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MINI-REVIEW

Recent trends and novel concepts in cofactor-dependent biotransformations

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Abstract Cofactor-dependent enzymes catalyze a broad range of synthetically useful transformations. However, the cofactor requirement also poses economic and practical challenges for the application of these biocatalysts. For three decades, considerable research effort has been devoted to the development of reliable in situ regeneration methods for the most commonly employed cofactors, particularly NADH and NADPH. Today, researchers can choose from a plethora of options, and oxidoreductases are routinely employed even on industrial scale. Nevertheless, more efficient cofactor regeneration methods are still being developed, with the aim of achieving better atom economy, simpler reaction setups, and higher productivities. Besides, cofactor dependence has been recognized as an opportunity to confer novel reactivity upon enzymes by engineering their cofactors, and to couple (redox) biotransformations in multi-enzyme cascade systems. These novel concepts will help to further establish cofactor-dependent biotransformations as an attractive option for the synthesis of biologically active compounds, chiral building blocks, and bio-based platform molecules.

Keywords Biocatalysis · Enzyme cofactor · Cofactor regeneration · Cascade reaction · Designer cells · Non-natural cofactors

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Introduction

Biocatalysis is increasingly recognized as a valuable synthetic tool in both industry and academia (Bornscheuer et al. 2012; Huisman and Collier 2013; Meyer et al. 2013; Muñoz Solano et al. 2012; Reetz 2013; Simon et al. 2013). Some synthetically relevant enzymes (e.g., hydrolases) are cofactor-independent and promote biotransformations via simple acid–base catalysis. On the other hand, enzymes that carry out more complex chemical reactions — such as group transfer or redox reactions — usually require one or more cofactors for catalytic activity (Richter 2013; Zhao and Van Der Donk 2003). These cofactors range in structural complexity from simple metal ions to organometallic complexes such as the cobalamines or heme, and they are more or less tightly bound to a specific site within an enzyme during catalysis. Tightly bound cofactors (e.g., flavins, thiamine diphosphate (ThDP), and pyridoxal phosphate (PLP)), also called *prosthetic groups*, remain in the enzyme's active site throughout many catalytic cycles, and are self-regenerating. In contrast, dissociable cofactors — e.g., nicotinamide cofactors (NAD(P)H), adenosine 5′-triphosphate (ATP), and (*S*)-adenosylmethionine (SAM) — also referred to as *coenzymes*, provide functional groups that are transferred onto the substrate during catalysis, and hence they are required in stoichiometric amounts (Richter 2013; Zhao and Van Der Donk 2003). For preparative-scale applications, however, cofactors of the latter type need to be regenerated in situ, as a stoichiometric use would be too expensive. Intensive research during the last decades has led to the development of several reliable regeneration methods for the most commonly employed cofactors (outlined in Table 1), and various reviews are available summarizing the state of the art (Berenguer-Murcia and Fernandez-Lafuente 2010; Faber 2011; Kara et al. 2013a; Liu and Wang 2007; Matsuda et al. 2009; Weckbecker et al. 2010; Wichmann and Vasic-Racki 2005; Wu et al. 2013; Zhao and Van Der Donk 2003).

Table 1 Selected cofactors applied in biocatalysis and the most common methods for their in situ regeneration

Cofactor	Type of reaction	Regeneration approach	Enzyme or substrate involved in regeneration
NAD(P)H	Hydride transfer to substrate	Coupled Enzyme	Hydrogenase Formate dehydrogenase Phosphite dehydrogenase Glucose dehydrogenase
		Coupled Substrate	Isopropanol Ethanol
		Electrochemical	Electrons from the anode, transferred via a redox mediator
$NAD(P)^+$	Hydride transfer from substrate	Coupled Enzyme	Glutamate dehydrogenase NAD(P)H oxidase
		Coupled Substrate	Acetone Acetaldehyde
		Electrochemical	Electrons to the cathode, transferred via a redox mediator
		Photochemical	Visible-light excitation of flavin catalyst
		Laccase–mediator system	Laccase in combination with redox mediators
ATP	Phosphorylation	Coupled enzyme	Pyruvate kinase Acetate kinase Polyphosphate kinase Carbamate kinase Phosphate ester hydrolase
SAM	Methylation	No established method available	
Flavins[a] (e.g., FAD, FMN)	Oxygenation, hydride transfer to/from substrate	Self-regeneration	
Metal porphyrins[a]	Peroxidation, oxygenation	Self-regeneration	
Pyridoxal phosphate (PLP)	Transamination, racemization	Self-regeneration	
Thiamine diphosphate (ThDP)	C–C bond formation or scission via 'Umpolung'	Self-regeneration	

Adapted from Faber (2011) and Zhao and Van Der Donk (2003)

FAD flavin adenine dinucleotide, *FMN* flavin mononucleotide

[a] Regeneration of the reduced cofactor usually requires additional NAD(P)H as an intermediary reducing agent, although direct regeneration is possible

Nevertheless, novel approaches are still explored for more (cost)effective and environmentally benign regeneration systems. From an environmental point of view, electrochemical approaches may seem particularly attractive; however, these fall back behind enzymatic methods in terms of total turnover numbers (TTNs) for cofactor, regeneration catalyst and mediator (Chenault and Whitesides 1987; Kara et al. 2013a). Furthermore, recent years have brought about a certain change in perspective, where the cofactor requirement of enzymes is no longer seen only as a challenge, but also as an opportunity — for instance, for coupling cofactor-dependent reactions in one-pot systems and for altering an enzyme's activity by supplying it with a modified cofactor.

In this mini-review, our main focus will be on recent developments and future perspectives in cofactor-dependent biotransformations. Therefore, we will discuss novel concepts, such as engineered cosubstrates, high-productivity biotransformations using whole cells, redox-neutral cascade systems, and engineered, non-natural cofactors. For established cofactor regeneration methods we refer to the above-mentioned reviews.

