Supporting Information for

Quaternary Ammonium Oxidative Demethylation: X-ray Crystallographic, Resonance Raman and UV-visible Spectroscopic Analysis of a Rieske-type Demethylase

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Supplementary Materials and Methods

Synthesis of Stachydrine (**1**). Stachydrine was synthesized with a slight modification of published procedures.[1] L-proline (4.951 g, 0.043 mol) and NaOH (5.160 g, 0.129 mol) were dissolved in 50 mL of dry methanol. To this solution was added methyl iodide (18.310 g, 0.129 mol, 8.0 mL), and the reaction mixture was refluxed for 6 h. Additional 6.10 g (0.043 mol, 2.7 mL) of methyl iodide was added and the mixture was refluxed for an additional 6 h. The solvent was then removed by rotatory evaporation and resulted in 27.0 g of light yellow solid. A portion of the crude product (10 g) was dissolved in water and applied to a cation-exchange column (H^+ form, AG 50W-X8, 100-200 mesh, 2.5 × 1.5 cm). The column was washed with 250 mL of water and then eluted with 250 mL of 1.5 N HC1 solution. Product-containing fractions were combined and the aqueous solvent was evaporated under reduced pressure. The desired product was extracted from the solid using dry methanol. The final pure stachydrine was obtained by re-crystallization using methanol/ether as the solvent. Stachydrine properties: $[\alpha]^{24}_{D}$ = - 20.4^{o} (c, 1.00, MeOH); ^{1}H NMR (D_2O, 400 MHz): 4.23 (dt, *J* = 9.6, 4.0 Hz, 1 H), 3.64-3.69 (m, 1 H), 3.48-3.55 (m, 1 H), 3.25 (s, 3 H), 3.06 (s 3 H), 2.46-2.49 (m, 1 H), 2.27-2.32 (m, 1 H), 2.10-2.16 (m, 2 H); ^{13}C NMR (D_2O, 75 MHz) 169.26, 74.04 (d, *J* =16.6 Hz), 67.91, 52.17 (d, *J* =12.6 Hz), 46.12 (d, *J* = 18.9 Hz), 24.31, 18.34. High resolution Mass Spectrometry (ESI^+): calculated for $C_7H_{14}NO_2^+$ 144.1025, found 144.1019.

Synthesis of N-methyl-proline **(2).** *N*-methyl-proline was synthesized according to the literature procedure.[2] L-Proline (2.0 g, 17.4 mmol) was dissolved in methanol (20 mL) and 40% aqueous formaldehyde (1.4 mL, 19.1 mmol) was added to this solution. Next, 10% Pd/C catalyst (500 mg) was added to the reaction mixture and the resulting slurry was stirred under hydrogen atmosphere overnight. The slurry was then filtered through a Celite pad to remove the catalyst. The pad was washed with methanol and the combined filtrates were concentrated under reduced pressure. The residue was dissolved in ethanol/benzene (1:1, 100 mL) and concentrated a second

time to provide a solid that was re-crystallized from methanol/diethyl ether solution as fine needles. (2.0 g, 90% yield). $[\alpha]^{24}_{D}$ = - 79.4 ^{o} (c, 2.00, MeOH); ^{1}H NMR (D_2O, 500 MHz): 3.71-3.75 (dd, *J* = 9.5, 7.5 Hz, 1 H), 3.55-3.59 (m, 1 H), 3.98-3.01 (m, 1 H), 2.77 (s, 3 H), 2.30-2.37 (m, 1 H), 1.81-2.01 (m, 3 H); ^{13}C NMR (D_2O, 100 MHz) 173.60, 70.49, 56.18, 40.56, 28.67, 22.70.

Stc2 ^{1}H-NMR Assay. The Stc2 assay mixture containing 0.4 mM Stc2 and 0.4 mM stachydrine (with equimolar NaOAc as internal standard), and 1.0 mM Fe(II) in 50 mM Tris pH 7.5 buffer was prepared under anaerobic condition and reduced by 1.0 mM dithionite in a Coy-anaerobic chamber. The mixture was then removed from the Coy chamber and exposed to oxygen at room temperature for 20 hours to initiate the oxidative demethylation reaction. To analyze the product formation, the reaction mixture was treated with $CHCl_3$ to denature the protein, and the resulting supernatant, after centrifugation at 14,000 g, was analyzed by proton NMR (500 MHz, Figure S1). In ^{1}H-NMR spectrum, the two methyl groups of stachydrine are at 3.16 and 2.97 ppm, respectively and the *N*-methyl-proline methyl group is at 2.80 ppm.

