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Fluorogenic kinetic assay for high-throughput discovery of stereoselective ketoreductases relevant to pharmaceutical synthesis

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ABSTRACT

Enantiomerically pure 1-(6-methoxynaphth-2-yl) and 1-(6-(dimethylamino)naphth-2-yl) carbinols are fluorogenic substrates for aldo/keto reductase (KRED) enzymes, which allow the highly sensitive and reliable determination of activity and kinetic constants of known and unknown enzymes, as well as an immediate enantioselectivity typing. Because of its simplicity in microtiter plate format, the assay qualifies for the discovery of novel KREDs of yet unknown specificity among this vast enzyme superfamily. The suitability of this approach for enzyme typing is illustrated by an exemplary screening of a large collection of short-chain dehydrogenase/reductase (SDR) enzymes arrayed from a metagenomic approach. We believe that this assay format should match well the pharmaceutical industry's demand for acetophenone-type substrates and the continuing interest in new enzymes with broad substrate promiscuity for the synthesis of chiral, non-racemic carbinols.

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1. Introduction

Biocatalysis offers excellent opportunities for the synthesis of chiral compounds in a non-racemic fashion.¹ Complementary to chemical synthesis or kinetic resolution of racemates, enzyme catalyzed direct asymmetric synthesis is a highly efficient technology, for example for the preparation of chiral alcohols, chiral amines, or chiral ketone derivatives by ketone reductions using carbonyl reductases,² reductive aminations using transaminases³ or carboli-gations using aldolases⁴ or transketolases,⁵ respectively, in up to 100% optical purity and 100% yield. Particularly, NAD(P)H dependent ketoreductases (KREDs) are a well-established and reliable source of chiral alcohols with high enantiomeric purity, which are of importance as chiral building blocks for the production of pharmaceutical intermediates (Fig. 1).⁶

While the application of baker's yeast and related whole cell catalysts is attractive for the low costs,⁸ most organisms were found to follow Prelog's rule in producing predominantly the (S)-configured products.⁹ In addition, microbial cells typically contain multiple enzymes having potentially conflicting specificities, thus eroding product yields and enantiomeric excess. The advent of larger commercial collections of recombinant KREDs significantly

facilitates the identification of an appropriate catalyst for the conversion of a specific substrate. Discovery of new KREDs from various sources, such as for less common enzymes with anti-Prelog selectivity,¹⁰ can be pursued in various ways. This includes theoretical approaches *in silico* such as data mining from recent whole genome sequencing, and experimental approaches *in vitro* such as screening in culture collections or in metagenomic campaigns, as well as rational engineering or directed evolution of known enzyme scaffolds.

A decisive factor for the further development of this platform technology is the ability for rapid identification of enzymes having high activity and high stereoselectivity for the desired product configuration. This is usually performed by screening for activity to reduce the ketone substrate, followed by determination of product alcohol configuration and enantiomeric excess using chiral chromatographic techniques (GC, HPLC), which can only be performed in a tedious serial fashion and are expensive. As most ketone reductions do not produce readily detectable changes in UV/VIS absorption or fluorescence of the product, the conversion is usually followed using either the associated depletion of reduced nicotinamide cofactor by UV ($\epsilon_{340\text{ nm}} = 6\,300\text{ M}^{-1}\text{ cm}^{-1}$), or by coupling redox cofactor recycling to a secondary product that can be monitored colorimetrically (e.g. INT-formazan; $\epsilon_{492\text{ nm}} = 19\,900\text{ M}^{-1}\text{ cm}^{-1}$).¹¹ However, significantly higher sensitivity and specificity are attainable by measurement of fluorescence emission rather than UV/VIS absorption.¹² Fluorogenic enzyme substrates are

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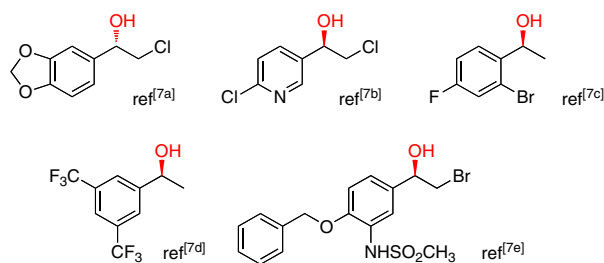


Fig. 1. Chiral pharmaceutical intermediates produced on large scale using KRED catalysis.⁷

convenient tools for screening because the signal produced is directly related to the enzyme-catalyzed reaction. Thus, a fluorometric assay for the discrimination of human alcohol dehydrogenase isoenzymes has been developed based on the consumption of fluorescent naphthaldehydes as reporter substrates.¹³ An indirect assay has been published by which a chiral secondary alcohol is oxidized to produce a ketone, which subsequently undergoes β -elimination in the presence of bovine serum albumin to release umbelliferone;¹⁴ however, because the blue fluorescent phenolate anion needs a shift to alkaline pH, the assay is not amenable to continuous kinetic measurements. To the best of our knowledge, no direct stereoselective fluorescence assay for measuring KRED activity has been reported.

