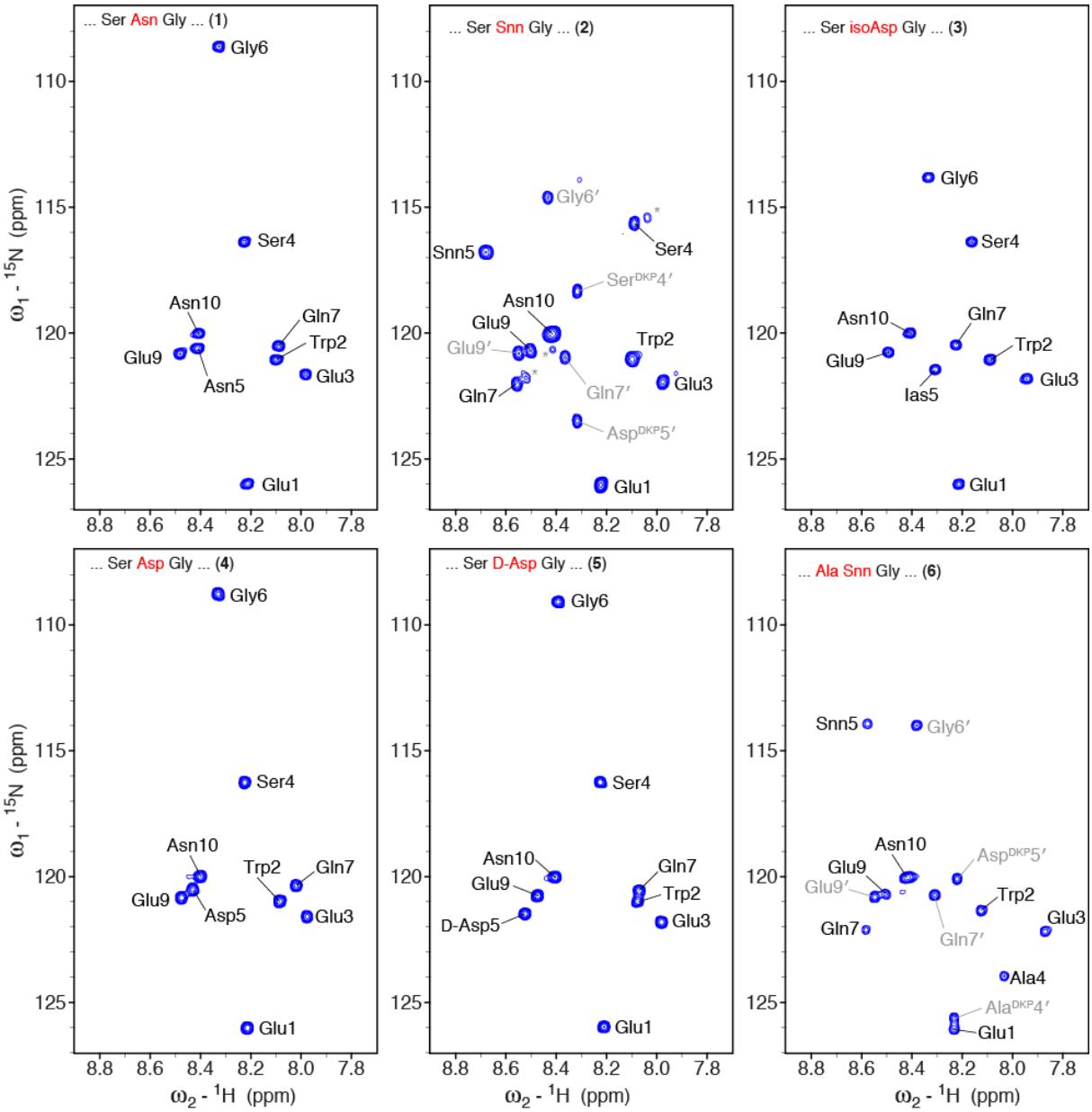


Figure S-5. Fingerprint ^{1}H-^{15}N-HSQC spectra of all synthesized peptides (2 mmol·L^{-1}) in H_2O/D_2O (93:7, v/v) using the natural abundance of ^{15}N. Spectra were recorded at 298 K using 500 transients. Whereas homogeneous peptides were obtained for those containing Asn-5, Asp-5, D-Asp-5 and isoAsp5, the two peptide preparations for obtaining Snn-5 displayed two sets of signals. In addition to the expected peptide signals, a second set was observed that originated from a truncated peptide lacking the N-terminus Ac-Glu-Trp-Glu and containing a diketopiperazine (DKP) – a side product of the synthesis. The second set of signals is labeled in grey and the residue numbers are marked with a prime. A third much weaker set of signals in **2** is marked with grey asterisks, originating from a piperidine linked to Cγ of an Asp-5, another possible side product.

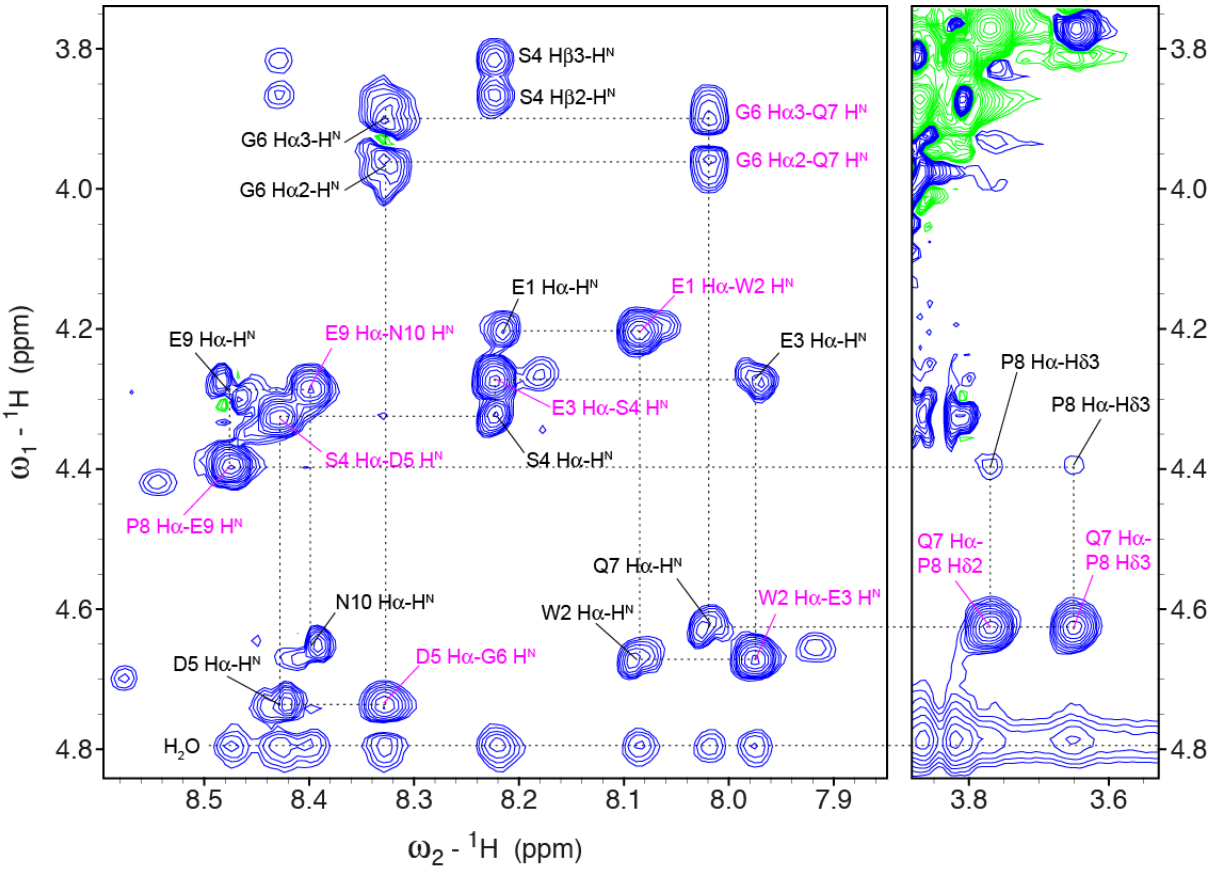


Figure S-6. Sequential assignment walk illustrated for peptide **4**. A 2D ^{1}H-^{1}H ROESY spectrum recorded of a sample in H_2O/D_2O (93:7, v/v) at 298 K with a mixing time of 200 ms and 96 transients. Intra-residue correlations are labeled in black, sequential correlations in magenta.

