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(*S*)-Selective MenD variants from *Escherichia coli* provide access to new functionalized chiral α-hydroxy ketones†

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We report the first rationally designed (*S*)-selective MenD from *E. coli* for the synthesis of functionalized α-hydroxy ketones. By mutation of two amino acids in the active site stereoselectivity of the (*R*)-selective *Ec*MenD (ee > 93%) was inverted giving access to (*S*)-5-hydroxy-4-oxo-5-phenylpentanoate derivatives with stereoselectivities up to 97% ee.

Optically active α-oxyfunctionalized carbonyl compounds are valuable synthetic building blocks in preparative organic chemistry.[1] Among them, α-hydroxy ketones are of particular value for the pharmaceutical as well as the fine chemistry sector.[2] In addition, α-hydroxy ketones are putative precursors of 2-amino alcohols, or 1,2-diols, among others.[3,4] Various non-enzymatic routes to chiral α-hydroxy ketones have been reported including organocatalytic strategies.[5,6] However, high stereoselectivities are rare. Furthermore, most of the syntheses require several steps, which impair the overall yields.[2]

Thiamine diphosphate (ThDP)-dependent enzymes are well known for their catalytic potential to form various α-hydroxy ketones. The mechanism has been studied extensively[7] and several ThDP-dependent lyases, such as pyruvate decarboxylases, branched-chain keto acid decarboxylase, benzoylformate decarboxylase, and benzaldehyde lyase, have been already characterized as powerful catalysts.[8] As most of the wild type (wt) enzymes are (*R*)-selective, access to (*S*)-α-hydroxy ketones is limited. Structural basis for stereoselectivity has been investigated with the benzoylformate decarboxylase from *Pseudomonas putida*, which shows (*S*)-selectivity in the carboligation of benzaldehyde and acetaldehyde.[9,10] The study revealed a structural element called "S-pocket", which allows an antiparallel arrangement of donor and acceptor substrates, the prerequisite for (*S*)-selectivity.[11] S-pockets are present in most of the (*R*)-selective enzymes, however, not accessible to acceptor substrates due to large amino acid side chains.[12,13]

The ThDP-dependent enzyme 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate synthase from *Escherichia coli* (*Ec*MenD) catalyzes the second step in the biosynthesis of menaquinones.[14] The carboligation potential of *Ec*MenD has recently been characterized concerning substrate diversity and stereoselectivity.[15,16] *Ec*MenD uses α-ketoglutarate (**1**) as the physiological donor, which is extraordinary among ThDP-dependent enzymes. This enables selective C4 chain elongation with a terminal carboxyl group. *Ec*MenD accepts a broad range of aldehydes as acceptors. Carboligation of **1** (upon decarboxylation) with different aromatic aldehydes gives α-hydroxy ketones with high enantiomeric excesses (ee) of >93% (*R*).[16] Here, we report the first rationally designed *Ec*MenD variants for the syntheses of functionalized (*S*)-α-hydroxy ketones starting from **1** and differently substituted benzaldehyde derivatives **2**.

We combined rational protein engineering with substrate engineering to get access to functionalized (*S*)-α-hydroxy ketones. Important residues of the S-pocket were identified based on the crystal structure of *Ec*MenD (2JLC).[17] Using docking studies (see ESI†) with benzaldehyde (**2a**), two residues were deduced to be crucial for stereoselectivity: I474 and F475 prevent the antiparallel arrangement of **2a** prior to C–C-bond formation. This explains the high (*R*)-selectivity of 99% ee of the wt enzyme in the carboligation reaction with **1** (Scheme 1A). Hence, in order to gain sufficient space for the phenyl ring of **2a**, both I474 and F475 were mutated to glycine and alanine, respectively.