Structural Homology Analysis

The CE algorithm[3] implemented in PyMol was used to align each structure with Stc2 using secondary structure matching. Visual inspection of each structure was used to determine the Rieske and catalytic domain boundaries, which were then independently aligned with the corresponding Stc2 domains using the CE algorithm (Table S1).

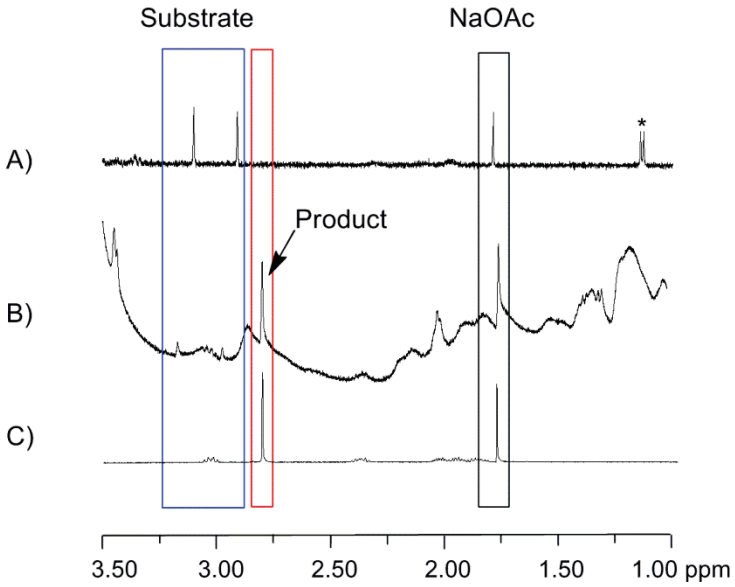
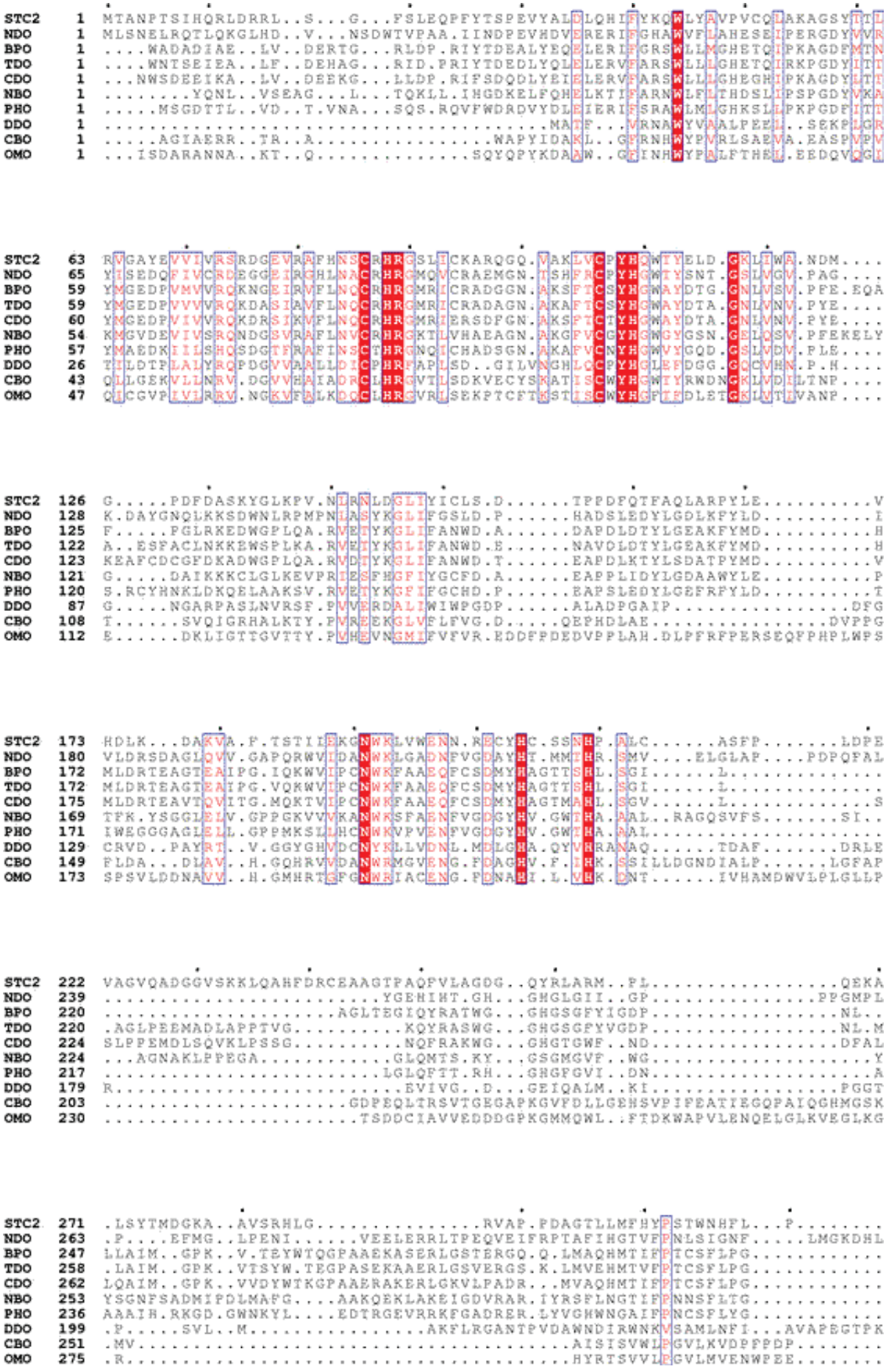


