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5,5,6-Fused tricycles bearing imidazole and pyrazole 6-methylidene penems as broad-spectrum inhibitors of β-lactamases

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Abstract—β-Lactamases are serine- and metal-dependent hydrolases, produced by the bacteria as defense against β-lactam antibiotics. Commercially available inhibitors such as clavulanic acid, sulbactam, and tazobactam, which are currently used in the hospital settings, have reduced activity against newly emerging β-lactamases. Bacterial production of diverse β-lactamases including class-A, class-C, and ESBLs has motivated several research groups to search for inhibitors with a broader spectrum of activity. Previously, several novel 6-methylidene penems bearing, [5, 5] [5, 6] and [5, 5, 5] heterocycles have been synthesized in our laboratory and were shown to be potent and broad-spectrum β-lactamase inhibitors. As a continuation of our previous work and in order to extend the structure–activity relationships, in this paper, we describe herein the synthesis and in vitro, in vivo activities of several novel 5,5,6-fused tricyclic heterocycles attached to the 6-methylidene penem core. The compounds presented in the current paper are potent and broad-spectrum inhibitors of the TEM-1 and AmpC β-lactamases. In combination with piperacillin, their in vitro activities showed enhanced susceptibility to class A- and C-resistant strains studied in various bacteria. Some of the newly synthesized compounds such as **12a–c** were shown to have in vivo activity in the acute lethal infection model against TEM-1 producing organisms. The 5,5,6-fused heterocyclic ring cores such as **21**, **25**, and **35** reported here are hitherto unknown in the literature.

1. Introduction

Development of resistance to β-lactam antibiotics by various bacteria severely limits the use of penicillins and cephalosporins in the treatment of bacterial infection.[1] The most significant known mechanism related to the development of bacterial resistance to the β-lactam antibiotics is the production of class A, class B, and class C β-lactamases.[2] These enzymes hydrolyze the β-lactam ring of the antibiotics and render them inactive. To overcome this, co-administering β-lactamase inhibitors with a β-lactam antibiotic is a general practice in the hospital setting. However, alterations and mutations within the β-lactamases have allowed bacteria to overcome the effects of the β-lactamase inhibitors. The currently available inhibitors such as clavulanic acid **1**, sulbactam **2**, and tazobactam **3** (Fig. 1) are effective inhibitors against class A producing organisms; but are not effective against either class C or class B producing organisms.[3a–c] Among these classes, the most problematic β-lactamases are the class C enzymes.[2] Bacterial genera such as *Citrobacter*, *Enterobacter*, *Hafnia*, *Pseudomonas*, *Morganella*, *Providencia*, and *Serratia* are known to express class C enzymes and confer resistance to the above-mentioned commercial products as well as extended-spectrum cephalosporins.[4] In addition to class C producing bacteria, there are strains which produce multiple enzymes of class A as well as extended-spectrum β-lactamases (ESBLs),[5,6a] which are of great concern in terms of their incidence and treatment options.[6b] Thus, the treatment of infection caused by strains producing ESBLs and or AmpC β-lactamases in hospital setting is a therapeutic challenge. Therefore,

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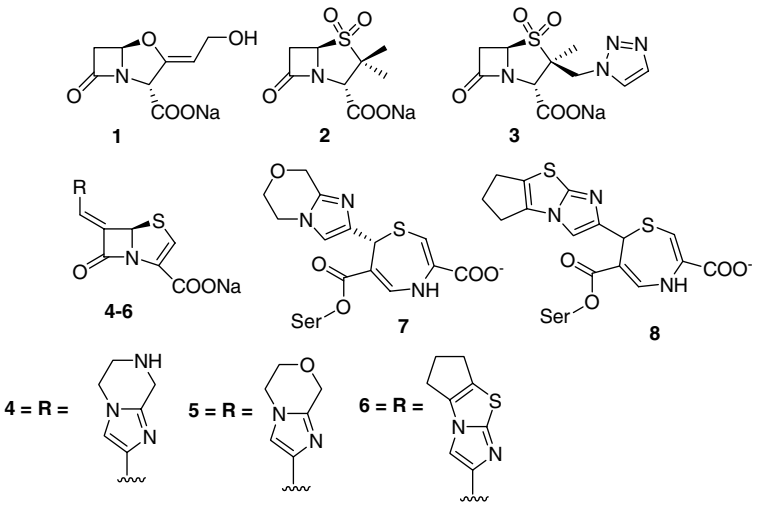


Figure 1. Commercial and previously reported β-lactamase inhibitors and 1,4-thiazepine complexes.

a continuous search for novel and broad-spectrum β-lactamase inhibitors is being actively pursued in several laboratories.[7–15]

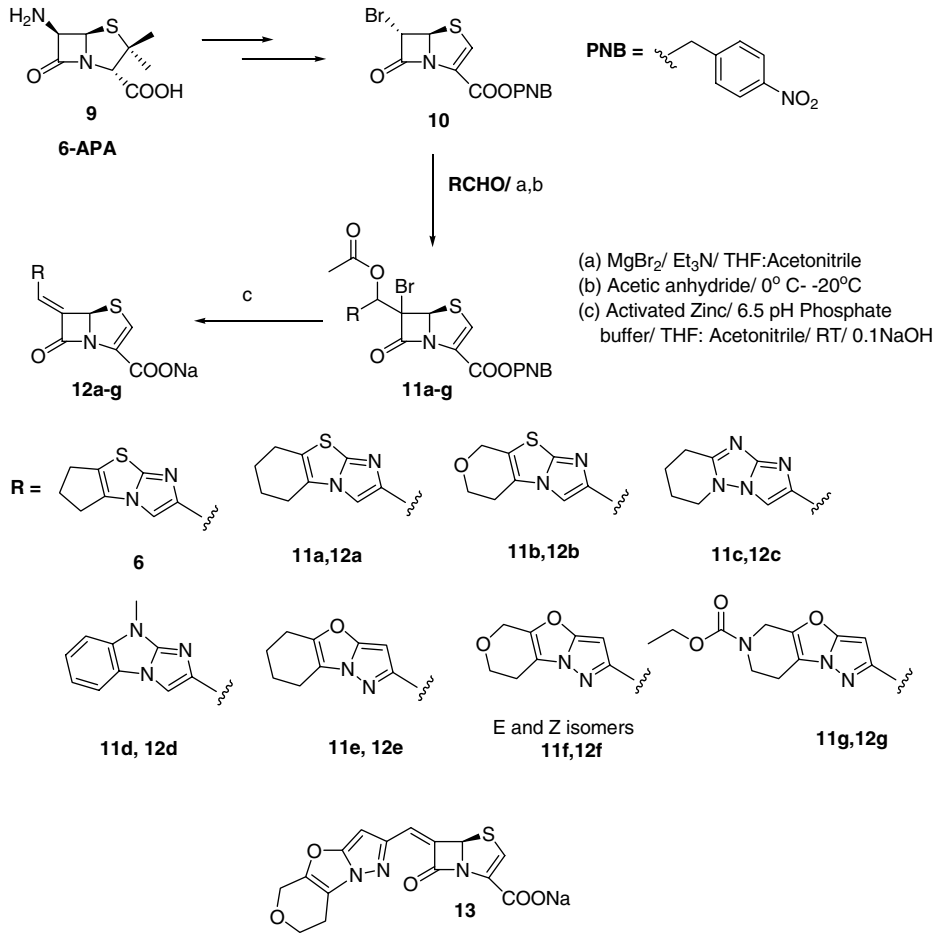
We have recently shown that several 6-methylidene penems bearing a monocyclic ring,[16a] a 6,5-fused bicyclic heterocycle or 5,5,5-fused tricyclic heterocycle[16b] are effective broad-spectrum β-lactamase inhibitors.[16c] Compounds such as **4**–**6** (Fig. 1) are of particular interest because of their broad-spectrum β-lactamase inhibitory activity. These compounds were synthesized on the basis of both modeling experiments and mechanistic considerations and were found to be potent both in vitro and in vivo. High resolution crystallographic structures of **5** and **6** with SHV-1 (class A) and GC1 enzymes revealed the formation of ring expanded seven-membered dihydro[1,4]thiazepines **7** and **8**, respectively. During these experiments, the stereochemistry of the C-7 carbon atom was also determined.[17,18] Subsequently, the formation of ring expanded dihydro[1,4]thiazepines **7** and **8** was also supported by electrospray-ionization mass spectrometry.[19] As mentioned above inhibitors such as **6** displayed good in vitro activities against ESBLs and class C expressing organisms as well as in vivo activity.[16b] This inhibitor **6** bears a 5,5,5-fused tricyclic system as a part of the 6-methylidene penem core. Hence, in order to extend the structure–activity relationships, the terminal cyclopentyl moiety in compound **6** was replaced with more lipophilic ring systems as in examples **12a**, **12d**, and **12e** and relatively polar ring system as depicted in example **12b**, **12c**, **12f**, and **12g**. The 5,5,6-fused heterocyclic ring cores such as **21**, **25**, and **35** described here are hitherto unknown in the literature. The structure–activity relationship in the series and the in vitro, in vivo data are presented in the current paper.

