MINIREVIEW

The impact of recent developments in technologies which enable the increased use of biocatalysts

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Abstract: While biocatalytic transformations are very powerful in enantioselective synthesis, frequently occurring under mild conditions and with extraordinary selectivity, there are practical challenges associated with the use of biocatalysis such as limited substrate scope, stability and reusability. Recent technological developments, for example immobilization, continuous flow and molecular biology, all contribute towards enhancing the use of enzymes in synthesis.

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Anita R. Maguire was born in 1964 in Cork. She undertook undergraduate and postgraduate studies at UCC (B.Sc., 1985; Ph.D., 1989), focusing during her studies on asymmetric catalysis in reactions of α-diazoketones. Following postdoctoral research in the Facultes Universitaires, Namur, Belgium, and subsequently at the University of Exeter, she returned to UCC in 1991 initially as a Lecturer in Organic Chemistry, then as Associate Professor of Organic Chemistry in 2002, and then as the first Professor of Pharmaceutical Chemistry in 2004. In 2011 she was appointed as Vice President for Research and Innovation at University College Cork. She was an Adjunct Professor at the University of Bergen from 2011-16. Her research interests include asymmetric synthesis, including biocatalysis and transition-metal catalysis, the development of novel synthetic methodology employing *α*-diazocarbonyl compounds, organosulfur chemistry, and continuous flow chemistry, and the design and synthesis of bioactive compounds with potential pharmaceutical applications and she is a Co-PI in the SSPC. She is the inaugural Chair of the National Forum on Research Integrity and was elected a Member of the Royal Irish Academy in 2014.

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Biocatalysis is a useful tool in chemical synthesis in particular in relation to enantioselective synthesis, and recent advances in technology provide the resources to expand use of biocatalysis beyond the natural substrates, as well as to enhance the stability of enzymes and scope of enzymatic transformations.[1]

1. Enabling technology

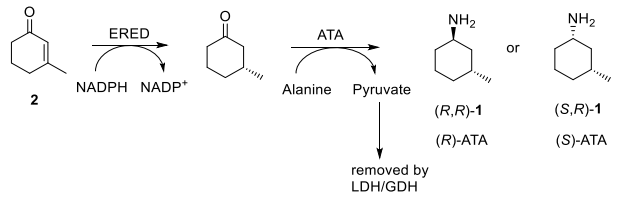
Rapid improvements in underpinning technology ranging from developments in molecular biology, which enables engineering of cells for the coexpression of multiple enzymes, thereby enabling cascade processes, discovery and design of novel biocatalysts, immobilization and use of continuous processing have significantly enhanced the synthetic utility of biocatalysis as discussed in the individual sections below.

1.1 Engineering cells for coexpression of multiple enzymes & cascade biocatalysis

Because of the extraordinary selectivity of biocatalysts, they can be particularly suited to use in cascade systems, where the product from one reaction is further reacted without the need for isolation. Biocatalysts can be used both with other biocatalysts or with chemocatalysts.[2] The topic of multi-enzymatic cascades has been reviewed recently.[3] *E. coli* and other host organisms can be used to coexpress multiple enzymes in a cascade bioreactor. All of the enzymes can be produced by the same cell by modification of the plasmid(s) which are inserted into the expression vector. The use of multiple enzymes in a one-pot reaction can be attractive, because of the specificity of enzymes, minimizing cross-reaction.

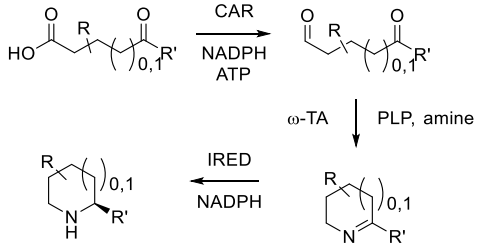
The combination of coexpressed enoate reductase (ERED) and amine transaminase (ATA) has been recently reported (Scheme 1).[4] The stereochemistry at the *β*-position is determined by the enoate reductase, using either an *(S)*- or *(R)*- selective enoate reductase and the use of the appropriate transaminase gives either *(S)*- or *(R)*- stereochemistry at the amine. All four diastereomers of amine **1** can be accessed by use of the appropriate combination of biocatalysts. Initially the diastereoselectivity of the transaminases was poor (14% *de*), but the proteins were modified using directed evolution. Two small changes gave improved diastereoselectivity (up to 97% *de*). Following this, the enoate reductase was modified to give better enantioselectivity in the reduction of **2**.[5] The stereochemistry at the beta-position is determined by the ene-reductase, and the

amine stereochemistry is controlled by the transaminase. Although this is a good example of coexpression and engineering of enzymes, lactate dehydrogenase and glucose dehydrogenase required for cofactor regeneration still had to be added separately.



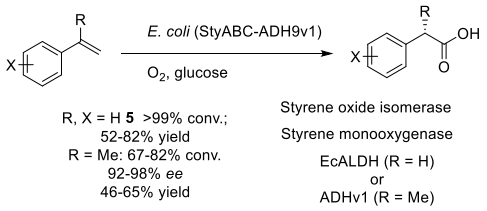
Scheme 1. Coexpression of multiple enzymes for the synthesis of cyclic amines

Turner *et al*. used a three enzyme one-pot cascade for the synthesis of substituted piperidines and pyrrolidines (Scheme 2). Reduction of the carboxylic acid by carboxylic acid reductase (CAR), gave an aldehyde substrate for the transaminases (ω-TA); the resulting imine was then reduced by an imine reductase (IRED). By changing the position of the substituent on the substrate, isomeric products were obtained.[6] Further work by the same group carried out the same reactions, but in this case coexpressing the required biocatalysts in the same cell expression system.[7]

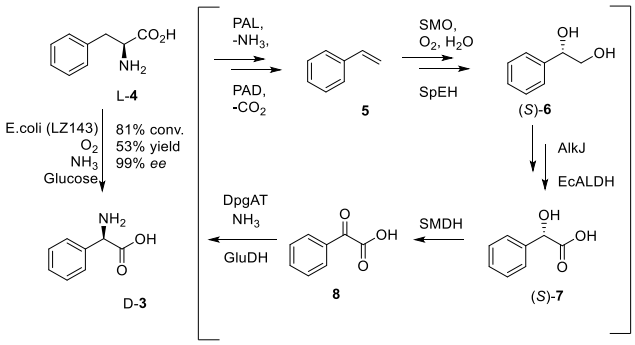


Scheme 2. Synthesis of disubstituted pyrrolidines and piperidines

A one-pot epoxidation-isomerization-oxidation to make terminal acids from terminal alkenes was recently reported (Scheme 3).[8] Workers from the same group, not satisfied with a three enzyme cascade, coexpressed up to nine enzymes in one host.[9] The eight-step nine enzyme reaction produced D-phenylglycine **3** from L-phenylalanine **4** in a cyanide-free synthesis (Scheme 4), using only ammonia, glucose and atmospheric oxygen. Phenylalanine ammonia lyase (PAL) and phenylacrylic acid decarboxylase (PAD) catalyze the loss of ammonia and carbon dioxide, respectively to produce styrene **5**. Epoxidation using a styrene monooxygenase (SMO) and hydrolysis using an epoxide hydrolase (SpEH) gives intermediate diol **6**. The diol **6** undergoes two oxidation steps, catalyzed by an alcohol dehydrogenase and an aldehyde dehydrogenase (AlkJ and EcALDH, respectively) to furnish *(S)*-mandelic acid *(S)*-**7**, which is further oxidized to the *α*-keto acid **8** by *(S)*-mandelate dehydrogenase (SMDH). Ammonia transfer is the final step in the eight-step cascade, catalyzed by D-phenylglycine ammonia transferase (DpgAT); glutamate dehydrogenase (GluDH) is used to regenerate the amine donor using ammonia. The specificity of each of these enzymes means that the product of one reaction is used by another enzyme, with the enzymes selected to avoid cross reaction.

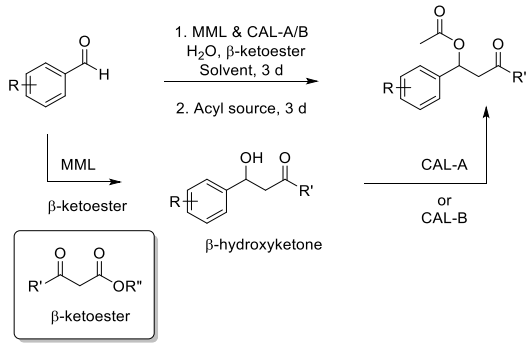


Scheme 3. Whole cell cascades: from alkenes to carboxylic acids



Scheme 4. Whole cells coexpressing nine enzymes, the synthesis of D-phenyl glycine **3**

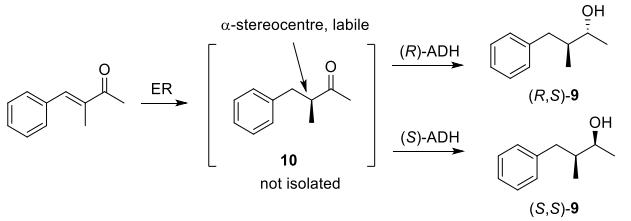
A series of *β*-hydroxy ketones and the acylated *β*-hydroxy ketones were synthesized by combination of two lipases (Scheme 5).[10] The first step is the decarboxylative aldol reaction in the presence of immobilized *Mucor miehei* lipase, followed by the kinetic resolution of the resulting *β*-hydroxy ketones. The catalysts were recycled up to three times, and some of the reactions were carried out at a >1 g scale.



