Solid-Phase Strategies for the Assembly of Template-Based Protein Mimetics[1]

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The template concept for overcoming the protein folding problem in protein de novo design has recently been extended for mimicking essential structural and functional features of proteins. Due to progress in synthetic methodologies, template-assembled constructs now become accessible for efficient solid-phase strategies, as exemplified for three prototype protein mimetics. As a key step, regioselectively addressable functional template molecules are prepared by convergent methods and immobilized on solid support (**2b** in Figure 1). Up to four orthogonal amino protecting groups, i.e., Fmoc, Alloc, Dde, and pNZ, allow for the step-by-step synthesis of protein surface mimetic **3**, template-assembled synthetic protein (TASP) **4**, and the convergent synthesis of receptor mimetic **5** in good overall yield. The elaborated protocols extend today's potential of solid-phase peptide synthesis for the construction of molecules of high structural complexity and open the way to template-based protein mimetics by combinatorial techniques.

Introduction

Protein design and mimicry plays a crucial role in deciphering the complex interplay between structure and function in native systems and constitutes a basis for constructing molecules of therapeutical use.[2] Some years ago, we introduced the concept of template-assisted protein design and mimicry for overcoming some intrinsic problems in protein de novo design.[3] As a key feature of this versatile approach, termed template-assembled synthetic proteins (TASP), a topological template, e.g., cyclic peptides, directs covalently bound peptide blocks or functional groups to well-defined spatial arrangements.[4] So far, a series of TASP molecules exhibiting a variety of structural (e.g., parallel and antiparallel 4-helix bundles[5] and $\beta\beta\alpha$-motifs[6]) and functional (e.g., membrane channel forming,[7] heme binding,[8] or surface mimetics[9]) features of proteins have been described.

Due to recent progress in the methodology of synthesis, the full potential of the template concept can be explored now. In particular, the availability of an increasing number of orthogonal protection techniques[10] together with improved methodologies in solid-phase peptide synthesis allows access of TASP molecules of increasing structural complexity.[11] So far, TASP molecules were constructed by stepwise solid-phase peptide synthesis[5a,7] (SPPS), fragment condensation,[12] or chemoselective ligation procedures[13] applying convergent strategies. Here, we extend the present state of the art of TASP synthesis in constructing resin-bound cyclic peptides exhibiting up to four orthogonally protected attachment sites.[9,11] The

(1) Abbreviations: AAA, amino acid analysis; Alloc, allyloxycarbonyl; Dde, 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl; DIEA, *N*,*N*-diisopropylethylamine; ES-MS, electrospray mass spectroscopy; Fmoc, 9-(fluorenylmethyloxycarbonyl); HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HBTU, *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; NMM, *N*-methylmorpholine; PAL, 5-(4-(aminomethyl)-3,5-dimethoxyphenoxy) valeric acid; PEG-PS, poly(ethylene glycol)-polystyrene graft resin support; pNZ, *p*-nitrobenzyloxycarbonyl; PS, polystyrene resin support; PyAOP, (7-azabenzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate; PyBOP, (benzotriazol-1-yloxy)tris (pyrrolidino)phosphonium hexafluorophosphate; RAFT, regioselectively addressable functionalized template; RP-HPLC, reversed-phase high performance liquid chromatography; SASRIN, 2-methoxy-4-alkoxybenzyl alcohol resin; SPPS, solid-phase peptide synthesis; Suc, succinyl; TASP, template-assembled synthetic protein; Trt, triphenylmethyl (trityl); XAL, 9-amino-xanthen-3-yloxymethyl; Xaa, Xbb, amino acid. Amino acid symbols denote the L-configuration.

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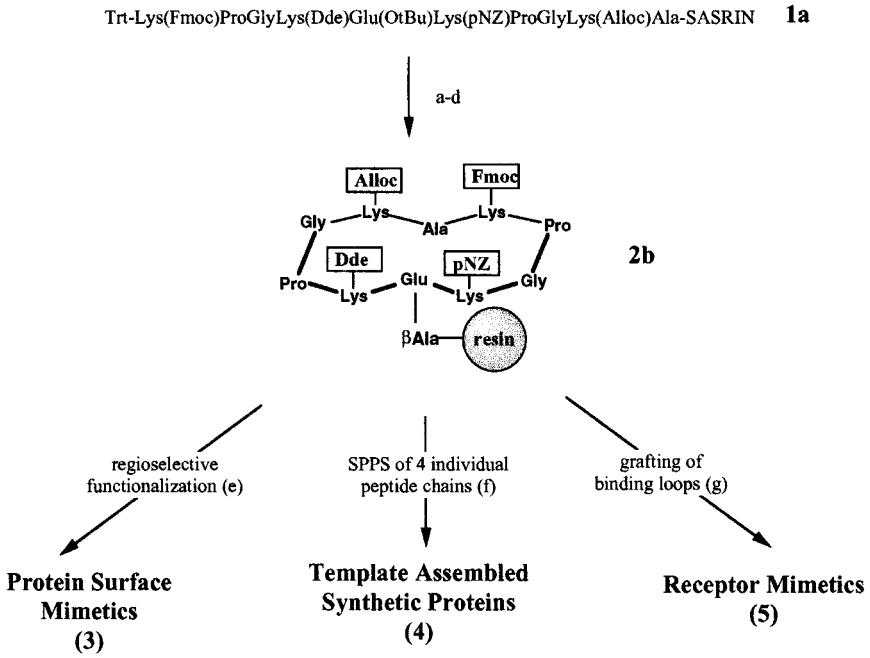

Figure 1. Synthesis scheme for the assembly of TASP molecules on solid phase: (a) stepwise synthesis of the linear template (**1a**); (b) simultaneous removal of the N-terminal Trt group and cleavage from the resin, subsequent cyclization in solution; (c) selective deprotection of the γ-carboxylic group of Glu; (d) attachment to the resin. The four orthogonal protecting groups of the lysine side chains can be removed sequentially and thus allow for the regioselective attachment of functional groups (e), peptides (f), or binding loops (g), making use of the advantages of solid phase peptide synthesis.

