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A Pioneering Career in Catalysis

Manfred T. Reetz

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A Pioneering Career in Catalysis: Manfred T. Reetz

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ABSTRACT: In this invited Account, we highlight the enormous scientific breadth of our mentor Professor Manfred T. Reetz. It stretches from the development of organometallic reagents and transition metal catalysts to the adventurous idea of directed evolution of chemo-, stereo-, and regioselective enzymes, which he considered to be most important. We hope to show that Reetz did not consider these research areas to be totally unrelated realms, and attempt to reveal his transdisciplinary way of thinking about methodology development. Since biocatalysis has become crucial for chemical synthesis, we mainly focus on Reetz's contributions in this area. Some personal reflections from some of his former co-workers are also included, which reveal the stimulating atmosphere in the Reetz group in terms of science, career advice, and the importance of ethical considerations.



KEYWORDS: biocatalysis, directed evolution, regioselectivity, saturation mutagenesis, stereoselectivity, protein engineering

1. MANFRED REETZ'S FIRST CAREER

Hearing the name Manfred T. Reetz, most readers will think about protein engineering and directed evolution. As former postdocs in the Reetz group working with directed evolution, we focus on this research area in the present Account. It is, however, interesting to note that biocatalysis was not Manfred Reetz's first love.

Manfred Reetz began his independent research in the field of organic chemistry in 1972 at Marburg University in Germany, following doctoral work with Ulrich Schöllkopf in Göttingen (1967–1969) on carbene reactivity and postdoctoral studies with Reinhard W. Hoffmann in Marburg on transannular olefinic π - π interactions.

In his first review article, Reetz coined the term "dyotropic rearrangements" as a previously not considered class of orbital symmetry-allowed transformations.¹ This concept is of particular relevance for natural products synthesis.² The combination of silicon and titanium chemistry subsequently allowed a longstanding problem to be solved, namely, the α -tertiary alkylation of ketones and esters by reacting S_N1 active alkyl halides with enolsilanes in the presence of TiCl₄, reviewed in 1982.³ TiCl₄ had been used as a Lewis acid in Mukaiyama aldol additions, which was probably the inspiration to test this particular Lewis acid first. In 1978, the group moved to the University of Bonn but returned two years later to Marburg, where Manfred Reetz became full professor, for German standards at a very early age (37 years old). Here, the group introduced ligand effects into classical carbanions by titanation, reviewed in a monograph^{5a} (see also review by Seebach^{5b}) and developed methods for chelation- and nonchelation controlled addition reactions to chiral α - and β -benzyloxy aldehydes⁶ and α, α -N,N-dibenzyl aldehydes with formation of pharmaceutically significant products.⁷ Early attempts at designing chiral Lewis acids as catalysts for asymmetric C–C bond formation were also made.⁸ Many of the studies on Lewis acidic reagents were flanked by QM investigations, often in collaboration with Gernot Frenking,⁹ whom Reetz had originally invited to Marburg as a "postdoctoral" research associate. Already then, the extraordinary breadth of Manfred Reetz's research interests became obvious.

On the basis of the above and other advances, Manfred Reetz had become an internationally renowned organic chemist and, in 1991, moved with his wife and four children from Marburg to Mülheim, where he accepted the position of the Director of the world-renowned Max-Planck-Institut für Kohlenforschung. First, he restructured the institute so that five catalysis departments were established, each headed by an independent codirector. These administrative efforts took 6–7 years for complete implementation. When reading Reetz's personal account of these fundamental changes,¹⁰ one can sense that they demanded from him a great deal of energy, tenacity, and a diplomatic spirit. On the scientific side, Reetz remained true to his broad interests and initiated several new projects going in different directions. Among others are the design of supramolecular catalysts,¹¹ electrochemical control of nanosized

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transition metal colloids and use in catalysis,¹² production of nanosized transition metal oxides,¹³ the role of ligand-free Pdclusters in Pd-catalysis,¹⁴ combinatorial asymmetric transition metal catalysis on the basis of mixtures of monodentate BINOLderived P-ligands,¹⁵ and sol–gel immobilization of lipases.¹⁶ By 1994, Reetz had already published more than 200 publications on the aforementioned topics securing him a front-row seat in the who-is-who of organic chemistry.

Unexpectedly, from the mid-1990 perspective, Reetz entered a fundamentally different category by pioneering the idea of directed evolution of stereoselective enzymes as catalysts in organic chemistry (Scheme 1).¹⁷ Later, Reetz considered this move to be the most important one in his scientific career. The standard mutagenesis methods in the field of directed evolution in general are error prone polymerase chain reaction (epPCR; a random shotgun technique),^{18a} DNA shuffling (a recombinant technique),^{18b} and saturation mutagenesis (focused randomization at a given residue or sets of residues as sites).^{18c}

Scheme 1. Concept of Directed Evolution of stereoselective enzymes for catalysis in Organic Chemistry and Biotechnology as developed by Reetz, iterative cycles of mutagenesis and screening being crucial [Reproduced with permission from reference 32. Copyright 2019, John Wiley and Sons]



2. DIRECTED EVOLUTION OF STEREO- AND REGIOSELECTIVE ENZYMES

2.1. Introductory Remarks. It is somewhat remarkable that as a synthetic organic chemist, Reetz happened to initiate this project. He already had some contact with the enzyme world studying sol gel immobilization of lipases to enhance their stability and sometimes stereoselectivity.¹⁶ The doctoral student working in this project, Albin Zonta, had a Master's degree in biochemistry. Some of this work was in collaboration with David Avnir^{16b} at Hebrew University in Jerusalem and a young assistant professor in microbiology at nearby Bochum University, Karl-Erich Jaeger.^{16c} Inspired by the seminal 1994 *Nature* paper of Pim Stemmer on DNA shuffling,^{18b} Reetz speculated that directed evolution could provide a reliable

means to control stereoselectivity (Scheme 1), not just protein stability as J. A. Wells,^{18c} R. Hageman,^{19a} and F. H. Arnold^{19b} had previously reported. It is essential to point out that Hageman, working on the thermostability of kanamycin nucleotidyltransferase,^{19a} and Arnold, focusing on increasing the robustness of a protease against hostile organic solvents,^{19b} both reported consecutive cycles of (random) mutagenesis, thereby clearly illustrating the evolutionary character of the procedure (not just an initial mutant library for increasing resistance to oxidative stress^{18c}).

