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1 Crystal Structures of BapA Complexes with β -Lactam-Derived Inhibitors Illustrate

2 Substrate Specificity and Enantioselectivity of β -Aminopeptidases

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Abstract

β -Aminopeptidases have exclusive biocatalytic potential because they react with peptides comprised of β -amino acids that serve as building blocks for the design of non-natural peptidomimetics. In the present study, we identified the β -lactam antibiotic ampicillin and the ampicillin-derived penicilloic acid as novel inhibitors of the β -aminopeptidase BapA from *Sphingosinicella xenopeptidilytica* (K_i values of 0.69 and 0.74 mM, respectively). We report high-resolution crystal structures of BapA in non-covalent complexes with these inhibitors and with the serine-protease inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride. All three inhibitors showed similar binding characteristics: The aromatic moiety extended into a hydrophobic binding pocket of the active site and the free amino group formed a salt bridge with Glu133 of BapA. The exact position of the inhibitors and structural details of the ligand-binding pocket illustrate the specificity and the enantioselectivity of BapA-catalyzed reactions with β -peptide substrates.

1 Introduction

2
3 The incorporation of backbone-elongated β -amino acids with proteinogenic side chains^[1]
4 (**Figure 1**) gives peptides that may form unique secondary structures and that are resistant to
5 degradation by most proteolytic enzymes.^[2, 3] These properties give rise to interesting new
6 biomedical applications for β -peptides as bioactive α -peptide mimetics.^[4-6] To date, five
7 sequence-related enzymes have been biochemically and functionally characterized that
8 possess the unusual property of cleaving N-terminal β -amino-acid residues from linear β - and
9 mixed β,α -peptides.^[7-11] Due to their common catalytic activity these β -peptide-converting
10 enzymes are referred to as β -aminopeptidases.^[12] The growing demand for β -amino-acid-
11 based building blocks^[13] has led to the development of biocatalytic applications for β -
12 aminopeptidases, such as the preparation of enantiopure β -amino acids by kinetic resolution
13 and the synthesis of β - and mixed β,α -peptides from mildly activated β -amino acids.^[14-17]
14
15 Alignment searches revealed many sequences from bacterial and eukaryotic genomes that
16 have similarity with the known β -aminopeptidases.^[12] Within these sequences, the
17 catalytically relevant amino acids are highly conserved, indicating that the ability to process
18 β -peptides is not restricted to the few described β -aminopeptidases (Merz et al., manuscript
19 submitted). The large abundance of β -aminopeptidase-like sequences suggests that these
20 enzymes have important cellular functions that are as yet unknown. Although peptides solely
21 composed of linear β -amino-acid residues do not occur in nature, many β -amino acids are
22 constituents of a wide variety of different, often highly bioactive substances, such as
23 microcystins,^[18, 19] taxol,^[20] bestatin,^[21, 22] and L-carnosine.^[23, 24] β -Aminopeptidases may

participate in the breakdown of such β -amino-acid-containing compounds to make them available for further metabolic conversions.

((INSERT FIGURE 1 HERE))

Antibiotics of the penicillin family constitute one particular group among the plethora of natural compounds that contain β -amino-acid substructures. They are of special interest for β -peptide research, because their characteristic β -lactam rings are cyclic derivatives of α,β -diamino-acid moieties (**Figure 2**). However, note that the antibiotic ampicillin, in addition to the β -lactam-containing core structure 6-aminopenicillanic acid (6-APA), also consists of the α -amino acid phenylglycine and may hence be viewed as a dipeptide with an N-terminal α -amino acid. The degradation of β -lactam-derived compounds is mainly catalyzed by two different classes of enzymes, namely by β -lactamases (EC 3.5.2.6) and by penicillin acylases (EC 3.5.1.11). β -Lactamases hydrolyze the amide bond of the β -lactam ring to a linear α,β -diamino-acid derivative (penicilloic acid).^[25, 26] Penicilloic acids are usually antimicrobially inactive, but interestingly they act as competitive inhibitors of the β -lactamase-catalyzed reaction.^[27] Several hundred β -lactamase variants, which are grouped into four different classes, have been described in the last decades.^[28, 29] Their genes are easily transferred between bacteria by horizontal gene transfer, causing the dissemination of resistance.^[30] The second class of β -lactam-converting enzymes comprises penicillin acylases, which hydrolyze the amide bond that links the β -lactam core to a specific side chain.^[31] Penicillin acylases are commercially important for the industrial chemo-enzymatic production of the β -lactam precursor 6-APA and of semisynthetic β -lactam antibiotics.^[32]

((INSERT FIGURE 2 HERE))

1 Although penicillin acylases and β -aminopeptidases do not share high sequence similarity,
2 both enzyme classes possess structural and catalytic features of the N-terminal-hydrolase
3 (Ntn) superfamily.^[33] The Ntn hydrolases adopt a characteristic four-layered $\alpha\beta\alpha$ -sandwich
4 structure. One Ntn-hydrolase unit is formed by two polypeptide chains (α and β) that result
5 from posttranslational autoproteolytic cleavage of an inactive precursor polypeptide.^[34, 35]
6 The classical autoproteolytic mechanism of the Ntn-hydrolase precursor is promoted by a
7 conserved serine, cysteine or threonine residue, which thereupon becomes the catalytic N-
8 terminal nucleophile of the newly formed β -polypeptide chain. Crystal structures of the β -
9 aminopeptidases DmpA from *Ochrobactrum anthropi* LMG7991 (EC 3.4.13.20; PDB ID:
10 1b65),^[36] BapA from *Sphingosinicella xenopeptidilytica* 3-2W4 (EC 3.4.11.25; PDB ID:
11 3n2w), and of the unprocessed S250A BapA mutant (proBapA; PDB ID: 3n5i) showed that
12 these enzymes are distinct from most other Ntn hydrolases by the orientation and
13 connectivity of the secondary-structure elements. Furthermore, the precursor structure of
14 BapA and an analysis active-site mutants revealed that self-processing of BapA is unlikely to
15 follow the classical autoproteolytic mechanism of Ntn hydrolases, but instead involves
16 different nucleophiles for self-processing and catalysis (Merz et. al, manuscript submitted).
17 On the basis of the available crystal structures it has been suggested that the topological and
18 functional properties of Ntn hydrolases and β -aminopeptidases resulted from convergent
19 evolution of different evolutionary origins.^[37]
20
21 Based on point mutations at the BapA active site, we have proposed a mechanism of the
22 BapA-catalyzed reaction (Merz et al., manuscript submitted). Essential functions were
23 assigned to the N-terminal catalytic nucleophile Ser250 that is activated by the
24 Ser288/Glu290 dyad. However, the molecular determinants of the unusual β -peptide
25 specificity of β -aminopeptidases have not been investigated in detail, since all attempts to

1 obtain crystal structures of β -aminopeptidase-ligand complexes and to identify β -
2 aminopeptidase-specific inhibitors have as yet been unsuccessful. In the present study, we
3 report the inhibition of BapA by the β -lactam antibiotic ampicillin and by the ampicillin-
4 derived penicilloic acid. Furthermore, we present crystal structures of non-covalent BapA
5 complexes with these inhibitors and with the broad-spectrum serine-protease inhibitor 4-(2-
6 aminoethyl) benzenesulfonyl fluoride (AEBSF, trade name: pefabloc SC), providing
7 structural details of ligand binding and mechanistic details of β -peptide transformations
8 catalyzed by β -aminopeptidases.

