**Supplementary Information**

# Sortase A-Mediated Hydrazinolysis Generates Homogeneous N-Terminal GL Proteins for OaAEP1-Catalyzed Bioconjugation

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# 1. Supplementary Methods

## 1.1 Reagents and materials

All Fmoc-amino acids and ethyl cyanoglyoxylate-2-oxime (Oxyma) was purchased from GL Biochem. AM-Rink resin was purchased from Tianjin Nankai HECHENG S&T Co., Ltd. N,N'-Diisopropylcarbodiimide (DIC), trifluoroacetic acid (TFA) were purchased from energy-chemical. HClO4, anhydrous diethyl ether (Et2O), piperidine were purchased from Sinopharm Chemical Reagent Co., Ltd. N,N-dimethylformamide (DMF), dichloromethane (DCM), acetonitrile (HPLC grade) were purchased from Chengdu Kelong Chemical Co., Ltd. PKH26 were purchased from Bidepharm. A-431 and MCF-7 live cells were purchased from Servicebio. Glycerol, guanidine hydrochloride, urea, and imidazole were purchased from Sangon Biotech. PreCot Q 6FF, Welch Ultimate XB-C4 and XB-C18 were purchased from Welch Materials.

## 1.2 High-performance liquid chromatography (HPLC) and Mass spectrometry (MS)

The AM-Rink resin (0.05 mmol) was swelled in N,N-dimethylformamide (DMF, 4 mL) for 10 min. Subsequently, all Fmoc-protected amino acids (0.5 mmol) were added to the resin, and the mixture was shaken at 75 °C for 20 min to complete peptide chain assembly. Upon completion of peptide elongation, the resin was treated with a cleavage cocktail consisting of trifluoroacetic acid/triisopropylsilane/water (TFA/TIPS/$H_2O$, v/v/v, 90:5:5) for 3 h. The resulting solution was concentrated under nitrogen blowing, followed by precipitation with cold anhydrous diethyl ether ($Et_2O$). The precipitate was collected by centrifugation at 4000 rpm to afford the crude peptide. The crude product was further identified and purified by high-performance liquid chromatography-mass spectrometry (HPLC-MS).

### 1.3 General procedure for solid phase peptide synthesis

The AM-Rink resin (0.05 mmol) was swollen in DMF (4 mL) for 10 min. All amino acids (0.5 mmol) were added to the resin and shaken at 75 °C for 20 minutes. After peptide assembly, the resin was treated with a solution (TFA/TIPS/water = 90/5/5) for 3 h. The solution was blown with $N_2$ and subsequently precipitated with cold $Et_2O$. After that, it was centrifuged at 4000 rpm to obtain the crude peptide. The crude peptides were identified and purified by HPLC-MS.

### 1.4 Cell confocal imaging

A431 and MCF-7 cells were seeded onto glass-bottomed culture dishes and incubated overnight at 37 °C in a 5% $CO_2$.A431 and MCF-7 cells were stained with 2 μM red fluorescent dye PKH26 at 37 °C for 10 min, respectively. The stained cells were then incubated with 233 nM nanobody-GFP at 37 °C for 1 h, respectively. After incubation, the cells were washed three times with PBS supplemented with 0.05% glycerol, followed by fixation with pre-chilled methanol at −20 °C for 10 min. Images of the fixed cells were acquired using an LSM 880 confocal laser scanning microscope (Zeiss, Germany).

The essential imaging parameters were set as follows: excitation wavelengths at 488 nm and 543 nm; emission detection windows at 493–560 nm and 548–685 nm, respectively.

## 2. Peptide synthesis methods

### 2.1 Synthesis of model peptide.

model peptide **1a**: YALPETGLTAPEY

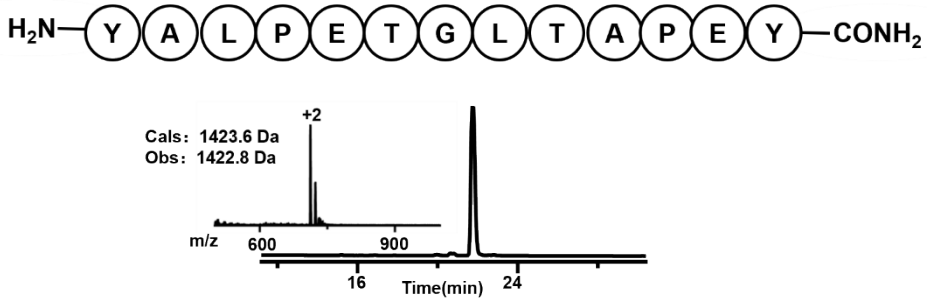


**Fig. S1**. Characterization of peptide **1a**: Structural diagram of peptide **1a**, RP-HPLC analysis of **1a** and ESI-MS spectrum of **1a.**

