SUPPORTING INFORMATION

Structure and Biocatalytic Scope of Thermophilic Flavin-Dependent Halogenase and Flavin Reductase Enzymes

Binuraj R. K. Menon, Jonathan Latham, Mark S. Dunstan, Eileen Brandenburger, Ulrike Klemstein, David Leys, Chinnan Karthikeyan, Michael F. Greaney, Sarah A. Shepherd and Jason Micklefield*

School of Chemistry and Manchester Institute of Biotechnology
The University of Manchester, 131 Princess Street, Manchester, M1 7DN, UK.

* E-mail: jason.micklefield@manchester.ac.uk

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1 Experimental Section

1.1 General

All chemical reagents and solvents were purchased from Sigma-Aldrich Company Ltd, Fisher Scientific UK Ltd, or Alfa Aesar. Chemicals were used without further purification.

1.2 Cloning, expression, and purification of enzymes

The tryptophan halogenase (named Th-Hal, GI: 740097853, WP_037947065.1) from *Streptomyces violaceusniger* strain SPC6 (also named as *Streptomyces thermolilacinus* SPC6) was synthesized (by GenScript, Piscataway, USA) and cloned into a pET28b(+) protein expression vector (Invitrogen) using restriction sites NdeI and XhoI. The tryptophan 6-halogenase KtzR from *Kutzneria* spp. 744 was amplified from *Kutzneria* spp. 744 genomic DNA and cloned into pET28a(+) vector using restriction sites KpnI and XhoI for this study. Expression vectors for PyrH (tryptophan 5-halogenase from *Streptomyces rugosporus*), PrnA (tryptophan 7-halogenase from *Pseudomonas fluorescens* BL915), RebH (tryptophan 7-halogenase from *Lechevalieria aerocolonigenes*), SttH (tryptophan 6-halogenase from *Streptomyces toxytricini*) and Fre (flavin reductase from *E. coli*), were all created as described previously.[29,31,32,53] The *ssuE* gene sequence was amplified from *E. coli* BL21 (DE3) genomic DNA and cloned into pET28a(+) vector using restriction sites NdeI and XhoI. The thermophilic flavin reductase (named Th-Fre) from *Bacillus subtilis* WU-S2B[46,47] was synthesised (by GenScript, Piscataway, USA) and cloned into a pACYCDUET-1 protein expression vector (Invitrogen) using restriction sites BamHI and EcoRI.

The plasmids containing tryptophan halogenase encoding genes were used to transform *E. coli* BL21 (DE3) cells (Novagen) for subsequent overproduction of halogenase enzymes with hexa histidine-tags at both the N- and C-terminus. LB medium containing kanamycin (50 μg/mL) and chloramphenicol (35 μg/mL) was inoculated with the transformed cells and then incubated at 37 °C for overnight for preparing the starter culture. The cells from the starter culture were diluted (1:100) in a 2xYT medium broth (Formedium) and incubated at 30 °C, with shaking at 180 rpm, until an optical density of 0.5 at 600nm (OD600) was reached. The temperature was then dropped to 24 °C until OD600 reached 0.6, and protein expression was induced by addition of IPTG (0.1 mM). The cells were grown for a further 14 hours at 20 °C and then harvested by centrifugation (4 °C, 30 min, 4000 rpm). The tryptophan halogenase enzymes were then purified from the cell pellets as reported previously.[29,31] Fre, SsuE, and Th-fre enzymes were also overproduced and purified following previously reported experimental protocols.[29,31,32]

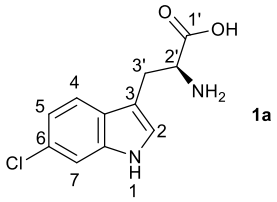
1.3 Activity and regioselectivity of halogenase enzymes

The following conditions were used for assays to determine regioselectivity and percentage of conversions. Purified halogenase enzyme (2.5 μM) was incubated at 30 °C (or 45 °C) with shaking for 30 minutes with Fre (1 μM), FAD (1 μM), NADH (2.5 mM), $MgCl_2$ (10 mM) and substrate (0.5 mM) in a total volume of 300 μL in 10 mM potassium phosphate buffer, pH 7.4. Reactions were stopped by heating at 95 °C for 5 min and the precipitated protein was removed by centrifugation before analysis of the supernatant via HPLC using an Agilent Technologies 1260 system and an Agilent Zorbax Eclipse Plus C18 4.6 × 100 mm × 3.5 μm column. For tryptophan, absorbance was measured at 280 nm. Kynurenine, anthranilamide and anthranilic acid absorbance were measured at 254 nm, with a 15 min gradient 5–75 % H_2O/acetonitrile + 0.1 % formic acid. Kinetic parameters were determined by measuring the reaction rate at different time intervals and by varying the

concentration of substrate tryptophan (0 – 0.75 mM). The purified halogenated products from the preparative scale biotransformation reactions were used to plot the calibration curve of each compound. For this, measured HPLC peak area (for tryptophan at 280 nm, for Kynurenine, anthranilamide and anthranilic acid at 254 nm wavelength) was plotted against the concentration of halogenated products (from 10 – 250 μM).

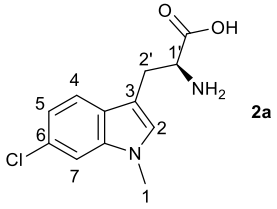
1.4 Preparative Scale Biotransformations

The preparative scale chlorination of tryptophan, *N*-methyltryptophan, kynurenine, anthranilic acid and anthranilamide was conducted in two 40 mL reactions, which were carried out simultaneously and combined prior to purification. Substrate (2.0 mM) was dissolved in 10 mM potassium phosphate buffer (pH 7.2, 30 mL) with sonication. $MgCl_2$ (10 mM), FAD (1 μM), thermo_hal (50 μM), Thermo_fre (5 μM) and NADH (5 mM) were then added to total volume of 40 mL in 10 mM potassium phosphate buffer. After incubation at room temperature for 24 hours, protein was precipitated with heating (70 °C, 15 min) and removed by centrifugation (10,000 rpm, 20 min) followed by filtration through a 2.0 μM cellulose syringe filter. The crude reaction mixture was then loaded onto an Agilent Mega BE Bond Elute, which had been activated with MeOH and equilibrated using H_2O. After washing with H_2O (3 column lengths), organics were eluted with MeOH (3 column lengths). Solvent was then removed *in vacuo* and the crude mixture was dissolved in H_2O/MeCN (1 mL) and purified by C18 preparative HPLC using a Phenomenex Luna® C18 HPLC column (10 μm packing, 250 x 21.2 mm). Starting conditions for preparative HPLC were 5 % H_2O/MeCN (plus 0.05 % TFA) held for 3 min prior to development to 95 % H_2O/MeCN over 23 min, 95 % H_2O/MeCN then held for 3 min prior to re-equilibration of starting conditions over 3 min. Flow rates were kept constant at 10 $mLmin^{-1}$. The UV absorbance was detected at 254 nm and 280 nm throughout.



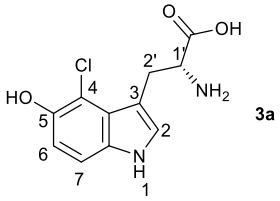
1a

6-chlorotryptophan (**1a**):[31] L-tryptophan was chlorinated, following the general method described above, to give **1a** as an off-white solid (12.4 mg, 62.5 %); ^{1}H NMR (400 MHz, MeOD) δ 8.48 (1H, s, H1), 7.63 (1H d, J = 8.5 Hz, H4), 7.33 (1H d, J = 1.8 Hz, H7), 7.18 (1H, s, H2), 6.99 (1H dd, J = 8.5, 1.8 Hz, H5), 3.80 (1H, m, H2'), 3.43 (1H, m, H3'), 3.12 (1H, m, H3'); ^{13}C NMR (101 MHz, MeOD) δ 174.3, 138.7, 128.7, 127.2, 126.2, 120.6, 120.5, 112.2, 110.0, 56.6, 28.2; m/z (ESI) 237 ([M^{35}Cl-H]$^-$, 75 %), 239 ([M^{37}Cl-H]$^-$, 25 %); HRMS (found: 237.0417, $C_{11}H_{10}N_2O_2Cl$) (expected: 237.0431).



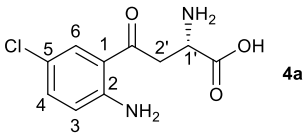
2a

1-methyl-6-chlorotryptophan (**2a**):[31] 1-Methyl-L-tryptophan **2** was chlorinated, as described above, to give **2a** as an off-white solid (4.8 mg, 12 %); ^{1}H NMR (400 MHz, D_2O) δ 7.60 (1H, d, *J* = 8.5 Hz, H5), 7.51 (1H, s, H7), 7.16 (1H, s, H2), 7.14 (1H, d, *J* = 8.6 Hz, H4), 4.06 (1H, s, H1'), 3.72 (3H, s, H1), 3.45 – 3.23 (2H, m, H2'); *m/z* (ESI) 251 (M^{35}Cl-H, 75 %), 253 (M^{37}Cl-H, 25), 363 ([M^{35}Cl+TFA-H]$^-$, 75 %), 365 ([M^{37}Cl+TFA-H]$^-$, 25 %); HRMS (found: 253.0737, $C_{12}H_{14}N_2O_2Cl$) (expected: 253.0744).



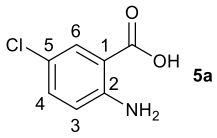
3a

4-chloro-5-hydroxy-L-tryptophan (3a): 5-hydroxy-L-tryptophan **3** was chlorinated in a single 40 mL reaction as described above. After removal of protein, the reaction mixture was lyophilised before being re-dissolved in H_2O (3.0 mL) and then purified by hydrophilic interaction liquid chromatography. The title compound **3a** was obtained as a brown solid (3.2 mg, 16 %); ^{1}H NMR (400 MHz, MeOD) δ 7.18 (1H, d, *J* = 8.7 Hz, H6), 7.15 (1H, s, H2), 6.83 (1H, d, *J* = 8.7 Hz, H7), 4.28 (1H, dd, *J* = 10.6, 4.5 Hz, H1'), 3.93 (1H, m, H2'), 3.13 (1H, m, H2'); *m/z* (ESI) 225 ([M^{35}Cl+H]$^+$, 257 ([M^{37}Cl+H]$^+$); HRMS (found: 225.0537, $C_{11}H_{12}N_2O_3{}^{35}Cl$) (expected: 225.0531).



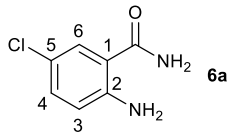
4a

5-chloro-L-kynurenine (4a):[29] L-kynurenine **4** was chlorinated, as described above, to give **4a** as a yellow solid (7.9 mg, 21 %); ^{1}H NMR (400 MHz, MeOD) δ 7.68 (d, *J* = 2.4 Hz, 1H), 7.22 (dd, *J* = 9.0, 2.4 Hz, 1H), 6.75 (d, *J* = 9.0 Hz, 1H), 4.32 (dd, *J* = 6.0, 4.3 Hz, 1H), 3.65 (dd, *J* = 5.1, 3.0 Hz, 2H); *m/z* (ESI) 241 ([M^{35}Cl-H]$^-$, 75 %), 243 ([M^{37}Cl-H]$^-$, 25 %); HRMS (found: 243.0536, $C_{10}H_{12}O_3N_2Cl$) (expected: 243.0531).



