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Crystal Structures of BapA Complexes with β-Lactam-Derived Inhibitors Illustrate Substrate Specificity and Enantioselectivity of β-Aminopeptidases

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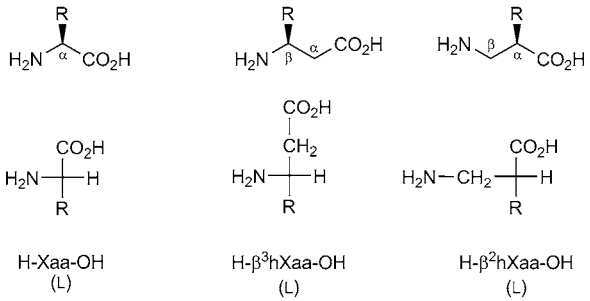
β-Aminopeptidases have exclusive biocatalytic potential because they react with peptides composed of β-amino acids, which serve as building blocks for the design of non-natural peptidomimetics. We have identified the β-lactam antibiotic ampicillin and the ampicillin-derived penicilloic acid as novel 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.

Introduction

The incorporation of backbone-elongated β-amino acids with proteinogenic side chains[1] (Scheme 1) yields peptides that might form unique secondary structures and are resistant to degradation by most proteolytic enzymes.[2,3] These properties give rise to interesting new biomedical applications for β-peptides as bioactive α-peptide mimetics.[4–6] To date, five sequence-related enzymes have been biochemically and functionally characterized; they possess the unusual property of cleaving N-terminal β-amino-acid residues from linear β- and mixed β,α-peptides.[7–11] Due to their common catalytic activity, these β-peptide-converting enzymes are referred to as β-aminopeptidases.[12] The growing demand for β-amino-acid-based building blocks[13] has led to the development of biocatalytic applications for β-aminopeptidases, such as the preparation of enantiopure β-amino acids by kinetic resolution and the preparation of β- and mixed β,α-peptides from mildly activated β-amino acids.[14–17]

Alignment searches revealed many sequences from bacterial and eukaryotic genomes that have similarity with the known β-aminopeptidases.[12] Within these sequences, the catalytically relevant amino acids are highly conserved, indicating that the



Scheme 1. Similarities of L-α-amino acids and β-amino acids of corresponding configurations. According to the nomenclature proposed by Seebach and co-workers, β-amino acids with proteinogenic side chains (R) are referred to as β-homoamino acids (βhXaa residues).[1] As with α-amino acids (Xaa residues), the notations H-β3hXaa-OH and H-β2hXaa-OH implicitly refer to the configurations of the stereocenters as shown in the formulae, that is, L in the Fischer projection (strictly applied, the rules for this projection would lead to D-configuration only in the case of β2hAla). The CIP nomenclature (*R*/*S* configuration) is not useful for amino acids in a biological context because it reverses, depending on the structures of the side chains. Thus, according to the CIP nomenclature, all Xaa residues have *S* configuration, except for Cys and Sec; β3hXaa residues have *S* configuration, except for Val, Ile, Ser, Thr, Cys, Sec, Met, and Asp; β2hXaa residues have *R* configuration, except for Ser and Thr.

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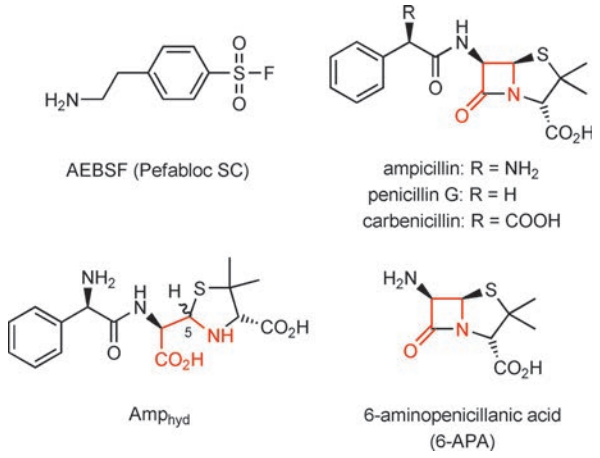
ability to process β-peptides is not restricted to the few described β-aminopeptidases.[18] The large abundance of β-aminopeptidase-like sequences suggests that these enzymes have important cellular functions that are as yet unknown. Although peptides composed solely of linear β-amino-acid residues do not occur in nature, many β-amino acids are constituents of a wide variety of different, often highly bioactive substances, such as microcystins,[19,20] taxol,[21] bestatin,[22,23] and L-carnosine.[24,25] β-Aminopeptidases might participate in the breakdown of such β-amino-acid-containing compounds to make them available for further metabolic conversions.

