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Supporting information for article:

Enzyme-substrate complex structures of CYP154C5 shed light on its mode of highly selective steroid hydroxylation

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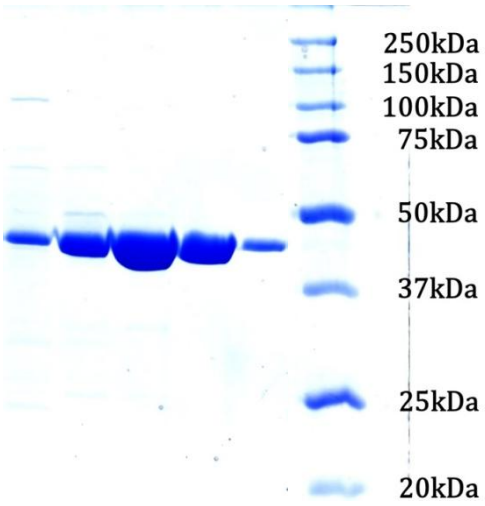

Figure S1 Nonreducing SDS-PAGE of previously alkylated CYP154C5 after purification. Bands represent monomeric CYP154C5 of respective gel filtration fractions that were applied in crystallisation experiments.

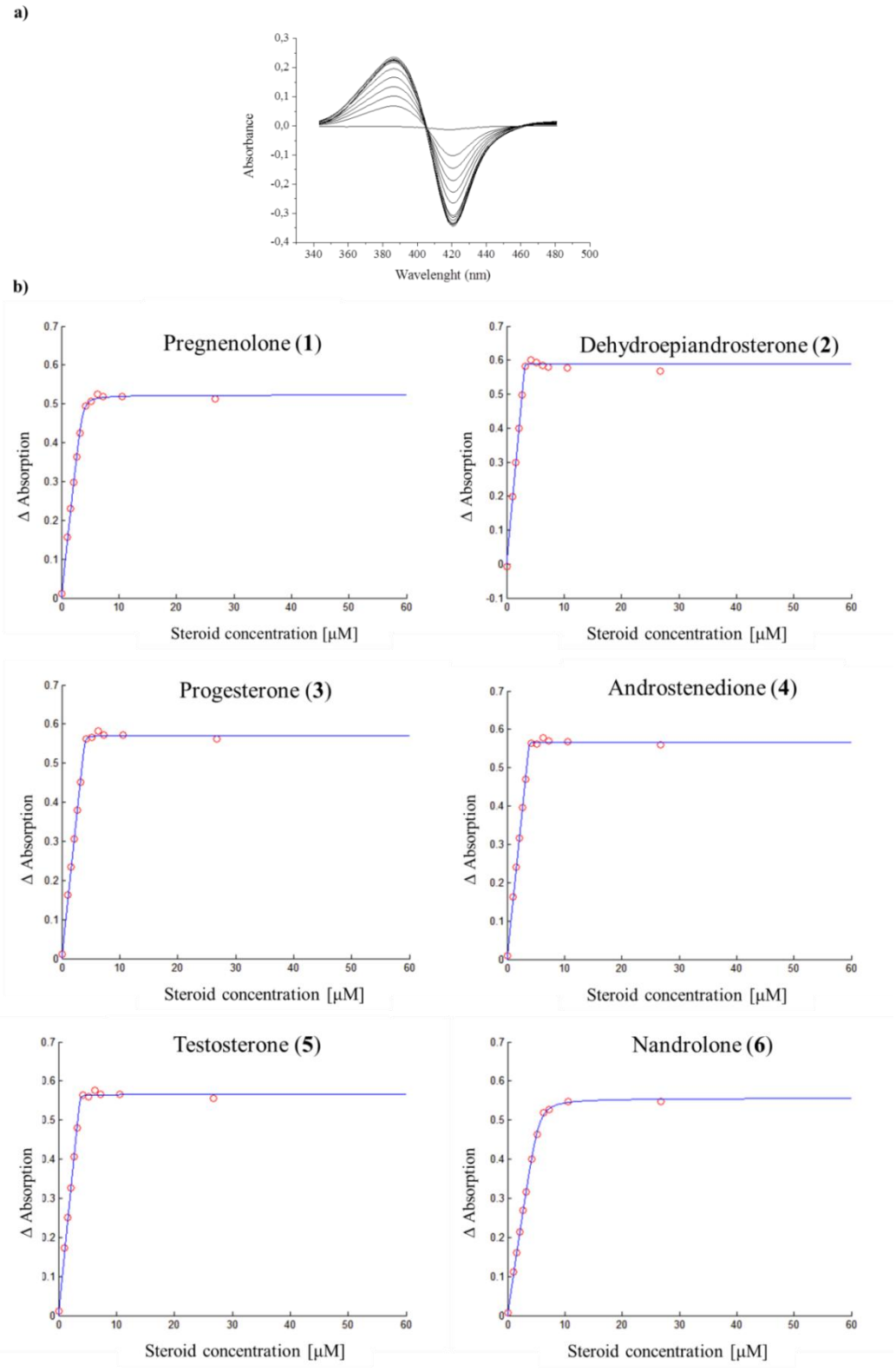

