2.1 Department of Synthetic Organic Chemistry

Director / External Group Leader

Manfred T. Reetz (born 1943)

Further group leaders:

Walter Leitner (born 1963)
external scientific member of the Institute

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)

1980-2010 > 155 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 (Denkmünze, 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 the Department of Synthetic Organic Chemistry

During the last three years the primary focus of research in the Reetz group was on methodology development in directed evolution of selective enzymes as catalysts in synthetic organic chemistry. The purpose was to make this Darwinian approach to asymmetric catalysis more efficient and therefore faster than in the past. Advanced gene mutagenesis methods and strategies were developed for the evolution of enhanced stereoselectivity, broader substrate scope (rate), higher thermostability and increased resistance to denaturing organic solvents. This involved the development of gene mutagenesis strategies characterized by high efficacy, improved molecular biological protocols, new approaches to high-throughput screening and selection as well as the design of bioinformatics-based and machine-learning techniques. Emphasis was also placed on 1) uncovering the reasons for increased efficacy, and 2) unveiling the source of enhanced stereoselectivity on a molecular level by means of mechanistic and theoretical studies.

Matthias Haenel, the only coal researcher in the Institute, retired in 2009. The External Member of the Institute, Walter Leitner (chair at TU Aachen), continued to run a small 2-3 person group here in Mülheim in the “Versuchsanlage”, studying catalytic reactions in non-conventional solvents such as ionic liquids and supercritical CO_2. During the last three year evaluation period, research by the local Leitner group led to 24 publications.

The Director of the Department, Manfred T. Reetz, was originally scheduled to retire in 2008 at the age of 65, but received special permission from the President of the Max Planck Society to continue until 68 (extension of contract until 31 August 2011). Due to the Institute’s plans regarding the successor and the concomitant extensive renovation of the respective floors in the high-rise laboratory building, the Reetz labs were closed in October 2010. Parallel to this development, Manfred Reetz accepted an offer from the University of Marburg to become the first Hans-Meerwein-Research-Professor starting 2011. The Marburg Chemistry Department will provide gene labs for about five coworkers as well as the general infrastructure, while the Max Planck Society has agreed to finance the research for five years following the formal termination of the Reetz-Directorship in August 2011. Thus, Manfred Reetz will head an external research group of the Max-Planck-Institut für Kohlenforschung, while also being a member of the Marburg faculty.

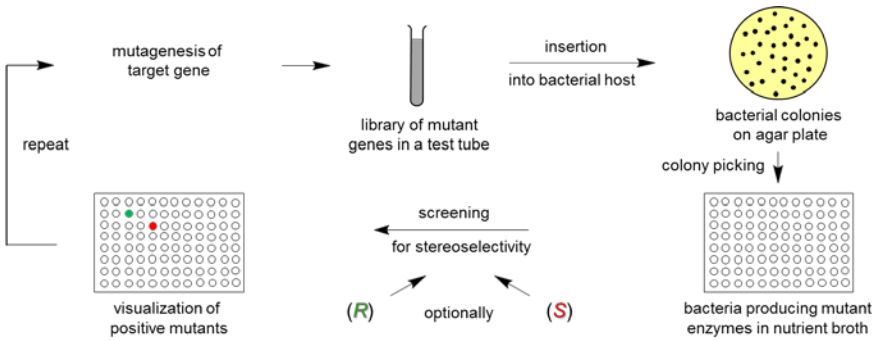
Due to the retirement of Manfred Reetz, new group leaders (assistant professors for Habilitation) were not recruited for the Department.

2.1.1 Research Area "Methodology Development in Directed Evolution" (M. T. Reetz)

Involved: C. G. Acevedo-Rocha, J. P. Acevedo, R. Agudo, Y. Gumulya, S. Kille, L. P. Parra, S. Prasad, J. Sanchis, E. Siirola, P. Soni, P. Torres Salas, Z.-G. Zhang, H. Zheng, F. E. Zilly

Objective: The goal was methodology development in the quest to make directed evolution more efficient than what it is was in 2010 with formation of higher-quality mutant libraries.

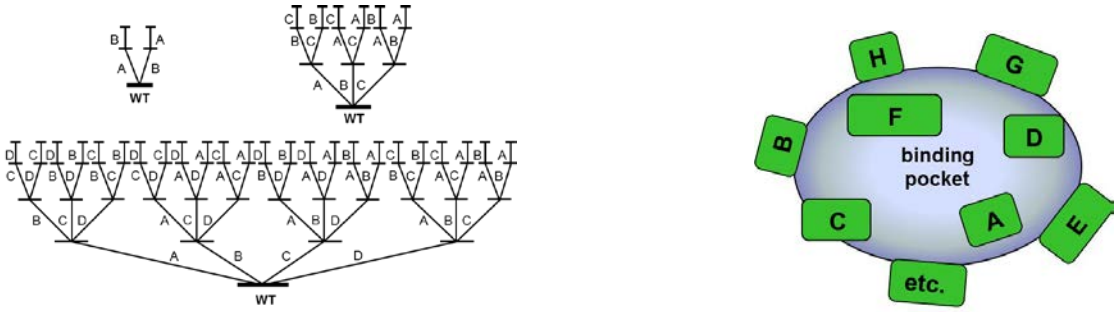
Results: Directed evolution involves repeating rounds of gene mutagenesis, expression and screening (or selection) of improved enzyme mutants. In the case of stereoselectivity, a fundamentally new approach to asymmetric catalysis has emerged (Scheme 1),[5,17,19] first demonstrated by our group in 1997 using a lipase as a catalyst in kinetic resolution.



Scheme 1. Steps in the concept of directed evolution of stereoselective enzymes.[5,17,19]

During the last three years we have not just continued to apply this concept using various types of enzymes for practical purposes (Report 2.1.2), but have also *focused on methodology development in the quest to make directed evolution more efficient and reliable.* In the previous Report (2008-2010), we suggested that *I*terative *S*aturation *M*utagenesis (ISM) may be the best way to generate highest-quality mutant libraries requiring less screening effort. Thanks to the advanced techniques and strategies that we recently introduced, notable progress has been made. *ISM is now a knowledge-driven approach to directed evolution which requires only small libraries and thus a minimum of screening, the latter now being performed by standard automated GC or HPLC.*[5] Sites labeled A, B, C, D, etc. in an enzyme comprising one or more amino acid

positions are subjected to saturation mutagenesis (introduction of all 20 natural amino acids), and the genes of the hits are then used as templates for randomization at the other sites (Scheme 2). When targeting stereoselectivity and/or substrate acceptance (rate), sites around the binding pocket are chosen on the basis of our previously developed *C*ombinatorial *A*ctive-Site *S*aturation *T*est (CAST) (Scheme 2).



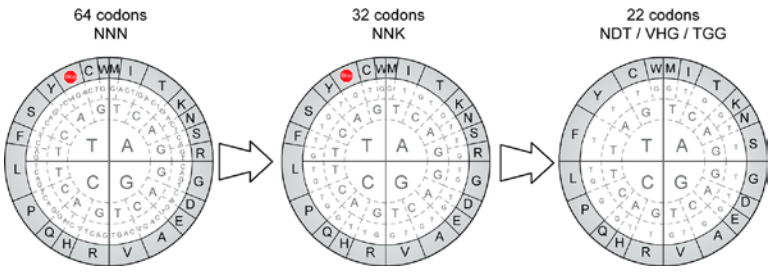
Scheme 2. Left: 2-, 3- and 4-site ISM systems; right: CAST sites for saturation mutagenesis.[5,17,19]

When applying saturation mutagenesis, it is necessary to consider statistical factors relating to the degree of oversampling necessary for covering a certain percentage of the library (number of transformants/mutants to be screened). Using the Patrick/Firth algorithms, we have devised a computer aid (CASTER) for designing mutant libraries and for calculating the respective oversampling number, e.g., for 95% library coverage.[5] The computed oversampling depends upon the number of amino acid positions at a given site and on the number of amino acids used as building blocks as set down by the codon degeneracy. The larger the randomization site and the larger the amino acid alphabet, the greater the screening effort. For example, instead of using the normal NNK codon degeneracy encoding all 20 canonical amino acids, we have shown by statistical analysis that NDT codon degeneracy encoding only 12 amino acids (Phe, Leu, Ile, Val, Tyr, His, Asn, Asp, Cys, Arg, Ser and Gly) requires in the case of a 2-residue site the screening of only 430 transformants, while classically NNK calls for 3,000. In the case of a 3-residue site the respective numbers are ≈5,000 (NDT) versus ≈ 100,000 (NNK). In the lab we have performed several studies using different enzymes and model reactions during the last three years comparing the classical NNK with NDT and with codon degeneracies encoding even smaller amino acid alphabets,[2,3,4,11,22,25,27,30,32,34,36] resulting in the conclusion that the *use of properly chosen amino acid alphabets constitutes one of the most proficient tools in present-day directed evolution.* Smaller amino acid alphabets reduce the structural diversity of the enzyme mutants in a given library, but the benefits in terms of dramatically reduced screening effort are enormous. *It is also useful to consider structural and mechanistic data as a*

guide in a bioinformatics approach. Application of the Nov-algorithms in our work has shown that screening can be reduced even more when targeting not the best mutant, but the second or nth best variant in a given library, while still leading to excellent mutants.[32]

