Supporting Information

Total chemical synthesis of the N-terminal domain of TIMP2

Yuanmei Sun[a], Yuqi Zhang[a], Chunmao He[*a]

[a] School of Chemistry and Chemical Engineering, South China University of Technology, Guangzhou 510640, China.

[*] Email: hecm@scut.edu.cn.

Catalogue

1. General methods

1.1 Materials and instruments

All chemicals were obtained from commercial sources: Bide Pharma, Adamas, Innochem, TCl, Alfred alfae, Thermo Fisher and were used without further purification unless otherwise stated. Fmoc protected amino acids, HATU, TBTU, HOBt, Rink amide MHBA and 2-Cl(Trt)-Cl resins were acquired from GL Biochem (Shanghai) and Bide Pharma. (ESI)-HRMS was recorded on Agilent 1290/Bruker maXis impact. The reagents acetylacetone (acac) and inslin (from bovine pancreas) were purchased from Aladdin. Sodium ascorbate, ethylene diamine tetraacetic acid (EDTA) and 2,2′(ethylenedioxy)diethanethiol (DODT) were purchased from TCI. Palladium chloride ($PdCl_2$) and 2,2'-Azobis[2-(2-imidazolin-2-yl) propane] dihydrochloride (VA-044) were obtained from J&K Scientific. N,N-diisopropylethylamine (DIPEA), N,N'-diisopropylcarbodiimide (DIC), dithiothreitol (DTT), tris(2-carboxyethyl)phosphine (TCEP), trifluoroacetic acid (TFA), 2-methyl-2-propanethiol (t-BuSH), and guanidine hydrochloride (Gdn·HCl) were obtained from Adamas. Methoxylamine (CH_3ONH_2·HCl) and triisopropylsilane (TIPS) were purchased from Energy Chemicals. Ribonuclease A from bovine pancreas (RNase A), trypsin (from bovine pancreas), tris(hydroxymethyl)aminomethane (Tris), glutathione reduced (GSH) and glutathione oxidized (GSSG) were purchased from Sangon. The reagents N,N-dimethylformamide (DMF) and dichloromethane (DCM) were purchased from GHTCH (Guangdong). 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and 4-mercaptophenylacetic acid (MPAA) were purchased from Alfa Aesar. His_6-Ulp1 was recombinantly expressed using a reported procedure. MALDI-TOF-MS was recorded on Shimadzu MALDI-TOF-MS 8020 instrument in the linear mode using a matrix of 10 mg mL^{-1} α-cyano-4-hydroxycinnamic acid (CHCA) in water/MeCN (1: 1, v/v) without TFA.

Analytical HPLC/MS: Agilent HPLC 1260, Ruihe® Tech analytical HPLC; Columns: Kromasil 200-5-C4 300Å (5 μm, 4.6 mm I.D. x 250 mm); Welch Ultimate® XB-C18 300Å (5 μm, 4.6 mm I.D. x 250 mm); Welch Ultimate® XB-C4 300Å (5 μm, 4.6 mm I.D. x 250 mm); flow rate: 1mL/min with UV detection at 214 and 254 nm; for LC-MS, Welch Ultimate® XB-C4 300Å (5 μm,

4.6 mm I.D. x 100 mm); flow rate: 0.4mL/min with UV detection at λ = 214 and 254 nm; Mobile phase A: 0.1% TFA in degassed miliQ H_2O; Mobile phase B: 0.1% TFA in HPLC grade acetonitrile. For LC-MS, the mobile phase additive was changed to 0.1% formic acid, flow rate: 0.8 mL/min with UV detection at λ = 214 nm.

Semi-preparative reverse-phase HPLC: Shimadzu HPLC (AR-20); *Column:* Waters XBridge® peptide BEH **C18** OBDTM Prep column 300Å (5 μm, 10 mm I.D. x 250 mm) running at a flow rate of 5.0 mL/min; Welch Ultimate XB-**C4** 120 Å (5 μm, 10 mm I.D. x 250 mm) columns at a flow rate of 4.7 mL/min with UV detection at 214 nm. Mobile phase: Solvent **A**: 0.1% TFA in degassed miliQ water; Solvent **B**: 0.1% TFA in HPLC grade acetonitrile.

Preparative reverse-phase HPLC: Ruihe® Tech prep-HPLC; *Column:* Daisogel **C18** 120 Å (10 μm, 20 mm I.D. x 250 mm) column at a flow rate of 35 mL/min; Welch Ultimate XB-**C4** 120 Å (5 μm, 20 mm I.D. x 250 mm) columns at a flow rate of 35 or 40 mL/min with UV detection at 214 nm. Mobile phase: Solvent **A**: 0.05% TFA in degassed miliQ water; Solvent **B**: 0.1% TFA in HPLC grade acetonitrile.

2. General synthesis procedures

2.1 Preloading of 2-Cl-(Trt)-$NHNH_2$ resin

2-Chlorotrityl chloride resin (0.9 mmol/g, 1 g) was swollen in DMF for 20 min and then washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2× 5 mL). The resin was treated with freshly prepared 5% hydrazine monohydrate in DMF (2× 20 mL) for 30 min and then washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2 × 5 mL). The resin was treated with freshly prepared 5% MeOH in DMF (20 mL) for 10 min and then washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2 × 5 mL). DIPEA (1.2 mmol) was added to a solution of Fmoc-AA-OH (0.6mmol) and TBTU (0.6 mmol) in DMF (5 mL). After 2 min of pre-activation, the mixture was added to the resin, which was then shaken for 2 h at 25 ℃. The resin was washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2 × 5 mL), and then capped with 20% acetic anhydride in DMF (10 mL) for 20 min, and washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2 × 5 mL) again

2.2 Estimation of amino acid loading

The resin (10 mg) loaded with the first amino acid was treated with 2% DBU/DMF (2 mL) for 30 min at 25 ℃ to remove the Fmoc group. The blank group was set up. The deprotection solution (2 mL) was diluted to 10 ml with MeCN, and then 0.8 mL was further diluted to 10 mL with MeCN. The UV absorbance of the resulting piperidine-fulvene adduct solution was measured (λ = 304 nm) to estimate the amino acid loading on the resin.

2.3 Fmoc deprotection

The resin was treated with 20% piperidine in DMF (5 mL, 2 × 10 min) at 25 ℃ and then washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2 × 5 mL).

2.4 Coupling of general amino acids

Peptides were synthesized on a CS Bio 136XT synthesizer using Fmoc solid phase peptide synthesis (SPPS) chemistry. The following Fmoc amino acids with side-chain protecting groups were used: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Val-OH, etc. SPPS was performed on 2-Cl-(Trt)-Cl or Rink amide MBHA resins. Fmoc deprotections were performed with 20% piperidine in DMF (10 min × 2). Couplings were performed with Fmoc amino acid (4.0 equiv to resin substitution), TBTU (3.9 equiv) and DIPEA (8.0 equiv) in DMF for 60 min (45 ℃). After coupling, unreacted free amine was capped by treatment with 20% acetic anhydride in DMF for 10 min and then washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2 × 5 mL).

2.5 Coupling of Fmoc-Ile-Thr(Psi(Me, Me)Pro)-OH or Fmoc-Val-Ser(Psi(Me, Me)Pro)-OH or Fmoc-Asp(OtBu)-Gly(Dmb)-OH

A solution of Fmoc-Ile-Thr(Psi(Me, Me)Pro)-OH or Fmoc-Val-Ser(Psi(Me, Me)Pro)-OH or Fmoc-Asp(OtBu)-Gly(Dmb)-OH (4 equiv), HOBT (3.9 equiv), and DIC (8 equiv) in DMF were added to the resin. The reaction was shaken for 4 h at 30 ℃. After coupling, unreacted free amine was capped by treatment with 20% acetic anhydride in DMF for 10 min and then washed with DMF (2 ×5 mL), DCM (2 ×5 mL), and DMF (2 ×5 mL).

2.6 Coupling of Fmoc-Cys(Trt)-OH or Fmoc-Cys(Acm)-OH

A solution of Fmoc-Cys(Trt)-OH or Fmoc-Cys(Acm)-OH (4 equiv), HOBT (3.9 equiv), and DIC (8 equiv) in DMF was added to the resin. The reaction was shaken for 1 h at 45 ℃. After coupling, unreacted free amine was capped by treatment with 20% acetic anhydride in DMF for 10 min and then washed with DMF (2 ×5 mL), DCM (2 ×5 mL), and DMF (2 ×5 mL).

