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Selective Modification of Sulfamidate-Containing Peptides

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Hybrid peptides whose *N*-terminal residues are activated in the form of α -methylisoserine-derived cyclic sulfamidates exhibit a rich reactivity as electrophiles, allowing site- and stereoselective modifications at different backbone and sidechain positions. The unique properties of this scaffold allow the stereocontrolled late-stage functionalization of the peptide backbone by nucleophilic ring opening with fluorescent probes, thiocarbohydrates and tags for strain-promoted azide-alkyne cycloaddition, as well as installing labile *N*-terminal affinity tags (biotin) and cytotoxic drugs (chlorambucil) for pH-controlled release. Finally, an unexpected base-promoted acyl group migration from the sulfamidate *N*-terminus allows fast and quantitative intramolecular modification of nucleophilic sidechains on the fully unprotected peptides.

Introduction

The site-selective modification of peptides with enriched biological functions, particularly in near-physiologic conditions, entails a significant challenge for chemical biology due to the similar reactivity of their amino acid constituents.^{1–3} Considerable progress has been made in this field by using tagged^{4–6} or customizable units,^{7–10} although differentiating among similar units within the same or coexisting peptides, commonly poses an additional problem. In this context, performing chemical reactions on a solid support has considerable advantages with respect to solution phase chemistry.^{11–13} Reaction mixtures are commonly cleaner, lower purification steps are needed, and, in a peptide context, solid-phase synthesis allows the controlled elongation of the peptide chain.^{14–16} Several types of reactions using a solid support have been described in addition to prototypical amide bond formation to obtain labelled peptides, such as copper(I) catalyzed azide-alkyne cycloadditions,¹⁷ Michael additions,¹⁸ cysteine *S*-alkylation,¹⁹ or ring-opening of aziridines.^{20,21}

Cyclic sulfamidates are versatile building blocks and highly customizable units for the regio- and stereoselective synthesis of amino acids, lactams, diazepines and valuable intermediates in drug development through nucleophilic ring-opening reactions.^{22,23} However, to date, only a few cases of heterogeneous reactions using cyclic sulfamidates have been reported. Cohen and Halcomb introduced sulfamidates derived from natural β -hydroxyamino acids, *L*-serine and *L*-threonine, in immobilized peptides and carried out ring-opening reactions on

resin using β -1-thioglucofuranose as a nucleophile to obtain thioglycopeptides.²⁴ Additionally, Lubell performed sulfamidate ring-opening reactions using a peptide bound to the resin to obtain β - and γ -lactams.^{25–27} We have recently reported the ring-opening of cyclic sulfamidates derived from α -methylisoserine with *S*- and *N*-nucleophiles in a peptide context in homogenous phase.^{28,29} These reactions proceeded at the sulfamidate tetrasubstituted carbon atom with complete inversion of the configuration at this stereocenter, giving rise to **hybrid peptides**. However, this methodology was limited to di- or tri-peptides. Herein, we report a methodology for on-resin site- and stereoselective chemical modification of longer **peptides** through the ring-opening of immobilized cyclic sulfamidates derived from α -methylisoserine, and further labeling of the resulting linear peptides. Additionally, the special reactivity of the *N*-terminus of sulfamidate-modified peptides is explored for potential pH-controlled drug release and selective side-chain modification via intramolecular *N*→*X* acyl shift.

Results and discussion

Selective peptide backbone modification

Following the strategy developed in our group for coupling cyclic sulfamidate **1** derived from α -methylisoserine to natural peptides in solid phase,[†] peptide **2** (1-Ala-Pro-Asp-Thr-Arg-Pro) was synthesized using solid-phase peptide synthesis (SPPS) with microwave activation. The sequence Ala-Pro-Asp-Thr-Arg-Pro is a well-known epitope of the MUC-1 mucin, which has been closely related to several types of cancer^{30,31} and used for the development of anticancer vaccines.^{32–35} Alternatively, acetylation at the *N*-terminus of the sulfamidate before resin cleavage using a 1:2 mixture of acetic anhydride (Ac_2O) and pyridine afforded peptide **3** (Ac-1-Ala-Pro-Asp-Thr-Arg-Pro) in a 78% global yield after HPLC purification (Scheme 1). As recently reported, capping of the *N*-terminus of the sulfamidate is necessary to enable nucleophilic ring-opening reactions.³⁶

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Scheme 1. Solid-phase synthesis of sulfamidate-containing peptides **2** and **3**. TFA = trifluoroacetic acid; TIS = triisopropylsilane; Pbf = 2,2,4,6,7-pentamethylidihydrobenzofuran-5-sulfonyl; yields refer to HPLC-purified compounds.

The azide functionality is widely used for bioorthogonal and bioconjugation applications given its superior reactivity in traceless reactions with phosphines (Staudinger ligation) and alkynes.^{37,38} A widely used strategy for labelling peptides and proteins is the so-called strain-promoted alkyne-azide cycloaddition (SPAAC).^{39,40} Thus, we assayed the potential of sulfamidate-containing peptides as transient electrophiles for the site-selective installation of the azide group and subsequent trapping with strained cycloalkynes. As a control reaction in solution, peptide **3** was dissolved in H₂O and reacted with 20 equiv. of sodium azide at room temperature. The reaction was monitored by HPLC (ESI fig. S1) observing a fast consumption of the starting material and the appearance of two new peaks after 2 h. Azide-labelled peptide **4'** was obtained as the main product (31%), while the minor peak (15%) corresponded to *N*-deacetylated peptide **2** (Scheme 2). Since the reaction mixture was not subjected to the typical acidic workup after sulfamidate ring-opening, the sulfamic acid moiety (SO₃⁻) remained attached at the *N*-terminus of the peptide. Reducing the amount of azide did not avoid the nucleophilic cleavage of the sulfamidate *N*-acetyl group and was detrimental for ring-opening (18% and 35% yields of **4'** and **2**, respectively, after 18 h using 2 equiv. of sodium azide). Such undesired partial deprotection has been recursively observed in our group in similar reactions in solution.^{28,41}

Scheme 2. Ring-opening reaction of peptide **3** with sodium azide in water solution. Conversion was estimated from the integration of HPLC signals in the reaction mixture (Fig. S1); yields refer to HPLC-purified compounds.

However, a substantial improvement was observed when the same ring-opening reaction was performed in the solid phase. After reacting the immobilized version of peptide **3** with 20 equiv. of sodium azide (relative to the used resin) for 3 h at

room temperature in DMF, and subsequent removal of protecting groups including the *N*-terminal sulfamic acid, resin cleavage and purification by HPLC, the desired azide-labelled peptide **4** was obtained in a 31% global yield (Scheme 3). Of note, *N*-deacetylation was completely suppressed under these conditions (ESI fig. S2). Peptide **4** was obtained with high diastereomeric purity (>95% as determined by HPLC and NMR), demonstrating that a S_N2-type mechanism, demonstrated previously for small-molecule variants,^{36,42} also operates in a solid-phase peptide context.

Peptide **4** was further reacted with 2 equiv. of strained cyclooctyne bicyclo[6.1.0]non-4-yn-9-ylmethanol (BCN-OH), a common handle used in SPAAC bioconjugations, in a 1:3 mixture of H₂O/acetonitrile at 25 °C. The 1,3-dipolar cycloaddition was completed in 2 h (>85% conversion after 30 min) and the expected azide-alkyne conjugate was obtained as a 1:1 mixture of diastereomers **5a** and **5b** in a 77% combined yield after HPLC purification (Scheme 3 and ESI fig. S3).

Scheme 3. On-resin ring-opening of peptide **3** with sodium azide and subsequent click cycloaddition with BCN-OH; yields refer to HPLC-purified compounds.

Fluorescent peptides are widely used as imaging agents for direct visualization of biochemical processes in cells.^{43–45} Hence, immobilized peptide **3** was labelled on-resin with fluorescent 7-mercapto-4-methylcoumarin **6** (10 equiv.) in the presence of *N,N*-diisopropylethylamine (DIPEA) as a base in DMF. After stirring at room temperature for 1.5 h and subsequent resin cleavage and HPLC purification, conjugated peptide **7** was obtained in a 45% global yield as a single product (Scheme 4). The fluorescent properties of peptide **7** were evaluated, observing an excitation maximum (λ_{exc}) at 331 nm and an emission maximum (λ_{em}) at 399 nm in DMSO (ESI fig. S4).

Scheme 4. On-resin ring-opening of peptide **3** with coumarin derivative **6**; yields refer to HPLC-purified compounds.

Glycosylation is one of the most prevalent post-translational modifications (PTM) occurring in peptides and proteins, with a defining impact on its structure and function.⁴⁶ Particularly, *S*-glycosylation of cysteine residues is observed in biologically active peptides such as antimicrobial sublancin.⁴⁷ We have previously used thiocarbohydrates as nucleophiles for the ring-opening of hindered cyclic sulfamidates as electrophiles to

synthesize **hybrid** glycopeptides in solution.^{28,29} Here, the glycosylation of immobilized peptide **3** was performed on-resin using tetra-*O*-acetyl- β -1-thioglucose **8** (10 equiv.) and DIPEA as a base. After stirring at room temperature for 1.5 h, removal of the carbohydrate *O*-acetyl protecting groups with a solution of 70% hydrazine in methanol, resin cleavage and HPLC purification, glycoconjugated peptide **9** was obtained in a 16% global yield (Scheme 5). Again, peptide by-products were not observed. Notably, the acetyl group at the peptide *N*-terminus was removed during the process likely by the action of hydrazine, which precluded the cleavage of the sulfamic acid normally removed upon acidic treatment.

Scheme 5. On-resin ring-opening of peptide **3** with thiocarbohydrate **8**; yields refer to HPLC-purified compounds.

