JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
pubs.acs.org/JACS

Enantiocomplementary Synthesis of γ-Nitroketones Using Designed and Evolved Carboligases

Xavier Garrabou, Rebecca Verez, and Donald Hilvert*

Laboratory of Organic Chemistry, ETH Zurich, 8093 Zurich, Switzerland

Supporting Information

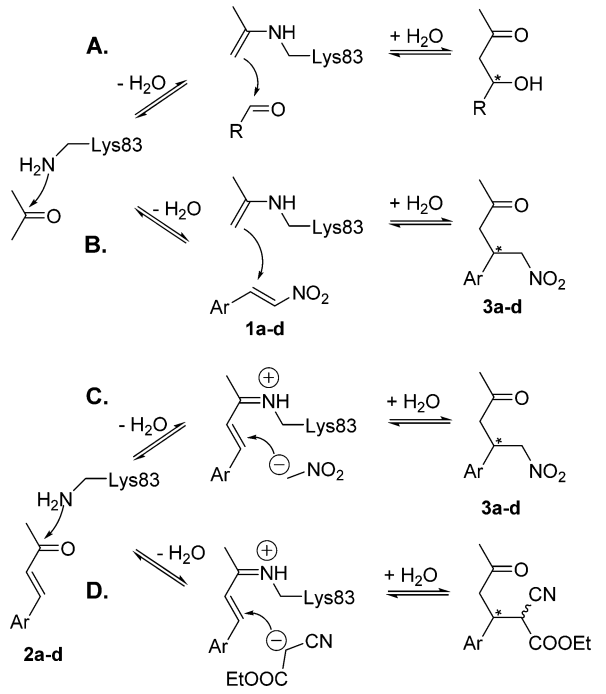
ABSTRACT: Artificial enzymes created by computational design and directed evolution are versatile biocatalysts whose promiscuous activities represent potentially attractive starting points for divergent evolution in the laboratory. The artificial aldolase RA95.5-8, for example, exploits amine catalysis to promote mechanistically diverse carboligations. Here we report that RA95.5-8 variants catalyze the asymmetric synthesis of γ-nitroketones via two alternative enantiocomplementary Michael-type reactions: enamine-mediated addition of acetone to nitrostyrenes, and nitroalkane addition to conjugated ketones activated as iminium ions. In addition, a cascade of three aldolase-catalyzed reactions enables one-pot assembly of γ-nitroketones from three simpler building blocks. Together, our results highlight the chemical versatility of artificial aldolases for the practical synthesis of important chiral synthons.

Asymmetric carbon−carbon bond formation is a key transformation in modern organic chemistry. Carboligase enzymes are attractive catalysts for such processes because of their high proficiency and selectivity under mild reaction conditions and potential utility in multistep reaction cascades.[1−3] Recently, artificial enzymes that promote diverse chemical transformations,[4,5] including asymmetric carboligations of synthetic interest,[6,7] have been computationally designed and experimentally optimized by directed evolution. These biocatalysts represent attractive starting points for further diversification into families of enzymes with distinct substrate and reactivity profiles.

Artificial aldolases are a case in point.[8,9] The promiscuous aldolase RA95.5-8,[10] created by extensive remodeling of an original computational design,[9] catalyzes mechanistically diverse carboligations. In addition to its original retro-aldolase activity (Scheme 1A), RA95.5-8 utilizes its active site lysine to promote asymmetric Michael additions[11] and Knoevenagel reactions[12] via Schiff base intermediates. Although the starting activities were modest, they could be substantially improved by multiple rounds of mutagenesis and screening.[6,11,12] Seeking new preparative applications for the RA95.5-8 enzyme family, we investigated the asymmetric synthesis of γ-nitroketones, which are valuable chiral synthons.[13−15]

Two types of Michael reaction may be envisioned for the stereocontrolled preparation of γ-nitroketones: addition of ketones to nitroolefins and addition of nitroalkanes to conjugated ketones. Simple amines as well as more sophisticated peptides have been used as catalysts for both types of reaction.[16,17] In this report, we show that RA95.5-8 variants can also accelerate these transformations to afford γ-nitroketones in practical yields and with complementary enantioselectivity.