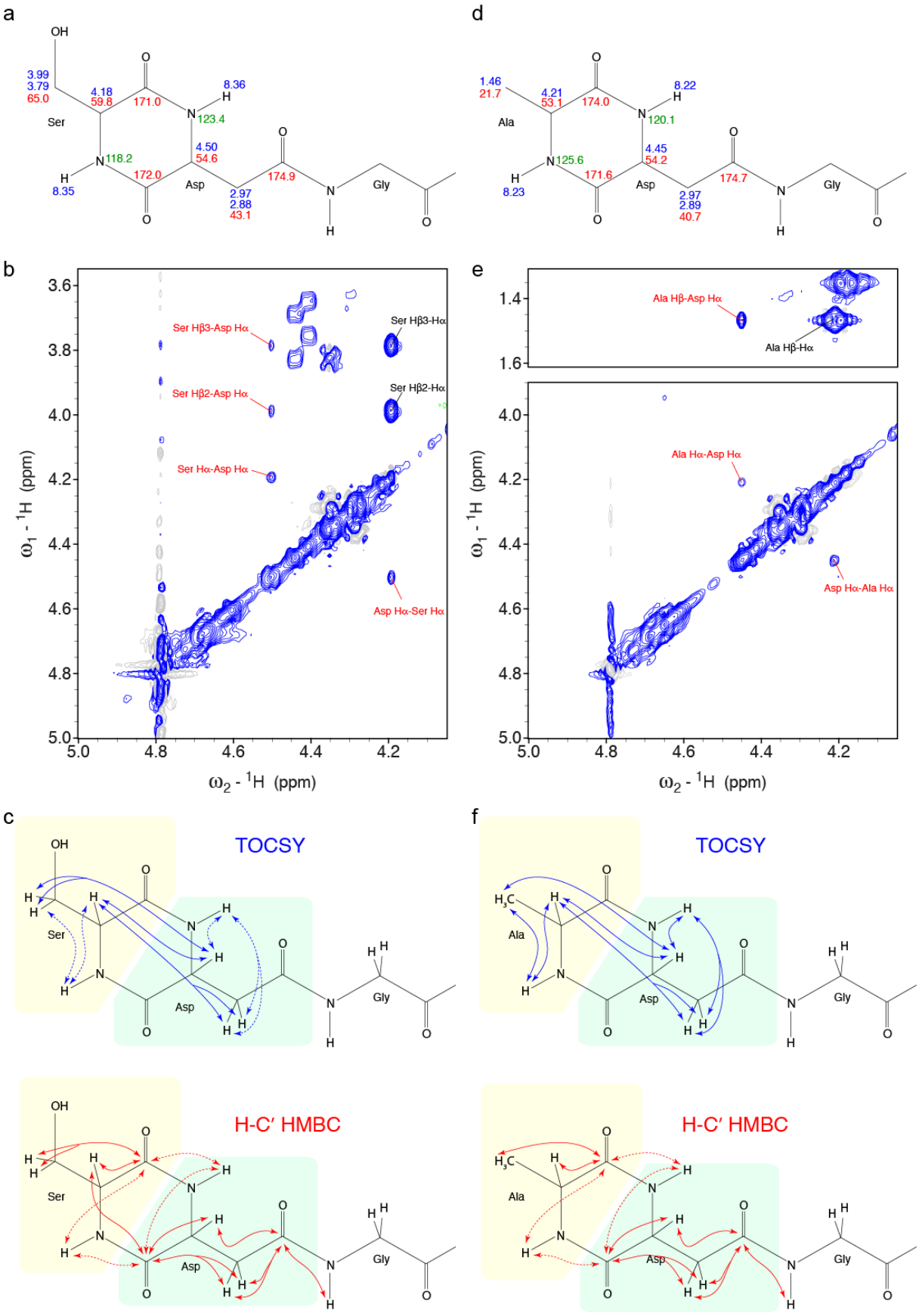

a
d
b
e
c
f
Ser
Asp
Gly
Ala
Ser Hβ3-Asp Hα
Ser Hβ2-Asp Hα
Ser Hα-Asp Hα
Asp Hα-Ser Hα
Ser Hβ3-Hα
Ser Hβ2-Hα
Ala Hβ-Asp Hα
Ala Hβ-Hα
Ala Hα-Asp Hα
Asp Hα-Ala Hα
ω1 - 1H (ppm)
ω2 - 1H (ppm)
TOCSY
H-C′ HMBC

Figure S-7. NMR characterization of the diketopiperazines cyclo(Ser-Asp)-... and cyclo(Ala-Asp)-... that are formed as side products during the peptide synthesis of the decapeptides **2** and **6**. (a) ^{1}H, ^{13}C, ^{15}N chemical shifts of cyclo(Ser-Asp) as part of the heptapeptide cyclo(Ser-Asp)-Gly-Gln-Pro-Glu-Asn-NH_2. The chemical shifts measured in 7 $mol \cdot L^{-1}$ urea at pH 2.3 are given. (b) Unusual sequential ^{1}H-^{1}H correlations of cyclo(Ser-Asp) observed in a 2D TOCSY spectrum measured at 298 K in D_2O with a mixing time of 120 ms and 4 transients. (c) NMR correlations confirming the presence of a diketopiperazine. Dotted arrows indicate correlations that suffered from chemical shift degeneracies (identical H^N chemical shifts), however, in spectra recorded in DMSO, in which the two amides of Ser and Asp show clearly distinct chemical shifts, the TOCSY signals were unambiguously observed. (d) ^{1}H, ^{13}C, ^{15}N chemical shifts of cyclo(Ala-Asp) as part of the heptapeptide cyclo(Ala-Asp)-Gly-Gln-Pro-Glu-Asn-NH_2 measured in D_2O and H_2O/D_2O at pH 2.5. (e) 2D ^{1}H-^{1}H TOCSY spectrum measured at 298 K in D_2O with a mixing time of 120 ms and 4 transients. (f) Summary of NMR correlations of the diketopiperazine cyclo(Ala-Asp).

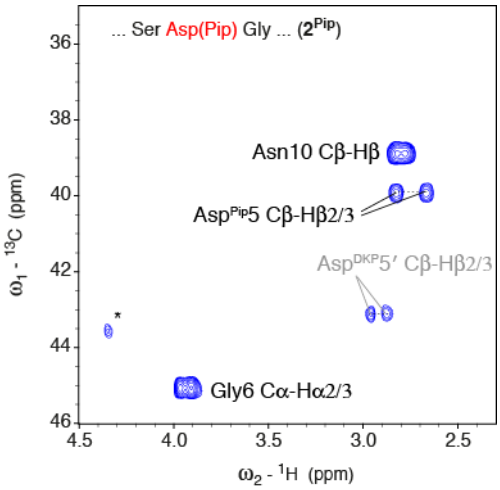


Figure S-8. NMR characterization of peptide **2** treated with piperidine. Shown is a is the fingerprint region 2D ^{1}H-^{13}C HSQC spectrum measured in D_2O displaying Cβ-Hβ correlations of Asp, Asn and isoAsp as well as Cα-Hα correlations of Gly. A comparison with Figure 3 revealed that the Cβ-Hβ correlations of Snn disappeared, whereas new Cβ-Hβ correlations of the Asp-piperidide showed up, showing that Snn was completely converted to the piperidide **2**$^{\mathbf{Pip}}$. However, the diketopiperazine **2**$^{\mathbf{DKP}}$ also present in sample of peptide **2** is unperturbed (signal labeled in grey) and thus unreactive to piperidine. An asterisk indicates a small amount of Gly, whose nitrogen is still part of an Snn.

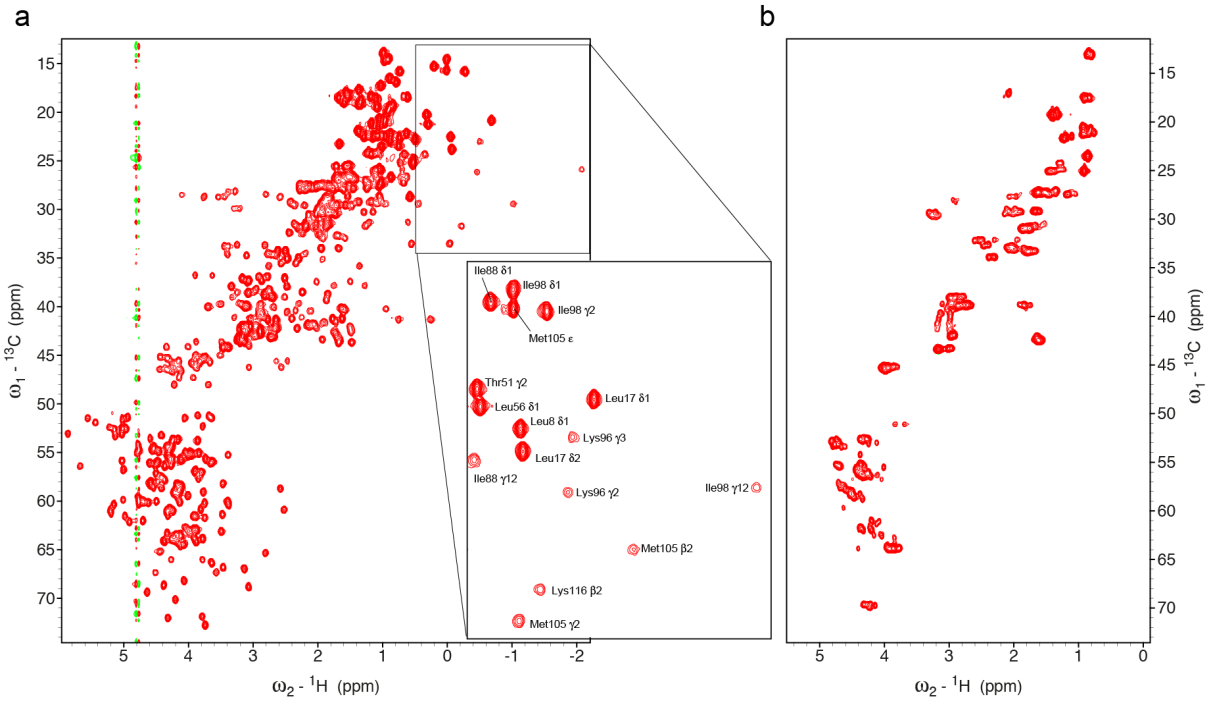


Figure S-9. 2D ^{1}H-^{13}C HSQC spectra of lysozyme at natural ^{13}C abundance (a) in the folded state and (b) denatured in 7 mol·L^{-1} urea-d_4 in D_2O (pH* 2.3). Spectra were recorded at 298 K, protein concentrations of 4.8 m mol·L^{-1} (folded) and 2 mmol·L^{-1} (denatured) and 72 (folded) or 156 transients (denatured). A part of the most upfield region of the folded protein spectrum is magnified and assignments are added, which were in accordance to previously reported data.[5]

