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Novel imidazole substituted 6-methylidene-penems as broad-spectrum β-lactamase inhibitors

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Abstract—β-Lactamases are serine and metallo-dependent enzymes produced by the bacteria in defense against β-lactam antibiotics. Production of class-A, class-B, and class-C enzymes by the bacteria make the use of β-lactam antibiotics ineffective in certain cases. To overcome resistance to β-lactam antibiotics, several β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam are widely used in the clinic in combination with β-lactam antibiotics. However, single point mutations within these enzymes have allowed bacteria to overcome the inhibitory effect of the commercially approved β-lactamase inhibitors. Although the commercially available β-lactamase inhibitor/β-lactam antibiotic combinations are effective against class-A producing bacteria and many extended spectrum β-lactamase (ESBL's) producing bacteria they are less effective against class-C enzymes expressing bacteria. To circumvent this problem, based on modeling studies several novel imidazole substituted 6-methylidene-penem derivatives were synthesized and tested against various β-lactamase producing isolates. The present paper deals with the synthesis and structure–activity relationships (SAR) of these compounds.

1. Introduction

β-Lactamases are serine-dependent enzymes produced by the bacteria in defense against all classes of β-lactam antibiotics, such as penicillins, cephalosporins, carbapenems, and monobactams. In order to overcome this resistance several β-lactamase inhibitor/β-lactam antibiotic combinations are currently being used in the clinical setting. However, single point mutations within these enzymes have rendered β-lactamases relatively resistant to inactivation.[1] Based upon the amino acid sequence variations, the β-lactamase enzymes have been divided into class-A, class-B, class-C, and class-D β-lactamases.[1d,2] Currently, only three β-lactamase inhibitors are used in the clinic in combination with β-lactam antibiotics (Fig. 1). Clinically employed β-lactamase inhibitors such as clavulanic acid[3a] **1** (given in combination with amoxicillin or ticarcillin), sulbactam[3b] **2** (given in combination with ampicillin), and tazobactam[3c] **3** (given in combination with piperacillin) are effective class-A enzyme inhibitors. However, these inhibitors/antibiotics combinations are the least effective against class-C and class-B producing bacteria including some extended spectrum β-lactamases (ESBL) producing bacteria.

During the last decade, several groups have focused on mechanistic considerations in designing new broad-spectrum inhibitors.[4,5] In this regard, boronates[6] have been reported as transition state inhibitors and acyl phosphonates and phosphonamidates[7] as active serine phosphorylating agents. In seeking insights into designing new 6-methylidene-penem inhibitors, we demonstrated that electrospray ionization mass spectrometry techniques provides a powerful method for characterizing transformations that occur upon the interaction of inhibitors with TEM-1, SHV-1, and AmpC β-lactamases.[8] High-resolution crystallographic structures of two novel penems carrying bicyclic or tricyclic heterocycles at the 6-methylidene moiety indicated the

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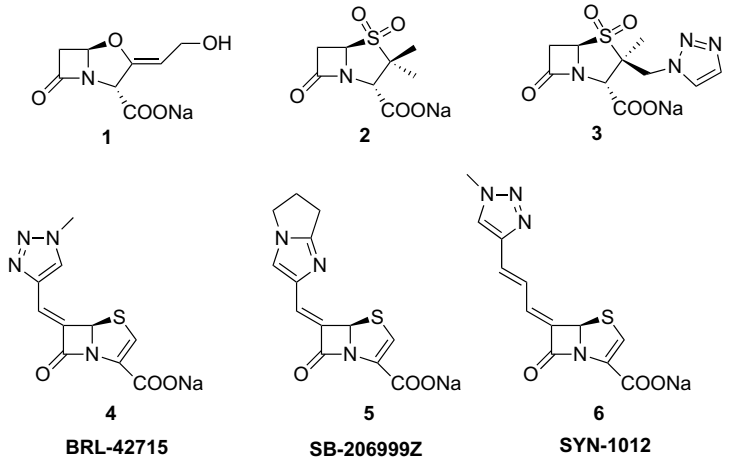


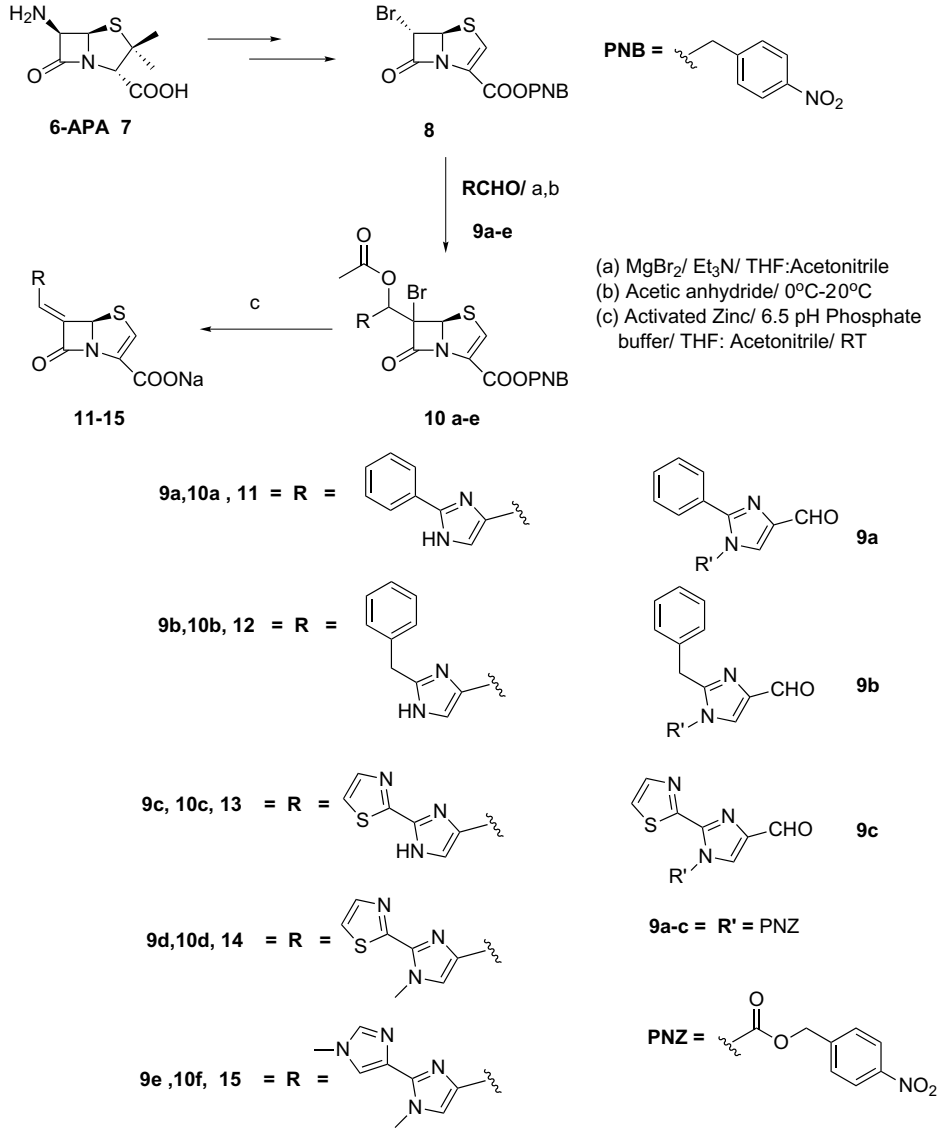
Figure 1. Currently used commercial β-lactamase inhibitors and penem inhibitors **4**–**6**.

