SUPPLEMENTARY INFORMATION

A conserved threonine prevents self-intoxication of NADPH-dependent enoyl-thioester reductase

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The Supplementary Information contains

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Supplementary Results

Supplementary Tables

Supplementary Table 1. Observed kinetic isotope effects in Etr1p and mutants as measured by incorporation ratios of H/D from buffers with defined H/D ratios**,** $^{D}k_{obs}$, $^{(NADPH/NADPD)}(k_{cat})$ and $^{(NADPH/NADPD)}(k_{cat}/K_m)$ were measured in a non-competitive fashion, for all the kinetic details see **material and methods**. All values represents averages of two independent values, based on at least four data points each. See methods section for details. Errors given as 95% confidence intervals. Abbreviations: n.d.= not determined, n.a.= not applicable.

Enzyme/reaction	$^{D}k_{obs}$	$^{(NADPH/NADPD)}(k_{cat})$	$^{(NADPH/NADPD)}(k_{cat}/K_m)$
WT	1.78 ± 0.08	4.3 ± 0.2	1.94 ± 0.95
S70A	1.11 ± 0.07	4.2 ± 0.2	1.1 ± 0.5
T324A	1.92 ± 0.15	3.6 ± 0.1	1.1 ± 0.3
S70A T324A	1.20 ± 0.08	n.d.	n.d.
Y79F	4.8 ± 0.8	n.d.	n.d.
T175V	4.4 ± 0.8	0.96 ± 0.04	2.0 ± 0.7
C2-ene adduct decay	4.5 ± 0.2	n.a.	n.a.
C4-adduct decay	4.4 ± 0.2	n.a.	n.a.

Supplementary Table 2. List of primers used to generate Etr1p and Ccr variants

Enzyme variant	Primer Name	Sequence
Etr1p S70A	Etr1p S70A fwd	CCGGTCAACCCGGCCGACATCAACCAG
	Etr1p S70A rev	CTGGTTGATGTCGGCCGGGTTGACCGG
Etr1p T324A	Etr1p T324A fwd	GGGCTTCTGGGTGGCCGAGCTGCTCAAG
	Etr1p T324A rev	CTTGAGCAGCTCGGCCACCCAGAAGCCC
Etr1p Y79F	Etr1p Y79F fwd	GATCCAGGGCGTCTTTCCGAGCAAGCCG
	Etr1p Y79F rev	CGGCTTGCTCGGAAAGACGCCCTGGATC
Etr1p T175V	Etr1p T175V fwd	CATCTCGGTCAATCCCCTGGTGGCCTACCTCATGCTCAC
	Etr1p T175V rev	GTGAGCATGAGGTAGGCCACCAGGGGATTGACCGAGATG
Ccr T195V	Ccr T195V fwd	GCTGACGCTCGCCGTCGCCTACCGCATGC
	Ccr T195V rev	GCATGCGGTAGGCGACGGCGAGCGTCAGC