Scheme 1. Promiscuous Transformations Catalyzed by a Designed and Evolved Retro-Aldolase Using a Reactive Lysine (Lys83)

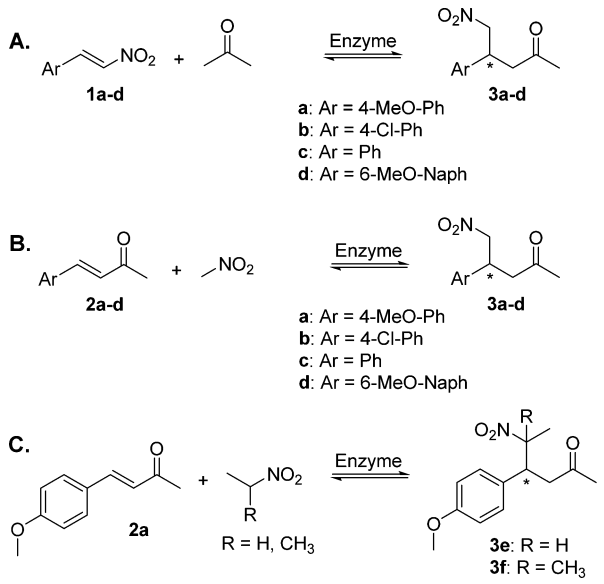


For the first approach, we envisaged that the catalytic Lys83 of RA95.5-8 would activate acetone as an enamine, which could then attack a nitroolefin in Michael fashion to give the desired γ-nitroketone product (Scheme 1B). We tested a panel of aldolase variants from previous studies (Table S1) as catalysts for the reaction of acetone with (*E*)-1-methoxy-4-(2-nitrovinyl)benzene (**1a**, Scheme 2A). HPLC analysis of the reaction products revealed that the choice of enzyme variant and the specific reaction conditions had a large influence on the relative yields of γ-nitroketone and various uncharacterized side products. Using low acceptor concentrations (<1 mM), a slightly acidic buffer (pH = 6.5), and conducting the reactions

Received: November 18, 2016
Published: December 19, 2016

DOI: 10.1021/jacs.6b11928
J. Am. Chem. Soc. 2017, 139, 103−106

Scheme 2. Nitro-Michael Reactions Catalyzed by RA95.5-8 Variants



in the dark, variants T53L/K210H RA95.5-8 and S233F RA95.5-8 were found to accelerate the formation of Michael product **3a** with minimal accumulation of side-products. They were therefore characterized further. For both catalysts, chiral HPLC analysis of the isolated reaction products showed a clear preference for formation of the "*S*"-configured **3a** adduct (Table 1, entries 1 and 2). S233F RA95.5-8 provided higher conversion in preparative reactions and was therefore chosen to catalyze the addition of acetone to a series of substituted nitrostyrenes (**1b–d**, Scheme 2A). The corresponding γ-nitroketones **3b–d** were isolated in moderate to good yields, and consistently exhibited a preference for the "*S*"-configured product (Table 1, entries 3–5). The stereoselectivity of the additions is opposite to that obtained with aldehyde acceptors,[6] indicating a distinct binding mode for the nitroolefins at the active site. The lower "*S*"-preference seen with **1d** may reflect this compound's 6-methoxynaphthyl group favoring a competing binding orientation more akin to that of 6-methoxy-2-naphthaldehyde for which the pocket was originally tailored.[9]

Mutagenesis and kinetic studies were carried out to provide insight into the mechanism of action and catalytic efficiency of these biocatalysts. Consistent with the proposed importance of enamine intermediates, negligible activity was observed when the catalytic Lys83 was replaced with methionine. Steady-state parameters for the reaction of **1a** in the presence of a large excess of acetone (2 M) were determined for both T53L/K210H RA95.5-8 (k_{cat}^{app} = 0.012 ± 0.002 s^{-1}, $K_{M,1a}^{app}$ = 610 ± 120 μM) and S233F RA95.5-8 (k_{cat}^{app} = 0.016 ± 0.001 s^{-1}, $K_{M,1a}^{app}$ = 510 ± 40 μM). The turnover numbers of the two variants are relatively modest, roughly an order of magnitude lower than the k_{cat} values for the retro-aldol reaction catalyzed by RA95.5-8,[10] possibly reflecting rate-determining Schiff base or enamine formation. This possibility prompted us to investigate RA95.5-8F[6] as a catalyst for the addition of acetone to **1a**. Although it is a 30-fold more efficient aldolase than RA95.5-8, chromatographic analysis of the reaction mixture revealed multiple side products and relatively little **3a**, which was produced with inverted stereochemistry (*R*/*S* = 77:23). These results suggest that RA95.5-8F binds **1a** in a different, less productive orientation than the other variants, outweighing its greater ability to activate acetone. Given the demonstrated evolvability of these systems, though, it should be possible to boost the efficiency and stereospecificity of these enzymatic reactions through additional rounds of mutagenesis and screening.