resulting immobilized regioselectively addressable functionalized templates[14] (RAFT **2b** in Figure 1) are used as scaffolds for the efficient solid-phase synthesis of prototype protein surface mimetics, TASP molecules disposing up to four different peptide blocks, and receptor mimetics featuring regioselectively attached ligand binding loops (Figure 1).[15]

Results and Discussion

Detailed conformational studies have shown that cyclic decapeptides of general sequence c[XaaXbbXaaProGly]$_2$ adopt a preferred β-sheeted conformation disposing the side chains of Xaa and Xbb to opposite faces of the template plane[16] (Xaa = Lys; Xbb = Ala, Glu in Figure 2). Consequently, in using one Xbb = Glu, the carboxylic group of Glu serves as a linker to fix the template molecule to the resin in a favorable orientation for the subsequent solid-phase assembly of TASP molecules. As side chain protecting groups of the four lysine residues (Xaa), the use of Alloc (cleavable by Pd^0),[17] Fmoc (piperidine),[18] pNZ ($SnCl_2$),[19] and Dde (hydrazine)[20] results in an orthogonal protection scheme for the regioselective functionalization of the polymer-bound template.

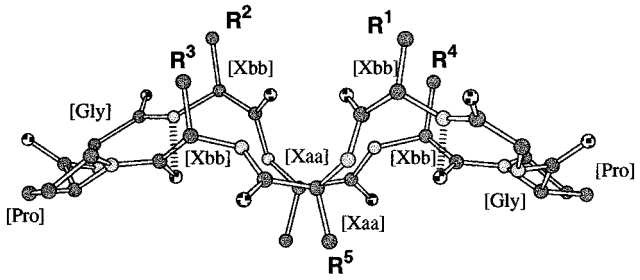

Figure 2. Molecular modeling picture of a template in a low energy conformation with R^1−R^4 as peptide attachment sites oriented above and R^5 below the plane of the template for the fixation onto the resin.

The synthesis of **2b** was achieved as follows. The linear sequence (**1**, Table 1) was assembled by standard SPPS[21] on the superacid-sensitive SASRIN resin using Trt-Lys-(Fmoc)OH as the N-terminal amino acid. Treatment of the resin with dilute acid allows simultaneous release of the linear-protected peptide and deprotection of the N-terminal moiety. Desalting by solid-phase extraction and lyophilization afforded the linear template **1** (Figure 1). Cyclization under high dilution in DMF using HATU as coupling reagent and DIEA as base proceeded rapidly in high yield and purity.[22] Subsequently, the carboxylic

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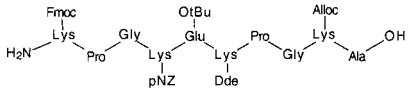
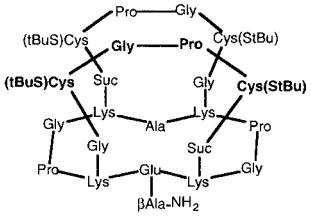
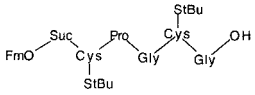
(22) Studies on this cyclization reaction indicated that HATU, PyBOP, and PyAOP are essentially equally efficient in terms of rapidity (total conversion in less than 10 min) and purity (95% by RP-HPLC).

Table 1. Template and Side Chain Peptide Sequences for the Synthesis of TASP Compounds

Compounds	Sequences
1	
2a	R^1 = Fmoc, R^2 = Alloc, R^3 = Dde, R^4 = pNZ, R^5 = OH
2b	R^1 = Fmoc, R^2 = Alloc, R^3 = Dde, R^4 = pNZ, R^5 = βAla-resin
3	R^1 = Ac-Ser, R^2 = Ac-Asp, R^3 = Ac-Glu, R^4 = Ac-His, R^5 = βAla-NH_2
4	R^1 = Ac-Ser-Thr-Val, R^2 = Ac-Asp-Thr-Val, R^3 = Ac-Glu-Thr-Val, R^4 = Ac-His-Thr-Val, R^5 = βAla-NH_2
5	
6	

function of glutamic acid was deprotected by treatment of the cyclic peptide with TFA:CH_2Cl_2 1:1 to give the COOH-containing template **2a** with the Lys side chains still protected.

The immobilization reaction was performed via amide bond formation. To prevent premature loss of the Fmoc protecting group from **2a** under extended exposure to strong base, collidine was preferred over DIEA. PyAOP then proved to be more efficient as an activating reagent compared to PyBOP, HATU, and HBTU. Despite the sterically demanding template molecule, relatively high subsitution levels were achieved independent of the physical and chemical nature of the polymer resin.[23] For example, using high loaded polystyrene-based resin H_2N-βAla-XAL-PS[24] (230 μmol/g) and 0.6 equiv of **2a**, 70% of the template was covalently linked to the polymer after 13 h. Similarly, an excess of **2a** (1.7 equiv) resulted in comparable substitution levels (80−100 μmol/g) under identical reaction conditions applying PEG-PS resin of lower capacity (118 μmol/g).

To evaluate the versatility of the orthogonally protected RAFT **2b** for the solid-phase synthesis of a variety of protein mimetics, the four lysine side chains were sequentially deprotected and functionalized to yield the prototype protein surface mimetic **3** and TASP **4** (Figure 1, Table 1). To this end, Fmoc was removed first in order to ensure compatibility with the Fmoc-based solid-phase strategies for the side chain peptide assembly; in addition, N-ϵ to N-ϵ migration of Dde was prevented using DBU:DMF 2:98 instead of piperidine:DMF 1:4.[25] Dde was removed with hydrazine/allyl alcohol/DMF 1/3/46, thus providing complete orthogonality to Alloc.[26] The latter was removed with $Pd(PPh_3)_4$ in CH_2Cl_2 using $PhSiH_3$ as scavenger.[27] Orthogonality of pNZ to Boc, Fmoc, Alloc, and Dde was achieved by using $SnCl_2$ (2 M in DMF) as reagent.

