Cell-free in vitro reduction of carboxylates to aldehydes: With crude enzyme preparations to a key pharmaceutical building block

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Abstract
The scarcity of practical methods for aldehyde synthesis in chemistry necessitates the development of mild, selective procedures. Carboxylic acid reductases catalyze aldehyde formation from stable carboxylic acid precursors in an aqueous solution. Carboxylic acid reductases were employed to catalyze aldehyde formation in a cell-free system with activation energy and reducing equivalents provided through auxiliary proteins for ATP and NADPH recycling. In situ product removal was used to suppress over-reduction due to background enzyme activities, and an N-protected 4-formylpiperidine pharma synthon was prepared in 61% isolated yield. This is the first report of preparative aldehyde synthesis with carboxylic acid reductases employing crude, commercially available enzyme preparations.

KEYWORDS
aldehyde, biocatalysis, carboxylic acid reductase (CAR), in vitro cofactor recycling, pharma synthon

1 INTRODUCTION

Aldehydes are key intermediates in medicinal chemistry. Their reactivity is a desired feature but also creates challenges during their synthesis. Classical methods for the synthesis of aldehydes encompass numerous examples of oxidations at specific reaction centers. Benzylic positions can be oxidized by using a NaClO/TEMPO/Co(OAc)₂ system or IBX. Terminal double bonds can be efficiently oxidized by O₂ applying Wacker conditions. Ozonolysis offers another facile, atom-efficient transformation of alkenes to aldehydes following the reductive workup of the resulting ozonides. Hydration of terminal triple bonds by appropriate choice of Ru-catalysts was also found to produce aldehydes in good to excellent yields. General chemical approaches towards aldehydes from the corresponding carboxylic acids typically require multi-step procedures as the direct reduction to aldehyde is not feasible, leading to over-reduction to the alcohol. To solve this issue, a complete reduction of the carboxylic acid or its derivatives to c is often employed with subsequent re-oxidation. Typical procedures involve either DIBAL-H or LiAlH₄ as reductant and chromium (VI) reagents, hypervalent iodine compounds, sulfonium species, TPAP/NMO or TEMPO/NaOCl systems as selective oxidants. Although DIBAL-H can offer direct access to aldehydes with the reduction of the corresponding esters and nitriles, the reaction generally results in low yields and requires strict and careful temperature control.
An enzymatic approach can circumvent this issue: carboxylic acid reductases (CARs) are a class of enzymes that selectively reduce carboxylic acids to aldehydes in a single step at the expense of ATP and NADPH (Scheme 1).\[19–21\] Thus, CARs are valuable tools for the synthesis of vanillin,\[22\] piperonal,\[23\] and other aldehydes\[24\] or related alcohols\[25\] with desired olfactory properties. Several applications of CARs have been shown that aim for follow-up products of aldehydes: among these are alcohols\[26\] and alkanes (for applications like lubricants or fuels),\[27–29\] amines,\[30\] diamines,\[31\] chiral amines, and so on, ranging from simple, small molecules\[32\] to complex chiral building blocks.\[33\]

Due to their co-factor demands, CARs are typically applied in whole-cell systems, taking advantage of cellular metabolism for the co-factor supply.\[34\] However, within today’s chemical synthesis community there is largely a reluctance to use reactions involving living cells. Specific equipment requirements, the complexity of fermentation optimization, and biomass removal challenges during product isolation have refocused industry towards isolated enzyme processes.\[35,36\] A strong need for cell-free methods for the preparation of valuable aldehyde intermediates from stable precursors therefore motivates the development of effective protocols. Given real-world applications in synthetic laboratories and later in production scale facilities, the clear goal is to design a method for preparative scale, single-step aldehyde synthesis with practical and commercially available crude enzyme preparations.

