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New β-lactam monocyclic inhibitors of human elastases: Synthesis and anti-elastase properties of 1-carbamoyl-4-methyleneaminoxyazetidinone derivatives†

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Summary — Some monocyclic β-lactam derivatives of types **II** (**1a–5a**) and **III** (**1b–5b**), designed as analogs of type **I** derivatives, in which the C-4 aryloxy group of **I** is replaced by an oximate moiety, were synthesized and tested in vitro for their inhibitory activity towards porcine pancreatic (PPE) and human leukocyte (HLE) elastases. All compounds were found to be inactive on PPE. While **1b–5b** did not display any appreciable activity towards HLE, compounds **1a–5a** exhibited a marked inhibitory activity on this enzyme. The most active in vitro type-**II** compound **4a** and the derivative **5a**, whose molecular structure presents a carboxylic moiety like type-**I** reference drug **14**, were tested in vivo for their human sputum elastase-inhibitory activity in elastase-induced lung hemorrhage in mice: they proved to possess an appreciable inhibitory activity even if somewhat lower than might have been expected on the basis of their activity indices obtained in the in vitro tests.

elastase inhibitor / β-lactam monocyclic inhibitor / 1-carbamoyl-4-methyleneaminoxyazetidinone derivative

Introduction

Human leukocyte elastase (HLE) is a serine protease whose involvement has been hypothesised in the connective-tissue destruction associated with several pulmonary diseases such as emphysema [2], acute respiratory distress syndrome [3], cystic fibrosis [4] and chronic bronchitis [5]. Compounds able to inhibit HLE may reduce the degradation of connective tissue and therefore may have considerable therapeutic potential for the treatment of these diseases. Several research studies have focused on the development of potent, selective HLE-inhibitors. Among them, some HLE-inhibitors of the class of monocyclic β-lactams of type **I** exhibit a specificity for HLE, together with biochemical and pharmacokinetic properties suitable for their utilization in human diseases [5, 6]. At present, compound L-680,833 [7], ie the chiral type-**I** compound in which R_1 is the carboxymethyl substituent and R_2 is the (*R*)-1-(*p*-tolyl)butyl group, is one of the most promising HLE-inhibitors under development.

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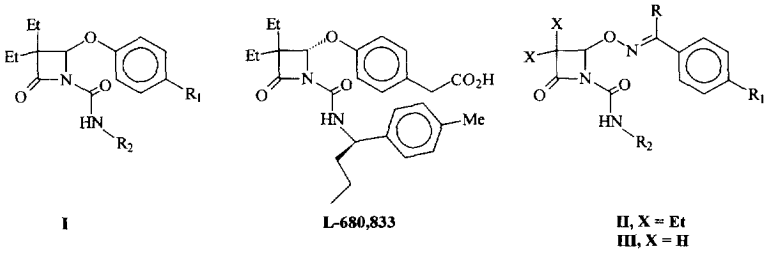
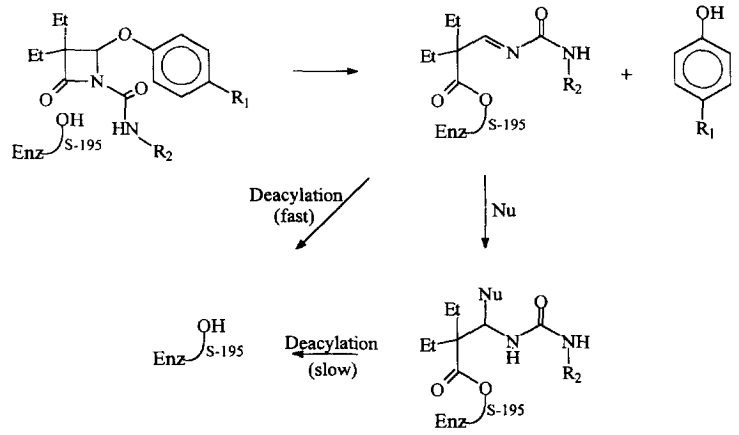


