

## SUPPORTING INFORMATION

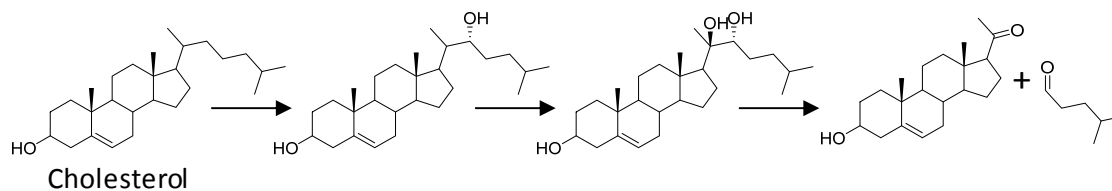
### A requirement for an active proton delivery network supports a Compound I mediated C-C bond cleavage mechanism in CYP51 catalysis

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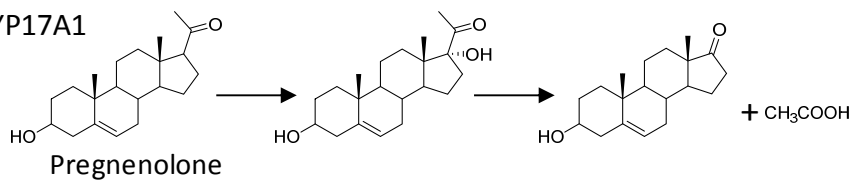
#### Table of Contents

Figure S1. Examples of cytochromes P450 catalyzing multistep reactions	S2
Figure S2. The asymmetric unit of 6UEZ	S3
Figure S3. Movement of the helix C and HI arm reshape the proximal surface of the CYP51 molecule	S4
Figure S4. Solvent molecules near the proton delivery area in D231A/H314A human CYP51	S5
Figure S5. The HPLC profile of a sterol mixture containing the 14 $\alpha$ -carboxaldehyde intermediate	S6
Figure S6. HPLC profiles of the aldehyde deformylation by WT and D231A/H314A human CYP51	S7
Table S1. Substrate-contacting residues ( $\leq 4.5$ Å) in human and <i>T. cruzi</i> CYP51	S8

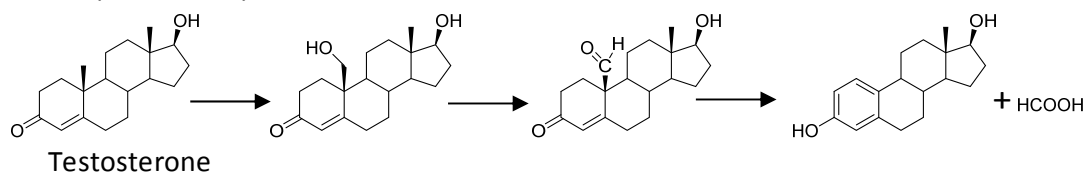
CYP11A1 (P450<sub>scc</sub>)