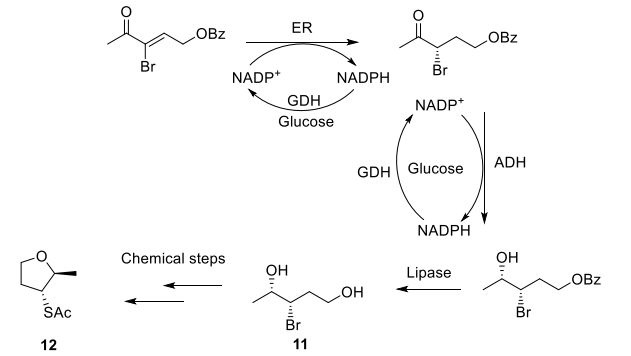
Scheme 5. Use of multiple lipases

Use of two immobilized enzymes, which are coexpressed by *E. coli*, in the synthesis of diastereomers of fragrance molecules has been described. Two reductions are carried out, initial reduction of the alkene, followed by reduction of the ketone (Scheme 6).[11] One of the main advantages of this is it is an economical route to the fragrance molecules **9**; another is that the unstable ketone **10** is not isolated. In fact, the reduction of the ketone proceeds with higher diastereoselectivity when combined with the ene-reductase in a one-pot reaction than when the reactions are carried out sequentially. This is because the compound with the labile *α*-stereocentre is not isolated when the two enzymes are used together. Workers from the same group also combined four

different enzymes, this time in addition to the ADH and ene-reductase, GDH and a lipase were used in the synthesis of bromodiol **11**, a precursor to the tetrahydrofuran **12**, which is also a fragrance molecule (Scheme 7).[12] The substrate was modified, so that after reaction with the ene-reductase and alcohol dehydrogenase, it would give a suitable substrate for the lipase, as well as furnishing enantiopure products from the bioreductions. Again, the one-pot strategy works better than sequential addition of the biocatalysts.



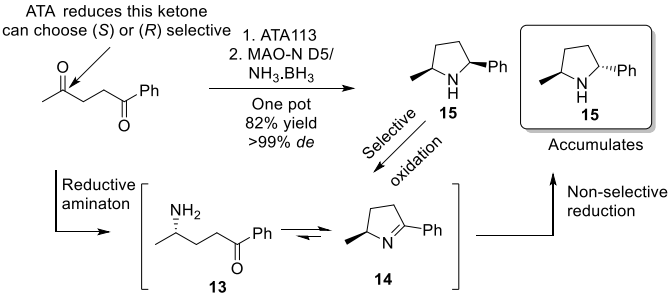
Scheme 6. Combination of an ene-reductase (ERED) with either (*R*)- or *(S)*-selective alcohol dehydrogenase (ADH)



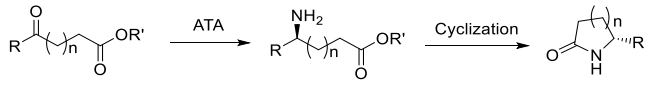
Scheme 7. Four enzyme cascade for the synthesis of **11**, an intermediate in the synthesis of odorant **12**

The coupling of reduction and oxidation reactions can be useful for a dynamic system, Turner *et al*. reported the use of a chemical reductant in conjunction with an oxidative biocatalyst (Scheme 8).[13] In this system, the initial step is mediated by either an *(S)*- or *(R)*-selective amine transaminase (ATA), and the resulting amine **13** can cyclize to give imine **14**. The imine **14** is non-selectively reduced to **15**. Only one diastereomer is oxidized back to **14**, eventually only one product is isolated, as the monoamine oxidase-favored diastereomer is continually oxidized, allowing the buildup of only one product. Gotor-Fernández *et al*. reported a related transamination, using *γ*- and *δ*- ketoesters. The ketone is reduced by a transaminase (Scheme 9) and the cyclization of the resulting amino esters result in *γ*- and *δ*- lactams.[14] The use of amino alcohols has been recently reported in the synthesis of *β*-, *γ*- or *δ*-lactams, in a bienzymatic cascade.[15]

The use of engineered cells to coexpress enzymes can allow more efficient reactions by eliminating the need of intermediate purification of potentially labile compounds. The use of enzymes in a one-pot reaction can be overall more efficient, as it can reduce the isolation required, which can potentially reduce the time needed for the overall process.



Scheme 8. Using a chemical reductant in combination with a biocatalyst



Scheme 9. Biocatalytic reduction, followed by spontaneous cyclization

1.2. Biotechnology: metagenomics, directed evolution and rational design

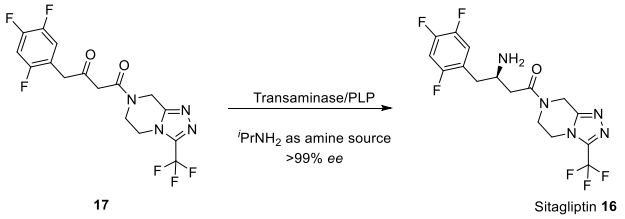
The modification of reaction conditions to optimize the performance of enzymes is widely used, but the use of molecular biology techniques, such as directed evolution, rational design and genome mining can increase the potential library of biocatalysts enormously.[16] The larger the library of genetic material there is to work from, the better the chance of finding an enzyme which will carry out novel reactions or which has synthetically diverse substrate specificity.[17] The amount of organisms which can be cultured under laboratory conditions is limited to approximately 1–2% of all organisms, meaning that traditional methods for accessing genetic diversity are limited to those organisms which can be cultured.[18] Genetic sequencing and metagenomic techniques can be used to identify genes which encode for proteins, which otherwise would be inaccessible to researchers *via* a culture dependent method.[19] The study of genetic material from unusual environments, such as high salt, high temperature, or low or high pH environments can give rise to biocatalysts which are suited to these environments *i.e.* high salt concentration, extreme temperatures and pH values.

Metagenomics allows evaluation of sequences which have evolved over millions of years. There are two types of analysis associated with metagenomic data: functional screening or sequence-driven analysis. Functional screening involves the cloning of the metagenomic DNA into a host organism followed by searching for the desired biocatalytic activity.[20] Several factors can affect the success of this method, the major one being that the host cell may not, in fact, express the proteins of interest; additionally a high throughput screen is usually required and a suitable detection method is not always available.[21] Sequence-driven analysis uses a characteristic conserved sequence as a "hook" or "probe" for picking out DNA which may code for a protein of interest. The catalytic triad is an example of a conserved motif which can be used in the identification of novel hydrolases.[22]

The identification of novel enzymes alone may not suffice in the context of finding an enzyme with desired activity/selectivity; for this, genetic mutation, either random or by design, can offer greater opportunities to tailor the enzymes for the required

functionality.[23] Directed evolution is a technique which has been successfully applied to the expression of modified biocatalysts.[24] By examination of the structure of the biocatalyst through molecular docking techniques, as well as by comparison to biocatalysts of known function and structure, homology modelling can be used to identify mutations to improve activity of the biocatalyst.[25] Random mutations can also be used to give information on the effect of changing various residues in the active site.[26]

An excellent example of directed evolution is the mutation of a transaminase for the asymmetric reduction step in the synthesis of Sitagliptin **16**. Sitagliptin **16** (marketed as Januvia) is an antidiabetic drug developed and marketed by Merck & Co. In conjunction with Codexis, a biocatalyst was identified for the reductive amination of the intermediate **17**.[27] Through the use of directed evolution, the poorly performing wild-type enzyme was extensively mutated until a biocatalyst with the desired activity was identified. In addition to this, the solvent tolerability and substrate scope were expanded beyond what is normally found in wild type transaminases, especially in the ability of the enzyme to accept a substrate with two bulky groups adjacent to the reaction site. Following this, Bornscheuer *et al*. reported the resolution of bulky substrates **18** and **19** (Figure 1) through rational design of a wild-type enzyme, requiring only four mutations.[28]



Scheme 10. Synthesis of sitagliptin **16**

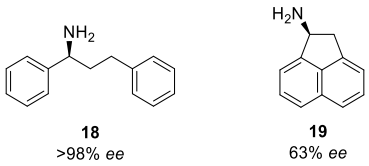
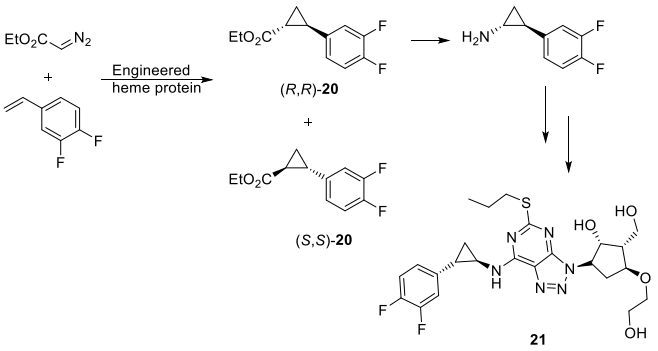
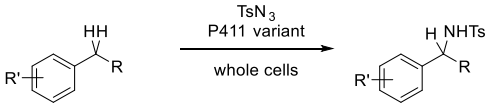


Figure 1. Bulky substrates

The use of mutated enzymes for non-natural substrates is a growing area of interest, and has recently been reviewed.[16a;29] It is potentially a very useful enabling strategy, and as the techniques available for enzyme modification and evolution are continuously improving, the possibilities are growing.[16a;29] The pioneering work of Arnold *et al*. showed the potential for the use of directed evolution.[30] Recently Arnold's group has reported the use of mutated P450 enzymes for olefin transformations, including cyclopropanation *via* carbene transfer, to furnish cyclopropane **20**, an intermediate in the synthesis of ticagrelor **21** (Scheme 11).[31] As well as this, workers from the same group have reported the functionalization of benzylic C–H bonds by a engineered iron-heme enzyme (Scheme 12).[32] Diazo and azide containing compounds are not traditionally accepted by enzymes; the use of directed evolution has allowed enzymes to be developed which make use of these useful intermediates.



