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Synthesis of new C-6 alkyliden penicillin derivatives as β-lactamase inhibitors

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Abstract

New penicillin, penicillin sulfone and sulfoxide derivatives bearing a C-6-alkyliden substituent were prepared. Their chemical synthesis, in vitro antibacterial activity and inhibition properties against two selected enzymes representing Class A and C β-lactamases are reported. Compounds **3a**–**c**, **7a**–**c** were able to inhibit either TEM-1 (a Class A enzyme, from *Escherichia coli*) or P-99 (a Class C enzyme, from *E. cloacae*), or both enzymes, when tested in competition experiments using nitrocefin as the reporter substrate. However, when tested in combination with amoxicillin, the same compounds did not show synergistic effects against *E. coli* and *E. cloacae* strains producing TEM-1 and P99 enzymes, respectively. This finding is most likely related to poor penetration through the bacterial cell wall, as shown by using a more permeable isogenic *E. coli* strain. Interestingly, a synergistic effect against a strain of *S. aureus* which produces PC1-enzyme (a Class A β-lactamase) was observed for compound **3a** when used in combination with amoxicillin.

Keywords: β-Lactams; β-Lactamase inhibitors; 6-Acetylmethylene penicillanic acid

1. Introduction

The clinical efficacy of β-lactam antibiotics is being compromised by the spreading of bacterial resistance due to bacterial production of different kinds of β-lactamases. These enzymes inactivate penicillin and cephalosporin-like antibiotics by hydrolyzing the β-lactam ring before they reach their natural target (PBPs, penicillin-binding proteins). Accordingly to Ambler's classification, β-lactamases can be divided into four molecular classes (A, B, C and D) on the basis of their primary structure and sequence homology [1–3]. Commercially available β-lactamase inhibitors (clavulanic acid, sulbactam and tazobactam), are effective above all against Class A β-lactamases. Due to the high rate of mutation of these enzymes, there is a need for more effective and broader spectrum inhibitors to recover the spectrum of activity of both penicillins and cephalosporins [4]. 6-acetylmethylene penicillanic acid (Ro 15-1903) is known to be a potent and broad spectrum β-lactamase inhibitor [5,6]. However, this compound has been shown to be chemically unstable in culture media and in water solutions at physiological pH [7,8]. In the present study we synthesised new C-6 alkylidene penicillanic acid derivatives in the attempt to obtain molecules with a wider spectrum and/or better affinity towards clinically relevant β-lactamases as well as improved chemical stability. The reported instability of Ro-15-1903 in the culture medium is likely due to the extended conjugation of the α,β-unsaturated ketone with the azetidone ring which becomes highly susceptible to nucleophilic attack even under mild conditions. We reasoned that the insertion of a methyl group α-to the ketone, as in compounds **3a** and **7a**, would disrupt the coplanarity of the system making it less reactive. The non coplanarity is supported by ab-initio calculation (data not shown) and by the increased stability of the system in the conditions described in the paper. The inhibitory properties of these compounds were

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determined using two isolated enzymes: TEM-1 and P-99. These enzymes were selected in that they are representative of Class A and C β-lactamases, respectively. Members of both classes of enzymes are clinically relevant, especially in the case of Gram-negative pathogens. In particular, the spectrum of hydrolysis of P99 includes the latest generation of cephalosporins. Furthermore, compounds **3a** and **7a** were tested in combination with amoxicillin, against isogenic *Escherichia coli* strains (a TEM-1 producer, a permeable TEM-1 producing strain and the parent strains), *E. cloacae* (a P99 producing strain and its parent) and one *S. aureus* strain producing PC1 β-lactamase (a Class A enzyme).

