Bioorganic Chemistry

Irreversible Inhibition of Metallo-β-lactamase (IMP-1) by 3-(3-Mercaptopropionylsulfanyl)-propionic Acid Pentafluorophenyl Ester**

Hiromasa Kurosaki, Yoshihiro Yamaguchi, Toshihiro Higashi, Kimitaka Soga, Satoshi Matsueda, Haruka Yumoto, Shogo Misumi, Yuriko Yamagata, Yoshichika Arakawa, and Masafumi Goto*

Pathogenic bacteria that produce metallo-β-lactamases (MBLs) are emerging as a new challenge to the medical community. These enzymes catalyze the hydrolysis of a wide spectrum of β-lactams, including carbapenems such as imipenem, some of which are coded in transferable plasmids.[1,2] Among the currently known MBLs, IMP-1, a member of subclass B1, which is encoded by the bla_{IMP} gene included in the integron structure,[2,3] rapidly spreads by facile horizontal gene transfer to other bacteria.[4] Moreover, many of the currently used serine β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam are ineffective against MBLs. Thus, the development of inhibitors of MBLs is important for the continuing application of such widely prescribed β-lactam antibiotics. Several such inhibitors have been reported to date;[5–11] for example, Payne et al. demonstrated[6] that mercaptoacetic acid, a hydrolysis product of mercaptoacetic acid thiol esters that are hydrolyzed by MBLs (β-lactamase II, CfiA, and CphA), binds irreversibly to the enzyme through formation of a disulfide bond with the active site cysteine residue under aerobic conditions, as evidenced

[*] Prof. Dr. H. Kurosaki, Y. Yamaguchi, T. Higashi, K. Soga, S. Matsueda, H. Yumoto, Prof. Dr. M. Goto
Department of Structure-Function Physical Chemistry
Graduate School of Pharmaceutical Sciences
Kumamoto University
Oe-honmachi 5-1, Kumamoto 862-0973 (Japan)
Fax: (+81) 96-371-4314
E-mail: ayasaya@gpo.kumamoto-u.ac.jp
Prof. Dr. S. Misumi
Department of Pharmaceutical Biochemistry
Graduate School of Pharmaceutical Sciences
Kumamoto University
Oe-honmachi 5-1, Kumamoto 862-0973 (Japan)
Prof. Dr. Y. Yamagata
Department of Structural Biology
Graduate School of Pharmaceutical Sciences
Kumamoto University
Oe-honmachi 5-1, Kumamoto 862-0973 (Japan)
Prof. Dr. MD. Y. Arakawa
Department of Bacterial Pathogenesis and Infection Control
National Institute of Infectious Diseases
4-7-1 Gakuen, Musashi-Murayama, Tokyo 208-0011 (Japan)

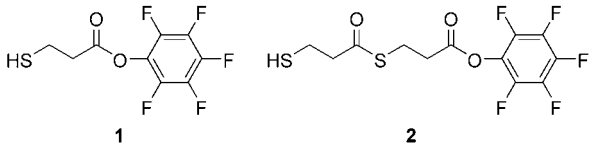
[**] This work was supported by H15-Shinkou-9 from the Ministry of Health Labor and Welfare of Japan and by a Grant-in-Aid for Scientific Research (B) (No. 16390017) from the Japan Society for the Promotion of Science.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

 DOI: 10.1002/ange.200500835

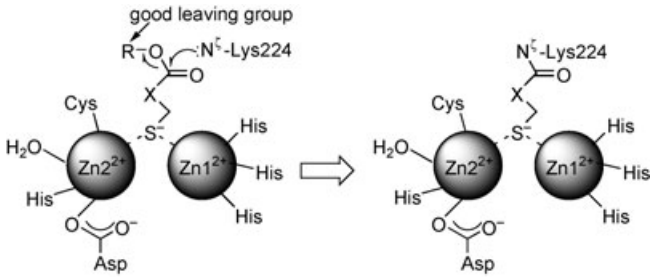
by both tryptic digestion and electrospray mass spectrometry studies.

