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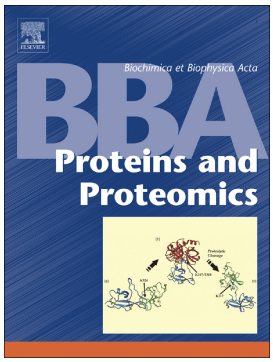

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Using enzyme cascades in biocatalysis: highlight on transaminases and carboxylic acid reductases

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Abstract

Biocatalysis, the use of enzymes in chemical transformations, is an important green chemistry tool. Cascade reactions combine different enzyme activities in a sequential set of reactions. Cascades can occur within a living (usually bacterial) cell; *in vitro* in 'one pot' systems where the desired enzymes are mixed together to carry out the multi-enzyme reaction; or using microfluidic systems. Microfluidics offers particular advantages when the product of the reaction inhibits the enzyme(s). *In vitro* systems allow variation of different enzyme concentrations to optimise the metabolic 'flux', and the addition of enzyme cofactors as required. Cascades including cofactor recycling systems and modelling approaches are being developed to optimise cascades for wider industrial scale use. Two industrially important enzymes, transaminases and carboxylic acid reductases are used as examples regarding their applications in cascade reactions with other enzyme classes to obtain important synthons of pharmaceutical interest.

Highlights

- Enzyme cascades represent an extension of biocatalysis to multiple reactions.
- Combining cascades allows cofactor regeneration and drives reactions to completion.
- Cascade modelling offers opportunities for optimisation and greater yields.
- Transaminase and carboxylic acid reductase based cascades are powerful examples of these principles.

Keywords: Enzyme cascades, Green chemistry, Cofactor regeneration, transaminase, carboxylic acid reductase.

1. Introduction

Biocatalysis is the enzymatic transformation of chemical substrates into a target product molecule. The metabolic enzymes and secondary metabolism of microorganisms have been exploited for food and beverage preparation for thousands of years. During the 20th century, whole cells and cell extracts were used to perform reactions previously synthesised by organic chemistry [1, 2]. The increasing use of purified or semi-purified enzymes and whole cells in these reactions has been driven by the developments of DNA sequencing, bioinformatics, gene synthesis, molecular biology techniques, and highly sensitive analytical techniques [1, 3]. Applications for bulk enzymes include cleaning products, the food and leather industries, bioremediation and biofuels. Biocatalysis is well suited to production of high value chiral compounds for cosmetics, fine chemicals and pharmaceuticals [4, 5]. The use of enzymes is expected to grow significantly with the drive to a more sustainable society and 'circular economy'.

The development of sustainable or 'green' chemical manufacturing is in increasing demand [6-9]. Public concerns and regulatory pressure for the reduction in the use of metal catalysts and reduced generation of hazardous waste substances will drive further development of biocatalysts [10]. This includes their use in recycling and minimising waste and energy use. Traditional organic syntheses generally require many successive reactions. Each reaction requires tightly defined, empirically determined conditions (temperature, pH, solvents, catalysts, substrates, etc.) to achieve a high yield. After each stage, unreacted substrates and side products must be removed [11]. These syntheses often therefore require the purification of the intermediates at each stage, which can produce further waste. The succession of reactions and purification reduces both atom economy and yield, and generates a plethora of wastes including toxic materials such as metals and organic solvents. Semi-synthetic methods utilize chemicals derived from microbial, plant and/or animal sources. The availability of these chemicals is often dependent on the growth rate of the specific organism and often provides low yields, leading to unsustainable batch production. Recycling and sustainability are key aspects of the 'circular economy'. Consequently, biological methods and chemical-biological pathways are becoming the choice to develop new 'green' chemical processes within industry [3].

Enzymes are ideally suited to overcome many of the challenges within synthetic or semi-synthetic chemistry [12, 13]. They typically display high enantio- and regioselectivity which is essential for the pharmaceutical industries [13]. Using enzymes reduces the number of steps required and eliminates the need for protection and de-protection of functional groups. Enzymes are rarely toxic or dangerous, and usually work in aqueous solution under mild reaction conditions. The principles underpinning single enzyme biotransformations can be extended to cascades of multiple enzymes, performing several successive enzymatic reactions towards the desired product. Industrial

companies such as Ginkgo Bioworks, GlaxoSmithKline and Evolva were early developers of enzymatic cascades. These cascades are being used to synthesise fragrances [14], medicinal compounds [15] and flavourings [16, 17].

2. Cascade reactions

2.1 Uses and categorisation of cascade reactions

Enzyme cascades typically concatenate several natural functionalities into an enhanced natural pathway or non-natural pathway (i.e. using promiscuous enzymes from diverse sources to transform chemicals not found in nature). This allows the production of chemical commodities through biotransformation, or phenotype generation within cell-based systems [8]. The reactions can be carried out in *in vivo* or *in vitro* (reviewed by [18]). Performing reactions *in vivo* eliminates the need for the addition of expensive cofactors. However, reaction intermediates can be toxic to the cell, product recovery can be more demanding, and additional side reactions often occur reducing the yield. An additional challenge is management of the metabolic flux (i.e. the passage of a metabolite through the reaction system over time) [19]. *In vivo*, unexpected metabolic bottlenecks, or toxic effects of intermediates, can require rebalancing of the pathway to optimise metabolite concentrations. This requires a pathway engineering approach to identify rate-controlling steps and re-optimise enzyme concentrations by changing promoters or/and ribosome binding sites (RBS) [20-22]. *In vitro* reactions use either crude lysates or enzymes that have been partially or highly purified. They can be carried out as 'one pot' reactions or with flow immobilised enzymes in tandem flow bioreactors [23-25]. The latter approach allows for the flux issues to be addressed since the enzyme concentrations used can be varied and product inhibition is avoided. 'One pot' reactions can be configured so that enzymes work simultaneously, catalysts can be added sequentially at different time points, or spontaneous reactions can be initiated by an enzymatic biotransformation and continue without further catalysis [26, 27]. Many examples of 'one pot' reactions have been reported in the literature such as the regio- and stereoselective synthesis of chiral amines (reviewed by [28]).

