Der Biokatalysator Cytochrom P450 und seine vielfältigen Anwendungsfelder

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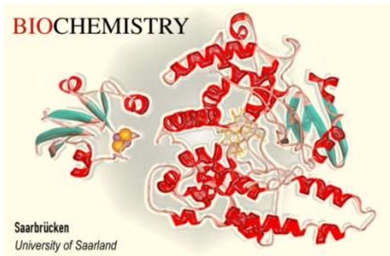
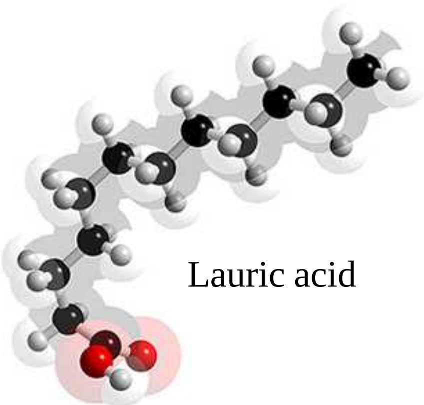


Reaction catalysed by cytochromes P450

P450 -systems

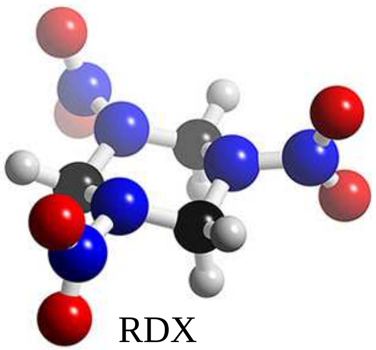
$$RH + O_2 \longrightarrow ROH + H_2O$$

$$NAD(P)H + H^{+} \longrightarrow NAD(P)^{+}$$



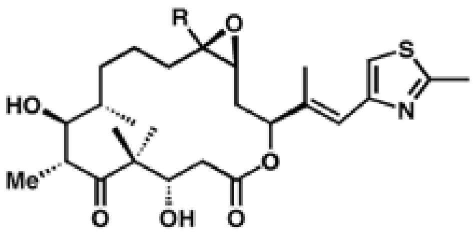
Lauric acid

CYP102A1



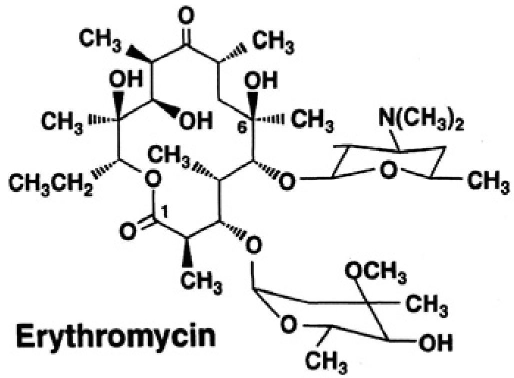
RDX

CYP177A1



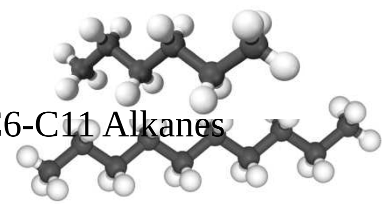
Epothilone A (R = H)
Epothilone B (R = CH3)

CYP167A1



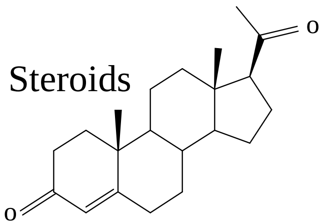
Erythromycin

CYP107A1 CYP107B1



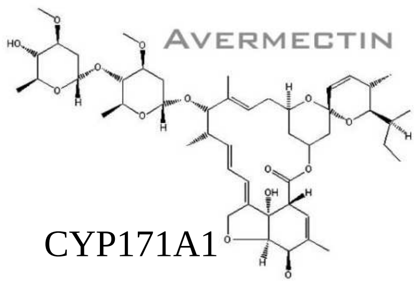
C6-C11 Alkanes

CYP153A6

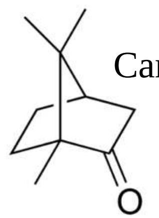


Steroids

CYP106A2, CYP21



CYP171A1



Camphor

CYP101A1

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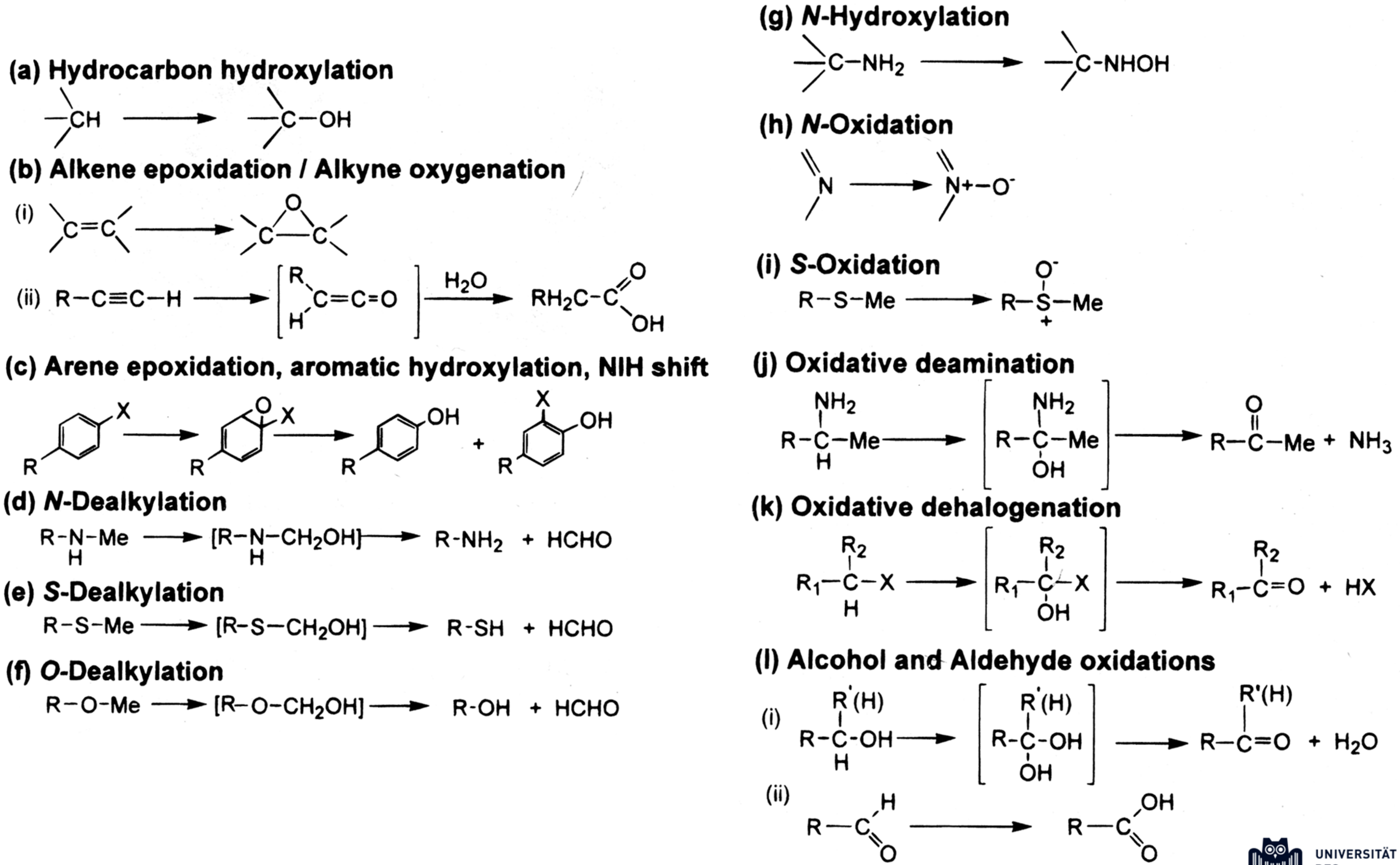
The Diversity of P450-Catalyzed Reactions (I)



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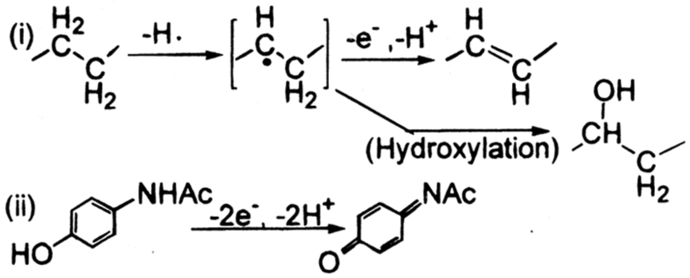
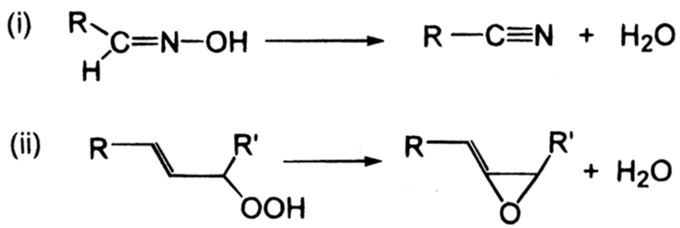
Chem. Rev. **1996**, *96*, 2841-2887.

The Diversity of P450-Catalyzed Reactions (II)

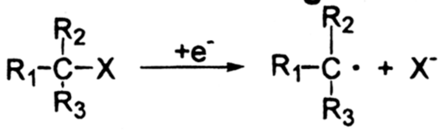
(m) Dehydrogenation



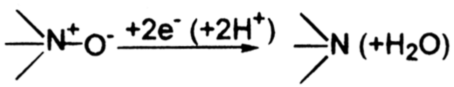
(n) Dehydrations



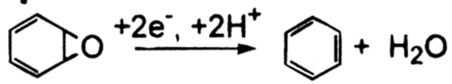
(o) Reductive dehalogenation



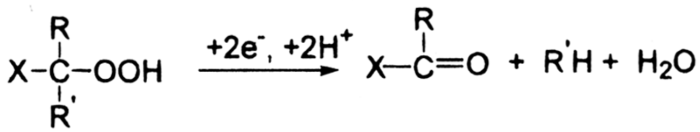
(p) *N*-Oxide reduction



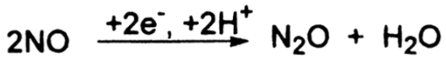
(q) Epoxide reduction



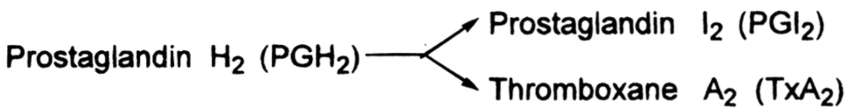
(r) Reductive β-scission of alkyl peroxides



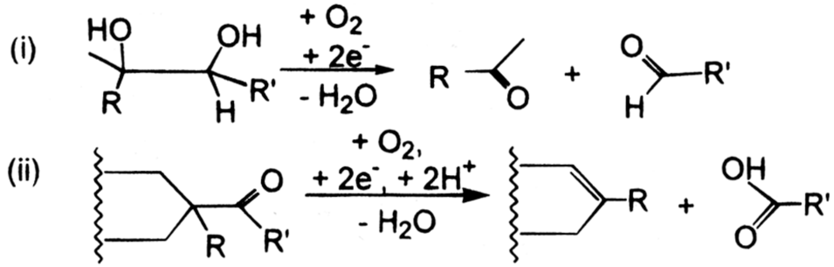
(s) NO reduction



(t) Isomerizations



(u) Oxidative C-C bond cleavage

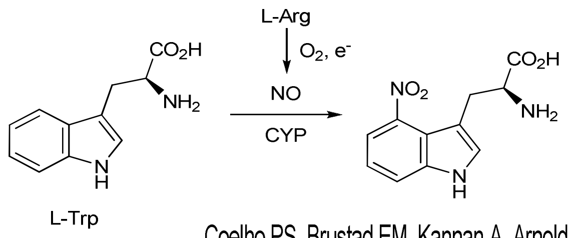


Chem. Rev. **1996**, *96*, 2841-2887.

