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Biotechnology in the Fine-Chemicals Industry: Cyclic Amino Acids by Enantioselective Biocatalysis

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Abstract. Enantiomerically pure cyclic amino acids are of increasing interest for the life-science industry. To meet this demand, *Lonza* has developed biotransformation processes with emphasis on the integration of chemistry and biocatalysis for the production of homochiral proline, piperidine-2-carboxylic acid and piperazine-2-carboxylic acid, and valuable derivatives of these compounds. The processes are designed for use on industrial scale; they have been optimised with respect to availability and cost of starting materials and biocatalyst, productivity of the process and a feasible product isolation procedure. Because of the extraordinarily high enantioselectivity of the enzymes, the (*R*)- and the (*S*)-enantiomers of the unnatural cyclic amino acids, which are both of commercial interest, can be produced in a single reaction. The examples presented demonstrate the benefit from interdisciplinary research for the production of fine chemicals by the combination of chemistry and biotechnology.

1. Introduction

Amino acids represent one of the fundamental classes of building blocks that nature uses for the construction of living matter. Therefore, they are interesting compounds for the life-science industry. The proteinogenic amino acids are important components used in parenteral nutrition and as animal feed supplements. Both proteinogenic and non-proteinogenic amino acids are also used as intermediates for pharmaceuticals, cosmetics, and pesticides. The increasing use of peptide-derived chemotherapeutics has enhanced the importance of enantiomerically pure non-proteinogenic amino acids. Among these, the cyclic amino acids have gathered a preferred position since they allow less rotational freedom, which results in a more defined ligand-receptor interaction. Amino acids are also versatile chiral building blocks for a wide range of fine chemicals [1].

The life-science industry has an ever increasing demand for more selective drugs and agents for the protection of plants and animals, with better defined modes of action and less toxic side effects. The application of homochiral compounds is a promising approach, since nature at the molecular level is intrinsically chiral. Therefore, the use of optically pure compounds in the pharmaceutical industry is increasing dramatically. As a consequence, it turns out that there is a growing demand for efficient industrial processes towards optically active compounds. Although the methodology of asymmetric synthesis in organic chemistry has markedly improved over the years, and although there is a rich chemistry for the preparation of amino acids with different side chains or stereochemistry, enzymatically catalysed processes will in the future play an even more important role than they do today: Enzymes have high catalytic activity, high substrate specificity and, in many cases, unmatched chemo-, regio- and enantioselectivity.

Biotechnological processes for the production of amino acids on an industrial scale have been well known for a long time. Many of the proteinogenic amino acids are produced cheaply and on a very large scale by sophisticated fermentative processes. In addition, there exist numerous routes that are based on the enzymatic resolution of racemic substrates, for example, using acylases, amidases or hydantoinases [2]. However, the situation is not so clear for cyclic amino acids. Since cyclic amino acids are normally non-proteinogenic, with the exception of (*S*)-proline, a fermentative process is difficult to realise. Also, the enzymes commonly used for the production of amino acids do not accept cyclic compounds as their substrates. As a consequence thereof and of the modest interest in cyclic amino acids in the past, there is only a very limited number of biocatalysts known that work on this special subclass of amino acids.

The aim of this article is to describe different routes for the enantioselective preparation of non-proteinogenic cyclic amino acids by the combination of chemistry and biotechnology. Since often both enantiomers are of industrial relevance, especially for non-proteinogenic amino acids, we used several hydrolases with exceptionally high enantioselectivity to resolve racemic substrates.

2. Preparation of Cyclic Amino Acids

2.1. *Proline*

Compared to the unnatural enantiomers of other proteinogenic amino acids, the environmental occurrence of (*R*)-proline is relatively rare. The free amino acid can be detected, mostly in very low amounts,

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in samples of marine sediments [3], food [4] and as a component of bacterial cells [5], plants [6] and animals [7][8]. Most of the (*R*)-proline found in the environment seems to originate from racemisation of (*S*)-proline either by chemical means [9] or enzymatically [10]. (*R*)-Proline, or a derivative thereof, has only in a few cases been identified as a constituent of more complex naturally occuring compounds. Examples are neoviridogrisein [11], a cyclic peptide antibiotic produced by *Streptomyces griseoviridis*, or ergocornine [12], an ergot alkaloid.

