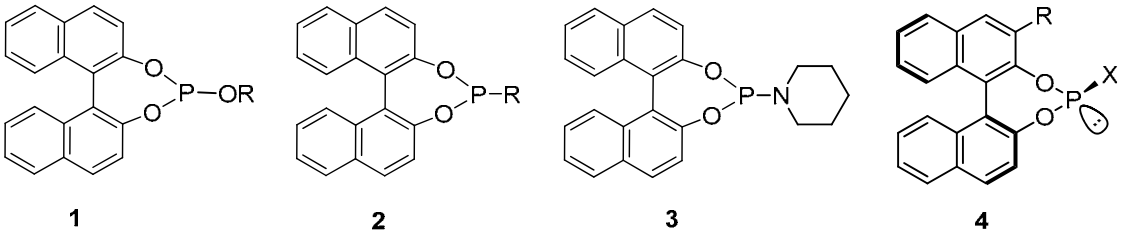
2.1.1 Research Area "Transition Metal Catalyzed Reactions" (M. T. Reetz)

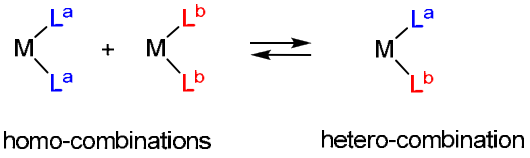
Involved: S. Alfs, S. G. Baca, O. G. Bondarev, Y. Fu, R. Goddard, H. Guo, X. Li, J.-A. Ma, G. Mehler, A. Meiswinkel, P. Scholz, K. Sommer, M. Surowiec, C.-S. Yang

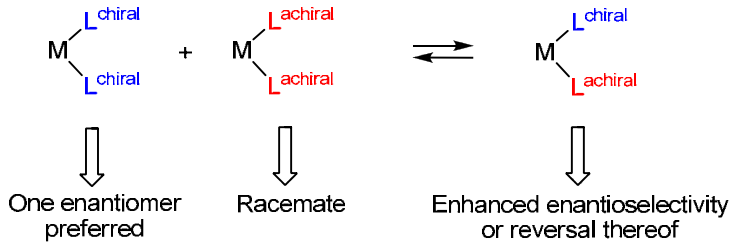
Objective: The objective of this research area is to design and test new types of ligands for enantio-, diastereo- and regioselective transition metal catalysis, and at the same time to develop novel concepts in catalysis. The challenge is to restrict the search for new catalyst systems to those ligands which are accessible in 2-3 simple steps from cheap starting materials, the specific goals being: 1) To understand the source of the previously observed high enantioselectivity of BINOL-derived monophosphites and monophosphonites as ligands in Rh-catalyzed olefin-hydrogenation. 2) To develop a new strategy in combinatorial transition metal catalysis based on the use of mixtures of chiral or achiral monodentate ligands. 3) To design readily available BINOL-derived chelating diphosphites and diphosphonites and to compare them to the respective monodentate counterparts in catalysis.

Results: In the last Report (2002-2004) we presented data showing that BINOL-derived monophosphites **1** and phosphonites **2** are surprisingly efficient ligands in Rh-catalyzed asymmetric olefin-hydrogenation, in many cases the *ee* being > 95%. Since BINOL is one of the cheapest chiral auxiliaries commercially available, this discovery, taken together with the work of B. Feringa/J. G. de Vries (DSM) regarding the use of the analogous phosphoramidites, has opened a new chapter in asymmetric transition metal catalysis. In the Mülheim lab and in many other groups new derivatives have been prepared and tested successfully during the last three years, including our discovery that the phosphoramidite piperidine-derivative **3** is considerably more active and enantioselective than all other ligands previously reported by the Dutch groups. We have also prepared mono-substituted P-ligands **4** which have an additional stereogenic center at phosphorus, and which often lead to even higher enantioselectivities than the simple ones (99% *ee*). These and other results show that the long-standing dogma regarding the necessity of using chelating diphosphines for obtaining high enantioselectivity no longer holds. In collaboration with W. Thiel and D. Blackmond a thorough mechanistic study regarding the source of high enantioselectivity was initiated, which includes kinetics, non-linear effects (NLEs), NMR studies and QM/MM calculations. The results show that an anti-Halpern system is operating, i. e., the major Rh-olefin intermediate is the more reactive one which leads to the product.

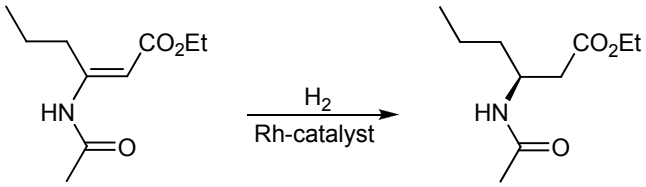


The Report 2002-2004 describes the initial the results of putting a new concept in combinatorial transition metal catalysis into practice, namely the use of mixtures of two chiral monodentate ligands or of mixtures comprising a chiral and an achiral monodentate P-ligand.



