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Substrate-Independent High-Throughput Assay for the Quantification of Aldehydes

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Abstract: The selective and direct reduction of carboxylic acids into the corresponding aldehydes by chemical methods is still a challenging task in synthesis. Several reductive and oxidative chemical methods are known to produce aldehydes, but most of them require expensive reagents, special reaction conditions, are two-step procedures and often lack chemoselectivity. Nature provides an elegant tool, so called carboxylic acid reductases (CARs) for the direct reduction of carboxylic acids to aldehydes. Discovery as well as engineering of novel CAR enzymes necessitates a robust, product selective high-throughput assay (HTA). We report a simple and fast HTA that allows the substrate-independent and chemoselective quantification of aldehydes (irrespective of their chemical structure) and is sensitive to the nM range. The HTA was validated by NMR and GC analyses and in microbial cells by reexamination of the substrate scope of CAR from Nocardia iowensis (CARiow). The results were fully consistent with reported data.

Keywords: aldehyde quantification; 2-amino-benzamidoxime; dihydroquinazoline; high-throughput screening; cell compatible assay

Introduction

Synthetic chemists have been intrigued by the structural complexity of natural compounds and always endeavored synthesizing them from readily available building blocks. To fulfill unprecedented needs, novel methodologies were developed, especially in the fields of catalysis (metal-, photo-, organo-, and biocatalysis)[1] to synthesize the most challenging molecules in as few steps as possible with satisfying yield and high optical purity.[2] Many syntheses rely on the availability of the highly reactive aldehyde functional group, which can be used for (asymmetric)-aldol-, and Mannich-type reactions as well as Grignard additions, Wittig reactions, oxidations and reductions. Aldehydes are not only essential for the synthesis of complex molecules for fine chemicals and pharmaceutical ingredients, they also play an important role in food and flavor ingredients.[3] Chemically, aldehydes are predominantly synthesized by oxidation of primary

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alcohols, by ozonolysis or the reduction of activated carboxylic acid derivatives (e.g. acid halides and esters).

Nature provides us with numerous enzymes that yield aldehydes as the products. Alcohol oxidases and alcohol dehydrogenases, for example, convert alcohols to aldehydes.[4] Carboxylic acid reductases (CARs) and aldehyde oxidoreductases (AORs) reduce carboxylates to aldehydes.[5] In order to screen for new and engineer known enzymes towards operational fitness for industrial applications, a fast, simple, structure independent, qualitative, and quantitative assay is required, enabling efficient identification of desired variants or novel enzymes in a reliable fashion by the first law of directed evolution: “you get what you screen for”. The design and accuracy of the assay is crucial for success and consequently, a robust high-throughput assay (HTA) is absolutely required to enable the generation of enzyme toolboxes with desired substrate scope, stereoselectivity, stability under process conditions etc.

Current analytical methods to quantify aldehydes rely on time-consuming GC and HPLC methods rather than on fast photometric assays. Commercial kits for the colorimetric detection of small aldehydes in μmol ranges in proprietary buffer solutions and serum samples are available. However, those kits are costly and have not been established for whole cell systems.[6] So far, no general application of these kits on structurally different aldehydes could be shown.

In the past, a number of HTAs have been developed to determine activity for various enzymes: esterases and lipases via fluorescence.[8] an in vivo selection method for esterases based on cell viability,[9] a method based on ‘fluorescence-activated droplet sorting’ for retro-aldolases,[10] colorimetric assays for the determination of racemase activity on agar plates,[11] for terpene synthases,[12] and for transaminases.[13] The latter ones rely on the indirect detection of side products formed during the actual target reaction, the addition of an auxiliary enzyme and a dye. Recently, Schnepel et al. published a fluorescence-based quantitative method for the determination of tryptophan halogenase activity in crude E. coli lysate after derivatization of their halogenated products via a Suzuki-Miyaura coupling.[14] Yan et al. developed an elegant method where product formation was directly analyzed on agar plates by using a qualitative multi-component analytical imaging method.[15] Lee et al. developed a growth dependent selection system for novel threonine aldolases based on the consumption of toxic acetaldehyde. Clones with high threonine aldolase activity consume acetaldehyde faster and exhibit a higher growth rate than others.[16] Most of the presented assays either require a specific “screening substrate”, include several operative steps, run at harsh conditions or they are incompatible with living cells.

The aim of this study was the development of a reliable high-throughput assay for aldehyde detection in the presence of resting cells (Scheme 1); the assay should allow for screening of large mutant libraries of aldehyde producing enzymes irrespective of the chemical structure of the substrate.