2. Chemistry

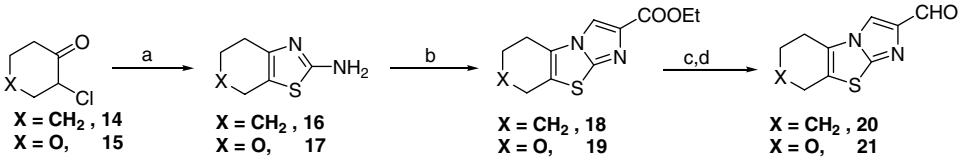
The tricyclic heterocycle bearing 6-methylidene penem carboxylic acid sodium salts **12a**–**g** (Scheme 1) were prepared by a novel two-step aldol condensation and reductive elimination procedures as described previously.[20] The pivotal starting material, namely (5*R*,6*S*)-6-bromo-7-oxo-4-thia-1-azabicyclo(3.2.0)hept-2-ene-2-carboxylic acid-4-nitrobenzyl ester **10**, required for this procedure was prepared from the commercially available 6-aminopenicilanic acid (6-APA) **9** by using a modified multi-step procedure.[21,22] Compound **10** was reacted with the appropriately substituted aldehydes **20**, **21**, **25**, **27**, and **33**–**35** in the presence of triethylamine and anhydrous $MgBr_2$ (Scheme 1).

The intermediate aldol products were trapped as their respective bromoacetoxy derivatives **11a**–**g**. Reaction of bromoacetoxy derivatives **11a**–**g** with activated zinc in phosphate buffer at pH 6.5 resulted in the introduction of the double bond with the '*Z*' geometry. During this process, the carboxyl functionality also underwent deprotection resulting in the formation of their respective sodium salts. In the case of compound **11f**, about 5% quantity of the '*E*' isomer **13** was also formed. The sodium salts of **12a**–**g** and **13** were obtained after purification on HP-21 resin (80 mL, Mitsubishi Kasei Co., Ltd) by reverse-phase column chromatography.

The aldehydes intermediates **20**, **21**, **25**, **27**, and **33**–**35** required to synthesize all the final compounds were prepared as described in the following section. Aldehydes **20** and **21** required to prepare compounds **12a** and **12b**, were synthesized starting from the 2-chlorocyclohexanone **14** and 3-chloro tetrahydro-4*H*-pyran-4-one **15**, respectively, by a four-step process as outlined in Scheme 2. The α-chloro ketone derivatives **14** and **15** were reacted with thiourea to afford the 2-aminothiazole derivatives **16** and **17**. The intermediates **16** and **17** were reacted with ethyl bromopyruvate in dimethoxyethane. The resulting tricyclic esters **18** and **19** were reduced to their corresponding alcohols using lithium aluminum hydride and then oxidized to their respective aldehydes **20** and **21** using activated manganese dioxide in refluxing chloroform for 48 h (Scheme 2). The aldehyde **25** required to synthesize compound **12c** was prepared by a



Scheme 1. General method to prepare compounds **12a**–**g**.

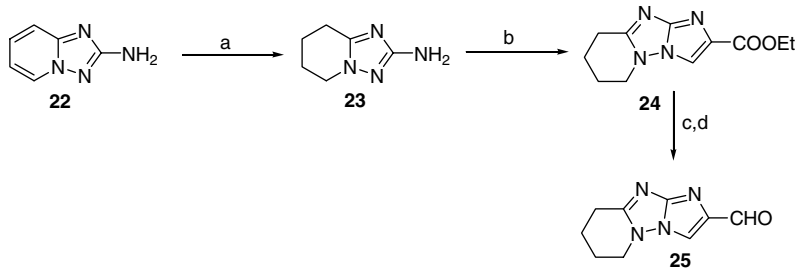


Scheme 2. General method to prepare aldehydes **20** and **21**. Reagents and conditions: (a) Thiourea, EtOH, THF, reflux; (b) $BrCH_2COCOOEt$, DME, reflux; (c) $LiAlH_4$, THF, 0 °C; (e) MnO_2, $CHCl_3$, reflux.

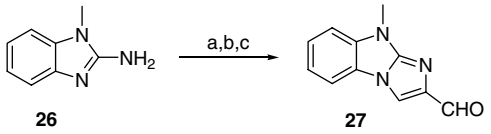
similar sequence of reactions starting from compound **23** and ethyl bromopyruvate (Scheme 3). Compound **23** required for this transformation was prepared by the hydrogenation of 3-amino-1,2,4-triazolopyridine **22**[23a] with Pd/C at room temperature at 40 psi pressure (Scheme 3). The tricyclic aldehyde **27** was synthesized starting from 1-methyl-2-amino benzimidazole **26** as indicated in Scheme 4. The tricyclic aldehydes **33**–**35** were synthesized by reacting ethyl-5-hydroxy-1*H*-pyrazole-3-carboxylate **28**[23b] and corresponding α-chloro cyclic ketones **14**, **15**, and **29** in the presence of K_2CO_3 in boiling acetonitrile as indicated in Scheme 5.

3. Biology

All the compounds synthesized were tested in vitro against TEM-1 and Amp-C enzymes for their inhibitory ability.[24] β-Lactamase inhibitory activities were determined spectrophotometrically as described by Bush et al.[24b] 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 antibiotic (piperacillin) and antibiotic plus inhibitors (compounds **12a**–**g** and **13**) were determined by the broth microdilution method as recommended by the Clinical Laboratory and Standards Institute (CLSI)[25] using Mueller–Hinton II broth (MHBII, BBL Cockeysville, MD). Microtiter plates containing serial dilutions of each antimicrobial agent or antimicrobial agent plus inhibitor were inoculated with each organism to yield the appropriate density (10^5 CFU/ml) in a 100-μl final volume. The plates were



Scheme 3. Synthesis of aldehyde **25**. Reagents and conditions: (a) H_2, Pd, C, 40 psi, RT; (b) $BrCH_2COCOOEt$, DME, sealed tube, reflux ; (c) DIBAL, THF, −78 to 0 °C; (e) MnO_2, $CHCl_3$, reflux.



Scheme 4. Synthesis of aldehyde **27**. Reagents and conditions: (a) $BrCH_2COCOOEt$, DME, reflux ; (b) $LiBH_4$, THF, MeOH, reflux; (c) MnO_2, $CHCl_3$, reflux.

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 piperacillin (PIP) alone, the inhibitor alone, and the inhibitor in combination with piperacillin 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 **12a**–**g** and **13**) at a ratio of 4:1 (Pip:Inhibitor). The seven days survival ratios from three separate tests were pooled for determination of median effective dose (ED_{50}) by probit analysis.

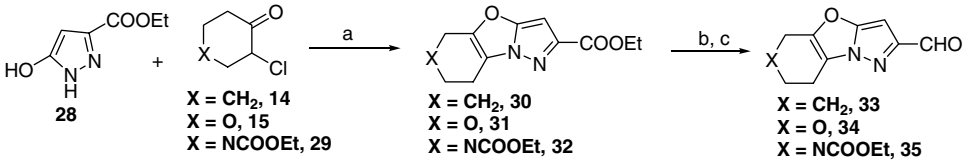
4. Structure–activity relationship

All the newly synthesized 6-methylidene penem inhibitors bearing tricyclic heterocycles (**6**, **12a**–**g**, and **13**) are listed in Table 1.

