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Kinetic Resolution of Aliphatic β-Amino Acid Amides by β-Aminopeptidases

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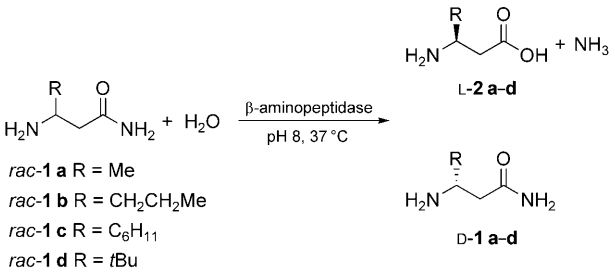
The growing demand for enantiomerically pure β-amino acids to be used in the pharmaceutical industry and as fine chemicals requires the development of new strategies for their synthesis. The β-aminopeptidases BapA from *Sphingosinicella xenopeptidilytica* 3-2W4, BapA from *Sphingosinicella microcystinivorans* Y2, and DmpA from *Ochrobactrum anthropi* LMG7991 are hydrolases that possess the unique ability of cleaving N-terminal β-amino acids from peptides and amides. Hydrolysis of racemic β^3-amino acid amides catalyzed by these enzymes displays enantioselectivity with a strong preference for substrates with the L-configuration and gives access to various aliphatic β^3-amino acids of high enantiopurity. This approach presents a new access to enantiopure β^3-amino acids under mild reaction conditions and complements chemical asymmetric synthesis strategies.

Introduction

The design of unnatural bioactive peptides has attracted increasing attention over the last years. Peptides containing β-amino acids are especially interesting, since they show enhanced resistance towards proteolytic enzymes. The promising pharmaceutical potential of compounds with β-peptidic substructures goes along with a growing demand for enantiopure β-amino acids as building blocks, which is an incentive to develop innovative approaches for their preparations.[1] Besides well-established chemical methods for the synthesis of enantiopure β-amino acids,[2] enzyme-catalyzed resolutions are an interesting alternative. Unlike kinetic resolution approaches to chiral α-amino acids,[3] the biocatalytic synthesis of enantiopure β-amino acids is generally limited by the availability of suitable enzymes that are able to catalyze reactions with β-amino acid containing compounds. Promising enzymatic approaches that provide access to enantiopure aromatic β^3-amino acids include the kinetic resolution of N-terminally modified β^3-amino acids by porcine kidney acylase[4] and by penicillin G amidase.[5] Furthermore, β^3- and $\beta^{2,3}$-amino acid esters of high enantiopurity were prepared in organic solvents with the lipases CAL-A and CAL-B from *Candida antarctica*.[6] However, there is still a lack of versatile enzymes that operate under mild reaction conditions and provide direct access to a broad range of enantiomerically pure β^3-amino acids by kinetic resolution.

The BapA enzymes from *Sphingosinicella xenopeptidilytica* 3-2W4 (3-2W4 BapA) and *S. microcystinivorans* Y2 (Y2 BapA) as well as DmpA from *Ochrobactrum anthropi* LMG7991 are aminopeptidases that catalyze the hydrolysis of N-terminal β-amino acid residues from amides and peptides.[7] Due to their unique substrate specificities and sequence similarities they are collectively referred to as β-aminopeptidases.[8] Despite being structurally distinct, β-aminopeptidases share the functional properties of the N-terminal nucleophile (Ntn) hydrolase family.[9] In recent investigations, we studied the hydrolytic properties of 3-2W4 BapA, Y2 BapA and DmpA,[10] and also used these three enzymes for the synthesis of β- and mixed β/α-peptides.[11] Herein, we investigated the enantiodifferentiation of enzyme-catalyzed amide hydrolysis, and employed β-aminopeptidases for the kinetic resolution of four aliphatic β^3-amino acid amides (*rac*-**1 a**–**d**) to the corresponding β^3-amino acids (**2 a**–**d**) in aqueous solution (Scheme 1).



Scheme 1. Kinetic resolution of the β^3-amino acid amides *rac*-**1 a**–**d** catalyzed by the β-aminopeptidases 3-2W4 BapA, Y2 BapA and DmpA.