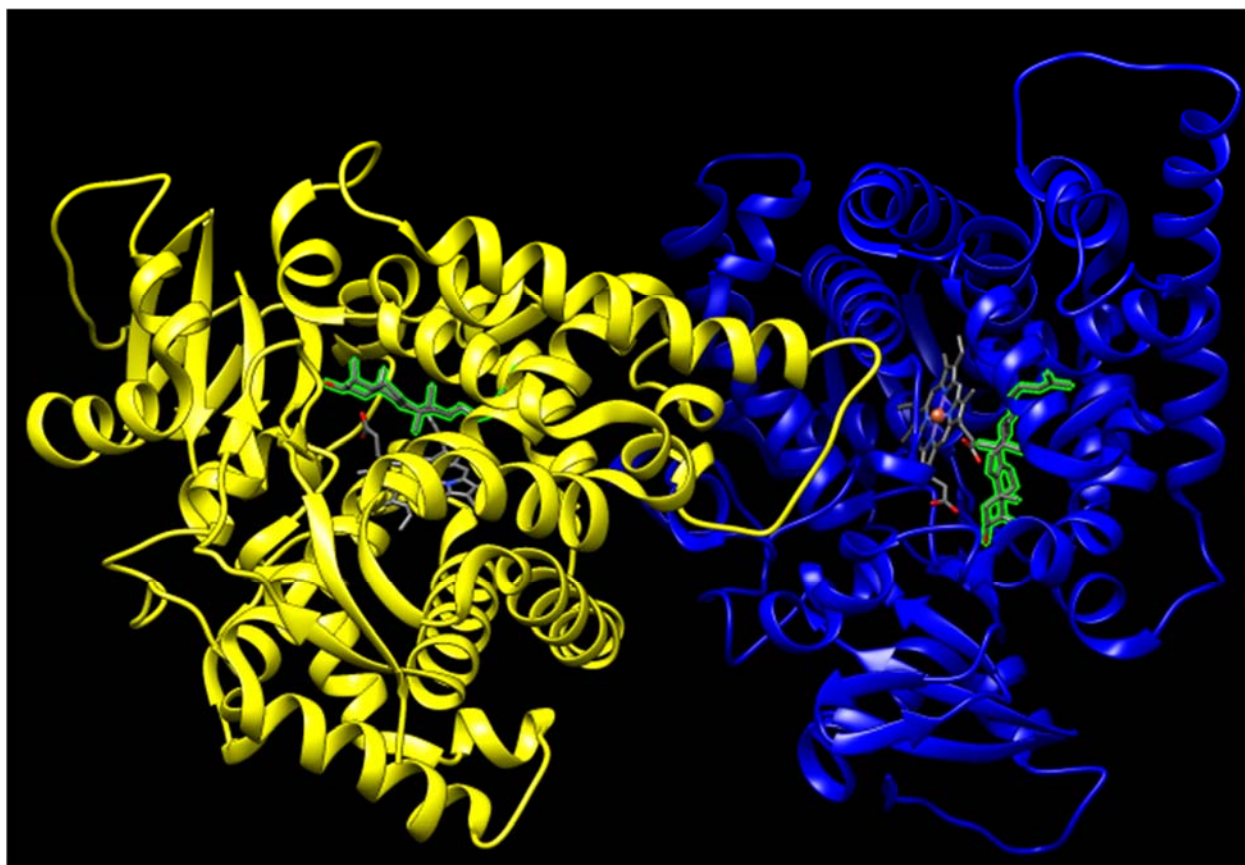
CYP17A1



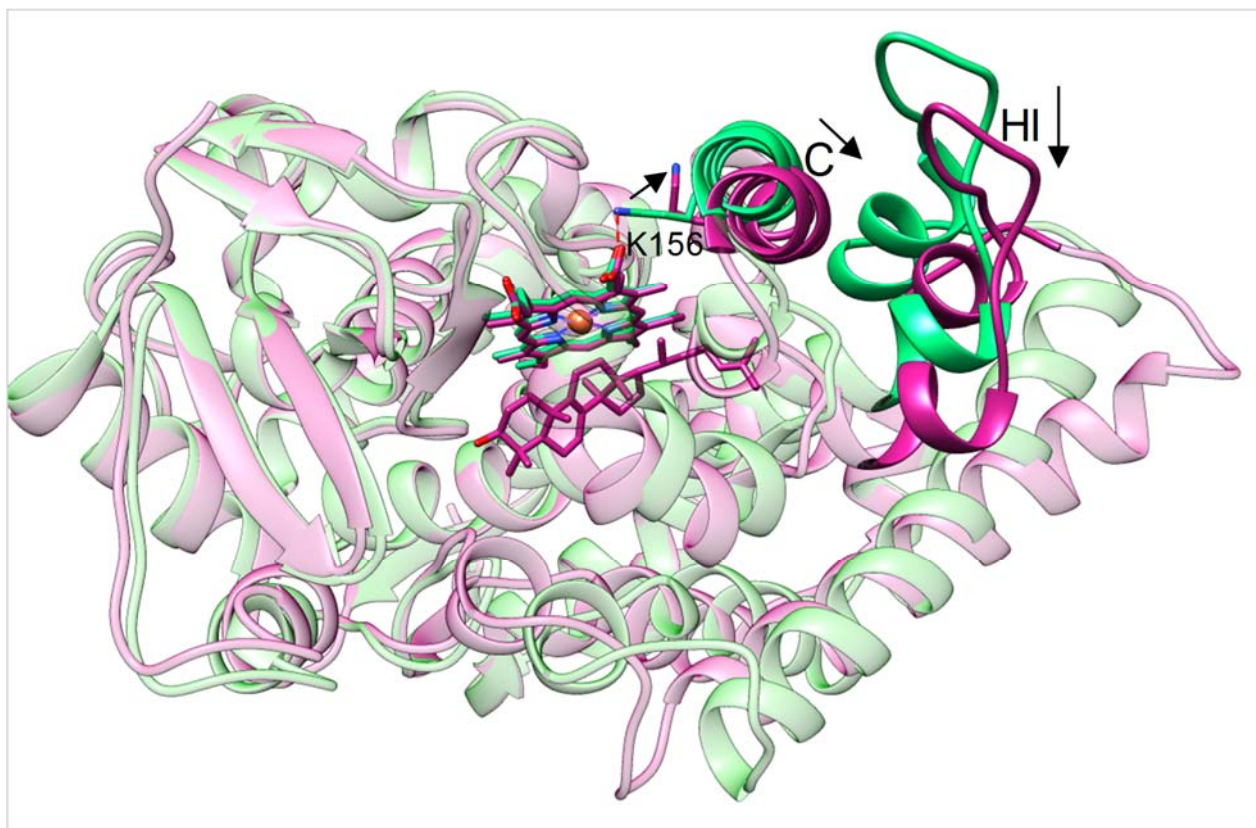
CYP19A1 (aromatase)



