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Modeling-Assisted Design of Thermostable Benzaldehyde Lyases from *Rhodococcus erythropolis* for Continuous Production of α-Hydroxy Ketones

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Enantiopure α-hydroxy ketones are important building blocks of active pharmaceutical ingredients (APIs), which can be produced by thiamine-diphosphate-dependent lyases, such as benzaldehyde lyase. Here we report the discovery of a novel thermostable benzaldehyde lyase from *Rhodococcus erythropolis* R138 (ReBAL). While the overall sequence identity to the only experimentally confirmed benzaldehyde lyase from *Pseudomonas fluorescens* Biovar I (PfBAL) was only 65%, comparison of a structural model of ReBAL with the crystal structure of PfBAL revealed only four divergent amino acids in the substrate binding cavity. Based on rational design, we generated two ReBAL variants, which were characterized along with the wild-type enzyme in terms of their substrate spectrum, thermostability and biocatalytic performance in the presence of different co-solvents. We found that the new enzyme variants have a significantly higher thermostability (up to 22 °C increase in T<sub>50</sub>) and a different co-solvent-dependent activity. Using the most stable variant immobilized in packed-bed reactors via the SpyCatcher/SpyTag system, (R)-benzoin was synthesized from benzaldehyde over a period of seven days with a stable space-time yield of 9.3 mmol·L<sup>-1</sup>·d<sup>-1</sup>. Our work expands the important class of benzaldehyde lyases and therefore contributes to the development of continuous biocatalytic processes for the production of α-hydroxy ketones and APIs.

**Introduction**

The production of active pharmaceutical ingredients (APIs) and natural compounds with multiple stereocenters is a highly topical field in research and development. Chiral α-hydroxy ketone moieties are key structural units of natural products and APIs of industrial relevance as they can be readily converted, for example, into diols, amino alcohols or esters.[11] The enantioselective synthesis of α-hydroxy ketones can be realized enzymatically by using oxidoreductases, like alcohol dehydrogenases[12] or benzil reductase,[13] or else, via thiamine-diphosphate (ThDP)-dependent lyases, such as pyruvate decarboxylase, benzoylformate decarboxylase or benzaldehyde lyase.[14]

Benzaldehyde lyases (BAL, EC 4.1.2.38) catalyze the stereoselective homo-coupling and cross-coupling of two aldehydes via benzoic ester condensation (Scheme 1) by employment of ThDP and Mg<sup>2+</sup> as cofactors. The BAL from *Pseudomonas fluorescens*[4,5] (PfBAL) is the only experimentally confirmed representative in this enzyme class recorded in the BRENDA[6] and TEED[7] database to date (for more detailed analysis of TEED database see supporting information page 20). It has a broad substrate spectrum[8,9] and can also catalyze C–N bond formations[10] cyclizations[11] and intramolecular Stetter reactions.[12] Moreover, via rational engineering of its binding site, PfBAL was successfully altered to perform benzoylformate decarboxylase[13] or formylase reactions.[14] However, current applications are limited by the relatively low stability of this enzyme. For example, PfBAL loses 40% of its initial activity within 27 h in phosphate reaction buffer at pH 8 and 25 °C.[15]

**Scheme 1.** Benzaldehyde lyase (BAL)-catalyzed formation of (R)-benzoin derivative (2) as homo-coupling product of aromatic aldehydes (1) and formation of (R)-2-hydroxy-3,3-dimethoxy-1-arylpropan-1-one (4) as cross-coupling product of aromatic aldehydes (1) and 2,2-dimethoxyacetaldehyde (3) investigated in this work. Ar: aromatic residue. For distinctive structures (1–p), see Scheme 2.

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This is especially critical for the realization of continuously operated flow reactors. Hence, continuous flow reactors using this enzyme based on retention by membranes,\textsuperscript{16,17}\textsuperscript{19} entrapment in polyvinyl alcohol\textsuperscript{[18]} or adsorption to silica particles\textsuperscript{19} showed limited success and were technically laborious. Likewise, several strategies for the direct, site-selective immobilization of PBAL on solid supports by employment of genetically-encoded tag systems, like the hexahistidine (His\textsubscript{6})-tag,\textsuperscript{19,20} HaloTag\textsuperscript{21} or aggregation-inducing tags,\textsuperscript{22} did not show the desired performance and only the His\textsubscript{6}-tag variant could be used in a fluidic setup.\textsuperscript{19}