2. Chemistry

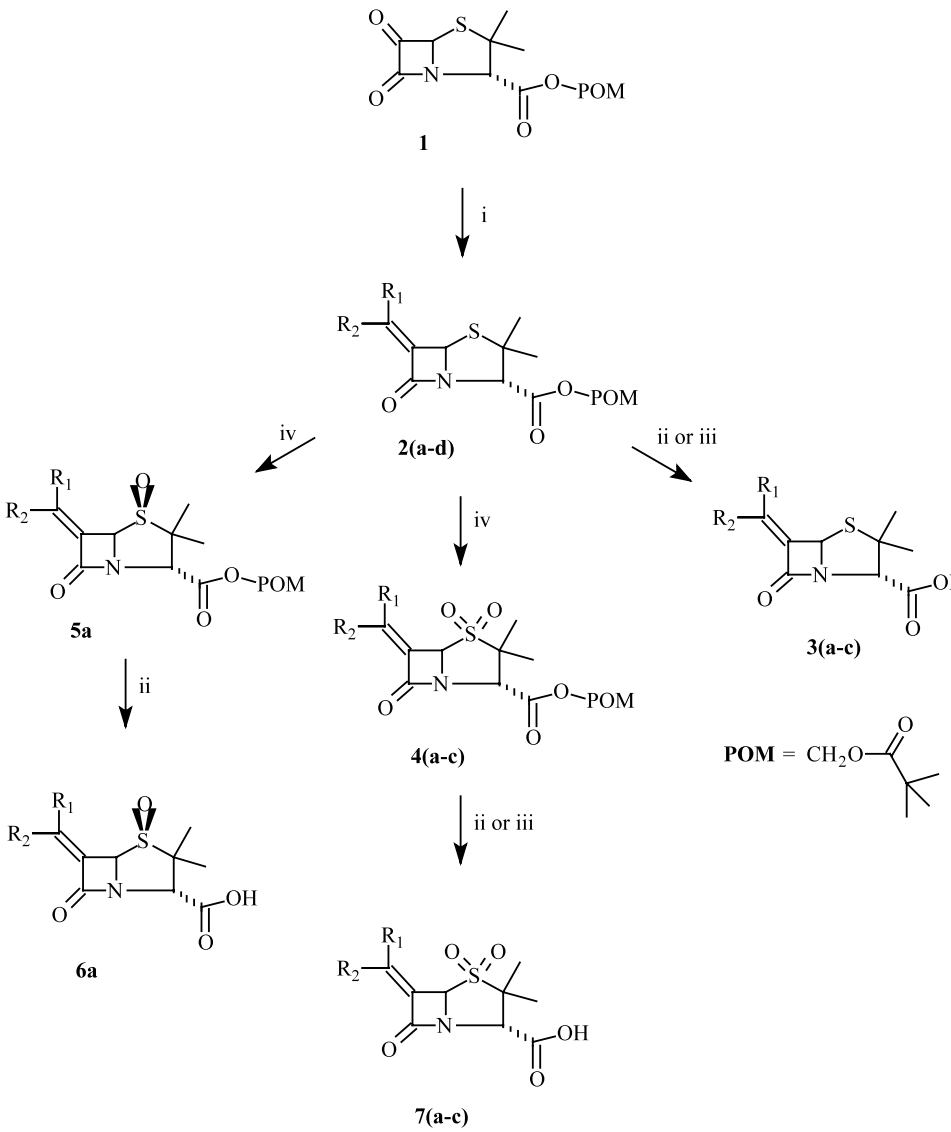
6-Oxoderivative **1** was synthesised, following published procedures, from 6-α-hydroxypenicillanate ester [9,10] and by subsequent oxidation with dimethyl sulfoxide-trifluoroacetic anhydride in CH_2Cl_2 [11,12]. Ketone **1** (Scheme 1) was then allowed to react with the proper triphenylphosphoranyliden derivative [13–15] in CH_2Cl_2 or THF at room temperature for about 30 min to obtain the alkyliden derivatives **2**(**a**–**d**). As reported for similar cases [16–19], the Wittig reaction gave predominantly or exclusively the Z isomer, easily purified by flash chromatography. The Z geometry assignment for compounds **2a**, **2b** and **2d** was confirmed by steady-state NOE experiments. In compound **2a**, mutual signal enhancements were observed only between the H-5 proton (5.95 ppm) and the CH_3 protons of the acetyl group (2.34 ppm) on the C-6 side chain: H-5{CH_3} = 4%, CH_3{H-5} = 2.7%. Moreover, no appreciable NOE effect was observed upon saturation of the signal at 2.26 ppm, suggesting that the two methyl groups in the C-6 side chain are predominantly in the *s-trans* conformation (Fig. 1). The resonances of the two methyl groups on the alkyliden chain, were assigned on the basis of the homoallylic long-range coupling ($J = 0.7$ Hz) between the hydrogen on C-5 and the allylic CH_3.

Concerning **2b**, the absence of any NOE effect between the H-5 proton (6.07 ppm) and the allylic proton (6.35 ppm) on the C-6 side chain confirmed the configuration of this fragment already proposed in the literature for similar compounds [19,20]. Finally, saturation of the signal at 9.72 ppm of compound **2d** (formyl proton) resulted in 5.8% enhancement of the resonance at 6.07 ppm (H-5 proton). Conversely, saturation of the signal at 6.07 ppm gave a 5.7% NOE on the proton at 9.72 ppm.

Compound **2d** was allowed to react with methoxyamine hydrochloride in CH_2Cl_2 in the presence of pyridine to give **12d** (Scheme 2). Reduction with $NaBH_4$ of **2**(**a**,**d**) gave the hydroxyl derivatives **8a** (diastereoisomeric mixture, 1:1 ratio) and **8d**. Oxidation of **2**(**a**–**c**) and **8d** with 1 or 2.5 equiv. of *m*-chloroperbenzoic acid (*m*-ClPBA) in CH_2Cl_2 at room temperature gave sulfoxide **5a** and sulfones **4**(**a**–**c**) and **10d**. After the reaction of **2a** with one equivalent of *m*-ClPBA we obtained a single sulfoxide (**5a**) and, by analogy with the work reported by Adam et al. [21], we assigned the S configuration to the monooxidized sulfur atom. Hydrolysis of compounds **2**(**a**–**c**), **5a**, **4**(**a**–**c**), **8**(**a**,**d**), **10d** and **12d** in the presence of pig liver esterase [22,23] in acetone/water (1:9) and phosphate-buffered medium (pH 8), or by bistributyltinoxide (BBTO) cleavage in Et_2O [24], afforded the acid derivatives **3**(**a**–**c**), **6a**, **7**(**a**–**c**), **9**(**a**,**d**), **11d** and **13d** (see Schemes 1 and 2).

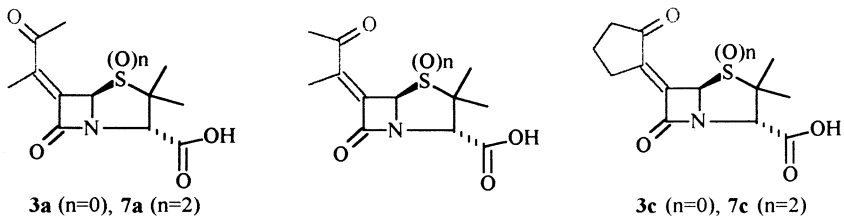
3. Results and discussion

Table 1 reports the IC_{50} values of the compounds determined with TEM-1 (Class A) and P99 (Class C) β-lactamases. Compound **3a** was able to inhibit TEM-1 with an IC_{50} value 2 times lower than that of clavulanic acid. On the other hand, it behaved as a P99 inhibitor with an IC_{50} of 24 μg ml^{-1}. This derivative can be considered as homologous to the previously reported compound 6-acetylmethylenepenicillanic acid [5,6], a potent and large spectrum β-lactamase inhibitor. However, the compound reported in the literature was chemically unstable in culture broth media and phosphate-buffered solutions at pH higher than 7 [7]. On the contrary, compound **3a** was stable under conditions similar to those reported in the literature (at pH 8 after 20 h at 30 °C) and in culture broth (as demonstrated by differential UV measurements on the tested mixtures). Despite this, **3a** did not show in vitro synergistic activity when used in combination with amoxicillin against TEM-1 and P99-producing *E. coli* and *E. cloacae* strains, respectively (Table 2). The experiment with a permeable *E. coli* mutant strain expressing the TEM-1 enzyme (*E. coli* 1919E) indicated that this lack of synergistic activity is due to poor uptake through the bacterial cell wall, a result similar to the findings obtained for sulfone analogue **7a** (Table 2). Oxidation of **3a** to its sulfone derivative **7a** only marginally affected the inhibitory activity on TEM-1, whereas an improved activity against P99 was observed. Sulfone **7c** also allowed a better inhibition of TEM-1 and P99 than did its parent sulfide **3c**. The same trend was observed for compounds **16b** and **19d,** whose sulfone analogues **18b** and **21d** were better P99-inhibitors than the parent compounds, which were totally inactive against this enzyme. This finding indicates that sulfone functionality is an important feature for the inhibition of the P99 enzyme. On the other hand, β-sulfoxide **6a** gave a significant reduction in activity.



