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Revealing Additional Stereocomplementary Pairs of Old Yellow Enzymes by Rational Transfer of Engineered Residues

Nathalie Nett,[a] Sabine Duewel,[a] Alexandra Annelis Richter and Sabrina Hoebenreich*

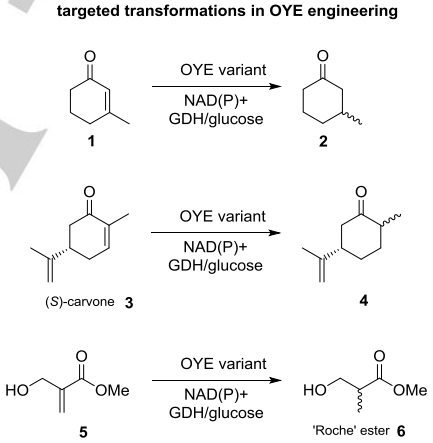
Abstract: Every year numerous protein engineering and directed evolution studies are published, increasing the knowledge which could be used by protein engineers. Here we test a protein engineering strategy that allows quick access to improved biocatalysts with very little screening effort. Conceptually it is assumed that engineered residues, previously identified by rational and random methods, induce similar improvements when transferred to family members. Applied to ene-reductases from the Old Yellow Enzyme family (OYE), the newly created variants were tested with three compounds revealing more stereocomplementary OYE pairs with potent turnover frequencies (up to 660 h^{-1}) and excellent stereoselectivities (up to >99%). Although systematic prediction of absolute enantioselectivity still remains for OYE variants, 'scaffold sampling' was confirmed as a promising addition to the protein engineers' collection of strategies.

Introduction

Implementing enzyme catalysis as a tool into our chemical synthesis toolbox is an important contribution to achieve sustainable synthesis routes for the 21th century.[1,2] Thus, the need to adapt natures given catalyst is high and as a result, protein engineering based on rational design as well as directed evolution is often inevitable.[3]

Finding the best residues at hotspot positions with almost no screening effort, be it *in silico* or experimentally, is currently the big challenge in protein engineering. Recently two groups simultaneously applied a fast protein engineering strategy accessing significantly improved biocatalysts with virtually no screening and creation of low numbers of variants. The strategy assumes that residues obtained earlier by protein engineering, either rational or random, will induce the same improvements of traits when transferred to their hotspot positions in other family members. Dunn *et al.*[4] transferred XNA polymerase activity to three polymerase scaffolds, overcoming the limitation of the originally evolved one. They named the strategy '*scaffold sampling'*. Gober *et al.*[5] quickly identified new olefin cyclopropanation biocatalysts that allow access to all four stereoisomers by transferring engineered residues into twelve P450 scaffolds. The overall success rate to obtain an improved biocatalyst by this protein engineering strategy appears high, as demonstrated by the results of both studies.

Here we examined the strategy with industrial interesting ene-reductases from the Old Yellow Enzyme family (OYE, EC 1.6.99.1). OYEs use flavin and a nicotinamide based hydride source to catalyse the trans-specific hydrogenation of C=C bonds of α,β-unsaturated carbonyl, nitro and cyano compounds. Hence, the family has attracted quite some attention as tool in organic synthesis over the last years.[1,6–8] Because only medium and low throughput screens based on gas or high-pressure liquid chromatography are suitable for most OYE transformations, effective strategies for engineering of family members by avoiding screening-intensive protein engineering or directed evolution are needed. The big challenge is the access of both enantiomers, since stereocomplementary pairs of OYE wild-types (wt) for control of facial selectivity are extremely rare and substrate specific among the OYE family. [9,10,11] Mining the existing OYE protein engineering literature reveals that OYEs have been evolved towards three common compounds (Scheme 1).



Scheme 1. Most common transformations in directed evolution and protein engineering studies of OYE for control of facial selectivity of hydride attack. The goal is to find complementary catalyst pairs allowing access to both enantiomers with excellent stereoselectivities.

The first discovered OYE variant able to switch facial selectivity was W116I in OYE1 from *Saccharomyces pastorianus* for the reduction of (*S*)-carvone ((*S*)-2-methyl-5-(prop-1-en-2-yl) cyclohex-2-en-1-one, **3**) to (2*S*,5*S*)-**4**.[12] Wt and W116F enzymes produce *(2R,5S)*-**4**, as well as all other reported OYE wild-types. Interestingly, W116X variants were identified by an activity based screening for the reduction of 3-methylcyclohex-2-en-1-one (**1**), but no flip in stereoselectivity was observed for **1**.[12] The second reported stereocomplementary pair was C26G and C26D/I69T variants of YqjM from *Bacillus subtilis,* allowing access to both enantiomers for the reduction of **1**.[13] They also found that variants with mutations in A104, homolog to W116 in hotspot position III (Figure 1), contribute to higher (*R*)-selectivity. When the precursor methyl-2-(hydroxymethyl)-acrylate (**5**) of the industrial important 'Roche ester' **6** was introduced as an OYE substrate, all tested wild-types produced (*R*)-**6**.[14] Access to (*S*)-**6** became possible upon discovery of OYE2.6 from *Pichia stipitis*.[15] The same study also showed that single site saturation mutagenesis of OYE1 at hotspot position W116

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likewise produced stereocomplementary variant pairs for **6**. In a follow up directed evolution study, additional complementary pairs with improved conversion levels were discovered for the OYE2.6 scaffold.[16] Lately, a rational design study identified variants C26N/I69A and I69A/H167A of YqjM to induce the same stereochemical flip.[17]