Results and Discussion

To overcome the above limitations and to identify BAL enzymes of higher stability that promise improved production of α-hydroxy ketones in a continuous reaction, we used the computational prediction tool Tome, which was recently developed by combining datasets of amino acid composition of individual enzymes with the optimal growth temperature of organism in a machine learning model.\textsuperscript{23} From the resulting set of sequences, we selected three candidate enzymes. A potential BAL from Rhodococcus erythropolis (ReBAL), which was selected based on the highest sequence identity (see Table S1). Although \textit{R. erythropolis} is a mesophilic organism, individual enzymes may exhibit much higher thermostability than the growth temperature of the organism, as previously reported.\textsuperscript{24} Furthermore, one potential BAL each was selected from Sphaerobacter thermophilus (StBAL) and Thermobacillus composti (TcBAL). The enzymes were obtained by heterologous expression in \textit{Escherichia coli} (Figure S1A, sequence data see Table S2) and the raw cell lysates were initially screened for aromatic ligation activity, using a literature-reported photometric assay\textsuperscript{25} based on the conversion of 2-furaldehyde (1\textnumero{}a, Scheme 2) into 2,2'-furin (2\textnumero{}a) (see also Figure S1B).

The enzyme from \textit{R. erythropolis} showed the desired activity with 1\textnumero{}a (Figure S1B), whereas the enzymes from \textit{S. thermophilus} and \textit{T. composti} did not show any significant activity with this substrate. This finding was reasonable since the selection of these enzymes was based on sequence homology alone. In order to better understand the results, we performed a multiple sequence alignment of the three enzymes along with other PBAL-related enzymes described in the literature. The resulting phylogenetic tree (Figure S2) revealed that the inactive StBAL and TcBAL are indeed closer related to acetohydroxyacid synthase (AHAS, E.C. 4.1.3.18) from \textit{Saccharomyces cerevisiae} than to PBAL. This result suggested that the potential catalytic activity of StBAL and TcBAL may include the decarboxylation of pyruvate like AHAS\textsuperscript{26} rather than the desired benzoin condensation. In contrast and in line with the activity observed for ReBAL, this enzyme is more closely related to PBAL on the sequence level. This finding also reflects the capability of both organisms of origin to metabolize aromatic compounds. As such, the organism of origin of PBAL (\textit{P. fluorescens Biovar I}) was specifically enriched in medium with anisoin as the sole carbon and energy source,\textsuperscript{27} and the gene encoding for ReBAL is originally located on the plasmid pLE138 from \textit{R. erythropolis} R138 that harbors several genes that were classified as involved in uptake and metabolism of aromatic compounds.\textsuperscript{27} The two enzymes are of comparable length (ReBAL, 557 amino acids and PBAL, 562 amino acids) and share 283 identical and 83 similar amino acids with only 9 gaps, indicating that 65% of the sequence is conserved between the two variants (Figure S3). Considering this high sequence similarity, we generated a 3D model of the protein structure of ReBAL (Figure 1) based on the crystal structure of PBAL\textsuperscript{28} using SWISS Model.\textsuperscript{29-33} The model suggests the same homotetrameric structure as found for the PBAL\textsuperscript{34} which can be described as a dimer of a dimers with four active sites (Figure 1, orange circles) located at the interface.

Comparison of the model of ReBAL (Figure 2A) with the structure of PBAL (Figure 2B) with respect to the residues considered to mainly constitute the binding site\textsuperscript{19} indicated that these residues were highly conserved. The only exceptions are Q112, E393, S416 and T479 in ReBAL, which are located in

