Electronic Supporting Information for

A Genetic Selection System for Evolving Enantioselectivity of Enzymes

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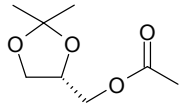
Synthesis of the compounds

Commercial compounds were purchased as indicated in parentheses and used as obtained: (*R*)-isopropylidene glycerol, (*S*)-isopropylidene glycerol, *rac*-isopropylidene glycerol, acetic anhydride, 4-(N,N-dimethylamino) pyridine, N,N-dimethyl formamide (all Fluka), sodium fluoroacetate (Merck) and triethylamine (Acros). All other chemicals were purchased from Aldrich.

All reactions were performed using dry solvents and magnetic stirring. Inert atmosphere was created by heating the glassware under vacuum (< $5*10^{-2}$ mbar) and three rounds of evacuation /Ar-flush. Flash column chromatography was performed using Merck silica gel 60 (230-400 mesh) and glass columns of appropriate size (resulting fill level: 50-100%). A Büchi GKR-51 apparatus was used for Kugelrohr-distillation. ^{1}H and ^{13}C (H-decoupled) nuclear magnetic resonance spectra (NMR) were recorded on a Bruker AV400. All chemical shifts (δ) were quoted in parts per million (ppm) and are reported relative to an internal standard (tetramethylsilane, δ = 0.0). The following abbreviations for multiplicities were used: s, singlet; d, doublet; m, multiplet.

Mass spectra (MS) were measured on a Finnigan MAT 8200 (Electron Ionization, 70 eV). Gas chromatography (GC) was performed on a Hewlett-Packard HP6890 with a flame ionization detector (FID). Compounds and absolute configuration of enantiomers were determined by comparison with standards.

(*S*)-Isopropylidene glycerol acetate [(*S*)-**1**]



5.20 g (*R*)-isopropylidene glycerol (IPG; 39.5 mmol) was dissolved in 75 ml dry dichloromethane. Then 8.00 g triethylamine (79.0 mmol), 8.06 g acetic anhydride (79.0 mmol) and 0.37 g 4-(N,N-dimethylamino) pyridine (DMAP) were added and the reaction stirred for 18 h at room temperature. The solvent was removed and the product isolated by flash column chromatography (200 g silica gel; hexane: ethyl acetate = 20:1; R_f = 0.13). For further purification the product was distilled (Kugelrohr; 23 mbar; 125 °C).

Yield: 5.29 g of a colorless liquid (76.8%).

^{1}H-NMR ($CDCl_3$) δ = 1.37 (s, 3H, $\underline{CH_3}$-C), 1.44 (s, 3H, $\underline{CH_3}$-C), 2.05 (s, 3H, $\underline{CH_3}$-COO), 3.73 (m, 1H, O-$\underline{CH}$(H) –CH-O), 4.05-4.20 (m, 3H), 4.33 (m, 1H, $\underline{CH}$-CH_2-OOC).

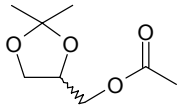
^{13}C-NMR ($CDCl_3$) δ = 21.0, 25.6, 26.5, 63.6, 66.6, 74.1, 110.0, 171.0.

MS (EI): m/z (rel%) = 43(85), 57(8), 72(15), 101(40), 159(100), 173 (<1).

GC: enantiopure

Program:	Instrument:	Hewlett Packard 6890
	Column:	25 m Ivadex 1/OV 1701 G/294
	Gas:	1.0 bar H_2
	Temperature program:	10 min iso. 100 °C.
	Inlet/ injector temp.:	250 °C / 320 °C
	Retention time:	4.19 min [(*S*)-IPG-Ac]

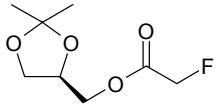
rac-Isopropylidene glycerol acetate (*rac*-**1**)
This compound was synthesized exactly analogous to (*S*)-isopropylidene glycerol acetate (see above).



Starting material: 5.00 g *rac*-isopropylidene glycerol (37.8 mmol), 7.70 g acetic anhydride (75.7 mmol), 7.70 g triethylamine (75.7 mmol) and 0.37 g DMAP.
Yield: 4.55 g of a colorless liquid (69.0%).

