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Potential of Pyrazolooxadiazinone Derivatives as Serine Protease Inhibitors

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As a part of an investigation on molecular hybrids as new serine protease inhibitors, the pyrazolo [4,3-c][1,2,5]oxadiazin-3(5H)-one ring system was selected as a model of potential mechanism-based inhibitors. Due to the inherent reactivity of this system an optimal balance between susceptibility to nucleophilic attack and stability in solvents was sought prior to development as therapeutic agents. Substitutions on N5 and C7 of the supporting pyrazole ring with either aliphatic or aromatic groups (compounds 2 *a-m*) and the replacement of the carbonyl oxygen on the reactive oxadiazinone ring with sulfur (compounds *3a,i*) were explored. Two members (2*i* and 2*k*) of this class of inhibitors displayed time-dependent inhibition of HLE suggesting mechanism-based inhibition. The observation that HLE generated a product(s) from compound 2*i* which displayed an identical UV-Visible spectrum to that observed during non-enzymatic hydrolysis further supports this proposal. FlexX-based docking of these compounds into a model of the human leukocyte elastase (HLE) active site produced a molecular model of the inhibitor–enzyme interaction.

Keywords: Pyrazolo[4,3-c][1,2,5]oxadiazin-3(5H)-ones, Mechanism-based inhibitors, Serine Proteases, FlexX docking

Abbreviations: α_1-PI, α_1-protease inhibitor; AA, amino acid; Cat-G, Cathepsin-G; DMSO, dimethylsulfoxide; FUT-175, 6-amidino-2-naphthyl-4-quinidinobenzoate dihydrochloride; HLE, human leukocyte elastase; PMN, polymorphonuclear neutrophils; ppm, parts per million; HPLC, high pressure liquid chromatography; TES, N-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid; THF, tetrahydrofuran; TMS, tetramethylsilane

INTRODUCTION

The degranulation of polymorphonuclear leukocytes (PMN) in inflammatory states results in the release of several enzymes including the proteolytic enzyme human leukocyte elastase (HLE).[1,2]

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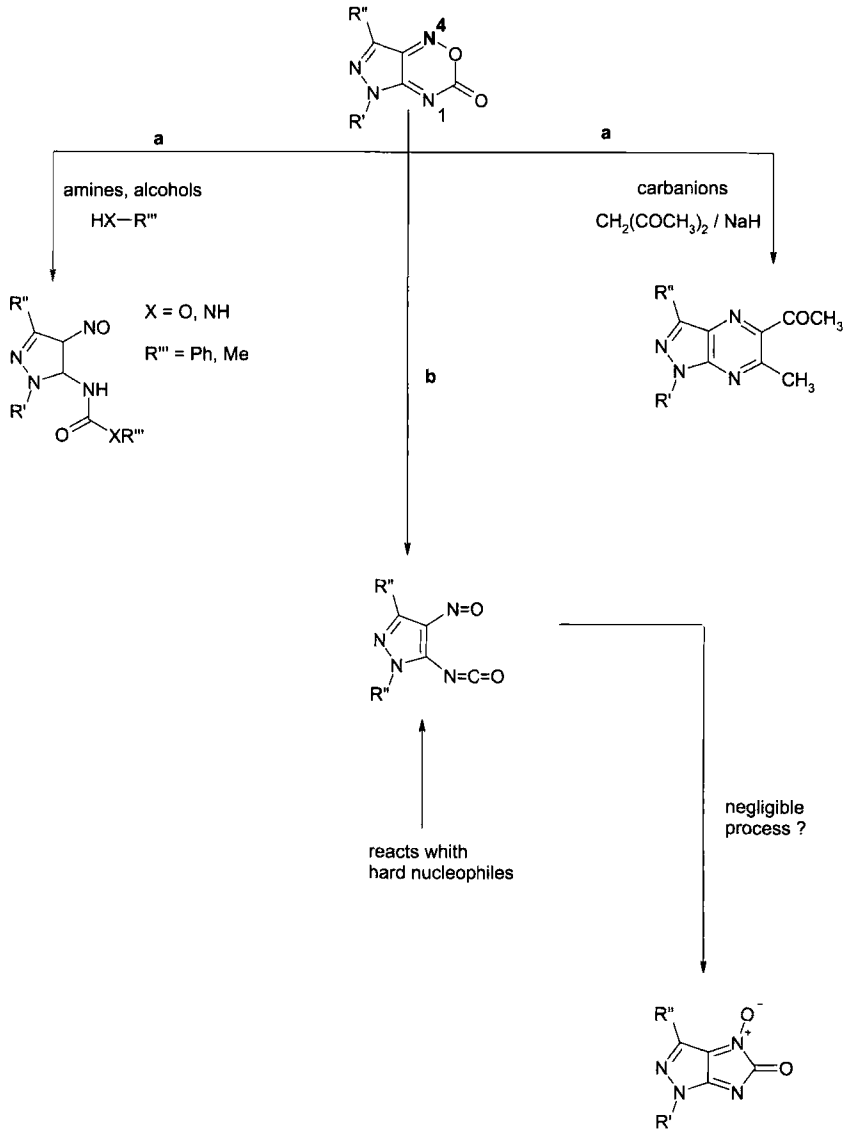


FIGURE 1 Predicted nucleophilic rearrangements of the oxadiazinone ring system.

HLE is stored in the azurophilic granules of PMN and on release its proteolytic activity is tightly regulated by endogenous protease inhibitors, such as α_1-PI. The chronic degradative action of HLE on elastin and other matrix components is believed to cause or exacerbate pathogenesis of the lung in diseases such as pulmonary emphysema and cystic fibrosis and other connective tissue ailments such as glomerulonephritis, adult respiratory distress syndrome and rheumatoid arthritis.[2–11] Therefore, HLE has been the target of extensive structural and mechanistic studies.[3,4] These efforts have included modulation of the enzyme activity through the use of synthetic and naturally occurring inhibitors.[5,6]

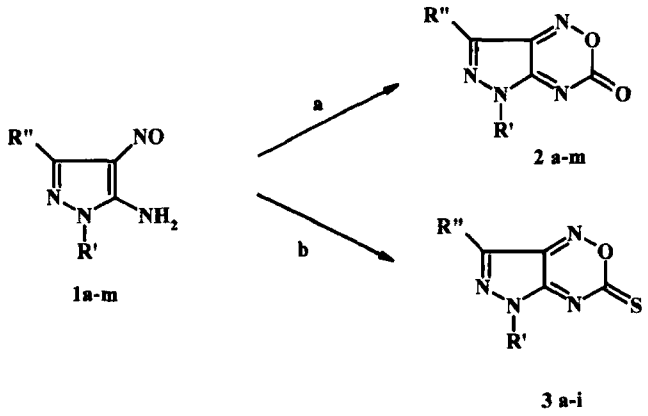
A contemporary strategy in drug design involves the use of latent reactive substrates as

enzyme inactivators.[5,6] This strategy features a relatively unreactive suicide substrate that is designed initially to bind to a specific target enzyme and then *via* a rearrangement is transformed during the normal course of catalysis to a reactive species. Ideally, the latter product is trapped by the enzyme and irreversibly inactivates it prior to dissociation from the active site. The reactive species is generated only in the enzyme' s active site, and thus suicide substrates promise greater *in vivo* selectivity than do conventional affinity reagents.[5–8]

Therefore this investigation focused on ring systems capable of functioning as support and reactive groups for inhibitors of serine proteases.[5–8] Starting with the investigation of the reactivity of the pyrazolooxadiazinone ring system (Figure 1, *pathway a*) (Scheme 1) by Guarneri *et al.*[9] and decomposition studies monitored by HPLC in nonenzymatic models[10] carried out in these laboratories, we proposed this electrophilic system as a bioisosteric functional and structural molecular hybrid between the 1,3-oxazine-2,6-dione and imidazole ring systems. These groups were demonstrated to function as support and reactive moieties in other serine protease inhibitors, respectively.[5,6,11]

In order to probe the topography of the HLE active site and ascertain the pattern of substitutions that result in maximal inhibitory activity i.e. optimal reactivity/stability ratio and specificity,[5,6,12,13] we carried out a number of modifications on both condensed rings. Specifically, we sought to balance the –M (π-acceptor) effect against that one +I (σ-donor) by substitution of either small aliphatic (**R′**)[14] and/or aromatic (**R″**) groups on the N5 and C7 positions of the pyrazole ring (compounds **2a-m**) (Scheme 1) so modulating the reactivity of the oxadiazinone ring (Figure 1).[14] In addition, the reactivity and size of the carbonyl was altered by substitution of the 3-carbonyl oxygen of the oxadiazinone ring with sulfur[15] (compounds **3a,i**).

The syntheses[9,10] stability studies and biological profiles of the title compounds **2a-m** and their 3-thione analogs **3a,i** are reported in this



Compd	R'	R"
2a	phenyl	methyl
2b	methyl	methyl
2c	4-chlorophenyl	methyl
2d	2,4-dichlorophenyl	methyl
2e	3,4-dichlorophenyl	methyl
2f	4-fluorphenyl	methyl
2g	2-nitrophenyl	methyl
2h	3-nitrophenyl	methyl
2i	4-nitrophenyl	methyl
2j	methyl	ethyl
2k	ethyl	methyl
2l	methyl	n-butyl
2m	tert-butyl	methyl
3a	phenyl	methyl
3i	4-nitrophenyl	methyl

a: phosgene,RT
b:thiophosgene,RT

Reaction solvent: anhydrous THF

SCHEME 1 Synthetic routes to pyrazolo[4,3-c][1,2,5]oxadiazin-3(5H)-ones **2 a-m** and pyrazolo[4,3-c][1,2,5]oxadiaxin-3(5H)-thiones **3 a,i**.

work. HPLC analysis of the stability as well as the enzyme kinetic studies support the proposal that the parent heterocyclic system is the active component.[10] The nonenzymatic and enzymatic reactions of these compounds are compared.

