Harnessing the Power of a Photoinitiated Thiol–ene "Click" Reaction for the Efficient Synthesis of *S*-Lipidated Collagen Model Peptide Amphiphiles

C.J. Aguilar[a,b], Makhdoom Sarwar[c,d], Sujay Prabakar[e], Wenkai Zhang[e] Paul W.R. Harris[a,b,c], Margaret A. Brimble[a,b,c*] and Iman Kavianinia[a,b,c*]

Supplementary Information

Contents

1.1 Peptide Synthesis

1.1.1 Reagents

All reagents were purchased as reagent grade and used without further purification. *N*,*N*-Diisopropylethylamine (DIPEA), piperidine, 1,2-ethanedithiol (EDT), diisopropylcarbodiimide (DIC), triisopropylsilane (TIPS), formic acid, acetic anhydride (Ac_2O), *N*-methylmorpholine (NMM), *N*-methyl-2-pyrrolidone (NMP), 2,2-dimethoxy-2-phenylacetophenone (DMPA), *tert*-nonyl mercaptan (mixture of isomers) were purchased from Sigma Aldrich (St. Louis, MO, USA). All Fmoc amino-acids were procured from CS Bio (Shanghai, China) and side chain protected as follows: Fmoc-Asp(tBu)-OH, Fmoc-Asn(Trt)-OH (Trt = triphenylmethyl), Fmoc-Cys(Trt)-OH, Fmoc-Lys(Boc)-OH (Boc= *tert*-butoxycarbonyl), Fmoc-Thr(*t*Bu)-OH Fmoc-Tyr(*t*Bu)-OH, Fmoc-Trp(Boc)-OH.*O*-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium, hexafluoro-phosphate (HATU) was purchased from CSBio (Shanghai, China) . Trifluoroacetic acid (TFA) was purchased from Oakwood Chemicals (Estill, SC, USA). 6-Chloro-1-hydroxybenzotriazole (6-Cl-HOBt) was purchased from Aapptec (Louisville, KY, USA). Dichloromethane (CH_2Cl_2) was purchased from ECP Limited (Auckland, New Zealand). Milli-Q high purity deionized water (MQ H_2O) was available from a Sartorius Arium® Pro Ultrapure Water System from Sartorius Stedim Biotech (Göttingen, Germany). 2-Iminothiolane (Traut's Reagent), *N,N*-dimethylformamide (DMF) (synthesis grade) and acetonitrile (HPLC grade) were purchased from Thermo Fisher Scientific (Hampshire, NH, USA). Diethyl ether (Et_2O) was purchased from Macron Fine Chemicals™, distributed by Avantar Performance Chemicals (Center Valley, PA, USA). Dimethyl sulfoxide (DMSO) was purchased from Romil Limited (Cambridge, United Kingdom). TentaGel® resin were purchased from RAPP Polymere (Tübingen, Germany).

1.1.2 General Procedure for Purification and Analysis

Yields refer to chromatographically homogeneous materials.

Analytical HPLC

Analytical reverse phase high-performance liquid chromatography (RP-HPLC) was performed on a Thermo Scientific Dionex UltiMate® 3000 equipped with a four channel UV Detector at 210, 225, 254 and 280 nm using a Phenomenex (Sunnyvale, CA) Gemini C18 column (110 Å, 5 µm, 4.6 mm × 150 mm) operated at room temperature, using a linear gradient of 5% B to 95% B over 30 min at 1 mL/min (*ca*. 3% B/min). Buffer A is H_2O with 0.1% TFA (*v/v*) and buffer B is MeCN with 0.1% TFA (*v/v*).

Semi-Prep HPLC

Semi-preparative RP-HPLC was performed on a Thermo Scientific Dionex Ultimate® 3000 HPLC equipped with a four channel UV Detector at 210, 225, 254 and 280 nm using a Phenomenex Gemini C18 110 Å 10.0 × 250 mm: 5 µm at a flow rate of 4 mL/min. A suitably adjusted gradient of 5% B to 95% B was used over 90 min (*ca*.1% B/min), where buffer A is H_2O with 0.1% TFA (*v/v*) and buffer B was MeCN with 0.1% TFA (*v/v*).

ESI-MS and LC-MS

Low-resolution mass spectrometry was performed on a Waters (Milford, Massachusetts, USA) Quattro micro-API Mass Spectrometer in ESI positive mode. LC-MS spectra were acquired using an Agilent Technologies (Santa Clara, CA, USA) Technologies 1260 Infinity LC equipped with an Agilent Technologies 6120 Quadrupole mass spectrometer. An analytical column (Agilent Zorbax C3, 3.5 µm; 3.0 × 150 mm) was used at a flow rate of 0.3 mL min^{-1} using a linear gradient of 5% B to 95% B over 30 min (*ca*. 3% B/min), where buffer A is H_2O with 0.1% formic acid (*v/v*) and buffer B was MeCN with 0.1% formic acid (*v/v*).

Kaiser Test

The Kaiser test was performed by retrieval of a few resin beads which were washed with 3 times with ethanol or dichloromethane. Three drops of each solution were added to the resin beads where solution A = 80% (*w/v*) phenol in ethanol, solution B = 2% (*w/v*) KCN in pyridine and solution C = 5% (*w/v*) ninhydrin in ethanol. The peptidyl resin was mixed well and heated at 90 °C for 3 minutes. The resin beads and

the solution turn dark blue when primary amine is present. The resin beads remain unchanged, and the solution stays yellow when no free primary amine is present (expected result after successful coupling). A re-coupling step is necessary when a slight blue colour is detected in the solution and/or on beads.

1.1.3 General Methods for Solid-Phase Peptide Synthesis (SPPS)

Method 1: General procedure for the attachment of Fmoc-Rink amide linker to a resin

To Tentagel S-NH_2 resin (0.1 mmol, 370 mg, loading: 0.27 mmol/g) pre-swollen in anhydrous CH_2Cl_2 (5 mL, 10 min), was added a solution of the Fmoc-Rink amide linker (4 eq., 0.4 mmol), DIC (4 eq., 0.4 mmol, 50 mg, 62.6 µL) and 6-Cl-HOBt (4 eq., 0.4 mmol, 68 mg) in a mixture of CH_2Cl_2/DMF (1:4, *v/v*, 5 mL). The reaction mixture was gently agitated at room temperature for 2 h twice with fresh set of reagents. The resin was filtered, washed with CH_2Cl_2 (3 × 3 mL) and repeated the same reaction with a fresh set of reagents for a further 3 h.

Method 2: General procedure for the removal of the N^{α}-Fmoc protecting group

The peptidyl resin was treated with a solution of 20% piperidine in DMF (*v/v*, 2 mL) and the mixture was agitated at room temperature for 5 min, filtered and the reaction repeated with a fresh set of reagents for a further 10 min. The resin was filtered and washed with DMF (3 × 3 mL).

Method 3: General procedure for the coupling of the amino acids using Fmoc-SPPS strategy

The peptidyl resin couplings were performed with a mixture of Fmoc-protected amino acid (5 eq., 0.5 mmol), HATU (4.8 eq., 0.48 mmol, 365 mg) and DIPEA (10 eq., 1.0 mmol, 348 µL) in DMF (*v/v*, 5 mL) in a single coupling cycle for 30 min at room temperature. The resin was filtered and washed with DMF (3 × 3 mL).

Method 4: General procedure for the removal of the N^{α}-Fmoc protecting group on Biotage®.

The peptidyl resin was treated with a solution of 20% piperidine in DMF (*v/v*, 2 mL) and the mixture was agitated at room temperature for 10 min, filtered and repeated the reaction with a fresh set of reagents for a further 10 min. The resin was filtered and washed with DMF (3 × 3 mL).

Method 5: General procedure for the automated coupling of the amino acids using Fmoc-SPPS strategy on Biotage®.

The peptidyl resin couplings were performed with solutions of Fmoc-protected amino acid (5 eq., 200 mM), HATU (4.8 eq., 500 mM) and NMM (10 eq., 1.0 mmol, 101 µL) in DMF in a single coupling cycle for 5 min at 75 °C with 25 W of microwave power. The resin was filtered and washed with DMF (2 × 3 mL).