dominance of hydrophobic stacking interactions between the heterocycle and amino acid residues in SHV-1 and GC1 enzymes.[9] The formation of 1,4-thiazepine ring structures[10] in both the enzymes was established with different orientation of the thiazepine ring and the heterocyclic moieties.[9] These findings for penems bearing bicyclic and tricyclic heterocyles at the methylidene moiety observed by crystallography[9] are different from modeling studies of a related monocyclic penem.[5,10,11] In the earlier modeling studies[5] the importance of hydrophobic interactions, the orientation of the thiazepine ring and the stereochemistry at the carbon bearing the heterocycle were not predicted. With these considerations in mind, we undertook the design and synthesis of penem inhibitors bearing a 6-methylidene monocyclic heterocyle. Earlier work has demonstrated that several penems containing furyl, oxazolyl, isoxazolyl, thiadiazolyl, and triazolyl rings are broad-spectrum inhibitors of class A and C enzymes.[12] However, 6-methylidene-penems bearing an imidazole nucleus have never been reported in the literature. Hence, it is the objective of this article to synthesize and elucidate the structure–activity relationships of several novel 6-methylidene-penems bearing imidazole derivatives against several bacteria expressing different β-lactamases. Unlike the other β-lactamase inhibitors,[3] the 6-methylidene structural feature of **4** imparts a unique mechanism of action for the β-lactamase inhibition.[5,8,11]

Based on the molecular modeling studies, and considerations for hydrophobicity, several novel 6-methylidene-penems bearing 2-substituted imidazole derivatives **11**–**15** were designed and synthesized. The inhibitor binding site is known in class-A and class-C β-lactamase enzymes.[13] However, due to the fast acylation of the active serine residues followed by ring opening, it has not been possible to use X-ray crystallography to capture the intact inhibitor structure with the enzyme as is also confirmed by mass spectrometric studies.[4,8] At the onset, a salt bridge between the inhibitor carboxylate and binding site lysine 234 in class A and 315 in class C, Amp-C helps in positioning the inhibitor β-lactam ring in the oxyanion hole.[13c,d] The residues around the penem moiety of the inhibitor present comparable interaction opportunities in TEM-1 and AmpC enzymes. However, there is significant difference between the remainder of the binding sites of TEM-1 and AmpC enzymes, which makes the design of broad-spectrum inhibitors challenging. Hypotheses for the atomic interactions were derived from several crystal structures available in the public domain with inhibitor fragments bound to the β-lactamase enzymes.[13c,d] In the present work these crystal structures were[13c,d] used to design new inhibitors. A binding mode for the penem moiety was predicted that was consistent with the derived hypotheses. Our molecular modeling work predicted higher potency for the *Z* configuration at the C6–C7 bond in the penem nucleus, which is also an observed fact with other 6-substituted methyledine penem derivatives. Subsequently, the triazole containing penem, BRL-42715, was manually docked in the TEM-1 (RCSB PDB code: 1BTL) and AmpC (RCSB PDB code: 1FSW) binding sites and a bioactive conformation was predicted. Keeping the penem moiety constant, novel methylidene substituents were designed that will be able to interact with amino acids of both, TEM-1 and AmpC binding sites.[13c,d] Due to sequence heterogeneity within each class, the designed inhibitors were refined against several experimentally determined and modeled mutant β-lactamase 3D structures. A hydrophobic substituent at the 2-position of the imidazole was designed that was predicted to enhance interaction with Glu 104 and Tyr 105 (in TEM-1) or Leu 119 and Gln 120 (in AmpC) residues. This hypothesis is reasonable since SYN-1012, compound **6**, demonstrated broad-spectrum activity and good synergy with many β-lactam antibiotics.[14] SYN-1012 is a penem with a methyltriazole substitution at the C6 position that differs from **4** by having an extended linker.

2. Chemistry

The 6-methylidene-penem carboxylic acid sodium salts **11**–**15** were prepared by a two step process (Scheme 1) starting from (5*R*,6*S*)-6-bromo-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid-4-nitro-benzyl



Scheme 1. General method to prepare compounds **11**–**15**.

ester **8** and the appropriately substituted aldehydes **9a**–**e**. The bromopenem derivative **8** required for this operation was prepared from the commercially available 6-amino penicillanic acid **7** (6-APA) by a modified multistep procedure as reported previously.[15] The first step involved a Lewis acid mediated (anhydrous $MgBr_2$) aldol condensation reaction between the appropriately substituted aldehyde **9a**–**e** and compound **8** in the presence of triethylamine as the base. The resulting diastereomeric mixture of bromohydrins was trapped as their respective acetylated derivatives **10a**–**e**. In the past,[15,16] this transformation was achieved by using strong bases such as LDA, LiHMDS, or Ph_2NLi. The use of the above mentioned strong bases led to poor yields and unpredicted side products. However, the use of a mild base in conjunction of a Lewis acid gave a better and reproducible yield without the formation of any side product. This is the first report, where $MgBr_2/Et_3N$ mediated procedure has been applied to 6-bromopenem derivative **8** with monocyclic aldehydes to form C6–C7 carbon bond. After standardizing this novel aldol condensation procedure, we next turned our attention to the reductive elimination procedure to introduce a Z-double bond between the C6 and C7, carbon atoms on the acetoxy bromohydrins **10a**–**e**. The resulting 6-methylidene-penem molecules **11**–**15** were prone to decomposition but stable at pH in the range of 5–9. Therefore, a neutral procedure was devised[17] using freshly prepared activated zinc and 0.5 M (pH 6.5) phosphate buffer. Zinc dust was activated using 0.1 N HCl for 15 min and filtered. It was washed well with deionized water and used as such. The amount of zinc required for this transformation was found to be four times the weight of the substrate bromohydrins. After several attempts with different solvent systems to carry out this transformation at room temperature, acetonitrile–THF (1:2) emerged as the most suitable combination.

The resultant crude products were purified by Diaion HP-21 resin (80 mL, Mitsubishi Kasei Co. Ltd) column

chromatography. After adsorbing, the column was eluted with water and then 5% acetonitrile aqueous solution. It is important to mention that at this juncture the use of activated zinc/phosphate buffer not only introduced the double bond between the C6 and C7 carbon atoms with the exclusive formation of *Z* isomer but also resulted in deprotection of the carboxyl functionality, thus avoiding an extra deprotection step. The reason for the exclusive formation of *Z* isomer is unknown and rather surprising. It might be due to the effect of the imidazole ring, which can chelate with the acetoxy-zinc enolate intermediate, which is formed during this reaction.[15,18] Osborne et al.[15] carried out this transformation in the preparation of **4** using Zn/NH_4Cl/TMEDA·2HCl in DMF at room temperature with a *E*:*Z* in the ratio of 1:13, respectively. Additionally, unlike the $MeAlCl_2$ procedure[15] (which was used previously to deprotect the carboxy functionality) the mild nature of this transformation avoided racemization at the C5 position. The aldehydes **9a**–**e** were prepared by the methods already reported in the literature.[19]