Selective *N*-terminal modification and pH-controlled release

Carbonyl functional groups (i.e. amides and carbamates) attached to the *N* atom of cyclic sulfamidates derived from amino acids have been observed to be highly reactive towards different types of nucleophiles, particularly in aqueous environments, often leading to undesired deprotection side reactions.^{24,28,41,48} Likewise, the special reactivity of *N*-acyl-*N*-alkylsulfonamides has been used to develop 'safety-catch' linkers for the solid-phase synthesis of peptide thioesters.^{49,50} We decided to exploit this special reactivity of *N*-carbonyl sulfamidates as an effective strategy to regulate the release of chemical functionalities in a controlled manner (i.e. chemical decaging or release).^{51–53} Therefore, two biologically relevant molecules such as a biotin (Btn-OH), a high-affinity label used in affinity chromatography and proteins/antibodies deflection,^{54,55} and chlorambucil (Clb-OH), a chemotherapeutic drug frequently used for the treatment of some types of cancer,^{56,57} were coupled on-resin to the *N*-terminus of immobilized peptide **3** using TBTU and DIPEA as coupling agents in DMF at -20°C for 45 min. Resin cleavage and purification by HPLC afforded peptides **10** and **11** in 33% and 62% yields, respectively (Figure 1a). A kinetic study of pH-dependent chlorambucil release was performed in different media. Hence, peptide **10** was dissolved in phosphate buffered saline (PBS, pH 7.4) simulating the physiological medium of a healthy cell environment, and a second sample was dissolved in a slightly acidic solution (pH 6.3), simulating cancer cells environments.^{58–61} Both samples were separately incubated at 25°C . Aliquots were taken periodically and monitored by analytical HPLC (ESI fig. S5). An exponential decay was observed for the concentration of peptide **10**, corresponding to pseudo-first order kinetics for the hydrolytic decaging reaction (Figure 1). In parallel, signals of increasing intensity corresponding to peptide **2** and free chlorambucil were observed. Notably, acidic medium accelerates payload cleavage by nearly 4-fold as reflected by the

measured kinetic rate constants ($k_{6.3} = 6.4 \cdot 10^{-5} \text{ s}^{-1}$; $k_{7.4} = 1.7 \cdot 10^{-5} \text{ s}^{-1}$, Figure 1b,c). These results suggest that the cytotoxic drug would remain attached to the sulfamidate-containing peptide for longer times in healthy cells, while it would be released slightly faster at the surroundings of more acidic cancer cells. This strategy, thus, poses potential for selective chemotherapy applications although the potential reactivity of the sulfamidate-modified peptides towards biological nucleophiles, particularly thiols (i.e. glutathione) might limit its practical use.

Fig. 1. a) Solid-phase synthesis of peptides **10** and **11** and subsequent pH-controlled release. b) Evolution of peptide **10** concentration at pH 7.4 (blue) and 6.3 (purple) at 25°C . c) Estimation of the pseudo-first order kinetic constants at pH 7.4 (blue) and 6.3 (purple) using the linearly fitted region of the $\text{Ln}[\mathbf{10}]$ vs. time plot. The reported apparent hydrolysis pseudo-first order constants (average $\pm$ standard deviation) include the concentration of bulk water (ca. 55 M). Experimental data shown in panels b and c were fitted using exponential ($[\mathbf{10}] = [\mathbf{10}]_0 e^{-kt}$) and linear ($\text{Ln}[\mathbf{10}] = \text{Ln}[\mathbf{10}]_0 - kt$) equations, respectively, using Microcal OriginPro 2019 software.

Selective side-chain modification via intramolecular *N*→X acyl shift

Certain proteins are able to excise part of their sequence (inteins) and join the remaining segments (exteins) by a process known as intein-mediated protein splicing.^{62–64} In class-II intein-like peptides, this process is initiated by an intramolecular *N*→S acyl shift followed by a trans(thio)esterification reaction.⁶⁵ Inspired by this biological transformation, and once the pH-controlled release of *N*-carbonyl groups from sulfamidate-containing peptides was demonstrated, we envisioned their selective side-chain modification by intramolecular *N*→X (X = S, N, O) transacylation of cleavable moieties to nucleophilic residues embedded in the peptide sequence. Thus, sulfamidate-modified peptides **12–15** bearing a Fmoc-protected alanine at the *N*-terminus were synthesized in 30–49% global yields after HPLC purification (Scheme 6). The Ala-His-Asn-Xxx-Gly (Xxx = Cys, Ser, Thr, Lys) sequence was selected since it is a common motif found in class-II intein-like peptides.⁶⁶ The influence of the

nucleophilic residue location relative to the sulfamidate linkage in the outcome of the acyl group migration, was also examined by synthesizing peptides **16–18** (30–35% global yields after HPLC purification). Since the peptides resulting from the hypothesized transacylation reactions would have identical molecular weights and very similar HPLC elution properties than those of the starting materials, we selected ^1H NMR to monitor reaction progress. First, peptide **12** was dissolved in a 24:1 $\text{CD}_3\text{CN}/\text{D}_2\text{O}$ mixture and a slight excess of triethylamine (TEA, 1.6 equiv.) was added as a base to activate Cys at room temperature. Complete conversion to a unique new product was observed immediately after increasing the pH of the solution (ESI fig. S6). Accordingly, HPLC analysis of the mixture showed a unique signal at a retention time very similar to that of the starting peptide, and the HPLC purified peptide **19** showed a mass identical to that of the starting material (ESI fig. S13). These results suggested that the Fmoc-Ala residue had been transferred from the nitrogen atom of the sulfamidate to the sulfur atom of the cysteine via intramolecular $\text{N}\rightarrow\text{S}$ transacylation, instead of the cysteine undergoing sulfamidate ring-opening as commonly observed in small-molecule reactions. This was further confirmed by recovering peptide **19** completely unreacted after treatment with a large excess of *N*-ethylmaleimide (NEM), which indicated complete cysteine modification unlike in Cys-unprotected control peptide **26** (ESI fig. S20). The transacylation reaction was demonstrated to be equally efficient irrespective of the relative positions of the reacting cysteine and the sulfamidate residue, and the peptide length, cleanly yielding sidechain-modified peptides **20–22** from activated peptides **16–18** (ESI figs. S10–12 and S17–S19).

Scheme 6. Chemical structure of intein-like sulfamidate-containing peptides **12–18** and the corresponding transacylated peptides **19–25**. Modified residues appear in boldface.

Yields (%) for peptides **12–18** were calculated as mmol of HPLC-purified compound divided by mmol of resin labelling content. Conversion values for peptides **19–25** were derived from the ^1H NMR spectra of reaction mixtures.

The acyl migration reaction was not limited only to cysteine and proceeded equally fast with peptide **15** bearing a nucleophilic lysine to cleanly yield sidechain-modified peptide **25** (90% conversion, ESI figs. S9 and S16). However, peptides **13** and **14** bearing much less acidic serine and threonine residues, respectively, the transacylation reaction was not completed after 3 h (ESI fig. S7–S8 and S14–S15). Of note, the histidine residues present in all assayed peptides remained unreacted in all cases. The discovery of this, to our knowledge, unprecedented $\text{N}\rightarrow\text{X}$ ($\text{X} = \text{S}, \text{N}, \text{O}$) transacylation reaction offers new possibilities for the efficient and selective peptide sidechain modification.

Conclusions

We have expanded the use of α -methylisoserine-derived cyclic sulfamidates for the synthesis and selective chemical modification of **hybrid peptides** in near-physiological conditions. Solid-phase peptide synthesis allowed the efficient coupling of such sulfamidates to the *N*-terminus of different peptide sequences, and subsequent site-selective and stereocontrolled installation of a fluorescent label, an azide tag for further bioconjugation and a post-translational modification. The special reactivity of carbonyl groups linked to the sulfamidate *N*-terminus allowed attaching labile affinity tags and an anticancer drug for controlled release in slightly acidic environments such as those found in cancer cells. Also, we describe for the first time the spontaneous transacylation reaction on nucleophilic sidechains in the peptide sequence. Overall, we demonstrate the value of chiral cyclic sulfamidates as versatile tools for bioconjugation and the selective chemical modification of peptides.

Experimental

Reagents and general procedures

Commercial reagents were used without further purification. Solvents were dried and redistilled prior to use. Thin-layer chromatography (TLC) was performed on silica gel plates (Polychrom SI F254), using UV-light, phosphomolybdic acid, potassium permanganate or ninhydrin as stains. Column chromatography was performed using silica gel (0.04–0.06 mm, 230–240 mesh). ^1H and ^{13}C NMR spectra were measured with Bruker Avance-400 and Bruker ARX-300 spectrometers in D_2O or $\text{H}_2\text{O}/\text{D}_2\text{O}$ (9:1). Multiplicities are quoted as singlet (s), doublet (d), or multiplet (m). Spectra were assigned by using COSY and HSQC. All chemical shifts (δ) were recorded in ppm and coupling constants (*J*) are reported in Hz. High-resolution electrospray mass (ESI) spectra were recorded on a microTOF-Q-Bruker spectrometer; accurate mass measurements were achieved by using sodium formate as an external reference. UV/Vis spectra were recorded in approximately 10^{-5} M solutions in DMSO by using an OceanOptics USB4000UV-Vis spectrometer and quartz cells (1 cm path length). Luminescence measurements were recorded with same solutions by using a Jobin-Yvon-Horiba Fluorolog 3-22 Tau-3 spectrofluorometer.

NMR experiments. NMR experiments were performed with 300 MHz and 400 MHz spectrometers at 298 K. Magnitude-mode ge-2D COSY spectra were acquired with gradients by using the cosygpqf pulse program with a pulse width of 90° . Phase-sensitive ge-2D HSQC spectra were acquired by using z-filter and selection before t1 removing the decoupling during the acquisition by use of the invigpndph pulse program with CNST2 (JHC)=145.

