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Structural Basis of the Inhibition of Class A β-Lactamases and Penicillin-Binding Proteins by 6-β-Iodopenicillanate

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Abstract: 6-β-Halogenopenicillanates are powerful, irreversible inhibitors of various β-lactamases and penicillin-binding proteins. Upon acylation of these enzymes, the inhibitors are thought to undergo a structural rearrangement associated with the departure of the iodide and formation of a dihydrothiazine ring, but, to date, no structural evidence has proven this. 6-β-Iodopenicillanic acid (BIP) is shown here to be an active antibiotic against various bacterial strains and an effective inhibitor of the class A β-lactamase of *Bacillus subtilis* BS3 (BS3) and the D,D-peptidase of *Actinomadura* R39 (R39). Crystals of BS3 and of R39 were soaked with a solution of BIP and their structures solved at 1.65 and 2.2 Å, respectively. The β-lactam and the thiazolidine rings of BIP are indeed found to be fused into a dihydrothiazine ring that can adopt two stable conformations at these active sites. The rearranged BIP is observed in one conformation in the BS3 active site and in two monomers of the asymmetric unit of R39, and is observed in the other conformation in the other two monomers of the asymmetric unit of R39. The BS3 structure reveals a new mode of carboxylate interaction with a class A β-lactamase active site that should be of interest in future inhibitor design.

Introduction

The antibacterial activity of penicillin rests upon its ability to inhibit the enzymatic activity of penicillin-binding proteins (PBPs), which are responsible for the late stages of peptidoglycan biosynthesis. PBPs are DD-peptidases that cleave the peptide bond between the C-terminal D-Ala-D-Ala of the peptidoglycan stem pentapeptide. In a similar way to that in which the active serine of PBPs attacks the peptidic amide bond, it also attacks the endocyclic amide bond of the penicillin β-lactam ring, leading to a long-lived acyl-enzyme PBP-penicillin that impairs the DD-peptidase activity.[1] Penicillin derivatives and other β-lactam antibiotics (cephalosporins, cephamycins, carbapenems, and monobactams) or γ-lactam antibiotics (lactivicin, pyrazolidinones) with enhanced antibacterial activity were either discovered as natural metabolites produced by bacteria and fungi or developed from them by direct chemical elaboration.[2−4]

A major mechanism of resistance of bacteria is the production of β-lactamases, which are able to hydrolyze the endocyclic amide bond of the β-lactam ring and release the hydrolyzed product.[5] Finding β-lactamase inhibitors has been an important field of research leading, for example, to the discovery and development of clavulanic acid, tazobactam, sulbactam, BRL42715, and 6-β-halogenopenicillanates.[6−11] 6-β-Iodopenicillanates and 6-β-bromopenicillanates are powerful inhibitors of β-lactamases.[10−14] Inactivation of the class A β-lactamase from *Bacillus cereus* by 6-β-iodopenicillanic acid (BIP) was shown to be accompanied by the formation of a new chromophore,[15,16] and analysis of the absorption, circular dichroic, and NMR spectra of the protein-bound chromophore or the isolated chromophore gave strong evidence that BIP

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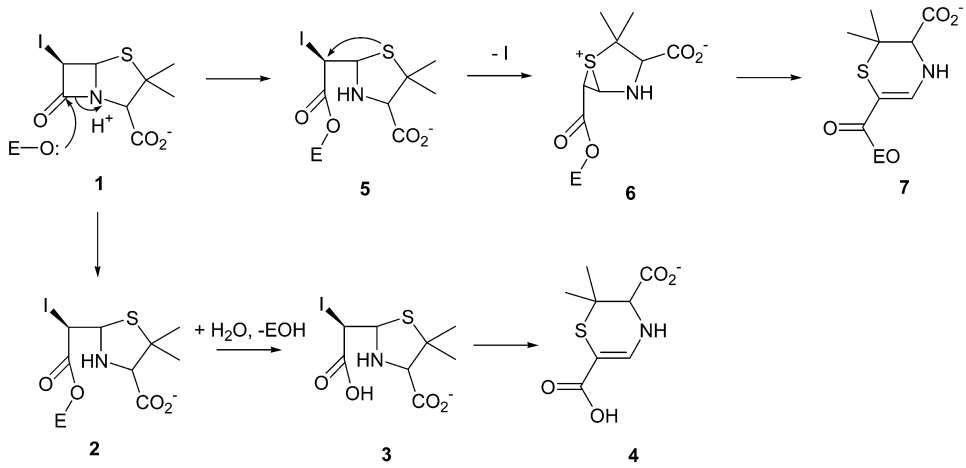
Scheme 1



covalently binds to the enzyme and undergoes a rearrangement of the penicilloyl-enzyme intermediate, possibly *via* ring-opening of the thiazolidine and reaction of the thiolate anion thereby formed.[17]

Inactivation of class A β-lactamases by BIP can be described by successive steps: Michaelis complex formation, acylation, iodide departure, and rearrangement of the penicilloyl moiety into a dihydrothiazine ring. Hydrolysis of the acyl-enzyme sometimes competes with this rearrangement (Scheme 1).[12,18] Iodide departure was proposed to be the rate-limiting step in the process. The strong influence of the ionic strength on the ratio of product turnover versus inactivation provided the rationale for a different rearrangement pathway proposal where opening of the β-lactam ring is followed by the transient formation of an episulfonium ion intermediate (Scheme 1).[19] A detailed physical organic analysis of the rearrangement independently came to the same conclusion concerning the mechanism.[20]