Usually we considered only 1-, 2- and 3-residue sites due to statistical considerations. However, recently we devised a project in which two "mega-sites" A and B were chosen, each consisting of five amino acid positions (residues). For 95% library coverage, one would have to screen the astronomical number of 10^8 transformants if all 20 canonical amino acids were to be used as building blocks (NNK codon degeneracy). Therefore we tested *the idea of using extremely reduced amino acid alphabets*, and screened only a few thousand mutants, the model system being a stereoselective Baeyer Villiger reaction catalyzed by a Baeyer-Villiger monooxygenase. Both pathways A → B and B → A were experimentally explored requiring only four mutant libraries, and both pathways proved to be successful.[32] Notably improved mutants were evolved, yet only 8-9% of the theoretical protein sequence space was covered. We suggest that the Nov-algorithm focusing not on the best, but on the nth best mutant in a given library is a better way to assess the statistics. *We also conclude that grouping single residues into "mega-sites" is currently the best way to perform ISM*. This reduces the number of possible evolutionary pathways (Scheme 2) and cuts the screening effort drastically.[32]

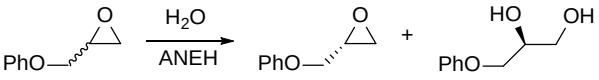
The phenomenon of amino acid bias due to the (natural) degeneracy of the genetic code constitutes a different problem which diminishes library quality when using all 20 canonical amino acids as building blocks. Our "trick" is simple and efficient: *Use of a designed mixture of three primers which create a degeneracy of 22 unique codons encoding all 20 canonical amino acid*s.[23] NDT, VHG and TGG codon degeneracies are chosen, which reduces the screening effort as shown by P450 experiments. Nature utilizes 64 codons (NNN); until recently we and others employed 32 codons (NNK) which is better, but now we are down to 22 codons for 20 amino acids which reduces amino acid bias considerably (Scheme 3).[32]



Scheme 3. Stepwise reduction of amino acid bias when using all 20 canonical amino acids.[32]

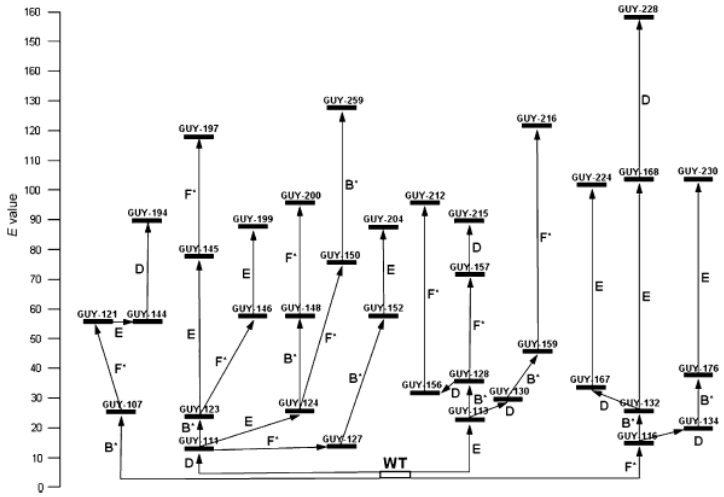
Another advancement during the 3-year assessment period is the systematic development of the Quick Quality control (QQC) of mutant libraries: *A pool of plasmid DNA belonging to all clones of a library is sequenced in a single run and analyzed to determine whether the designed degeneracy has been introduced and whether removal of the WT-sequence has been achieved.*[3,8,11,23,32,34,36] Low quality libraries should not be screened, e.g., those that contain a lot of WT enzymes or do not harbor many of the designed mutants. Should poor quality be indicated in a given case, such parameters as annealing temperature, primer length, and GC content as well as position and sequence of the target codon in the gene should be re-considered. In a chapter for *Methods in Molecular Biology* based on new and older experimental data, tips on how to apply ISM optimally while avoiding potential pitfalls are summarized as a guide for the experimenter. *In another contribution we focused on our previous method called Assembly of Designed Oligonucleotides (ADO), which is a type of synthetic DNA shuffling, but which can now be applied to saturation mutagenesis and thus to ISM.*[33]

A traditional problem in directed evolution arises when a newly created designed mutant library fails to contain any improved variant. *We have discovered a simple way to escape from such local minima in the fitness landscape*: *Simply use a non-improved or even an inferior mutant as a template for saturation mutagenesis in the following ISM cycle at a different randomization site.*[15] This seemingly "contra-intuitive" approach was illustrated using the epoxide hydrolase from *Aspergillus niger* (ANEH) All 24 evolutionary pathways of a 4-site ISM system (Scheme 2) were explored using the kinetic resolution featured in Scheme 4:



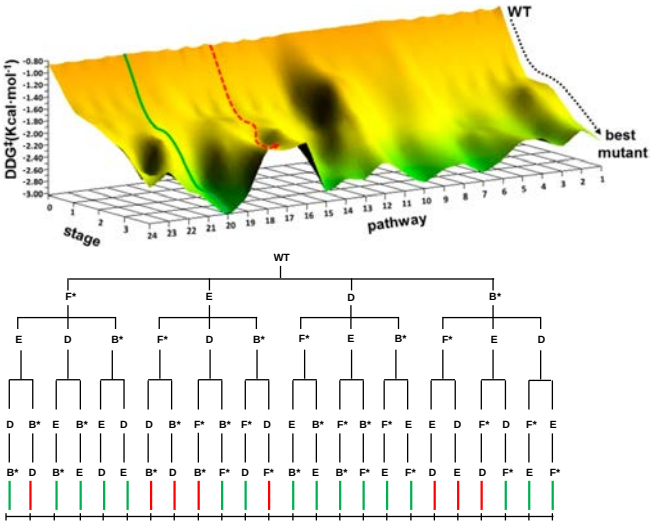
Scheme 4. Kinetic resolution of glycidyl phenyl ether as the model reaction.

Out of the 24 pathways, 8 exhibited local minima. In these cases an inferior mutant in a library lacking any hits was nevertheless used in the subsequent ISM round of saturation mutagenesis. Improved enantioselectivity of at least E = 30 was achieved in all such cases, which demonstrates that this approach constitutes a simple way to escape from local minima.[15] The 12 best pathways leading to selectivity factors of E > 88 are shown in Scheme 5 (the other 12 pathways provide mutants showing $E = \approx$ 30-70, not included here for clarity).



Scheme 5. Best 12 of the 24 pathways describing the fitness landscape.[15]

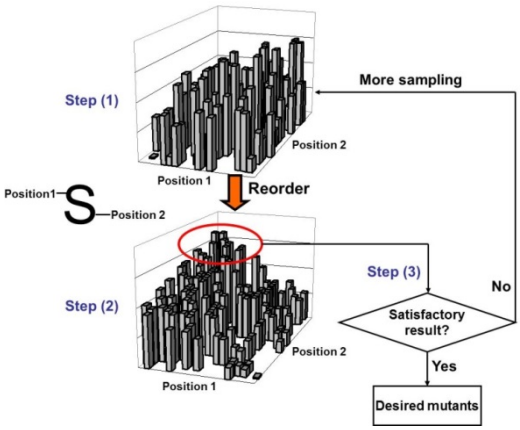
A fitness-pathway landscape was generated by systematic deconvolution of all sets of mutations that accumulated in every one of the 24 pathways (Scheme 6).[15] A typical pathway devoid of a local minimum is marked in green and one containing a local minimum is featured in red as part of the whole fitness landscape. There are 16 green and 8 red pathways.



Scheme 6. Left: Fitness landscape (see text); right: scheme of all 24 evolutionary ISM pathways.[15]

In collaboration with H. Rabitz (Princeton/USA), *an approach to in silico directed evolution was developed based on the adaptive substituent reordering algorithm (ASRA)* (Scheme 7).[14] Increased efficiency at every evolutionary stage was achieved. Potential enzyme mutants with desired properties from minimal sampling of focused libraries can be identified, which reduces the screening effort. This was demonstrated

using an epoxide hydrolase in the hydrolytic kinetic resolution of the chiral epoxide glycidyl phenyl ether (see above).