For a cell-free CAR mediated reaction system, ATP and NADPH recycling are required, and the key is to implement a balanced system of compatible enzymes and their respective substrates. We recently showed the proof of concept of CAR mediated aldehyde formation with cell-free co-factor recycling. Purified CAR was utilized in the presence of two purified polyphosphate kinases (PPKs) for ATP recycling, a purified pyrophosphatase (PPase) for the elimination of the CAR inhibiting pyrophosphate\[37\] and commercially available glucose dehydrogenase (GDH) for NADPH recycling (Scheme 1).\[38\]

Herein, we targeted the synthesis of a typical medicinal chemistry building block – the Cbz-protected 4-formyl piperidine aldehyde (1b). This compound has found many applications, and its prevalent use is in reductive aminations forming secondary or tertiary amines.\[39–41\] Spirocyclic indoles are also accessible via the acid-catalyzed Fischer reaction with substituted aryl hydrazines.\[42\] Applying appropriate work-up conditions further enables access to corresponding indolinones\[43\] and indolines.\[43–47\] Robinson-type annulation with methylvinylketone delivers 3-azaspirodecanones, which were used for the synthesis of several biologically active compounds.\[48–50\] Furthermore, the aldehyde moiety was addressed for classical condensation reactions with indanones,\[51\] Wittig reagents,\[52\] malonic esters, and ethyl cyanoacetate\[53\] (Scheme 2). A standard procedure of 1b synthesis is the reduction from the corresponding esters using DIBAL-H at −78°C requiring strict temperature control and typically resulting in mediocre yields of the product.\[54,55\] Alternatively, a Weinreb amide can be synthesized from an activated carboxylic acid species and directly reduced at ambient temperature employing LiAlH₄.\[42\] Known
back-oxidations of 1c to 1b are either carried out at low temperatures applying Swern-oxidation conditions,[56–58] using highly toxic chromium species,[46,59] or require expensive reagents (Dess-Martin periodinane)[60] (Scheme 2).

2 | MATERIALS AND METHODS

2.1 | Chemicals

N-Cbz-piperidine-4-carboxylic acid (1a) was purchased from Enamine or Fluorochem. 1b and 1c were purchased from Fluorochem. Piperonylic acid (2a, Figure S2) served as a model substrate for various optimization reactions. 2a, 2b, and 2c were purchased from Sigma Aldrich. Polyphosphates were purchased from Merck, Sigma-Aldrich, and Budenheim KG.

2.2 | Enzymes

GDH-105 was purchased from Codexis. Polyphosphate kinases from *Meothermus ruber* (MrPPK) and *Sinorhizobium melloti* (SmPPK) were prepared and purified as described,[38] or applied as crude lyophilized cell-free extracts [crude MrPPK, PRO-PPK(001), Prozomix, and crude SmPPK, PRO-PPK(002), Prozomix, resp.]. *Escherichia coli* pyrophosphatase (PPase) was prepared and purified as described,[61] or applied as crude lyophilized cell-free extract [crude PPase, PRO-PPase(001), Prozomix]. Crude lyophilized CFES containing a phosphopantetheinyl transferase and/or a carboxylic acid reductase were obtained from Prozomix [CAR 1: PRO-CAR(001); CAR 1A: PRO-CAR(001)-PPT(A); CARhis 1A: his-tagged PRO-CAR(001)-PPT(A); CAR 1B: PRO-CAR(001)-PPT(B); CAR 1D: PRO-CAR(001)-PPT(D); CARhis 2A: his-tagged PRO-CAR(002)-PPT(A); CARhis 3A: his-tagged PRO-CAR(003)-PPT(A); CAR 5: PRO-CAR(005); CAR 5A: PRO-CAR(005)-PPT(A); CARhis 5A: his-tagged PRO-CAR(005)-PPT(A); CAR SC: PRO-CAR(005)-PPT(C); CAR SD: PRO-CAR(005)-PPT(D)].

2.3 | Analytical methods

1a, 1b, and 1c were separated on a Macherey-Nagel EC 150/3 NUCLEODUR C18 Gravity, 3 µm column equipped with a C8 3/3 NUCLEODUR C18 Gravity, 3 µm, guard column. Conditions: 35°C, flow rate 1 mL/min, detection 210 nm UV, 10 µL injection volume, gradient (A) 5 mM NH₄HAc in H₂O, [B] acetonitrile 0–0.7 min: 10%; B: 0.7–3.1 min to 90%; B: 3.1–3.8 min 90%; B: 3.81–5.0 min: 10% B, tᵣ 1a: 2.34 min, 1b: two peaks at 3.10 and 3.35 min, 1c: 3.04 min. Conversions of 1a were calculated by linear interpolation of calibration curves. The product distribution was determined by area normalization. 2a, 2b, and 2c were analyzed by reversed-phase high-performance liquid chromatography (HPLC) as described previously.[38]

