Eur. J. Med. Chem. 35 (2000) 53−67

S0223523400001112/FLA

Original article

Synthesis, inhibitory activity towards human leukocyte elastase and molecular modelling studies of 1-carbamoyl-4-methyleneaminoxyazetidinones

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Received 6 May 1999; revised 7 July 1999; accepted 15 July 1999

Abstract – Some monocyclic β-lactam derivatives of type **3** (MAOAs) in which the leaving group (LG) on the C(4) is a methyleneaminoxy moiety, were synthesised and tested in vitro and in vivo for their inhibitory activity towards human leukocyte elastase (HLE). Some compounds showed an appreciable in vitro inhibitory activity against this enzyme. Effects on the anti-HLE activity due to the nature of the substituents R and R_1 present on their LG were observed and rationalised by means of molecular modelling techniques. The results of in vivo pharmacological tests indicated that MAOAs, while showing an inhibitory activity on the haemorrhage induced by HLE, did not exhibit any effects due to the R and R_1 substituents. © 2000 Éditions scientifiques et médicales Elsevier SAS

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

1. Introduction

Human leukocyte elastase (HLE) is a serine proteinase which is produced by neutrophils and is released from their azurophilic granules upon physiological stimulation [1]. This proteinase is one of the degradative enzymes involved in phagocytosis and plays a role in the homeostatic regulation systems. Its extracellular activity is normally regulated by circulating plasma and tissue-localised natural protease inhibitors. The principal role of intracellular HLE appears to be the degradation of foreign proteins ingested by leukocytes during phagocytosis, whereas the main target for extracellular elastase appears to be certain structural proteins such as elastin [2], laminin [3], fibronectin [4, 5], collagen and proteoglycan [6–10].

A pathological derangement of one of the natural controls of the activity of HLE may lead to its hyperexpession, which in turn may bring about an uncontrolled destruction of connective tissue, which is typical of various diseases. An excessive proteolysis of elastin by HLE has been considered to be responsible for several chronic pulmonary diseases such as emphysema, acute respiratory distress syndrome, cystic fibrosis and chronic bronchitis [11–14]. Compounds capable of inhibiting HLE may restore the balance between the free enzyme and endogenous antiproteinases, which is found to be altered in these pathologies, and therefore may have considerable therapeutic potential. Several studies have focused upon the development of potent, selective synthetic HLE-inhibitors, whether of a proteic [15–20] or non-proteic nature [21–33]. Among these last types of inhibitors, some monocyclic β-lactams **1**, substituted on the C-4 by an arylethereal moiety, have proved to possess HLE specificity and biopharmacological properties useful for their clinical utilisation (*figure 1*) [28–30].

For these compounds, as well as for other classes of non-proteic inhibitors, the anti-elastase activity appears to be linked to the presence, on their reactive nucleus, of an appropriate leaving group (LG) (*figure 2*). The expul-

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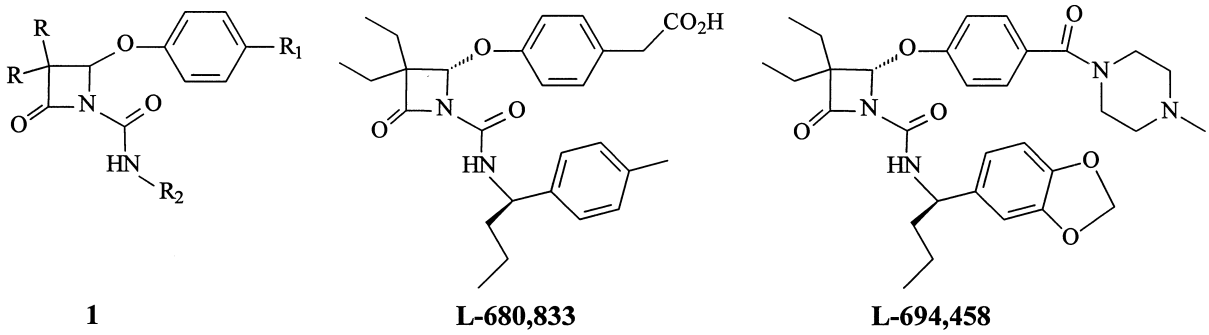


1 **L-680,833** **L-694,458**

Figure 1.

sion of this group leads to the formation of a new reactive electrophilic species, in some cases composed of an imine (**E-I**), capable of binding to the His-57 of the active site of the enzyme, thus creating an enzyme–inhibitor complex (**E-I***) stabilised by the presence of two covalent bonds between the two groups of the catalytic site, from which the enzyme can no longer be regenerated [21–33].

This type of mechanism suggested to us the idea of studying analogues of monocyclic β-lactams **1** in which their LG (i.e. the C-4 aryloxy group) is replaced by a substituent with different leaving-group characteristics, such as the oximate moiety. Accordingly some methyleneaminoxyazetidinones (MAOAs) of types **2** and **3** were synthesised and tested for their inhibitory activity towards HLE. While the type **2** derivatives proved to be inactive towards this enzyme, the 3,3-diethyl derivatives **3** proved to possess a good anti-HLE inhibitory activity, maintaining the same selectivity as the aryloxy type **1** compounds towards this enzyme (*figure 3*) [34].

These promising results obtained with a limited number of compounds suggested to us the idea of gaining insight into the structure–activity relationships in this class of β-lactams by studying the effects on the inhibitory activity towards HLE of substitution on the methyleneaminoxy molecular portion, which represents the LG in this class of derivatives (*figure 2*). On the basis of the observation that the MAOA **3b** (R = Me), previously studied, proved to possess a better inhibitory activity with respect to the unsubstituted MAOA **3a** (R = H), we decided to evaluate first the effects on the anti-HLE activity of the presence on the methyleneaminoxy group of substituents R with the greatest steric hindrance, while maintaining on the same molecular portion the phenyl group, which is present as the second substituent in the MAOAs **3**. A substituent R bulkier than the methyl group might interact with a suitable hydrophobic region of the enzyme active site, thus increasing the forces involved in the binding of the enzyme–inhibitor complex and consequently the activity. On this basis, we synthesised the new MAOAs **3c** and **3d** in which the substituent R is respectively an ethyl and a phenyl group. Unfortunately, these new MAOAs, tested in vitro for their inhibitory activity towards HLE, appeared to be less active than the methyl-substituted analogue **3b**. Therefore, at this point, we decided to verify the effects on the anti-HLE activity of the introduction of a polar group, R_1, in the *para* position of the phenyl group which is present as the second substituent on the methyleneaminoxy molecular portion in the more active MAOAs **3a** and **3b** previously studied. As R_1 substituents for these new MAOAs, we chose carboxyl (**3g** and **3h**) and the *O*-ethyl-*N*-morpholino group (**3i** and **3l**) (negatively and positively charged under physiological conditions, respectively) and a hydrogen bonding acceptor, like the fluorine atom of **3e** and **3f** (*figure 3*). This structural modification might improve the aqueous solubility of the new compounds, increasing their in vivo activity through an increase in their bioavailability. Also for type **1** compounds, the presence of an acidic or basic group, as in **L680,833** and **L694,458**, at present the leaders in this class of compounds, had appeared to improve the biopharmacological properties.

2. Chemistry

Compounds **3c–f** and **3h–l** were prepared as outlined in the scheme indicated in *figure 4*. Base-catalysed treatment of 3,3-diethyl-4-acetoxyazetidin-2-one **11** [35] with the benzophenone oxime **12d** [36] or the *E* [37] oximes **12c** [38], **12e** [39], **12f** [39] and **12h–l** gave the corresponding β-lactam intermediates **13c–l** which, by reaction with benzylisocyanate in the presence of triethylamine (TEA), afforded the benzylurea derivatives

