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Synthesis and pharmacological characterization of 2-aminobenzaldehyde oxime analogs as dual inhibitors of neutrophil elastase and proteinase 3

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ABSTRACT

Proteinase 3 (Pr3), and human neutrophil elastase (HNE) are two major neutrophilic serine proteases (NsPs) expressed in neutrophil azurophil granules. Emerging data suggest that excessive release of proteases mediates tissue damage, and therefore prolonged neutrophil accumulation has an important role in the pathogenesis of many diseases. Thus, HNE and Pr3 inhibitors may prove to be targets for the generation of agents in the treatment of neutrophilic inflammatory disease. Sivelestat is the only commercially available selective HNE inhibitor. Therefore, sivelestat was chosen as the model structure in an attempt to obtain more potent anti-NsPs agents. In the present study, a series 2-aminobenzaldehyde oxime and 2-aminobenzoate analogs were synthesized and their inhibitory effects on NsPs (CatG, Pr3, and HNE) were determined, respectively. The results of structure–activity relationships studies concluded that a hydroxyl oxime moiety plays an important role in ligand–enzyme affinity through hydrogen bonding. As compound **6** had more potency and showed dual inhibitory effects on NE and Pr3, both in vitro and in vivo experiments were carried out to evaluate its selectivity, effects in cell-based assays, and efficacy in models of inflammation and damage. Compound **6** had the potential to reduce paw edema induced by LPS and HNE, as well as acute lung injury, and may be approved as a candidate for the development of new agents in the treatment of neutrophilic inflammatory diseases.

1. Introduction

Neutrophils are a major type of blood leukocyte, and are essential for host defense against invading microorganisms.[1] In addition, neutrophils are involved in the activation, regulation and effector function of innate and adaptive immune cells.[2] In response to microbial attack, neutrophils are activated and recruited to the infection site, thereby releasing reactive oxygen species (ROS) and serine proteases into the extracellular space.[1,3] However, emerging data suggest that oxidant stress and excessive release of proteases mediate tissue damage. Thus, prolonged neutrophil accumulation has an important role in the pathogenesis of chronic inflammation, autoimmunity, and cancer.[1–4]

Serine proteases are the largest known family of protein-cleaving enzymes, and modulate blood coagulation, apoptosis and inflammation.[5] Of these, cathepsin G (CatG), proteinase 3 (Pr3), and human neutrophil elastase (HNE) are three major neutrophilic serine proteases (NsPs) expressed in neutrophil azurophil granules.[6,7] In particular, HNE and Pr3 are two major NsPs which play crucial roles in antimicrobial defense with overlapping and potentially redundant substrate specificity.[6] However, recent reports suggest that excess proteolysis which results from an influx of neutrophils into the airways is pivotal in the progressive destruction of lung parenchyma, for example, acute lung injury (ALI), acute respiratory distress syndrome (ARDS), and chronic obstructive pulmonary disease (COPD).[7–10] Therefore, HNE and Pr3 may prove to be targets for the generation of agents in the treatment of neutrophilic inflammatory disease.[6,11]

In 2000, an acetylating inhibitor, sivelestat, was developed for the treatment of ALI in Japan, and is the only nonpeptidic inhibitor

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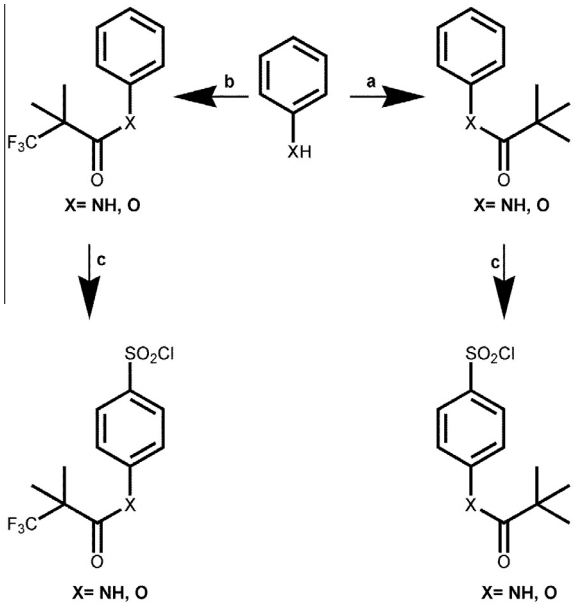


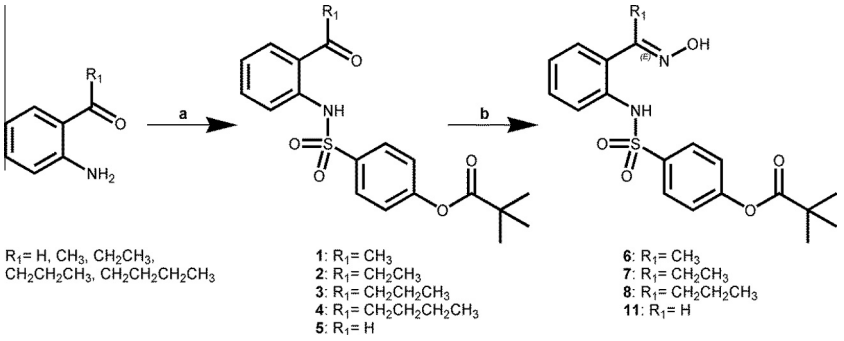
Scheme 1. Synthesis of Intermediates 4-(chlorosulfonyl)phenyl pivalate and 4-pivalamidobenzene-1-sulfonyl chloride. Reagents and conditions: (a) 3,3-dimethylbutyryl chloride, MeCN, 0 °C, 12 h; (b) pivaloyl chloride, Et_3N, 0 °C to rt, 2 h; (c) chlorosulfonic acid, MeCN, 0 °C to 75 °C, 2 h.

marketed.[12–14] However, the use of sivelestat was limited by its poor pharmacokinetics and serious risks of organ toxicity because it irreversibly inhibits HNE.[15] In addition, the clinical trials of sivelestat in the western world were terminated due to a lack in proof of therapeutic effect.[12] Therefore, sivelestat was chosen as the model structure in the present study in an attempt to obtain more potent anti-neutrophil serine protease agents. As sivelestat has hydrophobic and hydrogen-bonding interactions with HNE through pivalate ester, sulfonamide, and glycine moieties,[16] structural modification was carried out by changing the glycine moiety to hydrogen, aliphatic chains, or esters, and replacing the anthranilate moiety with 2-aminobenzaldehyde oximes, as well as exchanging pivalate ester with isostere. In the present study, we report the synthesis of target components (Schemes 1–7), as well as their structure–activity relationships (SARs) and both in vitro and in vivo pharmacologic data.

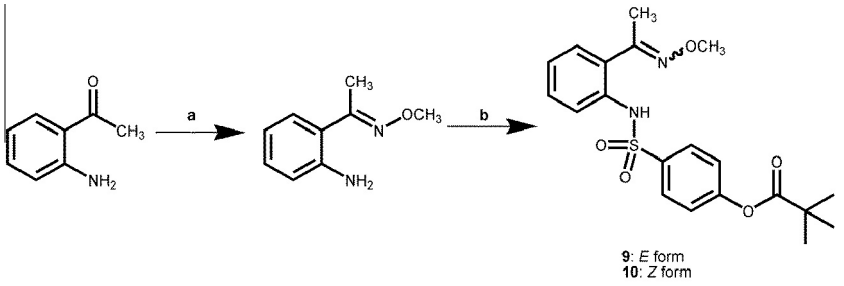
2. Results and discussion

2.1. Effects of synthetics on neutrophil serine proteases

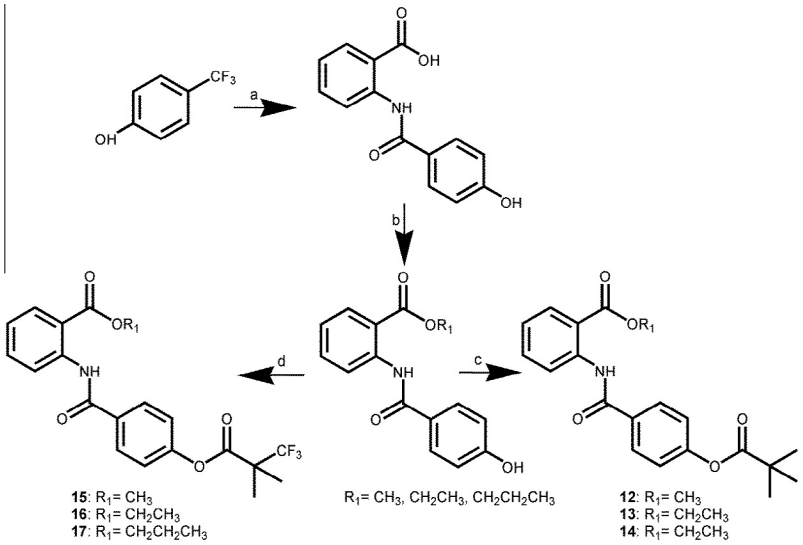
In attempt to investigate the SARs of the synthetics, the effects of all the synthetics on neutrophil serine proteases (HNE, Pr3 and CatG) were determined.[9,12,17] Most of these compounds had a potent and dual inhibitory effect on HNE and Pr3. Of these synthetics, a new 2-aminobenzaldehyde oxime analog, compound **6**, exhibited the most potent inhibitory effects on the activity of HNE and Pr3 with IC_{50} values of 0.05 and 0.22 μM, respectively, and was slightly potent than sivelestat (Tables 1 and 2). Further analysis of the SARs of the synthetics indicated that the activities of the compounds with *O*-methyl oxime (**9** and **10**) were markedly decreased compared to the compound with hydroxyl oxime (**6**), indicating that hydrogen bonding interactions play an important role in ligand–enzyme affinity. In 2012, Feng and his co-worker showed that sivelestat was able to bind to the S1 pocket of HNE through hydrophobic and hydrogen bonding interactions.[16] In particular, sivelestat was able to form hydrogen bonding interactions between the sulfonyl moiety and residues Gly 193 and Ser 195 of HNE, as well as between residue Ser 214 of HNE and the amine and carboxylic acid moieties of glycine in sivelestat.[16] In present study, the potential mode of binding of **6** and sivelestat to the



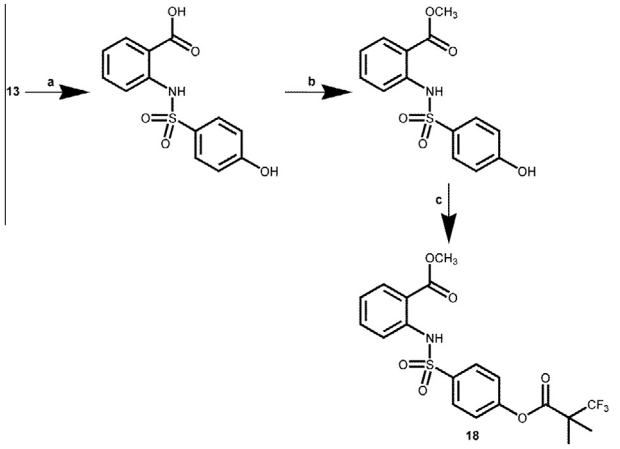
Scheme 2. The route for the preparation of compounds **1**-**8** and **11**. Reagents and conditions: (a) 4-(chlorosulfonyl)phenyl pivalate, pyridine, 4 h; (b) hydroxylamine hydrochloride, EtOH, reflux, 14 h.



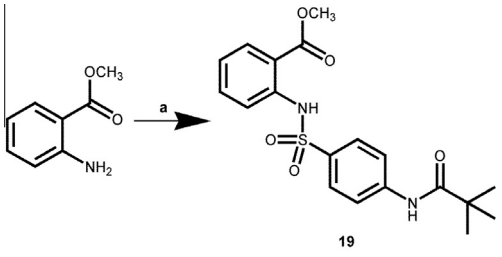
Scheme 3. The route for the synthesis of compounds **9** and **10**. Reagents and conditions: (a) methoxylamine hydrochloride, EtOH, reflux, 12 h; (b) 4-(chlorosulfonyl)phenyl pivalate, pyridine, 4 h.