2D-NOESY experiments. NOESY experiments were recorded with a Bruker Avance 400 MHz spectrometer at 298 K and pH 6.0–6.5 in $\text{H}_2\text{O}/\text{D}_2\text{O}$ (9:1). The experiments were conducted by using phase-sensitive ge-2D NOESY with WATERGATE for $\text{H}_2\text{O}/\text{D}_2\text{O}$ (9:1) spectra. NOEs intensities were normalized with respect to

the diagonal peak at zero mixing time. Distances involving NH protons were semi-quantitatively determined by integrating the volume of the corresponding cross-peaks. The number of scans used was 16 and the mixing time was 500 ms.

General protocol for microwave-assisted solid-phase peptide synthesis (MW-SPPS). Rink Amide MBHA resin (0.05 or 0.10 mmol) was dwelled with *N,N*-dimethylformamide for 5 min. DIC/Oxyma Pure® were used as coupling agents, a 20% (v/v) solution of piperidine in DMF for Fmoc deprotection, and a 1:2 mixture of acetic anhydride and pyridine for the final acetylation. Natural Fmoc-protected amino acids (5 equiv.) were coupled using the standard protocol. Sulfamidate building block **1** (5.0 equiv.) was coupled using Oxyma Pure® (5.5 equiv.), DIC (20.0 equiv.) in DMF (2 mL) under microwave at 75 °C for 20 min. Chlorambucil and biotin were manually coupled using TBTU and DIPEA in DMF at -20 °C for 45 min. Peptides were detached from the resin and acid-sensitive sidechain protecting groups by using a solution of TFA/TIS/H₂O (95:2.5:2.5) for 3 h at 25 °C. Peptides were then precipitated with cold diethyl ether and centrifuged, to afford the crude derivatives. Reaction crudes were analysed using a Waters 1525 HPLC system equipped with a diode array detector (210/254 nm) using the analytical column Phenomenex Luna® C18(2) column (5 µm, 250 mm × 4.6 mm) with a flow rate of 1.0 mL/min (linear gradient 5–95% B over 60 min). Peptides were purified using a Waters 2695 HPLC system equipped with a dual absorbance detector (210/254 nm) and the semi-preparative column Phenomenex Luna C18(2) (10 µm, 250 mm × 21.2 mm) with a flow rate of 10 mL/min and immediately lyophilised and stored at -20 °C. Method A: linear gradient 1–31% B over 30 min. Method B: linear gradient 1–61% B over 60 min. Method C: linear gradient 5–25% B over 5 min followed by linear gradient 25–50% over 25 min. Method D: linear gradient 5–30% B over 25 min. A: H₂O + 0.1% TFA. B: MeCN. Global yields (%) were calculated as mmol of pure peptide divided by mmol of resin labelling content (0.63–0.56 mmol/g loading) and include all amino acid couplings, Fmoc-deprotections, ring-opening reactions, protecting groups/resin cleavage and HPLC purification.

Sulfamidate **1** was synthesized following the methodology previously described by us.⁶⁷

Peptide **2** (1-Ala-Pro-Asp-Thr-Arg-Pro-NH₂) was synthesized and purified (method A) following the general protocol described above in a 62% global yield. Semi-preparative HPLC retention time: 25.33 min. HRMS (ESI) *m/z* = 840.3290, calculated for C₃₁H₅₁N₁₁O₁₃Na (M+Na⁺) = 840.3281. ¹H NMR (300 MHz, D₂O) δ (ppm): 1.08 (d, 3H, *J* = 6.4 Hz, CH₃ of Thr), 1.31 (d, 3H, *J* = 7.1 Hz, CH₃ of Ala), 1.51–1.67 (m, 6H, CH₃ of **1**, H_γ of Arg, H_β of Arg), 1.68–2.01 (m, 7H, H_β of Arg, H_β of Pro1, H_β of Pro2, H_γ of Pro1, H_γ of Pro2), 2.12–2.28 (m, 2H, H_β of Pro1, H_β of Pro2), 2.71–2.96 (m, 2H, H_β of Asp), 3.04–3.17 (m, 2H, H_δ of Arg), 3.45–3.62 (m, 3H, CH₂ of **1**, H_δ of Pro1, H_δ of Pro2), 3.63–3.76 (m, 2H, H_δ of Pro1, H_δ of Pro2), 3.81 (d, 1H, *J* = 12.8 Hz, CH₂ of **1**), 4.05–4.14 (m, 1H, H_β of Thr), 4.19–4.35 (m, 3H, H_α of Pro1, H_α of Pro2, H_α of Thr), 4.48–4.66 (m, 3H, H_α of Ala, H_α of Arg, H_α of Asp). ¹³C NMR (75 MHz, D₂O) δ (ppm): 15.1 (CH₃ of Ala), 18.8 (CH₃ of Thr), 22.5 (CH₃ of **1**), 24.0 (C_γ of Arg), 24.6 (C_γ of Pro1), 24.7 (C_γ of

Pro2), 27.5 (C_β of Arg), 29.2 (C_β of Pro1), 29.5 (C_β of Pro2), 34.9 (C_β of Asp), 40.5 (C_δ of Arg), 47.7 (C_δ of Pro1), 47.9 (C_δ of Pro2), 48.3 (C_α of Ala), 50.1 (C_α of Asp), 51.1 (C_α of Arg), 51.2 (CH₂ of **1**), 59.0 (C_α of Thr), 60.2 (C_α of Pro2), 60.7 (C_α of Pro1), 67.1 (C_β of Thr), 90.2 (C_α of **1**), 156.7 (HN=C of Arg), 171.3, 171.3, 171.4, 172.4, 172.6, 174.0, 176.8 (CO).

Peptide **3** (Ac-1-Ala-Pro-Asp-Thr-Arg-Pro-NH₂) was synthesized and purified (method A) following the general protocol described above in a 78% global yield. Semi-preparative HPLC retention time: 26.92 min. HRMS (ESI) *m/z* = 860.3561, calculated for C₃₃H₅₄N₁₁O₁₄S [M+H]⁺ = 860.3567. ¹H NMR (300 MHz, D₂O) δ (ppm): 1.05 (d, 3H, *J* = 6.4 Hz, CH₃ of Thr), 1.27 (d, 3H, *J* = 7.1 Hz, CH₃ of Ala), 1.47–1.63 (m, 3H, H_γ of Arg, H_β of Arg), 1.65–1.93 (m, 10H, CH₃ of **1**, H_β of Arg, H_β of Pro1, H_β of Pro2, H_γ of Pro1, H_γ of Pro2), 2.07–2.27 (m, 5H, Ac, H_β of Pro1, H_β of Pro2), 2.71–2.90 (m, 2H, H_β of Asp), 3.01–3.13 (m, 2H, H_δ of Arg), 3.43–3.72 (m, 4H, H_δ of Pro1, H_δ of Pro2), 4.02–4.14 (m, 2H, CH₂ of **1**, H_β of Thr), 4.16–4.31 (m, 3H, H_α of Pro1, H_α of Pro2, H_α of Thr), 4.47 (d, 1H, *J* = 10.5 Hz, CH₂ of **1**), 4.50–4.65 (m, 3H, H_α of Ala, H_α of Arg, H_α of Asp). ¹³C NMR (75 MHz, D₂O) δ (ppm): 15.1 (CH₃ of Ala), 18.8 (CH₃ of Thr), 22.2 (Ac), 22.5 (CH₃ of **1**), 23.9 (C_γ of Arg), 24.6 (C_γ of Pro1, C_γ of Pro2), 27.4 (C_β of Arg), 29.2 (C_β of Pro1), 29.5 (C_β of Pro2), 34.9 (C_β of Asp), 40.5 (C_δ of Arg), 47.7 (C_δ of Pro1), 47.8 (C_δ of Pro2), 48.3 (C_α of Ala), 50.1 (C_α of Asp), 51.1 (C_α of Arg), 52.7 (CH₂ of **1**), 59.0 (C_α of Thr), 60.2 (C_α of Pro2), 60.7 (C_α of Pro1), 66.9 (C_β of Thr), 90.1 (C_α of **1**), 156.6 (HN=C of Arg), 169.2, 171.3, 172.2, 172.3, 172.4, 172.5, 173.9, 176.7 (CO).

Peptide **4** (Ac-βAiba(N₃)-Ala-Pro-Asp-Thr-Arg-Pro-NH₂) [βAiba = beta-aminoisobutyric acid] was synthesized by reacting resin-linked peptide **3** (0.05 mmol of resin) with 20 equiv. of sodium azide in DMF at room temperature for 3 h. After that time, the resin was washed with DMF (5 × 3 mL) and CH₂Cl₂ (5 × 3 mL), treated with the cleavage cocktail, precipitated, and purified (method A) as described in the general method (31% global yield). Semi-preparative HPLC retention time: 23.39 min. HRMS (ESI) *m/z* = 823.4161, calculated for C₃₃H₅₅N₁₄O₁₁ [M+H]⁺ = 823.4169. ¹H NMR (300 MHz, D₂O) δ (ppm): 1.11 (d, 3H, *J* = 6.4 Hz, CH₃ of Thr), 1.31 (d, 3H, *J* = 7.1 Hz, CH₃ of Ala), 1.44 (s, 3H, CH₃ of βAiba), 1.52–2.04 (m, 13H, H_γ of Arg, H_β of Arg, H_β of Pro1, H_β of Pro2, H_γ of Pro1, H_γ of Pro2, Ac), 2.14–2.32 (m, 2H, H_β of Pro1, H_β of Pro2), 2.75–2.98 (m, 2H, H_β of Asp), 3.10–3.18 (m, 2H, H_δ of Arg), 3.40 (d, 1H, *J* = 14.2 Hz, CH₂ of βAiba), 3.48–3.80 (m, 5H, H_δ of Pro1, H_δ of Pro2, CH₂ of βAiba), 4.08–4.19 (m, 1H, H_β of Thr), 4.20–4.39 (m, 3H, H_α of Pro1, H_α of Pro2, H_α of Thr), 4.43–4.67 (m, 3H, H_α of Ala, H_α of Arg, H_α of Asp). ¹³C NMR (75 MHz, D₂O) δ (ppm): 15.2 (CH₃ of Ala), 18.8 (CH₃ of Thr), 19.0 (CH₃ of βAiba), 21.8 (Ac), 24.0 (C_γ of Arg), 24.6 (C_γ of Pro1), 24.7 (C_γ of Pro2), 27.4 (C_β of Arg), 29.3 (C_β of Pro1), 29.6 (C_β of Pro2), 35.0 (C_β of Asp), 40.5 (C_δ of Arg), 45.4 (CH₂ of βAiba), 47.8 (C_δ of Pro1), 47.9 (C_δ of Pro2), 48.2 (C_α of Ala), 50.1 (C_α of Asp), 51.1 (C_α of Arg), 59.0 (C_α of Thr), 60.3 (C_α of Pro2), 60.6 (C_α of Pro1), 67.0 (C_β of Thr), 118.2 (C_α of βAiba), 156.7 (HN=C of Arg), 171.3, 171.4, 172.4, 172.5, 173.0, 174.0, 174.4, 176.8 (CO).