The assay described herein is based on UV and fluorescence, can be operated from the nM to mM concentration range, requires minimal sample preparation (pH-adjustment & filtration or centrifugation) and it can be quantified.

Results and Discussion

The prerequisites for a suitable HTA are the fast reaction of the target aldehyde and the probe precursor in aqueous media at room temperature as well as the formation of a stable, UV active (high absorption coefficient) or fluorescent molecular probe, which is selective towards the aldehyde functionality but relaxed with respect to the overall chemical structure of the aldehyde. In a recent paper, Kitov et al. investigated different nucleophilic derivatization reagents to probe aldehyde containing proteins and to study protein functionalization.[17] Within their work, they investigated the rapid cyclization-reaction of 2-amino-benzamidoxime (ABAO) derivatives (e.g. 1 α - β) and a highly activated model aldehyde (2 α ) in acidic aqueous sodium acetate buffer, forming dihydroquinazoline products (e.g. 3 α - β) (Scheme 2). The latter species displayed a high absorption coefficient, showed fluorescence properties and could be detected down to 100 μM concentration. The study prompted us to investigate this reaction in more detail and to adapt it towards a
A general methodology for the detection of a large variety of chemically diverse aldehydes derived from enzyme mediated whole-cell transformations.

In a first step, we investigated the reaction of six ABAO derivatives \( 1 \alpha – \beta \) displaying different electronic properties with phenylacetaldehyde (2b) as aliphatic model aldehyde in aqueous buffer (100 mM sodium acetate, pH 4.5) at room temperature. Reaction progress was monitored via UV absorbance at 405 nm (1\( \alpha \), Supporting information Figure S4) and NMR measurements (1\( \alpha \)–\( \zeta \), Supporting information, Figure S5).

Quantitative product formation to 3\( \alpha – \zeta \beta \) was observed for all ABAO derivatives (1\( \alpha – \zeta \)) after 5 min. In the following, we investigated the reaction of six ABAO derivatives 1\( \alpha – \beta \) displaying different electronic properties with phenylacetaldehyde (2b) as aliphatic model aldehyde in aqueous buffer (100 mM sodium acetate, pH 4.5) at room temperature. Reaction progress was monitored via UV absorbance at 405 nm (1\( \alpha \), Supporting information Figure S4) and NMR measurements (1\( \alpha – \zeta \), Supporting information, Figure S5).

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After optimization of the reaction conditions (ABAO equivalents, organic solvents, Supporting information, Chapter 4.4) the versatility of the assay was demonstrated by converting more than 20 aldehydes in NaOAc-buffer (Scheme 2, e.g. aliphatic aldehydes (2b–d), benzaldehyde derivatives (2e–p), naphthaldehyde (2q), thiophene carboxaldehyde (2r), unsaturated (2s–v) and saturated cinnamic aldehydes (2w–x)). The corresponding dihydroquinazoline products were obtained in quantitative yields (3\( \alpha – \zeta \beta \), 3\( \alpha – x \), 3\( \beta i \)) within 3–20 min (Supporting information, Table S4) reaction time. The reaction kinetics were determined via UV measurements and the emerging cyclization products 3 were confirmed by NMR analysis (Supporting information, Chapter 4).

The assay can be used for quantification within the low μM range applying UV detection and semi-quantified down to nM concentrations by using fluorescence measurements. The latter were impeded by the noise of the fluorescence signal and the strong influence of the co-solvent. Furthermore, the quantification of the assay does not rely on tedious synthesis of reference compounds: we performed reactions with a constant amount of ABAO and varying concentrations of 2, and since all reactions proceeded to completion, the system can be calibrated “in situ”

Scheme 2. Aldehydes, ABAO-derivatives and chemical probes (dihydroquinazolines) investigated in this study. Top: Formation of dihydroquinazoline products (3) as reported by Kitov et al.\[^{[17]}\]. These compounds were used as a basis for the development of the HTA. Bottom: A set of 23 different aldehydes (2) was investigated with different ABAO derivatives (1) (only selected ABAO derivatives are shown, for a comprehensive list see Supporting information Figure S2). Different structures and substitutions were chosen to underline the general applicability and structural independence of the assay.
Next, the chemoselectivity of 1α was verified in the presence of different functional groups (alcohol, ketone, carboxylic acid). When monitoring the reaction at 405 nm, no product formation was observed with phenylacetic acid (4b), 2-phenylethanol (5b), phenylacetone (6b) or acetophenone (6e) (Supporting information, Figure S14). However, phenylacetaldehyde (2b) showed quantitative product formation in the presence of 4–6b, which confirmed the chemoselectivity of this assay for aldehydes.