Figure S1. ^{1}H-NMR Spectra from Stc2 assays. A) 0.25 mM stachydrine /NaOAc in D_2O *impurity from the NMR tube. B) Stachydrine oxidative demethylation reaction mixture after protein was removed by $CHCl_3$ treatment. C) 1.4 mM *N*-methyl proline and 1.0 mM NaOAc in D_2O.



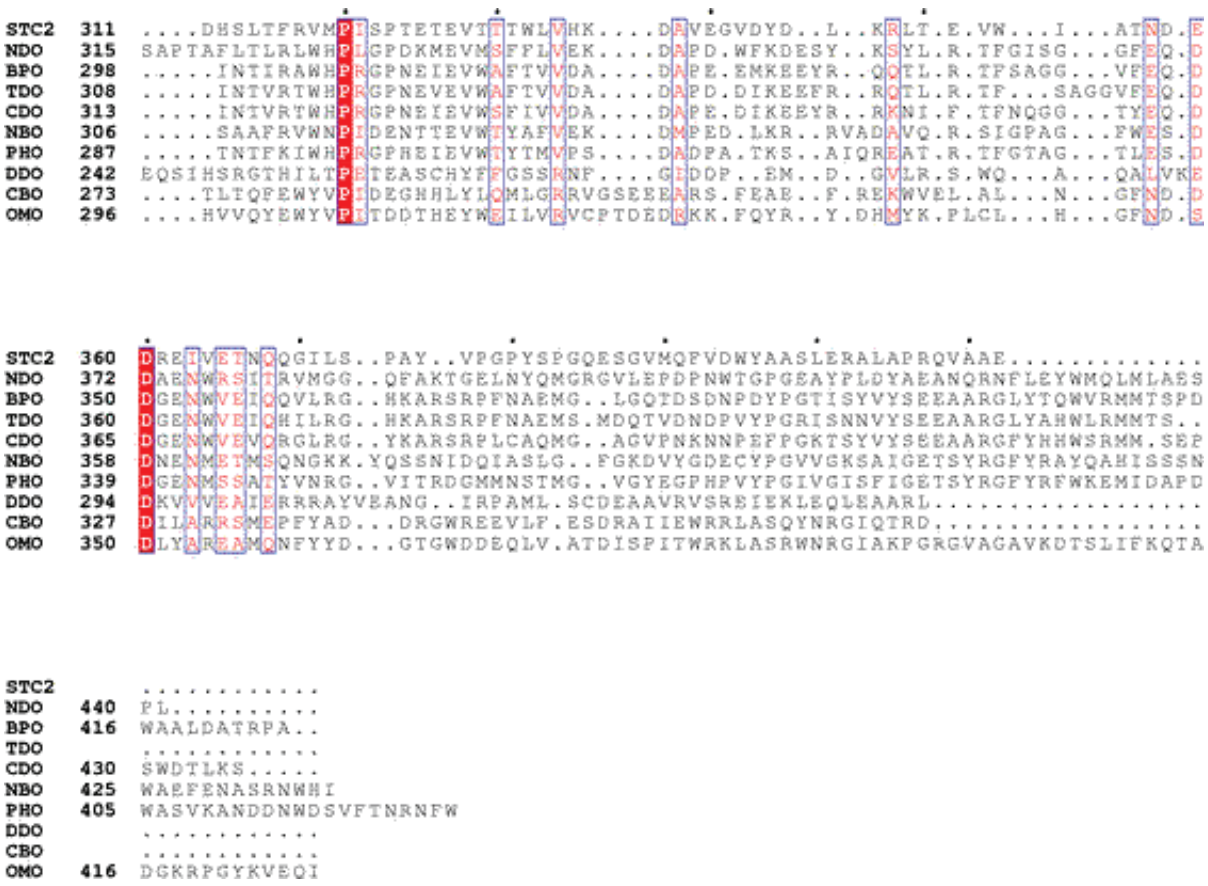


Figure S2. Sequence alignment of Rieske-type enzymes structurally homologous to Stc2. Details for these enzymes are listed in the following order: (enzyme name, abbreviation, PDB ID, sequence identity): naphthalene 1,2-dioxygenase, NDO, 1NDO, 21%; biphenyl dioxygenase, BPO, 1ULI, 21%; toluene 2,3-dioxygenase, TDO, 3EQQ, 20%; cumene dioxygenase, CDO, 1WQL, 21%; nitrobenzene dioxygenase, NBO, 2BMO, 24%; PAH-hydroxylating dioxygenase PHO, 2CKF, 21%; dicamba demethylase, DDO, 3GOB, 15%; carbazole 1,9α-dioxygenase, CBO, 3GKQ, 21%; 2-oxoquinoline 8-monooxygenase, OMO, 1ZO1, 17%.

Table S1. Structural similarity of Stc2 to other Rieske-type oxygenases.

Enzyme Name	Abbreviation	PDB ID	Sequence Identity (%)	RMSD Rieske /Catalytic (Overall)
naphthalene 1,2-dioxygenase	NDO	1NDO	21	2.03 / 5.19 (3.25)
biphenyl dioxygenase	BPO	1ULI	21	1.45 / 4.8 (4.6)
toluene 2,3-dioxygenase	TDO	3EQQ	20	1.94 / 5.78 (3.32)
cumene dioxygenase	CDO	1WQL	21	1.70 / 4.61 (3.07)
nitrobenzene dioxygenase	NBO	2BMO	24	2.02 / 5.11 (4.88)
PAH-hydroxylating dioxygenase	PHO	2CKF	21	1.59/ 4.95(3.28)
dicamba demethylase	DDO	3GOB	15	3.67/ 4.35 (4.55)
carbazole 1,9a-dioxygenase	CBO	3GKQ	21	3.67 / 5.29 (4.15)
2-oxoquinoline 8-monooxygenase	OMO	1ZO1	17	3.59 / 5.12 (4.57)