Our ultimate goal was to develop an irreversible inhibitor of IMP-1. We report here on the design and inhibition properties of three-dimensional structure-based irreversible inhibitors of IMP-1, **1** and **2**, and the crystal structure of a covalently bound complex formed between the hydrolysis product of **2** and IMP-1.



1 **2**

The strategy for the irreversible inhibition of IMP-1 is shown in Scheme 1: The thiol group in **2** coordinates to one or



Scheme 1. Strategy for the irreversible inhibition of thiol compounds with a good leaving group.

two Zn^{II} ion(s) in the active site as an anchor. Lys224 (following BBL numbering[12]) is conserved in almost all MBLs of subclass B1. In the first IMP-1 structure reported (PDB code 1DD6),[7] Lys224 is located at a distance of about 6 Å from the two Zn^{II} ions. Lys224 is thought to be important for substrate binding[6,13] and may attack an activated ester, thus forming a covalently bound inhibitor–enzyme adduct that irreversibly inhibits the enzyme. At this point, water molecules, from the solvation shell surrounding Lys224, may act as acceptors of H^+ ions from the positively charged N^{ζ} group of Lys224.

The coupling reaction of one equivalent of 3-mercaptopropionic acid (MPA) and 1-hydroxy-1*H*-benzotriazole (HOBt) with pentafluorophenol in the presence of one equivalent of *N*,*N*′-dicyclohexylcarbodiimide (DCC) in ethyl acetate at 0 °C afforded **1**, a synthetic intermediate of **2**, in 15 % yield (see Supporting Information). The desired inhibitor **2** was prepared in 45 % yield by treating **1** with MPA in the presence of DCC in ethyl acetate at 0 °C (see Supporting Information).

The time-dependent inactivation of IMP-1 by **1** and **2** was determined by incubating various amounts of inhibitor with 10 nM IMP-1 in 50 mM tris-HCl/0.5 M NaCl buffer (pH 7.4, tris = tris(hydroxymethyl)aminomethane) at 15 °C (see Supporting Information). The inactivation of IMP-1 by **1** and **2** was both time and concentration dependent. Plots of the natural logarithm of the residual activity against incubation time were linear, thus suggesting that the observed rate of inactivation follows pseudo-first-order kinetics (Figure 1 a and b). The double reciprocal plots of these slopes (k_{obs}) versus concentration of **1** and **2** gave straight lines (Figure 1 c

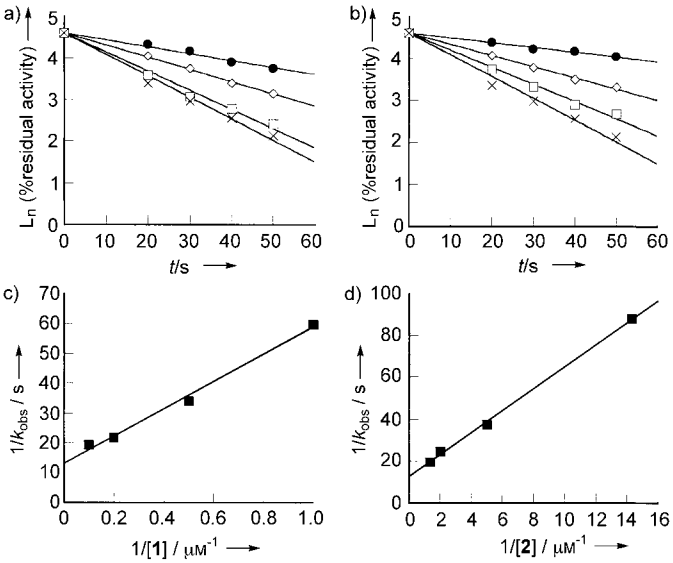


Figure 1. Time- and concentration-dependent inactivation of IMP-1 by **1** (a) and **2** (b) in 50 mM tris-HCl/0.5 M NaCl buffer (pH 7.4) at 15 °C. Inhibition concentrations: ●: 1 μM; ◇: 2 μM; □: 5 μM; and ×: 10 μM for **1**, and ●: 0.07 μM; ◇: 0.2 μM; □: 0.5 μM; and ×: 0.75 μM for **2**. Each point shown represents the mean of three experiments. Double reciprocal plots of k_{obs} versus concentration of **1** (c) and **2** (d).

