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Production of unnaturally linked chimeric proteins using a combination of sortase - catalyzed transpeptidation and click chemistry.

Martin D. Witte^{1,3}, Chris Theile¹, Tongfei Wu⁴, Carla P. Guimaraes¹, Annet E. M. Blom¹, Hidde L. Ploegh^{1,2}

¹ Whitehead Institute for Biomedical Research, Cambridge, United States of America

² Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

³ Current address: Bio-Organic Chemistry, Stratingh Institute for Chemistry, University of Groningen, Groningen, The Netherlands

⁴ Oncology Medicinal Chemistry, Janssen Research and Development, Turnhoutseweg 30, B-2340 Beerse, Belgium

Correspondence should be addressed to:

Hidde L. Ploegh

Whitehead Institute for Biomedical Research

9 Cambridge Center

Cambridge, MA 02142

Phone: (617) 324-2031

Fax: (617) 452-3566

e-mail: ploegh@wi.mit.edu

ABSTRACT

Chimeric proteins, including bi-specific antibodies, are biological tools with therapeutic applications. Genetic fusion and ligation methods allow the creation of N-to-C and C-to-N fused recombinant proteins, but not the majority of non-template encoded fusions. The present protocol describes a simple procedure for the production of unnaturally linked N-to-N and C-to-C chimeric proteins. Equipping the N-terminus or C-terminus of the proteins of interest with a set of click handles using sortase A, followed by a click reaction, establishes unnatural N-to-N and C-to-C (hetero)dimer linked fusions. If the peptides, sortase A, and the proteins of interest are in hand, the unnaturally fused proteins can be obtained in 3-4 days.

INTRODUCTION

Chimeric proteins are useful research tools as well as interesting therapeutic possibilities. Fusing a protein of interest with a fluorescent protein, such as GFP, allows the study of its intracellular localization and trafficking ¹. Fusions of toxins with cytokines or antibodies have been used to yield effective cancer treatments ².

Genetic fusion of protein-encoding genes of interest is the most common method for the production of chimeric proteins. While the genetic engineering required to produce such fusions is straightforward, the resulting product may not express or fold properly, causing it to lose its function. Sortase A-catalyzed transacylation reactions ³⁻⁵ can prevent such pitfalls and covalently link two already correctly folded proteins. In this strategy, the N-terminus of one of the proteins of interest is equipped with an oligoglycine sequence, while the C-terminus of the other protein is engineered with an LPXTG sortase-recognition motif. Sortase A from *Staphylococcus aureus* cleaves the LPXTG sequence between the threonine and the glycine residues to yield an acyl-enzyme intermediate. The protein with an N-terminal oligoglycine sequence can act as a nucleophile and resolve the acyl-enzyme intermediate to create the fusion protein. (For detailed information on sortase A, the reaction and references see the C-terminal sortase A labeling protocol [ref]). This strategy is site-specific and versatile and has been used to prepare a wide array of protein fusions ⁶⁻⁹. A disadvantage of sortase-mediated reactions and similar conjugation strategies, such as intein chemistry ¹⁰, split intein ¹¹, and native chemical ligation ¹² is that they exclusively afford N-to-C and C-to-N fused proteins. However, many proteins, including antibodies, require one of their termini to remain unmodified for retention of activity. Thus, a standard genetic fusion of two proteins of this type would diminish or abolish the activity of one of the proteins. A fusion of the same termini (i.e. N-to-N, or C-to-C) might then be preferable. In this protocol we describe a strategy that combines sortase A-catalyzed transacylation with a strain-promoted click reaction, to allow production of such unnaturally linked proteins (Fig 1)

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EXPERIMENTAL DESIGN

Production of unnaturally linked fusion proteins requires: proteins equipped with a sortase A recognition sequence, sortase A from *S.aureus* and probes equipped with click handles. The preparation of sortase A from *S.aureus* is described in “Site-specific C-terminal labeling of proteins using sortase-mediated reactions” [ref]. The design of proteins containing a sortase A recognition sequence is described in detail in “Site-specific C-terminal labeling of proteins using sortase-mediated reactions” and “Site-specific N-terminal labeling of proteins using sortase-mediated reactions” [ref].

Proteins that require their N-terminus for activity must be sortagged via their C-terminus with a click handle. Using standard molecular cloning techniques, the protein of interest is engineered with a C-terminal LPXTG sequence, followed by an optional His tag. The His tag is lost in the course of the sortase reaction and simplifies analysis of the sortase catalyzed transacylation of the click probe and purification of the modified protein. Both sortase A and input substrate can be His-tagged, and removed from the final reaction mixture by adsorption onto Ni-NTA agarose. For fusion proteins that require free C-termini for their function, the click handles are introduced at the N-terminus. For that purpose, one to five glycine residues are engineered at the protein’s N-terminus, using standard molecular cloning techniques.

