Supporting Information

Fibrillisation of Ring-Closed Amyloid Peptides

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Acknowledgements

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Experimental Methods

Fmoc- Lys(Boc)-Wang resin (150-300 μm, 0.1- 0.4 mmol g^{-1}) and Fmoc-Asp(All)-OH were purchased from Iris Biotech GMBH (Germany). Fmoc-Val-OH, Fmoc-Leu-OH, Fmoc-Phe-OH, Fmoc-Ala-OH and Fmoc-Glu-OtBu were purchased from Novabiochem (UK). Trifluoroacetic acid (TFA), piperidine, triisopropylsilane (TIPS), 1, 2-dichloroethane (DCE) and Hoveyda-Grubbs Catalyst (second generation) were purchased from Sigma-Aldrich. HOBt (1-hydroxybenzotriazole), HBTU ([2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate], DIPEA (diisopropylethylamine) and NMP (N-methylpyrrolidone) were obtained from AGCT Bioproduct Ltd (UK). Water (HPLC grade), acetonitrile (HPLC grade), dimethylformamide (DMF), dimethyl sulfoxide (DMSO), methanol (MeOH) and dichloromethane (DCM), acetone and diethyl ether were purchased from Fisher Scientific (UK). ^{1}H-NMR spectra were recorded on Bruker Nanobay 400 MHz or Bruker Avance III 700 MHz spectrometers with chemical shifts (δ) given as ppm relative to TMS (tetramethylsilane, 0.00 ppm) with a residual resonance of H_2O in CD_3OD or $(CD_3)_2SO$ present at *ca* 4.87 or 3.33 ppm respectively. Mass spectroscopy was performed using a ThermoFisher Scientific Orbitrap XL FTMS (Fourier Transform Mass Spectrometer) with an electrospray (ES) interface. A Discovery® BIO Wide Pore C18 SUPELCO HPLC Column (10 μm particle size, L × I.D. 15 cm × 10 mm), Perkin Elmer Series 200 Autosampler, Perkin Elmer Series 200 Peltier Column Oven and Perkin Elmer Series 200 UV/vis detector were set up for semi-

preparative reverse phase HPLC (RP-HPLC): 0.1% TFA in HPLC grade water and 0.1% TFA acetonitrile were employed as the eluents. During RP-HPLC process a gradient solvent was used: the acetonitrile portion increased linearly from 0% to 90% over 20 min and then decreased linearly to 0% over 10 min with flow rates 4 ml/min at 65 °C. The RP-HPLC process was monitored with UV/vis detector at 254 nm.

Synthesis of peptides I, II and III

The complete sequence of the peptide K(Boc)LVVD(OAll)D(OAll)FFAE(OtBu)Fmoc attached on Wang-resin shown in Scheme 1 was assembled from the C-terminus towards the N-terminus by solid phase peptide synthesis (SPPS), using standard FastMoc chemistry [Fmoc (9-fluorenylmethyloxycarbonyl) as protecting group and activation of Fmoc-amino acid with HBTU/HOBt/DIPEA in NMP to form the peptide bond].[1] The obtained resin-bound peptide was divided into two parts. The first part was used to prepare peptide **I** *via* removal of Fmoc groups with 20% piperidine in NMP, followed by treatment with cleavage cocktail (95% TFA, 2.5% triisopropylsilane, and 2.5% water)(SI Fig.1), and the second part was employed for ring-closing metathesis between the two adjacent *O*-allyl esters on the aspartic acid residues D(OAll)s of the resin-bound peptide catalyzed by (second generation) Hoveyda-Grubbs catalyst on the solid-phase.[2, 3] The metathesis was carried out at 60 °C in 1,2-dichloroethane (DCE) for 4d with *ca.* 0.6 mmol resin-bound peptide (based on Fmoc- Lys(Boc)-Wang resin) and 11 mol% of (second generation) Hoveyda-Grubbs catalyst. After the reaction, the N-terminal Fmoc group of the resin was removed with 20% piperidine in NMP and the crude products were cleaved from the resin by the method as above, followed by purification with semi-preparative reverse phase HPLC (RP-HPLC). HPLC (SI Fig. 2) shows that the crude products produced from ring-closing metathesis still contained some of peptide **I** that had not undergone metathesis. This is presumably due to the low loading of catalyst - 11 mol% of second generation Hoveyda-Grubbs catalyst was

employed in this work, while Arora[3] reported that the highest conversion of ring-closing metathesis based on solid-phase synthesis in the range of 60-75% could be achieved with at least 15 mol% of the catalyst. Another possible reason could be that the partially remaining peptide **I** was trapped in the pores within Wang-resin beads, and was thus unable to reach the catalyst and hence to undergo the metathesis reaction.

ESI-MS mass spectroscopy proved that peptide **II** was generated by intra-molecular ring-closing metathesis (SI Table 1). The geometry of the double bond of **II** was assigned by NMR spectrometry (coupling with multiple decoupling experiments, in which all methylene protons adjacent to the olefin protons in **II** were simultaneously irradiated using two frequencies to determine the coupling constants of the AB quartet formed by the two olefin protons). The corresponding J_{AB} value of the double bond for **II** is 15.4 Hz, indicating an *E*-isomer conformation (SI Fig. 4). Furthermore, the ESI-MS mass spectrum shows that the reaction also afforded alkene-bridged closed peptide dimer **III**. We believe that dimer **III** formed by double ring-closing metathesis between two resin-bound peptide molecules or *via* monocyclic peptides, such as peptide **I**, proceeding to undergo further metathesis during a long reaction time, typically four days in this work.[4] Dimer **III** contained inseparable mixtures of *E* and *Z* isomers and the olefin configuration has not been assigned in this work (SI Fig. 4 and SI Table 1). In addition, species, **IV** and **V**, were obtained (SI Fig. 5) with their main peaks centred at 2383.2211($[M+H]^+$) in both ESI-MS spectra (SI Fig. 6- SI Fig. 9, SI Table 1). This is ascribed to the presence of peptide K(Boc)LVVD(OAll)D(OAll)FFE(OtBu)Fmoc, i.e. the target product without the alanine residue, assembled on Wang-resin in the solid phase peptide synthesis (SI Scheme 1, shown in Route B). Thus, under the ring-closing metathesis condition, a

mixture of open peptide dimers could be formed from KLVVD(OAll)D(OAll)FFE. Due to the conformation (*E/Z*) around the double bond and the variation of alkene-bridged patterns between D(OAll) residues, *eg.* the alkene-bridged open peptide dimer *E*-isomer based on D1-D2, at least inseparable six isomers with open peptide dimer structures proposed in SI Scheme 2 could be associated with **IV** and **V**. However, identification of each isomer is outside the scope of this work.

Synthesis of resin-bound peptide

A fully automated peptide synthesizer (433A Applied Biosystems), which allowed for direct conductivity monitoring of Fmoc deprotection was used in preparing resin-bound peptide or a batchwise synthesis (> 0.50 mmol scale) was carried out using a fritted reaction glass vessel equipped with a positive nitrogen pressure or vacuum, whereby appropriate application of positive nitrogen pressure generated sufficient bubbles for effective mixing for a coupling reaction while washing was achieved by pumping solvent through the resin bed. Pre-loaded Fmoc- Lys(Boc)-Wang resin was used as the solid support, which was swollen in NMP, followed by removing the Fmoc protecting group with 20% piperidine in NMP. Four times excess of each Fmoc-amino acid (FmocXxx-OH) was used in each coupling cycles, in which the Fmoc-amino acid was dissolved in NMP and activated with HBTU/HOBt in DMF and DIEA, then transferred to reaction vessel to effect each coupling. After each coupling a solution of 20% piperidine/NMP was introduced and allowed to remove the Fmoc protecting group. Each coupling and Fmoc deprotection was followed by washing the resin with NMP. After the final Fmoc amino acid, Fmoc-Glu-OtBu, was attached, the resin-bound peptide was removed from reaction vessel, washed with NMP, DCM and MeOH, respectively,d ried under vacuum overnight and divided into two parts (Part 1 and 2) for the next reactions.

