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Effects of structure on inhibitory activity in a series of mechanism-based inhibitors of human neutrophil elastase

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ABSTRACT

A structurally-diverse series of carboxylate derivatives based on the 1,2,5-thiadiazolidin-one 1,1 dioxide scaffold were synthesized and used to probe the S′ subsites of human neutrophil elastase (HNE) and neutrophil proteinase 3 (Pr 3). Several compounds are potent inhibitors of HNE but devoid of inhibitory activity toward Pr 3, suggesting that the S′ subsites of HNE exhibit significant plasticity and can, unlike Pr 3, tolerate various large hydrophobic groups. The results provide a promising framework for the design of highly selective inhibitors of the two enzymes.

1. Introduction

The human neutrophil elastase (HNE) is believed to play an important role in the pathophysiology of an array of inflammatory diseases, including chronic obstructive pulmonary disease (COPD),[1] cystic fibrosis,[2] acute respiratory distress syndrome,[3] ischemia/reperfusion injury,[4] and others.[5,6] COPD is a multi-factorial disorder that is characterized by an oxidant/anti-oxidant imbalance,[7,8] alveolar septal cell apoptosis,[9,10], a protease/anti-protease imbalance,[1,11] and chronic inflammation.[7,12] The confluence and interplay of multiple processes and mediators have gravely hampered efforts aimed at elucidating the molecular mechanisms and biochemical events which underlie the initiation and progression of COPD.[13]

An array of proteases, including serine (neutrophil elastase, proteinase 3), cysteine (cathepsin S) and metallo- (MMP-12) proteases released by neutrophils, macrophages and T lymphocytes that are capable of degrading lung elastin and other components of the extracellular matrix,[14] have been implicated in COPD. Elucidation of the pathogenic mechanisms in COPD and, specifically, the role each protease plays in the disorder, would pave the way toward the development of novel COPD therapeutics.[15]

We have previously demonstrated that the 1,2,5-thiadiazolidin-3-one 1,1 dioxide scaffold is a powerful and versatile core structure that can be used in the design of potent mechanism-based inhibitors of serine proteases that exploit multiple binding interactions on either side of the scissile bond.[16] X-ray crystallography and ESI-MS studies have furthermore demonstrated that inhibitor (I) inactivates HNE via a mechanism that involves the initial formation of a relatively stable acyl enzyme that incorporates in its structure a conjugated sulfonyl imine functionality. Subsequent slow reaction with water leads to the formation of one or more acyl enzymes of variable stability (Fig. 1). We describe herein the results of exploratory studies related to the utilization of inhibitor (I) to probe the S′ subsites[17] of HNE and human proteinase 3 (Pr 3) that shares 54% sequence similarity with HNE.[18]

2. Chemistry

Compounds **3–18** were synthesized as shown in Scheme 1[16,19] starting with (L) leucine methyl ester hydrochloride. The heterocyclic ring **1** was readily assembled in three steps as previously described[20] and then further elaborated to yield *N*-chloromethyl intermediate **2** (R_1 = isobutyl, R_2 = methyl or benzyl) which was transformed into the desired compounds via reaction with sodium iodide in acetone and subsequently with a carboxylic acid in the presence of DBU.

3. Biochemical studies

The inhibitory activity of compounds **3–18** was determined using the progress curve method.[21] Typical progress curves for the hydrolysis of MeOSuc-AAPV-*p*-NA by HNE in the presence of inhibitor **18** are shown in Figure 2. Control curves in the absence of inhibitor were linear. 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 **18** as a function of time were determined according to Eq. (1), 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