Engineered cosubstrates

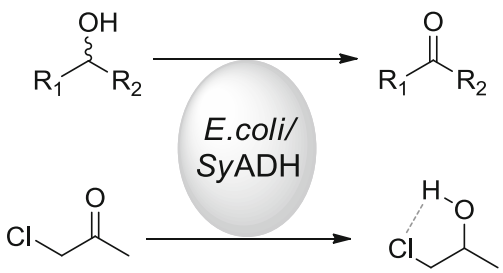
For in situ regeneration of oxidized or reduced nicotinamide cofactors in alcohol dehydrogenase (ADH)-catalyzed reactions the so called 'substrate-coupled' approach excels in simplicity, as only one biocatalyst is required for the whole reaction. However, this approach represents a biocatalytic version of the well-known Meerwein–Ponndorf–Verley reduction and hence reversibility and the poor thermodynamic driving force of the reaction necessitate significant molar surpluses (usually 10–20 equiv.) of the cosubstrate. From an environmental point of view this is not desirable as the waste thus generated has to be dealt with. In the following we will introduce novel approaches to render substrate-coupled biotransformations more efficient by exploiting structural features of the cosubstrate.

Thermodynamically stable coproduct via intramolecular hydrogen bonding

In ADH-catalyzed oxidations, $NAD(P)^+$ is most commonly regenerated by the reduction of a cosubstrate (e.g., acetone), which is used in excess. A more efficient alternative exploiting thermodynamic stabilization (Bisogno et al. 2010a) of the coproduct via intramolecular hydrogen bonding has recently been reported by Kroutil and coworkers: By employing activated ketones, for example, chloroacetone, 1,1- and 1,3-dichloroacetone, 1,1,1-trichloroacetone, or ethyl acetoacetate, only stoichiometric amounts of cosubstrate were required (Lavandera et al. 2008). Hence, this concept dramatically reduces the required cosubstrate loading and the associated waste generation. The biotransformations were catalyzed by lyophilized *Escherichia coli* cells overexpressing ADH from *Sphingobium yanoikuyae* (*Sy*ADH, Scheme 1). The oxidation of several structurally diverse alcohols proceeded to high conversion (90 % and higher) at 30 g/L substrate concentration using only 1.5 equiv. of chloroacetone as cosubstrate.

1,4-Butanediol as 'smart cosubstrate' for redox biocatalysis

The in situ regeneration of reduced nicotinamide cofactors employing ADHs usually requires excess amounts of sacrificial electron donors, e.g., ethanol or isopropanol. Recently, a 'smart cosubstrate' approach for NAD(P)H regeneration has been introduced, whereby 1,4-butanediol (1,4-BD) was shown to be an efficient cosubstrate to promote NADH-dependent biotransformations — ranging from reduction of C=O and C=C bonds to specific oxyfunctionalization (Scheme 2) (Kara et al. 2013b). The thermodynamically stable coproduct γ-butyrolactone (GBL) makes the regeneration reaction irreversible, and its formation from 1,4-BD by horse liver ADH (HLADH) supplies two equivalents of NADH. As a consequence, the cosubstrate demand is dramatically reduced (to 0.5 equiv.), and faster reaction rates are obtained. Due to the cofactor specificity of HLADH, this approach is currently limited to NADH regeneration, but further research might extend the scope to NADPH-dependent transformations.



Scheme 1 Quasi-irreversible oxidation of secondary alcohols catalyzed by lyophilized *E. coli*/*Sy*ADH coupled with chloroacetone as hydrogen acceptor

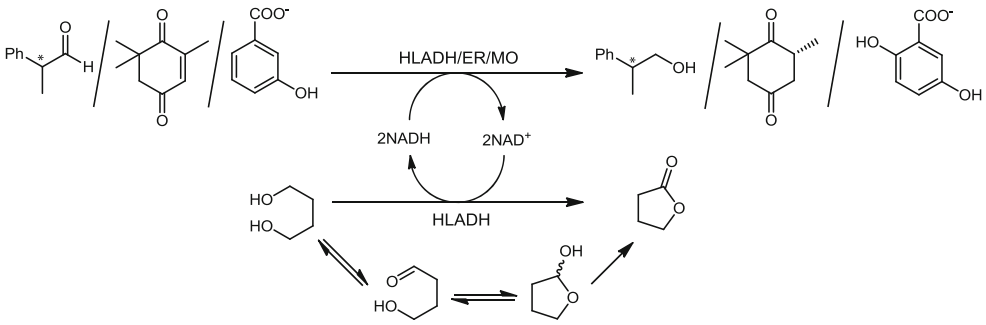
Using H_2 as stoichiometric reducing agent would represent the 'greenest' and the most atom efficient way for the regeneration of NAD(P)H. Ansorge-Schumacher and coworkers have recently demonstrated the enhancement in stability of O_2-tolerant [NiFe]-hydrogenase from *Ralstonia eutropha* H16 (Herr et al. 2013; Ratzka et al. 2012) which would enable the practical application of this promising enzyme. In a whole cell approach, the biocatalytic hydrogenation of carboxylic acids has recently been reported using endogenous hydrogenase of *Pyrococcus furiosus*, the reduction proceeding in a highly chemo-selective fashion (Ni et al. 2012a).

Enones as cosubstrates for NAD(P)H-independent bioreduction of C=C bonds

In flavoprotein-catalyzed reductions, the regeneration of reduced flavins is usually achieved by NAD(P)H, which in turn is regenerated in situ using the established 'enzyme-coupled' methods. In contrast, Faber and coworkers have recently reported the NAD(P)H-independent reduction of activated C=C bonds via direct hydrogen transfer from a sacrificial 2-enone or 1,4-dione as hydrogen donor (Stueckler et al. 2010). During the disproportionation (dismutation) of enones, an equivalent of [2H] is transferred by the enoate reductase (ER) between the two enone substrates, yielding an oxidized and a reduced product in equimolar amounts. The dienone thus formed spontaneously tautomerizes to a phenol and hence shifts the equilibrium toward the desired products (Scheme 3). While this disproportionation as such is of limited synthetic value, the authors could demonstrate that certain ketones (e.g., 3-methylcyclohex-2-en-1-one, cyclohexane-1,4-dione) are preferentially oxidized by ERs and hence can be used as sacrificial electron donors that promote the reduction of the substrate of interest.

While this approach is advantageous due to its simplicity, inhibition of the enzyme by the phenol coproduct poses a problem. To overcome this limitation, the phenol can be scavenged using a polymeric adsorbent (macroporous polystyrene carbonate). Under optimized conditions, conversions above 90 % and enantiomeric excess (*ee*) values of >99 % were achieved (Winkler et al. 2013).