2.4 | Analytical scale biotransformation reactions

A 2x buffer mix was prepared to give final concentrations of MOPS (100 mM, pH 7.5), Mg²⁺ (70 mM), and D-glucose (100 mM). Bromothymol blue (8 mM) was added (optionally) to buffer mix (125 µL). Next, polyP solution (1 M based on phosphate units in H₂O, pH 7) followed by 1a (10 mM final concentration) was added. NADP⁺ (0.5 mM final concentration), ATP (1 mM final concentration), and a master mix of co-enzymes (MrPPK, SmPPK, EcPPase, or the respective crude preparations, and GDH-105) was added. The total volume per reaction was 250 µL. The reactions were started by the addition of CAR preparation (125 µg mL⁻¹–3 mg mL⁻¹). Notably, lyophilizes contain salts and host proteins. The reactions proceeded in 1.5 mL Eppendorf tubes at 30°C and 700 rpm in an Eppendorf Thermomixer. When the pH decreased (as optionally indicated by a color change of bromothymol blue), NaOH was added. The reactions were terminated by the addition of 200 µL of MeOH/formic acid in a ratio of 19:1 to 50 µL of the reaction mix. After vigorous mixing and centrifugation, the supernatants were analyzed by reversed-phase HPLC.

2.5 | Preparative scale biotransformation reaction

The reaction was carried out in a 2 L glass bottle. 2x buffer mix (300 mL) was added to the polyP solution (0.5 M based on phosphate units in 400 mM MOPS, 240 mL). The buffer mix contained appropriate amounts of MOPS, MgCl₂, and glucose. After the addition of 3.3 g L⁻¹ 1a, the pH was adjusted to 7.7 at 30°C. Next, the cofactor solution and enzyme master-mix was added. Final concentrations were 100 mM MOPS, 12 mM 1a, 0.5 mM NADP⁺, 1 mM ATP, 70 mM Mg²⁺, 200 mM polyP, 100 mM glucose, 0.2 U mL⁻¹ GDH-105, 50 mg L⁻¹ crude MrPPK lyophilizate, 20 mg L⁻¹ crude SmPPK lyophilizate, 12.5 mg L⁻¹ crude PPase lyophilizate, and 0.5 g L⁻¹ crude PRO-CAR(005)-PPT(C) lyophilizate. Finally, the aqueous mixture was overlaid by 150 mL of cyclohexane. The bottle was equipped with a stopper and magnetic stirrer and placed in a temperature-controlled room at 30°C. Gentle stirring is important to avoid the formation of an emulsion. Samples were drawn from both layers and analyzed by HPLC as described in Section 2.3. After 7 h, 0.25 mM NADP⁺ and 0.5 mM ATP were added. After 9.5 h, the reaction mix was frozen at –20°C. For workup, the mixture was thawed, transferred into a separatory funnel and the phases were separated. The aqueous phase was saturated with NaCl and extracted seven times with Et₂O (200 mL). To avoid emulsification of the media, this was performed without vigorous shaking. The combined organic layers were washed with brine (300 mL), dried over Na₂SO₄, and concentrated to yield 1.62 g of a colorless oil. HPLC-MS showed minor contamination of the organic phase with 1a. The crude product was immediately purified by silica gel chromatography (200 g) eluting with Et₂O-light petroleum – 4:1 to 10:1 to yield aldehyde 1b (1.14 g, 61%) and 1c (75 mg, 4%) both as colorless, viscous oils. For NMR spectra and data see SI Section 5.2).
FIGURE 1  Cell free 1b synthesis with crude CARs, purified PPass and PPKs and commercial GDH. Substrate: 1a (10 mM); Reaction time: 16 h; 125 µg mL⁻¹ crude CAR lyophilizate; 100 µg mL⁻¹ MrPPK; 40 µg mL⁻¹ SmPPK; 25 µg mL⁻¹ EcPase; 0.2 U mL⁻¹ GDH-105