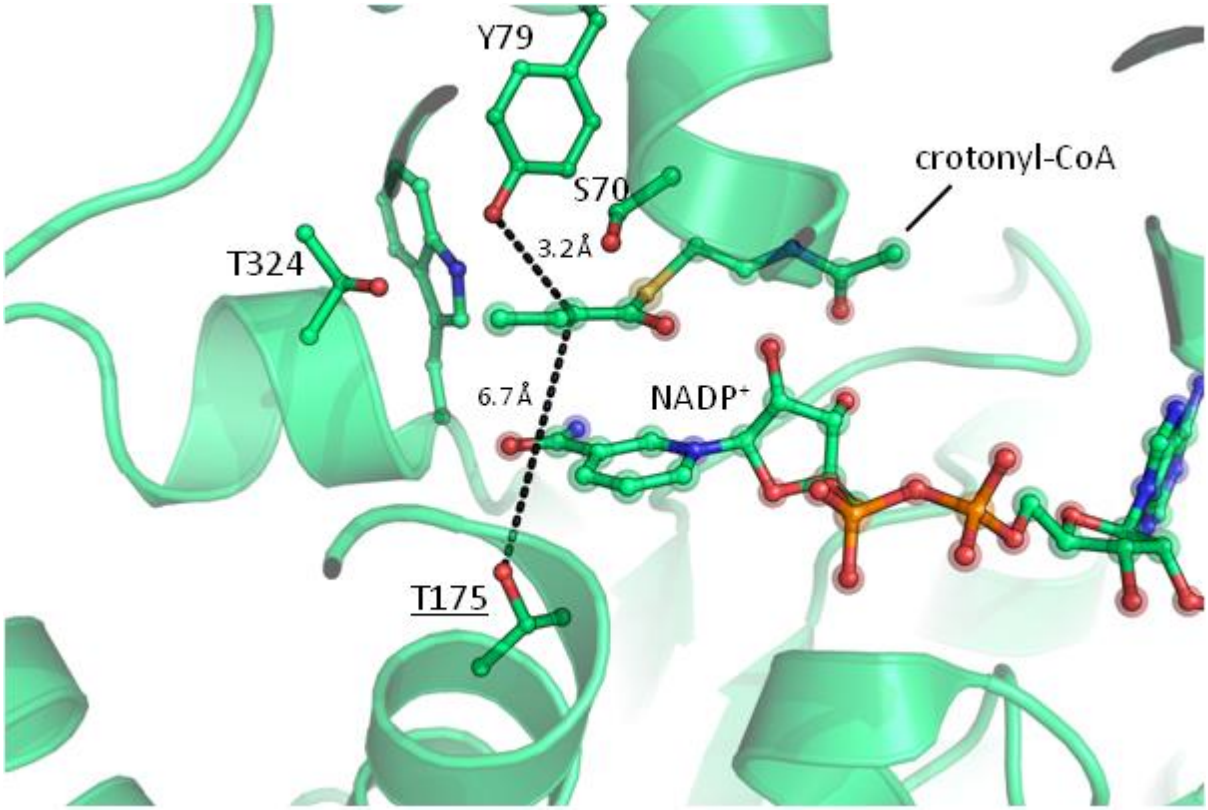
Supplementary Table 3. Etr1p T175V data collection and refinement statistics for both, the monoclinic and trigonal crystalline forms (see **Supplementary Figure 8**).

	Etr1p T175V monoclinic form	Etr1p T175V trigonal form
Data collection		
Wavelength (Å)	1.73913	1.73913
Space group	$P2_1$	$P3_12$
Resolution (Å)	49.47 – 2.10 (2.21 – 2.10)	47.04 – 2.50 (2.64 – 2.50)
Cell dimensions		
a, b, c (Å)	67.42, 101.86, 81.59	93.50, 93.50, 231.20
α, β, γ (°)	90, 101, 90	90, 90, 120
R_{merge} (%)[a]	5.9 (43.0)	12.4 (151.1)
R_{pim} (%)[a]	3.9 (28.6)	4.4 (55.7)
I/σ_I[a]	11.8 (2.5)	12.2 (1.5)
$CC_{1/2}$	99.6 (80.8)	99.7 (67.7)
Completeness (%)[a]	96.2 (91.5)	100.0 (100.0)
Redundancy[a]	3.3 (3.1)	8.6 (7.7)
Refinement		
Resolution (Å)	49.47 – 2.10	43.34 – 2.5
Number of reflections	60553	41370
R_{work}/R_{free}[b] (%)	15.85 / 19.70	19.16 / 21.96
Number of atoms		
Protein	5610	5586
Ligands/ions	137	209
Solvent	525	157
Average B-factors (Å^2)	40.42	53.04
Molprobity clash score, all atoms	1.93 (100th percentile)	2.8 (100th percentile)
Ramachandran plot		
Favoured regions (%)	97.94	96.69
Outlier regions (%)	0	0
rmsd[c] bond lengths (Å)	0.009	0.004
rmsd[c] bond angles (°)	1.097	0.787
PDB code	5LB9	5LBX

[a] Values relative to the highest resolution shell are within parantheses. [b] R_{free} was calculated as the R_{work} for 5% of the reflections that were not included in the refinement. [c] msd, root mean square deviation.