MATERIALS

Chemical

Melting points were determined on a Büchi capillary apparatus and are uncorrected. IR spectra of compound **1,2 a-m** and **3a,i** (Scheme 1) were obtained with potassium bromide discs on a Perkin-Elmer 299B spectrophotometer. IR spectra of compound **5 a,b** and **6,7 c-d** were obtained with potassium bromide discs on a Perkin-Elmer 1600 FT-IR series spectrophotometer. ^{1}H and ^{13}C NMR spectra of compounds **1,2 a-m** and **3a,i** were recorded on a Bruker AC 200 spectrometer (DMSO-d_6 or $CDCl_3$) operating at a frequency of 200 Mhz. ^{1}H NMR spectra of compounds **5 a,b** and **6,7 c-d** were recorded at 300.13 MHz on a Bruker AMX-R 300 spectrometer (DMSO-d_6) at 298 °K. All ^{1}H chemical shifts are given as δ ppm downfield from TMS using the residual solvent peak as internal reference. Coupling constants are reported in Hz. Column chromatography was performed on silica gel (Kieselgel 60, 70–230 mesh ASTM, Merck and Kieselgel 60, 230–400 mesh ASTM, Merck for compounds **5a,b**). Elemental combustion analyses were performed on a Carlo Erba Mod. EA 1108 Analyzer instrument by Dr S Di Marco of the Microanalysis Laboratory of Dipartimento di Scienze Farmaceutiche, Università di Catania.

Reactions were routinely followed by TLC on silica gel plates (60 F254 Merck), eluted with dichloromethane/MeOH ($\varphi = 20\%$)/toluene($\varphi =$ 10%) for compounds **1,2 a-m** and **3a,i**; ethyl acetate and ethyl acetate/MeOH ($\varphi = 20\%$) for compounds **5a,b**, **6c,d** and **7c,d**. The purity of each compound was assessed by TLC similarly. The presence of the compounds was detected by UV irradiation at 254–365 nm. All chemicals were purchased from Aldrich, Fluka, Merck and Carlo Erba Chemical Co and were used without further purification.

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) were taken on the Helwett-Packard HPG2025 A mass spectrometer operating in a positive linear mode.

HPLC Stability Experiments

HPLC stability experiments were conducted on a Jasco liquid chromatograph system equipped with a BIP-1 pump model and a Reodyne Model 7125 injector with a 20 µl sample loop. The eluents were monitored by a variable wavelength UV detector (JASCO-UVIDEC 100-V) connected to a VARIAN 4270 integrator. The detector wavelength was set at 254 nm with an integrator attenuation of 32. The chromatographic separations were performed isocratically at room temperature on a 5µm, HYPERSIL C18 column (250 × 4.5 mm I.D.) or a Phenomenex Bondclone C18 column (300 × 3.9 mm ID) using MeOH/water ($\varphi = 30$, 35 or 80 (**2k**) %) as a mobile phase, at a flow rate of 1 ml/min.

HPLC grade MeOH and THF were obtained from PROMOCHEM (FRG) and water was deionized and doubly distilled. The spectrophotometric analyses were performed on a Jasco UVIDEC-610 double beam spectrophotometer with a slit width of 1 nm, scan speed of 100 nm/min and chart speed of 20 nm/cm in the range 200–500 nm. Compounds **2a** (C = 31.1 µg/ml), **2c** (28.4 µg/ml), **2i** (35.1 µg/ml) were dissolved in MeOH or THF. The UV spectra were obtained after 1 h and 24 h for the MeOH solutions (MeOH as blank) and immediately and 1 week for the THF solutions (THF as blank). Fresh solutions at the same concentrations were prepared in either MeOH or THF and aliquots were analyzed by HPLC over time. Aliquots of the solutions after 24 h were run concurrently with MeOH as the blank. The degradation of the compounds was expressed

as the percentage decrease of the compound peak area *versus* time, using the following:

$$\% \text{ degradation} = \Delta t^0 - \Delta t'/\Delta t^0 \times 100 \qquad (1)$$

where Δt^0 = compound peak area at $t = 0$, as soon as the solution is prepared; $\Delta t'$ = compound peak area at t = one hour, one week, etc. Therefore, even though the degradation products were observed chromatographically, the compound peak was chosen as the reference peak to determine the rate of decomposition.[10]

METHODS

Chemistry

Synthesis of Pyrazolo[4,3-c][1,2,5]oxadiazin-3(5H)-ones

Compounds **2a-m** were prepared by treatment of compounds **1a-m**[16–19] with trichloromethyl chloroformate at room temperature following a previously reported procedure.[9,10] A similar synthetic pathway, using thiophosgene in place of diphosgene, led to the 3-thione analogs **3a,i**.[10] The syntheses of compounds **1a-m** were performed by nitrosation of the appropriate 1,3-disubstituted-5-aminopyrazoles according to conventional methods.[16–19]

Synthesis of 1,3-disubstituted-4-nitroso-5-aminopyrazoles 1a-m

Compounds **1a-e** were prepared according to reported methods.[16–19]

Compounds **1f-i** were prepared according to the following procedure.

A stream of ethyl nitrite was bubbled through a saturated solution of the appropriate 5-aminopyrazole (10 mmol) in EtOH for 10 min then a few drops of concentrated HCl was added and the ethyl nitrite bubbling was continued for 30 min. The red precipitate formed was collected and recrystallized from the appropriate solvent.

5-Amino-1-(4-fluorophenyl)-3-methyl-4-nitrosopyrazole **1f** Yield, 85%; mp 220–221°C (ETOH). IR (KBr)ν: 3300–2800, 1660, 1560, 1515 cm^{-1}. ^{1}H-NMR (DMSO-d$_6$): 2.63 (s, 3H, Me), 7.30–7.60 (m, 4H, Ar), 8.33 (br, 2H, NH_2). Found: C, 54.56; H, 4.14; N, 25.60. $C_{10}H_9FN_4O$ requires: C, 54.54; H, 4.11; N, 25.44%.

5-Amino-3-methyl-1-(2-nitrophenyl)-4-nitrosopyrazole **1g** Yield, 87%; mp 246–247 °C (ethanol). IR (KBr)ν: 3500–2800, 1650, 1580, 1530 cm^{-1}. ^{1}H-NMR (DMSO-d$_6$): 2.61 (s, 3H, Me), 7.70–7.90 (m, 3H, Ar), 8.20 (d, J = 7.9 Hz, 1H, Ar), 8.48 (br, 2H, NH_2). Found: C, 48.80; H, 3.40; N, 28.31. Anal $C_{10}H_9N_5O_3$ requires: C, 48.58; H, 3.66; N, 28.33%.

5-Amino-3-methyl-1-(3-nitrophenyl)-4-nitrosopyrazole **1h** Yield, 83%; mp 207–208.5 °C (ethanol). IR (KBr)ν: 3400–3000, 1650, 1530 cm^{-1}. ^{1}H-NMR (DMSO-d$_6$): 2.66 (s, 3H, Me), 7.60 (m, 1H, Ar), 7.98 (d, J = 8.7 Hz, 1H, Ar), 8.25 (d, J = 8.1 Hz, 1H, Ar), 8.30 (s, 1H, Ar), 8.65 (s, 2H, NH_2). Found: C, 48.45; H, 3.59; N, 28.21. $C_{10}H_9N_5O_3$ requires: C, 48.58; H, 3.66; N, 28.33%.

5-Amino-3-methyl-1-(4-nitrophenyl)-4-nitrosopyrazole **1i** Yield, 85%; mp 230–231.5 °C (dioxane). IR (KBr)ν: 3500–2900, 1650, 1600, 1520 cm^{-1}. ^{1}H-NMR (DMSO-d$_6$): 2.65 (s, 3H, Me), 7.8 (d, J = 8.9 Hz, 2H, Ar), 8.3 (d, J = 8.9 Hz, 2H, Ar), 8.7 (br, 2H, NH_2). Found: C, 48.75; H, 3.58; N, 28.26. $C_{10}H_9N_5O_3$ requires: C, 48.58; H,3.66; N, 28.33%.

Compounds 1j-m

A solution of sodium nitrite (2.11 g, 30 mmol) in water (10 ml) was added dropwise to a solution of the appropriate 5-aminopyrazole (30 mmol) in acetic acid (100 ml). After stirring for 1 h, the reaction mixture was diluted with water (100 ml), neutralized with 30% ammonia and extracted with ethyl acetate (3 × 50 ml). The combined organic extracts were dried over magnesium sulfate and rotary evaporated *in vacuo* to give a red solid residue that was recrystallized from the appropriate solvent.

5-Amino-3-ethyl-1-methyl-4-nitrosopyrazole **1j** Yield, 65%; mp 114–115 °C (ethyl acetate/light petroleum). IR (KBr)ν: 3370, 3200–2800, 1660, 1540, 1500, 1450, 1260 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.42 (t, J = 7.5 Hz, 3H, Me), 3.08 (q, J = 7.5 Hz, 2H, CH_2), 3.54 (s, 3H, N-Me), 7.75 (br, 2H, NH_2). Found: C, 46.57; H, 6.42; N, 36.55. $C_6H_{10}N_4O$ requires: C, 46.74; H, 6.53; N, 36.34%.

5-Amino-1-ethyl-3-methyl-4-nitrosopyrazole **1k** Yield, 82%; mp 168 °C (ethyl acetate/light petroleum). IR (KBr)ν: 3360, 3200–2500, 1680, 1550, 1510, 1240 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.36 (t, J = 7.2 Hz, 3H, Me), 2.64 (s, 3H, Me), 3.88 (q, J = 7.2 Hz, 2H, CH_2), 7.68 (br, 2H, NH_2). Found: C, 46.69; H, 6.49; N, 36.47. $C_6H_{10}N_4O$ requires: C, 46.74; H, 6.53; N, 36.34%.

5-Amino-3-n-butyl-1-methyl-4-nitrosopyrazole **1l** Yield, 55%; mp 74–75 °C (ethyl acetate/light petroleum). IR (KBr)ν: 3400–2800, 1660, 1535, 1500, 1460, 1260 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 0.94 (t, J = 7.2 Hz, 3H, Me), 1.46 (m, 2H, CH_2), 1.82 (m, 2H, CH_2), 3.02 (t, J = 7.9 Hz, CH_2), 3.54 (s, 3H, N-Me), 7.98 (br, 2H, NH_2). Found: C, 52.69; H, 7.78; N, 30.82. $C_8H_{14}N_4O$ requires: C, 52.73; H, 7.74; N, 30.74%.

5-Amino-1-tert-butyl-3-methyl-4-nitrosopyrazole **1m** Yield, 68%; mp 137–138 °C (ethyl ether). IR (KBr)ν: 3400–2900, 1630, 1550, 1470, 1230 cm^1. ^{1}H-NMR (DMSO-d_6): 1.49 (s, 9H, *t*-Bu), 2.50 (s, 3H, Me), 8.18 (s, 2H, NH2). Found: C, 52.74; H, 7.77; N, 30.48. $C_8H_{14}N_4O$ requires: C, 52.73; H, 7.74; N, 30.74%.