3. Biology

All the compounds synthesized were tested in vitro against TEM-1 and AmpC enzymes for their inhibitory ability.[20] β-Lactamase inhibitory activities were determined spectrophotometrically as described by Bush et al.[20] using nitrocefin as substrate. IC_{50} values were calculated using WinNonlin (Pharsight Corp., Mountainview, CA). In each experiment, tazobactam **3** was used as a standard. The potent compounds from the above mentioned enzyme inhibition in vitro assay, were tested in the antimicrobial susceptibility assay. In this assay, the in vitro activities of the antibiotics (piperacillin) and antibiotics+inhibitors (compound **11**–**15**) were determined by the broth microdilution method as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) using Mueller-Hinton II broth (MHBII, BBL Cockeysville, MD). Microtiter plates containing serial dilutions of each antimicrobial agents or antimicrobial agents+inhibitor were inoculated with each organism to yield the appropriate density (10^5 CFU/mL) in a 100 μL final volume. The plates were incubated for 18–24 h at 35 °C in ambient air. The minimum inhibitory concentration (MIC) for all isolates was defined as the lowest concentration of antimicrobial agent that completely inhibited the growth of the organisms as detected by the unaided eye. The MIC values presented in this article were determined for the inhibitor alone and in combination with piperacillin (Pip) at a constant concentration of 4 μg/mL of the inhibitor. The potent compounds in this assay were taken to an in vivo acute lethal infection model. In this model, five dose levels per compound with five female CD-1 (Charles River Laboratories) mice at each dose level were used. Each group was injected intraperitoneally with a lethal dose (10–100 LD_{50}) of the pathogen. The same group was injected intravenously with piperacillin alone or piperacillin combined with inhibitor (compounds **11**–**15**) at a ratio of 4:1 (Pip:inhibitor). The seven days survival ratio from three separate tests were pooled for determination of median effective dose (ED_{50}).

4. Structure–activity relationships

The inhibitory activities (IC_{50} values in nanomolar) of the five methylidene-penem derivatives **11**–**15** and tazobactam against TEM-1, Imi-1 (class-A), CcrA (class-B), and AmpC (class-C) are listed in Table 1. All the newly synthesized molecules were found to be potent inhibitors of both class-A and class-C enzymes except for compound **15**, which was found to be less active against Imi-1. All the new analogs were more potent than tazobactam. Against TEM-1, all the new compounds synthesized were 25–150-fold more potent than tazobactam. Tazobactam is a very weak inhibitor of AmpC enzyme and was 12,000–84,000-fold less active than all the newly synthesized inhibitors. But among these newly synthesized derivatives **11**–**15**, the *N*-methyl imidazolyl-2-thiazole derivative **13** and the corresponding desmethyl compound **14** were found to be about 10-fold less active than **12** against TEM-1. The 2-phenyl substituted imidazole derivative **11** was found to be less potent than the one carbon extended 2-benzyl substituted imidazole derivative **12** by about 10-fold. Molecular modeling suggested that the benzyl substituent in compound **12** forms a π–π stacking interaction with Tyr 105 in the TEM-1 active site (Fig. 2). In the case of phenyl analog **11** this interaction is predicted to be weaker, which might explain the differences in the potencies against TEM-1. Replacing the phenyl with 2-thiazole moiety (compound **13**) and subsequently methylating the imidazole nitrogen (compound **14**) did not alter the potency against TEM-1. However, replacing the 2-thiazole moiety in compound **14** with the *N*-methylimidazole group (compound **15**) increased the potency against TEM-1 and AmpC enzymes. But as mentioned before the inhibitory activity of compound

Table 1. In vitro activity of compounds **11**–**15** against different β-lactamases, IC_{50} (nM)

Compound	TEM-1 (IC_{50}, nM)	Imi-1 (IC_{50}, nM)	CcrA (IC_{50}, nM)	AmpC (IC_{50}, nM)
Tazobactam	100 ± 8	NA	400,000 ± 200	84,000 ± 300
11	4.0 ± 1	6 ± 2	250 ± 5	2.0 ± 1
12	0.4 ± 0.2	6 ± 3	230 ± 8	2.0 ± 1
13	6.0 ± 1	68 ± 4	260 ± 5	6.0 ± 1
14	5.0 ± 1	11 ± 50	220 ± 9	3.0 ± 0.2
15	1.0 ± 0.3	1300 ± 1	190 ± 7	1.0 ± 1

NA: Not available.

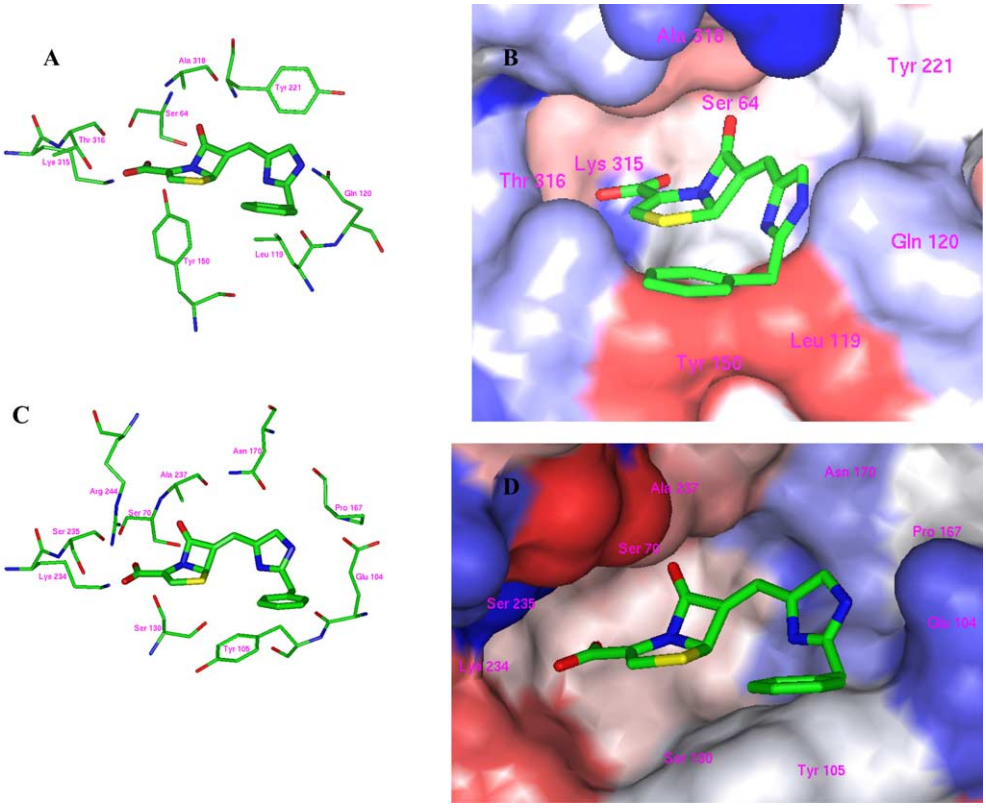


Figure 2. A and B: Compound **11** docked to Amp-C active site (class-C); C and D: compound **11** docked to TEM-1 active site (class-A).

15 against Imi-1, a carbapenem hydrolyzing enzyme[21] is less than the other molecules listed in Table 1. As can be seen from Table 1, all the newly synthesized compounds showed only a modest in vitro inhibitory activity against class-B enzymes. However, compounds **11**–**15** were found to be more potent than tazobactam against class-B enzyme.

