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Synthesis and biological evaluation of 6-bromo-6-substituted penicillanic acid derivatives as β-lactamase inhibitors

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Abstract

The synthesis of a selected set of 6-bromopenicillanic acid derivatives with an additional C6 substituent is reported. All these substances were tested as inhibitors of class A and C β-lactamase enzymes derived from *Escherichia coli* (TEM-1) and *E. cloacae* (P99). As 6-(1-hydroxyethyl) derivatives **4c** and **6c** were found to be weak β-lactamase inhibitors, they were further investigated in combination with amoxicillin against a series of β-lactamase-producing bacterial strains. Some structure-activity relationships are discussed.

Keywords: β-Lactams; β-Lactamase inhibitors; Brobactam

1. Introduction

The most common resistance mechanism adopted by bacteria against β-lactam antibiotics is the production of β-lactamases [1,2]. These enzymes can be divided into four classes (A, B, C and D) on the basis of their primary structure and homology [3] being class A (penicillinases) and C (cephalosporinases) the most clinically relevant. β-lactamases are able to catalyze the hydrolysis of the β-lactam ring, thus leading to inactive compounds [4]. Clavulanic acid, sulbactam and tazobactam (Fig. 1) are marketed and widely used in association with piperacillin (tazobactam, Zosyn®, American Home Products), amoxicillin (clavulanic acid, Augmentin®, SmithKline Beecham) and ampicillin (sulbactam, Unasyn®, Pfizer). Although these compounds are effective inhibitors of class A β-lactamases, they are ineffective or less effective against class C enzymes [1,5]. Because of the rapid spreading of bacteria that produce class C β-lactamases, there is an urgent need to widen the spectrum of the current β-lactamase inhibitors.

6β-Bromopenicillanic acid (brobactam) (Fig. 1) is an extensively studied β-lactamase inhibitor whose mechanism of action involves the nucleophilic displacement of bromide to give a dihydrothiazine derivative that is irreversibly bound to the active site [6,7] (Fig. 2). Brobactam is as potent as clavulanic acid and inhibits the same range of β-lactamase enzymes [6].

Herein we report an investigation of some 6-bromopenicillanic acid derivatives (Fig. 3) bearing a second substituent in C-6 to test if a further substituent in this position affects the inhibitory activity. The choice of the second substituent was made on the basis of the hypothesis that electron-withdrawing substituents could facilitate the displacement of bromine, which is the

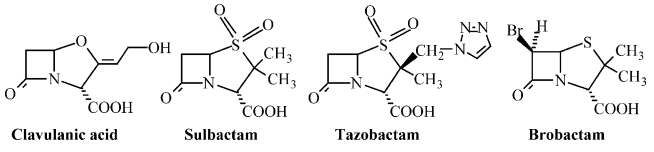

Fig. 1. Structure of representative β-lactam inhibitors of β-lactamases.

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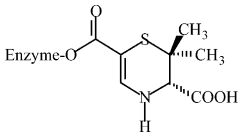

Fig. 2. Enzyme-bound dihydrothiazine derivative.

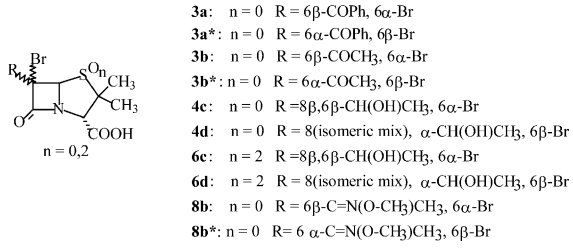

Fig. 3. New 6-bromopenicillanic acid derivatives studied.

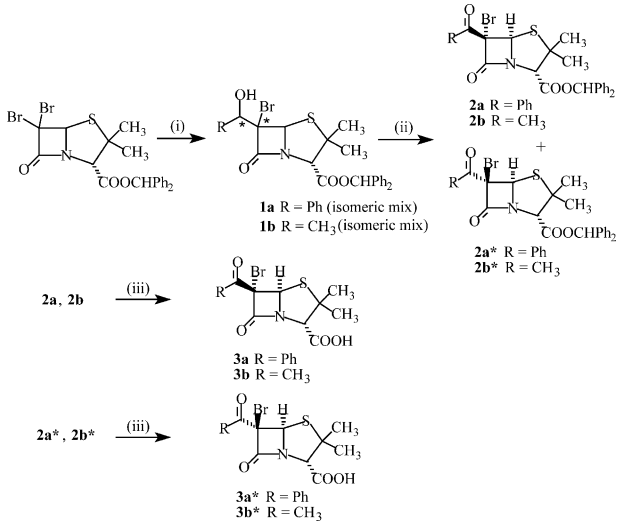

Fig. 4. Reagents and conditions: (i) (a) CH_3MgBr, THF, −78 °C, (b) benzaldehyde or acetaldehyde; (ii) pyridinium dichromate, CH_2Cl_2, r.t.; (iii) *m*-cresol; 50 °C for **3a**, **3a*** , **3b*** ; TFA, anisole for **3b**.

