16th International Symposium

CYTOCHROME P450
BIODIVERSITY & BIOTECHNOLOGY

23 - 27 June 2024
Torino, Italy

Conference Programme and Book of Abstracts

Accademia delle Scienze di Torino
1783

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Previous Meetings

2023	Copenhagen, Denmark
2018	York, UK
2016	Vancouver, Canada
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2012	Torino, Italy
2010	Woods Hole, USA
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REGISTRATION AND OPENING LECTURE, VENUE: ACCADEMIA DELLE SCIENZE
Sala dei Mappamondi
Via Accademia delle Scienze, 6 – 10123 Torino

MAIN CONFERENCE VENUE: TEATRO VITTORIA
Via Antonio Gramsci, 4 – 10123 Torino

Sunday June 23

Registration and opening

4:30 PM	**Registration** Venue: Accademia delle Scienze Location: Sala dei Mappamondi Via Accademia delle Scienze, 6 – 10123 Torino
5:00 PM	**Welcome address** Prof. Daniele Castelli, Direttore della Classe di Scienze Fisiche, Matematiche e Naturali, *Accademia delle Scienze di Torino* Dr.ssa Chiara Mancinelli, Cancelliera dell'*Accademia delle Scienze di Torino* Prof. Gianfranco Gilardi, *Accademia delle Scienze di Torino* e *Università di Torino*
5:15 PM	**OPENING KEYNOTE LECTURE** **Impact of P450s on the green transition** Birger Lindberg Møller *University of Copenhagen, Denmark*
6:00 PM	**Welcome reception**

Monday June 24

Morning sessions

Session 1: Bioengineering and biotechnology Session Chair: Sheila Sadeghi	
9:00 AM	**Looking back to the future of P450 biotechnology: ancestral P450s from across the biosphere as bio-bricks for synthetic biology** Elizabeth Gillam *University of Queensland, Australia*
9:20 AM	**Structure-function analysis and protein engineering of CYP105A1 for its practical uses** Toshiyuki Sakaki *Toyama Prefectural University, Japan*
9:40 AM	**Towards understanding the mechanism of action of P450 bioactivated nematicides** Peter Roy *University of Toronto, Canada*
10:00 AM	**Evolution of a 3DM structure-based class specific molecular information system of the cytochrome P450 monooxygenases** Martie Smit *University of the Free State, South Africa*
10:20 AM	Coffee break

Session 2: Plant and insects (A) Session Chair: Toshiyuki Sakaki	
11:00 AM	**Characterization of biosynthesis of solanoeclepin B, a novel hatching factor for potato cyst nematode** Masaharu Mizutani *Kobe University, Japan*
11:20 AM	**P450s evolution in the green lineage: the functions essential for plant terrestrialization** Danièle Werck-Reichhart *CNRS, University of Strasbourg, France*
11:40 AM	**Inactivation of cytochrome P450s involved in sesquiterpene lactone biosynthesis to accumulate medicinal terpenes in chicory taproots (*Cichorium intybus* L.)** Katarina Cankar *Wageningen University and Research, The Netherlands*
12:00 PM	**Structure-activity-relationships of triterpenoid saponins** Søren Bak *University of Copenhagen, Denmark*
12:20 PM	**Flash Presentations** Angela Hayward – *University of Exeter, United Kingdom* Sabrina Helmy Aly – *University of Torino, Italy* Tea Kuvek – *University of BOKU, Austria* Yana Toporkova – *Kazan Institute of Biochemistry and Biophysics, Russia*
12:55 PM	Lunch and Poster

Monday June 24

Afternoon sessions

Session 3: Plant and insects (B) Session Chair: Joerg Bohlmann	
2:30 PM	**Mosquito P450s; looking back at insecticide resistance marker identification and forwards to accelerating insecticide discovery** Mark Paine *Liverpool School of Tropical Medicine, United Kingdom*
2:50 PM	**Structural diversification of strigolactones driven by cytochrome P450s** Takatoshi Wakabayashi *The University of Tokyo, Japan*
3:10 PM	**Redox partners and electron transfer chains of cytochrome P450 systems in phenylpropanoid-lignin biosynthesis** Chang-Jun Liu *Brookhaven National Laboratory, USA*
3:30 PM	Coffee break

Session 4: Plant and insects (C) Session Chair: Giovanna Di Nardo	
4:05 PM	**The anti-diabetic metabolite Montbretin-A** Joerg Bohlmann *University of British Columbia, Canada*
4:25 PM	**Catalytic site constraints in *Camptotheca* p450s mediating alkaloid synthesis** Mary Schuler *University of Illinois Urbana, USA*
4:45 PM	**CYP5164B1: a key enzyme in the oxylipin pathway during a brown algal host-endophyte interaction** Gabriel Markov *Sorbonne Université, CNRS, France*
5:05 PM	**Exploring P450 superfamily diversity with P450Atlas - online tool for automated subfamily assignment** Dominik Gront *University of Warsaw, Poland*
5:20 PM	**Discovering new cytochromes P450 from cyanobacteria** Danilo Correddu *University of Torino, Italy*
5:35 PM 7:00 PM	Poster session

Tuesday June 25

Morning sessions

Session 5: Green catalytic processes Session Chair: Vlada Urlacher	
9:00 AM	**Investigating novel P450 function in ribosomal biarylitide biosynthesis pathways** Max Cryle *Monash University, Australia*
9:20 AM	**Effect of flexibility in P450 chimeric proteins** Gianluca Catucci *University of Torino, Italy*
9:40 AM	**Biocatalytic potential of fungal CYP505s** Dirk Opperman *University of the Free State, South Africa*
10:00 AM	**Cytochrome P450 enzyme family and paclitaxel biosynthesis in *Taxus*** Jianbin Yan *Chinese Academy of Agricultural Sciences, China*
10:20 AM	Coffee break

Session 6: P450 and non-P450 cascade reactions Session Chair: Amit Pandey	
11:00 AM	**P450s for lignan biosynthesis in recombinant *E. coli*** Vlada Urlacher *Heinrich-Heine University Düsseldorf, Germany*
11:20 AM	**Design of a cascade reaction for lignin valorisation** Giovanna Di Nardo *University of Torino, Italy*
11:40 AM	**Biosynthesis and diversification of complex triterpenes in plants** Zhenhua Liu *Shanghai Jiao Tong University, China*
12:00 PM	**Molecular interactomics of CYPs with other enzymes** Andrei Gilep *Institute of Bioorganic Chemistry, NASB, Belarus*
12:20 PM	**Flash Presentations** Jemma Gullick – *Monash University, Australia* Daniele Giuriato – *University of Torino, Italy* Yuki Sugai – *Nagoya University, Japan* Francesca Fata – *National Research Council (CNR), Italy*
12:55 PM	Lunch and Poster

Tuesday June 25

Afternoon

Social event	
4:00 PM	**Guided tour of the historical Orto Botanico of the University of Torino** Meeting point: in front of the **Castello del Valentino** Location: Viale Mattioli, 25 – 10125 Torino
6:00 PM	**Aperitivo**

Wednesday June 26

Morning sessions

Session 7: Biodiversity and Evolution Session Chair: Elizabeth Gillam	
9:00 AM	**The Tao of P450: grasping the 10,000 things** David Nelson *University of Tennessee Health Science Center, USA*
9:20 AM	**A mitochondrial CYP gene mediates the secondary metabolic resistance mechanism to ethiprole in brown planthopper *Nilaparvata lugens*** Bartlomiej Troczka *University of Exeter, United Kingdom*
9:40 AM	**Functional significance of P450 diversity in herbivorous rodents** Denise Dearing *University of Utah, USA*
10:00 AM	**Molluscan cytochromes P450** Jed Goldstone *Woods Hole Oceanographic Institution, USA*
10:20 AM	Coffee break

Session 8: Industrial Applications Session Chair: Gianfranco Gilardi	
11:00 AM	**Biosynthesis and engineering of plant tropane alkaloids hyoscyamine and cocaine** Sheng-Xiong Huang *Kunming Institute of Botany, CAS, China*
11:20 AM	**Cytochrome P450 engineering with mutability landscapes** Carlos Acevedo-Rocha *The Novo Nordisk Foundation Center for Biosustainability, Technical University Denmark, Denmark*
11:40 AM	**Repurposing a P450 reductase with [FeFe]-hydrogenase and BVMO as non-physiological partners for H_2-dependent NADPH regeneration in indigoids production** Francesca Valetti *University of Torino, Italy*
12:00 PM	**Selective C-H functionalization via P450-catalyzed abiological group transfer reactions** Rudi Fasan *University of Texas at Dallas, USA*
12:20 PM	**Flash Presentations** Elyse Frydendall – *University of Michigan, USA* Melissa De Angelis – *University of Torino, Italy* Lauren Murray – *Monash University, Australia* George Paul Voicu – *University of Torino, Italy*
12:55 PM	Lunch and Poster

Wednesday June 26

Afternoon sessions

Session 9: New Structural-Functional Insight (A) Session Chair: James De Voss	
2:30 PM	**50 Years working on cytochrome P450: What have we learned?** Stephen Sligar *University of Illinois, USA*
2:50 PM	**Rapid determination of cytochrome P450 binding affinities and their application in probing P450 structure/function** Emily Scott *University of Michigan, USA*
3:10 PM	**Crystal structure of CYP85A3, another key enzyme in brassinosteroid biosynthesis** Shingo Nagano *Tottori University, Japan*
3:30 PM	Coffee break

Session 10: New Structural-Functional Insight (B) Session Chair: Stephen Sligar	
4:05 PM	**C-C and C-N bond formation by cytochrome P450s** Thomas Makris *North Carolina State University, USA*
4:25 PM	**Unusual structural features of two CYP51s: a pathogenic amoeba and deep-water fish** Galina Lepesheva *Vanderbilt University, USA*
4:45 PM 6:00 PM	Poster session

SOCIAL DINNER	
8:00 PM	**Restaurant Esperia – Club Circolo Canottieri** Location: Corso Moncalieri, 2 – 10131 Torino

Thursday June 27

Morning sessions

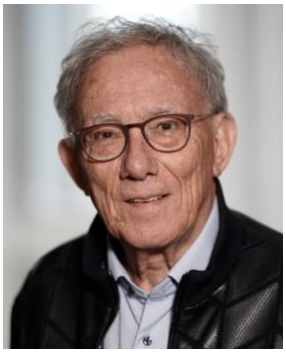
Session 11: Microbial and Fungal P450s Session Chair: Rita Bernhardt	
9:30 AM	**Thermophilic cytochromes P450 from an Australian hot spring** James De Voss *University of Queensland, Australia*
9:50 AM	**An ancient key player in steroidogenesis: The X-ray protein structure of the ancestral cholesterol metabolizing cytochrome P450** Simone Brixius-Anderko *University of Pittsburgh, USA*
10:10 AM	**Biochemical and genetic understanding of the role(s) of cytochromes P450 in giant viruses** David Lamb *Swansea University, United Kingdom*
10:30 AM	**P450-mediated oxidations trigger a unique non-enzymatic oxidation cascade completing celastrol biosynthesis** Yong Zhao *University of Copenhagen, Denmark*
10:50 AM	Coffee break

Session 12: P450-mediated Interactions Session Chair: Jed Goldstone	
11:30 AM	**Distinguishing the functions of canonical strigolactones through disruption of the rice CYP711A (MORE AXILLARY GROWTH 1) homologues** Jian You Wang *King Abdullah University of Science and Technology, Kingdom of Saudi*
11:50 AM	**Dual factors required for cytochrome P450 mediated hydrocarbon ring contraction in bacterial gibberellin phytohormone biosynthesis** Raimund Nagel *Leipzig University, Germany*
12:10 PM	**Variations in cytochrome P450 reductase (POR) and adrenodoxin reductase (FDXR) regulate P450 mediated interactions and metabolism** Amit Pandey *University of Bern, Switzerland*

12:30 PM	**CLOSING KEYNOTE LECTURE** John Stegeman *Woods Hole Oceanographic Institution, USA*
13:00 PM	**Concluding remarks** Rita Bernhardt - *Saarland University, Germany* Gianfranco Gilardi - *University of Torino, Italy*

OPENING KEYNOTE LECTURE

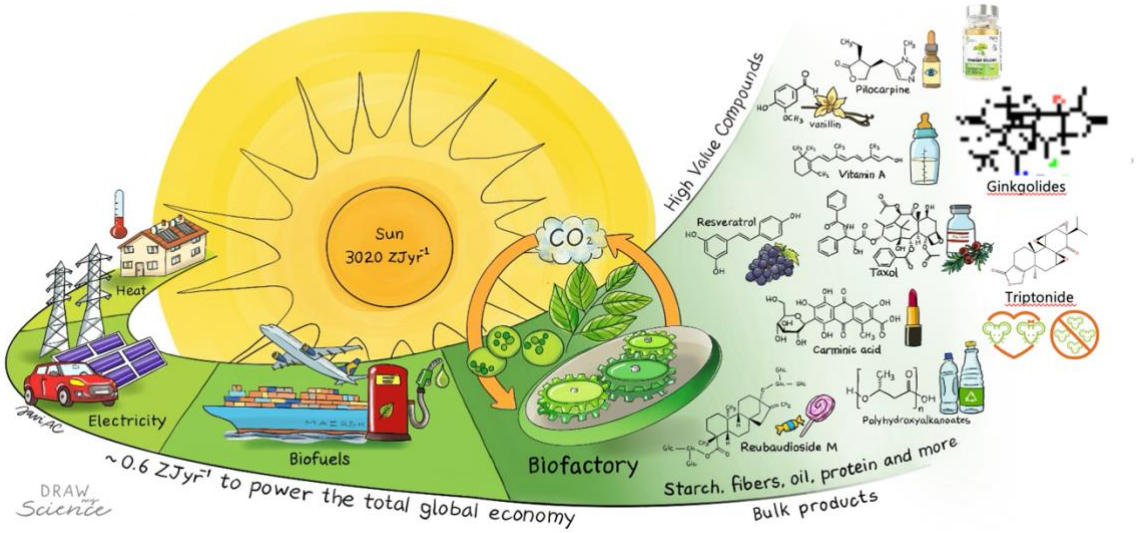
Circular biomanufacturing based on solar energy and use as CO_2 as the sole carbon source



Birger Lindberg Møller

University of Copenhagen,Denmark

Nearly all energy on our Earth comes from the Sun. No wonder, that the large scale sustainable production system chosen by Nature is photosynthesis, a process driven by solar light and enabling plants and algae to produce foods and materials with carbon dioxide as the sole carbon source. Cytochrome P450s are key players present in all kingdoms and add an additional layer of uniqueness to the production system. We should adhere to and be guided by Nature in our efforts to develop environmentally benign green production systems for the future. Examples of basic and applied research along these lines at our lab will be presented in the lecture to set the stage for the great presentations at the International Symposium on Cytochrome P450 Biodiversity and Biotechnology here in Torino.



Session 1: Bioengineering and biotechnology

Session chair: Sheila Sadeghi

Looking back to the future of P450 biotechnology: ancestral P450s from across the biosphere as bio-bricks for synthetic biology

Elizabeth M.J. Gillam

School of Chemistry and Molecular Biosciences, The University of Queensland, St. Lucia, Brisbane, Australia, 4072.

For many years, directed or artificial evolution has provided the principal means of engineering P450s for biotechnology. However recent studies have revealed the value of exploring the natural evolutionary history of P450s for inspiration, in terms of both the templates and methods for engineering catalysts for novel applications. Ancestral sequence reconstruction can be used to resurrect ancestors that are markedly more thermostable and solvent tolerant than their extant counterparts, yet retain similar catalytic activities and substrate specificity. These properties make ancestral P450s robust templates for diverse applications in the emerging field of synthetic biology. This presentation will show how ancestral P450s can be used as modular bio-bricks for creating novel bioreactors and exploiting non-natural redox support systems, using examples drawn from reconstructions of plant, animal and microbial P450 families.

Session 1: Bioengineering and biotechnology

Session chair: Sheila Sadeghi

Structure-function analysis and protein engineering of CYP105A1 for its practical uses

Toshiyuki Sakaki[1], Sachiyo Yoneda[1], Yuya Yogo[1], Moeka Wada[2], Teisuke Takita[2], Bunzo Mikami[2], Kiyoshi Yasukawa[2], and Kaori Yasuda[1]

1. Department of Pharmaceutical Engineering, Toyama Prefectural University, Imizu, Japan,
2. Graduate School of Agriculture, Kyoto University, Kyoto, Japan

We determined a crystal structure of *Streptomyces griseolus* CYP105A1[1]. Substrate-binding pocket of CYP105A1 contains three Arg residues Arg73, 84, and 193, which might play essential roles in the substrate recognition. As expected, substitution of Arg193 to Ala significantly reduced the hydroxylation activity towards vitamin D_3. In contrast, R73A and R84A showed much higher activity than the wild type, suggesting that these Arg residues give an inhibitory effect on the activity. Based on these results, we constructed double variants at 73 and 84 positions[3]. Of all the variants tested, both R73A/R84A and R73V/R84A showed the highest 25-hydroxylation activity for 1□-hydroxyvitamin D3 and 1□-hydroxylation activity for 25-hydroxyvitamin D_3, and the k_{cat}/K_m values of both variants were approximately 400- and 100-fold higher than the wild type enzyme, respectively[2].

CYP105A1 also metabolizes various non-steroidal anti-inflammatory drugs (NSAIDs), and showed 4′-hydroxylation activity towards diclofenac, mefenamic acid, flufenamic acid, tolfenamic acid, and meclofenamic acid. It should be noted that this reaction specificity was similar to that of human CYP2C9. Substitution of Arg at position 73 with Ala in CYP105A1 dramatically reduced the hydroxylation activity toward diclofenac, flufenamic acid, and ibuprofen, indicating that Arg73 is essential for the hydroxylation of these substrates. In contrast, substitution of Arg84 with Ala remarkably increased the hydroxylation activity towards diclofenac, mefenamic acid, and flufenamic acid[3].

