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Inactivation of human neutrophil elastase by 1,2,5-thiadiazolidin-3-one 1,1 dioxide-based sulfonamides

Yi Li, Qingliang Yang, Dengfeng Dou, Kevin R. Alliston and William C. Groutas*

Department of Chemistry, Wichita State University, Wichita, KS 67260, USA

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Abstract—The interaction of a series of 1,2,5-thiadiazolidin-3-one 1,1 dioxide-based sulfonamides with neutrophil-derived serine proteases was investigated. The nature of the amino acid component, believed to be oriented toward the S′ subsites, had a profound effect on enzyme selectivity. This series of compounds were found to be potent, time-dependent inhibitors of human neutrophil elastase (HNE) and were devoid of any inhibitory activity toward neutrophil proteinase 3 (PR 3) and cathepsin G (Cat G). The results of these studies demonstrate that exploitation of differences in the S′ subsites of HNE and PR 3 can lead to highly selective inhibitors of HNE.

1. Introduction

The neutrophil-derived serine endopeptidases human neutrophil elastase (HNE), proteinase 3 (PR 3), and cathepsin G (Cat G) have been implicated in a range of inflammatory diseases, including chronic obstructive pulmonary disease (COPD).[1] Although the pathogenesis of COPD is poorly understood, current studies indicate that this multifactorial disorder is characterized by a cigarette smoke-induced cycle of oxidative stress,[2] alveolar septal cell apoptosis,[3] a protease/antiprotease imbalance,[4] and chronic inflammation.[5] An array of serine (HNE, PR 3, and Cat G), cysteine (cathepsin S), and metallo- (MMP-9 and MMP-12) proteases released by neutrophils, macrophages, and T lymphocytes contribute to the degradation of lung connective tissue and mediate a multitude of signaling pathways associated with the pathophysiology of the disorder.[6] Consequently, pharmacological agents capable of abrogating or modulating the aberrant proteolytic activity of the aforementioned enzymes are of potential therapeutic value.[7]

We have recently described the design and in vitro biochemical evaluation of a novel class of mechanism-based inhibitors (I) that inactivate target serine proteases via an unprecedented enzyme-induced sulfonamide fragmentation process (Fig. 1).[8] Sulfonamide inhibitor (I) embodies in its structure a functionalized heterocyclic scaffold with appended recognition elements for optimal exploitation of binding interactions with the S_n and S'_n subsites[9] of the target enzyme. Derivatives of inhibitor (I) (R_1 = isobutyl and R_2 = methyl) were previously found to inactivate HNE efficiently, however they also showed significant inhibitory activity toward trypsin despite the lack of a basic P_1 residue. In an effort to optimize the inhibitory potency and selectivity of (I) toward HNE and PR 3, recognition element R_3 was varied using a series of amino acid esters and the inhibitory activity of the resulting compounds toward HNE, PR 3, Cat G, and bovine trypsin was then evaluated. The results of these studies are described herein.

2. Results

2.1. Chemistry

Compounds **4**–**11** were synthesized starting with (L) norvaline using the sequence of steps shown in Scheme 1. Key intermediate **1** was synthesized using similar procedures as those described previously.[8b] The synthetic methodology was straightforward, however the reaction sequence involving the conversion of the thioesters to the corresponding sulfinyl chlorides which, without isolation, were reacted with the amino acid esters was found to be capricious and gave low yields (10–20%)

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* Corresponding author. Tel.: +1 316 978 7374; fax: +1 316 978 3431; e-mail: bill.groutas@wichita.edu