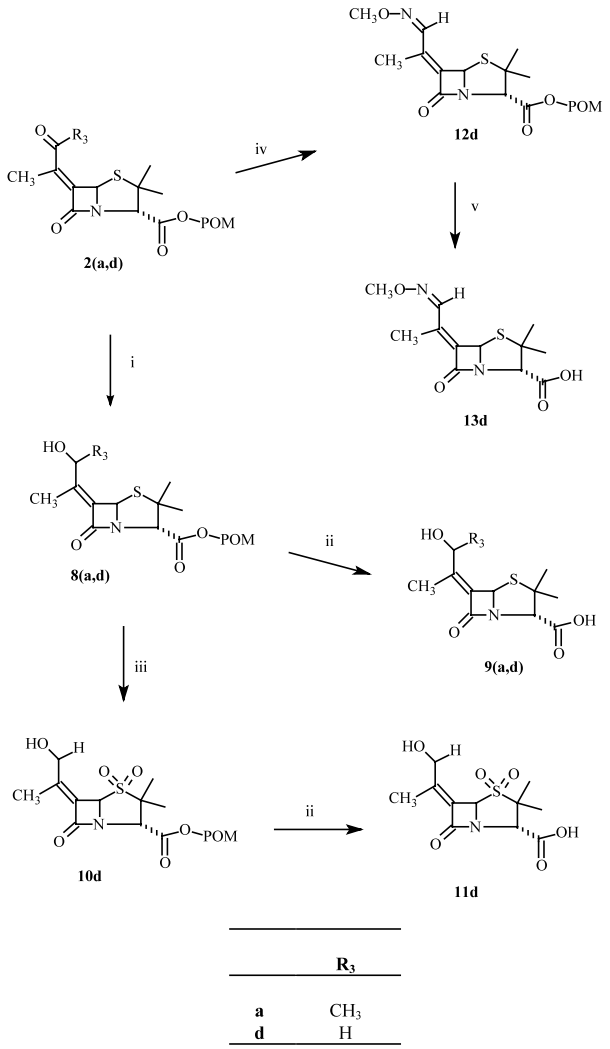
	R_1	R_2		R_1	R_2
a	$COCH_3$	CH_3	**c**		$-CH_2(CH_2)_3CO-$
b	CONH2	H	**d**	CHO	CH_3

Scheme 1. (i) $Ph_3P{=}R_1R_2$, CH_2Cl_2, 0 °C—r.t., 30 min; (ii) pig liver esterase, H_2O/acetone 9:1, phosphate buffer (pH 8), 30 °C, 24 h; (iii) bistributyltinoxide, Et_2O, r.t., 6 h; (iv) *m*-ClPBA, CH_2Cl_2, 0 °C—r.t.



s-*trans* s-*cis*

Fig. 1. *s-trans* and *s-cis* conformations of compounds **3a** and **7a** and the constrained analogs **3c** and **7c**.



Scheme 2. (i) $NaBH_4$, MeOH, r.t.; (ii) pig liver esterase, H_2O/acetone 9:1, phosphate buffer (pH 8), 30 °C, 24 h; (iii) *m*-ClPBA, CH_2Cl_2, 0 °C-r.t.; (iv) CH_3O–NH_2·HCl, CH_2Cl_2, pyridine, r.t. 30′; (v) bistributyltinoxide,Et_2O, r.t., 6 h.

Compounds **3c** and **7c** can be considered constrained analogues of **3a** and **7a**, respectively. As demonstrated by the NOE measurements described in the Section 2, the carbonyl group in the C-6 side chain of **3a** and **7a** is in a fixed *s-trans* conformation (Fig. 1) in the opposite direction from the exocyclic carbonyl of compounds **3c** and **7c**.

This different spatial disposition of the carbonyls seems to greatly influence the activity against TEM-1 but not that of P99. Thus, cyclic analogues **3c** and **7c** are less effective against TEM-1 than **3a** and **7a**. This could be due to the lack of a putative positive interaction between the C-6 lateral chain carbonyl, opportunely positioned in **3a** and **7a**, and some amino acidic residues in the active site of the TEM-1 enzyme. Arisawa and Adam proposed a possible mechanism of inactivation of TEM-1 β-lactamase by 6-acethylmethylenpenicillanic acid [25]. They postulated the formation of a stable enzyme-inhibitor complex via

Table 1
Inhibitory activity expressed as IC_{50} (μg ml^{-1}) against isolated TEM-1 and P99 β-lactamases

Comp.	TEM-1 (*E. coli*)	P99 (*E. cloacae*)
Sulbactam	2 [a]	>50 [a]
Clav. ac.	0.7 [a]	–
3a	0.33	24
3b	24	–
3c	130	27
6a	264	>355
7a	8	1
7b	1.3	107
7c	35	5
9a [b]		
9d		
11d	163	118
13d	>355	352

[a] Data derived from references [27,28].
[b] Diastereoisomeric mixture, 1:1 ratio.

reaction of the α,β-unsaturated ketone of the compound with an amino group in the active site of the TEM-1 acylated enzyme. The resulting Schiff-base could slowly rearrange into a pyrrole structure behaving as a vinylogous urethane resistant to hydrolysis. Our findings are consistent with this hypothesis, which would explain the lack of activity against the TEM-1 enzyme of the cyclic analogues **3c** and **7c**, as well as the asparenomycin-like compounds **9a** and **9d**. As already mentioned, in **3c** and **7c** the side chain ketone would not be in the correct position to form the imino complex with the enzyme, whereas, in compound **9a** and **9d**, the α,β-unsaturated keto group is missing. However, a negative steric interaction for the cyclopentanone analogues **3c** and **7c** within the TEM-1 active site cannot be excluded.