The in vitro enzyme inhibition studies were carried out against TEM-1, Imi-1 (class A), and AmpC (class C) enzymes and their IC_{50} values are enlisted in Table 1. In all our experiments, tazobactam was used as the standard and the comparator. As can be seen from Table 1, all the newly synthesized inhibitors **12a**–**g** with '*Z*' olefin geometry are potent inhibitors of both TEM-1 and AmpC enzymes with IC_{50} values of 1.2–24 and 0.62–16 nM, respectively. As compared to tazobactam, all the newly synthesized inhibitors were about 4- to 90-fold more potent against TEM-1 and about 5000- to 84,000-fold more active against AmpC enzymes. All the inhibitors are modestly potent against Imi. Among the seven 6-methylidene penems reported here, compounds **12a**–**d** represent the 'imidazole'-fused tricyclic compounds and the remaining compounds bear the pyrazole ring system as a part of their tricyclic appendage. It was shown earlier[16b] that compound **6**, which bears the imidazole ring, has broad spectrum of activities. Hence, in order to extend the structure-activity relationships the cyclopentyl moiety in compound **6** was replaced with more lipophilic cyclohexyl (example **12a**) and relatively polar pyran ring system (example **12b**). These modifications did not alter the in vitro activity against TEM-1. However, the cyclohexyl ring containing compound **12a** is about 3- to 4-fold more potent than **6** against class C enzyme. Com-

Table 1. In vitro activity of compounds **6**, **13**, and **12a**–**g** against different β-lactamases, IC_{50} (nM)

Compound	Class-A (IC_{50}) nM		Class-C (IC_{50}) nM
	TEM-1	Imi-1	Amp-C
Tazobactam	100 ± 8	NA	84,000 ± 300
6	1.4	72	2.1
12a	1.9	33	0.62
12b	2.8	100	1.5
12c	2.4	14	1.4
12d	24	50	16
12e	4	160	5.5
(Z)-12f	4.3	140	4
(E)-13	150	2900	53
12g	1.2	98	4



Scheme 5. General method to prepare compounds **33**–**35**. Reagents and conditions: (a) K_2CO_3, CH_3CN, reflux; (b) $LiBH_4$, THF, 0 °C; (c) MnO_2, $CHCl_3$, reflux.

pound **12a** is 2-fold more potent against Imi when compared to the lead compound **6**. When the thiazole ring in examples **6**, **12a**, and **12b** was replaced with 1,2,4-triazole ring system as in example **12c**, the potency remained almost identical to **12a** and **12b** against TEM-1 and AmpC, and exhibited excellent potency against Imi-1. The potency decreased as the terminal saturated rings of **6** and **12a–12c** were replaced with a phenyl. Thus, compound **12d** is almost 10-fold less potent against TEM-1 than compounds **6** and **12a–c**. 6-Methylidene penem derivatives **12e–g** bearing a pyrazole ring displayed good potency against TEM-1 and AmpC enzymes. The imidazole fused tricyclics **6** and **12a–c** are more potent inhibitors of TEM-1 and AmpC as compared to the pyrazole fused tricyclic bearing penem inhibitors **12e–g**. During the preparation of example **12f**, a small quantity of the corresponding '*E*' isomer **13** was isolated and tested for in vitro activity against all of the four enzymes. As can be seen in Table 1, the potency of **13** is >50-fold weaker against TEM-1, as compared to its corresponding '*Z*' isomer **12f**. This decrease in the potency can be explained by modeling of **12f** and **13** at the TEM-1 binding site[26] (Fig. 2).

Our optimized protein:ligand complex models (TEM-1:**12f**, TEM-1:**13**, AmpC:**12f**, and AmpC:**13**) indicated a preference for the *Z* isomer over *E* isomer (Fig. 2). β-Lactamase inhibition involves positioning of the lactam oxygen in the oxyanion hole, and subsequently, its acylation. β-Lactam and the tricyclic head groups are co-planar in the predicted bioactive conformations of **12f** and **13**. The shape of the *Z* isomeric form allows the tricyclic head group to be directed toward the opening of the binding site and still makes good H-bonding and vdW contacts with the binding site amino acids. This orientation of the tricyclic head group allows the lactam oxygen to be positioned deep into the oxyanion hole and facilitates nucleophilic attack by the active serine. In contrast, as shown in Figure 2B the *E* isomer places the tricyclic head group deeper into the binding site, and, thereby, pulls the lactam oxygen away from the oxyanion hole, making the acylation more difficult and resulting in weaker enzyme inhibition. It is also noteworthy to see from Figure 2B that the *E* isomer is less firmly embedded in the oxyanion hole and makes fewer contacts with the binding pocket residues as compared to the corresponding *Z* isomer (**12f**). These molecular modeling observations are consistent for the AmpC binding site as well.

5. In vitro antibacterial activity

The in vitro effectiveness of the newly synthesized compounds was evaluated in the cell-based assay by measuring their minimum inhibitory concentration (MIC). These data are summarized in Table 2, which also lists the expressing enzyme and its class. In all these experiments, piperacillin was combined with the newly synthesized inhibitors and tested against different piperacillin-resistant bacteria (MIC > 64 μg/mL) expressing various β-lactamases. The piperacillin + tazobactam combination was used for comparison. At the onset, it is important to note that, when tested alone, both the tazobactam and the newly synthesized inhibitors (**6**, **12a–g**, and **13**) did not exhibit any antibacterial activity (data not shown) against the isolates used in this study, which established the lack of inherent antibacterial activity. The newly synthesized compounds **6**, **12a–g**, and **13**, and tazobactam, when combined with piperacillin at a constant 4 μg/mL concentration, reduced the MIC values (except **12d** and **13**) of piperacillin (>64 μg/mL) against TEM-1 producing isolates. However, it is evident from Table 2 that even though the tazobactam plus piperacillin combination has synergy against class A producing isolates, this combination is less effective against most class C β-lactamase producing organisms (*S. marcescus* is an exception). The addition of the newly synthesized inhibitors such as **6**, **12a** and **12b** effectively reduced the MIC value and restored the susceptibility to piperacillin for the class C producing organisms. Among the newly synthesized inhibitors, the imidazole-fused tricyclic derivatives attached to the 6-methylidene penem moiety **12a–c** are more effective than the pyrazole ring-containing compounds such as **12e–g**. Compound **12g**, in spite of its low IC_{50} values, exhibited poor whole cell activity. It is noteworthy that all the newly synthesized compounds reported here exhibited good aqueous solution state stability in the pH range of 7–8.5.

6. In vivo efficacy

The in vivo efficacy data (ED_{50} values) of piperacillin and the most potent inhibitors **12a–c** (4:1 ratio) are listed in Table 3. These data were generated in a murine acute lethal infection model with *Escherichia coli* LSU 80-8 (a TEM-1 producing organism).[16b] In all these experiments, tazobactam was used for comparison. When piperacillin was administered alone (IV), it did not have efficacy (ED_{50} > 128 mg/kg) against *E. coli* LS80-8. However, when piperacillin was combined with the newly synthesized inhibitors **6** and **12a–c**, reduced ED_{50} values in the range of 19–32 mg/kg were observed. In the same experiment, tazobactam demonstrated similar effectiveness against *E. coli* LSU 80-8.

7. Conclusions

Guided by the mechanistic understanding, computational chemistry and as an extension to our previous work, several novel tricyclic heterocycle-substituted 6-methylidene penem molecules were synthesized. Many of these molecules are potent and broad-spectrum β-lactamase inhibitors. During the course of this investigation, several novel tricyclic aldehydes **20**, **21**, **25**, **27**, and **33–35** were synthesized. The newly synthesized 6-methylidene penem derivatives **6**, **12a–c**, and **12e–g** have a broader spectrum of activity than any of the currently available inhibitors on the market. They were shown to have good in vitro activity against both class A and class C enzymes. In vitro antimicrobial susceptibility testing studies demonstrated that, the newly synthesized compounds **12a–c**, **12e**, and **12f** reduced the MIC values of

