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Engineered artificial carboligases facilitate regioselective preparation of enantioenriched aldol adducts

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Supporting Information Placeholder

ABSTRACT: Controlling regio- and stereoselectivity of aldol additions is generally challenging. Here we show that an artificial aldolase with high specificity for acetone as the aldol donor can be reengineered via single active site mutations to accept linear and cyclic aliphatic ketones with notable efficiency, regioselectivity and stereocontrol. Biochemical and crystallographic data show how the mutated residues modulate the binding and activation of specific aldol donors, as well as their subsequent reaction with diverse aldehyde acceptors. Broadening the substrate scope of this evolutionarily naïve catalyst proved much easier than previous attempts to redesign natural aldolases, suggesting that such proteins may be excellent starting points for the development of customized biocatalysts for diverse practical applications.

The aldol reaction represents a methodological cornerstone of synthetic organic chemistry, allowing for direct C-C bond formation under mild conditions. Achieving regio- and stereocontrol in aldol additions of unmodified ketones, however, remains challenging with small-molecule catalysts. Enzymes offer a potentially promising alternative, due to their inherent chirality and precise substrate recognition, but engineering of natural aldolases to meet synthetic needs is far from trivial.[1–4] Natural class I aldolases are highly evolved for their native reaction and have proven resistant to efforts to change donor specficity.[1] Because artificial enzymes have little historical bias, they are potentially more amenable to engineering by laboratory evolution.[5–8]

A family of computationally designed carboligases has shown remarkably high and tunable activity for diverse reactions that proceed via Schiff base intermediates.[9–16] The aldolase RA95.5-8F (Figure 1), obtained by design and ultrahigh-throughput evolution,[17,18] offers native-like catalytic rates, good to excellent stereoselectivity and high total turnover numbers for the addition of acetone to a variety of aliphatic and aromatic aldehydes. As for natural aldolases, though, this artificial carboligase poorly tolerates alternative donors. Here we show that single active site mutations can considerably expand the donor specificity of RA95.5-8F. The resulting variants efficiently catalyze additions of cyclic ketones and *n*-alkan-2-ones to aldehydes with complete regioselectivity and control of up to three chiral centers, an otherwise formidable challenge for conventional synthetic methods requiring complex catalysts in relatively high loading.[19,20]

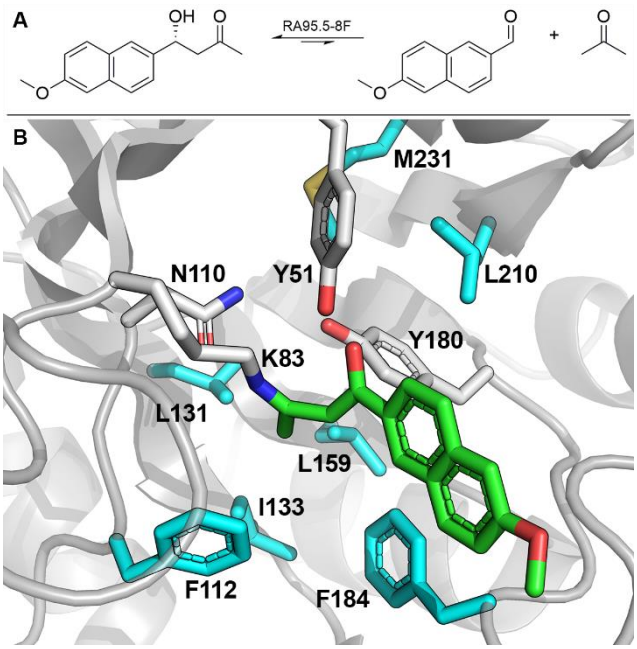
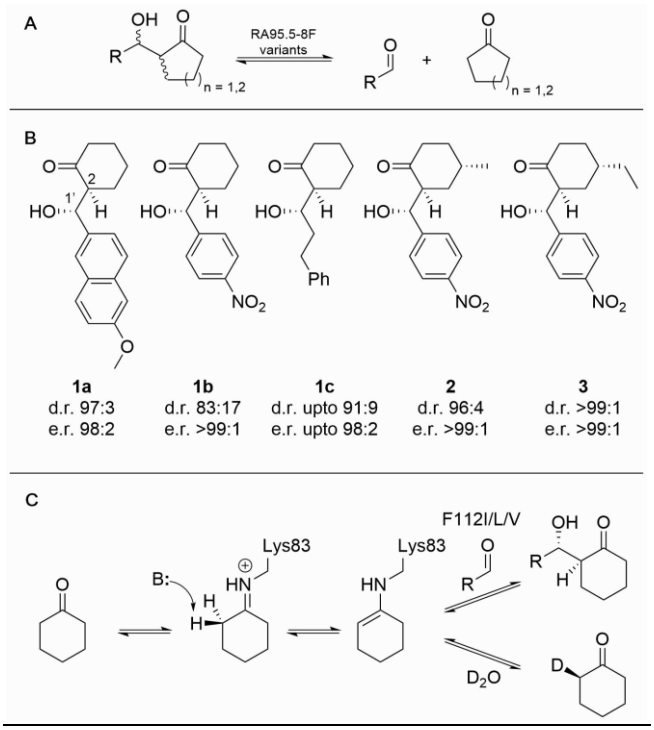