Alternative chemo-enzymatic approaches for the NAD(P)H-independent reduction of C=C bonds have been reported by Hollmann and coworkers. These rely on a rhodium (Rh) complex (Bernard et al. 2012) or on photo-excited flavins (Grau et al. 2009; Taglieber et al. 2008) as catalysts for the regeneration of the reduced flavin cofactor in ERs. A recent study demonstrated the combination of these approaches, employing a Rh-based mediator for shuffling electrons from a photo-excited flavin to NAD^+. The generated NADH was used in the L-glutamate dehydrogenase-catalyzed reduction of α-ketoglutarate (Nam and Park 2012).



Scheme 2 'Smart cosubstrate' 1,4-BD coupled biotransformations. *HLADH* horse liver ADH, *ER* enoate reductase, *MO* monooxygenase

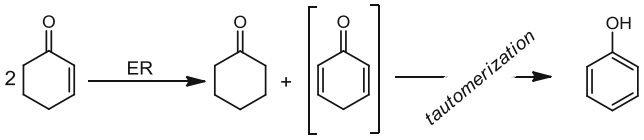
Alternative amino donors in transaminations

ω-Transaminases (ω-TAs) provide an efficient biocatalytic option for the synthesis of optically active amines, and have recently attracted increasing interest (Höhne and Bornscheuer 2009; Koszelewski et al. 2010; Mathew and Yun 2012). The PLP cofactor required by these enzymes is self-regenerating, but the reversibility and unfavorable equilibrium of the transamination reaction necessitate removal of the ketone (or ketoacid) coproduct. A very simple approach for irreversible transaminations was recently reported by Berglund and coworkers, who used 3-aminocyclohexa-1,5-dienecarboxylic acid as an alternative amino donor (Wang et al. 2013). The ketone coproduct spontaneously tautomerizes to 3-hydroxybenzoic acid, providing a strong thermodynamic driving force and shifting the equilibrium to the product side (Scheme 4). By this approach, (*S*)- or (*R*)-phenylethylamine derivatives are accessible in up to >99 % conversions and with *ee*'s of >99 %, depending on the transaminase chosen. This novel concept offers several advantages, such as requirement for only one enzyme, and for only one equivalent of the amino donor. On the other hand, 3-aminocyclohexa-1,5-dienecarboxylic acid is not commercially available, and more expensive than the commonly used amino donors isopropylamine and alanine.

Cosubstrates as an organic phase

An entirely different cosubstrate engineering concept has recently been reported by Hollmann and coworkers: The authors have developed a 'hydrophobized formates' approach, where formic acid esters (e.g., octyl formate) serve as organic cosolvent solubilizing hydrophobic reagents and as a sacrificial electron donor for formate dehydrogenase (FDH)-mediated in situ regeneration of NADH. The system was used to support 2-hydroxybiphenyl-3-monooxyygenase (HbpA)-catalyzed hydroxylation of 2-hydroxybiphenyl (Scheme 5) (Churakova et al. 2013). A two-liquid phase system (2PLS) was established since the cosubstrate octyl formate itself and the corresponding coproduct 1-octanol were sufficiently hydrophobic to form a water-immiscible phase. High substrate loadings were achieved by large volumetric ratios of organic phase to aqueous phase (9:1). The major limitation appeared to be substrate diffusion, since the enzymes' performance decreased drastically in the two-liquid phase system (2LPS).

The reported 'hydrophobized formates' concept possesses potential for biotransformations of lipophilic substrates in combination with FDH-mediated cofactor regeneration. However, the aforementioned mass transfer limitations need to be addressed, for instance using alternative reactor concepts.



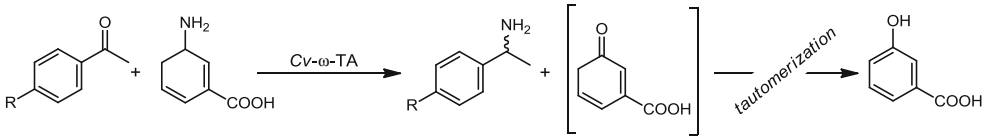
Scheme 3 ER-catalyzed disproportionation of cyclohex-2-enone and the spontaneous tautomerization of the oxidized product to phenol

High-productivity biotransformations using whole cells

The use of whole microbial cells instead of isolated enzymes offers major advantages, for example, no need for enzyme purification, relatively high stability against external stress factors (e.g., stirring, organic solvents), and no need for exogenous cofactor addition (Weckbecker et al. 2010). Whole cells may fall back behind the isolated enzymes with respect to specific activity and selectivity; however, this challenge can be considered to be solved. Nowadays, tailor-made genetically engineered microorganisms allow highly efficient biotransformations at high substrate concentrations, and this section will discuss the recent progress in this area.

Designer cells

Gröger and coworkers (2006) have introduced the concept of 'designer cells' — recombinant microorganisms expressing all enzymes necessary for a particular biotransformation. In their contribution, the authors report on the reduction of acetophenone and its derivatives catalyzed by an ADH, whereby the nicotinamide cofactor was in situ regenerated by glucose dehydrogenase (GDH), and both enzymes were expressed in the same *E. coli* strain (Scheme 6). The resulting whole-cell biocatalyst proved stable at high substrate



Scheme 4 Asymmetric synthesis of (*S*)- or (*R*)-phenylethylamine derivatives catalyzed by *Chromobacterium violaceum* ω-TA variant W60C (*Cv*-ω-TA W60C) using 3-aminocyclohexa-1,5-dienecarboxylic acid as the amino donor with spontaneous tautomerization of the ketone coproduct

concentrations (up to 200 g/L) and provided sufficient amounts of the nicotinamide cofactor to support the reaction, thus no external addition of NAD(P)H was required. By variation of the ADH expressed in the 'designer cell', a broad range of (*R*)- and (*S*)-alcohols was accessible with conversions of >90 % and *ee*'s of >99 %. In recent years, the 'designer cell' approach has been adopted by many research groups, and it has been used for various ADH-catalyzed reductions at high substrate concentrations (Berkessel et al. 2007; Eixelsberger et al. 2013; Ema et al. 2008; Ni et al. 2011, 2012b; Shen et al. 2012).