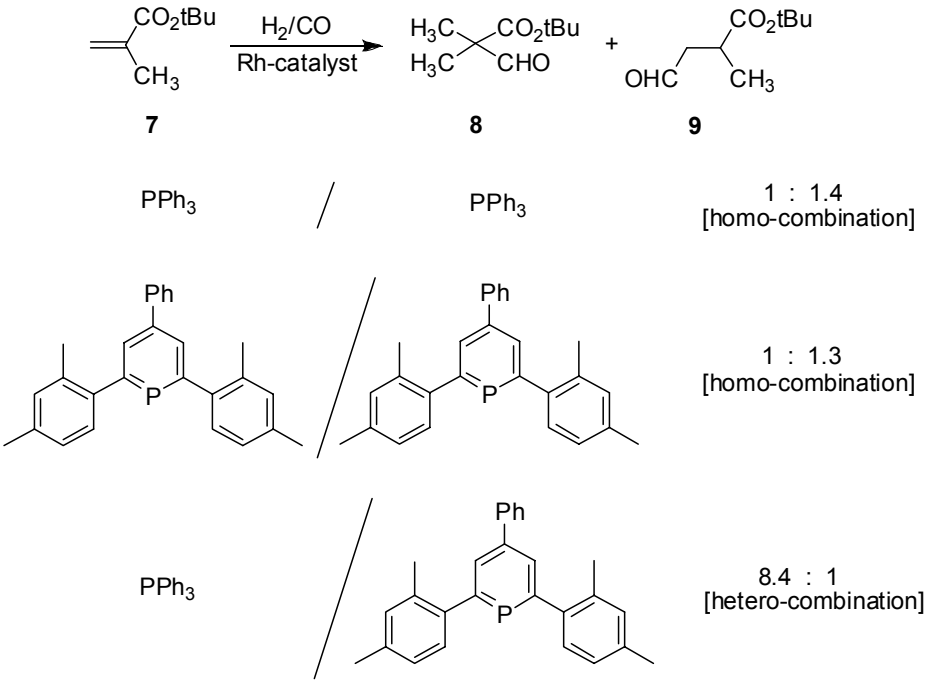


We have generalized this combinatorial approach to include the control of enantio-, diastereo- and regioselectivity. Two of many examples are shown here:

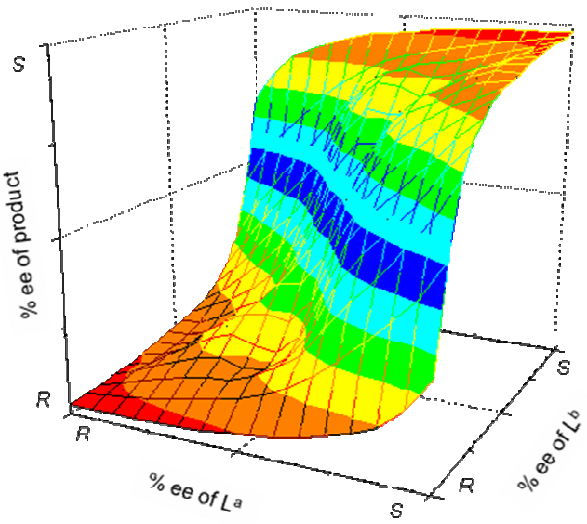


5 **6**

(*R*)-**1** (R=CH_3)/(*R*)-**1** (R=CH_3): 85% *ee* [homo-combination]
(*R*)-**2** (R=$C(CH_3)_3$/(*R*)-**2** (R=$C(CH_3)_3$: 31% *ee* [homo-combination]
(*R*)-**1** (R=CH_3)/(*R*)-**2** (R=$C(CH_3)_3$: 98% *ee* [hetero-combination]

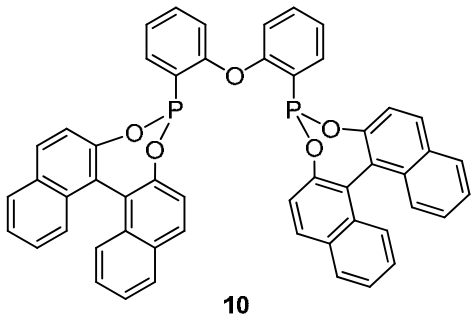


Mechanistic work regarding the mixture concept has also been initiated. In addition to NMR studies, novel non-linear effects were observed when using two different chiral P-ligands in a mixture, L^a and L^b, the enantiopurity of both ligands being varied simultaneously. These effects are strong evidence that in the transition state of hydrogenation two different ligands are bound to rhodium.

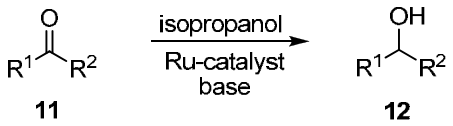


In earlier work we had observed that monodentate ligands such as **1**-**4** are not well suited for asymmetric transfer hydrogenation of prochiral ketones. We therefore studied

various diphosphonites in Ru-catalyzed transfer hydrogenation of prochiral ketones, especially with respect to the difficult class of alkyl/alkyl-ketones **11** which show poor enantioselectivity in the best literature systems. The xanthene-derived ligand **10** turned out to provide unprecedented degrees of enantioselectivity, e. g.:



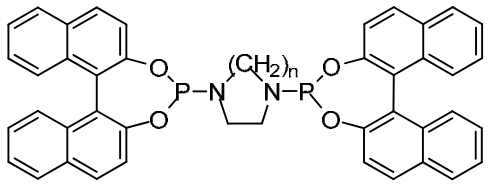
10



11 **12**

R^1 = *c*-C_6H_{11}; R^2 = CH_3 : 99% *ee*
R^1 = $CH(CH_3)_2$; R^2 = CH_3 : 99% *ee*
R^1 = *n*-C_6H_{13}; R^2 = CH_3 : 90% *ee*
R^1 = *n*-C_4H_9; R^2 = CH_3 : 90% *ee*

Finally, we discovered that certain diphosphonites such as **10** are excellent ligands in the asymmetric Ir-catalyzed hydrogenation of quinolines (*ee* = 92-94%), and that diphosphoramidites such as **13a-b** constitute surprisingly efficient ligands in Rh-catalyzed olefin-hydrogenation (*ee* > 95%).