Figure S2 a) Type I spectral shift in the absorbance spectrum of CYP154C5 using **1** as substrate. The absorbance difference ΔA (Abs 386 – 420 nm) increases until saturation of the active site with **1**. b) Resulting substrate-binding titrations for CYP154C5 using steroid substrates **1** to **6**. ΔA was plotted against the applied steroid concentration and the resulting data was fitted using the tight binding equation.

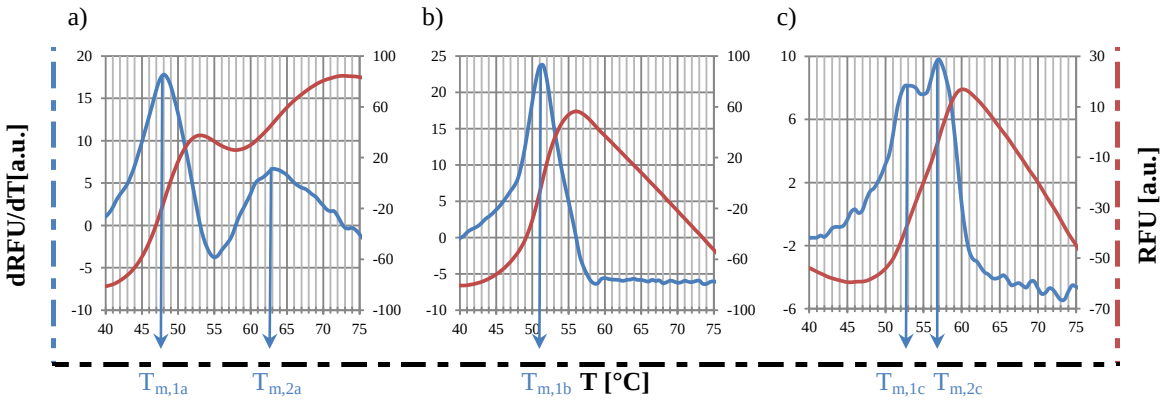

Figure S3 Thermal unfolding curves (red) of CYP154C5 and first derivatives (blue) from thermal shift experiments. a) Pure enzyme with DTT. b) Pure enzyme with TCEP. c) Enzyme with 1 eq **5**, DMSO and TCEP.

