

## Chemo-enzymatic synthesis of amino acids and derivatives

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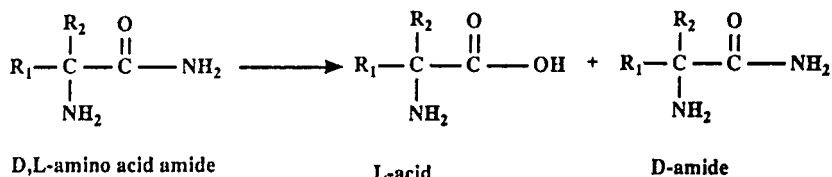
**Abstract-** A short overview is given of new developments in the chemo-enzymatic synthesis of amino acids. Amino acid resolution procedures based on stereoselective enzymatic hydrolysis of amino acid amides have been developed. Two enzymes, an aminopeptidase from *Pseudomonas putida* and an amidase from *Mycobacterium neoaurum* have been purified to homogeneity. In addition, methods for the stereoselective synthesis of  $\alpha$ -alkyl-substituted hydroxy acids are given.

### INTRODUCTION

Amino acids and derivatives are becoming increasingly important as intermediates for pharmaceuticals and agro-chemicals. Well-known examples include D-phenylglycine and D-p-hydroxy-phenylglycine as building blocks for the antibiotics Ampicillin respectively Amoxycillin and L-phenylalanine for the dipeptide sweetener Aspartame. More recently, L-homophenylalanine and derivatives are used for the synthesis of ACE-inhibitors, L-valine as a precursor for the immuno-depressant Cyclosporin A and D-valine in the production of Fluvalinate, a pyrethroid insecticide.

At DSM a generally applicable resolution process based on the enzymatic hydrolysis of amino acid amides with an aminopeptidase from *Pseudomonas putida* has been developed. The principle step of the process is depicted in scheme 1 ( $R_2 = H$ ). Also visualized in this scheme is the resolution of  $\alpha$ -disubstituted amino acids ( $R_2 = \text{alkyl}$ ) with the aid of an L-amidase preparation from *Mycobacterium neoaurum*. Both processes have been extensively reviewed (ref.1). Recent developments include new approaches towards the synthesis of the starting materials of the enzymic resolution ( $\alpha$ -H and  $\alpha$ -alkylsubstituted amino acid amides), enzyme purification and the development of other aminopeptidase or amidase preparations with different substrate and/or stereoselectivities. Some of these developments will be discussed in the sequel. Moreover, some applications of the amino acid derivatives will be given. Finally, the enzymatic resolution of  $\alpha$ -H and  $\alpha$ -alkyl substituted hydroxy-acids will be discussed.

Scheme 1



## ENZYME PURIFICATION

In the process for the production of  $\alpha$ -H amino acids crude enzyme preparations from *Pseudomonas putida* ATCC 12633 are used. From purification studies it became clear that the whole cells contained a number of aminopeptidase activities. Thus, after French pressure cell desintegration of the whole cells and ammonium sulfate fractionation a cell-free extract was obtained. This cell-free extract was subsequently submitted to Mono Q anion-exchange chromatography. Four distinct fractions with activity could be observed whilst one of these fractions showed a strictly  $Mn^{2+}$ -dependent enzyme activity.

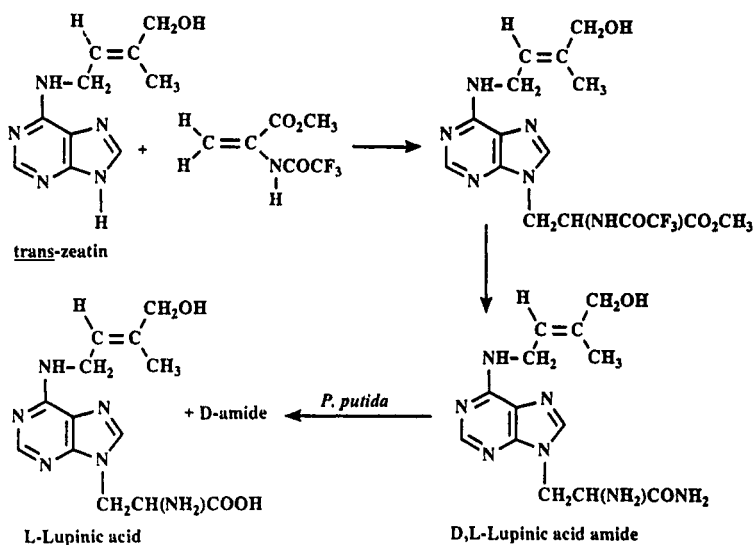
From the fact that the different classes of proteinase activity showed different relative activities towards two representative substrates (L-valine and L-phenylglycine) the presence of different L-aminopeptidase activities in the cell-free extract of *Pseudomonas putida* ATCC 12633 with overlapping substrate specificity could be inferred. Noteworthy, the  $Mn^{2+}$ -dependent enzyme still was capable to stereoselectively hydrolyze a large number of different substrates. The L-amidase enzyme in *Mycobacterium neoaurum* ATCC 25795 was purified to homogeneity by the following purification procedure: French pressure cell desintegration of the whole cells, ammonium sulfate fractionation of the crude extract, Mono Q anion-exchange chromatography and Mono S cation-exchange chromatography.