Synthesis of peptide I

The N-terminal Fmoc group on the obtained resin-bound peptide (Part 1) was removed with a solution of 20% piperidine/NMP for 60 min, washed with NMP, DCM and MeOH, respectively, then treated with a mixture of 95% TFA, 2.5% triisopropylsilane and 2.5% water. The mixture was stirred at room temperature for approximately 4 h, followed by filtration. The cleaved resin was washed three times with TFA. During the cleavage the side chain protecting groups (Boc) were removed by TFA. The obtained peptide solution was collected and concentrated by evaporating TFA under vacuum, followed by precipitating in cold diethyl ether. The crude product was separated by centrifugation and decanting the supernatant. The crude peptide was redissolved in HPLC grade water/acetonitrile co-solvent and then purified by reverse phase HPLC (RP-HPLC). The fractions of the peptide from RP-HPLC were collected, followed by lyophilization to give a white solid, which was characterized by ESIMS and ^{1}H NMR. **Peptide I** as a white powder (RP-HPLC, t_R = 10.74 min): MS: calc. $[M+HCO+2H]^{2+}$ = 646.7, $[M+HCO+H]^+$ = 1292.5, found $[M+HCO+2H]^{2+}$ = 645.4, $[M+HCO+H]^+$ = 1291.7; ^{1}H NMR (700 MHz, DMSO-d_6) δ (ppm): 0.82 (m, 18H), 1.15-1.73 (m, 12H), 1.88 (m, 4H), 2.16-2.39 (m, 3H), 2.73 (m, 5H), 2.94 (m, 2H), 3.75 (m, 2H), 4.12 (m, 3H), 4.31 (m, 2H), 4.49 (m, 4H), 4.67(m, 1H), 4.95 (m, 1H), 5.04 (m, 1H), 5.16 (m, 2H), 5.25 (m, 2H), 5.27 (m, 2H), 5.84 (m, 2H), 7.17 (m, 10H), 7.56, 7.76, 7.88, 7.94, 8.00, 8.07, 8.15 [amide-H].

Ring-closing metathesis based on the resin-bound peptide

The resin-bound peptide (0.6 mmol, based on Fmoc- Lys(Boc)-Wang resin) was swelled in 10.0 mL of degassed anhydrous 1, 2-dichloroethane (DCE) in a dried flask under an argon atmosphere for 50 min. The Hoveyda-Grubbs catalyst (GII) (43 mg, 0.069 mmol) in 3 ml DCE was added to the flask and the mixture was stirred at 60 °C. After 4 d, the resin was filtrated and treated with DMSO: DMF (1:1) overnight to remove catalyst-derived impurities. It was filtered, washed with dichloromethane, methanol, NMP and acetone, followed by treatment with a solution of 20% piperidine/NMP for 60 min to remove the Fmoc group on the N-terminus. The resin was then washed with NMP, dichloromethane and methanol and stirred in 10 ml of the cleavage cocktail (TFA:H_2O:triisopropylsilane, 95:2.5:2.5) for 4 h, followed by filtration. The obtained peptide solution was collected and concentrated by evaporating TFA under vacuum, followed by precipitating in cold diethyl ether. The

crude product was separated by centrifugation and decanting the supernatant. The crude peptide was redissolved in HPLC grade water/acetonitrile co-solvent and purified by reverse phase HPLC. The fractions of the peptide were collected, followed by lyophilization to give a white solid, which was characterized by ESIMS and ^{1}H NMR.

Peptide II as a white powder (4 mg, RP-HPLC, t_R = 14.26 min): MS: calc. $[M+2H]^{2+}$ = 618.2063, $[M+H]^+$ = 1234.3967, found $[M+2H]^{2+}$ = 617.8126, $[M+H]^+$ = 1234.6373; ^{1}H NMR (700 MHz, MeOH-d_4) δ (ppm): 0.86 (m, 18H), 1.27-1.89 (m, 12H), 2.03-2.21 (m, 4H), 2.32-2.48 (m, 4H), 2.88-3.10 (m, 8H), 3.90 (m, 1H), 4.03-4.12 (m, 2H), 4.17 (m, 1H), 4.24-4.46 (m, 4H), 4.50-4.69 (m, 4H), 5.20 (m, 2H), 5.77 (m, 1H), 5.87 (m, 1H), 7.18 (m, 10H), 8.08, 8.20, 8.24, 8.60 [amide-H].

Peptide III as a white powder (10 mg, RP-HPLC, t_R = 15.19 min): MS: calc. $[M+3H]^{3+}$ = 823.9391, $[M+2H]^{2+}$ = 1235.4047, $[M+H]^+$ = 2469.8014, found $[M+3H]^{3+}$ = 823.7610, $[M+2H]^{2+}$ = 1235.1383, $[M+H]^+$ = 2469.2680; ^{1}H NMR (700 MHz, MeOH-d_4) δ (ppm): 0.81-0.97 (m, 36H), 1.24-1.77 (m, 24H), 1.90-2.21 (m, 8H), 2.30-2.44 (m, 8H), 2.87-3.01 (m, 16H), 3.64 (brs, 2H), 4.03-4.72 (m, 12H), 5.19-5.25 (m, 6H), 5.82 (m, 2H), 7.15-7.30 (m, 20H), 7.80, 8.08, [amide-H].

Characterization Methods

Cryogenic Transmission Electron Microscopy (Cryo-TEM).

Images for peptide **I** were obtained at Unilever Research, Colworth, Bedford, UK. Sample preparation was carried out using a CryoPlunge 3 unit (Gatan Instruments) employing a double blot technique. Approximately 3 µl of sample was pipetted onto a plasma etched (15 s) 400 mesh holey carbon grid (Agar Scientific) held in the plunge chamber at approx 90% humidity. The samples were blotted, from both sides for 0.5, 0.8 or 1.0 s dependant on sample viscosity. The samples were then plunged into liquid ethane at a temperature of -170 ˚C. The grids were blotted to remove excess ethane

then transferred, under liquid nitrogen to the cryo TEM specimen holder (Gatan 626 cryo holder) at -170 ˚C. Samples were examined using a Jeol 2100 TEM operated at 200 kV and imaged using a Gatan Ultrascan 4000 camera and images captured using DigitalMicrograph software (Gatan).

Images for peptides **II** and **III** were measured at EMEZ, ETH Zurich, CH. For sample preparation a manual plunger was used. 2.5 µl of sample were pipetted onto a plasma etched (45 s) 400 mesh lacey carbon grid (EMS). The samples were blotted on one side according to sample viscosity and then plunged into liquid ethane at a temperature of -170 ˚C. The grids were transferred, under liquid nitrogen to the cryo TEM specimen holder and examined in a FEI CM12 TEM operated at 100 kV using a Gatan CCD 794 camera. Images were captured using the DigitalMicrograph software (Gatan).

Negative Stain Transmission Electron Microscopy (TEM).

For the visualization of peptide **III**, negative stain TEM was also performed at EMEZ, ETH Zurich. A 4 µl aliquot of solution (0.25 wt% or 0.5 wt% in water) was applied for one minute on carbon-coated 400 mesh copper-grids, which were freshly glow discharged. After washing twice in double-distilled water for 5 s, the sample was stained in 4 µl of 2 % uranyl acetate for 1 s, and in another 4 µl for 15 s. Following each step, the excess moisture was drained along the periphery using a piece of filter paper. Dried grids were examined by TEM (FEI, model Morgagni, NL), operated at 100 kV.

Small-Angle X-ray Scattering/Wide-Angle X-ray Scattering (SAXS/WAXS). Experiments were performed on beamline ID02 at the European Synchrotron Radiation Facility (ESRF), Grenoble France. The X-ray wavelength was $\lambda = 0.0995$ nm and the sample-detector distance was 1.30 m. The wavenumber $q = 4\pi\sin\theta/\lambda$ scale (2θ is the scattering angle) is calculated based on the pixel size of the CCD detector and the sample-detector distance.

X-ray Diffraction (XRD).

Measurements were performed on stalks prepared by drying filaments from solutions containing 1.7 – 3 wt% peptide. Solutions of the peptide were suspended between the ends of wax-coated capillaries and dried. The stalks were mounted (vertically) onto the four axis goniometer of a RAXIS IV++ x-ray diffractometer (Rigaku) equipped with a rotating anode generator. The XRD data was collected using a Saturn 992 CCD camera.

