General Information. Azf was purchased from Bachem (Torrance, CA, USA). 5(6)′-carboxytetramethylrhodamine was purchased from Novabiochem, EMD Millipore (Darmstadt, Germany). Dibenzylcyclooctyne-amine ($DBCO_A$) and dibenzylcyclooctyne-545 ($DBCO_{545}$) were purchased from Click Chemistry Tools (Scottsdale, AZ, USA). Methyl-sulfoxide-d_6 was purchased from Cambridge Isotopes Laboratories, Inc. (Andover, MA, USA). Ni-NTA resin was purchased from Qiagen (Valencia, CA, USA). *E. coli* BL21(DE3) cells were purchased from Stratagene (La Jolla, CA, USA). T4 DNA Ligase, XhoI and NdeI restriction enzymes were purchased from New England Biolabs (Ipswich, MA, USA). Max Efficiency® DH5α™ Competent Cells were purchased from Life Technologies (Grand Island, NY, USA). Sequencing-grade trypsin was purchased from Promega (Madison, WI, USA). Restriction-grade Factor Xa protease was purchased from Novagen (San Diego, CA, USA). QuikChange® site-directed mutagenesis kits were purchased from Stratagene. DNA oligomers were purchased from Integrated DNA Technologies, Inc (Coralville, IA, USA). Protease inhibitor cocktail was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were purchased from Fisher Scientific (Pittsburgh, PA, USA). Milli-Q filtered (18 MΩ) water was used for all solutions (Millipore; Billerica, MA, USA). The pAaT-WT (formerly pEG6) plasmid, containing His_{10}-tagged *E. coli* AaT, was a gift from Alexander Varshavsky (California Institute of Technology). *E. coli* TS351G cells were a gift from Emmanualle Graciet (Trinity College, Dublin). The pAzfRS (formerly $pDULE2_{Azf}$) plasmid was a gift from Ryan Mehl (Oregon State University). The pCDNA3.1-RS_{Azf} plasmid was a gift from Thomas Sakmar (The Rockefeller University).

Matrix-assisted laser desorption/ionization (MALDI) mass spectra were collected with a Bruker Ultraflex III MALDI-TOF/TOF mass spectrometer (Billerica, MA, USA). Electrospray ionization (ESI) mass spectra were collected with a Waters LCT Premier XE liquid chromatograph/mass spectrometer (Milford, MA, USA). UV/vis absorbance spectra were obtained with a Hewlett-Packard 8452A diode array spectrophotometer (Agilent Technologies, Santa Clara, CA). NMR spectra, ^{1}H and ^{13}C, were collected with a Bruker DRX 500 MHz instrument. DMSO-d_6 was calibrated at δ 2.51 for ^{1}H and δ 39.5 for ^{13}C. Gel images were obtained with a Typhoon FLA 7000 (GE Lifesciences; Princeton, NJ, USA). HPLC assays were performed on an Agilent 1100 HPLC using a Waters Symmetry Shield C18 column.

Synthesis of Dibenzocyclooctyne Tetramethylrhodamine ($DBCO_{TMR}$, S2). To a solution of 5(6)′-carboxytetramethylrhodamine (36.2 μmol) in 2 mL dimethylformamide was added 1-(3-Dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (72 μmol), H-hydroxybenzotriazole (72 μmol), and triethylamine (144 μmol). The reaction was stirred on ice for 10 minutes and then dibenzocyclooctyne-amine **S1** (36.2 μmol) was added. The reaction was stirred and allowed to warm to room temperature overnight. Solvent was removed under vacuum with a liquid nitrogen trap. The reaction was purified using 100 mL silica gel in 1:9 methanol/dichloromethane with 1 % acetic acid. Product fractions were concentrated by rotary evaporation and dried on high-vac overnight, affording $DBCO_{TMR}$ (**S2**) as a dark red solid in 72.5 % yield. ^{1}H and ^{13}C NMR spectra for 6′ isomer (isolated by HPLC purification) shown in Figure S2. Protons identified in the ^{1}H NMR are annotated below (tetramethylrhodamine assignments based on Kvach *et al.*).[1] HRMS (ESI) *m/z* calcd for $C_{43}H_{37}N_4O_5$ $[M]^+$ 689.2764, found: 689.2761.

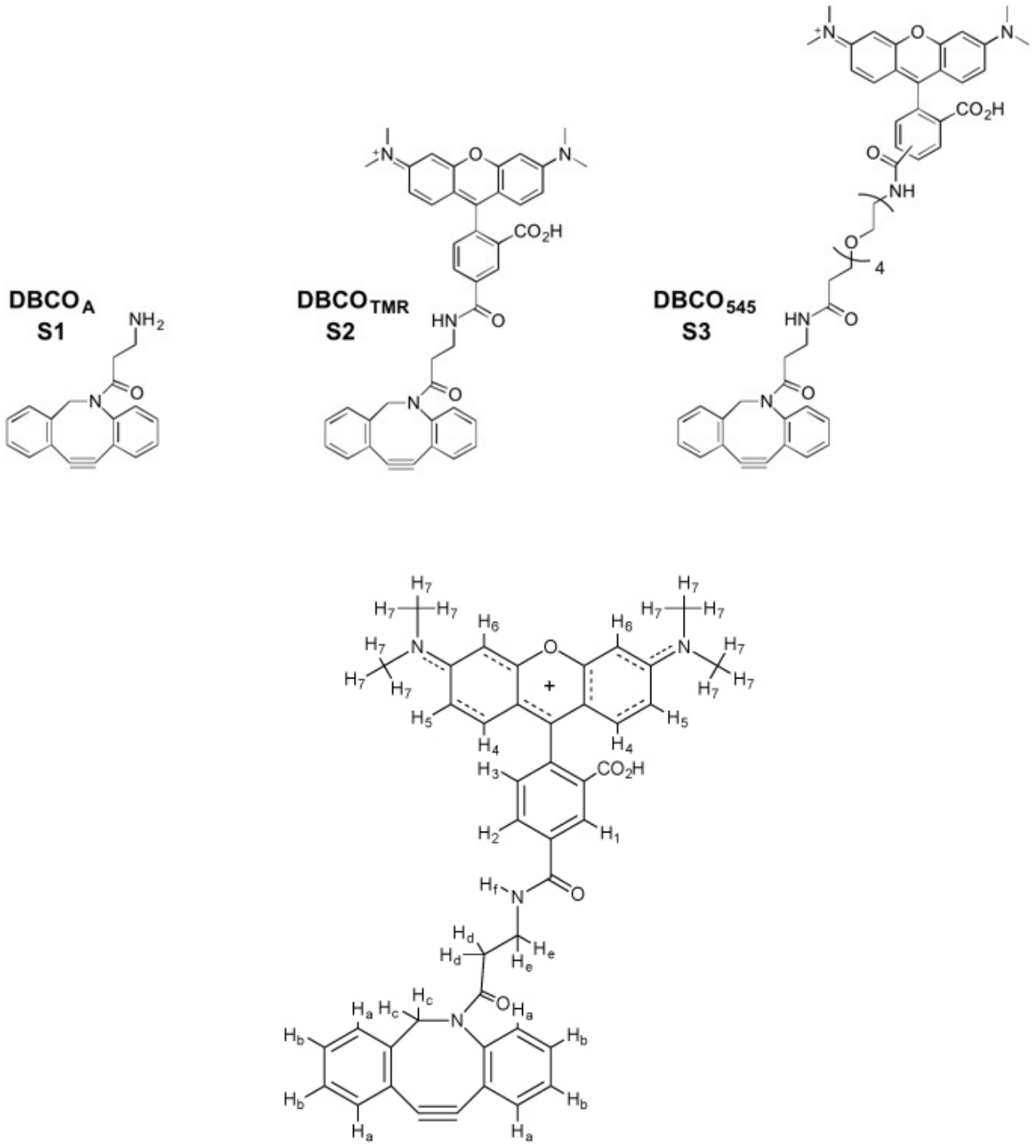

Figure S1. Top: Structures of dibenzocyclooctyne (DBCO) reagents used for inhibition. Bottom: Assignment of protons for ^{1}H NMR spectrum of $DBCO_{TMR}$.