Because early experiments showed that they generally had weak antimicrobial activity, 6-β-halogenopenicillanates are usually considered to be β-lactamase inhibitors. As such, they can be used to lower the minimum inhibitory concentration (MIC) of various β-lactams as antibiotics.[13,21,22] Few experiments, however, have been conducted to explore the inhibitory effect of 6-β-halogenopenicillanates on purified PBPs. BIP actually does inhibit the DD-peptidase activity of *Actinomadura* R39 (R39), but not the DD-peptidase from *Streptomyces* R61.[23]

R39 is a low-molecular-weight type-4 PBP with a structure homologous to *Escherichia coli* PBP4 and *Bacillus subtilis* PBP4a.[24] R39 is a multidomain water-soluble enzyme, loosely associated with the bacterial cell membrane. The penicillin-binding domain of R39 contains the active site responsible for the DD-peptidase activity and acylation by β-lactam antibiotics.

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The overall fold of the DD-peptidase domain is very similar to the fold of class A β-lactamases such as that from *Bacillus licheniformis* (BS3, Figure 1). The active site is at the interface of two subdomains, an all-α and an α/β domain, and is defined by three motifs common to all PBPs and serine β-lactamases. The main difference between PBPs and class A β-lactamases is the presence in the latter of a loop bearing an asparagine and a glutamic acid responsible for the rapid deacylation of most β-lactam antibiotics. In contrast, PBPs form long-lived acyl-enzymes with β-lactams.

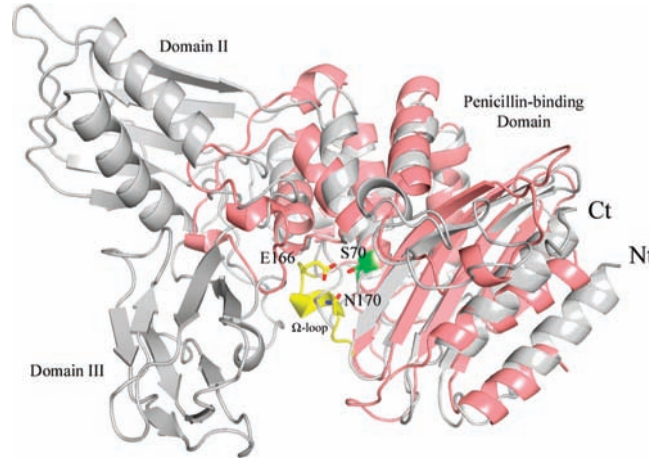

Figure 1. Structures of BS3 (salmon) and R39 (gray). The R39 penicillin-binding domain, domain II, and domain III are indicated. The BS3 active serine is shown in green. The BS3 Ω-loop is shown in yellow. BS3 E166 and N170 are shown as yellow sticks with oxygens in red and nitrogens in blue. Ct, C-terminal; Nt, N-terminal.

In this paper, we show that kinetic experiments, mass spectrometry, and the X-ray structure analysis of the complexes between the BIP and two enzymes, BS3 and R39, give direct evidence for the rearrangement of BIP in a dihydrothiazine ring (Scheme 1). To explore the inhibitory properties of BIP on the DD-peptidase activity of PBPs, MICs of BIP on different Gram-negative and Gram-positive strains of bacteria have been determined.

Materials and Methods

Determination of the Second-Order Rate Constant of Inhibition, k_2/K. The interaction of the PBPs (E) and BIP (I) can be described by model 1.

$$E + I \underset{}{\overset{K}{\rightleftharpoons}} EI \xrightarrow{k_2} EI^* \xrightarrow{k_3} E + P$$
$$EI^* \xrightarrow{k_4} EI^i$$

model 1

If $k_3 = 0$ and $k_2 \ll k_4$, the rate of the formation of EI* is the rate-limiting step, and the determination of the pseudo-first-order rate constants k_i allows the calculation of k_2/K by using the following equation:[25]

$$k_i = (k_2[I])/([I] + K(1 + [S]/Km))$$

If $[S] < K_m$ and $[I] \ll K$,

$$k_i = k_2/K[I]$$

S is the reporter substrate *N*-benzoyl-D-alanyl-thioglycolate (S2d) for the R39 and nitrocefin for BS3, respectively. The K_m values for S2d[26] and nitrocefin were determined initially by initial rate measurements. R39 can hydrolyze the thioester S2d. The activity is increased in the presence of D-alanine because deacylation is rate-determining and D-alanine intercepts the acyl enzyme. The activity toward the thioester was determined spectrophotometrically in the presence of 4,4′-dithiodipyridine, a sensitive thiol-specific reagent that has an absorption maximum at 324 nm ($\varepsilon_{324} = 20\,000$ M^{-1} s^{-1}) after reaction with 2 equiv of thiol. Assays were conducted at 30 °C in 10 mM sodium phosphate buffer with 100 mM NaCl pH 7.2, 100 μM S2d ($K_m = 0.3$ mM), 100 mM D-alanine, and 0.5 mM 4,4′-dithiodipyridine (Acros Organics). R39 (0.4 nM) was mixed with various concentrations of BIP (20−125 nM) in the assay described above; S2d hydrolysis was followed by absorbance at 324 nm using an Uvikon 860 spectrometer linked to a microcomputer through an RS232 interface. Once the time course was complete, values of pseudo-first-order rate constants of inactivation, k_i, were determined from total progress curve analysis, and the k_2/K values were determined as described above.

