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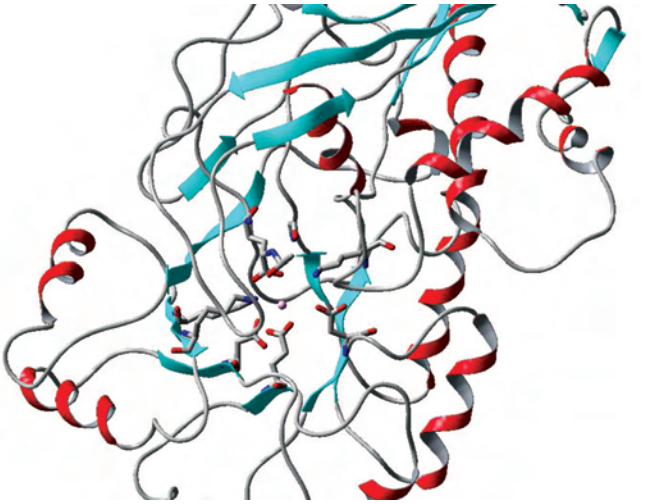

# SYNTHESIS OF ENANTIOPURE AMINOACIDS AND AMINES

## Concerted Use of a Biocatalyst and a Chemocatalyst

**Ingenza Ltd., an Edinburgh based company, that applies biocatalysis discovery, directed evolution and bioprocess optimisation in a large number of potentially useful industrial processes, for the market or for contract services, has developed an ingenious technology** for deracemization of racemic amines and aminoacids that affords the corresponding enantiopure derivatives with high chemical yield (often approaching 100%) and high e.e. (>99%) by the combined use of a bio- and a chemo-catalyst.

A new versatile and scalable method is emerging as a robust platform technology for the preparation of optically active amines and amino acids by deracemisation of the corresponding racemic mixtures. It is being optimised for industrial application by Ingenza Ltd., an Edinburgh based biocatalysis discovery and bioprocess development company. The approach employs the simultaneous use of a highly enantioselective oxidase biocatalyst and a non-selective chemical reducing agent, preferentially a hydrogen transfer donor in the presence of a heterogeneous precious metal catalyst (Fig. 1). A wide range of optically pure amines and amino acids can be prepared in yields often approaching 100%. The process involves no substrate recycling and affords the desired enantiomer typically in >99% e.e. starting from the racemic mixture using a one-pot process.

The key advantages of the technology lie in the co-ordinated action of already proven industrial catalysts and efficient methods of genetic screening to adapt the approach for the preparation of valuable industrial targets.

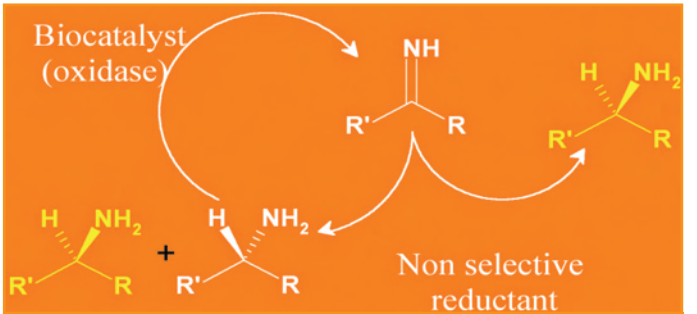


Fig. 1 - Deracemisation of amino acids or amines

## Enantiopure amines

Enantiomerically pure amines are of increasing industrial importance value in the fine chemical and pharmaceutical areas in view of their application as resolving agents [1], chiral auxiliaries [2] or catalysts for asymmetric synthesis [3]. Moreover, enantiopure amines often possess a more pronounced biological activity than the corresponding racemic ones in their own right and hence they are in significant demand as intermediates and/or active ingredients for pharmaceuticals [4] and agrochemicals (Fig. 2). However, the present methods used to prepare enantiomerically pure chiral amines are largely based upon the resolution of racemates, either by recrystallisation of diastereomeric salts [5] or by enzyme-catalysed kinetic resolution of racemic substrates using lipases and acylases [6]. Resolutions of this type are inherently inefficient (maximum 50% yield) and are increasingly viewed as uneconomic and non-competitive. In order to develop more efficient methods, attention is turning towards asymmetric approaches or their equivalent, for example the asymmetric hydrogenation of imines [7] or the conversion of ketones to amines using transaminases [8]. Asymmetric approaches have proven extremely successful in specific instances but due to restrictions in substrate range, have not to date offered a general solution. Attempts to develop dynamic kinetic resolutions, employing enzymes in combination with transition metal catalysts, have unfortunately been hampered by the harsh conditions required to racemise amines [9]. Deracemisation of racemic mixtures is an attractive alternative as a more general solution, yielding close to 100% product in a single pass and conducted in an aqueous environment.

