2.6 Biocatalysis – Emeritus Manfred T. Reetz

Emeritus:

Manfred T. Reetz (born 1943)

Curriculum Vitae: Manfred T. Reetz

1943	Born in Hirschberg (Germany) on August 13, 1943
1965	Bachelor degree, Washington University, St. Louis, USA
1967	Master degree, University of Michigan, Ann Arbor, USA
1969	Doctoral degree, Universität Göttingen with U. Schöllkopf
1971-72	Post-doc with R.W. Hoffmann at Universität Marburg
1973-1978	Assistant Professor at Universität Marburg (including Habilitation)
1978	Guest Professor at University of Wisconsin, USA
1978-1980	Associate Professor at Universität Bonn
1980-1991	Full Professor at Universität Marburg
1989-1990	Guest Professor at Florida State University, Tallahassee/USA
1991-2011	Director at the Max-Planck-Institut für Kohlenforschung, Mülheim/Ruhr
1993-2002	Managing Director of the Max-Planck-Institut für Kohlenforschung
1992-2011	Honorary Professor at Ruhr-Universität Bochum
1993-2011	Chairman of Studiengesellschaft Kohle mbH (SGK)

Awards and Honors

1976	Chemical Industries Prize (Dozentenstipendium des Fonds der Chemischen Industrie)
1977	Jacobus van't Hoff Prize (The Netherlands)
1978	Chemistry Prize of the Academy of Sciences Göttingen
1986	Otto-Bayer-Prize (Germany)
1989	Leibniz Award of the Deutsche Forschungsgemeinschaft
1997-	Member of German National Academy of Sciences Leopoldina
1997	Fluka-Prize "Reagent of the Year 1997"
2000	Nagoya Gold Medal of Organic Chemistry
2001-	Member of Nordrhein-Westfälische Akademie der Wissenschaften
2003	Hans Herloff Inhoffen Medal
2005-	Foreign Member of the Royal Netherlands Academy of Arts and Sciences
2005	Karl-Ziegler-Prize (Germany)
2005	Cliff S. Hamilton Award in Organic Chemistry (USA)
2006	Ernst Hellmut Vits-Prize (Germany)

2006	Prelog Medal (Switzerland)
2007	Honorary Professor at Shanghai Institute of Organic Chemistry (China)
2007	Ruhr-Prize for Arts and Science (Germany)
2009	Lilly Distinguished Lectureship Award (Czech Republic)
2009	Arthur C. Cope Award, ACS (USA)
2009	Yamada-Koga Prize (Japan)
2011	Honorary doctoral degree of Johann Wolfgang Goethe-Universität, Frankfurt (Germany)
2011	Tetrahedron Prize for Creativity in Organic Chemistry
2011	Otto-Hahn-Prize (Germany)
2012	IKCOC-Prize (Japan)
2013	Susi and Barry Trost Lectureship
2014	Chirality Medal
2014	Paul Tarrant Lectureship (University of Florida/USA)
2015	Schulich Distinguished Lectureship (Technion/Israel)
2016	Honorary Member of the Israeli Chemical Society

1980-2016 > 165 Plenary Lectures and Name Lectureships

Other Activities / Committees

1987-1988	Chairman of Chemistry Department, Universität Marburg
1989-1992	Committee Member of Fonds der Chemischen Industrie (Engeres Kuratorium)
1990-1995	Member of the Board, German Chemical Society (GDCh)
1992-1996	Chairman of Selection Committee, August-Wilhelm-von-Hofmann-Prize (Denkmünze, GDCh)
1993-2004	Member of the Scientific Advisory Board, Institut für Katalyseforschung Rostock
1994-1998	Member of Selection Committee, Carl-Duisberg-Prize (GDCh)
1994-1999	Member of Advisory Board, *Nachrichten aus Chemie, Technik und Laboratorium*
1994-2001	Member of Selection Committee, Karl Heinz Beckurts-Prize
1995	Vice-President of German Chemical Society (GDCh)
1997	President of Bürgenstock-Conference

1997-2001	Member of Board, Katalyseverbund NRW
1997-2012	Member of Advisory Board, *Topics in Organometallic Chemistry*
1998-2005	Member of Selection Committee, Emil-Fischer-Medaille (GDCh)
1999-2007	Member of Advisory Board, Catalysis NRSC (The Netherlands)
1999-2005	Chairman of Selection Committee, Adolf-von-Baeyer-Prize /GDCh
1999-	Member of Selection Committee, Alfried Krupp-Prize
1999-2011	Member of Selection Committee, Otto Bayer-Prize (Bayer AG)
2000-	Member of Advisory Board, *Russian Journal of Organic Chemistry*
2000-2005	Member of Advisory Board, *Advanced Synthesis & Catalysis*
2001-2005	Member of Scientific Advisory Board for the School of Engineering and Science, International University Bremen
2002-2010	Member of Editorial Board, *Angewandte Chemie*
2003-2017	Member of the Kuratorium der Alfried Krupp von Bohlen und Halbach-Stiftung
2003-	Member of the International Advisory Board, *QSAR & Combinatorial Science*
2005-2013	Member of the Editorial Advisory Board, *Bulletin of the Chemical Society of Japan*
2006-2011	Member of the Advisory Board, *Topics in Stereochemistry*
2006/2007	Member of the International Advisory Board of the Chemistry Department of Nagoya University (Japan)
2007-2015	Senator of the Chemistry Section, German National Academy of Sciences Leopoldina
2008-2010	Member of Advisory Board of the Karl Ziegler-Foundation (German Chemical Society)
2008-	Member of Ethics Committee of the Max Planck Society
2009-	Associate Editor of Chemistry and Biology
2009-2010	President of BOSS XII
2009-2010	Coordinator of ORCHEM 2010
2011-2013	Speaker of Class I of the German National Academy of Sciences Leopoldina
Since 1980	Member of Advisory Committees of numerous scientific conferences

Research in Biocatalysis: External Emeritus Group of M. T. Reetz

Following formal retirement as Director of the Department of Synthetic Organic Chemistry in August 2011 at age 68, Manfred T. Reetz continued research as the first Hans-Meerwein-Research-Professor in the Chemistry Department of Philipps-University in Marburg. As part of a 5-year contract with participation of the Max-Planck-Society, the MPI für Kohlenforschung and the Philipps-University, the Max-Planck-Society pledged to support 5-6 postdocs with stipends in addition to funds for consumables. The Mülheim MPI offered access to its infrastructure and some additional support for consumables, while Marburg provided lab space, infrastructure and likewise some support for consumables. During this time Reetz was also a member of the LOEWE network SynChemBio, financed by the state of Hessen.

On August 31st 2016 the 5-year contract terminated. Two of the four remaining postdocs with stipends are now financed by the Mülheim MPI until the summer and winter of 2017, respectively. The other two postdocs receive salary support from Reetz third party funding (A. C. Cope Fund/USA) until the summer and fall of 2017, respectively.

The primary topic in the Biocatalysis Group is directed evolution of stereo- and regioselective enzymes as catalysts in organic chemistry and biotechnology. Following proof-of-principle by the Reetz group in 1997, this Darwinian approach to asymmetric catalysis has been generalized, and indeed today numerous academic and industrial groups around the world are actively using the technology. During the last three years, the group has focused on four major projects:

- Methodology development with the aim of making directed evolution more efficient, faster, and therefore highly reliable.
- Applications in synthetic transformations that are problematic or even impossible using state-of-the-art transition metal catalysts or organocatalysts, thereby demonstrating complementarity of the three approaches.
- De novo design of one-pot cascade reactions enabled by directed evolution.
- Learning from directed evolution by performing mechanistic, structural and theoretical studies of selected mutants.

2.6.1 Research Area "Methodology Development in Directed Evolution of Selective Enzymes in Organic Chemistry" (M. T. Reetz)

Involved: C. G. Acevedo-Rocha, R. Agudo, S. Hoebenreich, A. Li, G. Li, A. Ilie, R. Lonsdale, Z. Sun, J. Wang, F. E. Zilly

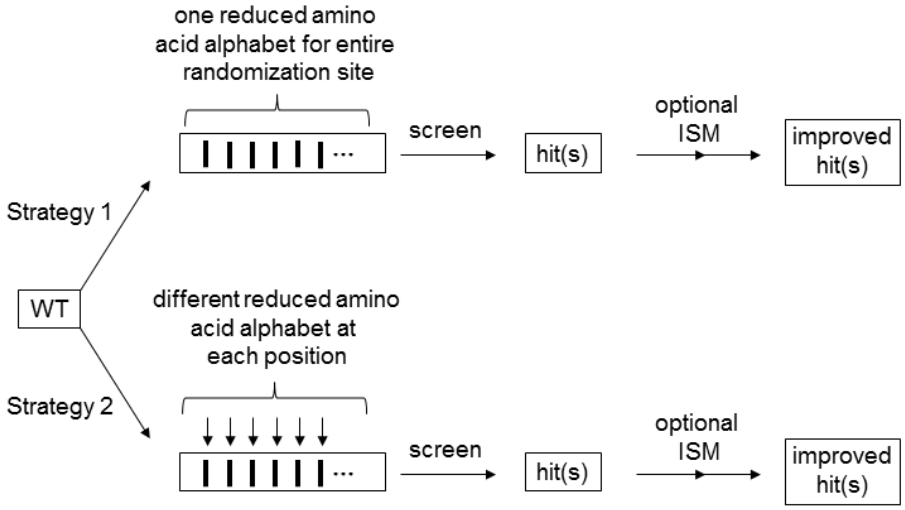
Objective: As part of our intensified continuation of methodology development in directed evolution, new methods and strategies were devised which enable the generation of highest-quality mutant libraries requiring minimal screening (traditionally the bottleneck of directed evolution). The ultimate goal is to develop a "mature" directed evolution in which screening is no longer the slow step in the overall process.
Results: Two fundamentally different approaches to methodology development were considered: 1) Advanced techniques for beating the numbers problem in directed evolution; and 2) Exploring the possibility of solid-phase gene synthesis in mutant library construction as an alternative to the usual molecular biological (genetic) approach.

