Supporting Information

A Radical Clock Probe Uncouples H-atom Abstraction from Thioether Crosslink Formation by the Radical SAM Enzyme SkfB

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Abbreviations

CID, collision-induced dissociation; CPG, cyclopropylglycine; DTT, dithiothreitol; *E. coli*, *Escherichia coli*; FMOC, Fluorenylmethyloxycarbonyl; HPLC, high performance liquid chromatography; SAM, S-adenosyl-L-methionine; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; UHPLC-MS, ultra high performance liquid chromatography-mass spectrometry

Table S1. Primers used for all cloning described in the Experimental Procedures. The bold and underlined nucleotides indicate the codon mutated to alanine.

Primer	Sequence
C351A Forward	GCAGATGGATACGTAACTCCT**GCC**CAATTAGAAGATTTGCCGCTAGG
C351A Reverse	CCTAGCGGCAAATCTTCTAATTG**GGC**AGGAGTTACGTATCCATCTGC
C385A Forward	GCTTCAGTTAAAATGTGAAGCAAAAAAT**GCC**AAATGTATAGGGAAAATTGAGCTATCTGAACC
C385A Reverse	GGTTCAGATAGCTCAATTTTCCCTATACATTT**GGC**ATTTTTTGCTTCACATTTTAACTGAAGC

Table S2. Iron and Sulfide Content of SkfB and its ΔAux variant.

Variant	mol Fe / mol protein	mol S /mol protein
WT	8.1	6.2
ΔAux	4.6	4.5

Table S3. Theoretical and observed ions from tandem MS of the SIAXTR & SIAZTR fragments.

Unmodified SIAXTR Peptide

Ion	Observed m/z	Calculated m/z	Error (ppm)	Sequence
b2	201.1234	201.1234	0.0	.SI.a
b3	272.1606	272.1605	0.5	.SIA.x
b4	369.2134	369.2132	0.5	.SIAX.t
b4 - H2O	351.2029	351.2027	0.6	.SIAX.t
b5	470.2610	470.2609	0.1	.SIAXTR.r
b5 - H2O	452.2505	452.2504	0.3	.SIAXT.r
y2	276.1668	276.1666	0.5	X.TR.
y3	373.2196	373.2194	0.5	a.XTR.
y4	444.2566	444.2565	0.3	i.AXTR.
z2	259.1402	259.1401	0.5	x.TR.
z2 - H2O	241.1296	241.1295	0.4	x.TR.
z3	356.1931	356.1928	0.6	a.XTR.
z3 - H2O	338.1825	338.1823	0.5	a.XTR.
z4	427.2301	427.23	0.4	i.AXTR.
z4 - H2O	409.2196	409.2194	0.4	i.AXTR.
a4	341.2185	341.2183	0.6	.SIAX.t

Modified SIAZTR Peptide

Ion	Observed m/z	Calculated m/z	Error (ppm)	Sequence
b2	201.1236	201.1234	1.0	.SI.a
b3	272.1607	272.1605	0.7	.SIA.z
b4	370.2199	370.2201	-0.5	.SIAZ.t
b4 - H2O	352.2094	352.2095	-0.3	.SIAZ.t
b5	471.2674	471.2678	-0.8	.SIAZT.r
b5 - H2O	453.2567	453.2572	-1.1	.SIAZT.r
y2	276.1670	276.1666	1.1	z.TR.
y3	374.2261	374.2262	-0.3	a.ZTR.
y4	445.2627	445.2634	-1.6	i.AZTR.
z2 - H2O	259.1403	259.1401	0.8	z.TR.
z2 - H2O	241.1298	241.1295	1.2	z.TR.
z3	357.1996	357.1997	-0.3	a.ZTR.
z3 - H2O	339.1889	339.1891	-0.6	a.ZTR.
z4	428.2367	428.2368	-0.2	i.AZTR.
z4 - H2O	410.2259	410.2262	-0.7	i.AZTR.
y5	558.3479	558.3474	0.9	s.IAZTR.

Figure S1. UHPLC-MS analysis of purified M40CPG–SkfA obtained from solid phase peptide synthesis. (**A**) Total ion chromatogram (TIC, black) and extracted ion chromatogram (red) of *m/z* of 1442.5 – 1443.5 corresponding to the +4 charge state mass envelope of CPG–SkfA. (**B**) Full mass spectrum averaged from 8.4-10.4 min in the extracted ion chromatogram depicted in (**A**). (**C**) Deconvoluted mass spectrum of the peptide. The peak with *m/z* of 5765.9974 corresponds to fully reduced CPG–SkfA ($[M+H]^+$ calculated = 5765.9984, 0.2 ppm error).