Here we report on the development of a direct fluorogenic assay format, which allows the rapid and highly sensitive quantification of enzyme activity in a high-throughput fashion in a highly parallel microtiter plate format. The assay can be used for simultaneous Prelog stereospecificity typing as well as the determination of the level of enantioselectivity of KRED enzymes in one step. The assay concept further allows a systematic modular variation of the substrate structure to facilitate a substrate morphing approach^{4a} for protein engineering projects.

2. Results and discussion

2.1. Assay principle

KREDs are nicotinamide cofactor-depending enzymes that reduce ketones into chiral secondary alcohols and *vice versa*.¹⁵ In the reverse mode, the carbinol functionality reacts by hydride transfer to NAD(P)⁺ with formation of NAD(P)H and release of a ketone. Substituted acetophenones comprise one of the most important substrate classes, for which most KREDs have been found to be active against, many exhibiting high stereoselectivity.^{8b} When the non-fluorescent 1-(6-methoxynaphth-2-yl) ethanol **1a** (R = CH₃) in the appropriate enantiomeric form acts as a KRED substrate, the corresponding acetophenone derivative **2a** would be formed, which shows a bright blue fluorescence

emission around 460 nm with quantum yield of about 0.2 that can be readily detected using a common plate reader (Scheme 1). Recently, we have developed a similar fluorogenic assay for transaminases based on the corresponding chiral amine, from which the same ketone is liberated upon transaminase catalysis.¹⁶ It is interesting to note that substrate *rac*-**1a** very recently has also been used in a confocal microscopic study to localize redox enzymes in the yeast *Candida parapsilosis* by occurrence of fluorescence upon oxidative generation of ketone **2a**.¹⁷

2.2. Substrate synthesis and resolution

For the synthesis of racemic alcohols **1** we recently described a modular approach comprising a Grignard addition to the commercial aldehyde **3** (Scheme 2),¹⁶ which directly furnishes the desired compounds for KRED assays. In addition, the individual carbinol **1a** was prepared by standard borohydride reduction of the commercial ketone **2a**.¹⁸ This synthetic strategy rapidly provides access to a family of assay compounds that can be precisely designed by selecting an appropriate Grignard reagent for introducing a broad variety of structural variations in the R group, which should facilitate an incremental sculpting of the small binding pocket of a given target KRED scaffold. Besides being highly efficient because of its modular nature, this synthesis also avoids the need for tedious separation of regioisomeric 6-methoxy-acynaphthalenes, which are obtained by Friedel-Crafts acylation.¹⁹

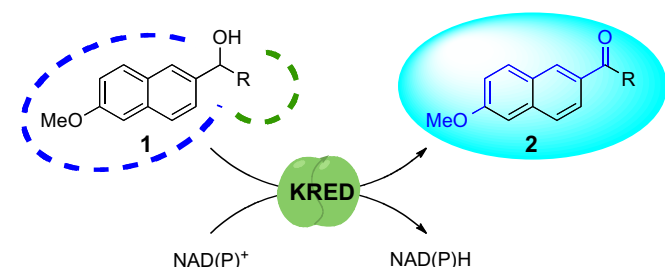
Enantiomers of **1a** were kinetically resolved by lipase-catalyzed transacylation in vinyl acetate using immobilized lipase B from *Candida antarctica* (CAL-B) according to literature precedent.²⁰ Both antipodes of the carbinol were obtained with >99% *ee* (HPLC analysis) in excellent yield, including the deprotection of the ester (R)-**4a**. Configurational assignments follow the general predictions of Kazlauskas rule.²¹