Scheme 4. The route for the preparation of 2-benzamidobenzoate esters (**12**–**17**) Reagents and conditions: (a) methyl anthranilate, 1 M NaOH, dioxane, reflux, 12 h; (b) DCC, DMAP, anhydrous methanol, 12 h; (c) pivaloyl chloride, Et_3N/DCM (1:3), 2 h; (d) 3,3,3-trifluoro-2,2-dimethylpropionic acid, DMAP, EDC, DCM, 2 h.

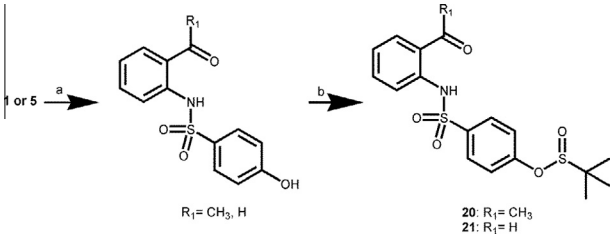


Scheme 5. The route for the preparation of compound **18**. Reagents and conditions: (a) 2 M NaOH, reflux, 6 h; (b) DCC, DMAP, anhydrous methanol, 12 h; (c) 3,3,3-trifluoro-2,2-dimethylpropionic acid, DMAP, EDC, DCM, 16 h.



Scheme 6. The route for the preparation of compound **19**. Reagents and conditions: (a) 4-pivalamidobenzene-1-sulfonyl chloride, pyridine, 4 h.

HNE were carried out docking studies.[16] As Figure S1, sivelestat and **6** are able to form hydrogen bonding interactions through their pivalate ester moiety and residues Gly 193 and Ser 195 of HNE, as well as between residues Pro 98 of HNE and oxime group of **6** or carboxylic acid moieties of glycine in sivelestat, respectively (Fig. S1). Accordingly, the hydroxyl oxime moiety in compound **6** consisted of a hydrogen bond, while the hydroxyl oxime moiety



Scheme 7. The route for the preparation of compounds **20** and **21**. Reagents and conditions: (a) 2 M $NaOH_{aq}$, 12 h; (b) *t*-butylsulfinyl chloride, pyridine, 0 °C, 1 h.

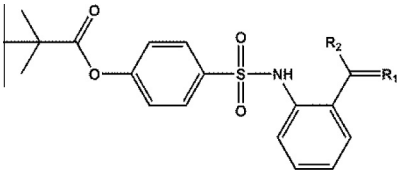
was modified to the *O*-methyl oxime moiety and the affinity to HNE was suppressed by blocking the role of the hydrogen bond donor. Moreover, those with extending carbon chains (e.g., compounds **2**–**4**, **7**, and **8**), or replacing pivalate ester with its isosteres (e.g., compounds **15**–**21**) had significantly reduced effects on proteases activities. Interestingly, the *E* form of the *O*-methyl oxime derivative (**9**) had a more favorable effect on Pr3 than the *Z* form (**10**).

2.2. Effects of synthetics on $O_2^{\cdot-}$ generation and elastase release in human neutrophils

Since neutrophil respiratory burst and degranulation are important in many inflammatory disorders, thus investigating the influence of synthetics on $O_2^{\cdot-}$ generation and elastase release in fMLF/CB-induced human neutrophils was determined.[18,19] The results showed that, with the exception of compounds **6** and **8**, most of the synthetics selectively inhibited elastase release in fMLF/CB-induced human neutrophils (Tables 3 and 4). Moreover, compounds **2**, **3**, **5**, **6**, and **11** showed equal potency to that of sivelestat in inhibiting neutrophil elastase release. The potency of compounds **3** and **11** in cell-based assays was approximately 5-fold greater than in enzyme-based assays, suggesting that these two compounds may not only suppress HNE directly, but also indirectly modulate HNE release through the signaling pathway.

Accordingly, the selectivity of compound **6** for HNE was confirmed by the cell-associated HNE assay, which showed that inhibition of HNE release was greater than superoxide generation in fMLF-induced human neutrophils. Moreover, the selectivity of

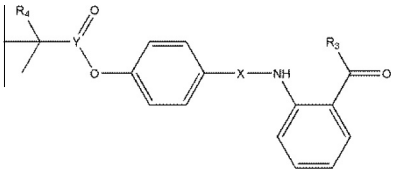
Table 1
Neutrophil serine proteases (HNE, Pr3 and CatG) inhibitory activities of synthetics (**1**–**11**)



Synthetics	R_1	R_2	HNE	Pr3	CatG
			IC_{50}[a] (μM)		
1	O	CH_3	0.11 ± 0.01	0.47 ± 0.03	>10
2	O	CH_2CH_3	0.19 ± 0.01	1.09 ± 0.08	>10
3	O	$CH_2CH_2CH_3$	0.37 ± 0.01	0.36 ± 0.03	>10
4	O	$CH_2CH_2CH_2CH_3$	2.24 ± 0.17	2.31 ± 0.10	>10
5	O	H	0.12 ± 0.04	2.05 ± 0.05	>10
6	N-OH	CH_3	0.05 ± 0.01	0.22 ± 0.02	>10
7	N-OH	CH_2CH_3	0.68 ± 0.03	2.50 ± 0.12	>10
8	N-OH	$CH_2CH_2CH_3$	0.36 ± 0.02	1.73 ± 0.03	>10
9[b]	N-OCH_3	CH_3	0.32 ± 0.04	0.61 ± 0.03	>10
10[b]	N-OCH_3	CH_3	0.37 ± 0.03	2.37 ± 0.11	>10
11	N-OH	H	0.39 ± 0.02	0.53 ± 0.03	>10
Sivelestat[c]	O	$NHCH_2COOH$	0.07 ± 0.00	0.34 ± 0.03	>10

[a] The IC_{50} values are presented as mean ± SEM (n = 3).
[b] Compounds **9** and **10** are *E*/*Z* form isomers, respectively.
[c] Sivelestat was used as positive control.

Table 2
Neutrophil serine proteases (HNE, Pr3 and CatG) inhibitory activities of synthetics (**12**–**23**)

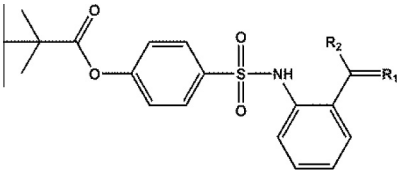


Synthetics	R_3	R_4	X	Y	HNE	Pr3	CatG
					IC_{50}[a] (μM)		
12	OCH_3	CH_3	CO	C	0.82 ± 0.03	2.05 ± 0.13	>10
13	OCH_2CH_3	CH_3	CO	C	0.48 ± 0.03	3.02 ± 0.07	>10
14	$OCH_2CH_2CH_3$	CH_3	CO	C	1.23 ± 0.09	3.91 ± 0.29	>10
15	OCH_3	CF_3	CO	C	>10	>10	>10
16	OCH_2CH_3	CF_3	CO	C	>10	>10	>10
17	$OCH_2CH_2CH_3$	CF_3	CO	C	>10	>10	>10
18	OCH_3	CF_3	SO_2	C	3.69 ± 0.12	4.94 ± 0.36	>10
19	OCH_3	CH_3	SO_2	N	>10	>10	>10
20	CH_3	CH_3	SO_2	S	>10	>10	>10
21	H	CH_3	SO_2	S	>10	>10	>10
22	$NH(CH_2)_2OH$	CH_3	SO_2	C	0.13 ± 0.01	0.31 ± 0.02	>10
23	$O(CH_2)_2OH$	CH_3	SO_2	C	0.29 ± 0.01	0.61 ± 0.06	>10
Sivelestat[b]	$NHCH_2COOH$	CH_3	SO_2	C	0.07 ± 0.00	0.34 ± 0.03	>10

[a] The IC_{50} values are presented as mean ± SEM (n = 3).
[b] Sivelestat was used as positive control.

sivelestat for Pr3 was better than compound **6**, but the inhibitory effect of compound **6** was more potent than sivelestat. To our knowledge, Pr3 and CatG are the other serine proteases in neutrophils, and have an important role in neutrophilic inflammation by multiple mechanisms often with significant overlap to HNE.[17,20] A recent study indicated that active NsPs collectively caused more severe lung damage and enhanced the activity of tissue destructive proteases than HNE alone.[21] Although HNE is a traditional therapeutic target in neutrophilic inflammation, these findings indicate that this doctrine may no longer be valid; CatG and Pr3 are pathogenic proteases that are associated with the initiation and/or chronicity of lung inflammation and tissue injury.

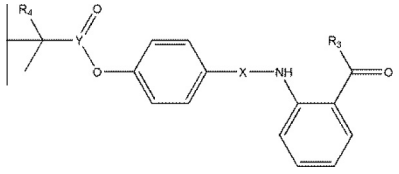
Table 3
Effects of synthetics (**1**–**11**) on $O_2^{\cdot-}$ generation and elastase release in fMLF-induced human neutrophils



Synthetics	R_1	R_2	$O_2^{\cdot-}$ generation	Elastase release
			IC_{50}[a] (μM)	
1	O	CH_3	>20	0.09 ± 0.02
2	O	CH_2CH_3	>20	0.04 ± 0.01
3	O	$CH_2CH_2CH_3$	>20	0.05 ± 0.02
4	O	$CH_2CH_2CH_2CH_3$	>20	0.11 ± 0.03
5	O	H	>20	0.05 ± 0.01
6	N-OH	CH_3	19.61 ± 3.49	0.03 ± 0.01
7	N-OH	CH_2CH_3	>20	0.11 ± 0.03
8	N-OH	$CH_2CH_2CH_3$	13.37 ± 0.89	0.13 ± 0.03
9[b]	N-OCH_3	CH_3	>20	0.17 ± 0.02
10[b]	N-OCH_3	CH_3	>20	0.18 ± 0.03
11	N-OH	H	>20	0.08 ± 0.02
Sivelestat[c]	O	$NHCH_2COOH$	>20	0.05 ± 0.02

[a] The IC_{50} values are presented as mean ± SEM (n = 3).
[b] Compounds **9** and **10** are *E*/*Z* form isomers, respectively.
[c] Sivelestat was used as positive control.

Table 4
Effects of synthetics (**12**–**23**) on $O_2^{\cdot-}$ generation and elastase release in fMLF-induced human neutrophils



Synthetics	R_3	R_4	X	Y	$O_2^{\cdot-}$ generation	Elastase release
					IC_{50}[a] (μM)	
12	OCH_3	CH_3	CO	C	>20	0.55 ± 0.11
13	OCH_2CH_3	CH_3	CO	C	>20	0.30 ± 0.02
14	$OCH_2CH_2CH_3$	CH_3	CO	C	>20	0.62 ± 0.16
15	OCH_3	CF_3	CO	C	>20	>20
16	OCH_2CH_3	CF_3	CO	C	>20	13.84 ± 4.16
17	$OCH_2CH_2CH_3$	CF_3	CO	C	>20	>20
18	OCH_3	CF_3	SO_2	C	3.69 ± 0.12	4.94 ± 0.36
19	OCH_3	CH_3	SO_2	N	>20	13.59 ± 3.16
20	CH_3	CH_3	SO_2	S	>20	3.44 ± 0.90
21	H	CH_3	SO_2	S	>20	9.41 ± 2.13
22	$NH(CH_2)_2OH$	CH_3	SO_2	C	>20	0.05 ± 0.02
23	$O(CH_2)_2OH$	CH_3	SO_2	C	>20	0.05 ± 0.01
Sivelestat[b]	$NHCH_2COOH$	CH_3	SO_2	C	>20	0.05 ± 0.02

[a] The IC_{50} values are presented as mean ± SEM (n = 3).
[b] Sivelestat was used as positive control.