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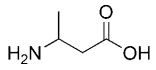
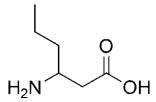
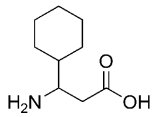
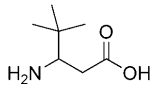
Results and Discussion

Separation of β³-amino acid enantiomers

The enzyme-catalyzed hydrolysis of the β³-amino acid amides *rac*-**1a–d** was followed by measuring the formation of the corresponding free β³-amino acids **2a–d** on a teicoplanin HPLC stationary phase. Under these separation conditions, β³-amino acids of D-configuration elute prior to the L-enantiomers (Table 1).[12,13]

Table 1. Elution order of the enantiomers of **2a–d** on a Chirobiotic T2 teicoplanin HPLC column with a mobile phase composition of MeOH (90%)/H_2O (10%) at 10 °C.

	β-Amino acid	Retention factor, k[a]	Separation factor, α[b]	Elution sequence[c]
2a		$k_D = 1.79$ $k_L = 2.03$	1.13	D < L ($R < S$)[d]
2b		$k_D = 1.47$ $k_L = 1.85$	1.26	D < L ($R < S$)
2c		$k_D = 1.32$ $k_L = 1.79$	1.36	D < L ($S < R$)
2d		$k_D = 0.91$ $k_L = 1.11$	1.22	D < L ($S < R$)

[a] Retention factor $k = (t_R - t_M)/t_M$, where t_R is the retention time of the compound and t_M is the retention time of an unretained compound. [b] Separation factor $\alpha = k_D/k_L$. [c] According to the proposed general elution sequence.[12] [d] According to elution of a standard of L-**2a**.

Kinetic resolution of β³-amino acid amides

The three β-aminopeptidases 3-2W4 BapA, Y2 BapA, and DmpA efficiently resolve the racemic β³-amino acid amides *rac*-**1a–d**. The enzyme-catalyzed reactions have L-enantioselectivity and form the respective L-β³-amino acids L-**2a–d** in high enantiomeric excesses (Table 2). With all three enzymes, *rac*-**1a** and *rac*-**1b** were the most efficiently resolved substrates ($E >$ 200) among the four tested compounds, and yielded amino acids L-**2a** and L-**2b** in high enantiomeric excesses of over 98%. Employing *rac*-**1b** as a substrate, we did not observe hydrolysis of the D-enantiomer until L-**1b** had been fully converted by the enzymes (Figure 1, left side). In the case of *rac*-**1a** we could not detect the formation of D-**2a** over 72 h, not even after L-**1a** was completely consumed. Alkaline hydrolysis of the remaining amide by the addition of sodium hydroxide and heating led to recovery of D-**1a**. This means that all enzymes completely converted *rac*-**1a** to enantiopure L-**2a**, leaving the amide D-**1a** unreacted.

To determine the influence of the acyl leaving group, we employed the β-aminopeptidases for the kinetic resolution of *rac*-**3a**, the *p*-nitroanilide analogue of **1a** (Scheme 2).[10,11] With all three enzymes, we observed full conversion of the L-enantiomer to the acid L-**2a**, whereas formation of D-**2a** could not be detected. Alkaline hydrolysis of the unreacted *p*-nitroanilide led to recovery of D-**2a**. Our data suggest that the enantioselectivity of β-amino-acid amide cleavage catalyzed by the β-aminopeptidases depends on the configuration of the stereogenic center in the β-position and not on the acyl leaving group. In addition to *rac*-**3a**, we had enantiopure L-**3a** and D-**3a** at our disposal, which allowed a more detailed analysis of the L-enantioselectivity. Hydrolysis experiments with 10 mM solutions of the enantiopure substrates showed that 3-2W4 BapA converts L-**3a** more than 2000-times faster than D-**3a** (11.8 and 0.0056 μmol min^{-1} per mg of protein, respectively).

