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Preliminary communication

Synthesis and inhibitory activity towards human leukocyte elastase of new 7α-methoxy and 7α-chloro (2-acyloxymethyl) cephem derivatives

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Abstract – Some new cephem derivatives of types **4** and **5**, viewed as analogues of type **I** esters in which the atomic sequence of the C-2 ester group is formally inverted, were synthesised and tested in vitro for their inhibitory activity towards human leukocyte elastase and porcine pancreatic elastase. An examination of the inhibition data obtained for the new type **4** and **5** derivatives, while exhibiting a considerable reduction in their activity against porcine pancreatic elastase, indicated that these compounds still maintain an appreciable inhibitory activity against human leukocyte elastase. On this basis the new type of C-2 substitution appears to contribute to the research of new, potentially interesting, cephalosporinic human leukocyte elastase inhibitors. © 2001 Éditions scientifiques et médicales Elsevier SAS

elastase inhibitor / HLE inhibitor / cephalosporin derivative / C-2 hydroxymethyl cephem derivative

1. Introduction

Human leukocyte elastase (HLE) is a serine protease [1–11] whose hyperexpession may lead to the degradation of the connective tissue associated with certain pulmonary, vascular and neoplastic pathologies [12–16]. Therefore, compounds capable of inhibiting HLE may restore the balance between the free enzyme and endogenous antiproteinases, and thus may have considerable therapeutic potential.

In the past decade, HLE inhibitors obtained from suitably derivatized β-lactam compounds such as cephalosporins [17], penicillins [18], monocyclic β-lactams [19, 20] and unconventional β-lactams [21, 22], have been proposed for the therapeutic treatment of patients affected by chronic bronchitis and cystic fibrosis. Among these β-lactam compounds studied, cephalosporins and synthetic β-lactams have been the most promising.

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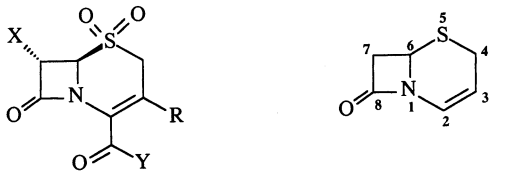
Cephalosporins of type **I** (*figure 1*) were the first β-lactam derivatives studied for their HLE inhibitory activity, and their inhibition mechanism has been analysed using chemical [23, 24], crystallographic [25] and kinetic [26] methods.

Molecular modelling studies on a cephem sulphone of type **I** have indicated the importance of the C-2 substituent, which appears to be involved in the binding process with the S1′-S2′ sites of HLE [27]. The character of this enzyme, an endopeptidase, offers the possibility of changing the chemical nature of the C-2 substituent on the cephem nucleus, where the C-2 carboxyl may be substituted with other groups without affecting the HLE binding process [21, 28]. Therefore, attention has focused on compounds obtained by modifying the substituents in the C-2, C-3, C-4 and C-7 positions of the cephem nucleus, with the aim of improving their inhibitory activity and enzyme specificity. These efforts have produced some new β-lactam HLE inhibitors such as the cephem compounds **II** [28], and the 2-spirocyclopropyl cephems

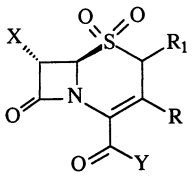
III [22], which, in spite of their structural differences, do not appear to differ substantially from **I**, as regards the reactivity of the β-lactam carbonyl involved in the initial step of the sequence of the enzymatic reactions, which consisted in the attack on the same carbonyl of the serine hydroxy group present in the catalytic site of this enzyme. However, for these compounds, the high reactivity of the β-lactam is likely to have a negative influence on their stability.

A major differentiation in the reactivity of the β-lactam carbonyl of new cephem inhibitors from that of derivatives **I**–**III** may be obtained by introducing into the C-2 position of the cephem nucleus substituents able to exert electronic effects different from those exerted by the carboxylic or carbonylic groups present in the type **I**–**III** structures. Normally in the cephem derivatives, the reactivity of the β-lactam nucleus may be considered to be linked to its low degree of amidic resonance shown in the **d** form (*figure 2*). Substituents on C-2 possessing high electron-withdrawing characteristics due to their conjugating effects, such as the carboxylic or carbonylic groups, reduce this amidic resonance by increasing the possibility of the enolic resonance form **b**, thus consequently improving the reactivity of the β-lactam



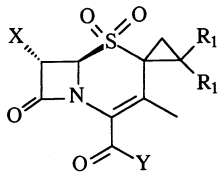
I

X = MeO, Cl
R = Me, CH_2OAc or CH_2SHet
Y = OAlkyl, OAryl, NHAlkyl, NHAryl



II

X = MeO, Cl
R = Me, CH_2OAc or CH_2SHet
Y = OAlkyl, Alkyl, Aryl
R_1= SHet



III

X = MeO, Cl
Y = OAlkyl, OAryl, NHAlkyl NHAryl, Alkyl, Aryl
R_1= Aryl, Alkyl

Figure 1.

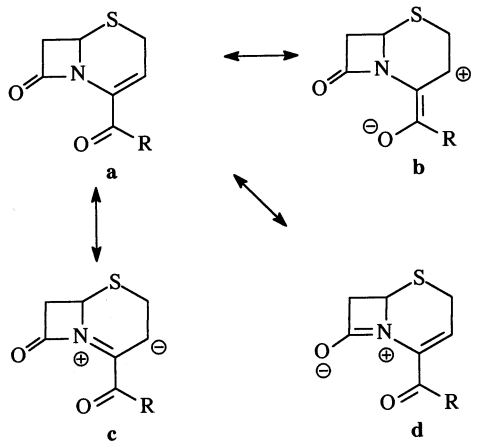


Figure 2.

ring to the nucleophile attack of the enzyme [29]. On the contrary, the presence of substituents devoid of any conjugating electron-withdrawing effect in C-2 does not allow the contribution of the resonance form **b**; as a consequence, the β-lactam maintains a certain degree of amidic resonance, resulting in a lower level of electrophilic reactivity of the β-lactam itself, and a higher level of nucleophilicity of the C-2 C-3 double bond of the dihydrothiazine ring.