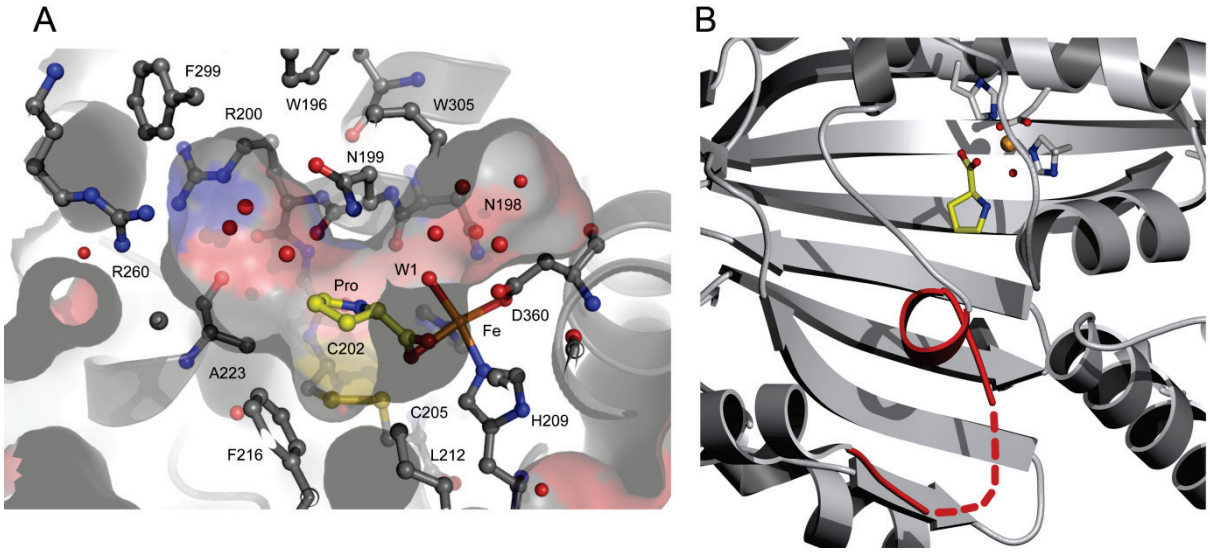


Figure S3. A) The bi-lobed, solvent excluded cavity at the mononuclear iron site of Stc2. Several solvent molecules (red, non-bonded spheres) occupy each lobe of the cavity. B) Ribbon diagram of the active Stc2 active site with Fe shown as orange sphere and Fe coordinating residues and product proline shown as ball and stick. The gating loop (residues 215 – 233) is colored red with relative location of disordered residues 226 – 229 indicated by a dashed line).

