Supplementary Information for

Aliphatic Ether Bond Formation Expands the Scope of Radical SAM Enzymes in Natural Product Biosynthesis

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Materials and strains. All materials were obtained from MilliporeSigma or Fisher Scientific unless otherwise specified. Restriction enzymes, T4 DNA ligase, Q5® High-Fidelity DNA Polymerase, Q5® Site-Directed Mutagenesis Kit, Trypsin-ultra™ Mass Spectrometry Grade, Endoproteinase GluC, Shrimp Alkaline Phosphatase (rSAP), and the corresponding buffers were purchased from New England BioLabs (NEB). All Fmoc- and side chain-protected amino acids, and other components for solid-phase peptide synthesis were purchased from MilliporeSigma, except for HOBt which was obtained from AnaSpec. DNA purification kits were purchased from Qiagen. The *tqqB* gene, codon-optimized for expression in *E. coli*, was purchased from GENEWIZ as a gene fragment in a pUC19 vector.

General procedures. UV-vis spectra were acquired on a Cary 60 UV-visible spectrophotometer (Agilent). HPLC separations were carried out on an Agilent 1260 Infinity Series analytical or preparative HPLC system equipped with a photodiode array detector and an automated fraction collector. Low resolution HPLC-MS analysis was performed on an Agilent instrument consisting of a liquid autosampler, a 1260 Infinity Series HPLC system coupled to a photodiode array detector and a 6120 Series ESI mass spectrometer. A Phenomenex® Luna C18 column (3 µm, 4.6 x 100 mm) was used with a flow rate of 0.5 mL/min and a gradient of 5% MeCN in H_2O to 100% MeCN over 12 min. Both MeCN and H_2O contained 0.1% (v/v) formic acid. High-resolution (HR) HPLC-MS was carried out on an Agilent UHD Accurate Mass Q-tof LC-MS system, equipped with a 1260 Infinity Series HPLC, an automated liquid sampler, a photodiode array detector, a JetStream ESI source, and the 6540 Series Q-tof. Samples were separated on a Phenomenex® Luna C18 column (3 µm, 4.6 x 100 mm) operating at 0.5 mL/min with a gradient of 5% MeCN in H_2O to 95% MeCN over 13 min (+0.1 % formic acid. NMR spectra were acquired at the Princeton University Department of Chemistry Facilities in D_2O or H_2O/D_2O (19:1) in the triple resonance cryoprobe of a Bruker A8 Avance III HD 800 MHz NMR spectrometer or a Bruker A1 Avance III 500 MHz NMR spectrometer. 1D/2D NMR data were analyzed with MestReNova software.

Cloning of TqqB. The *tqqB* gene (IMG gene ID: 2552353593, *S. suis* R735) was PCR-amplified from a pUC19 vector using Q5® DNA Polymerase in FailSafe™ Buffer D (Epicentre) and primers *tqqB*_F (NdeI) and *tqqB*_R (BamHI) (Table S2). The PCR product was purified using the QIAquick PCR Purification Kit and subsequently digested with NdeI and BamHI. The vector pET-28b(+) was digested with the same restriction enzymes and subsequently treated with rSAP. The digested fragment and plasmid were purified using the QIAquick Gel Extraction Kit and ligated in an insert:vector molar ratio of 5:1 using T4 DNA Ligase. The reaction was incubated for 3 hours at room temperature, heat inactivated for 10 min at 65°C, and transformed into chemically-competent *E. coi* DH5α cells. The transformants were selected on an LB agar plate containing kanamycin (50

μg/mL). The target vector pET-28b(+)_*tqqB* was isolated from a liquid culture of a single colony using the QIAprep Spin Miniprep Kit. Insertion of the gene fragment was confirmed by sequencing with the T7 promoter and terminator primers. Vectors pET-28b(+)_*tqqB* and pDB1282, which carries the *isc* operon required for proper assembly of the Fe-S clusters in TqqB, were subsequently co-transformed into chemically-competent *E. coli* BL21(DE3) cells for expression.

Mutagenesis of TqqB. Mutagenesis of the active-site [4Fe-4S] cluster and the putative auxiliary cluster was carried out on vector pET-28b(+)_*tqqB* using the Q5® Site-Directed Mutagenesis Kit. Two cysteine-to-alanine mutations were made in a single mutagenesis step for each cluster. Sites of mutation were selected based on the sequence alignment to SuiB (Figure S2). Primers (Table S2) were designed using the NEBaseChanger software. The standard protocol was followed with some modifications; a gel extraction step was included to isolate the correct plasmid band after the PCR step and the KLD reaction was incubated at room temperature for 3 hours rather than 15 min before transformation. Single colonies were selected for sequencing and plasmids with the correct double mutations were subsequently co-transformed with pDB1282 into chemically-competent *E. coli* BL21(DE3) cells for expression.

Expression, Purification, and Reconstitution of TqqB and Mutants. Expression, purification, and reconstitution of TqqB and TqqB mutants were carried out using previously published procedures without modifications.[1] Concentrations of purified TqqB and mutants were quantified using the method reported by Barr et al.[2] Purified TqqB and mutant enzymes were anaerobically reconstituted with 10-fold excess Fe^{2+} and S^{2-} as previously reported.[1]

Synthesis of Substrate (TqqA) and Substrate Variants by SPPS. The 37mer TqqA was synthesized via Fmoc-based solid phase chemistry using an automated microwave peptide synthesizer (CEM). Preloaded HMPB-Tyr(OtBu)-ChemMatrix® resin (0.1 mmol) and standard Fmoc and side chain-protected amino acids were used. The resin was swelled in DMF (10 mL) for 3 minutes. Fmoc deprotection reactions were performed using 10% piperazine (w/v) in a 10:90 solution of EtOH:NMP (3 mL) with 0.1 M HOBt. Fmoc-amino acids were coupled using 0.5 M DIC in DMF as an activating reagent. The resin was washed 3 times with a total volume of 7 mL DMF following deprotection. Amino acids 2-17 were coupled with a single coupling reaction for 4 minutes at 75 °C. with 5 equiv. of each amino acid. Amino acids 18-37 were double coupled with two successive 4 min coupling reactions at 75 °C with 5 equiv. of each amino acid. The terminal Fmoc group was deprotected in the last step of the synthesis. Upon completion of the synthesis, the resin was removed from the reaction vessel of the peptide synthesizer and transferred to an Econo-Pac column (BioRad). The resin was washed several times with DMF, followed

by DCM, then dried thoroughly under vacuum. The peptide was cleaved from the resin by incubation with freshly-prepared cleavage cocktail (5 mL) consisting of 92.5% TFA, 2.5% H2O, 2.5% TIS, and 2.5% DODT, which was added to prevent oxidation of Met. The reaction was stirred for 3 h at room temperature. The mixture was drained from the reaction tube and the resin was rinsed several times with TFA. The filtrate and rinses were combined and subsequently concentrated by evaporation of TFA under a stream of N_2. The peptide was precipitated by addition of 10 volumes of ice-cold diethyl ether. The ether/peptide suspension was incubated at -20°C for 20 minutes before isolating the precipitated peptide by centrifugation (4°C, 3500g, 15 min). The ether was poured off and the peptide was dried overnight.

Purification of TqqA and Variants. Crude TqqA was dissolved in 40% MeCN (+0.1% formic acid) and purified by preparative HPLC. Repeated injections were performed on a preparative Phenomenex® Luna C18 column (5 µm, 250 x 21.20 mmm), which was equilibrated in 15% MeCN in H_2O (+0.1% formic acid). The peptide was eluted with a gradient of 8-58% MeCN over 18 minutes to give pure TqqA, which was verified by HR-HPLC-MS (Table S4).

Enzymatic Activity Assays. Enzymatic assays were performed in an inert atmosphere in a glovebox (MBraun). The TqqA peptide substrate was brought into the glovebox as aliquots of lyophilized powder for the appropriate reaction volume. Reactions were typically carried out in 1.5 mL Eppendorf tubes on a 30 µL scale and contained assay buffer (100 mM HEPES, 300 mM KCl, 10% glycerol, pH 7.5), 30 µM TqqB, 5 mM sodium dithionite (DT), 5 mM dithiothreitol (DTT), 1 mM SAM and 500 µM TqqA. After 5 hours of incubation, reactions were removed from the glovebox and quenched by addition of an equal volume of MeCN (+ 1% formic acid). Precipitated protein was removed by centrifugation. Samples were then analyzed by HR-HPLC-MS as described above. Substrate and product typically eluted in a single peak at 40% MeCN. Relative quantification of product formation was conducted by analysis of extracted ion chromatograms.

Proteolytic Digestion of Reaction Product. To analyze the proteolysis fragments of the TqqA substrate and product, 100 µL–5mL reactions were set up and quenched as described above. For digestion by trypsin, $CaCl_2$ was added to the sample from a 2 M stock to achieve a final concentration of 20 mM. Trypsin-ultra™ Mass Spectrometry Grade was added to a final concentration of 100:1 substrate to trypsin. Reactions were incubated for 24 hours at 37°C. For digestion by GluC, an equal volume of 2x GluC Reaction Buffer (50 mM Tris-HCl, 0.5 mM Glu-Glu, pH 8) was added to the supernatant to a final concentration of 50:1 substrate to enzyme. Reactions were incubated for 24 hours at 37°C. Proteolysis reactions were directly analyzed by HPLC-Qtof-MS as

described above. In certain cases, reactions were first desalted using a Pierce™ graphite spin columns prior to MS analysis.

Large Scale Assays and Proteolysis of Product. Large-scale reactions to generate enough product for NMR analysis were carried out on a 5 mL scale with the same assay components and concentrations as described above. Reactions were allowed to incubate for 5 hours and then brought out the glovebox, quenched, and digested with trypsin as described above.

Isolation of Product Fragment. The 5mer product fragment (TQQTK) from the trypsin digestion was isolated by HPLC. Repeated injections were performed on a semi-preparative Phenomenex® Luna C18 100Å column (5 µm, 250 x 10 mm), which was equilibrated in 5% MeCN in H_2O (+0.1% formic acid). The peptide was eluted with a gradient of 5-49% MeCN over 20 minutes at a flow rate of 2.5 mL/min. Fractions were collected at 0.1 min intervals during the first 10 minutes. Each fraction was tested by HPLC-MS and fractions containing the expected product 5mer fragment were pooled and dried by lyophilization. The lyophilized material was resuspended in H_2O (+0.1% formic acid) and repeated injections were performed on an analytical Phenomenex® Synergi™ Fusion-RP 80Å column (4 µm, 100 x 4.6 mm), which was equilibrated with 100% H_2O (+0.1% formic acid). The peptide was eluted with a gradient of 0-20% MeCN over 5 minutes at a flow rate of 0.5 mL/min. Fractions containing the pure product were pooled and lyophilized.