5. In vitro antibacterial activity

The above mentioned in vitro potent compounds in the enzyme assay **11**–**15** were tested for their in vitro antibacterial activity alone and in combination with piperacillin against various bacteria expressing different β-lactamases. These data are summarized in Table 2. In all these experiments, piperacillin+tazobactam were used at a constant 4 μg/mL for comparison purpose. All the isolates enlisted in Table 2 were resistant to piperacillin alone (>64 μg/mL). When tested alone, neither tazobactam nor any of the newly synthesized penem inhibitors **11**–**15** exhibited antibacterial activity against the isolates used in this study, which established the lack of inherent antibacterial activity (except for *E. coli* GC 2804).

The newly synthesized compounds when combined with piperacillin enhanced its activity against piperacillin resistant β-lactamase producing organisms. The MIC values for piperacillin were reduced to 2–64 μg/mL in the presence of a constant 4 μg/mL of each of the inhibitors, including tazobactam against class-A producing isolates. It should be noted from Table 2 that even though the tazobactam+piperacillin combination has synergy against class-A producing organisms, this combination is less effective against class-C producing organisms (*E. cloacae* GC 2071, *E. cloacae* GC1475, *P. aeruginosa* GC 1764, and *S. marcescens*). However, the addition of the newly synthesized inhibitors **11**–**15** restored susceptibility to piperacillin for 50–90% of the strains. Especially the combination of compound **12** and piperacillin considerably reduced the MIC values of piperacillin against both class-A and class-C producing organisms rendering the susceptibility range to 90% of the organisms. This combination is very effective against *P. aeruginosa* GC 1764 as well.

6. In vivo efficacy

The in vivo data (ED_{50} values) of piperacillin and inhibitors (4:1 ratio) in a murine acute lethal infection model with *E. coli* LSU 80-8 (a TEM-1 producing organism) are listed in Table 3. When administered alone, piperacillin had no efficacy (ED_{50} > 128 mg/kg). When piperacillin was combined with tazobactam or compounds **11**, **13**–**15** the ED_{50} values were greater than 64 mg/kg. However, compound **12**, when co-administered intravenously, reduced the ED_{50} value of piperacillin to 43 mg/kg. It should be stressed here the in vivo data (ED_{50} values) of piperacillin reported here were only performed against a TEM-1 producing β-lactamase. The results of the ED_{50} values could be different if used against other hyperproducing extended spectrum

Table 2. In vitro antimicrobial activity of inhibitors[a] in combination with piperacillin (Pip) at a constant 4 μg/mL concentration

Species and strain	Expression	Tazo+Pip	Pip+**11**	Pip+**12**	Pip+**13**	Pip+**14**	Pip+**15**
E. coli GC 2844	None	2	2	2	2	2	2
E. coli GC 2847	TEM-1	2	16	2	64	32	64
E. coli GC 2920	IRT-2	4	2	2	2	1	0.5
E. coli GC 2883	OXA+10+PSE-2	2	4	2	8	8	16
E. coli GC 2894	AmpC	2	8	2	32	2	32
E. coli GC 2905	P99	2	4	1	1	4	2
E. coli GC 2906	Imi-1	2	4	2	64	4	64
E. coli GC 2804	Imp	0.12	<0.06	<0.06	<0.06	<0.06	<0.06
E. coli GC 2805	CcrA	>64	>64	>64	>64	>64	>64
E. coli GC 2252	IRT-2	2	16	16	32	16	>64
E. cloacae GC 1477	AmpC	>64	32	32	>64	32	>64
E. cloacae GC 2071	Imi+1+AmpC	>64	64	4	32	>64	>64
E. cloacae GC 1475	P99	>64	8	4	32	4	>64
K. pneumoniae GC 2825	4 bla's	>64	64	8	>64	>64	>64
S. marcescens GC 1781	Sme-1+AmpC	1	1	1	1	1	8
P. aeruginosa GC 1764	AmpC	>64	32	4	16	8	64
S. maltophilia GC 1712	L1	>64	>64	>64	>64	>64	>64
S. marcescens GC 4142	AmpC	>64	1	2	4	2	8
E. coli GC 2203	Control	>64	2	1	2	1	1
S. aureus GC 2216	Control	64	<0.06	<0.06	1	<0.06	0.5

[a] MIC values of piperacillin and the inhibitors when tested alone were found to be >64 μg/mL.

Table 3. In vivo efficacy (IV)[a] in a murine acute lethal infection model with *E. coli* LSU 80-8, a TEM-1 producing organism

Compound	ED_{50} (mg/kg)
Tazobactam	>64
11	>64
12	43 ± 5
13	>64
14	>64
15	>64

[a] Piperacillin; inhibitors = 4:1: ED_{50} value of piperacillin alone > 128 mg/kg.

β-lactamase strains or an inhibitor resistance β-lactamase strain. The in vivo efficacy of compound **12** in other acute lethal infection models against various bacteria, producing different classes of β-lactamases are under investigation.

7. Conclusion

In this paper, based on the modeling experiments and mechanistic understanding several novel imidazole substituted 6-methylidene-penem molecules were designed and synthesized as a potent β-lactamase inhibitors. Preparation of these compounds **11**–**15** was synthetically challenging and therefore a novel Lewis acid mediated aldol condensation reaction was standardized. This novel C6–C7 bond formation reaction was shown to be rather mild, efficient, and a useful variation of the aldol reaction. In this paper, we have also described a mild and efficient reductive elimination procedure that can be applied to other pH sensitive molecules such as penems. The newly synthesized compounds have a broader spectrum of activity than any of the currently available inhibitors in the market and were shown to have excellent in vitro activity against both class-A and class-C enzymes. In vitro antimicrobial susceptibility testings, when compounds **11**–**15** were combined with piperacillin, the MIC's values for piperacillin were reduced to susceptible range for most of the organisms enlisted in Table 1. Especially the 2-benzyl substituted imidazole derivative **12**, when combined with piperacillin, rendered 90% of the organisms listed here susceptible. The same compound **12**, in vivo enhanced the activity of piperacillin against *E. coli* LSU 80-8, a TEM-1 producing organism. Further investigations in various organisms are in progress to determine their potential as therapeutic agents.

8. Experimental section

8.1. General methods

Melting points were determined in an open capillary tube on a Meltemp melting point apparatus (Laboratory Devices, Cambridge, MA) and are uncorrected. ^{1}H NMR spectra were determined with Bruker DPX-400 spectrometer at 400 MHz. Chemical shifts δ are reported in parts per million (δ) relative to residual chloroform (7.26 ppm), TMS (0 ppm), or dimethylsulfoxide (2.49 ppm) as an internal reference with coupling constants (J) reported in hertz (Hz). The peak shapes are denoted as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. Electrospray (ES) mass spectra were recorded in positive or negative mode on a Micromass Platform spectrometer. Electron impact and high-resolution mass spectra were obtained on a Finnigen MAT-90 spectrometer. Combustion analysis were obtained using Perkin–Elmer Series II 2400 CHNS/O analyzer (Robertson Microlit, Maddison, NJ). Chromatographic purifications were performed by open chromatography using IBW-127ZH (Fuji Silysia). Thin-layer chromatography (TLC) was performed on Merck PLC prescored plates $_{60}F_{254}$. The terms 'concentrated' and 'evaporated' refer to the removal of solvents using a rotary evaporator at water aspirator pressure with

a bath temperature equal to or less than 40 °C. Unless otherwise noted, reagents were obtained from commercial sources and were used without further purification.