Recently, we found that CYP105A1 had a hydroxylation activity towards statins such as mevastatin, lovastatin, and simvastatin. The R84A variant showed a significantly higher hydroxylation activity towards the statins than WT. Some metabolites appear to be novel compounds, while some metabolites appear to coincide with those produced by CYP3A4. These results suggest that CYP105A1 and its variants are useful for producing metabolites of various drugs in the human body. Furthermore, the new metabolites may lead to drug discovery.

1- Sugimoto H et al., Biochemistry 47, 4017-4027 (2008)
2- Hayashi K et al., Biochemistry 47, 11964-11972 (2008)
3- Yogo Y et al., Drug Metab. Pharmacokinet., 45, 100455 (2022)

Session 1: Bioengineering and biotechnology

Session chair: Sheila Sadeghi

Towards understanding the mechanism of action of P450 bioactivated nematicides

Jessica Knox[*1,2], Andrew R. Burns[*1,2], Nazli H. Farshour[1], Brittany Cooke[1,2], Savina R. Cammalleri[1,2], Megan Kitner[3], Jack M.P. Castelli[1,2], Emily Puumala[1], Jamie Snider[2], Igor Stagljar[1,2], Leah E. Cowen[1], Inga Zasada[3] and Peter J. Roy[1,2,4]

1. Department of Molecular Genetics, University of Toronto, Toronto, ON
2. Terrence Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, Toronto, ON
3. United States Department of Agriculture - Agricultural Research Service (USDA-ARS) Horticultural Crops Research Laboratory, Corvallis, OR
4. Department of Pharmacology and Toxicology, University of Toronto, Toronto, ON

One of our areas of interest is the identification and characterization of compounds that kill parasitic nematodes. Towards this end, we previously identified 67 small molecule scaffolds with nematode selectivity (Burns *et al.*, 2015). From these nematicidal candidates, we identified several molecules that have potential utility to control plant parasitic nematodes and whose activity is cytochrome P450-dependent (Burns *et al.*, 2023; Knox *et al.*, *in review*; Knox *et al.*, *unpublished*). In this presentation, I show how these molecules are metabolized into lethal metabolites by nematode cytochrome P450s. I will also describe our efforts to understand how the bioactivated metabolites kill cells. We intend to exploit these strategies to better understand how other commonly used compounds are metabolized into adverse metabolites

Session 1: Bioengineering and biotechnology

Session chair: Sheila Sadeghi

Evolution of a 3DM structure-based class specific molecular information system of the cytochrome P450 monooxygenases

Martie Smit

University of the Free State, Department of Microbiology and Biochemistry, Bloemfontein, 9300, South Africa

3DM systems are structure-based information systems of protein super families, mainly privately owned, built by Bio-Prodict, a Dutch computational biotechnology company. 3DM systems rely on structure-based multiple alignments of X-ray structures of proteins belonging to targeted superfamilies to identify a "core" for which 90 % of structures contain at least three consecutive amino acids with alpha carbons ideally within 2.5 angstroms of the average position[1]. Residues belonging to the "core" are assigned 3DM numbers, which differ from the numbers in the original sequences, but allow that for a given position in the core information from different proteins can be linked. Subfamilies are created based on diverse representative structures selected by 3DM. Protein sequences obtained through BLAST searches are sorted into these subfamilies based on alignment of the "core" which should reach a minimum score. Three key features of 3DM are correlated mutation analysis (CMA) of residues in the "core"[2,3] the ability to group sequences based on amino acid distribution at a panel of selected "core" residues and the ability to search proteins for a position specific motif based on such a panel of selected "core" residues. Literature data, including mutagenesis data, can also be extracted for residues in the "core".

The Biocatalysis Group at UFS commissioned its first P450 3DM system in 2009, the first private 3DM system built by Bio-Prodict. The 3DM system was updated in 2011, 2013, 2021 and 2023 to include newly elucidated CYP structures. The latest 2023 P450 3DM iteration has 147 061 aligned sequences sorted into 123 structure-based subfamilies with 387 "core" positions. Five publications by three other groups have since 2018 referred to the use of P450 3DM systems[4,5,6,7,8]. However there has been no publication describing any of these P450 3DM systems in detail. Here we will describe the evolution of our P450 3DM system, what we have learned from it and how we have used it.

1- Kuipers, R. K. *et al. Proteins* 2010, 78, 2101–13
2- Kuipers, R. K. P. *et al. Proteins* 2009, 76, 608–16
3- Van Den Bergh, T. *et al. PLoS One* 2017, 12, 1–19
4- Tavanti, M. *et al. Biochem. Biophys. Res. Commun.* 2018, 501, 846–850
5- Klenk, J. M., Dubiel, P., Sharma, M., Grogan, G., Hauer, B. *Microb. Biotechnol.* 2019, 12, 377–391
6- Klenk, J. M. *et al. J. Biochem.* 2019, 166, 51–66
7- Grobe, S. *et al. Angew. Chemie - Int. Ed.* 2021, 60, 753–757
8- Rapp, L. R. *et al. ACS Catal.* 2021, 11, 3182–3189

Session 2: Plant and insects (A)

Session chair: Toshiyuki Sakaki

Characterization of biosynthesis of solanoeclepin B, a novel hatching factor for potato cyst nematode

Ryota Akiyama[1], Kosuke Shimizu[1], Karen Akanuma[1], Soichiro1, Makino[1], Itaru Sakata[2], Yukihiro Sugimoto[1], Atsuhiko Kushida[2], Keiji Tanino[3], Masaharu Mizutani[1]

1 Graduate School of Agricultural Science, Kobe University, Japan
2 Technology Application Research Team, Department of Research Promotion, Hokkaido Agricultural Research Center, Japan
3 Department of Chemistry, Faculty of Science, Hokkaido University, Japan

Cyst nematodes are highly evolved sedentary endoparasites that are considered as harmful pests worldwide. Potato cyst nematodes (PCNs: *Globodera rostochiensis* and *Globodera pallidas*) parasite only on Solanaceae plants such as potato and tomato. The eggs of PCNs in the cyst are protected from drought, cold, and insecticides in soil, and the eggs can survive without hatching for up to 20 years in the absence of the host plants. When the host plants appear nearby, the eggs hatch in response to specific compounds called hatching factors (HFs) secreted by host plant's roots. Solanoeclepin A (SEA) is a highly active HF for PCN, that was isolated from the root exudate in 1999.[1)] We conducted hatching assay guides purification of HF from 70,000 liters of potato hydroponic culture solution and discovered a novel HF called solanoeclepin B (SEB). We found that SEB can be easily detected in tomato hairy root culture medium and identified five SEB biosynthesis genes (*SOLA1*～*SOLA5*) in tomato. Furthermore, we found that SEB is converted to SEA in soil microorganisms.[2)]

In this study, we report on the expression analysis of SEB biosynthesis genes. We found conditions under which the expression of the SEB biosynthesis gene increased as a result of hydroponic cultivation of tomato plantlets under various nutritional conditions. Based on co-expression analysis, we selected candidate genes for SEB biosynthesis. Tomato hairy roots in which each of the candidate genes was disrupted were created and analyzed, and several *SOLA* genes were identified as SEB biosynthesis genes. Overall, this study sheds light on the complex mechanisms underlying cyst nematode parasitism and offers new avenues for their control.

1- Schenk, H., Driessen, R. A., de Gelder, R., Goubitz, K., Nieboer, H., Brüggemann-Rotgans, I. E., & Diepenhorst, P. Elucidation of the structure of Solanoeclepin A, a natural hatching factor of potato and tomato cyst nematodes, by single-crystal x-ray diffraction. Croatica Chemica Acta, 72(2-3), 593-606, 1999.
2- Shimizu, K., Akiyama, R., Okamura, Y., Ogawa, C., Masuda, Y., Sakata, I.,Watanabe, B., Sugimoto, Y., Kushida, A., Tanino, K., Mizutani, M.* Solanoeclepin B, a hatching factor for potato cyst nematode. Science Advances, 9(11): eadf4166, 2023.

Session 2: Plant and insects (A)

Session chair: Toshiyuki Sakaki

P450s evolution in the green lineage: the functions essential for plant terrestrialization

Danièle Werck-Reichhart

Institut de biologie moléculaire des plantes (IBMP), CNRS, University of Strasbourg, 12 rue du general Zimmer, 67084 Strasbourg, France. werck@unistra.fr

Plants started to colonize land around 500 million years ago. It meant dealing with new challenges like absence of buoyancy, water and nutrients shortage, protection from increased light radiation, reproduction on land, and interaction with new microorganisms. This obviously required the acquisition of novel functions and metabolic capacities. The different P450s families are typically associated with specific functions. P450 family emergence and evolution in the green lineage thus offer the opportunity to catch a glimpse of the timing of the evolution of the critical functions that were required (or became dispensable) for the plant transition to land. Based on the analysis of currently available genomic data, it is possible to propose an evolutionary history of plant P450s in the context of plant terrestrialization and an overview of the main associated functions in the different lineages. Without surprise, it highlights the importance of the biosynthesis of antioxidants, UV screens, and biopolymers, and of some critical signaling pathways controlling plant development and stress response. It also points to important unsolved questions that would deserve to be answered to improve our understanding of plant growth, structure, adaptation to current and upcoming challenging environments, as well as management of important agricultural traits.

Session 2: Plant and insects (A)

Session chair: Toshiyuki Sakaki

Inactivation of cytochrome P450s involved in sesquiterpene lactone biosynthesis to accumulate medicinal terpenes in chicory taproots (*Cichorium intybus* L.)

Katarina Cankar[1], Claudia Nunes Santos[2], Marjan de Mey[3], Dirk Bosch[1]

[1]*Wageningen University and Research, Wageningen, The Netherlands*
[2]*Nova medical school, Lisbon, Portugal*
[3]*Gent University, Gent, Belgium*

Chicory roots are a rich source of sesquiterpene lactones with medicinal properties. In the framework of the EU-CHIC project, genome editing protocols were developed and used to create new industrial chicory varieties trough precision breeding targeting biosynthetic pathways leading to bitter tasting terpenes. To allow more economical and more sustainable extraction of the food fibre inulin, chicory varieties in which the accumulation of bitter tasting terpenes was completely abolished were obtained by inactivation of the germacrene A synthase[1]. Rerouting of the biosynthesis by inactivating specific cytochrome P450 enzymes resulted in chicory varieties that each accumulate different specific terpenes with medicinal potential[2-3]. These could be positioned as secondary products from root chicory, next to inulin. Chicory roots predominantly accumulating the anti-cancer costunolide and the antiinflammatory 8-deoxylactucin were obtained. The anti-inflammatory activity was confirmed in an inflamed intestinal mucosa model. A sustainable process to extract sesquiterpene lactones using supercritical CO_2 extraction combined with fractionation was established.

Plant compounds can also be made available at commercial scale by introducing their biosynthesis into industrial micro-organisms. In the recently started EU-deCYPher[4] project artificial intelligence and machine learning techniques are used to identify plant cytochrome P450s and design optimal microbial cell factories for the fermentative bio-based production of oxygenised terpenoids and flavonoids.

1- Cankar et al. Inactivation of the germacrene A synthase genes by CRISPR/Cas9 eliminates the biosynthesis of sesquiterpene lactones in Cichorium intybus L. Plant Biotechnol J. 2021; 19(12):2442-24532
2- Cankar et al. CRISPR/Cas9 targeted inactivation of the kauniolide synthase in chicory results in accumulation of costunolide and its conjugates in taproots. Front Plant Sci. 2022; 29:13:940003
3- Cankar et al. Lactucin synthase inactivation boosts the accumulation of anti-inflammatory 8-deoxylactucin and its derivatives in chicory (Cichorium intybus L.). J Agric Food Chem. 2023; 10;71(15):6061-6072
4- www.decypher.bio

Session 2: Plant and insects (A)

Session chair: Toshiyuki Sakaki

Structure-activity-relationships of triterpenoid saponins.

Søren Bak*[a], Jan Günther[a], Pablo D. Cárdenas[a], Malboro Dervishi[a], Jincheng Shen[a], Rajeswari Gopal Geetha[a], Viviana Monje-Galvan[b], Anja Fuglsang[a], Nina Cedergreen[a], Hans Chr. Bruun Hansen[a]

[a] *University of Copenhagen, Department of Plant and Environmental Sciences, Denmark.*
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Triterpenoid saponins are present in more than 100 plant families. They are defensive compounds against insect pests and pathogens. More than 1,300 structures are described in the literature, which is only the tip of the iceberg of saponin chemical diversity in nature. Saponins are well known for a range of diverse biological functions including anti-microbial (anti-fungal, anti-bacterial), insecticidal, vermicidal, molluscicidal, and herbicidal activities. However, most publications focus their potential as pharmaceutical drugs due to their anti-tumorigenic, anti-inflammatory, and immunomodulatory activities. Saponins are specific to some organisms and not others determined by their chemical structure. Thus, an understanding of the intrinsic structure-activity-relationship of saponins provides an untapped potential to uncover the potential of saponins as e.g. biopesticides that are target specific and yet environmentally friendly.

Structurally, saponins are comprised of a triterpenoid backbone structure generated by a 2,3oxidosqualene cyclase (OSC), the backbone is then hydroxylated by P450s and finally glycosylated at different positions. Substrate and product promiscuity seems to drive structural diversity and thus plants often contain a blend or cocktail of different saponin structures with different bioactivities. The combination of triterpenoid backbone structure, level of hydroxylation, and glycosylation determines their physiochemical and biological properties and gives then amphipathic (surfactant) properties that are hypothesized to enable them to perforate biological membrane systems.

We are conducting mode-of-action studies to understand their structure-activity-relationships at the molecular, cellular, and at the ecosystems level by combining 2D and 3D structure analysis with bioactivity assays using test organisms, molecular dynamic simulations, and by using *in vitro* generated large unilamellar vesicles (LUVs) mimicking different biological membranes.

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Session 3: Plant and insects (B)

Session chair: Joerg Bohlmann

Mosquito P450s; looking back at insecticide resistance marker identification and forwards to accelerating insecticide discovery

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Mosquitoes transmit numerous lethal diseases including malaria, dengue, yellow fever, West Nile virus, zika, chikungunya and filariasis, which kill 100's of thousands of people a year and cause debilitating illnesses to 100's of millions with devastating health and economic consequences across the globe. In short, mosquitoes are the most dangerous animals on Earth. Insecticides are critical for disease control. Since the turn of the millenium, pyrethroid treated bed nets have been the front-line intervention for malaria control in Africa. However, pyrethroid resistance is now endemic, driving efforts by the Innovative Vector Control Consortium (IVCC) to repurpose agricultural pesticides and to develop new active ingredients for a new generation of mosquito control products. Insecticide resistance is complex and multifactorial. However, P450s play a central role in insecticide metabolism and disposition that is commonly associated with metabolic resistance. The major African malaria vectors, *Anopheles gambiae*, *An. coluzzi* and *An. funestus* have evolved elevated levels of pyrethoid metabolising P450s that have broad substrate specificity that can compromise the introduction of new vector control products. This presentation looks back at the early discovery of P450 resistance mechanisms in malaria vectors and looks forwards to the development of effective tools for the pre-emptive identification of metabolic resistance liabilities in new product pipelines.

Session 3: Plant and insects (B)

Session chair: Joerg Bohlmann

Structural diversification of strigolactones driven by cytochrome P450s

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Strigolactones (SLs) are carotenoid-derived plant specialized metabolites that function as endogenous phytohormones and exogenous signaling molecules in the rhizosphere. These compounds are involved in the regulation of shoot branching/tillering, facilitate hyphal branching in arbuscular mycorrhizal fungi, and act as germination stimulants for root parasitic weeds. The diversity of SLs is remarkable, with over 30 different structures identified, highlighting their biochemical and functional complexities. Canonical SLs are characterized by a tricyclic lactone embodying the ABC-ring system. Conversely, non-canonical SLs have incomplete ABC-ring systems. The biosynthesis of SLs involves a conserved upstream pathway in which all-*trans*-β-carotene is converted to carlactone through sequential catalysis by the enzymes carotenoid isomerase DWARF27 (D27), carotenoid cleavage dioxygenase 7 (CCD7), and CCD8. Subsequently, CL is converted to carlactonoic acid (CLA) by the action of the cytochrome P450 CYP711A subfamily, a critical step that precedes the structural diversification of SLs. The synthesis of different SL structures downstream of CLA involving cytochrome P450 enzymes underlines the complexity and specificity of this pathway. Our research has previously identified the CYP722C subfamily of enzymes as key players in the synthesis of canonical SL in dicot plants, in addition to cytochrome P450 enzymes that modify the basic skeleton of canonical SL structures. Elucidating how SL structures diversify is critical to understanding the molecular mechanisms underlying the effects of these diverse compounds on plant growth and plant–environment interactions. This presentation highlights the latest insights into the function of cytochrome P450 enzymes in SL biosynthesis.

