

Sortase-Mediated Protein Ligation: A New Method for Protein Engineering

Ansata Therapeutics, Inc. 505 Coast Boulevard South, La Jolla, CA 92037

Sortase—Primers (5'-GATATACATATGCAAGCTAAACCTCAAATTCGG-3' and 5'-GTGGTGCTCGATTGACTTCTGTAGCTACAAAGAT-3') were used to PCR amplify the *srtA* sequence corresponding to amino acids 60 to 204 from the genomic DNA of *Staphylococcus aureus*. The DNA fragment was digested with Nde I and Xho I and subsequently ligated into pET23b (Novagen) vector for expressing a sortase containing a C-terminal 6-His tag. The plasmid was transformed into *Escherichia coli* (*E. coli*) BL21(DE3) for protein expression. The cell culture was grown in LB medium containing 50 mg/L ampicillin at 37°C. The protein expression was induced with 0.2 mM isopropyl β-D-thiogalactoside (IPTG) at 30°C for 3 hours. The cell paste was then suspended in 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 1 mM β-mercaptoethanol and lysed by sonication. Nucleic acids were precipitated by the addition of 0.1% polyethylene imine (PEI). The supernatant of the lysate was applied to a 5-ml Ni-NTA (Qiagen) column. The column was washed with 10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 30 mM imidazole, and 1mM β-mercaptoethanol. The protein was eluted with 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 250 mM imidazole and 1mM β-mercaptoethanol (BME). Pooled fractions were buffer exchanged into 50 mM Tris-HCl, pH 7.5 and 150 mM NaCl through a 10DG desalting column (BioRad). The protein was subjected to MALDI-TOF mass spectroscopy (Bruker Daltonic Autoflex) analysis showing an average mass of 17,792 ± 5 (calculated 17,792).

GST-LPETG-6His—Oligos

AATTCCTTCCGGAAACCGGCCTCGAGCACCAACCACCACCACACTGATAGC-
3' and 5'
GGCCGCTATCAGTGGTGGTGGTGGTGGTGCTCGAGGCCGGTTTCCGGGAAGG-
3') were used to anneal and insert into pGEX-4T-1 vector between EcoRI and NotI sites encoding GST with a C-terminal with LPETGHHHHHH sequence. The plasmid was transformed into *E. coli* BL21 for protein expression. The cell culture was grown in LB medium containing 50 mg/L ampicillin at 37°C. The protein expression was induced with 0.2 mM IPTG at 30°C for 3 hours. The cell paste was then suspended in 35 ml of 1X phosphate buffered saline (PBS) buffer containing 1 mM TCEP [Tris(2-carboxyethyl}phosphine]. The lysate supernant was applied to a 5-ml GST-binding column (Novagen). The column was washed with 50ml PBS containing 1 mM TCEP. The protein was eluted with 10 mM reduced glutathione. Pooled fractions were buffer exchanged into 150 mM NaCl through a 10DG desalting column. The protein was subjected to MALDI-TOF mass spectroscopy analysis showing an average mass of 28,258 ± 20 (calculated 28,246).

GFP-LPETG-6His—Primers (5'-ATATACATATGGTGAGCAAGGGCG-3' and 5'-TGGTGCTCGAGACCGGTTCCGGAAGCTTGTACAGCTCGTCCATGC-3') were used to PCR amplify eGFP sequence from a pET24b-eGFP vector. The PCR product was digested and inserted between the Nde I and Xho I sites of pET23b vector to express eGFP with a C-terminal LPETGLEHHHHH sequence. The plasmid DNA was transformed into BL21(DE3) for protein expression. The protein expression and purification procedure is similar to that of sortase. The purified protein was desalted into 150 mM NaCl. The protein was subjected to MALDI-TOF mass spectroscopy analysis showing an average mass of $28,496 \pm 8$ (calculated 28,503).