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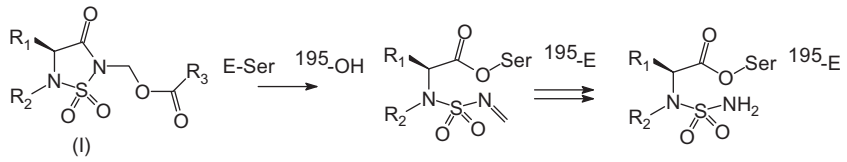
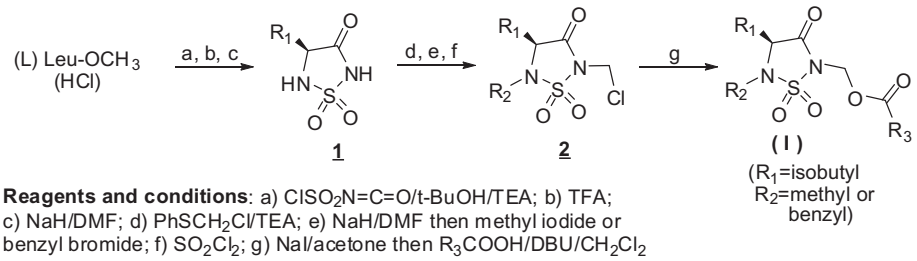


Figure 1. Mechanism of action of inhibitor (I).



Scheme 1. Synthesis of inhibitor (I).

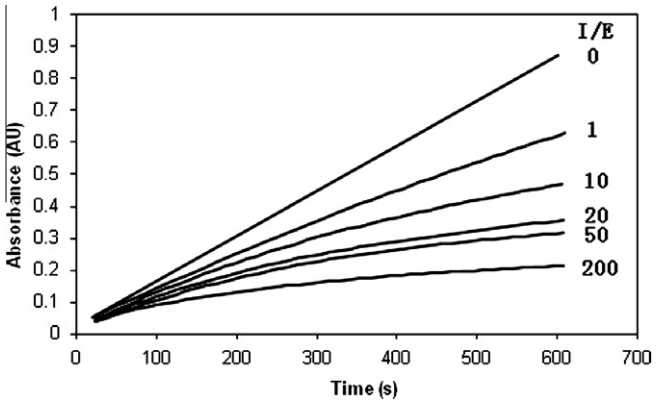


Figure 2. Progress curves for the inhibition of human neutrophil elastase (HNE) by inhibitor **18**. Absorbance was monitored at 410 nm for reaction solutions containing 10 nM HNE, MeOSuc-AAPV *p*-nitroanilide (105 μM), and the inhibitor at the indicated inhibitor to enzyme ratios in 0.1 M HEPES buffer 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.

fitting the A versus t data to 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). The apparent second-order rate constants (k_{inact}/K_I M^{-1} s^{-1}) were determined in duplicate and are listed in Table 1.

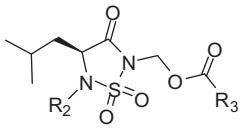
$$A = v_s t + \{(v_0 - v_s)\ (1 - e^{-k_{obs} t}) \setminus k_{obs}\} + A_0 \quad (1)$$

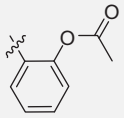
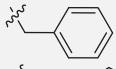
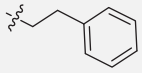
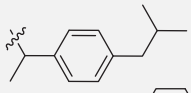
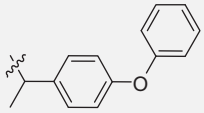
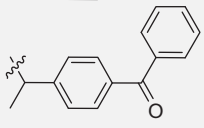
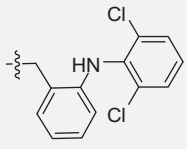
$$k_{obs} \setminus [I] = (k_{inact} \setminus K_I)[1 + [S] \setminus K_m] \quad (2)$$

4. Results and discussion

We have previously shown that carboxylate derivatives represented by general structure (I) are some of the most potent inhibitors of HNE with k_{inact}/K_I values close to 5×10^6 M^{-1} s^{-1} and apparent K_I values in the sub-nanomolar range.[16a,19] We have furthermore established that inhibitory potency is dependent on the pK_a of the leaving group (R_3COO^-), as well as its inherent structure. Thus, the structure of the leaving group can be modulated to probe the S′ subsites of closely-related serine proteases, such as HNE and Pr 3, potentially leading to enhanced binding affinity and enzyme selectivity.[20]