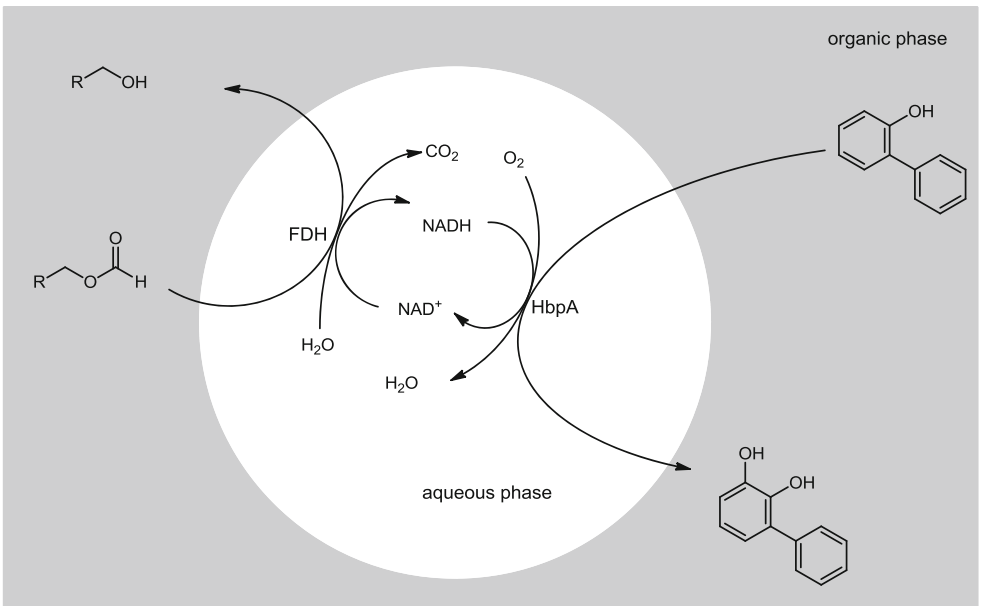
Whole-cell biocatalysis in neat substrates

The use of whole cells hosting overexpressed dehydrogenases for bio-reductions with substrate-coupled cofactor regeneration has been demonstrated in aqueous and organic media (Hollmann et al. 2011). However, the so-called 'neat substrates' approach of carrying out these biotransformations in a solvent-free system was only recently explored by Jakoblinnert et al. (2011). As a model system, reduction of acetophenone was coupled with isopropanol oxidation in neat substrates (acetophenone/isopropanol=30:70 v/v), and the reactions were catalyzed by lyophilized *E. coli* cells expressing carbonyl reductase from *Candida parapsilosis* (CPCR; Scheme 7). The 'neat substrates' approach gave the product (*S*)-phenylethanol in >98 % conversion, with an *ee* of >99 % and with high productivities (up to 500 g/L within 14 days). Furthermore, the proposed concept was also applied for the industrially more relevant product (*S*)-3-butyn-2-ol, a building block for several pharmaceutical drugs. It is worth mentioning that lyophilized cells and reaction mixtures were pre-equilibrated to control the water activity (a_w), as this parameter showed a significant influence on initial reaction rates.

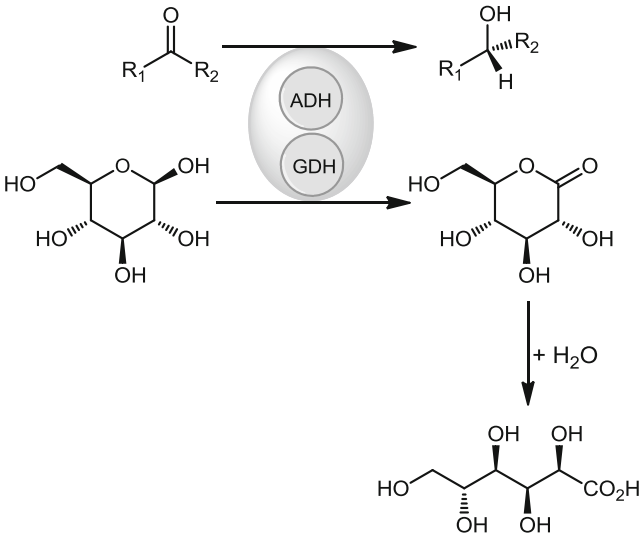
The developed approach excels in high productivity, offers a simple work-up since the cells are easily separated from the reaction mixture, and requires no external cofactor. Further increases in productivity might be achieved by recycling of the whole-cell biocatalyst.

Redox-neutral cascade reactions

The in situ regeneration of (redox) cofactors — whether it is implemented via a 'substrate-coupled' or an 'enzyme-coupled' approach — always requires the stoichiometric use of an auxiliary substrate. The corresponding product formed in the regeneration reaction is usually of little synthetic interest and is therefore discarded as waste. It would clearly be more efficient if two synthetically useful reactions with opposite cofactor demand (e.g., NAD^+ and NADH) were coupled, and indeed recent years have seen increasing research efforts in this direction. The resulting redox-neutral cascade reactions



Scheme 5 HbpA-catalyzed hydroxylation of 2-hydroxybiphenyl using formic acid esters as cosubstrate for FDH-catalyzed in situ cofactor regeneration. The cosubstrate, e.g., octylformate, serves as a substrate reservoir and product sink

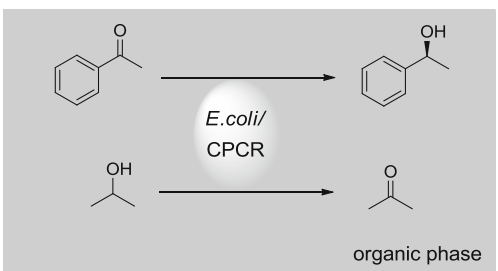


Scheme 6 General reaction scheme of the 'designer cells' catalyzed reduction of carbonyl compounds coupled with glucose as cosubstrate

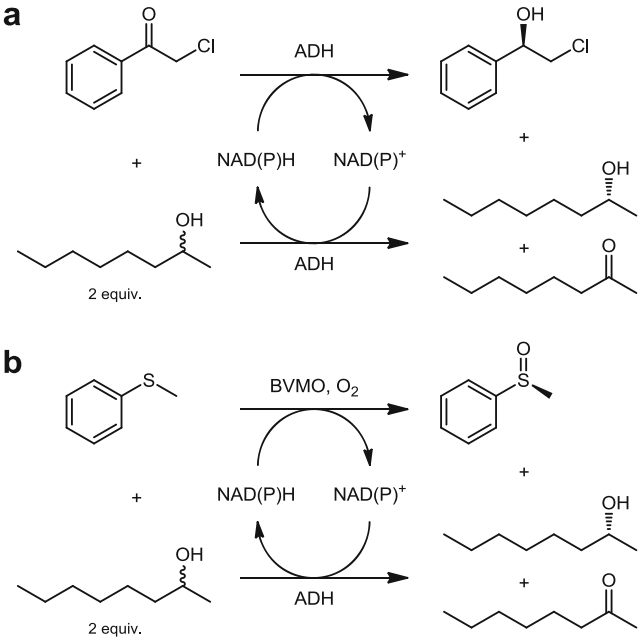
(Kara et al. 2013a; Ricca et al. 2011; Schrittwieser et al. 2011) fall into two categories: In *parallel cascades*, two reactions are connected only via the cofactor cycling, and the overall system affords two (or more) distinct products from two different substrates. In *linear cascades*, on the other hand, the reactions also share a common intermediate, which is produced in one reaction and consumed in another, and only one final product is formed.