Scheme 7. Representation of steps involved in an ASRA operation as applied to ISM.[15]

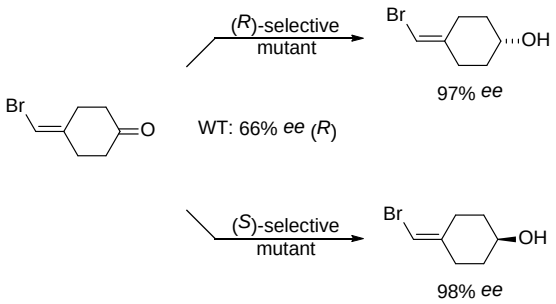
Finally, the overall experimental process based on ISM can be improved even more by optimizing the screening step. In collaboration with O. Trapp (Heidelberg), application of his multiplexing chromatography algorithm to the HPLC analysis of a 3,000-membered library of ANEH mutants was successful, requiring less than a few hours.

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

Involved: C. G. Acevedo-Rocha, J. P. Acevedo, R. Agudo, W. Augustyniak, M. Bocola, Y. Gumulya, A. Ilie, S. Kille, L. P. Parra, S. Prasad, G.-D. Roiban, F. Schulz, E. Siirola, P. Soni, P. Torres Salas, Q. Wu, Z.-G. Zhang, H. Zheng, F. E. Zilly

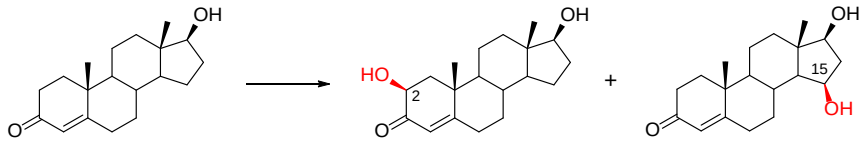
Objective: The goal was to apply the newly developed directed evolution methods to selected regio- and stereoselective transformations in synthetic organic chemistry not readily possible by state of the art transition metal catalysis or organocatalysis.

Results: One of several projects concerned enantioselective ketone reductions. Prochiral ketones $R^1R^2C=O$ can be reduced with high enantioselectivity to the respective alcohols using Noyori-type Ru-catalysts, provided the groups R^1 and R^2 are sterically sufficiently different. Alcohol dehydrogenases (ADHs) constitute an alternative. However, substrate scope and/or stereoselectivity are often limited, which calls for directed evolution. An example that we worked on in our group is the reduction of the ketone shown below, which leads to an axially chiral alcohol.[22] The robust ADH from *Thermoethanolicus brockii* (TbSADH), which had been characterized by X-ray crystallography, is only moderately *R*-selective (66% *ee*). As expected, chiral Ru-complexes as catalysts fail completely (< 5% *ee*). Upon applying ISM (see Report 2.1.1), *R*-selectivity was boosted to 97% *ee*, and reversal of enantioselectivity was likewise achieved with *S*-selectivity amounting to 98% *ee*. Other substrates were also tested successfully. T*he bromide is a key compound because transition Pd-catalyzed carbonylation and Suzuki coupling provided a number of derivatives, showing that the combination of biocatalysis and transition metal catalysis constitutes an attractive synthetic strategy.*[22]



Scheme 1. Induced axial chirality using evolved ADH mutants.[22]

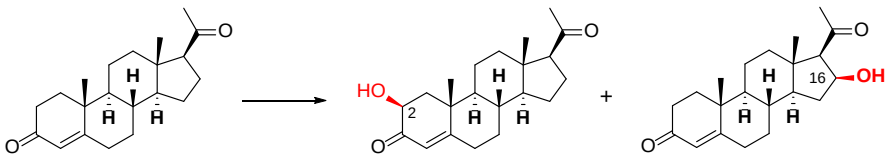
Another synthetically rewarding focus was on selective C-H activating oxidative hydroxylation catalyzed by P450 monooxygenases, especially in those cases in which synthetic reagents or catalysts fail. *Although in this area dozens of directed evolution studies had already appeared, up to 2010 no protein engineering method existed allowing the control of regio-* ***and*** *stereoselectivity.* The mechanism involves a high energy radical abstraction of an H-atom by the high-spin heme-Fe=O followed by rapid C-O bond formation of the intermediate radical. With ISM in hand,[5] we turned to this challenge by initially considering the hydroxylation of testosterone as the model reaction (Scheme 2).[3] Whereas the starting enzyme P450-BM3-F87A is not regioselective (1 : 1 mixture of alcohols at the 2- and 15-positions, plus small amounts of other alcohols, we succeeded in evolving 2ß- and 15ß-selective mutants, each showing > 99% diastereoselectivity (no trace of α-alcohols). A molecular dynamics (MD) investigation revealed for the 2ß-selective mutant a 2ß-pose directly above the catalytically active heme-Fe=O, and for the 15ß-selective mutant the respective 15ß-pose (see Report 2.1.3). Other steroids such as progesterone were also studied.[3]



Testosterone	2β-Hydroxytestosterone		15β-Hydroxytestosterone
P450-BM3(F87A)	50	:	50
2β-selective mutant	97	:	3
15β-selective mutant	4	:	96

Scheme 2. Regio- and stereoselective oxidative hydroxylation of testosterone using P450-BM3 mutants.[3]

Other steroids such as progesterone were also tested, generally with mutants already evolved for testosterone, i.e., without performing additional mutagenesis experiments.[3] A few of the results are shown in Scheme 3.

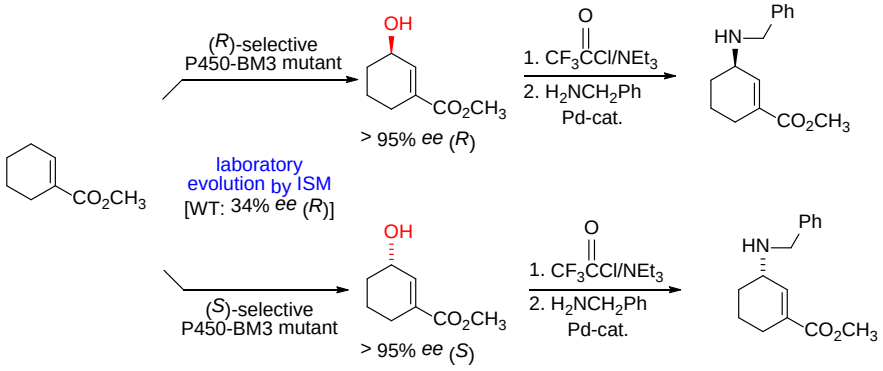


Progesterone			
P450-BM3(F87A)	18	:	82
2β-selective mutant	>99	:	<1
16β-selective mutant	9	:	91

Scheme 3. Regio- and stereoselective oxidative hydroxylation of progesterone using P450-BM3 mutants.[3]

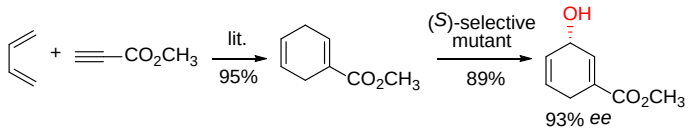
Most recently we have used testosterone as the model compound in order to target other positions and also to reverse diastereoselectivity (α-attack). For example, only two rounds of ISM led to 16α- and 16ß-selective mutants, respectively, showing 83-85% selectivity (which still needs to be improved by further mutagenesis experiments).

We originally surmised that in the binding pocket of P450 enzymes, known to be geometrically large, the selectivities of bulky compounds such as steroids are easier to control than of smaller substrates. In order to check this uncertainty, cyclohexene-1-carboxylic acid ester was tested (Scheme 4).[11] Gratifyingly, it was indeed possible to evolve regio- and enantioselective P450-BM3 mutants. Following the initial steroid examples, *this is the first case of directed evolution of a P450 enzyme in which regio- and enantioselectivity* (*in favor of either enantiomer) of a "small" substrate was shown to be possible.*[11] Here again subsequent transition metal catalyzed reactions were performed, in this case with formation of novel GABA-analogs.[11] However, upon testing 1-methylcyclohexene (which lacks a functional group helpful in ensuring a specific pose in the binding pocket), directed evolution was less successful (mixtures of products).[30] This shows that state the art directed evolution needs further improvement, at least in the case of P450-catalyzed oxidative hydroxylation of such non-functionalized substrates.