2.3. Specificity and species crossover evaluations of compound 6 on serine proteases

To evaluate inhibitor specificity, we analyzed the effects of the most potent compound, **6**, on three other serine proteases, bovine pancreatic trypsin, bovine plasmatic thrombin, and bovine pancreatic chemotrypsin. The results showed that compound **6** was at least 11-fold more selective for HNE compared with the other serine proteases (Table 5). Compared with the positive HNE inhibitor, compound **6** showed greater specificity than sivelestat for HNE

Table 5
Potency and Specificity of compound **6** and sivelestat for HNE and other proteases

Compounds Serine proteases	**6**	Sivelestat
	IC_{50}[a] (nM)/selective index (SI)	
HNE	51.26 ± 7.88/1	65.04 ± 4.26/1
Pr3	211.56 ± 17.31/4.32	338.22 ± 28.35/5.20
CatG	>10000/>195.08	>10000/>153.75
Trypsin	>10000/>195.08	>10000/>153.75
Chemotrypsin	593.56 ± 20.38/11.58	1947.06 ± 86.60/29.94
Thrombin	>10000/>195.08	>10000/>153.75

[a] The IC_{50} values are presented as mean ± SEM (n = 3).

Table 6
Potency of **6** for NE species crossover

Compounds	HNE	Mouse NE	Procine Pancreas NE
	IC_{50}[a] (nM)		
6	51.26 ± 7.88	190.75 ± 11.69	1881.98 ± 35.83
Sivelestat[b]	65.04 ± 4.26	145.39 ± 17.41	2185.17 ± 34.02

[a] The IC_{50} values are presented as mean ± SEM (n = 3).
[b] Sivelestat was used as positive control.

over the other serine proteases, CatG, trypsin, and thrombin, and exhibited more inhibitory effects on Pr3 and chymotrypsin than sivelestat. Furthermore, compound **6** showed good crossover potency for HNE from other species (Table 6).

2.4. Compound 6 attenuated HNE-induced paw edema in mice

To evaluate the in vivo activity of compound **6** against HNE, we determined the effect of this compound on HNE-induced paw edema in mice via intraperitoneal administration.[22] HNE (5 μg/site) was injected into the plantar region of the right hind paw thereby causing notable foot pad swelling, which reached a maximum at 10 min, and then slowly decreased, but was still significant at 4 h (Fig. 1A–C). Compared with the control group, intraperitoneal administration of compound **6** (100 or 50 mg/kg) resulted in a significant reduction in paw edema in a time- and dose-dependent manner. It should be noted that a reduction in paw edema was also observed in the sivelestat (100 mg/kg) treatment group; however, this reduction was less than that with the same dosage of compound **6**.

2.5. Compound 6 diminished LPS-induced paw edema in mice

As compound **6** had a potent effect on HNE-induced paw edema in mice, the effect of compound **6** on LPS-induced paw edema in mice was also determined.[23] Paw edema was induced by an intraplantar injection of 25 μl (20 μg/site) LPS into the right hind paw, and paw swelling was measured at 10, 20, 40, 60, 120, and 240 min after LPS administration. The results indicated that edema formation reached a maximum at 10 min, and decreased from 20 to 240 min (Fig. 2). Pretreatment with compound **6** (100 mg/kg) significantly suppressed the edema induced by LPS, and the reduction in paw edema was less, but not significantly different to that in mice pre-treated with sivelestat (100 mg/kg).

2.6. Compound 6 suppressed the increase of MPO activity in lung tissue from mice with LPS-induced ALI

Myeloperoxidase (MPO) is an index of neutrophil infiltration, and plays an important role in the development of ALI.[24] The result of this experiment showed that MPO level in lung tissue was

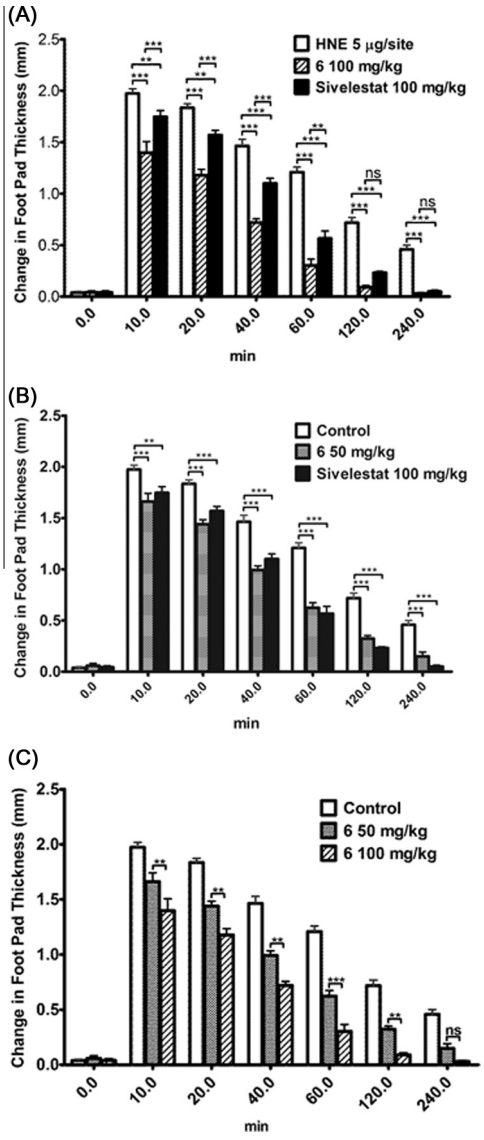


Figure 1. Effects of **6** on human neutrophil elastase-induced paw edema in mice. (A) Compound **6** and sivelestat (each 100 mg/kg body wt, ip), respectively; (B) compound **6** (50 mg/kg body wt, ip) and sivelestat (100 mg/kg body wt, ip), respectively; (C) compound **6** (50 mg/kg or 100 mg/kg body wt, ip, respectively). Values are shown as the means ± SEM (n = 6). $^{***}p$ <0.001, $^{**}p$ <0.01, $^{*}p$ <0.05, ns: p >0.05 compared to corresponding data.

significantly augmented compared with the sham group after intratracheal LPS administration (Fig. 3). In addition, both sivelestat and compound **6** significantly suppressed lung MPO activity in ALI mice compared with the LPS-induced group (Fig. 3).

Neutrophils form the first line of defense during infection and are essential in this function.[5] In pathological conditions, neutrophils upregulate the release of proteases into the extracellular space and cause host tissue damage.[20,25] Many clinical studies and animal models of ALI have shown that MPO overexpression, leukocyte infiltration, lung edema, endothelial and epithelial injury are accompanied by the invasion of neutrophils in the extracellular and bronchoalveolar space, as well as by overexpression of neutrophil proteases. Thus, neutrophil proteases are considered a target in the treatment of ALI.[9,20,25] Herein, the protective effects of compound **6** in lung injury following LPS treatment were examined. The results showed no significant differences in MPO activity and histological analysis between the compound **6**-treated group and the sham-treated animals. The administration of compound **6** or

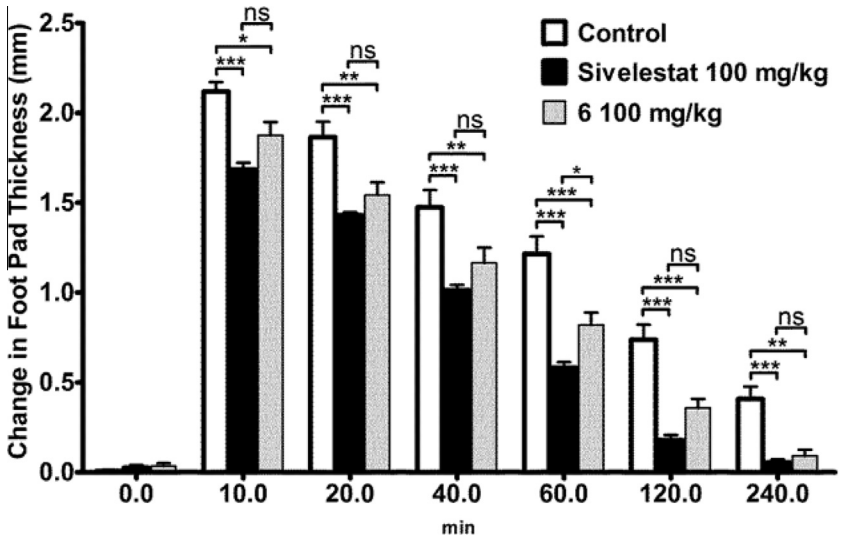


Figure 2. Effects of **6** on LPS-induced paw edema in mice. Compound **6** and sivelestat (each 100 mg/kg body wt, ip) were administered intraperitoneally, respectively. Values are shown as the means ± SEM (n = 6). $^{***}p$ <0.001, $^{**}p$ <0.01, $^{*}p$ <0.05, ns: p >0.05 compared to corresponding data.

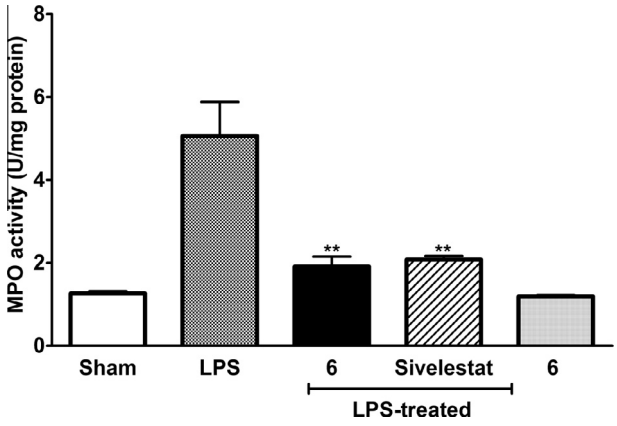


Figure 3. Effects of **6** and sivelestat (each 100 mg/ kg) on MPO activity in lung tissue from mice with LPS-induced ALI. Values are shown as the means ± SEM (n = 6). $^{**}p$ <0.01 compared with the LPS-treated group.

sivelestat (each 100 mg/kg of body weight) significantly attenuated the increase in MPO activity, leukocyte infiltration, and lung tissue destruction after administration of LPS (Figs. 3 and 4). These data indicated that compound **6** had similar potency to sivelestat and attenuated lung injury in LPS-induced ALI.

2.7. Compound 6 significantly attenuated histological changes in lung tissue from mice with LPS-induced ALI

To evaluate histological changes in lung tissues after pretreatment with sivelestat or compound **6** in LPS-treated mice, the animals were sacrificed 6 h after injection of LPS, and lung tissue was subjected to H&E staining. As shown in Figure 4, lung sections from the control and compound **6** (100 mg/kg) alone groups, showed intact structure and clear pulmonary alveoli, suggested compound **6** was safe at dosage of 100 mg/kg. Administration of LPS resulted in diffuse edematous changes, alveolar thickening, leukocyte infiltration, and lung tissue destruction (Fig. 4B). In contrast, the LPS-induced histological changes in lung were markedly attenuated by treatment with sivelestat or compound **6** (each 100 mg/kg) (Fig. 4C, D and E).

3. Conclusion

In conclusion, we synthesized twenty-three 2-aminobenzaldehyde oxime analogs and compared their inhibitory effects on NsPs (CatG, Pr3, and HNE), respectively. The results of SARs studies concluded that the hydroxyl oxime moiety was favorable for HNE and Pr3 activity, and indicated that hydrogen bonding interactions play an important role in ligand–enzyme affinity. As compound **6** had significant potency and showed dual inhibitory effects on HNE and Pr3, both in vitro and in vivo experiments were carried out to evaluate its selectivity, effects in cell-based assays, and efficacy in models of inflammation and damage. Particularly, compound **6** synthesized easier than sivelestat.[26] In summary, compound **6** has the potential to reduce ALI and associated inflammatory disorders, and may be approved as a candidate for the development of new agents in the treatment of neutrophilic inflammatory diseases.