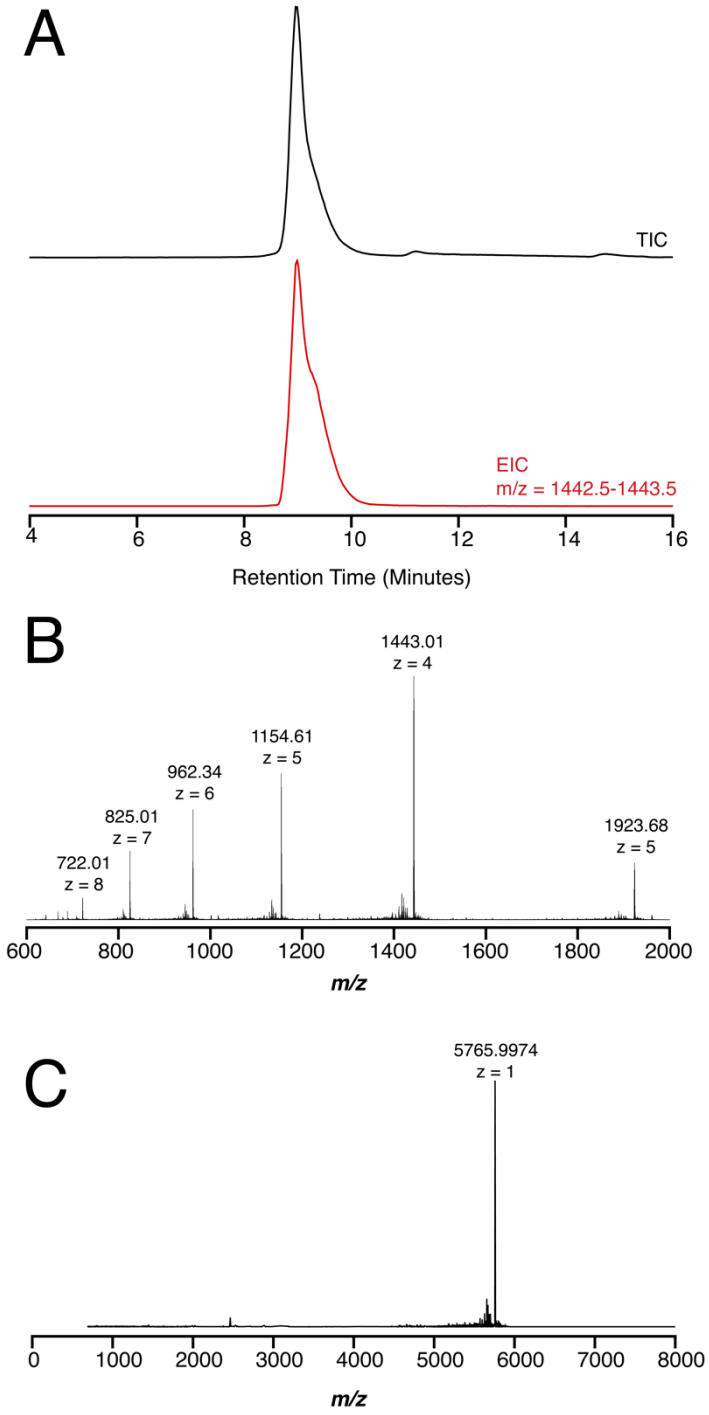


Figure S2. Full mass spectrum of wild type SkfA incubated with (**A**) no enzyme (**B**) ΔAux SkfB, and (**C**) wild-type SkfB. In all cases, modification reactions were carried out in the presence of SAM and dithionite, and the reactions were quenched by iodoacetamide to modify free thiols. The mass envelopes corresponding to unmodified SkfA containing three carbamidomethylated cysteines are highlighted by the gray boxes, while the mass envelopes corresponding to SkfA with only two carbamidomethylated cysteines and one thioether crosslink are highlighted by the red boxes. The additional peaks observed in (**C**) result from incomplete carbamidomethylation of the peptide.