Parallel cascade systems

Several examples of redox-neutral parallel cascades have recently been reported by Gotor's research group. In the simplest case, the asymmetric reduction of an α-halo ketone is combined with the kinetic resolution of a secondary alcohol (Scheme 8a), where both reactions are catalyzed by the same ADH (Bisogno et al. 2009). The favorable equilibrium of the halo ketone reduction (see above) ensures irreversibility of the entire reaction system, which yields two optically enriched alcohols and the ketone by-product of the kinetic resolution. High conversions (typically >80 %) and optical purities (up to



Scheme 7 Reduction of acetophenone coupled to isopropanol oxidation in neat substrates catalyzed by *E. coli*/CPCR. Organic phase represents the mixture of substrate and cosubstrate



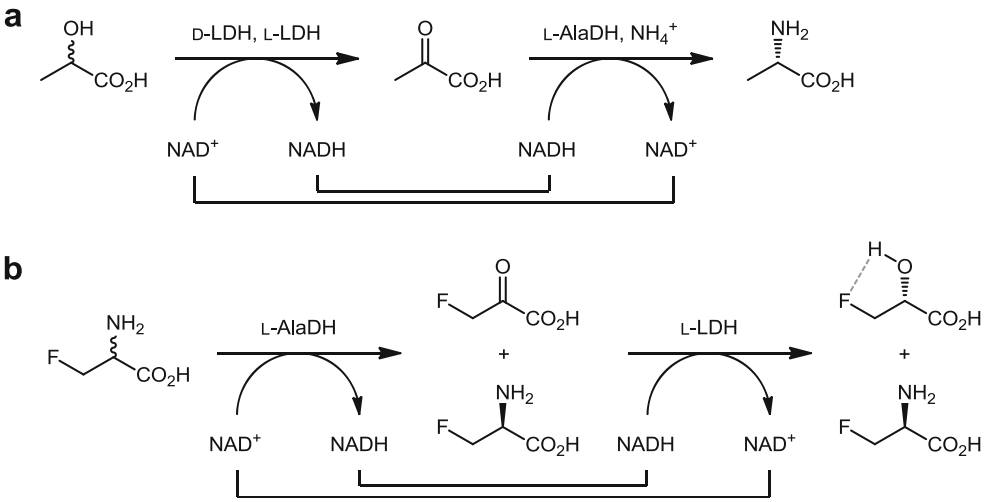
Scheme 8 Examples of redox-neutral parallel cascade systems. Combination of ADH-catalyzed asymmetric ketone reduction and kinetic resolution of alcohols (**a**), and combination of BVMO-catalyzed asymmetric sulfoxidation and ADH-catalyzed kinetic resolution of alcohols (**b**)

>99 % *ee* for both products) have been achieved with a broad range of substrate combinations.

In follow-up studies (Bisogno et al. 2010b; Rioz-Martínez et al. 2010), the kinetic resolution of alcohols has been coupled with asymmetric sulfoxidation or enantioselective Baeyer–Villiger oxidation reactions (Scheme 8b), catalyzed in both cases by Baeyer–Villiger monooxygenases (BVMOs). These systems, for which the authors have coined the term PIKAT (parallel interconnected kinetic asymmetric transformations), hence afford chiral alcohols along with chiral sulfoxides or chiral ketones and esters in varying degrees of optical enrichment. Although Baeyer–Villiger oxidation of the ketone by-product was also observed in these studies, the resulting 'redox decoupling' of the two desired transformations did not present a major problem. The parallel reactions showed good performance even when the NADPH cofactor was used only in low micromolar concentrations, reaching cofactor turnover numbers (TONs) above 5,000 (Bisogno et al. 2010b).

Linear cascade systems

The major disadvantage of the parallel cascade concept is the fact that the desired products need to be separated from each other, which becomes increasingly difficult when three or more compounds are formed. Linear cascades afford only a single product, and several elegant systems of this kind have been developed in recent years, of which some have also been applied on preparative scale.



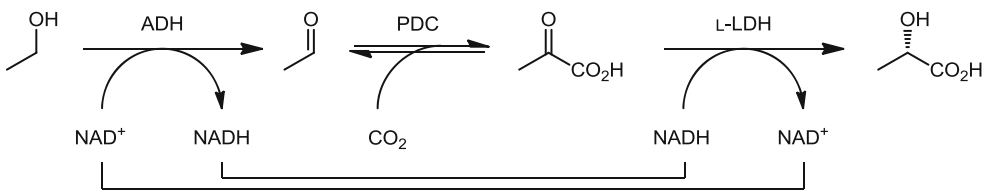
Scheme 9 Examples of redox-neutral linear cascade systems for the interconversion of amino acids and hydroxy acids. Transformation of D,L-lactate into L-alanine (**a**), and transformation of D,L-fluoroalanine into D-fluoroalanine and L-fluorolactate (**b**)

An early example of a redox-neutral linear cascade is the transformation of racemic lactate into L-alanine *via* the intermediate pyruvate (Scheme 9a), employing a combination of D- and L-lactate dehydrogenases (LDHs) and L-alanine dehydrogenase (AlaDH) as biocatalysts (Wandrey et al. 1984). Using polymer-bound NADH and a membrane reactor retaining the enzymes and cofactor, the process was run for more than 1 month, reaching a space-time-yield of 134 g/L per day for L-alanine and a ton of over 19,000 for NADH. Recently, a similar system has been employed by Kroutil and coworkers for the deracemisation of mandelic acid to L-phenylglycine, in this case using a combination of D-mandelate dehydrogenase and mandelate racemase to achieve complete conversion of *rac*-mandelic acid to phenyl glyoxylate. For the reductive amination step, commercial amino acid dehydrogenases were used (Resch et al. 2010). Interestingly, the reverse reaction, i.e., the conversion of an amino acid to a hydroxy acid in a redox-neutral system, has also been reported (Gonçalves et al. 2000, 2003, 2006). While this process is thermodynamically unfavorable for most amino acid–hydroxy acid couples (Gonçalves et al. 2006; Resch et al. 2010), 3-fluorolactic acid has been obtained in good yield from D,L-3-fluoroalanine, using LDH and AlaDH as biocatalysts. Possibly, an intramolecular hydrogen bonding interaction (Scheme 9b) is responsible for the unusual equilibrium position of this system.