13 a n = 2
b n = 3

Publications resulting from this research area: 14, 138, 139, 141, 142, 143, 144, 145, 146, 216, 347, 348, 350, 351, 352, 353, 355, 455, 508

External funding: Fonds der Chemischen Industrie

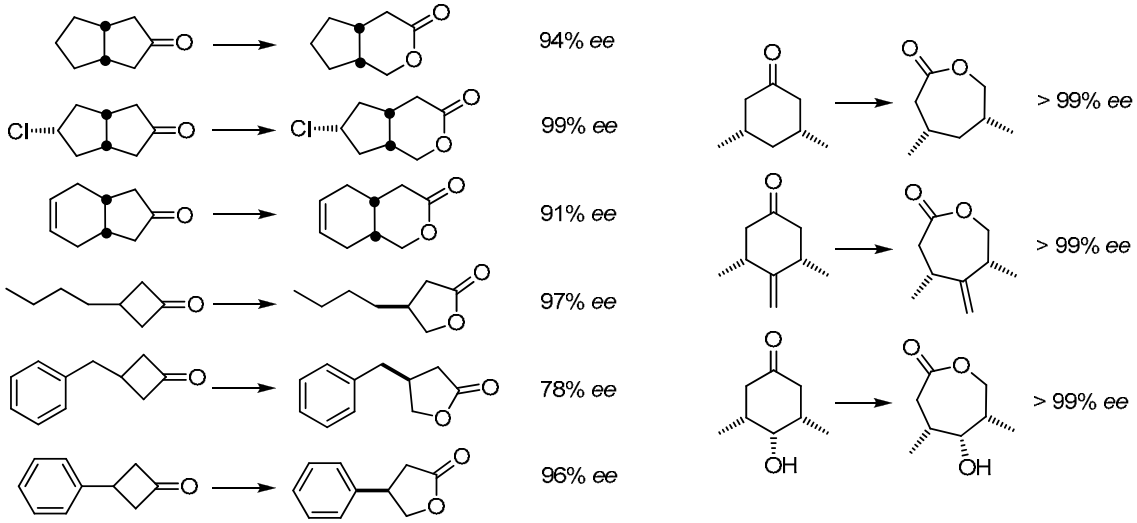
Cooperations: D. G. Blackmond (Imperial College, London, UK); K. Angermund, W. Thiel (Mülheim/Ruhr, DE); J. G. de Vries (DSM, Amsterdam, NL); B. L. Feringa (Groningen University, NL); K. N. Gavrilov (Ryazan State University, RU)

2.1.2 Research Area "Directed Evolution as a Means to Create Enantioselective Enzymes" (M. T. Reetz)

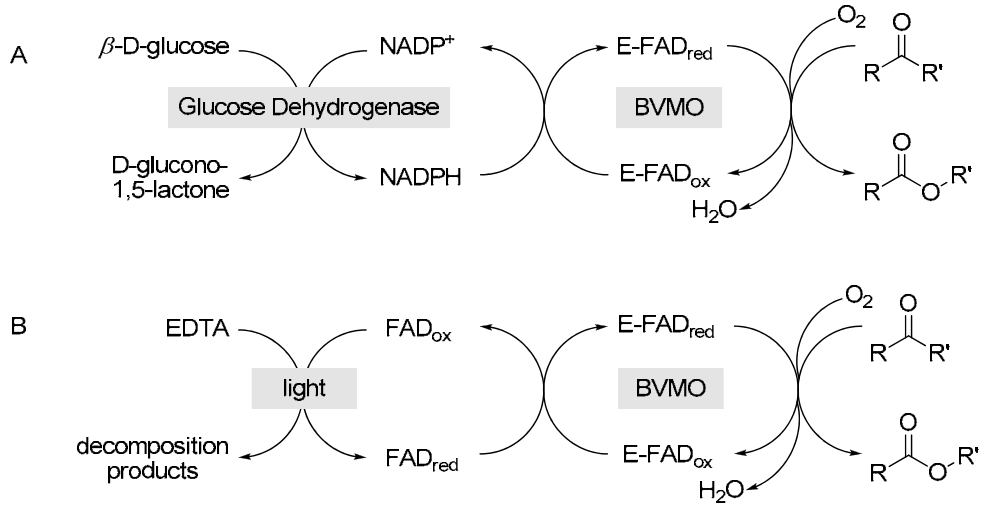
Involved: M. Bocola, J. D. Carballeira, C. M. Clouthier, M. Hermes, H. Höbenreich, F. Hollmann, F. Leca, M. Maichele, J. Peyralans, A. Taglieber, A. Vogel, S. Wu

Objective: During the last three years our long-term project regarding the directed evolution of functional enzymes as enantioselective and thermostable catalysts in organic chemistry has split into two parts. The first part is summarized here, the goals being 1) to explore how well certain mutants of monooxygenases and lipases, previously evolved by our earlier strategies based on error-prone PCR and DNA shuffling, perform when testing them as catalysts in the selective transformation of other substrates; 2) to develop new co-factor regeneration systems for the mono-oxygenases; 3) to understand the source of enhanced enantioselectivity of the evolved mutant enzymes; 4) to develop further high-throughput screening systems. The second part (Section 2.1.3) concerns methodology development in directed evolution.

Results: In a 2004 paper we reported the directed evolution of enantioselective Cyclohexanone Monooxygenase (CHMO) mutants as Baeyer-Villiger catalysts in the O_2-mediated desymmetrization of 4-hydroxycyclohexanone. The WT delivers an *ee* of only 9%. *R*- and *S*-selective mutants were evolved on an optional basis (*ee* = 90%). Based on structural considerations, certain mutants were expected to be good catalysts for other substrates. Since selective partial oxidation is certainly one of the major challenges in current organic chemistry, one of the mutants was tested as a catalyst in the following transformations. None of the presently known synthetic chiral transition metal catalysts using peroxides as oxidants are capable of such performance.