Variants of 4-oxalocrotonate tautomerase have been shown to catalyze analogous Michael-type reactions,[18−20] utilizing an *N*-terminal proline residue[21] to promote stereoselective additions of aliphatic aldehydes (but not ketones) to nitrostyrenes. The unique ability of RA95.5-8 to activate a ketone as the Michael nucleophile might be explained by the higher nucleophilicity of primary amines, which has been successfully exploited in organocatalysis to activate hindered or poorly electrophilic carbonyl groups.[22] As a consequence, RA95.5-8 variants might also catalyze ketone-derived enamine additions to other electrophiles such as maleimides, nitroso compounds, or stable imines.[17]

The second approach to γ-nitroketones involves Michael addition of nitroalkanes to conjugated ketones, activated as iminium ions (Scheme 1C). We tested a number of aldolase variants (Table S1) for the Michael addition of nitromethane to (*E*)-4-(4-methoxyphenyl)but-3-en-2-one (**2a**, Scheme 2B). The T53L/K210H RA95.5-8 variant, which was previously optimized for the reaction of 2-ethyl cyanoacetate to **2a** (Scheme 1D),[11] catalyzes the asymmetric synthesis of (*R*)-4-(4-methoxyphenyl)-5-nitropentan-2-one ((*R*)-**3a**) in high yield and enantioselectivity as determined by chiral HPLC analysis (Table 1, entry 6). This reaction was kinetically characterized by varying the concentration of the conjugated ketone at several fixed concentrations of nitromethane. Intersecting lines in the double reciprocal plot (Figure S2) suggest that both substrates bind independently. Global fitting of the data to a random binding mechanism gave the steady-state parameters

Table 1. Enantiocomplementary Preparation of γ-Nitroketones Using Variants of RA95.5-8 as Catalysts

entry	substrates		RA95.5-8variant	product	yield	*R*/*S* ratio
1	**1a**	acetone	T53L/K210H	**3a**	61%	13:87
2	**1a**	acetone	S233F	**3a**	84%	14:86
3	**1b**	acetone	S233F	**3b**	64%	12:88
4	**1c**	acetone	S233F	**3c**	65%	18:82
5	**1d**	acetone	S233F	**3d**	37%	44:56
6	**2a**	nitromethane	T53L/K210H	**3a**	82%	96:4
7	**2b**	nitromethane	T53L/K210H	**3b**	70%	94:6
8	**2c**	nitromethane	T53L/K210H	**3c**	65%	77:23
9	**2d**	nitromethane	T53L/K210H	**3d**	63%	99.3:0.7
10	**2a**	2-nitropropane	T53L/K210H	**3f**	81%	93.5:6.5

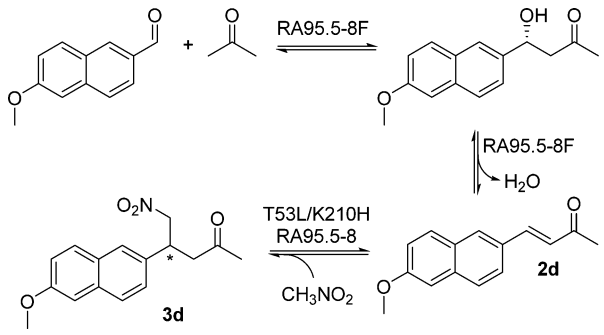
DOI: 10.1021/jacs.6b11928
J. Am. Chem. Soc. 2017, 139, 103−106

k_{cat} = 0.16 ± 0.01 s^{-1}, $K_{M,2a}$ = 220 ± 10 μM and $K_{M,nitromethane}$ = 45 ± 3 mM. This reaction is thus about ten times faster than the enzymatic addition of acetone to nitrostyrene **1a** catalyzed by the same aldolase variant and similar to the rates observed for the reaction of ethyl 2-cyanoacetate and **2a**.[11] Again, Michael activity is lost upon mutagenesis of Lys83 to methionine, supporting the hypothesis that this residue activates the conjugated ketone via iminium ion formation.[11] Like 2-ethyl cyanoacetate, nitromethane preferentially attacks the *Re* face of the resulting enzyme intermediate, giving rise to the *R*-configured γ-nitroketone. This route to **3a** is thus stereocomplementary to the reaction of acetone with nitrostyrene described above.