Enzymatic Activity Assays with TqqA Variants. Enzymatic assays for TqqA substrate variants were performed as described above but were incubated overnight. The peptides were then digested with the GluC protease as described above; subsequently HR-MS and HR-MS/MS analysis was carried out on the 17mer fragment. For quantification of conversion yield relative to wt TqqA, reactions were quenched with and equal volume of MeCN (+1% formic acid) and analyzed by HR-HPLC-MS. For each variant, the extracted ion chromatogram of the three most abundant charge states for the substrate and product was integrated and the product/substrate ratio determined relative to the ratio obtained for the wt TqqA reaction (see Figure 5 and Table S7). Yields reported are based on quantification of the most abundant charge state, though very similar values (± 10% of the reported yield) were obtained from quantification of other charge states.

Table S1. Strains and plasmids used and generated in this study.

Strain/Plasmid	Purpose	Source
E. coli DH5α	Host strain for cloning	NEB
E. coli BL21(DE3)	Host strain for expression	NEB
pET-28b(+)	Kan^R, Expression vector	Novagen
pDB1282	*isc* operon	Prof. Dennis Dean & Prof. Squire Booker
pET-28b(+)_*tqqB*	Kan^R, Expression of TqqB WT	This study
E. coli BL21(DE3) + pET-28b(+)_*tqqB* + pDB1282	Expression of TqqB WT	This study
pET-28b(+)_*tqqB_C335A/C353A*	Kan^R, Expression of TqqB C335A/C353A	This study
E. coli BL21(DE3) + pET-28b(+)_*tqqB_C335A/C353A* + pDB1282	Expression of TqqB C335A/C353A	This study
pET-28b(+)_*tqqB_C112A/C119A*	Kan^R, Expression of TqqB C112A/C119A	This study
E. coli BL21(DE3) + pET-28b(+)_*tqqB_C112A/C119A* + pDB1282	Expression of TqqB C112A/C119A	This study

Table S2. Primers used in this study.

tqqB _F (NdeI)	TCCTGGCATATGAAGTTCATGCTGAATTGTGAC
*tqqB*_R (BamHI)	TCGATTGGATCCTTATTTTTTCTCAACCATTTTGTAAATTTCC
*tqqB_C335A/C353A*_F	CAACGAGAGCGGCCATATCAAACCGGCCACCTTTTTTCCGGACGGC
*tqqB_C335A/C353A*_R	ATGGTCCATTCGATAACACCGGCGCCAGCGCGCATGGTGCCATCAAC
*tqqB_C112A/C119A*_F	TGTACCCATGCCTTCAAAGAAGCTGGTCCG
*tqqB_C112A/C119A*_R	CTGTAAATGGGCCTTGTTGGTCAGTTCAATAAC

Table S3. Iron and labile sulfide quantification for wt and mutant TqqB after the reconstitution reaction. Shown are the averages of three independent measurements with the standard deviation (S.D.) noted in parentheses.

Protein	**Fe/protomer (S.D.)**	**S^{2-}/protomer (S.D.)**
TqqB WT	8.66 (0.01)	8.49 (0.29)
TqqB C112A/C119A	4.69 (0.04)	4.91 (0.34)
TqqB C335A/C353A	4.44 (0.06)	3.90 (0.31)

Table S4. Substrate and product HR-MS data before and after protease digestion.

Fragment		Calc m/z	Obs m/z	Δppm
MSVIEFKKLSSKLLKVSKIETLSGCGTKRTQQTKGWY (Substrate)		$[M+3H]^{3+}$: 1401.77259	1401.78407	8.2
		$[M+4H]^{4+}$: 1051.58126	1051.59087	9.1
		$[M+5H]^{5+}$: 841.46646	841.47257	7.3
		$[M+6H]^{6+}$: 701.38993	701.39630	9.1
		$[M+7H]^{7+}$: 601.33527	601.34053	8.8
MSVIEFKKLSSKLLKVSKIETLSGCGTKRTQQTKGWY (Product)		$[M+4H]^{4+}$: 1051.07735	1051.08558	7.8
		$[M+5H]^{5+}$: 841.06333	841.06975	7.6
		$[M+6H]^{6+}$: 701.05399	701.05912	7.3
		$[M+7H]^{7+}$: 601.04732	601.05222	8.2
GluC Digestion	MSVIE	$[M+H]^{1+}$: 578.28543	578.28829	5.0
		$[M+2H]^{2+}$: 289.64635	289.64453	6.3
	FKKLSSKLLKVSKIE	$[M+2H]^{2+}$: 874.55585	874.55650	0.7
		$[M+3H]^{3+}$: 583.37299	583.37717	7.2
	TLSGCGTKRTQQTKGWY (Substrate)	$[M+2H]^{2+}$: 957.97817	957.97230	6.1
		$[M+3H]^{3+}$: 638.98787	638.98704	1.3
		$[M+4H]^{4+}$: 479.49272	479.49519	5.2
	TLSGCGTKRTQQTKGWY (Product)	$[M+2H]^{2+}$: 956.97035	956.97008	0.3
		$[M+3H]^{3+}$: 638.31595	638.31599	4.3
Trypsin Digestion	MSVIEFK	$[M+H]^{1+}$: 853.44880	853.45014	1.6
	LSSK	$[M+H]^{1+}$: 434.26092	434.26501	9.4
	LLK	$[M+H]^{1+}$: 373.28093	373.28367	7.3
	VSK	$[M+H]^{1+}$: 333.21325	333.21376	1.5
	IETLSGCGTK	$[M+H]^{1+}$: 1008.50302	1008.49776	5.2
	TQQTK (Substrate)	$[M+H]^{1+}$: 605.32532	605.32393	2.3
		$[M+2H]^{2+}$: 303.16634	303.16447	6.2
	TQQTK (Product)	$[M+H]^{1+}$: 603.30967	603.31166	3.3
		$[M+H]^{2+}$: 302.158515	302.15559	9.7
	GWY	$[M+H]^{1+}$: 425.18195	425.18582	9.1

Table S5. Complete NMR assignments (from spectra obtained in D_2O) for the synthetic 5mer substrate and the 5mer derived from the trypsin-cut product of the TqqB reaction. Carbonyl signals for all but residues Q31 and Q32 were not observed in the HMBC spectrum.

$TqqA_{30-34}$		**Substrate**		**Product**	
		δH	δC	δH	δC
T30	C	-	167.96	-	Not Obs.
	α	3.80	58.40	3.42	63.86
	β	4.06	66.15	4.09	64.94
	γ1	1.21	18.70	1.14	19.26
Q31*	C	-	172.77	-	173.79
	α	4.32	53.09	-	77.76
	β	2.01, 1.91	26.73	2.08	31.93
	γ	2.28	30.84	2.27	28.60
	Cδ	-	177.70	-	177.76
Q32*	C	-	172.77	-	Not Obs.
	α	4.32	53.09	4.29	53.44
	β	2.01, 1.91	26.73	2.01, 1.93	26.54
	γ	2.28	30.84	2.26	31.16
	Cδ	-	177.70	-	177.50
T33	C	-	170.79	-	Not Obs.
	α	4.24	59.08	4.19	59.12
	β	4.12	67.08	4.07	67.14
	γ1	1.11	18.79	1.05	18.80
K34	C	-	178.08	-	Not Obs.
	α	4.09	54.81	4.03	55.09
	β	1.73, 1.63	30.85	1.69, 1.59	31.01
	γ	1.29	21.86	1.24	22.01
	δ	1.57	26.25	1.54	26.33
	ε	2.88	39.08	2.84	39.26

***** Substrate glutamine signals are interchangeable

Table S6. Complete NMR assignments (from spectra obtained in 95:5 H_2O/D_2O) for the synthetic 5mer substrate and the 5mer derived from the trypsin-cut product of the TqqB reaction. An HMBC spectrum was not obtained so no shifts were assigned for quaternary carbons. Only TOCSY and ROESY spectra were recorded for the 5mer substrate.

$TqqA_{30-34}$		**Substrate**	**Product**	
		δH	δH	δC
T30	N	Not Obs.	Not Obs.	-
	α	3.78	3.45	64.48
	β	4.05	4.15	65.15
	γ1	1.22	1.18	19.43
Q31	N	8.94	8.91	-
	α	4.32	-	Not Obs.
	β	1.91, 1.57	2.11	31.95
	γ	2.28	2.31	28.71
	N	6.91, 7.59	6.86, 7.62	-
Q32	N	8.72	8.67	-
	α	4.32	4.32	53.45
	β	2.01, 1.91	2.04, 1.97	26.41
	γ	2.28	2.28	31.02
	N	6.91, 7.59	6.86, 7.62	-
T33	N	8.40	8.40	-
	α	4.24	4.21	59.37
	β	4.13	4.10	67.14
	γ1	1.11	1.08	18.80
K34	N	8.12	8.12	-
	α	4.07	4.06	55.09
	β	1.63, 1.71	1.62, 1.71	30.96
	γ	1.30	1.27	21.95
	δ	1.57	1.57	26.44
	ε	2.88	2.88	39.49
	N	7.50	Not Obs.	Not Obs.

Table S7. HR-MS data for the products of the TqqB reaction with T30 and Q31 variants. The average conversion yields (± S.D.) of three independent measurements are shown using the dominant charge state. In addition, the percent conversion was determined from the next two most abundant charge states and was found to be within 10% of the reported values below.

Peptide	$[M+5H]^{5+}$: Calc m/z -2H (Product)	$[M+5H]^{5+}$: Obs m/z	∆ppm	% Conversion Relative to WT
Q31D	838.45701	838.47229	18.2	35 ± 1
Q31E	841.26014	841.26705	8.2	73 ± 1
Q31N	838.26020	838.27214	14.2	81 ± 6
Q31A	829.65904	829.67362	17.6	101 ± 8
Q31G	826.85591	826.86856	15.3	66 ± 4
T30S	838.26020	838.27569	18.5	16 ± 1
T30C	841.85877	n/a	n/a	0
Q31N-Me-Q	843.86646	843.86180	5.5	97 ± 10

Table S8. HR-MS/MS data for the products of the TqqB reaction with wt TqqA as well as T30 and Q31 variants after GluC treatment.