NMR&MS: see (*S*)-isopropylidene glycerol acetate

(*R*)-Isopropylidene glycerol fluoroacetate [(*R*)-**4**]



2.84 g sodium fluoroacetate (28.4 mmol) and 2 drops of N,N-dimethyl formamide were put in 60 ml dry tetrahydrofurane (THF). The heterogeneous mixture was cooled to 0 °C and 2.88 g oxalyl chloride (22.7 mmol) was added giving significant evolution of gas. After 30 min, the ice bath was removed and the mixture stirred for another 2 h. Then 1.80 g pyridine (22.7 mmol) and 1.5 g (*S*)-isopropylidene glycerol (11.3 mmol) were added and the reaction stirred at room temperature for 48 h. The precipitate was filtered off and washed with DCM and THF. After that, the solvent was removed. The product was isolated by flash column chromatography (90 g silica gel; hexane: EtAc = 10:1; $R_f = 0.11$). For further purification, the product (1.08 g) was distilled (Kugelrohr; 20 mbar; 155 °C).
Yield: 0.44 g of a colorless liquid (20.1%).

^{1}H-NMR ($CDCl_3$) δ = 1.37 (s, 3H, $\underline{CH_3}$-C), 1.44 (s, 3H, $\underline{CH_3}$-C), 3.75 (m, 1H, O-$\underline{CH}$(H) – CH-O), 4.05-4.40 (m, 4H), 4.90 (d, 2H, F-$\underline{CH_2}$-COO).
^{13}C-NMR ($CDCl_3$) δ = 25.3, 26.6, 65.5, 66.1, 73.3, 78.3, 110.1, 167.7.
MS (EI): m/z (rel%) = 43(100), 61(20), 72(12), 101(17), 177(60), 193 (<1).

Creation of a pGAPZα-CALB-expression vector and transformation in *Pichia pastoris*

Genomic DNA from *Candida antarctica* ATCC 32657 was isolated using AquaPure DNA Isolation Kit (BioRad, Germany). Approximately, 50 ng of genomic DNA was used to amplify CALB wild-type gene by a PCR reaction using primers containing restriction enzymes-sites, *Xho*I-calb-F (TCT$\underline{CTCGAG}$AAAAGACTACCTTCCGGTTCGGACC) and *Not*I-calb-R ($\underline{GCGGCCGC}$TTAGGGGGTGACGATGCCGGA) (recognition sites are underlined) (Tables 3 and 4). The PCR reaction was analyzed by an agarose gel (0.8%) and the 977 bp amplicon was purified using QIAquick Gel Extraction Kit (QIAGEN). 1.5 µg of the amplified DNA were digested with these restriction enzymes (see Sambrook J, Fritsch EF, Maniatis T, 1989. Molecular Cloning: A Laboratory Manual. Second Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) for 2 h and then purified with QIAquick PCR Purification Kit (QIAGEN). 2 µg of pGAPZα vector (Invitrogen, Germany) were previously digested with the same enzymes (*Not*I and *Xho*I) for 2 hours and the fragment (3.101 kb) was purified from a 0.8% agarose gel (kit). Insert (amplified CALB gene) and vector (pGAPZα) were ligated leading to the construct pGAPZα-CALBwt (Figure 1) (ligation was performed with T4-ligase (New England Biolabs) following the guidelines for

cohesive ends in Sambrook J, Fritsch EF, Maniatis T, 1989. Molecular Cloning: A Laboratory Manual. Second Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). The gene was fused in-frame with the α-factor signal peptide of *Saccharomyces cerevisiae* to allow the secretion of the recombinant protein.

Table 1: Pipetting scheme of the PCR for CAL B-amplification

Volume	Component	Concentration
27 µl	Water	
5 µl	dNTPs (Novagen)	2 mM each
1 µl	Template	50 ng/µl
5 µl	Sense primer (Alfa-f)	2.5 µM
5 µl	Antisense primer (CALBX-r)	2.5 µM
5 µl	Buffer for KOD (Novagen)	10x
2 µl	$MgSO_4$	25 mM
0.5 µl	KOD Polymerase (Hot Start; Novagen)	

Table 2: Temperature program of the PCR for CAL B-amplification

Temperature	Time	
95 °C	3 min	
95 °C 57 °C 72 °C	1 min 1 min 2 min 30 sec	30 cycles
72 °C	10 min	

Pichia pastoris X-33 electrocompetent cells were prepared following the instructions of EasySelect™ Pichia Expression Kit (Invitrogen, Germany). Plasmid pGAPZα-CALBwt (1 µg) was digested with *BsmB*I (linearized) and then used to transform the yeast using a MicroPulser™ Electroporation Apparatus (BioRad, Germany) at 1,500 V, 25 µF, 200 Ω and 0.2 cm cuvettes. Transformants were selected on YPD agar plates (see EasySelect™ manual) containing 100 µg mL^{-1} of Zeocin at 30 °C for 72 hours.