Our previous results showed that the substitution of the C-2 carboxyl group of natural cephalosporins **1** (*figure 3*) with the hydroxymethyl group of compound **2** or the arylacyloxymethyl one of compounds **3** [30, 31] increases the nucleophile reactivity of the C-2 C-3 double bond of the cephalosporin system, thus indicating a smaller contribution of form **b** to the resonance and therefore a greater weight of the less reactive form **d**.

On the basis of these results, we decided to transfer the same type of substituent to the C-2 carbon atom of type **I** and **II** inhibitors, in order to evaluate the effects of this structural modification, potentially capable modulating the β-lactam system reactivity, on the anti-HLE properties of the cephalosporin derivatives.

This work describes the synthesis and the in vitro inhibitory activity towards HLE and porcine pancreatic elastase (PPE) of a series of new cephem esters of types **4** (**4a**–**c**) and **5** (**5a**,**b**), in which the esterified C-2 carboxylic group of the cephalosporins of type **I** is replaced by a hydroxymethyl group esterified by aromatic or aliphatic acyls. The esters were chosen so as to have a C-2 side chain sterically similar to that of

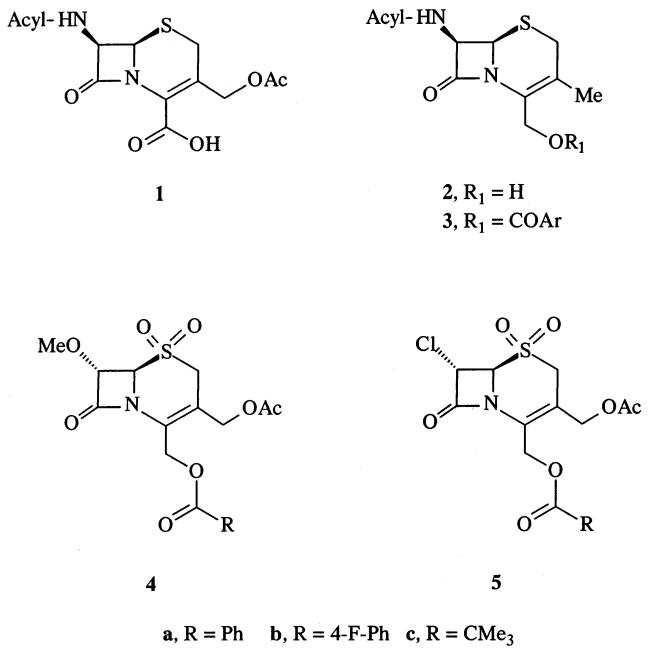


Figure 3.

the more interesting cephalosporin esters of type **I** (the benzyl and the *t*-butyl one [27]). Compounds **4** and **5** may therefore be viewed as analogues of type **I** drugs in which the atomic sequence of the ester group is formally inverted. The substituents on the C-7 carbon atom of **4** (the methoxy group) or **5** (the chlorine atom) were selected because they are present on the same carbon of the most interesting compounds of type **I** [32].

2. Chemistry

The cephem esters **4a**–**c** and **5b**–**c** were synthesised as shown in *figure 4*. The starting cephaloram **6** was transformed into the acid chloride **7** by treatment with oxalyl chloride $(ClCO)_2$ and catalytic dimethylformamide (DMF) in dichloromethane (DCM), and then, without the isolation of **7**, into the alcohol **8** by reduction of **7** with LiAl(*t*-BuO)$_3$H in THF at 0 °C. Reaction of alcohol **8** with the appropriate acid in anhydrous DCM, using 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDCI) as the condensing agent gave the esters **9a**–**c** which were converted into the *N*-7-*t*-Boc-imides **10a**–**c** by treatment with di-*tert*-butyl dicarbonate [*t*-$(BOC)_2O$] in anhydrous DCM, in the presence of triethylamine (TEA) as the base and 2,6-dimethylaminopyridine (DMAP) as the catalyst. Imides **10a**–**c**, without isolation, were treated with *N*,*N*-diethylethylendiamine in anhydrous DCM to give the crude *N*-7-*t*-(BOC)-amides **11a**–**c**, which without any purification were transformed into the desired 7β-amino-3-cephem esters **12a**–**c** by reaction with trifluoroacetic acid (TFA) in anhydrous DCM at room temperature. **12a**–**c** were purified by flash chromatography and then transformed into the 7-diazoketons **13a**–**c** (*figure 5*) by treatment with an aqueous sodium nitrite solution in the presence of 2 N sulphuric acid at 0 °C. For the preparation of the 7α-methoxy cephem esters **14a**–**c**, the appropriate 7-diazoketon **13a**–**c** was treated with rhodium acetate dimer (Rh_2OAc_4) in methanol at 0 °C. For the preparation of the 7α-chlorine derivatives **15a**,**b**, a crude mixture of **13a**,**b** was treated with concentrated hydrogen chloride in EtOH at room temperature for 20 s. The crude 7α-methoxy and 7α-chlorine derivatives, **14a**–**c** and **15a**,**b**, were purified by flash chromatography on silica gel and then oxidised to the sulfones **4a**–**c** and **5a**,**b** with *m*-chloroperoxybenzoic acid (MCPBA) in anhydrous DCM. Pure **4a**–**c** and **5a**,**b** were obtained by flash chromatography on silica gel of the crude products.