A. WT TqqA

Ion	Calc m/z	Obs m/z	∆ppm	Sequence
b_2^{+1}	215.13906	215.12682	56.9	TL
b_3^{+1}	302.17109	302.17256	4.9	TLS
b_9^{+1}	904.46696	904.46314	4.2	TLSGCGTKR
b_{11}^{+1} -2H	1131.55756	1131.55502	2.2	TLSGCGTKRTQ
b_{14}^{+2} -2H	744.883055	744.88018	3.9	TLSGCGTKRTQQTK
b_{15}^{+2} -2H	773.393785	773.38697	8.8	TLSGCGTKRTQQTKG
b_{16}^{+2} -2H	866.433435	866.43014	3.8	TLSGCGTKRTQQTKGW
b_{17}^{+2} -2H	947.965105	947.96209	3.2	TLSGCGTKRTQQTKGWY
y_1^{+1}	182.08121	182.0621	105	Y
y_2^{+1}	368.16053	368.15775	7.6	WY
y_4^{+1}	553.27695	553.27767	1.3	KGWY
y_5^{+1}	654.32463	654.32756	4.5	TKGWY

y_6^{+1}	782.38321	782.38531	2.7	QTKGWY
y_{15}^{+2} **-2H**	849.904515	849.89962	5.8	SGCGTKRTQQTKGWY

B. Q31D-TqqA

Ion	Calc m/z	Obs m/z	∆ppm	Sequence
b_2^{+1}	215.13906	215.12687	56.7	TL
b_{17}^{+2} **-2H**	941.449285	941.45138	2.2	TLSGCGTKRTEQTKGWY
y_1^{+1}	182.08121	182.06295	100.3	Y
y_2^{+1}	368.16053	368.16167	3.1	WY
y_4^{+1}	553.27695	553.28205	9.2	KGWY

C. Q31E-TqqA

Ion	Calc m/z	Obs m/z	∆ppm	Sequence
b_2^{+1}	215.13906	215.12738	54.3	TL
b_3^{+1}	302.17109	302.16833	9.13	TLS
b_9^{+1}	904.46696	904.46461	2.6	TLSGCGTKR
b_{14}^{+2} **-2H**	745.375055	745.37436	1.0	TLSGCGTKRTEQTK
b_{15}^{+2} **-2H**	773.885785	773.88648	0.9	TLSGCGTKRTEQTKG
b_{16}^{+2} **-2H**	866.925445	866.92506	0.4	TLSGCGTKRTEQTKGW
b_{17}^{+2} **-2H**	948.457115	948.45702	0.1	TLSGCGTKRTEQTKGWY
y_1^{+1}	182.08121	182.0639	95.1	Y
y_2^{+1}	368.16053	368.16211	4.3	WY
y_4^{+1}	553.27695	553.27930	4.3	KGWY
y_5^{+1}	654.32463	654.32616	2.3	TKGWY
y_{14}^{+2} **-2H**	806.880505	806.88034	0.2	GCGTKRTEQTKGWY
y_{15}^{+2} **-2H**	850.396525	850.39676	0.3	SGCGTKRTEQTKGWY
y_{16}^{+2} **-2H**	906.938555	906.94951	12.1	LSGCGTKRTEQTKGWY

D. Q31N-TqqA

Ion	Calc m/z	Obs m/z	∆ppm	Sequence
b_2^{+1}	215.13906	215.12737	54.3	TL
b_3^{+1}	302.17109	302.16959	5.0	TLS
b_{11}^{+1} -2H	1117.54191	1117.5327	8.2	TLSGCGTKRTN
b_{14}^{+2} -2H	737.875225	737.87481	0.6	TLSGCGTKRTNQTK
b_{15}^{+2} -2H	766.385955	766.38303	3.8	TLSGCGTKRTNQTKG
b_{16}^{+2} -2H	859.425615	859.42222	4.0	TLSGCGTKRTNQTKGW
b_{17}^{+2} -2H	940.957275	940.94929	8.5	TLSGCGTKRTNQTKGWY
y_1^{+1}	182.08121	182.06328	98.4	Y
y_2^{+1}	368.16053	368.16304	6.8	WY
y_4^{+1}	553.27695	553.27831	2.5	KGWY
y_6^{+1}	782.38321	782.38059	3.4	QTKGWY
y_{14}^{+2} -2H	799.380675	799.38042	0.3	GCGTKRTNQTKGWY
y_{15}^{+2} -2H	842.896685	842.89502	2.0	SGCGTKRTNQTKGWY
y_{16}^{+2} -2H	899.438725	899.45937	23.0	LSGCGTKRTNQTKGWY

E. Q31A-TqqA

Ion	Calc m/z	Obs m/z	∆ppm	Sequence
b_2^{+1}	215.13906	215.127	56.1	TL
b_9^{+1}	904.46696	904.46530	1.8	TLSGCGTKR
b_{12}^{+1} -2H	1202.59466	1202.58752	5.9	TLSGCGTKRTAQ
b_{14}^{+1} -2H	1431.73732	1431.7232	9.9	TLSGCGTKRTAQTK
b_{15}^{+2} -2H	744.883055	744.88183	1.6	TLSGCGTKRTAQTKG
b_{16}^{+2} -2H	837.922705	837.91313	11.	TLSGCGTKRTAQTKGW
b_{17}^{+2} -2H	919.454375	919.44982	5.0	TLSGCGTKRTAQTKGWY
y_1^{+1}	182.08121	182.06237	103.5	Y
y_2^{+1}	368.16053	368.16082	0.8	WY
y_4^{+1}	553.27695	553.27671	0.4	KGWY

y_{14}^{+2} -2H	777.877765	777.87447	4.2	GCGTKRTAQTKGWY
y_{15}^{+1} -2H	1641.78024	1641.76671	8.2	SGCGTKRTAQTKGWY

F. Q31G-TqqA

Ion	Calc m/z	Obs m/z	∆ppm	Sequence
b_2^{+1}	215.13906	215.12682	56.9	TL
b_9^{+1}	904.46696	904.45019	4.2	TLSGCGTKR
b_{14}^{+2} -2H	709.364495	709.36222	3.2	TLSGCGTKRTGQTK
b_{15}^{+2} -2H	737.875225	737.86978	7.4	TLSGCGTKRTGQTKG
b_{16}^{+2} -2H	830.914885	830.91549	0.7	TLSGCGTKRTGQTKGW
b_{17}^{+2} -2H	912.446545	912.44596	0.6	TLSGCGTKRTGQTKGWY
y_1^{+1}	182.08121	182.06292	100.4	Y
y_2^{+1}	368.16053	368.167012	17.6	WY
y_{15}^{+2} -2H	814.385955	814.382544	4.2	SGCGTKRTGQTKGWY

Table S9. Prevalence of TqqB. A BLAST analysis was carried out with TqqB. Several hits were identified with significant % identity to TqqB. The genomic context of each of was analyzed and several gene clusters were identified that contained both an ABC transporter and a precursor peptide with homology to TqqA.

Strain	NCBI Protein ID	% ID to TqqB	Precursor Sequence
Streptococcus azizii	ONK263421	58%	MVKMNLKNLSSKLLKIKSMPLSGCGTKRTQQTKGNLA
Atopobacter sp. AH10	WP_121565910.1	43%	MKQLSLYDLKAKLTIETGQSIRNCGTKRTKNTKLISF
Nosocomiicoccus massiliensis strain UMB0959	WP_102167102.1	37%	MSKKEFLKGLAERQSVKALGCGTSKTKNTKGPIPI
Anaerosalibacter massiliensis	WP_042679969.1	32%	MNKTNFFNYKASKITPKAKYCGPTVITICPDLVLSAARPKK
Bacillus cereus strain B4080	WP_046945647.1	31%	MNSNEFLALEDLLSRSLVDTLKRCGTRKTKRTS
Marinitoga hydrogenitolerans DSM 16785	WP_072866099.1	29%	MKNIKKFLKVVVYGGGCGTKRTKNTGSFTL

Figure S1: UV-Vis spectrum of purified and reconstituted TqqB. The absorption band at ~620 nm is most likely due to a contaminating protein, which we identified by in-gel digestion and mass spectrometry of the resulting fragments as YqjI.[3] This protein has been shown to bind Ni^{2+} and Fe^{2+}, which give the broad absorption feature at ~620 nm (after oxidation to Fe^{3+} in the case of iron), though at this point we cannot rule out that the feature is due to one or more auxiliary 2Fe-2S clusters.[2,3]

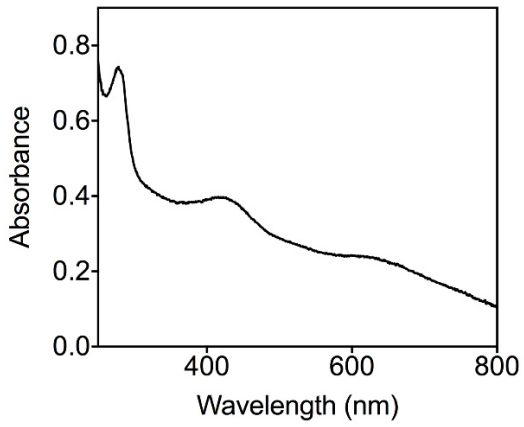


Figure S2: Alignment of C-terminal domains in TqqB and SuiB.[4] The Cys residues involved in ligation of AuxI and AuxII in SuiB are shown in red and blue, respectively. Residues proposed to be involved in ligation of the Aux cluster in TqqB are shown in red. All Cys residues in this domain are conserved between the 270 members of the TQQ subfamily. This alignment shows that TqqB has four Cys residues in its Twitch domain that could provide four ligands to the Aux cluster. However, crystallographic and/or spectroscopic studies are required to determine if all four available Cys ligands coordinate the Aux cluster or if there is an open coordination site for substrate binding, as depicted in our first mechanistic proposal.

```
TqqB 315-TWEEDNC321F--NYF----------EDIVDGTMRC335GAGVIEWTINESGHIKPC353TFFPDGEFSSYSLQNFEEYSM-374
SuiB 315-NFATEGC321HLFTAYPELINNSIEFSEFDEMYYGC347RAKYTKMEIMSNGDILPC365IAFLGVNQTKQN--AFEK----381

TqqB 375-QNHEQNIVRKINDWEGLLQTVG--------------L--STRNIC403EEIY--K--MVEKK----------------413
SuiB 382------D---LLDVWYDDPLYGGIRSFRTKNSKC406LSC409GLLKIC415EGGC419YVNLIKEKSPEYFRDSVC437QL---439

AuxI in SuiB
AuxII in SuiB
```

Figure S3. Time-dependent formation of 5'-dA in the reaction of TqqB with SAM and sodium dithionite in the absence of substrate. Quantification was carried out by integration of the area under the 5'-dA peak for a single replicate at each timepoint and comparison to a standard curve made in duplicate from an authentic standard.