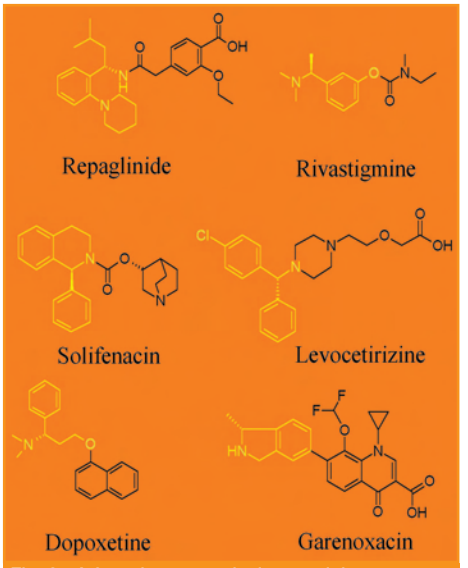

Fig. 2 - A few pharmaceuticals containing enantiopure amines

## Background to the deracemisation process

The deracemisation process derives from synthetic chemistry and biology established in the laboratory of Professor Nicholas Turner at the School of Chemistry at Edinburgh University. Based upon previous literature reports [10, 11] the Turner group originally explored the deracemisation of cyclic and acyclic amino acids using commercial D-amino acid oxidase (DAAO) from porcine kidney and the reducing agents, sodium borohydride ($NaBH_4$) and sodium cyanoborohydride ($NaBH_3CN$) [12].

Initial studies addressed limitations of the earlier work where 500 equivalents of $NaBH_4$ were necessary to achieve high yields from the deracemisation of proline in the aqueous environment required by the enzyme. By introducing $NaBH_3CN$ a milder and more water stable hydride reducing agent the Turner group began a concerted program of process improvement and were able to achieve a 99% yield and 99% e.e. of L-proline from DL-proline [13] using porcine kidney DAAO and only 3 molar equivalents of $NaBH_3CN$. Stereoinversion of D-proline to L-proline proved equally effective using these reagents. Similar results were observed in the deracemisation of DL-piperazine-2-carboxylic acid which could be converted to the L-enantiomer in 86% yield and 99% e.e. using DAAO and $NaBH_3CN$ [13].

Further significant enhancements to the versatility and economic potential of the deracemisation process followed in a 1999 collaboration between the Turner group and NSC Technologies, a unit of Great Lakes Fine Chemicals. In this work, amine-boranes [12] and catalytic transfer hydrogenation using Pd/C [14] were introduced to supercede borohydrides and proved more effective reducing agents in the deracemisation of cyclic and acyclic amino acids. The use of supported metal catalysts in transfer hydrogenation to prepare amino acids by deracemisation, introduced by Dr. Scott Laneman at Great Lakes, was shown as a useful and economical possible solution. Recombinant bacterial strains producing cloned microbial D-amino acid oxidase (DAAO) and L-amino oxidase (LAAO) have also been introduced. This has enabled the deracemisation of DL amino acids to yield the either enantiomer and has provided an opportunity to exploit recent advances in molecular biology and directed evolution methodology to further enhance the deracemisation process towards natural and unnatural amino acids. Deracemisation of DL-leucine to D-leucine was initially demonstrated using amine-boranes and cells expressing recombinant LAAO, with a 98% yield and 99% e.e. [12]. However our recent work in developing the deracemisation process has focused almost entirely upon the use of catalytic transfer hydrogenation due to the highly favourable process economics offered by this approach.