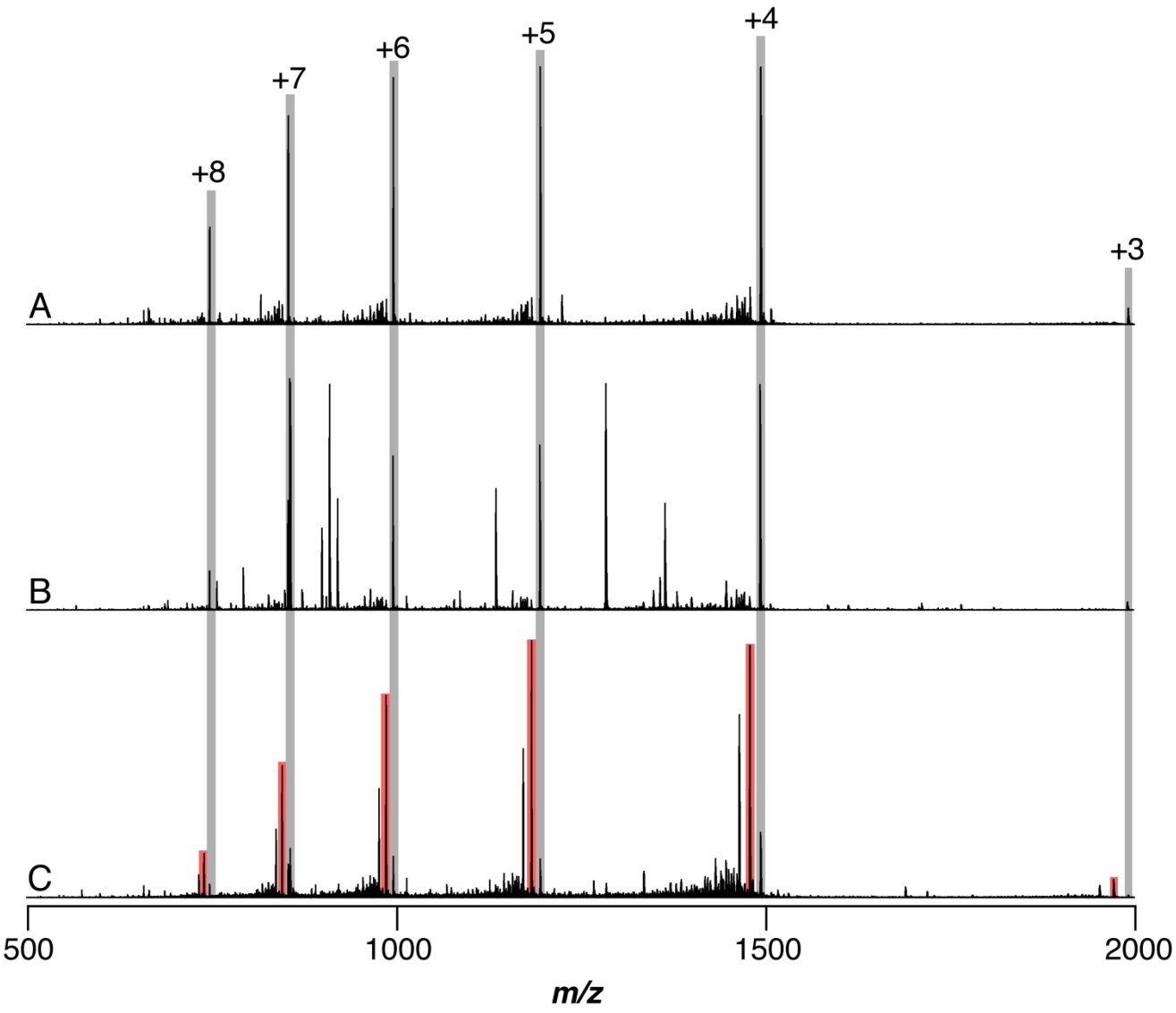


Figure S3. Full mass spectrum of CPG-SkfA incubated with (**A**) no enzyme, (**B**) ΔAux SkfB, and (**C**) wild-type SkfB. In all cases, modification reactions were carried out in the presence of SAM and dithionite, and the reactions were quenched by iodoacetamide to modify free thiols. The mass envelopes corresponding to no-thioether containing SkfA carrying three carbamidomethylated cysteines are highlighted by the gray boxes. We do not observe any peaks corresponding to 2 carbamidomethylations and a single thioether, such as those observed with wildtype SkfA in **Fig. S2** (red boxes).

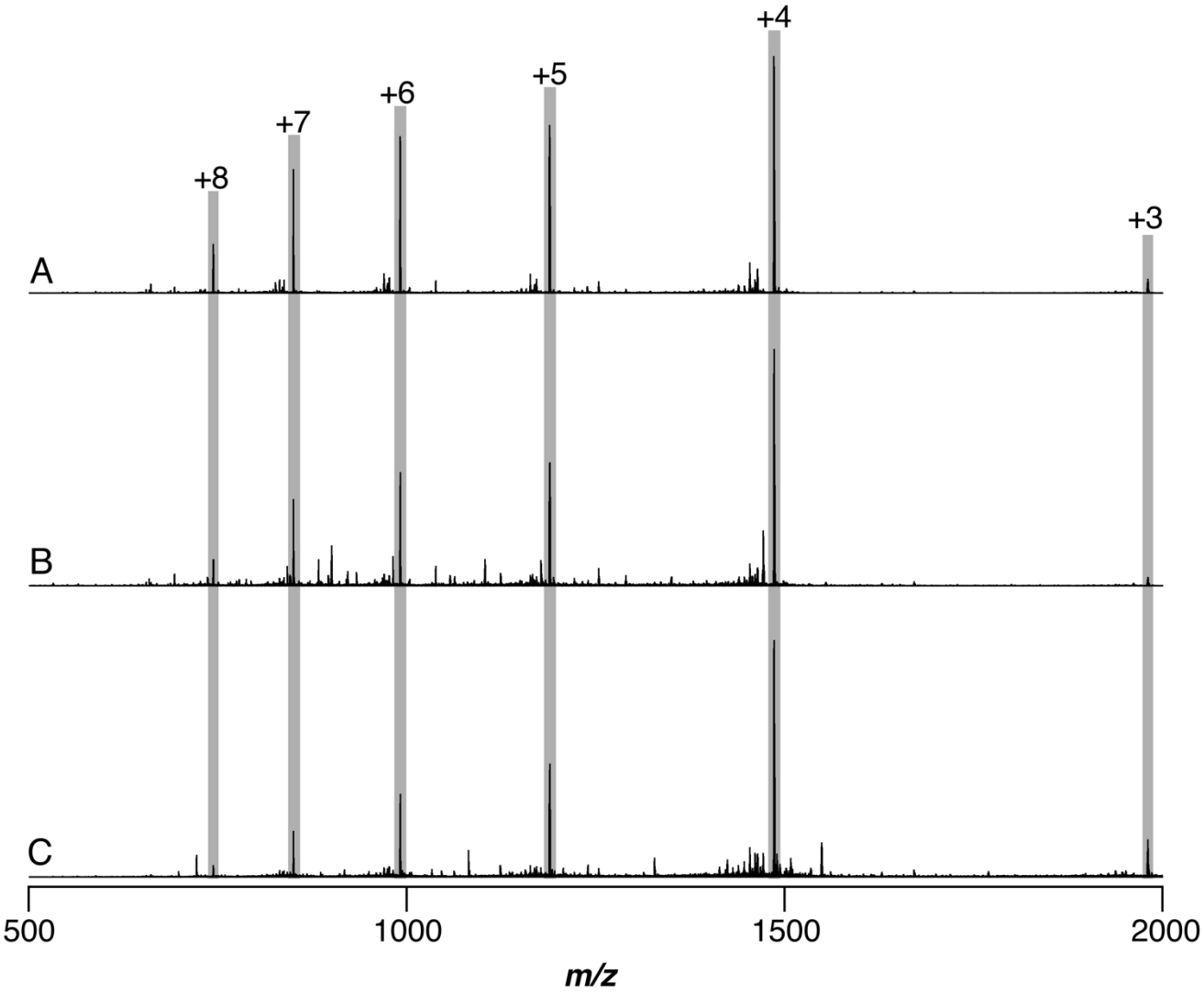


Figure S4. Production of 5'deoxyadenosine by wild-type and C351A/C385A (ΔAux) SkfB in the presence of native substrate (SkfA) and cyclopropyl glycine substituted substrate (CPG-SkfA).