Fourier Transform Infra-Red (FTIR) Spectroscopy

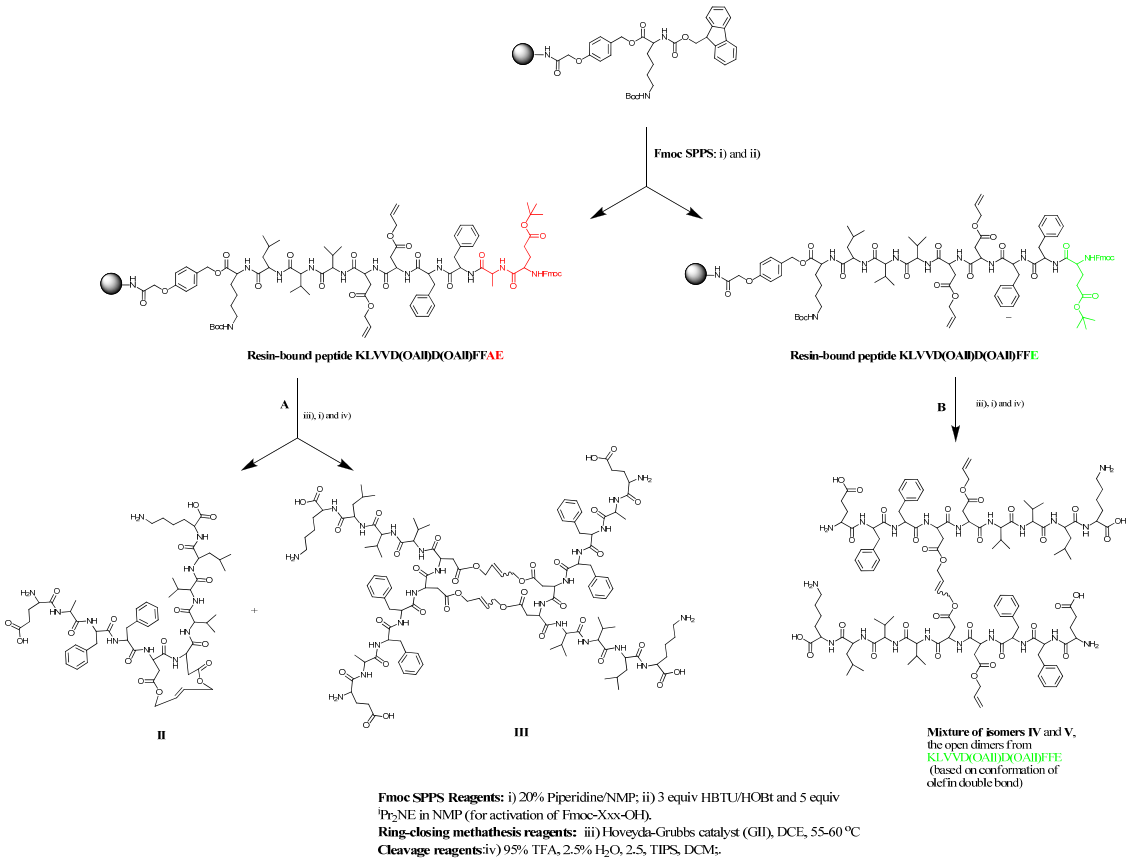
Spectra were recorded using a Nexus-FTIR spectrometer equipped with a DTGS detector and a multiple reflection attenuated total reflectance (ATR) system. Solutions of the three peptides in D_2O (0.5 wt.% or 2 wt%) were sandwiched in ring spacers between two CaF_2 plate windows (spacer 0.0125 mm). All spectra were scanned 128 times over the range of 4000-950 cm^{-1}.

Molecular Dynamics Computer Simulations.

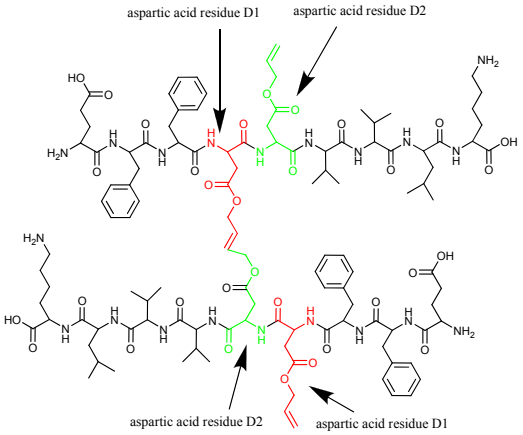
Computational modelling (molecular mechanics with charge-equilibration and hydrogen bonding) was carried out on an SGI O2 workstation, using the Dreiding II force field within Cerius2 (Accelrys Inc, San Diego, version 3.5). Local minima only were obtained.

SI Table 1 Mass spectrometry for peptides **II**, **III**, **IV** and **V**

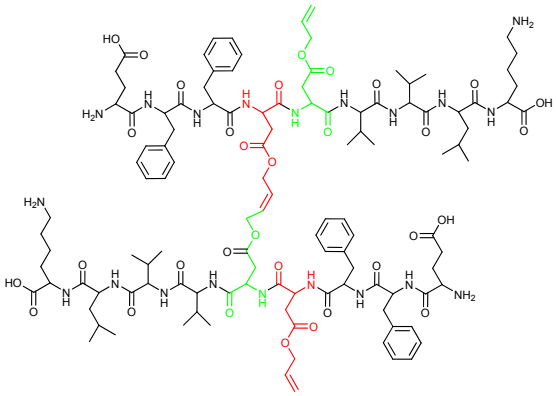
Peptide	Calcd. $[M+H]^{+}$	Calcd. $[M+2H]^{2+}$	Calcd. $[M+3H]^{3+}$	Calcd. $[M+4H]^{4+}$	Found $[M+H]^{+}$	Found. $[M+2H]^{2+}$	Found $[M+3H]^{3+}$	Found $[M+4H]^{4+}$
II	1234.40	618.20			1234.64	617.82		
III	2469.80	1235.40	823.94	618.21	2469.27	1235.14	823.76	618.32
IV	2383.71	1192.36	795.24	596.68	2383.22	1191.11	794.41	596.06
V	2383.71	1192.36	795.24	596.68	2383.22	1191.11	794.74	596.31



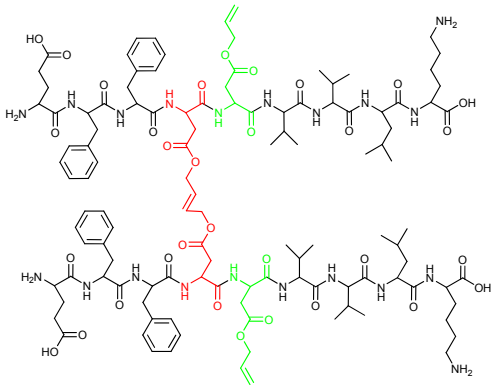
SI Scheme 1. Synthesis scheme for **II** and **III,** and proposed formation of by-products peptides **IV** and **V** (route B). The possible isomers in the mixture of **IV** and **V**, proposed in **SI Scheme 2**, were assumed to be open dimers, based on the conformation of olefin double bond and alkene-bridged patterns, generated from KLVVD(OAll)D(OAll)FFE in the reaction.



