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Isoxazoline Derivatives as Potential Inhibitors of the Proteolytic Enzymes Human Leukocyte Elastase, Cathepsin G and Proteinase 3: a Structure–Activity Relationship Study

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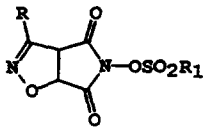
Abstract—A series of isoxazoline derivatives were synthesized and investigated for their *in vitro* inhibitory activity towards human leukocyte elastase, cathepsin G and proteinase 3.

Introduction

Chronic inflammatory diseases such as pulmonary emphysema,[1,2] cystic fibrosis,[3,4] psoriasis[5] and rheumatoid arthritis,[6] are associated with an influx of neutrophils and the extracellular release of lysosomal enzymes, including the serine proteinases human leukocyte elastase (HLE), cathepsin G (Cath G) and proteinase 3 (PR 3). Poor regulation of these enzymes by their endogenous protein inhibitors can lead to the degradation of the major components of the extracellular matrix.[7,8] Consequently, intense and ongoing efforts in this area have focused on the design and development of agents that are effective modulators of the activity of these enzymes.[9,10]

We have recently reported some preliminary findings related to the use of isoxazoline derivatives (represented by structure **I**) as mechanism-based inhibitors of HLE and Cath G.[11] Herein we wish to describe the results of a structure–activity relationship study, and related *in vitro* biochemical studies with HLE, Cath G and proteinase 3.



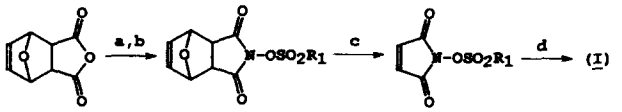
(I)

Chemistry

Compounds **1–12** were synthesized as shown in Scheme 1.[12,13] The physical and spectral data for compounds **3–12** are listed in Table 1.

Biochemistry

Enzyme assays and inhibition studies with HLE, Cath G and PR 3 were carried out as described previously.[14–16]



R1 = methyl (1a) (2a-b)
trans-styryl (1b)

[a]$NH_2OH.HCl$/methanol; [b]R_1SO_2Cl/pyridine; [c] 140–160 °C; [d] R-CH=N-OH/N-chlorosuccinimide, triethylamine/CH_2Cl_2.

Scheme 1.

Results and Discussion

HLE, Cath G and PR 3 are serine endopeptidases that have similar, extended binding sites and prefer hydrophobic substrates or inhibitors (Table 2). The primary specificity site S_1 of Cath G is larger than that of HLE and PR 3, showing a strong preference for Phe. Furthermore, while the observed similarity in the substrate specificities of PR 3 and HLE reflects a similar makeup of their active sites, there are subtle differences, including a more restrictive primary specificity site S_1 and S_n' domain in the case of PR 3.[17–19]

We have recently studied the interaction of 3-phenyl-substituted isoxazoline derivatives (**I**, R = phenyl, R_1 = methyl, phenyl, *trans*-styryl, *p*-(carboxy)phenyl) with HLE and Cath G. These compounds were found to function as alternate substrates of HLE, undergoing rapid acylation/deacylation, ultimately leading to full enzymatic activity being regained. In contrast, the corresponding Cath G-derived acyl enzymes had much greater stability, regaining about 50% activity after 24 h. Furthermore, the addition of excess hydroxylamine to fully-inactivated Cath G did not lead to any significant regaining in enzymatic activity. Based on these preliminary findings and the known substrate specificities of the enzymes under study, a series of 3-alkyl substituted derivatives of **I** were synthesized and investigated for their inhibitory activity towards HLE, Cath G and PR 3.

