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Introducing Branches into a Self-Assembling Peptide Fiber**

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Abbreviations: εAhx - ε-aminohexanoic acid; βAla - β-alanine; Aloc - allyloxycarbonyl; Boc - *tert*-butyloxycarbonyl; *t*-Bu - *tert*-butyl; CD - circular dichroism; DCM - dichloromethane; DIPEA - diisopropylethylamine; DMF - *N*,*N*-dimethylformamide; EDT - 1,2-ethanedithiol; EPPS - *N*-2-Hydroxyethylpiperazine-*N*'-3-propanesulphonic acid; HPLC - high pressure liquid chromatography; Fmoc - 9-fluorenylmethoxycarbonyl; MALDI-Tof MS - matrix assisted laser desorption/ionization-time of flight mass spectrometry; Mbh - dimethoxybenzhydryl; MOPS - 3-(*N*-morpholino)-propanesulfonic acid; Mtt - 4 methyltrityl; Pmc - 2,2,5,7,8-pentamethylchroman-6-sulfonyl; RP-HPLC - reversed phase high performance liquid chromatography; SEM - scanning electron microscopy; SPPS - solid-phase peptide synthesis; TBTU - 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TEM - transmission electron microscopy; TFA - trifluoroacetic acid; TIS - triisopropylsilane; Trt - trityl.

Material and Methods. Peptides were assembled on a Pioneer Peptide Synthesis System (PE Applied Biosystems, CA, USA). All reagents and resins were purchased from Applied Biosystems (Warrington, UK) or CN Biosciences (Nottingham, UK). Analytical and semi-preparative gradient RP-HPLC was performed on a JASCO HPLC system (Model PU-980, Tokyo, Japan) using Vydac C_{18} analytical (5 μm, 4.6 mm i.d. x 250 mm) and semi-preparative (5 μm, 10 mm i.d. x 250 mm) columns. Both analytical and semi-preparative runs used a 20-40% B gradient over 45 min at 3 mL/min (semi-preparative), or 1 mL/min (analytical) where buffer A was 5% aqueous CH_3CN, 0.1% TFA, and buffer B was 95% aqueous CH_3CN, 0.085% TFA. PD-10 columns (Sephadex G-25 M) were purchased from Amersham Biosciences (Buckinghamshire, UK). Mass spectra were recorded on a TofSpec E Matrix Assisted Laser Desorption Ionization (MALDI) spectrometer (Micromass Ltd, Manchester, UK). CD experiments were conducted on a JASCO J-715 spectropolarimeter fitted with a Peltier temperature controller (Tokyo, Japan).

Peptide Design. Peptide sequences were designed on the basis of principles described elsewhere.[3] T-SAF peptides were made to direct assembly of orthogonally conjoined fibers (Figure S1, left). A control T-shaped peptide (T-terminator) was designed to inhibit fibrillogenesis if co-assembled with the linear peptides (Figure S1, right).

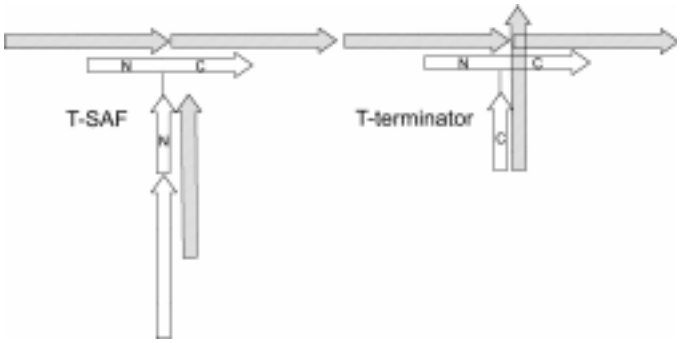

Figure S1. Schematic representation of co-assembly of linear and complementary SAF peptides with branch-forming (T-SAF, left) and assembly-terminating (T-terminator, right) T-shaped peptides.

Synthesis strategy. *Step-wise Fmoc-based solid phase approach*. Peptide **2** was synthesized and left on the resin. The lysinyl residue at position 14, which was protected by either an allyl[5d] or a 4-methyltrityl (Mtt)[5a] group, was selectively deprotected. The resulting free ε-amino group of lysine-14 was used to initiate the synthesis of peptide **3**: first, three βAla units were coupled sequentially, and then the remainder of the sequence of **3** was added by step-wise synthesis. Products were fully deprotected and cleaved from the resin for analysis by RP-HPLC and MALDI-TOF mass spectrometry. Using either protecting group at lysine-14, this approach gave a mixture of products, which did not yield any of the target T-SAF peptide.

