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Chemical Ligation to Obtain Proteins Comprising Helices with Individual Amino Acid Sequences

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Abstract—Development of the strategies for assembling multiple kinds of peptide segments would give new possibilities for the de novo design of functional proteins. We will introduce our approach for the selective assembly of helical peptide segments on a peptide template to give four-helix-bundle proteins comprising individual helices.

Introduction

De novo design of artificial peptides and proteins is one of the developing research fields for the creation of novel functional molecules and materials. In the past decade, certain progress has been achieved in this field.[1] Though the functions exerted by these artificial proteins are, generally speaking, not so sophisticated at the present stage as those of the natural proteins, it is amazing that peptides comprising at most 100 amino acids or so can manifest a significant part of functions of natural proteins. One idea to endow more sophisticated functions to artificial proteins is to generate proteins comprising multiple functional groups that can work in concert. Here we will discuss the strategies for constructing proteins with the above properties especially by chemical ligation. We will introduce our approach for assembling helical peptide segments using a peptide template to give four-helix-bundle proteins composed of individual helices.

Strategies for creating functional proteins composed of multiple kinds of peptide segments by chemical ligation

To construct proteins with individual peptide segments, it is important to choose efficient ligation systems and suitable protection systems for the selective assembly of peptide segments. The following are candidates for possible ligation approaches. Peptide bonds may be formed by silver ion-activated peptide thiocarboxylic acid or thioester,[2] by a ligation of a peptide thioester and a cysteinyl peptide,[3] or by a ligation of a peptide thiocarboxylic acid and a histidinyl peptide.[4] Also, approaches to use unnatural peptide bond substituents can be adopted. For example, thioether formation,[5,6] thioester formation,[7] disulfide formation,[8,9] pseudoproline formation,[10] and oxime formation[11] are all reasonable choices.

Another important factor that we should consider is the selection of the protecting groups. These protecting groups should be stable under ligation conditions and should be selectively cleavable when necessary. By choosing suitable combinations of ligations and protecting groups, various types of artificial proteins comprising multiple peptide constituents would be designable. Mutter et al. proposed the template-assembled synthetic proteins (TASP)[1c,11] where a mimic of the

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Abbreviations: A = alanine, E = glutamic acid, F = phenylalanine, G = glycine, I = isoleucine, K = lysine, L = leucine, M = methionine, N = asparagine, P = proline, Q = glutamine, R = arginine, S = serine, T = threonine, V = valine, W = tryptophan, Y = tyrosine, GABA = γ-aminobutyric acid, Fmoc = 9-fluorenylmethyloxycarbonyl, Ac = acetyl, ^{t}Bu = *t*-butyl, Boc = *t*-butyloxycarbonyl, Trt = trityl, MBzl = *p*-methoxybenzyl, Ad = adamantyl, Acm = acetamidomethyl, DICDI = diisopropylcarbodiimide, HOBt = 1-hydroxybenzotriazole, NMM = *N*-methylmorpholine, TFA = trifluoroacetic acid, EDT = 1,2-ethanedithiol, AgOTf = silver trifluoromethanesulfonate, DTT = dithiothreitol, AcOH = acetic acid, Tris = tris(hydroxymethyl)aminomethane, MOPS = 3-(*N*-morpholino)propanesulfonic acid, DMF = dimethylformamide, MeOH = methanol, HPLC = high performance liquid chromatography, LSIMS = liquid secondary ion mass spectrometry, TOFMS = time of flight mass spectrometry, CD = circular dichroism.

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complementary-determining region of a monoclonal antibody was constructible by assembling three kinds of peptide loops on a peptide template.[12]

Our approach to construct proteins comprising individual helices

We have developed three approaches to construct artificial proteins composed of four individual helices (Fig. 1).[6,9,13] Here we adopt the following ligation and protection systems. The first approach[9] is to use the disulfide cross-link as the ligation strategy. For the selective disulfide formation, dithiodipyridine is applied. The Acm group is employed for the selective protection of cysteines. The second connection strategy employs the thioether as the cross-link that is formed by the reaction between haloacetyl moiety and a cysteine.[6] The Acm group is also applied here as the protecting group of Cys. The third approach uses a peptide template that has four cysteines for the selective introduction of haloacetylated peptide segments.[12] The main feature of our approach is the feasibility of introducing four unique helices with different amino acid sequences. Moreover, the use of HPLC-purified unprotected peptide segments as intermediates facilitates the purification of the intermediates to afford proteins of very high purity. We have already reported the details of the first two approaches,[6b,9c] and here the details regarding the third synthetic approach[13] are reported.