## Development of amine deracemisation

The introduction of amino acid oxidase biocatalysts from recombinant strains reflected a similar initiative to adapt the deracemisation approach to the preparation of optically active chiral amines. In a col-

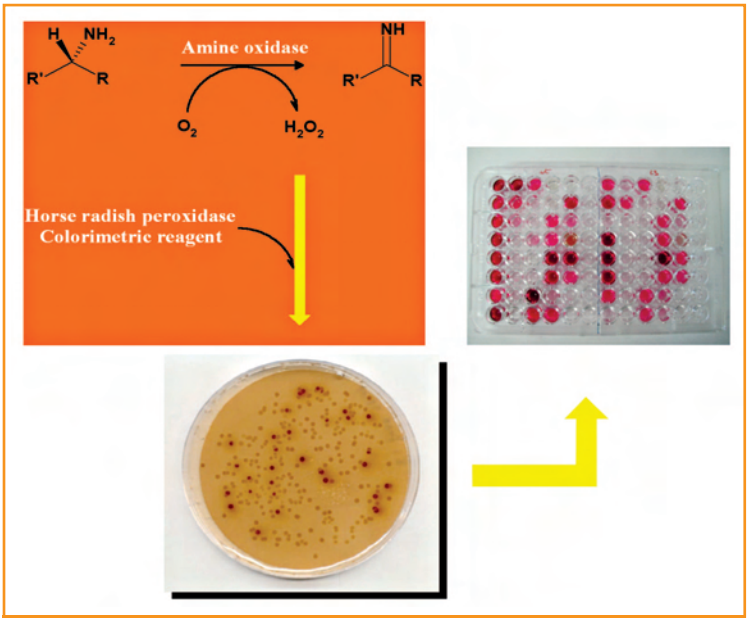


Fig. 3 - The cycle of laboratory evolution of new oxidase specificities and improved performance

laboration between the Turner group and pharmaceutical company GlaxoSmithKline, novel amine oxidase biocatalysts were isolated and developed for deracemisation applications. Whereas the chemo-enzymatic deracemisation of amino acids accessed known enantioselective amino acid oxidases, no suitable enantioselective amine oxidases were known which could be applied in a similar oxidation and reduction process with amines. However the cloned, *MaoN*, gene encoding an amine oxidase from *A. niger* was obtained. This enzyme is active upon simple aliphatic amine substrates such as amylamine and butylamine but also displays a low activity towards benzylamine, and was tested against α-methylbenzylamine as a model system for study. Despite the poor catalytic rate, the enzyme displayed a clear preference for oxidation of L-α-methylbenzylamine over the D-enantiomer and represented a potential target for improvement by methods of *in vitro* evolution.

## Directed evolution of amine oxidase biocatalysts and requirements for the chemocatalyst

Many efficient laboratory procedures now exist to introduce specific or random mutations into genes of interest as a mean to produce enzyme variants with altered properties. However, it is arguably the selectivity and throughput of the screening procedure which most impacts success in identifying improved biocatalysts. By coupling random mutagenesis with a powerful and very high-throughput *in vitro* and *in situ* selection it was possible to rapidly evolve, from the wild type *MaoN*, new amine oxidases which were both highly enantioselective and also possessed broad substrate specificity. The screening procedure takes advantage of the fact that amine oxidase like other members of the oxidase family evolves hydrogen peroxide as a reaction by-product. The presence of peroxide (and therefore oxidase activity) can be detected colorimetrically by the addition of peroxidase and a reagent which yields a coloured product. This can be carried out directly upon bacterial colonies carrying randomly mutated oxidase isolates, enabling up to 500,000 single isolates to be screened in a single experiment. As shown in Figure 3 the appearance of darker coloured colonies indicates increased oxidase biocatalyst activity against a particular amine substrate. Increases in activity, substrate range and stability can be achieved through multiple cycles of this process. Improved amine as well as amino acid oxidases are then characterised kinetically in micro-titre plate based assays. In this way the oxidase biocatalysts can be adapted towards commercial amine or amino acid targets of interest.

This screen, used in conjunction with a "mutator" host bacterium, which introduces random gene mutations at a high frequency, enabled the isolation of an amine oxidase variant with 47 fold enhanced catalytic activity and 6 fold enhanced enantioselectivity against α-methylbenzylamine compared to the wild type enzyme [15]. This biocatalyst is capable of completely oxidising (*S*)-α-methylbenzylamine in 24 hrs with no detectable oxidation of the (*R*)-enantiomer. The enhanced activity is due to a single amino acid change from asparagine to serine at position 336 in the peptide sequence. Subsequent rounds of directed evolution, using the Asn336Ser variant as the parent, resulted in the identification of a double mutant (Asn336Ser/Ile246Met) which has *ca*. 7-fold enhanced activity towards chiral secondary amines, compared to the Asn336Ser variant, and shows reasonable activity towards tertiary amines. In deracemisation reactions, employing the Asn336Ser variant and ammonia-borane as the reductant, (*R/S*)-α-methylbenzylamine was converted to the (*R*)-enantiomer with a yield of 77% and e.e. of 93%. Further rounds of directed evolution and the combination of individual beneficial mutations yielded additional improvements in activity and selectivity of the biocatalyst. More significantly for industrial application, in a subsequent screen of 80 diverse chiral amines, approximately 50% of the substrates were transformed by the enzyme with comparable rates and high enantioselectivity for the (*S*)-enantiomer [16].