Beating the numbers problem:
We have previously developed the *C*ombinatorial ***A***ctive-Site ***S***aturation ***T***est (CAST) in which sites comprising 1, 2, 3 or more residues lining the enzyme binding pocket are randomized using codon degeneracies ranging from NNK encoding all 20 canonical amino acids to those that encode reduced amino acid alphabets down to 5-6 amino acids as combinatorial building blocks for the whole site. Application of our CASTER computer aid, based on the Patrick/Firth-algorithm, shows that when using NNK codon degeneracy for saturation mutagenesis, the degree of oversampling for ensuring 95% library coverage rapidly reaches astronomical numbers. These numbers can be reduced sharply upon applying, for example, NDT codon degeneracy encoding only 12 representative amino acids, *but randomizing sites larger than 4-5 residues still requires excessive screening* (Table 1).[2,18,26,27,30]

	NNK (20 aa)		NDT (12 aa)	
number of amino acid positions at one site	codons	transformants needed	codons	transformants needed
1	32	94	12	34
2	1028	3066	144	430
3	32768	98163	1728	5175
4	1.05×10^6	3.14×10^6	20736	62118
5	3.36×10^7	1×10^8	2.49×10^5	7.45×10^5
6	$>1 \times 10^9$	$>3.2 \times 10^9$	$>2.9 \times 10^6$	$>8.9 \times 10^6$
7	3.4×10^{10}	1×10^{11}	3.5×10^7	1.1×10^8
8	1×10^{12}	3.3×10^{12}	4.2×10^8	1.3×10^9

Table 1

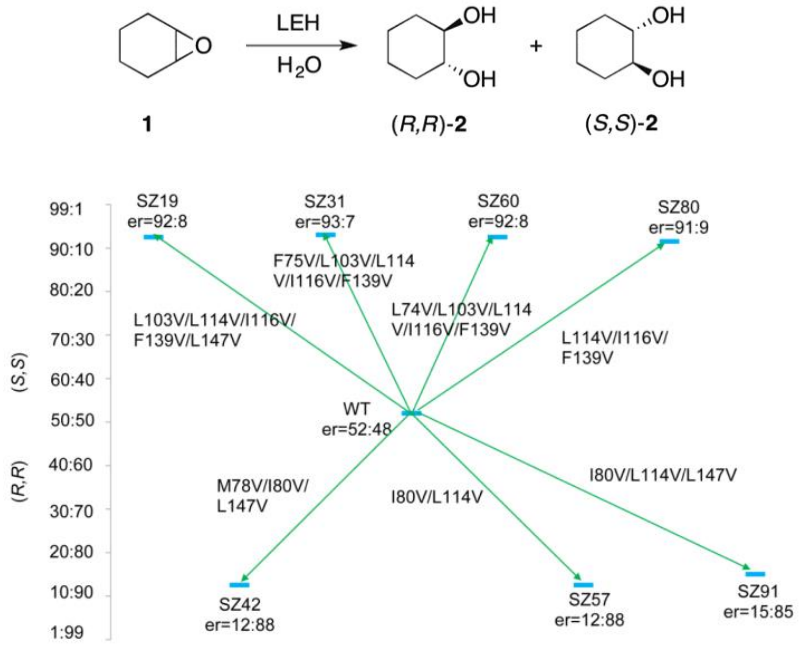
We systematized saturation mutagenesis according to the principle shown in Scheme 1 which features two different strategies.[30,40] Criteria for rationally choosing reduced amino acid alphabets were also developed using *1) Crystal structures, revealing the electronic and steric nature of CAST residues*[18,37]*; 2) bioinformatics consensus information derived from multiple sequence alignment in the CAST region* [18,26,27,37]*; 3) exploratory NNK-based saturation mutagenesis at individual positions at CAST sites requiring in each case only one 96-format microtiter plat*e, revealing which single mutations may be crucial in subsequent combinatorial mutagenesis at large randomization sites.[28,38] The most frequently "occurring" amino acids are then used as reduced amino acid alphabets.



Scheme 1

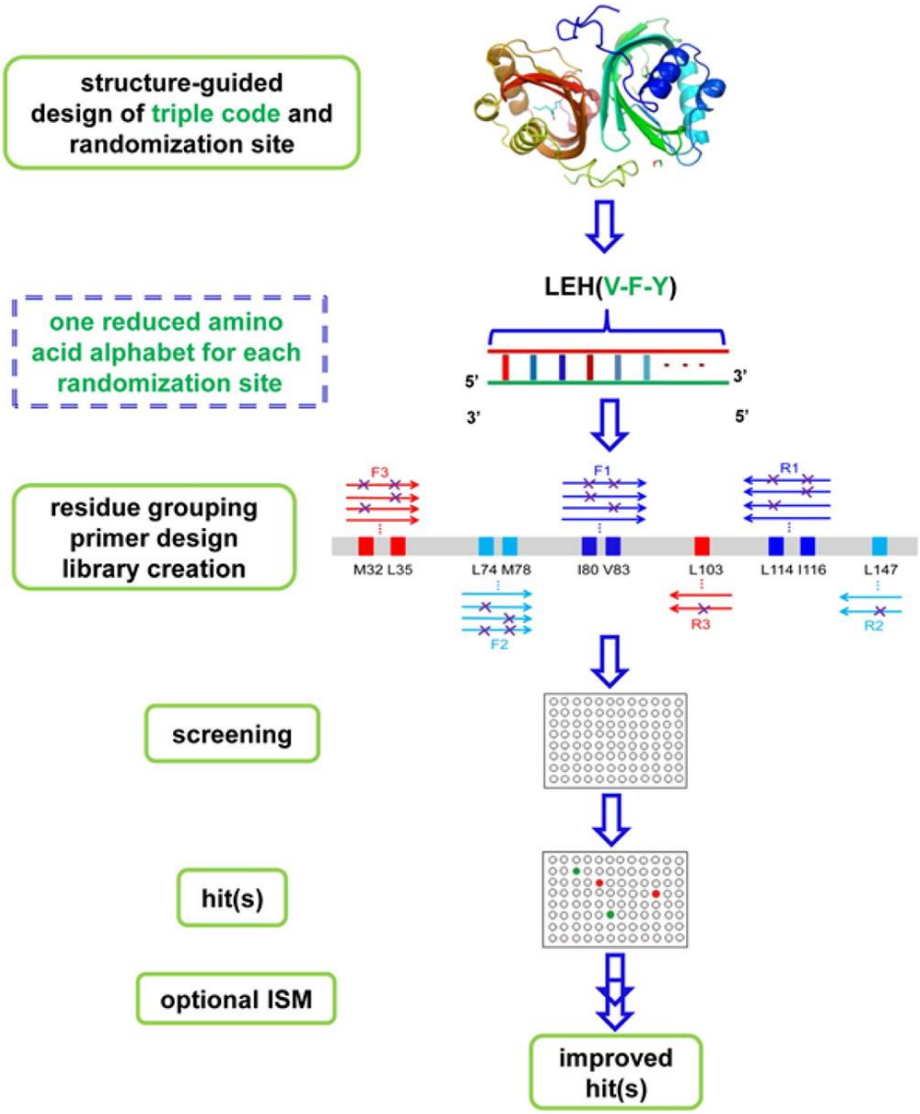
An example of strategy 1 (Scheme 1), in which a single amino acid was used to randomize 10 CAST positions in a single saturation mutagenesis experiment requiring only 3,000 transformants for 95% library coverage concerns the hydrolytic desymmetrization of cyclohexene oxide catalyzed by the limonene epoxide hydrolase (LEH) with formation of the (*R*,*R*)- and (*S*,S)-diols,[18] (Scheme 2). *NNK or NDT would*

have required the screening of $3x10^{15}$ or $2x10^{11}$ transformants, respectively! Since the crystal structure of wildtype (WT) LEH shows essentially only hydrophobic residues surrounding the binding pocket, *valine as a bulky hydrophobic amino acid was chosen as the sole building block.* Scheme 2 shows that excellent results were obtained in one and the same small library generated by single code saturation mutagenesis (SCSM). [18] In the case of the best (*R,R*)-selective mutant (er = 12:88), only one round of iterative saturation mutagenesis (ISM) boosted stereoselectivity to er = 2:98. [18] Typically 3 or 4 valines were introduced at different CAST positions in a given improved mutant, which causes notable changes in the shape of the binding pocket, thereby inducing a specific orientation as the reactive pose of the substrate. The best mutants were also tested in the reaction of other structurally different epoxides.