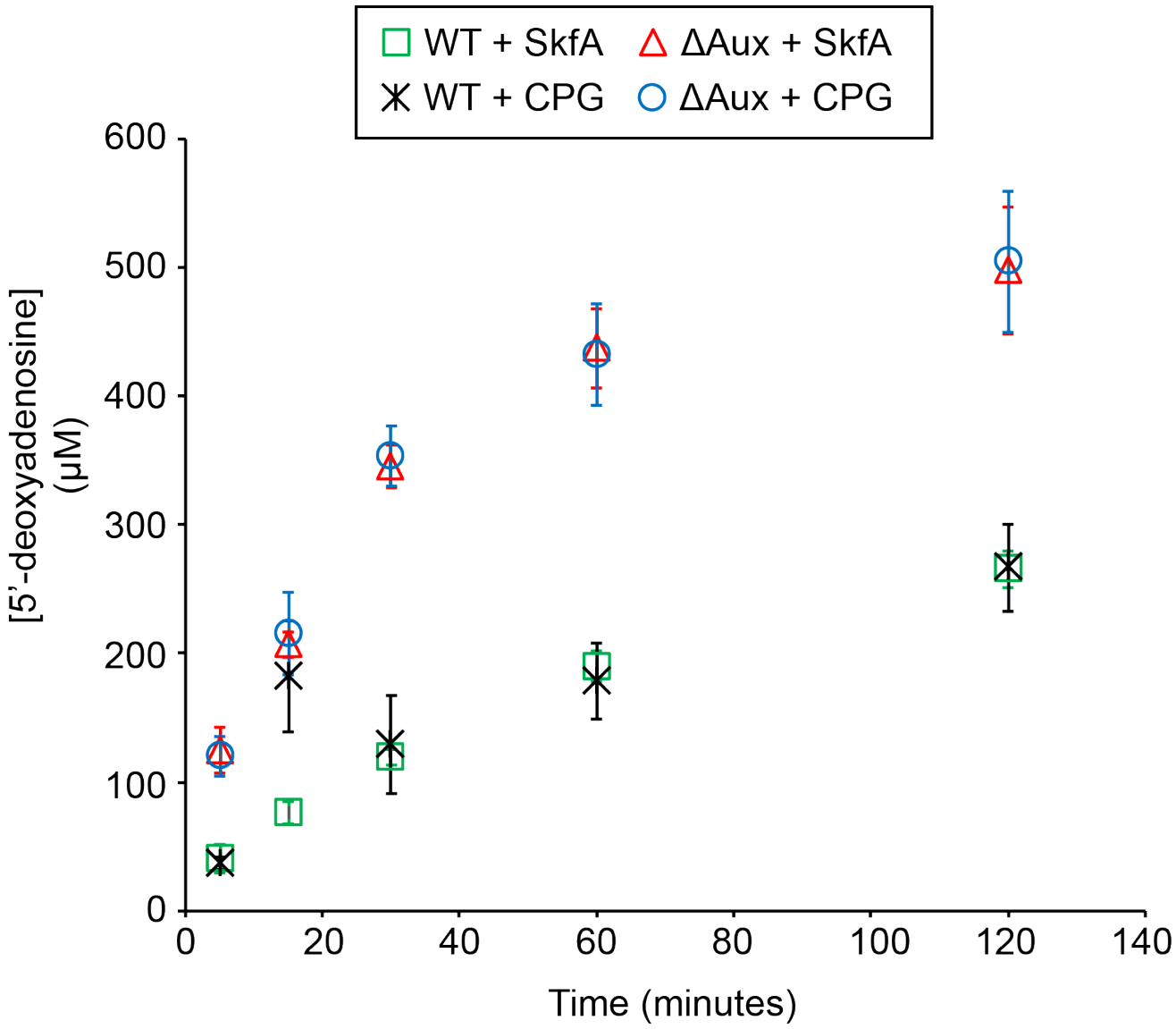


Figure S5. Full mass spectrum of wild type SkfA incubated with (**A**) no enzyme in H_2O (**B**) WT SkfB in H_2O, and (**C**) wild-type SkfB in D_2O. In all cases, modification reactions were carried out in the presence of SAM and dithionite, and the reactions were quenched by iodoacetamide to modify free thiols. The average *m/z* values corresponding to unmodified SkfA containing three carbamidomethylated cysteines are shown with black text and arrows, while the average *m/*z values corresponding to SkfA with only two carbamidomethylated cysteines and one thioether crosslink are shown with red text and arrows. Note that the *m/z* values for the peptide do not change whether incubated in H_2O or D_2O with enzyme. The additional peaks observed in (**B**) and (**C**) result from incomplete carbamidomethylation of the peptide.

