

Supporting Information

Light-driven hydroxylation of testosterone by *Synechocystis* sp. PCC 6803 expressing the heterologous CYP450 monooxygenase CYP110D1

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List of abbreviations

In alphabetical order

AGC	Automatic Gain Control
CDW	Cell Dry Weight
Chl <i>a</i>	Chlorophyll <i>a</i>
CYP450	Cytochrome P450 monooxygenase
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethyl Sulfoxide
GC-HRMS	Gas Chromatography – High Resolution Mass Spectrometry
HPLC	High Performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Coherence
HRP	Horseradish Peroxidase
LED	Light Emitting Diode
OD	Optical Density
PCR	Polymerase Chain Reaction
PdR	Putidaredoxin Reductase
Pdx	Putidaredoxin
PSI	Photosystem I
PSII	Photosystem II
RBS	Ribosome Binding Site
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Sub	Substrate
USD	United States Dollar

Experimental

Preparative-scale biotransformations with *Synechocystis*

Synechocystis cells were grown as described in the previous sub-section and concentrated to $OD_{730}=10$ ($1.8 \text{ g}_{\text{CDW}} \text{ L}^{-1}$) in antibiotic-free BG11 medium. The reaction was initiated by mixing 50 mL of concentrated cell suspension with 625 μL of 80 mM testosterone dissolved in DMSO, to reach a final concentration of 1 mM. The reaction mixtures were incubated within an open glass cylinder with a diameter of 9 cm and 11.5 cm high and placed within the photoreactor mentioned above for 42 hours. The product extraction was performed by thoroughly mixing the reaction mixture with an equal volume of ethyl acetate for three times. The organic phase was dried in a rotavapor and stored at 4 °C prior to analysis.

Samples derivatization and gas chromatography/high-resolution mass spectrometry (GC-HRMS)

Samples of 1 mM testosterone, 1 mM 15β -hydroxytestosterone as well as the extracted reaction mixture from the preparative scale biotransformation (with approximately 1 mM of the product) were analyzed *via* GC-HRMS. The samples were first derivatized using *N,O*-bis(trimethyl-silyl)-trifluoroacetamide and trimethylchlorosilane in pyridine to produce the respective OTMS-derivatives before GC-HRMS measurements.

For the GC separation, a Thermo Scientific Trace 1310 gas chromatograph (Thermo Scientific, Bremen, Germany) was equipped with a 30 m analytical column (Phenomenex ZB5-MS, 30 m x 0.25 mm ID, $t_f = 0.25 \mu\text{m}$). A split injection port at 270°C was used for sample introduction and the split ratio was set to 10:1. The temperature program was 50 °C (3 min) - 10°C min⁻¹ - 310 °C (3 min). The helium carrier gas was set to 1.0 mL min⁻¹ flow rate (constant flow mode). The transfer line was kept at 290°C.

For the mass spectrometry analysis, a Q Exactive™ GC orbitrap mass spectrometer (ThermoScientific, Bremen, Germany) was used. The resolution was set to 60,000 (FWHM; instrument setting at 200 u). Mass range was 50–650 u and 2 micro scans were averaged per data scan. Automated gain control (AGC target) was set to 1×10^6 and maximum inject time was set to “auto”. Auxiliary temperatures were set to 290 °C for both transfer lines 1 and 2. MS transfer line temperature was set to 290 °C and the temperature of the electron ionization source was set to 220 °C. EI was performed at 70 eV energy in positive mode. Helium (carrier gas) and nitrogen (supply for the C-Trap) were equipped with gas purification cartridges to trap moisture and organic impurities of the gases (Thermo Scientific, Bremen, Germany). Column bleed ion at 207.03235 u was used as lock mass for internal mass calibration of the data. High resolution masses of the derivatized 15β -hydroxytestosterone standard and the respective product peak of the reaction mixture (from the preparative scale biotransformation) after derivatization were $m/z = 520.32153$ and $m/z = 520.32159$, respectively, corresponding to the steroid with 3 OTMS groups.

Nuclear magnetic resonance (NMR) analysis

NMR analysis of the extracted reaction mixture from the preparative scale biotransformation were performed with a 300 MHz Bruker spectrometer using deuterated chloroform as solvent. Complete assignment of all ¹³C signals was done using literature data,³⁷ Distortionless Enhancement by Polarization Transfer (DEPT) and Heteronuclear Single Quantum Coherence (HSQC) analysis and reported in Figures S6-S8 and Tables S3 and S4 .

Supporting tables and figures

Table S1 List of primers used in this study.

Name	Sequence ^a	Purpose	References
NS-P- <i>Nde</i> I-FW	AAAAAAAA <u>CATATG</u> ACAGTCAC TCAAACCTACCC	Amplification of <i>alr4766</i> from <i>Nostoc</i> sp. PCC 7120 genomic DNA.	This study
NS-P- <i>Hind</i> III-RV	GGAATTAAGCTT <u>CACGAATTAC</u> GCATTC	Amplification of <i>alr4766</i> from <i>Nostoc</i> sp. PCC 7120 genomic DNA.	This study
CYP110D1_RBS0032_ <i>Spe</i> I_FW	GAC <u>ACTAGT</u> cacacaggaagT ACTAGATGACAGTCACTCAAAA CCTAC	Amplification of <i>alr4766</i> and addition of <i>Spe</i> I and RBS BBa_B0032 at 5' end.	This study
CYP110D1_His-tag_RBS0032_ <i>Spe</i> _FW	GAC <u>ACTAGT</u> cacacaggaagT ACTAGATG CACCATCACCATCA CCAT ACAGTCACTCAAAACCTA C	Amplification of <i>alr4766</i> and addition of <i>Spe</i> I, RBS BBa_B0032 and N-terminal His-Tag at 5' end.	This study
CYP110D1_ <i>Pst</i> I_RV	CTGCTGCAGTTACGAATTACGC ATTCTTTTA	Amplification of <i>alr4766</i> and addition of <i>Pst</i> I at 3' end.	This study
pJ201_prom_FW	GGCTCAGTCGAAAGACTG	Confirmation of construct assembly.	This study
pJ201_prom_RV	GATATCAAAATTATACATGTCA ACG	Confirmation of construct assembly.	This study
CYP110D1_central_FW	GCAATTGCTAACTACCCTGT	Confirmation of the presence of the CYP110D1 construct in <i>Synechocystis</i> .	This study
pSEVA251_FW	GCGGATAACAATTTACACAG	Confirmation of construct assembly. Confirmation of the presence of the CYP110D1 construct in <i>Synechocystis</i> .	This study
pSEVA251_RV	GAACAAATCCAGATGGAGTTC	Confirmation of construct assembly. Confirmation of the presence of the CYP110D1 construct in <i>Synechocystis</i> .	This study

^a Restriction sites are underlined; RBS sequence is in lower case; His tag coding sequence is bold.