model peptide **1b**: YALLPETGLTAPEY

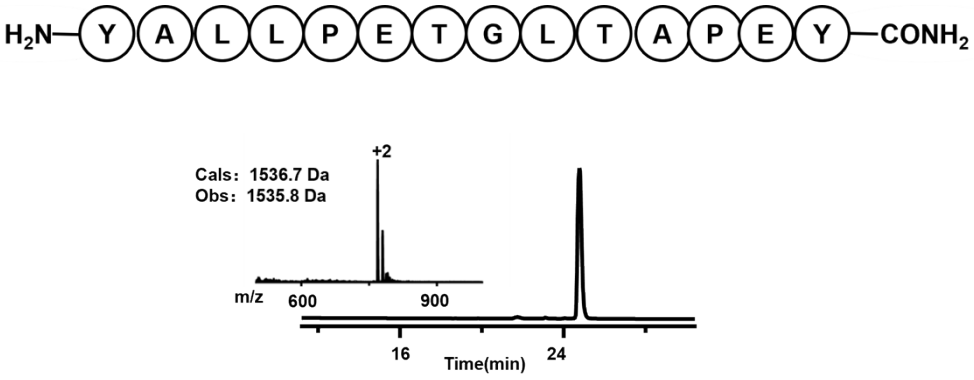


**Fig. S2**. Characterization of peptide **1b**: Structural diagram of peptide **1b**, RP-HPLC analysis of **1b** and ESI-MS spectrum of **1b.**

model peptide **1c**: YALLLPETGLTAPEY

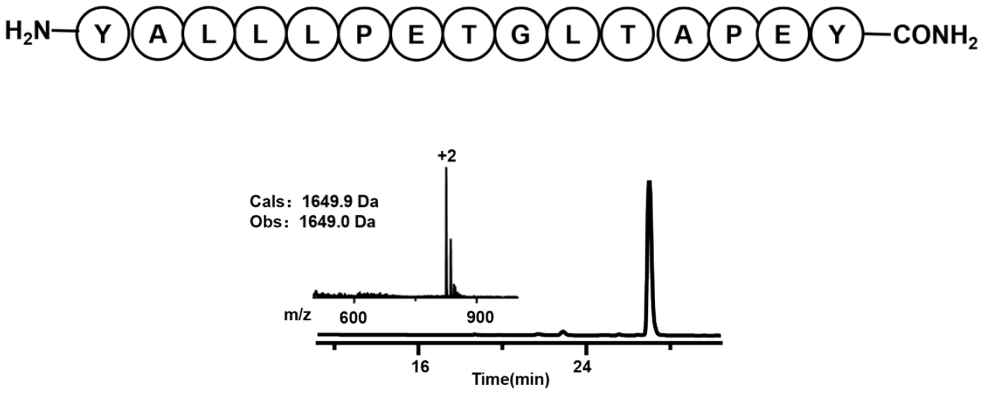

**Fig. S3**. Characterization of peptide **1c**: Structural diagram of peptide **1c**, RP-HPLC analysis of **1c** and ESI-MS spectrum of **1c.**

## 3. Protein expression and purification

### 3.1 Protein Sequence:

$His_6$-LLLPETGL-Ub: an $His_6$-GGS-LLLPETGL motif was conjugated to the N-terminus of Ub.

**Amino acid sequence:**

MHHHHHHGGSLLLPETGLMQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG

**Ub-NCL:** an NCL motif was conjugated to the C-terminus of Ub.

**Amino acid sequence:**

MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGGNCL

**$His_6$-LLLPETGL-GFP:** $His_6$-GGS-LLLPETGL-GGGSGGGS motif was conjugated to the N-terminus of GFP(D244A).

**Amino acid sequence:**

MHHHHHHGGSLLLPETGLGGGSGGGSVSKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKRHDFFKSAMPEGYVQERTISFKDDGTYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNFNSHNVYITADKQKNGIKANFKIRHNVEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSVLSKDPNEKRDHMVLLEFVTAAGITHGMAELYK

**nanobody-NCL-$His_6$**: an NCL-$His_6$ motif was conjugated to the C-terminus of nanobody.

**Amino acid sequence:**

MQVKLEESGGGSVQTGGSLRLTCAASGRTSRSYGMGWFRQAPGKEREF
VSGISWRGDSTGYADSVKGRFTISRDNAKNTVDLQMNSLKPEDTAIYYCAAA
AGSAWYGTLYEYDYWGQGTQVTVSSAAAEQKLISEEDLNGAANCLHHHHH
H