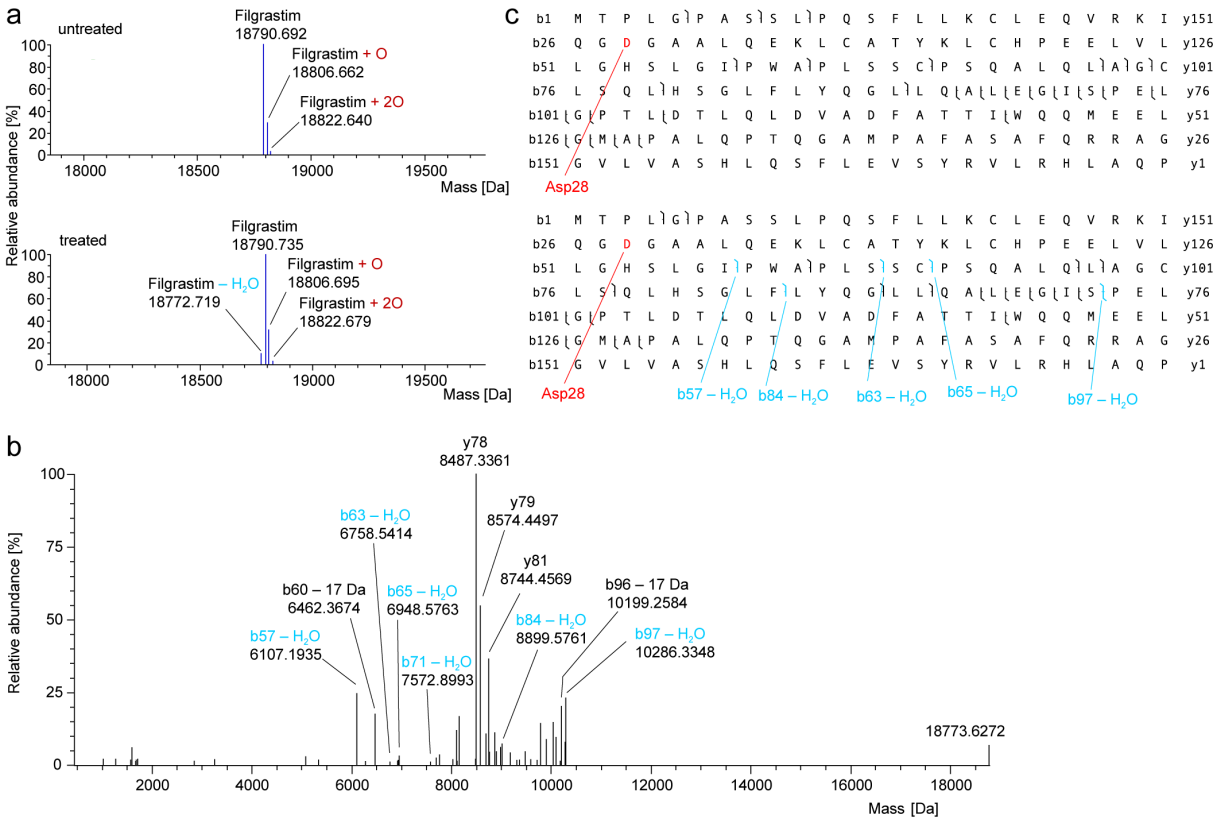


Figure S-10. Detection of succinimide in expired filgrastim by top-down mass spectrometry. (a) Deconvoluted mass spectra of reduced filgrastim as control (top) and of filgrastim treated at pH 4 (bottom): Intact mass spectrometry revealed filgrastim species with H_2O loss. (b) Fragments diagnostic for Snn-formation due to dehydration of Asp28 are labeled in light blue. (c) Sequence coverage map of untreated (top) and pH 4 treated filgrastim (bottom) generated with ProSight Lite. The diagnostic fragments b57, b65, b84 and b97 shown in light blue indicate Snn formation with water loss (-18 Da) at Asp28 (red). Additionally detected fragments with -17 Da mass shift may hint to additional modifications occurring during the treatment with TCEP at pH 4. A possibility is the deamidation of glutamine to glutamic acid (+ 1 Da) in combination with the succinimide formation.

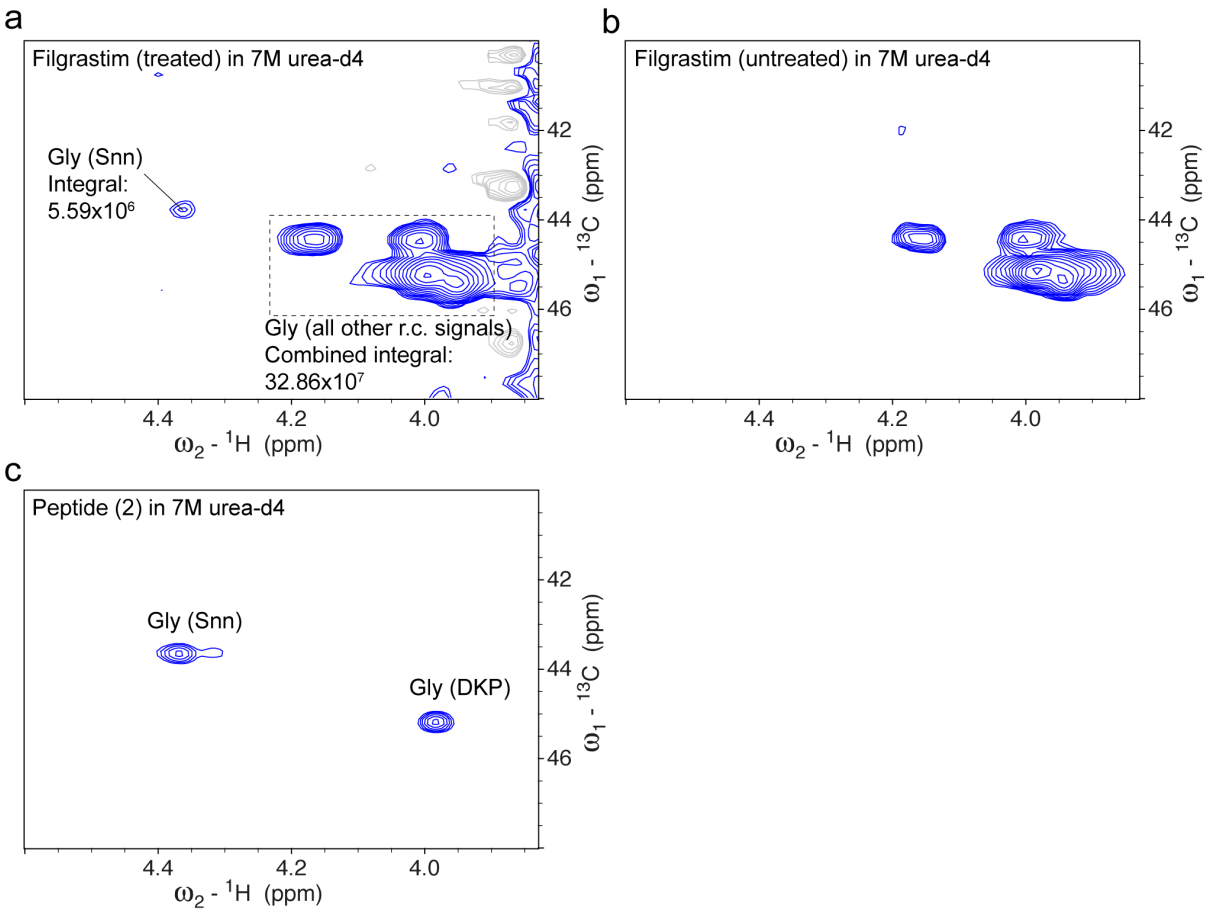
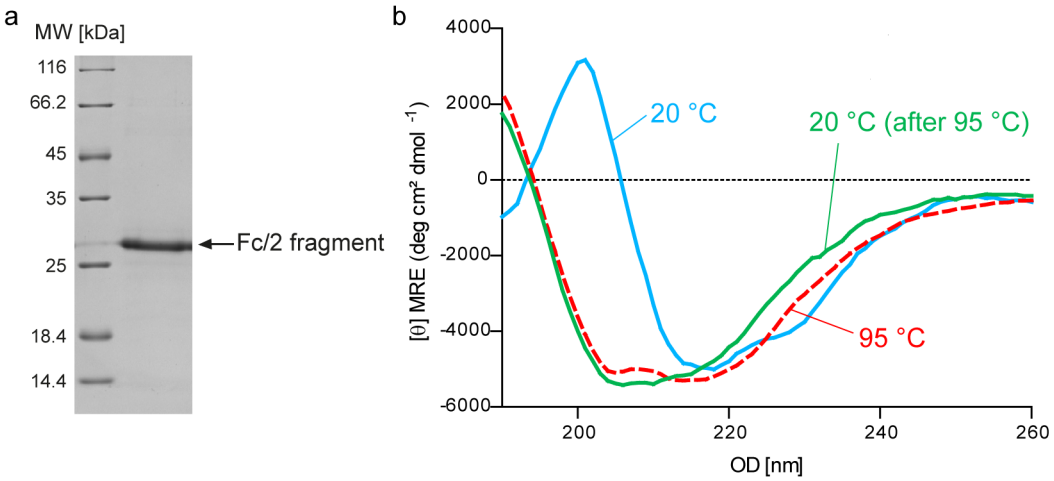


Figure S-11. Application of the NMR approach to the biotherapeutic filgrastim. (a) Representative region of a ^{13}C HSQC of filgrastim (expired in 2014) that was treated for 65 h at pH 4.0 in the presence of TCEP, lyophilized and denatured in fully deuterated 7 M urea-d_4 in D_2O (pH* 2.3) at a concentration of 1.2 mM, measured at 600 MHz and 298 K with ns=400, d1=2 s and td=350. Clearly a signal of a glycine next to a Snn is observed. Due to a small influence of the neighboring residue on the Cα and Hα chemical shifts, several random coil signals of glycines are visible: for example, a C-terminal Pro neighbor, which occurs twice in the protein sequence, shifts ^{13}C by -2.0 ppm and ^{1}H by 0.11 ppm.[6] The integrals indicate ~23% Snn per molecule. The artifacts on the right of the spectrum originate from t_1 noise of sorbitol that remained from the formulation in the sample. (b) Comparable region of a ^{13}C HSQC of untreated filgrastim (expired in 2014) in fully deuterated 7 M urea-d_4 (pH* 2.3) at a concentration of 0.7 mM, recorded at 600 MHz and 298 K with ns=600, d1=2 s and td=350. A signal indicating Snn is not observable. (c) Comparable region of a ^{13}C HSQC of peptide **2** measured in 7 M urea-d_4 (pH* 2.3) showing the random coil Cα-Hα cross-peaks of glycines for comparison.