The accessibility of the N-terminal oligoglycine sequence and the C-terminal LPXTG sequence has a dramatic influence on the reaction rate of the transpeptidation. Since this parameter is different for every protein, we recommend testing the substrates empirically, using fluorescently labeled or biotinylated peptides, prior to performing the reaction with the click handles. Design of a linker between the sortase A sequence (Gly_n and/or LPXTG) and the protein may be required to enhance the extent of conversion and reaction rate. In cases where the initiator methionine is not removed by methionyl-aminopeptidase in the course of protein expression, a thrombin cleavage site can be cloned directly upstream of the Gly_n nucleophile to ensure a clean and active N-terminus [ref]. The optimized protein substrates and conditions can be used with no further modification in transpeptidation reactions with click-handle containing peptides. After purification of the modified proteins, mixing of the azide-containing protein with the

cyclooctyne-containing protein in a suitable buffer is sufficient to initiate dimerization of the proteins.

SYNTHESIS OF THE CLICK-HANDLE CONTAINING PEPTIDES

To prepare N-to-N linked proteins, we synthesize peptides containing the LPXTGG sortase A recognition sequence at their C-terminus (X can be any residue but we prefer a polar residue, such as a glutamic acid) and an azido or a cyclooctyne (DIBAC)¹⁴ group at the N-terminus of the probe (Figure 2). While the azido group is stable under basic and acidic conditions and can be introduced in the course of synthesis on a solid support, the DIBAC is labile under strong acidic and alkaline conditions¹⁴ and is best introduced after cleaving the peptide from the resin.

Peptides for creating C-to-C linked proteins are synthesized with an N-terminal triglycine motif and an azide or cyclooctyne at the C-terminus (Figure 2). Again, the azido group is introduced on the resin by coupling Fmoc-azidolysine-OH or by modifying a lysine side-chain with azidohexanoic acid. The DIBAC group must be introduced after cleaving the peptide off the resin, using cysteine maleimide chemistry to selectively link the cyclooctyne in the presence of a free N-terminus. A fluorescent dye, such as the TAMRA group in peptide **1**, can be incorporated for visualization purposes.

MATERIALS

REAGENTS

- *N,N*-Dimethylformamide (DMF; Applied Biosystems, cat. no. GEN002007) ! **Caution**
Flammable/toxic
- Acetonitrile (ACN; JT Baker Analytical, cat. no. 9017-03) ! **Caution** Flammable/toxic
- *N*-methyl-2-pyrrolidone (NMP; Sigma Aldrich, cat. no. 328634-2L) ! **Caution**
Flammable/irritant/toxic
- Diisopropylethylamine (DIPEA; Fisher BioReagent, cat. no. BP592500) ! **Caution**
Highly flammable/corrosive
- Dichloromethane (DCM; VWR, cat. no. JT9305-3) ! **Caution** Carcinogen

- Dimethylsulfoxide (DMSO; EMD Chemicals Inc, cat. no. MX1458-6) ! **Caution** Irritant/flammable
- Piperidine (Sigma Aldrich, cat. no. 104094) ! **Caution** Flammable/corrosive
- Pyridine (Sigma Aldrich, cat. no. 270407) ! **Caution** Highly flammable/toxic
- Diethyl ether (EMD Chemicals Inc, cat. no. EX0185-8)! **Caution** Highly flammable/harmful
- Trifluoroacetic acid (TFA; Sigma Aldrich, cat. no. T6508) ! **Caution** Strongly corrosive/toxic
- Triisopropylsilane (TIS; Sigma Aldrich, cat. no. 233781) ! **Caution** Flammable.
- 5(6)-carboxy-tetramethylrhodamine (5(6)-TAMRA; Novabiochem, cat. no, 815030)
- Fmoc-Lysine(Mtt)-OH (EMD biosciences, cat. no. 04-12-1137)
- Fmoc-Cys(Trt)-OH (Novabiochem, cat. no. 852008)
- Fmoc-Gly-OH (Novabiochem, cat. no. 852001)
- Fmoc-Thr(tBu)-OH (Novabiochem, cat. no. 852000)
- Fmoc-Glu(OtBu)-OH(Novabiochem, cat. no. 852009)
- Fmoc-Pro-OH (Novabiochem, cat. no. 852017)
- Fmo-Leu-OH (Novabiochem, cat. no. 852011)
- Fmoc- ϵ -caproic acid (Novabiochem, cat. no. 852053)
- Fmoc-Azidolysine-OH (Novabiochem, cat. no. 852326)
- Fmoc-Gly-Gly-Gly-OH (Chem-Impex international, cat. no. 08072)
- 6-Azido-hexanoic acid (Novabiochem, cat. no. 851097)
- DBCO-maleimide (DIBAC-maleimide) (Click Chemistry Tools, cat. no. A108)
- DIBAC-OSu as used in () has been discontinued. DBCO-PEG4-OSu may be used as a substitution (Click Chemistry Tools, cat. no. A134)
- 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU; Novabiochem, cat. no. 851006) ! **Caution** Irritant/harmful
- benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP; Novabiochem, cat. no. 851009) ! **Caution** Irritant/harmful
- Ninhydrin (Eastman, cat. no. 2495) ! **Caution** Harmful

- Potassium cyanide (Sigma-Aldrich, cat. no. 31252) ! **Caution** Highly toxic/hazardous to the environment
- Phenol (J.T. Baker, cat. no.2858-04) ! **Caution** Toxic/corrosive
- Rink amide resin SS, 100-200 mesh, 1% DVB (Advanced Chemtech, cat. no. SA5030)