8.2. Materials and methods

8.2.1. Molecular modeling. Hydrogen atoms were added to the pdb files, 1BTL (TEM-1) and 1FSW (Amp-C), and energy minimized in Quanta/CHARMM29 (Accelrys Inc., San Diego, CA). BRL-42715 was modeled using Quanta and energy minimized with Gaussian 98 using 6-31G* basis set. CHARMM29 was parameterized to reproduce the inhibitor geometry obtained from quantum mechanics energy minimization. Inhibitor conformations that were up to 5 kcal/mol above the global minima were manually docked in the β-lactamase inhibitor binding pockets of TEM-1 and AmpC proteins. The inhibitor was docked placing the penem carboxylate oxygen 2.4 A away from the Lys 234 (TEM-1)/Lys 315 (AmpC) Nz. Assuming a short distance required for the nucleophillic attack, the lactam oxygen was placed 2.2 A away from the Ser 70 (TEM-1)/Ser 64 (AmpC). The bioactive inhibitor conformation was chosen based upon lowest interaction energy (calculated using CHARMM29) with the binding pocket amino acids. Inhibitors with the lowest interaction energies were recommended for synthesis.

8.3. Preparation of (5*R*),(6*Z*)-6-(2-phenyl-1*H*-imidazol-4-ylmethylene)-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid sodium salt (11)

8.3.1. Step 1: Preparation of 4-formyl-2-phenyl-imidazole-1-carboxylic acid 4-nitro-benzyl ester (9a). To a stirred mixture of 4-formyl-2-phenylimidazole (624 mg) and sodium hydrogen carbonate (791 mg) in dioxane (3.6 mL), THF (3.6 mL), and water (7.2 mL), 48.7% solution of *p*-nitrobenzyl chloroformate (PNZCl) in dioxane (2.08 mL) was added at room temperature and stirred for 2.5 h. The reaction mixture was diluted with ethyl acetate and washed with brine. The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The residue was crystallized from ethyl acetate and *n*-hexane to give the title compound (956 mg, 75%). ^{1}H NMR (δ, $CDCl_3$) 5.41 (s, 2H), 7.32 (d, 2H, $J = 8.6$ Hz), 7.40–7.51 (m, 3H), 7.56–7.58 (m, 2H), 8.17–8.20 (m, 2H), 8.22 (s, 1H), 9.97 (s, 1H).

8.3.2. Step 2: Preparation of (5*R*,6*RS*)-6-{(*RS*)-acetoxy-[1-(4-nitro-benzyloxycarbonyl)-2-phenyl-1*H*-imidazol-4-yl]-methyl}-6-bromo-7-oxo-4-thia-1-aza-bicyclo[3.2.0]-hept-2-ene-2-carboxylic acid 4-nitro-benzyl ester (10a). 4-Formyl-2-phenyl-imidazole-1-carboxylic acid 4-nitro-benzyl ester (568 mg) and the dry THF solution (15 mL) of (5*R*,6*S*)-6-bromo-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid 4-nitro-benzyl ester (587 mg) were added successively to the dry acetonitrile (15 mL) solution of anhydrous $MgBr_2$ (622.4 mg) under a nitrogen atmosphere at room temperature. After cooling the reaction mixture to −20 °C, triethylamine (0.494 mL) was added in one portion. The reaction vessel was covered with a foil to exclude light. The reaction mixture was stirred for 7 h at −20 °C and treated with acetic anhydride (0.277 mL) in one portion and warmed to 0 °C and stirred at that temperature for 27 h. The mixture was diluted with ethyl acetate and washed with 5% citric acid aqueous solution, saturated sodium hydrogen carbonate, and brine. The organic layer was dried ($MgSO_4$) and filtered through a pad of Celite. The pad was washed with ethyl acetate and the filtrate was concentrated under reduced pressure. The residue was applied to silica gel column chromatography and eluted with ethyl acetate–hexane (2/3–1/1). The title compound was obtained as three diastereomixture (6/4/1.5, a pale yellow amorphous solid, 986 mg, 86%). ^{1}H NMR (δ, $CDCl_3$) 2.04 (s, 0.35×3H), 2.10 (s, 0.13×3H), 2.30 (s, 0.52×3H), 5.25–5.43 (m, 4H), 6.09 (s, 0.52×1H), 6.22 (s, 0.13×1H), 6.31 (s, 0.35×1H), 6.33 (s, 0.35×1H), 6.87 (s, 0.13×1H), 6.92 (d, 0.52×1H, $J = 1.4$ Hz), 7.31–7.76 (m, 11H), 8.17–8.25 (m, 4H).

8.3.3. Step 3: Preparation of (5*R*),(6*Z*)-6-(2-phenyl-1*H*-imidazol-4-ylmethylene)-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid sodium salt (11). (5*R*,6*RS*)-6-{(*RS*)-Acetoxy-[1-(4-nitro-benzyloxycarbonyl)-2-phenyl-1*H*-imidazol-4-yl]-methyl}-6-bromo-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid 4-nitro-benzyl ester (**10a**) (1.15 g) was dissolved in THF (16.1 mL) and acetonitrile (7.5 mL). Freshly activated Zn dust [a mixture of Zn dust (15 g) and 0.1 N HCl was stirred at room temperature for 15 min and filtered. Washed well with water and 0.5 M phosphate buffer and used as such] (4.6 g) and 0.5 M phosphate buffer (pH 6.4, 23.6 mL) were added to the mixture. The reaction vessel was covered with a foil to exclude light and vigorously stirred for 3 h at room temperature. At the end, reaction solution was cooled to 10 °C and 1 M NaOH solution was added to adjust the pH to 7.5. Reaction mixture was mixed with ethyl acetate and filtered through a pad of Celite. The pad was washed with water and the aqueous layer was separated. The aqueous layer was concentrated under high vacuum at 35 °C and applied to Diaion HP-21 (100 mL, Mitsubishi Kasei Co. Ltd) resin column chromatography. After adsorbing, the column was eluted with water and then with 5–10% acetonitrile–water. The combined fractions were concentrated under high vacuum at 35 °C and lyophilized to give the title compound as a yellow amorphous solid (322 mg, 63%). Mp 281 °C (dec); ^{1}H NMR (δ, D_2O) 6.32 (s, 1H), 6.76 (s, 1H), 6.79 (s, 1H), 7.22 (s, 1H), 7.24–7.33 (m, 3H), 7.60–7.63 (m, 2H).

8.4. Preparation of (5*R*),(6*Z*)-6-(2-benzyl-1*H*-imidazol-4-ylmethylene)-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid sodium salt (12)

8.4.1. Step 1: Preparation of 2-benzyl-1*H*-imidazole-4-carbaldehyde. 2-Phenyl-acetoamidine (2 g) was added to the solution of 2-bromo-3-isopropoxy-propenal (2.88 g) in $CHCl_3$ (30 mL) and stirred for 1 h at room temperature. Triethylamine (2.09 mL) was added to the mixture and heated to reflux for 6 h. The mixture was cooled to room temperature, diluted with $CHCl_3$, and washed with 10% potassium hydrogen carbonate. The organic layer was dried ($MgSO_4$) and concentrated under reduced

pressure. The residue was purified by silica gel column chromatography and eluted with ethyl acetate–hexane (3/1). The crude compound was crystallized from ethyl acetate and *n*-hexane to give the title compound (1.10 g, 40%). ^{1}H NMR (δ, $CDCl_3$) 4.17 (s, 2H), 7.24–7.36 (m, 5H), 7.72 (br s, 1H), 9.60 (br s, 1H), 10.28 (br s, 1H).