In the 1990s, organic chemists were not interested in directed evolution or rational design²⁰ of protein stability, because much of this could be done by enzyme immobilization, which was the reason why Reetz focused on stereoselectivity. The collaboration with Zonta and Jaeger proved to be ideal. Initially, the chemistry and screening were performed in Mülheim, and mutagenesis in Bochum, but Reetz soon realized that for efficiency everything should be done in his laboratory. This required huge investments for establishing a molecular biology lab with new regulations (biosafety level 2) and for acquiring extensive robotics for high-throughput screening. *Thus, this research endeavor was not only a scientific adventure with an unknown outcome but also a considerable investment risk*.

2.2. Early Success. Having gained experience with lipases,¹⁶ it was logical to use this class of enzymes in a model transformation.¹⁷ One of the first challenges was to develop a high-throughput screening system for assessing the stereoselectivity of a lipase-catalyzed transformation involving kinetic resolution. Fortunately, Albin Zonta stayed in the group as a postdoc and succeeded in this ground-breaking task.^{17a} Subsequently, the Reetz group and others developed ee-assays for different enzyme classes.²¹ Using epPCR developed by Leung et al. in 1989,^{18a} initial experiments with a chiral α -phenyl substituted *p*-nitrophenylester as a substrate were not very promising, at the time a disturbing sign. Then, Zonta suggested p-nitrophenyl-2-methyldecanoate (Scheme 2).¹⁷ Upon performing four rounds of epPCR at a low mutation rate, which ensured a single point mutation in each cycle (generation), the enantioselectivity factor *E* increased from 1.1 to 11.3 in favor of the (S)-acid (Scheme 2).^{17a} At later group meetings, we (as second generation co-workers) could sense our mentor's excitement and joy of pushing the frontiers in those days!

At the time, a company published a note in an industrial journal on improving the enantioselectivity of a transaminase by applying random mutagenesis in a single experiment, but iterative cycles as defined in directed evolution (Scheme 1) were not considered.²² In subsequent work at the Mülheim MPI, the fifth cycle resulted only in a modest improvement. When Reetz reported these results at international conferences, some organic chemists and protein engineers were contending that such a "leveling off effect" or "diminishing returns effect," as already suggested in Scheme 2, is inevitable, constituting the weakness of this otherwise remarkable approach to creating enantioselective catalysts. But the Reetz group did not succumb and initiated systematic methodology development in directed evolution, initially using the original experimental platform (Scheme 2). This proved to be crucial for the entire field.^{23,24} The lipase from Pseudomonas aeruginosa (PaL) has become "the" model enzyme to test new evolutionary strategies, and indeed few other enzymes have been submitted to directed evolution as extensively as PaL. In 2001, the Reetz group reported the utility of several mutagenesis techniques, the combination of DNA shuffling and saturation mutagenesis

Scheme 2. (a) Model Hydrolytic Kinetic Resolution originally used in Directed Evolution of stereoselectivity¹⁷ and (b) first case of Directed Evolution of a stereoselective enzyme based on consecutive cycles of mutagenesis and screening, demonstrated by applying epPCR to the lipase from *P. aeruginosa* (PaL) as catalyst in the hydrolytic kinetic resolution of *rac-* α -Methyl(p-nitrophenol)ester^{17a,a} [Reproduced with permission from reference 17a. Copyright 1997, John Wiley and Sons]



 $^a\!E$ is the selectivity factor reflecting the relative rate of reaction of the starting material enantiomers.

leading to an unprecedented selectivity factor of E = 51 in the model system.^{17c} epPCR at a higher mutation rate was likewise tested with simultaneous introduction of more than one mutation per cycle.^{17c} The positive result contradicted other recommendations at the time calling for single mutational steps.²⁵ Using similar strategies, PaL's enantioselectivity was reversed in favor of (*R*).^{17b}

Significantly, in the 2001 PaL study, it was also shown for the first time that saturation mutagenesis at residues at the binding pocket of an enzyme leads to a significant increase in enantioselectivity (Scheme 3).^{17c} Later, the Reetz group wondered why no one had ever done this seminal step before in the quest to evolve stereoselectivity! In doing so, NNK codon degeneracy (N, adenine/cytosine/guanine/thymine; K, guanine/thymine) with 32 codons encoding all 20 canonical amino acids as building blocks was applied to a four-residue randomization site, this mutant library harboring a quadruple variant E160A/S161D/L162G/N163F with a selectivity factor of E = 30 (S) in the reaction of $rac - \alpha$ -methyl(p-nitrophenol)ester upon screening of 5000 mutants.^{17c} All four mutations influenced the shape of the binding pocket. It was also shown that the combination of saturation mutagenesis, epPCR, and DNA shuffling provided a mutant with remarkable E = 51 (S).^{17c} However, a total of 50 000 transformants had to be screened for enantioselectivity in labor-intensive work. As revealed by a later QM/MM study in collaboration with Walter Thiel, this variant had an important local mutation at the binding pocket in addition to remote mutations.²⁶ At that time, a lively discussion regarding remote versus close mutations suggested that much was still to be learned regarding optimal mutagenesis strategies.²⁷

Scheme 3. First example of Saturation Mutagenesis at residues at the binding pocket of an enzyme for influencing enantioselectivity, in this case Directed Evolution of *P. aeruginosa* lipase as catalyst in the kinetic resolution of 2-Methyl decanoic acid *p*-nitrophenyl ester^{17c,d}



For readers not active in protein engineering, it must be remembered that protein sequence space is astronomically large, millions or billions of possible combinations of mutations being possible (mutating four residues to 20 amino acids results in 160,000 possible protein variants), and *finding an adequate mutant without endless screening (bottleneck of directed evolution) is the central challenge*. Reetz also developed genetic selection systems, but these were limited to a few enzymes, and thus a general method was needed to address this bottleneck.^{21d} Various versions of saturation mutagenesis had been developed in the 1980s for other purposes. For example, J. A. Wells improved oxidative stability by focusing amino acid exchanges solely at rationally chosen remote residues.^{18c}

By 2004, directed evolution of enzyme stereoselectivity²⁸ (Scheme 1) was a hot subject at essentially all protein engineering conferences. Especially notable in this sense are the contributions by N. J. Turner on monoamine oxidases (MAOs) for the (industrial) synthesis of chiral amines using mutator strains as a random mutagenesis method²⁹ and C.-H. Wong's studies of stereoselective aldol addition reactions using epPCR and DNA shuffling.³⁰ A more comprehensive story of the history of directed evolution can be found in the 2016 monograph³¹ authored by Reetz and in the 2020 review on methodology development.³²

