Supplemental data:

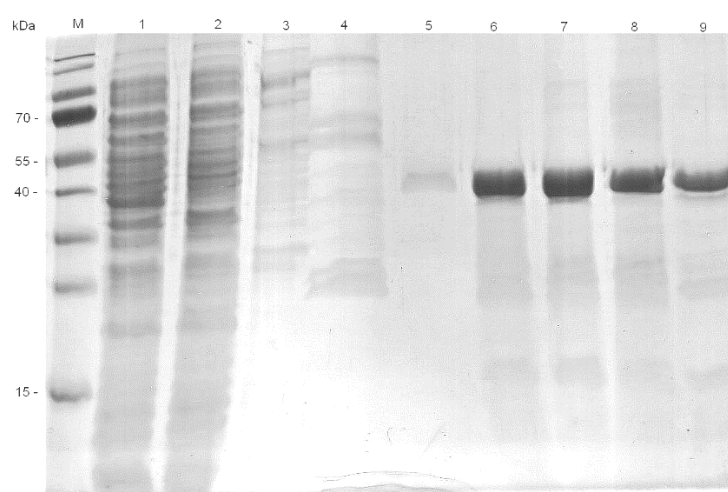
Supplemental Table 1: Foward and reversed primers used for the mutagenesis of CYP260B1.

Name	Sequence
T224A_For	gtggaggcgtgcgaggggGtcgtgctcGCGttgatcc
T224A_Rev	tCGtctcgtagctgccgaggatcaaCGCgagcacgaC
T232A_For	ctcACGttgatcctcggcagctacgagGCGaccagctg
T232A_Rev	caggttggccagcatccagctggtCGCctcgtagctg
T224A/T232A_For	ggggtcgtgctcGCGttgatcctcggcagctacgagGCGaccagctgga
T224A/T232A_Rev	tccagctggtCGCctcgtagctgccgaggatcaaCGCgagcacgacccc

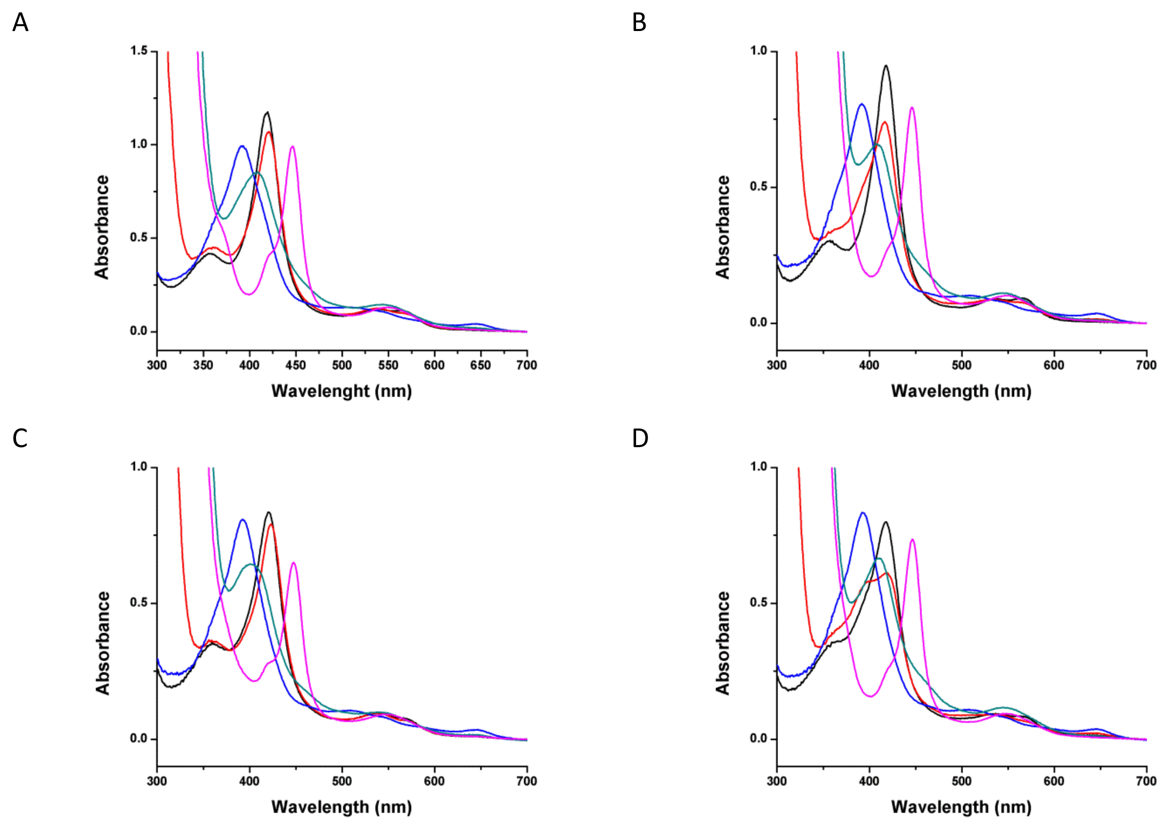
Supplemental Table 2: Soret-peaks of CYP260B1 variants in oxidized, reduced, CO-bound, cinnamaldehyde-bound and progesterone-bound state, respectively.