Next, a protocol for aldehyde detection in the presence of resting cells was developed. First, we investigated the importance of the pH value for the formation of the dihydroquinazoline product 3ab. Resting cell transformations are typically performed at neutral pH, whereas the assay requires acidic conditions (pH 4.5). In case of phenylacetaldehyde (2b), performing the reaction in resting cell media at pH 7 decreased the dihydroquinazoline formation rate tremendously and only 20% of the desired product 3ab was detected after 20 min, whereas quantitative product formation was observed in less than 5 min at pH 4.5. Thus, we introduced a dilution step to adjust the pH from 7.0 to 4.5 ensuring full conversion of aldehyde 2b and restoring the fast reaction kinetics observed at pH 4.5 (Supporting information, Figure S15). Additionally, we observed that the presence of glucose in the medium resulted in a background reaction caused by the reducing aldehyde group (Supporting information, Figure S16). This reaction was very slow,[17] mainly due to the very low concentration of the free aldehyde form of glucose in water (<0.01%). Nevertheless, reaction parameters such as dilution and ABAO (1α–β) amount were optimized, which resulted in a significant suppression of the background signal. The accuracy of the HTA was confirmed by comparison of reaction yields measured by GC and the ABAO assay. Aldehyde yields determined by both analytical methods were highly consistent, emphasizing that the assay can be used as quantitative analytical tool in the presence of resting cells (Supporting information, Figure S18). Finally, sample preparation for the HTA (centrifugation, dilution, pH adjustment & on-plate calibration) was adapted in view of whole-cell biotransformations.

Application of the ABAO Assay

Chemoselectivity and the freedom to apply any aldehyde of choice prompted us to perform a time-resolved substrate screening of the well-known CARNi[19] to validate the HTA. The expression of CARNi was performed in an engineered E. coli strain with Reduced Aldehyde Reduction[20] to minimize over-reduction of aldehydes to the corresponding alcohols caused by endogenous alcohol dehydrogenases and aldo-keto-reductases present in the host.[20–21] Initially, reaction conditions for CARNi mediated whole-cell reduction to phenylacetaldehyde (2b) were optimized. We investigated different concentrations of phenylacetic acid (4b) and identified a substrate concentration of 2.5 mM of carboxylic acid 4b as ideal (Supporting information, Figure S19). Different carboxylic acids (4) including aliphatic-, aromatic-, and olefinic acids were investigated and their aldehyde yields are shown in Figure 1. Benzoic acid derivatives were generally well reduced by CAR, but with increasing steric bulk in 2-position the yields decreased significantly (2f–i), which was in accordance to reported results.[22]

Conclusion

The lack of general high-throughput analytics for the enzymatic synthesis of aldehydes necessitates the

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Figure 1. Time dependent substrate screening of structural diverse carboxylic acids (4) in the presence of CARNi expressing whole cells (2.5 mM 4, resting cell OD590 = 10, 25°C). Aldehyde (2) yields were detected with the developed ABAO assay using 1α as the probe. Reaction yields were reported up to 5 h; prolonged reaction times led to significantly decreased yields due to aldehyde volatility or side reactions of the whole-cell host.
development of a fast and simple assay. The presented HTA is sensitive (up to nM range), robust, inexpensive, compatible to aqueous systems as well as microbial cells and can be performed with standard laboratory equipment. By comparison of the yields obtained via GC and this ABAO assay, sensitivity and accuracy were validated. The assay can be conducted in cuvettes or 96 well format and requires, for very unreactive aldehydes about 0.5 h for completion, including 10 min incubation time at room temperature. The ABAO assay is suitable for various aliphatic and aromatic aldehydes. Its selectivity towards aldehydes allows the quantitative determination of enzyme activity in the presence of cells, carboxylic acids, alcohols and ketones. Furthermore, this selectivity and the very fast reaction rates enable future in-depth mechanistic investigations for the reactivity of aldehydes in organic synthesis. In summary, we present a valuable and convenient tool for the detection of aldehydes in enzymatic as well as chemical reactions based on UV absorption and fluorescence. With this assay in hands first random mutagenesis studies of aldehyde producing enzymes become possible.\textsuperscript{[23]}

**Experimental Section**

For detailed experimental procedures and NMR spectra of novel compounds see Supporting information.