It took the Reetz group some time to fully realize that saturation mutagenesis is a particularly effective tool in the evolution of stereoselectivity, possibly to be preferred over epPCR, mutator strains, and DNA shuffling because these techniques are not focused. After all, in view of Emil Fischer's lock-and-key hypothesis and Linus Pauling's suggestion that enzyme transition states are stabilized by local interactions, what is more logical than to influence the shape of binding pockets via focused amino acid exchange events near the active site? *In 2005, systematization was achieved with the emergence of the Combinatorial Active-site Saturation Test (CAST) for extending substrate scope (activity) and influencing enantioselectivity.*³³ Not

just one site at the binding pocket was considered as previously reported (Scheme 4), 1^{17c} but more systematically other local

Scheme 4. Illustration of CAST and Iterative Saturation Mutagenesis (ISM):^{32,35} (a) Generalization of CAST sites; (b) restricting the number of CAST residues (Red); (c) ISM schemes for a two-site (Left), three-site (right), and four-site system (bottom), resulting in 2, 6, and 24 upward pathways^{*a*} [Reproduced with Permission from Reference 32. Copyright 2019, John Wiley and Sons]



^aSM, saturation mutagenesis; SSM, single site saturation mutagenesis; CSM, combinatorial saturation mutagenesis.

residues with generation of a set of mutant libraries. CAST is a useful acronym to distinguish it from saturation mutagenesis at remote sites.^{18c} When applying CAST, both first and second sphere residues can be targeted. When the method was first introduced, the Reetz group neglected to consider statistical factors, including the degree of oversampling in the screening step. Fortunately, this was done shortly thereafter, which indeed opened a new door in directed evolution (section 2.3).

The success of performing saturation mutagenesis around the binding pocket in the lipase study^{17c,33} and subsequently using

different enzymes types as reported by Reetz and then by other groups³⁴ was clearly visible internationally.^{17d} However, the question arose what to do if the initial mutant libraries do not satisfy the requirements set by the experimenter in terms of stereoselectivity and activity. The Reetz group then developed the idea of Iterative Saturation Mutagenesis (ISM),³⁵ which is particularly useful in CASTing for stereoselectivity (Scheme 4), for evolving enzyme promiscuity on the basis of artificial metalloenzymes (section 2.5), or when enhancing protein stability and other applications (section 2.6). Generally, not all ISM pathways need to be explored, but some may be more productive than others.³⁶ As summarized elsewhere,³¹ this was also confirmed by many successful ISM-based evolution studies on different enzyme types including lipases, epoxide hydrolases, enoate reductases, Baeyer-Villiger and cytochrome P450 monooxygenases, etc.

2.3. The Importance of Methodology Development. At the time of the first saturation mutagenesis experiments for evolving stereoselectivity (Schemes 3 and 4),^{17c,33} conventional statistical factors were not considered. Thereafter, the Reetz group used the Firth–Patrick algorithm³⁷ to calculate the degree of oversampling of a mutant library necessary to ensure 95% coverage (or any other % coverage) when applying NNK codon degeneracy.³⁸ This assumes the absence of amino acid bias, which means that the numbers are approximations. Experimentally, amino acid bias when applying any form of saturation mutagenesis (or any other mutagenesis method) is a problem in itself because it reduces the quality of the mutant libraries. The Reetz group therefore developed several approaches to minimize and even eliminate bias,^{39a-c} including the 22c-trick for saturation mutagenesis,^{39a} which is similar to the Tang-Gao technique,^{39d} and which has since been used by many groups.⁴⁰

Being an organic chemist, Reetz wondered whether all 20 canonical amino acids are really necessary for efficient saturation mutagenesis. Why not use, for example, NDT codon degeneracy (D, adenine/guanine/thymine; T, thymine) encoding a "cock-tail" of 12 polar/nonpolar, aromatic/nonaromatic, charged/ neutral, and small/large amino acids resulting in dramatically reduced screening effort? The CASTER computer aid was developed for user-friendly application of CAST and ISM (free of charge at the Reetz Web site: kofo.mpg.de/en/research/biocatalysis). The statistics of NNK versus NDT codon degeneracies are reproduced in Table 1.³⁸ Experimentally, the reduced amino acid alphabet corresponding to NDT proved to



	NNK (20 AA)		NDT (12 AA)		TCSM (4AA)		SCSM (2AA)	
no. of amino acid positions at one site	codons	transformants needed	codons	transformants needed	codons	transformants needed	codons	transformants needed
1	32	94	12	34	4	12	2	6
2	1024	3068	144	430	16	48	4	12
3	32 768	98 163	1728	5175	64	192	8	24
4	$>1.0 \times 10^{6}$	$>3.1 \times 10^{6}$	20 736	62 118	256	767	16	48
5	$>3.3 \times 10^{7}$	$>1.0 \times 10^{8}$	248 832	745 433	1024	3066	32	96
6	$>1.0 \times 10^{9}$	$>3.2 \times 10^{9}$	$>2.9 \times 10^{6}$	$> 8.9 \times 10^{6}$	4096	12 271	64	192
7	$>3.4 \times 10^{10}$	$>1.0 \times 10^{11}$	$>3.5 \times 10^{7}$	$>1.1 \times 10^{8}$	16 384	49 083	128	384
8	$>1.0 \times 10^{12}$	$>3.3 \times 10^{12}$	$>4.2 \times 10^{8}$	$>1.3 \times 10^{9}$	65 536	196 328	256	767
9	$>3.5 \times 10^{13}$	$>1.0 \times 10^{14}$	$>5.1 \times 10^{9}$	$>1.5 \times 10^{10}$	262 144	785 314	512	1534
10	$>1.1 \times 10^{15}$	>3.4 × 10 ¹⁵	$>6.1 \times 10^{10}$	$>1.9 \times 10^{11}$	$>1.0 \times 10^{6}$	$>3.1 \times 10^{6}$	1024	3068

^aTCSM: Triple-code saturation mutagenesis. SCSM: Single-code saturation mutagenesis.

be superior to NNK at identical screening effort as shown in two different studies. $^{\rm 38b,41}$

