Supporting information

Use of a tyrosine analog to modulate the two activities of a non-heme iron enzyme OvoA in ovothiol biosynthesis, cysteine oxidation versus oxidative C-S bond formation

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1. ^{13}C-NMR spectra of wild-type OvoA catalyzed reactions

For the wild-type OvoA **primary** isotope effect assay, three enzymatic reactions were conducted as described in the experimental section.

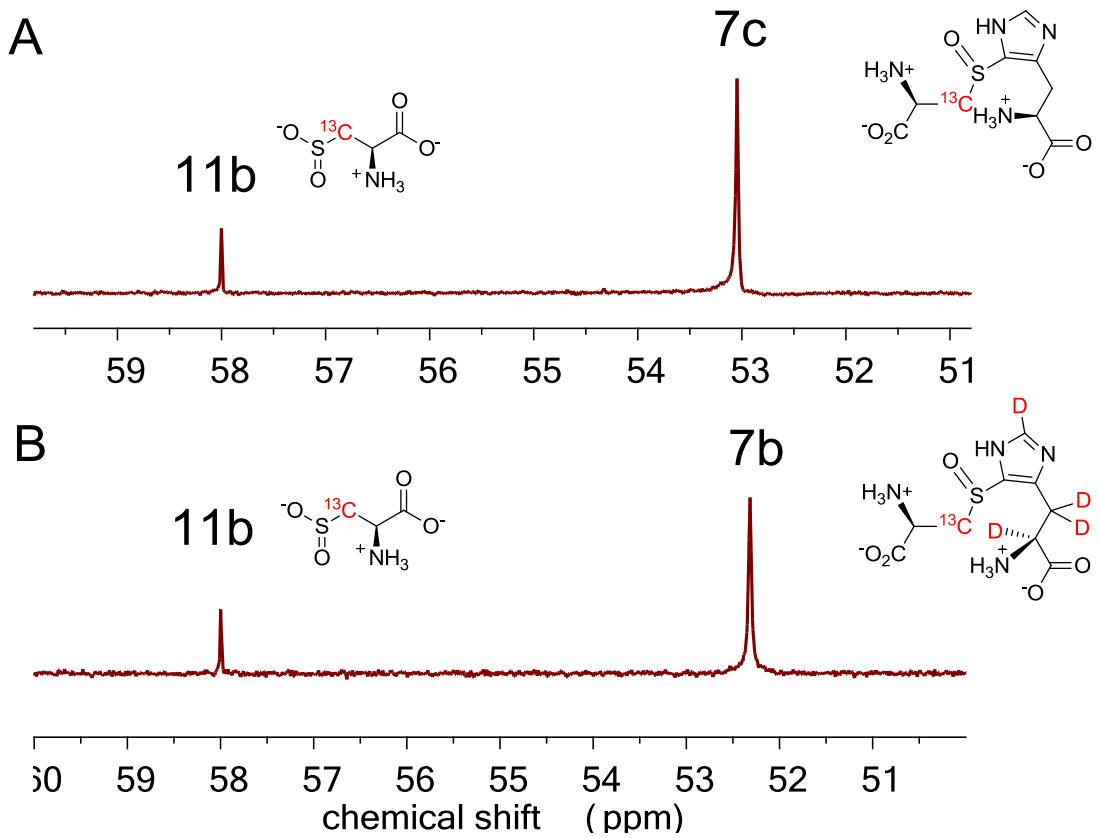


Figure S1. ^{13}C-NMR spectra of wild-type OvoA catalyzed reactions. A. ^{13}C-NMR spectrum of the reaction mixture using [β-^{13}C]-cysteine and unlabeled histidine as substrates; B. ^{13}C-NMR spectrum of the reaction mixture using [β-^{13}C]-cysteine and [U-$^{2}H_5$]-histidine as substrates.

For the wild-type OvoA **solvent** deuterium isotope effect assay, three enzymatic reactions were conducted as described in the experimental section.

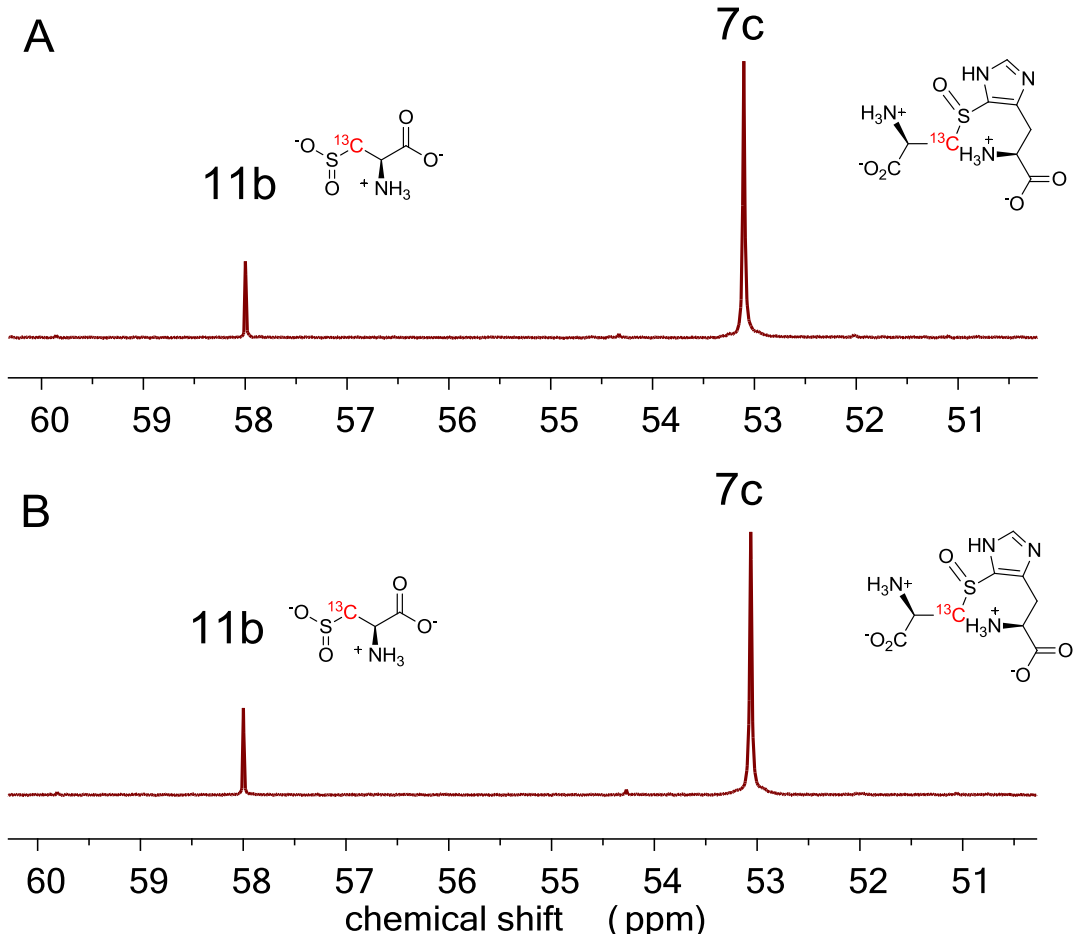


Figure S2. ^{13}C-NMR spectra of wild-type OvoA catalyzed reactions. **A**. ^{13}C-NMR spectrum of the reaction mixture using [β-^{13}C]-cysteine and unlabeled histidine as substrates in unlabeled buffer; **B**. ^{13}C-NMR spectrum of the reaction mixture using [β-^{13}C]-cysteine and unlabeled histidine as substrates in D_2O buffer.