Gly--emGFP—Primers (5'-GGCAGCCATATGATGGTGAGCAAGGGCGAG-3' and 5'-CGGATCCTCGAGTCACTTGTACAGCTCGTCCATGC-3') were used to PCR amplify emerald GFP (emGFP) from a pET24b-emGFP vector. The PCR product was digested and inserted between the Nde I and Xho I sites of pET15b vector to express emGFP with an N-terminal 6-His tag from the vector. The plasmid DNA was transformed into BL21(DE3) for protein expression. The protein expression and purification procedure is similar to that of sortase. MALDI-TOF analysis of the protein molecular weight (observed $28,926 \pm 6$; calculated 28,927) suggested that the N-terminal methionine of the purified protein has been removed by endogenous aminopeptidase in *E. coli*.

Peptide Synthesis

General. Fmoc-Gly-Gly-OH was purchased from Bachem (Torrance, CA). All other Fmoc-protected α -amino acids and Rink amide resin were purchased from EMD Biosciences/Novabiochem (San Diego, CA). N-Methylpyrrolidinone (NMP), dimethylformamide (DMF), diisopropylethylamine (DIEA), piperidine, trifluoroacetic acid (TFA), CH_3CN , and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from American Bioanalytical (Natick, MA). Acetic anhydride, CH_2Cl_2 , triisopropylsilane (TIPS), ethanedithiol, thioanisole, phenol, α -cyanohydroxycinnamic acid (CHCA), and folic acid dihydrate were purchased from Sigma-Aldrich (Milwaukee, WI). Et_2O was purchased from Pharmco Products (Brookfield, CT). Purification was performed on a Varian PrepStar HPLC (dual Model 218 pump modules and Model 320 UV-Vis detector) with a Thomson reversed-phase preparatory column (C_{18} , Advantage 300 Å, 5 μm , 20 mm x 250 mm, 15 mL/min flowrate). Final analysis was performed on a Waters analytical HPLC system (Waters 2695 separations module and Waters 2996 photodiode array detector) with a Vydac analytical HPLC column (C_{18} , 300 Å, 5 μm , 4.6 mm x 150 mm, 1 mL/min flowrate) using UV detection at 214 nm. Separations were performed using linear gradients of solvent B (95% CH_3CN in water, with 0.05% TFA) in solvent A (5% CH_3CN in water, with 0.05% TFA). Mass spectra were acquired on a Bruker Autoflex MALDI-TOF mass spectrometer (Bruker Daltonics, Inc.) in CHCA matrix.

Unless noted otherwise, peptides were synthesized on a Rainin/PTI Symphony automated peptide synthesizer using standard synthesis cycles (double coupling, Ac_2O capping) with HBTU activation and standard Fmoc-based amino acid derivatives. Peptide cleavage was in a cocktail consisting of 90% TFA, and 2% each of water, TIPS, ethanedithiol, thioanisole, and phenol for 2 h. Crude peptides were purified to homogeneity and lyophilized to dryness. Peptide identity was confirmed by MALDI-TOF mass spectrometry.

AT-P-022 ($\text{H}_2\text{N-RRQRRTSKLMKRAhx}$)₂KYK(GG-NH₂)-NH₂—Peptide assembly was achieved on an ABI433a automated peptide synthesizer using customized versions of the ABI Fastmoc 0.1 mmol coupling cycles to facilitate extended Fmoc deprotection and coupling times. In addition to standard washes with NMP, repeated cycles included the following steps: Fmoc deprotection was achieved with four successive six min washes of 20% piperidine in DMF, chain elongation was performed via two 20 min couplings in NMP with 20 equivalents (2 mmoles) of HBTU-activated Fmoc-amino acid relative to initial resin loading (Rink amide resin, 0.43 mmole/g, 232 mg, 100 μmoles), and unreacted peptide chains were then capped with a five min wash of 10% Ac_2O /10% DIEA in NMP. Standard Fmoc-amino acid derivatives were utilized in chain assembly with two exceptions: the C-terminal Lys and branch-point Lys were protected on the ϵ -amine with 4-methyltrityl (Mtt) and Fmoc, respectively. The C-terminal amine remained protected throughout chain elongation, while removal of the dual Fmoc protection of the