Between 2005 and 2015, most so-called first generation CAST studies reported by the Reetz lab and others focused on the randomization of 2 or 3 residues to NNK and/or NDT degeneracy because the number of transformants needed to cover sufficiently the library was practical. However, some libraries failed to contain improved variants or "hits," which is due to bias when building libraries and/or because the wrong residues were targeted. To avoid these pitfalls, many co-workers started applying a quick quality control (QQC) to check library diversity, while others applied site-saturation mutagenesis (SSM) to active site residues to identify and rank the most relevant residues for mutagenesis. These efforts led to the development of second generation CAST based on reduced amino acid alphabets because it was no longer necessary to apply combinatorial mutagenesis based on NNK or NDT degeneracy (see below). Other reduced amino acid alphabets in CASTing were also tested in the Reetz lab, and by 2015 many other groups had learned to appreciate the advantages of this approach.³² One of numerous examples concerns the directed evolution of a robust alcohol dehydrogenase (ADH) as the catalyst in the asymmetric reduction of ketones leading to axial chirality (Scheme 5).⁴² Since the α - and α' -residues next to the carbonyl

Scheme 5. ADH catalyzed asymmetric reduction of an axially prochiral ketone⁴²



function are sterically very similar, efficient asymmetric reduction using modern Ru-based catalysts is impeded,⁴³ which illustrates the complementarity of enzyme catalysis and transition metal catalysis, as Reetz emphasized in many group meetings. His appreciation of modern chemical techniques was also of particular value in this and other projects. For example, Pd-catalyzed carbonylation and Suzuki coupling with the enantiomerically pure bromo products were performed, leading to new members of axially chiral products of potential interest as liquid crystals.⁴²

Further advancements led to what can be called second generation CAST (2015–2020), which included, *inter alia*, solid-phase gene synthesis of saturation mutagenesis libraries, use of mutability landscapes, and machine learning (see below). This phase of research also led to the recommendation that three to four semirationally chosen building blocks in combination with randomization sites composed of about four to five residues constitutes a viable compromise between maximal structural diversity and minimal screening.⁴⁴ Such decisions depend on the particular enzyme under study and need to be guided by any data and techniques that may be available:

- Knowledge of enzyme mechanism and X-ray structure or homology model
- Mutational effects in previous studies
- Consensus data by multiple sequence alignments (MSA)
- Docking computations
- Molecular dynamics (MD) computations, ideally in combination with QM calculations
- Rosetta algorithms
- Machine learning techniques

It is interesting to note that these are the same guidelines in so-called rational protein design,²⁰ which is an alternative to directed evolution,^{23,24,32} but which was traditionally not generally successful when targeting stereoselectivity. To further reduce amino acid alphabets and screening efforts, Reetz proposed that saturation mutagenesis can be applied using two different strategies: simultaneous randomization at a multiresidue site using a given degenerate codon as usual or a different degenerate codon at each residue of the same multiresidue site in a single randomization experiment (Scheme 6).^{45a} Obviously, strategy 2 requires the maximum amount of guiding information.^{45b}

Scheme 6. Two different strategies when applying Saturation Mutagenesis with highly reduced amino acid alphabets⁴⁵ [Reproduced with permission from reference 45b. Copyright 2016, John Wiley and Sons]



Strategy 2 was first applied to the evolution of a stereoselective Baeyer–Villiger monooxygenase^{45a} and subsequently to a lipase as reported by Bäckvall et al.⁴⁶ and to limonene epoxide hydrolase (LEH).^{44a,b} In the initial LEH study, the Single Code Saturation Mutagenesis (SCSM) strategy applied to scanning 10 identified CAST residues could efficiently reshape the binding pocket leading to significantly optimize and invert the enantioselectivity.^{44a} In a further interesting LEH study, a three-membered reduced amino acid alphabet (in addition to wildtype) in the form of Triple Code Saturation Mutagenesis (TCSM)^{44b,c} was introduced, a method that can be applied using either strategy 1 or 2.^{44d} The basic steps of this approach may appear to be complicated (Scheme 7), but performing the molecular biology is in fact trivial.

TCSM was also used in the directed evolution of an alcohol dehydrogenase (ADH) as catalyst in the asymmetric reduction of difficult-to-reduce prochiral ketones such as tetrahydrofuran-3-one leading to products of high pharmaceutical interest,^{44c} which is not possible using modern transition metal catalysis.⁴³ *Guided by docking experiments, exploratory NNK based saturation mutagenesis was first applied separately at five CAST residues,* requiring in each case the screening of only a single 96-well plate. Mutations moderately favoring (*R*)- and (*S*)-selectivity were Scheme 7. Illustration of Triple Code Saturation Mutagenesis (TCSM) in the evolution of stereoselective LEH mutants^{44b}



identified, which served as a guide in the actual TCSM experiments. For 95% library coverage, TCSM would require the screening of about 3000 transformants. In order to minimize screening, two randomization sites comprising four residues, A and B, were designed, the triple codes being V–N–L for possible (*R*)-selectivity and V–Q–L for (*S*)-selectivity, requiring in each case the screening of only 576 transformants (six 96-well microtiter plates).^{44c} This two-phase semirational CAST approach was highly successful, not only for the model substrate but also for three other small prochiral ketones (Scheme 8).^{44c} The results of screening the two libraries A and B are shown in Scheme 9, the data being so good that ISM was not necessary. Furthermore, TCSM is systematically compared with

Scheme 8. Examples of difficult-to-reduce prochiral ketones used in the TCSM Study $^{\rm 44c}$







SCSM and Double Code Saturation Mutagenesis (DCSM) and was applied to multiparameter engineering of LEH^{44e,f} and other enzymes.

. Biocatalytic cascade processes,⁴⁷ particularly involving cytochrome P450-BM3 monooxygenase,⁴⁸ continued to be of great interest for the Reetz group. The first study combined engineered P450s and enoate reductases to reduce aromatic olefins.^{48a} In another study, the problem of chemo- and regioselective dihydroxylation of benzene was first solved by evolving cytochrome P450-BM3. The resulting mutant enabled selective transformation of benzene into hydroguinone without any overoxidation and only a trace of the catechol regioisomer.^{49a} QM/MM computations uncovered the reasons for selectivity. The experimental result contrasts with the known multistep chemical Hock-type process. Despite many attempts, transition metal hetero- or homogeneous catalysis fail due to uncontrolled overoxidation. The biocatalytic route, based on screening only a handful of semirationally evolved P450-BM3 mutants, was then extended in order to access the natural product arbutin, which is a long-known compound widely used in the treatment of skin ailments.⁴⁹ This required the additional use of arbutin synthase in a one-pot E. coli designer cell process (60% isolated yield; Scheme 10).^{49a} Protein engineering of cytochrome P450 monooxygenases by directed evolution or rational design continues to be an exciting research area for the production of pharmaceutically significant products as summarized in a review,^{48c} in which the important contributions of L.-L.

