Enzyme-Catalyzed Formation of β-Peptides: β-Peptidyl Aminopeptidases BapA and DmpA Acting as β-Peptide-Synthesizing Enzymes

by **Tobias Heck**[a])[1]), **Hans-Peter E. Kohler***[a]), **Michael Limbach**[b])[2]), **Oliver Flögel**[b])[3]), **Dieter Seebach***[b]), and **Birgit Geueke**[a])[4])

[a]) EAWAG, Swiss Federal Institute of Aquatic Science and Technology, CH-8600 Dübendorf (phone: +41 44 823 5521; e-mail: kohler@eawag.ch)

[b]) Laboratorium für Organische Chemie, Department für Chemie und Angewandte Biowissenschaften, ETH-Zürich, Hönggerberg HCI, Wolfgang-Pauli-Strasse 10, CH-8093 Zürich (phone: +41 44 632 2990; fax: +41 44 632 1144; e-mail: seebach@org.chem.ethz.ch)

Dedicated to Professor *Stefan Toma* on the occasion of his 70th birthday

In recent studies, we discovered that the three β-peptidyl aminopeptidases, BapA from *Sphingosinicella xenopeptidilytica* 3-2W4, BapA from *S. microcystinivorans* Y2, and DmpA from *Ochrobactrum anthropi* LMG7991, possess the unique feature of cleaving N-terminal β-amino acid residues from β- and α/β-peptides. Herein, we investigated the use of the same three enzymes for the reverse reaction catalyzing the oligomerization of β-amino acids and the synthesis of mixed peptides with N-terminal β-amino acid residues. As substrates, we employed the β-homoamino acid derivatives H-βhGly-*p*NA, H-β^{3}hAla-*p*NA, H-(*R*)-β^{3}hAla-*p*NA, H-β^{3}hPhe-*p*NA, H-(*R*)-β^{3}hPhe-*p*NA, and H-β^{3}hLeu-*p*NA. All three enzymes were capable of coupling the six β-amino acids to oligomers with chain lengths of up to eight amino acid residues. With the enzyme DmpA as the catalyst, we observed very high conversion rates, which correspond to dimer yields of up to 76%. The β-dipeptide H-β^{3}hAla-β^{3}hLeu-OH and the β/α-dipeptide H-βhGly-His-OH (carnosine) were formed with almost 50% conversion, when a five-fold excess of β^3-homoleucine or histidine was incubated with H-β^{3}hAla-*p*NA and H-βhGly-*p*NA, respectively, in the presence of the enzyme BapA from *S. microcystinivorans* Y2. BapA from *S. xenopeptidilytica* 3-2W4 turned out to be a versatile catalyst capable of coupling various β-amino acid residues to the free N-termini of β- and α-amino acids and even to an α-tripeptide. Thus, these aminopeptidases might be useful to introduce a β-amino acid residue as an N-terminal protecting group into a 'natural' α-peptide, thereby stabilizing the peptide against degradation by other proteolytic enzymes.

Introduction. – β-Peptides consisting of homologated proteinogenic amino acid residues βhXaa [1] are generally stable to common peptide-cleaving [2] and peptide-metabolizing [3] enzymes *in vitro* and *in vivo* (microorganisms [2b], plants [3b], insects

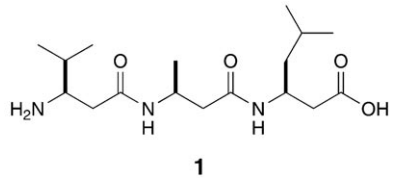
[1]) Part of the projected PhD thesis of *T. H.*, ETH-Zürich, financed by the *Deutsche Bundesstiftung Umwelt* (Project No. 13176-32).

[2]) Postdoctoral Fellow at ETH-Zürich (2004–2006), financed by *Novartis Pharma AG*, Basel.

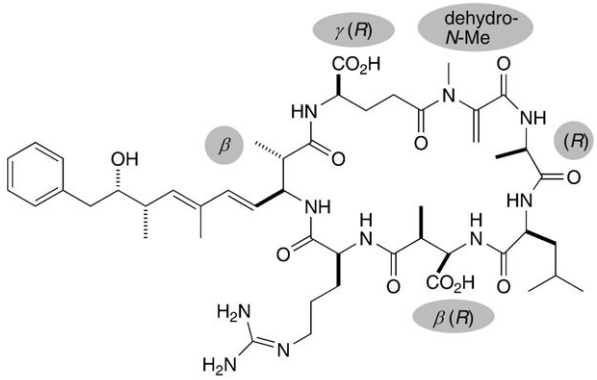
[3]) Postdoctoral Fellow at ETH-Zürich (2003–2006), financed by the *Deutsche Forschungsgemeinschaft*, by the *Swiss National Science Foundation* (Project No. 200020-100182), and by *Novartis Pharma AG*, Basel.

[4]) Postdoctoral Fellow financed by the *Swiss National Science Foundation* (Project No. 3152A0-100770).

[3b], mammals [2b][3a,c,d]). Recently, we showed that three structurally related aminopeptidases from *Proteobacteria* are able to cleave β-peptides [4]. The L-aminopeptidase D-Ala-esterase/amidase DmpA from *Ochrobactrum anthropi* LMG7991 was the first described enzyme of this class, and it was isolated due to its ability to hydrolyze both (*R*)- and (*S*)-α-amino acids from peptides, esters, and amides [5]. The foremost published substrate specificity of this enzyme was extended by the finding that it also cleaves peptides and amides containing small N-terminal β-homoamino acids with high activities [4c]. Two further β-peptidyl aminopeptidases originate from the environmental isolates *Sphingosinicella xenopeptidilytica* 3-2W4 and *S. microcystinivorans* Y2 [4a,b]. These bacterial strains were isolated because of their capability to metabolize the artificial β-tripeptide H-β^3hVal-β^3hAla-β^3hLeu-OH (**1**) and the highly toxic cyanobacterial metabolite microcystin (**2**), respectively [6][5]).



1



2 (microcystin-LR)

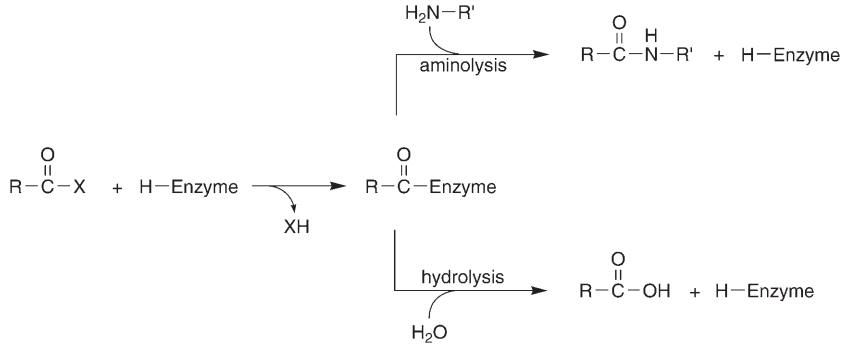
One distinctive feature of the β-peptidyl aminopeptidases from both *Sphingosinicella* strains (named 3-2W4 BapA and Y2 BapA, resp., according to the designation of their host strains) is that they exclusively cleave N-terminal β- but not α-amino acid residues from peptides [4]. A comprehensive investigation of the substrate specificities of recombinant DmpA, 3-2W4 BapA, and Y2 BapA revealed that the enzymes can hydrolyze a broad range of oligopeptides with N-terminal β-amino acids, yet exhibiting different amino acid preferences [4c]. The physiological substrates of these amino-

[5]) Note that the cyclo-heptapeptide microcystin-LR contains only two proteinogenic (*S*)-amino acid residues (Leu, Arg) and is otherwise composed of dehydro-*N*-Me-Ala, (*R*)-Ala, (*R*)-(3Me)-Asp (as β-amino acid residue), (*R*)-Glu (as γ-amino acid residue), and an α-Me-β-amino acid with a most unusual side chain. For total syntheses of microcystins, see [6e,f].

peptidases, however, are as yet unknown. For more details about this class of enzymes, we refer to a recent review article on bacterial β-peptidyl aminopeptidases, covering the literature up to the end of 2006 [7]. Further enzymes that react with peptides containing β-amino acids are very rare; exceptions are different carnosinases from bacterial [8] and mammal origin [9], as well as poly(aspartic acid) hydrolases from *Sphingomonas* sp. KT-1 that degrade the β-peptidic polymer of L-aspartic acid $H-[NH-CH(CO_2H)-CH_2-CO]_n-OH$ [10].