3. Synthesis of the peptide segments

3.1 Synthesis of TIMP-2(1–31) thioester segment 1-SR

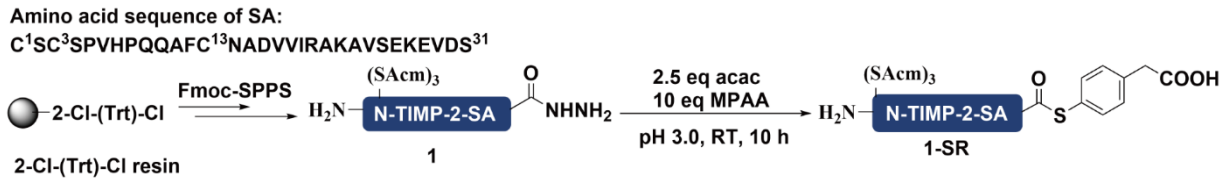

Figure S1. Synthesis of TIMP-2(1–31) thioester **1-SR**

TIMP-2(1–31)-$NHNH_2$ (SA) was synthesized on 2-Cl-(Trt)-Cl resin (theoretical loading: 0.9 mmol/g) using Fmoc-Ser(*t*Bu)-OH with 0.35 mmol/g loading and elongated according to standard Fmoc-SPPS protocols to afford resin-bound peptide. The peptide was cleaved using TFA/H_2O/DODT (95:2.5:2.5 v/v/v) (3 mL per 100 mg of resin) for 2 h a t 25 ℃. After filtration, the filtrate was concentrated by blowing with a gentle flow of N_2. Add the precooled diethyl ether to precipitate crude peptides. The resulted suspension was centrifuged (8000 rpm, 5 min, 4 ℃), and the ether layer was decanted. Air-dry the peptide product in the open centrifuge tube for about 30 min to

acquire the crude peptide TIMP-2(1–31)-$NHNH_2$ (SA). ESI-MS analysis of **1** with the observed mass 3545.0 Da, calcd 3545.0 Da (average isotopes).

The crude peptide SA (400 mg, assumed 100% purity) was dissolved to 40 mg/mL in 6 M Gdn·HCl, 0.2 M Na_2HPO_4, pH 3.0, with 10 equiv MPAA, 2.5 equiv acac (from a 0.1 M stock in water) were added to the mixture, and the reaction mixture was stirred for 10 h to form thioester fragment TIMP-2(1–31)-SR (**1-SR**). The mixture was centrifuged (8000 rpm, 5 min, 4 ℃), filtered and purified by preparative HPLC at 25 ℃ with a gradient of 20 to 50% MeCN (with 0.1% TFA) in 25 min to obtain 42 mg of segment **1-SR** (Isolated yield 10.1%). The purity and exact mass of the peptide was confirmed using analytical HPLC and ESI-MS, respectively. ESI-MS analysis of **1-SR** with the observed mass 3681.7 Da, calcd 3681.7 Da (average isotopes).

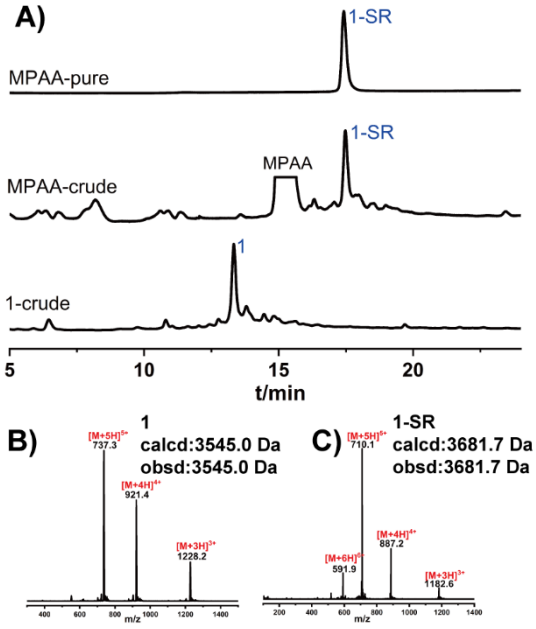

Figure S2. (A) Analytical HPLC traces (20 to 50% MeCN (with 0.1% TFA) in 25 min, λ = 214 nm) of crude TIMP-2(1–31)-$NHNH_2$ SA and purified TIMP-2(1–31) **thioester 1**. (B) ESI-MS analysis of **1** with the observed mass 3545.0 Da, calcd 3545.0 Da (average isotopes). (C) ESI-MS analysis of **1-SR** with the observed mass 3681.7 Da, calcd 3681.7 Da (average isotopes).

3.2 Synthesis of TIMP-2(32–71) segment B(M52Nle)

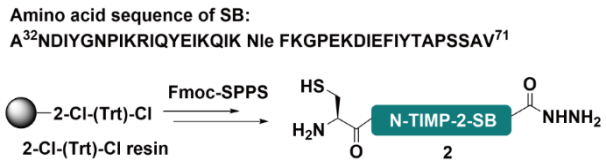

Figure S3. Synthesis of TIMP-2(32–71)-$NHNH_2$ segment B

TIMP-2(32–71)-$NHNH_2$ (M52Nle) was synthesized on 2-Cl-(Trt)-Cl resin (theoretical loading: 0.9 mmol/g) using Fmoc-Val-OH with 0.3 mmol/g loading and elongated according to standard Fmoc-SPPS protocols to afford resin-bound peptide. The peptide was cleaved using TFA/H_2O/DODT (95:2.5:2.5 v/v/v) (3 mL per 100 mg of resin) for 2 h a t 25 ℃. After filtration, the filtrate was concentrated by blowing with a gentle flow of N_2. Add the precooled diethyl ether to precipitate crude peptides. The resulted suspension was centrifuged (8000 rpm, 5 min, 4 ℃), and the ether layer was decanted. Air-dry the peptide product in the open centrifuge tube for about 30 min to acquire the crude peptide TIMP-2(32-71)-$NHNH_2$ (**2**). The crude peptide **2** (460 mg, assumed 100% purity) was dissolved to 40 mg/mL in 6 M Gdn·HCl, 0.2 M Na_2HPO_4, pH 3.0, filtered and purified by preparative HPLC at 25 ℃ with a gradient of 25 to 50% MeCN (with 0.1% TFA) in 25 min to obtain 50 mg of segment **2** (Isolated yield 10.9%) The purity and exact mass of the peptide was confirmed using analytical HPLC and ESI-MS, respectively. ESI-MS analysis of **2** with the observed mass 4645.4 Da, calcd 4645.4 Da (average isotopes).

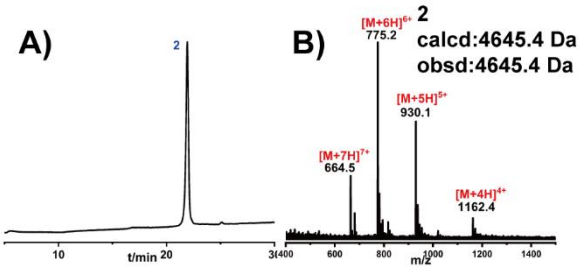

Figure S4. (A) Analytical HPLC traces (25 to 50% MeCN (with 0.1% TFA) in 25 min, λ = 214 nm) of purified **2**. (B) ESI-MS analysis of **2** with the observed mass 4645.4 Da, calcd 4645.4 Da (average isotopes).

3.3 Synthesis of TIMP-2(72–100) thioester segment 3-SR

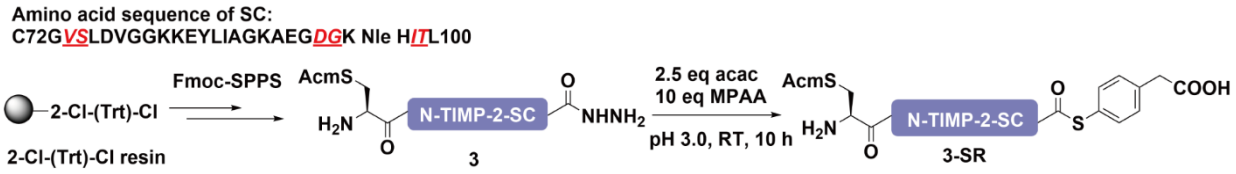

Figure S5. Synthesis of TIMP-2(72–100) thioester **3-SR**

TIMP-2(72–100)-$NHNH_2$ (SC1) was synthesized on 2-Cl-(Trt)-Cl resin (theoretical loading: 0.9 mmol/g) using Fmoc-Leu-OH with 0.35 mmol/g loading and elongated according to standard Fmoc-SPPS protocols to afford resin-bound peptide. The pseudoproline dipeptides of Fmoc-Ile-Thr(Psi(Me, Me)Pro)-OH or Fmoc-Val-Ser(Psi(Me, Me)Pro)-OH or dipeptides Fmoc-Asp(OtBu)-Gly(Dmb)-OH was coupled according to the protocol 2.5. The peptide was cleaved using TFA/phenol/H_2O/thioanisole/EDT (82.5:5:5:5:2.5 v/v/v/v/v) (3 mL per 100 mg of resin) for 2 h a t 25 ℃. After filtration, the filtrate was concentrated by blowing with a gentle flow of N_2. Add the precooled diethyl ether to precipitate crude peptides. The resulted suspension was centrifuged (8000 rpm, 5 min, 4 ℃), and the ether layer was decanted. Air-dry the peptide product in the open centrifuge tube for about 30 min to acquire the crude peptide TIMP-2(72–100)-$NHNH_2$ (**3**). ESI-MS analysis of **3** with the observed mass 3056.7.0 Da, calcd 3056.6 Da (average isotopes).