Soret-peaks [nm]				
P450	**CYP260B1**	**T224A variant**	**T232A variant**	**T224A/T232A double mutant**
Oxidized	**417**	**417**	**417**	**418**
Reduced	**410**	**410**	**410**	**410**
CO-bound	**448**	**448**	**448**	**448**
Cinnamaldehyde-bound	**417**	**417**	**417**	**418 (with a small shoulder at 393)**
Progesterone-bound	**393**	**393**	**393**	**393**

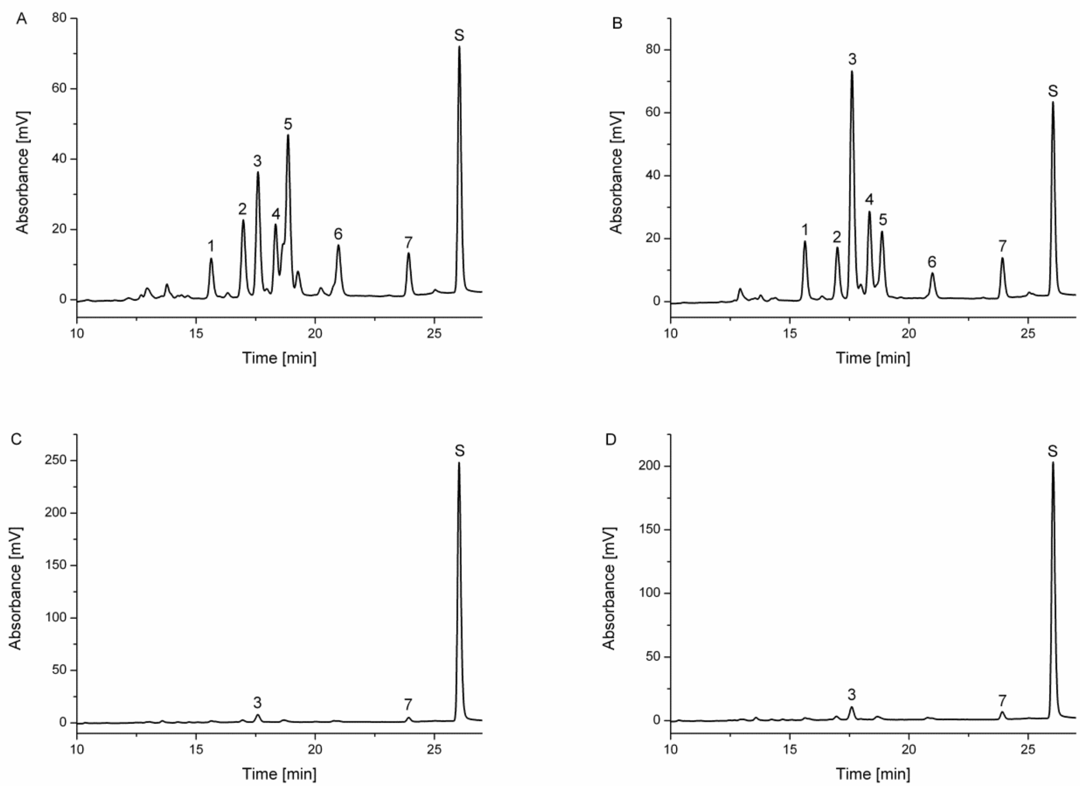
The spectra of the substrate free oxidized, reduced and reduced CO-complexed enzymes showed the typical maxima at 417, 410 and 448 nm, respectively, for all proteins (see Supplemental Figure 2). The cinnamaldehyde bound forms of the wild type and single mutants showed a peak maximum at about 417 nm that is characteristic for the low spin state [1]. This observation may be caused by the small size of cinnamaldehyde, which might not have replaced all the water molecules from the active site despite serving as a substrate. Likewise, the double mutant T224A/T232A showed a maximum at 417 nm, however, a little shoulder at 393 nm was also shown, which leads to the assumption that the conformation of its active site was influenced by the double mutation. In contrast, the addition of the known substrate progesterone [2] showed a type-I shift for all proteins with a peak maximum at 393 nm representing the high spin state [1]. Since progesterone is a relatively larger molecule compared to cinnamaldehyde, it might have replaced the water from the active site resulting in the high spin state.