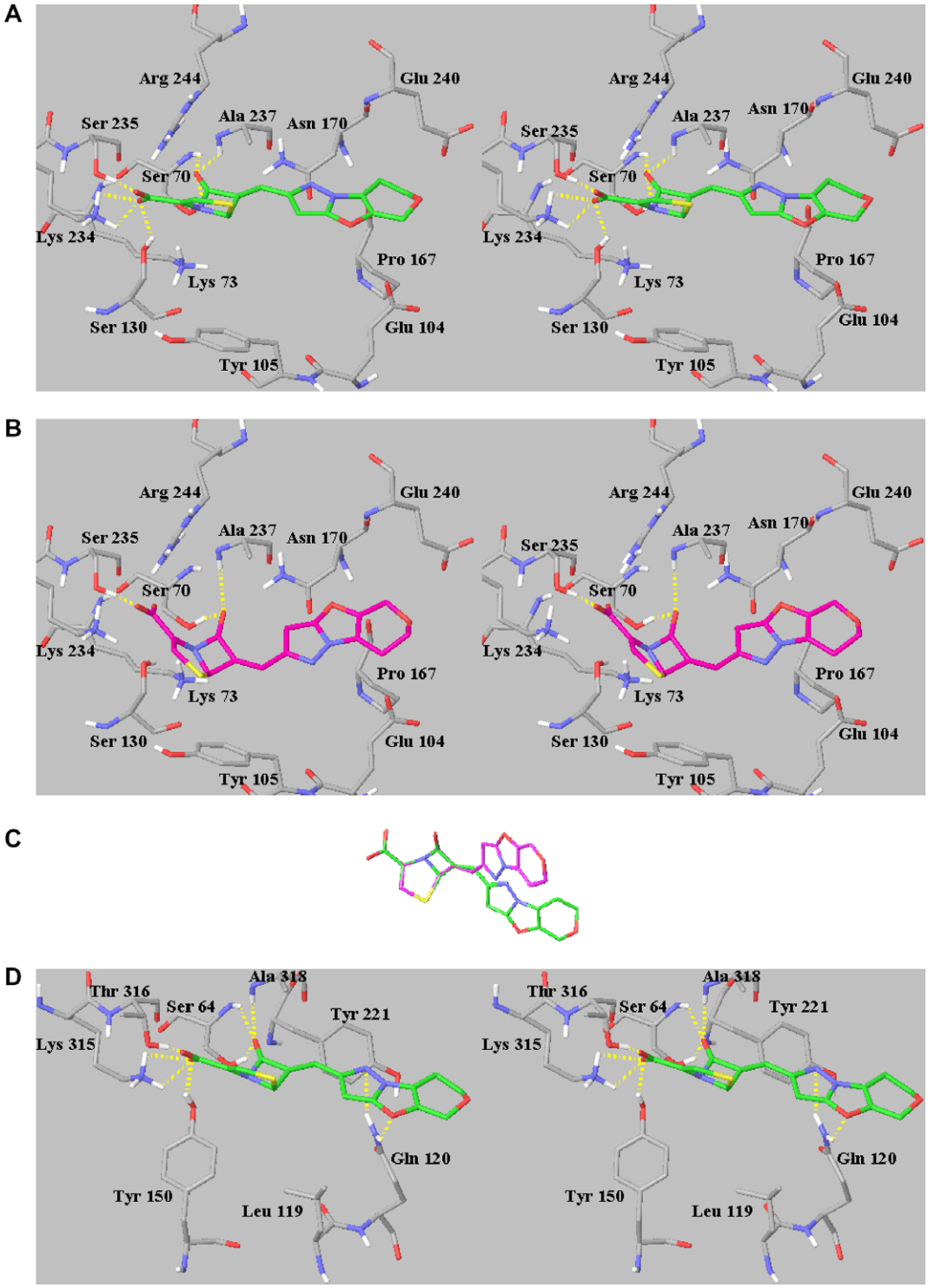


Figure 2. Nitrogen, oxygen, carbon, sulfur and hydrogen atoms are shown with blue, red, green, yellow and white colors, respectively. Carbons of compound 13 are colored magenta. The inter-molecular hydrogen bonds are shown with dotted yellow lines. There is a salt bridge interaction between carboxylate function of the ligand and lysine sidechain. (A) **12f** docked in TEM-1 binding site. (B) Compound **13** docked in TEM-1 binding site. This *E* isomer is less firmly embedded in the oxyanion hole and makes fewer contacts with the binding pocket residues as compared to the corresponding *Z* isomer (**12f**). (C) Overlay of predicted bioactive conformations of **12f** and **13** demonstrating how the tricyclic head groups are oriented differently when superimposed by the β-lactam ring. (D) Compound **12f** docked in AmpC binding site.

piperacillin to the susceptible range for the class A and some of the class C producing organisms. In particular, compounds, **12a**–**c**, which bear an imidazole ring in the tricyclic ring skeleton, when combined with piperacillin rendered the organisms listed here susceptible. The same compounds **6** and **12a**–**c** in vivo enhanced the activity of piperacillin against *E. coli* LSU 80–8, a TEM-1 producing organism. Further investigations in various other

Table 2. In Vitro Antimicrobial activity of Piperacillin (PIP) and the combined newly synthesized inhibitors (**12a–g, 13, 6**) or Tazobactam at a constant 4 μg/mL concentration

Organisms and their enzyme expression	Tazo	6	12a	12b	12c	12d	12e	12f	13	12g
E. coli GC 2844	2	2	2	2	2	4	2	2	2	2
E. coli GC 2847 (TEM-1)	2	4	8	4	8	>64	16	32	>64	32
E. coli GC 2920 (IRT-2)	4	2	2	2	2	4	4	2	8	4
E. coli GC 2883 (OXA-10 + PSE-2)	1	2	2	4	2	64	8	8	16	8
E. coli GC 2894 (AmpC)	32	2	4	2	2	32	32	16	32	64
E. coli GC 2905 (P99)	64	4	4	8	2	32	32	16	32	32
E. coli GC 2906 (Imi-1)	2	2	2	2	64	16	4	8	64	64
E. coli GC 2804 (imp)	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	0.12	<0.06
E. coli GC 2805 (CcrA)	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
E. coli GC 2253 (IRT-2)	2	8	16	16	16	32	16	16	32	16
E. cloacae GC 1477 (AmpC)	>64	16	32	16	>64	>64	64	64	>64	>64
E. cloacae GC 4142 (AmpC)	64	4	8	8	8	>64	32	32	>64	64
E. cloacae GC 6991 (AmpC)	>64	16	16	16	16	32	32	16	32	64
E. cloacae GC 1475 (P99)	64	4	8	8	2	64	32	32	32	64
S. marcescens GC 1781 (Sme-1 + AmpC)	0.50	1	1	1	0.50	2	2	2	4	2
P. aeruginosa GC 1764 (AmpC)	>64	2	2	4	32	64	64	32	64	>64
S. maltophilia GC 1712 (L1)	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
S. marcescens GC 4132 (AmpC)	64	2	2	2	1	16	8	8	32	8
E. coli GC 2203	1	2	2	4	1	2	2	4	2	2
S. aureus GC 2216	0.50	2	<0.06	2	0.25	64	16	16	64	16

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

Compound	ED_{50} (mg/kg)
Tazobactam	20
12a	23.3
12b	25.3
12c	32.5
6	19

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

organisms of related compounds are in progress to determine their potential as clinically viable therapeutic agents.

8. Experimental

8.1. General methods

Melting points were determined in 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. 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 Finnigan MAT-90 spectrometer. Combustion analysis was 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 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.

9. Molecular modeling

The lowest energy conformations of compounds **11f** and **12f** were manually docked in TEM-1 (RCSB PDB code: 1BTL) and AmpC (RCSB PDB code: 1FSW) as described previously.[16a,26] Hydrogen atoms were added and the four models were soaked in a box of water (TIP3P) applying periodic boundary conditions using MOE.[26] The complexes were optimized by energy minimizing at a constant dielectric of 1 with MMFF94 forcefield in MOE.

10. Compound 12a

10.1. Preparation of (5*R*),(6*Z*)-7-oxo-6-(5,6,7,8-tetrahydroimidazo[2,1-*b*][1,3]benzothiazol-2-ylmethylene)-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid (12a)

10.1.1. Step 1: Preparation of 2-Carbethoxy-{5,6,7,8-tetrahydro}-imidazo[2,1-*b*][1,3]benzothiazole (18). A mixture of 2-chlorocyclohexanone (13.2 g, 100 mmol) and thiourea (8.0 g, 101 mmol) was refluxed in ethanol:THF (1:2) for 16 h. The reaction mixture was cooled to room temperature and the separated white solid was filtered (12.0 g separated). This was dissolved in anhydrous ethanol (100 ml) and sodium methoxide (3.3 g, 63 mmol). To this ethyl bromopyruvate (15.0 g, 76 mmol) was added and stirred at room temperature for 2 h. It was

then refluxed for 48 h. At the end, reaction mixture was cooled to room temperature and concentrated. The residue was extracted with chloroform and washed well with water. The product was purified by silica-gel column chromatography by eluting it with 50% ethyl acetate:hexane. Red semi-solid; Yield: 6.2 g (39%); (M+H) 251.

The ester was reduced with $LiBH_4$ and the resultant alcohol was oxidized with active MnO_2. The aldehyde, namely 5,6,7,8-tetrahydroimidazo[2,1-*b*][1,3]benzothiazole-2-carbaldehyde **20**, obtained was taken to next step.

10.1.2. Step 2: Preparation of 4-nitrobenzyl (5*R*)-6-[(acetyloxy)(5,6,7,8-tetrahydroimidazo[2,1-*b*][1,3]benzothiazol-2-yl)methyl]-6-bromo-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylate (11a). To a stirred solution of 5,6,7,8-tetrahydroimidazo[2,1-*b*][1,3]benzothiazole-2-carbaldehyde **20** (412 mg, 2.0 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 **10** (770 mg, 2 mmol) in dry THF (10 mL)/acetonitrile (15 mL), anhydrous $MgBr_2{:}O(Et)_2$ (1.2 g, 3.0 mmol) was added under an argon atmosphere at room temperature. After cooling to −20 °C, Et_3N (2.0 mL) was added and the reaction vessel was covered with foil to exclude light. The reaction mixture was stirred for 4 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 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 eluted with ethyl acetate:hexane (1:1). Collected fractions were concentrated under reduced pressure and the mixture of diastereomers was taken to the next step. Pale yellow amorphous solid; Yield: 980 mg, 77%; (M+H) 634.