2.3. Assay development

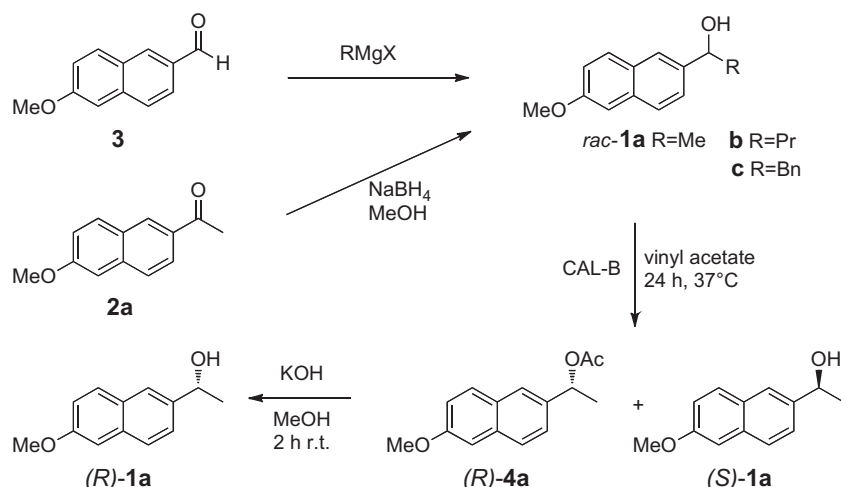
Orienting tests performed with commercial yeast alcohol dehydrogenase (YADH) and horse liver alcohol dehydrogenase (HLADH) proved negative, apparently the acetophenone moiety being too bulky to enter the substrate-binding sites. However, when racemic **1a** was incubated with the (R)-selective KRED from *Lactobacillus brevis* in the presence of its preferred cofactor NADP⁺ at an optimum pH of 7.5, the development of the bright blue fluorescence of ketone **2a** could be monitored against background, verifying the general assay concept. These experiments also indicated that the extended naphthyl fluorophore structure is instrumental in effectively discriminating enzymes that are substrate tolerant (e.g. the KRED from *L. brevis*) from those having a rather narrow specificity for smaller substrates (e.g. YADH and HLADH enzymes).

Indeed, the *L. brevis* KRED has previously been shown to have a broad substrate tolerance,²² in particular being highly adaptive to bulky substrates including substituted acetophenones.²³ This broad scope as well as its high stability and stereoselectivity make the enzyme very attractive for industrial applications.²⁴ The X-ray protein crystal structure has been solved to atomic resolution, including a ternary complex with nicotinamide cofactor and substrate (R)-1-phenyl ethanol bound to the active site.²⁵ Inspection of its structure indicates that the substrate binding site has a narrow pocket at the bottom of the groove, which binds the small alkyl moiety of an (R)-configured methyl carbinol, while the arene unit makes several hydrophobic contacts to side-chains but points towards the entrance of the active site.^{25b} The latter appears to be wide enough to also accommodate the substituted naphthyl moiety of the fluorogenic substrate (R)-**1a**.

Despite its polar hydroxyl function substrate **1a** is only sparingly soluble in plain water and must be applied from concentrated



Scheme 1. Fluorogenic assay principle for KREDs with release of the fluorescent ketone **2** upon oxidation of a chiral benzylic alcohol **1**.



Scheme 2. Synthesis of substrate alcohols **1a–c** and racemate resolution of **1a** by lipase catalyzed transacylation.

stock solution in DMSO or DMF. Dilution to a final assay concentration of 15% DMSO gave clear solutions containing up to 1.0 mM substrate **1a** or ketone **2a** that generated a consistent data quality in liquid assays. KRED enzymes require nicotinamide cofactors in stoichiometric quantities or a suitable *in situ* recycling system. In direction of oxidation, NAD(P)^+ recycling is typically achieved by either substrate-coupling in the presence of a sacrificial ketone, which thereby is reduced to the corresponding secondary alcohol, or by enzyme-coupling such as with the lactate dehydrogenase/pyruvate system.²⁶ For several reasons we decided on the substrate-coupling option with acetone as co-substrate, which takes advantage of the ability of many KREDs to react with small ketone substrates, and because conversion to isopropanol does not induce pH changes that are typically observed for enzyme-coupled approaches; also no extra enzyme is needed. An additional benefit is the fact that acetone can assist as a co-solvent to solubilize the substrate and usually is well tolerated by enzymes at concentrations of 10% or even higher.²⁷ An enzyme identified by this new procedure in turn will offer an economical advantage with respect to the necessity for cofactor recycling when planning preparative applications on larger scale.²⁸