Table 1
List of inhibitors and inhibitory activity toward human neutrophil elastase



Compound	R_3	R_2	k_{inact}/K_I (M^{-1} s^{-1})
3		Methyl	336,000
4		Benzyl	1090,000
5		Methyl	419,000
6		Benzyl	1060,000
7[a]		Methyl	357,100
8[a]		Benzyl	63,800
9[a]		Methyl	296,300
10[a]		Benzyl	3200
11[b]		Methyl	201,000
12[b]		Benzyl	1580
13[b]		Methyl	501,000
14[b]		Benzyl	1770
15[b]		Methyl	540,000
16[b]		Benzyl	31,000
17		Methyl	981,000
18		Benzyl	1330

[a] See Ref. 19.
[b] Compounds were screened as diastereomeric mixtures.

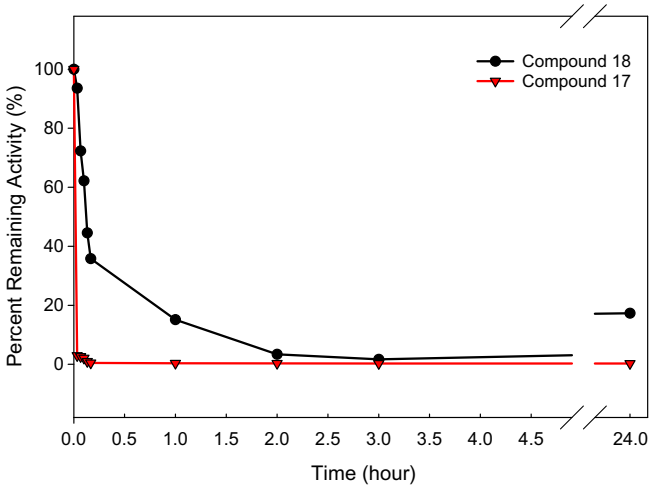


Figure 3. Time dependent loss of enzymatic activity. Percent remaining activity versus time plot obtained by incubating inhibitor **17** or **18** (7 μM) with human neutrophil elastase (700 nM) in 0.1 M HEPES buffer containing 0.5 M NaCl, pH 7.25, and 1% DMSO. Aliquots were withdrawn at different time intervals and assayed for enzymatic activity using MeOSuc-AAPV *p*-NA by monitoring the absorbance at 410 nm.

HNE and Pr 3 are similar in many respects (for instance, the two enzymes have an extended binding site and show a strong preference for small hydrophobic P1 residues), however, they exhibit significant differences in their S′ subsites. S′–P′ interactions play a more important role in catalysis in the case of Pr 3 than HNE.[18,22] The S′ subsites of HNE are generally hydrophobic while those of Pr 3 are relatively polar.[23] Specifically, the enzymes differ in their S_1'–S_3' (as well as the S_2 subsite), due to the replacement of Ala 213 and Leu 99 in HNE by Asp 213 and Lys 99, respectively, in Pr 3. Furthermore, the substitution of Leu 143 in HNE to Arg 143 in Pr 3, and the presence of Asp 61 make the S′ subsites of Pr 3 more polar and distinctly different from the hydrophobic HNE subsites. Based on these considerations, we initially set out to probe the S′ subsites of HNE by using a series of structurally-diverse carboxylic acids (R_3COOH) and subsequently extend these studies to Pr 3. Our long term objective is to obtain highly selective inhibitors of HNE and Pr 3 which can be used as probes to delineate the precise role(s) each protease plays in the pathophysiology of COPD.

Incubation of compound **17** (or **18**) with HNE led to rapid and time-dependent irreversible inactivation of the enzyme (Fig. 3). In both cases, the inactivated enzyme regained its activity very slowly, indicating the formation of fairly stable enzyme-inhibitor adducts. This is particularly true in the case of compound **17**, an observation that is consistent with previous findings[19] The potency of the synthesized inhibitors was determined by generating a series of progress curves (Fig. 2) and determining the k_{inact}/K_I values by analyzing the data (progress curve method), as described in the experimental section.