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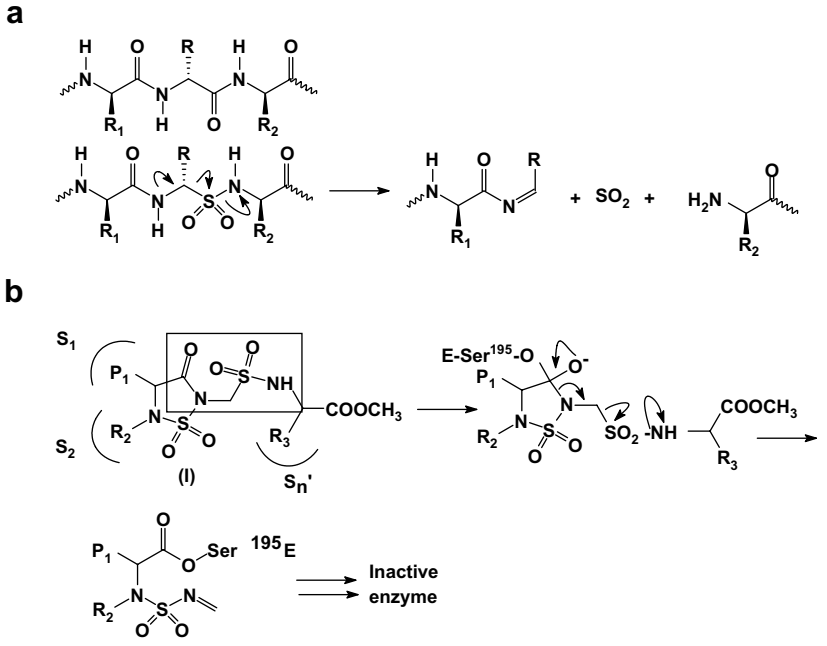
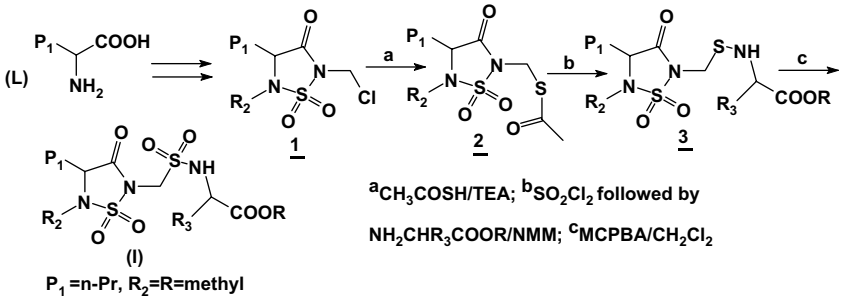


Figure 1. Design and mechanism of action of inhibitor (I).



Scheme 1. Synthesis of inhibitors **4**–**11**.

of the sulfinamide products. Optimization of the reaction conditions and monitoring product formation using ^{1}H NMR improved yields somewhat (30%).

2.2. Biochemical studies

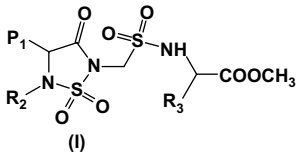
2.2.1. Progress curve method. The inhibitory activity of compounds **4**–**11** toward HNE was determined by the progress curve method.[10,8b] The apparent second-order rate constants (k_{inact}/K_I M^{-1} s^{-1}) were determined in duplicate and are listed in Table 1. Typical progress curves for the hydrolysis of MeOSuc-AAPV-*p*-NA by HNE in the presence of inhibitor **4** are shown in Figure 2. The release of *p*-nitroaniline was continuously monitored at 410 nm. The pseudo first-order rate constants (k_{obs}) for the inhibition of HNE by derivatives of (I) as a function of time were determined according to Eq. 1 below, where A is the absorbance at 410 nm, v_0 is the reaction velocity at $t = 0$, v_s is the final steady-state velocity, k_{obs} is the observed first-order rate constant, and A_0 is the absorbance at $t = 0$. The k_{obs} values were obtained by fitting the $A \sim t$ data into Eq. 1 using nonlinear regression analysis (SigmaPlot, Jandel Scientific). The second-order rate constants (k_{inact}/K_I M^{-1} s^{-1}) were then determined by calculating k_{obs}/[I] and then correcting for the substrate concentration using Eq. 2. Control curves in the absence of inhibitor were linear.

$$A = v_s t + \{(v_0 - v_s)(1 - e^{-k_{obs}t})/k_{obs}\} + A_0 \quad (1)$$

$$k_{obs}/[I] = (k_{inact}/K_I)[1 + [S]/K_m] \quad (2)$$

2.2.2. Incubation method. The inhibitory activity of compounds **4**–**11** toward trypsin was determined using the incubation method[8b] and is expressed in terms of the bimolecular rate constant k_{obs}/[I] M^{-1} s^{-1}, where k_{obs} is the pseudo-first-order rate constant of enzyme inactivation and [I] is the concentration of the inhibitor in the incubation mixture. Briefly, in this method the enzyme is incubated with excess inhibitor and the loss of enzymatic activity is followed by withdrawing aliquots at different time intervals and assaying for enzymatic activity. The observed rate constants (k_{obs}) are then determined by plotting ln([E_t]/[E_0]) versus t according to Eq. 3 below,