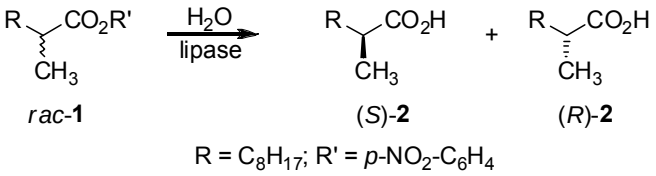


In 2004 the first X-ray structure of a Baeyer-Villiger, Monooxygenase (BVMO), namely Phenylacetone Monooxygenase (PAMO) was published by M. W. Fraaije, with whom we now collaborate. It is thermostable, but hardly accepts any substrates of synthetic interest. We therefore generated PAMO mutants P1, P2 and P3 which show a considerably broadened substrate scope and high enantioselectivity. Mechanistically, the oxidized flavin is reduced by the co-factor NADPH, which is traditionally regenerated by glucose dehydrogenase. In another project we have simplified the overall system considerably by replacing the regeneration-enzyme by light (sunlight or light-bulb), EDTA being the source of electrons. This is the first light-driven BVMO to be reported in the literature, and it is highly enantioselective in relevant cases (*ee* > 95%).



An intriguing facet of directed evolution concerns the source of enhanced enantioselectivity. In the case of the on-going project regarding the directed evolution of enantioselective mutants of the lipase from *Pseudomonas aeruginosa* as catalysts in the hydrolytic kinetic resolution of rac-**1**, the theoretical analysis has proved to be illuminating. The best mutant leads to a selectivity factor of $E = 51$ and is characterized by six mutations, only one of them being near the active site. Thus, for the first time remote effects were shown to influence the enantioselectivity of an enzyme. The QM/MM study uncovered an unusual relay mechanism (see report by W. Thiel). Moreover, it predicted that only two of the mutations are mainly responsible for enhanced enantioselectivity. We went back to the lab and prepared several of these "reduced" mutants. Indeed, the predicted double mutant is highly enantioselective in the model reaction ($E = 64$!). This is a triumph of theory, but it also indicates that in our original directed evolution study using repeating rounds of epPCR and DNA shuffling, superfluous mutations had accumulated. This "disturbing" observation is a clear sign

that our original strategies are successful, but certainly not as efficient as they could be (see Section 2.1.3).

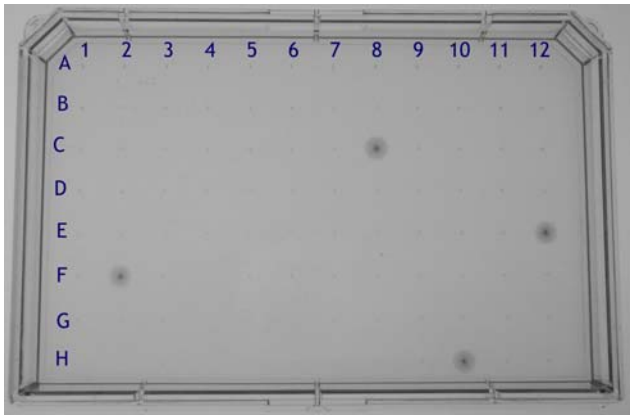


rac-**1** (*S*)-**2** (*R*)-**2**

R = C_8H_{17}; R' = *p*-NO_2-C_6H_4

Another challenge in directed evolution concerns the development of high-throughput assays needed in evaluating thousands of mutants, as for example when evolving enantioselectivity. We have previously devised several such screens, including the Mülheim MS-based *ee*-assay for kinetic resolution and desymmetrization. Nevertheless, we are continuing, two long-term goals being: 1) Enantioselective reactions and *ee*-analysis on a single chip (lab-on-a-chip), and 2) Selection systems on the basis of a growth advantage of the host microorganism, rather than screening. In collaboration with D. Belder (see his report for details), a device was designed and implemented which constitutes for the first time a lab-on-a-chip in which both the biocatalytic enantioselective reaction and the *ee*-analysis can be performed. Parallelization of the micro-channels in order to achieve high-throughput is the next goal. With regard to selection systems in the directed evolution of enantioselective enzymes, we have considered several approaches, some of which are beginning to be successful in this truly difficult endeavor: 1) Phage display (in collaboration with W. Quax/Groningen); 2) Cell sorting using FACS (in collaboration with H. Kolmar/Darmstadt); 3) Pre-selection test for evaluating the activity of epoxide hydrolases (EHs).

Pre-screens or pre-selection assays are ideal in directed evolution, because most libraries contain numerous non-active clones, which can be sorted out prior to elaborate *ee*-analysis. Since epoxides, but not the diols of a kinetic resolution thereof, are toxic to organisms, we devised an efficient pre-selection system. Accordingly, we showed that bacterial growth (*E. coli*) on agar plates relates directly to the presence of active EH mutants because they catalyze the detoxicating hydrolysis of the epoxide substrates. The photograph below features an agar plate harboring 96 *E. coli* colonies containing an epoxide and an EH, the four spots correctly signaling the presence of active enzyme mutants.



Preliminary work on promiscuity in enzyme catalysis has been initiated with the perspective of exploiting our methods of directed evolution in the quest to entice enzymes to catalyze certain classes of reaction types which are not possible with the respective wild-type. Among the early results is the finding that some lipases catalyze the Morita-Baylis-Hillman reaction. Our other discovery that the enzyme tHisF, which is instrumental in the biosynthesis of histidine, shows promiscuous behavior toward esters (esterase-like hydrolysis) is perhaps not so astonishing. What is really intriguing, however, is our surprising finding that this promiscuous catalysis does not occur in the active site where the natural reaction is known to take place. We call this unusual phenomenon "alternate-site promiscuity". Our discoverys appears to be the first known exception to the accepted dogma in the theory of evolution that natural and promiscuous reactions occur in one and the same binding pocket!