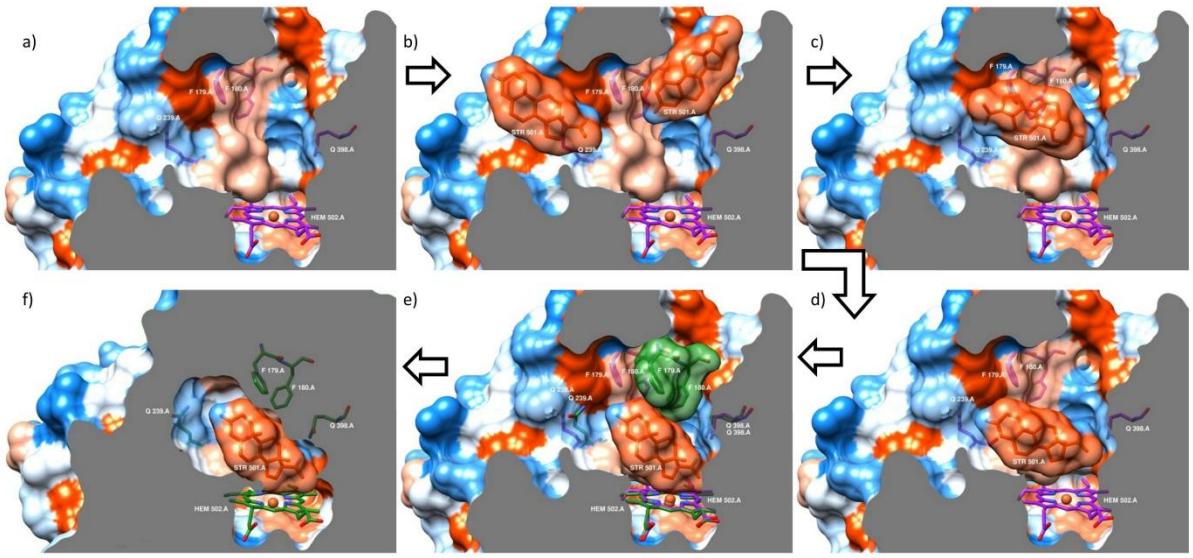

Figure S4 Cross-sections of CYP154C5's active site in the opened and closed conformation visualizing the predicted dynamic steroid binding model. Arrows indicate the sequential CYP154C5-steroid interaction steps. a) Substrate free active site in the opened conformation. b) The two possibilities for the steroid substrate to enter the active site. c) Nonproductive initial binding of the steroid between the two polar regions around Q239 and Q398. This binding mode arises from the close interaction of the steroid with F179 and F180, preventing the required F-G-helix movement for active site closure. In consequence the steroid is too far away from the heme for successful conversion. d) Correct initial binding of the steroid between Q239 and Q398, enabling the active site closure via F-G-helix movement. e) Superposition of opened (magenta) and closed (green) active site, indicating the F-G-helix movement during active site closure. The important role of F179 and F180 is highlighted by their surfaces (green). f) Conversion-enabling orientation of the steroid in the closed active site of CYP154C5.

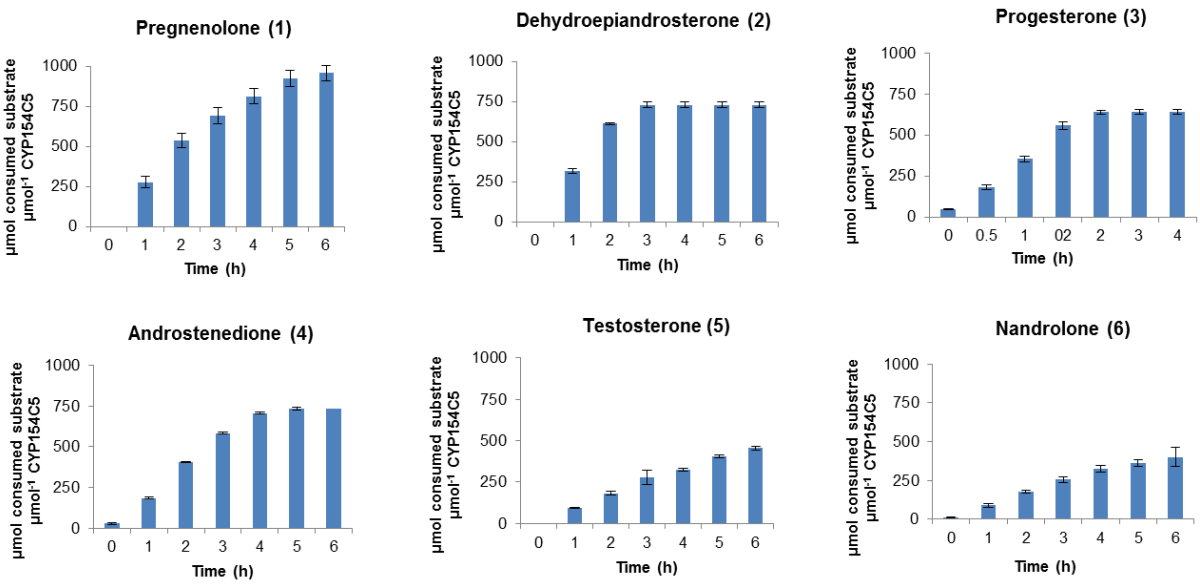

Figure S5 Turnover number (TON, µmol of consumed substrate per min per µmol of P450) determination for the conversion of steroids 1 to 6 by the three-component system composed of purified CYP154C5, Pdx and PdR. TON were calculated for the time period for which the highest substrate consumption rate was observed. Standard deviations of duplicate measurements are given.

Table S1 Initial NADH oxidation rates of the purified three-component system consisting of CYP154C5, Pdx and PdR in the conversion of steroids 1 to 6. Results represent mean values of duplicate measurements.

Steroid	NADH oxidation rate (min^{-1})
1	9.42 ± 0.07
2	6.25 ± 0.33
3	15.10 ± 0.06
4	9.19 ± 0.14
5	6.01 ± 0.00
6	6.98 ± 0.04