The estimated molecular weight of the L-amidase is approximately 136 kD (gelfiltration) and 40 kD on SDS page. The enzyme shows an optimal pH of 7.5-9.5. The temperature optimum is 45°C. In table 1,  $K_m$  and  $V_{max}$  values for the purified L-amino amidase from *Mycobacterium neoaurum* ATCC 25795 are given.

table 1

Substrate	$V_{max}$ ( $\mu\text{mol}/\text{min} \cdot \text{mg}$ )	$K_m$ ( $\text{mmol}/\text{L}$ )
D,L- $\alpha$ -Me-Val-NH <sub>2</sub>	75	100
D,L- $\alpha$ -Me-Leu-NH <sub>2</sub>	125	10
D,L- $\alpha$ -Me-Phe-NH <sub>2</sub>	100	5
D,L-Iso-Val-NH <sub>2</sub>	165	30
D,L- $\alpha$ -Allyl-Ala-NH <sub>2</sub>	240	5
D,L- $\alpha$ -Me-homo-Phe-NH <sub>2</sub>	(5)	(15)
D,L- $\alpha$ -Me-PG-NH <sub>2</sub>	(5)	(155)
D,L-Pro-NH <sub>2</sub>	175	40

Scheme 2



## APPLICATIONS

### $\alpha$ -H amino acids

A great number of applications of the aminopeptidase catalyzed resolution of  $\alpha$ -H amino acids has been reviewed (ref. 1). Typical examples include both aliphatic (valine, leucine, methionine etc) and aromatic (phenylglycine, homophenylalanine) amino acids. As an illustration of the remarkably relaxed substrate specificity of the aminopeptidase preparation the synthesis and resolution of lupinic acid is depicted in scheme 2 (ref. 2).

L-Lupinic acid is an isolate from *lupinus angustifolius* and is a principal metabolite of the phytohormone *trans*-zeatin, being one of the most effective natural stimulants of plant cell division.

#### $\alpha$ -Alkylsubstituted amino acids

A well-known application of the use of  $\alpha$ -alkylsubstituted amino acids in the pharmaceutical industry is L- $\alpha$ -methyl-3,4-dihydroxy-phenylalanine, which is used as a drug to treat patients suffering from high blood pressure. More recently, medicinal chemists have become interested in bio-active peptides containing  $\alpha$ -substituted amino acids, since they tend to freeze specific conformations and dramatically slow down enzymatic processes. Thus, the rational design of conformationally constrained compounds which mimic or block the biological effects of physiologically important peptides represents a major goal of modern medicinal chemistry. These conformational analogues should not only retain the pharmacological properties of the parent peptide, they should also exhibit improved bioavailability and pharmacokinetics.