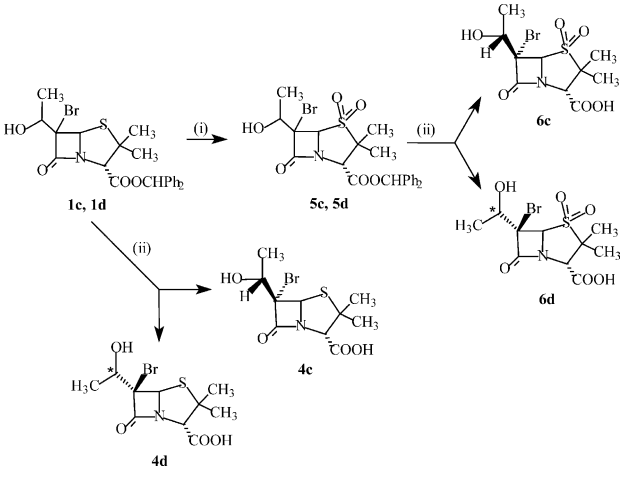

Fig. 5. Reagents and conditions: (i) *m*-chloroperoxybenzoic acid, CH_2Cl_2; (ii) TFA, anisole.

crucial step in the inhibition of β-lactamase; moreover, the 6β-(1-hydroxyethyl) group is known to improve the inhibitory activity of sulbactam against class C β-lactamases [5,8] and to be responsible for the high β-lactamase-inhibitory activity in carbapenems [9].

2. Chemistry

The synthesis of the 6-bromopenicillanic acid derivatives (**3a**, **3a*** , **3b**, **3b*** , **4c**–**d**, **6c**–**d**, **8b**, **8b***) was performed following the route shown in Fig. 4, 5 and 6 using benzhydryl 6,6-dibromopenicillanate as starting material [10]. This compound underwent metal–halogen exchange with methylmagnesium bromide in THF at −78 °C followed by reaction with freshly distilled benzaldehyde or acetaldehyde, giving isomeric mixtures of the corresponding bromohydrins (**1a** [11], **1b**).

1b was found to be a diastereomeric mixture of three different 6-(1-hydroxyethyl) derivatives and it was partially separated by chromatography, affording **1c** as a single diastereoisomer and **1d** as a mixture of two diastereoisomers (ratio **1c**/**1d** 2:1).

The configurations of the hydroxyethyl group and of the bromine atom were assigned according to previous crystallographic and ^1H NMR studies carried out on benzyl ester analogues [12].

1c and **1d** were separately oxidized with pyridinium dichromate in CH_2Cl_2 to the corresponding acetyl derivatives **2b** and **2b*** , which were then deprotected using trifluoroacetic acid–anisole [13] for **2b** or *m*-cresol [14] for **2b*** to obtain their corresponding acids **3b** and **3b*** (Fig. 4).

Hydroxybenzyl adducts (**1a** [11], diastereomeric ratio not determined) were directly oxidized with pyridinium dichromate in CH_2Cl_2 to the corresponding benzoyl derivatives **2a** and **2a*** which, after separation by flash-chromatography, were deprotected with *m*-cresol [14] to their corresponding acids **3a** and **3a*** (Fig. 4). The configuration of the 6-benzoyl derivatives was assigned as 6β for **2a** and 6α for **2a*** on the basis of the observed chemical shift of the proton in position C-5 in the ^1H NMR spectrum compared with the shift of the same proton in the 6-acyl derivatives **2b** and **2b*** .

1c and **1d** were deprotected with trifluoroacetic acid–anisole [13], affording **4c** and **4d**, respectively, or oxidized to their corresponding sulfones **5c** and **5d** using *m*-chloroperoxybenzoic acid and then deprotected to **6c** and **6d** (Fig. 5).