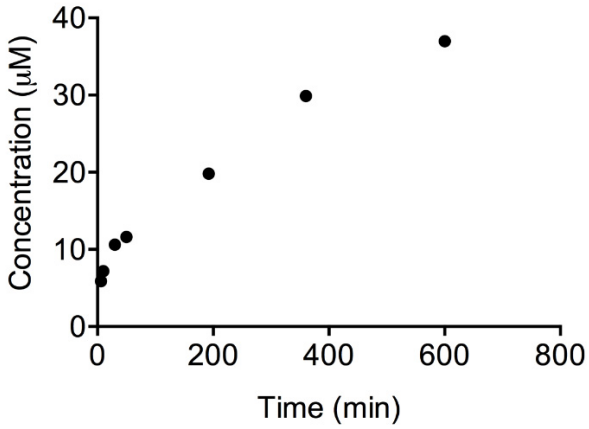


Figure S4. Time-dependent formation of product and 5'-dA in the presence of substrate from a single replicate at each timepoint. Shown is the integrated area of the extracted ion peak for the -2 Da product mass or 5'-dA as a function of time.

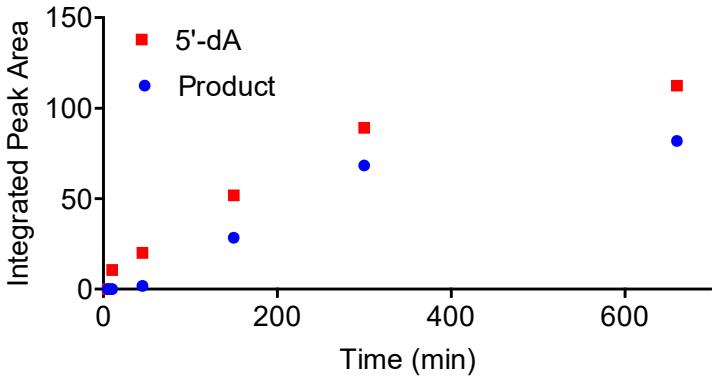


Figure S5. Enzymatic activity of TqqB active site cluster (C112A/C119A) and auxiliary cluster (C335A/C353A) mutants. Shown is the extracted ion count for the -2 Da product mass for each reaction.

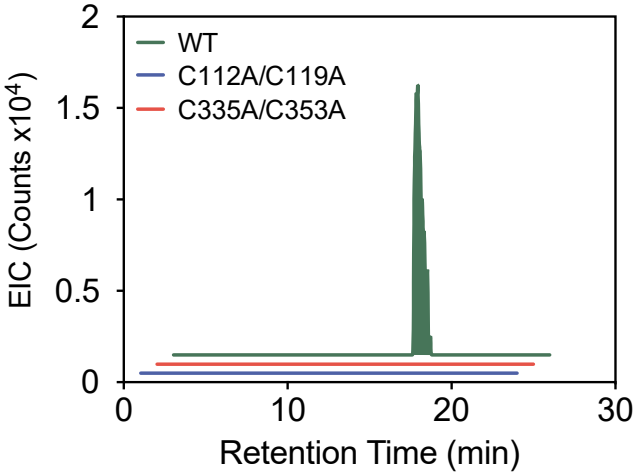


Figure S6. TOCSY slice (in H_2O/D_2O) for the substrate-T33 methyl group (A), product-T33 methyl group (B), substrate-K34 ε-^{1}Hs (C), product-K34 ε-^{1}Hs (D). No significant changes are observed between the substrate and product spectra for these residues.

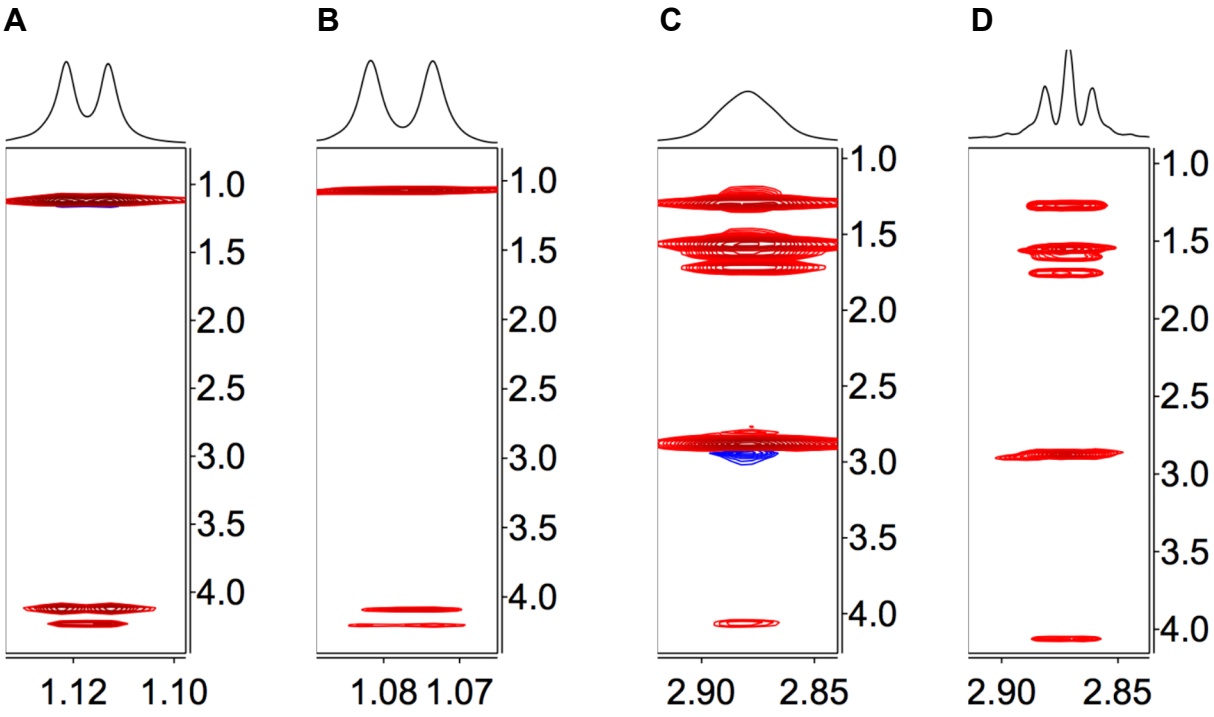
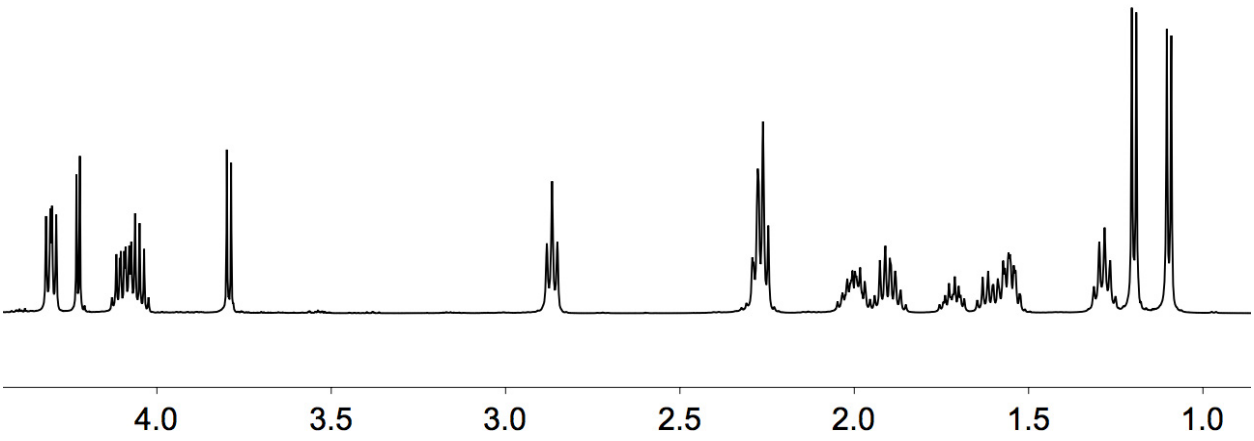
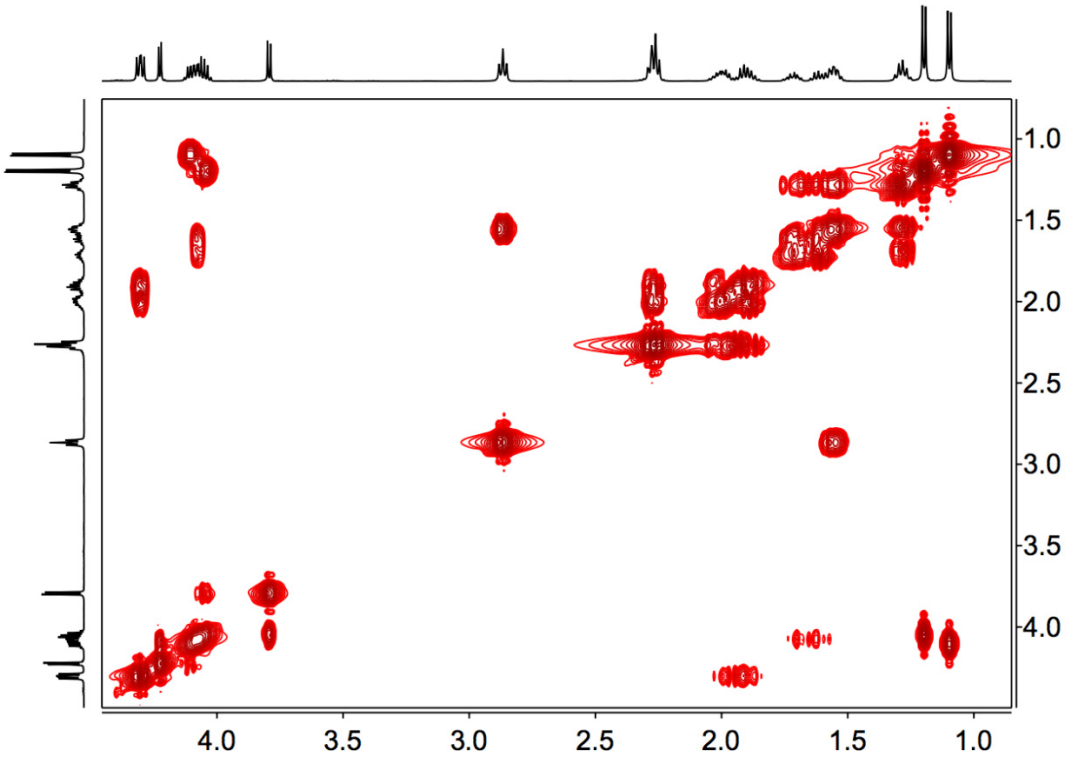


Figure S7. NMR spectra of the TqqA substrate in D_2O (pages S18–S20).