Scheme 2

Although the extreme case of SCSM proved to be successful,[18] we do not recommend it as a general guide. Instead, *triple code saturation mutagenesis (TCSM) involving three rationally chosen amino acids appears to be a viable compromise between structural diversity and screening effort*, provided appropriate choices of reduced amino acid alphabets are made.[27,28,38] Using the same LEH-based platform, but this time with valine/phenylalanine/tyrosine (V-F-Y) as building blocks in TCSM (Scheme 3), even better results were obtained with less screening.[27]



Scheme 3

Importantly, TCSM was also tested using two other enzyme types in reactions of synthetically challenging substrates, again requiring only small libraries screened by automated chiral GC:

• *ADH as catalyst in the reduction of difficult-to-reduce prochiral ketones.*[28]

• *P450-BM3 as catalyst in the multi-step transformation of cyclohexane into (R)- and (S)-2-dihydroxycyclohexanone.*[38]

On the basis of these developments, we conclude that TCSM, *being step-economical,* is currently the best strategy. It is superior to SCSM,[18] double code saturation mutagenesis (DCSM)[37] and binary patterning.[39] It should be noted that primer design is a rational molecular biological exercise,[1,2,40] but when making decisions in CAST-based directed evolution, the cost of primers also deserves attention, as delineated in our study focusing on techno-economical analyses.[20]

Finally, *an efficient technique for multi-parameter optimization of thermostability, enantioselectivity and activity was developed*, in which saturation mutagenesis was performed at CAST sites for enantioselectivity and activity and simultaneously at remote residues displaying the highest flexibility as judged by B-factors (B-FIT technique) for stability.[31]

Solid-phase gene synthesis in mutant library construction:
Driven by international technological developments, the cost of gene syntheses has dwindled in recent years, but synthesizing a thousand potentially enantioselective mutants may appear to be unreasonable. However, if this is done combinatorially, it proves to be feasible. We chose a mechanistically complex enzyme and first designed P450-BM3 CAST libraries for selective hydroxylation of a model compound, and generated them by the usual genetic manner. In parallel, the design schemes were sent to a company which applied their chemical technology called *Sloning, which involves solid-phase gene synthesis.* Subsequently we compared the libraries. *We discovered that the Sloning libraries are superior in terms of quality and diversity (less amino acid bias, less repetition of wildtype, etc.).* If the prices of such libraries continue to tumble, then the usual molecular biology applied in saturation mutagenesis will be replaced by such a *chemical approach*. Other companies are now offering commercial libraries prepared by solid-phase gene synthesis on chips which promise to have significantly higher library quality and diversity. Library design based on CASTing, ISM, reduced amino acid alphabets, etc. will then play the key role when applying such a chip-technology. *The future of directed evolution?*

Future directions: Proving the generality of TCSM is necessary, especially when combined with simultaneous multi-parametric optimization of stereoselectivity, activity and thermostability. (one of the remaining challenges in directed evolution). Particularly exciting would be the implementation of an alternative to Sloning-mediated gene synthesis, namely solid-phase based generation of mutant gene libraries on microchips, possibly in combination with microfluidic devices.

Publications resulting from this research area: 1, 2, 7, 14, 17, 18, 20, 26- 28, 30, 31, 37-40.

External funding: LOEWE Research Cluster SynChemBio (Hessen, DE); Arthur C. Cope Fund (US)

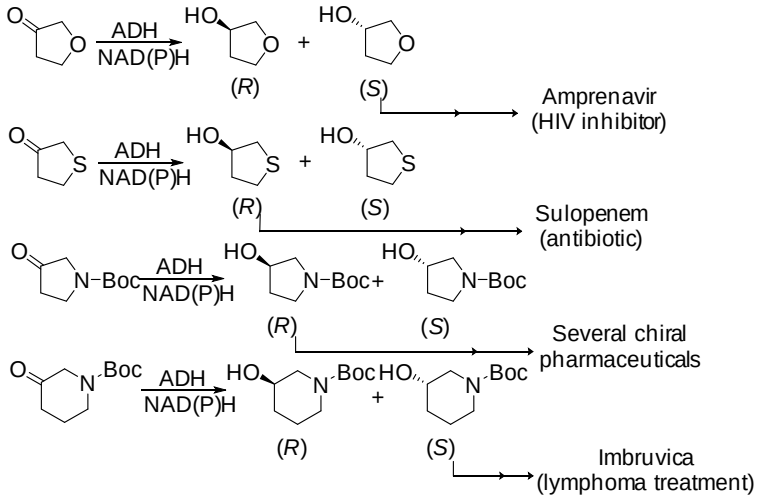
Cooperations: Y. Nov (Statistics Department, Haifa University/IL), M. Zilly (Physics Department, Duisburg-Essen University/DE), J. Zhou (Shanghai Institute of Organic Chemistry/CN); J.-H. Xu (East China University of Science and Technology, Shanghai/CN), J.-E. Bäckvall (Stockholm University/SE)

2.6.2 Research Area "Applications of Advanced Directed Evolution Methods" (M. T. Reetz)

Involved: C. G. Acevedo-Rocha, R. Agudo, A. Ilie, A. Li, R. Lonsdale, G.-D. Roiban, J. Sanchis, Z. Sun, J. Wang, Z.-G. Zhang

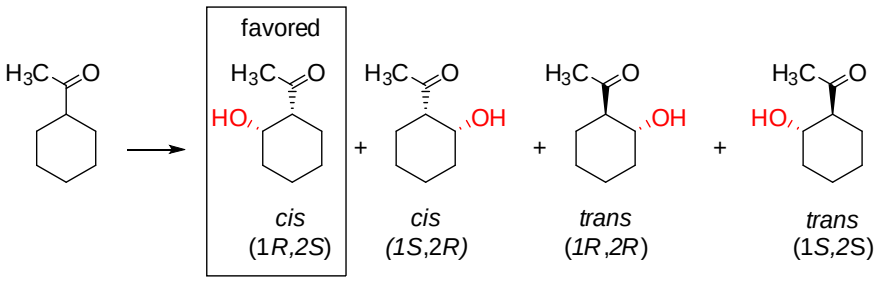
Objective: Application of newly developed directed evolution methods to selected stereo- and regioselective reactions in synthetic organic chemistry with primary emphasis on those transformations that are not readily possible using state-of-the-art transition metal catalysts or organocatalysts.

Results: Directed evolution generally involves a model substrate, the evolved mutant(s) being highly stereoselective for that particular compound. However, organic chemists need catalysts that are active and selective not just for a single compound (unless a pharmaceutical company is interested in only one specific transformation).[34,40] We have therefore tested our mutants not just for model substrates, but also for additional compounds. Typical examples follow: Upon applying triple code saturation mutagenesis (TCSM) as an advanced form of CASTing to the robust alcohol dehydrogenase from *Thermoethanolicus brockii* (TbSADH) using 3-oxo-tetrahydrofuran as substrate, *both (R)- and (S)-selective mutants were evolved step-economically (95-99% ee at full conversion) in a single library*.[28] *Commercial chiral Ru-catalysts failed to provide acceptable levels of enantioselectivity.* The mutants were also applied to other difficult-to-reduce ketones[28] (Scheme 1) and to structurally very different substrates,[35] likewise leading to 94-98% ee.