Results and Discussion

Crystal structure of BapA in complex with AEBSF

Biochemical studies with the β -aminopeptidase BapA demonstrated the inhibition of the enzyme in the presence of high concentrations of the broad-spectrum serine-protease inhibitor AEBSF.^[38] We determined the crystal structure of a non-covalent BapA-AEBSF complex (BapA-AEBSF) at 1.8 Å resolution; crystallographic data are summarized in **Table 1**. AEBSF was found in each of the four active sites at the interface of three adjacent subunits of the BapA ($\alpha\beta$)₄-heterooctamer. In contrast to the irreversible inhibition mechanism of AEBSF in serine, threonine and cysteine proteases, in which a covalent bond between the catalytically active nucleophile and the sulfonyl group of the inhibitor is formed,^[39] the structure of the BapA-AEBSF complex shows non-covalent binding of the inhibitor. The fluoro-sulfonyl terminus of the inhibitor points away from the catalytic nucleophile Ser250-O γ into the hydrophobic binding pocket and the amino group of AEBSF forms a salt bridge to the carboxyl group of Glu133. Thr76, Thr100, Leu135, and Leu287 (e.g. subunit A) together with residues of the two neighboring $\alpha\beta$ -subunits (e.g. subunit C: Thr316; subunit D: Leu84, Val88, Gln90, Leu92, Phe124, Ser125, Leu127, Leu128, and Leu303) constitute a hydrophobic pocket that supports ligand binding by *van der Waals* interactions (**Figure 3**). The aromatic ring of Phe124, which showed very weak electron density in the native structure of BapA (Merz et al., manuscript submitted), is well defined through a π -stacking interaction with the aromatic ring of AEBSF.

((INSERT FIGURE 3 HERE))

Inhibition of BapA by penicillin-derived β -lactam antibiotics

To investigate β -aminopeptidase-catalyzed biotransformations of cyclic β -amino acids and β -lactams, we examined the potential of the β -aminopeptidases BapA and DmpA to process the penicillin derivatives ampicillin, penicillin G and carbenicillin. These compounds contain a β -lactam ring as part of their common 6-APA core structure (**Figure 2**) and differ by their D-phenylglycyl, phenylacetyl and D-phenylmalonyl acyl groups, respectively. With neither enzyme we observed deacylation or hydrolysis of the β -lactam amide bond, demonstrating that both BapA and DmpA lack penicillin-acylase and β -lactamase activity with the tested lactams.

Furthermore, we tested the inhibitory effect of the three β -lactam antibiotics and of the ampicillin-derived penicilloic acid Amp_{hyd} (**Figure 2**) on the BapA- and DmpA-catalyzed conversion of the chromogenic reporter substrate H- β^3 hAla-*p*NA. Despite the structural similarity of ampicillin, penicillin G and carbenicillin with respect to their 6-APA core structure, only ampicillin inhibited substrate conversion by BapA with a K_i value of 0.69 mM (**Table 2**). Ampicillin differs from penicillin G and carbenicillin by the presence of an α -amino group in its D-phenylglycyl moiety (**Figure 2**). With the penicilloic acid Amp_{hyd} a K_i value of 0.74 mM was observed; thus, the structural change associated with the conversion of ampicillin to Amp_{hyd}, *i.e.* hydrolytic opening of the β -lactam ring, did not affect the inhibitory effect on substrate conversion by BapA. In contrast to BapA, DmpA was neither significantly inhibited by any of the three β -lactam derivatives nor by the hydrolyzed species Amp_{hyd}.

Crystal structures of BapA in complex with ampicillin and Amp_{hyd}

Crystal structures of BapA in non-covalent complex with ampicillin (BapA-Amp) and with the (5*S*)-epimer of enzymatically produced Amp_{hyd} (BapA-Amp_{hyd}) at 1.7 Å and 1.85 Å resolution, respectively, were obtained by soaking BapA crystals with the respective compounds; crystallographic data are summarized in **Table 1**. Like in the BapA-AEBSF complex, one inhibitor molecule bound to each of the four active sites of the BapA ($\alpha\beta$)₄-heterooctamer (**Figure 4**). Both ampicillin and Amp_{hyd} interact with the BapA active site in a similar manner. The α -amino group of the D-phenylglycyl moiety of ampicillin forms a salt bridge with Glu133 and a hydrogen bond with a water molecule in the active site (**Figure 5A**). As observed for the aromatic ring of AEBSF in the BapA-AEBSF complex, the phenyl ring of ampicillin extends into the hydrophobic pocket of the BapA active site (*cf.* **Figure 3** and **Figure 5**). The backbone nitrogen of Leu135 and the carbonyl oxygen of Leu287 form hydrogen bonds with the amide that links the D-phenylglycyl acyl group of ampicillin to the 6-APA core. Furthermore, the carboxyl group attached to the thiazolidine ring forms a salt bridge with the side chain of Arg138. In the case of Amp_{hyd}, the main interactions between the D-phenylglycyl acyl group and the active site of BapA are similar to those observed for the binding of ampicillin (*cf.* **Figure 5A** and **5B**). Because of the missing β -lactam substructure, the core of Amp_{hyd} is more relaxed than that of ampicillin and the position of the thiazolidine is less rigid. Due to this structural difference, the interaction of the carboxyl group attached to the thiazolidine ring with Arg138 is not present in the BapA-Amp_{hyd} complex. Instead, the additional carboxyl group of Amp_{hyd} is in close proximity of Asn207-NH₂ and the amino terminus of Ser250, compensating for the missing salt bridge with Arg138.

((INSERT FIGURE 4 AND FIGURE 5 HERE))

