Structure-Activity Relationship of 6-Methylidene Penems Bearing 6,5 Bicyclic Heterocycles as Broad-Spectrum β-Lactamase Inhibitors: Evidence for 1,4-Thiazepine Intermediates with C7 R Stereochemistry by Computational Methods

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The design and synthesis of a series of 6-methylidene penems containing [6,5]-fused bicycles (thiophene, imidazole, or pyrazle-fused system) as novel class A, B, and C β-lactamase inhibitors is described. These penems proved to be potent inhibitors of the TEM-1 (class A) and AmpC (class C) β-lactamases and less so against the class B metallo-β-lactamase CcrA. Their in vitro and in vivo activities in combination with piperacillin are discussed. On the basis of the crystallographic structures of a serine-bound reaction intermediate of **2** with SHV-1 (class A) and GC1 (class C) enzymes, compounds **14a**−**l** were designed and synthesized. Penems are proposed to form a seven-membered 1,4 thiazepine ring in both class A and C β-lactamases. The interaction energy calculation for the enzyme-bound intermediates favor the formation of the C7 R enantiomer over the S enantiomer of the 1,4-thiazepine in both β-lactamases, which is consistent with those obtained from the crystal structure of **2** with SHV-1 and GC1.

Introduction

Resistance to β-lactam antibiotics in Gram-negative bacteria due to the production of β-lactamases is a serious medical problem with global consequences. β-Lactams are broadly classified into enzymes with a serine residue at the active site (classes A, C, D) or a zinc ion as a cofactor in metalloenzymes (class B). To date, over 340 β-lactamases have been identified with various degrees of spectrum activity and substrate specificity.[1] Extensive clinical use of β-lactam antibiotics in combination with inhibitors, such as clavulanic acid, sulbactam, and tazobactam resulted in the emergence of new β-lactamases of several types, such as TEM, SHV, CTX-M (class A), VIM (class B), AmpC, GC (class C), and Oxa (class D) in both plasmid and chromosomal forms as well as the extended-spectrum β-lactamases (ESBLs) with broad substrate specificity. Therefore, an intensive search for a new generation of β-lactamase inhibitors with a broader spectrum of activity[2] than the clinically used inhibitors in β-lactam[3−12] or non-β-lactam[13] chemical classes continues unabated.

Functionally, β-lactamases hydrolyze β-lactam rings in all classes of β-lactam antibiotics, thus rendering these agents ineffective. The catalytic mechanism of β-lactamases is currently under active investigation using a plethora of biophysical methods.[14] In one such study, electrospray ionization mass spectrometry techniques were employed to characterize the kinetics of the reaction of 6-methylidene penems, including tetrahydroimidazo[1,2-*a*]pyrazine dihydroimidazo[2,1-*c*]oxazine and dihydropyrrolo[1,2-*b*]pyrazole penems **1**−**3**, respectively with TEM-1, SHV-1, and AmpC β-lactamases confirming their attachment to active serine residues and their rearrangement to dihydro [1,4]thiazepines **4**−**6** (Figure 1).[15] The high-resolution crystallographic structure determination of the reaction product of **2** with SHV-1 and GC1 β-lactamases revealed that 1,4 dihydrothiazepine intermediate **5** is oriented differently in each complex following the initial acylation reaction with Ser70 and Ser64, respectively.[16] The core dihydrothiazepine rings in the two complexes are generally in the same position but differ by a 180° rotation about the connecting bond to the acylated serine ester. Tyr105 in the SHV-1 complex was rotated 20° to establish hydrophobic π-stacking with the imidazo[2,1-*c*]oxazine ring, whereas the amino acid Tyrosine 224 in the GCI complex, is displaced ∼6 Å to establish such π-stacking with the heterocyclic ring. As a consequence to the binding mode in SHV-1

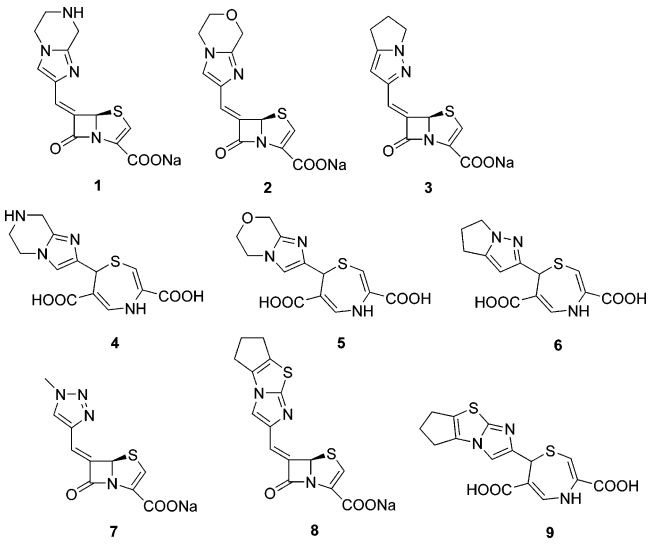

Figure 1. Structures of various penem inhibitors and rearranged seven-membered dihydro[1,4]thiazepine ring products.

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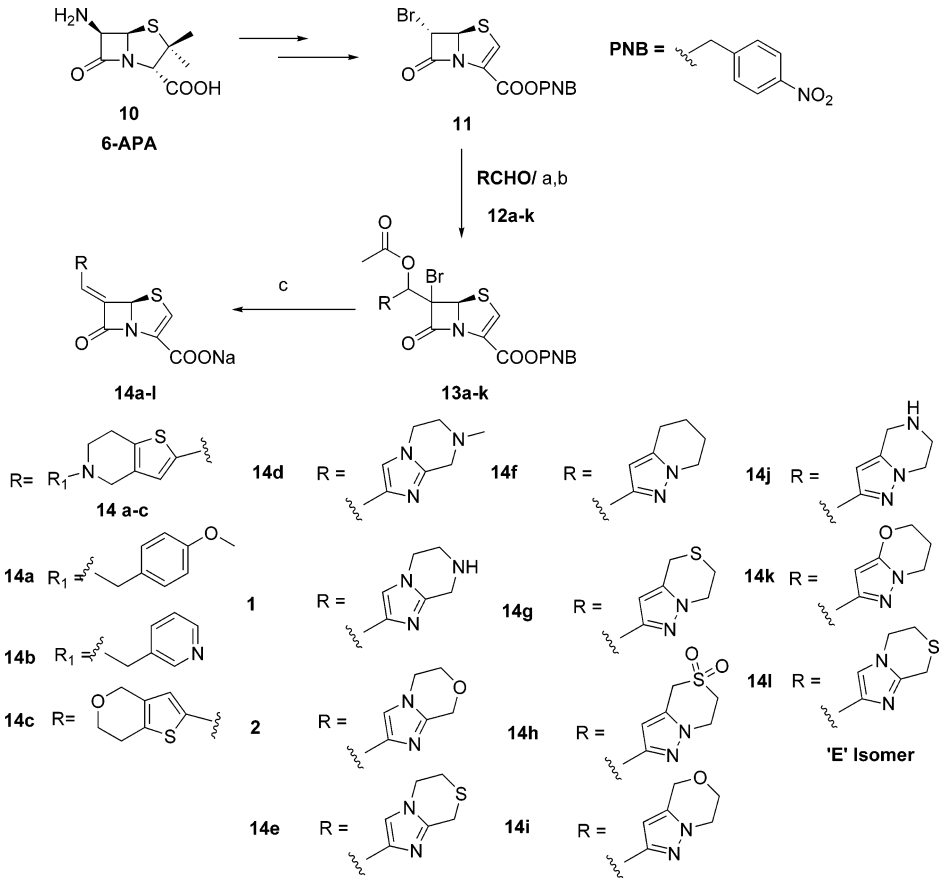
Scheme 1. General Method for the Preparation of Compounds **14a**−**l**[a]



[a] (a) $MgBr_2$/ Et_3N/ THF/acetonitrile; (b) acetic anhydride, 0 °C to −20 °C; (c) activated zinc/ 6.5 pH phosphate buffer/THF/acetonitrile, RT.

and GC1 a preferred R configuration at C7 of the dihydrothiazepine ring **5** was observed. This outcome differs from that predicted for **7** (BRL 42715)−enzyme complex intermediate by modeling studies.[4] Although the changes to the Ω-loop tyrosine residues were also noted in the high-resolution crystal structures of the dihydrocyclopenta[d]imidazo[2,1-*b*]thiazole penem (**8**) with SHV-1 and GC1, both R and S configurations are observed in the GC1 complex with **9**.[12] In this article, we report on the synthesis and SAR studies of novel 6,5-fused bicyclic 6-methylidene penems and their binding preference in class A and C β-lactamases on the basis of this new orientation of the Ω-loop tyrosines residues in class A and class C β-lactamases.

Chemistry. The bicyclic 6-methylidene penem carboxylic acid sodium salts **14a**−**l**, were prepared by a novel two step aldol condensation and reductive elimination procedure as reported previously.[17]

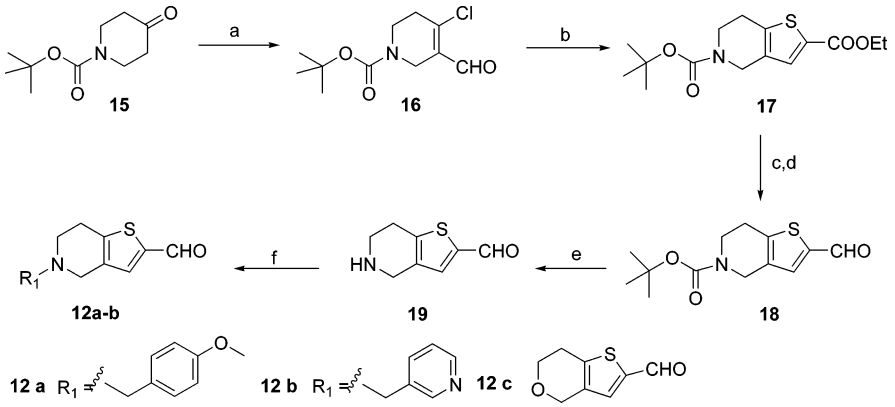
According to this procedure, appropriately functionalized aldehydes **12a**−**k** were reacted with (5*R*,6*S*)-6-bromo-7-oxo-4-thia-1-azabicyclo(3.2.0)hept-2-ene-2-carboxylic acid 4-nitrobenzyl ester **11** in the presence of triethylamine and anhydrous $MgBr_2$ (Scheme 1). Starting material **11** was prepared from the commercially available 6-aminopenicilanic acid (6-APA) **10** by a modified multistep procedure.[18,19]

The intermediate aldol products were trapped as their respective bromoacetoxy derivatives **13a**−**k**. To introduce the double bond at the 6-position of the penem nucleus, a mild reductive elimination procedure was devised, predominantly yielding the Z isomers. Intermediates **13a**−**k**, upon treatment with activated zinc in phosphate buffer at pH 6.5, not only resulted in the introduction of the double bond but also deprotected the carboxyl functionality. It should be pointed out here that in most cases only the Z isomer is formed during this transformation, likely due to the formation of a major diastereomer in **13a**−**k**.[17] In the case of **14e**, about a 5% quantity of the E isomer **14l** was also formed. The sodium salts of **14a**−**l** were obtained after purification by HP-21 reverse-phase column chromatography.

Aldehydes **12a**−**k** reported in this work were prepared by various procedures, which are described below. An overview of the structures of these aldehydes **12a**−**k** (Scheme 1) reveals that there are three categories of bicyclic aldehydes: (a) aldehydes based on the thiophene nucleus (Scheme 2, e.g., **12a**−**c**); (b) imidazole-fused aldehydes (Schemes 3 and 4, e.g., **12d** and **12e**); and (c) pyrazole-fused aldehydes (Schemes 6 and 7, e.g., **12 f**−**k**).

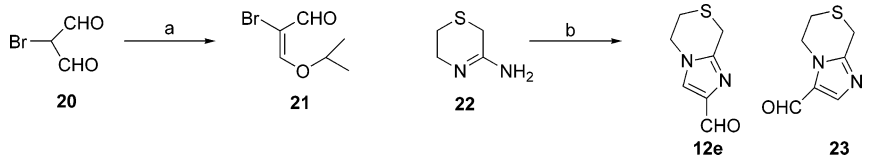
Thiophene-Fused Bicyclic Aldehydes. The bicyclic aldehydes based on the fused thiophene nucleus (e.g., **12a**−**c**) were prepared as shown in Scheme 2. 5-*tert*. Butyl-1-piperidinecarboxylate derivative **15** was chloroformylated using a Vilsmier−Hack reaction to yield intermediate **16** in good yields. This was reacted with ethyl mercaptoacetate in the presence of excess triethylamine to yield the 5-*tert*-butyl-2-ethyl 6,7-dihydrothieno-[3,2-c]pyridine-2,5(4*H*)-dicarboxylate derivative **17**. Ester derivative **17** was then converted via **18** to aldehyde **19** by a three step process as outlined in Scheme 2. The resulting aldehyde **19** thus prepared served as a key intermediate to prepare the N-alkylated compounds such as **12a** and **12b**. The corresponding 6,7-dihydro-4*H*-thieno[3,2-c]pyran-2-carbaldehyde **12c** was prepared by starting from the commercially available 4*H*-pyran-4-one and following the sequence of reaction similar to that outlined for the preparation of **18**.