3. Results and discussion

The new cephem esters **4a**–**c** and **5a**,**b** were evaluated for their ability to inhibit HLE- and PPE-catalysed hydrolysis of the substrate *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide. *Table I* shows the results obtained with these enzymes expressed as concentration-independent second-order rate constant k_{inact}/K_I [20]. *Table I* also shows the results obtained in the same tests with the cephem sulphone **16** [17], used as a reference type **I** drug. All the tested cephem esters **4a**–**c** and **5a**,**b** were found to possess a moderate inhibitory activity against HLE, with k_{inact}/K_I values ranging from 1 540 of **4a** to 7 600 $M^{-1}s^{-1}$ of **5b** which proved to be the most active in the series. On the contrary, in the case of PPE, the compounds tested showed a poor inhibitory activity, with k_{inact}/K_I values ranging from 240 of **4a** to 1 800 $M^{-1}s^{-1}$ of **4c**. In the same tests, the reference drug **16** showed a k_{inact}/K_I value of 25 000 $M^{-1}s^{-1}$ against HLE and 131 000 $M^{-1}s^{-1}$ against PPE.

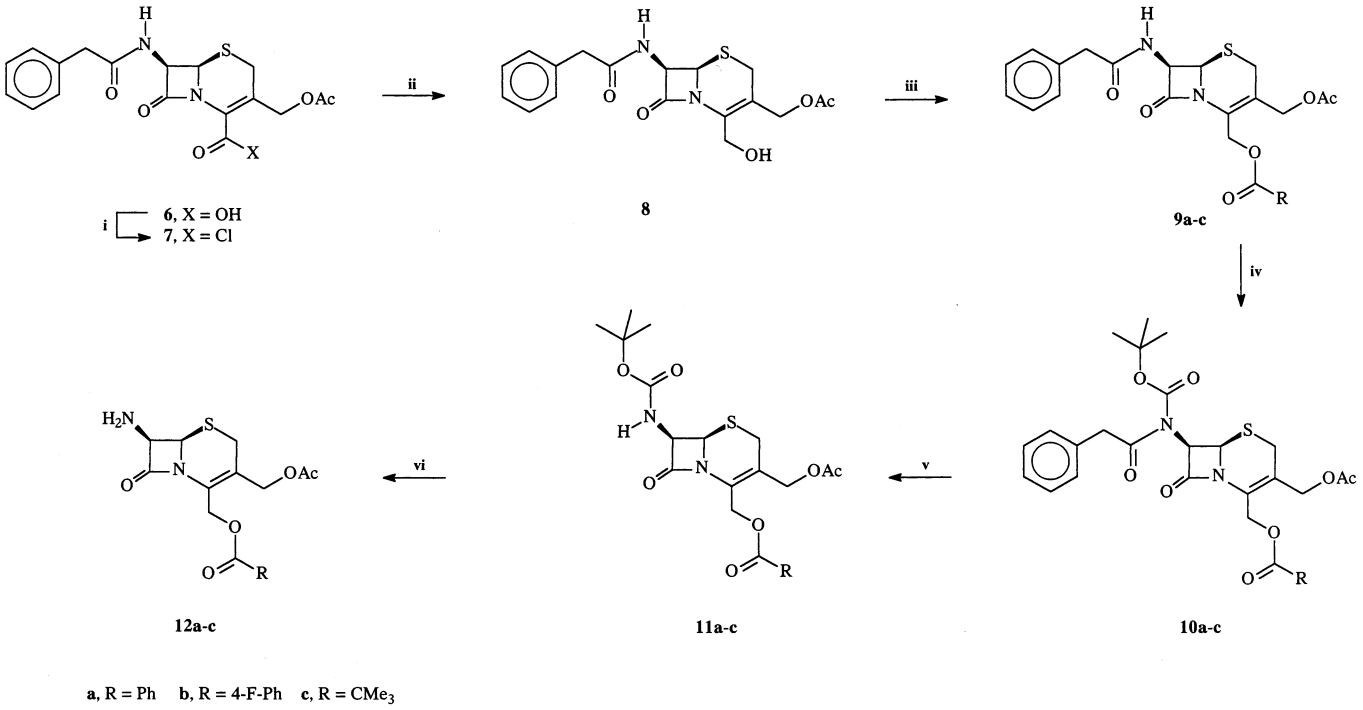


Figure 4. Conditions: **i**: $(ClCO)_2$, DCM, cat. DMF, 0 °C; **ii**: LiAl(*t*-BuO)$_3$H, THF, 0 °C; **iii**: RCO_2H, EDCL, cat. DMAP, DCM, −5 to 0 °C; **iv**: $(t\text{-BOC})_2O$, TEA, cat. DMAP, DCM, 0 °C; **v**: *N*,*N*-diethylethylenediamine, DCM, room temperature; **vi**: TFA, DCM, room temperature.

An examination of these results indicates that the newly synthesised cephem sulfones **4a**–**c** and **5a**,**b** maintain a still appreciable inhibitory activity against HLE, with the most active derivative in this series (the *p*-fluorobenzoate **5b**, substituted on the C-7 carbon with an α-chlorine atom) which is only 3 times less active than the reference drug **16**; on the contrary, the activity against PPE is considerably reduced, with the most active compound **4c** which is 73 times less active than **16**.

In conclusion, the results obtained with the new cephem derivatives of types **4** and **5** indicate that the substitution of the more electron-withdrawing carboxy group, linked to the C-2 double bond of type **I** drugs, with the less electron-withdrawing hydroxyl group esterified with aliphatic or aromatic acyls of **4** and **5** seems to reduce the ability of the new compounds to interact with PPE considerably. On the contrary, this type of substitution does not substantially hinder the inhibition process towards HLE if compared with the reference type **I** drug (**16**). Therefore, this new type of C-2 substitution, which does not have a too significant effect on the anti-HLE activity of the new compounds, appears to be able to contribute to the development of a new class of potentially interesting cephalosporinic HLE inhibitors.