1 Models of tetrahedral BapA-substrate complexes

2 Based on structural similarities of the N-terminal moiety of Amp_{hyd} to a linear α -peptide, we
3 modeled N-terminally unprotected L- and D- β^3 -homophenylalanine residues of peptidic BapA
4 substrates^[10, 14] onto the backbone of the ligand in the BapA-Amp_{hyd} complex. For model
5 building, we positioned the carbonyl group of the substrate into appropriate distance (1.6 Å)
6 and trajectory ($105 \pm 5^\circ$)^[40, 41] for nucleophilic attack by Ser250-O γ , and aimed for the best
7 possible match of the β^3 -homophenylalanine residues on the electron density of Amp_{hyd}.
8 Tetrahedral reaction intermediates were formed by the addition of Ser250-O γ to the carbonyl
9 carbon atom of the β^3 -homophenylalanine residues. Rotation around the C α -C β bond of the
10 β -amino-acid backbone gives rise to two synclinal conformers ((+)-sc and (-)-sc) and an
11 antiperiplanar conformer (ap) for a β -amino-acid residue.^[1] The models presented in **Figure**
12 **6** and **Figure S1** show that the (+)-sc conformer of L- β^3 -homophenylalanine and the (-)-sc
13 conformer of D- β^3 -homophenylalanine represent the only energetically favorable
14 conformations of the respective compounds that meet the steric demands for successful
15 formation of a tetrahedral enzyme-substrate intermediate. Complex formation with the other
16 conformers (*i.e.* (-)-sc and ap of L- β^3 -homophenylalanine, and (+)-sc and ap of D- β^3 -
17 homophenylalanine) can be excluded due to massive steric repulsions between the side chain
18 of the β -amino-acid residue and residues of the BapA active site (**Table 3** and **Figure S1**). In
19 the favored conformations, the hydrophobic side chains and the amino groups of the
20 covalently bound L- and D- β^3 -homophenylalanine residues match well with the position of
21 the phenylglycyl moiety of Amp_{hyd} in the BapA-Amp_{hyd} complex (**Figure 6**). The proximity
22 of Asn207-NH₂ and Leu135-NH to the oxyanion of the modeled tetrahedral intermediates
23 suggests that these residues stabilize the negatively charged tetrahedral intermediate during
24 the catalytic reaction (for mechanistic details see Merz et al., manuscript submitted).

1
2 ((INSERT FIGURE 6 HERE))
3

4 **Implications of ligand binding for β -peptide conversions by BapA and DmpA**

5 The structures of the BapA-inhibitor complexes show a good overlap of the amino group and
6 the aromatic ring of AEBSF with the positions of the phenylglycyl acyl groups of ampicillin
7 and Amp_{hyd} (**Figure 7**). In combination with previous information on the substrate specificity
8 of BapA for N-terminally unprotected β -peptides^[10, 38] and results obtained from an analysis
9 of BapA mutants (Merz et al., manuscript submitted), the crystal structures of the BapA-
10 ligand complexes (**Figure 3** and **Figure 5**) and the inhibition of BapA by ampicillin-derived
11 compounds (**Table 2**) provide further strong evidence that the presence of a free terminal
12 amino group plays a key role for ligand recognition and binding by BapA. Due to their amino
13 groups attached to aromatic rings through one or two CH₂-groups, the β -lactam-derived
14 ligands (ampicillin and Amp_{hyd}) and AEBSF have structural similarity to β^3 -
15 homophenylglycine and β^3 -homophenylalanine residues, respectively (*cf.* **Figure 2** and
16 **Figure 1**). Therefore, the formation of non-covalent complexes between BapA and these
17 ligands suggests that the binding of β -peptidic substrates is promoted by similar interactions
18 within the BapA active site, *i.e.* (i) salt-bridge formation between Glu133 and the amino
19 group of the substrate's N-terminal β -amino-acid residue, and (ii) hydrophobic interactions
20 between the ligand-binding pocket of BapA and the side chain of the substrate.

21
22 Based on the models of the tetrahedral BapA-substrate intermediates (**Figure 6**), we argue
23 that the exclusive specificity of the enzyme for peptides with backbone-elongated N-terminal
24 β -amino-acid residues is governed by the geometry of the active site, in particular by the
25 orientations and distances of the substrates' functional groups to Glu133-COO⁻ and to

Ser250-O γ . Our results show that compounds, such as ampicillin and Amp_{hyd}, mimicking the N-termini of conventional α -peptides bind to the active site of BapA, but are not converted. Hence, it seems that the lack of α -aminopeptidase activity of BapA is caused by the “rigidity” of the α -amino-acid backbone and by an insufficient distance between the substrate’s functional groups (NH₂ and CO). These limitations prevent appropriate positioning of the carbonyl group for nucleophilic attack under the restrictions given by the BapA active site. In contrast, the extended backbone of β -amino-acid residues confers a degree of rotational flexibility around the C α -C β bond, which is not present in α -amino-acid residues; due to the increase in backbone length and the additional degree of rotational freedom, a β -amino-acid residue is able to adopt a stable conformation allowing for nucleophilic attack by Ser250-O γ , while maintaining the salt bridge to Glu133 as well as the stabilization of the tetrahedral intermediate by the Leu135/Asn207 oxyanion hole.

In contrast to BapA, which exclusively cleaves peptides carrying N-terminal β -amino acids with aliphatic, aromatic or functionalized side chains, DmpA preferentially reacts with substrates carrying unsubstituted or sterically undemanding N-terminal β -amino acids (e.g. β -homoglycine and β^3 -homoalanine), but also α -amino acids.^[7, 10, 16, 38] The superposition of DmpA on the modeled BapA complexes with AEBSF and Amp_{hyd} perfectly illustrates this steric restriction of the DmpA active site (**Figure 7**). While BapA has a rather open ligand-binding pocket, the active site of DmpA is largely occluded by a loop ranging from Gln131 to Trp137; in particular, the side chain of Trp137 protrudes far towards the catalytic nucleophile Ser250 and could hence (i) force substrates with N-terminal α -amino acids into a position that allows for nucleophilic attack of Ser250-O γ , and (ii) restrict catalysis by DmpA to substrates with sterically undemanding side chains. In fact, the preference of DmpA to

process substrates with small N-terminal β -amino acids could be changed towards substrates with larger side chains by the single amino-acid mutation of Trp137 to alanine (Merz et al., manuscript submitted).

((INSERT FIGURE 7 HERE))

Enantioselectivity of the BapA-catalyzed reactions

The BapA-catalyzed kinetic resolution of racemic β^3 -amino-acid amides with different aliphatic side chains has recently been demonstrated to proceed with high L-enantioselectivity.^[17] As shown in the models presented in **Figure 6**, tetrahedral reaction intermediates could be formed between Ser250-O γ of BapA and β^3 -amino-acid residues of both L-configuration ((+)-sc conformer) and D-configuration ((-)-sc conformer). Our models indicate that the distance between Glu133-COO $^-$ and the substrate's amino terminus is larger for the complex with the (-)-sc conformation than for the one with the (+)-sc conformation of the substrate molecules, indicating a negative effect on the strength of the salt bridge for the former complex. Therefore, we propose that in the active site of BapA the (+)-sc conformation of an L- β^3 -amino-acid residue is energetically favored over the (-)-sc conformation of a D- β^3 -amino-acid residue. The enantiomeric excess of the L- β^3 -amino-acid product formed by the BapA-catalyzed kinetic resolution of racemic β^3 -amino-acid amides also depends on the size of the substrate's side chain; substrates with small aliphatic substituents are more efficiently resolved by BapA than substrates with sterically demanding side chains.^[17] We propose that the correlation between decrease of enantioselectivity and increase of side-chain size of the N-terminal β -amino-acid residue is due to additional non-polar interactions of the hydrophobic ligand-binding pocket with large side chains of the substrate. Such hydrophobic effects could improve the stability of complexes formed

1 between BapA and large D- β^3 -amino-acid residues of the (-)-sc conformation and partially
2 compensate for a weak salt bridge to Glu133.