I **L-680,833** **II, X = Et** **III, X = H**

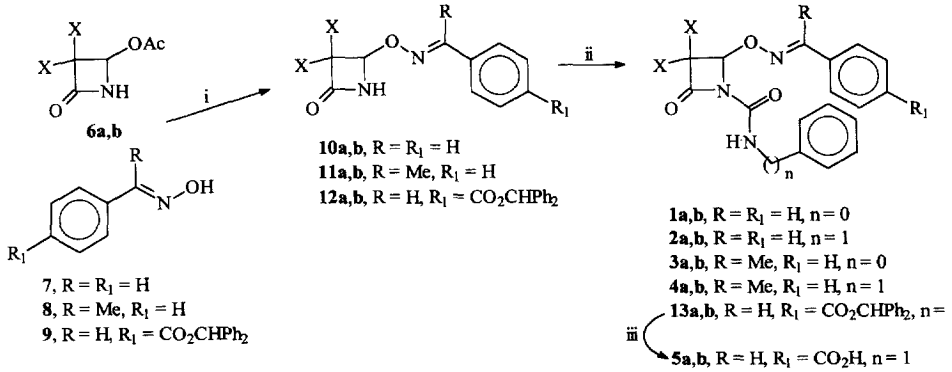
Studies on the mechanism of the inhibition process of HLE by type-**I** compounds have suggested that the first stage of the reaction sequence (scheme 1) which leads to the inactivation of the enzyme consists of the acylation of the active-site serine (S-195) hydroxyl group by the β-lactam carbonyl with subsequent or concerted loss of the C-4 phenol as the leaving-group, to yield an iminic acyl-enzyme intermediate [8]. Further reaction, such as the addition of a nucleophile (Nu) to the iminic moiety of this intermediate, may provide additional stabilization for the initially formed acyl-enzyme, from which, alternatively, the active enzyme might be regenerated by deacylation (scheme 1) [8].

This inhibition mechanism proposed for type-**I** compounds suggested the idea of a study of their analogs in which the C-4 aryloxy group was replaced by other substituents possessing a different order of leaving-group ability. Here we report the synthesis and the HLE-inhibitory properties of certain monocyclic β-lactams of type **II** (**1a–5a**), designed as analogs of type-**I** compounds in which the C-4 aryloxy substituent is replaced by a benzylideneaminoxy group, and of their corresponding analogs of type **III** (**1b–5b**) in which the C-3 ethyl groups of **1a–5a** are lacking (see scheme 2).

The choice for an oximate moiety as a substituent on the C-4 of type-**II** compounds was made in order



Scheme 1.



Reagents and conditions: (i) Oxime, NaOH, Me_2CO/H_2O 1:1, rt, 1h; (ii) PhNCO or BnNCO, DMAP, TEA, CH_2Cl_2, rt, 24h; (iii) TFA/anisole 1:2, 0 °C, 4h

Scheme 2.

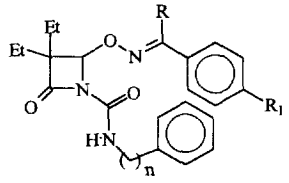
to verify whether the replacement of the aryloxy moiety of type-**I** compounds with a group with a better nucleophilicity, and therefore a worse leaving-group ability [9–12], might lead to positive results.

Chemistry

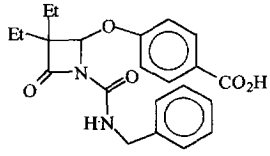
Compounds **1a,b–5a,b** were synthesized as outlined in scheme 2. Base-catalyzed treatment of acetoxyazetidin-2-ones **6a** [8] and **6b** [13] with the known *E* oximes **7**, **8** [14] or the new *E* oxime **9** (obtained by reaction of 4-carboxybenzaldehyde with diphenyldiazomethane and then with hydroxylamine hydrochloride, in the presence of K_2CO_3), gave the corresponding β-lactam derivatives **10–12** which, by reaction with phenyl- or benzylisocyanate, afforded the phenylurea (**1**, **3**) or benzylurea derivatives (**2**, **4**, **13**), respectively. Treatment of **13a,b** with trifluoroacetic acid and anisole afforded the corresponding free acids **5a,b**.