Table 1. Inhibitory activity of compounds **4**–**11** toward human neutrophil elastase, proteinase 3, cathepsin G, and bovine trypsin



(I)

Compound[a]	R_3	k_{inact}/K_I M^{-1} s^{-1}			
		HNE	PR 3	Cat G	Trypsin[b]
4	(L) CH_3	1050	Inactive[c]	Inactive[c]	ND[d]
5	(L) $CH_3(CH_2)_2$	34,650	Inactive	Inactive	19
6	(L) $PhCH_2$	41,750	Inactive	Inactive	50
7	(D) $PhCH_2$	9760	Inactive	Inactive	ND
8	(L) $PhCH_2OCH_2$	16,870	Inactive	Inactive	60
9	(L) CH_2OH	1100	Inactive	Inactive	12
10	(L) $(CH_2)_4NH_2$	12,900	Inactive	Inactive	ND
11	(L) $(CH_2)_4NHCbz$	6900	Inactive	Inactive	ND

[a] P_1 = *n*-propyl, R_2 = methyl.
[b] $k_{obs}/[I]$ M^{-1} s^{-1} values determined using the incubation method (see text for details).
[c] [I]/[E] = 250 for 30 min.
[d] Not determined.

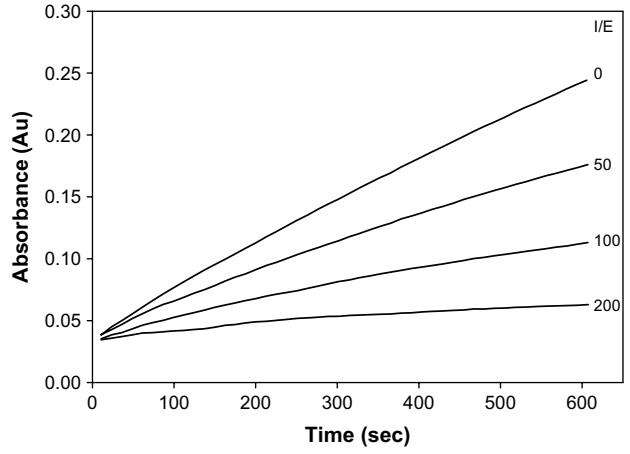


Figure 2. Progress curves for the inhibition of human neutrophil elastase (HNE) by inhibitor **4**. Absorbance was monitored at 410 nm for reaction solutions containing 10 nM HNE, 105 μM MeOSuc-AAPV *p*-nitroanilide, and the inhibitor at the indicated inhibitor to enzyme ratios in 0.1 M HEPES buffer containing 0.5 M NaCl, pH 7.25, and 2.5% DMSO. The temperature was maintained at 25 °C, and reactions were initiated by the addition of enzyme.

where $([E_t]/[E_0])$ is the amount of active enzyme remaining at time t. These are the average of two or three determinations.

$$\ln([E_t]/[E_0]) = k_{obs}\ t \qquad (3)$$

3. Discussion

3.1. Inhibitor design

The design of inhibitor (I) is briefly summarized below: (a) in contrast to ordinary peptides, α-sulfonopeptides (α-amido sulfonamides) undergo a spontaneous fragmentation reaction to yield a Michael acceptor (Fig. 1); (b) inhibitor (I) is a stable molecule which also embodies in its structure an α-sulfonopeptide motif (Fig. 1b). Based on the mechanism of action of serine proteases, it was envisaged that embellishment of (I) with appropriate recognition elements would allow (I) to bind to the active site of a target serine protease, leading to the formation of a tetrahedral intermediate that would subsequently collapse via a sulfonamide fragmentation-driven process to generate a Michael acceptor. Formation of a stable enzyme–inhibitor adduct was anticipated to led to inactivation of the enzyme; (c) HNE and PR 3 play an important role in inflammatory diseases such as COPD[1,11] and cystic fibrosis.[12] Furthermore, both enzymes hydrolyze lung elastin, the hydrophobic component of lung connective tissue, with high efficiency and also induce emphysema in hamsters when administered intratracheally.[13] Consequently, in order for (I) to inhibit both enzymes, an *n*-propyl group was chosen as the P_1 residue (vide infra); (d) an R_2 = methyl group was chosen at S_2 based on earlier studies which demonstrated that the presence of a methyl group at R_2 leads to the formation of highly stable acyl enzymes;[14] and (e) there are significant structural differences in the make up of the S'_n subsites of serine proteases, consequently it was anticipated that varying R_3 may enhance the selectivity of (I). Literature precedent regarding the successful exploitation of S′–P′ interactions in the design of potent and selective inhibitors of serine[15] and cysteine[16] proteases provided a strong measure of assurance about the soundness of the approach. As stated earlier, our primary objective was to obtain a derivative of (I) that would display high potency and selectivity toward HNE and PR 3 over other serine proteases.