Figure 1: (**A**) Retroaldol cleavage of (*R*)-methodol,[21] catalyzed by RA95.5-8F.[18] (**B**) Structure of RA95.5-8F in complex with a diketone inhibitor (green). Mechanistically relevant residues are shown in gray, residues mutated in this study in cyan. PDB code: 5an7.

To create RA95.5-8F variants with broader substrate scope, four residues lining the binding pocket for the aldol donor (Phe112, Leu131, Ile133, and Leu159) and three additional residues (Phe184, Leu210, and Met231) that pack against the catalytic quartet were targeted independently for cassette mutagenesis (Figure 1). The resulting libraries were screened for cleavage of diastereomeric mixtures of aldol adducts of cyclopentanone and cyclohexanone with 6-methoxy-2-naphthaldehyde (Scheme 1A). Reaction progress in crude cell lysates was monitored spectrophotometrically at 350 nm in multiwell plates. Although all of the libraries proved inactive toward the cyclopentanone adducts, the F112 library afforded several hits for the cyclohe-

Scheme 1: (A) Screening reactions (B) Aldol adducts produced biocatalytically with F112I/L/V RA95.5-8F. (C) Stereoselective deuteuration of cyclohexanone.



xanone derivatives. Rapid cleavage of the larger substrate was made possible by replacement of Phe112 with the smaller aliphatic amino acids valine, leucine, and isoleucine.

The F112V, F112L and F112I variants were overproduced and purified. The crystal structure of the apo F112L variant, solved to 2.16 Å resolution, confirmed that the effects of the large-to-small mutation are largely local. The protein backbone aligns well with the parent enzyme (cα r.m.s. deviation of 0.336 Å) and is only distinct in the previously unresolved L1 loop (Figure S28). The leucine substitution, combined with subtle repositioning of Phe89, opens up the binding pocket, providing space for sterically more demanding substrates.

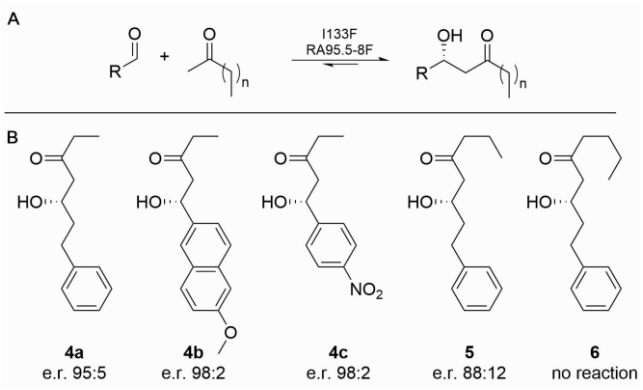
Kinetic measurements with aldol adduct **1a** (Scheme 1B), prepared enzymatically, showed that mutation of Phe112 improved catalytic efficiency by more than an order of magnitude, largely through improvements in k_{cat} (Table S5). Indeed, the steady-state parameters for this substrate (k_{cat} = 7 to 9 s^{-1} and k_{cat}/K_M = 7 x 10^4 to 1.1 x 10^5 M^{-1} s^{-1}) are comparable to those for the RA95.5-8F-catalyzed cleavage of (*R*)-methodol[18] (k_{cat} = 11 s^{-1} and k_{cat}/K_M = 3.4 x 10^4 M^{-1} s^{-1}).