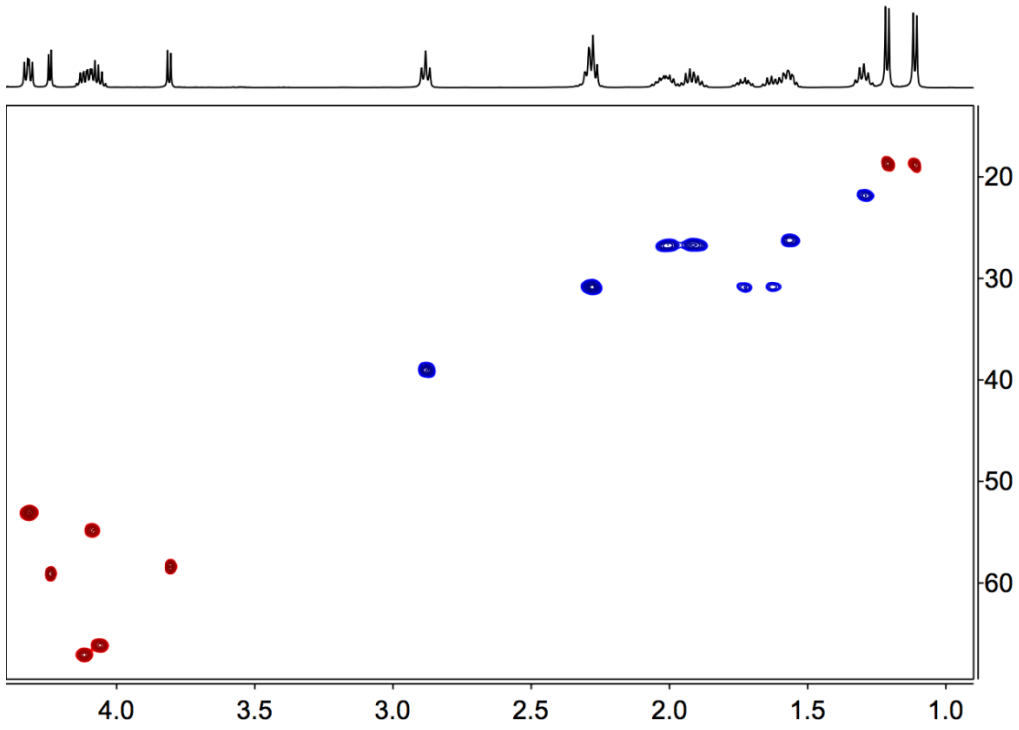
A. ^{1}H spectrum



B. COSY spectrum



C. HSQC spectrum



D. HMBC spectrum

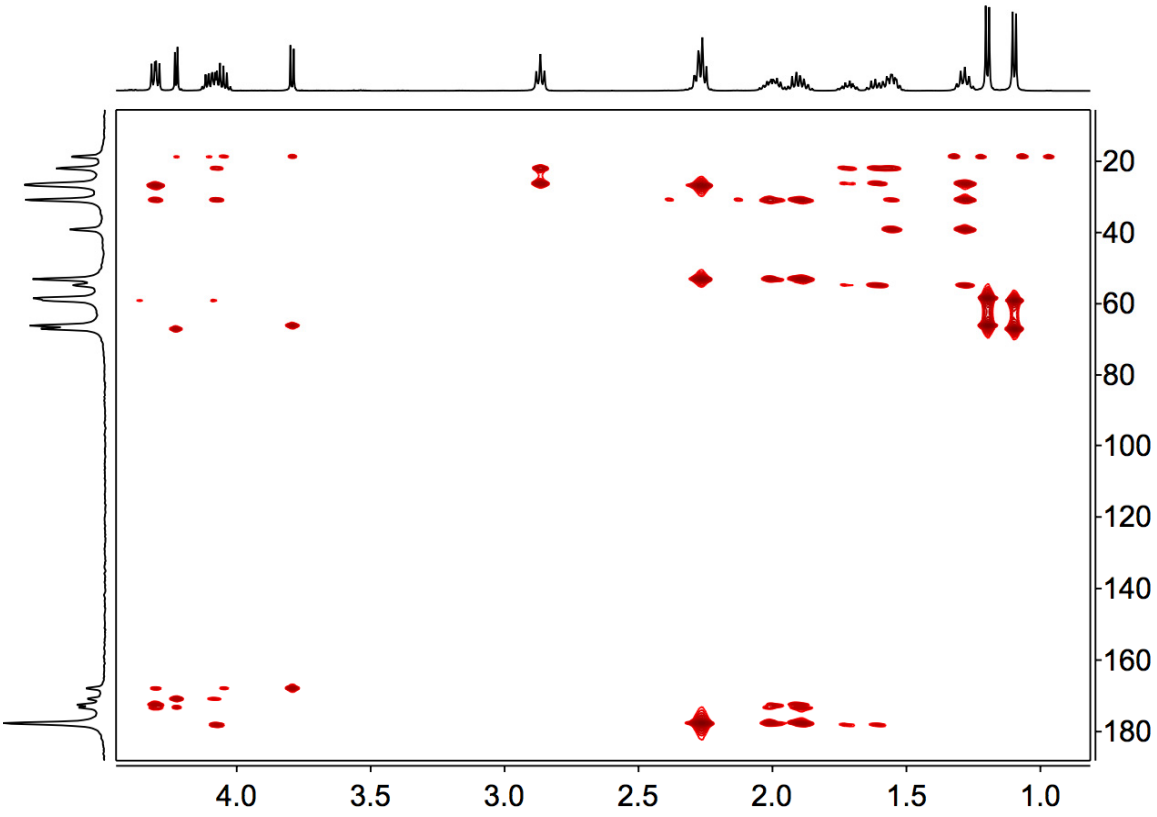
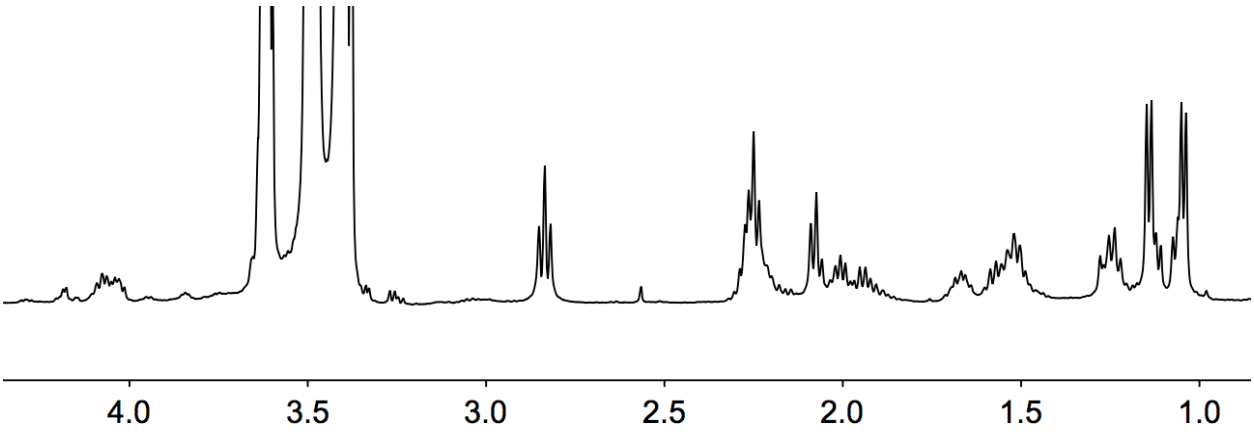
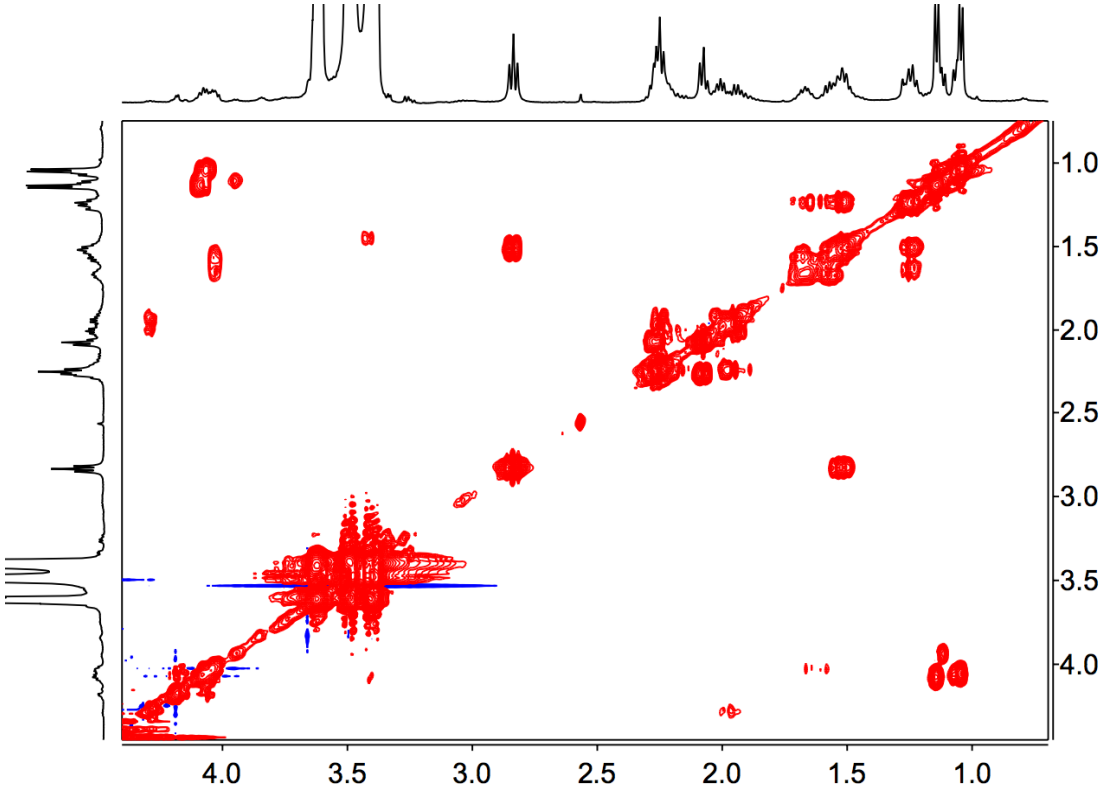


Figure S8. NMR spectra for the purified, cut product of TqqB in D_2O (pages S20–S22).

