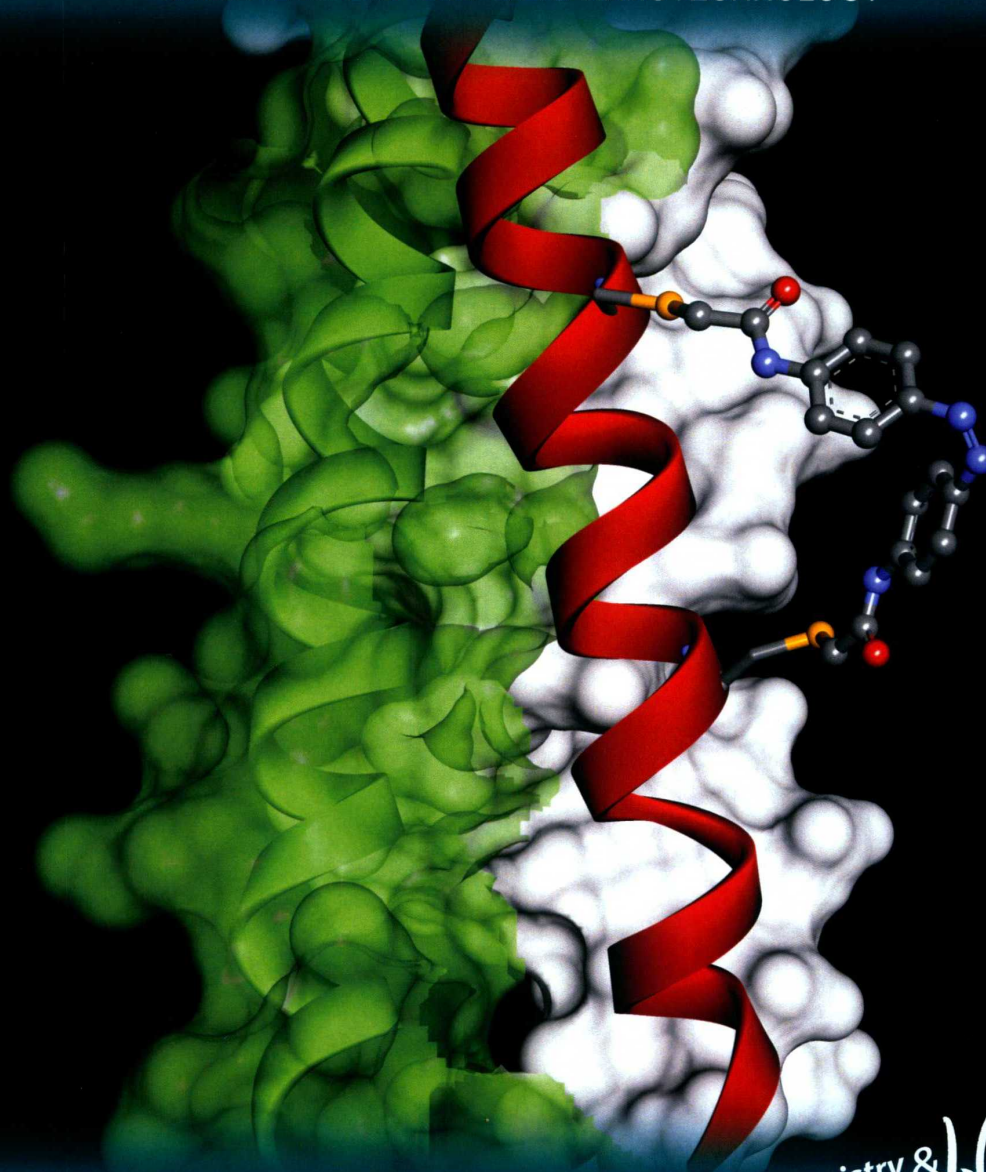


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Resolves the Hydride, but Not the Debate (D. Schilter)
Chemical Talking with Living Systems:
Molecular Switches Steer Quorum Sensing in Bacteria (H. A. Wegner)

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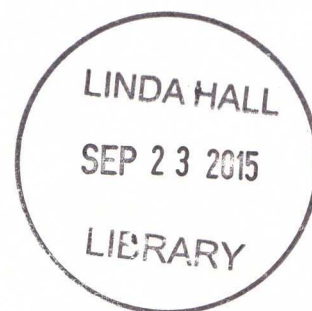
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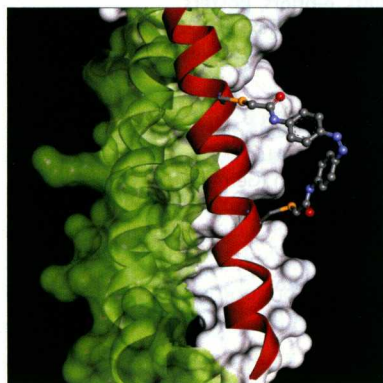
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COVER PICTURE

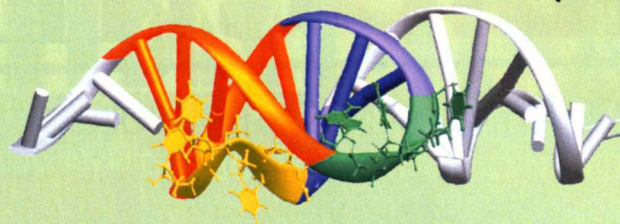


The cover picture shows a photoswitchable azobenzene crosslinked peptide (red) that acts as a dominant negative inhibitor of DNA binding by the transcription factor CREB (green). The DNA target is shown in white. On p. 1757 ff. of this issue, G. A. Woolley and co-workers show that the largest degree of conformational control is obtained when the photoswitchable crosslinker is placed at the site of highest intrinsic helical propensity in the inhibitor.

NEWS

Spotlights on our sister journals

1704 – 1707



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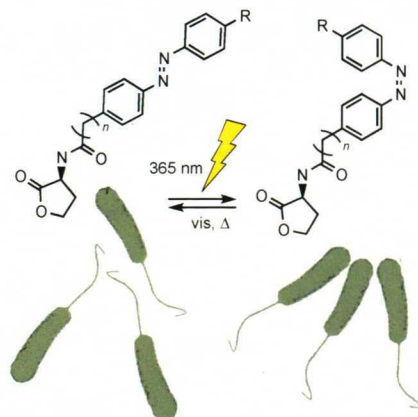
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Further masthead information follows directly
after the Table of Contents.

HIGHLIGHTS

New avenues in bacterial engineering:

An azobenzene molecular switch has been incorporated into an autoinducer for quorum sensing (QS) in bacteria. The authors demonstrated that irradiation with different wavelengths of light influences the QS system thereby controlling gene expression as well as the phenotype, as exemplified by pyocyanin production.



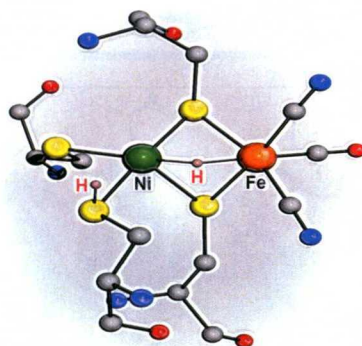
*L. Schweighauser, H. A. Wegner**

1709 – 1711

**Chemical Talking with Living Systems:
Molecular Switches Steer Quorum
Sensing in Bacteria**

An (X-ray) eye for detail:

Modern high-resolution protein crystallography allows H atoms to be located. Applied to nickel-iron hydrogenase, X-ray structural analysis has finally confirmed the presence of an active-site hydride and thiol, as well as unveiling the intricate pathways that protons take to and from the active site.