5a

5-chloroanthranilic acid (5a):[29] Anthranilic acid **5** was chlorinated, as described above, to give **5a** as a white solid (2.2 mg, 8 %); ^{1}H NMR (400 MHz, D_2O) δ 7.89 (1H, d, *J* = 2.5 Hz, H6), 7.42 (1H, dd, *J* = 8.7, 2.6 Hz, H4), 7.01 (1H, d, *J* = 8.7 Hz, H3); *m/z* (ESI) 125.9 ([M^{35}Cl-CO_2]$^-$, 75 %), 127.9 ([M^{37}Cl-CO_2]$^-$, 25 %), 169.9 ([M^{35}Cl-H]$^-$, 75), 171.9 ([M^{37}Cl-H]$^-$, 25); HRMS (found: 171.0075, $C_7H_6NO_2Cl$) (expected: 171.0082).



6a

5-chloroanthranilamide (6a):[29] Anthranilamide **6** was chlorinated, as described above (1.4), to give **6a** as a white solid (5.1 mg, 19 %); ^{1}H NMR (400 MHz, MeOD) δ 7.53 (1H, d, *J* = 2.4 Hz, H6), 7.15 (1H, dd, *J* = 8.8, 2.5 Hz, H4), 6.72 (1H, d, *J* = 8.8 Hz, H3); *m/z* (ESI) 193 ([M^{35}Cl+Na]$^{+}$, 100 %), 195 ([M^{37}Cl+Na]$^{+}$, 25 %); HRMS (found: 171.0323, $C_7H_8ON_2Cl$) (expected: 171.0320).

1.5 Kinetic characterisation of flavin reductase reaction

To measure flavin reduction rates, in flavin reductase enzymatic assays, reaction mixtures were prepared with 10 nM flavin reductase enzyme, 100 μM NADH (or NADPH) and varying concentration of FMN or FAD (0 – 100 μM) in a 10 mM potassium phosphate buffer at pH 7.4 (total volume 1 mL). To determine kinetic parameters for NADH oxidation, reaction mixture contained 10 nM flavin reductase enzyme, 100 μM FAD (or FMN) and varying concentration of NADH (0 – 250 μM) in a 10 mM potassium phosphate buffer at pH 7.4 (total volume 1 mL). The absorbance changes at 340 nm were measured using a Cary 50 UV-visible spectrophotometer (Varian Inc., California, USA). Spectral changes were recorded for 2 minutes at 30 °C or 45 °C after initiating the reaction by adding the flavin reductase enzyme.

1.6 Thermal stability assays for halogenases and flavin reductases

The thermal stability of enzymes was determined using a variable temperature circular dichroism (CD) spectrometer (Chirascan from Applied photophysics). A 10 μM solution of enzyme in 10 mM potassium phosphate buffer at pH 7.4 was placed into a 0.5 mm path length cuvette and the CD spectrum was measured every 2 °C, from 20 °C up to 80 °C maintaining the sample at fixed temperature for 2 minute with the temperature ramp of 0.2 °C per minute. The absorbance changes at 222 nm were plotted against temperature, and fitted with a nonlinear curve fitting equation (Boltzmann sigmoidal function) to determine the melting temperature of the proteins.

1.7 Enzyme preparation for protein crystallisation

For crystallographic trials, the enzymes (Th-Hal and Th-Fre) were overproduced in Origami^{TM} 2(DE3) Singles^{TM} competent cells from Novagen. Bacterial cells containing the individual plasmids were grown in 2YT medium at 37 °C for 2 h, before decreasing the temperature to 24 °C until an OD600 of 0.6. Recombinant protein production was induced with IPTG (0.1 mM) and the cells were incubated at 18 °C for a further 24 h prior to harvesting by centrifugation (4 °C, 20 min, 4000 rpm). Proteins were purified initially using an Ni^{2+} sepharose affinity tag procedure (as described previously), followed by loading onto a 140 ml Sephadex 200 size exclusion chromatography column (pre-equilibrated with 50 mM potassium phosphate, 500 mM NaCl, pH 7.2).[29,31,32] Homogeneous variant enzyme solution was subsequently diluted (12.5 mg/ml) in crystallization buffer (20 mM HEPES, 100 mM NaCl, pH 7.2 buffer) and mixed in a 1:1 ratio with the precipitant solution. For Th-Hal precipitant solution was commercially available protein crystallization screen solution Morpheus HT-96 screen single reagent, well number A3 (from Molecular Dimensions). The actual precipitant composition was 0.06 M divalents (0.3M Magnesium chloride hexahydrate; 0.3M Calcium chloride dehydrate) in 0.1 M buffer (1.0 M imidazole + MES monohydrate acid buffer) at pH 6.5 made up with a 50% v/v precipitant mix (that contained 25 % v/v MPD; 25 %

PEG 1000; 25 % w/v PEG 3350). For Th-Fre precipitant solution was commercially available protein crystallization screen solution Morpheus HT-96 screen single reagent, well number H1 (from Molecular Dimensions). The actual precipitant composition was 0.1 M amino acids in a 0.1 M buffer (1.0 M imidazole + MES monohydrate acid buffer) at pH 6.5 made up with a 50 % v/v precipitant mix (that contained 10 % w/v PEG 20,000; 20 % v/v PEG MME 550). Crystals were grown by vapour diffusion at 4 °C overnight.

1.8 Data Collection, Model Building, and Refinement

Data were collected at Diamond Light Source from single cryofrozen crystals. Th-hal diffracted to a resolution of 2.33 Å and Th-Fre to a resolution of 2.5 Å. All data were processed and scaled using XDS and the structures solved by molecular replacement in Phaser (Search models 2AQJ and 3F2V). Iterative cycles of rebuilding and refinement were carried out in COOT and Phenix, validation with MOLPROBITY was integrated into the iterative rebuild process. Further validation of the final model was carried out using PDB_REDO. The crystal structure of Th-hal and Th-Fre has been deposited in the protein data bank (PDB file 5LV9 and 5LVA).

2) Supplementary Figures

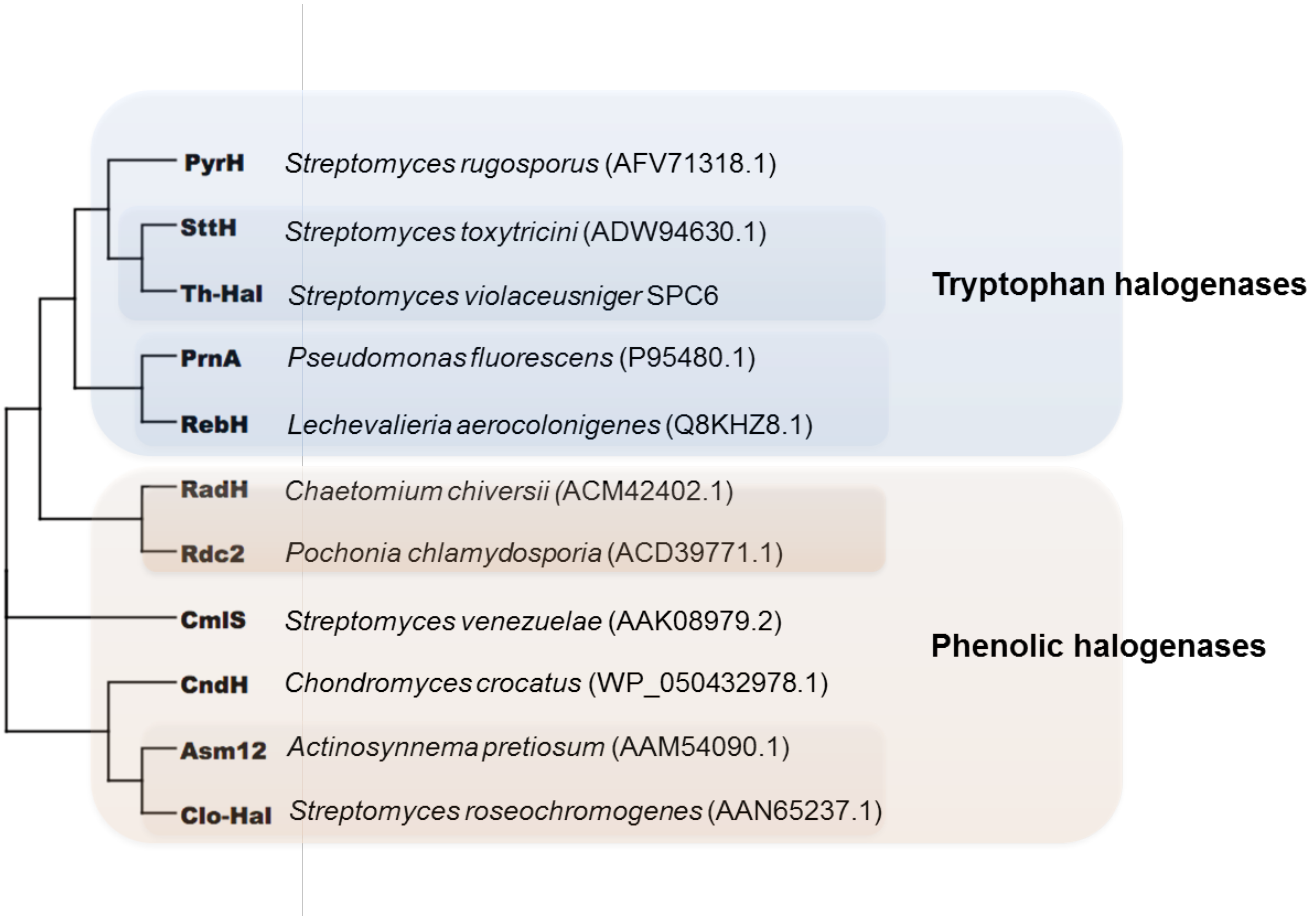


Figure S1. The phylogenetic tree showing the evolutionary relationship between tryptophan Fl-Hal and phenolic Fl-Hal enzymes.