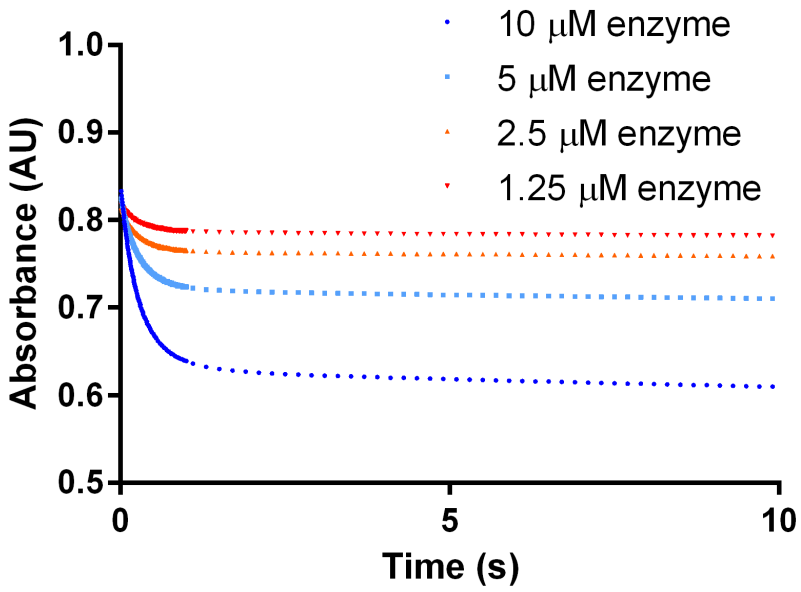
Supplementary Figures

Supplementary Figure 1. Active site of Etr1p. Distances from Y79 (3.2 Å) and T175 (6.7 Å) to the Cα of crotonyl-CoA, the protonation site. The distance from T175 to the Cα of crotonyl-CoA is too large to be directly involved in the protonation step.

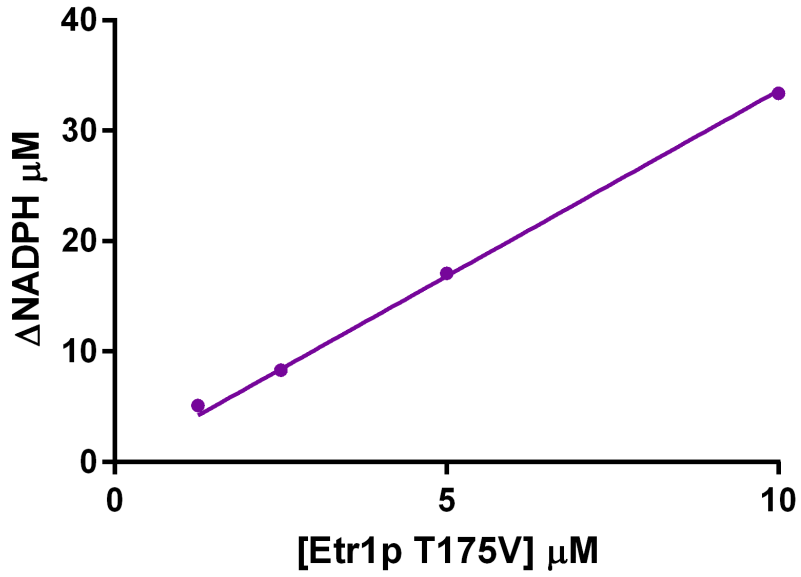


Supplementary Figure 2. Linearity of the burst phase in Etr1p T175V with amount of enzyme. a) Stopped flow traces of the burst phase with the indicated amount of enzyme. **b)** The amount of NADPH that is consumed in the first 2 seconds of the reaction plotted versus the amount of enzyme. The slope of the line is 3.27± 0.07.μM NADPH per μM enzyme, indicating multiple turnovers takes place before the enzyme is inhibited.

