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In vitro evaluation of the antielastase activity of polycyclic β-lactams

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ABSTRACT

A series of bi- and tricyclic β-lactam compounds was synthesized and evaluated as inhibitors of cleavage of synthetic substrates *in vitro* by the serine proteases Human Leukocyte Elastase (HLE), Human Leukocyte Proteinase 3 (HLPR3) and Porcine Pancreatic Elastase (PPE). The obtained results have permitted us to describe a homobenzocarbacephem compound as HLE and HLPR3 inhibitor, to observe the positive effect that the styryl group exerts on the HLE inhibitory activity in polycyclic β-lactam compounds and to conclude that the hydroxyl function decreases the HLE inhibitory activity or rules it out completely.

1. Introduction

The HLE (EC 3.4.21.37) [1], also called neutrophil elastase (HNE), polymorphonuclear leukocyte elastase or granulocyte elastase, is a 29 kDa glycoprotein with serine peptidase activity [2]. The HLE, together with other neutrophil serine peptidases, including HLPR3, hydrolyses the contents of phagolysosomes, degrades extracellular matrix proteins and regulates inflammation [3]. As a consequence of deregulation in enzyme synthesis or alteration of its physiological inhibition, the protease/antiprotease equilibrium is disrupted and the enzyme may exert its proteolytic activity on non-physiological targets.

The HLE hydrolytic activity is very potent and it is capable of degrading several substrates, including elastin, collagen or fibrin. The consequences of its exacerbated activity can be devastating and are associated to serious pathologies [4], such as, pulmonary emphysema [5], rheumatoid arthritis [6], psoriasis, acute respiratory distress syndrome, cystic fibrosis, and tumor progression [7]. In an analogous way, pancreatitis [8] can be provoked by pancreatic tissue digestion when the inactive form of HPE, Human Pancreatic Elastase (zymogen), becomes pathologically activated inside the pancreas instead of in the intestine.

Thus, HLE has been the object of extensive research to develop potent inhibitors [9] that target its destructive and pro-inflammatory action by restoring the protease/antiprotease imbalance. Alpha-1-antitrypsin and the secretory leukocyte protease inhibitor are some natural molecules showing antiprotease activity. However, the action of these inhibitors is also controlled and counteracted by mechanisms related to the formation of the enzyme-substrate complex and to the oxidation state of the environment [10]. A number of synthetic inhibitors of HLE has been produced over the last years and applied, for instance, in specific lung pathologies [11]. Various compounds have been used as the starting point for the synthesis of specific proteases inhibitors, such as acyl compounds, pyrrolpyrrolones, coumarins, azapeptides, chloromethylketones, trifluoromethylketones, and sulphonil-fluorides. β-Lactam compounds, traditionally used as antibacterial agents, have also been tested as inhibitors of mammalian serine proteases, such as HLE [12], thrombin [13], prostate specific antigen (PSA) [14], human cytomegalovirus (HCMV) protease [15], and human chymase [16].

The abilities of mono- and polycyclic β-lactams to inhibit serin-enzymes lie mainly in the β-lactam ring. Its action consists of kidnapping the Ser residue at the active site of the enzyme by a nucleophilic attack that opens the β-lactam ring (Fig. 1) and produces a stable acyl-enzyme covalent complex that turns the enzyme inactive [17].

β-Lactams could be classified as mechanism-based inhibitors of HLE which could promote irreversible inhibition via a suicide-type mechanism by the presence of a potential leaving group in the inhibitor structure. Thus, they could represent a good model base for designing powerful drugs able to inhibit HLE and restore the altered protease/antiprotease ratio at the inflammatory sites. There are a lot of examples in the literature of elastase inhibitors with a β-lactam skeleton [18], some of which have been proofed to be orally active [19].

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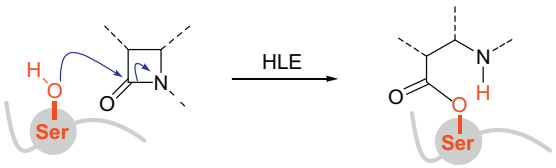


Fig. 1. Proposed mechanism for the inhibition of HLE by β-lactam compounds.

Our research group, with a vast experience in the synthesis of β-lactam compounds [20], has evaluated a series of monolactams against the enzymes Human Leukocyte Elastase (HLE), Porcine Pancreatic Elastase (PPE) and Rat Leukocyte Elastase (RLE) [21]. The obtained results revealed that only the monolactams **A** (Fig. 2) bearing the styryl and moreover the methylstyryl groups at the C4 position of the β-lactam ring and a D-glucosamine derived moiety (Sg) at the *N*-substituent, showed specific HLE inhibitory activity.

In this work, we analyse as lead inhibitors of HLE the polycyclic β-lactams **1–18** (Fig. 3) which have been obtained by cyclisation reactions of benzyl [22] and homobenzyl [23] radicals generated from epoxyolefin-, and epoxynitrile-2-azetidinones with titanocene monochloride (Cp_2TiCl) [24].