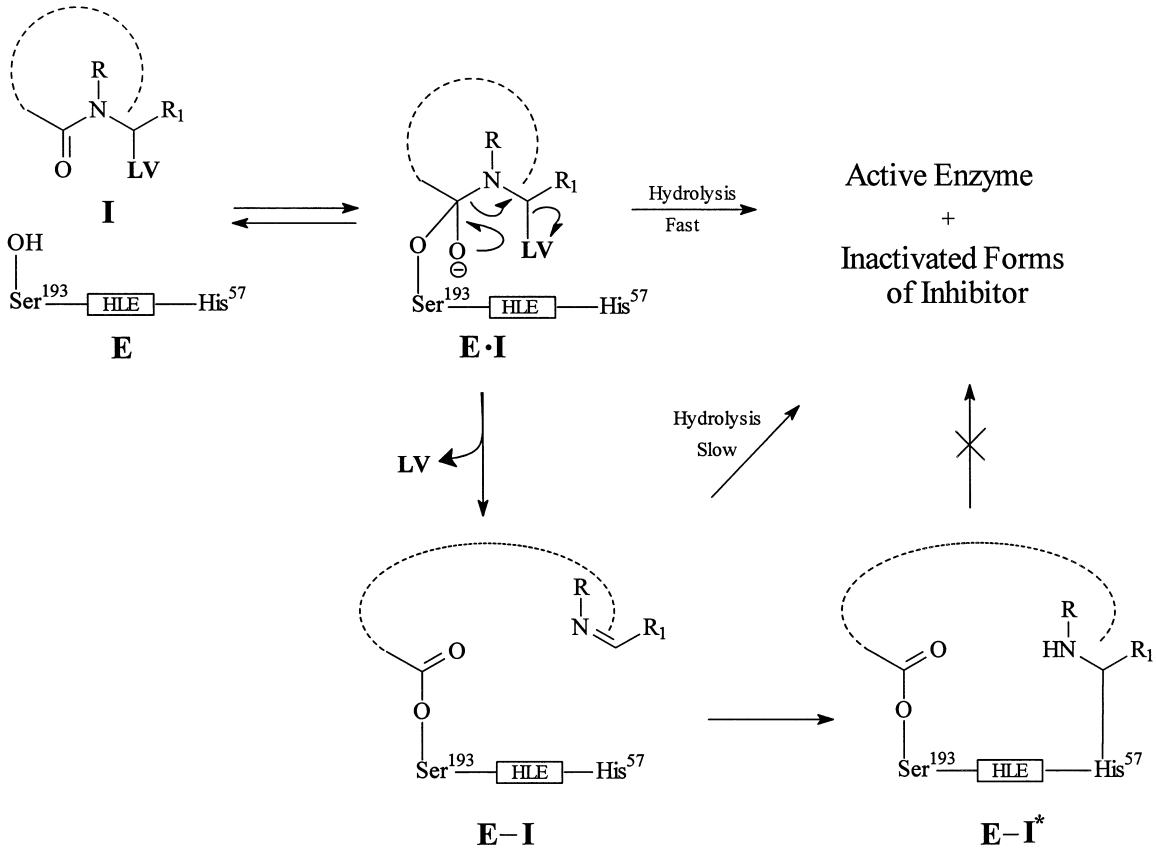
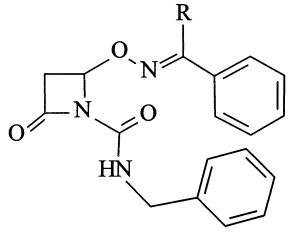


Figure 2.

3c–**14h**, respectively. Treatment of **14h** with trifluoroacetic acid and anisole afforded the corresponding free acid **3h**.

The *E* oxime **12h** was synthesised by reaction of 4-acetylbenzoic acid **15** with diphenyldiazomethane and then with hydroxylamine hydrochloride in the presence of potassium carbonate. The *E* oximes **12i** and **12l** were synthesised by Mitsunobu [40] reaction of phenols **17** and **18** with 4-(2-hydroxyethyl)morpholine and then by treatment of the carbonyl derivative **19** and **20** with hydroxylamine hydrochloride in basic media (*figure 5*).

The syn relationship around the double bond of the methyleneaminoxy molecular portion, between the oxime oxygen and the alkyl substituent linked to the iminic carbon atom, of the newly synthesised oximes **12h**–**l**, was assigned by ^{1}H-NMR study of the shielding induced by an anisotropic solvent (C_6D_6) on **12h**–**l** in the unprotonated and protonated forms of the oximic nitrogen (see Experimental section). For these compounds, in agreement with findings for aryl-alkyl or aryl-hydrogen analogues of unsymmetrical oxime ethers which present a syn relationship between the oximic oxygen and the alkyl or the hydrogen iminic substituent [41], the protonation of the oximic nitrogen determines an appreciable shift of the signal of the nearest protons of the alkyl groups or the signals of the hydrogen, linked to the adjacent iminic carbon atom, to a higher field, depending on the quantity of acid added to the oximic compound. The *E* geometry around the C=N double bond of the oxime ether intermediates **13c**–**l** and **14h** and therefore of the MAOAs **3c**–**l** was assigned on the basis of the knowledge of the *E* configuration of the starting oximes, bearing in mind that these oximes have proved to be configurationally stable under the different synthetic procedures used for the preparation both of **13c**–**l** and **14h** and of the desired new azetidinone derivatives **3c**–**f** and **3h**–**l** (see Experimental).



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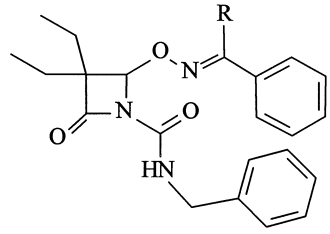
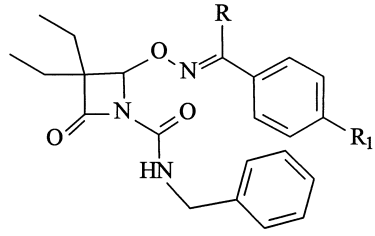


3a, R = H
3b, R = Me,



3c, R = Et, R_1 = H **3g**, R = H, R_1 = CO_2H
3d, R = Ph, R_1 = H **3h**, R = Me, R_1= CO_2H
3e, R = H, R_1 = F **3i**, R = H, R_1 = *O*-Ethyl-*N*-morpholino
3f, R = Me, R_1 = F **3l**, R = Me, R_1 = *O*-Ethyl-*N*-morpholino

Figure 3.

3. Biological results

The newly synthesised MAOAs **3c–f** and **3h–l**, like those previously described, **3a**, **3b** and **3g**, were evaluated for their ability to inhibit HLE-catalysed hydrolysis of the substrate *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide. *Table I* shows the inhibitory activity of MAOAs **3** towards this enzyme as a concentration-independent second-order rate constant k_{inact}/K_I. *Table I* also shows the inhibitory activity observed in the same test for two type **1** drugs used as references: compound **1a**, already used in our preceding paper [34], and compound **L680,833**, one of the two leaders in this class of derivatives. All the MAOAs **3** tested were found to possess an appreciable inhibitory activity, with values of k_{inact}/K_I ratio ranging from 3 400 $M^{-1}s^{-1}$ for **3d** to 98 300 $M^{-1}s^{-1}$ for **3f**.

In the MAOAs **3** unsubstituted on the phenyl group linked to the methyleneaminoxy molecular portion, the activity increased from **3a** (R = H, k_{inact}/K_I = 30 900 $M^{-1}s^{-1}$) to **3b** (R = Me, k_{inact}/K_I = 44 000 $M^{-1}s^{-1}$). In contrast, the activity decreased slightly going to **3c** (R = Et, k_{inact}/K_I = 8 000 $M^{-1}s^{-1}$) and to **3d** (R = Ph, k_{inact}/K_I = 3 400 $M^{-1}s^{-1}$), which represents the worst derivative in the whole new series.

Among the MAOAs **3e–l**, substituted on the phenyl group of the methyleneaminoxy molecular portion, **3f**, **3h** and **3l**, which contain a methyl on the iminic carbon of the LG, showed, in all cases, better values of the k_{inact}/K_I ratio than the corresponding analogues **3e**, **3g** and **3i**, in which a hydrogen is present in the place of the methyl on the same carbon atom. This difference is particularly marked in the *para*-fluorine substituted compounds where the methyl-substituted **3f**, the most interesting compound in the whole series, presents a value of k_{inact}/K_I = 98 300 $M^{-1}s^{-1}$, about sixteen times higher than the unsubstituted **3e** analogue.