The sequential functionalization of the lysine side chains was performed stepwise according to SPPS procedures, using PyBOP and DIEA. Release from the resin and final deprotection afforded TASP **3** and **4** in good yield and purity (Figure 3). After HPLC purification, the chemical integrity of TASP molecule **3** and **4** was confirmed by ES-MS, analytical HPLC, and NMR spectroscopies.

Although the branched architecture of TASP could lead to coupling difficulties associated with interchain clustering,[28] the grafting of the amino acid derivatives to the lysine side chains of the polymer-bound template proceeded quantitatively. Even sterically demanding coupling reactions did not give rise to incomplete coupling steps as monitored by UV measurements of the Fmoc release.

Critical steps were the removal of N-ϵ-lysine protecting groups. However, even in the presence of bulky neighboring groups, the removal of N-ϵ-lysine protecting groups proceeded to completion. The prototype molecules **3** and **4** were obtained in comparable overall yields using either conventional polystyrene- or PEG-PS-based resins. However, the impact of a more hydrophilic resin could be of utmost importance in applying chemoselective ligation techniques.

The scope of the elaborated strategies was further extended by the convergent assembly of receptor mimetic **5**, featuring two regioselectively attached peptide loops derived from the metal binding loops of the electron-carrier protein rubredoxin.[29]

The peptide fragment **6**, bearing selectively accessible C- and N-terminal carboxylic groups, was assembled by Fmoc-based SPPS on the superacid sensitive SASRIN resin, using FmOSucOH for the last coupling in order to introduce the protected N-terminal carboxylic group. This peptide fragment was grafted in two copies onto the immobilized RAFT **2b** as follows. The first loop was regioselectively attached (4 equiv of **6**, PyAOP, and collidine) and cyclized (PyAOP and DIEA) after removing, respectively, Fmoc and Alloc on the template attachment sites. Similarly, the second loop was grafted to the template upon the successive cleavages of Dde and pNZ. Regioselectivity in the intramolecular loop cyclizations was enabled by the OFm protecting group of peptide

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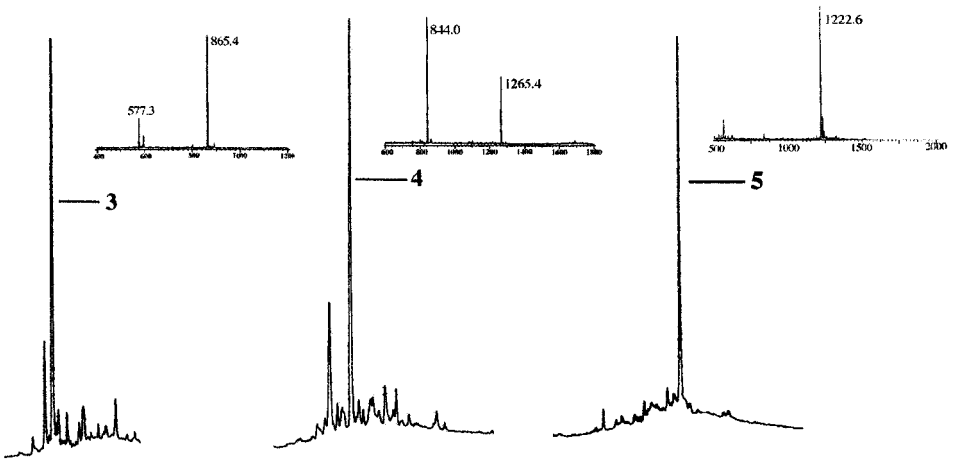

Figure 3. Analytical RP-HPLC chromatograms of the crude products (C_{18}, 214 nm, solvent B 0.09% TFA in 90% MeCN, solvent A 0.09% TFA): TASP **3** (t_R = 21.31 min, 5−20% B in 30 min); TASP **4** (t_R = 14.66 min, 15−30% B in 30 min), and 2Loops-TASP **5** (t_R = 22.50 min, 0−100% B in 30 min). Inserts: ES-MS of **3**, **4**, and **5**.

fragment **6**, removed by piperidine:DMF 1:4 prior to the cyclization reactions. Release from the resin afforded TASP **5** in good yield and purity. After HPLC purification, the chemical integrity of the 2-loop TASP molecule **5** was confirmed by ES-MS and analytical HPLC.[30]

As observed earlier, the cyclization reaction of the loops on the immobilized template molecule proceeded without any observable side reaction (e.g., dimerization, oligomerization), as monitored by analytical HPLC of crude TASP **5** (Figure 3). In contrast to solution methods, the extensive handling of orthogonal protecting groups is profoundly facilitated by applying solid-phase procedures. Most notably, due to the orthogonality of the chosen protecting groups, a number of alternative deprotection protocols seems to be feasible, depending on the target structure.

Conclusion

In conclusion, the elaborated protocols extend the present scope of solid-phase peptide synthesis and allow for the efficient and rapid access to template-based protein mimetics of high structural diversity. As demonstrated for the example of prototype TASP molecules, the immobilized regioselectively addressable template **2b** can be readily transformed to polybranched and polycyclic peptide architectures developed as putative functional mimetics of proteins. Due to the chemo- and regioselective access of four attachment sites, these immobilized templates represent versatile tools for combinatorial approaches in protein design and mimicry.