The crude peptide **3** (400 mg, assumed 100% purity) was dissolved to 40 mg/mL in 6 M Gdn·HCl, 0.2 M Na_2HPO_4, pH 3.0, with 10 equiv MPAA, 2.5 equiv acac (from a 0.1 M stock in water) were added to the mixture, and the reaction mixture was stirred for 10 h to form thioester fragment TIMP-2(72–100)-SR (**3-SR**). The mixture was centrifuged (8000 rpm, 5 min, 4 ℃), filtered and purified by preparative HPLC at 25 ℃ with a gradient of 20 to 38 to 42% MeCN (with 0.1% TFA) in 10+8 min to obtain 35 mg of segment **3-SR** (Isolated yield 8.7%). The purity and exact mass of the peptide was confirmed using analytical HPLC and ESI-MS, respectively. ESI-MS analysis of **3-SR** with the observed mass 3193.6 Da, calcd 3193.6 Da (average isotopes).

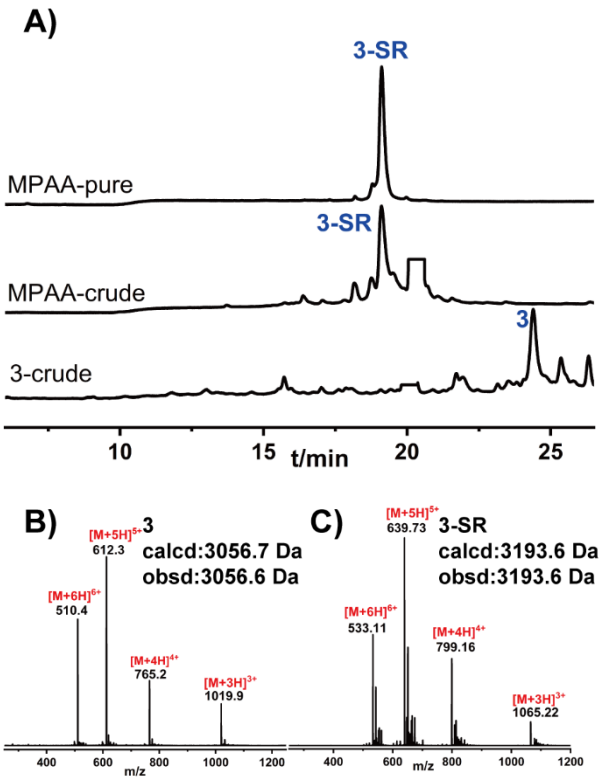

Figure S6. (A) Analytical HPLC traces (20 to 38 42% MeCN (with 0.1% TFA) in 10+8 min, λ = 214 nm) of crude TIMP-2(72–100)-$NHNH_2$ SC and purified TIMP-2(72–100) thioester **3-SR**. (B) ESI-MS analysis of **3** with the observed mass 3056.7 Da, calcd 3056.6 Da (average isotopes). (C) ESI-MS analysis of **3-SR** with the observed mass 3193.6 Da, calcd 3193.6 Da (average isotopes).

3.4 Synthesis of TIMP-2(101–127) segment D

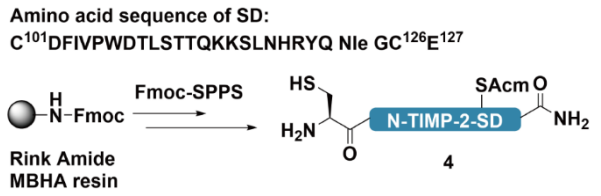

Figure S7. Synthesis of TIMP-2(101–127)-$CONH_2$ segment D

The TIMP-2(101-127)-$CONH_2$ segment D was synthesized on Rink amide MBHA resin (theoretical loading: 1.2 mmol/g) using Fmoc-Glu-OH with 0.42 mmol/g loading and elongated according to standard Fmoc-SPPS protocol to afford resin-bound peptide. The peptide was cleaved using TFA/H_2O/DODT (95:2.5:2.5 v/v/v) (3 mL per 100 mg of resin) for 2 h at 25 ℃. After

filtration, the filtrate was concentrated by blowing with a gentle flow of N_2. Add the precooled diethyl ether to precipitate crude peptides. The resulted suspension was centrifuged (8000 rpm, 5 min, 4 ℃), and the ether layer was decanted. Air-dry the peptide product in the open centrifuge tube for about 30 min to acquire the crude peptide. The crude peptide **4** (300 mg, assumed 100% purity) was dissolved to 40 mg/mL in 6 M Gdn·HCl, 0.2 M Na_2HPO_4, pH 3.0, filtered and purified by preparative HPLC at 25 ℃ with a gradient of 25 to 50% MeCN (with 0.1% TFA) in 25 min to obtain 45 mg of segment **4** (Isolated yield 15.0%) The purity and exact mass of the peptide was confirmed using analytical HPLC and ESI-MS, respectively. ESI-MS analysis of **4** with the observed mass 3181.7 Da, calcd 3181.6 Da (average isotopes).

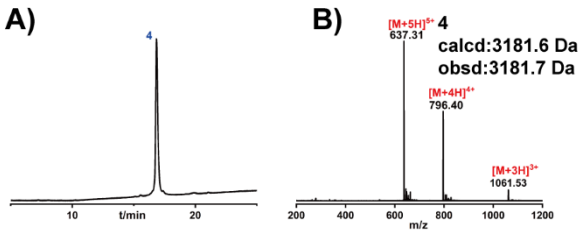

Figure S8. (A) Analytical HPLC traces (25 to 50% MeCN (with 0.1% TFA) in 25 min, λ = 214 nm) of purified **4**. (B) ESI-MS analysis of **4** with the observed mass 3181.7 Da, calcd 3181.6 Da (average isotopes).

4. Native Chemical Ligation, desulfurization, and Acm deprotection

4.1 Synthesis of segment 6 by one-pot NCL and desulfurization

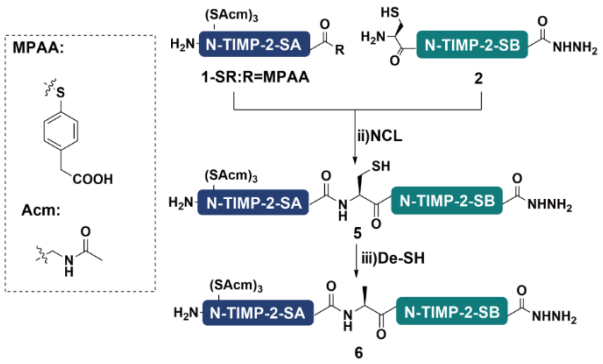

Figure S9. Synthesis of segment **6** by one-pot NCL and desulfurization

1-SR (12.0 mg, 0.0032611 mmol, 1.50 equiv.) and N-terminal Cys peptide **2** (10.10mg, 0.0021745 mmol, 1.00 equiv.) was weighted into a glass sample vial along with a micro stirrer magnet and dissolved in appropriate volume of pre-degassed reaction buffer (pH 6.5, 2.5 M imidazole, 6 M Gdn·HCl, 10 mM TCEP) (2174 μL) to make up final reaction concentration of 1.50 mM. The ligation reaction was stirred at room temperature. Aliquot of this reaction mixture was taken every 2 h, reduced with TCEP and analyzed by HPLC, and ESI-MS. Upon completion, solid TCEP (124.4 mg, 0.43482 mmol) was directly added into to this reaction mixture to make up final concentration of 200 mM and vortexed well to ensure complete solvation of the solid TCEP. Without further pH adjustment, *t*-BuSH (108.7 μL, 5% v/v), and an aqueous solution of 0.10 M VA-044 (42.17 mg, 0.130 mmol, 40.0 equiv.) was added into the reaction. The desulfurization reaction was incubated at 37 ℃ in a water bath in well-ventilated fume hood for 24 h. Aliquot of this reaction mixture was analyzed by HPLC and ESI-MS. Then, the mixture was centrifuged (8000 rpm, 5 min, 4 ℃), filtered and purified by preparative HPLC at 25 ℃ with a gradient of 20 to 50% MeCN (with 0.1% TFA) in 25 min to collect the desired fractions and immediately lyophilized, affording the desired fragment **6** as a white amorphous powder (11 mg, 46.2% isolated yield). The purity and exact mass of the peptide was confirmed using analytical HPLC and ESI-MS, respectively.

