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Solid-phase synthesis of peptide with azopyridine side-chain for Mn(I)–CO binding and red-light responsive CO release

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1 General considerations

1.1 Reagents and Instruments

All chemicals were obtained from commercial sources: Sigma-Aldrich, Adamas-beta, General-reagent without further purification. Boc-4-Amino-L-phenylalanine (Boc-Aph-OH) was purchased from Aladdin. 4-Amino-L-phenylalanine (H-Aph-OH), 2-(1-hydroxy-3-methylbutylidene)-5,5-dimethylcyclohexane-1,3-dione (ivDde-OH) and 1-(9-fluorenyl)methyl chloroformate (Fmoc-Cl) were purchased from Bidepharm. Allyl carbonochloridate (Alloc-Cl) was purchased from TCI. Fmoc-amino acids, 1-Hydroxybenzotriazole (HOBt), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate (TBTU), Rink Amide MBHA Resin were purchased from GL Biochem (Shanghai). Acetonitrile (MeCN) used in analytical HPLC and semi-preparative HPLC was obtained from Fisher and Sigma-Aldrich, respectively. CO probe 1 (COP-1) was synthesized and characterized as described previously.[1] Recombinant hemopexin-like (PEX) domain of MMP-2 was obtained as described previously.[2] Myoglobin (Mb, from equine heart) was from Sigma-Aldrich. Horse serum was obtained from Sangon. DMEM cell culture medium containing 5% FBS (BioInd), 4.5 g/L D-Glucose, 4 mM L-glutamine, and 0.9 mM sodium pyruvate was obtained from VivaCell Biosciences (Shanghai). Penicillin-Streptomycin (Gibco, Life Technologies, USA) was used for cell culture. Cell Counting Kit-8 (CCK-8) was obtained from GlpBio.

^{1}H NMR spectra were recorded on a 400 MHz NMR spectrometer (AVANCE III HD 400, Bruker) and a 600 MHz NMR spectrometer (AVANCE III HD 600, Bruker). Analytical HPLC (Agilent 1260) was performed on a Phenomenex Jupiter C18 column (4.6 × 250 mm, 4.6 μm particle size) running at a flow rate of 1 mL min^{-1} with UV detection at 214 nm. Semi-preparative HPLC (Shimadzu AR-20) was performed using a Waters XBridge® peptide BEH C18 OBD™ Prep column, 300 A, 5 μm, 10 × 250 mm) running at a flow rate of 5 mL min^{-1} with UV detection at 214 nm. Solvent A: 0.1 % TFA in water; Solvent B: 0.1% TFA in MeCN. LC-MS was performed on an Agilent LC/MSD (ESI) system on ACE 5 C4 column (150 × 4.6 mm). UV–visible spectra were recorded on a UV spectrophotometer (UV-1800, Shimadzu Corporation). FT-IR measurements were conducted using FT-IR spectrometer (Tensor 27, Bruker) using KBr pellet. A multi-Mode microplate reader (SpectraMax i3x, Molecular Devices) was used in CCK-8 assay. Flow cytometry analysis was performed using Annexin V-APC/7-AAD Apoptosis Detection Kit (KeyGEN BioTECH) and BD FACSCanto™ Clinical Flow Cytometry System (USA).

1.2 Light source

The light source used in the CO release study was red light (~625 nm, LED, 5 W), and it was used for the spectral analysis of CO release, myoglobin assay, image of living cells and cellular assay.

1.3 Two-compartment myoglobin assay

A two-compartment myoglobin assay developed by Liao et. al was used.[3] As shown in **Figure S1**, myoglobin solution was degassed and mixed with excess amount of $Na_2S_2O_4$ in a sealed flask. A series of azpy-CORM solution in PBS buffer was degassed and transferred into an air-tight syringe. The syringe was irradiated by red LED light at 4℃ and left for 3 min to increase the solubility of CO in solution. Pull

the plunger slowly to draw the deoxy-Mb solution into syringe, shake the syringe gently to make sure two solutions mixed fully. The final concentration of azpy-CORM in this assay was 10 µM, and final concentration of deoxy-Mb is 40 µM (~1.3 times of the theoretical yield of CO). Then absorbance of the mixture solution was recorded in a quartz cuvette. All experiments were performed in triplicate.

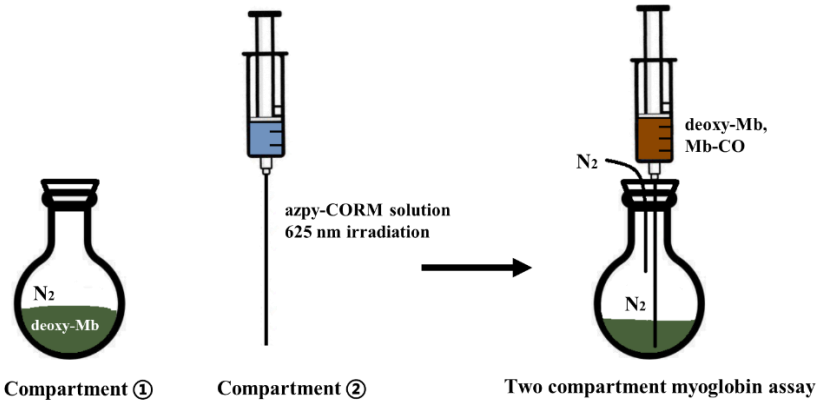

Figure S1. Two compartment Mb-CO assay of CO release from azpy-CORMs.

2 Synthesis and characterization of complexes

2.1 Amino acid monomer with the azpy ligand (1)

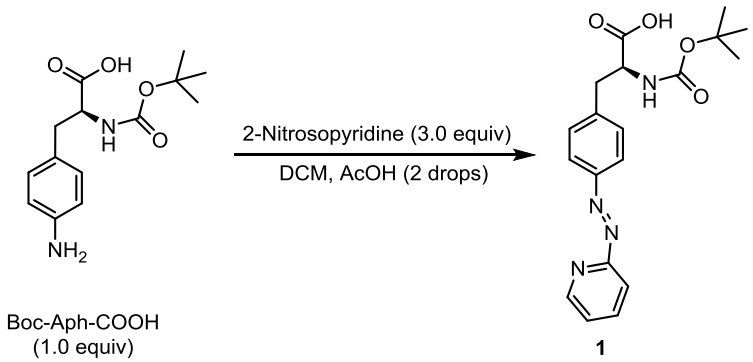

Figure S2. Synthesis of monomer (1)

To a solution of Boc-Aph-COOH (1.0 equiv, 0.071 mmol, 20.00 mg) in 2 mL of dichloromethane (DCM) containing 2 drops of acetic acid (AcOH), 2-nitrosopyridine (synthesized as described previously,[4] 3.0 equiv, 0.21 mmol, 23.12 mg) was added. The mixture was stirred fully at room temperature for 12 h, then was evaporated to dryness under high vacuum. The product was re-crystallized from MeCN to obtain monomer **1** as an orange powder. Yield: 21.23 mg (80.6%).

ESI-MS calculated for $[M+H]^+$ m/z = 371.41, found: 371.10. **^{1}H NMR (600 MHz, Chloroform-*d*)** δ 8.73 (dd, *J* = 4.9, 1.8 Hz, 1H), 7.98 – 7.90 (m, 3H), 7.84 (d, *J* = 8.0 Hz, 1H), 7.45 (dd, *J* = 17.6, 7.7 Hz, 3H), 7.26 (s, 1H), 5.26 (d, *J* = 7.4 Hz, 1H), 4.69 (q, *J* = 6.1 Hz, 1H), 3.30 (d, *J* = 5.5 Hz, 2H), 1.45 (s, 9H).

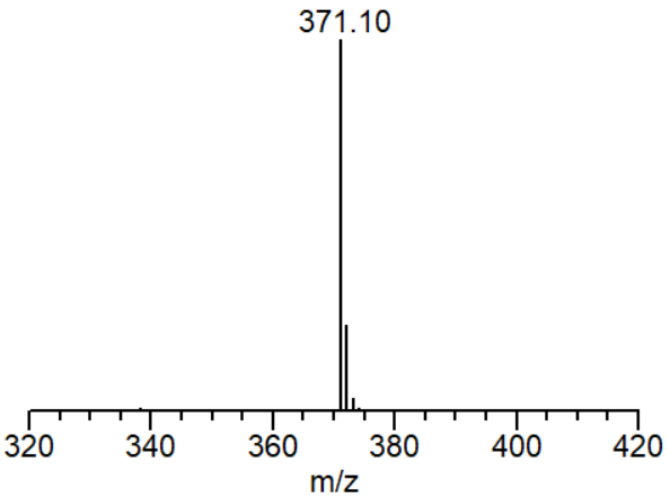

Figure S3. ESI-MS spectrum of monomer **1** (Mass calculated for $[M+H]^+$ = 371.41, found: 371.10).

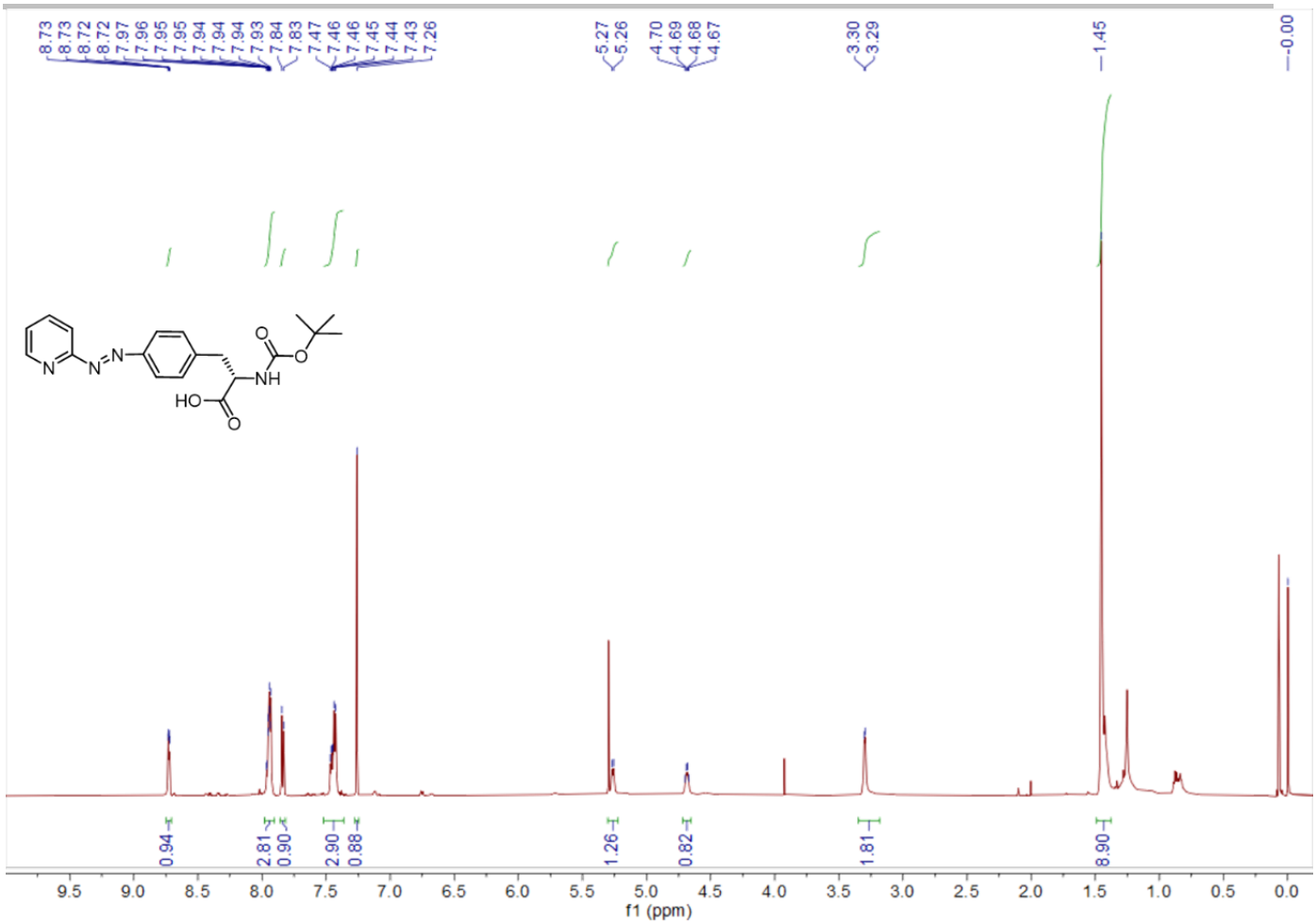

Figure S4. ^{1}H NMR spectrum of monomer **1**.

2.2 Azpy-CORM monomer (2)

Synthesis of azpy-CORM monomer (2)

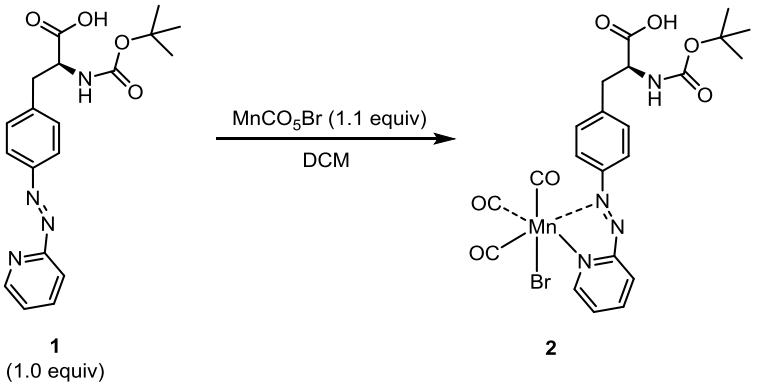

Figure S5. Synthesis of azpy-CORM monomer (**2**)

$Mn(CO)_5Br$ (1.1 equiv, 0.014 mmol, 3.9 mg) was added to a solution of monomer **1** (1.0 equiv, 0.013 mmol, 5.0 mg) in 1 mL DCM. The mixture was stirred fully for 4 h at 40 °C in the dark. The solution was dried under high vacuum and crystallised from Et_2O to afford azpy-CORM monomer **(2)** as a dark blue powder. Yield: 5.19 mg (68%). ESI-MS calculated for $[M\text{-}Br+CH_3CN]^+$ m/z = 550.11, found: 550.10.

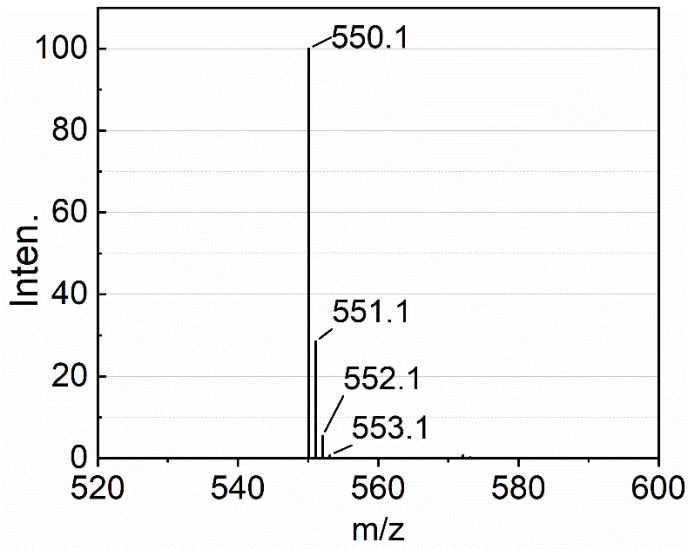

Figure S6. ESI-MS calculated for $[M\text{-}Br+CH_3CN]^+$ m/z = 550.11, found: 550.10.

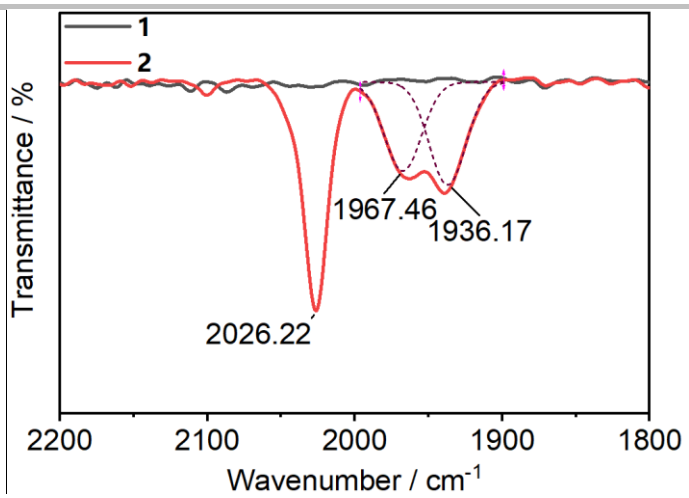

Figure S7. FT-IR spectra of **1** (black line) and **2** (red light) in KBr pellets. The Gaussian fittings allowed for the assignment of the two un-resolved ν_{CO} bands are shown as dashed lines.

CO quantification

The concentration of Mb-CO was calculated according to the following equations 1:[5]

$$\frac{A_{542}}{A_{iso}} = \frac{\varepsilon_{d542}[Mb] + \varepsilon_{CO542}[MbCO]}{\varepsilon_{iso}([Mb] + [MbCO])} = \frac{\varepsilon_{d542}}{\varepsilon_{iso}} - \frac{\varepsilon_{d542} - \varepsilon_{CO542}}{\varepsilon_{iso}} \times \frac{[MbCO]}{[Mb] + [MbCO]}$$

$$\therefore \frac{[MbCO]}{[Mb] + [MbCO]} = \left(\frac{\varepsilon_{d542}}{\varepsilon_{iso}} - \frac{A_{542}}{A_{iso}}\right) \times \frac{\varepsilon_{iso}}{\varepsilon_{d542} - \varepsilon_{CO542}} \quad \text{(Equation 1)}$$

Where A_{542} and A_{iso} are the absorbance at 542 and 552, ε_{d542}, ε_{CO542} and ε_{iso} are the extinction coefficients of Mb at 542 nm, MbCO at 542 nm and Mb (and MbCO) at 552 nm, respectively, and [Mb] and [MbCO] are the concentration of Mb and MbCO. $\varepsilon_{d542}/\varepsilon_{iso}$ and $\varepsilon_{CO542}/\varepsilon_{iso}$ are 0.836 and 1.227, respectively.

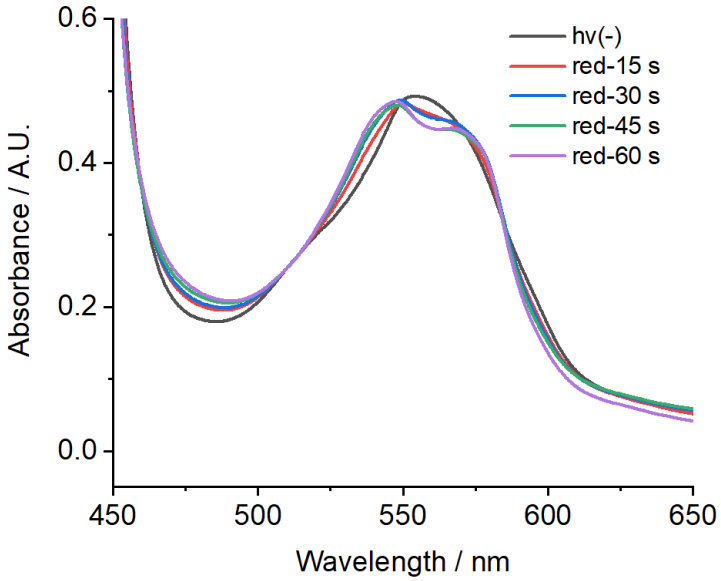

Figure S8. Time-resloved absorption spectral of deoxy-Mb (final concentration: 40 µM, in 10 mM PBS buffer, pH 7.4) in a solution containing **2** (final concentration 10 µM, in 10 mM PBS buffer, pH 7.4) after red light irradiation at RT.

Apparent rate of CO release

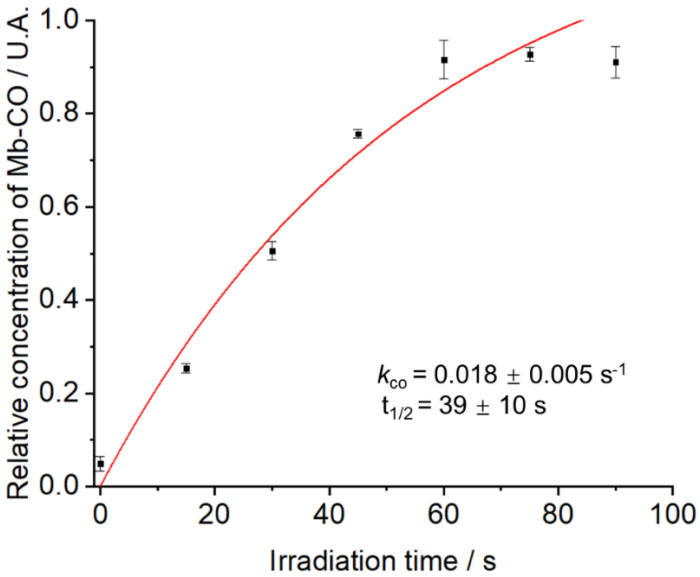

Figure S9. Apparent CO release rate (k_{CO}) and the half-time ($t_{1/2}$) of CO release from azpy-CORM monomer **2** (conc. 10 µM) through a nonlinear regression fitting. The conversion rate of Mb-CO vs. irradiation time (s) in PBS buffer at 298 K upon exposure to red light. All experiments were performed in triplicate.

