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.

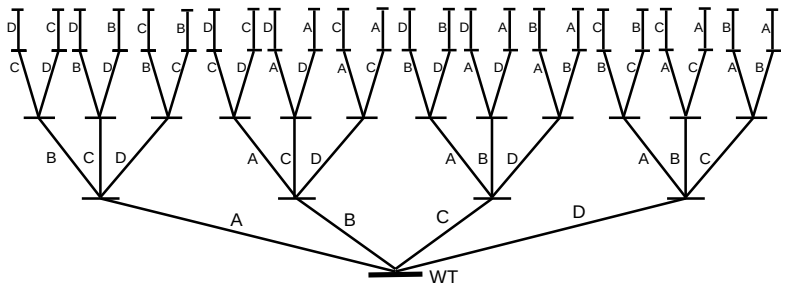
Due to the upcoming retirement of Manfred Reetz, new group leaders (assistant professors for Habilitation) were not recruited for the Department.

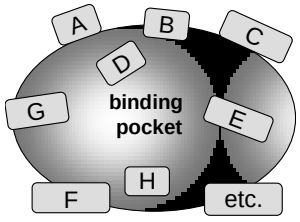
2.1.1 Research Area "Methodology Development in Directed Evolution" (M. T. Reetz)

Involved: J. P. Acevedo, M. Bocola, D. J. Bougioukou, J. D. Carballeira, J. Drone, L. Fernàndez, L. Gonzaga de Oliveira, Y. Gumulya, H. Höbenreich, F. Hollmann, N. Jiao, D. Kahakeaw, S. Kille, R. Lohmer, J. J.-P. Peyralans, J. Podtetenieff, S. Prasad, J. Sanchis, F. Schulz, M. Rusek, P. Soni, A. Taglieber, S. Wu, H. Zheng, F. E. Zilly

Objective: The goal was methodology development in the quest to make directed evolution more efficient and faster than the state of the art in 2007.

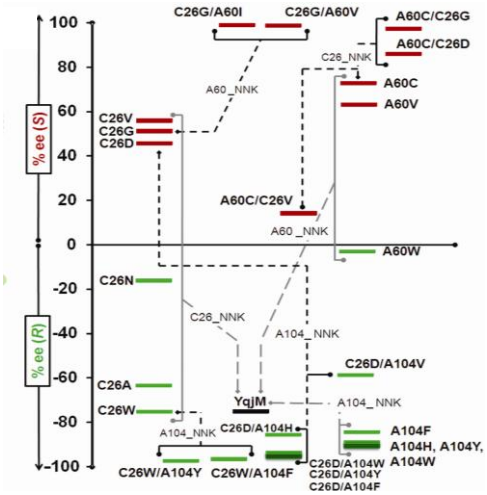
Some degree of catalyst improvement can always be expected from directed evolution, irrespective of the mutagenesis strategy or method, repeating rounds of error-prone PCR as a "shotgun method" being the most popular approach. However, especially in our group the focus of research has turned to methodology development in the quest to make directed evolution faster, more efficient and reliable. In the previous Report (2005-2007), we described initial results of what we termed Iterative Saturation Mutagenesis (ISM) as a means to generate high-quality mutant libraries, quality being defined in terms of the frequency of hits in a given mutant library and the degree of catalyst improvement, be it stereoselectivity, activity or thermostability. *ISM is a knowledge-driven approach to directed evolution which requires only small libraries and which has proven to be much more successful than originally anticipated.* Sites in an enzyme comprising one or more amino acid positions, labeled A, B, C, D, etc, are randomized by saturation mutagenesis, and the genes of the hits are then used as templates for randomization at the other sites. In the case of a 4-residue site, the scheme below pertains. It is not necessary to explore all pathways, but the appropriate choice of the sites is crucial. In the case of stereoselectivity and/or substrate acceptance, sites around the binding pocket are chosen (CASTing).

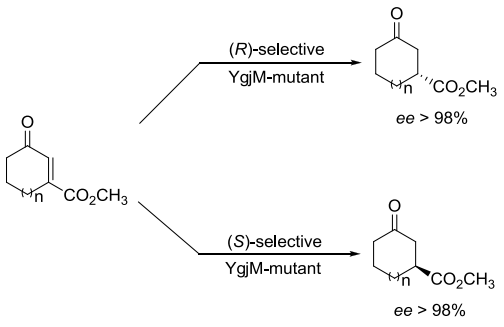




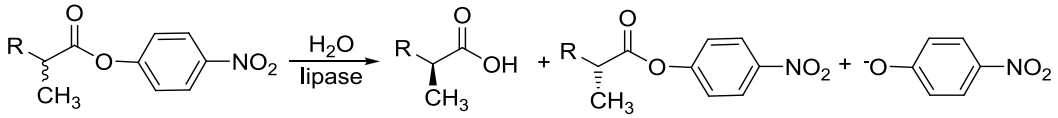
The first example of ISM in the embodiment of CASTing was described in the previous Report (2005-2007), in which the enantioselectivity of the epoxide hydrolase from *Aspergillus niger* (ANEH) as a catalyst in the hydrolytic kinetic resolution of a chiral epoxide was improved from E = 4.6 (WT) to E = 115, as compared to E = 11 using epPCR and screening the same number of transformants. This was the first indication that libraries resulting from ISM are "smart". During the last three years we have not only generalized this approach, the underlying reason for efficacy was also pinpointed: It is the absence of superfluous mutations coupled with the occurrence of cooperative epistatic effects operating between the point mutations within a site *and* between sets of mutations occurring at the sites A, B, C, D, etc. Cooperativity is the ideal form of epistasis in directed evolution because it means more than additive interactions. Moreover, we have shown in several studies that the utilization of reduced amino acid alphabets as ensured by the appropriate codon degeneracy reduces the amount of oversampling necessary for 95% library coverage drastically. For example, instead of using the normal NNK codon degeneracy encoding all 20 canonical amino acids, we have shown by statistical analysis (CASTER computer aid) 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 3000! The quality of an NDT library matches or exceeds that of an NNK counterpart!