Although proteases and peptidases are commonly associated with protein digestion, their use as biocatalysts in the synthesis of peptides was demonstrated a long time ago [11]. High regio- and stereospecificity, high coupling efficiency, minimal protective group requirements, and mild reaction conditions are advantages associated with the application of proteolytic enzymes that make them interesting alternatives to conventional chemical catalysts. A common approach to protease-catalyzed peptide synthesis, the so-called kinetic approach, is illustrated in *Scheme 1*.

Scheme 1. *Schematic Representation of the Kinetic Approach to Enzyme-Catalyzed Peptide Synthesis.* The acylated enzyme intermediate formed from the activated amino acid (*e.g.*, X = *p*NA, OMe, OEt) and the peptidase undergoes competing aminolysis and hydrolysis reactions.



In a kinetically controlled reaction system an 'acyl donor' component with a mildly activated carboxy terminus, such as an ester or amide, acylates serine or cysteine proteases to form a characteristic acyl–enzyme intermediate. Two nucleophiles, H_2O and the amino group of a second amino acid or a peptide ('acyl acceptor'), compete for the acyl moiety bound to the active site of the enzyme, which leads either to hydrolysis or to formation of the desired amide bond (aminolysis). The peptide product temporarily accumulates in the reaction mixture, but, as soon as the acyl donor component is completely consumed, the newly formed peptide bond is slowly hydrolyzed by the enzyme. Thus, it is important for preparative purposes to stop the reaction at the point of maximum product formation. For more detailed information on theory and applications of protease-catalyzed peptide synthesis, we refer to recent review articles [12].

Recently, *Yokozeki* and *Hara* applied the kinetic approach as an efficient and cost-effective protease-catalyzed method for the production of L-Ala-L-Gln and various other α-di- and oligopeptides in aqueous solution using unprotected starting materials

[13]. Apart from one lipase-catalyzed reaction [14], the synthesis of β-peptides, however, has thus far exclusively been accomplished by nonenzymatic methods.

With the three β-peptidyl aminopeptidases, 3-2W4 BapA, Y2 BapA, and DmpA, at our disposal (*Table 1*), we were able to test the enzymes as catalysts for β-peptide formation in a kinetically-controlled reaction system, and we report herein first, mostly analytical results.

Table 1. *Enzymes Used for Peptide Coupling in the Present Study*

Enzyme name	Abbreviation	Origin	Ref.
β-Peptidyl aminopeptidase	3-2W4 BapA	*Sphingosinicella xenopeptidilytica* 3-2W4	[4]
β-Peptidyl aminopeptidase	Y2 BapA	*Sphingosinicella microcystinivorans* Y2	[4b,c]
L-Aminopeptidase D-Ala-esterase/amidase	DmpA	*Ochrobactrum anthropi* LMG7991	[4c][5]

Results and Discussion. – *β-Amino Acid* p-*Nitroanilides* (pNA) **3**, *the Substrates for the Coupling Reactions Catalyzed by the Enzymes 3-2W4 BapA, Y2 BapA, and DmpA.* As activated substrates for the enzyme-catalyzed formation of β-peptides, we employed the β-homoamino acid *p*-nitroanilides **3a**, (*S*)- and (*R*)-**3b**, (*S*)- and (*R*)-**3c**, and (*S*)-**3d**, derived from glycine, alanine, phenylalanine, and leucine, respectively (*Scheme 2*). The use of *p*NA derivatives of the corresponding β-amino acids is convenient because of the easy detectability of substrates and reaction products[6]). The compounds (*S*)-**3b**, (*S*)-**3c**, and (*R*)-**3c** were prepared as described in [4c]. The preparation and characterization of the β^3hAla derivative (*R*)-**3b** and of the leucine-derived β-amino acid *p*-nitroanilide (*S*)-**3d**, is described in the *Exper. Part* of the present paper. The anilides **3** hydrolyzed very slowly in a nonenzymatic process under the applied experimental conditions.

Homo-Coupling of the p-*Nitroanilides* **3** *to Oligomer*-p*NAs* **4**. To investigate the enzyme-catalyzed oligomerization of β-amino acid residues, we incubated the corresponding β-amino acid derivatives **3** (5 mM) with the recombinant enzymes 3-2W4 BapA, Y2 BapA, or DmpA. In all experiments, the formation of oligomer-*p*NAs **4** was detected by LC/MS analyses. As typical examples, the HPLC diagrams and time–concentration curves for the β^3hAla derivatives (*S*)-**3b** and (*R*)-**3b** are depicted in *Fig. 1* and *Fig. 2*, respectively. From the figures, we conclude that *i*) all three enzymes generated longer oligomers from (*S*)-**3b** (up to six amino acid residues) than from (*R*)-**3b** (up to four amino acid residues); *ii*) for both substrates, oligomer-*p*NAs formed by DmpA were shorter than the corresponding ones generated by 3-2W4 BapA and Y2 BapA; *iii*) DmpA produced the highest dimer-*p*NA concentrations (1.2 mM for (*S*)-**4b**, $n=2$, and 1.9 mM for (*R*)-**4b**, $n=2$) of all three enzymes and also exhibited the highest specific dimerization activities (*cf. Table 2*).

As expected, all oligomers formed were eventually degraded by the enzymes to the β-amino acids **5**, so that after long reaction periods the only detectable *p*NA derivative was *p*-nitroaniline itself.

In general, the degree of oligomerization extended over a range and strongly depended on the employed substrate. The longest oligomer-*p*NAs **4** were formed from

[6]) The anilides **3** have an absorption maximum at 318 nm, *p*-nitroaniline itself at 383 nm.