In addition to aldol cleavage, all three variants promote aldol synthesis with high stereoselectivity. For example, F112I RA95.5-8F catalyzes the addition of cyclohexanone to 6-methoxy-2-naphthaldehyde, the microscopic reverse of the screening reaction, to produce **1a** with an *anti*/*syn* ratio of 97:3 and an enantiomeric ratio (e.r.) of 98:2. As for the parent enzyme, the observed stereochemistry can be rationalized by attack of the cyclohexanone donor, activated as an enamine by Lys83, on the *re*-face of the aldehyde acceptor. Similarly, the reaction of cyclohexanone with 4-nitrobenzaldehyde afforded **1b** as the dominant product stereoisomer. In this case, the *anti*/*syn* and enantiomeric ratios were 83:17 and >99:1, respectively. For the reaction of cyclohexanone and 3-phenylpropanal, depending on the catalyst employed**,** **1c** was obtained with *anti*/*syn* and enantiomeric ratios up to 91:9 and 98:2. For comparison, the parental enzyme achieved only modest yields (15%) and diastereoselectivities (85:16) for this reaction.[18] The F112V variant, which has the smallest side chain, even accepts substituted cyclohexanones as aldol donors. Reaction of 4-methyl- and 4-ethycyclohexanone with 4-nitrobenzaldehyde afforded aldol adducts **2** and **3** with excellent stereocontrol at three chiral centers (Scheme 1B).

Because the engineered aldolases exploit amine catalysis, cyclohexanone should undergo facile deuteration via its Schiff base and enamine adducts with Lys83 (Scheme 1C).[22] As expected, the F112I/L/V variants all promote H/D-exchange in buffered D_2O (Figure S17). Isolation of the resulting monodeuterated cyclohexanone and determination of its specific optical rotation ($[\alpha]_D^{26} = +2.2°$ (c = 0.41, $CDCl_3$)) revealed that one of the two pro-*R* α-hydrogens exchanges first.[23] The enzyme-bound enamine is thus preferentially deuterated on the same face as the aldehyde acceptors add in the synthetic aldol reactions. Unexpectedly, though, the rates of H/D exchange determined by ^{1}H-NMR spectroscopy were at most twofold faster than that observed for the parent enzyme RA95.5-8F, indicating that the enhanced synthetic ability of the variants derives from a beneficial effect on a later, slower step in the catalytic mechanism, most likely C-C bond formation.

Having successfully engineered RA95.5-8F to accept bulky, cyclic ketones as aldol donors, our attention turned to extended linear ketones. The regioselective preparation of linear aldol adducts from asymmetric ketones has been described, for example using stoichiometric amounts of polymer-bound lithium dialkylamides.[24] However, simultaneous control over regio- and stereochemistry remains challenging,[25–27] requiring costly organocatalysts at high catalyst loading.[28,29] We anticipated that the inexpensive and tailorable enzyme would be selective in both properties at sub-stoichiometric catalyst loadings. HPLC screening of the seven aldolase libraries directly for the reaction of 2-butanone and 3-phenylpropanal yielded a single hit, which had the I133F mutation located at the bottom of the donor bind-

Scheme 2: (A) Aldol synthesis with linear ketones. (B) Aldol adducts prepared at synthetic scale with I133F RA95.5-8F.