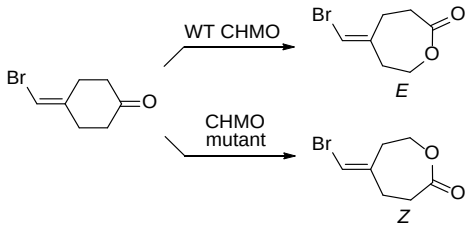
Scheme 4. Selective oxidative hydroxylation of a small molecule catalysed by P450-BM3 mutants.[11]

Although it can be argued that the above chiral alcohols can be obtained by other routes, this does not apply to the structurally more complex *S*-alcohol shown in Scheme 5. It was previously prepared by Berchtold et al. in 1987 in 11 steps and subsequently used in several natural products syntheses. Without performing additional mutagenesis experiments, the previously obtained *S*-selective mutant was tested in this reaction, leading to 93% *ee* (89% conversion)[11]:



Scheme 5. Hydroxylation catalyzed by a P450-BM3 mutant evolved for the substrate shown in Scheme 4.[11]

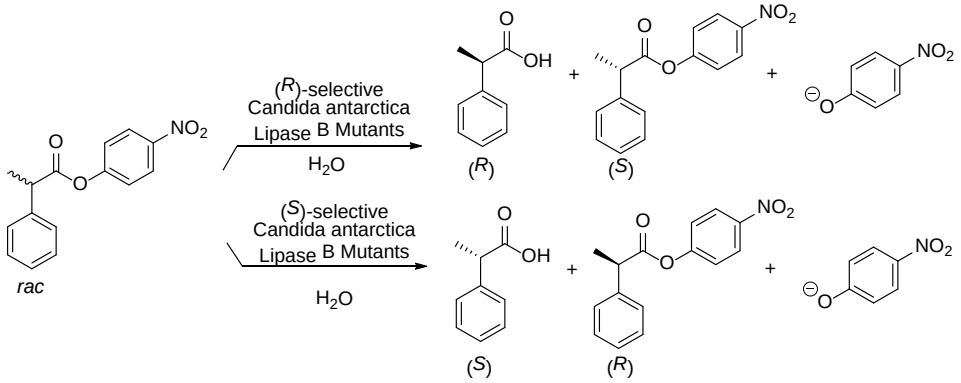
Yet another difficult synthetic transformation, not at all possible by standard synthetic organic reagents or catalysts, concerns the diastereoselective Baeyer-Villiger reaction of compounds of the type represented by 4-bromomethylene-cyclohexanone (Scheme 6). *This type of transformation has not been considered previously in organic chemistry nor in biocatalysis.* Whereas control experiments using such reagents as MCPBA led to 1 : 1 mixtures of *E*- and *Z*-products, as expected, we discovered that wild-type (WT) Baeyer-Villiger monooxygenase CHMO accepts the compound with 99% *E*-selectivity.[27] Application of ISM provided a mutant showing reversed diastereoselectivity (82% *Z*-selective). *Subsequent Pd-catalyzed carbonylation and Suzuki coupling reactions were performed, demonstrating once more the power of combining biocatalysis with transition metal catalysis.* The approach constitutes a novel way to selectively prepare *E*- and *Z*-olefins.[27] Further examples of directed evolution of stereoselective Baeyer-Villiger monooxygenases were also reported.[20,32]



Scheme 6. Diastereoselective Baeyer-Villiger reactions catalysed by CHMO.[27]

Hydrolytic enzymes such as lipases have been used successfully for decades in organic chemistry and biotechnology, kinetic resolution of chiral alcohols or desymmetrization of meso-diols being typical examples (which can also be performed to some extent by applying chiral organocatalysts). In contrast, chiral acids or esters are often more difficult to handle, e.g., the robust and industrially viable lipase *Candida antarctica* B (CALB) cannot be used for this type of substrates (slow reactions with poor stereoselectivity), and organocatalysts show only moderate stereoselectivity as recently shown by the groups of I. Shiina and V. B. Birman. Therefore, we initiated a project directed toward evolving CALB mutants with broad substrate scope.[25] ISM was first

applied to the model transformation involving the ibuprofen-type of substrate shown in Scheme 7, then other substrates were likewise tested.



Scheme 7. Hydrolytic kinetic resolution catalyzed by CALB mutants.[25]

Five CAST sites A, B, C, D and E around the binding pocket were chosen for saturation mutagenesis. Exploration of a select few upward/downward pathways provided *S*- and *R*-selective mutants, respectively (Figure 1).[25] The results were explained on a molecular level by extensive MD simulations and docking experiments. Enzyme kinetics were also performed, demonstrating notably enhanced catalytic activity in the reaction of this particular compound, but also when subjecting structurally related substrates to this kind of hydrolytic kinetic resolution.[25] The superiority of ISM was also demonstrated using other lipases.[4,13]

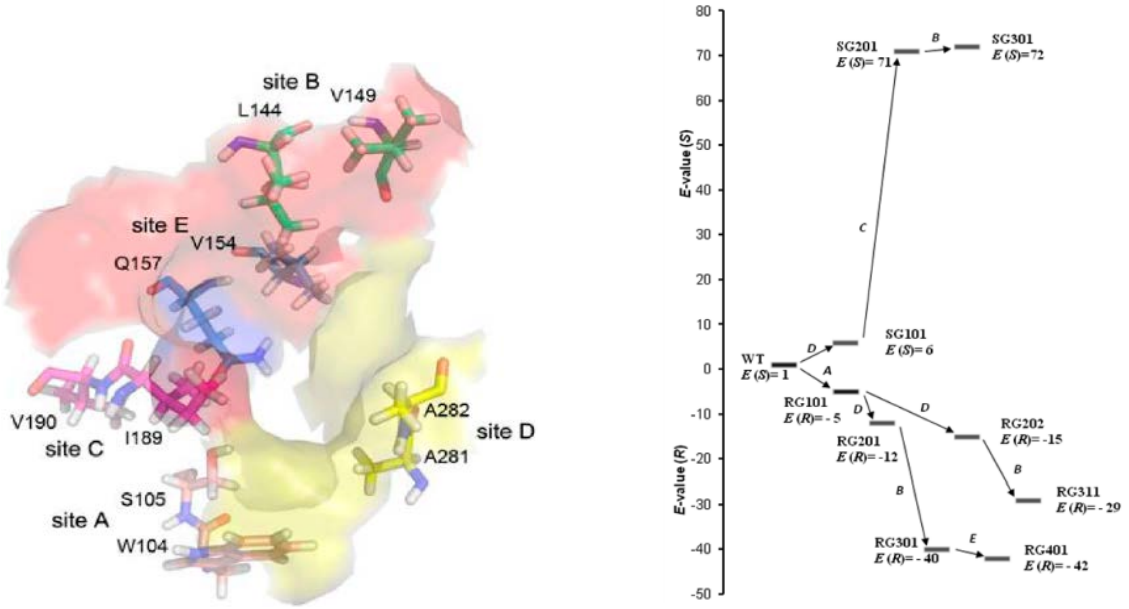
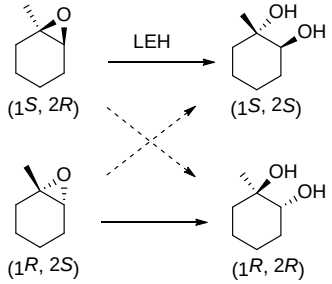


Figure 1. Left: CAST sites A-E in CALB; right: ISM results of the model reaction.[25]

In research regarding biocatalysis not related to directed evolution, we discovered that the activity and selectivity of P450-BM3 can be notably enhanced by the use of chemically inert additives of the type per-fluoro fatty acids $CF_3(CF_2)_nCO_2H$, which presumably enter the large binding pocket with concomitant reduction of its size, while also switching the low-spin form of heme-Fe to the catalytically active high-spin state.[9] A number of small alkanes not accepted by WT P450-BM3 such as propane are oxidatively hydroxylated regioselectively (only isopropanol) with high TON values (≈ 3000), which may have practical ramifications.[9] Methane as originally reported does not undergo oxidation to such an extent, if at all, which has been corrected. Nevertheless, the notion that an additive of this kind acts as a molecular switch, turning on the catalytically active state of the enzyme while effectively reducing the volume of the large binding pocket, constitutes a practical approach. It can be combined with directed evolution: We have shown that the selectivity of P450-BM3 mutants evolved by ISM as catalysts in steroid hydroxylation can be influenced by treatment with such additives (S. Kille, M.T. Reetz, unpublished results). Lipases also respond to such additives as recently demonstrated by our group, but the phenomenon behind such effects is a different one.[10]

Other work concerns thermostabilization of selected enzymes and higher expression rates using directed evolution and rational design as well as enzyme immobilization.[8,12,21] In collaboration with E. Meggers (Marburg), *we have also evolved the first P450-BM3 mutants for selective bioorthogonal deprotection reactions occurring in cells* (unpublished). Finally, *a synthetically intriguing goal is the question of stereoconvergent transformation of racemic compounds allowing for 100% conversion instead of the usual kinetic resolution*. This was tested using an epoxide hydrolase (EH), although epoxides cannot be racemized. While some examples of stereoconvergency are known in the literature using certain WT EHs and we had previously performed directed evolution of the EH from *Aspergillus niger* (ANEH) for this purpose in one case, we recently turned to the limonene epoxide hydrolase (LEH) due to its higher intrinsic activity towards bulky substrates. An example is shown in Scheme 8. Initial results appear promising, setting the stage for further ISM-based optimization. Currently we record an *ee*-value of 70% at 95% conversion in favor of the (1*S*, 2*S*)-diol (E. Siirola, M.T. Reetz, unpublished). It means that one enantiomeric form of the epoxide reacts with water regioselectively at one C-atom, while the enantiomer reacts at the other C-atom, both with inversion of configuration leading to the same enantiomeric product! *One and the same mutant induces two different reactions of*

opposite regioselectivity by enforcing two different poses of the two enantiomers in the enzyme's binding pocket.