Scheme 10. Synthesis of Hydroquinone from Benzene: (A) Traditional chemical cascade process compared to the newly designed direct double hydroxylation using engineered P450-BM3 (B); This route was also used to produce the natural product Arbutin (C)⁴⁹



Wong, H. Zhao, Z. Li, R. Bernhardt, V. Urlacher, F. H. Arnold, R. Fasan, U. Schwaneberg, A. J. Mulholland, S. Shaik, and others are included. At the time, Reetz joked that this is his first and only natural product synthesis! We add: thus far!

Other types of "impossible" reactions of interest in the Reetz group include abnormal versus normal Baeyer–Villiger reactions catalyzed by Baeyer–Villiger monooxygenases (BVMOs).⁵⁰ Decades of experimental and theoretical mechanistic work by organic chemists had revealed the dominance of stereoelectronic effects in Baeyer–Villiger reactions, leading to the conclusion that in the fragmentation of the Criegee intermediate an antiperiplanar conformation for maximal sigma-orbital overlap is crucial (Scheme 11, top). The migrating group that stabilizes the developing positive charge at the respective O-atom best is set in motion preferentially. In order to override the electronic effect, directed evolution based on ISM was successfully applied. A mutant of cyclohexanone mono-

Scheme 11. Normal versus abnormal Baeyer–Villiger reactions using WT and evolved BVMOs⁵⁰



oxygenase (CHMO) was evolved which catalyzes the sole formation of the abnormal product in the reaction of 4-phenyl-2-butanone (Scheme 12, bottom).⁵⁰ Impressively, in the case of benzyl ethyl ketone, two regio-complementary mutants were evolved by CAST/ISM for 97% benzyl or 98% ethyl migration, respectively. Particularly exciting is also the result of a deep-seated QM/MM study made possible by the collaboration with K. N. Houk (see also section 2.4).

Other recent methodology developments coming from the Reetz group are listed in Table 2.^{38b,c,39a-c,51,52a,b,53-60}

Since space limitation does not allow the elaboration of all of these advances and related contributions,^{52c-e} we mention here only two developments. The first case concerns the use of second generation CAST/ISM for solving a notoriously difficult synthetic problem in organic chemistry, namely, the targeted regio- and diastereoselective oxidative hydroxylation of steroids.^{61a} The model transformation was the P450-BM3 catalyzed hydroxylation of testosterone at position C16 with α - and β diastereoselectivity on an optional basis (Scheme 12). The Reetz group had previously observed that the known mutant F87A accepts this substrate, but with the formation of a 1:1 mixture of the 2β - and 15β -products. Following CAST/ISM and the screening of 9,000 transformants by HPLC, both 2β - and 15β selective mutants were evolved.⁶² These results aroused a great deal of attention, but pharmaceutical chemists were politely contending that these regioisomeric alcohols were of little practical interest, in contrast to steroids bearing hydroxyl groups at the C7, C11, or C16 positions. The decision for a C16 project was clearly a risky endeavor. Collaborators from four countries (Germany, UK, Israel, and China) included organic chemists, NMR spectroscopists, X-ray crystallographers, a mathematician, and biotechnologists.^{61a}

As a first step, exploratory NNK based saturation mutagenesis was performed individually at five CAST residues, similar to the first phase in the TCSM studies (Schemes 7–9).^{44c} But in this case, massive DNA sequencing, being the crucial part of mutability landscaping as reported by G. J. Poelarends,⁶³ was carried out in the quest to induce regio- and stereoselectivity as well as activity.^{61a} The information gained by this exercise as well as other available data was used to design reduced amino alphabets and to choose appropriate randomization sites and upward pathways for ISM. A typical mutability landscape in this study is shown in Scheme 13, which reflects a kind of fingerprint. It can be seen that highest activity does not always correlate with the highest selectivity. Such trade-offs in directed evolution are well-known and limit the improvement of two or more parameters.⁶⁴ Nevertheless, following the screening of only 3000 transformants by automated HPLC, highly selective mutants were identified for both the C16 α and C16 β products, thanks to a combination of mutability landscaping and MD simulations to guide the engineering process.^{59a,61a} This is still a lot of screening, but it must be remembered that the goal was extremely ambitious and that simultaneous optimization of three catalytic parameters is a persisting challenge in protein engineering. The best mutants were then tested successfully in the selective hydroxylation of four structurally different steroids (Scheme 14), which in contrast to the former study,⁶² represented a significant advancement.^{61a} Recently, Reetz reported a follow up of this study, again mutability landscaping was applied to engineer a highly selective and active mutant for various steroids at position 7β , which is of interest for the treatment of brain diseases.^{61b} In this case, <1600 mutants were screened from a large combinatorial library that was built with a

Scheme 12. Regio- and diastereoselective oxidative hydroxylation of Testosterone at position C16 catalyzed by mutants of P450-BM3, either diastereomer being accessible^{61a}



Table 2. Selected methodologies originating from the Reetz lab

method involved	comment	ref
22c-trick	a simple combination of primers creates unbiased libraries and reduces screening efforts significantly	39a
two-step PCR strategy	library generation can be tricky with some genes; this issue can be solved using a novel two-step PCR strategy	39b
ISM	importance of additive versus nonadditive cooperative or antagonistic mutational effects	51
assembly of designed oligonucleotides (ADO)	simultaneous mutagenesis of many codons can be achieved by the assembly of oligos into genes in a single step	52a, b
CAST	P450 catalyzed oxidative hydroxylation of achiral compounds with simultaneous creation of two chirality centers	53
cheaper and faster QQC	traditionally, library quality is based on the sequencing of pooled plasmids from agar plates, but its throughput can be increased by culturing in liquid media	54
NNK versus NDT codon degeneracy	using 12 instead of 20 amino acids significantly reduces the screening of combinatorial libraries	38b, c
CAST (TCSM) and B-FITTER	combination of two methods to simultaneously increase thermostability, enantioselectivity, and activity	55
photobiocatalysis	photochemical, NADPH-independent regeneration of oxidoreductases	56
one-step focused and random mutagenesis	combination of epPCR and saturation mutagenesis maximizes library diversity at the active site and removes locations	57
CAST, NNK	increasing the flexibility of active site residues of a thermophilic enzyme increases activity at mesophilic conditions	58
synthetic libraries	creation of combinatorial libraries of excellent quality using on-chip solid-phase gene synthesis	39c
ASRA, innov'SAR algorithm	machine learning as an aid in directed evolution of stereoselective enzymes	59
oxidase—peroxygenase mutual benefit system	exploiting designed oxidase-peroxygenase mutual benefit system for asymmetric cascade reactions	60

Scheme 13. Typical mutability landscape of P450-BM3 mutant F87A toward Testosterone 61a,a



^{*a*}The five target residues are indicated on the left side; the traits being considered are shown in percentage on the right: substrate conversion under defined conditions correlating with activity (black) and selectivity toward 2β - (red), 15β - (blue), or 16β -hydroxytestosterone (green).

novel DNA assembly method. The mutability landscape is a very promising approach for the targeted functionalization of C-H bonds in complex substrates.