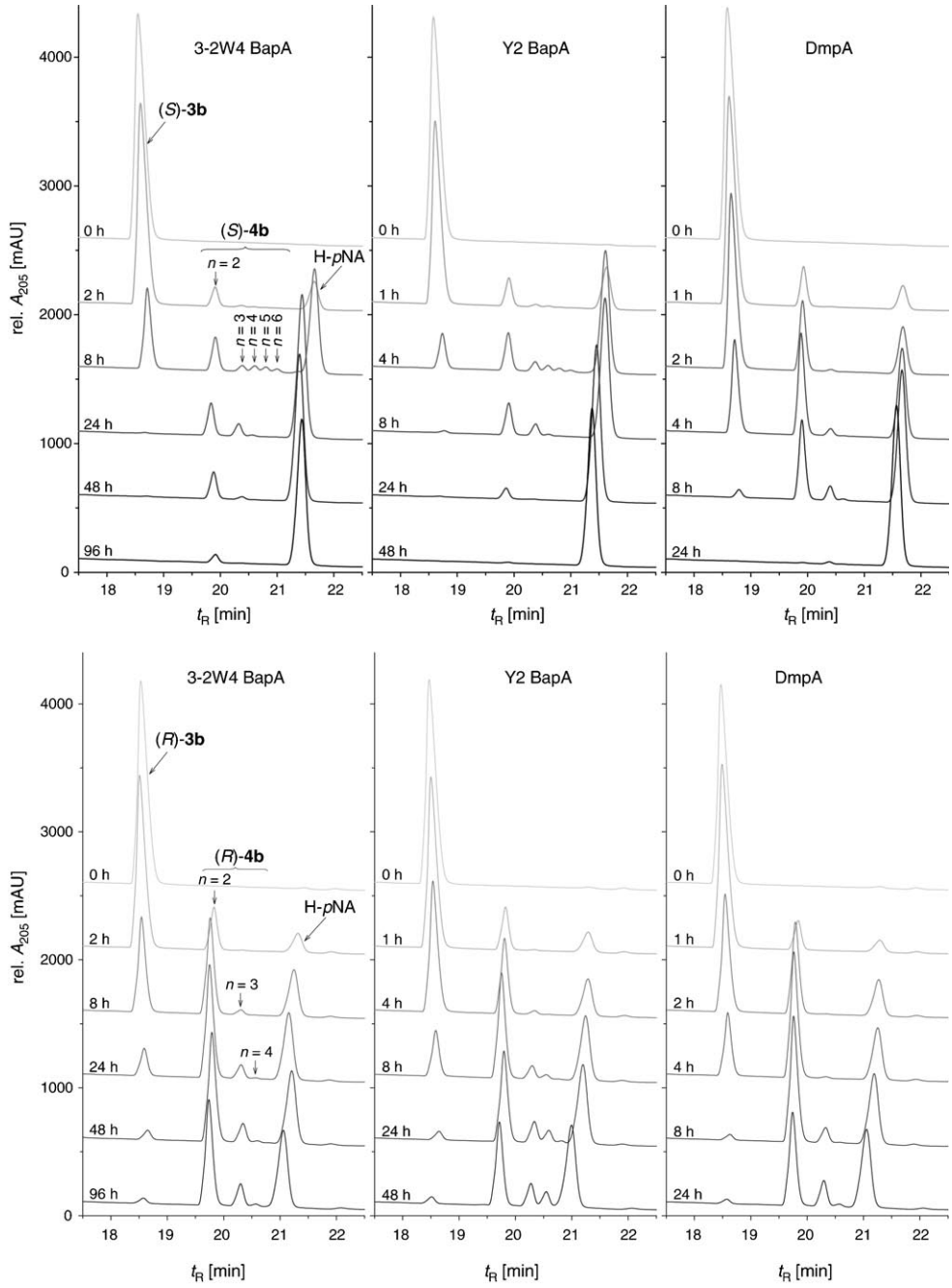


Fig. 1. *Oligomerization of the β³hAla-derived* p-*nitroanilides (*S*)-***3b*** (top) *and (*R*)-***3b*** (bottom) *in presence of the enzymes 3-2W4 BapA, Y2 BapA, and DmpA*. The figure shows the HPLC profiles of the assay mixtures after various incubation times. The oligomer-*p*NAs ***4*** ($n=2-6$) were identified by LC/MS analysis. $[M+H]^+$ for (*S*)-**4b**: 309.2 ($n=2$), 394.2 ($n=3$), 479.2 ($n=4$), 564.2 ($n=5$), 649.5 ($n=6$); $[M+H]^+$ for (*R*)-**4b**: 309.3 ($n=2$), 394.3 ($n=3$), 479.4 ($n=4$).

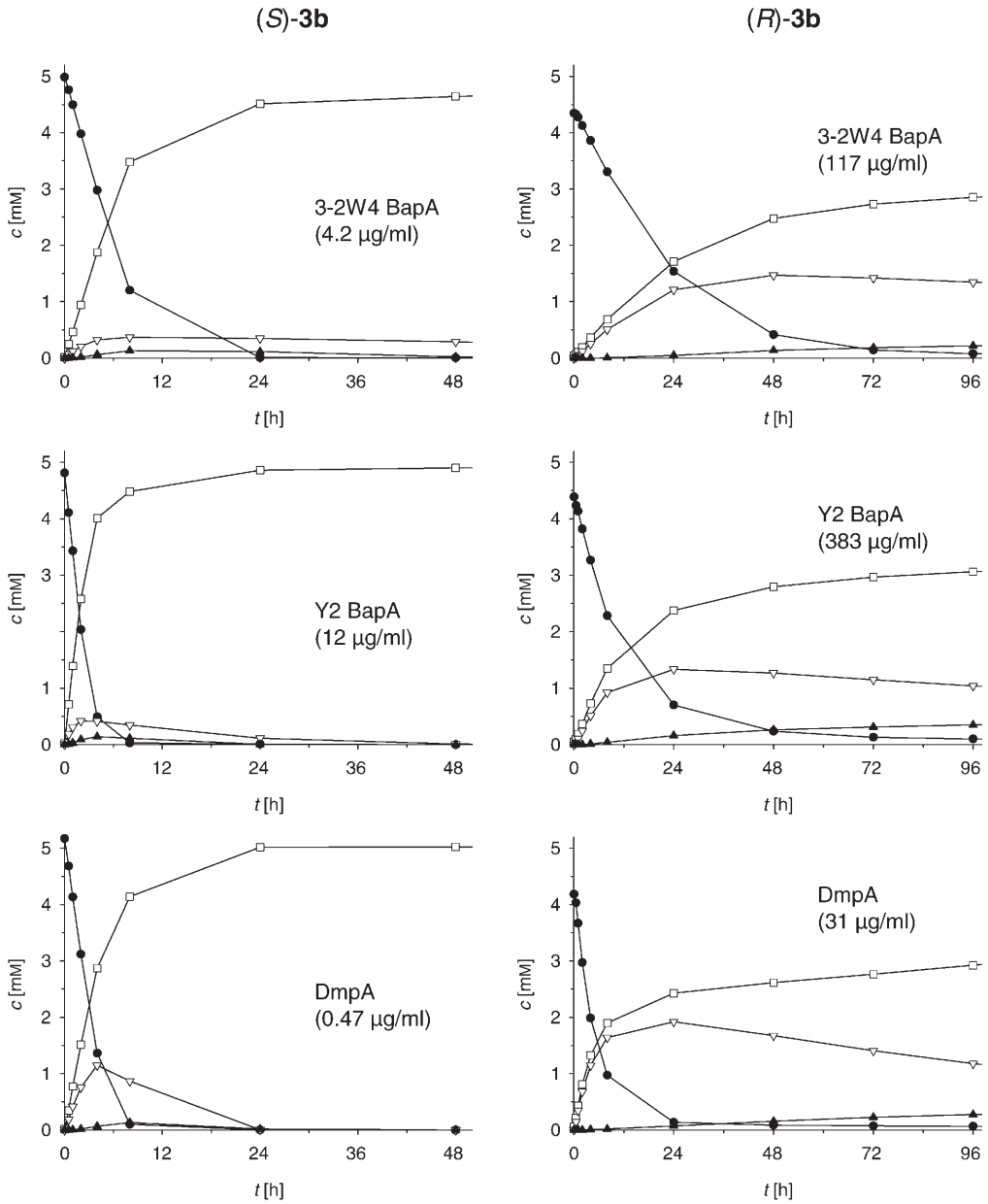
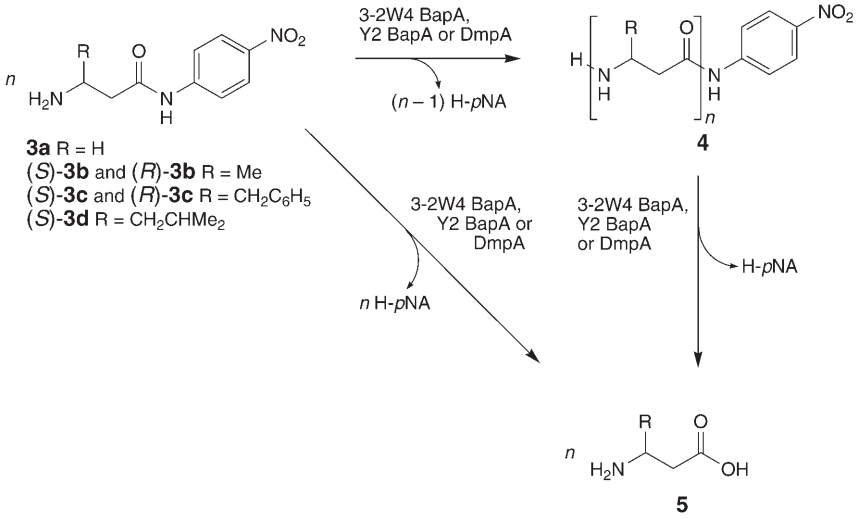