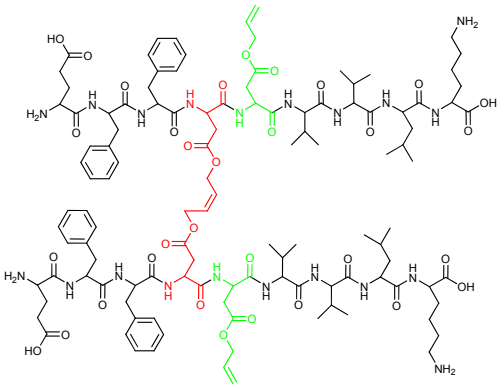
Alkene-bridged peptide E-isomer based on D1-D2



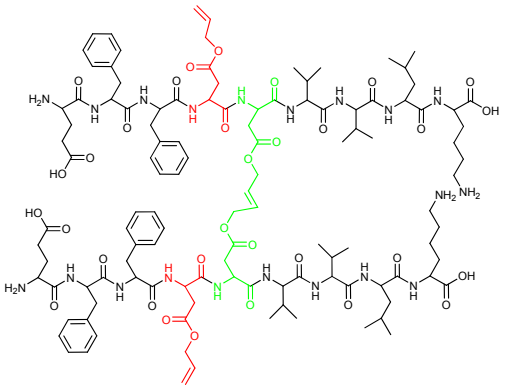
Alkene-bridged peptide Z-isomer based on D1-D2



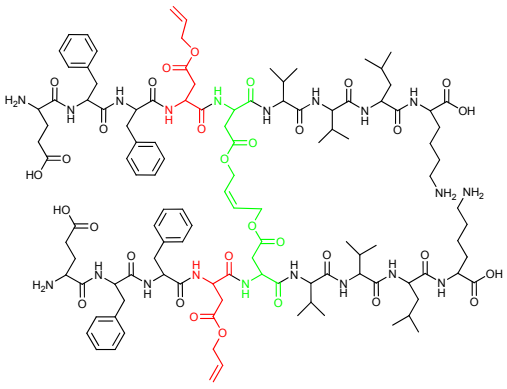
Alkene-bridged peptide E-isomer based on D1-D1



Alkene-bridged peptide Z-isomer based on D1-D1

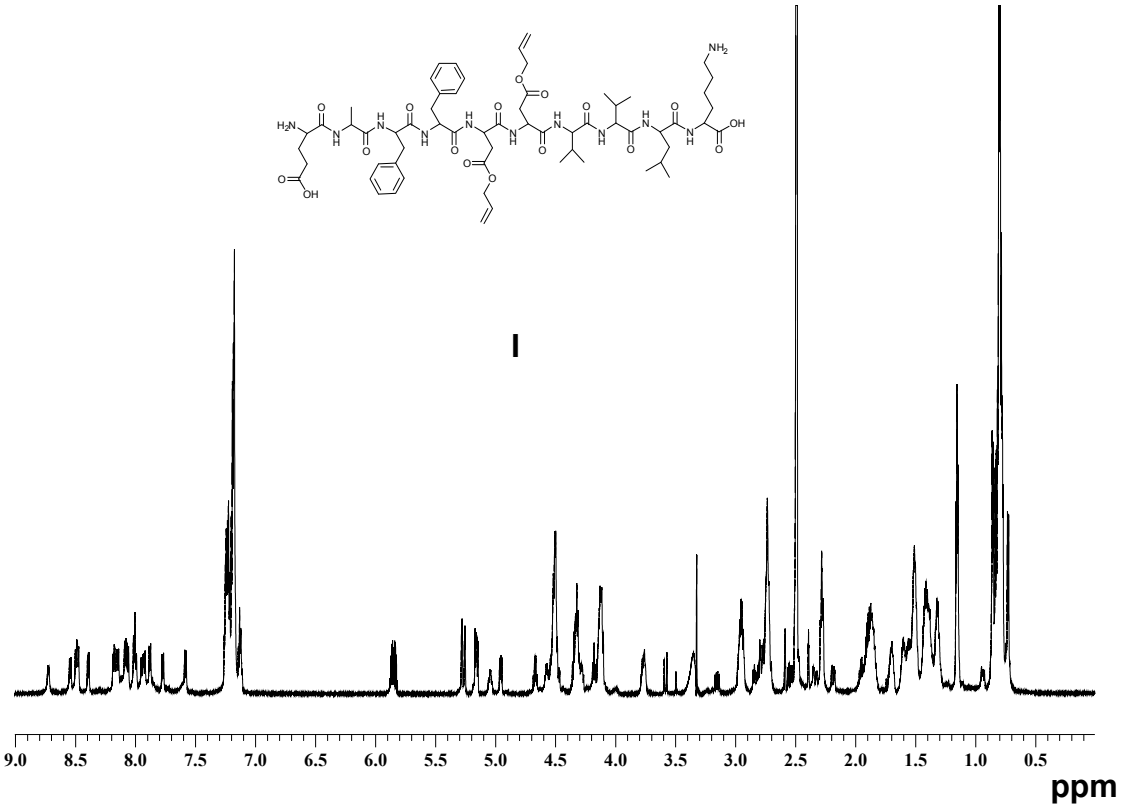


Alkene-bridged peptide E-isomer based on D2-D2

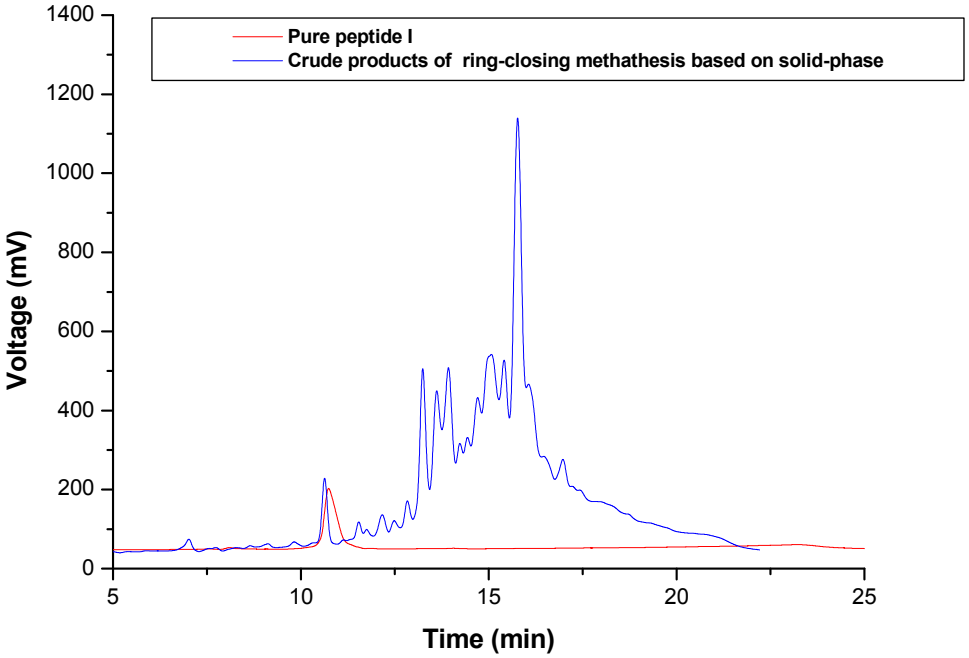


Alkene-bridged peptide Z-isomer based on D2-D2

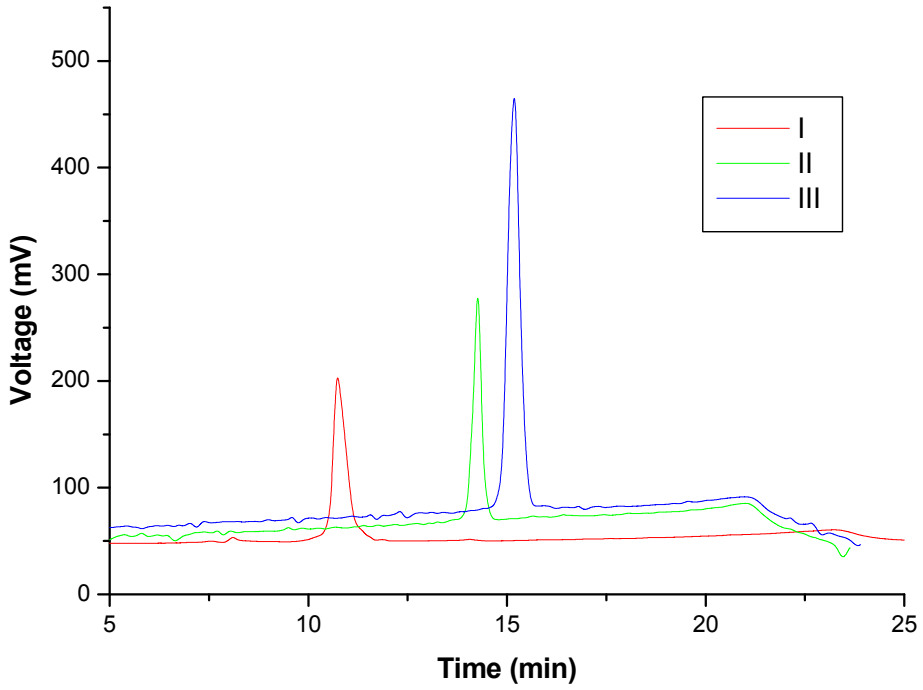
SI Scheme 2. Proposed isomers as open dimers generated from KLVVD(OAll)D(OAll)FFE in the reaction (**SI scheme 1**, route B) assigned as the mixture of peptides **IV** and **V**.