One-pot multi-enzymatic or chemoenzymatic reaction cascades can typically be classified into one of five types (linear, orthogonal, parallel, triangular and cyclical cascades as shown in Figure 1) [15, 29]. **Linear cascades** are the simplest cascade form [30]. They involve a linear sequence of biotransformations processing a single substrate into a single product *via* one or more intermediates. This type of cascade can help alleviate unfavourable equilibria, and provide rapid

processing of unstable or toxic intermediates, with an example in [23]. **Orthogonal cascades**, in contrast, consist of a single transformation that is coupled to auxiliary reactions to promote the formation of the desired product. Orthogonal cascades achieve this in diverse ways. A very common approach is the removal of unwanted co-products or regeneration of cofactors and/ or co-substrates (to drive equilibria towards the desired product; e.g. [31, 32]). Cofactor regeneration may also be achieved by using a **parallel cascade**. Whilst orthogonal cascades couple with one or more irrelevant auxiliary reactions, parallel cascades incorporate a second potentially useful reaction with a reciprocal demand for cofactor turnover (elegantly demonstrated for $NADP^+$ and pyridoxal phosphate regeneration in [33]). **Cyclical cascades** and **triangular cascades** are the least common forms of cascade, due to their specialized applications. Cyclic cascades require recycling of some intermediates to the starting materials, for example in deracemization reactions. An excellent recent example of this is the deracemization of tetrahydroisoquinolines [34]. This one-pot enzymatic cascade improved on a previously described chemoenzymatic reaction [35]. Triangular cascades involve the conversion of an initial substrate to an intermediate that then combines with the initial substrate in a later step (with an example in [36]).

The classification of enzyme cascades using the above designs affords the most basic representation of potential processes. Cascade design may involve combinatorial versions of these cascades giving rise to interesting properties and advantages. For example, a cyclical regeneration system may be generated through combining an orthogonal cascade with a linear cascade allowing for regeneration of a co-substrate though downstream processing of an intermediate from the initial enzyme reaction (Figure 1F [37]). This ultimately reduces input of co-substrate required, as well as driving the equilibria forward for the successive reaction and has been referred to a 'recycling' cascade [38]. The critical examples below (**5.1**/**5.2**) illustrate practical combinations of cascades.

2.2 Advantages and disadvantages of cascade reactions

There is a broad desire to carry out multiple chemical steps in a single reaction process. Where this is successful, it reduces challenges of intermediate stability, isolation/purification of desired compounds, and solvent exchange [6]. There are obvious benefits in terms of space and resource use, and often the time taken to complete a process [3]. Whilst there are many good examples of chemical "one-pot" syntheses that combine two or more steps, in the majority of cases, incompatibility of conditions (especially solvents, pH and co-catalysts) requires reactions to be performed consecutively.

Cascading reactions facilitated by multiple enzymes have the potential to overcome many of the challenges within synthetic or semi-synthetic chemistry [15]. As enzymes generally have compatible reaction conditions (neutral or slightly alkaline pH, mild temperature, presence of similar salts and mild reducing agents) [39], there are reduced barriers to having multiple reactions proceeding simultaneously in comparison to purely chemical reactions. For enzyme-catalysed reactions, this offers particular advantages. Enzyme reactions can generate partially unstable intermediates, and a cascade reaction can resolve this with a further reaction [40]. This has the particular advantage of facilitating schemes including reactive substrates (e.g. aldehydes). Enzymes catalyse both forward and reverse reactions, and so reactions operating closer to equilibrium result in a mixture of substrate and product. Using enzyme cascades offers the opportunity to drive reactions towards completion by coupling with another reaction to drive the reaction in the desired direction [18]. Side reactions are also minimised in cascade reactions, as successive reactions drive equilibria in favour of the desired product.

However, the disadvantages of enzyme cascades must be considered when designing new biotransformations. In particular, the majority of enzymes have a narrow operational range, and precise requirements for activity [39]. Enzymes generally show greater than 50% activity over a pH window of 2-5 pH units; enzyme activity generally increases with temperature until the protein unfolds; and enzymes require a collection of salts and buffers that may not be compatible (e.g. magnesium is both a cofactor and inhibitor of many enzymes). Particularly where enzymes in the cascade have differing cofactor requirements, cascades may require multi-factorial optimisation (e.g. using a design of experiments approach [41]) to obtain maximum activity.

A further challenge for incorporation of enzyme cascades into larger synthetic pipelines is that enzymes are generally evolutionarily optimised towards their biological role *in vivo,* rather than large scale industrial application. Challenges imposed by this include poor solvent compatibility with desired products [42], restricted substrate ranges [39], substrate flux sinks [43], and low turnover rates. Initially many enzymes were only available in small amounts and at high cost, being extracted from animal or plant tissues [8]. With developments of molecular biology techniques and discovery of new enzymes using bioinformatics it is possible to produce many enzymes in the quantities required for industrial applications. A limitation is still often encountered in enzyme expression in a soluble form and enzyme stability. However, this is improving with the development of new expression vectors and expression hosts allowing enzymatic applications for *in vitro* processes. These limitations apply not only to enzyme cascades but also chemoenzymatic cascades [26], primarily due to reaction condition incompatibility [44]. However, several examples of incorporation of chemical steps with biocatalysis for *in vitro* processes have been demonstrated [12, 44-48].

