CHEMISTRY
A European Journal

A Journal of
ChemPubSoc Europe

Accepted Article

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To be cited as: *Chem. Eur. J.* 10.1002/chem.201605757

Link to VoR: http://dx.doi.org/10.1002/chem.201605757

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10.1002/chem.201605757

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Chemoselective Henry condensations catalyzed by artificial carboligases

Xavier Garrabou, Duncan S. Macdonald, and Donald Hilvert*

Abstract: The promiscuity of *de novo* designed enzymes provides a privileged platform for diverse abiological reactions. In this work we report the first example of a nitroolefin synthase that catalyzes the Henry condensation between aromatic aldehydes and nitromethane. Significant catalytic activity was discovered in a computationally designed and evolved carboligase, RA95.5-8, and mutations around the active site were shown to improve the reaction rate, demonstrating the potential to optimize the enzyme by directed evolution. This novel nitroolefin synthase could participate in complex biological cascades, whereby the highly tunable chemoselectivity could afford useful synthetic building blocks.

Enzymes are gaining increasing recognition as valuable tools for the efficient and selective catalysis of chemical reactions in the laboratory. The repertoire of transformations promoted by natural enzymes is impressive, yet many fundamental reactions in organic chemistry remain beyond Nature's purview. Recently, the combination of computational design and directed evolution has emerged as a potentially general means to create *de novo* enzymes for any reaction of interest.[1] Because these catalysts often utilize catalytic motifs that are broad in scope, they may also serve as versatile starting points for functional diversification.

The computationally designed[2] and experimentally optimized[3] retro-aldolase RA95.5-8 exemplifies the inherent catalytic promiscuity of such enzymes. This catalyst uses a nucleophilic lysine to promote the retro-aldol reaction for which it was designed[3] (Scheme 1A). It can also exploit Schiff-base adducts to accelerate several other transformations,[4] including the addition of ethyl 2-cyanoacetone to iminium-activated enones and aldehydes to afford Michael[4a] and Knoevenagel products,[4b] respectively (Scheme 1B and 1C). RA95.5-8 also catalyzes Michael-type additions of nitromethane to iminium-activated enones, as well as enamine-mediated addition of acetone to electrophilic nitroolefins.[4d] Given the exceptional reactivity and versatility of nitro compounds, we wondered whether they might participate in other reactions catalyzed by the retro-aldolase. Here we report that this enzyme is able to promote the synthesis of nitroolefins via chemoselective Henry condensations of nitromethane and aromatic aldehydes.

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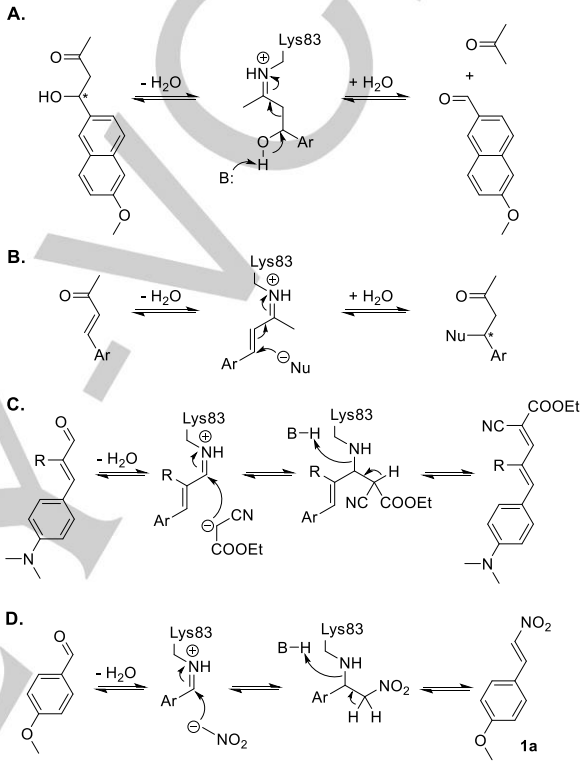


Scheme 1. Reactions catalyzed by RA95.5-8 using Lys83 as a nucleophilic amine. **A:** Retro-aldol cleavage of methodol.[3] **B:** Michael addition of conjugated ketones and carbon nucleophiles (Nu: ethyl 2-cyanoacetate, nitromethane).[4a, 4d] **C:** Knoevenagel condensation of electron-rich aldehydes (R = H, Me) and ethyl 2-cyanoacetate.[4b] **D:** Reversible Henry condensation of anisaldehyde and nitromethane to afford (*E*)-1-methoxy-4-(2-nitrovinyl)-benzene (**1a**).