Publications resulting from this research area: 17, 36, 136, 140, 162, 228, 252, 327, 342, 343, 344, 356, 460, 505, 506, 507, 510, 511, 512, 531

External funding: Idecat (EU); DFG (Schwerpunkt "Gerichtete Evolution"); Fonds der Chemischen Industrie

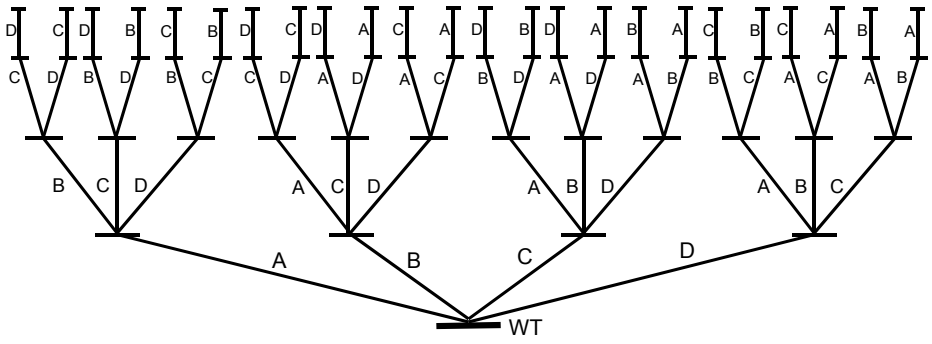
Cooperations: W. Thiel (Mülheim/Ruhr, DE); D. Belder (Mülheim/Ruhr, now Regensburg, DE); K.-E. Jaeger (Düsseldorf/Jülich, DE); J. E. Bäckvall (Stockholm, SE); M. M. Kayser (St. John, CA); J. Baratti and R. Furstoss (Marseilles, FR); B. W. Dijkstra and W. Quax (Groningen, NE); H. Kolmar (Darmstadt, DE)

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

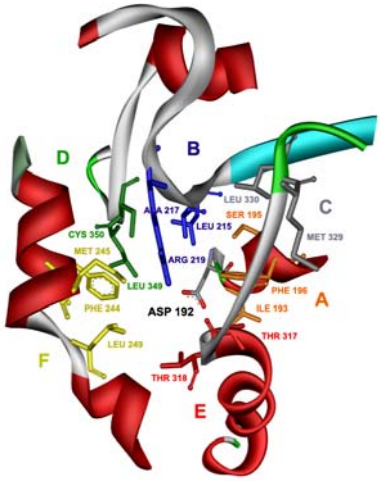
Involved: Y. An, S. Bastian, M. Bocola, J. D. Carballeira, C. M. Clouthier, J. Drone, L. Fernandez, Y. Gumulya, H. Höbenreich, D. Kahakeaw, S. Kille, R. Lohmer, L. Oliveira, S. Prasad, J. Sanchis, F. Schulz, P. Soni, A. Taglieber, L.-W. Wang, S. Wu, F. Zilly

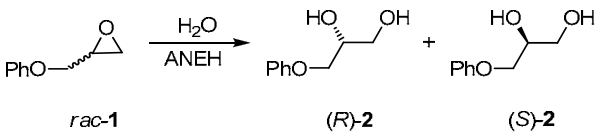
Objective: All previous papers on directed evolution emerging from our laboratory or from other groups describe successful experiments using such gene mutagenesis methods as epPCR (which is used most often), saturation mutagenesis, DNA shuffling and/or other molecular biological techniques. However, one current challenge revolves around the question of how to design and maximize the *quality* of mutant libraries, enabling "fast" directed evolution. Our goal was to find a method which ensures the generation of "smart" libraries in order to solve problems of 1) substrate scope of enzymes; 2) enantioselectivity; and 3) thermostability.

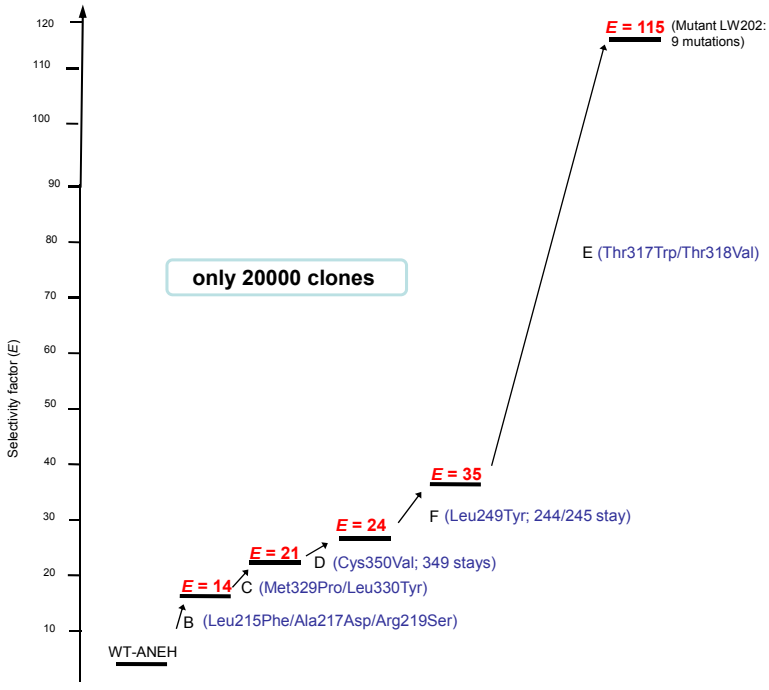
Results: Our contribution to the development of "fast" directed evolution is Iterative Saturation Mutagenesis (ISM). The first step requires a decision as to the appropriate sites A, B, C, D, etc. where saturation mutagenesis is to be performed, which means random introduction of all 20 proteinogenic amino acids. A given site can be comprised of one, two, three (or more) amino acid positions in the enzyme. This decision depends upon the nature of the property to be engineered. In the case of substrate acceptance and/or enantioselectivity, the choice is made on the basis of the Combinatorial Active-Site Saturation Test (CAST). When enhancing thermostability using ISM, the criterion is based on B-factors. Then the respective sites are each randomized with formation of focused libraries, and the mutant genes of the respective hits are used to perform further saturation mutagenesis at the other sites. ISM is illustrated here for the case of four sites A, B, C, and D:



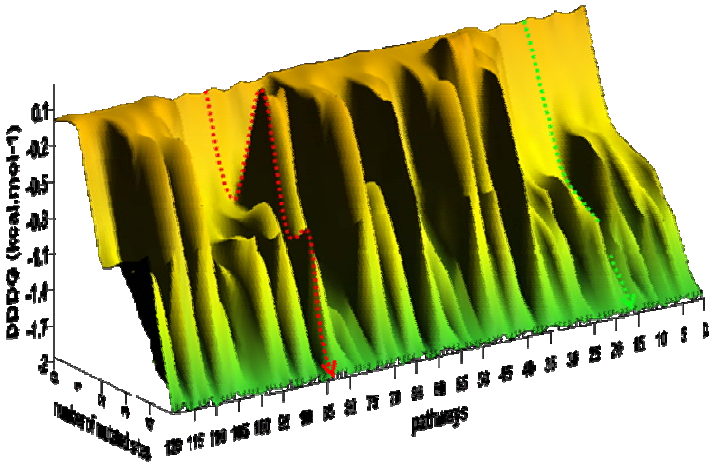
CASTing means the systematic generation of focused libraries by saturation mutagenesis at sites around the complete binding pocket. We have applied CASTing successfully in broadening the substrate scope of lipases and in enhancing the enantioselectivity of a Baeyer-Villigerase and of the epoxide hydrolase from *Aspergillus niger* (ANEH). The latter is illustrated here. It can be seen that the selectivity factor of the kinetic resolution increases from E = 4.6 (WT) to E = 115. The number of clones that were screened amounts to only 20000, which happens to be the same number required in our earlier study using the conventional approach based on epPCR, but which led to only E = 11. Thus, ISM *appears to be far superior to conventional ways of probing protein sequence space*.



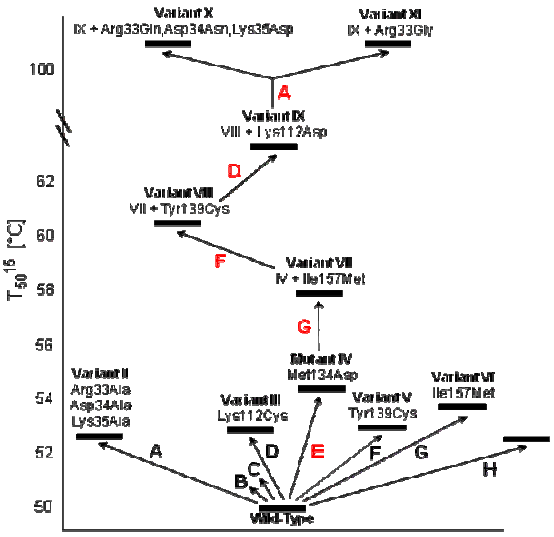




Other upward pathways have not yet been explored. However, the deconvolution of the five *sets* of observed mutations shows that numerous other feasible pathways leading to mutant LW202 exist. The energy site-mutation landscape of all of the theoretically possible 120 pathways to this *specific* mutant has been mapped, two different types becoming visible: Favored (green), less feasible (red):



The criterion for choosing the appropriate sites when attempting to increase the thermostability of enzymes is different. Since hyperthermophilic enzymes are known to be more rigid than the mesophilic counterparts, we chose those sites where mutations can be expected to influence rigidity, specifically by considering B-factors from X-ray data which reflect increased smearing of atomic electron densities relative to equilibrium positions. Using what we call the B-FIT method, we were able to increase the thermostability of the lipase from *Bacillus subtilis* by 45 °C. Such dramatic thermostabilization has no precedence in directed evolution. Only 8000 clones were screened in five iterative rounds of saturation mutagenesis, which again illustrates the power of ISM.



Variant X	Variant XI
T_{50}^{60} = 89 °C	T_{50}^{60} = 93 °C
ΔT_{50}^{60} = 41 °C	ΔT_{50}^{60} = 45 °C

Same catalytic profile at room temperature as WT

Only 8000 mutants screened!

We have also used statistical models from the literature to calculate the degree of oversampling that is necessary when going for 95% coverage of the respective protein sequence space in saturation mutagenesis (in our studies thus far such complete coverage was not strived for). A computer aid is available on our homepage. Part of the data is shown below in the table, which raises the crucial question as to the choice of the optimal codon degeneracy. All results so far show that employing NDT, meaning the use of only 12 amino acids as building blocks, is far superior to the conventional NNK degeneracy which encodes all 20 amino acids but which requires dramatically more oversampling. The respective mutant libraries have a much higher *density* as well as enhanced *quality* of hits. If this observation should prove to be general, which we suspect at this point, then ISM will turn out to be an even more powerful tool in directed evolution. The ISM-approach means a symbiosis of "rational design" and randomization at appropriately chosen sites. The unfortunate accumulation of superfluous or deleterious mutations, as observed in older studies using conventional tools such as repeating rounds of epPCR and DNA shuffling, is not likely to occur when using ISM correctly.