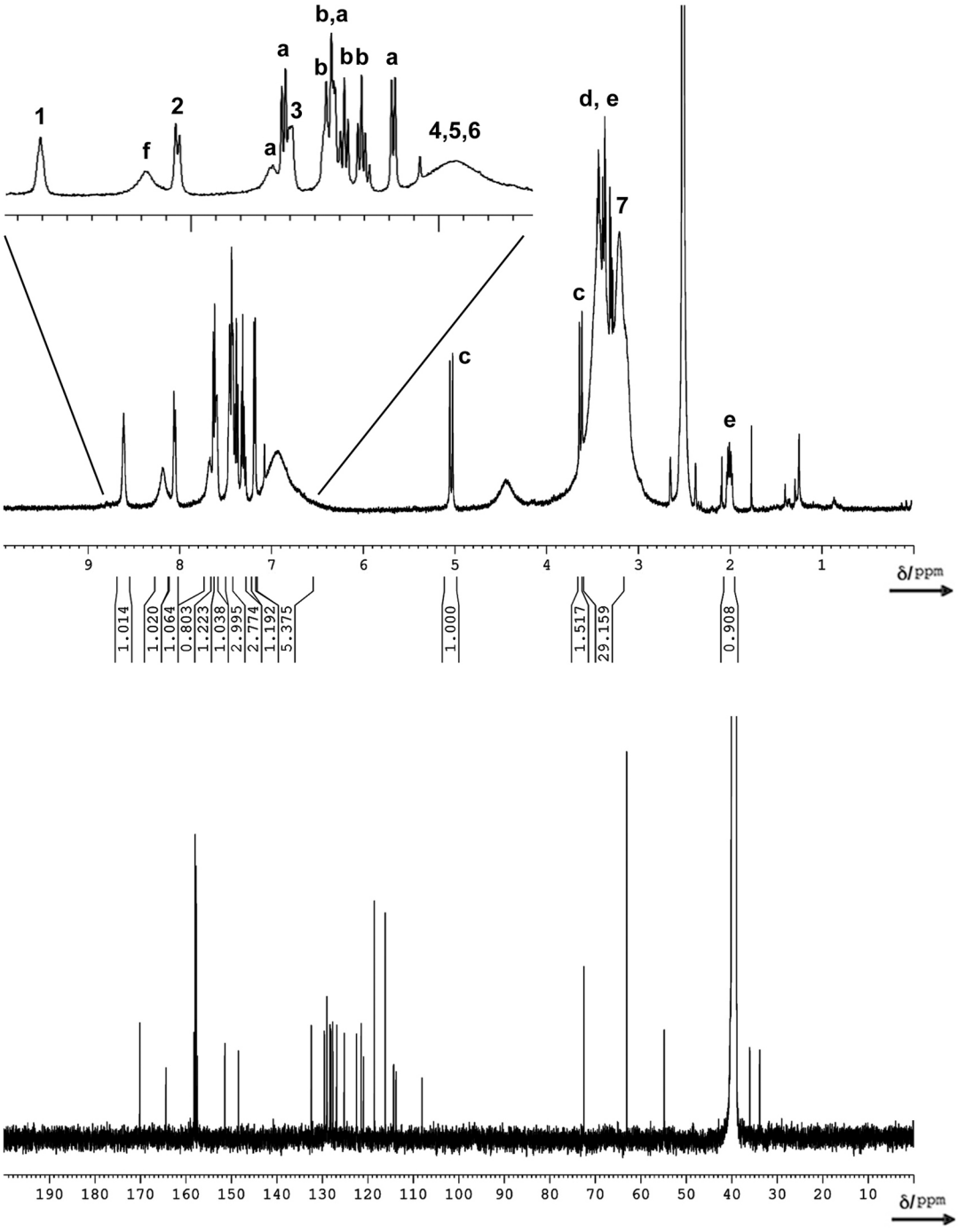

Figure S2. $DBCO_{TMR}$ ^{1}H and ^{13}C NMR in DMSO-d_6.

E. coli AaT Gene

5'ATGGGCCATCATCATCATCATCATCATCATCACAGCAGCGGCCATATCGAAGGTCGTCATATGCGCCTGGTT
CAGCTTTCTCGCCATTCAATAGCCTTCCCTTCCCCGGAAGGCGCATTACGTGAGCCTAACGGCCTGCTGGCACTTGG
GGGCGATCTTAGCCCTGCGCGCCTGTTAATGGCTTACCAGCGTGGTATTTTTCCGTGGTTTTCTCCAGGCGACCCCA
TCCTCTGGTGGTCGCCCGATCCCCGCGCGGTGCTATGGCCAGAATCACTGCATATCAGCCGTAGTATGAAGCGATTT
CATAAACGCTCGCCCTATCGTGTCACGATGAATTACGCTTTTGGTCAGGTCATTGAAGGCTGTGCCAGCGATCGCGA
AGAAGGAACCTGGATCACGCGTGGCGTGGTCGAAGCCTACCATCGCCTTCACGAACTCGGCCATGCCCACTCCATTG
AAGTCTGGCGTGAAGATGAGCTTGTCGGCGGTATGTACGGCGTGGCCCAGGGAACGCTATTTTGTGGCGAGTCCATG
TTCAGCCGGATGGAAAATGCGTCTAAAACGGCGCTTCTGGTATTCTGTGAGGAATTTATCGGTCATGGCGGTAAGCT
TATCGACTGCCAGGTCCTTAACGATCACACAGCATCGCTTGGTGCCTGCGAAATTCCCCGCCGGGATTACCTTAATT
ATCTCAATCAAATGCGCCTCGGACGATTGCCGAATAATTTCTGGGTACCACGATGCTTGTTTTCACCACAAGAATGA
—3'

E. coli AaT Amino Acid Sequence

MGHHHHHHHHHHSSGHIEGRHMRLVQLSRHSIAFPSPEGALREPNGLLALGGDLSPARLLMAYQRGIFPWFSPGDPI
LWWSPDPRAVLWPESLHISRSMKRFHKRSPYRVTMNYAFGQVIEGCASDREEGTWITRGVVEAYHRLHELGHAHSIE
VWREDELVGGMYGVAQGTLFCGESMFSRMENASKTALLVFCEEFIGHGGKLIDCQVLNDHTASLGACEIPRRDYLNY
LNQMRLGRLPNNFWVPRCLFSPQE

DNA Oligomers used for Quikchange® Mutagenesis

A. AaT-M1-TAG
Forward: 5'GGCCATATCGAAGGTCGTCATTAGCGCCTGGTTC-3'
Reverse: 5'GAACCAGGCGCTAATGACGACCTTCGATATGGCC-3'
B. AaT-Y42-TAG
Forward: 5′CGCCTGTTAATGGCTTAGCAGCGTGGTATTTTTCC-3′
Reverse: 5′GGAAAAATACCACGCTGCTAAGCCATTAACAGGCG-3′
C. AaT-F47-TAG
Forward: 5′CTTACCAGCGTGGTATTTAGCCGTGGTTTTCTCCAGGC-3′
Reverse: 5′GCCTGGAGAAAACCACGGCTAAATACCACGCTGGTAAG-3′
D. AaT-W59-TAG
Forward: 5′CCCATCCTCTGGTAGTCGCCCGATCCC-3′
Reverse: 5′GGGATCGGGCGACTACCAGAGGATGGG-3′
E. AaT-W68-TAG
Forward: 5′CCGCGCGGTGCTATAGCCAGAATCACTGC-3′
Reverse: 5′GCAGTGATTCTGGCTATAGCACCGCGCGG-3′
F. AaT-F81-TAG
Forward: 5′GCCGTAGTATGAAGCGATAGCATAAACGCTCGCCCTATC-3′
Reverse: 5′GATAGGGCGAGCGTTTATGCTATCGCTTCATACTACGGC-3′
G. AaT-W135-TAG
Forward: 5′CCACTCCATTGAAGTCTAGCGTGAAGATGAGCTTG-3′
Reverse: 5′CAAGCTCATCTTCACGCTAGACTTCAATGGAGTGG-3′

Figure S3. Construction of AaT Azf Mutants.