A recent application of ISM-based CASTing was the evolution of (*R*)- and (*S*)-selective mutants of the enoate reductase (YqjM) as catalysts in a model reaction involving the conjugate reduction of 3-methylcyclohexenone (scheme below, left), mutants that also catalyze the reduction of a wide variety of structurally different enones not at all accepted by WT YqjM, as for example illustrated on the right. *This shows once again that in directed evolution you can get more than what you evolved/screened for*.

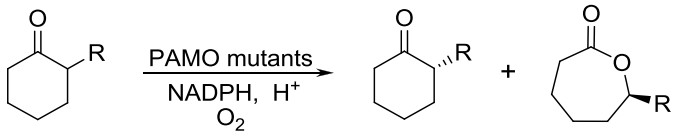




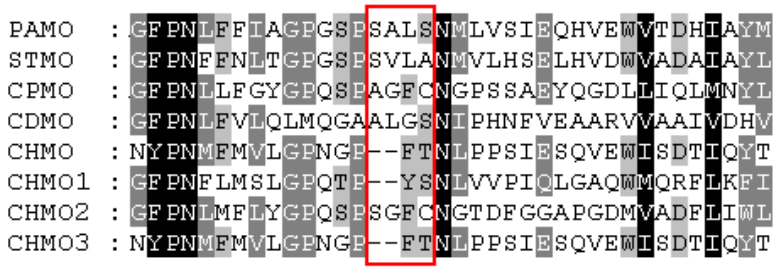
Perhaps the most impressive demonstration of the efficacy of ISM became apparent when we revisited our original system studied in 1995-2001 based on the lipase from *Pseudomonas aeruginosa* as a catalyst in the hydrolytic kinetic resolution of *rac*-2-methyldecanoic acid *p*-nitrophenyl ester. This is the most systematically studied enzyme in directed evolution. Among other attempts, epPCR at various mutation rates, DNA shuffling and non-systematic saturation mutagenesis, requiring the screening of 50,000 transformants, had resulted in a mutant with six point mutations, showing E = 51 compared to WT with E = 1.2. A theoretical analysis in cooperation with the Thiel group had predicted that only two of the six mutations are necessary, which was corroborated experimentally, a triumph of theory but also proving the inefficiency of such mutagenesis methods and strategies. With the new approach using a 3-site ISM scheme in which each site is composed of two residues and screening less than 10,000 mutants, a very active mutant showing E = 594 was rapidly evolved, characterized by only three point mutations. Here again, deconvolution studies uncovered dramatically strong cooperative effects. Superfluous mutations do not occur (in contrast to epPCR). The reason for enhanced activity and stereoselectivity was unveiled by extensive MD simulations.



In addition to the normal CASTing approach in which first sphere residues directly aligning the binding pocket are identified on the basis of the X-ray structure or homology model, second sphere residues can also be considered for saturation mutagenesis (extended CASTing), as shown in the evolution of active and stereoselective mutants of the Baeyer-Villiger-Monooxygenase PAMO. Oxidative kinetic resolution of the following substrates was found to occur with selectivity factors generally amounting to E > 100.



We also developed a *bioinformatics-based* approach to CASTing, by focusing on a loop region at the binding pocket of PAMO, but now aligning the respective sequences of eight different BV monooxygenases in order to identify conserved residues which were assigned as randomization sites. This information was utilized in designing greatly reduced amino alphabets which were subsequently used in saturation mutagenesis, leading to highly stereoselective oxidases.



***Further methodology development during the three year research period*:**

- ISM in the form of B-FIT was applied to an epoxide hydrolase by focusing on sites having high B-factors, resulting in a thermostabilization of 21°C.
- ISM was applied to an epoxide hydrolase as a catalyst for inducing *stereoconvergency* in the transformation of a racemic trans-1,2-disubstituted epoxide with formation of a single enantiomeric diol (99% *ee*).
- ISM was further generalized by evolving highly stereoselective mutants of the limonene epoxide hydrolase, showing broad substrate scope.
- The novel concept of saturation mutagenesis at a remote site which can be expected to induce *allosteric* effects with concomitant re-shaping of the binding pocket, therefore influencing substrate acceptance and stereoselectivity, was demonstrated for the first time using a Baeyer-Villiger Monooxygenase.
- A molecular biological method for saturation mutagenesis for difficult-to-amplify templates was developed based on the use of megaprimers.
- In collaboration with the Rabitz group (Princeton University), a *computational approach* was developed for identifying protein mutants with desired catalytic properties from minimal sampling of focused ISM libraries. The so-called Adaptive Substituent Reordering Algorithm (ASRA) was applied to the *Aspergillus niger* epoxide hydrolase, demonstrating notable predictive power regarding enantioselectivity. The algorithm reduces the screening effort, and therefore increases further the efficacy of ISM.
- A system for light-driven NADPH regeneration in monooxidases and reductases was developed.