2. OvoA structure prediction

Protein sequence alignment of *Erwinia tasmaniensis* OvoA (OvoA_erwinia, GenBank accession number WP_012439783) with EgtB from *Mycobacterium smegmatis* (EgtB_smegmatis, GenBank accession number WP_011731158) and *Mycobacterium thermoresistible* (EgtB_thermo GenBank accession number WP_050811957.1) were performed using T-Coffee.[1] The sequence alignment results show high sequencing similarity and the conserved active site tyrosine (highlighted in red box). OvoA structure prediction was carried out using I-TASSER[2] and the predicted OvoA structure was aligned with EgtB structure (PDB: 4X8B). The structural alignment results show an overall similarity between OvoA and EgtB active site with the conserved tyrosine active site in close proximity to the iron center.

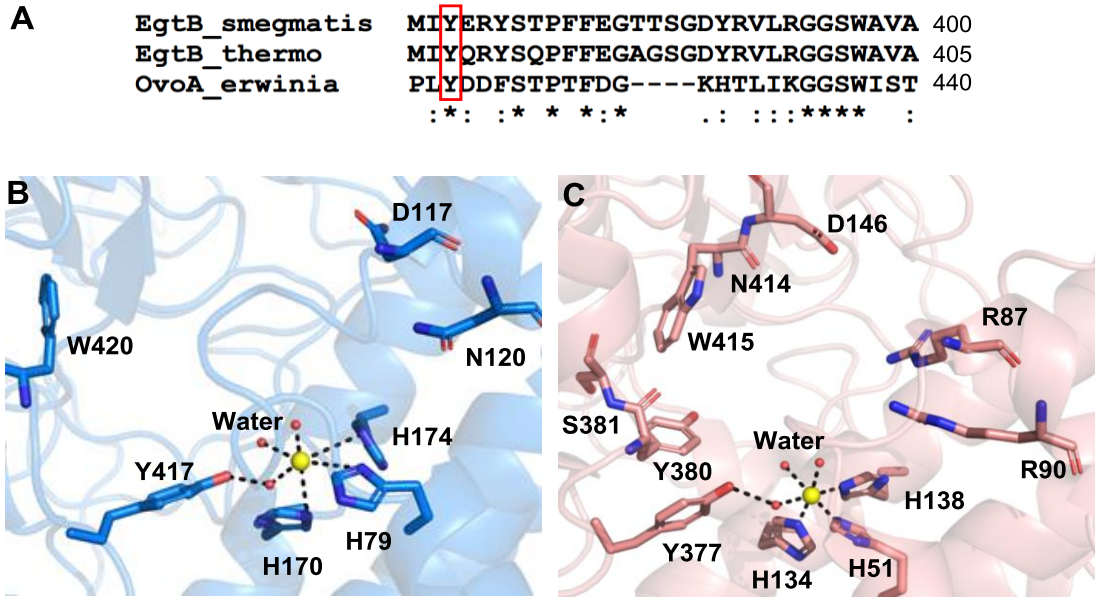


Figure S3. Sequence alignments of OvoA with EgtB and OvoA structure prediction. (A) Protein sequence alignment of OvoA with EgtB from *Mycobacterium smegmatis* and *Mycobacterium thermoresistible* showing high sequencing similarity and the conserved active site tyrosine (highlighted in red box). (B) Active site of predicted OvoA structure using I-TASSER, in comparison to that of EgtB (PDB: 4X8B) showing the conserved 3-histidines binding motif for the iron center and the active site tyrosine. OvoA is shown in blue while EgtB is in salmon. The iron center is in yellow and water ligand is presented in red sphere.

3. Construction and overexpression of OvoA Y417F

A Q5 site-directed mutagenesis kit was purchased from New England Biolabs. Site-directed mutagenesis based on the pPL-OvoA wild-type was carried out to generate OvoA Y417F using primer pair PL1431/PL1432 (primer sequences are shown in section 12). The protein (pPL-OvoA wild-type) was overexpressed and prepared with a same protocol of OvoA wild-type.[3]

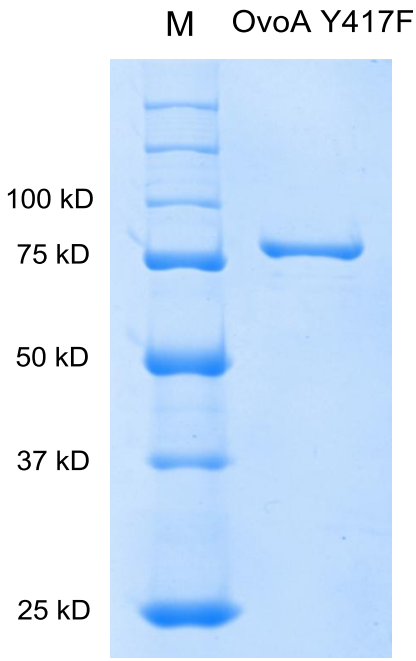


Figure S4. SDS-PAGE analysis of OvoA Y417F purified anaerobically.

4. Product profiles of OvoA and OvoA Y417F catalyzed reactions

Run reactions using unlabeled histidine and [β-^{13}C]-cysteine as substrates. The reaction mixture contained 20 mM air saturated KPi H_2O buffer at pH 8.0, 10 mM histidine, 10 mM cysteine, 3 mM DTT, 1 mM sodium ascorbate and 8 μM iron-reconstituted OvoA (or its variants). The reaction was stirred at 25℃ for 2 h. After the reaction was complete, the protein was removed by ultrafiltration. The filtrate was detected by ^{13}C-NMR.