Recent studies in our laboratory have demonstrated that inhibitor (I) is a highly efficient inhibitor of HNE that inactivates the enzyme via an enzyme-induced sulfonamide fragmentation process.[8b] However, derivatives of (I) were also found to inhibit trypsin, consequently this report details our studies aimed at gaining further insight into the interaction of (I) with (chymo)trypsin-like serine

proteases and optimizing inhibitory activity and enzyme selectivity by varying R_3, the recognition element believed to interact with the S'_n subsites. While all three neutrophil-derived serine proteases (HNE, PR 3, and Cat G) were studied with the synthesized inhibitors, our main efforts were focused on the identification of a dual HNE and PR 3 inhibitor suitable for further development.

Inhibitors **4**–**11** were all found to be efficient inhibitors of HNE (Table 1). For instance, incubation of compound **4** with HNE lead to rapid, time-dependent, irreversible loss of enzymatic activity (Fig. 3). The enzyme did not regain any activity after 24 h, suggesting the formation of a fairly stable enzyme–inhibitor adduct. With the exception of compound **10**, compounds bearing hydrophobic R_3 groups exhibited higher inhibitory activity toward HNE. While this is likely a reflection of the strong preference of HNE for hydrophobic inhibitors (and substrates), rather than the effect of any specific hydrophobic interactions, the high potency of compound **4** may arise from a π–π stacking interaction between Phe 41 in HNE and the phenyl group of the inhibitor.

Quite surprisingly, compounds **4**–**11** were all devoid of any inhibitory activity toward PR 3 (following incubation at an [I]/[E] ratio of 250), exhibiting absolute selectivity between HNE and PR 3. This remarkable observation is in sharp contrast to previously reported studies that showed that 1,2,5-thiadiazolidin-3-one 1,1 dioxide-based derivatives are highly effective inhibitors of both HNE and PR 3.[17] The fact that compounds **4**–**11** show no inhibitory activity toward PR 3 is probably a reflection of the significant structural differences in the make up of the S'_n subsites of the two enzymes.[18,19] While HNE and PR 3 are highly homologous (57%) and their active sites have some general features in common, namely, they both have extended binding sites and prefer hydrophobic substrates and inhibitors, nevertheless there are also significant differences between the two enzymes. Specifically, interactions beyond the S'_1 subsite have a profound effect on catalysis in the case of PR 3 but not HNE.[19a] Furthermore, the S'_n subsites of PR 3 are decidedly more polar than those of HNE due to the presence of Asp 61 and Arg 143, which may partly account for the ability of these compounds to discriminate between the two enzymes.[18] These observations are of particular relevance to the design of HNE and PR 3 inhibitors since their primary substrate specificities are very similar [HNE and PR 3 show a preference for (Val and Leu) and (Abu and Norval), respectively, at P_1], consequently varying the nature of the primary specificity residue P_1 may not be sufficient to attain high selectivity. Recently the differences in the S_2 and S'_1–S'_3 subsites of PR 3 and HNE have been successfully exploited in the design of highly specific substrates of HNE and PR 3.[19] Furthermore, recent kinetic and molecular modeling analysis studies have reinforced the importance of the charge distribution differences at the active sites of the two enzymes.[20] Although the reasons for the noteworthy selectivity exhibited by the compounds used in these studies are not intuitively obvious, their potential utility as pharmacological probes is clearly evident.