Measurements of CO release quantum yield

The quantum yield of a photoreaction is defined as followed:

$$\emptyset = \frac{number\ of\ reacted\ molecular\ per\ time\ unit}{number\ of\ photons\ absorbed\ per\ time\ unit} \times 20 \quad \text{(Equation 2)}$$

(1) CO quantification

The quantification of CO release from **2** was measured by two compartment myoglobin assay. Briefly, the solution of **2** (200 μL in PBS buffer, pH 7.4; final conc.: 10 μM) was placed in quartz cell to record the absorption at 625 nm, then exposed to 625 nm red light for 30 s. A solution of deoxy-myoglobin (deoxy-Mb) (300 μL in PBS buffer, pH 7.4; final conc.: 40 μM) containing sodium dithionite (2.4 mM) was added into the photoactivated solution of **2** and mixed fully. The absorbance of the mixture solution was recorded in a quartz cuvette and the concentration of Mb-CO was calculated according equation 1. All experiments were performed in triplicate.

(2) Photon quantification

The photon quantification was measured by Reinecke's salt actinometry at excitation wavelengths of 625 nm in an aqueous solution.[6] A solution (500 μL) of Reinecke's salt (10 mM) was irradiated by 625 nm red light for 30 s and another 500 μL of Reinecke's salt placed in the dark as a control. After irradiation, 100 μL of each solution was diluted with 900 μL of a solution containing 0.1 M $Fe(NO_3)_3$ and 0.5 M $HClO_4$. After 5 min incubation in the dark, the mixtures were diluted to 2 mL by dd-H_2O and the absorbance at 450 nm was measured. All experiments were performed in triplicate.

(3) Calculation of CO release quantum yield

The quantum yield of Reinecke's salt at the irradiation wavelength (625 nm) is as follow:

$$\emptyset(R) = \frac{moles\ of\ SCN^-/t}{F(1-10\text{^}(-OD(625))} \quad \text{(Equation 3)}$$

$$[SCN^-] = \frac{\Delta A(450\ nm)}{l\varepsilon(450\ nm)} \times 20 \quad \text{(Equation 4)}$$

Where ΔA(450 nm) is the absorption difference between the irradiated solution and the dark control; *l* is the optical path length of the cuvette; ε =4300 $M^{-1}cm^{-1}$ at a wavelength of 450 nm which is the extinction coefficient of $Fe(SCN)^{2+}$; $\emptyset(R)$ = 0.28; t is the irradiation time of Reinecke's salt solution.

The quantum yield of CO release is as follows:

$$\emptyset(CO) = \frac{moles\ of\ CO/t'}{F(1-10\text{^}(-OD'(625))} \quad \text{(Equation 5)}$$

Where the moles of CO was measured by two compartment myoglobin assay; OD' (625) is the absorption of the solution **2** at 625 nm; t' is the irradiation time in myoglobin assay, which is same with the irradiation time of Reinecke's salt solution.

Table S1. Measurements and calculation results of the CO release quantum yield.

Photons quantification	1st	2nd	3rd
A450	0.772	0.699	0.712
A450’	0.716	0.646	0.666
△A	0.056	0.053	0.046
[SCN^-] (M)	2.60E-04	2.47E-04	2.14E-04
OD 625	0.026	0.024	0.024
CO quantification	1st	2nd	3rd
A555	0.502	0.493	0.502
A552	0.512	0.502	0.511
A542	0.486	0.475	0.482
[CO] (M)	15.84E-06	14.98E-06	14.69E-06
OD’ 625	0.01	0.01	0.01
quantum yield	1st	2nd	3rd
Φ_{CO}	4.35E-02	4.01E-02	4.54E-02
Average	4.30E-02±0.0027		

Serum stability assay

A stock solution of azpy-CORM monomer **2** was prepared using methanol (2 mg/mL in MeOH). The solution of **2** was incubated together with 50% horse serum solution in PBS buffer (final concentration of **2** was 0.5 mg/mL) at RT in the dark. The resulting solution was centrifuged at 11 000 rpm for 10 min in the dark, and 10 μL of the supernatant was analysed by LC-MS. The percentage of the monomer **2** remained was calculated based on the area under its HPLC peak. The possible CO leakage during this process was also detected via the Mb assay.

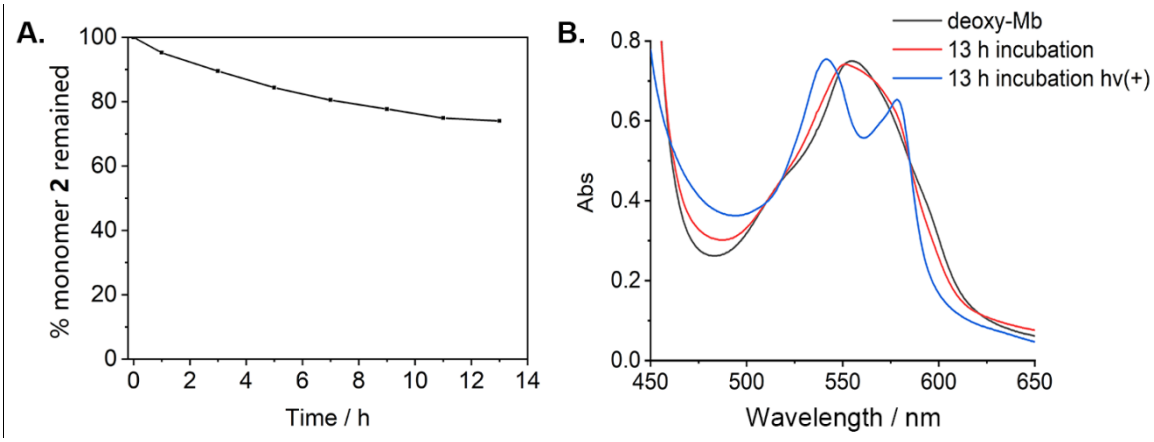

Figure S10. (A) Serum stability assay as indicated by the percentage of the monomer **2** remained vs. incubation time. (B) The absorption spectra of deoxy-Mb added into the solution containing **2** following 13 h of the serum stability test (conc. of **2**: 50 μM; conc. of Mb: 50 μM, in 50% horse serum solution in PBS buffer).

3 Preparation of azpy-peptide-CORM

3.1 Fmoc-L-Aph(PG)-OH synthesis

3.1.1 Fmoc-L-Aph(Alloc)-OH

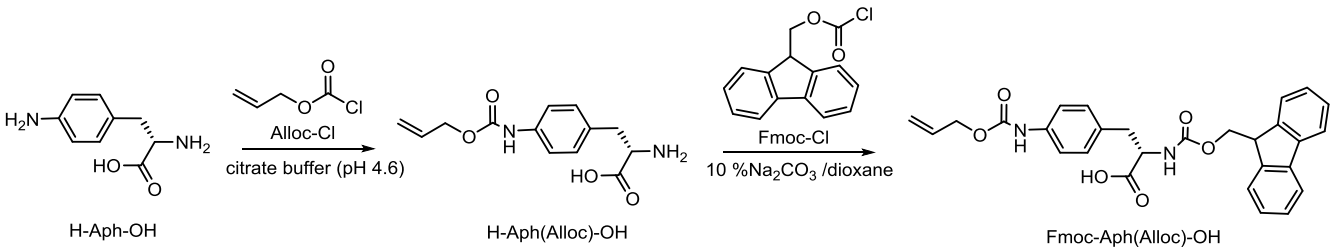

Figure S11. Synthesis of Fmoc-L-Aph(Alloc)-OH

Fmoc-L-Aph(Alloc)-OH was synthesized as described previously.[7] Alloc chloroformate (0.59 mL, 5.60 mmol) was added to a solution of H-Aph-OH (1.00 g, 4.63 mmol) in citrate buffer (pH 4.6). The mixture was stirred at room temperature and the reaction was monitored by analytical TLC. The product was collected by filtration and washed with citrate buffer (pH 4.6) and dd-H_2O, and dried under high vacuum to afford the H-Aph(Alloc)-OH as white powder (0.61 g, 49% yield). ESI-MS calculated $[M+H]^+$: 265.28, found: 265.10.

H-Aph(Alloc)-OH (0.61 g, 2.30 mmol) was dissolved in 10% Na_2CO_3 /dioxane (1:1, 15 mL), and a solution of Fmoc-Cl (0.64 g, 2.50 mmol) in dioxane (4 mL) was added drop wise at 0 °C for 1 h. The mixture was then stirred at room temperature overnight and monitored by analytical TLC. The solution was extracted with ether. The aqueous layer was acidified with HCl to pH 2.0 in ice bath. The white precipitate was collected and dissolved in EtOAc and washed with 0.1 M HCl and dd-H_2O, then dried with Na_2SO_4 and concentrated under reduced pressure. The product was re-crystallized from EtOAc/hexane to obtain Fmoc-L-Aph (Alloc)-OH (0.76 g, 68% yield).

ESI-MS calculated for $[M+H]^+$: 487.52, found: 487.20. **^{1}H NMR (400 MHz, DMSO-d_6)** δ 12.73 (s, 1H), 9.65 (s, 1H), 7.88 (d, *J* = 7.4 Hz, 2H), 7.75 – 7.46 (m, 3H), 7.41 – 7.26 (m, 5H), 7.23 – 7.11 (m, 2H), 5.97 (ddq, *J* = 15.5, 10.2, 5.1 Hz, 1H), 5.34 (dd, *J* = 17.3, 8.2 Hz, 1H), 5.23 (d, *J* = 10.3 Hz, 1H), 4.60 (d, *J* = 5.3 Hz, 2H), 4.16 (d, *J* = 25.8 Hz, 4H), 3.02 (dd, *J* = 13.9, 4.6 Hz, 1H), 2.81 (dd, *J* = 13.8, 10.4 Hz, 1H).

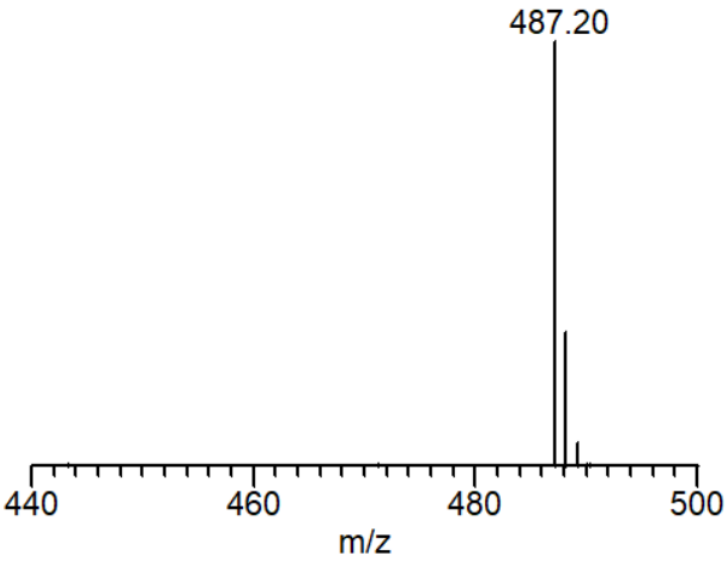

Figure S12. ESI-MS calculated for Fmoc-L-Aph(Alloc)-OH: $[M+H]^+$ m/z = 487.52, found: 487.20.

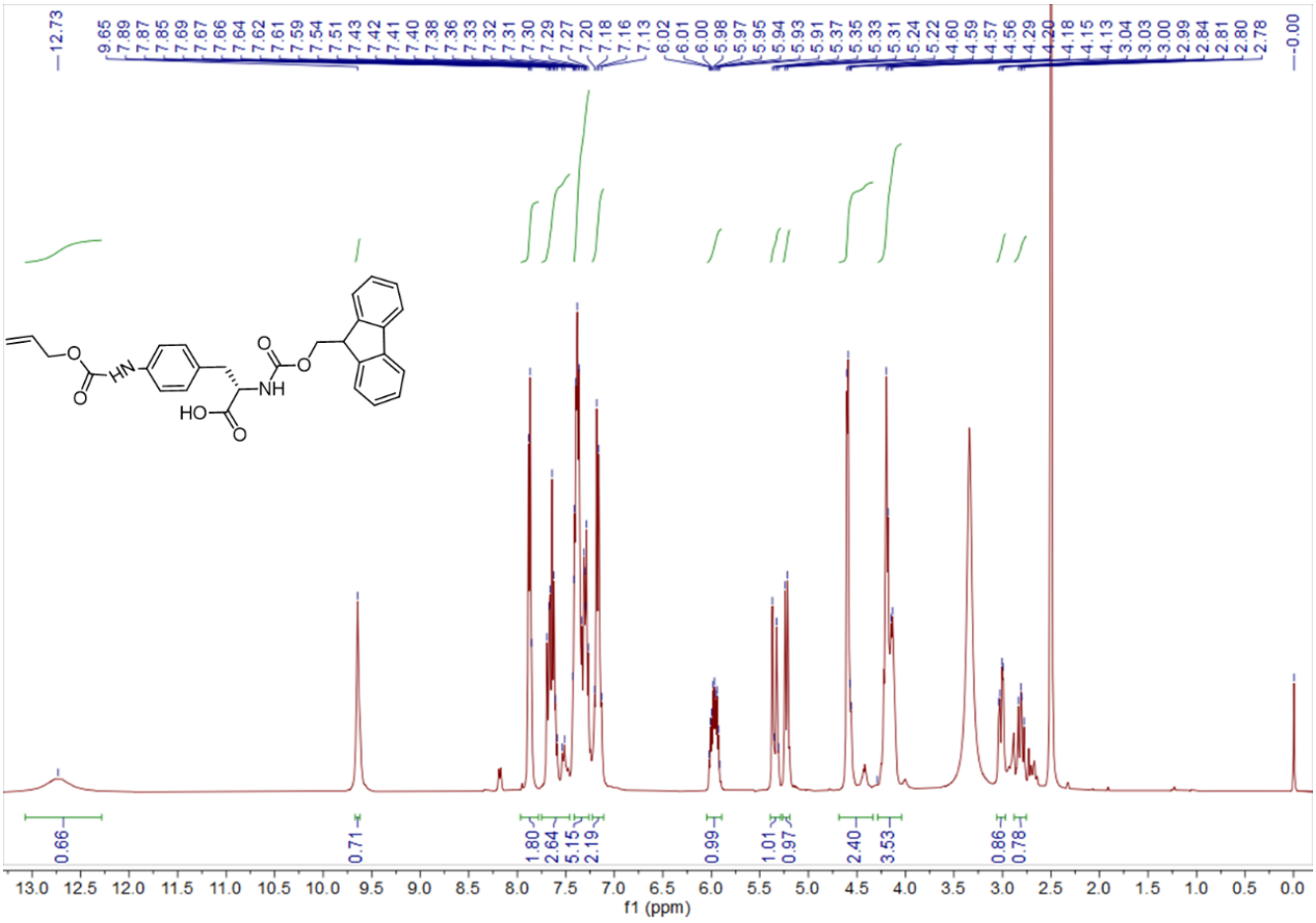

Figure S13. ^{1}H NMR spectra of Fmoc-L-Aph(Alloc)-OH.

3.1.2 Fmoc-L-Aph(ivDde)-OH[8-10]

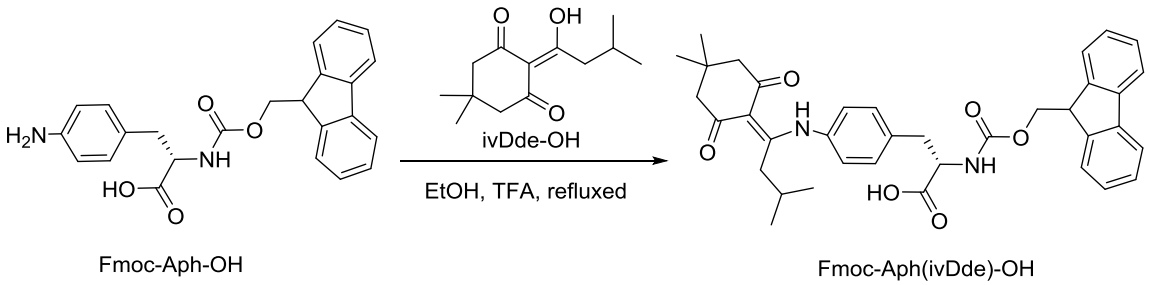

Figure S14. Synthesis of Fmoc-L-Aph(ivDde)-OH

Fmoc-Aph-OH (0.05 g, 0.11 mmol) and ivDde-OH (0.03 g, 0.12 mmol) was dissolved in EtOH (1 mL), and a solution of TFA (0.011 mmol) in EtOH (0.5 mL) was added. The mixture was refluxed and the reaction monitored by analytical TLC. The solution was dried under high vacuum to afford a brown oil. The oil was triturated with hexane to remove any unreacted ivDde-OH and then crystallised from EtOAc/hexane to obtain Fmoc-L-Aph(ivDde)-OH (0.048 g, 72% yield).

ESI-MS: calculated for $[M+H]^+$: 609.74, found: 609.30. **^{1}H NMR (400 MHz, Chloroform-*d*)** δ 7.76 (d, *J* = 7.6 Hz, 2H), 7.56 (dd, *J* = 7.6, 2.7 Hz, 2H), 7.40 (t, *J* = 7.5 Hz, 2H), 7.33 – 7.27 (m, 2H), 7.20 (d, *J* = 7.9 Hz, 2H), 7.01 (d, *J* = 8.1 Hz, 2H), 5.38 (d, *J* = 7.9 Hz, 1H), 4.69 (d, *J* = 7.0 Hz, 1H), 4.46 – 4.35 (m, 2H), 4.20 (t, *J* = 6.6 Hz, 1H), 3.18 (ddd, *J* = 43.8, 13.8, 5.8 Hz, 2H), 2.47 (s, 4H), 1.81 (hept, *J* = 6.7 Hz, 1H), 1.08 (s, 6H), 0.73 (dd, *J* = 6.7, 4.4 Hz, 6H).

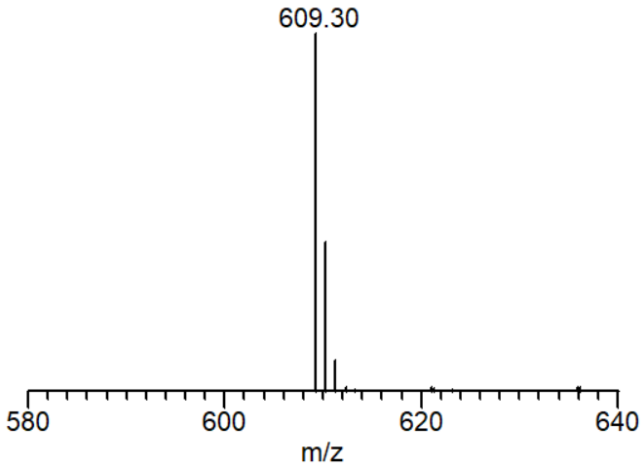

Figure S15. ESI-MS calculated for Fmoc-L-Aph (ivDde)-OH: $[M+H]^+$ m/z = 609.74, found: 609.30.

