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Supplementary information

Programmable enzymatic oxidation of tyrosine-lysine tetrapeptides

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Synthesis and characterisation of Fmoc-tyrosine-lysine containing tetrapeptides

Initial amino acid loading

2-chlorotrityl chloride resin (100-200 mesh; 1% DVB; 1.1 mmol/g) (500 mg, 0.55 mmol) was weighed into a 10 mL polypropylene syringe equipped with a porous polypropylene frit (Torviq SF-1000), which was used as the reaction vessel. The resin was washed with dichloromethane (3 × 5 mL) before being allowed to swell in dichloromethane (5 mL) for at least 0.5 h prior to the loading of the first amino acid.

A solution of Fmoc-AA-OH (3 equiv.) was dissolved in a mixture of dry dichloromethane (2 mL), *N,N*-dimethylformamide (2 mL) and *N,N*-diisopropylethylamine (DIPEA) (8 equiv., 0.8 mL) and taken up into the syringe with resin and stirred overnight using an orbital shaker. The resin was then washed with dichloromethane (3 x 4mL) and *N,N*-dimethylformamide (DMF) (3 × 4 mL).

N-terminal Fmoc deprotection

A solution of 20% (v/v) piperidine in DMF (2 × 4 mL) was added to the resin once for 1 min, then a fresh aliquot was taken up again and stirred for 10 mins. The solution was subsequently expelled and the resin washed with DMF (5 × 4 mL). The resulting resin-bound amine was used immediately in the next peptide coupling step.

Amino acid coupling

The next amino acid (3 equiv., masses as below) was dissolved in a 0.45 M DMF solution of 1-hydroxybenzotriazole hydrate (HOBt·H_2O)/*N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) (3 equiv.) and DIPEA (6 equiv., 0.6 mL) and this coupling solution added to the resin and stirred for 45 mins using an orbital shaker. The solution was expelled and the resin washed with DMF (5 x 4 mL).

Amino acid	Mass used (mg)
Fmoc-Tyr(O^tBu)-OH	758
Fmoc-Lys(Boc)-OH	773

After another N-terminal Fmoc deprotection, iterative couplings were performed in order to build up the required peptide sequence.

Cleavage of the peptide

After the final coupling step, the resin was washed with DMF (3 x 4 mL) and dichloromethane (3 x 4 mL). A solution of 1:9 dichloromethane: trifluoroacetic acid with three drops of water was then added to the resin, and the resin stirred for 2 h using an orbital shaker. The cleavage solution was then expelled, the resin washed with dichloromethane (2 x 4 mL) and the solvents evaporated under a stream of nitrogen. The resulting residue was lyophilised and purified by semi-preparative HPLC using gradient of acetonitrile/water with 0.1% formic acid to give a white fluffy solid.

Characterisation data for **Fmoc-YYKK**: IR: 3284 (w), 1635 (s), 1522 (s), 1446 (w), 1238 (m), 826 (m), 738 (s); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.32 (s, 1H), 8.14–2.97 (m, 2H), 7.84–7.76 (m, 3H), 7.70 (d, *J* = 7.5 Hz, 1H), 7.55 (t, *J* = 8.1 Hz, 1H), 7.37–7.31 (m, 2H), 7.30–7.19 (m, 2H), 6.99–6.91 (m, 2H), 6.89–6.78 (m, 2H), 6.61–6.50 (m, 4H), 4.47–4.36 (m, 1H), 4.26–4.16 (m, 1H), 4.14–4.00 (m, 3H), 3.91–3.83 (m, 2H), 2.73–2.59 (m, 6H), 1.67–1.19 (m, 12H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 175.00, 174.00, 171.04, 170.84, 165.82, 156.37, 143.03, 139.88, 137.89, 130.75, 130.70, 130.43, 129.40, 128.56, 127.77, 127.69, 121.86, 120.51, 115.49, 115.36, 115.26, 110.25, 59.26, 56.53, 54.38, 53.85, 52.58, 39.15, 38.79, 37.46, 31.98, 31.84, 27.37, 26.86, 22.86, 21.96, 15.06; HR-MS (ESI): calcd for $C_{45}H_{54}N_6O_9 + H^+$: 823.4025, found 823.4002.

Characterisation data for **Fmoc-YKYK**: IR: 2948 (w), 1654 (s), 1546 (m), 1509 (s),1386 (w), 1238 (s), 747 (s); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.50 (d, *J* = 8.3 Hz, 1H), 8.39 (s, 1H), 8.01 (d, J = 7.7 Hz, 1H), 7.81 (d, *J* = 7.7 Hz, 2H), 7.59–7.52 (m, 2H), 7.41 (d, *J* = 8.9 Hz, 1H), 7.37–7.31 (m, 2H), 7.28–7.21 (m, 3H), 7.00 (d, *J* = 8.7 Hz, 2H), 6.92 (d, *J* = 8.4 Hz, 3H), 6.59–6.53 (m, 5H), 4.33–4.26 (m, 1H), 4.25–4.19 (m, 1H), 4.13–4.02 (m, 4H), 3.77–3.70 (m, 3H), 2.80–2.72 (m, 2H), 2.68–2.59 (m, 4H), 1.67–1.08 (m, 16H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 174.31, 173.62, 171.36, 170.10, 165.79, 156.36, 156.30, 143.03, 139.88, 137.89, 130.75, 130.57, 130.39, 129.40, 128.52, 128.29, 127.77, 121.86, 120.51, 115.37, 110.25, 56.11, 55.90, 54.38, 52.20, 38.86, 37.08, 33.04, 32.62, 30.21, 30.00, 27.93, 27.78, 22.76, 22.42, 22.32; HR-MS (ESI): calcd for $C_{45}H_{54}N_6O_9 + H^+$: 823.4025, found 823.4003.