branch-point Lys facilitated dual chain elongation for the remaining N-terminal residues. Upon completion of chain assembly, the N-terminal Fmoc protecting groups remained on the resin and the resin was transferred to a glass peptide synthesis reaction vessel with fritted glass filters at the top and bottom (Ace Glass, Vineland, NJ). The resin was washed with CH₂Cl₂ and treated with a solution of 1% TFA/5% TIPS in CH₂Cl₂ to remove the Mtt group from the C-terminal Lys. Upon initial addition of the cocktail, the solution turned bright yellow. The solution was allowed to pass through the resin by gravity filtration through the fritted glass vessel at a rate of approximately 10 mL/min. Between 8-10 minutes, the solution exiting the reaction vessel turned light yellow, then clear, indicating complete removal of the Mtt group. The resin was washed with NMP and a solution containing Fmoc-Gly-Gly-OH (177 mg, 0.5 mmoles), HBTU (186 mg, 0.49 mmoles), and DIEA (217 μ L, 1.25 mmoles) in 10 mL NMP was added to the resin and agitated by N₂ flow for 40 min. The reaction vessel was drained, the resin was washed with NMP (5x 8 mL), and a solution of 20% piperidine in DMF (10 mL) was added. After 10 min, the reaction vessel was drained and the piperidine step was repeated. After final washes with NMP (5x 8 mL), CH₂Cl₂ (5x 8 mL), and MeOH (3x 8 mL), the resin was dried under a stream of N₂. A cocktail containing TFA (9.2 mL), ethanedithiol (0.2 mL), TIPS (0.2 mL), thioanisole (0.2 mL), water (0.2 mL), and phenol (100 mg) was added to the dry resin and the reaction vessel was placed on an orbital shaker for 2 h. The TFA cocktail containing crude, deprotected peptide was drained into a 50 mL polypropylene tube containing 35 mL Et₂O at -80° C to precipitate the crude peptide. The tube was centrifuged at 3000 rpm for 5 minutes and the supernatant was decanted away from the crude peptide pellet. Et₂O (-80° C) was added to the tube to a volume of 35 mL and the tube was vigorously shaken to distribute the crude peptide. The tube was again centrifuged and the supernatant decanted. A stream of N₂ was applied to the wet peptide pellet to remove excess Et₂O until cracks appeared on the surface. The crude peptide was dissolved in HPLC buffer A and purified via a linear 0-70% gradient of B in A over 30 min. Fractions identified by MALDI-TOF MS to contain the product were pooled, lyophilized, and repurified to yield the final product as a single peak by analytical HPLC. The material was lyophilized to dryness and used in experiments; final purified yield was not calculated.

Analysis: LRMS (MALDI-TOF) calculated M+H: 3973.84; found M+H: 3973.22. HPLC retention time: 12.15 min, single peak (0-70% B in A over 30 min, 1 mL/min).