Design of the template. Mutter and his co-workers have introduced the idea of a template assisted protein (TASP)[11] and stable artificial helical proteins have been constructed by this laboratory.[1c] Based on their concept, we have designed a template that has four cysteines with selectively detachable protecting groups [H-Cys(Acm)^1-Lys^2-Cys(SH)^3-Pro^4-Gly^5-Cys(MBzl)^6-Glu^7-Cys(Ad)^8-Gly^9-OH **1**] allowing four different peptides to be covalently attached. The Cys^3 residue was originally protected by the Trt group that was removed by TFA treatment after the peptide chain we constructed via the Fmoc-solid-phase peptide synthesis strategy. The MBzl group of Cys^6 is selectively cleavable with 1 M Me_3SiBr-thioanisole in TFA,[14] the Ad group[15] of Cys^8 cleavable with 1 M CF_3SO_3H-thioanisole in TFA,[16] and the Acm group of Cys^1 is stable to these acid treatments and removed with AgOTf treatment.[17] The main feature of the approach is the feasibility of introducing four different α-helices onto the template. This unsymmetrical helix bundle is prepared by: (i) substituting Lys in positions 1, 3, 6, and 9 (represented by *) of the Mutter template [Ac-Lys*-Lys-Lys*-Pro-Gly-Lys*-Glu-Lys*-Gly-OH] by Cys, (ii) employing four kinds of cysteine protecting groups that can be removed selectively, and (iii) covalently attaching the peptides to the template by the reaction between a halomethylated helical peptide and a cysteine. The template was prepared by Fmoc-solid-phase peptide synthesis[18] followed by TFA:EDT (95:5) treatment. To avoid the steric hindrance on the introduction of the helices onto the template, the protecting groups of cysteines were arranged in the order of helical segments being successively introduced from the center of the sequence towards the end, namely in the order of Cys^3→Cys^6→Cys^8→Cys^1, respectively.

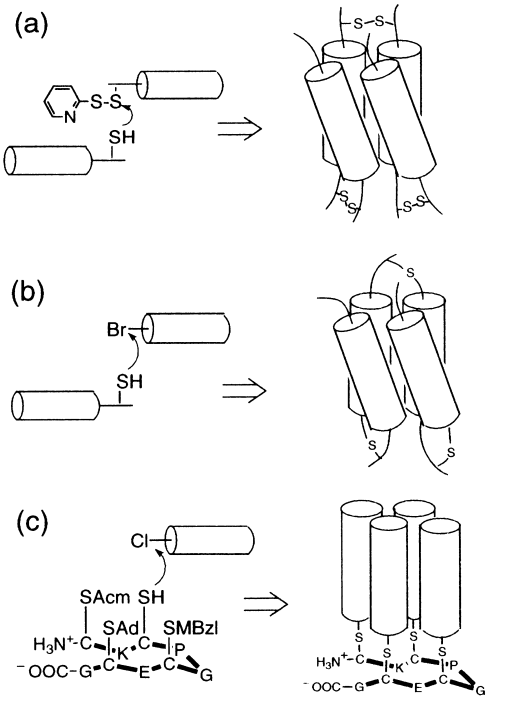

Figure 1. Three approaches to construct artificial proteins comprised of four individual helices. (a) Assembly via selective disulfide formation; (b) assembly via selective thioether formation; (c) assembly on the multi-component differentially protected template.

