Biocatalysis is a Key Technology for Successful Chiral Synthesis at Almac

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Dr Tom Moody, Head of Biocatalysis and Isotope Chemistry and Dr Stefan Mix, Biocatalysis Team Leader at Almac describe the latest biocatalytic technologies developed by the company and how they are assiting in API development. The technology is used to support its broader chemistry offerings for the synthesis of advanced chiral intermediates and APIs for pharmaceutical and biotech customers.

It is now well-recognised that the need for green, economic, robust and scaleable processes is at the forefront of customer research plans for the synthesis of chiral APIs and intermediates. The chemical industry is under severe pressure to make their chemical processes greener, lower costs, minimise waste and shorten existing syntheses.[1-3] At Almac, the selectAZyme™ platform provides a diverse, A-Z range of enzymes including reductases, transaminases, hydrolases, nitrilases and many others.

In collaboration with their customers, Almac selects the optimum enzyme to provide an efficient and cost-effective process for scale-up. These enzymes are finding uses in applications from A-Z in medicinal chemistry, metabolite synthesis and in the large-scale manufacture of speciality chemicals. "The power of enzymes is their unequalled selectivity for the chemical reactions they catalyze".[4]
Biocatalysis is becoming the workhorse of the chemist's toolbox for chiral chemistry[5] and is now at the epicentre of key drivers in process design and economics, including:

- Determination of key cost contributors
- Route scouting
- ID and prioritisation of routes to be investigated
- Key reaction optimisation and DoE
- Investigation into waste stream management

Discovery of new enzymes for a novel synthesis at Almac involves research scientists searching the chemical literature and carrying out bioinformatics to create a panel of enzymes that can be optimised through gene-informed diversity screening. Following on from this, desired mutations are introduced into the genes, resulting in novel enzymes with the desired biocatalytic activity. Each 'optimised' enzyme is then screened against a range of standard drug- and intermediate-like compounds to determine its selectivity and activity and whether further optimisation is required.

Screening, upscaling and biocatalyst production takes place at Almac's integrated facilities in Craigavon, UK, which includes molecular and microbiology, analytical, radiolabelling, process chemistry and pilot plant facilties. For any new molecule, the company can complete route invention, screening, enzyme production, process optimisation and manufacture to GMP standard. For production of biocatalysts beyond pilot plant capabilities, Almac uses its UK and European specialist fermentation partners for multi-thousand-litre-scale production.

Why has there been a surge in the application of this green technology? The answer is simple, says Dr Tom Moody: "success breeds success; the key difference today with biocatalysis compared to 10 years ago is - we now have all the supporting technologies that can really make a difference in enzyme development such as bioinformatics, enzyme evolution, high-throughput screening, etc. The list goes on and on; we can now get processes running in weeks and can evolve enzymes in months". The application of green selectAZyme™ technology is a first choice for Almac when looking at chiral synthesis, and enzymes now offer myriad chemical transformations, as shown in Figure 1.

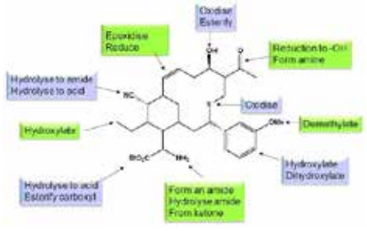


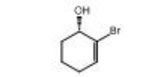
Figure 1. Examples of selectAZyme™ platform transformations

Another key advantage of running these processes is the timeline required for implementation. From selection of a selectAZyme™ catalyst to actual manufacture of product, timelines are similar to those of conventional chemistry optimisation and scale-up. Typical timelines are shown in Figure 2.