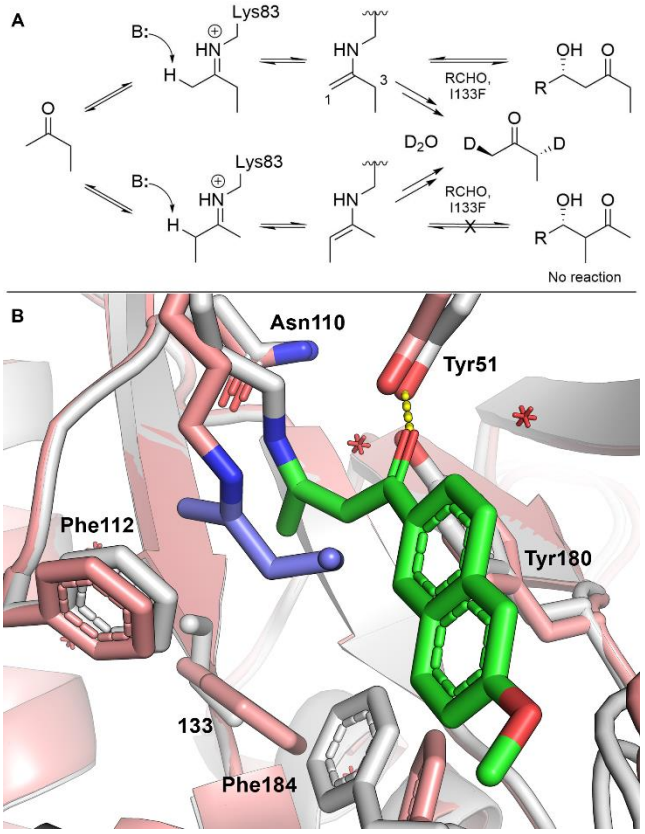


Figure 2: (**A**) Mechanism of regioselective enamine formation by the I133F enzyme, as observed in deuteuration studies. (**B**) Structure of I133F RA95.5-8F (PDB code: 6TF8) covalently bound to pentan-2-one (purple) with observed water molecules (red). The overlaid parental enzyme (gray) is complexed with a diketone inhibitor (green).

ing pocket. Biocatalytic transformations with the purified enzyme confirmed that the I133F variant confers complete regiocontrol over the addition of 2-butanone to 3-phenylpropanal, providing exclusively the normally disfavored linear regioisomer, adduct (*S*)-**4a**, with an e.r. of 95:5 (Scheme 2). Even higher e.r. values (98:2) were obtained with aromatic aldehydes such as 6-methoxy-2-naphthaldehyde and 4-nitrobenzaldehyde. Again, only the linear regioisomers **4b** and **4c** were observed. Notably, the stereoselectivities achieved are similar to those reported for acetone using RA95.5-8F as catalyst. With larger aldol donors, however, stereoselectivity erodes. 2-Pentanone reacts with 3-phenylpropanal to afford the linear aldol adduct (*S*)-**5** with an *e.r.* of 88:12, whereas 2-hexanone showed no conversion, suggesting that the engineered active site sterically restricts donor chain length.

Steady-state kinetics showed that (*R*)-**4b** is efficiently cleaved by I133F RA95.5-8F (k_{cat} = 4.8 s^{-1}, k_{cat}/K_M = 3.4 x 10^4 M^{-1} s^{-1}). Although the parent enzyme and the F112I variant are both poor catalysts for the synthesis of this aldol adduct, they unexpectedly promote its cleavage with comparable or even slightly higher catalytic efficiency (Table S6). Consistent with this observation, deuteration experiments with 2-butanone indicated that both RA95.5-8F and the I133F variant catalyze H/D-exchange at both C-1 and C-3 (Figures 2A and S25-S27). No difference was observed in the rate of deuteration of C-1 between the two enzymes (Table S7), so differential enolization cannot explain why I133F is the only variant that is useful for aldol synthesis with the extended linear ketones.

To understand the synthetic prowess of this variant, we determined its crystal structure in complex with 2-pentanone to 1.90 Å resolution. Comparison with the parent enzyme showed how the I133F mutation reshaped the donor binding pocket (Figure 2B). Although phenylalanine is larger than the original isoleucine at position 133, its sidechain adopts a rotameric conformation that creates additional space at the bottom of the cavity. Accompanying adjustments in the position of neighboring residues, particularly Phe184 which shifts by 2.5 Å, further opens the pocket.

In the complex, 2-pentanone is bound to Lys83 as a Schiff base, but its propyl group extends toward the mouth of the pocket where the acceptor aldehyde must bind (Figures 2B & S31). A water molecule, bound by the catalytic residues Tyr51, Asn110, and Tyr180, is well placed (3.2 Å) to either hydrolyze the Schiff base or abstract the C-3 pro-*R* hydrogen (Figure S29). However, since we only observed aldol additions at C-1 of this ketone, the enamine resulting from C-3 deprotonation cannot be productive for C-C bond formation, presumably because the propyl chain sterically blocks access of the aldehyde acceptor (Figure S31). To form **5**, the bound 2-pentanone would have to flip by ~180° to position the C-1 methyl for deprotonation and subsequent attack on the incoming 3-phenylpropanal. The same applies for the smaller 2-butanone, where the relative rates of H/D-exchange at C-1 and C-3 (Table S7) indicate that the ratio of productive to unproductive poses is ~1:3.