3
4 Besides its high catalytic activity with substrates composed of L- β^3 -amino acids, BapA also
5 slowly processes a diastereomeric mixture of the β^2 -dipeptide H- β^2 hPhe- β^2 hAla-OH with
6 high D-enantioselectivity.^[16] In order to elucidate the structural details of this enantioselective
7 reaction, we modeled the reaction intermediates formed by the nucleophilic addition of BapA
8 Ser250-O γ to the carbonyl group of the (+)-sc conformers of D- and L- β^2 -homophenylalanine
9 residues (**Figure S2**). Due to the previously mentioned flexibility of the Phe124 side chain,
10 the tetrahedral reaction intermediate of D- β^2 -homophenylalanine could be placed in the active
11 site of BapA. In contrast, reasonable placing of the tetrahedral reaction intermediate of the
12 corresponding L-enantiomer was not possible because of a steric conflict between C γ of the
13 substrate's side chain and Thr76 of BapA. This fully explains the high enantioselectivity of
14 the BapA-catalyzed reaction towards these substrates.

15
16 β^2 -Dipeptides with small and therefore less demanding side chains at the N-terminal amino-
17 acid residue are not converted by BapA, irrespective of their configuration.^[16] As suggested
18 for the conversion of substrates carrying N-terminal D- β^3 -amino-acid residues with side
19 chains of different sizes, the preference of BapA for bulky β^2 -amino-acid residues likewise
20 can be explained by the stabilization of the enzyme-substrate complex by increased
21 hydrophobic interactions between large amino-acid side chains and the ligand-binding
22 pocket.

Physiological significance of ampicillin binding and β -peptide conversion by BapA and other β -aminopeptidases

The position and the orientation of ampicillin in the crystal structure of the BapA-Amp complex (**Figure 5a**) explain why BapA exhibited neither penicillin-acylase nor β -lactamase activity. Most importantly, the observed distance of Ser250-O γ to the carbonyl-carbon atom of the phenylglycyl moieties (2.6 Å) or to the one of the β -lactam ring (4.3 to 4.6 Å) was too large to allow a nucleophilic attack. Moreover, the observed angle between Ser250-O γ and the carbonyl plane of the phenylglycyl acyl group (84.9 to 88.5°) and the one between Ser250-O γ and the carbonyl plane of the β -lactam (81.4 to 83.5°) was not in accordance with the required trajectory of $105 \pm 5^\circ$, at which nucleophilic attack of a carbonyl carbon may occur.^[40, 41] Based on these structural and biochemical observations, we have rationalized why BapA is an ampicillin-binding, but not an ampicillin-converting enzyme. Although the present study outlines the structural determinants of the exclusive β -peptide specificity of BapA, the natural substrates of this enzyme and of other β -aminopeptidases as well as the physiological significance of ampicillin recognition and inhibition are as yet unknown and remain to be unraveled. One similar enzyme of unknown physiological function is the D-aminopeptidase (DAP) from *O. anthropi*, another well-studied example of an aminopeptidase with an unusual substrate specificity that is inhibited by ampicillin and other β -lactam-derived compounds.^[42] DAP exhibits sequence and structural similarities to β -lactamases and D,D-carboxypeptidases, and was classified as a new member of the penicillin-recognizing enzyme family.^[43] It was converted to an enzyme with D,D-carboxypeptidase activity and new penicillin-binding properties by directed mutagenesis.^[44] Although there are no obvious sequence similarities between BapA and any other β -lactam-converting or -binding enzymes, future investigations will need to address the catalytic potential of the many uncharacterized

- 1 proteins that were retrieved from sequencing projects and that are similar to the characterized
- 2 β -aminopeptidases (Merz et al., manuscript submitted) towards transformations of β -lactams.

Experimental Section

General remarks

AEBSF and the β -lactam antibiotics ampicillin, penicillin G, and carbenicillin were purchased from Sigma-Aldrich (Buchs, Switzerland). H- β^3 hAla-*p*NA was prepared chemically following a published procedure.^[10] The enzymatic conversions of the β -lactams were followed by HPLC analysis 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). Samples (10 μ l) were analyzed on a reversed-phase HPLC stationary phase Nucleodur C18-Pyramid (250 $\times$ 4 μ m, 5 μ m particle size; Macherey-Nagel, Düren, Germany) that was equilibrated with 0.1% formic acid in water for 3 min at a column temperature of 20°C. The compounds were separated with a linear gradient of 0 to 60% acetonitrile within 15 min at a constant flow rate of 1 ml/min and detected at a wavelength of 205 nm. Mass spectra of all compounds were recorded with an API 4000 liquid chromatography/tandem MS system connected to an Agilent 1100 LC system. For protein determination, we used five-fold concentrated Bradford reagent (Bio-Rad, Rheinach, Switzerland) and bovine serum albumin (BSA) as a standard; absorbance measurements were performed at 595 nm with a Specord S 100 spectrophotometer (Analytik Jena, Jena, Germany). Proteins were analyzed by SDS-PAGE using pre-cast 10% Novex tricine gels (Invitrogen AG, Basel, Switzerland) according to the manufacturer's instructions. The protein gels were stained with Coomassie Brilliant Blue and the purities of the enzymes were estimated by calculating the relative intensities of the protein bands with a GS-800 calibrated imaging densitometer and the software Quantity One (Bio-Rad, Rheinach, Switzerland).

Enzymatic production of Amp_{hyd}

Amp_{hyd} was produced from ampicillin by enzymatic conversion with the β -lactamase that was encoded on the pET3c plasmid (Novagen, Madison, WI, USA), constitutively expressed in *E. coli* BL21(DE3) pLysS and partially purified by anion-exchange and hydrophobic-interaction chromatography. Ampicillin (50 mg) was dissolved in H₂O (5.2 ml) and the enzymatic reaction was started by the addition of the β -lactamase-containing solution to yield a final protein concentration of 15 μ g/ml. After 16 h of incubation at 30°C, the enzymatic reaction was stopped by removing all proteins > 10 kDa by centrifugation with Centricon YM-10 centrifugal devices (10 kDa MWCO; Millipore Corp., Billerica, CA, USA). The formation of Amp_{hyd} was verified by HPLC-MS analysis (t_R = 10.3 min, $[M + H]^+$: 368.0 (calcd. M : 367.1), purity after peak integration > 90%). NMR analysis showed that the primary reaction product (5*R*)-Amp_{hyd} undergoes epimerization in aqueous solution at C-5 to form (5*S*)-Amp_{hyd} (see Supporting Information). The solution containing Amp_{hyd} was frozen to -80°C and freeze-dried.

Expression and purification of BapA for protein crystallization

The β -aminopeptidases BapA from *S. xenopeptidilytica* 3-2W4 was expressed without its N-terminal 29-amino-acid signal sequence in a pET3c-expression system, purified from the *E. coli* host^[38] and crystallized (Merz et al., manuscript submitted). BapA crystals were soaked for 5 min in a solution of 1.5 M ammonium sulfate, 100 mM HEPES (pH 7.5) containing 50 mM freshly dissolved AEBSF and for 30 min in 1.5 M ammonium sulfate, 100 mM HEPES (pH 7.5) containing saturating concentrations of ampicillin or Amp_{hyd}. After cryo-protection in a solution containing 1.5 M ammonium sulfate, 100 mM HEPES (pH 7.5) and 30% glycerol, the crystals were flash-frozen in liquid nitrogen.