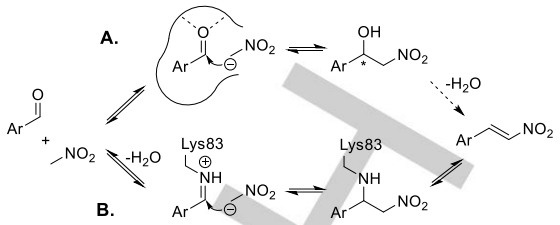
In recent studies of the RA95.5-8-catalyzed addition of acetone to (*E*)-1-methoxy-4-(2-nitrovinyl)benzene (**1a**), we observed a few uncharacterized side products in addition to the desired γ-nitroketone.[4d] To gain further insight into this process, we incubated the enzyme in a buffered solution of **1a** in the absence of acetone and observed efficient hydrolytic cleavage of the nitroolefin to anisaldehyde and nitromethane. A variant of the enzyme in which Lys83 was mutated to methionine exhibited negligible activity with the nitroolefin, suggesting a mechanism in which the ε-amine of the catalytic lysine promotes a retro-Henry reaction (Scheme 1D, right to left), analogous to the previously reported enzyme-catalyzed Knoevenagel condensation (Scheme 1C). The reverse reaction, enzymatic synthesis of **1a** from anisaldehyde and nitromethane (Scheme 1D, left to right), was also readily detected by UPLC-MS and spectroscopic analysis, although the nitroolefin product did not accumulate due to the

10.1002/chem.201605757

unfavorable equilibrium of the reaction in aqueous medium (K_{eq} ≈ 5 M^{-1}).[5] Efforts to increase the yield of **1a** by using a large excess of nitromethane were unsuccessful, as nitromethane spontaneously reacts with the newly formed Michael acceptor to afford the corresponding 1,3-dinitro compound.[6] In line with the proposed mechanism, the enzyme does not accelerate the cleavage of 1-(4-methoxyphenyl)-2-nitroethan-1-ol or its dehydration to **1a**.

The enzymatic Henry condensation was kinetically characterized using a spectrophotometric assay. The concentration of anisaldehyde was varied at several fixed concentrations of nitromethane (see Supporting Information). The intersecting lines in the resulting double-reciprocal plot suggest fast and reversible Schiff-base formation between anisaldehyde and Lys83. The data were fitted to a simplified equation for a bi-substrate reaction to determine the steady-state parameters. The Henry condensation has a ten-fold lower k_{cat} value (0.018 ± 0.001 s^{-1}) than the RA95.5-8-catalyzed Knoevenagel reactions, although the Michaelis constants ($K_{M,anisaldehyde}$ = 193 ± 12 µM; $K_{M,nitromethane}$ = 70 ± 6 mM) are relatively similar. Kinetic characterization of the enzymatic retro-Henry cleavage of nitrostyrene **1a** gave steady-state parameters k_{cat} = 0.056 ± 0.002 s^{-1} and $K_{M,\mathbf{1a}}$ = 213 ± 20 µM. Other nitroolefins such as trans-β-nitrostyrene (**1b**) and (*E*)-1-bromo-4-(2-nitrovinyl)benzene (**1c**) are also cleaved by RA95.5-8 with 1.3- and 2.7-fold higher k_{cat}/K_M values than **1a**.