Model study for constructing the artificial four-helix bundle proteins. To ascertain whether four-helix-bundle proteins can be constructed using this template, an artificial helical protein **6** was constructed (Fig. 2). Peptide **2** was designed as a model segment to introduce onto template **1**. Peptide **2** contains chloroacetyl–aminobutyl moiety at its N-terminus as a linker and the same α-helical sequence (ELEELLKKLKELLK) as DeGrado has used previously in a four-helix-bundle protein.[19] C-terminal Gly-Tyr-NH_2 was employed to facilitate purification by monitoring UV absorbance at 275 nm and to avoid a possible helix–dipole interaction. Peptide **2** was prepared by Fmoc-solid-phase peptide synthesis followed by 1 M Me_3SiBr-thioanisole:TFA treatment.[14]

Introduction of one equivalent of **2** onto the single free SH of template **1** was carried out in 6 M guanidine HCl (pH 8.0, overnight) (Fig. 2). The product **3** was treated with 1 M Me_3SiBr-thioanisole in TFA (0 °C, 2 h) to remove the MBzl protecting group. Under these conditions, the Ad group was stable. After introduction of the second equivalent of peptide **2**, the resulting peptide **4** was treated with 1 M CF_3SO_3H-thioanisole in TFA[16] (0 °C, 1.5 h) to remove the Ad protecting group. The third helix was then introduced to give **5**. Next, the Acm protecting group was removed by AgOTf:TFA treatment[17] (0 °C, 1.5 h). Final introduction of peptide **2** onto the template afforded the desired protein **6**. Here a certain amount of the dimer of the AgOTf-treated sample of **5** was formed, which diminished the yield of

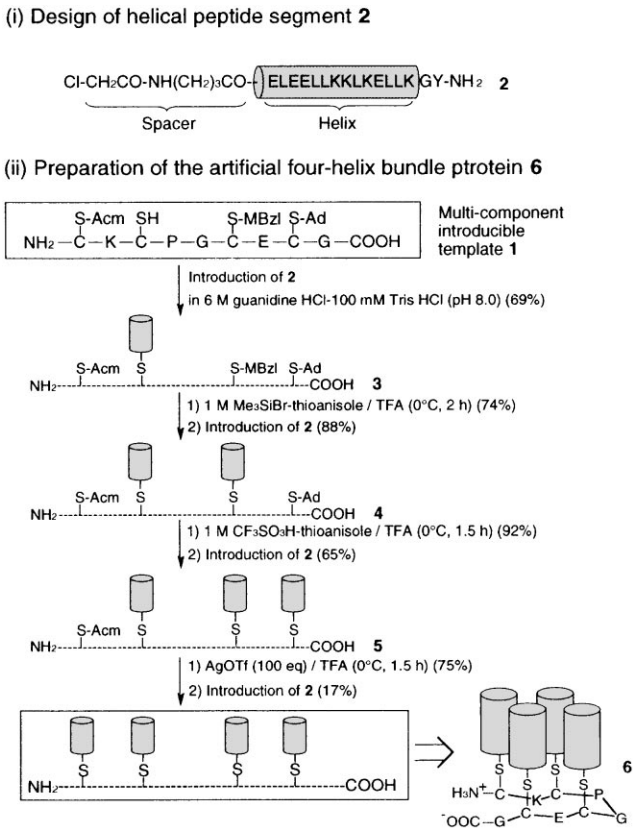

Figure 2. Model study for constructing the four-helix bundle proteins with the aid of the multi-component differentially protected template **1**.

this step. Fidelity of the final product **6** was confirmed by liquid secondary ion mass spectrometry (LSIMS). A CD spectrum of the protein was suggestive of α-helical secondary structure (Fig. 3). In the aqueous buffer, the $[\theta]_{222}$ of the protein was not very large [−12,900 deg·cm^2/dmol (47 μM protein in 18 mM MOPS-200 mM NaCl (pH 7.0)]. On the other hand, the minimum at 222 nM increased in intensity when CD was recorded in MeOH [−15,500 deg·cm^2/dmol (protein concentration: 71 μM)]. In water, repulsion of the helix dipole may destabilize the helical structure, whereas in MeOH, the repulsion would be alleviated when MeOH intrudes the hydrophobic core between the helices, making the helices dissociate.

Through this experiment, we have shown that proteins comprising as many as 70 residues would be constructible with the above approach. We then applied this strategy for assembling helical peptides corresponding to the voltage sensor of the sodium channel.