Method 6: General procedure for global deprotection and full cleavage of peptide from resin

After washing the resin with CH_2Cl_2 (3 × 3 mL), a cleavage cocktail of TFA/H_2O/TIPS (95:2.5:2.5, *v/v/v*, 5 mL) was added to the peptidyl resin and agitated for 3 h at r.t.. The cleavage mixture was drained from the resin, and the collected TFA solution was evaporated by a flow of N_2. The peptide was precipitated from cold diethyl ether (2 × 40 mL), isolated (centrifugation, 4000 rpm, 10 min), decanted the diethyl ether and then dissolved in H_2O/MeCN (1:1, *v/v,* 5 mL) containing 0.1% TFA and lyophilised.

<u>Method 6.1: General procedure for mini cleavage of peptide from resin</u>

Mini cleavage was performed with retrieval of a few resin beads (1-3 mg) and washed with 3 times with CH_2Cl_2 (2 mL) and dried. After washing the resin beads, a cleavage cocktail of TFA/H_2O/TIPS (95:2.5:2.5, *v/v/v*, 1 mL) was added to the peptidyl resin and agitated for 1 h at r.t. The cleavage mixture was drained from the resin, and

the collected TFA solution was evaporated by a flow of N_2. The peptide was precipitated from cold diethyl ether (2 × 40 mL), isolated (centrifugation, 4000 rpm, 10 min), decanted the diethyl ether and then dissolved in H_2O/MeCN (1:1, *v/v,* 5 mL) containing 0.1% TFA and analysed by HPLC and ESI-MS.

1.1.4 General Methods for *N*-Terminal Modifications
*All modifications were conducted based on a 0.1 mmol scale

Method 7: General procedure for the attachment of 2-iminothiolane/2-IT (Traut's reagent):

After the removal of the N^{α}-Fmoc protecting group from the final *N*-terminal peptidyl residue, the peptidyl resin (0.1 mmol scale) was treated with a solution of 2-IT (4 eq., 0.4 mmol, 55 mg) in PBS buffer (5 mL) to give a final peptide? concentration of 11 mg/mL at pH 7.4 for 1 h at 0 °C. The completion of the reaction was monitored by mini cleavage using the conditions from **Method 6.1** and analysed by analytical RP-HPLC and ESI-MS. PBS buffer was removed by washing the peptidyl resin with H_2O (3 × 3 mL).

*Upon reaction completion, acidifying the solution mixture to pH <7 is necessary to avoid side-product formation. In this case, after the reaction was complete, the mixture was acidified using the cleavage conditions outlined in Method 6, which also cleaved the peptide off the resin.

Method 8: General procedure for the attachment and formation of 4-mercaptobutyramide moiety (3 stepwise reactions):

1st step: Thiolation

After the removal of the N^{α}-Fmoc protecting group from the final *N*-terminal peptidyl residue, the peptidyl resin was treated with a solution of 2-IT (4 eq., 0.4 mmol, 55 mg) in PBS buffer (5 mL) at pH 7.4 for 1 h at r.t. The resin was filtered and washed with H_2O (2 × 3 mL) then DCM (2 × 3 mL). The completion of the reaction was monitored by mini cleavage using the conditions from **Method 6.1** and analysed by analytical RP-HPLC and ESI-MS.

2nd step: Decay

After the reaction was complete, the thiolated peptidyl residue was left in PBS buffer (pH 7.4) for 20 h to let the 4-mercaptobutyramidine (non-cyclic) decay to the cyclic non-thiol product, *N*-substituted 2-iminothiolane. The resin was filtered and washed with H_2O (2 × 3 mL) then DCM (2 × 3 mL). The formation of the *N*-substituted 2-iminothiolane was monitored by mini cleavage using the conditions from **Method 6.1** and analysed by analytical RP-HPLC and ESI-MS.

3rd step: Hydrolysis/Acidification

Hydrolysis of the *N*-substituted 2-iminothiolane was conducted by incubating the cleaved peptide in sodium acetate buffer (pH adjusted to pH 5 with 0.1 M NaOH) for 5 days at r.t. to generate the desired product, 4-mercaptobutyramide. A desalting column was used to separate the peptide from the buffer salts. The formation of the 4-mercaptobutyramide was monitored and analysed by analytical RP-HPLC and ESI-MS following a mini-cleavage as described in **Method 6.1**

Method 9: General procedure for the attachment of an acetyl group:

After the removal of the N^{α}-Fmoc protecting group from the final peptidyl residue, the peptidyl resin (0.1 mmol scale) was treated with a solution of acetic anhydride in DMF (20%, *v/v*, 1 mL), and DIPEA (10 eq., 1 mmol, 174 µL) in DMF 5 mL) in two coupling cycles for 10 min. at r.t. The resin was filtered and washed with DMF (2 × 3 mL) then DCM (2 × 3 mL). The completion of the reaction was monitored by Kaiser test where a negative result confirms a complete reaction.

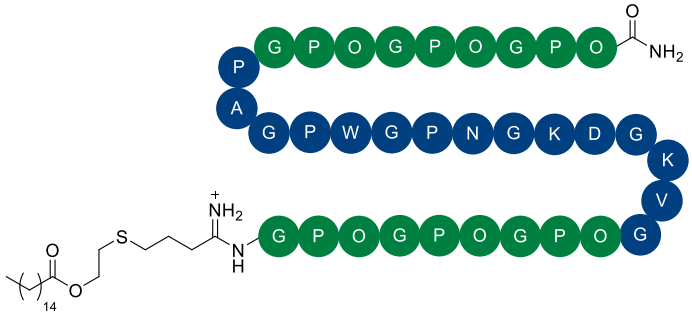
Method 10: General procedure for the attachment of palmitic acid:

After the removal of the N^{α}-Fmoc protecting group from the final peptidyl residue, the peptidyl resin (0.1 mmol scale) was treated with a solution of palmitic acid (4 eq., 0.4 mmol, 101 mg), COMU (4 eq., 0.4 mmol, 171 mg), Oxyma (4 eq., 0.4 mmol, 56 mg) and DIPEA (8 eq., 0.8 mmol, 139 µL) in DMF (*v/v*, 5 mL) for 3 h at r.t. The resin was filtered and washed with DMF (2 × 3 mL).

Method 11: Attachment of lipidated vinyl esters using solution-phase thiol-ene reaction:

The peptide containing the *N*-terminal thiol handle was added to a solution of vinyl ester (70 eq.), DMPA (1.5 eq., 9 mol), *tert*-nonyl mercaptan (80 eq., 0.74 mmol), TIPS (80 eq., 0.74 mmol) with 5% TFA in NMP (*v/v*, 4 mL) to give a final concentration of the peptide at *ca*. 10 mg/mL. The reaction mixture was irradiated under UV light (365 nm) for 60 min at r.t. Upon completion of the reaction, as determined by analytical RP-HPLC, the peptide was precipitated from cold diethyl ether (2 × 40 mL), isolated (centrifugation, 4000 rpm, 10 min), decanted the diethyl ether and then dissolved in H_2O/MeCN (1:1, *v/v,* 5 mL) containing 0.1% TFA and lyophilised.

1.1.5 Synthesis of **CMP1**



Peptide synthesis

Fmoc-Rink amide linker (4 eq., 0.4 mmol, 215 mg) was coupled to Tentagel S-NH_2 resin (0.1 mmol scale, loading= 0.27 mmol/g, 370 mg) according to **Method 1**. The first peptidyl residue, hydroxyproline Fmoc-Hyp(tBu)-OH, was manually coupled onto the linker-resin via protocol **Method 3**. Subsequent deprotection and amino acid couplings were performed via the automated peptide synthesiser Biotage® using **Method 4** and **Method 5**. Upon synthesis completion, mini cleavage of the peptidyl resin was conducted using **Method 6.1** and was analysed using LC-MS to ensure the correct peptide (**CMP 1**) was synthesised.

Direct thiolation by Traut's reagent of peptide **8** to peptide **9** was achieved using the conditions in **Method 7**. The peptidyl resin was dried, and the thiolated peptide was cleaved from the resin and deprotected using the conditions outlined in **Method 7** to afford peptide **9**.