Finally, compound **3a** showed a good synergic action in combination with amoxicillin against a PC1-producing (β-lactamase Class A) *S. aureus* strain. Against this strain, the MIC of amoxicillin, when used in combination with compound **3a** in a 1:1 ratio, was reduced from 256 to 8 μg ml^{-1}. This effect was probably due to the lack of the outer membrane in Gram-positive bacteria, which facilitates β-lactam penetration and allows the compound to exert its inhibitory activity.

4. Conclusions

Some of the new penicillanic acid derivatives reported in this study inhibit both the TEM-1 and P99 β-lactamase enzymes and, under similar experimental conditions, are more chemically stable than other compounds reported in the literature belonging to the same series. The compounds synthesised have a broader spectrum of activity as β-lactamase inhibitors than does clavulanic acid, in that they are active against both Class A and C enzymes. Seemingly, the compounds do not penetrate the outer membrane of Gram-negative bacteria well and further investigation will be required to address this issue.

5. Experimental

5.1. Chemical materials and methods

Melting points were determined on a Büchi SMP-510 capillary melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker AC 200 or a Bruker AMX 600 spectrometer; chemical shifts are reported in ppm and given in δ units; carboxilic acid protons chemical shifts are not reported because they are too broad to be seen or they exchance with the deuterated solvent used. EI MS spectra (70 eV) were taken on a Fison Trio 1000 mass spectrometer. ESI mass spectra were taken on a Thermo Quest LQC DUO ESI ion trap mass spectrometer. Infrared spectra were obtained on a Bruker FT-48 spectrometer; absorbance values are reported in ν (cm^{-1}). Ultraviolet (UV) spectra were recorded on a Beckman DU 640 spectrophotometer. Elemental analyses were performed on a Carlo Erba analyzer and the values obtained are within $\pm 0.4\%$ of the theoretical values. Flash chromatography was performed on silica gel 60 (230–400 mesh Merck). TLC was performed on Merck precoated $60F_{254}$ plates. 2-(triphenylphosphoranylidene)-propionaldehyde was purchased from Aldrich. 3-(triphenylphosphoranylidene)-butan-2-one, 2-(triphenylphosphoranylidene)-acetamide and 2-(triphenylphosphoranylidene)-cyclopentanone were prepared from their corresponding phosphonium salt as previously reported [13–15]. Pig liver esterase was purchased from Sigma.

5.2. General method for the Wittig reaction: 3,3-dimethyl-6-(1-methyl-2-oxo-propylidene)-7-oxo-4-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid 2,2-dimethyl-propionyloxymethyl ester (**2a**)

Crude pivaloyloxy 6-oxopenicillanate **1** (420 mg, 1.28 mmol) was dissolved in 5 ml of CH_2Cl_2 and the resulting solution stirred at 0 °C. 425 mg of 3-triphenylphosphanylidene-butan-2-one (1.28 mmol) was added to the solution and the reaction was stirred at 0 °C for 1 h. The solution was concentrated using a rotavapor apparatus and the crude product was purified by flash-chromatography (eluent EtOAc/cyclohexane 1:1) to obtain 380 mg of pure product as an oily compound (overall yield 66%) which solidified upon standing as an amorphous solid. MS (EI): m/z 383, 195; IR ($CDCl_3$) 1766, 1692 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 5.95 (m, 1H, $J = 0.7$ Hz, H-5), 5.88 (d, 1H, $J = 5.4$ Hz), 5.80 (d, 1H, $J = 5.4$ Hz), 4.57 (s, 1H, H-2), 2.34 (s, 3H), 2.26 (d, 3H, $J = 0.7$), 1.60 (s, 3H), 1.51 (s, 3H), 1.23 (s, 9H).

5.3. General method for the enzymatic hydrolysis: 3,3-dimethyl-6-(1-methyl-2-oxo-propylidene)-7-oxo-4-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid (**3a**)

8.4 μl of pig liver esterase (59 U, 10%) were added to a solution of 140 mg of **2a** (0.36 mmol) in 2 ml of acetone and 18 ml of 0.1 M phosphate buffer (pH 8). The mixture was left under stirring at 30 °C and pH was monitored and when necessary adjusted to 8 with a few drops of 1 N NaOH.

After 20 h the mixture was filtered to remove unreacted starting material and the filtrate was washed with EtOAc. The aqueous phase was acidified with 2% HCl and extracted three times with EtOAc. The organic layer was dried over Na_2SO_4 and concentrated in vacuo to afford 70 mg of crude product containing pivalic

Table 2
In vitro activity of amoxicillin in combination with compounds **3a–c**, **6a**, **7a–c**, **9a,d**, **11d**, **13d** (amoxicillin/compound, 2:1 and 1:1)

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] TEM1+[d]	*E. cloacae* 1051E P99+ [e]	*E. cloacae* 1321 E	*S. aureus* ATCC 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
3a	>256	>256	64	64	>256	>256	32	32
Amox.+**3a** 2:1	256	16	≤2	64	>256	4	16	8
Amox.+**3a** 1:1	256	8	≤2	64	>256	4	8	8
7a	128	256	16	32	>256	>256	128	256
Amox.+**7a** 2:1	128	4	≤2	64	>256	4	64	128
Amox.+**7a** 1:1	128	4	≤2	32	256	4	64	128

[a] β-Lactamase-producing strain.
[b] Wild type.
[c] Permeable strain.
[d] TEM-1-producing strain.
[e] P99-producing strain.
[f] PC1-producing strain.
MIC values are expressed as μg ml^{-1}.

acid as impurity. Crystallization from CH_2Cl_2/cyclohexane gave 36 mg of **3a** (yield 37%) as a white solid: melting point (m.p.): 150–153 °C (dec) 185 (melt); MS (EI) *m/z* 269, 181; IR (KBr) 3114, 1771, 1725, 1656 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.9 (s, 1H, H-5), 4,58 (s, 1H, H-2), 2.35 (s, 3H), 2.28 (s, 3H), 1.64 (s, 3H), 1.59 (s, 3H); *Anal.* ($C_{12}H_{15}NO_4S$) C, H, N.