2. Results and discussion

2.1. Chemistry

As it has been above reported, the synthesis of the target molecules **1–16** is based in a radical cyclisation induced by Cp_2TiCl in the epoxy-monolactams **19–25** (Scheme 1). These compounds were prepared by standard Staudinger reactions between methoxyacetyl chloride in the presence of TEA and the imines obtained by condensation of the amines **I** [21b], 2-amino-2,2-dimethyletanol and *o*-cyanoaniline with cinnamaldehyde or methyl-cinnamaldehyde. Several chemical transformations on the formed 2-azetidinones yielded the unsaturated epoxy-esters **19**, the epoxy-nitriles **20** and **21** and the *cis*/*trans* epoxy-benzonitriles **22–25**. Finally, the treatment of the epoxides **19–25** with Cp_2TiCl afforded the bi- and tricyclic β-lactams **1–16**.

The reported studies on the radical cyclisation reactions induced by Cp_2TiCl in epoxy-esters **19** [22b] were carried out by inverse addition of the reagents; that is, by addition of a solution of the epoxide in THF to a green suspension of Cp_2TiCl in THF. Under these conditions, only the isomers **19ab** and **19ba** afforded the carbacephams **3** and **6**, respectively. In this work, we report the physical properties of the carbacephams **1–8** and the carbapenam **9**, which were obtained by treatment of the four optically pure isomers **19** with Cp_2TiCl by direct addition of the reagents (see experimental section). The stereochemistry for these new bicyclic compounds (Fig. 3) was rigorously established by IR and NMR spectroscopy.

In order to evaluate if the role the styryl group plays on the antielastase activity of the polyciclic β-lactams is similar to that reported for the monolactams **A** [21], also the trilactams **17** and **18** were obtained by dehydratation of the epoxy-benzonitriles **14–16** with *p*-Me-$C_6H_4SO_2Cl$.

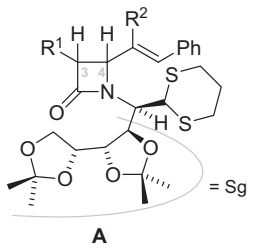


Fig. 2. Monocyclic β-lactams with specific HLE inhibitory activity [21].

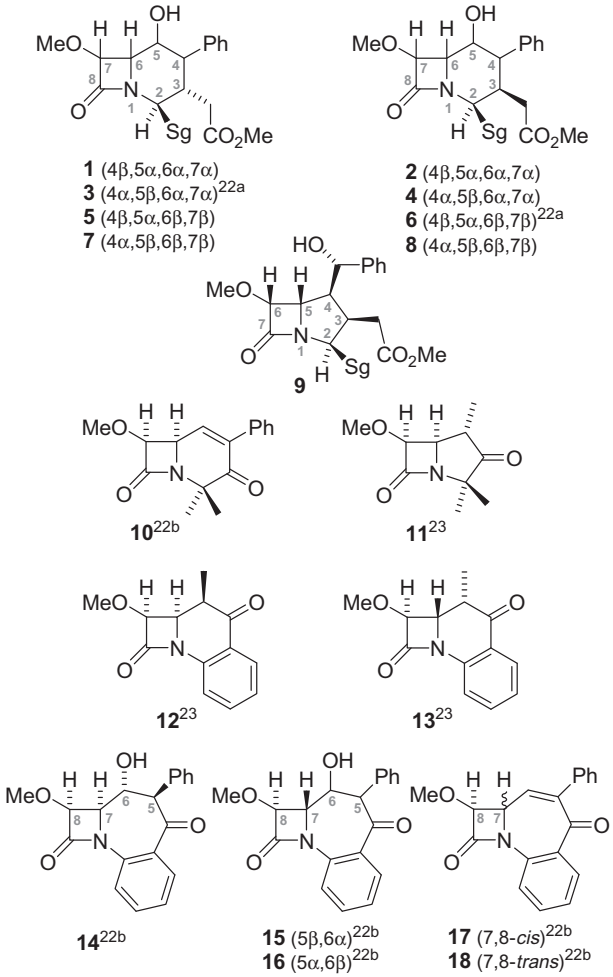


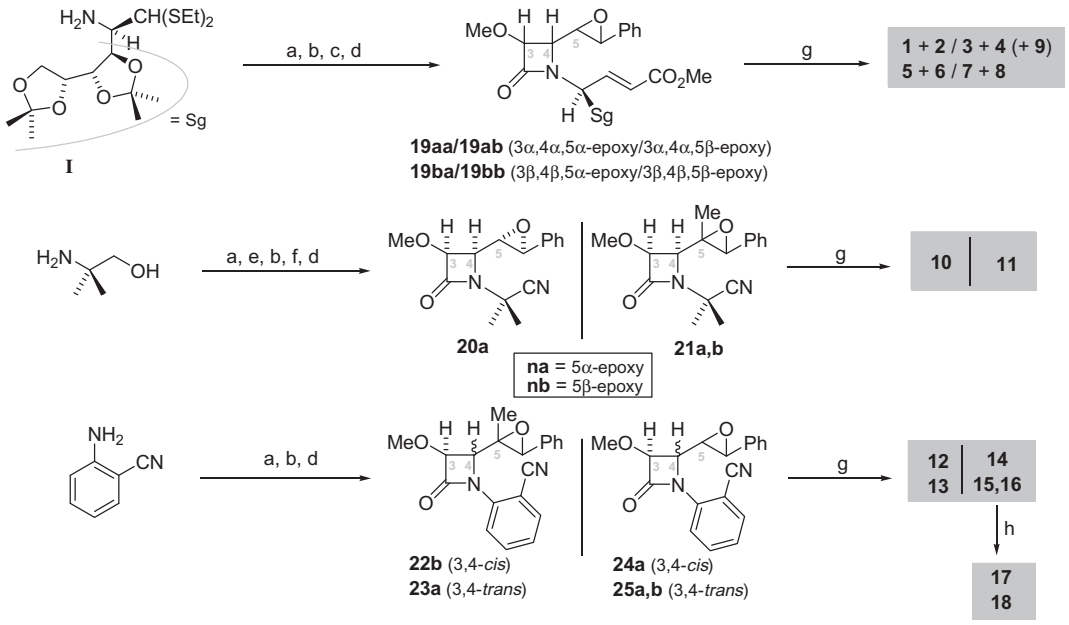
Fig. 3. Polycyclic β-lactams analyzed as candidate inhibitors of HLE.