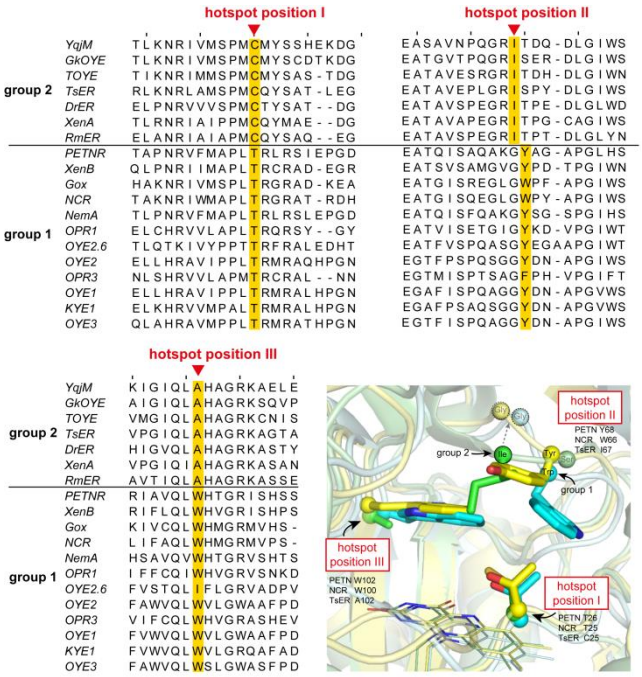


Figure 1. Sequence alignment showing position and residues of hotspots in primary and tertiary structure of wild-type OYEs, which have a low sequence homology across group members (23-34%) and consist of two subgroups, possessing distinct conserved residues at targeted hotspot positions.[6,8] An important aspect is the >98% sequence identity at these empirically determined hotspot positions, excluding the use of consensus based engineering strategies for residue identification.[18] Alignment was created with Clustal Omega[19] and sequences are sorted by pairwise identity. Structures of TsER (3hgj, green)[20], PETNR (3p81, yellow)[21] and NCR (4a3u, cyan)[22] have been aligned using PyMol. Residues at hotspot positions are shown as sticks, FMN as lines. The C_α atoms of residues are shown as spheres. The side chains of hotspot position II fill the same space, but residues have different positions in primary structure.

In addition to aforementioned studies, a few single-residue saturation mutagenesis,[21,23–26] rational design[27] or directed evolution studies[28] exist, which allow extraction of engineered residues. Taken all together, several hotspot positions have been identified and especially positions I, II and III (Figure 1) were found to control facial selectivity, activity and substrate scope in OYE1, OYE2.6, PETNR, KYE and YqjM.[12,13,16,23,26] Testing all combinatorial possibilities arising from empirical identified hotspot positions, their amino acid combinations, the screening substrates and available OYE scaffolds would be too resource intensive and we therefore restricted ourselves to seven scaffolds (Figure 2, green), three groups of variants (Figure 2, red) and the three substrates (**1**-**3**). Engineered residues were selected to represent both OYE groups.[6] Wild-type scaffolds were partially selected based on proven potential as biocatalyst in cascade reactions[29,30], large scale synthesis[9,31,32], use of NAD(P)H mimics[33] or stability.[20,34] With this setup, we were interested in testing OYE family specific response when applying the scaffold sampling strategy. We focused on accessing new stereocomplementary variants with preparative useful turnover numbers and if the new OYE biocatalysts are superior relative to their starting scaffolds and to the original engineered wild-types.

Results and Discussion

Mutability and Recombinant Expression

Mutagenesis of selected wild-type genes turned out to be challenging. High GC content and stable secondary DNA structures were found in the sequence areas of interest (65-70%, Table S3). We started with mutagenesis to introduce the aspartate/threonine combination into the seven scaffold genes. Surprisingly, *nemA and xenA* mutagenesis yielded either the wt gene or the aspartate mutation alone, even after several rounds of PCR optimization (see supporting information). Therefore we did not further pursue the engineering of NemA and XenA.
After introducing aspartate/threonine at hotspot position I+II into TsER, NCR, DrER, RmER and XenB, a first heterologous expression test with wt and mutants were performed. To avoid a later falsification of biotransformation data due to contaminations with *E. colis'* own OYE, we used a knockout strain for enzyme production.[35] The OD_{600} normalized SDS-PAGE analysis (Figure S2) reveals that tested variants were produced. Variant XenB T25D/Y66T did not yield any protein after affinity purification, confirming recent reports[30], XenB studies were not further pursued. Mutagenesis and expression problems can sometimes be solved by changing the plasmid backbone to smaller or newest generation plasmids. This might be especially promising for XenA, XenB (pGaston) and NCR (pET28).
Overall, site directed mutagenesis yielded 14 heterologous expressed variants from group 1 and 2 members, which were further tested for turnover and stereoselectivity.