Characterisation data for **Fmoc-YKKY**: IR: 2980 (m), 1641 (s), 1518 (s), 1399 (w), 1235 (s), 1065 (m), 738 (s); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.43 (d, *J* = 7.3 Hz, 1H), 8.29 (s, 1H), 7.95 (d, *J* = 7.9 Hz, 1H), 7.83–7.81 (m, 2H), 7.79–7.76 (m, 2H), 7.37–7.32 (m, 2H), 7.30–7.25 (m, 2H), 7.06 (d, *J* = 6.7 Hz, 1H), 6.90 (d, *J* = 8.4 Hz, 2H), 6.80 (d, *J* = 8.1 Hz, 2H), 6.57 (d, *J* = 8.6 Hz, 2H), 6.52 (d, *J* = 7.9 Hz, 2H), 6.21 (s, 2H), 4.22 (m, 2H), 3.94–3.83 (m, 4H), 2.89–2.78 (m, 3H), 2.76–2.55 (m, 6H), 1.63–1.04 (m, 12H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 173.84, 171.55, 170.54, 165.83, 156.33, 156.09, 143.03, 139.88, 137.89, 130.90, 130.72, 129.42, 129.42, 129.40, 128.92, 128.46, 127.79, 127.77, 127.76, 121.86, 120.51, 120.51, 115.36, 115.01, 110.25, 56.10, 55.82, 54.42, 52.45, 39.06, 38.75, 37.10, 33.09, 31.71, 28.01, 27.39, 22.88, 22.39; HR-MS (ESI): calcd for $C_{45}H_{54}N_6O_9 + H^+$: 823.4025, found 823.4002.

Characterisation data for **Fmoc-KYYK**: IR: 3284 (w), 1650 (s), 1518 (s), 1342 (w), 1248 (m), 747 (s); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.36 (s, 1H), 8.30 (d, *J* = 8.2 Hz, 1H), 8.16–8.05 (m, 1H), 7.85–7.75 (m, 3H), 7.65 (t, *J* = 8.2 Hz, 1H), 7.38–7.31 (m, 2H), 7.31–7.22 (m, 2H), 6.95 (t, *J* = 8.2 Hz, 2H), 6.86–6.75 (m, 2H), 6.61–6.48 (m, 4H), 4.35–4.23 (m, 3H), 4.22–4.11 (m, 3H), 3.92–3.86 (m, 2H), 3.80–3.73 (m, 3H), 2.95–2.78 (m, 3H), 2.75–2.57 (m, 6H), 1.70–1.01 (m, 12H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 174.76, 173.99, 171.66, 170.38, 166.21, 156.37, 143.03, 139.88, 137.89, 130.63, 130.54, 129.40, 128.35, 127.98, 127.77, 121.86, 120.51, 115.35, 115.31, 110.25, 55.21, 54.95, 54.64, 54.33, 39.22, 38.94, 37.52, 37.00, 33.86, 32.01, 27.56, 27.41, 22.44, 22.28; HR-MS (ESI): calcd for $C_{45}H_{54}N_6O_9 + H^+$: 823.4025, found 823.4003.

Characterisation data for **Fmoc-KYKY**: IR: 2995 (s), 2906 (m), 1522 (s), 1395 (m), 1248 (s), 1065 (s), 734 (m); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.62 (s, 1H), 8.24 (d, *J* = 8.2 Hz, 1H), 8.13 (d, *J* = 7.2 Hz, 1H), 8.07 (d, *J* = 7.6 Hz, 2H), 7.90 (t, *J* = 7.4 Hz, 2H), 7.77 (d, *J* = 6.9 Hz, 1H), 7.65 (d, *J* = 8.2 Hz, 1H), 7.63–7.56 (m, 2H), 7.54–7.47 (m, 2H), 7.18–7.08 (m, 4H), 6.84–

6.73 (m, 4H), 4.66–4.58 (m, 1H), 4.48–4.37 (m, 2H), 4.35–4.28 (m, 1H), 4.21–4.08 (m, 3H), 3.19–3.04 (m, 3H), 3.00–2.93 (m, 1H), 2.92—2.80 (m, 5H), 1.81–1.55 (m, 7H), 1.49–1.26 (m, 5H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 174.60, 174.42, 171.40, 170.73, 166.07, 156.42, 156.07, 143.03, 139.88, 137.89, 130.58, 129.40, 128.03, 127.77, 121.86, 120.51, 115.30, 115.15, 110.25, 56.42, 54.67, 54.51, 53.10, 39.00, 38.71, 37.33, 36.84, 34.27, 31.80, 31.56, 28.97, 27.52, 27.11, 22.28, 21.93; HR-MS (ESI): calcd for $C_{45}H_{54}N_6O_9 + H^+$: 823.4025, found 823.3999.

Characterisation data for **Fmoc-KKYY**: IR: 2982 (m), 1645 (s), 1512 (s), 1390 (m), 1257 (s), 1068 (s), 735 (s); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.51–8.37 (m, 1H), 8.32 (s, 1H), 7.91–7.75 (m, 3H), 7.68–7.62 (m, 1H), 7.39–7.31 (m, 3H), 7.30–7.23 (m, 2H), 7.22–7.17 (m, 1H), 6.91–6.82 (m, 4H), 6.59–6.48 (m, 4H), 4.24–4.04 (m, 5H), 3.92–3.83 (m, 3H), 2.93–2.85 (m, 2H), 2.84–2.77 (m, 2H), 2.77–2.68 (m, 2H), 2.68–2.54 (m, 5H), 1.57–1.05 (m, 12H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 173.85, 171.39, 170.30, 165.88, 156.36, 155.97, 143.03, 139.88, 137.89, 130.98, 130.21, 129.40, 129.05, 128.63, 127.77, 121.86, 120.51, 115.41, 115.06, 110.25, 56.47, 56.10, 54.08, 52.57, 39.10, 38.77, 37.10, 34.07, 33.06, 27.90, 27.38, 22.61, 22.42; HR-MS (ESI): calcd for $C_{45}H_{54}N_6O_9 + H^+$: 823.4025, found 823.4003.