Peptide APDTRP (Ac-Ala-Pro-Asp-Thr-Arg-Pro-NH₂) was obtained as a by-product in the synthesis of peptide **4**. Semi-preparative HPLC retention time (Method A): 19.10 min. MS (ESI) *m/z* = 697.3, calculated for C₂₉H₄₉N₁₀O₁₀ [M+H]⁺ = 697.3628. ¹H NMR (300 MHz, D₂O) δ (ppm): 1.11 (d, 3H, *J* = 6.3 Hz, CH₃ of Thr), 1.28 (d, 3H, *J* = 7.1 Hz, CH₃ of Ala), 1.53–2.10 (m, 13H, H γ of Arg, H β of Arg, H β of Pro1, H β of Pro2, H γ of Pro1, H γ of Pro2, Ac), 2.14–2.31 (m, 2H, H β of Pro1, H β of Pro2), 2.75–2.94 (m, 2H, H β of Asp), 3.10–3.18 (m, 2H, H δ of Arg), 3.48–3.81 (m, 4H, H δ of Pro1, H δ of Pro2), 4.09–4.20 (m, 1H, H β of Thr), 4.20–4.39 (m, 3H, H α of Pro1, H α of Pro2, H α of Thr), 4.43–4.68 (m, 3H, H α of Ala, H α of Arg, H α of Asp).

Peptides **5a** and **5b** (Ac- β Aiba(BCN-triazol)-Ala-Pro-Asp-Thr-Arg-Pro-NH₂) were synthesized by reacting peptide **4** with 2 equiv. of bicycle[6.1.0]non-4-yn-9-ylmethanol (BCN-OH) in a 1:3 mixture of H₂O and MeCN at room temperature for 2 h, and purified using method B (77% combined yield). Semi-preparative HPLC retention times: 18.60 and 20.00 min, respectively. HRMS (ESI) *m/z* = 973.5200 and 973.5211, calculated for C₄₃H₆₈N₁₄O₁₂ [M+H]⁺ = 973.5214. ¹H NMR for **5a** (300 MHz, D₂O) δ (ppm): 1.02–1.13 (m, 5H, CH₃ of Thr, H¹, H⁸), 1.16–1.31 (m, 4H, CH₃ of Ala, H⁹), 1.46–2.33 (m, 22H, CH₃ of β Aiba, H γ of Arg, H β of Arg, H β of Pro1, H β of Pro2, H γ of Pro1, H γ of Pro2, Ac, H², H⁷), 2.57–2.97 (m, 6H, H β of Asp, H³, H⁶), 3.08–3.16 (m, 2H, H δ of Arg), 3.50–3.81 (m, 7H, H δ of Pro1, H δ of Pro2, CH₂ of β Aiba, H¹⁰), 3.98 (d, 1H, *J* = 14.4 Hz, CH₂ of β Aiba), 4.05–4.13 (m, 1H, H β of Thr), 4.18–4.36 (m, 3H, H α of Pro1, H α of Pro2, H α of Thr), 4.42–4.67 (m, 3H, H α of Ala, H α of Arg, H α of Asp). ¹H NMR for **5b** (300 MHz, D₂O) δ (ppm): 0.71–0.90 (H¹, H⁸), 0.95–1.12 (m, 4H, CH₃ of Thr, H⁹), 1.22 (d, 3H, *J* = 7.1 Hz, CH₃ of Ala), 1.43–2.29 (m, 22H, CH₃ of β Aiba, H γ of Arg, H β of Arg, H β of Pro1, H β of Pro2, H γ of Pro1, H γ of Pro2, Ac, H², H⁷), 2.51–2.96 (m, 6H, H β of Asp, H³, H⁶), 3.08–3.16 (m, 2H, H δ of Arg), 3.48–3.83 (m, 7H, H δ of Pro1, H δ of Pro2, CH₂ of β Aiba, H¹⁰), 3.99 (d, 1H, *J* = 14.5 Hz, CH₂ of β Aiba), 4.05–4.14 (m, 1H, H β of Thr), 4.17–4.37 (m, 3H, H α of Pro1, H α of Pro2, H α of Thr), 4.50–4.64 (m, 3H, H α of Ala, H α of Arg, H α of Asp).

Peptide **7** (Ac- β Aiba(Coum)-Ala-Pro-Asp-Thr-Arg-Pro-NH₂) was synthesized by reacting resin-linked peptide **3** (0.05 mmol of resin) with 10 equiv. of 7-mercapt-4-methylcoumarin **6** and 9 equiv. of DIPEA in DMF at room temperature for 1.5 h. After that time, the resin was washed with DMF (5 x 3 mL) and CH₂Cl₂ (5 x 3 mL), treated with the cleavage cocktail, precipitated, and purified (method C) as described in the general method (45% global yield). Semi-preparative HPLC retention time: 16.47 min. HRMS (ESI) *m/z* = 972.4244, calculated for C₄₃H₆₂N₁₁O₁₃S [M+H]⁺ = 972.4244. ¹H NMR (300 MHz, D₂O) δ (ppm): 1.09 (d, 3H, *J* = 6.4 Hz, CH₃ of Thr), 1.27 (d, 3H, *J* = 7.0 Hz, CH₃ of Ala), 1.35 (s, 3H, CH₃ of β Aiba), 1.45–2.04 (m, 13H, H γ of Arg, H β of Arg, H β of Pro1, H β of Pro2, H γ of Pro1, H γ of Pro2, Ac), 2.13–2.30 (m, 2H, H β of Pro1, H β of Pro2), 2.36 (s, 3H, CH₃ of Coum), 2.77–2.96 (m, 2H, H β of Asp), 3.00–3.14 (m, 2H, H δ of Arg), 3.46–3.75 (m, 6H, H δ of Pro1, H δ of Pro2, CH₂ of β Aiba), 4.05–4.17 (m, 1H, H β of Thr), 4.21–4.67 (m, 6H, H α of Pro1, H α of Pro2, H α of Thr, H α of Ala, H α of Arg, H α of Asp), 6.30 (s, 1H, H³), 7.24–7.40 (m, 2H, H⁶, H⁸), 7.62 (d, 1H, *J* = 9.1 Hz, H⁵). ¹³C NMR (75 MHz, D₂O) δ (ppm):

15.3 (CH₃ of Ala), 17.9 (CH₃ of Coum), 18.8 (CH₃ of Thr), 21.0 (CH₃ of β Aiba), 21.8 (Ac), 24.0 (C γ of Arg), 24.6 (C γ of Pro1), 24.7 (C γ of Pro2), 27.5 (C β of Arg), 29.3 (C β of Pro1), 29.6 (C β of Pro2), 34.9 (C β of Asp), 40.5 (C δ of Arg), 45.5 (CH₂ of β Aiba), 47.8 (C δ of Pro1), 47.9 (C δ of Pro2), 48.3 (C α of Ala), 50.1 (C α of Asp), 51.0 (C α of Arg), 55.9 (C α of β Aiba), 59.1 (C α of Thr), 60.2 (C α of Pro2), 60.6 (C α of Pro1), 67.1 (C β of Thr), 114.4 (C³), 120.5 (C^{4a}), 122.8 (C⁸), 125.6 (C⁵), 131.4 (C⁶), 134.5 (C⁷), 152.1 (C^{8a}), 155.4 (C⁴), 156.6 (HN=C of Arg), 163.6 (C²), 171.2, 171.3, 172.5, 172.9, 173.5, 174.0, 174.3, 176.7 (CO).