**Full sequence of Sortase A:**

MQAKPQIPKDKSKVAGYIEIPDADIKEPVYPGPATREQLNRGVSFAEENE
SLDDQNISIAGHTFIDRPNYQFTNLKAAKKGSMVYFKVGNETRKYKMTSIRN
VKPTAVEVLDEQKGKDKQLTLITCDDYNEETGVWETRKIFVATEVKLEHHH
HHH

**Full sequence of OaAEP1:**

MGMAHHHHHHMQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPD
QQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGGARDGDYLHLPSEVSRF
FRPQETNDDHGEDSVGTRWAVLIAGSKGYANYRHQAGVCHAYQILKRGGLK
DENIVVFMYDDIAYNESNPRPGVIINSPHGSDVYAGVPKDYTGEEVNAKNFL
AAILGNKSAITGGSGKVVDSGPNDHIFIYYTDHGAAGVIGMPSKPYLYADELN
DALKKKHASGTYKSLVFYLEACESGSMFEGILPEDLNIYALTSTNTTESSWAY
YCPAQENPPPPEYNVCLGDLFSVAWLEDSDVQNSWYETLNQQYHHVDKRIS
HASHATQYGNLKLGEEGLFVYMGSNPANDNYTSLDGNALTPSSIVVNQRDA
DLLHLWEKFRKAPEGSARKEEAQTQIFKAMSHRVHIDSSIKLIGKLLFGIEKCT
EILNAVRPAGQPLVDDWACLRSLVGTFETHCGSLSEYGMRHTRTIANICNAGI
SEEQMAEAASQACASIP

### 3.2 Sortase A expression and purification

Sortase A expression and purification were consistent with previously described.[1,2]

### 3.2 OaAEP1 expression, purification and activation

OaAEP1 expression, purification and activation were consistent with previously described.[3]

### 3.3 Ub-NCL, $His_6$-LLLPETGL-Ub, nanobody-NCL-$His_6$, $His_6$-LLLPETGL-GFP expression and purification

Ub-NCL (vector: pET28a) and $His_6$-LLLPETGL-Ub (vector: pET28a) were prepared using a method similar to that for Ub.[4] The Ub-NCL was purified by HPLC, and the $His_6$-LLLPETGL-Ub was purified by a Ni-NTA affinity column. The nanobody-NCL-$His_6$ (vector pET-22b(+)) was prepared using a method similar to that for nanobody-LPETG-$His_6$.[5] The nanobody-NCL-$His_6$ was refolded through stepwise dialysis into a PBS buffer containing 4 M, 2 M, and 0 M urea concentrations. Subsequently, the nanobody-NCL-$His_6$ separated by size exclusion column chromatography. $His_6$-LLLPETGL-GFP was prepared using a method similar to that for EGFP.[6]

## 4. Sortase A-mediated hydrazinolysis reaction and OaAEP1-mediated ligation.

### 4.1 Hydrazinolysis of model peptides

Enzyme-mediated hydrazinolysis reaction were conducted in HEPES buffer containing 1 mM $CaCl_2$ at pH 7.0 and 37 °C, for various time courses, (molar ratios: $YA(L)_nPETGLTAPEY/NH_2NH_2$ = 1/40, n=1/2/3). The reaction was terminated with 1‰ TFA, and the hydrazinolysis product was then monitored by analytical reversed-phase high-performance liquid chromatography (RP-HPLC) and characterized by ESI-MS.

### 4.2 Hydrazinolysis of protein

The reactions were performed in HEPES buffer containing 1 mM $CaCl_2$ at pH 7.0 and 37 °C, for various time courses. The molar ratios of $His_6$-LLLPETGL-protein/$NH_2NH_2$ substrate were 1/40. The reactions were quenched using 6 M Guanidine·HCl (pH 3.0), and the progress of the reaction was monitored by analytical

RP-HPLC, followed by characterization of the ligated products using ESI-MS.

### 4.3 Protein-protein ligation

The ligation reactions were performed in PBS buffer at pH 7.0 and 25 °C, for various time courses. The molar ratios of GL-Ub/Ub-NCL substrate, and GL-GFP/nanobody-NCL-$His_6$ substrate ware 1/1.5. The reactions were quenched using 6 M Guanidine·HCl (pH 3.0), and the completion of the reaction was monitored by analytical RP-HPLC, followed by characterization of the ligated products using ESI-MS. The ligation reaction between nanobody-NCL-$His_6$ and GL-GFP was analyzed by SDS-PAGE under reducing conditions.

### 4.4 Purification of hydrazinolysis and ligation proteins

Hydrazinolyzed di-Ub and Ub-GFP were isolated and purified via Ni-NTA affinity chromatography. The ligated di-Ub was characterized by HPLC. In contrast, nanobody-GFP was purified using size-exclusion chromatography (SEC) in PBS (pH 7.0) and stored at −80 °C.