Scheme 2. General Method for the Preparation of Thiophene Based Aldehydes **12a**−**c**[a]



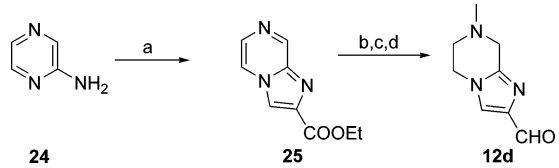
[a] (a) $POCl_3$/DMF/CH_2Cl_2/ 0 °C to RT; (b) ethyl mercaptoacetate/Et_3N/EtOH/ RT; (c) $LiAlH_4$/THF; (d) MnO_2/$CHCl_3$/reflux; (e) 1 N HCl in dioxane/ MeOH/ CH_2Cl_2/ RT; (f) RCl/ DIEA/DMF/ RT.

Scheme 3. Preparation of Compound **12e**[a]



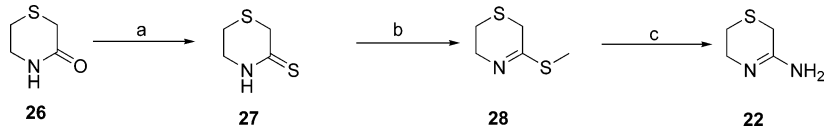
[a] (a) *p*-Toluenesulfonic acid/toluene/ 2-propanol/Dean−Stork; (b) compound **21**,EtOH/$CHCl_3$/ reflux.

Scheme 4. Preparation of **12d**[a]



[a] (a) Ethyl bromopyruvate/DME/ reflux; (b) Pd/C/H_2/HCl/EtOH/ 15 h; (c) HCHO/$NaBH_3CN$; (d) DIBAL/ −78 °C/ toluene.

Scheme 5. Preparation of **22**[a]



[a] (a) Lawesson's Reagent/THF/ reflux; (b) CH_3I/$CHCl_3$/Et_3N; (c) NH_4Cl/EtOH/ reflux.

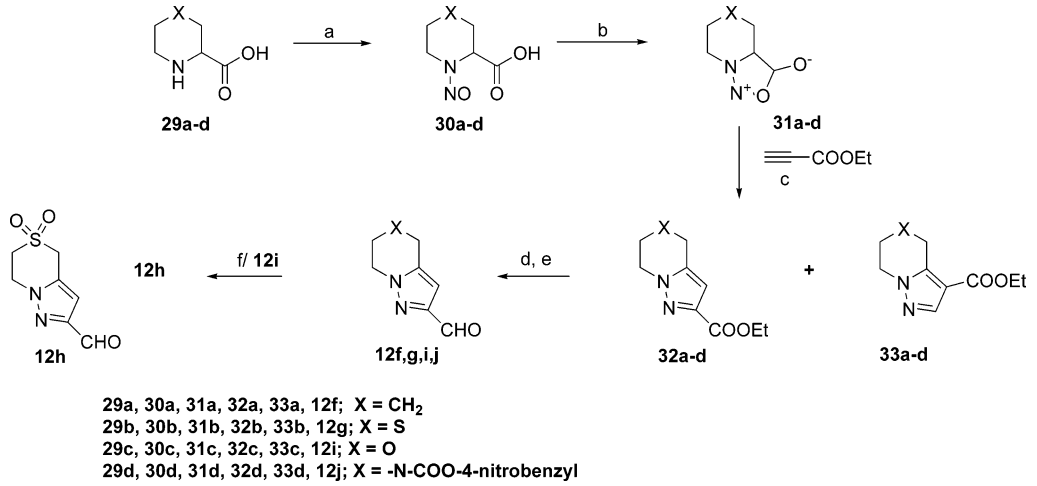
Imidazole-Fused Bicyclic Aldehydes. Bicyclic aldehydes based on imidazole-fused heterocycles **12d**,**e** were synthesized by the methods outlined in Schemes 3 and 4. The aldehyde **12e** was prepared by reacting 2-bromo-3-isopropoxypropenal **21** (Scheme 3) with the 2-amino substituted nitrogen heterocycle **22**. The resultant products from this reaction yielded two regio isomers, **12e** and **23**, which were separated by silica gel column chromatography. Compound **22**, which was required for this transformation, was prepared from **26** as depicted in Scheme 5. The required 2-bromo-3-isopropoxypropenal **21** was prepared by refluxing bromomalonaldehyde **20** and isopropyl alcohol in toluene/*p*-toluenesulfonic acid, in a Dean Stork apparatus by continuously removing the water formed during the course of this reaction. The aldehyde **12d** was prepared starting from 2-aminopyrazine **24**. 2-Aminopyrazine was reacted with ethyl bromopyruvate and the resulting bicyclic intermediate **25** was subsequently converted to **12d** as outlined in Scheme 4.

Pyrazole-Fused Aldehydes. Pyrazole-fused bicyclic aldehydes **12f**−**12g**, **12i**, and **12j** were prepared from their corresponding cyclic α-amino acid derivatives **29a**−**d** as outlined in Scheme 6.[21] The appropriately substituted cyclic α-amino acid derivatives **29a**−**d** were nitrosated using $NaNO_2$ in glacial acetic acid to yield **30a**−**d**. These compounds were reacted with trifluoroacetic anhydride to yield the sydnone derivatives **31a**−**d**. Upon reaction with ethyl propiolate, sydnones **31a**−**d** underwent a (3 + 2) cycloaddition reaction, with the simultaneous extrusion of CO_2 to mainly yield the desired regioisomers **32a**−**d.** The other isomers, namely, **33a**−**d**, were formed only to a minor extent. These two regio isomers can be distinguished from each other by ^{1}H NMR spectroscopy. The proton in the pyrazole ring of the undesired isomers **33a**−**d** is shifted more downfield compared to the aromatic proton of the desired isomers **32a**−**d**.

The ester derivatives **32a**−**d** were converted to the aldehydes **12f**,**g**,**i**,**j** by a two step reduction, oxidation process as outlined in Scheme 6. The aldehyde **12h** was prepared by oxidizing **12g** with *m*-chloroperbenzoic acid at 0 °C.

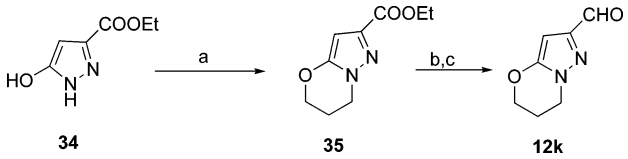
The aldehyde **12k** (Scheme 7) was synthesized starting from ethyl-5-hydroxy-1*H*-pyrazole-3-carboxylate (**34**).[22] Compound **34** was reacted with dibromopropane and K_2CO_3 in boiling acetonitrile to form the cyclized product **35**, which was converted to the aldehyde **12k** by a reduction/oxidation sequence of reactions as depicted in Scheme 7.

Scheme 6. General Method for the Preparation of Pyrazole-Fused Aldehydes **12f−12j**[a]



[a] (a) $NaNO_2$/acetic acid/ 0 °C; (b) $(CF_3CO)_2O$/ 0 °C/ RT; (c) ethyl propiolate/*o*-xylene/ reflux; (d) $LiBH_4$/THF/ reflux; (e) $MnO_2/CHCl_3$/reflux; (f) *m*-chloroperbenzoic acid.

Scheme 7. Preparation of **12k**[a]



[a] (a)K_2CO_3/CH_3CN/NH/dibromopropane/reflux; (b) $LiBH_4$/THF/reflux; (c) $MnO_2/CHCl_3$/ reflux.

Table 1. Activity in Vitro of Compounds **14a−l** against Different β-Lactamases, IC_{50} (nM)[a]

	class A (IC_{50}) nM		class B (IC_{50}) nM	class C (IC_{50}) nM
compd no.	TEM-1	Imi	CcrA	Amp-C
tazobactam	100 ± 8	NA	400000 ± 200	84000 ± 300
14a	2 ± 1	27 ± 2	78 ± 2	15 ± 3
14b	10 ± 2	40 ± 3	280 ± 5	18 ± 2
14c	1 ± 0.5	9 ± 1	14 ± 1	4 ± 1
14d	4 ± 1	22 ± 2	120 ± 5	6 ± 1
1	5 ± 1	28 ± 2	320 ± 15	6 ± 1
2	0.4 ± 0.2	8 ± 2	66 ± 6	5 ± 1
14e	3 ± 1	2300 ± 50	8 ± 1	2 ± 1
14f	2 ± 1	90 ± 10	62 ± 10	3 ± 1
14g	1 ± 0.5	18 ± 3	61 ± 5	1 ± 1
14h	1 ± 0.5	56 ± 10	43 ± 2	2 ± 1
14i	4 ± 1	20 ± 10	30 ± 10	6 ± 1
14 j	1 ± 0.5	123 ± 20	162 ± 20	3 ± 1
14k	6 ± 1	413 ± 10	140 ± 10	5 ± 2
14l	730 ± 10	>10000	110 ± 10	420 ± 20

[a] Compound **2**, IC_{50} value against SHV-1 is 9 nM and that of GC1 is 6.2 nM.

Results and Discussion

Structure−Activity Relationship. All of the newly synthesized inhibitors **14a−l** listed in Table 1 are representative examples of bicyclic 6,5-fused heterocyclic 6-methylidene penems. These heterocycles were incorporated into the penem nucleus on the basis of modeling studies and crystal structure studies.[12,16,23] The in vitro enzyme inhibition was carried out against TEM-1, Imi-1 (class A), CcrA (class B), and Amp C (class C) enzymes, and their respective IC_{50} values are listed in Table 1. In all of the experiments, tazobactam was used as the standard and the comparator. As can be seen from Table 1, all of the newly synthesized inhibitors with Z olefin geometry demonstrated excellent inhibition against TEM-1 and Amp C enzymes with IC_{50} values of 0.4−10 nM and 1−18 nM, respectively. In comparison with tazobactam, they all exhibited almost 10−250-fold greater potency against TEM-1 and were about 4600−84 000-fold more active against Amp C enzymes. All of the newly synthesized compounds were active against Imi-1, a carbapenem-hydrolyzing enzyme,[24] and CcrA, a metallo-β-lactamase.

Among the three categories of heterocycles attached to the 6-methylidene penem molecules, compounds **14a−c** fall into the class of thiophene-fused heterocyles; compounds **1**, **2**, **14d**, and **14e** are imidazole-fused and compounds **14f−k** are pyrazole-fused heterocyles, respectively. Penems based on the thiophene-fused heterocycles **14a−c** displayed good activity against TEM-1 and AmpC enzymes with **14c** having appreciable activity against the CcrA enzyme. Among the imidazole- and pyrazole-based compounds, derivatives **14e** and **14g**, which bear a 1,4-thiazene-fused to the five-membered rings are the most potent compounds against both TEM-1 and AmpC enzymes. Hence, a systematic SAR was carried out in both the imidazole- and pyrazole-based series. The sulfur atom in compound **14g** was replaced with $-CH_2$- **14f**, $-SO_2$- **14h**, -O- **14i**, and −NH- **14j** moieties, and these changes did not dramatically alter the in vitro activity against TEM-1 and AmpC. Similarly, in the imidazole-fused series, replacing the sulfur atom in compound **14e** with −N(Me)- **14d**, -NH- **1**, and −O- **2** resulted in a slight reduction in enhancement of potency with **2** against TEM-1; but the inhibition against AmpC remained almost unaltered. During the preparation of **14e**, a small quantity of the corresponding E isomer **14l** was isolated and tested for in vitro activity against all of the four enzymes. As can be seen from Table 1, the potency of **14l** diminished over 200-fold compared to that of its corresponding Z isomer **14e**.

To evaluate the effectiveness of these compounds in an in vitro cell-based assay, piperacillin was combined with the newly synthesized inhibitors and tested against various piperacillin resistant bacterial pathogens expressing different β-lactamases, and the minimum inhibitory concentration (MIC) values were

Table 2. Antimicrobial Activity in Vitro of Piperacillin plus Inhibitors (**14 a**−**l**) at a Constant Concentration of 4 μg/mL[a]

piperacillin MIC (μg/mL) + inhibitor	*Escherichia coli* TEM-1 (class A)	*Enterobacter cloacae* P99 (class C)	*Pseudomonas aeruginosa* AmpC (class C)	*Serratia marcescens* AmpC + Sme1[b] (class C)
tazobactam	2	64	64	1
14a	8	32	32	1
14b	32	64	64	1
14c	16	8	8	0.5
14d	16	1	4	1
1	2	2	16	1
2	2	8	1	1
14e	8	8	8	8
14f	2	4	4	1
14g	4	8	2	0.5
14h	4	16	2	1
14i	4	8	2	1
14j	4	16	0.5	1
14k	4	4	1	1
14l	64	32	64	4

[a] The MIC value of piperacillin and all of the inhibitors alone was >64 μg/mL. [b] Class A carbapenemase.

determined (Table 2). The combination of piperacillin−tazobactam was used as a comparator. Initially, the newly synthesized compounds when tested alone exhibit MIC values >64 μg/mL, which confirmed the fact that these compounds do not have any intrinsic antibacterial activity. However, when piperacillin was combined with compounds **1**,**2** and **14a**−**k**, all compounds exhibited greater activity than piperacillin alone against class A-producing *E. coli*. The more lipophillic thiophene-fused compounds **14a**−**b** exhibit less activity in the panel of both class A-producing *E.coli* and class C-expressing organisms compared to that of the other newly synthesized inhibitors. This is also consistent with their isolated enzymes IC_{50} values. However, against *Serratia marcescens*, compounds **14a**−**b** are as potent as the rest of newly synthesized inhibitors. In general, all of the newly synthesized inhibitors when combined with piperacillin showed good activity against *Serratia marcescens*, which may be attributed to good penetrability. It is also evident from Table 2 that even though the combination of piperacillin−tazobactam is potent against class A-producing bacteria, this combination is not effective against class C-producing organisms such as class C-producing *Enterobacter cloacae* and *Pseudomonas aeruginosa.* However, when the newly synthesized compounds **14a**−**l** (except for **14b** and **14l**) were combined with piperacillin, they enhanced its activity against piperacillin−tazobactam resistant class C-producing organisms. The MIC values of piperacillin were reduced to 0.5−32 μg/mL in the presence of a constant concentration of 4 μg/mL of each inhibitor. This was especially evident for the combination of piperacillin and imidazole-fused bicyclic derivatives **1**, **2**, **14d**, and **14e** and the pyrazole-fused bicyclic derivatives **14f**−**k**, which considerably reduced the MIC values of piperacillin (alone >64 μg/mL) against both class A- and class C-producing organisms. In an expanded set of class A- and class C-producing organism profiles (data not shown), 90% of the organisms were susceptible to the combination of piperacillin and the imidazole- and pyrazole-fused inhibitors **14d**−**k**. Penem **14l**, the E isomer of compound **14e**, is considerably less active than its corresponding Z isomer **14e**.