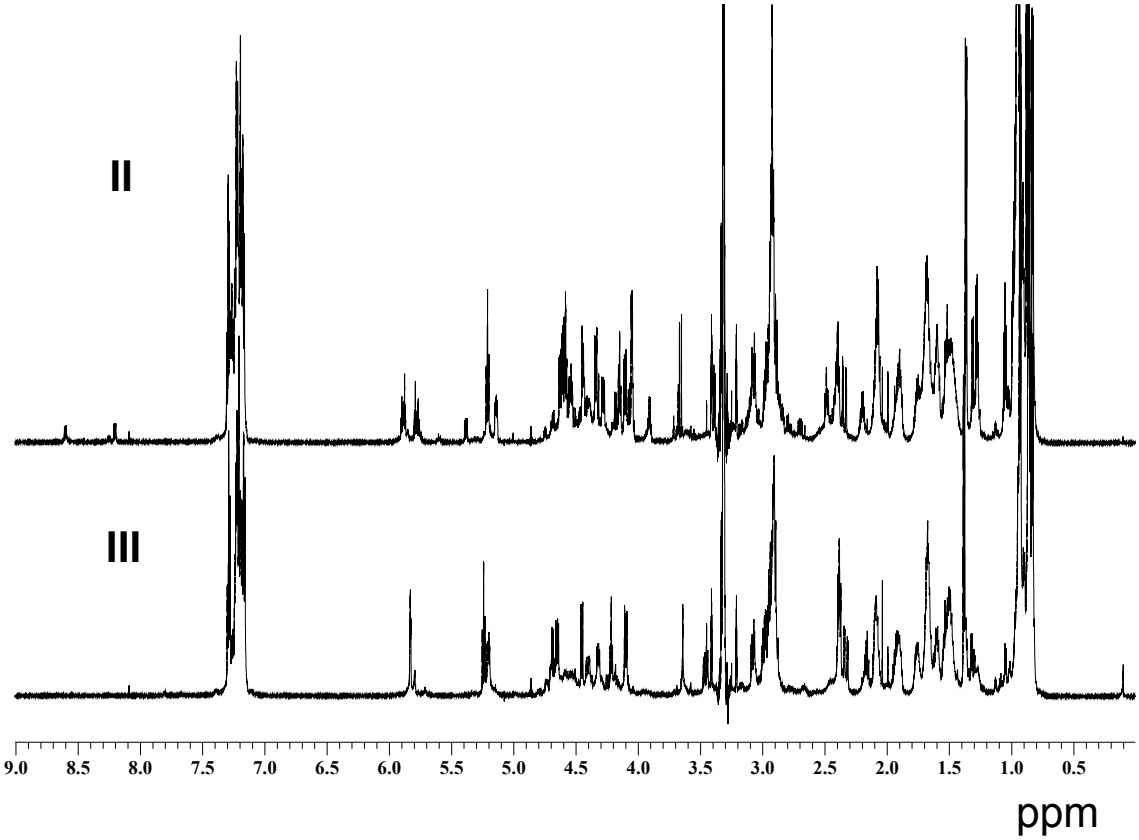
S1 Fig.1. ^{1}H NMR spectra of peptide **I** (solvent: DMSO-d_6, with H_2O suppressed).



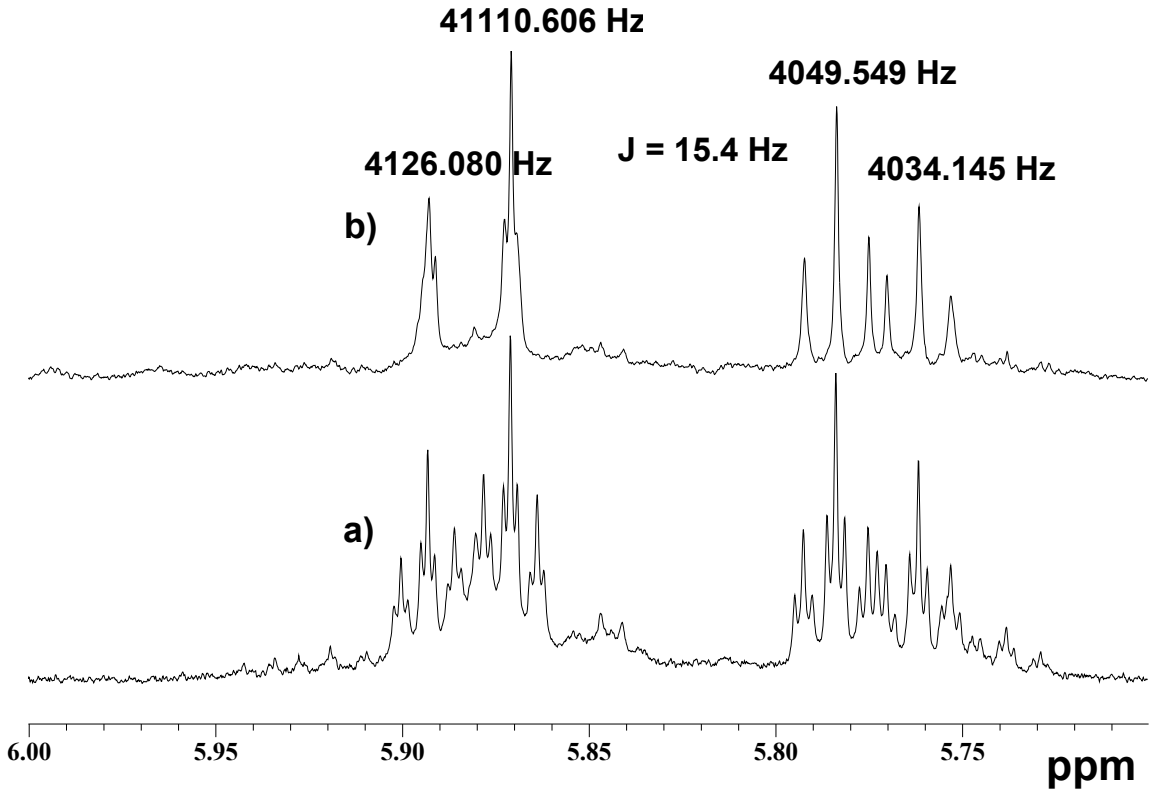
SI Fig. 2. RP-HPLC for peptide **I** and the crude products of ring-closing metathesis based on solid-phase synthesis.