Atomic force microscopy measurements

Peptide solutions were prepared at 0.5% (w/v) in water and cast onto a freshly cleaved mica substrate, followed by spreading of the drop over the mica using a glass slide, with the excess liquid wicked away using capillary action. These samples were left to dry in air overnight. Imaging was undertaken on a Bruker Multimode 8 atomic force microscope in Scanasyst mode in air, whereby the imaging parameters are constantly optimised through the force curves that are collected, preventing damage of soft samples. Bruker Scanasyst-Air probes were used, with a spring constant of 0.4 - 0.8 N/m and a tip radius of 2 nm.

Circular dichroism measurements

CD measurements were collected on a Jasco J-1500 spectrophotometer, with data collected between 180 – 500 nm with a bandwidth of 2 nm, digital integration time (D.I.T.) of 2 seconds, scan speed of 100 nm/min and data pitch of 0.1 nm. In a typical experiment, 1% (w/v) peptide sols were prepared as above and diluted as necessary in water. Temperature was kept constant at 25 °C and all experiments were repeated at least three times and averaged into a single plot.

pK_b measurements

Tetrapeptides were dissolved at a concentration of 0.1% (w/v) through the addition of MilliQ water and 3.5 equivalents of 0.1 M HCl. 0.1 M NaOH was added in 50 μL aliquots, and the pH allowed to stabilise for several minutes before a reading was taken. Each titration was repeated three times and averaged into a single plot. pK_b was calculated by determining the volume at which the equivalence point occurs through plotting the derivative of the graph, finding the pH at half of this volume and subtracting from 14.

Tyrosinase oxidation of Fmoc-tyrosine-lysine containing tetrapeptides

Tetrapeptides were dissolved in 100 mM HEPES (pH 7.1) at a concentration of 0.25% (w/v). Tyrosinase was dissolved in 100 mM HEPES at 2 mg/mL, and 50 μL tyrosinase stock was

added to 450 µL peptide solution, giving final peptide and tyrosinase concentrations of 0.225% (w/v) and 0.2 mg/mL, respectively. The samples were then incubated at room temperature. At each time point, an image of the sample was taken, then the sample was then centrifuged at 14,000 rpm for 10 mins, before being imaged again. For UV-Vis analysis, the supernatant was diluted twofold in MillQ water and its absorption spectrum between 200 nm and 800 nm recorded using a Thermo Nanodrop 2000. A calibration curve was constructed for each peptide prior to measuring unknown samples. All measurements were performed in triplicate, and the oxidations were carried out at least twice.

ATR-IR measurements on polymeric peptide precipitates

Measurements were made on a JASCO FT/IR 4700 spectrophotometer fitted with a PIKE MIRacle™ Single Reflection ATR accessory and ZnSe crystal plate. Polymeric peptide precipitates were washed twice with 100 mM HEPES (pH 7.1) and freeze dried. The resulting powder was applied to the ATR crystal using a MIRacle™ basic High-Pressure Clamp at approximately 400 psi. All spectra were scanned at least 16 times over the range of 4000 - 650 cm^{-1} and were acquired at a resolution of 4 cm^{-1}.

Mass spectrometry analysis of polymeric peptide precipitates

Polymeric peptide precipitates were washed twice with 100 mM HEPES (pH 7.1) and freeze dried. Samples were solubilised in HPLC grade methanol and analysed using an Agilent Technologies 6490 triple quadrupole mass spectrometer. Data were acquired in ESI positive mode, drying gas temperature was 250 °C at 14 L min^{-1}, sheath gas temperature was 250 °C at 11 L min^{-1}, and nebuliser pressure was 20 psi. Capillary voltage was 3000 V and nozzle voltage 1500 V.

Electrical impedance spectroscopy

The impedance behaviour of Fmoc-dityrosine-dilysine tetrapeptide solutions upon treatment with tyrosinase was assessed as follows. Solutions were poured into a plastic sample holder (acrylic 1 cm width, 1 cm height, 2.5 cm length containing two pieces of reticulated vitreous carbon (RVC, ERG Aerospace, USA, 20 pores per inch). A custom-designed instrument was used to measure electrical impedance spectroscopy for frequencies between 100 Hz and 100 kHz.[1] Briefly, an alternating current signal (1 V peak voltage) was applied using a waveform generator (Agilent U2761A) across a circuit consisting of a known resistor (10 kΩ) and the sample. Current was calculated across the known resistor and then used to measure the impedance by measuring the potential difference across the known resistor with an oscilloscope (Agilent U2701A).

Microscale thermophoresis (MST) measurements

Peptides were dissolved at 20 mg/mL in MilliQ water and serially diluted. Samples were taken up into Tycho NT.6 capillaries using capillary action and loaded into a NanoTemper Tycho NT.6. A standard measurement involved heating the sample from 35 °C to 95 °C at 30 °C/min and monitoring the ratio of intrinsic tyrosine fluorescence ΔF, where ΔF is defined as the intensity of fluorescence at 350 nm divided by the fluorescence intensity at 330 nm ($\Delta F = I_{350\ nm}/I_{330\ nm}$) upon excitation at 280 nm. At least three samples were measured at each concentration.