The configuration of the oxime **9** (*E*) was assigned on the basis of the chemical shift value of the proton linked to the iminic carbon, which is practically equal to that of the same proton in the unsubstituted (*E*)-benzaldoxime **7** of known configuration [14]. The configuration around the C=N double bond of the intermediate (**10–13**) and final compounds (**1–5**) was assigned on the basis of the knowledge of the configuration of the starting oximes **7–9** (*E*), bearing in mind that the value of the chemical shift of the signal of the proton(s) linked to the oxime (for **10**, **12**, **13**, **1**, **2**, **5**) or methyl (for **11**, **3**, **4**) carbon is practically unchanged on passing from the starting oximes **7–9** to **10–13** and then to **1–5**.

Table I. Inhibition of human elastases by **1a–5a**.



1-5



14

Compound	*n*	*R*	R_1	*HLE*[a] $k_{inact}/K_I \pm SD$[b] $(M^{-1}\ s^{-1})$	*Lung hem % inhib of HSE*[c]
1a	0	H	H	18000 ± 1200	
2a	1	H	H	33000 ± 1700	
3a	0	Me	H	7600 ± 600	
4a	1	Me	H	41000 ± 2500	38
5a	1	H	CO_2H	6100 ± 500	27
14				2200 ± 300	56[d]

[a]Substrate: *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide. [b]SD: standard deviation. [c]Percentage inhibition in elastase-induced lung hemorrhage in mice when the compound was dosed orally at 30 mg/kg 5 h before challenge with HSE. [d]Value reported in [8] for HLE-induced lung hemorrhage in the hamster.

Biological results and discussion

Compounds **1–5** were initially evaluated for their ability to inhibit in vitro the hydrolysis of the substrate *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide induced by porcine pancreatic elastase (PPE) and HLE, following the procedures described in the *Experimental protocols*. All compounds (**1–5**) were found to be completely inactive on PPE up to 100 μM. As regards HLE, while **1b–5b** did not display any appreciable activity towards this enzyme, compounds **1a–5a** exhibited an appreciable inhibitory activity, with k_{inact}/K_I values ranging from 6100 to 41000 $M^{-1}\ s^{-1}$ (table I). Under the same experimental conditions, compound **14**, taken as a type-**I** reference drug, showed a k_{inact}/K_I value (2200 $M^{-1}\ s^{-1}$) lower than those of all type-**II** compounds (**1a–5a**). The most active in vitro type-**II** compound **4a** and the acid derivative **5a**, which presents a carboxylic moiety like type-**I** reference drug **14**, were also evaluated in vivo for their human sputum elastase (HSE)[1]-inhibitory activity (table I) by the method of elastase-induced lung hemorrhage in mice, described in the *Experimental protocols*. At the dose screened, both compounds showed appreciable percent inhibition values, albeit somewhat lower than might have been expected on the basis of their activity indices obtained in the in vitro tests.

The results obtained for type-**II** compounds **1a–5a** indicate that the replacement of the aryloxy substituent of type-**I** drugs with a benzylideneaminoxy group, leads to an improvement in the in vitro inhibitory properties towards HLE; this claim might be tentatively explained by assuming that the worse leaving-group ability of the oximate moiety with respect to the aryloxy group might exert a positive influence on the kinetic factors which regulate the irreversible process of enzyme inhibition.

[1]It is reported in literature that HSE is both immunologically and catalytically indistinguishable from HLE [15].

The inactivity in the in vitro tests of type-**III** compounds with no substituents on C(3) is consistent with the hypothesis that the interaction of monocyclic β-lactam compounds with the HLE catalytic site requires the presence on the C(3) carbon of a small substituent able to occupy the S-1 specificity pocket of the same enzyme [16]. The inactivity of both type-**II** and type-**III** compounds towards PPE is in agreement with previous findings indicating a high degree of selectivity for human serine proteases on the part of monocyclic β-lactam compounds of type **I**.

Experimental protocols

Chemistry

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. IR spectra for comparison of compounds were taken as paraffin oil mulls or as liquid film on a Mattson 1000 FTIR spectrometer. ^{1}H-NMR spectra were obtained with a Varian CFT-20 instrument operating at 80 MHz in a ca 2% solution of $CDCl_3$ for all compounds, with the only exception of **5a** (Me_2CO–d_6) and **5b** (DMSO–d_6). The proton magnetic resonance assignments were established on the basis of the expected chemical shifts and the multiplicity of the signals. Analytical TLCs were carried out on 0.25 mm layer silica gel plates containing a fluorescent indicator; spots were detected under UV light (254 nm). Column chromatographs were performed using 230–400 mesh silica gel (Macherey-Nagel Silica Gel 60). Magnesium sulphate was always used as drying agent. Evaporations were done in vacuo (rotating evaporator). Elemental analyses were carried out by our analytical laboratory and were consistent with theoretical values to within ± 0.4%.