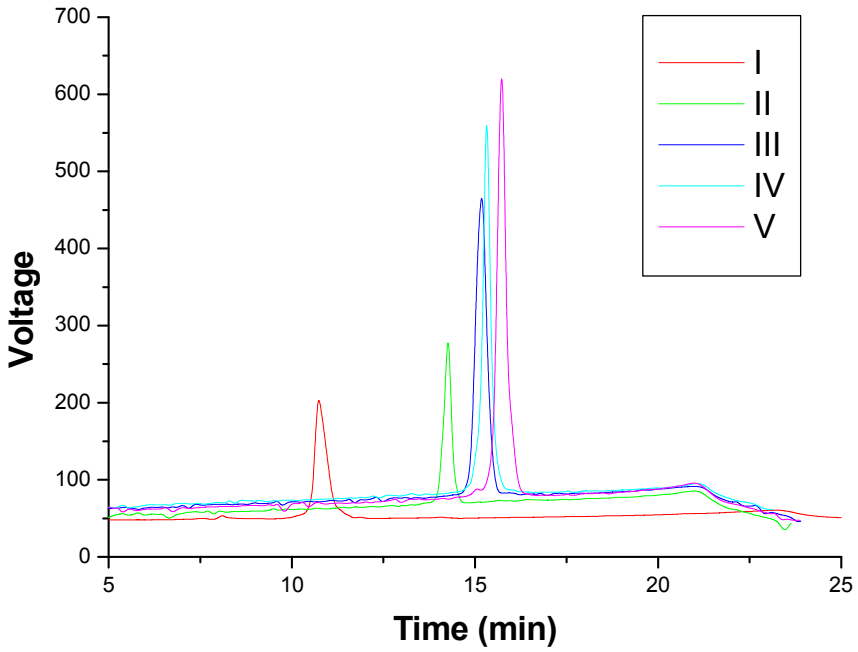
SI Fig. 3 RP-HPLC for peptide **I** and alkene-bridged peptides **II**, **III**.



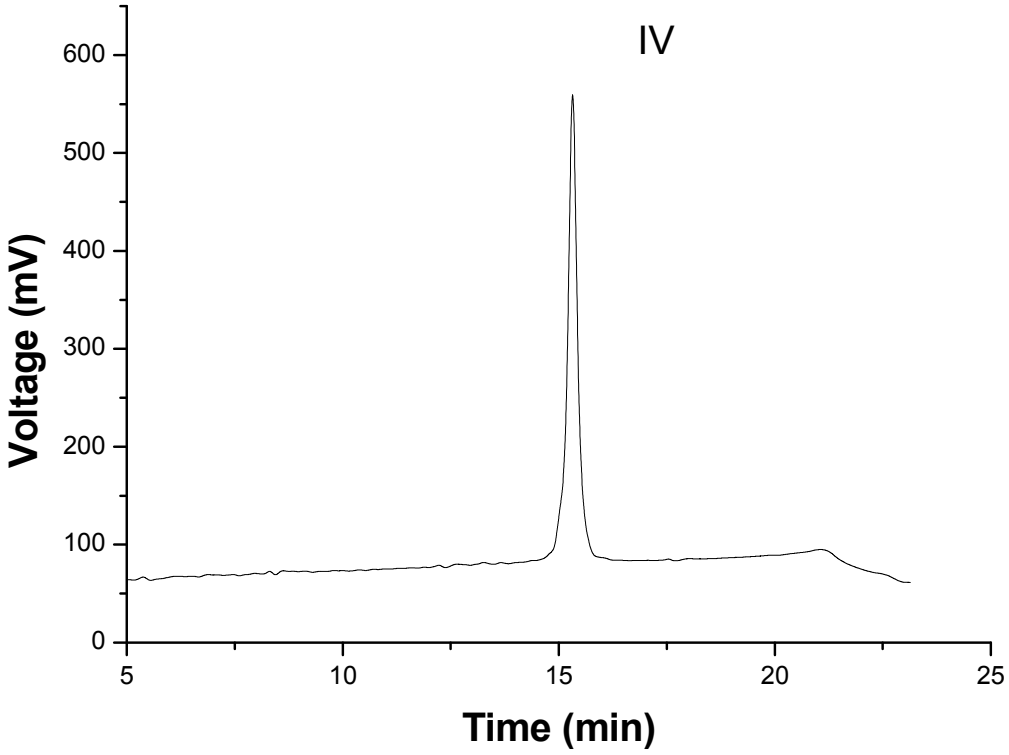
SI Fig. 4a. ^{1}H NMR spectra of peptide **II** and **III** (solvent: MeOH-d_4, with H_2O suppressed).



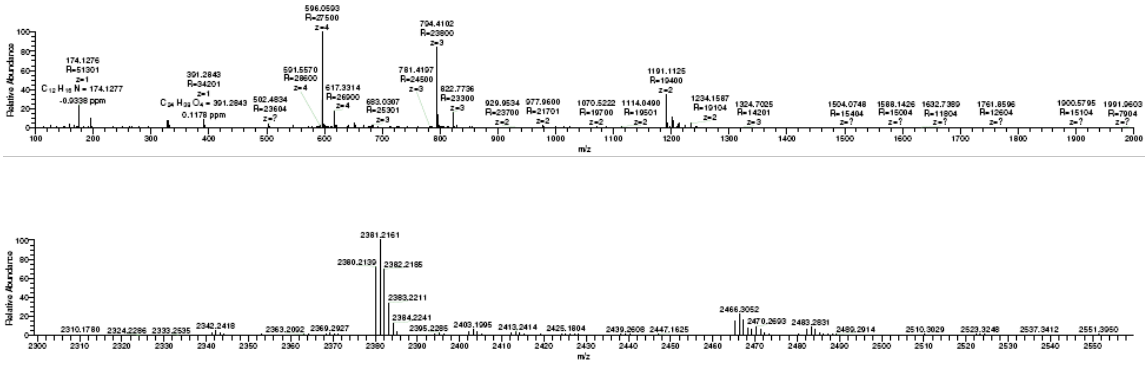
SI Fig. 4b. Determination of conformation of olefin double bonds in **II** by ^{1}H NMR homonuclear decoupled experiment a) Before homodecoupling ; b). After homodecoupling experiment, J_{AB} = 15.4 Hz).



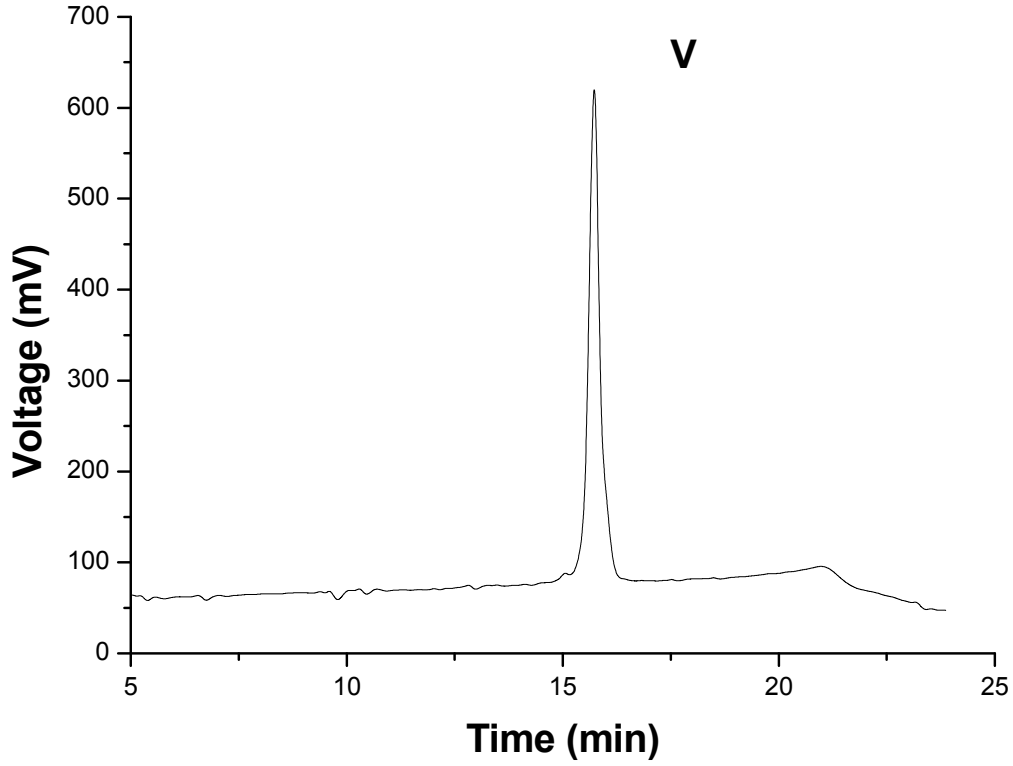
SI Fig. 5. RP-HPLC for peptide **I**, **II**, **III**, **IV**, and **V**.