REAGENT SETUP

- **Kaiser test solution A.** Dissolve 500 mg of ninhydrin in 10 mL ethanol.
- **Kaiser test solution B.** Dissolve 80 g of phenol in 20 mL of ethanol.
- **Kaiser test solution C.** Dissolve 1.3 mg potassium cyanide (20 μ mol) in 20 mL of water. Add 2 mL of the potassium cyanide solution to 100 mL of pyridine.
- **20% piperidine in NMP.** Mix 20 mL piperidine and 80 mL NMP.
- **Cleavage cocktail A** (95% TFA, 2.5% H₂O, and 2.5% TIS). Mix 4.75 mL TFA, 125 μ L H₂O and 125 μ L TIS (5 mL). **CRITICAL:** Prepare fresh.
- **Cleavage cocktail B** (93% TFA, 2.5% H₂O, 2.5% TIS, 2% ethanedithiol). Mix 4.65 mL TFA, 125 μ L H₂O, 125 μ L TIS, 100 μ L ethanedithiol (5 mL). **CRITICAL:** Prepare fresh.
- **Buffer A HPLC.** 0.1% TFA in H₂O.
- **Buffer B HPLC.** 0.1% TFA in ACN.
- **Buffer A LC/MS.** 0.1% formic acid in H₂O
- **Buffer B LC/MS.** 0.1% formic acid in ACN

EQUIPMENT

- 3 mL syringe equipped with filter frit (New England Peptide, AC0-003)
- Screw cap glass column with a fritted glass filter bottom (Peptides International, cat. no. SHG-20215-PI)
- Wrist Action shaker (St. John Associate Inc.)
- Swinging bucket tabletop/benchttop centrifuge (Beckman)
- HPLC system (e.g. Agilent 1100 series)
- Reverse phase C18 column (Waters Delta Pak 15 μ m, 100 Å, 7.8 x 300 mm)
- LC/MS

- NMR spectrometer
- Microfuge tubes
- Test tube racks
- Syringes
- Graduate cylinders
- Heating block
- Erlenmeyer flasks
- 50 mL polypropylene conical tubes (Corning)

Kaiser Test TIMING 5 min

Perform a Kaiser test to monitor the extent of coupling¹⁵.

1 Mix 2 μL of solution A, 2 μL of solution B and 4 μL of solution C in a microcentrifuge tube.

2 Add 5-10 beads of the dried resin (obtained after the final wash step after the coupling reaction) to the mixture.

3 Heat the tube to 95°C for 3 min. A dark blue color indicates incomplete coupling.

Note: This test works for primary amines and does not work for testing the attachment of an amino acid to a Pro residue. Alternative methods such as the acetaldehyde/*p*-chloroanil test or microcleavage can be used to monitor these reactions^{16,17}

Microcleavage Test TIMING 45 min

1 Prepare a 30 μL solution of 95% TFA and 5% H₂O.

2 Add 5-10 beads to the solution and cleave for 20 min at room temperature.

3 Take 5 μL of the supernatant and dilute in 30 μL of H₂O and analyze by mass spectrometry.

C-terminal probes

H₂N-GGGK(N₃)K(TAMRA)-CONH₂ (1)

Resin solvation TIMING 15 min

1 Place rink amide resin (167 mg, 100 μmol) to a capped fritted glass bottom column.

Note: A fritted syringe may be more suitable for smaller scaled synthesis.

2 Add DCM (6 mL) to the resin and solvate the resin for 15 min under continuous shaking in a wrist action shaker at room temperature. Remove the DCM by filtration.

Deprotection TIMING 17 min

3 Remove the N-terminal Fmoc-protecting group by shaking the resin with a 20% piperidine solution in NMP (6 mL) for 15 min at room temperature.

4 Remove the 20% piperidine solution by filtration and wash the resin with NMP (5 mL, 3×) and CH₂Cl₂ (5 mL, 3×).

Coupling TIMING 2-3 h until pause point, 3.5 h per coupling cycle

5 Prepare a solution of Fmoc-Lys(Mtt)-OH (187 mg, 300 μmol), HBTU (114 mg, 300 μmol) and DIPEA (104 μL, 600 μmol) in 6 mL of NMP, incubate this solution for 1 min and add it to the resin. Shake the resin for 2 h at room temperature.

6 Drain the shaker and wash the resin with NMP (5 mL, 3×) and DCM (5 mL, 3×).

7 Monitor the extent of coupling with the Kaiser test. Repeat the coupling reaction with half of the equivalents if the Kaiser test shows incomplete coupling (i.e. a blue color).
PAUSE POINT. Dried resin can be stored at 4 °C for months. CRITICAL. Store the peptide with the N-terminus protected with an Fmoc-group.

8 Repeat steps **3-7** with Fmoc-Azidolysine-OH (117 mg, 300 μmol) instead of Fmoc-Lys(Mtt)-OH.

9 Repeat steps **3-7** with Fmoc-Gly-Gly-Gly-OH (123 mg, 300 μmol) instead of Fmoc-Lys(Mtt)-OH.

10 Remove the 4-methyltrityl (Mtt) protecting group by treating the resin twice with 5 mL of 1% TFA, 1% TIS in CH₂Cl₂ for 30 min at room temperature. *Note:* The cleaved Mtt produces an orange color. Eventually the color will fade from reaction with the TIS. If the color remains after the second 30 min reaction, perform a third 30 min deprotection.