Publications resulting from this research area: 85, 88, 90, 94, 101, 119, 161, 173, 232, 234, 238, 240, 241, 283, 305, 367, 384, 385, 386, 387, 388, 407, 419, 425, 426

External funding: Deutsche Forschungsgemeinschaft (Schwerpunkt 1170 “Gerichtete Evolution”); Fonds der Chemischen Industrie

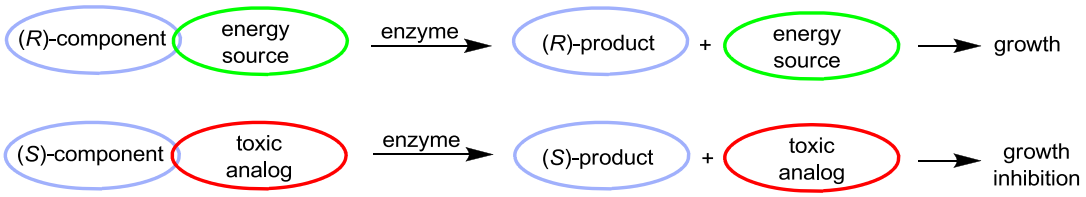
Cooperations: H. Rabitz (Princeton, USA)

2.1.2 Research Area "Methods for Enzyme Screening/Selection" (M. T. Reetz)

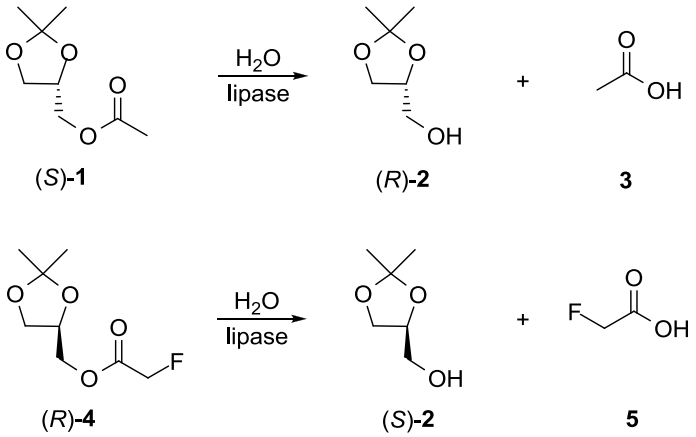
Involved: D. Bougioukou, L. Fernàndez, Y. Gumulya, H. Höbenreich, D. Kahakeaw, S. Kille, J. Sanchis, P. Soni, A. Taglieber, A. Vogel

Objective: The bottleneck of directed evolution is the screening/selection step, a problem that is particularly acute when evolving stereoselectivity. We therefore increased our efforts to develop high-throughput *ee*-screening assays. A conceptionally different goal was the development of the first selection system in which the host organism experiences a growth (survival) advantage because it harbors an enantioselective enzyme.

Results: The challenge of developing a selection system for the directed evolution of enantioselective enzymes was met by proposing the following concept:

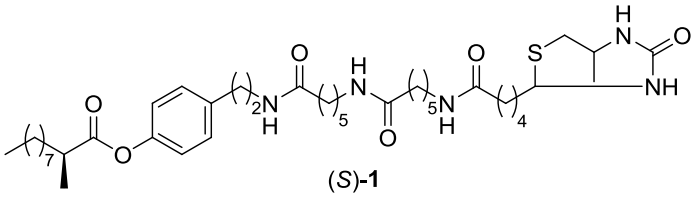


For proof-of-principle, the hydrolytic kinetic resolution of the pseudo-racemate (*S*)-**1**/(*R*)-**4** catalyzed by CALB lipase was chosen as the test reaction, generating either an energy source (**3**) for the chosen host organism (*Pichia pastoris*) or a poison (**5**). Following saturation mutagenesis, ideally only colonies harboring CALB mutants which favor substrate (*S*)-**1** should survive and therefore appear on the agar plates. Indeed, the anticipated effect was observed, > 80% expressing (*S*)-**1**-selective mutants.

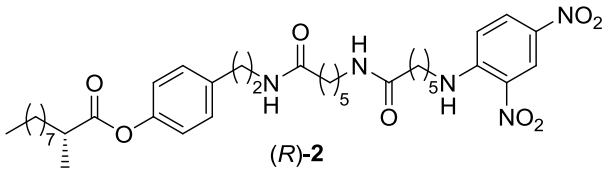


In another approach, the technique of fluorescence-activated cell sorting (FACS) was exploited for the first time in the quest to develop Super-High-Throughput *ee*-screening.

The basic idea was to label each of the two enantiomers conjugated to tyramide with a different fluorescent dye (green/red), FACS then allowing 10^8 cells and thus the number of enzyme clones to be evaluated on a single-cell basis!