As expected, compounds **4**–**11** showed no activity toward neutrophil cathepsin G (following incubation at an [I]/[E] ratio of 250 for 30 min). This is no doubt due to the strong preference of Cat G for a Phe residue at P_1.[21]

Compounds **4**–**11** were also found to inhibit bovine trypsin in a time-dependent fashion, albeit much less efficiently (Table 1). For instance, incubation of compound **4** with bovine trypsin led to time-dependent inactivation of the enzyme with partial recovery of enzymatic activity after 24 h (Fig. 4). The observed inhibitory activity toward trypsin may be due to partial

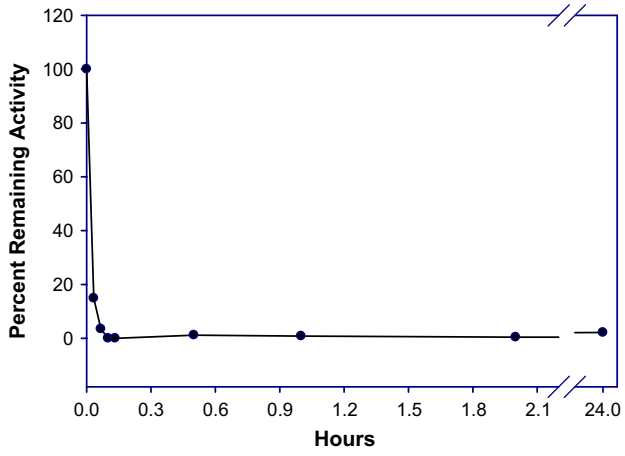


Figure 3. Time-dependent loss of enzymatic activity. Percent remaining activity versus time plot obtained by incubating inhibitor **4** (7 μM) with human neutrophil elastase (700 nM) in 0.1 M HEPES buffer containing 0.5 M NaCl, pH 7.25, and 1% DMSO. Aliquots were withdrawn at different time intervals and assayed for enzymatic activity using MeOSuc-AAPV-*p*-NA by monitoring the absorbance at 410 nm.

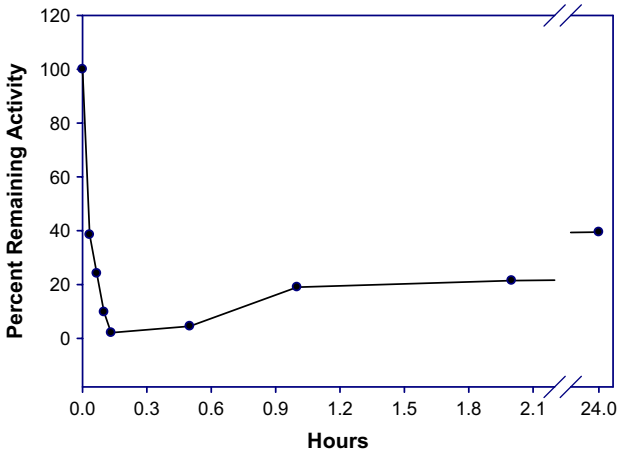


Figure 4. Time-dependent loss of enzymatic activity. Percent remaining activity versus time plot obtained by incubating inhibitor **4** (0.125 mM) with bovine trypsin (5 μM) in 0.025 M phosphate buffer containing 0.1 M NaCl, pH 7.51, and 1% DMSO. Aliquots were withdrawn at different time intervals and assayed for enzymatic activity using *N*-(*p*-tosyl)-Gly-Pro-Lys *p*-NA by monitoring the absorbance at 410 nm.

occupation of the S_1 pocket by the *n*-propyl group in a way that is conducive to binding, namely, by not clashing with the Asp residue at the bottom of the trypsin S_1 pocket.

In conclusion, a series of compounds that shows high potency and selectivity toward HNE but no activity toward PR 3 and Cat G is reported. Further studies aimed at enhancing the HNE/trypsin selectivity ratio and illuminating the structural basis for the selectivity observed with this class of compounds are currently in progress.

4. Experimental

4.1. General

Melting points were recorded on a Mel-Temp apparatus and are uncorrected. The ^1H and ^{13}C NMR spectra of the synthesized compounds were recorded on Varian XL-300 or XL-400 spectrometers. Human neutrophil elastase, proteinase 3, and Boc-Ala-Ala-Nva thiobenzyl ester were purchased from Elastin Products Co., Owensville, MO. Bovine trypsin, methoxysuccinyl Ala-Ala-Pro-Val *p*-nitroanilide, succinyl Ala-Ala-Pro-Phe *p*-nitroanilide, *N*-(*p*-tosyl)-Gly-Pro-Lys 4-nitroanilide, and 5,5′-dithio-bis(2-nitrobenzoic acid) were purchased from Sigma Chemicals Co., St. Louis, MO. Human neutrophil cathepsin G was purchased from Athens Research and Technology Co., Athens, GA. Silica gel (230–450 mesh) used for flash chromatography was purchased from Sorbent Technologies, Atlanta, GA. Thin layer chromatography was performed using Analtech silica gel plates. The TLC plates were visualized using iodine vapor and/or UV light. A Hewlett–Packard diode array UV/vis spectrophotometer was used in the enzyme assays and inhibition studies.