Recent evidence establishes the formation of seven-membered 1,4-thiazepine in the reaction of 6-methylidene penems with class A and C β-lactamases.[4,12,15,16] However, one issue of concern is the stereochemical outcome at the sterogenic C7 center. In the case of **36** (Figure 2), molecular modeling

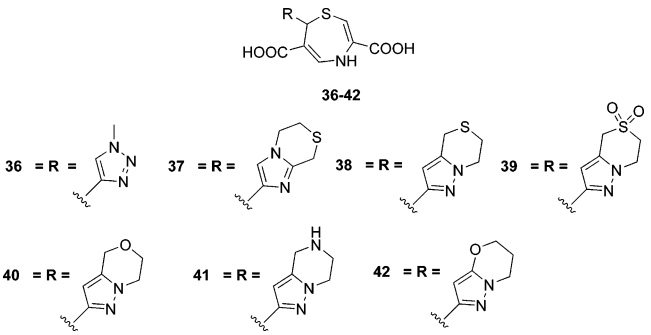


Figure 2. Structures of various rearranged seven-membered dihydro-[1,4]thiazepine ring products.

Table 3. Calculated Interaction Energy Difference between the 7R Isomer and the 7S Isomer in SHV-1 and GC1 Enzymes[a]

thiazepine derivative	ΔIE SHV-1	ΔIE GC1
4	−17.1	−6.1
5	−32.5	−43.3
37	−76.0	−35.4
38	−45.9	−68.2
39	−119.6	−15.4
40	−90.5	−68.0
41	−6.5	−68.3
42	−60.3	−89.3

[a] ΔIE = IE R isomer − IE S isomer in kcal/mol.

predictions favor the 7S enantiomer, whereas with **5**, the high-resolution crystal structure with both SHV-1 and GC1 indicates the formation of the 7R enantiomer. The stereochemistry in thiazepine **9** is 7R in SHV-1 and both 7R and 7S in GCI as determined by high-resolution X-ray crystallography.

On the basis of the experimentally determined enzyme **2** complex, a computational analysis was developed to investigate whether the poor interaction of 7*S*-thiazepine compared to that of the 7R enantiomer with the enzyme contributes to the enzyme preference for the latter because the fused bicyclic 6,5 penems were designed on this basis and proved to be potent inhibitors of β-lactamases (Table 1).

Preference for the 7R or S enantiomer is determined by comparing calculated interaction energies in SHV-1 and GC1 for eight thiazepine derivatives **4**, **5**, and **37**−**42** (Figures 1 and 2; Table 3) derived from their respective β-lacatamase inhibitors.

Distances observed in the energy optimized structures indicated several hydrogen bonding, van der Waals, and stacking interactions. In SHV-1, four heteroatoms from the R isomer of the thiazepine derivative **5** interact with the enzyme: hydrogen bonds between the sulfur and Asn132, N4 and Ala237, carbonyl at the 6-position and Ala237, and imidazole N and Ser130. In addition, the bicyclic moiety is coplanar and stacks with Tyr105 (Figure 3A). In contrast, the 7S enantiomer makes hydrogen bonding contacts with Ala237 and Asn132 but does not stack with Tyr105 (Figure 3B). Similarly, in GCI, the R isomer of thiazepine **5** derivative has four heteroatoms that interact with the enzyme: N4 and Asn152, C6 carboxylate with Asn152 and Gln120, and CO with Ser 321 as well as the interaction of the bicyclic moiety with Tyr224 (Figure 3C). The stronger hydrogen bonding interactions including the thiazepine carboxylate are missing altogether in the S enantiomer (Figure 3D).

There is significant stacking and van der Waals interactions between the Tyr105 of SHV-1 and bicyclic headgroups that have R stereochemistry at the C7 atom, demonstrating tighter binding than the S enantiomers. Likewise, in GC1, several hydrogen-bonding contacts and some nonpolar interactions of **5** with the enzyme binding site contribute to tighter binding, particularly,

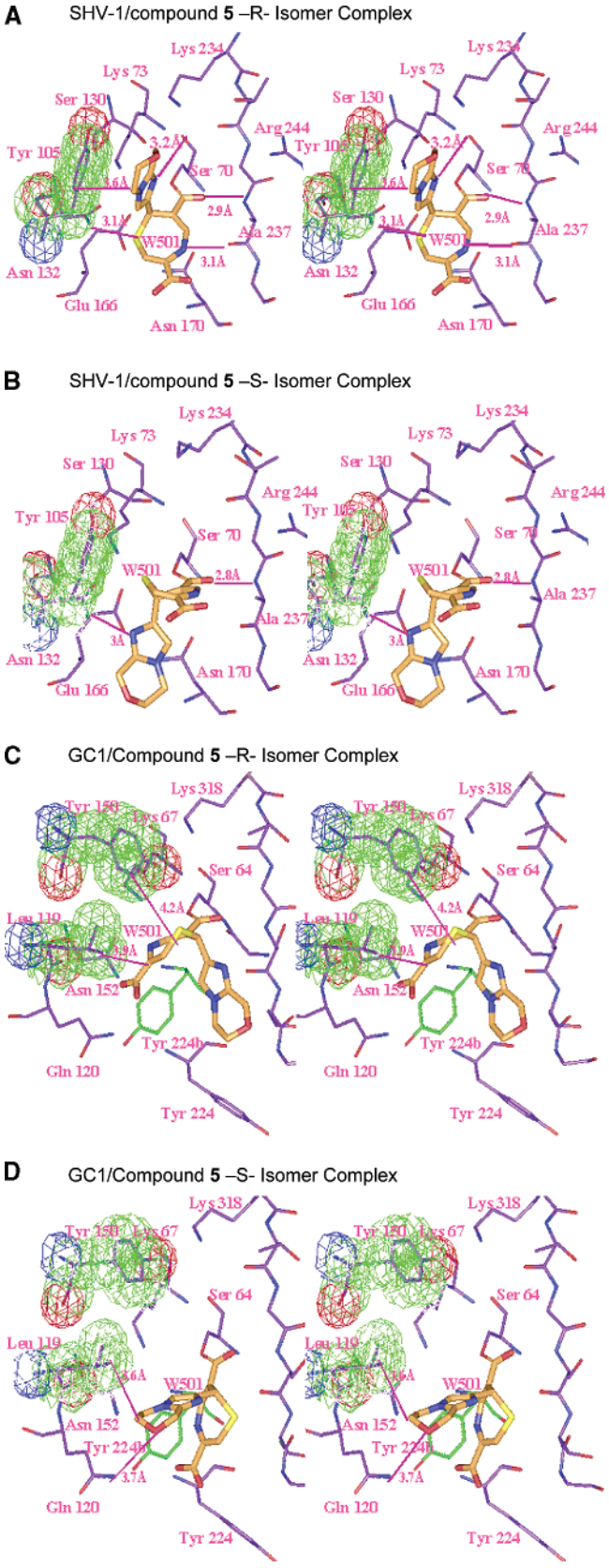


Figure 3. (A) SHV-1/compound **5**-*R*−isomer complex. (B) SHV-1/compound **5**-*S*−isomer complex. (C) GC1/Compound **5**-*R*−isomer complex. (D) GC1/compound **5**-*S*−isomer complex.

the electrostatic contact between the thiazepine carboxylate of **5** and Asn152 and Glu120.

To predict the preference for either the C7R or S enantiomer in the 6,5 bicyclic inhibitors, we analyzed the binding of each using molecular modeling and calculated the interaction energies for each pair (Table 3). The calculated interaction energies for compounds **4**, **5**, and **37**−**42** are listed in Table 3, in both SHV-1 and GC1 for each pair of enantiomers. In all cases, the R enantiomer is preferred in both enzymes. The preference indicated by modeling and corroborated by our calculations is consistent throughout this set and represents factors driving the binding of the R enantiomers.

Table 4. Relative% Hydrolysis of the DHP Enzyme[a]

species	1	2	14f	14g	14h	14i	14j	14k
hog	5	2	100	49	49	100	77	89
human	3	8	32	20	21	16	19	37
mouse	32	19	62	40	18	48	32	86

[a] Imipenem was set at 100%.

Encouraged by the good activity obtained in MIC tests, penems **1**, **2**, and **14f**−**k** were further selected for in vivo evaluation in an acute lethal infection model. To determine their in vitro efficacy, the above-mentioned compounds were evaluated for their stability toward renal dihydropeptidase (DHP) enzymes.[27] The relative stabilities of the selected penem molecules tested against DHP and are summarized in Table 4. Relative to that of imipenem, which was set as 100%, the selected newly synthesized molecules are more stable against human DHP than against hog or mouse enzymes. Compounds **1** and **2** were found to be as stable as tazobactam.

The in vivo data (ED_{50} values) in a murine acute lethal infection model against TEM-1-producing *E. coli* and AmpC-producing *Enterobacter aerogenes* with piperacillin and the inhibitor combinations are listed in Table 5. As seen in Table 5, when piperacillin was administered alone, it had only a moderate to poor efficacy against both TEM-1- and class C-producing organisms. However, when mixed with each of the inhibitors at a 4:1 ratio (piperacillin to inhibitor) and administered intravenously, they exhibited a marked reduction in ED_{50} values. Piperacillin plus inhibitors, such as **1**, **2**, **14f**−**g**, **14i**, and **14j**, appeared to be the most effective combinations for infection with class A-producing organisms. Similarly, compounds **1**, **2**, **14f**, and **14g** when combined with piperacillin in the ratio of 1:4 (piperacillin/inhibitors) exhibited good potency against AmpC-producing *Enterobacter aerogenes*.

Conclusion

On the basis of modeling experiments and mechanistic understanding, several novel thiophene-, imidazole-, and pyrazole-fused bicyclic-6-methylidene penem molecules were designed and synthesized. These compounds were found to be potent, broad spectrum, and in vivo active β-lactamase inhibitors. They were synthesized by an extension of a novel aldol condensation−reductive elimination procedure.[17] The novel aldehydes required for this coupling reaction were synthesized, and the procedures are described. When the newly synthesized compounds **14a**−**l** were combined with piperacillin in the whole cell assay, the MIC values for piperacillin were reduced to a susceptible range for both class A- and class C-producing organisms. It is also shown in the case of 6-methylidene penem molecules that the Z geometry across the double bond is important for potency. These newly synthesized molecules are superior to the commercially available inhibitors in terms of their potency and spectrum of activity. On the basis of the molecular modeling and interaction energies, the preference for the formation of 1,4-thiazepines in class A and class C β-lactamases favoring C7-R stereochemistry is predicted for the reported 6,5-fused bicyclic 6-methylidene penem inhibitors.

Table 5. Efficacy (IV)[a] in Vivo in a Murine Acute Lethal Infection Model with *E. coli*- and AmpC-Producing Organisms

organism	1	2	14f	14g	14h	14i	14j	14k
Escherichia coli TEM-1	27 ± 1	23 ± 3	28 ± 3	31 ± 1	64 ± 2	19 ± 1	30 ± 2	90 ± 4
Enterobacter aerogenes Amp C[b]	NT	96 ± 4	86 ± 1	123 ± 5	NT	NT	NT	NT

[a] Piperacillin−inhibitor at a ratio of 4:1. [b] The ED_{50} (mg/kg) value of piperacillin alone against *Escherichia coli* is 165 mg/kg with 95% confidence limit and against Amp C-producing *Enterobacter aerogenes* is 421 with 95% confidence limit.

Experimental Section

***β*-Lactamase Assays.** β-Lactamase inhibitory activity of the penem inhibitors was determined spectrophotometrically, as described by Bush et al.[26] Homogeneously purified class A β-lactamases TEM-1 from *E. coli* and Imi-1 from *Enterobacter cloacae*, class B enzyme CcrA from *Bacteroides fragilis*, and class C enzyme AmpC from *Enterobacter cloacae* were employed in the assay. The enzyme concentrations for TEM-1, Imi-1, CcrA, and AmpC were 4.3, 7.1, 1.2, and 2.1 nM, respectively. A wide range of inhibitor concentrations were prepared in 50 mM PO_4 at pH 7.0 to include the possible IC_{50} values. The substrate used to initiate the enzyme reaction was nitrocefin at 50 μg/mL in the same buffer as the inhibitor. Initially, the enzyme and inhibitor (20 μL each) were preincubated for 10 min at 25 °C prior to the addition of 160 μL volume of nitrocefin. Initial rates of hydrolysis were monitored for 5 min at 495 nm using a Molecular Devices Spectra Max 250 with the kinetic protocol of SoftMax Program. Readings from the Spectra Max 250 were exported and transferred to Microsoft Excel. The percent inhibition of each inhibitor concentration was calculated on the basis of the control enzyme activity. The inhibitor concentration that caused a 50% reduction in the enzymatic activity (IC_{50}) was determined graphically.