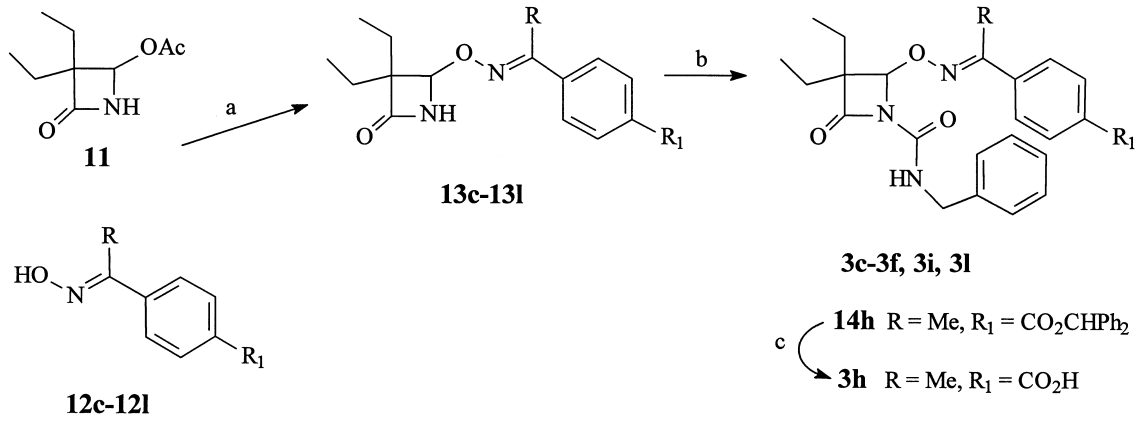


Figure 4. (a) Oxime, NaOH, Me_2CO/H_2O 1:1, r.t., 1h; (b) BnNCO, DMAP, TEA, CH_2Cl_2, r.t., 24h; (c) TFA/anisole 1:2, 0°C, 4h.

Under the same experimental conditions, the MAOAs **3** showed a similar inhibitory activity to those of the reference drugs. All the MAOAs studied, **3a–l**, showed activity from 1.5–44 times higher than that of compound **1a**. The most active MAOA **3f** displayed an inhibitory activity about 6 times lower than that of **L-680,833**.

All the MAOAs **3** and the reference drug **L-680,833** were also evaluated in vivo for their HLE-inhibitory activity by the method of elastase-induced lung haemorrhage in the mouse [34]. As confirmed by the data shown in *table I*, only some of the MAOAs **3** studied showed an appreciable inhibitory property on HLE-induced lung

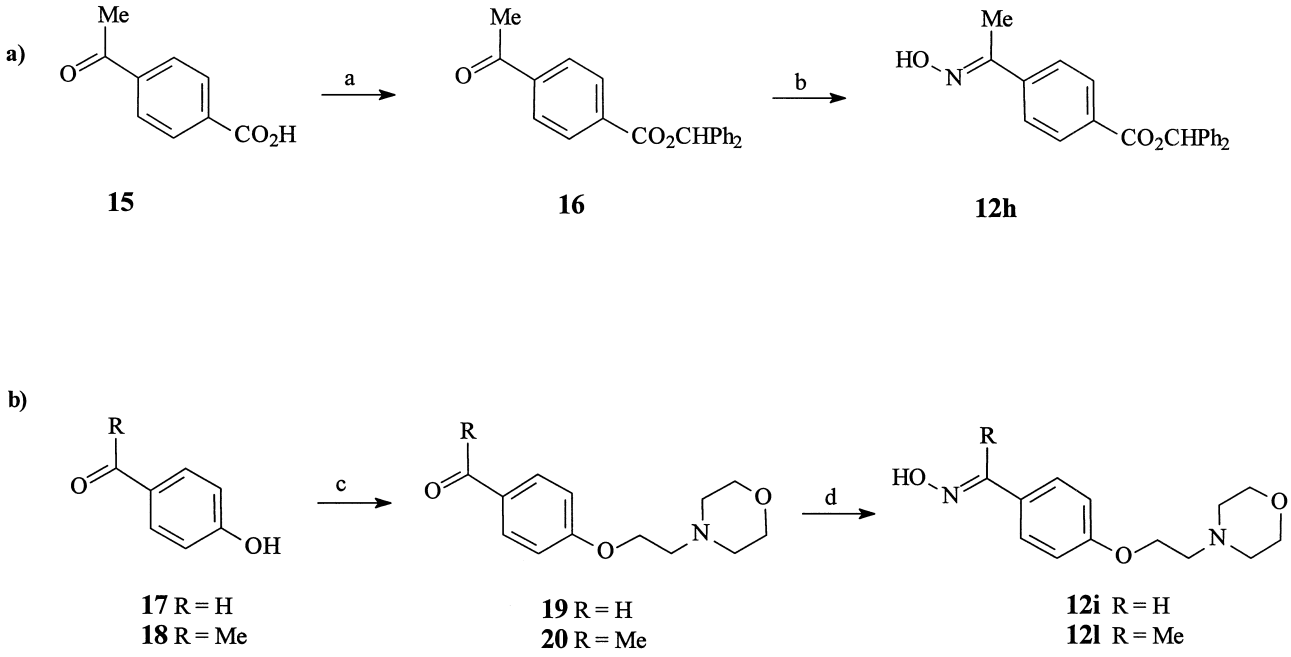
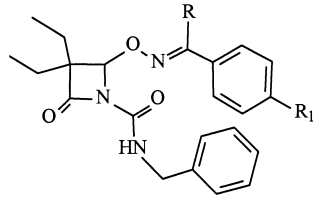


Figure 5. (a) Diphenyldiazomethane, $CHCl_3$/DMF, r.t.; (b) $NH_2OH{\cdot}HCl$, K_2CO_3 1 M sol./THF, 0°C, r.t. 24h; (c) 4-(2-hydroxyethylmorpholine), TPP, DEAD, dry THF, r.t., 24h; (d) $NH_2OH{\cdot}HCl$, K_2CO_3, $EtOH/H_2O$, r.t., 24h.

Table I. In vitro and in vivo inhibition of HLE by **3a**–**l**.



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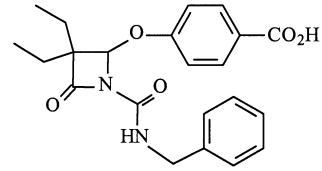
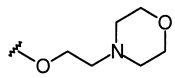
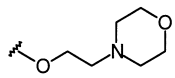


1a

Compound	R	R_1	HLE[a] k_{inact}/K_I $M^{-1}s^{-1}$ (± SD)[b]	Lung haem. % inhib. of HLE[c] (± SD)
3a	H	H	30 900 (3 100)	39 (12)
3b	Me	H	44 000 (3 900)	43 (17)
3c	Et	H	8 000 (700)	*
3d	Ph	H	3 400 (300)	34 (15)
3e	H	F	6 000 (800)	19 (15)
3f	Me	F	98 300 (9 000)	31 (18)
3g	H	COOH	6 100 (700)	14 (5)
3h	Me	COOH	13 500 (1 400)	*
3i $H_2C_2O_4$	H		17 900 (1 900)	21 (9)
3l $H_2C_2O_4$	Me		35 200 (3 300)	*
1a			2 200 (300)	56**
L680,833			590 000 (80 000)	100 (0.02)

[a]Substrate: *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide. [b]SD: standard deviation. [c]Percentage inhibition in elastase-induced lung haemorrhage in mice when the compound was dosed orally at 10 mg/kg 5 h before challenge with HLE. *Inactive at the dose screened. **Value reported in ref. [35] for HLE-induced lung haemorrhage in hamsters when dosed at 30 mg/kg.

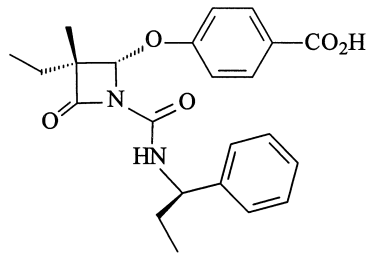
haemorrhage at the dose screened (10 mg/kg). Compounds **3a**, **3b**, **3d** and **3f** at this dose showed a percentage of inhibitory activity ranging from 31% of **3f**, *para*-fluorine phenyl-substituted, to 43% of its analogue **3b**, which is devoid of this type of substituent on the phenyl ring.

4. Molecular models

With the aim of investigating the role of each molecular moiety of the MAOAs **3** in determining interaction with the enzyme, a molecular model of HLE was constructed by means of molecular mechanics and molecular dynamics calculations. For this purpose, we started from the crystallographic structure of HLE complexed with the peptidic inhibitor MSACK (Metoxysuccinyl-Ala-Ala-Pro-Ala-Chloromethylketone) [42]. The MSACK was then substituted with compound **L680,833**, which was placed in the catalytic site, taking into account the experimental data [43] available for compound **L674,197** (*figure 6*), which is structurally similar to it. Subsequently, the complex thus obtained was minimised. At this point, all MAOAs **3a**–**l** were inserted in turn into the catalytic site in place of **L680,833** and every complex was minimised.

The HLE–inhibitor complexes were modelled in a situation in which no bonds were present between the inhibitor and the enzyme.

