

# Carboxylic acid reductase enzymes (CARs)

Margit Winkler<sup>1,2</sup>

<sup>1</sup> Institute of Molecular Biotechnology, Graz University of Technology, Petersgasse 14, 8010 Graz, Austria

<sup>2</sup> acib GmbH, Petersgasse 14, 8010 Graz, Austria

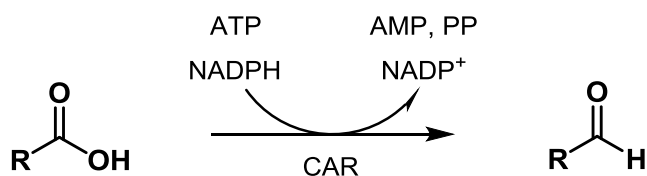
Carboxylate reductases (CARs) are emerging as valuable catalysts for the selective one-step reduction of carboxylic acids to their corresponding aldehydes. The substrate scope of CARs is exceptionally broad and offers potential for their application in diverse synthetic processes. Two major fields of application are the preparation of aldehydes as end products for the flavor and fragrance sector and the integration of CARs in cascade reactions with aldehydes as the key intermediates. The latest applications of CARs are dominated by *in vivo* cascades and chemo-enzymatic reaction sequences. The challenge to fully exploit product selectivity is discussed. Recent developments in the characterization of CARs are summarized, with a focus on aspects related to the domain architecture and protein sequences of CAR enzymes.

\*Corresponding author: M. Winkler, [margit.winkler@tugraz.at](mailto:margit.winkler@tugraz.at), +43 316 873 9333

*Abbreviations:* CAR: carboxylate reductase; ATP: adenosine 5'-triphosphate; AMP: adenosine 5'-monophosphate; NADPH:  $\beta$ -Nicotinamide adenine dinucleotide phosphate; A: adenylation; T: transthiolation; R: reductase;

## Introduction

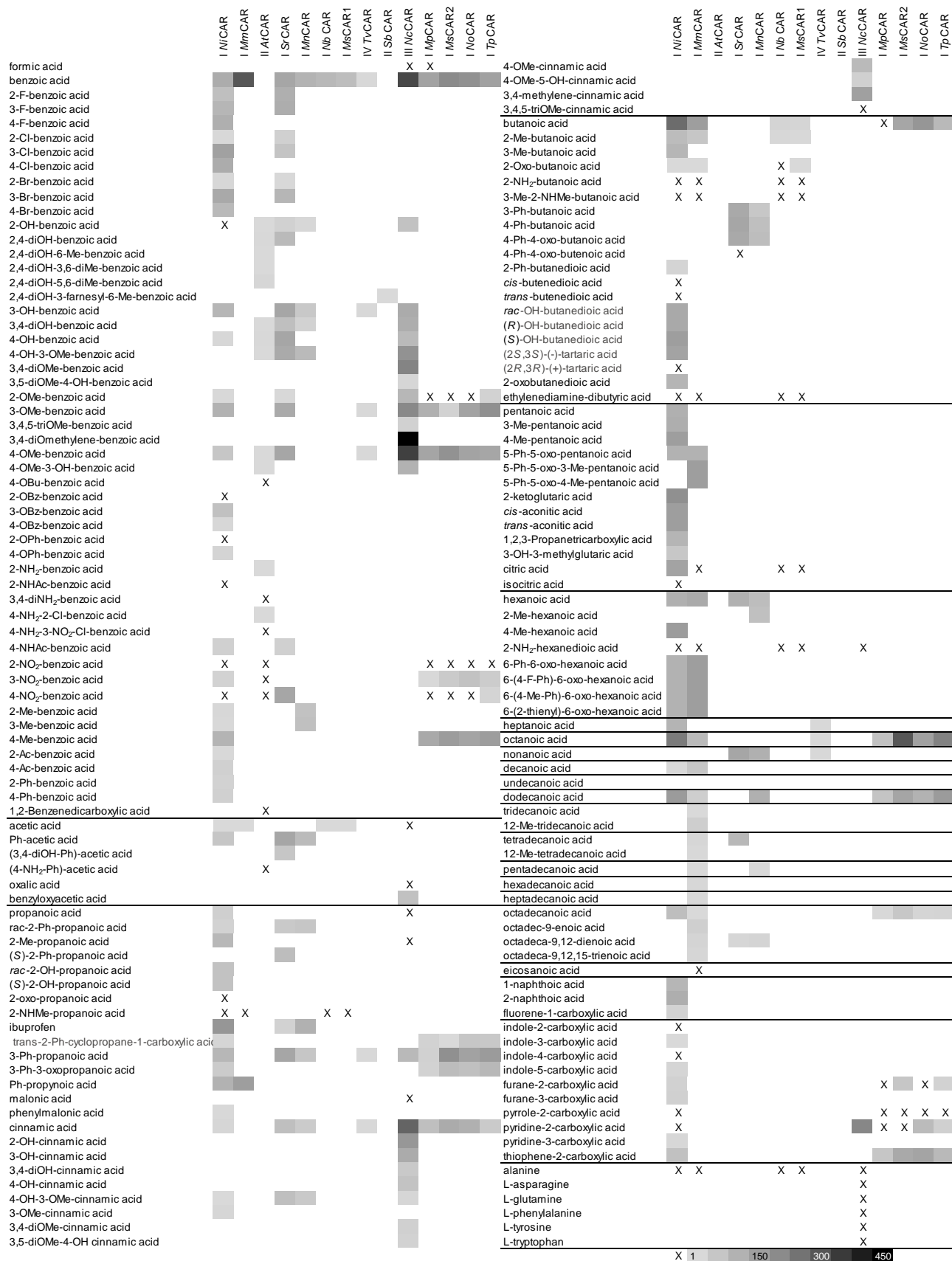
Carboxylic acid reductases (CARs) catalyze the reduction of a carboxylic acid substrate to the corresponding aldehyde (Scheme 1). EC 1.2.1.30 CARs activate the carboxylate substrate at the expense of ATP and catalyze the reduction step using NADPH as the hydride donor [1]. This activity has long been observed as a metabolic reaction of numerous fungi [2],[3] and pioneering work on the characterization of the first CAR protein, purified from the fungus *Neurospora crassa* (NcCAR), was reported in the 1970s [4]. The first amino acid sequence of a CAR was elucidated from a bacterial source (*Nocardia iowensis*, NiCAR) [5]. For years, NiCAR occupied a unique role in the scientific literature until the first homologous sequence was published in 2013 [6]. In the last two years, considerable interest in CARs is reflected by new enzymes being reported from various labs worldwide, and new concepts for the application of CARs in synthetic routes. This review focuses on the last three years of research on recombinant CARs, as only sequence information allows for the application of an enzyme in biocatalysis on scale, be it *in vitro* or *in vivo*.