Assembly of peptides corresponding to the voltage sensor of the *Electrophorus electricus* sodium channel. The S4 segments of the Na^+ channel are assumed to be involved in the voltage sensing of the channel.[20] Assembly of the peptides corresponding to this region may afford proteins that have voltage dependent functions.[21] Four peptide segments corresponding to the repeat I to IV of the S4 segments of the *Electrophorus electricus* sodium channel[20] were designed as shown in

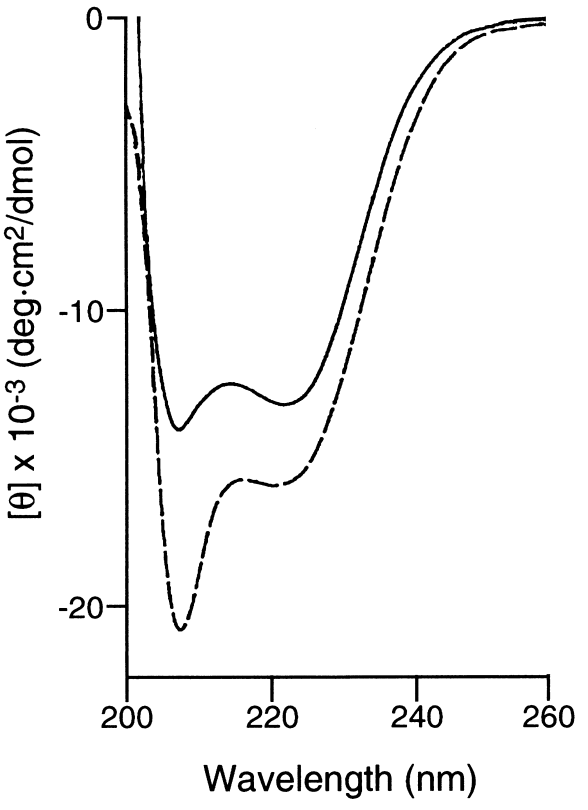

Figure 3. CD spectra of the four-helix bundle protein **6**. Solid line: spectrum in 18 mM MOPS-200 mM NaCl (pH 7.0) (protein concentration: 47 μM); dashed line: spectrum in MeOH (protein concentration: 71 μM). Mean residue ellipticity [θ] was calculated on the basis that the total number of amino acid residues involved in helix formation is 56.

Figure 4. As was mentioned above, the chloroacetyl-γ-aminobutyl moiety was introduced at the N-terminus of each segment as a linker. At the C-terminus of each peptide, Gly-Tyr-NH_2 was introduced for easy detection during purification. Though the S4 segments contain several basic amino acids (i.e. Arg and Lys) the segments themselves are not highly soluble in water as is typical for transmembrane peptides. In order to examine if four-helix-bundle proteins comprised of these segments can be constructed with the above approach, the S4 segment of the repeat IV, which contains seven Arg and one Lys residue and is expected to be most

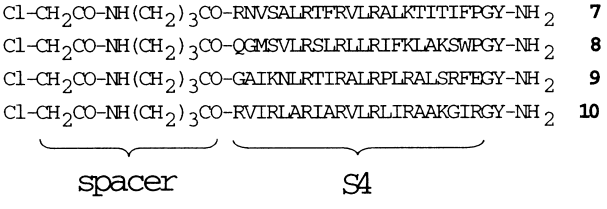

Figure 4. Design of the peptide segments corresponding to the voltage sensor segments of the *Electrophorus electricus* sodium channel. Each amino acid is represented by its one-letter code. Segment **7, 8, 9,** and **10** contain sequences corresponding to positions 204–225 (S4 in the repeat I), 651–672 (S4 in the repeat II), 1092–1113 (S4 in the repeat III), and 1414–1438 (S4 in the repeat IV) of the channel protein, respectively.