8.4.2. Step 2: Preparation of 2-benzyl-4-formyl-imidazole-1-carboxylic acid 4-nitro-benzyl ester (9b). To a stirred solution of 2-benzyl-1*H*-imidazole-4-carbaldehyde (1 g) and sodium hydrogen carbonate (1.13 g) in dioxane–water (1:1, 60 mL), 48.7% solution of *p*-nitrobenzyl chloroformate (PNZCl) in dioxane (2.62 g) was added at 0 °C and stirred for 2.5 h. The reaction mixture was diluted with ethyl acetate and washed with saturated sodium hydrogen carbonate and brine. The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The residue was applied to silica gel column chromatography and eluted with chloroform–acetone (9/1). The title compound was obtained as pale brown oil (1.97 g, 100%). ^{1}H NMR (δ, $CDCl_3$) 4.46 (s, 2H), 5.42 (s, 2H), 7.19–7.30 (m, 5H), 7.44–7.47 (m, 2H), 8.06 (s, 1H), 8.21–8.25 (m, 2H), 9.91 (s, 1H).

8.4.3. Step 3: Preparation of (5*R*,6*RS*)-6-{(*RS*)-acetoxy-[2-benzyl-1-(4-nitro-benzyloxycarbonyl)-1*H*-imidazol-4-yl]-methyl}-6-bromo-7-oxo-4-thia-1-aza-bicyclo[3.2.0]-hept-2-ene-2-carboxylic acid 4-nitro-benzyl ester (10b). To a stirred solution of 2-benzyl-4-formyl-imidazole-1-carboxylic acid 4-nitro-benzyl ester (1.9 g) in dry acetonitrile (10 mL) was added a dry acetonitrile (50 mL) solution of anhydrous $MgBr_2$ (2.41 g) under a nitrogen atmosphere at room temperature. The dry THF solution (60 mL) of (5*R*,6*S*)-6-bromo-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid 4-nitro-benzyl ester (2 g) was added to the mixture, cooled to −20 °C, and triethylamine (1.8 mL) was added in one portion. The reaction vessel was covered with a foil to exclude light. The reaction mixture was stirred for 6 h at −20 °C and treated with 4-dimethylaminopyridine (127 mg) and acetic anhydride (0.98 mL) in one portion. The reaction mixture was warmed to 0 °C and stirred for 16 h at 0 °C. The mixture was diluted with ethyl acetate and washed with H_2O, saturated sodium hydrogen carbonate, and brine. The organic layer was dried ($MgSO_4$) and filtered through a pad of Celite and washed with ethyl acetate. The filtrate was concentrated under reduced pressure. The residue was applied to silica gel column chromatography, then eluted with ethyl acetate–hexane (2/3–1/1). The title compound was obtained as two diastereomixture (3/2, a pale yellow amorphous solid, 2.8 g, 70%). ^{1}H NMR (δ, $CDCl_3$) 2.00 (s, 0.6 × 3H), 2.26 (s, 0.4 × 3H), 4.34–4.37 (m, 2H), 5.25–5.47 (m, 4H), 6.02 (s, 0.4 × 1H), 6.22 (s, 0.6 × 1H), 6.25 (s, 0.6 × 1H), 6.85 (d, 0.4 × 1H, J = 1.5 Hz), 7.14–7.62 (m, 11H), 8.21–8.25 (m, 4H).

8.4.4. Step 4: Preparation of (5*R*),(6*Z*)-6-(2-benzyl-1*H*-imidazol-4-ylmethylene)-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid sodium salt (12). (5*R*,6*RS*)-6-{(*RS*)-Acetoxy-[2-benzyl-1-(4-nitro-benzyloxycarbonyl)-1*H*-imidazol-4-yl]-methyl}-6-bromo-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid 4-nitro-benzyl ester (**10b**) (2.5 g) was dissolved in THF (35 mL) and acetonitrile (16.3 mL). Freshly activated Zn dust (10 g) and 0.5 M phosphate buffer (pH 6.4, 51.3 mL) were added to the mixture. The reaction vessel was covered with a foil to exclude light and stirred vigorously for 2 h at room temperature. The mixture was cooled to 10 °C, and 1 M NaOH aqueous solution was added to adjust pH to 7.5. The reaction solution was mixed with ethyl acetate and filtered through a pad of Celite. The pad was washed with water and the aqueous layer was separated. The aqueous layer was concentrated under high vacuum at 35 °C and applied to Diaion HP-21 (250 mL, Mitsubishi Kasei Co. Ltd) resin column chromatography. After adsorbing, the column was eluted with water and then with 5–10% acetonitrile–water. The combined fractions were concentrated under high vacuum at 35 °C and lyophilized to give the title compound as a yellow amorphous solid (780 mg, 67%). Mp 146 °C (dec); ^{1}H NMR (δ, D_2O) 3.92 (s, 2H), 6.39 (d, 1H, J = 0.8 Hz), 6.74 (s, 1H), 6.89 (s, 1H), 7.13–7.16 (m, 3H), 7.21–7.25 (m, 3H).

8.5. Preparation of (5*R*),(6*Z*)-6-(2-thiazol-2-yl-1*H*-imidazol-4-ylmethylene)-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid sodium salt (13)

8.5.1. Step 1: Preparation of 4-formyl-2-thiazol-2-yl-imidazol-1-carboxylic acid 4-nitro-benzyl ester (9c). To a stirred solution of 2-thiazol-2-yl-1*H*-imidazol-4-carbaldehyde (570 mg in dry CH_2Cl_2 (35 mL), *N*,*N*′-diisopropylethylamine and 48.7% solution of *p*-nitrobenzyl chloroformate (PNZCl) in dioxane (1.69 mL) were added slowly at 0 °C. The reaction mixture was stirred for 2 h and diluted with $CHCl_3$. The organic layer was washed with saturated sodium hydrogen carbonate in brine and dried over $MgSO_4$. It was concentrated under reduced pressure and the residue was crystallized from ethyl acetate and *n*-hexane to give the title compound (1.05 g, 92%). ^{1}H NMR (δ, $CDCl_3$) 5.49 (s, 2H), 7.50 (d, 2H, J = 8.8 Hz), 7.55 (d, 1H, J = 3.4 Hz), 7.89 (d, 1H, J = 3.1 Hz), 8.19 (s, 1H), 8.24 (dq, 2H, J = 8.8, 2.0 Hz), 9.97 (s, 1H).