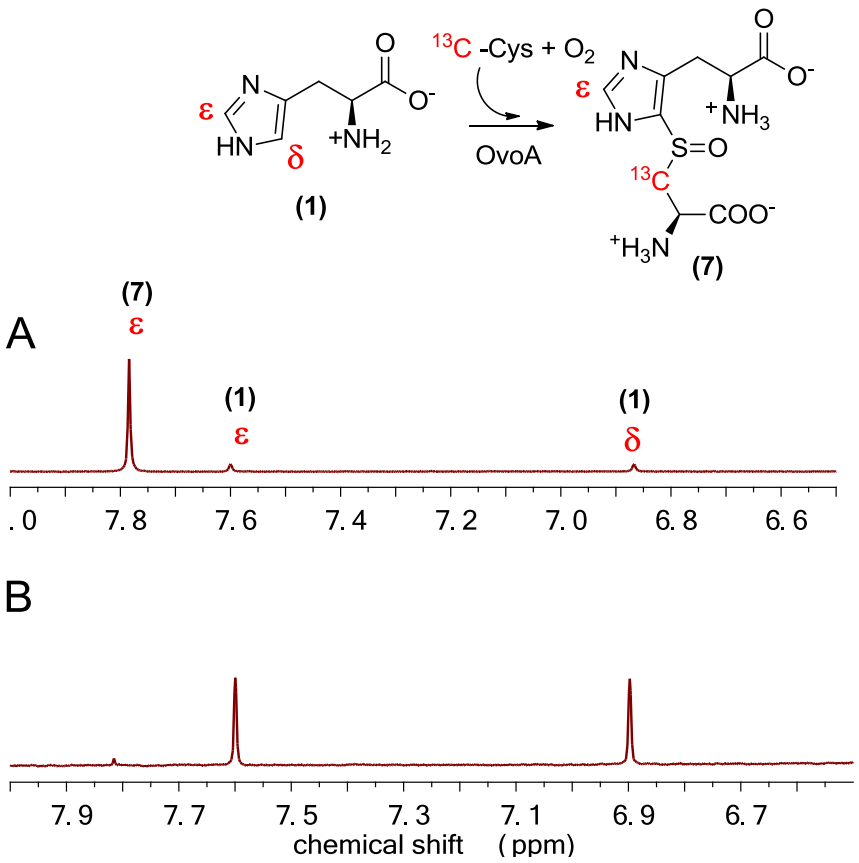


Figure S5. ^{1}H-NMR spectra of OvoA wild-type (A) and OvoA Y417F (B) reaction mixture.

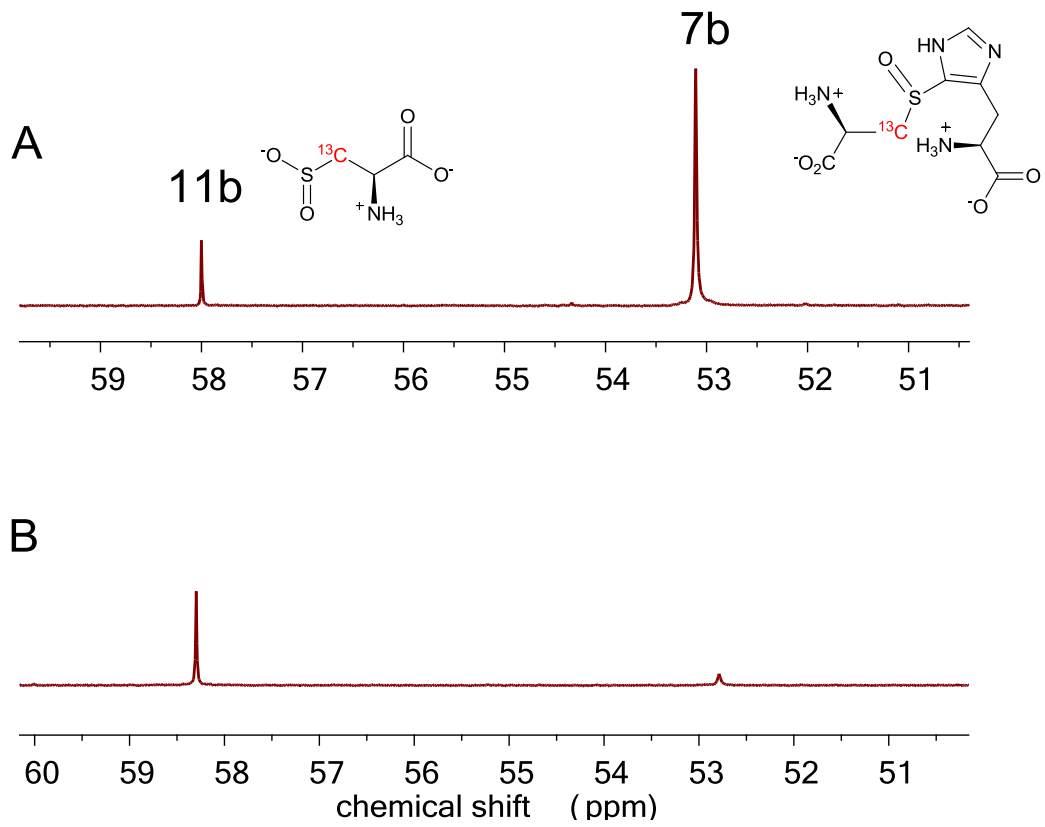
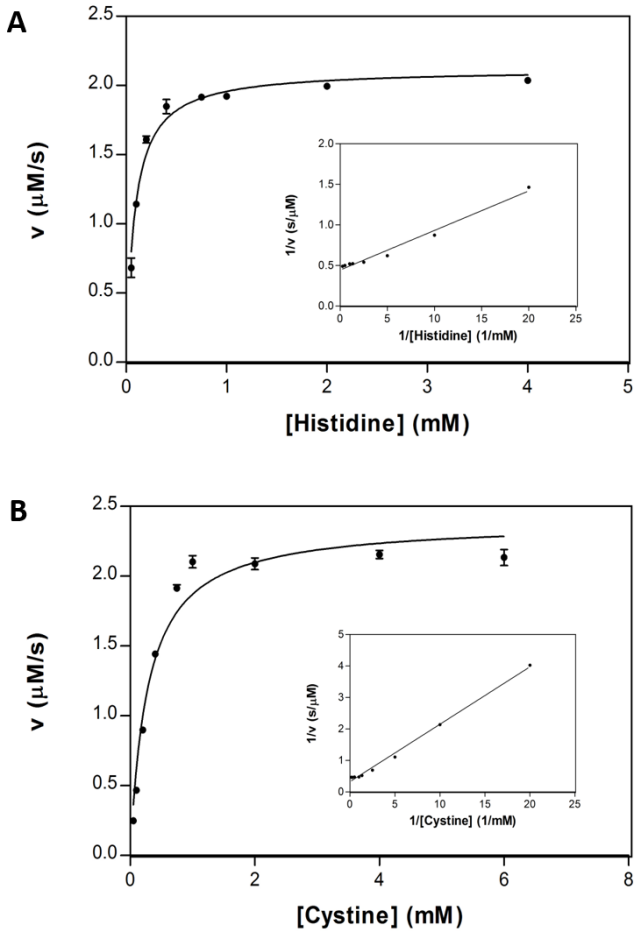


Figure S6. ^{13}C-NMR spectra of OvoA wild-type (A) and OvoA Y417F (B) reaction mixture.

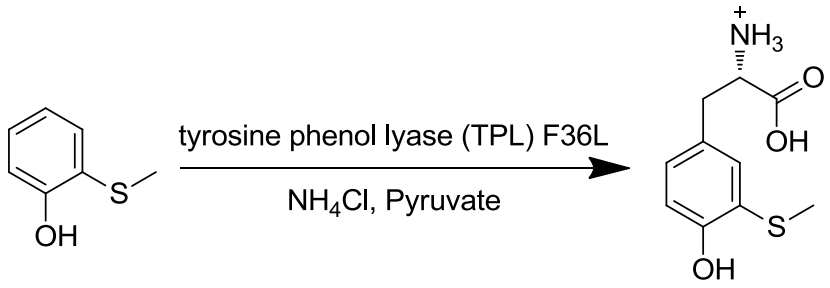
5. Kinetics of OvoA Y417F determined by Oxygen consumption assay



	K_m (histidine) μM	K_m (cystine) μM	k_{cat} (s^{-1})
OvoA Y417F	82.5± 7.1	279 ± 36	3.01± 0.05

Figure S7. OvoA steady-state kinetic analysis at 25℃. **A**. OvoA Y417F histidine concentration dependent kinetic analysis. **B**. OvoA Y417F Cysteine concentration dependent kinetic analysis. For OvoA Y417F, the assay mixtures contained 0.75 μM enzyme, 2 mM histidine (or 2 mM cysteine),1 mM DTT, 0.2 mM ascorbate in 50 mM air-saturated KPi buffer, pH 8.0 and various amounts of L-histidine or cysteine in a total volume of 1.0 mL. The data was fitted to a Michaelis-Menten equation using GraphPad Prism.