c

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ATGGGGCCCTCCGTATTCCTATTCCCCCCCAAGCCGAAAGACACCTTAATGATCAGCCGTACCCCG
 M  G  P  S  V  F  L  F  P  P  K  P  K  D  T  L  M  I  S  R  T  P
GAAGTTACGTGCGTGGTGGTGGACGTGAGCCATGAGGACCCGGAAGTTAAATTTAATTGGTATGTG
 E  V  T  C  V  V  V  D  V  S  H  E  D  P  E  V  K  F  N  W  Y  V
GACGGTGTGGAAGTGCACAATGCCAAAACGAAACCACGTGAAGAACAGTATAATAGCACATATCGA
 D  G  V  E  V  H  N  A  K  T  K  P  R  E  E  Q  Y  N  S  T  Y  R
GTGGTTAGCGTTTTAACCGTTCTGCATCAGGACTGGCTGAATGGTAAAGAATATAAATGCAAAGTT
 V  V  S  V  L  T  V  L  H  Q  D  W  L  N  G  K  E  Y  K  C  K  V
AGCAATAAAGCCTTACCGGCCCCGATCGAAAAAACCATCAGCAAAGCCAAAGGTCAGCCGCGAGAG
 S  N  K  A  L  P  A  P  I  E  K  T  I  S  K  A  K  G  Q  P  R  E
CCGCAGGTGTATACCCTGCCGCCGAGCCGTGACGAACTGACCAAAAATCAGGTTAGCCTGACCTGC
 P  Q  V  Y  T  L  P  P  S  R  D  E  L  T  K  N  Q  V  S  L  T  C
CTGGTTAAAGGTTTTTACCCGAGCGACATCGCCGTGGAATGGGAAAGCAATGGTCAGCCAGAAAAT
 L  V  K  G  F  Y  P  S  D  I  A  V  E  W  E  S  N  G  Q  P  E  N
AATTATAAAACCACACCGCCGGTGCTGGACAGCGACGGTAGCTTTTTTTTATATAGCAAATTAACC
 N  Y  K  T  T  P  P  V  L  D  S  D  G  S  F  F  L  Y  S  K  L  T
GTGGACAAAAGCCGTTGGCAGCAGGGTAATGTTTTTAGCTGCAGCGTGATGCACGAAGCACTGCAT
 V  D  K  S  R  W  Q  Q  G  N  V  F  S  C  S  V  M  H  E  A  L  H
AATCATTATACACAGAAAAGCTTAAGCCTGAGCCCAGGAAAATAA
 N  H  Y  T  Q  K  S  L  S  L  S  P  G  K stop
```

Figure S-12. Physicochemical characterization and sequence of the Fc/2 fragment. (a) Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified Fc/2 fragment followed by Coomassie staining. (b) Far UV CD spectra recorded at 20°C (blue line), after heating up to 95°C (red dashed lined) and after cooling down to 20°C (green line). The initial spectrum indicates the presence of secondary structure elements and upon heat denaturation, the Fc/2 fragment unfolded and was not able to refold upon cooling to 20°C. (c) DNA and protein sequence used for recombinant expression after the sequence was harmonized.

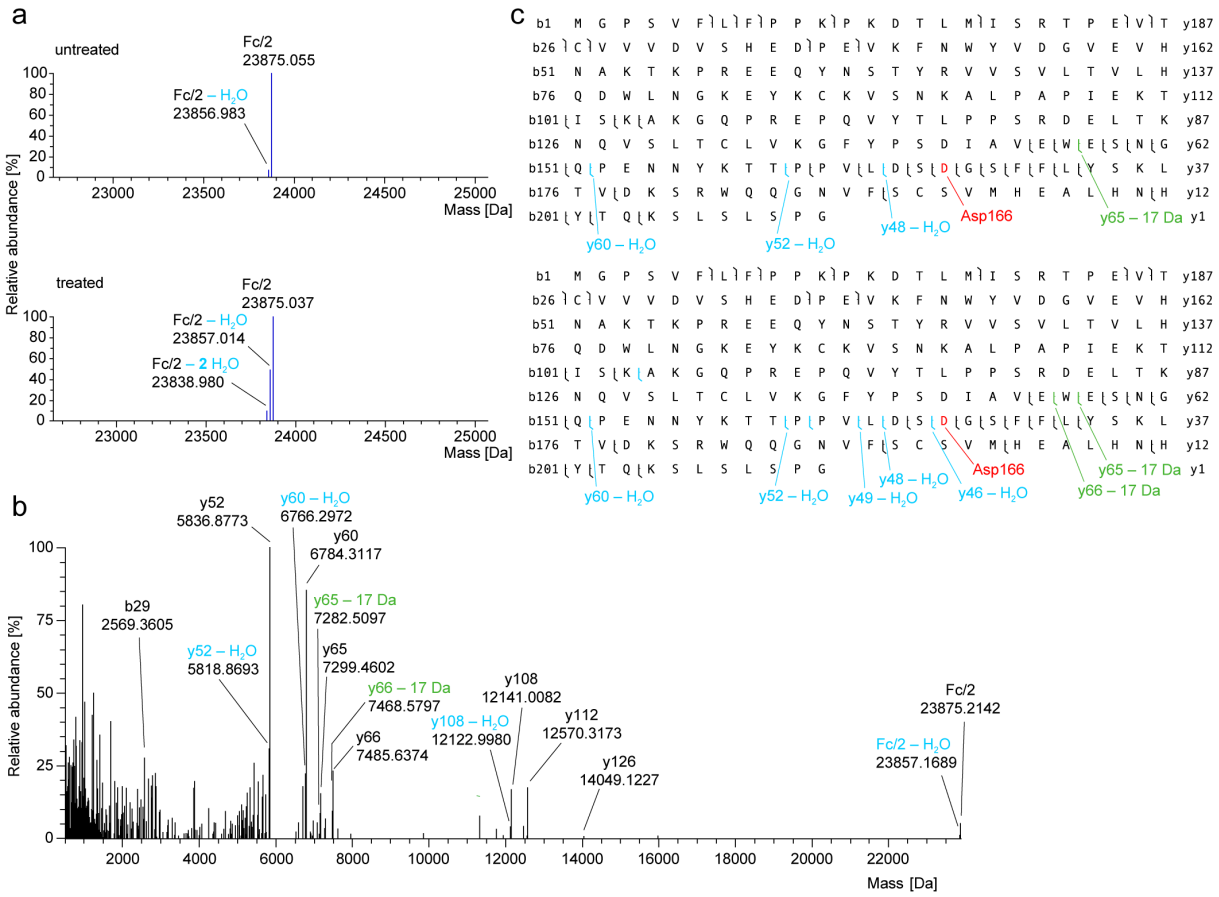

Figure S-13. Detection of succinimide in Fc/2 by top-down mass spectrometry. (a) Deconvoluted mass spectra of reduced Fc/2 as control (top) and of Fc/2 treated at pH 4 (bottom): Intact mass spectrometry revealed species with H_2O loss already in the control sample. (b) Fragments diagnostic for Snn-formation due to dehydration of Asp166 are labeled in light blue. (c) Sequence coverage map of untreated (top) and pH 4 treated Fc/2 (bottom) generated with ProSight Lite. The diagnostic fragments y46, y48, y49, y52 and y60, shown in light blue, indicate Snn formation with water loss (-18 Da) at Asp166 (red). Detected fragments with a -17 Da mass shift (y66 and y65) hint to additional modifications occurring during treatment at pH 4. A possibility is the deamidation of asparagine residues Asn149 within an NG-motif or Asn154/Asn155 within the PENNYK-motif.

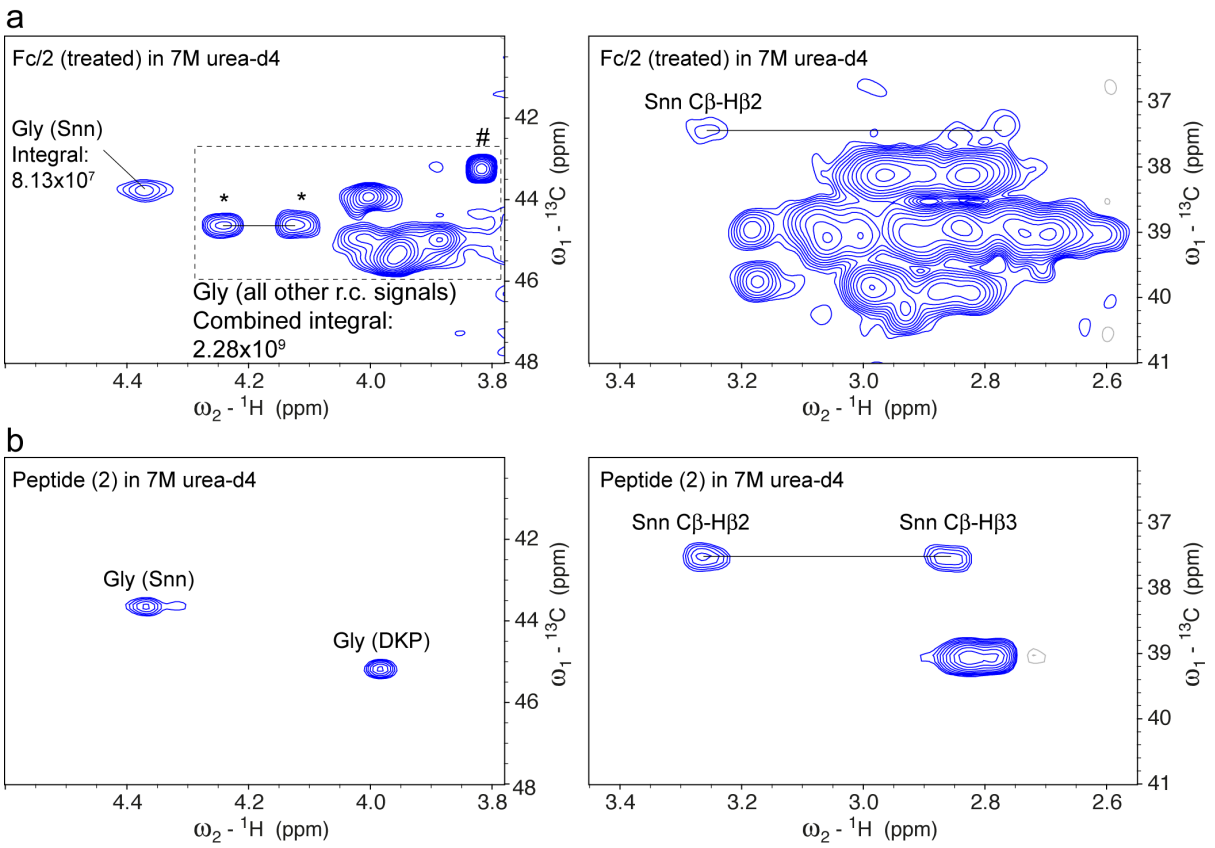

Figure S-14. Application of the NMR approach to Fc/2 recombinantly expressed in *E. coli*. a) Representative regions of a ^{13}C HSQC of Fc/2 that was treated for 138 h at pH 4.0, lyophilized and denatured in fully deuterated 7 M urea-d_4 in D_2O (pH* 2.3) at a concentration of 0.24 mM, measured at 700 MHz and 298 K with ns=312, d1=2 s and td=350. A signal of a glycine within a Snn-Gly motif is observed. The integrals indicate ~3.4 % Snn per Fc/2 molecule, which contains 9 Gly residues. The two signals indicated with an asterisk originate from the same Gly, because they share the same ^{13}C resonance and their integrals correspond to 1/18 of the combined integral of all Gly signals. The signal with an # originates from two protons. These signals with deviating random coil chemical shifts might originate from the two Gly residues at the N- and C-terminus. (b) Comparable regions of a ^{13}C HSQC of peptide **2** measured in 7 M urea-d_4 (pH* 2.3) showing a Gly Cα-Hα signal of the Snn-Gly motif and random coil Cα-Hα cross-peaks of glycines for comparison.

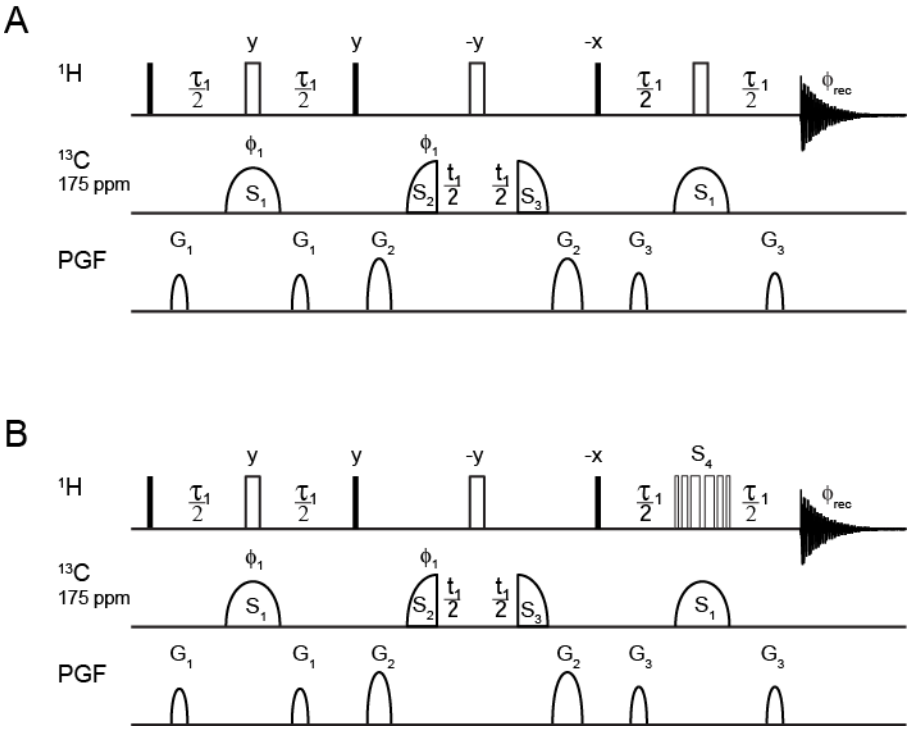

Figure S-15. Pulse sequences for the observation of proton-carbonyl correlations based on $^{n}J_{HC'}$ long-range couplings for measurements in (a) D_2O or (b) H_2O/D_2O. The delay τ_1 was 26, 52 and 126 ms, optimal for $^{n}J_{HC'}$ couplings of 19 Hz, 9.6 Hz and 4 Hz, respectively. Pulse phases are x if not stated otherwise. Shaped pulse S_1 is a 500 ms REBURP pulse,[4] S_2 a 500 ms Q5,[3] S_3 a 500 ms time-reversed Q5. The shaped pulse phase alignment (spoal) was set for S_1, S_2 and S_3 to 0.5 (middle), 1 (end) and 0 (beginning), respectively. S_4 stands for a 3-9-19 Watergate composite pulse. Gradient strengths were 19, 30 and 65 % for G_1, G_2 and G_3, respectively.

Table S-1. Protein crystal structures containing a Snn residue (22 cases).[a]

PDB code	Residue number of Snn	following residue type	protein name
1AT5	Snn101	Gly	Hen Egg White Lysozyme
1JBE	Snn75	Gly	CheY
1WL8	Snn112	Glu	Ph1346 from *Pyrococcus horikoshii*
2IMZ	Snn440	Terminus	Mtu Reca intein splicing domain
2OMK	Snn38	Gly	Thiamin pyrophosphokinase of *Bacteroides thetaiotaomicron*
2YHW	Snn575	Gly	N-Acetylmannosamine kinase
3C03	Snn262	Terminus	Escu C-Terminal Domain
3ESM	Snn149	Gly	uncharacterized protein from *Nocardia farcinica*
3H5R	Snn149	Terminus	*E. Coli* MCCB
3I4W	Snn332	Gly	PSD-95 PDZ domain
3IFJ	Snn440	Terminus	Mtu Reca Intein, splicing domain
3IGD	Snn440	Terminus	Mtu Reca Intein, splicing domain
3K82	Snn332	Gly	PSD-95 PDZ domain
3NZM	Snn159	Terminus	DNAE Intein
4CUO	Snn290	Asp	Banyan Peroxidase
4D3X	Snn152	His	Legumain from Cricetulus Griseus
4D3Y	Snn152	His	Prolegumain from Cricetulus Griseus
4D3Z	Snn152	His	Prolegumain from Cricetulus Griseus
4DX3	Snn319	Cys	Enolase from Agrobacterium tumefaciens
4DXK	Snn319	Cys	Enolase from Agrobacterium tumefaciens