Nevertheless, there is an increasing interest in using (*R*)-proline and its derivatives as building blocks for pharmaceuticals (*Fig. 1*). A very prominent example is *Eletriptan*, an anti-migraine drug developed by *Pfizer* [13]. Further examples are *Clemastine*, an antihistaminic drug of *Novartis* [14], *Ro-19-8022*, an anxiolytic compound developed by *Roche* [15], and an antineoplastic agent from *GlaxoWellcome* [16].

To stay abreast with these changes in drug development, *Lonza* decided to approach the production of (*R*)-proline with several strategies. Two of them are racemic resolutions using highly enantioselective hydrolases, whereas the third route relies on the chemo-enzymatic conversion of (*S*)-arginine to (*R*)-proline [17].

As a first choice, we decided to set up a racemic resolution based on the enantioselective hydrolysis of proline esters (*Scheme 1*) [18]. We isolated several new bacterial strains from soil samples that were able to fulfill our requirements. The highest selectivity and productivity was achieved using an *Aureobacterium* sp. strain as the biocatalyst with the isopropyl ester of proline as the substrate. Under optimal conditions, this process can be run with a substrate concentration of more than 200 g/l, yielding the (*R*)-isomer of proline isopropyl ester with an excellent enantiomeric excess (>98% ee at appropriate conversion). (*S*)-Proline, which is the second product of the esterase reaction, can be easily reintroduced into the process to improve the economics. Since the esterase of *Aureobacterium* sp. also accepts several other esters of proline (*Scheme 1*), it is possible to create tailor-made intermediates for further organic synthesis. The ester functions both as a protecting and activating group, so that chemical reactions of the amino group are facilitated as well as the reduction of the carbonyl group to obtain (*R*)-prolinol. Also, any ester can be easily cleaved by alkaline treatment to obtain (*R*)-proline without loss of optical purity.