Scheme 11. Cyclopropanation catalyzed by engineered heme protein



Scheme 12. Enzymatic C–H amination

The use of directed evolution and metagenomic analysis is a powerful combination for the modification and improvement of existing biocatalysts as well as for the discovery of new biocatalysts.

1.3. Immobilization

Immobilization of biocatalysts offers a number of distinct advantages from a synthetic perspective: enhanced stability of enzymes, greatly enhanced ease of use and potential to recycle, and accessibility to the non-expert, especially through the use of commercially available immobilized enzymes, as described in a series of recent reviews.[33] Immobilization of biocatalysts, free enzymes or whole cells, can protect the enzymes from product inhibition, variations in temperature or pH; it also adds economic value to the biocatalysts by allowing recovery and reuse.[34] Recent developments in the support media available (including biodegradable chitin and chitosan, magnetic supports, silica) provides greater choice for the immobilization of biocatalysts depending on the requirements of an individual process.[35]

There are three types of immobilization: binding to a support, entrapment (also called encapsulation), and cross linking (Figure 2, A, B and C, respectively).[36]

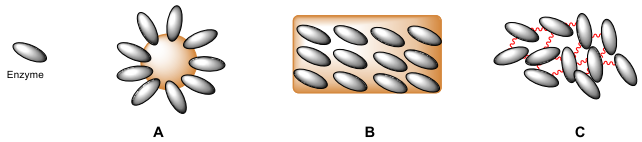


Figure 2. Modes of immobilization of biocatalysts

Lipases are commonly immobilized on hydrophobic supports; this can increase their activity by holding them in the open or active conformation.[37] Specific interactions, for example the use of fusion enzymes, allows better control over the activity of the

enzyme, as the part of the protein interacting with the support is essentially separate from the protein. Fusion proteins involve the addition of a sequence for a binding peptide to the enzyme to allow specific immobilization, and is highly useful.[38] The immobilization of affinity labelled (e.g. his-tagged) enzymes directly from the whole cell preparation is even more advantageous than the immobilization of purified proteins as it combines the immobilization step with the purification step.[39] The concept of a one-step purification immobilization is potentially very useful and has been reviewed previously.[39a] For some varieties of tags and immobilizing supports, similar results for enzyme activity can be attained when immobilizing the pure protein and when using the crude cell extract, thus eliminating the need for pre-purification of the enzyme (Figure 3).[40] The availability of specialized immobilization carriers designed for enzymes is growing.[41]

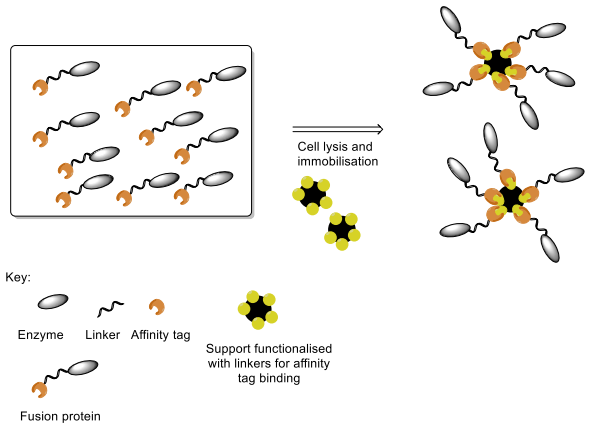


Figure 3. Simultaneous purification and immobilization of fusion proteins

The use of magnetic supports coated with ligands or peptides for interaction with the tagged proteins allows facile separation of the supported catalyst for reuse. Recently, lipases from *Thermomyces lanuginosus* and *Rhizopus oryzae* have been immobilized on magnetic nanoparticles with retention of activity and increase in stability relative to the free enzyme; this also allowed the facile separation and recovery of the enzyme.[42] Lipases have also previously been immobilized on magnetic lauric acid-stabilized particles for the resolution of menthol.[43] Horseradish peroxidase has been immobilized on iron oxide nanospheres on chemically reduced graphene oxide which has been shown to be an effective immobilization medium.[44] Marszałł *et al*. immobilized lipases onto magnetic chitosan nanoparticles, improving both the resolution of (*RS*)-atenolol **22** and the recoverability and reusability of the lipase.[45] The advantage of these systems is that the immobilized biocatalysts can be readily recovered by magnetic separation, removing the need for a filtration step. Lipases immobilized on magnetic nanoparticles have also been used in microtube reactors.[46]

Zhu *et al.* reported the use of a pH sensitive support for *Candida rugosa* lipase used in the hydrolysis of ketoprofen ester.[47] In this case, once the reaction was completed, a simple adjustment of the pH precipitated the support and allowed facile recovery (Figure 4). The immobilized enzyme survived several precipitation and dissolution cycles while retaining activity (46% activity after 8 cycles) and increased the enantioselectivity relative to the free enzyme by 1.5-8.7 times.

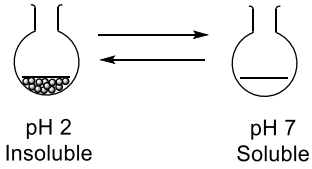


Figure 4. pH-Controlled solubility of enzyme support

Wever *et al*. reported a method for covalent immobilization of an acid phosphatase, which retained the activity and the majority of the efficiency of the enzyme.[48] The immobilized enzyme was shown to be stable after a year of storage in buffer. The catalyst was also used in a continuous flow system to produce a range of phosphorylated alcohols at a gram scale, using a cheap phosphate donor. The methodology could allow the facile integration of the reaction in a large, multi-enzyme, continuous system.

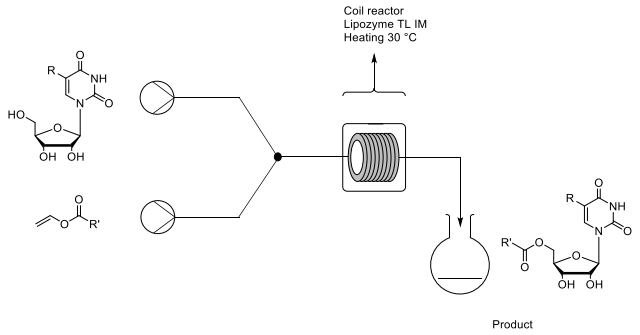
The use of epoxy-functionalized resins is advantageous because of their ease of use. They have been used to immobilize halohydrin dehydrogenases directly from the cell lysate, without need for prior purification.[49] Epoxy functionalized silica has been used as an efficient support for lipases in the resolution of ibuprofen esters.[50]

The wide range of support materials available offers a number of approaches for the immobilization of enzymes leading to enhanced stability, as well as imparting desirable properties for facile recovery of the biocatalyst and product purification.

1.4. Flow Chemistry in Combination with Biocatalysis

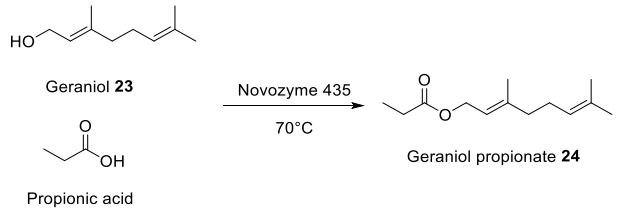
Flow chemistry or continuous processing is an important and rapidly growing area of research and is an increasing area of focus in the synthesis of pharmaceutical compounds and fine chemicals.[51] In addition to the synthetic advantages outlined above, the immobilization of biocatalysts enables their use in continuous processing. From the point of view of biocatalysis, an important advantage of continuous processing relative to batch processing is that the enzymes can be somewhat protected from product inhibition, by continually removing the inhibitors of the enzymes as they are formed, the productivity of these biocatalysts can be increased. While one of the most significant synthetic problems addressed by flow processing is the use of hazardous intermediates,[52] other problems which can be addressed by continuous flow technology are those associated with scale-up of processes, such as heat and mass transfer and sufficient mixing of reagents, which can limit the utility of some reactions. The productivity of oxidases can be limited by the rate of oxygen uptake, a problem which has been addressed by flow technology.[53] Biocatalysts used in combination with continuous flow technology has been recently reviewed by a number of groups.[54] Microreactors allow precise control of mixing and temperature, and their use, in conjunction with biocatalysts has been described.[55]

Recently, the enzymatic synthesis of nucleoside analogues using immobilized Lipozyme® catalyzed transesterification has been reported.[56] The regioselective reaction was carried out using a microflow reactor (Scheme 13). The use of microflow technology allowed the reduction in the amount of DMSO required to solubilize the uridine derivatives, as well as mild temperature and short reaction time. The use of this technology enabled large libraries of compounds to be generated quickly without the use of protecting groups.