Scheme 8. Stereoconvergent process catalyzed by an evolved LEH mutant (E. Siirola, M.T. Reetz).

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

Involved: J. P. Acevedo, C. G. Acevedo-Rocha, A. Augustyniak, W. Augustyniak, M. Bocola, Y. Gumulya, S. Kille, L. P. Parra, S. Prasad, J. Sanchis, F. Schulz, P. Soni, P. Torres Salas, Z.-G. Zhang, H. Zheng, F. E. Zilly

Objective: Two goals were defined: a) to unveil the reasons for increased selectivity and enhanced thermal robustness of mutant enzymes evolved in the laboratory, and b) to understand on a molecular level the efficacy of iterative saturation mutagenesis (ISM).

Results: Two fundamentally different lessons were learned from directed evolution, specifically because additional research efforts were invested following the actual directed evolution studies:

a) Upon performing biophysical characterization of the mutants including enzyme kinetics flanked by MD and QM investigations, the factors leading to improved catalyst improvement can be revealed, which also deepens our understanding of how enzymes function in general.
b) Upon deconvoluting the point mutations or sets of mutations that accumulate along an evolutionary ISM pathway, knowledge is generated which reveals whether additive or non-additive interactions (cooperative, deleterious or none) occur between the individual components (mutations) in the experimentally generated fitness landscape.

In a collaborative effort with the group of Walter Thiel regarding the origin of enantioselectivity in the oxidative desymmetrization of 4-methyl- and 4-hydroxycyclohexanone catalyzed by the Baeyer-Villiger monooxygenase CHMO, a QM/MM molecular dynamics study was performed.[16] Although the gross mechanism had been established decades earlier as involving a flavin-hydroperoxide anion ($FADHOO^-$) attacking the carbonyl function with formation of a Criegee-intermediate followed by fragmentation and migration of a σ-bond, details crucial for a true understanding were lacking. The computations showed that the Criegee-intermediate is stabilized by a strong H-bond due to Arg-329. It was also shown that the traditional stereoelectronic requirement for low-energy σ-bond migration in the Criegee intermediate of synthetic reactions, namely an anti-periplanar arrangement of the C-C-O-O moiety, is decisive for fast and stereoselective CHMO-catalyzed reactions. The

energy difference between the chair conformer of 4-methylcyclohexanone with equatorial methyl and the chair conformer with methyl in the axial position (≈ 2.3 kcal/mol) determines the outcome of the desymmetrization reaction! *Insight into the mechanism of CHMO as a typical Baeyer-Villiger monooxygenase was thereby generated, increasing our knowledge of how this class of enzyme functions.*[16] In the case of desymmetrization of 4-hydroxy-cyclohexanone catalyzed by a CHMO mutant, H-bonds to the hydroxyl group was found to play a major role.[29]

Another theoretical problem relates to the question *why certain P450-BM3 mutants obtained by ISM show pronounced regio- and stereoselectivity when used as catalysts in the oxidative hydroxylation of such substrates as steroids*, whereas the starting enzyme is unselective (Report 2.1.2). Since a high-energy radical process is involved, it is unlikely that the transition state is stabilized by the protein environment as it is in most other enzyme-catalyzed transformations (Pauling hypothesis of why enzymes are so active). Rather, any regio- and stereoselective P450-catalyzed reaction must involve a protein environment which simply holds the substrate in a certain pose with the respective C-H moiety pointing directly at the catalytically active high-spin heme-Fe=O. Therefore, the 2ß- and 15ß-selective P450-BM3 mutants as catalysts in the oxidation of testosterone were subjected to extensive MD simulations and docking experiments.[3] It turned out that in the case of the 2ß-selective mutant the energetically most favorable pose is indeed the one in which the 2ß-hydrogen atom points directly at heme-Fe=O, ready to be attacked via H-abstraction followed by rapid C-O bond formation of the short-lived radical. In the case of the 15ß-selective mutant, only one active pose is revealed in which the 15ß-hydrogen atom is poised for oxidative attack (Figure 1).

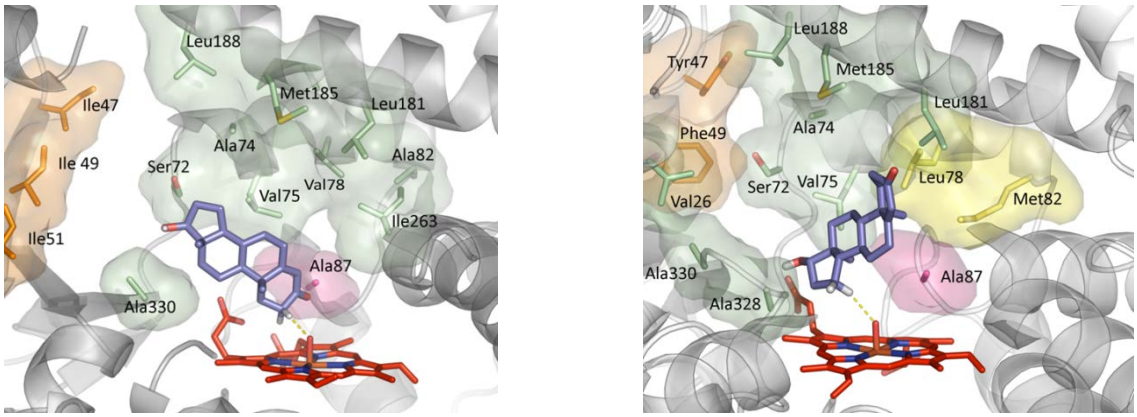


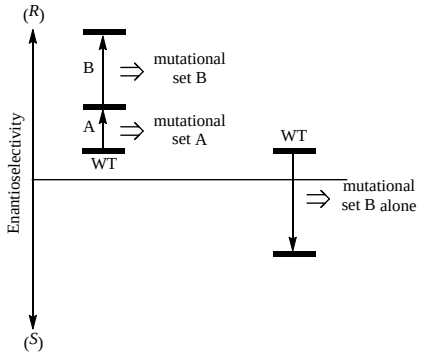
Figure 1. Left: Lowest energy pose of testosterone above catalytically active heme-Fe=O in 2ß-selective mutant; right: respective pose in 15ß-selective mutant.[3]

In contrast to P450 catalyzed reactions, those mediated by hydrolases such as lipases or epoxide hydrolases involve transition states in which the protein environment exerts a stabilizing effect due to many different interactions between the substrate and the binding pocket wall. Quite often these can be identified by MD simulations and docking experiments without the need to perform QM calculations. *We have utilized this option in our own group routinely, thereby enabling the identification of factors responsible for the observed mutational effects on catalysis, insights which deepen our understanding of how these enzymes function in detail.* One of several examples during the last three years concerns the study of (*R*)- and (*S*)-selective mutants of the lipase CALB as catalysts in the kinetic resolution of α-phenyl propionic acid ester (Report 2.1.2). Additional stabilizing H-bonds of the oxy-anion intermediate were identified in each case, thereby explaining the role of mutants.[25] Similar effects were uncovered in the directed evolution of *Ps. aeruginosa* lipase.[4]

A very different goal is to gain an understanding as to why ISM is so effective. *For this purpose we have performed extensive deconvolution studies with concomitant construction of fitness landscapes reflecting the energies of complete ISM pathways as determined by experimental* $\Delta\Delta G^{\neq}$ *values* (Report 2.1.2). In order to analyze the vast data more carefully, the question of additivity versus non-additivity of mutational interactions needs to be posed and answered at every step of an evolutionary pathway.[15,24,25,32,34] Consider a 4-step pathway in a 4-site ISM scheme (Report 2.1.1). The influence of the first set of mutations (or possibly single point mutation) in library A is known experimentally, and when visiting site B the effect of the sum of the first mutational set (A) and the second mutational set (B) is also accessible by measuring the catalytic property of interest (e.g., enantioselectivity). However, the effect of mutational set B alone is not known. Therefore, in a deconvolution step the mutational set B is introduced by site-specific mutagenesis of the original WT enzyme with formation of a new mutant.