4. Materials and methods

4.1. Chemicals and reagents

α-Chymotrypsin from bovine pancreas, trypsin from bovine pancreas, thrombin from bovine plasma, elastase from human leukocytes, *N*-succinyl-Ala-Ala-Pro-Phe-7-amido-4-methylcoumarin, N_{α}-benzoyl-L-arginine-7-amido-4-methylcoumarin, porcine pancreas elastase, lipopolysaccharides *(Escherichia coli* 055:B5), sodium azide, *o*-dianisidine dihydrochloride, phenol, and 2-aminoacetophenone were purchased from Sigma–Aldrich Chemicals (St. Louis, MO, USA). Human neutrophil elastase and Cathepsin G, and *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide were provided by Enzo (Farmingdale, NY, USA). Proteinase 3 (human neutrophils), *t*-butyloxycarbonyl-Ala-Ala-Nva-thiobenzyl ester, benzoyl-Phe-Val-Arg-7-amido-4-methylcoumarin hydrochloride, and methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide were purchased from Calbiochem (Darmstadt, Germany). Zoletil 50 was provided by Virbac Laboratories (France). Reactions were detected by thin-layer chromatography (TLC) using Merck 60 F254 silica gel aluminum backed plates; spots were recorded under ultraviolet irradiation (254 and 365 nm). Flash column chromatography was performed using silica gel (Silicycle, 70–230 mesh or 230–400 mesh). The nuclear magnetic resonance (NMR) spectra using $CDCl_3$ as the

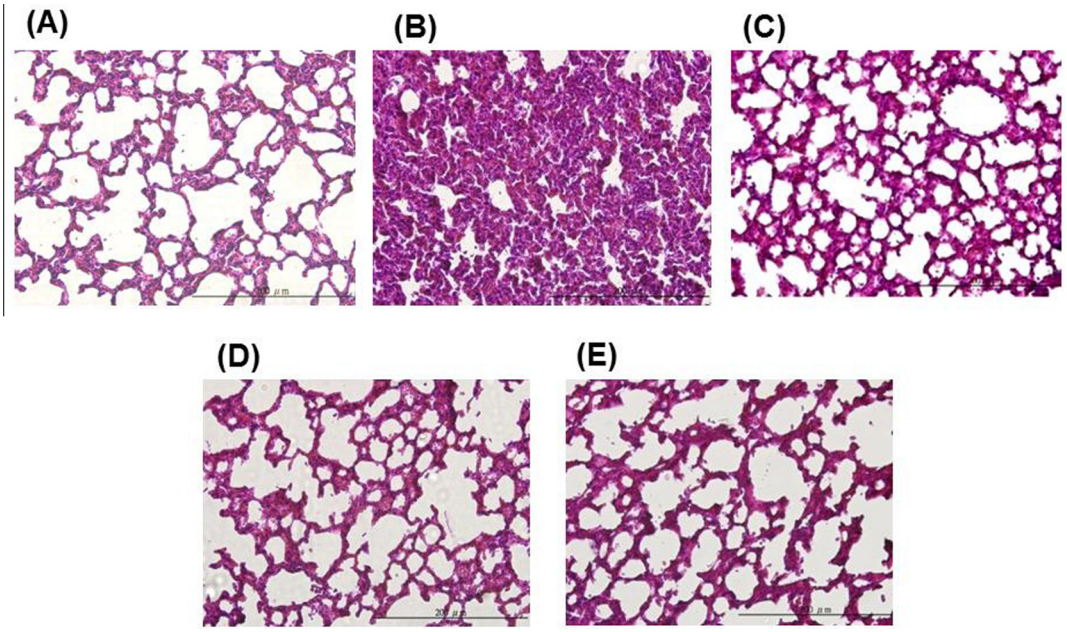


Figure 4. Histological effects of compound **6** and sivelestat on lungs of rats after a sham operation (Sham) or LPS-treatment. Representative photomicrographs of lung of (A) sham animals receiving vehicle; (B) LPS-treated animals receiving vehicle; (C and D) LPS-treated animals receiving **6** or sivelestat (each 100 mg/kg body wt, ip), respectively; (E) sham animals receiving **6** (100 mg/kg body wt, ip) Tissue sections were stained with hematoxylin–eosin, examined at an original magnification of ×400, and photographed.

solvent were obtained on a Bruker AVANCE-400 MHz FT-NMR spectrometer. Chemical shifts were internally referenced to the solvent signals in tetramethylsilane (TMS). Low-resolution EI-MS were recorded on a Quattro GC/MS spectrometer having a direct inlet system, low-resolution and high-resolution ESI-MS spectra on a Bruker Daltonics APEX II 30e spectrometer.

4.1.1. General procedure for synthesis of 2-aminobenzaldehyde analogs (1–5)

Initially, phenol (10 mmol) was dissolved in acetonitrile. The mixture solution was slowly added 3,3-dimethylbutyryl chloride (1.5 equiv) at 0 °C, and then stirred at room temperature for 12 h. The solvent was evaporated at reduced pressure. The residue was purified by silica gel column chromatography using a mixture of *n*-hexane and acetone to afford the phenyl pivalate. Subsequently, phenyl pivalate was dissolved in acetonitrile. The mixture solution was slowly added chlorosulfonic acid (1.5 equiv) at 0 °C and reacted at 0 °C for 15 min, and then refluxed at 75 °C for 2 h. The solutions were quenched by ice; the resulting mixtures were filtrated; and the residues was purified by silica gel column chromatography using a mixture of *n*-hexane/ethyl acetate to afford 4-(chlorosulfonyl)phenyl pivalate (Scheme 1). To a mixture solution of 2-aminoacetophenone, 1-(2-aminophenyl)propan-1-one, 1-(2-aminophenyl)butan-1-one, 1-(2-aminophenyl)pentan-1-one, or 2-aminobenzaldehyde (each 1.0 equiv) in pyridine, respectively, was added 4-(chlorosulfonyl)phenyl pivalate, and then stirred at room temperature for 4 h. The reaction mixture was concentrated and purified by silica gel column chromatography using a mixture of *n*-hexane/acetone solutions, to afford the products (Scheme 2, **1**–**5**).

4.1.1.1. 4-(*N*-(2-Acetylphenyl)sulfamoyl)phenyl pivalate (1). 89% yield. White powder, mp 157–159 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.45 (1H, s, NH), 7.86 (2H, d, J = 8.8 Hz, H-3′, 5′), 7.80 (1H, dd, J = 1.2, 8.4 Hz, H-3), 7.72 (1H, dd, J = 1.2, 8.4 Hz, H-6), 7.48 (1H, td, J = 1.2, 8.4 Hz, H-5), 7.14 (2H, d, J = 8.4 Hz, H-2′, 6′), 7.10 (1H, td, J = 1.2, 8.4 Hz, H-4), 2.56 (3H, s, CH_3), 1.33 (9H, s, $(CH_3)_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 202.5 (s, –$COCH_3$–), 176.2 (s, –OCO–), 154.6 (s, C-1′), 139.7 (s, C-1), 136.5 (s, C-4′), 134.9 (d, C-5), 131.9 (d, C-3), 128.8 (d, C-3′, 5′), 123.0 (d, C-4), 122.7 (s, C-2), 122.2 (d, C-2′, 6′), 119.5 (d, C-6), 39.2 (s, $C(CH_3)_3$), 28.1 (q, $COCH_3$), 27.0 (q, $(CH_3)_3$). ESI-MS (m/z, %): 373.8 $[M-H]^-$ (100). HRESI-MS m/z 374.1057 $[M-H]^-$ (calcd for $C_{19}H_{20}O_5NS$ 374.1066).

4.1.1.2. 4-(*N*-(2-Propionylphenyl)sulfamoyl)phenyl pivalate (2). 68% yield. White powder, mp 129–131 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.45 (1H, s, NH), 7.80–7.84 (3H, m, H-3′, 5′, 3), 7.70 (1H, br dd, J = 1.6, 8.4 Hz, H-6), 7.44 (1H, td, J = 1.6, 8.4 Hz, H-5), 7.11 (2H, d, J = 8.8 Hz, H-2′, 6′), 7.08 (1H, td, J = 1.6, 8.4 Hz, H-4), 2.92 (2H, q, J = 7.2 Hz, CH_2CH_3), 1.31 (9H, s, $(CH_3)_3$), 1.12 (3H, t, J = 7.2 Hz, CH_2CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 205.1 (s, –$COCH_2CH_3$–), 176.2 (s, –OCO–), 154.5 (s, C-1′), 139.4 (s, C-1), 136.5 (s, C-4′), 134.5 (d, C-5), 130.9 (d, C-3), 128.7 (d, C-3′, 5′), 123.1 (d, C-4), 122.6 (s, C-2), 122.1 (d, C-2′, 6′), 119.7 (d, C-6), 39.1 (s, $C(CH_3)_3$), 32.8 (t, CH_2CH_3), 26.9 (q, $(CH_3)_3$), 8.1 (q, CH_2CH_3). ESI-MS (m/z, %): 387.9 $[M-H]^-$ (100). HRESI-MS m/z 388.1213 $[M-H]^-$ (calcd for $C_{20}H_{22}O_5NS$ 388.1223).

4.1.1.3. 4-(*N*-(2-Butyrylphenyl)sulfamoyl)phenyl pivalate (3). 73% yield. White powder, mp 82–84 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.46 (1H, s, NH), 7.82 (2H, d, J = 8.8 Hz, H-3′, 5′), 7.81 (1H, dd, J = 1.2, 8.4 Hz, H-3), 7.71 (1H, dd, J = 1.2, 8.4 Hz, H-6), 7.45 (1H, td, J = 1.2, 8.4 Hz, H-5), 7.11 (2H, d, J = 8.8 Hz, H-2′, 6′), 7.08 (1H, td, J = 1.2, 8.4 Hz, H-4), 2.86 (2H, t, J = 7.6 Hz, $CH_2CH_2CH_3$), 1.65 (2H, sext., J = 7.6 Hz, $CH_2CH_2CH_3$), 1.32 (9H, s, $(CH_3)_3$), 0.94 (3H, t, J = 7.6 Hz, $CH_2CH_2CH_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 204.7 (s, –$COCH_2CH_3$–), 176.2 (s, –OCO–), 154.5 (s, C-1′), 139.6 (s, C-1), 136.6 (s, C-4′), 134.6 (d, C-5), 131.0 (d, C-3), 128.8 (d, C-3′, 5′), 123.1 (d, C-4), 122.8 (s, C-2), 122.1 (d, C-2′, 6′), 119.9 (d, C-6), 41.5 (t, $CH_2CH_2CH_3$), 39.1 (s, $C(CH_3)_3$), 26.9 (q, $(CH_3)_3$), 17.8 (t, $CH_2CH_2CH_3$), 13.6 (q, $CH_2CH_2CH_3$). HRESI-MS m/z 404.1526 $[M+H]^+$ (calcd for $C_{21}H_{26}O_5NS$ 404.1533).

4.1.1.4. 4-(*N*-(2-Pentanoyl phenyl)sulfamoyl)phenyl pivalate (4). 62% yield. White powder, mp 69–71 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.46 (1H, s, NH), 7.83 (2H, d, J = 8.8 Hz, H-3′, 5′), 7.82 (1H, dd, J = 1.2, 8.0 Hz, H-3), 7.72 (1H, dd, J = 1.6, 8.0 Hz, H-6), 7.46 (1H, td, J = 1.2, 8.0 Hz, H-5), 7.12 (2H, d, J = 8.8 Hz, H-2′, 6′), 7.09 (1H, td, J = 1.2, 8.0 Hz, H-4), 2.88 (2H, t, J = 7.2 Hz, $CH_2CH_2CH_2CH_3$), 1.60 (2H, Quint., J = 7.6 Hz, $CH_2CH_2CH_2CH_3$), 1.34 (2H, sext., J = 7.6 Hz, $CH_2CH_2CH_2CH_3$), 1.33 (9H, s, $(CH_3)_3$), 0.93 (3H, t, J = 7.2 Hz, $CH_2CH_2CH_2CH_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 204.9 (s, –COC–), 176.2 (s, –OCO–), 154.5 (s, C-1′), 139.6 (s, C-1), 136.6 (s, C-4′), 134.6 (d, C-5), 131.0 (d, C-36), 128.8 (d, C-3′, 5′), 123.1 (d, C-4), 122.8 (s, C-2), 122.1 (d, C-2′, 6′), 119.9 (d, C-6), 39.3 (t, CH_2CH_2 CH_2CH_3), 39.1 (s, $C(CH_3)_3$), 26.9 (q, $(CH_3)_3$), 26.5 (t, CH_2CH_2 CH_2CH_3), 22.2 (q, CH_2 $CH_2CH_2CH_3$), 13.8 (q, $CH_2CH_2CH_2CH_3$). ESI-MS (m/z, %): 440.2 $[M+Na]^+$ (100). HRESI-MS m/z 440.1502 $[M+Na]^+$ (calcd for $C_{22}H_{27}O_5NNaS$ 440.1511).