Synthesis of Pyrazolo[4,3-c][1,2,5]oxadiazin-3(5H)-ones 2a-m

Compounds **2a-e** were prepared according to the reported methods.[9,18] Compounds **2f-m** were prepared according to the following procedure. Trichloromethyl chloroformate (1.32 ml, 11 mmol) was added to a suspension of the starting 5-amino-4-nitrosopyrazole **1f-m** (10 mmol) in anhydrous THF (100 ml). The mixture was stirred at room temperature until the starting material could not be detected by TLC (1–3 h), then the solution was rotary evaporated *in vacuo* to dryness and the red residue was recrystallized from dichloromethane/light petroleum.

5-(4-Fluorophenyl)-7-methylpyrazolo[4,3-c][1,2,5] oxadiazin-3(5H)-one **2f** Yield, 74%; mp 138–140 °C. IR (KBr)ν: 1760, 1630, 1585, 1510 cm^{-1}. ^{1}H-NMR (DMSO-d_6): 2.50 (s, 3H, Me), 7.42 (m, 2H, Ar), 7.94 (m, 2H, Ar). ^{13}C-NMR (DMSO-d_6): 11.32 (q, J = 130.2 Hz, Me), 116.40 (dd, J_{CF} = 23.0 Hz, J_{CH} = 165.1 Hz, Ar), 121.90 (dd, J_{CF} = 8.5 Hz, J_{CH} = 165.6, Ar), 132.42 (s, Ar), 148.121 (s), 148.56 (s, C-7), 150.16 (s), 153.23 (s), 160.19 (d, J_{CF} = 243.4, Ar). Found: C, 53.78; H, 2.90; N, 22.60. $C_{11}H_7FN_4O_2$ requires: C, 53.66; H, 2.86; N, 22.75%.

7-Methyl-5-(2-nitrophenyl)pyrazolo[4,3-c][1,2,5] oxadiazin-3(5H)-one **2g** Yield, 93%; mp 141–142 °C. IR (KBr)ν: 1760, 1640, 1610, 1540, 1350 cm^{-1}. ^{1}H-NMR (DMSO-d_6): 2.50 (s, 3H, Me), 7.75–8.24 (m, 4H, Ar). ^{13}C-NMR (DMSO-d_6): 11.30 (q, J = 130.5 Hz, Me), 127.77 (d, J = 166.5 Hz, Ar), 127.07 (d, J = 162.0 Hz, Ar), 130.32 (d, J = 168.5 Hz, Ar), 134.49 (d, J = 167.0 Hz , Ar), 134.71 (s), 143.17 (s), 147.19 (s), 148.85 (s), 151.51 (s, C-7), 152.91 (s). Found: C, 48.00; H, 2.20; N, 25.81. $C_{11}H_7N_5O_4$ requires: C, 48.35; H, 2.58; N, 25.63%.

7-Methyl-5-(3-nitrophenyl)-pyrazolo[4,3-c][1,2,5] oxadiazin-3(5H)-one **2h** Yield, 89%; mp 132–133 °C. IR (KBr)ν: 1760, 1630, 1610, 1540, 1500/cm^{-1}. ^{1}H-NMR (DMSO-d_6): 2.56 (s, 3H, Me), 7.89 (m, 1H, Ar), 8.22 (m, 1H, Ar), 8.40 (m, 1H, Ar), 8.75 (m, 1H, Ar). ^{13}C-NMR (DMSO-d_6): 11.60 (q, J = 131.0 Hz, Me), 114.46 (d, J = 171.8 Hz, Ar), 122.24 (d, J = 168.3 Hz, Ar), 126.03 (d, J = 168.0 Hz, Ar), 132.37 (d, J = 169.3 Hz, Ar), 137.93 (s, Ar), 149.23 (s), 149.28 (s), 150.26 (s, C-7), 152.33 (s), 154.02 (s). Found: C, 48.10; H, 2.39; N, 25.61. $C_{11}H_7N_5O_4$ requires: C, 48.35; H, 2.58; N, 25.63%.

7-Methyl-5-(4-nitrophenyl)pyrazolo[4,3-c][1,2,5] oxadiazin-3(5H)-one **2i** Yield, 91%; mp 184–185 °C. IR (KBr)ν: 1760, 1630, 1580, 1510, 1500 cm^{-1}. ^{1}H-NMR (DMSO-d_6): 2.56 (s, 3H,

Me), 8.26 (d, J = 9.1 Hz, 2H, Ar), 8.46 (d, J = 9.1 Hz, 2H, Ar). ^{13}C-NMR (DMSO-d_6): 11.42 (q, J = 130.3 Hz, Me), 119.37 (d, J = 170.4 Hz, Ar), 125.43 (d, J = 171.0 Hz, Ar), 141.11 (s, Ar), 144.71 (s, Ar), 148.71 (s), 149.46 (s, C-7), 151.65 (s), 152.86 (s). Found: C, 48.45; H, 2.38; N, 25.66. $C_{11}H_7N_5O_4$ requires: C, 48.35; H, 2.58; N, 48.35%.

7-Ethyl-5-methylpyrazolo[4,3-c][1,2,5]oxadiazin-3(5H)-one **2j** Yield, 78%; mp 116.5–117.5 °C. IR (KBr)ν: 1760, 1630, 1550, 1510 cm^{-1}. ^{1}H-NMR (DMSO-d_6): 1.21 (t, J = 7.2 Hz, 3H, Me), 2.74 (q, J = 7.2 Hz, 2H, CH_2), 3.55 (s, 3H, N-Me). ^{13}C-NMR (DMSO-d_6): 10.50 (q, J = 127.5 Hz, Me), 19.50 (t, J = 129.1 Hz, Me), 32.37 (q, J = 141.2 Hz, Me), 146.98 (s), 150.94 (s), 151.22 (s, C-7), 153.59 (s). Found: C, 46.47; H, 4.62; N, 31.20. $C_7H_8N_4O_2$ requires: C, 46.66; H, 4.47; N, 31.09%.

5-Ethyl-7-methylpyrazolo[4,3-c][1,2,5]oxadiazin-3(5H)-one **2k** Yield, 75%; mp 115.5–116 °C. IR (KBr)ν: 1750, 1610, 1500 cm^{-1}. ^{1}H-NMR (DMSO-d_6): 1.31 (t, J = 7.2 Hz, 3H, Me), 2.40 (s, 3H, Me), 3.96 (q, J = 7.2 Hz, 2H, CH_2). ^{13}C-NMR (DMSO-d_6): 11.31 (q, J = 129.8 Hz, Me), 12.83 (q, J = 127.4 Hz, Me), 40.46 (t, J = 132.9 Hz, CH_2), 147.09 (s, C-7), 147.60 (s), 150.32 (s), 153.68 (s). Found: C, 46.76; H, 4.29; N, 31.10. $C_7H_8N_4O_2$ requires: C, 46.66; H, 4.47; N, 31.09%.

7-n-Butyl-5-methyl-pyrazolo[4,3-c][1,2,5]oxadiazin-3(5H)-one **2l** Yield, 77%; mp 103–104 °C. IR (KBr)ν: 1770, 1640, 1550, 1510 cm^{-1}. ^{1}H-NMR (DMSO-d_6): 0.90 (t, J = 7.2 Hz, 3H, Me), 1.38 (m, 2H, CH_2), 1.67 (m, 2H, CH_2), 2.76 (t, J = 7.5 Hz, CH_2), 3.56 (s, 3H, N-Me). ^{13}C-NMR (DMSO-d_6): 13.47 (q, J = 123.4 Hz, Me), 21.50 (t, J = 121.6 Hz, CH_2), 25.51 (t, J = 131.0 Hz, CH_2), 28.16 (t, J = 130.5Hz, CH_2), 30.25 (t, J = 141.5 Hz, CH_2), 32.36 (q, J = 141.15 Hz, Me), 147.10 (s), 150.24 (s), 150.92 (s, C-7), 153.64 (s). Found: C, 51.87; H, 11.24; N, 26.98. $C_9H_{12}N_4O_2$ requires: C, 52.16; H, 11.18; N, 27.03%.

5-tert-Butyl-7-methylpyrazolo[4,3-c][1,2,5]oxadiazin-3(5H)-one **2m** Yield, 82%; mp 129.5–130.5 °C. IR (KBr)ν: 1740, 1630, 1590, 1450 cm^{-1}. ^{1}H-NMR (DMSO-d_6): 1.56 (s, 9H, *t*-Bu), 2.36 (s, 3H, Me). ^{13}C-NMR (DMSO-d_6): 11.29 (q, J = 129.7 Hz, Me), 27.69 (q, J = 131.0 Hz, *t*-Bu), 60.20 (s, *t*-Bu), 145.81 (s, C-7), 147.59 (s), 150.30 (s), 152.92 (s). Found: C, 51.94; H, 11.27; N, 27.18. $C_9H_{12}N_4O_2$ requires: C, 52.16; H, 11.18; N, 27.03%.

Synthesis of Pyrazolo[4,3-c][1,2,5]oxadiazin-3(5H)-thiones 3a,i

To a suspension of the 5-amino-4-nitrosopyrazole **1a**[16]**,i** (6 mmol) in anhydrous THF (100 ml), thiophosgene (0.5 ml, 6.3 mmol) was added dropwise then the mixture was stirred at room temperature until the TLC analysis revealed the loss of the starting material (3–5 h). When these solutions were rotary evaporated *in vacuo* to dryness a red solid residue was obtained which was purified by column chromatography using light petroleum/ethyl acetate (φ = 3%) as an eluent.

7-Methyl-5-phenyl-pyrazolo[4,3-c][1,2,5]oxadiazin-3(5H)-thione **3a** Yield, 76%; mp 165–167 °C. IR (KBr)ν: 1660, 1590, 1570, 1500 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 2.53 (s, 3H, Me), 7.35–7.58 (m, 3H, Ar), 8.05–8.10 (m, 2H, Ar). ^{13}C-NMR ($CDCl_3$): 10.92 (q, J = 131.0 Hz, Me), 120.81(d, J = 165.0 Hz, Ar), 127.32 (d, J = 162.0 Hz, Ar), 129.21 (d, J = 162.0 Hz, Ar), 136.65 (s, Ar), 145.86 (s, C-4a or C-7a), 146.76 (s, C-7a or C-4a), 147.83 (s, C-7), 172.42 (s, C-3). Found: C, 54.20; H, 3.56; N, 22.60; S,12.89. $C_{11}H_8N_4OS$ requires: C, 54.09; H, 3.30; N, 22.94; S, 13.12%.