Figure 7 shows an overall view of HLE complexed with the MAOA **3b** (R = Me). Compound **3b** is inserted into the very hydrophobic pocket composed of the residues of the sites S1 (Val190, Phe192, Ala213, Val216 and Phe228), S1′ (Phe41, Cys42 and Gly43) and S2 (His57, Leu99 and Phe215). The innermost part of the pocket is occupied by the two ethyl groups linked to the C-3 of the β-lactam nucleus, which are enclosed by the



L-674,194

Figure 6.

residues of the S1 site. The two phenyl rings occupy a part of the S2 site and point out of the pocket towards the solution.

The structures of the interaction complexes of the other MAOAs **3** and that of **L680,833** were found to be quite similar to that of **3b**, shown in *figure 7*; the main differences were in the region of the iminoethereal portion and of the phenyl ring attached to it.

Figure 8 shows some details of the interaction between HLE and compound **3b**. The β-lactam carbonyl group of **3b** is H-bonded with Ser195 and Gly193; the iminoethereal portion of **3b** does not seem to interact strongly with any particular residue of the enzyme; however the closest residue to this polar group is His57, a residue considered very important for the catalytic action of HLE. Therefore, the distance between one of the two nitrogens of the imidazolic ring of His57 and the oxygen of the iminoethereal group of compound **3b** (3.5 Å) could allow a polar interaction and therefore could improve the interaction of **3b** with HLE.

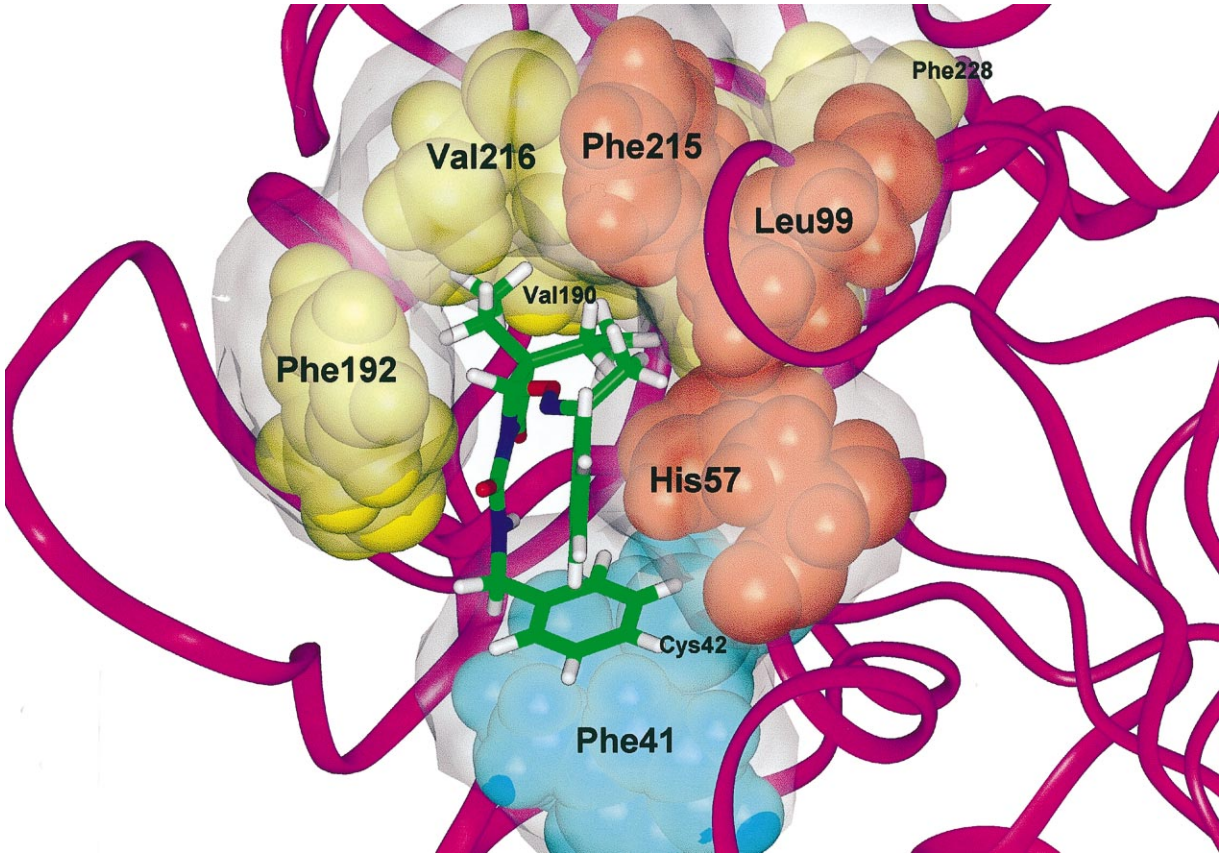


Figure 7. Compound **3b** inserted into the catalytic site of HLE. The ribbon of HLE is coloured magenta. The atoms of the residues which form the sites S1, S2 and S1′ are shown as CPK spheres coloured in yellow (residues of S1 site), brown (residues of S2 site) and cyan (residues of S1′ site). The solvent accessible surfaces of these sites are shown in grey.

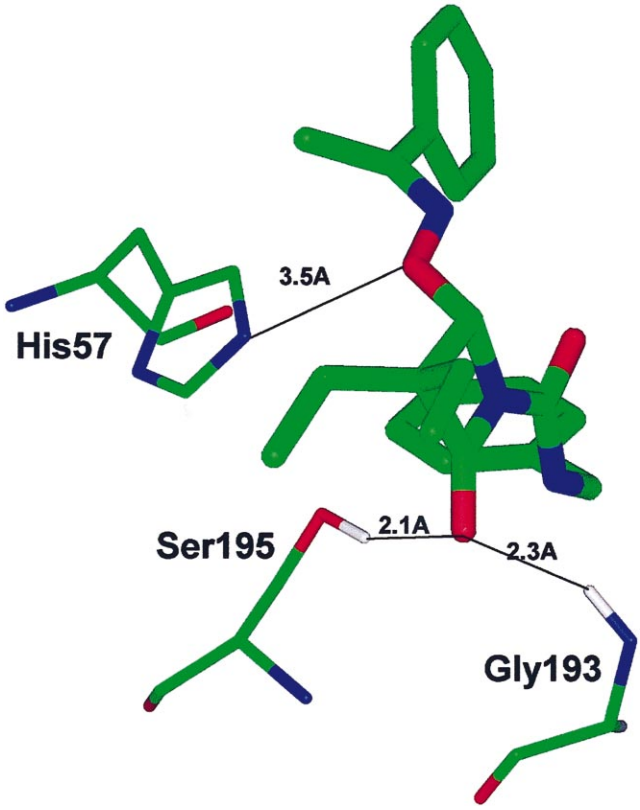


Figure 8. A detail of the interaction of compound **3b** with HLE; only the residues His57, Gly193 and Ser195 of HLE are shown.

Figure 9 shows another detail of the interaction of compound **3b** with HLE. It can be seen that the methyl group of **3b** occupies a little pocket in the S2 site of HLE, thus allowing a hydrophobic interaction of this group with the side chains of residues Leu99 and Phe215. This interaction is also found in the other MAOAs possessing a methyl group on the iminic carbon (**3f**, **3h** and **3l**) and could supply an explanation for the higher inhibitory activity of these compounds with respect to the corresponding ones without the methyl group on the iminic carbon (cfr. compound **3b** vs. **3a**, **3f** vs. **3e**, **3h** vs. **3g** and **3l** vs. **3i**).

Molecular modelling calculations also indicate that the size of this pocket is too small for a bulkier group like an ethyl or a phenyl, and that the presence of such bulky groups, as in compounds **3c** and **3d**, induces a modification of the arrangement of the molecule in the pocket mainly at the level of the phenyl ring linked to the iminic carbon. In this arrangement the phenyl rings of these compounds were found to be farther from the S2 site with a weaker ability, consequently, to interact. This fact could explain the lower activity of compounds **3c** and **3d** with respect to not only MAOA **3b**, but also **3a**.

Figure 10A indicates the possibility of a hydrogen bond between the fluorine atom in the *para* position of the phenyl ring linked to the iminic carbon of compound **3f** and the amidic group of the side chain of Asn61 situated on the external surface of the enzyme, with a distance F-H of about 2.5 Å. *Figure 10B* shows that in analogue **3e**, the different arrangement of the iminic phenyl ring in the enzyme pocket due to the lack of the methyl group on the iminic carbon makes the distance F-H greater (3.5 Å); for this reason the F-H interaction is weaker. The results obtained for the *para*-F substituted compounds **3e** and **3f** might therefore explain the synergic effect of this substitution and the introduction of a methyl on the iminic carbon which makes the inhibitory activity of compound **3f** particularly high.