## 5. Supplementary figures on hydrazinolysis reaction monitoring/analysis and product characteristics

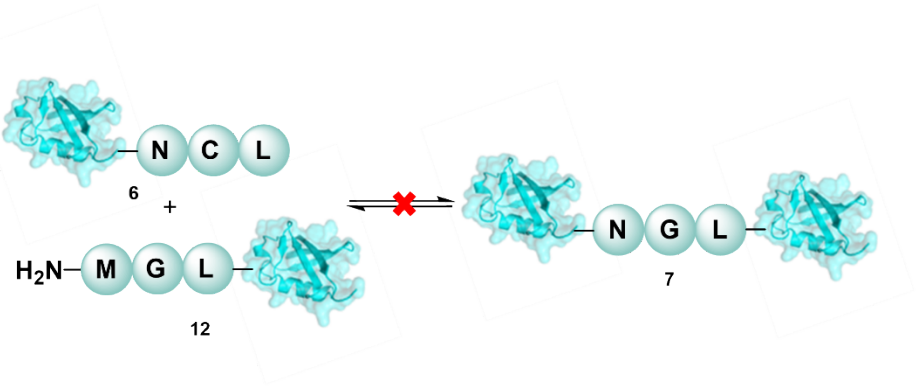


**Fig S4**. Synthetic route to **7**, the blocking of the N-terminal Met in **12** hinders its reaction with **6**.

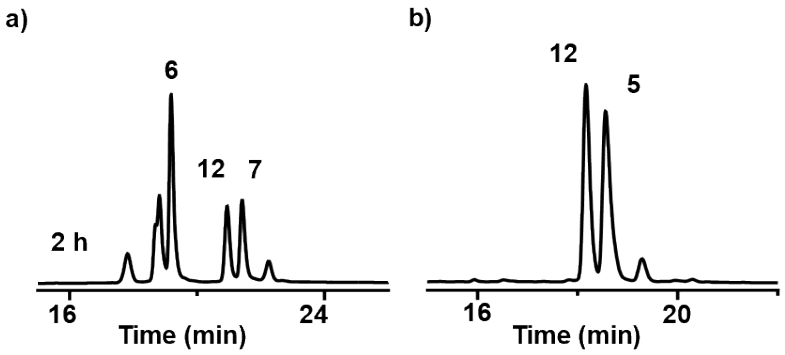


**Fig S5**. (a) HPLC analysis of reaction using a heterogeneous system of **5** and **12**. (b) HPLC analysis of a heterogeneous system of **5** and **12**.

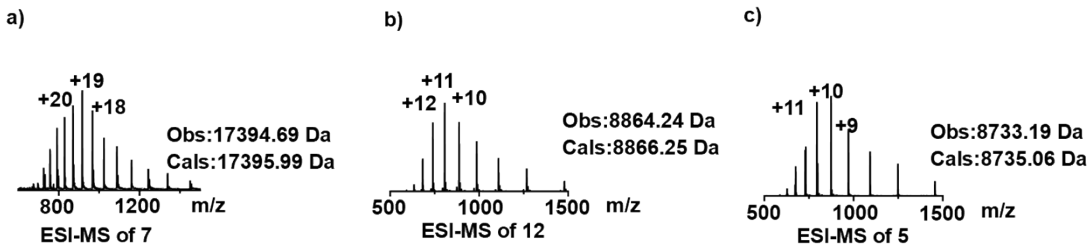


**Fig S6**. (a) ESI-MS spectrum of **7**. (b) ESI-MS spectrum of **12**. (c) ESI-MS spectrum of **5**.

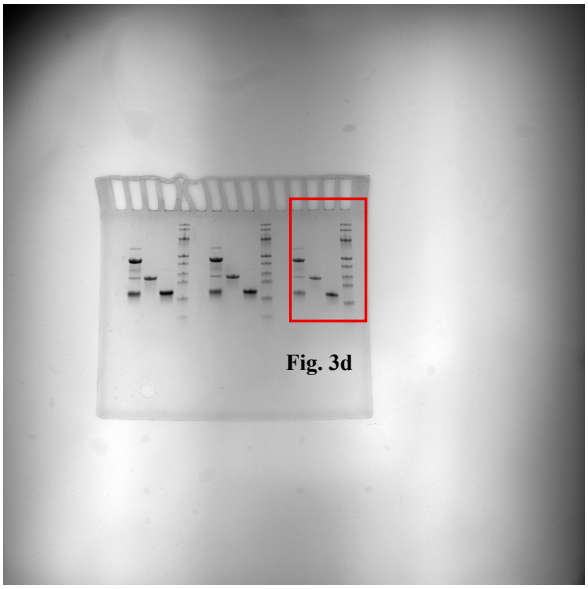


**Fig S7**. Unprocessed scans of SDS-PAGE gels with the cropped areas highlighted in boxes.

## References


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