10.1.3. Step 3: Preparation of (5*R*),(6*Z*)-7-oxo-6-(5,6,7,8-tetrahydroimidazo[2,1-*b*][1,3]benzothiazol-2-ylmethylene)-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid sodium salt (12a). 4-Nitrobenzyl (5*R*)-6-[(acetyloxy)(5,6,7,8-tetrahydroimidazo[2,1-*b*][1,3]benzothiazol-2-yl)-methyl]-6-bromo-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylate **11a** (980 mg, 1.55 mmol) was dissolved in THF (20 mL) and acetonitrile (10 mL). Freshly activated Zn dust (5.2 g) was rapidly added along with 0.5 M phosphate buffer (pH 6.5, 28 mL). The reaction vessel was covered with foil to exclude light and the reaction mixture was vigorously stirred for 2 h at room temperature. The reaction mixture was filtered, 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 later with 10% acetonitrile:water. The fractions containing the product were collected and concentrated under reduced pressure at room temperature. The yellow solid was washed with acetone, filtered, and dried. Yield: 120 mg, 20%; yellow solid; mp 250 °C (dec); (M+H+Na) 382. ^{1}H NMR (DMSO-d_6) δ 1.9 (m,2H), 2.5 (m, 2H), 3.2–3.4 (m, 4H), 6.6 (s, 1H), 7.1 (s, 1H), 7.5 (s, 1H), 8.1 (s, 1H).

$C_{16}H_{12}N_3NaO_3S_2$.

Calcd C 50.39%, H 3.17%, N 11.02%; found: C 50.10%, H 3.42, N 10.99.

11. Compound 12b

11.1. Preparation of (5*R*),(6*Z*)-6-(5,8-dihydro-6*H*-imidazo[2,1-*b*]pyrano[4,3-*d*][1,3]thiazol-2-ylmethylene)-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid (12b)

11.1.1. Step 1: Preparation of ethyl 5,8-dihydro-6*H*-imidazo[2.1-*b*]pyrano[4,3-*d*][1,3]thiazole-2-carboxylate (19). To a mixture of tetrahydro-4*H*-pyran-4-one (5.0 g, 50 mmol) in CCl_4 (100 ml) at 0 °C, SO_2Cl_2 (7.4 g, 55 mmol) was slowly added. After the addition, reaction mixture was stirred at room temperature for 4 h and carefully quenched with ice-cold water. Reaction mixture was washed well and dried over anhydrous $MgSO_4$. The organic layer was filtered and concentrated. The colorless oil obtained was dissolved in THF/EtOH (2:1) containing thiourea (4.0 g, 52 mmol) and refluxed for 8 h. At the end, reaction mixture was cooled to room temperature and the separated, 6,7-dihydro-4*H*-pyrano[4,3-*d*][1,3]thiazol-2-amine hydrochloride **17** white solid was filtered. Yield 4.5 g (47%); mp 115 °C, (M+H) 157.

To a stirred solution of, 6,7-dihydro-4*H*-pyrano[4,3-*d*][1,3]thiazol-2-amine hydrochloride (4.0 g, 20.8 mmol) in anhydrous ethanol (100 ml), sodium methoxide (1.1 g, 21 mmol) was added. This was stirred at room temperature for 30 min and to this ethyl bromopyruvate (10.0 g, 51 mmol) was added and stirred at room temperature for 2 h. Then it was refluxed for 48 h. At the end reaction mixture was cooled to room temperature and concentrated. The residue was basified with 2 M $NaHCO_3$ solution. extracted with chloroform, and washed well with water. The product was purified by silica-gel column chromatography by eluting it with 50% ethyl acetate:hexane. Red semi-solid; yield: 3.1 g (59%), (M+H) 253.

The ester **19** was reduced with $LiBH_4$ and the resultant alcohol was oxidized with active MnO_2. The aldehyde obtained was taken to next step.

11.1.2. Step 2: Preparation of 4-nitrobenzyl (5*R*)-6-[(acetyloxy)(5,8-dihydro-6*H*-imidazo[2,1-*b*][1,3]pyrano-[4,3-*d*][1,3]thiazol-2-yl)-6-bromo-7-oxo-4-thia-1-azabicyclo-[3.2.0]hept-2-ene-2-carboxylate (11b). Starting from 2-formyl-5,8-dihydro-6*H*-imidazo[2.1-*b*]pyrano[4,3-*d*][1,3]-thiazole (208 mg, 1.0 mmol), (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 (400 mg, 1.3 mmol), and anhydrous $MgBr_2{:}O(Et)_2$ (1.2 g, 3.0 mmol) and following the proce-

dure outlined for **12a**, (Step 2) 400 mg, (yield, 62%) of the titled product was isolated. Mp 78 °C; (M+H) 636.

11.1.3. Step 3: Preparation of (5*R*),(6*Z*)-6-(5,8-dihydro-6*H*-imidazo[2,1-*b*]pyrano[4,3-*d*][1,3]thiazol-2-ylmethylene)-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid (12b). Starting from 4-nitrobenzyl (5*R*)-6-[(acetyloxy)(5,8-dihydro-6*H*-imidazo[2,1-*b*][1,3]pyrano[4,3-*d*][1,3]-thiazol-2-yl)-6-bromo-7-oxo-4-thia-1-azabicyclo[3.2.0]-hept-2-ene-2-carboxylate (500 mg, 0.79 mmol) and following the procedure outlined for **12a** (Step 3) the titled compound was isolated. The yellow solid was washed with acetone, filtered, and dried. Yield: 85 mg, 30%; as yellow crystals; mp 205 °C; (M+H+Na) 383. ^{1}H NMR (DMSO-d_6) δ 2.8 (m, 2H), 4.0 (m,2H), 4.6 (s,2H), 6.4 (s,1H), 6.5 (s,1H), 7.0 (s,1H), 8.1 (s,1H).

$C_{15}H_{10}N_3NaO_4S_2$.

Calcd C 46.99%, H 2.63%, N 10.96%; found: C 46.91%, H 2.68, N 10.91.

12. Compound 12c

12.1. Preparation of (5*R*),(6*Z*)-6-(4,5,6,7-tetrahydro-1,3a,3b,8-tetraaza-cyclopenta[*a*]indene-2-ylmethylene)-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid sodium salt (12c)

12.1.1. Step 1: 5,6,7,8-Tetrahydro-[1,2,4]triazolo[1,5-*a*]pyridin-2-ylamine (23). The 12.7% solution of HCl in ethanol (5.35 mL) and 10% Pd–C (50% wet) (2.5 g) were added to the mixture of [1,2,4]triazolo[1,5-*a*]pyridin-2-ylamine **22** (2.5 g) in ethanol (72 mL). The reaction mixture was hydrogenated at 40 psi of H_2 for 3 days at room temperature. The mixture was filtered and concentrated under reduced pressure. The residue was treated with saturated potassium carbonate solution and extracted with chloroform. The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. The title compound **23** was obtained as a pale yellow solid (2.31 g, 90%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.88–1.94 (m, 2H), 1.98–2.05 (m, 2H), 2.77 (t, 2H, J = 6.2 Hz), 3.95 (t, 2H, J = 6.2 Hz), 4.09 (br s, 2H).

12.1.2. Step 2: 4,5,6,7-Tetrahydro-1,3a,3b,8-tetraaza-cyclopenta[*a*]indene-2-carboxylic acid ethyl ester (24). Ethyl bromopyruvate (10.23 g) was added to the mixture of 5,6,7,8-tetrahydro-[1,2,4]triazolo[1,5-*a*]pyridin-2-ylamine (5.8 g) in 1,2-dimethoxyethane (320 mL). The reaction mixture was stirred for 5 h at room temperature and concentrated to 100 mL under reduced pressure. To the stirred solution, diethyl ether (200 mL) was added and the separated solid was filtered. The precipitate was dissolved in ethanol (175 mL) and stirred for 20 h at 110 °C in sealed tube. The reaction mixture was cooled to room temperature and concentrated under reduced pressure. The residue was treated with saturated potassium carbonate solution and extracted with chloroform. The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. The residue was applied to silica gel column chromatography and eluted with ethyl acetate–methanol (1/1). The title compound **24** was obtained as a pale yellow solid (7.56 g, 77%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.42 (t, 3H, J = 7.1 Hz), 2.14–2.25 (m, 4H), 3.11 (t, 2H, J = 6.1 Hz), 4.37 (t, 2H, J = 5.7 Hz), 4.41 (q, 2H, J = 7.1 Hz), 7.57 (s, 1H); (M+H) 235.