2.3 Cofactor recycling

Many enzyme-catalysed transformations require cofactors. These are complex organic molecules that assist the protein with catalytic activity, facilitating reactions that are challenging using only the twenty natural amino acids [39]. In many cases these are catalytic and are required only at a concentration sufficient to ensure complete loading of enzymes (e.g. thiamine diphosphate, pyridoxal phosphate). However, the most common enzyme cofactors, NAD^+, $NADP^+$ and ATP, form part of the reaction and are modified as well as the substrates (e.g. NAD^+ accepts a hydride as a substrate is oxidised). These coenzymes are too costly to provide at stoichiometric levels [29], and are easily degraded under some conditions, for example the presence of oxygen presence, extremes of pH or high temperature [49]. This can be a problem for enzyme cascades comprising enzymes requiring cofactors and prevents their more widespread use in industry. For a biotransformation to be economically viable an efficient regeneration and reuse of the cofactors is essential [50]. The field of co-factor recycling has been extensively studied for oxidoreductases as the enzymes in this class require the NAD(H) or NADP(H) cofactors. Many chemical, enzymatic and electrochemical methods have been investigated to regenerate various cofactors [51, 52]. There are two main strategies for enzymatic cofactor regeneration. The first approach is a substrate-coupled reaction systems, in which one enzyme that uses both the reduced and oxidized forms of a cofactor is applied to catalyse both the synthesis of the product from one substrate and the cofactor regeneration reaction with a second substrate [50]. An example of substrate coupled regeneration is described by Tan and co-workers [53] using *Streptomyces griseus* carbonyl reductase for the conversion of prochiral ketones into highly optically active alcohols. Isopropanol was used as the co-substrate for the NADH recycling in the substrate-coupled reaction (Figure 2A).

The second strategy, enzyme-coupled reactions, makes use of a different enzyme to catalyse the cofactor regeneration reaction. This strategy provides much greater flexibility in term of choice of substrates and is widely used. For example, the most commonly used recycling enzymes for NADH and NADPH are glucose or formate dehydrogenases [54]. More recently, the P450 BM3 monooxygenase has been used to regenerate $NADP^+$ through the direct reduction of oxygen (Figure 2B; [55]). Also, excellent total turnover numbers have been achieved using a glutathione-based recycling system that combines glutaredoxin from *Escherichia coli* and the glutathione reductase from *Saccharomyces cerevisiae* for $NADP^+$ regeneration. This system uses organic disulfides (oxidized cysteine or 2-hydroxyethyl disulfide) as oxidizing agents and allows $NADP^+$ recycling with a total

turnover number in excess of 5×10^5 [56]. Phosphite dehydrogenases have also been employed, which offer the advantages of an inexpensive substrate and unreactive product (phosphate) [57].

ATP regeneration is less studied than other cofactor regeneration systems. In recent years several methods have emerged using enzymes like pyruvate kinase, creatine kinase, adenylate kinase and polyphosphate kinase [52, 58]. More recently, the use of *E. coli* cell lysate was shown to be an effective way to recycle ATP [59]. This method used the acetate kinase activity in the lysate to simultaneously support multiple ATP-dependent enzyme-catalysed chemical processes. The equilibrium was driven towards ATP recycling by the addition of cheap acetyl phosphate.

2.4 Kinetic modelling of cascades

A major challenge for the deployment of biocatalytic processes to industrial process plants is to determine the appropriate and optimal scales for operation and the quantities of each reactant to be used. Developing a reaction model early in the design process can demonstrate the practical and economic feasibility of a proposed process [60]. Models can further aid the critical evaluation of pathways and highlight potential issues (e.g. bottlenecks, inhibitors, side-product formation) [61, 62]. However, such reaction engineering is a particular concern for enzyme cascades, as the detailed modelling of even a single enzymatic process can be challenging [41]. The two main approaches to modelling cascades are constraint-based modelling and kinetic modelling. Constraint-based modelling abstracts the process into mathematical equations (e.g. flux-balance analysis, or a design of experiments approach [63, 64]). Parameters are fitted to these equations based on experimental measurement of the system. These models have the advantages of making no assumptions about the system, and being experimentally efficient. However, the abstract nature of these models renders them less able to predict or identify bottlenecks [60].

Kinetic models, in contrast, are based on kinetic rate laws for individual reactions. The relevant rate laws and parameters for each step are determined experimentally, using either a systematic approach [65, 66] or a design of experiments approach [41]. Alternatively, parameters have been fitted to the entire system for up to fifteen reactions [67]. The large number of parameters to be fitted leads to multiple solutions, reducing the predictive power of the model [67]. Kinetic models have proved highly insightful in the study of metabolic pathways in well-studied model organisms [68, 69]. However, the use of kinetic modelling has been poorly utilised or ignored in most process development. Indeed, kinetic models of enzymatic reactions have only been explored in a few studies. Key examples are cofactor recycling *in vitro* [65], simulation of a three-enzyme cascade

system for synthesis of 6-hydroxyhexanoic acid [70], modelling of the stability of an aldolase [71], or a three-enzyme cascade for the biotransformation of sucrose to cellobiose [72].

Both constraint-based and kinetic modelling have pitfalls that must be considered for application to industrial processes. In particular, industrial processes usually require high concentrations of reactants to be economic [41], and non-aqueous solvents are likely to be necessary to maintain substrates, products and intermediates at these concentrations. The conditions modelled may not be reflective of the final process. This is particularly the case for kinetic models, as the detailed parameters may well be affected by these conditions, and other reactants become inhibitory at the high concentrations.