1 **Structure determination**

2 Data were collected at the Swiss Light Source beamline PX on a 6M Pilatus detector in a
3 cryostream at 100 K at 1 Å wavelength. Diffraction data on a total of 720 frames were
4 recorded with an oscillation range of 0.5° per frame. The data was processed with the
5 program XDS.^[45] Initial phases were obtained by molecular replacement using the program
6 PHASER^[46] and the structure of BapA (PDB ID: 3n2w) as search model. The asymmetric
7 units each contained one molecule of the active BapA ($\alpha\beta$)₄-heterooctamer. The final model
8 of BapA-AEBSF contained residues 1-245 / 250-371 for chain A, 1-245 / 250-317 for chain
9 B, 1-245 / 250-371 for chain C, and 1-245 / 250-371 for chain D. The final model of BapA-
10 Amp contained residues 1-245 / 250-371 for chain A, 1-243 / 250-317 for chain B, 1-244 /
11 250-371 for chain C, and 1-245 / 250-371 for chain D. The final model of BapA-Amp_{hyd}
12 contained residues 1-245 / 250-371 for chain A, 1-242 / 250-317 for chain B, 1-244 / 250-371
13 for chain C, and 1-244 / 250-371 for chain D. The refinement of the structures was carried
14 out with the program PHENIX.^[47] Model building and superpositions for figures were
15 performed with the programs COOT^[48] and SSM,^[49] respectively. Figures were created with
16 the program Pymol.^[50]

17

18 **Expression of BapA and DmpA for enzymatic activity assays**

19 To exclude the possibility of β -lactamase cross-contamination from enzyme expression in a
20 β -lactamase-encoding pET3c expression system, the genes *bapA* from *S. xenopeptidilytica* 3-
21 2W4 and *dmpA* from *O. anthropi* LMG7991 were cloned into pET9c vectors yielding
22 pAR116 and pAR114, respectively.^[10, 16] The enzymes were recombinantly expressed in *E.*
23 *coli* BL21(DE3) pLysS and purified according to established procedures.^[10, 38] The
24 lyophilized enzyme powders were dissolved in a 10 mM potassium-phosphate buffer at pH
25 7.2, and the protein contents of the enzyme stocks were determined spectrophotometrically.

1 **Penicillin-acylase and β -lactamase activity assay**

2 The reaction mixtures contained 1 mM of the β -lactam antibiotics ampicillin, penicillin G or
3 carbenicillin in 100 mM potassium-phosphate buffer at pH 7.2 and 37°C. The enzymatic
4 reactions were initiated by the addition of one of the enzymes BapA or DmpA. Samples were
5 withdrawn at intervals over 24 h from the reaction mixtures and the enzymatic reactions were
6 terminated by heating at 90°C for 3 min. The samples were analyzed by reversed-phase
7 HPLC and HPLC-MS.

8

9 **Enzyme-inhibition assay**

10 The inhibitory effects of the β -lactam antibiotics ampicillin, penicillin G or carbenicillin and
11 of the penicilloic acid Amp_{hyd} on the β -aminopeptidase-catalyzed conversion of the reporter
12 substrate H- β^3 hAla-*p*NA were determined spectrophotometrically (Merz et al., manuscript
13 submitted). The assay mixtures contained the reporter substrate and the inhibitors in various
14 molar ratios. Experimental data was fitted to the competitive inhibition model (Eq. 1) by non-
15 linear regression analysis with the VisualEnzymics software (Softzymics, Princeton, NJ,
16 USA) for the program IGOR Pro (WaveMetrics, Oswego, OR, USA).

17

18
$$v = \frac{V_{\max} \cdot [S]}{K_m \cdot (1 + \frac{[I]}{K_i}) + [S]} \quad (\text{Eq. 1})$$

1 PDB accession numbers

2

3 BapA in complex with AEBSF (BapA-AEBSF, PDB ID: 3n33); BapA in complex with

4 ampicillin (BapA-Amp, PDB ID: 3ndv); BapA in complex with Amp_{hyd} (BapA-Amp_{hyd}, PDB

5 ID: 3nfb).

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7 program. H.P.K. and B.G. conceived the work. T.H. and B.G. carried out protein expression,
8 purification and crystallization experiments. T.H. and A.R. carried out kinetic analyses. T.M.
9 carried out crystallographic data collection, structure determination, refinement and
10 modeling. M.G.G. assisted the structure determination. D.R. carried out the NMR analysis of
11 the inhibitors. T.H., T.M. and C.B. wrote the paper and prepared the figures. D.S. contributed
12 to the β -peptide substrates. D.S., M.G.G., B.G. and H.P.K. contributed to discussion, data
13 interpretation, and manuscript preparation. M.G.G., B.G. and H.P.K. supervised the work.
14 We declare no competing financial interests.

1 Figures

2

3

4 **Figure 1: Similarities of L- α -amino acids and β -amino acids of corresponding**

5 **configurations.** According to the nomenclature proposed by Seebach and coworkers β -amino

6 acids with proteinogenic side chains (R) are referred to as β -homoamino acids (β hXaa

7 residues).^[1] As with α -amino acids (Xaa residues), the notations H- β^3 hXaa-OH and H-

8 β^2 hXaa-OH implicitly refer to the configurations of the stereocenters as shown in the

9 formulae, *i.e.* L in the *Fischer* projection (strictly applied, the rules for this projection would

10 lead to D-configuration only in the case of β^2 hAla). The CIP nomenclature (*R/S*-

11 configuration) is not useful for amino acids in a biological context because it reverses,

12 depending on the structures of the side chains. Thus, according to the CIP nomenclature all

13 Xaa residues have (*S*)-configuration, except for Cys and Sec; β^3 hXaa residues have (*S*)-

14 configuration, except for Val, Ile, Ser, Thr, Cys, Sec, Met and Asp; β^2 hXaa residues have

15 (*R*)-configuration, except for Ser and Thr.

Figure 2: Chemical formulae of AEBSF, and β -lactam-derived compounds. Hydrolysis of the β -lactam amide bond of ampicillin by a β -lactamase leads to formation of the linear penicilloic acid (5*R*)-Amp_{hyd}, which subsequently undergoes epimerization at C-5 of the thiazolidine ring to form (5*S*)-Amp_{hyd}, the prevailing epimer in aqueous solution (as shown by NMR, see Supporting Information). 6-APA is the common reaction product formed by the penicillin-acylase-catalyzed hydrolysis of penicillin-derived β -lactam antibiotics. The β -amino-acid moieties of the β -lactam-derived compounds are highlighted in red.