Fig. 2. *Oligomerization of (*S*)-***3b*** (left-hand side) and (*R*)-***3b*** (right-hand side) catalyzed by the enzymes 3-2W4 BapA, Y2 BapA, and DmpA.* Substrates **3** (●), dimer-pNAs **4**, $n=2$ (▽), and oligomer-*p*NAs **4**, $n>2$ (▲) were detected and quantified at 318 nm, H-*p*NA (□) at 383 nm. The assay mixtures contained different enzyme concentrations, which are given in the diagrams.

Scheme 2. *Formation of β-Amino Acid Oligomers* **4** *from β-Amino Acid* p-*Nitroanilides* **3** *in Presence of the Enzymes 3-2W4 BapA, Y2 BapA, and DmpA*. The amides **3** as well as the formed oligomer *p*-nitroanilides **4** undergo competing N-terminal cleavage reactions and are finally completely hydrolyzed by the enzymes to the free *β*-amino acids **5** and *p*-nitroaniline (H-*p*NA). Coupling of **3** with the N-terminus of the free amino acids **5** could lead to the formation of free *β*-oligopeptides H-[β^3hXaa]$_n$-OH; traces of such free oligopeptides were detected in the mass spectra of the samples derived from (*S*)-**3c** and (*R*)-**3c** (not described herein).



3a (up to eight *β*hGly-residues), followed by the β^3hAla-oligomers (*S*)-**4b** and (*R*)-**4b**, whereas only dimer-*p*NAs were generated from β^3hPhe- and β^3hLeu-building blocks (*Table 2*, and *Figs. 1* and *2*). This may be caused by the sterically less demanding side chains (H, Me) of the former two *β*-amino acid residues or by poor solubility of the oligomer-*p*NAs containing the hydrophobic *β*-amino acid residues β^3hPhe and β^3hLeu.

The specific dimerization activities of the enzymes as well as the yields obtained in the dimerization and oligomerization reactions are presented in *Table 2*. The specific activities vary over a range of five orders of magnitude (from less than 0.001 to 18.3 U/mg).

The enzyme DmpA acted much faster on the *β*-amino acid *p*-nitroanilides **3a**, and (*S*)-**3b** and (*R*)-**3b** than the other enzymes. On the other hand, 3-2W4 BapA and Y2 BapA converted substrates carrying the bulkier side chains of phenylalanine (*i.e.*, (*S*)-**3c** and (*R*)-**3c**) and leucine (*i.e.*, (*S*)-**3d**) faster than DmpA. These observations are in accordance with the expectations from our previous degradation experiments employing the same substrates in a different reaction system [4c].

Interestingly, both the (*S*)- and the (*R*)-forms of H-β^3hAla-*p*NA ((*S*)-**3b** and (*R*)-**3b**, resp.) and of H-β^3hPhe-*p*NA ((*S*)-**3c** and (*R*)-**3c**, resp.) were accepted as substrates by the enzymes. In the case of the β^3hAla derivatives, all three enzymes catalyzed the formation of dimer-*p*NAs of the (*R*)-form more slowly than those of the (*S*)-form; with the β^3hPhe derivatives, however, the (*R*)-form reacted faster than its enantiomer

Table 2. *Comparison of the Maximum Reached Concentrations and the Specific Activities of Dimer-*p*NA* (**4**, $n=2$) *Formation from the β-Amino Acid* p*-Nitroanilides* **3** *Catalyzed by the Enzymes 3-2W4 BapA, Y2 BapA, and DmpA*. The initially employed concentration of the substrates **3** was 5 mM. The dipeptide **6b** was generated from (*S*)-**3b** (5 mM) and β^3-homoleucine **5d** (25 mM), and carnosine (**7a**) from **3a** (5 mM) and histidine (25 mM) in the presence of the enzymes. One unit (U) is defined as the amount of enzyme that catalyzes the formation of 1 μmol of dimer-*p*NA (**4**, $n=2$), or of the dipeptides **6b** and **7a** per min.

Target peptide		3-2W4 BapA		Y2 BapA		DmpA	
		Max. conc. [mM]	Specific act. [U/mg protein]	Max. conc. [mM]	Specific act. [U/mg protein]	Max. conc. [mM]	Specific act. [U/mg protein]
4a	$n=2$	0.60	0.18	0.77	0.091	1.8	18.3
	$n=3-8$	0.51		0.50		0.35	
(*S*)-**4b**	$n=2$	0.37	0.54	0.42	0.52	1.2	13.5
	$n=3-6$	0.13		0.14		0.13	
(*R*)-**4b**[a])	$n=2$	1.5	0.008	1.3	0.005	1.9	0.18
	$n=3-4$	0.29		0.42		0.40	
(*S*)-**4c**[b])	$n=2$	0.08	0.092	0.17	0.006	0.14	<0.001
	$n>2$	n.d.[c])		n.d.		n.d.	
(*R*)-**4c**[b])	$n=2$	1.1	0.46	0.91	0.007	1.8	0.003
	$n>2$	n.d.		n.d.		n.d.	
(*S*)-**4d**[a])	$n=2$	0.28	1.0	0.15	0.015	0.15	0.002
	$n>2$	n.d.		n.d.		n.d.	
6b		1.6	1.3	2.4	1.0	0.41	1.4
7a		1.5	0.13	2.3	0.094	0.64	1.3

[a]) The assay mixture contained 10% DMSO. [b]) The assay mixture contained 30% DMSO. [c]) n.d.: Not detectable.

(*Table 2*). In degradation experiments employing H-β^3hPhe-*p*NA as the substrate, all enzymes hydrolyzed the (*S*)-form faster than the (*R*)-form [4c].

A comparison of the dimer-*p*NA yields reached with 3-2W4 BapA, Y2 BapA, and DmpA in the coupling of the six β-amino acid *p*-nitroanilides **3** to oligomer-*p*NAs **4** reveals the following facts *i*) the four substrates **3a**, (*S*)-**3b**, (*R*)-**3b**, and (*R*)-**3c** were converted in high yields to the corresponding dimer *p*-nitroanilides **4a**, (*S*)-**4b**, (*R*)-**4b**, and (*R*)-**4c**, $n=2$, respectively, to reach maximum concentrations between 0.37 and 1.9 mM, which correspond to yields of 15 to 76%; *ii*) in these four cases, the enzyme DmpA showed the best performance reaching maximum yields from 48 to 76%; *iii*) the maximum concentrations of the dimer *p*-nitroanilides (*S*)-**4c** and (*S*)-**4d**, $n=2$, generated from the phenylalanine- and leucine-derived precursors (*S*)-**3c** and (*S*)-**3d**, respectively, were very small with all three enzymes, *i.e.*, the rate of substrate cleavage exceeded the rate of coupling; *iv*) the enzymes converted the (*R*)-enantiomers of **3b** and **3c** to dimer-*p*NAs in 1.6- to 4-times, and 5.4- to 14-times higher yields, respectively, than the corresponding (*S*)-enantiomers.