Considering the fluorescence spectrum of ketone **2a** in water, kinetic measurements were performed with excitation at 330 nm ($\lambda_{\text{max}} = 312 \text{ nm}$) and emission observed at 460 nm ($\lambda_{\text{max}} = 441 \text{ nm}$).¹⁸ The ketone shows a linear fluorescence increase with rising concentration, which allows continuous quantification of enzyme activity by correlation with a standard curve. The limit of detection (LOD) and limit of quantification (LOQ) values derived by linear regression to characterize the performance of the assay were 0.35 nmol/min and 1.2 nmol/min (v_{max} for **2a**), respectively.²⁹ In order to shift the reaction equilibrium towards target ketone **2a** synthesis, assays were run under slightly alkaline conditions (pH 7.5), in the presence of the natural nicotinamide cofactor mixture present in the cell-free extract.

Resultant time curves for appearance of fluorescence due to formation of ketone **2a** showed excellent linearity, and varying the amount of protein confirmed a good correlation between signal intensity and enzyme activity (Figs. S3, S4). No fluorescence signal was recorded in either the absence of the biocatalyst or co-substrate, as long as no additional NAD(P)^+ cofactor was added (which could act as a co-substrate) beyond that bound to the enzyme active site. Measurements were tested in microtiter plate format with good reproducibility against parallel standard dilutions.

Reaction rates were easily determined even for concentrations of alcohol **1a** as low as 100 μM and short reaction times (20 min)

with good linearity between substrate concentration and signal intensity (Fig. S3). When the KRED from *L. brevis* was exposed to pure substrate enantiomers, only the **(R)-1a** isomer gave rise to fluorescence development while the opposite **(S)-1a** isomer did not react, even over extended reaction times (<1% activity). This confirms reports for acetophenone-type reductions that this enzyme has a high discrimination in the specificity constants k_{cat}/K_M for individual enantiomers^{22a,23a} or an excellent kinetic *E*-factor (or enantioselectivity).³⁰ For **(R)-1a** the *L. brevis* KRED showed Michaelis-Menten-type kinetics with a $K_M = 0.30 \text{ mM}$ in the presence of 15% DMSO (Fig. S6).

Although we have not yet explored the option for measuring the influence of further reaction parameters that might have an influence on enzyme activity, such as pH, temperature or addition of other co-solvents, it appears to be a rather simple task to study enzyme properties by serial variation of any such effectors or physical parameters under the standard assay conditions. Especially, the replacement of the co-substrate acetone by other (non-fluorescent) ketones or aldehydes as alternatives for the required *in situ* cofactor recycling seems a promising option to further determine the substrate specificity of a given KRED by using the otherwise identical, highly sensitive detection method.

In order to evaluate and validate the assay for its potential in high-throughput screening applications, we further determined the Z-factor,³¹ a dimensionless statistical parameter that reflects both the assay signal dynamic range and the data variation associated with the signal measurements. For a substrate concentration of 0.8 mM we determined an excellent Z-factor of 0.90, whereas even at lower substrate concentration of 0.2 mM a very high Z-factor of 0.72 resulted. Thus, the fluorogenic assay seems to be very appropriate for screening efforts of variant libraries in the directed evolution of KREDs with improved or altered protein properties, while consuming only very small substrate quantities (e.g., 0.5 mg of **1a** per 96-well microtiter plate at 0.25 mM substrate concentration).

2.4. Typing of novel KRED enzymes

As an exemplary application of the new assay we attempted to identify active KRED hits among a commercial collection of aldo/ketoreductase enzymes arrayed in 96-well plate format (Prozomix Ltd) by exposure to the **rac-1a** substrate under the optimized conditions (0.25 mM, 5% acetone, 90 min). The enzymes were recruited by a metagenomic approach employing environmental samples from the UK and are classified as belonging to the short-

chain dehydrogenases/reductases (SDR) type. SDR enzymes constitute one of the largest protein superfamilies known today, most of which are NAD(P)H-dependent oxidoreductases.³² SDRs present a common three-dimensional structure featuring an N-terminal Rossmann-fold motif for nucleotide binding. They form a large, functionally heterogeneous protein family that displays a wide substrate spectrum, including steroids, alcohols, sugars, aromatic compounds, and xenobiotics. When we discovered that indeed a high fraction of biocatalysts in this SDR collection proved active on the sterically demanding substrate **1a**, we conducted an enantioselectivity typing of these enzymes by applying the individual enantiomers (*S*)-**1a** and (*R*)-**1a** under the same conditions (Fig. 2A). The corresponding activity data allow calculating a rough estimate for the enantioselectivity of the KRED panelists before determining exact specificity constants.