4.2. Representative syntheses

4.2.1. Synthesis of compound 2. To a solution of 4-*n*-propyl-5-methyl-2-chloromethyl-1,2,5-thiadiazolin-3-one 1,1 dioxide **1** (3.38 g; 14.04 mmol) and thiolacetic acid (2.14 g; 28.08 mmol) in dry acetonitrile (30 mL) kept in an ice bath was added dropwise a solution of triethylamine (1.70 g; 16.8 mmol) in acetonitrile (5 mL). The mixture was allowed to warm to room temperature and stirred for 2 days. The solvent was removed on the rotovac and the residue was taken up in ethyl acetate (100 mL) and washed with 5% aqueous HCl (20 mL) and brine (20 mL). The organic layer was dried over anhydrous sodium sulfate. Removal of the solvent left a crude product which was purified by flash chromatography using hexane/ethyl acetate to yield 3.50 g (89% yield) of an oily product. ^1H NMR ($CDCl_3$): δ 0.95–1.00 (t, 3H), 1.38 (m, 1H), 1.45 (m, 1H), 1.80 (m, 1H), 1.90 (m, 1H), 2.40 (s, 3H), 2.90 (s, 3H), 3.82 (t, 1H), 5.10 (q, 2H).

4.2.2. Synthesis of compound 4. Thioester **2** (2.40 g; 8.56 mmol) was dissolved in $CDCl_3$ (7 mL) and cooled to 5 °C using an ice bath and kept under nitrogen. Sulfuryl chloride (1.6 mL; 17.12 mmol) was then added and the mixture was stirred for 3 h. NMR analysis showed that most of the starting material had reacted. The solvent and sulfuryl chloride were then removed at 5 °C using a vacuum pump (40 min). The residue was redissolved in dry methylene chloride (10 mL) and then cooled to −78 °C using a dry ice/acetone bath. A mixture of (L) Phe–OCH_3 hydrochloride (2.77 g; 12.84 mmol) and *N*-methyl morpholine (1.30 g; 12.84 mmol) in methylene chloride (15 mL) was added to the solution and the resulting mixture was stirred at −78 °C for 1 h and then allowed to warm up to 25 °C by stirring overnight. The precipitate was filtered off and the filtrate was concentrated, leaving a crude product (3.28 g) which was purified by flash chromatography using hexane/ether. A pure product **3** was obtained (1.04 g; 29% yield) as a colorless oil (^1H NMR: δ 1.98 (m, 3H), 1.38 (m, 1H), 1.50 (m, 1H), 1.80 (m, 1H), 1.98 (m, 1H), 2.90 (s, 3H), 3.00 (m, 2H), 3.70 (s, 3H), 3.80 (t, 1H), 3.90 (m, 1H), 4.50–4.78 (dd, 2H), 7.20–7.30 (m, 5H), however, sulfinamide **3**, as well as the rest of the synthesized sulfinamides, were found to color on standing, consequently they were used in the next step without purification.

A solution of sulfinamide **3** (1.98 g; 4.76 mmol) in dry methylene chloride (10 mL) was cooled in an ice bath and then treated with 77% *m*-chloroperbenzoic acid (4.27 g; 19.06 mmol). The mixture was stirred overnight and the solvent removed on the rotovac. The residue was taken up in ethyl acetate (50 mL) and washed with saturated sodium bicarbonate (3× 10 mL), brine (3× 10 mL) and the organic extract was dried over anhydrous sodium sulfate. Removal of the solvent left a crude product which was purified using flash chromatography (hexane/ethyl acetate eluents), yielding compound **4** as pure white solid (0.83 g; 39% yield), mp 129–132 °C. ^1H NMR ($CDCl_3$): δ 0.98 (t, 3H), 1.40 (m, 2H), 1.5 (d, 3H), 1.85 (m, 1H), 1.95 (m, 1H), 2.95 (s, 3H), 3.80 (s, 3H), 4.00 (t, 1H), 4.30 (m, 1H), 4.85 (d, 1H), 5.10 (d, 1H), 5.40 (d, 1H).