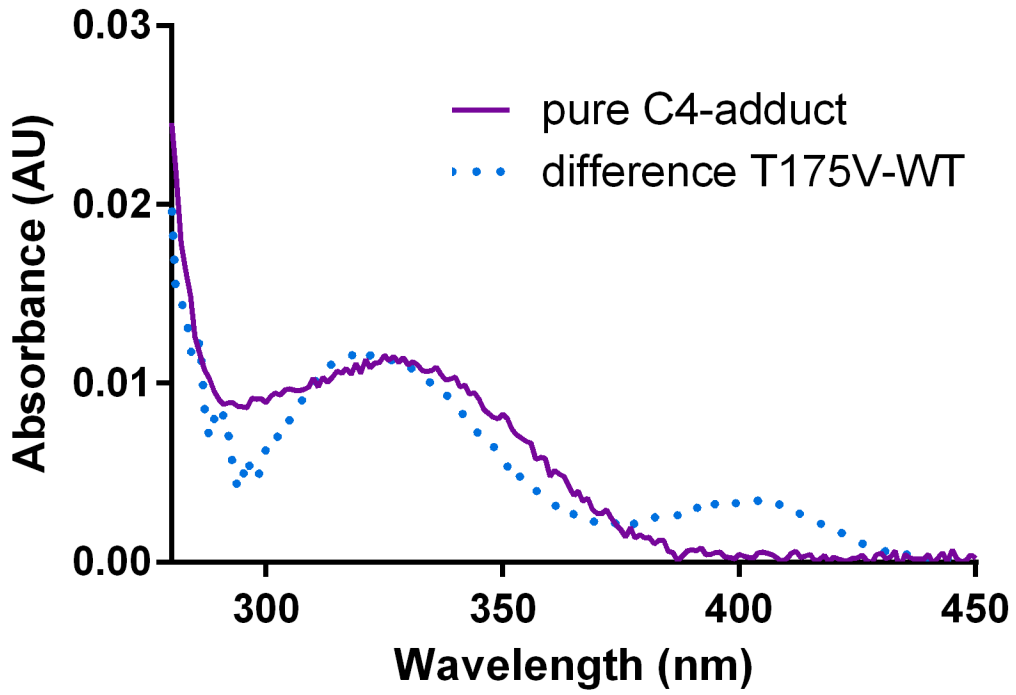
a)



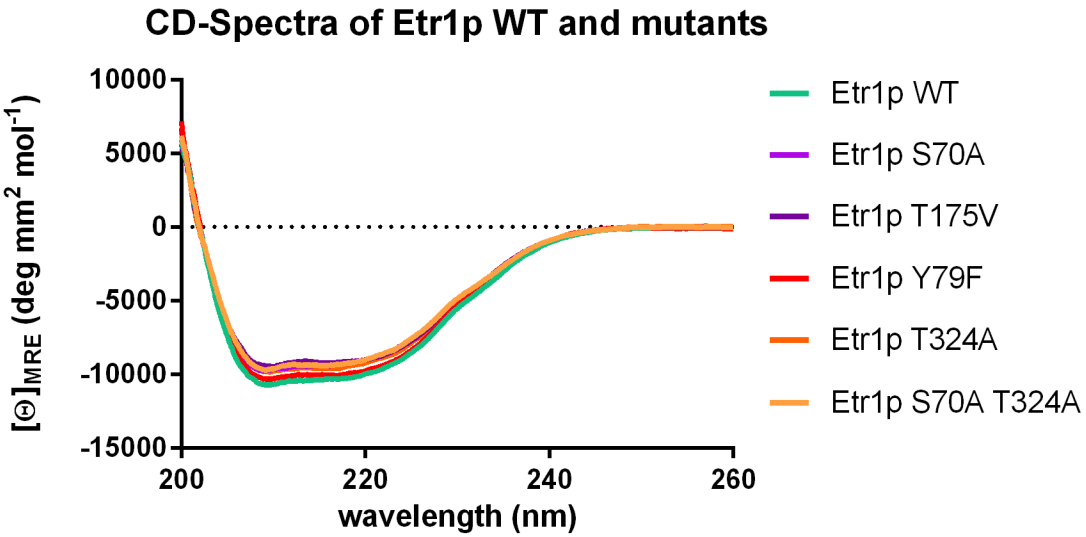
b)