Table 1. Physical constants and spectral data for compounds 3–12

Compd	R	R_1	MP °C	NMR ppm	MF (anal)
3	ethyl	methyl	96-97	1.25(t,3H),2.4-2.7(m,2H),3.48(s,3H) 4.58(d,2H),5.5(d,2H)	$C_8H_{10}N_2O_6S$ (C,H,N)
4	ethyl	trans-styryl	49-51	1.2(t,3H),2.3-2.6(m,2H),4.5(d,1H), 5.4(d,1H),6.9(d,1H),7.4-7.6(m,5H), 7.7(d,1H)	$C_{15}H_{14}N_2O_6S$ (C,H,N)
5	n-propyl	methyl	101-2	1.0(t,3H),1.56-1.80(m,2H),2.4-2.6 (m,2H),3.5(s,3H),4.5(d,1H),5.45(d,1H)	$C_9H_{12}N_2O_6S$ (C,H,N)
6	n-propyl	trans-styryl	44-45	0.98(t,3H),1.5-1.8(m,2H),2.3-2.55 (m,2H),4.4(d,1H),5.41(d,1H),6.9(d,1H), 7.45-7.60(m,5H),7.72(d,1H)	$C_{16}H_{16}N_2O_6S$ (C,H,N)
7	isopropyl	methyl	117-8	1.3(dd,6H),2.84(m,1H),3.48(s,3H), 4.58(d,1H),5.43(d,1H)	$C_9H_{12}N_2O_6S$ (C,H,N)
8	isopropyl	trans-styryl	56-67	1.25(dd,6H),2.75(m,1H),4.55(d,1H), 5.4(d,1H),6.9(d,1H),7.4-7.6(m,5H), 7.7(d,1H)	$C_{16}H_{16}N_2O_6S$ (C,H,N)
9	isobutyl	methyl	124-5	0.95-1.10(dd,6H),2.1(m,1H),2.40 (dd,2H),3.46(s,3H),4.45(d,1H), 5.43(d,1H)	$C_{10}H_{14}N_2O_6S$ (C,H,N)
10	isobutyl	trans-styryl	52-53	0.9-1.1(dd,6H),2.05(m,1H),2.30(d,2H), 4.42(d,1H),5.4(d,1H), 6.9(d,1H), 7.42-7.60(m,5H),7.70(d,1H)	$C_{17}H_{18}N_2O_6S$ (C,H,N)
11	benzyl	methyl	112-3	3.42(s,3H),3.75(d,1H),4.05(d,1H), 4.3(d,1H),5.32(d,1H),7.45(m,5H)	$C_{13}H_{12}N_2O_6S$ (C,H,N)
12	benzyl	trans-styryl	62-63	3.5(d,1H),3.92(d,1H),4.25(d,1H), 5.3(d,1H),6.9(s,1H),7.25-7.6(m,10H), 7.7(d,1H)	$C_{20}H_{16}N_2O_6S$ (C,H,N)

Table 2. Substrate specificity of leukocyte elastase, cathepsin G and proteinase 3

Subsite[a]	S_4	S_3	S_2	S_1	S_1'	S_2'	S_3'
HLE	Ala	Ala	Pro	Val[b]	–[c]	–	–
Cath G	Ala	Ala	Pro	Phe	–	–	–
PR 3	Ala	Ala	Pro	Abu[d]	–	–	–

[a]The individual amino acid residues of a peptide substrate are designated by P_n,....P_3, P_2, P_1, P_1', P_2', P_3',....P_n', and the corresponding subsites of the enzyme by S_n,....S_3, S_2, S_1,....S_1', S_2', S_3',....S_n'. S_1 designates the primary specificity site of an enzyme and P_1 - P_1' is the scissile bond.
[b]Norval and Norleu are also accommodated.
[c]Not rigorously established.
[d]2-3 Carbon chains fit best.

The interaction of compounds **3–12** with HLE led to time-dependent loss of enzymatic activity, followed by partial regaining of enzymatic activity (Fig. 1). In all cases, rapid acylation of the enzyme was followed by rapid deacylation, leading to partial recovery of enzymatic activity. Not surprisingly, the behavior of these compounds is similar to that of the succinimide series.[20] Figure 2 illustrates the apparent structural complementarity of these two classes of compounds. The extremely rapid acylation step, reflecting in part the greater reactivity of the isoxazoline nucleus, made the accurate determination of the bimolecular rate constants k_{obs}/[I] by sampling techniques difficult. The nature of the R and R_1 groups did not appear to affect the acylation and deacylation rates in a significant way.

The kinetic behavior of compounds **3–12** suggests that processing of **I** by the catalytic machinery of the enzyme triggers an instantaneous Lossen rearrangement, leading to the formation of a highly reactive isocyanate.[21,22] The latter partitions via two primary pathways, namely, a deacylation pathway (k_{deacyl})

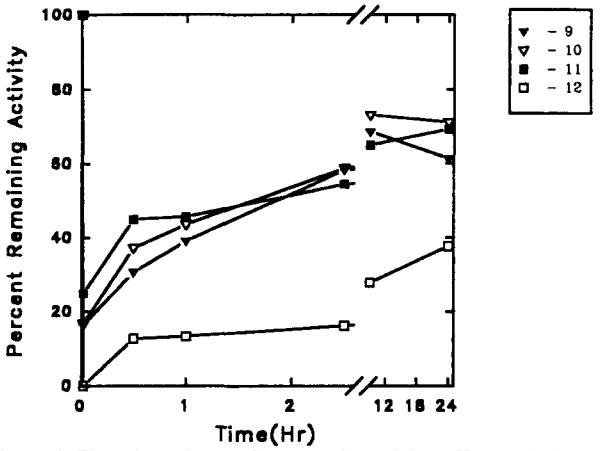


Figure 1. Time dependence of enzymatic activity. Human leukocyte elastase (395 nM) was incubated with inhibitors **9–12** (79 μM) in 0.1 M HEPES buffer, pH 7.2, 0.5 M NaCl and 1% dimethyl sulfoxide.