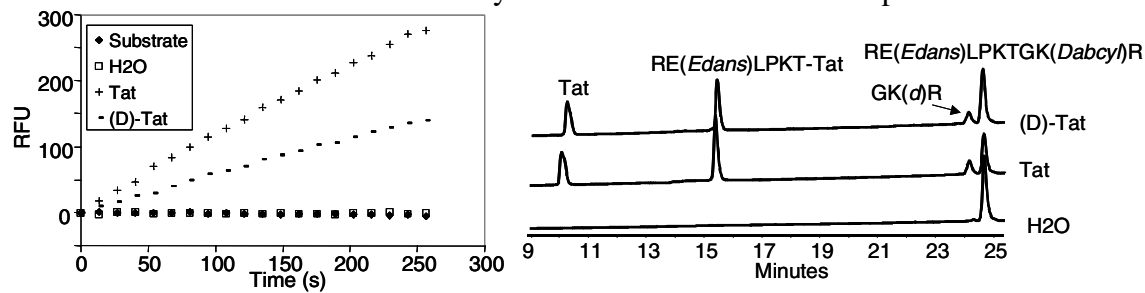
H₂N-G₃K(Folate)—Chain elongation was performed by standard Fmoc-based methods on Rink amide resin (0.43 mmole/g, 106 mg, 0.025 mmoles), using Fmoc-Lys(Mtt)-OH as the C-terminal amino acid. The N-terminal Fmoc group was left on the resin and the resin was transferred to a glass peptide synthesis reaction vessel with fritted glass filters at the top and bottom (Ace Glass, Vineland, NJ). The resin was washed with CH₂Cl₂ and treated with a solution of 1% TFA/5% TIPS in CH₂Cl₂. Upon initial addition of the cocktail, the solution turned bright yellow. The solution was allowed to pass through the resin by gravity filtration through the fritted glass vessel at a rate of approximately 10 mL/min. When the solution exiting the reaction vessel was no longer yellow (approximately 10 min), addition was halted and the resin was washed with NMP. A solution containing folic acid dihydrate (36 mg, 0.075 mmoles), HBTU (28 mg, 0.074 mmoles), and DIEA (33 μ L, 0.188 mmoles) in 5 mL NMP was added to the resin and agitated by N₂ flow. After 45 min, the reaction vessel was drained and the resin was washed with NMP (5x 5 mL). A solution of 20% piperidine (5 mL) was added and N₂ was used to agitate the reaction. After 10 min the reaction vessel was drained and the piperidine step was repeated. After final washes with NMP (5x 5 mL), CH₂Cl₂ (5x 5 mL), and MeOH (3x 5 mL), the resin was dried under a stream of N₂. Resin cleavage was carried out in 5 mL of a 95:3:2 solution of TFA:TIPS:H₂O for 2 h. Following two repeated cycles of precipitation-centrifugation (3000 rpm x 5 min) in cold Et₂O (35 mL),

the crude peptide pellet was dried with a stream of N₂ and dissolved in HPLC solvent A (5 mL). HPLC purification via a linear gradient of 0-70% B in A gave the final product as a mixture of isomers and was used without determination of yield.

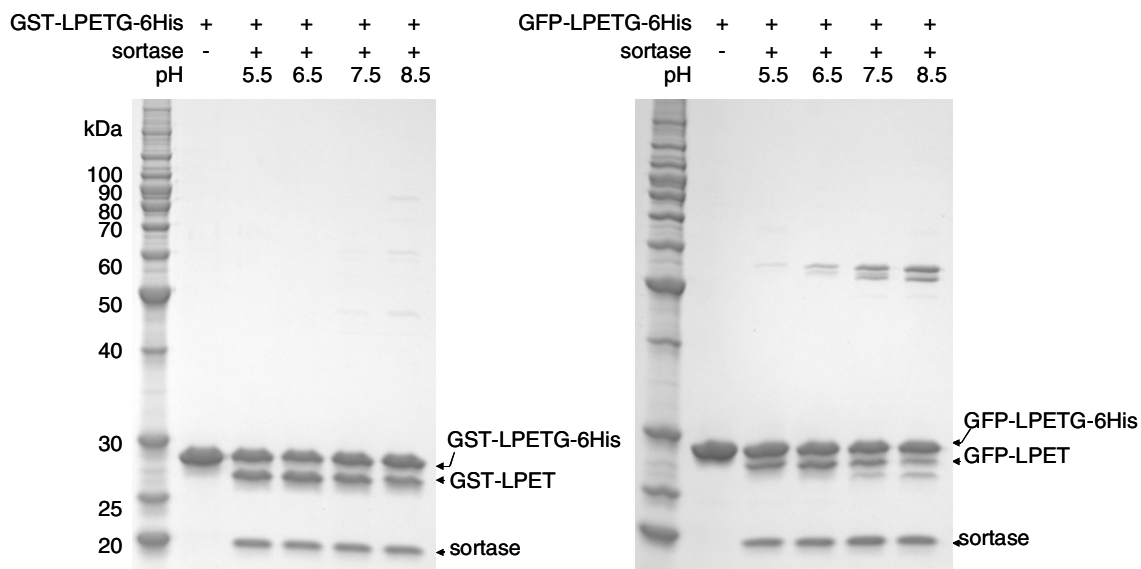
Analysis: LRMS (MALDI-TOF) calculated M+H: 740.74; found M+H: 741.15. HPLC retention time: 9.0-9.5 min, twin peaks (0-70% B in A over 30 min, 1 mL/min).