11 Wash the resin with DCM (5 mL, 3×), NMP (5 mL, 3×) and finally briefly with NMP (3 mL) containing DIPEA (87 μL, 500 μmol, 5 equiv.).

Note: This final wash step neutralizes the resin and improves the coupling efficiency in the next step. This wash step should be brief. Prolonged exposure to alkaline conditions may result in partial removal of the Fmoc-protecting group.

12 Couple 5(6)-TAMRA (128 mg, 300 μ mol) under the agency of PyBOP (157 mg, 300 μ mol, 3 equiv.) and DIPEA (104 μ L, 600 μ mol) in NMP (6 mL). Shake the reaction for 16 h at room temperature.

13 Repeat steps **6-7**.

14 Repeat steps **3-4**.

Cleavage of the peptide TIMING 3 h

15 Wash the resin twice with DCM (5 mL) and cleave the peptide off the resin by incubating the resin twice with 2.5 mL of cleavage cocktail A for 2 h at room temperature.

16 Add the cleavage solution (dropwise) into 90 mL of cold diethyl ether to precipitate the crude product. Rinse the beads with an additional 0.5 mL of cleavage solution and add dropwise into the ether solution.

17 Incubate the resulting suspension for 1 h at -20 °C.

18 Collect the crude product by centrifuging the suspension at 3,500g for 15 min at 4 °C.

19 Remove the supernatant and dry the pellet under reduced pressure.

PAUSE POINT. Store the product at -20 °C prior to purification.

Note: Analyze the crude product by LC/MS. If LC/MS shows the crude peptide is of sufficient purity, steps **20-22** may be omitted and the peptide may be used directly in sortase reactions.

HPLC purification:

20 Resuspend the peptide in water (2 mL). *Note:* Up to 50% (v/v) of *tert*-butanol may be added to increase solubility.

21 Purify the peptide by reverse phase HPLC on a Waters Delta Pak 15 μ m, 100 Å C18 column (7.8 \times 300 mm) using the following gradient: 25-34% B over 3 column volumes.

22 Lyophilize the purified peptides.

Critical steps. Monitor the extent of coupling with the Kaiser test. Verify the identity and purity by LC/MS analysis (linear gradient 5→45% B in 10 min) and NMR spectroscopy. PAUSE POINT. The purified lyophilized peptide can be stored at -20 °C indefinitely.

H₂N-GGGK(Azidohexanoic acid)-CONH₂ (2)

- 1** Solvate and load the rink amide resin (167 mg, 100 μmol) with Fmoc-Lys(Mtt)-OH (187 mg, 300 μmol) as described in steps **1-7** of peptide **1**.
- 2** Couple Fmoc-Gly-Gly-Gly-OH (123 mg, 300 μmol) using the conditions described in steps **3-7** of peptide **1**, except replace Fmoc-Lys(Mtt)-OH for Fmoc-Gly-Gly-Gly-OH.
- 3** Remove the Mtt protecting group as described in steps **10-11** of peptide **1**.
- 4** Couple 6-azido-hexanoic acid (62 mg, 400 μmol) under the agency of PyBOP (208 mg, 400 μmol) and DIPEA (140 μL, 800 μmol) in NMP (4 mL). Agitate the reaction for 2 h at room temperature.
- 5** Repeat steps **6-7** of peptide **1**, followed by steps **3-4** of peptide **1**.
- 6** Cleave the peptide from the resin as described in steps **15-19** of peptide **1**.
- 7** Purify H₂N-GGGK(azidohexanoyl)-CONH₂ as described in steps **20-22** of peptide **1**, but using the following HPLC gradient 15→24% B in 3 column volumes.

Critical step. Monitor the extent of coupling with the Kaiser test. Verify the identity and purity of **2** by LC/MS analysis (linear gradient 5→45% B in 10 min) and NMR spectroscopy.

PAUSE POINT. The lyophilized peptide can be stored at -20 °C indefinitely.

H₂N-GGGC(DIBAC)-CONH₂ (3)

- 1** Load rink amide resin (167 mg, 100 μmol) with Fmoc-Cys(Trt)-OH (175 mg, 300 μmol) as described in steps **1-7** of peptide **1**, but replacing Fmoc-Lys(Mtt)-OH for Fmoc-Cys(Trt)-OH.
- 2** Repeat steps **3-7** of peptide **1** using Fmoc-Gly-Gly-Gly-OH (123 mg, 300 μmol) instead of Fmoc-Lys(Mtt)-OH.
- 3** Cleave the peptide from the resin by incubating the resin twice with 2.5 mL of cleavage cocktail B for 2 h at room temperature.

CRITICAL STEP. Addition of ethanedithiol prevents formation of disulfide bridges.

4 Precipitate peptide **3** as described for peptide **1** in steps **16-19**.

5 Dissolve the pellet in 2 mL of methanol.

6 Repeat steps **16-19** using the methanolic solution rather than the cleavage solution.

CRITICAL STEP. Step **6** removes residual traces of ethanedithiol, which will interfere with the introduction of the DIBAC via a maleimide-thiol reaction.