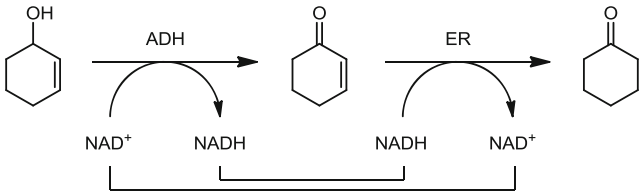
Another interesting linear cascade involving LDH has recently been reported by Ping Wang and coworkers (Tong et al. 2011): Their system comprises an ADH, pyruvate decarboxylase (PDC), and LDH, and transforms ethanol and CO_2 into L-lactate with internal NADH cycling (Scheme 10). This system establishes an elegant concept for using renewable carbon sources for the formation of a valuable compound; however, a huge excess of carbonate and continuous addition of ethanol had to be used to drive the transformation in the desired direction, and product yields (41 %) as well as NADH TONs (<9) were low.

The isomerization of allylic alcohols to saturated ketones has been realized in a biocatalytic redox-neutral system via the combination of alcohol oxidation by an ADH and alkene reduction by an enoate reductase (Boonstra et al. 2000, 2001; Gargiulo et al. 2012). While in the earlier studies by Boonstra et al. complex molecules were interconverted (morphine was transformed into hydromorphone), the recent work by Gargiulo et al. focused on the transformation of cyclohexenol into cyclohexanone (Scheme 11). In both cases, 'overreduction' of the saturated ketone product to the corresponding alcohol was observed as an undesired side reaction, which could be suppressed to some degree by careful optimization of the enzyme and cofactor concentrations. However, the yields of hydromorphone and cyclohexanone, respectively, did not exceed 60 %, and both systems required comparably high cofactor amounts (>4 mol%).

Monooxygenases (MOs) use O_2 to incorporate one oxygen atom into a substrate molecule, while the second one is reduced to water under the consumption of NAD(P)H. Hence,



Scheme 10 Synthesis of L-lactate from ethanol and CO_2 via a redox-neutral cascade comprising an alcohol dehydrogenase, pyruvate decarboxylase, and L-lactate dehydrogenase



Scheme 11 Biocatalytic redox isomerization of cylohexenol to cyclohexanone

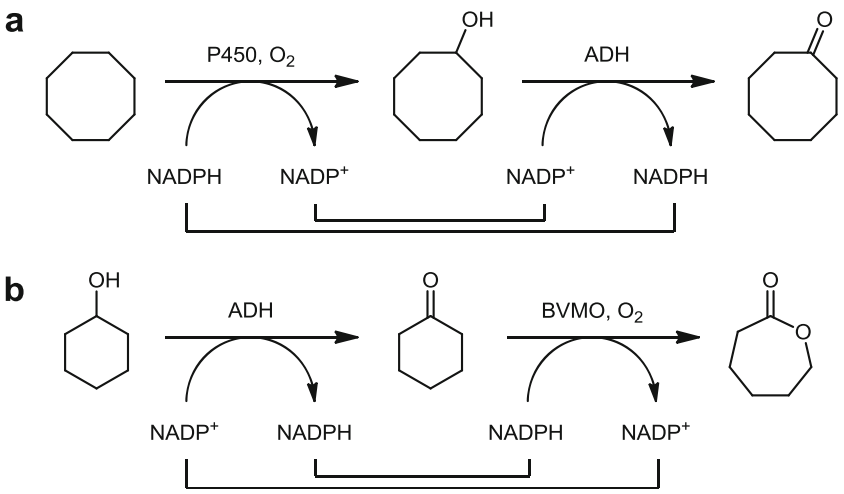
they catalyze substrate oxidation, but consume a reduced nicotinamide cofactor. Consequently, the 'NAD(P)H-neutral' combination with a second, $NAD(P)^+$-dependent oxidation reaction is possible, and two examples of such a biocatalytic cascade have recently been reported: Gröger and coworkers have developed a double oxidation system for the conversion of alkanes to ketones by combining cytochrome P450-catalyzed hydroxylation with ADH-catalyzed alcohol oxidation (Scheme 12a) (Müller et al. 2013; Staudt et al. 2013b). Although NAD(P)H is recycled in the process, the authors observed that additional reductants (2-propanol or glucose/GDH) increase the product yields, since not all NAD(P)H consumption by the P450 also results in substrate hydroxylation (a phenomenon known as 'decoupling'). Under optimized conditions, excellent TONs of up to 11,600 were reached for the P450 enzyme; however, only a few cofactor turnovers were achieved.

The 'inverse' concept of using an alcohol oxidation followed by a monooxygenation has been independently reported by the research groups of Harald Gröger and Uwe Bornscheuer (Mallin et al. 2013; Staudt et al. 2013a), apparently inspired by earlier work of Willetts et al. (1991). Both groups studied the conversion of cyclohexanol into ε-caprolactone via cyclohexanone as the intermediate, catalyzed by a combination of an ADH and a BVMO (Scheme 12b). In both cases, high conversions could only be attained at relatively low initial cyclohexanol concentrations, and inhibition as well as instability of the BVMO were identified as the limiting factors. While Staudt et al. focused on quantifying the inhibition and deactivation effects caused by elevated reactant and product concentrations, Mallin et al. investigated immobilization of the biocatalysts as a way to stabilize the MO. Unfortunately, immobilization did only improve the BVMO's stability towards elevated cyclohexanol concentrations, but could not prevent its rapid deactivation under the process conditions.