Four variants were prepared: I474G/F475G, I474G/F475A, I474A/F475G, and I474A/F475A. In all cases, the formation of (*S*)-5-hydroxy-4-oxo-5-phenylpentanoate (**3a**) was observed. Whereas variants I474G/F475G and I474G/F475A showed only low selectivity for the formation of (*S*)-**3a** (ee 34% and ee 27%), the variants I474A/F475A and I474A/F475G revealed higher (*S*)-selectivities of 66% ee and 75% ee, respectively. Thus, the S-pocket could be opened such that **2a** was able to arrange

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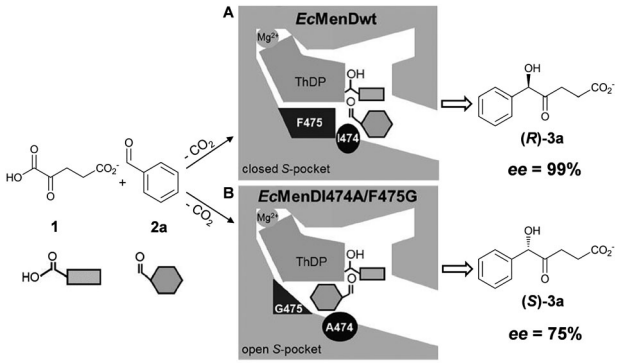
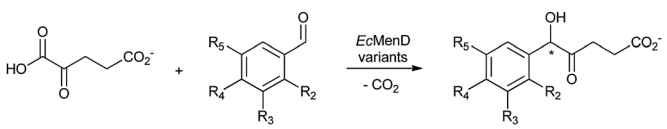


Scheme 1 Inversion of the stereoselectivity of wt *Ec*MenD by structure-based design of the active site.

predominantly in an antiparallel orientation relative to the ThDP-bound donor aldehyde (Scheme 1B). Consequently, the results support the previously developed S-pocket concept.[11,12] Besides accessibility of the S-pocket, optimal stabilization of the acceptor **2a** is crucial for high stereoselectivity. Best stabilization of antiparallely oriented **2a** seemed to be achieved in I474A/F475G. However, the catalytic activity, which may be estimated from the conversions of the *Ec*MenD variants, was lower compared to the wt enzyme (Table 1). Whereas I474G/F475A and I474A/F475A still showed a good conversion of 67% and 68%, respectively, variants I474G/F475G and I474A/F475G revealed only 26% and 15% conversion after 24 h under the tested conditions. Mutation of two adjacent amino acids in the active site might destabilize the binding of the substrate, in which the introduction of glycine at position 475 seemed to have a significant influence on the conversion.

The full catalytic potential of the three best (*S*)-selective *Ec*MenD variants I474G/F475G, I474A/F475G, and I474A/F475A was explored in the next step. Various *ortho*-, *meta*-, and *para*-substituted benzaldehydes were tested beginning with fluoro- and chlorobenzaldehydes (**2b–g**). As expected, stereoselectivity was affected with all substrates compared to the high (*R*)-selectivity of *Ec*MenDwt (ee > 93%, Table 1). *Ec*MenD variants showed the following trends: all tested *ortho*- and *para*-substituted benzaldehyde derivatives resulted in a higher amount of the (*R*)-enantiomer compared to **2a**, while the *meta*-substituted benzaldehydes led to higher (*S*)-selectivity relative to **2a**. Under non-optimized experimental conditions lowest conversions of <24% were observed for *para*-substituted derivatives **2d** and **2g**, which might be a consequence of weak substrate stabilization in the active site concomitant with low conversion rates. Compared to the *para*-substituted benzaldehydes higher conversions of 31–76% were observed with *ortho*-substituted derivatives. As in both cases the (*R*)-enantiomers are predominantly formed, a sub-optimal stabilization of *ortho*- and *para*-substituted benzaldehydes in the S-pockets of the three tested *Ec*MenD variants can be deduced. Remarkable results were obtained with *meta*-substituted benzaldehydes as acceptors: (*S*)-selectivity as well as conversion could be enhanced significantly with all variants compared to benzaldehyde **2a** as the acceptor. Employing **2c** and especially **2f** stereoselectivities of 59–89% ee (*S*) and conversions of 43–94% were obtained. Consequently, the nature of the substrate plays an important role. As *meta*-substituted benzaldehyde derivatives seem to be highly beneficial for (*S*)-selective carboligation using *Ec*MenD variants, **2h–2j** were also tested in the carboligation with **1**. Whereas substrate **2j** was also highly (*S*)-selectively transformed with an ee of 78–93%, the most striking result was obtained with **2h** and **2i**. With these substrates an almost complete inversion of stereoselectivity was possible, connected with high conversions of >86% under the tested conditions. For instance, I474A/F475A catalyzes the formation of **3h** with complete conversion and an ee of 95% (*S*). In order to examine