5. Conclusions

In a previous paper [34] it was observed that the substitution of the LG of monocyclic β-lactams **1**, the C-4 aryloxy group, with a methyleneaminoxy molecular portion leads to a new class of HLE inhibitors, the MAOAs **3**.

In the present work, the effects induced on the anti-elastase properties of the MAOAs **3** by the R and R_1 substituents present on the LG have been more accurately investigated. Among the compounds studied, **3a**, **3b**, **3f** and **3l** were found to possess an appreciable in vitro inhibitory activity against HLE, with kinetic constant values (k_{inact}/K_I) ranging from 30 900–98 300 $M^{-1}s^{-1}$.

The results made it possible to rationalise how the substitution on the iminic carbon present on the LG of MAOAs **3** can modulate the in vitro inhibitory activity towards the HLE of such compounds, showing, in particular, that the optimal substitution was the one in which R is a methyl group. Moreover it was observed that the inhibitory activity was markedly improved by a *para*-fluorine substitution on the phenyl ring of the LG (R_1 = F).

Molecular modelling calculations made it possible to understand the reason for the higher activity possessed by compounds in which the R substituent on the LG is a methyl group. In fact the R substituent occupies a little lipophilic pocket near the active site capable of accommodating groups not larger than a methyl. These theoretical studies also indicated that a fluorine substituent in the *para* position on the phenyl ring of the methyleneaminoxy LG (R_1) is able to improve the interaction with HLE, forming a hydrogen bond with the residue Asn61.

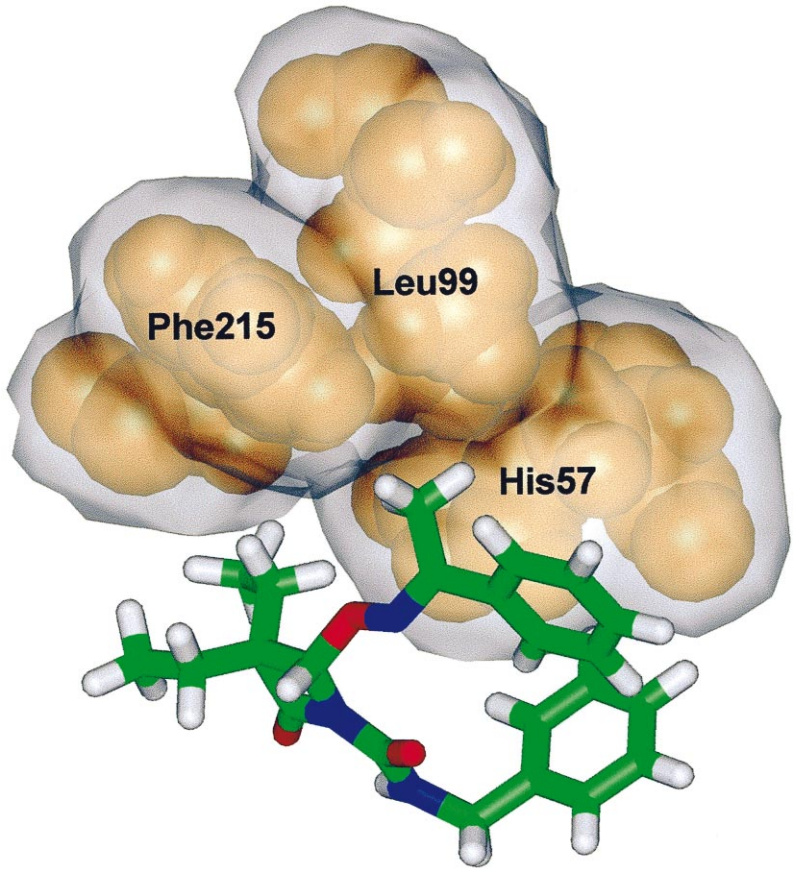


Figure 9. The methyl substituent on the iminic carbon of compound **3b** occupies a little liphophilic pocket of HLE formed by residues Leu99 and Phe215 (represented as CPK spheres coloured brown) of the S2 site.

The in vivo results indicated the absence of any relationship between the kind of R and R_1 substituent on the LG and the lung haemorrhage inhibition induced by HLE in mice. In any case, some of the MAOAs, **3a**, **3b**, **3d** and **3f**, showed a certain inhibitory activity towards haemorrhage induced by HLE.

6. Experimental protocols

6.1. Chemistry

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Infrared (IR) spectra for comparison of compounds were taken as paraffin oil mulls or as liquid film 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, with the only exceptions of **9**·$H_2C_2O_4$ and **10**·$H_2C_2O_4$ (DMSO-d_6). For configurational ^{1}H-NMR studies C_6D_6 and CF_3CO_2D were used as solvents. The proton magnetic resonance assignments were established on the basis of the expected chemical shifts and the multiplicity of the signals. Analytical TLC was carried out on 0.25 mm layer silica gel plates containing a fluorescent indicator, and spots were detected 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 appropri-

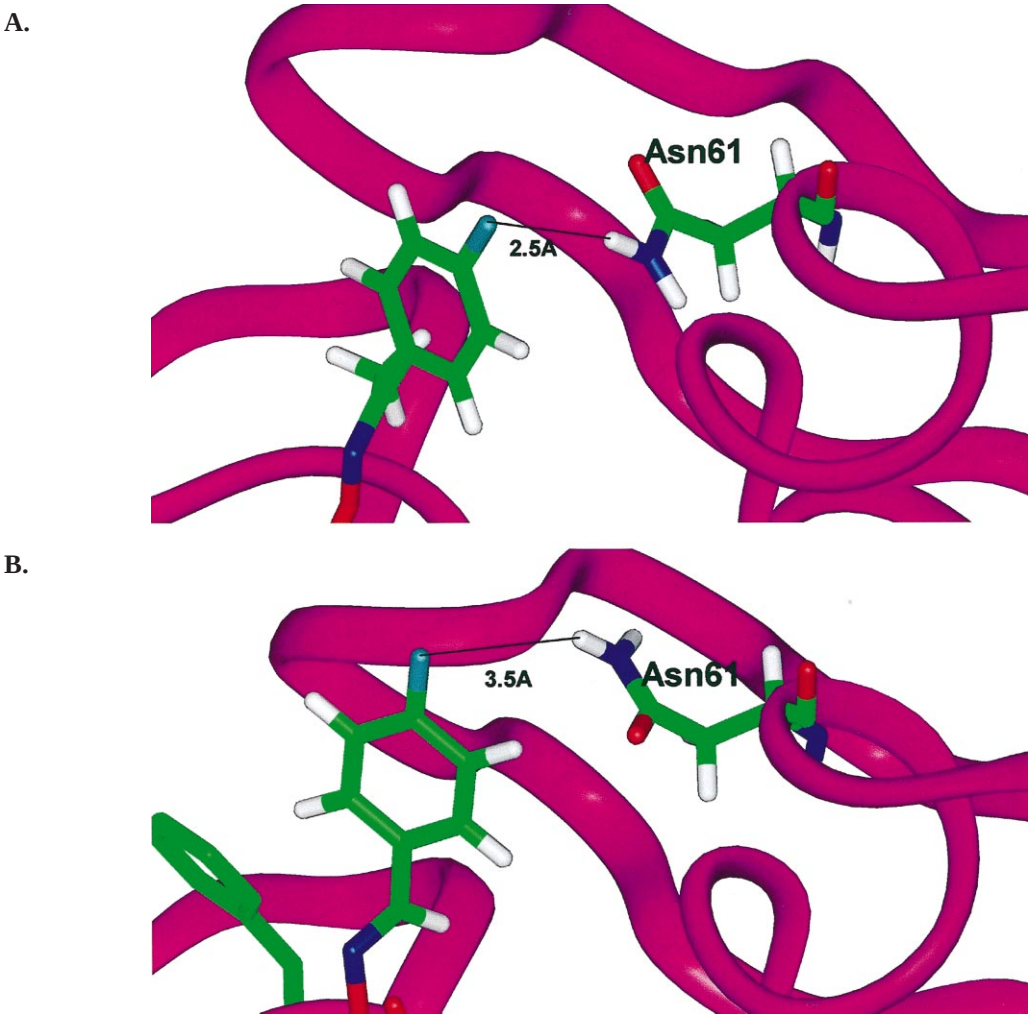


Figure 10. A. Interaction of the *para*-fluorine substituent present on compound **3f** with the residue Asn61 situated on the external surface of HLE; **B.** The same interaction, but for compound **3e**.

ate 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%.