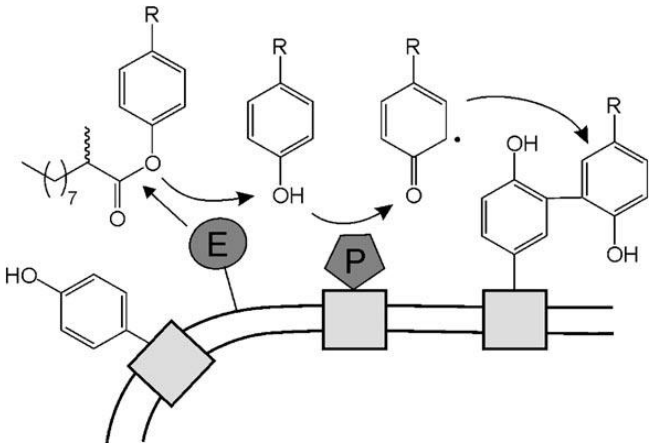


(*S*)-**1**

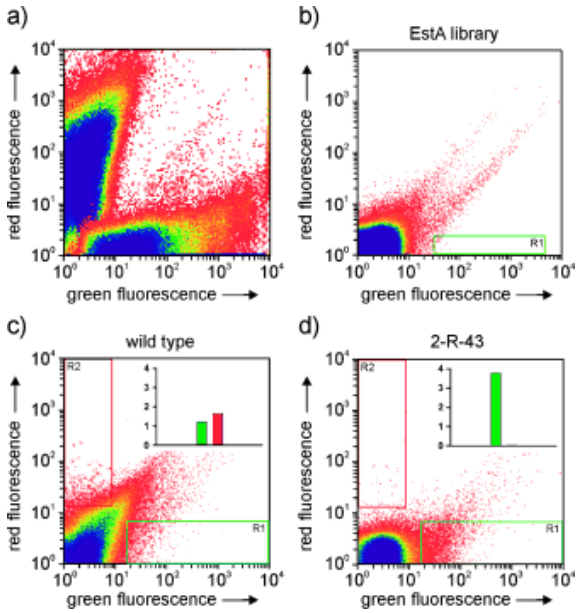


(*R*)-**2**

We chose the *Ps. aeruginosa* esterase-catalyzed hydrolytic kinetic resolution of a chiral ester, the challenge being the development of a method to trap the respective products on the surface of the bacterial cell. For this purpose, horse radish peroxidase-mediated radical formation was envisioned, which was designed to ensure the immediate covalent attachment of reaction products to the surface of the esterase-proficient bacterial cell:



In the case of the (*R*)-substrate, 2,4-dinitrophenolate labeling was detected by an Alexa Fluor 488-labeled antibody which mediates green fluorescence, whereas biotin deposition was detected using R-phycoerythrin conjugate (red fluorescence). The actual goal was to invert enantioselectivity (WT:E = 1.2 (*S*)). Indeed, this was achieved, the selectivity factor of the best mutant esterase amounting to E = 16 (*R*).



a) Overlay of flow-cytometry analyses of esterase-displaying cells that were incubated for 60 min with either *S* or *R* enantiomer of tyramide ester. **b**) EstA library sort. The green window indicates the sorting gate. **c**, **d**) FACS histogram of EstA wild-type; (**c**) and clone 2-R-43; (**d**) after 5 min incubation with a 1:1 mixture of both enantiomeric substrates and fluorescence staining. The inlet shows the percentage of cells within the respective green or red gate.

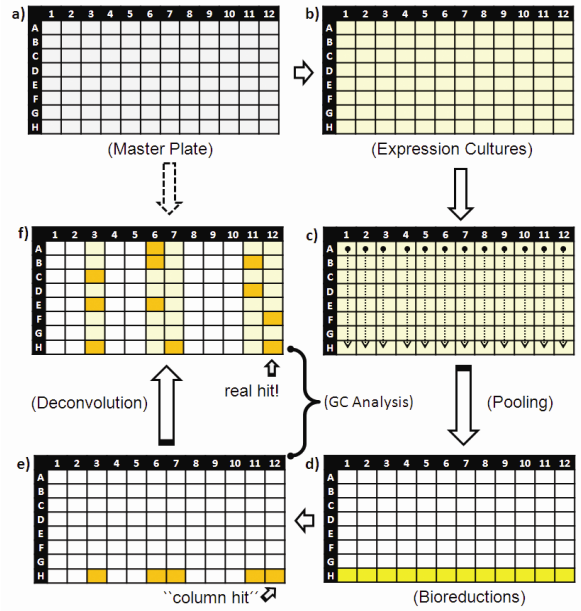
Further optimization was not strived for at this point, but it is obvious that the method could be used, for example, in simultaneous randomization at sites composed of 4-5 residues which would normally require the screening of 3 to 100 million transformants!

Rather than scanning larger portions of the (endless) protein sequence space by generating and screening by FACS or selecting large numbers of mutants (10^6-10^8) by appropriate systems, an alternative strategy is to strive for the opposite, namely to design strategies which generate small but highest-quality libraries (see previous Research Area), which can be screened by simple analytical assays. Most of our efforts are directed toward this goal, which in many cases has indeed been achieved, simply by generating small, but highest-quality libraries and then applying automated GC or HPLC. Nevertheless, we continued our efforts to design alternative medium- or high-throughput assays. One approach was the adaptation of multiplexing GC (or HPLC), an elegant technique which Oliver Trapp had first developed in the Institute in 2005. Before he left for the University of Heidelberg, a collaboration was initiated with the goal to assess within one day 3000 mutants of the epoxide hydrolase from *Aspergillus niger* as a catalyst in the hydrolytic kinetic resolution of a racemic epoxide. The envisioned system did in fact work because following optimization it was possible to rapidly separate all four compounds, (*R*)- and (*S*)-epoxide as well as (*R*)- and (*S*)-diol,

allowing for high-throughput determination of 3000 *ee*-values (mutants). Further optimization of the multiplexed system with respect to increasing the stability of the column material is in progress.

Two other strategies for increasing the throughput of enzyme evaluation were implemented. Whenever possible, it is desirable to develop on-plate assays as pre-tests for activity determination. We accomplished this in the case of epoxide hydrolases, specifically by adapting Reymond's assay (adrenaline color test) with the development of a cell-based color assay for automated high-throughput activity screening. A second more general strategy is to utilize pooling. In the case of manipulating the stereoselectivity of the enoate reductase YqjM in the asymmetric reduction of 3-methylcyclohexenone, a step-wise GC-based pooling protocol was devised, which led to the reduction of the overall screening effort by more than 50%!