4. Experimental protocols

4.1. Chemistry

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Infrared (IR) spectra for comparison of compounds were recorded on a Mattson 1000 FTIR spectrometer. Nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Varian CFT-20 (80 MHz) or on a Bruker AC-200 (200 MHz) in a ca. 2% solution of $CDCl_3$ for all compounds. The proton magnetic resonance assignments were established on the basis of the expected chemical shifts and the multiplicity of the signals. Analytical thin layer chromatography (TLC) was carried out on 0.25-mm layer silica gel plates containing a fluorescent indicator, and spots were de-

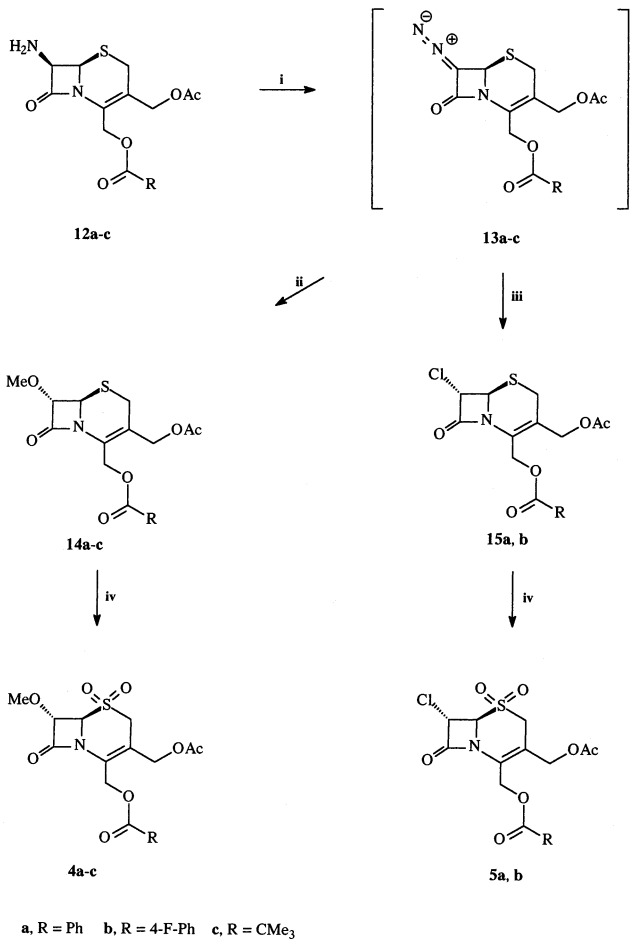


Figure 5. Conditions: **i**: $NaNO_2$, 2 N H_2SO_4, 0 °C; **ii**: Rh_2OAc_4, MeOH, 0 °C; **iii**: HCl, abs. EtOH, 0 °C; **iv**: MCPBA, DCM, 0 °C.

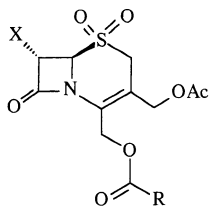
tected under UV light (254 nm). Flash chromatography or preparative medium pressure liquid chromatography (MPLC) were carried out through glass columns containing 40–63 μm silica gel (Macherey-Nagel Silica Gel 60) or reversed phase of octadecyl silica gel (C_{18} Polygosil 60-4063 Macherey-Nagel). The MPLCs were performed using a chromatographic apparatus consisting of a Buchi 681 pump, a Knauer differential refractometer detector, and a Philips PM 8220 pen recorder. Solvents and reagents were obtained from commercial vendors in the appropriate grade and were used without further purification unless otherwise indicated. Elemental analyses were carried out by our analytical laboratory and were consistent with theoretical values to within ±0.4%.

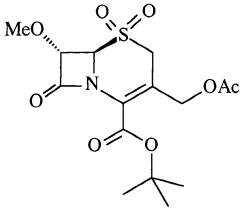
4.1.1. N-((6R)-3-acetoxymethyl-2-hydroxymethyl-8-oxo-(6rH)-5-thia-1-aza-bicyclo[4.2.0]oct-2-en-7t-yl)-2-phenyl-2-yl-acetamide **8**

A solution of acid **6** (13.0 mmol, 5.0 g), in anhydrous DCM (76 mL) and anhydrous DMF (0.19 mL), cooled at 0 °C, was treated dropwise with a solution of $(ClCO)_2$ (34.2 mmol, 4.3 g) in anhydrous DCM (6.7 mL). After stirring at 0 °C for 1 h, the reaction mixture was evaporated at 0–5 °C and the crude residue was washed with benzene to remove the excess of $(ClCO)_2$.

$LiAl(t\text{-}BuO)_3H$ (30.7 mmol, 7.3 g) was added portionwise over a period of 20 min to the residue, dissolved in anhydrous THF (133 mL) and cooled at 0 °C. The reaction mixture was stirred at the same temperature for another 30 min and then poured into 2 N aqueous HCl at pH 2.8, extracted with AcOEt, washed with 5% aqueous $NaHCO_3$ and brine and dried. The organic phase was evaporated to give a crude residue (20 g) which was purified by flash chromatography on silica gel, eluting with 4:1 AcOEt/toluene to obtain the pure alcohol **8** as an oil (1.7 g, 35%). IR ($CHCl_3$): ν 1778 cm^{-1}; ^{1}H-NMR: δ 1.97 (s; 3H); 3.11 and 3.45 (2d; 2H, $J = 17.8$ Hz); 3.56 (s; 2H); 3.60–4.80 (m; 4H); 4.88 (d;

Table I. Inhibition of HLE and PPE by **4a–c**, **5a**,**b** and reference drug **16**.