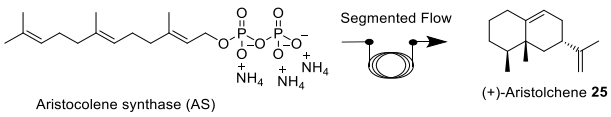
Scheme 13. Synthesis of nucleoside analogues using immobilized Lipozyme®

Using various carboxylic acids, esters of geraniol **23** were synthesized by lipase-catalyzed transesterification in a flow system, using commercially available immobilized lipases in a packed bed reactor (Scheme 14).[57] The process optimization was carried out in flow; a batch reaction mimicking the optimized conditions for the reaction was carried out to compare the efficiencies in both systems. The continuous synthesis of geranyl propionate **24** has been carried out.[58] The lipase for the transformation was identified through the use of batch reactions and a range of supports were screened to maximize the immobilization efficiency in order to facilitate the transfer of the reaction to a continuous system. As well as this, the support system chosen increased the activity of the lipase by almost 30%.



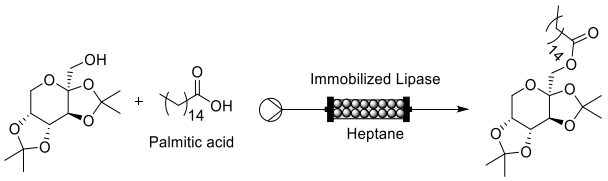
Scheme 14. Continuous synthesis of geranyl propionate **24**

Flow chemistry has been used to overcome the slow release of products in the synthesis of sesquiterpene **25** from farnesyldiphosphate using a terpene synthase as biocatalyst (Scheme 15).[59] Continuous extraction of the product from the aqueous layer is necessary to make the biocatalytic process viable. When compared to batch, the flow system offered many advantages. Under batch conditions high-speed mixing would be necessary to achieve continuous extraction of the terpene products from the aqueous layer; this can lead to denaturation of the enzyme by shearing. The flow system offered more control over the interactions of the biocatalyst with the solvent; correct solvent choice was crucial, as emulsion formation would impede phase separation and complicate product recovery.



Scheme 15. Synthesis of (+)-**25**

The combination of flow chemistry and biocatalysis has also been applied to the production of fats for the food industry, as an alternative to hydrogenation and chemical (trans)esterification.[60] An immobilized preparation of lipase from *Thermomyces lanuginosus* was used to convert soybean oil into semi-solid fats such as margarine and shortening, which are higher value products than the liquid oil starting materials. Biodiesel and other materials derived from vegetable oils or biobased oils are economically important, as they can be used as an alternative to petroleum based products.[61] Lipases can be used in the synthesis of biofuels from oil through (trans)esterification.[62] Immobilized CAL-B has been used in a continuous flow system for the synthesis of ceramides, important compounds in both the pharmaceutical and cosmetic industries.[63] Commercially available immobilized biocatalysts were evaluated for the synthesis of ketal protected fatty acid esters of fructose (Scheme 16).[64] The best conditions for the reaction were identified in batch reactors, and used as a starting point for the development of continuous flow conditions.

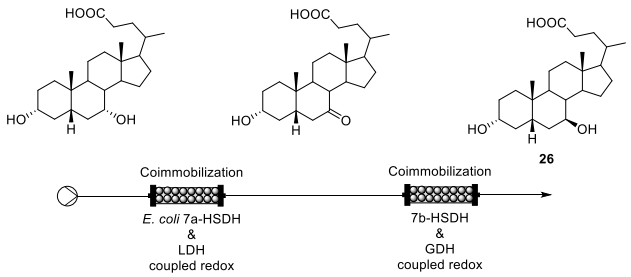


Scheme 16. Synthesis of ketal protected fatty acids

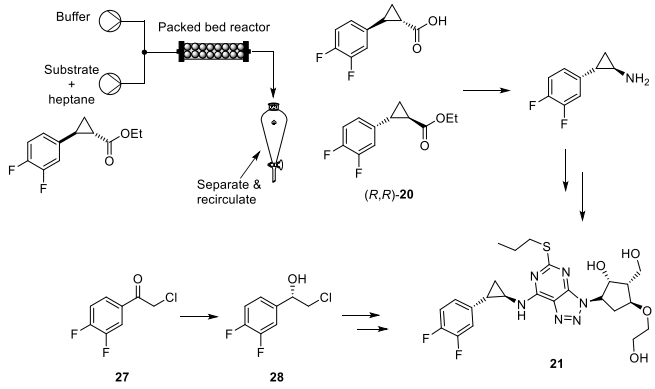
Another example of a biocatalytic process which is improved by transfer to continuous flow is the synthesis of ursodeoxycholic acid **26** (Scheme 17).[65] The process in batch involved the use of four enzymes in a two-step one-pot system. After the first step in batch, the enzymes had to be inactivated by heat treatment to avoid a reverse reaction occurring. Not only was this step potentially costly and time-consuming but it also meant that the enzymes could not be reused. The system was transferred to flow to spatially separate the enzymes required for the first and second steps. The two enzymes for each step were co-immobilized. This resulted in a continuous process which avoided the need for heat treatment to denature the enzymes by spatially separating the enzymes for each step, and allowed reuse of the enzymes.

Recently, Turner *et al*. have shown the use of flow chemistry for the lipase-catalyzed resolution of the cyclopropyl subunit **20** for the synthesis of ticagrelor **21** (Scheme 18).[66] The biphasic mixture of product and starting material was separated, and the organic material recirculated to increase the conversion. The asymmetric reduction of the chloroethyl precursor **27** was also reported, in this case using a ketoreductase from a metagenomic

library.[67] The process for the synthesis of alcohol **28** was optimized to give up to 500 g/L substrate concentration.

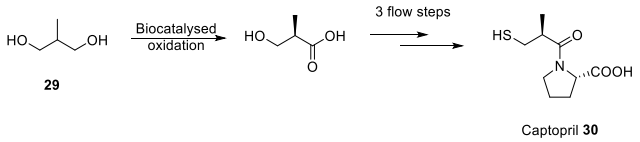


Scheme 17. Coimmobilization and combination of multiple enzymatic steps



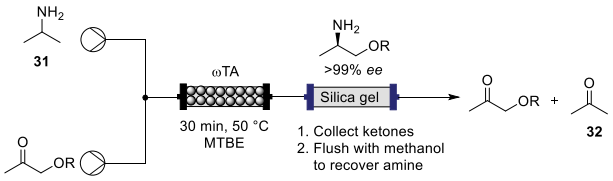
Scheme **18**. Synthesis of ticagrelor **21**

Enzymatic desymmetrization of the prochiral diol **29** in continuous flow has recently been reported, as the first step in an entirely flow-based synthetic route to captopril **30** (Scheme 19).[68] In this system, not only was the entire synthesis carried out using flow chemistry, but the chemoenzymatic step is carried out using immobilized whole cells, instead of purified protein, which decreases the overall cost of the product, as the expense associated with purification of proteins is eliminated.



Scheme 19. Synthesis of captopril **30**

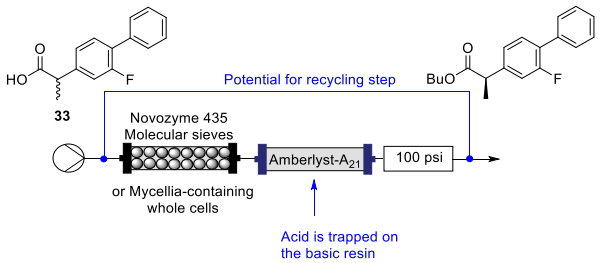
Purified transaminase from *Halomonas elongata* containing a poly His-tag was immobilized on a metal-derivatized epoxy resin and the substrate scope explored through the use of flow chemistry.[69] Although this process required purification of the enzyme, the use of the immobilized enzyme had the advantage of being able to withstand higher flow rates than the previously reported immobilization of a similar whole cell system (0.78 vs. 0.02 mL min^{-1}).[70] The ability to use a higher flow rate is important as it allows higher productivity. When the same immobilization procedure was applied to the crude cell extract, the transaminase was also successfully immobilized, eliminating the need for a separate enzyme purification step. In addition to this, a "catch-and-release" in-line product purification was added. By trapping the amine products on either a column of silica gel or an acidic resin the amine product was separated from unreacted starting material (Scheme 20).[69-70]



Scheme 20. Using continuous processing and in-line purification

In a complementary approach, covalent immobilization of HaloTag® modified proteins directly from the whole cell has been reported.[71] Here tagged fusion enzymes are immobilized directly from the crude cell extract onto a resin pre-packed into a flow reactor, eliminating a costly separate purification step by combination with the immobilization step (Figure 3). Using this approach, two biocatalysts were effectively combined in a cascade reactor.

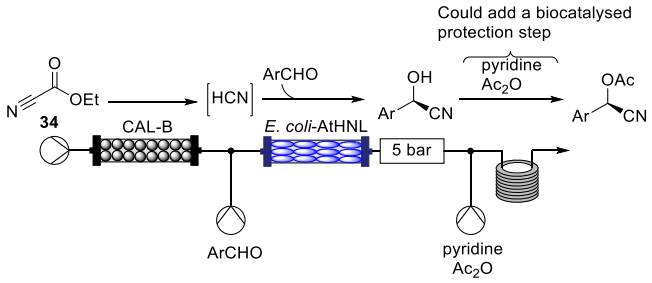
The biocatalyzed resolution of flurbiprofen **33** has been carried out in a flow system, using the commercially available immobilized lipase, Novozyme 435 or mycelium-containing whole cells, in both batch and flow reactors (Scheme 21).[72] When using either immobilized lipase, or mycelia, an in-line purification step was added; a basic resin was used to separate the unreacted acid from the ester product in the continuous flow system. When using the commercially available catalyst the excess water was absorbed by including molecular sieves. Use of the cell mycelia facilitates separation from the reaction mixture without the need for immobilization, in addition to this they also remove the excess water.[72d;73] When using both the commercially available catalyst, and the whole cell systems, the product was obtained in excellent enantiopurity and high conversion; the use of flow reactors reduced the reaction time in both cases.