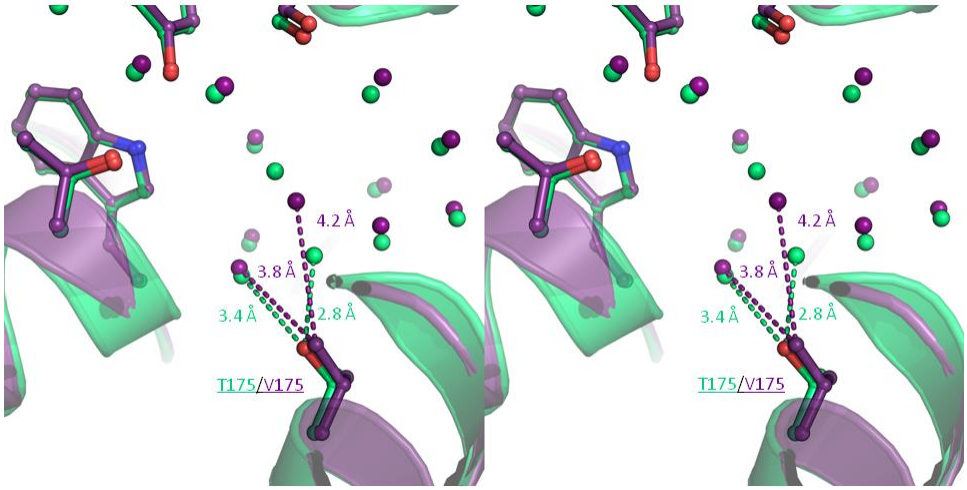
Supplementary Figure 3. Difference spectroscopy demonstrates C4-adduct formation in the Etr1p T175V mutant. Spectrum of pure C4- adduct (purple) and difference spectrum (blue dotted lines) of two assays containing either 4 μM Etr1p WT or 4 μM T175V. Both assays contained 40 μM NADPH and 50 μM crotonyl-CoA in 100mM Na_2HPO_4 pH 7.9. Spectra were taken after both reactions depleted NADPH (within manual mixing time).



Supplementary Figure 4. Circular dichroism spectra of Etr1p WT and mutants employed in this study. Each measurement was performed with 10 µM of protein and was performed in triplicate to yield the mean representative scans. Molar ellipticity was subtracted from a control buffer scan. The spectra were collected on a JASCO 815 CD spectrometer scanned from 200–260 nm in a 1 mm quartz cuvette.

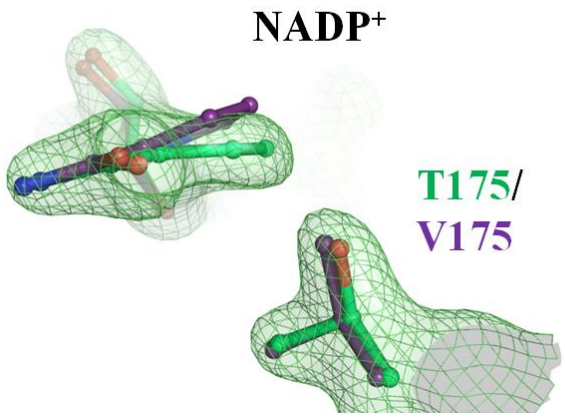


Supplementary Figure 5. Stereo image of the active site of apo-Etr1p WT and T175V. Most of the water network in the active site superimposes between the WT and T175, only the crystal water in direct contact with position 175 is shifted in the mutant.