The kinetic resolutions of compounds *rac*-**1c** (Figure 1, right side) and *rac*-**1d**, which carry bulky cyclohexyl and *tert*-butyl substituents at the β-carbon, respectively, yielded the corresponding L-β³-amino acids L-**2c** and L-**2d** in enantiomeric excesses of over 92%. The DmpA-catalyzed reaction displayed the highest enantioselectivity ($E > 100$ and $ee_L > 97\%$) for the resolution of both, *rac*-**1c** and *rac*-**1d**. Although the substrates **1a–d** could not be analyzed on the employed teicoplanin stationary phase, our results suggest that kinetic resolution of *rac*-**1a–d** with the three β-aminopeptidases not only leads to

Table 2. Kinetic resolution of *rac*-**1a–d** by 3-2W4 BapA, Y2 BapA and DmpA at an initial substrate concentration of 20 mM.

Substrate	Enzyme	Enzyme concentration [μg mL^{-1}]	Reaction rate [μmol min^{-1} per mg of protein]	t [h]	ξ [%][a]	ee of L-**2a–d** [%][b]	E[c]
rac-**1a**	3-2W4 BapA	6.2	13	8	48	> 98	> 300
	Y2 BapA	13	7.3	24	48	> 98	> 200
	DmpA	1.6	34	4	49	> 98	> 400
rac-**1b**	3-2W4 BapA	9.9	16	6	48	> 98	> 300
	Y2 BapA	21	4.3	4	48	98	> 300
	DmpA	520	0.079	32	50	> 98	> 500
rac-**1c**	3-2W4 BapA	9.9	8.5	1.5	38	> 97	> 100
	Y2 BapA	64	0.38	4	28	95	61
	DmpA	520	0.0033	120	36	> 97	> 100
rac-**1d**	3-2W4 BapA	31	2.5	1	24	95	53
	Y2 BapA	94	0.75	4	51	92	91
	DmpA	520	0.043	8	39	> 97	> 100

[a] Conversion $\xi = 1 - \left(c_P^L + c_P^D\right) \Big/ \left(c_{S_0}^L + c_{S_0}^D\right)$ at time, t. [b] Enantiomeric excess of L-**2a–d** $ee = \left(c_P^L - c_P^D\right)/\left(c_P^L + c_P^D\right)$. [c] Enantiomeric ratio $E = \ln[1 - \xi(1 + ee)]/\ln[1 - \xi(1 - ee)]$.[14]