2.2. Inhibition of Elastases

In vitro analysis of the antielastase activity [25] of the compounds **1–18** was carried out by a spectrophotometric microassay based on the use of multi-well plates (see experimental section for details). With this procedure several compounds can simultaneously be screened by recording absorbances over defined periods of time with a Multiscan spectrophotometer connected to a computer that records all data. Another advantage is the use of small reaction volumes, with significant saving of reactants. Kinetic and dose-response experiments were done.

In a preliminary screening, all compounds shown in Fig. 3, except **7**, **15** and **16**, were evaluated at a 50 μM concentration. Selected compounds showing more than 25% inhibition of enzymatic activity were further assayed and the IC_{50} values (half maximal inhibitory concentration) determined (Table 1).

As shown in Table 1, only the trilactams **17** and **18** exhibited higher than 25% HLE-inhibition activities. All the studied bilactams, except **10** and **11**, bear a hydroxyl group in the second ring of their structures. Since none of them has a significant HLE inhibitory activity, we guess that the hydroxyl function decreases the anti-HLE activity. This conclusion was supported by the inhibition displayed by the tricyclic β-lactam **14**, the hydroxylated precursor of **17**.



Scheme 1. *Reagents and conditions:* (a) cinnamaldehyde, for **19**, **20**, **24** and **25** or *trans*-α-methyl-cinnamaldehyde, for **21–23**; CH_2Cl_2 (toluene for **19**), rt. (b) methoxyacetylchloride, TEA, CH_2Cl_2, rt. (c) 1: $PhI(CF_3CO_2)_2/NaHCO_3$, 9:1 CH_3CN/H_2O, rt. 2: $Ph_3P{=}CH{-}CO_2Me$, THF, rt. (d) *m*-CPBA, $NaHCO_3$, CH_2Cl_2, rt. (e) $^tBu(Me)_2SiCl$, pyr/DMAP, rt. (f) 1: HCl (0.1 M), MeOH, rt. 2: $(COCl)_2$, DMSO, CH_2Cl_2, −78 °C, then TEA. 3: $Me_2N\text{-}NH_2$, MeOH, rt; then MMPP, 0 °C. (g) 1: Cp_2TiCl, THF, rt; 2: KH_2PO_4 aq. (h) *p*-Me-$C_6H_4SO_2Cl$, TEA, DMAP, CH_2Cl_2, rt.

Table 1
Percentage inhibition of HLE for compounds **1–18** at 50 μM. IC_{50} (μM) measurements and K_i (μM) values for **17** and **18**.[a]

Comp. no.	% Inhibition at 50 μM	Comp. no.	% Inhibition at 50 μM	IC_{50} (μM)	K_i (μM)
1	13	10	0		
2	10	11	14		
3	8	12	16		
4	9	13	9		
5	1	14	8		
6	5	17	97	21.0 ± 1.2	12.6 ± 0.7
8	1	18	98	22.1 ± 2.6	13.4 ± 1.6
9	12				

[a] IC_{50} and K_i are expressed as mean ± SEM (standard error of the mean).

The trilactam **14**, with a hydroxyl group in its structure, shows only an 8% inhibition of the enzymatic activity. However, the enones **17** and **18**, with a styryl moiety in their structures, resulted to be the most active compounds at a concentration of 50 μM, showing 97% and 98% inhibition of the enzymatic activity, respectively. On the contrary, their benzocarbacephem homologues, compounds **12** and **13**, gave lower values of inhibition, probably due to the absence of a double bond in the second ring [26].

Although the styryl group seems to be responsible for the greater inhibitory activity shown by the tricyclic systems **17** and **18**, bilactam **10** showed no inhibition at all, being even less active than the carbacephams **1–8**, which are influenced by the negative effect of the hydroxyl groups present in their structures. Perhaps the *gem*-dimethyl moiety that the carbacepham **10** bears at the C2 position has a bad influence on the anti-elastase activity in this kind of bilactams, overruling the positive effect of the styryl group. It was also evident that the positive effect of the D-glucosamine derived moiety, Sg, is apparently stronger than the negative influence of the hydroxyl group.