Modeling the Schiff base adducts of **4c** and **5** using the Rosetta software suite[30] confirmed that both linear products are readily accommodated in the remodeled active site. These molecules can adopt extended conformations similar to that seen for the diketone inhibitor in the parent enzyme (Figure S32-S33), with slight adjustments in the position of the catalytic lysine helping to accommodate the bulkier donor in the expanded pocket. The docking models further indicate that a productive hydrogen bond between the product hydroxyl group and Tyr51 likely dictates the enzyme's stereochemical preferences. Although the disfavored enantiomer also fits in the active site, it cannot make an analogous interaction for steric reasons.

Carboligases are attractive catalysts for preparing chiral building blocks for complex natural products and other molecules.[31–36] In practical terms, however, the application of natural aldolases in industrial processes has been limited relative to other enzyme classes such as hydrolases, oxidoreductases, and transaminases,[8] owing in part to their narrow donor specificity.[2,37] Despite some successes,[3,38] expanding the substrate scope of these catalysts has proved challenging because substrate recognition and catalysis are usually entangled in complex networks of polar residues.[1,39]

The unprecedented promiscuity[15,16,40] and mutational tolerance[14,18] of artificial aldolases created by design and evolution offers a potential alternative. In these systems, substrate recognition is largely independent of the catalytic apparatus, so successful discrimination between different aldol adducts only requires creation of complementary pockets of appropriate size, shape and polarity for the appended groups, a considerably easier task than redesigning

hydrogen bonding networks. In favorable cases, as shown in the current study, large changes in substrate preference can even be realized by single active site mutations. The ensuing control over multiple stereocenters and regioselective formation of normally disfavored linear aldol adducts from linear ketones adds to the repertoire of tools available to synthetic chemists looking for efficient ways to control C-C bond formation. Although more extensive engineering may be needed to adapt RA95 aldolases for other substrates, particularly polar ketones, ultrahigh-throughput screening methods may expedite the search for useful variants.[18,41–43]

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Complete experimental procedures, with sequence information, additional kinetic data, crystallographic data, code for running Rosetta modeling, as well as Figures S1-S33 and Tables S1-S9 (PDF)

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Notes

The authors declare no competing financial interests.

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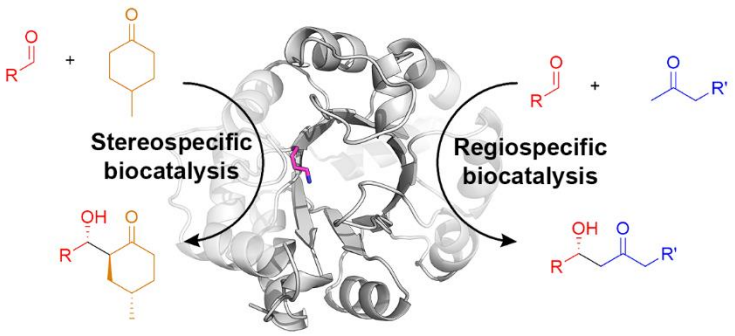