7 Lyophilize the crude peptide in a pre-weighted microfuge tube, determine the yield based on resin loading and analyze the purity of the crude H₂N-GGGC-CONH₂ by LC/MS analysis.

8 Dissolve the crude peptide (38 mg, 83 μmol) in PBS (0.25 mL) and add DIBAC-maleimide (17 mg, 40 μmol in DMF (0.25 mL).

9 Incubate the reaction overnight and subsequently quench the reaction by adding 0.1% aqueous TFA (2 mL).

10 Purify and lyophilize the peptide as described in steps **21-22** using the following gradient: 20-35% B in 5 column volumes.

PAUSE POINT. Store the purified product at -20 °C.

Critical step. Monitor the extent of coupling with the Kaiser test. The coupling reaction is to be repeated in case of a positive Kaiser test. Verify the identity and purity of **3** by LC/MS analysis (linear gradient 5→45% B in 10 min) and NMR spectroscopy.

Note: Prolonged storage of the maleimide probes in aqueous solution can result in hydrolysis of the maleimide. The resulting ring-opened product is still an excellent probe for the sortase reaction, but may cause difficulty during ion-exchange purification of the sortagged proteins. It is therefore crucial that probes containing maleimide-coupled functional groups are stored as lyophilized powders.

Synthesis of 6-azido-hexanoyl LPETGG-CONH₂ (4)

1 Solvate and load rink amide resin (167 mg, 100 μmol) with Fmoc-Gly-OH (99 mg, 300 μmol) as described in steps **1-7** for peptide **1**.

2 Elongate the peptide by repeating steps **3-7** with Fmoc-Gly-OH (99 mg, 300 μmol), Fmoc-Thr(OtBu)-OH (119 mg, 300 μmol), Fmoc-Glu(OtBu)-OH (128 mg, 300 μmol), Fmoc-Pro-OH (101 mg, 300 μmol), Fmoc-Leu-OH (106 mg, 300 μmol), and 6-azido-hexanoic acid (47 mg, 300 μmol). *Note:* The Kaiser test does not work for the Leu

coupling since Pro is a secondary amine. To test this coupling, one can perform a micro cleavage. Mix five to ten resin beads with TFA (20 μ L) for 30 min at room temperature. Add 5 μ L of the cleavage mixture to 20 mL of H₂O and analyze by LC/MS. Note that the orthogonal protecting groups may not be fully removed during this abbreviated cleavage step.

3 Cleave the peptide from the resin using the conditions described in steps **15-19** for peptide **1**.

4 Purify and lyophilize the peptide as described in steps **20-22** for peptide **1**, but using the following gradient: 26-35% B in 3 column volumes.

Critical step. Monitor extent of coupling with the Kaiser test. Verify the identity and purity of **4** by LC/MS analysis (linear gradient 5 \rightarrow 45% B in 10 min) and NMR spectroscopy.

PAUSE POINT. The lyophilized peptide can be stored at -20 $^{\circ}$ C indefinitely.

Synthesis of DIBAC-LPETGG-CONH₂ (5)

1 Solvate and load rink amide resin (84 mg, 50 μ mol) with Fmoc-Gly-OH (48 mg, 150 μ mol) as described in steps **1-7** for peptide **1**.

2 Elongate the peptide by repeating steps **3-7** with Fmoc-Gly-OH (48 mg, 150 μ mol), Fmoc-Thr(OtBu)-OH (60 mg, 150 μ mol), Fmoc-Glu(OtBu)-OH (64 mg, 150 μ mol), Fmoc-Pro-OH (51 mg, 150 μ mol), Fmoc-Leu-OH (53 mg, 150 μ mol).

3 Remove the Fmoc-protecting group as described in steps **3-4** of peptide **1**.

4 Cleave the peptide from the resin as described in steps **15-19** of peptide **1**. **Critical step.** The peptide must be cleaved off the resin before introducing the DIBAC. The cleavage cocktail can cause a ring rearrangement reaction, which forms a product that does perform a strain-promoted click reaction.

5 Dissolve the crude product in DMF (0.5 mL). Add DIBAC-OSu (14 mg, 20 μ mol) and DIPEA (7 μ L, 40 μ mol) and incubate the reaction overnight at room temperature.

6 Dilute the solution in 2 mL of 0.1% aqueous TFA and purify and lyophilize as described in steps **21-22** of peptide **1** using the following gradient: 25-34% B in 3 column volumes.

Critical step. Monitor the peptide couplings with the Kaiser test. Verify the identity and purity of **3** by LC/MS analysis (linear gradient 5→45% B in 10 min) and NMR spectroscopy.

PAUSE POINT. The lyophilized peptide can be stored at -20 °C indefinitely.

Protocols for the preparation of sortase A from *S.aureus* and for the N- and C-terminal labeling of proteins are described in detail in other protocols [ref]. Below follows a description for the N- and C-terminal labeling of proteins with the click handles.