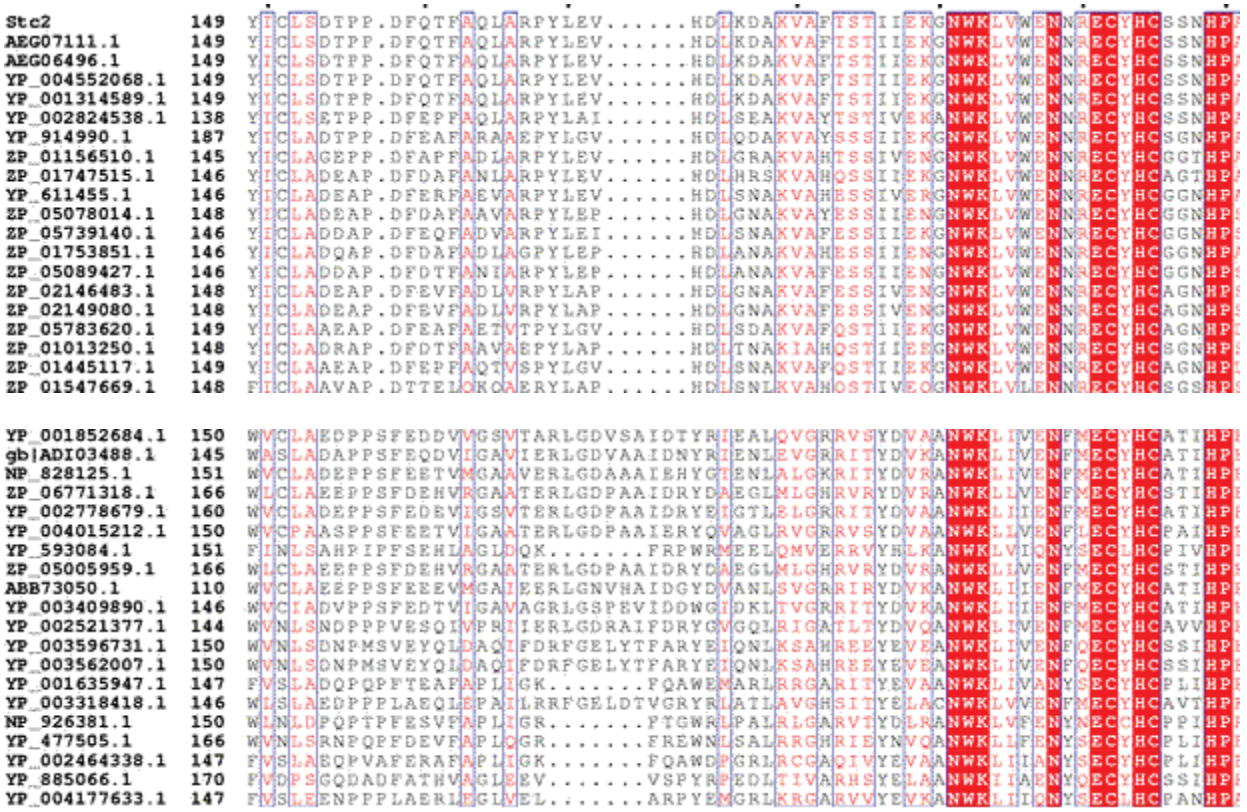


Figure S4. Alignment of the portion of the sequences containing the strictly conserved CxHC motif (signature of disulfide proximal to the mononuclear Fe site) identified using as query the Stc2 sequence (SMa0751, NP_435626). An example set of 20 sequences with high overall identity (top, sequence identity range 57 – 98%) and low overall sequence identity (bottom; sequence identity 31 – 24%) are shown with alignment between them retained.

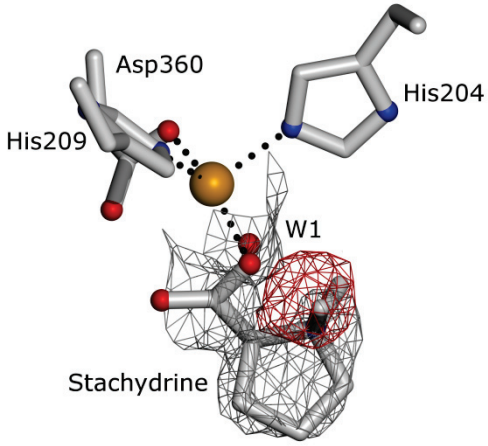


Figure S5. Structure of Stc2 mononuclear Fe active site with stachydrine modeled showing electron density calculated between 39.7 - 2.2 Å resolution with coefficients 2Fo-Fc (gray cages) contoured at 1.0 σ and calculated with coefficients Fo-Fc (red cages) contoured at -3 σ.