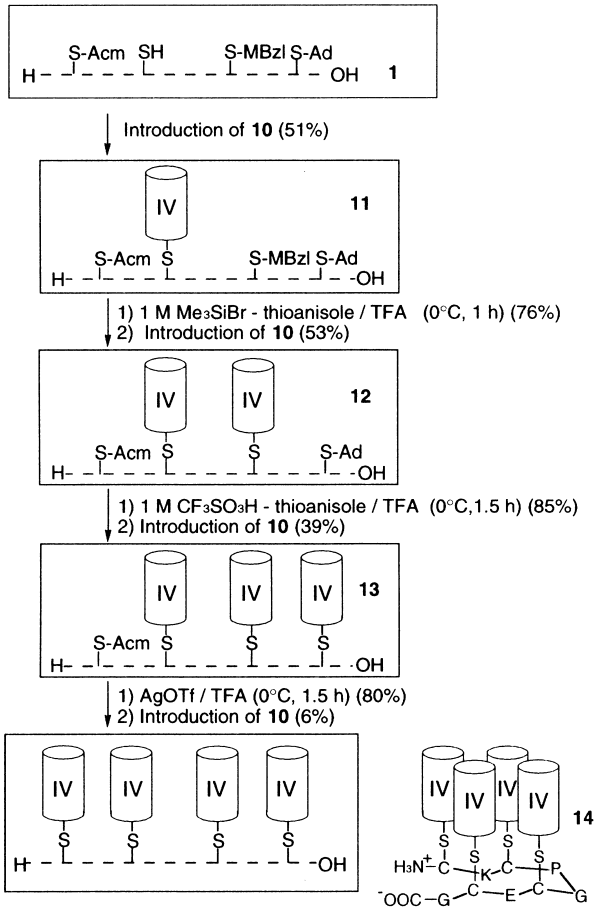

Figure 5. Assembly of peptide segment **10** corresponding to the voltage sensor segment (S4) in the repeat IV of the sodium channel. The roman numerals of the helices emphasize that all the helices used here are derived from S4 in repeat IV.

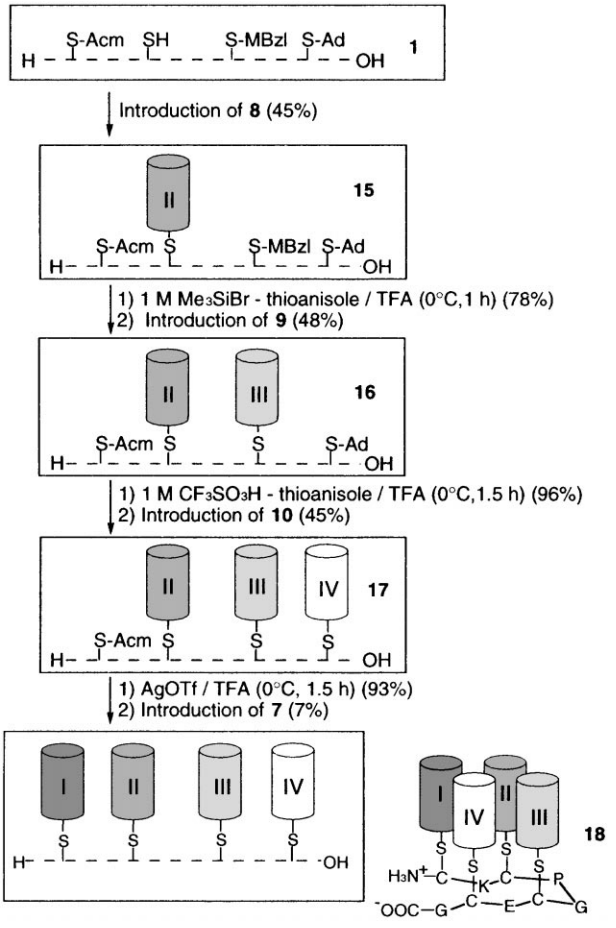

Figure 6. Construction of the four-helix bundle protein **18** comprised of the peptides corresponding to the S4 segments in the repeat I to IV of the sodium channel. The roman numerals on the helices denote the repeat number of the respective segments.