The positive effect exerted by the styryl group could be due to the fact that the phenyl ring may stabilize the enzyme-inhibitor complex previous to the β-lactam ring opening because this phenyl group could form hydrophobic bonds with the residues at the oxyanion hole of the enzyme, which is responsible for the charges stabilization during the enzymatic catalysis process.

Compounds **17** and **18**, which showed higher than 25% HLE-inhibition values, were tested at lower concentration (1, 5, 10 and 20 μM) in order to determine the IC_{50} parameters shown in Table 1 (21.0 ± 1.2 μM, for **17**; 22.1 ± 2.6 μM, for **18**). According to these results, the relative *cis/trans* stereochemistry of the β-lactam ring seems to have no effect on the HLE inhibitory activity of these tricyclic compounds.

The inhibitory effect of compounds **17** and **18** against HLPR3 was tested under the same experimental conditions used for HLE in the presence of the same substrate concentration (100 μM methoxysuccinyl–Ala–Ala–Pro–Val–*p*-nitroanilide). Both compounds significantly inhibited HLPR3, rendering the following IC_{50} values: 16.2 ± 3.9 μM for trilactam **17** and 24.6 ± 4.9 μM for trilactam **18**. K_i values were 18.3 ± 5.2 μM for compound **17** and 27.9 ± 11.2 μM for compound **18**. The measurements (expressed as mean ± SEM) are similar to the ones obtained for HLE (Table 1). Therefore, compounds **17** and **18** are mutual inhibitors of the tested neutrophil serine proteases.

Both trilactams **17** and **18** were used to undertake the specificity study with the enzymes HLE and Porcine Pancreatic Elastase (PPE). The assays with PPE were carried out under the same experimental conditions as the previous HLE–inhibition activity assays, except the buffer pH value, that in this case was pH = 8.0. The obtained results were 35% and 70% of enzyme inhibition at 50 μM for **17** and **18**, respectively.

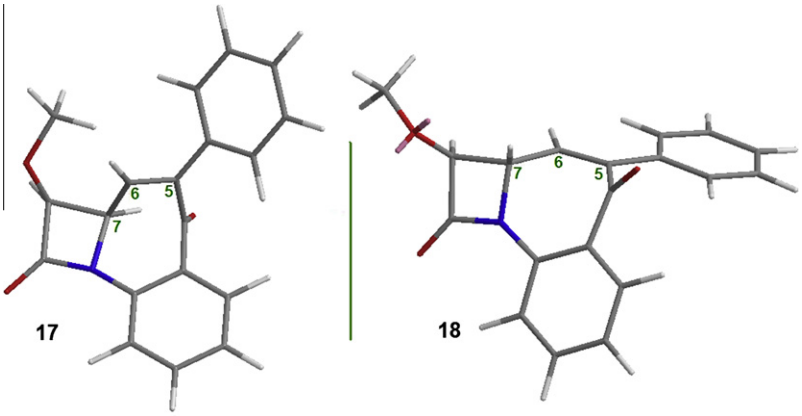


Fig. 4. Lower energy conformers of the tricyclic β-lactams **17** and **18**.

These latter values, as compared with those obtained against HLE (Table 1), indicate that compound **18** at 50 μM shows high inhibition values for both elastases, whereas compound **17** presents lower inhibition of PPE at the same concentration. These results seem to point out that the *cis*-trilactam **17** can be a selective inhibitor of the HLE (compared to PPE). Furthermore, the IC_{50} value of compound **17** in the presence of PPE was 170 μM (range: 140–201 μM), almost nine times higher than the IC_{50} calculated for HLE.

The low toxicity of β-lactam compounds makes homobenzocarbacephem **17** an attractive HLE inhibitor despite the obtained IC_{50} for compound **17** against HLE is much higher than those exhibited by the natural inhibitors studied under the same experimental conditions [10].

The observed specific effect of compound **17** against the HLE (compared to PPE) is probably due to minor differences in the residues of both the catalytic active site environment in the enzymes HLE and PPE and the oxyanion hole [27], which therein helps molecule adaptation and charge stabilization. These slight differences give rise to different interactions with those substituents of the selected compound that are mainly involved in the enzyme-inhibitor complex stability.

On the other hand, as the HLE-inhibitory effect observed for compound **17** (compared to PPE) appears to be related to the *cis* disposition of the substituents of the 2-azetidinone ring, it seems clear that the configuration of this ring affects the interactions occurring at the enzyme catalytic site and its environment.

The minimized structures [28] of both isomeric compounds **17** and **18** show a very unlike spatial disposition in each case (Fig. 4). This different spatial disposition may be responsible for the specific HLE-inhibitory activity (compared to PPE) of compound **17**, which should fit better in the catalytic site environment of the HLE enzyme.

3. Conclusions

From the results reported here, we conclude that the homobenzocarbacephem **17** may be an interesting HLE inhibitor and that the styryl group also exerts a positive effect on the HLE inhibitory activity in polycyclic β-lactam compounds as the formerly observed for monolactams **A** (Fig. 2) [21].