Table S2 List of plasmids used/generated in this study.

Designation	Plasmid	Description	References
pIT2	pIT2-MCS	Broad host-range expression vector, TetR, ori pBBR1.	1
pACYC::camAB	pACYC-Duet1	Plasmid for the expression of putidaredoxin and putidaredoxin reductase in <i>E. coli</i> .	1
pIT2::CYP110D1	pIT2-MCS	Plasmid for the expression of CYP110D1 in <i>E. coli</i> .	This study
pJ201::P _{psbA2} *	pJ201	Promoter based on <i>Synechocystis</i> native P _{psbA2} promoter.	2

pJ201::P _{trc.x.tetO2}		pJ201	Synthetic promoter containing a modified tetO operator.	3
pJ201::P _{psbA2*} ::B0032::CYP110D1		pJ201	CYP110D1 coding sequence under the control of P _{psbA2*} promoter and the Bba_B0032 RBS.	This study
pJ201::P _{psbA2*} ::B0032::CYP110D1 N-terminal His-Tag	N-	pJ201	N-terminal His-tagged CYP110D1 coding sequence under the control of P _{psbA2*} promoter and the Bba_B0032 RBS.	This study
pJ201::P _{trc.x.tetO2} ::B0032::CYP110D1		pJ201	CYP110D1 under the control of P _{trc.x.tetO2} promoter and the Bba_B0032 RBS.	This study
pJ201::P _{trc.x.tetO2} ::B0032::CYP110D1 N-terminal His-Tag	N-	pJ201	N-terminal His-tagged CYP110D1 coding sequence under the control of P _{trc.x.tetO2} promoter and the Bba_B0032 RBS.	This study
pSEVA251		pSEVA251	Replicative plasmid/shuttle vector, KmR, ori RSF1010	4
pSEVA251::P _{psbA2*} ::B0032::CYP110D1		pSEVA251	CYP110D1 coding sequence under the control of P _{psbA2*} promoter and the Bba_B0032 RBS, for the expression in <i>Synechocystis</i> .	This study
pSEVA251::P _{psbA2*} ::B0032::CYP110D1 N-terminal His-Tag	N-	pSEVA251	N-terminal His-tagged CYP110D1 coding sequence under the control of P _{psbA2*} promoter and the Bba_B0032 RBS, for the expression in <i>Synechocystis</i> .	This study
pSEVA251::P _{trc.x.tetO2} ::B0032::CYP110D1		pSEVA251	CYP110D1 coding sequence under the control of P _{trc.x.tetO2} promoter and the Bba_B0032 RBS, for the expression in <i>Synechocystis</i> .	This study
pSEVA251::P _{trc.x.tetO2} ::B0032::CYP110D1 N-terminal His-Tag		pSEVA251	N-terminal His-tagged CYP110D1 coding sequence under the control of P _{trc.x.tetO2} promoter and the Bba_B0032 RBS, for the expression in <i>Synechocystis</i> .	This study

Table S3 Main ¹H chemical shifts and assignments of 15β-hydroxytestosterone, and comparison with published data.

Hydrogen	δ (ppm)		
	This work	15β ⁷	15α ⁶
H17 (HOH)	3.58 (J=8.6 Hz)	3.48 (J=8.8 Hz, 8.8 Hz) 17α-16α/β	3.91 (J=9 Hz)
H15 (HOH)	4.19	4.14 (J=7.9 Hz, 2.6 Hz) 15α-16α/β	4.14. (J=9.5 Hz)
H 4	5.74	5.71	5.74
H 18	1.23	1.26	1.21
H 19	1.07	1.02	0.83

15β-Hydroxytestosterone: ¹H NMR (300 MHz, CDCl₃): δ=5.74 (1H, s, 4-H), 4.24–4.15 (1H, m, 15α-H), 3.58 (1H, dd, J= 8.6 Hz, 17α-H), 1.23 (s, 19-H), 1.07 (s, 18-H), 2.67–0.75 (m).

Table S4 ¹³C chemical shifts and assignments of 15β-hydroxytestosterone, and comparison with published data.

Carbon	δ (ppm)			Carbon	δ (ppm)		
	This work	15β ⁵	15α ⁶		This work	15β ⁵	15α ⁶
C1	199,49	199.8	199.5	C12 H ₂	37,81	38.0	36.6
C5	171,07	171.3	170.8	C1 H ₂	35,75	35.9	35.8
C4 H	123,88	124.0	123.9	C2 H ₂	33,92	34.0	33.9
C17 HOH	81,03	81.2	78.8	C6 H ₂	32,66	32.8	32.7
C15 HOH	69,00	69.2	72.6.	C8 H	31,46	31.6	35.4
C14 H	55,18	55.3	58.6	C7 H ₂	31,03	31.2	32.8
C9 H	54,27	54.4	53.8	C11 H ₂	20,55	20.7,	20.6
C16 H ₂	43,39	nd	42.7	C19 H ₃	17,29	17.5	17.5
C13	42,21	42.4	44.4	C18 H ₃	13,68	13.9	12.6
C10	38,76	38.9	38.7				

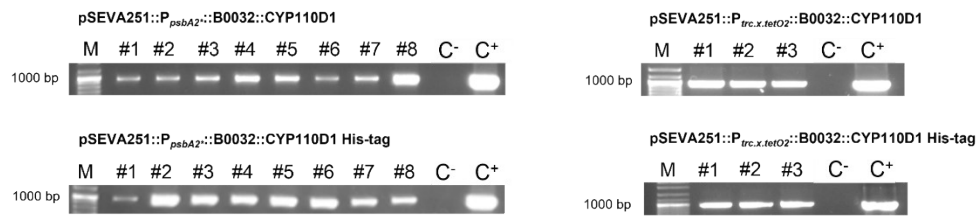


Fig. S1 Confirmation of the presence of each of the synthetic modules $P_{psbA2}::B0032::CYP110D1$ (with or without His-tag), or $P_{trc.x.tetO2}::B0032::CYP110D1$ (with or without His-tag) in *Synechocystis* strains by PCR using the primer pair (CYP110D1_central_FW and pSEVA251_RV). C⁻, negative control; C⁺, positive control using the purified plasmid as a template; #, clone identification number; M, Gene Ruler DNA Ladder Mix (Thermo-Fisher Scientific).