FIGURE 2  (A) Cell free 1a reduction with crude, commercially available lyophilizates. Substrate: 1a (10 mM); Reaction time: 16 h; 1.0 mg mL⁻¹ crude CAR; 200 µg mL⁻¹ MrPPK; 80 µg mL⁻¹ SmPPK; 50 µg mL⁻¹ EcPase; 0.2 U mL⁻¹ crude GDH-105. (B) Optimization of conditions for cell free 1a reduction with crude, commercially available lyophilizates. Substrate: 1a (10 mM); Reaction time: 16 h; standard: 1.0 mg mL⁻¹ crude CARhis 5A; 200 µg mL⁻¹ MrPPK lyophilizate; 80 µg mL⁻¹ SmPPK lyophilizate, 50 µg mL⁻¹ EcPase lyophilizate in a total volume of 250 µL; ISPR with cyclohexane (25% v/v); reduced: 0.75 mg mL⁻¹ crude CARhis 5A, 100 µg mL⁻¹ MrPPK; 40 µg mL⁻¹ SmPPK, 25 µg mL⁻¹ EcPase; minimal: 0.5 mg mL⁻¹ crude CARhis 5A, 50 µg mL⁻¹ MrPPK; 20 µg mL⁻¹ SmPPK, 12.5 µg mL⁻¹ EcPase. Bars show accumulated product contribution of both phases

3  |  RESULTS

3.1  |  Cell-free reduction with crude CARs and purified auxiliary enzymes

By challenging a panel of eight different CAR crude cell-free extract lyophilizates (Prozomix, Haltwhistle UK) with the Cbz protected piperidine carboxylic acid 1a, we were delighted to observe up to 65% conversion to the aldehyde in the presence of purified auxiliary proteins (Figure 1). Since CARs release aldehydes and over-reduction should not be triggered by the CARs themselves, the small amounts of alcohol 1c were attributed to alcohol dehydrogenase and/or aldo-keto reductases⁶² that were present in the crude lyophilized cell-free-extracts. The differences in product distribution were a result of varying combinations of CAR and phosphopantetheinyltransferases in combination with varying amounts of soluble CAR in the enzyme preparations.

3.2  |  The alcohol challenge in reductions with crude enzymes

When in addition to crude CARs, the auxiliary proteins were also applied as crude lyophilized cell-free extracts and the CAR content increased to achieve full conversion, the formation of undesired alcohol (1c) became predominant and none of the reductions consumed
the substrate exhaustively (Figure 2A). 1b and 1c formation occurred simultaneously and even after very short reaction times significant 1c amounts were detected (Figure S4).

In the case of piperonylic acid, which was employed as a model substrate in the development of the reaction conditions herein (2a, Supporting information), negligible formation of alcohol (2c) was observed with purified enzymes and commercial GDH at full conversion.[38,63] When purified CARs were substituted for the same crude lyophilizates as described in Figure 1 under identical conditions for the reduction of 2a, full conversion was achieved by two of the described CAR preparations (Figure S3) with similarly modest alcohol generation to that observed with 1a (Figure 1), indicating that background enzymes in the crude CAR lyophilizates do not dominate for either of the two compounds.

We exploited the lipophilicity of 1b, applying different water-immiscible organic phases for in situ product removal (ISPR),[23,64] and found cyclohexane as the most compatible solvent for this system. The fraction of 1b tripled in the presence of the second phase (Figure 2B) as compared to the single-phase reaction because the lipophilicity of 1b caused its partial removal from the aqueous enzyme solution.[64] The conversion of 1a was 76%–77% in both reactions, indicating no negative impact of cyclohexane on the applied enzymes. Further optimization of the reaction conditions included a comparison of several polyphosphates with different chain lengths (Figure S5) and fine-tuning the amounts of auxiliary enzymes (Figure S6–S9). As summarized in Figure 2B, the reduction of lyophilizate input decreased the formation of 1c, at nearly the same total conversion (Figure 2B, ISPR, minimal [66% conversion of 1a] vs ISPR, reduced [69% conversion of 1a]). The middle bar in Figure 2B shows the effect of co-factor feed after a reaction time of 5 h to compensate for the potential decomposition of ATP/ADP/AMP and NADP(H) throughout the reaction. Indeed, co-factor supplementation had a positive influence. His-tagged CAR 5A had been chosen for this optimization because the potential to increase low aldehyde contents by reaction engineering was high.