**Scheme 1** CARs catalyze the direct and selective two-electron reduction of organic acids to aldehydes. Four-electron reduction to alcohols has not been observed with wild-type CARs.

### The broad substrate scope of CARs

The potential of CARs for bioorganic synthesis is underlined by the relaxed substrate tolerance of this enzyme class. Figure 1 gives a semi-quantitative comparison of CAR activities for diverse substrates, ranging from aliphatic acids to aromatic, poly- and heterocyclic acids. This summary includes data from recombinant CARs (type I: *Nocardia iowensis* NiCAR #Q6RKB1.1; *Mycobacterium marinum* MmCAR #WP\_012393886.1; *Segniliparus rotundus* SrCAR #WP\_013138593.1; *Mycobacterium neoaurum* MnCAR #WP\_019810583.1; *Nocardia brasiliensis* NbCAR #AFU02004.1; *Mycobacterium smegmatis* MsCAR1 #WP\_015306631.1 and MsCAR2 #AFP42026.1; *Mycobacterium phlei* MpCAR #WP003889896.1; *Nocardia otitidiscaviarum* NoCAR #WP\_029928026.1; *Tsukamurella paurometabola* TpCAR #WP\_013126039.1; type II: *Aspergillus terreus* AtCAR #XP\_001212808.1 *Stachybotrys bisbyi* SbCAR #BAV19380.1; type III: *Neurospora crassa* NcCAR #YP\_955820.1; type IV: *Trametes versicolor* TvCAR #XP\_008043822.1;) and data from NiCAR and NcCAR isolated from their natural hosts [2,5–22]. Reported non-substrates are also included in Figure 1. The substrate specificities of CARs largely overlap, but there are exceptions. SrCAR, e.g., was the only CAR reducing a nitro-substituted benzoic acid derivative [14]. CARs were historically named aryl-aldehyde dehydrogenase (NADP<sup>+</sup>), indicating selectivity for aromatic substrates, however, short to medium chain aliphatic acids are also efficiently reduced. Octanoic acid, e.g., is reduced by recombinant NiCAR with a  $k_{cat}$  of 233 min<sup>-1</sup> [16], which is even higher than typical reports for the reduction of the aromatic standard substrate benzoic acid. In general, the substrate scope of CAR is exceptionally broad, ranging from aliphatic compounds (from C2 to C18) to (hetero)aromatic compounds and a wide range of substituents is tolerated. A clear limitation, so far, seem to be polar groups adjacent to the carboxylate moiety. This conclusion is corroborated by results of the Yakunin group [23].