Some samples at certain concentrations (i.e. Fmoc-KYKY at concentrations above 0.1 mg/mL) were unable to be measured as their fluorescence intensity overwhelmed the detector. As such, a sample being "too bright" to measure was used as an indicator of the presence of monomers. Increasing sample concentration results in self-assembly, which in turn decreases fluorescence intensity, this is observed for all peptides excepting Fmoc-KYKY at 20 mg/mL.

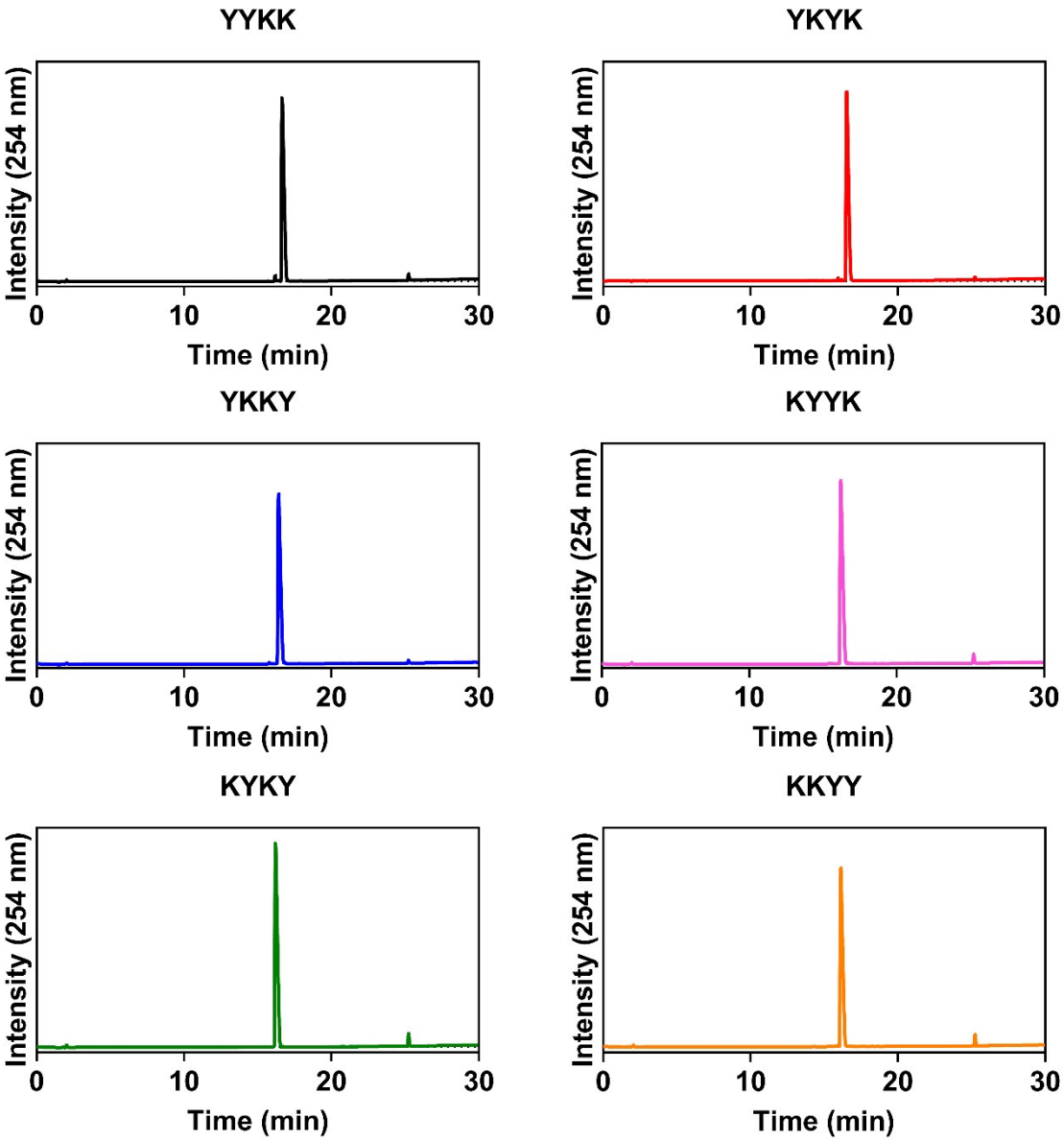

Fig. S1 – Analytical HPLC traces of Fmoc-dityrosine-dilysine tetrapeptides.