Codon usage	No. of codons	No. of AA	No. of stops	AA encoded	95% coverage for 2 pos.	95% coverage for 3 pos.
NNK	32	20	1	All 20	3066	98163
NDT	12	12	0	RNDCGHILFSYV	430	5175
DBK	18	12	0	ARCGILMFSTWV	969	17470
NRT	8	8	0	RNDCGHSY	190	1532

N: adenine / cytosine / guanine / thymine; K: guanine / thymine;
D: adenine / guanine / thymine; T: thymine

Publications resulting from this research area: 136, 137, 247, 344, 345, 346, 505, 506, 509, 514

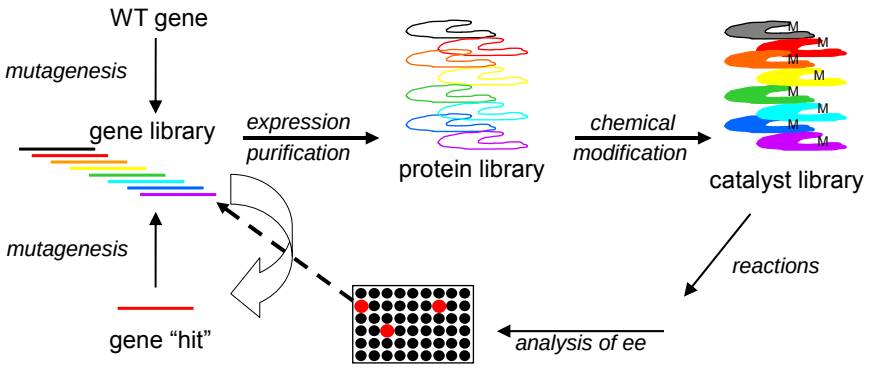
External funding: DFG (Schwerpunkt "Directed Evolution"); Fonds der Chemischen Industrie

Cooperations: none

2.1.4 Research Area "Directed Evolution of Hybrid Catalysts" (M. T. Reetz)

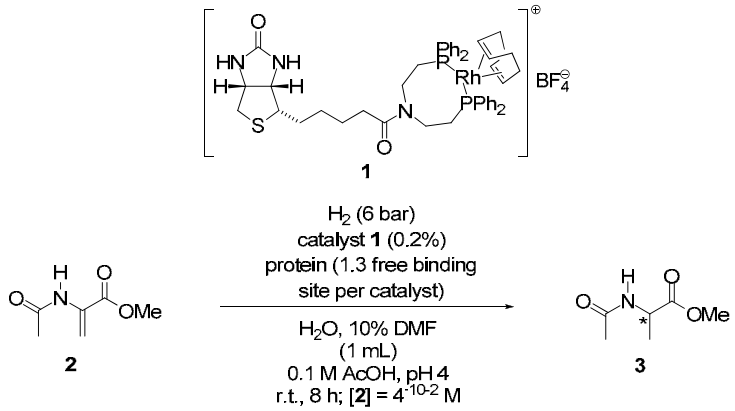
Involved: Y. Fu, F. Hollmann, N. Jiao, A. Maichele, P. Maiwald, M. Maywald, R. Mondiere, J.-P. Peyralans, A. Pletsch, J. Podtetenieff, B. Rasmussen, M. Rentzsch, A. Taglieber

Objective: Enzymes are capable of catalyzing a wide variety of selective bond-forming reactions of interest in synthetic organic chemistry, but they cannot catalyze the majority of reactions in organic chemistry mediated by synthetic transition metal catalysts incorporating such metals as Pd, Pt, Rh, Ru, Au, etc., transformations that are often known to work even in aqueous medium. On the other hand, it has been known for a long time that a ligand/metal entity can be anchored to proteins covalently or non-covalently, providing a hybrid catalyst. Since there is no reason to believe that the particular environment around the newly introduced transition metal provided by the WT protein should already be ideal for the purpose at hand, e. g., high enantio- or regioselectivity of a given reaction of interest, we proposed in 2002 the idea of *directed evolution of hybrid catalysts*:

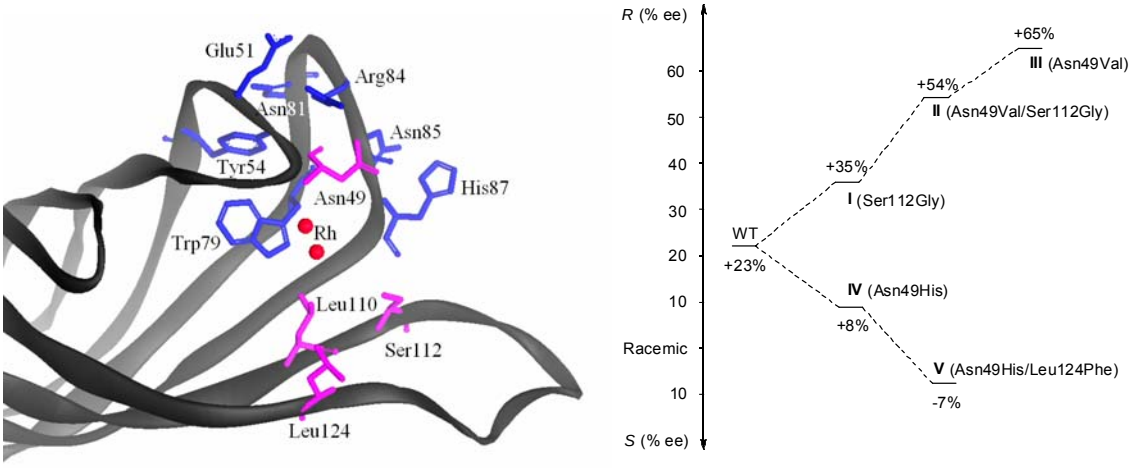


In the last Report 2002-2004 we outlined the technological problems in putting this concept into practice, which includes the necessity of *en masse* purification following the expression of thousands of mutant proteins (which is not necessary in normal directed evolution!). It also requires an excellent expression system which can be miniaturized and parallelized, producing enough protein for *en masse* bioconjugation and catalysis. This is a particularly sensitive issue, because synthetic catalysts are generally several orders of magnitude less active than enzymes. Finally, the host protein needs to be robust. During the last three years in this long-term project we followed two objectives: 1) To provide for the first time proof-of-principle of this novel way to tune a transition metal catalyst; and 2) To continue to develop platforms upon which the concept can be implemented in a practical manner.