Trypsin Digest and MALDI MS Analysis of Unnatural Amino Acid Incorporation into Aminoacyl Transferase. Proteins were expressed from 100 mL LB cultures as described above. Elution fractions were checked by SDS-PAGE and combined. Protein was precipitated using 1:4 trichloroacetic acid/protein sample and incubated at - 20 °C for 15 minutes. Precipitate was centrifuged for 15 minutes at 13,200 rpm to pellet protein. The protein pellet was then washed three times with cold acetone to remove co-precipitated imidazole. Trace acetone was removed by incubating protein pellets in a 95 °C water bath for 5 minutes. Protein pellets were then resuspended in 6 M guanidinium hydrochloride with 50 mM Tris pH 8.0. Protein was further denatured by boiling at 95 °C for 10 minutes. Digestions were performed by diluting protein samples to 0.75 M guanidinium hydrochloride with 50 mM Tris pH 7.6 and 1 mM calcium chloride. Sequencing grade modified trypsin (0.6 μg, Promega) was used to digest samples for 24 hours at 37 °C. Trypsin digest aliquots (1 μL) were combined with α-cyano-4-hydroxycinnamic acid (1 μL of a saturated solution in 1:1 H_2O/CH_3CN with 1 % TFA) and analyzed by MALDI MS. Representative MALDI MS data for AaT-Z_{47} are shown in Figure S4, data for AaT-WT, AaT-Z_{47}, AaT-Z_{68}, and AaT-Z_{135} are collected in Table S1. We were not able to observe the $DBCO_{TMR}$ adduct for Z_{135}, but no residual Azf-containing peak was observed.

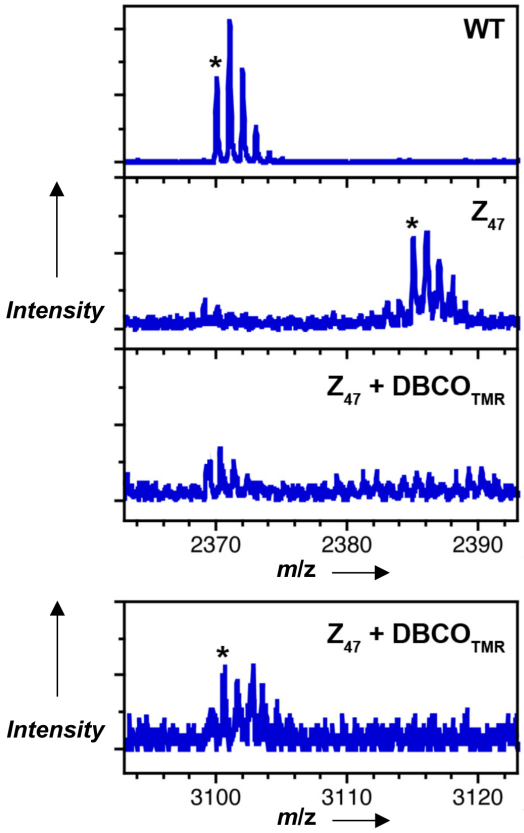

Figure S4. MALDI MS Analysis of AaT-Z_{47} $DBCO_{TMR}$ Labeling. Mass region corresponding to the trypsinized 45-64 region of WT or AaT-Z_{47} with or without $DBCO_{TMR}$ modification. The expected mass peak is denoted by an asterisk in each spectrum.

Table S1. Trypsin Digest AaT Mutant Sequence Coverage. Underline indicates observed fragment.

AaT Sequence

MGHHHHHHHHHHSSGHIEGR/HMRLVQLSR/HSIAFPSPEGALREPNGLLALGGDLSPAR/LLMAYQR/GI**F**PWFSPGDPILWWSPDPR/AVL**W**PESLHISR/SMKRFHKRSPYRVTMNYAFGQVIEGCASDREEGTWITRGVVEAYHR/LHELGHAHSIEV**W**R/EDELVGGMYGVAQGTLFCGESMFSRMENASKTALLVFCEEFIGHGGK/LIDCQVLNDHTASLGACEIPR/RDYLNYLNQMR/LGRLPNNFWVPR/CLFSPQE

AaT-WT			
Position	**Peptide**	**Calcd (M+H)$^+$**	**Obsvd (M+H)$^+$**
45-64	GI**F**PWFSPGDPILWWSPDPR	2370.17	2371.00
45-64	GI**Z**$_{-N2}$PWFSPGDPILWWSPDPR	2385.17	--
65-76	AVL**W**PESLHISR	1407.77	1407.68
65-76	AVL**Z**$_{-N2}$PESLHISR	1383.77	--
123-136	LHELGHAHSIEV**W**R	1683.87	1683.76
123-136	LHELGHAHSIEV**Z**$_{-N2}$R	1659.87	--
AaT-F_{47}Z			
Position	**Peptide**	**Calcd (M+H)$^+$**	**Obsvd (M+H)$^+$**
45-64	GI**F**PWFSPGDPILWWSPDPR	2370.17	--
45-64	GI**Z**$_{-N2}$PWFSPGDPILWWSPDPR	2385.17	2386.09
65-76	AVL**W**PESLHISR	1407.77	1407.64
65-76	AVL**Z**$_{-N2}$PESLHISR	1383.77	--
123-136	LHELGHAHSIEV**W**R	1683.87	1683.71
123-136	LHELGHAHSIEV**Z**$_{-N2}$R	1659.87	--
AaT-W_{68}Z			
Position	**Peptide**	**Calcd (M+H)$^+$**	**Obsvd (M+H)$^+$**
45-64	GI**F**PWFSPGDPILWWSPDPR	2370.17	2370.97
45-64	GI**Z**$_{-N2}$PWFSPGDPILWWSPDPR	2385.17	--
65-76	AVL**W**PESLHISR	1407.77	--
65-76	AVL**Z**$_{-N2}$PESLHISR	1383.77	1383.66
123-136	LHELGHAHSIEV**W**R	1683.87	1683.74
123-136	LHELGHAHSIEV**Z**$_{-N2}$R	1659.87	--

Table S1 continued. Trypsin Digest AaT Mutant Sequence Coverage.