Experimental Section

Protected amino acids were purchased from Calbiochem-Novabiochem AG (Läufelfingen, Switzerland) and Isochem (Strasbourg, France) or synthesized (Fmoc-Lys(pNZ)OH and Trt-Lys(Fmoc)OH) according to published procedures. Fmoc-Ala-SASRIN resin was obtained from BACHEM Feinchemikalien AG (Bubendorf, Switzerland), Fmoc-XAL-PEG-PS resin was from PerSeptive Biosystems GmbH (Hamburg, Germany), and Fmoc-XAL-PS resin was from Calbiochem-Novabiochem. PyAOP and HATU were purchased from PerSeptive Biosystems, PyBOP from Calbiochem-Novabiochem; other reagents and solvents were from Fluka (Buchs, Switzerland). RP-HPLC spectra were run on Waters equipment using columns packed with Vydac Nucleosil 300 Å 5 mm C_{18} particles. The analytical column (250 × 4.6 mm) was operated at 1 mL/min and the preparative column (250 × 21 mm) was at 18 mL/min, with UV monitoring at 214 or 280 nm. Solvent B consisted of 0.09% TFA in 90% MeCN, and solvent A consisted of 0.09% TFA. Solid-phase extractions were carried out using Sep-Pak Vac cartridge tC_{18} 35 cm^3 from Waters (Milford, Massachusetts). Mass spectra were obtained by electron spray ionization (ES-MS) on a Finnigan MAT SSQ 710C. NMR spectra were recorded at 400 MHz using a Bruker ARX spectrometer at 300 K. Samples of around 5−8 mg were dissolved in 0.4−0.5 mL of DMSO-d_6. Chemical shifts were calibrated on the solvent isotopic impurity (2.49 ppm at 300 K). 2D experiments were typically acquired using 2K × 512 matrixes over a 4000 Hz sweep width in both dimensions. Quadrature detection in the indirect dimension was achieved by using the TPPI procedure.[31] Scalar connectivities were recovered from 2D TOCSY[32] and 2D double quantum filtration (DQF) COSY.[33] For the TOCSY experiments, the scheme proposed by Rance[34] was followed with a mixing time typically of the order of 50 ms. The isotropic mixing was performed by means of the WALTZ16[35] pulse train applied with $\gamma B_2/2\pi = 8$ kHz. Dipolar connectivities were obtained either through the conventional NOESY sequence[36] or the ROESY sequence[37] with mixing times from 100 to 200 ms. A randomization of the mixing length (±5%) was introduced in the NOESY experiments in order to minimize coherence transfer. The spin lock mixing interval of the ROESY sequence was applied by coherent CW irradiation at $\gamma B_2/2\pi = 2$ kHz. Experimental data processing was performed using Bruker software package. The standard sinebell squared routine was employed for apodization with a shift range of 60−90° and zero filling in both dimensions before 2D transformations were applied to end up with square matrixes of 2K × 2K real point data. Assignments were achieved by examination of the sequential NOESY connectivities after identifying all of the spin systems in the DQF−COSY and TOCSY maps.[38]

(30) Detailed structural and functional properties of **5** are currently under investigation in our laboratory and will be the subject of a future report.

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RAFT Synthesis. A. H_2NLys(Fmoc)ProGlyLys(pNZ)-Glu(OtBu)Lys(Dde)ProGlyLys(Alloc)AlaOH, 1. Assembly of the protected peptide was carried out manually in a glass reaction vessel fitted with a sintered glass frit. All of the reaction vessels were silylated using Me_2SiCl_2:toluene 1:4 for 24 h and then washed with toluene, MeOH, and CH_2Cl_2. Fmoc-Ala-SASRIN (3 g, 0.47 μequiv/g) was washed and swollen with CH_2Cl_2 (2 × 50 mL × 15 min) and DMF (2 × 50 mL × 15 min). Coupling reactions were performed using, relative to the resin loading, 2 equiv of N-α-Fmoc-protected amino acid activated in situ with 2 equiv of PyBOP and 4 equiv of DIEA in 30 mL of DMF for 30 min. The completeness of each coupling was confirmed by TNBS[39] and/or Kaiser[40] tests. N-α-Fmoc protecting groups were removed by treatment with piperidine:DMF 1:4 (30 mL × 5 + 10 + 10 min), the completeness of each deprotection being verified by the UV absorption of the piperidine washings at 299 nm.

Peptide resin **1a** was treated repeatedly with TFA:CH_2Cl_2 1:99 until the resin beads became dark purple (10 × 50 mL × 3 min). Each washing solution was neutralized with pyridine:MeOH 1:4 (5 mL). The combined washings were concentrated under reduced pressure, and 12.09 g of white solid was obtained by precipitation from EtOAc/petroleum ether. This solid was dissolved in EtOAc, and pyridinium salts were extracted with water. The organic layer was dried over Na_2SO_4, filtered, and concentrated to dryness. Precipitation from CH_2Cl_2/Et_2O afford 2.75 g of white solid which was further desalted by solid-phase extraction and lyophilized to afford 2.35 g (93%) of the linear peptide. This material was used in the next step without further purification. $C_{87}H_{121}N_{15}O_{23}$ (1745.0). RP-HPLC: t_R = 23.6 min (Vydac, C_{18}; 0−100% B in 30 min). ES-MS: $[M + 2H]^{2+}$= 873.9.