Peptide **9** (HSO₃- β Aiba(β Glc-S)-Ala-Pro-Asp-Thr-Arg-Pro-NH₂) was synthesized by reacting resin-linked peptide **3** (0.05 mmol of resin) with 10 equiv. of tetra-*O*-acetyl- β -thioglucose **8** and 9 equiv. of DIPEA in DMF at room temperature for 1.5 h. After that time, the resin was washed with DMF (5 x 3 mL) and methanol (5 x 3 mL) and treated with a 70% solution of hydrazine in methanol (3 x 45 min). Then, the resin was washed with methanol (5 x 3 mL), DMF (5 x 3 mL) and CH₂Cl₂ (5 x 3 mL), treated with the cleavage cocktail, precipitated, and purified (method A) as described in the general method (16% global yield). Semi-preparative HPLC retention time: 19.77 min. HRMS (ESI) *m/z* = 1014.3829, calculated for C₃₇H₆₄N₁₁O₁₈S₂ [M+H]⁺ = 1014.3867. ¹H NMR (400 MHz, D₂O) δ (ppm): 1.08 (d, 3H, *J* = 6.4 Hz, CH₃ of Thr), 1.29 (d, 3H, *J* = 7.1 Hz, CH₃ of Ala), 1.46 (s, 3H, CH₃ of β Aiba), 1.50–2.00 (m, 10H, H γ of Arg, H β of Arg, H β of Pro1, H β of Pro2, H γ of Pro1, H γ of Pro2), 2.12–2.23 (m, 2H, H β of Pro1, H β of Pro2), 2.74–2.92 (m, 2H, H β of Asp), 3.04–3.32 (m, 7H, H δ of Arg, H², H⁴, H⁵, H⁶), 3.36–3.44 (m, 1H, H³), 3.46–3.79 (m, 6H, H δ of Pro1, H δ of Pro2, CH₂ of β Aiba), 4.05–4.16 (m, 1H, H β of Thr), 4.17–4.34 (m, 3H, H α of Pro1, H α of Pro2, H α of Thr), 4.40–4.64 (m, 4H, H α of Ala, H α of Arg, H α of Asp, H¹). ¹³C NMR (75 MHz, D₂O) δ (ppm): 15.0 (CH₃ of Ala), 18.8 (CH₃ of Thr), 22.1 (CH₃ of β Aiba), 24.0 (C γ of Arg), 24.6 (C γ of Pro1, C γ of Pro2), 27.3 (C β of Arg), 29.2 (C β of Pro1), 29.5 (C β of Pro2), 35.0 (C β of Asp), 40.4 (C δ of Arg), 47.7 (C δ of Pro1), 47.8 (C δ of Pro2), 48.5 (C α of Ala), 49.4 (C⁶), 50.2 (C α of Asp), 51.0 (C α of Arg), 52.8 (C α of β Aiba), 59.0 (C α of Thr), 60.2 (C α of Pro2, C α of Pro1), 60.6 (CH₂ of β Aiba), 66.9 (C β of Thr), 69.1 (C⁴), 72.2 (C²), 77.1 (C³), 79.7 (C⁵), 83.0 (C¹), 156.6 (HN=C of Arg), 171.3, 171.4, 172.6, 173.3, 174.1, 174.5, 176.8 (CO).

Peptide **10** (Chlorambucil-1-Ala-Pro-Asp-Thr-Arg-Pro-NH₂) was synthesized and purified (method D) following the general protocol described above in a 33% global yield. Semi-preparative HPLC retention time: 52.07 min. HRMS (ESI) *m/z* = 1103.4148, calculated for C₄₅H₆₉Cl₂N₁₂O₁₄S [M+H]⁺ = 1103.4148. ¹H NMR (300 MHz, D₂O) δ (ppm): 1.08 (d, 3H, *J* = 6.3 Hz, CH₃ of Thr), 1.31 (d, 3H, *J* = 7.0 Hz, CH₃ of Ala), 1.51–2.01 (m, 15H, CH₃ of **1**, H γ of Arg, H β of Arg, H β of Pro1, H β of Pro2, H γ of Pro1, H γ of Pro2, H β of chlorambucil), 2.08–2.31 (m, 2H, H β of Pro1, H β of Pro2), 2.45–2.58 (m, 2H, H α of chlorambucil), 2.60–2.73 (m, 2H, H γ of chlorambucil), 2.74–2.96 (m, 2H, H β of Asp), 3.05–3.15 (m, 2H, H δ of Arg), 3.45–3.77 (m, 8H, H δ of Pro1, H δ of Pro2, H¹), 3.92–4.02 (m, 4H, H²), 4.03–4.16 (m, 2H, CH₂ of **1**, H β of Thr), 4.17–4.35 (m, 3H, H α of Pro1, H α of Pro2, H α of Thr), 4.43–4.66 (m, 4H, CH₂ of **1**, H α of Ala, H α of Arg, H α of Asp), 7.36–7.50 (m, 4H, Arom.). ¹³C NMR (75 MHz, D₂O) δ (ppm): 15.2 (CH₃ of Ala),

18.8 (CH₃ of Thr), 22.4 (CH₃ of **1**), 24.0 (C γ of Arg), 24.6 (C γ of Pro1, C γ of Pro2), 25.5 (C β of chlorambucil), 27.4 (C β of Arg), 29.2 (C β of Pro1), 29.5 (C β of Pro2), 32.9 (C α of chlorambucil), 33.5 (C γ of chlorambucil), 34.9 (C β of Asp), 37.2 (C¹), 40.5 (C δ of Arg), 47.7 (C δ of Pro1), 47.9 (C δ of Pro2), 48.3 (C α of Ala), 50.1 (C α of Asp), 51.1 (C α of Arg), 52.2 (CH₂ of **1**), 58.9 (C²), 59.1 (C α of Thr), 60.2 (C α of Pro2), 60.7 (C α of Pro1), 67.0 (C β of Thr), 86.3 (C α of **1**), 122.1, 122.3, 130.7, 133.0, 144.8 (Arom.), 156.7 (HN=C of Arg), 169.1, 171.1, 172.1, 172.4, 173.9, 174.0, 176.7 (CO).

Peptide **11** (Biotin-1-Ala-Pro-Asp-Thr-Arg-Pro-NH₂) was synthesized and purified (method D) following the general protocol described above in a 62% global yield. Semi-preparative HPLC retention time: 31.49 min. HRMS (ESI) *m/z* = 1044.4221, calculated for C₄₁H₆₆N₁₃O₁₅S₂ [M+H]⁺ = 1044.4237. ¹H NMR (300 MHz, D₂O) δ (ppm): 1.06 (d, 3H, *J* = 6.4 Hz, CH₃ of Thr), 1.24–1.37 (m, 6H, CH₃ of Ala, H γ of biotin, H β of biotin), 1.49–1.63 (m, 7H, H β of biotin, H δ of biotin, H γ of Arg, H β of Arg), 1.68–1.97 (m, 10H, CH₃ of **1**, H β of Arg, H β of Pro1, H β of Pro2, H γ of Pro1, H γ of Pro2), 2.11–2.26 (m, 2H, H β of Pro1, H β of Pro2), 2.46–2.57 (m, 2H, H α of biotin), 2.65 (d, 1H, *J* = 13.0 Hz, H⁴), 2.73–2.93 (m, 3H, H β of Asp, H⁴), 3.03–3.15 (m, 2H, H δ of Arg), 3.16–3.25 (m, 2H, H¹), 3.47–3.74 (m, 4H, H δ of Pro1, H δ of Pro2), 4.05–4.14 (m, 2H, CH₂ of **1**, H β of Thr), 4.17–4.37 (m, 4H, H α of Pro1, H α of Pro2, H α of Thr, H²), 4.46–4.65 (m, 5H, CH₂ of **1**, H α of Ala, H α of Arg, H α of Asp, H³). ¹³C NMR (75 MHz, D₂O) δ (ppm): 15.2 (CH₃ of Ala), 18.8 (CH₃ of Thr), 22.3 (CH₃ of **1**), 24.0 (C γ of Arg), 24.6 (C γ of Pro1, C γ of Pro2), 27.4 (C β of Arg), 27.5 (C δ of biotin), 27.6 (C β of biotin), 27.8 (C γ of biotin), 29.2 (C β of Pro1), 29.5 (C β of Pro2), 34.3 (C α of biotin), 34.8 (C β of Asp), 39.6 (C⁴), 40.5 (C δ of Arg), 47.7 (C δ of Pro1), 47.9 (C δ of Pro2), 48.3 (C α of Ala), 50.1 (C α of Asp), 51.1 (C α of Arg), 52.3 (CH₂ of **1**), 55.2 (C¹), 59.1 (C α of Thr), 60.2 (C α of Pro2), 60.7 (C α of Pro1), 60.8 (C α of biotin), 62.0 (C²), 67.0 (C β of Thr), 86.3 (C α of **1**), 156.6 (HN=C of Arg), 165.2, 169.1, 171.3, 171.4, 172.1, 172.4, 172.6, 173.9, 174.0, 176.7, 178.8 (CO).

Peptide **12** (Fmoc-Ala-1-Ala-His-Asn-Cys-Gly-NH₂) was synthesized and purified (method C) following the general protocol described above in a 40% global yield. Semi-preparative HPLC retention time: 25.39 min. HRMS (ESI) *m/z* = 956.3012, calculated for C₄₀H₅₀N₁₁O₁₃S₂ [M+H]⁺ = 956.3025. ¹H NMR (300 MHz, CD₃CN/D₂O (95:5)) δ (ppm): 1.18–1.47 (m, 6H, CH₃ of Fmoc-Ala, CH₃ of Ala), 1.79 (s, 3H, CH₃ of **1**), 2.71–2.74 (m, 2H, H β of His), 2.86–2.98 (m, 2H, H β of Cys), 3.01–3.28 (m, 2H, H β of Asn), 3.66–3.89 (m, 2H, H α of Gly), 4.15 (d, 1H, *J* = 10.4 Hz, CH₂ of **1**), 4.20–4.53 (m, 6H, H α of Cys, CH₂ of Fmoc, CH of Fmoc, H α of Fmoc-Ala, H α of Ala), 4.54–4.68 (m, 2H, H α of His, H α of Asn), 4.74 (d, 1H, *J* = 10.4 Hz, CH₂ of **1**), 7.21 (s, 1H, H δ of His), 7.32–7.52 (m, 4H, Arom.), 7.60–7.71 (m, 2H, Arom.), 7.79–7.90 (m, 2H, Arom.), 8.43 (s, 1H, H ϵ of His). ¹³C NMR (75 MHz, CD₃CN/D₂O (95:5)) δ (ppm): 15.8, 16.6 (CH₃ of Fmoc-Ala, CH₃ of Ala), 22.5 (CH₃ of **1**), 25.3 (C β of Cys), 26.2 (C β of Asn), 35.9 (C β of His), 42.1 (C α of Gly), 46.9 (CH of Fmoc), 49.8 (C α of Fmoc-Ala, C α of Ala), 50.6 (C α of His), 52.2 (CH₂ of **1**), 52.5 (C α of Asn), 56.0 (C α of Cys), 66.9 (CH₂ of Fmoc), 85.9 (C α of **1**), 120.0, 125.3,

127.2, 127.8, 128.9, 133.5, 141.1, 143.9, 144.0 (Arom), 156.3 (CO of Fmoc), 168.1, 170.4, 170.8, 171.7, 172.5, 173.0 (CO).