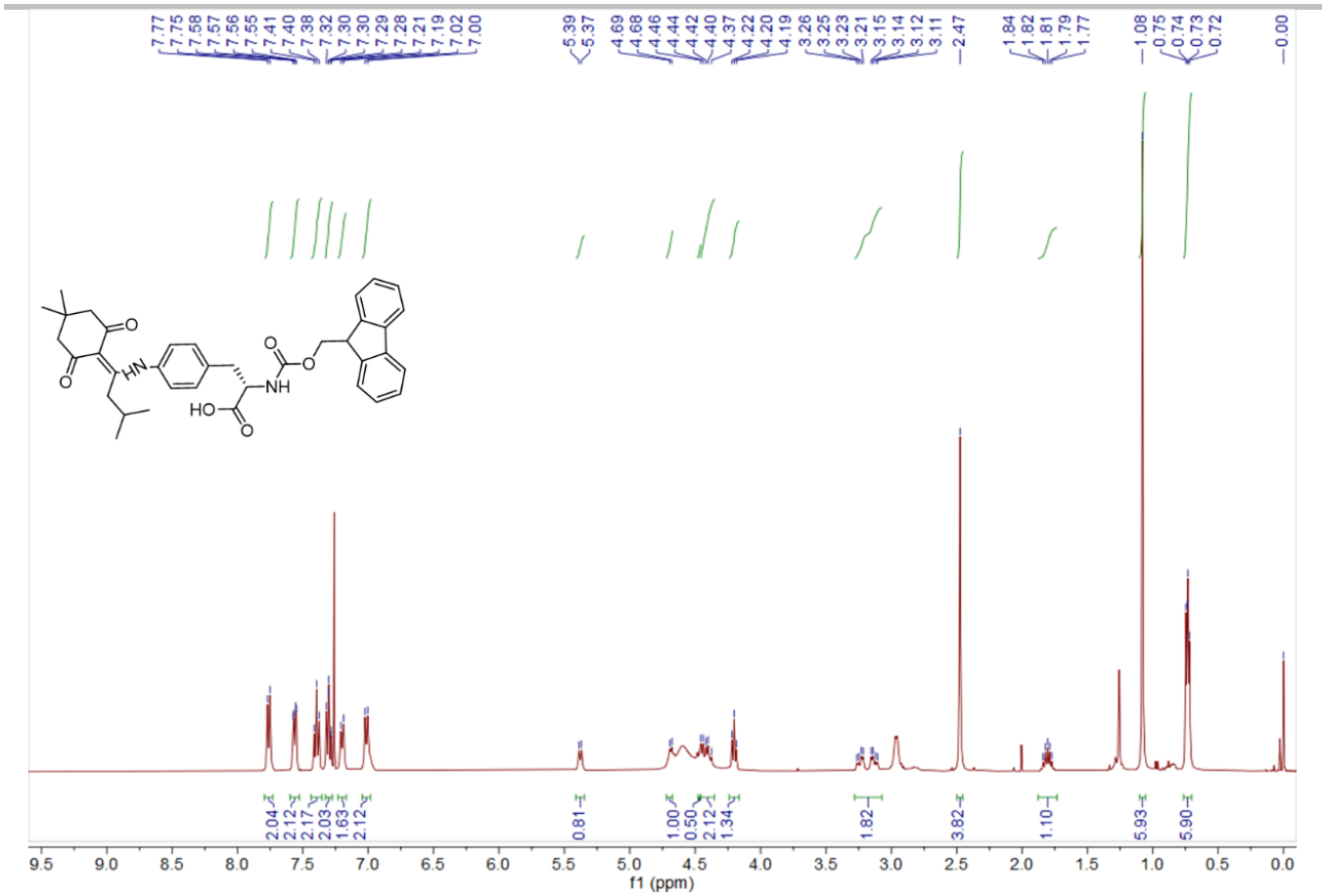

Figure S16. ^{1}H NMR spectra of Fmoc-L-Aph(ivDde)-OH.

3.2 Azpy-peptide synthesis

3.2.1 General procedure for azpy-peptide synthesis

Solid-phase peptide synthesis

All the peptides were synthesized on Rink Amide MBHA resin using standard solid-phase Fmoc chemistry. Amino acid couplings were performed using 4 eq. of activated amino acid (HOBT: DIC: amino acid, 1: 2: 1 or TBTU: DIEA: amino acid, 1: 2: 1) relative to the resin for about 2 h, and 2 eq. of Fmoc-Aph(Alloc)-OH or Fmoc-Aph(ivDde)-OH was used during its coupling.

On resin Alloc group removal

On-resin Alloc groups removal was using $Pd(PPh_3)_4/CHCl_3$/AcOH/NMM as described previously.[11]

On resin iv-Dde group removal

On-resin iv-Dde groups removal of Aph(ivDde) was using 2% N_2H_4·H2O in DMF for 30 min two times at RT as described previously.[12]

On resin Mills reaction

The amino-protection group of Aph was first deprotected completely and the on-resin Mills reaction was initiated by the addition of 2-nitrosopyridine (10 eq, 0.4 M) in DCM containing 2 drops of AcOH to the peptide resins and shaking at 25 ℃ overnight. The reaction was monitored by the Kaiser test and the resulting orange-red peptide resin was washed with DCM, dried under vacuum.

Peptide cleavage and purification

The peptides were cleaved from the resins with a TFA cocktail (TFA, DODT and H_2O, 95: 2.5: 2.5, v/v/v, 10 mg resin/ mL) at 25 ℃ for about 2 h. The crude peptides which contain azpy side-chain were firstly irradiated by green light (~540 nm, LDE, 5W), and then purified by prep-HPLC, pooled and lyophilized to afford the desired peptides as organ-yellow powder.

3.2.2 The synthesis and characterization details of azpy model peptides

P53 (3c)

Peptide resin **3a** was synthesized using standard solid-phase Fmoc chemistry. Then, peptide resin **3b** was obtained by on-resin Alloc removal as described in the general procedure. And peptide **3c** was obtained by on resin Mills reaction and peptide cleavage from resin as described in the general procedure.

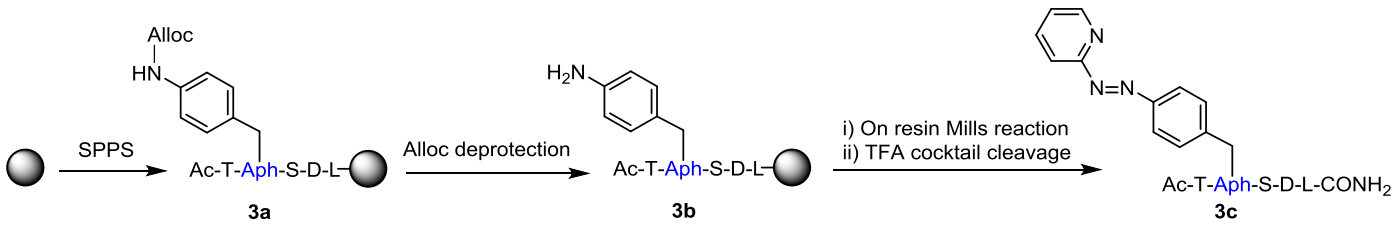

Figure S17. The synthetic route for **3c**.

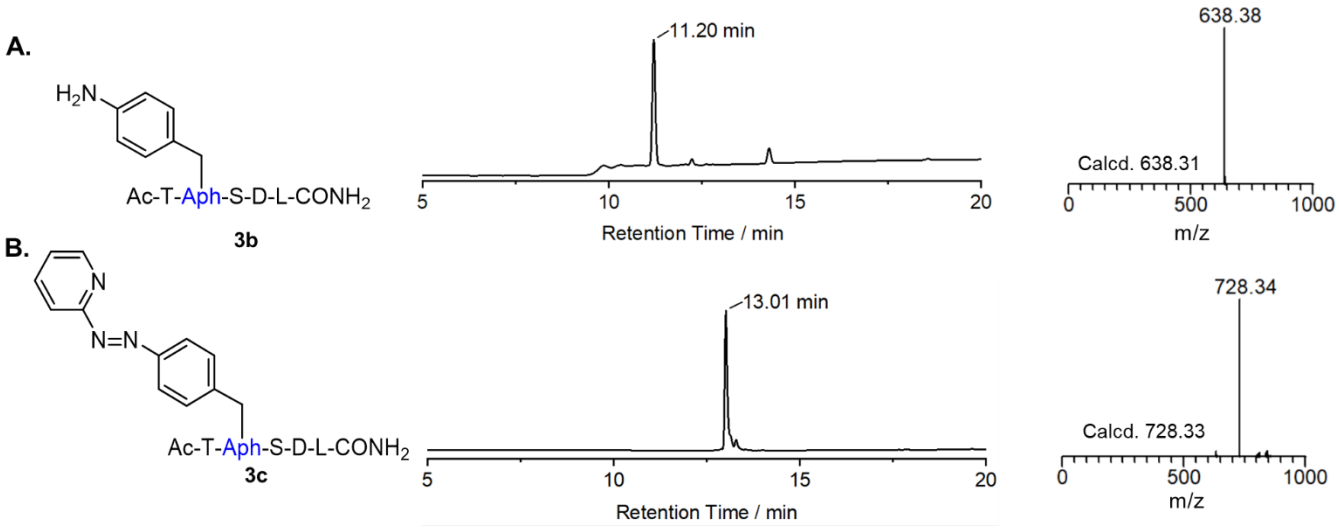

Figure S18. The analytical cleavage of **3b** and **3c**. (A) HPLC: t_R = 11.20 min (5-90% solvent B in solvent A over 15 min, λ = 214 nm, C18 column); ESI-MS calculated for $[M+H]^+$: 638.31, found: 638.38. (B) HPLC: t_R = 13.01 min (5-90% solvent B in solvent A over 15 min, λ = 214 nm, C18 column); ESI-MS calculated for $[M+H]^+$: 728.33, found: 728.34.

Leu-enkephalin (4c)

Peptide resin **4a** was synthesized using standard solid-phase Fmoc chemistry. Then, peptide resin **4b** was obtained by on-resin Alloc removal as described in the general procedure. And peptide **4c** was obtained by on resin Mills reaction and peptide cleavage from resin as described in the general procedure.

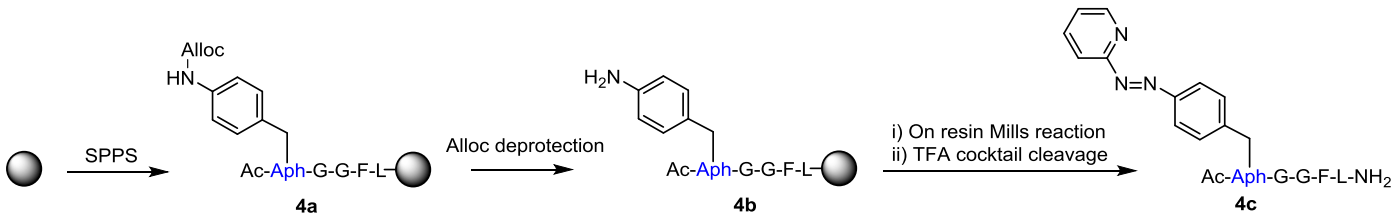

Figure S19. The synthetic route for **4c**.

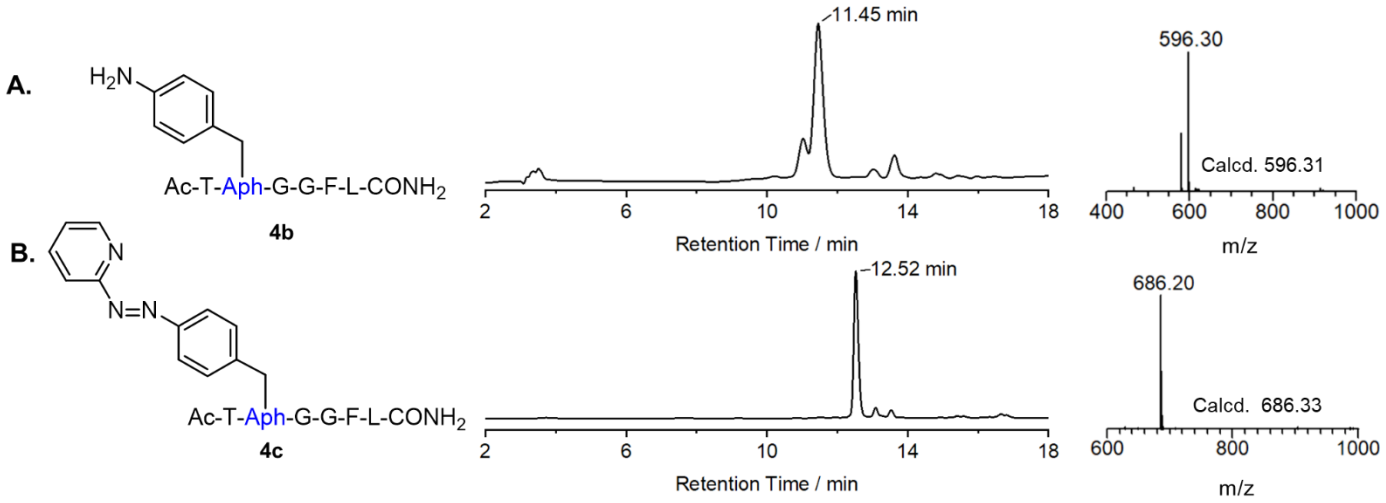

Figure S20. The analytical cleavage of **4b** and **4c**. (A) HPLC: t_R = 11.45 min (10-50% solvent B in solvent A over 15 min, λ = 214 nm, C4 column); ESI-MS calculated for $[M+H]^+$: 596.31, found: 596.30. (B) HPLC: t_R = 12.52 min (10-50% solvent B in solvent A over 15 min, λ = 214 nm, C4 column); ESI-MS calculated for $[M+H]^+$: 686.33, found: 686.20.

Neurotensin (5c)

Peptide resin **5a** was synthesized using standard solid-phase Fmoc chemistry. Then, peptide resin **5b** was obtained by on-resin Alloc removal as described in the general procedure. And peptide **5c** was obtained by on resin Mills reaction and peptide cleavage from resin as described in the general procedure.

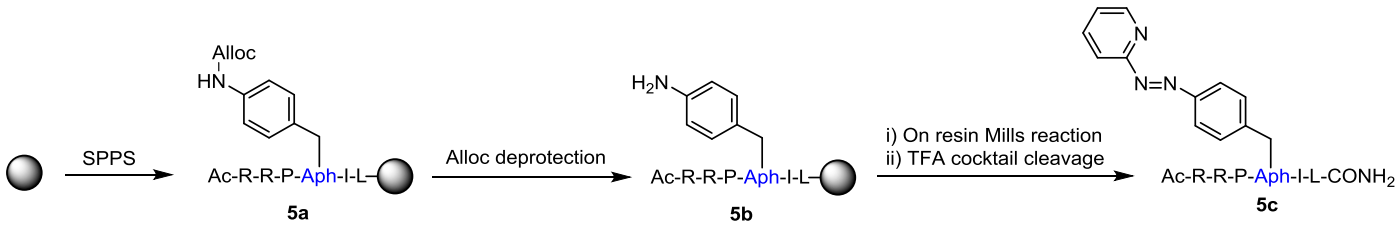

Figure S21. The synthetic route for **5c**.

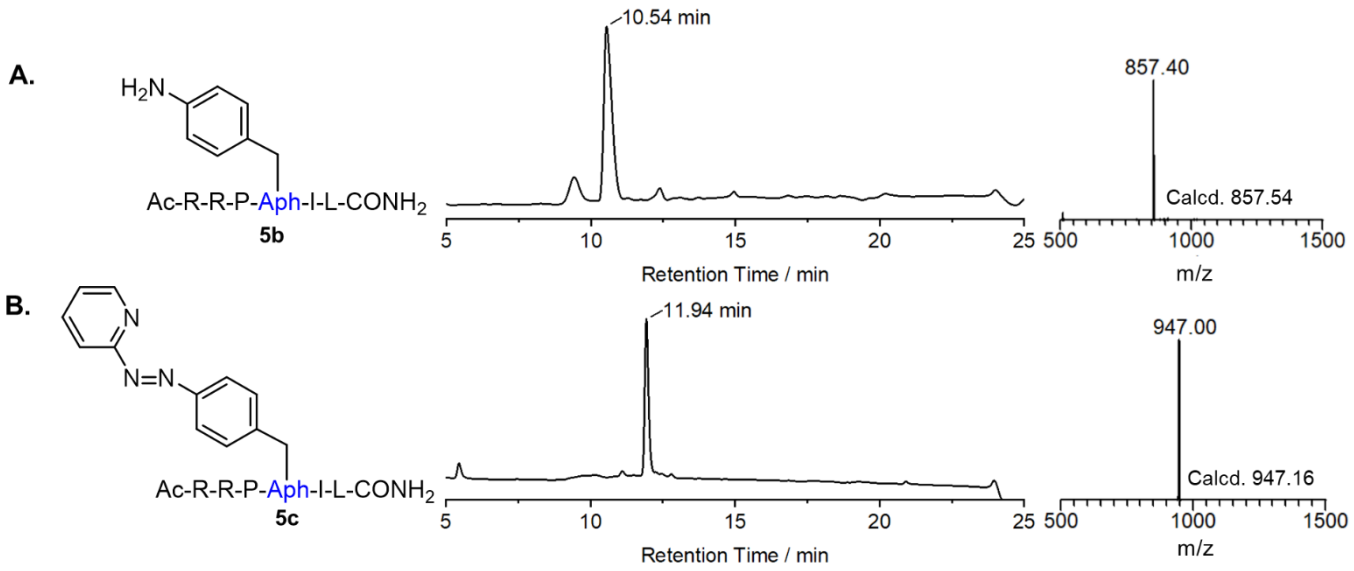

Figure S22. The analytical cleavage of **5b** and **5c**. (A) HPLC: t_R = 10.54 min (10-50% solvent B in solvent A over 15 min, λ = 214 nm, C4 column); ESI-MS calculated for $[M+H]^+$: 857.54, found: 857.40. (B) HPLC: t_R = 11.94 min (10-50% solvent B in solvent A over 15 min, λ = 214 nm, C4 column); ESI-MS calculated for $[M+H]^+$: 947.16, found: 947.00.

TAT (6c)

Peptide resin **6a** was synthesized using standard solid-phase Fmoc chemistry. Then, peptide resin **6b** was obtained by on-resin Alloc removal as described in the general procedure. And peptide **6c** was obtained by on resin Mills reaction and peptide cleavage from resin as described in the general procedure.

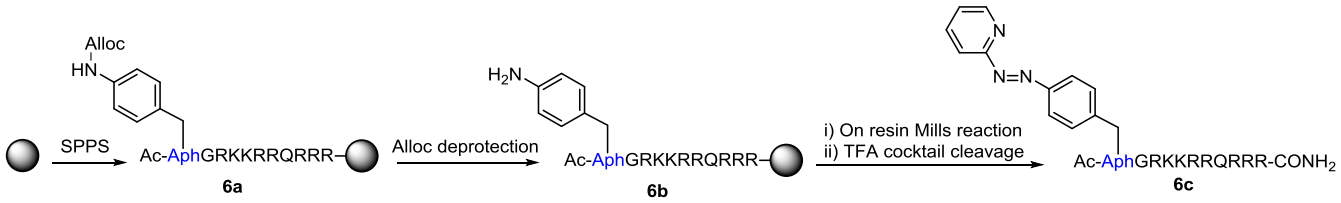

Figure S23. The synthetic route for **6c**.

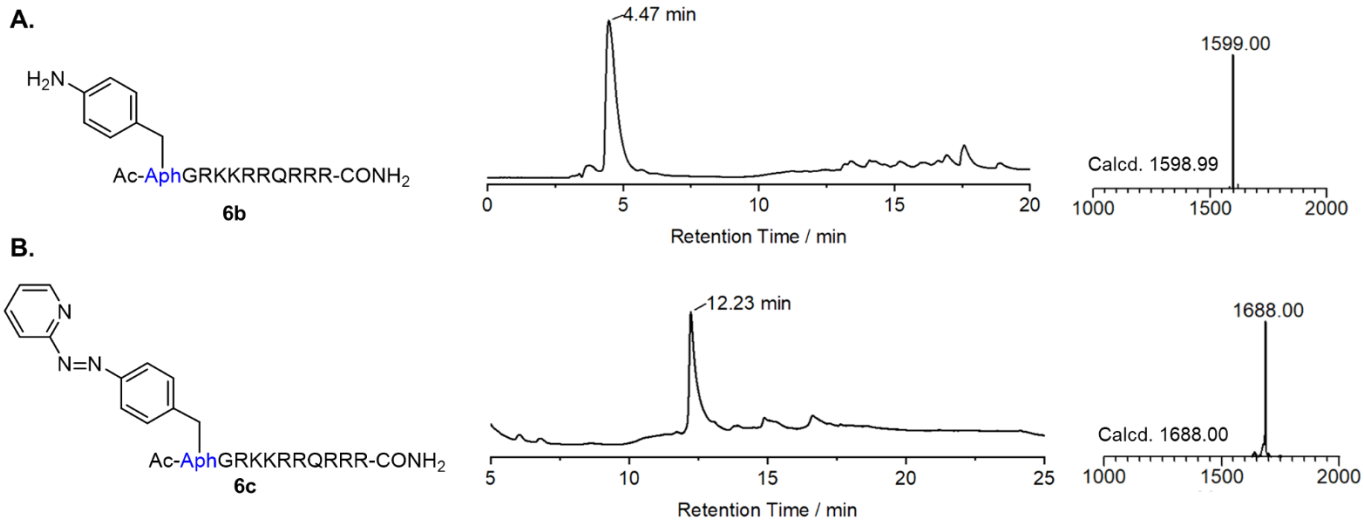

Figure S24. The analytical cleavage of **6b** and **6c**. (A) HPLC: t_R = 4.47 min (5-55% solvent B in solvent A over 15 min, λ = 214 nm, C4 column); ESI-MS: Average isotopes mass calculated 1598.99, found: 1599.00. (B) HPLC: t_R = 11.94 min (5-55% solvent B in solvent A over 15 min, λ = 214 nm, C4 column); ESI-MS: Average isotopes mass calculated 1688.00, found: 1688.00.

5-FAM-TAT (7c)

Peptide resin **7a** was synthesized using standard solid-phase Fmoc chemistry. Then, peptide resin **7b** was obtained by on-resin Alloc removal as described in the general procedure. And peptide **7c** was obtained by on resin Mills reaction and peptide cleavage from resin as described in the general procedure.