Benzhydryl (E)-benzaldoxime-4-carboxylate ***9***

A solution of diphenyldiazomethane (6.5 g, 0.033 mol) in $CHCl_3$ (10 mL) was added portionwise to an unstirred solution of 4-carboxybenzaldehyde (5 g, 0.033 mol) in DMF (25 mL). The resulting mixture was poured into a saturated aqueous $NaHCO_3$ solution (100 mL) at 0 °C and then extracted with $CHCl_3$ (2 x 100 mL). The organic phase was dried and evaporated to give an oily residue, which after crystallization with AcOEt–hexane, yielded 4.95 g of pure benzhydryl benzaldehyde-4-carboxylate [48%; mp 87–88 °C; ^{1}H-NMR δ 7.11 (s, 1H), 7.21–7.36 (m, 10 H), 7.90 and 8.20 (AA'BB' system, 4H, J = 8.8 Hz), 10.04 (s, 1H). Anal $C_{21}H_{16}O_3$ (C, H)]. Benzhydryl benzaldehyde-4-carboxylate (4.8 g, 0.015 mol) was dissolved in THF (90 mL) and the resulting solution, after being cooled at 0 °C, was treated with an aqueous solution of 1 M K_2CO_3 (10 mL) and then, portionwise, with an aqueous solution of 0.55 M hydroxylamine hydrochloride (36 mL). The resulting mixture was stirred at room temperature for 24 h and then acidified at 0 °C (pH 5) with aqueous 5% HCl. After evaporation of THF, the aqueous layer was diluted with water (50 mL) and extracted with Et_2O (2 x 100 mL). The organic phase was dried and evaporated to give a solid residue which, after crystallization with AcOEt–hexane yielded pure **9** (4.47 g, 90%): mp 124–125 °C; ^{1}H-NMR δ 7.15 (s, 1H), 7.27–7.39 (m, 10 H), 7.65 and 8.15 (AA'BB' system, 4H, J = 8.8 Hz), 8.17 (s, 1H). Anal $C_{21}H_{17}NO_3$ (C, H, N).

General procedure for the preparation of the (E)-4-(arylideneaminoxy)-2-azetidinones ***10a,b–12a,b***

1 N NaOH (6.5 mL) was added to a stirred solution of **7** [14], **8** [14], or **9** (6.5 mmol) in acetone (6.5 mL). After stirring for 10 min at room temperature, a solution of **6a** [8] or **6b** [13] (5.8 mmol) in acetone (3.5 mL) was added, and the resulting mixture was stirred at the same temperature for 40 min. The acetone was then removed in vacuo and the aqueous layer was extracted with ether. The organic phase was washed with brine, dried and evaporated to give an oily residue which, after purification by flash chromatography eluting with 2:1 hexane–AcOEt mixture, yielded the appropriate pure azetidinone **10–12**.

10a (47%): mp 69–70 °C; ^{1}H-NMR δ 1.01 (t, 3H, J = 7 Hz), 1.03 (t, 3H, J = 7 Hz), 1.63–1.97 (m, 4H), 5.50 (s, 1H), 7.33 (br, 1H), 7.38–7.64 (m, 5H), 8.15 (s, 1H). Anal $C_{14}H_{18}N_2O_2$ (C, H, N).

10b (54%): mp 74–75 °C; ^{1}H-NMR δ 3.11–3.17 (m, 2H), 5.74 (dd, 1H, J = 2.4 and 4.0 Hz), 7.25 (br, 1H), 7.33–7.62 (m, 5H), 8.14 (s, 1H). Anal $C_{10}H_{10}N_2O_2$ (C, H, N).

11a (45%): mp 76–77 °C; ^{1}H-NMR δ 1.02 (t, 3H, J = 7 Hz), 1.05 (t, 3H, J = 7 Hz), 1.65–1.99 (m, 4H), 2.30 (s, 3H), 5.60 (s, 1H), 6.70 (br, 1H), 7.40–7.77 (m, 5H). Anal $C_{15}H_{20}N_2O_2$ (C, H, N).