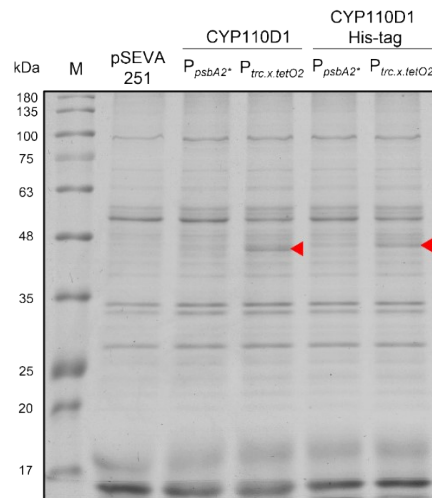


Fig. S2 CYP110D1 protein expression in engineered *Synechocystis* sp. PCC 6803 strains. SDS-PAGE analysis of protein extracts isolated from *Synechocystis* mutants harboring the empty vector pSEVA251 or each of the synthetic modules for the expression of CYP110D1 with or without a His-tag. Cells were cultivated in BG11 medium supplemented with $50 \mu\text{g mL}^{-1}$ kanamycin, at 30°C and under a 12 hours light ($30 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) / 12 hours dark regimen, until an $\text{OD}_{730} \sim 3$, harvested, suspended in lysis buffer and disrupted by sonication (for details see Experimental Section). $10 \mu\text{g}$ of proteins were loaded in each lane. M, Molecular mass standards are indicated on the left (kDa). Red arrowheads indicate the band corresponding to the heterologous protein CYP110D1. A single representative experiment of three biological replicates is shown.

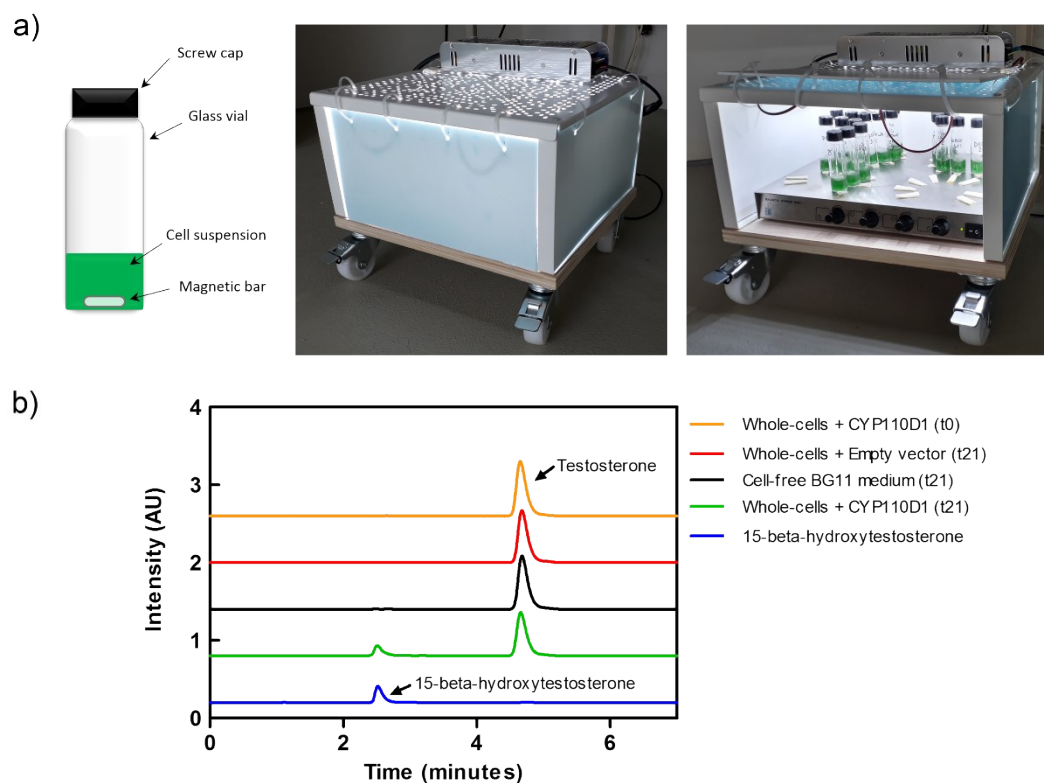


Fig. S3 Biotransformation of testosterone into 15 β -hydroxytestosterone by *Synechocystis* whole-cells expressing the heterologous CYP450 monooxygenase CYP110D1. (a) Schematic representation of the biotransformation setup and the photoreactor used for the assays. (b) HPLC chromatograms showing the substrate and product formation after extraction (for details see Experimental Section) and a commercial standard of 15 β -hydroxytestosterone. Reactions were performed by incubating 0.5 mM testosterone in 2 mL reaction volume of cells concentrated to $OD_{730}=5$ ($0.9 \text{ g}_{\text{CDW}} \text{ L}^{-1}$) in BG11 medium, in closed vials, at 30 °C, with a light intensity of $150 \mu\text{mol photons s}^{-1} \text{ m}^{-2}$. Whole-cells + CYP110D1 (t0) - *Synechocystis* cells expressing CYP110D1 under the control of P_{psbA2^*} (via the replicative plasmid pSEVA251) at the beginning of the reaction. Whole-cells + empty vector (t21) - *Synechocystis* cells harboring the empty plasmid after 21 hours of incubation. Cell-free BG11 medium (t21) - negative control. Whole-cells + CYP110D1 (t21) - *Synechocystis* cells expressing CYP110D1 under the control of P_{psbA2^*} (via the replicative plasmid pSEVA251) after 21 hours of incubation. For each condition tested, 3 biological replicates and 2 technical replicates were made.