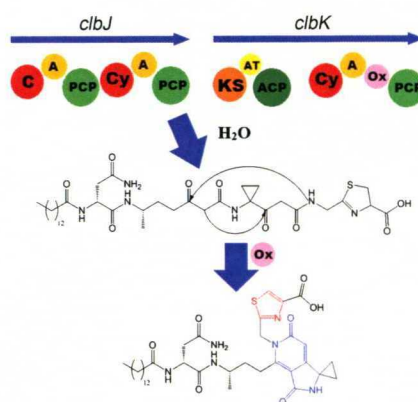
*D. Schilter**

1712 – 1714

**Nickel-Iron Hydrogenases: High-
Resolution Crystallography Resolves
the Hydride, but Not the Debate**

COMMUNICATIONS

A vital precolibactin intermediate was isolated and identified as a new 1*H*-pyrrolo[3,4-*c*]pyridine-3,6(2*H*,5*H*)-dione- and thiazole-containing compound. Based on its structure together with gene-knockout experiments, we mapped its logical biosynthetic pathway. Our results lay a foundation for further work to determine the enzymatic events in this mysterious pathway.



*Z.-R. Li, Y. Li, J. Y. H. Lai, J. Tang, B. Wang,
L. Lu, G. Zhu, X. Wu, Y. Xu, P.-Y. Qian**

1715 – 1719

**Critical Intermediates Reveal New
Biosynthetic Events in the Enigmatic
Colibactin Pathway**



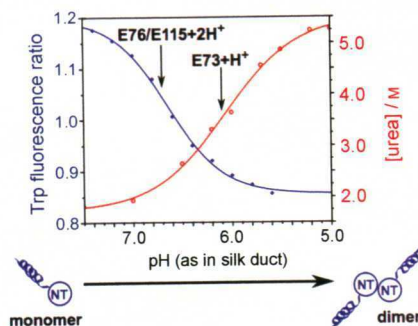
Inside Cover



M. Otikovs, G. Chen, K. Nordling,
M. Landreh, Q. Meng, H. Jörnvall,
N. Kronqvist, A. Rising, J. Johansson,*
K. Jaudzems*

1720 – 1724

 **Diversified Structural Basis of a Conserved Molecular Mechanism for pH-Dependent Dimerization in Spider Silk N-Terminal Domains**



Spinning webs: Conversion of spider silk proteins from soluble dope to insoluble fibers involves pH-dependent dimerization of the N-terminal domain (NT). We have analyzed the pH dependence of the *A. ventricosus* minor ampullate spidroin NT and determined solution structures at pH 7.2 and 5.5, revealing that the dimerization is mediated by protonation of three glutamic acid residues.

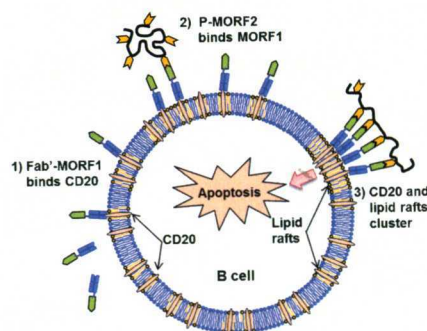


Back Cover

J. M. Hartley, T.-W. Chu, E. M. Peterson,
R. Zhang, J. Yang, J. Harris, J. Kopeček*

1725 – 1729

 **Super-Resolution Imaging and Quantitative Analysis of Membrane Protein/Lipid Raft Clustering Mediated by Cell-Surface Self-Assembly of Hybrid Nanoconjugates**

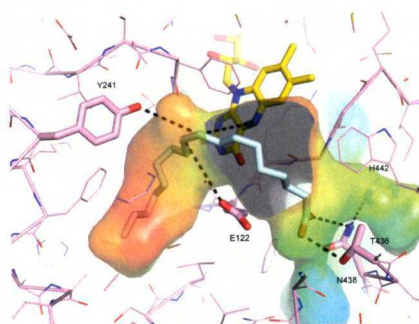


Hybrid nanomaterials: Super-resolution imaging was used to quantify organizational changes in the plasma membrane after treatment with hybrid nanoconjugates. The images were analyzed by using pair-correlation analysis to determine the density of bound conjugates, cluster size, and average number of molecules per cluster.

M. Engleder, T. Pavkov-Keller,
A. Emmerstorfer, A. Hromic, S. Schrempf,
G. Steinkellner, T. Wriessnegger, E. Leitner,
G. A. Strohmeier, I. Kaluzna, D. Mink,
M. Schürmann, S. Wallner, P. Macheroux,
K. Gruber,* H. Pichler*

 1730 – 1734

 **Structure-Based Mechanism of Oleate Hydratase from *Elizabethkingia meningoseptica***

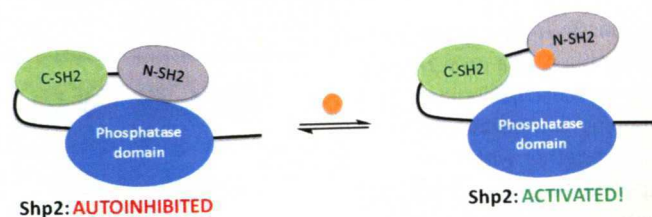


We report the first crystal structure of an oleate hydratase in complex with the essential flavin cofactor. Elucidation of the active-site amino acid residues allowed us to propose a structure-based reaction mechanism for this class of hydratases that accounts for the role of the flavin moiety. Our findings should help employment of this enzyme class in future biocatalytic applications.

C. M. Chio, K. W. Cheng, A. C. Bishop*

1735 – 1739

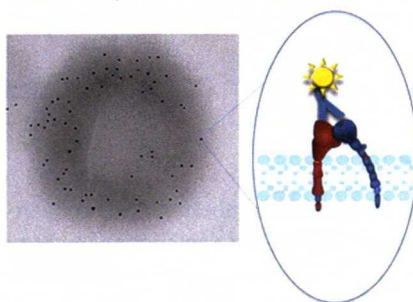
 **Direct Chemical Activation of a Rationally Engineered Signaling Enzyme**



Turn it on: Rational engineering of a loop at Shp2's interdomain interface yields a protein phosphatase that retains its mechanism of autoinhibitory regulation but can be target-specifically activated with a small molecule. This

strategy for achieving direct chemical control over enzyme activity may be applicable to a range of signaling proteins, many of which are regulated through domain-domain interactions.

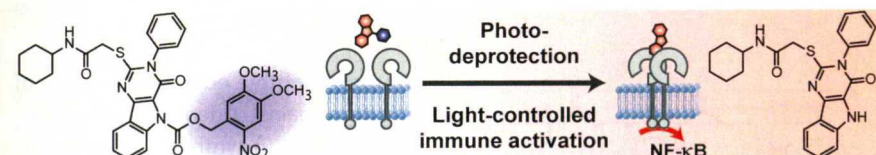
A heterodimeric membrane protein species, integrin $\alpha_v\beta_3$, was synthesised by ribosomes derived from cell lysates in the presence of an amphiphilic polymeric membrane. Immunolabelling with a function-blocking antibody in combination with biochemical analysis revealed functional folding of integrin $\alpha_v\beta_3$ in a chemically defined and robust synthetic membrane architecture.



C. Zaba, S. Ritz, C.-W. D. Tan, S. Zayni, M. Müller, U. Reuning,* E.-K. Sinner*

1740 – 1743

Functional Cell Adhesion Receptors (Integrins) in Polymeric Architectures



Controlling the spatial and temporal aspects of immune signaling would enable further study of immune synergies and assist in the development of new vaccines. Here, a caged TLR4 ago-

nist was synthesized, affording a photo-controlled agonist that was inactive while caged, yet effected NF-κB activity in cells following UV photocontrolled deprotection.

L. Stutts, A. P. Esser-Kahn*

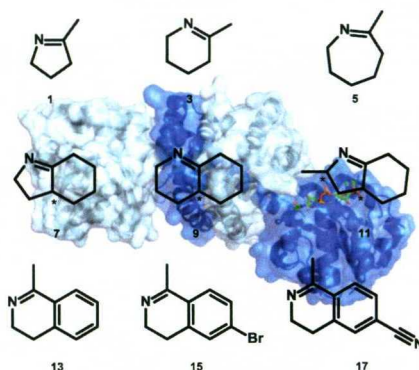
1744 – 1748

A Light-Controlled TLR4 Agonist and Selectable Activation of Cell Subpopulations



FULL PAPERS

IREd in the face: Twenty novel imine reductases (IREdS) were characterized chemically by screening them against a set of cyclic imines and by applying bioinformatic methods. High yields of pure product were obtained from all of them. The IREdS were subgrouped by an active-site clustering according to the experimental results.