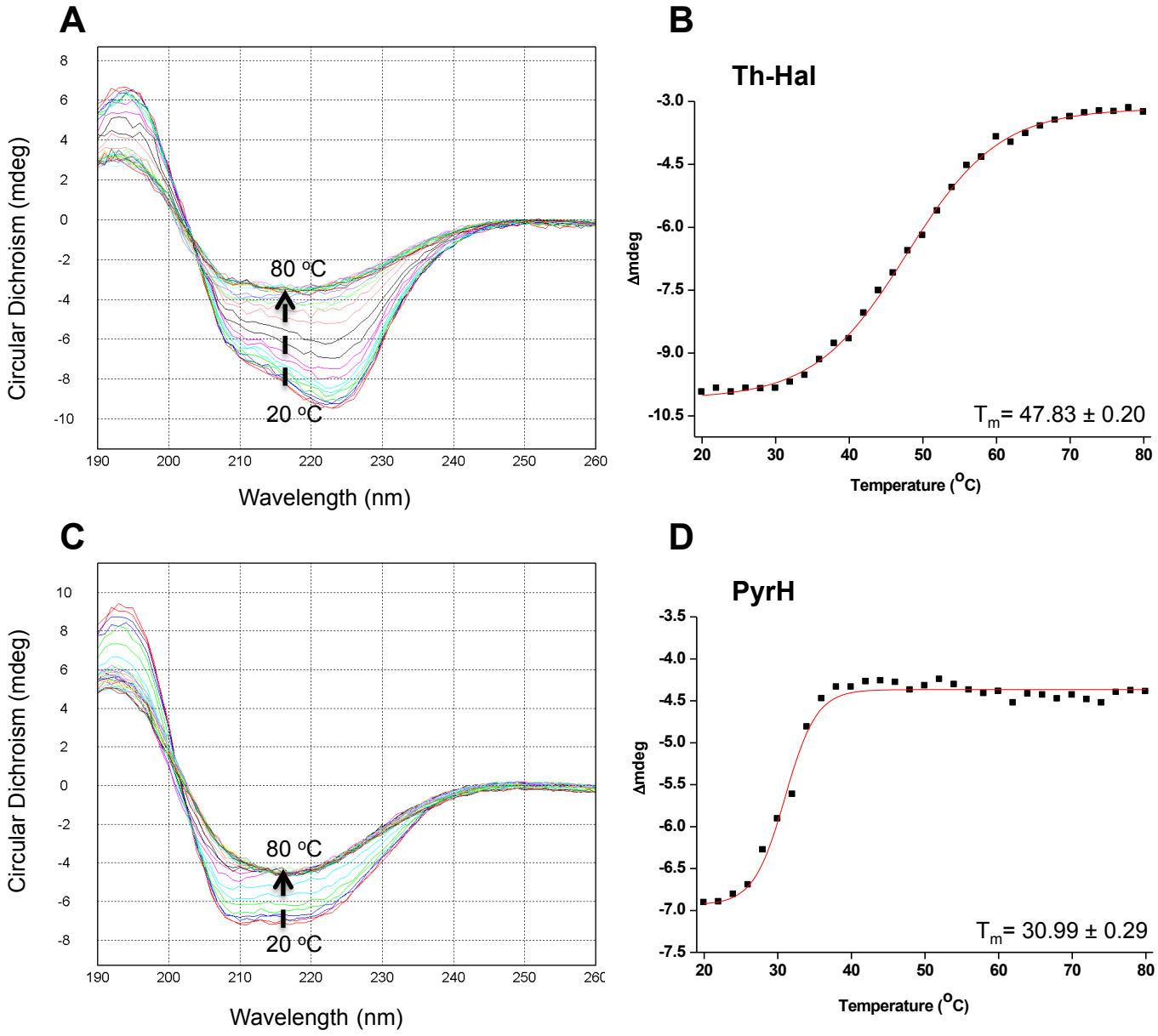


Figure S2. Thermal stability assays and melting temperatures (T_m) of Th-Hal and PyrH halogenases. (**A**) Variable temperature CD and (**B**) melting curve and T_m of Th-Hal. (**C**) Variable temperature CD and (**D**) melting curve and T_m of PyrH. The CD spectrum was measured every 2 °C, from 20 °C up to 80 °C by keeping 10 μM enzyme in a 0.5 mm path length cuvette at fixed temperature for 2 minute with the temperature ramp of 0.2 °C per minute. The absorbance changes at 222 nm was plotted against temperature, and fitted with a nonlinear curve fitting function to find melting point of proteins

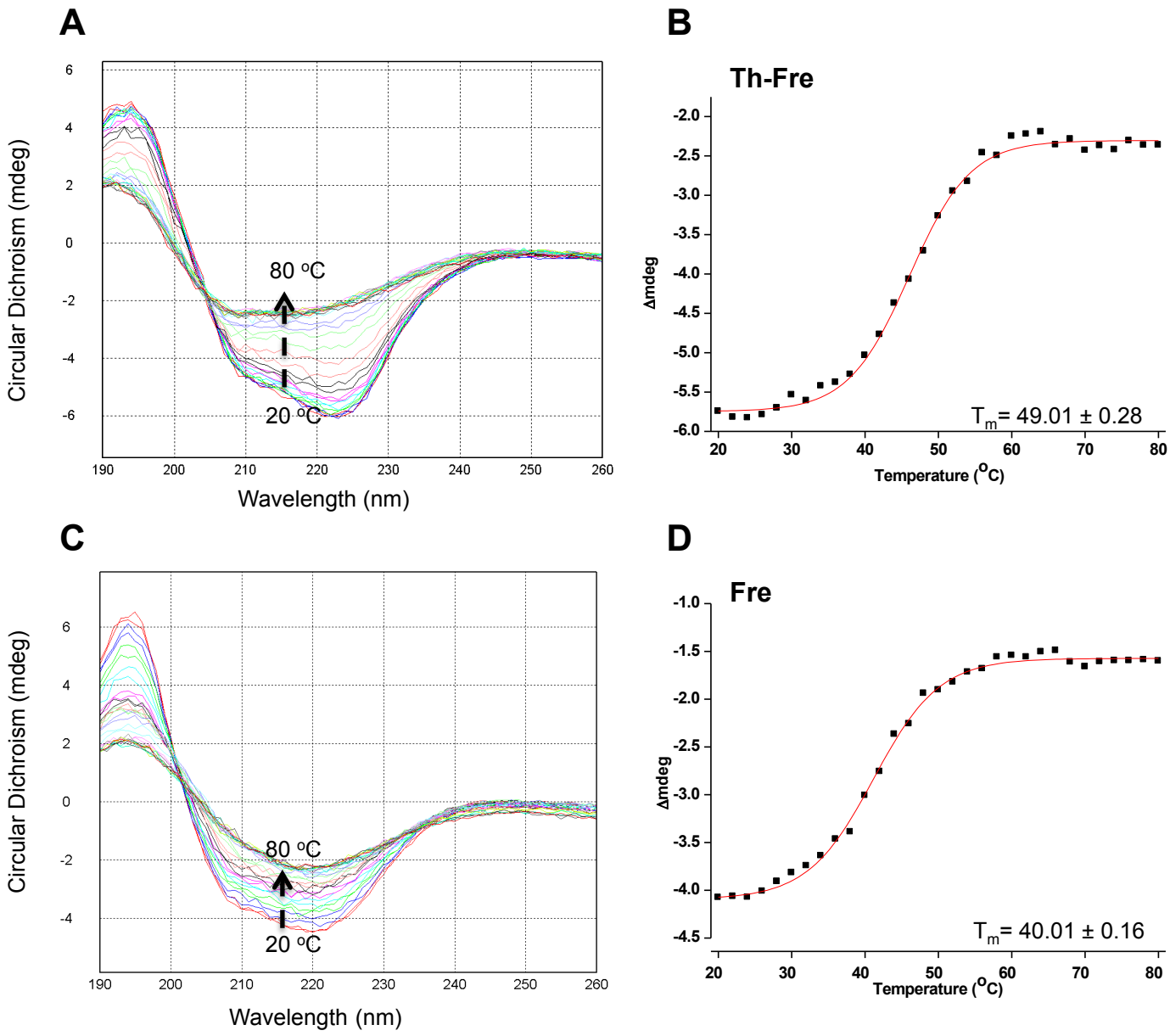


Figure S3. Thermal stability assays and melting temperatures (T_m) of Th-Fre and the *E. coli* Fre (**A**) Variable temperature CD and (**B**) melting curve and T_m of Th-Fre. (**C**) Variable temperature CD and (**D**) melting curve and T_m of the *E. coli* Fre. The CD spectra were acquired and processed as described above.

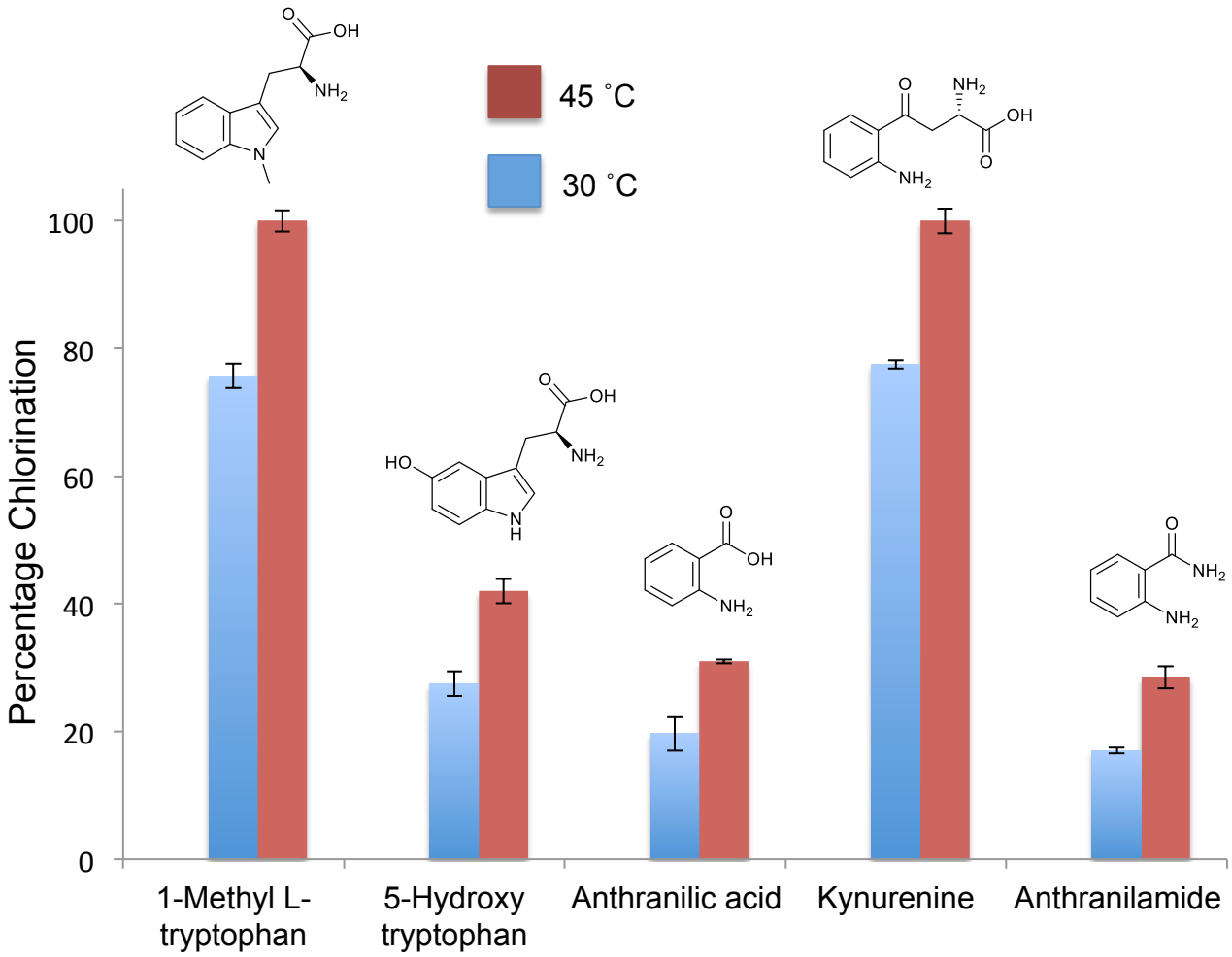


Figure S4. Chlorination of non-native substrates **2-6** to the chlorinated derivatives **2a-6a** at different temperatures with Th-Hal and Th-Fre. Reaction were carried out with Th-Hal (2.5 µM), Th-Fre (1 µM), FAD (1 µM), NADH (2.5 mM), $MgCl_2$ (10 mM) and substrate (0.5 mM) in 10 mM potassium phosphate buffer, pH 7.4. After 180 minutes incubation at 30 °C (or 45 °C) and 200 rpm, the reactions were analysed by HPLC.