A second achievement which we want to highlight here is the efficient and unique fusion of rational design²⁰ and directed

evolution^{23,24} in the form of Focused Rational Iterative Saturation Mutagenesis (FRISM).^{32,65} At a given CAST residue, a small number of suggestions, typically three to four, are made for single mutations based on the known tools used in rational design. They are the same guides as in second generation CAST/ISM, but FRISM would be by itself a third generation strategy. The predicted few mutants are then tested experimentally in the reaction of interest, and the best one is identified, which is then used as a template for performing the same procedure at another single CAST residue, and so on. Several pathways are theoretically possible, depending upon the number of chosen mutational sites (CAST residues). Not all of the pathways can be expected to provide one and the same final mutant, even in the case of only two or three sites (Scheme 15a/ b).^{32,65} Note that in each step a single mutation is introduced, but more than one amino acid exchange may be more promising, as recommended previously for CAST/ISM. Such generalization is indeed possible, but it requires additional design and experimental work. The idea of FRISM was inspired by the efficiency of CAST/ISM, the advantage being that the generation and screening of mutant libraries is no longer necessary.

FRISM was first applied in the ambitious attempt to generate four stereocomplementary mutants of the lipase from *Candida antarctica* B (CALB) as the catalyst in the nonstandard transacylating kinetic resolution of a racemic ester using a racemic alcohol with formation of four different stereochemical products, each harboring two chirality centers (Scheme 16).⁶⁵ WT CALB is unselective and delivers a mixture of the four ester products, two diastereomers and their enantiomers. Therefore, four different FRISM pathways were needed, leading to the discovery of four unique mutants showing \geq 95% overall Scheme 14. Additional steroids used to test the best P450-BM3 mutants evolved for enabling 16α - and 16β -selectivity in the model reaction of Testosterone^{61a}



stereoselectivity. It remains to be seen whether FRISM can be applied to such transformations as P450 catalyzed regio- and stereoselective oxidative hydroxylation of steroids and other challenging transformations using other enzyme types. In principle, application of this method for the enhancement of thermostability or binding affinity should also be possible, provided appropriate guides are available for making rational decisions at all stages of the iterative process.

2.4. Learning from Directed Evolution. Mechanistic studies always played a central role in the 50-year career of Manfred Reetz. Indeed, he was convinced that directed evolution is ideally suited for this purpose (see review),^{26d} collaborating not only with Walter Thiel and Ken Houk, as already mentioned, but also with Sason Shaik, Marc Garcia-Borràs, Meilan Huang, and Sílvia Osuna. Also, Reetz hired theoreticians as postdocs in the group for several years, who were valuable daily discussion partners of the experimentally working co-workers. Having coined the term "learning from directed evolution,"26a Reetz has often and impressively demonstrated the benefits arising from such theoretical studies. The group published the first MD study of an enantioselective enzyme mutant created by directed evolution,²⁶ namely, the PaL lipase mutant with E = 51 (S) and a 250-fold activity enhancement in the hydrolytic kinetic resolution of rac-2methyldecanoic acid p-nitrophenyl ester (Scheme 2). It was followed by the first QM/MM studies of the stereoselectivity of a Bayer-Villiger monooxygenase (BVMO), also in collaboration with W. Thiel,^{66a} and of an enantioselective enoate reductase (YqjM), in this case together with a postdoc in the group.66b

Although in several projects the *in silico* data provided insight into the source of the superior catalytic performance of the best mutants, a more fundamental question still had to be addressed. In the early stages, ISM had proven to be an effective method to find best mutants, but Reetz was very interested in estimating to what extent pure luck played a role in the process, for instance in the selection of the order in which the sites were mutated. To answer this fundamental question, the idea of deconvolution of multimutational variants was implemented experimentally.^{67a} In doing so, LW202, an Aspergillus niger epoxy hydrolase (ANEH) mutant,³⁵ was deconvoluted. LW202 had been obtained after five rounds of ISM from the ANEH WT (E = 4) leading to an Evalue of 192 in the kinetic resolution of a racemic epoxide. The synthesis of the intermediate evolutionary mutants, corresponding to all the permutations in the order of saturation mutagenesis of the sites, enabled Reetz to build what he called a fitnesspathway landscape. The analysis of the surface revealed that at least half of the pathways lead to LW202 without local minima. It is interesting to note that LW202 was originally evolved by ISM after screening a total of 20 000 transformants.³⁵ The probability to obtain nine mutations (not even with the right amino acids) in the positions of LW202 by a completely random method such as epPCR would have been 1 in 5×10^{23} mutants!

Following the advent of CAST/ISM, the experimental PaL platform was revisited.^{26c} Aided by the PaL crystal structure, six CAST residues (first generation) were grouped into three tworesidue randomization sites A (M16/L17), B (L159/L162), and C (L231/V232), which were subjected to NNK based saturation mutagenesis. Whereas libraries A and C failed to contain improved mutants, library B harbored single mutant L162N, which then served as a template for DNT (encoding 11 amino acids) based saturation mutagenesis. This provided the amazingly active and highly stereoselective mutant 1B2 (M16A/ L17F/L162N) with E = 594 (S; Scheme 17, top).^{26c} Upon viewing this ISM scheme, one could conclude that the initial mutation L162N contributes only to a very small extent to the overall result, and that the simultaneous introduction of M16A/ L17F in the ISM step constitutes the dominant factor. However, this assumes that the effect of mutations is additive, which on the basis of earlier experience in the Reetz group is not all justified. Scheme 15. FRISM as a method for unifying Directed Evolution and Rational Design: 32,65 (a) The case of two mutagenesis sites involving only two pathways $1 \rightarrow 2$ and $2 \rightarrow$ 1; (b) The case of three sites, illustrated by two of the 3! = 6possible pathways $1 \rightarrow 2 \rightarrow 3$ and $1 \rightarrow 3 \rightarrow 2$ [Reproduced with permission from reference 32. Copyright 2019, John Wiley and Sons]



Scheme 16. Stereodivergent transacylation between racemic Phenylpropionic acid *p*-nitrophenyl ester and racemic 1-Phenylethanol catalyzed by four different FRISM-generated CALB mutants⁶⁵