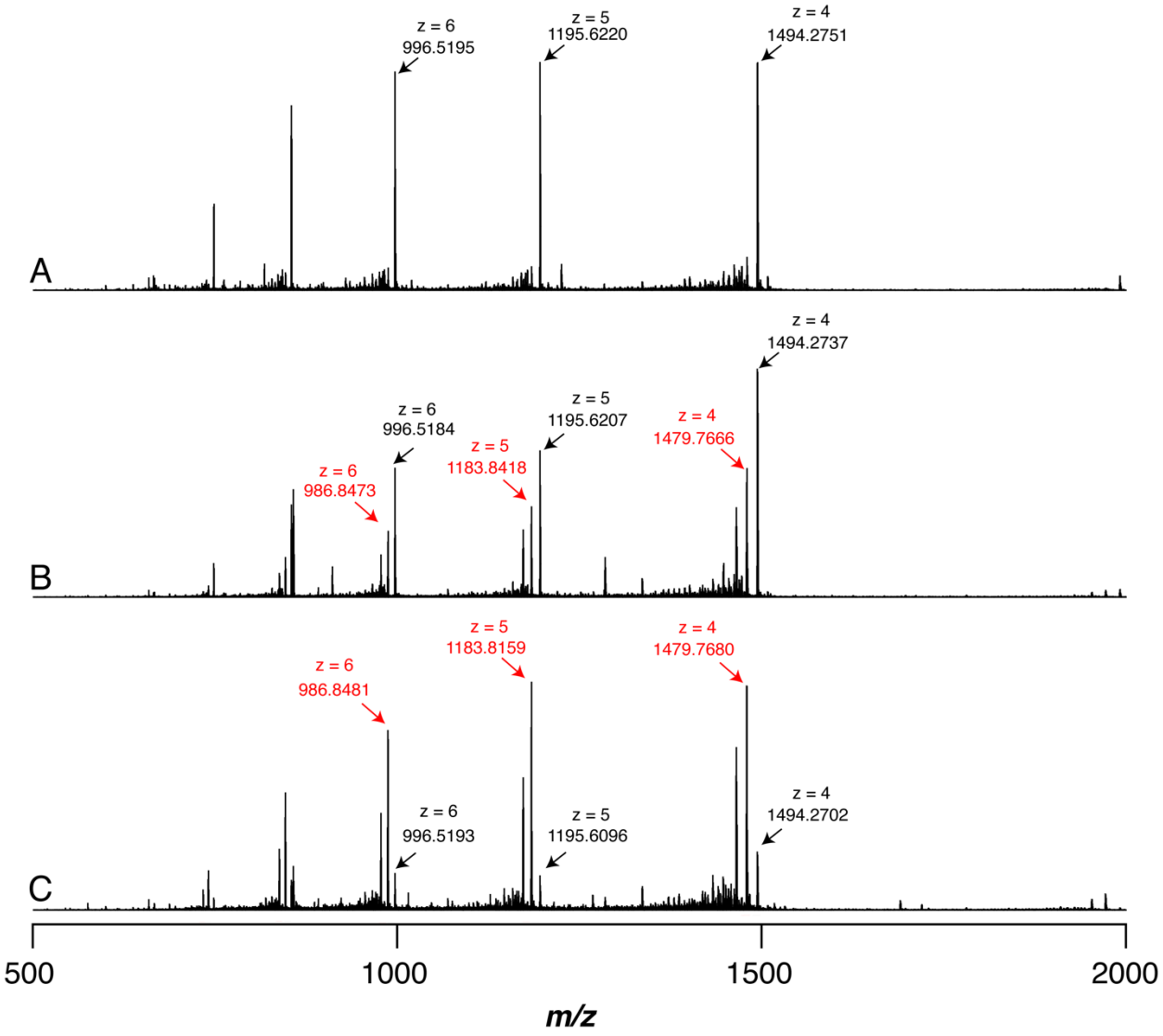


Figure S6. Full mass spectrum of CPG-SkfA incubated with (**A**) no enzyme in D_2O (**B**) WT SkfB in H_2O, (**C**) ΔAux SkfB in D_2O, and (**D**) wild-type SkfB in D_2O. In all cases, modification reactions were carried out in the presence of SAM and dithionite, and the reactions were quenched by iodoacetamide to modify free thiols. All the peaks when SkfB is present correspond to the mass of CPG-SkfA, except that when the reactions are carried in D_2O the peptides are 1 amu higher in average mass. The text above each peak corresponds to average *m/z*.