Interestingly, while the most active candidate (G03 = KRED363) in the specific collection examined reached up to 90% total substrate conversion, more than half of the enzymes (53/96) caused at least 10% relative conversion, and more than one quarter (27/96) even reached at least 50% conversion, with either substrate enantiomer. Concerning their enantiopreference, (*S*)-selective Prelog-type enzymes were by far predominating (42/96; almost 80% of active catalysts), with very few enzymes showing anti-Prelog (*R*)-specificity (2/96; <4% of active catalysts); some fraction of the enzymes (9/96; 17% of active catalysts) showed only low enantioselectivity because they had ambiguous activity with both substrate enantiomers.

For an assay validation, in addition to the (*R*)-selective KRED from *L. brevis* one representative hit of an (*S*)-selective (plate entry A12 = KRED(00300)) and non-stereoselective SDR (plate entry B03 = KRED(00303)) were expressed in *E. coli*, purified by NTA chromatography and re-evaluated with respect to their enantioselectivity. In each case, the stereopreference could be unequivocally confirmed. All enzymes showed Michaelis-Menten-type kinetics with high substrate affinity (e.g., K_M = 0.076 mM of KRED(00300) for substrate (*S*)-**1a** and 0.025 mM of KRED(00303) for substrate (*R*)-**1a**).

An attempt to correlate the activity and stereoselectivity data with a phylogenetic analysis of the SDR panel revealed that

properties were spread throughout the respective protein family (PF00106) without any clear pattern (Fig. 3). This result emphasizes the need for novel assays to rapidly characterize novel KREDs experimentally as their stereo-preference cannot be predicted by genetic cluster analysis alone, even within the primary sequence boundaries of an individual pfam.

We also conducted an exemplary test to evaluate the potential of the fluorogenic assay method to determine the substrate tolerance of individual KRED enzymes. For this purpose, the arrayed panel was probed against the homologous propyl carbinol substrate *rac*-**1b** (Fig. 2B).¹⁶ When compared to the activity with substrate **1a** carrying the smallest methyl substituent, interestingly the carbinol **1b** having a larger alkyl moiety was converted by about one fifth of all enzymes (20/96), or more than one third of the active catalysts (20/53) with a relative velocity of >10%, indicating that many catalyst in the SDR category will offer an interesting substrate promiscuity. Still, the majority of the active enzymes in the SDR panel seems to have a rather small pocket for adapting only a methyl moiety. Particularly, the option for systematic structural variation of the substrate by altering the R group in the modular synthesis of carbinols **1** should be a valuable asset to probe and expand the small substrate pocket of a selected KRED by protein engineering, if directed at residues building up the small substrate binding pocket. The potential utility of such a biocatalyst engineering approach is supported by the observation that when challenged with the benzyl derivative *rac*-**1c**, an example of a substrate carrying two very bulky moieties next to the reaction site, however none of the native SDRs in the selected panel showed detectable activity.

2.5. Sensitivity improvement

The assay principle is based on the blue fluorescence emission of the methoxy substituted ketone **2**, for which a certain limitation might be seen in the incomplete quantum yield and the potential interference of the UV irradiation wavelength with other photoactive components in the assay solution, e.g. when using crude cell extracts. In order to enhance the sensitivity of signal detection, we also tested the corresponding acedan system (Scheme 3),