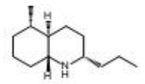
Figure 2. Timelines for using the selectAZyme™ platform

Biocatalysis and API Clinical Trials Supply

In the timely delivery of batches of API to support clinical trials, options to simplify or shorten the synthetic route are always welcome. (S)-2-bromocyclohex-2-enol(1) is frequently encountered as the starting point in a number of natural product syntheses, including (+)-trans-195A (2), the name assigned to a decahydroquinoline alkaloid isolated from the skin of dendrobatid frogs.[6]
Blechert and coworkers prepared **1** on a 0.5 g scale in 95% yield and 99% e.e. using a CBS reduction followed by chromatographic purification. Almac had a requirement to synthesise 100 g quantities of **1** for a novel therapeutic agent currently under development, and wanted to evaluate the use of a CRED enzyme for this. Screening of the selectAZyme™ CRED kit identified an enzyme that exhibited high conversion and high enantioselectivity, albeit using a glucose / glucose dehydrogenase coupled system.[7] There are a number of reaction parameters to consider when developing a CRED reduction, including temperature, pH, cofactor regeneration and % substrate loading. Systematic evaluation of these parameters identified good reaction progress at 300C (lower temperatures gave slower reaction progress; higher temperatures also gave slower reaction progress, presumably due to denaturation of the enzyme), pH 6-6.5 (pH 8 gave significantly slower progress) and 20 volumes of solvent.



(*S*)-2-bromocyclohex-2-enol
(1)



(+)-*trans*-195A
(2)

IPA (20% v/v wrt substrate) was shown to be effective at regenerating the cofactor. Lower (sub-stoichiometric) concentrations of IPA gave incomplete reaction, while higher concentrations also produced a detrimental effect. Convenient as IPA is to use for cofactor regeneration, it does lead to an equilibrium that doesn't favour complete substrate reduction. This problem was overcome by applying a partial vacuum to the reaction mixture to remove acetone, while sparging in IPA to maintain a sufficient concentration in the reaction mixture. Application of these conditions on 100 g input substrate scale, using the CRED as a cell paste, generated the desired product as a colourless oil in 88% yield with an enantiomeric excess of 99.8%. Significantly, the product was of sufficiently high purity to use directly in the subsequent step without any further purification.

For many projects, biocatalysis can be purposely employed from the outset. However, biocatalysis is also a highly valuable technique when problems are encountered with a process. One example of this is shown in Figure 3.

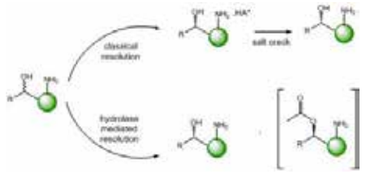


Figure 3. Examples of selectAZymeTM platform transformations

Delivery of this project required access to a chiral amino alcohol of high enantiomeric purity. On lab scale, this had been readily achieved by a traditional diastereomeric resolution. However, as this chemistry was developed for scale-up it quickly became apparent that this resolution wasn't working as required, with low yields and challenging filtration issues being observed. To ensure that the committed delivery date for the API was met, work started on an enzymatic resolution approach, while continuing to work on improving the crystallisation. Following a selectAZyme™ hydrolase enzyme screen, a lipase was identified that converted the undesired enantiomer to an acetate ester, simply by running the reaction in ethyl acetate (both as acyl donor and solvent). The two product components (desired enantiopure alcohol and undesired ester) were readily separated and, following some focussed development work, the lipase approach was successfully applied on scale, leading to on-time delivery of API of the required purity.
Another typical project at Almac includes, for example, a Phase IIb compound where nine steps of chemistry resulted in the formation of three chiral centres from a registered starting material with a global yield of 7.4%. Key to winning the project was the marriage of Almac's synthetic, analytical and solid state chemistry and introduction of green selectAZyme™ chemistry for induction of chirality.
The project was initiated with clear objectives:

1) Increase productivity of the process >50% kg/[L/day].
2) >20% reduction in waste.
3) Removal of expensive and toxic solvents.
4) Removal of heavy metals and subsequent contamination.
5) Removal of necessity for specialised equipment (currently high-pressure hydrogenation).
6) Development of a process with consistent quality of product.
7) Lower the cost per kg of product.