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 (Scheme 2).



Scheme 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 C5 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 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.

However, note that the antibiotic ampicillin, in addition to the β-lactam-containing core structure 6-aminopenicillanic acid (6-APA), also contains 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 β-lactamases (EC 3.5.2.6) and 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).[26,27] Penicilloic acids are usually antimicrobially inactive, but interestingly, they act as competitive inhibitors of β-lactamase-catalyzed reactions.[28] Several hundred β-lactamase variants, which are grouped into four different classes, have been described in the last decades.[29,30] Their genes are easily transferred between bacteria by horizontal gene transfer, causing dissemination of resistance.[31] 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.[32] Penicillin acylases are commercially important for industrial chemo-enzymatic production of the β-lactam precursor 6-APA and semisynthetic β-lactam antibiotics.[33]

Although penicillin acylases and β-aminopeptidases do not share high sequence similarity, both enzyme classes possess structural and catalytic features of the N-terminal-hydrolase (Ntn) superfamily.[34] The Ntn hydrolases adopt a characteristic four-layered αββα-sandwich structure. One Ntn hydrolase unit is formed by two polypeptide chains (α and β) that result from post-translational autoproteolytic cleavage of an inactive precursor polypeptide.[35,36] The classical autoproteolytic mechanism of the Ntn hydrolase precursor is promoted by a conserved serine, cysteine, or threonine residue, which thereupon becomes the catalytic N-terminal nucleophile of the newly formed β-polypeptide chain. Crystal structures of the β-aminopeptidases DmpA from *Ochrobactrum anthropi* LMG7991 (EC 3.4.13.20; PDB ID: 1B65),[37] BapA from *Sphingosinicella xenopeptidilytica* 3-2W4 (EC 3.4.11.25; PDB ID: 3N2W), and the unprocessed S250A BapA mutant (proBapA; PDB ID: 3N5I) showed that these enzymes are distinct from most other Ntn hydrolases by the orientation and connectivity of the secondary-structure elements. Furthermore, the precursor structure of BapA and an analysis of active-site mutants revealed that self-processing of BapA is unlikely to follow the classical autoproteolytic mechanism of Ntn hydrolases but instead involves different nucleophiles for self-processing and catalysis.[18] On the basis of the available crystal structures, it has been suggested that the topological and functional properties of Ntn hydrolases and β-aminopeptidases resulted from convergent evolution of different evolutionary origins.[38]

Based on point mutations at the BapA active site, we have proposed a mechanism of the BapA-catalyzed reaction.[18] Essential functions were assigned to the N-terminal catalytic nucleophile Ser250, which is activated by the Ser288/Glu290 dyad. However, the molecular determinants of the unusual β-peptide specificity of β-aminopeptidases have not been investigated in detail, as all attempts to obtain crystal structures of β-aminopeptidase–ligand complexes and to identify β-aminopeptidase-specific inhibitors have as yet been unsuccessful. In the present study, we report the inhibition of BapA by the β-lactam antibiotic ampicillin and by the ampicillin-derived penicilloic acid. Furthermore, we present crystal structures of non-covalent BapA complexes with these inhibitors and with the broad-spectrum serine protease inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF, trade name: pefabloc SC), providing structural details of ligand binding and mechanistic details of β-peptide transformations catalyzed by β-aminopeptidases.

Results and Discussion

Crystal structure of BapA in complex with AEBSF

Biochemical studies with β-aminopeptidase BapA demonstrated inhibition of the enzyme in the presence of high concentra-

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

	BapA–AEBSF	BapA–Amp	BapA–Amp_{hyd}
Data collection			
space group	*P*21	*P*21	*P*21
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_{work}/R_{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.d.			
bond lengths [Å]	0.007	0.008	0.007
bond angles [°]	1.05	1.104	1.05

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

tions of the broad-spectrum serine protease inhibitor AEBSF.[39] 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,[40] 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-Oγ into the hydrophobic binding pocket, and the amino group of AEBSF forms a salt bridge to the carboxy group of Glu133. Thr76, Thr100, Leu135, and Leu287 (e.g., subunit A), together with residues of the two neighboring αβ-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 1). The aromatic ring of Phe124, which showed very weak electron density in the native structure of BapA,[18] is well defined through a π-stacking interaction with the aromatic ring of AEBSF.