Attempts to oxidize the benzhydryl 6-acyl sulfide derivatives to the corresponding sulfones by standard oxidation procedures (*m*-CPBA or $KMnO_4$) lead to extensive decomposition and the same result was obtained in attempts to oxidize **5c** or **5d** with pyridinium dichromate to obtain 6-bromo-6-acetyl derivatives.

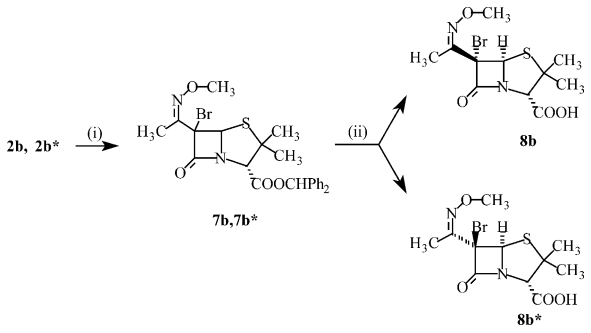

Fig. 6. Reagents and conditions: (i) methoxylamine hydrochloride, pyridine, Na_2SO_4, r.t.; (ii) TFA, anisole.

Table 1
β-Lactamase-inhibitory activity of 6-bromopenicillanic acid derivatives against isolated TEM-1 and P99 enzymes, IC_{50} (μg ml^{-1})

Comp.	TEM-1 (*E. coli*)	P99 (*E. cloacae*)
Clav. acid	0.7 [a]	>4000 [a]
3a	N I [b]	N I [b]
3a*	N I [b]	N I [b]
3b	N I [b]	N I [b]
3b*	N I [b]	N I [b]
4c	825	N I [b]
4d	N I [b]	N I [b]
6c	760	61
6d	1000	N I [b]
8b	N I [b]	N I [b]
8b*	N I [b]	N I [b]

[a] See ref. [6,15].
[b] N I: non-inhibiting.

Methoxyimino derivatives **8b** and **8b*** were synthesized from 6-bromo-6-acetyl derivatives **2b** and **2b*** by treatment with methoxylamine hydrochloride and pyridine; intermediates **7b** and **7b*** were subjected to deprotection by means of trifluoroacetic acid–anisole (Fig. 6).

3. Results and discussion

The β-lactamase-inhibitory activity of the 6-bromopenicillanic acid derivatives studied as inhibitors of P99 and TEM-1 β-lactamase derived from *E. cloacae* and *Escherichia coli*, respectively, is reported in Table 1. Compounds **4c**, **6c** and **6d** were found to be weak inhibitors of TEM-1 β-lactamase, and **6c** was able to inhibit the class-C β-lactamase P99.

Table 2 reports the in vitro activity of **4c** and **6c** both alone and in 1:2 or 1:1 combinations with the known antibiotic amoxicillin. A similar combination of clavulanic acid and amoxicillin was used as a reference standard. These data show that neither **4c** nor **6c** possess antibacterial activity against the selected bacterial strains. These combinations with amoxicillin did not provide the synergic effect against class A β-lactamase-producing bacteria that was observed with amoxicillin–clavulanic acid, nor did they give a synergic effect against class C β-lactamase-producing strains.

These results show that the presence of a carbonyl or methoxyimino moiety in position C-6 in addition to the bromine is detrimental for β-lactamase inhibitory activity regardless of their configuration.

The three substances active as weak inhibitors of isolated β-lactamase enzymes (Table 1) each bear a hydroxyethyl group in C-6, giving further confirmation

Table 2
In vitro synergy study of compounds **4c** and **6c** in combination with amoxicillin

Comp.	*E. coli*, ATTC, 35218, β-lact.+ [a]	*E. coli*, 1850E, W.T. [b]	*E. coli*, 18520E, P.M. [c]	*E. coli*, 1919E, P.M. [c], TEM-1 [d]	*E. cloacae*, 1051E, P99+ [e]	*E. cloacae*, 1321E, P99−	*S. aureus*, ATTC, 43387, β-lact.+ [a]	*S. aureus*, 853E, PC1+ [f]
Clavulanic acid	32	64	64	64	64	64	32	32
Amoxicillin	>256	8	8	>256	>256	4	256	128
Amox.+clav. 2:1	8	8	4	16	128	4	<=2	<=2
Amox.+clav. 1:1	8	8	4	8	64	4	<=2	<=2
4c	>256	>256	>256	>256	>256	>256	>256	>256
Amox.+**4c** 2:1	>256	16	8	>256	>256	4	128	128
Amox.+**4c** 1:1	>256	8	8	>256	>256	4	128	128
6c	>256	>256	>256	>256	>256	>256	>256	>256
Amox.+6c 2:1	>256	16	4	>256	>256	4	128	128
Amox.+**6c** 1:1	>256	8	4	>256	>256	4	128	128

