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Enzymatic resolution of α,α-Disubstituted α-Amino Acid Esters and Amides

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Abstract: The scope and limitations of the enzymatic resolution of α,α-disubstituted α-amino acid amides by an amino acid amidase from *Mycobacterium neoaurum* and of the corresponding ethyl esters with Pig liver esterase (PLE) have been studied. Moderate enantiomeric excesses were obtained with PLE, with only a narrow substrate specificity. *Mycobacterium neoaurum* on the contrary yields a broad range of S-α,α-disubstituted α-amino acids **1** and the corresponding R-amides **2**.

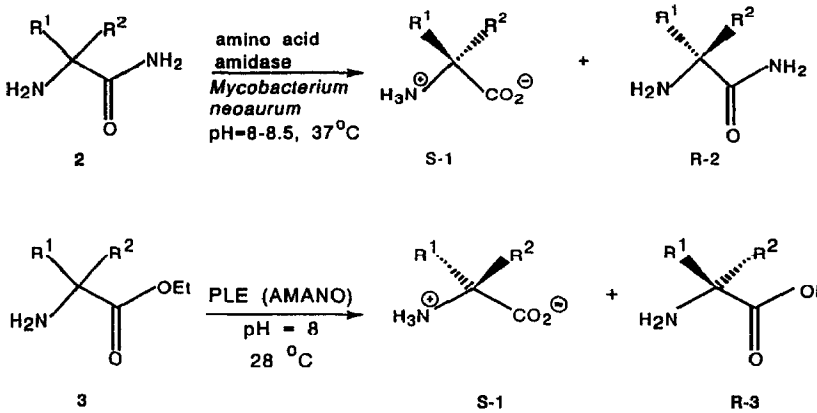
Optically pure α,α-disubstituted α-amino acids **1**, especially α-methyl substituted amino acids, are of increasing interest for the agro chemical and pharmaceutical industry[1]. More recently, higher disubstituted amino acids have also been described for this use[2]. These compounds may act as enzyme inhibitors and several are antagonists of receptors. Also, the influence of these disubstituted amino acids on peptide structure is of current interest[3]. Peptides containing the disubstituted amino acids show a strong tendency for 3_{10}-helix formation instead of α-helix formation. Moreover, the change of handedness of the helical screw sense has been observed by the introduction of α-methyl substituents in peptides[4]. Disubstituted amino acids can also stabilize peptide bonds. For example, the dipeptide sweetener aspartame (α-L-aspartyl-L-phenylalanine methyl ester) exhibits higher stability under acidic conditions by introdution of an α-methyl substituent at the phenylalanine moiety[5].

α,α-Disubstituted amino acids can be prepared by asymmetric synthesis using either chiral auxiliaries[6] or asymmetric phase transfer catalysts[7]. These methods yield disubstituted amino acids in moderate to excellent enantiomeric purities but are rather labourious. The majority of α,α-disubstituted amino acids are prepared by enzymatic resolution. Esterases[8], acylases[9] or hydantoinases[10] are used for the stereoselective hydrolysis of various esters and amides. Although a vast amount of work has been performed on the α-H-amino acid derivatives, the activity for the α,α-disubstituted compounds is only moderate to low.

We at DSM concentrated on the enzymatic route for the resolution of α,α-disubstituted amino acid amides **2** using an amino acid amidase from *Mycobacterium neoaurum* ATCC 25795[11].

Since the racemic α,α-disubstituted amino acid amides **2** are readily available[12], this enzymatic route offers a straight forward route to enantiomerically pure R- and S-α,α-disubstituted amino acids. In order to investigated the scope and limitations of this enzymatic amide hydrolysis we have varied the substituents at the α-position. The results are compared with the Pig Liver Esterase (PLE) catalyzed hydrolysis of α,α-disubstituted α-amino acid ethyl esters **3** which are also described in this article.

The disubstituted substrates, *i.e.* the α,α-disubstituted amino acid ethyl esters **3** and amides **2**, are easily prepared by either esterification of the corresponding racemic amino acids in H_2SO_4/ethanol or by phase transfer catalyzed alkylation of the N-benzylidene-α-H-amino acid amides[12], respectively. The amides can also be prepared by strongly acidic hydrolysis of the Strecker reaction product of ketones[11].



Enzymatic hydrolysis of the α,α-disubstituted amino acid ethyl esters **3a-f** was performed with PLE since from screening experiments on α,α-disubstituted-α-hydroxy esters we found this enzyme to give the best results[13]. The substrates (2% aqueous solution) were subjected to hydrolysis with 5-10 weight% PLE (Amano) at pH 8.0 and 28°C. The conversion was monitored by TLC and at approximately 50% the reactions were worked-up by filtration of the enzyme through Celite, followed by separation of the acid and the ester by acid-base extraction. The results of the hydrolysis are summarized in Table 1.

For **3a-c** the S-enantiomer of the ester is preferably hydrolyzed but only **3c** and **3e** are hydrolyzed with acceptable enantioselectivity. All α-methyl-α-alkyl substituted esters tested (R^1= methyl and R^2=benzyl, phenethyl, i-propyl, i-butyl or neo-pentyl) showed only a very low preference for hydrolysis of the S-enantiomer ($E < 3$).