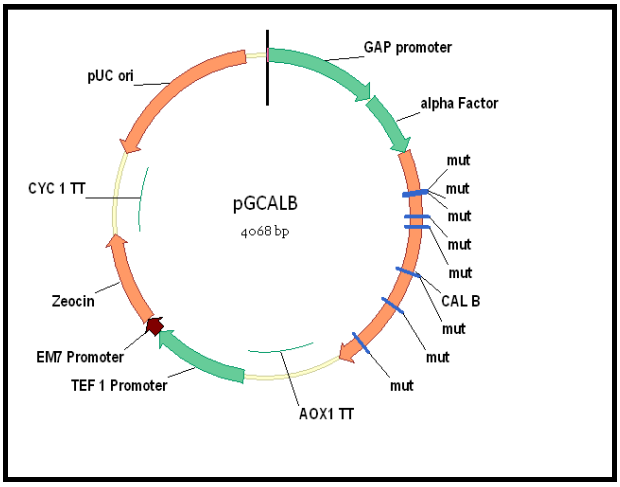


Figure 1: pGAPZα-CALB plasmid. mut: nucleotides changes found in the CALB sequence compare with sequence Accession Number Z30645 in GenBank.

Library creation

Saturation mutagenesis libraries were generated using the QuikChange™ mutagenesis strategy (QuikChange™ mutagenesis kit Instructions manual 2003, Stratagene, USA) by using primers having NNK and NDT degeneracy (N = A, T, C, G; K = T, G; D = A, T, G) for amino acid positions 278 and 281 respectively. The sequence of the primers used were
For- GCCGCGGCTGCGCTCNNKGCGCCGNDTGCTGCAGCCATCGTG
Rev- CACGATGGCTGCAGCAHNCGGCGCMNNGAGCGCAGCCGCGGC
The created libraries were electroporated in *E.coli* XL-1 Blue electrocompetent cells (Stratagene, USA) (prepared following the protocol described in MicroPulser™ Electroporation Apparatus Operating Instructions and Applications Guide, Biorad). Selection was carried out on LB agar plates containing 25 μg ml^{-1} of Zeocin. Then the colonies were pooled and after miniprep using QIAGEN Plasmid Mini Kit (QIAGEN, Germany), the plasmids were linearized with *BsmB*I (approximately 8 μg of DNA). Finally, the DNA was electroporated in *Pichia pastoris* and the transformants selected on YPD-Zeocin plates.

Effects of pH on toxicity of fluoroacetate with acetate as carbon source

Comparison of pH 4, 5 &6

20 ml minimal medium (yeast nitrogen base; pH 6.0) with 0.5% sodium acetate was inoculated with *Pichia pastoris* X33 (untransformed) from the plate and grown overnight (30 °C, 140 rpm) in the shaker. The preculture was used to inoculate several 20 ml cultures (YNB minimal medium; 100 mM citrate-buffer):

0.2% sodium acetate (pH 6.0, citrate buffer)
0.2% sodium acetate + 0.05% sodium fluoroacetate (pH 6.0)
0.2% sodium acetate (pH 5.0, citrate buffer)
0.2% sodium acetate + 0.05% sodium fluoroacetate (pH 5.0)
0.2% sodium acetate (pH 4.0, citrate buffer)
0.2% sodium acetate + 0.05% sodium fluoroacetate (pH 4.0)

The cells of the preculture were harvested by centrifugation (1500 g; 5 min) and washed once with sterile NaCl-solution (0.9%). After resuspension (0.9% NaCl-solution), all cultures were inoculated to an optical density (OD_{600}) of 0.05 and grown in the shaker at 28 °C and 140 rpm.
After one day, all positive controls (pH 4.0, 5.0 and 6.0) and cultures with fluoroacetate at pH 5.0 & 6.0 were already grown significantly. The fluoroacetate-culture at pH 4.0 was incubated for another four days, but no growth was observed.