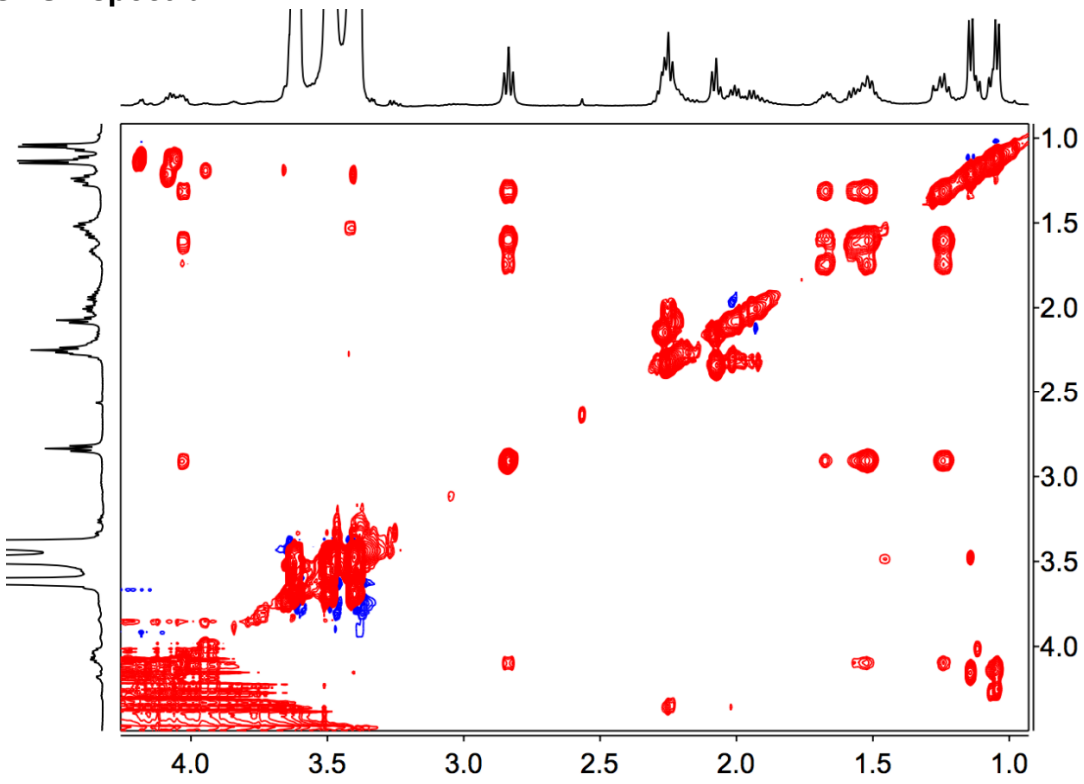
A. ^{1}H spectrum



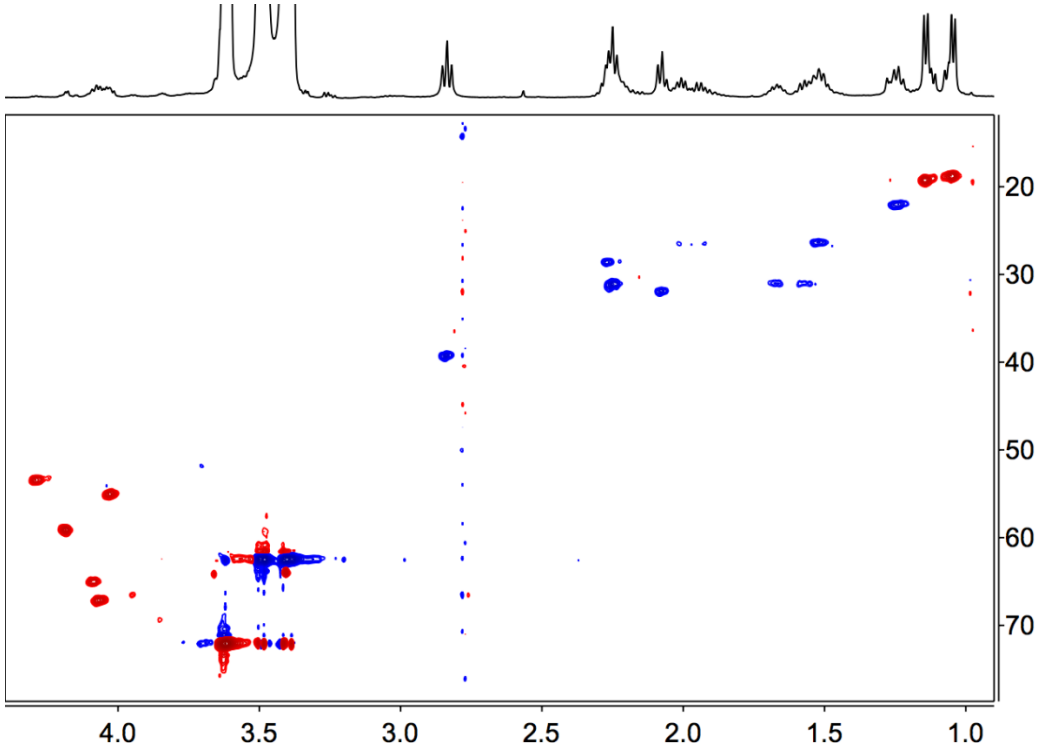
B. COSY spectrum



C. TOCSY spectrum



D. HSQC spectrum



E. HMBC spectrum

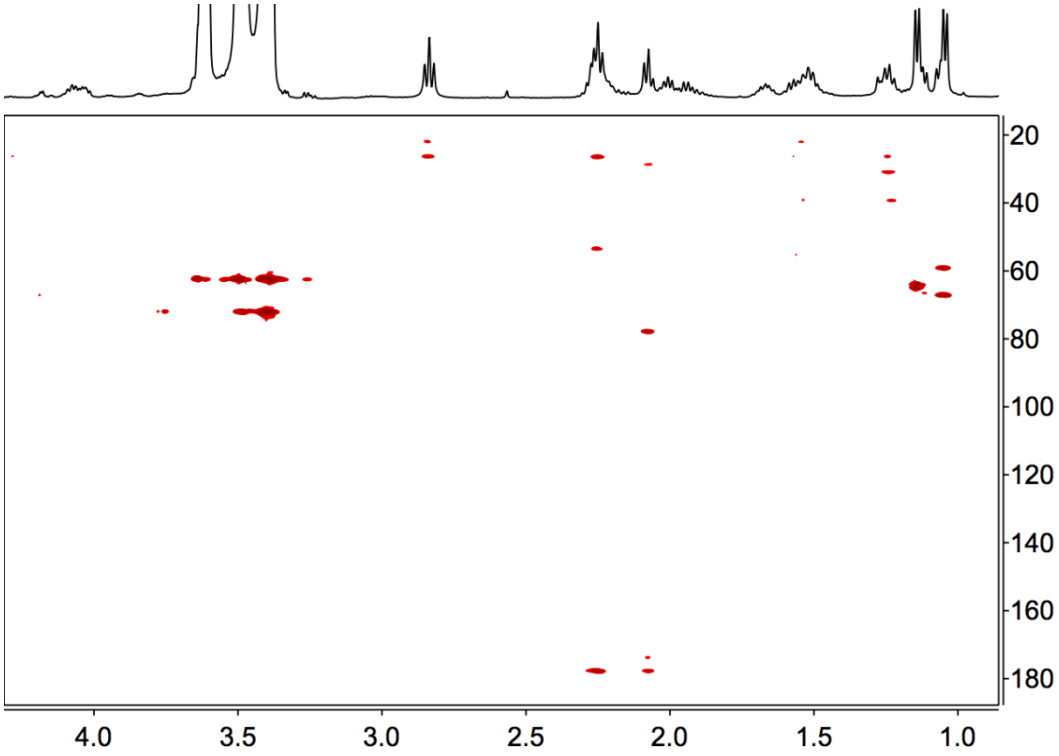
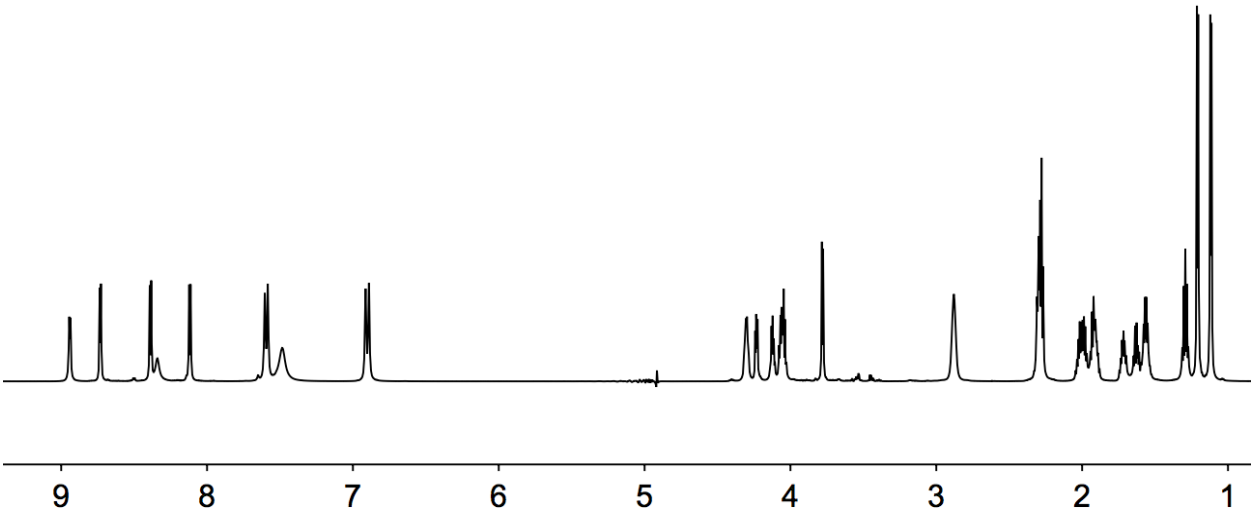