*5.4. General method for the oxidation reaction: 3,3-dimethyl-6-(1-methyl-2-oxo-propylidene)-4,4,7-trioxo-4λ⁶-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid 2,2-dimethyl-propionyloxymethyl ester (**4a**)*

A solution of 80% *m*-ClPBA (298 mg, 1.45 mmol) in CH_2Cl_2 (7 ml) was added dropwise to a solution of **2a** (224 mg, 0.58 mmol) in CH_2Cl_2 (7 ml) under stirring at 0 °C. After the addition was completed, the ice bath was removed and the solution left under stirring at room temperature (r.t.) overnight. The solution was then diluted with CH_2Cl_2 and washed three times with K_2HPO_4 0.25 M (pH 8). The organic layer was treated with Na_2SO_4 and concentrated in vacuo. The residue was purified by flash-chromatography (eluent EtOAc/cyclohex. 1:1) to yield 188 mg of **4a**. (78%): m.p. 175–177 °C (white solid from EtOAc/cyclohex.); MS (EI) *m/z* 415; IR (KBr) 1774, 1751, 1666 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.97 (d, 1H, $J = 5.5$ Hz), 5.74 (d, 1H, $J = 5.5$ Hz), 5.40 (m, 1H, $J = 0.9$ Hz, H-5), 4.45 (s, 1H, H-2), 2.42 (s, 3H), 2.38 (d, 3H, $J = 0.9$), 1.59 (s, 3H), 1.46 (s, 3H), 1.24 (s, 9H).

*5.5. 3,3-Dimethyl-6-(1-methyl-2-oxo-propylidene)-4,4,7-trioxo-4λ⁶-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid (**7a**)*

As for **3a**.

Yield 20%; m.p. 182–185 °C C (white solid from CH_2Cl_2); MS (EI) *m/z* 283 (301–18), 193 (301-SO_2–CO_2); IR (nujol) 3464, 1767, 1733 cm^{-1}; ^1H NMR ($CDCl_3 + CD_3OD$) δ 5.31 (m, 1H, $J = 0.8$ Hz H-5), 4,25 (s, 1H, H-2), 2.28 (s, 3H), 2.23 (d, 3H, $J = 0.8$), 1.49 (s, 3H), 1.36 (s, 3H); *Anal.* ($C_{12}H_{15}NO_6S$) C, H, N.

*5.6. 3,3-Dimethyl-6-(1-methyl-2-oxo-propylidene)-4,7-dioxo-4λ⁴-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid 2,2-dimethyl-propionyloxymethyl ester (**5a**)*

A solution of 80% *m*-ClPBA (65 mg, 0.29 mmol) in CH_2Cl_2 (2 ml) was added dropwise to a solution of **2a** (100 mg, 0.26 mmol) in CH_2Cl_2 (2 ml) under stirring at 0 °C. After the addition was complete, the ice bath was removed and the solution left under stirring at r.t. for 2 h. The solution was then diluted with CH_2Cl_2 and washed three times with K_2HPO_4 0.25 M (pH 8). The organic layer was treated with Na_2SO_4 and concentrated in vacuo. The residue was purified by flash-chromatography (eluent EtOAc/cyclohex. 1:1) to yield 70 mg of **5a**. (67%): m.p. 147–149 °C (white solid from EtOAc/cyclohex.); MS (EI) *m/z* 400 (399 + 1), 285 (400-POM); IR (nujol) 1766, 1751 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.99 (d, 1H, $J = 5.5$ Hz), 5.73 (d, 1H, $J = 5.5$ Hz), 5.62 (m, 1H, $J = 0.8$ Hz, H-5), 4.66 (s, 1H, H-2), 2.40 (s, 3H), 2.38 (d, 3H, $J = 0.8$), 1.66 (s, 3H), 1.28 (s, 3H), 1.23 (s, 9H).

*5.7. 3,3-Dimethyl-6-(1-methyl-2-oxo-propylidene)-4,7-dioxo-4λ⁴-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid (**6a**)*

As for **3a**.

Yield 44%; m.p. 199–201 °C (white solid from EtOAc/cyclohex.); MS (EI) *m/z* 193 (285-SO–CO_2), 123; IR (nujol) 1766, 1740 cm^{-1}; ^1H NMR ($CDCl_3 + CD_3OD$) δ 5.60 (m, 1H, $J = 0.8$ Hz H-5), 4.57 (s, 1H, H-2), 2.36 (s, 3H), 2.33 (d, 3H, $J = 0.8$), 1.66 (s, 3H), 1.28 (s, 3H). *Anal.* ($C_{12}H_{15}NO_5S$) C, H, N.

*5.8. 6-Carbamoylmethylene-3,3-dimethyl-7-oxo-4-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid 2,2-dimethyl-propionyloxymethyl ester (**2b**)*

As for **2a**.

Purification by flash-chromatography (eluent EtOAc/cyclohexane 8:2); overall yield 26%; MS (EI) *m/z* 370, 256; IR (nujol) 3416, 3342, 3300, 3150, 1772 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.35 (d, 1H, $J = 1.1$ Hz), 6.11 (bs, 2H), 6.07(d, 1H, $J = 1.1$ Hz), 5.87 (d, 1H, $J = 5.5$ Hz), 5.79 (d, 1H, $J = 5.5$ Hz), 4.55 (s, 1H, H-2), 1.58 (s, 3H), 1.50 (s, 3H), 1.22 (s, 9H).