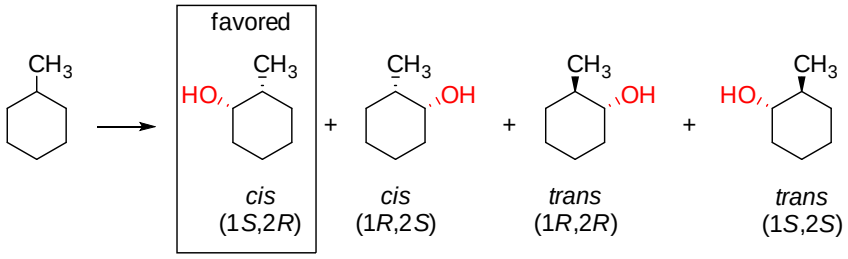


Scheme 1

Extensive work with P450-BM3 was performed in various applications.[6,9,11,12,15,17,21,38] An example is simultaneous control of regio-, enantio- and diastereoselectivity in the hydroxylation of achiral compounds with concomitant creation of two chirality centers, catalyzed by mutants evolved by applying CASTing based on the use of a 6-membered reduced amino acid alphabet. Scheme 2 shows two substrates, six others reacted similarly.[6] *Notice that the potentially most reaction positions with the weakest CH-bonds were not attacked!*



Best mutant: Regioselectivity: 78 : 22
Diastereoselectivity: 98 : 2
Enantioselectivity: 98 : 2
Conversion: >85%



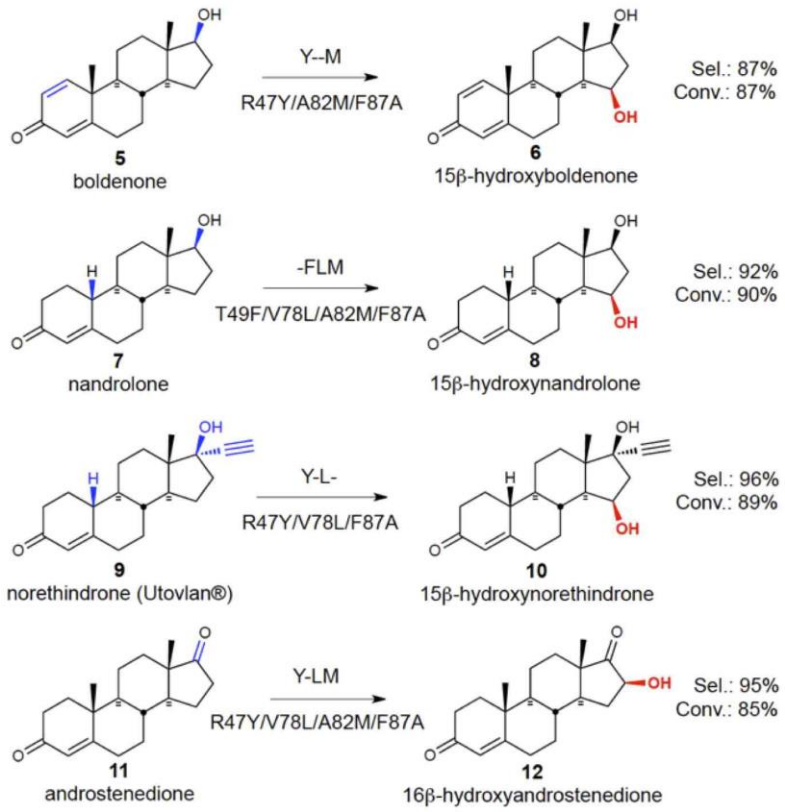
Best mutant: Regioselectivity: 71 : 29
Diastereoselectivity: 97 : 3
Enantioselectivity: 96 : 4
Conversion: >85%

Scheme 2

Enantioselective α-hydroxylation of ketones can be performed in many cases with transition metal catalysts or organocatalysts. Nevertheless, we performed P450-CASTing with a rationally chosen reduced amino acid, which provided small mutant libraries harboring hits with 96-99% ee for (*R*)- and inverted (*S*)-selectivity (Scheme 3).[17] *The mutants were also tested with 19 other substrates,* often (but not always) leading to >90% ee.

Scheme 3

In the last 3-Year Report (2011-2013) we described the first case of P450 directed evolution for controlling regioselective hydroxylation of testosterone at the 2ß- and 15ß-positions on an optional basis as part of "late-stage hydroxylation". Since then the CAST-approach has been improved significantly as part of methodology development (see Reetz report 1), also with the aim of targeting other positions in steroids. Typical examples are featured in Scheme 4, the first three cases involving 15ß-selectivity of further steroids, *the last one illustrating an evolved switch to hydroxylation at position C16*.[41] We have since investigated a total of 15 structurally different steroids, which is of significance for the pharmaceutical industry.

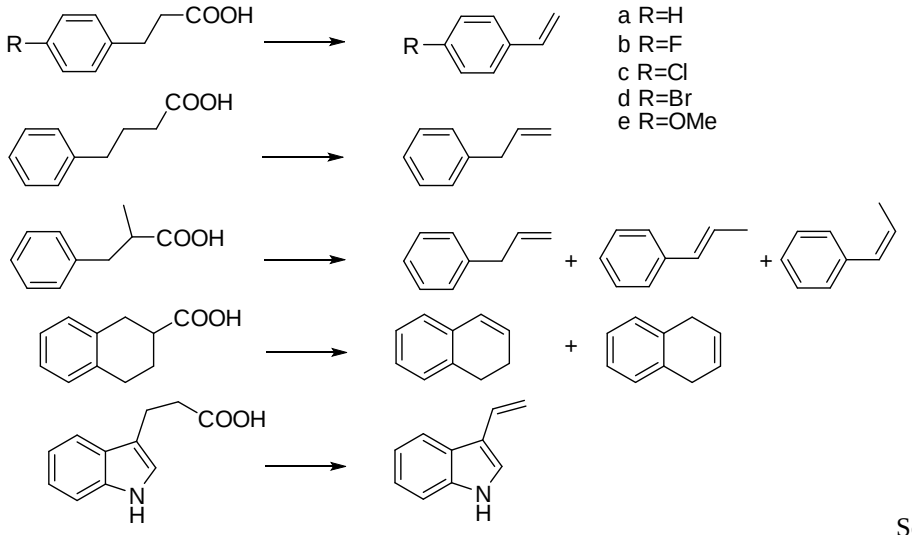


Scheme 4

Further work with P45-BM3 in selective oxidation reactions involved:

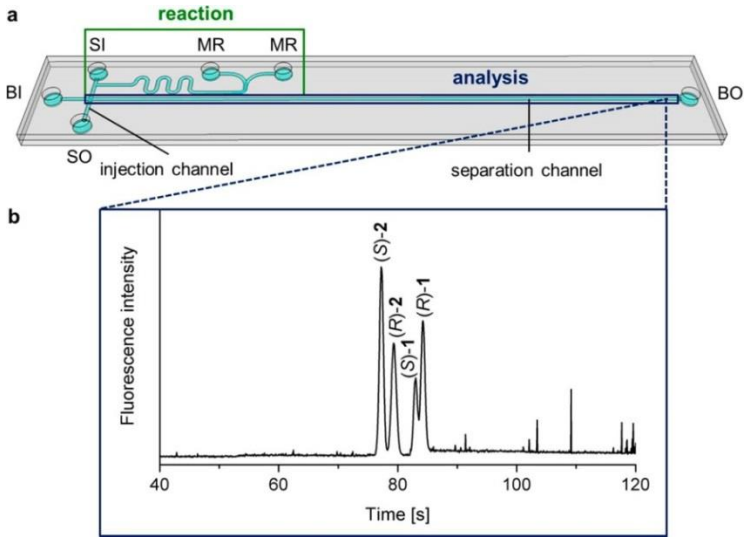
- *Regio- and enantioselective hydroxylation of substituted 1-tetralones at the 4-position with formation of alcohols that are chiral components of prominent Chinese folk medicines.*[9]
- *Regio-, diastereo- and enantioselective hydroxylation of achiral alkanes with formation of alcohols having three centers of chirality in a single CH-activation event. (The principle was illustrated, but in the absence of further mutagenesis, the degree of overall selectivity remains low to moderate in this case).*[11]
- *Diastereoselective epoxidation of cyclic alkenes involving anti- versus syn preference.*[12]
- *Chemo- and regioselective hydroxylation as a means to perform bioorthogonal activation of caged compounds in living cells.*[21]

Further projects involved altogether different goals. Inspired by the recently reported P450-peroxydase $OleT_{JE}$, which in nature catalyzes the oxidative decarboxylation of fatty acids $RCH_2CH_2CO_2H \rightarrow RCH{=}CH_2$, a directed evolution study was initiated in which structurally very different carboxylic acids were tested at room temperature using air as the oxidant (Scheme 5).[32] Since practical problems were encountered in setting up optimal expression and screening systems, only very small rationally designed libraries were generated. Increased activity in broadening substrate acceptance as well as high regioselectivity but moderate diastereoselectivity favoring trans-olefins were observed. This is the *first time that such an enzyme has been used to produce di-substituted olefins from branched carboxylic acids*.[32] It is a mild alternative to the Pd-mediated Gooßen-method at 120 °C (best chemical method).



Scheme 5

Other projects focused on the substrate range of mutants of epoxide hydrolases,[18,27,30,37,39] of promiscuous Baeyer-Villiger monooxygenases as sulfoxidation catalysts,[10] and in the practical one-step transformation of cyclohexanone into caprolactone.[16] A phosphotriesterase was evolved iteratively as a robust degrader of the widely used pesticide malathion,[36] which can probably be used for the detoxification of other more dangerous phosphorous-based compounds. In a different type of application, *Aspergillus niger* epoxide hydrolase mutants were applied to a hydrolytic kinetic resolution and *used for the first time in an integrated microfluidic device in which only a few hundred cells were necessary for enantioselectivity quantification* (Scheme 6).[29]



Scheme 6

Future directions: Late-stage P450 hydroxylation in a natural products synthesis would be one interesting topic, as is advanced directed evolution of $OleT_{JE}$ for generalizing oxidative decarboxylation of structurally different carboxylic acids as a mild olefin synthesis. Evolving desaturases for introducing double bonds regioselectively in any given organic compound is yet another challenging goal. Also of interest, an alcohol dehydrogenase such as TbSADH could be subjected to directed evolution so that it becomes a transaminase with ammonia being the amine source (ketones exist in equilibrium with small amounts of the ketimine), with significant advantages over transaminases. Directed evolution of cascade sequences is another "hot" topic.