The enzymatic hydrolysis of the α,α-disubstituted α-amino acid amides **2a-l** was performed with the amino acid amidase from freeze dried *Mycobacterium neoaurum* ATCC 25795 as previously described[11]. In addition to the amides described previously, now a wider range of substrates was tested with this micro-organism. The amides (2-10 weight%, depending on the solubility) were subjected to the amidase (10 weight% calculated on the substrate) in an aqueous solution at pH 8-8.5 at 37°C.

Table 1: PLE catalyzed hydrolysis of 3a-f with R^2 = phenyl.

	R^1	conv %[a]	recovered ester 3		product 1		E[b]
			chem yield %	% ee	chem yield %	% ee	
3a	$-CH_3$	14	67	5(R)	6.3	31(S)	2
3b	$-CH_2-CH_3$	66	28	25(R)	40	13(S)	2
3c	$-CH_2-CH=CH_2$	57	41	95(R)	57	72(S)	23
3d	$-CH_2-CH_2-CH_3$	54	50	20	23	17	2
3e	$-CH_2-CH_2-CH_2-CH_3$	51	31	97	41	93	114
3f	$-CH_2-C_6H_5$	55	10	6	39	54	6

a) The conversion was calculated from ee(S)/(ee(S)+ee(P))

b) The enantiomeric ratio E according to Sih et al. (ref 14)

At approximately 50% conversion the reactions were stopped by centrifugation of the cells, the amino acid and the amide separated on a strongly basic ion-exchange column[15] or by acid-base extraction. The results are shown in Table 2.

As can be seen from Table 2 the selectivity for amino acid amides with two small substituents is only moderate. For substrates with sterically more demanding substituents the enzyme is almost completely stereospecific. However, the reactivity decreased from 4-5 hours for **2a** and **2b** to no reaction after 10 days for **2l**. Typically the reaction was complete after 24-40 hours (**2c-g**, **2i** and **2j**). Only **2h** and **2k** needed reaction times of 7 days to reach the conversion in Table 2.

To account for all the observed results we propose an active site model for the amidase(s) in *Mycobacterium neoaurum* as reflected in Figure 1. From biochemical studies we know that the amidase is a cysteine

Table 2: Enzymatic hydrolysis of **2a-l** with *Mycobacterium neoaurum* ATCC 25795 at pH 8-8.5, 37°C.

	R^1	R^2	conv %	recovered amide R-2		product S-1		E
				chem yield %	% ee	chem yield %	% ee	
2a	$-CH_2-CH_3$	$-CH_3$	47	50	73	45	80	19
2b	$-CH_2-CH=CH_2$	$-CH_3$	41	51	54	44	78	14
2c	$-CH(CH_3)_2$	$-CH_3$	50	41	>98	44	>98	>200
2d	$-(CH_2)_5-CH_3$	$-CH_3$	48	37	89	36	>99	>200
2e	$-(CH_2)_8-CH_3$	$-CH_3$	50	39	99	36	>99	>200
2f	$-CH_2-C_6H_5$	$-CH_3$	50	37	99	42	>99	>200
2g	$-CH_2-C_6H_5$	$-CH_2-CH_3$	39	27	62	30	>96	100
2h	$-CH_2-C_6H_5$	$-CH_2-CH=CH_2$	19	70	20	18	85	15
2i	$-CH_2-CH=CH-C_6H_5$	$-CH_3$	33	61	48	25	>98	150
2j	$-C_6H_5$	$-CH_3$	48	42	86	35	95	110
2k	$-C_6H_5$	$-CH_2CH_3$	13	59	14	9	94	50
2l	$-C_6H_5$	$-CH_2-CH=CH_2$	<5	--	--	--	--	--

containing hydrolase and that only α-NH_2 containing compounds with no substituents at the nitrogen atom are suitable substrates[16]. A small hydrophobic region which can contain the maximum of a C3 unit must be present. For the large substituent no constraints are observed. Only direct attachment of an aromatic substituent to the chiral centre restricts the activity (compare **2g** *vs.* **2k** and **2h** *vs.* **2l**). For the more hydrophobic substrates the solubility in water decreases. A similar active-site model was used by Jones[17] to explain the stereospecific hydrolyses by PLE. The use of this model for the hydrolysis of the disubstituted amino acid esters **3** does not however explain the observed stereospecificity. Moreover, only **3c** and **3e**, in which an aromatic and a C3 or C4 moiety is present, gave acceptable enantiomeric excesses. We therefore consider PLE to be a less favourable enzyme for the synthesis of enantiomerically pure disubstituted amino acids and prefer the use of *Mycobacterium neoaurum*, also because the racemic substrates are more readily available.

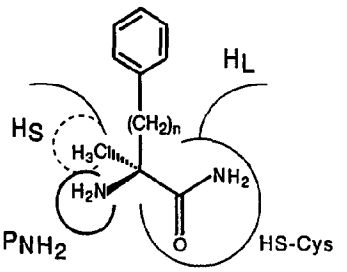


Figure 1

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