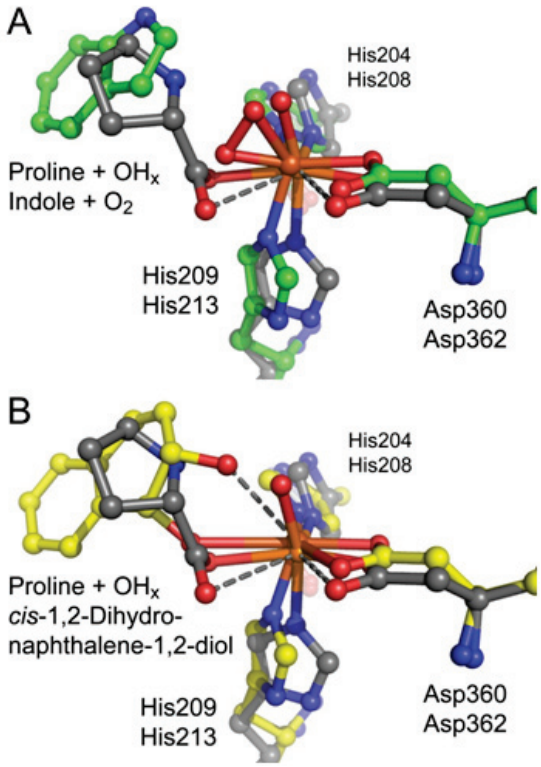


Figure S6: Overlay of the Stc2 ligand complex with naphthalene 1,2-dioxygenase ligand complexes. Superposition was based upon the best overlay of the Cβ atoms from three protein residues that coordinate the mononuclear iron center (His204/208, His 209/213 and Asp360/362). Long interactions are indicated with gray dashed lines. A) Stc2-Pro (C, N, O, and Fe atoms colored gray, blue, red and orange, respectively; residue are labeled in the top line) superimposed with the ternary complex of naphthalene 1,2-dioxygenase, indole and O2 (C atoms colored green, others by atom type; residue are labeled in the bottom line; PDB 1O7N). B) Stc2-Pro overlayed on the naphthalene 1,2-dioxygenase product complex (C atoms colored yellow, others by atom type; PDB 1O7P).