water-soluble, was assembled using the above approach (Fig. 5). Assembly was conducted in the same manner as described above (Fig. 2). Each reaction proceeded without difficulty, with the exception of the final ligation, where the yield remained poor owing to the dimer formation of the AgOTf-treated sample of **13**. The fidelity of the peptides used for segment condensations and the final product were ascertained by LSIMS or TOFMS. The synthesis of the protein with four S4 segments was judged to be successful with the above approach. The protein that consists of S4 segments of repeat I to IV was then constructed using the same strategy (Fig. 6). The resulting protein **18** was highly pure based on HPLC analysis. The estimated molecular weight [*m*/*z*: 12596.8 $(M+Na)^+$] from TOFMS was in good agreement with the theoretical average mass (12596.2). The CD spectrum of the protein in MeOH suggested that the protein adopts a helical structure in the membrane [$[\theta]_{222}$: −13,100 deg·cm^2/dmol (14 μM protein/MeOH)]. The protein formed an ion channel in a diphytanoylphosphatidylcholine membrane. Moreover, increase in the conductance at the positive voltage (i.e. rectification) was observed.[14] An artificial ion channel protein that functions according to the voltage change was thus created.

Conclusions

The approach we have outlined here enables protein chemists to assemble multiple helices having individual functional groups that are expected to work in harmony with each other affording proteins exerting sophisticated functions. We have recently reported that the thioester ligation strategy is also well applicable to the construction of highly pure helix-bundle proteins on a Mutter template [Ac-Lys*-Lys-Lys*-Pro-Gly-Lys*-Glu-Lys*-Gly-OH (*: where a peptide segment is introduced)].[22] If we adopt selectively detachable protecting groups for these Lys residues, proteins with four different helices would be also constructible. In the cysteine-containing template we report here, each peptide segment is introduced to the template via its N-terminus. On the other hand, the approach using the thioester ligation method affords proteins where peptide segments are attached to the template by their C-termini. We expect these approaches to be used in concert when the N-terminus or the C-terminus is important for exerting protein functions, or when antiparallel helical bundles are required.

Experimental

H-Cys(Acm)-Lys-Cys-Pro-Gly-Cys(MBzl)-Glu-Cys(Ad)-Gly-OH (1). H-Cys(Acm)-Lys(Boc)-Cys(Trt)-Pro-Gly-Cys(MBzl)-Glu(O^{t}Bu)-Cys(Ad)-Gly-(*p*-benzyloxybenzyl-alcohol resin) was prepared using Fmoc-solid-phase peptide synthesis. TFA:EDT (95:5) treatment of the peptide-resin (rt, 2 h) followed by HPLC-purification afforded the desired template **1**. Yield 13% (from the initial introduction of Fmoc-Gly on the resin). LSIMS: *m*/*z* 1224.5 $(M+H)^+$ (expected 1224.5).

Cl-CH$_2$CO-NH(CH$_2$)$_3$CO-Glu-Leu-Glu-Glu-Leu-Leu-Lys-Lys-Leu-Lys-Glu-Leu-Leu-Lys-Gly-Tyr-NH$_2$ (2). The title peptide was prepared using Fmoc-solid-phase peptide synthesis on a Rink amide resin[23] using DICDI-HOBt coupling method followed by 1 M Me_3SiBr-thioanisole:TFA treatment (0 °C, 1.5 h) and HPLC-purification, basically in the same manner as reported earlier.[18b] The GABA residue was introduced using Fmoc-GABA, and the N-terminal chloroacetyl moiety using choloacetic acid with the aid of DICDI-HOBt. Yield 31% [from the initial introduction of Fmoc-Tyr(^{t}Bu) on the resin]. LSIMS: *m*/*z* 2107.9 $(M+H)^+$ (expected 2108.0).

Introduction of the first helix onto the template (3). 1 (4.4 mg, 3.6 mmol) was added to the solution of **2** (7.5 mg, 3.6 mmol) in 6 M guanidine HCl containing 100 mM Tris HCl (pH 8.0) (600 μL). The mixture was stirred at room temperature overnight. The solution was desalted by gel-filtration on Sephadex® G-10 using 1 N AcOH as an eluent and lyophilized. The resulting powder was purified by reverse phase HPLC to afford the desired hybrid. Yield 5.4 mg (69%). LSIMS: *m*/*z* 3295.4 $(M+H)^+$ (expected 3296.1).