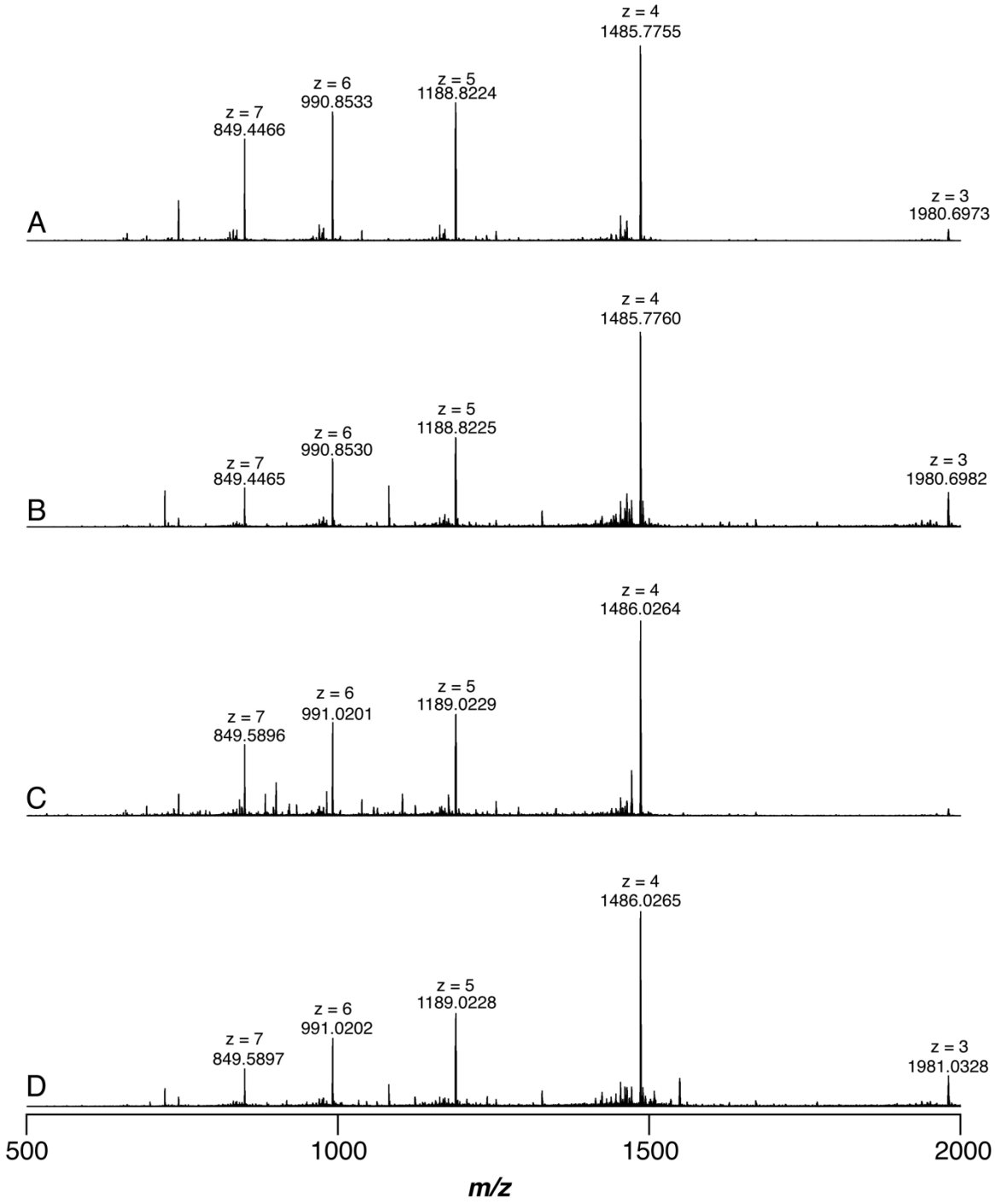


Figure S7. EIC traces showing that ring opening is dependent on presence of reaction components. Left column corresponds to *m/z* of 644.36-644.38, and the right column corresponds to *m/z* of 645.37-645.38. The conditions for assays are listed in the Methods above. (**A**) and (**B**) are assays without peptide substrate; (**C**) and (**D**) are without SkfB enzyme; (**E**) and (**F**) are without dithionite; (**G**) and (**H**) are without SAM; (**I**) and (**J**) are with all components and wild-type SkfB; (**K**) and (**L**) are with all components and ΔAux SkfB variant.

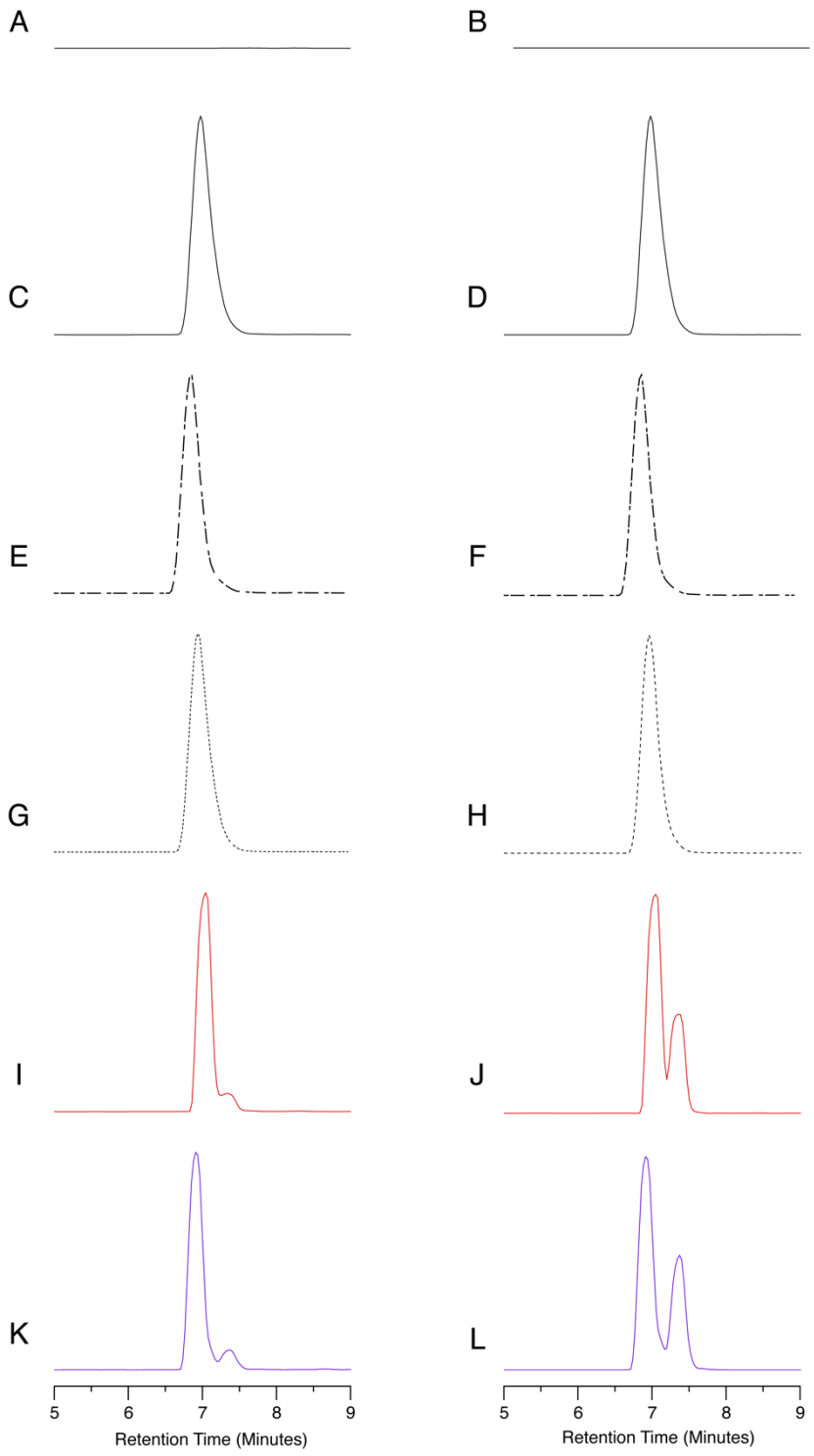


Figure S8. Purified SIAXTR peptide standard. (**A**) Top: TIC trace (black), bottom: EIC trace for m/z 644.36-644.38 (red). (**B**) full spectrum for SIAXTR sample scanned over the EIC peak.