6. Preparation of 2-amino-3-(4-hydroxy-3-(methylthio) phenyl) propanoic acid (MtTyr)



Scheme S1. Biosynthesis of MtTyr, catalyzed by TPL F36L.[4]

One liter of Tyrosine phenol lyase (TPL) F36L enzymatic reaction mixture contained 10 mM 2-(methylthio) phenol, 30 mM ammonium acetate, 60 mM sodium pyruvate, 5 mM β-mercaptoethanol, 40 μM PLP, 50 mM KPi buffer (pH=8.0) and 30 μM (final concentration) TPL F36L mutant enzyme. The reaction was incubated in the dark with stirring at 25℃ for 24 h. The reaction mixture was acidified to pH=3.0 with TFA and the precipitated protein was removed by filtering the mixture through a 3 cm thick celite pad. The filtrate was extracted by 500 mL of ethyl acetate. The aqueous layer was loaded onto a 100 mL Dowex 50WX8-100 cation exchange column (Sigma-Aldrich). After loading, the column was washed by 500 mL of 30% ACN. The 2-amino-3-(4-hydroxy-3-(methylthio) phenyl) propanoic acid was then eluted by 10 % ammonium hydroxide solution. Fractions with positive ninhydrin tests were combined. The target compound MtTyr was crystallized when the elution was neutralized. The crystals were lyophilized and stored at 4℃.

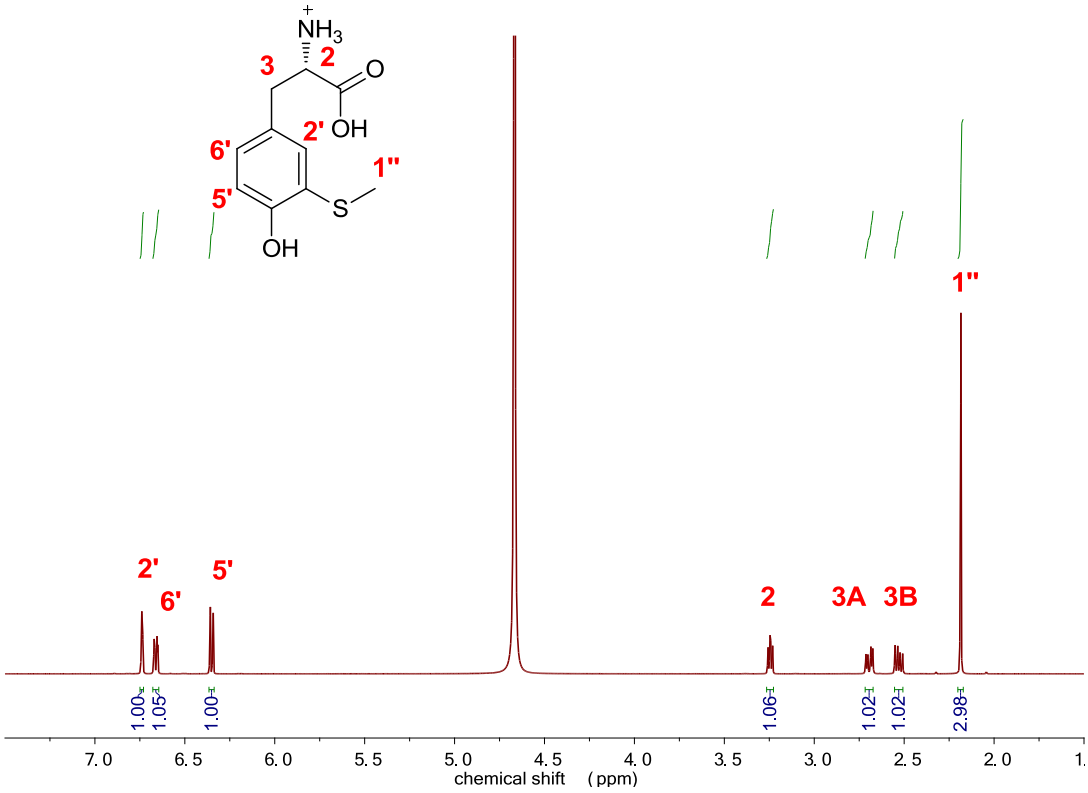


Figure S8. ^{1}H NMR Spectrum of 2-amino-3-(4-hydroxy-3-(methylthio) phenyl) propanoic acid. (500 MHz, D_2O) δ 2.17 (s, 3H), 2.51 (dd, J = 13.8, 7.3 Hz, 1H), 2.68 (dd, J = 13.8, 5.3 Hz, 1H), 3.23 (dd, J = 7.3, 5.3 Hz, 1H), 6.33 (d, J = 8.1 Hz, 1H), 6.65 (dd, J = 8.1, 2.2 Hz, 1H), 6.72 (d, J = 2.3 Hz, 1H).

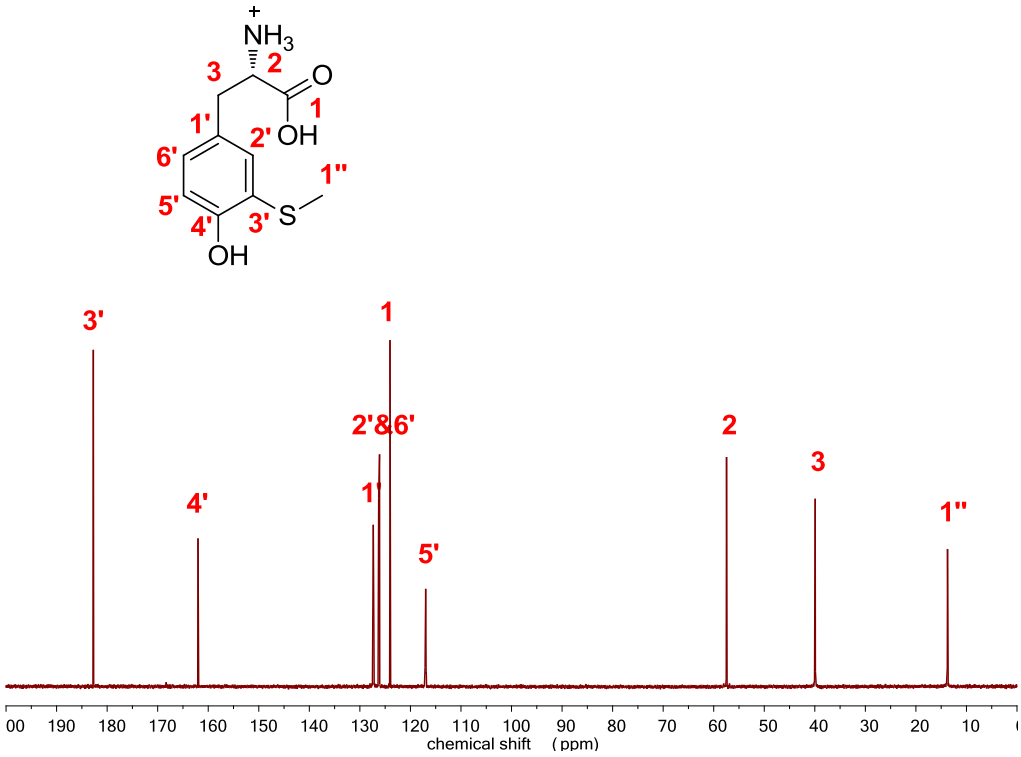


Figure S9. ^{13}C NMR spectrum of 2-amino-3-(4-hydroxy-3-(methylthio) phenyl) propanoic acid. (500 MHz, D_2O) δ 13.72, 39.93, 57.44, 117.12, 124.05, 126.10, 126.30, 127.33, 162.03, 182.74.