Use of the Enzymes 3-2W4 BapA, Y2 BapA, and DmpA for Coupling of Two Different Amino Acids and for Attachment of β-Amino Acid Residues to an α-Tripeptide. To see whether we can selectively generate dipeptides containing two different β-amino acid residues, or a β- and an α-amino acid residue, we first tested two

combinations (*Scheme 3*). Thus, to the aqueous solution containing the β^3-homoalanine acid *p*-nitroanilide (*S*)-**3b** ('acyl donor') and the free amino acid β^3-homoleucine **5d** ('acyl acceptor'), with a molar ratio of 1:5, we added one of the enzymes 3-2W4 BapA, Y2 BapA, or DmpA (*Scheme 3*,*a*). The time–concentration curves resulting from the HPLC analyses of withdrawn samples are presented on the left-hand side of *Fig. 3*, clearly showing formation of the β-dipeptide **6b**.

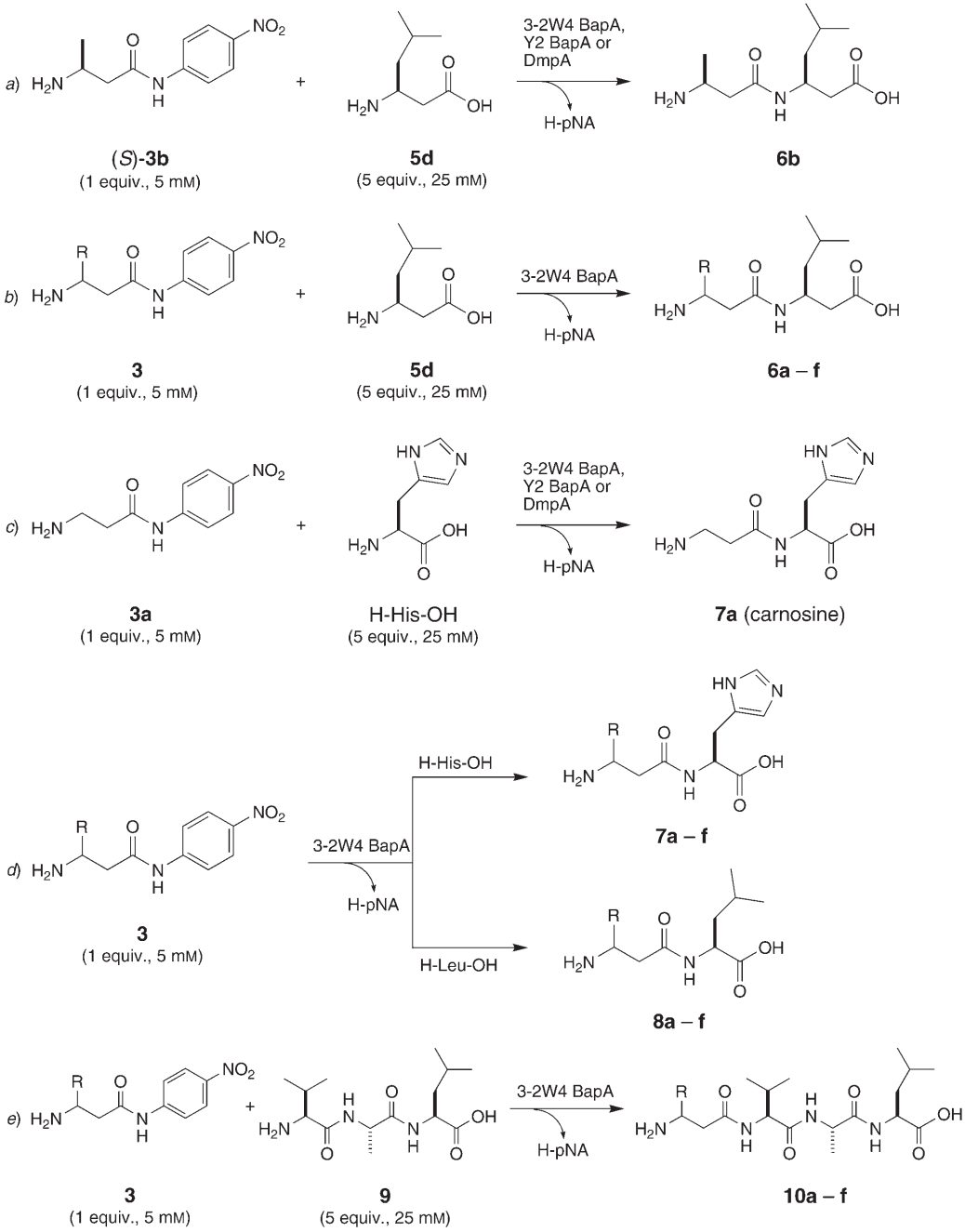
As a second target peptide, we chose the naturally occurring β/α-dipeptide carnosine, H-βhGly-His-OH (**7a**). Under the same conditions as applied for the formation of the dipeptide **6b**, carnosine (**7a**) was generated from the *p*-nitroanilide derivative of β-homoglycine (**3a**) and histidine (*Scheme 3*,*c* and *Fig. 3*, right-hand side). The specific enzyme activities as well as the maximum reached concentrations of the dipeptides **6b** and **7a** are included in *Table 2*. It is interesting to note that the enzyme Y2 BapA, the slowest of the three catalysts, performed best as far as the maximum concentrations of the dipeptides **6b** and **7a** (corresponding to product yields of 48 and 46%, resp.) are concerned, whereas DmpA was the poorest catalyst in this respect with maximum yields of 8 and 13%, respectively (*cf. Fig. 3*). Apart from the desired dipeptides **6b** and **7a**, we also observed the formation of the oligomer-*p*NAs (*S*)-**4b** and **4a**, respectively, as by-products. We made sure that spontaneous, nonenzymatic peptide coupling between the reactants did not occur under the conditions applied in the enzyme-catalyzed reactions.

Additionally, we selected the enzyme 3-2W4 BapA to study reactions of the six β-amino acid *p*-nitroanilides **3** with a five-fold molar excess of free β^3-homoleucine (**5d**; yielding compounds **6a**–**6f**), histidine (yielding **7a**–**7f**), leucine (yielding **8a**–**8f**), or the α-tripeptide H-Val-Ala-Leu-OH (**9**; yielding **10a**–**10f**). By subjecting withdrawn samples to LC/MS analysis, we could establish the formation of the β-dipeptides **6**, of the β/α-dipeptides **7** (apart from **7c**, the mother ion of which could not be detected) and **8**, as well as of the β/α-tetrapeptides **10** (*Table 3* and *Scheme 3*). The quantification of the peptide products **6a**, **6c**–**6f**, **7b**–**7f**, **8a**–**8f**, and **10a**–**10f** was not possible because authentic standards were not available.

These results clearly show that the β-peptidyl aminopeptidase 3-2W4 BapA can be used as a versatile catalyst, coupling a broad range of β-amino acids of (*S*)- or (*R*)-configuration to the *N*-terminus of a second β- or α-amino acid and even to an α-peptide.