Turnover number and frequency

All five purified wild-types and 14 variants were obtained as active enzymes, being able to convert 10 mM of **3** containing an electronic activated C=C double bond with turnover numbers (TON) between 60 and 1000. With our catalyst loading of 10 µM (0.1 mol%), the chosen reaction condition maximally lead to a TON of 1000. Reduction of **3** achieved turnover frequencies (TOF) between 40 and 667 molecules per hour, showing that most of our new variants are faster than literature reported variants (TOF, 18 - 300 h^{-1}).[12,23,36,37] With our standardized, non-optimized conditions, our TOF numbers are already close to the best reported TOF for (*S*)-carvone (~1000 h^{-1}) by LacER.[31] As expected, the wild-types of group 2, TsER, RmER, DrER and XenA, had a rather low activity (≤ 1%) for **1**.[13,32,37,38,39] NCR wt, TsER C25D/I67T, C25G/I67T and DrER C40D/I81T reach the best TOF of 82-190 h^{-1} for reduction of **1**. The electronic deactivation of the beta position reduced TOF almost ten-fold. A more significant effect on TOF was observed, when the hydrogen bond acceptor potential of the anchoring group is altered by switching from ketones to esters, as in the case of **5**, where only six (Table 1) of the 19 tested enzymes showed detectable formation of **6**. A reasonable active enzyme catalyst

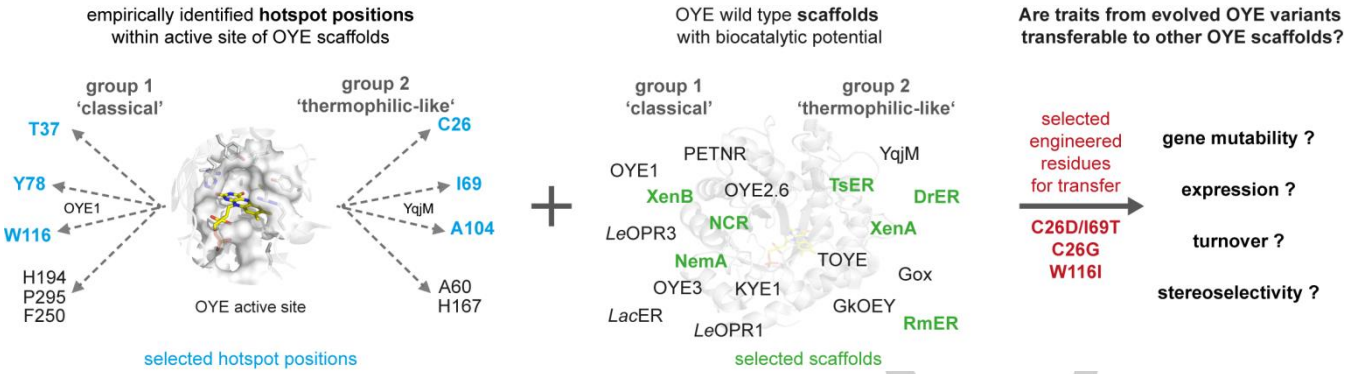


Figure 2. Conceptual overview of protein engineering strategy applied in this study. Choosing engineered residues (red) identified in directed evolution and protein engineering studies in hotspot positions (blue) and transferring them to OYE scaffolds (green).

should have at least a TON of 10-100 and a conversion of >90% for industrial applications.[40] TsER wt and TsER C25D/I67T achieved this with 94 and 98% conversion after 24h, albeit with a moderate TOF of 39 and 41 h^{-1}, respectively. Transfer of engineered residues into group 1 OYE scaffold NCR did increase conversion of **5**, since NCR W100I improved from no detectable to 46% production of **6**. A similar trend was observed for OYE1 W116X variants, but not for OYE2.6 variants, even when mutating at the same three hotspot positions discussed here.[16,41] In group 2, conversion of **5** was significantly reduced for engineered variants of YqjM, where substrate engineering circumvented the problem.[17] More trends are apparent; C26G homologs showed either unaltered or decreased conversion, irrespective of the substrate. Literature also reports the same trend for YqjM and XenA, especially when substrates without C_α activation are used,[13,32] and saturation mutagenesis studies of OYE2.6[41] or PETNR[23] did not yield a homolog glycine variant when selected by conversion. Since we noted during our data mining that double/triple variants with mutations in hotspot position II generally favour higher conversion levels,[13,16,42] a threonine at the respective position was introduced in all C26G homologs and indeed conversion of **1** and **3** for glycine/threonine variants increased compared to the single C26G variants.

Interestingly the C26D/I69T pair did generally increase conversion and TOF in group 2 members (Table 1). The introduction into group 1 members NCR and XenB however resulted in a loss of conversion from 47 to 3% or insolubility, respectively. A similar phenomenon was observed when hotspot position II in OYE2.6 (Y78) was exchanged to smaller residues and abolished carvone conversion.[16] Vice versa, incorporation of W116I homologs into group 2 member TsER was not able to induce increased conversion levels. Mixed reports are apparent in the literature demonstrating that the influence of hotspot III on conversion might be weaker than other hotspots and highly substrate dependent. No activity for **1** was observed with PETNR W102I, but other variants at this hotspot position and other substrates showed increased conversion.[23,43] Incorporation of W116I into circular permutated OYE1 variants lowered conversion of **3**,[25,44] a saturation mutagenesis library at W116 showed no hits for **1**[45] and biocatalytic characterisation of all 20 variants showed that W116I is not the best engineered residue for this position.[15,46] Nevertheless, our NCR data supports the importance of hotspot position III itself. Additionally, data mining also reveals an importance for fine-tuning reactivity, either alone[15,23,41,46] or in context with variations at other hotspot positions.[13,16,36]

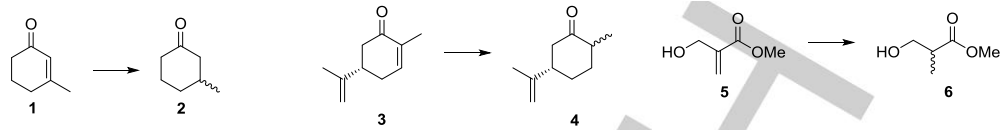
Results for reduction of **1** and **3** reflect that evolution of the active site towards **1** was performed in a group 2 member. Therefore, it was surprising that variant TsER C25D/I67T converts the structurally different **5** equally well as the wt, and the aspartate/threonine variant of DrER showed increased conversions, but only marginally (from 2 to 6%).