AaT-$W_{135}Z$			
Position	**Peptide**	**Calcd $(M+H)^+$**	**Obsvd $(M+H)^+$**
45-64	GI**F**PWFSPGDPILWWSPDPR	2370.17	2370.97
45-64	GI**Z**$_{-N2}$PWFSPGDPILWWSPDPR	2385.17	--
65-76	AVL**W**PESLHISR	1407.77	1407.65
65-76	AVL**Z**$_{-N2}$PESLHISR	1383.77	--
123-136	LHELGHAHSIEV**W**R	1683.87	--
123-136	LHELGHAHSIEV**Z**$_{-N2}$R	1659.87	1659.71

AaT-$F_{47}Z$ + $DBCO_{TMR}$			
Position	**Peptide**	**Calcd $(M+H)^+$**	**Obsvd $(M+H)^+$**
45-64	GI**F**PWFSPGDPILWWSPDPR	2370.17	--
45-64	GI**Z**$_{-N2}$PWFSPGDPILWWSPDPR	2385.17	--
45-64	GI**Z(DBCO$_{TMR}$)**PWFSPGDPILWWSPDPR	3100.45	3101.59
AaT-$W_{68}Z$ + $DBCO_{TMR}$			
Position	**Peptide**	**Calcd $(M+H)^+$**	**Obsvd $(M+H)^+$**
65-76	AVL**W**PESLHISR	1407.77	--
65-76	AVL**Z**$_{-N2}$PESLHISR	1383.77	--
65-76	AVL**Z(DBCO$_{TMR}$)**PESLHISR	2073.05	2073.98
AaT-$W_{135}Z$ + $DBCO_{TMR}$			
Position	**Peptide**	**Calcd $(M+H)^+$**	**Obsvd $(M+H)^+$**
123-136	LHELGHAHSIEV**W**R	1683.87	--
123-136	LHELGHAHSIEV**Z**$_{-N2}$R	1659.87	--
123-136	LHELGHAHSIEV**Z(DBCO$_{TMR}$)**R	2349.15	--

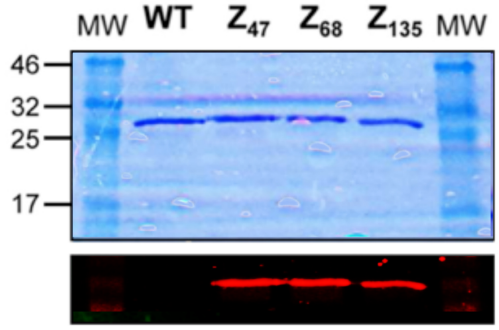

Figure S5. PAGE Gel Analysis of AaT $DBCO_{545}$ Labeling. AaT WT and mutants were treated with $DBCO_{545}$ in DMSO. Both images depict the same gel, stained with Coomassie dye (top) or imaged using 532 nm excitation (bottom). MW: Molecular weight markers (kDa).

Investigation of AaT Cys-Thiolate DBCO Adducts. Wild type AaT and AaT-Z_{47} were purified as described above, but during affinity purification and dialysis either 5 μM β-ME or 5 mM β-ME was used for each protein. Purified proteins were then incubated with 100 μM $DBCO_{TMR}$ to assess the degree of Cys-DBCO non-specific labeling. SDS-PAGE analysis and fluorescence imaging was used to determine the degree of labeling (Fig. S6, Left). WT AaT and AaT-Z_{47} from batches purified using 5 μM β-ME were treated with or without 2 mM iodoacetamide (IAM) for 30 min at room temperature. Following IAM pre-treatment, samples were incubated with 100 μM $DBCO_{TMR}$ for 1 h at 37 °C. SDS-PAGE analysis and fluorescence imaging was used to determine the degree of labeling (Fig. S6, Right). These data indicate that the DBCO reagents undergo some reactions with Cys nucleophiles, as has been previously observed by van Geel *et al*.[2]

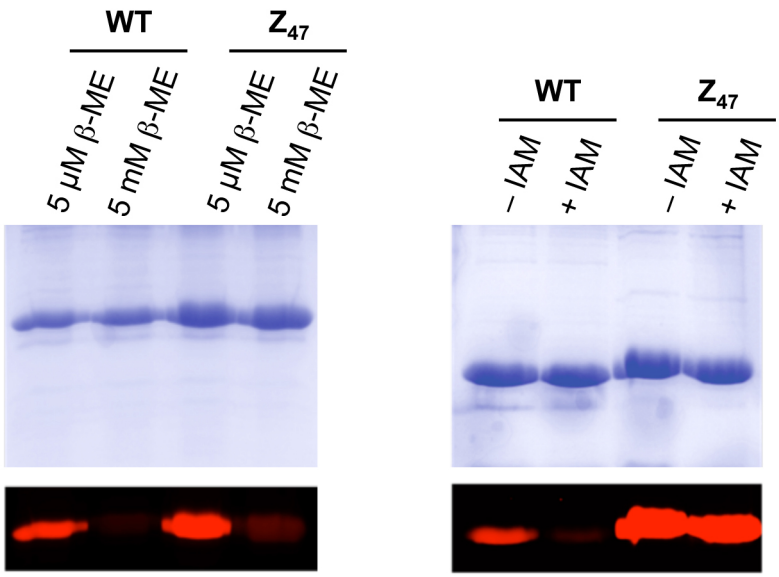

Figure S6. Blocking AaT Cys-DBCO Adducts. Left: Lane 1: WT AaT (5 μM β-ME) incubated with 100 μM $DBCO_{TMR}$; lane 2: WT AaT (5 mM β-ME) incubated with 100 μM $DBCO_{TMR}$; lane 3: AaT-Z_{47} (5 μM β-ME) incubated with 100 μM $DBCO_{TMR}$; and lane 4: AaT-Z_{47} (5 mM β-ME) incubated with 100 μM $DBCO_{TMR}$. Right: Lane 1: WT AaT (5 μM β-ME) incubated with 100 μM $DBCO_{TMR}$; lane 2: WT AaT (5 μM β-ME) pre-treated with 2 mM IAM, followed by 100 μM $DBCO_{TMR}$; lane 3: AaT-Z_{47} (5 μM β-ME) incubated with 100 μM $DBCO_{TMR}$; and lane 4: AaT-Z_{47} (5 μM β-ME) pre-treated with 2 mM IAM, followed by 100 μM $DBCO_{TMR}$.

HPLC Analysis of PheLysAlaAcm Ligations. All HPLC analyses of PheLysAlaAcm ligations were monitored on an Agilent 1100 HPLC using a Waters C18 column (Milford, MA). The solvents used for peptide purification were the following: 0.1 % trifluoroacetic acid in water (Solvent A) and 0.1 % trifluoroacetic acid in acetonitrile (Solvent B). The HPLC method had the following solvent gradient (Gradient 1): 0 min 1 % B, 5 min 1 % B, 10 min 30 % B, 15 min 40 % B, 20 min 100 % B, 25 min 100 % B, 27 min 1 % B, 30 min 1% B. Peptides were monitored at two absorption wavelengths during HPLC analysis, 215 nm for peptide absorption, and 325 nm for Acm absorption.

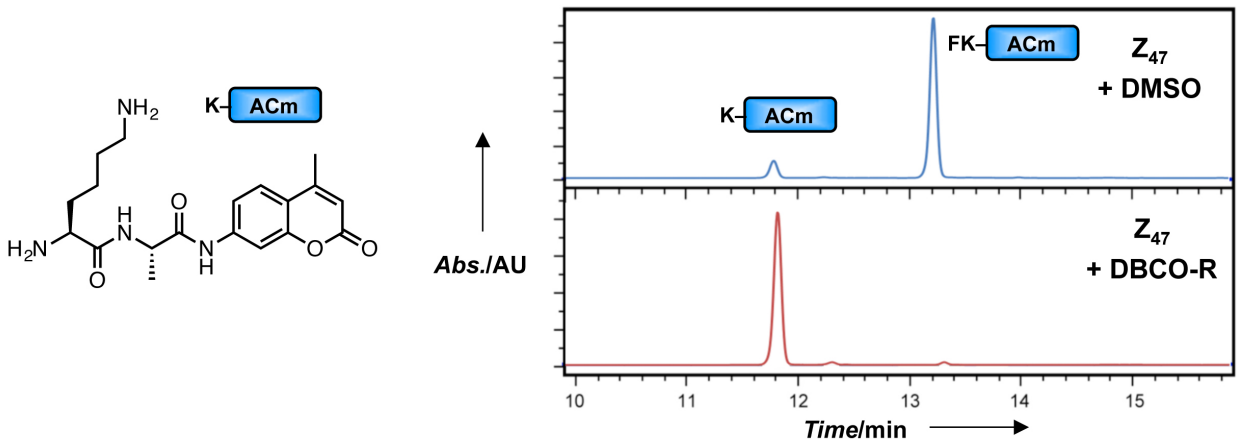

Figure S7. Representative HPLC Analysis of LysAlaAcm Transfer Reactions. Top: Conversion of LysAlaAcm starting material to PheLysAlaAcm product in the presence of AaT-Z_{47} treated with DMSO vehicle; Bottom: Conversion of LysAlaAcm starting material to PheLysAlaAcm product in the presence of AaT-Z_{47} treated with $DBCO_{545}$.