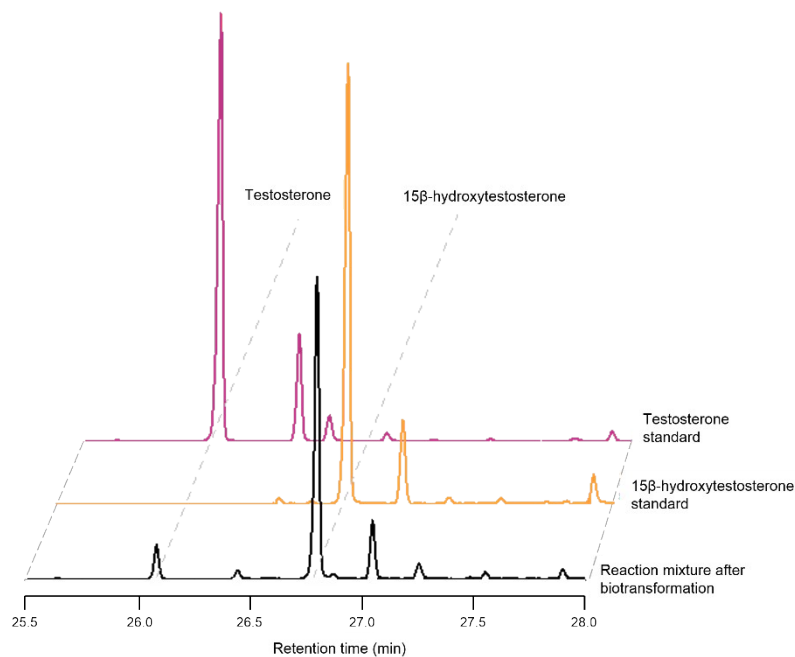
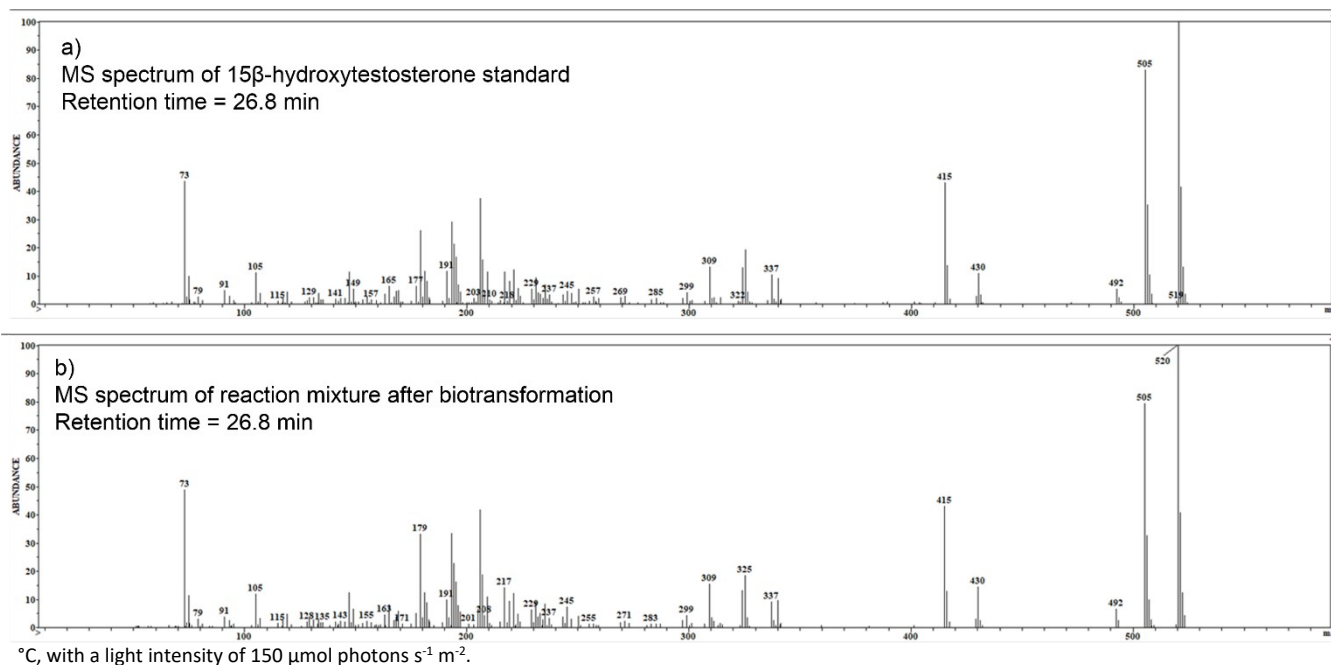


Fig. S4 GC analysis of the product present in the extracted reaction mixture after biotransformation (black) compared with standards of testosterone (purple) and 15β-hydroxytestosterone (orange). The reaction was performed by incubating 1 mM testosterone in 50 mL reaction volume of *Synechocystis* cells (expressing CYP110D1 under the control of $P_{trc.x.tetO2}$ promoter) concentrated to $OD_{730}=10$ ($1.8 \text{ g}_{CDW} \text{ L}^{-1}$) in BG11 medium, in open glass cylinders, at 30



$^{\circ}\text{C}$, with a light intensity of $150 \mu\text{mol photons s}^{-1} \text{ m}^{-2}$.

Fig. S5 Comparison of the MS fragmentation spectra of the peak at 26.8 min for (a) the standard 15β-hydroxytestosterone and (b) the product present in the reaction mixture after biotransformation (b). The reaction was performed by incubating 1 mM testosterone in 50 mL reaction volume of *Synechocystis*

cells (expressing CYP110D1 under the control of $P_{trc.x.tetO2}$ promoter) concentrated to $OD_{730}=10$ ($1.8 \text{ g}_{CDW} \text{ L}^{-1}$) in BG11 medium, in open glass cylinders, at 30 °C, with a light intensity of $150 \mu\text{mol photons s}^{-1} \text{ m}^{-2}$.