The following inferences can be made by careful perusal of the results shown in Table 1: (a) several of the compounds represented by general structure (I) inhibit HNE potently; (b) compounds **3–18** were devoid of any inhibitory activity toward human neutrophil proteinase 3, suggesting that the S′ subsites of HNE are hydrophobic and more tolerant of size, as opposed to the more polar S′ subsites of Pr 3.[18,22,23] (c) a range of structurally-diverse hydrophobic elements (R_3) can be accommodated at the S′ subsites of HNE. This is in accord with the structure of HNE and the results of previous studies.[19,20,24] (d) a noteworthy increase in potency is observed upon replacement of the methyl group at R_2 with a benzyl group (compounds **3–6**). These observations are consistent with previous studies which have shown that the nature of R_2 influences potency, as well as the stability of the enzyme-inhibitor complex,[16c,19,20] namely, compounds with small R_2 groups (such as methyl) are less potent than the corresponding compounds with larger R_2 groups (such as benzyl) and also form acyl enzyme complexes that deacylate very slowly (Fig. 2); (e) an unexpected and significant *decrease* in potency is observed when R_2 = benzyl and a spacer of 1–2 carbons connects the carboxylate and aromatic moieties (Table 1, compounds **8,10**, **12**, **14**, **16**, and **18**). A similar drop in potency was observed in a series of simpler congeners of (I), represented by $R_3(CH_2)_nCOOH$, with n = 1–2, and R_2 = benzyl (exemplified by compounds **8** and **10**, Table 1) but not when n = 0 or n = 3–4. Though speculative, these results suggest that the decrease in the inhibitory activity of compounds **8, 10**, **12**, **14**, **16**, and **18** (Table 1) may be the result of an unproductive hydrophobic collapse,[25] namely, the clustering of the hydrophobic groups in the inhibitor molecule in aqueous solution to minimize exposed hydrophobic surface area. All these molecules share a common feature associated with the flexibility and hydrophobicity of their leaving groups. Specifically, the presence of one (or two) sp^3-hybridized carbon atoms between the carboxylate group and the aromatic moiety in the leaving group appears to play a critical role in the hydrophobic collapse which forms part of the flexible turn structure of the aromatic moiety, allowing it to fold back and interact hydrophobically with the isobutyl and benzyl groups. The driving force for this phenomenon is thought to be the exclusion of water by such hydrophobic collapse in an aqueous environment.[25] Such conformation-directing hydrophobic effects result in an unproductive conformation and have been previously observed as well as exploited in the design of potent ligands.[26,27]

In conclusion, exploitation of structural differences in the S′ subsites of HNE and Pr 3 has led to the identification of highly selective and potent mechanism-based inhibitors of HNE. Inhibitory activity toward HNE in this series of compounds is influenced by multiple factors, including the possible formation of solvent-induced unproductive conformations.

5. Experimental

5.1. General

The ^1H spectra were recorded on a Varian XL-300 or XL-400 NMR spectrometer. A Hewlett–Packard diode array UV/vis spectrophotometer was used in the in vitro evaluation of the inhibitors. Human neutrophil elastase, proteinase 3 and Boc-Ala-Ala-Nva thiobenzyl ester were purchased from Elastin Products Company, Owensville, MO. Methoxysuccinyl Ala-Ala-Pro-Val *p*-nitroanilide and 5,5′-dithio-bis (2-nitrobenzoic acid) were purchased from Sigma Chemicals, St. Louis, MO. Melting points were determined on a Mel-Temp apparatus and are uncorrected. Reagents and solvents were purchased from various chemical suppliers (Aldrich, Acros Organics, TCI America, and Bachem). 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 and/or UV light.