3. Critical examples

3.1 Transaminase cascades

Transaminases (TAs; EC 2.6.1) are enzymes whose use in the industrial biocatalysis pipeline is mature [73, 74]. Since many drugs have chiral amines within their structures the TAs have been used extensively by pharmaceutical companies to provide these important building blocks (Figure 3A). The TAs are part of a large group of enzymes that use the pyridoxal phosphate cofactor (PLP). This forms a covalent bond (a Schiff base) with the N_{ζ} atom of the active site lysine during the reaction mechanism [75]. The TA family of enzymes have been classified into six different Pfam groups on the basis of their secondary/ternary structure and substrate specificities [76]. The TAs most commonly used in biocatalysis are the TA group 3 enzymes. These are the so-called ω-TAs that catalyse the amination of substrates with a ketone/aldehyde component distal to the carboxyl group [77]. These TAs generally have a broad substrate specificity, and are all *(S)*-selective. Key examples are the TAs from *Vibrio fluvialis* [78], *Chromobacterium violaceus* and *Pseudomonas aeruginosa* [79], all of which have been biochemically and structurally characterised [80-82]. There are many examples where these TAs have been employed in cascade reactions to produce chiral amines by both kinetic resolution via enantioselective deamination or by asymmetric synthesis using reductive amination (reviewed by [29, 75]). The reductive amination allows the product to be produced in 100% yield. However, there is an unfavourable equilibrium which lies on the side of the starting material. An early approach utilised other enzymes such as a lactate dehydrogenase coupled to glucose dehydrogenase to shift the equilibrium to product formation [83, 84]. Related approaches use pyruvate decarboxylase [85]. The essential PLP cofactor can potentially be recycled *in situ* on the enzyme. However, for some TAs, especially for the commonly used *C. violaceus* enzyme, additional

PLP needs to be present in the biocatalytic reaction [86]. For these reasons and to enable to control the flux through the enzyme cascade these reactions are carried out *in vitro*. The linking enzymes that drive the equilibrium to completion were used together as part of a larger cascade by Pickl and colleagues to prepare a series of functionalised amines from the corresponding alcohols (Figure 3B) [87], and by Monti and colleagues to prepare functionalised amines from unsaturated ketones [88].

The group 4 TAs have also been of specific interest since they catalyse transamination of branched chain L-amino acids (BCAAs), D-amino acids or ω-amino acids. These enzymes are naturally *(R)* selective and the catalysis within this group occurs on the *re*-side of the cofactor, which differs from most of the other PLP enzymes where the reactions occur on the *si*-side. By comparing the sequence for group 4 TAs activity a sequence fingerprint has been established [89]. In addition to the commercially available enzymes, many groups have modified TA enzymes using rational and semi-rational protein engineering and directed evolution methods [90]. More thermally stable and robust TA enzymes of the groups 3 and 4 have been identified and characterised from thermophilic genomes and metagenomes [91, 92].

An industrial milestone for using the *(R)* selective enzymes was achieved by Merck and Codexis who applied an engineered TA for the large scale biocatalytic production of the Sitagliptin, an important anti-hypertensive drug [93]. Many new cascade reactions have been developed using the TA enzymes and this mini-review can only provide a few examples. These cascades involve other enzymes to access biocatalytic routes to an increasing number of biologically active important compounds. TAs have been used in combination with monoamine oxidase variants to enable the regio- and stereo-selective synthesis of chiral 2,5-disubstituted pyrrolidine building blocks and the deracemization and dealkylation of a range of methylbenzylamine derivatives (Figure 3C) [28, 94]. Another elegant example of the advantages of TAs is the synthesis of the vasopressor methoxamine from 2,5-dimethoxybenzaldehyde and pyruvate [95]. The product, generated from two achiral substrates, contains two newly formed chiral centres. With careful selection of the enzymes, the preferred stereoisomer was over 99% of the product, with a yield of 64% (Figure 3D). A similar approach extended the cascade with norcoclaurine synthase to convert the product to form industrially valuable tetrahydroisoquinolines (Figure 3E [44]). These reactions were most efficient when performed sequentially without purification of intermediates, to prevent the enzymes forming side products or catalysing unwanted reactions. This highlights one of the challenges of extending well characterised reactions into cascades where other reactants may interfere with one enzyme's function.

An interesting cascade reaction using an ω-TA together with the following highlighted enzymes (carboxylic acid reductases) together with an imine reductase enzyme has been used in a one pot synthesis of mono and di-substituted piperidines and pyrrolidines, which are found in many biologically active natural products and pharmaceuticals [96]. This further illustrates how the application of the TA enzymes has been vital to accelerate the reduction of traditional chemistry by more sustainable biocatalytic reactions.

3.2 Carboxylic acid reductase cascades

Carboxylic acid reductases (CARs; EC 1.2.1.30) are a family of enzymes with increasing importance in 'green' chemistry [97, 98]. They are large (~130 kDa) multi-domain enzymes that catalyse the reduction of both aromatic and aliphatic carboxylic acids to their aldehydes [40, 99]. This thermodynamically unfavourable reaction is driven by the hydrolysis of ATP to AMP and pyrophosphate, and the oxidation of NADPH to $NADP^+$ [40]. CARs offer an environmentally attractive alternative to the use of harsh reducing agents such as $LiAlH_4$. Their regioselective reduction of carboxylic groups eliminates the need for protection, and they operate under mild conditions. CARs are particularly attractive for cascade reactions, as they generate the reactive aldehyde group in a single step (avoiding the full reduction and selective oxidation often necessary with chemical methods). This group is a substrate for a wide range of synthetically useful reactions (for example reductive aminases [100]), whilst many chemical and enzymatic reactions produce carboxylic acid moieties (Figure 4A; [65, 98, 100]). *In vivo* pathways using CARs to connect reactions have been successfully achieved. Examples include the production of the flavour vanillin by yeast [101], the drug building block 3-hydroxytyrosol [102] and a synthetic pathway for the production of propane in *E. coli* [103].