Compd.	R	X	HLE[a]	PPE[a]
			$k_{inact}/K_I \pm$ S.D.[b] (M^{-1} s^{-1})	$k_{inact}/K_I \pm$ S.D.[b] (M^{-1} s^{-1})
4a	Ph	MeO	1 540 ± 125	240 ± 15
4b	*p*-F-Ph	MeO	1 600 ± 145	350 ± 30
4c	CMe_3	MeO	4 000 ± 410	1 800 ± 145
5a	Ph	Cl	1 600 ± 170	432 ± 35
5b	*p*-F-Ph	Cl	7 600 ± 910	580 ± 50
16			25 000 ± 2 250	131 000 ± 7 860

[a] Substrate: *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide.
[b] S.D.: standard deviation.

1H, $J = 4.8$ Hz); 5.67 (dd; 1H, $J = 4.8$ and 8.9 Hz); 6.23 (d; 1H, $J = 8.9$ Hz, D_2O exchangeable); 7.24 (brs; 5H). Anal. $C_{18}H_{20}N_2O_5S$ (C, H, N).

4.1.2. General procedure for esters **9a**–**c**

A solution of alcohol **8** (3.0 mmol, 2.5 g) in anhydrous DCM (26 mL), stirred and cooled at −5 °C, was treated with the appropriate carboxylic acid (5.3 mmol) in the presence of EDCI (10.6 mmol) and DMAP (2.6 mmol). The solution was stirred at 0 °C for 3 h and then washed with cooled solutions of 10% HCl, 5% $NaHCO_3$ and H_2O. Evaporation of the dried and filtered extracts gave the crude esters **9a**–**c**, which were purified as follows. **9a** was purified by flash chromatography on silica gel, eluting with a 2:1 hexane/AcOEt mixture and was obtained it as an oil (42%); *N-((6R)-3-acetoxymethyl-2-benzoyloxymethyl-8-oxo-(6rH)-5-thia-1-aza-bicyclo[4.2.0]oct-2-en-7t-yl)-2-phenyl-2-yl-acetamide* **9a**. m.p.: 60 °C (benzene). IR ($CHCl_3$): ν 1775 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 2.02 (s; 3H); 3.20 and 3.48 (2d; 2H, $J = 17.7$ Hz); 3.64 (s; 2H); 4.58 (d; 1H, $J = 12.8$ Hz); 4.89 (d; 1H, $J = 4.8$ Hz); 4.90 (d; 1H, $J = 12.8$ Hz); 5.12 and 5.40 (2d; 2H, $J = 12.6$ Hz); 5.70 (dd; 1H, $J = 4.8$ and 9.0 Hz); 6.02 (d; 1H, $J = 9.6$ Hz, D_2O exchangeable); 7.06–8.07 (m; 10H). Anal. $C_{25}H_{24}N_2O_6S$ (C, H, N). Crude **9b** was purified by trituration with petroleum ether (40–60 °C) to give pure **9b** as an oil (70%); *N-((6R)-3-acetoxymethyl-2-(4-fluorobenzoyl)-oxymethyl-8-oxo-(6rH)-5-thia-1-aza-bicyclo[4.2.0]oct-2-en-7t-yl)-2-phenyl-2-yl-acetamide* **9b**. IR ($CHCl_3$): ν 1773 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 2.06 (s; 3H); 3.20 and 3.55 (2d; 2H, $J = 17.6$ Hz); 3.67 (s; 2H); 4.68 and 4.96 (2d; 2H, $J = 12.8$ Hz); 4.97 (d; 1H, $J = 4.8$ Hz); 5.25 and 5.42 (2d; 2H, $J = 8.8$ Hz); 5.79 (dd; 1H, $J = 4.8$ and 8.8 Hz); 6.38 (d; 2H, $J = 8.8$ Hz, D_2O exchangeable); 6.99–7.35 and 7.94–8.27 (2m; 9H). Anal. $C_{25}H_{23}FN_2O_6S$ (C, H, N). **9c** was directly obtained as a pure oil (90%); *N-((6R)-3-acetoxymethyl-2-pivaloyloxymethyl-8-oxo-(6rH)-5-thia-1-aza-bicyclo[4.2.0]oct-2-en-7t-yl)-2-phenyl-2-yl-acetamide* **9c**. IR ($CHCl_3$): ν 1773 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 1.77 (s; 9H); 2.07 (s; 3H); 3.16 and 3.63 (2d; 2H, $J = 17.9$ Hz); 3.89 (s; 2H); 4.76 (d; 1H, $J = 4.8$ Hz); 5.03 (m; 2H); 5.82 (dd; 1H, $J = 4.8$ and 9.4 Hz); 6.59 (d; 1H, $J = 9.4$ Hz, D_2O exchangeable); 7.32 (brs; 5H). Anal. $C_{23}H_{28}N_2O_6S$ (C, H, N).

4.1.3. General procedure for amines **12a**–**c**

t-$(BOC)_2O$ (15.6 mmol), DMAP (9.2 mmol) and TEA (8.9 mmol) were added to a solution of the appropriate ester **9a**–**c** (8.9 mmol) in anhydrous DCM (190 mL), stirred and cooled at 0 °C. After stirring for 1 h at room temperature, the reaction mixture was washed with cooled 1 N HCl (2×25 mL) and brine (2×50 mL), dried and evaporated to give the crude imide **10a**–**c**. The residue was dissolved in anhydrous DCM (300 mL) and *N*,*N*-diethylethylendiamine (10.8 mmol) was added to the resulting solution. After stirring for 48 h at room temperature, the reaction mixture was washed with cooled 1 N HCl (2×25 mL) and brine (2×25 mL), dried and evaporated to give the crude *t*-BOC-amide **11a**–**c**, which was dissolved in anhydrous DCM (182 mL). TFA (50 mL) was added to the resulting solution and the mixture was stirred for 20 min at room temperature and then evaporated. The residue, dissolved in $CHCl_3$, was poured into ice and 5% $NaHCO_3$ (100 mL), cooling at 0 °C and keeping it in these conditions for 1 h. The mixture was then extracted with AcOEt (3×50 mL), washed with brine (2×50 mL), dried and evaporated to give a crude residue which was purified by flash chromatography on silica gel or used for the next reaction.