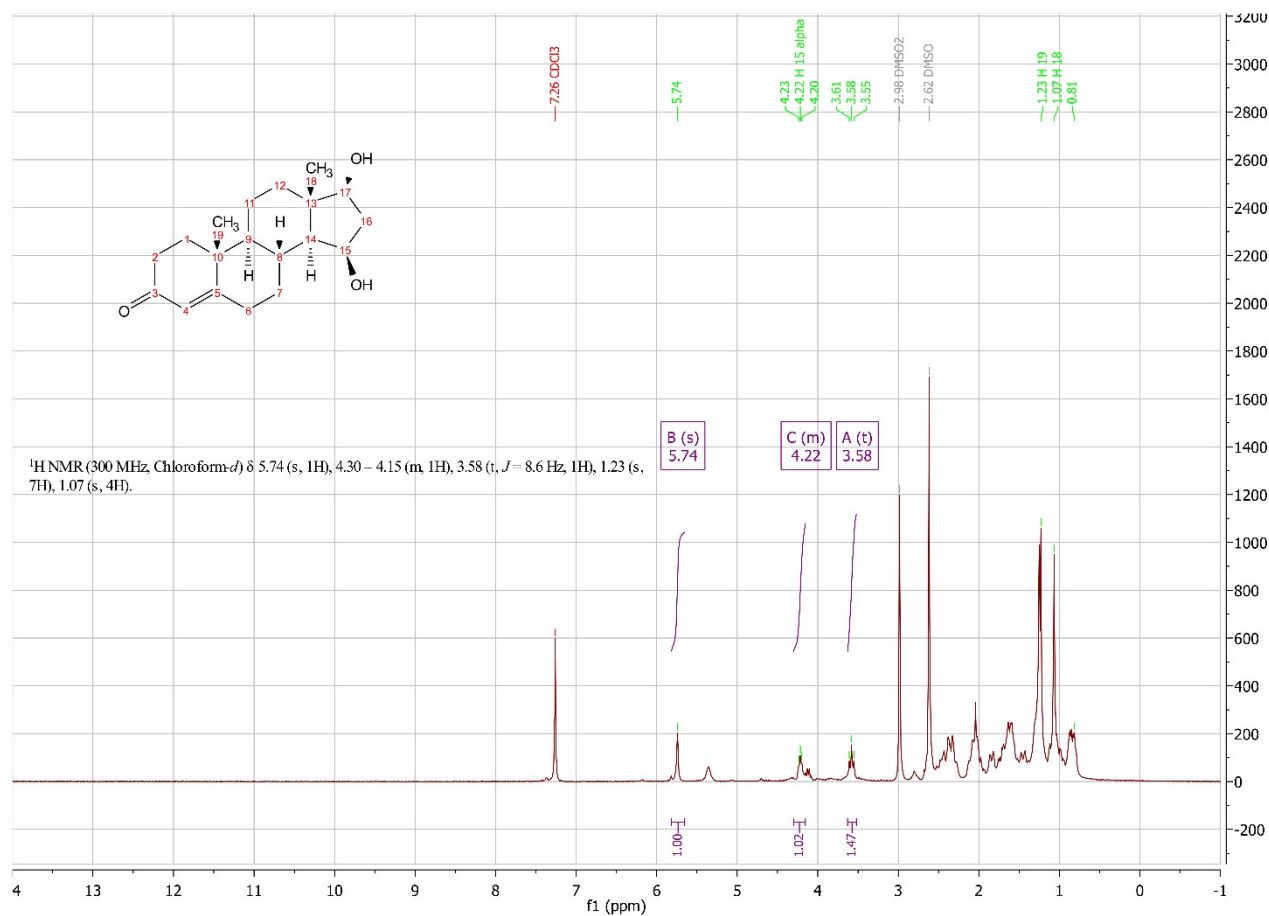


Fig. S6 $^1\text{H NMR}$ spectrum of the product (15 β -hydroxytestosterone) present in the reaction mixture after biotransformation (see also Table S3). The reaction was performed by incubating 1 mM testosterone in 50 mL reaction volume of *Synechocystis* cells (expressing CYP110D1 under the control of $P_{trc.x.tetO2}$ promoter) concentrated to $OD_{730}=10$ ($1.8 \text{ g}_{CDW} \text{ L}^{-1}$) in BG11 medium, in open glass cylinders, at 30 °C, with a light intensity of $150 \mu\text{mol photons s}^{-1} \text{ m}^{-2}$.

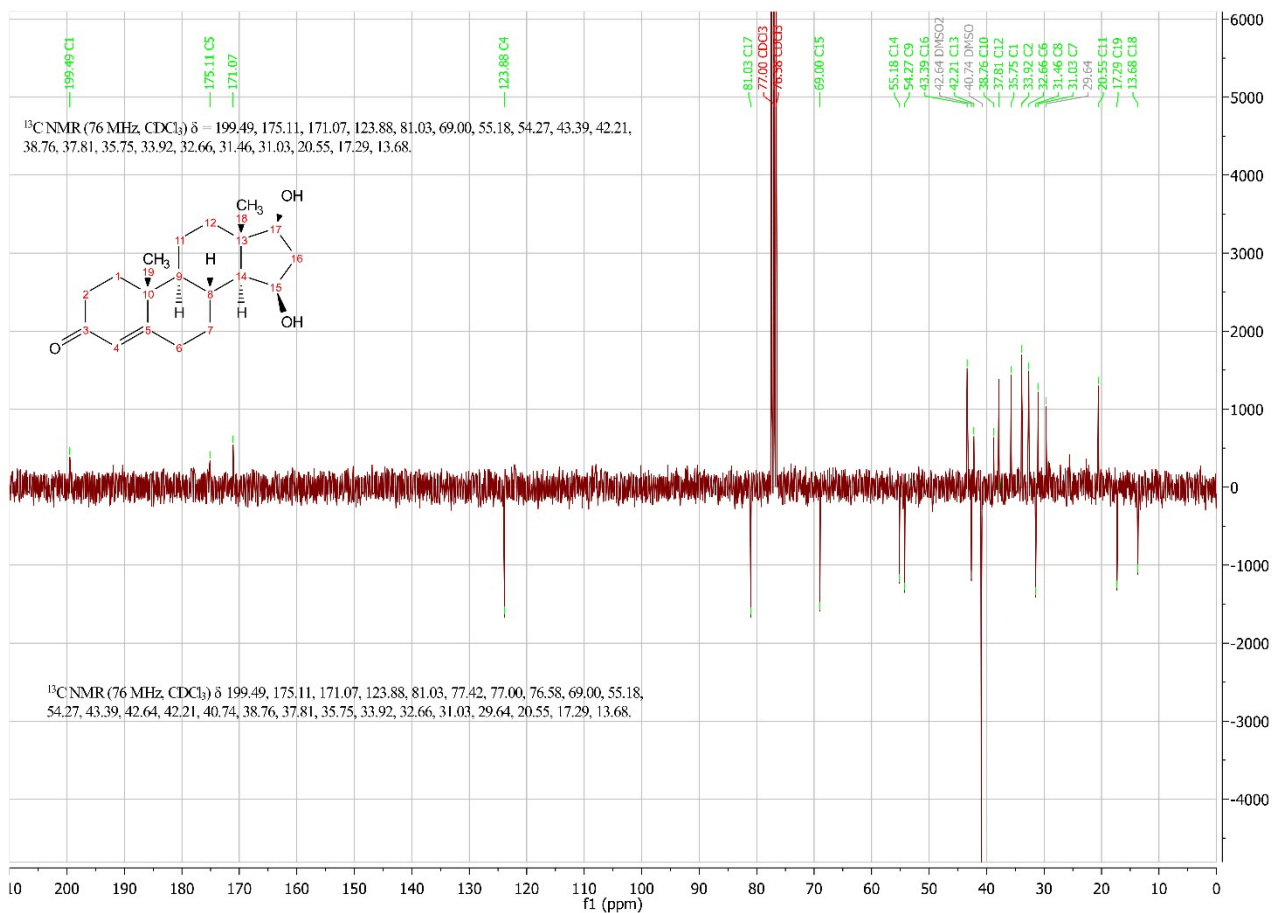


Fig. S7 ¹³C NMR spectrum of the product (15β-hydroxytestosterone) present in the reaction mixture after biotransformation (see also Table S4). The reaction was performed by incubating 1 mM testosterone in 50 mL reaction volume of *Synechocystis* cells (expressing CYP110D1 under the control of $P_{trc,x.tetO2}$ promoter) concentrated to $OD_{730}=10$ ($1.8 \text{ g}_{CDW} \text{ L}^{-1}$) in BG11 medium, in open glass cylinders, at 30 °C, with a light intensity of $150 \mu\text{mol photons s}^{-1} \text{ m}^{-2}$.