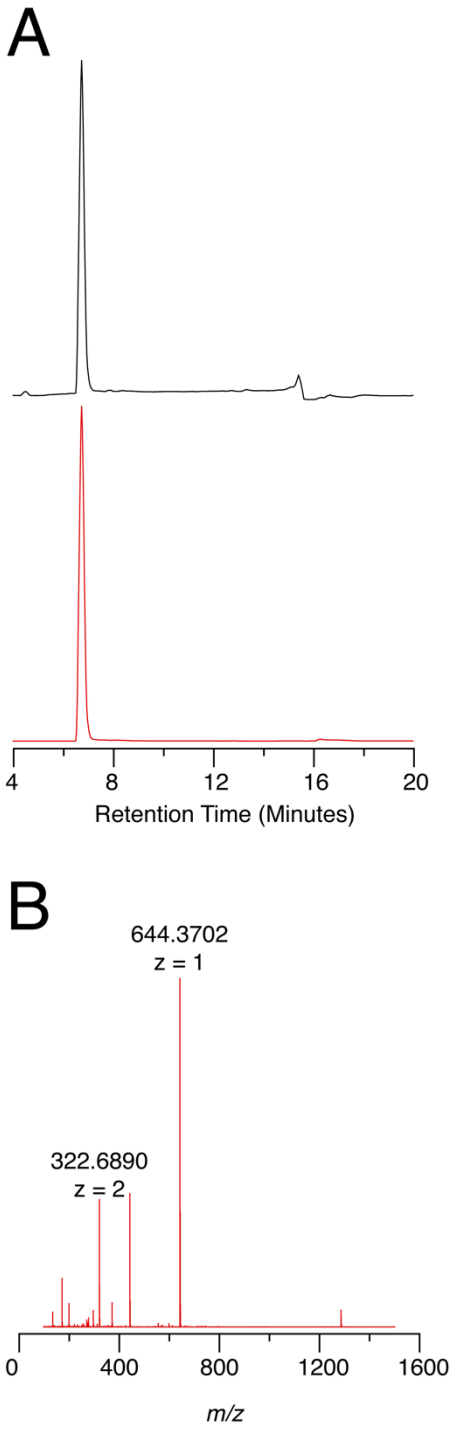


Figure S9. Purified SIAZTR peptide standard. (**A**) Top: TIC trace (black), bottom: EIC trace for m/z 646.38-646.39 (blue). (**B**) full spectrum for SIAZTR sample scanned over the EIC peak.

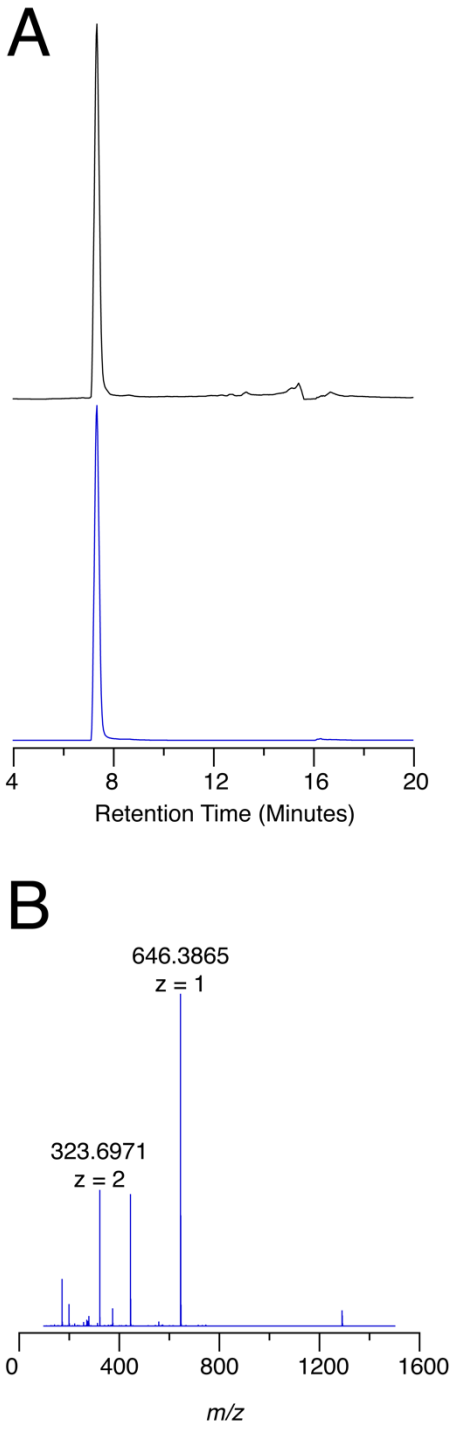


Figure S10. Co-injection of SIAXTR and SIAZTR peptides. Top: TIC trace (black), middle: EIC trace for *m/z* 644.36-644.38 (red), bottom: EIC trace for m/z 646.38-646.39 (blue).