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Proposed molecular structure of the benzaldehyde lyase ReBAL from \textit{Rhodococcus erythropolis}. The model is based on the crystal structure of the tetrameric benzaldehyde lyase PBAL from \textit{Pseudomonas fluorescens} (PDB: 3D7K),\textsuperscript{29} with three twofold axes P, Q and R. The locations of the four active sites are indicated as orange circles (two in the front plane and two in the back).}
\end{figure}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
Species & Enzyme Activity \\
\hline
\textit{Rhodococcus erythropolis} & 1a, 1b, 1c, 1d, 1e, 1f, 1g, 1h, 1i, 1j, 1k, 1l, 1m, 1n, 1o, 1p \\
\textit{Sphaerobacter thermophilus} & 2a, 2b, 2c, 2d, 2e, 2f, 2g, 2h, 2i, 2j, 2k, 2l, 2m, 2n, 2o, 2p \\
\textit{Thermobacillus composti} & 3a, 3b, 3c, 3d, 3e, 3f, 3g, 3h, 3i, 3j, 3k, 3l, 3m, 3n, 3o, 3p \\
\hline
\end{tabular}
\caption{Distinctive aromatic aldehydes (1a–p) investigated in this work.}
\end{table}
Q112A, E393A, S416G and T479A (for the corresponding primer was also validated using CASTp. Pf BAL, but to also generate selectivity of the wild-type and Re BAL, such knowledge is necessary to design effective inhibitors. We therefore decided to not only compare the activity and thermostability of the here investigated enzymes with published data, we directly determined the turnover number (TON) for the conversion of benzaldehyde (1b) into benzoin (2b, Scheme 1) for all four BAL variants using an HPLC assay (Figure S10). For the PIBAL we obtained a TON of 234 s⁻¹ ± 11, which is comparable to previously reported values. For Re BAL we found an about 15 times lower conversion of 1b of only 15 s⁻¹ ± 1. As expected, matching the amino acids in the binding site of Re BAL to the residues found in PIBAL led to a twofold increase of the TON to 36 s⁻¹ ± 1 for the ReBALmat variant, while the larger binding site of ReBALnab showed a slightly higher TON of 45 s⁻¹ ± 1. This finding can be correlated with the replacement of polar residues in the binding site with nonpolar residues, which should lead to the formation of an increased affinity for the nonpolar (aromatic) substrate. This hypothesis is in line with the activity of ReBALnab having the smallest number of polar residues and showing the highest turnover of the ReBAL variants. However, the significantly lower activity of the ReBAL variants is compensated by their thermostability at elevated temperature. For example, the residual activity of ReBAL significantly exceeded that of PIBAL after incubation at 50°C for 1 h (Figure S11).

Figure 2. Structure model of A) wild-type ReBAL and B) crystal structure of PIBAL (PDB: 3D7K) as well as models of C) ReBALmat and D) ReBALnab. Models were generated using SWISS Model. Stick models of all amino acid residues of PIBAL, wild-type, matched and widened ReBAL within a range of 6 Å of the catalytic site containing thiamine diphosphate (ThDP) and benzoylphosphonic acid methyl ester (MBP) are represented as orange sticks. Coordinates of the ThDP-MBP adduct in the modeled structures were superimposed from the crystal structure of PIBAL. For a surface representation of the binding sites, see Figure S4.

Figure 3. A) Histagged PIBAL (60 kDa, 1), ReBAL (61 kDa, 2), ReBALmat (61 kDa, 3) and ReBALnab (61 kDa, lanes 4) were produced as pure enzymes in biological triplicates and analyzed using a Coomassie stained 15% SDS tris-glycine polyacrylamide gel. M: PageRuler Prestained Protein Ladder (Thermo Scientific). B) Average thermal inactivation curves with 95% confidence interval of all four purified BAL enzymes at an identical concentration of 5 μM after 10 min incubation in 50 mM KP, reaction buffer pH 8 containing 2.5 mM MgSO₄ and 0.25 mM ThDP (for individual data sets see Figures S6–S9). Analyses were carried out in technical duplicates and biological triplicates.

2.5 mM MgSO₄ as cofactors for 10 min at defined temperatures in the range of 30–80°C. The above described 2-furaldehyde-based activity assay was then used to determine the remaining enzyme activity at 30°C. T50 values, defined as the temperature at which 50% of the initial enzyme activity still remained, were determined from the plots of the residual activities against the incubation temperatures (Figures S6–S9). The analysis (Figure 3B) showed a significantly increased T50 value for ReBAL (59.7°C ± 0.6) as compared to PIBAL (45.9°C ± 0.4). Interestingly the ReBALmat (66.0°C ± 0.2) and the ReBALnab (68.4°C ± 0.3) variants showed an even higher T50 value. These findings could be explained by the location of the binding site at the dimer interface, where an exchange of amino acids might also affect the interaction between both monomers resulting in an increased overall stability as it has been previously reported for other enzyme classes.