Figure 3: Stereo representation of the BapA active site in complex with the serine-protease inhibitor AEBSF. The essential catalytic residues of BapA (subunit A) and selected residues contributing to the hydrophobic ligand-binding pocket are shown as sticks in yellow and grey, respectively; the subunit localizations of the residues are indicated with capital letters. AEBSF is depicted as sticks in light blue. The catalytic residues and AEBSF are contoured with a 2 F_oc electron-density map at 1.5 σ . Interactions between the amino group of AEBSF and the active site of BapA are indicated by dotted lines with distances given in Å.

Figure 4: Quaternary structure of catalytically active BapA in complex with ampicillin (BapA-Amp). The four subunits of the BapA ($\alpha\beta$)₄-heterooctamer, each comprising a large α - and a small β -polypeptide chain, are depicted in different colors. One ampicillin molecule and the catalytic residues of subunit A (grey) are shown as sticks in cyan and yellow, respectively.

Figure 5: Stereo representations of the BapA active site in complex with the β -lactam inhibitor ampicillin (A) and the penicilloic acid Amp_{hyd} (B). The catalytic residues of BapA (subunit A) and selected residues of the ligand-binding pockets are shown as sticks in yellow and grey, respectively; the subunit localizations of the residues are indicated with capital letters. Ampicillin and Amp_{hyd} are depicted as sticks in light blue and dark blue, respectively, and contoured with a 2 Fofc electron-density map at 1.5 σ . Interactions between the bound compounds and active-site residues of BapA are indicated by dotted lines with distances given in Å.

Figure 6: Stereo representation of models of tetrahedral BapA-substrate intermediates. Tetrahedral reaction intermediates between Ser250-O γ and N-terminal β^3 -homophenylalanine residues of L-configuration (preferred (+)-sc conformer) and D-configuration (preferred (-)-sc conformer) are shown in orange and magenta, respectively. The models are based on the BapA-Amp_{hyd} crystal structure (grey); the ligand Amp_{hyd} is indicated as blue lines contoured with a 2 Fofc electron-density map at 1.5 σ . Interactions between active-site residues of BapA (yellow) and Amp_{hyd} are indicated by dotted lines. Models of all three energetically favorable conformers of the bound residues ((+)-sc, (-)-sc and ap) are depicted in **Figure S1**.

Figure 7: Stereo representation of the BapA-Amp_{hyd} active site (grey) superposed on the crystal structure of DmpA (PDB ID: 1b65; dark red). The catalytic residues Glu133, Ser250, Ser288 and Glu290 of BapA (yellow sticks) correspond to Glu144, Ser250, Ser288 and Asp290 of DmpA (red sticks). The side chain of Trp137 of DmpA clashes with the BapA ligands Amp_{hyd} (dark blue) and AEBSF (light blue).

1 Tables

2

3 **Table 1: Data collection and refinement statistics (molecular replacement).**

	BapA-AEBSF	BapA-Amp	BapA-Amp _{hyd}
Data collection			
Space group	P21	P21	P21
Cell dimensions			
a, b, c (Å)	87.4, 96.8, 101.3	86.9, 96.4, 101.5	88.3, 97.1, 102.2
α, β, γ (°)	90, 108.4, 90	90, 108.4, 90	90, 108.7, 90
Resolution (Å) ^[a]	48.0 – 1.8 (1.9 – 1.8)	48.8 – 1.7 (1.8 – 1.7)	49.3 – 1.85 (1.9 – 1.85)
R_{sym} or R_{merge}	10.0 (49.7)	11.6 (58.1)	8.6 (45.5)
$I / \sigma I$	12.9 (3.1)	9.2 (2.2)	13.0 (4.0)
Completeness (%)	97.6 (94.7)	98.7 (94.4)	98.4 (99.2)
Redundancy	3.78 (3.70)	3.32 (2.73)	3.78 (3.77)
Refinement			
Resolution (Å)	1.8	1.7	1.85
No. reflections	144691	172065	137077
$R_{\text{work}} / R_{\text{free}}$	16.0 / 19.0	16.7 / 19.2	15.0 / 17.8
No. atoms			
Protein	10708	10668	10680
Ligand/ion	238	168	194
Water	1265	1138	1229
B -factors			
Protein	19.61	18.81	19.58
Ligand/ion	27.85	25.61	26.17
Water	29.48	26.53	29.50
R.m.s. deviations			
Bond lengths (Å)	0.007	0.008	0.007
Bond angles (°)	1.05	1.104	1.05

4 ^[a] Values in parenthesis correspond to the highest-resolution outer shell. One crystal was
5 used for each dataset.

Table 2: Inhibition of BapA by the β -lactam derivatives ampicillin and Amp_{hyd}. The kinetic parameters K_m , V_{max} and K_i of the reactions were obtained after fitting initial velocities of the β -aminopeptidase-catalyzed conversion of the chromogenic substrate H- β^3 hAla-*p*NA (pH 7.2, 37°C)^[10] to a competitive inhibition model (Eq. 1, Experimental Section). Penicillin G and carbenicillin did not inhibit substrate conversion by BapA.

Inhibitor	K_i [mM]	K_m [mM]	V_{max} [$\mu\text{mol}\cdot\text{min}^{-1}\text{mg}^{-1}$]
Without	-	1.1 ± 0.15	16.4 ± 0.86
Ampicillin	0.69 ± 0.05	1.1 ± 0.09	16.3 ± 0.50
Amp _{hyd}	0.74 ± 0.11	0.98 ± 0.16	16.8 ± 1.0

Table 3: Survey of the conformers of BapA-substrate intermediates. The tetrahedral reaction intermediates are formed through nucleophilic attack of Ser250-O γ of BapA to β^3 -homophenylalanine residues of L- or D-configuration (see also **Figure 6** and **Figure S1**).

Conformation ^[a]		Color code in Figure S1	Clashes between C γ / C δ atoms of the bound substrate and BapA residues	Electrostatic interactions with Glu133
L-β^3-Homophenylalanine residue				
(+)-sc		Orange	C γ : - C δ : -	+
(-)-sc		Magenta	C γ : - C δ : Thr76, Gly289	-
ap		Green	C γ : Leu135 C δ : Leu135, Thr100, Glu133	-
D-β^3-Homophenylalanine residue				
(+)-sc		Orange	C γ : Glu133 C δ : Glu133, Thr134, Leu135	-
(-)-sc		Magenta	C γ : - C δ : -	+
ap		Green	C γ : Thr76, Gly289 C δ : Thr76, Gly289	-

^[a] (+)-sc = (+)-synclinal, (-)-sc = (-)-sc synclinal, ap = antiperiplanar conformer of the bound β^3 -homophenylalanine residue. Dotted lines represent non-covalent interactions. X indicates

- 1 steric repulsions between the side chain of the substrate and residues of the BapA active site.
- 2 R_y represents the C-terminal part of the substrate prior to formation of the acyl enzyme (see
- 3 Merz et al., manuscript submitted). Bn = benzyl. C_γ and C_δ refer to carbon atoms of the β³-
- 4 amino-acid side chain.