Ligation

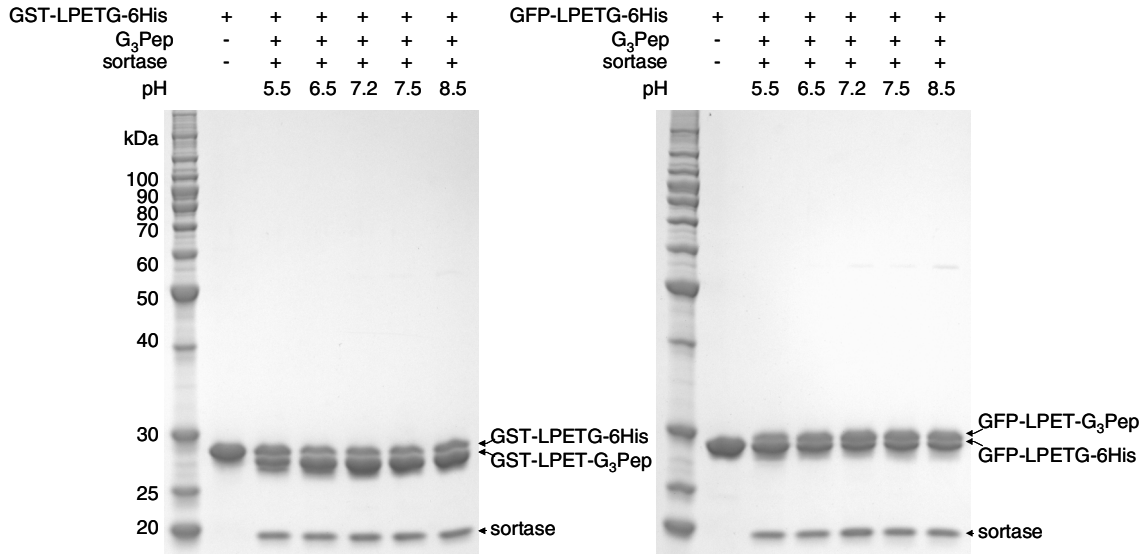
Peptide-peptide ligation—RE(*Edans*)LPKTGK(*Dabcyl*)R (*Edans*, 5'-[(2'-aminoethyl)amino]naphthalene-1-sulfonic acid); and *Dabcyl*, 4-[[4-(dimethylamino)phenyl]azo]benzoic acid) (synthesized by the Keck facility at Yale Univ) was dissolved in H₂O and added to a final concentration of 100 μM. The peptide was incubated at 37°C in the absence or presence of 100 μM various peptide G_nPep {G_nRRNRRTSKLMLR (n = 1, 2, 3, or 5); (L)- or (D)-Tat (GYGRKKRRQRRR)} and 10 μM SrtA in buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5mM CaCl₂, and 2mM BME. The rate of substrate cleavage was measured with the fluorescence increase of *Edans* at emission wavelength of 460 nm and excitation wavelength of 360 nm on a fluorometer (Applied Biosystems CYTOFLUOR Series 4000). The product formation was monitored by a C-18 reverse phase HPLC (Vydac, Cat 218TP54) over the course of 28 hrs, using a gradient of 0.5% to 38% CH₃CN in 0.1% trifluoroacetic acid in 40 minutes at a flow rate of 1 ml/min. Elution of peptides was monitored at 214 nm and fractions were collected for mass analysis on a MALDI-TOF mass spectrometer.