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 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 (Scheme 2) and differ with regard to their D-phenylglycyl, phenylacetyl, and D-phenylmalonyl acyl groups, respectively. With neither enzyme did we observe deacylation or hydrolysis of the β-lactam amide bond, thus 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} (Scheme 2) on BapA- and DmpA-catalyzed conversion of the chromogenic reporter substrate H-β^3hAla-*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 (Scheme 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}, that is, hydrolytic opening of the β-lactam ring, did not affect the inhibitory effect on substrate conversion by BapA. In contrast

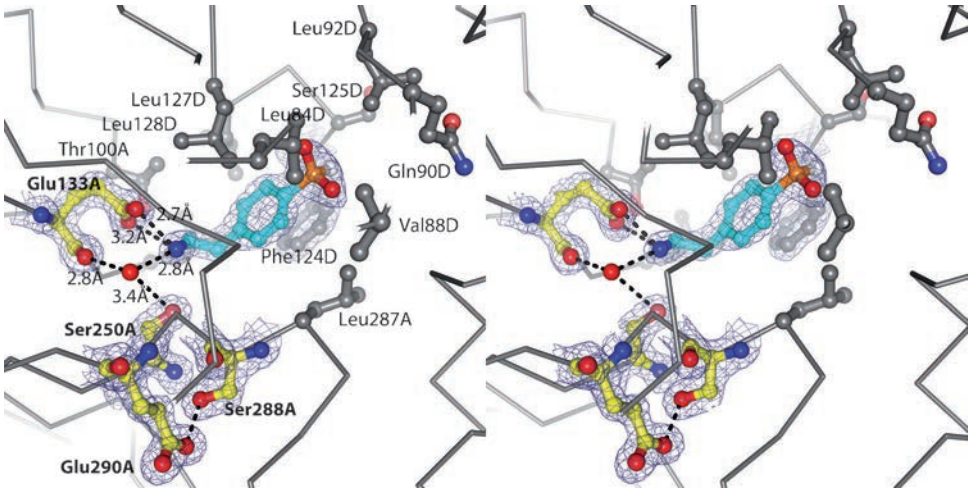


Figure 1. Stereo representation of the BapA active site in complex with the serine protease inhibitor AEBSF. The essential catalytic residues of BapA (subunit A) and selected residues contributing to the hydrophobic ligand-binding pocket are shown as sticks in yellow and grey, respectively; subunit localizations 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 $2F_o-F_c$ 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.

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} [μmol min^{-1} mg^{-1}]
no inhibitor	–	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

to BapA, DmpA was not significantly inhibited by any of the three β-lactam derivatives or by the hydrolyzed species Amp_{hyd}.

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

Crystal structures of BapA in non-covalent complexes 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. As in the BapA–AEBSF complex, one inhibitor molecule was bound to each of the four active sites of the BapA $(\alpha\beta)_4$-heterooctamer (Figure 2). Both ampicillin and

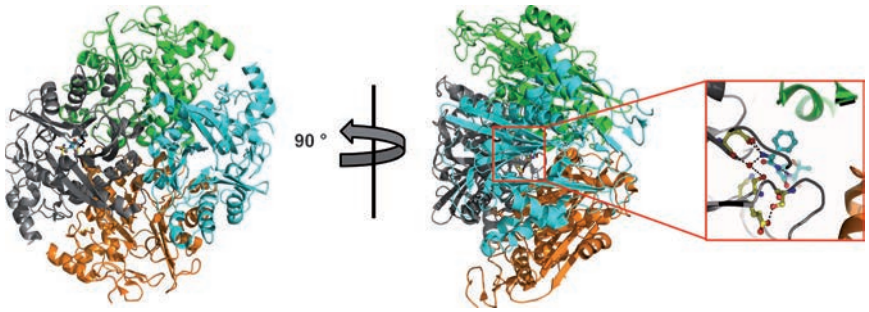


Figure 2. Quaternary structure of catalytically active BapA in complex with ampicillin (BapA–Amp). The four subunits of the BapA $(\alpha\beta)_4$-heterooctamer, each comprising a large α- and a small β-polypeptide chain, are depicted in different colors. One ampicillin molecule and the catalytic residues of subunit A (gray) are shown as sticks in cyan and yellow, respectively.