MATERIALS AND REAGENTS

MATERIALS

REAGENTS

- Purified target protein in buffer (no phosphate-based buffer)
- Purified sortase A in buffer (no phosphate-based buffer) prepared as described in “Site-specific C-terminal labeling of proteins using sortase-mediated reactions” [ref]
- Oligoglycine peptide **1-3** (if creating C-to-C fused proteins) or LPETGG peptide **4** and **5** (if creating N-to-N fused proteins) stock solution: 10 mM in DMSO or water (10× stock).
- Imidazole (Alfa Aesar, cat. no. A10221) ! **Caution** Corrosive
- 4× loading LDS-buffer (Invitrogen NP0008)
- Common reagents for SDS-PAGE analysis
- Brilliant blue R (Sigma-Aldrich, cat. no. B7920)
- Methanol, MeOH (EMD, cat. no. MX0488-1) ! **Caution** Toxic/highly flammable
- Acetic acid, AcOH (VWR, cat. no. BDH3094) ! **Caution** Corrosive
- Hydrochloric acid, HCl (EMD, cat. no. HX0603-4) ! **Caution** Strongly corrosive
- Ethanol (Pharmco-AAPER, cat. no. 111000190) ! **Caution** Highly flammable/toxic
- BCA-protein reagent assay kit (Pierce cat. no. 23227)

EQUIPMENT

- Micropipettes (5-1000 µL)

- 1.5 mL centrifuge tubes
- Centrifuge for 1.5 mL centrifuge tubes
- 37 °C water bath
- End-over-end shaker
- FPLC (Äkta system or similar; GE Healthcare)
- HiLoad 16/60 Superdex 75 prep grade size exclusion chromatography column
- Superdex™ 75 HR 10/30 size-exclusion column
- Amicon ultra concentrators (Millipore)
- 50 mL polypropylene conical tubes (Corning or similar)

REAGENT SETUP

- **Sortase buffer:** 500 mM Tris-HCl pH 7.5, 1.5 M NaCl, 100 mM CaCl₂ (not required if using a Ca²⁺-independent sortase) (10× stock).
- **S75 eluent.** 50 mM Tris, 150 mM NaCl, pH 7.4 in H₂O.

N-terminal sortagging.

1 Add Sortase A of *S.aureus* (150 µM final concentration), and probe **4** or **5** (0.5 mM final concentration) to GGG-protein in sortase buffer (1×).

2 Incubate the resulting mixture at 37 °C for 3 h.

3 Purify the product on a Pharmacia ÄKTA Purifier system equipped with HiLoad 16/60 Superdex™ 75 size exclusion column. Flow-rate 1 mL/min, fraction-size 1.3 mL.

Critical step. The sortase A product must be purified prior to dimerization. Excess of probe interferes with the dimerization reaction. Other purification methods can be used (dialysis, desalting columns) for purification, but size exclusion chromatography in our hands performs best from a speed, yield and purity perspective.

Note: The addition of 10% glycerol to the eluent may be beneficial for proteins that show a tendency to precipitate.

4 Collect the peak fractions as judged by the UV trace.

5 Concentrate the protein in Amicon ultra concentrators with the appropriate MW cutoff and quantify the product by SDS-PAGE and staining.

Critical step. Verify the identity and purity of the product by SDS-PAGE and by LC/MS (linear gradient 5→45% B in 10 min).

Pause point. The pure product can be stored at -20 °C for several months and at 4 °C for several weeks (protein dependent).

C-terminal sortagging.

1 Add sortase A of *S.aureus* (150 μM final concentration) and probe **1**, **2** or **3** (0.5 mM final concentration) to the protein-LPETGGHHHHHH (15 μM final concentration) in sortase buffer (1×).

2 Incubate the resulting mixture for 16 h at 37 °C.

3 Purify the protein as described in steps **3-5** of the N-terminal labeling.

Critical step. The sortase A reaction product must be purified prior to execution of the dimerization reaction. Excess probe interferes with the dimerization reaction. Other purification methods can be used (dialysis, desalting columns) for purification, but size exclusion chromatography in our hands performs best from a speed, yield and purity perspective.

Critical step. Verify the identity and purity of the product by SDS-PAGE gel electrophoresis and by LC/MS analysis (linear gradient 5→45% B in 10 min).

Pause point. The pure product can be stored at -20 °C for several months and at 4 °C for several weeks.

Procedure for the dimerization of click handle containing proteins.

1 Add 100 μL azido-containing protein (80 μM in 50 mM Tris pH 7.4, 150 mM NaCl) to a microfuge tube containing 100 μL cyclooctyne-containing protein (85 μM in 50 mM Tris pH 7.4, 150 mM NaCl). *Note:* Other buffers may be used as well.

2 Incubate the resulting mixture for 16 h at 25 °C.

3 Centrifuge at 14,000 rpm for 10 min to remove any precipitate formed.

4 Apply the supernatant to a Pharmacia ÄKTA Purifier system equipped with a SuperdexTM 75 HR 10/30 size-exclusion column. Flow-rate 0.4 mL/min, fraction-size 0.5 mL.

Note: The addition of 10% glycerol to the eluent may be beneficial for proteins that show a tendency to precipitate.

5 Collect the peak fractions.

6 Concentrate the fractions in Amicon ultra concentrators (Millipore).

Critical step. Analyze the identity and purity of the product by SDS-PAGE and by LC/MS (linear gradient 5→45% B in 10 min).