Crude mixture of peptide **9** (50 mgs, 16% yield, 70% purity) was directly used for *S*-lipidation without further purification. Attachment of the vinyl ester; vinyl palmitate was conducted using the thiol-ene conditions outlined in **Method 11**. Upon completion of the reaction, the peptide was precipitated from cold diethyl ether (2 × 40 mL), isolated (centrifugation, 4000 rpm, 10 min), decanted the diethyl ether and then dissolved in H_2O/MeCN (1:1, *v/v,* 5 mL) containing 0.1% TFA and lyophilised.

Purification of **CMP1** was conducted on a semi-preparative RP-HPLC using a Dionex Ultimate® 3000 on a Phenomenex Gemini C18 110 Å 10.0 × 250 mm; 5 µm using a gradient of 5-95% B at 1% B/min at a flow rate of 4 mL/min. Fractions of the correct peptide were collected and identified using ESI-MS and analytical RP-HPLC (**Figure S1**), combined and lyophilised to afford **CMP1** as an amorphous white solid (10 mg, 3% yield, 98% purity).

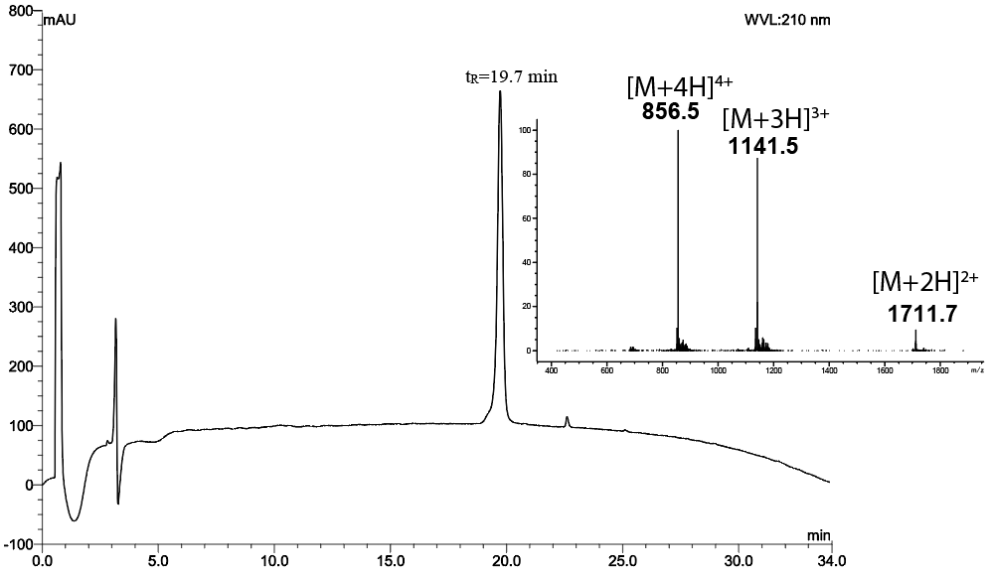


Figure S1. Analytical RP-HPLC (210 nm) of pure **CMP1** performed with a Phenomenex Gemini C18 column (110 Å, 5 µm, 4.6 mm × 150 mm), operated at room temperature, using a linear gradient of 5% B to 95% B over 30 min at 1 mL/min (*ca*. 3% B/min). Buffer A is H_2O with 0.1% TFA (*v/v*) and buffer B is MeCN with 0.1% TFA (*v/v*). ESI-MS of **CMP1**, mass deconvolution calculated at 3421.70 Da (standard deviation of 0.42 Da); theoretical mass calculated at 3421.76 Da.

Monitoring photoinitiated thiol-ene reaction for **CMP1**

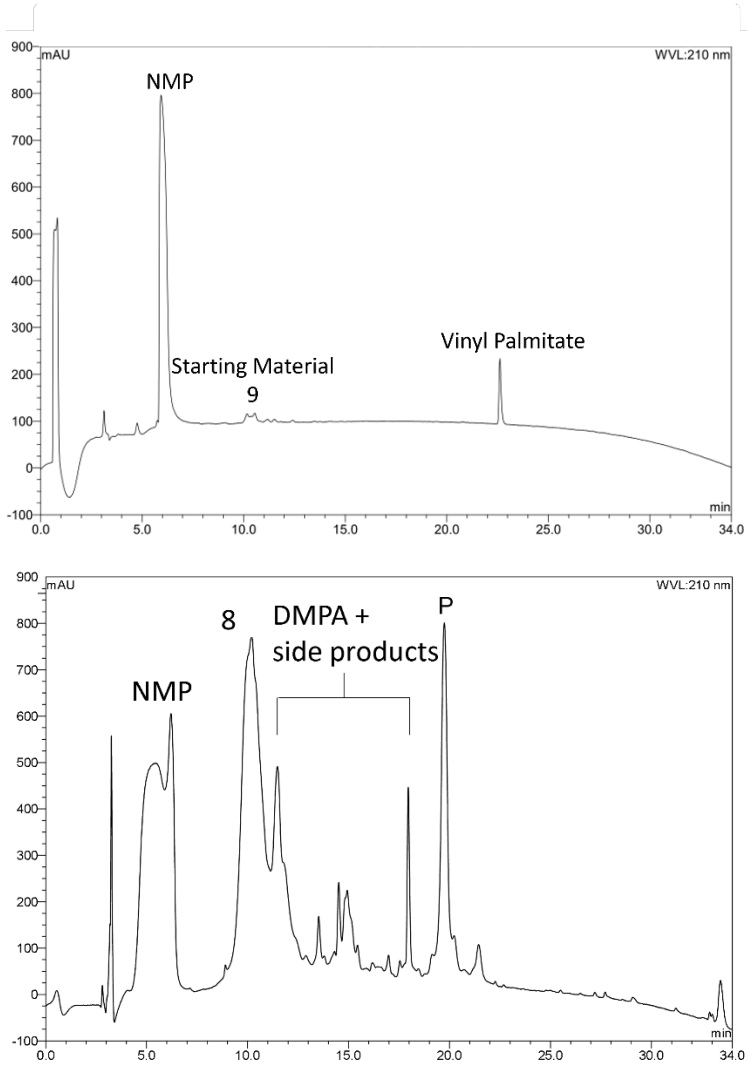
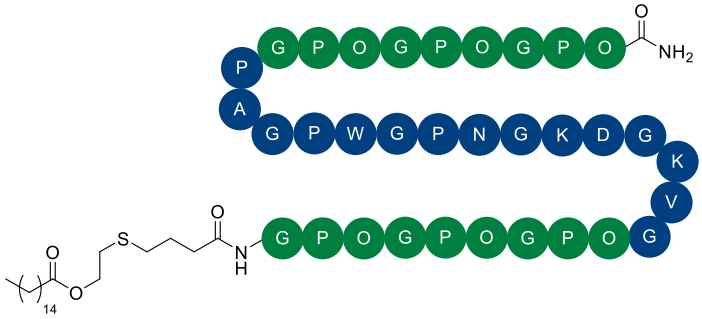


Figure S1.5. Analytical RP-HPLCs (210 nm) of crude **CMP1** (labelled as **P**) before (top) and after (bottom) *S*-lipidation via thiol-ene reaction. No starting material was observed after irradiation. Peptide **8** was also observed after *S*-lipidation which could indicate that the thiol handle was released by the radical initiator DMPA under UV exposure. Performed with a Phenomenex Gemini C18 column (110 Å, 5 μm, 4.6 mm × 150 mm), operated at room temperature, using a linear gradient of 5% B to 95% B over 30 min at 1 mL/min (*ca*. 3% B/min). Buffer A is H_2O with 0.1% TFA (*v/v*) and buffer B is MeCN with 0.1% TFA (*v/v*).

1.1.6 Synthesis of **CMP2**



Peptide synthesis

Fmoc-Rink amide linker (4 eq., 0.4 mmol, 215 mg) was coupled to Tentagel S-NH_2 resin (0.1 mmol scale, loading = 0.27 mmol/g, 370 mg) according to **Method 1**. The first peptidyl residue, hydroxyproline Fmoc-Hyp(tBu)-OH, was manually coupled onto the linker-resin via protocol **Method 3**. Subsequent deprotection and amino acid couplings were performed via the automated peptide synthesiser Biotage® using **Method 4** and **Method 5**. Upon synthesis completion, mini cleavage of the peptidyl resin was conducted using **Method 6.1** and was analysed using LC-MS to ensure the correct peptide (peptide **8**) was synthesised.