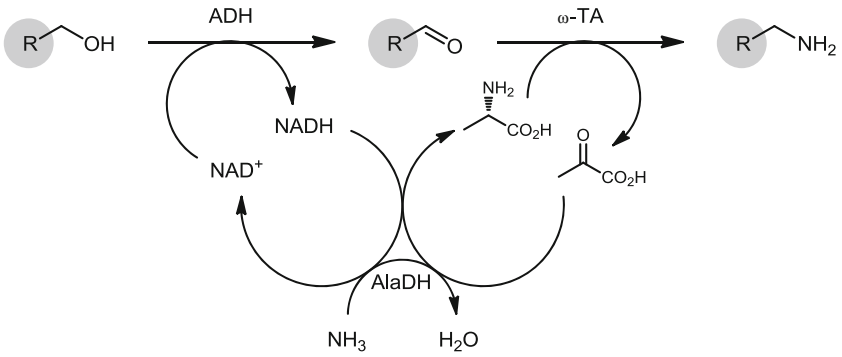
Very recently, the concept of redox-neutral linear cascade systems has been extended beyond the exclusive use of nicotinamide-dependent enzymes: Sattler and coworkers (2012) have combined the ADH-catalyzed oxidation of primary alcohols with the ω-TA-catalyzed reductive amination of the resulting aldehyde to form a primary amine. Since the former reaction generates NADH from NAD^+, while the latter converts the cosubstrate alanine into pyruvate, a third biocatalyst was needed to connect the two transformations. AlaDH provided this 'missing link' and enabled the use of ammonia as the terminal nitrogen source (Scheme 13). The cascade system was employed for the deamination of long-chain terminal diols, reaching conversions of >99 %, 70 % isolated product yield, and an NAD^+ turnover of more than 160 cycles. In later studies, the concept has also been applied to the amination of secondary alcohols, including the renewable platform chemical isosorbide (Lerchner et al. 2013; Tauber et al. 2013), and it has been implemented in whole-cell biocatalysis (Schrewe et al. 2013).

Non-natural cofactors

Since several decades, researchers have sought to expand the scope of cofactor-dependent biotransformations by introducing synthetic cofactor analogues, either to address the cost, availability, and stability issues associated with the natural compounds, or to confer novel reactivity upon enzymes.



Scheme 12 Examples of biocatalytic double oxidation systems featuring internal cofactor cycling. Transformation of cyclooctane into cyclooctanone (**a**), and transformation of cyclohexanol into ε-caprolactone (**b**)



Scheme 13 Amination of primary alcohols via a biocatalytic network comprising an alcohol dehydrogenase, an ω-transaminase, and alanine dehydrogenase

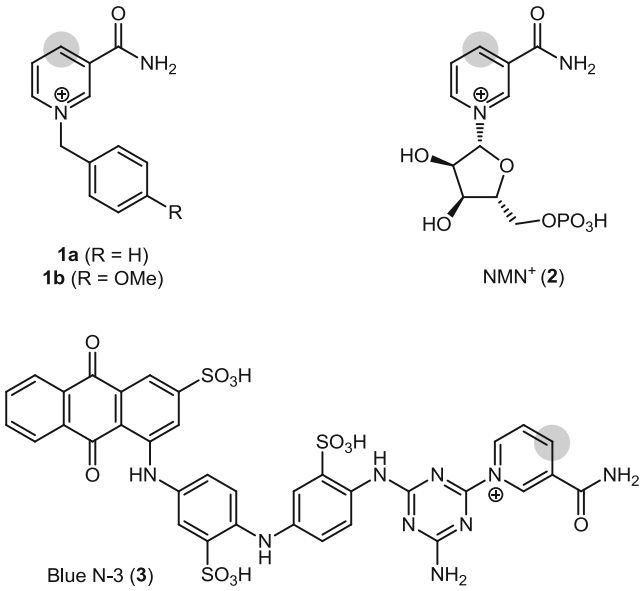
Recent years have brought about some interesting developments in this context.

NAD(P)H analogues

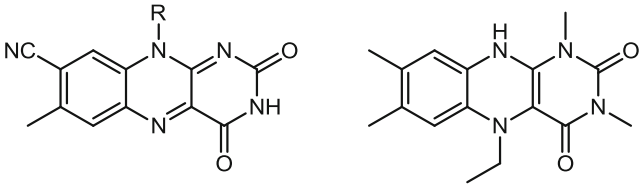
The nicotinamide cofactors are widely employed in biocatalysis and several reliable methods are available for their regeneration. On the other hand, they are rather expensive, quite unstable towards hydrolysis (in both acidic and basic media), and the redox-active part of their structure (the nicotinamide ring) is readily available and cheap. As a consequence, considerable effort has been devoted to the development of suitable synthetic NAD(P)H analogues for use in redox biocatalysis. Early studies have focused on the replacement of NADH in ADH reactions (typically using horse liver ADH) by compounds ranging in structural complexity from simple *N*-benzylnicotinamide (**1a**) to triazine dyes (e.g., **3**) whose molecular weight is higher than that of NADH itself (Scheme 14). A general limitation of these approaches arises from the fact that in ADHs the nicotinamide cofactor needs to be bound and properly positioned in the active site during the whole catalytic cycle. In addition, cofactor binding often induces subtle structural changes that facilitate catalysis. The non-natural NAD(P)H analogues lack most of the essential binding interactions, resulting both in increased K_m values and reduced reaction rates (Ansell and Lowe 1999; Campbell et al. 2012; Lo and Fish 2002).

This problem should be less severe for enzymes that only use nicotinamide cofactors for reducing a tightly bound prosthetic group such as flavin or heme. Indeed, studies on an FAD-dependent hydroxybiphenyl monooxygenase (HbpA) indicated that **1a** reduces free FAD approximately 40 times faster than NADH does (Lutz et al. 2004). On the other hand, **1a** showed a 130-fold increased K_m value with HbpA relative to NADH, and led to 'decoupling' of the flavin reduction and the substrate hydroxylation (resulting in hydrogen peroxide formation). Reduced cofactor affinity and lower rates have also been observed in a study that used **1a** and its *para*-methoxy derivative **1b** to support P450 MO reactions (Ryan et al. 2008). However, when using a P450 BM3 variant with a modified cofactor binding site, and a 200 μM concentration of cofactor, the rate attained with the two synthetic nicotinamide derivatives was comparable to the one observed with NADH or NADPH (15–23 vs. 30–35 nmol s^{-1} mg^{-1}). The most promising results so far have been obtained by using *N*-alkylnicotinamides in C=C reductions catalyzed by enoate reductases: Hollmann and coworkers have recently demonstrated that several synthetic nicotinamide derivatives actually allow faster reactions than NAD(P)H, while the enantioselectivity of the biotransformations is fully retained (Paul et al. 2013). In addition, the use of non-natural cofactors improved the chemoselectivity in reactions where competing carbonyl reduction by 'contaminating' ADHs present in the crude enzyme preparation is usually observed.