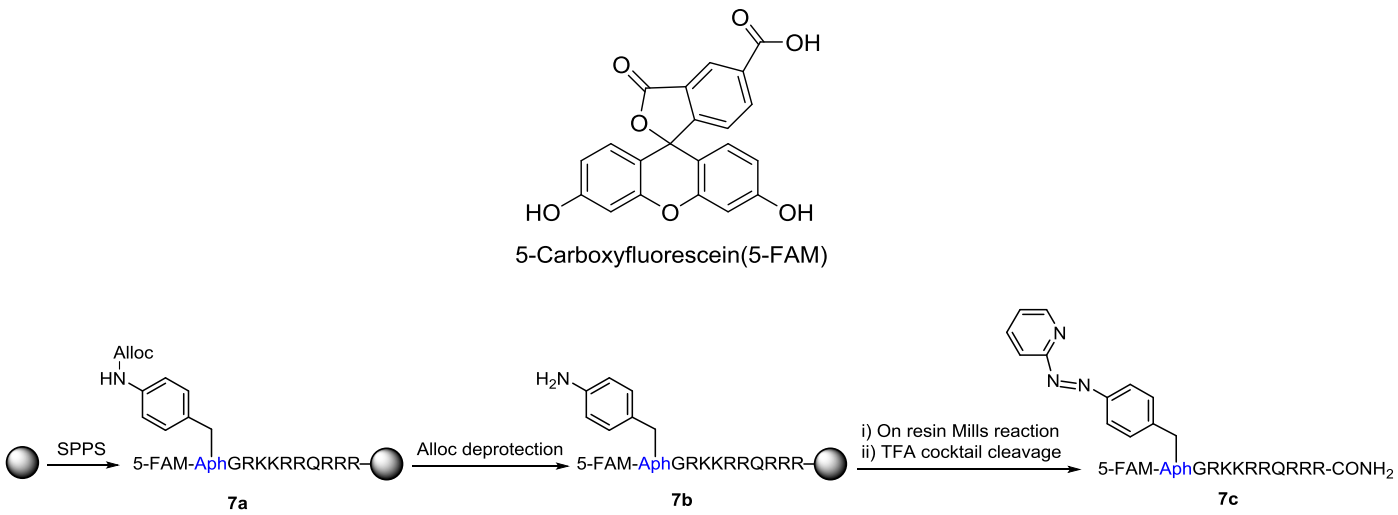

Figure S25. The synthetic route for **7c**.

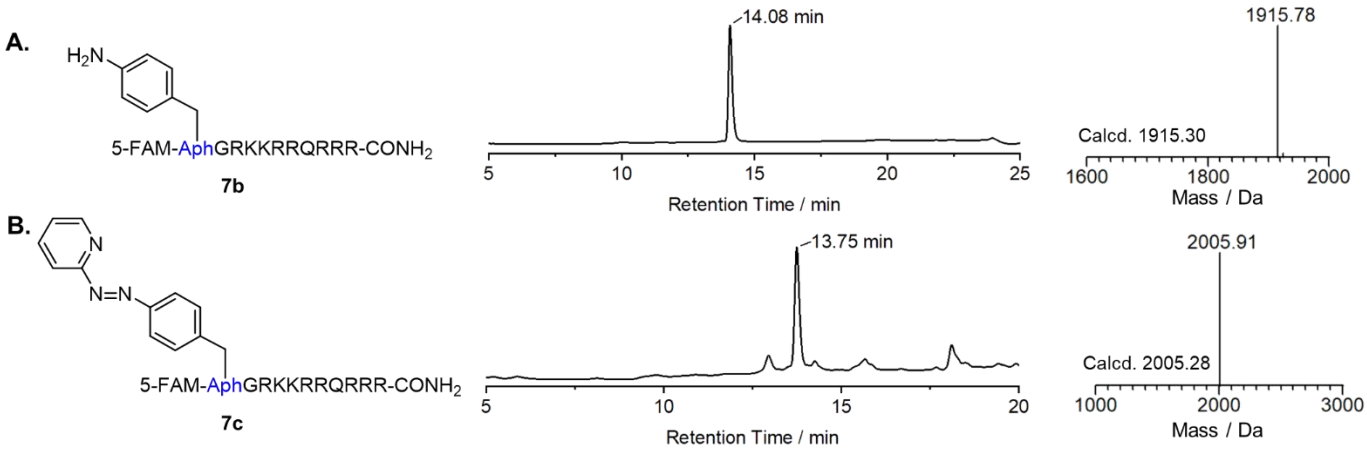

Figure S26. The analytical cleavage of **7b** and **7c**. (A) HPLC: t_R = 14.08 min (10-50% solvent B in solvent A over 15 min, λ = 214 nm, C4 column); ESI-MS: Average isotopes mass calculated: 1915.30, found: 1915.78. (B) HPLC: t_R = 13.75 min (10-50% solvent B in solvent A over 15 min, λ = 214 nm, C4 column); ESI-MS: Average isotopes mass calculated: 2005.28, found: 2005.91.

C-TIMP-2-L (8c)

Peptide resin **8a** was synthesized using standard solid-phase Fmoc chemistry. Then, peptide resin **8b** was obtained by on-resin Alloc removal as described in the general procedure. Upon the complete assembly of the Alloc deprotection, peptide **8c** was obtained by on resin Mills reaction and peptide cleavage from resin as described in the general procedure.

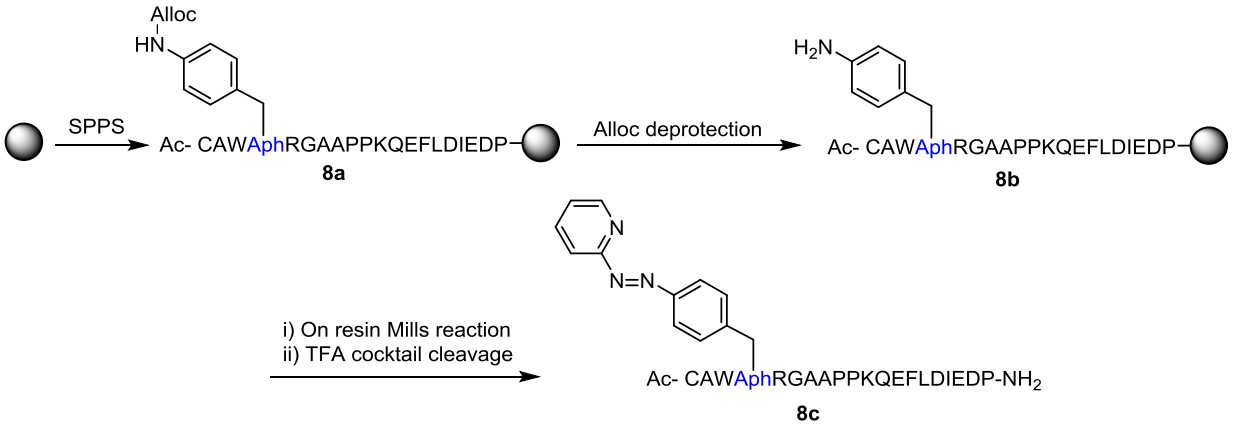

Figure S27. The synthetic route for **8c**.

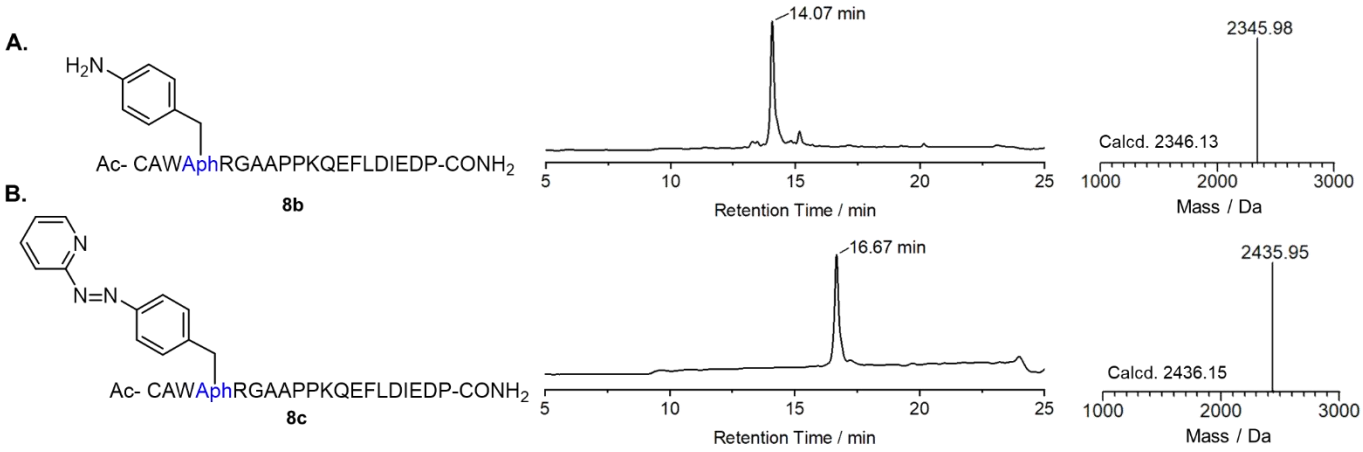

Figure S28. The analytical cleavage of **8b** and **8c**. (A) HPLC: t_R = 14.07 min (15-60% solvent B in solvent A over 15 min, λ = 214 nm, C18 column); ESI-MS: Average isotopes mass calculated: 2346.13, found: 2345.98. (B) HPLC: t_R = 16.67 min (15-60% solvent B in solvent A over 15 min, λ = 214 nm, C4 column); ESI-MS: Average isotopes mass calculated: 2436.15, found: 2435.95.

C-TIMP-2-L(C20S) (9c)

Peptide resin **9a** were synthesized using standard solid-phase Fmoc chemistry. Then, peptide resin **9b** was obtained by on-resin Alloc removal as described in the general procedure. Upon the complete assembly of the Alloc deprotection, peptide **9c** was obtained by on resin Mills reaction and peptide cleavage from resin as described in the general procedure.

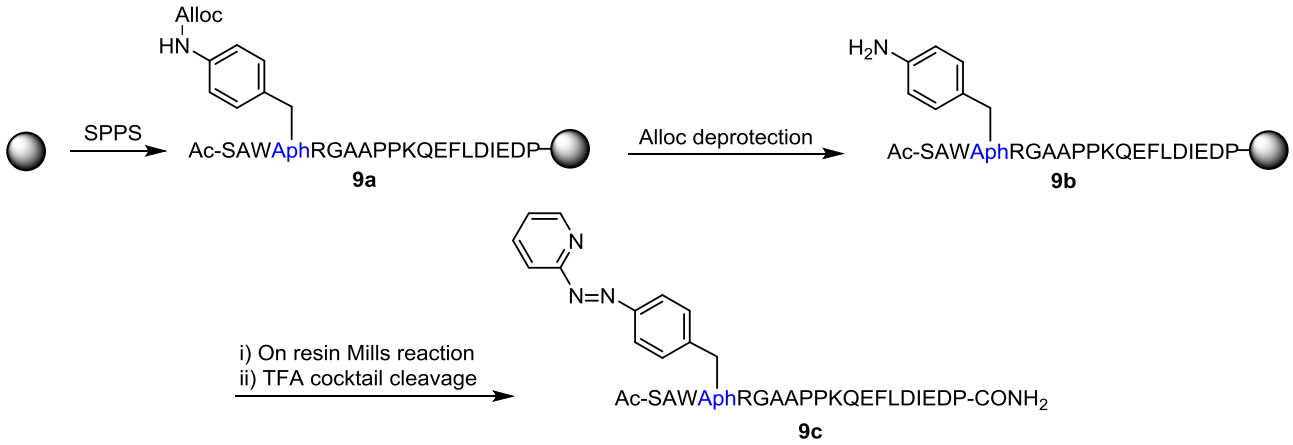

Figure S29. The synthetic route for **9c**.

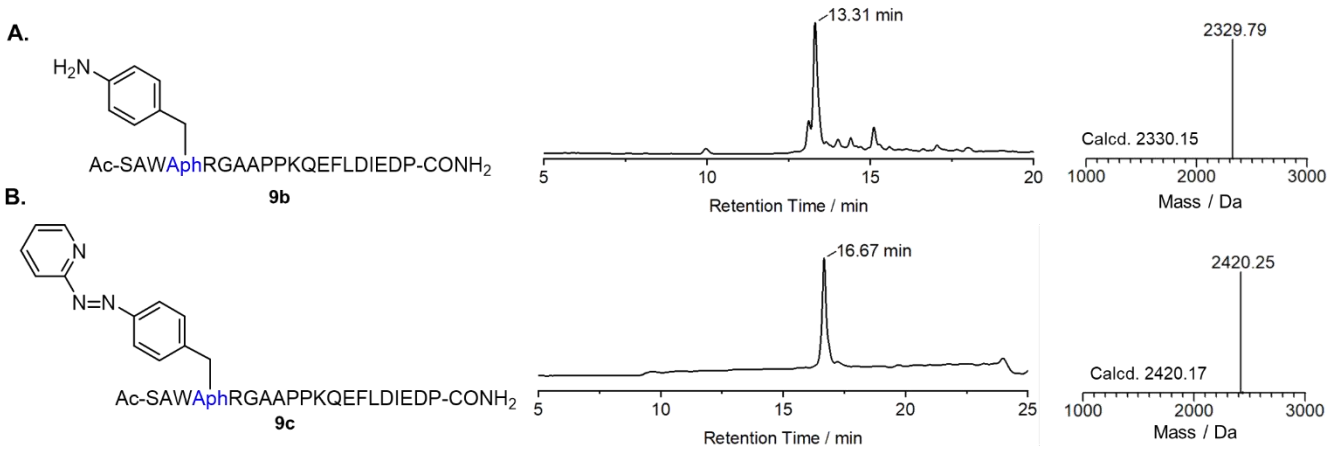

Figure S30. The analytical cleavage of **9b** and **9c**. (A) HPLC: t_R = 13.31 min (15-65% solvent B in solvent A over 15 min, λ = 214 nm, C4 column); ESI-MS: Average isotopes mass calculated: 2330.15, found: 2329.79. (B) HPLC: t_R = 16.67 min (15-65% solvent B in solvent A over 15 min, λ = 214 nm, C4 column); ESI-MS: Average isotopes mass calculated: 2420.17, found: 2420.25.

NEDD8 (10c)

Peptide resin **10a** was synthesized using standard solid-phase Fmoc chemistry. Then, peptide resin **10b** was obtained by on-resin Alloc removal as described in the general procedure. And peptide **10c** was obtained by on resin Mills reaction and peptide cleavage from resin as described in the general procedure.

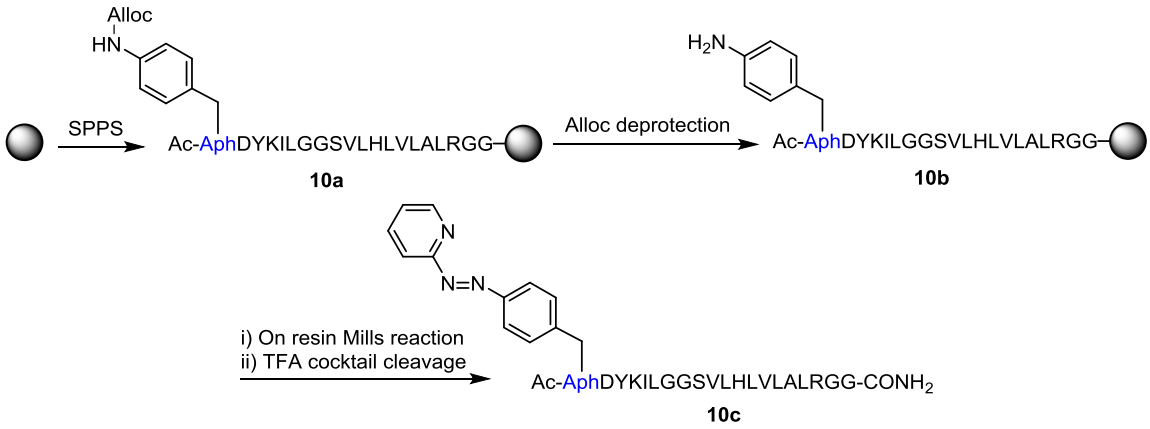

Figure S31. The synthetic route for **10c**.

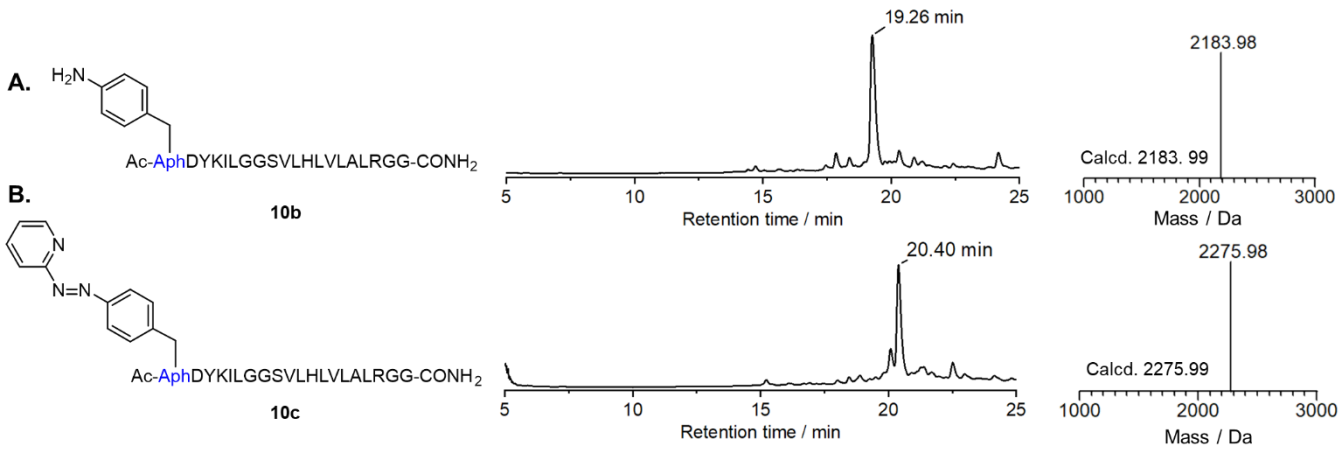

Figure S32. The analytical cleavage of **10b** and **10c**. (A) HPLC: t_R = 19.26 min (15-65% solvent B in solvent A over 15 min, λ = 214 nm, C4 column); ESI-MS: Average isotopes mass calculated: 2183.99, found: 2183.98. (B) HPLC: t_R = 20.40 min (15-60% solvent B in solvent A over 15 min, λ = 214 nm, C4 column); ESI-MS: Average isotopes mass calculated: 2275.99, found: 2275.98.

Octreotide (11c)

Peptide resin **11a** was synthesized using standard solid-phase Fmoc chemistry. Then, peptide resin **11b** was obtained by on-resin Alloc removal as described in the general procedure. And peptide **11c'** was obtained by on resin Mills reaction and cleavage from resin. Then, the peptide **11c** was refolded using air oxidation in 10 mM ammonium acetate, pH 7.8 buffer.

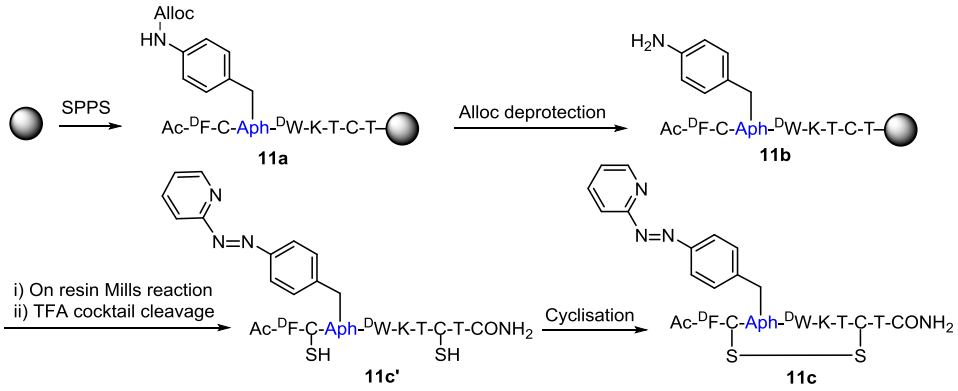

Figure S33. The synthetic route for **11c**.