Optimization of pH concerning growth on acetate and toxicity of fluoroacetate

20 ml minimal medium (YNB; pH 6.0) with 0.5% sodium acetate was inoculated with *Pichia pastoris* X33 from the plate and grown overnight (28 °C, 160 rpm) in the shaker. The preculture was used to inoculate overall twelve 20 ml cultures (YNB minimal medium; 100 mM citrate-buffer):
A test and a positive control for the following pH: 4.0, 4.2, 4.4, 4.6, 4.8 and 5.0.

0.5% sodium acetate (positive control)
0.5% sodium acetate + 0.05% sodium fluoroacetate (test)

The cells were harvested by centrifugation (1500 g; 5 min) and washed once with sterile NaCl-solution (0.9%). After resuspension (0.9% NaCl-solution), all cultures were inoculated to an OD_{600} of 0.05 and grown in the shaker at 28°C and 160 rpm.

Table 3: Growth(OD_{600}) on acetate in presence of fluoroacetate (pH 4.6- 5.0)

Incubation time(days)	pH 5.0		pH 4.8		pH 4.6	
	Pos. Contr.	Test	Pos. Contr.	Test	Pos. Contr.	Test
0.5	0.69	0.58	0.61	0.166	0.41	0.054
3	3.52	3.75	3.34	3.43	3.155	0.285
4					3.16	3.3

Table 4: Growth(OD_{600}) on acetate in presence of fluoroacetate (pH 4.0- 4.4)

Incubation time(days)	pH 4.4		pH 4.2		pH 4.0	
	Pos. Contr.	Test	Pos. Contr.	Test	Pos. Contr.	Test
0.5	0.145	0.060	0.040	0.070	0.045	0.060
3	1.50	0.065	0.041	0.045	0.033	0.040
4	3.5	0.050	0.190	0.0454	0.040	0.042
5	3.25	0.025	2.15	0.043	0.039	0.35
6	3.40	0.036	3.10	0.046	0.606	0.027
11	3.5	0.025	3.2	0.020	3.0	0.019

Plate selection

Selection for enantioselectivity of *Pichia pastoris* X33- CALB (library)

A 20 ml minimal medium (YNB; pH 4.6)-preculture with 0.2% sodium acetate was inoculated with a suspension of *Pichia pastoris*-CALB library-transformants (after Zeocin-selection; see above) and grown 30 h (28 °C, 200 rpm) in the shaker. The experiment was performed on a solid plate with a small Petri dish (plastic) containing 20 ml solid medium (YNB minimal medium; pH 4.6). The agarose was melted in the microwave oven, cooled down to 55 °C, all components added and the resulting medium thoroughly mixed before casting the solid plate.

Composition of the plate:

0.5% Agarose (Eurogentec)
1.34% Yeast nitrogen base (Sigma-Aldrich)
100 mM Citrate-buffer (pH 4.6)
0.3% (*S*)–IPG-acetate (17 mM)
0.003% (*R*)–IPG-fluoroacetate (0.16 mM; stock solution: 0.5% in H_2O)

The cells of the preculture were harvested by centrifugation (1500 g; 5 min) and washed five times with sterile NaCl-solution (0.9%). After resuspension (0.9% NaCl-solution), the library-mixture was diluted with sterile 0.9% NaCl-solution to an optical density at 600 nm of $2x10^{-3}$. 50 µl of the diluted cell-suspension were plated out on one Petri dish and the plate incubated at 30 °C for nine days. Approximately 70-80 colonies could be observed on the plate. The ten biggest colonies of the plate were used to inoculate 2ml YPD–cultures (+50 mg/l chloramphenicol) in 15 ml Greiner tubes, the mutants grown in the shaker for three days (250 rpm; 30 °C), sequenced and the enantioselectivity of the lipase variants analyzed in kinetic resolution experiments (*rac*-IPG acetate; see below).

Sequencing of mutants

200 µl cell suspension of the culture (see above) was transferred to an Eppendorf-tube and the cells centrifuged. The supernatant was discarded. The pellet was resuspended in 300 µl "Cell Lysis Solution" (AquaPure DNA Isolation Kit, BioRad) and 1.5 µl proteinase K (20 mg/ml) added. Then 200 µl acidified seed beads were added, the heterogeneous mixture was vortexed for 3 min and incubated at 55 °C in a thermomixer for 1 h. After cooling down to room temperature, 1.5 µl of RNAse-solution (kit) were added, briefly vortexed and again incubated