D. Wetzl, M. Berrera, N. Sandon, D. Fishlock, M. Ebeling, M. Müller, S. Hanlon, B. Wirz, H. Iding*

1749 – 1756

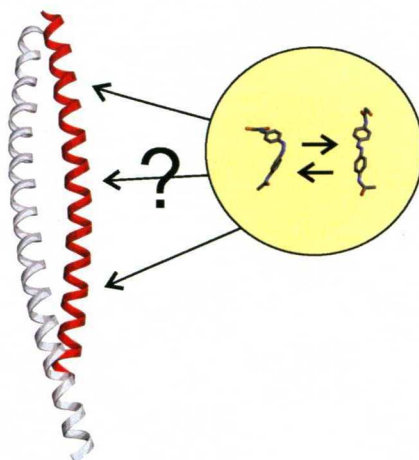
Expanding the Imine Reductase Toolbox by Exploring the Bacterial Protein-Sequence Space



Inside
Back Cover



Systematic testing of potential sites for *cis/trans* photoswitchable azobenzene-based crosslinkers leads to effective light-controlled inhibitors of CREB transcription factors. The largest degree of photocontrol is achieved when the crosslinker is in the zipper region between Cys residues in the heptad segment showing the highest intrinsic helical propensity.



A. M. Ali, M. W. Forbes, G. A. Woolley*

1757 – 1763

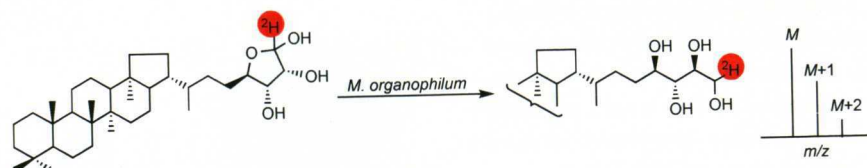
Optimizing the Photocontrol of bZIP Coiled Coils with Azobenzene Crosslinkers: Role of the Crosslinking Site



A. Bodlenner, W. Liu, G. Hirsch,
P. Schaeffer, M. Blumenberg, R. Lendt,
D. Tritsch, W. Michaelis, M. Rohmer*

1764 – 1770

**C₃₅ Hopanoid Side Chain Biosynthesis:
Reduction of Ribosylhopane into
Bacteriohopanetetrol by a Cell-Free
System Derived from
*Methylobacterium organophilum***

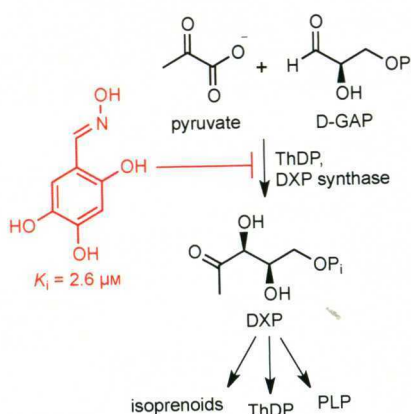


²H-labelled ribosylhopane was converted into ²H-labelled bacteriohopanetetrol by a cell-free system derived from *Methylobacterium organophilum* in the presence of NADPH. This afforded the first direct evidence of its intermediacy in the C₃₅ hopanoid biosynthetic pathway.

D. Barteo, F. Morris, A. Al-khouja,
C. L. Freel Meyers*

1771 – 1781

**Hydroxybenzaldoximes Are D-GAP-
Competitive Inhibitors of *E. coli*
1-Deoxy-D-Xylulose-5-Phosphate
Synthase**

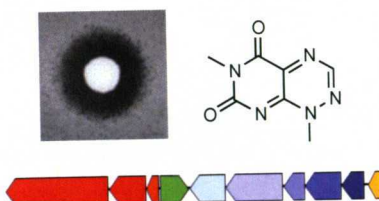


An uncompetitive edge: A new class of inhibitors against 1-deoxy-D-xylulose-5-phosphate (DXP) synthase, an essential enzyme in bacterial central metabolism, was discovered. Kinetic analysis reveals that these inhibitors are competitive against D-GAP and uncompetitive or noncompetitive against pyruvate, thus suggesting that they target the uniquely stable lactyl-thiamin diphosphate intermediate.

B. Philmus,* B. T. Shaffer, T. A. Kidarsa,
Q. Yan, J. M. Raaijmakers, T. P. Begley,
J. E. Loper*

1782 – 1790

**Investigations into the Biosynthesis,
Regulation, and Self-Resistance of
Toxoflavin in *Pseudomonas protegens*
Pf-5**

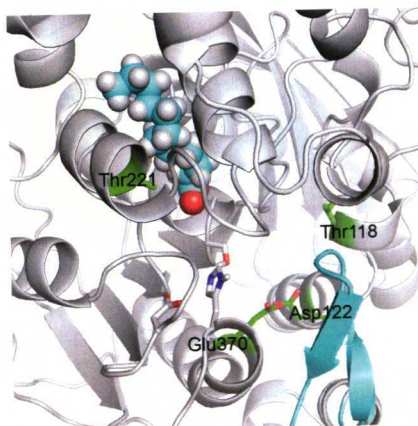


Toxoflavin talk: The soil bacterium *P. protegens* Pf-5 produces toxoflavin, a triazine antibiotic. Through gene knockouts, we showed that the initial steps in toxoflavin biosynthesis are identical to those in riboflavin biosynthesis; this led to a revised biosynthetic scheme. The protein encoded by *toxM*, found in the Pf-5 cluster, confers resistance to toxoflavin by degrading it.

J. Müller, M. A. Sowa, B. Fredrich,
H. Brundiek, U. T. Bornscheuer*

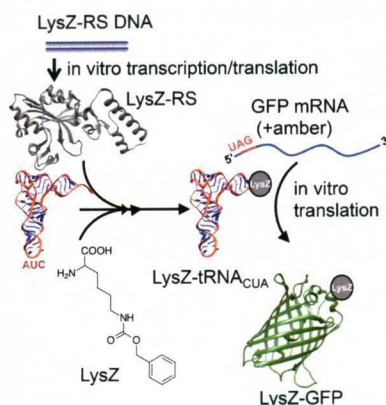
1791 – 1796

**Enhancing the Acyltransferase Activity
of *Candida antarctica* Lipase A by
Rational Design**



Acyltransferase activity of CAL-A: Analysis of the CAL-A structure suggested 28 single-mutant variants to favor the desired acyltransferase activity. After screening, variant Asp122Leu exhibited significantly reduced production of free fatty acids, whereas the catalytic properties (e.g., thermostability, pH optimum) were not significantly altered.

Enzyme evolution: Using a liposome-based in vitro compartmentalization approach, we screened a randomly mutagenized library of *N*-benzyloxycarbonyl-L-lysine (LysZ) tRNA synthetases and obtained one that was able to increase LysZ incorporation both in vitro and in vivo. The ease and flexibility of this approach should enable evolution of not only the aminoacyl-tRNA synthetases but also of other enzymes involved in protein translation.