8.5.2. Step 2: Preparation of (5*R*,6*RS*)-6-{(*RS*)-acetoxy-[1-(4-nitro-benzyloxycarbonyl)-2-thiazol-2-yl-1*H*-imidazol-4-yl]-methyl}-6-bromo-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid 4-nitro-benzyl ester (10c). 4-Formyl-2-thiazol-2-yl-imidazol-1-carboxylic acid 4-nitro-benzyl ester (940 mg) was added to the dry acetonitrile (35 mL) solution of anhydrous $MgBr_2$ (1.26 g) under a nitrogen atmosphere at room temperature. A dry THF solution (28 mL) of (5*R*,6*S*)-6-bromo-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid 4-nitro-benzyl ester (1.01 g) was added to the mixture, cooled to −20 °C, and triethylamine (0.942 mL) was added in one portion. The reaction vessel was covered with a foil to exclude light and stirred for 4 h at −20 °C and treated with 4-dimethylaminopyridine (64 mg) and acetic anhydride (0.495 mL) in one portion. The reaction mixture was warmed to 0 °C and stirred for 20 h at 0 °C. The mixture was diluted with ethyl acetate and washed with 0.1 M phosphate buffer (pH 7), saturated sodium hydrogen carbonate, and brine. The

organic layer was dried over anhydrous $MgSO_4$ and filtered through a pad of Celite and washed with ethyl acetate. The filtrate was concentrated under reduced pressure and the residue was purified by silica gel column chromatography (ethyl acetate–hexane = 1/1 ~ethyl acetate only) and (5*R*,6*RS*)-6-(*RS*)-acetoxy-[1-(4-nitro-benzyloxycarbonyl)-2-thiazol-2-yl-1*H*-imidazol-4-yl]-methyl-6-bromo-7-oxo-4-thia-1-aza-bicyclo[3.2.0]-hept-2-ene-2-carboxylic acid 4-nitro-benzyl ester (860 mg) was obtained as a spongy solid.

8.5.3. Step 3: Preparation of (5*R*),(6*Z*)-6-(2-thiazol-2-yl-1*H*-imidazol-4-ylmethylene)-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid sodium salt (13). To a stirred solution of (5*R*,6*RS*)-6-{(*RS*)-acetoxy-[1-(4-nitro-benzyloxycarbonyl)-2-thiazol-2-yl-1*H*-imidazol-4-yl]-methyl}-6-bromo-7-oxo-4-thia-1-aza-bicyclo[3.2.0]-hept-2- ene-2-carboxylic acid 4-nitro-benzyl ester (860 mg) in THF (12 mL) and acetonitrile (5.6 mL) freshly activated Zn dust (3.44 g) and 0.5 M phosphate buffer (pH 6.4, 17.6 mL) were added. The reaction vessel was covered with a foil to exclude light and stirred vigorously for 2 h at room temperature. At the end, reaction mixture was cooled to 10 °C and 1 M NaOH solution was added to adjust the pH to 7.5. The reaction mixture was mixed with ethyl acetate and filtered through a pad of Celite. The pad was washed with water and the aqueous layer was separated. The aqueous layer was concentrated under high vacuum at 35 °C and applied to Diaion HP-21 (90 mL, Mitsubishi Kasei Co. Ltd) resin column chromatography. After adsorbing, the column was eluted with water and then with 2.5–5% acetonitrile–water. The combined fractions were concentrated under high vacuum at 35 °C and lyophilized to give the title compound as a yellow amorphous solid (126 mg, 11%). Mp 145 °C (dec); ^{1}H NMR (δ, D_2O) 6.40 (d, 1H, J = 0.7 Hz), 6.81 (s, 1H), 6.89 (s, 1H), 7.38 (s, 1H), 7.48 (d, 1H, J = 3.1 Hz), 7.71 (d, 1H, J = 3.3 Hz).

8.6. Preparation of (5*R*),(6*Z*)-6-(1-methyl-2-thiazol-2-yl-1*H*-imidazol-4-ylmethylene)-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid sodium salt (14)

8.6.1. Step 1: Preparation of 1-methyl-2-thiazol-2-yl-1*H*-imidazol-4-carbaldehyde (9d). To a stirred solution of potassium *tert*-butoxide (494 mg) in dry THF–DMF (3:1, 40 mL) 2-thiazol-2-yl-1*H*-imidazol-4-carbaldehyde (717 mg) and 18-crown-6 (106 mg) was added at room temperature. The reaction mixture was stirred for 10 min and treated with methyl iodide (0.274 mL). After stirring for 17.5 h at room temperature, it was concentrated under reduced pressure. The residue was dissolved with ethyl acetate and filtered and the filtrate was evaporated under reduced pressure. The crude material was purified with silica gel column chromatography (ethyl acetate–hexane=1/1). The title compound (410 mg, 53%) and its regio-isomer 1-methyl-2-thiazol-2-yl-1*H*-imidazol-5-carbaldehyde (240 mg, 31%) were obtained as a white solid.

1-Methyl-2-thiazol-2-yl-1*H*-imidazol-4-carbaldehyde: ^{1}H NMR (δ, $CDCl_3$) 4.21 (s, 3H), 7.44 (d, 1H, J = 3.2 Hz), 7.68 (s, 1H), 7.89 (d, 1H, J = 3.2 Hz), 9.91 (s, 1H).

1-Methyl-2-thiazol-2-yl-1*H*-imidazol-5-carbaldehyde: ^{1}H NMR (δ, $CDCl_3$) 4.48 (s, 3H), 7.51 (d, 1H, J = 3.1 Hz), 7.82 (s, 1H), 7.97 (d, 1H, J = 3.1 Hz), 9.82 (s, 1H).

8.6.2. Step 2: Preparation of (5*R*,6*RS*)-6-[(*RS*)-acetoxy-(1-methyl-2-thiazol-2-yl-1*H*-imidazol-4-yl)-methyl]-6-bromo-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid 4-nitro-benzyl ester (10d). 1-Methyl-2-thiazol-2-yl-1*H*-imidazol-4-carbaldehyde (380 mg) was added to the dry acetonitrile (28 mL) solution of anhydrous $MgBr_2$ (1.03 g) under a nitrogen atmosphere at room temperature. A colorless powder was deposited over a period of 10 min. To this suspension a dry THF solution (28 mL) of (5*R*,6*S*)-6-bromo-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid 4-nitro-benzyl ester (760 mg) was added and cooled to −20 °C. To this stirred solution triethylamine (0.768 mL) was added in one portion. The reaction vessel was covered with a foil to exclude light and was stirred for 5 h at −20 °C and treated with 4-dimethylaminopyridine (48 mg) and acetic anhydride (0.371 mL) in one portion. The reaction mixture was warmed to 0 °C and stirred for 22 h at this temperature. At the end, the mixture was diluted with ethyl acetate and washed with 5% citric acid aqueous solution, saturated sodium hydrogen carbonate, and brine. The organic layer was dried ($MgSO_4$) and filtered through a pad of Celite and the pad was washed with ethyl acetate. The filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography and eluted with ethyl acetate–hexane (3/2). The title compound was obtained as two diastereomeric mixture (1/1, a pale yellow amorphous solid, 673.4 mg, 52%). ^{1}H NMR (δ, $CDCl_3$) 2.00 (s, 0.5 × 3H), 2.28 (s, 0.5 × 3H), 4.12 (s, 3H), 5.28 (d, 0.5 × 1H, J = 13.5 Hz), 5.29 (d, 0.5 × 1H, J = 13.5 Hz), 5.44 (d, 0.5 × 1H, J = 13.5 Hz), 5.47 (d, 0.5 × 1H, J = 13.5 Hz), 6.16 (s, 0.5 × 1H), 6.30 (s, 0.5 × 1H), 6.42 (s, 0.5 × 1H), 6.87 (d, 0.5 × 1H, J = 0.8 Hz), 6.92 (d, 0.5 × 1H, J = 0.8 Hz), 7.19 (s, 0.5 × 1H), 7.34–7.36 (m, 1H), 7.47 (s, 0.5 × 1H), 7.50 (s, 0.5 × 1H), 7.60–7.64 (m, 2H), 7.83–7.85 (m, 1H), 8.23–8.26 (m, 1H).