Conclusions. – The β-peptidyl aminopeptidases BapA isolated from the *Gram*-negative microorganisms *Sphingosinicella xenopeptidilytica* 3-2W4 and *S. microcystinivorans* Y2, and the enzyme DmpA from *Ochrobactrum anthropi* LMG7991 are able to cleave β^3-homoamino acid moieties from the N-terminus of peptides, and were now tested for peptide coupling. All three enzymes were capable of converting *p*-nitroanilide derivatives of (*S*)- and (*R*)-β^3-homoamino acids to oligomer-*p*NAs of various chain lengths, with the dimers generally prevailing. In the case of the smallest substrate (*i.e.*, H-βhGly-*p*NA (**3a**)), oligomers containing up to eight β-amino acid residues were detected, whereas only dimers were formed from the sterically demanding substrates (*S*)-**3c**, (*R*)-**3c**, and (*S*)-**3d**, which carry phenylalanyl and leucyl side chains. The BapA enzymes and DmpA differed in their substrate specificities, and the specific dimerization activities of the enzymes varied over a range of five orders of

Scheme 3. *Formation of Dipeptides* **6**–**8** *and Tetrapeptides* **10** *Catalyzed by the Enzymes 3-2W4 BapA, Y2 BapA, and DmpA*. Due to competing hydrolytic cleavage, the peptides are eventually completely hydrolyzed by the enzymes. For details, see *Table 3*.



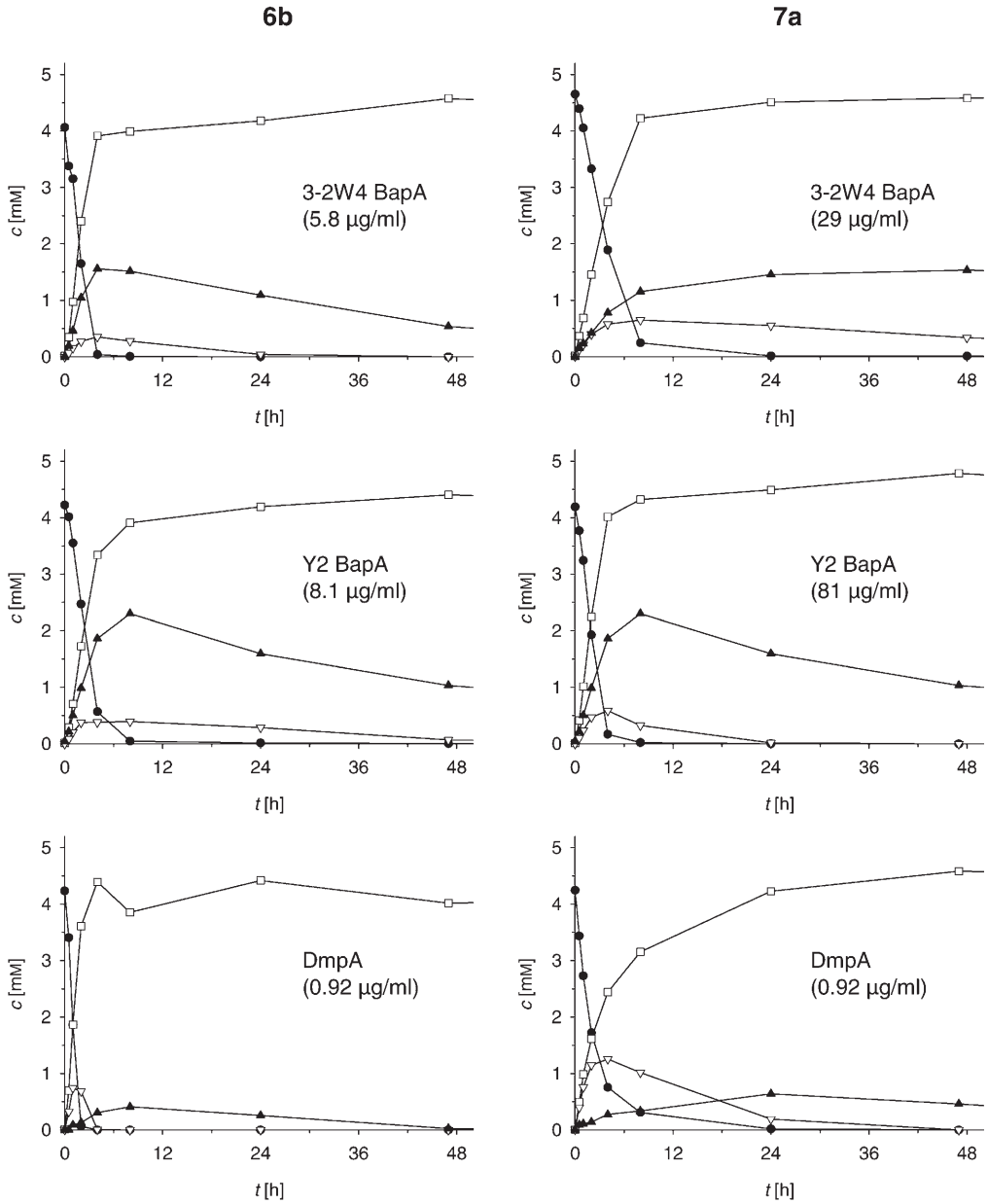


Fig. 3. *Formation of the β-dipeptides H-β³hAla-β³hLeu-OH* (**6b**; left-hand side) *and of carnosine* (**7a**; right-hand side) (▲) *catalyzed by the enzymes 3-2W4 BapA, Y2 BapA, and DmpA*. The β-amino acid *p*-nitroanilides **3a** and (*S*)-**3b** (●) as well as the oligomer-*p*NAs **4a** and (*S*)-**4b** (▽), which were formed as by-products, were detected and quantified at 318 nm, H-*p*NA (□) at 383 nm. The assay mixtures contained different enzyme concentrations, which are given in the diagrams.

Table 3. *List of Peptides Generated from the β-Amino Acid* p-*Nitroanilides* **3** (5 mM) *and Various 'Acyl Acceptor' Compounds* (25 mM) *by the Enzyme 3-2W4 BapA*. The formation of the target peptides **6**–**8** and **10** was established by LC/MS analysis; the detected parent ions are given.

'Acyl donor'		'Acyl acceptor'			
		H-β^3hLeu-OH (**5d**)	H-His-OH	H-Leu-OH	H-Val-Ala-Leu-OH (**9**)
3a	Product	H-βhGly-β^3hLeu-OH (**6a**)	H-βhGly-His-OH (**7a**)	H-βhGly-Leu-OH (**8a**)	H-βhGly-Val-Ala-Leu-OH (**10a**)
	$[M+H]^+$	217.0	227.1	203.2	373.1
(*S*)-**3b**	Product	H-β^3hAla-β^3hLeu-OH (**6b**)	H-β^3hAla-His-OH (**7b**)	H-β^3hAla-Leu-OH (**8b**)	H-β^3hAla-Val-Ala-Leu-OH (**10b**)
	$[M+H]^+$	231.3	241.0	217.2	387.3
(*R*)-**3b**[a])	Product	H-(*R*)-β^3hAla-β^3hLeu-OH (**6c**)	H-(*R*)-β^3hAla-His-OH (**7c**)	H-(*R*)-β^3hAla-Leu-OH (**8c**)	H-(*R*)-β^3hAla-Val-Ala-Leu-OH (**10c**)
	$[M+H]^+$	231.3	n.d.[b])	217.3	387.4
(*S*)-**3c**[c])	Product	H-β^3hPhe-β^3hLeu-OH (**6d**)	H-β^3hPhe-His-OH (**7d**)	H-β^3hPhe-Leu-OH (**8d**)	H-β^3hPhe-Val-Ala-Leu-OH (**10d**)
	$[M+H]^+$	307.2	317.1	293.1	463.3
(*R*)-**3c**[c])	Product	H-(*R*)-β^3hPhe-β^3hLeu-OH (**6e**)	H-(*R*)-β^3hPhe-His-OH (**7e**)	H-(*R*)-β^3hPhe-Leu-OH (**8e**)	H-(*R*)-β^3hPhe-Val-Ala-Leu-OH (**10e**)
	$[M+H]^+$	307.2	317.1	293.0	463.2
(*S*)-**3d**[a])	Product	H-β^3hLeu-β^3hLeu-OH (**6f**)	H-β^3hLeu-His-OH (**7f**)	H-β^3hLeu-Leu-OH (**8f**)	H-β^3hLeu-Val-Ala-Leu-OH (**10f**)
	$[M+H]^+$	273.3	283.1	259.2	429.4

[a]) The assay mixture contained 10% DMSO. [b]) n.d.: not detectable. [c]) The assay mixture contained 30% DMSO.

magnitude. In some cases, the maximum concentrations of dimer-*p*NA product corresponded to high yields of up to 76%. After long reaction times, the coupling products were subsequently cleaved, and the reaction mixtures consisted entirely of the free amino acids and *p*-nitroaniline, as expected.