**Figure 1 Overview of substrate specificities of recombinant as well as purified CARs.** The heat map represents CAR activities reported in the literature. Black indicates highest activities ( $k_{cat} 450 \text{ min}^{-1}$ ) and light grey (1) indicates lowest activities ( $k_{cat} \leq 1 \text{ min}^{-1}$ ). X represents reports of non-activity under the tested conditions. White: not described. For some substrate/CAR combinations,  $k_{cat}$  could not be calculated because reported data was based on endpoint

measurements (e.g. [10][18,19]); the respective entries are estimations. In case of *Sr*CAR and *Mn*CAR, e.g., kinetic data were available for benzoic acid. Activities for all other compounds were estimated based on reported relative yields and may be underestimated [14,15]. CAR types are indicated as roman numbers in front of their abbreviations in the headline.

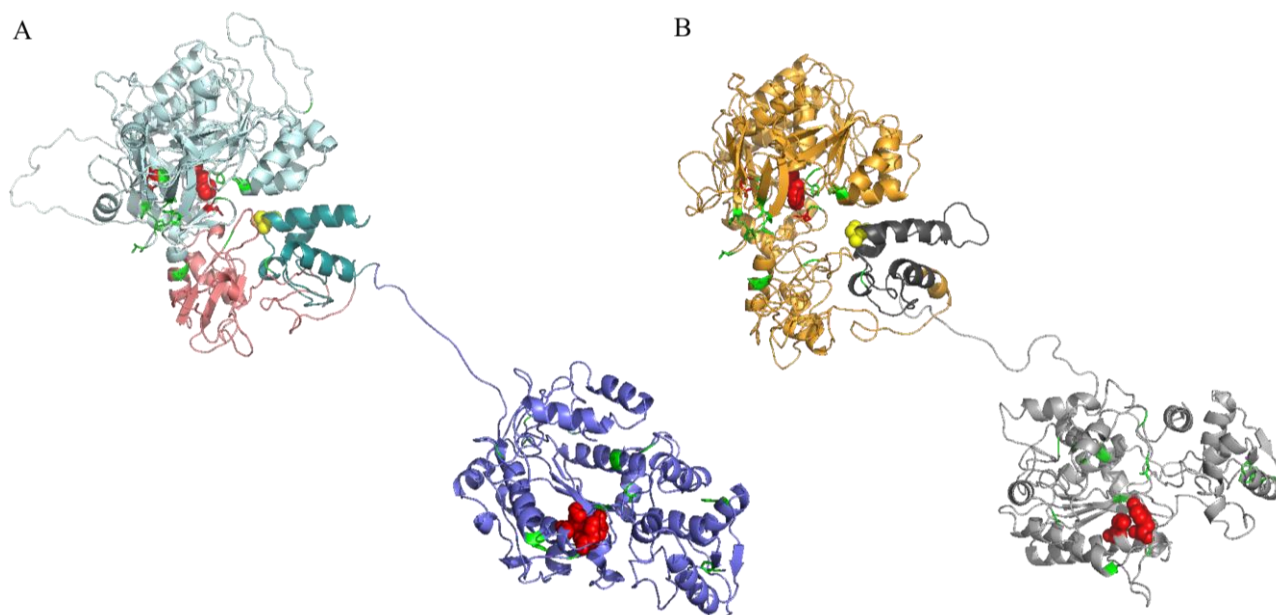
### **Domain architecture and mechanistic features of CARs**

EC 1.2.1.30 CARs consist of three domains: an adenylation domain (A-domain), a transthioation domain (T-domain), and a reductase-domain (R-domain) (Figure 2). A conserved serine in the T-domain (Figure 2, yellow spheres) is post-translationally modified by a phosphopantetheinyl transferase [24]. The modular domain architecture with A- and T-domains shows similarities to non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS), in which the domains are also connected by flexible linkers, and a substrate is shuttled from the *N*- to the *C*-terminus. Owing to this modularity, a recent phylogenetic study suggested four distinct CAR subgroups. One group consists of bacterial (type I) and the other three groups of fungal CARs (type II-IV). The bacterial group is characterized by an extra C-terminal stretch in the A-domain (Figure 2A, salmon region) [25,26]. Figure 2 shows models of the type I *Ni*CAR in comparison to the type III *Nc*CAR. Despite subtle differences, the overall architecture is similar. Notably, the enzymatic activity of Type II and IV CARs appears to be significantly lower in comparison to type I and III CARs