Antimicrobial Susceptibility Testing. The in vitro activities of the antibiotics were determined by the microbroth dilution method as recommended by the National Committee for Clinical Laboratory Standards (NCCLS). (NCCLS. 2003. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standards: M7-A6, vol. 20. National Committee for Clinical Laboratory Standards, Wayne, PA.) Mueller−Hinton II broth (MHBII, BBL Cockeysville, MD) was used for the testing procedure. Microtiter plates containing 50 μL per well of 2-fold serial dilutions of piperacillin combined with a constant amount (8 μg/mL) of a β-lactamase inhibitor were inoculated with 50 μL of inoculum to yield the appropriate density (10^5 CFU/mL) in 100 μL final volume. The plates were incubated for 18−22 h at 35 °C in ambient air. The minimal inhibitory concentration (MIC) for all isolates was defined as the lowest concentration of antimicrobial agent that completely inhibits the growth of the organism as detected by the unaided eye.

Molecular Modeling. The C7 R enantiomer of enzyme−penem **2** complex was built in tautomeric form **5** in the pdb files 1ONG and 1ONH. Models with the C7 S enantiomer were prepared by modifying the experimentally observed C7 S enantiomer of thiazepine **9** (1Q2Q) and replacing the R enantiomers with the modeled S enantiomers in 1ONG and 1ONH. Hydrogen atoms were added in all four models using Sybyl7.0/ Biopolymer module and solvated with the Tripos water model by applying periodic boundary conditions. All protein−ligand complexes were energy minimized at a constant dielectric of 1 with the conjugated gradient method and the MMFF94 force field. The energy optimization resulted in an rms deviation of 0.39 and 0.45 Å with respect to Cα atoms of 1ONG and 1ONH, respectively. Protein−ligand interaction energies were calculated by subtracting the internal energies of the protein and ligand from the internal energy of the protein−ligand complex. Preference for C7 R or S enantiomer was determined by comparing calculated interaction energies in SHV-1 or GC1 enzymes. The thiazepine intermediates of a set of eight β-lactamase inhibitors were modeled starting from 1ONG, 1ONH, and the aforementioned C7 S enantiomer complexes, and the relative interaction energies were calculated for the C7 R and S enantiomers following the same procedure.

DHP Stability. Dehydropeptidases (DHPs) of hogs, mice, and humans were obtained from their respective kidneys, and the levels of hydrolysis of the penem molecules by these enzymes were determined as previously described.[25,28]

Antibacterial Protection in Vivo. Mice were challenged by injecting 0.5 mL intraperitoneally or 0.05 mL intranasally a predetermined bacterial inoculum suspended in broth, saline, or hog gastric mucin. The bacterial inoculum is equivalent to 10−100 LD_{50} values of the specific infecting strain and will result in death of the nontreated control animals within 7 days, which determine the bacterial virulence in untreated mice. Antibacterial doses (dose concentration prepared by 2-fold serial dilutions of piperacillin and the newly synthesized inhibitors **14a**−**l**) were dissolved or suspended in 0.2% aqueous agar or methocel, phosphate buffered saline or an adjuvant and were administered intravenously in the following manner. Intravenously: dose volume of 0.2 mL, administered 30 min after infection. For the treatment of infections with more virulent organisms, more doses of up to 48 h may be administered.[25] (Intravenous dosing will not exceed 3 doses/24 h period.) The protective effects of the piperacillin plus the newly synthesized inhibitors **14a**−**l** were measured by the survival of the infected untreated animals compared to that of the treated animals. For this determination, animals were observed for 7 days after treatment. A census of survivors was taken twice daily, and at that time, dead as well as moribund animals were removed. The 7 day survival ratio from three separate tests were pooled for the estimation of median effective dose (ED_{50}) by a computerized program for probit analysis.[28] The test was performed three times on separate days to provide a statistically valid number of animals and to minimize variation in test results on a day-to-day and test-to-test basis.

General Methods. Melting points were determined in an open capillary tubes on a Meltemp melting point apparatus and are uncorrected. ^{1}H NMR spectra were determined with a Bruker DPX-400 spectrometer at 400 MHz. The chemical shifts δ are reported in parts per million (δ) relative to that of the residual chloroform (7.26 ppm), TMS (0 ppm), or dimethyl sulfoxide (2.49 ppm) as internal references 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. 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, the reagents were obtained from commercial sources and were used without further purification. Compounds **1** and **2** were synthesized by following the procedure reported by us previously.[15]

Preparation of (5*R*,6*Z*)-6-{[5-(4-Methoxybenzyl)-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridin-2-yl)]methylene}-7oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic Acid (14a). Step1: 5-*tert*-Butyl 2-ethyl 6,7-dihydrothieno[3,2-*c*]pyridine-2,5(4*H*)-dicarboxylate (17). To a stirred dry DMF (7.3 g, 100 mmol), $POCl_3$ (12.25 g, 80 mmol) was slowly added between 0−5 °C. After the addition, the solidified mass was dissolved in CH_2Cl_2 (20 mL) and stirred at room temperature for 2 h. The temperature was lowered to 0 °C and *tert*-butyl-1-piperidinecarboxylate **15** (9.9 g, 50 mmol) in CH_2Cl_2 was added slowly. After the addition, the reaction mixture

was stirred at room temperature for 2 h and poured over crushed ice and sodium acetate. It was stirred for 30 min at room temperature, extracted with CH_2Cl_2, washed well with water, dried over anhydrous $MgSO_4$, and concentrated. The crude product was dissolved in CH_2Cl_2, and ethylmercaptoacetate (9.6 g, 80 mmol)/Et_3N (10.1 g, 100 mmol) was added slowly at room temperature. The reaction mixture was refluxed for 2 h and quenched with water. The CH_2Cl_2 layer was washed well with water, dried over anhydrous $MgSO_4$, filtered, and concentrated. The product was purified by silica gel column chromatography by eluting it with 3:1 ethyl acetate−hexane. Yield: 8.7 g, 56%; white liquid. $(M + H)^+$ 312.

Step 2: *tert*-Butyl 2-(hydroxymethyl)-6,7-dihydrothieno[3,2-*c*]pyridine-5(4*H*)-carboxylate. To stirred suspension of $LiAlH_4$ (500 mg) a solution of 5-*tert*-butyl 2-ethyl 6,7-dihydrothieno[3,2-*c*]pyridine-2,5(4*H*)-dicarboxylate **17** (1.0 g, 3.21 mmol) was added, and the reduced alcohol was isolated as white liquid. Yield: 807 mg (92%); $(M + H)^+$ 270.

Step 3: *tert*-Butyl 2-(formyl)-6,7-dihydrothieno[3,2-*c*]pyridine-5(4*H*)-carboxylate(18). To a stirred solution of *tert*-butyl 2-(hydroxymethyl)-6,7-dihydrothieno[3,2-*c*]pyridine-5(4*H*)-carboxylate (1.0 g 3.7 mmol) in CH_2Cl_2 (300 mL), active MnO_2 (20 g, excess) was added and stirred at room temperature for 18 h. At the end, the reaction mixture was filtered through Celite and washed with $CHCl_3$. The combined organic layer was washed well with water, dried, and concentrated. The product was found to be pure and taken to the next step without purifications. Then, 800 mg (81% Yield) of the aldehyde derivative was isolated as brown solid. $(M + H)^+$ 268.

Step 4: 2-(Formyl)-6,7-dihydrothieno[3,2-*c*]-5(4*H*)-pyridine (19). 2-(Formyl)-6,7-dihydrothieno[3,2-*c*]-5(4*H*)-pyridine was prepared starting from *tert*-butyl 2-(formyl)-6,7-dihydrothieno[3,2-*c*]-pyridine-5(4*H*)-carboxylate **18** (1.0 g 3.7 mmol), which was dissolved in CH_2Cl_2 (20 mL), MeOH (90% 20 mL), and 1 N HCl in dioxane (10 mL). The reaction mixture was stirred at room temperature for 48 h and concentrated to dryness, which was taken to the next step without purification. Yield: 750 mg (HCl salt, quantitative); $(M + H)^+$ 168.

Step 5: 2-Formyl [5-(4-methoxybenzyl)-4,5,6,7-tetrahydrothieno][3,2-*c*]pyridine (12a). To a stirred solution of 2-(formyl)-6,7-dihydrothieno[3,2-*c*]-5(4*H*)-pyridine **19** (1.4 g, 5.2 mmol) in DMF (20 mL), 4-methoxybenzyl chloride (0.94 g, 6.2 mmol) and *N*,*N*-diisopropylethylamine (10 mL, excess) were added at room temperature. The reaction mixture was stirred for 24 h and quenched with water. The reaction mixture was extracted with chloroform, washed well with water, dried over anhydrous $MgSO_4$, filtered, and concentrated. The product was purified by silica gel column chromatography by eluting it with ethyl acetate; pale yellow oil. Yield: 470 mg (35%); $(M + H)^+$ 288.

Step 6: 4-Nitrobenzy-6-[(acetyloxy)[5(4-methoxybenzyl)-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridin-2-yl)methyl]-6-bromo-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylate (13a). 2-Formyl [5-(4-methoxybenzyl)-4,5,6,7-tetrahydrothieno][3,2-*c*]pyridine **12a** (574 mg, 2.0 mmol) and the 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 **11** (772 mg, 2.0 mmol) were added successively to the dry acetonitrile (15 mL) solution of anhydrous $MgBr_2/O(Et)_2$ (390 mg, 1.5 mmol) under an argon atmosphere at room temperature. After cooling to −20 °C, Et_3N (2.0 mL) was added in one portion. The reaction vessel was covered with foil to exclude light and stirred for 8 h at −20 °C. At the end, the reaction mixture was treated with acetic anhydride (1.04 mL) and kept for 15 h at 0 °C. The mixture was diluted with ethyl acetate and washed with brine solution. 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 (1:1). Collected fractions were concentrated under reduced pressure, and the mixture of diastereoisomers were taken to the next step; pale yellow amorphous solid. Yield: 550 mg (40%); $(M + H)^+$ 714 and 716.

Step-7: (5*R*,6*Z*)-6-{[5-(4-Methoxybenzyl)-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridin-2-yl)]methylene}-7oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic Acid Sodium Salt (14a). 4-Nitrobenzy-6-[(acetyloxy)[5(4-methoxybenzyl)-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridin-2-yl)methyl]-6-bromo-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylate **13a** (300 mg, 0.42 mmol) was dissolved in THF (20 mL) and acetonitrile (10 mL). Freshly activated Zn dust (5.2 g) was added rapidly with a 0.5 M phosphate buffer (pH 6.5, 28 mL). The reaction vessel was covered with foil to exclude light and stirred vigorously for 2 h at room temperature. The reaction mixture was filtered, and the filtrate was cooled to 3 °C and the pH adjusted to 8.5 using 0.1 N NaOH. The aqueous layer was washed with ethyl acetate and concentrated under high vacuum at 35 °C to give a yellow precipitate. The product was purified by HP21 resin reverse-phase column chromatography. Initially, the column was eluted with deionized water (2 L) and the latter with 10% acetonitrile/water. The fractions containing the product were collected and concentrated at reduced pressure at room temperature. The yellow solid was washed with acetone and filtered. It was crystallized from a 3:1 acetone/water mixture. Yield: 50 mg (18%); as yellow crystals; mp 127 °C; $(M + H)^+$ 441.

Preparation of (5*R*,6*Z*)-7-Oxo-6-{[5-(pyridin-3-ylmethyl)-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridin-2-yl)]methylene}-7oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic Acid Sodium Salt (14b). Step 1: 2-Formyl [5-(pyridin-3-ylmethyl)-4,5,6,7-tetrahydrothieno][3,2-*c*]pyridine (12b). To a stirred solution of 2-(formyl)-6,7-dihydrothieno[3,2-*c*]-5(4*H*)-pyridine (**19**) (1.05 g, 5.2 mmol) in DMF (20 mL), 3-picolyl chloride hydrochloride (0.852 g, 5.2 mmol) and *N*,*N*-diisopropylethylamine (10 mL, excess) was added at room temperature. The reaction mixture was stirred for 24 h and quenched with water. It was extracted with chloroform, washed well with water, and dried over anhydrous $MgSO_4$. It was filtered and concentrated. The product was purified by silica gel column chromatography by eluting it with ethyl acetate; pale yellow semisolid. Yield: 800 mg (59%); $(M + H)^+$ 259.

Step 2: 4-Nitrobenzy-6-[(acetyloxy)[5(pyridin-3-ylmethyl)-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridin-2-yl)methyl]-6-bromo-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylate (13b). 2-Formyl [5-(pyridin-3-ylmethyl)-4,5,6,7-tetrahydrothieno][3,2-*c*]-pyridine **12b** (516 mg, 2.0 mmol) and the 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 **11** (772 mg, 2.0 mmol) were added successively to the dry acetonitrile (15 mL) solution of anhydrous $MgBr_2/O(Et)_2$ (390 mg, 1.5 mmol) under an argon atmosphere at room temperature. After cooling to −20 °C, Et_3N (2.0 mL) was added in one portion. The reaction vessel was covered with foil to exclude light. The reaction mixture was stirred for 2 h at −20 °C and treated with acetic anhydride (1.04 mL) in one portion. The reaction mixture was warmed to 0 °C and stirred for 15 h at 0 °C. The reaction mixture was worked-up as described for the preparation of **14a**. The residue was applied to silica gel column chromatography, and the column was eluted with ethyl acetate/hexane (1:1). Collected fractions were concentrated under reduced pressure, and the mixture of diastereoisomers were taken to next step; pale yellow amorphous solid. Yield: 700 mg (51%); $(M + H)^+$ 685 and 687.