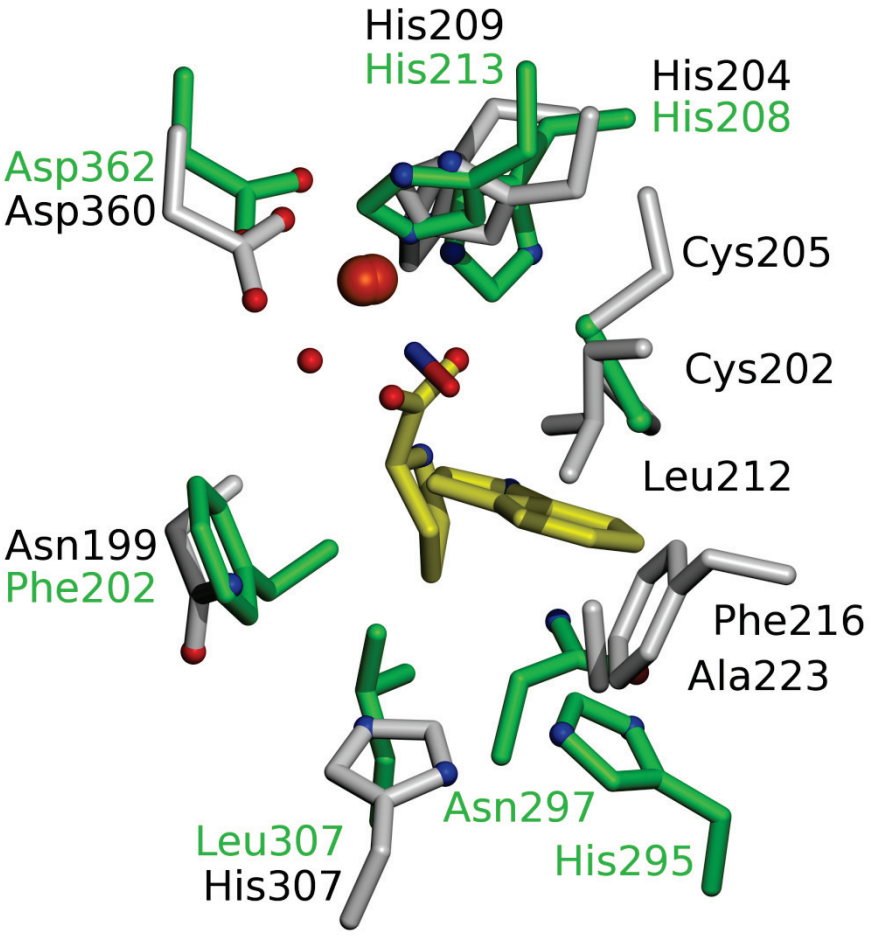


Figure S7: Superposition of the hydrophobic active sites of Stc2 and NDO. Side chains lining the active site of the complex of Stc2 (grey sticks) with proline (yellow sticks) are shown superimposed with those of the complex of naphthalene 1,2-dioxygenase (green sticks) with indole (yellow sticks) and NO (PDB 1UUV). The water molecule (red sphere) is from the Stc2 structure. Fe is shown as an orange sphere.