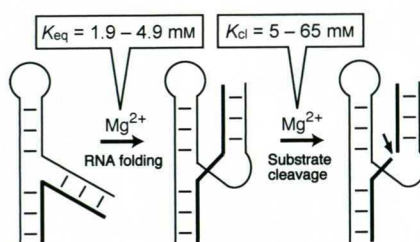
A. Uyeda, T. Watanabe, Y. Kato,
H. Watanabe, T. Yomo, T. Hohsaka,*
T. Matsuura*

1797 – 1802

Liposome-Based In Vitro Evolution of Aminoacyl-tRNA Synthetase for Enhanced Pyrrolysine Derivative Incorporation



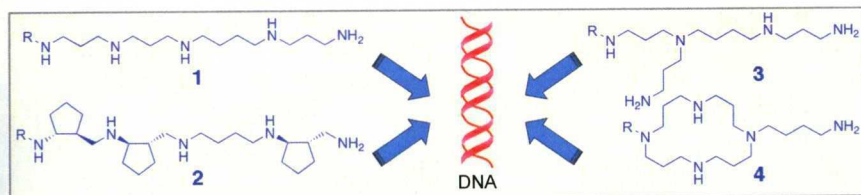
The RNA cleavage activity of the hammerhead ribozyme is increased in mixed aqueous solutions containing cosolvents, owing to the reduction in the Mg^{2+} concentration required for efficient catalysis. This is contrary to the effects on the Mg^{2+} -induced structural transitions.



S. Nakano,* Y. Kitagawa, H. Yamashita,
D. Miyoshi, N. Sugimoto

1803 – 1810

Effects of Cosolvents on the Folding and Catalytic Activities of the Hammerhead Ribozyme



Versatile solid-phase synthesis of polyamines: A peptide chemistry approach was used to construct linear, partially constrained, branched, and cyclic polyamines. Examination of the effects on

DNA duplex stability and structure revealed that the polyamines exhibit distinct activities, thus highlighting the importance of polyamine backbone structure.

N. Umezawa,* Y. Horai, Y. Imamura,
M. Kawakubo, M. Nakahira, N. Kato,
A. Muramatsu, Y. Yoshikawa,
K. Yoshikawa, T. Higuchi*

1811 – 1819

Structurally Diverse Polyamines: Solid-Phase Synthesis and Interaction with DNA



* Author to whom correspondence should be addressed.

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Expanding the Imine Reductase Toolbox by Exploring the Bacterial Protein-Sequence Space

Dennis Wetzl,^[a] Marco Berrera,^[a, c] Nicolas Sandon,^[a] Dan Fishlock,^[a] Martin Ebeling,^[a, c] Michael Müller,^[b] Steven Hanlon,^[a] Beat Wirz,^[a] and Hans Iding^{*,[a]}

Recent investigations on imine reductases (IREDs) have enriched the toolbox of potential catalysts for accessing chiral amines, which are important building blocks for the pharmaceutical industry. Herein, we describe the characterization of 20 new IREDs. A C-terminal domain clustering of the bacterial protein-sequence space was performed to identify the novel IRED candidates. Each of the identified enzymes was characterized against a set of nine cyclic imine model substrates. A refined

clustering towards putative active-site residues was performed and was consistent both with our screening and previously reported results. Finally, preparative scale experiments on a 100 mg scale with two purified IREDs, IR_20 from *Streptomyces tsukubaensis* and IR_23 from *Streptomyces viduochromogenes*, were carried out to provide (*R*)-2-methylpiperidine in 98% ee (71% yield) and (*R*)-1-methyl-1,2,3,4-tetrahydroisoquinoline in > 98% ee (82% yield).

Introduction

Chiral amines are important building blocks for pharmaceutical and agrochemical target compounds, because they are abundant structural and functional elements in natural chemical scaffolds with biological activities.^[1] Yet despite their importance and the continued need for chiral amines, their accessibility by asymmetric synthesis is still rather limited. Non-enzymatic approaches for the asymmetric synthesis of chiral amines include transition-metal and organocatalyzed reactions.^[2] Nevertheless, for industrial applications the chiral resolution of optically active amines remains very important, despite the limitation of a maximum yield of 50%.^[3] In this case, biocatalysis could help to fill the gap, which would give an asymmetric synthetic approach towards chiral amines.^[3] A number of enzyme classes have recently been investigated to assess their utility for the preparation of optically active amines.^[4] These classes include transaminases, ammonia lyases, and monoamine oxidases, of which some catalysts have been engineered to a degree of sophistication that enables their use in industrial settings.^[5] Recently, Mitsukura et al. described imine-reducing enzymes from *Streptomyces* sp. GF3546 and *Streptomyces* sp. GF3587 that have been shown to reduce

a broad range of substrates.^[6] Turner and co-workers have demonstrated the utility of these imine reductases (IREDs) for the asymmetric reduction of a broad panel of cyclic imines producing chiral secondary and tertiary amines, which was also highlighted in the review of Gamanera and Dominguez.^[7,8] By resolving the first crystal structure of an NADPH-dependent IRED, Grogan and co-workers have paved the way for a deeper understanding of this class of enzymes.^[7] The first description of a direct reductive alkylamination of ketones catalyzed by IREDs gave a hint of their applicability to the reduction of acyclic imines.^[9] In this approach, the imine could be formed in situ, and chemical synthesis of the substrate prior to the reduction step could be omitted. In spite of these results, the synthetic potential and substrate scope of IREDs has not yet been fully explored, especially because the number of available, well-described IREDs remains low. Various strategies exist for the discovery of novel enzymes with a desired function.^[10] Given that there is a known and well-described enzyme as a starting point, additional enzymes can easily be identified to give similar reaction types by using bioinformatic tools, such as BLAST search, to mine the steadily expanding protein-sequence data space. In combination with the decreasing prices of synthetic genes, this approach opens the way for the fast and efficient discovery of novel biocatalysts with desired catalytic properties. Hauer and co-workers gave an example of how these tools can be used to identify further enzymes with imine reducing properties and shed light on the catalytic properties of these biocatalysts.^[11] They set up a database of proteins that have the potential to bear imine-reducing properties (Imine Reductase Engineering Database; abbreviated IDB) and demonstrated the usefulness of this approach by identifying three new enzymes from that database and giving a hint on the catalytic properties in terms of their selectivity.^[11] With over 400 bacterial protein sequences being annotated as putative

[a] Dr. D. Wetzl, Dr. M. Berrera, Dr. N. Sandon, Dr. D. Fishlock, Dr. M. Ebeling, Dr. S. Hanlon, Dr. B. Wirz, Dr. H. Iding
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Albertstrasse 25, 79104 Freiburg (Germany)

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Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/cbic.201500218>.

IREDs in the IDB and only ten enzymes having been characterized so far, the surface of the IRED sequence space has barely been scratched. Herein, we present the characterization of 20 new IREDs, thus significantly expanding the IRED toolbox.

Results and Discussion

Identification of novel IRED candidates for synthetic applications

The sequences of six IREDs known from literature (IREds from *Streptomyces* sp.GF3587, *Streptomyces* sp. GF3546, *Streptomyces kanamyceticus*, *Streptosporangium roseum*, *Streptomyces ipomoeae*, *Paenibacillus elgii*) were used as a starting point for searching similar protein sequences in the available bacterial protein-sequence space.^[6a,b,8a,b,9,11–12] We hypothesized that the C-terminal domain of the IREDs, as opposed to the NADPH co-factor that is bound to the N-terminal domain, might more significantly influence the catalytic properties of the enzymes in terms of substrate scope and selectivity. The N-terminal Rossman fold is, unlike the C terminus, rather conserved between the members of this class of enzymes.^[9] Based on this hypothesis we built a Hidden Markov model (HMM) of the C-terminal domain of the IREDs reported in the literature and performed a clustering of all bacterial protein sequences that matched this initial HMM. The corresponding clustering resulted in 85 clusters denoted as RC1–RC85 (see Supporting Information, Table S1), of which only 15 clusters contained more than three candidates. The majority of the IRED proteins were grouped into eight of the 85 clusters, including all of the IREDs known in the literature being located within the first two clusters. Given the previously described clustering and annotation from Hauer and co-workers, we compared the cluster annotation of all literature-known enzymes with our clustering. Interestingly, the enzymes that were described as *R*-selective enzymes in the literature were all located within cluster RC1, while the enzymes that were denoted as *S*-selective were located within cluster RC2. For setting up our IRED toolbox, we chose 14 new IRED candidates from the first round (Table 1). Two previously known IREDs from *Streptomyces virginiae* and from *Streptomyces* sp. GF3546 were added to our library as reference enzymes (IR_7 and IR_8, respectively).^[6a,8b,9] The novel enzyme candidates were chosen to be diverse with respect to the C-terminal clustering and the IDB, because we wanted to gain an overview of the IRED sequence space. Additionally, we selected enzymes from different organisms to further increase the diversity. According to the IDB, four of our enzyme candidates were annotated to be *S*-selective (IR_1–IR_4) and six as *R*-selective (IR_9–IR_14). Some of our candidates (IR_5, IR_6, IR_15, and IR_16) were not listed in the IDB, therefore no annotation for the stereo-preference was known (see Table S3).