Direct thiolation by Traut's Reagent of peptide **8** to peptide **9** was achieved using the conditions in **Method 9**. The peptidyl resin was dried, and the thiolated peptide was cleaved from the resin and deprotected using the conditions outlined in **Method 7** to afford peptide **9**.

Decay and hydrolysis of the 4-mercaptobutyramidine moiety of peptide **9** to peptide **11** was achieved by leaving the peptidyl residue in PBS buffer (pH 7.4) at r.t. followed by incubation in sodium acetate buffer (pH 5) as instructed in **Method 8**.

peptide **11** was first purified (8 mg, 2.5% yield, 95% purity) before *S*-lipidation (**Figure S2**). Attachment of the vinyl ester; vinyl palmitate was conducted using the thiol-ene conditions outlined in **Method 11**. Upon completion of the reaction, the peptide was precipitated using diethyl ether (40 mL), isolated (centrifugation, 4000 rpm, 10 min), concentrated under a light stream of N_2, repeated for 3 times, then dissolved in H_2O/MeCN (1:1, *v/v,* 5 mL) containing 0.1% TFA and lyophilised.

Purification of **CMP2** was conducted on a semi-preparative RP-HPLC using a Dionex Ultimate® 3000 on a Phenomenex Gemini C18 110 Å 10.0 × 250 mm; 5 µm using a gradient of 5-95% B at 1% B/min at a flow rate of 4 mL/min. Fractions of the correct peptide were collected and identified using ESI-MS and analytical RP-HPLC (**Figure S3**), combined and lyophilised to afford **CMP2** as an amorphous white solid (10 mg, 2.9% yield, 98% purity).

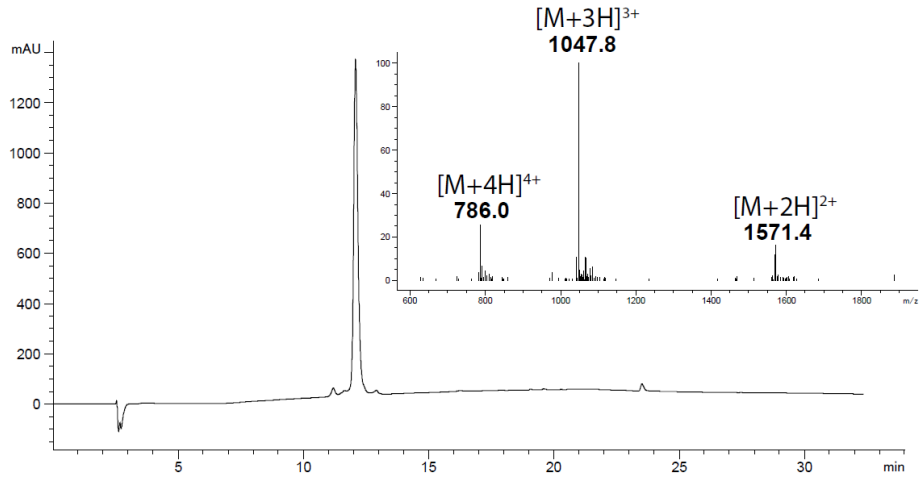


Figure S2. (**A**) Analytical RP-HPLC (210 nm) of pure peptide **11** performed with a Phenomenex Gemini C18 column (110 Å, 5 µm, 4.6 mm × 150 mm), operated at room temperature, using a linear gradient of 5% B to 95% B over 30 min at 1 mL/min (*ca*. 3% B/min). Buffer A is H_2O with 0.1% TFA (*v/v*) and buffer B is MeCN with 0.1% TFA (*v/v*) (**B**) ESI-MS of peptide **11**, mass deconvolution calculated at 3140.40 Da (standard deviation of 0.4 Da); theoretical mass calculated at 3139.48 Da.

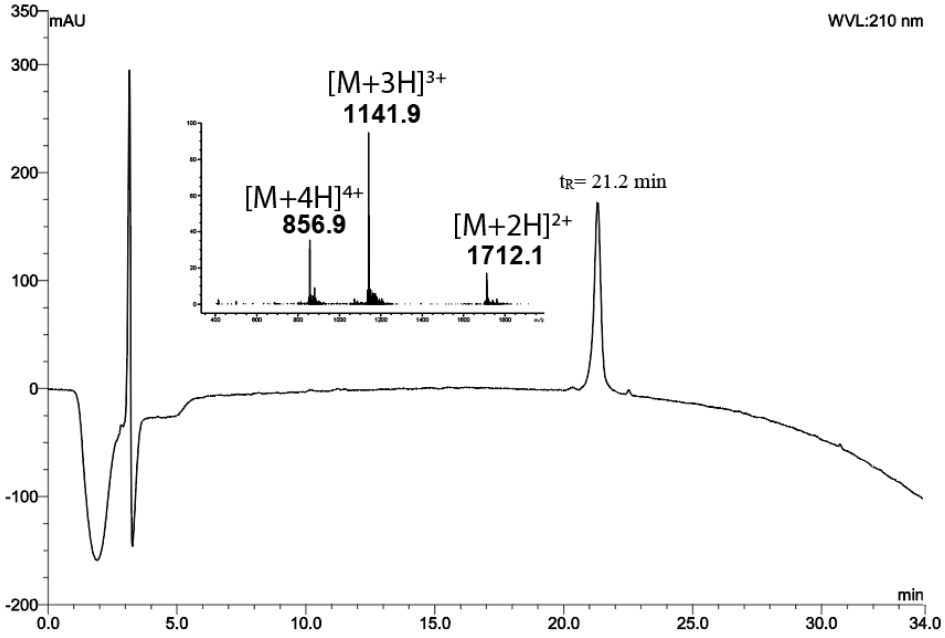


Figure S3. Analytical RP-HPLC (210 nm) of pure **CMP2** performed with a Phenomenex Gemini C18 column (110 Å, 5 µm, 4.6 mm × 150 mm), operated at room temperature, using a linear gradient of 5% B to 95% B over 30 min at 1 mL/min (*ca*. 3% B/min). Buffer A is H_2O with 0.1% TFA (*v/v*) and buffer B is MeCN with 0.1% TFA (*v/v*). ESI-MS of **CMP2**, mass deconvolution calculated at 3422.90 Da (standard deviation of 0.99 Da); theoretical mass calculated at 3421.74 Da.

Monitoring photoinitiated thiol-ene reaction for **CMP2**

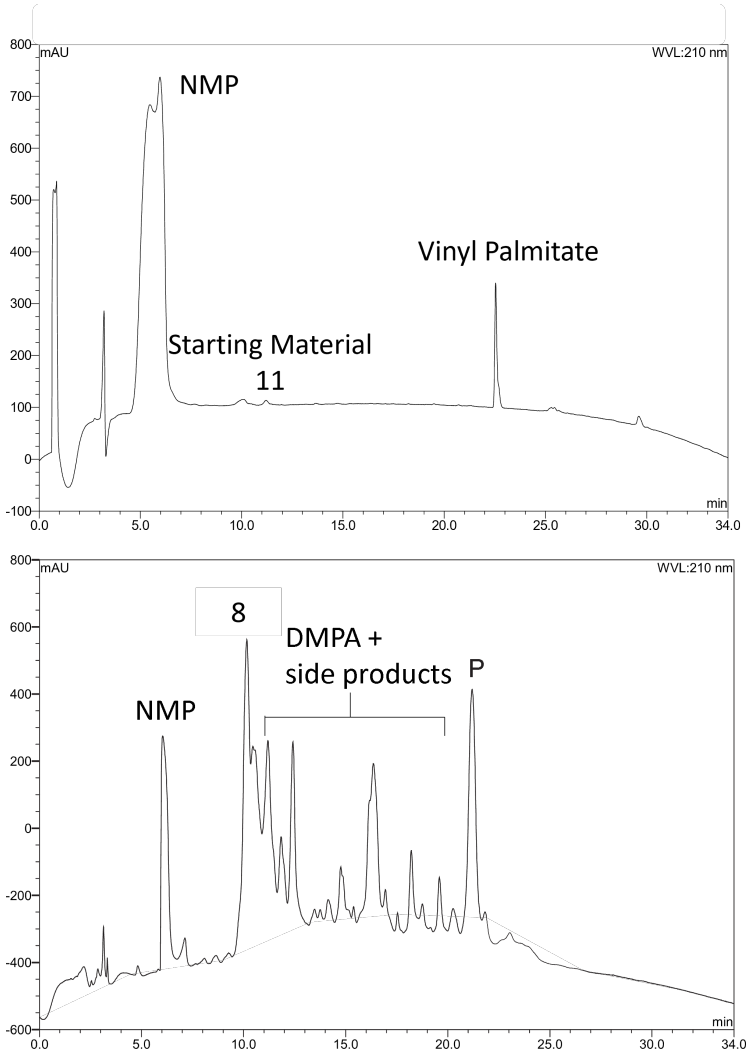
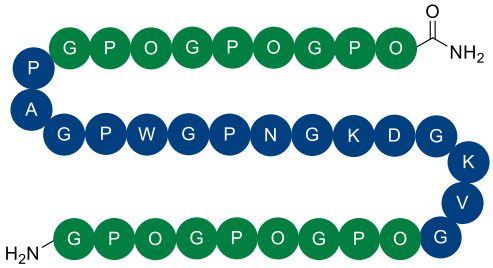