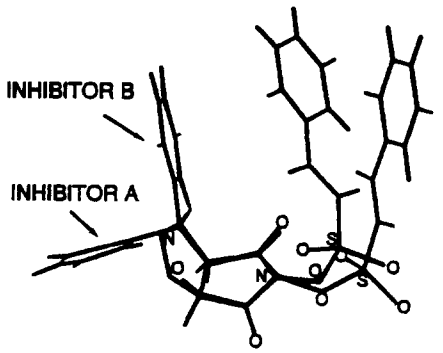


Figure 2. Superimposition of the energy-minimized structures of inhibitors A (*cis*-*N*-[(*trans*-styrylsulfonyl)oxy]-3-phenyl-2-isoxazoline-4,5-dicarboxamide) and B (3-benzyl-*N*-[(*trans*-styrylsulfonyl)oxy]-succinimide.

leading to turned over product(s), and an inactivation pathway (k_{inact}), as illustrated in Figure 3. This mechanistic interpretation, while somewhat speculative, is consistent with and analogous to previous findings with succinimide derivatives[20,21] and the established chemical behavior of isoxazoline derivatives I with nucleophilic species.[23]

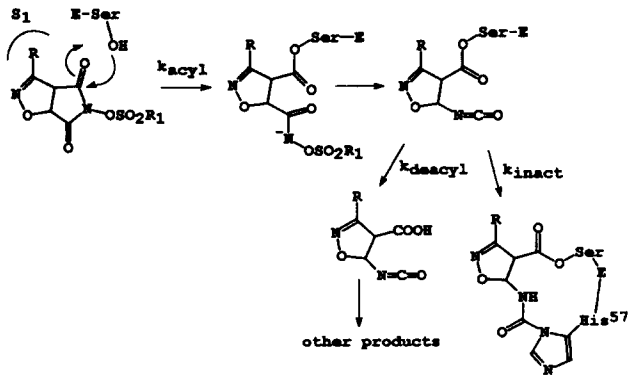


Figure 3. Postulated mechanism of action of I.

The binding of compounds **3–12** to the active site of Cath G led to rapid acylation of the enzyme, followed by partial regaining of enzymatic activity (Fig. 4). It is noteworthy that small, but subtle, differences in the active site geometry appear to determine to a large extent how efficiently the reactive species is trapped.

The interaction of PR 3 with compound **I** is similar to that of HLE and Cath G. For example, incubation of compound **5** with PR 3 leads to rapid acylation, followed by full recovery of enzymatic activity after 24 h (Fig. 5).

Finally, the stability in 0.1 M HEPES buffer, pH 7.2, of a representative member of this class of compounds was determined by HPLC using a 1:1 acetonitrile:water mixture as the mobile phase. The half-life of the compound was determined to be 1.5 h, attesting to the high chemical reactivity and low stability of this class of compounds.

In conclusion, a series of substituted isoxazoline derivatives has been synthesized and shown to form acyl enzymes with HLE, Cath G and PR 3 of variable stability.

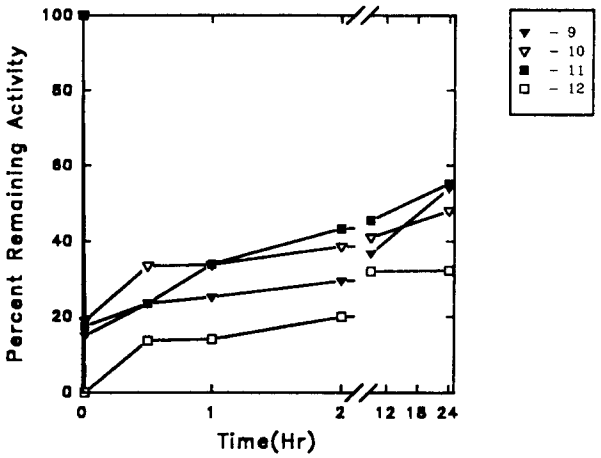


Figure 4. Time dependence of enzymatic activity. Human leukocyte cathepsin G (1.46 μM) was incubated with inhibitors **9–12** (192 μM) in 0.1 M HEPES buffer, pH 7.5, 0.5 M NaCl, and 1% dimethyl sulfoxide.

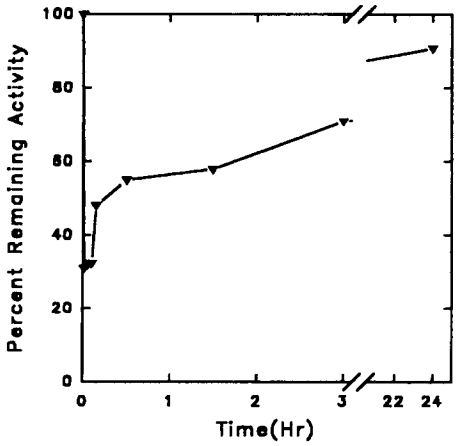


Figure 5. Time dependence of enzymatic activity. Human leukocyte proteinase 3 (373 nM) was incubated with inhibitor **5** (74.6 μM) in 0.1 M HEPES buffer, pH 7.5, 0.5 M NaCl and 1% dimethyl sulfoxide.