Overall RmER wt and variants turned out to be the least active OYE, possibly due to dimer-dimer instabilities.[39] Since, activity improvements are reported to trade off against stability, melting temperatures of RmER C25D/I66T, DrER C40D/I81T were analysed and found to drop between 4 and 10°C compared to the wild-types (see Figure S7). We could further confirm that NCR wild-type and variants have a high biocatalytic potential and shown that TsER variants are equally good as NCR variants when used for biotransformation. Additionally, all shown TsER variants are still obtainable as active, when purified at 70°C for 90 min and ratios of conversion levels obtained with purified (heat treated) or cell lysate (no heat treatment, see supporting information) were maintained.

Control of Facial Selectivity

It appears that group 1 wild-types, with their bulky residues in hotspot position II and III prefer a binding mode of **1** that leads to (*S*)-**2**.[11,47] Reports of facial selectivity for group 2 members are rare. XenA wt favours (*S*)-**2** (>99%)[30] like all other OYE wt and only YqjM wt produces (*R*)-**2** with 76% ee.[13] We expected that TsER wt and DrER wt will likewise produce the (*R*)-enantiomer with their structurally almost identical active sites.[34] To access accurate stereoselectivities for entries with such low conversions (≤ 2%, Table 1), their reactions were performed in large scale (10 mL). Surprisingly, group 2 members DrER wt and TsER wt produced (*S*)-**2** with 93% and 76% ee, respectively, and YqjM wt reproducibly (*R*)-**2** with 78% ee (Figure 3). It would be interestingto understand what determines the re-face orientation of **1** in YqjM wt compared to DrER and TsER. Turning to the fact that all current known OYE wild-types reduced **3** with a facial selectivity yielding exclusively (*2R,5S*)-**4**.[31,48] Our data confirms this trend for TsER, DrER, RmER and XenB, and newly shows

Table 1. OYE wt and variant activities under defined conditions given as turnover numbers (TON) and turnover frequencies (TOF) as product formation rate, using 10 µM OYE and 10 mM substrate concentration.



	conv. [a] (%GC)	TON	TOF[b] (h^{-1})	conv. [a] (%GC)	TON	TOF[c] (h^{-1})	conv. [a] (%GC)	TON	TOF[d] (h^{-1})
wild-types									
NCR	47	465	93	84	840	560	n.c.	n.a.	n.a
XenB	10	100	20	68	680	453	n.c.	n.a.	n.a
RmER	n.c.	n.a.	n.a.	93	930	620	n.c.	n.a.	n.a
DrER	1	10	2	99	990	660	2	20	1
TsER	1	10	2	100	1000	667	94	940	39
C26G homologs									
NCR T25G	9	93	19	43	430	287	n.c.	n.a.	n.a
RmER C25G	n.c.	n.a.	n.a.	16	160	107	n.c.	n.a.	n.a
DrER C40G	n.c.	n.a.	n.a.	28	280	187	n.c.	n.a.	n.a
TsER C25G	7	70	14	97	970	647	n.c.	n.a.	n.a
C26G/I69T homologs									
NCR T25G/W66T	1	14	3	6	60	40	n.c.	n.a.	n.a
RmER C25G/I66T	2	20	4	19	190	127	n.c.	n.a.	n.a
DrER C40G/I81T	5	50	10	85	850	567	n.c.	n.a.	n.a
TsER C25G/I67T	41	410	82	99	990	660	11	110	5
C26D/I69T homologs									
NCR T25D/W66T	3	34	7	69	690	460	n.c.	n.a.	n.a
RmER C25D/I66T	7	70	14	96	960	640	n.c.	n.a.	n.a
DrER C40D/I81T	73	730	146	99	990	660	6	60	3
TsER C25D/I67T	95	950	190	100	1000	667	98	980	41
W116I homologs									
NCR W100I	1	6	1	31	310	207	46	460	19
TsER A102I	<1	4	1	81	810	540	n.c.	n.a.	n.a

(a) mean from triplicates, standard deviation ±5%; (b) 5h; (c) 1.5h; (d) 24 h; n.c. no conversion under assay conditions, n.a. not applicable