4.1.1.5. 4-(*N*-(2-Formylphenyl)sulfamoyl)phenyl pivalate (5). 64% yield. White powder, mp 121–123 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.81 (1H, s, NH), 9.81 (1H, s, H), 7.90 (2H, d, J = 8.8 Hz, H-2′, 6′), 7.69 (1H, br dd, J = 8.4 Hz, H-3), 7.60 (1H, dd, J = 1.2, 8.4 Hz, H-6), 7.51 (1H, td, J = 1.2, 8.4 Hz, H-4), 7.17 (1H, td, J = 1.2, 8.4 Hz, H-5), 7.16 (2H, d, J = 8.8 Hz, H-3′, 5′), 1.33 (9H, s, $(CH_3)_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 195.0 (s, C=O), 176.1 (s, –OCO–), 154.7 (s, C-4′), 139.5 (s, C-2), 136.2 (s, C-1′), 136.1 (d, C-6), 135.8 (d, C-4), 128.8 (d, C-2′, 6′), 123.2 (d, C-5), 122.3 (s, C-3′, 5′), 121.9 (d, C-1), 117.7 (d, C-3), 39.1 (s, $C(CH_3)_3$), 26.9 (q, $(CH_3)_3$). ESI-MS (m/z, %): 360.3 $[M-H]^-$ (100). HRESI-MS m/z 360.0900 $[M-H]^-$ (calcd for $C_{18}H_{18}O_5NS$ 360.0909).

4.1.2. General procedure for synthesis of 2-aminobenzaldehyde oxime analogs (6–11)

To a mixture solution of compounds **1**–**3** or **5** (each 1 equiv) in ethanol, respectively, was added hydroxylamine hydrochloride (1.5 equiv), and refluxed for 14 h.[27] The reaction mixture was concentrated and purified by silica gel column chromatography using a mixture of *n*-hexane/acetone (4:1 or 5:1) solutions, to afford compounds **6**–**8**, and **11** (Scheme 2).

To a mixture solution of 2-aminoacetophenone (1 equiv) in ethanol was added methoxylamine hydrochloride (1.5 equiv), and refluxed for 12 h.[27] The reaction mixture was concentrated, partitioned in dichloromethane and water, and gave the 1-(2-aminophenyl)ethanone *O*-methyl oxime. Subsequently, 1-(2-aminophenyl)ethanone *O*-methyl oxime was dissolved in pyridine, and added 4-(chlorosulfonyl)phenyl pivalate. The mixture solution was stirred for 4 h at room temperature, and then purified by silica gel column chromatography using a mixture of *n*-hexane/acetone (4:1) solutions, to afford compounds **9** and **10** (Scheme 3).

4.1.2.1. (*E*)-4-(*N*-(2-(1-(Hydroxyimino)ethyl)phenyl)sulfamoyl)phenyl pivalate (6). 27% yield. White powder, mp 166–168 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.86 (1H, s, NH), 8.51 (1H, s, OH), 7.73 (2H, d, J = 8.8 Hz, H-2′, 6′), 7.65 (1H, dd, J = 1.2, 8.0 Hz, H-3), 7.33 (1H, dd, J = 1.2, 8.0 Hz, H-6), 7.28 (1H, td, J = 1.2, 8.0 Hz, H-4), 7.11 (1H, td, J = 1.2, 8.0 Hz, H-5), 7.09 (2H, d, J = 8.8 Hz, H-3′, 5′), 2.05 (3H, s, CH_3), 1.33 (9H, s, $(CH_3)_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.9 (s, –OCO–), 157.3 (s, C=N), 154.9 (s, C-4′), 136.5 (s, C-1′), 135.7 (s, C-2), 130.0 (d, C-4), 129.2 (d, C-2′, 6′), 129.0 (d, C-6), 125.9 (s, C-1), 125.1 (d, C-5), 122.9 (d, C-3), 122.5 (d, C-3′, 5′), 39.6 (s, $C(CH_3)_3$), 27.4 (q, $(CH_3)_3$), 12.8 (q, CH_3). ESI-MS (m/z, %): 390.9 $[M+H]^+$ (100). HRESI-MS m/z 391.1322 $[M+H]^+$ (calcd for $C_{19}H_{23}O_5N_2S$ 391.1330).

4.1.2.2. (*E*)-4-(*N*-(2-(1-(Hydroxyimino)propyl)phenyl)sulfamoyl)phenyl pivalate (7). 38% yield. White powder, mp 167–169 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.04 (1H, s, NH), 8.40 (1H, s, OH), 7.76 (2H, d, J = 8.8 Hz, H-2′, 6′), 7.67 (1H, dd, J = 1.2, 8.0 Hz, H-3), 7.37 (1H, dd, J = 1.2, 8.0 Hz, H-6), 7.27 (1H, td, J = 1.2, 8.0 Hz, H-4), 7.10 (1H, td, J = 1.2, 8.0 Hz, H-5), 7.09 (2H, d, J = 8.8 Hz, H-3′, 5′), 2.64 (2H, q, J = 7.6 Hz, CH_2CH_3), 1.32 (9H, s, $(CH_3)_3$), 0.99 (3H, t, J = 7.6 Hz, CH_2CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.4 (s, –OCO–), 161.8 (s, C=N), 154.4 (s, C-4′), 136.3 (s, C-1′), 135.9 (s, C-2), 129.6 (d, C-4), 128.8 (d, C-2′, 6′), 128.3 (d, C-6), 124.4 (s, C-1), 123.6 (d, C-5), 122.0 (d, C-3, 3′, 5′), 39.2 (s, $C(CH_3)_3$), 27.0 (q, $(CH_3)_3$), 19.5 (t, CH_2CH_3), 10.8 (q, CH_2CH_3). ESI-MS (m/z, %): 405.2 $[M+H]^+$ (100). HRESI-MS m/z 405.1500 $[M+H]^+$ (calcd for $C_{20}H_{25}O_5N_2S$ 405.1500).

4.1.2.3. (*E*)-4-(*N*-(2-(1-(Hydroxyimino)butyl)phenyl)sulfamoyl)phenyl pivalate (8). 70% yield. White powder, mp 168–170 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.07 (1H, s, NH), 8.34 (1H, s, OH), 7.77 (2H, d, J = 8.8 Hz, H-2′, 6′), 7.68 (1H, dd, J = 1.2, 8.0 Hz, H-3), 7.36 (1H, dd, J = 1.2, 8.0 Hz, H-6), 7.27 (1H, td, J = 1.2, 8.0 Hz, H-4), 7.10 (2H, d, J = 8.8 Hz, H-3′, 5′), 7.09 (1H, td, J = 1.2, 8.0 Hz, H-5), 2.61 (2H, t, J = 7.6 Hz, $CH_2CH_2CH_3$), 1.35 (2H, sext., J = 7.6 Hz, $CH_2CH_2CH_3$), 1.32 (9H, s, $(CH_3)_3$), 0.89 (3H, t, J = 7.6 Hz, $CH_2CH_2CH_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.3 (s, –OCO–), 160.8 (s, C=N), 154.4 (s, C-4′), 136.4 (s, C-1′), 136.0 (s, C-2), 129.6 (d, C-4), 128.7 (d, C-2′, 6′), 128.4 (d, C-6), 124.3 (s, C-1), 123.7 (d, C-5), 122.0 (d, C-3′, 5′), 121.8 (d, C-3), 39.2 (s, $C(CH_3)_3$), 27.9 (t, $CH_2CH_2CH_3$), 27.0 (q, $(CH_3)_3$), 19.9 (t, $CH_2CH_2CH_3$), 14.2 (q, $CH_2CH_2CH_3$). ESI-MS (m/z, %): 419.2 $[M+H]^+$ (100). HRESI-MS m/z 419.1635 $[M+H]^+$ (calcd for $C_{21}H_{27}O_5N_2S$ 419.1643).

4.1.2.4. (*E*)-4-(*N*-(2-(1-(Methoxyimino)ethyl)phenyl)sulfamoyl)phenyl pivalate (9). 60% yield. White powder, mp 88–90 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.77 (1H, s, NH), 7.69 (2H, d, J = 8.8 Hz, H-2′, 6′), 7.65 (1H, br dd, J = 1.2, 8.4 Hz, H-3), 7.30 (1H, br td, J = 1.2, 8.4 Hz, H-4), 7.27 (1H, d, J = 8.4 Hz, H-6), 7.11 (1H, td, J = 1.2, 8.4 Hz, H-5), 7.08 (2H, d, J = 8.8 Hz, H-3′, 5′), 4.06 (3H, s, OCH_3), 2.02 (3H, s, CH_3), 1.33 (9H, s, $(CH_3)_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.2 (s, –OCO–), 156.1 (s, C=N), 154.4 (s, C-4′), 136.4 (s, C-1′), 135.4 (s, C-2), 129.7 (d, C-6), 128.7 (d, C-2′, 6′), 128.5 (d, C-4), 125.0 (s, C-1), 124.5 (d, C-5), 122.3 (d, C-3′, 5′), 121.9 (d, C-3), 62.6 (q, OCH_3), 39.2 (s, $C(CH_3)_3$), 27.0 (q, $(CH_3)_3$), 13.2 (q, CH_3). ESI-MS (m/z, %): 403.4 $[M-H]^-$ (100). HRESI-MS m/z 403.1322 $[M-H]^-$ (calcd for $C_{20}H_{23}O_5N_2S$ 403.1331).

4.1.2.5. (*Z*)-4-(*N*-(2-(1-(Methoxyimino)ethyl)phenyl)sulfamoyl)phenyl pivalate (10). 2.5% yield. White powder, mp 146–148 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.66 (2H, d, J = 8.8 Hz, H-2′, 6′), 7.64 (1H, br dd, J = 1.2, 7.6 Hz, H-3), 7.40 (1H, td, J = 1.2, 7.6 Hz, H-4), 7.23 (1H, td, J = 1.2, 7.6 Hz, H-5), 7.10 (2H, d, J = 8.8 Hz, H-3′, 5′), 7.05 (1H, d, J = 1.2, 7.6 Hz, H-6), 3.91 (3H, s, OCH_3), 1.57 (3H, s, CH_3), 1.36 (9H, s, $(CH_3)_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.3 (s, –OCO–), 154.6 (s, C=N), 153.6 (s, C-4′), 136.7 (s, C-1′), 132.7 (s, C-2), 130.3 (d, C-1), 130.1 (s, C-6), 128.8 (d, C-2′, 6′), 127.3 (d, C-4), 126.5 (d, C-3), 126.3 (d, C-3), 122.3 (d, C-3′, 5′), 62.1 (q, OCH_3), 39.2 (s, $C(CH_3)_3$), 27.0 (q, $(CH_3)_3$), 21.3 (q, CH_3). ESI-MS (m/z, %): 427.1 $[M+Na]^+$ (100). HRESI-MS m/z 427.1298 $[M+Na]^+$ (calcd for $C_{20}H_{24}O_5N_2SNa$ 427.1303).

4.1.2.6. (*E*)-4-(*N*-(2-(1-(Hydroxyimino)methyl)phenyl)sulfamoyl)phenyl pivalate (11). 52% yield. White powder, mp 129–131 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.52 (1H, s, NH), 8.06 (1H, s, H), 7.87 (2H, d, J = 8.8 Hz, H-2′, 6′), 7.64 (1H, br dd, J = 8.0 Hz, H-3), 7.26 (1H, td, J = 1.2, 8.0 Hz, H-4), 7.14 (2H, d, J = 8.8 Hz, H-3′, 5′), 7.13 (1H, dd, J = 1.2, 8.0 Hz, H-6), 7.05 (1H, td, J = 1.2, 8.0 Hz, H-5), 1.33 (9H, s, $(CH_3)_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.4 (s, –OCO–), 154.6 (s, C=N), 152.1 (s, C-4′), 136.3 (s, C-1′, 2), 132.1 (d, C-4), 130.5 (d, C-6), 128.9 (d, C-2′, 6′), 123.7 (d, C-5), 122.2 (d, C-3′, 5′), 119.5 (s, C-1), 119.1 (d, C-3), 39.2 (s, $C(CH_3)_3$),

27.0 (q, $(CH_3)_3$). ESI-MS (*m*/*z*, %): 399.1 $[M+Na]^+$ (100). HRESI-MS *m*/*z* 399.0985 $[M+Na]^+$ (calcd for $C_{18}H_{20}N_2O_5SNa$ 399.0983).