An elegant example of a cascade reaction to exploit CARs was demonstrated by France et al. [96]. They employed a three enzyme cascade (CAR, transaminase - TA, imine reductase - IRED) to convert keto acids into piperidines (Figure 4B), which are common pharmaceutical building blocks. The final step in this cascade creates a new stereocentre, and substrates can have additional chirality. The product enantiomers depend on the choice of stereoselective TAs and IREDs. This work also included an orthogonal cascade, using lactate dehydrogenase to remove the TA product pyruvate and so drive the intermediate step to completion. One limitation of this cascade is the need for two reducing equivalents per reaction. To overcome this, the same team successfully added the main cascade for expression in *E. coli* (which has abundant lactate dehydrogenase) [104]. They achieved conversion rates of up to 93% for a three step *in vivo* transformation. This demonstrates the concept of designing and building biosynthetic cascades into *E. coli* host cells, with the cells able to meet all

cofactor requirements and synthesise enzymes. The cells can then produce pharmaceutically and industrially relevant chemical scaffolds.

An alternative approach to the industrial application of CARs is to utilise recycling cascades to regenerate cofactors *in vitro*. Strohmeier et al. [105] used two parallel recycling cascades to regenerate the ATP and NADPH cofactors of CARs (Figure 4C). They used the commonly employed polyphosphate kinase pathway to regenerate ATP, and glucose dehydrogenase to regenerate NADPH. Using these recycling cascades, a range of aliphatic and aromatic carboxylic acids were converted to aldehydes with over 99% conversion, when using ATP and NADPH at a 1:10 and 1:20 ratio with respect to the substrate.

Finnigan et al. combined these two ideas to develop a three step enzymatic cascade from an ester to an alcohol *in vitro* [65]. This transformation included two parallel recycling cascades and an orthogonal cascade to remove an inhibitory by-product, requiring seven enzymes (Figure 4D). This cascade was demonstrated for the reduction of methyl 4-toluate to 4-tolyl alcohol, and achieved approximately 75% yield of product. This study developed and validated a kinetic model of the entire seven enzyme system, and demonstrated that this could be used to optimise conditions for increased turnover. This work highlights the value of kinetic models for developing enzymatic cascades, and that this is eminently possible for systems even with complex cascades.

4. Conclusions and perspective

Biocatalysis has a proven track record of offering replacements for individual chemical reactions with a lower environmental impact. Cascade reactions are an extension of biocatalysis to couple a series of reactions and so provide replacement for more than one chemical step. Cascade reactions offer several further advantages in comparison to conventional biocatalysis. In particular, they provide a route to utilising enzymes that operate near equilibrium, or which have unstable substrates or products. Effective recycling cascades have been developed to recover costly cofactors using inexpensive sacrifice substrates. Modelling approaches are being developed to predict conditions for optimal turnover, especially at the high concentrations that are required for economic scale-up. The carboxylic acid reductases are an important example of enzymes that have been successfully deployed *in vivo*, and which are now being developed for biocatalytic cascades. They demonstrate the advantages of using cascades by linking reactions and providing a highly reactive product utilising the development of cofactor recycling cascades. Transaminases demonstrate the value of cascades for driving reactions towards the desired products. Their successful integration with several

different partner enzymes highlights the value of “modular” enzyme sets. Successful industrial deployment of cascade reactions illustrates their rich potential as part of the industrial synthetic biocatalytic ‘toolkit’.

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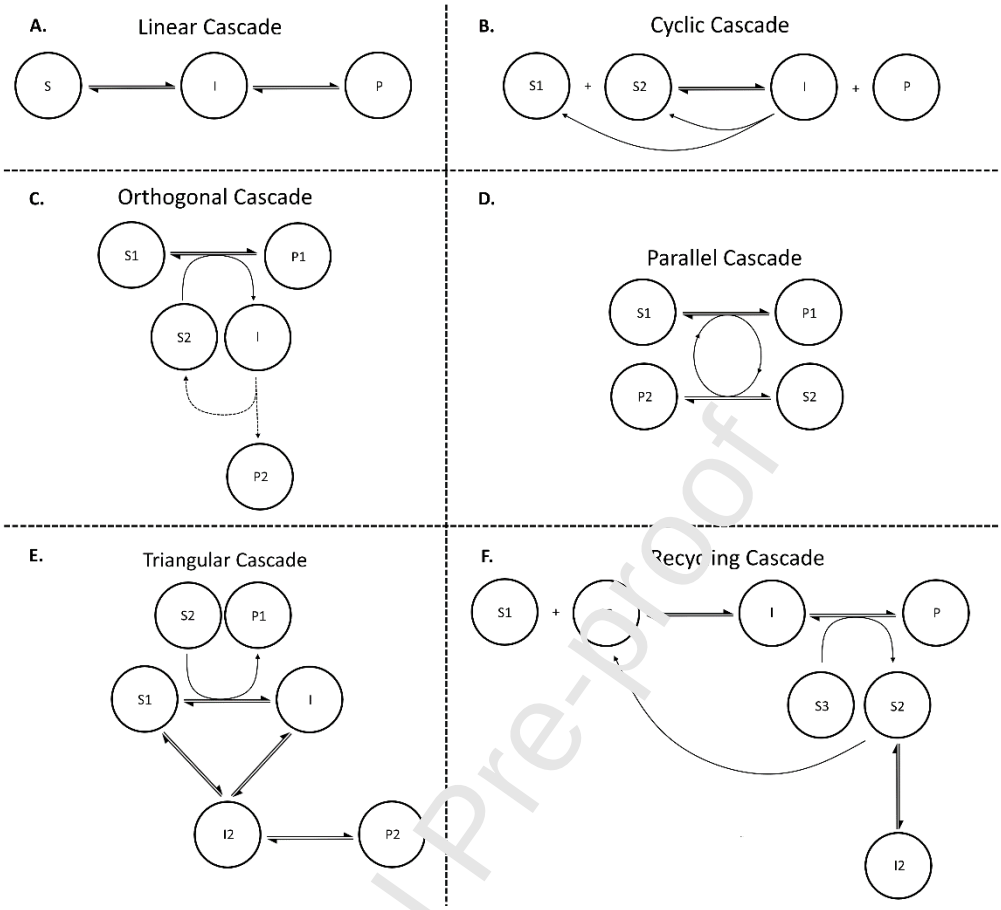