4.2.3. Compound 5. Mp 87–90 °C. ^1H NMR ($CDCl_3$): δ 0.95 (m, 6H), 1.45 (m, 4H), 1.80 (m, 3H), 1.95 (m, 1H), 2.92 (s, 3H), 3.80 (s, 3H), 3.96 (t, 1H), 4.20 (q, 1H), 4.95–5.10 (dd, 2H), 5.40 (d, 1H).

4.2.4. Compound 6. Mp 119–121 °C. ^1H NMR ($CDCl_3$): δ 0.95 (t, 3H), 1.35 (m, 1H), 1.45 (m, 1H), 1.82 (m, 1H), 1.90 (m, 1H), 2.90 (s, 3H), 3.17 (t, 2H), 3.75 (s, 3H), 3.93 (t, 1H), 4.50 (t, 1H), 4.65–4.85 (dd, 2H), 7.22 (m, 5H).

4.2.5. Compound 7. Mp 99–101 °C. ^1H NMR ($CDCl_3$): δ 0.95 (t, 3H), 1.35 (m, 1H), 1.45 (m, 1H), 1.82 (m, 1H), 1.90 (m, 1H), 2.90 (s, 3H), 3.17 (t, 2H), 3.75 (s, 3H), 3.93 (t, 1H), 4.50 (t, 1H), 4.65–4.85 (dd, 2H), 7.20 (m, 5H).

4.2.6. Compound 8. Mp 74–76 °C. ^1H NMR ($CDCl_3$): δ 0.95 (t, 3H), 1.41 (m, 1H), 1.50 (m, 1H), 1.85 (m, 1H), 1.95 (m, 1H), 2.98 (s, 3H), 3.75 (s, 3H), 3.90 (m, 2H), 3.95 (t, 1H), 4.40 (m, 1H), 4.55 (dd, 2H), 5.05 (dd, 2H), 5.70 (d, 1H), 7.32 (m, 5H).

4.2.7. Compound 9. Mp 80–82 °C. ^{1}H NMR ($CDCl_3$): δ 0.95 (t, 3H), 1.40 (m, 1H), 1.50 (m, 1H), 1.85 (m, 1H), 1.95 (m, 1H), 2.75 (br s, 1H), 2.95 (s, 3H), 3.80 (s, 3H), 3.98 (d, 2H), 4.02 (t, 1H), 4.32 (s, 1H), 5.00 (dd, 2H), 5.90 (br s, 1H).

4.2.8. Compound 10. Oil. ^{1}H NMR ($CDCl_3$): δ 0.95 (t, 3H), 1.45 (m, 6H), 1.80 (m, 4H), 2.95 (s, 3H), 3.20 (m, 2H), 3.80 (s, 3H), 4.00 (t, 1H), 4.20 (m, 1H), 4.80–5.10 (dd, 2H), 5.00 (m, 2H), 5.50 (d, 1H), 7.40 (m, 5H).

4.2.9. Compound 11. Oil. ^{1}H NMR ($CDCl_3$): δ 0.95 (t, 3H), 1.48 (m, 6H), 1.85 (m, 4H), 2.90 (s, 3H), 3.20 (q, 2H), 3.75 (s, 3H), 3.95 (t, 1H), 4.19 (q, 1H), 4.95 (q, 2H), 5.10 (s, 2H), 5.49 (d, 1H), 7.32 (m, 5H).

5. Enzyme assays and inhibition studies

5.1. Human neutrophil elastase

HNE was assayed by mixing 10 μL of a 70 μM enzyme solution in 0.05 M sodium acetate/0.5 M NaCl buffer, pH 5.5, 10 μL dimethylsulfoxide, and 980 μL of 0.1 M HEPES buffer containing 0.5 M NaCl, pH 7.25, in a thermostated cuvette. A 100-μL aliquot was transferred to a thermostated cuvette containing 880 μL of 0.1 M HEPES/0.5 M NaCl buffer, pH 7.25, and 20 μL of a 70-μM solution of MeOSuc-Ala-Ala-Pro-Val *p*-nitroanilide, and the change in absorbance was monitored at 410 nm for 60 s. In a typical inhibition run, 10 μL of inhibitor (700 μM) in dimethylsulfoxide was mixed with 10 μL of 70 μM enzyme solution and 980 μL of 0.1 M HEPES/0.5 M NaCl buffer, pH 7.25, and placed in a constant temperature bath. Aliquots (100 μL) were withdrawn at different time intervals and transferred to a cuvette containing 20 μL of MeOSuc-Ala-Ala-Pro-Val *p*-nitroanilide (7 mM) and 880 μL of 0.1 M HEPES/0.5 M NaCl buffer. The absorbance was monitored at 410 nm for 60 s.