5.2. Synthesis of Compounds 3–18

5.2.1. General procedure

To a solution of 2-chloromethyl compound **2** (2 mmol) in 6 mL dry acetone was added sodium iodide (2.2 mmol), and the reaction mixture was stirred overnight at room temperature. The by-product, sodium chloride, was removed by filtration through a small amount of silica gel in a disposable glass pipette. The solvent was then removed and the iodomethyl intermediate re-dissolved in 4 mL dry methylene chloride. To this solution was added a solution

of the appropriate carboxylic acid (2.2 mmol) and DBU (2.2 mmol) in 4 mL additional methylene chloride, and the resulting mixture was stirred overnight at room temperature. The solvent was removed; the residue dissolved in methylene chloride (40 mL) and washed successively with 5% HCl (20 mL), saturated $NaHCO_3$ (20 mL), and finally with saturated sodium chloride (20 mL). The organic layer was then dried over sodium sulfate. The drying agent and solvent were removed, leaving a crude product which was purified by flash chromatography (hexane/methylene chloride or hexane/ethyl acetate) to give compounds **3–18**.

5.2.2. Compound 3

Oil (84% yield). ^{1}H NMR ($CDCl_3$) 0.98 (dd, J = 9.5, 6.4 Hz, 6H), 1.82–1.98 (m, 3H), 2.92 (s, 3H), 3.92 (t, J = 6.3 Hz, 1H), 5.90 (q, J = 14.3, 10.0 Hz, 2H), 6.84–7.86 (m, 4H), 10.28 (s, 1H). HRMS (ESI) calcd for $C_{15}H_{20}N_2O_6SNa$ $[M+Na]^+$ 379.0940, found 379.0953.

5.2.3. Compound 4

Oil (63% yield). ^{1}H NMR ($CDCl_3$) 0.75 (dd, J = 31.5, 6.1 Hz, 6H), 1.62–1.80 (m, 3H), 3.98 (t, J = 7.3 Hz, 1H), 4.42 (q, J = 60.5, 14.5 Hz, 2H), 5.91 (d, J = 2.3 Hz, 2H), 6.88–7.88 (m, 9H), 10.30 (s, 1H). HRMS (ESI) calcd for $C_{21}H_{24}N_2O_6SNa$ $[M+Na]^+$ 455.1253, found 455.1252.

5.2.4. Compound 5

Oil (70% yield). ^{1}H NMR ($CDCl_3$) 0.99 (dd, J = 9.6, 6.3 Hz, 6H), 1.80–1.86 (m, 2H), 1.86–2.00 (m, 1H), 2.38 (s, 3H), 2.90 (s, 3H), 3.90 (t, J = 6.3 Hz, 1H), 5.84 (dd, J = 18.6, 11.4 Hz, 2H), 7.12 (d, J = 7.8 Hz, 1H), 7.32 (t, J = 7.8 Hz, 1H), 7.59 (t, J = 7.5 Hz, 1H), 8.05 (d, J = 7.8 Hz, 1H). HRMS (ESI) calcd for $C_{17}H_{26}N_3O_7S$ $[M+NH_4]^+$ 416.1491, found 416.1500; $C_{17}H_{22}N_2O_7SNa$ $[M+Na]^+$ 421.1045, found 421.1048.

5.2.5. Compound 6

Oil (79% yield). ^{1}H NMR ($CDCl_3$) 0.73 (dd, J = 33.2, 6.1 Hz, 6H), 1.55–1.80 (m, 3H), 2.35 (s, 3H), 3.92 (t, J = 7.2 Hz, 1H), 4.25–4.55 (dd, J = 75.6, 15.6 Hz, 2H), 5.83 (q, J = 21.0, 16.0 Hz, 2H), 7.10–8.06 (m, 9H). HRMS (ESI) calcd for $C_{23}H_{30}N_3O_7S$ $[M+NH_4]^+$ 492.1804, found 492.1821; $C_{23}H_{26}N_2O_7SNa$ $[M+Na]^+$ 497.1358, found 497.1360.