An unsolved problem is still the in situ regeneration of non-natural nicotinamide cofactors. Although reduction of *N*-benzylnicotinamide **1a** by a rhodium (II) complex and formate as reducing agent has been demonstrated, this catalyst has proven prone to detrimental interactions with enzymes, which result in deactivation of both the biocatalyst and the metal complex (Lutz et al. 2004; Poizat et al. 2010; Ryan et al. 2008).



Scheme 14 Examples of NAD(P)H analogues. The hydride-accepting site of the molecules (C4 of the nicotinamide ring) is highlighted



Scheme 15 Examples of synthetic flavin derivatives

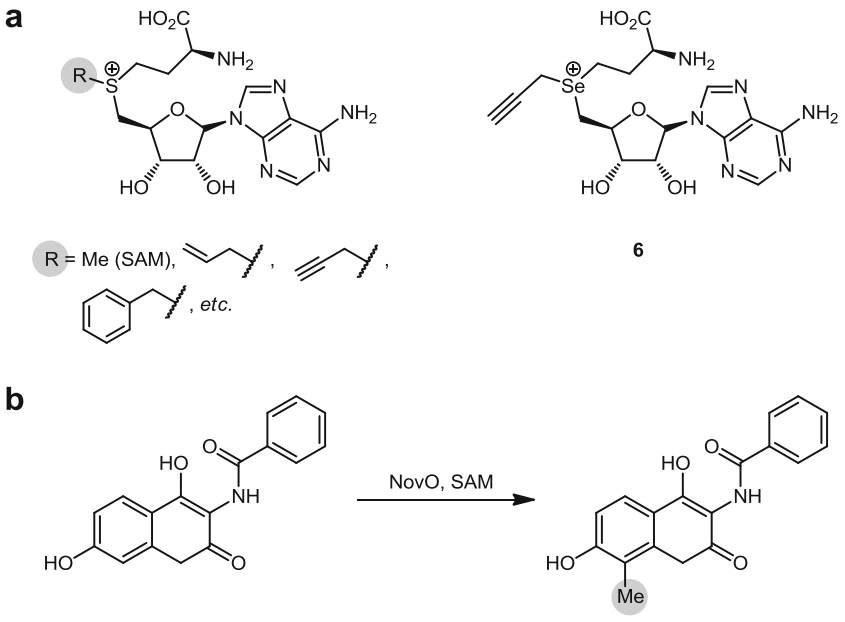
Flavin analogues

Non-natural flavin derivatives bearing various substituents on the isoalloxazine ring have found broad use as active site probes for flavoproteins and as organocatalysts for oxidation reactions (de Gonzalo and Fraaije 2013; Hefti et al. 2003). Due to their altered redox potential, they can also have a profound influence on the catalytic activity of an enzyme. Massey and coworkers have succeeded in conferring desaturase activity upon 'old yellow enzyme' (OYE) from yeast by supplying the apo-protein with 8-cyano-8-demethyl-FMN (8-CN-FMN, **4**; Scheme 15) as a cofactor (Murthy et al. 1999). The raised redox potential (−50 mV, compared to −207 mV for native riboflavin) of **4** rendered the aerobic oxidation of carbonyl compounds to the α,β-unsaturated derivatives faster than the NADPH-dependent C=C reduction that is usually observed with OYEs. Hence, through manipulation of the cofactor the natural activity was reversed.de Gonzalo and coworkers (2011) have recently taken this approach one step further by incorporating *N*-alkylated flavins (e.g., **5**, Scheme 15) into riboflavin-binding protein (RfBP) from chicken eggs, which does not show any natural catalytic activity. The RfBP-flavin complexes prepared in this study exhibited moderate activity and stereoselectivity in sulfoxidation reactions using H_2O_2 as the oxidant.

SAM analogues

SAM is the cofactor required by most methyltransferases (MTases), which incorporate the *S*-methyl group into their substrate (Klimašauskas and Weinhold 2007; Struck et al. 2012). Recent studies have shown that these enzymes are not restricted to the natural cofactor and can also accept various synthetic SAM derivatives bearing larger alkyl groups. Stecher et al. (2009) have reported on the 'Friedel–Crafts' alkylation of coumarins, using MTases from *Streptomyces* spp. and synthetic SAM analogues carrying vinylic, propargylic, or benzylic substituents. The non-natural alkyl donors were transferred onto a range of coumarin derivatives with high efficiency (Scheme 16), and kinetic analysis demonstrated that the vinyl analogue even reacts faster than SAM (Tengg et al. 2012). A related approach was followed by Winter and coworkers (2013), who have used the propargyl-seleno analogue **6** (Scheme 16a) of SAM as cofactor for the in-line MTase domain of a polyketide synthase, resulting in the formation of novel, propargylated polyketide products. In addition, synthetic SAM derivatives have found wide application for the functional labeling of biomacromolecules and other MTase substrates (Islam et al. 2011; Lee et al. 2010; Motorin et al. 2011; Peters et al. 2010; Wang et al. 2011; Willnow et al. 2012).

Overall, alkylation using SAM analogues has been demonstrated for an impressive variety of substrates and transferable groups. The main limitation of the method is the lack of



Scheme 16 Examples of synthetic SAM analogues (**a**), and biocatalytic methylation of a coumarin derivative by the SAM-dependent methyltransferase NovO (**b**)

an in situ regeneration system for SAM and its derivatives, which presents both a practical and an economic challenge.

Conclusions and outlook

Emerging strategies in cofactor-dependent biotransformations are clearly focussing on high atom efficiencies and productivities, and on the synergistic combination of enzyme-catalyzed reactions. These developments enable novel biocatalytic synthesis routes or provide resource-saving alternatives for existing biotransformations. From an environmental point of view, more quantitative analyses (e.g., life cycle assessments) are highly desirable to evaluate the 'greenness' of the novel approaches. The concepts discussed in this review arouse our curiosity to see their large-scale application, which will expand the scope of cofactor-dependent biotransformations for the production of building blocks in pharmaceutical, agrochemical, and food industries.

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