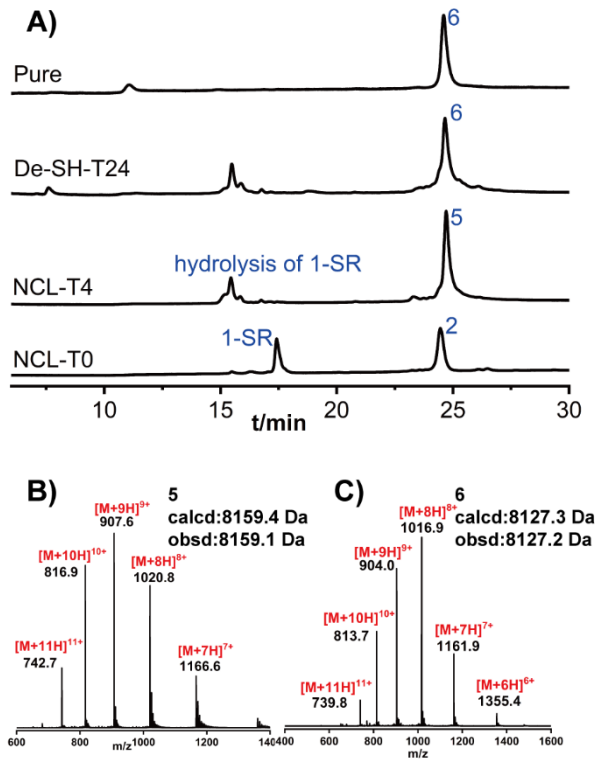

Figure S10. (A) Analytical HPLC traces (20 to 50% MeCN (with 0.1% TFA) in 25 min, λ = 214 nm) of **NCL** and purified **6.** (B) ESI-MS analysis of **5** with the observed mass 8159.1 Da, calcd 8159.4 Da (average isotopes). (C) ESI-MS analysis of **6** with the observed mass 8127.2 Da, calcd 8127.3 Da (average isotopes).

4.2 Synthesis of segment 8 by one-pot NCL and Acm deprotection

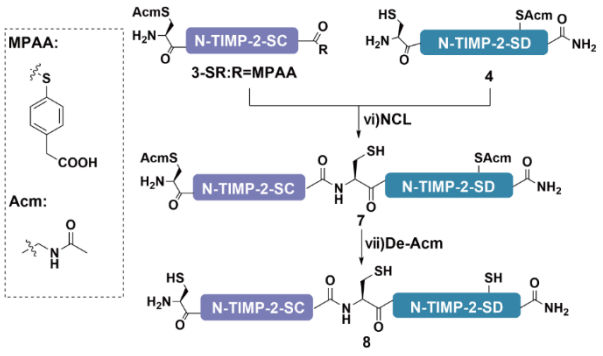

Figure S11. Synthesis of segment **8** by one-pot NCL and Acm deprotection

3-SR (12.7 mg, 0.0039787 mmol, 1.00 equiv.) and N-terminal Cys peptide **4** (11.4mg, 0.0035815 mmol, 0.90 equiv.) was weighted into a glass sample vial along with a micro stirrer magnet and dissolved in appropriate volume of pre-degassed reaction buffer (pH 6.5, 0.2 M NaH_2PO_4, 6 M Gdn ⋅HCl, 10 mM TCEP) (2652 μL) to make up final reaction concentration of 1.50

mM. The ligation reaction was stirred at room temperature. Aliquot of this reaction mixture was taken every 2 h, reduced with TCEP and analyzed by HPLC, and LC-MS. Upon completion, 10.6 mg of $PdCl_2$ dissolved in 0.2 mL of 6 M Gdn·HCl, 0.2 M NaH_2PO_4, pH 6.5 buffer and incubated at 37 ℃ for 15 min to assist dissolution. Then $PdCl_2$ solution were added to the peptide solution, which were incubated at 37 ℃ for 1 h. Progress of the reaction was monitored by taking aliquot from the reaction mixture and treated with a small amount of DTT, followed by centrifugation. The precipitate was repeatedly washed with 6 M Gdn·HCl solution and reduced by the addition of the buffer (6 M Gdn·HCl, 0.2 M NaH_2PO_4, 50 mM TCEP, pH 6.5), incubated for 30 min. And then, the mixture was centrifuged (8000 rpm, 5 min, 4 ℃), filtered and purified by preparative HPLC at 25 ℃ with a gradient of 25 to 50% MeCN (with 0.1% TFA) in 25 min to collect the desired fractions and immediately lyophilized, affording the desired fragment **8** as a white amorphous powder (11.2 mg, 45.9% isolated yield). The purity and exact mass of the peptide was confirmed using analytical HPLC and ESI-MS, respectively.

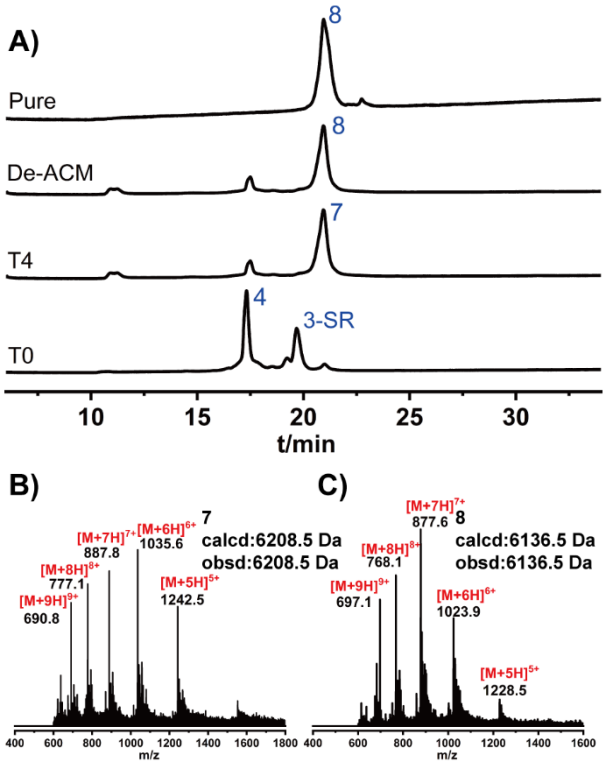

Figure S12. (A) Analytical HPLC trace (25 to 50% MeCN (with 0.1% TFA) in 25 min, λ = 214 nm) of **NCL** and purified **8.** (B) ESI-MS analysis of **7** with the observed mass 6208.5 Da, calcd 6208.5 Da (average isotopes). (C) ESI-MS analysis of **8** with the observed mass 6136.5 Da, calcd 6136.5 Da (average isotopes).

4.3 Synthesis of segment 10 by one-pot NCL and Acm deprotection followed by optimised rapid dilution folding method

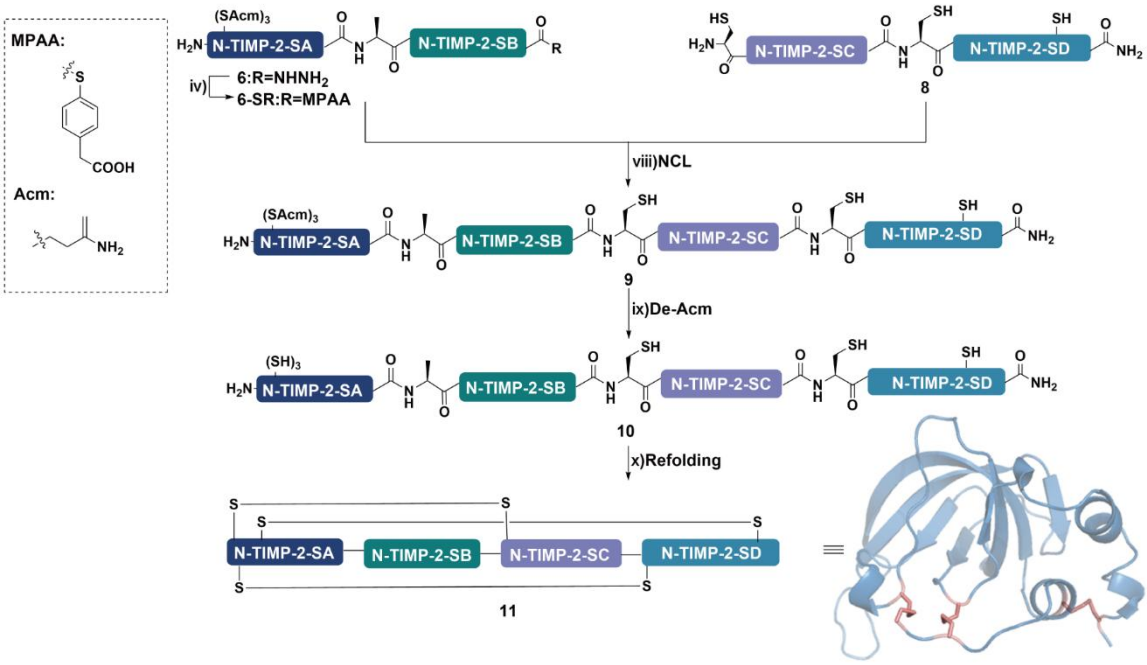

Figure S13. Synthesis of segment **10** by one-pot NCL and Acm deprotection

Peptide **6** (10.2 mg, 0.0012550 mmol, 1.0 equiv.) was dissolved to 2 mM in 6 M Gdn·HCl, 0.2 M Na_2HPO_4, pH 3.0, with 200 equiv MPAA, 2.5 equiv acac (from a 0.1 M stock in water) were added to the mixture, and the reaction mixture was stirred for 10 h to form **6-SR**. Then N-terminal Cys peptide TIMP-2-SCD-$CONH_2$ (23.1 mg, 0.0037643 mmol, 3.0 equiv.) was weighted into a glass sample vial along with a micro stirrer magnet and dissolved in appropriate volume of pre-degassed reaction buffer (pH 6.5, 0.2 M NaH_2PO_4, 6 M Gdn·HCl, 10 mM TCEP) (1255 µL) to make up final reaction concentration of 1.00 mM. The ligation reaction was stirred at room temperature. Aliquot of this reaction mixture was taken every 2 h, reduced with TCEP and analyzed by HPLC, and LC-MS. After 4h, the mixture was exchanged into a 1000 µL buffer containing 6 M Gdn·HCl, 0.2 M Na_2HPO_4, at pH 6.5 (Amicon® Ultra-0.5 concentrator, 0.5 mL, 3 K MWCO, 11000 rpm, 4 ℃, 12 times). Whereafter 10.0 mg of $PdCl_2$ dissolved in 0.2 mL of 6 M Gdn·HCl, 0.2 M NaH_2PO_4, pH 6.5 buffer and incubated at 37 ℃ for 15 min to assist dissolution. Then $PdCl_2$ solution were added to the peptide solution, which were incubated at 37 ℃ for 1 h. Progress of the reaction was monitored by taking aliquot from the reaction mixture and treated with a small amount of DTT, followed by