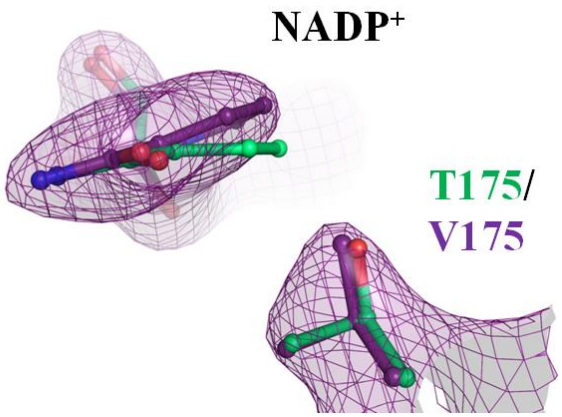


Supplementary Figure 6. Superimposition of NADP$^+$ and amino acid residue 175 for Etr1p WT (PDB 4W99) and the T175V variant (PDB: 5LBX) including electron densities. Overlay of NADP$^+$ and the amino acid residue 175 in comparison to electron density maps. **a)** 2F_o-F_c electron density map contoured at 1.5 σ, as well as ball and stick representation of the WT in green, with the T175V variant overlaid as ball and stick in purple **b)** 2F_o-F_c electron density map contoured at 1.5 σ, as well as ball and stick representation for the T175V variant in purple, with the WT overlaid only as ball and stick in green.

a)

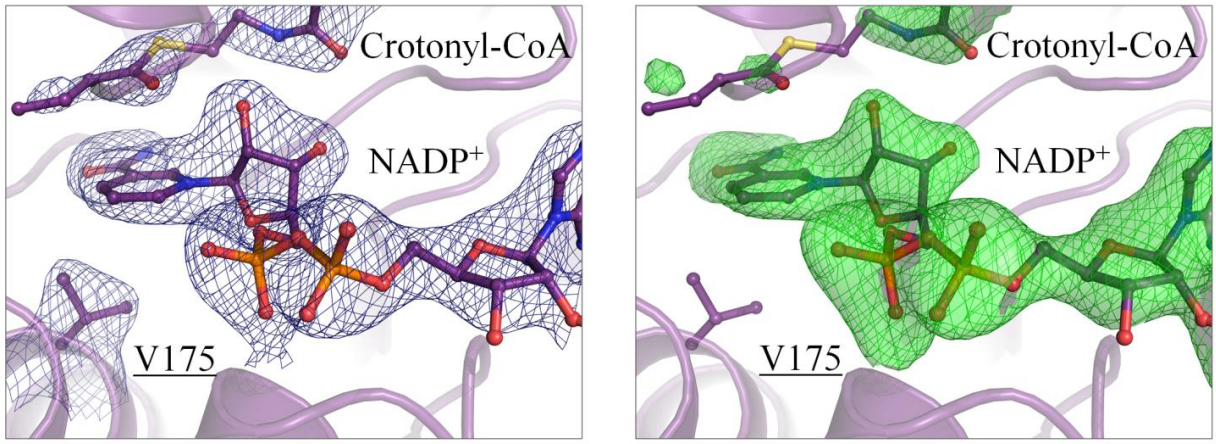


b)



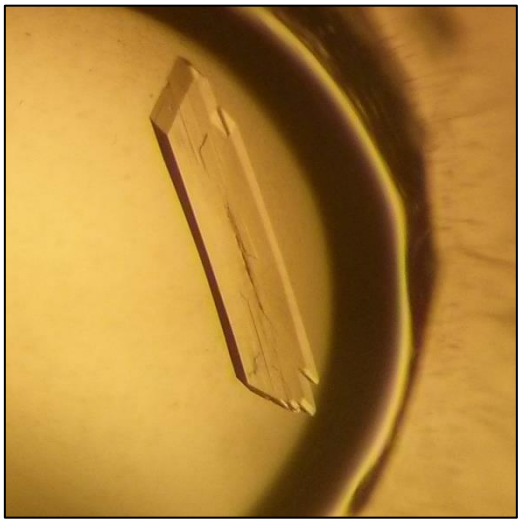
Supplementary Figure 7. 2F_o-F_c electron density maps for NADP$^+$ and crotonyl-CoA bound at T175V after and before refinement. a) 2F_o-F_c electron density map for the T175V variant with NADP$^+$ and crotonyl-CoA contoured at 1.0 σ. **b)** F_o-F_c electron density map with NADP$^+$ and crotonyl-CoA omitted prior to refinement. The difference map is contoured at 3.0 σ.