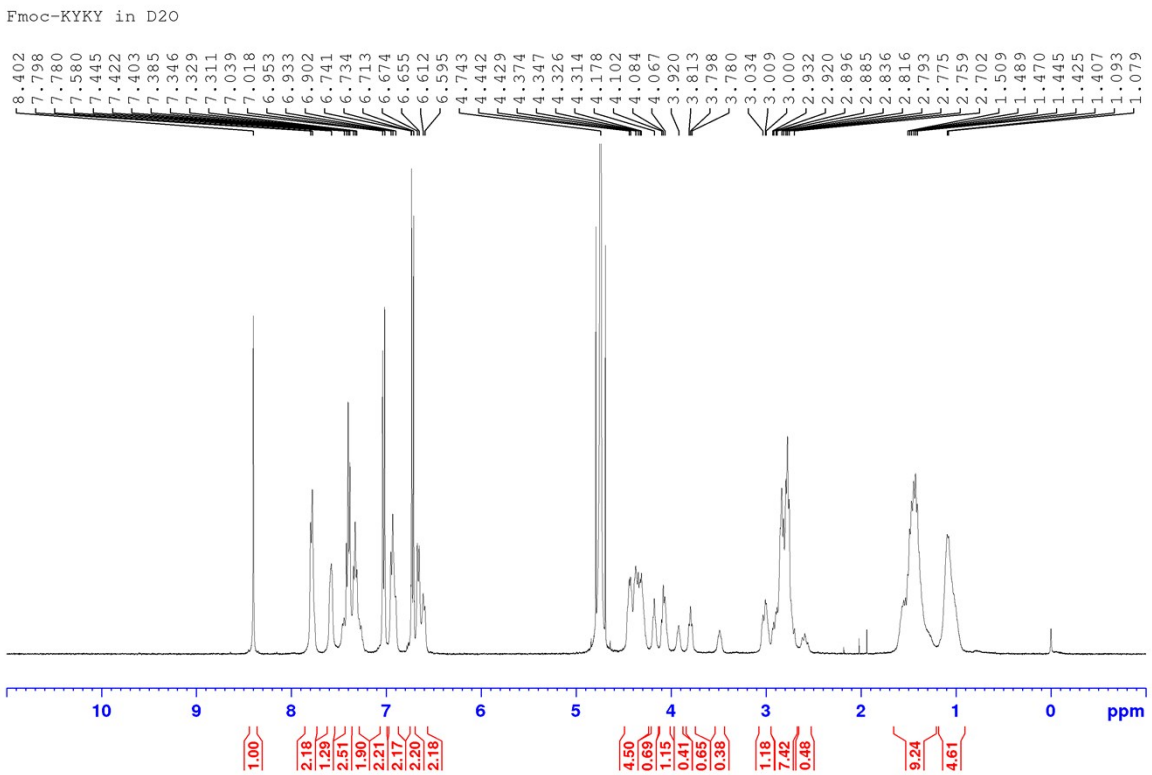

Fig. S2 – ^{1}H NMR of Fmoc-KYKY performed in D_2O at a concentration of 10 mg/mL, showing the presence of monomers, either due to a lack of self-assembly, or rapid exchange of any self-assembled structures with their monomeric counterparts.

Table S1 – Nanofibre diameters of Fmoc-dityrosine-dilysine tetrapeptides, as determined through AFM imaging. In all cases, at least 20 nanofibre diameters were measured across multiple images.

Peptide	Diameter (nm)
Fmoc-YYKK	2.7 ± 0.6
Fmoc-YKYK	2.5 ± 0.7
Fmoc-YKKY	N/A
Fmoc-KYYK	2.4 ± 0.5
Fmoc-KYKY	N/A
Fmoc-KKYY	2.5 ± 0.7