For BS3, nitrocefin was used as reporter substrate. BS3 (0.7 nM) was mixed with various concentrations of BIP (10−70 nM) in 50 mM sodium phosphate buffer pH 7.2 with 5 μM nitrocefin ($K_m =$ 11 μM) at 30 °C. Nitrocefin hydrolysis was followed by absorbance at 482 nm ($\varepsilon_{482} = 15\,000$ M^{-1} s^{-1}), and the k_i and k_2/K values were determined as described above.

Hydrolysis of β-Iodopenicillanate. The product of BIP turnover is 2,3-dihydro-2,2-dimethyl-1,4-thiazine-3,6-dicarboxylate (Scheme 1, **4**), which has a maximum of absorbance at 305 nm ($\varepsilon_{305} = 8150$ M^{-1} s^{-1}).[12] In order to show the presence of **4**, the hydrolysis of BIP was followed by absorbance at 305 nm. Therefore, 10 μM BS3 was incubated with 100 μM BIP in 50 mM sodium phosphate buffer pH 7.2 at 30 °C. The same experiment was done with 1 μM R39 in the presence of 10 μM BIP in 10 mM sodium phosphate buffer with 100 mM NaCl pH 7.2.

In the presence of stoichiometric amounts of BIP and protein, a new absorption band can be observed at 323 nm ($\varepsilon_{323} \approx 10\,500$ $M^{-1}cm^{-1}$), which can be attributed to the formation of the inactivation complex (EI^i).[27] Difference spectra between the complex formed in the presence of BIP and the free enzyme were measured in the presence of 10 μM protein (R39 or BS3) in buffer (10 mM sodium phosphate buffer with 100 mM NaCl pH 7.2 with R39 and 50 mM sodium phosphate buffer pH 7.2 with BS3, respectively) in the presence of different BIP concentrations (10, 20, 30, and 100 μM).

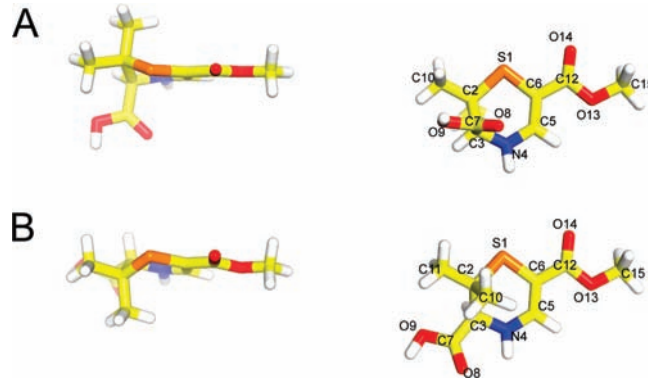

Figure 2. (A) C-up and (B) S-up conformers of the model compound 2,2-dimethyl-2,3-dihydro-1,4-thiazine-6-methoxycarbonyl-3*S*-carboxylic acid (rearranged BIP with the methyl ester mimicking the serine bond). The right view is rotated 90° from the left view. Oxygens are red, nitrogens blue, sulfur orange, and hydrogens white.

Mass Spectrometry. Covalent adducts were identified by mass spectrometry. BIP (100 μM) was incubated with 1 μM protein in buffer (R39, 10 mM sodium phosphate buffer with 100 mM NaCl pH 7.2; BS3, 50 mM sodium phosphate buffer pH 7.2) at 25 °C for 60 min (volume, 1.2 mL). The volume was decreased to 10 μL by ultrafiltration using a Microcon YM-10 instrument (Millipore). Sample preparation was done by two additional ultrafiltration steps with 500 μL of water with R39, and by two additional ultrafiltration steps with 500 μL of 500 mM ammonium acetate and 500 μL of 25 mM ammonium acetate with BS3, respectively. The samples were concentrated to 30 μL. They were mixed with acetonitrile (50% v/v) and trifluoroacetic acid (0.1% v/v). Mass spectrometry was then conducted using an ESI-Q-TOF micromass instrument (Waters) operated in positive mode, with a capillary voltage of 3.0 kV, a sample cone voltage of 100 V, and a temperature of 100 °C; RF lens 1 was set at 100 V, and a 500 L h^{-1} desolvation gas flow was used. Sample was injected into the source for desolvation at 4 μL min^{-1} flow.

BIP Conformation. The geometry of the 2,2-dimethyl-2,3-dihydro-1,4-thiazine-6-methoxycarbonyl-3*S*-carboxylic acid (Figure 2) was fully optimized with the B3LYP functional[28] using the double-ζ basis set 6-31G(d).[29] All the calculations were performed with the Gaussian 03 program.[30]

BS3 β-Lactamase Crystallography. Protein Purification and Crystallization. The expression and purification of the BS3 enzyme were performed as described previously.[31] Crystals were grown at 20 °C by hanging drop vapor diffusion with drops containing 5 μL of a protein solution (38 mg mL^{-1}), 1 μL of ethanol 30%, and 4 μL of 10% PEG 6000 in 100 mM sodium citrate buffer (pH 3.4), equilibrated against 1 mL of the latter solution at 20 °C. The BS3-BIP adduct was obtained by diffusing into the crystals increased concentrations of BIP over the course of 24 h at room temperature. A fresh solution of BIP was continuously added up to a final concentration of 68 mM.