Pooling protocol:



Publications resulting from this research area: 6, 47, 89, 161, 384

External funding: Deutsche Forschungsgemeinschaft (Schwerpunkt 1170/"Gerichtete Evolution"); Fonds der Chemischen Industrie

Cooperations: K.-E. Jaeger (Düsseldorf / Jülich, DE); H. Kolmar (Darmstadt, DE); O. Trapp (Mülheim/Ruhr / Heidelberg, DE)

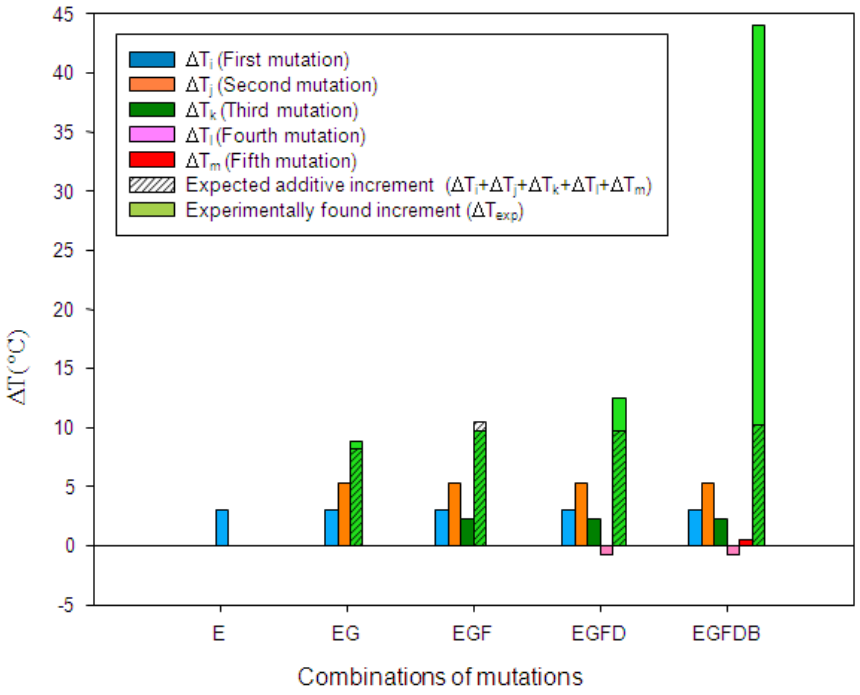
2.1.3 Research Area "Learning from Directed Evolution" (M. T. Reetz)

Involved: J. P. Acevedo, W. Augustyniak, D. J. Bougioukou, J. D. Carballeira, Y. Gumulya, D. Kahakeaw, S. Kille, R. Lohmer, S Prasad, J. Sanchis, P. Soni, A. Taglieber, L.-W. Wang, S. Wu, H. Zheng

Objective: 1) To uncover the reason for the observed efficacy of the ISM method; 2) to unveil the source of enhanced stereoselectivity or increased thermostability of evolved mutants on a molecular level.

Results: Considerable efforts were invested in the quest to understand why Iterative Saturation Mutagenesis (ISM) as a gene mutagenesis strategy in directed evolution provides superior results while requiring smaller libraries. It is more than an intellectual exercise, because such analyses point the way to achieving even greater efficacy. This research encompassed:

1) *Sequencing and characterizing as many improved enzymes in a given mutant library as possible, not just the very best hits*. This showed us that a) very different sequences lead to similarly improved functions, and that b) when attempting to improve two different properties such as activity and enantioselectivity, it is *not* optimal to select the very best hit for the subsequent cycle of ISM.
2) *Deconvoluting a given mutant characterized by x point mutations into all possible permutational combinations of single, double, triple, etc. mutants*. This allows epistatic interactions between point mutations and sets of mutations to be analyzed quantitatively in terms of $\Delta\Delta G^{\ddagger}$ values. We discovered that ISM is consistently accompanied by cooperative effects, i.e., the interactions are more than additive. In directed evolution this is the ideal form of epistasis. As an example, a *Bacillus* lipase mutant with five point mutations, evolved by ISM in five steps for increased thermostability (48°C → 93°C!), shows pronounced cooperativity, especially in the last evolutionary step of the ISM-sequence E → EG → EGF → EGFD → EGFDB (involving sites B, D, E, F and G):



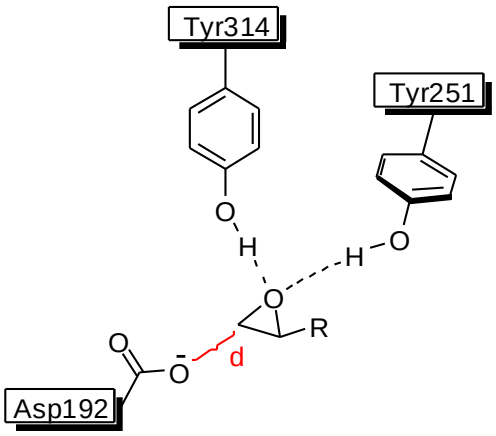
3) *Constructing fitness landscapes featuring all pathways leading from WT to a given evolved mutant, as in the case of the enantioselective Aspergillus niger epoxide hydrolase*. In the five-step process it means visualizing by experimental $\Delta\Delta G^{\ddagger}$ increments all 5! = 120 pathways. We found that not just the originally discovered pathway leads to the specific mutant, but about 50% of the others do as well showing no local minima (favored trajectories). In the case of disfavored trajectories characterized by local minima, backtracking provides a means to escape local minima. Such fitness landscapes allow for new mutants, but not for new mutations as in an actual ISM scheme. Construction of a corresponding fitness landscape of a complete ISM scheme is in progress, and preliminary results show that here too many pathways lead to improved enzymes.