Publications resulting from this research area: 6, 9, 10-12, 15-18, 21, 27-29, 32, 35-38, 40

External funding: LOEWE Research Cluster SynChemBio (Hessen, DE); Arthur C. Cope Fund (US)

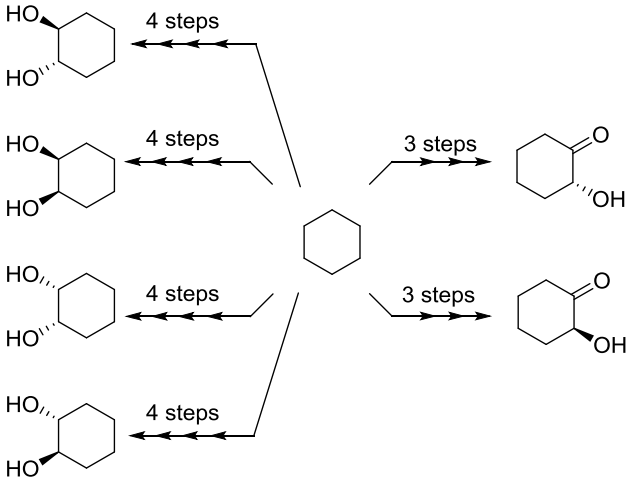
Cooperations: E. Meggers (Marburg, DE); I. Korendovych (Syracuse, US), D. Belder (Leipzig, DE); J. Zhou (Shanghai Institute of Organic Chemistry, CN); J-H. Xu (East China University of Science and Technology, Shanghai, CN); J. Sanchis (Monash University, AU)

2.6.3 Research Area "Creation of Designer Cells for Redox-based Enzymatic Cascade Reactions Enabled by Directed Evolution" (M. T. Reetz)

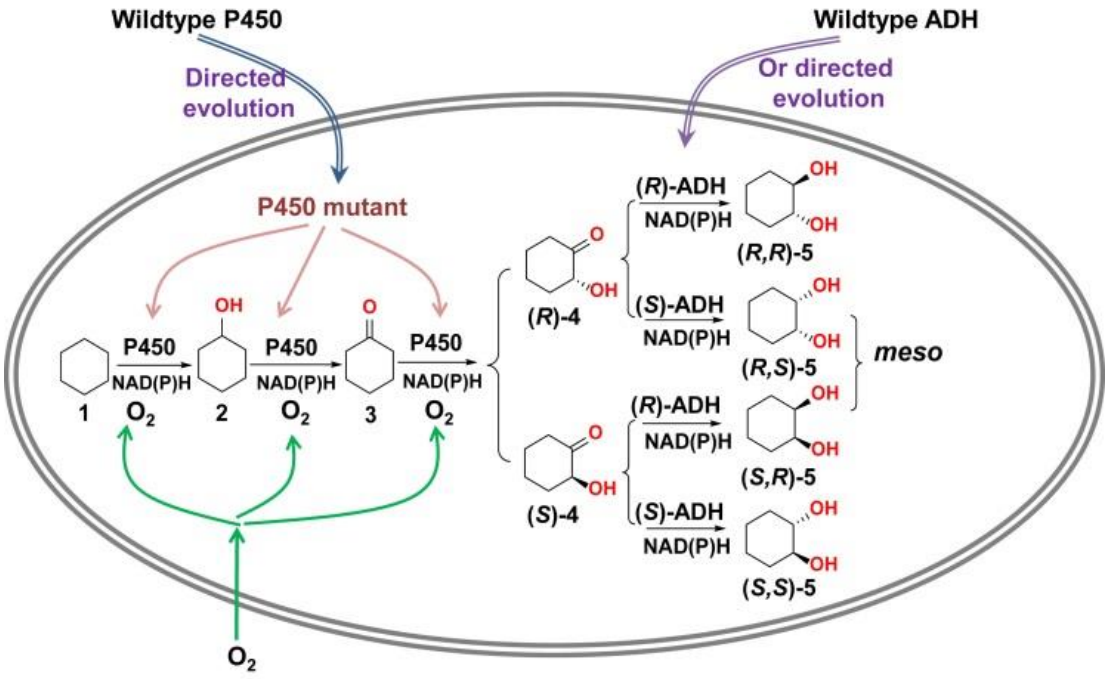
Involved: A. Ilie, A. Li, R. Lonsdale, Z. Sun, J. Wang

Objective: The purpose was the use of directed evolution in the construction of *E. coli* designer cells that enable one-pot regio- and stereoselective multi-step redox reactions in cascade sequences not possible by transition metal catalysts or organocatalysts.

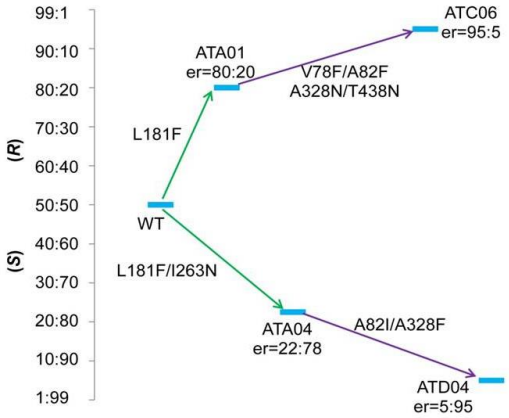
Results: Nature orchestrates the buildup of structural complexity stepwise *in vivo* in the cytosol of cells in which a multitude of enzymes function as selective catalysts. In contrast to metabolic pathway engineering which is based on renewable feedstocks such as glucose as the starting material for accessing special compounds of interest (derivatives), we are interested in the creation of designer cells which enable one-pot multi-step reaction sequences based on cheap petrochemicals. *The de novo construction of designer cells with the aim of realizing any given reaction sequence that an organic chemist might envision is a different type of problem not covered by metabolic engineering*. Particularly challenging are those cases of cascade reactions in designer cells that involve several sequential redox steps, especially when the control of regio-, chemo- and stereoselectivity is required which is impossible using wildtype enzymes. Indeed, very few examples have been reported to date, and only one utilized directed evolution (our own study highlighted in the 2011-2013 Report). We envisioned several cascade sequences utilizing cyclohexane (**1**) as the starting material: Two designed *E. coli* cells that consume cyclohexane as an energy source with formation of the acyloins (*R*)- and (*S*)-2-hydroxycyclohexanone, respectively, as well as more complex cells that take cyclohexane all the way to (*R*,*R*)-, (*S*,*S*)- or *meso*-cyclohexane-1,2-diols:



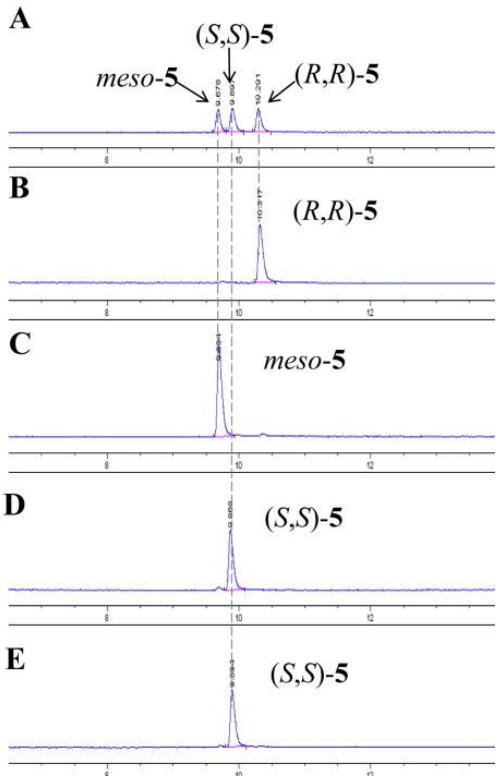
In order to reach both goals, a flexible unified concept was devised according to[38]:



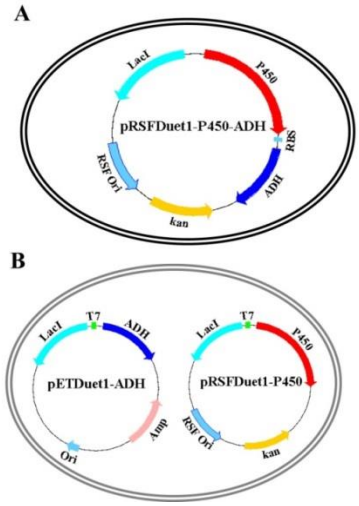
P450-BM3, a well-known monooxygenase having a fused reductase domain, was chosen as the enzyme for the first three CH-activating steps, followed by appropriate alcohol dehydrogenases (ADH)s for the final step(s) on the way to the diols. P450-BM3 does not accept cyclohexane (**1**), nor cyclohexanol (**2**) or cyclohexanone (**3**), probably due the small size of these substrates in the large binding pocket of the enzyme. The difficult part was evolving single P450-BM3 mutants which catalyze all three steps starting from cyclohexane (**1**), one leading to (*R*)-**4**, the other to (*S*)-**4**. This was achieved by applying triple code saturation mutagenesis (TCSM) in iterative form (ISM) (see Reetz Report 1), initially using cyclohexanone (**3**) as substrate. Enantioselectivities of er = 95 : 5 were achieved in the formation of (*R*)- and (*S*)-**4**. These two mutants also catalyzed the two first steps **1**→ **2**→**3**, thereby *making the one-pot conversion of cyclohexane (1) into the acyloins (R)- and (S)-4 possible.*[38]



The remaining goal was the production of the three stereoisomeric diols **5** in a one-pot process starting from cyclohexane (**1**). Using a bioinformatics approach, ADHs as appropriate enzymes in the reduction of C4-acyloins were readily identified. Gratifyingly, they also proved to be excellent catalysts in the diastereoselective reduction of the acyloins (*R*)- and (*S*)-**4**, with formation of (*R*,*R*)-, (*S*,*S*)- and *meso*-**5**, respectively, as shown by the GC plots of the crude products resulting from the different designer cells (*top chromatogram A: standard mixture of the three stereoisomers; B, C, D and E: crude products from E. coli designer cells using cyclohexane as starting material.*):



Thus, in the case of the ADHs, directed evolution was not necessary, since the wildtypes did the job. The best plasmid configuration, important for minimizing cell stress, inter alia, was also explored by testing two versions: Putting both P450 and ADH genes in one plasmid led to significantly better results than placing two separate plasmids in a designer cell[38]:



This work demonstrates that directed evolution is highly useful in the construction of designer cells which enable redox-based multi-step cascade sequences. Such one-pot processes requiring only a single workup are not possible using state-of-the-art transition metal catalysts or organocatalysts.

Future directions: This study opens the door for many synthetically novel possibilities, e.g., testing in one-pot processes other starting materials as substrates, utilizing (evolved) transaminases in the last step in order to obtain all four stereoisomeric amino-alcohols, and extending the directed evolution concept to include other enzymes such as mutants of Baeyer-Villiger monooxygenases.

Publications resulting from this research area: 22, 38

External funding: LOEWE Research Cluster SynChemBio (Hessen, DE)

Cooperations: Jian-He Xu (East China University of Science and Technology, Shanghai, CN)

2.6.4 Research Area "Learning from Directed Evolution" (M. T. Reetz)

Involved: C. G. Acevedo-Rocha, A. Ilie, A. Li, G. Li, R. Lonsdale, J. Sanchis, Z. Sun, J. Wang

Objective: a) Examine the mechanistic details of an ene-reductase by QM/MM; b) Uncover the reasons for enhanced or inverted stereoselectivity or altered regioselectivity of selected evolved enzymes; c) Explore additive versus cooperative or deleterious mutational effects by constructing and analyzing multidimensional fitness landscapes, showing when epistasis is crucial in directed evolution.

Results:

QM/MM study of the ene-reductase YqjM

Ene-reductases catalyze the conjugate reduction of α/ß-unsaturated ketones and other activated alkenes, but stereoselectivity is often poor, or reversal is necessary. We have previously applied directed evolution to the ene-reductase YqjM for enhancing and inverting enantioselectivity of several prochiral substrates, but were unable to postulate sound interpretations because crucial mechanistic details of this class of enzymes were lacking. We performed the first QM/MM study of this class of enzymes, cyclohexenone serving as the model substrate.[24] Figures 1a and 1b show the highest ranked poses of the "normal" and "flipped" orientations, respectively, and Fig. 1c features the QM region used in QM/MM computations.

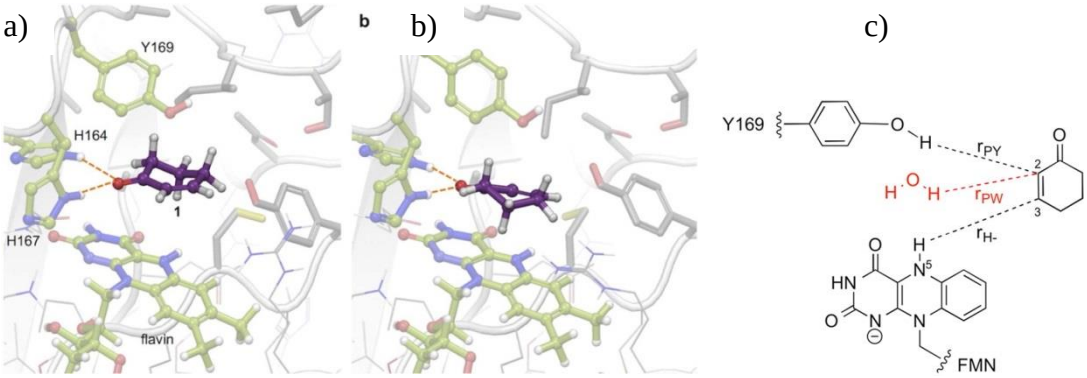


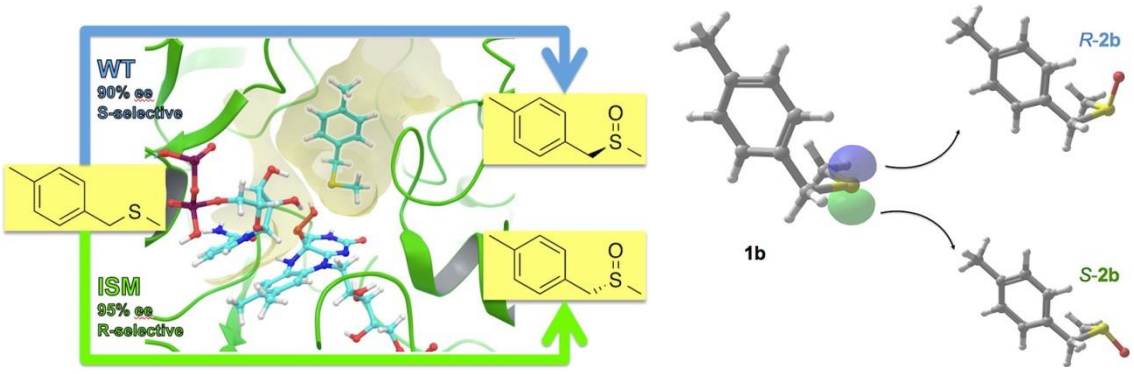
Fig. 1

Reaction pathways at B3LYP-D/OPLS2005 level suggest that *the hydride from FMN and the proton are added in distinct steps, not concertedly* as often postulated. The substrate is bound weakly, two active site histidines inducing polarization of the

carbonyl function (not LUMO-lowering by strong H-bonds to the carbonyl O-atom!), with Tyr196 providing the proton in the final fast step. This study not only provides *detailed insights into the mechanism of an ene-reductase as generated by QM/MM, the results can also be used to interpret the enantioselectivity of evolved mutants as catalysts in the reduction of prochiral substrates*.[24]

Shedding light on the origin of enhanced and inverted stereoselectivity of enzyme mutants

In most of our experimental studies of directed evolution of stereo- and regioselective enzymes, we apply docking and molecular dynamics simulations (MD) simulations in order to shed light on the reasons for altered catalytic profiles. The lessons from directed evolution flanked by such theoretical studies enable fundamental mechanistic insights. Typical examples are featured here, beginning with the Baeyer-Villiger monooxygenase PAMO which we used in an asymmetic promiscuous sulfoxidation reaction employing *p*-methylbenzyl methyl thioether as substrate.[10] WT PAMO favors formation of the (*S*)-sulfoxide (90% ee), while CAST-based iterative saturation mutagenesis (ISM) led in two steps to the quadruple mutant I67Q/P440F/A442N/L443I showing complete reversal of enantioselectivity (95% ee in favor of the (*R*)-sulfoxide (Fig. 2a). This switch entails an energy change of $\Delta\Delta G^{\neq} = 16.4$ kJ/mol !