Scheme 21. Biocatalyzed continuous synthesis of flurbiprofen **33**

Continuous flow has been utilized in the synthesis of cyanoacetates using two distinct biocatalysts, CAL-B and a hydroxynitrile lyase (HNL) in a linked multistep flow process (Scheme 22).[74] The CAL-B hydrolyses **34** to release HCN, which is then utilized by the lyase in the synthesis of cyanohydrins, which are chemically acylated before isolation. The advantage of this continuous flow system is that it avoids the need to use hydrogen cyanide gas. The use of a surrogate which is reacted *in situ*, means that there should only ever be a very small amount of the HCN in the system. The use of a flow-system also allowed an additional protecting group to be easily added to improve the overall stability of the target cyanohydrins. This could potentially

be effected using a biocatalyzed transesterification step to add the protecting group, rather than the chemical step; lipases have previously been used to resolve cyanohydrins *via* both hydrolysis and transesterification.[75]



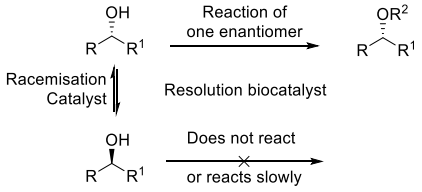
Scheme 22. *In situ* generation of HCN gas for asymmetric synthesis of cyanohydrins

It is evident that the use of biocatalysis in organic synthesis is greatly facilitated through immobilization and continuous flow and enhanced activity in this area is anticipated. Advances in support materials enable the use of higher flow rates and a wider range of solvents to enable telescoping of biocatalyzed reaction steps as part of an overall synthetic process.

2. Dynamic kinetic resolution and combining chemo- and biocatalysts

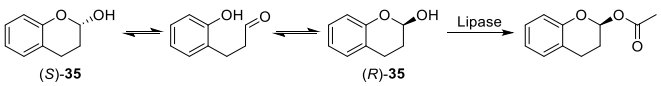
The use of multiple biocatalysts or a combination of chemo- and biocatalysts in the same reaction vessel is an attractive method of synthesis, since it can reduce the reaction time and product purification required. Dynamic kinetic resolution is an attractive method of increasing the yield of kinetic resolution reactions (Scheme 23). While biocatalysis has enormous potential for dynamic kinetic resolution, in practice, ensuring the biocatalyst is compatible with the conditions employed for racemization can be limiting.[2b;2c;76] [77] However, with the availability of a wide range of immobilization techniques, biocatalysts can be stabilized so they can tolerate harsher reaction conditions and indeed can potentially be protected from these conditions through compartmentalization especially in flow systems.

The dynamic kinetic resolution of alcohols using metal-based racemization catalysts in combination with lipases is well reported and there have been several reviews on the use of complexes of metals such as iron, ruthenium, and iridium.[77b-e;78] Acids and bases, zeolite, even light activated catalysts have also been used as racemization catalysts for the dynamic kinetic resolution of alcohols.[79]

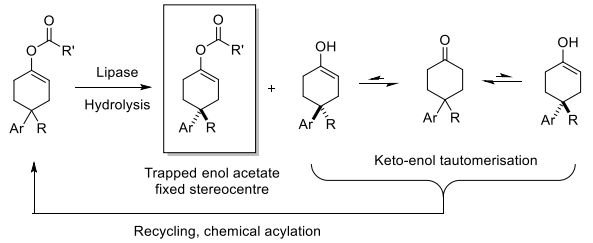


Scheme 23. Dynamic kinetic resolution, using alcohols as an example

The lipase-catalyzed resolution of the hemiacetal moiety of sugars is widely reported and utilizes the dynamic ring opening/closing of the hemiacetal in place of the addition of an external catalyst.[80] The same ring-opening and ring-closing was utilized within our group for the dynamic kinetic resolution of 2-chromanol **35** (Scheme 24).[81] The use of such systems takes advantage of the instability of the ring structure, or the reversibility of reaction that occurs naturally, without the need for an external catalyst. Another example of this is the use of prochiral ketones (Scheme 25). The enol can be trapped by chemical acylation, taking advantage of the keto-enol tautomerization, and the resulting enol acetate can be selectively hydrolyzed using a hydrolytic enzyme.[82] Spontaneous racemization has also been applied to the synthesis of 1,2-diaminopentane derivatives and 3,4-dihydroisocoumarins (Figure 5).[83] The use of prochiral substrates (desymmetrization) is an attractive alternative to kinetic resolution; some examples of such compounds which have been used as intermediates in the synthesis of pharmaceutical compounds are shown in Scheme 26.[84]

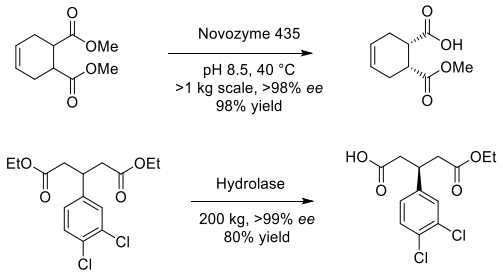


Scheme 24. Spontaneous racemization



Scheme 25. Dynamic kinetic resolution with no external catalysts: using equilibria for racemization

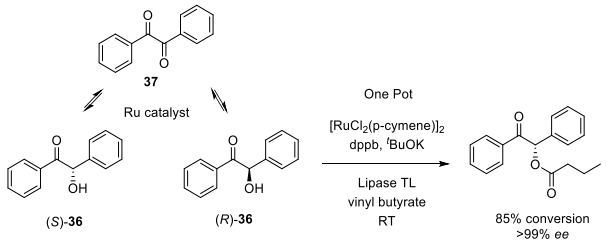
Figure 5. 1,2-Diaminopentane derivatives, and 3,4-dihydroisocoumarin derivatives



Scheme 26. Lipase-mediated resolution of prochiral compounds

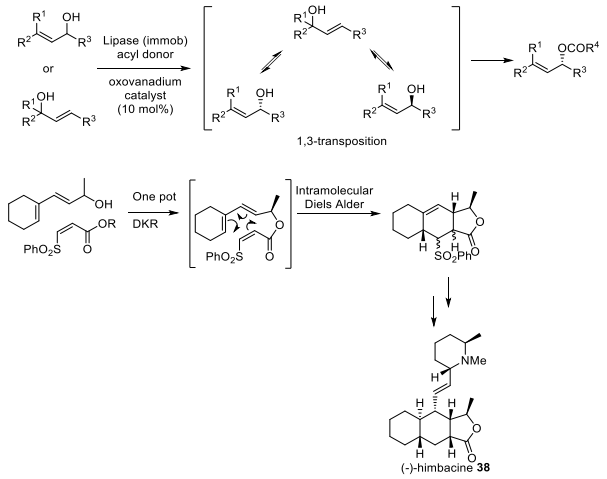
The dynamic kinetic resolution of *α*-hydroxy ketones, such as benzoin **36**, *via* a diketone intermediate **37** has been reported (Scheme 27).[85] This dynamic kinetic resolution has been carried out in both batch and continuous mode by variation of support used for lipases in combination with various chemocatalysts, as

well as examination of solvent effect, to make the reaction greener.[86] The use of a readily available ruthenium catalyst in combination with a base was initially considered at 50 °C. However, it was shown that the right combination of catalyst and ligand allowed the reaction to be carried out at a lower temperature (room temperature).



Scheme 27. Dynamic kinetic resolution of benzoin **26**

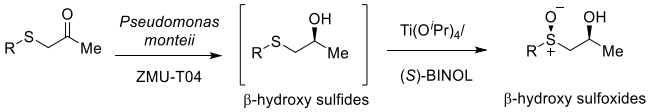
Akai *et al*. reported the lipase/metal-catalyzed dynamic kinetic resolution of racemic allylic alcohols (Scheme 28).[87] The acyl group installed in the lipase-catalyzed transesterification was carefully chosen so that it would undergo further reaction, making the reaction more atom economical, as the acyl group did not require removal after the resolution step.[88] The oxovanadium catalyst carried out a racemization/isomerization of the allylic alcohol starting material. As an illustrative example, this methodology was applied to the total synthesis of (−)-himbacine (−)-**38**, furnishing the product with excellent enantioselectivity.[89]



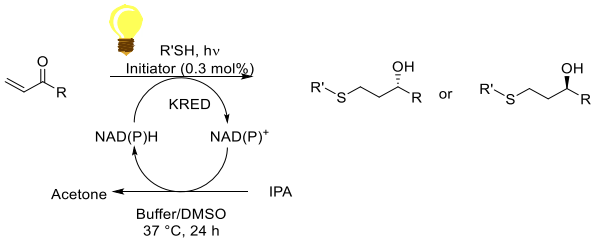
Scheme 28. Combining lipase-catalyzed resolution and an intramolecular Diels-Alder reaction

The combination of a biocatalyzed reduction and a chemical oxidation was also reported. The bioreduction was carried out first, followed by sulfur oxidation using a chiral chemocatalyst (Scheme 29).[90] Potentially, the bioreduction could be combined with a biocatalytic sulfur oxidation step in place of the chemocatalyzed step. Biocatalyzed sulfur oxidation can avoid the over oxidation to the sulfone which can be a problem for chemical catalysis.[91] The synthesis of sulfur containing ketones and subsequent bioreduction to give the corresponding alcohols has also been reported, in a one-pot photobiocatalytic cascade (Scheme 30).[92]

The initial Michael reaction is light activated, forming a sulfide from a thiol and an *α,β*-unsaturated ketone. Varying the choice of ketoreductase gave access to both enantiomers of the 1,3-mercaptoalcohol products.