Data Collection, Structure Determination, and Refinement. Data were collected at room temperature at LURE (Orsay, France) on beamline DW32 at a wavelength of 0.9474 using a MarResearch Mar345 imaging plate. Indexing and integration were carried out using Mosflm,[32] and the scaling of the intensity data was ac-

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Table 1. Data Collection and Refinement Statistics

	BS3-BIP	R39-BIP
PDB code	2wk0	2wke
	Data Collection	
resolution range (Å)[a]	27−1.65 (1.74−1.65)	42.8−2.20 (2.32−2.20)
no. of unique reflections	72 436	94 213
R_{merge} (%)[a,b]	5.7 (50.3)	6.4 (26.8)
redundancy[a]	3.8 (3.8)	3.1 (2.4)
completeness (%)[a]	98.3 (97.3)	91.5 (73.0)
$\langle I\rangle/\langle\sigma I\rangle$[a]	14.7 (2.5)	13.6 (2.9)
	Refinement	
resolution range	17.7−1.65	29−2.2
no. of protein atoms	4014	13394
no. of water molecules	364	600
R_{cryst} (%)	19.0	20.6
R_{free} (%)	21.1	26.1
	rms deviations from ideal stereochemistry	
bond lengths (Å)	0.009	0.012
bond angles (°)	1.31	1.51
mean *B* factor, all atoms (Å^2)	16.6	21.8
protein	12.8-17.5[c]	20.1-22.0-19.9-23.5[d]
BIP	14.9-21.6[c]	38.0-36.2-36.4-36.0[d]
Cl ion	14.5-17.8[c]	
water	31.4	32.3
estimated maximal error (Å)	0.070	0.220
rmsd of Cα atom with native structure (Å)	0.25[e]	0.43[e]

[a] Statistics for the highest resolution shell are given in parentheses. [b] $R_{merge} = \sum|I_i - I_m|/\sum I_i$, where I_i is the intensity of the measured reflection and I_m is the mean intensity of all symmetry-related reflections. Figures within brackets are for the outer resolution shell. [c] Monomers A-B. [d] Monomers A-B-C-D. [e] Monomer A.

complished with SCALA of the CCP4 program suite.[33] Refinement was carried out using REFMAC5,[34] TLS,[35] and Coot.[36] The structure of the BS3 β-lactamase bound to the rearranged BIP (2,2-dimethyl-2,3-dihydro-1,4-thiazine-6-carbonyl-3*S*-carboxylic acid, BIPr) was refined to 1.65 Å with R_{cryst} and R_{free} values of 19.0% and 21.1%, respectively; 92.7% of the residues are in the most favored region of the Ramachandran plot and 6.8% of the residues in the additionally allowed region. The ligand occupancy was refined as 1.0. Data statistics and refinement are given in Table 1.

R39 DD-Peptidase Crystallography. Protein Purification and Crystallization. The R39 DD-peptidase was expressed and purified as described previously.[37] Crystals were grown at 20 °C by hanging drop vapor diffusion. Crystals were obtained by mixing 4 μL of a 25 mg mL^{-1} protein solution (also containing 5 mM $MgCl_2$ and 20 mM Tris, pH 8), 2 μL of well solution (2.0 M ammonium sulfate and 0.1 M MES, pH 6), 0.5 μL of 0.1 M $CoCl_2$ solution, and 0.2 μL of 0.1 M BIP.

Data Collection, Structure Determination, and Refinement. Data were collected at 100 K on an ADSC Q315r CCD detector at a wavelength of 0.9763 Å on beamline BM30A at the European Synchrotron Radiation Facility (ESRF, Grenoble, France). X-ray diffraction experiments were carried out under cryogenic conditions (100 K) after transferring the crystals into 100% glycerol. Intensities were indexed and integrated using Mosflm.[32] Data were scaled with SCALA of the CCP4 program suite.[33] Refinement was carried out using REFMAC5,[34] TLS,[35] and Coot.[36] The structure of the R39 DD-peptidase bound to BIPr was refined to 2.3 Å with R_{cryst} and R_{free} values of 20.6% and 26.1%, respectively; 91.6% of the residues are in the most favored region of the Ramachandran plot and 7.7% of the residues in the additionally allowed region. The ligand occupancy was refined as 1.0. Data statistics and refinement are summarized in Table 1.

Minimum Inhibitory Concentration (MIC) Tests. Tests were made using microtitration plates in 200 μL (final volume) of Müller−Hinton broth (MHB), following the EUCAST (European Committee on Antimicrobial Susceptibility Testing)/CLSI (Clinical and Laboratory Standard Institute) recommended procedure. BIP was solubilized in MHB just before utilization. Inocula were prepared for each strain, resuspending isolated colonies from 18 h cultured plates. Equivalents of 0.5 McFarland turbidity standard (approximately 1×10^8 CFU mL^{-1}) were prepared in saline solution (NaCl 0.085%) and then diluted 200-fold in MBH (initial population). MIC was determined as the lowest dilution of product showing no visual turbidity.

Results

Kinetic Analysis and Mass Spectrometry. The product of BIP turnover is 2,3-dihydro-2,2-dimethyl-1,4-thiazine-3,6-dicarboxylate (Scheme 1, **4**), which has a maximum absorbance at 305 nm.[12] No increase of the absorbance at 305 nm was observed during an incubation of 60 min of R39 and BS3 with 10 equiv of BIP, respectively. Difference spectra between the complex formed in the presence of BIP and the free enzymes show a maximum at 323 nm which is characteristic for the formation of this complex (Scheme 1, **7**). A maximum absorption at 323 nm was observed directly after the first addition of BIP (1:1 ratio of BIP to enzyme), indicating the rapid formation of the inactivation complex. No shift of the maximum of absorbance to lower wavelength after further additions of BIP was observed as it has been described for the *Streptomyces albus* G β-lactamase.[12] This indicated the absence of turnover of the inactivator. In the absence of turnover ($k_3 = 0$) and a rapid formation of the inactivation complex ($k_4 \gg k_2$), the formation of the acyl-enzyme is the rate-limiting step,[27] and the second-order rate constants of the inactivation of R39 ($k_2/K = 52\,300 \pm 6400$ M^{-1} s^{-1}) and BS3 ($k_2/K = 58\,900 \pm 7900$ M^{-1} s^{-1}) were determined. These values indicated that BIP is a good inactivator of both proteins.