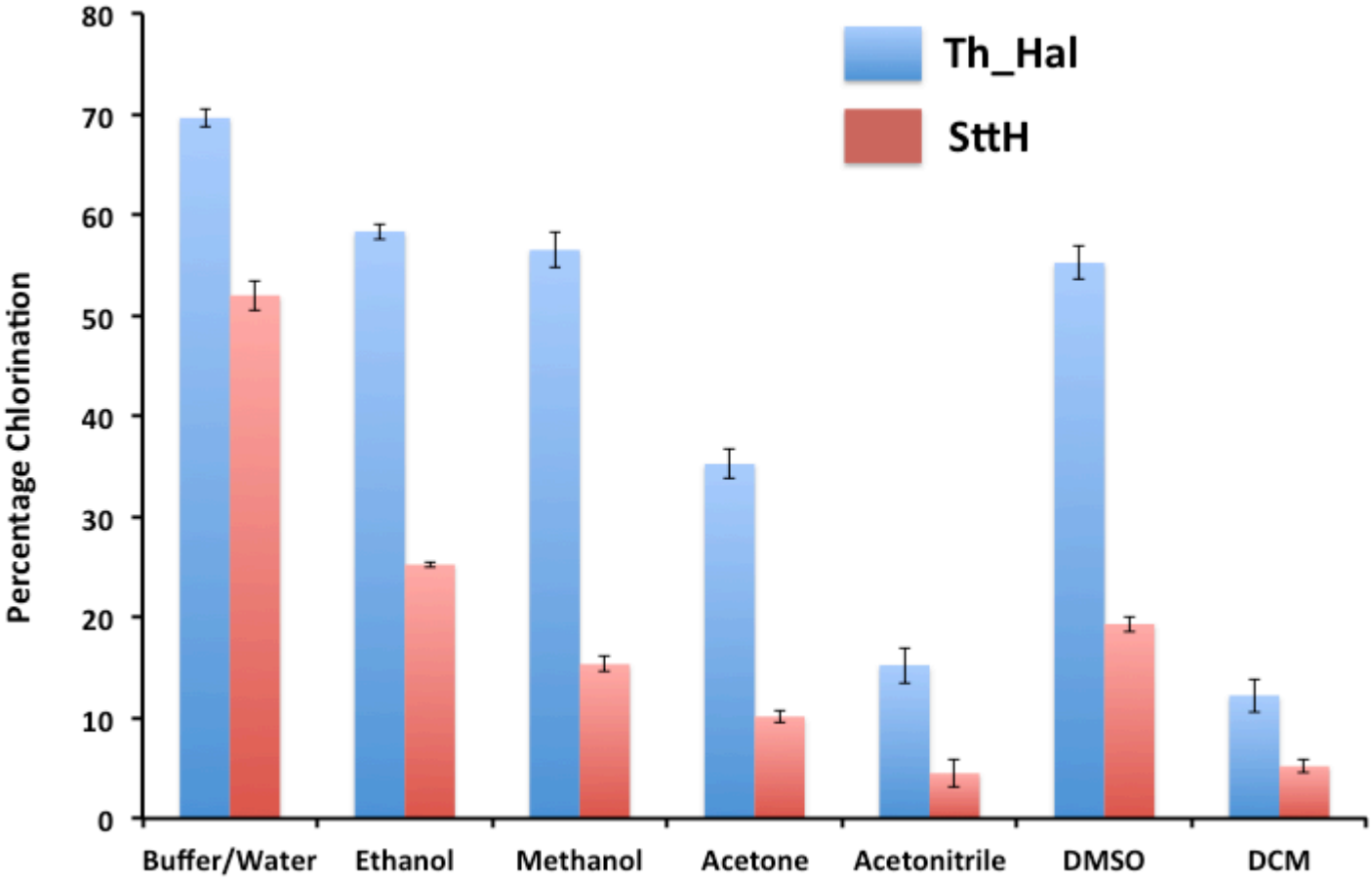


Figure S5. Comparison of solvent tolerance by Th_hal and its mesophilic counter part SttH upon halogenation of tryptophan. Reaction were carried out with Th-Hal or SttH (2.5 µM), Th-Fre (1 µM), FAD (1 µM), NADH (2.5 mM), $MgCl_2$ (10 mM) and tryptophan (0.5 mM) in 10 mM potassium phosphate buffer, pH 7.4 in the presence of 10 % of corresponding organic solvent. After 30 minutes incubation at 30 °C and 200 rpm, the reactions were analysed by HPLC.

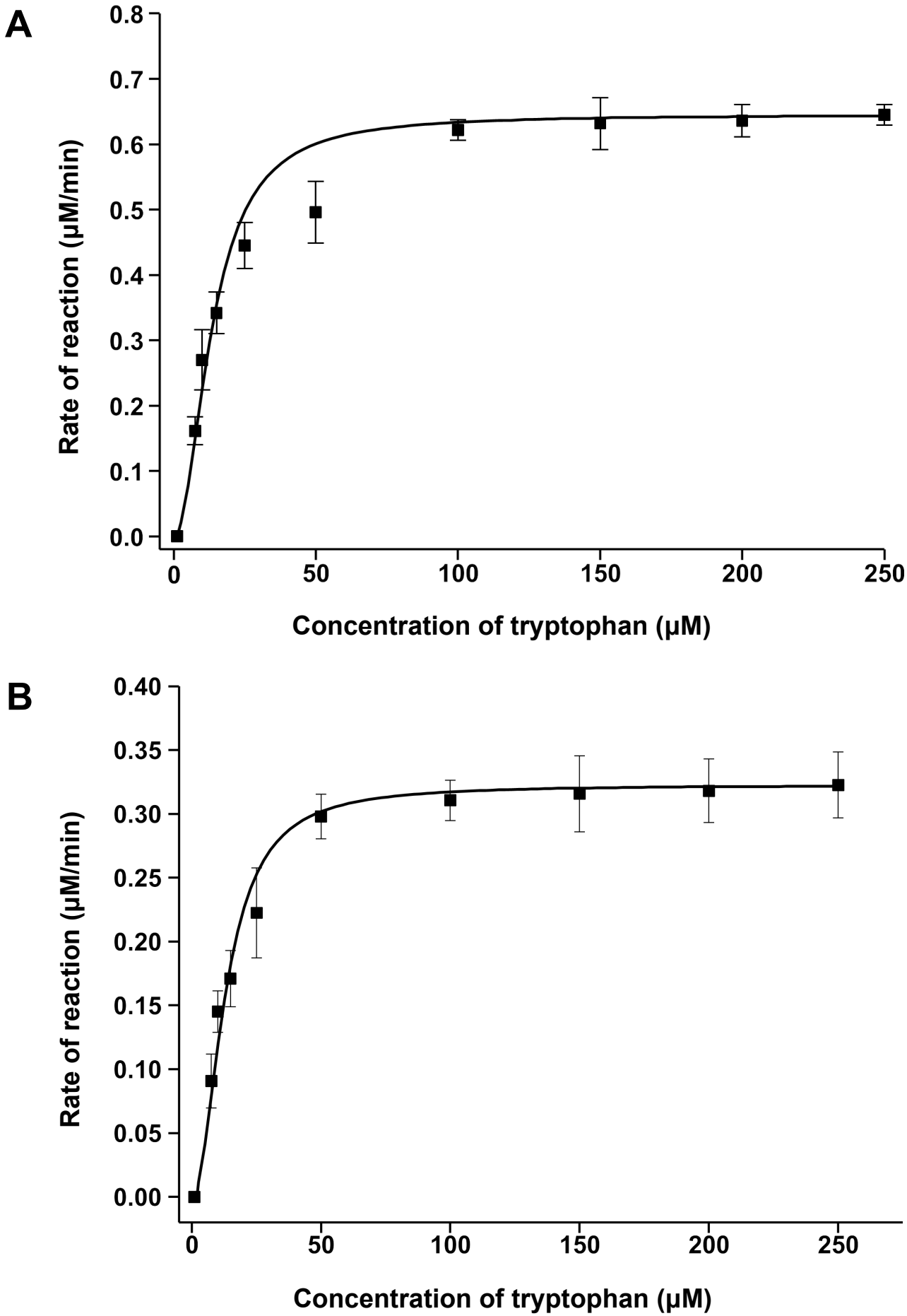
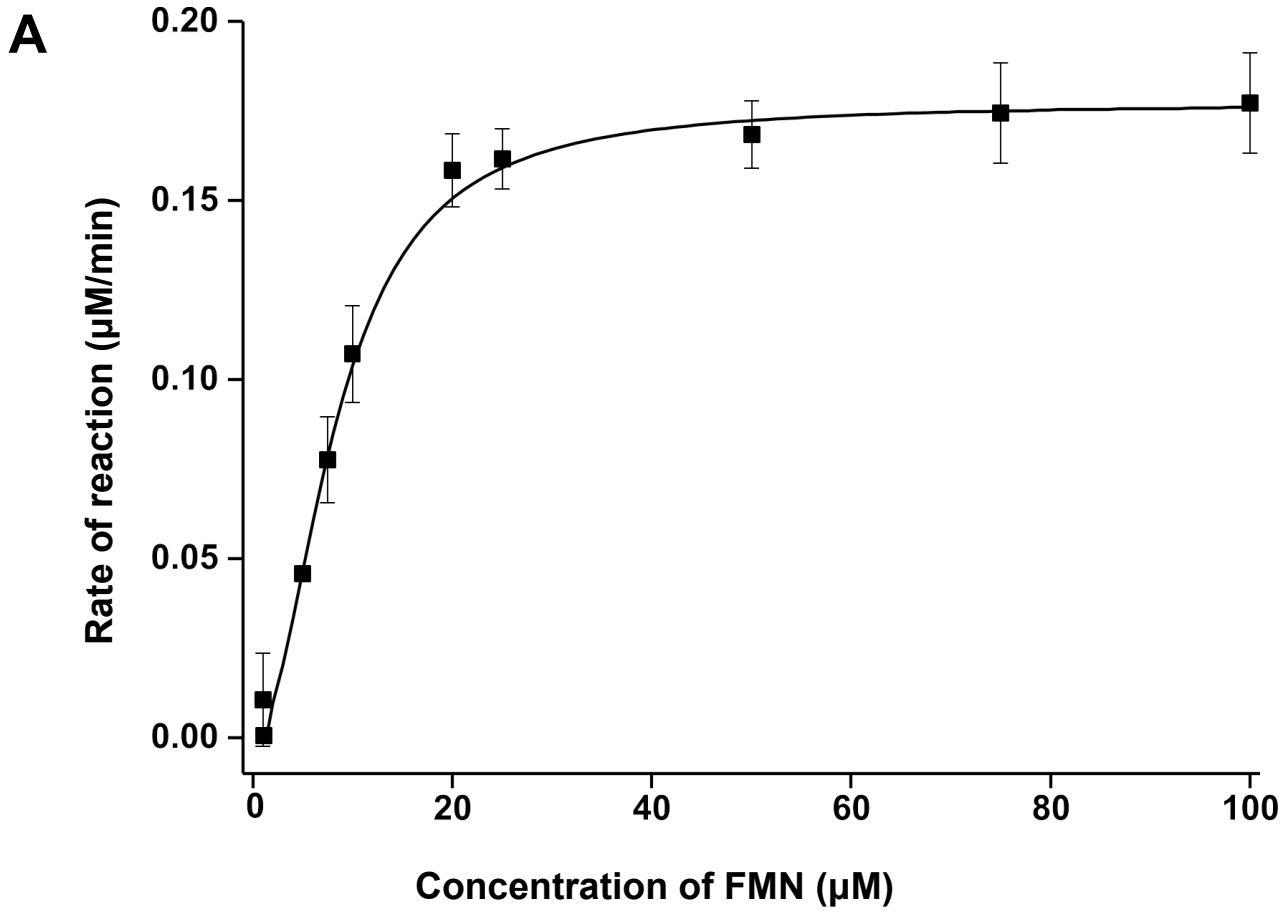


Figure S6. Michaelis Menten curve for **(A)** Th-Hal and **(B)** PyrH halogenase enzymes.