Amp_{hyd} interact with the BapA active site in a similar manner. The α-amino group of the D-phenylglycine moiety of ampicillin forms a salt bridge with Glu133 and a hydrogen bond with a water molecule in the active site (Figure 3 A). 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 1 and Figure 3). The backbone nitrogen of Leu135 and the carbonyl oxygen of Leu287 form hydrogen bonds with the amide that links the D-phenylglycine acyl group of ampicillin to the 6-APA core. Furthermore, the carboxy 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-phenylglycine acyl group and the active site of BapA are similar to those observed for the binding of ampicillin (cf. Figure 3 A and B). 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 carboxy group attached to the thiazolidine ring with Arg138 is not present in the BapA–Amp_{hyd} complex. Instead, the additional carboxy group of Amp_{hyd} is within close proximity of Asn207-NH_2 and the amino terminus of Ser250, compensating for the missing salt bridge with Arg138.

Models of tetrahedral BapA–substrate complexes

Based on structural similarities of the N-terminal moiety of Amp_{hyd} to a linear α-peptide, we modeled N-terminally unprotected L- and D-β^3-homophenylalanine residues of peptidic BapA substrates[10,14] onto the backbone of the ligand in the BapA–Amp_{hyd} complex. For model building, we positioned the carbonyl group of the substrate at an appropriate distance (1.6 Å) and trajectory (105 ± 5°)[41,42] for nucleophilic attack by Ser250-Oγ, and aimed for the best possible match of the β^3-homophenylalanine residues on the electron density of Amp_{hyd}. Tetrahedral reaction intermediates were formed by the addition of Ser250-Oγ to the carbonyl carbon atom of the β^3-homophenylalanine residues. Rotation around the Cα–Cβ bond of the β-amino-acid backbone gives rise to two synclinal conformers ((+)-sc and (−)-sc) and an antiperiplanar (ap) conformer for the β-amino-acid residue.[1] The models presented in Figure 4 and Figure S1 show that the (+)-sc conformer of L-β^3-homophenylalanine and the (−)-sc conformer of D-β^3-homophenylalanine represent the only energetically favorable conformations of the respective compounds that meet the steric demands for successful formation of a tetrahedral enzyme–substrate intermediate. Complex formation with the other conformers (i.e., (−)-sc and ap of L-β^3-homophenylalanine, and (+)-sc and ap of D-β^3-homophenylalanine) can be excluded due to massive steric repulsions between the side chain of the β-amino-acid residue and residues of the BapA active site (Table 3 and Figure S1). In the favored conformations, the hydrophobic side chains and the amino groups of the covalently bound L- and D-β^3-homophenylalanine residues match well with the position of the phenylglycyl moiety of Amp_{hyd} in the BapA–Amp_{hyd} complex (Figure 4). The proximity of Asn207-NH_2 and Leu135-NH to the oxyanion of the modeled tetrahedral intermediates suggests that these residues stabilize the negatively charged tetrahedral intermediate during the catalytic reaction (for mechanistic details, see ref. [18]).