$DBCO_A$ Inhibition. Ni-NTA purified AaT-WT and AaT-Z_{47} (0.20 mg/mL) were incubated with 100 μM $DBCO_A$ for 2 hours at 37 °C. As a vehicle control, AaT and AaT-Z_{47} mutants were also treated with DMSO. Clicked proteins were then stored at - 80 °C. LysAlaAcm ligation assays were performed as descried above. All HPLC analyses of PheLysAlaAcm ligations were monitored on an Agilent 1100 HPLC using a Waters C18 column.

Concentration Dependence of AaT in LysAlaAcm Ligations. Ligations were carried out in triplicate as described above for 30 minutes at 37 °C. AaT concentrations were used at 2.26, 1.13, 0.565, and 0.283 μM. All HPLC analyses of PheLysAlaAcm ligation assays were monitored on an Agilent 1100 HPLC using a Waters C18 column.

Table S2. HPLC Analysis of $DBCO_{545}$ LysAlaAcm Reactions. Performed in the presence and absence (DMSO vehicle only) of $DBCO_{545}$ labeling.

AaT Mutant	+ Vehicle Conversion %	+ $DBCO_{545}$ Conversion %
WT	100.0 ± 1.7	108.9 ± 0.5
1/2X WT	71.2 ± 0.5	
1/4X WT	49.1 ± 0.6	
1/8X WT	30.6 ± 0.3	
Z_1	X	X
Z_{42}	4.8 ± 1.0	3.1 ± 0.2
Z_{47}	67.4 ± 5.4	0.9 ± 0.2
Z_{59}	31.3 ± 2.6	11.8 ± 0.4
Z_{68}	26.7 ± 2.4	4.7 ± 0.6
Z_{81}	40.8 ± 3.1	25.0 ± 2.3
Z_{135}	52.8 ± 2.1	25.6 ± 2.3

Table S3. HPLC Analysis of $DBCO_A$ LysAlaAcm Reactions. Performed in the presence and absence (DMSO vehicle only) of $DBCO_A$ labeling.

AaT Mutant	+ Vehicle Conversion %	+ $DBCO_A$ Conversion %
WT	98.6 ± 0.3	98.7 ± 0.1
Z_{47}	60.7 ± 2.4	0.4 ± 0.2
Z_{68}	17.3 ± 1.0	2.3 ± 0.6
Z_{135}	23.8 ± 0.3	1.6 ± 0.2

Dose Dependent Inhibition of AaT-Z_{47}. Ni-NTA purified AaT-Z_{47} (0.20 mg/mL) was incubated with $DBCO_{545}$ at the following concentrations: DMSO, 0.5, 1.0, 5.0, 10.0, 17.5, 25.0, 50.0, 100.0, and 200.0 μM. SPAAC reactions were incubated for 1 hour at 37 °C and then immediately used in LysAlaAcm ligations. Ligations were carried out in triplicate as described above for 2 hours at 37 °C. All HPLC analyses of PheLysAlaAcm ligations were monitored on an Agilent HPLC using a Waters C18 column.

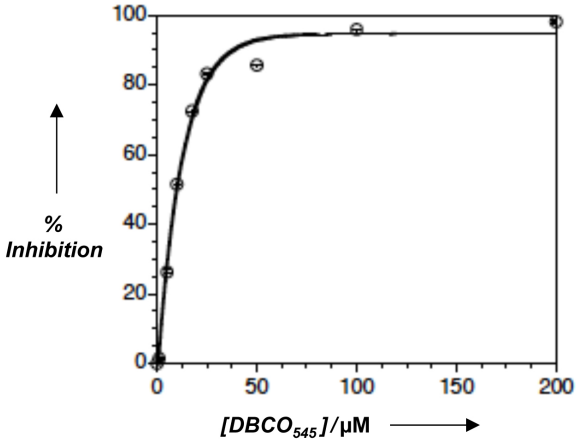

Figure S8. Dose Dependent Inhibition of AaT-Z_{47}. Activity of AaT-Z_{47} after modification with various concentrations of $DBCO_{545}$ for 1 hour.

Computational Modeling. Computational models were constructed in PyMol (Schrödinger, LLC; New York, NY, USA) and Spartan (Wavefunction Inc.; Irvine, CA, USA). A fragment corresponding to the residues within 8 Å of the amino acid to be mutated to Azf (F_{47}, W_{68}, or W_{135}) was excised from the phenyalanyl adenosine AaT complex (PDB 2Z3K) in PyMol. This fragment was imported as a PDB file into Spartan. Molecular models of the triazole products of Azf with $DBCO_A$ or $DBCO_{TMR}$ were constructed in Spartan. The position of the α-carbon of the resulting modified amino acid sidechain was frozen at the position of the α-carbon of the mutated amino acid. The geometries of the modified sidechains were optimized using the MMF94 forcefield while keeping the positions of the protein atoms frozen. The resulting modified amino acid structures were then imported into PyMol as PDB files. Since the Spartan geometry optimization resulted in the position of the TMR ring facing outward for the Z_{135}-$DBCO_{TMR}$ structure, we also manually rotated the TMR moiety to approach the substrate pocket as closely as possible. All of our $DBCO_{TMR}$ models are shown in Figure S9.

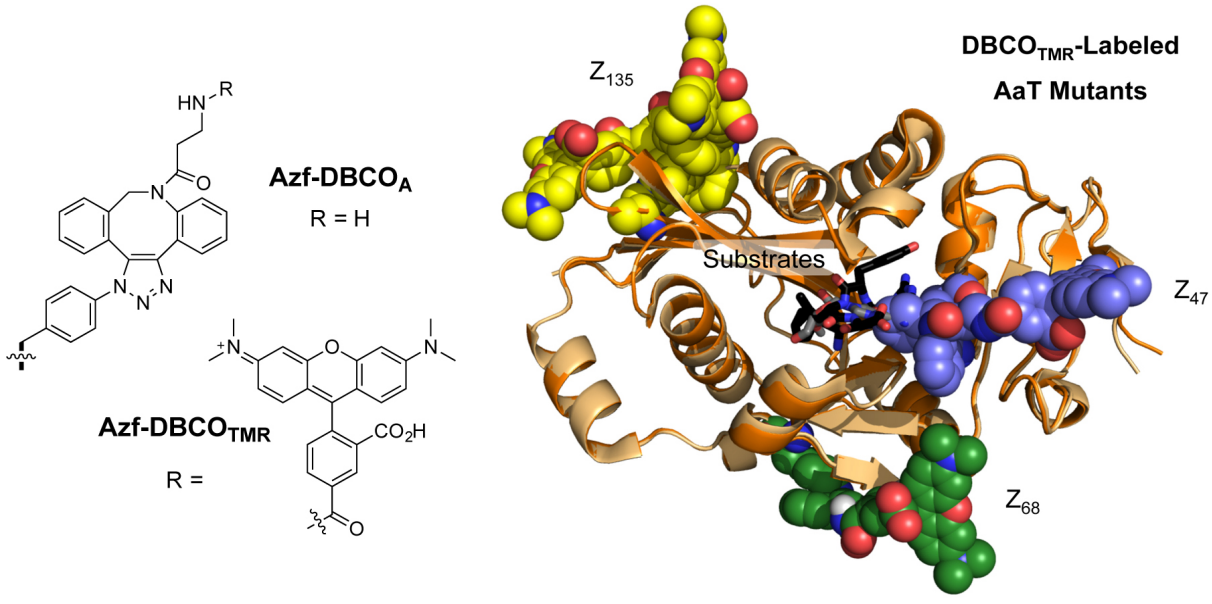

Figure S9. Models of $DBCO_{TMR}$-Labeled AaT Mutants. Geometry-optimized models of Azf-$DBCO_{TMR}$ adducts at positions 47, 68, and 135 of AaT are shown overlain on the protein (PDB 2Z3K and 2Z3N).