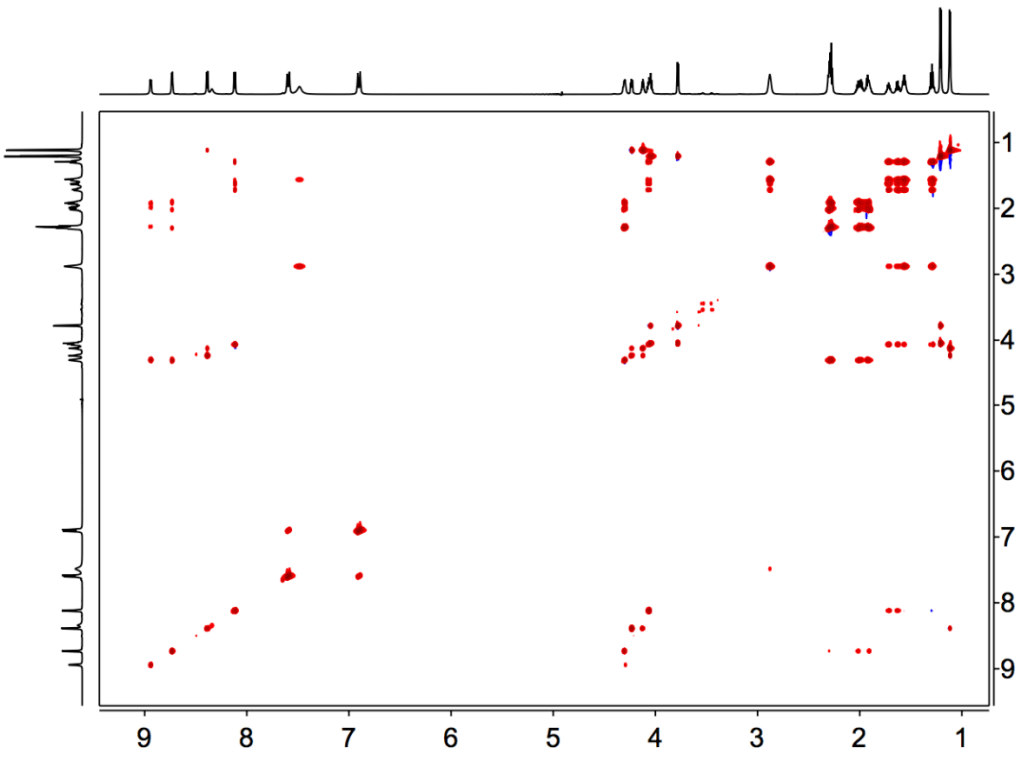


Figure S9. NMR spectra of TqqA in H_2O/D_2O (19:1, pages S23–S24) at 4°C.

A. ^{1}H spectrum



B. TOCSY spectrum



C. ROESY spectrum

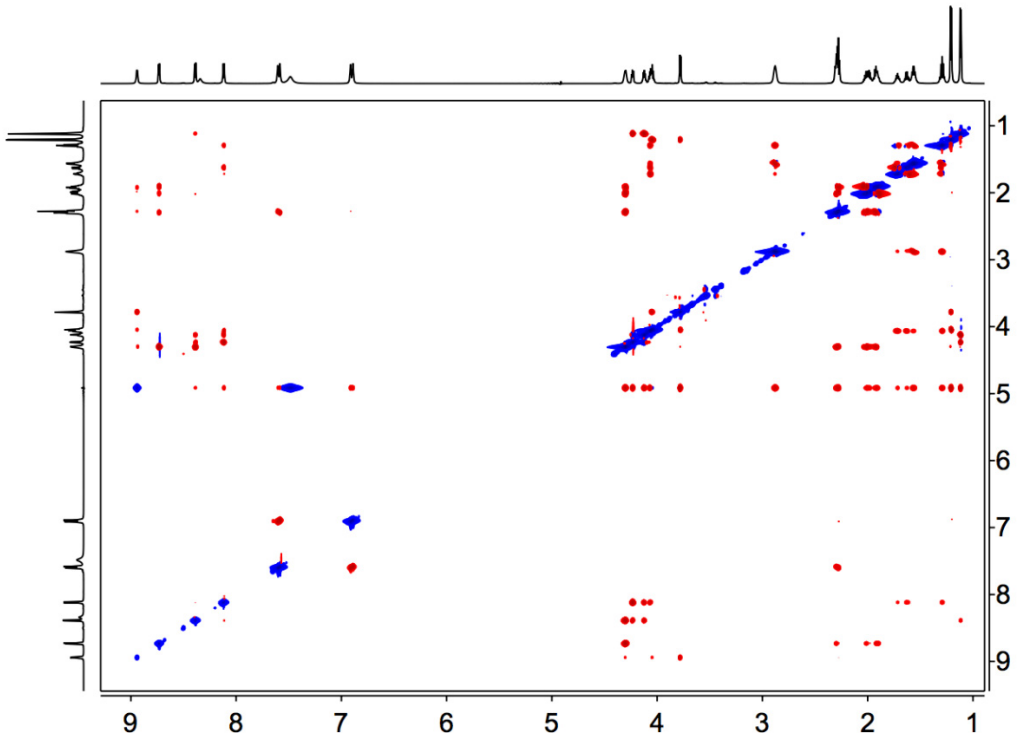
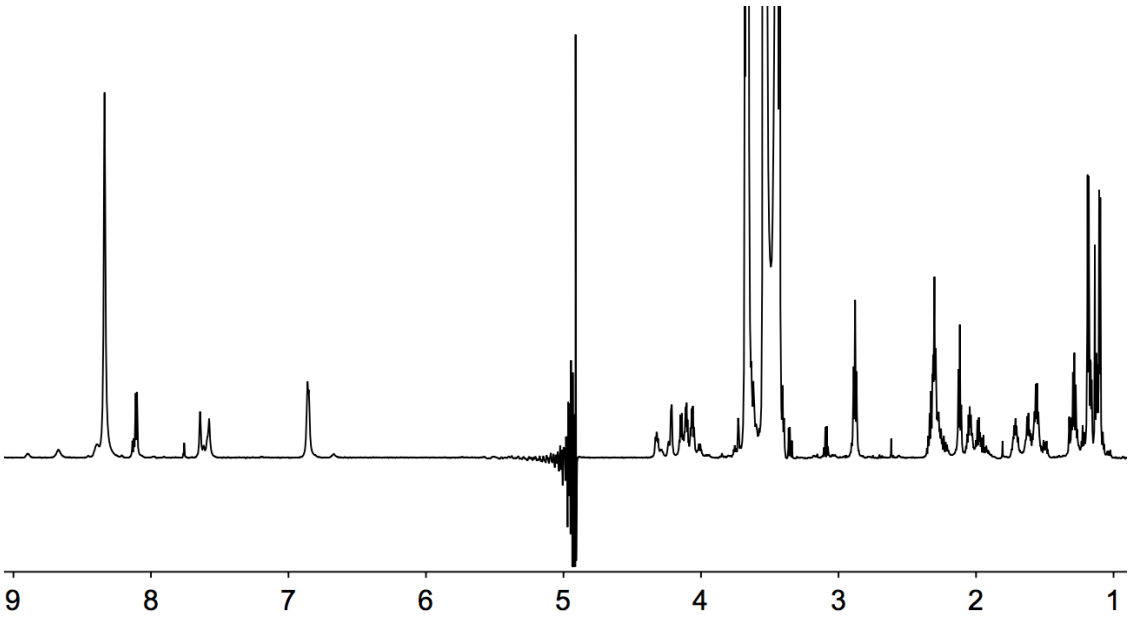
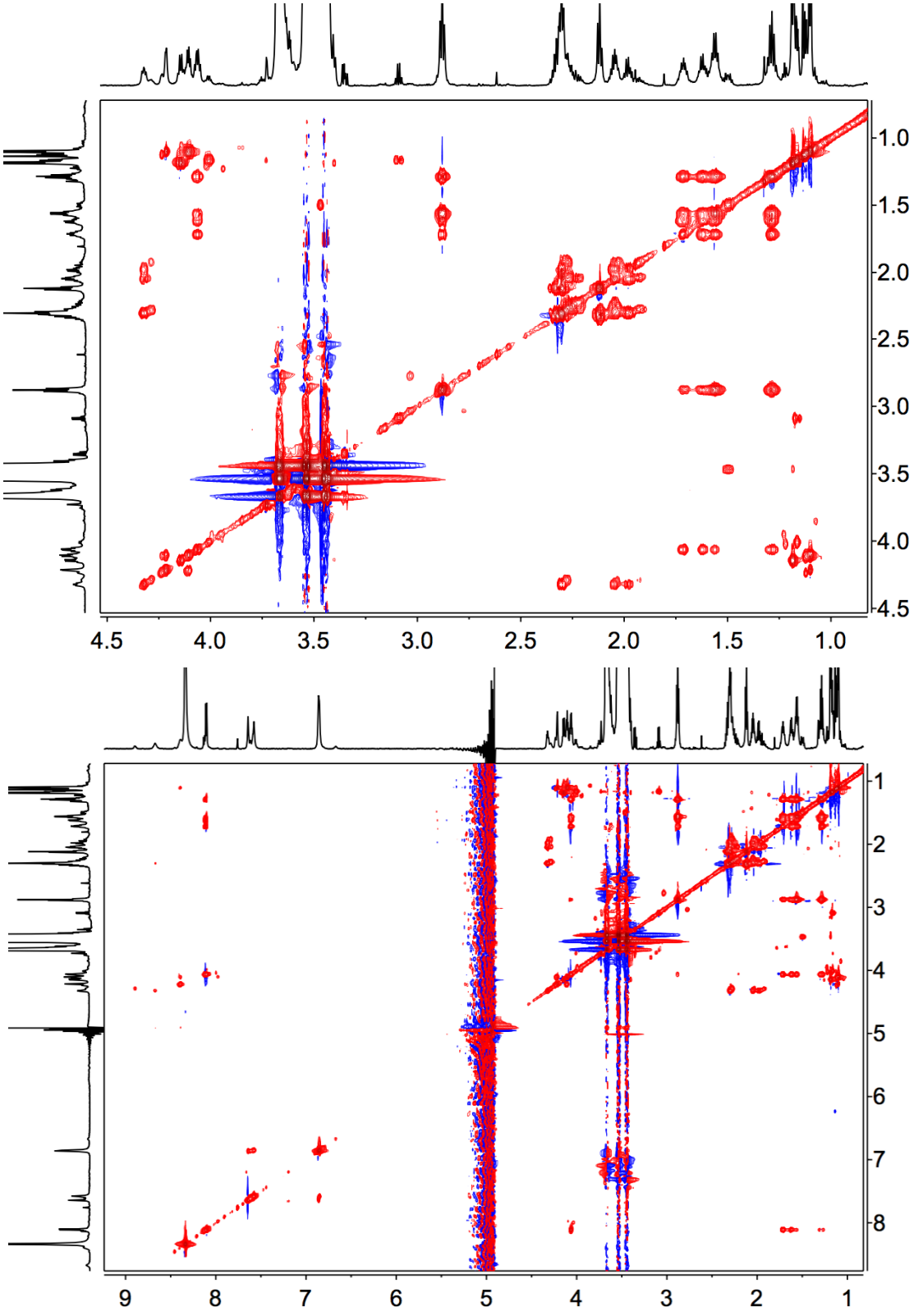