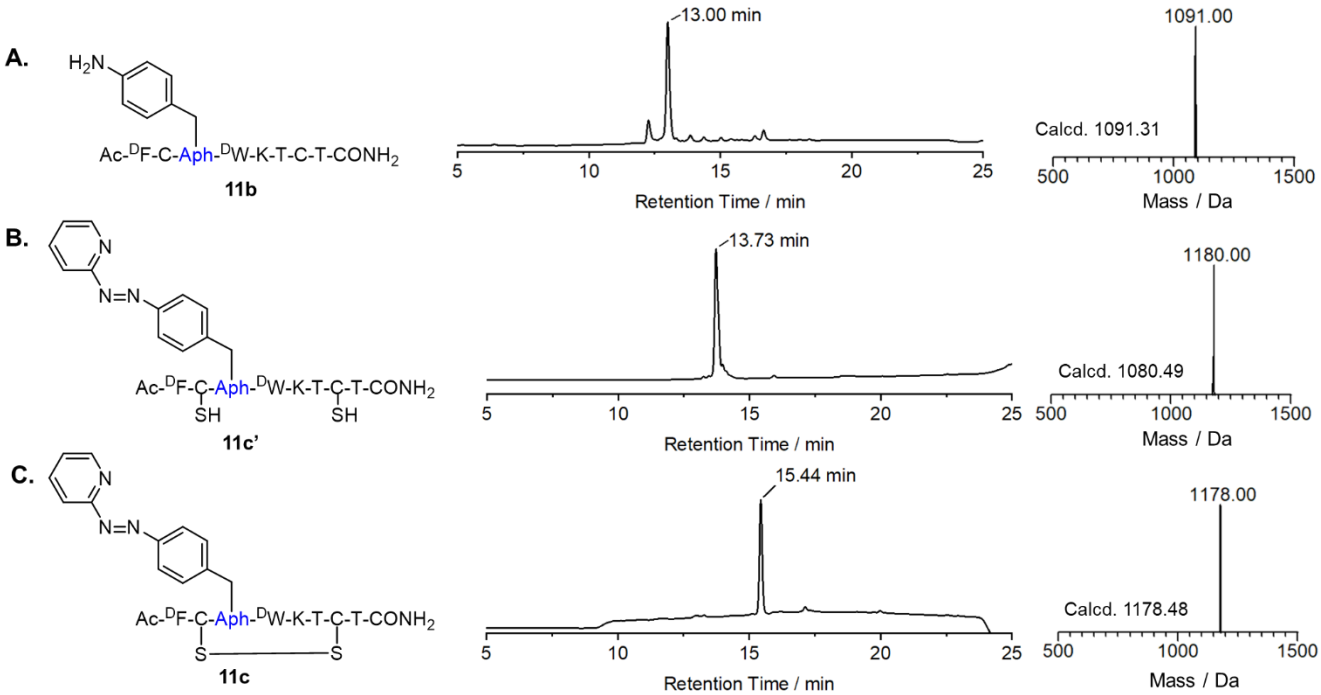

Figure S34. The analytical cleavage of **11b**, **11c'** and **11c**. (A) HPLC: t_R = 13.00 min (15-60% solvent B in solvent A over 15 min, λ = 214 nm, C4 column); Average isotopes mass calculated: 1091.31, found: 1091.00. (B) HPLC: t_R = 13.73 min (15-60% solvent B in solvent A over 15 min, λ = 214 nm, C4 column); Average isotopes mass calculated: 1080.49, found: 1180.00. (C) HPLC: t_R = 15.44 min (15-60% solvent B in solvent A over 15 min, λ = 214 nm, C4 column); Average isotopes mass calculated: 1178.48, found: 1178.00.

MiniCTX3 (12c)

Peptide resin **12a'** was synthesized using standard solid-phase Fmoc chemistry and Allyl and Alloc groups were removed as described previously. The deprotection was assessed by the kaiser test.

Peptide resin **12a** was obtained by on resin cyclisation with DIC (0.392 M) and HOBt (0.8 M) in DMF at r.t as described previously.[13] The reaction was assessed by the kaiser test.

Peptide resin **12b** was obtained by on-resin ivDde removal of Aph(ivDde) was using as described previously. And peptide **12c** was obtained by on resin Mills reaction and cleavage from resin.

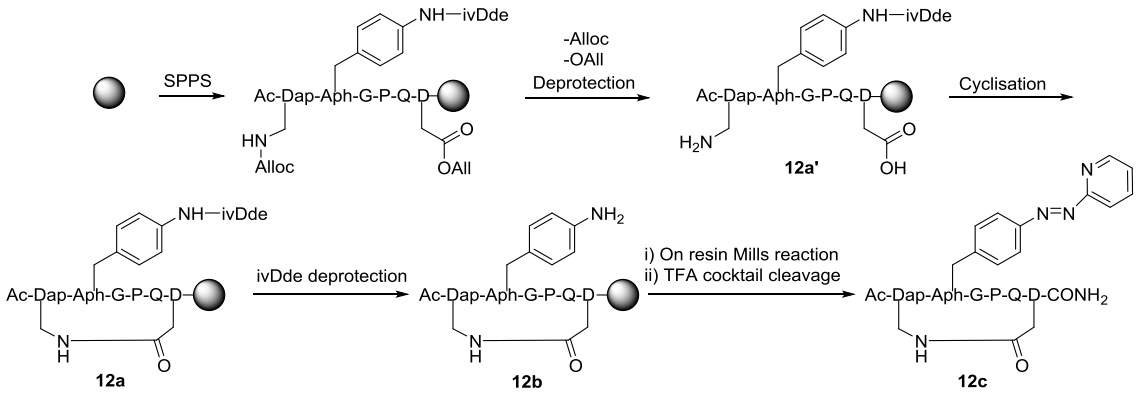

Figure S35. The synthetic route for **12c**.

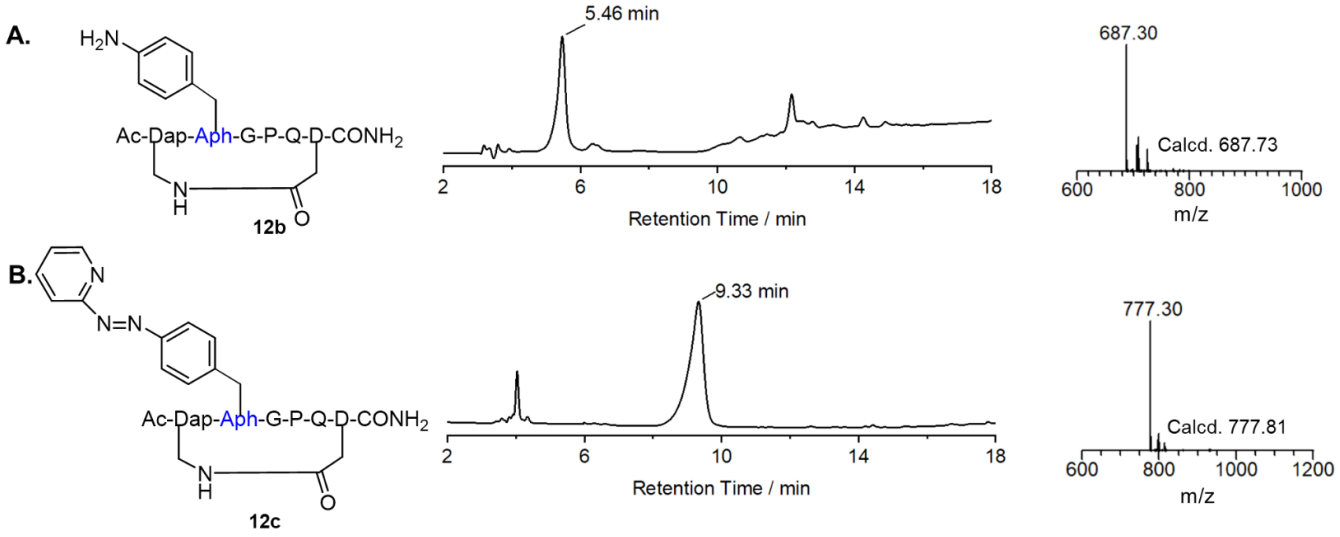

Figure S36. The analytical cleavage of **12b** and **12c**. (A) HPLC: t_R = 5.46 min (5-50% solvent B in solvent A over 15 min, λ = 214 nm, C4 column); ESI-MS calculated for $[M+H]^+$: 687.73, found: 687.30. (B) HPLC: t_R = 9.33 min (5-50% solvent B in solvent A over 15 min, λ = 214 nm, C4 column); ESI-MS calculated for $[M+H]^+$: 777.81, found: 777.30.

Ac-TAT(13)

The wild-type Ac-TAT(**13**) was obtained by standard procedure of Fmoc SPPS.

Peptide sequence: Ac-YGRKKRRQRRR-$CONH_2$.

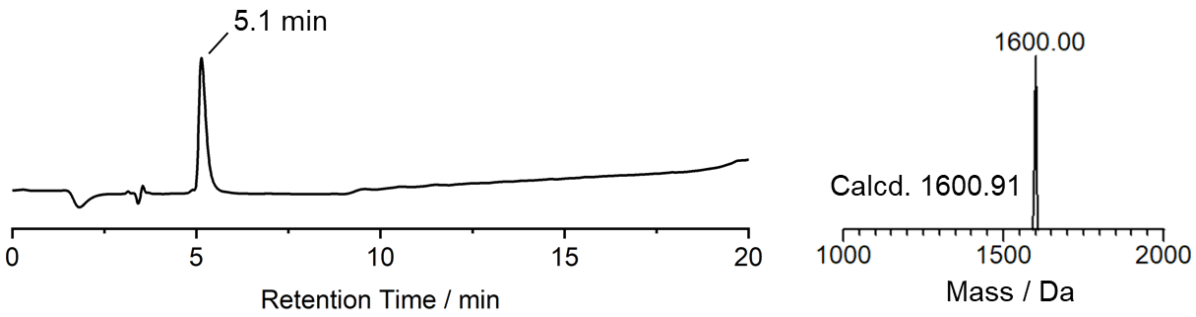

Figure S37. HPLC and ESI-MS traces of **13**. HPLC: t_R = 5.1 min (5-50% solvent B in solvent A over 15 min, λ = 214 nm, C4 column). Average isotopes mass calculated: 1600.91, found: 1600.00.

5-FAM-TAT (14)

The wild-type 5-FAM-TAT(**14**) was obtained by standard procedure of Fmoc SPPS.

Peptide sequence: 5-FAM-YGRKKRRQRRR-$CONH_2$.

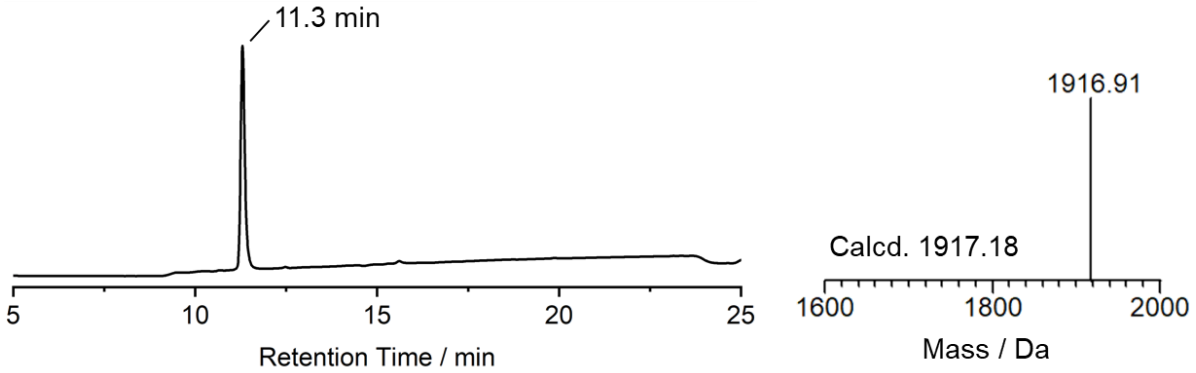

Figure S38. HPLC and ESI-MS traces of **14**. HPLC: t_R = 11.3 min (15-65% solvent B in solvent A over 15 min, λ = 214 nm, C4 column). Average isotopes mass calculated: 1917.18, found: 1916.91.

C-TIMP-2 (15)

The wild-type C-TIMP-2(**15**) was obtained by standard procedure of Fmoc SPPS.

Peptide sequence: Ac-CAWYRGAAPPKQEFLDIEDP-$CONH_2$.

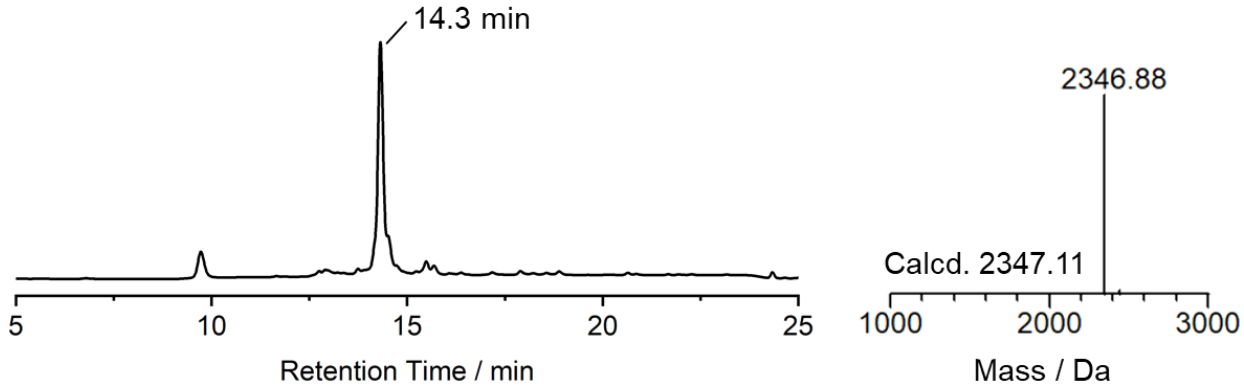

Figure S39. HPLC and ESI-MS traces of **15**. HPLC: t_R = 14.3 min (15-65% solvent B in solvent A over 15 min, λ = 214 nm, C4 column). Average isotopes mass calculated: 2347.11, found: 2346.88.

3.3 Azpy-peptide-CORM synthesis

3.3.1 Off-target Mn-CO conjugation

Azpy-based and off-target Mn-CO conjugation

A series of azpy-peptide-CORMs were obtained by the addition of $Mn(CO)_5Br$ (5 eq, 0.4 M) to a solution of azpy-peptide (1.0 equiv) in DCM/MeOH. The mixture was stirred fully for 4 h at 40 °C in the dark. The solution was dried under high vacuum and precipitated with ice-cold Et_2O to remove the excess $Mn(CO)_5Br$. The off-target Mn-CO conjugation could potentially happen for the peptide side-chains containing heteroatoms, like Cys, Trp, and His.

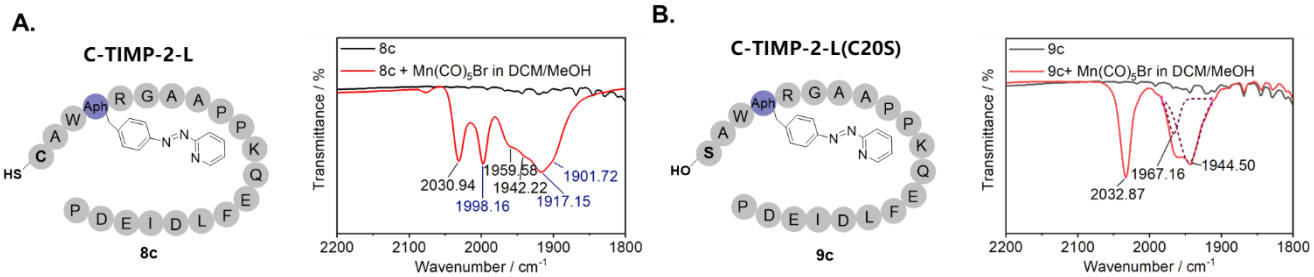

Figure S40. The different situation after coordinating with $Mn(CO)_5Br$ in solution phase diretly on **8c** (A, containing cysteine) and **9c** (B, replace cysteine with serine).

Off-target Mn-CO conjugation cleavage

The precipitate was further treated with 20% TFA in DCM/MeOH for 10 min to prevent the potential off-target Mn-CO conjugation at sensitive residues (see below). After the final precipitation by ice-cold Et_2O, azpy-peptide-CORMs were obtained as dark blue powder without further purification.

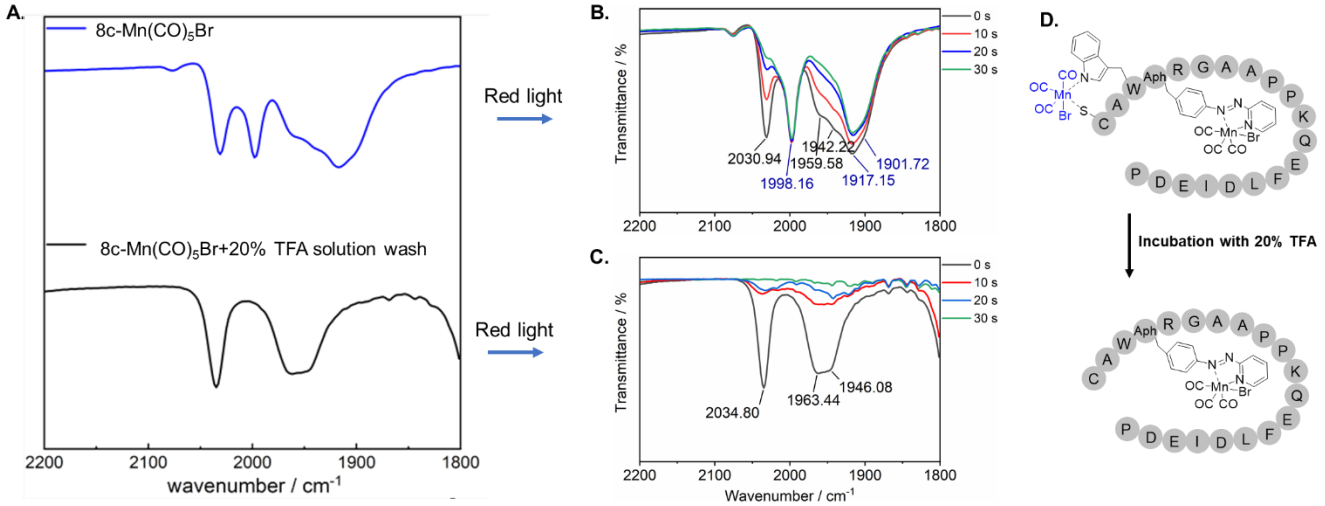

Figure S41. The off-target Mn-CO conjugation on **8c** (containing cysteine). (A) The FT-IR spectra of the product obtained by addition of $Mn(CO)_5Br$ to **8c** solution (blue line) and the product obtained with an extra washing step with a 20% TFA solution after excess $Mn(CO)_5Br$ were removed fully (black line). (B-C) Change in the solid-state FT-IR spectra upon red light irradiation of the product. (D) The predicted structure before and after 20 % TFA solution washing.

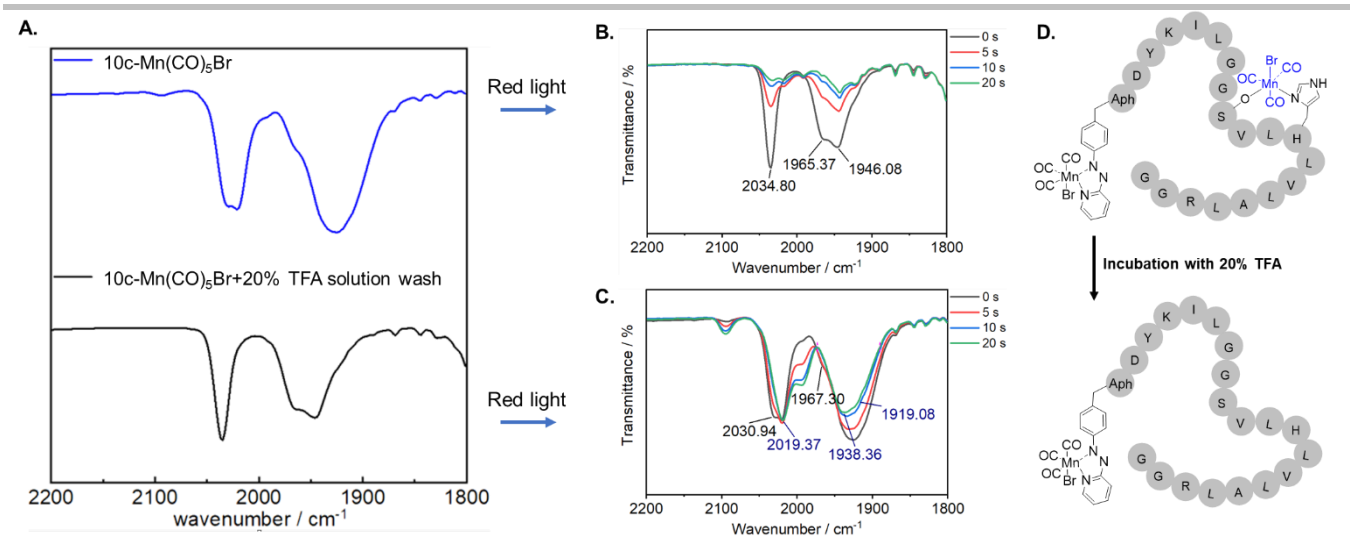

Figure S42. The off-target Mn-CO conjugation on **10c** (containing histidine). (A) The FT-IR spectra of the product obtained by addition of $Mn(CO)_5Br$ to **10c** solution (blue line) and the product obtained with an extra washing step with a 20% TFA solution after excess $Mn(CO)_5Br$ were removed fully (black line). (B-C) Change in the solid-state FT-IR spectra upon red light irradiation of the product. (D) The predicted structure before and after 20 % TFA solution washing.