Screening of the novel IRED library

The enzymes of the initial library were heterologously expressed in *Escherichia coli* and 13 out of 14 novel candidates were well expressed and could be purified by using standard

Table 1. IRED candidates used in the initial screening.

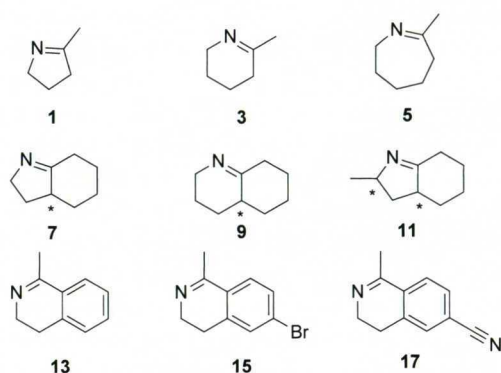
IREd	Organism	Protein identifier	Cluster ^[a]
IR_1	<i>Streptomyces</i> sp. Mg1	WP_008741284.1	RC2
IR_2	<i>Streptomyces fulvissimus</i>	WP_015611605.1	RC2
IR_3	<i>Bacillales</i> sp.	WP_007131315.1	RC2
IR_4	<i>Kribbella flavida</i> DSM 17836	WP_012921542.1	RC2
IR_5	<i>Cupriavidus</i> sp. HPC(L)	WP_023264430.1	RC5
IR_6	<i>Saccharomonospora xinjiangensis</i>	WP_006236994.1	RC8
IR_7	<i>S. aurantiacus</i>	WP_016644884	RC2
IR_8	<i>Streptomyces</i> sp. GF3546	BAM99301.1	RC2
IR_9	<i>Frankia</i> sp. QA3	WP_009737838.1	RC1
IR_10	<i>Mycobacterium smegmatis</i>	WP_011731218.1	RC10
IR_11	<i>Verrucosipora maris</i>	WP_013733165.1	RC11
IR_12	<i>Streptomyces</i> sp. CNB091	WP_018958796.1	RC4
IR_13	<i>Saccharopolyspora erythraea</i> NRRL 2338	YP_001108711.1	RC1
IR_14	<i>Nocardia cyriacigeorgica</i> GUH-2	YP_005263141.1	RC1
IR_15	<i>Actinomadura rifamycini</i>	WP_026403156.1	RC16
IR_16	<i>Actinomadura oligospora</i>	WP_026413575.1	RC16

[a] Clustering, as determined in our study, based on the C-terminal sequence.

immobilized-metal-ion affinity chromatography (IMAC). For the expression of these enzymes, we used a standardized protocol without any further optimization. The IR_14 was the only enzyme not expressed under the tested conditions and was thus excluded from further characterization. The screening of the novel IRED candidates was performed with purified enzymes, to ensure unambiguous characterization of catalytic properties, and with the application of a cofactor recycling system. All IREDs were screened against a set of substrates (depicted in Scheme 1) by using a deep-well based screening method that allowed the screening of one substrate against the entire library. Our model substrates comprised different monocyclic (**1**, **3**, and **5**) and bicyclic aliphatic imines (**7**, **9**, and **11**) as well as arylc substrates with a benzylic imine functional group (**13**, **15**, and **17**).

The results of this screen are summarized in Table 2 (for detailed conversions see Table S4). We found that 10 of the 13 new IRED candidates were active against most of the screened substrates, while only a few candidates seemed to have a rather limited substrate scope with low conversions under the screened conditions. The vast majority of the tested IRED candidates displayed good conversions (>90%) against nearly all of the tested substrates as well as good to excellent enantioselectivities, often yielding the corresponding product with an enantiomeric excess >90%. These results emphasized the versatility of these enzymes and encouraged us to use our library as a basis for further investigations. IR_2, IR_3, and IR_6, however, seemed to represent exceptions; they showed a more limited substrate scope than the other IREDs and displayed poor conversions and selectivities for most of the aliphatic substrates, although both the conversions and selectivities were significantly higher for the arylc substrates (**15** and **17**).

Interestingly, the substrates themselves seemed to play an important role in the stereochemical outcome of the reaction for some of the tested enzymes. For example, IR_14 produced



Scheme 1. Substrates used in the screening of the novel IRED candidates.

the corresponding *R*-amine with substrates **1**, **3**, **5** and **13** with good selectivities, while also forming the *S*-amine with substrates **15** and **17** (bearing a bromo- or cyano substituent), which were sterically as well as electronically dissimilar to **13**. Additionally, the enantiomeric excess of the corresponding products formed by IR₁₁, IR₁₂, and IR₁₅ for these substrates seemed to decrease drastically in comparison to the biotransformations with the other substrates. Inversion of the stereochemistry of the corresponding amine for **15** and **17** compared to **13** means that the hydride transfer for the enzymes showing this phenomenon, such as IR₁₄, seems to be directly dependent on the imine substrate.

To yield the product amine with inverted stereochemistry, the hydride needs to be transferred from the opposite face of the prochiral substrates (*re* face, as opposed to *si*), as was found in the case of the above-mentioned biotransformation

with IR₁₄. Similar findings were reported, by the groups of Grogan and Turner for an IRED from *Nocardiopsis halophila*.^[13] This enzyme converted sterically less-demanding substrates to the corresponding *S*-amines, while for sterically more-demanding substrates the hydride attack occurred from the opposite face of the prochiral substrate, which resulted in either the corresponding *R*-amines or, due to an inverted priority of the substituents, again in the formation of the corresponding *S*-amine.

Expanding the IRED library

Characterization of the library showed that the initially selected candidates had a strong bias towards the formation of the corresponding *S*-product. To counteract this bias, we enlarged our library by adding five more enzymes (IR₁₉–IR₂₃) from the cluster RC1, which contained the enzymes that were reported in the literature to display *R* selectivity on **1**.

Furthermore, although IR₁₀ was annotated to have *R* selectivity in the IDB, it was found to strictly produce the *S*-amine for all the tested substrates, thus indicating that clustering of IREDs based on their global sequence might suffer from a bias of conserved domains that are not responsible for selectivity. This enzyme was part of cluster RC10, one of the smaller clusters, from which we chose two more candidates (IR₁₇ and IR₁₈) to check for consistency in the catalytic properties of this cluster.

The novel candidates (IR₁₇–IR₂₃) of the second screen were expressed and purified according to the method of the initial library and tested against the same set of substrates. All of the selected enzymes were well expressed and active against most of the screened substrates. The results of this

Table 2. Screen of the initial IRED library. Conversions have been determined based on percent area of the product and are indicated by color. The enantiomeric excess of the formed product is indicated by numbers and given in % ee. Positive values indicate the predominant formation of the *S* enantiomer while negative values indicate *R* enantiomer as the main product. The enantiomeric excess of biotransformations with conversions below 25% was not determined. Conversions are coded as follows: green = 100%–≥ 75%, yellow < 75%–≥ 50%, orange < 50%–≥ 25%, and red < 25%–0%.