Figure S3.5. Analytical RP-HPLCs (210 nm) of crude **CMP2** (labelled as **P**) before (top) and after (bottom) *S*-lipidation via thiol-ene reaction. No starting material was observed after irradiation. Peptide **8** was also observed after *S*-lipidation which could indicate that the thiol handle was released by the radical initiator DMPA under UV exposure. Performed with a Phenomenex Gemini C18 column (110 Å, 5 µm, 4.6 mm × 150 mm), operated at room temperature, using a linear gradient of 5% B to 95% B over 30 min at 1 mL/min (*ca*. 3% B/min). Buffer A is H_2O with 0.1% TFA (*v/v*) and buffer B is MeCN with 0.1% TFA (*v/v*).

1.1.7 Synthesis of **CMP3**



Peptide synthesis

Fmoc-Rink amide linker (4 eq., 0.4 mmol, 215 mg) was coupled to Tentagel S-NH_2 resin (0.1 mmol scale, loading = 0.27 mmol/g, 370 mg) according to **Method 1**. The first peptidyl residue, hydroxyproline Fmoc-Hyp(tBu)-OH, was manually coupled onto the linker-resin via protocol **Method 3**. Subsequent deprotection and amino acid couplings were performed via the automated peptide synthesiser Biotage® using **Method 4** and **Method 5**. The peptidyl resin was dried, and the peptide was cleaved from the resin and deprotected using the conditions outlined in **Method 6** to afford **CMP3**.

Purification of **CMP3** was conducted on a semi-preparative RP-HPLC using a Dionex Ultimate® 3000 on a Phenomenex Gemini C18 110 Å 10.0 × 250 mm; 5 µm using a gradient of 5-95% B at 1% B/min at a flow rate of 4 mL/min. Fractions of the correct peptide were collected and identified using ESI-MS and analytical RP-HPLC (**Figure S4**) combined and lyophilised to afford **CMP3** as an amorphous white solid (55 mg, 18% yield, >97% purity).

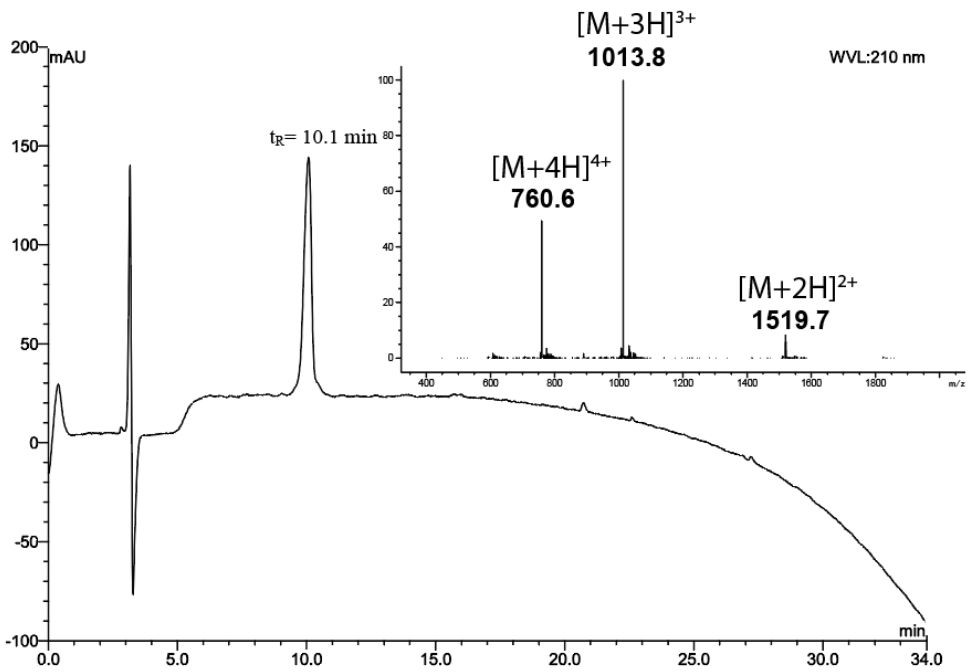
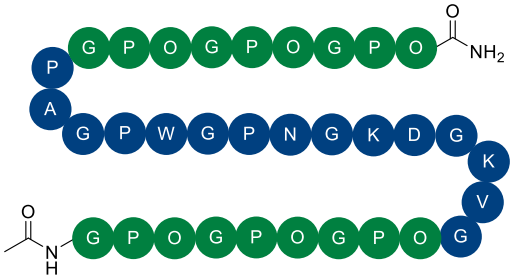


Figure S4. Analytical RP-HPLC (210 nm) of pure **CMP3** performed with a Phenomenex Gemini C18 column (110 Å, 5 µm, 4.6 mm × 150 mm), operated at room temperature, using a linear gradient of 5% B to 95% B over 30 min at 1 mL/min (*ca*. 3% B/min). Buffer A is H_2O with 0.1% TFA (*v/v*) and buffer B is MeCN with 0.1% TFA (*v/v*). ESI-MS of **CMP3**, mass deconvolution calculated at 3037.90 Da (standard deviation of 0.71 Da); theoretical mass calculated at 3037.47 Da.

1.1.8 Synthesis of **CMP4**



Peptide synthesis

Fmoc-Rink amide linker (4 eq., 0.4 mmol, 215 mg) was coupled to Tentagel S-NH_2 resin (0.1 mmol scale, loading = 0.27 mmol/g, 370 mg) according to **Method 1**. The first peptidyl residue, hydroxyproline Fmoc-Hyp(tBu)-OH, was manually coupled onto the linker-resin via protocol **Method 3**. Subsequent deprotection and amino acid couplings were performed via the automated peptide synthesiser Biotage® using **Method 4** and **Method 5**. Upon synthesis completion, mini cleavage of the peptidyl resin was conducted using **Method 6.1** and was analysed using LC-MS to ensure the correct peptide (CMP **1**) was synthesised.

Acetylation of **peptide 8** was achieved using the conditions in **Method 9**. The peptidyl resin was dried, and the N^{α}-acetylated peptide was cleaved from the resin and deprotected using the conditions outlined in **Method 6** to afford **CMP4**.

Purification of **CMP4** was conducted on a semi-preparative RP-HPLC using a Dionex Ultimate® 3000 on a Phenomenex Gemini C18 110 Å 10.0 × 250 mm; 5 µm using a gradient of 5-95% B at 1% B/min at a flow rate of 4 mL/min. Fractions of the correct peptide were collected and identified using ESI-MS and analytical RP-HPLC (**Figure S5**), combined and lyophilised to afford CMP **4** as an amorphous white solid (40 mg, 13% yield, >97% purity).

