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

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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-pNA, H-βh3hAla-pNA, H-βh4hPhe-pNA, H-βh3hLeu-pNA, and H-βhLeu-pNA. 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-β3hAla-β3hLeu-OH and the βα-dipeptide H-βhGly-His-OH (carnosine) were formed with almost 50% conversion, when a five-fold excess of β3-homoleucine or histidine was incubated with H-β3hAla-pNA and H-βhGly-pNA, 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.


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4) Postdoctoral Fellow financed by the Swiss National Science Foundation (Project No. 3152A0-100770).
Recently, we showed that three structurally related aminopeptidases from *Proteobacteria* are able to cleave $\beta$-peptides [4]. The $\lambda$-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 $(S)$-$\alpha$-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 $\beta$-homoamino acids with high activities [4c]. Two further $\beta$-peptidyl aminopeptidases originate from the environmental isolates *Sphingosinibactera xenopeptidilytica* 3-2W4 and *S. microcystinivorans* Y2 [4a,b]. These bacterial strains were isolated because of their capability to metabolize the artificial $\beta$-tripeptide H-$\beta^3$Val-$\beta^3$Ala-$\beta^3$Leu-OH (1) and the highly toxic cyanobacterial metabolite microcystin (2), respectively [6]5).

One distinctive feature of the $\beta$-peptidyl aminopeptidases from both *Sphingosinibactera* strains (named 3-2W4 BapA and Y2 BapA, resp., according to the designation of their host strains) is that they exclusively cleave N-terminal $\beta$- but not $\alpha$-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 $\beta$-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, $(R)$-$(3\text{Me})$-Asp (as $\beta$-amino acid residue), $(R)$-Glu (as $\gamma$-amino acid residue), and an $\alpha$-Me-$\beta$-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 carnosinas 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(CO₂H)–CH₂–CO]₄–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 = pNA, 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, H₂O 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
Apart from one lipase-catalyzed reaction [14], the synthesis of $\beta$-peptides, however, has thus far exclusively been accomplished by nonenzymatic methods.

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

### Results and Discussion

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#### $\beta$-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 $\beta$-peptides, we employed the $\beta$-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 pNA derivatives of the corresponding $\beta$-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 $\beta$-hAla derivative (R)-3b and of the leucine-derived $\beta$-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-pNAs 4.

To investigate the enzyme-catalyzed oligomerization of $\beta$-amino acid residues, we incubated the corresponding $\beta$-amino acid derivatives 3 (5 mM) with the recombinant enzymes 3-2W4 BapA, Y2 BapA, and DmpA. In all experiments, the formation of oligomer-pNAs 4 was detected by LC/MS analyses. As typical examples, the HPLC diagrams and time–concentration curves for the $\beta$hAla 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-pNAs formed by DmpA were shorter than the corresponding ones generated by 3-2W4 BapA and Y2 BapA; iii) DmpA produced the highest dimer-pNA 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 $\beta$-amino acids 5, so that long reaction periods the only detectable pNA 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-pNAs 4 were formed from

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

<table>
<thead>
<tr>
<th>Enzyme name</th>
<th>Abbreviation</th>
<th>Origin</th>
<th>Ref.</th>
</tr>
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<tbody>
<tr>
<td>$\beta$-Peptidyl aminopeptidase</td>
<td>3-2W4 BapA</td>
<td>Sphingosinicella xenoptidilytica 3-2W4</td>
<td>[4]</td>
</tr>
<tr>
<td>$\beta$-Peptidyl aminopeptidase</td>
<td>Y2 BapA</td>
<td>Sphingosinicella microcystinivorans Y2</td>
<td>[4b,c]</td>
</tr>
<tr>
<td>L-Aminopeptidase d-Ala-esterase/amidase</td>
<td>DmpA</td>
<td>Ochrobactrum anthropi LMG7991</td>
<td>[4c][5]</td>
</tr>
</tbody>
</table>

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[6] The anilides 3 have an absorption maximum at 318 nm, p-nitroaniline itself at 383 nm.
Fig. 1. Oligomerization of the β′-Ala-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-pNAs 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-pNAS 4, \( n > 2 \) (▲) were detected and quantified at 318 nm, H-pNA (▼) at 383 nm. The assay mixtures contained different enzyme concentrations, which are given in the diagrams.
3a (up to eight βH Gly-residues), followed by the βH Ala-oligomers (S)-4b and (R)-4b, whereas only dimer-pNAs were generated from βH Phe- and βH Leu-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-pNAs containing the hydrophobic β-amino acid residues βH Phe and βH Leu.