*5.9. 6-Carbamoylmethylene-3,3-dimethyl-7-oxo-4-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid (**3b**)*

As for **3a**.

Yield 43%; m.p. 154–157 °C (white solid from EtOAc/cyclohex.); MS (EI) *m/z* 256, 154; IR (nujol) 3417, 3328, 3266, 3218, 1772 cm^{-1}; ^1H NMR ($CDCl_3 + CD_3OD$) δ 6.32 (d, 1H, $J = 1.1$ Hz), 6.05 (d, 1H, $J = 1.1$ Hz), 4.47 (s, 1H, H-2), 1.58 (s, 3H), 1.53 (s, 3H); *Anal.* ($C_{10}H_{12}N_2O_4S$) C, H, N.

*5.10. 6-Carbamoylmethylene-3,3-dimethyl-4,4,7-trioxo-4λ⁶-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid 2,2-dimethyl-propionyloxymethyl ester (**4b**)*

As for **4a**.

Purification by flash-chromatography (eluent EtOAc/cyclohexane 8:2); yield 50%; m.p. 172–175 °C (white solid from EtOAc/cyclohex.); MS (EI) *m/z* 338 (402-SO_2); IR (nujol) 3024, 1792, 1745, 1725, 1679 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.67 (d, 1H, $J = 1.3$ Hz), 5.97 (d, 1H,

$J = 5.4$ Hz), 5.96 (bs, 2H), 5.75 (d, 1H, $J = 5.4$ Hz), 5.64 (d, 1H, $J = 1.3$ Hz, H-5), 4.49 (s, 1H, H-2), 1.59 (s, 3H), 1.47 (s, 3H), 1.24 (s, 9H).

*5.11. General method for the BBTO catalyzed hydrolysis: 6-Carbamoylmethylene-3,3-dimethyl-4,4,7-trioxo-4λ⁶-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid (**7b**)*

0.28 ml of bistributyltinoxide (0.56 mmol) were added to a solution of **4b** (111 mg, 0.28 mmol) in 7 ml of Et_2O, and the mixture was left under stirring at r.t. for 6 h. The mixture was then diluted with EtOAc and extracted two times with 20 ml of 0.25 M K_2HPO_4. The aqueous phase was acidified with 2% HCl and extracted three times with EtOAc. The organic phase was treated with Na_2SO_4 and concentrated in vacuo to afford 45 mg of crude product, which was triturated with Et_2O and crystallized from EtOAc/cyclohex to give 18 mg of **7b** (yield 22%) as a yellowish solid: m.p. 115 °C (dec) 185–195 °C (melt); MS (EI) m/z 288; IR (nujol) 3417, 3348, 3266, 3198, 1791 cm^{-1}; ^1H NMR ($CDCl_3$ + CD_3OD) δ 6.67 (d, 1H, $J = 1.4$ Hz), 5.59 (d, 1H, $J = 1.4$ Hz, H-5), 4.37 (s, 1H, H-2), 1.58 (s, 3H), 1.46 (s, 3H); *Anal.* ($C_{10}H_{12}N_2O_6S$) C, H, N.

*5.12. 3,3-Dimethyl-7-oxo-6-(-2-oxo-cyclopentylidene)-4-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid 2,2-dimethyl-propionyloxymethyl ester (**2c**)*

As for **2a**.

Purification by flash-chromatography (eluent EtOAc/cyclohexane 3:7); overall yield 46%; MS (EI) m/z 395; IR ($CDCl_3$) 1766, 1686 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.00 (s, 1H, H-5), 5.87 (d, 1H, $J = 5.5$ Hz), 5.80 (d, 1H, $J = 5.5$ Hz), 4.55 (s, 1H, H-2), 2.95 (m, 2H), 2.43 (m, 2H), 2.09 (m, 2H) 1.58 (s, 3H), 1.50 (s, 3H), 1.23 (s, 9H).

*5.13. 3,3-Dimethyl-7-oxo-6-(-2-oxo-cyclopentylidene)-4-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid (**3c**)*

As for **7b**.

Purification by flash-chromatography (eluent EtOAc); yield 32%; m.p.: 160 °C (dec.) (triturated with Et_2O); MS (EI) m/z 237 (281-CO_2); IR ($CDCl_3$) 1763, 1680 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.00 (s, 1H, H-5), 4.54 (s, 1H, H-2), 2.95 (m, 2H), 2.44 (m, 2H), 2.09 (m, 2H) 1.62 (s, 3H), 1.58 (s, 3H); *Anal.* ($C_{13}H_{15}NO_4S$) C, H, N.

*5.14. 3,3-Dimethyl-4,4,7-trioxo-6-(-2-oxo-cyclopentylidene)-4λ⁶-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid 2,2-dimethyl-propionyloxymethyl ester (**4c**)*

As for **4a**.

Purification by flash-chromatography (eluent EtOAc/cyclohexane 1:1); yield 54%; m.p.: 130–132 °C (Crystallized from Et_2O/light petroleum); MS (EI) m/z 427, 363 (427-SO_2); IR ($CDCl_3$) 1786, 1755, 1698 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.97 (d, 1H, $J = 5.5$ Hz), 5.75 (d, 1H, $J = 5.5$ Hz), 5.54 (s, 1H, H-5), 4.47 (s, 1H, H-2), 3.01 (m, 2H), 2.52 (m, 2H), 2.14 (m, 2H) 1.58 (s, 3H), 1.47 (s, 3H), 1.24 (s, 9H).

*5.15. 3,3-Dimethyl-4,4,7-trioxo-6-(-2-oxo-cyclopentylidene)-4λ⁶-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid (**7c**)*

As for **7b**.

Purification by flash-chromatography (eluent EtOAc); yield 25%; MS MS (ESI) m/z 312, 268 (–CO_2), 204 (–SO_2); IR (nujol) 3011, 1785, 1738, 1692 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.56 (s, 1H, H-5), 4.48 (s, 1H, H-2), 3.04 (m, 2H), 2.52 (m, 2H), 2.17 (m, 2H) 1.63 (s, 3H), 1.54 (s, 3H); *Anal.* ($C_{13}H_{15}NO_6S$) C, H, N.