7-Methyl-5-(4-nitrophenyl)pyrazolo[4,3-c][1,2,5]oxadiazin-3(5H)-thione **3i** Yield, 67%; mp 170–172 °C. IR (KBr)ν: 1700, 1625, 1600, 1525, 1505 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 2.58 (s, 3H, Me), 8.35 (d, J = 9.4 Hz, 2H, Ar), 8.45 (d, J = 9.4 Hz, 2H, Ar). ^{13}C-NMR (DMSO-d_6). 10.19 (q, J = 130.0 Hz, Me), 119.75 (d, J = 171.0 Hz, Ar), 124.67 (d, J = 172.0 Hz, Ar), 141.34 (s, Ar), 144.17 (s, Ar), 147.40 (s, C-4a or C-7a), 147.75 (s, C-7a or C-4a), 148.73 (s, C-7), 171.82 (s, C-3). Found: C, 45.87; H, 2.20; N, 24.51; S,11.12. $C_{11}H_7N_5O_3S$ requires: C, 45.67; H, 2.43; N, 24.21; S, 11.08%.

Synthesis of 6-aminopyrazolo[3,4-d][1,3] oxazin-4(1H)-one 5a

A solution of 3-aminopyrazolo-4-carboxylic acid (2.5 g, 19.66 mmol)[21] and NaOH (0.78 g, 20, 16 mmol) in 20 ml of water was stirred at room temperature. A saturated solution of CNBr (4.8 g, 45.32 mmol, 97% pure, Aldrich) was added dropwise over 1 h. After stirring 24 h with random additions of 5% sodium hydroxide to maintain a pH of 8 the insoluble solid was filtered and washed vigorously with water. The resulting brown solid was dried and further purified by column chromatography (ethyl acetate-methanol ($\varphi = 20\%$)). Yield, 30%; mp > 300 °C. IR (KBr)ν: 3360, 3160 cm^{-1} NH; 1790 cm^{-1} C = O. TLC system: ethyl acetate or ethyl acetate-methanol ($\varphi = 20\%$). Found: C, 39.17; H, 2.56; N, 36.68. $C_5H_4N_4O_2$ requires: C, 39.47; H, 2.63; N, 36.84%.

Synthesis of 3-methyl 6-aminopyrazolo[3,4-d] [1,3]oxazin-4(1H)-one 5b

Compound **5b** was prepared from **4b**[22] according to the procedure for preparation of **5a** by increasing the reaction time up to 30 h. The resulting brown solid was heated in ethanol and the clear solution after filtration was evaporated under reduced pressure. The white solid residue was gently heated in DMF/water ($\varphi = 50\%$), dried and further purified by column chromatography (ethyl acetate). Yield, 35%; mp > 300 °C. IR (KBr)ν: 3373, 3195 cm^{-1} NH; 1761 cm^{-1} C = O. ^{1}H NMR(DMSO-d_6): δ 12.69 (br, 1H, NH); 7.73 (br, 2H, NH_2); 2.28 (s, 3H, methyl). TLC system: ethyl acetate or ethyl acetate-methanol ($\varphi = 20\%$). Found: C, 43.29; H, 3.67; N, 33.45. $C_6H_6N_4O_2$ requires: C, 43.37; H, 3.61; N, 33.73%.

Synthesis of N-(4-carboethoxypyrazol-3-yl)Ní-(benzoyl)thiourea 6c

To a stirred solution of commercially available or *in situ* prepared benz-oyilisothiocyanate (6.76 g, 35.46 mmoles)[23,24] in anhydrous acetone (10 ml when commercially available benzoylisothiocyanate was used), a saturated 3-amino-4-carboethoxypyrazole[21] **4c** solution (6.2 g, 39.96 mmol) in anhydrous acetone (50 ml), was slowly added dropwise. The mixture was gently refluxed for 3 h. The precipitate was collected by filtration, repeatedly washed with cold water and dried. Compound **6c** can be processed without recrystallization. An analytically pure sample can be obtained by recrystallization from ethanol.

Yield, 80%; mp 198–200 °C. IR (KBr)ν: 3230 cm^{-1} NH; 1710, 1680 cm^{-1} C = O. TLC system: ethyl acetate. Found: C, 52.59; H, 4.67; N, 17.75; S, 10.04. $C_{14}H_{14}N_4O_3S$ requires: C, 52.81; H, 4.43; N, 33.73; S, 10.07%.

Synthesis of 6-aminopyrazolo[3,4-d] [1,3]thiazin-4(1H)-one 7c

A suspension of N-(4-carboethoxypyrazol-3-yl)N′-(benzoyl)thiourea **6c** (0.6 g., 1.88 mmol) in 98% sulphuric acid (4 ml) was left at room temperature for 4 days. The clear solution resulting was slowly added to ice water (30 ml) and the solid residue was collected, washed with sodium bicarbonate ($\omega = 5\%$) and cold water, dried and recrystallized from ethanol. Yield, 80%; mp > 300 °C. IR(KBr)ν: 3409, 3098 cm^{-1} NH; 1692 cm^{-1} C = O. TLC system: ethyl acetate-methanol ($\varphi = 20\%$). Found: C, 35.77; H, 2.40; N, 33.31; S, 19.12. $C_5H_4N_4OS$ requires: C, 35.70; H, 2.39; N, 33.51; S, 19.06%.

Hydrolysis of 7-Methyl-5-(4-nitrophenyl) pyrazolo[4,3-c][1,2,5]oxadiazin-3(5H)-one 2i

(0.1 g., 0.366 mmol) was dissolved in methanol (50 ml). The colour turned from orange to green. After 24 h, evaporation of the reaction mixture to dryness yielded a solid which was collected with ethyl ether. Yield, 90%; mp 151.5–152.5 °C.

IR (KBr)ν: 3206 (br), 1707, 1683, 1591, 1518, 1501 cm^{-1}. ^{1}H-NMR (DMSO-d_6): 2.38 (s, 3H, Me), 3.66 (s, 3H, Me), 8.07 (d, J = 8.8 2H, Ar),

8.39 (d, J = 8.8, 2H, Ar), 14.30 (br, 1H, NH). MALDI-MS $(M+H)^+$ calcd. for $C_{12}H_{11}N_5O_5$ 305.25197, found m/z 306.6.

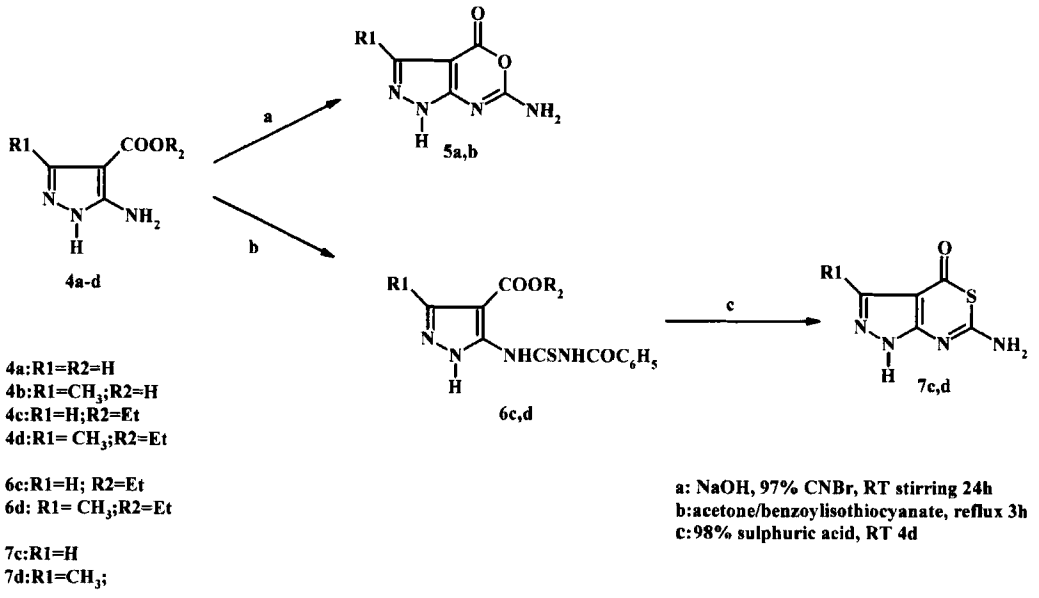
Hydrolysis of 5-tert-butyl-7-methyl pyrazolo [4,3-c] [1,2,5] oxadiazin-3(5H)-one 2m

Yield, 85%; mp 113–115 °C. IR (KBr)ν: 3243, 1711, 1561 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.66 (s, 9H, *t*-Bu), 2.43 (s, 3H, Me), 3.78 (s, 3H, OMe), 7.28 (s, 1H, NH). MALDI-MS $(M+H)^+$ calcd. for $C_{10}H_{16}N_4O_3$ 240.26, found m/z 241.8. $(M+K)^+$, found m/z 280.3.

Synthesis of 6-aminopyrazolo[3,4-d][1,3]oxazin-4(1H)-ones 5a, b

The 6-aminopyrazoloxazinones **5a,b** were available from the reaction of the corresponding 3-aminopyrazolo-4-carboxylic acid **4a,b**[20,21] with cyanogen bromide in sodium hydroxide.[14] The products were insoluble in aqueous media and could easily be purified by filtration and column chromatography. The isosteric thiazinone derivatives **7c,d** were prepared according to our previously reported method by ring closure in 98% sulphuric acid of the pertinent N-(4-carboethoxypyrazol-3-yl) N′-benzoylthiourea **6c, d**.[10,20] These latter intermediates produced the isomeric 4-hydroxy-6-mercaptopyrazolo[3,4-d] pyrimidine derivatives when heated with a methanolic 2N solution of sodium hydroxide.[10,20] Intermediates **6c, d**[10,20] were obtained by reaction of the 3-amino-4-carboethoxypyrazolo **4c**[21] or its 5-methyl substituted analog **4d**[22] with an equimolar amount of benzoylisothiocyanate, which was obtained either commercially or prepared *in situ*,[23,24] in acetone at reflux for 3 h. The reaction with benzoylisothiocyanate was necessary because of the lack of reactivity of compounds **4c,d** with ammonium or potassium thiocyanate.[26,29,30] The synthetic routes are outlined in Schemes 1 and 2.

All spectral data (IR, ^{1}H and ^{13}C NMR) are in accordance with the assigned structures and are consistent with the literature data for the compounds noted.[9,10,22–28] They are listed below for each of the new compounds synthesized.



SCHEME 2 Synthetic routes to 6-aminopyrazolo[3,4-d][1,3]oxazin-4(1H)-ones **5 a,b** and 6-aminopyrazolo[3,4-d][1,3]thiazin-4-(1H)-one **7 c,d**.