centrifugation. The precipitate was repeatedly washed with 6 M Gdn·HCl solution and reduced by the addition of the buffer (6 M Gdn·HCl, 0.2 M NaH_2PO_4, 50 mM TCEP, pH 6.5), incubated for 30 min. And then, the mixture was centrifuged (8000 rpm, 5 min, 4 ℃), filtered and purified by size exclusion chromatography(SEC) at 25 ℃ in 6 M Gdn·HCl, 0.2 M NaH_2PO_4, pH 6.5 (with 2 mM TCEP) to collect the desired fractions. And then the mixture was exchanged into a 150 μL buffer containing 6 M Gdn·HCl, 0.2 M Na_2HPO_4, at pH 5.0 (Amicon® Ultra-0.5 concentrator, 0.5 mL, 3 K MWCO, 11000 rpm, 4 ℃, 3 times). The resulting ligation mixture (~500 μL) was added dropwise to a 2 mL refolding buffer (0.45 M Gdn·HCl, 50 mM Tris, 0.44 mM GSSG, 0.78 mM GSH, pH 8.75) (final protein conc. was ~0.25 mg/mL) stir at RT for 2 h. Store the refolding mixture at 4 ℃ overnight without stirring(18 h). The mixture was centrifuged (8000 rpm, 5 min, 4 ℃), filtered and purified by semi-preparative HPLC with a gradient 25 to 50% MeCN in 25 min(with 0.1% TFA) to collect the desired fractions and lyophilized, and the desired folded protein N-TIMP2 (**11**) powder (0.8 mg, 4.5% isolated yield) was obtained. The purity and exact mass of the N-TIMP2 was confirmed using analytical HPLC and ESIMS, respectively.

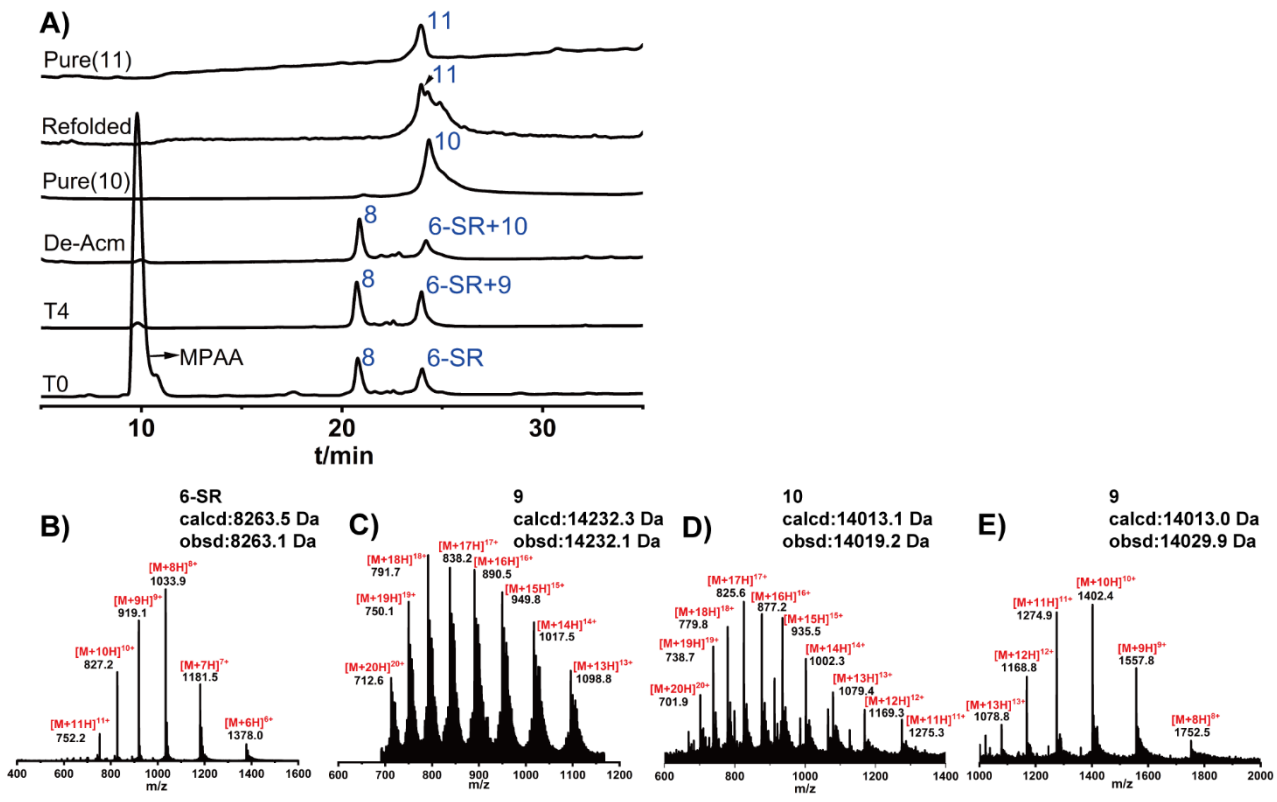

Figure S14. (A) Analytical HPLC traces (25 to 50% MeCN (with 0.1% TFA) in 25 min, λ = 214 nm) of **NCL**, Acm deprotection, refolding and purified 11 **.** (B) ESI-MS analysis of **6-SR** with the observed mass 8263.1 Da, calcd 8263.5 Da (average isotopes). (C) ESI-MS analysis of **9** with the observed mass 14232.1 Da, calcd 14232.3 Da (average isotopes). (D) ESI-MS analysis of **10** with the observed mass 14019.2 Da, calcd 14019.1 Da (average isotopes). (E) ESI-MS analysis of **11** with the observed mass 14012.9 Da, calcd 14013.0 Da (average isotopes).

5. Enzyme digestion

N-TIMP2(11) was dissolved in a solution of 25 mM NH_4HCO_3 (1 mg/mL, 100 μL). Trypsin (0.5 mg/mL, 10 μL) was firstly added to the mixture to digest the protein. The reaction was carried out at 37 ℃ for 2 h. Next, thermolysin (0.16 mg/mL, 11 μL) was added to the reaction mixture for further digestion. The reaction was carried out at 70 ℃ for 10 h. Finally, the enzymatic reaction was analysed by LC-MS. As shown in Figure S18, following trypsin and the rmolysin treatment of the protein N-TIMP2, a peptide fragment bearing the two disulfide bond: Cys1–Cys72 and Cys3-Cys101 can be clearly observed mass 1118.59 Da (calcd mass 1118.27 Da); another peptide fragment containing Cys13–Cys126 can be clearly observed mass 1092.32 Da (calcd mass 1092.21 Da). Attempted MS/MS analysis of the disulfide pairing mode was not successful due to low resolution of data.