B. c[Lys(Fmoc)ProGlyLys(pNZ)GluLys(Dde)ProGlyLys-(Alloc)Ala], 2a. The above linear peptide (1.11 g, 600 μmol) was dissolved in DMF (600 mL), and the pH was adjusted to 8−9 by addition of DIEA. HATU (0.26 g, 1.1 equiv) was added, and the solution was stirred at room temperature for 3 h. Solvent was removed in a vacuum; the residue was dissolved in TFA:CH_2Cl_2 1:1 (70 mL) and allowed to stand for 45 min at room temperature. The solution was then concentrated under reduced pressure, and the residue was triturated with Et_2O and filtered to yield 0.94 g of crude RAFT molecule **2a** of more than 90% purity. One-hundred-sixty milligrams of this solid was purified by preparative RP-HPLC to yield 101 mg (59% yield) of pure **2a**. The remaining 780 mg was dissolved in MeCN:H_2O 1:1, and the pH was raised to 7−8 by addition of 180 μL of collidine. Solid-phase extraction and lyophilization afforded 0.75 g of the collidine salt of compound **2a** sufficiently pure to be engaged in the immobilization reaction. $C_{83}H_{111}N_{15}O_{22}$ (1670.9). RP-HPLC: t_R = 24.09 min (Vydac, C_{18}, 0−100% B in 30 min). ES-MS: $[M + 2H]^{2+}$ = 836.5. NMR ^{1}H, 400 MHz, DMSO-d_6, c[Lys^1(Fmoc)Pro^{2}Gly^{3}Lys^4(pNZ)Glu^{5}Lys^6(Dde)Pro^7-Gly^{8}Lys^9(Alloc)Ala^{10}], δ (ppm): 13.24 (m, 1H, NHε-Lys^6), 8.62 (br, 2H, NH-Gly^3 and NH-Gly^8), 8.20 (d, 2H, J = 8.8 Hz, ArH-pNZ), 8.07 (br, 2H, NH-Glu^5 and NH−Ala^{10}), 7.87 (d, 2H, J = 7.3 Hz, ArH-Fmoc), 7.66 (d, 2H, J = 7.3 Hz, ArH-Fmoc), 7.55 (d, 2H, J = 8.8 Hz, ArH-pNZ), 7.40 (t, 2H, J = 7.3 Hz, ArH−Fmoc), 7.40 (m, 2H, NHα-Lys^1 and NHα-Lys^6), 7.31 (m, 2H, NHα-Lys^4 and NHα-Lys^9), 7.30 (t, 2H, J = 7.3 Hz, ArH-Fmoc), 7.30 (m, 1H, NHε-Lys), 7.21 (t, 1H, J = 4.7 Hz, NHε-Lys), 7.09 (t, 1H, J = 5.0 Hz, NHε-Lys), 5.85 (m, 1H, CH_2═C*H*-CH_2-Aloc), 5.21 (dd, 1H, J = 1.5 Hz, J = 17.4 Hz, *trans*-CH_2═CH-CH_2-Aloc), 5.14 (dd, 1H, J = 1.5 Hz, J = 8.3 Hz, *cis*-CH_2═CH-CH_2-Aloc), 5.11 (s, 2H, CH_2-pNZ), 4.70 (br, 2H, α-Lys^1 and α-Lys^6), 4.34 (d, 2H, J = 5.1 Hz, CH_2═CH-CH_2-Aloc), 4.27 (m, 2H, α-Lys^4 and α-Lys^9), 4.24 (m, 2H, CH_2-Fmoc), 4.18 (m, 1H, CH-Fmoc), 4.13 (m, 1H, α-Ala^{10}), 4.04 (br, 2H, α-Pro^2 and α-Pro^7), 4.03 (br, 1H, α-Glu^5), 3.77 (m, 2H, α-Gly^3 and α-Gly^8), 3.66 (m, 2H, α′-Gly^3 and α′-Gly^8), 3.58 (m, 2H, δ-Pro^2 and δ-Pro^7), 3.42 (m, 2H, δ′-Pro^2 and δ′-Pro^7), 3.33 (m, 2H, ε-Lys^6), 2.94 (m, 6H, ε-Lys^1, ε-Lys^4, and ε-Lys^9), 2.45 (s, 3H, C═CMe-Dde), 2.24 (s, 4H, CH_2-Dde), 2.20 (m, 2H, γ-Glu^5), 2.09 (m, 4H, β-Pro^2 and β-Pro^7), 1.97 (m, 2H, β-Glu^5), 1.90 (m, 4H, γ-Pro^2 and γ-Pro^7), 1.84−1.55 (m, 8H, β-Lys^1, Lys^4, Lys^6, and Lys^9), 1.55−1.35 (m, 8H, δ-Lys^1, Lys^4, Lys^6, and Lys^9), 1.33 (d, 3H, J = 6.8 Hz, β-Ala^{10}), 1.29−1.13 (m, 8H, γ-Lys^1, Lys^4, Lys^6, and Lys^9), 0.91 (s, 6H, Me_2-Dde).

General Procedure for RAFT Immobilization. A. c-[Lys(Fmoc)ProGlyLys(pNZ)Glu(βAla-resin)Lys(Dde)Pro-GlyLys(Alloc)Ala], 2b. Fmoc-HN-XAL-PS resin (1.0 g, 230 μequiv/g) was washed and swollen with CH_2Cl_2 (2 × 20 mL × 15 min) and DMF (2 × 20 mL × 15 min) in a silanisated glass reaction vessel fitted with a sintered glass frit. The resin was treated with piperidine:DMF 1:4 (10 mL × 5 + 10 + 10 min) and washed with DMF (4 × 20 mL × 1 min) in order to remove the Fmoc protecting group. Fmoc-β-Ala-OH (0.47 g, 1.5 mmol) was coupled using PyBOP (0.78 g, 1.5 mmol) and DIEA (0.5 mL, 3 mmol) in 10 mL of DMF for 30 min, and the resin was washed with DMF (2 × 20 mL × 1 min) and CH_2Cl_2 (2 × 20 mL × 1 min). A TNBS test then ensured the completeness of the reaction. Removal of the Fmoc protecting group was performed as described above. The resin was reacted with a solution of **2a** (0.27 g, 150 μmol), PyAOP (0.12 g, 230 μmol), and collidine (0.3 mL, 2.3 mmol) in 10 mL of DMF for 13 h and then washed with DMF (2 × 15 mL × 1 min) and CH_2Cl_2 (2 × 15 mL × 1 min). Unreacted amine moieties were then quenched using 1-pyrenebutyric acid[41] (0.43 g, 1.5 mmol), PyBOP (0.78 g, 1.5 mmol), and DIEA (0.5 mL, 3 mmol) in 10 mL of DMF for 30 min, and the resin was washed with DMF (2 × 15 mL × 1 min) and CH_2Cl_2 (2 × 15 mL × 1 min). The immobilization reaction of RAFT molecule **2a** proceeded in 69% yield (starting from **2a**) as determined by UV measurement of Fmoc release from the attached template, affording a resin with a substitution level of 104 μmol/g.

Stepwise Solid-Phase Synthesis of TASP 3 and 4. General Procedures for Removal of N-ε-Protecting Groups. A. N-ε-Fmoc. The resin was sequentially swollen in DMF, treated with DBU:DMF 2:98 (5 × 10 mL/g of resin × 3 min) and finally washed with DMF (5 × 10 mL/g of resin × 3 min). The progress of the reaction was followed by the decrease of UV measurements of the DBU solution washings at 299 nm.