HPLC Stability Studies

Stability of the Pyrazolooxadiazinones in MeOH Solution

Monitoring the process by HPLC, the degradation in MeOH solutions starts immediately with the rate of decomposition of **2i** > **2c** > **2a** (Table I). Compound **2k** decomposed in MeOH with a rate constant of 0.006 min^{-1}. The UV spectrum of compound **2i** in MeOH registered after 1 h showed a similar profile as the one registered after 24 h (λ max = 314 nm). On the other hand the spectra of compounds **2a** and **2c** registered after 1 h were overlapping and showed three bands with maxima at 420, 310, 256 nm. After one day in MeOH the UV-Visible spectra of the three compounds were similar with λ max at 314–8 nm. The peaks in the HPLC chromatograms due to the parent compounds disappeared and only those due to the degradation products were evident after 24 h in MeOH. The latter were evident at lower retention times relative to the parent compound indicative of the formation of a more hydrophilic product, corresponding to the 3-methyl-1-p-nitrophenyl-4-nitroso-5methoxycarbonylaminopyrazole (MALDI-MS $(M+H)^+$ calcd. for $C_{12}H_{11}N_5O_5$ 305.25197, found m/z 306.6.), i.e. the isocyanate masking derivative as shown in Figure 1 (*pathways a and b*). Similarly compound **2m** produced the corresponding 3-methyl-1-tert-butyl-4-nitroso-5-methoxycarbonylaminopyrazole (MALDI-MS $(M+H)^+$ calcd. for $C_{10}H_{16}N_4O_3$ 240.26, found m/z 241.8. $(M+K)^+$, found m/z 280.3.

Stability of the Pyrazolooxadiazinones in THF Solution

The HPLC retention times for the degradation products of **2a**, **2c**, and **2i** were 3.34, 4.0 and 2.4 min, respectively. Decomposition of the pyrazolo-oxadiazinones in THF is slower (HPLC analysis (Table I) of the solutions still shows the presence of **2c** and **2a** even after 1 week[10]) but it might be more extensive and less compatible with a simple hydrolysis of the parent ring system. It may correspond to capture of the isocyanate by adventitious water in the THF and loss of carbonic anhydride (CO_2) with formation of the corresponding 5-amino-3-methyl-1-p-nitrophenyl-4-nitrosopyrazoles **1a**, **1c**, **1i**. No decomposition was observed in a sealed NMR tube until 14 days.

Energy Calculations

All procedures were performed using SYBYL molecular modelling software version 6.5 (1999, Tripos Associates Inc., Saint Louis, MO, USA)[25] installed on a SGI O2-R5000 and −R10000 workstations (Silicon Graphics Computer Systems, Mountain View, CA, USA) operating under IRIX

TABLE I Degradation of pyrazolooxadiazinones in MeOH and THF

Compound	MeOH		THF	
	Time	% Degradation[a]	Time	% Degradation[a]
2a	60 min	64	week	87
2c	20 min	36	week	88
2i	10 min	39	13 min	3
	k min^{-1}	$t_{1/2}$ min	k min^{-1}	$t_{1/2}$ min
2k[b]	0.006 ± 0.0002	115	0.049 ± 0.0003	141

[a] The compounds were incubated in each solvent and monitored by HPLC. The % degradation was calculated from the decrease in the area of the parent peak in each chromatogram (see Experimetal). [b] Sufficient time points were collected with this compound to obtain rate constants for the first order decomposition in both solvents.

6.5. Energy calculations were carried out using the Powell conjugate gradient minimizer within the MAXIMIN procedure. Electrostatic contributions were calculated with the dictionary charges for the protein structure originating from the AMBER force field as implemented in the actual Sybyl version. Ligand partial charges were calculated using an *ab initio* calculation within the quantum chemical program package Gaussian 92.[26] For *ab initio* charge calculation a Hartree-Fock operator with the STO-3G basis set was used.[27]

Docking studies

HLE is a glycosylated, strongly basic serine protease consisting of a single peptide chain of 218 AA residues and four disulfide bridges (42–58, 136–201, 168–182, 191–220).[28] The 3-D structure of HLE has been elucidated using a combination of peptide sequencing and crystallographic methods.[29,30] The sequence alignment was based on tertiary structure similarities and follows the numbering system defined for bovine chymotrypsin-A.[31]

To date the native stucture of HLE has not been forthcoming since the uncomplexed form of the enzyme produces crystals unsuitable for X-ray analysis, but the structures of a number of HLE-inhibitor complexes have been determined crystallographically.[31,32] Therefore, the three-dimensional structural model of HLE used in these studies was derived from the coordinates registered as 1PPF in the Brookhaven Protein Data Bank which were obtained from HLE complexed to the third domain of Turkey (OMTKY 3) ovomucoid ligand.[32,33] The sugar moieties were not used in this structure, but since the glycosylation sites are sufficiently far away from the substrate binding sites, they are not likely to affect ligand binding.

The docking analysis was performed using the FlexX default parameters and the complex exhibiting the highest interaction energy was selected.[34] Water molecules were removed as they were found not to be located within the active site region and hydrogen atoms on the backbone and side chains were added based on the standard average bond angles and lengths. Dictionary charges from the AMBER force field were added. A sphere of 10Å around SER195 was defined as binding region for the docking experiments.

Since crystal structures of the ligands under investigation were not available their structures were created manually starting from geometrically optimized fragments. The potential energies were minimized in order to obtain a comparable starting point for each compound. The Powell method of the MAXIMIN 2 subroutine was invoked for minimization of the strain energy of each molecule[26,35] using the parameters of the TRIPOS standard force field.[25] During the energy minimization process contributions from bond stretching, angle bending, torsional, out of plane bending, bonded and non-bonded van der Waals and electrostatic energy were calculated.

Enzymology

The enzymes were assayed spectrophotometrically at 410 nm with *p*-nitroanilide substrates[36] at 25 °C. MeOsucc-AAPV-pNA[(a)] was purchased from Calbiochemical Company. Succ-AAPA-pNA was purchased from Chemical Dynamics Corporation. N-Succ-AAPF-pNA was purchased from Bachem. All substrate stock solutions were prepared in DMSO. Peptide *p*-nitroanilide stock concentrations were either determined from the absorbance at 315 nm ($\varepsilon = 15{,}000$) or by complete hydrolysis to free p-nitroanilide ($\varepsilon = 9{,}350$ at

[(a)] Peptide based substrates were abbreviated using the standard letter representation of the amino acids. Additional functionalities present were abbreviated as follows; MeOsucc, Methoxysuccinyl; pNA, para-nitroanilide.

410 nm). Buffers (Sigma Chemical Company) were titrated to the appropriate pH with either NaOH or HCl prior to use. Human Cat-G and bovine α-chymotrypsin were purchased from Athens Research and Technology, Inc. (Athens, GA). HLE (Elastin Products, St. Louis, MO) was assayed in 1 ml of buffer A (450 mM NaCl, DMSO ($\varphi = 10\%$) and 45 mM TES at pH 7.5) versus either 0.2 mM succ-AAPA-pNa or 1 mM MeOsucc-AAPV-pNa. The concentration of HLE stock solutions was determined based upon the activity *versus* 1 mM MeOsucc-AAPV-pNa. The turnover number under these conditions determined by Green *et al.*[37] was $472 \pm 30\,min^{-1}$. Progress curves for the hydrolysis of substrates were linear in the absence of inhibitors over the time course of the reaction (data not shown). The slopes were proportional to the rate of the reaction. The activity of inhibitors *versus* the 0.04 μM HLE catalyzed hydrolysis of 0.2 mM succ-AAPA-pNa was determined at concentrations of inhibitor designed to yield 30–50% inhibition when possible according to the method of Knight *et al.*[36] The activity of the time-dependent inhibitors (k_{inact}/K_i) *versus* the HLE catalyzed hydrolysis of 0.2 mM succ-AAPA-pNa was determined at inhibitor concentrations that produced maximum inhibition over either 15 or 7.5 min. A typical concentration used for **2i** was 18 μM.

The stability of 46 μM **2i** was monitored by UV-Visible spectroscopy in buffer A at 25 °C. The first order rate constants for the disappearance of parent and appearance of a new species were determined by fitting the change in absorbance over time to equation (2) UV-Visible spectroscopy was conducted on a Varian DMS-300 specrophotometer.

Kinetic data were collected either on Cary 210 and Varian DMS-300 spectrophotometers or a Molecular Devices Thermomax 96 well plate reader. The data were fit by linear regression to equation (3) to obtain the initial rates. The K_i for the inhibition of HLE by inhibitors was estimated assuming competitive inhibition using equation (4). This equation predicts that $v_i/v_0 = 0.5$ when $[I] = K_i$. The nonlinear progress curves observed with time-dependent inhibitors of HLE were fitted equation (5)[38] to obtain the first order rate constant k_{obs}. Second order rate constants (k_{inact}/K_i) were estimated by calculating $k_{obs}/[I]$ and then correcting for the substrate concentration and Michaelis constant according to equation (6).

$$y = a * e(-kt) + c \tag{2}$$

$$y = vX + B \tag{3}$$

$$v_i/v_0 = 1/(1 + [I]/K_i) \tag{4}$$

$$y = v_s * t + ((v_0 - v_s)(1 - e(-k_{obs} * t))/k_{obs})A_0 \tag{5}$$

$$k_{obs}/[I] = k_{inact}/(K_i(1 + [S]/K_m)) \tag{6}$$

In equations (2, 3) and (5) y refers to absorbance. In equation (2), a and c refer to the pre-exponential and a constant respectively. In equation (3) X is time and v is the slope ($\Delta A/\Delta t$) which is proportional to the rate of substrate hydrolysis. B is the y-intercept. In equations (4) and (6) K_i is the inhibition constant which is equivalent to the dissociation constant for formation of the E–I complex. In equations (4) (5), v_0 and v_i refer to the initial uninhibited and inhibited velocities respectively. In equation (5) v_s and A_0 refer to the final steady state velocity and initial absorbance respectively. In the case of irreversible inhibition v_s approaches zero. In equations (5) and (6) k_{obs}, and k_{inact} refer to the observed and actual rate constants respectively. The former is dependent on the inhibitor concentration while the latter is independent.