Pause point. The pure product can be stored at -20 °C for several months and at 4 °C for several weeks.

Troubleshooting

Problem	Possible reason	Solution
Incomplete conversion for C-terminal protein modification	Wrong buffer	The buffer pH should be around pH 7.5. If <i>S. aureus</i> Sortase A is used the buffer should contain 1 mM CaCl ₂ . Avoid using phosphate-based buffers when using <i>S. aureus</i> sortase.
	The ratio between the C-terminal protein-LPXTGG and GGG-label is not optimal.	Increase the amount of nucleophile. The conditions need to be determined empirically.
	Sortase is degraded	Prepare a fresh batch of Sortase
	Sortase is inactive	Test sortase on GFP containing a C-terminal LPETGG motif.
C-terminus is not adequately exposed		Extend the C-terminus of the protein by introducing a linker (Gly ₄ Ser) _n immediately upstream of the sortase motif.

Incomplete conversion for N-terminal protein modification

Wrong buffer

The pH of the reaction mixture should be ~ pH 7.5. If *S. aureus* Sortase A is used, the buffer should contain 1mM CaCl₂. Avoid using phosphate-based buffers when using *S. aureus* sortase.

The ratio between the N-terminal protein-LPETGG and GGG-label is not optimal.

Decrease the amount of protein or increase the amount of LPETGG probe. These conditions need to be determined empirically.

Sortase is not active

Test activity of sortase with oligoglycine GFP

N-terminus is not exposed

Increase the length of the Gly_n N-terminus

<p>Incomplete dimerization</p>	<p>Excess probe from the sortase A reaction remains present.</p> <p>Concentration of proteins is not sufficient.</p> <p>The ratio of azide and cyclooctyne is not correct.</p> <p>The cyclooctyne has been modified.</p>	<p>Purify the reaction mixture by size exclusion column chromatography or an alternative method to resolve protein from click handles.</p> <p>Check concentration of the starting proteins. Concentrations typically used for dimerization are around 10-80 μM.</p> <p>Check the concentrations and make sure azide and cyclooctyne containing proteins are mixed 1:1.</p> <p>Make sure that in the course of peptide synthesis, the cyclooctyne is introduced</p>
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		<p>in the final step to prevent ring rearrangements.</p> <p>Exclude the cyclooctyne from azides and/or thiols to prevent unwanted side reactions</p>
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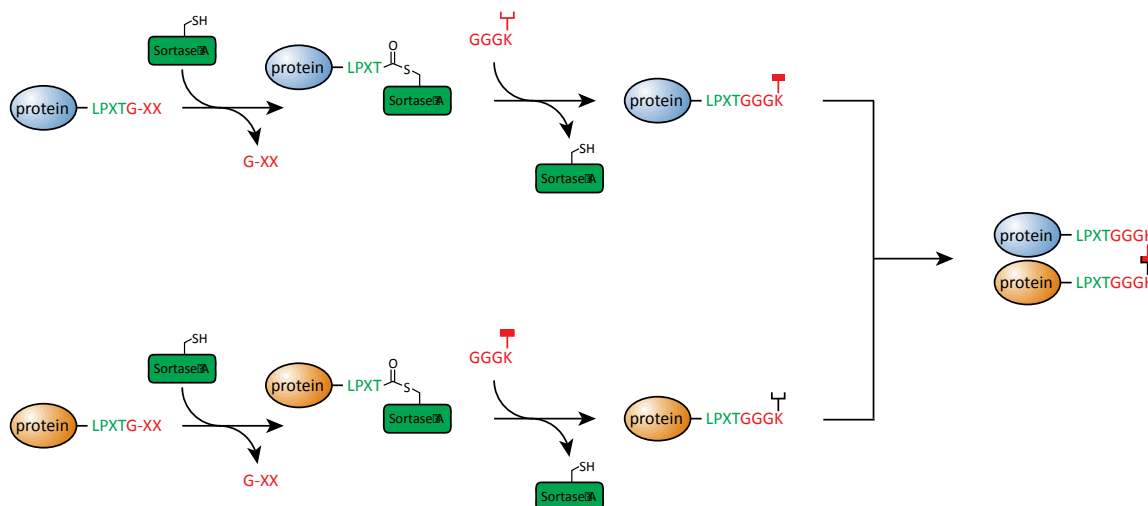


Figure 1. Schematic representation of the strategy to produce unnaturally linked chimeric proteins. Using sortase A the first protein is equipped with a click-handle (i.e. azide). The second protein is equipped with a complementary click-handle (i.e. cyclooctyne) in similar fashion. Combining the purified proteins produces the unnaturally linked chimeric protein.