By using a five-fold molar excess of one β-amino acid or a peptide over the *p*NA derivative of a β-amino acid, we observed the enzyme-catalyzed formation of mixed β-dipeptides, β/α-dipeptides, and β/α-tetrapeptides.

Finally, it is important to note that the enzymes described in the present publication are the first peptidases which were applied for coupling of various β-amino acids to peptides. The unique feature of catalyzing reactions with many N-terminal β-amino acid residues of peptides may suggest interesting practical applications for the aminopeptidases 3-2W4 BapA, Y2 BapA, and DmpA, which are not amenable to conventional enzymes. Especially attractive is the enzyme-catalyzed attachment of a β-amino acid to the N-terminus of a 'natural' α-peptide because N-terminal β-amino acid residues may be considered as protective groups against proteolytic enzymes *in vitro* and *in vivo* [2][3].

Experimental Part

General. We analyzed substrates and peptide products quantitatively by reversed-phase HPLC on a *Dionex* HPLC system equipped with a *P680* pump, an *ASI-100* automated sample injector, and a *UVD340U* photodiode array detector (*Dionex*, Sunnyvale, CA, USA). Separation of the compounds was achieved on a *Nucleosil 100-5 C_{18}* column (250 × 4 mm; *Macherey-Nagel*, D-Düren), which was equilibrated with 0.1% CF_3COOH (TFA) in H_2O. Oligomer-*p*NAs and peptides generated from the amino acid *p*-nitroanilides **3a**, (*S*)-**3b**, and (*R*)-**3b** were separated with a linear gradient from 0 to 30% MeCN. A gradient from 0 to 60% MeCN was applied for the separation of all compounds derived from (*S*)-**3c**, (*R*)-**3c**, and (*S*)-**3d**. We detected *p*NA derivatives of β-amino acids and oligomer-*p*NAs at a wavelength of 318 nm, free *p*-nitroaniline at 383 nm, and free peptides at 205 nm. Quantification of *p*NA derivatives, free *p*-nitroaniline, and of the dipeptides **6b** and **7a** was achieved on the basis of standard curves with H-βhGly-*p*NA (**3a**), *p*-nitroaniline, H-β^3hAla-β^3hLeu-OH (**6b**), and carnosine (**7a**), resp. We recorded mass spectra of the products with an *API 4000* liquid chromatography/tandem MS system connected to an *Agilent 1100 LC* system. *p*-Nitroaniline and carnosine (**7a**) were purchased from *Sigma-Aldrich* (CH-Buchs), and compound **3a** from *Bachem* (CH-Bubendorf). The preparation of the β-amino acid *p*-nitroanilides (*S*)-**3b**, (*S*)-**3c**, and (*R*)-**3c** [4c], and of the peptides **6b** and **9** [6a] had been described previously. For protein determination, we used *Bradford* reagent (5 ×) from *Bio-Rad* (CH-Reinach); absorbance measurements were performed at 595 nm with a *Specord S 100* spectrophotometer (*Analytik Jena*, D-Jena).

General Procedure for the Preparation of p-*Nitroanilides of β^3-Homoamino Acids* (*GP 1*). To a soln. of the Boc-protected amino acid (1 equiv.) and *p*-nitroaniline (1.5 equiv.) in pyridine (0.2M) at −20° (MeOH/ice), phosphoroxychloride (1.3 equiv.) was slowly added, and the suspension was stirred under rewarming to 20° for 6 h. The mixture was diluted with AcOEt (200 ml), ice was added, and the org. phase was extracted with 6N HCl (3 × 50 ml), H_2O (50 ml), and brine (50 ml), and dried (Na_2SO_4). The residue was suspended in CH_2Cl_2 (50 ml), TFA (5 ml) was added at 20°, and the soln. was stirred for 12 h. Then, the volatiles were removed in the vacuum. The residue was co-evaporated with toluene (3 ×) and with CH_2Cl_2 (3 ×). The remaining residue was precipitated from THF/pentane to give the desired β-amino acid *p*-nitroanilides as slightly yellow solids.

TFA·H-(R)*-β^3hAla-pNA* ((*R*)-**3b**). Reaction of Boc-(R)-β^3hAla-OH (1.01 g, 5.00 mmol), *p*-nitroaniline (1.04 g, 7.5 mmol), and phosphoroxy chloride (0.6 ml, 6.55 mmol) according to *GP 1* yielded 877 mg (52%) of (*R*)-**3b**. M.p. 150–154°. $[\alpha]_D^{20} = -22.9$ (*c* = 2.0, MeOH). ^{1}H-NMR (300 MHz, CD_3OD): 1.39 (*d*, *J* = 6.3, Me); 2.70–2.95 (*m*, CH_2); 3.69–3.73 (*m*, CH); 7.80 (*d*, *J* = 8.9, 2 arom. H); 8.16 (*d*, *J* = 8.9,

2 arom. H). ^{13}C-NMR (75 MHz, CD_3OD): 19.0; 41.1; 45.2; 120.7; 126.0; 144.9; 146.0; 171.1. MALDI-MS: 235 (27), 224 (100, M^+), 208 (30). HR-MS: 224.1029 ($[M+H]^+$, $C_{10}H_{13}N_3O_3^+$; calc. 224.1030).

*TFA·H-(*S*)-β³hLeu-pNA* ((*S*)-**3d**). Reaction of Boc-(S)-β³hLeu-OH (3.83 g, 15.6 mmol), *p*-nitroaniline (3.23 g, 23.4 mmol), and phosphoroxy chloride (1.86 ml, 6.55 mmol) according to *GP 1* yielded 2.61 g (44%) of (*S*)-**3d**. M.p. 177–178°. $[\alpha]_D^{20} = +22.6$ ($c = 2.0$, MeOH). ^{1}H-NMR (300 MHz, CD_3OD): 0.97 (*d*, *J* = 4.1, Me); 0.99 (*d*, *J* = 4.1, Me); 1.47–1.70 (*m*, CH_2); 1.70–1.89 (*m*, CH); 2.74 (*dd*, *J* = 16.6, 7.7, CH_2); 2.90 (*dd*, *J* = 16.6, 3.4, 1 H, CH_2); 3.61–3.80 (*m*, CH); 7.81 (*d*, *J* = 9.1, 2 arom. H); 8.16 (*d*, *J* = 9.1, 2 arom. H). ^{13}C-NMR (75 MHz, CD_3OD): 22.7; 22.9; 25.6; 38.3; 43.0; 120.6; 125.9; 144.8; 145.9; 171.2. MALDI-MS: 266 (100, M^+), 237 (27). HR-MS: 266.1501 ($[M+H]^+$, $C_{13}H_{19}N_3O_3^+$; calc. 266.1499).