Cat-G and α-chymotrypsin were assayed in 0.2 ml buffer A (45 mM TES, pH 7.5, 135 mM NaCl and DMSO ($\varphi = 10\%$)) in a 96 well plate format *versus* 0.2 mM succ-AAPF-pNa as substrate. Inhibitors were tested at 10 and 20 and in some cases 50 μg/ml. Fut-175 (1. 5 and 20 μg/ml) was used as a positive control for the serine proteases.[39]

RESULTS AND DISCUSSION

The compounds discussed in this work contain moieties potentially susceptible to addition of the catalytic hydroxyl of Ser-195[b] to produce an acylenzyme.[5,6,11] Formation of the latter intermediate can be facilitated by the anchimeric assistance of an unmasked amino group which can serve either as an electron donor or attractor[14] prior to irreversible inactivation by addition of a nucleophile such as histidine in the enzyme active site.[6] Improved inhibitor binding at the enzyme S_1[3(c)] site or other subsites may be gained from additional interactions with the ring heteroatoms.[40–44]

The reaction of the oxadiazinone ring that occurs in nonenzymatic models with nucleophiles such as amines, alcohols and carbanions is outlined in Figure 1 (*pathway a*), the possible reaction mechanism being under discussion.[9,45] Assuming reactions occur through first order processes, which is highly likely, an alternative proposal concerning the compounds reported in this paper is outlined in Figure 1 (*pathway b*) and discussed below. Whatever the mechanism is, both the parent compounds (i.e. the methoxycarbonilamino or the isocyanate derivative) possess polyfunctional electrophilic and/or nucleophile character during the initial attack and irreversible inactivation of the enzyme, respectively[9] if reaction with water occurs during enzyme reactions (*see* text) unmasking of the isocyanate, a functionality known to serve as a modifier of serine protease active sites, may be of particular significance.[5,6,11]

Due to the new topology of the inhibitors, docking experiments were carried out to obtain insight into the possible mechanism of action of the compounds and to explain the differences in mechanism of inhibition that some of the molecules displayed for HLE.

Docking Studies of Pyrazolooxadiazinones

A general problem in the prediction of three dimensional structures of flexible drugs is that the conformations *in vacuum* (i.e. the conformations calculated by force field, semiempirical or *ab initio* methods) may be significantly different from the ones in *aqueous* solution, in the crystal and in a protein binding site. Computer graphics-aided docking analysis based on molecular mechanics calculations, however, has proven useful for the determination of structures of supramolecular complexes of inhibitors bound to enzyme active sites.[46,47]

In Figures 2, 3, and 4 the resulting complex structures for compounds **2i**, **2k**, **2a**, and HLE are represented. As can be seen, all compounds investigated are located in proximity of two amino acids of the catalytic triad (Ser195, His57). The most active mechanism-based inhibitor in our series **2k** (Figure 2) was found to be closest to both amino acids. From the complex obtained it seems possible that the nitroso group formed after nucleophilic attack by the serine OH and subsequent ring opening reaction could react with the imidazole ring of His57. This is also the case for compound **2i** (Figure 3), whereas the non-mechanism based inhibitor **2a** is located in a unfavourable position.

Stability of the Pyrazolooxadiazinones and Hammett Analysis

HPLC analysis of fresh MeOH and THF solutions of the pyrazoloox-adiazinones showed only one characteristic peak for each compound. The HPLC retention times for **2a**, **2c**, and **2i** were 4.2, 5.7, and 4.8 min, respectively. The stability observed differed in MeOH and THF solutions and with structure. The rate of decomposition was greater in MeOH than in THF for all of the

[b] The sequence numbering system is that of α-chymotrypisn.[31]
[c] The enzyme subsites and peptide substrates are numbered according to the nomenclature of Schechter and Berger (1967).[40]

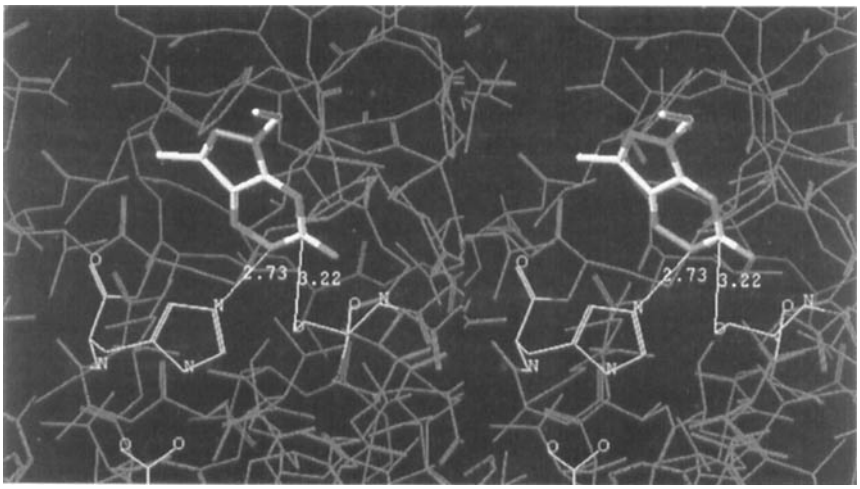


FIGURE 2 Compound **2k** docked into the HLE active site by FlexX. (See Color Plate I).

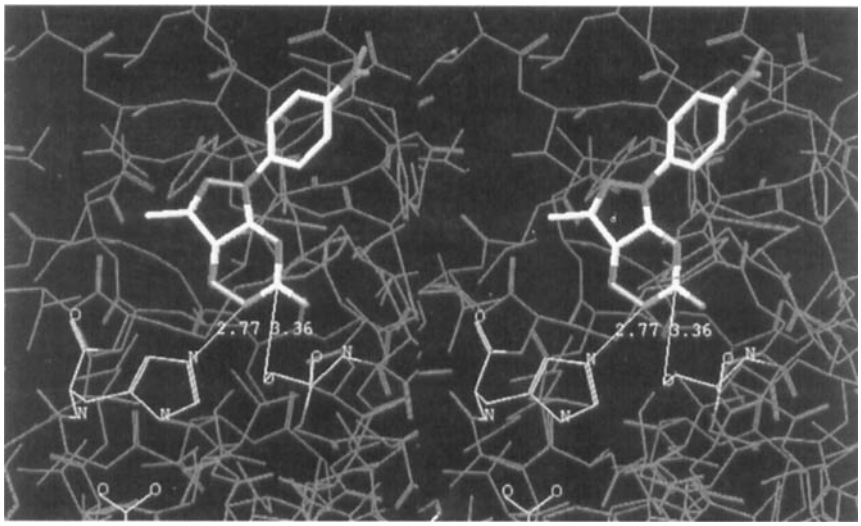


FIGURE 3 Compound **2i** docked into active site by FlexX. (See Color Plate II).

compounds tested and it is dependent on the N5 pyrazole substitution (Table I). A strong electron withdrawing group such as a p-nitrophenyl group (compound **2i**) increases the reactivity of the system. The decreased nucleophile reactivity of the N5-methyl substituted derivative **2b** compared to that of the N5-aryl substituted analogs is in accordance with this conclusion.

Assuming reactions occurs through first order processes, which is highly likely, the collected data allow approximate rate constants for degradation of **2a**, **2c** and **2i** to be estimated as 0.016, 0,022 and 0.049 min^{-1} respectively. The relative rates are 0.0, 0.14 and 0.49. Sigma para values for H, p-Cl and p-NO_2 are 0.0, 0.23 and 0.78 while the sigma-minus values are 0.0, 0.27 and 1.17 for the anilines. A Hammett analysis (*graphs not shown*) leads to ρ values (slopes) of 0.5–0.6. Thus the slope is similar or greater than those that typically apply to reactions at benzoate-type cen-

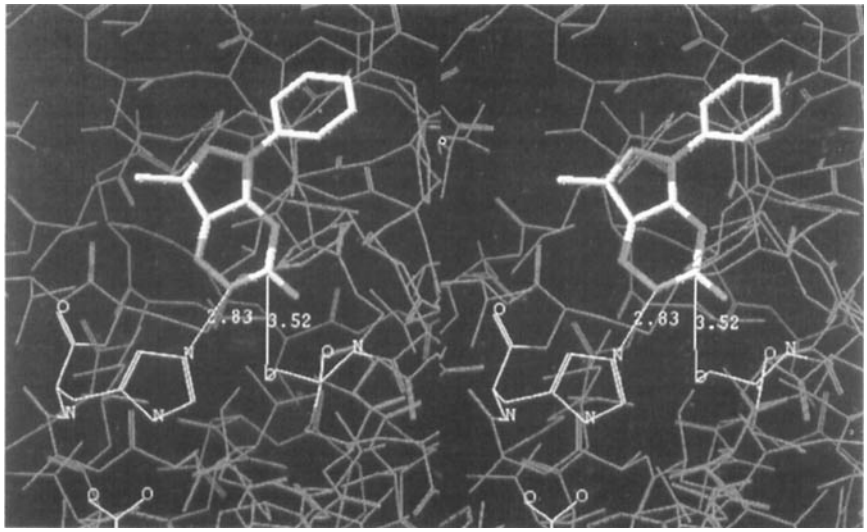


FIGURE 4 Compound **2a** docked into active site by FlexX. (See Color Plate III).

tres, one bond displaced from the aromatic ring. The reaction here is postulated to occur at an atom which is four bonds away from the aromatic ring. An alternative proposal which is compatible with the above observations is outlined in (Figure 1, *pathway b*) if reaction with water occurs during enzyme reactions. The inhibitory effect might be due to the capture of the isocyanate isomer by adventitious water and loss of CO_2 leading to compounds and then possibly their corresponding ureas **1a**, **1c**, **1i**. The latter mechanism might suggest a possible additional second hit role of the nitroso group as nucleophile, similar to the powerful α-effect nucleophile used as antidote to nerve gases through nucleophilic removal of covalently bound ligand from the active site-OH of acetylcholinesterase. Actually, NMR studies revealed that compound **2i** was stable in THF over several weeks when left in a sealed tube whereas the same compound decomposed to give the corresponding 3-methyl-1-p-nitrophenyl-4-nitroso-5-aminopyrazolo **1i** when reaction with adventitious water was possible. However, it must be pointed out that the character of the nitroso group as nucleophile in the intermediates here is reduced because of the p-NO_2 electron withdrawing effect on the heterocyclic ring whereas the same π-acceptor effect (-M) enhances the electrophilic character of the isocyanate (C=N=O) group.

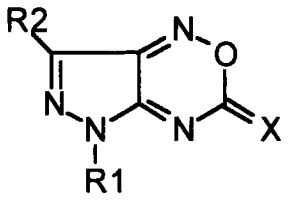
As an extension of the study we explored the potential of the structurally related 6-aminopyrazolo[3,4-d][1,3]oxazin-4(1H)-ones (compounds **5a,b**) and 6-aminopyrazolo[3,4-d][1,3]thiazin-4-(1H)-one (compounds **7c,d**)[10,20] as HLE inhibitors.