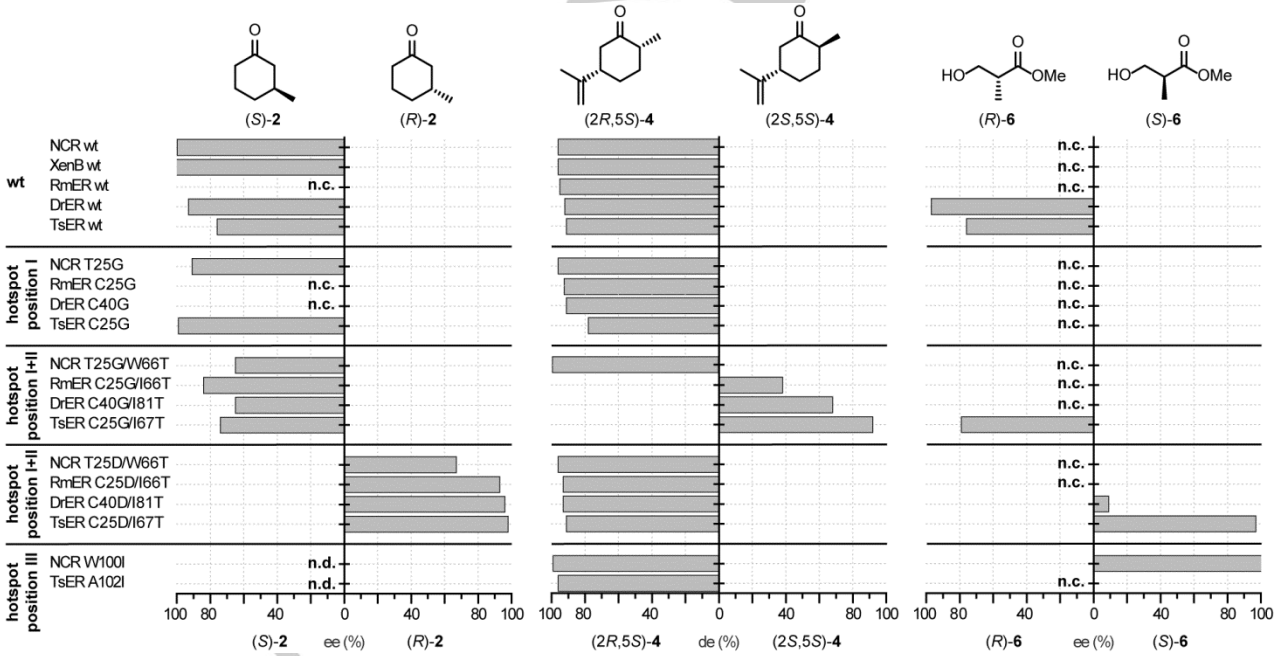


Figure 3. Overview of stereoselectivity of purified OYE variants producing **2**, **4** and **6**. The selectivity on the left is the prevailing selectivity accessible with wt OYEs. A selectivity switch for all three substrates is observed with the created variants in hotspot positions I+II. Assays were performed aerobically, in 200 µl containing 10 µM OYE, 10 mM substrate, a $NADP^+$, glucose, GDH based cofactor recycling system for 5 h (**1**), 1.5 h (**3**) or 24 h (**5**) in 100 mM KPi, pH = 7.4. Enantiomeric excess was determined by GC. Error bars omitted for clarity, error from n=3 given in supporting information. n.c. no conversion, n.d. not determined.

that NCR wt is no exception. Additionally, our data newly showed 76% or 97% ee for (*R*)-**6** for TsER and DrER wt, respectively, and confirms the trend that all current known OYE wt scaffolds exclusively yield the (*R*)-enantiomer of **6**,[14] where the only known exception is OYE2.6 wt.[15]

We found that control of facial selectivity previously observed for the YqjM variant pair C26G and C26D/I69T is transferable to NCR, RmER, DrER and TsER for the reduction of **1**. Glycine variants in hotspot position I give access to (*S*)-**2** and the aspartate/threonine combination in hotspot position I+II allows

access to (*R*)-**2**, irrespective of the scaffold or the selectivity of the wild-type. It appears that for the substrate/residue pair 3-methylcyclohex-2-en-1-one (**1**) and aspartate/threonine in hotspot position I+II the flipped orientation is predominant and the glycine based variants showed the same facial selectivity as the majority of wt. Notably, TsER C25G, DrER C40D/I81T and TsER C25D/I67T showed increased selectivity compared to YqjM variants. Achieved selectivities are excellent, up to 98% (*R*)-**2**, which is one of the best results obtained so far with OYEs. Only engineering of group 2 achieved good TON, TOF and enantioselectivity values for (*R*)-**2** production. Since literature reported engineered group 1 members all favour (*S*)-**2**.[12,23,36], our NCR T25D/W66T variant is the first (*R*)-**2** selective group 1 example, but with rather low activity. An exception might be XenA C25G, where a report by Yanto *et al.*[32] indicates >99% (*R*)-**2** with a TOF of 53 h^{-1}, but stands in sharp contrast to the systematic appearance of (*S*)-**2** preference of hotspot I glycine variants reported here.

Further, the same pattern of stereocomplementarity for the glycine/threonine and aspartate/threonine variants was observed for the reduction of **3**, allowing access to (*2S,5S*)-**4** or (*2R,5S*)-**4** with excellent diastereoselectivity of up to 92 or 96% *de*, respectively. This is the first report of group 2 members producing (*2S,5S*)-**4** and besides OYE1 W116X variants from group 1,[24] the only other set of variants so far for this reaction.[48] All our other new variants led to (*2R,5S*)-**4,** even the hotspot position III homologs NCR W100I and TsER A102I. Lastly, reduction of **5** confirms the generality of the stereocomplementary pair, since TsER C25G/I67T and C25D/I67T gave 79% (*R*)-**6** or 97% (*S*)-**6** *ee*, respectively. The combination of **5** and glycine based variants showed the same facial selectivity as the wt and the aspartate/threonine combination flipped it. This represents the second variant of group 2 that is able to access (*S)*-**6**. Rüthlein *et al.* discovered YqjM variants C26N/I69A and H167A/I69A for production of (*S)*-**6**,[17] albeit with TOFs of 10.3 and ~0.3 h^{-1}, respectively, compared to 41 h^{-1} (TsER C26D/I67T) and 19 h^{-1} (NCR W100I). A combinatorial library of TsER (unpublished data) contained some of the *in silico* designed variants of Rüthlein *et al*., but they showed no conversion of **5** with our reaction conditions (Table S6).