B. N-ε-Dde. The resin was swollen in DMF, treated with hydrazine hydrate:allyl alcohol:DMF 1:3:46 (5 × 10 mL/g of resin × 3 min) and finally washed sequentially with CH_2Cl_2, MeOH, DMF, and CH_2Cl_2 (2 × 10 mL/g of resin × 1 min). The progress of the reaction was followed by the decrease of UV measurements of the hydrazine solution washings at 299 nm.

C. N-ε-Alloc. The resin was swollen in CH_2Cl_2 (5 mL/g of resin) under nitrogen, and 25 equiv of $PhSiH_3$ was added. After 3 min, a solution of 0.2 equiv of $Pd(PPh_3)_4$ in CH_2Cl_2 (5 mL/g of resin) was added, and the mixture was shaken under nitrogen for 20 min. The whole operation was repeated 4 times, and the resin was washed sequentially with CH_2Cl_2, DMF:H_2O 9:1, DMF and CH_2Cl_2 (2 × 10 mL/g of resin × 1 min).

D. N-ε-pNZ. The resin was swollen in DMF and treated 3 times with a solution of $SnCl_2$ (2 M), AcOH (1.6 mM), and phenol (0.01 M) in DMF (10 mL/g of resin) for 2 h. The resin was then washed sequentially with DMF, CH_2Cl_2:TEA 9:1, DMF, and CH_2Cl_2 (2 × 10 mL/g of resin × 1 min).

General Procedure for Branched Peptide Formation onto the Immobilized Template. Branched peptides were assembled on the immobilized template upon sequential removal of N-ε-protecting groups, following conventional stepwise SPPS procedures according to Fmoc/tBu strategy. Coupling reactions were performed using, relative to the resin loading, 5 equiv of N-α-Fmoc-protected amino acid activated in situ with 5 equiv of PyBOP and 5 equiv of DIEA in DMF (10 mL/g of resin) for 30 min. Acetylations were performed

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(41) Alternatively, this could be performed using Ac_2O:pyridine in DMF.

using, relative to the resin loading, 10 equiv of acetic anhydride and 10 equiv of pyridine in DMF (10 mL/g of resin) for 30 min. The completeness of each coupling was confirmed by TNBS and/or Kaiser tests. N-α-Fmoc protecting groups were removed by treatment with piperidine:DMF 1:4 (20 mL/g of resin × 5 + 10 + 10 min), the completeness of each deprotection being verified by the UV absorption of the piperidine washings at 299 nm.

General Procedure for Final Cleavage and Deprotection. The dry resin was reacted with TFA:TIS:CH_2Cl_2 5:1:94 (3 × 10 mL/g of resin × 15 min) and washed with CH_2Cl_2 (3 × 10 mL/g of resin × 1 min). The cleavage solution was concentrated under reduced pressure, and the residue was treated with TFA:CH_2Cl_2 1:1 for 1 h. The solution was concentrated under reduced pressure, and the residue was treated with Et_2O to precipitate a crude material which was purified by preparative RP-HPLC.

c[Lys(SerAc)ProGlyLys(HisAc)Glu(βAlaNH$_2$)Lys-(AspAc)ProGlyLys(GluAc)Ala], 3. TASP molecule **3** was synthesized on solid phase according to general procedures described above; 104 μmol of PS-bound RAFT **2b** afforded, after preparative RP-HPLC (5−20% solvent B in 30 min) and lyophilization, 33 μmol (57 mg, 32%) of pure **3**. $C_{75}H_{118}N_{22}O_{25}$ (1727.9). RP-HPLC: t_R = 21.31 min (Vydac, C_{18}, 5−20% B in 30 min). ES-MS: $[M + 2H]^{2+}$ = 865.4, $[M + 3H]^{3+}$ = 577.3. NMR ^{1}H 400 MHz, DMSO-d_6, [Lys^1(Ser^{11}Ac^{12})Pro^{2}Gly^{3}Lys^4-(His^{41}Ac^{42})Glu^5(βAla^{51}NH$_2$)Lys^6(Asp^{61}Ac^{62})Pro^{7}Gly^{8}Lys^9(Glu^{91}Ac^{92})-Ala^{10}], δ (ppm): 8.94 (s, 1H, Im4-His^{41}), 8.51 (m, 2H, NH-Gly^3 and NH-Gly^8), 8.14 (d, 1H, J = 8.3 Hz, NH-His^{41}), 8.13 (-, 1H, NH-Glu^5), 8.12 (-, 1H, NH-Ala^{10}), 8.10 (d, 1H, J = 8.3 Hz, NH-Asp^{61}), 7.97 (m, 1H, NH-Glu^{91}), 7.96 (m, 1H, NHε-Lys^4), 7.87 (m, 1H, NHε-Lys^9), 7.85 (m, 1H, NH-Ser^{11}), 7.83 (m, 1H, NHε-Lys^6), 7.82 (m, 1H, NHε-Lys^1), 7.77 (t, 1H, J = 5.4 Hz, NHε-βAla^{51}), 7.44 (br, 2H, NHα-Lys^4 and NHα-Lys^9), 7.43 (br, 2H, NHα-Lys^1 and NHα-Lys^6), 7.31 (s, 1H, Im2-His^{41}), 7.30 (s, 1H, βAla^{51}NH_2), 6.79 (s, 1H, βAla^{51}NH_2), 4.83 (br, 1H, HO-Ser^{11}), 4.63 (br, 2H, α-Lys^1 and α-Lys^6), 4.55 (m, 1H, α-Asp^{61}), 4.53 (m, 1H, α-His^{41}), 4.25 (m, 1H, α-Ser^{11}), 4.25 (m, 2H, α-Lys^4 and α-Lys^9), 4.21 (m, 1H, α-Glu^{91}), 4.18 (m, 1H, α-Ala^{10}), 4.11 (br, 2H, α-Pro^2 and α-Pro^7), 4.06 (br, 1H, α-Glu^5), 3.79 (m, 2H, α-Gly^3 and α-Gly^8), 3.68 (m, 2H, α′-Gly^3 and α′-Gly^8), 3.56 (m, 2H, δ-Pro^2 and δ-Pro^7), 3.54 (d, 2H, J = 5.6 Hz, β-Ser^{11}), 3.48 (m, 2H, δ′-Pro^2 and δ′-Pro^7), 3.20 (m, 2H, α-βAla^{51}), 3.08 (m, 1H, β-His^{41}), 3.01 (m, 8H, ε-Lys^1, Lys^4, Lys^6, and Lys^9), 2.87 (dd, 1H, J = 8.7 Hz, J = 15.2 Hz, β′-His^{41}), 2.64 (dd, 1H, J = 5.8 Hz, J = 16.3 Hz, β-Asp^{61}), 2.45 (dd, 1H, J = 8.2 Hz, J = 16.3 Hz, β′-Asp^{61}), 2.20 (m, 2H, γ-Glu^{19}), 2.20 (t, 2H, J = 7.0 Hz, β-βAla^{51}), 2.09 (m, 4H, γ-Pro^2 and γ-Pro^7), 2.05 (br, 2H, γ-Glu^5), 1.90 (m, 4H, β-Pro^2 and β-Pro^7), 1.89 (m, 2H, β-Glu^5), 1.87 (s, 3H, Ac^{12}), 1.86 (m, 1H, β-Glu^{19}), 1.85 (s, 3H, Ac^{92}), 1.84 (s, 3H, Ac^{62}), 1.83 (s, 3H, Ac^{42}), 1.70 (m, 1H, β′-Glu^{19}), 1.61 (br, 8H, β-Lys^1, Lys^4, Lys^6, and Lys^9), 1.43 (m, 8H, δ-Lys^1, Lys^4, Lys^6, and Lys^9), 1.29 (d, 3H, J = 6.8 Hz, β-Ala^{10}), 1.19 (m, 8H, γ-Lys^1, Lys^4, Lys^6, and Lys^9).

