Electronic Supplementary Material (ESI) for RSC Advances.


# Supporting Information

**Employing non-canonical amino acids towards the immobilization of a hyperthermophilic enzyme to increase protein stability**

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## Experimental

**General:** Solvents and reagents were obtained from either VWR, Sigma Aldrich, or Fischer Scientific and used without further purification. Plasmids for genetic incorporation of ncAAs were provided by the laboratory of Dr. Peter Schultz at The Scripps Research Institute. The SSo P1 Carboxylesterase plasmid was obtained from Dr. Robert Kelley at NCSU. All proteins were purified according to manufacturer's protocols using a Qiagen Ni-NTA Quik Spin Kit. SDS-PAGE gels were imaged on a BioRad Molecular Imager (Gel Doc XR+). All PCR reactions were performed a BioRad icycler, with the Qiagen Quik-Change II Mutagenesis kit. All assays were performed on a BioTek synergy HT microplate reader. Microwave irradiation was performed using a CEM Discover microwave reactor.

***p*-propargyloxyphenylalanine (*p*PrF**). Boc-tyrosine-OMe (114 mg, 2 eq, 0.4 mmol) was added to a flame-dried vial. Cesium carbonate (254 mg, 3 eq, 0.6 mmol) was added, followed by dry DMF (3 mL). This mixture was stirred at 100°C for 20 mins. Propargyl alcohol (20 µL, 1 eq, 0.2 mmol) was then added to the mixture, as well as a catalytic potassium iodide. The reaction was stirred overnight at 100°C, then cooled to room temperature and extracted with brine (10 mL x 3) and diethyl ether (10 mL x 3). The organic layers were combined, dried with magnesium sulfate, filtered, and excess solvent was removed in vacuo. Column chromatography (silica gel, 5:1 hexanes/ethyl acetate) was performed to yield the protected amino acid. The crude product was dissolved in 1,4-dioxane (2 mL) and 1 M lithium hydroxide (2 mL) was added and the reaction was stirred at room temperature for 2 hours. 1,4-dioxane was then removed in vacuo and the resulting water solution was acidified through the dropwise addition of 6 M HCl. The reaction was then extracted into ethyl acetate and the organic layer dried with magnesium sulfate and filtered. Excess solvent was removed *in vacuo* to yield a colorless oil. The oil was dissolved in dichloromethane (DCM, 1.5 mL). Trifluoroacetic acid (TFA, 0.5 mL) was added and the reaction was stirred at room temperature for 1 hour. Excess solvent was removed in vacuo to yield *p*PrF as a white crystal (22 mg, 0.06 mmol, 31.6% yield). 1H NMR (400 MHz, CDCl3): δ 7.02 (d, J = 12 Hz, 2 H), 6.82 (d, J = 12 Hz, 2 H), 4.95 (d, J = 8 Hz, 1 H), 4.53 (d, J = 8 Hz, 1 H), 4.03 (t, J = 4 Hz, 2 H), 3.71 (s, 3 H), 3.02 (m, J = 8 Hz, 1 H), 2.39 (t, J = 4 Hz, 2 H), 1.97 (m, J = 8 Hz, 2 H), 1.55 (s, 1 H), 1.41 (s, 9 H). 13C NMR (400 MHz, CDCl3): δ 172.4, 157.9, 130.3, 127.9, 114.5, 83.5, 79.9, 68.8, 66.0, 54.5, 52.2, 37.4, 28.3, 28.2, 21.1, 15.1.

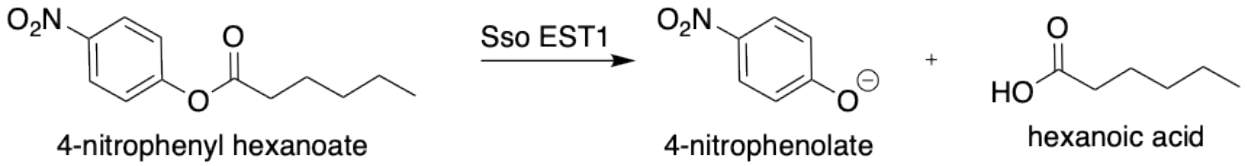
**Quick-Change PCR Protocol:** Four sites were selected for insertion of the TAG mutation: Y90, Y116, Y191, and Y214. A pQE-30 plasmid harboring SSo P1