The Diversity of P450-Catalyzed Reactions (III)

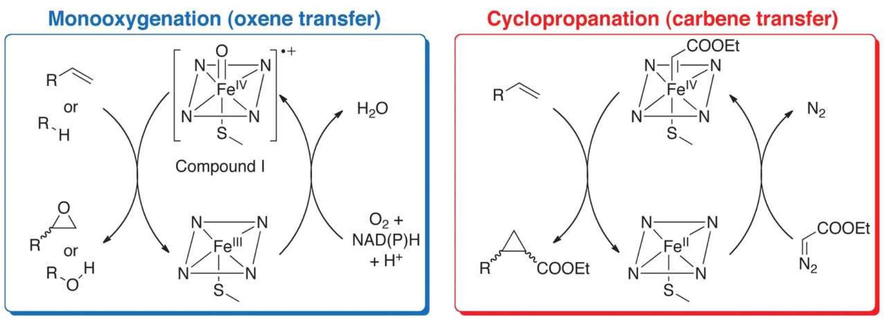
Barry SM, Kers JA, Johnson EG, Song L, Aston PR, Patel B, Krasnoff SB, Crane BR, Gibson DM, Loria R, Challis GL (2012) Cytochrome P450-catalyzed L-tryptophan nitration in thaxtomin phytotoxin biosynthesis. Nat Chem Biol 8:814-816.

Nitration of tryptophan



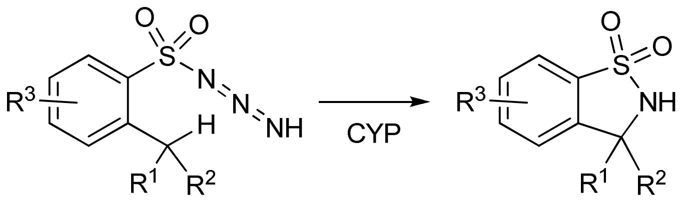
Coelho PS, Brustad EM, Kannan A, Arnold FH (2013a) Olefin cyclopropanation via carbene transfer catalyzed by engineered cytochrome P450 enzymes. Science 339:307-310.

Cyclopropanation



McIntosh JA, Coelho PS, Farwell CC, Wang ZJ, Lewis JC, Brown TR, Arnold FH (2013) Enantioselective intramolecular C-H amination catalyzed by engineered cytochrome P450 enzymes in vitro and in vivo. Angew Chem Int Ed Engl 52:9309-9312.

Intramolecular C-H amination



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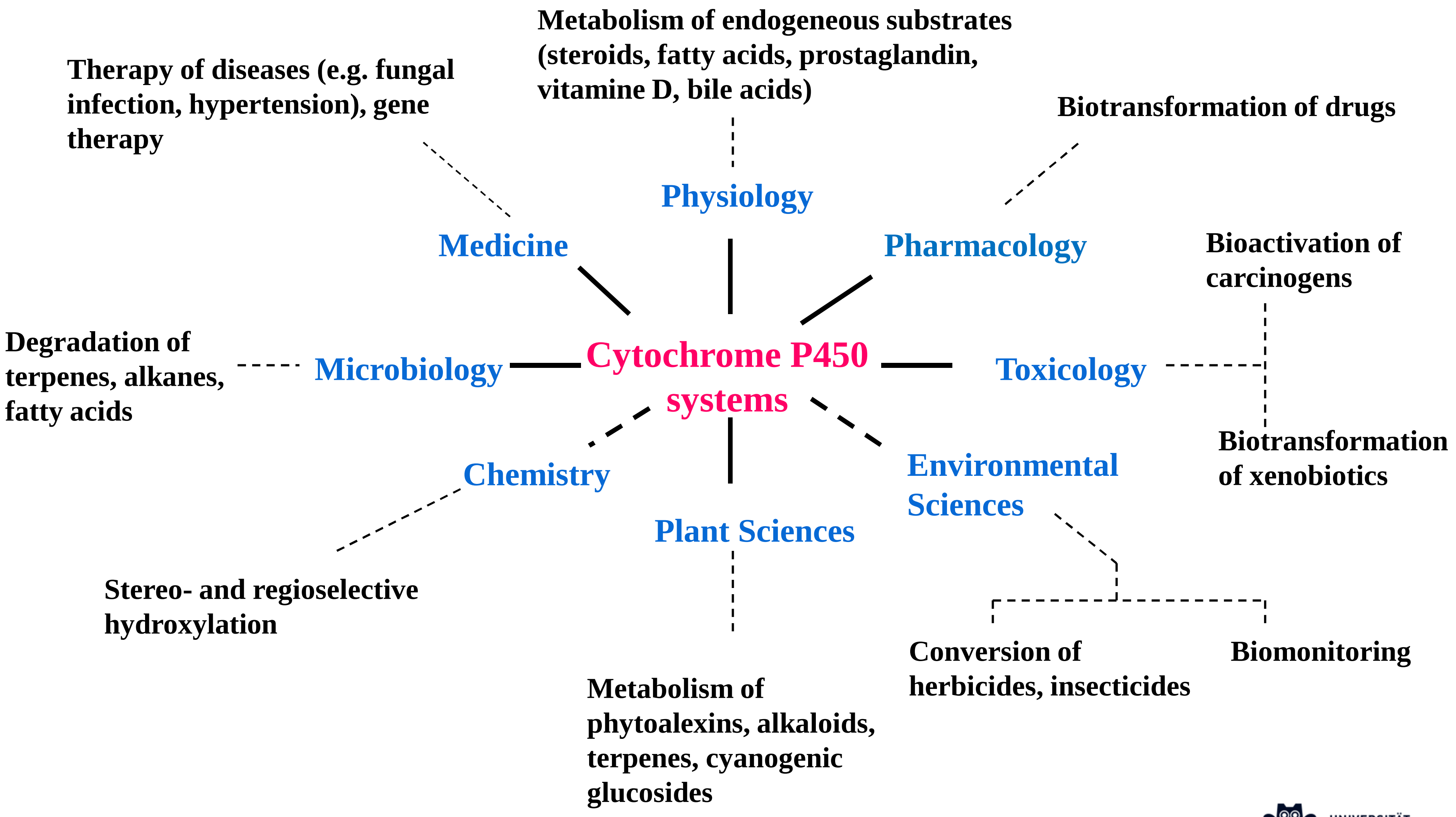
Function and potential applications of cytochrome P450 systems



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2013: > 21.000 Isoenzymes (Genes)

CYPom = all CYPs of an organism

- *Escherichia coli*: 0
- *Bacillus subtilis*: 7
- *Mycobacterium tuberculosis*: 20
- *Saccharomyces cerevisiae*: 3
- *Arabidopsis thaliana*: 275
- *Caenorhabditis elegans*: 80
- *Drosophila melanogaster*: 90
- *Homo sapiens* 57

http://drnelson.utmem.edu
/CytochromeP450.html

CYTOCHROME P450

Specific isoform

CYP109C1

Family
(>40% identity)

Subfamily
(>55% identity)

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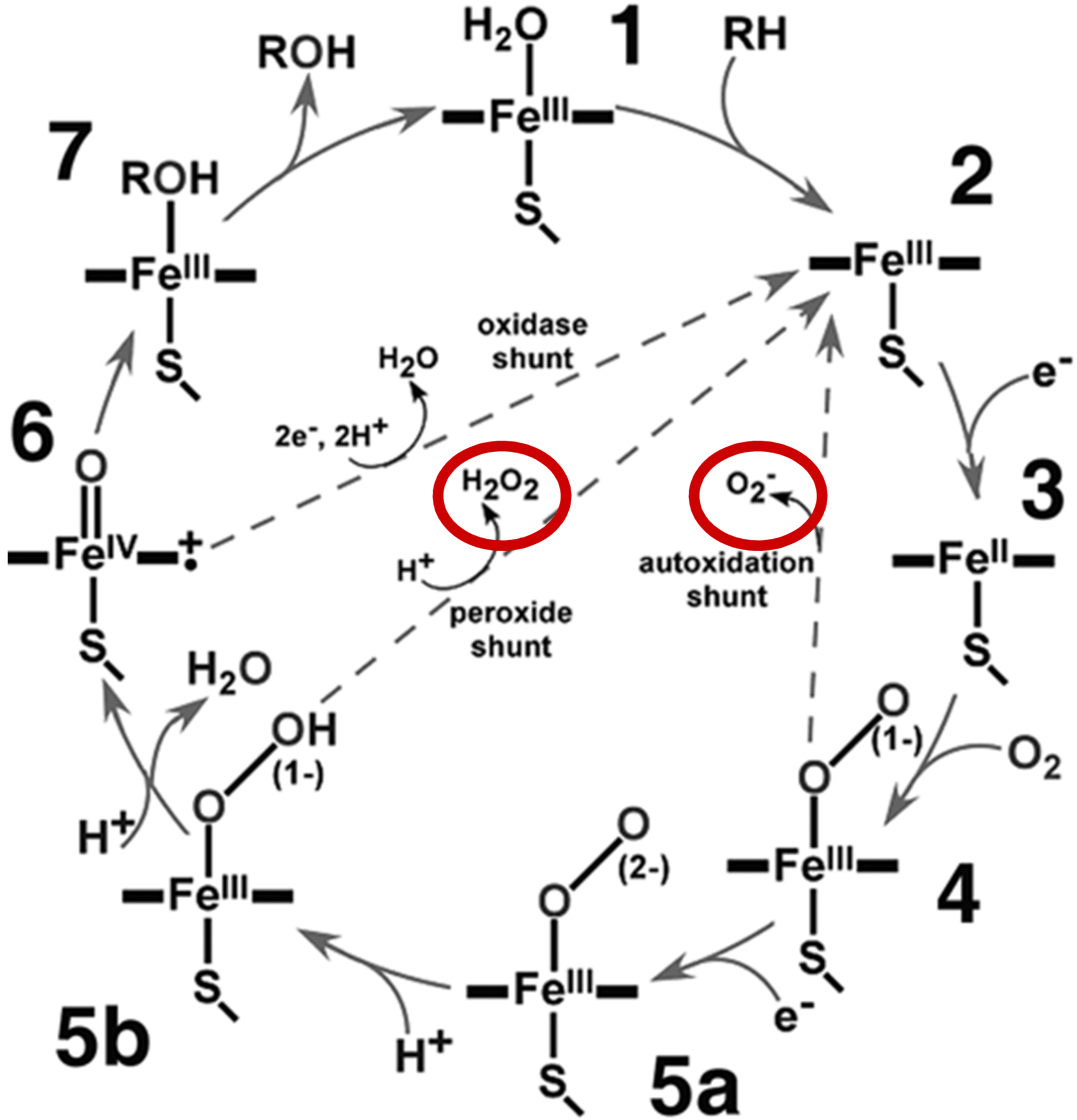