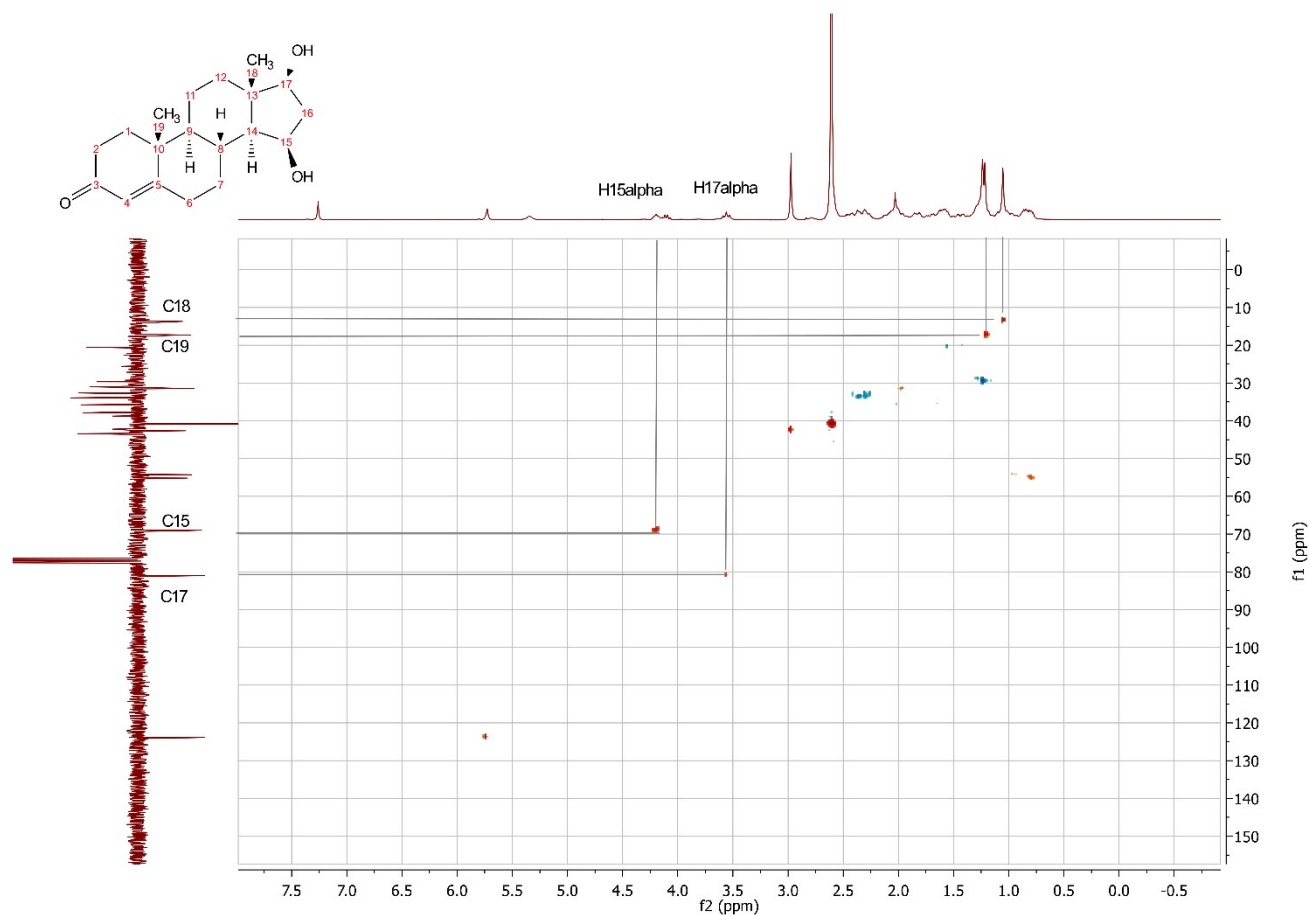


Fig. S8 2D-NMR spectrum of the product (15 β -hydroxytestosterone) present in the reaction mixture after biotransformation (see also Table S4). The reaction was performed by incubating 1 mM testosterone in 50 mL reaction volume of *Synechocystis* cells (expressing CYP110D1 under the control of $P_{trc,x,tetO2}$ promoter) concentrated to $OD_{730}=10$ ($1.8 \text{ g}_{CDW} \text{ L}^{-1}$) in BG11 medium, in open glass cylinders, at 30 °C, with a light intensity of $150 \mu\text{mol photons s}^{-1} \text{ m}^{-2}$.

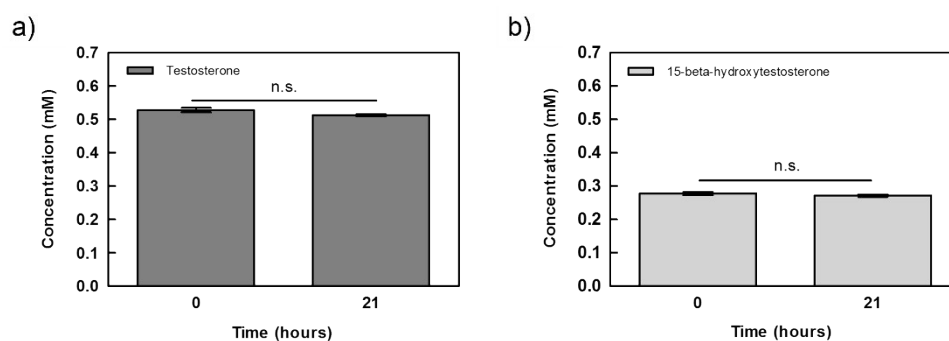


Fig. S9 Control reactions performed incubating purified testosterone or 15 β -hydroxytestosterone with *Synechocystis* whole-cells harboring the empty pSEVA251 vector (a) Concentration of testosterone in the reaction mixtures at the beginning of the reaction and after 21 hours incubation. (b) Concentration of 15 β -hydroxytestosterone in the reaction mixtures at the beginning of the reaction and after 21 hours incubation. Reactions were performed in 2 mL reaction volume, with cells concentrated to $OD_{730}=10$ ($1.8 \text{ g}_{CDW} \text{ L}^{-1}$) in BG11 medium supplemented with 0.5 mM testosterone or 0.25 mM 15 β -hydroxytestosterone, in closed vials, at 30 °C, with a light intensity of $150 \mu\text{mol photons s}^{-1} \text{ m}^{-2}$. Error bars represent the standard error of the mean (SEM) of 3 biological replicates and 2 technical replicates (n.s., not significant, p -value > 0.05).