4. Experimental

4.1. Chemistry

4.1.1. General methods

Flash chromatographies were run on silica gel (Merck 60 230–400 mesh) and thin layer chromatographies (TLC), on commercial silica gel plates (Merck F-254). Mass spectra (MS) were recorded on an Applied Biosystems QSTAR XL (HRMS, 5 kV). IR spectra were recorded as neat film on a Nicolet IR-100 instrument. ^1H and ^{13}C NMR spectra were obtained on Bruker WP200SY and Bruker Avance 400DRX instruments (200 and 400 MHz respectively) in $CDCl_3$ solutions with tetramethylsilane as internal standard. Solvents and reagents were purified according to standard techniques.

4.1.2. Preparation of epoxy-monolactams ***19–25***

Epoxy-monolactams **19–25** were prepared according to the protocols reported in references 22 (esters **19**, 22a; nitriles **20**, ***24*** and **25**, 22b) and 23 (nitriles **21–23**).

4.1.3. Preparation of bicyclic β-lactams ***1–9***

***In situ* Generation of Titanocene Monochloride (Cp_2TiCl)**. To 548 mg (2.2 mmol) of titanocene dichloride in anhydrous and strictly deoxygenated THF (12.5 mL, *c* 0.176 M), 262 mg (4.0 mmol) of activated zinc granules were added. The resulting red mixture was then vigorously stirred under argon atmosphere with rigorous exclusion of oxygen until a green color was observed (about 20 min.).

Radical Reactions with Cp_2TiCl. A THF green suspension of Cp_2TiCl, generated *in situ* from Cp_2TiCl_2 and Zn^0 as described above, was added drop-wise through cannula to a solution of the corresponding epoxide **19** (1.0 mmol) in THF (17.0 mL, *c* 0.058 M). The resulting reaction mixture was stirred until a color change from green to orange occurred or until the disappearance of the starting material was observed by TLC. Then the reaction was quenched with 10% v/v aqueous KH_2PO_4 (30.0 mL) and the resulting aqueous phase was extracted with ethyl acetate. The organic combined extracts were filtered through Celite®, dried (anhydrous Na_2SO_4) and concentrated *in vacuo*. The crude product obtained was purified by column chromatography using hexanes/EtOAc mixtures as the eluent.

Carbacephams 1 and 2: From the epoxide **19aa** (262 mg, 0.51 mmol), 69 mg of **1** (26%) and 85 mg of **2** (32%) were isolated in 3 h.

1: R_f (4:6 hexanes/EtOAc) 0.26; $[\alpha]_D^{25}$ +56 (c 1.0, $CHCl_3$); IR (film) υ 3509, 1761, 1738, 1215, 1070, 700 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 1.26 (3H, s), 1.34 (3H, s), 1.35 (3H, s), 1.54 (3H, s), 2.31 (1H, dd, *J* = 4.6 and 16.8 Hz), 2.58 (1H, dd, *J* = 9.0 and 16.8 Hz), 3.25–3.32 (2H, m), 3.58 (3H, s), 3.65 (3H, s), 3.90 (1H, d, *J* = 8.0 Hz), 3.95–4.00 (3H, m), 4.11 (1H, t, *J* = 4.7 Hz), 4.14 (1H, dd, *J* = 5.2 and 9.2 Hz), 4.37 (1H, d, *J* = 7.2 Hz), 4.50 (1H, dd, *J* = 4.7 and 9.4 Hz), 4.76 (1H, d, *J* = 4.7 Hz), 7.20–7.40 (5H, m) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.4, 26.3, 26.8 (2C), 32.9, 34.5, 49.7, 50.6, 51.6, 53.1, 59.7, 67.8, 68.6, 77.3, 78.9, 79.1, 85.3, 109.4, 110.1, 126.8, 128.2 (2C), 129.6 (2C), 138.6, 168.6,

172.6 ppm; HRMS (Q-TOF) calcd for $C_{27}H_{38}NO_9$ ($M^+ + 1$) 520.2541, found 520.2573.

2: R_f (4:6 hexanes/EtOAc) 0.30; $[\alpha]_D^{25}$ +64 (c 1.0, $CHCl_3$); IR (film) υ 3511, 1761, 1738, 1165, 1020 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 1.32 (3H,s), 1.39 (3H,s), 1.43 (3H,s), 1.47 (3H,s), 2.20–2.35 (2H,m), 2.55–2.65 (1H,m), 2.82 (1H,t,*J* = 11.2 Hz), 3.47 (1H,d,*J* = 10.8 Hz), 3.54 (3H,s), 3.68 (3H,s), 3.70 (1H,d, *J* = 8.4 Hz), 3.96 (1H,dd,*J* = 4.2 and 8.6 Hz), 4.01–4.06 (2H,m), 4.08 (1H,d,*J* = 6.4 Hz), 4.13 (1H,dd,*J* = 6.4 and 8.6 Hz), 4.24 (1H,dd,*J* = 4.4 and 7.2 Hz), 4.41 (1H,ddd,*J* = 7.2, 10.8 and 11.2 Hz), 4.81 (1H,d,*J* = 4.4 Hz), 7.20–7.35 (5H,m) ppm; ^{13}C NMR (100 MHz,$CDCl_3$) δ 25.2, 26.5, 26.7, 27.0, 38.3, 40.6, 50.7, 51.5, 53.8, 54.3, 59.7, 67.7, 72.9, 77.3, 77.7, 81.7, 86.6, 109.6, 109.8, 127.2, 128.6 (2C), 128.7 (2C), 139.4, 171.0, 172.1 ppm; HRMS (Q-TOF) calcd for $C_{27}H_{38}NO_9$ ($M^+ + 1$) 520.2541, found 520.2539.