The MIC is expressed as μg ml^{-1}.
[a] β-Lactamase-producing strain.
[b] W.T. = wild type.
[c] P.M. = permeable membrane.
[d] TEM-1-producing strain.
[e] P99-producing strain.
[f] PC1-producing strain.

of the importance of this moiety in the inhibitory activity [16]. In the case of **6c**, the presence of the sulfone extends this activity to class C enzymes. The 6β-configuration of the hydroxyethyl group in derivative **6c** is in accordance with the configuration of class C inhibitor derivatives lacking the bromine atom [16]. In any case, the reduced activity of our compounds with respect to that of their debrominated analogues shows that the double substitution we attempted is detrimental for β-lactamase inhibitory activity.

4. Experimental

4.1. Chemistry

Melting points were taken on a Buchi SMP-510 capillary apparatus and are uncorrected. Infrared spectra (IR) were recorded on a Bruker FT-48 spectrometer and absorbances are reported in ν (cm^{-1}). Elemental analyses were performed on a Carlo Erba analyzer and were within $\pm 0.4\%$ of the theoretical values (when solvent is reported in the analytical formula, it has been detected even by ^1H NMR analysis). EI-MS spectra (70 eV) were taken on a Fison-Trio 1000 instrument. ^1H NMR spectra were recorded on a Bruker 200 spectrometer, chemical shifts are reported in ppm (δ scale). Column chromatography purifications were performed under 'flash' conditions using Merck 230–400 mesh silica gel. Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel 60 F_{254} plates.

*4.1.1. 6β-Benzoyl-6α-bromo-3,3-dimethyl-7-oxo-4-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid benzhydryl ester (**2a**) and 6α-benzoyl-6β-bromo-3,3-dimethyl-7-oxo-4-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid benzhydryl ester (**2a***)*

Pyridinium dichromate (306 mg, 0.81 mmol) was added to a solution of **1a** [10] (150 mg, 0.27 mmol) in 2 ml of dry CH_2Cl_2 at 0 °C. The reaction mixture was stirred for 18 h at r.t., filtered on Celite, washed with hot EtOAc and then evaporated to give a crude residue. The two isomers were separated by flash-chromatography on silica gel (cyclohexane-EtOAc 9:1).

Compound **2a**: 26% yield; amorphous solid. IR (Nujol): 1799, 1734, 1683 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.20 (s, 3H); 1.51 (s, 3H); 4.64 (s, 1H); 5.88 (s, 1H); 6.97 (s, 1H); 7.39–7.32 (m, 10H), 7.63–7.47 (m, 3H); 7.99–7.97 (m, 2H). MS (m/z): 224–226, 167 (100%), 152, 105.

Compound **2a***: 54% yield; m.p. 145 °C (dec.) (EtOAc–hexane). IR (Nujol): 1785, 1734, 1669 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.31 (s, 3H); 1.72 (s, 3H); 4.69 (s, 1H); 6.15 (s, 1H); 6.92 (s, 1H); 7.35–7.33 (m, 10H), 7.63–7.48 (m, 3H); 8.32–8.28 (m, 2H). MS (m/z): 470, 167 (100%), 152, 105.

*4.1.2. 6α-Bromo-6β-(1-hydroxy-ethyl)-3,3-dimethyl-7-oxo-4-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid benzhydryl ester (**1c**) and 6β-bromo-6α-(1-hydroxy-ethyl)-3,3-dimethyl-7-oxo-4-thia-1-aza-bicyclo[3.2.0]-heptane-2-carboxylic acid benzhydryl ester (**1d**)*

Benzhydryl 6,6-dibromo penicillanate [10] (800 mg, 1.52 mmol) in dry THF (14 ml) at −78 °C under nitrogen was treated, dropwise, with a solution of methylmagnesium bromide in diethyl ether (3 M; 0.64 ml, 1.92 mmol) and the resulting solution was allowed to react for 20 min under stirring. Freshly distilled acetaldehyde (0.6 ml, 10.7 mmol) in dry THF (2.5 ml) was then added. After 20 min saturated aqueous ammonium chloride was added and the reaction mixture extracted with EtOAc. The organic phases were combined, washed with water and brine, dried on Na_2SO_4 and the solvent was evaporated to yield a crude material which was subjected to flash-chromatography on silica gel (cyclohexane–EtOAc 7:3).