Figure 1. Types of enzyme cascade. A: Linear cascade: a single product is synthesized via one or more intermediates states from an initial substrate. **B:** Cyclic cascade: a combination of substrates is transformed into an intermediate that is subsequently converted back to substrate, shifting the equilibrium towards one of the initial substrates. **C:** Orthogonal cascade: product formation is coupled with a co-factor/co-substrate regeneration step or to destructive by-product reactions. **D:** Parallel cascade: two distinct enzymatic reactions are coupled through co-factors/co-substrates. **E & F**: Combinations: these enzymatic cascades can be combined to produce novel cascades. For example, **F** [38] links a linear cascade and an orthogonal cascade. Legend: S, Substrate; P; Product; I, Intermediate.

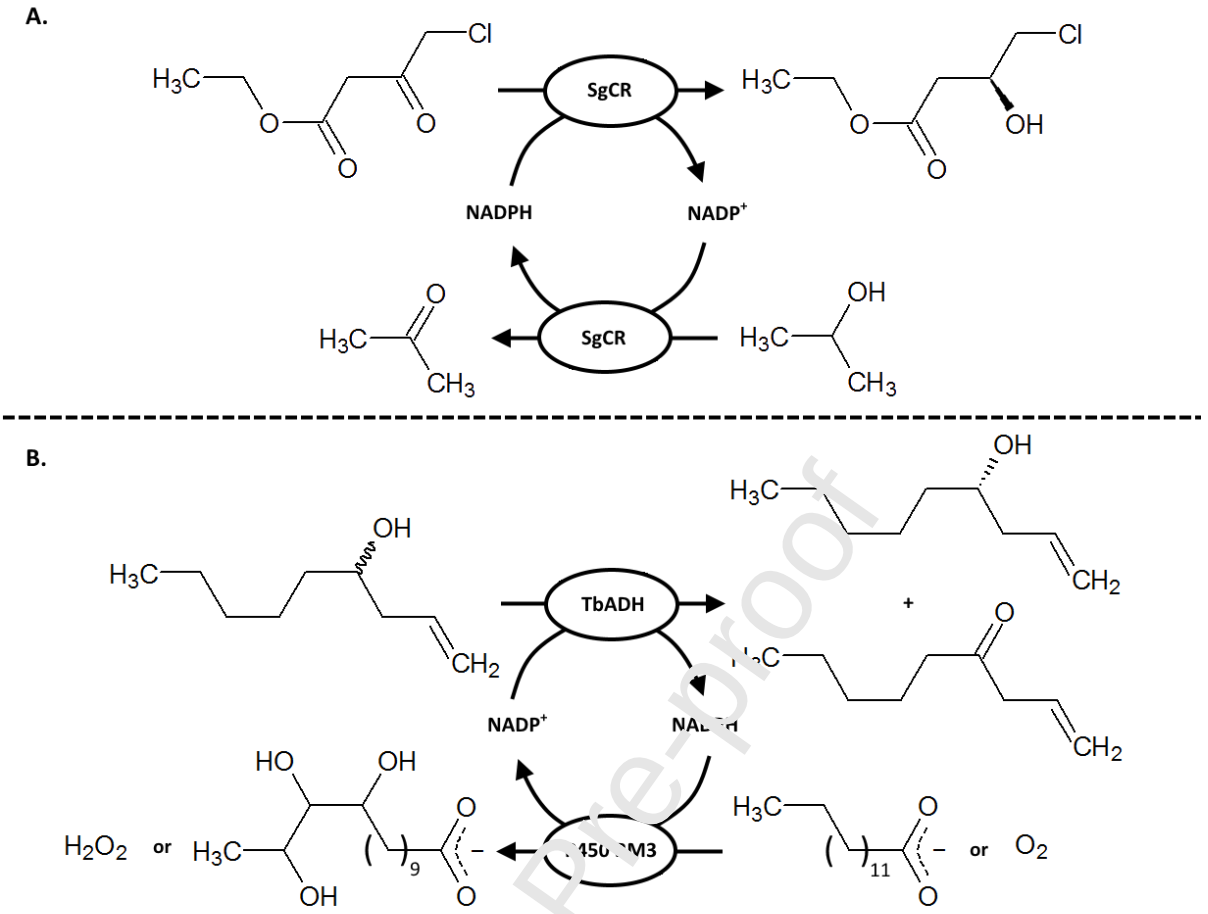

Figure 2: Examples of substrate and enzyme coupled cofactor regeneration strategy. A: Conversion of 4-chloro-3-oxobutanoate (COBE) to *(S)*-4-chloro-3-hydroxybutanoate (*(S)*CHBE) via *Streptomyces griseus* carbonyl reductase (SgCR), the recycling of NADH is an example of substrate coupled conversion with 1.6 equivalent of isopropanol to COBE [53]. **B:** The *Thermoanaerobacter brockii* alcohol dehydrogenase (TbADH) catalyses the conversion of the racemic alcohol 1-nonen-4-ol to *(S)*-1-nonen-4-ol and the corresponding ketone. The reaction is coupled to P450 BM3 for $NADP^+$ regeneration. Surprisingly, the P450 BM3 cofactor regeneration enzyme can uncouple from its usual myristic acid substrate and instead reduce oxygen [55].