6.1.1. General procedure for the preparation of the (E)-4-(arylideneaminoxy)-2-azetidinones **13c–f** *and* **13h**

1 N NaOH (6.5 mL) was added to a stirred solution of one of the appropriate *E* oximes **12c**–**f** or **12h** (6.5 mmol) in acetone (6.5 mL). After stirring for 10 min at room temperature, a solution of **11** (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 a 2:1 hexane-AcOEt mixture, yielded the appropriate pure azetidinone **13c–f** and **13h**. **13c** (52% yield), m.p. 72–73 °C, ^{1}H-NMR ($CDCl_3$) δ 0.96–1.24 (m, 9H), 1.67–2.04 (m, 4H), 2.80 (q, 2H, *J* = 9.6 Hz), 5.52 (s, 1H), 6.51 (br, 1H), 7.38–7.56 (m, 5H). Anal. $C_{16}H_{22}N_2O_2$ (C, H, N). **13d** (47% yield), m.p. 69–70 °C, ^{1}H-NMR ($CDCl_3$) δ 0.90 and 1.13 (2t, 6H, *J* = 7 Hz), 1.47–1.97 (m, 4H), 5.63 (s, 1H), 6.52 (br, 1H), 7.29–7.62 (m, 10H). Anal. $C_{20}H_{22}N_2O_2$ (C, H, N). **13e** (52% yield), m.p. 75–78 °C, ^{1}H-NMR ($CDCl_3$) δ 1.01 and 1.03 (2t, 6H, *J* = 7.2 Hz), 1.55–1.90 (m, 4H), 5.45 (s, 1H), 6.50 (br, 1H), 6.95–7.70 (m, 4H), 8.09 (s, 1H). Anal. $C_{14}H_{17}FN_2O_2$ (C,

H, N). **13f** (60% yield), m.p. 77–79 °C, ^{1}H-NMR ($CDCl_3$) δ 1.02 and 1.11 (2t, 6H, *J* = 7.2 Hz), 1.53–1.98 (m, 4H), 2.26 (s, 3H), 5.49 (s, 1H), 6.90–7.85 (m, 4H). Anal. $C_{15}H_{19}FN_2O_2$ (C, H, N). **13h** (43% yield), m.p. 125–126 °C, ^{1}H-NMR ($CDCl_3$) δ 1.09 and 1.11 (2t, 6H, *J* = 7 Hz), 1.62–1.91 (m, 4H), 2.27 (s, 3H), 5.51 (s, 1H), 6.35 (br, 1H), 7.09 (s, 1H), 7.22–7.42 (m, 10H), 7.68 and 8.11 (2d, 4H, *J* = 8.8 Hz). Anal. $C_{29}H_{30}N_2O_4$ (C, H, N).

6.1.2. General procedure for the preparation of the (E)-4-(arylideneaminoxy)-2-azetidinones **13i** *and* **13l**

1 N NaOH (12.7 mL) was added to a stirred solution of **12i** or **12l** (3.7 mmol) in acetone (3.5 mL). After stirring for 10 min at room temperature, a solution of **11** (3.3 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 MPLC on C_{18} reversed phase using a 6:4 mixture of MeOH-H_2O with 8% of an aqueous solution of NH_4OH (30%) and 4% of 2-propanol, yielded the appropriate pure azetidinone **13i** or **13l** as oils. **13i** (28% yield),^{1}H-NMR ($CDCl_3$) δ 1.06 and 1.08 (2t, 6H, *J* = 7 Hz), 1.64–1.86 (m, 4H), 2.45–2.70 (m, 4H), 2.85 (t, 2H, *J* = 5.6 Hz), 3.60–3.90 (m, 4H), 4.18 (t, 2H, *J* = 5.6 Hz), 5.50 (s, 1H), 6.43 (br, 1H), 6.93 (d, 2H, *J* = 8.8 Hz), 7.53 (d, 2H, *J* = 8.8 Hz), 8.12 (s, 1H). Anal. $C_{20}H_{29}N_3O_4$ (C, H, N). **13l** (31% yield),^{1}H-NMR ($CDCl_3$) δ 1.07 and 1.10 (2t, 6H, *J* = 7 Hz), 1.62–1.81 (m, 4H), 2.23 (s, 3H), 2.45–2.60 (m, 4H), 2.80 (t, 2H, *J* = 5.6 Hz), 3.55–3.73 (m, 4H), 4.18 (t, 2H, *J* = 5.6 Hz), 5.47 (s, 1H), 6.45 (br, 1H), 6.85 (d, 2H, *J* = 8.8 Hz), 7.51 (d, 2H, *J* = 8.8 Hz). Anal. $C_{21}H_{31}N_3O_4$ (C, H, N).

6.1.3. General procedure for the preparation of the (E)-4-(arylideneaminoxy)-1-N-(benzylaminocarbamoyl)-2-azetidinones **3c**–**f**, **3i**–**l** *and* **14h**

A stirred solution of the appropriate azetidinone **13** (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 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 crystallisation with *i*-PrOH (in the case of **3c**–**f** and **14h**) or by MPLC on C_{18} reversed phase using a 6:4 mixture of MeOH-H_2O with 8% of an aqueous solution of NH_4OH (30%) and 4% of 2-propanol (in the case of **3i**–**l**). **3c** (62% yield), m.p. 94 °C, ^{1}H-NMR ($CDCl_3$) δ 0.94–1.25 (m, 9H), 1.64–1.86 (m, 4H), 2.77 (q, 2H, *J* = 7.2 Hz), 4.46 (d, 2H, *J* = 5.6 Hz), 5.90 (s, 1H), 6.84 (br, 1H), 7.20–7.75 (m, 10H). Anal. $C_{24}H_{29}N_3O_3$ (C, H, N). **3d** (47% yield), m.p. 70–71 °C, ^{1}H-NMR ($CDCl_3$) δ 1.02 and 1.06 (2t, 6H, *J* = 7 Hz), 1.53–1.85 (m, 4H), 4.44 (d, 2H, *J* = 5.6 Hz), 5.94 (s, 1H), 6.81 (br, 1H), 7.24–7.51 (m, 15H). Anal. $C_{28}H_{29}N_3O_3$ (C, H, N). **3e** (53% yield), m.p. 122–124 °C, ^{1}H-NMR ($CDCl_3$) δ 1.01 and 1.03 (2t, 6H, *J* = 7.2 Hz), 1.55–1.99 (m, 4H), 4.47 (d, 2H, *J* = 5.6 Hz), 5.86 (s, 1H), 6.80–7.84 (m, 10H), 8.09 (s, 1H). Anal. $C_{22}H_{24}FN_3O_3$ (C, H, N). **3f** (55% yield), m.p. 83–85 °C, ^{1}H-NMR ($CDCl_3$) δ 1.01 and 1.03 (2t, 6H, *J* = 7.2 Hz), 1.52–1.98 (m, 4H), 2.25 (s, 3H), 4.44 (d, 2H, *J* = 5.6 Hz), 5.90 (s, 1H), 6.75–7.75 (m, 10H). Anal. $C_{23}H_{26}FN_3O_3$ (C, H, N). **3i** (55% yield), m.p. 150–151 °C (**3i**·$H_2C_2O_4$), ^{1}H-NMR ($CDCl_3$) δ 1.04 and 1.07 (2t, 6H, *J* = 7 Hz), 1.71–1.92 (m, 4H), 2.45–2.70 (m, 4H), 2.79 (t, 2H, *J* = 5.6 Hz), 3.50–3.70 (m, 4H), 4.12 (t, 2H, *J* = 5.6 Hz), 4.51 (d, 2H, *J* = 5.6 Hz), 5.86 (s, 1H), 6.85 (d, 2H, *J* = 8.8 Hz), 7.20–7.32 (m, 5H), 7.48 (d, 2H, *J* = 8.8 Hz), 8.07 (s, 1H). Anal. $C_{28}H_{36}N_4O_5$ (C, H, N). **3l** (62% yield), m.p. 155–157 °C (**3l**·$H_2C_2O_4$), ^{1}H-NMR ($CDCl_3$) δ 1.05 and 1.08 (2t, 6H, *J* = 7 Hz), 1.64–1.80 (m, 4H), 2.30 (s, 3H), 2.50–2.70 (m, 4H), 2.75 (t, 2H, *J* = 5.6 Hz), 3.65–3.80 (m, 4H), 4.17 (t, 2H, *J* = 5.6 Hz), 4.52 (d, 2H, *J* = 5.6 Hz), 5.95 (s, 1H), 6.85 (d, 2H, *J* = 8.8 Hz), 7.25–7.32 (m, 5H), 7.56 (d, 2H, *J* = 8.8 Hz). Anal. $C_{29}H_{38}N_4O_5$ (C, H, N). **14h** (83% yield), m.p. 112–113 °C, ^{1}H-NMR ($CDCl_3$) δ 1.01 and 1.04 (2t, 6H, *J* = 7 Hz), 1.61–1.89 (m, 4H), 2.29 (s, 3H), 4.51 (d, 2H, *J* = 5.6 Hz), 5.51 (s, 1H), 6.87 (br, 1H), 7.09 (s, 1H), 7.22–7.42 (m, 5H), 7.68 and 8.11 (2d, 4H, *J* = 8.8 Hz). Anal. $C_{37}H_{37}N_3O_5$ (C, H, N).