5.2.6. Compound 11

Oil (66% yield). ^{1}H NMR ($CDCl_3$) 0.88 (d, J = 7.0 Hz, 6H), 0.92–0.98 (dd, J = 11.6, 6.5 Hz 6H), 1.50 (d, J = 6.4 Hz 3H), 1.72–1.92 (m, 4H), 2.45 (d, J = 7.2 Hz, 2H), 2.85 (s, 3H), 3.74 (q, J = 6.9 Hz, 1H), 3.82 (t, J = 5.6 Hz, 1H), 5.52–5.72 (m, 2H), 7.06–7.22 (dd, J = 31.9, 7.2 Hz, 4H). HRMS (ESI) calcd for $C_{21}H_{36}N_3O_5S$ $[M+NH_4]^+$ 442.2376, found 442.2377; $C_{21}H_{32}N_2O_5SNa$ $[M+Na]^+$ 447.1930, found 447.1935.

5.2.7. Compound 12

Oil (95% yield). ^{1}H NMR ($CDCl_3$) 0.63–0.78 (dd, J = 38.6, 5.7 Hz, 6H), 0.88 (d, J = 6.6 Hz, 6H), 1.51 (d, J = 7.3 Hz, 3H), 1.51–1.89 (m, 4H), 2.42 (d, J = 7.4 Hz, 2H), 3.75 (q, J = 7.0 Hz, 1H), 3.86 (t, J = 6.9 Hz, 1H), 4.20–4.50 (dd, J = 84.9, 13.7 Hz, 2H), 5.62 (m, 2H), 7.09–7.20 (dd, J = 34.8, 6.4 Hz, 4H), 7.36 (s, 5H). HRMS (ESI) calcd for $C_{27}H_{40}N_3O_5S$ $[M+NH_4]^+$ 518.2689, found 518.2670; $C_{27}H_{36}N_2O_5SNa$ $[M+Na]^+$ 523.2243, found 523.2232.

5.2.8. Compound 13

Oil (33% yield). ^{1}H NMR ($CDCl_3$) 0.91–0.98 (dd, J = 17.8, 6.6 Hz, 6H), 1.49–1.51 (d, J = 7.2 Hz, 3H), 1.74–1.92 (m, 3H), 2.85 (s, 3H), 3.74 (q, J = 7.1 Hz, 1H), 7.77–3.83 (m, 1H), 5.55–5.70 (m, 2H), 6.84–7.38 (m, 9H). HRMS (ESI) calcd for $C_{23}H_{32}N_3O_6S$ $[M+NH_4]^+$ 478.2012, found 478.1999; $C_{23}H_{28}N_2O_6SNa$ $[M+Na]^+$ 483.1566, found 483.1559.

5.2.9. Compound 14

Oil (59% yield). ^{1}H NMR ($CDCl_3$) 0.63 (d, J = 5.9 Hz, 3H), 0.76 (d, J = 5.9 Hz, 3H), 1.51 (d, J = 7.14 Hz, 3H), 1.50–1.72 (m, 3H), 3.74 (q, J = 6.4 Hz, 1H), 3.85 (t, J = 5.8 Hz, 1H), 4.18 (d, J = 13.8 Hz, 1H), 4.50 (d, J = 13.8 Hz, 1H), 5.62–5.65 (m, 2H), 6.98–7.35 (m, 14H). HRMS (ESI) calcd for $C_{29}H_{36}N_3O_6S$ $[M+NH_4]^+$ 554.2325, found 554.2349; $C_{29}H_{32}N_2O_6SNa$ $[M+Na]^+$ 559.1879, found 559.1899.

5.2.10. Compound 15

Oil (68% yield). ^{1}H NMR ($CDCl_3$) 0.88–0.98 (dd, J = 11.6, 6.4 Hz, 6H), 1.54 (d, J = 7.1 Hz, 3H), 1.72–1.94 (m, 3H), 2.86 (s, 3H), 3.80–3.89 (m, 2H), 5.58–5.71 (m, 2H), 7.42–7.84 (m, 9H). HRMS (ESI) calcd for $C_{24}H_{32}N_3O_6S$ $[M+NH_4]^+$ 490.2012, found 490.2051; $C_{24}H_{28}N_2O_6SNa$ $[M+Na]^+$ 495.1566, found 495.1548.