Session 3: Plant and insects (B)

Session chair: Joerg Bohlmann

Redox partners and electron transfer chains of cytochrome P450 systems in phenylpropanoid-lignin biosynthesis

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Phenylpropanoid-monolignol biosynthesis leads to the formation of heteropolymer lignin and a variety of methanolic soluble phenolics in different plant tissues. Three endoplasmic reticulum (ER) membrane-resident cytochrome P450 enzymes, cinnamate 4-hydroxylase (C4H, CYP73A5), coumaroyl ester 3′-hydroxylase (C3'H, CYP98A3) and ferulate 5-hydroxylase (F5H, CYP84A1), catalyze regio-specific hydroxylation of the phenyl ring of monolignol precursor, which determines the key structural characteristics of lignin subunits. Eukaryotic P450 monooxygenases require redox partner(s) to deliver reducing power, i.e., electrons, to their catalytic center, thereby driving their reactivity. The eukaryotic endomembrane contains two electron transport systems: NADPH-cytochrome P450 oxidoreductase (CPR) and NADH-cytochrome b_5 reductase (CBR)-cytochrome b_5 (CB5). CPR has been recognized as a typical electron donor for P450 catalysis and lignin biosynthesis. Interestingly, we discovered that in addition to CPR, cytochrome b_5 family member, CB5D, also serves as an indispensable electron donor for F5H-catalyzed syringyl lignin biosynthesis in Arabidopsis.[1] Moreover, monolignol biosynthetic P450s recruit distinct electron transfer chains to support their hydroxylation reactions. C4H employs conventional NADPH-CPR chain for its *para*-hydroxylation of aromatic ring, while F5H pairs with CB5D, shuttling electrons from both NADPH-CPR and NADHCBR routes to the phenyl ring 5-hydroxylation reaction. Specifically, in Arabidopsis stem F5H recruits NADPH-CPR-CB5 as the prime electron transfer chain for syringyl lignin synthesis, while in seeds it dominantly employs NADH-CBR-CB5 for the syntheses of the 5-hydroxylated phenolics sinapoyl esters and seed coat suberin aromatics.[2] In contrast, the independently evolved F5H in primitive vascular plant *Selaginella moellendorffii* does not require CB5 electron donor protein at all; instead, like conventional P450 C4H and C3'H, it solely requires CPR for catalysis. Interestingly, the emergence of CB5D function occurs before plant colonization, predating the invention of F5Hs in vascular plants.[3] This suggests that the recently evolved angiosperm F5H enzymes co-opted the anciently invented CB5D, forming a modern cytochrome P450 monooxygenase system for aromatic ring meta-hydroxylation.

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Session 4: Plant and insects (C)

Session chair: Giovanna Di Nardo

The anti-diabetic metabolite Montbretin-A
From plant biodiversity to metabolic pathway discovery and bioengineering with applications for human health related to food security

Joerg Bohlmann

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Plants and their diverse specialized metabolites have been used by humans for centuries as flavors and fragrances as well as in traditional and modern medicines. They remain an important source for the discovery of new nutraceuticals and pharmaceuticals. Montbretin A (MbA) is a complex flavonoid metabolite; a highly potent and selective inhibitor of the human pancreatic αamylase (HPA); and a potential new treatment option for type 2 diabetes and obesity. The only known source for MbA are the below-ground storage organs of montbretia (Crocosmia) species, which are native to the grasslands of southern and eastern Africa and commonly grown in gardens around the world. Due to the low abundance of MbA in montbretia plants, and due its complex chemical structure, natural product extraction and chemical synthesis are insufficient for scalable MbA production. Our goal is to develop an improved bio-production system for MbA using genes, enzymes and regulating factors of MbA biosynthesis in montbretia. In recent work, we discovered the complete biosynthetic pathway of MbA using an approach that combined knowledge of montbretia biology, metabolite profiling, differential transcriptome analysis, cDNA cloning, heterologous gene expression, and enzyme biochemistry. This includes the discovery of genes encoding UDP-sugar dependent glycosyltransferase and acyltransferase enzymes, which catalyze the assembly of MbA from its different building blocks. We are using these genes to bioengineer the production of MbA in yeast and plants. The presentation discusses challenges and opportunities of exploring plant biosynthetic systems for the development of bioproducts.

Session 4: Plant and insects (C)

Session chair: Giovanna Di Nardo

Catalytic site constraints in *Camptotheca* p450s mediating alkaloid synthesis

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Terpene indole alkaloids represent a class of plant-derived medicinal compounds produced in low levels in medicinal plants such as *Catharanthus roseus* (Cra) and *Camptotheca acuminata* (Caa). The early (and common) TIA pathways in these species utilize several CYP72A subfamily members to form loganic acid from 7-deoxyloganic acid (via a simple monooxygenation) and, subsequently, to form secologanin and secologanic acid from loganin and loganic acid (via a C-C bond scission). Divergences in the specificities of these early alkaloid P450s have allowed *Camptotheca secologanic* acid synthases (SLASs) to become bifunctional enzymes capable of two sequential steps converting 7-deoxyloganic acid to secologanic acid. In contrast, *Catharanthus* 7-deoxyloganic acid hydroxylase (7DLH) and secologanin synthase (SLS) have remained monofunctional enzymes capable either of monooxygenation or C-C bond scission. In vitro reconstitutions have demonstrated that *Camptotheca* also contains a monofunctional 7DLH capable only of hydroxylating 7-deoxyloganic acid. Mutageneses aimed at evaluating residues important for the tight specificity of *Camptotheca* 7DLH (CYP72A729) and the broad specificity of its SLAS (CYP72A564) have identified several residues where reciprocal switches substantially affect their activities. Among these: Lys128His in the B'-C loop in 7DLH increases hydroxylation of 7-deoxyloganic acid and His132Lys in SLAS decreases this hydroxylation as well as C-C bond scissions of loganic acid and loganin; Gly321Ser in the beginning of the I-helix in 7DLH does not affect hydroxylation of 7-deoxyloganic acid while Ser324Gly in SLAS significantly increases C-C bond scission of loganic acid; Asp332Glu in the acid-alcohol pair within the I-helix of 7DLH increases hydroxylation of 7-deoxyloganic acid while Glu335Asp in SLAS completely eliminates both of its activities.

The late TIA pathways in these species utilize a different array of CYP71 and CYP81 family members to form near final species-specific alkaloids such as camptothecin (*Camptotheca*) and vinblastine (*Catharanthus*). These late alkaloid P450s include CYP71BE subfamily proteins that epoxidate strictosamide (the conjugated product formed from secologanic acid and tryptamine) and isoliquiritigenin (a phenylpropanoid) as well as CYP81BQ subfamily proteins that decorate camptothecin (a product subsequently methoxylated). The identification of mutations that enhance or eliminate these respective activities aid engineering efforts aimed at modifying camptothecin synthesis in *Camptotheca* cell cultures, microbial systems and/or other plants.

The identification of the residues essential for the broad substrate scope of the SLASs presents opportunities for more tailored heterologous production of TIAs.

Session 4: Plant and insects (C)

Session chair: Giovanna Di Nardo

CYP5164B1: a key enzyme in the oxylipin pathway during a brown algal host-endophyte interaction

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The evolution of organisms results in continuous changes in metabolic pathways. This phenomenon is described as metabolic drift. We hypothesize that biotic interactions may be a driving force by fostering metabolic plasticity. Brown algae offer unique model systems to address this question, with host-endophyte species pairs belonging to the same phylum. These pairs enable to reconstruct the establishment of interactions between the metabolic pathways of both partners dating back to their common ancestor[1]. The phylogenetic proximity between Laminariales and their endophytic Ectocarpales increases the probability that orthologous enzymes have identical catalytic activities, allowing compensation phenomena and loss of genes by endophytes. Comparison of genome-scale metabolic networks has identified metabolic reactions that may have been lost in the endophyte[2]. Some are part of the oxylipin pathway which is induced during defense responses in biotic interactions[3]. One concerns a gene presumably acting just downstream a CYP5164 enzyme, homologous to plant CYP74, a member of a multigenic subfamily that has diversified in Laminariales and Ectocarpales[4]. Metabolic profiling of a CRISPR knock-out mutant for the CYP5164B1 gene in a free-living Ectocarpale indicates that there are differences in the global metabolomic profile compared to the wild-type strain, and ongoing analysis will indicate if mutant oxylipin profiles are consistent with the previously determined catalytic activity of the recombinant enzyme[5], and if there is evidence for further metabolization consistent with the candidate role proposed for the downstream enzyme.

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Session 4: Plant and insects (C)

Session chair: Giovanna Di Nardo

Exploring P450 superfamily diversity with P450Atlas - online tool for automated subfamily assignment

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The P450 superfamily, comprising 9723 known families further divided into over 24,000 subfamilies, showcases a staggering diversity within the realm of these enzymes. In this contribution we introduce the P450Atlas website: the ultimate source of information about all named P450 families and subfamilies. The primary functionality of the website is automated assignment of a query sequence to one of known subfamilies. Additionally, users can browse the list of families across the tree of life and access up-to-date statistics describing the P450 universe.

The novel subfamily assignment method relies on Hidden Markov Models (HMMs). For each subfamily a separate HMM profile has been constructed; a query sequence is then assigned by hmmscan tool of the HMMER package. Extensive validation that has been carried out with a benchmark set of over 11000 test sequences showed almost perfect assignment with only a very few sequences placed in an incorrect subfamily. When compared to BLAST program, the new approach proven to be more sensitive and accurate. We hope that the P450Atlas website will serve as a valuable tool for the P450 community, providing deeper insights into the diverse landscape of P450 enzymes.

Session 4: Plant and insects (C)

Session chair: Giovanna Di Nardo **Poster 32**

Discovering new cytochromes P450 from cyanobacteria

Danilo Correddu, Gianluca Catucci, Samuele Rosso, Valentina Aleo, Giovanna Di Nardo and Gianfranco Gilardi

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Cyanobacteria are the oldest photosynthetic organisms, known to be the responsible for the oxygenation of our planet. From these unicellular organisms descend chloroplasts of plants and algae, with whom they share similarities in genes coding for enzymes involved in the biosynthesis of an extraordinary number of natural and bioactive compounds.[1] Recent bioinformatic studies show that, also in cyanobacteria, cytochromes P450 play a significant role in these metabolic pathways.[2] However, since the first pioneering study of CYP120A1 from *Synechocystis* sp. PCC 6803 about 20 years ago,[3,4] little effort has been directed towards new functional and structural characterizations.[5]

Recent challenges on P450 biocatalysis are the use of efficient and stable enzymes, and the replacement of expensive electron donors such as NAD(P)H by using alternative molecules, directing the electrons flow from photosystems of the chloroplasts to the P450 heme, or by exploiting the peroxygenase activity of some P450 families.

Our research focuses on the identification of novel cyanobacterial P450s with these characteristics. More specifically, P450s that share about 50 % similarity with members of the CYP152 family were identified in different cyanobacterial species. We expressed and characterized the first cyanobacterial P450 peroxygenase from *Rivularia* sp. MS3 ($P450_{Riv}$). Sequence and structural alignments indicated $P450_{Riv}$ as a putative fatty acid hydroxylase with peroxygenase activity. In our experiments we confirmed that $P450_{Riv}$ perform its catalytic activity towards medium chain fatty acids (C8:0, C10:0, C12:0, C14:0 and C16:0) using H_2O_2 as electron donor. GC-MS analysis revealed the specificity to produce their corresponding α-hydroxylated forms, with a conversion of up to 80 % for hexadecanoic acid. The enzyme showed a high resistance to H_2O_2 and a high thermal stability, which is greatly enhanced by about 10 °C in presence of its substrate hexadecenoic acid.

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Session 5: Green catalytic processes

Session chair: Vlada Urlacher

Investigating novel P450 function in ribosomal biarylitide biosynthesis pathways

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Cytochrome P450s are capable of a wide range of oxidative transformations across diverse biosynthetic processes. Given this synthetic utility and combination of oxidative power and regiochemical precision, it is little surprise that this P450s have been widely implicated as potential biocatalysts. Here, I will report our recent investigations of peptide crosslinking P450 enzymes from biarylitide biosynthesis, which can generate a range of cyclic tripeptide species from minimal pentapeptide substrates.

In this presentation, I will detail the results of our ongoing research to characterise new and engineered members of this P450 family, which can perform crosslinking between a range of different aromatic residues. I will also detail our investigations into unexpected chemical transformations performed by biarylitide P450 enzymes and our efforts to understand the molecular basis for these unexpected chemical modifications. Given the utility of peptide crosslinks - and other modifications - in important natural products and the synthetic challenge that these can represent, these P450 enzymes have the potential to play roles as important synthetic tools in the generate of high-value cyclic tripeptides and synthons.

Session 5: Green catalytic processes

Session chair: Vlada Urlacher

Effect of flexibility in P450 chimeric proteins

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Previous work demonstrated that soluble bacterial reductase from Bacillus megaterium, BMR can be used as an artificial electron transfer partner fused to the human P450 domain in a single polypeptide chain. This fusion strategy, known as 'Molecular Lego'[1,2] was employed by our group for the biochemical characterization of the 3A4-BMR chimera. Different constructs have been tested and the data clearly indicated that a five glycines extension of the loop connecting the two domains resulted in improved the overall performance of the enzyme in terms of activity, coupling efficiency and flexibility due to a higher flexibility of the system[3–5]. Here we used differential scanning calorimetry to evaluate stabilizing role of BMR. We have also applied the same approach to CYP19A1 (aromatase) and the data in this case show that the activity of the chimeras is very low (<0.003 min−1) for all the constructs tested with a different linker loop length: ARO-BMR, ARO-BMR-3GLY, and ARO-BMR-5GLY. In terms of thermal stability BMR fusions showed an increase in T_{onset} by 10 °C and the presence of a cooperative unfolding process driven by the BMR protein that is absent when the isolated purified human proteins are analysed by DSC. Previously characterized 3A4-BMR constructs show the same behaviour of ARO-BMR constructs in terms of thermal stabilization, but a higher activity as a function of the loop length. Overall we have demonstrated that, when using the *Molecular Lego* approach, the chimeras of P450-BMR work well when the P450 domain can compensate its own internal flexibility and plasticity for the physical constraint given by the presence of an extra polypeptide chain, as in the case of 3A4BMR[6]. Careful attention should be paid in the design of P450-BMR chimeras because the functionality of the P450 domain can either benefit (3A4) or be impaired (aromatase) by the BMR fusions depending on intrinsic biochemical features of the P450 domain.

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Session 5: Green catalytic processes

Session chair: Vlada Urlacher

Biocatalytic potential of fungal CYP505s

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CYP505s belongs to a sub-class of fungal self-sufficient CYPs, with the N-terminal CYP domain fused to a cytochrome P450 reductase (CPR) domain. The closest related sub-family to the CYP505s are the bacterial CYP102s. CYP505s are probably best known for CYP505B1 (Fum6), involved in fumonisin production, and CYP505A1 (P450foxy), implicated in fungal pathogenicity.

Most CYP505s characterized to date hydroxylate fatty acids sub-terminally (ω-1 to ω-3), with regioselectivities similar to those displayed by CYP102A1 (P450BM3). Structural investigation of CYP505A30[1] revealed an active site architecture alike to that of BM3, but as with BM3, the cocrystallized structure of CYP505A30 gave a non-productive binding conformation of the fatty acid. CYP505A30 can also hydroxylate fatty alcohols, often with higher catalytic efficiencies than with the corresponding fatty acids, and *n*-alkanes. With *n*-alkanes, mixtures of non-vicinal diols can be synthesized via sequential subterminal hydroxylation.[2] Due to the high similarly of the active sites of CYP505A30 and BM3, despite the low sequence identity, we have rationally transferred mutations known to alter the regioselectivity of BM3 to CYP505A30 for more selective production of symmetrical diols, due to improved C2 (ω-1) selectivity.[1] Although some members of the CYP505 family such as CYP505D6 give in-chain hydroxylation as minor products, members of the CYP505Es, such as CYP505E3, preferentially hydroxylate *n*-alkanes, fatty alcohols and fatty acids in-chain.[3,4] Unlike CYP116B46, this regioselectivity is determined from the methyl end and not the carboxyl group, suggesting different mechanisms in substrate coordination. This unique ω-7 hydroxylation of fatty acids and alcohols opens unique routes to produce δ-dodecalactone.

Ancestral sequence reconstruction revealed that the CYP505E and CYP505P clades, with ω-7 vs ω-3 regioselectivity respectively, share a common ancestor. Comparison of the common ancestor with the ancestors of the CYP505E and CYP505P clades shows that the CYP505Es diverged to evolve this ω-7 regioselectivity, as the common ancestor gives a more diverse hydroxylation pattern which is also substrate dependent.

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Session 5: Green catalytic processes

Session chair: Vlada Urlacher

Cytochrome P450 enzyme family and paclitaxel biosynthesis in Taxus

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Plants have evolved complex metabolic pathways that produce a multitude of natural small molecules with unique structures and functions. These compounds have played a crucial role in plant growth, development, and environmental adaptation, making them the largest reservoir of bioactive substances on Earth. Moreover, they have been the primary source of drugs for disease prevention and treatment throughout human history. The metabolic pathway of Taxus plants, a species dating back to the Quaternary glacial period and known as the "plant giant panda," produces a structurally unique diterpenoid compound called paclitaxel. Significantly, almost half of the biosynthetic enzymes that contribute to the paclitaxel biosynthesis pathway belong to the CYP450 protein family. Here, we will delve into the latest advancements made in the CYP450 family, provide an in-depth analysis of the paclitaxel biosynthesis pathway, explore metabolic regulation, and investigate its synthetic biology manufacturing.

Session 6: P450 and non-P450 cascade reactions

Session chair: Amit Pandey

P450s for lignan biosynthesis in recombinant *E. coli*

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The lignans (-)-deoxypodophyllotoxin and (-)-podophyllotoxin from the endangered plant *Sinopodophyllum hexandrum* are used as precursors in the synthesis of the chemotherapeutics etoposide and teniposide. To overcome its limited availability, alternative sustainable routes are needed. In our previous work, we introduced a heterologous biosynthetic pathway to lignans in *Escherichia coli*.[1,2] The reconstituted cascade involved several genes from four plants, including two genes encoding cytochromes P450 and their redox partner, a cytochrome P450 reductase (CPR).

Cytochromes P450 are recognized as important bio-bricks for synthetic biology. However, when expressed heterologously in *E. coli*, poor P450 activity often limits the entire biosynthetic pathway. In the established cascade, two P450-catalyzed reactions were found to limit the efficient bioconversion of the lignan pinoresinol to the final products. To alleviate this problem, we applied several approaches, including i) rational engineering of signal peptides to target membrane-anchoring of these enzymes; ii) search for the optimal P450:CPR pair; and iii) regulation of the expression of genes encoding both P450s and CPR.