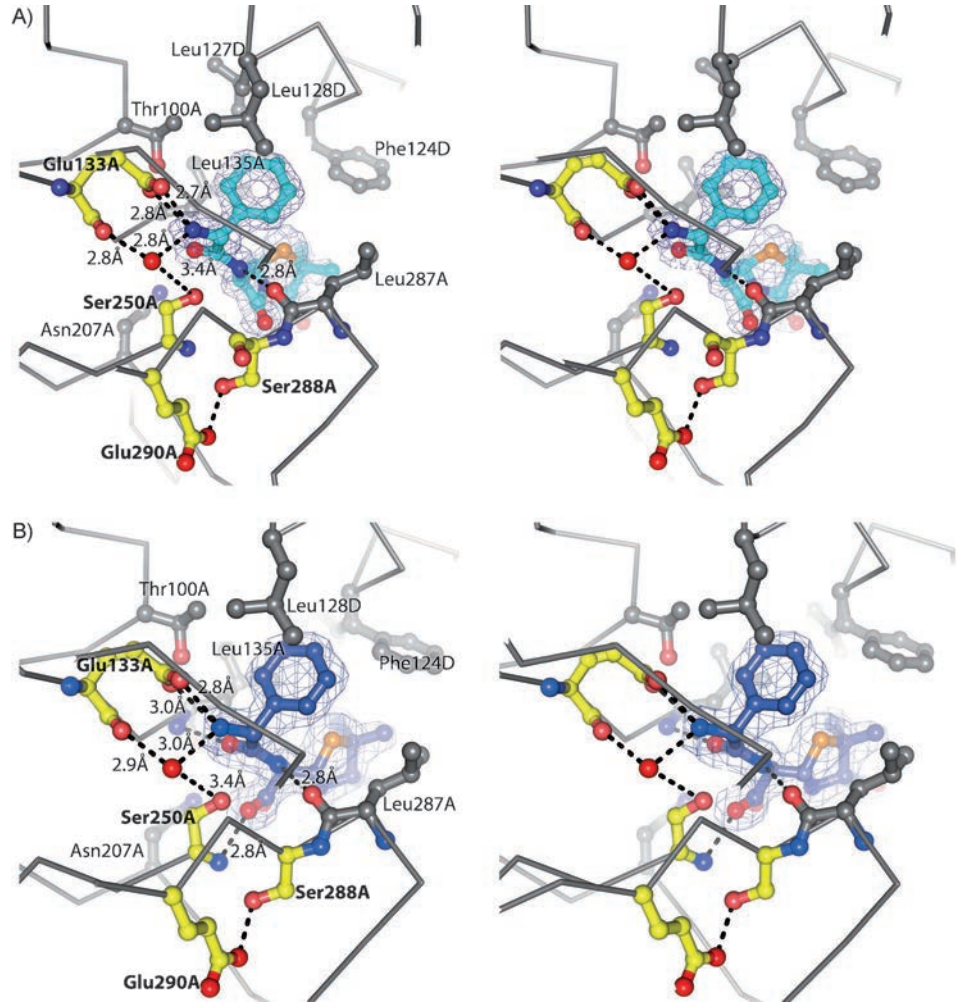


Figure 3. Stereo representations of the BapA active site in complex with A) the β-lactam inhibitor ampicillin and B) the penicilloic acid Amp_{hyd}. The catalytic residues of BapA (subunit A) and selected residues of the ligand-binding pockets are shown as sticks in yellow and grey, respectively; subunit 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 $2F_o-F_c$ 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.