8.6.3. Step 3: Preparation of (5*R*),(6*Z*)-6-(1-methyl-2-thiazol-2-yl-1*H*-imidazol-4-ylmethylene)-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid sodium salt (14). To a stirred solution of (5*R*,6*RS*)-6-[(*RS*)-acetoxy-(1-methyl-2-thiazol-2-yl-1*H*-imidazol-4-yl)-methyl]-6-bromo-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid 4-nitro-benzyl ester (**10d**) (604 mg) in THF (8.5 mL)–acetonitrile (3.9 mL) freshly activated Zn dust (2.41 g) and 0.5 M phosphate buffer (pH 6.4, 23.6 mL) were added. The reaction vessel was covered with a foil to exclude light and vigorously stirred for 3 h at room temperature. At the end, reaction mixture was cooled to 10 °C and 1 M NaOH solution was added to adjust the pH to 7.5. The reaction solution was mixed with ethyl acetate and filtered through a pad of Celite. The pad was washed with water and the aqueous layer was separated and concentrated under high vacuum at

35 °C. The concentrate was applied to Diaion HP-21 (60 mL, Mitsubishi Kasei Co. Ltd) resin column chromatography. After adsorbing, the column was eluted with water and then with 5% acetonitrile–water and 10% acetonitrile–water. The combined fractions were concentrated under high vacuum at 35 °C and lyophilized to give the title compound as a yellow amorphous solid (231 mg, 64%). Mp 214 °C (dec); ^{1}H NMR (δ, D_2O) 3.91 (s, 3H), 6.44 (s, 1H), 6.84 (s, 1H), 6.91 (s, 1H), 7.43 (s, 1H), 7.60 (d, 1H, J = 3.3 Hz), 7.84 (d, 1H, J = 3.3 Hz).

8.7. Preparation of (5*R*),(6*Z*)-6-(3,1′-dimethyl-3H,1′H-[2,4′]biimidazolyl-4-ylmethylene)-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid sodium salt (15)

8.7.1. Step 1: Preparation of 3,1′-dimethyl-3H,1′H-[2,4′]biimidazolyl-4-carbaldehyde (9e). To a stirred solution of potassium *tert*-butoxide (3.23 g) in dry DMF (140 mL),1H,1′H-[2,4′]biimidazolyl-4-carbaldehyde (1.95 g) and 18-crown-6 (634 mg) were added at 0 °C. The reaction mixture was stirred for 10 min and treated with methyl iodide (1.85 mL). After stirring for 30 min at 0 °C the reaction mixture was stirred at room temperature for 17 h. The reaction mixture was concentrated under reduced pressure and the residue was extracted with chloroform, washed well with water and dried over anhydrous $MgSO_4$. The organic layer was filtered and evaporated under reduced pressure. The crude material was purified by silica gel column chromatography (chloroform–methanol = 9/1). The title compound (887 mg, 39%) and its regio-isomer 1,1′-dimethyl-1H,1′H-[2,4′]biimidazolyl-4-carbaldehyde (296 mg, 13%) were obtained as a pale pink solid.

3,1′-Dimethyl-3H,1′H-[2,4′]biimidazolyl-4-carbaldehyde: ^{1}H NMR (δ, $CDCl_3$) 3.78 (s, 3H), 4.37 (s, 3H), 7.51 (d, 1H, J = 1.0 Hz), 7.62 (d, 1H, J = 1.0 Hz), 7.76 (s, 1H), 9.71 (s, 1H).

1,1′-Dimethyl-1H,1′H-[2,4′]biimidazolyl-4-carbaldehyde: ^{1}H NMR (δ, $CDCl_3$) 3.76 (s, 3H), 4.10 (s, 3H), 7.47 (d, 1H, J = 0.9 Hz), 7.57 (s, 1H), 7.59 (d, 1H J = 0.9 Hz), 9.85 (s, 1H).

8.7.2. Step 2: Preparation of (5*R*,6*RS*)-6-[(*RS*)-acetoxy-(3,1′-dimethyl-3H,1′H-[2,4′]biimidazolyl-4-yl)-methyl]-6-bromo-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid 4-nitro-benzyl ester (10e). To a stirred solution of 3,1′-dimethyl-3H,1′H-[2,4′]biimidazolyl-4-carbaldehyde (171 mg) in dry acetonitrile (20 mL) a solution of anhydrous $MgBr_2$ (800 mg) under a nitrogen atmosphere at room temperature was added. It was stirred at room temperature for 10 min and a dry THF solution (20 mL) of (5*R*,6*S*)-6-bromo-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid 4-nitro-benzyl ester (346 mg) was added to the mixture, cooled to −20 °C, and triethylamine (0.768 mL) was added in one portion. The reaction vessel was covered with a foil to exclude light. The reaction mixture was stirred for 6 h at −20 °C and treated with 4-dimethylaminopyridine (22 mg) and acetic anhydride (0.17 mL) in one portion and subsequently warmed to 0 °C and stirred for 17 h at that temperature. The mixture was diluted with ethyl acetate and washed with 5% citric acid aqueous solution, saturated sodium hydrogen carbonate, and brine. The organic layer was dried ($MgSO_4$) and filtered and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography and eluted with chloroform–methanol (9/1). The title compound was obtained as a spongy yellow solid. Yield: 507.6 mg, 91.4%. ^{1}H NMR (δ, $CDCl_3$) 2.19 (s, 3H), 3.75 (s, 3H), 4.03 (s, 3H), 5.29 (d, 1H, J = 13.5 Hz), 5.42 (d, 1H, J = 13.5 Hz), 6.03 (s, 1H), 6.65 (s, 1H), 7.34 (s, 1H), 7.48 (d, 1H, J = 1.2 Hz), 7.55 (d, 1H, J = 1.2 Hz), 7.59–7.61 (m, 3H), 8.23–8.26 (m, 2H).

8.7.3. Step 3: Preparation of (5*R*),(6*Z*)-6-(3,1′-dimethyl-3H,1′H-[2,4′]biimidazolyl-4-ylmethylene)-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid sodium salt (15). To a stirred solution of (5*R*,6*RS*)-6-[(*S*)-acetoxy-(3,1′-dimethyl-3H,1′H-[2,4′]biimidazolyl-4-yl)-methyl]-6-bromo-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid 4-nitro-benzyl ester (**10e**) (596 mg) in THF (8.3 mL) and acetonitrile (3.9 mL), freshly activated Zn dust (2.38 g) and 0.5 M phosphate buffer (pH 6.5, 12.2 mL) were added. The reaction vessel was covered with a foil to exclude light and stirred vigorously for 2 h at room temperature. At the end, reaction mixture was cooled to 10 °C and 1 M NaOH solution was added to adjust the pH to 7.5. The reaction solution was mixed with ethyl acetate and filtered through a pad of Celite. The pad was washed with water and the aqueous layer was separated and concentrated under high vacuum at 35 °C. The concentrate was applied to Diaion HP-21 (60 mL, Mitsubishi Kasei Co. Ltd) resin column chromatography. After adsorbing, the column was eluted with water and then with 5–10% acetonitrile–water. The combined fractions were concentrated under high vacuum at 35 °C and lyophilized to give the title compound as a yellow amorphous solid (66.3 mg, 19%). Mp 113 °C (dec); ^{1}H NMR (δ, D_2O) 3.31 (s, 3H), 3.52 (s, 3H), 5.74 (s, 1H), 6.43 (s, 2H), 6.70 (s, 1H), 7.27 (s, 1H), 7.46 (s 1H).

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References and notes

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