for 15 min at 37 °C. The content was cooled down to room temperature and mixed with 100 µl "Protein precipitation solution" (Kit). After that, the mixture was vortexed for 30 sec and centrifuged at 13.000 rpm for 3 min. The supernatant was transferred to a new 1.5 ml Eppendorf-tube. 300 µl isopropanol were added to the solution and mixed by inverting the tube several times. After centrifugation (13.000 rpm; 1 min), the supernatant was removed with a pipette and 300 µl ethanol (70%) were added. Again the tube was inverted several times and centrifuged (13.000 rpm; 1 min). Then the ethanol was discarded and the pellet dried for 30 min. The pellet was resuspended in 50 µl water and incubated overnight at room temperature. The solution was used as template (1:5-dilution) for the PCR-amplification (Table 5). Four PCR-reactions per mutant were performed with different annealing temperature (56-70 °C) (Table 6).

Table 5: Pipetting scheme of the PCR for CAL B-amplification (four reactions)

Volume	Component	Concentration
54 µl	Water	
10 µl	dNTPs (Novagen)	2 mM each
1 µl	Template (1:5)	50 ng/µl
10 µl	Sense primer (Alfa-f)	2.5 µM
10 µl	Antisense primer (CALBX-r)	2.5 µM
10 µl	Buffer for KOD (Novagen)	10x
4 µl	$MgSO_4$	25 mM
1 µl	KOD Polymerase (Hot Start; Novagen)	

Table 6: Temperature program of the PCR for CAL B-amplification

Temperature	Time	
95 °C	3 min	
95 °C 56 °C (gradient) 72 °C	1 min 1 min 2 min 30 sec	30 cycles
72 °C	10 min	

The results of the PCR-reactions were checked by agarose gel electrophoresis (0.8% agarose) for single band purity (length of the fragment: approximately 1300 bp). Successful amplification reactions were dialyzed (Milipore) for 30 min against water and diluted to 20-50 ng/µl DNA (measurement of double-strain DNA at 260 nm). The diluted solutions were sent for sequencing (Medigenomix, Martinsried, Germany) with CALBX-r as sequencing-primer (Table 7).

Table 7: Primers used for PCR-amplification and sequencing of CAL B

Primer	DNA-sequence
Alfa-f	5'-ATAGGATCCATGAGATTTCCTTCAATTTTTACTGCTGTT-3'
CALBX-r	5'-TCTCTCGAGTTAGGGGGTGACGATGCCGGA-3'

Analysis of lipase-enantioselectivity

For the biotransformations, the supernatants of the YPD-cultures were used (see above).
The reactions were performed in 1.5 ml Eppendorf-tubes using the following composition:

100 µl	Expression supernatant
350 µl	Phosphate-buffer (100 mM; pH 7.0)
50 µl	*rac*-IPG acetate (10 mg/ml in acetonitrile)

The reaction mixtures were kept on 30 °C and 800 rpm in the shaker for seven hours. After the reaction, the aqueous mixture was extracted with 300 µl of dichloromethane per tube and the organic layers transferred to 96-microtiterplates for the GC-autosampler. The samples were analyzed by GC.

GC-program:	See (*S*)-isopropylidene glycerol acetate	
Retention times:	2.91 min	(*R*)-IPG [(*R*)-**2**]
	3.05 min	(*S*)-IPG [(*S*)-**2**]
	4.19 min	(*S*)-IPG acetate [(*S*)-**1**]
	4.38 min	(*R*)-IPG acetate [(*R*)-**1**]

Screening of CALB-library for enantioselectivity

Expression of CAL B-mutants in 96-deep well plates

Two 96-deep well plates (2.2 ml storage plate mark II, Thermo scientific) were filled with 800 µl YPD-medium (+ 50 mg/l chloramphenicol) per well and the cultures inoculated from single colonies of *Pichia pastoris* after the transformation of the pGAPZα-CALB library (from Zeocin selection-plates). Each deep well plate was covered with two gas-permeable adhesive seals (Thermo scientific) and incubated in the shaker at 28 °C and 200 rpm for 72 h (Multitron, HT IFORS). The cells were centrifuged down (1500 g; 5 min) and the supernatant used to perform the biotransformation.

Biotransformation and GC-analysis

The biotransformations were performed in 96-well-plates using the following composition:

100 µl	Expression supernatant
350 µl	Phosphate-buffer (100 mM; pH 7.0)
50 µl	*rac*-IPG acetate (10 mg/ml in acetonitrile)

The reaction mixtures were kept on 30 °C and 800 rpm in the shaker for seven hours. After the reaction, the aqueous mixture was extracted with 300 µl of dichloromethane per well and the organic layers transferred to 96-microtiterplates for the GC-autosampler. The samples were analyzed by GC.