*5.16. 3,3-Dimethyl-6-(1-methyl-2-oxo-ethylidene)-7-oxo-4-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid 2,2-dimethyl-propionyloxymethyl ester (**2d**)*

As for **2a**.

Purification by flash-chromatography (eluent EtOAc/cyclohexane 3:7); yield 58%; MS (EI) m/z 369; IR ($CDCl_3$) 1766, 1694 cm^{-1}; ^1H NMR ($CDCl_3$) δ 9.72 (s, 1H), 6.07 (m, 1H, $J = 0.7$ Hz, H-5), 5.88 (d, 1H, $J = 5.5$ Hz), 5.81 (d, 1H, $J = 5.5$ Hz), 4.61 (s, 1H, H-2), 2.12 (d, 3H, $J = 0.7$), 1.61 (s, 3H), 1.53 (s, 3H), 1.23 (s, 9H).

*5.17. General method for the reduction with $NaBH_4$: 6-(2-Hydroxy-1-methyl-ethylidene)-3,3-dimethyl-7-oxo-4-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid 2,2-dimethyl-propionyloxymethyl ester (**8d**)*

Compound **2d** (200 mg; 0.54 mmol) was dissolved in 3.2 ml of THF and 1.6 ml of MeOH after which 49 mg of $NaBH_4$ (1.3 mmol) were added. The solution was left under stirring for 30 min, then acidified with a saturated solution of NH_4Cl and extracted three times with EtOAc. The organic phases were collected, dried over Na_2SO_4 and concentrated under vacuum. The crude product was purified by flash-chromatography (eluent EtOAc/cyclohexane 1:1) to obtain 180 mg of **8d** as an oily product (yield 58%); MS (EI) m/z 371; IR ($CHCl_3$) 3498, 1752 cm^{-1}; ^1H NMR ($CDCL_3$) δ 5.91 (s, 1H, H-5), 5.88 (d, 1H, $J = 5.4$ Hz), 5.78 (d, 1H, $J = 5.4$ Hz), 4.50 (s, 1H, H-2), 4.30 (bs, 2H), 2.01 (s, 3H), 1.59 (s, 3H), 1.48 (s, 3H), 1.22 (s, 9H).

*5.18. 6-(2-Hydroxy-1-methyl-ethylidene)-3,3-dimethyl-7-oxo-4-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid (**9d**)*

As for **3a**.

Purification by flash-chromatography (eluent EtOAc); yield 64%; oily product; MS (EI) *m/z* 257; IR (KBr) 3411, 1749, 1703 cm^{-1}; ^1H NMR ($CDCL_3$) δ 5.92 (s, 1H, H-5), 4.48 (s, 1H, H-2), 4.31 (bs, 2H), 2,28 (bs, 1H, OH) 2.01 (s, 3H), 1.65 (s, 3H), 1.56 (s, 3H); *Anal.* ($C_{11}H_{15}NO_4S$) C, H, N.

5.19. 6-(2-Hydroxy-1-methyl-ethylidene)-3,3-dimethyl-4,4,7-trioxo-4λ⁶-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid 2,2-dimethyl-propionyloxymethyl ester (10d)

As for **4a**.

Purification by flash-chromatography (eluent EtOAc/cyclohexane 1:1); yield 82%; m.p.: 134 °C (crystallized from Et_2O/light petroleum); MS (EI) *m/z* 339 (403-SO_2), 180 (339-CO_2POM); IR (KBr) 3506, 1775, 1759, 1718 cm^{-1}; ^1H NMR ($CDCL_3$) δ 5.96 (d, 1H, $J = 5.5$ Hz), 5.75 (d, 1H, $J = 5.5$ Hz), 5.40 (s, 1H, H-5), 4.40 (s, 1H, H-2), 4.41 (bd, 1H, $J = 18.2$ Hz), 4.29 (bd, 1H, $J = 18.2$ Hz), 2.29 (bs, 1H, OH), 2.07 (s, 3H), 1.57 (s, 3H), 1.43 (s, 3H), 1.23 (s, 9H).

*5.20. 6-(2-Hydroxy-1-methyl-ethylidene)-3,3-dimethyl-4,4,7-trioxo-4λ⁶-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid (**11d**)*

As for **3a**

Yield 50%; m.p.: 143 °C (dec.) (crystallized from EtOAc/Et_2O); MS (EI) *m/z* 180 (289-SO_2–CO_2H); IR (KBr) 3525, 1775, 1721 cm^{-1}; ^1H NMR ($CDCl_3$ + MeOD) δ 5.40 (s, 1H, H-5), 4.29 (s, 1H, H-2), 4.31 (d, 2H, $J = 18.2$), 4.20 (d, 2H, $J = 18.2$), 2.03 (s, 3H), 1.58 (s, 3H), 1.44 (s, 3H); *Anal.* ($C_{11}H_{15}NO_6S$) C, H, N.

*5.21. 6-(2-Hydroxy-1-methyl-propylidene)-3,3-dimethyl-7-oxo-4-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid 2,2-dimethyl-propionyloxymethyl ester (**8a**)*

As for **8d**.

Purification by flash-chromatography (eluent EtOAc/cyclohexane 1:1); yield 76% (diastereoisomeric mixture 1:1 ratio from NMR integration); oily product; MS (EI) *m/z* 385; IR ($CDCl_3$) 3534, 1764, 1751, 1712 cm^{-1}; ^1H NMR ($CDCL_3$) δ 5.88 (d, 1H, $J = 5.5$ Hz), 5.88 (s, 1H, H-5), 5.78 (d, 1H, $J = 5.5$ Hz), 4.50 (s, 1H, H-2), 4.40 (bm, 1H, $J = 6.8$), 2.03 (s, 3H), 1.59 (s, 3H), 1.48 (s, 3H), 1.38 (2d, 1:1 ratio, 3H, $J = 6.8$), 1.22 (s, 9H).