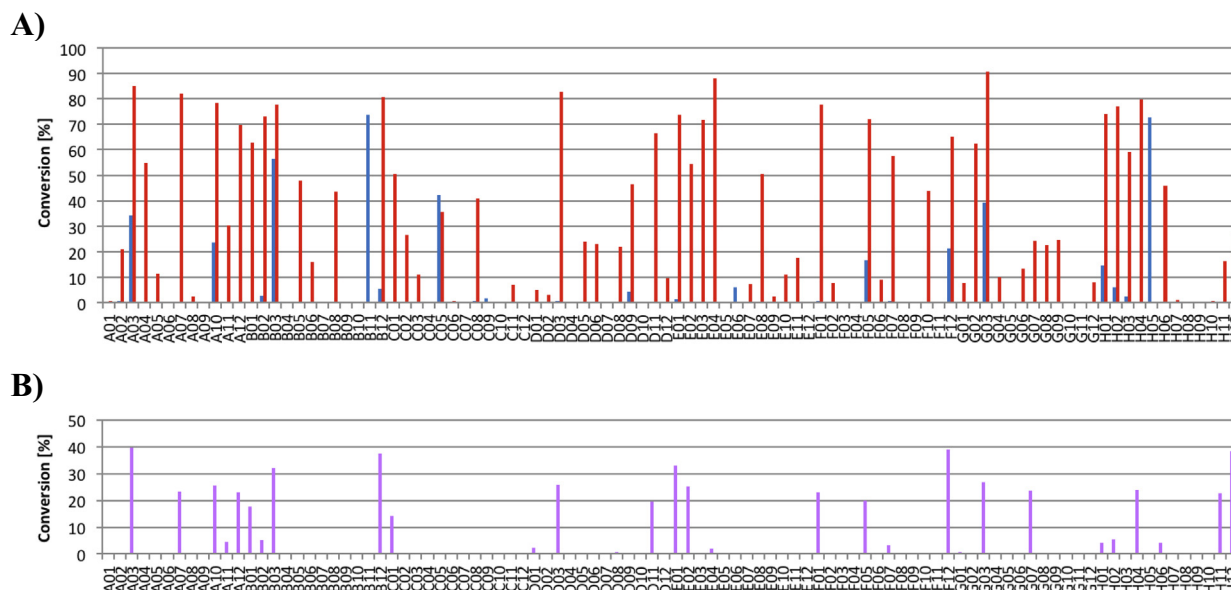


Fig. 2. A) Exemplary screening of a highly diverse metagenomic short-chain dehydrogenase/reductase (SDR) array in 96-well plate format (Prozomix Ltd, Cat No. PRO-KRED (00289–00384)), by using individual enantiomers (*S*)-**1a** and (*R*)-**1a** (■ and ■, respectively). Maximum substrate conversion after 1.5 h was 90% as determined by comparison against a fluorescence standard curve measured with ketone **2a** under identical assay conditions. B) Screening of the same plate by using *rac*-**1b** as substrate (■).

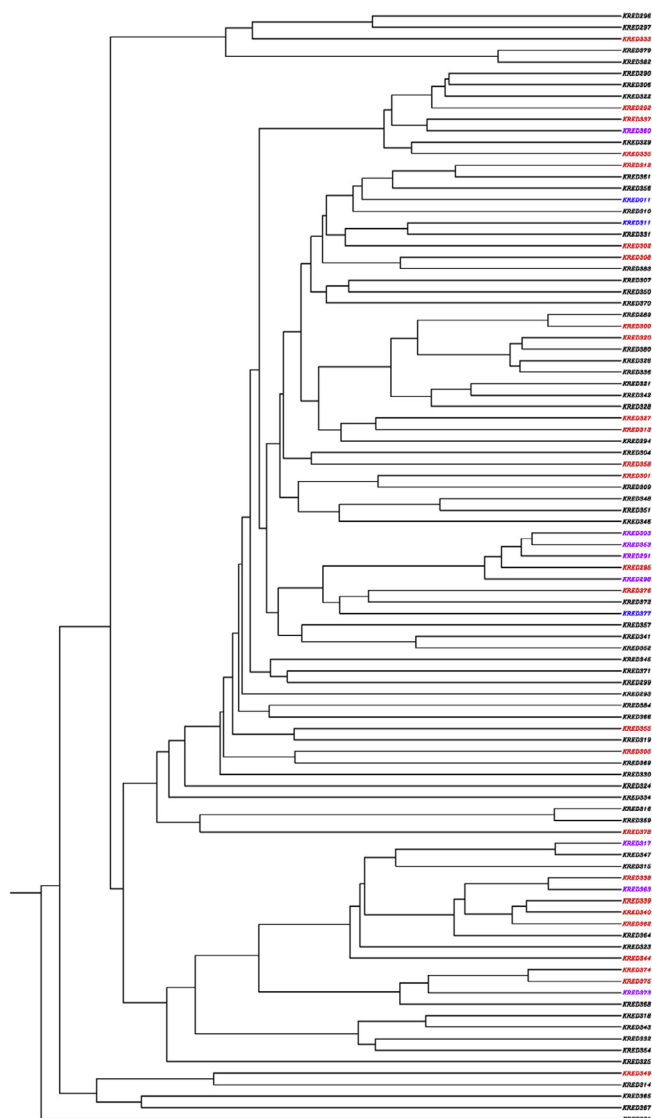
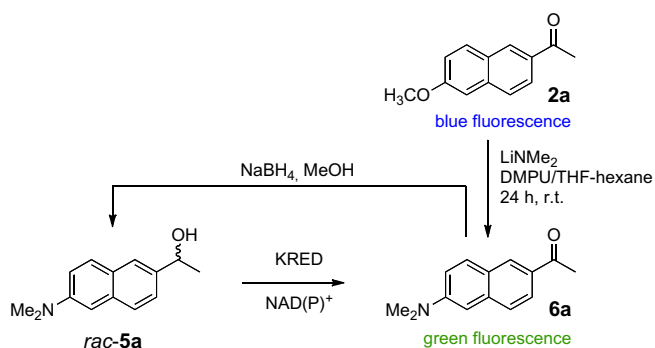