Carbacephams 3 and 4; carbapenam 9: From the epoxide **19ab** (220 mg, 0.43 mmol), 45 mg of **3** (20%), 16 mg of **4** (7%) and 11 mg of **9** (5%) were isolated in 4 h.

3: The properties of this compound were in agreement with those reported in Ref. [22a].

4: R_f (4:6 hexanes/EtOAc) 0.28; $[\alpha]_D^{25}$ +26 (c 1.0, $CHCl_3$); IR (film) υ 3480, 1761, 1738, 1212, 1065, 700 cm^{-1}; ^1H NMR (400 MHz,$CDCl_3$) δ 1.31 (3H,s), 1.39 (3H,s), 1.46 (3H,s), 1.50 (3H,s), 2.10 (1H,dd,*J* = 4.8 and 12.8 Hz), 2.20 (1H,dd,*J* = 10.8 and 12.8 Hz), 2.33 (1H,br s), 2.65–2.75 (1H,m), 3.05 (1H,t, *J* = 10.6 Hz), 3.55 (3H,s), 3.59 (3H,s), 3.70 (1H,t,*J* = 7.9 Hz), 3.85 (1H,dd,*J* = 4.1 and 8.6 Hz), 3.95–4.00 (3H,m), 4.12–4.16 (2H,m), 4.27 (1H,d,*J* = 6.4 Hz), 4.68 (1H,d,*J* = 4.1 Hz), 7.20–7.40 (5H,m) ppm; ^{13}C NMR (100 MHz,$CDCl_3$) δ 25.3, 26.3, 26.8, 27.0, 35.5, 40.2, 48.2, 50.6, 51.7, 58.8, 59.0, 67.7, 70.4, 77.3, 78.4, 78.8, 85.0, 110.0, 110.3, 127.8 (2C), 128.7, 129.0, 129.2, 138.5, 167.5, 171.7 ppm; HRMS (Q-TOF) calcd for $C_{27}H_{38}NO_9$ ($M^+ + 1$) 520.2541, found 520.2527.

9: R_f (4:6 hexanes/EtOAc) 0.32; $[\alpha]_D^{25}$ +14 (c 1.0, $CHCl_3$); IR (film) υ 3488, 1761, 1738, 1215, 1070, 700 cm^{-1}; ^1H NMR (400 MHz,$CDCl_3$) δ 1.32 (3H,s), 1.45 (3H,s), 1.47 (3H,s), 1.49 (3H,s), 2.25–2.35 (2H,m), 2.62 (1H, dt, *J* = 5.7 and 11.3 Hz), 2.85–2.95 (1H,m), 3.65 (6H,s), 3.77 (1H,t,*J* = 8.2 Hz), 3.98 (1H,dd,*J* = 4.3 and 8.5 Hz), 4.00 (1H,dd,*J* = 4.9 and 11.3 Hz), 4.05–4.10 (1H,m), 4.10 (1H,d,*J* = 11.3 Hz), 4.14 (1H,dd,*J* = 1.5 and 5.5 Hz), 4.15 (1H,t,*J* = 8.5 Hz), 4.39 (1H,dd,*J* = 1.5 and 8.2 Hz), 4.70 (1H,d,*J* = 4.9 Hz), 7.20–7.35 (5H,m) ppm; ^{13}C NMR (100 MHz,$CDCl_3$) δ 25.2, 26.6, 26.8, 27.0, 32.5, 39.6, 48.8, 51.6, 54.3, 59.5, 60.1, 67.6, 68.6, 76.9, 77.5, 83.3, 85.0, 109.2, 109.9, 127.1, 128.5 (3C), 129.2, 139.6, 172.0, 172.6 ppm; HRMS (Q-TOF) calcd for $C_{27}H_{38}NO_9$ ($M^+ + 1$) 520.2541, found 520.2560.

Carbacephams 5 and 6: From the epoxide **19ba** (400 mg, 0.77 mmol), 88 mg of **5** (22%) and 152 mg of **6** (38%) were isolated in 16 h.

5: R_f (4:6 hexanes/EtOAc) 0.30; $[\alpha]_D^{25}$ −20 (c 1.0, $CHCl_3$); IR (film) υ 3466, 1761, 1750, 1215, 1065, 700 cm^{-1}; ^1H NMR (400 MHz,$CDCl_3$) δ 1.31 (3H,s), 1.32 (3H,s), 1.35 (3H,s), 1.59 (3H,s), 2.08 (1H, dd, *J* = 3.2 and 16.2 Hz), 2.37 (1H, dd, *J* = 8.0 and 16.2 Hz), 2.45–2.55 (1H,m), 3.00 (1H,t,*J* = 11.2 Hz), 3.35 (1H,dd,*J* = 4.3 and 7.5 Hz), 3.52 (3H,s), 3.55–3.65 (1H,m), 3.58 (3H,s), 3.95–4.05 (3H,m), 4.05–4.15 (2H,m), 4.20 (1H, dd, *J* = 3.4 and 8.1 Hz), 4.50 (1H,d,*J* = 4.3 Hz), 7.20–7.40 (5H, m) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.4, 26.5, 26.6, 26.8, 32.7, 40.2, 51.6, 53.3, 58.4, 58.5, 59.0, 68.0, 70.2, 76.7, 77.3, 77.7, 83.3, 109.4, 109.7, 127.8 (2C), 129.1 (3C), 138.6, 163.2, 171.7 ppm; HRMS (Q-TOF) calcd for $C_{27}H_{38}NO_9$ ($M^+ + 1$) 520.2541, found 520.2568.