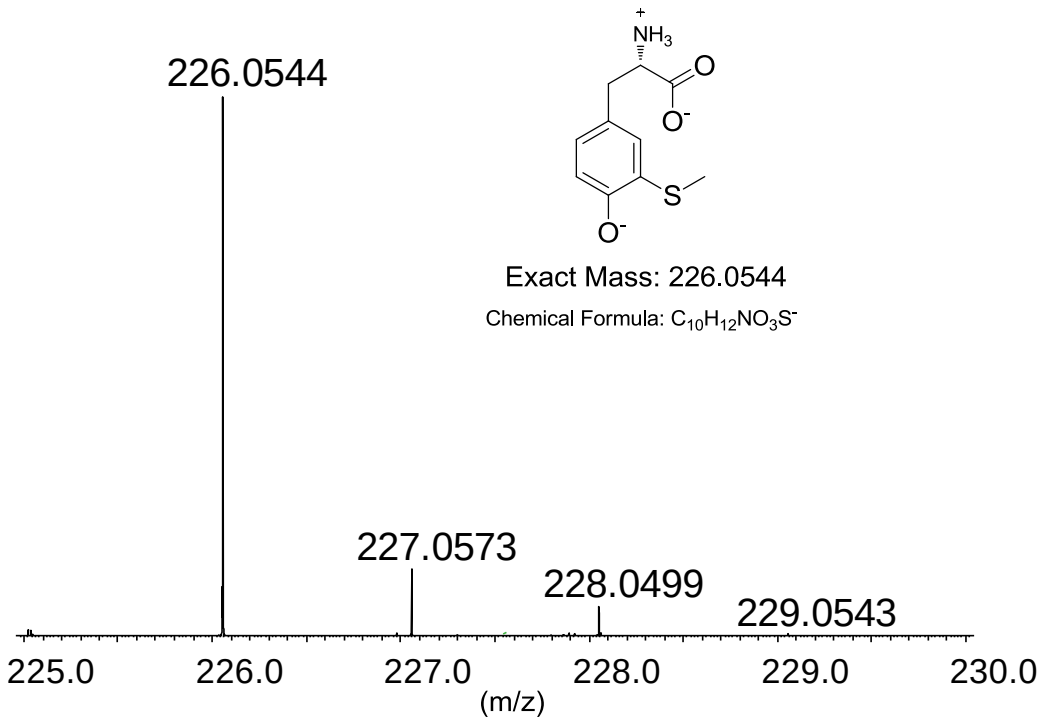


Figure S10. Molecular ion region in the HRMS spectrum of 2-amino-3-(4-hydroxy-3-(methylthio) phenyl) propanoic acid. The calculated $[M-H]^-$ in negative mode for compound was 226.0544, and found 226.0544.

7. Detailed protocols for the purification of OvoA Y417MtTyr variant.

Cells (8 g) were resuspended in 80 mL of anaerobic buffer (100 mM Tris-HCl, 500 mM NaCl and 10% glycerol, pH 8.0) in an anaerobic coy chamber. Lysozyme (0.2 mg/mL) was then added into the cell suspension. The cell mixture was incubated at 25℃ for 20 min and on ice for 10 min with gentle agitation. The cells were disrupted by sonication and the cell debris were removed by high speed centrifugation. Then the supernatant was mixed with the Strep-Tactin resin (20 mL) and incubate on ice for 40 min with gentle shaken. After incubation, the supernatant was drained out and the column was washed with 80 mL anaerobic buffer. The OvoA Y417MtTyr protein was eluted with the elution buffer (100 mM Tris-HCl, 100 mM NaCl and 2.5 mM desthiobiotin, pH 8.0). For those OvoA protein using for reaction in D_2O, the elution buffer was making in D_2O (100 mM Tris-HCl, 100 mM NaCl and 2.5 mM desthiobiotin, pH 7.6). The protein was then concentrated anaerobically by ultrafiltration to approximately 40 μM. After that, it was frozen by liquid nitrogen and stored at -80℃

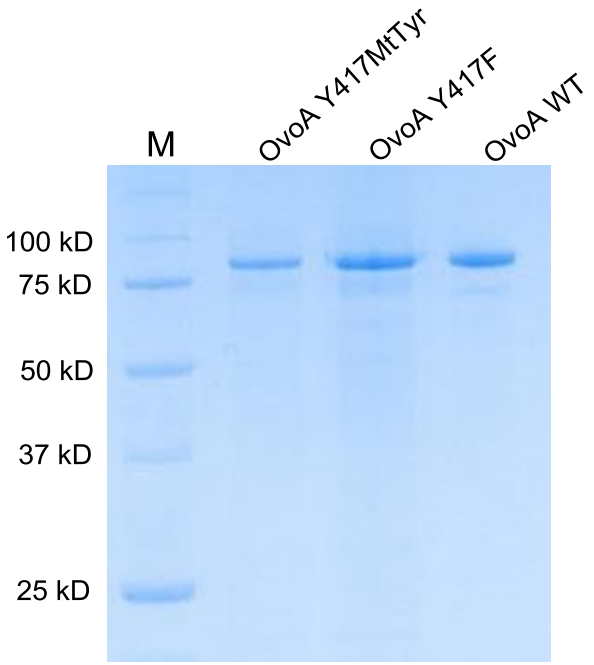


Figure 11. SDS-PAGE analysis of recombinant OvoA wild-type, OvoA Y417F and OvoA Y417MtTyr purified anaerobically.