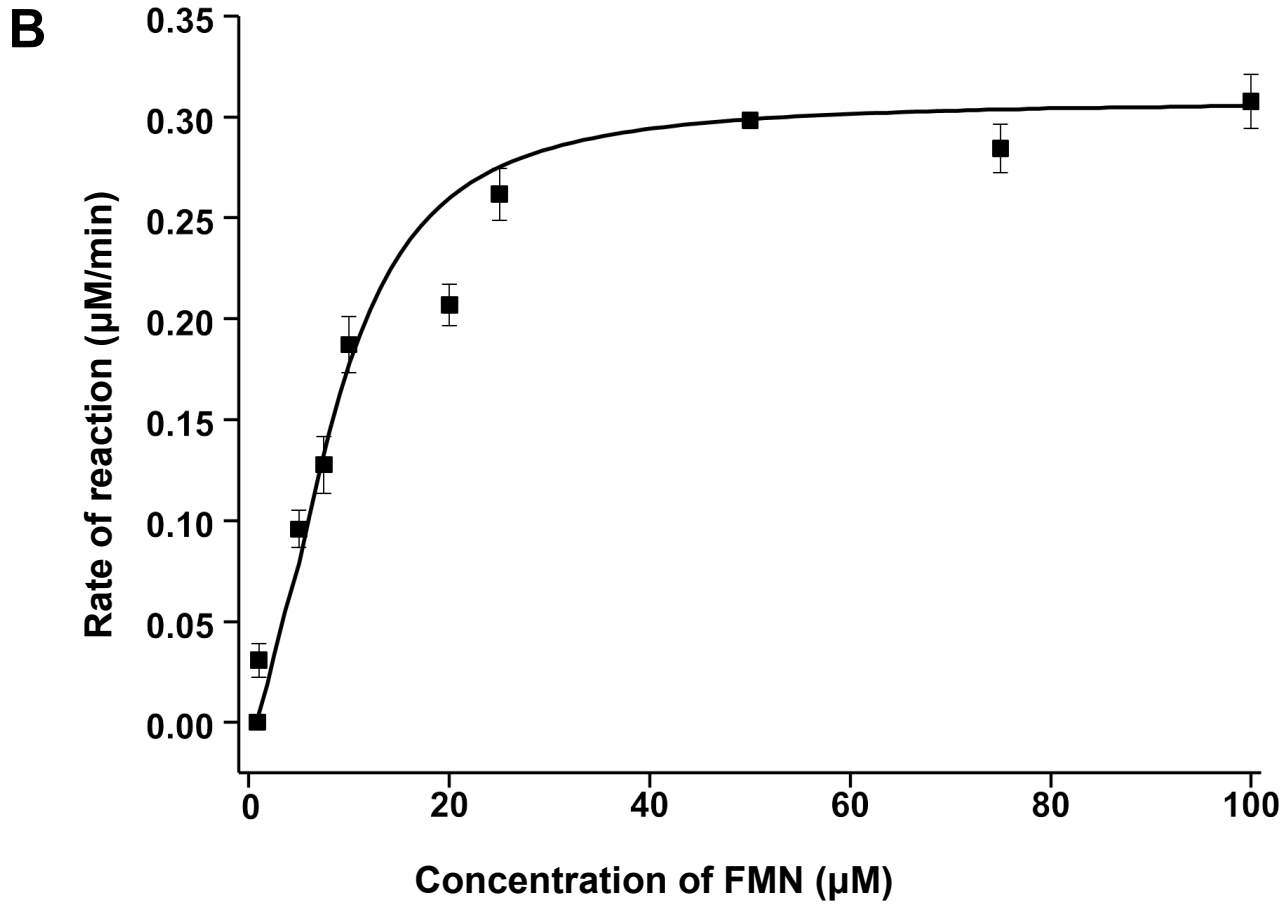


Figure S7. Michaelis Menten curve for FMN reduction by Th-Fre **(A)** at 30 °C and **(B)** at 45 °C.

Figure S8. Amino acid sequence alignment between Th-Hal and SttH halogenase enzyme. Conserved amino acids are in black and non-conserved residues are colour coded as shown above.

A

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Th-Fre          --MKVLVLAFHP---NMEQSVVNRAFADTLKDAPGITLRDLY------------------
Human           AGKKVLIVYAHQEPKSFNGSLKNVAVDELSRQGCTVTVSDLYAMNFEPRATDKDITGTLS
                   ***::  *    .:: *: * *. :  .:.  :*: ***

Th-Fre          ------------QEYPDEAI--DVEKEQKLCEEHDRIVFQFPLYWYSSPPLLKKWLDHVL
Human           NPEVFNYGVETHEAYKQRSLASDITDEQKKVREADLVIFQFPLYWFSVPAILKGWMDRVL
                            : * : ::  *: .***   * * ::*******:* *.:** *:*.**

Th-Fre          LYGWAYGTNG----TALRGKEFMVAVSAGAPEEAYQAGGSNHYAISELLRPFQATSNFIG
Human           CQGFAFDIPGFYDSGLLQGKLALLSVTTGGTAEMYTKTGVNGDSRYFLWPLQHGTLHFCG
                  *:*:.  *      *.**  :::*::*.. * *   * *  :   *    :.* :* *

Th-Fre          TTYLPPYVFYQAGTAGKSELAEGATQYREHVLKSF---------------
Human           FKVLAPQISFAPEIASEEERKGMVAAWSQRLQTIWKEEPIPCTAHWHFGQ
                 . *.* : : .  *.:.*    .: : :.: . :
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B

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Th-Fre          MKVLVLAFHPNMEQSVVNRAFADTLKDAPGITLRDLYQEYPDEAIDVEKEQKLCEEHDRI
P.pentosaceus   MQTLIIVAHPELARSNTQPFFKAAIENFSNVTWHPLVADF-----NVEQEQSLLLQNDRI
                *:.*::. **:: .* .:  *  :::: ..:* . *  ::     :**:**.*  ::***

Th-Fre          VFQFPLYWYSSPPLLKKWLDHVLLYGWAYGTNGTALRGKEFMVAVSAGAPEEAYQAGGSN
P.pentosaceus   ILEFPLYWYSAPALLKQWMDTVMTTKFATGHQ-YALEGKELGIVVSTGDNGNAFQAGAAE
                :::*******:*.***:*:* *:   :* * :  ** ***: :.**:*   :*:***.::

Th-Fre          HYAISELLRPFQATSNFIGTTYLPPYVFYQAGTAGKSELAEGATQYREHVLKSF
P.pentosaceus   KFTISELMRPFEAFANKTKMMYLPILAVHQFLYLEPDAQQRLLVAYQQYATNVG
                :::****:***:* :*     ***  ..:*      .      . *.::. :
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Figure S9. Amino acid sequence alignments of **(A)** Th-Fre and the human quinone reductase 2 (Nqo2) (PDB 4FGL) and (**B**) Th-Fre and the NADPH-quinone reductase from *P. pentosaceus* (PDB 3HA2) are shown. The extra amino acid insertion at the N-terminal region of human quinone reductase 2 is shown with red coloured dash lines.

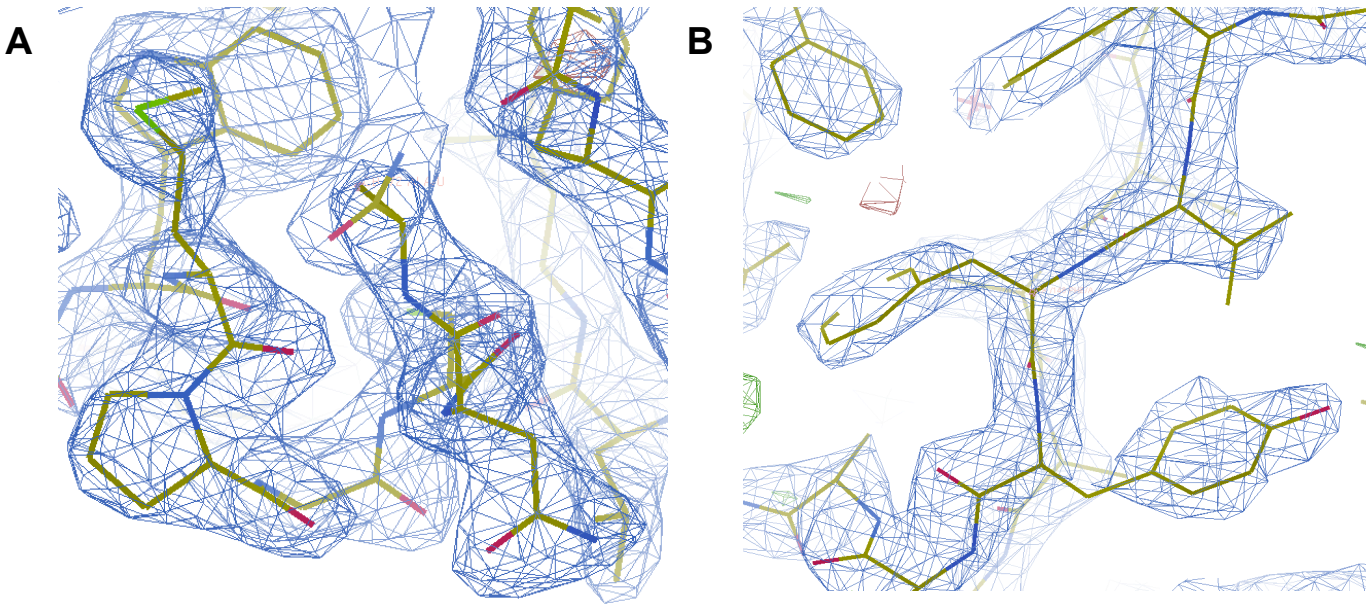


Figure S10. 2Fo-Fc electron density map of resolved crystal structures of (A) Th-Hal and (B) Th-Fre enzymes at randomly selected amino acid residues. The protein backbone and amino acid residues are shown as sticks. Th-Fre structure contains an extra electron density above FMN cofactor (corresponds to an unknown aromatic moiety), which was spared in the model.