Substrate	IR_1	IR_2	IR_3	IR_4	IR_5	IR_6	IR_7 ^[a]	IR_8 ^[a]	IR_9 [*]	IR_10	IR_11	IR_12 [*]	IR_13	IR_14	IR_15	N ^[b]
1	99	n.d.	n.d.	95	n.d.	n.d.	99	99	-95	97	-98	-97	44	-97	99	-
3	98	99	n.d.	85	12	-9	99	99	-90	98	-69	-81	97	-98	99	-
5	99	n.d.	n.d.	97	18	n.d.	96	96	-99	95	-88	-73	98	-96	99	-
7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-
9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-
11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-
13	99	n.d.	86	99	-68	n.d.	99	92	-99	99	-88	-85	68	-87	99	-
15	96	11	37	98	51	95	97	91	-98	98	-21	-33	90	67	18	-
17	99	69	93	99	n.d.	98	99	98	-92	94	-6	-56	79	35	17	-

n.d.: not determined owing to low conversions (< 25%) or complex stereochemistry (substrates **7**, **9**, and **11**) with two or more stereocenters; [a] IR₇ and IR₈ are the known enzymes from *S. aurantiacus* (IR₇) and *Streptomyces* sp. GF3546 (IR₈);^[8] [b] Negative control biotransformations, with a complete reaction mixture where the IRED is replaced by potassium phosphate buffer. * due to the low stability, these enzyme candidates were omitted from the screen using deep-well plates and characterized directly after purification.

Table 3. Screen of the second IRED library. Conversions have been determined based on percent area of the product and are indicated by color. The enantiomeric excess of the formed product is indicated by numbers and given in % ee. Positive values indicate the predominant formation of the *S* enantiomer while negative values indicate *R* enantiomer as the main product. The enantiomeric excess of biotransformations with conversions below 25% was not determined. Conversions are coded as follows: green = 100%–≥75%, yellow <75%–≥50%, orange <50%–≥25%, and red <25%–0%.

Substrate	IR_17	IR_18	IR_19	IR_20	IR_21	IR_22	IR_23
1	98	98	-80	-99	n.d.	-99	-93
3	99	99	-82	-98	-99	-99	-92
5	97	93	-25	-99	-99	-99	-99
7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
13	97	99	-89	-80	n.d.	-82	-96
15	97	98	-98	74	-97	58	-76
17	92	99	-90	24	-97	-4	-72

n.d.: not determined owing to low conversions (<25%) or complex stereochemistry (substrates 7, 9, and 11) with two or more stereocenters.

second screen of enzymes are summarized in Table 3 (for detailed conversions see Table S5). We were pleased to find that IR_17 and IR_18 displayed similar activities and selectivities to the initially characterized IR_10, which supported our initial hypothesis of the clustering approach. IR_17 and IR_18 converted all of the tested substrates to the corresponding *S* enantiomer with good to excellent selectivities. The conversions for the biotransformations were found to be generally good, exceeding 90% for all aryl substrates as well as for most of the aliphatic substrates.

The five additional candidates from cluster RC1 (IR_19–IR_23) were found to convert all of the tested substrates with good conversions, predominantly forming the *R*-product. Interestingly, IR_20 and IR_22, like IR_14, seemed to be influenced by the substituents of the substrates 15 and 17. Once again 15 was found to be converted preferentially to the corresponding *S* enantiomer, whereas for the other substrates both enzymes formed the corresponding *R* enantiomer with good to excellent selectivities.

Clustering refinement based on active-site residues

Encouraged by the results of the screen, we were interested in refining the initial C-terminal clustering to better explain and predict the catalytic properties of the enzyme candidates, such as IR_13. IR_13 was located in cluster RC1, grouped together with enzymes that were found or reported to produce almost exclusively the corresponding *R* enantiomers of the tested substrates (IR_14, IR_20, and IR_22 were exceptions to this, and produced the *S* enantiomer of 15 and/or 17). In addition, we

were interested in identifying positions in the active site of the enzymes that might be responsible for the catalytic properties. Therefore, the clustering was refined by reducing the number of residues considered to only those that are in close proximity to the enzyme's hydride donor, namely the C4 atom of the NADPH cofactor. Residues that lined the interdomain active-site cleft were selected by visual inspection of the X-ray structure from the *S. kanamyceticus* IRED (see Figure 1). By using these positions, a pseudo-sequence was created and aligned for all IRED candidates (see Figure S1). Thus, the corresponding positions of the other enzymes from this study were also extracted and pseudo-sequences were created, just as for the *S. kanamyceticus* IRED. We found from the sequence alignment of the IREDs in this study (Figure S1) that some of the enzymes possessed residues at the putative "catalytic" position 187 (according to the numbering in *S. kanamyceticus* IRED) that seem to contradict some of the previous considerations about IREDs. While IREDs having an aspartate at that position were reported to display *R* selectivity with substrate 1, IR_10 formed the corresponding *S* enantiomer of all of the substrates tested in this screen with medium to good selectivities, despite being an "aspartate-IRED".^[11] Furthermore, IR_6 and IR_13 possessed, according to our sequence alignment, a phenylalanine and an asparagine at the respective position, where the previously characterized enzymes possessed a tyrosine or an aspartate residue. Certainly these findings need to be underpinned by further investigations to conclusively prove the replacement of these putative catalytically active residues by the residues found in the sequence alignment. IR_6, for example, possesses a glutamate at the position next to the phenylalanine putatively capable of taking the function of the catalytically "active" residue at position 187. In the clustering, according to the calculated sequence distances of the pseudo-sequences, all enzymes displaying distinct selectivities towards the tested substrates were well grouped together and separated from the enzymes displaying opposing selectivities (see Figure 1). To challenge our clustering approach, we included the literature known IREDs from the groups of Mitsukura, Turner, Hauer, Müller, and Höhne (IREDs Sgf3587, Sgf3546, *S. kanamyceticus* (Sk), *S. roseum* (Sr), *S. ipomoeae* (Si), *P. elgii* (Pe), *Streptomyces turgidiscabies* (St), *Streptomyces aurantiacus* (Sa)) to see whether their allocation within the clustering fits the reported experimental data.^[6a,b,8a,b,9,11–12] Gratifyingly, the previously reported IREDs were grouped together with enzymes that behaved similarly in terms of their stereopreference towards the tested substrates. The refined clustering also revealed that the IREDs described in the literature were not so diverse in terms of sequence distance of the active site, while the IREDs from our study were found to be distributed more broadly over the sequence space (Figure 1). The residues contained in these pseudo-sequences might include amino acid positions that significantly influence the substrate scope and selectivity of the enzymes. Based on the experimental results, a consensus sequence was extracted from the IREDs displaying *R*- and *S*-stereopreference, respectively, for the substrates tested in this study as well as from the IREDs reported in the literature (Figure S2).