Peptide **13** (Fmoc-Ala-1-Ala-His-Asn-Ser-Gly-NH₂) was synthesized and purified (method C) following the general protocol described above in a 30% global yield. Semi-preparative HPLC retention time: 24.14 min. HRMS (ESI) *m/z* = 940.3217, calculated for C₄₀H₅₀N₁₁O₁₄S [M+H]⁺ = 940.3254. ¹H NMR (300 MHz, CD₃CN/D₂O (95:5)) δ (ppm): 1.29–1.40 (m, 6H, CH₃ of Fmoc-Ala, CH₃ of Ala), 1.79 (s, 3H, CH₃ of **1**), 2.66–2.78 (m, 2H, H β of His), 3.02–3.26 (m, 2H, H β of Asn), 3.71–3.93 (m, 4H, H α of Gly, H β of Ser), 4.14 (d, 1H, *J* = 10.4 Hz, CH₂ of **1**), 4.21–4.54 (m, 6H, H α of Ser, CH₂ of Fmoc, CH of Fmoc, H α of Fmoc-Ala, H α of Ala), 4.57–4.67 (m, 2H, H α of His, H α of Asn), 4.72 (d, 1H, *J* = 10.4 Hz, CH₂ of **1**), 7.21 (s, 1H, H δ of His), 7.31–7.46 (m, 4H, Arom), 7.62–7.70 (m, 2H, Arom), 7.82–7.88 (m, 2H, Arom), 8.44 (s, 1H, H ϵ of His). ¹³C NMR (75 MHz, CD₃CN/D₂O (95:5)) δ (ppm): 15.8, 16.6 (CH₃ of Fmoc-Ala, CH₃ of Ala), 22.5 (CH₃ of **1**), 26.2 (C β of Asn), 36.1 (C β of His), 42.0 (C α of Gly), 46.9 (CH of Fmoc), 49.7 (C α of Fmoc-Ala, C α of Ala), 50.4 (C α of His), 52.2 (CH₂ of **1**), 52.4 (C α of Asn), 55.9 (C α of Ser), 61.3 (C β of Ser), 66.9 (CH₂ of Fmoc), 85.9 (C α of **1**), 120.0, 125.3, 127.2, 127.8, 128.7, 133.5, 141.1, 143.9, 144.0, (Arom), 156.4 (CO of Fmoc), 168.2, 170.4, 170.8, 171.0, 171.8, 172.5, 172.6 (CO).

Peptide **14** (Fmoc-Ala-1-Ala-His-Asn-Thr-Gly-NH₂) was synthesized and purified (method C) following the general protocol described above in a 40% global yield. Semi-preparative HPLC retention time: 23.70 min. HRMS (ESI) *m/z* = 954.3418, calculated for C₄₁H₅₂N₁₁O₁₄S [M+H]⁺ = 954.3410. ¹H NMR (300 MHz, CD₃CN/D₂O (95:5)) δ (ppm): 1.13 (d, 3H, *J* = 6.6 Hz, CH₃ of Thr), 1.29–1.41 (m, 6H, CH₃ of Fmoc-Ala, CH₃ of Ala), 1.79 (s, 3H, CH₃ of **1**), 2.68–2.79 (m, 2H, H β of His), 3.02–3.30 (m, 2H, H β of Asn), 3.69–3.93 (m, 2H, H α of Gly), 4.15 (d, 1H, *J* = 10.3 Hz, CH₂ of **1**), 4.21–4.55 (m, 7H, H β of Thr, H α of Thr, CH₂ of Fmoc, CH of Fmoc, H α of Fmoc-Ala, H α of Ala), 4.61–4.79 (m, 3H, H α of His, H α of Asn, CH₂ of **1**), 7.22 (s, 1H, H δ of His), 7.28–7.49 (m, 4H, Arom), 7.61–7.72 (m, 2H, Arom), 7.78–7.89 (m, 2H, Arom), 8.46 (s, 1H, H ϵ of His). ¹³C NMR (75 MHz, CD₃CN/D₂O (95:5)) δ (ppm): 15.9, 16.6 (CH₃ of Fmoc-Ala, CH₃ of Ala), 18.9 (CH₃ of Thr), 22.5 (CH₃ of **1**), 26.3 (C β of Asn), 36.1 (C β of His), 42.1 (C α of Gly), 46.9 (CH of Fmoc), 49.7, 49.8 (C α of Fmoc-Ala, C α of Ala), 50.6 (C α of His), 52.2 (CH₂ of **1**), 52.5 (C α of Asn), 59.1 (C α of Thr), 66.8 (CH₂ of Fmoc, C β of Thr), 85.9 (C α of **1**), 120.0, 125.3, 127.2, 127.8, 128.8, 133.6, 141.1, 143.9, 144.0, (Arom), 156.3 (CO of Fmoc), 168.1, 170.5, 170.8, 171.3, 171.9, 172.6, 173.2 (CO).

Peptide **15** (Fmoc-Ala-1-Ala-His-Asn-Lys-Gly-NH₂) was synthesized and purified (method C) following the general protocol described above in a 49% global yield. Semi-preparative HPLC retention time: 20.67 min. HRMS (ESI) *m/z* = 981.3849, calculated for C₄₃H₅₇N₁₂O₁₃S [M+H]⁺ = 981.3883. ¹H NMR (300 MHz, CD₃CN/D₂O (95:5)) δ (ppm): 1.25–1.48 (m, 8H, CH₃ of Fmoc-Ala, CH₃ of Ala, H γ of Lys), 1.52–1.91 (m, 7H, CH₃ of **1**, H δ of Lys, H β of Lys), 2.65–2.76 (m, 2H, H β of His), 2.86–2.94 (m, 2H, H ϵ of Lys), 3.07–3.30 (m, 2H, H β of Asn), 3.70–3.88 (m, 2H, H α of Gly), 4.15 (d, 1H, *J* = 10.4 Hz, CH₂ of **1**), 4.18–4.53 (m, 6H, H α of Lys, CH₂ of Fmoc, H α of Fmoc-Ala, H α of Ala), 4.56–

4.67 (m, 2H, H α of His, H α of Asn), 4.72 (d, 1H, J = 10.4 Hz, CH $_2$ of **1**), 7.23 (s, 1H, H δ of His), 7.29–7.50 (m, 4H, Arom), 7.62–7.71 (m, 2H, Arom), 7.78–7.89 (m, 2H, Arom), 8.48 (s, 1H, H ϵ of His). ^{13}C NMR (75 MHz, CD $_3$ CN/D $_2$ O (95:5)) δ (ppm): 15.8, 16.6 (CH $_3$ of Fmoc-Ala, CH $_3$ Ala), 21.9 (C γ of Lys), 22.5 (CH $_3$ of **1**), 26.0 (C β of Asn, C δ of Lys), 30.0 (C β of Lys), 35.9 (C β of His), 39.2 (C ϵ of Lys), 42.0 (C α of Gly), 46.9 (CH of Fmoc), 49.8 (C α of Fmoc-Ala, C α of Ala), 50.5 (C α of His), 52.2 (CH $_2$ of **1**), 52.6 (C α of Asn), 53.3 (C α of Lys), 66.9 (CH $_2$ of Fmoc), 86.0 (C α of **1**), 120.0, 125.3, 127.2, 127.8, 128.9, 133.5, 141.1, 143.9, (Arom), 156.4 (CO of Fmoc), 168.2, 170.7, 170.9, 171.9, 172.6, 172.7, 172.9, 173.4 (CO).

Peptide **16** (Fmoc-Ala-**1**-Ala-His-Cys-Asn-Gly-NH $_2$) was synthesized and purified (method C) following the general protocol described above in a 30% global yield. Semi-preparative HPLC retention time: 26.00 min. HRMS (ESI) m/z = 956.3029, calculated for C $_{40}$ H $_{50}$ N $_{11}$ O $_{13}$ S $_2$ (MH $^+$) = 956.3025. ^1H NMR (300 MHz, CD $_3$ CN/D $_2$ O (95:5)) δ (ppm): 1.29–1.40 (m, 6H, CH $_3$ of Fmoc-Ala, CH $_3$ of Ala), 1.79 (s, 3H, CH $_3$ of **1**), 2.73–2.76 (m, 2H, H β of His), 2.83–2.89 (m, 2H, H β of Cys), 3.03–3.30 (m, 2H, H β of Asn), 3.69–3.90 (m, 2H, H α of Gly), 4.14 (d, 1H, J = 10.4 Hz, CH $_2$ of **1**), 4.20–4.54 (m, 6H, H α of Cys, CH $_2$ of Fmoc, CH of Fmoc, H α of Fmoc-Ala, H α of Ala), 4.62–4.75 (m, 3H, H α of His, H α of Asn, CH $_2$ of **1**), 7.23 (s, 1H, H δ of His), 7.30–7.46 (m, 4H, Arom), 7.57–7.70 (m, 2H, Arom), 7.78–7.89 (m, 2H, Arom), 8.47 (s, 1H, H ϵ of His). ^{13}C NMR (75 MHz, CD $_3$ CN/D $_2$ O (95:5)) δ (ppm): 15.9, 16.7 (CH $_3$ of Fmoc-Ala, CH $_3$ Ala), 22.5 (CH $_3$ of **1**), 25.2 (C β of Cys), 26.5 (C β of Asn), 36.1 (C β of His), 42.2 (C α of Gly), 46.9 (CH of Fmoc), 49.8 (CH $_3$ of Fmoc-Ala, CH $_3$ Ala), 50.3 (C α of His), 52.3 (CH $_2$ of **1**, C α of Asn), 56.0 (C α of Cys), 66.8 (CH $_2$ of Fmoc), 86.0 (C α of **1**), 120.0, 125.3, 127.2, 127.8, 129.0, 133.6, 141.1, 143.9, 144.0 (Arom), 156.2 (CO of Fmoc), 168.1, 168.3, 170.5, 170.8, 171.3, 172.3, 173.5 (CO).