**General Procedure for the Preparation of 2-Aminobenzamide Oximes (1α–ζ)**

2-Aminobenzonitrile derivatives (4.00–14.00 mmol) were dissolved in ethanol (0.54 mmol/mL) and hydroxylamine hydrochloride (1.1 eq.) was added. NaHCO\textsubscript{3} (1.2 eq.) in water (1 mL/125 mg hydroxylamine hydrochloride) was added and the solution was stirred for 15 min at room temperature. The solution was refluxed overnight. The mixture was concentrated, diluted with water (2 mL/mmol 2-aminobenzonitrile) and neutralized with 1 N HCl. The aqueous solution was extracted with EtOAc, then the combined organic layers were dried over Na\textsubscript{2}SO\textsubscript{4} and concentrated. The obtained solid was washed with DCM/PE to obtain 2-aminobenzamide oxime derivatives 1\textsubscript{α–ζ}.

**General procedure for plate reader assays:** To 190 μL of a 5.3 mM solution of different ABAO derivatives 1α–ζ dissolved in NaOAc buffer (100 mM, pH = 4.5, Supporting information chapter 4.1) in a 96-well plate, 10 μL of phenylacetaldehyde (2b) in acetonitrile were added to obtain final concentrations of 1 mM aldehyde 2b and 5 mM unsubstituted ABAO 1α. After 10 minutes incubation at room temperature, the absorbance was measured at 405 nm.

**General procedure for product validation by NMR:** Phenylacetaldehyde (2b, 350 μL of a 2 mM solution in deuterated NMR buffer) and ABAO derivatives (1α–ζ, 350 μL of a 10 mM solution in deuterated buffer, Supporting information chapter 4.1) were mixed in a NMR tube to obtain final concentrations of 1 mM aldehyde 2b and 5 mM ABAO 1α–ζ and these were immediately measured.

**General procedure for the preparation of resting cells in deep well plates:** A single colony of the desired E. coli RARE transformant was incubated in 4 mL LB-0.8G pre-culture medium supplemented with ampicillin (100 μg/mL) at 37°C and 200 rpm for 14 h. The main culture medium LB-5052 supplemented with ampicillin (100 μg/mL) was inoculated with 0.2% (v/v) of the pre-culture and incubated at 37°C and 150 rpm for 4 h. Then the temperature was lowered to 25°C and the main culture shaken at 150 rpm for 20 h.\textsuperscript{[20]}

For resting cell preparation, cells were harvested by centrifugation (6,000 × g at 4°C, 15 min). The pellet was re-suspended in 1/10 volume of the main culture in resting cell medium (M9 medium without nitrogen) to a cell density of OD\textsubscript{590} = 10. Resting cells were stored at 4°C up to one day (Supporting information chapter 5.3).

**Biotransformations in screw-cap vials:** A screw-cap vial (8 mL) was loaded with resting cells (1,975 μL, OD\textsubscript{590} = 10) and carboxylic acid (4, 25 μL, 200 mM) dissolved in acetonitrile or an equimolar amount of KOH was added, to obtain a final acid concentration of 2.5 mM (optimized conditions, Supporting information chapter 5.3). The vials were shaken at 200 rpm at 25°C in a Multitron shaker and samples were taken immediately, after 1, 3, 5, and 24 h. Reactions were performed in triplicates. Samples were analyzed at 405 nm using the ABAO reaction in cuvettes and/or by GC as described below.

**GC sample preparation:** The reaction mixture (100 μL) was treated with 20 μl of 2 N HCl and extracted into 200 μL of ethyl acetate containing 1 mM methyl benzoate as internal standard in an Eppendorf vessel. The organic layer was removed after centrifugation for 10 s at 5,000 × g. The extraction was repeated once. The combined organic layers were dried over anhydrous Na\textsubscript{2}SO\textsubscript{4} and analyzed via GC. Conversions were calculated by linear interpolation of calibration with authentic standards (Supporting information chapter 5.3).

**Acknowledgements**

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**References**


The ISOFR is an established and highly recognized meeting organized every four years. The objective of this event is to gather international leading scientists in the flourishing field of radical chemistry. Along with 7 plenary lectures, 31 invited speakers will present their latest findings. Further, this meeting will provide a platform for young scientists (PhD and postdoctoral students) to share their own results in a poster presentation and to get useful ideas for their own scientific work. As a highlight of this meeting, the 80th birthday of Prof. Bernd Giese will be celebrated with a mini symposium. In addition, *Advanced Synthesis & Catalysis* will publish a special issue on radical chemistry dedicated to Prof. Bernd Giese.

The ISOFR provides an ideal platform to critically discuss up to date development in this “hot” area and to initiate new directions.

Beyond the borders, the potential of radicals will be discussed in this week in June. In particular, young scientists (PhD and postdoctoral students) will get the chance to interact with the leading scientists of the field.

### Plenary Speakers

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