Construction of Recombinant $\alpha S_{2\text{-}140}K_2$ Reporter Plasmid. A plasmid containing a wild type αS gene cloned between NdeI and HindIII in pT7-7 vector was provided by Dr. Elizabeth Rhoades (Department of Molecular Biophysics and Biochemistry, Yale University). An XhoI site (CTCGAG) was introduced 3' to the WT αS gene in the pT7-7 vector using QuikChange® mutagenesis. The αS gene was digested by NdeI and XhoI and cloned into the pET-16b prokaryotic expression vector (EMD Biosciences; San Diego, CA, USA), which contained an N-terminal His-tag followed by a Factor Xa recognition site (IEGR).[3] QuickChange® mutagenesis was then used to delete residues H_{-1} and M_1 followed by a D_2K mutation to yield the H_{10}-$\alpha S_{2\text{-}140}K_2$ plasmid. The plasmid identity was confirmed using DNA sequencing with a T7 promoter primer.

Human αS Gene

5′ATGGGCCATCATCATCATCATCATCATCATCACAGCAGCGGCCATATCGAAGGTCGTCATATGGATGTATTC
ATGAAAGGACTTTCAAAGGCCAAGGAGGGAGTTGTGGCTGCTGCTGAGAAAACCAAACAGGGTGTGGCAGAAGCAGC
AGGAAAGACAAAAGAGGGTGTTCTCTATGTAGGCTCCAAAACCAAGGAGGGAGTGGTGCATGGTGTGGCAACAGTGG
CTGAGAAGACCAAAGAGCAAGTGACAAATGTTGGAGGAGCAGTGGTGACGGGTGTGACAGCAGTAGCCCAGAAGACA
GTGGAGGGAGCAGGGAGCATTGCAGCAGCCACTGGCTTTGTCAAAAAGGACCAGTTGGGCAAGAATGAAGAAGGAGC
CCCACAGGAAGGAATTCTGGAAGATATGCCTGTGGATCCTGACAATGAGGCTTATGAAATGCCTTCTGAGGAAGGGT
ATCAAGACTACGAACCTGAAGCCTAA-3′

DNA Oligomers used for Quikchange® Mutagenesis

A. Deletion of Residues H_{-1} & M_1

Forward: 5′GGCCATATCGAAGGTCGT$^{\downarrow}$GATGTATTCATGAAAGGA-3′

Reverse: 5′TCCTTTCATGAATACATC$^{\downarrow}$ACGACCTTCGATATGGCC-3′

B. Mutation of D_2 to K_2

Forward: 5′GCGGCCATATCGAAGGTCGTAAAGTATTCATGAAAGGACTTTC-3′

Reverse: 5′GAAAGTCCTTTCATGAATACTTTACGACCTTCGATATGGCCGC-3′

Human H_{10}-$\alpha S_{2\text{-}140}K_2$ Amino Acid Sequence

MGHHHHHHHHHHSSGHIEGRK$_2$VFMKGLSKAKEGVVAAAEKTKQGVAEAAGKTKEGVLYVGSKTKEGVVHGVATVAEK
TKEQVTNVGGAVVTGVTAVAQKTVEGAGSIAAATGFVKKDQLGKNEEGAPQEGILEDMPVDPDNEAYEMPSEEGYQD
YEPEA

Figure S10. Construction of αS Reporter Plasmid.

Expression and Purification of $\alpha S_{2\text{-}140}K_2$. The plasmid containing the αS gene was used to transform *E. coli* BL21(DE3) cells. Transformed cells were selected on the basis of ampicillin resistance. Single colonies were used to inoculate 5 mL of LB media supplemented with ampicillin (100 μg/mL). The primary 5 mL culture was incubated at 37 °C with shaking at 250 rpm for 5 hours. 500 mL of LB media supplemented with ampicillin was inoculated with 1 mL of the primary culture. The 500 mL culture was incubated at 37 °C with shaking at 250 rpm until an OD_{600} of 0.6 was reached. Expression was induced with the addition of 1.0 mM IPTG and cells were incubated at 25 °C for 16 hours. The cells were harvested by centrifugation at 5000 x g for 15 minutes and the resulting pellet was resuspended in a Factor Xa cleavage buffer (100 mM NaCl, 50 mM Tris pH 8.0, 5 mM $CaCl_2$) that included protease inhibitor cocktail, 1 mM PMSF, and 10 units/mL DNAse1–Grade II) and sonicated. Following sonication, the cell lysate was boiled for 30 minutes prior to centrifugation for 20 minutes at 30,000 x g, 4 °C. Collected soluble protein was gently shaken for 1 hour at ambient temperature with Ni-NTA resin. Protein was purified by rinsing with Factor Xa cleavage buffer followed by washing with Factor Xa wash buffer (100 mM NaCl, 50 mM Tris pH 8.0, 5 mM $CaCl_2$, and 50 mM imidazole). Protein was eluted with Factor Xa elution buffer 100 mM NaCl, 50 mM Tris pH 8.0, 5 mM $CaCl_2$,and 250 mM imidazole). SDS-PAGE analysis was performed to analyze the purity of αS elution fractions. Collected protein fractions were dialyzed against Factor Xa cleavage buffer at 4 °C. The His-10 purification tag was removed by incubation of purified H_{10}-$\alpha S_{2\text{-}140}K_2$ (0.5 mg/mL) with FactorXa, Restriction Grade, Bovine Plasma (20 μL, Novagen; EMD Millipore Corporation, Billerica, MA) overnight at 37°C. Uncleaved H_{10}-$\alpha S_{2\text{-}140}K_2$ was removed by treatment with Ni-NTA resin. Factor Xa was removed by boiling for 30 minutes and centrifugation for 20 minutes at 30,000 x g, 4 °C. $\alpha S_{2\text{-}140}K_2$ was dialyzed into modified AaT

buffer (50 mm HEPES pH 8.0, 150 mM KCl, 10 mM $MgCl_2$) at 4 °C. Protein concentration was determined by BCA assay (Thermo Scientific Pierce) and purity was confirmed by SDS-PAGE and MALDI mass spectrometry. Protein was stored at - 80 °C until further use.

Cloning of *E. coli* AzfRS from into pET21a. An NdeI cut site was introduced into pCDNA3.1-RS_{Azf} 5' of the *E. coli* AzfRS gene and an XhoI cut site was introduced 3' to the *E. coli* AzfRS gene using site-directed mutagenesis. The sequences and mutagenic primers are shown in Figure S11. The *E. coli* AzfRS gene was excised from pCDNA3.1-RS_{Azf} using NdeI and XhoI restriction enzymes. The pET21a plasmid, containing a C-terminal 6-His tag, was digested with NdeI and XhoI. The pET21a vector and *E. coli* AzfRS insert were purified on a 0.8 % agarose gel and isolated using a Qiagen Gel Extraction Kit. T4 DNA ligase reactions were performed using a 1:3 vector:insert ratio at 16 °C for 1 hour. Library efficiency DH5α cells were then transformed with 5 μL of the ligation. Colonies were "mini-prepped" and preliminarily screened using NdeI and XhoI restriction enzyme digests. The DNA sequence identity of the product, pET21-RS_{Azf}, was confirmed using T7 primers.