*5.22. 6-(2-Hydroxy-1-methyl-propylidene)-3,3-dimethyl-7-oxo-4-thia-1-aza-bicyclo[3.2.0]-heptane-2-carboxylic acid (**9a**)*

As for **3a**.

Purification by flash-chromatography (eluent EtOAc); yield 57% diastereoisomeric mixture 1:1 ratio from NMR integration); oily product; MS (EI) *m/z* 271, 253 (271-H_2O); IR ($CDCl_3$) 3461, 1750 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.89 (s, 1H, H-5), 4.47 (2s, 1:1 ratio, 1H, H-2), 4.40 (bm, 1H, $J = 6.6$), 2.50 (bs, OH) 2.04 (s, 3H), 1.65 (s, 3H), 1.56 (s, 3H), 1.38 e 1.39 (2d, 1:1 ratio, 3H, $J = 6.6$); *Anal.* ($C_{12}H_{17}NO_4S$) C, H, N.

*5.23. 6-(2-Methoxyimino-1-methyl-ethylidene)-3,3-dimethyl-7-oxo-4-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid 2,2-dimethyl-propionyloxymethyl ester (**12d**)*

Methoxylamine hydrochloride (101 mg, 1.2 mmol) and pyridine (0.12 ml) were added to a solution of **2d** (75 mg, 0.2 mmol) in CH_2Cl_2 (2.5 ml) at r.t. The reaction was left under stirring for 30 min, diluted with water and extracted with CH_2Cl_2 (3x). The organic phase was treated with Na_2SO_4, filtered and concentrated under vacuum. The crude product was purified by flash-chromatography (cyclohexane/EtOAc 9:1) to obtain 64 mg of **12d** as an oily product (yield 80%). MS (EI) *m/z* 398, 367 (398-OCH_3); IR ($CDCl_3$) 1762, 1680 cm^{-1}; ^1H NMR ($CDCL_3$) δ 7.62 (s, 1H), 5.87 (d, 1H, $J = 5.4$ Hz), 5.80 (d, 1H, $J = 5.4$ Hz), 5.77 (s, 1H, H-5), 4.53 (s, 1H, H-2), 3.98 (s, 3H), 2.20 (s, 3H), 1.58 (s, 3H), 1.48 (s, 3H), 1.22 (s, 9H).

*5.24. 6-(2-Methoxyimino-1-methyl-ethylidene)-3,3-dimethyl-7-oxo-4-thia-1-aza-bicyclo[3.2.0]-heptane-2-carboxylic acid (**13d**)*

As for **7b**.

Purification by flash-chromatography (eluent EtOAc); yield 50%; oily compound; MS (EI) *m/z* 284, 253; IR ($CHCl_3$) 3450, 1755 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.62 (s, 1H), 5.77 (s, 1H, H-5), 4.52 (s, 1H, H-2), 3.99 (s, 3H), 2.20 (s, 3H), 1.62 (s, 3H), 1.57 (s, 3H); *Anal.* ($C_{12}H_{16}N_2O_4S$) C, H, N.

5.25. β-lactamase inhibition experiments

The inhibitory activity of compounds **3**(**a**–**c**), **6a**, **7**(**a**–**c**), **9**(**a**,**d**), **11d** and **13d** on TEM-1 (Class A) and P-99 (Class C) ß-lactamases was assessed in competition experiments using nitrocefin as the reporter substrate. Nitrocefin was used at a final concentration of 180 μM for TEM-1 and of 250 μM for P99, respectively. 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) were added to the reaction mixture (100 μl final volume: 50 μl substrate + 50 μl compound) and nitrocefin hydrolysis was monitored spectrophotometrically at 482 nm, for 3 min, at 30 °C. Each compound was assayed at 14 different

concentrations, ranging from 0.015 to 1.25 mM. The rate of ß-lactamase hydrolysis in the presence of different compound concentrations was calculated. The IC_{50} is defined as the concentration of compound, in μg ml^{-1}, that reduced the rate of nitrocefin hydrolysis by 50% under the experimental conditions described above.

5.26. Bacterial strains and culture conditions

Eight reference strains were used in these studies. *E. coli* ATCC 35218, *E. coli* 1850E (wild type = W.T.), *E. coli* 1852E (permeable membrane = P.M.; TEM-1-negative strain = TEM-1^-), *E. coli* 1919E (permeable membrane = P.M.; TEM-1-producing strain = TEM-1^+), *E. cloacae* 1051E (P99-producing strain = P99 +), *E. cloacae* 1321E (P9 − negative strain = P99 −), *S. aureus ATCC 43387* 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 studies of in vitro pharmacodynamics, 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}.

5.27. Determination of the MIC

Compounds **3a** and **7a** (see Section 2) 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 were also tested alone and in combination as reference agents. MICs were determined using the NCCLS standard broth microdilution method [26]. The final compound concentration ranged from 0.25 to 128 μg ml^{-1}. The MIC was defined as the lowest compound concentration which inhibited visible bacterial growth after 24 h of incubation at 37 °C.

5.28. Stability determinations of *3a* and *7a* in MHB (Mueller Hinton Broth)

The stability of **3a** and **7a** in culture broth was checked by differential UV spectrometry. 0.3 ml of aqueous solution of **3a** and **7a,** respectively (128 μg ml^{-1} of final compound concentration), were incubated with an equal volume of MHB at 37 °C. 50 μl from each sample were withdrawn at different time points and diluted with 1350 μl of distilled water. Absorbance at 250 nm (A_{250}) of the obtained samples was measured. The blank solution was prepared by adding 50 μl of a 1:1 (v/v) MHB:water solution to 1350 μl of distilled water. No decrease in A_{250} was observed over a period of 18 h, demonstrating the strong stability of compounds **3a** and **7a** in culture medium. The two compounds were also treated with sodium hydroxide to observe, for comparison, the disappearance of the absorbance peak at 250 nm after decomposition.

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