and d). The regression line did not pass through the origin, but intercepted the positive *y*-axis, thus indicating the initial formation of a dissociable complex between IMP-1 and inhibitor before inactivation.[14] The k_{inact} and K_i values were calculated from these plots to be $0.076 \pm 0.002\ s^{-1}$ and 3.452 ± 0.030 μM for **1** and $0.080 \pm 0.002\ s^{-1}$ and 0.423 ± 0.013 μM for **2**. These results show that the second-order rate constant for inactivation (k_{inact}/K_i) with **2** increases by about ninefold over that with **1**.

To determine whether **1** or **2** inhibits IMP-1 irreversibly, 10 μM IMP-1 was incubated with 1 mM **1** or **2** at 0 °C for various periods of time (0–2 h) and the mixtures were then filtered through gel (Sephadex G-25) to separate the protein from the excess inhibitor (see Supporting Information). The activity of the resulting protein was measured using nitrocefin as the substrate. The control, without inhibitor, shows no loss of activity of IMP-1 after filtration through the gel. On the other hand, the inactivation of IMP-1 by **1** and **2** resulted in a nearly 100 % inhibition, clearly showing that both inhibitors inhibited IMP-1 very rapidly and irreversibly. Furthermore, the irreversibility of the binding of **1** and **2** was also confirmed by dialysis at 4 °C for 16 h (see Supporting Information): in neither of these cases was any activity recovered, thus verifying that **1** and **2** are irreversible inhibitors.

After the incubation of 100 μM **2** with 10 μM IMP-1 for 30 minutes and gel filtration, the sample was analyzed by MALDI-TOF mass spectrometry (see Supporting Information). The MALDI-TOF mass spectrum of the intact IMP-1

showed the parent signal at *m*/*z* 25113.2 while that treated with **2** showed a peak at *m*/*z* 25290.1 (see Supporting Information). The increase in mass of *m*/*z* 176.9 corresponds to the mass of $SCH_2CH_2COSCH_2CH_2CO$ (this unit is denoted **2**-P), which indicates that this moiety of **2** is covalently attached to IMP-1 in a ratio of 1:1.

To identify the site of amino acid attachment and to determine the three-dimensional structure, crystals of IMP-1 treated with **2** were prepared by the hanging-drop method and the molecular structure was determined (see Supporting Information). The structure of the inhibitor bound IMP-1 (**2**-P/IMP-1) at a resolution of 2.63 Å was refined to an *R*-factor value of 22.8 % and an R_{free} value of 24.3 % using 2.63- to 44.7-Å data.[15] There are two **2**-P/IMP-1 molecules, A and B, in the asymmetric unit. The two molecules are almost identical with a root-mean-square deviation of 0.31 Å when all C^{α} atoms between A and B are superimposed. The overall structure of each molecule adopts a αβ/βα sandwich structure as found in the native enzyme, the three-dimensional structure of which was not greatly perturbed by the inhibitor. The overall structure of molecule A is shown in Figure 2a.

The thiolate group in **2**-P bridges between the two Zn^{II} ions in the active site (distances Zn1···inhibitor(S), 2.2/2.2; Zn2···inhibitor(S), 2.3/2.5 Å in molecules A/B; Figure 2b). The geometry around Zn2 is changed from a trigonal bypyramid in the native structure to a distorted tetrahedral structure, as previously reported by Concha et al.[7] (angles (Asp120)$O^{\delta 2}$-Zn2-Cys221(S^{γ}), 101/101°; (Asp120)$O^{\delta 2}$-Zn2-His263($N^{\varepsilon 2}$), 99/104°; Asp120 $O^{\delta 2}$-Zn2-inhibitor(S), 122/112°; (Cys221)S^{γ}-Zn2-His263($N^{\varepsilon 2}$), 109/116°; (Cys221)S^{γ}-Zn2-inhibitor(S), 107/113°; (His263)$N^{\varepsilon 2}$-Zn2-inhibitor(S), 117/111° in molecules A/B.