In the last decades it has been shown that C $\alpha$ -methyl, C $\alpha$ -alkyl-glycyl residues represent an interesting new type of conformational constraint in peptides. Thus, peptides rich in the achiral C $\alpha$ , $\alpha$ -dimethylglycine (Aib) residue strongly prefer regularly folded backbone conformations. More recently, the prototype of chiral  $\alpha$ -amino acids of this family, C $\alpha$ -methyl, C $\alpha$ -ethylglycine, has been examined and its conformational behaviour found close to that of Aib, including an unexpected screw-sense indifference (ref. 3). In order to extend this work with chiral  $\alpha$ -amino acids of this family, a variety of L-( $\alpha$ Me)Val derivatives and peptides (upto the pentamer level) have been synthesized and characterized at the University of Padua in the group of Prof. Toniolo (ref. 4). The results indicate that (i) the ( $\alpha$ Me)Val residue is a strong type I/III  $\beta$ -turn and helix former and (ii) the relationship between ( $\alpha$ Me)Val chirality and helix screw sense is the same as that of C $\alpha$ -monosubstituted protein amino acids (ref. 5). Similar studies have been performed with L-( $\alpha$ Me)Phe analogues of physiologically active peptides like the formyl methionyl tripeptide chemoattractant (ref. 6).

L-( $\alpha$ Me)Phe was also incorporated in two classes of dipeptide sweeteners as a substitute for L-phenylalanine. Thus, the ( $\alpha$ Me)Phe analogue of aspartame (figure 1, R<sub>1</sub> = H) was synthesized. The compound had about the same sweetness as aspartame but showed superior stability at pH 4. Also, the corresponding N-formylcarbamoyl-( $\alpha$ Me)Phe-analogue of aspartame (figure 1, R<sub>1</sub> = CO-NH-CHO) exhibited a comparable sweet taste. N-formylcarbamoylaspartame (ref. 7) is a recently discovered aspartame derivative which is very stable at high pH values (pH 6-8). In contrast, the corresponding N-formyl (R<sub>1</sub> = CHO) and N-carbamoyl (R<sub>1</sub> = CO-NH<sub>2</sub>) derivatives of both aspartame and the ( $\alpha$ Me)Phe analogue of aspartame were not sweet.

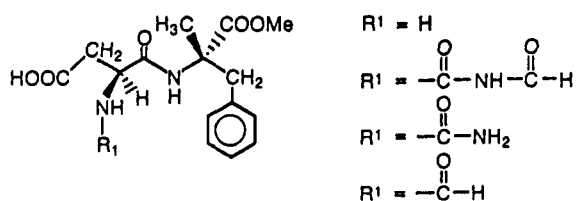
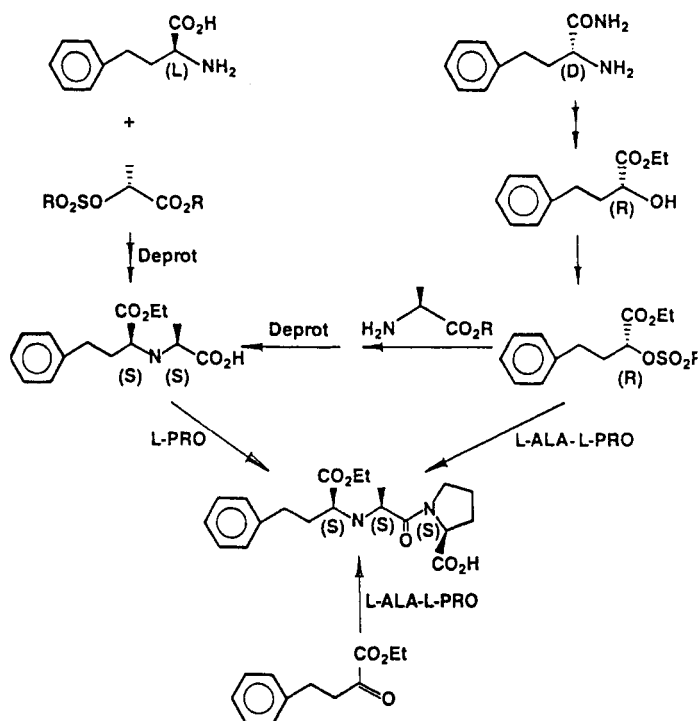


figure 1  
L-( $\alpha$ -Me)Phe aspartame analogues

#### $\alpha$ -Hydroxy acids

The use of homophenylalanine as a building block for the synthesis of Angiotensin-Converting-Enzyme (ACE-)inhibitors like Enalapril has been well-established. However, not only this amino acid is a good starting material for these products, also other related compounds can be used (ref. 8). Some examples are depicted in scheme 3.

Scheme 3



From this scheme it is evident that also  $\alpha$ -hydroxy acids can be versatile intermediates in the synthesis of ACE-inhibitors or other interesting pharmaceuticals. Therefore, we embarked on a program for the asymmetric synthesis and/or kinetic resolution of both  $\alpha$ -H and  $\alpha$ -substituted hydroxy acids.