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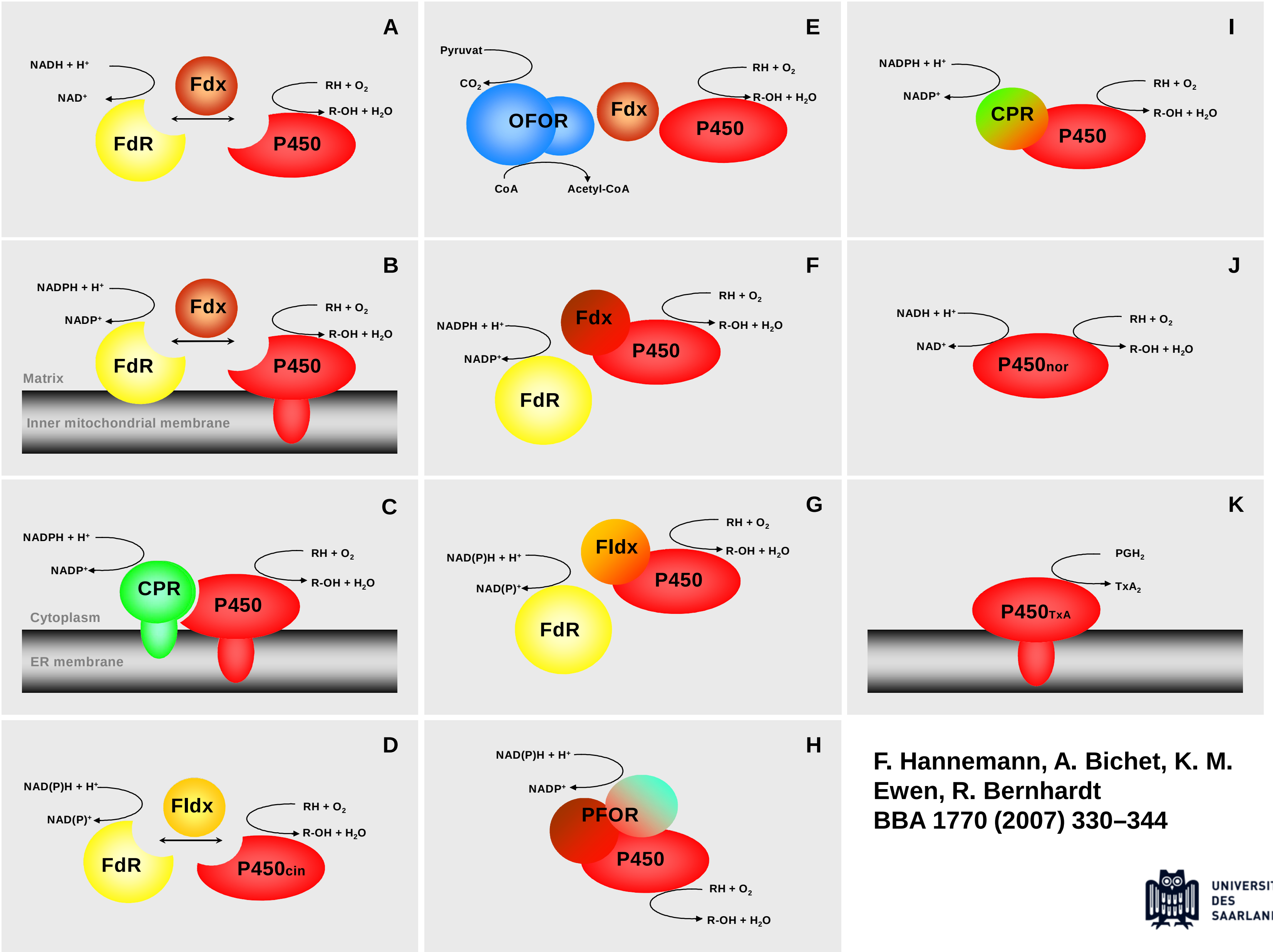
Biological variations of electron transport chains in P450 systems



F. Hannemann, A. Bichet, K. M. Ewen, R. Bernhardt
BBA 1770 (2007) 330–344

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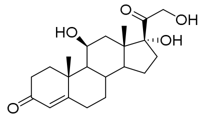
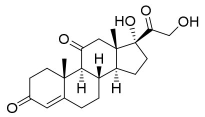
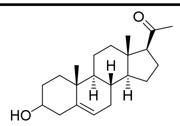
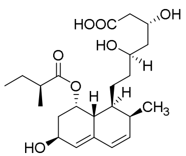
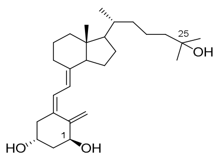
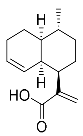
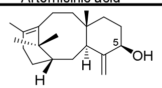
Successful examples of P450 application

Product	Microorganism and/or CYPs involved	Reference / Company
 Hydrocortision	Biotransformation with *Curvularia* sp.	(Petzoldt et al. 1982) Schering (1982), now Bayer
	de novo synthesis in *S. cerevisiae*; mammalian CYP11A1, CYP17A1, CYP21, CYP11A1, 3β-HSD	(Szczebara et al. 2003) Sanofi
 Cortisone	Biotransformation with *Rhizopus* sp.	(Hogg 1992; Peterson et al. 1952) Upjohn (1952), now Pharmacia and Upjohn Company, Pfizer
 Pregnenolone	*de novo* synthesis in *S. cerevisiae;*mammalian CYP11A1,	(Duport et al. 1998) Sanofi
 Pravastatin	Biotransformation with *Streptomyces carbophilus*	(Arai et al. 1988) Daiichi Sankyo Inc. and Bristol-Myers Squibb
 1α,25-Dihydroxyvitamin D3	Microbial biotransformation; Recombinant *E. coli* with e.g. CYP105A1 from *Streptomyces griseolus*	(Sasaki et al. 1992) (Sakaki et al. 2011)
 Artemisinic acid	*de novo* synthesis in *S. cerevisiae*; CYP71AV1 from *Artemisia annua*.	(Ro et al. 2006) (Paddon et al. 2013) Sanofi
 Taxadien-5α-ol	*de novo* synthesis in *E. coli*; taxadiene-5α-hydroxylase from *Taxus brevifolia*	(Ajikumar et al. 2010)
Blue roses	Transgenic plants; petunia CYP75B and CYP75A	(Holton et al. 1993; Katsumoto et al. 2007) Suntory Ltd, Calgene Pacific (now Florigene Pty Ltd)

Successful examples of P450 application

For details see:

Bernhardt, R and Urlacher, V. “Cytochromes P450 as promising catalysts for biotechnological application: chances and limitations“
Applied Microbiol Biotechnol., in press

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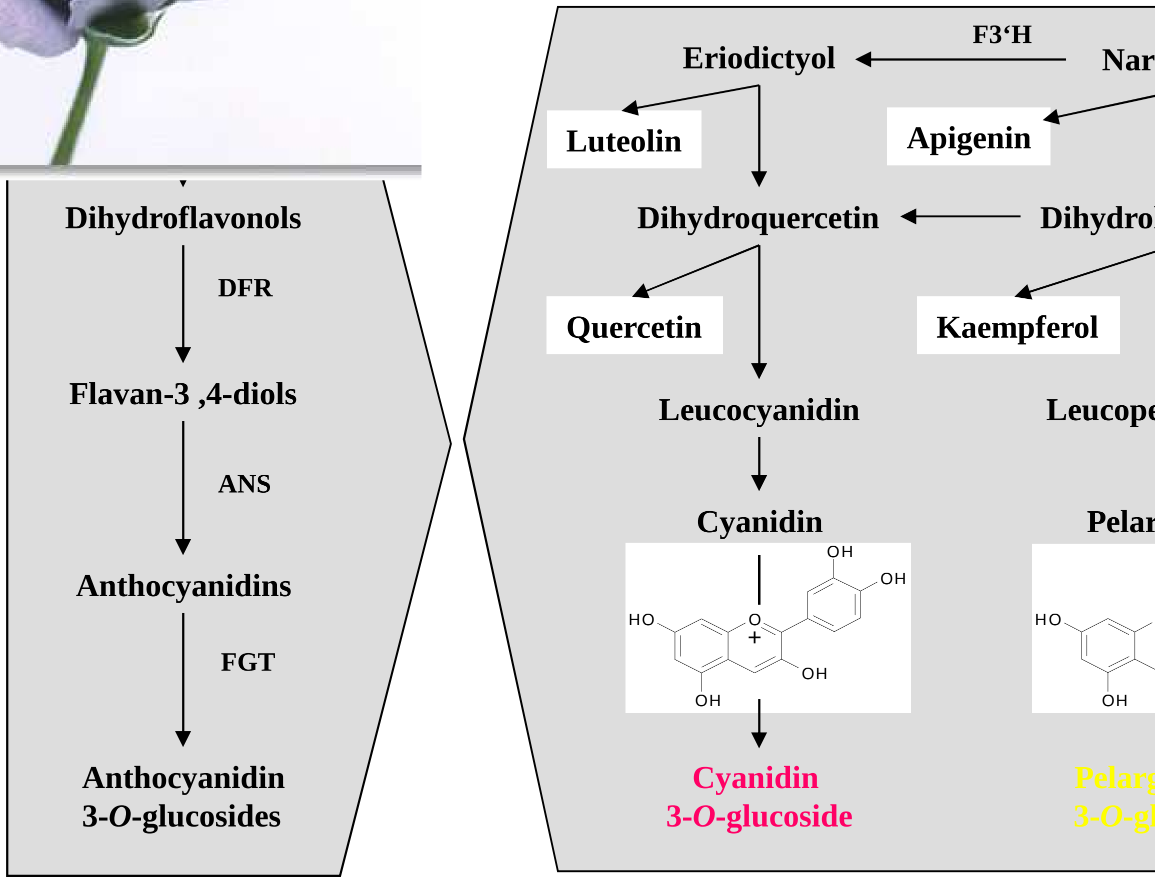
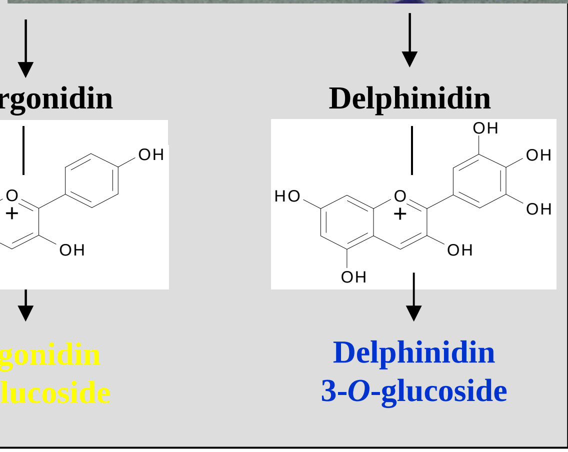
Creation of blue ro





Forkmann G and Martens S (2001) Metabolic engineering and applications of flavonoids. Curr. Opin. Biotechnol. 12: 155-160

Holton, T.A., Brugliera, F. Lester, D.R., Tanaka, Y. Hyland, C.D. Menting, J.G.T., Lu, C., Farcy, E., Stevenson, T.W. and Cornish, E.C. (1993) Cloning and expression of cytochrome P450 genes controlling flower colour. Nature, 366, 276-279

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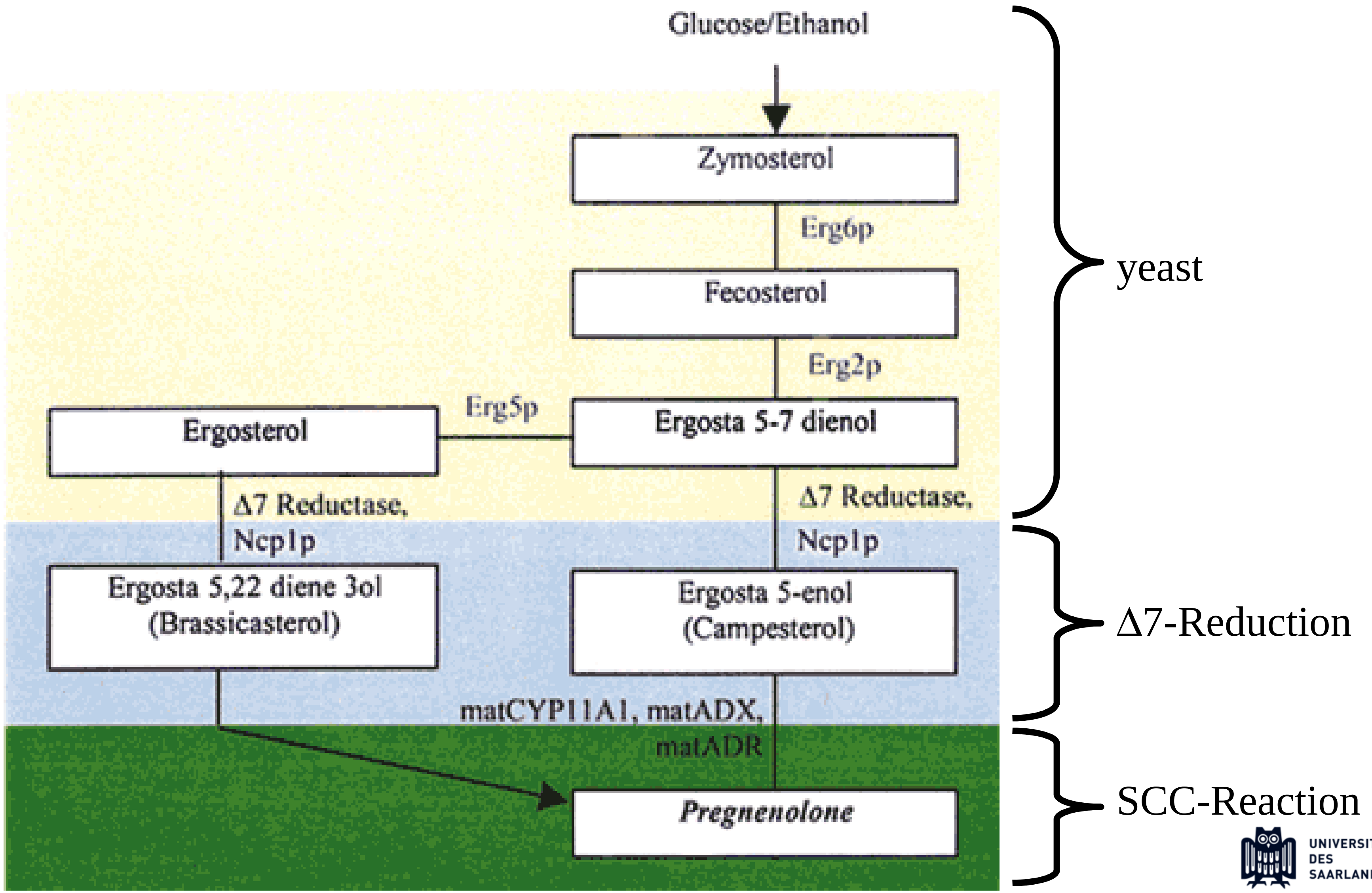
Connection between yeast sterol metabolism and mammalian steroids (*Sanofi*)