The $2|F_o|-|F_c|$ electron density map clearly indicates the formation of a covalent amide bond between the ester and side chain N^{ζ} atom of Lys224 with the concomitant displacement of the pentafluorophenolate group, which is not seen in the electron density.

The carbonyl oxygen atom of the amide group formed between the inhibitor and IMP-1 forms a hydrogen bond with the main-chain nitrogen atom of Asn233 (ca. 3.1/3.3 Å in molecules A/B) while the carbonyl oxygen atom of the thioester group of the inhibitor is hydrogen bonded to the side chain nitrogen atom of Asn233 (ca. 3.0/3.5 Å in molecules A/B).

In summary, the synthesis of a covalent, irreversible inhibitor **2** of IMP-1 is described. An X-ray crystal structure of the inhibitor covalently bound to IMP-1 confirmed this strategy. These findings shed further light on the design of inhibitors based on the three-dimensional structure of MBLs related to IMP-1.

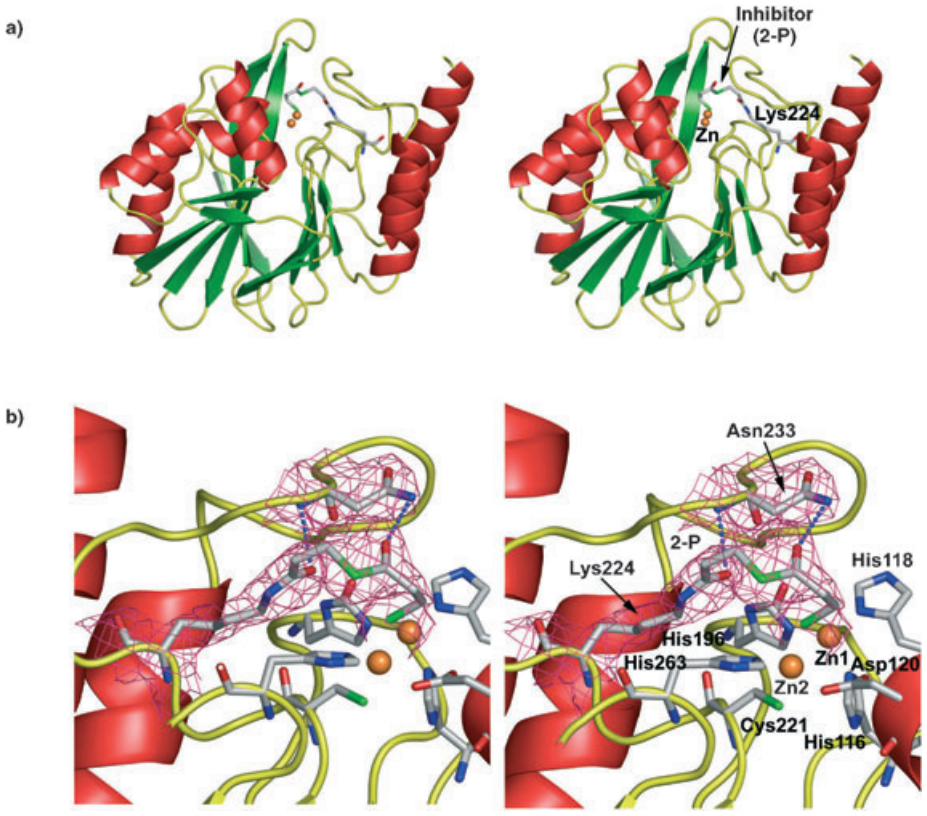


Figure 2. a) The overall structure of IMP-1 complexed with 2-P. α-Helices, β-strands, and loops are shown in red, green, and yellow, respectively. Zn^{2+} ions are represented as orange spheres. The inhibitor and Lys224 are displayed as sticks (C, N, O, and S atoms colored gray, blue, red, and green, respectively). b) The crystal structure of IMP-1 modified by **2**-P. The electron density of Asn233 as well as Lys224 and its covalently attached inhibitor molecule is shown countoured at 1.0σ in a $2|F_o|-|F_c|$ map. Hydrogen bonds are indicated by blue dashed lines.