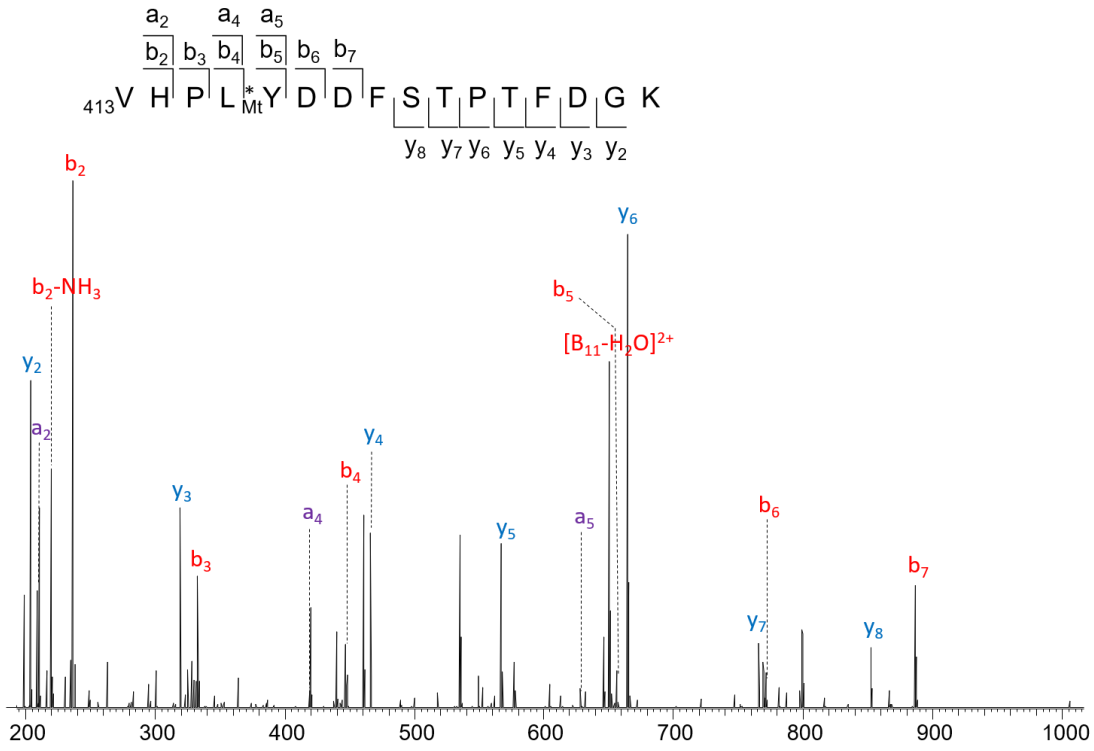
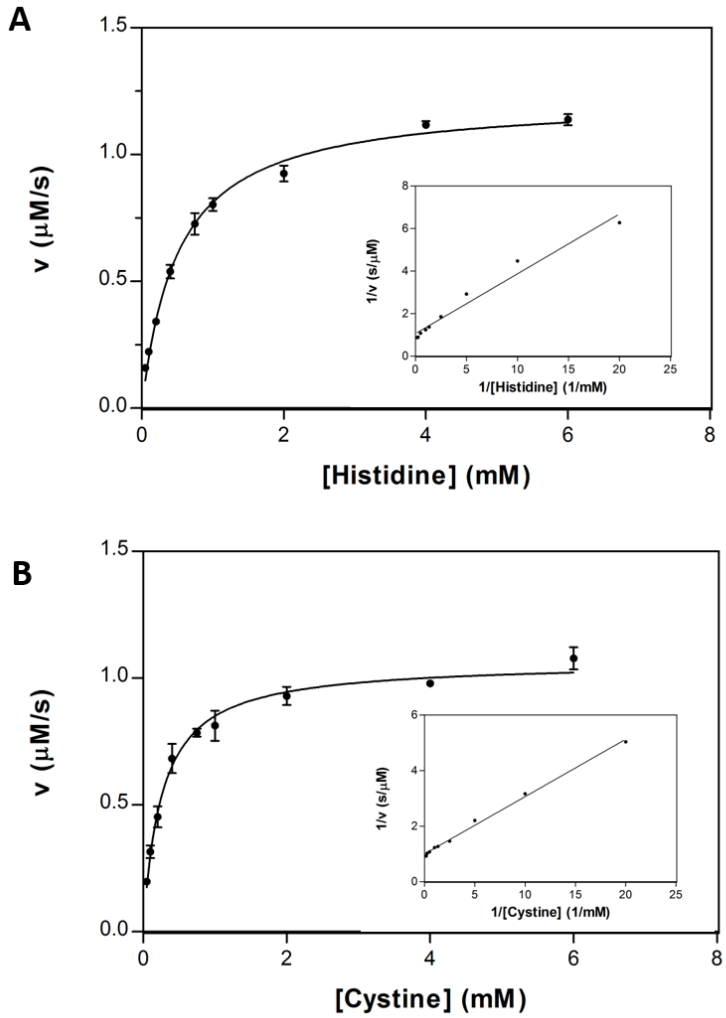


Figure S12. Electrospray ionization MS/MS spectrum of a tryptic peptide of the OvoA Y417MtTyr (residue 413-428). The parent ion has the signal with *m/z* 628.9539 (3 charges).

8. Kinetics of OvoA Y417MtTyr determined by Oxygen consumption assay



	K_m (histidine) μM	K_m (cystine) μM	k_{cat} (s^{-1})
OvoA Y417MtTyr	506± 35	253± 25	1.14± 0.02

Figure S13. OvoA Y417MtTyr steady-state kinetic analysis at 25℃. **A**. OvoA Y417MtTyr histidine concentration dependent kinetic analysis. **B**. OvoA Y417MtTyr cysteine concentration dependent kinetic analysis. For OvoA Y417MtTyr, the assay mixtures contained 1 μM enzyme, 2 mM histidine (or 2 mM cysteine), 1 mM DTT, 0.2 mM ascorbate in 50 mM air-saturated KPi buffer, pH 8.0 and various amounts of L-histidine or cysetine in a total volume of 1.0 mL. The data was fitted to a Michaelis-Menten equation using GraphPad Prism.

9. Product profiles of wild-type OvoA and OvoA Y417MtTyr catalyzed reactions

Run reactions using unlabeled histidine and [β-^{13}C]-cysteine as substrates. The reaction mixture contained 20 mM air saturated KPi buffer in H_2O at pH 8.0, 10 mM histidine, 10 mM cysteine, 3 mM DTT, 1 mM sodium ascorbate and 8 μM iron-reconstituted OvoA wild-type (or OvoA Y417MtTyr). The reaction was stirred at 25℃ for 2 h. After the reaction was complete, the protein was removed by ultrafiltration. The filtrate was detected by ^{13}C-NMR.

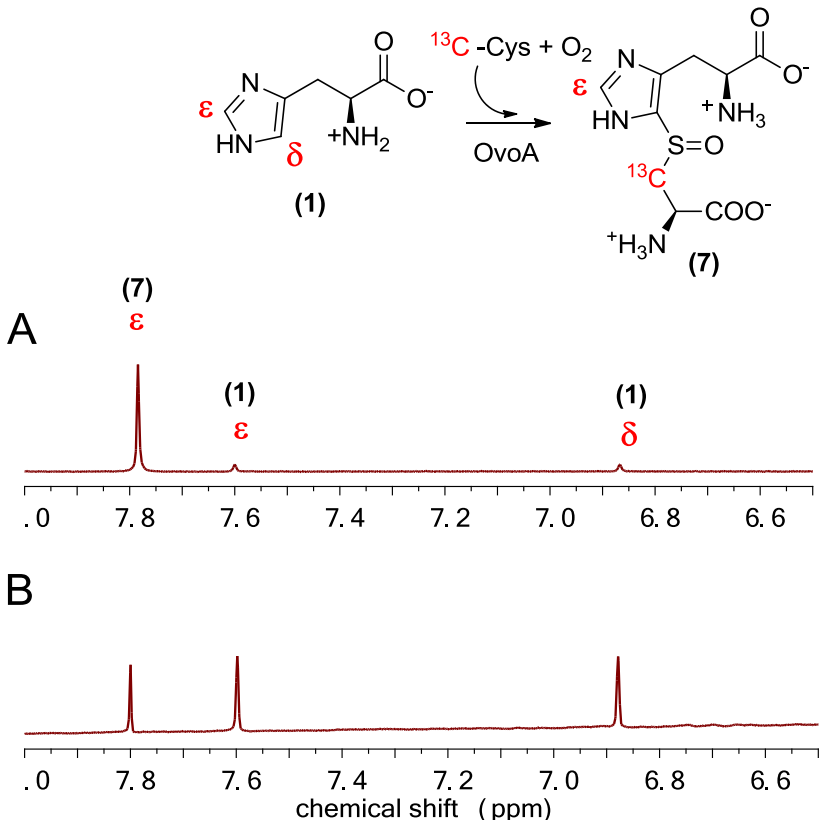


Figure S14. ^{1}H-NMR spectra of wild-type OvoA (A) and OvoA Y417MtTyr (B) reaction mixture.