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2

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11

Supporting Information

Figure S1: Models of tetrahedral reaction intermediates formed between BapA Ser250-O γ and the three energetically favorable conformations of L- β^3 -homophenylalanine (A) and D- β^3 -homophenylalanine residues (B). The models are based on the crystal structure of BapA-Amp_{hyd} (grey). The catalytic residues of BapA (subunit A) are highlighted in yellow; the subunit localizations of the labeled residues are indicated with capital letters. The (+)-sc, (-)-sc) and ap conformers of the ligands are shown in orange, magenta and green, respectively; the conformer that best matches the active site of BapA is highlighted as sticks. Predicted electrostatic interactions with Glu133 and hydrogen bonds of the oxyanion hole are indicated by dotted lines. For a schematic overview of the depicted conformers we refer to **Table 3** in the main text.

Figure S2: Models of tetrahedral reaction intermediates formed between BapA Ser250-O γ and β^2 -homophenylalanine residues. The models are based on the crystal structure of BapA-Amp_{hyd} (grey). The catalytic residues of BapA (subunit A) are shown in yellow; the subunit localizations of the labeled residues are indicated with capital letters. The (+)-sc conformers of the bound D- and L- β^2 -homophenylalanine residues are shown as sticks in magenta and orange, respectively. The predicted electrostatic interactions with Glu133 and hydrogen bonds of the oxyanion hole are indicated by straight, steric repulsions by bent dotted lines.

1 NMR analysis of Amp_{hyd}

2 The ¹H, ¹³C and ¹⁵N NMR spectra used for stereochemical assignments were recorded at
3 400.13, 100.61 and 40.56 MHz on a Bruker Avance-400 NMR spectrometer (Bruker Biospin
4 AG, Fällanden, Switzerland). The ¹H, the 1D NOESY spectra and the 1D ¹³C spectra, and the
5 ¹H ¹³C HSQC, ¹H ¹³C HMBC, ¹H ¹H DQF-COSY and ¹H ¹⁵N HMQC (recorded with ¹⁵N at
6 natural abundance) 2D correlation NMR experiments were performed at 298 K on a 5 mm
7 broadband inverse probe with z-gradient (100% gradient strength of 53.5 Gcm⁻¹) and 90°
8 pulse lengths of 6.8 (¹H), 14.5 (¹³C) and 21.3 μs (¹⁵N). All NMR experiments were performed
9 in D₂O solutions using the Bruker standard parameter sets and pulse programs selecting
10 coupling constants of 145 Hz (HSQC), 10 Hz (HMBC), 3 Hz (HMQC) and mixing times of 1
11 s (1D NOESY, applying 50 ms selective 180° inversion pulses). The ¹H and ¹³C chemical
12 shifts were referenced relative to the signals of 3-trimethylsilyl tetradeutero sodium
13 propionate (TSP) dissolved in D₂O at 0.0 and -1.6 ppm, respectively. The ¹⁵N chemical shifts
14 were referenced to a capillary containing neat nitromethane at 0.0 ppm. The relative amounts
15 of products were determined by integration of ¹H NMR signals of the H-9 / H-10 methyl
16 groups.

17

18 The ¹H and ¹³C chemical shifts of the starting material ampicillin were readily assigned over
19 the 2D correlated NMR experiments (**Table S1** and **S2**), and the relative configuration of H-9
20 and H-10 determined by NOE enhancements found between the pairs of protons H-3 / H-10
21 and H-5 / H-9, whereas no close contacts were found for H-5 / H-10 (**Table S3**). The ¹H
22 chemical shifts evaluated for H-9 and H-10 completely agree with an earlier study.^[1] The ¹⁵N
23 chemical shift of N-4 at -213 ppm is typical for the β-lactam nitrogen (**Table S1**). For the
24 structurally closely related penicillin G a value of -212 ppm was observed.^[2] After some days
25 in solution, additional ¹H NMR resonances belonging to diastereotopic methyl groups were

1 observed. These signals were assigned to a primary ((5*R*)-Amp_{hyd}, amount in the 1% region)
2 and a secondary hydration product ((5*S*)-Amp_{hyd}, traces). The resonances of these two
3 products were observed as the main signals in the ¹H NMR spectrum, when ampicillin was
4 converted enzymatically by a β-lactamase (see Experimental Procedures). Two additional
5 sets of ¹H and ¹³C NMR resonances similar to the data found for ampicillin were found for
6 the hydration products (5*R*)- and (5*S*)-Amp_{hyd} (**Table S1**). Whereas all ¹³C chemical shifts of
7 the individual carbons and also the ¹⁵N NMR data of N-11 and N-18 in these molecules
8 remained very similar to the starting material ampicillin (**Table S1**), a low frequency shift of
9 approximately 100 ppm was observed for N-4 of the hydration products compared with
10 ampicillin, apparently induced by the ring opening reaction at of the β-lactam moiety. The
11 relative configuration of the primary hydration product (5*R*)-Amp_{hyd} was established by NOE
12 enhancements found between the pairs of protons H-3 / H-10 and H-5 / H-9, whereas no
13 close contacts were found for H-5 / H-10 (the same NOE's have previously been observed for
14 ampicillin; **Table S3**). The NOE contact observed between H-3 and H-6 implies a rotation
15 around the C-5 / C-6 bond of the compound (**Figure S3**). For the second hydration product
16 (5*S*)-Amp_{hyd} an NOE enhancement was observed between H-3 and H-5 (and also to H-10),
17 indicating an epimerization at C-3 or C-5. In fact, this kind of reaction is already mentioned
18 in the literature;^[3, 4] the postulated reaction mechanism is shown in **Scheme S1**. The ratio of
19 (5*R*)-Amp_{hyd} / (5*S*)-Amp_{hyd} shifted from 36 / 64 % (initial) to 15 / 85 % over 9 days. It has to
20 be mentioned that further, not identified by-products were formed during this time period
21 (amounts in the low % range).

1 **Table S1: ^1H , ^{13}C and ^{15}N chemical shifts of ampicillin and its derivatives (5*R*)-Amp_{hyd}**
2 **and (5*S*)-Amp_{hyd}.**

Position ^[a]	δ (^1H) / ppm			δ (^{13}C or ^{15}N) / ppm		
	Ampicillin	(5 <i>R</i>)- Amp _{hyd}	(5 <i>S</i>)- Amp _{hyd}	Ampicillin	(5 <i>R</i>)- Amp _{hyd}	(5 <i>S</i>)- Amp _{hyd}
2				65.3	59.7	59.6
3	4.19	3.09	3.32	74.3	76.4	76.7
5	5.48	5.07	5.04	67.7	66.7	67.6
6	5.47	4.29	4.89	59.0	60.9	56.4
7				175.6	176.8	175.6
8				175.6	176.4	175.5
9	1.44	1.14	0.52	27.5	27.2	28.2
10	1.52	1.21	1.48	31.3	27.0	29.0
12				176.4	169.9	170.9
13	4.68	5.21	5.28	59.2	58.1	58.3
14				140.1	133.5	133.5
15	7.43	7.58	7.58	128.1	129.2	129.1
16	7.45	7.55	7.55	130.2	130.8	131.3
17	7.40	7.52	7.52	129.6	131.5	131.5
4				-213.2	-322.6	-310.6
11				-269.8	-258.4	-264.0
18				-349.8	n.d. ^[b]	-339.4

^[a] For numbering of carbon atoms see **Figure S3**.