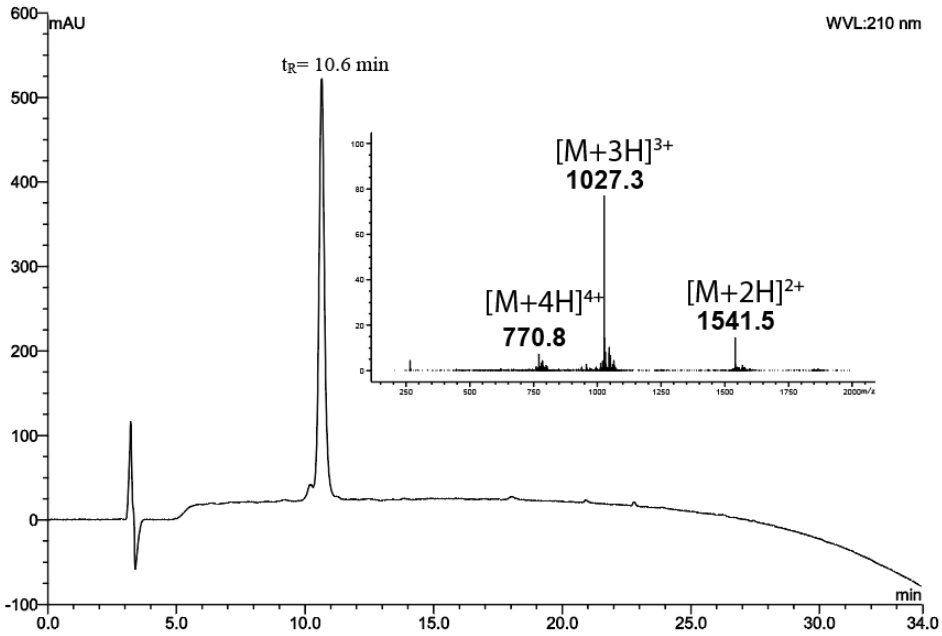
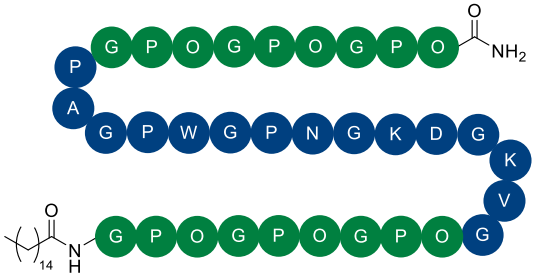


Figure S5. Analytical RP-HPLC (210 nm) of pure **CMP4** performed with a Phenomenex Gemini C18 column (110 Å, 5 µm, 4.6 mm × 150 mm), operated at room temperature, using a linear gradient of 5% B to 95% B over 30 min at 1 mL/min (*ca*. 3% B/min). Buffer A is H_2O with 0.1% TFA (*v/v*) and buffer B is MeCN with 0.1% TFA (*v/v*). ESI-MS of **CMP4**, mass deconvolution calculated at 3080.10 Da (standard deviation of 1.27 Da); theoretical mass calculated at 3079.48 Da.

1.1.9 Synthesis of **CMP5**



Peptide synthesis

Fmoc-Rink amide linker (4 eq., 0.4 mmol, 215 mg) was coupled to Tentagel S-NH_2 resin (0.1 mmol scale, loading= 0.27 mmol/g, 370 mg) according to **Method 1**. The first peptidyl residue, hydroxyproline Fmoc-Hyp(tBu)-OH, was manually coupled onto the linker-resin via protocol **Method 3**. Subsequent deprotection and amino acid couplings were performed via the automated peptide synthesiser Biotage® using **Method 4** and **Method 5**. Upon synthesis completion, mini cleavage of the peptidyl resin was conducted using **Method 6.1** and was analysed using LC-MS to ensure the correct peptide (peptide**8**) was synthesised.

N-Lipidation of peptide **8** was achieved with (C_{16}) palmitic acid using the conditions in **Method 10** The peptidyl resin was dried, and the N^{α}-lipidated peptide was cleaved from the resin and deprotected using the conditions outlined in **Method 6** to afford **CMP5**.

Purification of **CMP5** was conducted on a semi-preparative RP-HPLC using a Dionex Ultimate® 3000 on a Phenomenex Gemini C18 110 Å 10.0 × 250 mm; 5 µm using a gradient of 5-95% B at 1% B/min at a flow rate of 4 mL/min. Fractions of the correct peptide were collected and identified using ESI-MS and analytical RP-HPLC (**Figure S6**). The clean fractions were combined and lyophilised to afford **CMP5** as an amorphous white solid (33 mg, 10% yield, >95% purity).

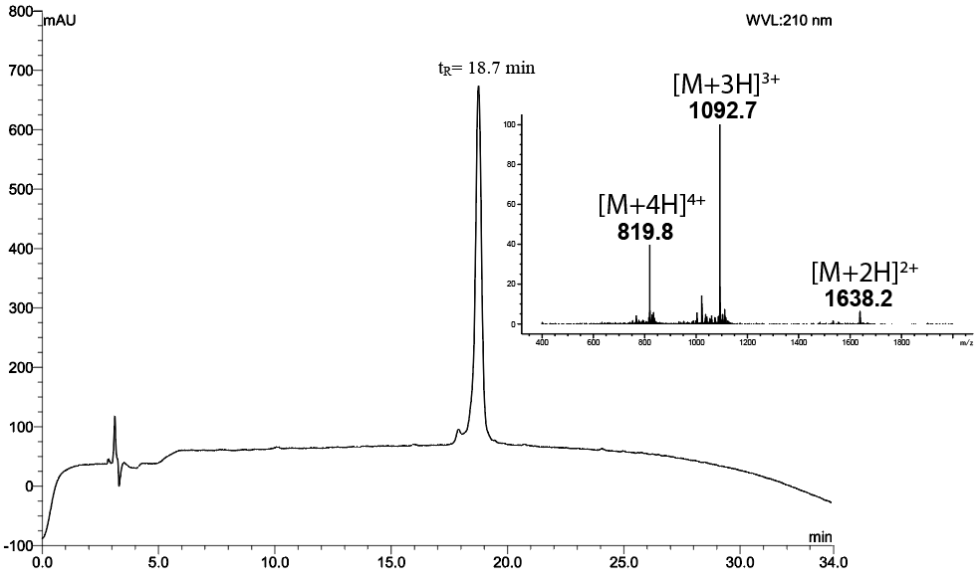


Figure S6. Analytical RP-HPLC (210 nm) of pure **CMP5** performed with a Phenomenex Gemini C18 column (110 Å, 5 µm, 4.6 mm × 150 mm), operated at room temperature, using a linear gradient of 5% B to 95% B over 30 min at 1 mL/min (*ca*. 3% B/min). Where buffer A is H_2O with 0.1% TFA (*v/v*) and buffer B is MeCN with 0.1% TFA (*v/v*). ESI-MS of **CMP5**, mass deconvolution calculated at 3275.20 Da (standard deviation of 0.01 Da); theoretical mass calculated at 3275.70 Da.

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1.2 Circular Dichroism Spectra and Thermal Denaturation Studies

1.2.1 General Procedure for CD Spectra Recording

A Chirascan (Applied Photophysics Ltd, Leatherhead, UK) was used for CD characterisations. CD spectra were recorded on peptide solutions at a concentration of 0.2 mM in 50 mM AcOH. The peptide solutions were pre-incubated at 5°C for at least 24 h to ensure sufficient structure folding. Each CD spectrum measurement was acquired with 1 s integrations and a step size of 1 nm from 180 nm to 320 nm at 20 °C using a 1 mm path quartz cuvette. The reported spectra represent the average of five scans.

1.2.2 General Procedure for the Determination of T_m-values

The thermal denaturation experiments were performed with peptide solutions (0.2 mM) that were pre-incubated at 5°C for at least 24 h in 50 mM AcOH. The samples were then heated from 5 to 80 °C, monitoring the molar ellipticity at 224 nm. Each thermal denaturation experiment was acquired with a heating rate of 2 °C/min and a step size of 5 nm using a 1 mm path quartz cuvette.

1.2.3 General Procedure for Molar Residual Ellipticity (MRE) calculations

The molar residual ellipticity (MRE) is calculated from the measured ellipticity using the equation:

$$[\theta] = \frac{\theta \times m}{c \times l \times n_r}$$

Where θ is the measured ellipticity in millidegrees, m is the molecular weight in g/mol, c is the concentration in mg/ml, l is the path length of the cuvette in cm and $n_r$is the number of amino acids in the peptide.

Thermal denaturation curves were used to calculate the first derivative to determine the thermal temperature stability (T_m) in °C of the sample. The equation used to calculate the first derivative is shown below:

$$\frac{dy}{dx} = \frac{\Delta MRE}{\Delta Temperature}$$

Where $\frac{dy}{dx}$ is the first derivative, ΔMRE is the change in MRE and $\Delta Temperature$ is the change in temperature.