The preparative utility of T53L/K210H RA95.5-8 for γ-nitroketone synthesis was examined with a series of substituted conjugated ketones (**2b–d**, Scheme 2B). In all cases, addition of nitromethane gave the corresponding γ-nitroketones (**3b–d**) in good to excellent yields and high selectivity for the *R*-configured product (Table 1, entries 7–9). Bulkier nitroalkanes such as nitroethane and 2-nitropropane were also assayed as nucleophiles in the reaction with **2a** (Scheme 2C). Although product accumulation with nitroethane was severely hampered by enzyme deactivation, 2-nitropropane reacted smoothly to afford the substituted nitroketone **3f** in good yield and high stereoselectivity (Table 1, 10). This type of tertiary nitro compound would not be accessible by addition of acetone to a nitroolefin.

Because of their inherent compatibility, enzymes are ideally suited for the construction of complex molecular scaffolds from simpler starting materials via one-pot, multistep reaction sequences.[23−26] Accordingly, we wondered whether the diverse activities observed for the RA95.5-8 aldolase variants might be combined to prepare γ-nitroketones in a three-step biocatalytic cascade involving sequential aldol addition of acetone and a suitable aldehyde, dehydration, and Michael addition of a nitroalkane (Scheme 3). In previous work, we showed that

Scheme 3. One-Pot, Three-Step Synthesis of Michael Adduct 3d Catalyzed by RA95.5-8 Variants



RA95.5-8F is an efficient catalyst for the first step in this sequence, namely the synthesis of (*R*)-methodol from acetone and 6-methoxy-2-naphthaldehyde.[6] During characterization of this highly active enzyme variant, we observed that under certain reaction conditions (i.e., relatively high catalyst loadings, low donor concentrations, and extended reaction times) dehydration of the aldol adduct, the second step in the cascade, occurred. These parameters were therefore optimized to enable preparative-scale enzymatic synthesis of the conjugated ketone **2d** (see SI). Inclusion of T53L/K210H RA95.5-8 and nitromethane in the reaction mixture completed the sequence and enabled efficient one-pot synthesis of γ-nitroketone **3d**. Under optimized conditions, the final product was produced in similar yield and stereoselectivity (64% isolated yield, *R*/*S* = 97.7:2.3) as the reaction using purified **2d** as starting material (Table 1, entry 9). This simple and effective biocatalytic cascade attests to the synthetic opportunities enabled by the combined action of related artificial enzymes.

In summary, we have developed two stereocomplementary biocatalytic routes to γ-nitroketones. Our results highlight the utility and versatility of artificial aldolases as practical catalysts for asymmetric carbon−carbon bond forming reactions. Chemical promiscuity, combined with inherent evolvability, make these and other computationally designed enzymes attractive starting points for the development of new catalysts with broadened substrate scope and tailored substrate specificities and stereoselectivities.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b11928.

Complete experimental procedures and additional kinetic data, including Table S1 and Figures S1–S3 (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*hilvert@org.chem.ethz.ch

ORCID

Xavier Garrabou: 0000-0003-3663-8420

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors are grateful to the Swiss National Science Foundation (SNSF) and the ETH Zurich for generous support of this work.