Organism	Sequence identity with Th_Hal	Reported optimum growth temperature	Accession number
Streptomyces violaceusniger SPC6	100 %	45 °C	WP_037947065.1
Streptomyces thermoautotrophicus	61 %	65 °C	KWX04309.1
Microbulbifer thermotolerans	36 %	43 – 49 °C	AMX03406.1
Rubellimicrobium thermophilum	36 %	45 – 54 °C	WP_021098953.1
Sulfobacillus thermosulfidooxidans	21 %	50-55°C	WP_020375004.1

Table S1. The list of potential thermophilic organisms that carry tryptophan halogenase enzyme.

	FMN			NADH		
Enzyme	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (μM.s^{-1})	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (μM.s^{-1})
T.Fre	16 ± 1	2.5 ± 0.3	6.4 ± 0.9	9.2 ± 0.1	15 ± 3	0.61 ± 0.12
SsuE	8.1 ± 0.2	8.3 ± 0.1	0.98 ± 0.03	4.3 ± 0.1	43 ± 8	0.10 ± 0.02
Fre	4.3 ± 0.4	14.7 ± 0.3	0.29 ± 0.03	2.2 ± 0.5	35 ± 2	0.06 ± 0.01

Table S2. The kinetic parameters for Th-Fre reduction of FMN with NADH at 30 °C.

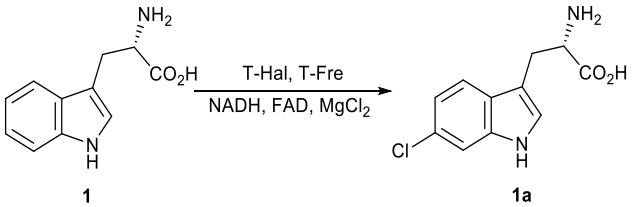
	FAD			NADH		
Enzyme	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (μM.s^{-1})	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (μM.s^{-1})
T.Fre	9.5 ± 0.6	16.3 ± 0.2	0.58 ± 0.04	6.1 ± 0.4	25 ± 3	0.24 ± 0.03
SsuE	4.3 ± 0.6	12.3 ± 0.9	0.35 ± 0.06	3.1 ± 0.1	53 ± 2	0.06 ± 0.003
Fre	7.2 ± 0.3	7.7 ± 0.5	0.94 ± 0.07	6.2 ± 0.5	25 ± 1	0.25 ± 0.02

Table S3. The kinetic parameters for Th-Fre reduction of FAD with NADH at 30 °C

	Thermo Hal	Thermo Fre
Data collection		
Wavelength	0.979	0.976
Space group	**P 65**	**P 61 2 2**
a, *b*, *c* (Å)	67.14 67.14 477.37	6.840 66.840 335.000
a, b, g (°)	90.00 90.00 120.00	90.00 90.00 120.00
Resolution (Å)	2.33-56.51(2.39-2.33)	54.7- 2.53 (2.77- 2.53)
R_{sym} or R_{merge}	0.145 (0.63)	0.15 (0.87)
I / s*I*	11.7 (2.5)	19.7 (4.4)
Completeness (%)	99.7 (99.4)	99.9 (99.6)
Redundancy	9.9 (6.6)	18.4 (18.4)
Refinement		
Resolution (Å)	**2.33**	**2.53**
R_{work} / R_{free}	0.204/ 0.247	0.190 /0.251
No. atoms	8110	2953
Average *B*-factors (all atoms)	38.01	30.95
R.m.s. deviations		
Bond lengths (Å)	0.009	0.009
Bond angles (°)	1.26	1.254

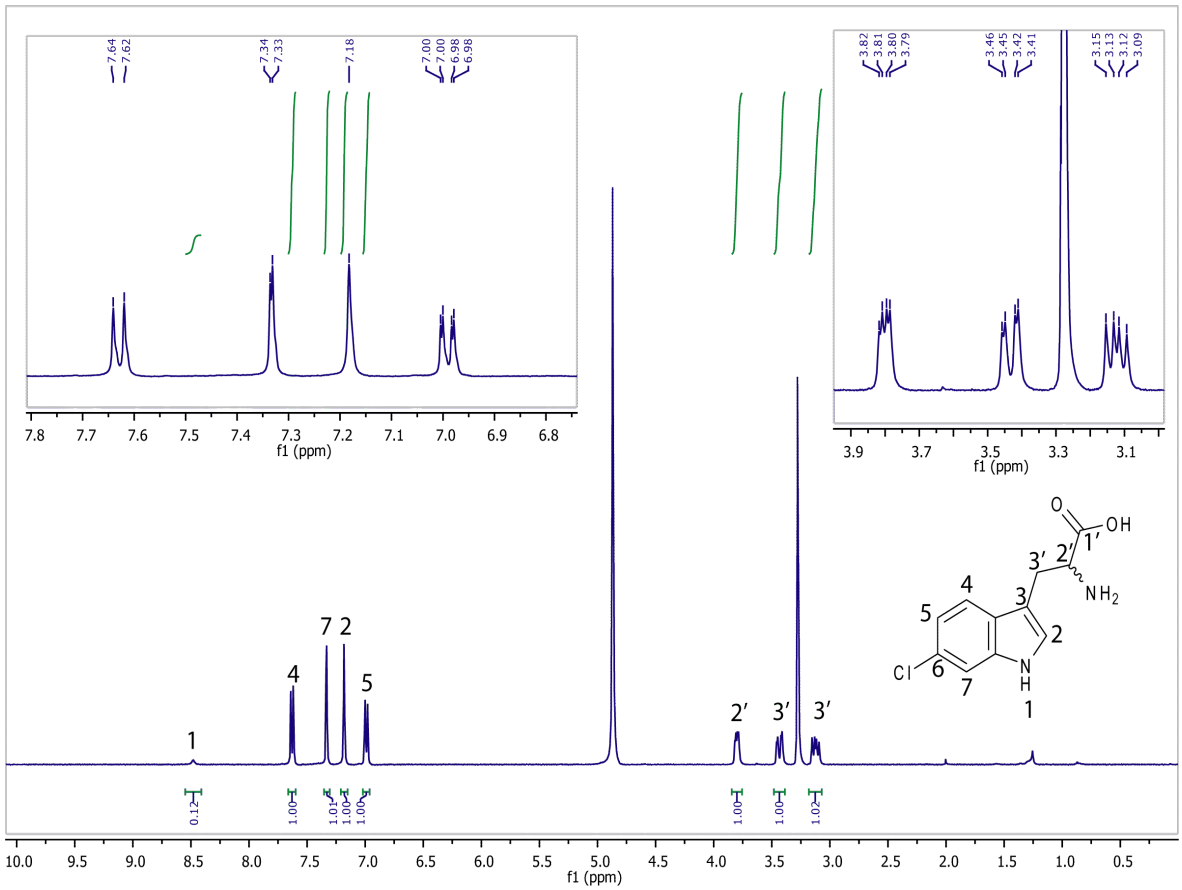
Table S4. Data collection and refinement table for Th-hal and Th-Fre. (* Statistics for the highest-resolution shell are in parentheses)