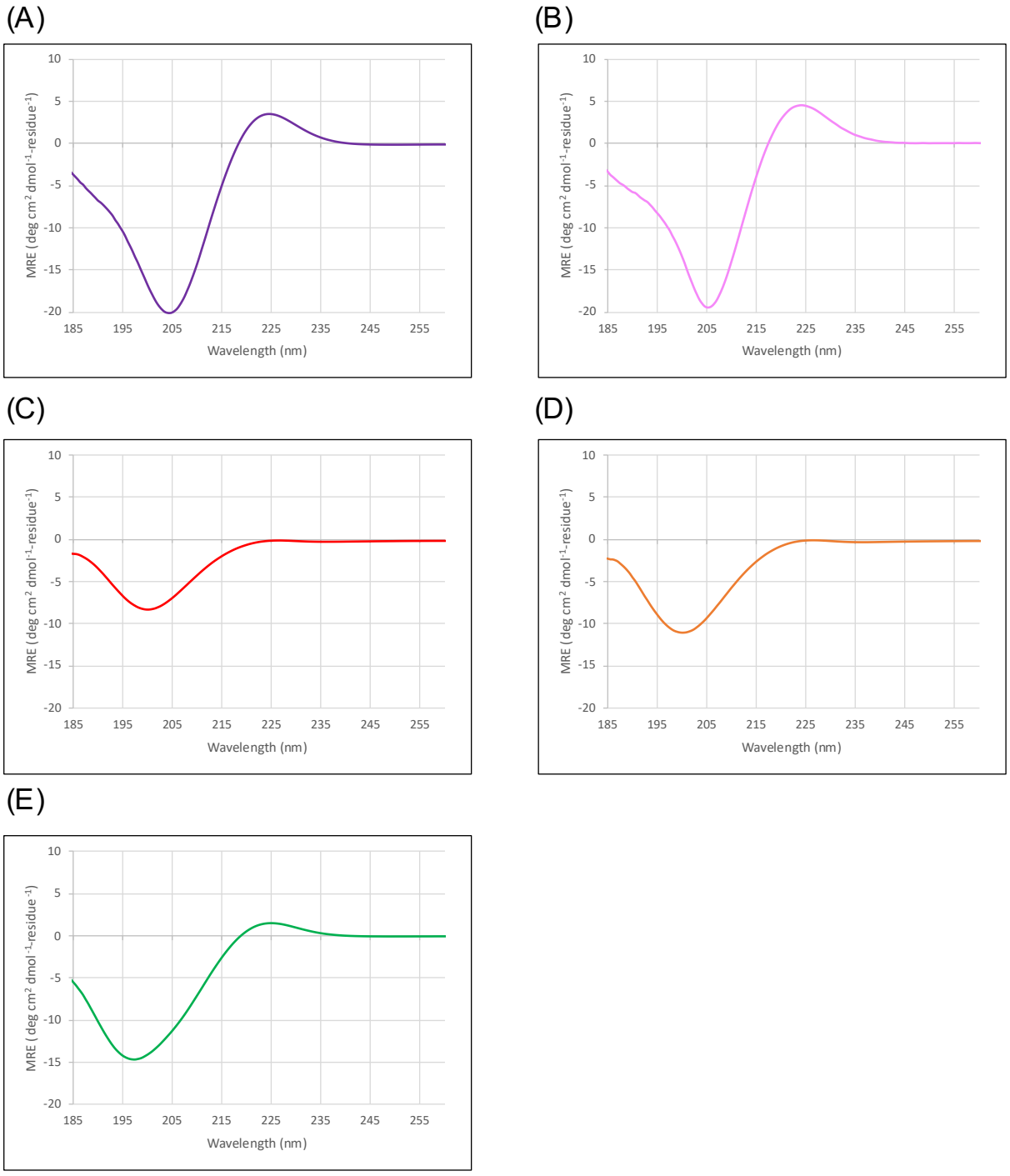


Figure S7. Circular Dichroism Spectra of **CMP1-5**. (**A**) CD spectra of **CMP1**. (**B**) CD spectra of **CMP2**. (**C**) CD spectra of **CMP3**. (**D**) CD spectra of **CMP4**. (**E**) CD spectra of **CMP5**.

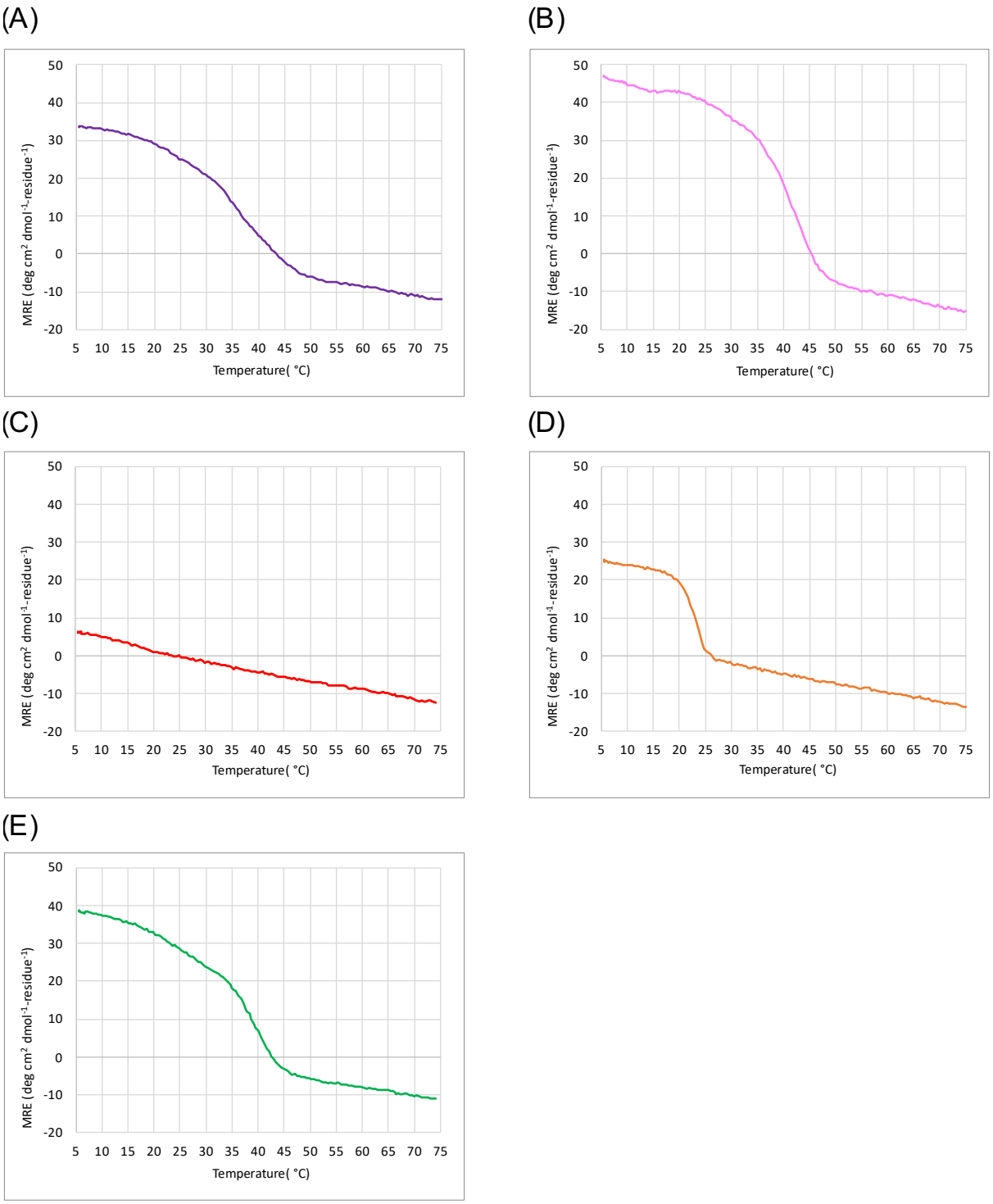


Figure S8. Thermal Denaturation (TD) curves of **CMP1-5**. (**A**) TD spectra of **CMP1**. (**B**) TD spectra of **CMP2**. (**C**) TD spectra of **CMP3**. (**D**) TD spectra of **CMP4**. (**E**) TD spectra of **CMP5**.