Compound **1c**: 68% yield; amorphous solid. IR (KBr): 3474, 1781, 1745 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.27 (s, 3H); 1.28 (d, 3H, $J = 5.96$ Hz); 1.43 (s, 3H); 4.22 (m, 1H), 4.61 (s, 1H); 5.63 (s, 1H); 6.94 (s, 1H); 7.38–7.35 (m, 10H). MS (m/z): 225–223, 167 (100%), 152, 114.

1d: 31% yield (diastereomeric mixture 3:1 ratio from ^1H NMR integration); amorphous solid. IR (KBr): 3423, 1765, 1736 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.26 (s); 1.44 (d); 1.66 (s); 4.16 (m, major isomer); 4.27 (m, minor isomer); 4.61 (s); 5. 51 (s); 6.94 (s); 7.36–7.34 (m). MS (m/z): 410, 167 (100%), 152, 114.

*4.1.3. 6α-Bromo-6β-(1-hydroxy-ethyl)-3,3-dimethyl-4,4,7-trioxo-4λ⁶-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid benzhydryl ester (**5c**) and 6β-bromo-6α-(1-hydroxy-ethyl)-3,3-dimethyl-4,4,7-trioxo-4λ⁶-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid benzhydryl ester (**5d**)*

Hydroxy sulfides **1c** and **1d** were separately oxidized to sulfones. A solution of 57–86% *m*-chloroperoxybenzoic acid (376 mg) in 6 ml of CH_2Cl_2 was added dropwise to a solution of **1c** (or **1d**; 150 mg, 0.3 mmol) in 6 ml of CH_2Cl_2 at 0 °C. The mixture was stirred for 20 h at r.t., then washed with 3% Na_2SO_3 solution in saturated aqueous $NaHCO_3$, then with brine. The organic layer was dried and the solvent evaporated to give a residue which was purified by flash-chromatography on silica gel (cyclohexane–EtOAc 8:2).

Compound **5c**: 69% yield; m.p. 140 °C (Et_2O–hexane). IR (KBr): 3536, 1808, 1734, 1325 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.14 (s, 3H); 1.37 (d, 3H, $J = 6.2$ Hz), 1.58 (s, 3H); 4.61 (s, 1H); 4.81 (m, 1H), 4.81 (s, 1H); 6.97 (s, 1H); 7.40–7.32 (m, 10H). MS (m/z): 167 (100%), 152.

5d: 76% yield (diastereomeric mixture 2:1 ratio from ^1H NMR integration); amorphous solid. IR (KBr): 3487, 1805, 1749, 1334 cm^{-1}. ^1H NMR ($CDCl_3$) δ:

1.12 (s); 1.46 (d, minor isomer, $J = 5.98$ Hz); 1.50 (d, major isomer, $J = 6.4$ Hz); 1.60 (s); 4.11 (m, minor isomer); 4.27 (m, major isomer); 4.59 (s); 4.71 (s); 6.94 (s, major isomer); 6.96 (s, minor isomer); 7.36–7.32 (m). MS (m/z): 167 (100%), 152.

*4.1.4. 6β-Acetyl-6α-bromo-3,3-dimethyl-7-oxo-4-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid benzhydryl ester (**2b**) and 6α-acetyl-6β-bromo-3,3-dimethyl-7-oxo-4-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid benzhydryl ester (**2b***)*

Activated molecular sieves and pyridinium dichromate (288 mg, 0.76 mmol) were added to a solution of the appropriate alcohol (**1c** or **1d**; 125 mg, 0.225 mmol) in 2 ml of dry CH_2Cl_2 at 0 °C. The mixture was stirred for 18 h at r.t., filtered on celite, washed with hot EtOAc and then evaporated to give a residue which was purified by flash-chromatography on silica gel (cyclohexane–EtOAc 6:4).

Compound **2b**: 74% yield; m.p. 121 °C (CH_2Cl_2–hexane). ^{1}H NMR ($CDCl_3$) δ: 1.25 (s, 3H); 1.54 (s, 3H); 2.42 (s, 3H); 4.57 (s, 1H); 5.57 (s, 1H); 6.94 (s, 1H); 7.38–7.30 (m, 10H). MS (m/z): 487–489 (M^+), 320–322, 167 (100%), 152, 114.