Experimental

Melting points were recorded on a Mel-Temp apparatus and are uncorrected. The infrared and NMR spectra were recorded on a Perkin-Elmer 1330 infrared spectrophotometer and a Varian XL-300 NMR spectrometer, respectively. A Gilford UV-vis spectrophotometer was used to perform the enzyme assays and inhibition studies. The stability of the inhibitors was investigated using a high-pressure liquid chromatograph equipped with a refractive index detector. Human leukocyte elastase was purchased from Elastin Products Co., Owensville, MO. Methoxysuccinyl Ala-Ala-Pro-Val *p*-nitroanilide and Boc-L-Ala *p*-nitrophenol methoxysuccinyl Ala-Ala-Pro-Phe *p*-nitroanilide were purchased from Sigma Chemical Co., St. Louis, MO. Human leukocyte cathepsin G and proteinase 3 were purchased from Athens Research & Technology Co.,

Athens, GA. The commercially available computational chemistry software SYBYL 5.41D, Tripos Associates (St. Louis, MO, U.S.A.) was used in conjunction with a Silicon Graphics Personal IRIS workstation.

Syntheses

cis-*5-Norbornene*-endo-*2,3*-N-*hydroxyimide.* A solution of potassium hydroxide (6.8 g, 0.17 mol) in methanol (25 mL) was added to a solution of hydroxylamine hydrochloride (8.4 g, 0.12 mol) in methanol (80 mL). The solution was cooled in an ice bath and then treated with *cis*-5-norbornene-*endo*-dicarboxylic anhydride (20 g, 0.12 mol) in 200 mL methanol. The solution was stirred overnight at room temperature. The precipitated solid was collected and air-dried, yielding 17.5 g (80% yield) of pure product, mp 184–189°C. NMR (DMSO-d_6): δ 10.85 (*s*, 1H), 6.53 (*s*, 2H), 5.11 (*s*, 2H), 2.82 (*s*, 2H).

Synthesis of N-*methyl (1a) and* N-*(styrylsulfonyl-oxy)maleimide (1b) adducts of furan.* Methanesulfonyl or *trans*-styryl sulfonyl chloride (0.05 mol) in dry acetonitrile (20 mL) was added dropwise to a solution of pyridine (0.05 mol) and *cis*-5-norbornene-*endo*-2,3-*N*-hydroxyimide (0.05 mol) in acetonitrile (50 mL). The mixture was stirred at room temperature for 5 h. The solvent was removed *in vacuo* and the oily residue was treated with 5% hydrochloric acid (30 mL). The precipitate was collected and dried, giving a pure product. Compound **1a**: mp 155–157 °C; NMR (DMSO-d_6): δ 6.60 (*s*, 2H), 5.25 (*s*, 2H), 3.60 (*s*, 3H), 3.08 (*s*, 2H). Compound **1b**: mp 139–142 °C; NMR (DMSO-d_6): δ 7.45–7.80 (*m*, 7H), 6.55 (*s*, 2H), 5.05 (*s*, 2H), 3.0 (*s*, 2H).

Synthesis of N-*methyl (2a) and* N-*(styrylsulfonyloxy) maleimides (2b).* Compound **1a** (or **1b**) was heated in an oil bath to 140–160 °C for 15 min under vacuum. The residue was cooled to room temperature and taken up in methylene chloride. Evaporation of the solvent left a crude product which was twice recrystallized from hexane:methylene chloride (1:1). Compound **2a**: ($CDCl_3$): δ 6.82 (*s*, 2H), 3.2 (*s*, 3H). Compound **2b**: NMR ($CDCl_3$): δ 7.72 (*d*, 1H), 7.6–7.4 (*m*, 6H), 6.8 (*s*, 2H).

Preparation of compounds 3–12. General procedure.[13] The appropriate alkyl oxime (5.5 mmol) was added to a suspension of *N*-chlorosuccinimide (5.0 mmol) in methylene chloride (10 mL). After all the solid had gone into solution, compound **2a** (or **2b**) (5.0 mmol) was added, followed by the dropwise addition of triethylamine (5.5 mmol) over 20 min. The mixture was stirred at room temperature overnight. The reaction mixture was transferred to a separatory funnel, washed with water (2 × 20 mL), dried and evaporated. The crude product was purified by repeated recrystallization from hexane:methylene chloride (1:1).

Acknowledgement

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