6: The properties of this compound were in agreement with those reported in Ref. [22a].

Carbacephams 7 and 8: From the epoxide **19bb** (200 mg, 0.39 mmol), 30 mg of **7** (15%) and 16 mg of **8** (8%) were isolated in 5 h.

7: R_f (4:6 hexanes/EtOAc) 0.29; $[\alpha]_D^{25}$ −23 (c 1.0, $CHCl_3$); IR (film) υ 3480, 1759, 1738, 1215, 1070, 700 cm^{-1}; ^1H NMR (400 MHz,$CDCl_3$) δ 1.34 (3H,s), 1.36 (3H,s), 1.42 (3H,s), 1.59 (3H,s), 2.17 (1H, dd, *J* = 8.1 and 15.7 Hz), 2.44 (1H, dd, *J* = 5.3 and 15.7 Hz), 3.35–3.45 (1H,m), 3.50 (1H, dd, *J* = 3.2 and 4.8 Hz), 3.57 (3H,s), 3.60 (3H,s), 3.85 (1H, dd, *J* = 1.8 and 4.8 Hz), 4.00–4.12 (4H,m), 4.15 (1H, dd, *J* = 3.5 and 6.0 Hz), 4.19 (1H, t, *J* = 8.3 Hz), 4.30 (1H, dd, *J* = 1.9 and 8.3 Hz), 4.51 (1H, d, *J* = 4.8 Hz), 7.20–7.40 (5H,m) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.2, 25.3, 26.7, 26.8, 32.1, 34.4, 49.9, 51.5, 51.8, 56.6, 59.4, 68.0, 69.1, 76.5, 77.3, 77.7, 84.0, 109.3, 109.7, 127.2, 128.7 (2C), 130.1 (2C), 138.4, 163.7, 172.1 ppm; HRMS (Q-TOF) calcd for $C_{27}H_{38}NO_9$ ($M^+ + 1$) 520.2541, found 520.5980.

8: R_f (4:6 hexanes/EtOAc) 0.25; $[\alpha]_D^{25}$ −6 (c 1.0, $CHCl_3$); IR (film) υ 3480, 1761, 1738, 1215, 1070, 700 cm^{-1}; ^1H NMR (400 MHz,$CDCl_3$) δ 1.26 (3H,s), 1.30 (3H,s), 1.33 (3H,s), 1.37 (3H,s), 2.75–2.85 (2H,m), 3.00–3.10 (1H,m), 3.35 (1H,br s), 3.63 (3H,s), 3.66 (3H,s), 3.74 (1H, d, *J* = 6.4 Hz), 3.90 (1H, dd, *J* = 2.8 and 4.8 Hz), 3.93 (1H, dd, *J* = 6.4 and 8.8 Hz), 4.00–4.05 (2H,m), 4.15 (1H, dd, *J* = 6.0 and 8.7 Hz), 4.23 (1H,d,*J* = 3.2 Hz), 4.45 (1H, dd, *J* = 2.8 and 3.2 Hz), 4.55 (1H, t, *J* = 6.4 Hz), 4.76 (1H, dd, *J* = 0.9 and 4.8 Hz), 7.20–7.35 (5H, m) ppm; ^{13}C NMR (100 MHz,$CDCl_3$) δ 25.3, 26.3, 27.3, 27.4, 29.6, 37.4, 51.4, 53.7, 55.2, 59.4, 59.5, 68.0, 69.5, 77.2, 77.9, 79.4, 84.5, 109.9, 110.1, 126.7, 127.7, 128.7 (3C), 141.3, 165.5, 173.3 ppm; HRMS (Q-TOF) calcd for $C_{27}H_{38}NO_9$ ($M^+ + 1$) 520.2541, found 520.2568.

*4.1.4. Preparation of polycyclic β-lactams **10–18***

Compounds **10–18** were prepared according to the protocols reported in references 22b (bilactam **10** and trilactams **14–18**) and 23 (compounds **11–13**).