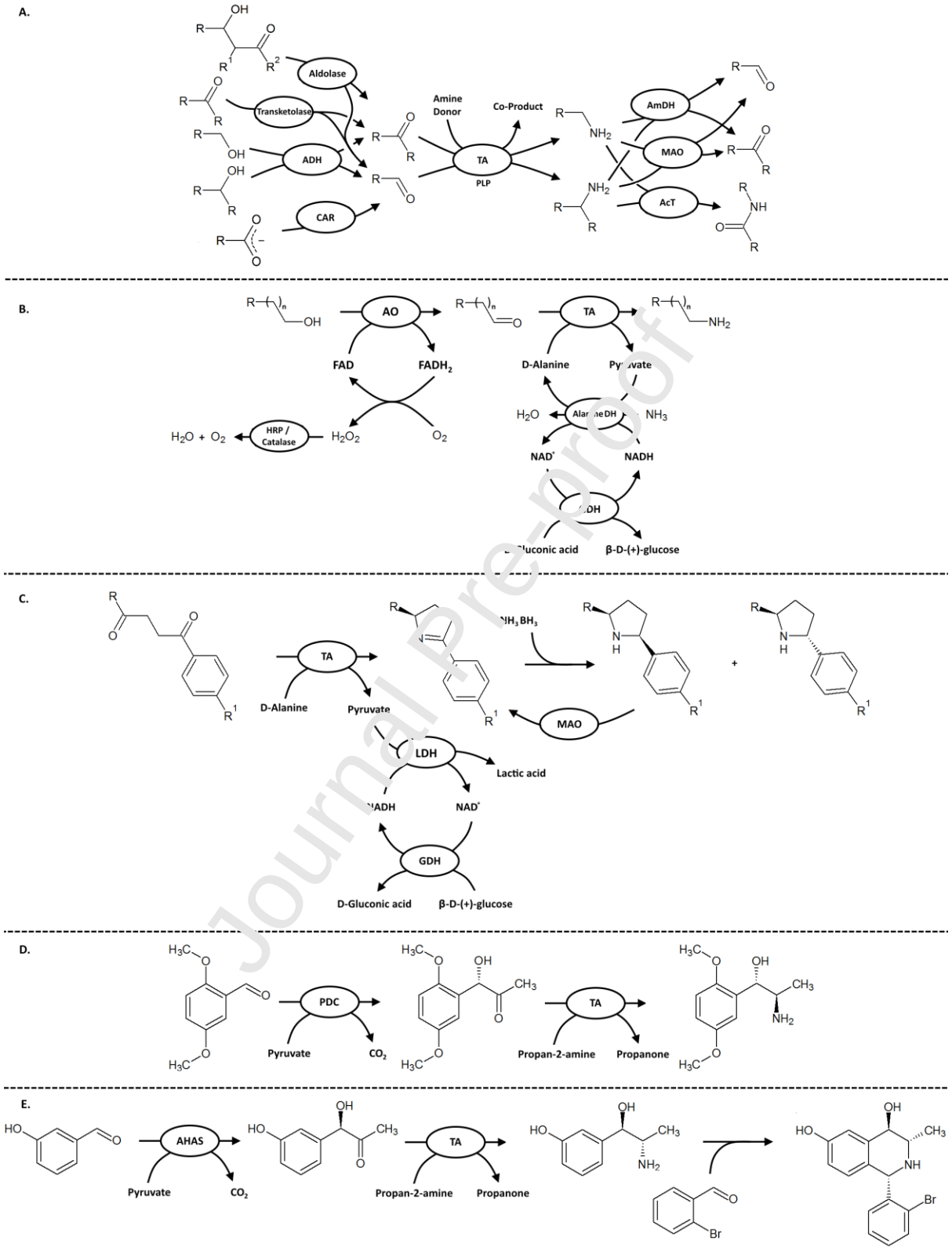

Figure 3. Utilizing transaminases in multi-step enzyme reactions. **A:** TA can use products from many industrially relevant enzyme reactions as substrates, and their products are substrates for other

enzymes. Cofactors and additional substrates are not shown for clarity. AcT: acetyltransferase [106]; ADH: alcohol dehydrogenase; AmDH: amine dehydrogenase; CAR: carboxylic acid reductase; MAO: monoamine oxidase; TA: transaminase. **B**: Transaminase based cascade to aminate primary alcohols. Several auxiliary enzymes drive this reaction to completion through by-product and cofactor recycling mechanisms utilizing HRP/catalase, AlanineDH and GDH. AO: Alcohol Oxidase, HRP: Horse radish peroxidase, AlanineDH: Alanine dehydrogenase, GDH: Glucose dehydrogenase. [67] **C**: Chiral 2,5-disubstituted pyrrolidine building blocks were synthesised using a chemoenzymatic cascade. This approach exploits several biocatalytic processes and takes advantage of the complementary regioselectivity displayed by the ω-TA and MAO variants to establish two stereogenic centres, minimising waste generation. LDH: Lactate dehydrogenase. [28, 94] **D**: (1*S*,2*R*)-methoxamine synthesis using a biocatalytic 1-pot two-step cascade approach. PDC: pyruvate decarboxylase. [95] **E**: Example of a enzymatic and chemoenzymatic three-step cascade for the synthesis of the trisubstituted tetrahydroisoquinoline (1*S*,3*S*,4*R*)-1-(2-bromophenyl)-1,2,3,4-tetrahydroisoquinoline-4,6-diol. AHAS: acetohydroxyacid synthase. [44]