Table 1 Carboligation of α-ketoglutarate (**1**) and differently substituted benzaldehydes (**2**) to functionalized chiral α-hydroxy ketones (**3**) by *Ec*MenDwt and variants[a]



1 **2a-k** **3a-k**

					wt		I474G/F475G		I474A/F475A		I474A/F475G	
Product	R_2	R_3	R_4	R_5	Conv.[b] (%)	ee[c] (%)	Conv.[b] (%)	ee[c] (%)	Conv.[b] (%)	ee[c] (%)	Conv.[b] (%)	ee[c] (%)
3a	H	H	H	H	>99	>99 (*R*)	26	34 (*S*)	68	66 (*S*)	15	75 (*S*)
3b	F	H	H	H	>99	94 (*R*)	66	≤5	76	20 (*S*)	55	48 (*S*)
3c	H	F	H	H	>99	96 (*R*)	58	59 (*S*)	75	80 (*S*)	43	82 (*S*)
3d	H	H	F	H	>99	99 (*R*)	10	32 (*R*)	24	30 (*S*)	14	46 (*S*)
3e	Cl	H	H	H	98	93 (*R*)	31	70 (*R*)	66	40 (*R*)	57	≤5
3f	H	Cl	H	H	>99	96 (*R*)	75	85 (*S*)	82	88 (*S*)	94	89 (*S*)
3g	H	H	Cl	H	>99	93 (*R*)	21	89 (*R*)	12	70 (*R*)	15	48 (*R*)
3h	H	Br	H	H	99	99 (*R*)	99	94 (*S*)	99	95 (*S*)	87	97 (*S*)
3i	H	I	H	H	>99	94 (*R*)	>99	91 (*S*)	99	93 (*S*)	86	93 (*S*)
3j	H	OCH_3	H	H	99	>99 (*R*)	99	78 (*S*)	89	87 (*S*)	64	93 (*S*)
3k	H	OCH_3	H	OCH_3	99	98 (*R*)	99	85 (*S*)	98	96 (*S*)	83	96 (*S*)

[a] Carboligation reaction was performed in 50 mM potassium phosphate buffer, pH 8.0, (2 mM $MgSO_4$, 0.1 mM ThDP, and 5 vol% MTBE) with 0.70 mg of the respective *Ec*MenD variant at 30 °C and 300 rpm for 24 h using 50 mM α-ketoglutarate (**1**) and 20 mM benzaldehyde derivatives (**2a–k**). [b] Conversion was determined by GC-MS [DB-5MS Agilent] based on benzaldehyde consumption. [c] Determined by chiral HPLC analysis using a Chiralpak IC, and Chiralcel OB and OD-H, respectively. The absolute configuration was determined by circular dichroism.

the full potential of the S-pocket, 3,5-di-methoxybenzaldehyde (**2k**) was finally tested. The results obtained with variants I474A/F475G and I474A/F475A, which catalyze the formation of (*S*)-**3k** with an ee of 96% and conversions of >83%, demonstrate that even this bulky substrate was able to enter the S-pocket properly.

The designed S-pocket of the *Ec*MenD variants seems to be tailored for *meta*-substituted benzaldehydes. High stereoselectivities as well as high conversions suggested good stabilization of the acceptor in the S-pocket. Furthermore, the parallel acceptor orientation, leading to the respective (*R*)-product, might be destabilized, too (Scheme 1). Reaction engineering might further improve stereoselectivities and conversions. To explain these results as well as the selectivities with *ortho*- and *para*-substituted substrates, structural studies with these (*S*)-selective *Ec*MenD variants are currently underway. Together with molecular dynamics simulations of the binding states (parallel and antiparallel orientation of the acceptor), we want to get a deeper insight into *Ec*MenD selectivity and carboligation activity. Furthermore, we want to explore the scope of *Ec*MenD catalysis with other acceptor substrates, *e.g.* aliphatic aldehydes.

In conclusion, we have rationally designed the first MenD variants as powerful biocatalysts for the (*S*)-selective synthesis of functionalized α-hydroxy ketones starting from differently substituted benzaldehydes. Mutation of two amino acids in the active site could invert the stereoselectivity of the wt enzyme for most of the investigated substrates. Particularly the use of *meta*-substituted substrates resulted in high (*S*)-selectivities accompanied by good to excellent conversions. Therewith, we provide access to new, yet not reported (*S*)-5-hydroxy-4-oxo-5-phenylpentanoate derivatives.

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