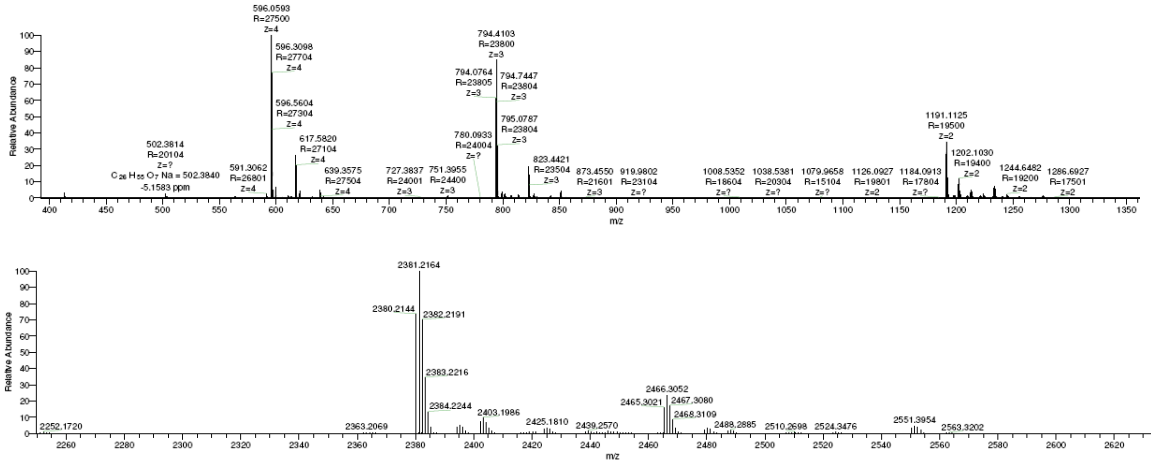
SI Fig.6. RP-HPLC for peptide **IV**.



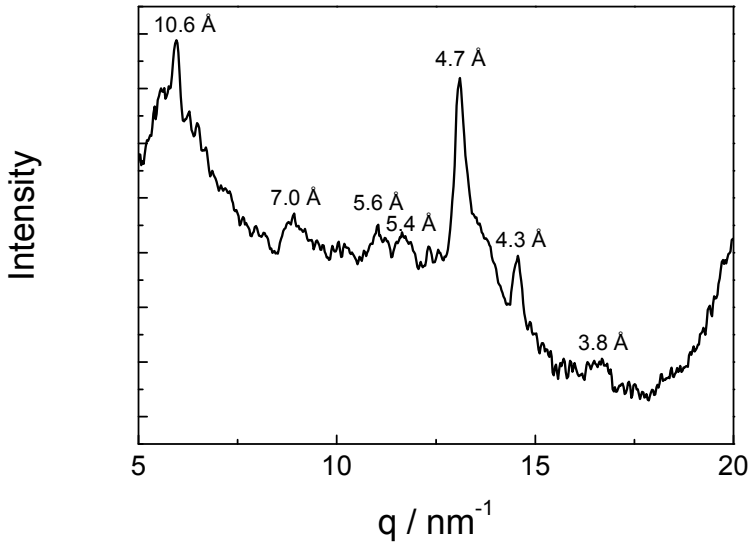
SI Fig. 7 FTMS-ESI mass spectra for peptide **IV**



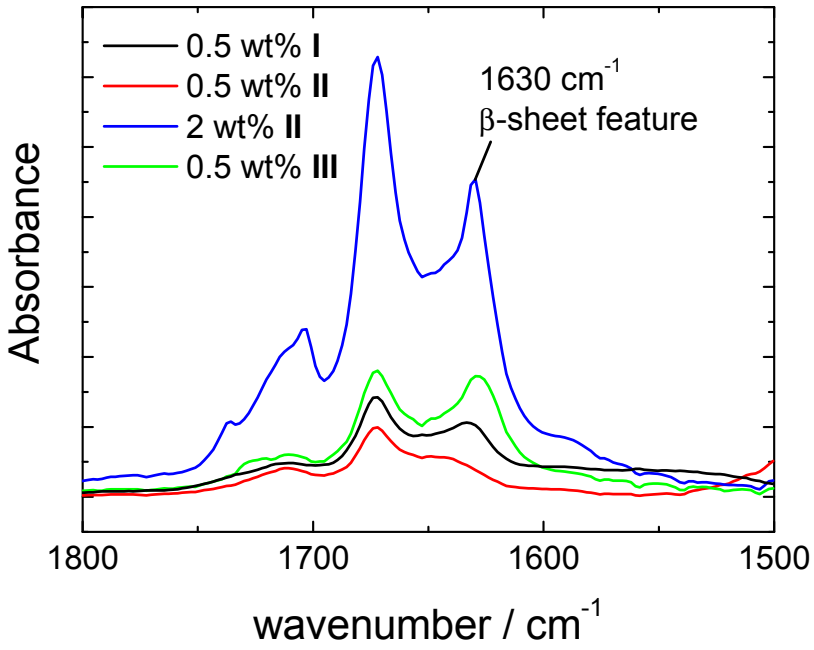
SI Fig. 8. RP-HPLC for peptide **V**.



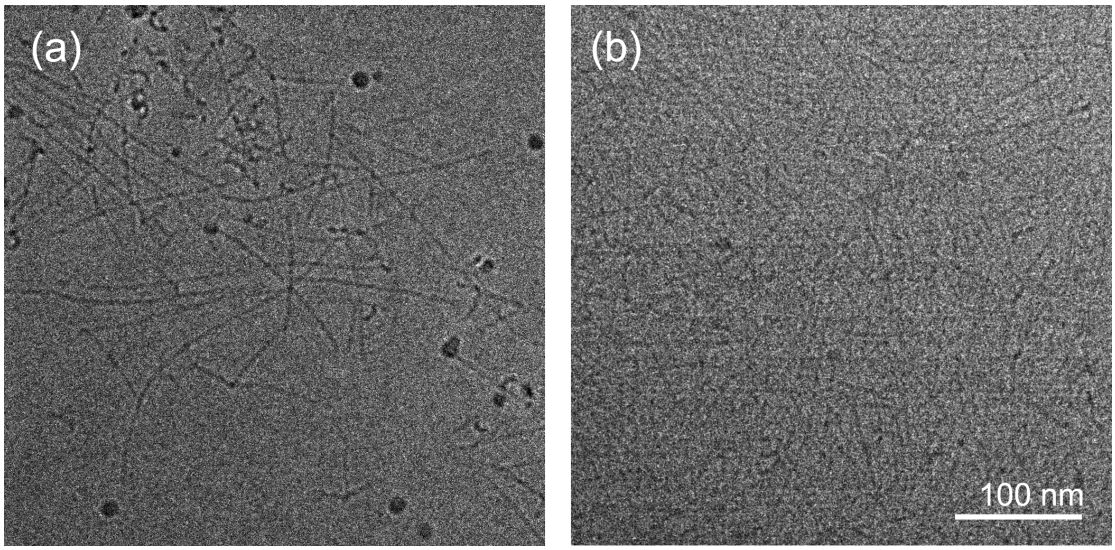
SI Fig. 9. FTMS-ESI mass spectra for peptide **V**.



SI Fig.10. *In situ* WAXS data (expanded from Fig.1b, and plotted on a linear-linear scale).