DNA Oligomers used for Quikchange® Mutagenesis

A. NdeI Site Insertion

Forward: 5′CTTAAGCTTGGTACCCC**CAT**ATGGCAAGCAGTAAC-3′

Reverse: 5′GTTACTGCTTGCCAT**ATG**GGGGTACCAAGCTTAAG-3′

B. XhoI Site Insertion

Forward: 5′GCTGGAAAGACTACAAA**CT**CGA**G**GACGACAAGTAA-3′

Reverse: 5′TTACTTGTCGTC**C**TCG**AG**TTTGTAGTCTTTCCAGC-3′

***E. coli* AzfRS Gene**

5′ATGGCAAGCAGTAACTTGATTAAACAATTGCAAGAGCGGGGGCTGGTAGCCCAGGTGACGGACGAGGAAGCGTTA
GCAGAGCGACTGGCGCAAGGCCCGATCGCGCTCCTGTGCGGCTTCGATCCTACCGCTGACAGCTTGCATTTGGGGCA
TCTTGTTCCATTGTTATGCCTGAAACGCTTCCAGCAGGCGGGCCACAAGCCGGTTGCGCTGGTAGGCGGCGCGACGG
GTCTGATTGGCGACCCGAGCTTCAAAGCTGCCGAGCGTAAGCTGAACACCGAAgAAACTGTTCAGGAGTGGGTGGAC
AAAATCCGTAAGCAGGTTGCCCCGTTCCTCGATTTCGACTGTGGAGAAAACTCTGCTATCGCGGCGAACAACTATGA
CTGGTTCGGCAATATGAATGTGCTGACCTTCCTGCGCGATATTGGCAAACACTTCTCCGTTAACCAGATGATCAACA
AAGAAGCGGTTAAGCAGCGTCTCAACCGTGAAGATCAGGGGATTTCGTTCACTGAGTTTTCCTACAACCTGTTGCAG
GGTTATAGCATGGCCTGTGCGAACAAACAGTACGGTGTGGTGCTGCAAATTGGTGGTTCTGACCAGTGGGGTAACAT
CACTTCTGGTATCGACCTGACCCGTCGTCTGCATCAGAATCAGGTGTTTGGCCTGACCGTTCCGCTGATCACTAAAG
CAGATGGCACCAAATTTGGTAAAACTGAAGGCGGCGCAGTCTGGTTGGATCCGAAGAAAACCAGCCCGTACAAATTC
TACCAGTTCTGGATCAACACTGCGGATGCCGACGTTTACCGCTTCCTGAAGTTCTTCACCTTTATGAGCATTGAAGA
GATCAACGCCCTGGAAGAAGAAGATAAAAACAGCGGTAAAGCACCGCGCGCCCAGTATGTACTGGCGGAGCAGGTGA
CTCGTCTGGTTCACGGTGAAGAAGGTTTACAGGCGGCAAAACGTATTACCGAATGCCTGTTCAGCGGTTCTTTGAGT
GCGCTGAGTGAAGCGGACTTCGAACAGCTGGCGCAGGACGGCGTACCGATGGTTGAGATGGAAAAGGGCGCAGACCT
GATGCAGGCACTGGTCGATTCTGAACTGCAACCTTCCCGTGGTCAGGCACGTAAAACTATCGCCTCCAATGCCATCA
CCATTAACGGTGAAAAACAGTCCGATCCTGAATACTTCTTTAAAGAAGAAGATCGTCTGTTTGGTCGTTTTACCTTA
CTGCGTCGCGGTAAAAAGAATTACTGTCTGATTTGCTGGAAAGACTACAAACTCGAGCACCACCACCACCACCACTG
A-3′

***E. coli* AzfRS Amino Acid Sequence**

MASSNLIKQLQERGLVAQVTDEEALAERLAQGPIALLCGFDPTADSLHLGHLVPLLCLKRFQQAGHKPVALVGGATG
LIGDPSFKAAERKLNTEETVQEWVDKIRKQVAPFLDFDCGENSAIAANNYDWFGNMNVLTFLRDIGKHFSVNQMINK
EAVKQRLNREDQGISFTEFSYNLLQGYSMACANKQYGVVLQIGGSDQWGNITSGIDLTRRLHQNQVFGLTVPLITKA
DGTKFGKTEGGAVWLDPKKTSPYKFYQFWINTADADVYRFLKFFTFMSIEEINALEEEDKNSGKAPRAQYVLAEQVT
RLVHGEEGLQAAKRITECLFSGSLSALSEADFEQLAQDGVPMVEMEKGADLMQALVDSELQPSRGQARKTIASNAIT
INGEKQSDPEYFFKEEDRLFGRFTLLRRGKKNYCLICWKDYKLEHHHHHH

Figure S11. Construction of AzfRS Expression Plasmid.

Purification of *E. coli* AzfRS. His_6-tagged *E. coli* AzfRS was expressed from the pET21-RS_{Azf} plasmid described above in *E. coli* BL21-Gold (DE3) cells. Cells were grown in 4 mL LB containing ampicillin (100 μg/mL) at 37 °C to an OD_{600} of 0.5. A secondary 500 mL LB culture was inoculated with 1 mL of the primary culture and grown to an OD_{600} of 0.6. Protein expression was induced with 1.0 mM IPTG and grown at 25 °C for 16 hours. Cells were pelleted at 6,000 rpm using a GS3 rotor and Sorvall RC-5 centrifuge. Cell pellets were

resuspended in Ni-NTA binding buffer (50 mM Tris, 10 mM imidazole, and 300 mM KCl, pH 8.0) that included protease inhibitor cocktail, 1 mM PMSF, and 10 units/mL DNAse-Grade II. Following resuspension, the cells were lysed using sonication. Soluble proteins were collected *via* centrifugation at 13,200 rpm for 15 minutes. Collected soluble protein was gently shaken for 1 hour on ice with Ni-NTA Superflow. Protein was purified by rinsing with Ni-NTA binding buffer and then washing with Ni-NTA was buffer (50 mM Tris, 50 mM imidazole, and 300 mM KCl, pH 8.0). Protein was eluted with elution buffer (50 mM Tris, 250 mM imidazole, and 300 mM KCl, pH 8.0). Fractions containing *E. coli* AzfRS were dialyzed overnight in synthetase buffer (50 mM Tris, 50% glycerol, and 1 μM β-ME, pH 7.5). The dialyzed enzymes were stored at – 80 °C.

Cleared *E. coli* TS351G Lysate Preperation. *E. coli* TS351G cells were transformed with pUC19 plasmid DNA. Cells were grown in 4 mL LB containing ampicillin (100 μg/mL) at 37 °C to an OD_{600} of 0.5. A secondary 500 mL LB culture was inoculated with 1 mL of the primary culture and grown overnight at 25°C. Cells were pelleted at 6,000 rpm using a GS3 rotor and Sorvall RC-5 centrifuge. Cell pellets were resuspended in 50 mM Tris and 300 mM KCl, pH 8.0 that included protease inhibitor cocktail, 1 mM PMSF, and 10 units/mL DNAse-Grade II. Following resuspension, the cells were lysed using sonication. Soluble proteins were collected *via* centrifugation at 13,200 rpm for 15 minutes. Cleared lysates were then stored at – 80 °C.