Scheme 29. Combining a bioreduction and a chemical oxidation



Scheme 30. One-pot photobiocatalytic cascade

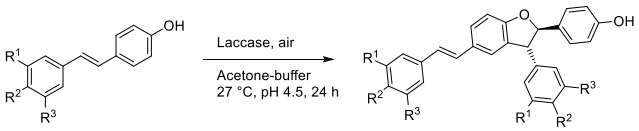
These selected examples highlight the potential of combining biotransformations and other reactions as a powerful synthetic strategy. Kinetic resolution is useful in the synthesis of enantiopure compounds, but an inherent disadvantage of this technique is the limited yield. The combination of biocatalysts with racemization catalysts to give a dynamic kinetic resolution is a very useful technique, but the conditions under which the two catalysts work can be very different. The use of immobilized catalysts which are, by-design, more stable under the desired conditions can facilitate the combination of biocatalysts and organo- or metal-catalysts. The physical separation of the catalysts, in a continuous flow system can also enable the combination of “incompatible” catalysts.

3. Examples of enzymes in pharmaceutical & natural product synthesis

There have been several reviews in the past 10 years of the applications of biocatalysts in the synthesis of drug products and intermediates.[93] The focus of this section is recent examples of synthetic routes which utilize biocatalytic steps specifically in relation to the use of enabling technology.

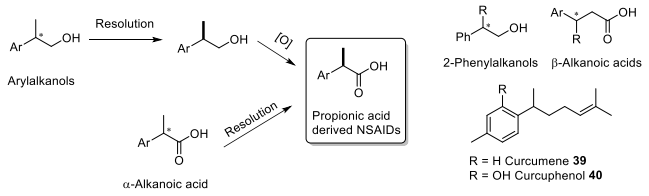
Biocatalytic retrosynthesis is a relatively new concept, pioneered by Turner *et al.*, which recognizes the need for the integration of biocatalysis in retrosynthetic analysis, choosing disconnections with consideration for reactions which can be catalyzed by enzymes.[94] There have been a number of reported total syntheses using biocatalysis either as a single resolution step, or a multistep sequence. More importantly, the use of biocatalysts in the drug discovery stage has significant potential. For example, recently the use of a laccase-mediated enantioselective oxidation was reported in the synthesis of a series of dihydrobenzofurans for *in vitro* testing (Scheme ***31***).[95] Biocatalysis is particularly suited to this use as the essentially by-product free reactions means that subsequent

purification is not necessary; a biological oxidant means that the use of costly metal and/or chiral oxidants can be avoided.[96]

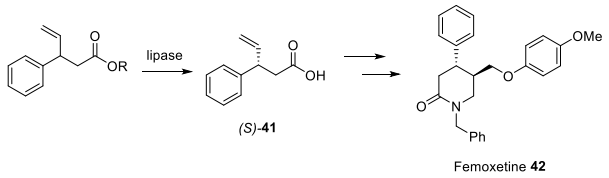


Scheme 31. Biocatalyzed medicinal chemistry route to highly substituted dihydrobenzofurans

Lipases have been used for the resolution of propanoic acid derived non-steroidal anti-inflammatory drugs through both the resolution of the 2-arylalkylalcohol precursors and the direct resolution of the *α*-alkyl carboxylic acids (Scheme 32).[97] Structurally related compounds, 2-phenylalkanols and 3-arylalkanoic acids (also called *β*-aryl alkanoic acids) have previously been resolved using hydrolases by our group, initially focusing on commercially available hydrolases but more recently, demonstrating the power of exploiting a metagenomic approach to identify a novel hydrolase with a complimentary substrates scope.[98] Their resolution and use for the synthesis of natural products *(R)*- and *(S)*-curcumene **39** and curcuphenol **40** has also been reported (Scheme 32).[99] A related compound, alkenoic acid **41** has been resolved and used in the chemoenzymatic synthesis of femoxetine **42** (Scheme 33), with one of the two stereocentres installed by lipase-catalyzed resolution.[100] A tandem metal-lipase dynamic kinetic resolution was also developed for the resolution of alkenoic acid **40** to further increase the yield of the resolution.[101]

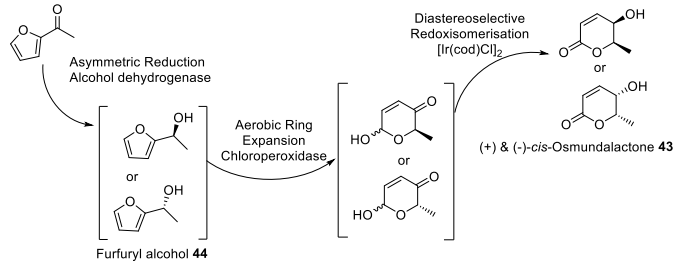


Scheme 32. Use of arylalkanols and arylalkanoic acids in synthesis



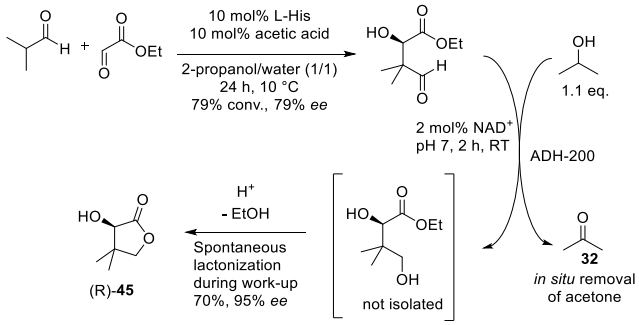
Scheme 33. Synthesis of femoxetine **42**

Recently, the total synthesis of *δ*-lactones (+)- and (−)-*cis*-osmundalactone **43** by a three step chemoenzymatic cascade from a readily available starting material has been described (Scheme 34).[102] By selecting an *(S)*- or *(R)*- selective alcohol dehydrogenase, both enantiomers of the furfuryl alcohol **44** can be accessed, which are then subjected to a biocatalyzed-aerobic ring expansion using a chloroperoxidase, which can be carried out as a one-pot transformation. The lactone intermediates are then diastereoselectively reduced using a transition metal catalyst. A "one-pot-like" synthesis of vitamin B6 intermediate *(R)*-pantolactone *(R)*-**45** was reported, combining an organocatalyzed aldol reaction and a biocatalyzed reduction, with *in situ* removal of the volatile by-products before the biocatalyzed step and continuous removal of the acetone **32** by-product from the biocatalyzed reaction (Scheme 35).[104][103] In contrast to the other reactions discussed in this section, the stereochemistry is provided by the organocatalyst, and further enhanced by the biocatalyst.

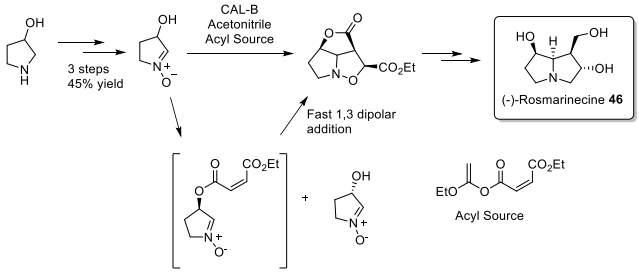


Scheme 34. One-pot chemoenzymatic cascade for the synthesis of osmundalactone **43**

Scheme 35. Synthesis of (R)-**45** by combined chemo- and biocatalysis



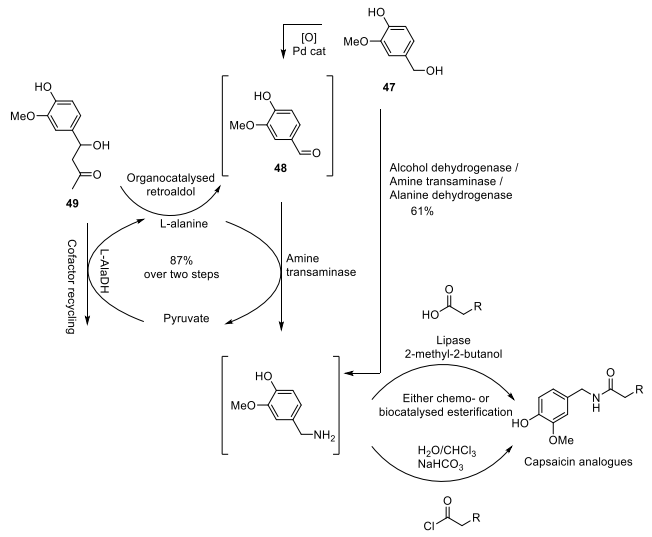
Akai *et al*. reported a one-pot domino biocatalytic transesterification/1,3-dipolar cycloaddition. The biocatalytic resolution is the key diastereoselective step in the synthesis of (−)-rosmarinecine **46** (Scheme 36).[105] It is also the first example of both a domino transesterification/1,3-dipolar cycloaddition and a total synthesis of (−)-**46** which does not use chiral pool starting materials or chiral auxiliaries.