CARs start the catalytic cycle by activation of the carboxylate substrate. The precise mechanism of the reaction cascade from acid to aldehyde is not yet fully proven experimentally and represents an area for further studies. In our current understanding, the first step is the activation of the carboxylate as an AMP-ester at the expense of ATP. The second step is a transesterification of the AMP-ester by the thiol of the phosphopantetheine to a thioester, which binds the acyl moiety covalently to the CAR. Next, the T-domain turns and transfers the substrate from the A-domain towards the R-domain in a swinging arm fashion [27]. Finally, the thioester is reduced in the R-domain by NADPH [5]. The dynamics and driving force of each single step on the molecular level needs experimental confirmation and may be rationalized by mutational and kinetic studies similarly to comprehensive NRPS domain alteration studies [28]. At present, first mutational studies are becoming available with the aim to identify key residues. The reductase domain of NRPS proteins was proposed to consist of the catalytic triad T, Y and K in similarity to a tyrosine-dependent short chain dehydrogenase family [29]. The tyrosine and lysine were confirmed to be essential in *At*CAR [30] and *Nc*CAR [25] (Y863 and K867 as well as Y844 and K848, respectively), however, threonines are not conserved in CAR reductase domains. In the A-domain of *Nc*CAR, the exchange of H237 and E433 for alanine abolished activity, indicating a catalytic role of these residues. Structural information for full length CARs is not yet available, but a NRPS crystal structure was solved in 2016 [31]. These new insights provide a sound basis to understand CARs in more detail on the molecular level and are expected to inspire CAR engineering. Until very recently, mutational studies were guided by molecular models. A Phyre2 [32] model of type I CARs shown in Figure 2A is based on five templates for the A-domain (pdb codes 5ja2, 5t3d, 4zxi, 5es8 and 2vsq; all are NRPS ligase modules) and one for the R-domain (pdb 4dqv; a reductase domain of a NRPS from *Mycobacterium tuberculosis*). These templates form also the basis for the *Nc*CAR model, but an additional sequence (pdb 4r0m) was used as a template for the A-domain. It needs to be stressed, however, that due to low similarities of these templates and CARs, particularly in the A-domain, the quality of these models is poor. Only in July 2017, first crystal structures of individual CAR domains with and without T-domain and a full length model of a new CAR from *Segniliparus rotundus* were published [26].

A sequence alignment of all experimentally confirmed CARs allowed to retrieve a characteristic CAR signature consisting of four regions with conserved residues in the A-domain, the region around the phosphopantetheinylated serine in the T-domain and three regions with conserved residues in the R-domain (Figure 2, green and red residues) [25]. This information is valuable to guide the identification of new CAR sequences. Owing to the modular domain architecture, domain- or subdomain-swapping may be a viable engineering approach for CARs, alternative to classical protein engineering methods [33,34].



**Figure 2 Phyre2 models of carboxylate reductases depict the three-domain architecture. A:** *NiCAR* (type I CAR). The A-domain is depicted top left in pale cyan, the mobile C-terminal domain in salmon, the T-domain in deep teal, and the R-domain bottom right in blue; All templates <55% identity to *NiCAR*. **B:** *NcCAR* (type III CAR). The A-domain is depicted top left in orange, the T-domain in dark grey, and the R-domain bottom right in medium grey. Yellow serines in the T-domains represent the position of the phosphopantetheine attachment site. Green residues are conserved within the superfamily of CARs. Red residues mark catalytic residues (experimentally confirmed in *NcCAR*, putative in *NiCAR*). All templates <25% identity to *NcCAR*.

### Synthetic applications of CARs

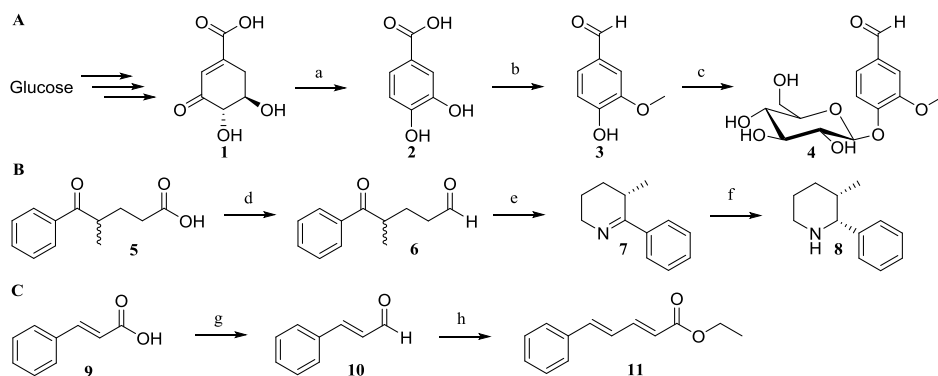
The most extensively studied application of CARs is the preparation of vanillin (**3**) [10,11,35,36] [37]. Like many other aldehydes, vanillin plays an important role as a flavor and fragrance component of food as well as body- and home-care products [38,39]. An elegant *de novo* route in yeasts utilizes *NiCAR* for synthesis of **3**. Undesired product removal by host enzymes was tackled by a selective gene knock out or *in-situ* product removal by inserting a glycosyltransferase in order to mask **3** as vanillin  $\beta$ -D-glucoside (**4**) (Figure 3A) [36]. Methyl branched aliphatic aldehydes, for example, impart the flavor impression of savory foods. *MmCAR* was used in form of a whole cell biocatalyst to prepare these target compounds from fungal lipid extracts with particularly prominent amounts of the respective methyl branched aliphatic acids [17]. Due to the demand for ATP and NADPH, microbial cells are currently considered to be the most economic option for the use of CARs for preparative purposes [21]. These cells may be equipped with additional enzymes to immediately convert the reactive and sometimes toxic aldehyde species further. *NiCAR* expressed in an engineered *E. coli* strain was coupled with a pyruvate decarboxylase to prepare L-phenylacetylcarbinol from the condensation product of benzaldehyde and pyruvate [37]. Another *in vivo* cascade took advantage of endogenous ADH activity of *E. coli* for the *NiCAR* mediated