Peptide **17** (Fmoc-Ala-**1**-Ala-His-Asn-Gly-Cys-NH $_2$) was synthesized and purified (method C) following the general protocol described above in a 31% global yield. Semi-preparative HPLC retention time: 26.14 min. HRMS (ESI) m/z = 956.3006, calculated for C $_{40}$ H $_{50}$ N $_{11}$ O $_{13}$ S $_2$ [M+H] $^+$ = 956.3025. ^1H NMR (300 MHz, CD $_3$ CN/D $_2$ O (95:5)) δ (ppm): 1.20–1.40 (m, 6H, CH $_3$ of Fmoc-Ala, CH $_3$ of Ala), 1.79 (s, 3H, CH $_3$ of **1**), 2.80–2.93 (m, 4H, H β of His, H β of Cys), 3.00–3.27 (m, 2H, H β of Asn), 3.69–3.99 (m, 2H, H α of Gly), 4.15 (d, 1H, J = 10.4 Hz, CH $_2$ of **1**), 4.19–4.52 (m, 6H, H β of Cys, CH $_2$ of Fmoc, CH of Fmoc, Fmoc-Ala, H α of Ala), 4.53–4.65 (m, 2H, H α of His, H α of Asn), 4.73 (d, 1H, J = 10.4 Hz, CH $_2$ of **1**), 7.20 (s, 1H, H δ of His), 7.31–7.50 (m, 4H, Arom), 7.62–7.74 (m, 2H, Arom), 7.81–7.85 (m, 2H, Arom), 8.42 (s, 1H, H ϵ of His). ^{13}C NMR (75 MHz, CD $_3$ CN/D $_2$ O (95:5)) δ (ppm): 15.8, 16.6 (CH $_3$ of Fmoc-Ala, CH $_3$ of Ala), 22.5 (CH $_3$ of **1**), 25.5 (C β of Cys), 26.1 (C β of Asn), 35.9 (C β of His), 42.8 (C α of Gly), 46.9 (CH of Fmoc), 49.8 (CH $_3$ of Fmoc-Ala, CH $_3$ Ala), 50.3 (C α of His), 52.2 (CH $_2$ of **1**, C α of Asn), 55.5 (C α of Cys), 66.9 (CH $_2$ of Fmoc), 85.9 (C α of **1**), 120.0, 125.3, 127.2, 127.8, 128.9, 133.5, 141.1, 143.9 (Arom), 156.4 (CO of Fmoc), 168.2, 170.0, 170.5, 170.8, 172.1, 172.6, 172.9, 173.3 (CO).

Peptide **18** (Fmoc-Ala-**1**-His-Asn-Cys-Gly-NH $_2$) was synthesized and purified (method C) following the general protocol

described above in a 35% global yield. Semi-preparative HPLC retention time: 25.83 min. HRMS (ESI) m/z = 885.2623, calculated for C $_{37}$ H $_{45}$ N $_{10}$ O $_{12}$ S $_2$ [M+H] $^+$ = 885.2654. ^1H NMR (300 MHz, CD $_3$ CN/D $_2$ O (95:5)) δ (ppm): 1.36 (d, 3H, J = 7.0 Hz, CH $_3$ of Ala), 1.69 (s, 3H, CH $_3$ of **1**), 2.71–2.75 (m, 2H, H β of His), 2.87–2.94 (m, 2H, H β of Cys), 3.11–3.43 (m, 2H, H β of Asn), 3.66–3.91 (m, 2H, H α of Gly), 4.10 (d, 1H, J = 10.4 Hz, CH $_2$ of **1**), 4.21–4.49 (m, 5H, H α of Cys, CH $_2$ of Fmoc, CH of Fmoc, H α of Ala), 4.61–4.80 (m, 3H, H α of His, H α of Asn, CH $_2$ of **1**), 7.24 (s, 1H, H δ of His), 7.29–7.53 (m, 4H, Arom), 7.60–7.69 (m, 2H, Arom), 7.79–7.90 (m, 2H, Arom), 8.48 (s, 1H, H ϵ of His). ^{13}C NMR (75 MHz, CD $_3$ CN/D $_2$ O (95:5)) δ (ppm): 15.9 (CH $_3$ of Ala), 22.5 (CH $_3$ of **1**), 25.2 (C β of Cys), 26.1 (C β of Asn), 35.9 (C β of His), 42.1 (C α of Gly), 46.9 (CH of Fmoc), 49.7 (C α of Ala), 50.8 (C α of His), 52.2 (CH $_2$ of **1**), 52.5 (C α of Asn), 55.9 (C α of Cys), 66.8 (CH $_2$ of Fmoc), 85.9 (C α of **1**), 120.0, 125.3, 127.2, 127.8, 129.1, 133.7, 141.1, 143.9, 144.0, (Arom), 156.2 (CO of Fmoc), 168.4, 169.8, 170.2, 170.4, 170.6, 171.7, 172.2, 173.0 (CO).

General protocol for the transacylation reaction. Peptides **12–25** were dissolved in a 24:1 mixture of CD $_3$ CN/D $_2$ O in an NMR tube. A slight excess of triethylamine (1.5–2.0 equiv.) was then added and a ^1H NMR spectrum was immediately acquired. When needed, further spectra were recorded at different times. Conversion values were derived from the ^1H NMR spectra of reaction mixtures.

Peptide **19** (1-Ala-His-Asn-Cys(Fmoc-Ala-S)-Gly-NH $_2$) was quantitatively obtained after treatment of peptide **12** (4 mg, 0.004 mmol) with TEA (1.0 μL , 0.007 mmol) in a 24:1 mixture of CD $_3$ CN/D $_2$ O at room temperature for 5 min. MS (ESI) m/z = 956.3, calculated for C $_{40}$ H $_{50}$ N $_{11}$ O $_{13}$ S $_2$ [M+H] $^+$ = 956.3025. ^1H NMR (300 MHz, CD $_3$ CN/D $_2$ O (95:5)) δ (ppm): 1.25–1.35 (m, 6H, CH $_3$ of Fmoc-Ala, CH $_3$ of Ala), 1.62 (s, 3H, CH $_3$ of **1**), 2.50–2.60 (m, 4H, H β of His, H β of Cys), 3.17–3.30 (m, 1H, H β of Asn), 3.35–3.48 (m, 2H, H β of Asn, CH $_2$ of **1**), 3.62 (d, 1H, J = 17.0 Hz, H α of Gly), 3.81 (d, 1H, J = 17.0 Hz, H α of Gly), 4.04 (d, 1H, J = 12.7 Hz, CH $_2$ of **1**), 4.19–4.44 (m, 7H, H α of Cys, CH $_2$ of Fmoc, CH of Fmoc, H α of Fmoc-Ala, H α of Ala, H α of Asn), 4.49–4.56 (m, 1H, H α of His), 6.84 (s, 1H, H δ of His), 7.29–7.46 (m, 4H, Arom.), 7.53 (s, 1H, H ϵ of His), 7.62–7.72 (m, 2H, Arom.), 7.77–7.87 (m, 2H, Arom.).

Peptide **20** (1-Ala-His-Cys(Fmoc-Ala-S)-Asn-Gly-NH $_2$) was quantitatively obtained after treatment of peptide **16** (5 mg, 0.005 mmol) with TEA (1.0 μL , 0.007 mmol) in a 24:1 mixture of CD $_3$ CN/D $_2$ O at room temperature for 5 min. MS (ESI) m/z = 956.3, calculated for C $_{40}$ H $_{50}$ N $_{11}$ O $_{13}$ S $_2$ [M+H] $^+$ = 956.3025. ^1H NMR (300 MHz, CD $_3$ CN/D $_2$ O (95:5)) δ (ppm): 1.24–1.39 (m, 6H, CH $_3$ of Fmoc-Ala, CH $_3$ of Ala), 1.61 (s, 3H, CH $_3$ of **1**), 2.60–2.81 (m, 4H, H β of His, H β of Cys), 3.08–3.34 (m, 2H, H β of Asn), 3.43 (d, 1H, J = 12.7 Hz, CH $_2$ of **1**), 3.68 (d, 1H, J = 17.1 Hz, H α of Gly), 3.85 (d, 1H, J = 17.1 Hz, H α of Gly), 4.01 (d, 1H, J = 12.7 Hz, CH $_2$ of **1**), 4.19–4.43 (m, 7H, H α of Cys, CH $_2$ of Fmoc, CH of Fmoc, H α of Fmoc-Ala, H α of Ala, H α of Asn), 4.53–4.60 (m, 1H, H α of His), 6.84 (s, 1H, H δ of His), 7.29–7.44 (m, 4H, Arom.), 7.56 (s, 1H, H ϵ of His), 7.61–7.70 (m, 2H, Arom.), 7.77–7.87 (m, 2H, Arom.).