12.1.3. Step 3: 4,5,6,7-Tetrahydro-1,3a,3b,8-tetraaza-cyclopenta[*a*]indene-2-carbaldehyde (25). Diisobutylaluminum hydride (1.01 M) in toluene (1.06 mL) was added dropwise to the solution of 4,5,6,7-tetrahydro-1,3a,3b,8-tetraaza-cyclopenta[*a*]indene-2-carboxylic acid ethyl ester (100 mg) in dry THF (5 mL) at −78 °C under a nitrogen atmosphere. The reaction mixture was stirred for 30 min at −78 °C and treated with ethanol (ca 1 mL). The mixture was warmed to 0 °C and stirred for 1 h at that temperature. The reaction solution was diluted with ethyl acetate (20 mL), treated with 0.5 mL saturated ammonium chloride solution, and sonicated for ca. 5 min (until a precipitate was deposited enough). The reaction mixture was filtered and the organic layer was dried (Na_2SO_4) and filtered through a pad of Celite. The filtrate was concentrated under reduced pressure. The residue was crystallized from dichloromethane and diethyl ether to give the title compound (47.4 mg, 58%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.16–2.27 (m, 4H), 3.14 (t, 2H, J = 6.1 Hz), 4.39 (t, 2H, J = 5.7 Hz), 7.53 (s, 1H), 10.01 (s, 1H), (M+H) 191.

12.1.4. Step 4: (5*R*,6*RS*)-6-{(*RS*)-Acetoxy-[4,5,6,7-tetrahydro-1,3a,3b,8-tetraaza-cyclopenta[*a*]indene-2-yl]-methyl}-6-bromo-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid 4-nitro-benzyl ester (11c). Starting from 4,5,6,7-Tetrahydro-1,3a,3b,8-tetraaza-cyclopenta[*a*]-indene-2-carbaldehyde (2.97 g, 5 mmol), (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 **(10)** (2.97 g, 7.7 mmol), and anhydrous $MgBr_2$ and following the procedure as outlined for **12a** (Step 2) the title compound was obtained as a brown amorphous solid. Yield: 651.6 mg, 13%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.10–2.24 (m, 4H), 2.29 (s, 3H), 3.04–3.07 (m, 2H), 4.28–4.32 (m, 2H), 5.27 (d, 1H, J = 13.7 Hz), 5.43 (d, 1H, J = 13.7 Hz), 6.19 (s, 1H), 6.91 (s, 1H), 7.01 (s, 1H), 7.49 (s, 1H), 7.59–7.62 (m, 2H), 8.23–8.25 (m, 2H); (M+H) 619.

12.1.5. Step 5: (5*R*),(6*Z*)-6-(4,5,6,7-tetrahydro-1,3a,3b,8-tetraaza-cyclopenta[*a*]indene-2-ylmethylene)-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid sodium salt (12c). Starting from (5*R*, 6*RS*)-6-{(*RS*)-Acetoxy-[4,5,6,7-tetrahydro-1,3a,3b,8-tetraaza-cyclopenta[*a*]indene-2-yl]-methyl}-6-bromo-7-oxo-4-thia-1-aza-bicyclo[3.2.0]-hept-2-ene-2-carboxylic acid 4-nitro-benzyl ester (643.6 mg, 1.03 mmol) and following the procedure outlined for **12a** (Step 3) the title compound was obtained as a yellow amorphous solid (68 mg, 18%, pH 7.4). Mp 175 °C (dec); (M+H+Na) 366; ^{1}H NMR (400 MHz, D_2O) δ 1.85–2.03 (m, 4H), 2.85–2.99 (m, 2H), 4.07–4.14 (m, 2H), 6.34 (s, 1H), 6.74 (s, 1H), 6.76 (s, 1H), 7.28 (s, 1H).

$C_{15}H_{12}N_5NaO_3S_2$.

Calcd 49.31%, H 3.31%, N 19.17%; found: C 49.01%, H 3.40, N 19.08.

13. Compound 12d

13.1. Preparation of (5*R*),(6*Z*)-8-[(9-methyl-9*H*-imidazo-[1,2-*a*]benzimidazol-2-yl)methylene]-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid (12d)

13.1.1. Step 1: Preparation of 9-methyl-9*H*-imidazo[1,2-*a*]benzimidazole-2-carbaldehyde (27). To a stirred solution of $LiBH_4$ (1.79 g, 82 mmol) in THF at 0 °C, ethyl 9-methyl-9*H*-imidazo[1,2-*a*]benzimidazole-2-carboxylate (2.5 g, 10.3 mmol) was added dropwise. The reaction mixture was refluxed for 2 h and cooled to room temperature and carefully quenched with ice-cold water and acidified with concd HCl to pH 4. The reaction mixture was stirred at room temperature for 1 h and basified with K_2CO_3. The residue was extracted with chloroform;methanol (3:1) and dried over anhydrous $MgSO_4$. It was filtered and concentrated to yield: 1.3 g of its corresponding alcohol.

The residue (1.3 g, 6.4 mmol) was oxidized with MnO_2 (5.0 g) in $CHCl_3$ under refluxing condition. After the completion, reaction mixture was filtered and concentrated. It was purified by SiO_2 column chromatography by eluting it with 1:1 ethyl acetate:hexane. Brown solid, yield: 330 mg (25%); (M+H) 200.

13.1.2. Step 2: Preparation of 4-nitrobenzyl(5*R*)-6-[(acetyloxy)(9-methyl-9*H*-imidazo[1,2-*a*]benzimidazole-2-)-methyl]-6-bromo-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylate (11d). Starting from 9-methyl-9*H*-imidazo[1,2-*a*]benzimidazole-2-carbaldehyde **27** (330 mg, 1.65 mmol), (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 (770 mg, 2 mmol), and anhydrous $MgBr_2{:}O(Et)_2$ (1.2 g, 3.0 mmol) and following the procedure as outlined for compound **12a** (Step 2) the titled compound (as mixture of diastereomers) was isolated as pale yellow amorphous solid; yield: 330 mg, 31%; (M+H) 628.

13.1.3. Step 3: Preparation of (5*R*),(6*Z*)-8-[(9-methyl-9*H*-imidazo[1,2-*a*]benzimidazol-2-yl)methylene]-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid sodium salt (12d). Starting from 4-nitrobenzyl(5*R*)-6-[(acetyloxy)(9-methyl-9*H*-imidazo[1,2-*a*]benzimidazole-2-)methyl]-6-bromo-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylate **11d** (1 g, 1.6 mmol) and following the procedure as outlined for **12a** (Step 3) the titled compound was separated as a yellow solid. Yield: 140 mg, 23%; as yellow solid; mp 220 °C (Dec); (M+H+Na) 375. ^{1}H NMR (DMSO-d_6) δ 3.4 (s,3H), 6.54 (s, 1H), 6.56 (s, 1H), 7.01 (s, 1H), 7.21 (t, 1H), 7.3 (t, 1H), 7.56 (d, 1H), 7.85 (d,1H), 8.1 (s,1H).

$C_{17}H_{11}N_4NaO_3S$.

Calcd 54.54%, H 2.96%, N 14.97%; found: C 54.51%, H 2.91, N 14.86.

14. Compound 12e

14.1. Preparation of (5*R*,6*Z*)-7-oxo-6-(5,6,7,8-tetrahydropyrazolo[5,1-*b*][1,3]benzoxazol-2-ylmethylene)-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid sodium salt (12e)

14.1.1. Step 1: Preparation of ethyl-5-[(2-oxocyclohexyl)oxy]-1*H*-pyrazole-3-carboxylate. To the stirred suspension of ethyl 5-hydroxy-1*H*-pyrazole-3-carboxylate **28** (6.25 g, 40 mmol) and potassium carbonate (22.1 g, excess) in 500 ml of acetonitrile was added 2-chlorocyclohexanone **14** (6.35 g, 48 mmol) and refluxed for 16 h. The reaction mixture was allowed to cool to room temperature and filtered. The residue was washed with acetonitrile and the filtrate was concentrated to an oil. The oil was dissolved in ethyl acetate and extracted with water. The organic phase was dried over $MgSO_4$ and evaporated to dryness. The product was purified by silica-gel column chromatography by eluting it with 1:1 ethyl acetate:hexane. The desired product (4.92 g (49%)) was obtained as a white solid. Mp 122–124 °C; (M+H) 253.

14.1.2. Step 2: Preparation of ethyl 5,6,7,8-tetrahydropyrazolo[5,1-*b*][1,3]benzoxazole-2-carboxylate (30). A mixture of ethyl-5-[(2-oxocyclohexyl)oxy]-1*H*-pyrazole-3-carboxylate (127.6 mg, 0.5 mmol), methane sulfonic acid (95 mg), and acetic acid 9.5 mL in toluene (50 ml) was refluxed for 18 h using a Dean–Stark trap to remove water. The reaction mixture was allowed to cool to room temperature and it was filtered. The filtrate was concentrated to an oil. The residue was dissolved in ethyl acetate and aqueous bicarbonate solution. The organic layer was washed with water and dried over $MgSO_4$. After removal of the ethyl acetate, the residue was purified by silica gel chromatography eluting with 1:1 ethyl acetate/hexane to give 69.7 mg (59%) of the desired product **30** as white solid. Mp 55–57 °C; (M+H) 235.0.