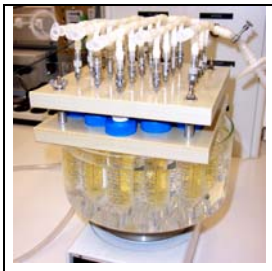
Results: Early on we opted for the Whitesides system, who had shown in 1978 that the biotinylated Rh/diphosphine complex **1** binds strongly in a non-covalent manner to avidin, as expected, providing a bioconjugate (hybrid catalyst) which catalyzes the asymmetric hydrogenation of *N*-acylamino acrylic acid (*ee* = 33-44%). We reasoned that since an efficient expression for avidin was not known, streptavidin should be the better choice for the host protein. Indeed, several expression systems for this protein had been reported, one of them being particularly efficient and providing enough material calculated to be necessary in each deep-well of appropriate microtiter plates. We also chose the ester **2** as the substrate (not the acid) because it can be extracted in a continuous manner.



Unfortunately, it was not possible to reproduce the published expression system for streptavidin production, which threatened the whole project. A molecular biologist in the group spent almost two years trying to develop a new expression system, but only a moderately improved version of a different known system could be implemented in the lab. This forced us to generate only very small mutant libraries, each fermentation being carried out in a 500 ml flask (instead of the planned 2-ml deep-wells!). This tedious procedure in combination with a ChemSpeed Accelerator SLT 100 Synthesizer allowed us to screen about 200 mutants per library. We modeled the Rh into the streptavidin cavity (two major conformations), and designed appropriate CAST libraries. In this way proof-of-principle was achieved after three iterative rounds of CASTing. In this "mini" directed evolution, work was terminated after reaching 65% *ee* in the product **3**:



Should a highly efficient and reproducible expression system for streptavidin be reported in the future, it will be logical to return to this platform for other reaction types such as hydroformylation (regio- and enantioselectivity!). In the meantime we have concentrated our efforts on alternative systems. One of them is tHisF as the host protein, a thermostable enzyme active in the biosynthesis of histidine. The development of a platform for the directed evolution based on this protein is almost finished. One of the crucial steps is *en masse* purification in parallized form on microtiter plates, which we have achieved by a simple heating procedure in a PCR instrument, a process which leads to the denaturization and precipitation of all protein material in the supernatant except that of the robust tHisF. Covalent bioconjugation is proceeding well.



19-fold fermentation unit

- 19 × 40 mL sterile LB-medium
- Inoculation with 0.5 mL preculture, incubation at 37 °C
- Induction with 40 µL 1 M IPTG at OD_{600} = 0.4 - 0.7
- Centrifugation after 6 h at 5000 rpm/10 min/4 °C in rotor for30 tubes
- Rinsing of cell pellet with 1 mL phosphate buffer (100 mM, pH = 7.8)
- *Resuspension in 400 µL phosphate buffer (10 mM, pH = 7.8 containing 2 mM DTT and 12.5 units/mL benzonase)*

24-deep-well plate

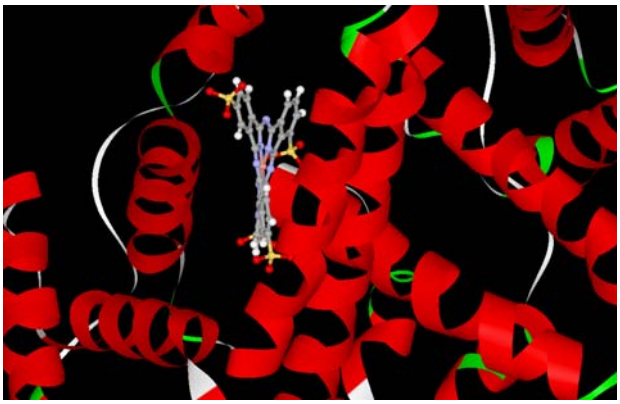
- 24 × 5 mL sterile LB-medium
- Corresponds to 576 cultures per incubator
- Inoculation with 0.1 mL preculture, incubation at 37 °C
- Induction with 5 µL 1 M IPTG at OD_{600} = 0.4 - 0.7
- Centrifugation after 6 h at 4000 rpm/40 min/4 °C using rotor for 4 plates
- Rinsing of cell pellet with 200 µL phosphate buffer (100 mM, pH = 7.8)
- Resuspension in 100 µL phosphate buffer (10 mM, pH = 7.8 containing 2 mM DTT and 12.5 units/mL benzonase)

↓

- Transfer into 96-well plates suitable for PCR-block
- 30 min heat-treatment at 72 °C in PCR-block (combined cell lysis and thermoprecipitation of *E. coli* host proteins)
- Centrifugation at 4000 rpm/120 min/4 °C in rotor for plates
- Collecting supernatant and freezing at –78 °C for storage

- Yield: approx. 3 mg in 200 µL Buffer
- Yield: approx. 0.3 mg in 40 µL Buffer
- Immediately before chemical modification: precipitation with 400 / 80 µL sat. ammonium sulfate, centrifugation, 2 × rinsing with 200 / 40 µL ammonium sulfate, resuspension of protein precipitate in phosphate buffer (10 mM, pH = 7.0).

We have also considered serum albumins such as BSA and HSA to which we have anchored site-selectively water-soluble sulfonylated Cu(II)-phthalocyanine complexes. The WT biojunjugate catalyzes some Diels-Alder reactions with amazingly high enantioselectivity (85-98% *ee*). In other cases the *ee*s are poor, which is not surprising. Since a good expression system is known for HSA, this may constitute another platform, although *en masse* protein purification still needs to be achieved.



Publications resulting from this research area: 349, 354, 513

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