11b (57%): mp 79–80 °C; ^{1}H-NMR δ 2.20 (s, 3H), 3.10–3.18 (m, 2H), 5.79 (dd, 1H, J = 2.4 and 4.0 Hz), 6.83 (br, 1H), 7.30–7.80 (m, 5H). Anal $C_{11}H_{12}N_2O_2$ (C, H, N).

12a (40%): mp 137–138 °C; ^{1}H-NMR δ 1.10 (t, 3H, J = 7 Hz), 1.12 (t, 3H, J = 7 Hz), 1.63–2.02 (m, 4H), 5.56 (s, 1H), 6.48 (br, 1H), 7.16 (s, 1H), 7.22–7.41 (m, 10H), 7.68 and 8.17 (AA'BB' system, 4H, J = 8 Hz), 8.24 (s, 1H). Anal $C_{28}H_{28}N_2O_4$ (C, H, N).

12b (50%, oil): ^{1}H-NMR δ 3.11–3.21 (m, 2H), 5.54 (dd, 1H, J = 2.4 and 4.0 Hz), 6.45 (br, 1H), 7.09 (s, 1H), 7.21–7.43 (m, 10H), 7.60 and 8.10 (AA'BB' system, 4H, J = 8.8 Hz), 8.13 (s, 1H). Anal $C_{24}H_{20}N_2O_4$ (C, H, N).

General procedure for the preparation of the (E)-4-(arylideneaminoxy)-1-N-(phenylaminocarbamoyl)-2-azetidinones ***1a,b, 3a,b*** *and (E)-4-(arylideneaminoxy)-1-N-(benzylaminocarbamoyl)-2-azetidinones* ***2a,b, 4a,b, 13a,b***

A stirred solution of the appropriate azetidinone **10a,b–12a,b** (1 mmol), Et_3N (0.14 mL, 1 mmol) and a catalytic amount of 4-(dimethylamino)pyridine (DMAP, 2 crystals) in anhydrous CH_2Cl_2 (5 mL), was treated under nitrogen with phenylisocyanate (0.33 mL, 3 mmol) or benzylisocyanate (0.37 mL, 3 mmol), and the mixture was stirred at room temperature for 24 h. The solvent was evaporated and the residue was purified by flash chromatography (in the case of **1**, **3**, using a 3:1:2 hexane–AcOEt–CH_2Cl_2 mixture as eluent) and then by crystallization with *i*-PrOH, or by crystallization with *i*-PrOH (in the case of **2**, **4**, **13**) to give pure **1–4** and **13**.

1a (56%): mp 100–101 °C; ^{1}H-NMR δ 1.05 (t, 6H, J = 7 Hz), 1.71–2.03 (m, 4H), 5.96 (s, 1H), 6.94–7.65 (m, 10H), 8.14 (s, 1H), 8.45 (br, 1H); IR ν 1776 cm^{-1} (β-lactam C=O). Anal $C_{21}H_{23}N_3O_3$ (C, H, N).

1b (67%): mp 136–137 °C; ^{1}H-NMR δ 3.42 (m, 2H), 6.15 (dd, 1H, J = 4 and 2 Hz), 7.07–7.65 (m, 10H), 8.20 (s, 1H), 8.38 (br, 1H); IR ν 1775 cm^{-1} (β-lactam C=O). Anal $C_{17}H_{15}N_3O_3$ (C, H, N).

2a (65%): mp 108–109 °C; ^{1}H-NMR δ 1.02 (t, 6H, J = 7 Hz), 1.66–2.00 (m, 4H), 4.46 (d, 2H, J = 6 Hz), 6.48 (br, 1H), 7.22–7.70 (m, 10H), 8.13 (s, 1H); IR ν 1776 cm^{-1} (β-lactam C=O). Anal $C_{22}H_{25}N_3O_3$ (C, H, N).

2b (81%): mp 94–95 °C; ^{1}H-NMR δ 3.38 (m, 2H), 4.49 (d, 2H, J = 6 Hz), 6.12 (dd, 1H, J = 4 and 2 Hz), 6.80 (br, 1H),

7.28–7.68 (m, 10H), 8.23 (s, 1H); IR ν 1779 cm^{-1} (β-lactam C=O). Anal $C_{18}H_{17}N_3O_3$ (C, H, N).