14.1.3. Step 3: Preparation of 5,6,7,8-tetrahydropyrazolo[5,1-*b*][1,3]benzoxazol-2-ylmethanol. To the stirred solution of ethyl 5,6,7,8-tetrahydropyrazolo[5,1-*b*][1,3]-benzoxazole-2-carboxylate **30** (3.84 g, 16.4 mmol) in 100 ml of THF were added 3.05 g of $LiBH_4$ and 3 ml of methanol. The solution was heated at 40 °C for 2.5 h. The reaction was quenched with 1 N HCl, the pH was adjusted to 1.0, and stirred at room temperature for 1 h. The reaction mixture pH was again adjusted to 8 with K_2CO_3 and extracted with ethyl acetate. The organic layer was dried over $MgSO_4$ and concentrated to an oil. The product was purified by SiO_2 column chromatographed to give 2.62 g of the desired product (83%). Mp 82–84 °C; (M+H) 193.

14.1.4. Step 4: Preparation of 5,6,7,8-tetrahydropyrazolo[5,1-*b*][1,3]benzoxazole-2-carbaldehyde (33). To the stirred solution of 5,6,7,8-tetrahydropyrazolo[5,1-*b*][1,3]-benzoxazol-2-ylmethanol (2.30 g, 11.97 mmol) in 60 ml of $CHCl_3$ was added 10 g of MnO_2. The suspension was refluxed for 1.5 h under a nitrogen atmosphere. The reaction mixture was filtered through a pad of Cel-

ite and concentrated to give yellow solid. The product was purified by SiO_2 column chromatography to give the titled aldehyde. Yield: 1.95 g 85.5%, (M+H) 191.

14.1.5. Step 5: 4-Nitrobenzy (5*R*)-6-[(acetyloxy)(5,67,8-tetrahydropyrazolo[5,1-*b*][1,3]benzoxazol-2-yl)methyl-6-bromo-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylate (11e). Starting from 5,6,7,8-tetrahydropyrazolo[5,1-*b*][1,3]benzoxazole-2-carbaldehyde **33** (589 mg, 3.1 mmol), (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 **10** (1.54 g, 4.6 mmol) and anhydrous $MgBr_2$:$O(Et)_2$ (2.21 g, 8.5 mmol) and following the procedure as outlined for **12a** (Step 2) the titled compound was isolated as the mixture of diastereo isomers. Pale yellow amorphous solid. Yield: 792 mg, 42%; mp 160–162 °C; (M+H) 618.

14.1.6. Step 6: Preparation of (5*R*,6*Z*)-7-oxo-6-(5,6,7,8-tetrahydropyrazolo[5,1-*b*][1,3]benzoxazol-2-ylmethylene)-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid sodium salt (12e). Starting from 4-Nitrobenzy (5*R*)-6-[(acetyloxy)(5,67,8-tetrahydropyrazolo[5,1-*b*][1,3]benzoxazol-2-yl)methyl-6-bromo-7-oxo-4-thia-1-azabicyclo-[3.2.0]hept-2-ene-2-carboxylate **11e** (318 mg, 0.5 mmol), and following the procedure as outlined for **12a** (Step 3) the titled product was obtained as a yellow solid. Yield 150 mg, (76%); Yellow solid; (M+H+Na) 365.2. H NMR (D_2O): δ 6.92 (1H, s), 6.91 (1H, s), 6.32 (1H, s), 5.85 (1H, s), 2.59 (4H, m), 1.80 (4H, m).

$C_{16}H_{12}N_3NaO_4S_2$.

Calcd 52.60%, H 3.31%, N 11.50%; found: C 52.48%, H 3.29, N 11.46.

15. Compound 12f

15.1. Preparation of (5*R*,6*Z*)-6-(7,8-dihydro-5*H*-pyrano[4,3-*d*]pyrazolo[5,1-*b*][1,3]oxazol-2-ylmethylene)7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid sodium salt (12f)

15.1.1. Step 1: Preparation of ethyl-5-[(4-oxotetrahydro-2H-pyran-3-yl)oxy]-1*H*-pyrazole-3-carboxylate. To the stirred suspension of ethyl 5-hydroxy-1*H*-pyrazole-3-carboxylate **28** (7.0 g, 45 mmol) and potassium carbonate (24.0 g excess) in 500 ml of acetonitrile was added 3-bromo-tetrahydro-pyran-4-one (8.0 g, 45 mmol) **15**, and refluxed for 16 h. The reaction mixture was allowed to cool to room temperature and filtered. The residue was washed with acetonitrile and the filtrate was concentrated to an oil. The residue was dissolved in ethyl acetate and washed with water. The organic phase was dried over $MgSO_4$ and evaporated to dryness. The desired product (9.0 g, 78%) was obtained as a white solid. Mp 121–123 °C; (M+H) 255.

15.1.2. Step 2: Preparation of ethyl 7,8-dihydro-5*H*-pyrano[4,3-*d*]pyrazolo[5,1-*b*][1,3]oxazole-2-carboxylate (31). A mixture of ethyl-5-[(4-oxotetrahydro-2*H*-pyran-3-yl)oxy]-1*H*-pyrazole-3-carboxylate (254 mg, 1 mmol) and methane sulfonic acid (192 mg) in acetic acid (7 ml) and toluene (50 ml) was refluxed for 18 h using a Dean–Stark trap to remove water. The reaction mixture was allowed to cool to room temperature. The reaction mixture was filtered. The filtrate was concentrated to an oil. The residue was dissolved in ethyl acetate aqueous bicarbonate solution. The organic layer was washed with water and dried over $MgSO_4$. After removal of the ethyl acetate, the residue was purified by silica gel chromatography eluting with ethyl acetate/hexane to give 120 mg (51%) of the desired product as white solid. Mp 116–118 °C; (M+H) 237.0

15.1.3. Step 3: Preparation of 7,8-dihydro-5*H*-pyrano[4,3-*d*]pyrazolo[5,1-*b*][1,3]oxazol-2-ylmethanol. To the stirred solution of 7,8-dihydro-5*H*-pyrano[4,3-*d*]pyrazolo[5,1-*b*][1,3]oxazole-2-carboxylate **31** (1.5 g, 6.3 mmol) in 100 ml of THF were added $LiBH_4$ (1.05 g) and methanol (2 ml). The solution was heated at 40 °C for 2.5 h. The reaction mixture was quenched by 1 N HCl, and the mixture adjusted to pH 1.3 and stirred at room temperature for 1 h. The reaction mixture was adjusted to pH 8 with K_2CO_3 and was extracted with ethyl acetate. The organic layer was dried over $MgSO_4$, concentrated to an oil, and column chromatographed to give the desired product. Yield 740 mg, 60%; (M+H) 196.

15.1.4. Step 4: Preparation of 7,8-dihydro-5*H*-pyrano[4,3-*d*]pyrazolo[5,1-*b*][1,3]oxazol-2-carbaldehyde (34). To the stirred solution of 7,8-dihydro-5*H*-pyrano[4,3-*d*]pyrazolo[5,1-*b*][1,3]oxazol-2-ylmethanol (1.0 g, 5.1 mmol) in 60 ml of $CHCl_3$ was added 8 g of activated MnO_2. The suspension was refluxed for 1.5 h under a nitrogen atmosphere. The reaction mixture was filtered through a pad of Celite. The filtrate was concentrated to give yellow oil. The product was purified by chromatography. Yield 790 mg, 80 (M+H) 193.

15.1.5. Step 5: 4-Nitrobenzy (5*R*)-6-[(acetyloxy)(7,8-dihydro-5*H*-pyrano[4,3]pyrazolo[5,1-*b*][1,3]oxazol-2-yl)-methyl]-6-bromo-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylate (11f). Starting from 7,8-dihydro-5*H*-pyrano[4,3-*d*]pyrazolo[5,1-*b*][1,3]oxazol-2-carbaldehyde **34** (600 mg, 3.1 mmol), (5R, 6S)-6-bromo-7-oxo-4-thia-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylic acid 4-nitro-benzyl ester **10** (1.54 g, 4.6 mmol) and anhydrous $MgBr_2$:$O(Et)_2$ (2.21 g, 8.5 mmol), and following the procedure as outlined for **12a** (Step 2) the titled compound was isolated as the mixture of diastereo isomers. Pale yellow amorphous solid; Yield: 1.35 g, 70%; (M+H) 619.