The presence of BIPr in the active site of both proteins has been confirmed by mass spectrometry. The mass increments between R39 (47 759 ± 2 Da) and BS3 (29 470 Da ± 2) and the inhibitor complexes (R39 complex, 47 953 ± 2 Da; BS3 complex, 29 665 ± 2 Da) were respectively 194 and 195, consistent with the formation of the covalently attached dihydrothiazine **7**.

BIP Conformation. Computations suggest that in the model compound 2,2-dimethyl-2,3-dihydro-1,4-thiazine-6-methoxycarbonyl-3*S*-carboxylic acid (rearranged molecule with the methyl ester mimicking the serine bond), the dihydrothiazine group can adopt two stable conformations (Figure 2). The C-up conformer (in which the carbon C2, bonded to the sulfur atom, is above the plane formed by the other atoms of the ring) has an axially oriented carboxylate group. The S-up conformer (in which the sulfur atom is above the plane formed by the other atoms of the ring) is rather flat with an equatorially associated carboxylate. The C-up conformer is 3.51 kcal mol^{-1} more stable. By reference to the S-up conformer, the energy barrier between them is 7.70 kcal mol^{-1}. For both minima, a different conformation of the ester mimicking the serine bond can be trapped after

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rotation of 180° around the C6−C12 bond. The energy stabilization is roughly 0.82 kcal.

BS3-BIP Structure. The electron density in the BS3-BIP crystal is well defined except for that of some glutamic acids that suffered radiation damage, lysines exposed to the solvent, loop 253−257 in monomer A, and loops 86−100, 200−209, and 253−257 in monomer B. The rms deviation between the monomers is 0.25 Å. The overall fold of the BS3-BIP complex is similar to the previously reported BS3 structure (apo-enzyme, PDB 1I2S[38]), with an rms deviation between the apo and complex structure of 0.25 Å for the Cα atoms of BS3 monomer A.

The asymmetric unit of BS3 crystals contains two protein molecules. The electron density map calculated in the absence of ligand provides a very clear density into which BIPr is covalently linked to the enzyme active serine (Figure 3a). The density in each monomer shows a covalent adduct resulting from the acylation of the active-site serine by BIP, followed by the intramolecular rearrangement of the latter. A chloride ion lies between Ser130Oγ, Lys234Nζ, Thr235Oγ, and Ser70Oγ. The density cannot account for the iodide ion released during catalysis, and the iodide remains unobserved. The β-lactam-derived ester carbonyl of the covalent adduct hydrogen-bonds to Ser70 and Ala237 nitrogens that define the oxyanion hole. BIPr adopts the C-up conformation with the sulfur oriented on the same side as the ester carbonyl.

One BIPr carboxylate oxygen is hydrogen-bonded to Asn132Nδ and Asn104Nδ, and its partner is hydrogen-bonded to Asn170Nδ. Asn170 normally interacts with a nucleophilic water molecule conserved in the structures of class A β-lactamases. The interactions between BS3 and BIPr induce a slight displacement of the Ω-loop that eventually reduces the space left for the deacylating water molecule. In monomer A, the total absence of electron density clearly precludes the presence of a water molecule. In monomer B, a small amount of density suggests the presence of a water molecule between Glu166, Asn170, and BIPr. Comparison of the position of this water molecule with its conserved position in other class A β-lactamases shows that the water molecule in BS3 monomer B is shifted some 0.4 Å, which might reflect a deficient positioning of the deacylation machinery of BS3 toward BIPr.

R39-BIP Structure. The electron density in the R39-BIP crystal is well defined except for the N-terminal residue and some arginine, lysine, and glutamic acid side chains pointing into the solvent. The overall fold of the R39-BIP complex is similar to the previously reported R39 structure, with an rms deviation between the apo and complex structures of 0.43 Å for the 466 Cα atoms of R39 monomer A (PDB 1W79).[39]

The asymmetric unit of R39 crystals contains four protein molecules. The environment is identical for monomers A and D and for monomers B and C, but different for both pairs. Although not preventing BIP from acylating the enzyme (see below), the active sites of monomers A and D in the apo-enzyme structure are obstructed by the loop 175−178 of a symmetric molecule, whereas monomers B and C show a freely accessible active site.