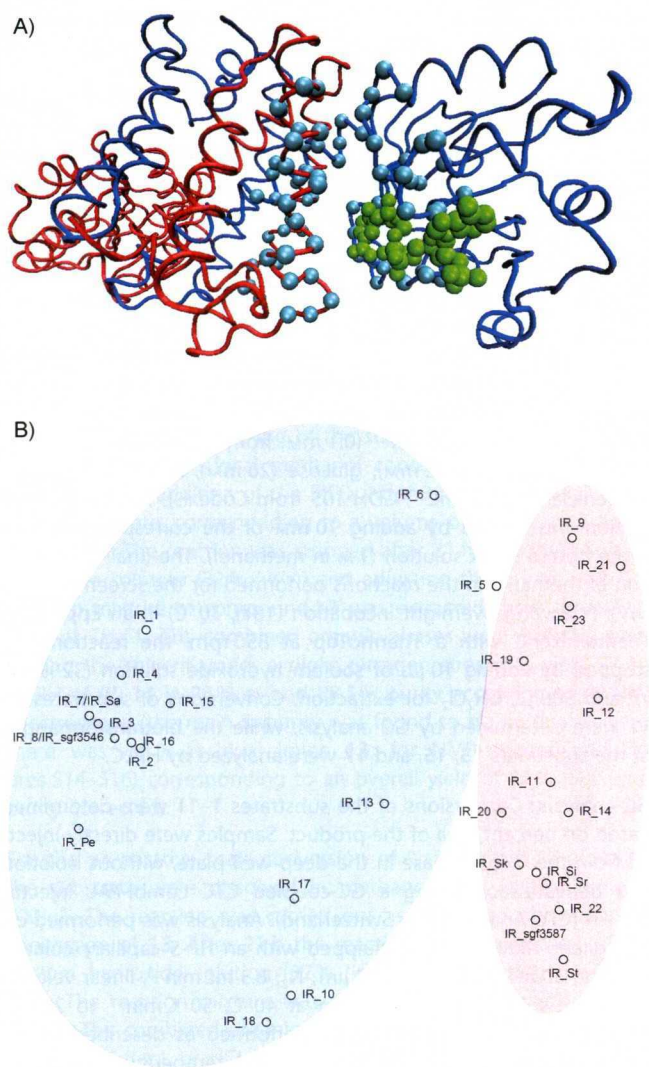


Figure 1. Refined clustering based on the defined active site residues: A) Residues that were taken into account for the refined clustering, highlighted in the crystal structure of *S. kanamyceticus* (3ZHB) as cyan balls.^[7] The enzyme consists of two subunits that are depicted as blue and red ribbons here. The NADP cofactor is depicted as green balls. B) Sequence-distance plot of the refined clustering. Enzymes producing the *S* and *R* enantiomers of the tested substrates are indicated by a blue or red patch, respectively. IR_5 was found to be located at the interface of the clusters.

Future investigations on these amino acid positions by mutational analysis might shed light on the residues responsible for the selectivity of these enzymes. Yet, even with this refined clustering, phenomena such as the altered hydride-transfer (as observed for the conversion 15 and 17 with IR_14, IR_20, and IR_22, which lead to the formation of the opposite enantiomer) are difficult to explain.

While this manuscript was in preparation, Turner and co-workers published the further characterization of the IRED from *Streptomyces* sp. GF3587.^[8a] In this work, the authors identified some residues being conserved among the two IREDs from *Streptomyces* sp. GF3587 and *S. kanamyceticus*.^[8a] These residues, including the positions Leu137, Asp187, Trp232, and Thr258 (with Asp187 formerly having been assigned to be the catalytically active residue) are also present in our sequences,

corroborating our assumption on the function of the active-site residues chosen for our refined clustering. Shortly the publication by Turner and co-workers, Grogan and co-workers solved the crystal structures of two new IREDs. They emphasized that the differences between IREDs displaying *R* and *S* selectivity towards substrates like **1** are very subtle. They hypothesized that the stereoselectivity of the IREDs might be governed by a more complex mode of action possibly involving motion of the different domains, as has been observed upon binding of the cofactor, NADPH.^[13]

Preparative-scale experiments

To demonstrate the suitability of our biocatalysts for large-scale chemical synthesis, biotransformations on a preparative 100 mg scale were conducted. We first investigated how to increase the potential substrate loading and solvent concentrations to better meet the requirements of preparative applications. For this, IR_1, IR_10, IR_20, and IR_23 were chosen as model enzymes owing to their good activities and selectivities. We found that methanol concentrations up to 5% did not have a negative effect on the overall conversions of the biotransformations for these IREDs (data not shown). This is in good accordance with what was reported by Mitsukura et al., who demonstrated that methanol was well tolerated by IRED *S. sp.* GF3546.^[6a] Additionally, we showed for substrates **3** and **13** that even at substrate concentrations of 20 mM the reaction was almost complete within 3 h, with conversions exceeding 90% (Table S8). This prompted us to follow a feeding strategy for these biotransformations, avoiding possible substrate inhibition that was reported for some substrates and had also been observed in our lab (data not shown).^[7,8b,11] Preliminary experiments showed that feeding a reaction that contained 20 mM **3** with another equivalent of substrate equal to 20 mM after 3 h still led to full conversion. Thus, we tested substrates **3** and **13** at a synthetically relevant substrate concentration of 1% w/v on 100 mg scale (see the Experimental Section). Starting with 0.4% w/v of the substrate (40 mg) and feeding another 0.6% w/v (60 mg) after 3.5 h, the biotransformation of **3** using IR_20 as well as the biotransformation of **13** using IR_23 led to >99% conversion. With this outcome these IREDs have taken another step on their way to being synthetically useful enzymatic tools for the asymmetric synthesis of amines.

Conclusion

We created a library of 20 novel IREDs, significantly enlarging the number of characterized IREDs. These enzymes were screened against a set of nine model substrates, representing different classes of imines, comprising aliphatic (monocyclic as well as bicyclic) and aryl substrates. Most of the enzymes showed a broad substrate scope and yielded the corresponding products at good to excellent conversions with high enantioselectivity. By choosing the appropriate enzyme candidates and applying them in biotransformations under preparative conditions, we demonstrated that the library can serve as a starting point for potential synthetic applications. Additionally,

we refined our initial C-terminal domain clustering of the IREDs. Therefore the number of residues taken into account was significantly reduced to those lining the active-site cleft. These residues include positions that are possibly involved in the stereochemical control of the reaction. By using this method, the enzymes described in this study and those previously known from the literature, could be clustered to better fit the experimental results for their described stereopreference. Additionally a consensus sequence was extracted for enzymes forming the corresponding *R* and *S* enantiomers of the tested substrates. Further investigations on these positions might help to unravel the catalytic mode of action for IREDs in general.

Experimental Section

General remarks: Chemicals were purchased from Sigma–Aldrich at the highest purity grade available. The cofactor NADPH was sourced from Roche Diagnostics. Protein purification steps were carried out at 4 °C and, if not stated otherwise, all steps involving protein samples were carried out on ice. All biotransformations were carried out in quadruplicate. The stereochemistry of the products was assigned by using reference substances. In the case of substrates **15** and **17**, no reference substances were available and the absolute configuration was assigned by Mosher's amide NMR analysis, as described in the Supporting Information.

Gene synthesis: The genes of the novel IREDs characterized in this work were synthesized by GenScript (GenScript Inc., USA) with codons optimized for expression in *E. coli*. The full sequences of the corresponding IRED genes, as well as their protein sequences and identifiers can be found in the Table S3. All genes were purchased as plasmids with the genes and an additional N-terminal His₆-tag having been subcloned in a pET22b(+) vector. The resulting plasmids pRDW_IR1–23 were used directly without any further purification or processing to transform *E. coli* BL21(DE3), which gave the final expression strains.

Protein expression and purification: Expression and purification of the His₆-tagged IREDs were carried out in *E. coli* BL21(DE3) cells harboring the corresponding pET22b(+)-based plasmid using ampicillin (100 µg mL⁻¹) as a selection marker. The cells were grown at 37 °C in LB-medium (low salt) until the OD₆₀₀ reached 0.4–0.6 and isopropyl β-thio-galactoside (IPTG) was added to a final concentration of 0.5 mM. After induction, the cells were incubated at 25 °C overnight and harvested by centrifugation. Cells were resuspended in 2 mL binding buffer (potassium phosphate buffer (50 mM, pH 7), imidazole (20 mM), sodium chloride (300 mM)) per gram of cell wet weight. Disruption of the cells was performed by sonication (Branson sonifier W250) with 3 × 60 s bursts and 60 s intervals with both steps being carried out on ice. The resulting solution was clarified by centrifugation (JA 18 rotor at 35000 × *g* for 45 min) and the targeted protein was purified on His GraviTrap TALON columns (GE Healthcare) according to the manufacturer's instructions. The elution buffer contained potassium phosphate (50 mM), sodium chloride (300 mM), imidazole (300 mM) and was adjusted to pH 7. After purification, the enzyme preparation was desalted against potassium phosphate buffer (50 mM, pH 7), using PD-10 desalting columns (GE Healthcare). If necessary, the enzyme preparation was concentrated using a VivaSpin concentrator columns (10 kDa molecular weight cut-off; GE Healthcare) until the targeted protein concentration was reached. Protein concentrations were deter-

mined using a Pierce BCA protein kit (Thermo Scientific) according to the manufacturer's instructions.