The second type of lesson to be learned from directed evolution, namely uncovering the source of enhanced stereoselectivity, activity or thermostability, is important in its own right, and it also extends our knowledge of how enzymes function. One project concerned stereoselective Baeyer-Villiger-Monooxygenases, in collaboration with the Thiel group using a QM/MM approach (unpublished). In other cases our group utilized molecular dynamics (MD) simulations and induced docking experiments as well as algorithms allowing for the construction of covariance maps as indicators of correlated versus anti-correlated domain motions in enzymes.

An example concerns the mechanistic and structural study of a highly stereoselective mutant of the *Aspergillus niger* epoxide hydrolase, LW202, as a catalyst in the hydrolytic kinetic resolution of a chiral epoxide (E = 115 versus E = 4.6 of WT). The mutant LW202 had previously been obtained in five ISM steps. The mechanistic study for uncovering the source of enantioselectivity at each evolutionary stage included:

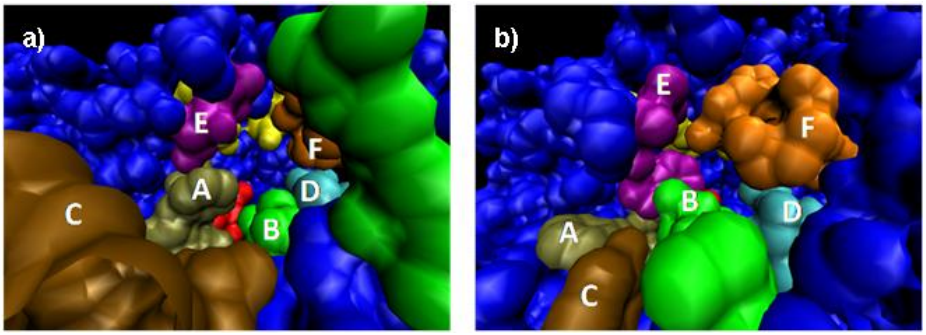
- Enzyme kinetics
- Inhibition experiments
- MD simulations and induced fit docking experiments
- X-ray structural analysis of the evolved enantioselective mutant (for the first time in directed evolution!)

The mechanism involves proper positioning of the epoxide and activation by Tyr314/Tyr251 in the binding pocket, followed by rate- and stereochemistry-determining nucleophilic attack of Asp192 with formation of a short-lived enzyme-ester intermediate which is rapidly hydrolyzed. The MD simulations showed that in the case of the best mutant the preferred (*S*)-substrate is perfectly aligned so that tyrosine activation is maintained and the distance, d, between the Asp-O-atom and the C-atom of the epoxide amounts to about 3.8 Å. In contrast, in the case of the disfavored (*R*)-enantiomer, d = 5.4 Å, too long for smooth nucleophilic attack to be possible. In the WT, the two respective d-values are similar (≈ 4 Å), but as the evolutionary process proceeds, the d_R value increases, until in the final mutant the reaction of this enantiomer is completely shut down, as in an ideal kinetic resolution. This prediction proved to be in line with the results of kinetics and inhibition experiments.



Mutant	d_R	d_S	Δd_{R-S}	*E* (expl.)
WT	4.3	3.5	0.8	4.6
LW081	4.8	4.0	0.8	14
LW086	4.9	4.0	0.9	21
LW123	5.1	4.0	1.1	24
LW44	5.1	3.9	1.3	35
LW202	5.4	3.8	1.6	115

The comparison of the crystal structures of WT and best mutant likewise proved to be eye-opening. Whereas the secondary and tertiary structures are essentially identical, the shape of the respective tunnel-like binding pockets are dramatically different as illustrated below (left: X-ray close-up of WT binding pocket; right: X-ray close-up of binding pocket of best mutant). Docking the (*R*)- and (*S*)-substrate into the respective binding pockets unambiguously shed light on the source of enhanced enantioselectivity, fully in line with the results of kinetics, inhibition experiments and MD simulations: In the best mutant it is impossible to position the disfavored (*R*)-substrate so that Tyr-activation and close distance d are both maintained.



Finally, in the directed evolution of the Baeyer-Villiger Monooxygenase PAMO, saturation mutagenesis at a remote site induced an allosteric effect leading to high activity and stereoselectivity. This was studied by MD simulations and covariance maps which revealed correlated and anti-correlated motions within the enzyme. This proved that the dynamics of the protein is essential for catalysis.

Publications resulting from this research area: 90, 93, 161, 235, 238, 239, 241, 386, 419, 425

External funding: Deutsche Forschungsgemeinschaft (Schwerpunkt 1170 “Gerichtete Evolution”); Fonds der Chemischen Industrie; EU Marie-Curie-Project

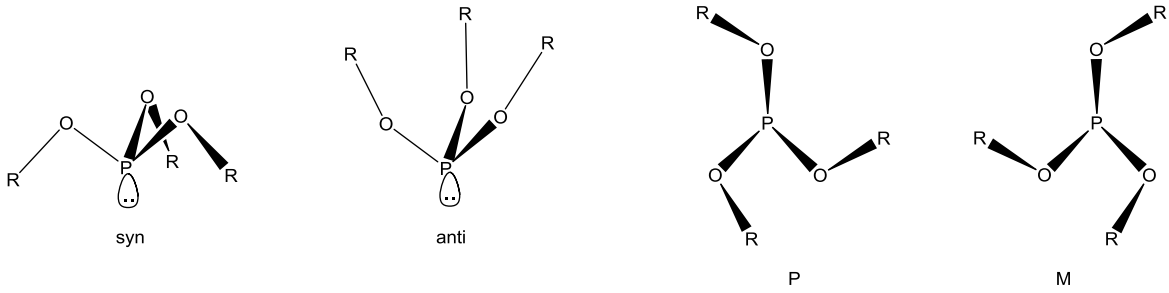
Cooperations: M. Arand (Zurich, CH); A. Archelas (Marseille, FR); M. Bocola (Regensburg, DE); S. L. Mowbray (Uppsala, SE); W. Thiel (Mülheim/Ruhr, DE)