Relative rates of oxidation of a range of these substrates are shown in Figure 4. These developments suggested that a toolbox of enzymes could be constructed and used with generally applicable reducing agents to establish deracemisation as a broad reaching com-

mercial technology to prepare chiral amines at high optical purity.
As shown, during this process atmospheric oxygen from air is the reagent for the oxidation step with the production of hydrogen peroxide as the by-product, but for the simultaneous process hydrogen, generated at the correct rate from ammonium formate by a precious metal catalyst, is necessary. Furthermore a minimal concentration of hydrogen peroxide seems important. It is clear that also the heterogeneous chemical catalyst has to be tuned to satisfy all of these requirements: nature of the metal, particle size and distribution, type of support, stability at the alkaline pH. Also no or little poisoning by ammonia seems to be of significant relevance. Certain Pd catalysts, on carbon or alumina support, have for the moment been found to offer the best performance based on screening, although a deeper analysis will be important in the future.

## Optimisation of the deracemisation process

Successful industrial bioprocesses must meet aggressive cost targets and for broad acceptance should be sufficiently robust to be compatible with existing equipment and manufacturing practices. In order to establish deracemisation as a competitive manufacturing route for chiral amines as well as for amino acids, especially for unnatural amino acids, for which industrial application is growing, many process parameters required optimisation in addition to the successful biocatalyst evolution described above. Ingenza is now actively engaged in the development and commercial application of the deracemisation process. Using state-of-the-art facilities and equipment for biocatalysis research and bioprocess scaling-up, the company is developing deracemisation bioprocesses on two levels. First, working closely with customers in the fine chemicals, agrochemicals and pharmaceuticals industries to adapt the deracemisation process to specific target compounds of interest. Second, by establishing specific manufacturing partnerships for scale-up following in-house development of deracemisation processes towards targets identified from Ingenza's market research. The company has established a portfolio of intellectual property on the deracemisation process and now spearheads the optimisation of the operating parameters for deracemisation. Principally these parameters include, substrate and catalyst loading, reaction conditions such as temperature, pH, aeration and agitation, catalyst formulation (free or immobilised enzyme and reducing catalyst), catalyst recycling, process scale-up and product recovery and purification.

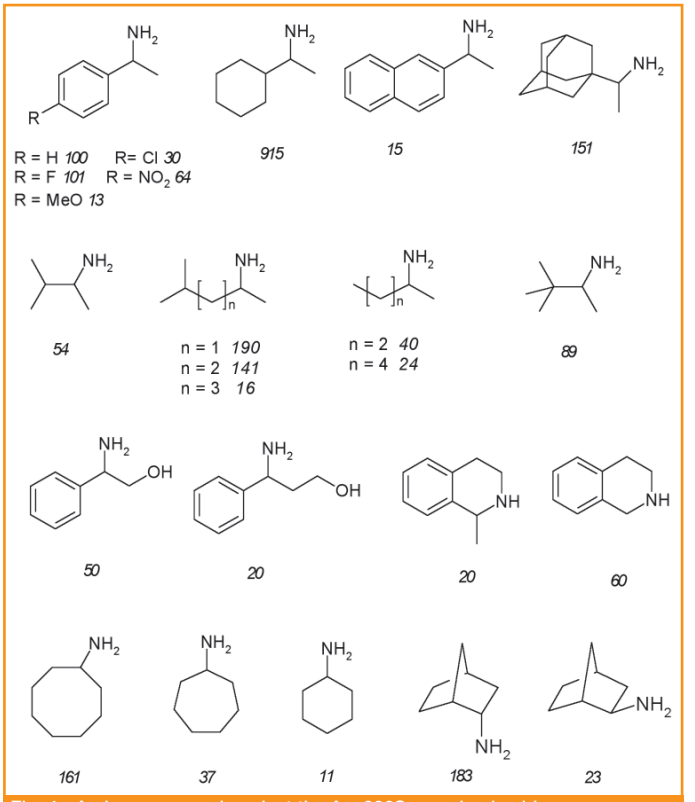


Fig. 4 - Amines screened against the Asn336Ser evolved oxidase. The numbers in italics show relative rates of oxidation compared to α-methylbenzylamine