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-βH Ala-pNA ((S)-3b and (R)-3b, resp.) and of H-βH Phe-pNA ((S)-3c and (R)-3c, resp.) were accepted as substrates by the enzymes. In the case of the βH Ala derivatives, all three enzymes catalyzed the formation of dimer-pNAs of the (R)-form more slowly than those of the (S)-form; with the βH Phe derivatives, however, the (R)-form reacted faster than its enantiomer.
In degradation experiments employing H-ββ3hPhe-pNA as the substrate, all enzymes hydrolyzed the (S)-form faster than the (R)-form [4c].

A comparison of the dimer-pNA yields reached with 3-2W4 BapA, Y2 BapA, and DmpA in the coupling of the six β-amino acid p-nitroanilides 3 to oligomer-pNAs 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-pNAs 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

### Table 2. Comparison of the Maximum Reached Concentrations and the Specific Activities of Dimer-pNA (4, n = 2) Formation from the β-Amino Acid p-Nitroanilides 3 Catalyzed by the Enzymes 3-2W4 BapA, Y2 BapA, and DmpA.

<table>
<thead>
<tr>
<th>Target peptide</th>
<th>3-2W4 BapA</th>
<th>Y2 BapA</th>
<th>DmpA</th>
</tr>
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<tbody>
<tr>
<td>4a</td>
<td>n = 2</td>
<td>0.60</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>n = 3–8</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td>(S)-4b</td>
<td>n = 2</td>
<td>0.37</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>n = 3–6</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>(R)-4b</td>
<td>n = 2</td>
<td>1.5</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td>n = 3–4</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>(S)-4c</td>
<td>n = 2</td>
<td>0.08</td>
<td>0.092</td>
</tr>
<tr>
<td></td>
<td>n &gt; 2</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>(R)-4c</td>
<td>n = 2</td>
<td>1.1</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>n &gt; 2</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>6b</td>
<td>n = 2</td>
<td>0.28</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>n &gt; 2</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>7a</td>
<td>1.6</td>
<td>1.3</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>0.13</td>
<td>2.3</td>
</tr>
</tbody>
</table>

a) The assay mixture contained 10% DMSO. b) The assay mixture contained 30% DMSO. c) n.d.: Not detectable.
combinations (Scheme 3). Thus, to the aqueous solution containing the β3-homoalanine acid p-nitroanilide (S)-3b (‘acyl donor’) and the free amino acid β3-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-pNAs (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 β3-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 anthrophi LMG7991 are able to cleave β3-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)-β3-homoamino acids to oligomer-pNAs of various chain lengths, with the dimers generally prevailing. In the case of the smallest substrate (i.e., H-βhGly-pNA (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 magnitude.
Scheme 3. Formation of Dipeptides 6–8 and Tetrarypeptides 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-β³-hAla-β³-hLeu-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-pNAs 4a and (S)-4b (○), which were formed as by-products, were detected and quantified at 318 nm, H-pNA (■) 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.

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</thead>
<tbody>
<tr>
<td>3a</td>
<td>Product</td>
<td>H-βhGly-βhLeu-OH (6a)</td>
<td>217.0</td>
<td>227.1</td>
<td>H-βhGly-Leu-OH (8a)</td>
</tr>
<tr>
<td>(S)-3b</td>
<td>Product</td>
<td>H-βhala-βhLeu-OH (6b)</td>
<td>231.3</td>
<td>241.0</td>
<td>H-βhala-Leu-OH (8b)</td>
</tr>
<tr>
<td>(R).-3b</td>
<td>Product</td>
<td>H-(R)-βhala-βhLeu-OH (6c)</td>
<td>231.3</td>
<td>n.d.</td>
<td>H-(R)-βhala-Leu-OH (8c)</td>
</tr>
<tr>
<td>(S)-3c</td>
<td>Product</td>
<td>H-βhPhe-βhLeu-OH (6d)</td>
<td>307.2</td>
<td>317.1</td>
<td>H-βhPhe-Leu-OH (8d)</td>
</tr>
<tr>
<td>(R)-3c</td>
<td>Product</td>
<td>H-(R)-βhPhe-βhLeu-OH (6e)</td>
<td>307.2</td>
<td>317.1</td>
<td>H-(R)-βhPhe-Leu-OH (8e)</td>
</tr>
<tr>
<td>(S)-3d</td>
<td>Product</td>
<td>H-βhLeu-βhLeu-OH (6f)</td>
<td>273.3</td>
<td>283.1</td>
<td>H-βhLeu-Leu-OH (8f)</td>
</tr>
</tbody>
</table>