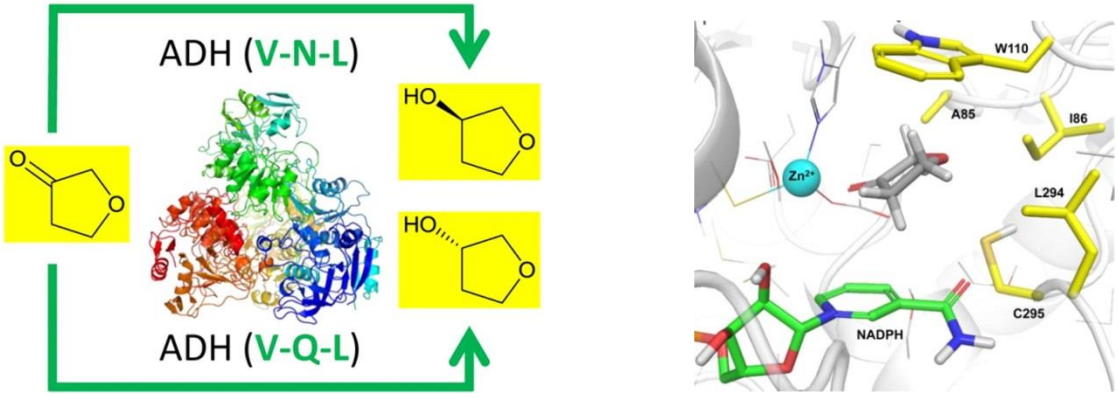
a) b)

Fig. 2

A previous QM/MM study (W. Thiel, M. T. Reetz, et al, *JACS* **2012**) focused on the mechanism of enzymatic Baeyer-Villiger oxidations, showing that the flavin anion Fl-OO^- is the oxidant. In the sulfoxidation reaction, Fl-OOH is the active electrophilic species.[10] the closest of the two sulfur lone electron pairs to the –OOH moiety will be the one that undergoes oxidation preferentially (Fig. 2b). Highest docking scores were computed, leading to a plausible model. *In this way the intricacies of binding were illuminated for the quadruple mutant I67Q/P440F/A442N/L443I, in line with the observed (R)-selectivity*.[10] Similar calculations were performed at all evolutionary steps

in the upward climb within all 24 pathways of the fitness landscape (see next section below).

Mutants of the alcohol dehydrogenase TbSADH, evolved by TCSM with 3-oxo-tetrahydrofuran as substrate (Fig. 3a) were also analyzed.[28] Mutant SZ2074 (I86N/C295N) shows 99% ee/(*R*) and mutant SZ2172 (I86V/W110L) ensures 95% ee/(*S*); WT 23% ee (*R*). The Prelog-rule, a model referring to a large binding cavity for the carbonyl α-group and a small one for the α'-group, cannot be applied, since in the present case steric differences are hardly discernible. TbSADH is a Zn-dependent ADH in which the carbonyl O-atom binds to the Lewis acid with concomitant activation for hydride attack by NADPH (Fig. 3b).[28]



a) b)

Fig. 3

The furan ether O-atom undergoes *different H-bonding interactions which positions the substrate in opposite orientations with respect to NADPH as hydride source* (Figure 4). [28]

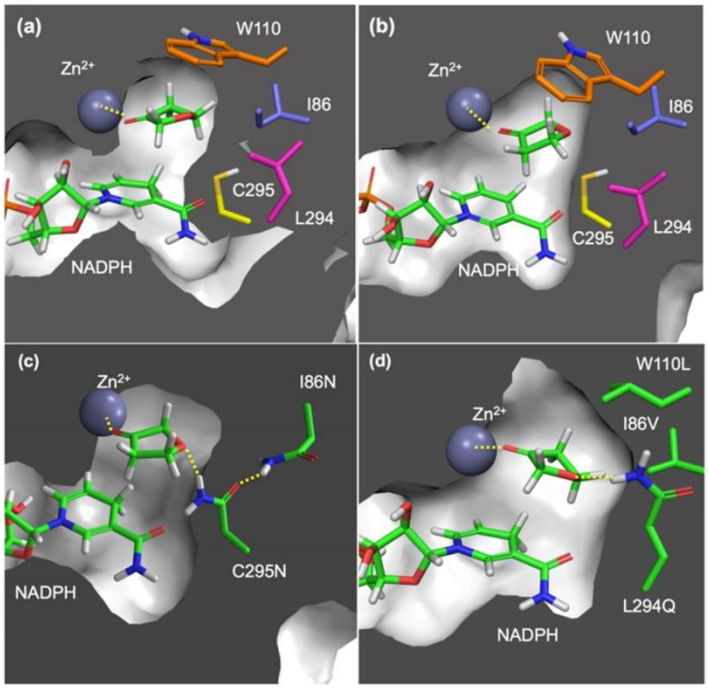


Fig. 4. (Docking poses of 3-oxo-tetrahydrofuran: a) WT (*S*)-selective pose; b) WT (*R*)-selective pose; c) SZ2074 (*R*)-selective pose; d) SZ2172 (S)-selective pose) [28]

Docking and MD computations were also performed in other directed evolution studies, focusing on enantioselective epoxide hydrolase,[27,31,39] regio- and stereoselective P450 enzymes,[6,9,12,17,21,38,41] and a P450-peroxydase as a diastereoselective catalyst in oxidative decarboxylation.[32] In the case of single code saturation mutagenesis (SCSM) of limonene epoxide hydrolase (LEH) with evolution of (*S*,*S*)-selective mutant SZ92 (95% ee) and reversed (*R*,*R*)-selective mutant SZ338 (96% ee) in the desymmetrization of cyclohexene oxide with formation of the respective diols, crystal structures harboring the (*S*,*S*)- and (*R*,*R*)-products were obtained (Fig. 5).[18] The *reshaped binding pockets* are clearly visible (see binding cavities located above D101), which were analyzed by docking/MD computations. *This is the first case of crystal structures of both enantiomeric mutants produced by directed evolution, thereby nicely illustrating Emil Fischer's lock-and-key hypothesis.*

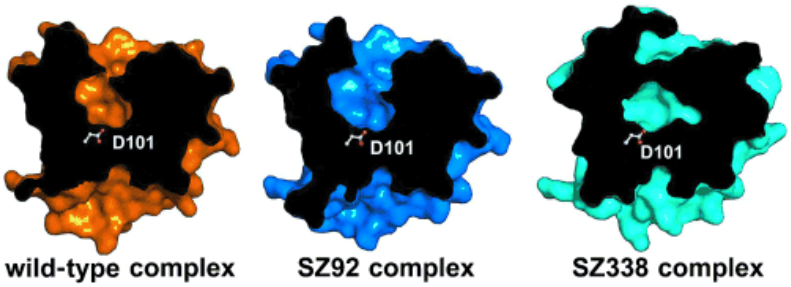
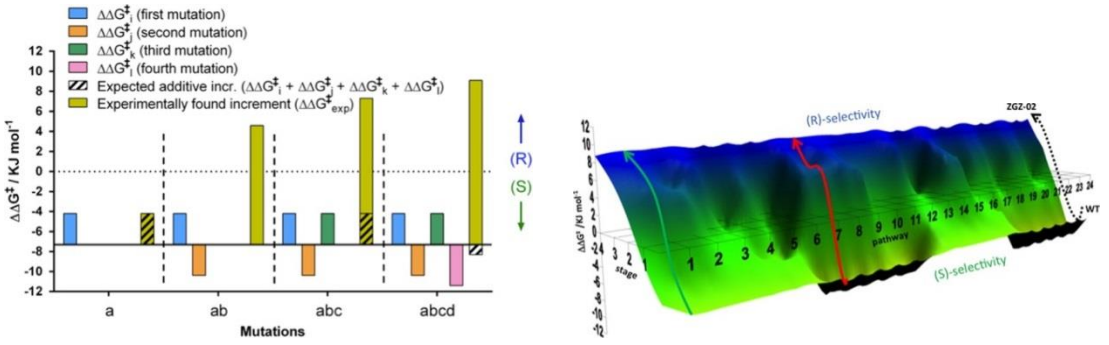


Fig. 5.

Fitness landscapes for exploring additive versus cooperative mutational effects

Additive mutational effects are those in which the respective point mutations do not influence each other, whereas non-additive mutational effects can be cooperative (more than additive) or deleterious (less than additive). Fitness landscapes needed to identify such effects are accessible by deconvoluting a multi-mutational variant produced by directed evolution. This requires formidable lab work (which explains why such studies are rare), but the results are illuminating. *We find cooperative (more than additive) and deleterious effects (less than additive) to be the rule, not the exception!* Two deconvolution studies were performed during the past three years: The first concerned reversal of enantioselectivity of PAMO in the sulfoxidation of *p*-methylbenzyl methyl thioether via two ISM steps WT (*S*) → P440F/A442N/L443I → I67Q/ P440F/A442N/L443I (*R*) (Fig. 2a).[10] As a first deconvolution step of the (*R*)-selective quadruple mutant, all four point mutations were prepared and tested in the asymmetric sulfoxidation reaction along 24 pathways, one trajectory defined by a→ b→c→d being shown in Scheme 1a.[10] *Surprisingly, all four point mutations proved to be (S)-selective, but in concert they are highly (R)-selective!* Alternatively speaking, the combination of four (*S*)-selective mutations results in a highly (*R*)-selective mutant. The same pattern was discovered in the other 23 evolutionary pathways. These and other trends at various evolutionary stages were explained by induced docking pose computations. A fitness landscape comprising 4! = 24 pathways was constructed experimentally (Scheme 1b), [10] which revealed that 6 trajectories lack local minima, 18 being characterized by local minima but with built-in escape events and continuous upward climbs to the same (R)-selective quadruple mutant. *In all cases strong cooperative (not just additive) mutational effects proved to be pervasive.*



a) b)

Scheme 1

Obviously, Scheme 1b features a fitness landscape based on a single catalytic trait in the upward climb. In a second eye-opening project utilizing P450-BM3 as catalyst in the oxidative hydroxylation of steroids, *part of the first multiparametric fitness landscape was constructed by considering both regioselectivity and activity as the definition of fitness*. Even at this early stage of the project it was observed that the two catalytic traits are interrelated.