c[Lys(ValThrSerAc)ProGlyLys(ValThrHisAc)Glu-(βAlaNH$_2$)Lys(ValThrAspAc)ProGlyLys-(ValThrGluAc)-Ala], 4. TASP molecule **4** was assembled on both PS- and PEG-PS-based resisn according to general procedures described above. Starting from 49 μmol of PS-bound RAFT **2b**, the procedure afforded, after preparative RP-HPLC (15−30% solvent B in 30 min) and lyophilization, 15 μmol (38 mg, 30%) of pure **4**. Starting from 117 μmol of PEG-PS-bound RAFT **2b**, it gave rise to 30 μmol (77 mg, 26%) of pure **4**. $C_{111}H_{182}N_{30}O_{37}$ (2528.8). RP-HPLC: t_R = 14.66 min (Vydac, C_{18}, 15−30% B in 30 min). ES-MS: $[M + 2H]^{2+}$ = 1265.4, $[M + 3H]^{3+}$ = 844.0. NMR ^{1}H, 400 MHz, DMSO-d_6, c[Lys^1(Val^{11}Thr^{12}Ser^{13}Ac^{14})Pro^2-Gly^{3}Lys^4(Val^{41}Thr^{42}His^{43}Ac^{44})Glu^5(βAla^{51}NH$_2$)Lys^6(Val^{61}Thr^{62}-Asp^{63}Ac^{64})Pro^{7}Gly^{8}Lys^9(Val^{91}Thr^{92}Glu^{93}Ac^{94})Ala^{10}] δ (ppm): 8.95 (s, 1H, Im4-His^{43}), 8.46 (br, 2H, NH-Gly^3 and NH-Gly^8), 8.29 (d, 1H, J = 7.6 Hz, NH-Asp^{63}), 8.20 (d, 1H, J = 8.3 Hz, NH-His^{43}), 8.12 (m, 1H, NH-Glu^5), 8.09 (d, 1H, J = 7.8 Hz, NH-Glu^{93}), 8.09 (m, 1H, NH-Ala^{10}), 8.04 (d, 1H, J = 7.6 Hz, NH-Ser^{13}), 7.95 (m, 1H, NHε-Lys*), 7.89 (m, 3H, 3 × NHε-Lys*), 7.88 (m, 1H, NH-Thr^{42}), 7.81 (m, 1H, NH-Val*), 7.79 (m, 1H, NH-βAla^{51}), 7.78 (m, 1H, NH-Thr^{12}), 7.74 (d, 1H, J = 8.0 Hz, NH-Thr^{92}), 7.65 (m, 1H, NH-Val*), 7.62 (m, 1H, NH-Thr^{62}), 7.60 (m, 2H, 2 × NH-Val*), 7.55 (br, 2H, NHα-Lys^4 and NHα-Lys^9), 7.44 (br, 2H, NHα-Lys^1 and NHα-Lys^6), 7.36 (s, 1H, Im2-His^{43}), 7.31 (s, 1H, βAla^{51}NH_2), 6.81 (s, 1H, βAla^{51}NH_2), 4.96 (br, 5H, HO-Ser^{13} and HO-Thr^{12}, Thr^{42}, Thr^{62}, and Thr^{92}), 4.73 (m, 1H, α-His^{43}), 4.62 (m, 1H, α-Asp^{63}), 4.62 (m, 2H, α-Lys^1 and α-Lys^6), 4.40 (m, 1H, α-Ser^{13}), 4.33 (m, 1H, α-Glu^{93}), 4.28 (m, 4H, α-Thr^{12}, Thr^{42}, Thr^{62}, and Thr^{92}), 4.20 (m, 2H, α-Lys^4 and α-Lys^9), 4.14 (m, 4H, α-Val^{11}, Val^{41}, Val^{61}, and Val^{91}), 4.14 (m, 1H, α-Ala^{10}), 4.14 (m, 2H, α-Pro^2 and α-Pro^7), 4.00 (m, 1H, α-Glu^5), 4.00 (m, 4H, β-Thr^{12}, Thr^{42}, Thr^{62}, and Thr^{92}), 3.76 (br, 4H, α-Gly^3 and α-Gly^8), 3.57 (m, 1H, β-Ser^{13}), 3.54 (m, 4H, δ-Pro^2 and δ-Pro^7), 3.20 (m, 2H, α-βAla^{51}), 3.10 (m, 4H, ε-Lys^1, Lys^4, Lys^6, and Lys^9), 3.06 (m, 1H, β-His^{43}), 2.96 (m, 1H, β′-His^{43}), 2.92 (m, 4H, ε′-Lys^1, Lys^4, Lys^6, and Lys^9), 2.70 (dd, 1H, J = 5.1 Hz, J = 16.4 Hz, β-Asp^{63}), 2.49 (m, 1H, β′-Asp^{63}), 2.25 (m, 2H, γ-Glu^{93}), 2.20 (m, 2H, β−βAla^{51}), 2.11 (br, 2H, β-Pro^2 and β-Pro^7), 2.05 (br, 2H, γ-Glu^5), 1.93 (m, 4H, β-Val^{11}, Val^{41}, Val^{61}, and Val^{91}), 1.90 (m, 4H, γ-Pro^2 and γ-Pro^7), 1.88 (s, 3H, Ac^{14}), 1.86 (s, 3H, Ac^{94}), 1.85 (m, 1H, β-Glu^{93}), 1.85 (s, 3H, Ac^{64}), 1.84 (m, 2H, β-Glu^5), 1.84 (s, 3H, Ac^{44}), 1.84 (m, 2H, β′-Pro^2 and β′-Pro^7), 1.74 (m, 1H, β′-Glu^{93}), 1.60 (br, 8H, β-Lys^1, Lys^4, Lys^6, and Lys^9), 1.36 (br, 8H, δ-Lys^1, Lys^4, Lys^6, and Lys^9), 1.29 (d, 3H, J = 7.1 Hz, β-Ala^{10}), 1.24 (br, 8H, γ-Lys^1, Lys^4, Lys^6, and Lys^9), 1.03 (m, 12H, γ-Thr^{12}, Thr^{42}, Thr^{62}, and Thr^{92}), 0.83 (m, 24H, γ-Val^{11}, Val^{41}, Val^{61} and Val^{91}).