In order to compare the activity of the here investigated enzymes with published data, we directly determined the turnover number (TON) for the conversion of benzaldehyde (1b) into benzoin (2b, Scheme 1) for all four BAL variants using an HPLC assay (Figure S10). For the PIBAL we obtained a TON of 234 s⁻¹ ± 11, which is comparable to previously reported values. For Re BAL we found an about 15 times lower conversion of 1b of only 15 s⁻¹ ± 1. As expected, matching the amino acids in the binding site of Re BAL to the residues found in PIBAL led to a twofold increase of the TON to 36 s⁻¹ ± 1 for the ReBALmat variant, while the larger binding site of ReBALnab showed a slightly higher TON of 45 s⁻¹ ± 1. This finding can be correlated with the replacement of polar residues in the binding site with nonpolar residues, which should lead to an increased affinity for the nonpolar (aromatic) substrate. This hypothesis is in line with the activity of ReBALnab having the smallest number of polar residues and showing the highest turnover of the ReBAL variants. However, the significantly lower activity of the ReBAL variants is compensated by their thermostability at elevated temperature. For example, the residual activity of ReBAL significantly exceeded that of PIBAL after incubation at 50°C for 1 h (Figure S11).
In order to further explore the differences in the molecular structure of the binding site on the enzymatic properties, we characterized the four BAL variants regarding their individual substrate spectrum using 16 aromatic aldehydes (1a–p,Scheme 2) for aromatic homo-coupling and aromatic-aliphatic cross-coupling reactions with 2,2-dimethoxyacetalddehyde (3, Scheme 1). Since many of these substrates are only poorly soluble in aqueous buffers, we used 20% DMSO as co-solvent, which had been previously reported for the PbBAL.\(^{[15]}\) The conversion of substrate and formation of product was determined by thin-layer chromatography (TLC, Figure S12) after 20 h incubation at 30°C (Table 1). Compared to PbBAL, ReBAL showed a similar but less broad substrate spectrum for the homo-coupling reactions with considerably lower formation of 2h, 2i, 2l and 2p. This indicates that ReBAL does not accept substrates with polar, aprotic para-substitutions as well as pyridine compounds. As expected, matching the binding site of ReBAL (ReBAL_{mat}) to the residues found in PbBAL restored an almost identical substrate pattern while losing reactivity against 2d. In contrast, the simple widening of the binding site by the introduction of alanine and glycine residues did not significantly alter the substrate spectrum. When compared to the wild-type ReBAL, the ReBAL_{wid} no longer accepts 2d but gains some reactivity towards 2p.

However, the impact of the mutations was found to be different for the cross-coupling reactions with substrate 3. While ReBAL performed quite poorly in these reactions, only accepting 1h with >10% conversion, the introduction of the mutations into the ReBAL binding site improved the ability to catalyze the cross-coupling. Again, the substrate scope of the ReBAL_{mat} showed a high similarity to PbBAL albeit with an overall lower conversion. Like in the case of the homo-coupling reactions, here the ReBAL_{wid} only showed a slightly altered reactivity compared to the wild-type ReBAL with better acceptance of substrate 1a, 1b, 1j and 1o, although with loosing acceptance of 1h and 1l. These results clearly illustrate that the substrate spectrum of the wild-type ReBAL can be aligned with the PbBAL enzyme by replacing specific amino acids within the binding site. Of note, similar as PbBAL, these ReBAL variants also showed a clear (R)-selectivity for the formation of the homo- and cross-coupling products of 1b. The stereoselectivity of the products was confirmed by determining the enantiomeric excess of 2b (Figure S13) as well as preparation and analysis of Mosher ester derivatives\(^{[39]}\) of the reaction product 4b with Mosher acid chloride (Figures S14–S16) using the known stereoselectivity of PbBAL with 4b as reference.\(^{[36]}\)

In order to expand the range of hydrophobic substrates of the BAL variants, we also wanted to investigate the effects of possible co-solvents. To this end, we analyzed the change in reactivity of the four BAL variants in ten different reaction mixtures (Figure 4A). Since we found only minor differences for the reaction in co-solvent-free buffer at either pH 7 or pH 8, we chose pH 8 as the reference for the assessment of the effect of co-solvent addition, since it was employed also in earlier studies (for direct comparison of the activities of the different enzymes with each other, see Figure 4B).\(^{[15,38,40]}\) We investigated reaction mixtures containing 40% choline-based deep eutectic solvents (DES) because this type of solvents has been reported to enable the conversion of hydrophobic substrates.\(^{[41]}\) We found an overall lower conversion for all four enzymes in the presence of choline: glycerol (G-DES). Addition of 20% tert-butanol, 5% tetrahydrofuran (THF), 5% 2-methyltetrahydrofuran (2-MTHF) or 5% acetonitrile led to similar or a decreased enzymatic activity, and 20% dimethyl sulfoxide (DMSO) led to the already reported increased performance of PbBAL.\(^{[15,36,42,43]}\) Interestingly, ReBAL was not significantly affected by this solvent and the effect of 20% DMSO on enzymatic performance appears to be selective for the architecture of the binding site of PbBAL, as the activity