Peptide **21** (1-Ala-His-Asn-Gly-Cys(Fmoc-Ala-S)-NH $_2$) was obtained (70% conversion) after treatment of peptide **17** (4 mg,

0.004 mmol) with TEA (1.0 μ L, 0.007 mmol) in a 24:1 mixture of CD₃CN/D₂O at room temperature for 5 min. MS (ESI) m/z = 956.3, calculated for C₄₀H₅₀N₁₁O₁₃S₂ [M+H]⁺ = 956.3025. ¹H NMR (300 MHz, CD₃CN/D₂O (95:5)) δ (ppm): 1.21-1.37 (m, 6H, CH₃ of Fmoc-Ala, CH₃ of Ala), 1.57 (s, 3H, CH₃ of **1**), 2.80-3.18 (m, 5H, H β of His, H β of Cys, H β of Asn), 3.25-3.49 (m, 2H, H β of Asn, CH₂ of **1**), 3.68 (d, 1H, J = 16.9 Hz, H α of Gly), 3.85 (d, 1H, J = 17.0 Hz, H α of Gly), 3.92 (d, 1H, J = 12.1 Hz, CH₂ of **1**), 4.15-4.50 (m, 7H, H α of Cys, CH₂ of Fmoc, CH of Fmoc, H α of Fmoc-Ala, H α of Ala, H α of Asn), 4.51-4.58 (m, 1H, H α of His), 6.86 (s, 1H, H δ of His), 7.28-7.46 (m, 4H, Arom.), 7.53 (s, 1H, H ϵ of His), 7.62-7.72 (m, 2H, Arom.), 7.77-7.87 (m, 2H, Arom.).

Peptide **22** (1-His-Asn-Cys(Fmoc-Ala-S)-Gly-NH₂) was quantitatively obtained after treatment of peptide **18** (6 mg, 0.006 mmol) with TEA (1.0 μ L, 0.007 mmol) in a 24:1 mixture of CD₃CN/D₂O at room temperature for 5 min. MS (ESI) m/z = 885.2, calculated for C₃₇H₄₅N₁₀O₁₂S₂ [M+H]⁺ = 885.2654. ¹H NMR (300 MHz, CD₃CN/D₂O (95:5)) δ (ppm): 1.35 (d, 3H, J = 7.2 Hz, CH₃ of Fmoc-Ala), 1.58 (s, 3H, CH₃ of **1**), 2.80-2.90 (m, 2H, H β of His), 3.05-3.14 (m, 2H, H β of Cys), 3.18-3.49 (m, 3H, H β of Asn, CH₂ of **1**), 3.67 (d, 1H, J = 17.0 Hz, H α of Gly), 3.83 (d, 1H, J = 17.0 Hz, H α of Gly), 4.01 (d, 1H, J = 12.7 Hz, CH₂ of **1**), 4.20-4.59 (m, 7H, H α of Cys, CH₂ of Fmoc, CH of Fmoc, H α of Fmoc-Ala, H α of Asn, H α of His), 6.91 (s, 1H, H δ of His), 7.30-7.48 (m, 4H, Arom.), 7.58 (s, 1H, H ϵ of His), 7.64-7.76 (m, 2H, Arom.), 7.79-7.90 (m, 2H, Arom.).

Peptide **23** (1-Ala-His-Asn-Ser(Fmoc-Ala-S)-Gly-NH₂) was obtained (90% conversion) after treatment of peptide **13** (5 mg, 0.005 mmol) with TEA (1.0 μ L, 0.007 mmol) in a 24:1 mixture of CD₃CN/D₂O at room temperature for 3 h. MS (ESI) m/z = 940.3, calculated for C₄₀H₅₀N₁₁O₁₄S [M+H]⁺ = 940.3254. ¹H NMR (300 MHz, CD₃CN/D₂O (95:5)) δ (ppm): 1.26-1.38 (m, 6H, CH₃ of Fmoc-Ala, CH₃ of Ala), 1.62 (s, 3H, CH₃ of **1**), 2.95-3.06 (m, 2H, H β of His), 3.28-3.49 (m, 2H, H β of Asn), 3.64-4.09 (m, 4H, CH₂ of **1**, H α of Gly), 4.16-4.68 (m, 9H, H β of Ser, CH₂ of Fmoc, CH of Fmoc, H α of Fmoc-Ala, H α of Ala, H α of Asn, H α of His), 5.09-5.22 (m, 1H, H α Ser), 6.87 (s, 1H, H δ of His), 7.31-7.49 (m, 4H, Arom.), 7.54 (s, 1H, H ϵ of His), 7.62-7.72 (m, 2H, Arom.), 7.79-7.87 (m, 2H, Arom.).

Peptide **24** (1-Ala-His-Asn-Thr(Fmoc-Ala-S)-Gly-NH₂) was obtained (45% conversion) after treatment of peptide **14** (5 mg, 0.005 mmol) with TEA (1.0 μ L, 0.007 mmol) in a 24:1 mixture of CD₃CN/D₂O at room temperature for 3 h. MS (ESI) m/z = 954.3, calculated for C₄₁H₅₂N₁₁O₁₄S [M+H]⁺ = 954.3410. ¹H NMR (300 MHz, CD₃CN/D₂O (95:5)) δ (ppm): 1.10-1.20 (m, 3H, CH₃ of Thr), 1.27-1.48 (m, 6H, CH₃ of Fmoc-Ala, CH₃ of Ala), 1.63 (s, 3H, CH₃ of **1**), 2.95-3.21 (m, 4H, H β of His, H β of Asn), 3.46 (d, 1H, J = 12.7 Hz, CH₂ of **1**), 3.71 (d, 1H, J = 17.0 Hz, H α of Gly), 3.83 (d, 1H, J = 17.0 Hz, H α of Gly), 4.03 (d, 1H, J = 12.7 Hz, CH₂ of **1**), 4.14-4.68 (m, 9H, H β of Thr, CH₂ of Fmoc, CH of Fmoc, H α of Fmoc-Ala, H α of Ala, H α of Asn, H α of His, H α Ser), 6.86 (s, 1H, H δ of His), 7.31-7.48 (m, 4H, Arom.), 7.54 (s, 1H, H ϵ of His), 7.63-7.74 (m, 2H, Arom.), 7.80-7.89 (m, 2H, Arom.).

Peptide **25** (1-Ala-His-Asn-Lys(Fmoc-Ala-S)-Gly-NH₂) was quantitatively obtained after treatment of peptide **15** (5 mg,

0.005 mmol) with TEA (1.0 μ L, 0.007 mmol) in a 24:1 mixture of CD₃CN/D₂O at room temperature for 5 min. MS (ESI) m/z = 981.3, calculated for C₄₃H₅₇N₁₂O₁₃S [M+H]⁺ = 981.3883. ¹H NMR (300 MHz, CD₃CN/D₂O (95:5)) δ (ppm): 1.25-1.51 (m, 8H, CH₃ of Fmoc-Ala, CH₃ of Ala, H γ of Lys), 1.53-1.91 (m, 7H, CH₃ of **1**, H δ of Lys, H β of Lys), 2.68-2.80 (m, 2H, H β of His), 2.86-2.99 (m, 2H, H ϵ of Lys), 3.07-3.28 (m, 2H, H β of Asn), 3.47 (d, 1H, J = 12.8 Hz, CH₂ of **1**), 3.68-3.85 (m, 2H, H α of Gly), 4.00-4.70 (m, 9H, CH₂ of **1**, H α of Lys, CH₂ of Fmoc, CH of Fmoc, H α of Fmoc-Ala, H α of Ala, H α of His, H α of Asn), 6.86 (s, 1H, H δ of His), 7.31-7.47 (m, 4H, Arom.), 7.56 (s, 1H, H ϵ of His), 7.62-7.73 (m, 2H, Arom.), 7.80-7.89 (m, 2H, Arom.).

Peptide **26** (1-Ala-His-Asn-Cys-Gly-NH₂) was synthesized and purified (method D) following the general protocol described above in a 72% global yield. Semi-preparative HPLC retention time: 17.28 min. HRMS (ESI) m/z = 663.1681, calculated for C₂₂H₃₅N₁₀O₁₀S₂ [M+H]⁺ = 663.1974. ¹H NMR (300 MHz, CD₃CN/D₂O (95:5)) δ (ppm): 1.35 (d, 3H, J = 7.2 Hz, CH₃ of Ala), 1.66 (s, 3H, CH₃ of **1**), 2.72-2.81 (m, 2H, H β of His), 2.87-2.98 (m, 2H, H β of Cys), 3.03-3.33 (m, 2H, H β of Asn), 3.48 (d, 1H, J = 12.7 Hz, CH₂ of **1**), 3.69-3.91 (m, 2H, H α of Gly), 3.99 (d, 1H, J = 12.7 Hz, CH₂ of **1**), 4.29-4.37 (m, 1H, H α of Ala), 4.41-4.50 (m, 1H, H α of Cys), 4.58-4.72 (m, 2H, H α of His, H α of Asn), 7.28 (s, 1H, H δ of His), 8.51 (s, 1H, H ϵ of His). ¹³C NMR (75 MHz, CD₃CN/D₂O (95:5)) δ (ppm): 16.6 (CH₃ of Ala), 22.4 (CH₃ of **1**), 25.3 (C β of Cys), 26.4 (C β of Asn), 36.0 (C β of His), 42.1 (C α of Gly), 49.9 (C α of Ala), 50.7 (C α of His), 51.8 (CH₂ of **1**), 52.4 (C α of Asn), 56.0 (C α of Cys), 90.2 (C α of **1**), 128.9, 133.6 (Arom), 170.5, 170.6, 171.7, 172.2, 173.1 (CO).

pH-Controlled release analysis. Peptide **10** was dissolved in PBS (50 mM, pH 7.4 or 6.3, final peptide concentration 100 μ M) and incubated at 25 °C. Aliquots were taken periodically and analysed by RP-HPLC using a Waters 1525 HPLC system equipped with a diode array detector (210/254 nm) using the analytical column Phenomenex Luna® C18(2) column (5 μ m, 250 mm $\times$ 4.6 mm) with a flow rate of 1.0 mL/min (linear gradient 5-95% B over 30 min). Retention times: 21.1 (peptide **10**) and 18.8 (peptide **2**) min. Chlorambucil was decomposed into various unidentified by-products.

Conflicts of interest

There are no conflicts to declare.

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Notes and references

‡ The details on optimizing this key synthetic step, which is far from trivial, will be reported elsewhere.

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