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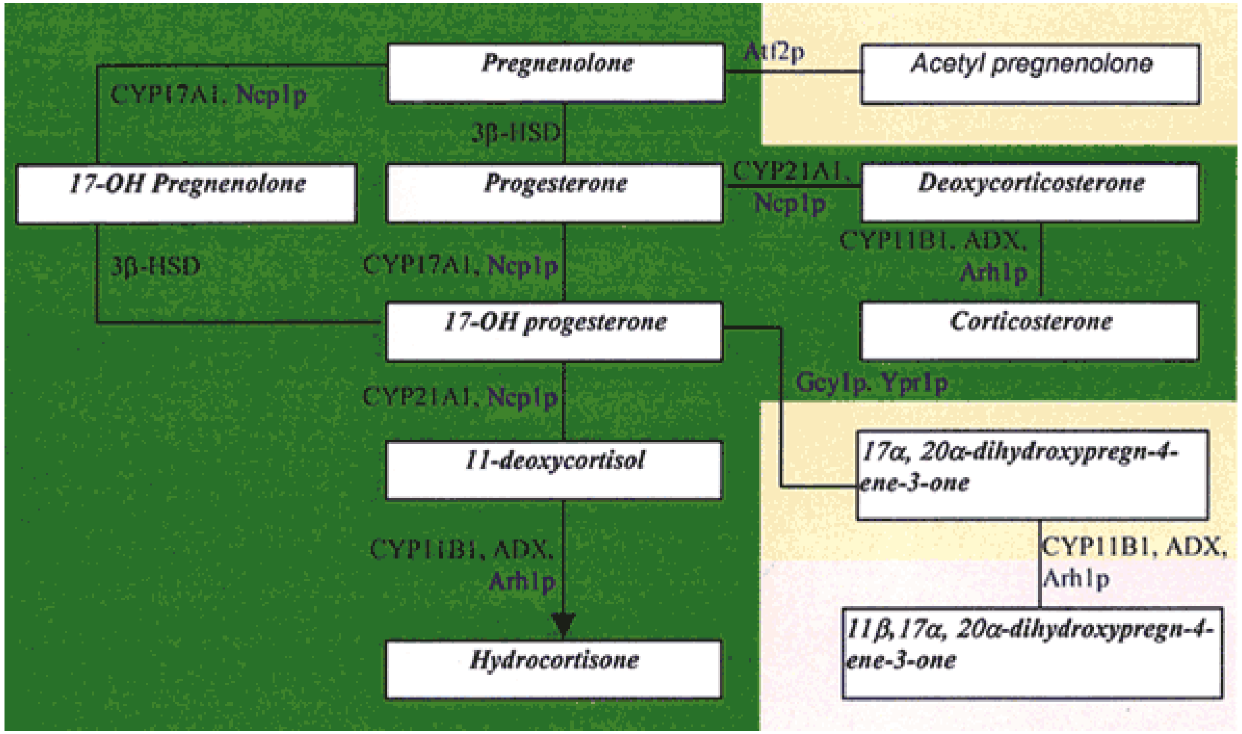
Production of hydrocortisone from pregnenolone



Yeast made derivatives from steroids

CYP11B1-made side-product

13 engineered genes in a single yeast strain: cortisol from simple carbon source

Total biosynthesis of hydrocortisone from a simple carbon source in yeast. Szczebara et al., Nat Biotechnol 21, 143-149, 2003

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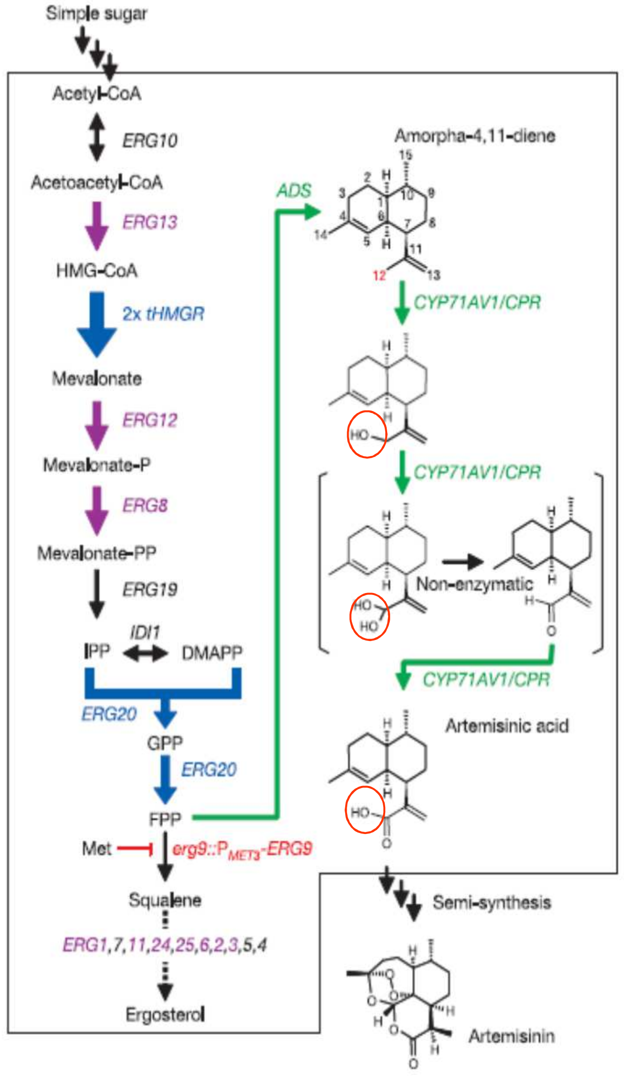
Production of artemisinin precursor in engineered hosts



JD. Keasling

Supported by the Gates Foundation (43 Mio$)

Head of the Joint BioEnergy Institute in California (150 Scientists)

Founder & Chairman of Amyris Biotechnology

Ca. 150 mg/l Amorphadiene
Ca. 100 mg/l Artemisinic acid

blue: *S. cerevisiae* mevalonate genes upregulated
purple: *S. cerevisiae* mevalonate genes indirectly upregulated
red: repressed
green: genes from *Artemisia*

Ro et al., 2006, Nature, 440, 940-943

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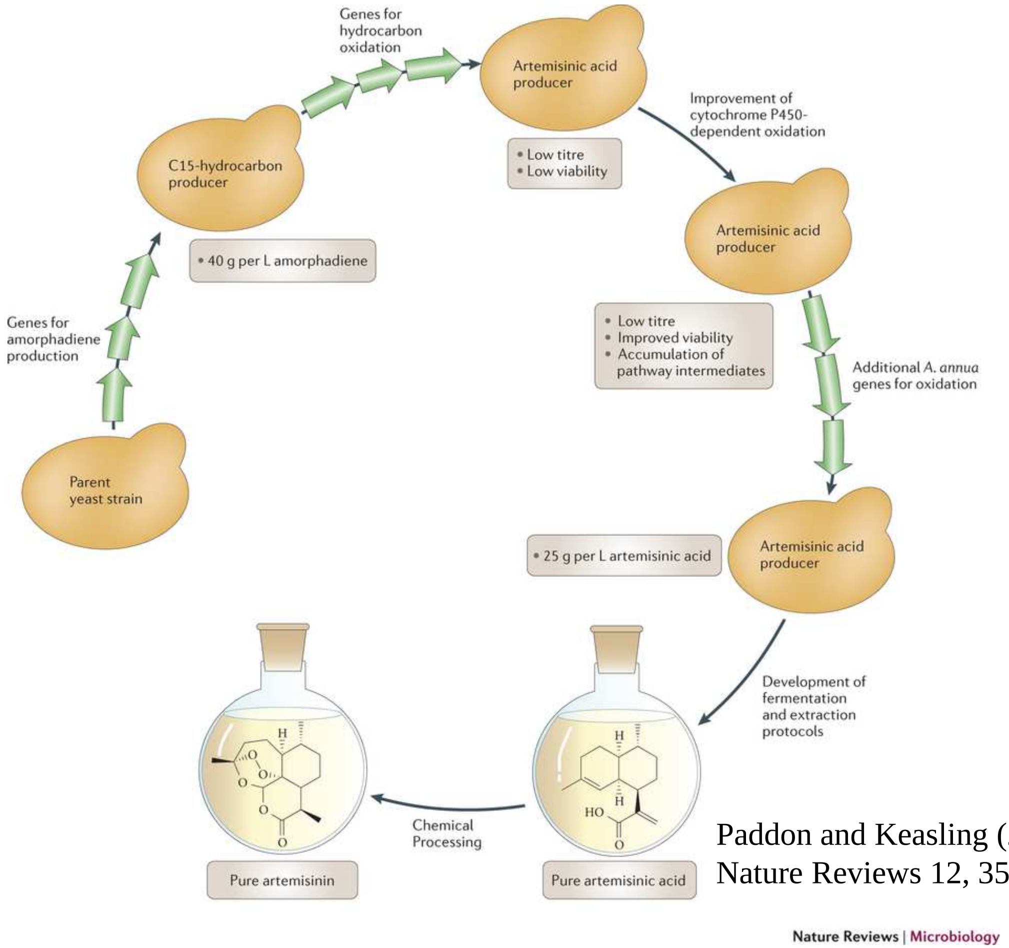
Production of artemisinin precursor in engineered hosts



Paddon and Keasling (2014) Nature Reviews 12, 355-367

Initial stage: engineering of the *Saccharomyces cerevisiae* to produce > 25 g per L amorphadiene (overexpression of nine genes of the mevalonate pathway and expression of the *Artemisia annua* amorphadiene synthase). Subsequent steps: expression of *A. annua* CYP71AV1, its cognate reductase CPR1, cytochrome b_5 and two dehydrogenases to convert amorphadiene to artemisinic acid, which was extracted from fermentation broth and chemically converted to artemisinin. Maximum titre achieved using this procedure was **25 g per L artemisinic acid**.

→ Developments of these systems:10-20 years

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Why not used more often in biotechnology?