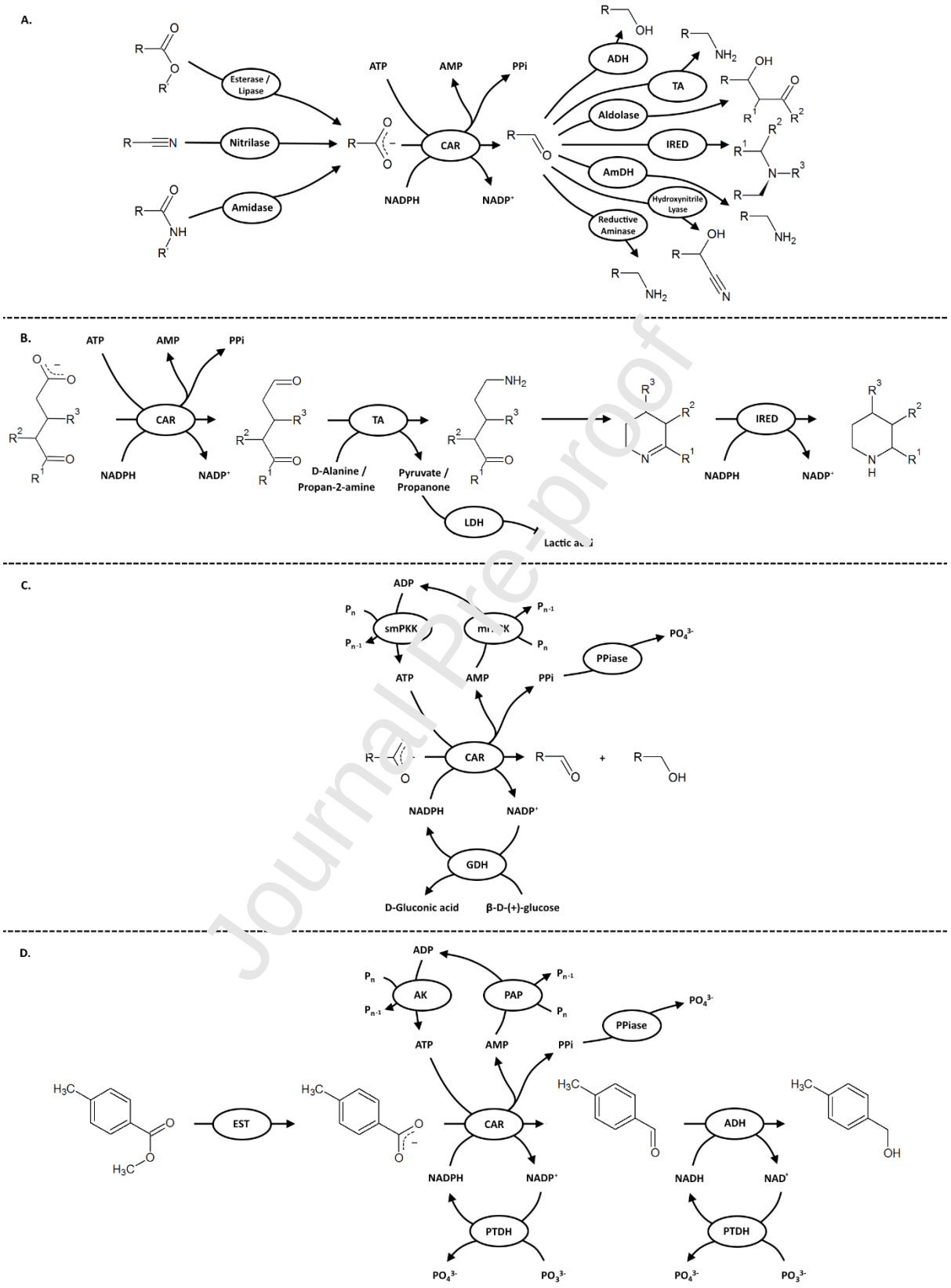

Figure 4. Utilizing CARs in multi-step enzyme reactions. **A**: CARs join many industrially relevant enzyme reactions, making them useful for the construction of novel multi-step enzyme reactions. Cofactors and additional substrates are not shown for enzymes other than CAR for clarity. ADH:

alcohol dehydrogenase, TA: transaminase, IRED: imine reductase, AmDH: amine dehydrogenase, CAR: carboxylic acid reductase. [65, 96, 107, 108] **B**: Whole cell *de novo* enzyme cascade for the diastereoselective and/or enantioselective conversion of linear keto acids into cyclic amine products. This cascade proceeds through carboxylic acid reduction, transamination, imine formation, and imine reduction. The cascade required a keto acid substrate and an amine donor. TA: Transaminase, (R/S)-IRED: imine reductase, LDH: lactate dehydrogenase. The TA cofactor pyridoxal-5'-phosphate is not shown. [96] **C**: Reaction Scheme for the *in vitro* reduction of carboxylic acids with full recycling of all cofactors. PPK: polyphosphate kinase, GDH: glucose dehydrogenase, Pn: polyphosphate, PPi: pyrophosphate, PPiase: pyrophosphatase, PO_4: phosphate. [105] **D**: A modelled seven enzyme reaction for the production of 4-tolylalcohol from methyl 4-toluate via the intermediates 4-toluic acid and 4-tolualdehyde. EST: Esterase, ADH: Alcohol dehydrogenase, PTDH: phosphite dehydrogenase, PAP: Polyphosphate AMP phosphotransferase, AK: Adenylate kinase, PO_3: Phosphite. R: R-group, limited by enzyme substrate specificity. For clarity, phosphopantetheinyl transferases required by CARs and the consumption or production of water and protons, is not shown. [65]

Using enzyme cascades in biocatalysis: highlight on transaminases and carboxylic acid reductases

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Declaration of interest statement:

The authors declare that they have no competing interests.