4.1.3. General procedure for synthesis of 2-benzamidobenzoate esters (12–17)

Initially, dissolved 4-(trifluoromethyl)phenol (1.0 equiv) and methyl anthranilate (2.0 equiv) in dioxane (9 mL), and then 1 M NaOH (4 equiv) was added. The reaction mixture was refluxed for 12 h, and then removed dioxane in vacuum. The residue was suspended in water, and adjusted pH value to 14, and then partitioned in dichloromethane (DCM) and water. The water layer was acidified to pH 1.0 with 1 M hydrochloric acid, and a precipitate formed. The precipitate was collected by filtration affording 2-(4-hydroxybenzamido)benzoic acid.[28] Secondarily, A dispersion of 2-(4-hydroxybenzamido)benzoic acid (1 equiv) and *N*,*N*′-dicyclohexylcarbodiimide (DCC, 1 equiv) in anhydrous methanol (10 ml) was stirred at room temperature for 1 h. 4-dimethylaminopyridine (DMAP, 0.5 equiv) was added and stirred at room temperature for 12 h, and gave 2-(4-hydroxybenzamido)benzoate esters. Finally, compounds **12**–**14** were synthesized through dissolving corresponding 2-(4-hydroxybenzamido)benzoate esters, respectively, in triethylamine/DCM (1:3) solution, and esterified through reacting with pivaloyl chloride for 2 h, and then purified by silica gel column chromatography using a mixture of *n*-hexane/ethyl acetate (2:1) solutions (Scheme 4). On the other hand, compounds **15**–**17** were synthesized through dissolving 3,3,3-trifluoro-2,2-dimethylpropionic acid and DMAP in DCM, and esterified through reacting with *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) and corresponding 2-(4-hydroxybenzamido)benzoate esters for 2 h, and then purified by silica gel column chromatography using a mixture of *n*-hexane/ethyl acetate (2:1) solutions (Scheme 4).

4.1.3.1. Methyl 2-(4-(pivaloyloxy)benzamido)benzoate (12). 48% yield. White powder, mp 118–120 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.04 (1H, s, NH), 8.91 (1H, d, *J* = 8.4 Hz, H-3), 8.09 (3H, m, H-6, 2′, 6′), 7.61 (1H, td, *J* = 1.2, 8.4 Hz, H-4), 7.22 (2H, dd, *J* = 1.6, 6.8 Hz, H-3′, 5′), 7.12 (1H, td, *J* = 1.2, 8.4 Hz, H-5), 3.96 (1H, s, CH_3), 1.38 (9H, s, $(CH_3)_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.6 (s, –OCO–), 169.1 (s, –COO–), 164.8 (s, –CONH–), 154.1 (s, C-4′), 141.8 (s, C-2), 134.8 (d, C-4), 132.2 (s, C-1′), 130.9 (d, C-6), 128.8 (d, C-2′, 6′), 122.6 (d, C-5), 121.9 (d, C-3′, 5′), 120.4 (d, C-3), 115.2 (s, C-1), 52.5 (q, OCH_3), 39.2 (s, $C(CH_3)_3$), 27.1 (q, $(CH_3)_3$). ESI-MS (*m*/*z*, %): 378 $[M+Na]^+$ (100). HRESI-MS *m*/*z* 378.1312 $[M+Na]^+$ (calcd for $C_{20}H_{21}O_5NNa$ 378.1323).

4.1.3.2. Ethyl 2-(4-(pivaloyloxy)benzamido)benzoate (13). 61% yield. White powder, mp 121–123 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.10 (1H, s, NH), 8.91 (1H, d, *J* = 8.4 Hz, H-3), 8.10 (3H, m, H-6, 2′, 6′), 7.60 (1H, td, *J* = 1.2, 8.4 Hz, H-4), 7.22 (2H, d, *J* = 8.4 Hz, H-3′, 5′), 7.12 (1H, t, *J* = 8.4 Hz, H-5), 4.42 (2H, q, *J* = 7.2 Hz, OCH_2CH_3), 1.43 (3H, t, *J* = 7.2 Hz, OCH_2CH_3), 1.38 (9H, s, $(CH_3)_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.6 (s, –OCO–), 168.6 (s, –COO–), 164.9 (s, –CONH–), 154.0 (s, C-4′), 141.8 (s, C-2), 134.7 (d, C-4), 132.3 (s, C-1′), 130.9 (d, C-6), 128.9 (d, C-2′, 6′), 122.6 (d, C-5), 121.9 (d, C-3′, 5′), 120.4 (d, C-3), 115.5 (s, C-1), 61.54, (t, OCH_2CH_3), 39.2 (s, $C(CH_3)_3$), 27.1 (q, $(CH_3)_3$), 14.19 (q, OCH_2CH_3). ESI-MS (*m*/*z*, %): 392 $[M+Na]^+$ (100). HRESI-MS *m*/*z* 392.1468 $[M+Na]^+$ (calcd for $C_{21}H_{23}O_5NNa$ 392.1472).

4.1.3.3. Propyl 2-(4-(pivaloyloxy)benzamido)benzoate (14). 60% yield. White powder, mp 84–86 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.10 (1H, s, NH), 8.91 (1H, dd, *J* = 0.4, 8.0 Hz, H-3), 8.09 (3H, m, H-6, 2′, 6′), 7.60 (1H, td, *J* = 1.2, 8.4 Hz, H-4), 7.22 (2H, dd, *J* = 2.0, 8.4 Hz, H-3′, 5′), 7.13 (1H, td, *J* = 1.2, 8.4 Hz, H-5), 4.31 (2H, t, *J* = 7.2 Hz, $OCH_2CH_2CH_3$), 1.84 (2H, sext., *J* = 7.2 Hz, $OCH_2CH_2CH_3$), 1.37 (9H, s, $(CH_3)_3$), 1.06 (3H, t, *J* = 7.2 Hz, $OCH_2CH_2CH_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.6 (s, –OCO–), 168.7 (s, –COO–), 164.8 (s, –CONH–), 154.0 (s, C-4′), 141.8 (s, C-2), 134.7 (d, C-4), 132.2 (s, C-1′), 130.8 (d, C-6), 128.8 (d, C-2′, 6′), 122.6 (d, C-5), 121.9 (d, C-3′, 5′), 120.4 (d, C-3), 115.5 (s, C-1), 67.1, (t, $OCH_2CH_2CH_3$), 39.2 (s, $C(CH_3)_3$), 27.1 (q, $(CH_3)_3$), 21.9 (t, $OCH_2CH_2CH_3$), 10.5 (q, $OCH_2CH_2CH_3$). ESI-MS (*m*/*z*, %): 406 $[M+Na]^+$ (100). HRESI-MS *m*/*z* 406.1625 $[M+Na]^+$ (calcd for $C_{22}H_{25}O_5NNa$ 406.1633).

4.1.3.4. Methyl 2-(4-(3,3,3-trifluoro-2,2-dimethylpropanoyloxy)benzamido)benzoate (15). 31% yield. White powder, mp 63–65 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.08 (1H, s, NH), 8.91 (1H, d, *J* = 8.0 Hz, H-3), 8.11 (3H, m, H-6, 2′, 6′), 7.62 (1H, td, *J* = 1.2, 8.0 Hz, H-4), 7.27 (2H, dd, *J* = 1.6, 7.2 Hz, H-3′, 5′), 7.14 (1H, td, *J* = 1.6, 8.0 Hz, H-5), 3.97 (1H, s, OCH_3), 1.60 (6H, s, $(CH_3)_2$). ^{13}C NMR (100 MHz, $CDCl_3$) δ169.1 (s, –COO–), 168.6 (s, –OCO–), 164.5 (s, –CONH–), 153.1 (s, C-4′), 142.0 (s, C-2), 134.8 (d, C-4), 133.0 (s, C-1′), 130.9 (d, C-6), 129.0 (d, C-2′, 6′), 126.1 (s, J_{C-F} = 281 Hz, C-9), 122.0 (d, C-5), 121.6 (d, C-3′, 5′), 120.3 (d, C-3), 115.1 (s, C-1), 52.4 (q, OCH_3), 49.0 (s, J_{C-F} = 27 Hz, C-8), 19.6 (q, J_{C-F} = 2 Hz, C-10, 11). ESI-MS (*m*/*z*, %): 408 $[M-H]^-$ (100). HRESI-MS *m*/*z* 432.1029 $[M+Na]^+$ (calcd for $C_{20}H_{18}O_5NF_3Na$ 432.1039).

4.1.3.5. Ethyl 2-(4-(3,3,3-trifluoro-2,2-dimethylpropanoyloxy)benzamido)benzoate (16). 34% yield. White powder, mp 70–72 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.14 (1H, s, NH), 8.91 (1H, d, *J* = 8.0 Hz, H-3), 8.11 (3H, m, H-6, 2′, 6′), 7.61 (1H, td, *J* = 1.6, 8.0 Hz, H-4), 7.26 (2H, br dd, *J* = 8.4 Hz, H-3′, 5′), 7.14 (1H, td, *J* = 1.6, 8.0 Hz, H-5), 4.43 (2H, q, *J* = 7.2 Hz, OCH_2CH_3), 3.97 (1H, s, OCH_3), 1.60 (6H, s, $(CH_3)_2$), 1.44 (3H, t, *J* = 7.2 Hz, OCH_2CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.1 (2s, –COO–, –OCO–), 165.0 (s, –CONH–), 153.5 (s, C-4′), 142.2 (s, C-2), 135.1 (d, C-4), 133.4 (d, C-1′), 131.4 (d, C-6), 129.4 (d, C-2′, 6′), 126.6 (s, J_{C-F} = 281 Hz, C-9), 123.1 (d, C-5), 122.0 (d, C-3′, 5′), 120.8 (d, C-3), 115.1 (s, C-1), 62.0 (t, OCH_2CH_3), 49.4 (s, J_{C-F} = 53 Hz, C-8), 20.1 (q, J_{C-F} = 2 Hz, C-10, 11), 14.6 (q, OCH_2CH_3). ESI-MS (*m*/*z*, %): 422 $[M-H]^-$ (100). HRESI-MS *m*/*z* 422.1210 $[M-H]^-$ (calcd for $C_{21}H_{19}O_5NF_3$ 422.1200).

4.1.3.6. Propyl 2-(4-(3,3,3-trifluoro-2,2-dimethylpropanoyloxy)benzamido)benzoate (17). 41% yield. White powder, mp 90–92 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.14 (1H, s, NH), 8.91 (1H, d, *J* = 7.6 Hz, H-3), 8.10–8.12 (3H, m, H-6, 2′, 6′), 7.61 (1H, td, *J* = 1.6, 7.6 Hz, H-4), 7.25 (2H, dd, *J* = 8.8 Hz, H-3′, 5′), 7.14 (1H, td, *J* = 1.6, 7.6 Hz, H-5), 4.32 (2H, t, *J* = 7.2 Hz, $OCH_2CH_2CH_3$), 1.83 (3H, q, *J* = 7.2 Hz, $OCH_2CH_2CH_3$), 1.60 (6H, $(CH_3)_2$), 1.06 (3H, t, *J* = 7.2 Hz, $OCH_2CH_2CH_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.7 (s, –COO–), 168.6 (s, –OCO–), 164.5 (s, –CONH–), 153.0 (s, C-4′), 141.7 (s, C-2), 134.7 (d, C-4), 133.0 (s, C-1′), 130.8 (d, C-6), 129.0 (d, C-2′, 6′), 126.1 (s, J_{C-F} = 281 Hz, C-9), 122.7 (d, C-5), 121.6 (d, C-3′, 5′), 120.3 (d, C-3), 115.4 (s, C-1), 67.1 (t, $OCH_2CH_2CH_3$), 49.0 (s, J_{C-F} = 26 Hz, C-8), 21.9 (t, $OCH_2CH_2CH_3$), 19.6 (q, J_{C-F} = 2 Hz, C-10, 11), 10.4 (q, $OCH_2CH_2CH_3$). ESI-MS (*m*/*z*, %): 436 $[M-H]^-$ (100). HRESI-MS *m*/*z* 436.1366 $[M-H]^-$ (calcd for $C_{22}H_{21}O_5NF_3$ 436.1370).