Microbial recombinant systems with multiple plasmids often suffer from plasmid instability and reduced cell growth. As a next step, we integrated the genes encoding this multi-step cascade into the *E. coli* chromosome using CRISPR/Cas technology. Comparative results for the episomal and chromosomal expression systems harboring multiple plant genes as well as the effect of energy and carbon sources on product titers will be described.

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Session 6: P450 and non-P450 cascade reactions

Session chair: Amit Pandey

Design of a cascade reaction for lignin valorisation

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Lignin is a three-dimensional, aromatic polymer generated through the radical polymerization of phenylpropane units (*p*-coumaryl, syringyl, and guaiacyl alcohols). Despite constituting approximately 15–40% of lignocellulose and being the primary renewable source of aromatics, lignin remains significantly underutilized.

Lignin valorisation can be achieved through its thermochemical or biological depolymerization into monomeric aromatic compounds. These compounds can serve as building blocks for the sustainable synthesis of chemicals, including *cis,cis*-muconic acid, a high value-added bio-product with potential applications in the manufacture of new functional resins, bio-plastics, food additives, agrochemicals, and pharmaceuticals.

In recent years, Dye-Decolorising Peroxidases (DyPs) have been gathering attention because they have been found to oxidize lignin. Bacterial DyPs represent rare enzymes able to degrade lignin which is usually attacked by fungal enzymes. Here, we present the structural and functional characterization of a novel bacterial DyP peroxidase from *Acinetobacter radioresistens* (ArDyp), an attractive microbial source of enzymes involved in aromatic degradation. The crystal structure of ArDyp was solved at 1.8 Å resolution and functional characterization revealed the ability of the enzyme to act on Kraft lignin and produce different products, including guaiacol. Thus, we are designing of a cascade reaction that includes three enzymes: ArDyP, a cytochrome P450 and catechol 1,2-dioxygenase, to develop a reactor for the biosynthesis of *cis,cis*-muconic acid in a continuous flow. In particular, catechol 1,2-dioxygenase from *Acinetobacter radioresistens* was already used in combination with CYP116B5 from the same microorganism to produce *cis,cis*-muconic acid from phenol. Unfortunately, CYP116B5 enzyme does not act on guaiacol but the demethylation of this aromatic compound has been reported for some bacterial cytochromes P450, including variants of P450 BM3 that generate catechol.[1,2] This last compound is the substrate of catechol 1,2-dioxygenase that was successfully immobilized on functionalized poly(dimethylsiloxane) in an active form and could produce *cis,cis*-muconic acid in a continuous flow reactor.

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Session 6: P450 and non-P450 cascade reactions

Session chair: Amit Pandey

Biosynthesis and diversification of complex triterpenes in plants

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Triterpenes are among the most structurally and functionally diversified small molecules found in plants. Furthermore, they represent a significant class of plant natural products with pharmaceutical and agronomic applications. Gene duplication is commonly regarded as a fundamental process for the diversification of triterpenoids. Recently, we demonstrated that an extreme form of gene duplication, known as whole genome duplication (WGD), plays crucial roles in co-opting and diversifying pentacyclic triterpenes in loquat (Su *et al.*, 2021, PNAS). During the presentation, I will share unpublished data concerning how WGD facilitates the diversification of triterpenes through genomic structural variations that lead to the formation of gene clusters. These examples highlight the remarkable metabolic plasticity at the genomic level and contribute to our understanding of how WGD drives the diversification of metabolic pathways. Additionally, I will present and discuss the reconstitution of complex triterpenoid pathways in *Nicotiana benthamiana*.

Session 6: P450 and non-P450 cascade reactions

Session chair: Amit Pandey

Molecular interactomics of CYPs with other enzymes

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CYPs play crucial roles in various biosynthetic and metabolic pathways. Our study utilized interactomics, structural analysis, functional assays, display technology, and bioinformatics to investigate the functional interactions of both mammalian and microbial CYPs with redox partners and other proteins, such as sulfotransferases and steroid dehydrogenases [1-7].

We characterized the interaction patterns for components of CYP-dependent monooxygesae systems [1-4]. We also demonstrated how protein-ligand interactions influence the formation of protein complexes [3,4].

Additionally, we investigated the functional interactomics of self-sufficient enzymes, namely CYP5A1 (thromboxane synthase) and CYP8A1 (prostacyclin synthase), with other proteins involved in the fate of eicosanoid biosynthesis, including enzymes and transporters [5,6]. We hypothesize that these interactions play pivotal roles in regulating the biosynthesis of bioactive eicosanoids.

Furthermore, we identified functional interactions between CYP17A1 and SULT2A1 or SULT1E1 [7]. Additionally, SULTs exhibited interactions with CYB5A and CPR [7]. The interaction dynamics of SULT2A1/CYP17A1 and SULT2A1/CYB5A complexes appeared to be modulated by 3'-phosphoadenosine-5'-phosphosulfate (PAPS). Importantly, we demonstrated that the enzymatic activity of SULTs increased in the presence of either CYP17A1 alone or in combination with CYB5A.

In conclusion, our findings underscore the significance of functional protein interactions in the regulation of CYP-containing multienzyme cascades.

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Session 7: Biodiversity and Evolution

Session chair: Elizabeth Gillam

The Tao of P450: grasping the 10,000 things.

David Nelson

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According to the Tao, the material diversity of the universe is represented by the "10,000 things". In the P450 universe we now have defined 10,000 P450 families (and counting). The vertebrate families are all known, the land plant families are all known (except the rarest ones). The unknown diversity is the dark matter of the P450 universe, and we are seeking to find it and define it. It lies in the invertebrates, the green and red algae, the fungi, the protists, and the bacteria (and a few viruses). Headway is being made on all these fronts. The statistical possibilities for novel P450 sequences are nearly uncountable, but the reality of evolving from a common ancestor places practical limits on the real number of CYP families. Each lineage has a parent as we see from the present day P450 clans. If the goal of the Earth Biogenome Project to sequence all eukaryotic diversity can be met, then all the P450 families in Eukaryotes can be discovered. Multiple projects are underway such as the Darwin Tree of Life. One area currently being explored is the deep-sea metagenome. King Abdullah University of Science and Technology (KAUST) has created a database of over 300 million protein seqs from 2100 sites in the deep ocean (a 60 Gb file). The sequences that were annotated as P450s by their pipeline have been extracted and further filtered to remove non-P450 seqs. The remainder is 115,000 P450 sequences. Approximately 65,000 of these are sorted into known CYP families in known kingdoms of life. Yet, 49,000 sequences did not fit into known families. It is even uncertain in what kingdoms these sequences belong. Here is a galaxy of dark matter to be resolved. Progress will be reported on this work. Another new avenue in P450 evolution is the application of Alphafold structures. Approximately 53 million high quality Alphafold structures have been clustered based on their 3D structures in a map of the protein Universe. The sequences fall into communities, some with over 1000 members. The communities are joined by their structural similarity into networks. These networks reflect P450 evolution. There are ~3900 P450 communities. These communities are like CYP families but not 1:1. For example, there are 26 vertebrate communities and only 19 vertebrate families. It may be possible using the predicted 3D structures to peer back farther in time to determine evolutionary relationships not detected by sequence comparison. This may be useful for piercing the transition of green plants to the land where so many new families suddenly evolved

Session 7: Biodiversity and Evolution

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A mitochondrial CYP gene mediates the secondary metabolic resistance mechanism to ethiprole in brown planthopper *Nilaparvata lugens*

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Brown planthopper *Nilaparvata lugens* is one of the most economically important pests of cultivated rice in south-east Asia. It causes damage through both direct phloem-feeding and transmission of plant viruses. Extensive use of insecticide treatments such as imidacloprid, fipronil and ethiprole resulted in the emergence of multiple resistant strains of *N. lugens*. Investigation of molecular mechanisms of resistance pointed to overexpression and qualitative changes in P450 gene CYP6ER1.

Metabolic resistance to insecticides is usually mediated by overexpression of cytochrome P450 genes belonging to either CYP 3 or 4 clades. However, from re-analysing RNAseq data of ethiprole resistant *N. lugens* strains we have identified a secondary mechanism enhancing ethiprole tolerance mediated by differential splicing and overexpression of CYP419A1, a member of a planthopper-specific family of mitochondrial CYP genes. We have functionally validated the protective effect of CYP419A1 using transgenic drosophila and report some unusual features of both CYP419A1 gene and protein, which include non-canonical heme-binding motif and extreme 5' end extension of the open reading frame.

Session 7: Biodiversity and Evolution

Session chair: Elizabeth Gillam

Functional significance of P450 diversity in herbivorous rodents

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The vast diversity of cytochrome P450 enzymes in herbivorous mammals is thought to stem from plantanimal warfare, whereby evolution of plant defenses such as phenolics and terpenoids led to duplication and divergence of P450 genes in herbivores. However, the majority of our knowledge in this area stems from studies of the metabolism of drugs and model compounds rather than studies on wild mammalian herbivores and their respective plant secondary metabolites. A problem of particular interest centers on the role of individual P450 enzymes in the ability of certain herbivores to specialize on plants that are lethal to most other species. My research team and collaborators have been working on this problem by taking a comparative approach using a tractable natural system consisting of herbivorous woodrats, genus *Neotoma,* that consume a variety of toxic plants. We compared the P450s of the specialist *N. stephensi*, the facultative specialist *N. lepida*, and the generalist *N. albigula*, by employing a crossdisciplinary approach involving ecology, biochemistry, pharmacology, structural biology, and genomics[1]. Multiple lines of evidence support the importance of P450 enzymes in enabling woodrats to adapt to different toxic diets. CYP2B enzymes appear to play a key role in the ingestion of juniper foliage and its major constituent, alpha-pinene. Differences in CYP2B gene copy number may contribute to differential tolerance of PSMs among woodrat species. The significant expansion of CYP3A enzymes in some populations of *N. lepida* within the last 17,000 years seem to have enabled some populations of woodrats to switch from diets of juniper to creosote bush, which produces a complex mixture of phenolic compounds on its leaves[2, 3]. Our work to date has revealed the tremendous diversity of P450 enzymes in woodrats and underscored the importance of gene duplication in enabling adaptation to novel dietary toxins.

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Session chair: Elizabeth Gillam

Molluscan cytochromes P450

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Mollusks, including gastropods, cephalopods, bivalves, and others, are second only to arthropods in the number of living animal species. Despite the diversity of physiology and ecotypes, mollusks have been very undersampled in genome and transcriptome analyses, and there remain huge gaps in our understanding of cytochrome P450 diversity. Similar to animals in other phyla, mollusk CYP numbers range from about 70 to 155, although there is insufficient taxonomic sampling to determine if there is are systematic differences between molluscan classes. As in other animals, significant lineage-specific 'blooms' are evident, particularly in Clan 2 and Clan 3. Notably, mollusks have CYP51, unlike insects, and CYP20s, and while steroid diversity in bivalves is different than in vertebrates it is likely that P450s play key roles. Functions of molluscan P450s are far less studied. We have examined the gene expression in several different bivalve species, including oysters and mussels, and observed that few P450s are differentially regulated in response to environmentally-relevant pollutant exposures. Knowledge of the diversity and regulation of molluscan P450s will facilitate both ecological and ecotoxicological studies in this incredibly varied phylum.

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Session 8: Industrial Applications

Session chair: Gianfranco Gilardi

Biosynthesis and engineering of plant tropane alkaloids hyoscyamine and cocaine

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Tropane alkaloids such as hyoscyamine and cocaine are important plant-derived natural drugs with a rich history of medicinal application, and they remain widely employed in modern clinical therapy. The elucidation of their biosynthetic pathways has long been a hotly debated and challenging topic during the last century[1]. Here are highlights of our findings in this field: (i) the characterization of polyketide synthases[2, 3], CYP450[4], and methyltransferase[4] in tropane alkaloid biosynthetic pathway solved the long-standing mechanistic conundrum concerning the tropane skeleton construction of hyoscyamine and cocaine, which has been cited as a work that may rewrite the textbook of natural product chemistry; (ii) the identification of 3-oxo-glutaric acid[2, 3] and ecgonone[4] intermediates in tropane alkaloid biosynthesis illustrated that the Robinson's chemical synthesis of tropinone in 1917[1] is biomimetic; (iii) finally, the yeast and plant cells were used for the production of tropane alkaloids , providing an alternative way to get the Tas.

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Session 8: Industrial Applications

Session chair: Gianfranco Gilardi

Cytochrome P450 engineering with mutability landscapes

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Controlling the regio- and stereoselective hydroxylation of organic molecules is a very useful yet challenging reaction in synthetic organic chemistry.[1] Directed evolution has emerged as one of the most successful methods to engineer proteins, a fact that has been recognized with the 2018 Nobel Prize in Chemistry.[2] It consists of iterative cycles of gene mutagenesis, expression and screening or selection until the desired objectives are accomplished. Integrating both experimental and computational methods into directed evolution workflows can accelerate enzyme engineering. In this talk, we will introduce the concept of mutability landscapes (ML) to identify hotspot residues involved in selectivity and activity in BM3, the most active cytochrome P450 enzyme described to date.[3] The combination of ML with molecular dynamics (MD) simulations was very successful to engineer the diastereoselective hydroxylation of steroids at position C16 that are useful as glucocorticoids, but the ML only included 5 residues.[4] The expansion of MLs to >30 active site residues lead to the discovery of mutants selective towards C7 position and others (unpublished), enabling the biosynthesis of steroids that could be useful to treat chronic neuronal damage.[5] Data-driven directed evolution combined with machine learning will accelerate enzyme engineering including P450s for a wide range of applications in biotechnology.[6]

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Session 8: Industrial Applications

Session chair: Gianfranco Gilardi

Repurposing a P450 reductase with [FeFe]-hydrogenase and BVMO as non-physiological partners for H2-dependent NADPH regeneration in indigoids production

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The modular and multicomponent structure of P450s is here exploited as an inspiration (similarly to the already successful "Molecular Lego" approach[1,2]) for a non-physiological cascade to perform biotechnologically relevant reactions employing gaseous hydrogen. The latter enables efficient and cost-effective NADPH recycling without the formation of byproducts that could alter pH or hinder simple product recovery. Additionally, the availability of low-cost H_2 (from dark fermentation of waste or solar and wind powered electrolysis) ensures the sustainability of the process and aligns with the principles of bio- and circular economy.

The system proposed is the first of its kind, repurposing a P450 reductase (BMR) from *P. megaterium* to perform the reverse of its canonical reaction, i.e. as NADPH regeneration module, combined with a unique, highly active and oxygen resilient [FeFe]-hydrogenase CbA5H from *C. beijerinckii*[3,4], used as H_2-driven electron supplier. The system was demonstrated[5] to achieve 28±2 nmol NADPH regenerated *s^{-1} *mg of hydrogenase^{-1} (TOF: 126±9 min^{-1}), and was tested with a mutated Baeyer Villiger monooxygenase (BVMO R292A)[6], confirming the ability to support indigo production across multiple reaction.

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Session chair: Gianfranco Gilardi

Selective C-H functionalization via P450-catalyzed abiological group transfer reactions

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Expanding the reaction scope of biological catalysts beyond the realm of enzymatic transformations occurring in nature can offer new opportunities for the exploitation of biocatalysis for chemical synthesis. In this contribution, we will present recent progress made by our group toward the design, investigation, and application of engineered cytochrome P450s for catalyzing 'new-to-nature' C(sp3)– H functionalization reactions via carbene and nitrene transfer chemistries. This work has enabled the development of efficient and stereoselective biocatalysts for the asymmetric synthesis and C–H functionalization of N-containing heterocycles and other valuable scaffolds for medicinal chemistry. These methodologies provide an attractive approach to the chemoenzymatic synthesis and late-stage C–H functionalization of pharmaceuticals and other biologically active molecules.

Session 9: New Structural-Functional Insight (A)

Session chair: James De Voss

50 Years working on cytochrome P450: What have we learned?

Stephen Sligar

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It is amazing, and somewhat unusual, that a field of science enjoys over five decades of significant federal funding. I began work as a physics graduate student, "seconded" to the Gunsalus laboratory, joining the P450 community in 1972. This is the 50th anniversary of my first publication in the field of cytochrome P450:

Sligar, S., Lipscomb, J., Debrunner, P., and Gunsalus, I.C. (1974) "Superoxide Anion Production from the Autooxidation of Cytochrome P-450." Biochemical and Biophysical Research Communications 61, 290-296.

The autoxidation of the soluble P450cam (CYP101A1) revealed a simple first order reaction producing superoxide. But when we tried the same experiment with purified P450 LM2 provided by Coon Laboratory, the reaction had complex kinetics and produced hydrogen peroxide directly. We now understand that this was due to the aggregate state of protein-lipid-detergent in the LM2 preparation. Fast forward to the use of Nanodiscs to provide a soluble membrane environment for the membrane associated P450s (and a vast spectrum of other membrane proteins) and the autoxidation reaction of CYP2B4 becomes simple first order. Other fundamental mechanistic insights revealed in P450cam, and first thought to be different in mammalian P450s, were found to be directly applicable when the membrane protein is assembled into a bilayer structure. The use of Nanodiscs also opened the door of using cryoenzymology to trap and investigate the reactivity of heme-oxygen intermediates in the P450 reaction cycle. In my talk today I will discuss a comparison of the bacterial P450cam and Nanodisc incorporated human CYP17A1 in terms of the role of substrate positioning to control proton delivery to the heme-oxygen active center, and thus dictate the reaction mechanism.