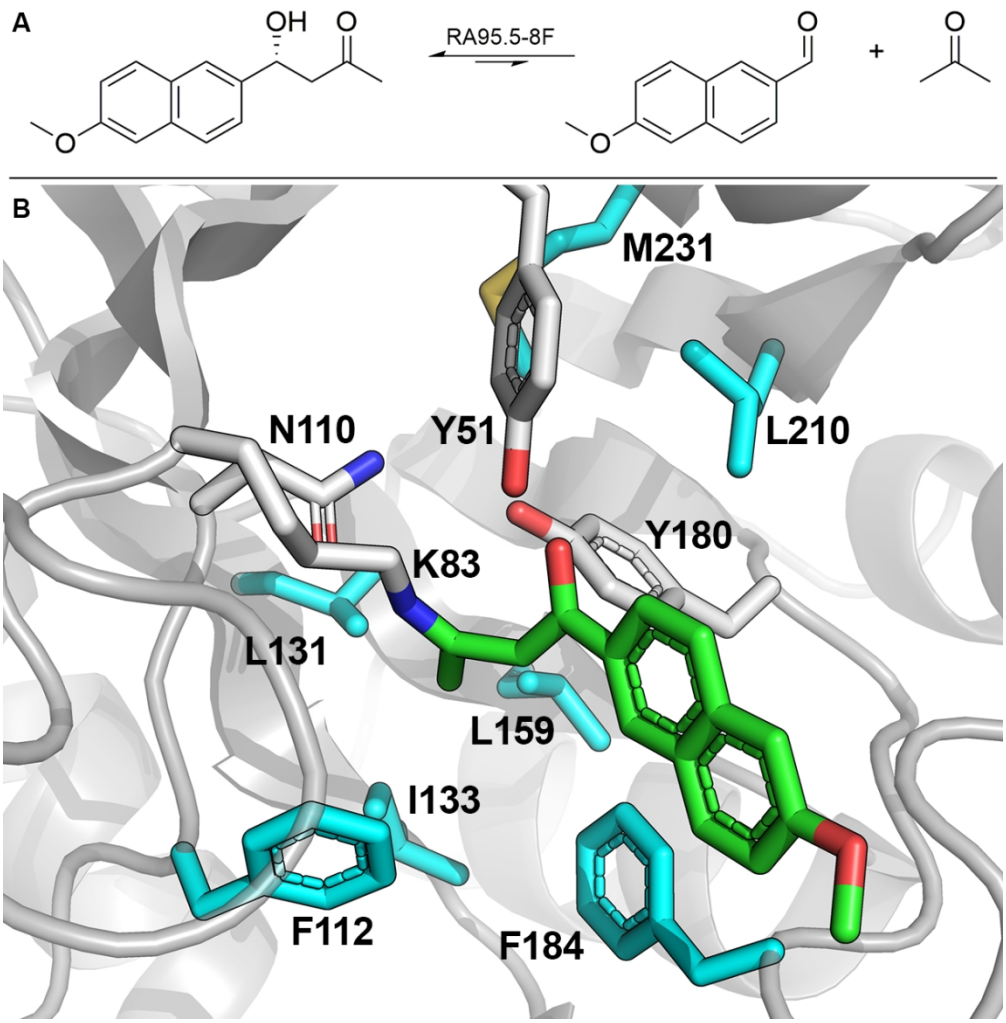
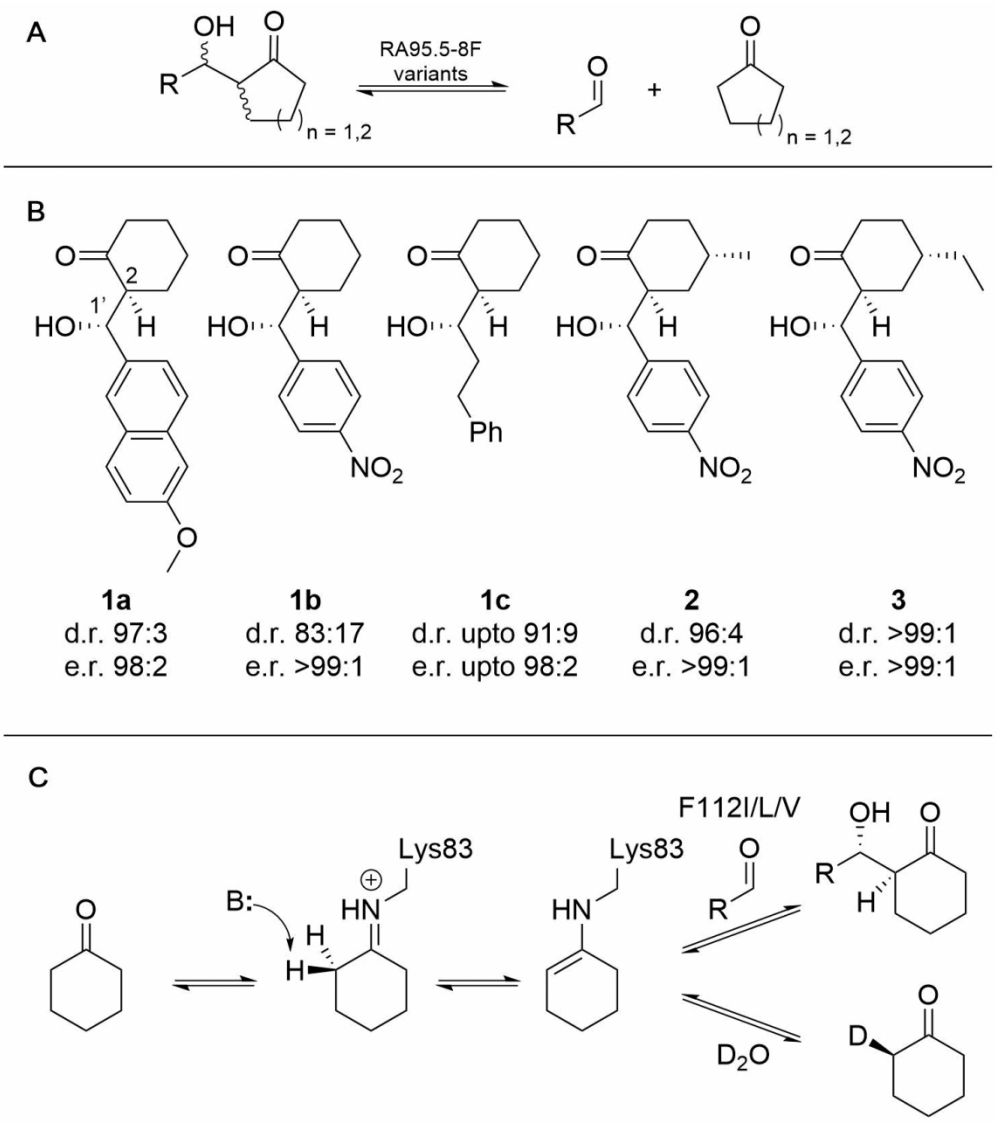