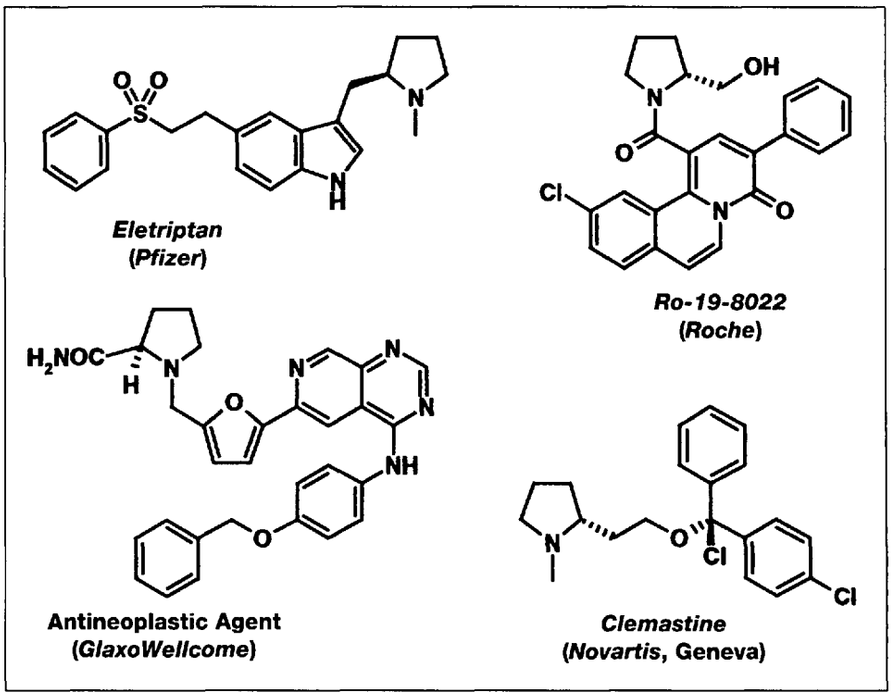
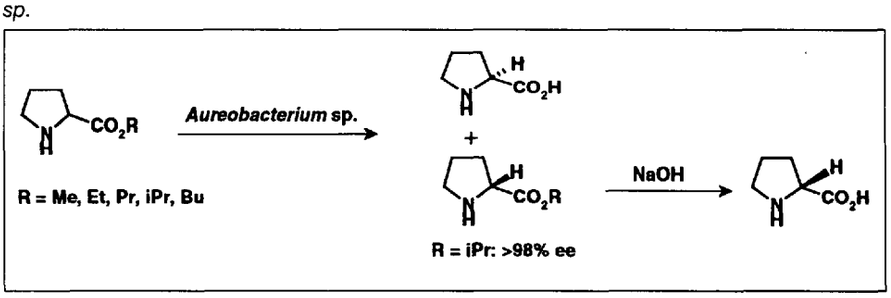
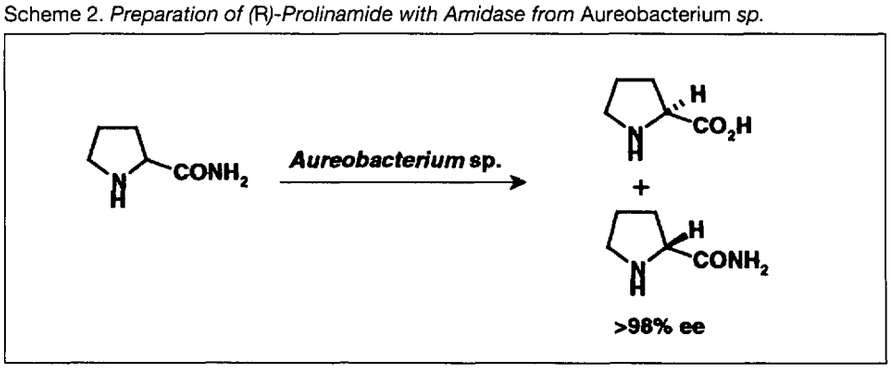


Fig. 1. *Pharmaceuticals containing (R)-proline*

Scheme 1. *Preparation of (R)-Proline Esters and (R)-Proline with Esterase from* Aureobacterium *sp.*



Scheme 2. *Preparation of (R)-Prolinamide with Amidase from* Aureobacterium *sp.*



Aureobacterium sp. also contains a prolinamide-hydrolysing activity that is highly enantioselective for the (*S*)-enantiomer (*Scheme 2*). The resolution of the racemic substrate yields optically pure (*R*)-prolinamide (98% ee at appropriate conversion) [18].

As an alternative to the esterase route, we designed another process that produces amino-protected derivatives of (*R*)-proline by the selective enzymatic cleavage of a common amino-protecting group [19]. Using enrichment techniques, we isolated from soil samples several new strains that were able to cleave *N*-(benzyloxycarbonyl)-(*S*)-proline (*N*-CBZ-(*S*)-proline) enantioselectively (*Scheme 3*). This reaction is catalysed by a proline acylase. In contrast to the enzymes described in the literature, which react slowly or not at all with *N*-