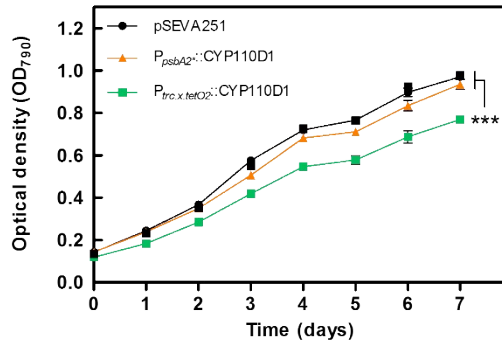


Fig. S10 Growth curves of engineered *Synechocystis* strains harboring the empty pSEVA251 vector or expressing CYP110D1 under the control of P_{psbA2}* or P_{trc.x.tetO2} promoters. Cells were grown in microtiter plates at 30 °C and under a 12 hours light (30 μmol photons m⁻² s⁻¹)/ 12 hours dark regime. Growth was monitored by measuring optical density at 790 nm (OD₇₉₀). Error bars correspond to the standard error of the mean (SEM) of 3 biological replicates and 4 technical replicates (***) *p*-value < 0.001). When not visible, the error bars are smaller than the size of the symbol.

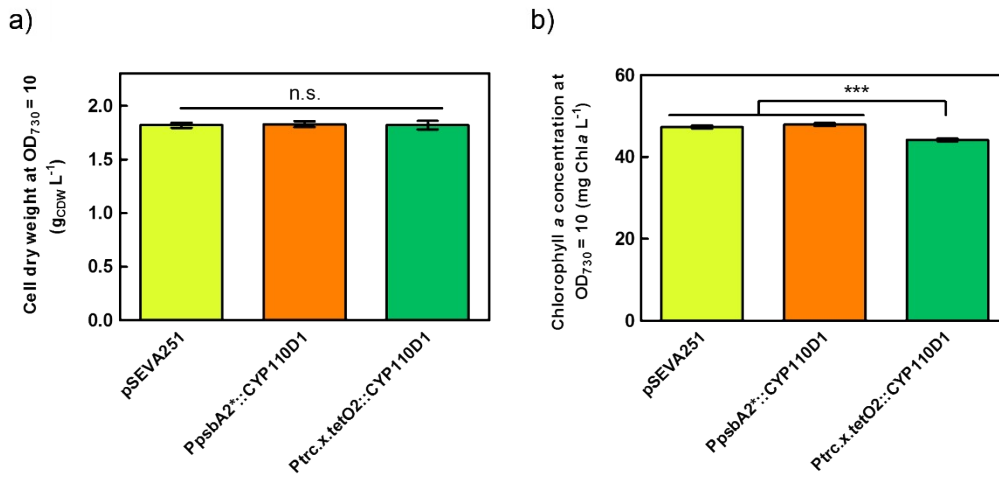


Fig. S11 (a) Cell dry weight and (b) chlorophyll *a* concentration of *Synechocystis* cells harboring the empty pSEVA251 vector or expressing CYP110D1 under the control of P_{psbA2}* or P_{trc.x.tetO2} promoters at OD₇₃₀=10. Cells were grown under standard conditions to an OD₇₃₀ of ~3 before being concentrated to OD₇₃₀ of 10 in fresh BG11 medium. Error bars represent the standard error of the mean (SEM) of 3 biological replicates and 2 technical replicates (n.s., not significant, *p*-value > 0.05; ***) *p*-value < 0.001).

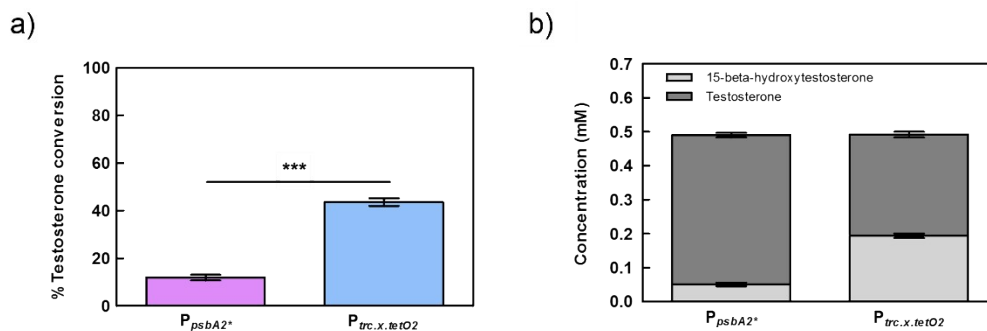
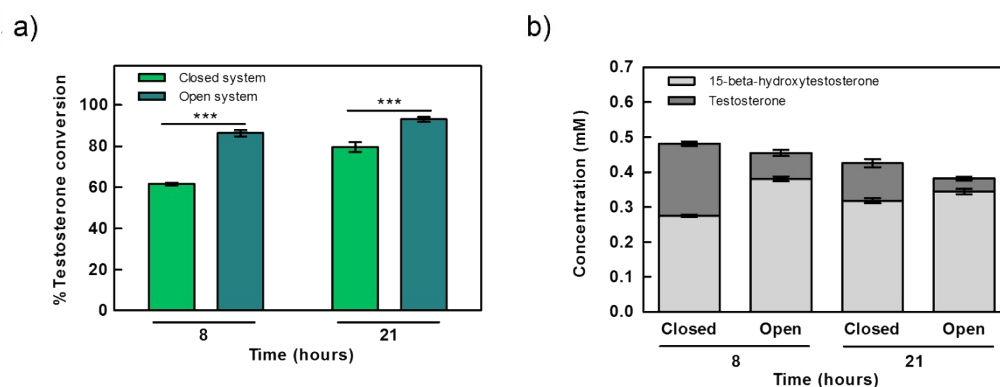


Fig. S12 Bioconversion of testosterone into 15 β -hydroxytestosterone by *Synechocystis* whole-cells expressing the His-tagged CYP110D1 under the control of the P_{psbA2^*} or $P_{trc.x.tetO2}$ promoter. (a) % of testosterone converted after 21 hours incubation. (b) Concentration of testosterone and 15 β -hydroxytestosterone in the reaction mixtures after 21 hours incubation. Reactions were performed in 2 mL reaction volume, with cells concentrated to $OD_{730}=5$ ($0.9 \text{ g}_{CDW} \text{ L}^{-1}$) in BG11 medium supplemented with 0.5 mM testosterone, in closed vials, at 30 °C, with a light intensity of $150 \mu\text{mol photons s}^{-1} \text{ m}^{-2}$.