Figure S10. NMR spectra of the purified, cut TqqB product in H_2O/D_2O (19:1) at 4ºC (pages S24–S27).

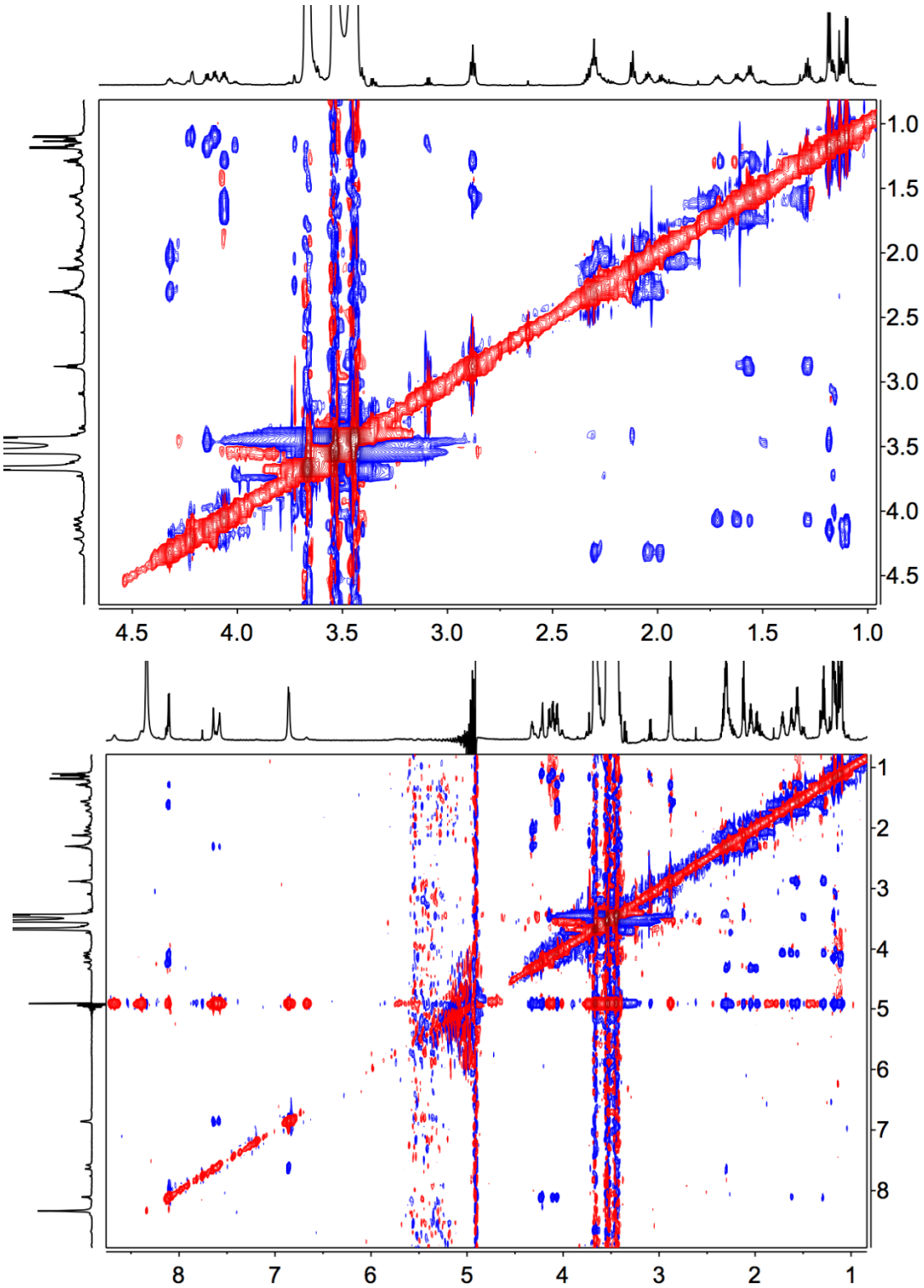
A. ^{1}H spectrum



B. TOCSY spectra



C. ROESY spectra



D. HSQC spectrum

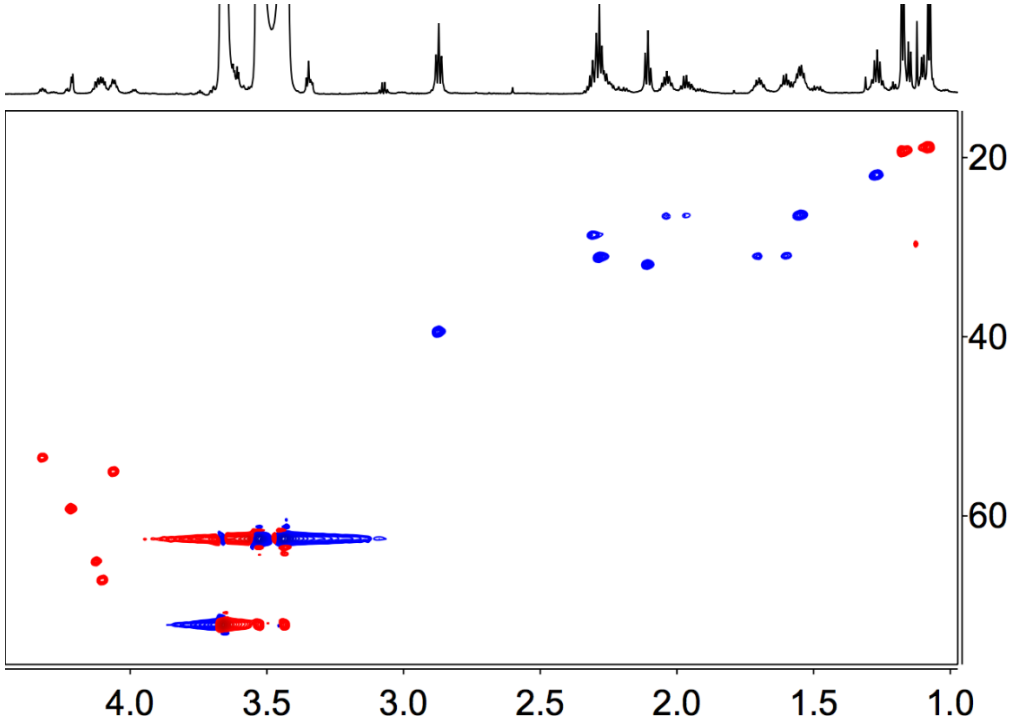
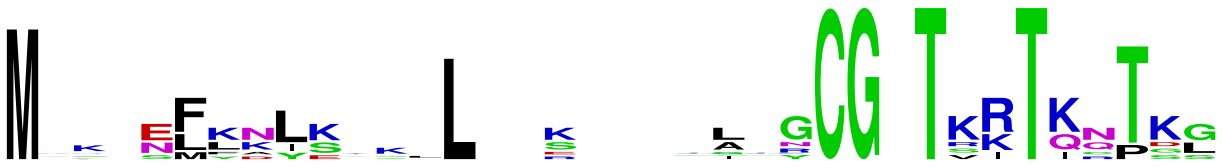


Figure S11. Logo plot of TqqA homologs. The precursor peptides from gene clusters homologous to the TQQ cluster (shown in Table S8) were aligned and a logo plot was generated.[5] The Thr residue involved in ether bond formation is strictly conserved along with a CG motif that may be the site of proteolysis to generate the mature peptide.



Supplementary References

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3. Wang, S.; Wu, Y.; Outten, F. W. Fur and the novel regulator YqjI control transcription of the ferric reductase gene *yqjH* in *Escherichia coli*. *J. Bacteriol.* **2011**, *193*, 563–574.
4. Davis, K. M.; Schramma, K. R.; Hansen, W. A.; Bacik, J. P.; Khare, S. D.; Seyedsayamdost, M. R.; Ando, N. Structures of the peptide-modifying radical SAM enzyme SuiB elucidate the basis of substrate recognition. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 10420–10425.
5. Crooks, G. E.; Hon, G.; Chandonia, J.-M.; Brenner, S. E. WebLogo: a sequence logo generator. *Genome Res.* **2004**, *14*, 1188–1190.