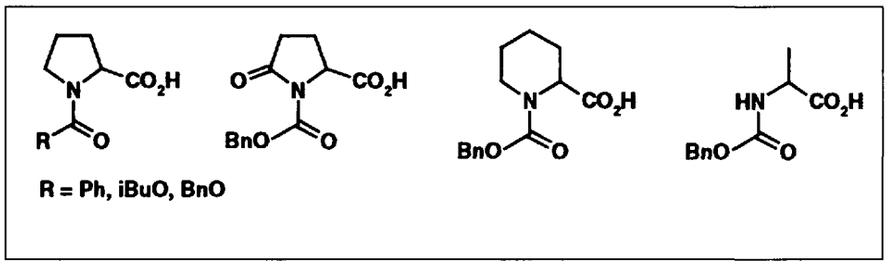
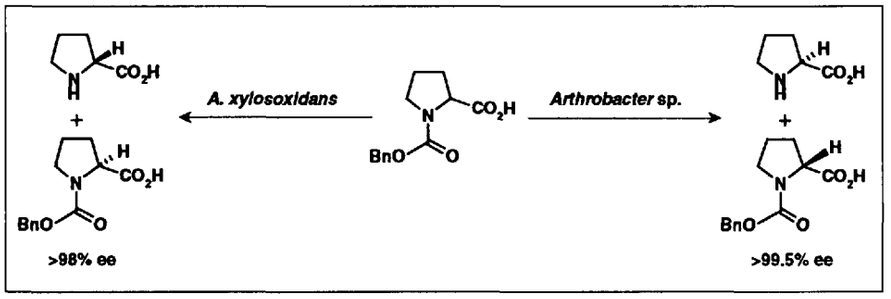
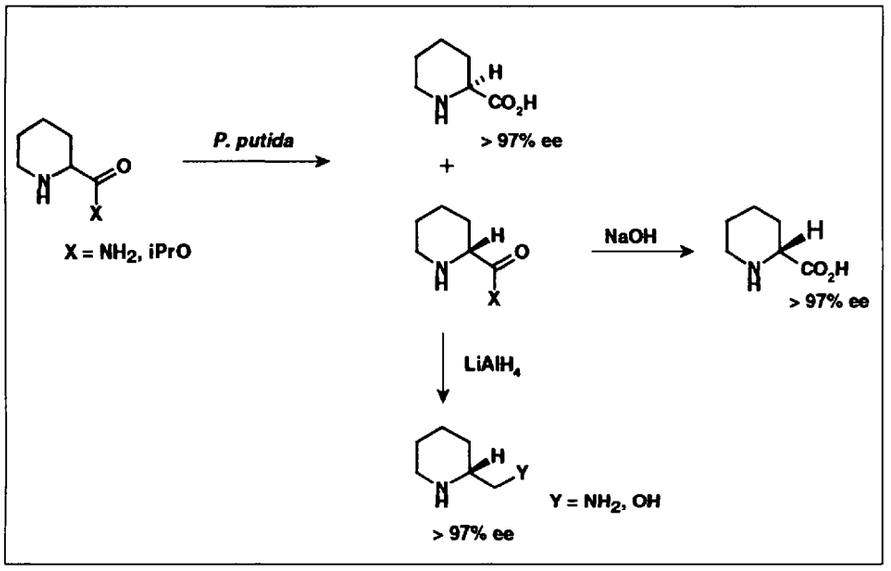


Fig. 2. *Substrate spectrum of (S)-proline acylase from* Arthrobacter *sp.*

Scheme 3. *Toolbox for the Preparation of (S)- and (R)-N-CBZ-Proline: (R)-Proline Acylase from* Achromobacter xylosoxidans *and (S)-Proline Acylase from* Arthrobacter *sp.*



Scheme 4. *Preparation of (S)-Piperidine-2-carboxylic Acid, (R)-Piperidine-2-carboxamide and (R)-Piperidine-2-carboxylic Acid Isopropyl Ester with Amidase from* Pseudomonas putida *and Examples of Chemical Conversions to Other Chiral Piperidine Building Blocks*



CBZ-(*S*)-proline [20][21], the proline acylase of the newly isolated *Arthrobacter* sp. strain shows a high reaction velocity. It also has an exceptionally high enantioselectivity and yields optically very pure product (ee >99.5% at appropriate conversion). After optimisation of the process, mainly by improving the enzyme expression, a product concentration of up to 70 g/l was reached.

Special attention was given to the overall design of the process. Therefore, all the chemistry needed for the preparation of racemic *N*-CBZ-proline from (*S*)-proline is performed in water to avoid unnecessary solvent changes. Also, the final isolation procedure is a simple extraction yielding not only optically pure *N*-CBZ-(*R*)-proline, but also an aqueous solution of (*S*)-proline that can be directly re-used as starting material. This process was stable and reproducibly productive at the 100-kg scale.