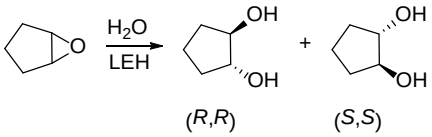
In traditional protein engineering, scientists expected mathematical additivity of the two sets of mutations (or two different single point mutations). *What we find in our work is the "surprising" fact that most often (mathematical) additivity does not pertain.*[24] Rather, non-additivity is observed in terms of pronounced cooperative effects, i.e., more than additivity. For example, the effect at mutational set B alone can be very small, but in concert with the mutational set A as observed in the ISM step an enormous increase in desired catalytic property results at stage A-B of the evolutionary pathway. Sometimes this type of deconvolution leads to an even more surprising effect, namely that A increases for example (*R*)-selectivity as does the accumulation of the mutational

set B in the subsequent ISM step, but deconvolution of A-B reveals that the mutational set B alone is actually (*S*)-selective. Mutational set B alone can formally be considered to be deleterious, but in concert with mutational set A, strong communication on a molecular level leads to enhanced (*R*)-selectivity (Scheme 1).[15,25,32]



Scheme 1. Example of strong non-additivity characterized by a pronounced cooperative effect.[15, 25,32]

Data of this kind also throws light on the efficacy of ISM and is relevant to protein engineering in general. We routinely encountered even more surprising results. For example, upon deconvoluting an evolved (*S*,*S*)-enantioselective double mutant (95% *ee*) of an epoxide hydrolase as a catalyst in the desymmetrization of cyclopentane oxide (Scheme 2), "strange" observations were made (E. Siirola, M. T. Reetz, unpublished): Each of the two single point mutations alone induce (*R*,*R)*-selectivity, i.e., opposite enantioselectivity! MD studies directed toward understanding these cooperative effects on a molecular level are underway.



Scheme 2. Hydrolytic desymmetrization catalyzed by LEH mutants (E. Siirola, M.T. Reetz, unpublished).

More data emerging from deconvolution studies is being collected in our group.[24] Today we believe that such phenomena occur more often than previously thought (also in Nature?). *The amino acids in any given protein interact with one another (unpredictably? in a non-linear manner*. We have performed an analysis of our extensive laboratory data accumulated thus far and of rare results of other groups, which has opened a new research area.[24]

Finally, *in order to uncover the reasons for enhanced thermal robustness of a Bacillus subtilis lipase mutant on a molecular level, protein NMR studies and other biophysical investigations were performed in collaboration with R. Boelens and B. Dijkstra*

(Groningen/Netherlands).[12,21] The effect of the mutations was traced to a notably reduced deleterious protein/protein interaction which in the WT lipase leads to aggregation and undesired precipitation upon heat treatment. Such behavior in the WT lipase shuts down catalytic performance, but in the mutant it is sharply reduced! We have learned a lot from this unusual phenomenon.[12,21]

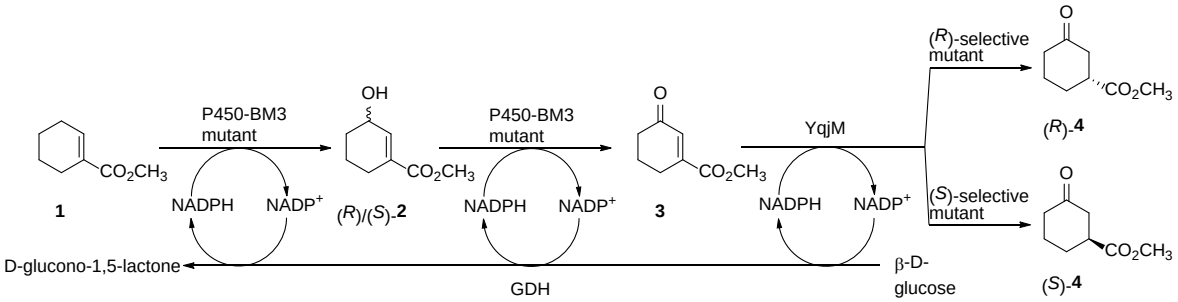
2.1.4 Research Area "Constructing Designer Cells for Enzymatic Cascade Reactions Based on Directed Evolution" (M. T. Reetz)

Involved: R. Agudo

Objective: The goal was to construct designer cells for stereocomplementary *de novo* enzymatic cascade reactions based on directed evolution.

Results: The benefits of exploiting sequential organic transformations in a single reaction vessel using synthetic reagents, catalysts or enzymes without isolating intermediate products have been documented in many studies. Nature orchestrates the buildup of structural complexity by enabling such reaction sequences *in vivo* in the cytosol of cells in which a multitude of enzymes function as selective catalysts. In the quest to produce complex natural products or biofuels, researchers have used whole cells, state of the art metabolic engineering offering many opportunities. These systems are considerably more complex than ordinary so-called designer cells such as engineered *E. coli* cells harboring, for example, an alcohol dehydrogenase (ADH) and the necessary NAD(P)H regeneration enzyme as a platform for asymmetric reduction of non-natural prochiral ketones. Industry often prefers whole cell catalysis relative to the *in vitro* use of isolated enzymes.
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, and indeed few examples have been reported to date and none utilizing directed evolution. 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.

Along these lines we have conceived, as a proof-of-principle, the following redox reaction sequence to be performed in a one-pot manner in stereocomplementary designer cells.[35] Starting from a simple compound **1** a value-added structurally more complex product **4** was envisioned to be formed in either of the two enantiomeric forms on an optional basis. One whole cell system was designed to provide the (*R*)-configurated final product (*R*)-**4**, while the other cell system was supposed to lead to the enantiomeric (*S*)-**4** (Scheme 1).



Scheme 1. Stereodivergent designer *E. coli* cells for converting starting compound **1** into either (*R*)-**4** or (*S*)-**4** in one-pot processes, respectively.[35]

The genes of three different enzymes were considered in this plan, namely a P450 monooxygenase for regio- and chemoselective *oxidation*, an enoate reductase for chemo- and stereoselective *reduction* and glucose dehydrogenase (GDH) for *cofactor regeneration*. In traditional synthetic organic chemistry the simultaneous use of oxidizing and reducing agents is generally problematic, but in biocatalysis it is more likely to succeed.[28] The major challenge in the present system is, *inter alia*, the two-step chemo- and regioselective C-H activating oxidative hydroxylation of compound **1** with formation of ketone **3** in the absence of alternative oxidation products resulting from **1** or **2** as well as the absence of likewise undesired oxidation events possibly occurring with **3** or **4**. We used P450-BM3 as the monooxygenase for the first two steps in *E. coli* cells, and therefore first carried out a study aimed at creating a mutant that catalyzes the **1** → **2** → **3** conversion with maximum efficiency. Since WT P450-BM3 fails (mixture of many oxidation products), we screened several mutant libraries obtained by saturation mutagenesis (CAST), the best mutant being Val28Leu/Ala82Phe/Phe87Ala. The gene of this mutant was then incorporated in *E. coli* designer cells (BOU730) harboring glucose dehydrogenase (GDH) necessary for NADPH regeneration. We had previously engineered the *E. coli* BOU730 cells in a study concerning stereoselective enoate reductases, and indeed this cell strain can be used conveniently in any enzymatic redox reaction that requires NADPH (or NADH) regeneration. For the last reduction step we envisioned the use of the enoate reductase YqjM from the Old Yellow Enzyme family. In our laboratory we had already applied ISM for evolving (*R*)- and (*S*)-selective mutants, respectively.[36] In control experiments it was ensured that these mutants are also chemoselective in that they do not catalyze the reduction of the starting material **1** which is not accepted by the enzymes.

In this proof-of-principle study we wanted to gain as much experience as possible regarding the choice of the molecular biological setup in relation to the final synthetic outcome, and therefore designed three different experimental platforms[35]:

(1) Use of two different engineered *E. coli* cells in a one-pot process, i.e., BOU730 cells containing an appropriate P450-BM3 mutant gene and BOU730 cells containing the respective YqjM mutant genes. This enables a convenient control element over the multistep process, because the ratio of the two cells can be appropriately adjusted, and after an appropriate time-lag the second cells can be added strategically once the first cell strain has performed its function as revealed by the reaction progress.

(2) Use of a one-pot two plasmid system based on BOU730 cells transformed with two different plasmids that encode for P450-BM3 and YqjM mutants, all in *E. coli* cells. This may lead to a metabolic burden influencing bacterial growth rate and/or protein expression rates.