4N6N	Snn147	His	Legumain (oxidized)
4N6O	Snn147	His	Legumain (reduced)

[a] obtained by OCA,[7] URL *http://oca.weizmann.ac.il*

Table S-2. Protein crystal structures containing an isoAsp (Ias) residue (44).[a]

PDB code	Residue number of Snn	following residue type	protein name
1AT6	Ias101	Gly	Hen Egg White Lysozyme
1C9P	Ias115	Ser	C115d from Enterobacter
1DLG	Ias67	Gly	MurA from *E. cloacae*
1DY5	Ias67	Gly	Bovine pancreatic ribonuclease
1EJC	Ias67	Gly	MurA from *E. cloacae*
1EJD	Ias67	Gly	MurA from *E. cloacae*
1EYN	Ias67	Gly	MurA from *E. cloacae*
1H0I	Ias3	Ias	Argadin, cyclid pentapeptide
1H0I	Ias4	Dal	Argadin, cyclid pentapeptide
1JG3	Ias4	His	L-isoaspartyl (D-aspartyl) O-methyltransferase
1LSQ	Ias67	Gly	Ribonuclease A
1Q3G	Ias67	Gly	MurA from *E. cloacae*
1RTU	Ias45	Glu	Ribonuclease U2 from *Ustilago sphaerogena*
1RYW	Ias67	Gly	MurA from *E. cloacae*
1W9V	Ias1436	Ias	Argifin, cyclid pentapeptide
1W9V	Ias1437	Dal	Argifin, cyclid pentapeptide
1WB0	Ias1390	Ias	Argifin, cyclid pentapeptide
1WB0	Ias1391	Dal	Argifin, cyclid pentapeptide
1YBG	Ias67	Gly	MurA from *E. cloacae*
2FI4	Ias115	Ser	BPTI variant
2FI5	Ias115	Ser	BPTI variant
2FTL	Ias115	Ser	BPTI+Trypsin
2FTM	Ias115	Ser	BPTI+Trypsin
2JV0	Ias141	Gly	Riz1 tumor suppressor
2Z2C	Ias67	Gly	MurA from *E. cloacae*
3AHS	Ias32	Gly	Ribonuclease U2B from *Ustilago sphaerogena*
3KQA	Ias67	Gly	MurA from *E. cloacae*
3KQJ	Ias67	Gly	MurA from *E. cloacae*
3KR6	Ias67	Gly	MurA from *E. cloacae*
3LTH	Ias67	Gly	MurA from *E. cloacae*
3SPB	Ias67	Gly	MurA from *E. cloacae*
3SU9	Ias67	Gly	MurA from *E. cloacae*
3SWA	Ias67	Gly	MurA from *E. cloacae*
3SWD	Ias67	Gly	MurA a from *E. cloacae*
3SWI	Ias67	Gly	MurA from *E. cloacae*
3SWQ	Ias67	Gly	MurA from *E. cloacae*
3UPK	Ias67	Gly	MurA from *E. cloacae*
3V4T	Ias67	Gly	MurA from *E. cloacae*
3V5V	Ias67	Gly	MurA from *E. cloacae*
4E7B	Ias67	Gly	MurA from *E. cloacae*
4E7C	Ias67	Gly	MurA from *E. cloacae*
4E7D	Ias67	Gly	MurA from *E. cloacae*
4E7E	Ias67	Gly	MurA from *E. cloacae*
4E7F	Ias67	Gly	MurA from *E. cloacae*
4E7G	Ias67	Gly	MurA from *E. cloacae*
4EII	Ias67	Gly	MurA from *E. cloacae*
4O0H	Ias401	Thr	human L-Asparaginase

[a] obtained by OCA,[7] URL *http://oca.weizmann.ac.il*

Table S-3. Comprehensive NMR assignment of Snn, isoAsp and Asp in the peptides in D_2O, H_2O/D_2O and under denaturing conditions in comparison with values previously reported.

Amino acid (modified or unmodified)	Nucleus type	Nucleus	δ in D_2O or H_2O/ D_2O pH 2.5	δ in 7 $mol \cdot L^{-1}$ urea pH 2.3	δ (HA) literature	δ in DMSO ref. TMS	δ in DMSO literature
Snn	^{1}H	HN	8.68	8.72		8.61	
		Hα	4.76	4.78		4.64	
		Hβ2[e]	3.25	3.26	~3.04[d]	3.05	
		Hβ3[e]	2.85	2.87	~2.50[d]	2.58	
	^{13}C	Cα	51.9	52.0		48.2	
		Cβ	37.4	37.5	37.9[d]	34.8	
		Cγ	179.5	179.9		174.3	
		CO	179.8	179.5		175.2	
	^{15}N	N	116.8	116.3		114.6	
isoAsp	^{1}H	HN	8.31	8.37		8.14	
		Hα	4.79	4.79		4.54	
		Hβ2[e]	2.92	2.92		2.65	
		Hβ3[e]	2.92	2.92		2.60	
	^{13}C	Cα	52.2	52.3		48.7	
		Cβ	39.4	39.5		36.6	
		Cγ	175.3	175.3		169.4	
		CO	176.5	177.0		172.5	
	^{15}N	N	121.5	120.9		117.2	
Asp	^{1}H	HN	8.43	8.50	8.55[a]	8.35	8.21[b]
		Hα	4.75	4.78	4.78[a]	4.62	4.63[b]
		Hβ2[e]	2.98	2.96	2.93[a]	2.72	2.70[b]
		Hβ3[e]	2.98	2.96	2.93[a]	2.56	2.44[b]
	^{13}C	Cα	52.9	53.0	52.9[a]	49.4	
		Cβ	37.8	38.1	38.0[a]	35.8	
		Cγ	176.9	177.1	177.1[a]	171.7	
		CO	175.5	175.5	175.8[a]	170.6	
	^{15}N	N	120.5	120.8	118.7[a]	119.0	
Gly after Snn	^{1}H	Hα2[e]	4.34	4.37		4.10	
		Hα3[e]	4.29	4,32		3.97	
	^{13}C	Cα	43.6	43.6		40.2	
		CO	170.8	170.7		165.3	
	^{15}N	N	n.d.	n.d.		n.d.	
Gly after isoAsp	^{1}H	HN	8.34	8.32		8.15	
		Hα2	3.95	3.94		3.73	
		Hα3[e]	3.89	3.94		3.73	
	^{13}C	Cα	45.1	45.2		41.6	
		CO	173.9	174.0		168.6	
	^{15}N	N	113.8	113.6		109.8	
Gly after Asp	^{1}H	HN	8.33	8.32	8.33[c]	7.95	8.15[b]
		Hα2	3.97	3.95	3.96[c]	3.70	3.76[b]
		Hα2	3.90	3.95	3.96[c]	3.70	3.76[b]
	^{13}C	Cα	45.3	45.3	45.1[c]	41.8	
		CO	173.7	173.7	174.9[c]	168.2	
	^{15}N	N	108.8	108.8	108.8[c]	104.5	

[a] values of the acidic form from Platzer et al.[8]
[b] values from Bundi et al.[9]
[c] random coil shifts from Wishart et al.[10]
[d] values from Flora et al.,[11] the ^{13}C shift was explicitly given in a figure, the ^{1}H shifts were read out of spectra in the Supplementary Figures
[e] resonances were not stereochemically assigned

Table S-4. Comprehensive NMR assignment of two side products – the diketopiperazine and the piperidide of Asp in the decapeptide in aqueous solution and under denaturing conditions.

Nucleus type	Nucleus	δ in D_2O or H_2O/D_2O pH 2.5 (ref. to DSS)	δ in 7 mol·L^{-1} urea pH 2.3 (ref. to DSS)	δ in DMSO referenced to TMS
Asp^{Pip}				
^1H	HN	8.40		
	Hα	5.18		
	Hβ2[e]	2.81		
	Hβ3[e]	2.66		
^{13}C	Cα	49.6		
	Cβ	39.9		
	Cγ	171.6		
	CO	175.3		
^{15}N	N	ov.		
Asp as part of cyclic diketopiperazine (Ser-Asp) with peptide extension at Cγ				
^1H	HN	8.32	8.36	7.88
	Hα	4.50	4.50	4.18
	Hβ2	2.96	2.97	2.74
	Hβ3	2.87	2.88	2.67
^{13}C	Cα	54.5	54.6	51.7
	Cβ	43.1	43.1	40.2
	Cγ	174.9	174.9	169.9
	CO	172.0	172.0	167.2
^{15}N	N	123.5	123.4	119.1
Ser as part of cyclic diketopiperazine (Ser-Asp)				
^1H	HN	8.32	8.35	8.03
	Hα	4.19	4.18	3.80
	Hβ2	3.98	3.99	3.73
	Hβ3	3.78	3.79	3.54
	Hγ1			5.11
^{13}C	Cα	59.7	59.8	56.9
	Cβ	64.8	65.0	62.1
	CO	171.0	171.0	166.1
^{15}N	N	118.3	118.2	114.5
Gly after Asp^{Pip}				
^1H	HN			
	Hα2	3.97		
	Hα3	3.91		
^{13}C	Cα	45.1		
	CO	174.1		
^{15}N	N			
Gly attached at Cγ of Asp as part of cyclic diketopiperazine (Ser-Asp)				
^1H	HN	8.32	8.40	8.27
	Hα2	3.97	3.98	3.73
	Hα3	3.91	3.98	3.73
^{13}C	Cα	45.1	45.2	41.9
	CO	174.1	174.1	168.7
^{15}N	N	114.6	114.5	110.9

Table S-5. Chemical shift assignments of peptide **1** measured in plain D_2O or 93% H_2O/7% D_2O at pH 2.5 and 298 K, referenced to DSS.

Residue	H	N	C	HA	CA	HB	CB	HG	CG	Others