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**Table 1.** Evaluation of BAL-catalyzed conversion of 1a–p into homo-coupling product 2 and aromatic-aliphatic cross-coupling products 4 with 3 (see Scheme 1) by TLC analysis (see Figure S12) after 20 h.\(^{[a]}\) The numbers indicate %-values of total conversion, determined via grayscale analysis of spot intensities at 245 nm (Figure S11A). In some cases (*), the TLC method used could not clearly confirm product formation. In other cases, production formation could be clearly excluded (n.d., not detectable).

| Entry | 1a | 1b | 1j | 1l | 2 | 3 | 4 | 4b | 4d |
|-------|--|--|--|--|--|--|--|--|--|
| a     | 34 | 57 | 62 | 55 | 87 | n.d. | 5 | 20 |
| b     | 60 | 68 | 66 | 64 | 87 | n.d. | 6 | 20 |
| c     | 41 | 17 | 22 | 16 | 87 | n.d. | n.d. | n.d. | n.d. |
| d     | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| e     | 35 | 17 | 22 | 18 | 94 | n.d. | n.d. | n.d. |
| f     | 59 | 25 | 17 | 10 | 89 | n.d. | n.d. | n.d. |
| g     | 89 | 4 | 71 | 11 | 45 | n.d. | 9 | n.d. |
| h     | n.d. | n.d. | n.d. | n.d. | 9 | n.d. | n.d. | n.d. |
| i     | 3 | 19 | 5 | 33 | n.d. | n.d. | 9 | n.d. |
| j     | n.d. | n.d. | n.d. | n.d. | 9 | n.d. | n.d. | n.d. |
| k     | 3 | n.d. | 3 | n.d. | 36 | n.d. | n.d. | n.d. |
| l     | 99 | 16 | 45 | 11 | 46 | n.d. | 3 | n.d. |
| m     | 56 | 52 | 53 | 38 | 81 | n.d. | n.d. | n.d. |
| n     | n.d. | n.d. | n.d. | n.d. | 9 | n.d. | n.d. | n.d. |
| o     | 75 | 63 | 66 | 70 | 51 | n.d. | 43 | 10 |
| p     | 28 | 9 | 13 | 20 | n.d. | n.d. | n.d. | n.d. |