Introduction of the second helix (4). 3 (3.2 mg, 0.96 mmol) was treated with 1 M Me_3SiBr-thioanisole in TFA (1 mL) in the presence of *m*-cresol and EDT (20 mL each) in an ice-bath for 2 h. The product was purified by gel-filtration on Sephadex® G-10 using 1 N AcOH as an eluent and lyophilized (2.3 mg). The obtained powder and **2** (1.7 mg, 1.1 equiv) were dissolved in 6 M guanidine HCl containing 100 mM Tris HCl (pH 8.0) (300 μL) and stirred at room temperature overnight. The product was purified as stated above to give **4**. Yield 3.4 mg (65%). LSIMS: *m*/*z* 5246.7 $(M+H)^+$ (expected 5246.4).

Introduction of the third helix (5). 4 (4.9 mg, 1.0 mmol) was treated with 1 M CF_3SO_3H-thioanisole in TFA (400 μL) in the presence of *m*-cresol and EDT (8 μL each) in an ice-bath for 1.5 h. The product was purified by gel-filtration on Sephadex® G-10 using 1 N AcOH as an eluent and lyophilized (4.4 mg). The obtained powder and **2** (2.0 mg, 1.1 equiv) were dissolved in 6 M guanidine HCl containing 100 mM Tris HCl (pH 8.0) (370 μL) and stirred at room temperature overnight. The product was purified as stated above to give **5**. Yield 4.0 mg (60%). LSIMS: *m*/*z* 7182.0 $(M+H)^+$ (expected 7182.7).

Introduction of the final helix (6). 5 (4.0 mg, 0.55 mmol) was treated with AgOTf (14 mg, 100 equiv) in TFA (0.7 mL) in the presence of *m*-cresol (10 μL) in an ice-bath for 1.5 h, then treated with DTT (8.5 mg, 100 equiv) at room temperature overnight. The product was purified by gel-filtration on Sephadex® G-10 using 1 N AcOH as an eluent and lyophilized (3.2 mg). The obtained powder and **2** (1.5 mg, 1.5 equiv) were dissolved in 6 M guanidine HCl containing 100 mM Tris HCl (pH 8.0) (300 μL) and stirred at room temperature for 48 h. The product was purified as stated above to give the desired four-helix-bundle protein **6**. Yield 0.71 mg (13%). LSIMS: *m*/*z* 9182.0 $(M+H)^+$ (expected 9182.1).

Preparation of the S4 peptide segments (7–10). These peptide segments were prepared by Fmoc-solid-phase peptide synthesis using a Rink amide resin basically in the same manner as stated in the preparation of **2**. Yield: **7**, 23%; **8**, 12%; **9**, 17%; **10**, 17%. LSIMS: *m*/*z* **7**, 2954.1 $(M+H)^+$ (expected 2953.9); **8**, 2981.3 $(M+H)^+$ (expected 2981.0); **9**, 2932.9 $(M+H)^+$ (expected 2932.9); **10**, 2952.6 $(M+H)^+$ (expected 2952.0). Fidelity of the products was also confirmed by amino acid analysis after 6 N HCl hydrolysis (110 °C, 24 h).

Assembling of the S4 segment in repeat IV on the template (14). Assembling of the S4 peptide segment **10** was conducted basically in the same manner as in the construction of **6**. Yields in the respective steps are as shown in Figure 5. LSIMS: *m*/*z* **11**, 4139.4 $(M+H)^+$ (expected 4140.1). TOFMS: *m*/*z* **12**, 6939.1 $(M+H)^+$ (expected 6947.5); **13**, 9740.0 $(M+Na)^+$ (expected 9739.9); **14**, 12584.7 $(M+H)^+$ (expected 125584.4).

Assembling of the S4 segments in repeat I to IV on the template (18). Assembling of the S4 peptide segments **7** to **10** was conducted basically in the same manner as in the construction of **6**. Yields of the respective steps are as shown in Figure 6. LSIMS: *m*/*z* **11**, 4170.0 $(M+H)^+$ (expected 4170.1); **12**, 6946.4 $(M+H)^+$ (expected 6946.4). TOFMS: *m*/*z* **13**, 9771.4 $(M+H)^+$ (expected 9727.8); **14**, 12596.8 $(M+H)^+$ (expected 2952.0).

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