(6R)-3-acetoxymethyl-2-benzoyloxymethyl-8-oxo-(6rH)-5-thia-1-aza-bicyclo[4.2.0]oct-2-en-7t-amine **12a**. The residue was purified by flash chromatography on silica gel eluting with a 9:1 AcOEt/hexane mixture to obtain the pure amine **12a** as an oil (30%).

IR ($CHCl_3$): ν 1772 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 1.86 (brs; 2H); 2.05 (s; 3H); 3.35 and 3.55 (2d; 2H, $J = 17.6$ Hz); 4.40–5.50 (m; 6H); 7.30–8.20 (brs; 5H). Anal. $C_{17}H_{18}N_2O_5S$ (C, H, N).

(6R)-3-acetoxymethyl-2-(4-fluorobenzoyl)oxymethyl-8-oxo-(6rH)-5-thia-1-aza-bicyclo[4.2.0]oct-2-en-7t-amine **12b**. IR ($CDCl_3$): ν 1773 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 1.89 (brs; 2H); 2.10 (s; 3H); 3.39 and 3.60 (2d; 2H, $J = 17.6$ Hz); 4.65–5.50 (m; 6H); 7.01–7.25 and 7.98–8.18 (m; 4H). Anal. $C_{17}H_{17}FN_2O_5S$ (C, H, N).

(6R)-3-acetoxymethyl-2-pivaloyloxymethyl-8-oxo-(6rH)-5-thia-1-aza-bicyclo[4.2.0]oct-2-en-7t-amine **12c**. IR ($CDCl_3$): ν 1770 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 1.19 (s; 9H); 1.89 (brs; 2H, D_2O exchangeable); 2.09 (s; 3H); 3.16 and 3.40 (2d; 2H, $J = 17.8$ Hz); 3.60–5.16(m; 8H). Anal. $C_{15}H_{22}N_2O_5S$ (C, H, N).

4.1.4. General procedure for the synthesis of the 7α-methoxy cephem derivatives **14a**–**c**

A solution of $NaNO_2$ (0.56 mmol) in H_2O (4.1 mL) was added to a solution of the appropriate amine **12a**–**c** (0.55 mmol) in DCM (4.1 mL). The resulting mixture was cooled in an ice bath and stirred vigorously, then 2 N aqueous H_2SO_4 (0.42 mL) was added dropwise over 30 min. Stirring was continued for 1 h in the same conditions and the reaction was monitored by IR spec-

troscopy controlling the appearance of the diazo group characteristic stretching-band at 2 100 cm^{-1}.

The two phases were separated and the aqueous medium was extracted with more DCM (5 mL). The combined organic layers were washed with brine (10 mL) and dried over anhydrous Na_2SO_4, previously dried in muffle. The resulting anhydrous DCM solution of the diazo derivative **13a**–**c**, stirred and cooled at 0 °C, was diluted with anhydrous MeOH (4.3 mL) and treated with Rh_2OAc_4 (4.05×10^{-3} mmol). After stirring in these conditions for 90 min, the mixture was filtered through silica gel, concentrated, and dried in vacuo to give a crude residue which was purified by flash chromatography on silica gel eluting with a 2:1 hexane/AcOEt mixture.

(6R)-3-acetoxymethyl-8-oxo-(6rH)-5-thia-1-aza-bicyclo[4.2.0]oct-2-en-7c-methyloxy-2-benzoyloxy methylester **13a**. IR ($CDCl_3$): ν 1780 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 2.04 (s; 3H); 3.33 (d; 1H, $J=17.7$ Hz); 3.55 (s; 3H); 3.59 (d; 1H, $J=17.7$ Hz); 4.56 (d; 1H, $J=1.9$ Hz); 4.69 (d; 1H, $J=1.9$ Hz); 4.73 and 4.99 (dd; 2H, $J=12.6$ Hz); 5.31 and 5.49 (dd; 2H, $J=12.9$ Hz); 7.30–7.60 and 8.05–8.12 (2m; 5H). Anal. $C_{18}H_{19}NO_6S$ (C, H, N).

(6R)-3-acetoxymethyl-8-oxo-(6rH)-5-thia-1-aza-bicyclo[4.2.0]oct-2-en-7c-methyloxy-2-(4-fluorobenzoyl)-oxymethylester **13b**. IR ($CDCl_3$): ν 1780 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 2.08 (s; 3H); 3.32 (d; 1H, $J=18.2$ Hz); 3.59 (s; 3H); 3.68 (d; 1H, $J=18.2$ Hz); 4.57 (d; 1H, $J=2.0$ Hz); 4.72 (d; 1H, $J=2.0$ Hz); 4.74 and 5.01 (dd; 2H, $J=12.8$ Hz); 5.32 and 5.55 (dd; 2H, $J=12.8$ Hz); 6.99–7.32 and 7.95–8.22 (2m; 4H). Anal. $C_{18}H_{18}FNO_6S$ (C, H, N).

(6R)-3-acetoxymethyl-8-oxo-(6rH)-5-thia-1-aza-bicyclo[4.2.0]oct-2-en-7c-methyloxy-2-pivaloyloxymethylester **13c**. IR ($CDCl_3$): ν 1780 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 1.19 (s; 9H); 2.05 (s; 3H); 3.18 (d; 1H, $J=18.9$ Hz); 3.54 (s; 3H); 3.56 (d; 1H, $J=18.0$ Hz); 4.51 (d; 1H, $J=2.0$ Hz); 4.56 (d; 1H, $J=12.6$ Hz); 4.66 (d; 1H, $J=2.0$ Hz); 4.83 (d; 1H, $J=12.6$ Hz). Anal. $C_{16}H_{23}NO_6S$ (C, H, N).