Fig. 3. Phylogenetic analysis for the novel and primary sequence diverse metagenomic SDR array (Fig. 2) showing a correlation of the enantiospecificity for the most active enzymes ($> 40\%$ rel. activity). Highly (*S*)-selective and (*R*)-selective enzymes ($> 90\%$ selectivity) are color coded as in Fig. 2, while non-stereoselective ones ($< 90\%$ selectivity) are shown in magenta.



Scheme 3. Synthesis of substrate alcohol **5a** for KRED activity screening with higher sensitivity due to longer wavelength fluorescence emission.

where the methoxy substituent of ketone **2a** is replaced by a dimethylamino group (**6a**). The latter not only causes a shift of

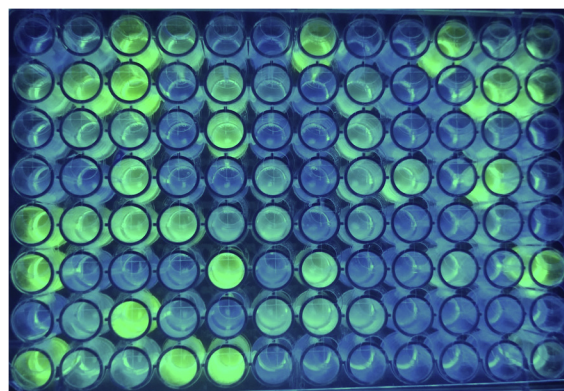


Fig. 4. Exemplary fluorogenic screening of a KRED array in 96-well plate format (Prozomix Ltd, Cat No.s PRO-KRED(00289-00384)) against *rac-5a*.

the fluorescence emission towards a longer green wavelength ($\lambda_{\text{abs}} = 362 \text{ nm}$ and $\lambda_{\text{em}} = 524 \text{ nm}$)¹⁸ but also significantly improves upon the fluorescence brightness ($\epsilon \times \Phi_F = 2240$). The synthesis of **6a** was first pursued via the phenol derived from **2a** by using the Bucherer reaction,³³ which suffered from incomplete conversion and separation problems. Much preferred is the direct substitution according to a modified literature procedure using LiNMe_2 in DMPU.³⁴ Following borohydride reduction of ketone **6a**, the resulting carbinol *rac-5a* could then be easily resolved into its enantiomers (*S*)-**5a** and (*R*)-**5a** by CAL-B catalyzed transacylation in analogy to the separation of methoxy compound *rac-1a* (Scheme 2).

Although substrate **5a** itself is fluorescent with emission in the blue range of the visible spectrum, there is no overlap with the long-wavelength green emission of ketone **6a**. Signal development was recorded at 520 nm without background interference. For an exemplary test, compound *rac-5a* was thus applied to the fluorogenic screening of the same diverse SDR array in 96-well plate format (Fig. 4), using the same conditions previously developed for the application of **1a**. For (*R*)-**5a** the KRED from *L. brevis* showed a high substrate affinity ($K_M = 0.22 \text{ mM}$), which is almost identical to that for (*R*)-**1a** (Fig. S7).