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Session 9: New Structural-Functional Insight (A)

Session chair: James De Voss

Rapid determination of cytochrome P450 binding affinities and their application in probing P450 structure/function

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Determining which small molecule ligands bind to a given cytochrome P450 enzyme and the affinity thereof is high value information in applications ranging from discovering P450 substrates and their roles in biological pathways, engineering P450 enzymes for industrial applications, evaluating drug metabolism, and inhibiting undesirable catalysis. The historically-dominant and perhaps most accessible method for evaluating P450 ligand binding are shifts in the absorbance Soret peak that occur when substrates or inhibitors are titrated in and access the P450 active site. A single careful manual titration of a P450 with a ligand observed using a spectrophotometer often takes ~3.5 hours of handson work for the experimentalist, yielding the maximum binding (A_{max}) and the dissociation constant (K_d). This absorbance shift assay has been adapted to a high-throughput environment, generating the same information content, but allowing an ~50-fold acceleration in data generation. Validated with both type I (substrate-like) and type II (inhibitor-like) compounds and for between-day variation, this assay does not use any more protein than the manual version. An additional advantage is that solvent concentrations remain constant throughout the titration, which is not the case with the manual spectrophotometer version. This assay has now been used to identify ligands and rank them by affinity for a number of human cytochrome P450 enzymes. Analysis of high-affinity ligand profiles for P450 enzymes with well-described ligands and active sites raises confidence that this approach can be used to provide ligand compatibility information for other P450 enzymes where the ligand profile and active site size and shape are not well known.

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Session 9: New Structural-Functional Insight (A)

Session chair: James De Voss

Crystal structure of CYP85A3, another key enzyme in brassinosteroid biosynthesis

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Several cytochrome P450s (P450s) are found in the biosynthetic system of brassinosteroid (BR), which regulates plant growth and development. In 2014, we determined the crystal structure of several forms of CYP90B1, which is the first and rate-determining enzyme in BR biosynthesis, and elucidated the stereo-specific hydroxylation mechanism to produce 22(*S*)-hydroxycampesterol.[1] During the biosynthetic reactions, the plant growth and development activity of BR is dramatically increased in the final two-step reactions catalysed by CYP85A family members.[2,3] In the first step of CYP85A reactions, two consecutive hydroxylations on 6-deoxocastasterone (6dCS), which are mediated by compound I, and subsequent spontaneous dehydration produce castasterone (CS). The second reaction catalysed by CYP85A is an atypical P450 reaction, a Baeyer-Villiger oxidation, which introduces an oxygen atom between C6 and C7 of CS to produce brassinolide, the most potent natural BR. Although peroxo heme has been proposed to be an active species of the second reaction, no experimental evidence has been provided so far. We determined the crystal structure of uniconazole-bound CYP85A3 and found that CYP85A3 was the most similar to cholesterol-bound CYP90B1 (RMSD: 1.79 Å). In the crystal structure of uniconazole-bound CYP90B1, SRS6 was unexpectedly located further away from the substrate binding site. However, the SRS6 of CYP85A3 was found to be close to the substrate binding site, as seen for substrate-bound CYP90B1 and many other P450s. We also performed Ala-scanning mutagenesis to find amino acid residues that interact with 6dCS. In this presentation, further structural details of this enzyme and a potential substrate binding mode will be discussed.

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Session 10: New Structural-Functional Insight (B)

Session chair: Stephen Sligar

C-C and C-N Bond formation by Cytochrome P450s

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CYP catalyzed intra- and inter-molecular crosslink formation via C-C and C-N bond installation is prevalent in the biosynthesis of many pharmacophores. Among these, tryptophan-linked dimeric diketopiperazine (DKP) alkaloids exhibit a wide range of biological activities and have now been found in numerous natural product biosynthesis pathways. A common strategy for DKP-dimerization involves a cyclodipeptide synthase which first converts two amino-acid charged tRNAs to form a cyclic dipeptide (CDP). Several cytochrome P450s (CYPs), prevalent in many bacterial DKP-containing biosynthetic gene clusters (BGCs), have been identified to catalyze the downstream intermolecular dimerization of two CDPs (or a CDP with a free nucleotide). To resolve the rationale for this atypical CYP transformation, we have examined a panel of "symmetric" and "asymmetric" CYP dimerases through a combination of steady-state and transient kinetics, spectroscopic methods, and x-ray crystallography. Our results suggest multiple pathways can operate within these enzymes and be leveraged for targeted DKP-alkaloid synthesis in vitro or in vivo.

Session 10: New Structural-Functional Insight (B)

Session chair: Stephen Sligar

Unusual structural features of two CYP51s: a pathogenic amoeba and deep-water fish

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CYP51 (sterol 14α-demethylase) is the most conserved cytochrome P450. Found in all biological kingdoms, it catalyzes the same three-step stereo-specific reaction, removing the 14α-methyl group from the core of cyclized precursors in sterol biosynthetic pathways. Because sterols are essential membrane components and regulatory molecules involved in various developmental processes, including cellular growth, division, and multiplication, CYP51 orthologs must preserve their catalytic role, even at very low sequence identities. Strict functional conservation is reflected in high structural similarity, so that the RMS deviations for all Cα atoms of the ligand-free/inhibitor bound CYP51 orthologs, from bacteria to human, are usually within 2 Å. There are, of course, some phyla-specific structural differences, which will be mentioned briefly. But mostly the talk will be about the peculiarities that we observed in our new X-ray structures, of the CYP51 enzymes from the opportunistic human pathogen *Acanthamoeba castellanii* (the A' and A helices and a new entry channel) and from the deep-water fish *Coryphaenoides armatus* (the unusual but apparently "allowed" FG arm inward movement). Deeper knowledge about this drug target P450 structure-function relationships should accelerate the development of new therapies to treat various human diseases.

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Session 11: Microbial and Fungal P450s

Session chair: Rita Bernhardt

Thermophilic cytochromes P450 from an Australian hot spring

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High throughput DNA sequencing coupled to bioinformatic anInteresrtinglyalyses (metagenomics) has revolutionised the field of microbial ecology. Such a 'metagenomic' approach has resulted in the discovery of new microbial lineages, metabolic processes and enzymes from uncharacterised and importantly uncultivated microorganisms in environmental samples. Using such an approach is particularly attractive in studying microorganisms from ecosystems with high temperature or pH extremes as their genes likely encode for enzymes that have evolved to resist conditions in harsh environments. As such, metagenomics has been suggested as a way in which specific biocatalysts with improved or desirable properties may be obtained. We have undertaken sequencing of DNA from the microbial biomass of an Australian hot spring in northern Queensland. Computer software mediated "bioprospecting" of the assembled metagenomes has allowed us to create a database of >200 potentially thermostable P450s. Some of these were found in bacterial families/genera known to be thermophilic, whilst some were apparently associated with typically mesophilic bacteria. We thus undertook the expression and characterisation of four of these P450s from a variety of P450 families and bacterial lineages. All are indeed thermostable with $^{15}T_{50}$'s (temperature at which 50% protein is intact after 15 mins) of 48-65 ºC; a mesophilic P450 has a $^{15}T_{50}$ < 40ºC. One of the challenges of employing thermostable P450s biotechnologically is obtaining suitable thermostable redox partners. Interestingly, none of the P450s expressed were associated with separate, functional redox partners. However, one of these P450s is a CYP116, a family that usually has both redox partners and hemoprotein as domains on a single polypeptide. This protein demonstrated good activity and thermal stability ($^{15}T_{50}$ 60 ºC). The characterisation and properties of these expressed P450s will be discussed.

Session 11: Microbial and Fungal P450s

Session chair: Rita Bernhardt **Poster 33**

An ancient key player in steroidogenesis: The X-ray protein structure of the ancestral cholesterol metabolizing cytochrome P450 11A1

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Steroid hormones play a fundamental role in the evolution of higher animals by regulating stress and immune responses, salt homeostasis, and reproduction. The first catalytic step in steroidogenesis is performed by the cytochrome P450 11A1 (CYP11A1) which kicks off a cascade of subsequent reactions by cleaving the side chain of cholesterol yielding pregnenolone. Pregnenolone serves as a precursor for all steroid hormones including glucocorticoids, mineralocorticoids, and sex hormones. Thus, the evolution of steroid hormone biosynthesis is tightly connected to the evolution of CYP11A1. Previous studies have used ancestral sequence reconstruction (ASR) to resurrect ancestral CYP11A1 to understand the evolution of this crucial enzyme. The vertebrate ancestor showed high thermostability and drastically altered substrate specificity. While the bovine isoform prefers cholesterol as substrate, the ancestral CYP11A1 prefers the cholesterol precursor desmosterol which hints at a different enzyme and, ultimately, active site architecture.

To examine the structural basis for both thermostability and substrate preference, we used X-ray protein crystallography to solve a three-dimensional structure of ancestral CYP11A1. For this, we generated highly pure and monodisperse ancestral CYP11A1 and conducted a preliminary screening for suitable conditions promoting crystal growth. Hits resulting from this screening were further optimized and yielded cubic-shaped protein crystals. Crystals were sent to the Stanford Synchrotron Light Source (SSRL) for diffraction experiments. With the data we collected we could solve a crystal structure to 2.4 Å resolution. Compared to human and bovine CYP11A1, the ancestral isoform exhibits substantial rearrangements of the substrate access channel and substrate recognition site which might be the cause for altered substrate preference.

The structural elucidation of ancestral CYP11A1 will unravel how evolution shaped crucial key players in steroidogenesis to facilitate the development of higher animals.

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Session 11: Microbial and Fungal P450s

Session chair: Rita Bernhardt

Biochemical and genetic understanding of the role(s) of cytochromes P450 in giant viruses

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Discovered just over 20 years ago, Giant Viruses (GVs) are enigmatic members of the global microbiome. They are distributed ubiquitously across diverse ecosystems and are particularly abundant in aquatic environments. Infecting hosts that range from algae to mammals, they have a particular affinity for microbial hosts. The remarkable diversity of metabolic genes described in GVs include enzymes involved in glycolysis, gluconeogenesis, TCA cycle, photosynthesis, and lipid β-oxidation. Our aim is to understand the implications of such genes for virus and host. As a beginning we have undertaken a functional genomics approach to understand the lipid metabolism pathways of the GV *Pandoravirus massiliensis* and their impacts on its amoeba host, *Acanthamoeba castellanii*. We have identified P450 and other proteins potentially associated with this GV lipid pathway. These include various hydrolase and lipase proteins (carboxylic ester hydrolase, patatin-like phospholipase, esterase/lipase), cytochromes P450 and two potential lipid transporters (lipocalin domaincontaining protein and lipid raft-associated protein). Transcriptomic analysis revealed the identified P450, lipase and hydrolase proteins to be well transcribed, as are the two transporters. These P450 genes are conserved in other pandoraviruses, as well as having homologs in Mollivirus and Faustovirus. Liquid Chromatography/Mass Spectrometry of extracted lipids from purified *P. massiliensis* virions and *A. castellanii*, pre- and postinfection, revealed an abundance of phosphatidylcholine and phosphatidylethanolamine along with betaine lipid diacylglyceryltrimethylhomoserine, ceramides and triacylglycerols (TAGs). Furthermore, C72 TAGs were observed to accumulate in amoeba membranes following viral replication and are not seen in amoeba pre-infection nor in purified virions. In contrast unique C24, C25 and C30 ceramides accumulate in pre-infection and purified virions but are not observed in amoeba membranes post-replication. Additionally, new classes of long chain fatty acids (LCFAs), including C30:2 and C30:3 LCFAs, were identified in a GV for the first time. We also describe another GV P450 gene, Mimivirus CYP5253, which is inserted into the genome of the soil arthropod, the springtail *Folsomia candida*. Recombinant *F. candida* CYP5253 gives a typical P450 spectrum. This finding highlights the possibility for horizontal gene transfer of GV P450s to or from other organisms from Mimivirus with potential impacts on host biochemistry. (Support: US NIH & NSF; Leverhulme, UK).

Session 11: Microbial and Fungal P450s

Session chair: Rita Bernhardt

P450-mediated oxidations trigger a unique non-enzymatic oxidation cascade completing celastrol biosynthesis

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Obesity is a major health risk still lacking effective pharmacological treatment. A remarkably potent anti-obesity agent, celastrol, has been identified in the root of *Tripterygium wilfordii*.[1] However, its application has been hampered by the lack of an efficient synthesis method. Here, we achieve the *de novo* biosynthesis of celastrol in yeast by elucidating the 11 missing steps in its biosynthesis.[2] First, we discover the cytochrome P450 enzymes that catalyse the four oxidation steps that produce the key intermediate celastrogenic acid. Subsequently, we reveal that non-enzymatic decarboxylation-triggered activation of celastrogenic acid leads to a cascade of tandem catechol oxidation-driven double-bond extension events that generate the characteristic quinone-methide moiety of celastrol. We use this knowledge to develop an efficient method to produce celastrol starting from sugar. Our work highlights the effectiveness of combining plant biochemistry with metabolic engineering and chemistry for the sustainable and scalable synthesis of complex specialized metabolites.

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Session 12: P450-mediated Interactions

Session chair: Jed Goldstone

Distinguishing the functions of canonical strigolactones through disruption of the rice CYP711A (MORE AXILLARY GROWTH 1) homologues

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Strigolactones (SLs) exert various biological functions as a plant hormone, best known for regulating shoot architecture and inhibiting branching/tillering, and as rhizospheric signaling molecules to attract symbiotic mycorrhizal fungi, particularly under phosphate starvation[1,2]. However, released SLs are also sensed by seeds of root parasitic weeds, such as *Striga hermonthica*, which depend on and abuse them as a germination signal ensuring the presence of a suitable host in the close vicinity[3]. SLs originate from β-carotene through a core pathway that leads to the central intermediate carlactone (CL). Structural modifications of CL by various cytochrome P450 (CYP450) enzymes, including CYP711A that is represented by Arabidopsis MORE AXILLARY GROWTH 1 (MAX1) and its homologs, result in canonical and non-canonical SLs[4]. Herein, CRISPR/Cas9-mediated rice mutants that lack canonical SLs due to the disruption of *MORE AXILLARY GROWTH1* (*MAX1*) homologous do not exhibit the dwarf and high-tillering phenotypes characteristic for SL-deficient plants, but release root exudates with a significantly decreased Striga seed-germinating activity and show a delay in the mycorrhization establishment[5]. Obtained results suggest that canonical SLs have a particular function as rhizospheric signals and do not significantly contribute to the regulation tillering. Moreover, these mutants show that the canonical SL 4-deoxyorobanchol is involved in the regulation of root and shoot development as well as in rhizosphere communications[6]. Therefore, our work uncovers that canonical SLs are not the major regulators for shoot tillering/branching but important rhizospheric signals mediating intraspecific communication, and deciphers specific hormonal functions of canonical SLs. In addition, reducing the amount and/or changing the pattern of released SLs is a promising strategy for targeted engineering of rice architecture as well as for increasing crop's resistance to root parasitic weeds that represent a global agricultural problem and one of the seven major threats to global food security, which particularly impacts cereal production in sub-Saharan Africa.

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Session 12: P450-mediated Interactions

Session chair: Jed Goldstone

Dual factors required for cytochrome P450 mediated hydrocarbon ring contraction in bacterial gibberellin phytohormone biosynthesis

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Cytochromes P450 (CYPs) are heme-thiolate monooxygenases that prototypically catalyze the insertion of oxygen into unactivated C–H bonds but are capable of mediating more complex reactions. One of the most remarked-upon alternative reactions occurs during biosynthesis of the gibberellin A (GA) phytohormones, involving hydrocarbon ring contraction with coupled aldehyde extrusion of ent-kaurenoic acid to form the first gibberellin intermediate. While the unusual nature of this reaction has long been noted, its mechanistic basis has remained opaque. Building on identification of the relevant CYP114 from bacterial GA biosynthesis, detailed structure–function studies are reported here, including development of in vitro assays as well as crystallographic analyses both in the absence and presence of substrate. These structures provided insight into enzymatic catalysis of this unusual reaction, as exemplified by identification of a key role for the "missing" acid from an otherwise highly conserved acid–alcohol pair of residues. Notably, the results demonstrate that ring contraction requires dual factors, both the use of a dedicated ferredoxin and absence of the otherwise conserved acidic residue, with exclusion of either limiting turnover to just the initiating and more straightforward hydroxylation. The results provide detailed insight into the enzymatic structure–function relationships underlying this fascinating reaction and support theuse of a semipinacol mechanism for the unusual ring contraction reaction.

Session 12: P450-mediated Interactions

Session chair: Jed Goldstone

Variations in cytochrome P450 reductase (POR) and adrenodoxin reductase (FDXR) regulate P450 mediated interactions and metabolism

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Multiple human diseases, including abnormalities in steroid and drug metabolism, are caused by mutations in the redox partners of cytochrome P450s including P450 reductase (POR)[1,2] and Adrenodoxin reductase (FDXR/ADXR). The POR transfers electrons from NADPH to cytochrome P450 proteins in endoplasmic reticulum for the metabolism of steroids, drugs, and other xenobiotics and in mitochondria FDX/FDXR system supports the cytochrome P450 activities. We identified human patients with defects in P450 redox partners and analyzed over 300 different variations found in patients and from large-scale sequencing projects.[3,4]

We identified potentially disease-causing variations and performed their characterization by functional studies using recombinant proteins, CRISPR edited cell lines, and re-programmed patient derived fibroblasts into induced pluripotent stem cells (iPSCs). Assays of steroid and drug metabolizing P450s as well as protein stability studies, flavin content analysis were conducted and analyzed together with clinical laboratory values. Interactions with cytochrome P450s and other redox partners are studied using single molecule microscopy, co-immunoprecipitation, fast proteolysis and computational modeling.