Figure 1: (A) Retroaldol cleavage of (R)-methodol,21 catalyzed by RA95.5-8F.18 (B) Structure of RA95.5-8F in complex with a diketone inhibitor (green). Mechanistically relevant residues are shown in gray, residues mutated in this study in cyan. PDB code: 5an7.



Scheme 1: (A) Screening reactions (B) Aldol adducts pro-duced biocatalytically with F112I/L/V RA95.5-8F. (C) Stereoselective deuteuration of cyclohexanone.

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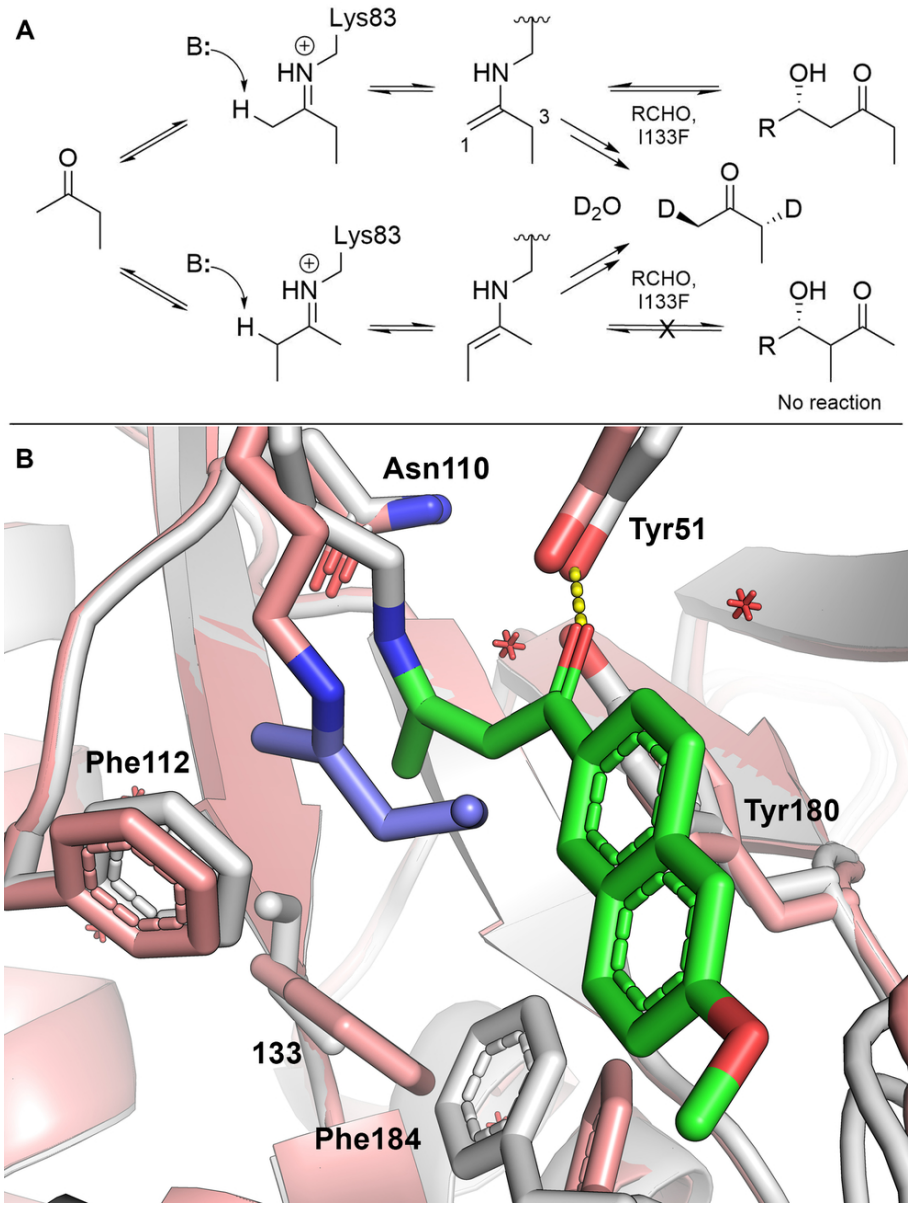