Progress curves were generated by adding 5 μL of a 2.0-μM HNE solution in 0.05 M sodium acetate buffer, pH 5.5, to 10 μL of inhibitor (50 μM solution in DMSO), 15 μL of substrate (MeOSuc-Ala-Ala-Pro-Val *p*-NA, 7 mM in DMSO) and 970 μL of 0.1 M HEPES buffer/0.5 M NaCl buffer, pH 7.25, and the absorbance was monitored at 410 nm for 10 min.

5.2. Human neutrophil proteinase 3

Twenty microliters of a 32.0-mM 5,5′-dithio-bis(2-nitrobenzoic acid) in DMSO and 10 μL of a 3.45-μM solution of human proteinase 3 in 0.1 M phosphate/0.25 M NaCl buffer, pH 6.50 (final enzyme concentration: 34.5 nM) were added to a cuvette containing a solution of 940 μL of 0.1 M HEPES/0.5 M NaCl buffer, pH 7.25, 10 μL of a 0.86 mM inhibitor solution in DMSO (final inhibitor concentration: 8.6 μM), and 20 μL of a 12.98-mM solution of Boc-Ala-Ala-NVa-SBzl in DMSO, and the change in absorbance was monitored at 410 nm for 2 min. A control (hydrolysis) was also run under the same conditions by adding 20 μL of a 32.0-mM 5,5′-dithio-bis(2-nitrobenzoic acid) solution in DMSO and 10 μL of a 3.45-μM solution of human proteinase 3 in 0.1 M phosphate buffer, pH 6.50 (final enzyme concentration: 34.5 nM) to a cuvette containing a solution of 940 μL of 0.1 M HEPES/0.5 M NaCl buffer, pH 7.25, 10 μL DMSO, and 20 μL of a 12.98-mM solution of Boc-Ala-Ala-NVa-SBzl in DMSO, and the change in absorbance was monitored at 410 nm for 2 min.

5.3. Human neutrophil cathepsin G

Ten microliters of a 6.8 μM solution of human neutrophil cathepsin G in 0.05 M sodium acetate/0.05 M NaCl buffer, pH 5.38, was added to a solution containing 10 μL of inhibitor (1.7 mM solution in DMSO) and 970 μL of 0.1 M HEPES buffer, pH 7.50. The mixture was incubated for 30 min, 10 μL of substrate (*N*-succinyl Ala-Ala-Pro-Phe *p*-nitroanilide, 85 mM in DMSO) was added, and the absorbance was monitored at 410 nm for 10 min.

5.4. Bovine trypsin

5.4.1. Incubation method. Bovine trypsin was assayed by mixing 10 μL of a 500-μM enzyme solution in 0.1 M Tris–HCl/0.01 M $CaCl_2$ buffer, pH 7.2, 10 μL DMSO, and 980 μL of 0.025 M phosphate/0.1 M NaCl buffer, pH 7.51, in a thermostated test tube. A 100-μL aliquot was transferred to a thermostated cuvette containing 890 μL 0.025 M phosphate/0.1 M NaCl buffer, pH 7.51, and 10 μL of a 60-mM solution of *N*-*p*-tosyl-Gly-Pro-Lys *p*-nitroanilide in DMSO and the change in absorbance was monitored at 410 nm for 60 s. In a typical inhibition run, 10 μL of inhibitor (12.5 mM) in DMSO was mixed with 10 μL of a 500-μM enzyme solution in 0.1 M Tris–HCl/0.01 M $CaCl_2$ buffer, pH 7.2, and 980 μL of 0.1 M Tris–HCl/0.01 M $CaCl_2$ buffer, pH 7.51, and placed in a constant temperature bath. Aliquots (100 μL) were withdrawn at different time intervals and transferred to a cuvette containing 10 μL of a 60 mM solution of *N*-*p*-tosyl-Gly-Pro-Lys *p*-nitroanilide in DMSO and 890 μL of 0.025 M phosphate/0.1 M NaCl buffer, pH 7.51. The absorbance was monitored at 410 nm for 60 s. The pseudo first-order rate constants were obtained from plots of $\ln(v/v_0)$ versus time and are the average of two determinations.

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