Convergent Solid-Phase Synthesis of TASP 5. FmO-SucCys(StBu)ProGlyCys(StBu)GlyOH, 6. The linear peptide sequence was assembled on Fmoc-Gly-SASRIN resin (2 g, 0.50 μequiv/g) according to the procedure described for **1**. FmOSucOH (0.59 g, 2 mmol) was coupled on the last amino acid using PyBOP (1.04 g, 2 mmol) and DIEA (700 μL, 4 mmol) in 20 mL DMF for 30 min. Peptide resin was treated repeatedly with TFA:CH_2Cl_2 5:95 until the resin beads became dark purple (3 × 20 mL × 3 min). The solution was concentrated to dryness. Precipitation from EtOAc:petroleum ether afforded 0.90 g of crude **6**. This compound was dissolved in MeCN:H_2O 2:1, and the pH was raised to 7−8 by addition of 400 μL of collidine. Solid-phase extraction and lyophilization afforded 0.88 g (87%) of the collidine salt of compound **6**. $C_{41}H_{55}N_5O_9S_4$ (890.1). RP-HPLC: t_R = 27.54 min (Vydac, C_{18}, 0−100% B in 30 min). ES-MS: $[M + H]^+$ = 890.7.

General Procedure for OFm Removal. The resin was swollen in DMF, treated with piperidine:DMF 1:4 (3 × 10 mL/g of resin × 5 + 10 + 10 min) and finally washed with DMF (5 × 20 mL/g of resin × 1 min). The progress of the reaction was followed by the decrease of UV measurements of the piperidine solution washings at 299 nm.

General Procedure for Peptide Loop Formation onto the Immobilized Template. After removal of the first N-ε protecting group according to the above procedure, the resin was reacted with a solution of **6** (4 equiv), PyAOP (4 equiv), and collidine (20 equiv) in DMF (10 mL/g) for 1 h and then washed with DMF (2 × 10 mL/g of resin × 1 min) and CH_2Cl_2 (2 × 10 mL/g of resin × 1 min). Completeness of the fragment condensation was confirmed by a TNBS test. After removal of OFm and the second N-ε protecting group according to the above procedures, the resin was reacted with a solution of PyAOP (4 equiv) and DIEA (10 equiv) in DMF (10 mL/g) for 30 min and then washed with DMF (2 × 10 mL/g of resin × 1 min) and CH_2Cl_2 (2 × 10 mL/g of resin × 1 min). Completeness of the loop cyclization was confirmed by a TNBS test.

SucCys(StBu)ProGlyCys(StBu)Gly SucCys(StBu)Pro-GlyCys(StBu)Glyc[Pro-Gly-Lys Ala Lys-Pro-Gly-Lys Glu-(βAlaNH$_2$) Lys], 5. Peptide fragment **6** was grafted in two copies on 32 μmol of PS-bound RAFT **2b**, first from Fmoc to Alloc-protected attachment sites, second from Dde to pNZ ones, according to general procedures described above. The dry resin was reacted with TFA:CH_2Cl_2 5:95 (3 × 10 mL/g of resin × 15 min) and washed with CH_2Cl_2 (3 × 10 mL/g of resin × 1 min). The cleavage solution was concentrated under reduced pressure, and the residue was precipitated with Et_2O to obtain crude material which was purified by preparative RP-HPLC (40−70% B in 30 min) to afford after lyophilization 3.5 μmol

(9 mg, 11%) of pure **5**. $C_{103}H_{168}N_{26}O_{26}S_8$ (2443.1). RP-HPLC: t_R = 22.50 min (Vydac, C_{18}, 0−100% B in 30 min). ES-MS: $[M + 2H]^{2+}$ = 1222.6.

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Supporting Information Available: Analytical HPLC and ES-MS data of **3**−**5** and ^{1}H NMR data of **3** and **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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