Identification of severe effects of POR and other redox partner mutations on steroid metabolizing cytochromes P450 indicates that likely pathogenic mutations may be found in the normal (non-clinical) population. Combination of redox partner variants as compound heterozygotes or homozygous may lead to a severe impact on steroid and drug metabolism by modulation of interactions with cytochrome P450 proteins. Therefore, variations in P450 redox partners need to be evaluated individually. Often, genetic mutations in P450 redox partners cause conformational changes in protein that can be targeted with small molecules.[5]

Genetic variations in POR as well as mitochondrial redox partner systems reveal a complex pathophysiological mechanism governed by modulation of protein-protein interactions involving cytochrome P450 proteins.

These studies were funded by grants to Amit V Pandey from the Swiss National Science Foundation, Cancer Research Switzerland, Novo Nordisk SA, Merck SA, Eli Lilly, Servier SA, Roche Research Foundation, and Novartis Foundation for Medical Biological Research.

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Cytochrome P450: Whither goest thou?

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Cytochrome P450, at 70+ years, has been called a “mature science”. Kuhn[1] uses the terms ‘exemplar’ or ‘paradigm’, according to which puzzles are solved in the “normal science” (observational) phase, maturing eventually to a revolution and new paradigm, in effect an exemplar that suggests (i) new puzzles, (ii) approaches to solving those puzzles, and (iii) a standard against solutions can be measured. The “P450 paradigm” arose from observations made in the 1940’s and 1950’ culminating with a name, and a function, opening an approach to massive puzzles and puzzle solving. Has the P450 field matured to the point of needing a new paradigm? What are the attributes of a mature science? When engineering grows out of it? Is it "mature" when the puzzles pursued have become narrower, more circumscribed, when excitement of discovery wanes as knowledge of the field grows toward being full? Does a mature science find fewer new investigators drawn to the field? In some ways, P450 can be seen as mature. But the puzzles, and the implications, are no less interesting nor less important. P450 inquiry can be seen in large puzzle “spheres”. This talk is intended to spark conversation on the P450 puzzles, and ways to promulgate the excitement. P450 puzzles abound in applied areas (disease, new chemicals), in structural understanding, in environmental connections, and in evolution, where P450 may be a target and a driver of evolution. These puzzle spheres all are interconnected. Unanticipated P450 features may be found in extreme environments, and the massive unexplored diversity of life. Overall, adaptation is perhaps a major driving force under which P450 gene origin, gene gain (including stochastically), gene loss, and gene regulation play out in these spheres.

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Poster 01

Characterization of enzymes involved in bacterial estrogens degradation pathway: a focus on *Novosphingobium tardaugens* CYP450 responsible for estrone 4-hydroxilation

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Estrogens (such as 17β-estradiol, estrone and the synthetic ethinylestradiol) are steroid hormones with a key regulative role in female reproductive system and in circulatory and neuronal systems. These compounds can be detected in surface waters, in addition to other contaminants, including drugs and pesticides, which can exert estrogenic activity. As these molecules can act as endocrine disrupting chemicals, the prolonged exposure can impair animal physiologic mechanisms and lead to development of estrogen-associated cancer, thus representing a public concern for aquatic ecosystems health and human well-being.

The biodegradation of estrogens can be achieved through the 4,5-*seco* pathway by bacteria isolated in natural environment and activated sludges. The principal metabolic steps are the 17β-dehydrogenation of 17β-estradiol to obtain estrone, followed by estrone 4-hydroxylation and cleavage of its A aromatic ring, leading to an unstable intermediate which is converted in pyridinestrone acid.[1]

The genes encoding for the enzymes catalysing the biodegradation reactions in *Novosphingobium tardaugens* NBRC16725, previously isolated in a sewage treatment plant in Tokyo, have been annotated and a cytochrome P450 is involved.[2]

In our work, we express (in *E. coli* BL21) and characterize *N. tardaugens* cytochrome P450 (gene *EGO55_13525*; *edcA*) involved in the 4-hydroxylation of estrone. Binding assays, molecular docking and activity assays on the total lysate are exploited to uncover enzyme interaction and activity not only with estrone, but also with other estrogens (17β-estradiol and ethinylestradiol) to expand the range of substrates the protein can exploit, uncovering the possibility to avoid the 17β-dehydrogenation step. This study constitutes the first step of a wider project that aims to characterize and optimize the enzymes involved in estrogen degradation pathway, with the final aim to develop a green system exploitable to reduce estrogens persistence in water and their endocrine disrupting activity.

This work is part of the project PNRR-NGEU which has received funding from the MUR – DM 118/2023.

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Poster 02

Filling out P450 phylogenies using transcriptomic sequences from underexplored animal taxa

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Cytochromes P450 are superfamily of enzymes represented in animals by twelve groupings, known as clans and further classified into families based on a 40% amino acid sequence identity threshold. Vertebrate animals have 18 P450 families which perform conserved functions ranging from steroid hormone biosynthesis to xenobiotic metabolism. It has been difficult to determine if these families are orthologous to P450 families in other animal lineages in order to investigate when these functions arose. Complete phylogenies with representation from all animal taxa have been difficult to assemble from protein sequences on publicly available databases such as NCBI and UniProt due lack of representation of sequences from understudied animal phyla. To address this problem, the NCBI Transcriptome Shotgun Assembly (TSA) database was used as a resource to discover new P450 sequences. tBLASTn_VDB was used to search each animal phyla using representative sequences from each animal P450 clan to assembled and curated to a set of 39,933 P450 sequences from 18 of the 32 searched animal phyla. Six animal phyla, which had no sequences available in the non-redundant protein databases, had several hundreds of P450 sequences present in the TSA. Each sequence was assigned to a clan and clanspecific phylogenies were constructed with IQ-TREE and FastTree. The results provided an overview of the P450 clans present across the animal kingdom, including six previously unexplored phyla, as well as identifying possible ancient orthologs to vertebrate P450 families and a P450 clan which was previously unidentified.

Poster 03

Exploitation of bacterial cytochromes P450s for improving the biological properties of plant natural compounds

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Within the framework of circular economy, there is a growing need for the evolution of green synthesis approaches and biocatalysis has been already validated in many examples that range from compounds of everyday use to pharmaceuticals. In this context, cytochromes P450 are biotechnologically useful enzymes, that can be used in the native state or can be engineered to improve their properties. Here we present diverse approaches to explore P450s utility for the modification of natural compounds, aimed at improving their activity and relevance to biomedical applications. First, we observed high conversion rate of bergamottin and piperine by using P450 BM3 A2 was obtained through random mutagenesis where change of two amino acids resulted in broader substrate specificity and reactivity. The isolated metabolites produced by P450 catalysis were tested in terms of biological activity and epoxybergamottin and piperine catechol showed higher antioxidant and antimicrobial activity than the original compounds.

Another approach is to characterize substrates and reactions of new and "orphan" cytochromes P450 towards natural compounds and compare it to closely related enzymes. Here we show insights into the characterization of first time expressed and purified P450 105D7 from *S. mirabilis* and comparison of its activity with already studied P450 105D7 from *S. avermitilis*. There is 85% of amino acid identity between both proteins. In our work we aimed to investigate how this small structural change can influence the activity of enzyme. For this reason, based on up-to-date scientific literature we constructed library of P450 105D7 substrates and tested them with new variant both with computation and direct methods.

The last presented enzyme, P450 105F2, was isolated from *S.peucetius* was described to be able to carry out hydroxylation of oleandomycin but the whole picture of its substrate specificity is still unknown. Here we apply the computational and spectroscopic methods to look for its possible substrates.

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Poster 04

Multi enzymatic cascade reaction with bacterial TMAO demethylase, formaldehyde dehydrogenase and human FMO3 for TMAO detection

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Multi-enzymatic cascade reactions, i.e., the concurrence of two or more enzymatic transformations, that can take place in one-pot, offer considerable advantages: the demand of time, costs and chemicals for product production is reduced, control of intermediates and reaction equilibria, and hazardous or unstable compounds production can be avoided. Among different class of drug-metabolizing enzymes the human flavin monooxygenases are the most important ones with cytochromes P450. Flavin-containing monooxygenase 3 (hFMO3) is involved in the metabolism of diet metabolites, xenobiotics, including therapeutic drugs, and thus important in interactions between humans and their chemical environment. Loss-of-function mutations in the gene coding for the enzyme cause trimethylaminuria, or fish-odour syndrome, a disorder that consists in the inability to transform the toxic trimethylamine (TMA) into the odourless N-oxide, namely TMAO. Furthermore, this molecule was recently found to be an important biomarker for predicting cardiovascular and kidney diseases, but its detection lacks quick quantification methodologies. To assess this problem, in this work the bacterial TMAO demethylase (Tdm), from *Methylocella silvestris* was used as an intermediate enzyme in the cascade reaction for its ability to use TMAO as substrate. The multi enzymatic cascade reaction used to create a quantification method for TMAO involved the following enzymes: hFMO3, oxidises the toxic TMA to TMAO and consumes NADPH as a cofactor, Tdm produces dimethylamine and formaldehyde from TMAO and commercial formaldehyde dehydrogenase (from *Pseudomonas putida*) Faldh reduces formaldehyde to formate using NAD^+ as cofactor. Since the stoichiometry of the three reactions is 1:1:1 this method can provide a quantification of the hFMO3 metabolite by measuring the formation of NADH as product of the three-enzyme cascade reaction. Since Tdm is a poorly characterized enzyme it was expressed in *E. coli*, purified with high degree of purity, and characterized biochemically using both differential scanning calorimetry and isothermal titration calorimetry.

Poster 05

Critical assessment and future perspectives of Cytochrome P450 engineering for direct bio-electrochemistry

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Protein engineering of cytochromes P450 can be considered as a well consolidated tool for the development of electrochemically efficient artificial multidomain enzyme systems. Many studies report on the successful use of cytochromes P450 as catalytic domain when coupled with bacterial reductases as electron transfer partners for the construction of chimeric enzymes. On electrode based systems, these P450/reductase chimeric enzymes show an overall improvement in terms of electron transfer tuning towards the catalytic site, coupling efficiency and electrocatalysis, as extensively reported by our group in the last decades.

The present work provides a critical overview of recent results reported on the development of bio-electrochemical systems based on chimeric P450/reductase enzymes. A comparison of our "molecular lego" approach with the literature data provides a clear scenario on the achievements that can be used to guide strategies for improvement of protein and electrode engineering. We take as a test case three P450 based chimeric enzymes obtained by genetic fusion with *Bacillus megaterium* BMR or *Desulfovibrio vulgaris* FLD reductases. These are here exploited with a view to elucidate both advantages and disadvantages of the different molecular design strategy with respect to bio-electrochemistry results.

As major findings of this critical evaluation, several elements have proven to be crucial for the development of efficient enzyme/electrode systems based on P450/reductase chimeras, including the selection of a suitable reductase domain, the optimisation of the electrode immobilisation strategy and the modulation of inter-domain flexibility. All experimental evidences here presented clearly demonstrate that chimeric P450 enzymes are feasible as biocomponent for the development of effective bio-electrochemical systems. Taking into account all the results reported, the combination of "molecular lego" and bio-electrochemistry can be esteemed as a powerful strategy for the development of enzymologically, biotechnologically and clinically relevant P450 based electrochemical tools.

Poster 06

Immobilization of human liver drug-metabolizing enzymes on a multi-channel microfluidic device for the prediction of the metabolic fate of xenobiotics

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In the last few years, microfluidic immobilized enzyme reactors (μ-IMERs) have become more and more widespread due to their numerous advantages, including reusability, prolonged lifetime, and substrate specificity. Their application in the drug development process can help in reducing time and costs throughout the implementation of quick and efficient assays that can predict the turnover of newly discovered drug molecules by major drug-metabolizing enzymes (DMEs). For this reason, we designed a multi-channel microfluidic device based on human flavin-containing monooxygenase 3 (hFMO3) for in vitro high-throughput screening of new drug candidates.[1] hFMO3 is a human liver enzyme that has a prominent role in drug metabolism and two of its common genetic variants are E158K and V257M. Both variants, along with the wild-type enzyme, were covalently immobilized via glutaraldehyde-mediated cross-linking on the surface of polylysine-coated serpentine-shaped channels. Different channel surface areas and several flow rates were tested. The differences observed in the conversion of tamoxifen (used as a model drug) by each polymorphic variant on the chip was found to be fully in line with previously published data in solution. Finally, this proof-of-concept device will be further developed for the prediction of the metabolic fate of other xenobiotics whose metabolism and clearance requires both cytochromes P450 and hFMOs.[2]

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Poster 07

Enzymatic H2-driven CO_2 capture and formate production in a cascade reaction

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The increase in greenhouse gas emissions and the limited reserves of fossil fuels are pushing towards the establishment of clean energy sources and processes for carbon dioxide (CO_2) mitigation in the atmosphere. In this context, hydrogen (H_2) has generated significant interest, although its storage and transport remain challenging. A potential solution is offered by the natural hydrogen-dependent CO_2 reductase (HDCR) of *Acetobacterium woodii*[1]. In this complex a [FeFe] hydrogenase and a formate dehydrogenase, connected by two electron transfer subunits, interact with each other to fix carbon dioxide to formate which can be exploited as a hydrogen carrier.

Herein, the *A. woodii* HDCR complex was mimicked by coupling the oxygen-resistant [FeFe] hydrogenase from *Clostridium beijerinckii* (CbA5H)[2] and the metal-dependent formate dehydrogenase from *Clostridium ljungdahlii* (ClFDH)[3]. Both enzymes were heterologously expressed and purified with affinity chromatography and the *in vitro* system activity was tested. CO_2 hydrogenation was obtained directly in solution without additional electron transfer subunits. The H_2 uptake reaction of the CbA5H was exploited to generate protons and electrons that are then transferred to the active site of ClFDH for CO_2 conversion to formate. Moreover, bio-hydrogen produced from waste biomasses by a consortium of *Lactobacillus helveticus* and *Clostridium beijerinckii* was exploited for this cascade reaction. These results offer new possibilities for high value-added molecules production using clean energy sources while capturing carbon dioxide.

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Poster 08

Tandem reactions of FMO3 and CYP2C19 with fenthion

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This study investigates the use of bioelectrodes to mimic the metabolic pathway involved in the biotransformation of the pesticide fenthion. Pesticides are suitable models for sequential reactions, initially metabolized by human flavin-containing monooxygenases (hFMO) followed by cytochrome P450s (CYP).[1] To evaluate the feasibility of an *in vitro* platform, hFMO3 and CYP2C19 were both expressed in *E. coli* and purified through affinity chromatography. The activity of the two purified enzymes was determined in solution in the presence of NADPH. In the case of hFMO3, the expected fenthion sulfoxide was obtained with a calculated K_M value of 25 ± 5 µM. Subsequently, both proteins were immobilized on glassy carbon electrodes (3 mm diameter) modified with gold nanoparticles (AuNps) stabilised with the cationic surfactant didecyldimethylammonium bromide (DDAB). AuNps were used to enhance the electron transfer and therefore increase the amount of detectable product(s). A potential bias of -0.6 V was applied to the hFMO3 bioelectrode for 20 minutes at room temperature. This was then followed by change to the CYP2C19 bioelectrode and a further 20 minutes of the reaction. The electrochemical products were separated and identified by LC-MS. The data obtained demonstrate that fenthion was converted to fenthion sulfoxide by hFMO3 and the latter was further converted to fenthion sulfone and fenthion oxon sulfone by CYP2C19. These results demonstrate the potential of using an electrochemical platform to replicate the complex in vivo metabolic reactions of xenobiotics.

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Poster 09

Engineering of fungal CYP505 for selective fatty acid hydroxylation

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The γ- and δ-lactones are compounds with various commercial applications as flavors and fragrances. Natural lactones occur in plants and fruits but their extraction from original sources is economically not feasible due to low abundance.[1] Their chemical synthesis raises environmental concerns as transition metal catalysts and peroxy acids are used. One attractive alternative for lactone synthesis involves the direct hydroxylation of bioavailable fatty acids at respective sites.

Recently, CYP505E3 from *Aspergillus terreus* and a number of other CYP505Es were reported that catalyze □-7 hydroxylation of 1-dodecanol, tetradecanoic acid, and dodecanoic acid.[2] Fatty acid hydroxylases from the fungal CYP505 family are self-sufficient flavocytochromes composed of a heme monooxygenase domain and a diflavin reductase domain, which additionally makes these P450s attractive candidates for lactone synthesis.

However, the target regioselectivity of the catalyzed hydroxylation reactions ranges from 12% to 44% for the different CYP505 enzymes, additionally impaired by high rates of over-hydroxylation. Accordingly, regioselectivity should be increased and over-hydroxylation decreased for a more efficient process. Furthermore, the activity towards decanoic and dodecanoic acids is lower than towards the corresponding alcohols.[2,3]

Therefore, structure-guided protein engineering was applied to CYP505 enzymes in this study to obtain higher activity and regioselectivity for γ- and δ-hydroxylation of decanoic and dodecanoic acid. To allow a time-efficient generation of mutant libraries, soluble CYP505 expression in *Escherichia coli* was optimized.

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Poster 10

Binding of steroid substrates reveals the key to the productive transition of the cytochrome P450 OleP

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OleP is a bacterial cytochrome P450 that catalyzes a rare epoxidation reaction during the biosynthesis of the macrolide antibiotic oleandomycin. The enzyme holds potential for biotechnological applications as the reaction is challenging to be replicated through conventional synthesis protocols. Furthermore, OleP exhibits substrate promiscuity, reacting with multiple intermediates of the biosynthesis[1]. The versatility of the enzyme was explored by examining its activity on alternative substrates, such as testosterone (TES) and lithocholic acid (LCA)[2,3]. These molecules mainly differ for the substituent group at the C17. This difference affects the OleP reactivity: the reaction with TES is non-specific, producing a mixture of hydroxylated derivatives, while the reaction with LCA is highly selective, producing only the 6β-hydroxylated product, murideoxycholic acid.