Acetyl				1.93	24.3					
1 Glu	8.21	126.0		4.21	56.1	1.89	28.6	2.34 2.27	32.5	
2 Trp	8.10	121.1		4.68	57.1	3.28	29.3			HD1: 7.25; CD1: 127.2; HE1: 10.15; NE2: 129.6; HE3: 7.60; CE3: 120.9; HH2: 7.24; CH2: 124.7; HZ2: 7.48; CZ2: 114.7; HZ3: 7.16; CZ3: 122.1
3 Glu	7.98	121.6		4.29	55.6	1.99 1.82	28.8	2.20	32.3	
4 Ser	8.23	116.4		4.32	58.4	3.86 3.82	63.5			
5 Asn	8.41	120.6		4.71	53.3	2.85	38.6			HD21: 7.56; HD22: 6.87; ND2: 112.3
6 Gly	8.33	108.6		3.97 3.90	45.2					
7 Gln	8.09	120.5		4.63	53.5	2.10 1.95	28.7	2.35	33.4	CD: 180.6; HE21: 7.51; HE22: 6.87; NE2: 112.4
8 Pro				4.41	63.2	2.27 1.92	32.0	2.03	27.4	HD: 3.79, 3.67; CD: 50.6
9 Glu	8.48	120.8		4.30	55.7	2.07 1.92	28.6	2.45	32.5	
10 Asn	8.41	120.0		4.66	52.9	2.82 2.77	38.9			HD21: 7.59; HD22: 6.90; ND2: 112.8
Amide	7.46 7.13	107.3								

Table S-6. Chemical shift assignments of peptide **2** (including also **2^{DKP}**) measured in plain D_2O or 93% H_2O/7% D_2O at pH 2.5 and 298 K, referenced to DSS.

Residue	H	N	C	HA	CA	HB	CB	HG	CG	Others
Acetyl			177.2	1.93						
1 Glu	8.21	126.0	176.1	4.21	56.1	1.91 1.88	28.5	2.33 2.25	32.4	CD: 179.6
2 Trp	8.10	121.0	176.2	4.68	57.1	3.28	29.2			HD1: 7.26; CD1: 127.2; HE1: 10.13; NE2: 129.3; HE3: 7.62; CE3: 120.9; HH2: 7.24; CH2: 124.7; HZ2: 7.48; CZ2: 114.7; HZ3: 7.16; CZ3: 122.2
3 Glu	7.98	121.9	175.6	4.27	55.7	1.99 1.81	28.7	2.20	32.3	CD: 179.6
4 Ser	8.09	115.6	174.6	4.35	58.0	3.82	63.5			
5 Snn	8.68	116.8	179.8	4.76	51.9	3.25 2.85	37.4		179.5	
6 Gly			170.8	4.34 4.29	43.6					
7 Gln	8.56	122.0	174.0	4.59	53.8	2.09 1.94	28.7	2.36	33.3	CD: 180.6; HE21: 7.49; HE22: 6.86; NE2: 112.5
8 Pro			177.1	4.40	63.3	2.29 1.92	32.0	2.00 2.02	27.4	HD: 3.75, 3.64; CD: 50.6
9 Glu	8.50	120.7	175.8	4.33	55.8	2.11 1.97	28.7	2.49	32.5	CD: 179.8
10 Asn	8.41	120.0	177.6	4.68	52.9	2.83 2.78	38.9		177.3	HD21: 7.60; HD22: 6.90; ND2: 112.8
Amide	7.47 7.13	107.3								
1 Ser^{DKP}	8.32	118.3	171.0	4.19	59.7	3.98 3.78	64.8			
2 Asp^{DKP}	8.32	123.5	172.0	4.50	54.5	2.96 2.87	43.1		174.9	
3 Gly^{DKP}	8.43	114.6	174.1	3.97	45.1					
4 Gln^{DKP}	8.37	121.0	174.2	4.65	53.7	2.11 1.96	28.7	2.39	33.4	CD: 180.7; HE21: 7.54; HE22: 6.86; NE2: 112.6
5 Pro^{DKP}			177.2	4.43	63.3	2.31 1.94	32.1	2.03	27.4	HD: 3.82, 3.68; CD: 50.6
6 Glu^{DKP}	8.55	120.8	175.8	4.34	55.9	2.12 2.00	28.6	2.51	32.5	CD: 179.8
7 Asn^{DKP}	8.41	119.6	177.6	4.68	52.9	2.83 2.78	38.9		177.3	HD21: 7.60; HD22: 6.90; ND2: 112.8

Table S-7. Chemical shift assignments of peptide **2** (including also $\mathbf{2^{DKP}}$) measured in 7 $mol \cdot L^{-1}$ urea-d_4 dissolved in D_2O at pH* 2.3 (uncorrected) or 7 $mol \cdot L^{-1}$ urea in 93% H_2O/7% D_2O at pH 2.3 and 298 K, referenced to DSS.

Residue	H	N	C	HA	CA	HB	CB	HG	CG	Others
Acetyl			177.1	1.94						
1 Glu	8.21	125.9		4.22	56.3	1.88	28.5	2.35 2.30	32.7	
2 Trp	8.15	121.1	176.2	4.67	57.3	3.29	29.4			
3 Glu	8.06	122.0	175.7	4.31	55.7	1.98 1.83	28.9	2.24	32.5	
4 Ser	8.18	116.0	174.6	4.37	58.1	3.84	63.7			
5 Snn	8.72	116.3	179.9	4.78	52.0	3.26 2.87	37.5		179.5	
6 Gly			170.7	4.37	43.6					
7 Gln	8.66	122.0		4.61	54.0	2.09 1.94	28.7	2.39	33.5	HE21: 7.48; HE22: 6.90; NE2: 111.9
8 Pro				4.44	63.3	2.29 1.93	32.2	2.03	27.6	HD: 3.80, 3.67; CD: 50.7
9 Glu	8.55	120.9	175.8	4.34	56.0	2.11 1.97	28.7	2.52	32.7	
10 Asn	8.46	120.1	177.6	4.70	53.0	2.83 2.79	39.0		177.2	HD21: 7.56; HD22: 6.97; ND2: 112.6
Amide	7.48 7.20	107.1								
1 Ser^{DKP}	8.35	118.2	171.0	4.18	59.8	3.99 3.79	65.0			
2 Asp^{DKP}	8.36	123.4	172.0	4.50	54.6	2.97 2.88	43.1		174.9	
3 Gly^{DKP}	8.40	114.5	174.1	3.98	45.2					
4 Gln^{DKP}	8.41	120.9		4.64	53.9			2.41	33.5	

Table S-8. Chemical shift assignments of peptide **2** (including also $\mathbf{2^{DKP}}$) measured DMSO at 298 K, referenced to TMS.

Residue	H	N	C	HA	CA	HB	CB	HG	CG	Others
Acetyl			169.4	1.81	22.1					
1 Glu	8.01	122.7	171.2	4.21	51.8	1.83 1.67	27.0	2.19	30.0	CD: 173.9
2 Trp	7.96	118.5	171.5	4.51	53.2	3.14 2.97	27.2			HD1: 7.14; HE3: 7.59
3 Glu	8.13	117.5	170.9	4.32	51.9	1.92 1.77	27.1	2.25	30.0	CD: 174.0
4 Ser	7.90	113.0		4.29	54.7	3.58	61.4			
5 Snn	8.61	114.6	175.2	4.64	48.2	3.05 2.58	34.8		174.3	
6 Gly			165.3	4.10 3.97	40.2					
7 Gln	8.46	118.8		4.51	50.9	1.90 1.67	26.9	2.13	30.6	CD: 173.8; HE21: 7.27; HE22: 6.81; NE2: 109.1
8 Pro			172.3	4.33	59.3	2.05 1.90	29.0	1.91 1.85	24.4	HD: 3.63, 3.60; CD: 46.8
9 Glu	8.26	117.0	170.6	4.12	52.5	1.91 1.76	26.6	2.30	30.0	CD: 173.9
10 Asn	7.94	116.3	172.7	4.41	49.4	2.50	36.5		171.7	HD21: 7.37; HD22: 6.92; ND2: 109.9
Amide	7.08 6.99	103.6								
1 Ser^{DKP}	8.03	114.5	166.1	3.80	56.9	3.73 3.54	62.1			
2 Asp^{DKP}	7.88	119.1	167.2	4.17	51.7	2.74 2.57	40.2		169.9	
3 Gly^{DKP}	8.27	110.9	168.7	3.73	41.9					
4 Gln^{DKP}	8.23	118.4		4.51		1.91 1.70				

Table S-9. Chemical shift assignments of peptide **3** measured in plain D_2O or 93% H_2O/7% D_2O at pH 2.5 and 298 K, referenced to DSS.

Residue	H	N	C	HA	CA	HB	CB	HG	CG	Others
Acetyl			177.2	1.92						
1 Glu	8.21	126.0	176.0	4.22	56.1	1.91 1.88	28.6	2.35 2.30	32.5	CD: 179.6
2 Trp	8.09	121.1	176.0	4.68	57.1	3.28	29.2			HD1: 7.24; HE3: 7.61
3 Glu	7.94	121.8	175.4	4.32	55.4	2.01 1.81	29.0	2.23	32.3	CD: 179.6
4 Ser	8.16	116.4	173.9	4.35	58.2	3.84	63.7			
5 isoAsp	8.31	121.5	176.5	4.79	52.2	2.92	39.4		175.3	
6 Gly	8.34	113.8	173.9	3.95 3.89	45.1					
7 Gln	8.23	120.5	174.1	4.61	53.6	2.09 1.91	28.8	2.35	33.3	CD: 180.6; HE21: 7.50; HE22: 6.85; NE2: 112.5
8 Pro			177.1	4.39	63.2	2.27 1.91	32.0	2.01	27.4	HD: 3.75, 3.63; CD: 50.6
9 Glu	8.49	120.8	175.7	4.32	55.8	2.10 1.95	28.6	2.48	32.5	CD: 179.7
10 Asn	8.41	120.0	177.5	4.67	52.9	2.81 2.76	38.9		177.3	HD21: 7.59; HD22: 6.90; ND2: 112.8
Amide	7.47 7.13	107.3								

Table S-10. Chemical shift assignments of peptide **3** measured in 7 $mol \cdot L^{-1}$ urea-d_4 dissolved in D_2O at pH* 2.3 (uncorrected) or 7 $mol \cdot L^{-1}$ urea in 93% H_2O/7% D_2O at pH 2.3 and 298 K, referenced to DSS.