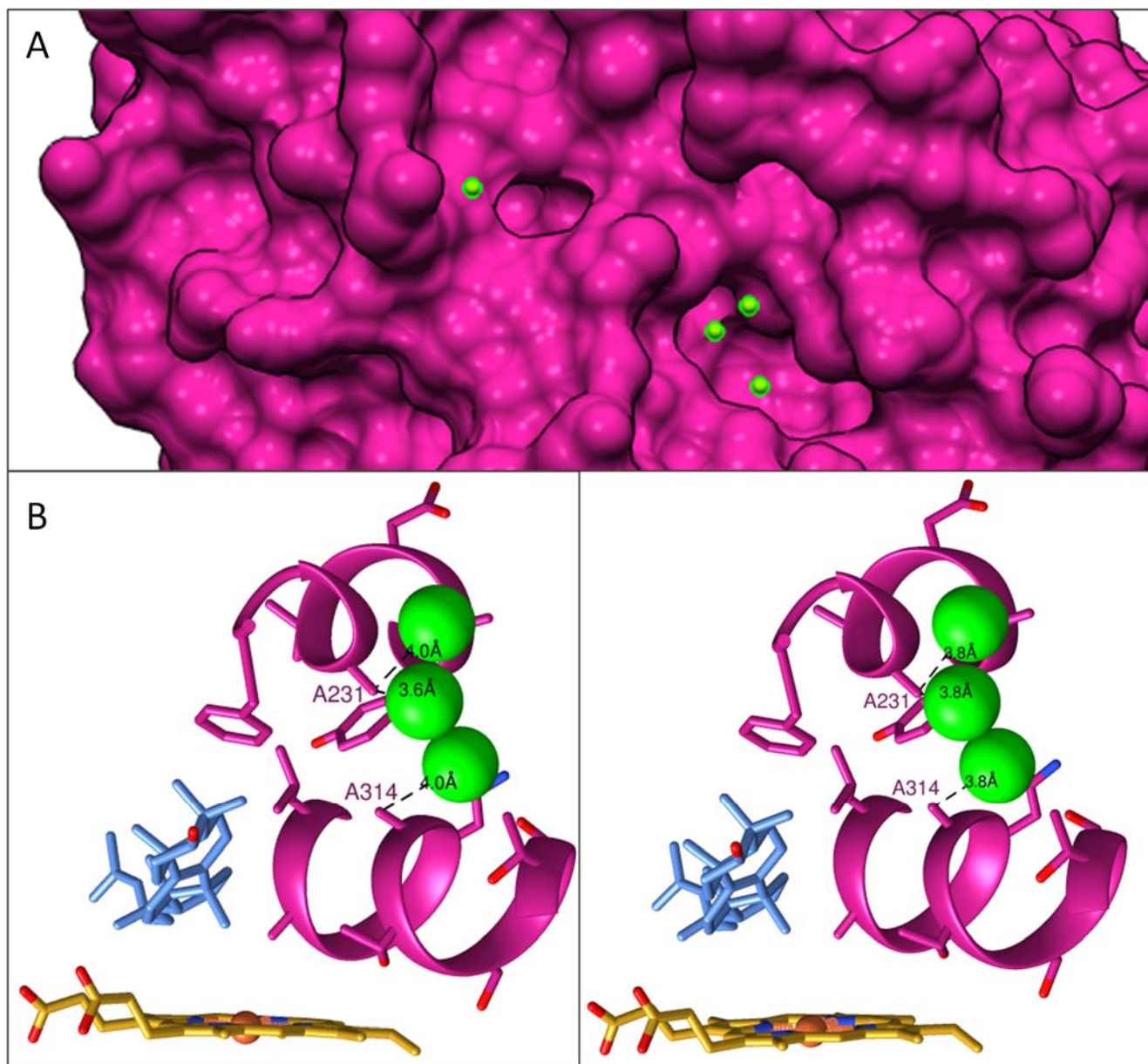
**Figure S1.** Examples of some other cytochromes P450 catalyzing multistep reactions leading to C-C bond cleavage.