In the R39-BIP complex, the electron density map calculated in the absence of ligand shows clear density for the rearranged BIP (BIPr), which is covalently linked to the enzyme active serine in all four monomers (Figure 3b,c). As expected, the carbonyl oxygen lies in the oxyanion hole. In monomers A and D, BIPr displays the C-up conformation and the dihydrothiazine ring is oriented with its sulfur on the side of the acyl-serine carbonyl (Figure 3b). The BIPr conformation is, therefore, similar to that in the BS3 β-lactamase. The dihydrothiazine carboxylate is oriented toward the phenyl group of Tyr147 but does not make any hydrogen bond with the protein. The carboxylate O8 distance to Asn300Nδ (from the SxN motif) is 3.9 Å in monomer A and 4.5 Å in monomer D. The sulfur atom of BIPr is at 3.5 Å from the backbone oxygen of Thr413. In monomers B and C, BIPr displays the S-up conformation and the dihydrothiazine ring is oriented with its sulfur on the side opposite to acyl-serine carbonyl (Figure 3c). The dihydrothiazine carboxylate points out from the active site without making interactions with the surrounding amino acids. The sulfur atom of BIPr is at 3.6 Å from the backbone oxygen of Ser298 and 3.5 Å (monomer B; 3.7 Å in monomer C) from the Asn300Oδ. In all four monomers, a water molecule is found instead of the sulfate ion observed in the apo-R39 structure between residues Ser298, Lys410, Thr411, and Thr413. As in BS3, the iodide atom released during catalysis remains unobserved in R39.

Superposition of R39 monomer B onto monomer A shows that BIPr cannot adopt in monomer A the S-up conformation found in monomer B because of steric hindrance between the BIPr carboxylate and the side-chain methyl group of Ala175, which belongs to a symmetry loop slightly inserted in the active site of monomer A. Similarly, superposition of the structure of BS3-BIPr with monomer B of R39-BIPr shows that BIPr cannot adopt in BS3 the S-up conformation found in R39 monomer B because of steric hindrance between the two BIPr methyl groups and the side chain of Tyr105. Conversely, BIPr could adopt in R39 monomer B the C-up conformation found in R39 monomer A or in BS3, indicating that BIPr conformation in R39 monomer B (S-up) is the preferred one in an unhindered active site.

Minimum Inhibitory Concentration Tests. BIP shows antimicrobial activity when tested on a broad range of bacterial strains (Table 2), indicating that one or more essential PBPs is probably inhibited. No significant differences in MIC values could be detected between Gram-positive and Gram-negative bacteria, suggesting that the external membrane of the latter organisms does not constitute a barrier for BIP to reach its target. Sensitive Gram-positive *Staphylococcus aureus* bacteria are inhibited at low BIP concentration (<2 μg/mL). The relatively high concentrations of BIP needed to inhibit bacterial growth in the other tested strains, however, may be due to PBP-unrelated factors needed for triggering bacterial killing. It is important to note that BIP does not act as a good resistance inducer in the *S. aureus* PL1 strain. This strain produces, in the presence of β-lactams, inducible low-affinity PBP2a but remains sensitive to BIP mostly because PBP2a is not expressed in the presence of BIP. In contrast, *S. aureus* ATCC 43300, which constitutively expresses low-affinity PBP2a, shows a high MIC value. The other tested strains constitutively producing low-affinity PBPs (*Enterococcus faecalis*, *E. faecium*, *E. hirae*) show similar high MIC values. This result is in good agreement with the low inhibitory activity measured against purified low-affinity *S. aureus* PBP2a and *E. faecium* PBP5 (data not shown).

Discussion

Class A β-Lactamase Inhibition and BIP Conformation in BS3. Kinetic results have previously evidenced that 6-β-iodopenicillanates and 6-β-bromopenicillanates are good ir-

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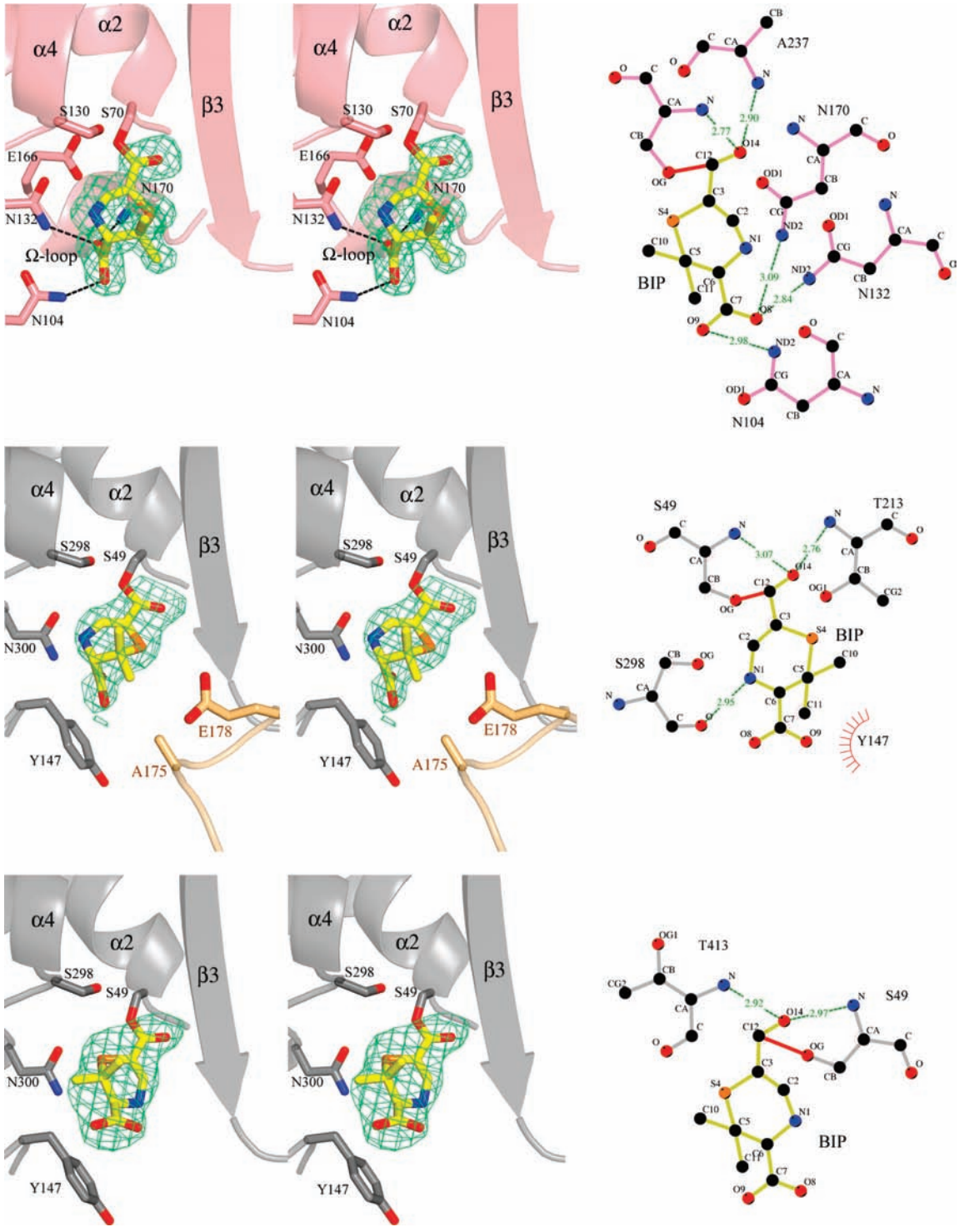