In this work we used X-ray crystallography, molecular dynamic (MD) simulations, and equilibrium binding assays to understand how the binding of different substrates affects the selectivity of the OleP reaction. We report the first structural description of OleP in complex with non-physiological substrates, identifying the key contacts that allow the recognition of these molecules as substrates[4]. Among those, direct hydrogen bonds established between the substrate and N-terminal residues of the I helix of OleP appears to be responsible for the efficient activation of the open-to-closed structural transition that enables the enzyme to adopt a productive conformation. The comparison of the two-steroid bound OleP structures, interpreted in the light of the results of the binding assays and MD simulations, provide a structural explanation for the different selectivity of the enzyme against these compounds.

Our results represent a significant advance in the understanding of the molecular rules that govern substrate recognition by OleP. The structures of OleP bound to TES and LCA provides a basis for future engineering work of the enzyme aimed at enhancing regio- and stereoselectivity of hydroxylation towards valuable steroids.

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Poster 11

Genetic and non-genetic factors influencing olanzapine metabolism by CYP1A2

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The drug-metabolizing CYP1A2 enzyme abundantly expressed in the liver, are highly polymorphic resulting in substantial variability in pharmacokinetics of CYP1A2 substrate drugs. We observed significant inter-individual differences in CYP1A2 activities and mRNA levels in human liver tissue samples as well as in psychiatric patients belonging to the Caucasian population. This variability is often attributed to genetic factors; however, the contribution of *CYP1A2* polymorphisms to CYP1A2 phenotype (CYP1A2 enzyme activity) is controversial. Therefore, we aimed to investigate the effect of *CYP1A2* single nucleotide polymorphisms (-3860G>A, -2467delT, - 739T>G, -163C>A, 2159G>A) on the CYP1A2 selective phenacetin *O*-dealkylation and mRNA expression as well as on CYP1A2substrate olanzapine blood concentrations. The *CYP1A2*1F* allele considered to be associated with increased CYP1A2 inducibility is generally identified by the presence of -163C>A polymorphism; however, -163C>A existed in several haplotypes (*CYP1A2*1F*, *CYP1A2*1L*, *CYP1A2*1M*, *CYP1A2*1V*, *CYP1A2*1W*); consequently, *CYP1A2*1F* was a less prevalent variant than reported in Caucasian populations (0-0.4% *vs* 32-57%). None of the tested *CYP1A2* polymorphisms had a significant effect either on CYP1A2 activity or on olanzapine plasma concentrations in patients with psychiatric disorders. Furthermore, significant correlation was observed between CYP1A2 activities and mRNA levels in the liver and those in the leukocytes, as well as between the CYP activities/expression and some non-genetic factors. CYP1A2-inducing smoking, chronic alcohol consumption and amoxicillin+clavulanic acid are known to have a significant effect on CYP activity, and consequently on pharmacokinetics of CYP-substrate drugs. We have clearly demonstrated that CYP1A2 expression and related smoking showed a strong correlation with olanzapine plasma concentrations in patients with psychiatric disorders. These results demonstrated that revealing the relevant factors in CYP activities and considering both genetic and non-genetic factors can contribute to personalized pharmacotherapy adjusted to the patients' drug-metabolizing ability, and can facilitate to avoid adverse drug reaction or the lack of therapeutic effect due to inappropriate dosing.

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Poster 12

Development of a High Throughput screen for cytochrome P450 ligand binding assays

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Human cytochrome P450 enzymes are membrane-embedded monooxygenases often playing roles as drug targets or drug metabolizing enzymes. For both, characterization of allowable interactions between an individual P450 active site and small molecule physicochemical features is key to understanding and potentially manipulating enzymatic functionality. The most common method for characterizing small molecule binding is by quantifying absorbance changes that commonly occur when substrates bind the P450 active site and cause spin stage changes in the heme iron. Traditional ligand titrations monitored by a spectrophotometer require significant manual time and increasing solvent concentrations. This assay has therefore been adapted for semi-automated high throughput screening, which increases the compounds examined 50-fold with constant solvent concentrations, while keeping total protein required equal. The 384-well assay was validated for both type I and II shifts typically observed for substrates and heme-coordinating inhibitors respectively. A library of ~100 azoles was assembled and screened with three human drug- and sterol-metabolizing P450 enzymes: CYP2A6, CYP2D6, and CYP8B1. Absolute spectra were collected across an 11-point titration for each compound. An R script corrects spectra for baseline absorbance and generates difference spectra by subtracting ligand-free P450 absorbance from each absolute spectrum, illustrating the change in absorbance due to ligand binding. Since azoles are frequent P450 ligands, it is not surprising that 42% to 57% bound to the different P450 enzymes. Absorbance differences plotted against ligand concentration yield dissociation constants (K_d). K_d values are used to generate pharmacophores for each P450 active site, for comparison to known structures for these three enzymes in the Protein Data Bank. The high throughput screen is thus useful for efficiently identifying and ranking ligands for P450 enzymes, facilitating generation of pharmacophores, identifying ligand profiles for P450 deorphanization, and screening potential drugs either to establish P450s as drug targets or to avoid P450 metabolism.

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Poster 13

Pesticides as endocrine disrupting chemicals: effect on aromatase activity and on HT29 cells viability

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In the European Union around 355000 tons of pesticides were sold in 2021[1]. Pesticides are used to protect crops and to increase production, but less than the 1% of them reach the target organisms, while the rest pollute water or soil ecosystems: residues of pesticides or their metabolites have been found in soil, air, water, drinking water, indicating an increasing risk of human exposure[2]. In a recent study, glyphosate, its metabolite AMPA and terbuthylazine were found in a high number of water samples in Italy (83%, 67% and 67%, respectively)[1]. An emerging risk related to pesticides exposure is due to the evidence that some of them may act as endocrine disrupting chemicals, molecules that interfere with hormone synthesis and signalling: for example, glyphosate has been shown to inhibit aromatase activity and to induce estrogenic activity[3].

Aim of this study was to test selected pesticides (glyphosate and its metabolite AMPA, quinclorac, terbuthylazine) for their ability to interfere with aromatase (CYP19A1), a key enzyme in estrogen synthesis, and to investigate their effect on human colorectal adenocarcinoma cell line (HT29). The pesticides were selected among those most found in water, while the cell line was chosen because it expresses aromatase[4] and because the gastrointestinal tract is a target of pesticide toxicity.

The interaction between pesticides and aromatase was assessed by spectroscopic binding assay: results showed type I binding by glyphosate[3] and AMPA; in the aromatase activity assay AMPA and terbuthylazine did not induce a significant effect, while quinclorac exerted an inhibitory effect only at high concentrations. In HT29 cells, dose- and time-dependent cytotoxic effects of the pesticides were evaluated by crystal violet staining and MTT assay. Results showed that glyphosate and its metabolite AMPA induced proliferation of HT29 cells under the experimental conditions tested, while quinclorac and terbuthylazine inhibited cell viability dose-dependently.

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Poster 14

Design of self-sufficient protein chimeras improves the catalytic performance and stability of P450-SPα and CYP116B5

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The exploitation of enzymes as industrial biocatalysts is appealing but often unfeasible because of the relatively low turnover and poor stability of these systems. Fusion proteins are a powerful tool to combine two or more enzyme functionalities in a cascade reaction, which may result in the increase of the catalytic performance of a multi-enzyme system. Our laboratory pioneered the *Molecular Lego* approach, by which we engineer enzyme "chimeras" where two non-physiological partner enzymes are joined together in a single polypeptide chain. Herein, we joined two P450 peroxygenases - P450-SPα[1] and CYP116B5-hd[2,3] - to sarcosine oxidase (SOX) through different protein-protein linkers, which differed for the aminoacidic sequence and structural rigidity. We showed that not only the peroxygenase activity of the two CYPs can be enhanced thanks to the continuous and controlled production of H_2O_2 in-situ guaranteed by SOX activity, but also the stability in solution of the multi-enzyme system can be improved acting on the protein-protein linker rigidity. Indeed, the k_{cat} for the conversion of *p*-nitrophenol increased by 7.5 and 12.1 times using the CYP116B5-flexible-SOX (20.1 ± 0.6 min^{-1}) and CYP116B5-rigid-SOX (32.1 ± 0.5 min^{-1}) respectively compared to the isolated CYP116B5-hd (2.7 ± 0.1 min^{-1}) [4]. The SPα-flexible-SOX outperformed by 3 times the isolated SPα[5] in terms of TON for lauric acid hydroxylation (TON: 6,800 and 2,300 respectively). Furthermore, the SPα-rigid-SOX displayed an increase in the T_{onset} of 10°C and an increase of 227 cal/mol of the unfolding enthalpy measured by differential scanning calorimetry (DSC). Residual activity experiments also demonstrated a 5.7 °C increase of the T_{50} for the SPα-rigid-SOX and an increase of the folding cooperativity for CYP116B5-rigid-SOX compared to the respective flexible constructs. Taken together our results demonstrate that the newly designed multi-enzyme system can beneficially affect the catalytic performances and stability of the P450.

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Poster 15

Discovery of 16(S)-Lipoxygenase/16-hydroperoxide lyase pathway in green tissues of cucumber (Cucumis sativus L.) plants

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Hydroperoxide lyase (HPL) belongs to the non-classical cytochromes P450 of the CYP74 family, which, unlike most P450 monooxygenases, do not require molecular oxygen and redox partners for their catalytic activity. Substrates, hydroperoxides of polyunsaturated fatty acids, act as donors of oxygen and electrons. HPL and lipoxygenase (LOX) form the HPL-branch of the LOX pathway, leading to the formation of C6 and C9 aldehydes and alcohols, as well as ω-oxoacids. These compounds play important roles in plant-plant and plant-herbivore interactions, function as signals activating systemic defense, and are involved directly in plant defense as antimicrobials and fungicides. A number of these compounds are specifically named Green Leaf Volatiles (GLVs). They cause a characteristic odor that occurs when leaves are crushed or otherwise injured. They are valuable chemicals that are used in the food and perfume industries.

Plant LOXs are classified as 9- or 13-LOXs according to the products of positional specific oxygenation. Thus, there are 9- and 13-HPL branches of the LOX pathway. GLVs are formed during the 13-LOX/HPL branch.

We have discovered a new branch of metabolism in the transformation of linole(n)ic acids, namely the 16-LOX/HPL branch. The cucumber plants as well as the recombinant cucumber HPL CYP74B6 possessed unprecedented 16-HPL activity, cleaving 16-HPOT into a C15 fragment, 15-oxo-9,12-pentadecadienoic acid, and a complementary volatile C3 fragment, propionic aldehyde. The 16-LOX/16-HPL route of oxylipin biosynthesis presents a novel facet of the plant LOX pathway.

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Poster 16

Investigating the tolerance of crosslinking cytochrome P450s in the chemoenzymatic synthesis of vancomycin analogues

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Glycopeptide antibiotics (GPAs), including vancomycin, are antibiotics of last resort against Grampositive bacteria. Unfortunately, antimicrobial resistance towards GPAs has developed, creating a need to develop effective treatments against resistant pathogens. Currently the only scalable method for GPA production is via their natural biosynthetic pathway, in part due to the side chain crosslinking present in GPAs. Given this, we sought to evaluate the tolerance of the cytochrome P450s responsible for this crosslinking in novel substrates *in vitro*. This work aimed to explore the acceptance of nitrogencontaining vancomycin analogues by crosslink forming cytochrome P450s (Oxys) involved in vancomycin biosynthesis. The previous development of an *in vitro* chemoenzymatic strategy allowed substrate tolerance of modified vancomycin peptide substrates by Oxy enzymes to be assessed. Recently, we have shown that eight new nitrogen-containing linear analogues were well tolerated by the first crosslinking enzyme of the vancomycin biosynthetic cascade, $OxyB_{bal}$. Analogues with an aniline involved in crosslinking were better tolerated by $OxyB_{bal}$ compared to those where nitrogen was incorporated as a pyridyl ring. The second crosslinking enzyme, $OxyA_{ris}$, showed lower activity towards the analogues tested, in line with previously reported $OxyA_{ris}$ tolerance towards modifications in this region. In this work, novel crosslinks that extend beyond the anticipated products were generated by the cytochrome P450 enzymes, providing insights into the crosslinking capabilities of these enzymes. Furthermore, the formation of unusually crosslinked products has implications on the proposed mechanism of action and interactions between the cytochrome P450s involved in this biosynthetic pathway. This work demonstrates the scope for nitrogen-containing modifications to be made to the Nterminal region of vancomycin to generate novel antibiotics with the potential to overcome antimicrobial resistance.

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Poster 17

Phylogenomic and functional characterisation determine the extent of conservation of a cytochrome P450-based insecticide detoxification mechanism in bees

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Recent work has demonstrated that many bee species have specific cytochrome P450 enzymes that can efficiently detoxify certain insecticides. The presence of these P450s, belonging to or closely related to the *CYP9Q* subfamily (*CYP9Q*-related), is generally well conserved across the diversity of bees [1]. However, the alfalfa leafcutter bee, *Megachile rotundata,* lacks *CYP9Q*-related P450s and is 170-2500 times more sensitive to certain insecticides than bee pollinators with these P450s [2]. The extent to which these findings apply to other Megachilidae bee species was uncertain. To resolve this knowledge gap, we sequenced the transcriptomes of four *Megachile* species and leveraged the data obtained, in combination with publicly available genomic data, to investigate the evolution and function of P450s in this poorly resolved family. Our analyses revealed that several Megachilidae species, belonging to the Lithurgini, Megachilini and Anthidini tribes, including all species of the *Megachile* genus, lack *CYP9Q*-related genes [3]. In place of these genes *Megachile* species have evolved phylogenetically distinct *CYP9* genes, the *CYP9DM* lineage. Functional expression of these P450s from *M. rotundata* reveal they lack the capacity to metabolise the neonicotinoid insecticides thiacloprid and imidacloprid. In contrast, species from the Osmiini and Dioxyini tribes of Megachilidae have *CYP9Q*-related P450s belonging to the *CYP9BU* lineage that are able to detoxify thiacloprid. These findings provide insight into the evolution of P450s that act as key determinants of insecticide sensitivity and have applied implications for pesticide risk assessment.

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Poster 18

Study on the interaction between fungicides and plant cytochromes P450.

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Synthetic pesticides are toxic compounds used in order to control pests and protect plants from diseases. Their use has increased as climate change negatively affects plants by decreasing their resistance to diseases and favouring the spreading and emergence of pathogens[1–3]. However, these substances might be toxic also for plants themselves by altering their metabolism. In particular, they might interfere with cytochromes P450 (CYPs) activity[4] which are especially important for production of secondary metabolites and in plant defence mechanisms[5,6].

In this study the interaction of fungicides with plants CYPs was investigated in two relevant crop plants, namely grape and rice. Grape is one of the most consumed fruits and it is especially important to produce high quality wine with health-promoting properties[7]. Rice is one of the three major crops with wheat and maize representing the base of human diet. From both plants, two enzymes belonging to atypical CYP74 family have been chosen. These enzymes are involved in lipoxygenase (LOX) pathway which leads to the synthesis of oxylipins. These molecules are remarkably important in defence, development, and growth.[8,9] In particular, CYP74C3 (VvAOS3) from grape and CYP74A2 (OsAOS2) from rice have been selected. Both CYP74C3 and CYP74A2 are two Allene Oxide Synthases (AOSs) which catalyse the dehydration of fatty acid hydroperoxides to epoxides[10]. In this work, these enzymes have been successfully expressed in *Escherichia coli* and purified through affinity chromatography. Pure enzymes have been used to screen a list of currently used fungicides which might interfere with enzymatic activity of CYPs. These compounds have been also tested on bacterial BM3 (CYP102A1) which has been chosen as CYPs model.

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Poster 19

The flexibility and dynamics of binding sites in plant cytochrome P450s

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Binding site flexibility and dynamics strongly affect the ability of proteins to accommodate substrates and inhibitors. The significance of these properties is particularly pronounced for proteins that are inherently flexible, such as cytochrome P450 (CYPs). These enzymes, found in a wide range of organisms, have been most thoroughly characterized in humans. Previous studies have demonstrated that the versatility of reactions catalyzed by human CYPs and their promiscuity towards substrate and inhibitors, depends on the characteristics of their binding site, including its dynamics and malleability. Accordingly, understanding the intricacies of CYP binding sites allows for accurate assessments of enzyme-substrate interactions.

Bearing in mind that it is a highly conserved class of proteins, this research aims to provide a concise overview of plant CYPs, the understudied counterparts to the human ones. The dynamics of selected CYPs is observed by performing molecular dynamics simulations with a special focus on the binding site. Due to the limited number of experimentally determined structures, the initial structures for the simulations were predicted by AlphaFold 2. The changes in the substrate binding site through simulation snapshots are recorded in terms of size, flexibility, dispersiveness, accessibility and hydrophobicity. The same pipeline was applied to human CYP3A4, CYP1A2, and CYP2A6 to validate the findings.

After collecting the binding site characteristics, the plant CYPs were categorized based on their (dis)similarities into a "flexibility map". These results lead a way to evaluate whether a certain small molecule (e.g. substrate or inhibitor) might be accommodated by a CYP of interest and how selective the enzyme is in its interactions.