Limitations of CYPs	Strategies to overcome them
Low activities	**Protein engineering**
Need for redox partners	**Heterologous partners, peroxide shunt, fusion proteins**
Uncoupling	**Protein engineering**
NAD(P)H limitation	**Cofactor regeneration**
Low substrate solubility	**2-phase systems, co-solvents**
Toxicity (substr. or prod.)	**Alternative host, 2-phase systems**
Selectivity of hydroxylation	**Protein engineering, screening of CYPs and substrates**

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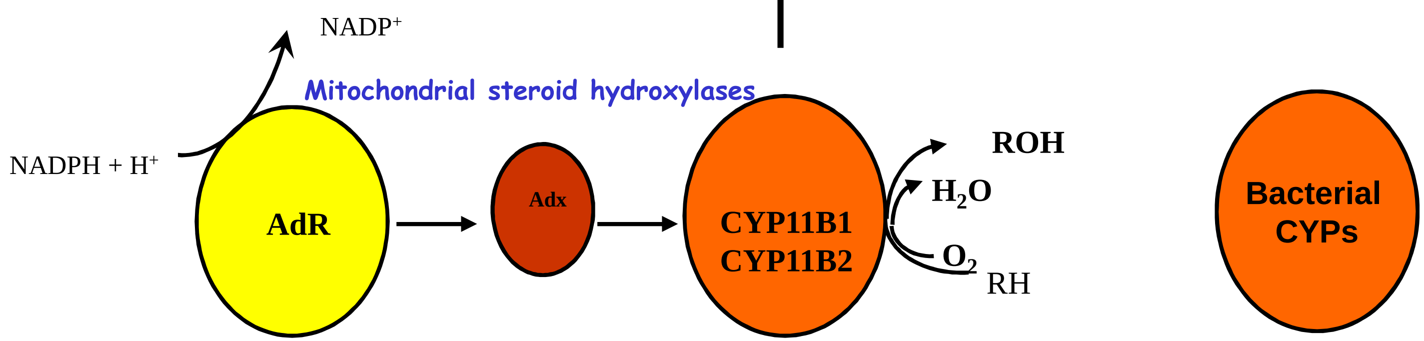
AIM OF OUR STUDIES

Diagnostics:
Molecular genetic analysis of hyperaldosteronism

Sequencing of genes from patients with steroid hydroxylase defects



Rate of catalysis: investigation of protein protein interactions and regulation of intra- and intermolecular electron transfer

Biocatalysis

Structural basis for the stereo- and regioselectivity of steroid hydroxylation

Development of isoenzyme specific inhibitors

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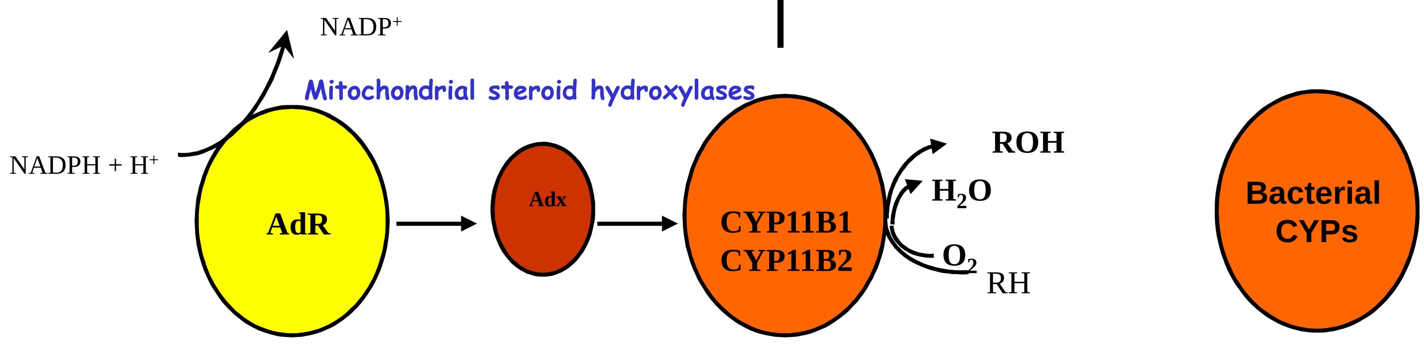

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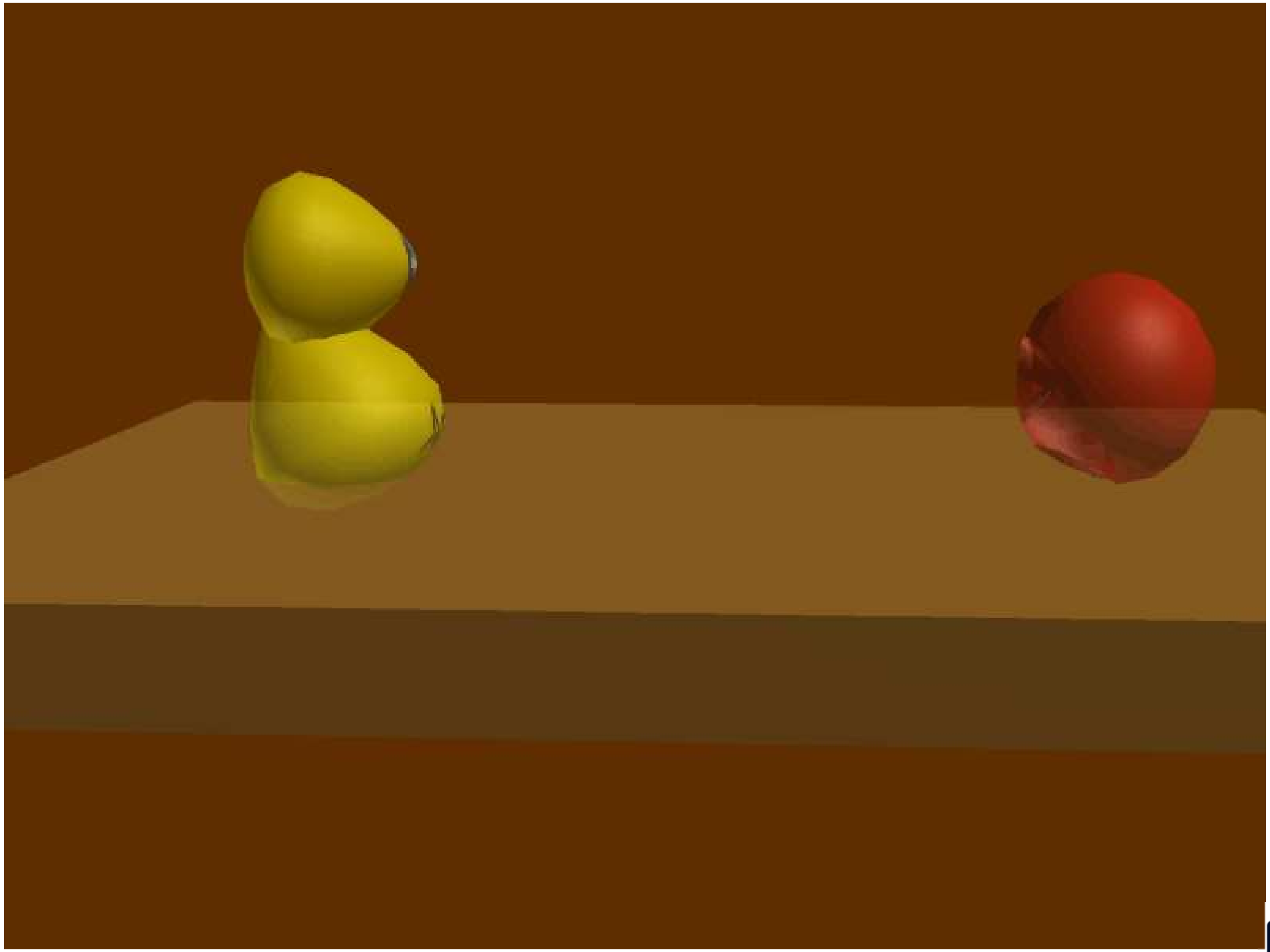
Central role of Adx in mitochondrial ET and steroid biosynthesis