Error bars represent the standard error of the mean (SEM) of 3 biological replicates and 2 technical replicates (***) p -value < 0.001).

Fig. S13 Bioconversion of testosterone into 15 β -hydroxytestosterone by *Synechocystis* whole-cells expressing CYP110D1 under the control of $P_{trc.x.tetO2}$ promoter, performed using either closed or open vials (closed or open system). (a) % of testosterone converted after 8 and 21 hours incubation. (b) Concentration of testosterone and 15 β -hydroxytestosterone in the reaction mixtures after 8 and 21 hours incubation. Reactions were performed by incubating 0.5 mM testosterone in 2 mL reaction volume, with cells concentrated to $OD_{730}=10$ ($1.8 \text{ g}_{CDW} \text{ L}^{-1}$) in BG11 medium, at 30 °C, with a light intensity of $150 \mu\text{mol photons s}^{-1} \text{ m}^{-2}$. Error bars represent the standard error of the mean (SEM) of 3 biological replicates and 2 technical replicates (***) p -value < 0.001).

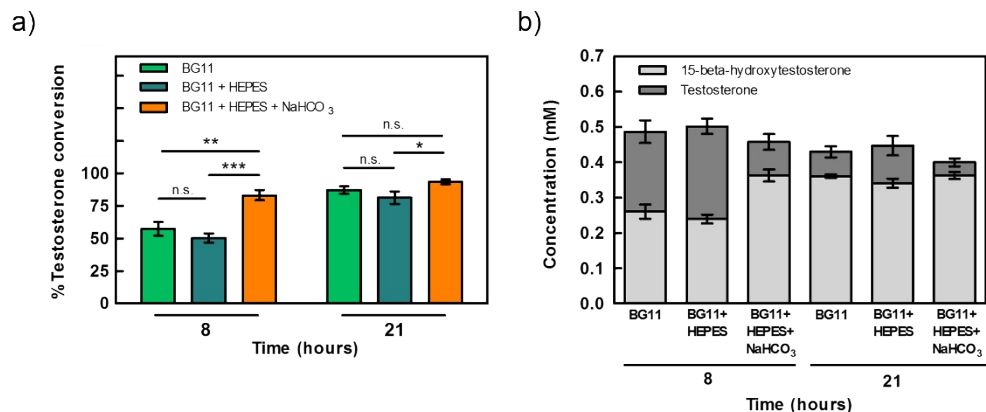


Fig. S14 Bioconversion of testosterone into 15 β -hydroxytestosterone by *Synechocystis* whole-cells expressing CYP110D1 under the control of $P_{trc.x.tetO2}$, performed in standard BG11 medium (BG11) or BG11 buffered with 50 mM HEPES pH 8.0 (BG11 + HEPES) or BG11 buffered with HEPES and supplemented with 50 mM NaHCO₃ (BG11 + HEPES + NaHCO₃). (a) % of testosterone converted after 8 and 21 hours incubation. (b) Concentration of testosterone and 15 β -hydroxytestosterone in the reaction mixture after 8 and 21 hours incubation. Reactions were performed by incubating 0.5 mM testosterone in 2 mL reaction volume, with cells concentrated to OD₇₃₀=10 (1.8 g_{CDW} L⁻¹) in BG11 medium, in closed vials, at 30 °C, with a light intensity of 150 μ mol photons s⁻¹ m⁻². Error bars represent the standard error of the mean (SEM) of 3 biological replicates and 2 technical replicates (n.s., not significant, p -value > 0.05; * p -value < 0.05; ** p -value < 0.01).

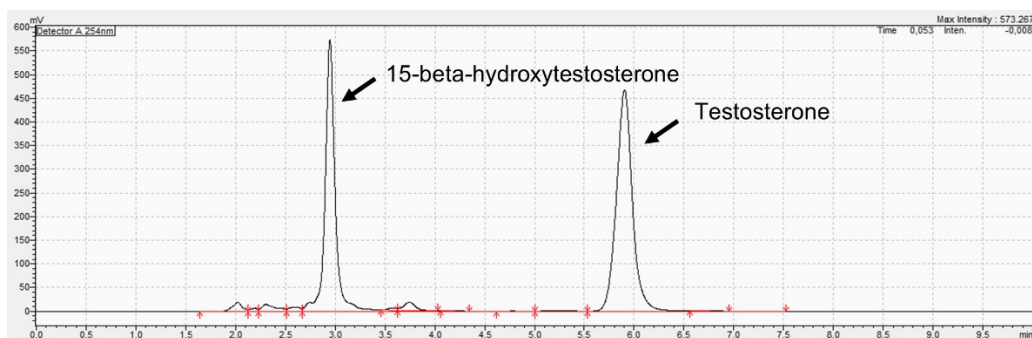


Fig. S15 Chromatogram showing the conversion of testosterone into 15 β -hydroxytestosterone by *E. coli* C43 (DE3) whole-cells expressing the monooxygenase CYP110D1, and the electron carriers putidaredoxin (Pdx) and putidaredoxin reductase (PdR). Reactions were performed by incubating 1 mM testosterone in 1 mL reaction volume, with cells concentrated to OD₆₀₀=40, in 100 mM potassium phosphate buffer pH 7.4 with 30 mM glucose, at 30 °C, for 1 hour. For this experiment 3 biological replicates were made.

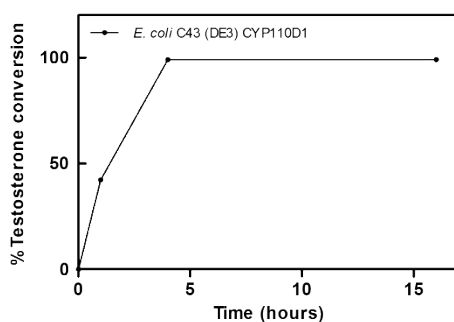


Fig. S16 % of testosterone conversion by *E. coli* C43 (DE3) whole-cells expressing the monooxygenase CYP110D1, putidaredoxin (Pdx) and putidaredoxin reductase (PdR) at different time points. Reactions were performed by incubating 1 mM testosterone in 1 mL reaction volume, with cells concentrated to OD₆₀₀=40, in 100 mM potassium phosphate buffer pH 7.4 with 30 mM glucose, at 30 °C. Error bars correspond to the standard error of the mean (SEM) of 3 biological replicates. When not visible, the error bars are smaller than the size of the symbol.

References

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