Received: March 7, 2005
Published online: May 13, 2005

Keywords: bioorganic chemistry · inhibitors · lactams · metalloenzymes · structure elucidation

[1] Y. Arakawa, M. Murakami, K. Suzuki, H. Ito, R. Wacharotayankun, S. Ohsuka, N. Kato, M. Ohta, *Antimicrob. Agents Chemother.* **1995**, *39*, 1612–1615.
[2] M. L. Riccio, N. Franceschini, L. Boschi, B. Caravelli, G. Cornaglia, R. Fontana, G. Amicosante, G. M. Rossolini, *Antimicrob. Agents Chemother.* **2000**, *44*, 1229–1235.
[3] N. Laraki, M. Galleni, I. Thamm, M. L. Riccio, G. Amicosante, J. M. Frère, G. M. Rossolini, *Antimicrob. Agents Chemother.* **1999**, *43*, 890–901.
[4] D. M. Livermore, N. Woodford, *Curr. Opin. Microbiol.* **2000**, *3*, 489–495.
[5] M. Goto, T. Takahashi, F. Yamashita, A. Koreeda, H. Mori, M. Ohta, Y. Arakawa, *Biol. Pharm. Bull.* **1997**, *20*, 1136–1140.
[6] D. J. Payne, J. H. Bateson, B. C. Gasson, D. Proctor, T. Khushi, T. H. Farmer, D. A. Tolson, D. Bell, P. W. Skett, A. C. Marshall, R. Reid, L. Ghosez, Y. Combret,

J. Marchand-Brynaert, *Antimicrob. Agents Chemother.* **1997**, *41*, 135 – 140.
[7] N. O. Concha, C. A. Janson, P. Rowling, S. Pearson, C. A. Cheever, B. P. Clarke, C. Lewis, M. Galleni, J. M. Frère, D. J. Payne, J. H. Bateson, S. S. Abdel-Meguid, *Biochemistry* **2000**, *39*, 4288 – 4298.
[8] J. H. Toney, G. G. Hammond, P. M. Fitzgerald, N. Sharma, J. M. Balkovec, G. P. Rouen, S. H. Olson, M. L. Hammond, M. L. Greenlee, Y. D. Gao, *J. Biol. Chem.* **2001**, *276*, 31913 – 31918.
[9] S. Bounaga, M. Galleni, A. P. Laws, M. I. Page, *Bioorg. Med. Chem.* **2001**, *9*, 503 – 510.
[10] S. Siemann, D. P. Evanoff, L. Marrone, A. J. Clarke, T. Viswanatha, G. I. Dmitrienko, *Antimicrob. Agents Chemother.* **2002**, *46*, 2450 – 2457.
[11] I. García-Sáez, J. Hopkins, C. Papamicael, N. Franceschini, G. Amicosante, G. M. Rossolini, M. Galleni, J. M. Frère, O. Dideberg, *J. Biol. Chem.* **2003**, *278*, 23868 – 23873.
[12] M. Galleni, J. Lamotte-Brasseur, G. M. Rossolini, J. Spencer, O. Dideberg, J. M. Frère, *Antimicrob. Agents Chemother.* **2001**, *45*, 660 – 663.
[13] S. Haruta, E. T. Yamamoto, Y. Eriguchi, T. Sawai, *FEMS Microbiol. Lett.* **2001**, *197*, 85 – 89.
[14] R. Kitz, I. B. Wilson, *J. Biol. Chem.* **1962**, *237*, 3245 – 3249.
[15] The X-ray coordinates for the **2**-P/IMP-1 complex have been deposited in the Protein Data Bank under the code number: 1VGN.