Enzyme Preparation and General Procedure for the Enzyme-Catalyzed Formation of β-Amino Acid Oligomers and of Mixed β- and β/α-Peptides. The recombinant β-peptidyl aminopeptidases 3-2W4 BapA, Y2 BapA, and DmpA were purified from their *E. coli* hosts as described in [4b,c]. The lyophilized enzyme powders were dissolved in a universal buffer [15] at pH 10, and the protein content of the enzyme solns. was determined according to *Bradford* [16] on basis of a standard curve with bovine serum albumine (BSA). To study the oligomerization of β-amino acids, we used a reaction mixture containing 5 mM H-β³hXaa-*p*NA (**3**) in universal buffer (pH 10) at 37°. For the generation of mixed β- and β/α-peptides, a five-fold molar excess (25 mM) of β³-homoleucine (**5d**), histidine, leucine, or of the α-tripeptide H-Val-Ala-Leu-OH (**9**) was added to the oligomerization assay mixtures. The reaction was started by the addition of a limiting amount of one of the enzymes 3-2W4 BapA, Y2 BapA, or DmpA. Samples were withdrawn regularly and the enzymatic reaction was stopped by the addition of 0.25% (*v*/*v*) 1M HCl. The compounds present in the samples were analyzed by HPLC, and their masses were confirmed by LC/MS.

REFERENCES

[1] D. Seebach, A. K. Beck, D. J. Bierbaum, *Chem. Biodiv.* **2004**, *1*, 1111.

[2] a) D. Seebach, M. Overhand, F. N. M. Kühnle, B. Martinoni, L. Oberer, U. Hommel, H. Widmer, *Helv. Chim. Acta* **1996**, *79*, 913; b) D. Seebach, S. Abele, J. V. Schreiber, B. Martinoni, A. K. Nussbaum, H. Schild, H. Schulz, H. Hennecke, R. Woessner, F. Bitsch, *Chimia* **1998**, *52*, 734; c) J. Frackenpohl, P. I. Arvidsson, J. V. Schreiber, D. Seebach, *ChemBioChem* **2001**, *2*, 445; d) G. Lelais, D. Seebach, *Helv. Chim. Acta* **2003**, *86*, 4152; e) D. F. Hook, F. Gessier, C. Noti, P. Kast, D. Seebach, *ChemBioChem* **2004**, *5*, 691; f) D. F. Hook, P. Bindschädler, Y. R. Mahajan, R. Sebesta, P. Kast, D. Seebach, *Chem. Biodiv.* **2005**, *2*, 591.

[3] a) H. Wiegand, B. Wirz, A. Schweitzer, G. P. Camenisch, M. I. Rodriguez Perez, G. Gross, R. Woessner, R. Voges, P. I. Arvidsson, J. Frackenpohl, D. Seebach, *Biopharm. Drug Dispos.* **2002**, *23*, 251; b) R. Lind, D. Greenhow, S. Perry, T. Kimmerlin, D. Seebach, *Chem. Biodiv.* **2004**, *1*, 1391; c) H. Wiegand, B. Wirz, A. Schweitzer, G. Gross, M. I. Rodriguez Perez, H. Andres, T. Kimmerlin, M. Rueping, D. Seebach, *Chem. Biodiv.* **2004**, *1*, 1812; d) M. Weiss, B. Wirz, A. Schweitzer, R. Amstutz, M. I. Rodriguez Perez, H. Andres, Y. Metz, J. Gardiner, D. Seebach, *Chem. Biodiv.* **2007**, *4*, 1413.

[4] a) B. Geueke, K. Namoto, D. Seebach, H.-P. E. Kohler, *J. Bacteriol.* **2005**, *187*, 5910; b) B. Geueke, T. Heck, M. Limbach, V. Nesatyy, D. Seebach, H.-P. E. Kohler, *FEBS J.* **2006**, *273*, 5261; c) T. Heck, M. Limbach, B. Geueke, M. Zacharias, J. Gardiner, H.-P. E. Kohler, D. Seebach, *Chem. Biodiv.* **2006**, *3*, 1325.

[5] L. Fanuel, I. Thamm, V. Kostanjevecki, B. Samyn, B. Joris, C. Goffin, J. Brannigan, J. Van Beeumen, J. M. Frère, *Cell. Mol. Life Sci.* **1999**, *55*, 812; L. Fanuel, C. Goffin, A. Cheggour, B. Devreese, G. Van Driessche, B. Joris, J. Van Beeumen, J. M. Frère, *Biochem. J.* **1999**, *341*, 147.

[6] a) J. V. Schreiber, J. Frackenpohl, F. Moser, T. Fleischmann, H.-P. E. Kohler, D. Seebach, *ChemBioChem* **2002**, *3*, 424; b) B. Geueke, H. J. Busse, T. Fleischmann, P. Kämpfer, H.-P. E. Kohler, *Int. J. Syst. Evol. Microbiol.* **2007**, *57*, 107; c) H. D. Park, Y. Sasaki, T. Maruyama, E. Yanagisawa, A. Hiraishi, K. Kato, *Environ. Toxicol.* **2001**, *16*, 337; d) T. Maruyama, H. D. Park, K. Ozawa, Y. Tanaka, T. Sumino, K. Hamana, A. Hiraishi, K. Kato, *Int. J. Syst. Evol. Microbiol.* **2006**, *56*, 85; e) J. M. Humphrey, J. B. Aggen, A. R. Chamberlin, *J. Am. Chem. Soc.* **1996**, *118*, 11759; f) B. M. Gulledge, J. B. Aggen, A. R. Chamberlin, *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2903.

[7] B. Geueke, H.-P. E. Kohler, *Appl. Microbiol. Biotechnol.* **2007**, *74*, 1197.
[8] K. F. Vongerichten, J. R. Klein, H. Matern, R. Plapp, *Microbiology* **1994**, *140*, 2591.
[9] M. Teufel, V. Saudek, J.-P. Ledig, A. Bernhardt, S. Boularand, A. Carreau, N. J. Cairns, C. Carter, D. J. Cowley, D. Duverger, A. J. Ganzhorn, C. Guenet, B. Heintzelmann, V. Laucher, C. Sauvage, T. Smirnova, *J. Biol. Chem.* **2003**, *278*, 6521.
[10] K. Tabata, K.-I. Kasuya, H. Abe, K. Masuda, Y. Doi, *Appl. Environ. Microbiol.* **1999**, *65*, 4268; K. Tabata, M. Kajiyama, T. Hiraishi, H. Abe, I. Yamato, Y. Doi, *Biomacromolecules* **2001**, *2*, 1155; T. Hiraishi, M. Kajiyama, K. Tabata, H. Abe, I. Yamato, Y. Doi, *Biomacromolecules* **2003**, *4*, 1285; T. Hiraishi, M. Kajiyama, I. Yamato, Y. Doi, *Macromol. Biosci.* **2004**, *4*, 330.
[11] M. Bergmann, H. Fraenkel-Conrat, *J. Biol. Chem.* **1938**, *124*, 1; M. Bergmann, J. S. Fruton, *J. Biol. Chem.* **1938**, *124*, 321.
[12] F. Bordusa, *Chem. Rev.* **2002**, *102*, 4817; D. Kumar, T. C. Bhalla, *Appl. Microbiol. Biotechnol.* **2005**, *68*, 726; C. Lombard, J. Saulnier, J. M. Wallach, *Protein Pept. Lett.* **2005**, *12*, 621.
[13] K. Yokozeki, S. Hara, *J. Biotechnol.* **2005**, *115*, 211.
[14] X.-G. Li, L. T. Kanerva, *Org. Lett.* **2006**, *8*, 5593.
[15] T. Teorell, E. Stenhagen, *Biochem. Z.* **1938**, *299*, 416.
[16] M. M. Bradford, *Anal. Biochem.* **1976**, *72*, 248.

Received August 3, 2007