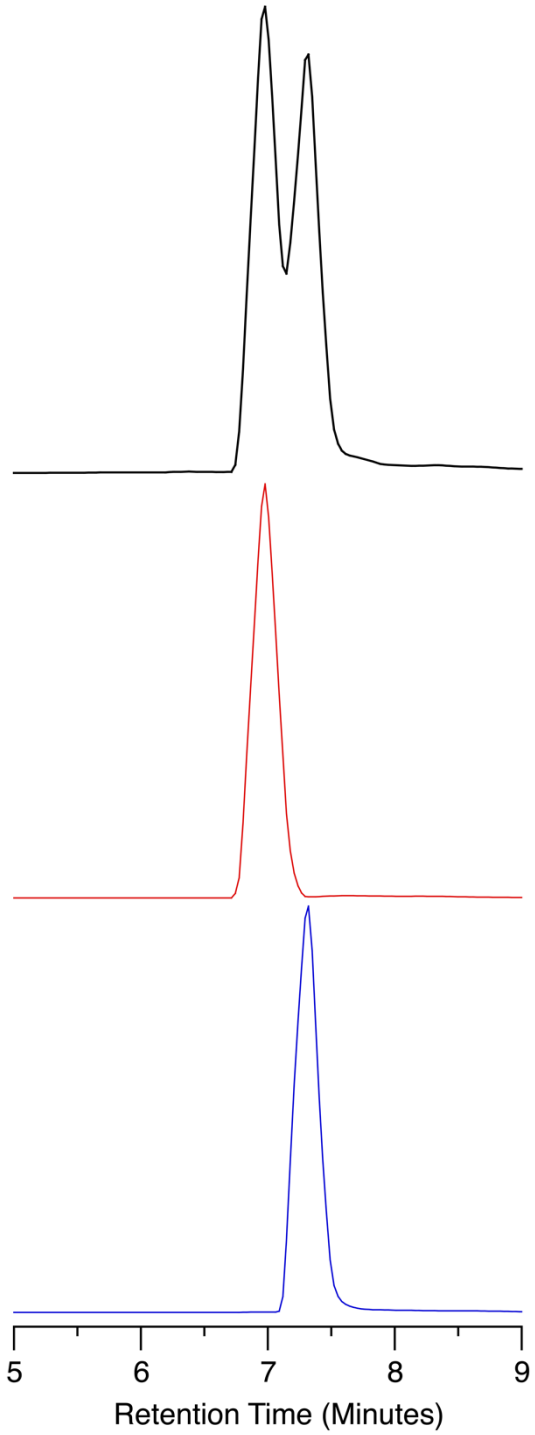
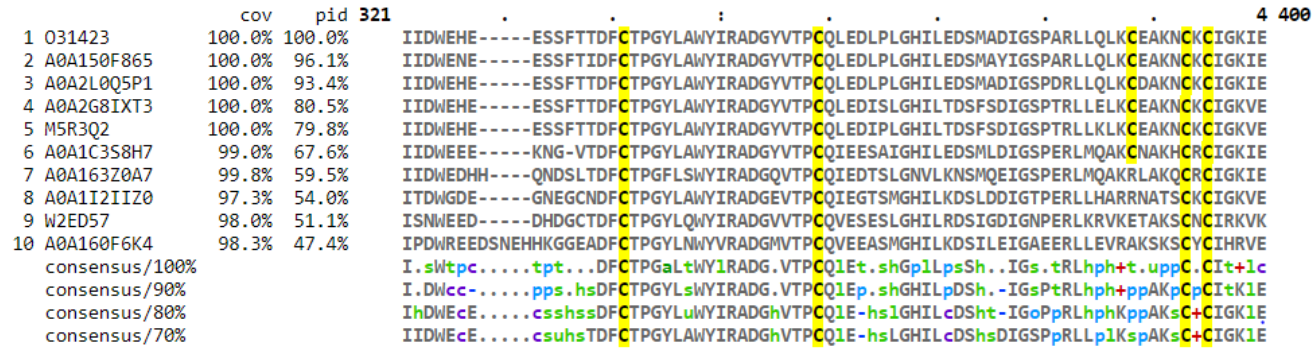


Figure S11. Sequence alignment of the putative Aux cluster binding domain (approximately residues 321-400) of 10 selected SkfB homologs. Cys residues are highlighted in yellow. Consensus sequences are supplied below the alignment, ranging from 100% to 70% alignment. Accession numbers are provided in the far left column. The percent coverage and percent identity listed are in reference to the entire sequence alignment beyond the narrow window we are displaying for the Aux domain. The alignment was generated using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and reformatted for color display with MView (https://www.ebi.ac.uk/Tools/msa/mview/).



References

(1) Thoden, J. B.; Holden, H. M. The Molecular Architecture of Human N-Acetylgalactosamine Kinase. *J. Biol. Chem.* **2005**, *280* (38), 32784–32791.

(2) Bruender, N. A.; Bandarian, V. SkfB Abstracts a Hydrogen Atom from Cαon SkfA to Initiate Thioether Cross-Link Formation. *Biochemistry* **2016**, *55* (30), 4131–4134.

(3) Beinert, H. Semi-Micro Methods for Analysis of Labile Sulfide and of Labile Sulfide plus Sulfane Sulfur in Unusually Stable Iron-Sulfur Proteins. *Anal. Biochem.* **1983**, *131* (2), 373–378.

(4) McCarty, R. M.; Krebs, C.; Bandarian, V. Spectroscopic, Steady-State Kinetic, and Mechanistic Characterization of the Radical SAM Enzyme QueE, Which Catalyzes a Complex Cyclization Reaction in the Biosynthesis of 7-Deazapurines. *Biochemistry* **2013**, *52* (1), 188–198.