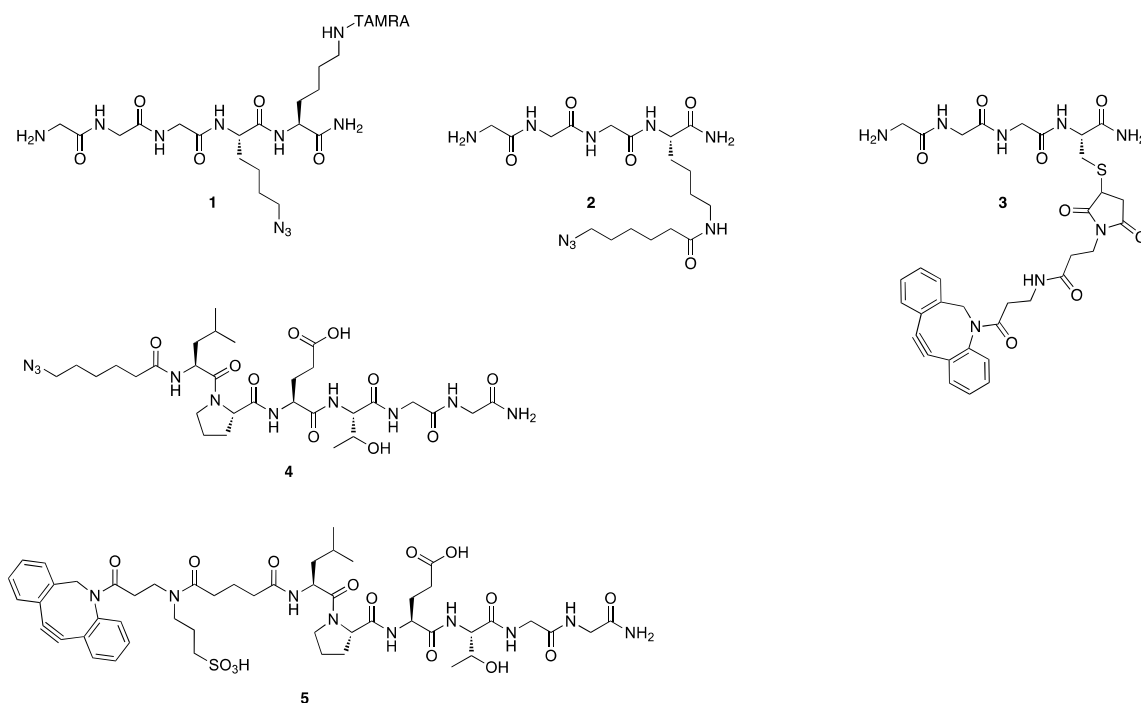


Figure 2. Structures of peptides 1-5

REFERENCES

1. Lippincott-Schwartz, J. & Patterson, G. H. Development and Use of Fluorescent Protein Markers in Living Cells. *Science* **300**, 87–91 (2003).
2. Manoukian, G. & Hagemester, F. Denileukin diftotox: a novel immunotoxin. *Expert Opin. Biol. Ther.* **9**, 1445–1451 (2009).
3. Popp, M. W. & Ploegh, H. L. Making and Breaking Peptide Bonds: Protein Engineering Using Sortase. *Angew. Chem. Int. Ed.* **50**, 5024–5032 (2011).
4. Tsukiji, S. & Nagamune, T. Sortase-Mediated Ligation: A Gift from Gram-Positive Bacteria to Protein Engineering. *ChemBioChem* **10**, 787–798 (2009).
5. Mao, H., Hart, S. A., Schink, A. & Pollok, B. A. Sortase-Mediated Protein Ligation: A New Method for Protein Engineering. *J Am Chem Soc* **126**, 2670–2671 (2004).
6. Guimaraes, C. P. *et al.* Identification of host cell factors required for intoxication through use of modified cholera toxin. *J Cell Biol* **195**, 751–764 (2011).
7. Hess, G. T. *et al.* M13 Bacteriophage Display Framework That Allows Sortase-Mediated Modification of Surface-Accessible Phage Proteins. *Bioconjug Chem* **23**, 1478–1487 (2012).
8. Popp, M. W., Antos, J. M. & Ploegh, H. L. *Current Protocols in Protein Science*. (John Wiley & Sons, Inc., 2009).doi:10.1002/0471140864.ps1503s56
9. Levary, D. A., Parthasarathy, R., Boder, E. T. & Ackerman, M. E. Protein-Protein Fusion Catalyzed by Sortase A. *PLoS ONE* **6**, e18342 (2011).
10. Chong, S. *et al.* Single-column purification of free recombinant proteins using a self-cleavable affinity tag derived from a protein splicing element. *Gene* **192**, 271–281 (1997).
11. Vila-Perelló, M. *et al.* Streamlined Expressed Protein Ligation Using Split Inteins. *J Am Chem Soc* **135**, 286–292 (2013).
12. Kent, S. B. H. Total chemical synthesis of proteins. *Chem. Soc. Rev.* **38**, 338 (2009).
13. Witte, M. D. *et al.* Preparation of unnatural N-to-N and C-to-C protein fusions. *P Natl Acad Sci Usa* **109**, 11993–11998 (2012).
14. Debets, M. F. *et al.* Aza-dibenzocyclooctynes for fast and efficient enzyme PEGylation via copper-free (3+2) cycloaddition. *Chem Commun* **46**, 97–99 (2010).
15. Kaiser, E. *et al.* Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* **34**, 595–598 (1970)
16. Vojkovsky, T. Detection of secondary amines on solid phase. *Pept. Res.* **8**, 236–237 (1995).
17. Gisin, B. F. The monitoring of reactions in solid-phase peptide synthesis with picric acid. *Anal. Chim. Acta* **58**, 248–249 (1972).