6.1.4. (E)-4-(arylideneaminoxy)-1-N-(benzylaminocarbamoyl)-2-azetidinone **3h**

Trifluoroacetic acid (2 mL) was added dropwise to a stirred and cooled solution of **14h** (0.30 g, 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 × 25 mL). The aqueous layer, after being washed with $CHCl_3$ (2 × 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 × 30 mL). Evaporation of the washed (brine) organic extracts yielded a white solid residue, which, after crystallisation with AcOEt-hexane, gave pure **3h**: 0.12 g as a white solid (72% yield), m.p. 144–145 °C, ^{1}H-NMR ($CDCl_3$) δ 1.09 and 1.10 (2t, 6H, *J* = 7 Hz), 1.74–2.03 (m, 4H), 2.35 (s, 3H), 4.53 (d, 2H, *J* = 5.6 Hz), 6.05 (s, 1H), 6.98 (br, 1H), 7.33–7.41 (m, 5H), 7.72 and 8.04 (2d, 4H, *J* = 8 Hz). Anal. $C_{24}H_{27}N_3O_5$ (C, H, N).

6.1.5. Benzhydryl (E)-acetophenonoxime-4-carboxylate **12h**

A solution of diphenyldiazomethane (6.5 g, 33 mmol) in $CHCl_3$ (10 mL) was added portionwise to an unstirred solution of 4-acetylbenzoic acid **15** (5.5 g, 33 mmol) 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 × 100 mL). The organic phase was dried and evaporated to give an oily residue, which after crystallisation with AcOEt-hexane, yielded 4.5 g of pure benzhydryl 4-acetylbenzoate **16** (41% yield), m.p. 106–107 °C, ^{1}H-NMR ($CDCl_3$) δ 2.62 (s, 3H), 7.10 (s, 1H), 7.22–7.35 (m, 10 H), 7.97 and 8.19 (2d, 4H, *J* = 8.8 Hz). Anal. $C_{22}H_{18}O_3$ (C, H). **16** (4.2 g, 13 mmol) was dissolved in THF (85 mL) and the resulting solution, after being cooled at 0 °C, was treated with an aqueous solution of 1 M K_2CO_3 (9 mL) and then, portionwise, with an aqueous solution of 0.55 M hydroxylamine hydrochloride (31 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 (45 mL) and extracted with Et_2O (2 × 100 mL). The organic phase was dried and evaporated to give a solid residue which, after crystallisation with AcOEt-hexane, yielded pure **12h**, 3.2 g (71% yield), m.p. 148 °C, ^{1}H-NMR ($CDCl_3$) δ 2.30 (s, 3H), 7.09 (s, 1H), 7.23–7.36 (m, 10 H), 7.68 and 8.11 (2d, 4H, *J* = 8.8 Hz). Anal. $C_{22}H_{19}NO_3$ (C, H, N).

6.1.6. General procedure for the preparation of 4-[2′-ethyloxy-N-morpholino]-benzaldehyde **19** *and 4-[2′-ethyloxy-N-morpholino]-acetophenone* **20**

A solution of the appropriate 4-hydroxyphenylcarbonyl derivative **17**, **18** (12 mmol), 4-(2-hydroxyethylmorpholine) (1.57 g, 12 mmol), triphenylphosphine (3.15 g, 12 mmol) and diethylazodicarboxylate (21.0 g, 12 mmol) in dry THF (70 mL) was stirred at room temperature for 24 h under N_2 atmosphere. The THF was removed under reduced pressure and the crude residue was dissolved with AcOEt (100 mL) and extracted with 10% HCl (2 × 50 mL). The aqueous phase was alkalinised to pH 9 with solid K_2CO_3, and extracted with AcOEt (2 × 100 mL). Evaporation of the dried and filtered extracts gave a crude residue which was purified by MPLC on silica gel using a 1:1 mixture of hexane-AcOEt with 2% of triethyamine to afford the desired compounds as oils. **19** [44]: 1.7 g (60% yield), ^{1}H-NMR ($CDCl_3$) δ 2.65–2.70 (m, 4H), 2.88 (t, 2H *J* = 5.6 Hz), 3.55–3.70 (m, 4H), 4.23 (t, 2H, *J* = 5.6 Hz), 7.05 and 7.89 (2d, 4H, *J* = 9 Hz), 9.97 (s, 1H). Anal. $C_{13}H_{17}NO_3$ (C, H, N). **20** [45]: 1.6 g (53% yield), ^{1}H-NMR ($CDCl_3$) δ 2.41–2.90 (m, 9H), 3.75–3.80 (m, 4H), 4.18 (t, 2H, *J* = 5.6 Hz), 6.87 and 7.88 (2d, 4H, *J* = 8.8 Hz). Anal. $C_{14}H_{19}NO_3$ (C, H, N).

6.1.7. General procedure for the preparation of 4-[2′-ethyloxy-N-morpholino]-(E)-benzaldoxime **12i** *and 4-[2′-ethyloxy-N-morpholino]-(E)-acetophenonoxime* **12l**

Solid K_2CO_3 (2.80 mmol) and then a solution of $NH_2OH{\cdot}HCl$ (5.60 mmol) in H_2O (9.5 mL) were added to a stirred and cooled (0 °C) solution of the appropriate phenylcarbonyl derivative **19**, **20** (4.25 mmol) in EtOH (25 mL). The resulting mixture was stirred at room temperature for 24 h and then neutralised with 21% H_3PO_4. The EtOH was removed under reduced pressure and the resulting mixture was extracted with Et_2O (50 mL). Evaporation of the dried and filtered extracts gave a solid residue which was crystallised by a 2:1 hexane-AcOEt mixture. **12i**, 0.93 g (57% yield), m.p. 114–115 °C, ^{1}H-NMR ($CDCl_3$) δ 2.55–2.75 (m, 4H), 2.88 (t, 2H, *J* = 5.6 Hz), 3.70–3.90 (m, 4H), 4.23 (t, 2H, *J* = 5.6 Hz), 6.90 and 7.46 (2d, 4H, *J* = 8.8 Hz), 8.08 (s, 1H). Anal. $C_{13}H_{18}N_2O_3$ (C, H, N). **12l**, 0.62 g (56% yield), m.p. 124–125 °C, ^{1}H-NMR ($CDCl_3$) δ 2.23 (s, 3H), 2.50–2.70 (m, 4H), 2.82 (t, 2H, *J* = 5.6 Hz), 3.58–3.80 (m, 4H), 4.18 (t, 2H, *J* = 5.6 Hz), 6.85 and 7.50 (2d, 4H, *J* = 8.8 Hz). Anal. $C_{14}H_{20}N_2O_3$ (C, H, N).

6.1.8. Configurational ^{1}H-NMR studies on oximes **12h–l**

^{1}H-NMR studies of the shielding effect induced by an anisotropic solvent on **12h–l** were carried out with 5% (w/w) in C_6D_6 (0.75 mL), adding increasing amounts (5, 10, 15 and 25 μL) of CF_3CO_2D. The spectra were recorded for the free bases and after each addition of acid. The chemical shifts of the protons of the methyl group or the proton linked to the iminic carbon atom in deuterobenzene solutions were respectively 2.30 ppm (Me protons, **12h**), 8.05 ppm (H, **12i**) and 2.23 ppm (Me protons, **12l**) as free bases. After the addition of the above-mentioned amounts of CF_3CO_2D, the chemical shift of the same protons of **12h–l** changed to 2.28, 2.26, 2.24 and 2.21 ppm for **12h**, 7.83, 7.65, 7.63 and 7.61 ppm for **12i** and 1.99, 1.87, 1.77 and 1.74 ppm for **12l** for their protonated forms.