4.1.5. General procedure for the synthesis of 7α-chloro cephem derivatives **15a**,**b**

A solution of $NaNO_2$ (0.35 mmol) in H_2O (2.5 mL) was added to a solution of the appropriate amine **12a**,**b** (0.32 mmol) in DCM (2.5 mL). The resulting mixture was cooled in an ice bath and stirred vigorously, then 2 N aqueous H_2SO_4 (0.28 mL) was added dropwise over 30 min. Stirring was continued for 1 h in the same conditions and the reaction was monitored by IR spectroscopy controlling the appearance of the diazo group characteristic stretching-band at 2 100 cm^{-1}.

The two phases were separated and the aqueous medium was extracted with more DCM (2.5 mL). The combined organic layers were washed with brine (5 mL) and dried over anhydrous Na_2SO_4, previously dried in muffle. The resulting anhydrous DCM solution of the diazoderivative **13a**,**b**, stirred and cooled at 0 °C, was diluted with absolute EtOH (0.65 mL) and concentrated aqueous HCl (0.026 mL). After stirring at room temperature for 20 s, the reaction mixture was poured into a 1 M KH_2PO_4 solution (15 mL) and extracted with DCM (5 mL). The organic phase was washed with brine (10 mL), dried and evaporated to obtain **15a**,**b** as crude residues.

(6R)-3-acetoxymethyl-8-oxo-(6rH)-5-thia-1-aza-bicyclo[4.2.0]oct-2-en-7c-chloro-2-benzoyloxymethylester **15a**. The crude residue was purified by flash chromatography on silica gel eluting with 2:1 hexane/AcOEt, obtaining **15b** as an oil (32%). IR ($CDCl_3$): ν 1780 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 2.04 (s; 3H); 3.39 and 3.59 (dd; 2H, $J=18.0$ Hz); 4.73 (d; 1H, $J=12.7$ Hz); 4.76 (d; 1H, $J=2.0$ Hz); 4.78 (d; 1H, $J=2.0$ Hz); 4.99 (d; 1H, $J=12.7$ Hz); 5.30 and 5.49 (dd; 2H, $J=13.2$ Hz); 7.40–7.60 and 8.00–8.12 (2m; 5H). Anal. $C_{17}H_{16}ClNO_5S$ (C, H, N). *(6R)-3-acetoxymethyl-8-oxo-(6rH)-5-thia-1-aza-bicyclo[4.2.0]oct-2-en-7c-chloro-2-(4-fluorobenzoyl)oxymethylester* **15b**. The residue was exclusively composed of the desired compound (90%). IR ($CDCl_3$): ν 1780 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 2.03 (s; 3H); 3.30 and 3.62 (dd; 2H, $J=18.0$ Hz); 4.76 and 4.92 (2d; 2H, $J=12.7$ Hz); 5.22 and 5.45 (2d; 2H, $J=13.2$ Hz); 6.90–7.30 and 7.82–8.20 (2m; 4H). Anal. $C_{17}H_{17}ClFNO_5S$ (C, H, N).

4.1.6. General procedure for the synthesis of sulphones **4a**–**c** *and* **5a**,**b**

Solid MCPBA (0.65 mmol, 70% pure) and $NaHCO_3$ (0.65 mmol) were added to a stirred solution, cooled at 0 °C, of the appropriate sulphur derivative **14a**–**c**, **15a**,**b** (0.26 mmol) in anhydrous DCM (5.3 mL). After stirring in these conditions for 2 h, the mixture was diluted with DCM (5 mL) and washed with cooled 10% aqueous $Na_2S_2O_3$ (2×10 mL), 5% aqueous $NaHCO_3$ (2×10 mL) and brine. The extracts were then dried and evaporated in vacuo to give a crude residue, which was purified by flash chromatography on silica gel, eluting with a hexane/AcOEt 2:1 mixture.

(6R)-3-acetoxymethyl-2-benzoyloxymethyl-8-oxo-(6rH)-5-thia-1-aza-bicyclo[4.2.0]oct-2-en-7c-methyloxy-

5,5-dioxide **4a**. IR ($CHCl_3$): *v* 1796 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 2.06 (s; 3H); 3.55 (s; 3H); 3.73 and 3.99 (2d; 2H, $J = 17.9$ Hz); 4.61 (d; 1H, $J = 13.0$ Hz); 4.71 (d; 1H, $J = 1.9$ Hz); 4.98 (d; 1H, $J = 13.0$ Hz); 5.19 (d; 1H, $J = 1.9$ Hz); 5.26 and 5.44 (2d; 2H, $J = 13.2$ Hz); 7.40–7.65 and 7.95–8.20 (2m; 5H). Anal. $C_{18}H_{19}NO_8S$ (C, H, N).

(6R)-3-acetoxymethyl-2-((4-fluorobenzoyl)oxymethyl)-8-oxo-(6rH)-5-thia-1-aza-bicyclo[4.2.0]-oct-2-en-7c-methyloxy-5,5-dioxide **4b**. IR ($CHCl_3$): ν 1796 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 2.06 (s; 3H); 3.55 (s; 3H); 3.73 and 3.99 (2d; 2H, J = 18.0 Hz); 4.61 (d; 1H, J = 13.0 Hz); 4.69 (d; 1H, J = 1.6 Hz); 5.25 and 5.42 (2d; 2H, J = 13.3 Hz); 7.01–7.19 and 8.0–8.12 (2m; 4H). Anal. $C_{18}H_{18}FNO_8S$ (C, H, N).