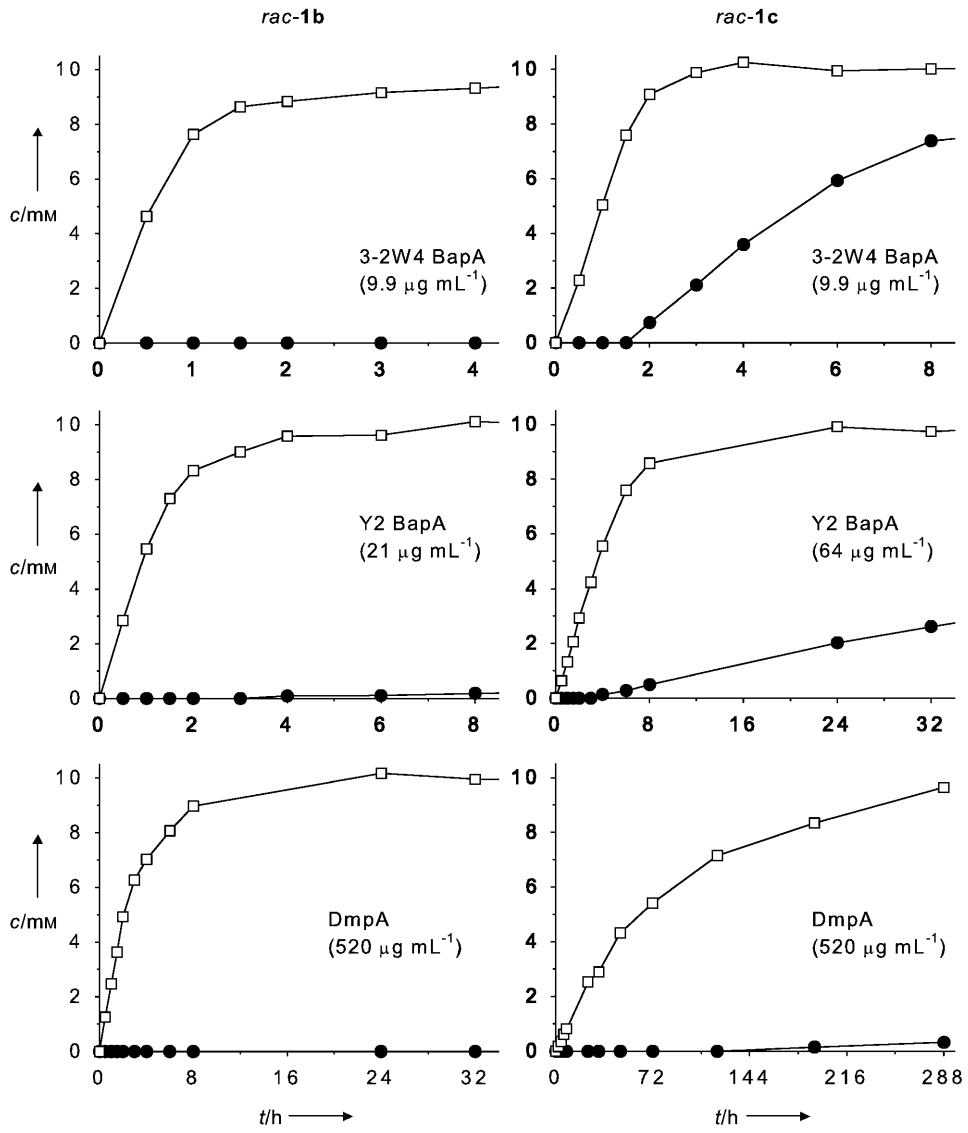
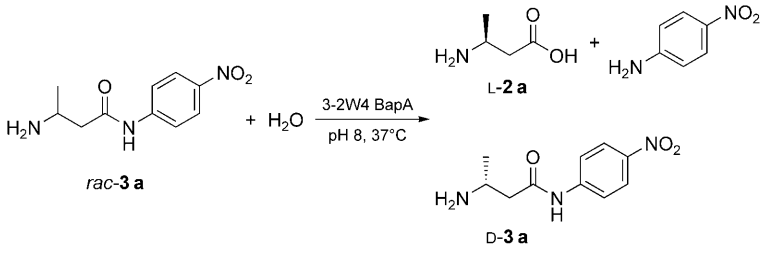


Figure 1. Time concentration curves for the kinetic resolution of *rac*-**1b** and *rac*-**1c** by 3-2W4 BapA, Y2 BapA, and DmpA at an initial substrate concentration of 20 mM. The graphs show the formation of the hydrolysis products L-**2b,c** (□) and D-**2b,c** (●).



Scheme 2. Kinetic resolution of the β^3-amino acid *p*-nitroanilide *rac*-**3a** catalyzed by 3-2W4 BapA.

formation of L-**2a–d**, but also gives access to the unreacted D-β^3-amino acid amides D-**1a–d** in high enantiopurity.

The reaction rates of the β-aminopeptidases were determined for L-**1a–d** because in all cases the L-enantiomers were hydrolyzed at much higher rates than the D-enantiomers (Table 2). As expected from previous degradation experiments,[10] DmpA rapidly hydrolyzed compound **1a** (34 μmol min^{-1} per mg of protein). Amides **1b–d**, which carry sterically more demanding propyl, cyclohexyl, and *tert*-butyl substituents, were converted very slowly by DmpA. In contrast, the BapA enzymes showed broad substrate specificity and converted all four of the tested substrates with good catalytic rates (0.38 to 13 μmol min^{-1} per mg of protein). Our results indicate that the nature of the β-carbon substituent controls the rate of the reaction: The lower the degree of branching of the substituent the higher was the rate of enzyme-catalyzed hydrolysis of the respective amide.