Table S1. Rpn Values for CMPs **1**-**5**.

CMP #	Peptide	Rpn
1	C_{16}-$S^{amidine}$-$(GPO)_3$-[IV-H1]-$(GPO)_3$-$CONH_2$	0.17
2	C_{16}-S^{amide}-$(GPO)_3$-[IV-H1]-$(GPO)_3$-$CONH_2$	0.24
3	H_2N-$(GPO)_3$-[IV-H1]-$(GPO)_3$-$CONH_2$	-
4	Ac-$(GPO)_3$-[IV-H1]-$(GPO)_3$-$CONH_2$	0.01
5	C_{16}-$(GPO)_3$-[IV-H1]-$(GPO)_3$-$CONH_2$	0.11

*Values were calculated from the ratios between the positive ellipticity peak over the negative ellipticity peak.[31,34,36,37] $S^{amidine}$ refers to the amidine moiety found at the *N*-terminus bearing the thiol handle, while S^{amide} refers to the amide moiety found at the *N*-terminus bearing the thiol handle.

Table S2. T_m Values for CMP **1**-**5** Triple Helix ↔ Coil Transitions

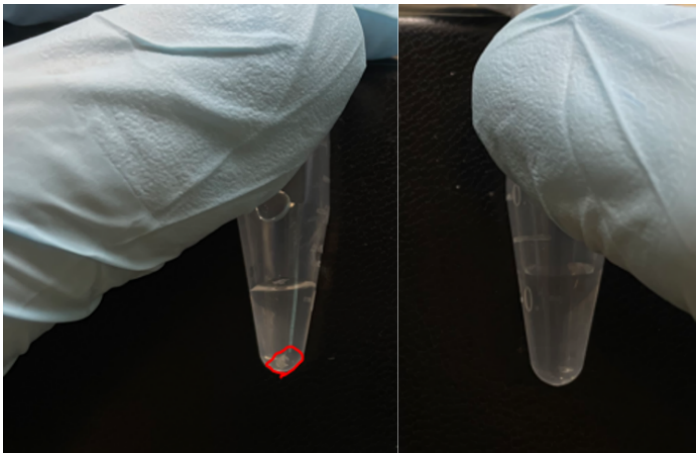
CMP #	Peptide	T_m (°C)
1	C_{16}-$S^{amidine}$-$(GPO)_3$-[IV-H1]-$(GPO)_3$-$CONH_2$	36.0
2	C_{16}-S^{amide}-$(GPO)_3$-[IV-H1]-$(GPO)_3$-$CONH_2$	42.0
3	H_2N-$(GPO)_3$-[IV-H1]-$(GPO)_3$-$CONH_2$	-
4	Ac-$(GPO)_3$-[IV-H1]-$(GPO)_3$-$CONH_2$	22.5
5	C_{16}-$(GPO)_3$-[IV-H1]-$(GPO)_3$-$CONH_2$	37.0

*Values were calculated from the first derivative of the thermal denaturation curves. Collagen peptide concentration used for this experiment is 0.2 mM. $S^{amidine}$ refers to the amidine moiety found at the *N*-terminus bearing the thiol handle, while S^{amide} refers to the amide moiety found at the *N*-terminus bearing the thiol handle.

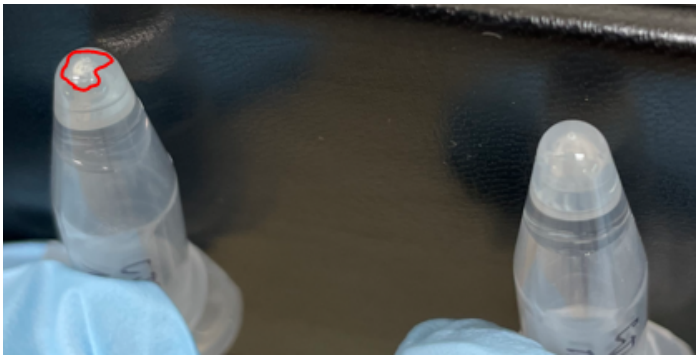
1.3 Solubility Testing

The solubilities of the respective CMPs were investigated by dissolving the peptides in 100% MQ H_2O (200 µl) to give a final 5 mg/ml concentration. **CMP1** and **CMP5** were used as subjects for this study to investigate the influence of the positively charged amidine moiety. After the addition of H_2O (pH 7), both mixtures were centrifuged (centrifugation, 4000 rpm, 5 min).

(A)



(B)



(C)

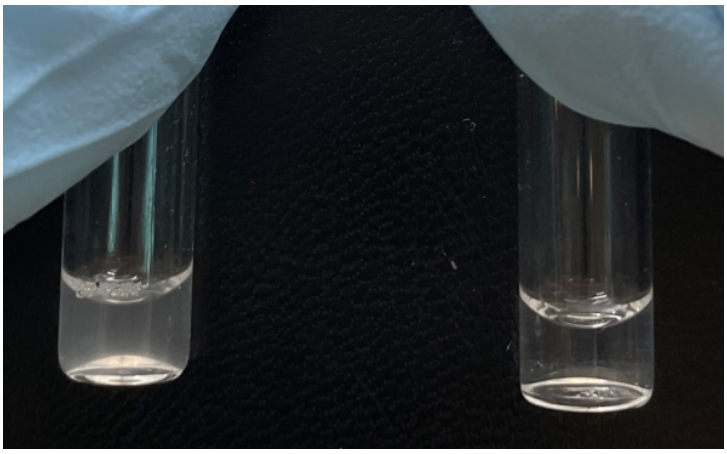


Figure S9. Pictures taken from **CMP5** (Left) and **CMP1** (Right) to show their solubility capabilities in H_2O. A red highlight shows the pellet that was formed after centrifugation.

1.4 Cell viability Assays

Cell culture:

For MCF 10A cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM) F12 (GIBCO, Life Technologies, New Zealand) supplemented with 5% horse serum (Invitrogen, New Zealand), 20 ng/mL EGF, 0.5 mg/mL Hydrocortisone, 100 ng/mL Cholera toxin, 10 µg/mL Insulin, 100 U/mL Penicillin G, 100 U/mL Streptomycin 20 ng/mL, 100 U/mL Pen/Strep (GIBCO, Life Technology, New Zealand). All culture experiments were performed in a humidified 5% CO_2 incubator at 37^{o}C.

Crystal violet assay for cytotoxicity measurements

Cells were seeded at a density of $3x10^3$ cells per well in a 96 well plate. Peptides were then added at a final concentration of 2.5 µM, 5 µM, 10 µM, 20 µM and 40 µM and cells were incubated for 72hr. After discarding the media, cells were washed with pH 7.4 PBS and then stained with crystal violet dye for 30 minutes at RT. After Staining, crystal violet was removed, and the excess dye was discarded by washing. The cell-bound dye was redissolved in 1% SDS and the optical density was measured at λ = 590 nm using a spectrophotometer (Varioskan, Thermo Fischer, New Zealand).

Cell adhesion:

96 well plates were coated with different concentrations of peptides and allowed to adsorb overnight. Nonspecific binding sites were blocked with 2 mg/mL BSA in PBS for 2 h at 37°C. Cells were seeded at a density of $1x10^4$ cells per well in a 96 well plate and incubated for 1 hr at 37°C. After Nonadherent cells were removed by washing with pH 7.4 PBS. Adherent cells were then stained with crystal violet and quantified using the protocol discussed under crystal violet assay.

Cell Spreading:

Cover slips were coated with 40 µM of peptides and then placed in the 24 well plate after UV sterilisation. After blocking the nonspecific binding sites, $2.5x10^4$ cells per well were seeded and incubated for 1 hr at 37°C. After Nonadherent cells were removed by washing with pH 7.4 PBS. After fixation, cells were stained with CytoPainter Phalloidin-iFluor 488 and then with Hoechst 33342. Cells were washed with cold 0.1% v/v PBS in Tween 20 for 10 minutes. A coverslip was then mounted using an anti-fading solution keeping the clean surface upwards. Cells were then visualised with a Zeiss Axio epifluorescence microscope and quantified using Image J.