Future directions: A QM/MM study of the (*R*)- and (*S*)-selective TbSADH mutants would strengthen the present qualitative model. Complete multiparametric fitness landscapes coupled with theoretical analyses of dynamic effects in enzymes also constitute future research topics.

Publications resulting from this research area: 6, 9, 10, 12, 15, 17, 18, 21, 24, 27, 28, 31-33, 37, 38

External funding: LOEWE Research Cluster SynChemBio (Hessen, DE); Arthur C. Cope Fund (US)

Cooperations: J. Sanchis (Monash University, AU), Y. Nov (Statistics Department, Haifa University, IL), M. Zilly (Physics Department, Duisburg-Essen University, DE), J. Zhou (Shanghai Institute of Organic Chemistry, CN); J.-H. Xu (East China University of Science and Technology, Shanghai, CN)

2.6.5 Publications 2014-2016 from the Emeritus Group Biocatalysis M. T. Reetz

(1) Acevedo-Rocha, C. G.; Reetz, M. T. In *Directed Evolution Library Creation: Methods and Protocols*, *Methods in Molecular Biology*; Ackerley, D., Copp, J., Gillam, E., Eds.; 2nd ed.; Humana Press: Totowa, **2014**, Vol 1179, pp 189-206.

(2) Acevedo-Rocha, C. G.; Kille, S.; Reetz, M. T. In *Directed Evolution Library Creation: Methods and Protocols*, *Methods in Molecular Biology*, Ackerley, D., Copp, J., Gillam, E., Eds.; 2nd ed.; Humana Press: Totowa, **2014**, Vol. 1179, pp 103-128.

(3) Roiban, G.-D.; Reetz, M. T. *Eur. J. Org. Chem.* **2014**, 2070-2076.

(4) Roiban, G.-D.; Ilie, A.; Reetz, M. T. *Chem. Lett*. **2014**, *43*, 2-10.

(5) Tielmann, P.; Kierkels, H.; Zonta, A.; Ilie, A.; Reetz, M. T. *Nanoscale* **2014**, *6*, 6220-6228.

(6) Roiban, G.-D.; Agudo, R.; Reetz, M. T. *Angew. Chem., Int. Ed*. **2014**, *53*, 8659-8663.

(7) Acevedo-Rocha, C. G.; Agudo, R.; Reetz, M. T. *J. Biotechnol*. **2014**, *191*, 3-10.

(8) Reetz, M. T. *Angew. Chem., Int. Ed.* **2014**, *53*, 8562-8586.

(9) Roiban, G.-D.; Agudo, R.; Ilie, A.; Lonsdale, R.; Reetz, M. T. *Chem. Commun.* **2014**, *50*, 14310-14313.

(10) Zhang, Z.-G.; Lonsdale, R.; Sanchis, J.; Reetz, M. T. *J. Am. Chem. Soc.* **2014**, *136*, 17262-17272.

(11) Ilie, A.; Agudo, R.; Roiban, G.-D.; Reetz, M. T. *Tetrahedron* **2015**, *71*, 470-475.

(12) Ilie, A.; Lonsdale, R.; Agudo, R.; Reetz, M. T. *Tetrahedron Lett.* **2015**, *56*, 3435-3437.

(13) Ilie, A.; Reetz, M. T. *Isr. J. Chem*. **2015**, *55*, 51-60.

(14) Hoebenreich, S.; Zilly, F. E.; Acevedo Rocha, C. G.; Zilly, M.; Reetz, M. T. *ACS Synth. Biol.* **2015**, *4*, 317-331.

(15) Roiban, G.-D.; Reetz, M. T. *Chem. Comm*. **2015**, *51*, 2208-2224.

(16) Parra, L. P.; Acevedo, J. P.; Reetz, M. T. *Biotechnol. Bioeng*. **2015**, *112*, 1354-1364.

(17) Agudo, R.; Roiban, G.-D.; Lonsdale, R.; Ilie, A.; Reetz, M. T. *J. Org. Chem.* **2015**, *80*, 950-956.

(18) Sun, Z.; Lonsdale, R.; Kong, X.-D.; Xu, J.-H.; Zhou, J.; Reetz, M. T. *Angew. Chem., Int. Ed*. **2015**, *54*, 12410-12415.

(19) Sun, Z.; Ilie, A.; Reetz, M. T. *Angew. Chem., Int. Ed.* **2015**, *54*, 9158-9160.

(20) Acevedo-Rocha, C. G.; Reetz, M. T.; Nov, Y. *Sci. Rep.* **2015**, *5*: 10654.

(21) Ritter, C.; Nett, N.; Acevedo-Rocha, C. G.; Lonsdale, R.; Kraling, K.; Dempwolff, F.; Hoebenreich, S.; Graumann, P. L.; Reetz, M. T.; Meggers, E. *Angew. Chem., Int. Ed*. **2015**, *54*, 13440-13443.

(22) Wang, J. B.; Reetz, M. T. *Nat. Chem.* **2015**, *7*, 948-949.

(23) Keinan, U.; Reetz, M. T. *Isr. J. Chem*. **2015**, *55*, 1154-1155.

(24) Lonsdale, R.; Reetz, M. T. *J. Am. Chem. Soc.* **2015**, *137*, 14733-14742.

(25) Wu, Z.; Reetz, M. T.; Harms, K. *Acta Crystallogr., Sect. E: Crystallogr. Commun.* **2016**, *72*, 106-108.

(26) Acevedo-Rocha, C. G.; Reetz, M. T. In *Understanding Enzymes: Function, Design, Engineering and Analysis*; Svendsen, A., Ed.; Pan Standord Publishing Pte. Ltd.: Singapore, **2016**; pp. 613-642.

(27) Sun, Z.; Lonsdale, R.; Wu, L.; Li, G. Y.; Li, A. T.; Wang, J. B.; Zhou, J. H.; Reetz, M. T. *ACS Catal*. **2016**, *6*, 1590-1597.

(28) Sun, Z.; Lonsdale, R.; Ilie, A.; Li, G. Y.; Zhou, J. H.; Reetz, M. T. *ACS Catal*. **2016**, *6*, 1598-1605.

(29) Krone, K. M.; Warias, R.; Ritter, C.; Li, A.; Acevedo-Rocha, C. G.; Reetz, M. T.; Belder, D. *J. Am. Chem. Soc*. **2016**, *138*, 2102-2105.

(30) Sun, Z.; Wikmark, Y.; Bäckvall, J.-E.; Reetz, M. T. *Chem.–Eur. J.* **2016**, *22*, 5046-5054.

(31) Li, G. Y.; Zhang, H.; Sun, Z.; Liu, X. Q.; Reetz, M. T. *ACS Catal*. **2016**, *6*, 3679-3687.

(32) Wang, J. B.; Lonsdale, R.; Reetz, M. T. *Chem. Commun.* **2016**, *52*, 8131-8133.

(33) Li, G.; Reetz, M. T. *Org. Chem. Front.* **2016**, *3*, 1350-1358.

(34) Reetz, M. T. *Chem. Rev.* **2016**; DOI: 10.1002/tcr.201600040.

(35) Sun, Z. T.; Li, G. Y.; Ilie, A.; Reetz, M. T. *Tetrahedron Lett*. **2016**, *57*, 3648-3651.

(36) Luo, X. J.; Zhao, J.; Li, C. X.; Bai, Y. P.; Reetz, M. T.; Yu, H. L.; Xu, J. H. *Biotechnol. Bioeng*. **2016**, *113*, 2350-2357.

(37) Sun, Z.; Lonsdale, R.; Li, G.; Reetz, M. T. *ChemBioChem* **2016**, *17*, 1865-1872.

(38) Li, A.; Ilie, A.; Sun, Z.; Lonsdale, R.; Xu, J. H.; Reetz, M. T. *Angew. Chem., Int. Ed.* **2016**, *55*, 12026-12029.

(39) Sun, Z.; Torres Salas, P.; Siirola, E.; Lonsdale, R.; Reetz, M. T. *Bioresour. Bioprocess*. **2016**, 3:44.

(40) Reetz, M. T. *Directed Evolution of Selective Enzymes: Catalysts for Organic Chemistry and Biotechnology*. Wiley-VCH: Weinheim, **2016**.