Step-3: (5*R*,6*Z*)-6-{[5-(Pyridin-3-ylmethyl)-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridin-2-yl)]methylene}-7oxo-4-thia-1-azabicyclo-[3.2.0]hept-2-ene-2-carboxylic Acid Sodium Salt (14b). Starting from 4-Nitrobenzy-6-[(acetyloxy)[5(pyridin-3-ylmethyl)-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridin-2-yl)methyl]-6-bromo-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylate (686 mg, 1.0 mmol) and following the procedure outlined for the preparation of **14a**, compound **14b** was isolated as a yellow solid. This was washed with acetone, filtered, and dried. Yield: 50 mg (12%) as yellow crystals; mp 134−136 °C; $(M + H)^+$ 412.

Preparation of 6-(6,7-Dihydro-4*H*-thieno[3,2-*c*]pyran-2-ylmethylene)-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic Acid Sodiums Salt (14c). Step 1: Preparation of 6,7-Dihydro-4*H*-thieno[3,2-*c*]pyran-2-carbaldehyde (12c). $POCl_3$ (3.83 mL, 50 mmol) was added dropwise to ice-cold DMF (3.85

mL, 50 mmol) within 3 min. CH_2Cl_2 (DCM) (20 mL) was added, and the bath was removed when the reaction media appeared to be pasty. The reaction was kept at room temperature for 2 h. Then it was cooled to 0 °C and 4*H*-pyran-4-one (5 g, 50 mmol) in 10 mL CH_2Cl_2 was added dropwise within 3 min. The reaction was kept at 0 °C for 2 h and poured into a mixture of ice and sodium acetate solution. After stirring for 2 h at room temperature, it was extracted with CH_2Cl_2 (2 × 200 mL), and the combined organic layers were dried over magnesium sulfate. The organic layer was filtered and concentrated to give 5.0 g of the crude product. This was dissolved in DCM (200 mL) and added to ethyl mercaptoacetate (4.2 g, 35 mmol) and Et_3N (10 mL). The mixture was refluxed for 18 h and cooled to room temperature. It was washed with water and dried over magnesium sulfate. The organic layer was filtered, concentrated, and flash chromatographed with 20% ethyl acetate in hexane. The collected material was dissolved in THF (100 mL), and $LiAlH_4$ (150 mL, 0.5M in THF) was injected and left at room temperature for 10 min. It was refluxed for 18 h and quenched at 23 °C by adding water and eventually 1 N HCl to clear up the mixture. It was extracted with ethyl acetate (2 × 200 mL), and the combined organic layers were dried over magnesium sulfate. It was filtered and concentrated to give 2.3 g of the product. The crude material was dissolved in DCM (300 mL) and manganese dioxide (15 g) was added and stirred at room temperature for 0.5 h. At the end, an additional 30 g of MnO_2 was added and refluxed for 1 h. The material was filtered through a pad of Celite and concentrated. Flash column chromatography gave 1.2 g of (14% yield) an oily product; $(M + H)^+$ 169.

Step 2: Preparation of 6-(6,7-Dihydro-4*H*-thieno[3,2-*c*]pyran-2-ylmethylene)-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic Acid Sodium Salt (14c). A mixture of 6,7-Dihydro-4*H*-thieno[3,2-*c*]pyran-2-carbaldehyde **12c** (336 mg, 2 mmol) and magnesium bromide (516 mg, 2 mmol) was dissolved in 20 mL of acetonitrile and stirred under an N_2 atm for 0.5 h. 6-Bromo-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid 4-nitrobenzyl ester **11** (770 mg, 2 mmol) in 20 mL of THF and triethylamine was added all at once, and the mixture was cooled to −20 °C and stirred for 8 h. Then acetic anhydride (0.4 mL) was added, and the mixture was stirred at 0 °C for 18 h. At the end, the reaction mixture was worked-up as described in the procedure for **14a.** Flash column chromatography using 20% ethyl acetate in hexane gave 491 mg (41%) of the product. This product was then dissolved in 15 mL of THF and 15 mL of aqueous phosphate buffer (pH 6.5). The mixture was then subjected to 45 psi of hydrogen for 1 h with 0.5 g of 10% palladium on carbon. The reaction mixture was filtered through a pad of Celite and concentrated in vacuo to remove most of the THF. The solution was then cooled to 0 °C and basified to pH 8.5 with 0.1 N sodium hydroxide. Then, it was purified by HP21 resin reverse-phase column chromatography. Initially, the column was eluted with deionized water (2 L) and the latter with 5% acetonitrile/water. The fractions containing the product were collected and concentrated at reduced pressure at room temperature. The yellow solid was washed with acetone and filtered. It was crystallized from a 3:1 acetone/water mixture, and 100 mg (38%) of the product was isolated; mp >250 °C; $(M-H)^-$ 320.3.

Preparation of (5*R*),(6*Z*)-6-(7-Methyl-5,6,7,8-tetrahydroimidazo[1,2-*a*]pyrazin-2-ylmethylene)-7-oxo-4-thia-1-aza-bicyclo-[3.2.0]hept-2-ene-2-carboxylic Acid, Sodium Salt (14d). Step 1: Imidazo[1,2-*a*]pyrazine-2-carboxylic Acid Ethyl Ester (25). To a stirred solution of 2-aminopyrazine (24.8 g, 258 mmol) in dimethoxyethane DME, ethyl bromopyruvate (62.9 g, 320 mmol) was added at room temperature and stirred for 2.5 h. The reaction mixture was cooled to 0 °C and stirred for 30 min to afford a pale brown precipitate. The precipitate was filtered and washed with Et_2O to give a pale brown solid. The precipitate (66.1 g) was suspended in EtOH (1.29 L) and heated at reflux temperature to turn to a clear solution. After refluxing for 2 h, the reaction mixture was concentrated under reduced pressure and then mixed with $CHCl_3$ and saturated aqueous $NaHCO_3$ solution. The mixture was filtered through a pad of Celite, and the separated organic layer was dried ($MgSO_4$) and filtered. The filtrate was concentrated under reduced pressure. The residue was applied to silica gel column chromatography, and the column was eluted with $CHCl_3$/MeOH (99/1−97/3), and the collected fractions were concentrated under reduced pressure. The separated solid was purified by crystallization from $CHCl_3/Et_2O$. The title compound was obtained as pale pink crystals. Yield: 10.9 g (22%).

Step 2: 5,6,7,8-Tetrahydroimidazo[1,2-*a*]pyrazine-2-carboxylic Acid Ethyl Ester, Hydrochloride. A mixture of imidazo[1,2-*a*]pyrazine-2-carboxylic acid ethyl ester **25** (13.7 g, 65.23 mmol) in 0.5 M HCl/EtOH (169 mL) and 10% Pd-C (50% wet) (1.37 g) was hydrogenated at 40 psi at room temperature for 15 h. The reaction mixture was filtered, and Pd-C was washed with EtOH. The filtrate was concentrated under reduced pressure. The residue was applied to silica gel column chromatography, and the column was eluted with $CHCl_3$/MeOH (9/1−2/1). The title compound was obtained as a brown solid. Yield: 10.4 g (63%).

Step 3: 7-Methyl-5,6,7,8-tetrahydroimidazo[1,2-*a*]pyrazine-2-carboxylic Acid Ethyl Ester. Et_3N (3.44 mL, excess), 37% HCHO aq (2.02 mL, excess), and $NaBH_3CN$ (1.78 g, excess) were added successively to the MeOH (75 mL) solution of 5,6,7,8-tetrahydroimidazo[1,2-*a*]pyrazine-2-carboxylic acid ethyl ester hydrochloride (5.2 g, 21 mmol) at room temperature and stirred for 3.5 h under a nitrogen atmosphere. The mixture was diluted with CH_2Cl_2 and washed with a 50% aqueous K_2CO_3 solution. The organic layer was dried (K_2CO_3) and filtered. The filtrate was concentrated under reduced pressure. The residue was applied to silica gel column chromatography, and the column was eluted with $CHCl_3$/acetone (1/1−1/2). The title compound was obtained as an orange oil. Yield: 2.68 g (57%).

Step 4: 7-Methyl-5,6,7,8-tetrahydroimidazo[1,2-*a*]pyrazine-2-carbaldehyde (12d). A 1 M solution of DIBAL in toluene (13.6 mL, 13.6 mmol) was added to the dry CH_2Cl_2 (86 mL) solution of 7-methyl-5,6,7,8-tetrahydroimidazo[1,2-*a*]pyrazine-2-carboxylic acid ethyl ester (1.8 g, 7.9 mmol) under a nitrogen atmosphere at −78 °C and stirred for 2 h. The mixture was quenched with 1 M HCl. The reaction mixture was filtered through a pad of Celite. The filtrate was washed with 50% aqueous K_2CO_3 solution, and the aqueous layer was extracted with CH_2Cl_2 (3 × 200 mL). The combined organic layer was dried (K_2CO_3) and filtered and concentrated under reduced pressure. The residue was applied to silica gel column chromatography, and the column was eluted with $CHCl_3$/MeOH (19/1−9/1). The title compound was obtained as colorless crystals. Yield: 591 mg 42%).

Step 5: (5*R*, 6*RS*)-6-[(*RS*)-Acetoxy(7-methyl-5,6,7,8-tetrahydroimidazo[1,2-*a*]pyrazin-2-yl)methyl]-6-bromo-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic Acid 4-Nitrobenzyl Ester (Diastereomers). Starting from 7-Methyl-5,6,7,8-tetrahydroimidazo[1,2-*a*]pyrazine-2-carbaldehyde (1.9 g, 10.3 mmol) and (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 (3.32 g, 8.6 mmol) and following the procedure outlined for the preparation of **14a**, a mixture of two diastereoisomers **13d** were obtained as red oil. The products were purified by silica gel column chromatography and eluted with $CHCl_3$/acetone (9/1−2/1). The title compound was obtained as a two diastereomeric mixture; red oil, Yield: 1.13 g (21%).

Step 6: (5*R*),(6*Z*)-6-(7-Methyl-5,6,7,8-tetrahydroimidazo[1,2-a]pyrazin-2-ylmethylene)-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic Acid, Sodium Salt (14d). (5*R*, 6*RS*)-6-[(*RS*)-Acetoxy(7-methyl-5,6,7,8-tetrahydroimidazo[1,2-*a*]pyrazin-2-yl)methyl]-6-bromo-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid 4-nitrobenzyl ester (1.11 g, 1.8 mmol) was dissolved in THF (32 mL) and acetonitrile (32 mL). Freshly activated Zn dust (4.46 g) was added rapidly with a 0.5 M phosphate buffer (pH 6.5, 48 mL). The reaction vessel was covered with foil to exclude light and vigorously stirred for 2 h at room temperature. The reaction mixture was filtered through a pad of Celite and cooled to 3 °C, and 0.1 N NaOH was added to adjust the pH to 8.5. The filtrate was washed with ethyl acetate, and the aqueous layer was separated. The aqueous layer was concentrated under high vacuum at 35 °C. The concentrate was applied to Diaion HP-21 (20 mL, Mitsubishi Kasei Co., Ltd.) resin column chromatography. After

adsorbing, the column was eluted with H_2O/MeCN (1/0−95/5). The combined fractions were concentrated under high vacuum at 35 °C and lyophilized to give the title compound as a yellow amorphous solid. Yield: 417 mg (65%); mp 200 °C (dec).

Preparation of (5*R*), (6*Z*)-6-(5,6-Dihydro-8*H*-imidazo[2,1-*c*]-[1,4]thiazin-2-ylmethylene)-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic Acid, Sodium Salt (14e) and (5*R*), (6*E*)-6-(5,6-Dihydro-8*H*-imidazo[2,1-*c*][1,4]thiazin-2-ylmethylene)-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic Acid, Sodium Salt (14l). Step 1: 5-Methylthio-3,6-dihydro-2*H*-[1,4]-thiazine hydroiodide (28). 5-Methylthio-3,6-dihydro-2*H*-[1,4]-thiazine hydroiodide was prepared by the method outlined by Ishibashi et al.[31]

Step 2: 3-Iminothiomorpholin Hydrochloride (22). 5-Methylthio-3,6-dihydro-2*H*-[1,4]thiazine hydroiodide (**28**) (7.1 g, 48.2 mmol) was dissolved in an 10% K_2CO_3 aqueous solution (150 mL) and extracted with CH_2Cl_2 (5 × 70 mL). The combined organic layer was dried ($MgSO_4$), filtered, and concentrated under reduce pressure. Ammonium chloride (1.7 g, 32 mmol) was added to the obtained residue in dry ethanol (128 mL) and heated to reflux for 1 h. The reaction mixture was cooled to room temperature and concentrated, and the iminothiomorpholin hydrochloride (**22**) was obtained as a brown solid (4.3 g, quant.).

Step 3: 5,6-Dihydro-8*H*-imidazo[2,1-*c*][1,4]thiazine-2-carbaldehyde (12e) and 5,6-Dihydro-8*H*-imidazo[2,1-*c*][1,4]thiazine-3-carbardehyde (23). A mixture of 2-bromo-3-hydroxypropenal (4.3 g, 22 mmol), *p*-toluenesulfonic acid monohydrate (52 mg), and 2-propanol (5.3 mL) in cyclohexane (43 mL) was azeotroped in a Dean-Stork apparatus, until no more water distilled over. The reaction mixture was concentrated under reduce pressure, and the residue was dissolved in dry ethanol (28 mL). This was added to a mixture of the dry ethanol (143 mL) solution of 3-iminothiomorpholin hydrochloride (**22**) (4.3 g, 28.3 mmol) and 28% methanol solution of sodium methylate (5.0 mL) and stirred at room temperature for 1 h. At the end, the reaction mixture was concentrated and dissolved in chloroform (128 mL) and triethylamine (3.6 mL). It was heated to reflux for 2.5 h and concentrated under reduce pressure. The residue was dissolved with dichloromethane (300 mL) and washed with 50% aqueous K_2CO_3 solution (2 × 100 mL), and the organic layer was dried ($MgSO_4$) and filtered. The filtrate was concentrated under reduced pressure and purified by silica gel column chromatography by eluting it with $CHCl_3$/acetone (10:1). 5,6-Dihydro-8*H*-imidazo[2,1-*c*][1,4]thiazine-2-carbardehyde (**12e**) (brown solid, 445 mg, 10.3%) and 5,6-Dihydro-8*H*-imidazo[2,1-*c*][1,4]thiazine-3-carbardehyde (**23**) (brown solid, 872 mg, 20.2%) were obtained.