carboxylesterase was diluted to 24.7 ng/μL using sterilized deionized water. The PCR reaction mixture was prepared by adding the diluted plasmid (5 μL) to 10 mM DNTPs (0.75 μL), KAPA Hi-Fi Polymerase (0.5 μL), forward primers (10 mM, 0.75 μL), reverse primer (10 mM, 0.75 μL), KAPA buffer (5 μL), and Milliq water (12.25 μL). A negative control PCR mixture was prepared without the addition of KAPA Hi-Fi polymerase. The reaction mixture was subjected to the following heating protocol: 95 °C (1 min.), eighteen cycles of melting (95 °C, 30 s), annealing (55 °C, 30 s), and extension (68 °C, 6 min), followed by an additional extension cycle (68 °C, 6 min) then an infinite hold at 4 °C. For both the reaction and control mixtures, the parent plasmid was digested and the mutant plasmid was ligated by the addition of DPN1 (20,000 units/μL, 2 μL), 5x T4 Ligase buffer (4 μL), and T4 Ligase (1 μL). The mixtures were then heated at 37 °C for 2 hours followed by one cycle of heat deactivation at 80 °C for 15 minutes. The reaction and control mixtures were cleaned and concentrated using a Zymo DNA Clean and Concentrator kit. The reaction and control mixtures were transformed (5 μL) into BL21 DE3 *Escherichia coli* cells by heat shock using an Eppendorf eporator electroporator. The transformed cells were plated and cultured (500 μL) onto LB agar containing ampicillin (50 μg/mL) and incubated at 37 °C overnight. Differential growth between the reaction and control plates was observed. One colony from the reaction plate was used to inoculate 10 mL of LB media containing ampicillin (50 μg/mL). This culture was incubated and allowed to shake at 37 °C overnight. The resulting cells were mini-prepped using an IBI High Speed Plasmid Mini Kit to isolate the DNA. The isolated plasmids were analyzed for successful insertion of the TAG mutation by sequencing at Genewiz. The primers were obtained from IDT DNA Technologies Inc. and are as follows: Y90TAG 5'-TGT AAT AGG CGA TGT GGA ATC TTA GGA CCC ATT ATG TAG AG-3' (forward) and 5'-CTC TAC ATA ATG GGT CCT AAG ATT CCA CAT CGC CTA TTA CA-3' (reverse), Y116TAG: 5'- CTA TAG GTT AGC TCC AGA ATA GAA GTT TCC TTC TGC AGT-3' (forward) and 5'-ACT GCA GAA GGA AAC TTC TAT TCT GGA GCT AAC CTA TAG-3' (reverse), Y191TAG 5'-CAA GAT CCA TGA TAG AGT CTG ATG GGT TCT TCC T- 3' (forward) and 5'-AGG AAG AAC CCA TCA GAC TAC TCT ATC ATG GAT CTT G-3' (reverse). The sequencing primer was 5'-TTC TGC TGA GCG GAT AAC-3'.

**Expression and Purification of GFP-151-*p*PrF.** Escherichia coli BL21(DE3) cells were co-transformed with a pET-GFP-TAG-151 plasmid (2.0 μL) and a pEvol-pCNF plasmid (2.0 μL) using an Eppendorf electroporator. Cells were then plated on LB-agar plates supplemented with ampicillin (50 mg/mL) and chloramphenicol (34 mg/mL) and grown at 37°C. After 16 hours, a single colony was used to inoculate LB media (10 mL) supplemented with ampicillin and chloramphenicol. The culture was grown to confluence at 37 °C over 16 hours. This culture was then used to begin an expression culture in LB media (250 mL) at OD600 = 0.1, then incubated at 37°C until it reached an OD600 of between 0.7 and 0.8. At this point, mutant protein expression was induced through the addition of 1 M ITPG (250 μL) and 20% arabinose (250 μL), as well as 100 mM pPrF (2.5 mL). Induced cells were grown for an additional16 hours at 30°C, then harvested via centrifugation (10 mins, 5000 rpm). The media was decanted, and the cell pellet was stored in a -80°C freezer for 20 minutes. Mutant GFP was then purified using commercially available Ni-NTA spin columns according to the manufacturer's protocol.

Protein yield and purity was then assessed via SDS-PAGE and spectrophotometrically via a Nanodrop spectrophotometer.