KN.4, an optimized RA95.5-8 variant with 30-fold enhanced activity for the Knoevenagel reaction,[4b] was also examined as a catalyst for the Henry condensation, in light of the mechanistic similarities between the two reactions. To our surprise, the steady-state parameters (Tables S2 and S3) showed that the optimized enzyme is actually about 20-fold less active for the reaction than the parent retro-aldolase RA95.5-8. Because some of the mutations present in KN.4 could conceivably still benefit the Henry condensation, we used gene shuffling to dilute the number of substitutions within the parent sequence (see SI). Approximately three hundred members of the corresponding library were assayed in multi-well plates using a spectrophotometric assay to monitor condensation of anisaldehyde and nitromethane at 370 nm. A variant with improved activity under the assay conditions was sequenced and characterized as pure protein. Of the original fifteen mutations in KN.4, four were retained in the improved catalyst (S21R, I107V, I113V and N135G), which exhibited a 2 to 3-fold higher k_{cat}^{app} than RA95.5-8 but a similar k_{cat}^{app}/K_M^{app} (Tables S2 and S3). This result demonstrates that some KN.4 mutations are generally beneficial for condensation reactions, whereas other changes boosted Knoevenagel activity at the expense of alternative reaction pathways. It also suggests that, as seen for other RA95.5-8 activities,[4b, 4c] it should be possible to improve the efficiency of the enzyme-catalyzed Henry condensation by further rounds of directed evolution. In fact, by simply screening our previously engineered retro-aldolase variants, we found that the K210R mutation alone increases k_{cat}^{app} for the condensation of anisaldehyde and nitromethane 4 to 6 fold (Table S2). The guanidinium cation of the arginine side chain may promote



Scheme 2. Henry additions vs. condensations. **A:** Diverse enzymes catalyze Henry additions of aldehydes and nitroalkanes to afford β-hydroxynitro compounds by activating the carbonyl group of the aldehyde (for example, by binding in an oxyanion hole). **B:** In contrast, RA95.5-8 utilizes amine catalysis to promote Henry condensation of the same substrates, affording exclusively nitrostyrenes as products.

binding and ionization of nitromethane via double hydrogen bonding, a strategy for activating nitro groups that is widely used in organocatalysis.[7] A more systematic evolutionary campaign can be expected to enhance this novel activity considerably.

Promiscuous additions of aldehydes and nitroalkanes to afford β-hydroxynitro compounds have been reported for diverse classes of enzyme (Scheme 2A).[8] Hydroxycyanide lyases, which naturally promote the reversible attack of cyanide on aromatic aldehydes, are particularly effective and stereoselective catalysts for these reactions.[9] In fact, the addition of nitromethane to aldehydes is so favorable in aqueous medium that it may be readily attained using suitable buffers at close-to-neutral pH. On the other hand, the subsequent dehydration of the initial Henry adducts to afford nitrostyrenes generally requires high temperatures or strongly basic conditions – a far more challenging prospect for biocatalytic systems.[6, 10] RA95.5-8 circumvents this problem by providing an alternative route to nitroolefins involving amine catalysis (Scheme 2B). As a consequence, this enzyme could be a valuable source of these useful synthetic building blocks. In contrast, catalysis of Henry reactions by bovine serum albumin, a model protein for promiscuous lysine-mediated catalysis,[11] only affords the corresponding β-hydroxynitro compounds via non-specific interactions.[12] Although the enzymatic synthesis of nitrostyrenes is thermodynamically unfavorable in aqueous medium, effective biocatalytic cascades can be envisioned in which the initially formed nitroolefins are transformed downstream either by reduction of the nitro group[13] or functionalization of the double bond[4d, 14] Preparative Henry condensations might also be achievable using suitable enzyme preparations in organic solvents.

In summary, we reported the first biocatalytic synthesis of nitrostyrenes in aqueous medium — a new activity for the artificial enzyme RA95.5-8. We have shown that single active site mutations can boost this activity, or, in turn, mitigate its competition with other enzymatic reactions involving nitrostyrenes.[4d] Optimized retro-aldolase variants for the Henry condensation could offer exciting opportunities for the design of innovative enzymatic synthetic routes for producing or diversifying a wide range of nitro compounds. Extrapolating from the functional versatility of these substances in combination with RA95.5-8, other activated substrates might be profitably

examined as a means of further broadening the reaction scope of this enzyme.

Acknowledgements

The authors are grateful to the Swiss National Science Foundation (SNSF) and the ETH Zurich for generous support of this work. We also thank Marius Furter for assistance with directed evolution experiments.

Keywords: artificial enzyme • directed evolution • enzyme promiscuity • Henry reaction • nitrostyrene synthesis

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10.1002/chem.201605757

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