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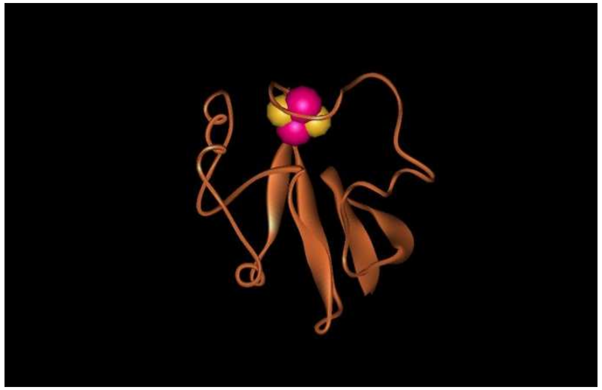
Putidaredoxin: high ET efficiency (TN ~ 3000 min-1)
Adrenodoxin: low ET efficincy (TN ~ 70 min-1)



```
                10        20        30        40        50
Adx:   SSSEDKITVHFINRDGETLTTKGKIGDSLLDVVVQNNLDIDGFGACEGTL
                : ....::.     .     : ::.....: :..   :   :.:.:.
Pdx:         SKVVYVSHDGTRRQLDVADGVSLMQAAVSNGI-YDIVGDCGGSA
                      10        20        30          40

                 60        70        80        90       100
       ACSTCHLIFEQHIFEKLEAITDEENDMLD-LAYGLTDRSRLGCQICLTKAM
       .:.:::   .. . .:. : .. : .::. .. .:. .::: ::: .: ..
       SCATCHVYVNEAFTDKVPAANEREIGMLECVTAELKPNSRLCCQIIMTPEL
           50        60        70 *      80     *  90

              110       120
       DNMTVRVPDAVSDARESIDMGMNSSKIE
       :....: :::
       DGIVVDVPDRQW
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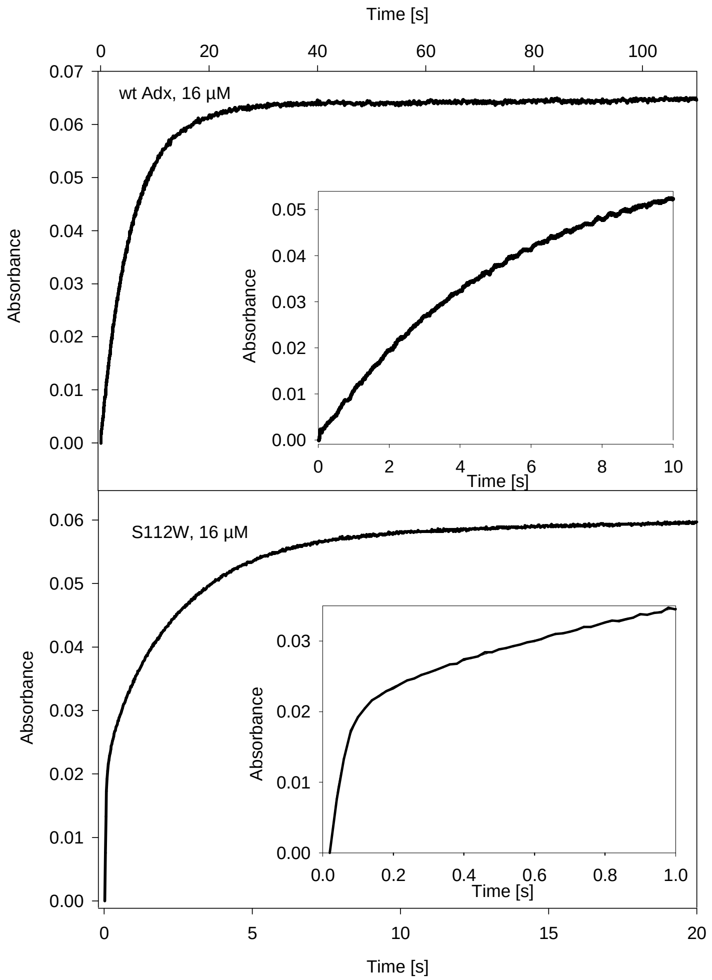
Stopped-flow measurements/Activity measurements



Protein	kcat $s^{-1}*10^{-3}$	Km µM	kcat/Km
CYP11A1			
Adx WT	11.0±0.2	3.24±0.5	3.4
Adx S112W	74.0±4.0	0.36±0.06	**205**
Adx Y82F/S112W	105.0±2.7	0.33±0.02	**318**
CYP11B1			
Adx WT	70.0±2.3	2.39±0.12	29
Adx S112W	97.0±5.8	1.05±0.05	**92**
Adx Y82F/ S112W	107.0±9.1	0.97±0.03	**110**

Truncation and one (two) point mutation(s) (S112W or Y82F/S112W) increase the efficiency of Adx by a factor of 75 (100)

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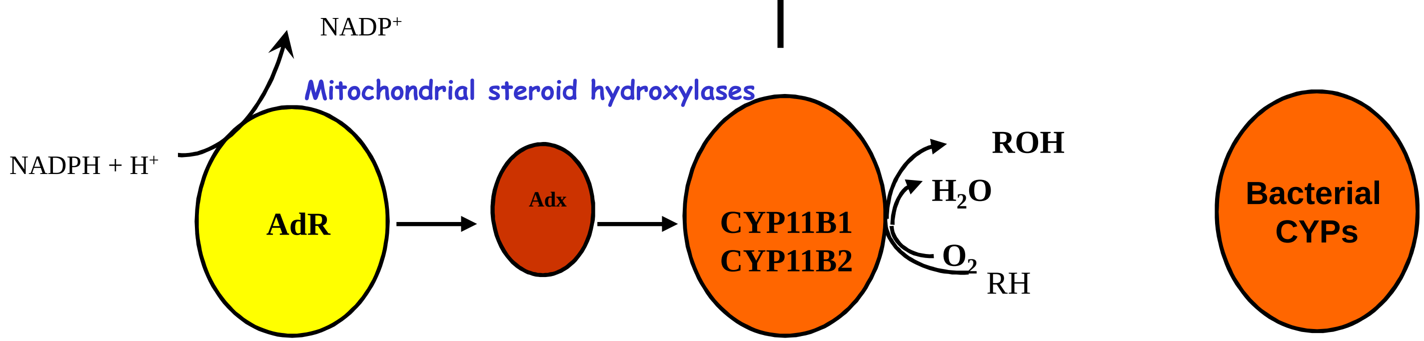

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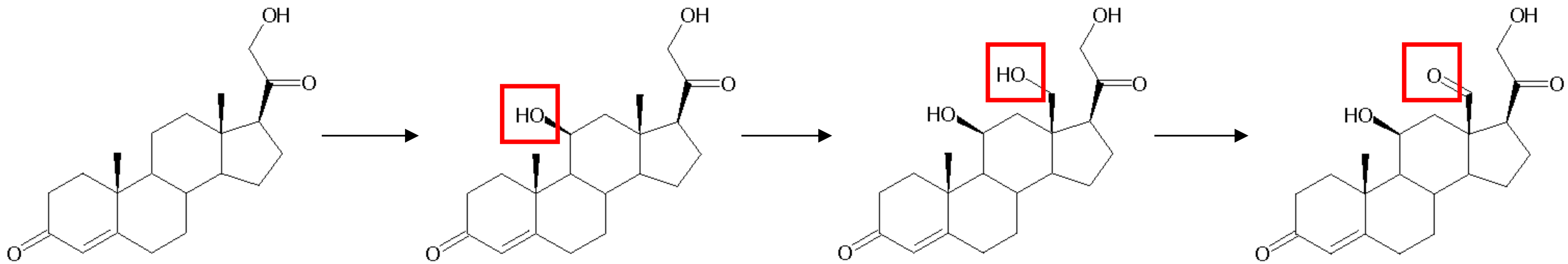
Engineering of mitochondrial steroid hydroxylases

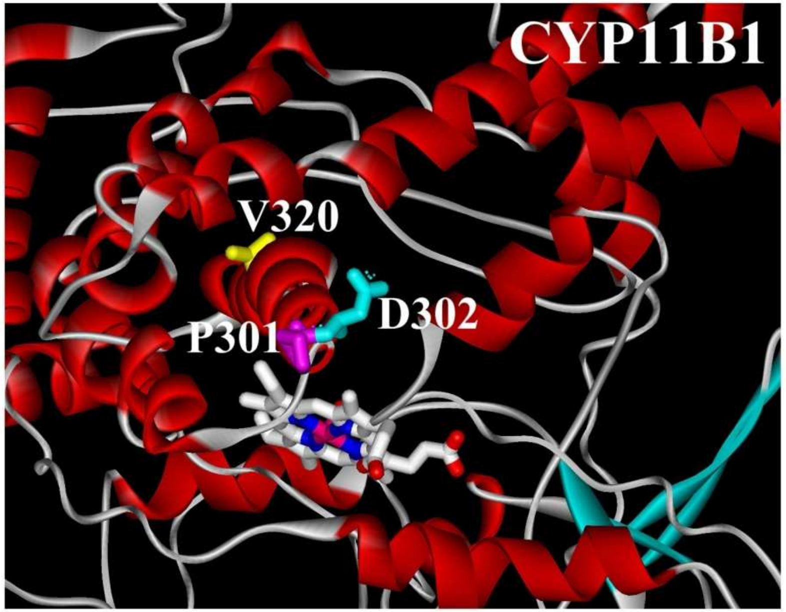
CYP11B1

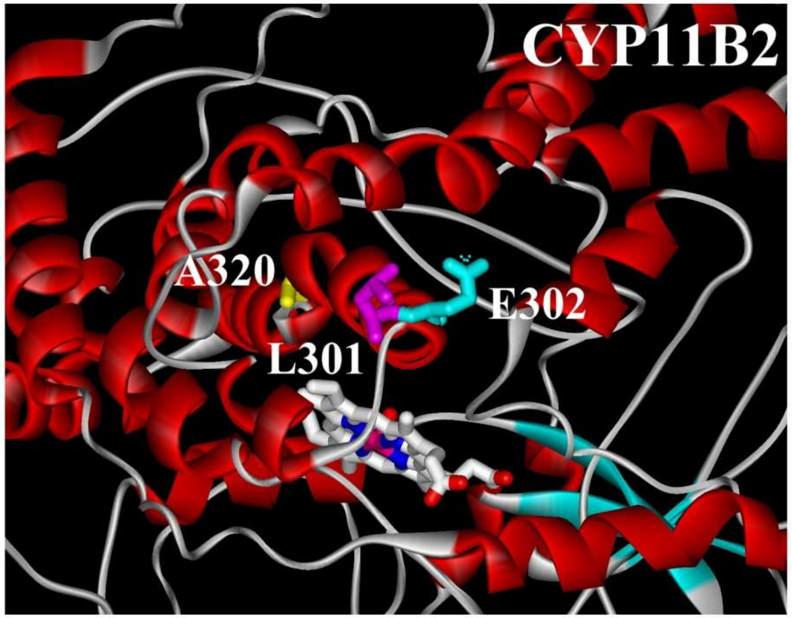
11-Deoxycortisol → Cortisol

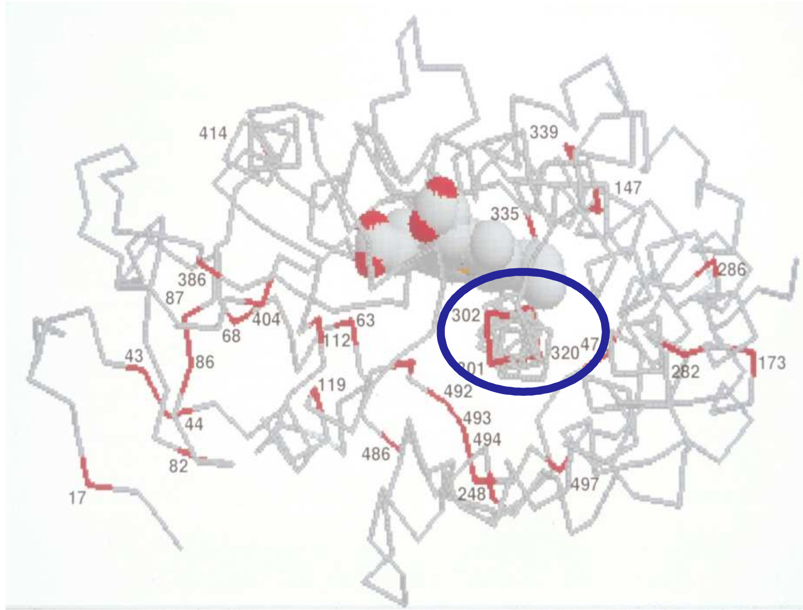
CYP11B2



11-Deoxycorticosterone	Corticosterone	18-Hydroxycorticosterone	Aldosterone
DOC	B	18OH-B	Aldo







→10 single, 6 double, 2 triple mutants
in/close to the the I-helix
produced and analysed

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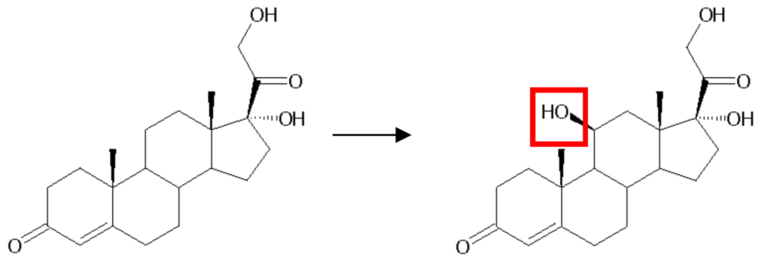
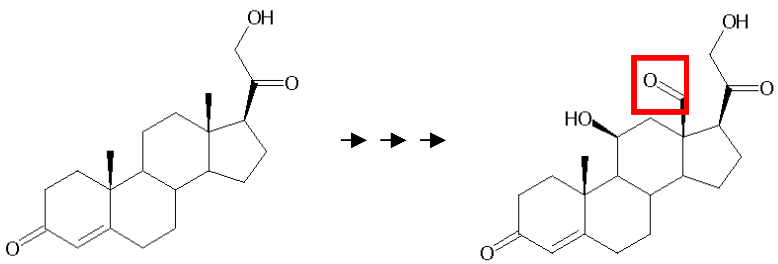
CYP11B1



11-Deoxycortisol → Cortisol

	WT	L301P/E302D/A320V
CYP11B2		
Cortisol	5-10%	85%
Aldosterone	100%	10%

CYP11B2



11-Deoxycorticosterone → Aldosterone

	WT	V320A
CYP11B1		
Cortisol	100%	100%
Aldosterone	0%	20%

We identified regions necessary for substrate selectivity: CYP11B2 got a glucocorticoid-synthesizing activity (like CYP11B1) and CYP11B1 became an aldosterone synthase

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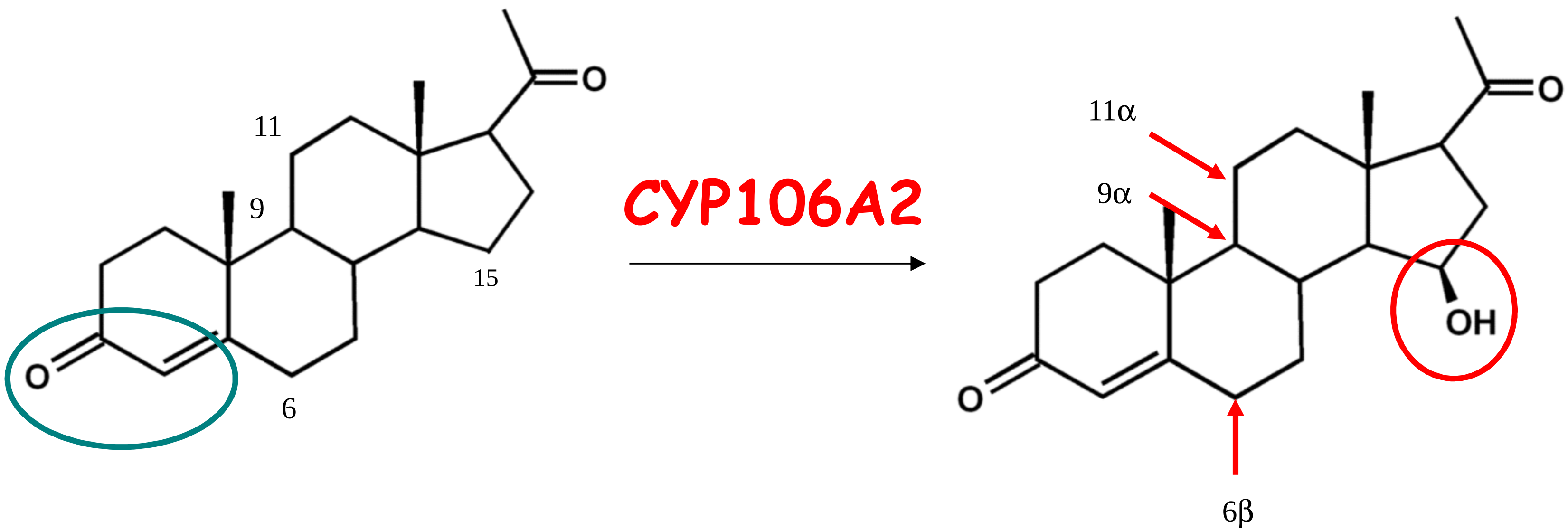
Engineering of mitochondrial steroid hydroxylases



CYP106A2 from *Bacillus megaterium* ATCC 13368

one of the few characterized bacterial steroid converting cytochromes P450

described to hydroxylate 3-oxo-Δ^4-steroids mainly in 15β-position

Goal: increase 11α hdroxylation

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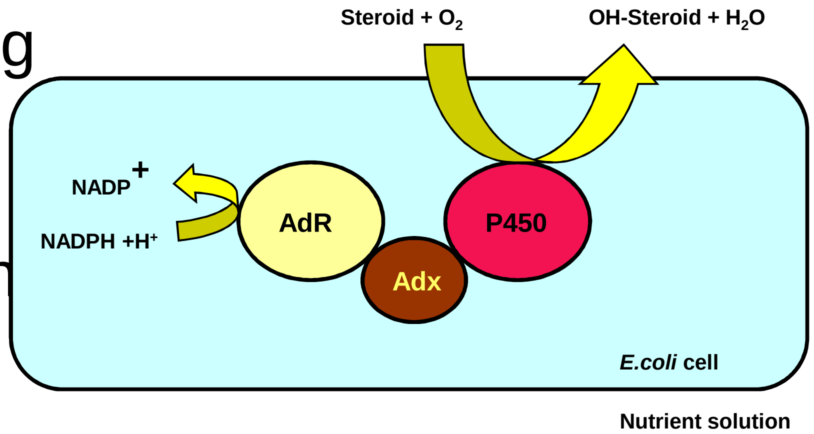
Changing the regio-selectivity of hydroxylation using site-directed and saturation mutagenesis

1) Computer modeling, substrate docking, alignment with CYP11B1, SDM