GC-program:	See (*S*)-isopropylidene glycerol acetate	
Retention times:	2.91 min	(*R*)-IPG [(*R*)-**2**]
	3.05 min	(*S*)-IPG [(*S*)-**2**]
	4.19 min	(*S*)-IPG acetate [(*S*)-**1**]
	4.38 min	(*R*)-IPG acetate [(*R*)-**1**]

Experiments with selected mutants in liquid culture

The following genetically modified organisms (GMO) were compared:

> *Pichia pastoris* X33 transformed with the mutants E1-D6, E2-G2, E2-A12 and wild type of pGAPZα-CAL B

Per GMO a 20 ml minimal medium (YNB; pH 4.6)-culture with 0.2% sodium acetate was inoculated from the plate and grown 24 h (28 °C, 200 rpm) in the shaker. The preculture was used to inoculate a 20 ml main-culture (YNB minimal medium; pH 4.6) in a 100 ml Erlenmeyer flask. To assure homogeneous conditions, the medium for all cultures was mixed in one pot. (*R*)–IPG-fluoroacetate was then added (stock solution: 0.5% in H_2O) to the test-cultures separately.

Composition of the medium:

1.34% Yeast nitrogen base
100 mM Citrate-buffer (pH 4.6)
0.3% (*S*)–IPG-acetate (17 mM)
0.004% (*R*)–IPG-fluoroacetate (0.21 mM; stock solution: 0.5% in H_2O)

The cells of the preculture were harvested by centrifugation (1500 g; 5 min) and washed five times with sterile NaCl-solution (0.9%). After resuspension (0.9% NaCl-solution), all cultures were inoculated to an OD_{600} of 0.05 and grown in the shaker at 28 °C and 200 rpm. The increase of OD_{600} was measured (Table 8).

Table 8: Growth of cultures with (*S*)–IPG acetate and 0.004% (*R*)-IPG fluoroacetate

Incubation time(hours)	WT	E1-D6	E2-G2	E2-A12
26	0.044	0.071	0.152	0.214
40	0.066	0.145	0.483	1.017
51	0.070	0.218	0.991	1.415
64	0.071	0.299	1.372	1.405

Examples of focused libraries:

(*a*) Y. Koga, K. Kato, H. Nakano and T. Yamane, *J. Mol. Biol.*, 2003, **331**, 585–592;
(*b*) M. S. Warren and S. J. Benkovic, *Protein Eng.*, 1997, **10**, 63–68;
(*c*) G. P. Horsman, A. M. F. Liu, E. Henke, U. T. Bornscheuer and R. J. Kazlauskas, *Chem.-Eur. J.*, 2003, **9**, 1933–1939;
(*d*) J. Yang, Y. Koga, H. Nakano and T. Yamane, *Protein Eng.*, 2002, **15**, 147–152;
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(*g*) C. Nowlan, Y. Li, J. C. Hermann, T. Evans, J. Carpenter, E. Ghanem, B. K. Shoichet and F. M. Raushel, *J. Am. Chem. Soc.*, 2006, **128**,15892–15902;
(*h*) K. Miyazaki and F. H. Arnold, *J. Mol. Evol.*, 1999, **49**, 716–720;
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(*j*) J. Chelliserrykattil and A. D. Ellington, *Nat. Biotechnol.*, 2004, **22**, 1155–1160;
(*k*) E. M. Gabor and D. B. Janssen, *Prot. Eng., Des. Sel.*, 2004, **17**, 571–579;
(*l*) M. Fa, A. Radeghieri, A. A. Henry and F. E. Romesberg, *J. Am. Chem. Soc.*, 2004, **126**, 1748–1754;
(*m*) A. Juillerat, T. Gronemeyer, A. Keppler, S. Gendreizig, H. Pick, H. Vogel and K. Johnsson, *Chem. Biol.*, 2003, **10**, 313–317;
(*n*) N. U. Nair and H. Zhao, *ChemBioChem*, 2008, **9**, 1213–1215;
(*o*) M. T. Reetz, S. Wilensek, D. Zha, K.-E. Jaeger, *Angew. Chem.* 2001, **113**, 3701–3703; *Angew. Chem. Int. Ed.* 2001, **40**, 3589–3591.