Interestingly, the proline acylase of *Arthrobacter* sp. does not accept derivatives of CBZ-proline without a free carboxy group (*e.g.*, amides, esters). However, the enzyme is able to hydrolyse substrates different from CBZ-proline. Variation of the aycl group is possible and also the use of other CBZ-protected amino acids such as piperidine-2-carboxylic acid, pyroglutamate and alanine (*Fig. 2*).

To complement the enzymatic activity of the (*S*)-specific proline acylase, we also identified an (*R*)-specific enzymatic activity [22]. Adaptation of our enrichment procedure led to the successful isolation of new strains, *e.g.*, *Achromobacter xylosoxidans* and *Arthrobacter ramosus*, that are able to enantioselectively hydrolyse the (*R*)-isomers of *N*-acylproline and *N*-acylpiperidine-2-carboxylic acid to give optically pure products (ee >98% at appropriate conversion). This enzymatic activity is, to the best of our knowledge, now described for the first time in the literature. As a consequence of our work, we have now the possibility to selectively produce either the (*S*)- or the (*R*)-isomer of different *N*-acylated cyclic amino acids simply by choosing the appropriate biocatalyst (*Schemes 3* and *5*).

2.2. *Piperidine-2-carboxylic Acid*

Piperidine-2-carboxylic acid, also called pipecolic acid or homoproline, occurs naturally in several plants and as a building block of macrolides such as rapamycin, FK-506 and ascomycin, which are (together with many derivatives) of pharmaceutical interest due to their immunosuppressive properties [23].

Many other pharmacologically active compounds contain (*S*)- or (*R*)-piperidine-2-carboxylic acid. Examples are: *Incel* (biricodar dicitrate) [24] for the treatment of cancer multidrug resistance from *Vertex Pharmaceutical*, *Naropin* (ropivacaine) [25] and *Chirocaine* (levobupivacaine) [26], local anaesthetics from *Astra-Zeneca* and *Chiroscience*, respectively, matrix-metalloproteinase inhibitors from *Agouron Pharmaceuticals* [27], and *L-164013*, a growth-hormone release-promoting agent from *Merck & Co* [28] (*Fig. 3*).

Both enantiomers of piperidine-2-carboxylic acid are of commercial value. Consequently, it is desirable to have access to both isomers. Therefore, we used a biocatalyst with high enantioselectivity that allowed the isolation of both enantiomers with good enantiomeric excess from the racemate. For the racemic resolution of piperidine-2-carboxylic acid, the corresponding amide was chosen (*Scheme 4*). It is easily synthesised from 2-cyanopyridine by hydration and hydrogenation, and it is stable against spontaneous hydrolysis. Several microorganisms, mainly of the genus *Pseudomonas* and *Klebsiella*, that

could hydrolyse the (*S*)-piperidine-2-carboxamide preferentially from the racemate, were isolated starting from soil samples [29][30].

For process development, we chose a *Pseudomonas putida* strain that showed high stereoselectivity for the cleavage of piperidine-2-carboxamide (E >200). After appropriate conversion, (*S*)-piperidine-2-carboxylic acid is obtained with high optical purity (>97% ee) together with (*R*)-piperidine-2-carboxamide. (*R*)-piperidine-2-carboxylic acid is liberated easily from the amide under alkaline conditions. Isolated (*R*)-piperidine-2-carboxamide can also be reduced to obtain (*R*)-2-(aminomethyl)piperidine (*Scheme 4*).

The same strain (possibly the same enzyme) is able to cleave a series of piperidine-2-carboxylic acid esters. The (*S*)-isopropyl ester is cleaved enantioselectively allowing the preparation of (*R*)-piperidine-2-carboxylic acid isopropyl ester (*Scheme 4*). The ester can be used as a piperidine-2-carboxylic acid equivalent in semi-protected form, or it can be reduced to (*R*)-2-(hydroxymethyl)piperidine. This building block is useful for the preparation of anticoagulants [31] and neuroprotective agents [32]. The corresponding (*S*)-enantiomers of 2-(hydroxymethyl)-piperidine and 2-(aminomethyl)piperidine can be prepared from (*S*)-piperidine-2-carboxylic acid *via* esterification or amidation, respectively, and reduction [33].