Enzymology

The inhibitory activities of the pyrazolooxadiazinones **2a-m** against HLE, chymotrypsin and Cat-G were determined spectrophotometrically using p-nitroanilide substrates (see the *Experimental*).[36] The compounds **2a-m** were either non-inhibitory or relatively weak, apparently reversible inhibitors of HLE except in the case of **2i** and **2k**.[(d)] These two compounds were clearly

[(d)] The progress curves for substrate hydrolysis were linear and the slopes were either equal to that of the control reaction (non-inhibitory) or less than the control reaction (reversible inhibition).

TABLE II Inhibition of HLE by compounds **2a-m** and **3a,i**



Compound	Inhibitor concentration μM	K_i μM	k_{inact}/K_i $M^{-1}s^{-1}$
2a	33	94	
2b	181	207	
2c	38	87	
2d	100	NI	
2e	100	NI	
2f	100	NI	
2g	181	>8400	
2h	181	>8400	
2i	18		**235**
2j	100	199 ± 34	
2k	100		**124 ± 13**
2l	100	113 ± 10	
2m	100	NI	
3a	NI		
3i	NI		

ND = compound yielded 44% inhibition but precipitated at this concentration therefore, K_i could not be calculated. NI = no inhibition at the highest concentration tested (limit of solubility).

time-dependent inhibitors of HLE (Table II) as shown in Figure 5 for **2i**. The second order rate constants for inactivation of HLE by **2i** and **2k** are 235 $M^{-1}s^{-1}$ and 124 $M^{-1}s^{-1}$, respectively.[e] The first order rate constant for the inactivation of HLE by 18 μM **2i** was 0.26 min. The mechanism for this process could be either slow binding inhibition or formation of a covalent complexe(s). Unfortunately if a complex was formed, its stability precluded isolation because upon removal of excess inhibitor only active enzyme was recovered. Compound **2i** was unstable under the assay conditions as well (see *Experimental*). The UV-Visible spectrum of 46 μM **2i** was monitored over time in the presence and absence of HLE. As can be seen from the overlayed UV-visible spectra in Figure 6, there are maxima at both 320 and 395 nm. At zero time, the starting material has a maximal absorbance at 395 nm and a lower absorbance at 320 nm. Over time (70 min), the absorbance at 320 nm increases while the absorbance at 395 nm decreases. The first order rate constants for the appearance of absorbance at 320 nm and disappearance at

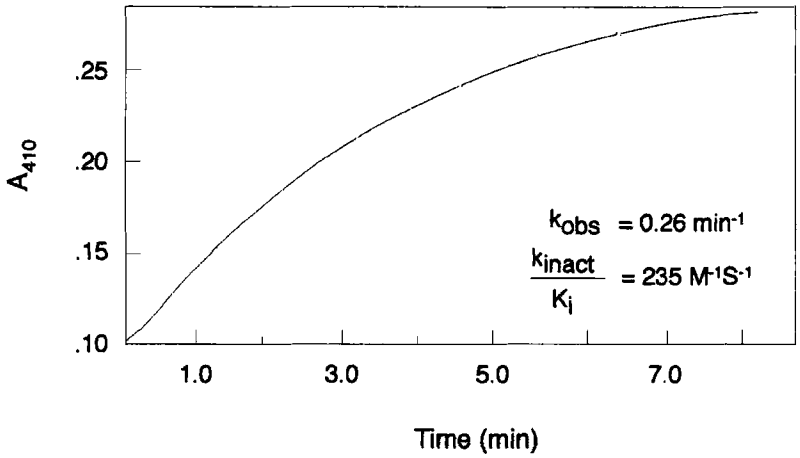


FIGURE 5 Time-dependent inhibition of HLE by compound 18 μM **2i**.

[e] The half-life of the E–I complexes could affect the interpretation of the second order rate constants. Under the conditions (40 nM HLE and 0.2 mM succ-AAPA-pNa) used to measure the rate constants for inhibition the amount of enzyme-catalyzed turnover of the inhibitor due to the instability of the E–I complex will be minimal when compared to the non-enzymatic hydrolysis. The latter will decrease the concentration of inhibitor in the assay, therefore, the second order rate constants may underestimate the actual activity by as much as a factor of 2.

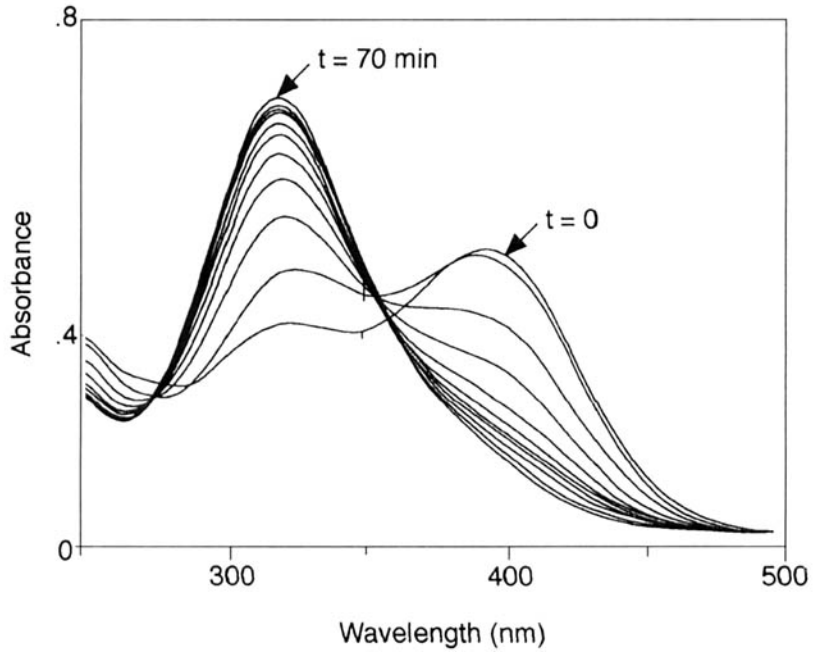


FIGURE 6 UV-Visible spectra of compound **2i** after incubation in buffer A at pH 7.5 as a function of time. There are 2 isobestic points if the data from time zero is excluded. The failure of the data at time zero to cleanly intercept the 2 isobestic points is likely due to mixing and equilibration of the reaction solution. The compound was added from a cold DMSO stock to room temperature buffer and then placed in the 25 °C chamber of the spectrophotometer. In addition, the fact that the rate constants calculated form the decomposition of starting material and appearance of product are in good agreement supports the proposal that the $t = 0$ curve is an anomaly. The calculations of the rate constants are essentially the same if the $t = 0$ data is omitted.

395 nm were essentially equivalent, 0.044 and 0.041 min^{-1}, respectively. The decomposition of **2i** was also monitored in the presence of 1 μM HLE. The first order rate constants for the appearance of absorbance at 320 nm was 0.038 min^{-1} in the presence of 1 μM HLE which suggests that enzyme catalysis is not required (data not shown). The observation that the first order rate constants for decomposition are an order of magnitude less than the first order rate constant for inactivation suggests that it is the parent compound that is active and not the decomposition product. When 10 μM HLE was incubated with 46 μM **2i**, the appearance of O.D. at 320 nm and the disappearance of O.D. at 395 nm was immediate and remained the same over 70 min suggesting that the enzyme produces a similar product(s) to that observed in the non-enzymatic reaction. Therefore **2i** is also a slow substrate for HLE.[f] Furthermore, this observation and the known mechanism of HLE suggest that the enzyme utilizes the catalytic serine to accomplish hydrolysis of **2i**. These results suggest that **2i** is a mechanism-based inhibitor and a slow substrate. The enzyme was inhibited to approximately 30% throughout this last experiment. These data suggest that the partition ratio for inactivation to initial turnover of the inhibitor is greater than 15:1. The final complex produced is unstable and slowly hydrolyzes to regenerate active enzyme. The thione derivatives **3a,i** were inactive at the limit of solubility.

[f] The observation that at low concentrations of HLE complete inhibition of HLE activity can be achieved, demonstrates that the enzyme can be temporarily inactivated, but the complex produced is unstable. Therefore, it is appropriate to refer to the rate constant as k_{inact}.

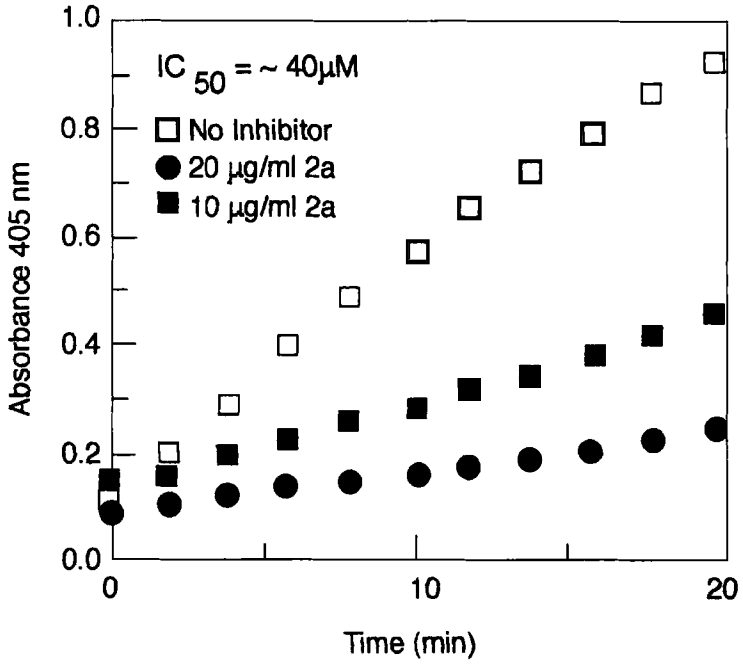


FIGURE 7 Inhibition of α-Chymotrypsin by compound **2a**.

Given the nonpolar character of these pyrazolooxadiazinones it was likely that they would inhibit serine proteases which have specificity for hydrophobic residues. While none of the compounds displayed significant inhibition (> 20%) *versus* Cat-G, **2a** displayed significant activity *versus* chymotrypsin at 20, 10 and 5 μg/ml (the compound was insoluble at 50 μg/ml). The progress curves were linear over a 20 min period and the slope was concentration dependent. The data in Figure 7 suggest a K_i of approximately 10 μg/ml or 44 μM if competitive inhibition is assumed. A freshly dissolved sample of **2i** displayed very weak inhibition of Cat-G.