15.1.6. Step 6: Preparation of (5*R*,6*Z*)-6-(7,8-dihydro-5*H*-pyrano[4,3-*d*]pyrazolo[5,1-*b*][1,3]oxazol-2-methylene)7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid sodium salt (12f) and (5*R*,6*E*)-6-(7,8-dihydro-5*H*-pyrano[4,3-*d*]pyrazolo[5,1-*b*][1,3]oxazol-2-ylmethylene)7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid sodium salt (13). Starting from 4-Nitrobenzy(5*R*)-6-[(acetyloxy)(7,8-dihydro-5*H*-pyrano[4,3]pyrazolo[5,1-*b*]-[1,3]oxazol-2-yl)methyl]-6-bromo-7-oxo-4-thia-1-azabi-cyclo[3.2.0]hept-2-ene-2-carboxylate (1.2 g, 1.9 mmol) and following the procedure as outlined for **12a** (Step 3) the titled compounds were isolated as a yellow solid.

In this reaction both *E* and *Z* isomers were formed and they were separated by prep. HPLC.

(5*R*,6*Z*)-6-(7,8-Dihydro-5*H*-pyrano[4,3-*d*]pyrazolo[5,1-*b*]-[1,3]oxazol-2-ylmethylene)7-oxo-4-thia-1-azabicyclo[3.2.0]-hept-2-ene-2-carboxylic acid sodium salt **12f**; yield 87 mg, (25%); yellow solid; (M+H+Na) 368.2.

H NMR (D_2O): 7.04 (1H, s), 7.01 (1H, s), 6.45 (1H, s), 6.09 (1H, s), 4.76 (2H, m), 4.12 (2H, m), 2.96 (2H, m).

(5*R*,6*E*)-6-(7,8-dihydro-5*H*-pyrano[4,3-*d*]pyrazolo[5,1-*b*]-[1,3]oxazol-2-ylmethylene)7-oxo-4-thia-1-azabicyclo[3.2.0]-hept-2-ene-2-carboxylic acid sodium salt **13**; yield 75 mg, (21%); yellow solid; (M+H+Na) 368.2.

H NMR (D_2O): 7.08 (1H, s), 6.81 (1H, s), 6.71 (1H, s), 6.40 (1H, s), 4.68 (2H, m), 4.03 (2H, m), 2.87 (2H, m).

Compound **12f**: $C_{15}H_{10}N_3NaO_5S$.

Calcd 49.05%, H 2.74%, N 11.44%; found: C 48.98%, H 2.72, N 11.48.

16. Compound 12g

16.1. Preparation of (5*R*,6*Z*)-6-{[6-(ethoxycarbonyl)-5,6,7,8-tetrahydropyrazolo[5′,1′:2,3][1,3]oxazolo[5,4-c]pyridin-2-yl]methylene}-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid sodium salt (12g)

16.1.1. Step 1: Preparation of ethyl 3-{[(3-ethoxycarbonyl)-1*H*-pyrazol-5-yl]oxy}-4-oxopiperidine-1-carboxylate. To the stirred suspension of ethyl 5-hydroxy-1*H*-pyrazole-3-carboxylate **28** (19.5 g, 127 mmol) and potassium carbonate (50.0 g, excess) in 500 ml of acetonitrile was added 3-bromo-4-oxo-piperidine-1-carboxylic acid ethyl ester **29** (37.45 g, 149 mmol), and refluxed for 16 h. The reaction mixture was allowed to cool to room temperature, and filtered. The residue was washed with acetonitrile and the filtrate was concentrated to an oil. The residue was dissolved in ethyl acetate and washed with water. The organic phase was dried over $MgSO_4$ and evaporated to dryness. The product was purified by silica-gel column chromatography by eluting it with 1:1 ethyl acetate:hexane. 8.5 g (19%) of the desired product was obtained as a yellow oil; (M+H) 326.

16.1.2. Step 2: Preparation of diethyl 7,8-tetrahydropyrazolo[5′,1′:2,3][1,3]oxazolo[5,4-c]pyridine-2,6(5H)-dicarboxylate (32). A mixture of ethyl 3-{[(3-ethoxycarbonyl)-1*H*-pyrazol-5-yl]oxy}-4-oxopiperidine-1-carboxylate (325 mg, 1 mmol) and methane sulfonic acid (95 mg) in acetic acid (5 ml) and toluene (50 ml) was refluxed for 18 h using a Dean–Stark trap to remove water. The reaction mixture was allowed to cool to room temperature and the reaction mixture was filtered. The filtrate was concentrated to an oil. The oil was dissolved in ethyl acetate and aqueous bicarbonate solution. The organic layer was washed with water and dried over $MgSO_4$. After removal of the ethyl acetate, the residue was purified by silica gel chromatography by eluting with 1:1 ethyl acetate/hexane to give the desired product as an yellow oil; yield: 175 mg, 57%; (M+H) 308.0.

16.1.3. Step 3: Preparation of ethyl 2-(hydroxymethyl)-7,8-dihydropyrazolo [5′,1′:2,3][1,3]oxazolo[5,4-c]pyridine-6(5*H*)-carboxylate. To the stirred solution of diethyl 7,8-tetrahydropyrazolo[5′,1′:2,3][1,3]oxazolo[5,4-c]pyridine-2,6(5H)-dicarboxylate (307 mg, mmol) of 40 ml of THF were added $LiBH_4$ (305 mg, excess) and 1 ml of methanol. The solution was heated at 40 °C for 2.5 h. The reaction mixture was quenched by 1 N HCl, and adjusted to pH 1.3 and stirred at room temperature for 1 h. The reaction mixture was again adjusted pH to 8 with K_2CO_3 and extracted with ethyl acetate. The organic layer was dried over $MgSO_4$, concentrated to an oil, and column chromatographed to give the desired product. Yield: 172 mg, 65%; (M+H) 266.

16.1.4. Step 4: Preparation of ethyl 2-formyl-7,8-dihydropyrazolo [5′,1′:2,3][1,3]oxazolo[5,4-*c*]pyridine-6(5*H*)-carboxylate (35). To the stirred solution of ethyl 2-(hydroxymethyl)-7,8-dihydropyrazolo [5′,1′:2,3][1,3]oxazolo-[5,4-c]pyridine-6(5*H*)-carboxylate (1.76 g, 6.6 mmol) in $CHCl_3$ (60 ml) was added activated MnO_2 (10 g, excess). The suspension was refluxed for 1.5 h under a nitrogen atmosphere. The reaction mixture was filtered through a pad of Celite and the filtrate was concentrated to give a yellow solid. The product was purified by chromatography. 1.43 g of the product was obtained (82%); Mp 97–99 °C, (M+H) 264.

16.1.5. Step 5: Preparation of ethyl 2-[(acetyloxy)(5R)-6-bromo-2-Z{[(4-nitrobenzyl)oxy]carbonyl}-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-en-6-yl)methyl]-7,8-dihydropyrazolo[5′,1′:2,3][1,3]oxazolo[5,4-c]pyridine-6(5H)-carboxylate (11g). Starting from ethyl 2-formyl-7,8-dihydropyrazolo [5′,1′:2,3][1,3]oxazolo[5,4-c]pyridine-6(5*H*)-carboxylate **35** (790 mg, 3. mmol), (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 **10** (1.54 g, 4.6 mmol), and anhydrous $MgBr_2$: $O(Et)_2$ (2.21 g, 8.5 mmol) and following the procedure as outlined for **12a** (Step 2) the titled compound was separated as the mixture of diastereo isomers. Pale yellow amorphous solid; yield: 1.67 g, 81%; (M+H) 690.

16.1.6. Step 6: Preparation of (5*R*,6*Z*)-6-{[6-(ethoxycarbonyl)-5,6,7,8-tetrahydropyrazolo[5′,1′:2,3][1,3]oxazolo-[5,4-c]pyridin-2-yl]methylene}-7-oxo-4-thia-1-azabicyclo-[3.2.0]hept-2-ene-2-carboxylic acid sodium salt (12g). Ethyl 2-[(acetyloxy)(5*R*)-6-bromo-2-*Z*{[(4-nitrobenzyl)-oxy]carbonyl} -7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-en-6-yl)methyl)]-7,8-dihydropyrazolo[5′,1′:2,3][1,3]-oxazolo[5,4-c]pyridine-6(5*H*)-carboxylate **11g** (828 mg, 0.5 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 (200 mg) at 40 psi pressure. After 4 h the reaction mixture was filtered, cooled to 3 °C, and 0.1 M NaOH was added to adjust 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

yellow precipitate. The product was purified by HP21 resin reverse phase column chromatography. Initially the column was eluted with deionized water (2 L) and later 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. Yield 375 mg (71%); yellow solid; (M+H+Na) 438.4.

H NMR (D_2O): δ 6.96 (1H, s), 6.94 (1H, s), 6.41 (1H, s), 6.00 (1H, s), 4.53 (2H, m), 4.13 (2H,q), 3.78 (2H,m), 2.78 (2H, m), 1.21 (3H, t).

$C_{15}H_{15}N_4NaO_6S$.

Calcd 49.32%, H 3.45%, N 12.78%; found: C 49.30%, H 3.41, N 12.67.

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