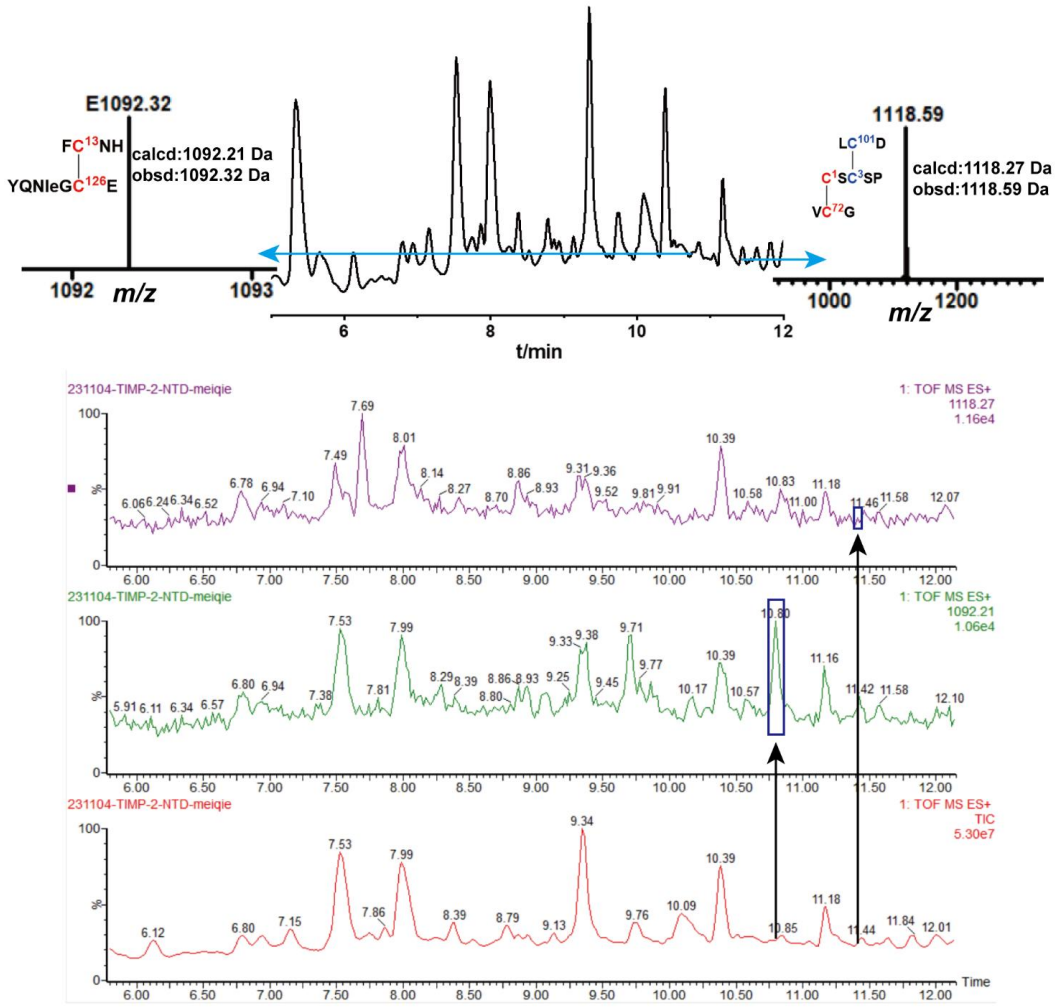

Figure S15. LC-MS analysis of the trypsin and thermolysin digest of N-TIMP2 (11).

Table 1: Peptide fragment observed following trypsin and thermolysin treatment of the protein N-TIMP2.

Fragment resulting from enzyme digestion	Calculated	Observed
$VC^{72}G\text{-}C^{1}SC^{3}SP\text{-}LC^{101}D$	1118.27	1118.59
$FC^{13}NH\text{-}YQNleGC^{126}E$	1092.21	1092.32
$V^{24}SEK^{27}$	461.52	461.99
$I^{43}QYE^{46}$	551.60	551.28
$D^{59}IEF^{62}$	522.56	522.29
$L^{75}DVGGK^{80}$	587.67	587.37
$L^{110}STTQK^{115}$	676.77	676.37

6. Attempted synthesis of TIMP2 full length

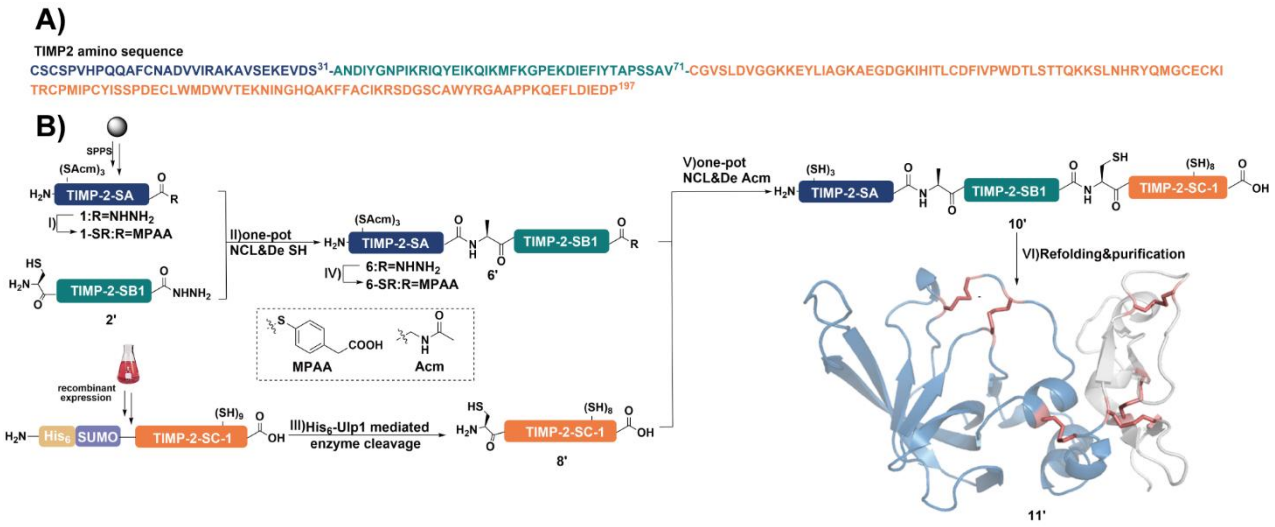

Figure. S16 Semi-synthesis strategy of TIMP2(1-194). (A) The amino acid sequence of TIMP2. (B) Scheme for the facile synthesis of the TIMP2.

6.1 Synthesis of TIMP-2(32–71) segment B1(M52)

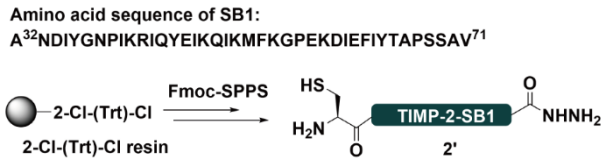

Figure S17. Synthesis of TIMP-2(32–71)-$NHNH_2$ segment B1 (**2'**)

TIMP-2(32–71)-$NHNH_2$ was synthesized on 2-Cl-(Trt)-Cl resin (theoretical loading: 0.9 mmol/g) using Fmoc-Val-OH with 0.35 mmol/g loading and elongated according to standard Fmoc-SPPS protocols to afford resin-bound peptide. The peptide was cleaved using TFA: phenol: Thioanisole: ethanedithiol**:** H_2O: dimethylsulfide: ammonium iodide = 81: 5: 5: 5: 2.5: 3: 2: 1.5 w/w (3 mL per 100 mg of resin) for 2.5 h at 25 ℃. After filtration, the filtrate was concentrated by blowing with a gentle flow of N_2. Add the precooled diethyl ether to precipitate crude peptides. The resulted suspension was centrifuged (8000 rpm, 5 min, 4 ℃), and the ether layer was decanted. Air-dry the peptide product in the open centrifuge tube for about 30 min to acquire the crude peptide TIMP-2(32-71)-$NHNH_2$ (M52Nle). The crude peptide **2'** (478 mg, assumed 100% purity) was dissolved to 40 mg/mL in 6 M Gdn·HCl, 0.2 M Na_2HPO_4, pH 3.0, filtered and purified by

preparative HPLC at 25 ℃ with a gradient of 15 to 40% MeCN (with 0.1% TFA) in 20 min to obtain 67 mg of segment **2'** (Isolated yield 14.0%) The purity and exact mass of the peptide was confirmed using analytical HPLC and ESI-MS, respectively. ESI-MS analysis of **2'** with the observed mass 4661.4.4 Da, calcd 4661.4 Da (average isotopes).

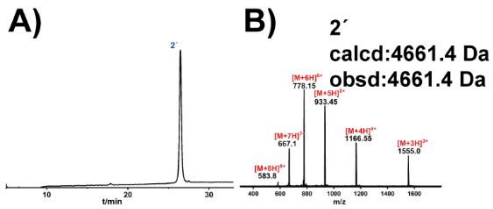

Figure S18. (A) Analytical HPLC traces (15 to 40% MeCN (with 0.1% TFA) in 20 min, λ = 214 nm) of purified **2'**. (B) ESI-MS analysis of **2'** with the observed mass 4661.4 Da, calcd 4661.4 Da (average isotopes).