Conclusions

In summary, we have described a novel and practical procedure for the preparation of L-β^3-amino acids in high enantiopurities using three β-aminopeptidases as catalysts. The nonhydrolyzed amide enantiomers are welcome precursors to the D-β^3-amino acids. The enzymes efficiently hydrolyzed all of the tested racemic β^3-amino acid amides that carried aliphatic substituents with different degrees of branching. The results show that the β-aminopeptidases are especially useful for the kinetic resolution of methyl- and propyl-substituted β^3-amino acid amides ($E > 200$ and $ee_L \geq 98\%$), but also substrates with bulky substituents are efficiently resolved by at least one of the enzymes ($E > 100$ and $ee_L > 97\%$). Our previous investigations on the hydrolytic properties of the enzymes[10] suggest that 3-2W4 BapA, Y2 BapA, and DmpA might also be useful for resolving other β^3-amino acid amides with aliphatic, aromatic, or functionalized β-carbon substituents.

Experimental Section

General remarks: The amino acids **2a–d** were analyzed by reversed-phase HPLC on a Dionex HPLC system equipped with a

P680 pump, an ASI-100 automated sample injector, an UltiMate 3000 thermostatted column compartment and a UVD 340U photodiode array detector (Dionex, Sunnyvale, CA, USA). Enantiomers were separated without further derivatization on the chiral teicoplanin stationary phase Chirobiotic T2 (250×4.6 mm; Astec, Whippany, NJ, USA) at a constant temperature of 10 °C and detected by measuring the absorbance at 205 nm. The mobile phase was composed of MeOH (90%)/H_2O (10%) and the applied flow rate was 1 mL min^{-1}. Under the described separation and detection conditions, the detection limit for compounds **2a**–**d** was 0.1 mM. For the determination of protein concentrations, we used Bradford reagent (5× concentrated) from Bio-Rad (Rheinach, Switzerland) and compared samples to a standard curve of bovine serum albumin; absorbance measurements were performed at 595 nm with a Specord S100 spectrophotometer (Analytik Jena, Jena, Germany). The *p*-nitroanilide derivatives *rac*-**3a**, L-**3a** and D-**3a** were synthesized according to known procedures.[10,11]

Enzyme expression and purification: The recombinant enzymes 3-2W4 BapA, Y2 BapA, and DmpA were purified from their *E. coli* hosts as described previously.[10,7c] The lyophilized enzyme powders were dissolved in an appropriate volume of Tris-HCl buffer (10 mM; pH 8) and the protein content of the enzyme stocks was determined.

General procedure for the kinetic resolution of *rac*-1a–d: The reaction mixtures contained 20 mM 3-aminobutanamide (*rac*-**1a**), 3-aminohexanamide (*rac*-**1b**), 3-amino-3-cyclohexylpropanamide (*rac*-**1c**) or 3-amino-4,4-dimethylpentanamide (*rac*-**1d**) in Tris-HCl buffer (100 mM; pH 8). Kinetic resolution of the substrates was initiated by the addition of an appropriate amount of one of the enzymes 3-2W4 BapA, Y2 BapA, or DmpA. Samples were withdrawn regularly from the reaction mixtures and the enzymatic reaction was quenched by heating the samples at 95 °C for 5 min. The hydrolysis products **2a**–**d** were analyzed by HPLC and quantified by relating the UV_{205} absorbance to a sample of the respective compound that was fully hydrolyzed at NaOH (1 M) and heating at 80 °C for 2 h.

General procedure for the enzyme-catalyzed hydrolysis of 3a: The reaction mixtures contained *rac*-**3a** (20 mM) or enantiopure L- or D-**3a** (10 mM) in Tris-HCl buffer (100 mM; pH 8) and DMSO (10%). Kinetic resolution of the substrates was initiated by the addition of an appropriate amount of 3-2W4 BapA. Samples were withdrawn regularly and analyzed as described in the previous section.

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Keywords: aliphatic beta-amino acids · beta-aminopeptidases · enzyme catalysis · kinetic resolution · Ntn-hydrolases

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[13] In the CIP nomenclature L-**2a** and **b** have (*S*)-, L-**2c** and **d** (*R*)-configuration.

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