The original chemistry involved myriad steps including a late-stage classical resolution using an expensive amine resolving agent and high-pressure hydrogenation using metal catalysis. The late-stage resolution resulted in huge volumes having to be processed until step 7. Almac's challenge was to make a scaleable lower-volume route that had green and cost incentives for change. Almac completed route invention, proof-of-concept demonstration followed by 100s of kgs scale-up prior to tonne manufacture. The revised route consisted of five steps using three different selectAZyme™ enzymes with a global yield of 23.4%.

Innovation was achieved by delivering a green process that was scaleable, derived from readily-available feed stocks, and used "off-the-shelf" selectAZyme™ enzymes with no "strings" attached. From retrosynthetic analysis it was demonstrated that the registered starting material could be made from feed stocks that would not have any long-term supply issues and could be sourced readily from India and China. Having the proposed route on paper, the next step was to synthesise the key intermediates and begin enzyme screening. The project involved an early-stage bioresolution that resulted in a monoacid product with >96% ee. From this, a bioreduction step introduced another chiral centre. Key to this enzyme screening was to find a carbonyl reductase (CRED) enzyme that was able to stereospecifically reduce the ketone of the desired enantiomer feedstock and not the undesired (2%) enantiomer from the bioresolution step.
The CRED identified resulted in a stereospecific reduction and subsequent biopolish of the diastereomeric mixture. The remaining undesired ketone was easily removed using conventional work-up at the next step. The process ran from start to finish using two solvent combinations. Having developed the process, all stereoisomers (seven different products) were synthesised readily from other key selectAZymeTM enzymes so that analytical development could be undertaken to determine the fate of these potential impurities. The summarised advantages of the green enzyme process are shown in the table below –

	Chemical Route	selectAZyme™ Route
No. of Steps	9	5
Catalyst Type	Rhodium-based Natural product amine	1x Liquid enzyme 2x Powdered enzymes
Volume Efficiency	83 g/L	151 g/L
Solvent Usage	DCM, MeTHF, EtOAc, DMF,	EtOAc, Toluene
Global Yield	7.4%	23.4%

It is clear from the example described herein, that biocatalysis offers an attractive approach for synthesis resulting in greener processes with real benefits for both the environment and costs of your APIs.

The increased demand for oxidative metabolite synthesis has resulted in more and more research and published literature in the area of P_{450} enzymes.
Their utilisation in practical synthesis of Phase I metabolites has led to a greater diversity of off-the-shelf catalysts that

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can be used by the synthetic chemist. The ease of access of P_{450} enzymes even from published gene sequences meant Almac was able to complete a carbon-13 project where the customer required the corresponding carbon-13 metabolite (Figure 4). Almac simply identified the P_{450} gene sequence from the literature and obtained the synthetic gene. The gene was cloned and expressed and the isolated enzyme obtained from fermentation was used in the isotope labs to easily access the customer metabolite incorporating the desired carbon-13 labelled sites.

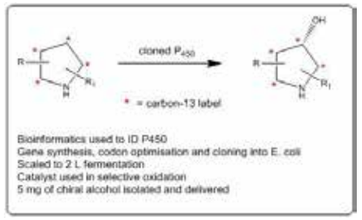

Figure 4: P_{450} mediated hydroxylation of a carbon-13 API.

Partnerships and Collaboration

The Almac/DSM agreement grants each company access to their respective enzyme platform technologies, services and expertise for the manufacturing of APIs. Almac's expertise in rapid enzyme identification, scale-up and implementation into early-phase projects complements DSM's experience of more than 30 commercial manufacturing bioprocesses run on a multi-ton scale, providing green processing from preclinical to commercial manufacturing scale. The collaboration also enables Almac to offer its customers a preferred partner for large-scale production.

Moody states: "The success of the projects undertaken between Almac and DSM already demonstrates there is a market for scaleable green technologies to access difficult-to-make chiral chemicals."