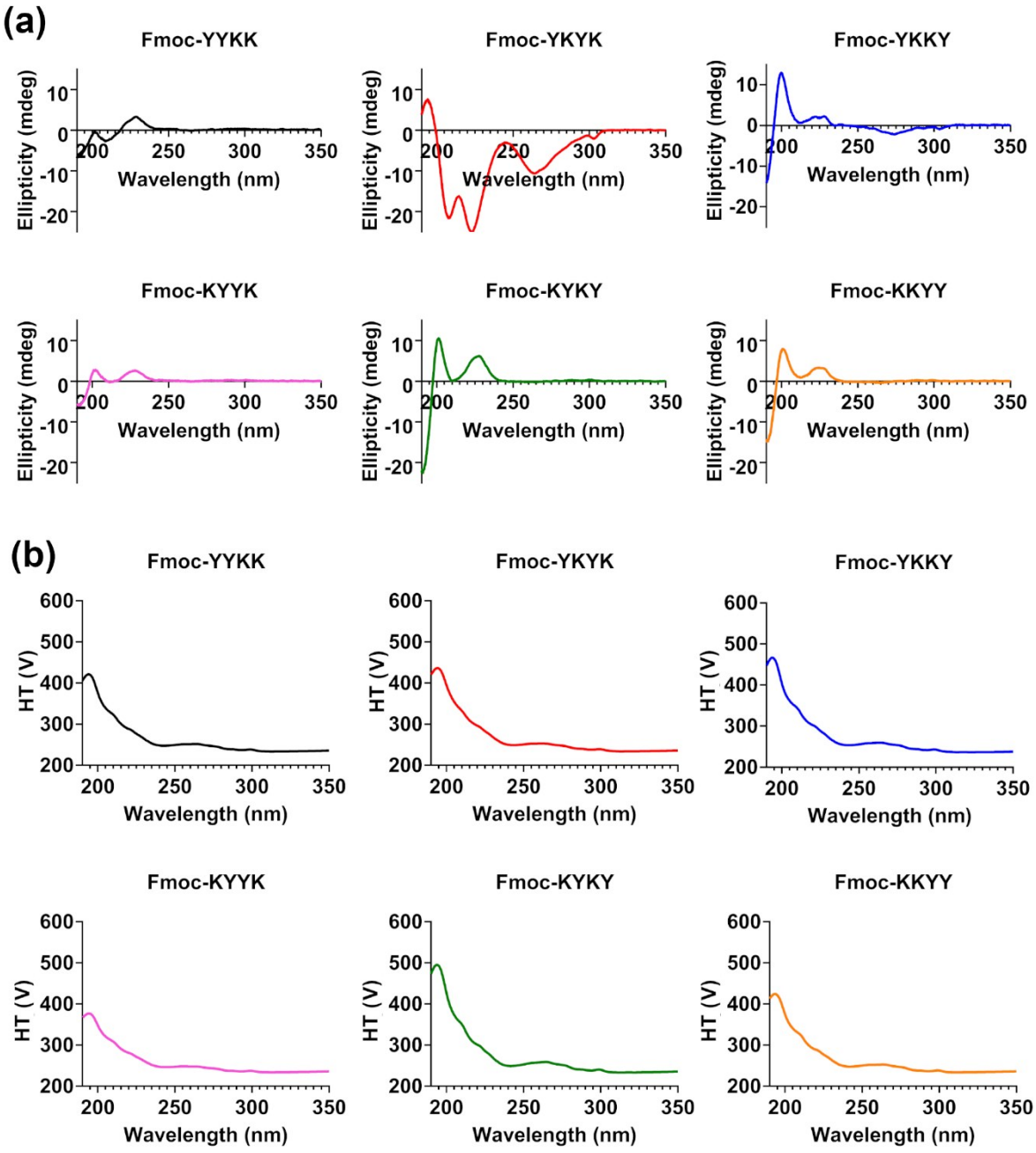

Fig. S3 – (a) Circular dichroism and (b) HT spectra of Fmoc-dityrosine-dilysine tetrapeptides. The alpha helical secondary structure of Fmoc-YKYK is clearly visible. Samples were prepared at 1% (w/v), before 8× dilution in order to maintain a HT value of under 600. Samples were measured in a 1mm quartz CD cuvette at room temperature, with each sample measured three times and averaged into a single plot.

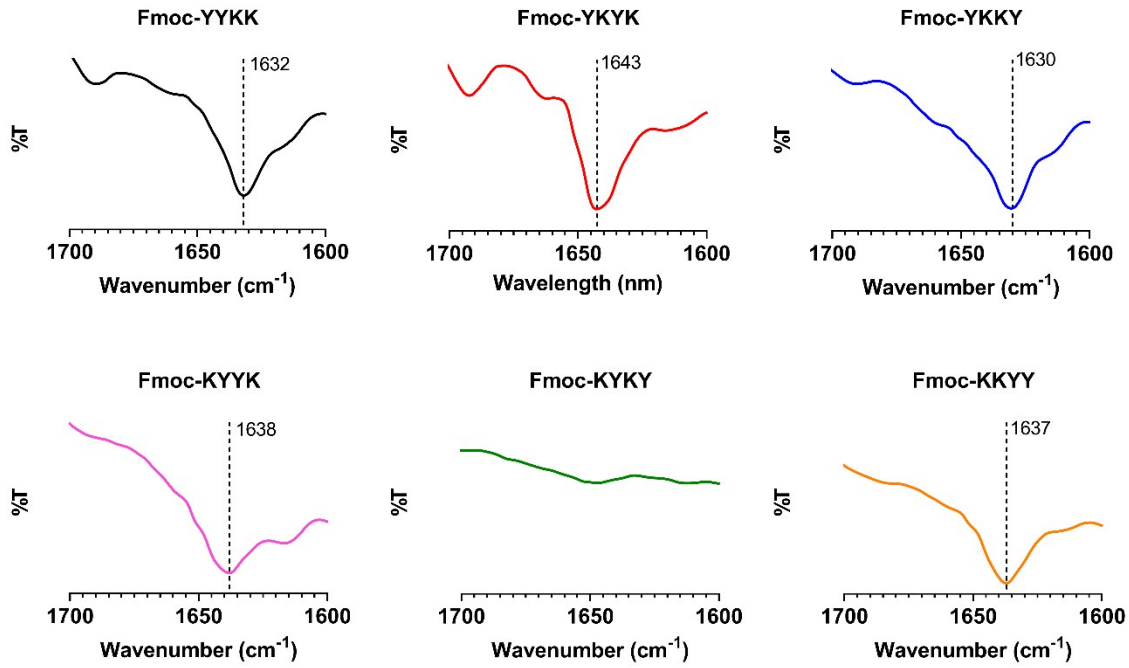

Fig. S4 – ATR-IR spectra of Fmoc-dityrosine-dilysine tetrapeptides, specifically their Amide I region. It can be seen that the Amide I peak for Fmoc-YKYK is shifted relative to the other tetrapeptides, consistent with the formation of an alpha helical structure.