(3) Use of engineered *E. coli* strains that harbor one of the YqjM genes inserted into the genome with P450-BM3 remaining in a plasmid. Problems similar to approach (2) may occur.

Approach (1): Upon adding in separate experiments *R*- or *S*-selective YqjM strains, respectively, to the reaction vessels in which the P450-BM3-catalyzed reaction had occurred for about one hour, both enantiomeric products were readily obtained after an additional 15 minutes as shown by GC analysis: ≈ 85% overall conversion to (*R*)-**4** (99% *ee*) and (*S*)-**4** (99% *ee*). Upon upscaling the reaction to 7.3 mM of starting compound **1**, the two-cell systems led to 72% of (*R*)-**4** and 75% of (*S*)-**4**, each enantiomerically pure. Control experiments testing the use of a mixture of isolated enzymes *in vitro* resulted in poor conversion to the desired products (< 24 %), which clearly demonstrates the superiority of using whole cells as nanoreactors.[35]

Approach (2): When applying the two-plasmid system, BOU730 cells transformed with two different plasmids, pRSF-P450BM3 and pACYC-YqjM, were used. These encode the P450-BM3 and YqjM mutants, respectively, under regulation of the bacteriophage T7 promoter. Upon adding IPTG to the medium, both proteins were successfully expressed as shown by control experiments. Although optimization was not strived for, the results proved to be reasonable: ≈ 50% regioselectivity and 99% enantioselectivity. The undesired side-products are due to overoxidation of the intermediate compounds and partially of **4**.[35]

Approach (3): This is the most challenging option in terms of controlling all necessary parameters, in which a one-plasmid system encoding the P450-BM3 mutant necessary in the first two steps is utilized while the YqjM coding sequences of the *R*-selective mutant (or *S*-selective mutant) are likewise inserted into the *E. coli* chromosome using BOU730 cells. Nevertheless, surprisingly good results were observed, although here again full optimization was not strived for: 50-52% conversion to the desired final products **4** in enantiomerically pure form.[35]

The limiting factor with regard to the formation of (*R*)-**4** or (*S*)-**4**, respectively, is the obvious possibility of indiscriminate oxidation of four different compounds, namely **1**, **2**, **3** and **4**, which complicates matters in the present system. Since the oxidation steps are rate-limiting as shown by control experiments, the use of two designer cells according to approach (1) with an optimal time-lag between their uses constitutes a useful tool. Nevertheless, all three approaches deserve attention in future studies using structurally more demanding substrates. Other types of enzymes also need to be studied in such an approach which minimize the possibility of conflicting side-reactions as in our first attempt to enter this challenging new field. *Finally, we conclude that the approach described herein nicely complements present day metabolic engineering.*

2.1.5 Research Area "Development of Novel Catalyst Systems for Epoxidation Reactions" (W. Leitner, N. Theyssen)

Involved: Y. Qiao

Objective:

The development of efficient catalytic epoxidation protocols for olefins remains an attractive target. Especially, if environmentally benign oxygen sources like hydrogen peroxide are used and catalyst systems with good recyclability are realized.

Results: (Figure 1).

We have synthesized a variety of heterogeneous epoxidation catalysts with a ferromagnetic core in nanoscale allowing a most easy catalyst recovery in a multi-batch procedure by a permanent magnet. Each ferrit core is covered by a dense SiO_2 layer to prevent unproductive iron ion-initiated decomposition of hydrogen peroxide.

In a first project, the catalytically active peroxotungstate anion, $[W_2O_{11}]^{2-}$, was immobilised via ionic forces to positively charged imidazolium containing entities (Figure 1). In accordance with results from diffuse reflectance IR-spectroscopy (DRIFTS) and ^{29}Si cross polarization magic angle spinning NMR-spectroscopy (CP/MAS), the imidazolium motives seem to be bounded in the envisaged manners to the coated ferrit core: either by hydrogen bonds (catalyst **1**) or covalent linkages (catalyst **2**).

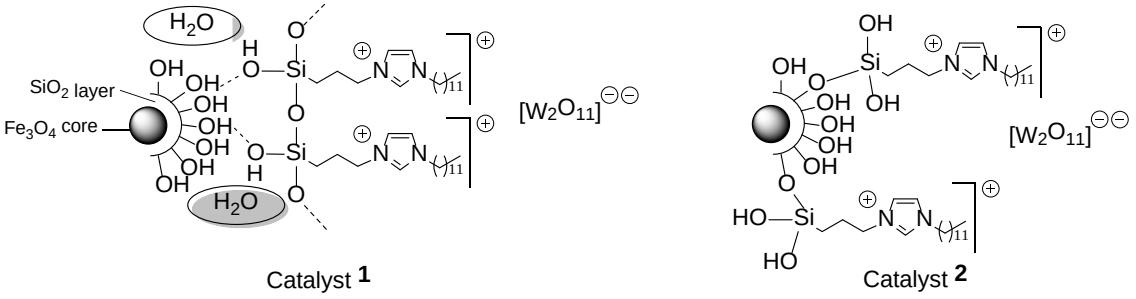


Figure 1: Magnetically recoverable catalysts for epoxidation based on peroxotungstate anions.

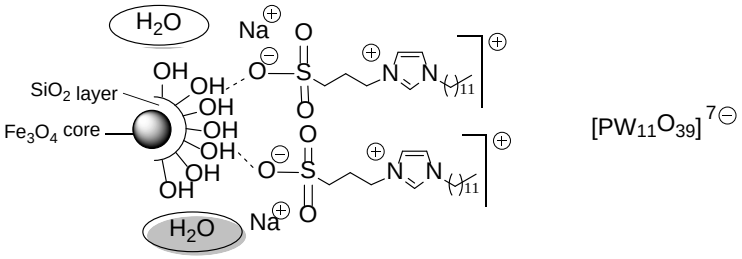
The epoxidation of cyclooctene was chosen as a benchmark reaction for both catalytic systems. A summary of the obtained results is given in the following:

- A methanol/water mixture (volume ratio 10:1) was found to be most suited in terms of catalyst activity, selectivity and stability. The small water fraction was shown to be crucial for long life stability of both systems.

- Surprisingly, the hydrogen bonded catalyst **1** turned out to be more stable in comparison with **2**, which immobilisation is reached by covalent bonds: under optimised conditions, 10 respective 7 consecutive runs could be performed using catalysts **1** and **2** without any performance loss. Complete conversion and perfect selectivity was detected in every cycle.
- Hot filtration experiments support the presence of heterogeneous catalysis. However, it cannot be excluded that the solid catalyst fraction might function as a reservoir for dissolvable active species. If this is the case, a quite fast deactivation of dissolved species would happen.
- Derived catalyst of simpler structures shows much weaker performances. For example, an impregnation of the shielded ferrit cores with $K_2W_2O_{11}$ resulted in a conversion of only 9%. Likewise, a physical mixture of the ionic components $[DMIm]_2$ (D = dodecyl) and $[W_2O_{11}]$ with the shielded ferrit cores gave only 34% conversion.

A substrate screening identifies cycloheptene and allylic alcohols as additional suitable substrates. 3-methyl-2-buten-1-ol, geraniol and cinnamyl alcohol were converted completely with good to perfect selectivities. Cyclohexene and styrene gave product mixtures, whereas 1-decene gave poor conversions but perfect selectivity.

In a follow-up project we have tried to enhance both the catalytic activity and immobilisation stability via the hydrogen bond mode. Therefore a catalyst was designed in which a heteropoly anion, $[PW_{11}O_{39}]^{7-}$ (a lacanury-type polyoxometalate), was used as the catalytically active moiety (Figure 2).



Catalyst **3**

Figure 2: Magnetically recoverable catalyst based on a lacunary-type polyoxometalate

Further differences were implemented in the imidazolium cation structure which now contains sulfonate groups in order to increase the bond strength of the hydrogen bonds to the silanol groups of the covered ferrit nanoparticles (Figure 2). Catalyst **3** works best under solvent-free conditions. 10 recycles without any activity loss in the epoxidation of

cyclooctene were monitored. Noteworthy, the leaching rate was reduced from 870 ppm in methanol to 3 ppm under solvent-free conditions which is so far the lowest value detected in our studies. Hot filtration experiments argue for heterogeneous catalysis only in the latter case.

During the reaction, **3** is present in an emulsion between aqueous H_2O_2 and cyclooctene. The addition of ethyl acetate after reaction breaks the emulsion and the catalyst can be separated easily by a magnetic field. In comparison to catalyst **1**, the substrate scope with **3** under solvent-free conditions is more limited. In addition to cyclooctene, allylic alcohols like 3-methyl-2-buten-1-ol, geraniol and crotyl alcohol were converted to more than 85% with over 93% selectivity.