Step 4: (5*R*), (6*Z*)-6-(5,6-Dihydro-8*H*-imidazo[2,1-*c*][1,4]-thiazin-2-ylmethylene)-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic Acid, Sodium Salt (14e) (5*R*), (6*E*)-6-(5,6-Dihydro-8*H*-imidazo[2,1-*c*][1,4]thiazin-2-ylmethylene)-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic Acid, Sodium Salt (14l). A dry acetonitrile (20 mL) solution of 5,6-dihydro-8*H*-imidazo[2,1-*c*][1,4]thiazine-2-carbaldehyde (392 mg, 2.3 mmol) was added to an acetonitrile solution of $MgBr_2$ (1.1 g, 5.9 mmol) under a nitrogen atmosphere at room temperature, and the mixture was stirred for 10 min. A dry THF (40 mL) solution 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 (**11**) (1.0 g, 2.5 mmol) was added, and the mixture was cooled to −20 °C, and then triethylamine (0.8 mL) was added in one portion. The reaction vessel was covered with foil to exclude light. It was stirred for 8 h at −20 °C and treated with 4-dimethylamino pyridine (30 mg) and acetic anhydride (0.44 mL) in one portion. The reaction mixture was warmed to 0 °C and stirred for 14 h. The reaction mixture was worked-up as before, and the residue was purified by silica gel column chromatography by eluting it with CH_2Cl_2/acetone (50:1). The crude (5*R*)-6-[acetoxy-(5,6-dihydro-8*H*-imidazo[2,1-*c*][1,4]thiazin-2-yl)methyl]-6-bromo-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid *p*-nitrobenzyl ester (**13e**) was obtained as a solid, which was dissolved in THF/acetonitrile (1:1, 30 mL), and freshly activated Zn dust (2.2 g) was added rapidly along with 0.5 mol/L of phosphate buffer (pH 6.5, 17 mL). The reaction vessel was covered with foil to exclude light and vigorously stirred for 2 h. The reaction mixture was worked-up as described in the previous experiments and purified by Diaion HP-21 resin (30 mL, Mitsubishi Kasei Co., Ltd.) column chromatography. After adsorbing, the column was eluted with water and then 10% acetonitrile aqueous solution. The combined active fractions were concentrated under high vacuum at 35 °C and lyophilized to give (5*R*), (6*Z*)-6-(5,6-dihydro-8*H*-imidazo[2,1-*c*][1,4]thiazin-2-ylmethylene)-7-oxo-4-thia-1-azabicyclo-[3.2.0]hept-2-ene-2-carboxylic acid, sodium salt (**14e**) as a yellow amorphous solid (168 mg, 20.9%). A small amount of the E isomer **14l** was also formed as a byproduct; mp 135 °C (dec).

Preparation of (5*R*)(6*Z*)-7-Oxo-6-(4,5,6,7-tetrahydropyrazolo-[1,5-*a*]pyridin-2-ylmethylene)-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic Acid, Sodium Salt (14f). Step 1: Tetrahydropyridino[1,2-*c*][1,2,3]oxadiazolone (31a). Concentrated HCl (1.96 mL) and $NaNO_2$ (2.2 g, 32 mmol) were added to the H_2O (21 mL) solution of *DL*-pipecolic acid (**29a**) (3.04 g, 23.38 mmol) under a nitrogen atmosphere at 0 °C and stirred for 1 h. The solution was extracted with CH_2Cl_2, and the organic layer was washed with brine. The mixture was dried over Na_2SO_4 and concentrated under reduced pressure to afford crude (2*RS*)-1-nitrosopiperidine-2-carboxylic acid (**30a**) as pale yellow crystals.

Trifluoroacetic anhydride (1.93 g, 9.1 mmol) was added to the THF (92 mL) solution of crude (2*RS*)-1-nitrosopiperidine-2-carboxylic acid (**30a**) (1.26 g, 8 mmol) under a nitrogen atmosphere at 0 °C and stirred for 5 h and, subsequently, for another 2 h at room temperature. The solution was concentrated under reduced pressure. The residue was applied to silica gel column chromatography, and the column was eluted with *n*-hexane/AcOEt (1/1−0/1). The title compound was obtained as colorless crystals (1.10 g, 98%).

Step 2: 4,5,6,7-Tetrahydropyrazolo[1,5-*a*]pyridine-2-carboxylic Acid Ethylester (32a). Ethyl propiolate (804 mg, 8.2 mmol) was added to an *o*-xylene (15 mL) solution of tetrahydropyridino-[1,2-*c*][1,2,3]oxadiazolone (**31a**) (1.04 g, 7.4 mmol) under a nitrogen atmosphere and refluxed for 16 h. The solution was concentrated under reduced pressure. The residue was applied to silica gel column chromatography, and the column was eluted with *n*-hexane/AcOEt (2/1−1/1). The title compound was obtained as a yellow oil (871 mg, 65%), and 4,5,6,7-tetrahydropyrazolo[1,5-*a*]pyridine-3-carboxylic acid ethyl ester (**32a**) was obtained as a yellow oil (345 mg, 26%).

Step 3: (4,5,6,7-Tetrahydropyrazolo[1,5-*a*]pyridin-2-yl)-methanol. To a stirred solution of MeOH (0.29 mL) and a THF (19 mL) solution of $LiBH_4$ (cont. 90%) (174 mg), 4,5,6,7-tetrahydropyrazolo[1,5-*a*]pyridine-2-carboxylic acid ethyl ester (**32a**) (862 mg, 4.4 mmol) was added and stirred for 1 h at room temperature and 1.5 h at 40 °C. The mixture was quenched with 1 M HCl to pH 1 and stirred for 1 h at room temperature. Solid K_2CO_3 was added to the solution to adjust the pH to 8, and the mixture was extracted with AcOEt. The organic layer was dried ($MgSO_4$) and filtered. The filtrate was concentrated under reduced pressure to afford the title compound as a pale yellow oil (691 mg, 95%).

Step 4: 4,5,6,7-Tetrahydropyrazolo[1,5-*a*]pyridine-2-carbaldehyde (12f). Activated MnO_2 (3.36 g) was added to the $CHCl_3$ (44 mL) solution of (4,5,6,7-tetrahydropyrazolo[1,5-*a*]pyridin-2-yl)methanol (673 mg, 4.6 mmol) and refluxed for 1 h under a nitrogen atmosphere. The reaction mixture was filtered through a pad of Celite and concentrated. The residue was applied to silica gel column chromatography, and the column was eluted with *n*-hexane/AcOEt (2/1−1/2). The title compound was obtained as a pale yellow oil (510 mg, 77%).

Step 5: (5*R*)(6*Z*)-7-Oxo-6-(4,5,6,7-tetrahydropyrazolo[1,5-*a*]-pyridin-2-ylmethylene)-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic Acid, Sodium Salt (14f). 4,5,6,7-Tetrahydropyrazolo [1,5-*a*] pyridine-2-carbaldehyde (**12f**) (483 mg, 3.3 mmol) was reacted with a THF solution of (5*R*, 6*S*)-6-bromo-7-oxo-4-thia-1-azabicyclo [3.2.0] hept-2-ene-2-carboxylic acid 4-nitrobenzyl ester (**11**) (1.28 g, 3.3 mmol) in the presence of anhydrous $MgBr_2$ (1.81 g, 9.7 mmol) as mentioned in the previous examples, and the

product **13f** was isolated as a crude residue. The crude product **13f** was dissolved in THF (35 mL) and acetonitrile (16 mL). Freshly activated Zn dust (7.43 g) was added rapidly with a 0.5 M phosphate buffer (pH 6.5, 51 mL). The reaction vessel was covered with foil to exclude light and vigorously stirred for 1.5 h at room temperature. The reaction mixture was filtered through a pad of Celite, and the filtrate was washed with ethyl acetate. The aqueous layer was cooled to 3 °C and 0.1 M NaOH was added to adjust the pH to 8.0. The mixture was concentrated under high vacuum at 35 °C, and the concentrate was applied to Diaion HP-21 (105 mL, Mitsubishi Kasei Co., Ltd.) resin column chromatography. After adsorbing, the column was eluted with H_2O/MeCN (1/0−85/15). The combined fractions were concentrated under high vacuum at 35 °C and lyophilized to give the title compound as a yellow amorphous solid (427 mg, 41%, pH 7.7); mp 190 °C (dec).

Preparation of (5*R*)(6*Z*)-6-(6,7-Dihydro-4*H*-pyrazolo[5,1-*c*]-[1,4]thiazin-2-ylmethylene)-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic Acid, Sodium Salt (14g). Step 1: (3*R*)-Thiomorpholine-3-carboxylic Acid (29b). The title compound was prepared by using the procedure described by Shiraiwa and coworkers.[29]

Step 2: 3-Oxo-3a,4,6,7-tetrahydro-3*H*-2-oxa-5-thia-1-aza-7a-azonioindenide (31b). $NaNO_2$ (3.14 g, 46 mmol) was added to the 1 mol/L HCl (33.7 mL) solution of (3*R*)-thiomorpholine-3-carboxylic acid (**29b**) (4.96 g, 33.74 mmol) under a nitrogen atmosphere at 0 °C and stirred for 0.5 h. The solution was extracted with $CHCl_3$ (5 times), and the organic layer was washed with brine. The mixture was dried over $MgSO_4$ and concentrated under reduced pressure to afford crude (3*R*)-4-nitrosothiomorpholine-3-carboxylic acid (**30b**) as a pale yellow solid.

Trifluoroacetic anhydride (7.07 g, 33.6 mmol) was added to the THF (169 mL) solution of crude (3*R*)-4-nitrosothiomorpholine-3-carboxylic acid (**30b**) under a nitrogen atmosphere at 0 °C and stirred for 3 h at 0 °C and for 17 h at room temperature. The solution was concentrated under reduced pressure. The residue was applied to silica gel column chromatography, and the column was eluted with *n*-hexane/AcOEt (1/1−0/1). The title compound was obtained as pale brown crystals (3.41 g, 64%).

Step 3: 6,7-Dihydro-4*H*-pyrazolo[5,1-*c*][1,4]thiazine-2-carboxylic Acid Ethyl Ester (32b). Ethyl propiolate (2.33 g, 23.7 mmol) was added to the *o*-xylene (72 mL) solution of 3-oxo-3a,4,6,7-tetrahydro-3*H*-2-oxa-5-thia-1-aza-7a-azonioindenide (**31b**) (3.41 g, 21 mmol) under a nitrogen atmosphere and refluxed for 15 h. The solution was concentrated under reduced pressure, and the residue was applied to silica gel column chromatography, and the column was eluted with *n*-hexane/AcOEt (2/1−1/1). The title compound was obtained as a yellow oil (3.13 g, 68%), and the other unwanted regio isomer **33b** 6,7-dihydro-4*H*-pyrazolo[5,1-*c*]-[1,4]thiazine-3-carboxylic acid ethyl ester was also obtained a as yellow oil (556 mg, 12%).

Step 4: (6,7-Dihydro-4*H*-pyrazolo[5,1-*c*][1,4]thiazin-2-yl)-methanol. $LiBH_4$ (cont. 90%) (536 mg) and MeOH (0.9 mL) were added to the THF (59 mL) solution of 6,7-dihydro-4*H*-pyrazolo-[5,1-*c*][1,4]thiazine-2-carboxylic acid ethyl ester (**32b**) (3.13 g, 14.76 mmol) under a nitrogen atmosphere at room temperature and stirred for 3 h at 40 °C. The mixture was quenched with 1 mol/L HCl at pH 1 and stirred for 1 h at room temperature. Solid K_2CO_3 was added to the solution to adjust the pH to 8, and the mixture was extracted with AcOEt. The organic layer was dried over K_2CO_3 and filtered. The filtrate was concentrated under reduced pressure to afford title compound as a pale yellow oil (2.51 g, quant.).

Step 5: 6,7-Dihydro-4*H*-pyrazolo[5,1-*c*][1,4]thiazine-2-carbaldehyde (12g). Activated MnO_2 (11.46 g, excess) was added to the $CHCl_3$ (135 mL) solution of (6,7-dihydro-4*H*-pyrazolo[5,1-*c*]-[1,4]thiazin-2-yl)methanol (2.31 g, 14 mmol) and refluxed for 1 h under a nitrogen atmosphere. The reaction mixture was filtered through a pad of Celite, and the filtrate was concentrated under reduced pressure. The residue was applied to silica gel column chromatography, and the column was eluted with *n*-hexane/AcOEt (1/1). The title compound was obtained as pale yellow crystals (1.78 g, 78%).

Step 6: (5*R*)(6*Z*)-6-(6,7-Dihydro-4*H*-pyrazolo[5,1-*c*][1,4]-thiazin-2-ylmethylene)-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic Acid, Sodium Salt (14g). 6,7-Dihydro-4*H*-pyrazolo[5,1-*c*][1,4]thiazine-2-carbaldehyde (**12g**) (841 mg, 5 mmol) was condensed with a dry THF solution (39 mL) of (5*R*, 6*S*)-6-bromo-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid 4-nitrobenzyl ester (**11**) (1.93 g, 10.3 mmol) as mentioned in the previous examples, and the product **13g** was isolated as a crude residue.