Therefore, partial deconvolution was performed by generating and testing M16A/L17F separately. The result proved to be eyeopening since this double mutant alone hardly leads to any improvement of enantioselectivity (E = 2.6 (S)). In concert with the initial mutation L162N, the actual final selectivity jumps to E = 594,^{26c} which proves that an enormous cooperative effect (i.e., synergistically nonadditive) is operating.⁵¹ In order to unveil this Scheme 17. CAST/ISM-based Directed Evolution of PaL as catalyst in the kinetic resolution of 2-Methyl decanoic acid *p*-nitrophenylester (Scheme 2):^{26c} (a) Optimal ISM pathway WT \rightarrow B \rightarrow A leading to triple mutant 1B2 (L162N/M16A/L17F) with *E* = 594; (b) MD based interpretation of WT PaL (Left) and mutant 1B2 (Right)



epistatic effect, extensive MD simulations were performed using the (S)- and the (R)-substrate separately.^{26c} In the case of the WT PaL oxyanion intermediate occurring in the ratedetermining step, the long alkyl group of the ester clashes severely with residue L162 (Scheme 17, bottom left). In the final quadruple mutant 1B2, exchanged residue Asn162 is "pulled away" by H-bonding to S158, thereby relieving the steric clash. At the same time, Asn162 also hydrogen bonds to H83, which becomes activated for undergoing additional H-bond mediated stabilization of the oxyanion (Scheme 17, bottom right). Finally, F17 forms a π/π interaction with the *p*-nitrophenyl group of the ester substrate.^{26c}

Note that these insights were made possible by partial deconvolution. Full deconvolution by also generating and testing single mutants M16A and L17F as well as all double combinations would have provided even more information on possible epistatic effects. Indeed, as already indicated above, the Reetz group pioneered the use of complete deconvolution as a method for uncovering cooperative, neutral, and antagonistic mutational effects on stereoselectivity at all evolutionary stages in terms of $\Delta \Delta G^{\ddagger, 67}$

At that time, other protein engineers also investigated epistatic effects, but from a different perspective. For instance, Arnold observed that the introduction of a stabilizing mutation into a β -lactamase increased the fraction of active proteins upon adding random single amino acid mutations, while D.S. Tawfik reported negative epistasis linked to protein robustness by randomly mutating a similar enzyme.⁶⁸ Another possibility is to create all possible (single, double, triple, etc.) mutant combinations of a multimutational variant (deconvolution) to determine epistatic effects and pathway accessibility for evolution, thus exploring the protein "fitness landscape" in a stepwise manner.^{32,67a} This approach was widely used earlier in the area of evolutionary biology, as developed by D. M. Weinreich, M. A. DePristo, and D. L. Hartl.⁶⁹ Reetz did not enter the field of evolutionary biology but considered the results of deconvolution/fitness-pathway landscapes to belong to the most important lessons learned in the field of directed evolution.^{26d,32} As a consequence of this work, he emphasized that caution must be taken in all directed evolution studies when attempting to interpret mutational effects. Typical evolutionary ladders, as in Scheme 2, seem to suggest to some protein engineers that traditional additive effects are involved,⁷⁰ but this is a serious misinterpretation.³² In fact, they reveal nothing about mutational additivity or nonadditivity. Extensive information on this unique branch of directed evolution is available, featuring the details of evolutionary stereoselectivity pathways in terms of $\Delta\Delta G^{\ddagger}$ values.^{26c,32,51,61,67}

Accordingly, we highlight another Reetz study, in this case, involving a BVMO (PAMO) as the catalyst in the asymmetric sulfoxidation of *p*-methylbenzyl methyl thioether.⁷¹ The goal was the reversal of enantioselectivity of WT (90% *ee* in favor of (*S*)-sulfoxide) to (*R*)-selectivity. Upon applying first generation CAST/ISM, the quadruple mutant ZGZ-02 (I67Q/P440F/ A442N/L443I) was evolved, enabling a stereoselectivity of 95% ee (*R*), which means, relative to WT PAMO, a dramatic change in free energy amounting to $\Delta\Delta G^{\ddagger} = 16.4$ kJ/mol (7.3 kJ/mol +9.1 kJ/mol). All 4! = 24 upward pathways from WT PAMO to ZGZ-02 in a "constrained fitness pathway landscape" were then constructed in the laboratory (Scheme 18, top).⁷¹ It represents a five-dimensional surface in which four point mutations

Scheme 18. Fitness-pathway landscape featuring 24 pathways leading from (S)-selective WT Baeyer–Villiger

Monooxygenase PAMO in asymmetric sulfoxidation to the best (R)-selective quadruple mutant ZGZ-02 (I67Q/P440F/A442N/L443I)^{71,a}



^{*a*}A typical pathway lacking local minima is the green trajectory, and one having a local minimum is represented by the red pathway. Mutations a, b, c, and d correspond to I67Q, P440F, A442N, and L443I, respectively.

characterizing mutant ZGZ-02 are independent vectors and $\Delta\Delta G^{\ddagger}$ constitutes the dependent variable. Docking experiments explained the most important effects on a molecular level. Several truly astounding epistatic effects⁶⁴ were discovered in all favored and disfavored pathways.⁷¹ This included the finding that the four mutations, I67Q, P440F, A442N, and L443I, when tested individually, all turned out to be (*S*)-selective, yet in concert induce extreme (*R*)-selectivity (95% *ee*; Scheme 18, bottom)! Partial deconvolution of an esterase as reported by Bornscheuer et al. also revealed unexpected results, supporting the contention that deconvolution of multimutational variants is a rewarding task.⁷²

We note that a 2012 publication reported a different goal, namely, CAST/ISM-based "non-constrained" fitness landscapes were constructed,³⁶ as had been suggested by Pim Stemmer to Manfred Reetz in an e-mail exchange. This basically has to do with the question of which ISM pathway is best, because in all ISM studies at the time, arbitrarily chosen pathways had been explored, as summarized in the 2020 review.³² It was discovered in a model study that all theoretically possible upward pathways lead to notably enhanced stereoselectivity, but some more than others.³⁶ When a given saturation mutagenesis library failed to harbor improved mutants, a simple strategy was developed for escaping from such a local minimum (normally a dead-end): use the "best" inferior mutant in the library as a template for the next ISM step. Dead-ends are normal in directed evolution,²⁴ as in the case of epPCR or DNA shuffling (although not always reported). The counterintuitive suggestion for considering an inferior mutant for the next cycle³⁶ has yet to be tested when using these alternative mutagenesis techniques. Indeed, epPCR and DNA shuffling continue to be used by many groups today.^{23,24}