Turning to isoleucine based variants at hotspot position III, we found no flip in facial selectivity for the reduction of **3**, which is contrary to the original report,[12] but consistent with cpOYE1 and OYE2.6 engineering results.[36,44] Nevertheless, NCR W100I showed >99% (*S*)-**6** selectivity, presumably representing a flipped facial selectivity compared to the wt.[14] This supports follow up studies by Stewart and coworkers, where it was shown that hotspot III in group 1 indeed controls stereoselectivity, but highly depending on amino acid substitution in this position and tested substrate.[15,16,46] A combinatorial library of NCR (unpublished data) yielded W100V, a homolog of one of the most promising OYE W116X variants.[48] Interestingly, this NCR W100V variant was not able to switch facial selectivity for reduction of **1** or **3** (Table S5).

Conclusion

Until we are able to understand on molecular level *why* engineered variants of OYE are better biocatalysts and thereby will be able to rationally design them, it should be possible to simply learn *how to use* the current knowledge of engineered residues and hotspot positions for future OYE protein engineering.

Here we discovered that the Asp/Thr and Gly/(Thr or wt) set in hotspot position I+II represent a reliable stereocomplementary pair of OYE variants, best suitable for group 2 members. Further, we showed that Ile incorporation into hotspot position III has more pronounced effect in group 1 member NCR than with group 2. Our results indicate that we need two sets of engineered residues for the two OYE subfamilies. We already have data for group 2 combination of hotspots I+II+III, but a dataset for hotspot position I in context with the other two positions for group 1 members is currently missing. But even with the bigger systematisation of the OYE engineering knowledge that our study provides, models based on empiric data for systematic prediction of *absolute* facial selectivity seem out of reach for OYEs.

Though, it remains to be seen if other recently identified stereocontrolling hotspot positions[45] and engineered residues[16,36] will be equally transferable among OYE scaffolds. We could confirm that scaffold sampling by transfer of engineered residues into wild-types is an extremely valuable protein engineering strategy with high success rates, allowing quick access to improved biocatalysts. It allows us protein engineers to start using the knowledge obtained earlier by protein engineering and evolution systematically, thereby avoiding a waste of resources when creating new and improved biocatalysts.

Experimental Section

Details about mutagenesis PCR, protein expression and biotransformation reactions can be found in the supporting information.

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Keywords: ene reductases • enzyme based stereocontrol • protein engineering • residue transfer • scaffold sampling

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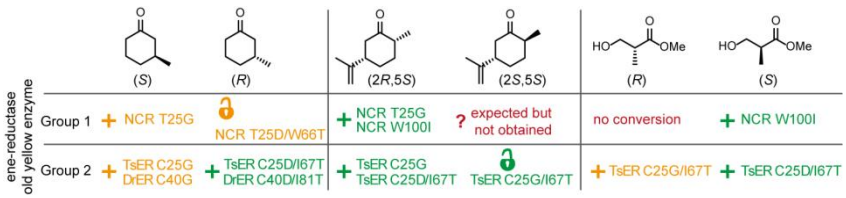

Transfer of engineered residues in ene-reductase wild-type scaffolds revealed additional stereocomplementary pairs and provide a fast engineering method for Old Yellow Enzymes.

*Nathalie Nett, Sabine Duewel, Alexandra Annelis Richter and Sabrina Hoebenreich**

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Revealing Additional Stereocomplementary Pairs of Old Yellow Enzymes by Rational Transfer of Engineered Residues

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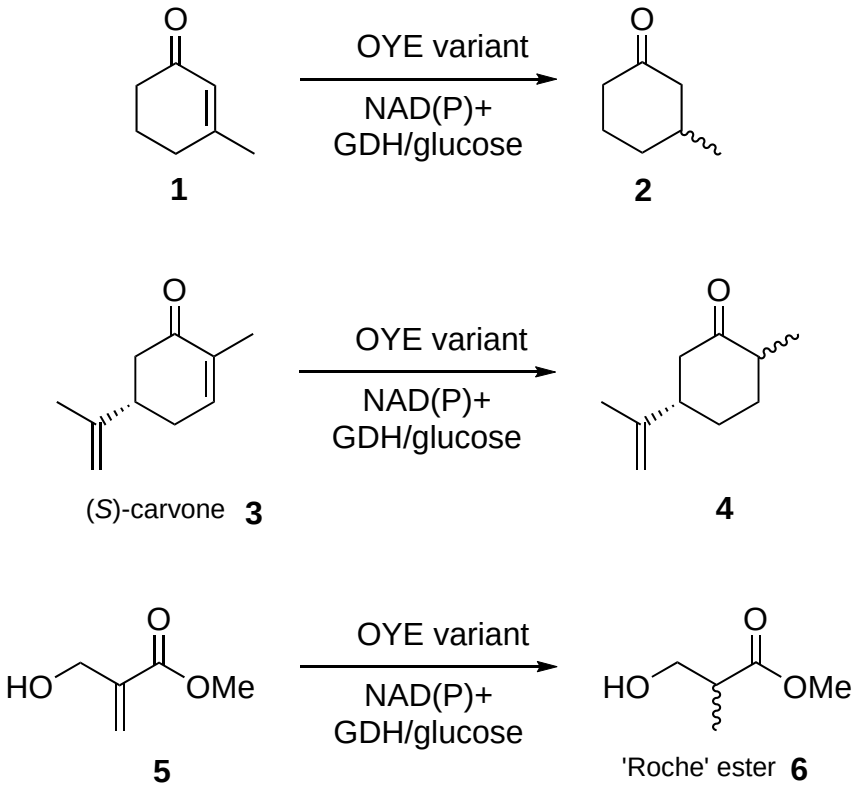
targeted transformations in OYE engineering