2.1.4 Research Area "Transition Metal Catalysis" (M. T. Reetz)

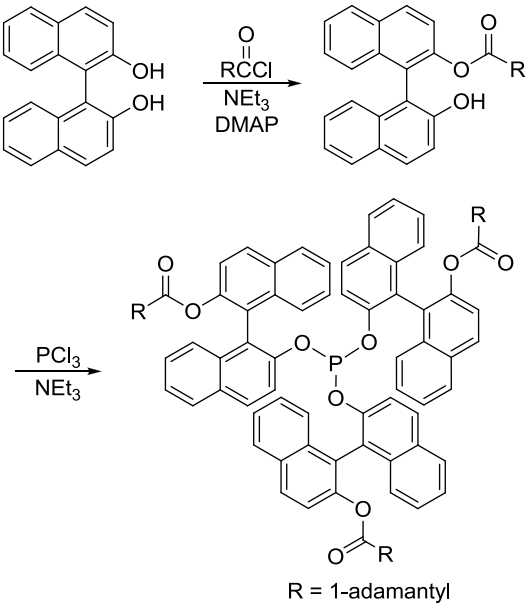
Involved: F. Berkermann, H. Guo, J.-A. Ma, G. Mehler, P. Wedemann, F. Hollmann, A. Taglieber, R. J. G. Mondière

Objectives: 1) Synthesis and application of the first helical phosphite; 2) Preparation of aqueous iridium oxide colloids/deposition on electrodes; 3) Design and preparation of a synthetic metalloenzyme.

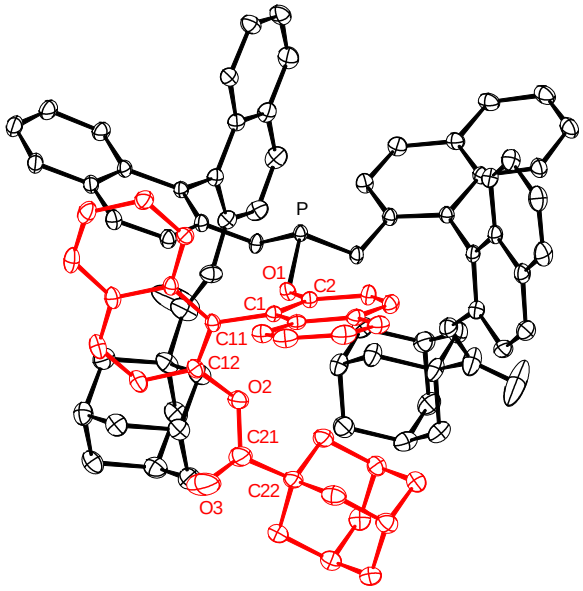
Results: Whereas a multitude of ligands for asymmetric transition metal catalysis based on central, axial and planar chirality have been described, very little is known regarding helical ligands. We have synthesized the first configurationally stable helical phosphite $P(OR)_3$. Such compounds can in principle exist as *syn* or *anti* conformers, and when helicity is involved either in the P or M forms:

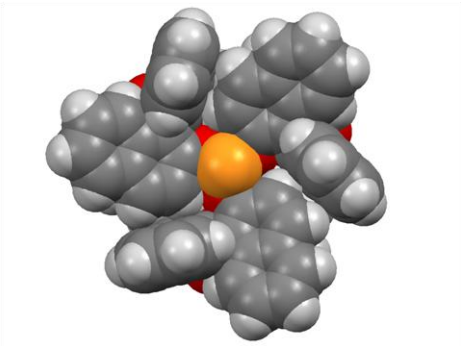


The challenge is to "lock in" a given conformer coding for a defined helicity, so that stereoisomerizing P ⇌ M interconversion cannot occur on energetic grounds. We devised a simple two-step sequence starting either from (*R*)- or (*S*)-BINOL:

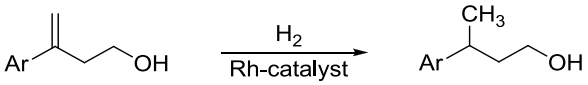


As X-ray analyses proved, (*R*)-BINOL leads to the (*R*,*R*,*R*)- compound with P-helicity, while (*S*)-BINOL provides the (*S*,*S*,*S*)-ligand characterized by M-helicity. The ligands are so sterically locked in that in each case the helicity does not interconvert, e.g., (*R*,*R*,*R*)/P $\rightleftharpoons$ (*R*,*R*,*R*)/M was not detected in the solid state (X-ray) nor in solution (NMR, CD). Only the *syn*-form was observed, as for example the crystal structure of the *syn*-(*S*,*S*,*S*)/M ligand shows:





The ligand was tested in Rh-catalyzed asymmetric hydrogenation of 14 different homo-allylic alcohols (which are known to be "difficult" substrates), leading to *ee* = 88-98%.