3a (67%): mp 91–92 °C; ^{1}H-NMR δ 0.97–1.25 (m, 6H), 1.70–2.03 (m, 4H), 2.28 (s, 3H), 5.99 (s, 1H), 7.02–7.71 (m, 10H), 8.50 (br, 1H); IR ν 1778 cm^{-1} (β-lactam C=O). Anal $C_{22}H_{25}N_3O_3$ (C, H, N).

3b (70%): mp 133–134 °C; ^{1}H-NMR δ 2.30 (s, 3H), 3.40 (m, 2H), 6.22 (dd, 1H, J = 4 and 2 Hz), 7.02–7.68 (m, 10H), 8.41 (br, 1H); IR ν 1774 cm^{-1} (β-lactam C=O). Anal $C_{18}H_{17}N_3O_3$ (C, H, N).

4a (59%): mp 113–114 °C; ^{1}H-NMR δ 1.02 (t, 6H, J = 7 Hz), 1.59–2.00 (m, 4H), 2.27 (s, 3H), 4.46 (d, 2H, J = 6 Hz), 5.92 (s, 1H), 6.88 (br, 1H), 7.18–7.69 (m, 10H); IR ν 1774 cm^{-1} (β-lactam C=O). Anal $C_{23}H_{27}N_3O_3$ (C, H, N).

4b (87%): mp 111–112 °C; ^{1}H-NMR δ 2.34 (s, 3H), 3.37 (m, 2H), 4.48 (d, 2H, J = 6 Hz), 6.16 (dd, 1H, J = 4 and 2 Hz), 6.88 (br, 1H) 7.28–7.72 (m, 10H); IR ν 1773 cm^{-1} (β-lactam C=O). Anal $C_{19}H_{19}N_3O_3$ (C, H, N).

13a (40%): mp 104–105 °C; ^{1}H-NMR δ 1.09 (t, 6H, J = 7 Hz), 1.71–1.92 (m, 4H), 4.52 (d, 2H, J = 5.6 Hz), 5.50 (s, 1H), 6.82 (br, 1H), 7.15 (s, 1H), 7.28–7.41 (m, 10H), 7.70 and 8.15 (AA'BB' system, 4H, J = 8 Hz), 8.23 (s, 1H); IR ν 1780 cm^{-1} (β-lactam C=O). Anal $C_{36}H_{35}N_3O_5$ (C, H, N).

13b (54%): mp 100–101 °C; ^{1}H-NMR δ 2.78–3.50 (m, 2H), 4.44 (d, 2H, J = 5.6 Hz), 6.10 (dd, 1H, J = 2.4 and 4 Hz), 7.08 (s, 1H), 7.26–7.43 (m, 10H) 7.63 and 8.10 (AA'BB' system, 4H, J = 8.8 Hz), 8.21 (s, 1H); IR ν 1780 cm^{-1} (β-lactam C=O). Anal $C_{32}H_{27}N_3O_5$ (C, H, N).

General procedure for the preparation of the (E)-4-(arylidene-aminoxy)-1-N-(benzylaminocarbamoyl)-2-azetidinones ***5a,b***

Trifluoroacetic acid (2 mL) was added dropwise to a stirred and cooled solution of **13a,b** (0.5 mmol) in anisole (4 mL) and the resulting mixture was stirred at 0 °C for 4 h and extracted with an ice-cooled 10% aqueous $NaHCO_3$ solution (3 x 25 mL). The aqueous layer, after being washed with $CHCl_3$ (2 x 30 mL), was acidified to pH 3.5 at 0 °C by the addition of 10% aqueous H_3PO_4 and then extracted with $CHCl_3$ (3 x 30 mL). Evaporation of the washed (brine) organic extracts yielded a white solid residue, which, after crystallization with AcOEt–hexane, gave pure **5a**, **5b**.

5a (72%): mp 170–171 °C; ^{1}H-NMR δ 1.08 (t, 6H, J = 8 Hz), 1.71–1.95 (m, 4H), 4.52 (d, 2H, J = 6 Hz), 5.99 (s, 1H), 7.10 (br, 1H), 7.34 (s, 5H), 7.82 and 8.12 (AA'BB' system, 4H, J = 8 Hz), 8.40 (s, 1H); IR ν 1774 cm^{-1} (β-lactam C=O). Anal $C_{23}H_{25}N_3O_5$ (C, H, N).