4.1.4. General procedure for synthesis of methyl 2-(4-(3,3,3-trifluoro-2,2-dimethylpropanoyloxy)phenylsulfonamide)benzoate (18)

Initially, dissolved compound **13** (1.0 equiv) in 2 M NaOH solution (4 mL), and refluxed for 6 h, and then partitioned in chloroform and water. The organic layer was concentrated and purified by silica gel column chromatography using a mixture of chloroform/methanol (5:1) solutions to yield 2-(4-hydroxyphenylsulfonamido)benzoate. 2-(4-Hydroxyphenylsulfonamido)benzoate was further esterified by DMAP and DCC as described in Scheme 4, and afford methyl 2-(4-hydroxyphenylsulfonamido)benzoate.

Finally, compound **18** were obtained through dissolving 3,3,3-trifluoro-2,2-dimethylpropionic acid and DMAP in acetonitrile (1 mL), and esterified through reacting with *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) and methyl 2-(4-hydroxyphenylsulfonamido)benzoate for 16 h, and then purified by silica gel column chromatography using a mixture of *n*-hexane/acetone (4:1) solutions (Scheme 5).

35% yield. White powder, mp <25 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.66 (1H, s, NH), 7.93 (1H, dd, J = 1.6, 8.0 Hz, H-6), 7.88 (2H, d, J = 8.8 Hz, H-2′, 6′), 7.70 (1H, br dd, J = 8.0 Hz, H-3), 7.47 (1H, td, J = 1.6, 8.0 Hz, H-4), 7.17 (2H, d, J = 8.8 Hz, H-3′, 5′), 7.06 (1H, td, J = 1.6, 8.0 Hz, H-5), 3.87 (3H, s, OCH_3), 1.53 (6H, s, $(CH_3)_2$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.7 (2s, –COO–, –OCO–), 154.0 (s, C-4), 140.5 (s, C-2), 137.7 (s, C-1′), 135.0 (d, C-4), 130.6 (d, C-6), 129.4 (d, C-2′, 6′), 127.8 (d, C-5), 126.4 (s, J = 281 Hz, C-9), 123.7 (d, C-5), 122.4 (d, C-3′, 5′), 119.7 (d, C-3), 116.6 (s, C-1), 52.9 (q, OCH_3), 49.4 (s, J = 27 Hz, C-8), 20.0 (q, C-10, 11). ESI-MS (*m*/*z*, %): 446 $[M+H]^+$ (100). HRESI-MS *m*/*z* 446.0880 $[M+H]^+$ (calcd for $C_{19}H_{19}O_6NF_3S$ 446.0887).

4.1.5. General procedure for synthesis of methyl 2-(4-pivalamidophenylsulfonamido)benzoate (19)

Initially, aniline (10 mmol) was dissolved in DCM. The mixture solution was slowly added pivaloyl chloride (1.1 equiv) and triethylamine (2.0 equiv) at 0 °C, and then stirred at room temperature for 2 h. The mixture was partitioned with DSM and water, and collected organic layer to afford the *N*-phenylpivalamide. Subsequently, *N*-phenylpivalamide was dissolved in DCM (5 mL), and chlorosulfonic acid (5 equiv) was slowly added at 0 °C, and then refluxed for 2 h. The solutions were quenched by ice, the mixture was partitioned with DSM and water, and collected organic layer to afford 4-pivalamidobenzene-1-sulfonyl chloride (Scheme 1). To a mixture solution of 4-pivalamidobenzene-1-sulfonyl chloride (0.05 mmol) in pyridine (3 mL) was added methyl anthranilate (0.45 mmol), and then stirred at room temperature for 4 h. The reaction mixture was concentrated and purified by silica gel column chromatography using a mixture of *n*-hexane/acetone (4:1) solutions, to afford compound **19** (35%, Scheme 6), white powder, mp 125–127 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.67 (1H, s, $NHSO_2$), 7.88 (1H, dd, J = 1.2, 8.0 Hz, H-6), 7.74 (1H, s, NHCO), 7.71 (2H, d, J = 8.8 Hz, H-2′, 6′), 7.61 (3H, d, J = 8.8 Hz, H-3, 3′, 5′), 7.39 (1H, td, J = 1.2, 8.0 Hz, H-4), 6.70 (1H, br td, J = 8.0 Hz, H-5), 3.85 (3H, s, OCH_3), 1.25 (9H, s, $(CH_3)_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.6 (s, –COO–), 168.8 (s, –OCO–), 143.1 (s, C-4′), 140.7 (s, C-2), 134.9 (d, C-4), 134.0 (s, C-1′), 131.7 (d, C-6), 128.8 (d, C-3′, 5′), 123.4 (d, C-5), 120.1 (d, C-2′, 6′), 119.2 (d, C-3), 116.2 (s, C-1), 52.9 (q, OCH_3), 40.3 (s, $C(CH_3)_3$), 27.8 (q, $(CH_3)_3$). ESI-MS (*m*/*z*, %): 403 $[M-H]^-$ (100). HRESI-MS *m*/*z* 403.1322 $[M-H]^-$ (calcd for $C_{20}H_{23}O_5N_2S$ 403.1335).

4.1.6. General procedure for synthesis of compounds 20 and 21

Sodium hydroxide aqueous solution (2 M, 3 mL) was added to a suspension of **1** or **5** (1 mmol), respectively. The reaction mixture was stirred for 12 h at room temperature; then, the mixture was diluted with sodium bicarbonate aqueous solution and extracted with ethyl acetate. The organic layer was washed with brine, dried, and filtered. Removal of the solvent gave intermediates *N*-(2-acetylphenyl)4-hydroxybenzenesulfonamide or *N*-(2-formylphenyl)4-hydroxybenzenesulfonamide, respectively. Each intermediate was dissolved in pyridine (2 mL), respectively, and *t*-butylsulfinyl chloride (5 equiv) was added slowly at 0 °C, then; the mixture was stirred at same temperature for 1 h. The mixture was diluted with sodium bicarbonate aqueous solution and extracted with ethyl acetate. Removal of the solvent of organic layer gave a residue that was purified by column chromatography (silica gel, *n*-hexane/acetone = 4:1 as eluent) to furnish **20** and **21**, respectively (Scheme 7).

4.1.6.1. 4-(*N*-(2-Acetylphenyl)sulfamoyl)phenyl 2-methylpropane-2-sulfinate (20).

90% yield. White powder, mp 137–139 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.48 (1H, s, NH), 7.83 (2H, d, J = 8.8 Hz, H-2′, 6′), 7.81 (1H, dd, J = 1.2, 8.4 Hz, H-6), 7.70 (1H, dd, J = 1.2, 8.4 Hz, H-3), 7.47 (1H, td, J = 1.2, 8.4 Hz, H-4), 7.19 (2H, d, J = 8.8 Hz, H-3′, 5′), 7.10 (1H, td, J = 1.2, 8.4 Hz, H-5), 2.57 (3H, s, CH_3), 1.33 (9H, s, $(CH_3)_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 202.5 (s, C=O), 158.1 (s, C-4′), 139.7 (s, C-2), 135.8 (s, C-1′), 135.0 (d, C-4), 132.0 (d, C-6), 129.4 (d, C-2′, 6′), 123.0 (d, C-5), 122.5 (s, C-1), 120.0 (d, C-3′, 5′), 119.2 (d, C-3), 59.2 (s, $C(CH_3)_3$), 28.1 (q, CH_3), 21.6 (q, $(CH_3)_3$). ESI-MS (*m*/*z*, %): 418 $[M+Na]^+$ (100). HRESI-MS *m*/*z* 418.0753 $[M-H]^-$ (calcd for $C_{18}H_2O_5NS_2Na$ 418.0753).

4.1.6.2. 4-(*N*-(2-Formylphenyl)sulfamoyl)phenyl 2-methylpropane-2-sulfinate (21).

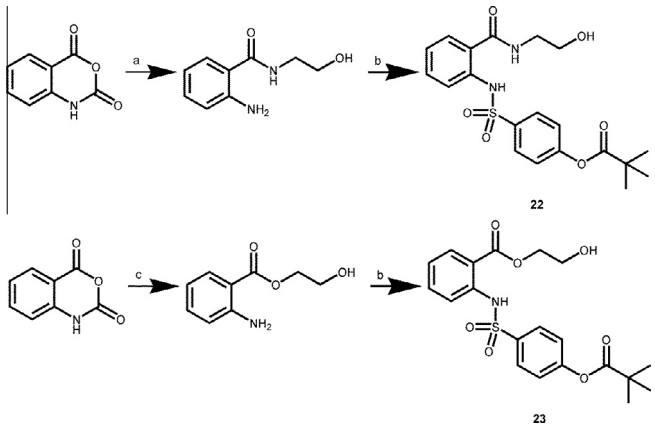
91% yield. White powder, mp 98–100 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.81 (1H, s, NH), 9.84 (1H, s, COH), 7.88 (2H, d, J = 8.8 Hz, H-3′, 5′), 7.69 (1H, br dd, J = 8.0 Hz, H-3), 7.62 (1H, dd, J = 1.2, 8.0 Hz, H-6), 7.53 (1H, td, J = 1.2, 8.0 Hz, H-5), 7.21 (2H, d, J = 8.8 Hz, H-2′, 6′), 7.20 (1H, br t, J = 8.8 Hz, H-4), 1.33 (9H, s, $(CH_3)_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 195.1 (s, CHO), 158.3 (s, C-1′), 139.6 (s, C-1), 136.2 (d, C-5), 135.9 (d, C-3), 135.5 (d, C-4′), 129.4 (d, C-3′, 5′), 123.3 (d, C-4), 122.0 (s, C-2), 120.0 (d, C-2′, 6′), 117.8 (d, C-6), 59.3 (s, $C(CH_3)_3$), 21.5 (q, $(CH_3)_3$). ESI-MS (*m*/*z*, %): 404 $[M+Na]^+$ (100). HRESI-MS *m*/*z* 404.0597 $[M+Na]^+$ (calcd for $C_{17}H_{19}O_5NS_2Na$ 404.0592).

4.1.7. Synthesis of 4-(*N*-(2-(2-Hydroxyethylcarbamoyl)phenyl) sulfamoyl)phenyl pivalate (22)

To a solution of isoatic anhydride (1 mmol) in terahydrofuran (THF, 5 mL) were added ethylanolamine (1.5 mmol), and the mixture was stirred at room temperature for 12 h. The resulting solution was concentrated in vacuum to yield a crude residue which was purified by chromatography on silica gel (acetone/hexane = 1:1) to provide intermediate 2-amino-*N*-(2-hydroxyethyl)benzamide. The intermediate (0.2 mmol) was dissolved in 10% pyridine/DCM solution (5 mL), and 4-(chlorosulfonyl)phenyl pivalate, which obtaining as Scheme 1 described, was added slowly at room temperature, then; the mixture was stirred at same temperature for 4 h. The resulting solution was concentrated and purified by chromatography on silica gel (acetone/hexane = 1:1) to furnish **22** (yielding 72%, Scheme 8), white powder, mp 112–114 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.52 (1H, s, NH), 7.70 (2H, d, J = 8.8 Hz, H-3′, 5′), 7.69 (1H, br d, J = 8.0 Hz, H-6), 7.42 (1H, br td, J = 8.0, 8.0 Hz, H-5), 7.39 (1H, br d, J = 8.0 Hz, H-6), 7.10 (1H, br td, J = 8.0, 8.0 Hz, H-4), 7.05 (2H, d, J = 8.8 Hz, H-2′, 6′), 3.68 (2H, t, J = 4.8 Hz, CH_2CH_2), 3.40 (2H, q, J = 4.8 Hz, CH_2CH_2), 1.33 (9H, s, $(CH_3)_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.6 (s, –OCO–), 168.73 (s, –COO–), 154.2 (s, C-1′), 137.9 (s, C-1), 136.6 (s, C-4′), 132.6 (d, C-5), 128.8 (d, C-3′, 5′), 126.9 (d, C-3), 125.0 (d, C-4), 124.3 (d, C-6), 124.0 (s, C-2), 122.0 (d, C-2′, 6′), 61.4 (t, CH_2CH_2), 42.2 (t, CH_2CH_2), 39.3 (s, $C(CH_3)_3$), 27.0 (q, $(CH_3)_3$). ESI-MS (*m*/*z*, %): 421 $[M+H]^+$ (100). HRESI-MS *m*/*z* 421.1425 $[M+H]^+$ (calcd for $C_{20}H_{25}N_2O_6S$ 421.1433).