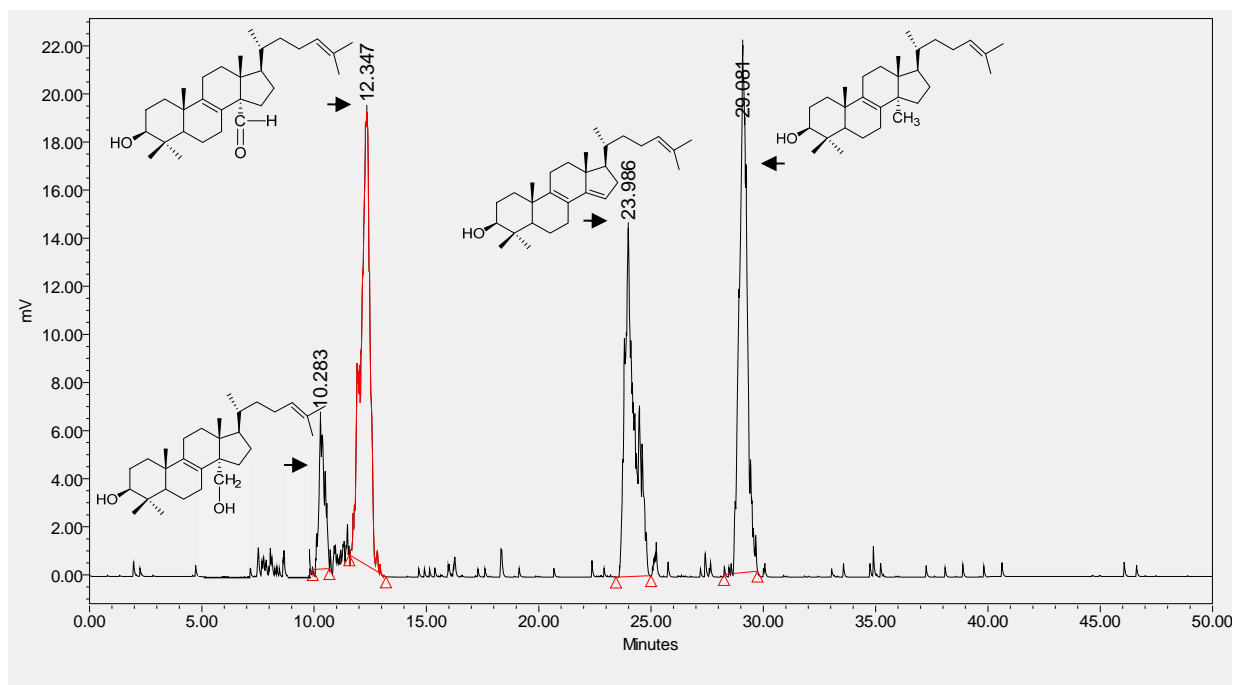
**Figure S2. The asymmetric unit of human CYP51 (D231A/H314A) (PDB code 6UEZ).** Two protein molecules (yellow and blue) are shown in a ribbon representation, two molecules of heme, and two molecules of lanosterol are shown in a stick representation. Lanosterol molecules are highlighted in green. RMSD of C $\alpha$  = 0.39 Å.