Compound **2b***: 95% yield; m.p. 150 °C (CH_2Cl_2–hexane). IR (Nujol): 1785, 1733, 1716 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ: 1.27 (s, 3H); 1.66 (s, 3H); 2.52 (s, 3H); 4.61 (s, 1H); 5.89 (s, 1H); 6.93 (s, 1H); 7.39–7.30 (m, 10H). MS (m/z): 487–489 (M^+), 320–322, 167 (100%), 152, 114.

*4.1.5. 6α-Bromo-6β-(1-methoxyimino-ethyl)-3,3-dimethyl-7-oxo-4-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid benzhydryl ester (**7b**) and 6β-bromo-6α-(1-methoxyimino-ethyl)-3,3-dimethyl-7-oxo-4-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid benzhydryl ester (**7b***)*

Na_2SO_4 (250 mg), pyridine (0.24 ml) and methoxylamino hydrochloride (200 mg, 2.4 mmol) were added to a solution of the 6-acetyl derivative (**2b** or **2b***; 195 mg, 0.4 mmol) in 5 ml of dry CH_2Cl_2. The mixture was stirred 5 days at r.t. then added of water, extracted with CH_2Cl_2 and dried on Na_2SO_4. The solvent was evaporated to dryness and the residue purified by flash-chromatography on silica gel (cyclohexane–EtOAc 9:1).

Compound **7b**: 42% yield; m.p. 145 °C (hexane). IR (KBr): 1786, 1744 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ: 1.21 (s, 3H); 1.53 (s, 3H); 2.12 (s, 3H); 3.92 (s, 3H); 4.59 (s, 1H); 5.77 (s, 1H); 6.94 (s, 1H); 7.37–7.33 (m, 10H). MS (m/z): 349–351, 208–210, 167 (100%), 152, 114.

Compound **7b***: 72% yield; oil. ^{1}H NMR ($CDCl_3$) δ: 1.27 (s, 3H); 1.66 (s, 3H); 2.11 (s, 3H); 3.89 (s, 3H); 4.60 (s, 1H); 5.59 (s, 1H); 6.92 (s, 1H); 7.36–7.33 (m, 10H). MS (m/z): 349–350, 208–210, 167 (100%), 152, 114.

4.1.6. General procedure for the deprotection of benzhydryl esters to acids

Method A was used to deprotect **2a**, **2a*** and **2b*** and method B to deprotect all the other benzhydryl esters.

4.1.6.1. Method A. Suitable ester (0.18 mmol) were dissolved in 1 ml of *m*-cresol and the solution was stirred at 50 °C for 6 h. The reaction mixture was extracted 2 × with $NaHCO_3$ solution; the water phases were combined and washed 2 × with EtOAc. The aqueous phase at 0 °C was acidified to pH 2/3 with 2 N HCl and then extracted with EtOAc. The organic phases were combined, dried on Na_2SO_4 and evaporated to give the desired compound.

4.1.6.2. Method B. TFA (0.17 ml; 2.2 mmol) was added to a solution of 0.146 mmol of the suitable ester in 0.17 ml of anisole at 0 °C. The mixture was stirred at r.t. for 3 h.

*4.1.7. 6β-Benzoyl-6α-bromo-3,3-dimethyl-7-oxo-4-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid (**3a**)*

Yield 45%; amorphous solid. ^{1}H NMR ($CDCl_3$) δ: 1.53 (s, 3H); 1.56 (s, 3H); 4.55 (s, 1H); 5.83 (s, 1H); 7.64–7.48 (m, 3H), 8.04–7.99 (m, 2H). MS (m/z): 383–385 (M^+); 105 (100%), 77. *Anal.* ($C_{15}H_{14}BrNO_4S$·0.5EtOAc) C, H, N.

*4.1.8. 6α-Benzoyl-6β-bromo-3,3-dimethyl-7-oxo-4-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid (**3a***)*

Yield 72%; amorphous solid. IR (KBr): 3381, 1731, 1720, 1676 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ: 1.60 (s, 3H); 1.75 (s, 3H); 4.60 (s, 1H); 6.07 (s, 1H); 7.66–7.43 (m, 3H), 8.31–8.27 (m, 2H). MS (m/z): 304, 105 (100%), 77. *Anal.* ($C_{15}H_{14}BrNO_4S$·0.5EtOAc) C, H, N.