In a different project, we considered the 150 year old publication by Berzelius describing the NaOH-induced hydrolysis of iridium chloride with formation of blue solutions, presumably stable colloids even though no stabilizer had been added which are normally required today. We optimized the procedure, starting from either $IrCl_3$ or $IrCl_4$:

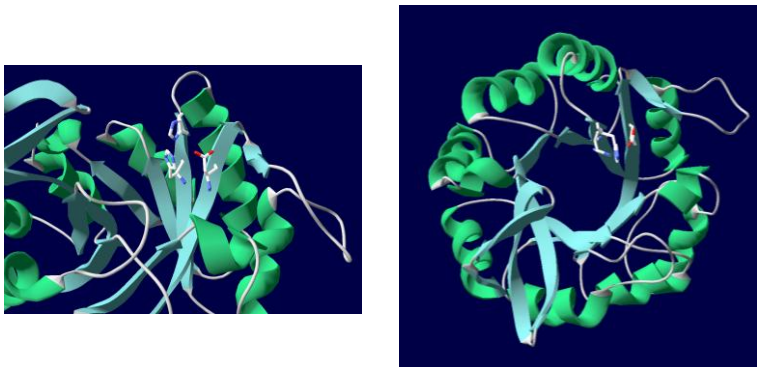
$$IrCl_x + NaOH \rightarrow Ir_yO_z \text{ (blue colloidal aqueous solution)}$$

The colloids were characterized by TEM (≈ 1 nm particles) and by other methods. Why such iridium-oxide nanoparticles in the absence of stabilizers are stable for months (electrostatic effects), and other transition metal oxides such as those of Pt, Pd, Os, are not, is not clear to us. We did, however, succeed in polymerizing other transition metals

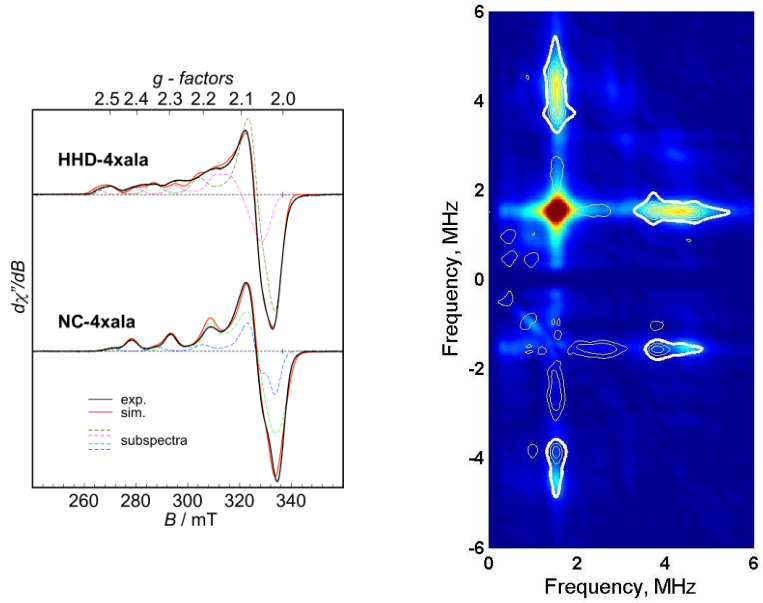
into the iridium oxide matrix with formation of mixed metal oxide colloids, which proved to be stable under the basic aqueous conditions.

We have used these materials in order to coat metal surfaces such as Pt-electrodes, which indeed show interesting electrocatalytic properties. They may be of interest in water splitting reactions or in classical electrochemical chlorine production. In the latter case, the state of the art describes Pt electrodes as being protected by a dip-coating process involving suspensions of bulk iridium oxide followed by heat treatment at high temperatures (750°C), a procedure that is repeated about 17 times for optimal effects in chlorine production. In sharp contrast, using the aqueous colloidal solutions, our dip-coating process needs to be performed only 3-4 times, and at much lower temperatures (≈ 275°C), leading to comparable stability/activity of the coated electrodes.

In a final project, transition metal catalysis was combined with protein science. In 2001/2002 we proposed the concept of directed evolution of hybrid catalysts, in which a synthetic ligand/transition metal entity is anchored covalently or non-covalently to a robust protein host, which as such delivers a single catalyst as previously sporadically described in the literature (e.g., Whitesides' system based on a biotinylated Rh-diphosphine complex in avidin as host). The novel part of our concept was the proposal that directed evolution can be performed on the protein host in general, *thereby providing a molecular biological tool for tuning a synthetic transition metal catalyst*. In the last Report (2005-2007), we described proof-of-principle of this Darwinian approach to asymmetric catalysis using the Whitesides' system, three ISM steps raising the *ee* of a Rh-catalyzed hydrogenation stepwise from 23% (WT) to 65% (final mutant). However, practical problems prevented us from exploring sufficiently large libraries. Recently we proposed an alternative approach, namely to utilize appropriate amino acids in a robust protein host for complexing transition metals directly (synthetic metalloenzyme). We chose a particularly stable enzyme, tHisF, as a robust host protein, which has a TIM-barrel eightfold α/β structure with a narrow "bottom" and a wide "top". At the top we applied site-specific mutagenesis with the creation of a potential transition metal binding site composed of His/His/Asp, the amino acids being "placed" geometrically in a correct (computed) manner for subsequent Cu(II)-complexation.



The design of this robust artificial metalloenzyme proved to be correct, because the mutant tHisF does indeed “soak up” Cu(II) specifically at the desired metal binding motif as proven by standard EPR measurements and sophisticated HYSCORE EPR experiments. The complex catalyzes the Diels-Alder reaction of a model compound (*ee* = 46%). This opens the door for directed evolution, for other Cu(II)-catalyzed reactions and for the complexation of other transition metals such as Fe, Mn or Co.



Publications resulting from this research area: 84, 86, 87, 91, 92, 233, 236, 237, 377

External funding: Fonds der Chemischen Industrie

Cooperations: E. Bill, R. Goddard, R. Mynott, E. J. Reijerse, W. Thiel (Mülheim/Ruhr, DE); R. Sterner (Regensburg, DE)