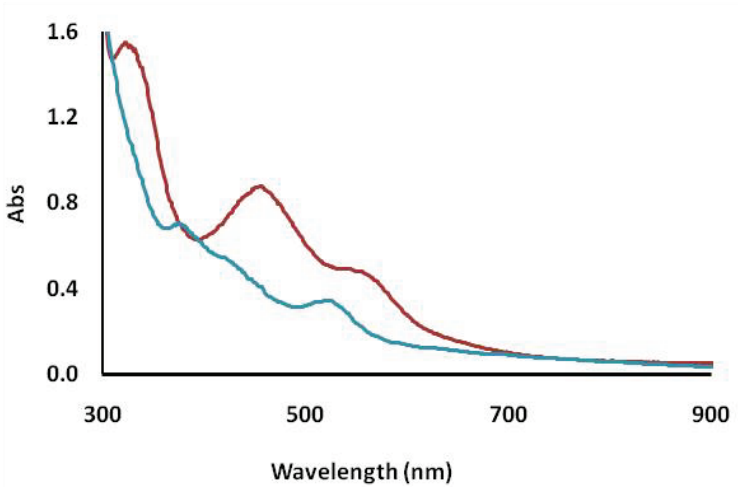
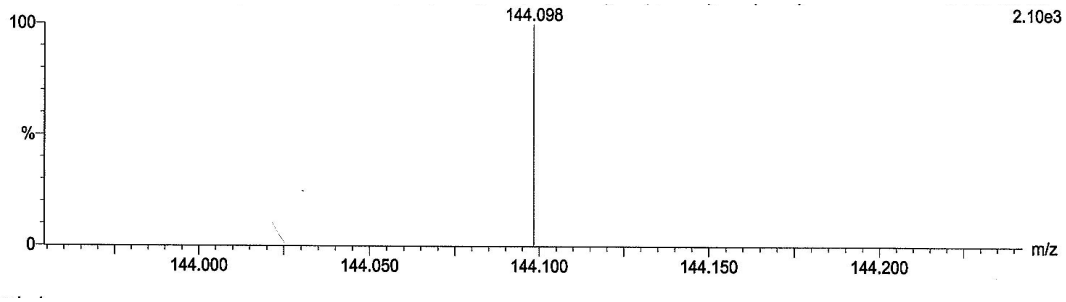


Figure S8: Solution spectra of Stc2 (0.4 mM) in 100 mM Tris, pH 7.5 buffer with 2.5 mM desthiobiotin used to elute protein from the Streptavidin column (red trace, as-isolated Stc2; blue trace, Stc2 reduced by 0.6 mM sodium dithionite).

A



B

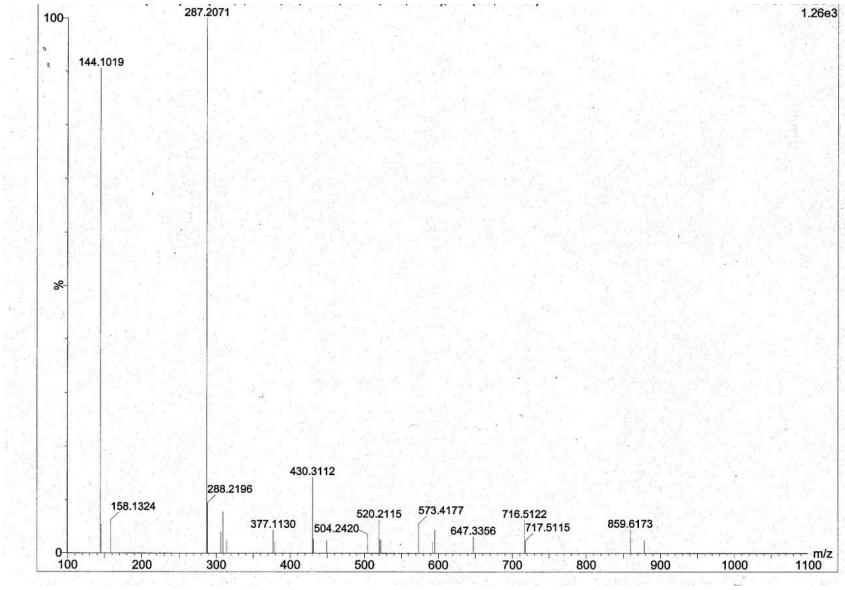


Figure 9: Mass spectrometry data for the stachydrine A) before x-ray irradiation (expected mass of stachydrine ([C7H14NO2]+ = 144.1025 [M]+), observed mass 144.098) and B) after exposure of 100 mM stachydrine (in water) at room temperature to approximately ten times the X-ray dose used for x-ray diffraction studies ([C7H14NO2]+ = 144.1025 [M]+) and observed 144.1019 and 287.2071 [2M-H]+.

References

(1) Woster, P. M.; Murray, W. J. *J. Med. Chem.* **1986**, *29*, 865.
(2) Han, Z.-j.; Wang, R.; Zhou, Y.-f.; Liu, L. *ChemInform* **2005**, *36*.
(3) Shindyalov, I. N.; Bourne, P. E. *Protein Eng* **1998**, *11*, 739.