preparation of the antioxidant 3-hydroxytyrosol [40]. The ability of *MmCAR* to reduce aliphatic acids enabled the development of a multi enzyme cascade reaction starting from aliphatic keto acids. *MmCAR*, in form of a whole cell biocatalyst, catalyzed the first step in this cascade, followed by enzymatic transamination, intramolecular cyclization and enantioselective enzymatic imine reduction to give chiral mono- or disubstituted piperidines and pyrrolidines. A preparative example is shown in Figure 3B [18]. When biosynthetic pathways to fatty acids are coupled to CARs and decarboxylases, short to medium chain alkanes become accessible[6,41,32]. Since these alkanes are bulk products in the fuel sector, it remains elusive if this technology may become competitive in future.

The selectivity of aldehyde preparation fuels the progress on CAR research. However, this product selectivity can not yet be fully exploited. The reason is the co-factor demand, which is mostly fulfilled by cellular systems to avoid the high costs of these co-substrates. The use of cells, however, is accompanied by undesired reactions which are catalyzed by the organism's protein machinery. Using CARs in cellular systems leads to a trade-off between ATP/NADPH supply and product selectivity, because cellular background reactivities tend to remove reactive aldehyde species. Strain engineering was used to reduce overreduction of aromatic substrates by host proteins [42,43]. This strategy was extremely effective for particular substrates but much less for others [21]. *In vivo* cascades may be optimized by tuning the aldehyde amount to low levels e.g. by balancing the reaction rates of subsequent transformations [32]. Another strategy to optimize yields in cascades involving aldehyde intermediates is channelling [44], for example by scaffolding proteins [45] or using microcompartments [46]. Ultimately, the full potential of CARs product selectivity may also be exploited in cell free systems. To reach this goal, the recycling of NADPH and ATP *in vitro* need to be implemented.

Apart from the use of CARs in cascades *in vivo*, a one-pot chemoenzymatic *in vitro* cascade from 4-hydroxybenzoic acid to 4-hydroxybenzaloxime was reported with *AtCAR* [13]. Similarly, *MnCAR* reduction provided aldehydes for a subsequent Wittig reaction to yield  $\alpha,\beta$ -unsaturated esters, such as the ester derived from cinnamic acid **9** (Figure 3C) [15]. The *in vitro* use of CARs was so far hampered by a lack of efficient methods for the recycling of ATP, however, recent progress in ATP recycling will certainly facilitate the development of *in vitro* systems in the near future [47].





**Figure 3 Selected examples of CAR mediated syntheses.** **A:** *de novo* synthetic pathway from glucose in yeasts. a) 3-dehydroshikimic acid **1** is transformed to 3,4-dihydroxybenzoic acid **2** by 3-dehydroshikimate dehydratase. b) NiCAR and a O-methyltransferase reduce the acid and introduce a methyl group, resp., to give vanillin **3**. c) glucose is attached to **3** by a glycosyltransferase to yield **4** (adapted from Ref [36]); **B:** Example for one-pot reaction cascade d) ketoacid **5** is reduced by *Mm*CAR in form of a whole cell biocatalyst to aldehyde **6**. e) **6** is converted to an amine by an ω-transaminase and the product undergoes intramolecular cyclization to imine **7**. f) (*S*) selective imine reductase affords (2*R*,3*S*)-**8** (adapted from Ref [18]); **C:** Example for sequential chemo-enzymatic route. g) cinnamic acid **9** is reduced to cinnamaldehyde **10** using *Mn*CAR *in vitro* with NADPH recycling. h) **10** is coupled to ethylacrylate in a Wittig type reaction (adapted from Ref [15]).