At the enzyme discovery stage, the collaboration between Almac and UCL in the area of metagenomics has boosted enzyme throughput into Almac's growing collection of enzyme platforms. Metagenomics, a culture-independent technique used to extract the total DNA from environmental samples, can allow access to 99% of enzyme genes in these samples. Work previously carried out at UCL has allowed a series of metagenomes to be obtained from various unusual sources. The use of bioinformatic tools will allow the metagenomes concerned to be mined for enzymes usable in both synthetic chemistry and synthetic biology projects.

Moody further commented, "The need for more diverse enzymes has never been greater, and this research programme further emphasises Almac's commitment to UK research and to biocatalysis development. Almac is working with Celbius (an ultrasound company) in the development of ultrasound-assisted biotransformations to increase throughput and ultimately drive down the cost of manufacturing. Having access to this technology is finding real benefits for our customers from bioremediation projects, through chemical synthesis to increased fermentation titres. Cost remains a prevalent issue within the industry, and the introduction of enzymes into processes earlier in the drug discovery pipeline helps to drive cost down as projects move forward. Our partnerships and collaborations are delivering real rewards to clients from both a technical and financial perspective."

Future Directions

"The growth of the biocatalysis business has been the direct result of increased acceptance and application of the new enzyme platforms used both in-house by Almac and by their customers for the identification of enzyme hits," says Moody. "The biocatalyst is now being applied by Almac in the optimisation and delivery of both chiral and achiral intermediates, as well as in GMP manufacturing supporting their customers worldwide.

"There are increasingly more and more chiral molecules in the drug development pipeline, and biocatalysis is increasingly the technology of choice for their synthesis," he adds. "Almac started employing biocatalysis in its custom chemistry services five years ago, initially using biocatalysts supplied by external vendors, and has since been developing its own IP-free enzymes for chiral synthesis. At Almac, biocatalysis has been show to be a technology that can reduce costs due to the potential of minimising processing steps at production, as well as minimising waste and impurity removal costs."

Mix comments: "As our customer demands changed, we rapidly recognised the best technology to access chiral compounds was through biocatalysis technology. Historically we did not have control of the technology and we subsequently realised that we needed to improve our knowledge in this area and to develop in-house biocatalysts. Critical to the biocatalysis business is to ensure to the customer security of supply of the catalysts for repeat manufacture. Today, we are now in a position to implement a biocatalysis-based synthesis for a new compound right from medicinal chemistry through to GMP manufacture – biocatalysis is our 'first point of call' for any project involving chiral moieties because it's as quick as or quicker than other processes to develop, and is predictable upon scale-up."

Moody adds: "In the past, due to customer demands for rapid supply, we often had to employ an inferior technology to produce the compound of interest, resulting in a sub-optimum process that 'we were stuck with'. This resulted in a process that was not easily changed and lacked the potential for futher cost reductions using the biocatalysitic option. Nowadays, biocatalytic methods can be developed and employed just as quickly as chemocatalytic methods. Therefore development timelines are now the same as in a chemocatalytic approach, and biocatalysis is the first method explored when developing a synthetic route to a new compound at Almac."

Summary

The existing pool of recombinant enzymes, both in the literature and available commercially, provides an ample resource from which to develop this technology further. In our opinion, the future focus for enzyme research needs to be on applying new technologies at the molecular level. However, this should also be integrated with methodologies aimed at improving biotransformations at the reaction level, including both physical and chemical approaches.[5] Future development of enzymic reaction systems could investigate and integrate the use of technologies that are known to speed up catalysis in other systems. Almac's continued investment into evolution technologies and metagenomic programmes further confirm the company's commitment to green technologies that offer solutions for their customers. This commitment is being driven by market needs and stresses, and the key to future success is flexibility and rapid response to change. Biocatalysis is a maturing technology and is certainly aiding future success stories for the rapid supply and delivery of chiral intermediates, fine chemicals and APIs by Almac.

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