The residue was dissolved in THF (83 mL) and acetonitrile (39 mL), and freshly activated Zn dust (7.72 g) was added rapidly along with a 0.5 M phosphate buffer (pH 6.5, 122 mL). The reaction vessel was covered with foil to exclude light, and the reaction mixture was vigorously stirred for 1.5 h at room temperature. The reaction mixture was filtered through a pad of Celite. The filtrate was washed with ethyl acetate, and the aqueous layer was separated. The aqueous layer was cooled to 3 °C, and 0.1 N NaOH was added to adjust the pH to 8.0. The mixture was concentrated under high vacuum at 35 °C. The concentrate was applied to Diaion HP-21 (150 mL, Mitsubishi Kasei Co., Ltd.) resin column chromatography. After adsorbing, the column was eluted with H_2O/MeCN (1/0−85/15). The combined fractions were concentrated under high vacuum at 35 °C and lyophilized to give the title compound as a yellow amorphous solid (371 mg, 22%, pH 8.0). Mp 190 °C (dec).

Preparation of (5*R*),(6*Z*)-6-(5,5-Dioxo-4,5,6,7-tetrahydro-5λ^6-pyrazolo[5,1-*c*][1,4]thiazin-2-ylmethylene)-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic Acid, Sodium Salt (14h). Step 1: 5,5-Dioxo-4,5,6,7-tetrahydro-5λ^6-pyrazolo[5,1-*c*][1,4]-thiazine-2-carbaldehyde (12h). *m*-Chloroperbenzoic acid (cont. 69%) (6.36 g, excess) was added to the CH_2Cl_2 (111 mL) solution of 6,7-dihydro-4*H*-pyrazolo[5,1-*c*][1,4]thiazine-2-carbaldehyde (**12g**) (1.86 g, 11 mmol) at 0 °C, and the reaction mixture was stirred for 18 h at room temperature. At the end, the reaction mixture was concentrated under reduced pressure. The residue obtained was triturated with 10 mL of THF and filtered to obtain crystals. The filtrate was concentrated under reduced pressure. The residue was triturated with 5 mL of THF and filtered to obtain more crystals. The combined crystals were dried under reduced pressure to give the title compound as a colorless solid (1.96 g, 89%).

Step 2:(5*R*, 6*RS*)-6-[(*RS*)-Acetoxy-(5,5-dioxo-4,5,6,7-tetrahydro-5λ^6-pyrazolo[5,1-*c*][1,4]thiazin-2-yl)-methyl]-6-bromo-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic Acid 4-Nitrobenzyl Ester (13h). 5,5-Dioxo-4,5,6,7-tetrahydro-5λ^6-pyrazolo[5,1-*c*][1,4]thiazine-2-carbaldehyde (**14h**) (1.95 g, 9.75 mmol) was condensed with a dry THF solution of (5*R*, 6*S*)-6-bromo-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid 4-nitrobenzyl ester (3.88 g, 10 mmol) in the presence of anhydrous $MgBr_2$ and Et_3N at −20 °C as outlined in the previous experiments. The reaction vessel was covered with foil to exclude light. The reaction mixture was stirred for 3 h at −20 °C and treated with acetic anhydride (cont. 97%) (3.79 mL) and DMAP (cont. 99%) (120 mg) in one portion. 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 ($MgSO_4$), followed by concentration under reduced pressure. The residue was purified with silica gel column chromatography ($CHCl_3$/acetone, 19:1−4:1) to give the title compound as a pale brown amorphous solid (diastereomeric mixture (8:2)); 1.35 g (22%).

Step 3: (5*R*),(6*Z*)-6-(5,5-Dioxo-4,5,6,7-tetrahydro-5λ^6-pyrazolo[5,1-*c*][1,4]thiazin-2-ylmethylene)-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic Acid, Sodium Salt (14h). (5*R*,6*RS*)-6-[(*RS*)-Acetoxy-(5,5-dioxo-4,5,6,7-tetrahydro-5λ^6-pyrazolo[5,1-*c*][1,4]-thiazin-2-yl)-methyl]-6-bromo-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid 4-nitrobenzyl ester (**13h**) (1.33 g, 2.1 mmol) was dissolved in THF (19 mL) and acetonitrile (9 mL). Freshly activated Zn dust (5.32 g) was added rapidly with a 0.5 M phosphate buffer (pH 6.5, 27 mL). The reaction

vessel was covered with foil to exclude light. The reaction mixture was vigorously stirred for 1.5 h at room temperature. The insoluble material was filtered off and washed with H_2O (27 mL). The filtrate was washed with ethyl acetate (27 mL), and the aqueous layer was cooled to 3 °C, and 0.1 N NaOH was added to adjust the pH to 8. The mixture was concentrated under high vacuum at 35 °C. The concentrate was treated to Diaion HP-21 (80 mL, Mitsubishi Kasei Co., Ltd.) resin column chromatography. After adsorbing, the column was eluted with H_2O/MeCN (1/0−9/1). The combined fractions were concentrated under high vacuum at 35°C and lyophilized to give the title compound as a yellow amorphous solid (306 mg, 38%).mp 180 °C (dec).

Preparation of (5*R*,6*Z*)-6-(6,7-Dihydro-4*H*-pyrazolo[5,1-*c*]-[1,4]oxazin-2-ylmethylene)-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic Acid Sodium Salt (14i): Step 1: 4-Nitrosomorpholine-3-carboxylic Acid (30c). To a solution of morpholine-3-carboxylic acid (**29c**) (6.96 g, 52 mmol) in water (20 mL) at 0 °C under a nitrogen atmosphere was added concentrated hydrochloric acid (4 mL), followed by sodium nitrite (5.0 g, 72 mmol) in small portions. The mixture was stirred at 0 °C for 1 h and concentrated under vacuum at 30−35 °C. The residue was stirred with 200 mL of acetone and filtered. The filtrate was evaporated and the residue treated with 50 mL of THF and concentrated. The process was repeated with 2 × 50 mL of THF to give 11.87 g of light yellow foam.

Step 2: 6,7-Dihydro-4*H*-[1,2,3]oxadiazolo[4,3-c][1,4]oxazin-8-ium-3-olate (31c). The crude 4-nitrosomorpholine-3-carboxylic acid (**30c**) (11.0 g, 68 mmol) from step 1 was dissolved in THF (250 mL) and cooled to 0 °C. A solution of trifluoroacetic anhydride (7.4 mL, 52 mmol) in THF (20 mL) was added with stirring over 10 min. The resulting mixture was stirred at 0 °C for 5 h, and warmed to room temperature for 16 h. The solvent was evaporated, and the residue was diluted with 250 mL of ethyl acetate and stirred with 30 g of anhydrous potassium carbonate. The mixture was filtered through a pad of silica gel, and the filtrate was evaporated. The residue was washed with a mixture of ethyl acetate-ether to give 3.80 g of a white solid.

Step 3: Ethyl 6,7-Dihydro-4*H*-pyrazolo[5,1-*c*][1,4]oxazine-2-carboxylate (32c). To a stirred solution of 6,7-dihydro-4*H*-[1,2,3]-oxadiazolo[4,3-*c*][1,4]oxazin-8-ium-3-olate (**31c**) (3.41 g, 24 mmol) in *o*-xylene (80 mL) was added ethyl propiolate (2.7 mL, 26 mmol) and heated at 140 °C for 3 h. An additional 2.0 mL (19 mmol) of ethyl propiolate was then added, and the mixture was stirred at reflux for 18 h. The reaction mixture was evaporated under vacuum, and the residue was dissolved in a mixture of methylene chloride and hexanes (1:5). The solution was passed through a pad of silica gel, and the filter pad was eluted with methylene chloride−hexanes, followed by ethyl acetate. The ethyl acetate was evaporated, and the residue washed with hexanes to give 4.10 g of a white solid; mp 63 °C; $(M + H^+)$ 197.1.

Step 4: 6,7-Dihydro-4*H*-pyrazolo[5,1-*c*][1,4]oxazin-2-ylmethanol. To a solution of ethyl 6,7-dihydro-4*H*-pyrazolo[5,1-*c*][1,4]-oxazine-2-carboxylate 32c (1.57 g, 8.0 mmol) in methylene chloride (30 mL) was added 24 mL of a 1.0 M solution of diisobutylaluminum hydride in methylene chloride at 0 °C under nitrogen. After stirring for 0.5 h at 0 °C, the mixture was warmed to room temperature for 2 h. The reaction mixture was quenched with 30 mL of saturated ammonium chloride solution and extracted with ethyl acetate. The organic solution was washed with brine, dried over anhydrous sodium sulfate, filtered, and evaporated to give 1.27 g of a colorless oil.

Step 5: 6,7-Dihydro-4*H*-pyrazolo[5,1-*c*][1,4]oxazine-2-carbaldehyde (12i). To a solution of 6,7-dihydro-4*H*-pyrazolo[5,1-*c*]-[1,4]oxazin-2-ylmethanol (1.08 g, 7.0 mmol) in 1,2-dichloroethane (30 mL) was added 5.4 g of activated manganese dioxide at room temperature with stirring. The mixture was heated to 60 °C for 1 h and then stirred at room temperature for 16 h. The reaction mixture was filtered through a column of silica gel topped with Celite. The filter pad was eluted with methylene chloride, followed by ethyl acetate. The ethyl acetate was evaporated, and the residue triturated with hexanes to give 0.81 g of a white solid.

Step 6: 4-Nitrobenzyl (5*R*)-6-[(Acetyloxy)(6,7-dihydro-4*H*-pyrazolo[5,1-*c*][1,4]oxazin-2-yl)methyl]-6-bromo-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylate (13i). To a stirred solution of $MgBr_2$ (0.94 g, 5.1 mmol) in acetonitrile (25 mL) under nitrogen was added 6,7-dihydro-4*H*-pyrazolo[5,1-*c*][1,4]oxazine-2-carbaldehyde (**12i**) (0.26 g, 1.7 mmol) at room temperature. A solution of (5*R*,6*S*)-6-bromo-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid 4-nitrobenzyl ester (**11**) (0.58 g, 1.5 mmol) in THF (25 mL) was added, and the mixture was cooled to −20 °C. Triethylamine (0.71 mL, 5.1 mmol) was introduced, and the mixture was stirred at −20 °C in the dark for 5 h. It was then treated with acetic anhydride (0.6 mL, 6.0 mmol) and 4-*N*,*N*-dimethylaminopyridine (24 mg, 0.2 mmol) and kept at 0 °C for 18 h. The mixture was concentrated, and the residue was dissolved in ethyl acetate. The ethyl acetate solution was washed with 5% citric acid followed by a saturated sodium bicarbonate solution and brine. The organic layer was dried over anhydrous sodium sulfate and evaporated. The crude material was chromatographed with silica gel (EtOAc/DCM, 1:5) to give 0.77 g of a white foam.

Step 7: (5*R*,6*Z*)-6-(6,7-Dihydro-4*H*-pyrazolo[5,1-*c*][1,4]oxazin-2-ylmethylene)-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic Acid Sodium Salt (14i). To a solution of 4-nitrobenzyl (5*R*)-6-[(acetyloxy)(6,7-dihydro-4*H*-pyrazolo[5,1-*c*][1,4]oxazin-2-yl)methyl]-6-bromo-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylate (**13i**) (0.35 g, 0.6 mmol) in THF (20 mL) under nitrogen was added 20 mL of a phosphate buffer solution (0.5 M, pH 6.5) and 120 mg of 10% Pd/C. The mixture was hydrogenated at 40−50 psi for 3 h and then filtered through Celite. The filter pad was washed with THF and concentrated to 25 mL. Using 0.1 N NaOH, the pH was adjusted to 8.5, and the aqueous solution was washed with ethyl acetate. The product was purified by Diaion HP-21 resin column chromatography. After loading the column, it was eluted with H_2O/MeCN (1/0−9/1). The combined fractions containing the product was concentrated under high vacuum at 35 °C and lyophilized to give the title compound as a yellow amorphous solid. $(M + H)^+$ 306.0.

Preparation of (5*R*),(6*Z*)-7-Oxo-6-(4,5,6,7-tetrahydropyrazolo-[1,5-a]pyrazin-2-ylmethylene)-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic Acid, Sodium Salt (14j). Piperazine-2-carboxylic Acid, Dihydrochloride. The title compound was prepared by the procedure described by Wu and co-workers. [30]

Step 1: Piperazine-1,3-dicarboxylic acid 1-(4-nitrobenzyl) Ester (29d). $CuCO_3 \cdot Cu(OH)_2 \cdot H_2O$ (15.8 g, 66 mmol) was added to a stirred aqueous solution (275 mL) of piperazine-2-carboxylic acid and dihydrochloride (22.3 g, 110 mmol), and the mixture was refluxed 10 min. The insoluble material was filtered off and washed with hot H_2O (165 mL). The filtrate was cooled to room temperature, and $NaHCO_3$ (9.2 g) and 1,4-dioxane (220 mL) was added to the dark blue solution. The mixture was cooled to 0 °C, and additional $NaHCO_3$ (18.5 g) was added. To the stirred solution, a 50% solution of 4-nitrobenzyl chloroformate in 1,4-dioxane (61.7 g) was added to the reaction mixture and stirred at 0 °C for 1.5 h. The precipitate that formed was filtered and washed with cold H_2O (140 mL), EtOH (100 mL), acetone (200 mL), and Et_2O (100 mL) and allowed to dry under reduced pressure to obtain the pale blue crystals. The crystals were mixed with 1 N HCl (330 mL) and EDTA·2Na (20.5 g). This was stirred for 2 h at room temperature. The suspension was filtered, and the filtrate was diluted with EtOH/H_2O (7:3, 550 mL) and refluxed for 10 min. The separated solid was filtered, and the combined solids were crystallized from ethanol/water (7:3) and dried under reduced pressure to obtain the title compound (26.25 g, 77%) as colorless crystals.