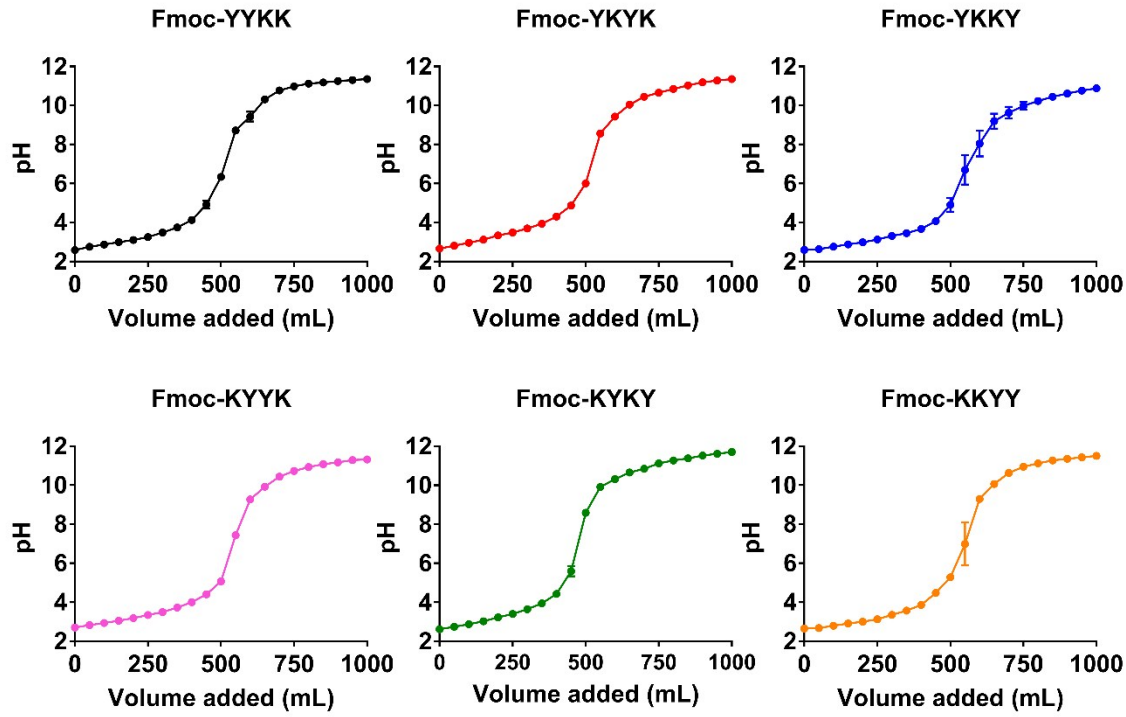

Fig. S5 – pK_b measurements performed for each of the Fmoc-dityrosine-dilysine tetrapeptides, showing almost identical pK_b values. Titrations were undertaken at room temperature, at a sample concentration of 0.1% (w/v), by dissolving the peptide in acidic water before adding 0.1 M NaOH in 50 µL aliquots.

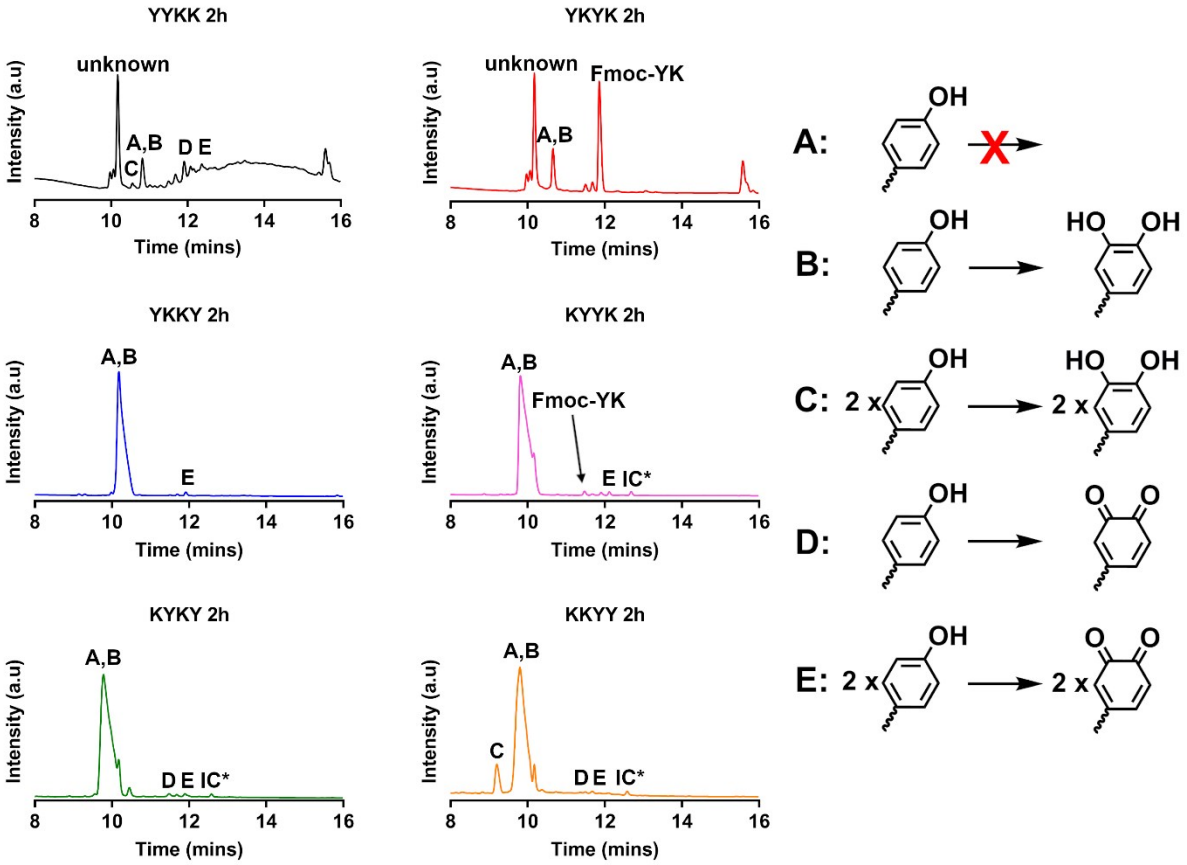

Fig. S6 – LC-MS traces of the supernatant solution after treatment of tetrapeptides with the enzyme tyrosinase after 2h (left) and potential oxidation products of the tyrosine residue (right). IC* corresponds a mass loss of 18 Da, which is likely due to intramolecular condensation of a lysine residue with a tyrosine hydroxyl.

Table S2 – *m/z* ions detected for Fmoc-dityrosine dilysine tetrapeptide supernatant after 2h and 24h treatment with tyrosinase

	Peak 1	**Peak 2**	**Peak 3**	**Peak 4**	**Peak 5**
YYKK	*Major*	*Minor*	*Minor*	*Minor*	*Minor*
	671 (unknown)	855 (C)	823, 839 (A, B)	837 (D)	851 (E)
YKYK	*Major*	*Minor*	*Major*		
	671 (unknown)	823, 839 (A, B)	531 (Fmoc-YK)		
YKKY	*Major*	*Minor*			
	823, 839 (A, B)	851 (E)			
KYYK	*Major*	*Minor*	*Minor*	*Minor*	
	823, 839 (A, B)	531 (Fmoc-YK)	851 (E)	804 (IC*)	
KYKY	*Major*	*Minor*	*Minor*	*Minor*	
	823, 839 (A, B)	837 (D)	851 (E)	804 (IC*)	
KKYY	*Medium*	*Major*	*Minor*	*Minor*	*Minor*
	855 (C)	823, 839 (A, B)	837 (D)	851 (E)	804 (IC*)

Table S3 – *m/z* ions detected with MALDI-MS for Fmoc-dityrosine dilysine tetrapeptide polymeric precipitate after 24h treatment with tyrosinase.