6.1.9. Configurational stability test for the asymmetric oximes **12c–f** *and* **12h–l**

The above-mentioned oximes **12c–f** and **12h–l** (2 mmol) in acetone (6.9 mL) were treated with a 1 N NaOH solution (6.9 mL) and the resulting solutions were stirred for 1 h at room temperature. The acetone was then removed in vacuo and the aqueous layers were then neutralised with H_3PO_4 solution (10%) and extracted with ether. The organic phases were washed with brine, dried and evaporated to give quantitatively the unchanged

starting oximes **12c–f** and **12h–l** (^{1}H-NMR analysis) as residues. These residues were then solubilised with anhydrous CH_2Cl_2 (10mL) and treated under stirring with Et_3N (2 mmol) and DMAP (2 crystals) for 24 h at room temperature. The mixture was then neutralised with H_3PO_4 solution (10%) and the organic phase was washed with brine, dried and evaporated to quantitatively give the unchanged starting oximes **12c–f** and **12h–l** as residues (^{1}H-NMR).

6.2. 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 [46] 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 = 13 000 M^{-1} cm^{-1}) with a Beckman DU-7 UV/visible spectrophotometer thermostated at 37 °C [35]. Both substrate and inhibitor stock solutions were made in DMSO. 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, corresponding to an enzyme concentration of about 10 nM; one milliunit releases one nmol of *p*-nitroaniline per min from MAAPVNA) was added to a reaction mixture containing MAAPVNA (0.2–0.4 mM) and the increase in absorbance was recorded for 3 min (time course A); one of the inhibitors dissolved in DMSO was subsequently added to a final concentration ranging from 0.05–4.0 μM and the absorbance change was further recorded up to 30 min (time course B). Control assays, in which the inhibitor was omitted, ran linearly in time up to an absorbance of 1.0–1.1 AU, before the slope started to change due to substrate consumption; assays in which the inhibitor was added showed a swift time-dependent fall in slope, and the absorbance levelled off between 0.4–1.0 AU at 30 min, depending on the inhibitor concentration and potency.

The first-order decay in HLE activity was evaluated by taking the slope every 60 s over time course B and dividing it by the slope in time course A (*figure 11*). 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). Pseudo first-order conditions were checked by evaluating total HLE molar concentration from the rate in time course A, substrate concentration and the enzyme–substrate kinetic parameters at 37 °C (k_{cat} = 20 s^{-1}, K_M = 0.12 mM, our data, not shown). 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 5% (i.e. over 3 natural log units). $1/k_{obs}$ values obtained at the same substrate concentration and at varying concentrations of a single inhibitor were plotted vs. 1/[I]. The reciprocal of the slope of this plot yields $\frac{k_{inact}}{K_I\,(1 + [S] \,/\, K_M)}$, from which the concentration-independent second-order rate constant k_{inact}/K_I is obtained. Similar values of this parameter were obtained for the same inhibitor at different substrate concentrations, in agreement with a competitive mechanism of inhibition.

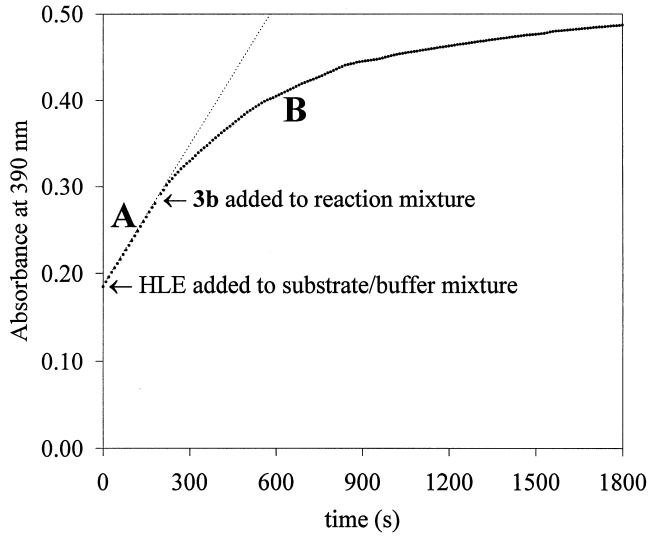


Figure 11. A typical time course of the absorbance change in the HLE inhibition assay, using compound **3b** as the inhibitor, is shown to illustrate the kinetic analysis method used herein; arrows and labels mark the addition of components to the reaction mixture. Black dots are absorbance readings every 10 s; the dotted straight line extrapolates the time course of the absorbance in the absence of added inhibitor. Initial conditions: $[S]_0$ = 0.35 mM (A_0 = 0.181); $[HLE]_0$ = 3.3 nM; [**3b**] = 0.2 μM. The reaction mixture (final volume 500 μL) was made in 0.05 M sodium phosphate buffer, pH 7.8, with 4% DMSO; *T* = 37 °C. Values of the changing slope in phase **B**, taken over 60 s intervals, were divided by the slope in phase **A**. The natural log of these ratios were plotted against time to get the k_{obs} value. The reciprocal of several k_{obs} values, obtained at different inhibitor concentrations and fixed substrate concentration, were eventually plotted against 1/[I] to get the inhibition apparent second-order rate constant; this was finally multiplied by $\left(1 + [S] \,/\, K_M\right)$ to get the k_{inact}/K_I value.

6.3. Pharmacology

6.3.1. HLE-induced lung haemorrhage in mice

Mice, anaesthetised with a mixture of ketamine and xylazine i.p., were instilled intratracheally with 50 μL of saline (vehicle) or human leukocyte elastase obtained from purulent sputum (Elastin Products Company inc.) (3 200 U/mL dissolved in saline, corresponding to a solution of 16 μg/50 μL). Test compounds were orally administered at 10 mg/kg as a suspension of DMSO in Methocel, 3 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 by using sonicated whole mouse blood supplemented with Na_2CO_3 2% w/v. The haemorrhage 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 haemorrhage in treated versus control animals. The basal value (vehicle) was subtracted from each treatment group.

6.4. Computational analysis

All the molecular mechanics (MM) and dynamics (MD) calculations were made by using the program Discover with the CVFF forcefield [47]. A distance-dependent dielectric constant equal to 4 was used in order to simulate an aqueous environment, no explicit water molecules were considered. All the ionisable groups of HLE were considered in their most stable form at a pH of 7.4, thus obtaining a structure with an excess of 11 positive-charged groups. The minimisation methods were steepest descents and conjugate gradient, and the MD runs were made with a 1 fs time step. All the structures were modelled and visualised through the software Insight II [47]. The starting-point for the modelling of the inhibitor active site was the X-ray structure of HLE complexed with the peptidic inhibitor MSACK (Metoxysuccinyl-Ala-Ala-Pro-Ala-Chloromethylketone) [42] whose co-ordinates were obtained from the Protein Data Bank [48, 49]. In the experimental structure, the hydrogens were added, the bond between MSACK and Ser195 was formed, and then a minimisation was performed with a tether constraint on the heavy atoms gradually lowered till complete relaxation.

At this point the molecule of MSACK was removed from the active site and substituted with the molecule of **L680,833**, again bound to Ser195 in accordance with the Michaelis complex structure. The molecule was manually adapted in such a manner as to satisfy the findings from Finke et al. [43] for an analogue of **L680,833** (**L674,197**), through X-ray crystallography; however, they do not supply the experimental co-ordinates. In particular, suitable constraints were introduced in order to take into account the interaction of the carbonylic oxygen of the β-lactamic cycle with the amidic hydrogens of Gly193 and Ser195 [43]. A run of MD at a temperature of 900°K was then carried out with quite a high tether constraint on the backbone of the enzyme (with the exception of residues 190–200, the nearest ones to the active site) in order to prevent the unfolding of the protein. After a 10 ps equilibration, a 100 ps run was carried out selecting one conformation every 10 ps. Each of these conformations was then minimised, gradually cancelling all constraints. The minimised conformations thus obtained were found to be similar to each other in the region of the active site and the one in which the conformation of **L680,833** was energetically favoured was selected.

Subsequently the bond between the inhibitor and Ser195 was broken and the structure was again minimised through MD and MM in a way similar to that used for the Michaelis complex.

At this point the molecule of **L680,833** was substituted in sequence with MAOAs **3a–l** using the same strategy of minimisation by means of MD and MM.

Acknowledgements

Part of this work was supported by the contract ‘Programma Nazionale di Ricerca sui Farmaci (seconda Fase), Tema 4°, granted by the Italian Ministry of University and Scientific and Technological Research.

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