Segment-condensation approach. Peptide **3** (including the three *C*-terminal βAla units) was synthesized and cleaved from the resin (H-βAla-2-ClTrt resin) under mild conditions (0.5% TFA in DCM) to leave all side chains protected, the *N*-terminus Boc-protected and only the *C*-terminal carboxylate free. Peptide **2** was synthesized, left on the resin and lysine-14 was again selectively deprotected. Peptide **3** was then coupled to the resin-bound peptide **2** using standard Fmoc coupling conditions to form an isopeptide bond between the ε-amino group of the deprotected lysine of **2** and the free *C*-terminus of **3**. Yields, <30% (homogeneous product).

Orthogonal extension of lysine-14. The *C*-terminal half of peptide **2** was synthesized up to and including lysine-14, which was protected with an Mtt group. At this point the standard Fmoc linear synthesis was punctuated as follows: the Mtt was selectively removed keeping all other protecting groups and the peptide-resin linkage intact. The newly formed free ε-amino group was neutralized with pyridine and reacted with activated carboxyl groups of Fmoc-βAla, or Fmoc-εAhx. Subsequent treatment of the resin with 20% piperidine in dimethylformamide removed both Fmoc groups leaving free α- and side-chain amino groups of the modified lysine. This was reacted with Mtt chloride to give lysine bi-protected with Mtt. As N^{α}-Mtt groups are more acid-sensitive than side-chain Mtt groups,[5a] the *N*-terminal Mtt was selectively removed using 0.5% TFA in dichloromethane for 60 min at 0°C with stirring. The second (*N*-terminal) half of **2** was then synthesized leaving the *N*-terminus Boc protected. The side-chain Mtt group of the extended lysine-14 was then selectively removed and the resulting amino group used to initiate the synthesis of a slight variant of peptide **3** that had two *C*-terminal βAla units rather than three. Yields, <60%.

Incorporating a pre-synthesized Fmoc-Lys(Mtt-βAla)-OH, or Fmoc-Lys(Mtt-εAhx)-OH in place of lysine-14 during the punctuated synthesis of **2** improved yields to >80%, and >90%, respectively.

Thioalkylation. The lysine-14 of **2** was activated with an α-bromoacetyl group, and **3** was remade with a *C*-terminal cysteine to set up the reaction with the formation of a thioether linkage. The cysteine variant of **3** was made using standard Fmoc-SPPS. Modified versions of peptide **2** were made with and without the βAla-extended lysine. The α-bromoacetyl moiety was added to the free side chain amino group of the resin-bound peptide with yields of >90% and <60%, respectively. Ligations were carried out at pH 7.5-8.2 with a three-fold excess of the cysteine-containing peptide **3**. With the unextended bromoacetyl activated **2** the reaction took 26 hours to complete (none of the bromoacetyl could be detected). With bromoacetyl moiety spaced from **2** with βAla the time was reduced to 20 hours. In both cases a homogeneous product was obtained in about the same ~70% yield (at pH 8.2).

Routine peptide synthesis was carried out by the combination of standard Fmoc/*t*Bu solid phase protocols on a PEG-PS-resin with TBTU/DIPEA as coupling reagents. Boc and Pmc were used as side-chain protecting groups for all lysines and arginines, except the branch-forming lysine which was protected by Mtt or Aloc. For asparagines and glutamines the more acid-stable Mbh protecting groups were preferred to Trt. Removal of Mtt groups was carried out by treatment with 2% TFA cleavage mixture (93:2:5 DCM/TFA/TIS) three times for 10 min each. Aloc groups were removed according to the published protocol.[5d] A 95% TFA mixture (95:2.5:2.5 TFA/water/TIS) was used as the post-synthesis cleavage cocktail. For the deprotection of the cysteine-containing branch segment another 93.5% TFA mixture (93.5:2.5:2.5:1.5 TFA/water/EDT/TIS) was used. The purification of all peptides was realized by semi-preparative RP-HPLC, and their purities confirmed by analytical RP-HPLC. The peptide identities were verified by MALDI-Tof mass spectrometry with α-cyano-4-hydroxycinnamic acid as the matrix: for the Lys(βAla)-based T-shaped peptides $[M+H]^+$ m/z 5172 (calc), 5173.4 (found); $[M+Na]^+$ m/z 5195.7 (found); for the Lys(εAhx)-based peptides $[M+H]^+$ m/z 5214 (calc), 5215.2 (found), $[M+Na]^+$ m/z 5237.2 (found). The doubly $[M+2H]^{2+}$ and triply $[M+3H]^{3+}$ charged ions were also present in both cases. The Lys(βAla)-based T-shaped control peptide (the tyrosine residue of the

C-terminal half of peptide **2** in peptide **3** was replaced by glutamine residue) $[M+H]^+$ m/z 5047 (calc), 5049 (found), $[M+K]^+$ m/z 5086.2 (found).