*4.1.9. 6β-Acetyl-6α-bromo-3,3-dimethyl-7-oxo-4-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid (**3b**)*

The product crystallized spontaneously after concentration of the reaction mixture and addition of hexane. Yield 70%; m.p. 125 °C (dec.). IR (KBr): 3141, 1774, 1735, 1710 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ: 1.56 (s, 3H); 1.60 (s, 3H); 2.43 (s, 3H); 4.49 (s, 1H); 5.53 (s, 1H). MS (m/z): 321–323 (M^+), 114, 100 (100%). *Anal.* ($C_{10}H_{12}BrNO_4S$) C, H, N.

*4.1.10. 6α-Acetyl-6β-bromo-3,3-dimethyl-7-oxo-4-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid (**3b***)*

Yield 39%; amorphous solid. IR (KBr): 3243, 1792, 1764, 1721 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ: 1.58 (s, 3H); 1.70 (s, 3H); 2.54 (s, 3H); 4.55 (s, 1H); 5.83 (s, 1H). MS (m/z): 321–323 (M^+), 114, 100 (100%). *Anal.* ($C_{10}H_{12}BrNO_4S$·0.1EtOAc) C, H, N.

*4.1.11. 6α-Bromo-6β-(1-hydroxy-ethyl)-3,3-dimethyl-7-oxo-4-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid (**4c**)*

The product crystallized after concentration of the reaction mixture and addition of light petroleum. Yield 75%; m.p. 148 °C (dec.). IR (KBr): 3392, 1772, 1748 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.28 (d, 3H, $J = 6.12$ Hz); 1.58 (s, 3H); 1.68 (s, 3H); 4.24 (m, 1H); 4.55 (s, 1H); 5.57 (s, 1H). MS (m/z): 160 (100%), 114. *Anal.* ($C_{10}H_{14}BrNO_4S$) C, H, N.

*4.1.12. 6β-Bromo-6α-(1-hydroxy-ethyl)-3,3-dimethyl-7-oxo-4-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid (**4d**)*

The product crystallized after concentration of the reaction mixture and addition of light petroleum then it was recrystallized. Yield 86% (diastereomeric mixture 2:1 ratio from ^1H NMR integration); m.p. 134 °C (dec.) (CH_2Cl_2-light pet.). IR (KBr): 3295, 1780, 1760, 1737 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.45 (d, minor isomer, $J = 5.48$ Hz); 1.48 (d, major isomer, $J = 6.1$ Hz); 1.56 (s); 1.70 (s); 4.22 (m, minor isomer); 4.28 (m, major isomer); 4.53 (s); 5.47 (s, major isomer); 5.49 (s, minor isomer). MS (m/z): 323–325, 160 (100%), 114. *Anal.* ($C_{10}H_{14}BrNO_4S$) C, H, N.

*4.1.13. 6α-Bromo-6β-(1-hydroxy-ethyl)-3,3-dimethyl-4,4,7-trioxo-4λ⁶-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid (**6c**)*

The product crystallized after concentration of the reaction mixture and addition of CH_2Cl_2-light pet. Yield 66%; m.p. 170 °C (dec.). IR (KBr): 3247, 1801, 1774, 1325 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.38 (d, 3H, $J = 6.04$ Hz); 1.52 (s, 3H); 1.65 (s, 3H); 4.55 (s, 1H); 4.83 (m, 1H); 4.85 (s, 1H). *Anal.* ($C_{10}H_{14}BrNO_6S$) C, H, N.

*4.1.14. 6β-Bromo-6α-(1-hydroxy-ethyl)-3,3-dimethyl-4,4,7-trioxo-4λ⁶-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid (**6d**)*

The product crystallized after concentration of the reaction mixture and addition of light pet. Yield 34% (diastereomeric mixture 5:1 ratio from ^1H NMR integration). IR (KBr): 3420, 1799, 1756, 1327 cm^{-1}. ^1H NMR ($CDCl_3$–DMSO-d_6) δ: 1.25 (d, minor isomer, J = 6.14 Hz); 1.33 (d, major isomer, $J = 6.14$ Hz); 1.36 (s); 1.49 (s); 3.97 (m, minor isomer); 4.08 (m, major isomer); 4.29 (s); 4.96 (s, major isomer); 5.00 (s, minor isomer). MS (m/z): 312–314, 202–204, 148–150 (100%). *Anal.* ($C_{10}H_{14}BrNO_6S{\cdot}0.1C_6H_{12}$) C, H, N.