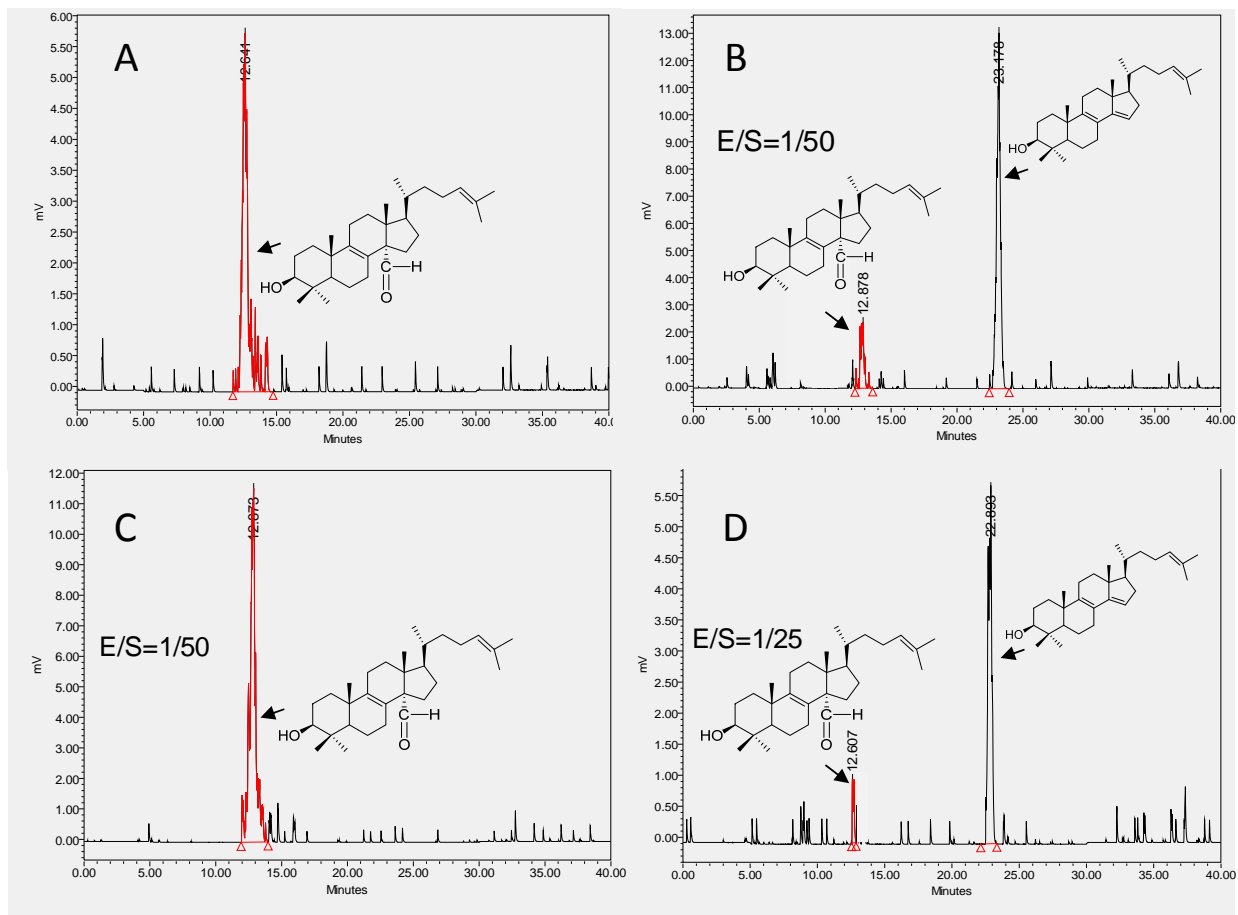
**Figure S3. Movement of the helix C and HI arm reshape the proximal surface of the CYP51 molecule.** Upper P450 face. Green: VFV-bound human CYP51 (PDB code: 4UHI), violet: lanosterol-bound D231A/H314A human CYP51. The arrows show the directions of changes upon binding of the substrate.