4.2. Biological evaluation

4.2.1. Materials

Human Leukocyte Elastase (HLE) and Human Leukocyte Proteinase 3 (HLPR3) were obtained form *Calbiochem–Novabiochem*; Porcine Pancreatic Elastase (PPE) was from *Elastin Products Co. Inc.*; substrate methoxysuccinyl–Ala–Ala–Pro–Val–*p*-nitroanilide, reference inhibitor 3,4–dichloroisocoumarin (DCI) and HEPES [4-(2-hydroxyethyl)-1-piperazinethanesulphonic acid] were from *Sigma–Aldrich*. All other reagents were from *Panreac Química S.A.*

Biological assays [29] were carried out on flat bottom 96-well microtiter plates (BIOTECH, S.L.) in a final reaction volume of 300 μL. Reactions were monitorized at 405 nm using an *Ascent Multiskan Spectrophotometer* (*Thermo Electron Corporation*) connected to a computer (*Main Program of Ascent Software*, 2.6 Version, *Thermo Labsystems*).

4.2.2. Enzyme assays for the inhibition of HLE and HLPR3

[30] Enzymatic activities of HLE and HLPR3 were assayed with the chromogenic substrate, methoxysuccinyl–Ala–Ala–Pro–Val–*p*-nitroanilide, in 50 mM HEPES buffer at pH = 7.8, containing 10% DMSO (dimethyl sulphoxide), by continuous reading of the release of *p*-nitroaniline from the peptide at 405 nm [30]. Enzyme concentrations were 20.4 nM (0.0038 IU per well) for HLE and 70 nM (0.0038 IU per well) for HLPR3.

The reaction evolution was recorded every 30 s for 10 min and the inhibitory activity was calculated at a time of 5 min, when the reaction is still linear and assuming that a 100% of anti–enzyme activity, or 0% of inhibition, corresponds to the absorbance value registered for the control well containing only enzyme + substrate. All experiments were conducted at 37 °C, in triplicate, and repeated at least three times.

3,4-Dichloroisocoumarin (DCI), at a concentration of 10 μM, was throughout used as a reference inhibitor.

4.2.3. Specificity assays

Only the most effective HLE inhibitors among the target molecules were selected for the specificity assays. These assays were

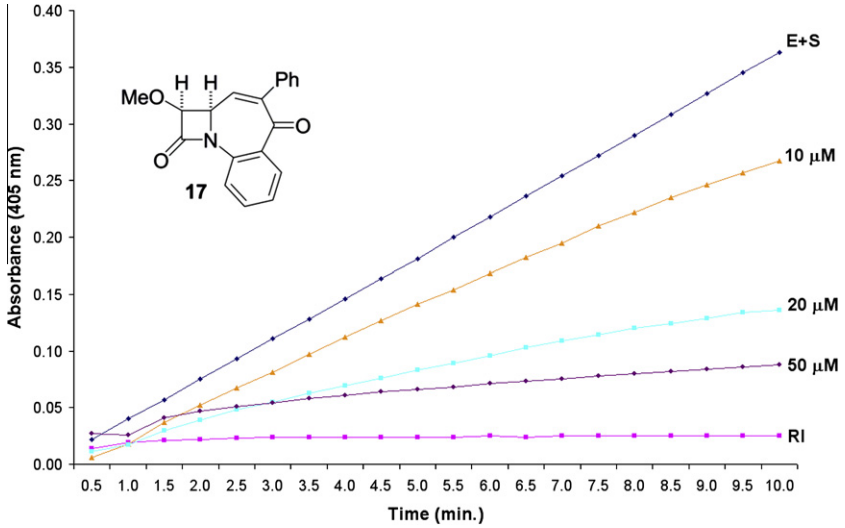


Fig. 5. Graphic plots of absorbance *versus* time for trilactam **17** (E+S: enzyme + sustrate; RI: reference inhibitor 3,4-dichloroisocoumarin).

carried out following the same experimental procedure described for the HLE and HLPR3 inhibition assays but at pH = 8.0, instead of 7.8, following the recommendations of the commercial supplier of the enzyme PPE (HLE activity was not significantly affected by the change in the pH value). HLE and PPE concentration was 20.4 nM.

4.2.4. Data analysis

All the obtained data were treated and analyzed with *Microsoft® Office Excel® 2007 Software* (12.0.6504.5011 Version, *Microsoft Corporation®*). The backgrounds were subtracted from the crude data and the data corresponding to a medium time among the linear region (reaction time of 5 min) were chosen to calculate inhibition percentages. The observed enzymatic activity in the absence of inhibitor (control well) was considered 100% of activity (0% inhibition) and the inhibition percentages were calculated in respect to that value. Progress graphic plots of absorbance *versus* time were obtained for each assay. A representative one is shown in Fig. 5.

IC_{50} values were calculated from dose-response studies performed in triplicate and repeated at least three times. A calculus sheet designed for that purpose with *Microsoft® Office Excel® 2007 Software* (12.0.6504.5011 Version, *Microsoft Corporation®*) was used for these calculations. Both, forecasting analysis based on linear regressions and calculations from the equations of trend curves in each case, produced the same results. K_i values were calculated from IC_{50} measurements according to the expression for a competitive inhibition in the presence of tightly bound inhibitors [31].

$$K_i(\mu M) = (IC_{50} - [E]/2)/([S]/K_m + 1)$$

The concentrations of the reagents in our microassays were as follows: [HLE] = 0.02 μM, [HLPR3] = 0.07 μM, [S] = 100 μM. K_m ($S_{0.5}$) determined for HLE was 152 μM and K_m ($S_{0.5}$) determined for HLPR3 was 112 μM.

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