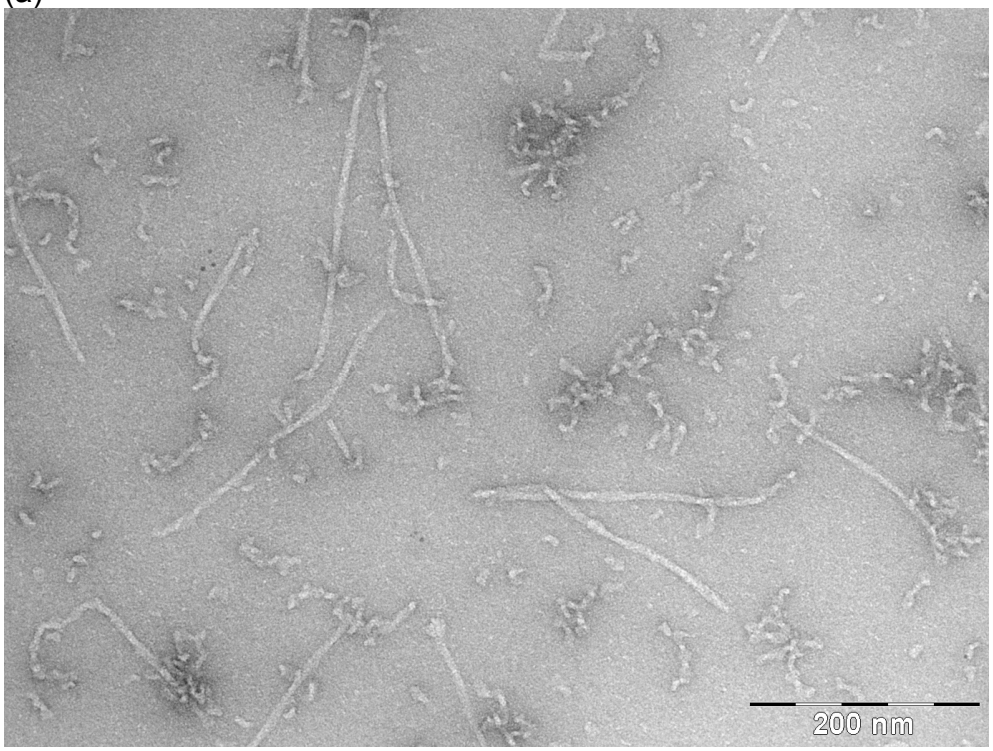


SI Fig.11. FTIR data obtained for peptide **II** at the two concentrations indicated.

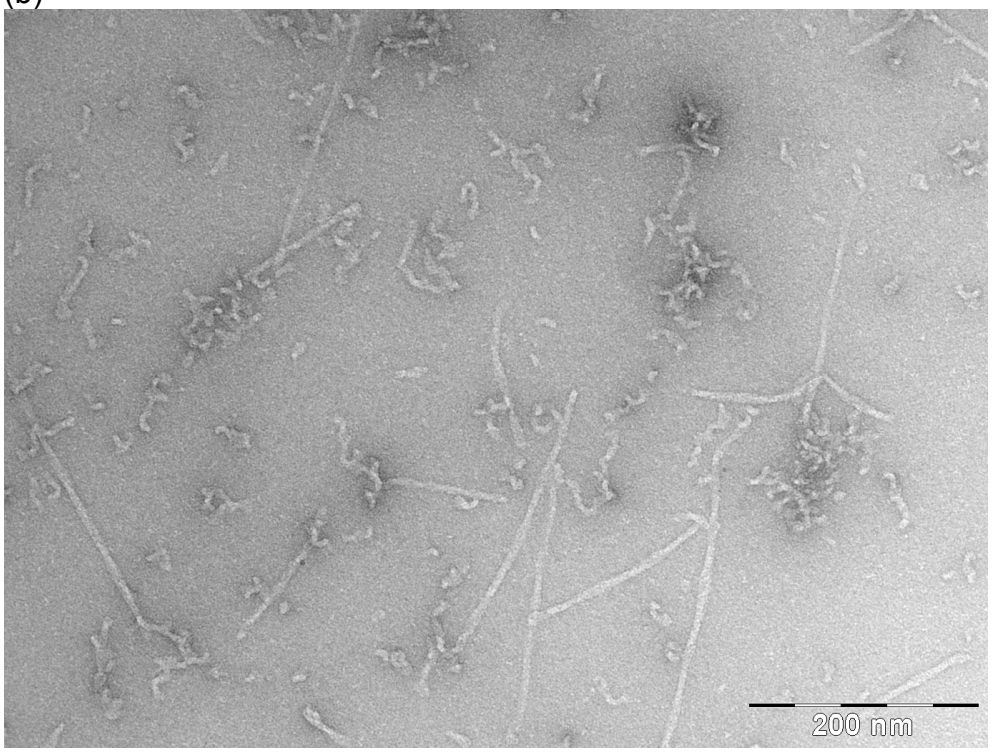


SI Fig.12. Cryo-TEM images of Peptide **II**, prepared in a 2 wt% solution in H_2O and diluted to 0.5 wt%.

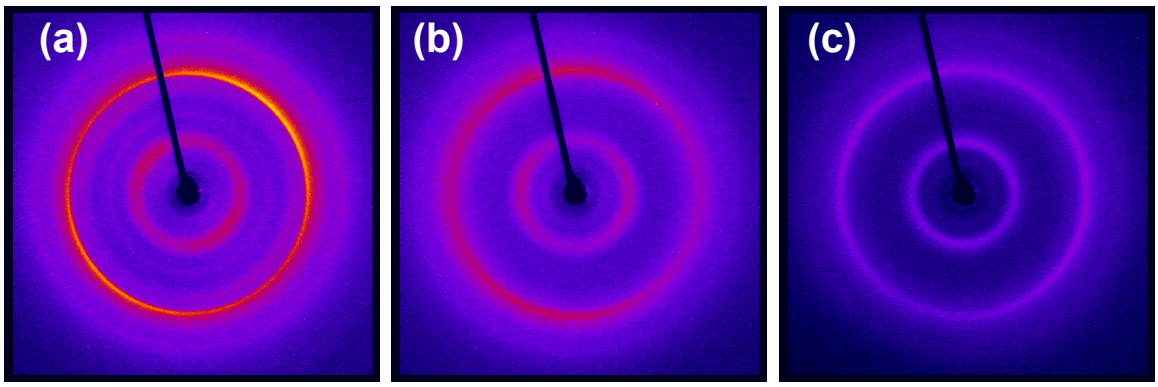
(a)



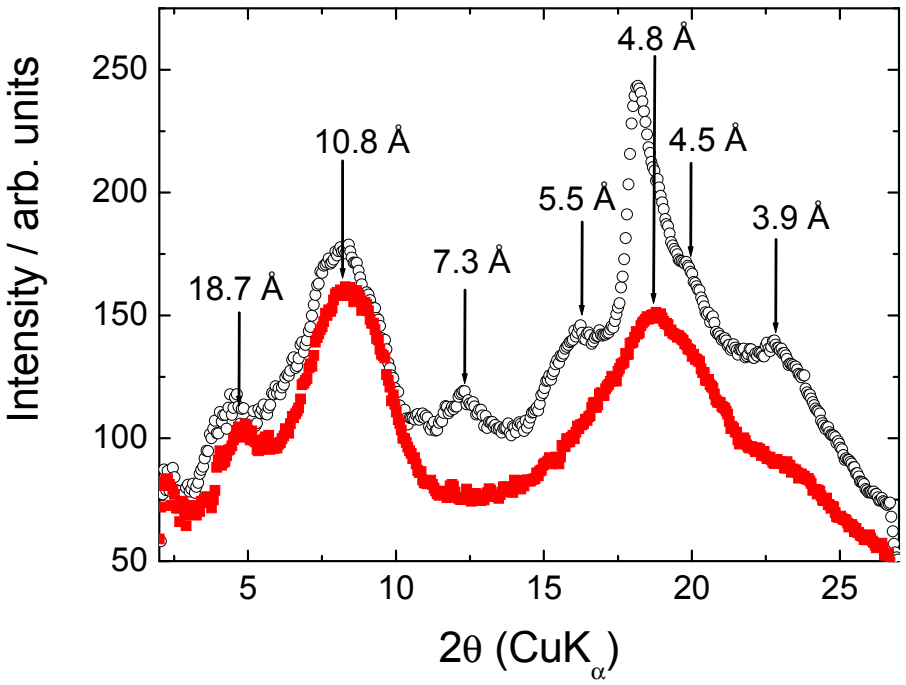
(b)



SI Fig.13. Representative negative stain TEM images of peptide **III**, prepared from an 0.5 wt% aqueous solution.



SI Fig.14. Fibre XRD images obtained from dried stalks. (a) Peptide **I**, (b) Peptide **II**, (c) Peptide **III**.



SI Fig. 15. WAXS intensity profiles (integrated equatorial sections) from dried stalks for (o) Peptide **I**, (■) Peptide **II**.

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