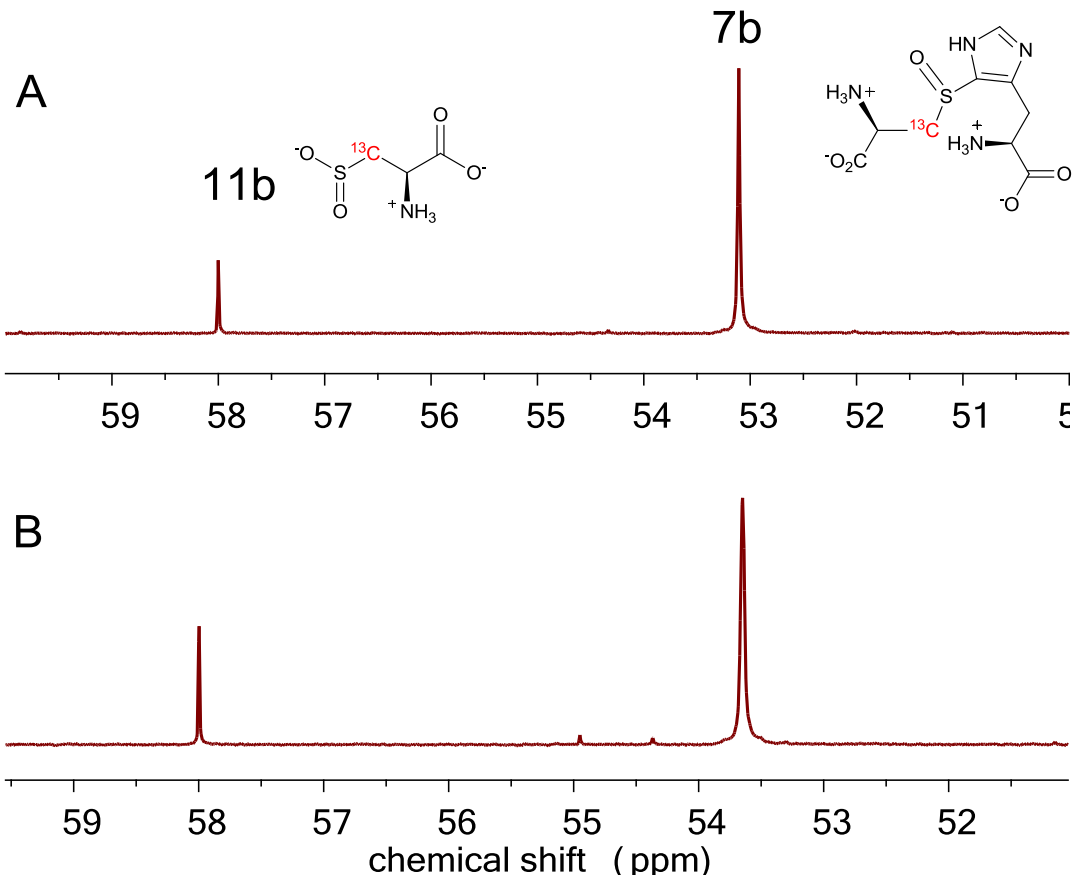


Figure S15. ^{13}C-NMR spectra of wild-type OvoA (A) and OvoA Y417MtTyr (B) reaction mixture.

10. **^{13}C-NMR spectra of OvoA Y417MtTyr catalyzed reactions**

For the OvoA Y417MtTyr **primary** isotope effect assay, three enzymatic reactions were conducted the same as OvoA wild-type measurement described in the experimental section.

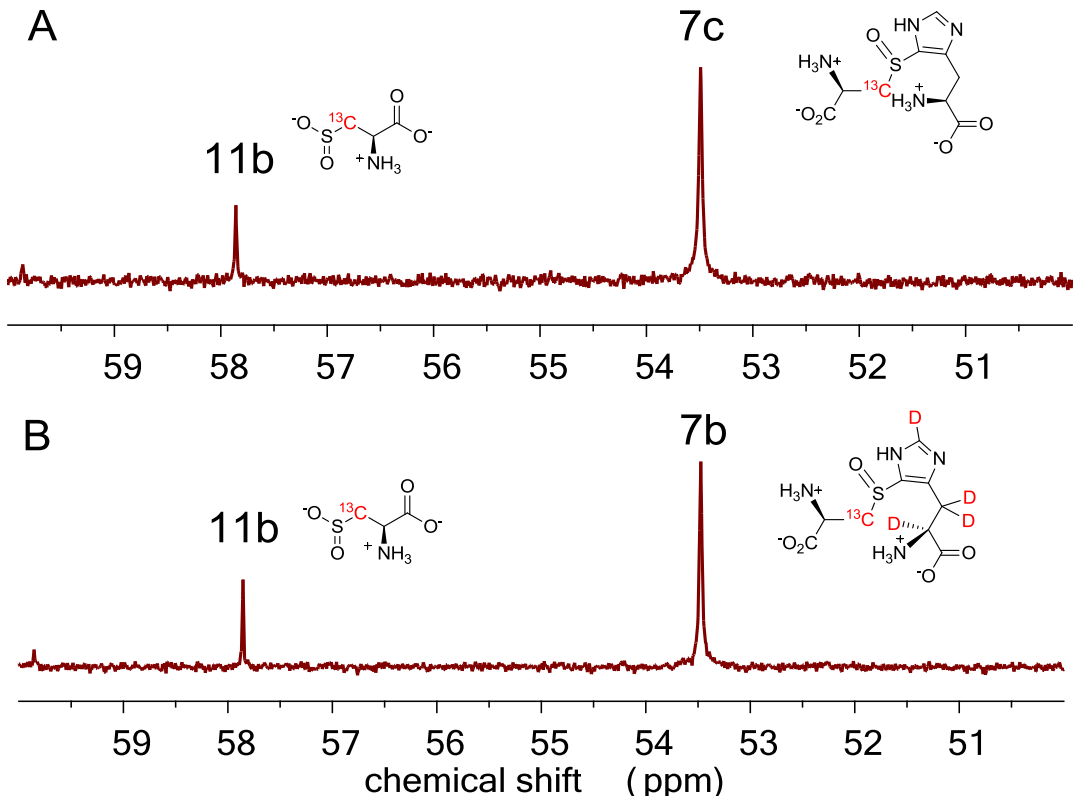


Figure S16. ^{13}C-NMR spectra of OvoA Y417MtTyr catalyzed reactions. A. ^{13}C-NMR spectrum of the reaction mixture using [β-^{13}C]-cysteine and unlabeled histidine as substrates; B. ^{13}C-NMR spectrum of the reaction mixture using [β-^{13}C]-cysteine and [U-$^{2}H_5$]-hestidine as substrates.