**Figure S4. Solvent molecules near the proton delivery area in the structure of D231A/H314A human CYP51.** *A*, enlarged fragment of the surface shown in Figure 6B, *B*, sick/ribbon representation of the involved segments in molecules A and B, left and right, respectively, the waters are depicted as green spheres, the distances to A231 and A314 are shown. The heme is yellow, lanosterol is blue.



**Figure S5. HPLC profile of a sterol mixture containing lanosterol 14 $\alpha$ -carboxaldehyde.** The sterols were extracted from a reaction mixture containing 10  $\mu$ M *T. brucei* CYP51, 5  $\mu$ M CPR, 1 mM NADPH, and 50  $\mu$ M lanosterol after 2 hours of incubation at 37  $^{\circ}$ C. The aldehyde intermediate peak is indicated in red.



**Figure S6.** HPLC profiles of aldehyde deformylation by WT and D231/H314 human CYP51. *A*, purified lanosterol 14 $\alpha$ -carboxaldehyde intermediate; *B*, 1-min reaction with WT enzyme; *C*, 1-min reaction with the D231A/H314A mutant; *D*, 30-min reaction with the D231A/H314A mutant.

**Table S1. Substrate-contacting residues ( $\leq 4.5$  Å) in human (6UEZ) and *T. cruzi* CYP51 (6FMO) sequence identity 27%).**

Secondary structural element	D231A/H314A human CYP51-lanosterol	I105F <i>T. cruzi</i> CYP51 - obtusifoliol
B' helix	Y131* L134 T135 F139	Y103 F105 M106 F110
B'' $\eta$ -turn (B'C loop)	V143 A144 Y145	- A115 Y116
C helix	F152 Q155 - L159	- Q126 L127 L130
F' helix	F234	-
I helix	G303 M304 G307 L308 L310 A311	- M284 A287 A288 F290 A291
K helix/ $\beta$ 1-4 strand	I377 I379 M381	L356 M358 M360
$\beta$ 4 hairpin	M487 I488	M487 M508
Total number of residues	22	19

\*Residues that align in the multiple sequence alignment are positioned on the same line.