In addition to the synthesis of both enantiomers of piperidine-2-carboxylic acid, we also developed syntheses for protected derivatives. Besides the above-described route yielding enantiomerically pure piperidine-2-carboxylic acid ester, we have found complementary biocatalysts able to produce the amino-protected forms *N*-CBZ-(*S*)- or *N*-CBZ-(*R*)-piperidine-2-carboxylic acid with high enantioselectivity (*Scheme 5*). These biocatalysts enable us to prepare enantiomerically pure piperidine-2-carboxylic acid building blocks tailor-made for further application in chemical synthesis for the final active compound.

2.3. *Piperazine-2-carboxylic Acid*

Piperazine-2-carboxylic acid is a building block used in the synthesis of many pharmacologically active compounds such as *Indinavir* (*Crixivan*) [34], *Midafotel* [35], and matrix-metalloproteinase inhibitors from different companies [36][37] (*Fig. 4*).

Since pyrazine-2-carboxamide is commercially available in ton amounts, it is very convenient to combine a hydrogenation to piperazine-2-carboxamide and a

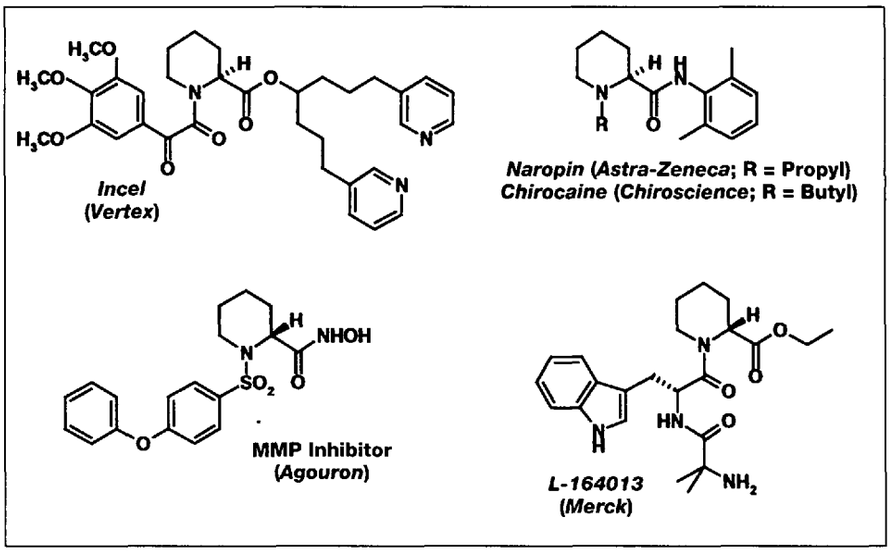


Fig. 3. *Pharmaceuticals containing (S)- or (R)-piperidine-2-carboxylic acid*

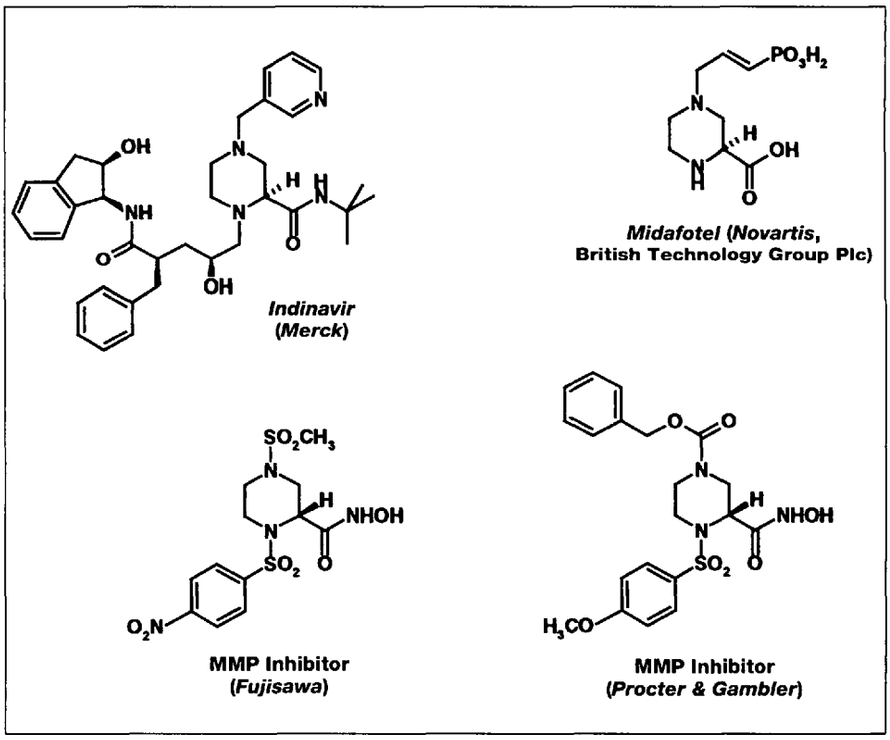
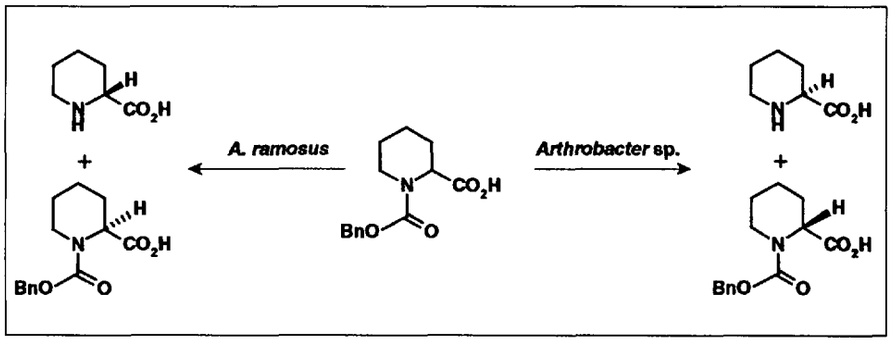


Fig. 4. *Pharmaceuticals containing (S)- or (R)-piperazine-2-carboxylic acid*

Scheme 5. *Toolbox for the Preparation of* N-*CBZ-(R)- and* N-*CBZ-(S)-Piperidine-2-carboxylic Acid: (R)-Proline Acylase from* Arthrobacter ramosus *and (S)-Proline Acylase from Arthrobacter sp.*