4.1.8. Synthesis of 2-hydroxyethyl 2-(4-(pivaloyloxy)phenylsulfonamido)benzoate (23)

To a solution of isoatic anhydride (1 mmol) in terahydrofuran (THF, 5 mL) were added ethylene glycol (1.5 mmol) and DMAP (1.0 mmol), the mixture was stirred at 75 °C for 12 h. The resulting solution was concentrated in vacuum to yield a crude residue which was purified by chromatography on silica gel (acetone/hexane = 1:4) to provide intermediate 2-hydroxyethyl-2-aminobenzoate. The intermediate was reacted with 4-(chlorosulfonyl)phenyl pivalate (2 mmol), which obtaining as Scheme 1 described, was added slowly at room temperature, then; the mixture was stirred at same temperature for 4 h. The resulting solution was concen-



Scheme 8. The route for the preparation of compounds **22** and **23**. Reagents and conditions: (a) ethylanolamine, THF, 12 h; (b) 4-(chlorosulfonyl)phenyl pivalate, 10% pyridine/DCM, 2 h; (c) ethylene glycol, DMAP, THF, 75 °C, 12 h.

trated and purified by chromatography on silica gel (acetone/hexane = 1:1) to furnish **23** (38%, Scheme 8), white powder, mp 106–108 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.43 (1H, s, NH), 7.94 (1H, dd, J = 1.2, 8.0 Hz, H-6), 7.80 (2H, d, J = 8.8 Hz, H-2′, 6′), 7.69 (1H, br d, J = 8.0 Hz, H-3), 7.46 (1H, td, J = 1.2, 8.0 Hz, H-4), 7.10 (2H, d, J = 8.8 Hz, H-3′, 5′), 7.06 (1H, t, J = 8.0 Hz, H-5), 4.36 (2H, t, J = 4.4 Hz, CH_2CH_2), 3.87 (2H, t, J = 4.4 Hz, CH_2CH_2), 2.36 (1H, s, OH), 1.31 (9H, s, $(CH_3)_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.5 (s, –OCO–), 167.8 (s, –COO–), 154.5 (s, C-4′), 140.0 (s, C-2), 136.3 (s, C-1′), 134.6 (d, C-4), 131.3 (d, C-6), 128.8 (d, C-2′, 6′), 123.5 (d, C-5), 122.1 (d, C-3′, 5′), 120.2 (d, C-3), 116.7 (s, C-1), 66.9 (t, CH_2CH_2), 60.7 (t, CH_2CH_2), 39.2 (s, $C(CH_3)_3$), 26.9 (q, $(CH_3)_3$). ESI-MS (m/z, %): 444 $[M+Na]^+$ (100). HRESI-MS m/z 422.1268 $[M+H]^+$ (calcd for $C_{22}H_{24}NO_7S$ 422.1273).

4.2. Biological assays

4.2.1. In vitro tests

4.2.1.1. Neutrophil serine proteases (HNE, Pr3 and CatG) inhibition assay. The selectivity and potency of synthetics were modified and determined by observing the hydrolysis of the cleavage of peptide substrates to products by serine protease.[9,12,17] HNE (50 nM in 20 mM Tris–HCl, pH 7.4 containing 0.1% sodium azide buffer solution) and different concentrations of synthetics (10 nM–10 μM in 0.2% DMSO in 20 mM Tris–HCl, pH 7.4 containing 0.1% sodium azide and 5 mM calcium chloride buffer solution) were incubated in assay buffer for 5 min at 30 °C. The substrate (125 μM in 0.2% DMSO in 20 mM Tris–HCl, pH 7.4 containing 0.1% sodium azide and 5 mM calcium chloride buffer solution) was added to the wells (assays were run in triplicates in a 96-well plate in a total volume of 100 μl), and the rate of hydrolysis was determined by monitoring the absorbance at 405 nm at 30 °C for 30 min. In addition, all synthetics and inhibitors were also tested their effects on inhibiting Pr 3 (50 nM in 100 mM HEPES buffer, pH 7.5 containing 500 mM NaCl, 10% DMSO, 170 μM DTNB) and CatG (100 nM in 100 mM Tris–HCl buffer containing 1.6 M NaCl, pH 7.5) activities with corresponding substrates *t*-butyloxycarbonyl-Ala-Ala-Nva-thiobenzyl ester (100 μM) and *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (200 μM), and the rate of hydrolysis was determined by monitoring the absorbance at 405 nm at 30 °C for 180 min.

4.2.1.2. Selectivity and species crossover of lead components. The selectivity of lead components were also determined by measuring the cleavage of substrates to products by serine proteases: chymotrypsin, trypsin, and thrombin as described.[13,17] Species crossover was evaluated in the similar way using NE from pig and mouse.[17] The final concentrations of serine proteases and their corresponding substrates as follows: chymotrypsin (5 nM)/*N*-succinyl-Ala-Ala-Pro-Phe-7-amido-4-methylcoumarin (5 μM), trypsin (50 nM)/N_α-benzoyl-L-arginine-7-amido-4-methylcoumarin (50 μM), thrombin (100 nM)/Benzoyl-Phe-Val-Arg-7-amido-4-methylcoumarin hydrochloride (100 μM), porcine pancreas elastase (400 nM)/methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (250 μM), and recombinant mouse neutrophil elastase (50 nM)/methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (125 μM).

4.2.1.3. Preparation of human neutrophils[18]. Blood was taken from healthy human donors (20–34 years old) by venipuncture, following a protocol approved by the Institutional Review Board at Chang Gung Memorial Hospital. Neutrophils were isolated with a standard method of dextran sedimentation prior to centrifugation in a Ficoll Hypaque gradient and the hypotonic lysis of erythrocytes. Purified neutrophils that contained >98% viable cells, as determined by the trypan blue exclusion method, were resuspended in Ca^{2+}-free HBSS buffer at pH 7.4 and maintained at 4 °C before use.

4.2.1.4. Measurement of O_2^- generation. The assay of O_2^- generation was described as literatures.[18] Briefly, after supplementation with 0.5 mg/ml ferricytochrome *c* and 1 mM Ca^{2+}, neutrophils were equilibrated at 37 °C for 2 min and incubated with drugs for 5 min. Cells were activated with formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLF, 30 nM) for 10 min in the pretreatment of cytochalasin B (CB, 1 μg/ml) for 3 min. Changes in absorbance with the reduction of ferricytochrome *c* at 550 nm were continuously monitored in a double-beam, six-cell positioner spectrophotometer with constant stirring (Hitachi U-3010, Tokyo, Japan). Calculations were based on differences in the reactions with and without SOD (100 U/ml) divided by the extinction coefficient for the reduction of ferricytochrome *c* (ε = 21.1/mM/10 mm).

4.2.1.5. Measurement of elastase release. The method to determine of elastase release of degranulation of azurophilic granules in human neutrophil as described previously.[18,19] Experiments were performed using methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide as the elastase substrate. Briefly, after supplementation with methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (100 μM), neutrophils (5×10^5/ml) were equilibrated at 37 °C for 2 min and incubated with drugs for 5 min. Cells were activated by 30 nM FMLP for 10 min in the pretreatment of 0.5 μg/ml CB for 3 min, and changes in absorbance at 405 nm were continuously monitored to assay elastase release. Results are expressed as a percent of elastase release in the drug-free control group.

4.2.2. In vitro tests

4.2.2.1. Animals. Male C57BL/6 mice (6–8 weeks old) weighing 20–25 g were used in this study. The animal protocol was approved by the Institutional Animal Care and Use Committee of Chang Gung University (Taoyuan, Taiwan, IACUC Approval no.: CGU12-011, period of protocol: valid from Jun. 01, 2012 to May. 31, 2015). Animals were anesthetized with pentobarbital (50 mg/kg) before intraperitoneal or intranasal administration.

4.2.2.2. LPS- and HNE-induced paw edema. Lipopolysaccharides (LPS) from *Escherichia coli* 055:B5 and human neutrophil elastase (HNE) were purchased from Sigma–Aldrich and dissolve in saline. Acute inflammation of mice hind paws was induced as literature.[22,23] Briefly, LPS (20 μg in 25 μl of saline) or HNE (5 μg in 25 μl of saline) were injected subcutaneously into the plantar region of the right hind paw. The thickness of the LPS-/or

HNE-treated paws was measured before and after LPS/or HNE injection at the time points, using digital calipers (Insize, Austria). Each measurement was repeated three times, and the mean values were calculated and expressed to the nearest ±0.01 mm.[6] The inflamed animals were pretreated intraperitoneally with **6** (100 and 50 mg/kg) or sivelestat (100 mg/kg), respectively, 1 h before induced by LPS- or HNE-injected.

4.2.2.3. LPS-induced acute lung injury. As described LPS from *Escherichia coli* 055:B5 was dissolve in saline. Acute inflammation of mice hind paws was induced as literature.[24,29,30] Thirty male C57BL/6 mice were randomly divided into five groups ($n = 6$): sham group (without LPS treated); LPS treated; LPS + **6** (100 mg/kg); LPS + sivelestat (100 mg/kg); and **6** alone. Mice were given intraperitoneal injection of **6** or sivelestat, respectively. Mice from the sham group and LPS groups were treated with an equal volume of vehicles instead. Except for sham group, all test animals were pretreated intraperitoneally with 50 μl saline, **6** (100 mg/kg) or sivelestat (100 mg/kg), respectively, 1 h before LPS-injected (800 μg in 25 μl of saline).

4.2.2.4. Measurement of myeloperoxidase (MPO) activity. MPO activity in whole-lung homogenates was determined as described.[10] Briefly, equal weights (100 mg wet weight) of lung from various groups were added 1 ml buffer (0.5% hexadecyltrimethylammonium bromide in a 50 mM phosphate buffer; pH 6.0) and sonicated at 3 cycles, twice, for 30 s on 4 °C. Homogenates were centrifuged at 2000*g* and 4 °C for 10 min, and the supernatants were stored at −80 °C. Determining the protein contents of samples using the Bio-Rad (Hercules, CA) assay kit, and samples were incubated with the substrate, *o*-dianisidine hydrochloride. The analyses was carried out in a 96-well plate by adding 290 μl of 50 mM phosphate buffer, 3 μl substrate solution (containing 20 g/l o-dianisidine hydrochloride), and 3 μl H_2O_2 (20 mM). The sample (10 μl) was added to each well to start the reaction. Standard MPO (Sigma, St. Louis, MO) was used in parallel to determine the MPO activity in the sample. The reaction was quenched by adding 3 μl sodium azide (30%). The light absorbance at 460 nm was read. MPO activity was determined using a curve obtained from the MPO standard.

4.2.2.5. Histological evaluation. Each specimen was dehydrated using a 10% buffered formaldehyde solution, PBS solution, and an ethanol solution, and were then embedded in paraffin wax. These specimens were stained with hematoxylin and eosin stain (H&E stain). For each sample, at least three different sites were examined and evaluated under light microscopy (Eclipse 4000, Nikon, Tokyo, Japan). Digital photomicrography was then processed with Adobe PhotoDeluxe (Adobe System, San Jose, CA, USA).

4.2.3. Statistical analysis

Results are expressed as the mean ± SEM. Data were analysed using the GraphPad Prism software (GraphPad Software, San Diego, CA). Statistical analysis was performed using Student's *t*-test or two-way analysis of variance (ANOVA) followed by Bonferroni post-tests. A value of $p < 0.05$ was considered statistically significant.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.12.056.

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