3) NMR spectra

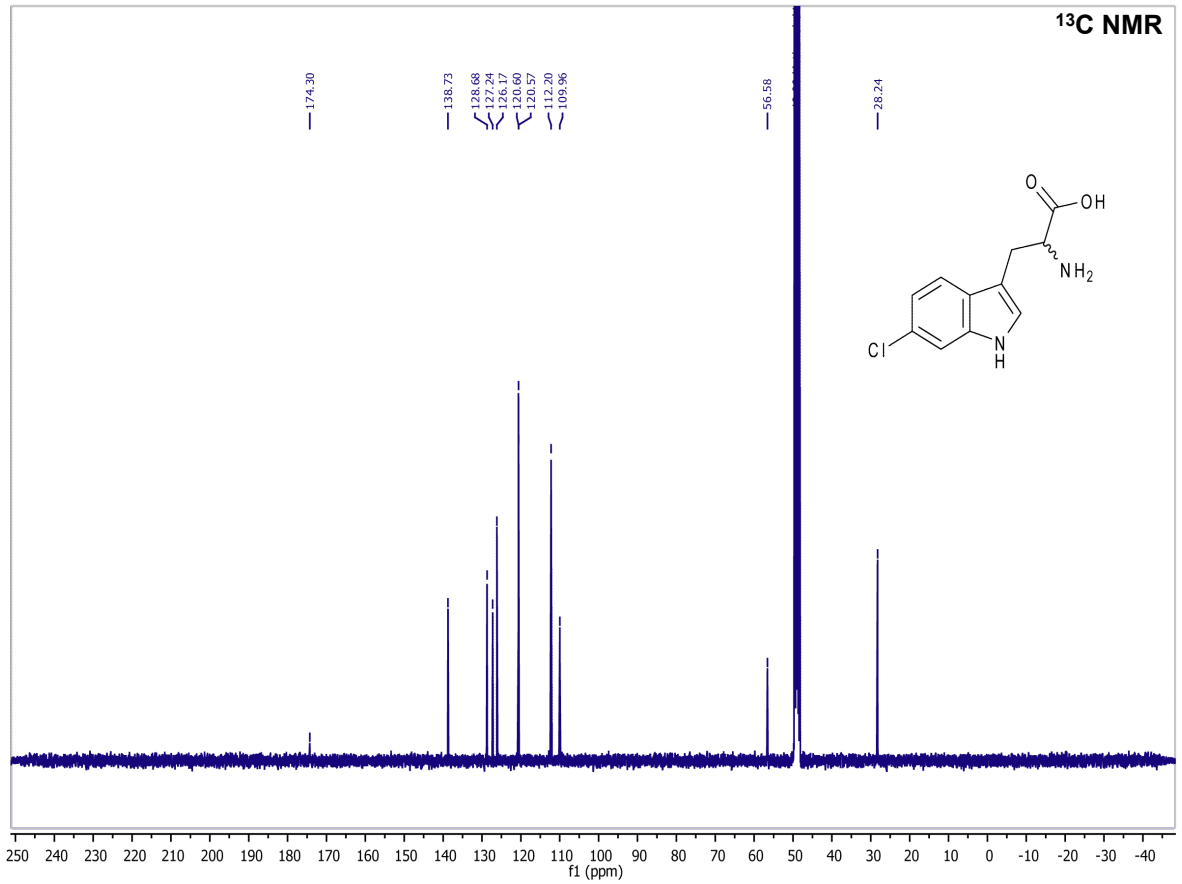


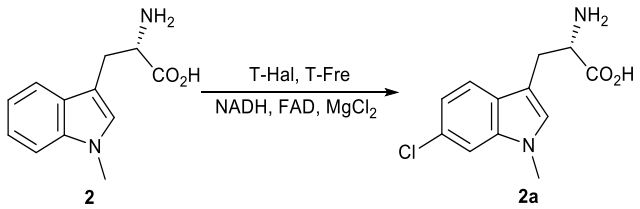
1 1a

^{1}H NMR

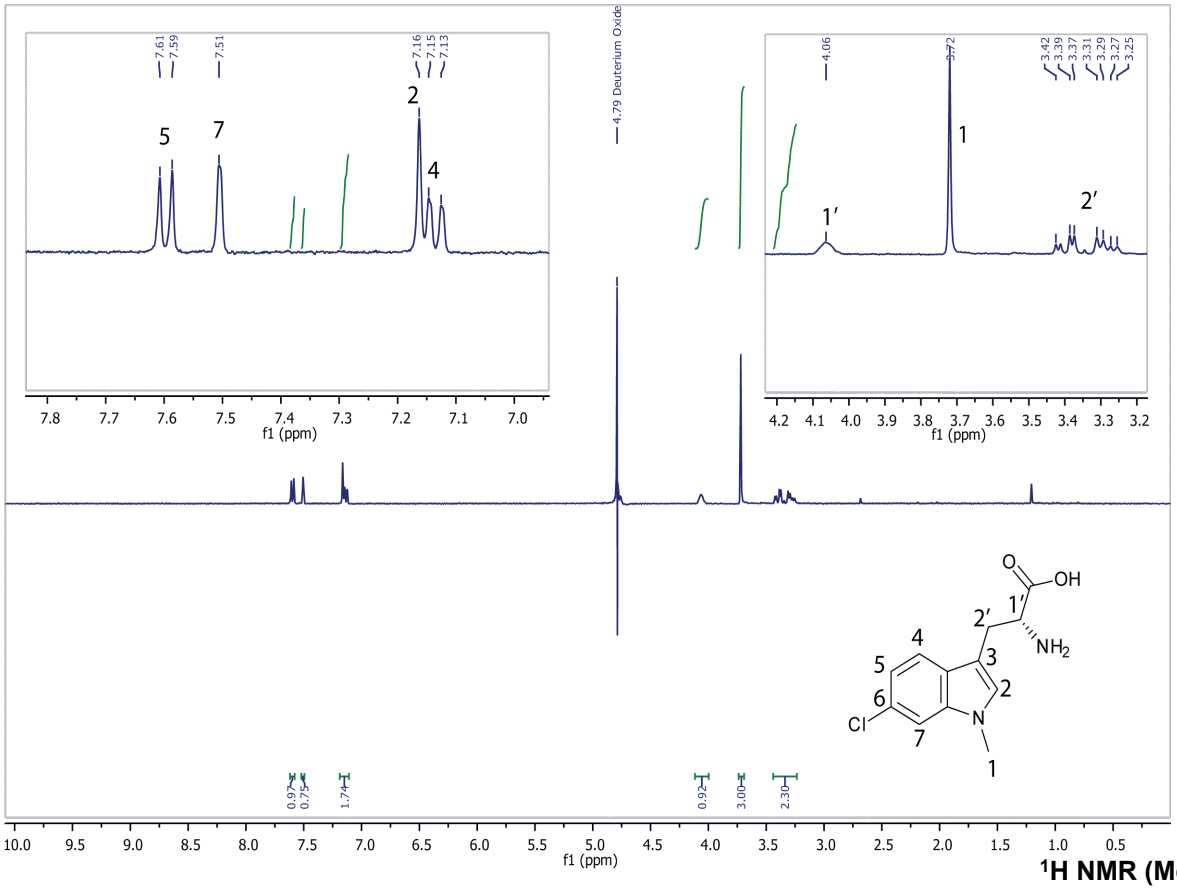


^{13}C NMR

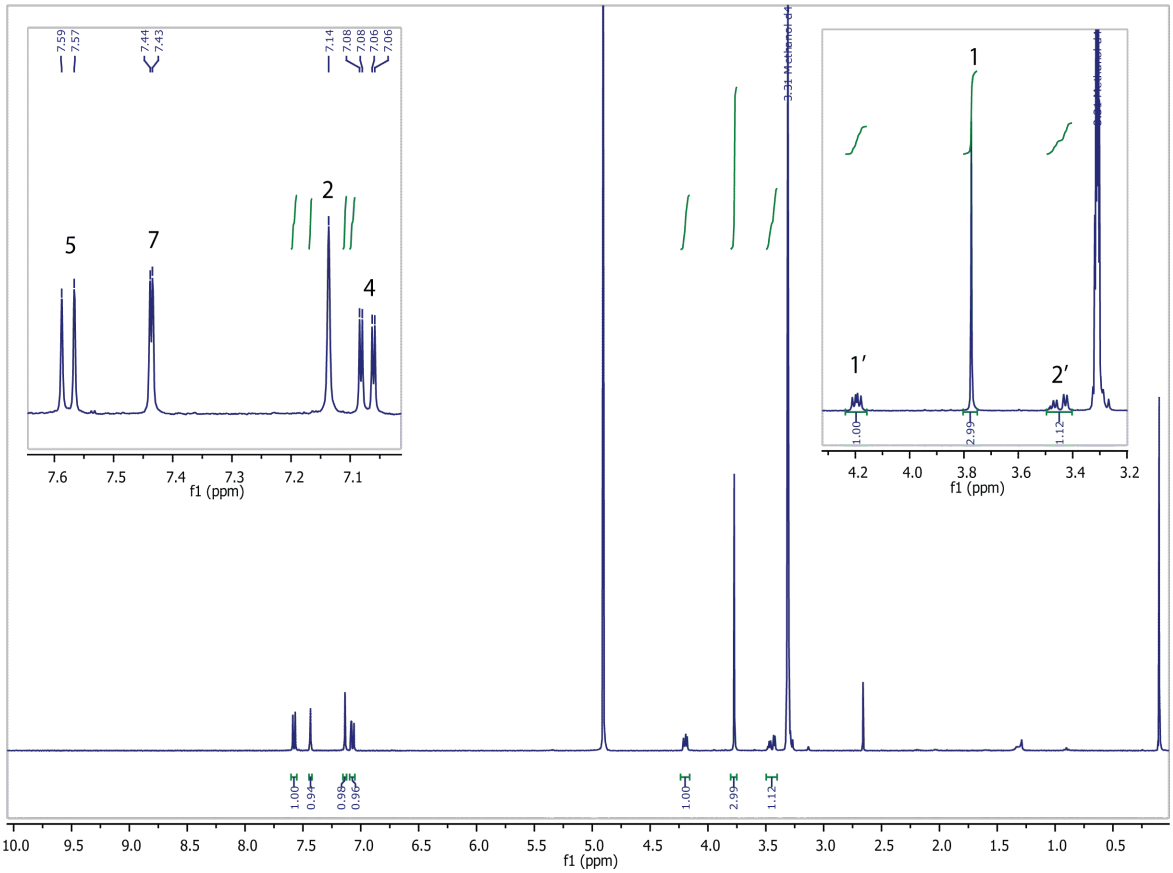


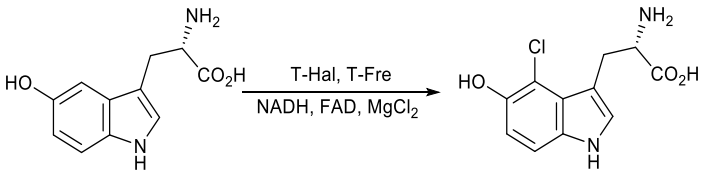


^{1}H NMR (D_2O)



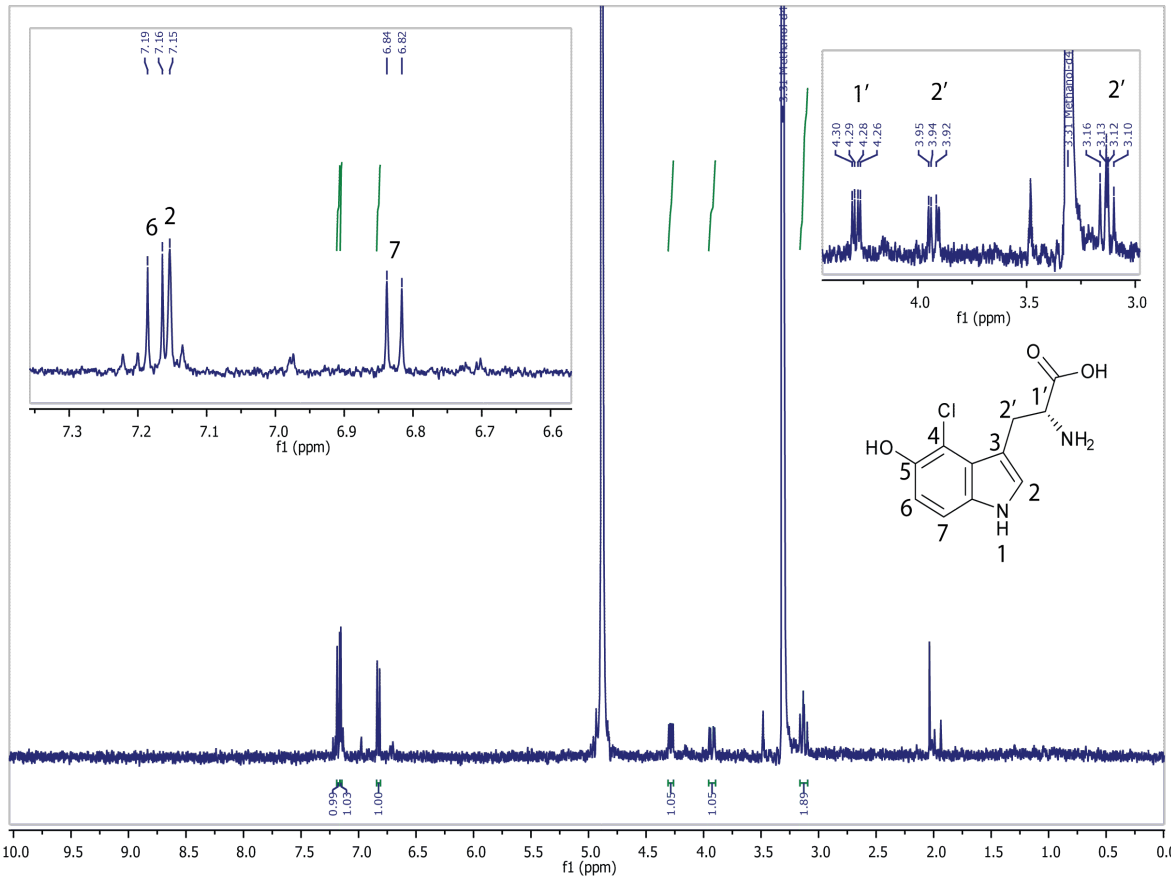
^{1}H NMR (MeOD)