■ REFERENCES

(1) Müller, M. *Adv. Synth. Catal.* **2012**, *354*, 3161−3174.
(2) Fesko, K.; Gruber-Khadjawi, M. *ChemCatChem* **2013**, *5*, 1248−1272.
(3) Schmidt, N. G.; Eger, E.; Kroutil, W. *ACS Catal.* **2016**, *6*, 4286−4311.
(4) Kries, H.; Blomberg, R.; Hilvert, D. *Curr. Opin. Chem. Biol.* **2013**, *17*, 221−228.
(5) Kiss, G.; Çelebi-Ölçüm, N.; Moretti, R.; Baker, D.; Houk, K. N. *Angew. Chem., Int. Ed.* **2013**, *52*, 5700−5725.
(6) Obexer, R.; Godina, A.; Garrabou, X.; Mittl, P. R. E.; Baker, D.; Griffiths, A. D.; Hilvert, D. *Nat. Chem.* **2017**, *9*, 50−56.
(7) Preiswerk, N.; Beck, T.; Schulz, J. D.; Milovník, P.; Mayer, C.; Siegel, J. B.; Baker, D.; Hilvert, D. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 8013−8018.
(8) Jiang, L.; Althoff, E. A.; Clemente, F. R.; Doyle, L.; Röthlisberger, D.; Zanghellini, A.; Gallaher, J. L.; Betker, J. L.; Tanaka, F.; Barbas, C. F.; Hilvert, D.; Houk, K. N.; Stoddard, B. L.; Baker, D. *Science* **2008**, *319*, 1387−1391.
(9) Althoff, E. A.; Wang, L.; Jiang, L.; Giger, L.; Lassila, J. K.; Wang, Z.; Smith, M.; Hari, S.; Kast, P.; Herschlag, D.; Hilvert, D.; Baker, D. *Protein Sci.* **2012**, *21*, 717−726.
(10) Giger, L.; Caner, S.; Obexer, R.; Kast, P.; Baker, D.; Ban, N.; Hilvert, D. *Nat. Chem. Biol.* **2013**, *9*, 494−498.
(11) Garrabou, X.; Beck, T.; Hilvert, D. *Angew. Chem., Int. Ed.* **2015**, *54*, 5609−5612.

DOI: 10.1021/jacs.6b11928
J. Am. Chem. Soc. 2017, 139, 103−106

(12) Garrabou, X.; Wicky, B. I. M.; Hilvert, D. *J. Am. Chem. Soc.* **2016**, *138*, 6972−6974.
(13) Barnes, D. M.; Wittenberger, S. J.; Zhang, J.; Ji, J.; Fickes, M. G.; Fitzgerald, M. A.; King, S. A.; Morton, H. E.; Plagge, F. A.; Preskill, M.; Wagaw, S. H. *J. Am. Chem. Soc.* **2002**, *124*, 13097−13105.
(14) Tian, J.; Zhong, J.; Li, Y.; Ma, D. *Angew. Chem., Int. Ed.* **2014**, *53*, 13885−13888.
(15) Guo, X.-T.; Shen, J.; Sha, F.; Wu, X.-Y. *Synthesis* **2015**, *47*, 2063−2072.
(16) Erkkilä, A.; Majander, I.; Pihko, P. M. *Chem. Rev.* **2007**, *107*, 5416−5470.
(17) Mukherjee, S.; Yang, J. W.; Hoffmann, S.; List, B. *Chem. Rev.* **2007**, *107*, 5471−5569.
(18) Zandvoort, E.; Geertsema, E. M.; Baas, B.-J.; Quax, W. J.; Poelarends, G. J. *Angew. Chem., Int. Ed.* **2012**, *51*, 1240−1243.
(19) Geertsema, E. M.; Miao, Y.; Tepper, P. G.; Dehaan, P.; Zandvoort, E.; Poelarends, G. J. *Chem. - Eur. J.* **2013**, *19*, 14407−14410.
(20) Van Der Meer, J. Y.; Poddar, H.; Baas, B. J.; Miao, Y.; Rahimi, M.; Kunzendorf, A.; Van Merkerk, R.; Tepper, P. G.; Geertsema, E. M.; Thunnissen, A. M. W. H.; Quax, W. J.; Poelarends, G. J. *Nat. Commun.* **2016**, *7*, 10911.
(21) Poddar, H.; Rahimi, M.; Geertsema, E. M.; Thunnissen, A. M. W. H.; Poelarends, G. J. *ChemBioChem* **2015**, *16*, 738−741.
(22) Melchiorre, P. *Angew. Chem., Int. Ed.* **2012**, *51*, 9748−9770.
(23) Schmidt-Dannert, C.; Lopez-Gallego, F. *Microb. Biotechnol.* **2016**, *9*, 601−609.
(24) Oroz-Guinea, I.; García-Junceda, E. *Curr. Opin. Chem. Biol.* **2013**, *17*, 236−249.
(25) Santacoloma, P. A.; Woodley, J. M. In *Cascade Biocatalysis*; Riva, S., Fessner, W.-D., Eds.; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, Germany, 2014; pp 231−248.
(26) Szekrenyi, A.; Garrabou, X.; Parella, T.; Joglar, J.; Bujons, J.; Clapés, P. *Nat. Chem.* **2015**, *7*, 724−729.

DOI: 10.1021/jacs.6b11928
J. Am. Chem. Soc. 2017, 139, 103−106