**Aqueous Esterase Activity Assay:**




4-nitrophenyl hexanoate — 4-nitrophenolate — hexanoic acid

**Preparation of Epoxy Sepharose 6B Resin:** Epoxy-activated 6B Sepharose (GE Healthcare, 1.0 g) was added to a double filter syringe and washed with deionized water (200 mL). The resin was dried via vacuum for 1 hour. Alkyn-ol (1 mL) and alcohol coupling buffer (5 mL, pH 13) were added to a vial. Dried resin was added to the vial and the vial was shaken at 30 °C at 200 rpm for 16 hours. The resin was transferred to a double filter syringe and washed 4 times with 5 mL of alcohol coupling buffer. The resin was dried for 1 hour via vacuum. The sepharose was transferred to a vial and capped with ethanolamine (2 M, 844 µL) and 7 mL of deionized water. The resin was shaken at 37 °C at 200 rpm overnight then washed in a filter syringe with acetate buffer (0.1 M, pH 4) and tris-HCl buffer (0.1 M, pH 8) using 3 alternating washes of 10 mL.

**Organic Solvent Based Assays.** For solution-based solvent assays, the total assay volume, amount of 4-Nitrophenyl hexanoate, and SSo EST1 protein/resin were preserved, but the 0.1M sodium acetate buffer and PBS pH 6 were replaced with THF as shown in the table below.

| Buffer: THF ratio | 0.1M acetate buffer µL) | PBS pH 6 (WT/control, µL) | THF (µL) |
|---|---|---|---|
| **100:0** | **76** | **46/26** | **0** |
| **90:10** | **69.2** | **41.7/21.7** | **12.2** |
| **75:25** | **57** | **34.5/14.5** | **30.5** |
| **0:100** | **0** | **0** | **122** |

**Microwave Irradiation Assay.** THF (500 µL) was added to SSo EST1 resin. The resulting solution was transferred to a microwave vial and 4-Nitrophenyl hexanoate (10 µL) was added. Using a CEM Discover microwave in Power mode, the vial was subjected to 300 W \ or 50 W for variable times with a safe temperature of 80ºC. After incubation, the vial contents were transferred to an eppendorf tube to which saturated sodium bicarbonate (100 µL) was added. The absorbance of each assay was measured by UV-

vis spectroscopy via a BioTek Synergy HT microplate reader equipped with a BioTek take3 plate.

## Supplementary Figures:

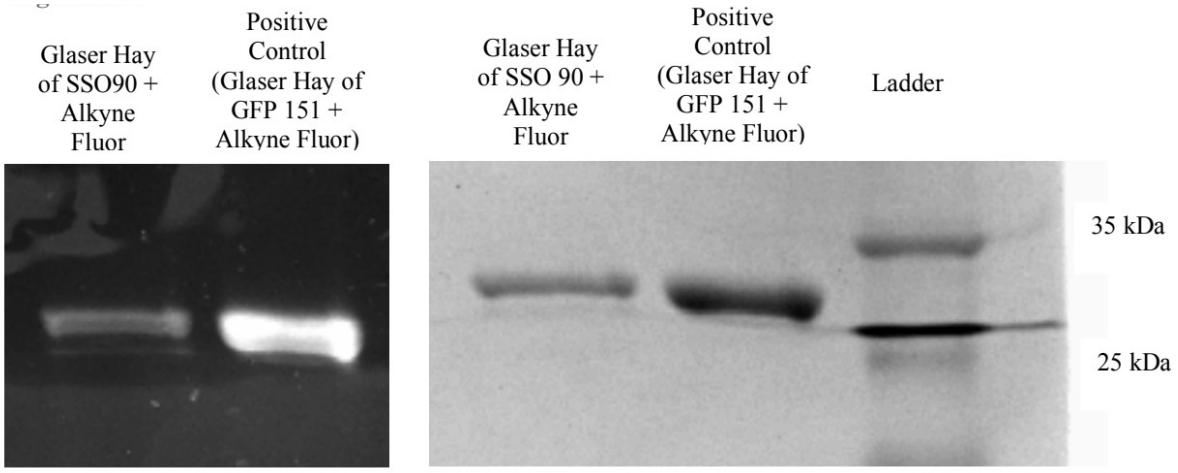



**Figure S1**. SDS PAGE confirmation of successful incorporation of *p*PrF ncAA. Fluorescence imaging (left) of Glaser-Hay coupling of a fluorophore (AlexaFluor-488 alkyne) to both GFP-151-*p*PrF and SSo EST1-90*p*PrF indicating a covalent attachment of the fluorophore. The same gel (right) after a coomassie stain indicating the presence of the two proteins. Glaser-Hay coupling would not be successful if the protein did not contain an alkynyl functionality for modification.