Residue	H	N	C	HA	CA	HB	CB	HG	CG	Others
Acetyl			177.1	1.93						
1 Glu	8.20	125.9	176.1	4.22	56.2	1.93 1.90	28.8	2.35 2.29	32.7	CD: 179.7
2 Trp	8.13	121.1	176.1	4.67	57.2	3.28	29.5			
3 Glu	8.05	121.9	175.5	4.33	55.5	2.01 1.83	29.2	2.26	32.6	CD: 179.8
4 Ser	8.24	116.7	174.0	4.36	58.3	3.85	63.9			
5 isoAsp	8.37	120.9	177.0	4.79	52.3	2.92	39.5		175.3	
6 Gly	8.32	113.6	174.0	3.95 3.93	45.2					
7 Gln	8.29	120.5	174.2	4.61	53.8	2.10 1.92	28.9	2.36	33.5	CD: 180.4; HE21: 7.46; HE22: 6.89; NE2: 112.0
8 Pro			177.0	4.41	63.3	2.27 1.91	32.2	2.01	27.6	HD: 3.75, 3.63; CD: 50.7
9 Glu	8.52	120.9	175.8	4.33	56.0	2.11 1.97	28.8	2.50	32.7	CD: 179.8
10 Asn	8.46	120.1	177.5	4.69	53.0	2.82 2.77	39.1		177.2	HD21: 7.56; HD22: 6.97; ND2: 112.6
Amide	7.48 7.19	107.1								

Table S-11. Chemical shift assignments of peptide **3** measured in DMSO at 298 K, referenced to TMS.

Residue	H	N	C	HA	CA	HB	CB	HG	CG	Others
Acetyl			169.4	1.80	22.3					
1 Glu	7.98	122.6	171.1	4.21	51.8	1.83 1.66	27.1	2.19	30.0	CD: 173.9
2 Trp	7.95	118.6	171.3	4.52	53.1	3.14 2.96	27.2			HD1: 7.14
3 Glu	8.11	117.4	170.9	4.34	51.8	1.94 1.77	27.3	2.26	30.0	CD: 173.9
4 Ser	7.89	113.5	169.5	4.32	54.7	3.59	61.7			
5 isoAsp	8.14	117.2	172.5	4.54	48.7	2.65 2.60	36.6		169.4	
6 Gly	8.14	109.8	168.6	3.73	41.6					
7 Gln	8.16	118.5	170.0	4.52	49.7	1.90 1.67	27.0	2.13	30.6	CD: 173.8; HE21: 7.25; HE22: 6.80; NE2: 109.1
8 Pro			172.3	4.33	59.3	2.05 1.90	29.0	1.92 1.85	24.4	HD: 3.64; CD: 46.8
9 Glu	8.23	116.9	170.6	4.12	52.5	1.91 1.76	26.6	2.29	30.0	
10 Asn	7.94	116.4	172.6	4.41	49.4	2.49	36.4		171.7	HD21: 7.35; HD22: 6.92; ND2: 109.8
Amide	7.08 6.98	103.6								

Table S-12. Chemical shift assignments of peptide **4** measured in plain D_2O or 93% H_2O/7% D_2O at pH 2.5 and 298 K, referenced to DSS.

Residue	H	N	C	HA	CA	HB	CB	HG	CG	Others
Acetyl			177.2	1.93	24.3					
1 Glu	8.21	126.0	176.1	4.21	56.1	1.89	28.5	2.34 2.28	32.5	
2 Trp	8.09	121.0	176.1	4.68	57.2	3.28	29.3			
3 Glu	7.97	121.6	175.9	4.28	55.6	1.99 1.82	28.8	2.20	32.3	
4 Ser	8.22	116.3	174.6	4.33	58.5	3.87 3.82	63.5			
5 Asp	8.43	120.5	175.5	4.75	52.9	2.98	37.8		176.9	
6 Gly	8.33	108.8	173.7	3.97 3.90	45.3					
7 Gln	8.02	120.4	174.0	4.64	53.5	2.11 1.96	28.8	2.36	33.4	HE21: 7.51; HE22: 6.87; NE2: 112.60
8 Pro			177.1	4.40	63.2	2.27 1.91	32.0	2.03	27.4	HD: 3.78, 3.66; CD: 50.6
9 Glu	8.48	120.8	175.7	4.29	55.8	2.07 1.92	28.6	2.46	32.5	
10 Asn	8.40	120.0	177.4	4.67	52.9	2.82 2.77	38.9		177.3	HD21: 7.59; HD22: 6.90; ND2: 112.80
Amide	7.46 7.13	107.3								

Table S-13. Chemical shift assignments of peptide **4** measured in 7 mol·L^{-1} urea-d_4 dissolved in D_2O at pH* 2.3 (uncorrected) or 7 mol·L^{-1} urea in 93% H_2O/7% D_2O at pH 2.3 and 298 K, referenced to DSS.

Residue	H	N	C	HA	CA	HB	CB	HG	CG	Others
Acetyl			177.1	1.93						
1 Glu	8.20	125.9	176.2	4.22	56.3	1.93 1.90	28.8	2.34 2.30	32.7	
2 Trp	8.13	121.1	176.2	4.67	57.3	3.28	29.5			
3 Glu	8.05	121.8	175.9	4.30	55.7	1.99 1.83	29.0	2.23	32.5	
4 Ser	8.26	116.5	174.6	4.35	58.4	3.90 3.82	63.8			
5 Asp	8.50	120.8	175.5	4.78	53.0	2.96	38.1		177.1	
6 Gly	8.32	108.8	173.7	3.95	45.3					
7 Gln	8.14	120.4	174.1	4.62	53.7	2.11 1.95	28.9	2.36	33.5	CD: 180.6; HE21: 7.47; HE22: 6.90; NE2: 112.0
8 Pro			177.0	4.42	63.3	2.28 1.92	32.2	2.02	27.6	HD: 3.77, 3.65; CD: 50.7
9 Glu	8.52	120.9	175.8	4.32	56.0	2.10 1.95	28.9	2.49	32.7	
10 Asn	8.46	120.1	177.5	4.69	53.0	2.82 2.78	39.0		177.2	HD21: 7.56; HD22: 6.97; ND2: 112.6
Amide	7.48 7.20	107.1								

Table S-14. Chemical shift assignments of peptide **4** measured in DMSO at 298 K, referenced to TMS.

Residue	H	N	C	HA	CA	HB	CB	HG	CG	Others
Acetyl			169.4	1.80	22.3					
1 Glu	7.98	122.6	171.2	4.21	51.8	1.83 1.66	27.1	2.19	30.0	CD: 173.9
2 Trp	7.94	118.5	171.4	4.52	53.1	3.14 2.96	27.2			
3 Glu	8.13	117.4	171.0	4.34	51.8	1.92 1.76	27.2	2.26	30.0	CD: 174.0
4 Ser	7.91	113.1	170.0	4.34	54.5	3.66 3.55	61.8			
5 Asp	8.35	119.0	170.6	4.62	49.4	2.72 2.56	35.8		171.7	
6 Gly	7.95	104.5	168.2	3.70	41.8					
7 Gln	8.07	118.1	168.3	4.50	49.7	1.90 1.67	27.0	2.13	30.6	CD: 173.8; HE21: 7.26; HE22: 6.82; NE2: 109.2
8 Pro			172.3	4.33	59.3	2.05 1.90	29.0	1.91 1.85	24.4	HD: 3.63; CD: 46.8
9 Glu	8.23	117.0	170.6	4.12	52.5	1.90 1.76	26.6	2.29	30.0	CD: 174.0
10 Asn	7.94	116.3	172.6	4.41	49.4	2.49	36.4		171.7	HD21: 7.35; HD22: 6.92; ND2: 109.8
Amide	7.08 6.98	103.6								

Table S-15. Chemical shift assignments of peptide **5** measured in plain D_2O or 93% H_2O/7% D_2O at pH 2.5 and 298 K, referenced to DSS.

Residue	H	N	C	HA	CA	HB	CB	HG	CG	Others
Acetyl										
1 Glu	8.21	126.0		4.21	56.1	1.89				
2 Trp	8.08	121.0		4.68	57.1	3.28	29.2			
3 Glu	7.98	121.8		4.28	55.6	1.99 1.82	28.8			
4 Ser	8.23	116.3		4.32	58.5	3.86 3.83	63.5			
5 Asp	8.52	121.5		4.81	52.9	2.97	38.0			
6 Gly	8.39	109.1		3.97 3.89	45.3					
7 Gln	8.07	120.5		4.52	53.6	1.90 2.06	28.8	2.31	33.3	HE21: 7.47; HE22: 6.84; NE2: 112.40
8 Pro				4.37	63.2	2.26 1.90	32.0	2.00	27.4	HD: 3.69, 3.58; CD: 50.6
8' Pro				4.71	62.7	2.18 1.85		1.97		HD: 3.61, 3.47; CD: 50.1
9 Glu	8.47	120.8		4.30	55.8					
10 Asn	8.41	120.0		4.67	52.9	2.82 2.77	38.9			HD21: 7.59; HD22: 6.90; ND2: 112.80
Amide	7.47 7.13	107.3								

Table S-16. Chemical shift assignments of peptide **6** (including also $\mathbf{6^{DKP}}$) measured in plain D_2O or 93% H_2O/7% D_2O at pH 2.5 and 298 K, referenced to DSS.

Residue	H	N	C	HA	CA	HB	CB	HG	CG	Others
Acetyl			177.3	1.94						
1 Glu	8.23	126.1	176.2	4.22	56.1	1.91	28.6	2.34 2.28	32.5	CD: 179.7
2 Trp	8.13	121.3	176.2	4.64	57.3	3.27	29.2			HD1: 7.26; HE1: 10.13; NE2: 129.6; HE3: 7.60
3 Glu	7.87	122.2	175.1	4.20	55.4	1.94 1.77	28.8	2.19	32.2	CD: 179.7
4 Ala	8.04	123.9	177.7	4.17	52.2	1.34	19.0			
5 Snn	8.58	113.9	179.8	4.73	51.7	3.24 2.81	37.4		179.5	