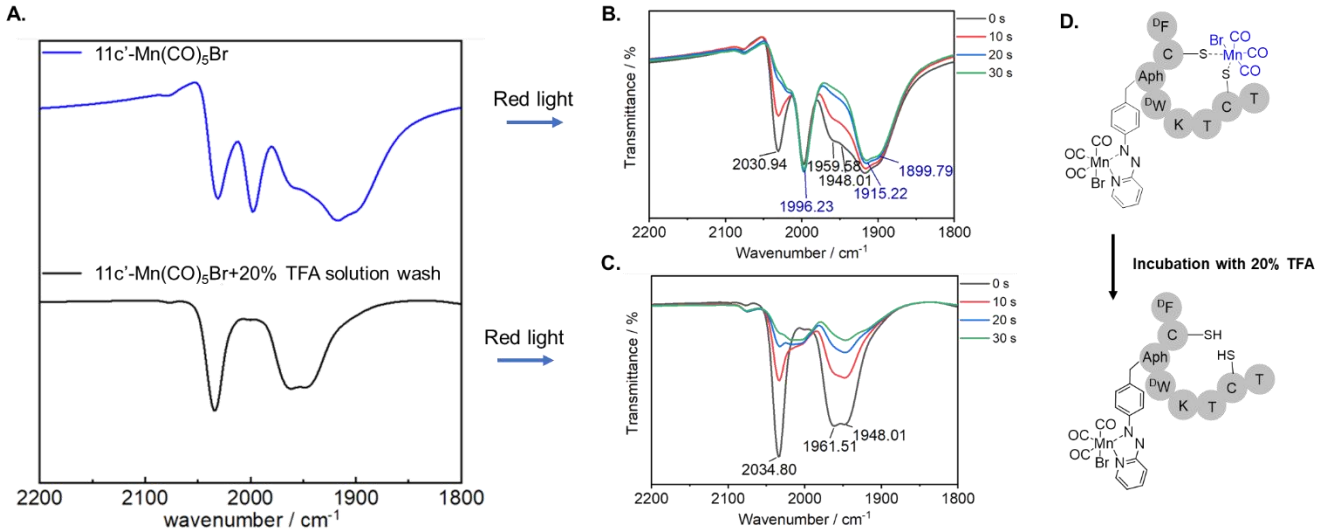

Figure S43. The off-target Mn-CO conjugation on **11c'**(containing cysteine). (A) The FT-IR spectra of the product obtained by addition of $Mn(CO)_5Br$ to **11c'** solution (blueline) and the product obtained with an extra washing step with a 20% TFA solution after excess $Mn(CO)_5Br$ were removed fully (black line). (B-C) Change in the solid-state FT-IR spectra upon red light irradiation of the product. (D) The predicted structure before and after 20 % TFA solution washing.

3.3.2 General procedure for azpy-peptide-CORM synthesis

P53-azpy-CORM (3d)

Peptide **3d** was obtained by the addition of $Mn(CO)_5Br$ (5 eq, 0.4 M) to a solution of purified peptide **3c** (1.0 equiv) in DCM/MeOH as mentioned above . The mixture was stirred fully for 4 h at 40 °C in the dark and was washed with 20% TFA solution as mentioned above.

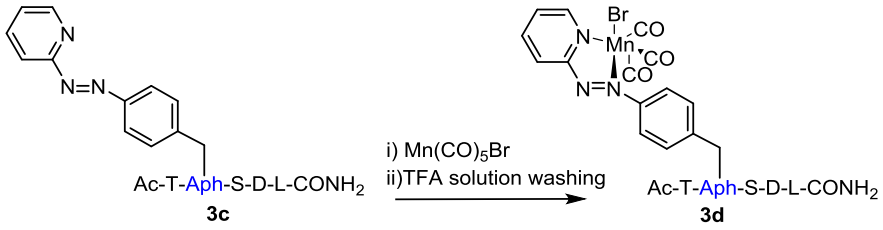

Figure S44. The binding between $Mn(CO)_5Br$ and **3c** in solution.

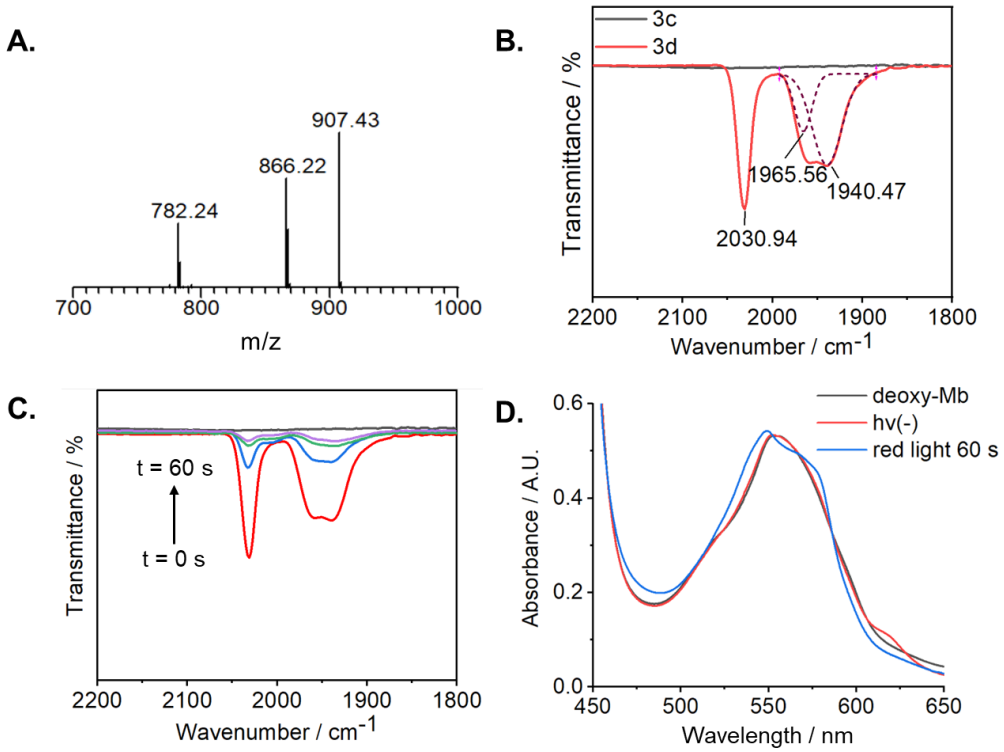

Figure S45. The final product **3d** and its CO release were analyzed by ESI-MS, FT-IR and Mb assay. (A) ESI-MS calculated for $[M\text{-}Mn(CO)_3\text{-}CH_3CN]^+$: 907.28, found: 907.43; calculated for $[M\text{-}Mn(CO)_3]^+$: 866.25, found: 866.22 and calculated for $[M\text{-}Mn]^+$ Calc: 782.27, found: 782.24; (B) FT-IR spectra of **3d**; (C) Change in the solid-state FT-IR spectra of **3d** upon red light irradiation. (D) The absorption spectral change of deoxy-Mb in Mb assay.

Leu-enkephalin-azpy-CORM (4d)

Peptide **4d** was obtained by the addition of $Mn(CO)_5Br$ (5 eq, 0.4 M) to a solution of purified peptide **4c** (1.0 equiv) in DCM/MeOH as mentioned above . The mixture was stirred fully for 4 h at 40 °C in the dark and further washing with 20% TFA solution as mentioned above.

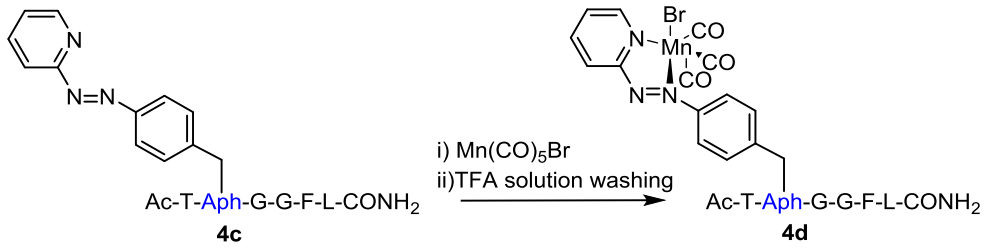

Figure S46. The binding between $Mn(CO)_5Br$ and **4c** in solution.

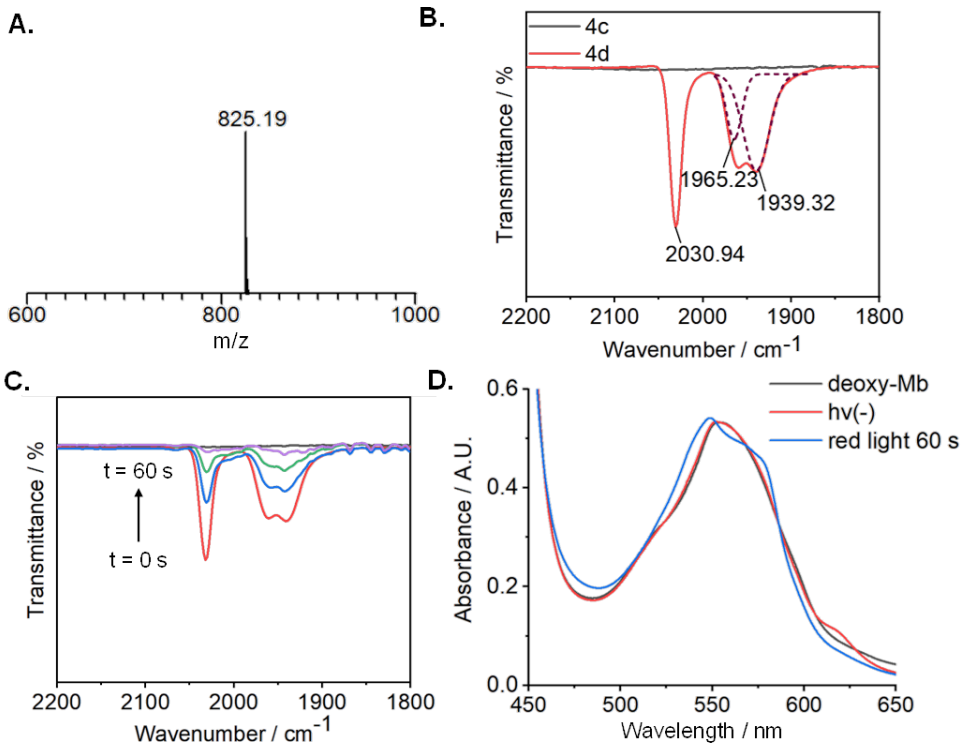

Figure S47. The final product **4d** and its CO release were analyzed by ESI-MS, FT-IR and Mb assay. (A) ESI-MS calculated for $[M\text{-}MnCO_3]^+$: 824.26, found: 825.19; (B) FT-IR spectra of **4d**; (C) Change in the solid-state FT-IR spectra of **4d** upon red light irradiation. (D) The absorption spectral change of deoxy-Mb in Mb assay.

Neurotensin-azpy-CORM (5d)

Peptide **5d** was obtained by the addition of $Mn(CO)_5Br$ (5 eq, 0.4 M) to a solution of purified peptide **5c** (1.0 equiv) in DCM/MeOH as mentioned above. The mixture was stirred fully for 4 h at 40 °C in the dark and further washing with 20% TFA solution as mentioned above.

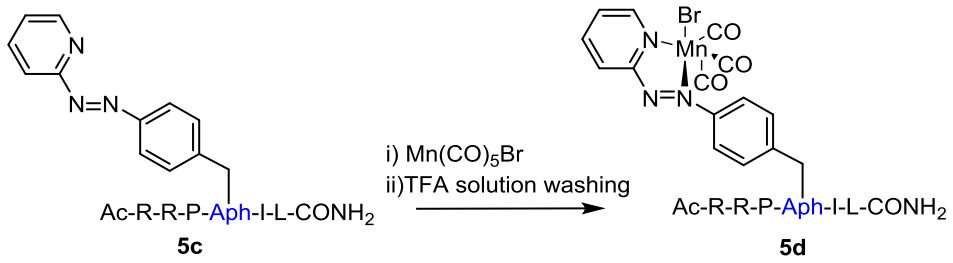

Figure S48. The binding between $Mn(CO)_5Br$ and **5c** in solution.

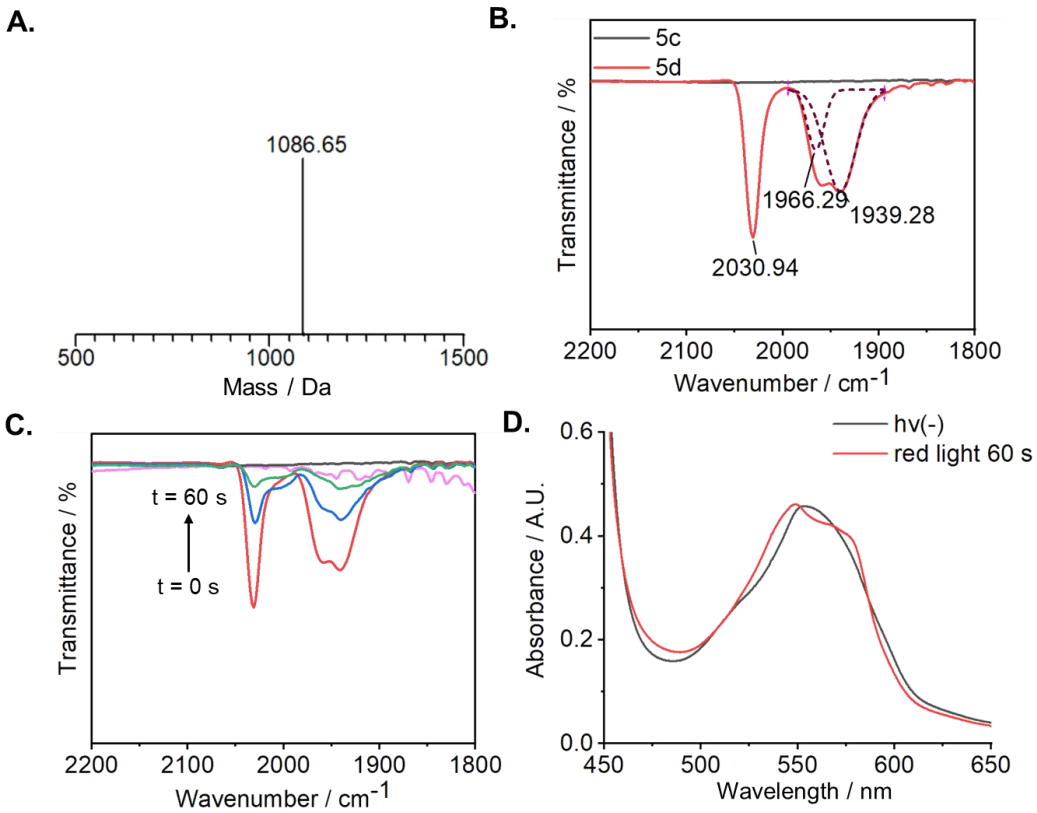

Figure S49. The final product **5d** and its CO release were analyzed by ESI-MS, FT-IR and Mb assay. (A) ESI-MS: Average isotopes mass calculated for [M-$Mn(CO)_3$]: 1086.48, found: 1086.65; (B) FT-IR spectra of **5d**; (C) Change in the solid-state FT-IR spectra of **5d** upon red light irradiation. (D) The absorption spectral change of deoxy-Mb in Mb assay.

TAT-azpy-CORM (6d)

Peptide **6d** was obtained by the addition of $Mn(CO)_5Br$ (5 eq, 0.4 M) to a solution of purified peptide **6c** (1.0 equiv) in DCM/MeOH as mentioned above. The mixture was stirred fully for 4 h at 40 °C in the dark and further washing with 20% TFA solution as mentioned above.

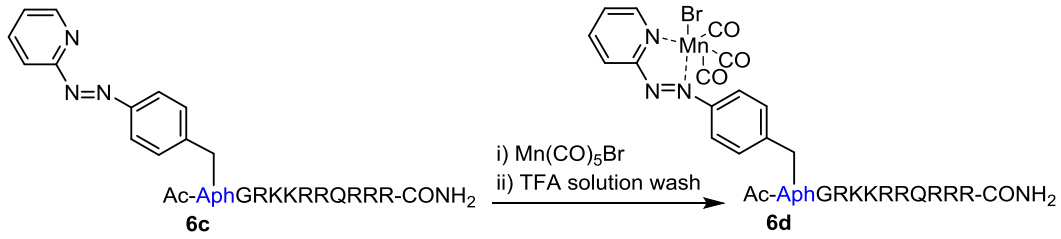

Figure S50. The binding between $Mn(CO)_5Br$ and **6c** in solution.

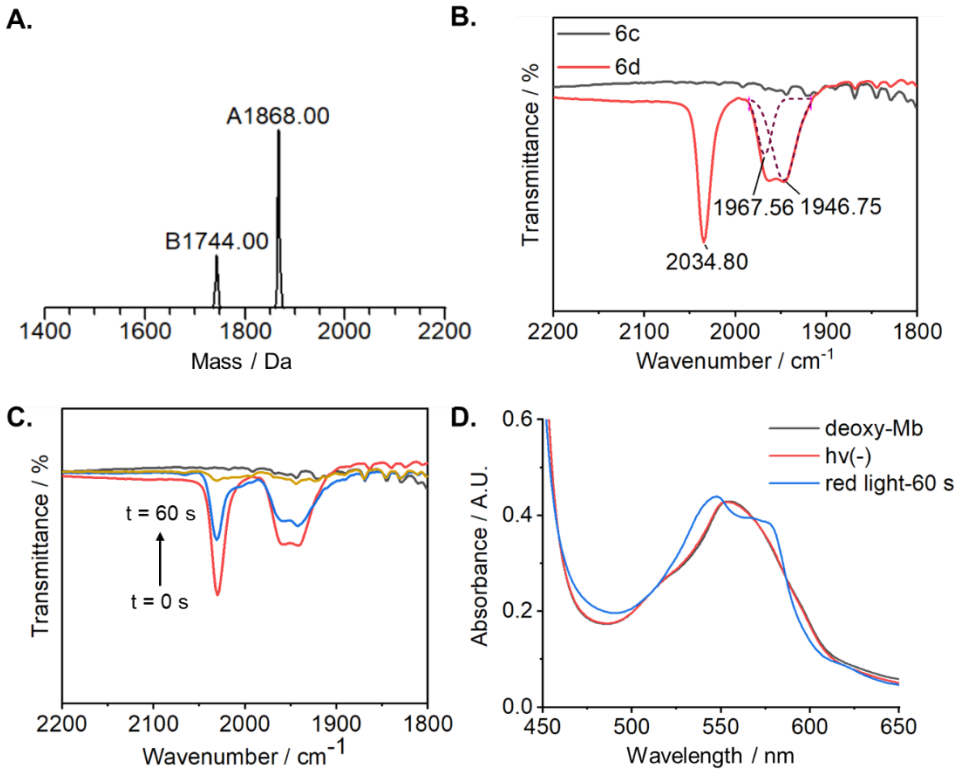

Figure S51. The final product **6d** and its CO release were analyzed by ESI-MS, FT-IR and Mb assay. (A) ESI-MS: Average isotopes mass calculated for [M-$Mn(CO)_3$]: 1743.35, found: 1744.00 and [M-$Mn(CO)_3$-CH_3CN]: 1868.96, found: 1868.00; (B) FT-IR spectra of **6d**; (C) Change in the solid-state FT-IR spectra of **6d** upon red light irradiation. (D) The absorption spectral change of deoxy-Mb in Mb assay.

5-FAM-TAT-azpy-CORM (7d)

Peptide **7d** was obtained by the addition of $Mn(CO)_5Br$ (5 eq, 0.4 M) to a solution of purified peptide **7c** (1.0 equiv) in DCM/MeOH as mentioned above.

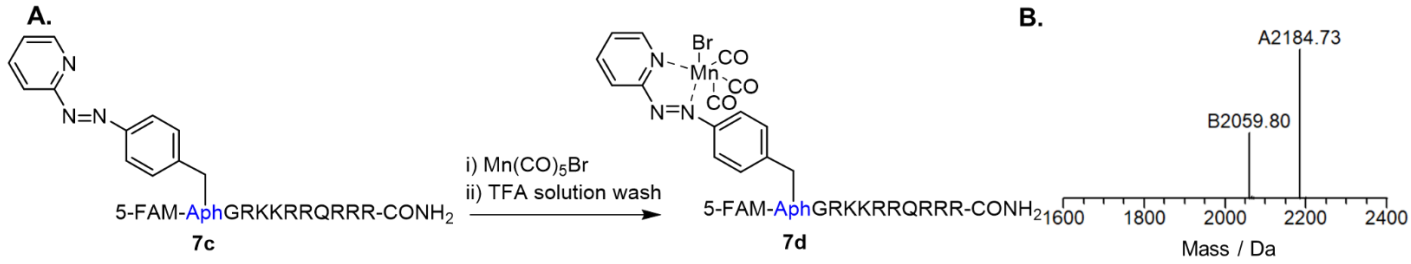

Figure S52. (A) The binding between $Mn(CO)_5Br$ and **7c** in solution; (B) ESI-MS: Average isotopes mass calculated for [M-Mn]: 2059.99, found: 2059.80, [M-$Mn(CO)_3$-CH_3CN]: 2185.00, found:2184.73.