5b (76%): mp 194–195 °C; ^{1}H-NMR δ 3.48 (m, 2H) 4.42 (d, 2H, J = 6 Hz), 6.12 (dd, 1H, J = 4 and 2 Hz), 6.98 (br, 1H), 7.23 (s, 5H), 7.61 and 8.00 (AA'BB' system, 4H, J = 9 Hz), 8.24 (s, 1H); IR ν 1775 cm^{-1} (β-lactam C=O). Anal $C_{19}H_{17}N_3O_5$ (C, H, N).

Enzyme inhibition assays

HLE inhibition

The activity of HLE (EC 3.4.21.37, Calbiochem) was assayed spectrophotometrically using *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (MAAPVNA) as the substrate [17] in 0.05 M sodium phosphate buffer, pH 7.8; the hydrolytic release of *p*-nitroaniline was monitored on the basis of the increase in absorbance at 390 nm (ε for MAAPVNA = 500 M^{-1} cm^{-1}; ε for *p*-nitroaniline = 13000 M^{-1} cm^{-1}) with a Beckman DU-7 UV/visible spectrophotometer. Absorbance values were collected every 10 sec over a 30 min period and stored for subsequent analysis. Both control and inhibitor test reaction mixtures were run; the substrate was dissolved in MeOH, whereas the inhibitors were dissolved in DMSO.

Apparent first-order decay rate constants (k'_{obs}) for HLE were evaluated by the time-dependent change in enzyme activity in assay mixtures containing the inhibitor in molar excess (from 0.1 to 2 μM concentration) with respect to HLE (16 nM), by comparison with the activity of an assay from which the inhibitor was omitted. $1/k'_{obs}$ values obtained at constant substrate concentration were plotted vs 1/[I]. The reciprocal of the slope of this secondary plot yields $k_{inact}/K_I(1 + [S]/K_M)$; this value was multiplied by $(1 + [S]/K_M)$ to get k_{inact}/K_I values.

PPE inhibition

The potential effect of HLE inhibitors on porcine pancreatic elastase (PPE, EC 3.4.21.36, Sigma) was assayed at pH 7.8 in 0.05 M sodium phosphate buffer, using MAAPVNA as the substrate [18]. Assay mixtures contained, in a final volume of 1.00 mL, 50 μg of PPE, 0.3 μmol of substrate and 2 nmol of inhibitor, which was omitted in parallel control assays. The increase in absorbance at 390 nm due to the hydrolytic release of *p*-nitroaniline was followed with a Beckman DU-7 UV-visible spectrophotometer. Neither zero-time inhibition nor time-dependent inactivation over a 30 min interval was observed on PPE by any of the five inhibitors tested.

HSE-induced lung hemorrhage in mice

Mice were anaesthetized with a mixture of ketamine and xylazine ip, and 50 μL of saline (vehicle) or human sputum elastase (Elastin Products Company inc) (3200 U/mL dissolved in saline, corresponding to a solution of 16 μg/50 μL) was instilled intratracheally. Test compounds were orally administered at 30 mg/kg as a suspension of DMSO in Methocel, 5 h before enzyme challenge. 1.5 h after elastase instillation, the animals were sacrificed by CO_2 asphyxiation and the trachea was exposed. The lungs were washed using a 600 μL saline-filled syringe connected to a tracheal cannula by gently expanding the lungs and then withdrawing the saline. This operation was repeated 3 times, yielding a final volume of about 1.5 mL of bronchoalveolar lavage fluid (BAL) for each animal. The BAL fluids were diluted with Na_2CO_3 and sonicated to ensure cell disruption. The blood content was determined spectrophotometrically at 414 nm. The amount of blood in each BAL sample was calculated by a standard curve obtained using sonicated whole mouse blood supplemented with Na_2CO_3 2% p/v. The hemorrhage was expressed as μL equivalents of blood in 1 mL of BAL fluid. The protective effect of the compounds was calculated as a percentage of inhibition of lung hemorrhage in treated versus control animals. The basal value (vehicle) was subtracted from each treatment group.

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