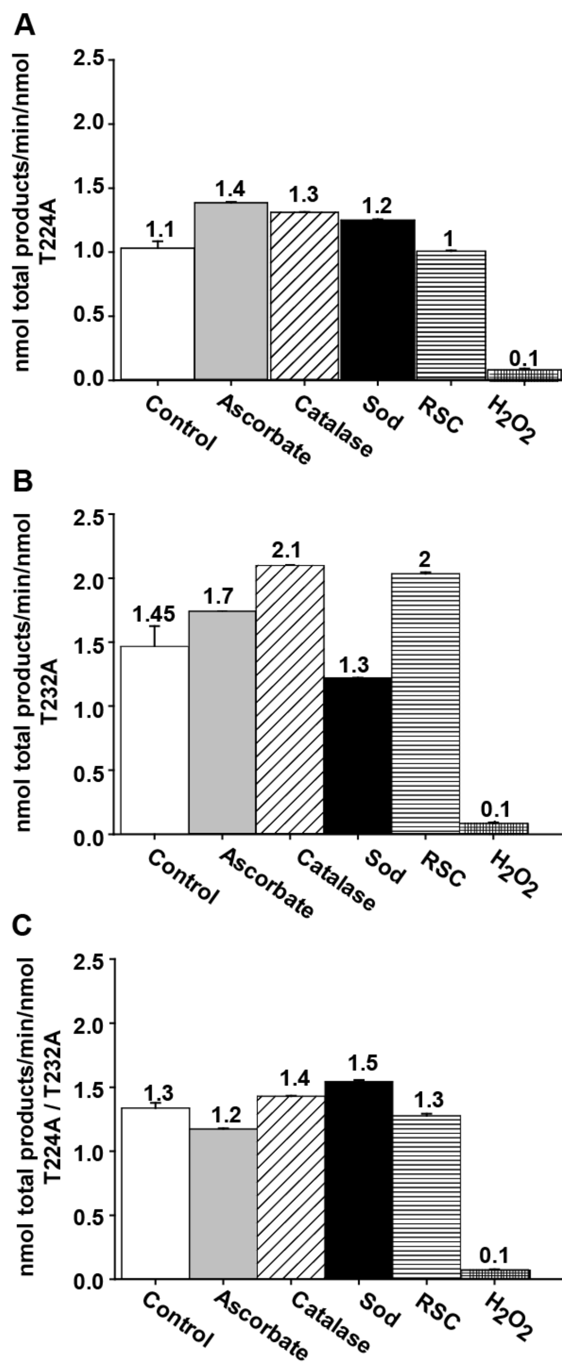
Supplemental Figure 1: SDS-PAGE of the purification of CYP260B1 as well as purified single mutants (T224A and T232A) and double mutant (T224A/T232A). M, protein marker IV (VWR Peqlab, Darmstadt, Germany); lane 1, the cell lysate; lanes 2, 3, and 4 show the flow through during the washing step of the purification; lane 5, the eluted protein of the wild type CYP260B1. Lanes 6, 7, 8, and 9 show the purified and concentrated proteins CYP260B1, T224A, T232A, and the double mutant, respectively.



Supplemental Figure 2: UV-Vis characterization of wild type CYP260B1 (A), T224A (B), T232A (C) and T224A/T232A double mutant (D). Black lines show the absorbance of pure protein, red lines show the absorbance of proteins bound with cinammaldehyde, blue lines show the absorbance of the proteins bound with progesterone, green lines show the absorbance of proteins in reduced-state, and magenta lines show the absorbance of the proteins in reduced and CO-complexed state.



Supplemental Figure 3: Chromatograms of the conversion of progesterone by wild type CYP260B1 (A), T224A mutant (B), T232A mutant (C), and T224A/T232A double mutant (D), respectively. 1-7 represent the main products and S represents the substrate progesterone.



Supplemental Figure 4: A, B and C show the effect of radical scavengers on the catalytic rate of T224A, T232A and T224A/T232A, respectively. Results are obtained from triplicate experiments ± standard deviation.

References:

[1] Luthra, A., Denisov, I.G. and Sligar, S.G. (2011). Spectroscopic features of cytochrome P450 reaction intermediates. Arch Biochem Biophys 507, 26-35.

[2] Salamanca-Pinzon, S.G., Khatri, Y., Carius, Y., Keller, L., Muller, R., Lancaster, C.R. and Bernhardt, R. Structure-function analysis for the hydroxylation of Delta4 C21-steroids by the myxobacterial CYP260B1, FEBS Lett. 2016 Jun;590(12):1838-51. doi: 10.1002/1873-3468.12217. Epub 2016 Jun 3.