Figure 3. (Left) Stereoviews of omit difference electron density maps ($F_o - F_c$) covering the BIP moiety covalently linked to the active serine. (Right) Schematic representation of the interactions between the BIP and the residues of the active site in (a, top) BS3 monomer A, (b, middle) R39 monomer A, and (c, bottom) R39 monomer B. The electron density maps are contoured at 2.5σ. The carbon atoms are shown in salmon for BS3, in gray for R39, in light orange for the R39-symmetric molecule, and in yellow for BIP. Oxygens are red, nitrogens blue, and sulfur orange.

reversible inhibitors of class A β-lactamases. Our kinetic, mass spectrometric, and crystallographic results assess the nature of the final product formed from BIP upon its reaction with the β-lactamase BS3. The BIP-BS3 structure provides a clear view of the acyl-enzyme formed between the enzyme and the inhibitor. It shows a dihydrothiazine ring, which is likely formed from the rearrangement shown in Scheme 1, in its energetically most favorable conformation, with the sulfur-linked carbon lying over the plane formed by the other ring atoms and the ring oriented with the sulfur on the side opposite to the acyl-serine carbonyl.

The formation of the Michaelis complex and acylation of the enzyme active site serine that have been described for penam derivatives of β-lactams should be similar for BIP.[40−42] The presence of the iodine atom induces a positive charge on C6, and after hydrolysis of the β-lactam amide bond, a 90° rotation

Table 2. BIP Minimum Inhibitory Concentration Tests on Bacterial Strains

bacterial strain	MIC (μg/mL) BIP	ampicillin
Proteus mirabilis ATCC 29936	64	32
Escherichia coli ATCC 8739	256	<2
Citrobacter freundii ATCC 8090	64	32
Pseudomonas aeruginosa ATCC 27853	>512	>512
Klebsiella pneumoniae ATCC 13883	256	64
Enterococcus faecalis ATCC 7937	256	4
Enterococcus faecalis ATCC 29212	16	<2
Enterococcus faecium ATCC 19434	128	32
Enterococcus hirae ATCC 8790	128	32
Micrococcus luteus ATCC 9341	<2	<2
Listeria monocytogenes ATCC 14780	64	<2
Listeria innocua ATCC 33090	128	<2
Staphylococcus epidermidis ATCC 12228	<2	<2
Staphylococcus aureus ATCC 25923	<2	<2
Staphylococcus aureus ATCC 43300 (MRSA)	256	32
Staphylococcus aureus PL 1 (inducible MRSA)	<2	<2

around the C6−C5 bond, enforced by steric interactions across this bond, would allow the sulfur to get close to the positive charge on C6. Formation of the episulfonium ion S−C5−C6 would then be concerted with the departure of iodide. The labile S−C5 bond breaks and C5 becomes sp^2. The rearrangement involves a large movement of the carboxylate group from its likely position in the pre-acylation state, where it probably hydrogen-bonds to Thr235, to its observed position in the crystal structure, close to the Ω-loop where it interacts with Asn170 and Asn132. These interactions lead to the observed axial C-up conformation of the rearranged BIP.

The interaction between the BIP-dihydrothiazine carboxylate and Asn170 induces a slight shift in the Ω-loop position that shortens the distance between Glu166 and the acyl-enzyme ester bond. Deacylation is impaired because the space left to the water molecule necessary for deacylation is reduced, which results in the absence of water in monomer A and, in monomer B, a shift of the water molecule compared to its position in the apo structure of BS3 or in other class A β-lactamases.

The class A β-lactamase inhibitory property of the BIP results from its rearrangement after enzyme acylation and the interaction of the dihydrothiazine carboxylate with both Asn132 and Asn170. These residues are conserved as essential amino acids for the β-lactam hydrolysis activity of class A β-lactamases. The mechanism should therefore be common to all class A β-lactamases. The chemical stability of the vinylogous amide moiety in **6** is another contributor to the inhibition.[43] Moreover, the few interactions of BIP with residues not critical for catalysis reduce the possibilities of development of resistance.

The most interesting feature of the BS3 structure may be the above-mentioned positioning of the inhibitor carboxylate. This represents a new mode of interaction of a carboxylate with the class A β-lactamase active site, a motif that could be incorporated into future inhibitors.