C-TIMP-2-L-azpy-CORM (8d)

Peptide **8d** was obtained by the addition of $Mn(CO)_5Br$ (5 eq, 0.4 M) to a solution of purified peptide **8c** (1.0 equiv) in DCM/MeOH for 4 h at 40 °C in the dark and further washing with 20% TFA solution as mentioned above.

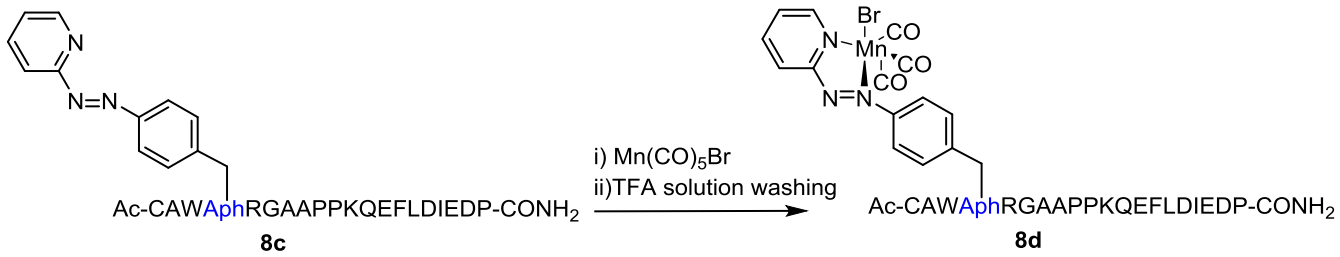

Figure S53. The binding between $Mn(CO)_5Br$ and **8c** in solution.

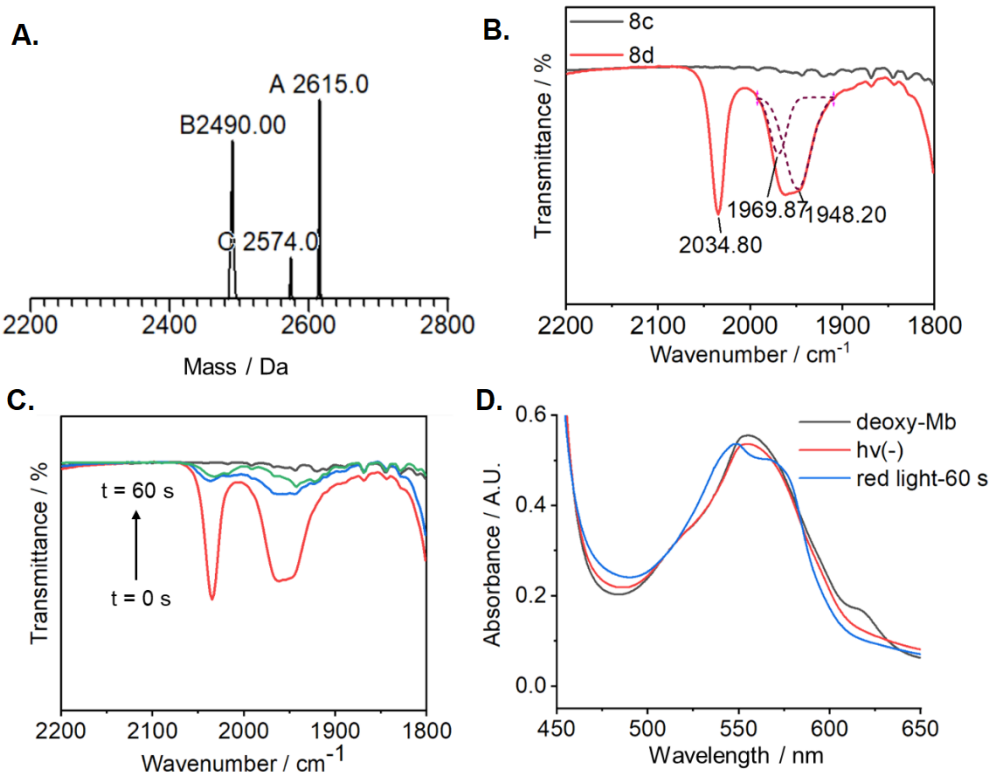

Figure S54. The final product **8d** and its CO release were analyzed by ESI-MS, FT-IR and Mb assay. (A) ESI-MS: Average isotopes mass calculated for [M-$Mn(CO)_3$-CH_3CN]: 2615.10, found: 2615.00, [M-$Mn(CO)_3$]: 2574.07, found: 2574.00 and [M-Mn]: 2490.09, found: 2490.00; (B) FT-IR spectra of **8d**; (C) Change in the solid-state FT-IR spectra of **8d** upon red light irradiation. (D) The absorption spectral change of deoxy-Mb in Mb assay.

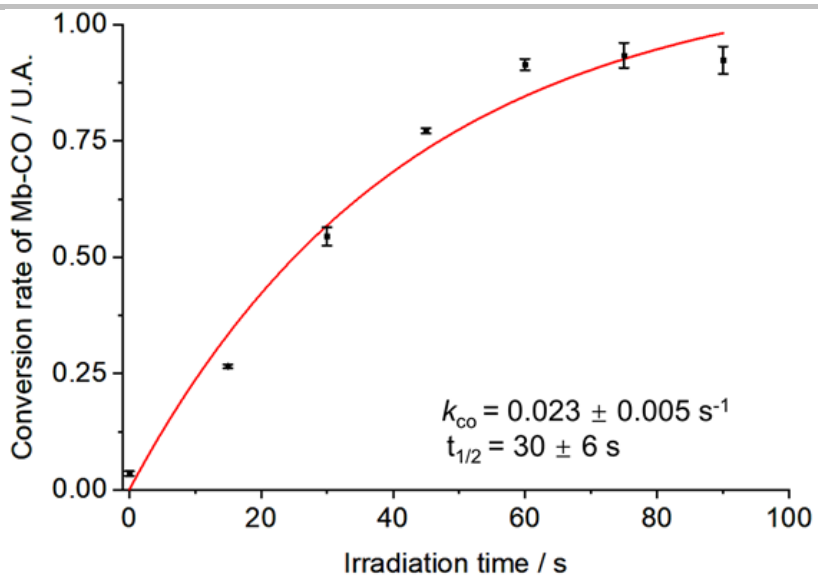

Figure S55. Apparent CO release rate (k_{CO}) and the half-time ($t_{1/2}$) of CO release from azpy-peptide-CORM **8d** (conc. 10 µM) through a nonlinear regression fitting. The conversion rate of Mb-CO vs. irradiation time (s) in PBS buffer at 298 K upon exposure to red light. All experiments were performed in triplicate.

C-TIMP-2-L(C20S)-azpy-CORM (9d)

Peptide **9d** was obtained by the addition of $Mn(CO)_5Br$ (5 eq, 0.4 M) to a solution of purified peptide **9c** (1.0 equiv) in DCM/MeOH for 4 h at 40 °C in the dark and further washing with 20% TFA solution as mentioned above.

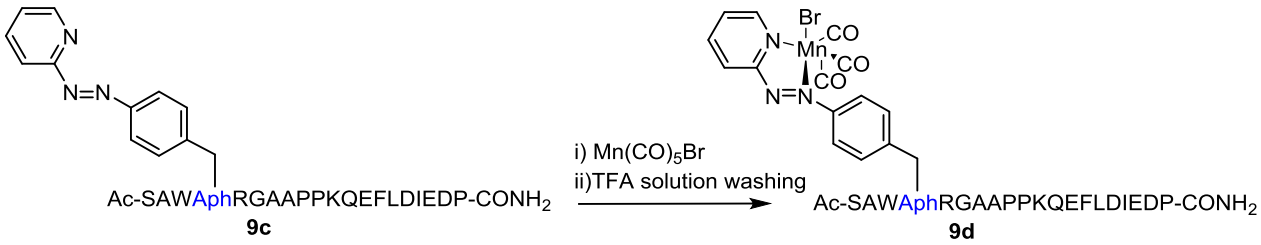

Figure S56. The binding between $Mn(CO)_5Br$ and **9c** in solution.

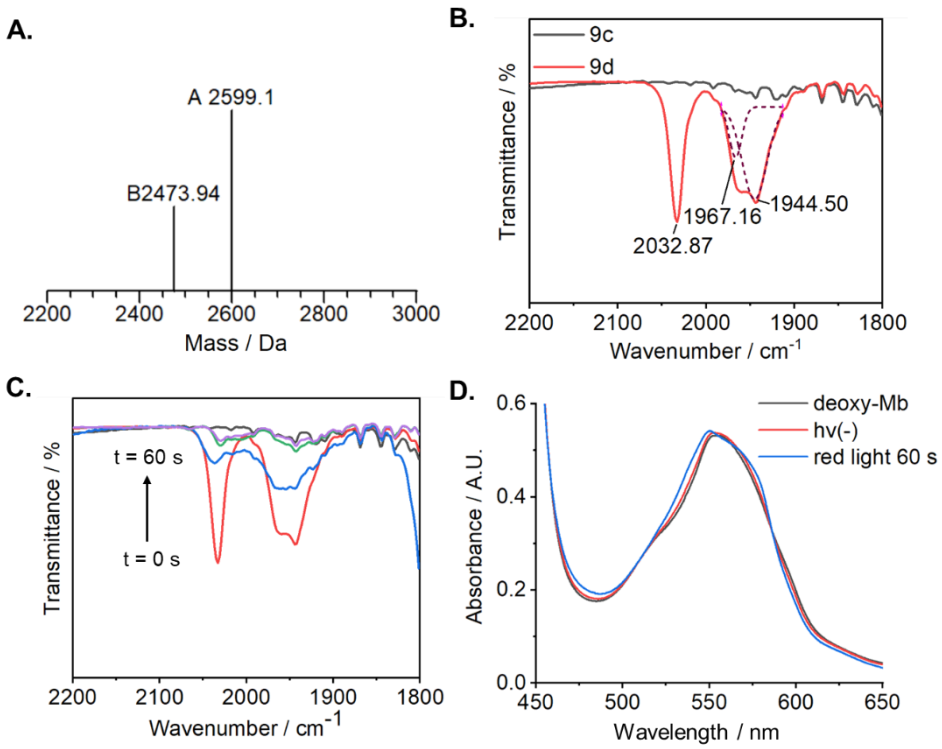

Figure S57. The final product **9d** and its CO release were analyzed by ESI-MS, FT-IR and Mb assay. (A) ESI-MS: Average isotopes mass calculated for [M-$Mn(CO)_3$-CH_3CN]: 2599.12, found: 2599.10 and [M-Mn]: 2474.11, found: 2473.94; (B) FT-IR spectra of **9d**; (C) Change in the solid-state FT-IR spectra of **9d** upon red light irradiation. (D) The absorption spectral change of deoxy-Mb in Mb assay.

NEDD8-azpy-CORM (10d)

Peptide **10d** was obtained by the addition of $Mn(CO)_5Br$ (5 eq, 0.4 M) to a solution of purified peptide **10c** (1.0 equiv) in DCM/MeOH as mentioned above . The mixture was stirred fully for 4 h at 40 °C in the dark and further washing with 20% TFA solution as mentioned above.

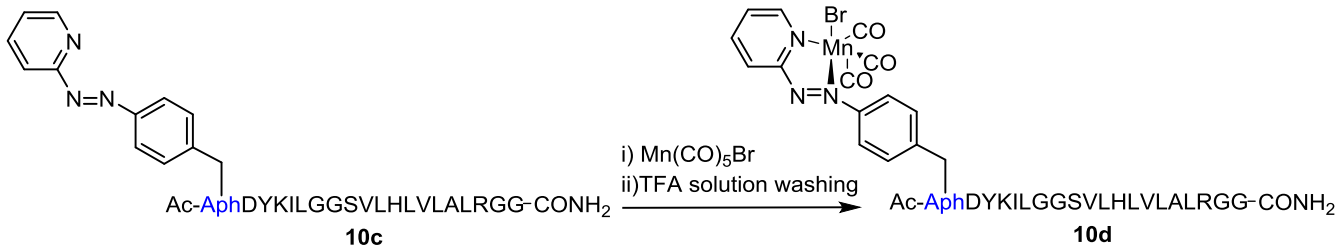

Figure S58. The binding between $Mn(CO)_5Br$ and **10c** in solution.

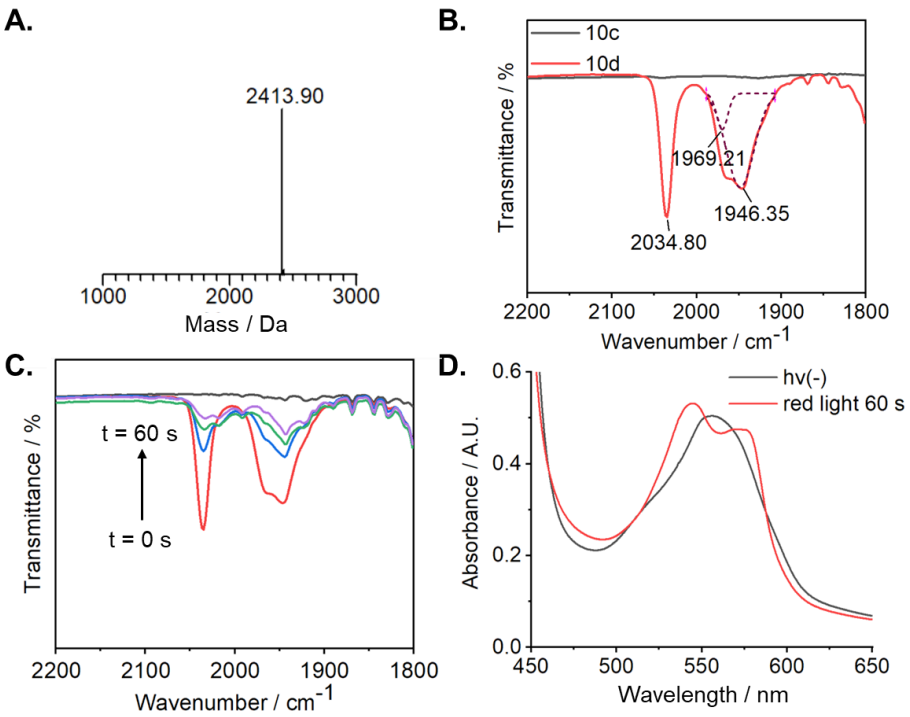

Figure S59. The final product **10d** and its CO release were analyzed by ESI-MS, FT-IR and Mb assay. (A) ESI-MS: Average isotopes mass calculated for [M-$Mn(CO)_3$]: 2413.19, found: 2413.90; (B) FT-IR spectra of **10d**; (C) Change in the solid-state FT-IR spectra of **10d** upon red light irradiation. (D) The absorption spectral change of deoxy-Mb in Mb assay.

Octreotide-azpy-CORM (11d)

Peptide **11d** was obtained by the addition of $Mn(CO)_5Br$ (5 eq, 0.4 M) to a solution of purified peptide **11c** (1.0 equiv) in DCM/MeOH for 4 h at 40 °C in the dark and further washing with 20% TFA solution as mentioned above.

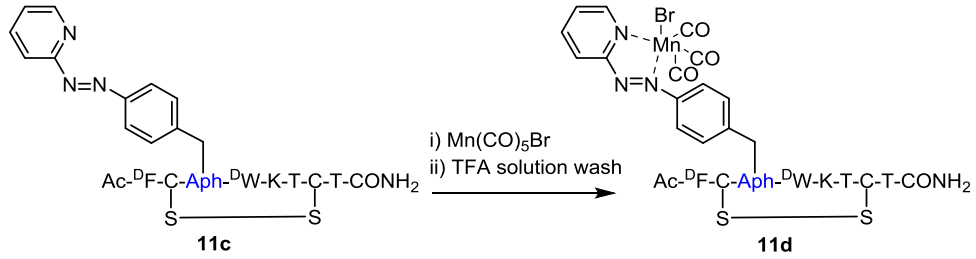

Figure S60. The binding between $Mn(CO)_5Br$ and **11c** in solution.

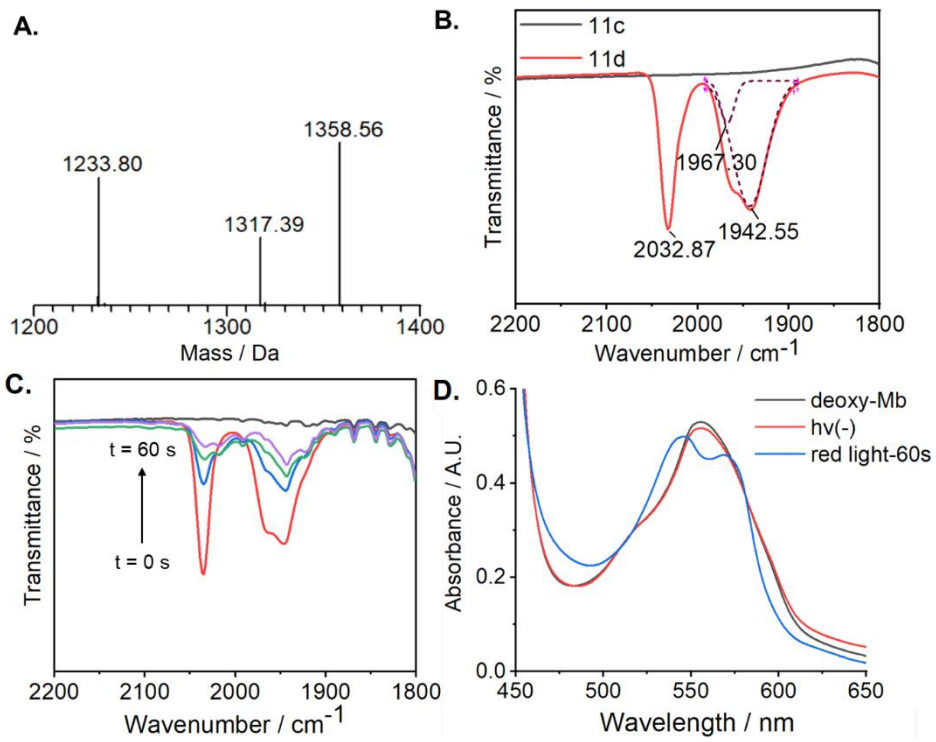

Figure S61. The final product **11d** and its CO release were analyzed by ESI-MS, FT-IR and Mb assay. (A) Average isotopes mass calculated for [M-$Mn(CO)_3$-CH_3CN]: 1358.48, found: 1358.56, [M-$Mn(CO)_3$]: 1317.40, found: 1317.39 and [M-Mn]: 1233.42, found: 1233.80; (B) FT-IR spectra of **11d**; (C) Change in the solid-state FT-IR spectra of **11d** upon red light irradiation. (D) The absorption spectral change of deoxy-Mb in Mb assay.

MiniCTX3-azpy-CORM (12d)

Peptide **12d** was obtained by the addition of $Mn(CO)_5Br$ (5 eq, 0.4 M) to a solution of purified peptide **12c** (1.0 equiv) in DCM/MeOH for 4 h at 40 °C in the dark and further washing with 20% TFA solution as mentioned above.

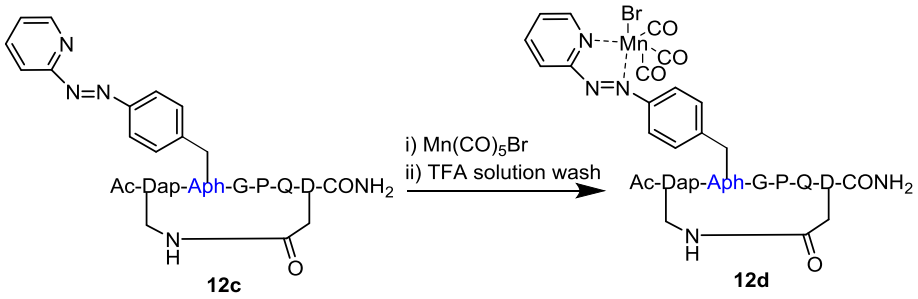

Figure S62. The binding between $Mn(CO)_5Br$ and **12c** in solution.