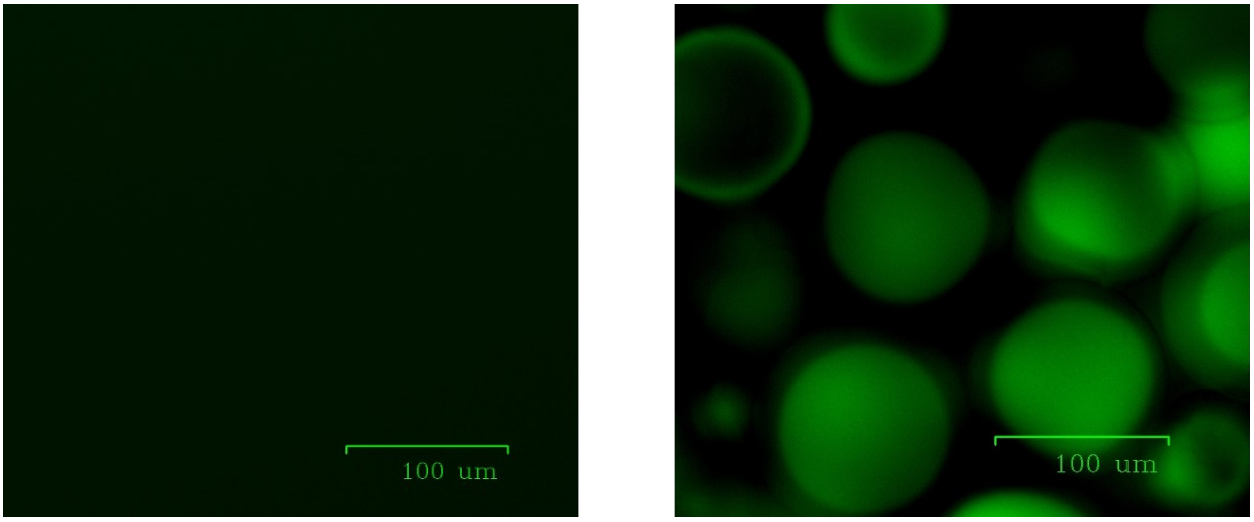



**Figure S2.** Confirmation of enzyme immobilization via Glaser-Hay coupling. GFP-WT (left) and GFP-151-*p*PrF (right) were reacted with propargyl alcohol modified Sepharose 6B resin under Glaser-Hay bioconjugation conditions. Following the washing of the resins they were imaged on a BioRad Zoe inverted fluorescence microscope. No fluorescence was observed with WT GFP due to the lack of an alkyne moiety for reaction. Successful

coupling was observed with GFP-151-*p*PrF due to the presence of the alkynyl ncAA. Fluorescence was quantified on a BioTek plate reader to ascertain relative concentrations of immobilized protein.

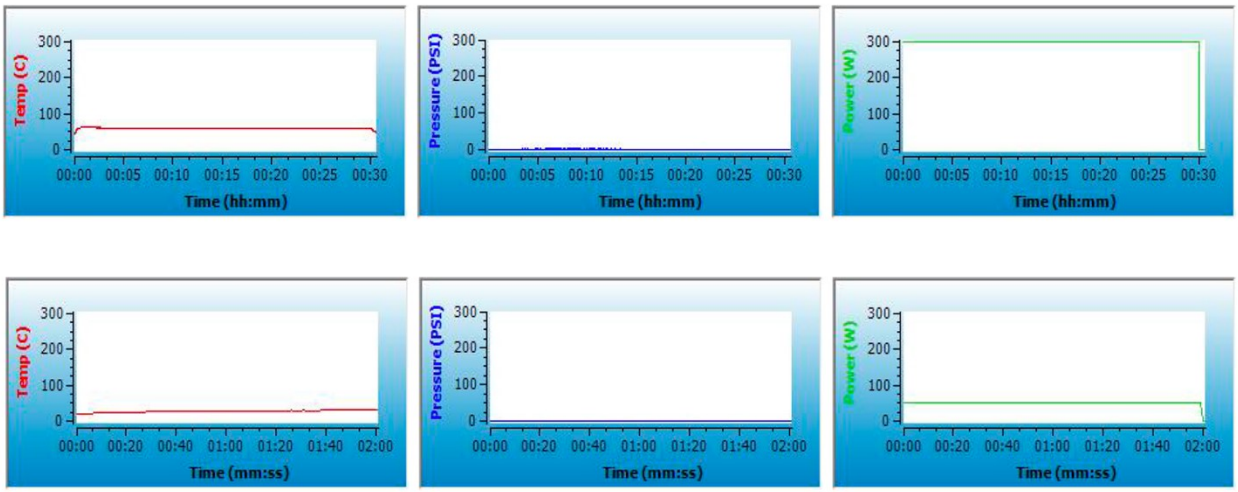



**Figure S3.** Microwave irradiation of resins. Sample outputs of microwave reaction profiles for the activation of immobilized SSo EST1. The 300 W/30 min reaction (top) reached approximately 56 °C and the 50W/2 min reaction (bottom) attained a maximum temperature of 38 °C.