^[b] No correlation observed (signal too weak or ^1H , ^{15}N coupling constant too small for efficient polarization transfer).

3

1 **Table S2: ^1H ^{13}C HMBC and ^1H ^{15}N HMQC correlations used for NMR shift**
 2 **assignment in Table S1.**

Compound	Observed HMBC and HMQC correlations (w: weak)
Ampicillin	H-3 $\rightarrow$ C-(2, 5, 8, 9w, 10); H-5 $\rightarrow$ C-(6, 7); H-6 $\rightarrow$ C-(5, 7, 12w); H-9 $\rightarrow$ C-(2, 3, 9); H-10 $\rightarrow$ C-(2, 3, 10); H-13 $\rightarrow$ C-(12, 14, 15); H-15 $\rightarrow$ C-(13, 14, 15, 17); H-16 $\rightarrow$ C-(14, 16); H-17 $\rightarrow$ C-(15) H-(3, 5, 6) $\rightarrow$ N-(4); H-13 $\rightarrow$ N-(11, 18)
(5 <i>R</i>)-Amp _{hyd}	H-3 $\rightarrow$ C-(2, 8, 9, 10w); H-5 $\rightarrow$ C-(2w, 3, 7); H-6 $\rightarrow$ C-(5, 7, 12w); H-9 $\rightarrow$ C-(2, 3, 10); H-10 $\rightarrow$ C-(2, 3, 9); H-13 $\rightarrow$ C-(12, 14, 15); H-16 $\rightarrow$ C-(14, 16); H-17 $\rightarrow$ C-(15) H-3 $\rightarrow$ N-(4); H-5 $\rightarrow$ N-(4); H-6 $\rightarrow$ N-(4, 11)
(5 <i>S</i>)-Amp _{hyd}	H-3 $\rightarrow$ C-(2, 8, 9, 10w); H-5 $\rightarrow$ C-(7w); H-6 $\rightarrow$ C-(5, 7, 12w); H-9 $\rightarrow$ C-(2, 3, 10); H-10 $\rightarrow$ C-(2, 3, 9); H-13 $\rightarrow$ C-(12, 14, 15); H-15 $\rightarrow$ C-(13, 15, 17); H-16 $\rightarrow$ C-(14, 16); H-17 $\rightarrow$ C-(15) H-3 $\rightarrow$ N-(4); H-(5, 6) $\rightarrow$ N-(4, 11); H-13 $\rightarrow$ N-(18)

3

4

5 **Table S3: ^1H 1D NOESY signals used for assignments of stereochemistry.**

Compound	Observed 1D-NOESY correlations (w: < 1% NOE observed)
Ampicillin	H-3 $\rightarrow$ H-(10); H-9 $\rightarrow$ H-(3, 5); H-10 $\rightarrow$ H-(3, 9)
(5 <i>R</i>)-Amp _{hyd}	H-3 $\rightarrow$ H-(6, 10, 15); H-5 $\rightarrow$ H-(3w, 6, 9w); H-6 $\rightarrow$ H-(3, 5); H-9 $\rightarrow$ H-(3, 5, 10); H-10 $\rightarrow$ H-(3, 9); H-13 $\rightarrow$ H-(15)
(5 <i>S</i>)-Amp _{hyd}	H-3 $\rightarrow$ H-(5, 10); H-5 $\rightarrow$ H-(3, 6); H-6 $\rightarrow$ H-(5, 13w); H-9 $\rightarrow$ H-(3, 10, 15); H-10 $\rightarrow$ H-(3, 9); H-13 $\rightarrow$ H-(6w, 15)

Ampicillin

(5*R*)-Amp_{hyd}

(5*S*)-Amp_{hyd}

Figure S3: Numbering of carbon atoms used for the NMR signal assignment of ampicillin and its derivatives (5*R*)-Amp_{hyd} and (5*S*)-Amp_{hyd} (as employed by Kiener and Waley, 1978)^[5].

(5*R*)-Amp_{hyd}

(5*R*)-Amp_{hyd} + (5*S*)-Amp_{hyd}

Scheme S1: Proposed reaction mechanism of the epimerization at C-5 of Amp_{hyd}

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1 **Keywords**

2

3 AEBSF, ampicillin, β -peptides, Ntn hydrolases, pefabloc SC

1 Table of Contents

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5 **The β -aminopeptidase BapA** is an unusual member of the Ntn hydrolases that converts β -
6 peptide substrates and is inhibited by the β -lactam antibiotic ampicillin and the ampicillin-
7 derived penicilloic acid. We solved three crystal structures of BapA-inhibitor complexes that
8 allowed modeling of bound β -peptide substrates in the active site of the enzyme. The models
9 show that the conformational flexibility of the β -peptide backbone is essential for substrate
10 conversion by BapA.

Justification for Publishing in *ChemBioChem*

β -Amino acids are key building blocks of many bioactive compounds, peptidomimetics and β -peptides, which resist breakdown by most common proteases and peptidases. In previous investigations, we described three unusual enzymes, namely the β -aminopeptidases 3-2W4 BapA, Y2 BapA and DmpA, which catalyze the hydrolysis of N-terminal β -amino-acid residues from peptides (*Chem. Biodiversity* **2006**, *3*, 1325-1348), the coupling of β -amino acids to peptides (*Chem. Biodiversity* **2007**, *4*, 2016-2030; *Adv. Synth. Catal.* **2010**, *352*, 407-415; *ChemBioChem* **2010**, *11*, 1129-1136), and the highly enantioselective kinetic resolution of racemic β -amino-acid amides (*ChemBioChem* **2009**, *10*, 1558-1561).

The biotechnological potential of the described β -aminopeptidases encouraged us to conduct structural work on the enzyme BapA from *Sphingosinicella xenopeptidilytica* 3-2W4. In the course of these investigations, we solved crystal structures of BapA and an unprocessed BapA precursor and proposed catalytic mechanisms for the autoproteolytic maturation of the enzyme, an unusual member of the Ntn-hydrolase enzyme family, and of β -peptide hydrolysis (Merz et. al., manuscript submitted). In the present study, we identified ampicillin and the ampicillin-derived penicilloic acid as novel inhibitors of the BapA-catalyzed reaction. We present further high-resolution crystal structures of non-covalent BapA complexes with these molecules and the serine-protease inhibitor AEBSF. The positions and interactions of the bound inhibitors within the BapA active site reveal interesting details that explain the enantioselectivity and the specificity of the enzyme for β -peptide substrates. Our results are a very promising starting point to engineer the available β -aminopeptidases towards biocatalytic applications, which is of broad interest for scientists from different fields of research, including biotechnology, biochemistry and organic chemistry. Therefore, we

- 1 believe that our present study fits excellently within the scope of *ChemBioChem* and we
- 2 consider this journal as the appropriate platform to share our results with other researchers.