Scheme 36. Synthesis of (−)-rosmarinecine **46** *via* a tandem transesterification/ dipolar cycloaddition

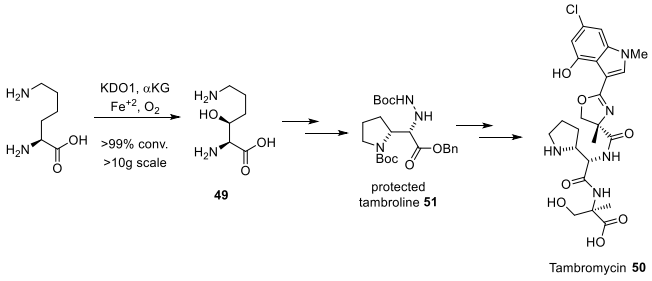
In an excellent example of the flexible use of multiple biocatalysts, Berglund *et al*. reported a total synthesis of capsaicin analogues from a primary alcohol **47** using a metal-catalyzed oxidation to produce aldehyde **48**, followed by a one-pot amine transaminase-catalyzed reductive amination and a lipase-catalyzed amidation, using a fatty acid as the acyl donor (Scheme 37).[106] The authors initially investigated the use of a biocatalyzed

oxidation, which could be coupled with the amine transaminase; the alcohol dehydrogenase could function as a redox partner for the regeneration of cofactors for the L-alanine dehydrogenase (L-AlaDH) as well as the oxidation of the alcohol **47**. However, this step was less efficient than the palladium catalyzed oxidation, giving 61% conversion as opposed to 87% conversion. In the development of this route, both the chemocatalyzed steps and the biotransformations were considered, and the two methods were combined in different ways. This is an excellent example of the combination of biocatalysts and chemocatalysts in the synthesis of a natural product and its analogues. Another step considered was the retroaldol reaction of **49** to produce aldehyde **48** *in situ*, catalyzed by the L-alanine which is already present in the reaction mixture as it is used as a cofactor for the amine transaminase reaction.



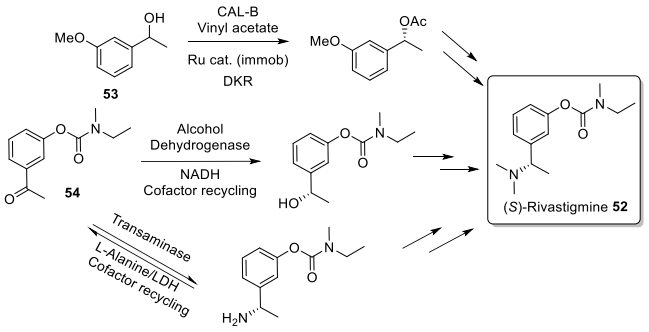
Scheme 37. Combined chemo- and biocatalyzed routes to capsaicin analogues

Renata *et al*. report the use of an overexpressed lysine hydroxylase as a clarified cell lysate, eliminating the need for costly protein purification.[107] The use of co-expressed chaperone proteins increased the expression and stability of the protein of interest. The lysine hydroxylase was used to make multigram quantities of the chiral material **49** which was further reacted to give natural product tambromycin **50** *via* the protected tambroline **51** (Scheme 38). By modifying the host cell, extra stability was imparted on the system.



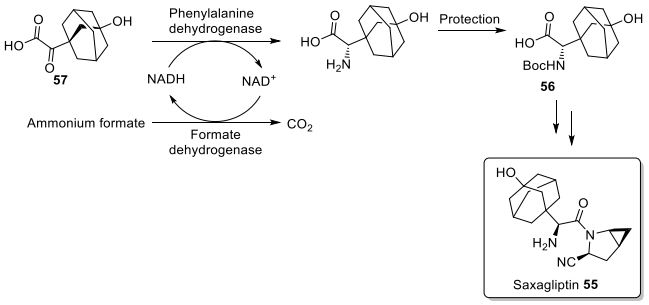
Scheme 38. Synthesis of natural product tambromycin **50**

The chemoenzymatic synthesis of *(S)*-rivastigmine **52** was reported, using a lipase to impart the stereochemistry, through CAL-B-mediated transesterification of intermediate **53** (Scheme 39).[108] The reuse of the CAL-B in this process was also explored, where the enzyme was shown to retain activity through 6 reaction cycles. The racemization of the alcohol **53** was carried out using a ruthenium catalyst for a dynamic kinetic resolution, further enhancing the value of this synthesis.[109] The use of baker's yeast-derived alcohol dehydrogenase for the stereoselective reduction of the ketone **54**, has also been reported, where the biocatalyst was identified by screening a large range of commercially available biocatalysts.[110] A novel transaminase has also been used for the reductive amination of **54** and this is a more direct route to *(S)*-**52** than the alcohol dehydrogenase.[111]

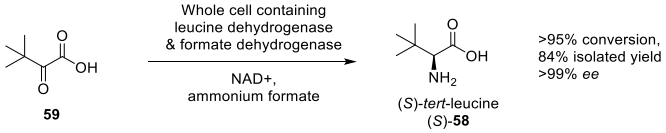


Scheme 39. Synthesis of *(S)*-rivastigmine **52**

Saxagliptin **55** was developed by Bristol-Meyers Squibb as a treatment for diabetes.[112] A key intermediate in the synthesis of **55** is the *N*-protected glycine derivative **56**, which was prepared by reductive amination of the keto acid **57** using a mutated phenylalanine dehydrogenase, a NAD dependent transferase (Scheme 40).[113] The process was coupled with formate dehydrogenase for cofactor recycling. A whole cell catalyst, which co-expresses multiple enzymes can be useful for processes which require NAD dependent enzymes; the redox coupling partners can be produced in the same cell system, negating the need for an external enzyme to be added. *(S)-tert*-Leucine *(S)*-**58**, an intermediate in the synthesis of atazanavine, boceprevir and telaprevir, has been synthesized in a whole cell system producing both the required leucine dehydrogenase, which carries out the reductive amination on the keto acid **59**, and the formate dehydrogenase, for cofactor recycling (Scheme 41).[114]

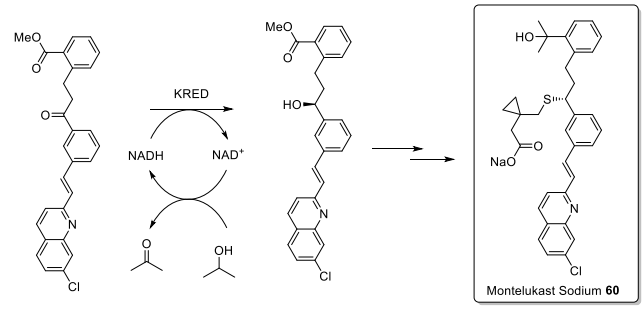


Scheme 40. Synthesis of Saxagliptin **55**

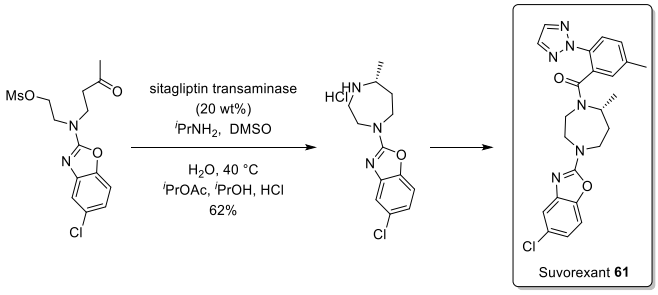


Scheme 41. Synthesis of *(S)-tert*-leucine **58**

The integration of biocatalysed steps in pharmaceutical synthesis is more challenging with more structurally complex targets. With added structurally complexity, the compatibility with wild-type enzymes is less likely. However, the advent of directed evolution can greatly with the resolution of these compounds. The key enantioselective step in the synthesis of montelukast **60** (Singulair®, Merck & Co.) has been achieved using an engineered ketoreductase in place of (–)-DIP-Cl and was run on a >200 kg scale (Scheme 42).[115] Another drug developed by Merck & Co., suvorexant **61**, requires only a short synthesis involving a tandem transamination and ring annulation using an evolved transaminase (Scheme 43) replacing the classical resolution in the previous synthetic route.[116] Introducing the stereocentre at a more advanced stage means that the synthesis can be carried out racemically, and that the racemization of the stereocentre can be limited.