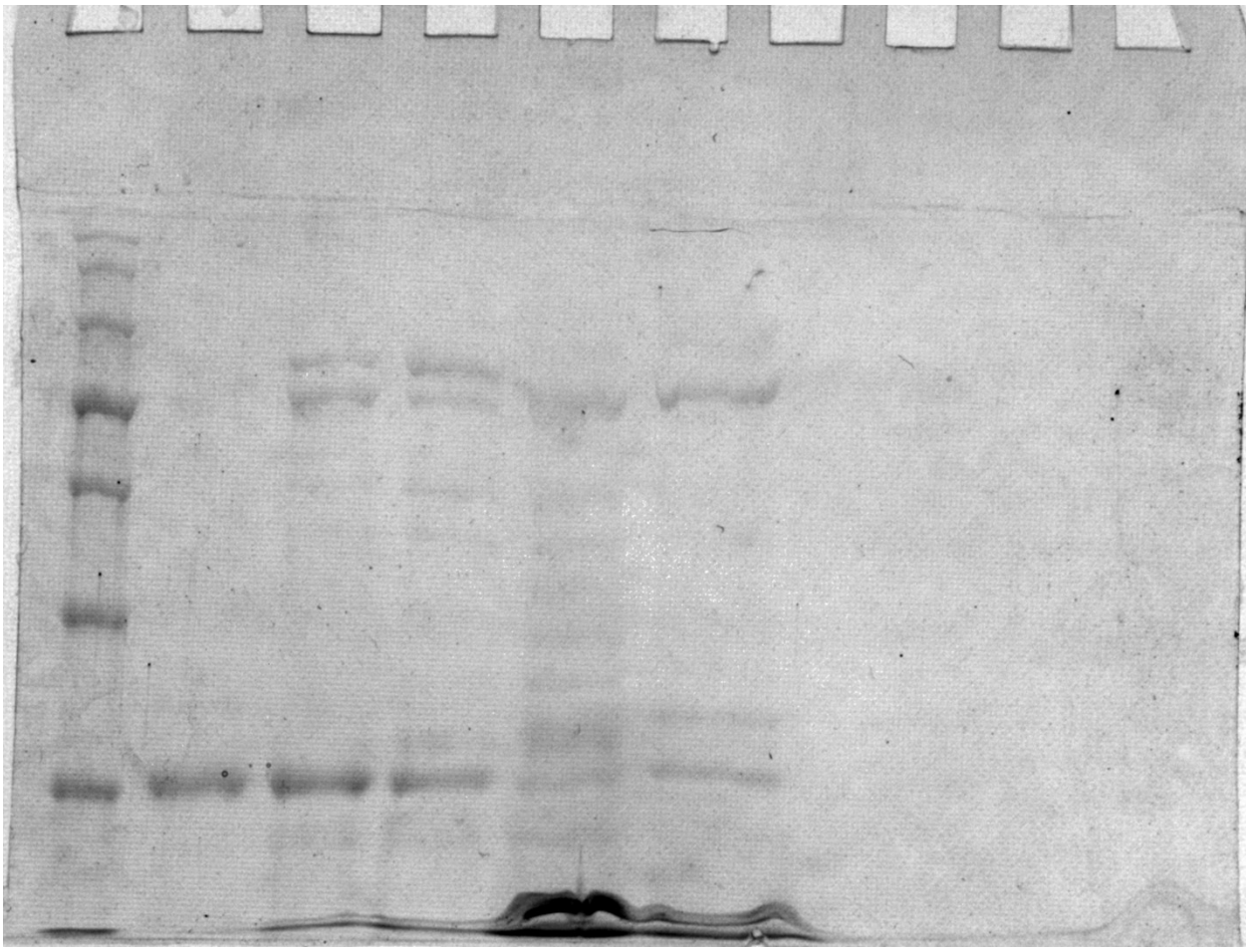


**Figure S4.** Full SDS-PAGE of SSo EST1 expressions.