a) **b)**

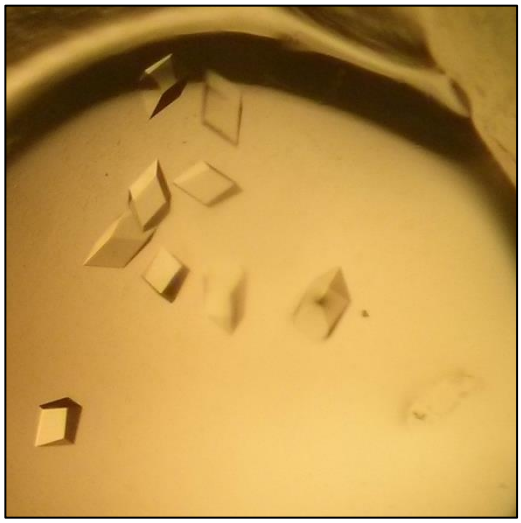


Supplementary Figure 8. Picture of the **a)** trigonal and **b)** monoclinic crystalline forms (see **Supplementary Table 2**).

a)



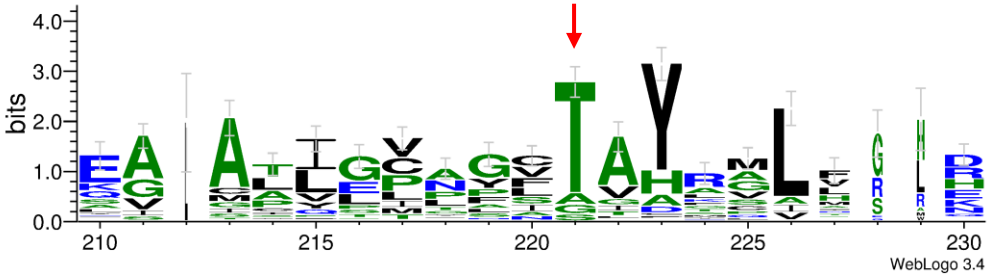
b)