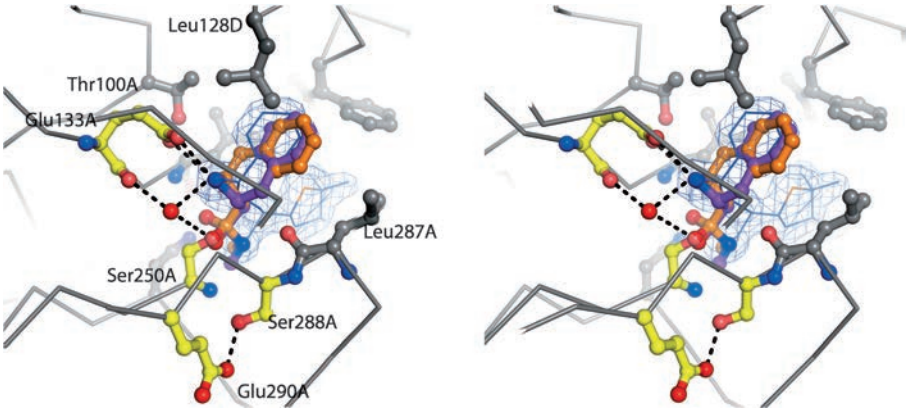


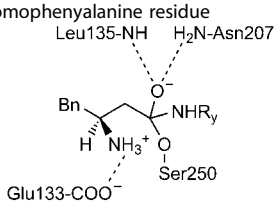
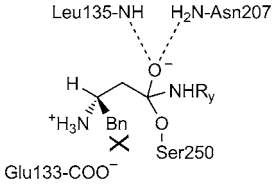
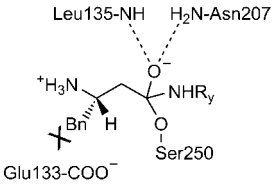
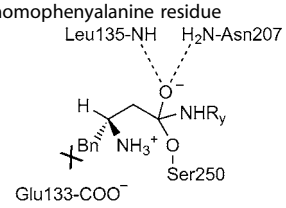
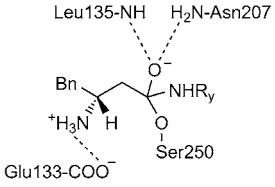
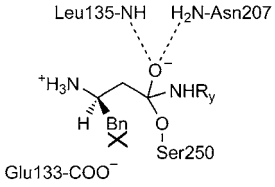
Figure 4. Stereo representation of models of tetrahedral BapA–substrate intermediates. Tetrahedral reaction intermediates between Ser250-Oγ and the N-terminal β^3-homophenylalanine residues of the L-configuration (preferred (+)-sc conformer) and D-configuration (preferred (−)-sc conformer) are shown in orange and magenta, respectively. These models are based on the BapA–Amp_{hyd} crystal structure (grey); the ligand Amp_{hyd} is indicated as blue lines contoured with a $2F_o-F_c$ electron density map at 1.5 σ. Interactions between active site residues of BapA (yellow) and the modeled β^3-homophenylalanine residues 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.

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

The structures of the BapA–inhibitor complexes show a good overlap of the amino group and the aromatic ring of AEBSF with the positions of the phenylglycine acyl groups of ampicillin and Amp_{hyd} (Figure 5). In combination with previous information regarding the substrate specificity of BapA for N-terminally unprotected β-peptides[10,39] and results obtained from an analysis of BapA mutants,[18] the crystal structures of the BapA–ligand complexes (Figures 1 and 3) and the inhibition of BapA by ampicillin-derived compounds (Table 2) provide further strong evidence that the presence of a free terminal amino group plays a key role in ligand recognition and binding by BapA. Due to the attachment of their amino groups to aromatic rings through one or two CH_2 groups, the β-lactam-derived ligands (ampicillin and Amp_{hyd}) and AEBSF have structural similarity to β^3-homophenylglycine and β^3-homophenylalanine residues, respectively (cf. Schemes 1 and 2). Therefore, the formation of noncovalent complexes between BapA and these ligands suggests that the binding of β-peptidic substrates is promoted by similar interactions within the BapA active site, that is, 1) salt-bridge formation between Glu133 and the amino group of the substrate's N-terminal β-amino-acid residue, and 2) hydrophobic interactions between the ligand-binding pocket of BapA and the side chain of the substrate.

Based on the models of the tetrahedral BapA–substrate intermediates (Figure 4), we argue that the exclusive specificity of the enzyme for peptides with backbone-elongated N-terminal β-amino acid residues is governed by the geometry of the

Table 3. Survey of the conformers of BapA–substrate intermediates. The tetrahedral intermediates are formed by nucleophilic attack of Ser250-Oγ of BapA to β^3-homophenylalanine residues of L- or D-configuration (see also Figure 4 and Figure S1).

Conformation[a]	Color code in Figure S1	Clashes between Cγ/Cδ atoms of the bound substrate and BapA residues	Electrostatic interactions with Glu133 COO^-
L-β^3-homophenylalanine residue			
(+)-sc 	orange	Cγ: – Cδ: –	+
(−)-sc 	magenta	Cγ: – Cδ: Thr76, Gly289	–
ap 	green	Cγ: Leu135 Cδ: Leu135, Thr100, Glu133	–
D-β^3-homophenylalanine residue			
(+)-sc 	orange	Cγ: Glu133 Cδ: Glu133, Thr134, Leu135	–
(−)-sc 	magenta	Cγ: – Cδ: –	+
ap 	green	Cγ: Thr76, Gly289 Cδ: Thr76, Gly289	–

[a] (+)-sc = (+)-synclinal, (−)-sc = (−)-synclinal, ap = antiperiplanar conformer of the bound β^3-homophenylalanine residue. Dotted lines represent non-covalent interactions. **X** indicates steric repulsions between the side chain of the substrate and residues of the BapA active site. R_y represents the C-terminal region of the substrate prior to formation of the acyl enzyme.[18] Bn = benzyl. Cγ and Cδ refer to carbon atoms of the β^3-amino acid side chain.