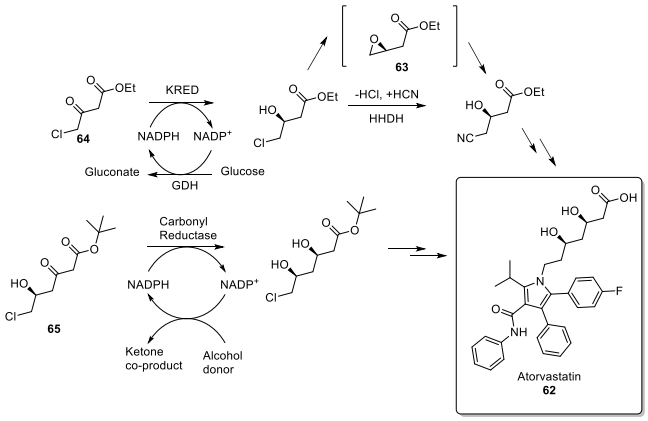


Scheme 42. Synthesis of montelukast sodium **60**



Scheme 43. Synthesis of suvorexant **61**

For atorvastatin **62** (Lipitor®) there are several intermediates which can be produced in enantiomerically pure forms using biocatalyzed reactions and this area has been reviewed.[117] Since this review, a two-step three enzyme process has been reported using a ketoreductase (KRED) and glucose dehydrogenase (GDH) in a redox coupling step, and a halohydrin dehydrogenase (HHDH) to carry out a functional group conversion *via* an intermediate epoxide **63** (Scheme 44).[118] The use of reductase to resolve the intermediate **64** can suffer from product inhibition, *i.e.* the product can slow down the reaction. Wang *et al*. reported the use of a dual-purpose functionalized resin for the immobilization of the enzyme. The support material also adsorbs the product, which prevents product inhibition, allowing increased substrate loading.[119] Recently, the biocatalyzed synthesis of intermediate ester **65** has been reported using a carbonyl reductase, identified through directed evolution.[120]

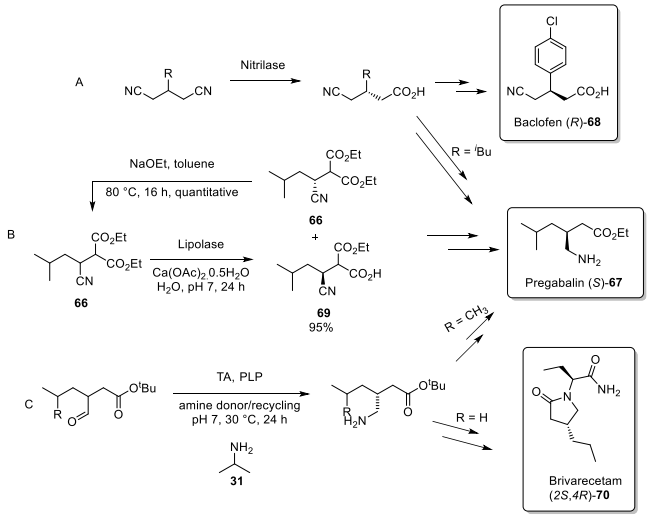


Scheme 44. Chemoenzymatic synthesis of intermediates of atorvastatin **62**

The diester intermediate **66** in the synthesis of pregabalin **67** was resolved using a commercially available lipase.[121] A recycling step was also developed, where the unreacted ester **66** from the resolution could be recycled to enhance the efficiency of the reaction (Scheme 45, **B**). The route is particularly well set up for the potential transfer to continuous flow, for the resolution/recycling. Another route to **67** utilized nitrilases for an initial desymmetrization step, followed by a Curtius rearrangement and hydrolysis (Scheme 45, **A**).[122] This route allowed access to both pregabalin **67** and baclofen **68**. The use of a mutated lipase from *Thermomyces lanuginosus* has also been reported in the resolution of **66**, giving increased activity against **69**.[123] Recently, the use of transaminases has been applied to the resolution of pregabalin **67** and brivaracetam **70** intermediate *α*-chiral aldehydes (Scheme 45 **C**).[124] The aldehydes racemize under the biotransformation conditions, specifically in the presence of the amine donor isopropylamine **31**, furnishing an effective DKR by taking advantage of the inherent equilibrium under the reaction conditions.

Several biocatalytic routes have been developed to access rasagiline mesylate *(R)*-**71**, a commercially marketed treatment for Parkinson's disease (Scheme 46). The resolution of *(S)*-1-indanol **72**, followed by a stereoinversion using Mitsunobu chemistry and a Staudinger reduction gives the enantiopure amine **73**.[125] The resolution of the amine has also been developed, combining the CAL-B-catalyzed transesterification of amine **73** with an *in situ* racemization step.[126] A route to rasagiline **71** has also been reported using imine reductases (IREDs) to convert the ketone **74** directly to the target compound *(R)*-**71**.[127] The enantiomer *(S)*-**71**, can also be accessed through the use of an *(S)*-selective imine reductase. While the IRED route seems the most logical route to use, factors such as scalability and knowledge of the chemistry involved would need to be carefully considered; CAL-B is a very well-studied lipase, and has been used in industrial processes previously, thus, may be more readily employed than the lesser known biocatalyst. Screening of

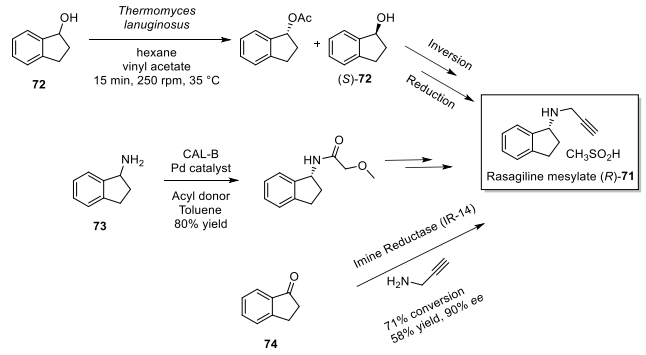
a library of mutant biocatalysts (IRED) gave excellent results here. Equally, the use of a well-characterized biocatalyst in conjunction with a racemization step also gives the product with excellent enantiopurity.



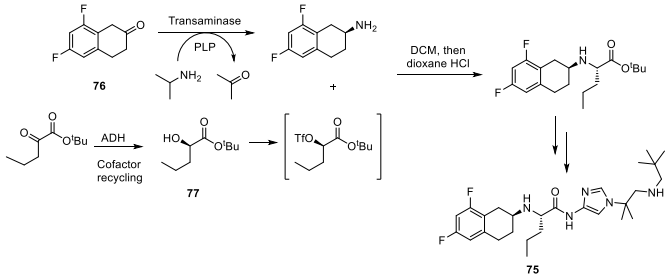
Scheme 45. Resolution of pregabalin precursors and synthesis of related compounds **68** & **70**

Enzymatic reactions have been used in the enantioselective synthesis of two enantiopure advanced intermediates of γ-secretase inhibitor **75**, on a multikilogram scale (Scheme 47).[128] The transaminase was used in conjunction with isopropylamine to effect enantioselective reductive amination of tetralone **76**; while an additional cofactor recycling step was required for the alcohol dehydrogenase-mediated reaction to produce the enantiopure ester **77**.

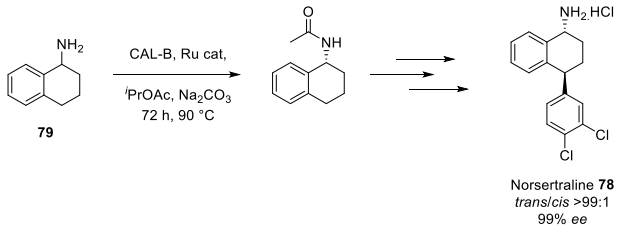
A chemoenzymatic route to norsertraline **78**, an API structurally related to the Pfizer antidepressant sertraline, has been reported through the resolution of 1-aminotetralin **79** by transesterification, and retention of the acyl group throughout the synthesis as a protecting group (Scheme 48).[129] The use of CAL-B at elevated temperatures in conjunction with a racemization catalyst is the first step in a six- step synthesis from commercially available **79**.



Scheme 46. Enzymatic routes to rasagiline mesylate **71**



Scheme 47. Synthesis of γ-secretase inhibitor **75**



Scheme 48. Dynamic kinetic resolution of 1-aminotetralin **79**

4. Conclusions

Biocatalytic transformations offer a very powerful approach for enantioselective synthesis, frequently occurring under mild conditions and with extraordinary selectivity obviating the need for complex protecting group strategies due to functional group tolerance. Nevertheless, there are practical challenges associated with the use of biocatalysis such as limited substrate scope, stability and reusability. Recent technological advances enable the enhanced use of biocatalytic approaches for enantioselective synthesis: including molecular biology techniques leading to modified or novel biocatalysts with improved properties, enzymes stabilization through immobilization, and the potential to use biocatalysts as part of a reaction cascade, either in traditional batch reactors or in continuous flow.

Acknowledgments

The collaboration with Fergal O'Gara's research team enabled through SSPC (funded by Science Foundation Ireland, grant number: 12/RC/2275) is gratefully acknowledged. Discussions with Dr Declan Gavin and Dr Alan Ford are gratefully acknowledged.

Keywords: biocatalysis • immobilization • flow chemistry • molecular biology

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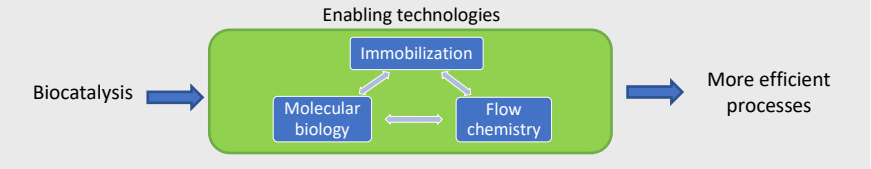

Recent technological advances which facilitate increased use of biocatalysts in organic synthesis are discussed, including immobilization, the use of biocatalysis in continuous flow and the impact of molecular biology on biocatalyst discovery and modification.

Biocatalysis*

*Aoife M. Foley, Anita R. Maguire**

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The impact of recent developments in technologies which enable the increased use of biocatalysts