6.2 Expression of TIMP-2(72–194) segment C-1

The gene encoding for the His_6-SUMO-TIMP-2(72–194 fusion protein was synthesized and codon-optimized for *E. Coli* expression(GenScript Inc., Nanjing). The synthetic gene was cloned into the pET-30a expression vector using the NdeI/EcoRI restriction sites. The cleavage site between Gly and Cys is marked in bold. The full amino acid sequence of His_6-SUMO-TIMP-2(72–194) was:
MNWSHPQFEKSSGSSGGHHHHHHGGSGGSGSDSEVNQEAKPEVKPEVKPETHINLKVSDGS
SEIFFKIKKTTPLRRLMEAFAKRQGKEMDSLRFLYDGIRIQADQAPEDLDMEDNDIIEAHREQ
IGGCGVSLDVGGKKEYLIAGKAEGDGKMHITLCDFIVPWDTLSTTQKKSLNHRYQMGCEC
KITRCPMIPCYISSPDECLWMDWVTEKNINGHQAKFFACIKRSDGSCAWYRGAAPPKQEFL
DIEDP

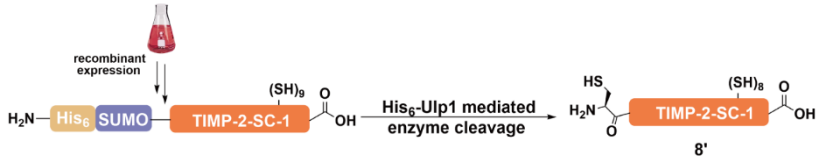

Figure S19. Expression and purification of TIMP-2(72–194) **8'**

Expression and purification of His_6-SUMO-TIMP-2(72–194):

Attempt #1

LB (2 L): 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7

Cell lysis buffer: 0.9% NaCl, pH 7

Extraction buffer: 8 M Urea, 20 mM imidazole, 0.5 M NaCl, 20 mM Tris, pH 8

Ni-NTA binding buffer: 8 M Urea, 5 mM Imidazole, 0.5 M NaCl, 20 mM Tris, 2 mM DTT pH 8

Ni-NTA eluting buffer: 8 M Urea, 250 mM Imidazole, 0.5 M NaCl, 20 mM Tris, 2 mM DTT pH 8

Refolding buffer: 6 M Urea, 0.15 M NaCl, 20 mM Tris, 10% glycerol, pH 8; 4 M Urea, 0.15 M NaCl, 20 mM Tris, 10% glycerol, pH 8; 2M Urea, 0.15 M NaCl, 20 mM Tris, 10% glycerol, pH 8; 1 M Urea, 0.15 M NaCl, 20 mM Tris, 10% glycerol, pH 8; 0.15 M NaCl, 20 mM Tris, 10% glycerol, pH 8, these dialysates were changed every 12 h.

Ni-NTA binding buffer after Ulp1 digestion: 6 M Gn ⊣HCl, 5 mM imidazole, 50 mM Tris, 2mM DTT, pH 7.8

Ni-NTA eluting buffer after Ulp1 digestion: 6 M Gn ⊣HCl, 250 mM imidazole, 50 mM Tris, 2mM DTT, pH 7.8

Attempt #2

LB (2 L): 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7

Cell lysis buffer: 0.9% NaCl, pH 7

Extraction buffer: 8 M Urea, 20 mM imidazole, 0.5 M NaCl, 20 mM Tris, pH 8

Ni-NTA binding buffer: 8 M Urea, 20 mM Imidazole, 0.5 M NaCl, 20 mM Tris, pH 8

Ni-NTA eluting buffer: 8 M Urea, 250 mM Imidazole, 0.5 M NaCl, 20 mM Tris, pH 8

Refolding buffer: 6 M Urea, 0.15 M NaCl, 20 mM Tris, 10% glycerol, pH 8; 4 M Urea, 0.15 M NaCl, 20 mM Tris, 10% glycerol, pH 8; 2M Urea, 0.15 M NaCl, 20 mM Tris, 10% glycerol, pH 8; 1 M Urea, 0.15 M NaCl, 20 mM Tris, 10% glycerol, pH 8; 0.15 M NaCl, 20 mM Tris, 10% glycerol, pH 8, these dialysates were changed every 12 h.

Ni-NTA binding buffer after Ulp1 digestion: 6 M Gn ⊣HCl, 5 mM imidazole, 20 mM NaH_2PO_4, 5 mM TCEP, pH 5.5

Ni-NTA eluting buffer after Ulp1 digestion: 6 M Gn ⊣HCl, 250 mM imidazole, 20 mM NaH_2PO_4, 5 mM TCEP, pH 5.5

The synthetic gene was cloned into the pET-30a expression vector using the *NdeI/EcoRI* restriction sites. The plasmid was firstly transformed into BL21(DE3) *E. coli* cells chemically. An overnight culture of the cells harbouring an expression vector was inoculated (1:50 dilution) in a 4 L flask containing 30 μg/mL Kanamycin in 2 L LB at 37 ℃. After reaching an OD_{600} of 0.6-0.8 overexpression of His_6-SUMO-TIMP-2-SC (72-194) was induced by the addition of 2 mL 1 M IPTG stock solution (final conc. 1 mM) at 37 ℃ for 4 hours. Cells were harvested by centrifugation (8000 rpm, 4 ℃, 15 min). Typically, 6 g cells were resuspended in 50 mL of cell lysis buffer and lysed by ultrasonication (30-40 % power, 3 s on 5 s off, 25 min). The crude lysate was centrifuged (16000 rpm, 4 °C, 20 min) and the supernatant was discarded. The precipitate was stirred at 4 ℃ overnight with 10 ml of Ni-NTA binding buffer to extract His_6-SUMO-TIMP-2-SC(72-194). The precipitate was removed by centrifugation (16000 rpm, 30 min, 4 ℃, 5 cycles) and the supernatant applied to a HisTrapTM FF column (5 mL) at 1 mL/min with a AKTA pure chromatography system. Absorption was monitored at 280 nm. The column was washed with 20 mL of Ni-NTA binding buffer. His_6-SUMO-TIMP-2(72-194) was eluted with 25 mL of Ni-NTA eluting buffer in fractions of 5 mL. The 10 mL fractions with A280 > 0.1 was gradually dialyzed to the 0.5 L refolding buffer to complete the refolding of SUMO domain. 400 μL of a stock solution of His_6-Ulp1 (A280 = 0.5) were added to the folded His_6-SUMO-TIMP-2(72-194) (10 mL, A280=1.8) (Ulp1: protein = 2:50, v/v) and the reaction was incubated for 2 hours at 30 ℃, filtered and purified by HisTrapTM FF column (5 mL) at 1 mL/min with a AKTA pure chromatography system. Equal volume of the buffer containing 6 M Gn·HCl, 200 mM Na_2HPO_4, 100 mM CH_3ONH_2·HCl, 10 mM TCEP (pH 3), was added to the mixture and the pH was adjusted to 4.0 to remove any Thz byproduct. After overnight incubation, the mixture was centrifuged (8000 rpm, 5 min, 4 ℃), filtered and purified by preparative HPLC at 25 ℃ with a gradient of 25 to 37% MeCN (with 0.1% TFA) in 30 min.

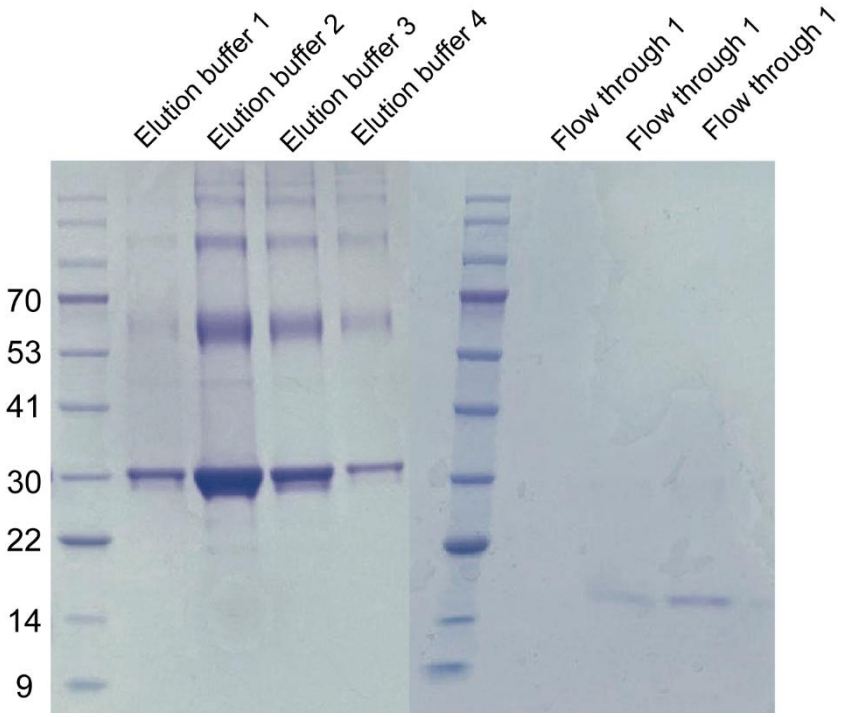

Figure S20. SDS-PAGE of the His_6-SUMO-TIMP-2(72–194) cell lysis, Ni-NTA purification of SC by cleavage of the His_6-SUMO tag

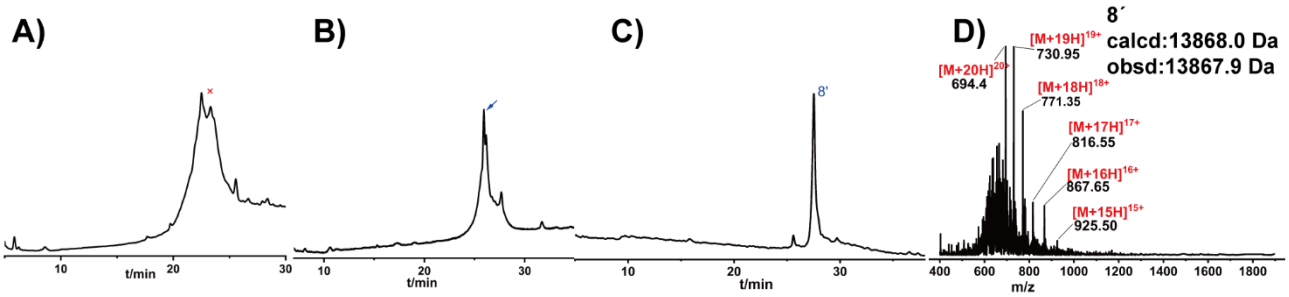

Figure S21. (A) Analytical HPLC traces (25 to 37% MeCN (with 0.1% TFA) in 30 min, λ = 214 nm) of **8'** (Attempt #1) after the cleavage of the His_6-SUMO tag. (B) Analytical HPLC traces (25 to 37% MeCN (with 0.1% TFA) in 30 min, λ = 214 nm) of SC (Attempt #2) after the cleavage of the His_6-SUMO tag. (C) Analytical HPLC traces of purified SC-1. (D) ESI-MS analysis of **8'** with the observed mass 13867.9 Da, calcd 13868.0 Da (average isotopes).