```
                 β-3-3/β-4-1       β-4-2/β-3-2
CYP106A2 381 AVPSFQLEENLTDSATGQTLTSLPLKASRM 410
CYP11B1  475 TLTQEDIKMVYSFILRPSMFPLLTFRAIN  503
             :::. :::   :     . :. *.::* .
```

2) Creation of a whole cell screening system



3) Creation of mutants by saturation mutagenesis and EP-PCR

4) Analysis of the mutants

5) Improvement of mutants by SDM

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Why not used more often in biotechnology?

Limitations of CYPs	Strategies to overcome them
Low activities	**Protein engineering**
Need for redox partners	**Heterologous partners, peroxide shunt, fusion proteins**
Uncoupling	**Protein engineering**
NAD(P)H limitation	**Cofactor regeneration**
Low substrate solubility	**2-phase systems, co-solvents**
Toxicity (substr. or prod.)	**Alternative host, 2-phase systems**
Selectivity of hydroxylation	**Protein engineering, screening of CYPs and substrates**

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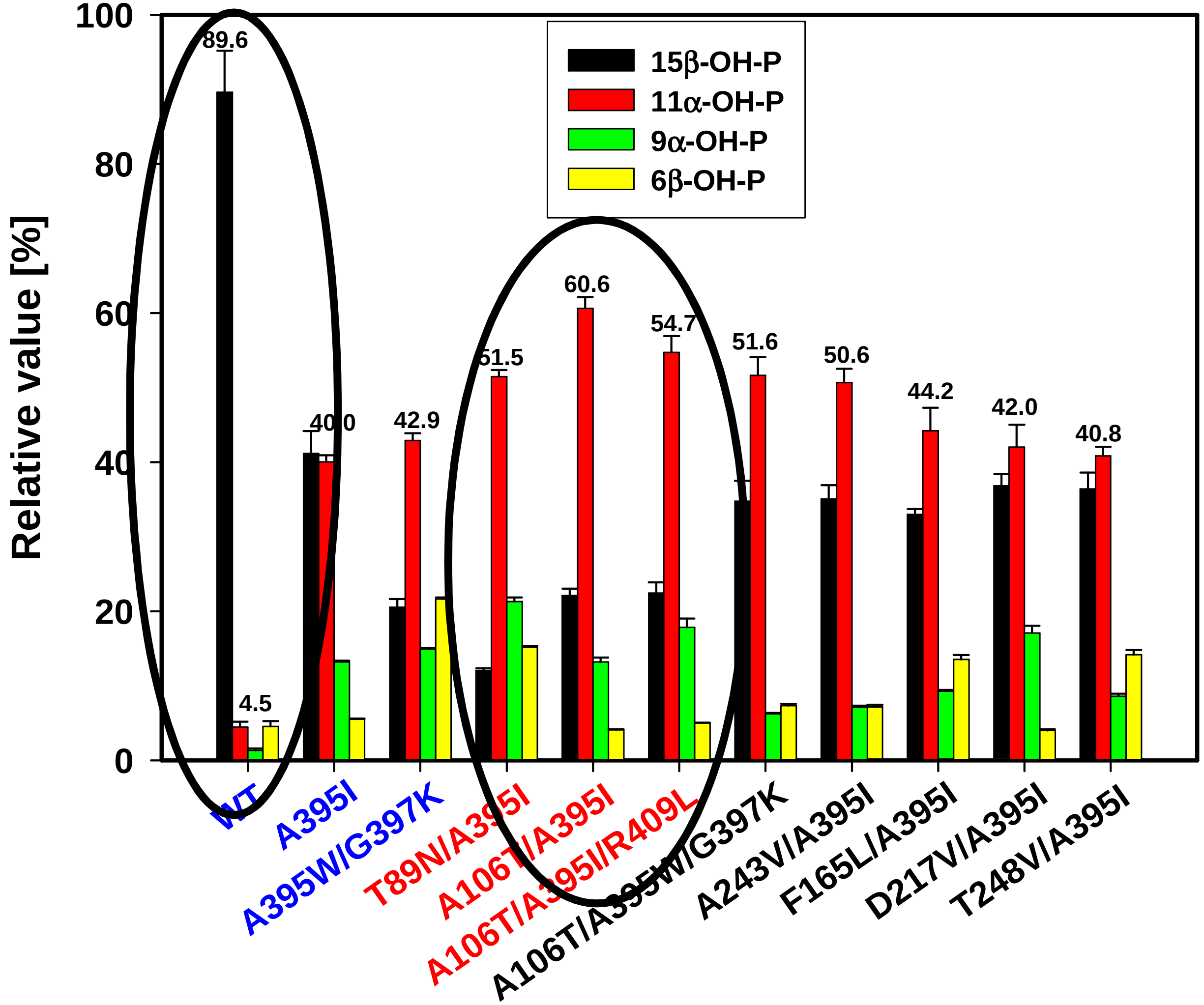
Regio-selectivity of CYP106A2 mutants towards progesterone



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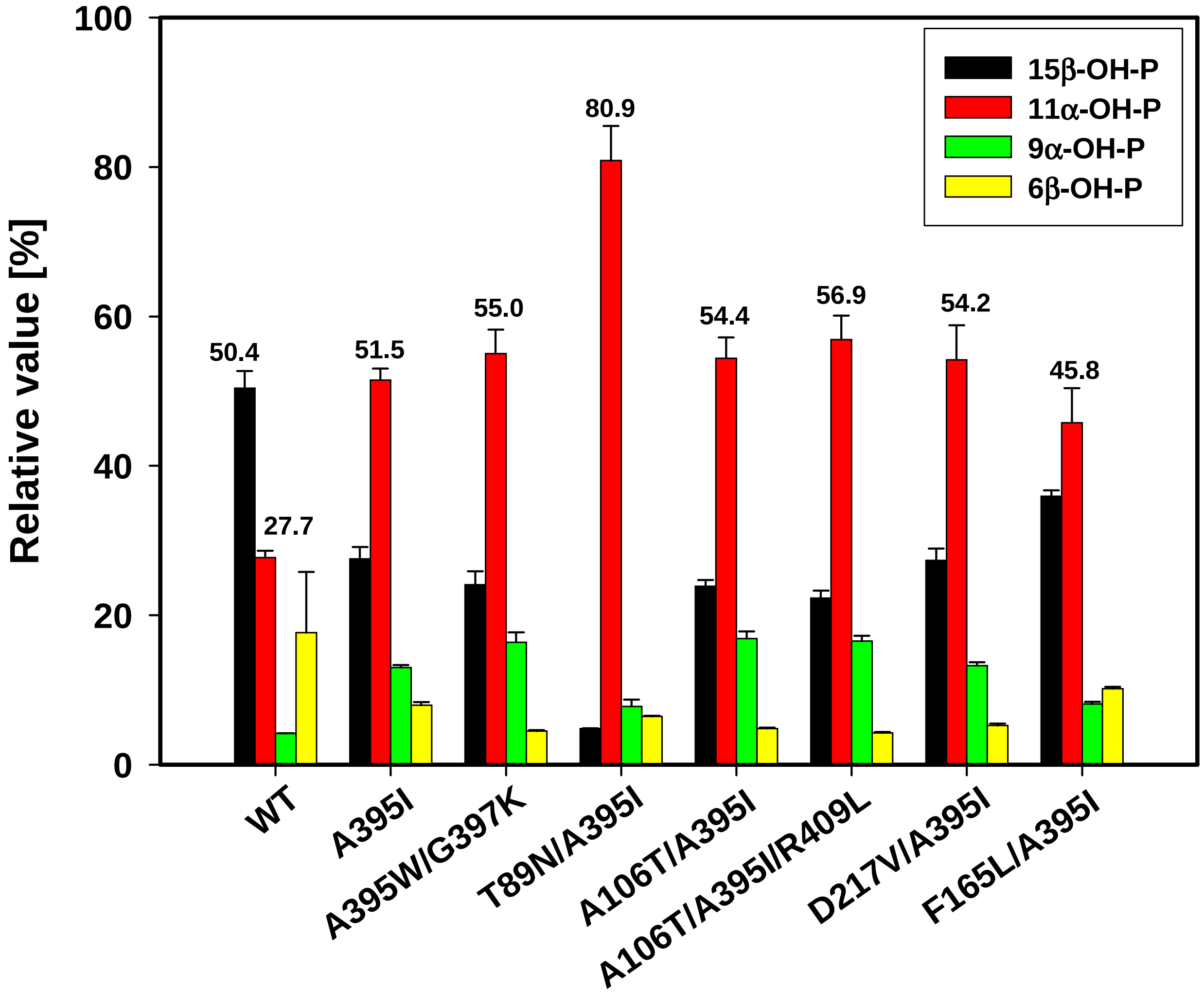
Regio-selectivity of CYP106A2 mutants towards progesterone conversion using a whole-cell system



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Selectivity of hydroxylation	Protein engineering, **screening of CYPs and substrates**

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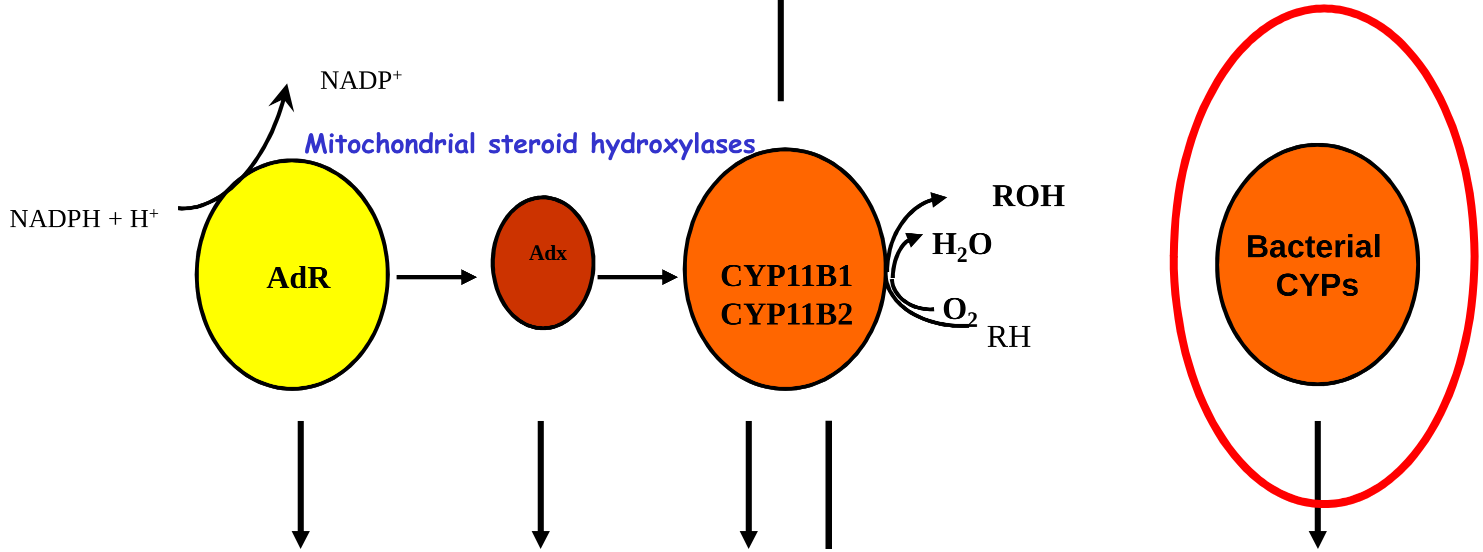
AIM OF OUR STUDIES

Diagnostics:
Molecular genetic analysis of hyperaldosteronism

Sequencing of genes from patients with steroid hydroxylase defects



Rate of catalysis: investigation of protein protein interactions and regulation of intra- and intermolecular electron transfer

Biocatalysis

Structural basis for the stereo- and regioselectivity of steroid hydroxylation

Development of isoenzyme specific inhibitors

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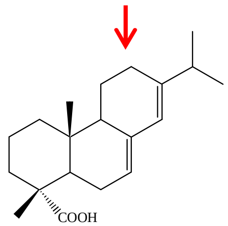
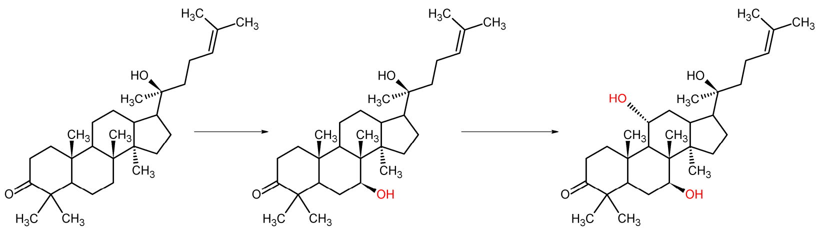
CYPs from *Bacillus megaterium*

CYP106A2 (ATCC 13368)
Steroid 15beta hydroxylation
Shift to 11alpha hydroxylation

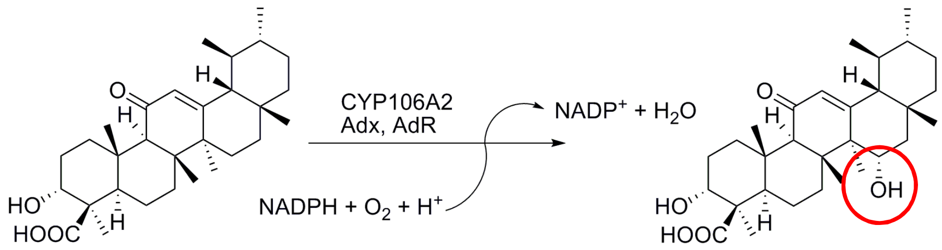


Abietic acid

Expanding substrate space:
abietic acid,
dipterocarpol,
11-keto boswellic acid



Dipterocarpol   7β-hydroxydipterocarpol   7β,11α-hydroxydipterocarpol



KBA   15α-hydroxy-KBA

CYPome from MS941
CYP106A1
CYP109
CYP109
CYP-BM3 analogue

→ New substrates and products found
3D of CYP106A2 ready, 1 CYP109: diffracting crystals

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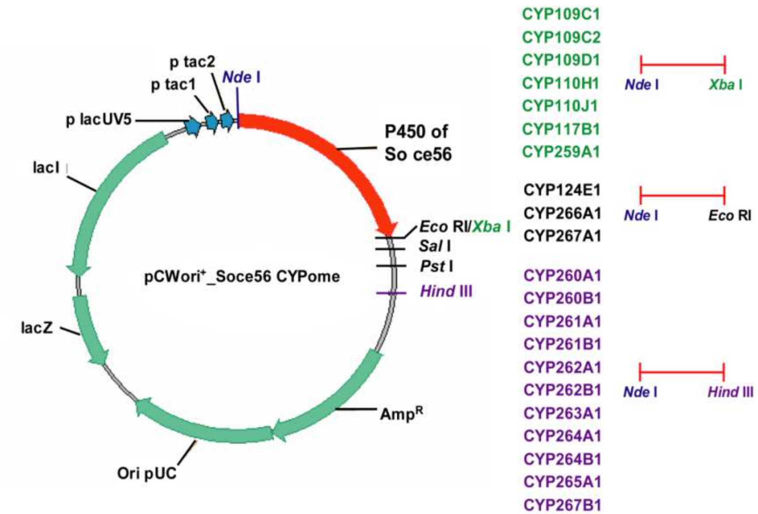
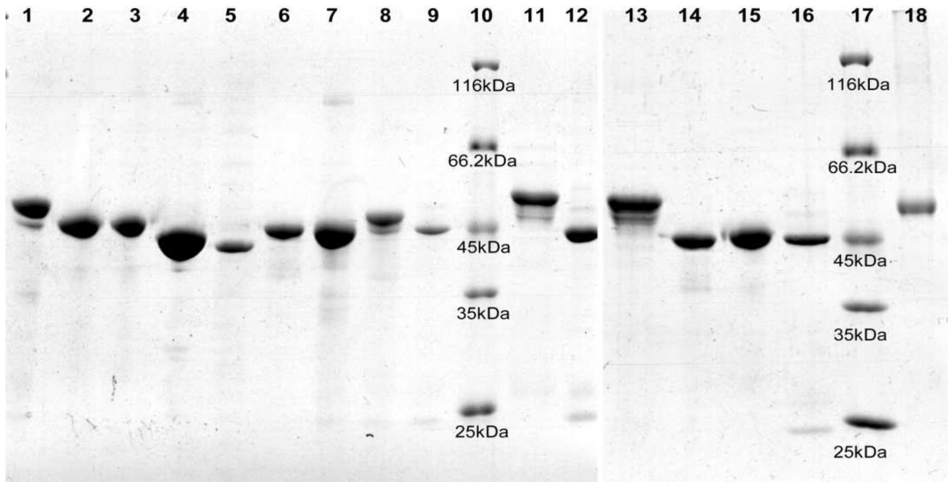