Screen plate preparation and biotransformations: To determine the substrate range of the IRED candidates (IR_1–IR_23), biotransformations were performed using the purified enzyme with NADPH-recycling. Biotransformations were performed on a 0.5 mL scale in deep-well plates (Eppendorf). Plates for the screen were prepared with 0.3 mg of the respective IRED in potassium phosphate buffer (50 mM), NADP⁺ (0.25 mM), and ddH₂O. The plates were stored at –20 °C until further use. Prior to use, plates were thawed overnight at 4 °C and the biotransformations reaction mixture was completed by adding the residual components. The final biotransformation of the IRED screen contained 0.6 mg mL⁻¹ of the pure IRED in potassium phosphate buffer (100 mM, pH 7) containing NADPH (0.5 mM), NADP⁺ (0.1 mM, from the plate preparation), magnesium chloride (2 mM), glucose (20 mM), and glucose dehydrogenase (0.26 mg mL⁻¹, GDH-105 from Codexis). The biotransformation was started by adding 10 mM of the corresponding substrate from a stock solution (1 M in methanol). The final concentration of methanol in the reactions performed for the screen was 1 % (v/v). Following overnight incubation (18 h, 30 °C) in an Eppendorf Themomixer C with a ThermoTop at 850 rpm, the reaction was stopped by adding 10 µL of sodium hydroxide solution (32 % (w/v)) and 300 µL CH₂Cl₂ for extraction. Conversions of substrates **1–11** were determined by GC analysis, while the biotransformations of the substrates **13**, **15**, and **17** were analyzed by HPLC.

GC-analysis: Conversions of the substrates **1–11** were determined based on percent area of the product. Samples were directly injected from the CH₂Cl₂ phase in the deep well plate, without isolation and derivatization, using a GC-coupled CTC combi-PAL injector system (CTC Analytics AG, Switzerland). Analysis was performed on an Agilent 7890B system equipped with an HP-5 capillary column (Agilent, 30 m × 0.25 mm × 0.25 µm; N₂; 8.3 mL min⁻¹, linear velocity 30 cm s⁻¹; split mode 1:10; 2 min at 40 °C, 50 °C min⁻¹ to 200 °C, hold 1 min). Chiral analyses were performed as described in the Supporting Information (for columns and temperature programs see Table S6).

HPLC-analysis: Biotransformations of the imine compounds **13**, **15**, and **17** were analyzed using a combined approach for the determination of conversions as well as chiral analysis. For sample preparation, the reaction mixtures were extracted as described above and 100 µL of the CH₂Cl₂ phase were transferred to an HPLC vial with insert. After evaporating the sample to dryness, the sample volume was filled up to 100 µL with ethanol and subjected to analysis. The analysis was performed on Agilent 1100 and 1290 HPLC systems equipped with either a UV or a DAD detector by normal phase chiral HPLC using varying isocratic mixtures of running solvents (*n*-heptane/ethanol, containing 0.1 % diethylamine as additive) with the following columns: Daicel CHIRALPAK IE 150 × 4.6 mm, 5 µm for the analysis of the biotransformations of compounds **13** and **17** and Daicel CHIRALPAK IF 150 × 4.6 mm, 5 µm for **15**. The flow rates were set to 1.5 mL min⁻¹ and 1.75 mL min⁻¹, respectively. For detailed information about the chiral HPLC methods (wavelengths and solvent systems), see Table S7.

Substrate-feeding experiments: Feeding experiments were carried out by using a modified biotransformation method. Therefore varying substrate concentrations were added to the reaction stepwise to a total volume of 25 µL of a methanolic substrate solution, corresponding to a final methanol concentration of 5 % (v/v) in every reaction. The corresponding substrate solutions were prepared from 2 M substrate stock solutions in methanol. Glucose con-

centrations were adjusted accordingly to have an excess of glucose compared to the imine substrate in the reaction.

Preparative scale biotransformations: The preparative biotransformations on a 100 mg scale were performed in a 10 mL reaction volume at 30 °C using the above-mentioned feeding approach for the IREDs.

For the conversion of **13**, 104 mg (716 μmol) of the substrate was dissolved in methanol to a total volume of 500 μL . The final reaction mixture contained purified IRED (0.6 mg mL^{-1}), NADPH (0.5 mM), potassium phosphate buffer (100 mM, pH 7), glucose dehydrogenase (0.26 mg mL^{-1} , GDH-105 from Codexis), magnesium chloride (2 mM), and glucose (120 mM). After adding all reaction components besides the IRED, 200 μL of the substrate solution, which corresponds to 42 mg of the substrate, were added in portions to avoid high local methanol concentrations. The reaction was started by addition of the IRED. After 3.5 h the residual 62 mg of the substrate, corresponding to a volume of 300 μL , was added in portions. The reaction was stopped after 21 h by adding sodium hydroxide solution (32% (w/v)) and adjusting the pH to > 12. The reaction mixture of compound **13** was extracted three times with 4 mL of CH_2Cl_2 . The combined organic phases were dried by evaporating the solvent under a slight nitrogen stream to yield 97 mg (92%) of (*R*)-**14** in 98% ee and 88.5% purity according to ^1H NMR spectroscopy (the main impurity was found to be residual solvent; there was < 1% residual imine **13**; for NMR spectra see Figures S14–S16) corresponding to an overall yield of 82% (588 μmol purity corrected).

For the preparative scale conversion of **3**, 100 mg (1.03 mmol) of the substrate were dissolved in methanol to a total volume of 500 μL . The reaction was carried out as described above for the conversion of **13**. After 21 h the reaction was stopped by adding sodium hydroxide solution (32% (w/v)) and adjusting the pH to > 12. The reaction mixture was extracted six times with 4 mL of CH_2Cl_2 . The combined organic phases were dried over magnesium sulfate and 5 mL of HCl (2 M) in diethylether was added. The solvent was evaporated under a slight nitrogen stream to yield 99 mg (728 μmol ; 71%) of (*R*)-**4** as the corresponding HCl salt in 98% ee and > 95% purity according to ^1H NMR spectroscopy (the main impurity was found to be residual solvent; < 1% residual imine **3**; for the chiral analysis and NMR spectra see Figures S17 and S18).

Substrates: The substrates **1**, **3**, **9**, and **13** were commercially available and sourced from the Sigma–Aldrich, Enamine (Kiev, Ukraine), and ChemStep (Martillac, France) at the highest purity grade available. The other substrates that were not commercially available have been synthesized as described in the Supporting Information.

Position-specific sequence alignment and enzyme clustering: The C-terminal domain was defined based on the crystal structure from *S. kanamyceticus* (IR_Sk) in complex with the nicotinamide co-factor (NADPH) and solved at a resolution of 2.73 Å (PDB ID: 3ZHB).

A profile Hidden Markov model (HMM) of the C-terminal domain, which in IR_Sk starts from His180, was built using the program HMMER v.3.0 (<http://hmmer.janelia.org>) from the sequence alignment of a set of six proteins known in the literature (comprising WP_006374254, WPV010497949, BAM99301, BAM99302, YP_003336672, and 3ZGY_A); this alignment was obtained using the program MUSCLE.^[14] The 702 proteins from the RefSeq non-redundant bacterial protein database that matched the C-terminal HMM profile were grouped based on sequence similarity into 85 clusters,

by using the program BlastClust (<ftp://ftp.ncbi.nih.gov/blast/documents/blastclust.html>).^[15]

For the refined, active-site-based clustering, the residues from both domains lining the interdomain active-site cleft were extracted from the x-ray structure of IR_Sk by visual inspection. Positions of the novel IREDs from this study as well as the IREDs from the literature were extracted from the aligned sequences. Pairwise sequence distances were calculated by using the PROTDIST program from the PHYLIP package (Phylogeny Inference Package (version 3.2). Cladistics 5: 164–166) and visualized by using the R-function, cmdscale.^[16]

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