active site, in particular by the orientations and distances of the substrate's functional groups to Glu133-COO^- and Ser250-Oγ. Our results show that compounds such as ampicillin and Amp_{hyd}, which mimic 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 functional groups (NH_2 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 Cα–Cβ 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-Oγ while maintaining the salt bridge to Glu133, as well as stabilizing 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) as well as α-amino acids.[7,10,16,39] Superposition of DmpA on the modeled BapA complexes with AEBSF and Amp_{hyd} perfectly illustrates this steric restriction of the DmpA active site (Figure 5). Although 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 therfore 1) force substrates with N-terminal α-amino acids into a position that allows for nucleophilic attack of Ser250-Oγ, and 2) 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.[18]

Enantioselectivity of 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 4, tetrahedral reaction intermediates could, in principle, be formed between Ser250-Oγ 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 be-

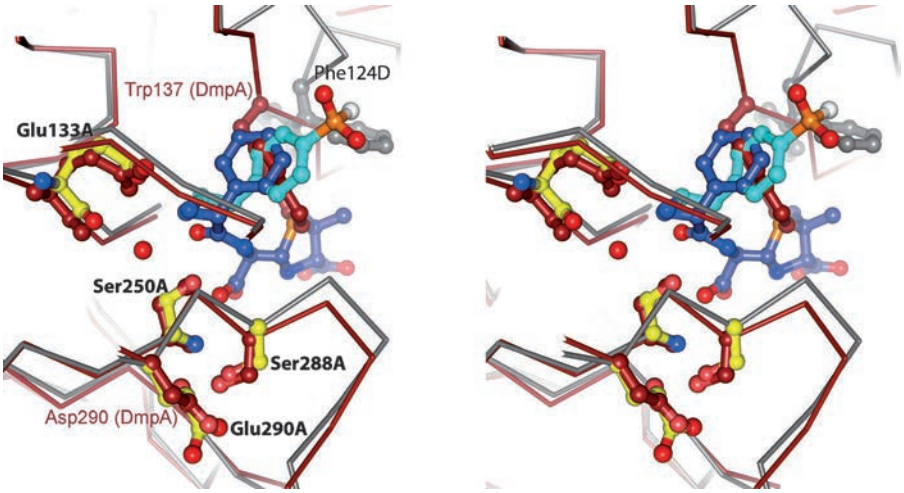


Figure 5. Stereo representation of the BapA–Amp_{hyd} active site (grey) superposed on the crystal structure of DmpA (PDB ID: 1B65; dark red). The catalytic residues Glu133, Ser250, Ser288, and Glu290 of BapA (yellow sticks) correspond to Glu144, Ser250, Ser288 and Asp290 of DmpA (red sticks). The side chain of Trp137 of DmpA clashes with BapA ligands Amp_{hyd} (dark blue) and AEBSF (light blue).

tween Glu133-COO^- and the amino terminus of the substrate is larger for the complex with a (−)-sc conformation than for the one with a (+)-sc conformation of the substrate molecules; this indicates 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 in enantioselectivity and increase in side chain size of the N-terminal β-amino-acid residue is due to additional nonpolar interactions of the hydrophobic ligand binding pocket with large side chains of the substrate. Such hydrophobic effects could improve the stability of complexes formed between BapA and large D-β^3-amino-acid residues with (−)-sc conformation and partially compensate for a weak salt bridge to Glu133.

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

β^2-Dipeptides with small and therefore less demanding side chains at the N-terminal amino-acid residue are not converted by BapA, irrespective of their configuration.[16] As suggested for the conversion of substrates carrying N-terminal D-β^3-amino-acid residues with side chains of different sizes, the preference of BapA for bulky β^2-amino-acid residues may likewise be explained by the stabilization of the enzyme–substrate complex by increased hydrophobic interactions between large amino acid side chains and the ligand binding 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 3A) indicate why BapA exhibits neither penicillin-acylase nor β-lactamase activity. Most importantly, the observed distance from Ser250-Oγ to the carbonyl carbon atom of the phenylglycyl moieties (2.6 Å) or of the β-lactam ring (4.3 to 4.6 Å) was too large to allow for a nucleophilic attack. Moreover, the observed angle between Ser250-Oγ and the carbonyl plane of the phenylglycine acyl group (84.9 to 88.5°) and the angle between Ser250-Oγ and the carbonyl plane of the β-lactam (81.4 to 83.5°) were not in accordance with the required trajectory of $105 \pm 5°$ at which nucleophilic attack of a carbonyl carbon can occur.[41,42] 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. A 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.[43] 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.[44] It was converted to an enzyme with D,D-carboxypeptidase activity and new penicillin-binding properties by directed mutagenesis.[45] 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 proteins that were retrieved from sequencing projects and that are similar to the characterized β-aminopeptidases[18] 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] 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 μL) were analyzed on a reversed-phase HPLC stationary phase Nucleodur C18-Pyramid (250×4 μm, 5 μm 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% CH_3CN within 15 min at a constant flow rate of 1 mL min^{-1} and were 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) with 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. 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 Quantity One software (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 *Escherichia coli* BL21(DE3) pLysS and partially purified by anion-exchange and hydrophobic-interaction chromatography. Ampicillin (50 mg) was dissolved in water (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 μg mL^{-1}. After 16 h 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 C5 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: β-Aminopeptidase BapA from *S. xenopeptidilytica* 3-2W4 was expressed without the N-terminal 29-amino-acid signal sequence in a pET3c-expression system, purified from the *E. coli* host,[39] and crystallized.[18] BapA crystals were soaked for 5 min in a solution of 1.5 M ammonium sulfate and 100 mM HEPES (pH 7.5) containing 50 mM freshly dissolved AEBSF, and for 30 min in 1.5 M ammonium sulfate and 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.