These observations suggest that members of this class of compounds might be developed as inhibitors of serine proteases with differing specificities.[48]

Pyrazolooxazinone **5a** appears to be a weak time-dependent inhibitor of HLE (k_{inact}/K_i $M^{-1}s^{-1}$ is 15 ± 2 at 657 μM) while its 3-methyl analog **5b** and the isosteres **7c,d** are inactive showing estimated K_is of 394 ± 20 μM and >8400 μM respectively. The latter value was indeterminate due to the solubility limit (1.13 mM).

CONCLUSION

A general approach toward the design of a new type of mechanism-based inhibitor of serine proteases involving the linkage of a reactive heterocycle moiety to an appropriate support element is described. The rationale underlying the design of these inhibitors is based upon the solution chemistry of the pyrazolooxadiazinone ring system. The cleavage of the oxadiazinone ring is a pre-requisite to unmasking the reactive inactivating species. Thus, it was anticipated that compounds such as **2i** and **2k** would inactivate human leukocyte elastase (HLE) *via* an enzyme induced ring opening and generation of a reactive electrophilic species which upon further reaction with an active site nucleophile (for example His57) would lead to irreversible inactivation of the enzyme.[4–8,13] The inhibition of HLE

by compounds **2i** and **2k** was time-dependent meeting one of the criteria expected for mechanism-based inactivation.[5,6] In addition, the observation that the reaction of **2i** with high concentrations of HLE produces instantaneously a product(s) with an identical UV-Visible spectrum to that generated in the non-enzymatic reaction supports at least the first step in the proposed mechanism of inhibition i.e. ring opening by the catalytic serine. Compound **2k** showed an improved stability. The potency of the compounds reported in this work is less than that of other known mechanism-based inhibitors of HLE[4–8,13] however, this approach could lead to a series of versatile mechanism-based inactivators with specificities for other serine proteases.[48] The flexibility of the synthetic procedures used to generate the compounds **2a-m** makes additional structural modifications readily feasible.[9,20,49] Comparative studies with the active site of other related serine proteases (Cat-G, Chymotrypsin, Factor Xa) as well as further docking with other related compounds will be part of future modeling efforts.

Combination of the data derived from modeling studies will allow the design of more directed novel molecules.

NOTE ADDED IN REVISION

After submission of this manuscript, a paper on the synthesis of 1,3-disubstituted-4-nitroso-5-aminopyrazoles **1g-i** was published.[50]

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References

[1] M. Baggiolini, U. Bretz, B. Dewald and M.E. Feigenson (1978) *Agents and Action*, **8**, 3.
[2] P. Birrer, N.G. McElvaney, A. Rudeburg, C. Wirz-Sommer, S. Liecht-Gallati, R. Kraemer, R. Hubbard and R.G. Crystal (1994) *Am. Respir. Crit. Care Med.*, **150**, 207–213.
[3] G. Weinbaum and W.C. Groutas (1990) *Focus on Pulmonary Pharmacology and Toxicology*. Boca Raton, Florida: CRC Press.
[4] D.J. Hlasta and E.D. Pagani (1994) Human Leukocyte Elastase Inhibitors. In: *Annu. Rep. Med. Chem.* (Bristol, J.A., Editor), p. 195. San Diego: Academic Press, Inc.
[5] R.B. Silverman (1988) *Mechanism-Based Enzyme Inactivation: Chemistry and Enzymology* (Coxon, J.M. Editor). Boca Raton, Florida: CRC Press Inc.
[6] A.D. Abell (1992) *Advances in Detailed Reaction Mechanisms*, Vol. 2, p. 243. Greenwich, Connecticut: JAI PRESS INC.
[7] I. Vergely, N. Boggetto, V. Okochi, S. Golpayegani, M. Reboud-Ravaux, R. Kobaiter, R. Joyeau and M. Wakselman (1995) *Eur. J. Med. Chem.*, **30**, 199.
[8] P.A. Finke, K.S. Shrenik, D.S. Fletcher, B.M. Ashe, K.A. Brause, G.O. Chandler, P.S. Dellea, K.M. Hand, A.L. Maycock, D.G. Osinga, D.J. Underwood, H. Weston, P. Davies and J.B. Doherty (1995) *J. Med. Chem.*, **38**, 2449.
[9] P. Giori, A.C. Veronese, T. Poli, C.B. Vicentini, M. Manfrini and M. Guarneri, (1986) *J. Heterocyclic Chem.*, **23**, 585.
[10] S. Guccione (1992) *Tesi di Dottorato di Ricerca (Italian PhD)*.
[11] W.E. Brown and F. Wold (1973) *Biochemistry*, **12**, 828.
[12] A.R. Moorman and R.H. Abeles (1982) *J. Am. Chem. Soc.*, **104**, 6785.
[13] W.B. Knight, A.L. Maycock, B.G. Green, B.M. Ashe, P. Gale, H. Weston, P.E. Finke, W.K. Hagmann, S.K. Shah and J.B. Doherty (1992) *Biochemistry*, **31**, 4980.
[14] A. Krantz, R.W. Spencer, T. Fat Tam, T.J. Liak, L.J. Copp, E.M. Thomas and S.P. Rafferty (1990) *J. Med. Chem.*, **33**, 464 and references therein.
[15] S. Patai and Z. Rappopart (1993) *The Chemistry of Functional Groups, Supplement S: the chemistry of sulphur-containing functional groups*, Chichester: John Wiley and Sons Ltd, Baffins Lane.
[16] E. Mohr (1909) *J. Pr. Chem.* **2**, 79, 1.
[17] E.C. Taylor and K.S. Hartke (1959) *J. Am. Chem. Soc.*, **81**, 2456.
[18] P. Giori, D. Mazzotta, G. Vertuani, M. Guarneri, D. Pancaldi, A. Brunelli (1981) *Il Farmaco Ed. Sc.*, **36**, 1019.
[19] R. Ferroni, L. Milani, D. Simoni, P. Orlandini, T. Poli, M. Guarneri and U. Taddeo (1988) *Il Farmaco Ed. Sc.*, **43**, 891.
[20] S. Guccione, L. Monsù-Scolaro and F. Russo (1996) *J. Heterocyclic Chem.*, **33**, 459 and references therein.
[21] P. Schmidt and J. Druey (1956) *Helv. Chim. Acta.*, **39**, 986.
[22] H. Baba, I. Hori, T. Hayashi and K. Midorikawa (1969) *Bull. Chem. Soc. Jpn.*, **42**, 1653.
[23] R.L. Frank and P.V. Smith (1948) *Org. Syntheses* **28**, 89 (H.R. Snyder, Ed.). New York: John Wiley and Sons, Inc.
[24] I.B. Douglass and F.B. Dains (1934) *J. Am. Chem. Soc.*, **56**, 1408.
[25] The program SYBYL 6.5 (1999) is available from Tripos Associates, 1699 South Hanley Road, St Louis, MO 63144, USA.

[26] Gaussian Inc, Pittsburgh, Pasadena, USA.
[27] D. Feller and E.R. Davidson *Chem. Rev.*, **86**, 681.
[28] S. Sinha, W. Watorek, S. Karr, J. Giles, W. Bode and J. Travis (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 2228.
[29] W. Bode, E. Papmokos, D. Musil, U. Seem and ller (1986) *EMBO J.*, **5**, 813.
[30] B.S. Hartley and D.L. Kauffman (1966) *Biochem. J.*, **101**, 229 and references therein.
[31] W. Bode, A. Wei, R. Huber, E. Meyer, J. Travis and S. Neumann, (1986) *EMBO J.*, **5**, 2453.
[32] Internet Address: *Gopher://pdbpdbbnlgov.*
[33] A. Wei, I. Mayr and W. Bode (1988) *FEBS Lett.*, **234**, 367.
[34] M. Rarey, S. Wefing and T. Lengauer, 1996 *J. Comput-Aided Mol. Des.*, **10**, 41.
[35] M. Clark, R. Cramer III and N. Van Opdenbosh (1989) *J. Comput. Chem.*, **10**, 982.
[36] W.B. Knight, B.G. Green, R. Chabin, P. Gale, A.L. Maycock, H. Weston, D. Kuo, W.M. Westler, C.P. Dorn, P.E. Finke, W.K. Hagmann, J.J. Hale, J. Liesch, M. MacCoss, M. Navia, S.K. Shah, D. Underwood and J.B. Doherty (1992) *Biochemistry*, **31**, 8160.
[37] B.G. Green, H. Weston, B.M. Ashe, J.B. Doherty, P. Finke, W. Hagmann, M. Lark, J. Mao, A. Maycock, R. Mumford, L. Walakovits and W.B. Knight (1991) *Arch. Biochem. Biophys.*, **286**, 284.
[38] J.F. Morrison and C.T. Walsh (1983) *Adv. Enzymol.*, **61**, 201.
[39] S. Fujii and Y. Hitomi (1981) *Biochim. Biophys. Acta*, **661**, 342.
[40] I. Schechter and A. Berger (1967) *Biochem. Biophys. Res. Commun.*, **27**, 157.
[41] M.A. Hernandez, J.C. Powers, J. Glinski, J. Oleksyszyn, J. Vijayalakshmi and E.F. Meyer, Jr (1992) *J. Med. Chem.*, **35**, 1121.
[42] H. Umeyama and S. Nakagawa (1985) In *Biomolecules* 21, (C. Nagata Ed.). Amsterdam: Elsevier.
[43] S.C. Zimmerman, J.S. Korthals and K.D. Cramer (1991) *Tetrahedron*, **47**, 2649.
[44] P.D. Edwards, D.J. Wolanin, D.W. Andisik and M.W. Davis (1995) *J. Med. Chem.*, **38**, 76.
[45] A. Ganesan and C.H. Heathcock (1993) *J. Org. Chem.*, **58**, 6155.
[46] H. Kubinyi (1993) *3D QSAR in Drug Design: Theory, Methods and Applications.* Leiden: ESCOM.
[47] H. Kubinyi (1993) *QSAR; Hansch Analysis and Related Approaches, Methods and Principles in Medicinal Chemistry.* Weinheim: VCH.
[48] A. Barth, M. Wahab, W. Brandt and K. Frost (1993) *Drug Design and Discovery*, **10**, 297.
[49] C.B. Vicentini, A.C. Veronese, M. Manfrini, S. Guccione, M. Guarneri and P. Giori (1993) *Heterocycles*, **36**, 2291.
[50] P.G. Baraldi, M.J. Pineda de las Infantas, S. Manfredini and R. Romagnoli (2000) *Synthesis*, **1**, 72.