biotechnological resolution of the racemate by amidases. *Lonza* has isolated microorganisms containing amidases that have a complementary preference for the two enantiomers of the carboxamide: a *Klebsiella terrigena* strain for the production of (*S*)-piperazine-2-carboxylic acid, and a *Burkholderia* sp. strain for the preparation of (*R*)-piperazine-2-carboxylic acid (*Scheme 6*) [29][38]. Both enantiomers are obtained as crystalline bis(hydrochloride) salts with very high optical purity (ee ≥ 99%). The remaining amide can be hydrolysed easily under alkaline or acidic conditions, thus allowing the production of both enantiomers with each strain.

3. Conclusion

The growing interest of the pharmaceutical and agrochemical industry for enantiomerically pure drugs and pesticides has led to an enhanced need for enantiomerically pure cyclic amino acids. This interest initiated a research program at *Lonza* to develop processes for the production of various optically pure cyclic amino acids based on a combination of chemistry and biotechnology. This approach combined the strengths of the high chemo-, regio- and enantioselectivity of the biocatalyst under mild reaction conditions with the existing arsenal of organic chemistry. As a result of our efforts, we have now access to a toolbox of biocatalysts for the preparation of homochiral cyclic amino acids and their derivatives. Therefore, our final conclusion is that optimal tuning between the biocatalytic and the chemical steps is of crucial importance for the manufacturing of fine chemicals on an industrial scale.

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Scheme 6. *Toolbox for the Preparation of* (R)- *and* (S)-*Piperazine-2-carboxylic Acid with Amidases from* Klebsiella terrigena *and* Burkholderia *sp.*