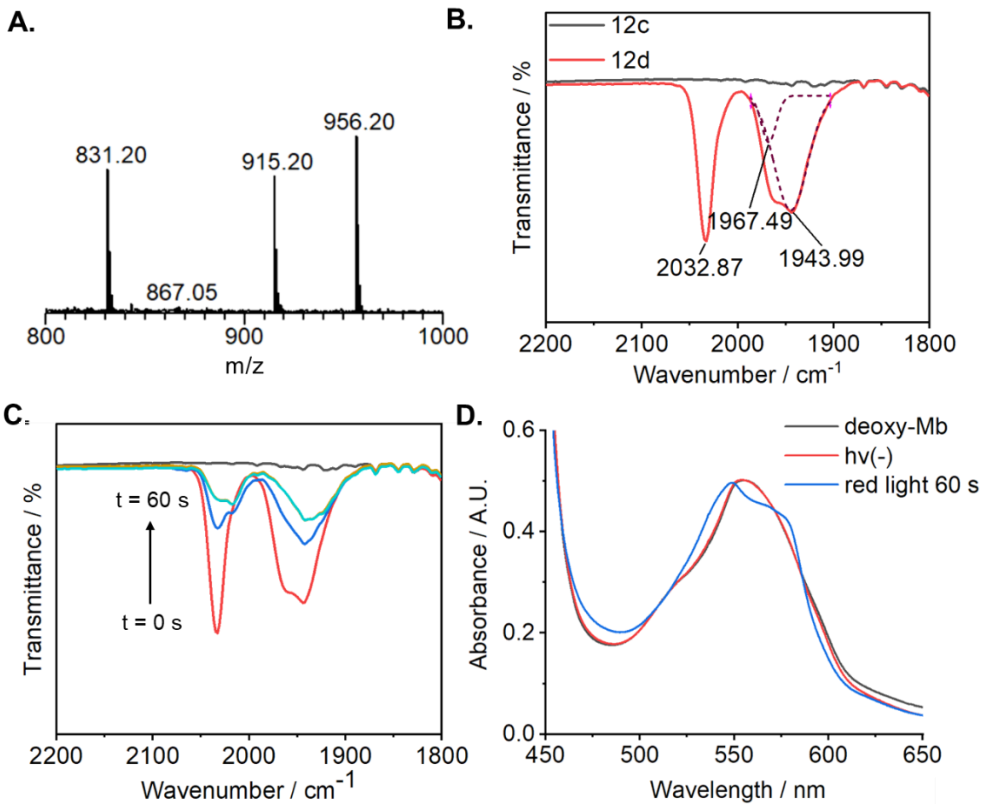

Figure S63. The final product **12d** and its CO release were analyzed by ESI-MS, FT-IR and Mb assay. (A) ESI-MS calculated for $[M\text{-}MnCO_3\text{-}CH_3CN]^+$: 956.28, found: 956.20, $[M\text{-}MnCO_3]^+$: 915.26, found: 915.20 and $[M\text{-}Mn]^+$: 831.27, found: 831.20; (B) FT-IR spectra of **12d**; (C) Change in the solid-state FT-IR spectra of **12d** upon red light irradiation. (D) The absorption spectral change of deoxy-Mb in Mb assay.

4 Biological experiments

4.1 Isothermal titration calorimetry (ITC)

The ITC assay was carried out using a PEAQ-ITC system (MicroCal™, Malvern Panalytical, UK). The solutions of PEX (48 µM), peptide **8d (**3.53 µM) and **15** (3.0 µM) were prepared in the same titration buffer (50 mM Tirs, 200 mM NaCl, 5 mM $CaCl_2$, pH 7.5). 40 µL PEX solution was added over 19 injections (2 µL, every 150 s) to **8d** or **15** at 25°C in the dark. The mixing paddle was rotating at a recommended speed from the manufacture. All experiments were performed in triplicate and the K_D and N values in the main text are shown as mean ± SD.

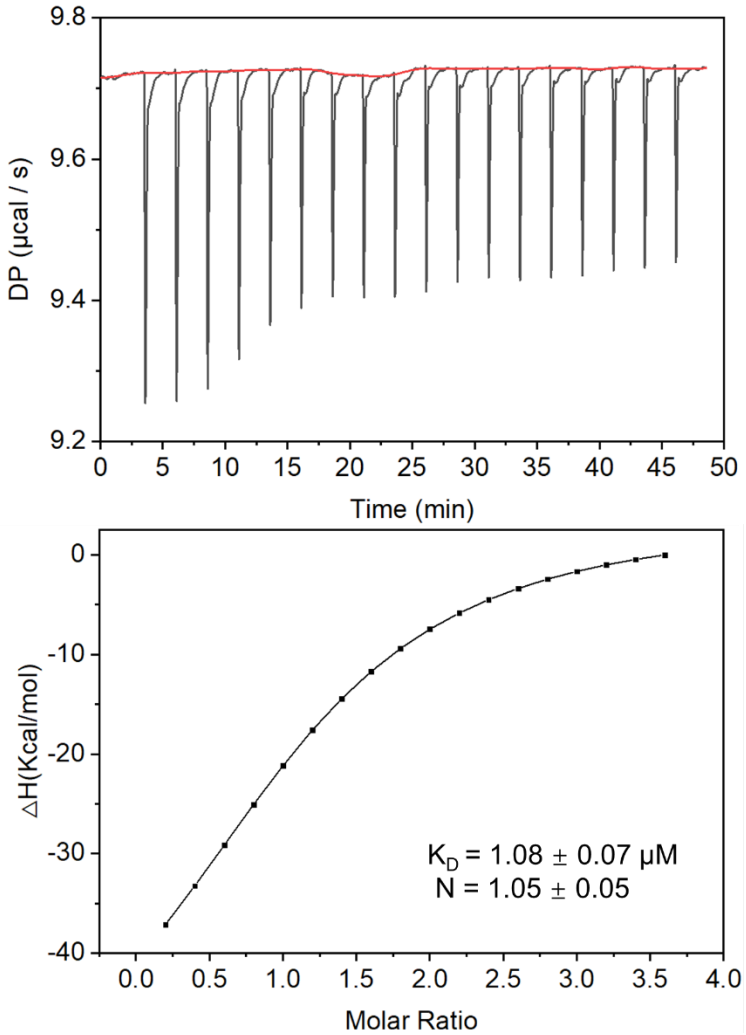

Figure S64. ITC titration curves for the addition of PEX (48 µM) into peptide **8d** (3.53 µM) in Tris buffer pH 7.5. A ~1:1 protein/peptide-CORM binding ratio was obtained from the titration.

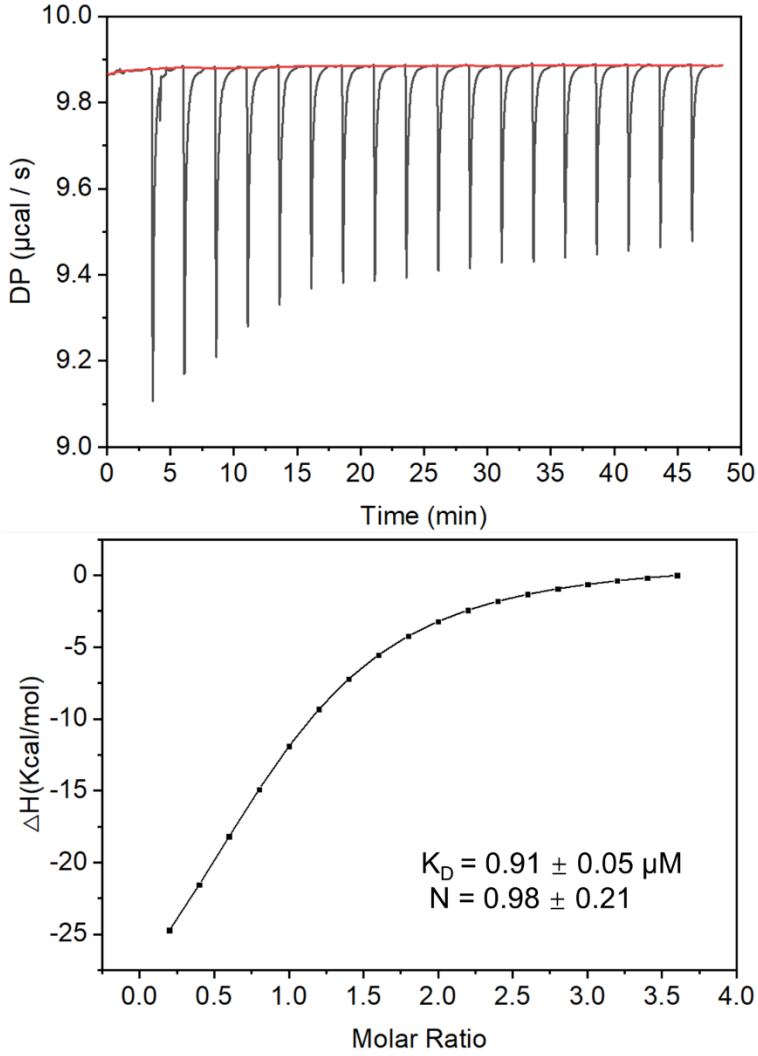

Figure S65. ITC titration curves for the addition of PEX (48 µM) into peptide **15** (3.0 µM) in Tris buffer, pH 7.5. A ~1:1 protein/peptide-CORM binding ratio was obtained from the titration.

4.2 Cell uptake and intracellular CO release

Cell culture

Hela cells, U87 cells and MCF-7 cells were incubated in complete medium (Dulbecco's modified Eagle's Medium (DMEM) and supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin) at 37°C in a humidified and 5% CO_2 incubator. For all experiments, cells were harvested by using trypsin (Gibco™) in PBS buffer and resuspended in fresh medium before plating.

Cell uptake

Hela cells were seeded into glass-bottom confocal dishes (20×10^3 cells per dish) and incubated for 24 h at 37 °C under 5% CO_2. The cells were treated with wild-type 5-FAM-TAT (**14**) or 5-FAM-TAT-azpy-CORM (**7d)** at the same final concentration of 10 μM in EBSS buffer and incubated for 1 h at 37 °C under 5% CO_2 in the dark. After the incubation, the cells were washed 3 times with PBS buffer to remove the residual peptides. Then, DAPI (4, 6-diamidino-2-phenylindole) solution (ab104139, Abcam) was added, and incubated for 5 min to fix the cells and stain the nuclei. After the incubation, the cells were gently washed twice to remove excess DAPI.

Confocal imaging was conducted using a confocal laser scanning microscope (Zeiss LSM980). The fluorescence images were taken under Plan-Apochromat 40×/0.95 Korr M27. Blue and yellow luminescent emissions from DAPI and 5-FAM labelled peptides were excited at the wavelengths of 353 nm and 493 nm, respectively, and the emission wavelengths were ranged from 353 to 601 nm for DAPI and 490 to 677 nm for 5-FAM labelled peptides, respectively.

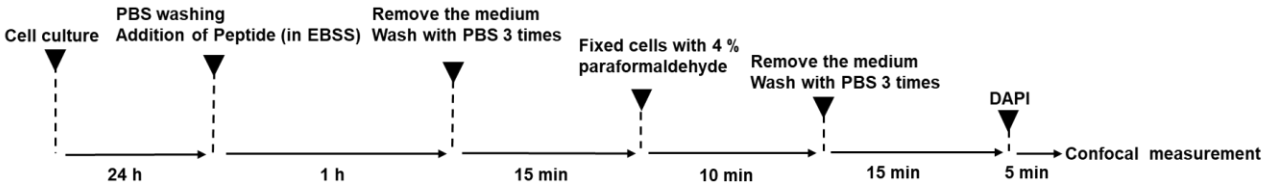

Figure S66. The experimental protocol of confocal fluorescence imaging of cellular uptake and nuclear localization of **14** and **7d** in HeLa cells.

Intracellular CO release

HeLa cells were seeded into glass-bottom confocal dishes (20×10^4 per dish) and incubated for 24 h at 37 °C under 5% CO_2. TAT-azpy-CORM (**6d**, the final concentration was 1 μM in EBSS) was added and incubated for 1 h at 37 °C under 5% CO_2 in the dark. After the incubation, the cells were washed 3 times with PBS buffer to remove the residual peptides. And then, 625 nm red light was irradiated to the dishes for 10 min (or 0 min). After the removal of the medium, fresh EBSS containing COP-1 solution (1 μM) was added to the dishes. The cells were incubated for 30 min at 37°C before the observation with confocal microscope. The fluorescence images were taken under Plan-Apochromat 63×/1.40 Oil DIC M27. Blue and green luminescent emissions from DAPI and COP-1 were excited at the wavelength of 353 nm and 493 nm and the emission wavelengths were ranged from 410 nm to 610 nm for DAPI and 490 nm to 677 nm for COP-1, respectively. Same preparation and observation were employed for no CORMs in EBSS as control.

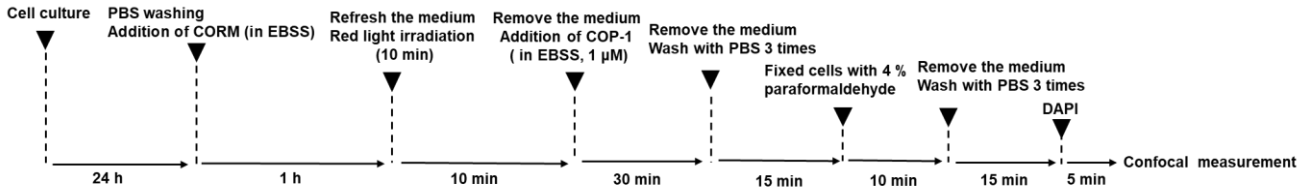

Figure S67. The experimental procedure of the confocal fluorescence imaging of intracellular red-light-responsive CO release from **6d** in HeLa cells.

4.3 In vitro cytotoxicity of intracellular CO release

Cell Counting Kit-8 (CCK-8) assay

In vitro cytotoxicity was assessed by the standard CCK-8 assay. The statistical evaluation of data was performed using a two-tailed unpaired Student's *t*-test. A *P*-value of less than 0.05 was considered statistically significant. Each data point is represented as mean ± standard deviation (SD) of three independent experiments (*n* = 3, *n* indicates the number of wells in a plate for each experimental condition). The dose dependence of the cytotoxicity was investigated at different peptide concentrations.

U87 cells, MCF-7 cells or HeLa cells were seeded in flat-bottomed 96-well plates (2×10^3 per well). After incubated for 48 h at 37°C under 5% CO_2, the medium was removed and the cells were washed by PBS for three times. Then, peptide-azpy-CORM **6d** and peptide **13** solution (dissolved in EBSS buffer) were added to the well, and the cells were incubated for 1 h at 37°C under 5% CO_2 in the dark. The concentration of **6d** and **13** was set at 0, 3.125, 6.25, 12.5 and 50 μM. After the EBSS was refreshed, the cells were irradiated by red light for 10 min (or 0 min). When the red-light irradiation finished, the cells were incubated for another 1 h. Next, the ESBB were replaced by complete medium and incubated for 24 h at 37°C under 5% CO_2. At the end of the incubation, the culture media were removed and the cells were washed with PBS for three times. Then, fresh medium containing CCK-8 solution was added to the cells and incubated for another 2 h. The absorbance at 450 nm was monitored using a multi-Mode microplate reader. The cytotoxicity was expressed as the percentage of cell viability compared to untreated sample.

Flow cytometry analysis

U87 cells ($\sim3\times10^6$ cells/well) were plated in a 6-well plate overnight and thereafter, incubated with **6d** (50 μM containing 1 mL EBSS) without and with 10 min red light irradiation. Further, the cells were trypsinized, washed (2 × 1 mL) with PBS, and centrifuged at 2000 rpm for 5 min. Then, the cell pellet was incubated with binding buffer (500 μL), Annexin-V FITC solution (5 μL) and 7-amino-actinomycin D solution (5 μL) in a dark place for 15 min. Thereafter, the samples were analyzed using flow cytometry (BD FACSCanto™ Clinical Flow Cytometry System, USA).

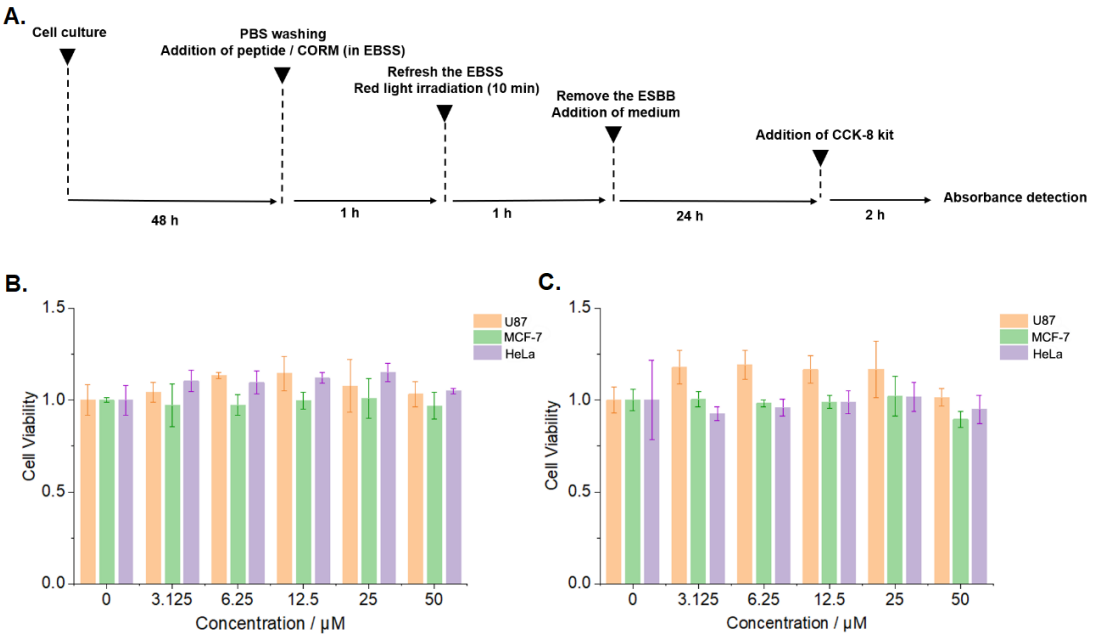

Figure S68. (A) The experimental protocol of cells viability assay; Cell viability of MCF-7, U87 and HeLa cells treated with **13** in the dark (B) or under red light irradiation (C). **13** did not significantly reduce cell viability with or without light irradiation. Data representative of *n* = 3 independent experiments.

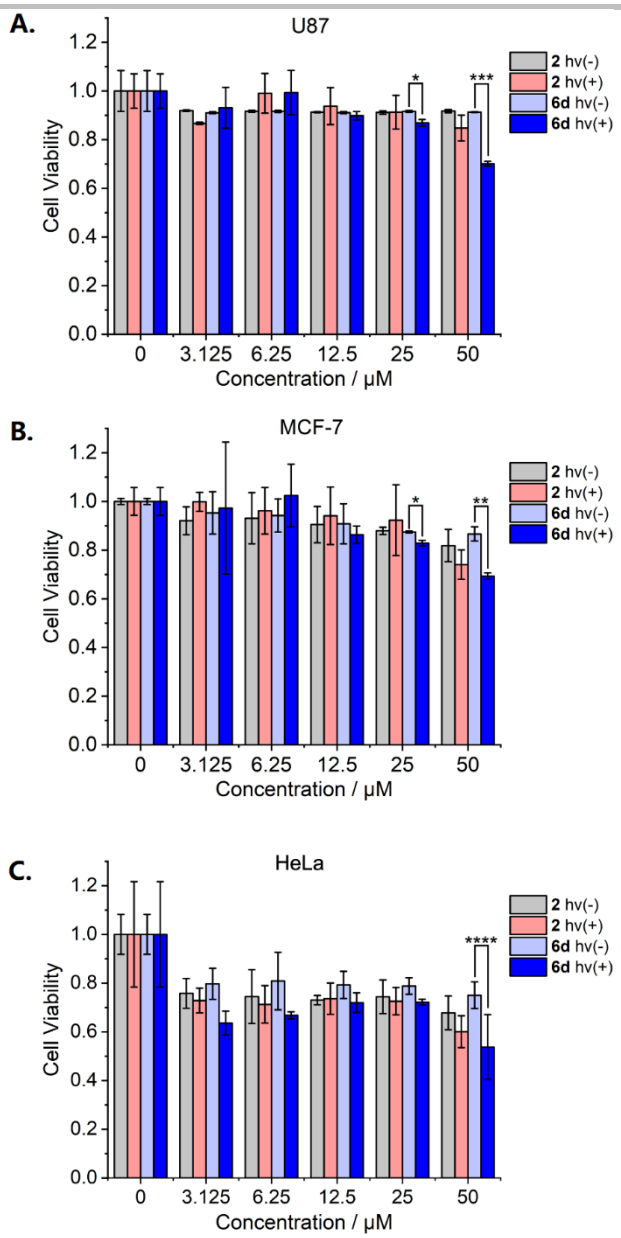

Figure S69. The dose-dependent dark/red-light cytotoxicity of **6d** vs. the monomer **2** in U87 (A), MCF-7 (B) and HeLa (C) cell lines. Data are shown as mean ± SD of *n* = 3 independent experiments, and analyzed with *t* test (**P* < 0.05 vs. the non-irradiated group; ***P* < 0.01 vs. the non-irradiated group; *****P* < 0.001 vs. the non-irradiated group).

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