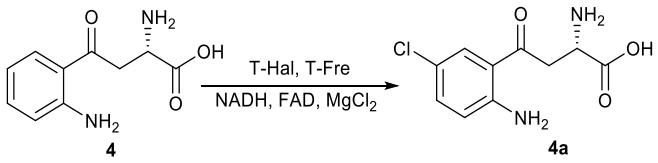



NH2
CO2H
HO
N
H
T-Hal, T-Fre
NADH, FAD, MgCl2
Cl

3 3a

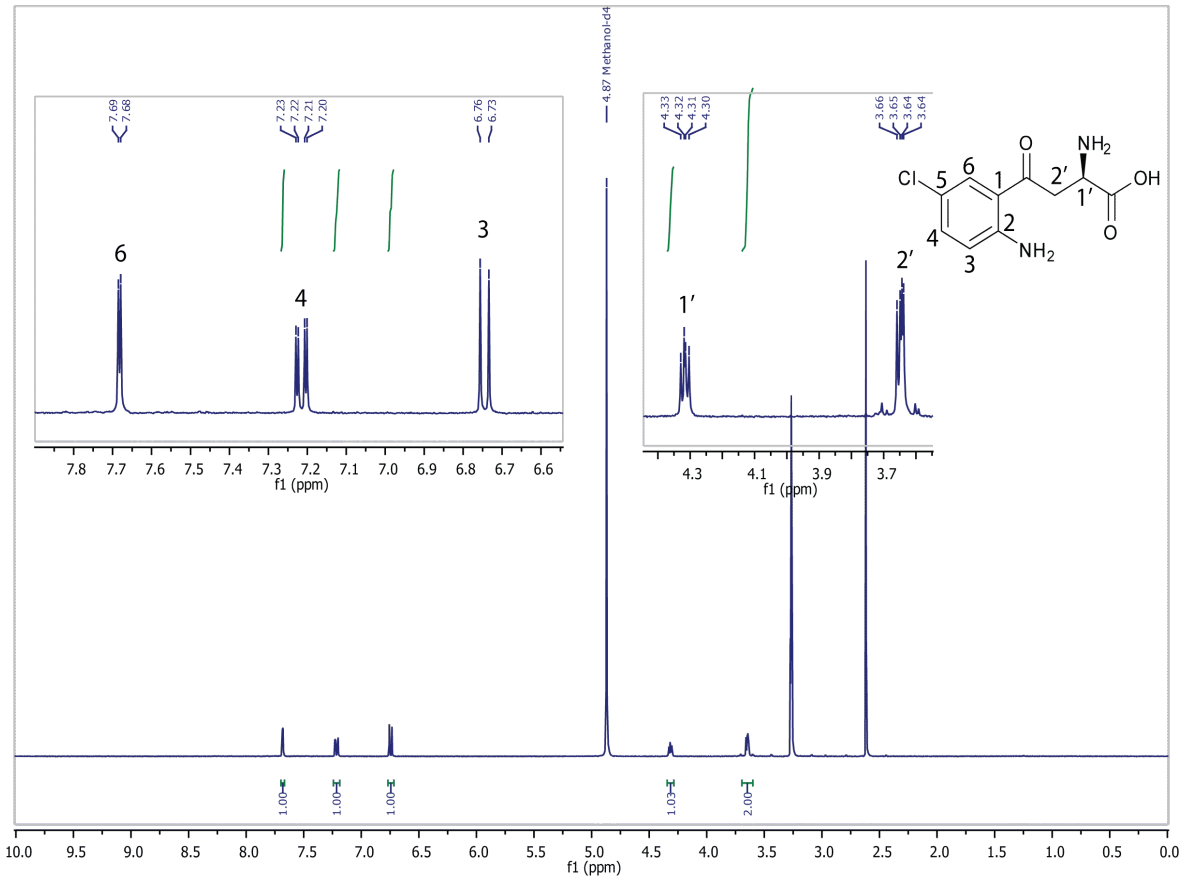
^{1}H NMR

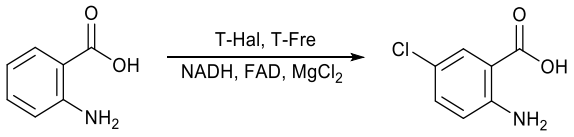

7.19
7.16
7.15
6.84
6.82
6
2
7
f1 (ppm)
1′
2′
4.30
4.29
4.28
4.26
3.95
3.94
3.92
3.31 Methanol-d4
3.16
3.13
3.12
3.10
4.0
3.5
3.0
O
OH
Cl
HO
NH2
4
3
5
2
6
N
H
7
1
0.99
1.03
1.00
1.05
1.05
1.89
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0



4 4a

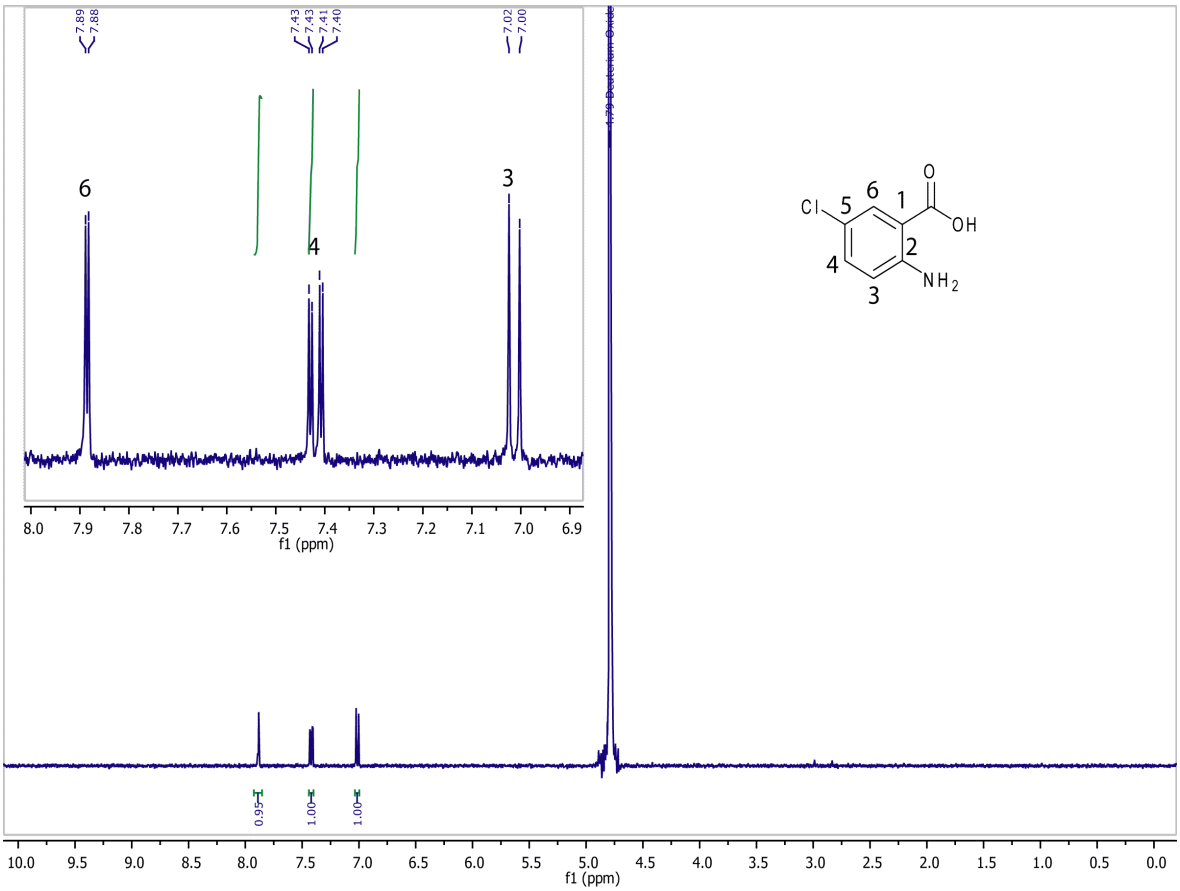
^{1}H NMR

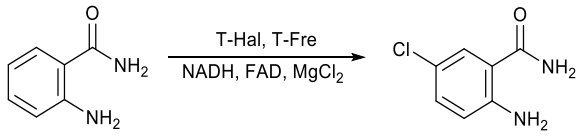



O
OH
NH2
T-Hal, T-Fre
NADH, FAD, MgCl2
Cl
O
OH
NH2

5 **5a**

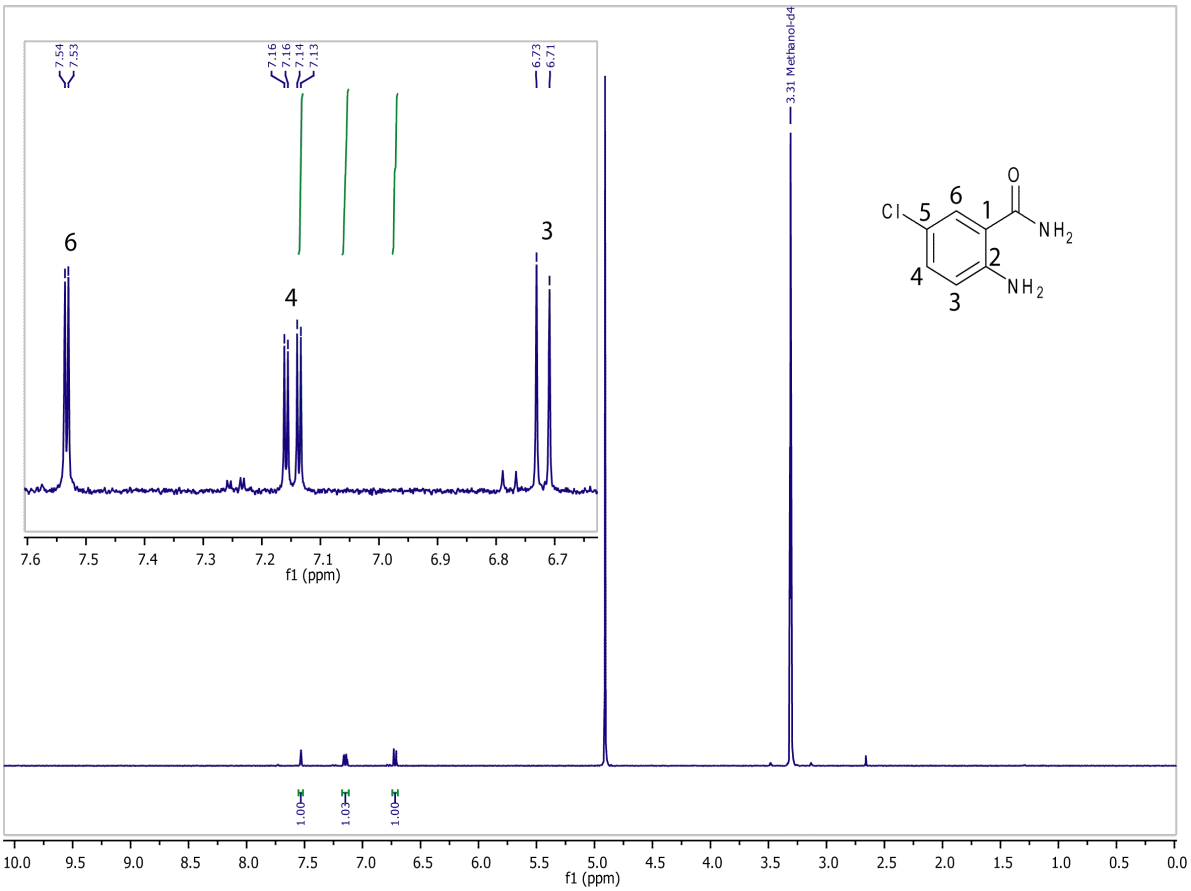
^{1}H NMR


7.89
7.88
7.43
7.43
7.41
7.40
7.02
7.00
6
4
3
8.0 7.9 7.8 7.7 7.6 7.5 7.4 7.3 7.2 7.1 7.0 6.9
f1 (ppm)
Cl
5
6
1
O
OH
2
4
NH2
3
0.95
1.00
1.00
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)


O
NH2
NH2
T-Hal, T-Fre
NADH, FAD, MgCl2
Cl
O
NH2
NH2

6 **6a**

^{1}H NMR


7.54
7.53
7.16
7.16
7.14
7.13
6.73
6.71
3.31 Methanol-d4
6
4
3
f1 (ppm)
Cl
O
NH2
NH2
1.00
1.03
1.00
f1 (ppm)