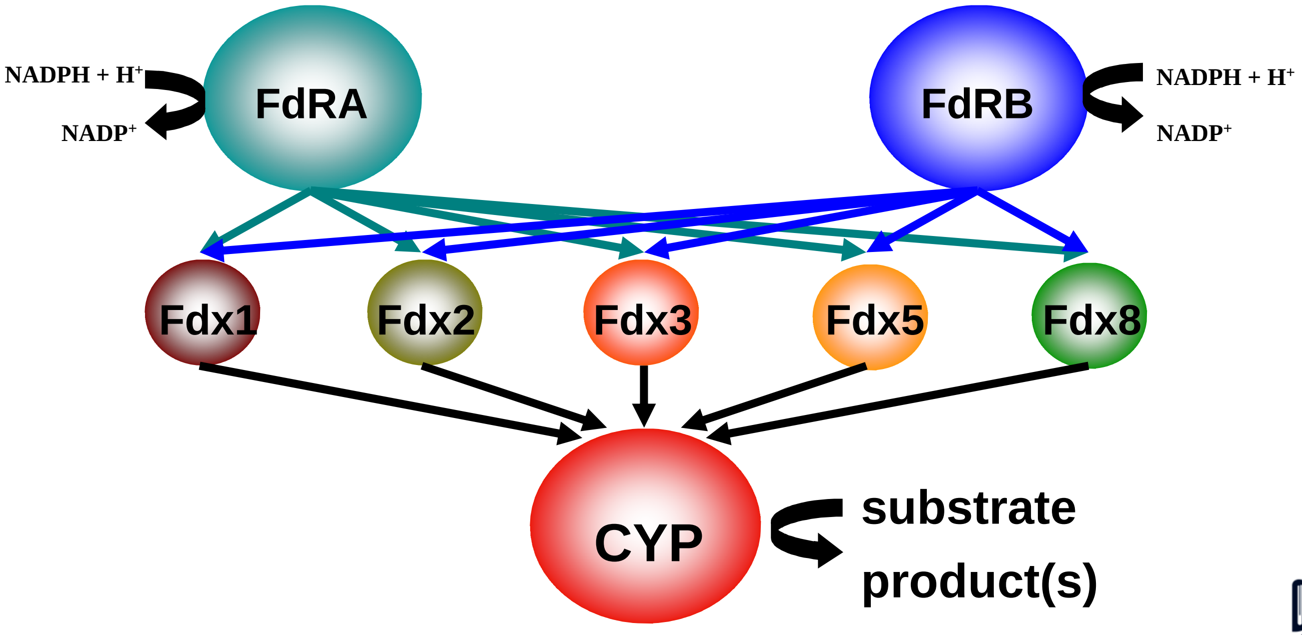
Cloning and expression of the Soce 56 CYPome







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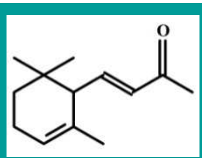
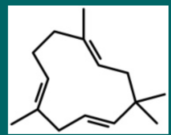
Examples of substrates for myxobacterial CYPs

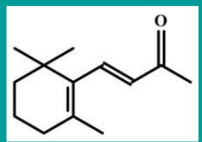
Norisoprenoids | **Sesquiterpenes**



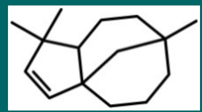
α-ionone



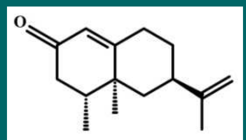
humulene



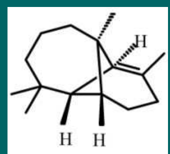
β-ionone



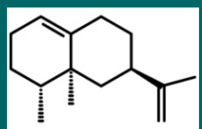
clovene



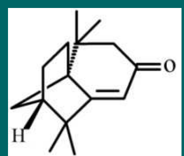
nootkatone



α-longipinene



valencene



isolongifolene-9-one

3D of one CYP ready, 2 more nearly ready → rational design

Different steroids

Different drugs

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Why not used more often in biotechnology?

Limitations of CYPs	Strategies to overcome them
Low activities	Protein engineering
Need for redox partners	Heterologous partners, **peroxide shunt, fusion proteins**
Uncoupling	**Protein engineering**
NAD(P)H limitation	**Cofactor regeneration: ADH co-expressed**
Low substrate solubility	**co-solvents: cyclodextrins**
Toxicity (substr. or prod.)	**Alternative host: Bacillus for terpenes**
Selectivity of hydroxylation	Protein engineering, screening of CYPs and substrates

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Summary bacterial CYPs

1) Two CYPomes available: all CYPs as well as redox partners cloned and expressed

2) Novel substrates identified (steroids, terpenes, fatty acids)

3) New products identified (NMR)

4) Novel reaction types found

5) Whole-cell systems developed in *E. coli* and *B. megaterium* (toxicity of terpenes in *S.cerevisiae*)

6) NADPH regeneration provided in whole cells

7) Efficient ways to solubilize hydrophobic substrates

→ Broad applicability for degradation of products in soil as well as for biotechnological application

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Evolution creates novel CYPs via adaptation to environment

From: Bernhardt and Urlacher, Appl.Microbiol.Biotech., in press

Perspectives for CYPs

Genome mining
Enzyme engineering
Recombinant whole-cell systems
Synthetic biology
Chemo-enzymatic processes
Cascade multi-enzymes reactions

Advantages of CYPs

Activation of O_2
Oxidation of inert C-atoms
Regio- and stereoselectivity
Broad substrate spectrum
Different reaction types

Limitations of CYPs

Low activities
Need for redox partners
Uncoupling
NAD(P)H limitation
Low substrate solubility

Thank you for your attention

BMBF, DBU, DFG, EU, Saarland

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