Finally, a quasi-homogeneous catalyst **4** was developed on the made experiences. Here, the same imidazolium cation as in catalyst **1**, **2** and **3** was combined with the lacunary-type polyoxometalate of **3**. The study revealed that the optimal ratio of the two components is 7:1 which reflects the charge ratio of these ions. Moreover, a variation in the alkyl chain length of the imidazolium cation showed that the dodecyl group is crucial. Its substitution by a methyl or hexyl group resulted in a very low cyclooctene conversion of only 1% and 2% respectively.

4 builds a fully homogeneous phase with aqueous hydrogen peroxide. The addition of cyclooctene results again in the formation of a stable emulsion, which makes an additional solvent unnecessary. Under such conditions this catalyst is extremely active allowing an epoxidation of cyclooctene already at 0 °C. Quantitative yield was obtained after 3 h reaction time. The activation energy was determined to be 34 kJ/mol. This is unprecedented low in comparison to values reported in the literature for epoxidation of cyclooctene (49-86 kJ/mol). Recycling of **4** is of course not as easy and elegant as in case of catalysts **1**-**3**, but possible via extraction with cyclohexane. This procedure allows at least three runs without activity loss.

External Funding: Chinese Scholarship Council (CSC)

Cooperations: Z. Hou (Research Institute of Industrial Catalysis, East China University of Science and Technology, Shanghai, P.R. China)

2.1.6 Publications 2011-2013 from the Department of Synthetic Organic Chemistry

Reetz Group

(1) Braunstein, P.; Reetz, M. T.; Sun, W.-H. *C. R. Chimie* **2011**, *14*, 787-788.

(2) Gumulya, Y.; Reetz, M. T. *ChemBioChem* **2011**, *12*, 2502-2510.

(3) Kille, S.; Zilly, F. E.; Acevedo, J. P.; Reetz, M. T. *Nat. Chem.* **2011**, *3*, 738-743.

(4) Prasad, S.; Bocola, M.; Reetz, M. T. *ChemPhysChem* **2011**, *12*, 1550-1557.

(5) Reetz, M. T. *Angew. Chem., Int. Ed.* **2011**, *50*, 138-174.

(6) Reetz, M. T. Die Evolutionsmaschine als Quelle für selektive Biokatalysatoren. In *Molekule aus dem All*; Al-Shamery, K., Ed.; Wiley-VCH, Weinheim, 2011, pp. 243-273.

(7) Reetz, M. T.; Krebs G. P. L. *C. R. Chimie* **2011**, *14*, 811-818.

(8) Reetz, M. T.; Zheng, H. *ChemBioChem* **2011**, *12*, 1529-1535.

(9) Zilly, F. E.; Acevedo, J. P.; Augustyniak, W.; Deege, A.; Häusig, U. W.; Reetz, M. T. *Angew. Chem., Int. Ed.* **2011**, *50*, 2720-2724; Corrigendum: *Angew. Chem. Int. Ed*. **2013**, *52*, 13503.

(10) Acevedo-Rocha, C. G.; Reetz, M. T. *Catal. Sci. Technol.* **2012**, *2*, 1553-1555.

(11) Agudo, R.; Roiban, G.-D.; Reetz, M. T. *ChemBioChem* **2012**, *13*, 1465-1473.

(12) Augustyniak, W.; Brzezinska, A. A.; Pijning, T.; Wienk, H.; Boelens, R.; Dijkstra, B. W.; Reetz, M. T. *Protein Sci.* **2012**, *21*, 487-497.

(13) Cesarini, S.; Bofill, C.; Pastor, F. I. J.; Reetz, M. T.; Diaz, P. *Process Biochem.* **2012**, *47*, 2064-2071.

(14) Feng, X.; Sanchis, J.; Reetz, M. T.; Rabitz, H. *Chem.-Eur. J.* **2012**, *18*, 5646-5654.

(15) Gumulya, Y.; Sanchis, J.; Reetz, M. T. *ChemBioChem* **2012**, *13*, 1060-1066.

(16) Polyak, I.; Reetz, M. T.; Thiel, W. *J. Am. Chem. Soc.* **2012**, *134*, 2732-2741.

(17) Reetz, M. T. Directed Evolution of Enzymes. In *Enzyme Catalysis in Organic Synthesis*; Drauz, K., Gröger, H., May, O., Eds.; 3rd Edition, Vol. 1-3, Wiley-VCH, Weinheim, 2012, Vol. 1, pp.119-190.

(18) Reetz, M. T. *Chem. Rec.* **2012**, *12*, 391-406.

(19) Reetz, M. T. *Tetrahedron* **2012**, *68*, 7530-7548.

(20) Zhang, Z.-G.; Parra L. P.; Reetz, M. T. *Chem. Eur. J.* **2012**, *18*, 10160-10172.

(21) Augustyniak, W.; Wienk, H.; Boelens, R.; Reetz, M. T. *Biomol. NMR Assign.* **2013**, *7*, 249-252.

(22) Agudo, R.; Roiban, G.-D.; Reetz, M. T. *J. Am. Chem. Soc.* **2013**, *135*, 1665-1668.

(23) Kille, S.; Acevedo-Rocha, C. G.; Parra, L. P.; Zhang, Z.-G.; Opperman, J. J.; Reetz, M. T.; Acevedo J. P. *ACS Synth. Biol*. **2013**, *2*, 83-92.
(24) Reetz, M. T. *Angew. Chem., Int. Ed*. **2013**, *52*, 2658-2666.
(25) Wu, Q.; Soni, P.; Reetz, M. T. *J. Am. Chem. Soc.* **2013**, *135*, 1872-1881.
(26) Roiban G.-D.; Reetz, M. T. *Angew*. *Chem., Int. Ed*. **2013**, *52*, 5439-5440.
(27) Zhang, Z.-G.; Roiban, G.-D.; Acevedo, J. P.; Polyak, I.; Reetz, M. T. *Adv. Synth. Catal.* **2013**, *355*, 99-106.
(28) Reetz, M. T. *J. Am. Chem. Soc*. **2013**, *135*, 12480-12496.
(29) Polyak, I.; Reetz, M. T.; Thiel. W. *J. Phys. Chem. B*, **2013**, *117*, 4993-5001.
(30) Roiban, G.-D.; Agudo, R.; Reetz, M. T. *Tetrahedron*, **2013**, *69*, 5306-5311.
(31) Reetz, M. T. Practical Protocols for Lipase Immobilization Via Sol-Gel Techiques. In *Methods in Biotechnology*; Guisan, J. M., Ed.; 2nd Edition, Humana Press, Totowa, 2013, Vol. 241, pp. 241-254.
(32) Parra, L. P.; Agudo, R.; Reetz, M. T. *ChemBioChem* **2013**, *14*, 2301-2309.
(33) Acevedo-Rocha, C. G.; Reetz, M. T. Assembly of Designed Oligonucleotides: A Useful Tool in Synthetic Biology for Creating High Quality Combinatorial DNA Libraries. In *Directed Evolution Library Creation: Methods and Protocols*; *Methods in Molecular Biology*; Ackerley, D., Copp, J., Gillam, E., Eds.; 2nd Edition, Humana Press, Totowa, 2013.
(34) Acevedo-Rocha, C. G.; Kille, S.; Reetz, M. T. Iterative Saturation Mutagenesis: A Powerful Approach to Engineer Proteins by Simulating Darwinian Evolution. In *Directed Evolution Library Creation: Methods and Protocols; Methods in Molecular Biology;* Ackerley, D., Copp, J., Gillam, E., Eds.; 2nd Edition, Humana Press, Totowa, **2013**.
(35) Agudo, R.; Reetz, M. T. *Chem. Comm.* **2013**, *49*, 10914-10916.
(36) Kille, S., Reetz, M. T. Protein Engineering: Development of Novel Enzymes for the Improved Reduction of C=C Double Bonds. In *Synthetic Methods for Biologically Active Molecules-Exploiting the Potential of Bioreductions*; Brenna, E., Ed.; Wiley-VCH, Weinheim, **2013**.

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(37) Qiao, Y.; Li, H.; Hua, L.; Orzechowski, L.; Yan, K.; Feng, B.; Pan, Z.; Theyssen, N.; Leitner, W.; Hou, Z. *ChemPlusChem* **2012**, **77**, 1128-1138.
(38) Qiao, Y., Hua, L.; Chen, J.; Theyssen, N.; Leitner, W.; Hou, Z. *J. Mol. Catal. A: Chem.* **2013**, 380, 43-48.
Further papers of W. Leitner regarding other topics see publication list in the appendices.