†) The assay mixture contained 10% DMSO. ‡) n.d.: not detectable. ‡‡) The assay mixture contained 30% DMSO.
magnitude. In some cases, the maximum concentrations of dimer-pNA 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 \( \beta \)-amino acid or a peptide over the pNA derivative of a \( \beta \)-amino acid, we observed the enzyme-catalyzed formation of mixed \( \beta \)-dipeptides, \( \beta/\alpha \)-dipeptides, and \( \beta/\alpha \)-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 \( \beta \)-amino acids to peptides. The unique feature of catalyzing reactions with many N-terminal \( \beta \)-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 \( \beta \)-amino acid to the N-terminus of a ‘natural’ \( \alpha \)-peptide because N-terminal \( \beta \)-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 C18 column (250 \( \times \) 4 mm; Macherey-Nagel, D-Duren), which was equilibrated with 0.1% CF3COOH (TFA) in H2O. Oligomer-pNAs 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 pNA derivatives of \( \beta \)-amino acids and oligomer-pNAs at a wavelength of 318 nm, free p-nitroaniline at 383 nm, and free peptides at 205 nm. Quantification of pNA derivatives, free p-nitroaniline, and of the dipeptides 6b and 7a was achieved on the basis of standard curves with H2Hgly-pNA (3a), p-nitroaniline, H\( \beta/\alpha \)hAla-\( \beta/\alpha \)hLeu-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 \( \beta \)-amino acid p-nitroanilides (S)-3b, (S)-3c, and (R)-3e [4c], and of the peptides 6b and 9 [6a] had been described previously. For protein determination, we used Bradford reagent (5 \( \times \)) from Bio-Rad (CH-Reinach); absorbance measurements were performed at 595 nm with a Spectord S100 spectrophotometer (Analytik Jena, D-Jena).

General Procedure for the Preparation of p-Nitroanilides of \( \beta \)-Homoamino Acids (GP 1). To a soin.

The mixture was diluted with AcOEt (200 ml), ice was added, and the org. phase was extracted with 6n HCl (3 \( \times \) 50 ml), H2O (50 ml), and brine (50 ml), and dried (Na2SO4). The residue was suspended in CH2Cl2 (50 ml), TFA (5 ml) was added at 20°, and the soin. was stirred for 12 h. Then, the volatiles were removed in the vacuum. The residue was co-evaporated with toluene (3 \( \times \)) and with CH2Cl2 (3 \( \times \)). The remaining residue was precipitated from THF/pentane to give the desired \( \beta \)-amino acid p-nitroanilides as slightly yellow solids.

TFA H\( \beta/\alpha \)hAla-pNA (9.3b). Reaction of Boc-(R)-\( \beta/\alpha \)hAla-OH (1.01 g, 5.00 mmol), p-nitroaniline (1.04 g, 7.5 mmol), and phosphoroxyl chloride (0.6 ml, 6.55 mmol) according to [7] yielded 877 mg (52%) of (R)-3b. M.p. 150–154°. [\( \alpha \)]D20 = −22.9 (c = 2.0, MeOH). 1H-NMR (300 MHz, CD3OD): 1.39 (d, J = 6.3, Me); 2.70 – 2.95 (m, CH2); 3.69 – 3.73 (m, CH); 7.80 (d, J = 8.9, 2 arom. H); 8.16 (d, J = 8.9,
arom. H). $^{13}$C-NMR (75 MHz, CD$_3$OD): 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 + H]+), C$_{13}$H$_{19}$N$_3$O$_3$; calc. 266.1499.

Enzyme Preparation and General Procedure for the Enzyme-Catalyzed Formation of β-Amino Acid Oligomers and of Mixed β- and βαβ-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 albumin (BSA). To study the oligomerization of β-amino acids, we used a reaction mixture containing 5 mM H-bpβhLeu-0H (3) in universal buffer (pH 10) at 37°C. For the generation of mixed β- and βαβ-peptides, a five-fold molar excess (25 mM) of βαβ-homoleucine (5d), histidine, leucine, or of the α-tripeptide H-Val-Ala-Leu-0H (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% trifluoroacetic acid · H$_2$O (TFA·H2O) and 0.99 (0.1 M NaCl, 5 mM EDTA, pH 8). 1H-NMR (300 MHz, CD$_3$OD): 2.90 (1H, CH$_2$); 7.81 (2H, CH); 9.1, 2H, arom. H). $^{13}$C-NMR (75 MHz, CD$_3$OD): 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 + H]+), C$_{13}$H$_{19}$N$_3$O$_3$; calc. 266.1499.

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