### 3.3 Gram-scale cell-free preparation of 1b

Finally, we selected two CAR preparations for preparative scale reactions (Scheme 3): the best performing preparation with respect to aldehyde levels CAR 5C and a less efficient CAR preparation for comparison (his tagged CAR 3A, Figure 2A). 2.2 g of 1a were treated with the latter CAR in the presence of co-substrates and crude auxiliary proteins in 1 L aqueous phase with 250 mL of cyclohexane. The reaction was stirred gently at 30°C and reaction progress was monitored by HPLC. Within the first 5 h 45% 1b and 10% of 1c were formed (Figure S10). Similarly, 2 g of 1a were converted with CAR 5C in 0.6 L aqueous phase and 150 mL of cyclohexane for ISPR. 1a was consumed faster (>70% within 5 h) and the product consisted of 67% of 1b and only 6% 1c (Figure 3). As both reactions stalled out after about 6 h, a fresh portion of NADP⁺ (0.25 mM) and ATP (0.5 mM) was added to each after 7 h, which restored reduction activity to some extent. For the less efficient CAR, the aldehyde did not exceed 60% in situ yield and 40% 1c was finally formed (Figure S10).

The reaction with the efficient CAR was progressed for 9 h (Figure 3) and then terminated by storage at −20°C since the reaction profile indicated that 1b generation had leveled off and longer reaction times would thus lead to over-reduction. The products were isolated by gentle extraction to avoid emulsification and purified by column chromatography. Pleasingly, 1b was obtained as a colorless viscous oil (1.14 g, 61% yield). Details and NMR spectra are given in the supporting information.

### 4 DISCUSSION

The toolbox of carboxylic acid reductases is increasing, as their value for single-step transformations from stable carboxylic acids to highly active aldehydes has been recognized both in academic and industrial
When all enzymes were produced in labs. Despite the CAR’s perfect selectivity, its typical application in form of whole-cell catalysts leads to by-product formation—especially over-reduction of aldehyde to the respective alcohol.[65] Engineered host organisms with gene knock-outs to avoid this detoxification response are highly useful tools[62,66] and may drastically improve aldehyde titers,[67,68] but the degree of residual alcohol formation depends on the substrate structure. Mono-methyl terephthalate, for example, has been converted to >90% of its respective alcohol in the presence of commercial GDH and purified CAR, PPKs, and PPase.[38] Alcohol formation had been triggered by protein impurities showing alcohol dehydrogenase (ADH)/ketoreductase activity, mainly introduced through the commercial GDH. The extent of alcohol formation again strongly depended on the substrate structure. Mono-methyl terephthalate, for example, has been converted to >90% of its respective alcohol in the presence of commercial GDH and purified CAR, PPK, and PPase, even when all enzymes were produced in E. coli RARE.[63]

Host background enzymes are of course present in abundance in typical crude enzyme preparations: crude lyophilizes typically consist of 25%–60% total protein, alongside salts and other low molecular weight compounds of unknown composition. The amounts of the desired enzyme may also vary considerably. Figure 2 emphasizes that the wish to apply simple, commercially available enzyme preparations is paid for by significantly increased alcohol formation. We reasoned that the separation of 1b from the dissolved enzymes would suppress this undesired over-reduction and applied in situ product removal, an effective strategy to minimize the contact of lipophilic compounds from the enzymes in the aqueous phase. Focusing on N-protected piperidine carbaldehyde 1b, gram scale reactions were carried out with the best CAR (CAR 5C) and a CAR with medium performance according to the analytical scale results (his-tagged CAR 3A). The efficient CAR gave very good conversions and a low alcohol formation rate. The less efficient CAR, his-tagged CAR 3A, catalyzed acid reduction much more sluggishly. Due to the availability of reducing equivalents, the desired product 1b was over-reduced to 1c. Long reaction times promoted 1c formation further. Monitoring the reaction progress is advisable to choose a reaction end-point with maximal aldehyde content, thus avoiding the over-reduction of the reaction progress is advisable to choose a reaction end-point with maximal aldehyde content.

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**CONFLICT OF INTEREST**
None declared.

**AUTHOR CONTRIBUTIONS**
A.S. and S.H. Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing; F.R. Supervision, Writing – original draft, Writing – review & editing; J.T.K. Project administration, Resources, Writing – review & editing; R.M.H. Conceptualization, Resources, Validation, Writing – review & editing; M.W. Conceptualization, Data Curation, Funding acquisition, Methodology, Project administration, Supervision, Writing – original draft.

**DATA AVAILABILITY STATEMENT**
Data are available in article supplementary material. Further data that support the findings of this study are available from the authors.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article.

SCHWARZ ET AL.

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