YYKK		1640	1655	1672	1683		2445	2461	2475	2493
		A + D	2D	B + E	2E		2A + D	A + 2D	3D	2C + E
YKYK	1626	1643	1658	1672	1683			2463	2477	2492
	2A	A + D	2D	B + E	2E			A + 2D	3D	2C + E
YKKY	1626	1643	1655	1672		2430	2446	2460	2475	2493
	2A	A + D	2D	B + E		3A	2A + D	A + 2D	3D	2C + E
KYYK		1644	1655	1673	1683			2461	2474	2492
		A + D	2D	B + E	2E			A + 2D	3D	2C + E
KYKY	1624	1641	1655	1672	1683			2461	2478	2494
	2A	A + D	2D	B + E	2E			A + 2D	3D	2C + E
KKYY			1655							
			2D							

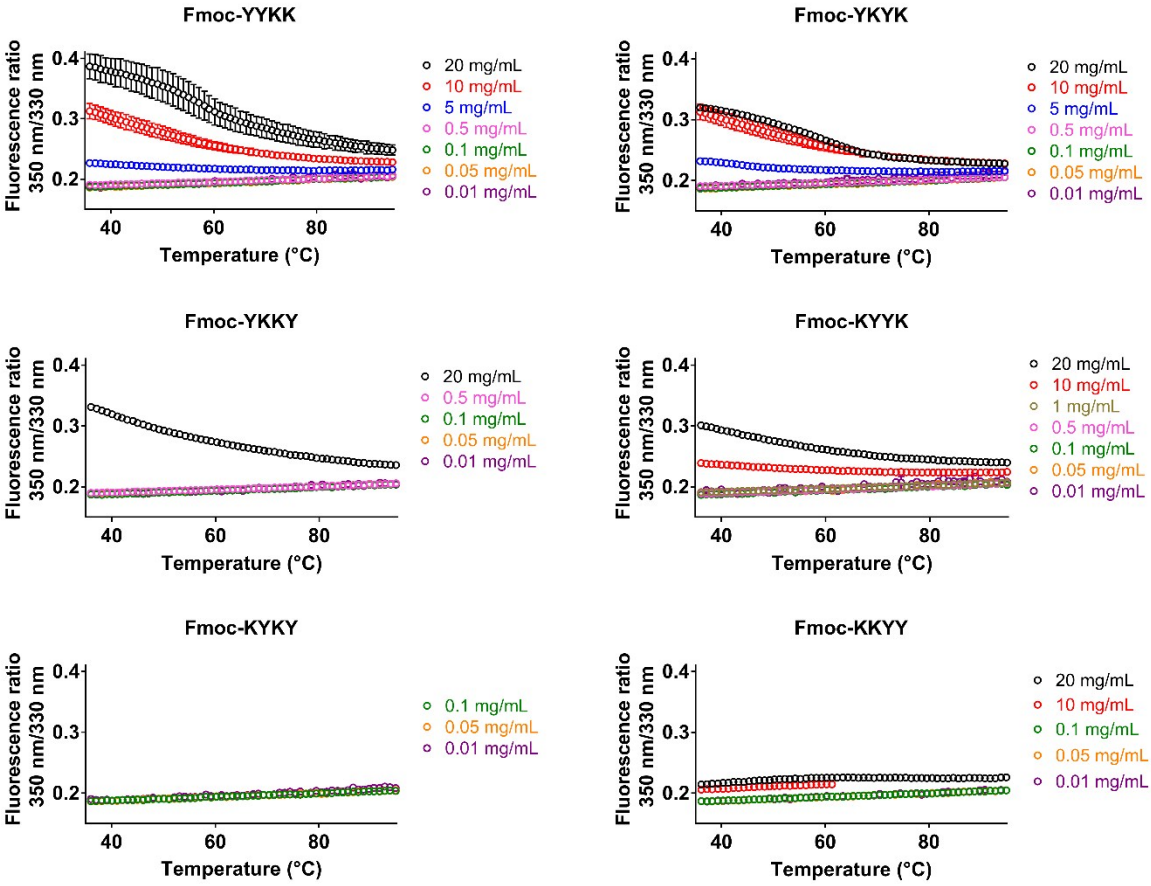

Fig. S7 – Microscale thermophoresis (MST) data using tyrosine intrinsic fluorescence for Fmoc-dityrosine-dilysine tetrapeptide solutions. Self-assembly can be seen for all peptides except Fmoc-KYKY at 20 mg/mL, however below this concentration a significant portion of Fmoc-YKKY exists as monomers, rendering tyrosine fluorescence of these solutions unable to be measured due to their brightness. Fmoc-KYYK and Fmoc-KKYY remain self-assembled at 10 mg/mL, while Fmoc-YYKK and Fmoc-YKYK are still self-assembled at 5 mg/mL. The fluorescence ratio ΔF (y-axis) can be used to approximate the solvent accessibility of tyrosine residues at a given concentration, with a larger value correlating with less exposed solvent residues. For solutions of monomers (all peptides below 0.1 mg/mL), similar values for ΔF are obtained.

References

1. H. Warren, R. D. Gately, P. O'Brien, R. Gorkin and M. in het Panhuis, *J. Polym. Sci. Pol. Phys.*, 2014, **52**, 864.