Step 2: 5-(4-Nitrobenzyloxycarbonyl)-3-oxo-3a,4,6,7-tetrahydro-3*H*-2-oxa-1,5-diaza-7a-azoniainden-3a-ide (31d). The aqueous (300 mL) solution of $NaNO_2$ (cont. 98.5%) (6.66 g, 98 mmol) was added to the acetic acid (864 mL) solution of piperazine-1,3-dicarboxylic acid 1-(4-nitrobenzyl) ester (26.72 g, 86.4 mmol) under a nitrogen atmosphere at 0 °C for 0.5 h and stirred for 1 h. An additional aqueous solution of $NaNO_2$ (cont. 98.5%) (2.41 g in 15 mL) was added to the reaction mixture at 0 °C and stirred for 1 h. The solution was concentrated under reduced pressure, and the

residue was extracted with ethyl acetate (5 times) and washed with brine. The mixture was dried over $MgSO_4$, filtered, and concentrated under reduced pressure to afford crude 4-nitrosopiperazine-1,3-dicarboxylic acid 1-(4-nitrobenzyl) ester (**30d**) as a pale brown amorphous solid (25.77 g, 88.2%).

The solid obtained above was dissolved in THF (371 mL), and a solution of trifluoroacetic anhydride in THF (24.0 g in 10 mL THF) was added slowly under nitrogen atmosphere at 0 °C for 15 min. The solution was stirred for 1.5 h at 0 °C and for 1h at room temperature and further stirred for 20 h at room temperature. The precipitated solid was filtered and washed with THF and Et_2O. The second crop of the product was obtained by concentrating the filtrate under reduced pressure and triturating with THF. The separated solids were combined and dried under reduced pressure to afford the title compound as colorless crystals (22.3 g, 91%).

Step 3: 6,7-Dihydro-4*H*-pyrazolo[1,5-*a*]pyrazine-2,5-dicarboxylic Acid 2-Ethyl Ester 5-(4-Nitrobenzyl) Ester (32d). Ethyl propiolate (cont. 99%)(8.28 g, 84.5 mmol) was added to the *o*-xylene (348 mL) solution of 5-(4-nitrobenzyloxycarbonyl)-3-oxo-3a,4,6,7-tetrahydro-3*H*-2-oxa-1,5-diaza-7a-azoniainden-3a-ide (**31d**) (22.3 g, 69 mmol) under a nitrogen atmosphere and refluxed for 16 h. The solution was concentrated under reduced pressure, followed by silica gel column chromatography (*n*-hexanesEtOAc, 2/1−1/3) and yielded the title compound as pale yellow crystals (16.78 g, 64%). 6,7-Dihydro-4*H*-pyrazolo[1,5-*a*]pyrazine-3,5-dicarboxylic acid 3-ethyl ester 5-(4-nitrobenzyl) ester (**33d**) was also obtained as pale yellow crystals (6.18 g, 24%).

Step 4: 2-Hydroxymethyl-6,7-dihydro-4*H*-pyrazolo[1,5-*a*]pyrazine-5-carboxylic Acid 4-Nitrobenzyl Ester. $LiBH_4$ (640 mg, 29 mmol) and MeOH (1.2 mL) were added to the THF (267 mL) solution of 6,7-dihydro-4*H*-pyrazolo[1,5-*a*]pyrazine-2,5-dicarboxylic acid 2-ethyl ester 5-(4-nitrobenzyl) ester (**32d**) (10 g, 26.5 mmol) under a nitrogen atmosphere at room temperature and stirred for 3 h at 40 °C. Additional $LiBH_4$ (523 mg) and MeOH (1.0 mL) were added to the solution and stirred for 2 h at 50 °C and 1 h. The mixture was cooled and acidified with 3 mol/L of HCl to pH 2 and stirred for 1 h at room temperature, and then solid K_2CO_3 was added to the solution to adjust the pH to 8. The insoluble material was filtered off, and the filtrate was extracted with EtOAc. The organic layer was dried (K_2CO_3) and concentrated under reduced pressure. The residue was purified with silica gel column chromatography ($CHCl_3$/MeOH, 49/1−19/1) to afford the title compound as a pale yellow solid (8.44 g, 95%).

Step 5: 2-Formyl-6,7-dihydro-4*H*-pyrazolo[1,5-*a*]pyrazine-5-carboxylic Acid 4-Nitrobenzyl Ester (12j). Activated MnO_2 (84.2 g, excess) was added to the $CHCl_3$/MeOH (95:5, 253 mL) solution of 2-hydroxymethyl-6,7-dihydro-4*H*-pyrazolo[1,5-*a*]pyrazine-5-carboxylic acid 4-nitrobenzyl ester (8.42 g, 25.2 mmol), and the mixture was refluxed for 1 h under a nitrogen atmosphere. The reaction mixture was filtered through a pad of Celite. Silica gel (20 g) was added to the filtrate, and the solvent was removed under reduced pressure to give the silica gel coating with the crude product. The above silica gel was loaded to silica gel column and eluted with $CHCl_3$/MeOH (49/1−19/1). The title compound was obtained asa yellow solid (2.82 g, 34%).

Step 6: 2-{(*RS*)-Acetoxy-[(5*R*, 6*RS*)-6-bromo-2-(4-nitrobenzyloxycarbonyl)-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-en-6-yl]-methyl}-6,7-dihydro-4*H*-pyrazolo[1,5-*a*]pyrazine-5-carboxylic Acid 4-Nitrobenzyl Ester. 2-Formyl-6,7-dihydro-4*H*-pyrazolo[1,5-*a*]-pyrazine-5-carboxylic acid 4-nitrobenzyl ester (**12j**) (2.71 g, 8.1 mmol) was condensed with (5*R*, 6*S*)-6-bromo-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid 4-nitrobenzyl ester (**11**) (3.27 g, 8.4 mmol) in the presence of anhydrous $MgBr_2$ and Et_3N at −20 °C. After 8 h, reaction mixture was treated with acetic anhydride (cont. 97%) (3.19 mL) and DMAP (203 mg). The reaction mixture was warmed to 0 °C and stirred for 18 h. At the end, the reaction mixture was diluted with ethyl acetate and worked-up as described before. The residue was purified with silica gel column chromatography (*n*-hexane/EtOAc (1/1−2/3), $CHCl_3$/acetone (29/1−9/1), and $CHCl_3$/acetone (29/1)), and the title compound was obtained as a yellow amorphous solid (diastereomeric mixture (64:36), 3.30 g, 53%).

Step 7: (5*R*),(6*Z*)-7-Oxo-6-(4,5,6,7-tetrahydropyrazolo[1,5-a]-pyrazin-2-ylmethylene)-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic Acid, Sodium Salt (14j). To a stirred solution of 2-{(*RS*)-acetoxy-[(5*R*,6*RS*)-6-bromo-2-(4-nitrobenzyloxycarbonyl)-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-en-6-yl]-methyl}-6,7-dihydro-4*H*-pyrazolo[1,5-*a*]pyrazine-5-carboxylic acid 4-nitrobenzyl ester (3.3 g, 4.3 mmol) in THF (43 mL) and acetonitrile (20 mL), was rapidly added Zn dust (12.36 g) with a 0.5 M phosphate buffer (pH 6.5, 63 mL). The reaction vessel was covered with foil to exclude light and vigorously stirred for 1.5 h at room temperature. The insoluble material was filtered off and washed with H_2O (63 mL). The filtrate was washed with ethyl acetate (63 mL), and the aqueous layer was cooled to 3 °C, and 1 N HCl was added to adjust the pH to 2.5. The mixture was stirred for 4 h, and an additional 63 mL of water and 1 N HCl were added to adjust the pH to 2.5. It was stirred for 17 h at 3 °C. The reaction mixture was cooled to 0 °C, and 1 N NaOH was added to adjust the pH to 8. The mixture was concentrated under high vacuum at 35 °C. The concentrate was treated to Diaion HP-21 (124 mL, Mitsubishi Kasei Co., Ltd.) resin column chromatography. After adsorbing, the column was eluted with H_2O/MeCN (1/0−95/5). The combined fractions were concentrated under high vacuum at 35 °C and lyophilized to give the title compound as a yellow amorphous solid (288 mg, 22%).

Preparation of (5*R*)(6*Z*)-6-(6,7-5*H*-Dihydropyrazolo[5,1-*b*]-oxazin-2-ylmethylene)-7-oxo-4-thia-1- azabicyclo[3.2.0]hept-2-ene-2-carboxylic Acid, Sodium Salt (14k). Step 1: Preparation of Ethyl 6,7-Dihydro-5*H*-pyrazolo[5,1-*b*][1,3]oxazine-2-carboxylate (35). To the stirred suspension of ethyl 5-hydroxy-1*H*-pyrazole-3-carboxylate 34 (10.34 g, 0.66 mol) and 36.62 g of potassium carbonate in 500 mL of acetonitrile was added 14.7 g of 1,3-dibromopropane and refluxed for 16 h. The reaction mixture was allowed to cool to room temperature and filtered, and the solid was washed with acetonitrile. The filtrate was concentrated to an oil. The residue was dissolved in ethyl acetate and extracted with water. The organic phase was dried over $MgSO_4$ and evaporated to dryness; 8.80 g of the desired product was obtained (68%); mp 44−46 °C $(M + H)^+$ 197.1.

Step 2: Preparation of 2,3-Dihydro-5*H*-pyrazolo[5,1-*b*][1,3]-oxazin-2-yl-methanol. To the stirred solution of 6,7-dihydro-5*H*-pyrazolo[5,1-*b*][1,3]oxazine-2-carboxylate (**35**) (4.0 g, 20 mmol) in 100 mL of THF was added 0.71 g of lithium borohydride and 1.03 g of methanol. The solution was heated at 40 °C for 2.5 h. The reaction was quenched by 1 N HCl, adjusted to pH 1.3, and stirred at room temperature for 1 h. The reaction mixture was adjusted pH to 8 with K_2CO_3. The reaction mixture was extracted with ethyl acetate. The organic layer was dried over $MgSO_4$ and concentrated to an oil and column chromatographyed to give 2.08 g of the desired product (67%); $(M + H)^+$ 155.

Step 3: Preparation of 6,7-Dihydro-5*H*-pyrazolo[5,1-*b*][1,3]-oxazine-2-carbaldehyde (12k). To the stirred solution of 2,3-dihydro-5*H*-pyrazolo[5,1-*b*][1,3]oxazin-2-yl-methanol (2.08 g, 13.5 mmol) in 60 mL of $CHCl_3$ was added 9.38 g of MnO_2. The suspension was refluxed for 2 h under a nitrogen atmosphere. The reaction mixture was filtered through a pad of Celite, and the filtrate was concentrated to give a yellow oil. The product was purified by chromatography to yield 2.15 g of the product (78%); $(M + H)^+$ 153.

Step 4: 4-Nitrobenzy(5*R*)-6-[(acetyloxy)(6,7-dihydro-5*H*-pyrazolo[5,1-*b*][1,3]oxazin-2-yl)methyl]-6-bromo-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylate (13k). 6,7-Dihydro-5*H*-pyrazolo[5,1-*b*][1,3]oxazine-2-carbaldehyde (**12k**) (330 mg, 2 mmol) and the 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-nitrobenzyl ester (0.794 g, 2.2 mmol) were added successively to the dry acetonitrile (15 mL) solution of anhydrous $MgBr_2/O(Et)_2$ (1.2 g) under an argon atmosphere at room temperature. After cooling to −20 °C, Et_3N (2.0 mL) was added in one portion. The reaction vessel was covered with foil to exclude light. The reaction mixture was stirred for 2 h at −20 °C and treated with acetic anhydride

(1.04 mL) in one portion. The reaction mixture was warmed to 0 °C and stirred for 15 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. The filtrate was concentrated under reduced pressure. The residue was applied to silica gel column chromatography, and then the column was eluted with ethyl acetate/hexane (1:1). The collected fractions were concentrated under reduced pressure, and the mixture of diastereoisomers were taken to the next step; pale yellow amorphous solid. Yield: 0.76 g (65%); $(M + H)^+$ 579.

Step-5: (5*R*)(6*Z*)-6-(6,7-5*H*- Dihydropyrazolo[5,1-*b*]oxazin-2-ylmethylene)-7-oxo-4-thia-1- azabicyclo[3.2.0]hept-2-ene-2-carboxylic Acid, Sodium Salt (14k). 4-Nitrobenzy(5*R*)-6-[(acetyloxy)-(6,7-dihydro-5*H*-pyrazolo[5,1-*b*][1,3]oxazin-2-yl)methyl]-6-bromo-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylate (**13k**) (350 mg, 0.6 mmol) was dissolved in THF (20 mL), acetonitrile (10 mL), and 0.5 M phosphate buffer (pH 6.5, 28 mL) and hydrogenated over 10% Pd/C at 40 psi pressure. After 4 h, the reaction mixture was filtered and cooled to 3 °C, and 0.1 N NaOH was added to adjust the pH to 8.5. The filtrate was washed with ethyl acetate, and the aqueous layer was separated. The aqueous layer was concentrated under high vacuum at 35 °C to give a yellow precipitate. The product was purified by HP21 resin reverse-phase column chromatography. Initially, the column was eluted with deionized water (2 L) and the latter with 10% acetonitrile/water. The fractions containing the product were collected and concentrated at reduced pressure at room temperature. The yellow amorphous solid was washed with acetone and filtered. Yield: 103 mg (52%).

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Supporting Information Available: Elemental analysis data and ^1H NMR data for compounds **14a**−**k**. This material is available free of charge via the Internet at http://pubs.acs.org.

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