3. Conclusion and outlook

The generation of brightly fluorescent acetophenone derivatives **2** and **6** from chiral 1-(6-methoxynaphth-2-yl)carbinols **1** and its 6-dimethylamino analog **5**, respectively, is a new principle to prospect for useful KRED catalysis. We demonstrated that this new assay format is applicable to the reliable determination of activity and kinetic constants of known and unknown enzymes. When employing enantiomerically pure individual antipodes of carbinols **1a/5a**, the method allows for the rapid and immediate and cost-effective enantioselectivity typing of enzymes. The assay is characterized by high sensitivity in microtiter plate format, which qualifies for the discovery of novel KREDs of yet unknown specificity among this vast enzyme superfamily. The suitability of this approach for enzyme typing was confirmed by an exemplary screening of a large collection of SDR enzymes arrayed from a metagenomic approach. We believe that this assay format should match well the pharmaceutical industry's demand for acetophenone-type substrates and the continuing interest in new enzymes with broad substrate promiscuity, particularly those tolerating bulky substituents, for the synthesis of chiral, non-racemic carbinols. We note that our fluorogenic assay also avoids problems from an interference of UV absorbing pharmaceutical intermediates with conventional NAD(P)H dependent measurements.

Its simplicity and low substrate consumption renders the assay particularly suitable for large-scale liquid screening campaigns in the directed evolution of optimized KREDs. The latter purpose is facilitated by the modular scheme of substrate synthesis that allows a simple and rapid generation of broad structural variations in the acyl/carbinol moiety, required in particular for a targeted adaptation of the small substrate-binding pocket. Not least, such discovery and optimization of desired KREDs is also important for the integration of redox conversion modules into advanced reaction cascades along the concept of *Systems Biocatalysis*.³⁵

4. Experimental section

4.1. Fluorogenic assay set-up and determination of kinetic constants

The assay was developed in Sarsted 96-well plates using a BMG Optima plate reader equipped with 330 nm filters for excitation and 460 nm filters for emission. The standard curve of the assay was determined by using a dilution series of the ketone **2a** and **6a** in a concentration range of 0.0–1.0 mM in the corresponding assay buffer. Different enzyme-substrate pairs were measured as triplicates using a serial dilution of 0.0–3.0 mg mL⁻¹ of the ADH from *Lactobacillus brevis* (lyophilized cell free extract in 50 mM phosphate buffer, pH 7.5). Each well contained 30 µL substrate stock in a 2:1 mixture of DMSO and acetone, 40 µL enzyme stock and 130 µL phosphate buffer (50 mM, pH 7.5).

Kinetic constants were calculated by varying the substrate concentration of (R)-**1a** and (R)-**5a** between 0.0 and 1.0 mM using a fixed enzyme concentration. Each well contained 0–30 µL substrate stock in a 2:1 mixture of DMSO and acetone, 30–0 µL of a DMSO–acetone mixture, 40 µL enzyme stock and 130 µL phosphate buffer (50 mM, pH 7.5). All measurements were performed in triplicate at 30 °C. Raw data were exported to MS Excel and were processed with Origin 9.1 for hyperbolic fitting.

LOD and LOQ were determined for both **2a** and **6a**. Assay reaction was performed using ADH from *L. brevis* cell free extract (200 µg per well) for incubation of substrate (R)-**1a** at 0.5 mM concentration and negative control without substrate. All data were measured as triplicates. The LOD was defined as LOD = 3.3 σ/S and LOQ was defined as LOQ = 10 σ/S, where σ is the standard deviation of the control and S is the slope of the standard curve. They were calculated for LOD = 6 µM and 1.7 µM, LOQ = 18 µM and 5 µM for **2a** and **6a**, respectively.

4.2. Microplate screening assays using commercial plate

Preparation of screening plates for KRED enzymes was performed as described.¹¹ Plates were kept frozen at -20 °C for storage until use. Using the cross-hairs in the microplate sealing film as a guide, 100 µL of an aq 0.25 mM solution of enantiomerically pure (R)-**1a** or (S)-**1a** containing 5% DMSO and 5% acetone were added to each well of the microplate (Prozomix Ltd, Cat No.s PRO-KRED (00289-00384)) using a multi-channel pipette. The contents were mixed carefully to dissolve the freeze-dried enzyme pellets. The plates were briefly shaken on a plate shaker, then pulse centrifuged, followed by removal of the cross-hair film before the kinetic analysis using a microplate reader.

4.3. Screening for substrate promiscuity

The microplate was tested using the assay conditions as described above but replacing the substrate with 1-(6-methoxynaphthalen-2-yl)butanol **rac-1b** or 1-(6-methoxynaphthalen-2-yl)-2-phenylethanol **rac-1c**.

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A. Supplementary data

Supplementary data (synthetic procedures, compound characterization and NMR spectra, HPLC analysis, kinetic charts) associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmc.2017.05.024>.

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