Poster 20

Engineering P450-cascade reactions for lignin biodegradation: the role of laccase from *Acinetobacter radioresistens*

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Lignin is a complex aromatic polymer cementing the cell walls of plants, giving them rigidity and resistance to decay. Mainly produced as a waste from the pulp and paper industry, it is normally used as a fuel to power paper mills, despite having a huge potential as a source of high value aromatic compounds.[1] Its controlled biodegradation requires many enzymes, among which cytochromes P450 play a fundamental role in the aromatic compound hydroxylation and demethylation reactions.[2] However, in order to achieve this type of degradation, the lignin architecture must be efficiently deconstructed to obtain aromatic units or aromatic monomers. These reactions can only take place thanks to the action of the lignin modifying enzymes (LME) which are the core lignin degrading enzymes such as the multicopper oxidase laccase, one of the extracellular glycoprotein enzymes that helps to degrade lignocellulosic material in cooperation with other enzymes as lignin peroxidase (LiP), manganese peroxidase (MnP). [3]

Laccase acts on phenolic substrates by catalysing the oxidation of their phenolic hydroxyl groups to phenoxy radicals while O_2 is reduced to water. Here laccase from *Acinetobacter radioresistens* was cloned in a plasmid encoding for the wild type protein with a C-terminal His-tag, the clones were expressed in *Escherichia coli* BL-21; nickel-affinity purification protocols for the recombinant protein were developed and optimized, giving a good yield of the active form of the His-tag WT laccase. The activity of the laccase was studied with two substrates, 2,2'-azino-bis3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and Syringaldazine (SGZ) using the purified protein. A selection of crystallization screening is tested with the robot Crystal Gryphon LCP (Art Robbins Instrument), to obtain laccase crystals for structure determination.

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Poster 21

P450 library enabled synthesis of biaryl natural products

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Biaryl scaffolds are prevalent in a vast array of drug molecules and natural products. The unique structures and promising bioactivities of biaryl natural products make them highly valuable compounds, however this structural feature contributes to their difficulty as synthetic targets.[1-3]

To overcome the challenges associated with constructing biaryl compounds through traditional synthesis, we propose a chemoenzymatic approach toward this class of molecules. This work describes the development of P450 enzymes into tunable biocatalysts for convergent oxidative coupling reactions with catalyst-controlled selectivity. In particular, the discovery and characterization of previously unknown P450s form a diverse library of sequences has led to the site-selective generation of biaryl bonds, relevant for the total synthesis of many classes of natural products with known therapeutic potentials. This approach allows for the rapid generation of molecular complexity in natural and novel biaryl compounds, with increased accessibility and structural diversity, thereby accelerating the therapeutic development of these bioactive molecules.

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Poster 22

Expression of CYP153A6 monoxygenase in Acetic Acid Bacteria allows the production of carboxylic acids starting from alkanes

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Enzymatic hydroxylation mediated by cytochrome P450 monooxygenases has been extensively studied since only molecular oxygen and reducing equivalents from NAD(P)H are requested for the reaction, which often occurs with high chemo- and regioselectivity. Even if many genetic approaches have been developed for the functional expression of CYP450 (together with cofactor regeneration systems and alkane transporter[1]), low biocatalytic efficiency and low enzymatic stability were frequently reported. Here we present the development of genetically modified acetic acid bacteria (AAB) as biocatalysts for the functionalization of methyl groups in the allylic position of different terpene derivatives (e.g., limonene, carveol, carvone) and non-functionalized methyl groups of aromatic substrates (e.g., toluene, xylenes, *p*-cymene, *p*-ethyl toluene).

We engineered two acetic acid bacteria (AAB) strains (*Acetobacter malorum* and *Komagataeibacter xylinus*) with a plasmid (based on the pSEVA331Bb vector backbone) encoding for a limonene monoxygenase operon from *Mycobacterium* spp. [1], including genes for the expression of a CYP153A6, a ferredoxin and a ferredoxin reductase for cofactor recycling.

The resulting recombinant strains were able to hydroxylate different terpenes thanks to the monooxygenase activity and to further oxidize alcohols into the corresponding aldehydes and carboxylic acids, exploiting the action of the AAB's unspecific membrane-bound alcohols (ADH) and aldehydes (ALDH) dehydrogenases[2]. Their activity towards *(S)*- and *(R)*-limonene was compared with a recombinant strain of *E. coli* expressing the same genes[2]: whereas the use of the *E. coli* strain allowed for the preparation of perillyl alcohol, recombinant AABs gave complete oxidation of both the enantiomers of limonene to perillic acid, with transient formation of perillyl alcohol and perillaldehyde.

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Poster 23

Tracing the evolutionary path of CYP4B1: Structural and functional adaptions/changes across species

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CYP4B1 exhibits activity towards endobiotic and xenobiotic substrates, as it mediates the hydroxylation of medium-chain fatty acids and the activation of protoxins.[1,2] Remarkably, while the rabbit ortholog hydroxylates fatty acids at multiple positions and with high turnover rates, the human ortholog is catalytically inactive, which was attributed at least partially to an amino acid substitution of an evolutionary conserved proline in the meander region by serine (p.P427S).[3]

Our research aims to trace the apparent evolutionary divergence in CYP4B1, to clarify at which point(s) adaptations/changes in the *cyp4b1*-gene occurred that led to changes in - or complete loss of - enzyme activity. Therefore, CYP4B1 orthologs from prosimians, old world monkeys and great apes were expressed in *E. coli* and purified, and their activity was tested by *in vitro* reconstitution with a variety of endobiotic substrates. Additionally, the orthologs were tested *in vivo* in human cell lines against protoxic substrates, distinguishing between active and inactive orthologs in cell survival assays.

Within the selected primate orthologs, we identified an evolutionary point where CYP4B1 lost its catalytic activity. The active recombinant orthologs revealed marked differences in activity and positional selectivity during fatty acid hydroxylation, in particular with regard to the ω/ω_{-1} ratio. Sequence and structure provided insights into the respective changes underlying the observed product distributions resulting from lauric acid hydroxylation.

This study highlights the importance of evolutionary divergence in shaping enzyme functionality and sheds light on the substrate selectivity and product diversity of CYP4B1 across species. Such comparative analyses provide valuable insights into the molecular mechanisms underlying metabolic pathways and highlight the interplay between sequence variation and enzymatic activity.

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Poster 24

The function of P450 enzymes in malaria and other vector-borne infectious diseases

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Vector-borne infectious diseases such as malaria, Zika virus disease, dengue fever, yellow fever disease, West Nile virus disease, Japanese encephalitis, leishmaniasis, and others continue to pose a significant global health concern. Malaria is standing out as the foremost threat, particularly affecting pediatric populations and posing life-threatening risks. Parasites, bacteria, and viruses transmitted by vectors contribute to a substantial burden on public health and incur significant economic costs. The vector control, with consideration for ecological and biodiversity conservation, has the potential to prevent most vector-borne diseases. While chemical control using pesticides and insecticides is commonly utilized as a prevention measure, the escalating resistance to insecticides poses a significant challenge in vector control efforts. Metabolic resistance, primarily through insect enzyme systems such as CYP P450 enzymes (e.g. Anopheles gambiae CYP4, 6, 9, 12, 314 and 325 families)[1], presents a major obstacle. These enzymes are crucial for metabolizing insecticides, leading to resistance. The identification and utilization of natural inhibitors or blockers specific to vector P450 enzymes, alongside conventional pesticides, offer a promising avenue for environmentally friendly insecticide practices. The exploitation of host CYP enzymes, which possess detoxification properties and are involved in immune responses and other biological processes (e.g. CYP1, 2, 3 and 4 families)[2,3], offers an additional strategy for combating vector-borne diseases.

Here, we summarize the known data on P450 enzymes from all contributors to vector-borne infections, including pathogens, vectors, and hosts, exploring the potential involvement of CYPs in disease progression[4].

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Poster 25

In silico docking of dihydrobacillaene to CYP107K orthologs

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The CYP107K enzymes in *Bacillus subtilis* are responsible for the biosynthesis of the known antibiotic bacillaene[1]. In this study, we examined interactions between dihydrobacillaene, the substrate in this process, and the CYP107K enzyme. In addition to the protein from *B. subtilis*, we also investigated its homologs found in *B. atrophaeus* and *B. amyloliquefaciens*. While there is an experimental 3D structure available for *B. subtilis* protein, homolog modeling was used for *B. atrophaeus* and *B. amyloliquefaciens*. Ligand docking was performed with Autodock Vina[2] and for the best docking poses molecular dynamics simulations were run in Gromacs[3] with Amber force field[4]. Analysis of the simulation results revealed the positioning of the ligand within the binding pocket and provided insights into the ligand-enzyme interactions.

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Poster 26

The CYP74 enzymes of lancelets: the way from plants to animals

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The oxidative metabolism of polyenoic fatty acids in aerobic organisms via the lipoxygenase pathway produces diverse oxylipins. Fatty acid hydroperoxides, the lipoxygenase products, undergo numerous secondary conversions. In plants, these conversions are mainly controlled by the CYP74 enzymes, including allene oxide synthase (AOS), hydroperoxide lyase (HPL), divinyl ether synthase (DES), and epoxyalcohol synthase (EAS). Recently, related enzymes were also discovered in a-proteobacteria, stony corals, sea anemones, and lancelets. All these CYP74-related proteins possessed less than 40% homology to CYP74 family members and thus were attributed to other novel P450 families, constituting the CYP74 clan alongside the CYP74 family [1].

Many marine organisms possess a great diversity of oxylipins. Lancelet oxylipins are poorly studied yet. Nevertheless, lancelet genomes encode various putative enzymes involved in oxylipin biosynthesis (including octadecanoids). Lancelets are so far the only Chordata known to possess the CYP74 clan genes. The genomes of the lancelets *Branchiostoma floridae* and *B. belcheri* possess 20 and 10 genes, respectively. Two CYP74 clan genes of lancelets have been previously cloned. CYP440A1 of *B. floridae* was identified as an EAS. CYP440A18 of *B. belcheri* was shown to possess EAS/AOS activity [2].

The genome of the European lancelet (*B. lanceolatum*) also possesses genes for CYP74 enzymes. In our laboratory, transcriptomic data for this organism under osmotic stress were obtained, and the profile of oxylipins was analyzed. Based on these data, as well as on data from the analysis of catalytically essential domains, a gene was selected for cloning (Bl10054), and the corresponding recombinant enzyme was obtained (CYP440A19). CYP440A19 exhibits versatile catalytic capabilities towards different C18 fatty acid hydroperoxides and produces several oxylipins, including some unusual ones (macrolactones and diols (leukotriene-like products)). The results of this study make a significant contribution to the understanding of the functioning of the lipoxygenase cascade in members of different kingdoms.

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Poster 27

Engineering *E. coli* for hydroxylation of propane with cytochrome P450BM3 and decoy molecules

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Gaseous alkanes are expected to be used as starting materials in industrial chemistry by introducing functional groups such as hydroxyl groups. To hydroxylate gaseous alkanes directly under mild conditions, our research group has focused on cytochrome P450BM3 (CYP102A1, P450BM3). P450BM3 is a self-sufficient P450 isolated from *P. megaterium* and it catalyses hydroxylation of longchain fatty acids at an extremely high rate. Due to these desirable properties, this enzyme has been engineered to catalyse the oxidation of non-native substrates. While many researchers attempt to achieve non-natural reactions by P450BM3 mutants, we have succeeded in altering the substrate specificity of wild-type P450BM3 by employing synthesized dummy substrates named decoy molecules. Decoy molecules bind to P450BM3 in the same manner as native substrates and activate the enzyme. Since decoy molecules are shorter than native substrates, non-native substrates such as benzene and propane can be taken into the reaction space and hydroxylated.[1]

However, the reaction requires a stoichiometric amount of expensive cofactor, NADPH, as electron donors. To overcome this problem, we conceived of employing whole-cell catalysis and developed a biotransformation system of benzene derivatives by E. coli overexpressing P450BM3.[2]

In this research, we adapted whole-cell catalysis to hydroxylation of propane. Since propane is a gas and the reaction mechanism is slightly different from that of benzene, we adjusted the reaction method and conditions to hydroxylation of propane and succeeded in the biotransformation of propane to propanol.[3] Then we developed a screening method for decoy molecules. Because it is practically difficult to maintain a high concentration of propane dissolved in water during microplate-based screening, we have introduced an aqueous/ionic liquid biphasic system in which ionic liquid can store large amounts of propane and work as a propane pool. Also, we optimised the expression levels of the enzyme and the regeneration rate of the coenzyme.

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Poster 28

Atypical cytochromes P450 of the CYP74 clan and their role in evolutional history

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Cytochromes P450 are widespread in nature. Classical cytochromes P450 are monooxygenases, which catalyze electron transfer from NAD(P)H to molecular oxygen and the regio- and stereospecific incorporation of an oxygen atom into the substrate. In contrast, the nonclassical P450s of the CYP74 clan require neither molecular oxygen nor external electron donors for their catalytic activity. They use fatty acid hydroperoxides, which serve both as a substrate and an oxygen donor. The CYP74 clan includes allene oxide synthase (AOS), hydroperoxide lyase (HPL), and divinyl ether synthase (DES). These enzymes play a key role in the lipoxygenase cascade, one of the pathways of oxidative metabolism of unsaturated fatty acids, the products of which are oxylipins, one of the most important classes of bioregulators in various aerobic organisms. Oxylipins are bioregulators that maintain homeostasis at the cellular and organismal levels. The most important oxylipins are mammalian eicosanoids and plant octadecanoids. The most well-studied plant oxylipins are jasmonates (AOS products) and traumatin and green leaf volatiles (HPL products), whereas other oxylipins remain outside of the focus of researchers' attention. Among them, there is a large group of epoxy hydroxy fatty acids (epoxyalcohols), whose biosynthesis has remained unclear for a long time.

In 2008, the first epoxyalcohol synthase (EAS) of lancelet *Branchiostoma floridae*, BfEAS (CYP440A1), was discovered. The present report collects data on EASs discovered after BfEAS and enzymes exhibiting EAS activity along with other catalytic activities. This report also presents the results of a study on the evolutionary processes possibly occurring within the P450 superfamily as a whole.

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Poster 29

Ar-CYP153A, a new cytochrome P450 system from *Acinetobacter radioresistens*: biochemical characterization and development of green catalytic processes

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Cytochromes P450 153 (CYP153) represent a family of bacterial enzymes capable of medium to longchain *n*-alkane hydroxylation. These heme-containing enzymes allow the activation of carbon rich but highly stable molecules, which then can enter different metabolic pathways. CYP153 genes evolved in those microorganisms able to grow in harsh environments, such as oil polluted areas. In this study, we identified a new CYP153A in *Acinetobacter radioresistens* genome, Ar-CYP153. As its homologs CYP153A71 and CYP153A33, Ar-CYP153 is also a three-protein system. In this system a ferredoxin reductase (Ar-FdR) collects the electrons from NAD(P)H, passes them to a ferredoxin (Ar-Fd) which eventually triggers the catalytic cycle of the cytochrome. Based on the homology with previously characterized CYP153 systems, Ar-CYP153 is supposed to perform not only the terminal hydroxylation of medium-chain alkanes to 1-alkanols[1], but also the terminal and subterminal oxy-functionalization of various fatty acids[2].

The system was expressed in *Escherichia coli* following a dual plasmid strategy. Lysates of the 3component enzymatic system were employed to assess the activity towards different substrates. We also tested the ability of Ar-CYP153 other classes of compounds, such as indole, which can be exploited for indigo dye production. Ar-CYP153 was then characterized in a whole cell setup. To ensure a constant regeneration of the reducing cofactors, a glucose dehydrogenase (GDH) was co-expressed alongside with the Ar-CYP153A catalytic system. A second approach was also considered, involving the metabolic production of reducing equivalents by boosting of *E. coli* TCA cycle[3]. Resting cells were employed to produce higher quantities of the industrial important substrate.

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Poster 30

Non-steroidal CYP17A1 inhibitors with dual CYP/AKR activity against prostate cancer

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This examination focuses on synthesizing and evaluating compounds that replace the nitrogen-containing heterocyclic ring in the chemical structure of cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1) inhibitors[1] with a phenyl group containing a sulfur-based substituent. Initial screening identified compounds capable of inhibiting CYP17A1 activity. Subsequently, their selectivity against cytochrome P450 21hydroxylase was tested. Additionally, these compounds exhibited modest inhibitory effects on aldo-keto reductase 1C3 (AKR1C3). Their impact on steroid hormone levels was also assessed, revealing synergistic modulatory effects. This research sets the groundwork for developing more potent dual inhibitors targeting both CYP17A1 and AKR1C3.[2]

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Poster 31

Functional characterization of variants in CYP21A2 linked to congenital adrenal hyperplasia

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The 21-hydroxylase (*CYP21A2*) is a cytochrome P450 enzyme involved in glucocorticoid, and mineralocorticoid biosynthesis. The 21-hydroxylase deficiency due to mutations in *CYP21A2* account for majority of cases of Congenital Adrenal Hyperplasia (CAH). The Non-classic CAH (NC-CAH) is a mild condition manifesting as hirsutism, acne, menstrual irregularities, and infertility in females. The NC-CAH is observed when CYP21A2 activity is 20-50% of normal due to *CYP21A2* mutations.

We studied structural and functional changes due novel mutations in *CYP21A2*. We analyzed enzyme activity by transfecting cDNA encoding WT and mutated *CYP21A2* gene into HEK-293 cells and calculated the progesterone to deoxycorticosterone conversion by recombinant CYP21A2. Western blot analysis was used to check protein expression levels. In addition, we assessed changes in the Gibbs free energy of the mutants versus wild type protein using foldX.

Among the mutants from patients, 8 showed less than 50% of WT activity; Our results suggest that presence of these mutations could be associated with NC-CAH phenotype. Only the Leu198Phe mutant is identified as stabilizing showing -1.7 kcal/mol shift in the Gibbs free energy and 129% of WT activity, and is unlikely to be associated with NC-CAH.

In the Samples from ClinVar genome database, of 5 selected mutations located on the surface of 21hydroxylase, all are identified as the de novo and deleterious. Among these mutants, 3 showed less than or around 50% of WT activity; Leu308Val 26% had of WT activity, Arg401Gly 26%, Arg436Cys 21%, and Glu162Gly had 53% of WT activity. Our results suggest that the presence of these mutations may be linked to the NC-CAH phenotype. Only the Ser373Asn mutation showed 75% of WT activity and is less likely to be associated with NC-CAH.

The data obtained provide valuable insights for the prospective diagnosis of NC-CAH due to CYP21A2 deficiency.

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