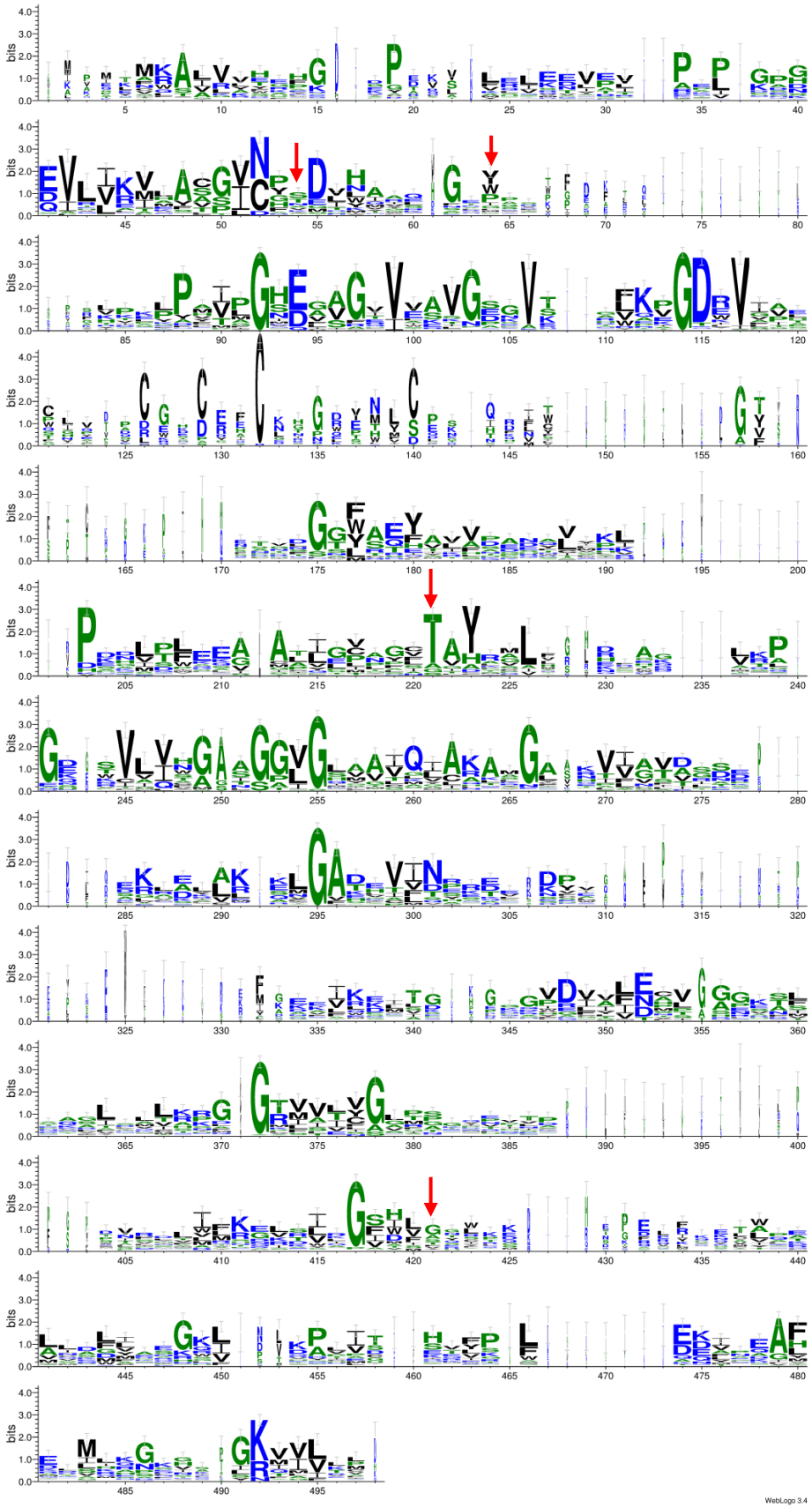
Supplementary Figure 9. Weblogo analysis of conserved residues in the MDR enzyme superfamily. Sequence logo of **a**) the residues around the conserved threonine 175 (red arrow) and **b**) the complete sequence alignment. The sequence numbering is shifted due to gaps in the alignment and the presence of a histidine-tag in the crystal structure that is used for the numbering throughout the paper. S70 is at position 54, Y79 at position 64, T175 at position 221, T324 at position 421. Sequences from referenceX were aligned with MAFFT v7.273 (with iterative refinement method E-INS-i) and the sequence logo was created with the Weblogo 3.4 server [1]. Columns with many gaps or unknown residues are narrow.

[1] Crooks, G.E., Hon, G., Chandonia, J.M. & Brenner, S.E. WebLogo: a sequence logo generator. *Genome Res* **14**, 1188-90 (2004).

a)



b)



Supplementary Figure 10. Detection of C4-adduct in the Ccr T195V variant and WT. **a)** UPLC-MS analysis of Ccr WT (green) and Ccr T195V variant (purple) containing 1 µl 30µM enzyme, 1 µl 500 mM Na_2HPO_4 (pH 7.9), 1 µl 200 mM NADPH, 1 µl 200 mM crotonyl-CoA, 1 µl 60 µM crotonic anhydrase and 1µL of 200 mM KHCO3. The T195V variant shows accumulation of a C4-adduct, while the Ccr WT did not accumulate detectable amounts of the C4-adduct. Both variants show accumulation of the catalytically competent C2-adduct. **b)** UPLC-MS analysis of authentic standards for the C2 adduct (blue) and the C4 adduct (orange). Data shown are representatives of at least three independent experiments.