In one example Ingenza is developing the deracemisation process to prepare L-2-aminobutyric acid from inexpensive D,L-2-aminobutyric acid (Fig. 5). Careful statistical design of the experimental procedures enabled a rapid screen of over 40 supported metal catalysts to be conducted with variable levels of ammonium formate and biocatalyst. These experiments established the optimal metal catalyst for the reaction and began to identify the optimal process conditions. The biocatalyst is prepared by high cell density fed-batch fermentation and used directly as whole cells in the reaction with the metal catalyst. At the current substrate loading of 100 mM the L-2-ami-


**Impiego concertato di un biocatalizzatore e di un catalizzatore chimico per la sintesi di ammine e amminoacidi enatiopuri**

RIASSUNTO

*Viene qui presentata una tecnologia efficiente, economica ed applicabile industrialmente per la sintesi di ammine ed amminoacidi enantiopuri a partire dai corrispondenti prodotti racemi (resa chimica ca.100% ed e.e. >99%). Il metodo, che usa un biocatalizzatore di ossidazione enantioselettivo, ammonio formiato come donatore di idrogeno e un catalizzatore metallico eterogeneo, è stato sviluppato da una società scozzese, Ingenza Ltd., specializzata nell'individuazione ed ottimizzazione di bioprocessi.*

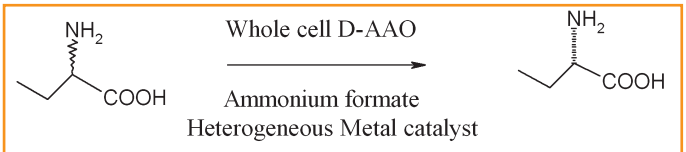


Fig. 5 - Deracemisation of D,L-2-aminobutyric acid

nobutyric acid is recovered at 99.9% e.e. with 95% isolated yield. Both the metal catalyst and biocatalyst can be recovered and reused. The process development is currently establishing conditions for the operation of this process at 500 mM substrate loading. Similar processes, including the preparation of L-pipecolic acid are following similar development strategies.

The enabling technologies required for deracemisation, including recombinant production of oxidases, high density fermentation and biocatalyst immobilisation are also being vigorously pursued. Ingenza's participation in the 6th European Framework Programme DATAGENOM project is supporting this development by enabling the identification and implementation of novel oxidase biocatalysts from genomic sources to further expand the application of oxidase based deracemisation. In addition a novel 1-step purification/immobilisation of the oxidase biocatalysts is offering highly economical production and re-use of the biocatalysts.

Ingenza has optimised amino acid deracemisation up to 10 L scale in-house and with substrate concentrations up to 20% and is currently scaling amine deracemisation to the same level. Ingenza has the internal capability to conduct fermentation and biotransformations at up to 15 L scale and works closely with customers and manufacturing partners for further process scale-up. The company has entered development and technology licensing agreements with commercial partners to deliver deracemisation processes for several target amines and amino acids and continues to identify specific compound targets for in-house development.

## Market overview and conclusive remarks

Recent market analysis has indicated that the sector of enantiopure active ingredients and intermediates currently stands at $15 billion and is growing at 9.4% per annum, with unnatural amino acids and chiral amines playing an increasingly significant role [17]. A large number of commercial routes, including many involving biocatalysts, exist for the preparation of unnatural amino acids for pharmaceutical development. However, each of these processes has some drawback and has been successfully demonstrated for the large scale preparation of only a restricted number of amino acids. Therefore a significant need remains for new platform technologies to provide access to the increasingly diverse range of these products being sought for pharmaceutical manufacture. Chemo-enzymatic deracemisation offers a potential effective and economic route to many of them.

The current competitive landscape for chiral amine production is dominated by chemical catalysis, which is under increasing pressure from cost, waste disposal and environmental constraints and has some difficulty to remain competitive. As such there is increasing interest in biocatalytic routes to chiral amines and two such routes have reached commercial application, namely the Basf process for amine resolution with hydrolase enzymes which results in a maximum yield of 50% and a route to a small number of specific amines from the prochiral ketone developed by Celgene (now Chiragene). In contrast, Ingenza's deracemisation technology can give yields up to 100% and can be widely applied to a large number of structurally diverse amines offering a more general and, in many cases, more cost effective route.

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