6 Gly			170.9	4.34 4.30	43.5					
7 Gln	8.58	122.1	174.1	4.61	53.8	2.10 1.95	28.4	2.38	33.3	CD: 179.6; HE21: 7.54; HE22: 6.88; NE2: 112.6
8 Pro			177.2	4.41	63.3	2.29 1.93	32.0	2.02	27.4	HD: 3.76, 3.65; CD: 50.6
9 Glu	8.51	120.7	175.8	4.33	55.9	2.10 1.97	28.6	2.49	32.5	CD: 179.9
10 Asn	8.42	120.0	177.6	4.68	52.9	2.83 2.79	38.9		177.4	HD21: 7.60; HD22: 6.90; ND2: 112.8
Amide	7.48 7.13	107.3								
1 Ala^{DKP}	8.23	125.6	173.9	4.21	54.1	1.46	21.7			
2 Asp^{DKP}	8.22	120.1	171.6	4.44	54.2	2.97 2.88	40.7		174.6	
3 Gly^{DKP}	8.38	114.0	174.1	3.94	45.0					
4 Gln^{DKP}	8.31	120.7	174.3	4.64	53.7	2.12 1.96	28.8	2.39	33.4	CD: 180.8; HE21: 7.49; HE22: 6.86; NE2: 112.5
5 Pro^{DKP}			177.2	4.43	63.3	2.32 1.94	32.1	2.05	27.4	HD: 3.81, 3.68; CD: 50.6
6 Glu^{DKP}	8.55	120.8	175.8	4.34	55.9	2.12 1.99	28.5	2.51	32.6	CD: 179.9
7 Asn^{DKP}	8.42	120.0	177.6	4.68	52.9	2.83 2.79	38.9		177.4	HD21: 7.60; HD22: 6.90; ND2: 112.8

Table S-17. Chemical shift assignments of peptide **2**$^{\mathbf{Pip}}$ (including also **2**$^{\mathbf{DKP}}$) measured in plain D_2O or 93% H_2O/7% D_2O at pH 2.5 and 298 K, referenced to DSS.

Residue	**H**	**N**	**C**	**HA**	**CA**	**HB**	**CB**	**HG**	**CG**	**Others**
Acetyl			177.2	1.92						
1 Glu	8.24	126.2	176.1	4.20	56.2	1.91 1.88	28.7	2.32 2.25	32.8	CD: 180.0
2 Trp	8.08	120.9	176.2	4.67	57.1	3.30	29.2			HD1: 7.25; HE1: 10.14; NE2: 129.6
3 Glu	7.96	121.7	175.5	4.27	55.6	1.98 1.81	28.9	2.20	32.6	CD: 179.9
4 Ser	8.13	116.1	173.3	4.33	58.2	3.81	63.7			
5 Asp^{Pip}	8.41	122.6	175.3	5.19	49.6	2.82 2.66	39.9		171.6	
6 Gly	8.32	113.9	174.0	3.90	45.1					
7 Gln	8.25	120.7	174.2	4.60	53.7	2.10 1.94	28.7	2.36	33.4	CD: 180.6; HE21: 7.53; HE22: 6.86; NE2: 112.5
8 Pro			177.1	4.39	63.3	2.28 1.91	32.0	2.01 2.03	27.4	HD: 3.76, 3.64; CD: 50.6
9 Glu	8.55	120.9	175.9	4.31	55.9	2.10 1.96	28.8	2.46	32.8	CD: 180.1
10 Asn	8.41	120.0	177.5	4.67	52.9	2.83 2.77	38.9		177.3	HD21: 7.60; HD22: 6.90; ND2: 112.8
Amide	7.47 7.13	107.3								
1 Ser^{DKP}	8.32	118.3	171.0	4.19	59.7	3.98 3.78	64.8			
2 Asp^{DKP}	8.32	123.5	172.0	4.49	54.5	2.96 2.87	43.1		174.9	
3 Gly^{DKP}	8.43	114.6	174.1	3.97	45.1					
4 Gln^{DKP}	8.37	121.0	174.2	4.66	53.6	2.11 1.96	28.7	2.40	33.4	CD: 180.6; HE21: 7.53; HE22: 6.86; NE2: 112.5
5 Pro^{DKP}			177.2	4.43	63.3	2.32 1.94	32.0	2.05	27.4	HD: 3.81, 3.68; CD: 50.6
6 Glu^{DKP}	8.61	121.0	175.8	4.32	55.9	2.12 2.00	28.7	2.48	32.9	CD: 180.2

	H2	C2	H3	C3	H4	C4	H5	C5	H6	C6			
Pip	3.58 3.40	46.8	1.52	28.0	1.64	26.4	1.58	28.6	3.53 3.44	49.8			

Table S-18. Content of the several peptide samples derived from the integrals of NMR signals and HPLC.

Peptide	Snn	diketopiperazine	Asp	IsoAsp	Asp^{Pip}	Asn
1 (Asn)[a]	n.d.	n.d.	n.d.	n.d.	n.d.	100%
2 (Snn)[a]	56%	36%	n.d.	n.d.	8%	–
$\mathbf{2^{Pip}}$ (Asp^{Pip})[a,b]	11%	30%	n.d.	n.d.	59%	–
2Δ[c,d]	65%	34%	–	–	2%	–
3 (isoAsp)[a]	11%	n.d.	n.d.	89%	n.d.	–
4 (Asp)[a]	n.d.	n.d.	100%	n.d.	n.d.	–
5 (D-Asp)[a]	n.d.	n.d.	100%	n.d.	n.d.	–
6 (Ala-Snn)[a]	43%	49%	n.d.	n.d.	8%	–
6Δ[c,d]	43%	54%	–	–	3%	–

[a] determined from integrals in a ^{1}H-^{13}C HSQC spectrum; n.d. stands for not detectable
[b] peptide **2** treated with piperidine leads to conversion of Snn into piperidide
[c] truncated peptide **2** or **6** lacking Glu1-Trp2-Glu3 obtained by peptide synthesis
[d] determined by HPLC separation

Table S-19. Mass spectrometry of untreated and pH 4-treated filgrastim displaying the deconvoluted masses of intact, oxidized and Snn-containing protein, an estimation of abundance and mass deviation..

Filgrastim	Measured mass [Da]	Mass deviation [Da]	Intensity [counts]	Abundance [%]	Identification	Mass deviation [ppm]
Untreated	18790.69	–0.02	6.79E+06	75.90	Filgrastim	-1.06
Untreated	18806.66	+15.95	1.96E+06	21.94	Filgrastim +O	-2.13
Untreated	18822.64	+31.93	1.93E+05	2.16	Filgrastim +2O	-3.19
Treated	18790.74	+0.03	1.13E+07	68.72	Filgrastim	-1.60
Treated	18806.70	+15.99	3.62E+06	22.04	Filgrastim +O	0
Treated	18772.72	–17.99	1.15E+06	7.01	Filgrastim $-H_2O$	+1.07
Treated	18822.68	+31.97	3.67E+05	2.23	Filgrastim +2O	-1.06

Table S-20. Mass spectrometry of untreated and pH 4-treated Fc/2 displaying the deconvoluted masses of intact and Snn-containing protein, fragments and an estimation of abundance and mass deviation.

Fc/2	Measured mass [Da]	Mass deviation [Da]	Intensity [counts]	Abundance [%]	Identification	Mass deviation [ppm]
Untreated	23875.06	0.06	4.90E+04	93.25	Fc/2	+2.51
Untreated	23856.98	–18.02	3.55E+03	6.75	Fc/2 – H_2O	–0.42
Treated	23875.04	+0.04	2.13E+04	62.64	Fc/2	+1.68
Treated	23857.02	–17.98	1.05E+04	30.95	Fc/2 – H_2O	+1.26
Treated	23839.00	–36.00	2.18E+03	6.41	Fc/2 – 2 H_2O	+0.84

Table S-21. HPLC and MS characterization of the synthetic crude peptides

Peptide no.	$M_{calc.}$ (Da), monoisotopic	M_{found} (Da), negative mode	M_{found} (Da), positive mode	t_R (min)
1	1229.49	1228.7 $[M-H]^-$	1252.8 $[M+Na]^+$ 1268.7 $[M+K]^+$	13.5[a]
2	1212.47	1211.7 $[M-H]^-$	1235.8 $[M+Na]^+$ 1251.7 $[M+K]^+$	13.6[a]
2^{Pip}	1297.56	1296.8 $[M-H]^-$	1320.8 $[M+Na]^+$ 1336.8 $[M+K]^+$	15.7[a]
2^{DKP}	726.29	725.8 $[M-H]^-$	-	4.2[b]
2Δ	726.29	725.8 $[M-H]^-$	-	4.9[b]
Ac-2Δ	768.30	767.7 $[M-H]^-$	-	7.0[b]
$2Δ^{Pip}$	811.38	810.9 $[M-H]^-$	-	13.0[b]
3	1230.48	1229.6 $[M-H]^-$	1270.3 $[M+K]^+$	12.7[a]
4	1230.48	1229.6 $[M-H]^-$	1270.3 $[M+K]^+$	13.3[a]
5	1230.48	1229.6 $[M-H]^-$	1254.4 $[M+Na]^+$ 1270.4 $[M+K]^+$	13.1[a]
6	1196.47	1195.9 $[M-H]^-$	1219.7 $[M+Na]^+$ 1235.7 $[M+K]^+$	14.5, 14.7[a]
6^{Pip}	1281.56	1281.0 $[M-H]^-$	1304.8 $[M+Na]^+$ 1320.8 $[M+K]^+$	16.5, 17.0[a]

[a] HPLC gradient: 10% B for 5 min., followed by 10-70% B in 40 min, [b] HPLC gradient: 5% B for 5 min, followed by a gradient of 5-60% B in 45 min

Supplementary References

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