5.2.11. Compound 16

Oil (82% yield). ^{1}H NMR ($CDCl_3$) 0.63–0.76 (dd, J = 35.2, 6.2 Hz, 6H), 1.57 (d, J = 7.3 Hz, 3H), 1.45–1.80 (m, 3H), 3.82–3.88 (m, 2H), 4.16–4.52 (dd, J = 84.2, 13.8 Hz, 2H), 5.65 (t, J = 3.6 Hz, 2H), 7.35–7.82 (m, 14H). HRMS (ESI) calcd for $C_{30}H_{36}N_3O_6S$ $[M+NH_4]^+$ 566.2325, found 566.2330; $C_{30}H_{32}N_2O_6SNa$ $[M+Na]^+$ 571.1879, found 571.1879.

5.2.12. Compound 17

Oil (60% yield). ^{1}H NMR ($CDCl_3$) 0.93–0.99 (dd, J = 9.8, 6.5 Hz, 6H), 1.76–1.94 (m, 3H), 2.87 (s, 3H), 3.82–3.87 (m, 3H), 5.70 (dd, J = 14.5, 9.7 Hz, 2H), 6.58 (m, 1H), 6.94–7.35 (m, 7H). HRMS (ESI) calcd for $C_{22}H_{25}Cl_2N_3O_5SNa$ $[M+Na]^+$ 536.0790, found 536.0781.

5.2.13. Compound 18

Oil (67% yield). ^{1}H NMR ($CDCl_3$) 0.66–0.79 (dd, J = 36.3, 6.4 Hz, 6H), 1.52–1.76 (m, 3H), 3.88 (m, 3H), 4.35 (dd, J = 79.9, 12.3 Hz, 2H), 5.70 (s, 2H), 6.52–7.39 (m, 13H). HRMS (ESI) calcd for $C_{28}H_{30}Cl_2N_3O_5S$ $[M+NH_4]^+$ 590.1283, found 590.1279; $C_{28}H_{29}Cl_2N_3O_5SNa$ $[M+Na]^+$ 612.1103, found 612.1108.

5.2.14. 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 dimethyl sulfoxide 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 0.1 M HEPES/0.5 M NaCl buffer, pH 7.25, and 20 μL of a 70 μM solution of MeO-Suc-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 (3.5 mM) in dimethyl sulfoxide was mixed with 10 μL of 70 μM enzyme solution and 980 μL 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 0.1 M HEPES/0.5 M NaCl buffer. The absorbance was monitored at 410 nm for 60 s.

5.2.15. Human neutrophil proteinase 3

Twenty microliters of 32.0 mM 5,5′-dithio-bis(2-nitrobenzoic acid) in dimethyl sulfoxide 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) were added to a cuvette containing a solution of 940 μL 0.1 M HEPES buffer, pH 7.25, containing 0.5 M NaCl, 10 μL 862.5 μM inhibitor in dimethyl sulfoxide (final inhibitor concentration: 8.62 μM) and 20 μL 12.98 mM Boc-Ala-Ala-NVa-SBzl and the change in absorbance was monitored at 410 nM for 2 min. A control (hydrolysis run) was also run under the same conditions by adding 5,5′-dithio-bis(2-nitrobenzoic acid) in dimethyl sulfoxide and 10 μL of a 3.45 μM solution of human proteinase 3 in 0.1 M phosphate buf-

fer, pH 6.50 (final enzyme concentration: 34.5 nM) to a cuvette containing a solution of 940 μL 0.1 M HEPES buffer, pH 7.25, containing 0.5 M NaCl, 10 μL dimethyl sulfoxide and 20 μL 12.98 mM Boc-Ala-Ala-NVa-SBzl and the change in absorbance was monitored at 410 nM for 2 min. Pr 3 activity remaining was determined using % remaining activity = $(v/v_0) \times 100$ and is the average of duplicate or triplicate determinations.

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