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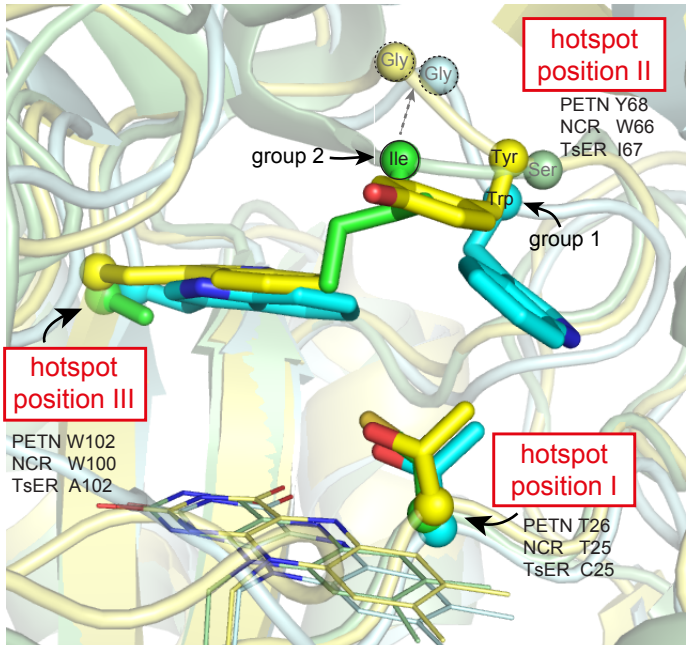
		hotspot position I	hotspot position II
group 2	*YqjM*	TLKNRIVMSPMCMYSSHEKDG	EASAVNPQGRITDQ-DLGIWS
	GkOYE	TLKNRIVMSPMCMYSCDTKDG	EATGVTPQGRISER-DLGIWS
	TOYE	TIKNRIMMSPMCMYSAS-TDG	EATAVESRGRITDH-DLGIWN
	TsER	RLKNRLAMSPMCQYSAT-LEG	EATAVEPLGRISPY-DLGIWS
	DrER	ELPNRVVVSPMCTYSAT--DG	EATAVSPEGRITPE-DLGLWD
	XenA	TLRNRIAIPPMCQYMAE--DG	EATAVAPEGRITPG-CAGIWS
	RmER	ELANRIAIAPMCQYSAQ--EG	EATAVSPEGRITPT-DLGLYN
group 1	*PETNR*	TAPNRVFMAPLTRLRSIEPGD	EATQISAQAKGYAG-APGLHS
	XenB	QLPNRIIMAPLTRCRAD-EGR	EATSVSAMGVGYPD-TPGIWN
	Gox	HAKNRIVMSPLTRGRAD-KEA	EATGISREGLGWPF-APGIWS
	NCR	TAKNRIWMAPLTRGRAT-RDH	EATGISQEGLGWPY-APGIWS
	NemA	TLPNRVFMAPLTRLRSLEPGD	EATQISFQAKGYSG-SPGIHS
	OPR1	ELCHRVVLAPLTRQRSY--GY	EATVISETGIGYKD-VPGIWT
	OYE2.6	TLQTKIVYPPTTRFRALEDHT	EATFVSPQASGYEGAAPGIWT
	OYE2	ELLHRAVIPPLTRMRAQHPGN	EGTFPSPQSGGYDN-APGIWS
	OPR3	NLSHRVVLAPMTRCRAL--NN	EGTMISPTSAGFPH-VPGIFT
	OYE1	ELLHRAVIPPLTRMRALHPGN	EGAFISPQAGGYDN-APGVWS
	KYE1	ELKHRVVMPALTRMRALHPGN	EGAFPSAQSGGYDN-APGVWS
	OYE3	QLAHRAVMPPLTRMRATHPGN	EGTFISPQAGGYDN-APGIWS

		hotspot position III
group 2	*YqjM*	KIGIQLAHAGRKAELE
	GkOYE	AIGIQLAHAGRKSQVP
	TOYE	VMGIQLAHAGRKCNIS
	TsER	VPGIQLAHAGRKAGTA
	DrER	HIGVQLAHAGRKASTY
	XenA	VPGIQIAHAGRKASAN
	RmER	AVTIQLAHAGRKASSE
group 1	*PETNR*	RIAVQLWHTGRISHSS
	XenB	RIFLQLWHVGRISHPS
	Gox	KIVCQLWHMGRMVHS-
	NCR	LIFAQLWHMGRMVPS-
	NemA	HSAVQVWHTGRVSHTS
	OPR1	IFFCQIWHVGRVSNKD
	OYE2.6	FVSTQLIFLGRVADPV
	OYE2	FAWVQLWVLGWAAFPD
	OPR3	VIFCQLWHVGRASHEV
	OYE1	FVWVQLWVLGWAAFPD
	KYE1	FVWVQLWVLGRQAFAD
	OYE3	FAWVQLWSLGWASFPD