6.3 Synthesis of segment 6 ´by one-pot NCL and desulfurization

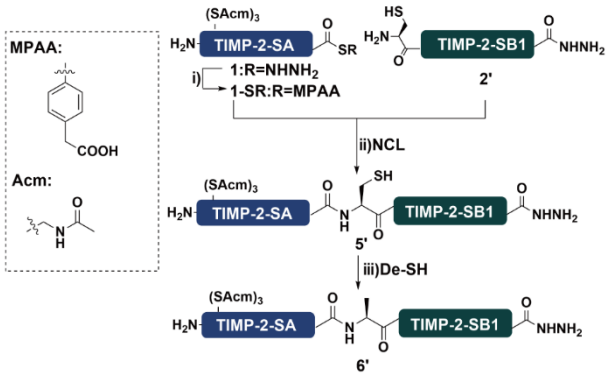

Figure S22. Synthesis of segment **6'** by one-pot NCL and desulfurization

TIMP-2-SA-MPAA ((14.7 mg, 0.003994 mmol, 1.00 equiv.) and N-terminal Cys peptide TIMP-2-SB1-$NHNH_2$ (14.32 mg, 0.003072307 mmol, 0.77 equiv.) was weighted into a glass sample vial along with a micro stirrer magnet and dissolved in appropriate volume of pre-degassed reaction buffer (pH 6.5, 2.5 M imidazole, 6 M Gdn·HCl, 10 mM TCEP) (2663 μL) to make up final reaction concentration of 1.50 mM. The ligation reaction was stirred at room temperature. Aliquot of this reaction mixture was taken every 2 h, reduced with TCEP and analyzed by HPLC, and ESI-MS. Upon completion, solid TCEP (152.7 mg, 0.5326 mmol) was directly added into to this reaction mixture to make up final concentration of 200 mM and vortexed well to ensure complete solvation of the solid TCEP. Without further pH adjustment, t-BuSH (133.15 μL, 5% v/v), and an aqueous solution of 0.10 M VA-044 (51.6 mg, 0.15976 mmol, 40.0 equiv.) was added into the reaction. The desulfurization reaction was incubated at 37 ℃ in a water bath in well-ventilated fume hood for 24 h. Aliquot of this reaction mixture was analyzed by HPLC and ESI-MS. Then, the mixture was centrifuged (8000 rpm, 5 min, 4 ℃), filtered and purified by preparative HPLC at 25 ℃ with a gradient of 15 to 45% MeCN (with 0.1% TFA) in 25 min to collect the desired fractions and immediately lyophilized, affording the desired fragment **6'** as a white amorphous powder (13 mg, 40.0% isolated yield). The purity and exact mass of the peptide was confirmed using analytical HPLC and ESI-MS, respectively.

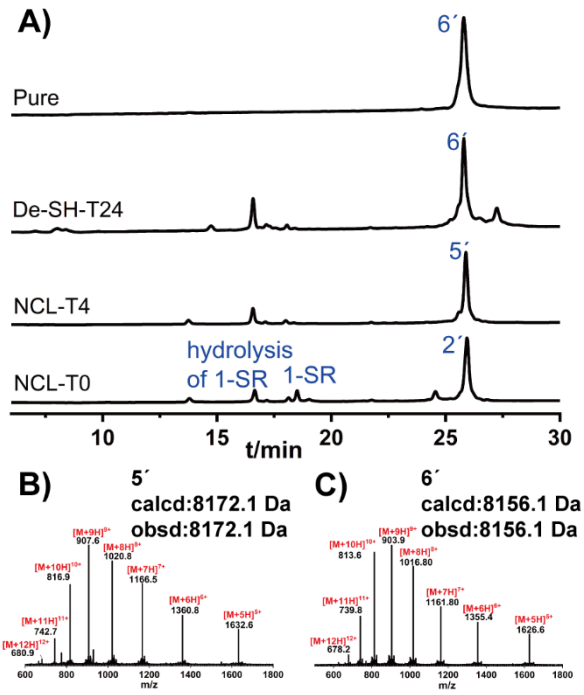

Figure S23. (A) Analytical HPLC traces (15 to 45% MeCN (with 0.1% TFA) in 25 min, λ = 214 nm) of **NCL** and purified **6'.** (B) ESI-MS analysis of **5'** with the observed mass 8172.1 Da, calcd 8172.1 Da (average isotopes). (C) ESI-MS analysis of **6'** with the observed mass 8156.1 Da, calcd 8156.1 Da (average isotopes).

6.4 Initial attempt to synthesis of segment 8' by NCL

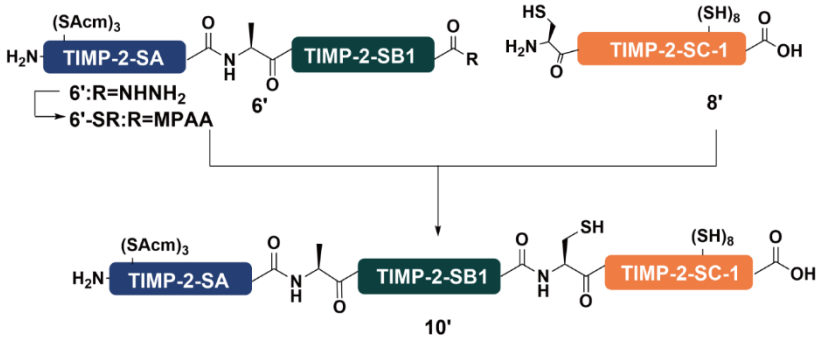

Figure S24. Synthesis of segment 10' by NCL

6' (2.90 mg, 0.3504 μmol, 1.00 equiv.) and protein segment **8'** (3.886 mg, 0.28032 μmol, 0.80 equiv.) was weighted into a 0.5 ml centrifuge tube, and suspended in pre-degassed reaction buffer (233.6 μL, 1.50 mM, pH 6.40, 200 mM NaH_2PO_4, 6.0 M Gdn·HCl, 100 mM MPAA, 20 mM TCEP). The reaction was incubated at 30 ℃. Aliquot of this reaction mixture was taken every 1 h and analyzed by HPLC and MALDI-TOF-MS. Upon completion of the ligation reaction, the reaction mixture was reduced with TCEP (20 mg) for 60 min and was then filtered through 0.22 μm syringe

filter and then purified by semi-preparative HPLC. Target product containing fraction was then pooled, combined and lyophilized to give target **10'** as white puffy solid.

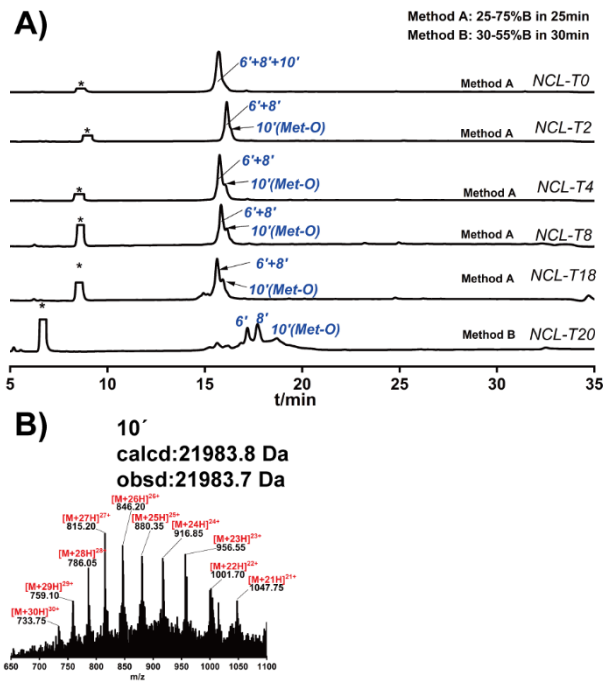

Figure S25. (A) Analytical HPLC traces (Method A: 25 to 75% MeCN (with 0.1% TFA) in 25 min, Method B: 25 to 50% MeCN (with 0.1% TFA) in 30 min, λ = 214 nm) of **10'.** (B) ESI-MS analysis of **10'(Met-O)** with the observed mass 21983.7 Da, calcd 21983.8 Da (average isotopes).