For a OvoA Y417MtTyr **solvent** deuterium isotope effect assay, three enzymatic reactions were conducted the same as OvoA wild-type measurement described in the experimental section.

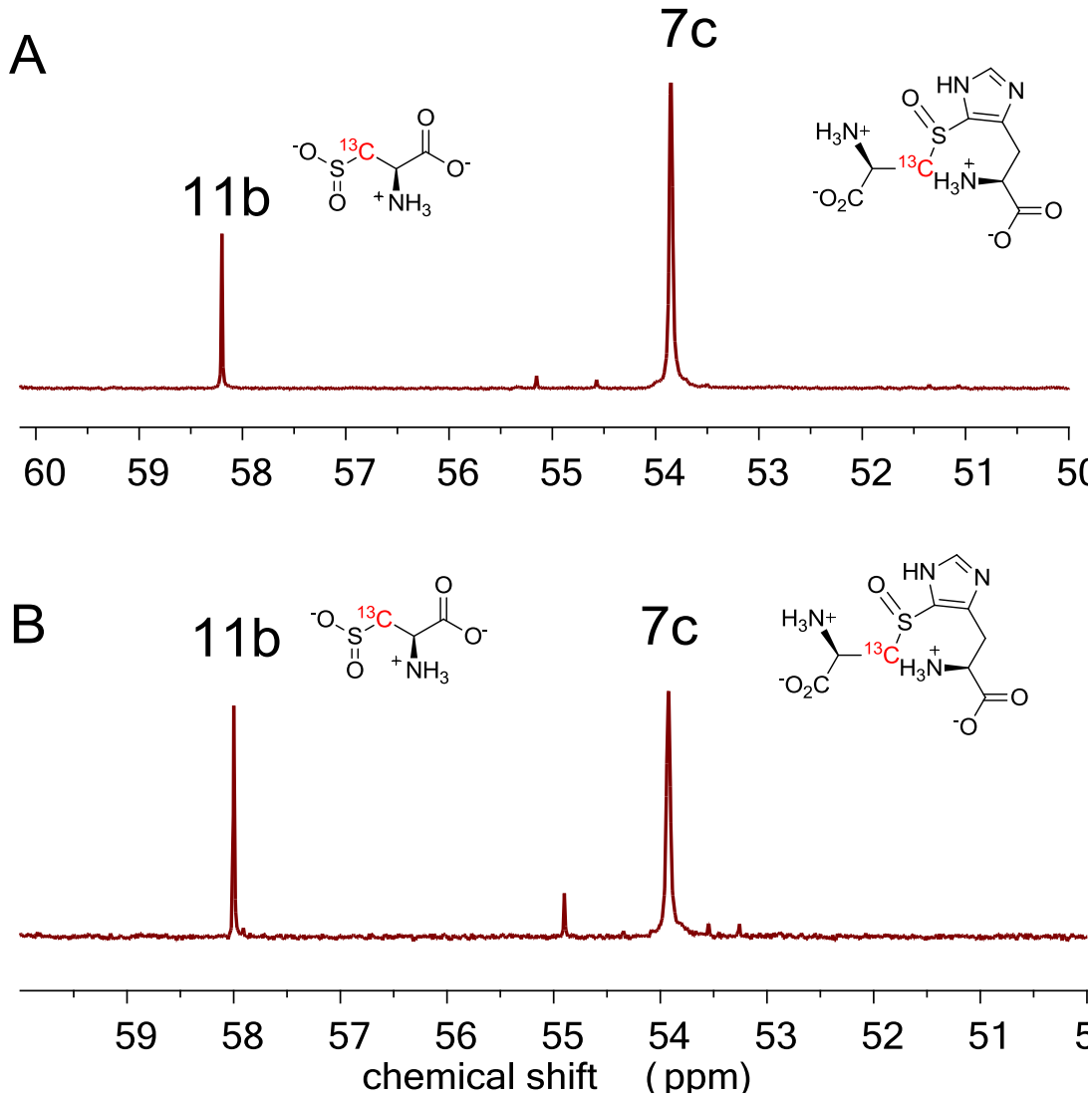


Figure S17. ^{13}C-NMR spectra of OvoA Y417MtTyr catalyzed reactions. **A**. ^{13}C-NMR spectrum of the reaction mixture using [β-^{13}C]-cysteine and unlabeled histidine as substrates in unlabeled buffer. **B**. ^{13}C-NMR spectrum of the reaction mixture using [β-^{13}C]-cysteine and unlabeled histidine as substrates in D_2O buffer.

11. **PCR primers and codon optimized gene sequences**

Table S1. PCR primers used in this study

Primer	Sequence	Usage
PL-1316	ACCCGCTGTAGGACGATTTCAG	Forward primer for OvoA Y417MtTyr
PL-1317	GAACACGAAAGCCCGGGA	Reverse primer for OvoA Y417MtTyr
PL-1431	GCTGAAATCGTCAAACAGCGGGTGAAC	Forward primer for OvoA Y417F
PL-1432	GTTCACCCGCTGTTTGACGATTTCAGC	Reverse primer for OvoA Y417F

DNA sequence of Codon optimized OvoA gene:

ATGGCGAAATGGGAACATGACGTGACCGCGCAACAACATCAAACCGGCC
TGCCGGCGCCGACCCGTATGCTGACCCTGAGCGGTGGTGACCCGCAGCAA
AAGCGTCTGCAGATCCTGAGCGATTTCAGCAAAACCTGGGAGCTGTACGA
AAGCCTGTTTGATTGCCTGACCGACGAACGTGCGTGGTATACCAAGGCGA
TCAGCCTGCGTCACCCGCTGATTTTCTACTATGGTCACACCGCGACCTTTT
ACATCAACAAACTGATGGCGGGTGGCCTGATTGACGCGCGTGTGGACGAT
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TCTGGACAACAGCCATTATAGCTGGCCGAGCCTGGCGGAGCTGCGTGATT
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AACTGCCGCTGGCGTGGGTTAGCGCGCAACCGCACTGGCCGGCGTGCCCG
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AGGAACTGCGTCTGCGTCTGCTGGCGGAGGAAGTGGCGATGCCGTGGGAC
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CGCCGATGATTCGTCCGGGTGGCGTTCTGATGCTGAGCAGCCCGTACACC
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GAACGGTGAAGCGCTGAGCACCTATCAGGCGCTGCAACGTCTGCTGGCGG
CGGATTTTGAGGAACTGGCGCCGCCGCAAGATGTGCCGTTTGTTATTCGTG
AGACCGCGCGTAAGTATCAACATAGCGTGGCGCAACTGACCCTGTGGCGT
AAGCGTCTCGAGGTCGACCTGCAGGGGGACCATGGTCTCTGA

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(3) Song, H.; Her, A. S.; Raso, F.; Zhen, Z. B.; Huo, Y. D.; Liu, P. H., *Org. Lett.* **2014,** *16*, 2122-2125.

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