## Conclusions

A current trend is to generate diversity in the CAR toolbox. The goals are to identify and characterize CARs with outstanding properties such as thermostability or high activities for particular substrates. There is a need to understand more about the structure-function relationship of CARs to allow focused engineering of known protein scaffolds towards these goals.

CARs reduce carboxylates to aldehydes and do not reduce the aldehyde any further, although aldehydes are significantly more prone to reduction than acids. From the application viewpoint, this product selectivity of carboxylate reducing enzymes is a highly appealing feature, which can, however, not yet be fully exploited. The demand for ATP and NADPH is mostly fulfilled by cellular systems to save costs, but cellular background reactivities diminish theoretically perfect aldehyde levels by side reactions. *In vitro* applications may become a solution to this dilemma.

It seems likely that CAR enzymes will find further applications in the production of volatiles for the flavor and fragrance sector. Another trend and major field of application is the integration of CARs in cascade reactions with aldehydes as the key intermediate. Hence, CARs can be expected to contribute in various reaction cascades towards pharmaceutical products and fine chemicals in the near future.

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## References

[3] \*This review summarizes carboxylate reductions comprehensively. Not only bioreductions catalyzed by the EC1.2.1.30 enzyme family but also by non-homologous isofunctional enzymes of other protein families are discussed.

[4] \*\*First report on acid reduction activity. Fungal mycelium was incubated with aromatic acids and the conversion to aldehyde and alcohol was observed.

[5] \*\* Identification and recombinant expression of the first CAR protein. This paper is the cornerstone for enzymatic carboxylate reduction technology

[6] \* First report of a bacterial CAR with the ability to reduce aliphatic acids

[30]\* This paper links CAR activity to NRPS-like proteins and reports the first sequence of a fungal CAR

[36] \*\* Demonstration of *de novo* biosynthesis of vanillin using NiCAR to catalyze vanillic acid reduction in yeasts, a strategy patented by Evolva.

1. Gross GG: **Evidence for enzyme-substrate intermediates in the aryl-aldehyde: NADP oxidoreductase catalysed reduction of salicylate.** *FEBS Lett.* 1969, **5**:177–179.
2. Gross GG, Bolkart KH, Zenk MH: **Reduction of cinnamic acid to cinnamaldehyde and alcohol.** *Biochem. Biophys. Res. Commun.* 1968, **32**:173–178.
3. Napora-Wijata K, Strohmeier GA, Winkler M: **Biocatalytic reduction of carboxylic acids.** *Biotechnol. J.* 2014, **9**:822–843.
4. Farmer VC, Henderson MEK, Russell JD: **Reduction of certain aromatic acids to aldehydes and alcohols by *Polystictus versicolor*.** *Biochim. Biophys. Acta* 1959, **35**:202–211.
5. He A, Li T, Daniels L, Fotheringham I, Rosazza JPN: ***Nocardia* sp . Carboxylic Acid Reductase : Cloning , Expression , and Characterization of a New Aldehyde Oxidoreductase Family.** *Appl. Environ. Microbiol.* 2004, **70**:1874–1881.
6. Akhtar MK, Turner NJ, Jones PR: **Carboxylic acid reductase is a versatile enzyme for the conversion of fatty acids into fuels and chemical commodities.** *Proc. Natl. Acad. Sci. U. S. A.* 2013, **110**:87–92.
7. Gross GG, Zenk MH: **Reduktion aromatischer Säuren zu Aldehyden und Alkoholen im zellfreien System.** *Eur. J. Biochem.* 1969, **8**:413–419.