(6R)-3-acetoxymethyl-2-pivaloyloxymethyl-8-oxo-(6rH)-5-thia-1-aza-bicyclo[4.2.0]-oct-2-en-7c-methyl-oxy-5,5-dioxide **4c**. IR ($CHCl_3$): ν 1796 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 1.21 (s; 9H); 2.09 (s; 3H); 3.57 (s; 3H); 3.71 and 3.98 (2d; 2H, J = 18.4 Hz); 4.54 (d; 1H, J = 13.0 Hz); 4.66 (d; 1H, J = 1.8 Hz); 4.86 (d; 1H, J = 13.0 Hz); 5.01–5.10 (2d; 2H, J = 13.3 Hz); 5.17 (d; 1H, J = 1.8 Hz). Anal. $C_{16}H_{23}NO_8S$ (C, H, N).

(6R)-3-acetoxymethyl-2-benzoyloxymethyl-8-oxo-(6rH)-5-thia-1-aza-bicyclo[4.2.0]-oct-2-en-7c-chloro-5,5-dioxide **5a**. IR ($CHCl_3$): ν 1812 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 2.07 (s; 3H); 3.79 and 4.02 (2d; 2H, J = 18.1 Hz); 4.62 (d; 1H, J = 13.1 Hz); 4.80 (d; 1H, J = 1.9 Hz); 4.98 (d; 1H, J = 13.1 Hz); 5.24 (d; 1H, J = 13.5 Hz); 5.36 (d; 1H, J = 1.9 Hz); 5.45 (d; 1H, J = 13.5 Hz); 7.40–7.65 and 7.98–8.19 (2m; 5H). Anal. $C_{17}H_{16}ClNO_7S$ (C, H, N). *(6R)-3-acetoxymethyl-2-((4-fluorobenzoyl)oxymethyl)-8-oxo-(6rH)-5-thia-1-aza-bicyclo[4.2.0]-oct-2-en-7c-chloro-5,5-dioxide* **5b**. IR ($CHCl_3$): ν 1812 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 2.12 (s; 3H); 3.78 and 4.09 (2d; 2H, J = 18.50 Hz); 4.65 (d; 1H, J = 12.7 Hz); 4.82 (d; 1H, J = 2.0 Hz); 4.99 (d; 1H, J = 12.7 Hz); 5.25 (d; 1H, J = 13.2 Hz); 5.38 (d; 1H, J = 2.0 Hz); 5.48 (d; 1H, J = 13.2 Hz). Anal. $C_{17}H_{15}ClFNO_7S$ (C, H, N).

4.2. HLE and PPE inhibition

The activities of HLE (EC 3.4.21.37, Calbiochem) and PPE (EC 3.4.21.36, Sigma Chemical Company) were assayed spectrophotometrically, using *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (MAAPVNA) as the substrate [33] in 0.05 M sodium phosphate buffer, pH 7.8. Both substrate and inhibitor solutions were prepared in DMSO. The hydrolytic release of *p*-nitroaniline was monitored from the increase in absorbance at 390 nm (ε for MAAPVNA = 500 M^{-1} cm^{-1}; ε for *p*-nitroaniline = 13 000 M^{-1} cm^{-1}) with a Beckman DU-7 UV–visible spectrophotometer thermostated at 37 °C [34]. The K_M values of HLE and PPE for MAAPVNA at 37 °C were evaluated and found to be 0.12 and 0.43 mM, respectively; the latter value is strongly at variance with that reported at 25 °C [33, 35]. A typical inhibition assay (carried out in a final volume of 500 μL of the buffer containing 4% DMSO) was run as follows: HLE (about 5 milliunits; one milliunit releases one nmol of *p*-nitroaniline per min from MAAPVNA) was added to a reaction mixture containing MAAPVNA (0.2–0.6 mM), and the increase in absorbance was recorded for 3 min (time course A); one of the inhibitors dissolved in <2 μL of DMSO was subsequently added to a final concentration ranging from 0.1 to 20 μM and the absorbance was further recorded up to 30 min (time course B). As a reference, Fig. 11 in [20] can be used. Control assays, in which the inhibitor was omitted, ran linearly in time up to an absorbance of 1.0 to 1.1 AU, before the slope started to change due to substrate consumption; assays in which the inhibitor was added readily showed a time-dependent decline in the slope, and the absorbance reading levelled off between 0.5 and 1.0 AU at 30 min, depending on the inhibitor concentration and potency.

The first-order decay in HLE or PPE activity was evaluated by taking the slope value every 60 s over time course B and dividing it by that of time course A. The natural log of this ratio was plotted as a function of time, the negative slope of this plot yielding a k_{obs} value (apparent first-order rate constant). The inactivation time course of the enzyme in the presence of both inhibitor and substrate strictly obeyed pseudo-first-order kinetics up to a residual activity lower than 10% (i.e., over more than 2 ln units). Since k_{obs} values did not increase linearly with [I], $1/k_{obs}$ values obtained at the same substrate concentration and at varying concentrations of a single inhibitor were plotted versus 1/[I] (double-reciprocal plot). The reciprocal of the slope of this plot yielded

$$\frac{k_{inact}}{K_I(1+[S]/K_M)},$$

from which the concentration-independent second-order rate constant k_{inact}/K_I was obtained, using the appropriate K_M value (for HLE or PPE) in computing the factor $(1+[S]/K_M)$. Similar values of this parameter were ob-

tained for the same inhibitor at different substrate concentrations, in agreement with a competitive mechanism of inhibition [20].

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[35] The K_M value of MAAPVNA for PPE was experimentally determined both under our assay conditions (37 °C, 0.05 M sodium phosphate buffer, pH 7.8) and under those of Nakajima et al. [33], always getting a value in the 0.4–0.6 mM range. We never obtained a value as high as that reported in [33] (6 mM), and therefore $K_M = 0.4$ mM (the value obtained under our assay conditions) was used for the correct evaluation of the inhibition parameters against PPE of the compounds under study in this work. The K_M value of MAAPVNA for HLE, experimentally determined both under our assay conditions and under those of Nakajima et al. [33], was always found to be in agreement with the value reported by these authors.