\([a]\) Reaction conditions: 5–10 mM aromatic aldehyde (1a–p) and 100 mM 3 in 50 mM KP, buffer (0.25 mM ThDP, 2.5 mM MgSO\(_4\), 20% DMSO, pH 8), 2 h at 30°C; BAL final concentration: 5 \(\mu\)M; Reaction volume: 100 \(\mu\)L. [b] Possible overlay of homo-coupling, oxidation or autocatalytic product on the TLC. [c] Indication of product conversion due to appearance of spots upon irradiation at 365 nm (see Figure S12B).
of ReBAL<sub>mut</sub> increased while ReBAL<sub>nsd</sub> remained unaffected. The activity of PBal also increased with the addition of 20% acetone, which had no significant effect on ReBAL but also increased the activity of ReBAL<sub>mut</sub> and ReBAL<sub>nsd</sub>. Surprisingly, the presence of 20% isopropanol also significantly increased the activity of ReBAL<sub>nsd</sub> and ReBAL<sub>mut</sub>. These results suggest a better interaction between the substrate and the less polar binding site architecture of ReBAL<sub>mut</sub> and ReBAL<sub>nsd</sub> in the presence of these solvents. This is consistent with previously reported data in which a modulation of catalytic properties for thiamine-diphosphate-dependent enzymes was found due to a direct interaction of small co-solvent molecules with the nonpolar binding site. In addition, the effects observed here may also be related to an interdependent effect of binding site architecture and surface hydrophobicity that affects enzyme performance (for a detailed discussion, see Figure S17). The finding of an increased performance with isopropanol was very promising for the application of the enzymes in flow reactors since the use of DMSO as a co-solvent can lead to problems in which a modulation of catalytic properties for thiamine-diphosphate-dependent enzymes was found due to a direct interaction of small co-solvent molecules with the nonpolar binding site. In addition, the effects observed here may also be related to an interdependent effect of binding site architecture and surface hydrophobicity that affects enzyme performance (for a detailed discussion, see Figure S17). The finding of an increased performance with isopropanol was very promising for the application of the enzymes in flow reactors since the use of DMSO as a co-solvent can lead to problems in which a modulation of catalytic properties for thiamine-diphosphate-dependent enzymes was found due to a direct interaction of small co-solvent molecules with the nonpolar binding site. In addition, the effects observed here may also be related to an interdependent effect of binding site architecture and surface hydrophobicity that affects enzyme performance (for a detailed discussion, see Figure S17). The finding of an increased performance with isopropanol was very promising for the application of the enzymes in flow reactors since the use of DMSO as a co-solvent can lead to problems in which a modulation of catalytic properties for thiamine-diphosphate-dependent enzymes was found due to a direct interaction of small co-solvent molecules with the nonpolar binding site. In addition, the effects observed here may also be related to an interdependent effect of binding site architecture and surface hydrophobicity that affects enzyme performance (for a detailed discussion, see Figure S17). The finding of an increased performance with isopropanol was very promising for the application of the enzymes in flow reactors since the use of DMSO as a co-solvent can lead to problems in which a modulation of catalytic properties for thiamine-diphosphate-dependent enzymes was found due to a direct interaction of small co-solvent molecules with the nonpolar binding site. In addition, the effects observed here may also be related to an interdependent effect of binding site architecture and surface hydrophobicity that affects enzyme performance (for a detailed discussion, see Figure S17). The finding of an increased performance with isopropanol was very promising for the application of the enzymes in flow reactors since the use of DMSO as a co-solvent can lead to problems in which a modulation of catalytic properties for thiamine-diphosphate-dependent enzymes was found due to a direct interaction of small co-solvent molecules with the nonpolar binding site. In addition, the effects observed here may also be related to an interdependent effect of binding site architecture and surface hydrophobicity that affects enzyme performance (for a detailed discussion, see Figure S17). The finding of an increased performance with isopropanol was very promising for the application of the enzymes in flow reactor systems via numbering-up. In conclusion, our work emphasizes that modeling-assisted rational design of ReBAL enzymes in combination with robust immobilization strategies is a powerful approach to create novel biocatalytic processes for continuous production of valued-added molecules. Specifically, we herein demonstrated the immobilization of a thermostable benzaldehyde lyase in a continuous packed-bed microreactor for the production of α-hydroxy ketones with constant activity over days. In this context, we screened three previously uncharacterized ThDP-dependent enzymes for aromatic ligase activity and identified...
ReBAL as a novel benzaldehyde lyase. After modeling the protein structure of ReBAL, two rationally designed variants with altered binding sites were generated. Comparing these three benzaldehyde lyase variants with the literature described PmBAL, we showed that ReBAL is more thermostable while being less active and having a narrower substrate spectrum. However, the ReBAL-mat variant whose binding site was designed to match PmBAL recovered the substrate scope. The most thermostable ReBAL-mat variant was then immobilized for the continuous flow biocatalysis where it showed a stable conversion over seven days. Our experiments expand the availability of enzymes for the continuous production of \( \alpha \)-hydroxy ketones and therefore can enhance the usability of this important enzyme class in biotechnological production processes. Since thermostable enzymes in general tolerate more mutations,\(^{[2]}\) we envision that the more stable ReBAL backbone identified in this work can be further modified using protein engineering to create completely novel reactivities and produce important enantiopure building blocks for the synthesis of APIs.

**Experimental Section**

Experimental details can be found in the Supporting Information.

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**Conflict of Interest**

The authors declare no conflict of interest.
A novel benzaldehyde lyase for the production of chiral α-hydroxy ketones was identified by a machine learning model and two variants with altered substrate scopes were created by rational design. All three variants exhibit significantly higher thermostability compared to the only previously described benzaldehyde lyase. When immobilized in a packed-bed reactor, the most thermostable variant showed stable substrate turnover over days.