2.5. Directed Evolution of Artificial Metalloenzymes for Promiscuous Reaction Types. In a reflective manner, Reetz realized early on that the group's concept of directed evolution of stereoselective enzymes (Scheme 1) had the limitation that WT enzymes cannot catalyze the plethora of reaction types known in transition metal catalysis. He then suggested and patented in 2000 the idea of directed evolution of artificial metalloenzymes, 73a,b originally termed hybrid catalysts, but its experimental implementation took longer than expected.^{73c} Using the Whitesides platform based on noncovalent attachment of a biotinylated achiral diphosphine Rh-complex to (strept)avidin,⁷⁴ and applying CAST/ISM, enantioselectivity of a model olefin-hydrogenation increased stepwise from 23% to 65% ee in three cycles.^{73c} The initial experimental difficulties have been described in reviews, but the concept opened the door to a new research area.^{73d,e} While the group of T. R. Ward extensively exploited the Whitesides system with impressive applications using other reaction types,⁷⁵ Reetz developed other ideas for artificial metalloenzymes.^{73d} One interesting scaffold was the supramolecular complexation of a commercially available Cu(II) phthalocyanine in serum albumins, which creates a binding pocket in these transport proteins with a potentially active transition metal center (Scheme 19).^{76a} Indeed, in this 2005 study, Diels-Alder reactions with typically 95% enantioselectivity and high endoselectivity were observed.^{76a} In his further study, Reetz chose a thermostable protein tHisF from Thermotoga maritima as a scaffold and creation of a designed copper binding site for turning Diels-Alder cycloadditions; a geometrical catalytic triad C9A/L50H/I52H was created for chelating copper to generate the artificial metalloenzyme. The designed hybrid catalyst was further optimized

Scheme 19. Water-soluble Cu(II)-Phthalocyanine (Upper Right) placed supramolecularly in Serum Albumins as catalyst in the shown asymmetric Diels—Alder reaction [Reproduced with permission from reference 76a. Copyright 2006, John Wiley and Sons]



by replacing four surface histidine residues on 84, 209, 228, and 244 positions to alanine (designed HHD-4xala) to reduce the competition for Cu(II). Therefore, the model Diels–Alder reaction was studied again, leading to an *endo/exo* product ratio of 13:1 with moderate enantioselectivity (46% ee), calling for directed evolution.^{76b}

A fundamentally different approach to directed evolution of stereoselective artificial metalloenzymes was reported by Arnold et al., who employed P450 enzymes as catalysts for a number of fascinating transformations.^{24a,40a,b,77} Fe carbenes are involved, and whenever the parent enzyme failed to deliver acceptable results, directed evolution was used, often by applying CAST/ ISM. Along quite different lines, the Reetz group created an artificial P450 leading to a redox-mediating Kemp eliminase^{78a} and constructed an artificial cysteine-lipase having an altered mechanism.^{78b}

2.6. Directed Evolution of Protein Stability. Following the establishment of CAST/ISM for stereo- and regioselectivity as well as activity, it was demonstrated that ISM can also work for evolving protein stability by using B-factors as a guide for choosing remote randomization sites.^{38a} A high B-factor value indicates poor resolution for this amino acid in the 3D protein crystal structure and reveals where the most flexible parts of the protein are. This idea turned out to be remarkably successful. Using lipase A from Bacillus subtilis as the model system, a computer program (B-FITTER) was developed to identify and rank the residues. Residues with the highest average B-factor values based on the 3D crystal structure were chosen for ISM. The 10 residues with highest B-factor values were grouped into eight individual sites A to H (except for site A composing three nearby residues). Saturation mutagenesis was applied to all sites finding the best variant IV at site E, which was taken as a template for ISM to sites $G \rightarrow F \rightarrow D \rightarrow A$. A total of six ISM cycles yielded mutants XI and X with essentially the same catalytic activity but an improved T_{50}^{15} value of 50 °C compared to the WT (Scheme 20). This resulted in the successful development of the B-FIT method,^{38a,79} which constitutes a semirational alternative to earlier^{20c,68b} and current approaches to directed evolution of protein robustness.^{20d,79,80}

Scheme 20. Engineering thermostability using ISM based on B Values:^{38b} (Top) ISM tree showing the evolution of *B. subtilis* LipA WT towards variant IX and X; (Bottom) Residual activity after 15 min heat treatment [Reproduced with permission from reference 38b. Copyright 2006, John Wiley and Sons]



3. CONCLUSIONS AND PERSONAL REMARKS

We have composed this Account with the intention of illuminating the enormous creativity of Manfred Reetz, putting his achievements in the proper context with respect to the developments in the field. *The primary emphasis is in the area of directed evolution, which Reetz considered to be the most important achievement in his 50-year career as an independent researcher.* Manfred Reetz has significantly shaped biocatalysis as we know it today. A comprehensive summary of some of his seminal contributions is shown in Scheme 21.

Thanks to Manfred Reetz, we now have effective tools at hand to tailor enzymes to suit the needs of chemists and molecular biologists in academia and industry. *This has established biocatalysis as an important tool in organic chemistry and biotechnology*. He consistently acknowledged the earlier contributions to directed evolution by other protein engineers,^{23,24} and was shocked upon hearing of the tragic and untimely death of his friend Pim Stemmer, whom he greatly admired.³² Except for the highest possible award, our mentor has thus far received many national and international prizes.

Reetz believed that science and ethical issues are inseparable, see for example reference 81. On the personal side, we first note that

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Account





Manfred Reetz considered the well-being of all his co-workers (technicians, secretaries, doctoral students, and postdocs) to be of the utmost importance. Many of us have profited from his continuing support many years after working with him.

As a supervisor, he is a role model for a good scientist. *He* asked everyone to remain self-critical when assessing their own experimental results, and he always stressed the importance of a sound and neutral language when writing papers or presenting lectures/posters at conferences. We very much appreciate his openness and rejoicing in out-of-the-box thinking and suggestions, giving his co-workers the space to try out their own ideas. Open minded and unbiased certainly describe Manfred Reetz's scientific attitude and personality. Indeed, many of his former co-workers share our appraisal of Manfred Reetz's role in their professional development (see the SI for some personal statements). We are incredibly grateful and privileged for having worked with an outstanding scientist and supporting mentor, and we wish Manfred Reetz many more happy and healthy years!

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscatal.0c04108.

A collection of personal statements from former Reetz coworkers (PDF)

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Notes

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