The mechanism of inhibition of class A β-lactamases by BIP is different from the mechanism used by penicillin sulfones such as tazobactam. In the reaction pathway of tazobactam, Ser70 acylation is followed by thiazolidine ring-opening between the sulfur atom and carbon C5. Attack on the thus-generated reactive imine by Ser130 results in a vinyl carboxylic acid that is covalently attached to Ser130Oγ and thereby inactivates the enzyme.[44,45]

Improvement of BIP inhibitory efficiency against class A β-lactamases may be achieved, in principle, by substitution on C6-α. In cefoxitin or imipenem, the C6-α substituent is essential for inhibition in preventing a water molecule from approaching the acyl-enzyme ester bond for deacylation. But a C6-α substituent may clash with the side chain of Asn132 and reduce the affinity of the enzyme for the ligand. A C6-α substituent may thus be detrimental to BIP inhibition. Attempts to improve 6-bromopenicillanic acid by incorporation of such substituents proved unsuccessful.[46] For the same reason, the low efficiency of 6-α-halogenopenicillanates as inhibitors of class A β-lactamases could result from a poorer affinity due to steric hindrance between the halogen atom and the side chain of Asn132. As an exception, 6-α-halogenopenicillanates could be inhibitors of the class A β-lactamase of *Mycobacterium tuberculosis*, which bears an Asn132Gly substitution. As well, the *Enterobacter cloacae* NMCA class A β-lactamase, a carbapenemase with enlarged cavity and additional space near Asn132,[47] could perhaps accommodate 6-α-halogenopenicillanates or 6-β-halogenopenicillanates substituted on position α. The rearrangement of 6-α-halogenopenicillanates may, however, also be inhibited by intramolecular steric interactions.[20] Another possibility, not yet explored, would be substitution at the 2-methyl groups where further interaction with the protein may be induced; such functionalization has proved effective with penicillin sulfones such as tazobactam and other 2′-β-substituted penam sulfones.[48]

Inhibition of PBPs and the BIP Conformation in R39. R39 is a type-4 PBP not essential to bacterial survival in laboratory conditions, but essential PBPs may also be sensitive to BIP, as shown by the MIC tests on a wide range of bacteria (Table 1). The structural results (Figure 3b,c) suggest that PBPs react with BIP in the same way as β-lactamases, yielding the same rearranged inhibitor. The structure of R39 in complex with BIP

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reveals two conformations of the dihydrothiazine. In monomers B and C, the active site is free of steric hindrance by the symmetric enzyme molecule, and the conformation of the dihydrothiazine is likely the one that should be observed in solution. The more stable dihydrothiazine conformation is observed in monomers A and D, where a symmetry loop prevents BIP from adopting the conformation found in the free active site. The difference between the two conformations involves not only the puckering of the dihydrothiazine ring but also its rotation around the C6−C12 bond with regard to the enzyme structure. The S-up conformer of the dihydrothiazine ring that is observed in monomers B and C may be stabilized by the interaction between the sulfur atom and the backbone oxygen of Ser298 and the side chain oxygen of Asn300.[49]

The calculated less stable conformation of the BIP dihydrothiazine ring in the unhindered active site of R39 monomer B resembles the situation of the thiazolidine ring of penicillins, which can adopt an axially oriented carboxylate group or an equatorially disposed conformation. Quantum chemical calculations have shown that the axial conformation is the most stable, and the X-ray structures available in the Cambridge Structural Database reveal that the majority of the penicillins are in the axial orientation. Crystal structures of acyl-enzymes derived from penicillins, however, present the thiazolidine in its equatorial conformation. Those crystallographic results suggest that the equatorial conformer of the BIP thiazolidine ring is the biologically active conformer. The results show that PBPs are inhibited by BIP. Its efficiency as an inhibitor could probably be improved, however, as in the case of class A β-lactamases, by substitution on C6-α or substitution at the 2-methyl groups. The C3′ substituent of ceftobiprole is responsible for the good antibacterial activity of this third-generation cephalosporin against methicillin-resistant *S. aureus*,[50] and one could take advantage of the information obtained with the C3′ substituent of cephalosporins to substitute one of the 2-methyl groups. Such information is available with the crystal structure of the complex between ceftidoren and *Streptococcus pneumoniae* PBP2x.[51] Highly resistant PBPs appear to be only weakly sensitive to BIP, but BIP could represent a lead compound to more effective inactivators of these PBPs.

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Conclusions

Structural study of the BS3-BIP acyl-enzyme has led to direct proof of the unique rearrangement involved in inhibition of class A β-lactamases by BIP (Scheme 1), and the nature of the interactions of the rearranged inhibitor with conserved active-site residues has been elucidated. The position of the inhibitor carboxylate and its interactions with Asn132, Asn104, and Asn170 are especially noteworthy.

PBPs were shown to be sensitive to BIP to various degrees. The crystal structure of the R39-BIP complex is the first of a PBP acylated by a 6-halogenopenicillanate. The detailed observation of the two possible conformations of the dihydrothiazine ring of the rearranged BIP linked to the enzyme-active serine provides a structural basis for the development of substituted 6-halogenopenicillanate as inhibitors of PBPs. BIP-based inhibitors of PBPs not only would escape the hydrolyzing action of β-lactamases but also are potential inhibitors of β-lactamases themselves.

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Supporting Information Available: Complete ref 30. This material is available free of charge via the Internet at http://pubs.acs.org.

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