*4.1.15. 6α-Bromo-6β-(1-methoxyimino-ethyl)-3,3-dimethyl-7-oxo-4-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid (**8b**)*

The product crystallized after concentration of the reaction mixture and addition of hexane, and subsequently recrystallized. Yield 74%; m.p. 141 °C (dec.) (Et_2O–hexane). IR (KBr): 3168, 1762, 1746, 1734 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.54 (s, 3H); 1.58 (s, 3H); 2.12 (s, 3H); 3.93 (s, 3H); 4.53 (s, 1H); 5.74 (s, 1H). MS (m/z): 350–352 ($M+$), 208–210, 191–193, 160, 84 (100%). *Anal.* ($C_{11}H_{15}BrN_2O_4S{\cdot}0.15Et_2O$) C, H, N.

*4.1.16. 6β-Bromo-6α-(1-methoxyimino-ethyl)-3,3-dimethyl-7-oxo-4-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid (**8b***)*

The reaction mixture was concentrated and then subjected to flash chromatography on silica gel (EtOAc). Yield 52%; m.p. 124 °C (dec.) (CH_2Cl_2-light pet.). IR (KBr): 3306, 1784, 1751, 1718 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.58 (s, 3H); 1.72 (s, 3H); 2.05 (s, 3H); 3.94 (s, 3H); 4.57 (s, 1H); 5.88 (s, 1H). MS (m/z): 350–352 (M^+), 208–210, 178–180, 84 (100%). *Anal.* ($C_{11}H_{15}BrN_2O_4S$) C, H, N.

4.2. Biology

4.2.1. β-Lactamase inhibition assay

The activity of compounds **3a**, **3a***, **3b**, **3b***, **4c**–**d**, **6c**–**d**, **8b**, **8b*** as β-lactamase inhibitors was assessed by determining the concentrations needed to obtain a 50% inhibition of the hydrolysis of nitrocefin, which was used as the reporter substrate in competition experiments. The tests were performed on TEM-1 and P99 as representative enzymes for class A and class C β-lactamases, respectively. Nitrocefin was used at final concentrations of 180 μM for TEM-1 and of 250 μM for P99.

The compounds were dissolved in 50 mM sodium phosphate buffer, pH 7.0, at a final concentration of 2.5 mM. 1.5 μl of 1:10 diluted enzyme (in 50 mM sodium phosphate buffer, pH 7.0) were added to the reaction mixture (100 μl final volume: 50 μl substrate + 50 μl compound) and nitrocefin hydrolysis was recorded at 482 nm for 3 min at 30 °C, using a Spectramax spectrophotometer. Compounds were assayed at 14 different concentrations ranging from 0.015 to 1.25 mM. The rates of hydrolysis at each inhibitory concentration were calculated and the IC_{50} values (expressed as μg ml^{-1}) were determined by plotting the percentage of inhibition against the inhibitor concentration.

4.2.2. Bacterial strains and culture conditions

Eight reference strains were used in these studies: *E. coli* ATTC 35218, *E. coli* 1850E (wild type = W.T.), *E.coli* 1852E (permeable membrane = P.M.), *E. coli* 1919E (TEM-1), *E. cloacae* 1051E (P99 enzyme producer = P99 +), *E. cloacae* 1321E (P99 enzyme non-producer = P99 −) and *S. aureus* 853E (penicillinase producer = PC1).

E. coli and *S. aureus* were grown overnight at 37 °C on Triptycase Soya Agar and on Baird–Parker Agar Base, respectively. For in vitro studies of pharmacody-

namics, logarithmic growth-phase cultures were prepared by inoculating colonies into Mueller Hinton Broth (Oxoid), to obtain a final inoculum of approximately 10^5 CFU ml^{-1}.

4.2.3. MIC determination

Compounds **4c** and **6c** were tested alone or in combination with amoxicillin (ratio 1:1 and 1:2) against a selected series of β-lactamase-producing strains. Amoxicillin and clavulanic acid alone and in combination were also tested as references. The MICs were determined by a standard broth microdilution method (NCCLS) [17]. The final compound concentrations ranged from 0.25 to 128 μg ml^{-1}. The MIC was defined as the lowest drug concentration, which inhibited visible growth of micro-organisms after 24 h of incubation at 37 °C.

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