Protein hydrolysis—Approximately 70 μM GFP-LPETG-6His was incubated 10 μM sortase, 150 mM NaCl, 5mM CaCl₂, and 2mM BME. The buffer pH was controlled by 50 mM sodium acetate (pH 5.5), 50 mM MES (pH 6.5), 50 mM TrisHCl (pH 7.5) or 50 mM TrisHCl (pH 8.5). The reactions were incubated at 37°C for 20 hours and analyzed on a 12% Tris-Glycine SDS-PAGE gel.



Protein-peptide ligation—Approximately 70 μ M GFP-LPETG-6His was incubated with 350 μ M G₃Pep (GGRRNRRTSKLMLR) in the presence of 10 μ M sortase, 150 mM NaCl, 5mM CaCl₂, and 2mM BME. The buffer pH was controlled by 50 mM sodium acetate (pH 5.5), 50 mM MES (pH 6.5), 50 mM TrisHCl (pH 7.5) or 50 mM TrisHCl (pH 8.5). The reactions were incubated at 37°C for 20 hours and analyzed on a 12% Tris-Glycine SDS-PAGE gel. It appears that in the presence of an N-terminal glycine-containing peptide at pH greater than 6.0, sortase catalyzes specific ligation reaction.



Approximately 70 μ M GFP-LPETG-6His was incubated with 350 μ M G_nPep (G_nRRNRRTSKLMLR, n = 1, 2, or 3), (D)-Tat, G₃K(Folate), G₂Y-PTD5 (GGYRRQRRTSKLMKR), or a branched peptide AT-P-022 in the presence of 20 μ M sortase, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM CaCl₂, and 2 mM BME. After 6 hours and 24 hours at 37°C, the ligation reactions were aliquoted out and analyzed on a 12% Tris-Glycine SDS-PAGE gel. The molecular weights of the ligation products were also confirmed by MALDI-TOF mass spectroscopy.

The protein ligation products GFP-LPET-[G₂Y-PTD5] and GFP-LPET-[AT-P-022] were isolated by passing each reaction mixture through a 0.5 ml Ni-NTA column equilibrated with 50 mM Tris-HCl pH 7.5 and 150 mM NaCl. The column flow-through containing the ligation product was further purified on a Superdex 75 gel filtration column (Amersham Pharmacia) equilibrated with phosphate buffer saline.

Protein-protein ligation—Approximately 35 μ M protein substrates (GFP-LPETG-6His or GST-LPETG-6His) was mixed with 70 μ M N-terminal glycine-containing protein (Gly--emGFP). The reactions were incubated at 37°C for 24 in the presence of 20 μ M sortase, 50 mM Tri-HCl, pH 7.5, 150 mM NaCl, 5 mM CaCl₂, and 2 mM BME. The results were analyzed on a 12% Tris-Glycine SDS-PAGE gel. The molecular weights of the ligation products were confirmed by MALDI-TOF mass spectroscopy.

Cell Culture.

NIH3T3 cells were seeded into twelve-well plates at a density that achieved approximately 80% confluence after an overnight incubation. The cells were incubated with approximately 1 μ M of protein in 500 μ l of DMEM (Dulbecco's modified Eagle's medium) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) or in 500ul of Opti-MEM (Invitrogen). After incubation for 2 hours at 37°C in a 5% CO₂ incubator, the cells were washed with PBS and treated with Trypsin (Invitrogen). The detached cells were pelleted and analyzed on a Becton&Dickinson flow cytometry (BD Bioscience).