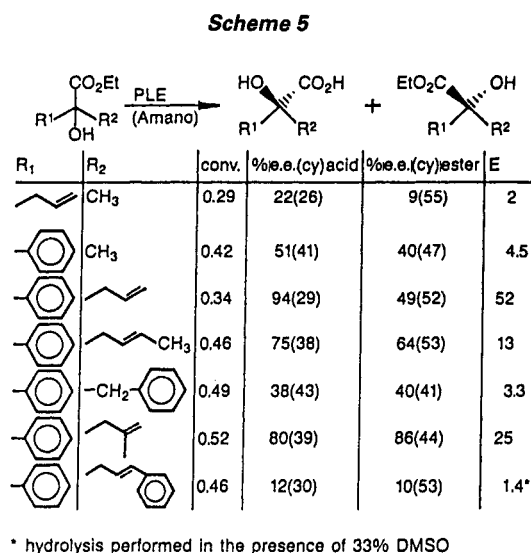
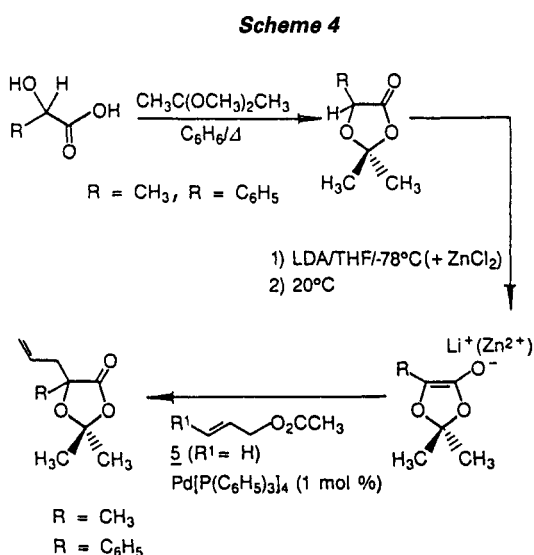
Thus, we have found that the lipase from *Pseudomonas fluorescens* can be used for the kinetic resolution of  $\alpha$ -hydroxy acid ethyl esters. Whereas, enzyme-catalyzed hydrolysis of ethyl (R,S)-2-hydroxy-2-phenylacetate was found to proceed without any stereoselectivity, ethyl (R)-2-hydroxyhexanoate and ethyl (R)-2-hydroxy-4-phenylbutanoate could be obtained in high enantiomeric excess from the corresponding racemic esters (ref. 9).

For the synthesis of  $\alpha$ -alkyl substituted hydroxy acids two approaches have been developed in cooperation with Prof. Kellogg at the University of Groningen. The first one was based on the transition metal catalyzed allylation of mandelic and lactic acid derivatives (scheme 4).

Although a catalytic process could be developed for the synthesis of racemic material, the asymmetric catalyzed variant, however, did not lead to optically pure products. The enantiomeric excess did not exceed 30% (ref. 10). We then turned our attention to the use of an enzymatic kinetic resolution procedure. Although conceptually less elegant (the unwanted isomer can not be racemized) this approach has led to the development of a procedure for the relatively facile synthesis of multigram quantities of a number of optically pure  $\alpha$ -substituted hydroxy acids using pig liver esterase. Moreover, in some cases both enantiomers could be obtained optically pure using a simple recrystallisation as the final purification step (ref.11). In scheme 5 some examples are given.

### CONCLUSION

In conclusion it can be stated that very efficient procedures have been developed for the synthesis of optically pure unusual (and unnatural) amino acids. Both enantiomers of  $\alpha$ -H and  $\alpha$ -substituted amino acids can be obtained. These amino acids can be incorporated in a number of compounds with interesting conformational and physiological properties.



Also kinetic resolution procedures have been developed for the synthesis of enantiomerically pure  $\alpha$ -H and  $\alpha$ -substituted hydroxy acids. Unfortunately, the scope of the latter resolutions is not as wide as in the case of the amino acids. Still, a number of  $\alpha$ -hydroxy acids now are accessible in enantiomerically pure form on a multigram scale. Further research in this fascinating and versatile area is in progress in our laboratories.

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