8. Gross GG: **Formation and Reduction of Intermediate Acyladenylate by Aryl-Aldehyde. NADP Oxidoreductase from *Neurospora crassa*.** *Eur. J. Biochem.* 1972, **31**:585–592.
9. Li T, Rosazza JP: **Purification, characterization, and properties of an aryl aldehyde oxidoreductase from *Nocardia* sp. strain NRRL 5646.** *J. Bacteriol.* 1997, **179**:3482–7.
10. Li K, Frost JW: **Synthesis of Vanillin from Glucose.** *J. Am. Chem. Soc.* 1998, **120**:10545–10546.
11. Venkitasubramanian P, Daniels L, Das S, Lamm AS, Rosazza JPN: **Aldehyde oxidoreductase as a biocatalyst: Reductions of vanillic acid.** *Enzyme Microb. Technol.* 2008, **42**:130–137.
12. Sheppard MJ, Kunjapur AM, Wenck SJ, Prather KLJ, Rosazza JPN: **Retro-biosynthetic screening of a modular pathway design achieves selective route for microbial synthesis of 4-methyl-pentanol.** *Nat. Commun.* 2014, **5**:5031.
13. Wang M, Zhao H: **Characterization and Engineering of the Adenylation Domain of a NRPS-Like Protein: A Potential Biocatalyst for Aldehyde Generation.** *ACS Catal.* 2014, **4**:1219–1225.
14. Duan Y, Yao P, Chen X, Liu X, Zhang R, Feng J, Wu Q, Zhu D: **Exploring the synthetic applicability of a new carboxylic acid reductase from *Segniliparus rotundus* DSM 44985.** *J. Mol. Catal. B Enzym.* 2015, **115**:1–7.
15. Duan Y, Yao P, Du Y, Feng J, Wu Q, Zhu D: **Synthesis of  $\alpha,\beta$ -unsaturated esters via a chemo-enzymatic chain elongation approach by combining carboxylic acid reduction and Wittig reaction.** *Beilstein J. Org. Chem.* 2015, **11**:2245–2251.
16. Finnigan W, Thomas A, Cromas H, Gough B, Snajdrova R, Adams J, Littlechild JA, Harmer N: **Characterization of carboxylic acid reductases as enzymes in the toolbox for synthetic chemistry.** *ChemCatChem* 2017, **9**:899–1143.
17. Fraatz MA, Goldmann M, Geissler T, Gross E, Backes M, Hilmer J-M, Ley J, Rost J, Francke A, Zorn H: **Biotechnological Production of Methyl-Branched Aldehydes.** *J. Agric. Food Chem.* 2016, doi:10.1021/acs.jafc.6b04793.
18. France SP, Hussain S, Hill AM, Hepworth LJ, Howard RM, Mulholland KR, Flitsch SL, Turner NJ: **One-Pot Cascade Synthesis of Mono- and Disubstituted Piperidines and Pyrrolidines using Carboxylic Acid Reductase (CAR),  $\omega$ -Transaminase ( $\omega$ -TA), and Imine Reductase (IRED) Biocatalysts.** *ACS Catal.* 2016, **6**:3753–3759.
19. Li C, Matsuda Y, Gao H, Hu D, Yao XS, Abe I: **Biosynthesis of LL-Z1272 $\beta$ : Discovery of a New Member of NRPS-like Enzymes for Aryl-Aldehyde Formation.** *ChemBioChem* 2016, **17**:904–907.
20. Moura M, Pertusi D, Lenzini S, Bhan N, Broadbelt LJ, Tyo KEJ: **Characterizing and predicting carboxylic acid reductase activity for diversifying bioaldehyde production.** *Biotechnol. Bioeng.* 2016, **113**:944–952.
21. Schwendenwein D, Fiume G, Weber H, Rudroff F, Winkler M: **Selective Enzymatic Transformation to Aldehydes *in vivo* by Fungal Carboxylate Reductase from *Neurospora crassa*.** *Adv. Synth. Catal.* 2016, **358**:3414–3421.
22. Winkler M, Winkler CK: ***Trametes versicolor* carboxylate reductase uncovered** *Monatshefte für Chemie - Chem. Mon.* 2016, **147**:575–578.