Fmoc-Lys(Mtt-βAla)-OH and Fmoc-Lys(Mtt-εAhx)-OH. The syntheses of pre-stretched Fmoc-Lys(Mtt-βAla)-OH and Fmoc-Lys(Mtt-εAhx)-OH were accomplished on a pre-loaded Fmoc-Lys(Mtt)-Wang-resin with the use of Boc-βAla-OH or Boc-εAhx-OH respectively followed by resin cleavage with the 95% TFA cocktail. The amorphous Fmoc-Lys(NH_2-βAla)-OH and Fmoc-Lys(NH_2-εAhx)-OH obtained were converted to the corresponding Mtt-protected derivatives using the published protocol.[5a] MS $[M+H]^+$: Fmoc-Lys(Mtt-βAla)-OH, m/z 695.8 (calc), 696.3 (found); Fmoc-Lys(Mtt-εAhx)-OH, m/z 737.9 (calc), 739.1 (found).

Thioalkylation. The ligations were preformed in 0.1 M MOPS-KOH (pH 7.5-7.8) or in 0.1 M EPPS-NaOH (pH 7.9-8.2) at 0.5 mM peptides (1 mL) at room temperature (22°C) and were monitored by MALDI-Tof (2 μL aliquots). After completion of the reactions, mixtures were desalted using PD-10 columns eluted with 1N acetic acid. The final purification of the conjugates was realized by RP-HPLC. Yields for all the conjugates obtained at pH 8.2 were within 63-70%. MS $[M+H]^+$: T-shaped with Lys(βAla), m/z 5332 (calc.), 5332.8 (found); with Lys, m/z 5261 (calc.), 5262.1 (found). The triply $[M+3H]^{3+}$ charged ions were also presented.

Figure S2. TEM (A-H) and SEM (I-K) images of SAF-peptide fibers formed in the absence (A-D) and the presence (E-K) of the branch-forming T-SAF peptides.

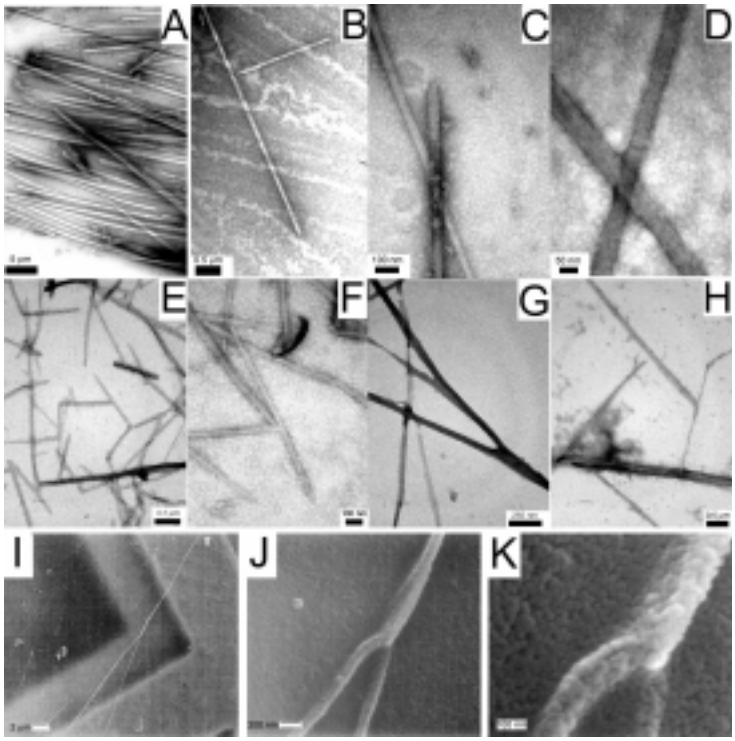

Table S1. HPLC analysis of incorporation of T-SAF peptides into fibers.

Starting T-SAF concentration (μM)	Peptide incorporated into fibers (μM)		
	SAF-1	SAF-2	T-SAF
100	60.7± 4.52	57.3 ± 4.1	6.94 ± 0.13
1	62.1± 3.7	60.6 ± 3.2	0.87 ± 0.06