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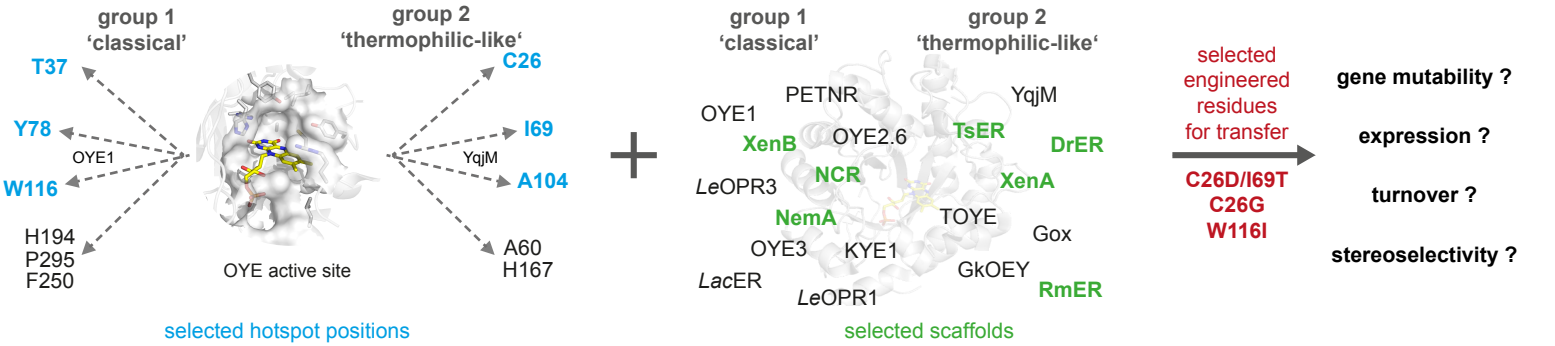

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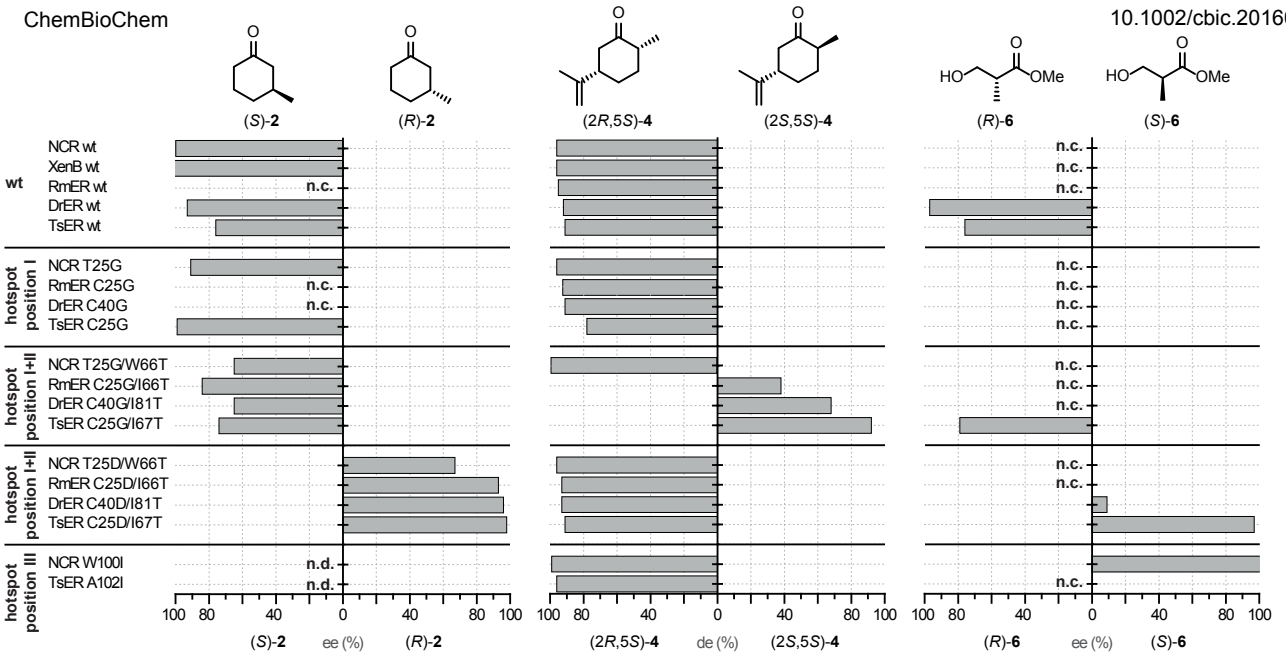

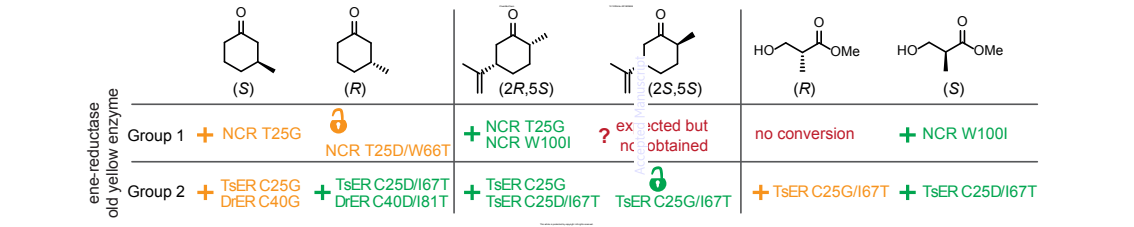
ene-reductase
old yellow enzyme
(S)
(R)
(2R,5S)
(2S,5S)
(R)
(S)
HO
OMe
Group 1
+ NCR T25G
NCR T25D/W66T
+ NCR T25G
NCR W100I
? expected but not obtained
no conversion
+ NCR W100I
Group 2
+ TsER C25G
DrER C40G
+ TsER C25D/I67T
DrER C40D/I81T
+ TsER C25G
TsER C25D/I67T
TsER C25G/I67T
+ TsER C25G/I67T
+ TsER C25D/I67T