Structure determination: Data were collected at the Swiss Light Source beamline PX on a 6 M Pilatus detector in a cryostream at 100 K at 1 Å wavelength. Diffraction data on a total of 720 frames were recorded with an oscillation range of 0.5° per frame. Data were processed with the program XDS.[46] Initial phases were obtained by molecular replacement using the program PHASER[47] and the structure of BapA (PDB ID: 3N2W) as a search model. The asymmetric units each contained one molecule of the active BapA $(\alpha\beta)_4$-heterooctamer. The final model of BapA–AEBSF contained residues 1–245/250–371 for chain A, 1–245/250–371 for chain B, 1–245/250–371 for chain C, and 1–245/250–371 for chain D. The final model of BapA–Amp contained residues 1–245/250–371 for chain A, 1–243/250–371 for chain B, 1–244/250–371 for chain C, and 1–245/250–371 for chain D. The final model of BapA–Amp_{hyd} contained residues 1–245/250–371 for chain A, 1–242/250–371 for chain B, 1–244/250–371 for chain C, and 1–244/250–371 for chain D. Refinement of the structures was carried out with the program PHENIX.[48] Model building and superpositions for figures were performed with COOT[49] and SSM,[50] respectively. Figures were created with PyMOL.[51]

Expression of BapA and DmpA for enzymatic activity assays: To exclude the possibility of β-lactamase cross-contamination from enzyme expression in a β-lactamase-encoding pET3c expression system, the genes *bapA* from *S. xenopeptidilytica* 3-2W4 and *dmpA* from *O. anthropi* LMG7991 were cloned into pET9c vectors to yield pAR116 and pAR114, respectively.[10,16] The enzymes were recombinantly expressed in *E. coli* BL21(DE3) pLysS and purified according to established procedures.[10,39] The lyophilized enzyme powders were dissolved in a 10 mM potassium-phosphate buffer at pH 7.2, and the protein contents of the enzyme stocks were determined spectrophotometrically.

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

Enzyme inhibition assay: The inhibitory effects of the β-lactam antibiotics ampicillin, penicillin G, or carbenicillin, and of the penicilloic acid Amp_{hyd}, on the β-aminopeptidase-catalyzed conversion of the reporter substrate H-β^{3}hAla-*p*NA were determined spectrophotometrically.[18] The assay mixtures contained the reporter substrate and the inhibitors in various molar ratios. Experimental data were fitted to the competitive inhibition model [Eq. (1)] by nonlinear regression analysis with the VisualEnzymics software (Softzymics, Princeton, NJ, USA) and the program IGOR Pro (WaveMetrics, Oswego, OR, USA).

$$v = \frac{v_{max} \cdot [S]}{K_m \cdot (1 + [I]/K_i) + [S]} \quad (1)$$

PDB accession numbers: BapA in complex with AEBSF (BapA–AEBSF, PDB ID: 3N33); BapA in complex with ampicillin (BapA–Amp, PDB ID: 3NDV); BapA in complex with Amp_{hyd} (BapA–Amp_{hyd}, PDB ID: 3NFB).

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Keywords: AEBSF · ampicillin · beta-peptides · Ntn hydrolases · pefabloc SC

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