23. Khusnutdinova AN, Flick R, Popovic A, Brown G, Tchigvintsev A, Nocek B, Correia K, Joo JC, Mahadevan R, Yakunin AF: **Exploring bacterial carboxylate reductases for the reduction of bifunctional carboxylic acids** *Biotechnol. J.* 2017, doi:10.1002/biot.201600751.
24. Venkitasubramanian P, Daniels L, Rosazza JPN: **Reduction of carboxylic acids by *Nocardia* aldehyde oxidoreductase requires a phosphopantetheinylated enzyme.** *J. Biol. Chem.* 2007, **282**:478–485.
25. Stolterfoht H, Schwendenwein D, Sensen CW, Rudroff F, Winkler M: **Four distinct types of E.C. 1.2.1.30 enzymes can catalyze the reduction of carboxylic acids to aldehydes.** *J. Biotechnol.* 2017, **257**:222–232.
26. Gahloth D, Dunstan MS, Quaglia D, Klumbys E, Lockhart-Cairns MP, Hill AM, Derrington SR, Scrutton NS, Turner NJ, Leys D: **Structures of carboxylic acid reductase reveal domain dynamics underlying catalysis.** *Nat. Chem. Biol.* 2017, **13**:975–981.
27. Venkitasubramanian P, Daniels L, Rosazza JPN: **Biocatalytic Reduction of Carboxylic Acids: Mechanism and Applications.** In *Biocatalysis in the Pharmaceutical and Biotechnology Industries*. Edited by Patel RN. CRC Press; 2006:425–438.
28. Gulick AM: **Conformational Dynamics in the Acyl-CoA Synthetases, Adenylation Domains of Non-ribosomal Peptide Synthetases, and Firefly Luciferase.** *ACS Chem. Biol.* 2009, **4**:811–827.
29. Chhabra A, Haque AS, Pal RK, Goyal A, Rai R, Joshi S, Panjekar S, Pasha S, Sankaranarayanan R, Gokhale RS: **Nonprocessive [2 + 2]<sub>e</sub>- off-loading reductase domains from mycobacterial nonribosomal peptide synthetases.** *Proc. Natl. Acad. Sci. U. S. A.* 2012, **109**:5681–6.
30. Wang M, Beissner M, Zhao H: **Aryl-aldehyde formation in fungal polyketides: discovery and characterization of a distinct biosynthetic mechanism.** *Chem. Biol.* 2014, **21**:257–63.
31. Drake EJ, Miller BR, Shi C, Tarrasch JT, Sundlov JA, Leigh Allen C, Skiniotis G, Aldrich CC, Gulick AM: **Structures of two distinct conformations of holo-non-ribosomal peptide synthetases.** *Nature* 2016, **529**:235–238.
32. Kallio P, Pásztor A, Thiel K, Akhtar MK, Jones PR: **An engineered pathway for the biosynthesis of renewable propane.** *Nat. Commun.* 2014, **5**:4731.
33. Reetz MT: **Laboratory Evolution of Stereoselective Enzymes: A Prolific Source of Catalysts for Asymmetric Reactions.** *Angew. Chemie Int. Ed.* 2011, **50**:138–174.
34. Kries H: **Biosynthetic engineering of nonribosomal peptide synthetases.** *J. Pept. Sci.* 2016, **22**:564–570.
35. Falconnier B, Lapierre C, Lesage-Meessen L, Yonnet G, Brunerie P, Colonna-Ceccaldi B, Corrieu G, Asther M: **Vanillin as a product of ferulic acid biotransformation by the white-rot fungus *Pycnoporus cinnabarinus* I-937: Identification of metabolic pathways.** *J. Biotechnol.* 1994, **37**:123–132.
36. Hansen EH, Møller BL, Kock GR, Bünner CM, Kristensen C, Jensen OR, Okkels FT, Olsen CE, Motawia MS, Hansen J: **De novo biosynthesis of vanillin in fission yeast (*Schizosaccharomyces pombe*) and baker's yeast (*Saccharomyces cerevisiae*).** *Appl. Environ. Microbiol.* 2009, **75**:2765–74.
37. Kunjapur AM, Tarasova Y, Prather KLJ: **Synthesis and accumulation of aromatic aldehydes in an engineered strain of *Escherichia coli*.** *J. Am. Chem. Soc.* 2014, **136**:11644–11654.

38. Sell C: *The chemistry of fragrances : from perfumer to consumer*. RSC Publishing; 2006.
39. Carroll AL, Desai SH, Atsumi S: **Microbial production of scent and flavor compounds**. *Curr. Opin. Biotechnol.* 2016, **37**:8–15.
40. Napora-Wijata K, Robins K, Osorio-Lozada A, Winkler M: **Whole-Cell Carboxylate Reduction for the Synthesis of 3-Hydroxytyrosol**. *ChemCatChem* 2014, **6**:1089–1095.
41. Sheppard MJ, Kunjapur AM, Prather KLJ: **Modular and selective biosynthesis of gasoline-range alkanes**. *Metab. Eng.* 2016, **33**:28–40.
42. Rodriguez GM, Atsumi S: **Toward aldehyde and alkane production by removing aldehyde reductase activity in *Escherichia coli***. *Metab. Eng.* 2014, **25**:227–37.
43. Kunjapur AM, Prather KLJ: **Microbial engineering for aldehyde synthesis**. *Appl. Environ. Microbiol.* 2015, **81**:1892–1901.
44. Wheeldon I, Minter SD, Banta S, Barton SC, Atanassov P, Sigman M: **Substrate channelling as an approach to cascade reactions**. *Nat. Chem.* 2016, **8**:299–309.
45. Dueber JE, Wu GC, Malmirchegini GR, Moon TS, Petzold CJ, Ullal A V, Prather KLJ, Keasling JD: **Synthetic protein scaffolds provide modular control over metabolic flux**. *Nat. Biotechnol.* 2009, **27**:753–759.
46. Lee MJ, Brown IR, Juodeikis R, Frank S, Warren MJ: **Employing bacterial microcompartment technology to engineer a shell-free enzyme-aggregate for enhanced 1,2-propanediol production in *Escherichia coli***. *Metab. Eng.* 2016, **36**:48–56.
47. Mordhorst S, Siegrist J, Müller M, Richter M, Andexer JN: **Catalytic Alkylation Using a Cyclic S - Adenosylmethionine Regeneration System**. *Angew. Chemie Int. Ed.* 2017, doi:10.1002/anie.201611038.