Figure 2: (A) Mechanism of regioselective enamine formation by the I133F enzyme, as observed in deuteuration studies. (B) Structure of I133F RA95.5-8F (PDB code: 6TF8) covalently bound to pentan-2-one (purple) with observed water molecules (red). The overlaid parental enzyme (gray) is complexed with a diketone inhibitor (green).

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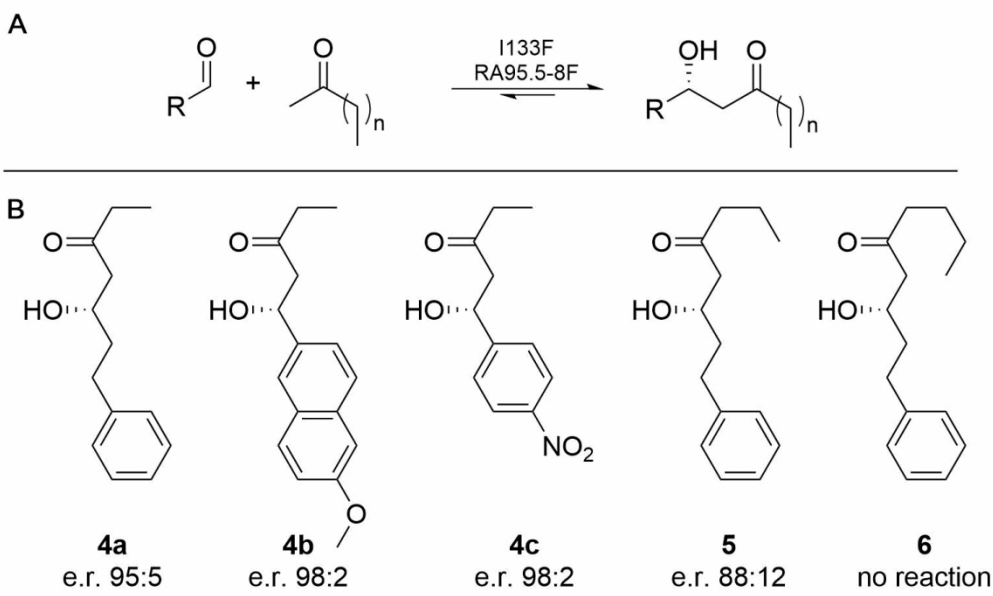

Scheme 2: (A) Aldol synthesis with linear ketones. (B) Aldol adducts prepared at synthetic scale with I133F RA95.5-8F.

404x243mm (96 x 96 DPI)