***In vitro* AaT Labeling Activity Assay.** $\alpha S_{2\text{-}140}K_2$ was N-terminally modified with Azf (Azf-$\alpha S_{2\text{-}140}K_2$) using either DMSO or $DBCO_{TMR}$ treated AaT-WT, AaT-Z_{47}, AaT-Z_{68}, or AaT-Z_{135}. AaT activity was assayed by fluorescently modifying Azf-$\alpha S_{2\text{-}140}K_2$ with fluorescein-alkyne (synthesized as previously described).[4] $\alpha S2_{\text{-}140}K_2$ (24.5 μg) was modified with Azf (1.0 mM) in cleared *E. coli* TS351G lysate using *E. coli* total tRNA (125 μg), ATP (2.5 mM), and *E. coli*

AzfRS (2.5 μg) in a reaction volume of 62.5 μL in modified AaT buffer (50 mm HEPES pH 8.0, 150 mM KCl, 10 mM MgCl2) and AaT (DMSO or $DBCO_{TMR}$ treated, 1.56 μg). ATP was omitted and replaced with an equivalent amount of MilliQ water for negative controls. The reaction mixture was incubated at 37 °C for 4 hours. Then Azf was removed by five buffer exchanges into phosphate buffered saline (PBS, 12 mM NaH_2PO_4, 50 mM NaCl, 4.7 mM KCl, pH 8.0) using Amicon Ultra 0.5 Centrifugal 10 kDa spin columns (EMD Millipore Corporation; Billerica, MA). Azf-$\alpha S_{2\text{-}140}K_2$ was then labeled with fluorescein-alkyne (33 μM) using tris(3-hydroxypropyltriazolylmethyl)amine (THPTA, 3.5 mM), $CuSO_4$ (681 μM), and sodium ascorbate (5.4 mM) at 37 °C for 1 hour. Aliquots (15 μL) of fluorescein labeled Azf-$\alpha S_{2\text{-}140}K_2$ were boiled with gel loading dye LDS (Pierce; Rockford, IL, USA) for ten minutes at 95 °C and analyzed by SDS-PAGE. Fluorescence images were obtained with a Typhoon FLA 7000 (GE Lifesciences). Fluorescein was imaged using 473 nm excitation and an Y520 filter. TMR was imaged using 532 nm excitation and an O580 filter. Images were collected using a 100 μm pixel size. Total protein content was visualized using Coomassie Brilliant Blue.

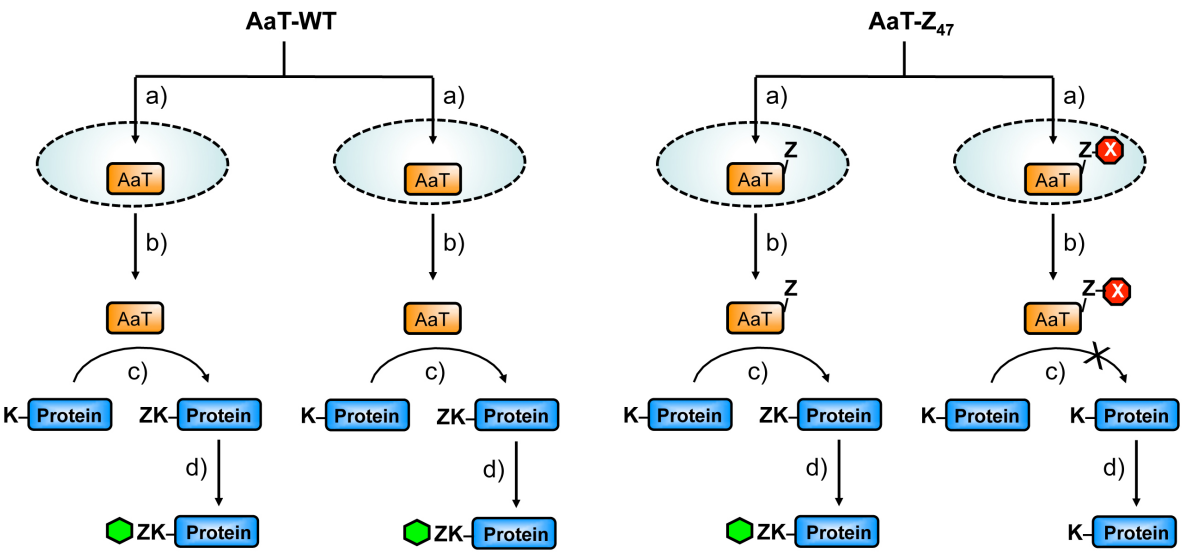

Figure S12. In Lysate Assay Protocol. Purified AaT proteins (WT or Azf mutants such as Z_{47}) are subjected to the following conditions: a) reaction with $DBCO_{545}$ or DMSO vehicle; b) mixing with TS351G cell lysate; c) Azf transfer assay using the αS reporter protein, *E. coli* AzfRS, and AaT; d) Azf transfer levels determined by Cu-catalyzed reaction with a fluorescein alkyne probe. WT control experiments determine the level of transfer activity that should be expected from uninhibited AaT as well as the levels of non-specific binding of the $DBCO_{TMR}$ reagent. Vehicle control experiments determine the level of transfer activity that should be expected from uninhibited AaT mutants (i.e., What is the effect of the Azf mutation alone?). Inhibition by the $DBCO_{TMR}$ reaction is determined by comparing the levels of labeling in the final step to the levels seen in the vehicle control.

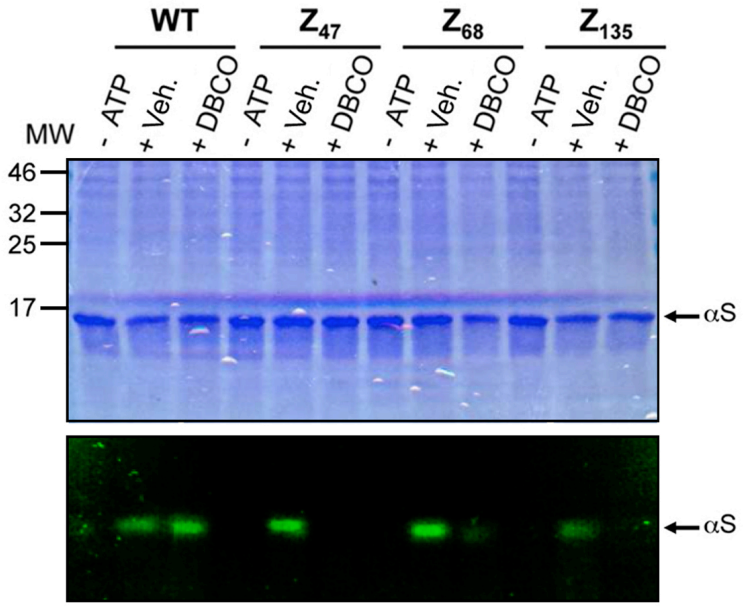

Figure S13. PAGE Gel Analysis of αS Transfer Reactions. AaT WT and mutants were subjected to one of three conditions, + DBCO: Purified AaT enzymes were treated with $DBCO_{545}$ in DMSO, then used to transfer Azf to the αS reporter protein in TS351G lysate; + Veh.: Purified AaT enzymes were treated with DMSO only, then used to transfer Azf to the αS reporter protein in TS351G lysate; - ATP: Purified AaT enzymes treated with DMSO only, then used in a mock transfer assay in which ATP was withheld to prevent Azf transfer. In all cases, Azf transfer was detected with a fluorescein alkyne. Transfer yields calculated from fluorescence intensity of αS bands were used to determine percent inhibition. Both images depict the same gel, stained with Coomassie dye (top) or imaged using 473 nm excitation (bottom). MW: Molecular weight markers (kDa).

References

[1] M. V. Kvach, I. A. Stepanova, I. A. Prokhorenko, A. P. Stupak, D. A. Bolibrukh, V. A. Korshun, V. V. Shmanai, *Bioconj. Chem.* **2009**, *20*, 1673-1682.

[2] R. van Geel, G. J. M. Pruijn, F. L. van Delft, W. C. Boelens, *Bioconj. Chem.* **2012**, *23*, 392-398.

[3] S. Batjargal, Y. J. Wang, J. M. Goldberg, R. F. Wissner, E. J. Petersson, *J. Am. Chem. Soc.* **2012**, *134*, 9172-9182.

[4] A. M. Wagner, M. W. Fegley, J. B. Warner, C. L. J. Grindley, N. P. Marotta, E. J. Petersson, *J. Am. Chem. Soc.* **2011**, *133*, 15139-15147.