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# Synthesis and in vitro evaluation of pseudosaccharinamine derivatives as potential elastase inhibitors

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**Abstract**—Pseudosaccharinamine derivatives were evaluated for elastase inhibitory activity. Ester derivatives of pseudosaccharinamine displayed reversible and high inhibition of human leukocyte elastase (HLE) as compared to porcine pancreatic elastase (PPE). Cyanomethyl (*2S*,*3S*)-*2*-(1,1-dioxobenzo[*d*]isothiazol-3-ylamino)-3-methylpentanoate was found to inhibit HLE at $K_i = 0.8$ μM.


## 1. Introduction

Human leukocyte elastase (HLE, EC 3.4.21.37) is a member of the serine protease family of enzymes produced by polymorphonuclear leukocytes (PMNs) and has a molecular weight of about 30 kDa. It is released from PMNs in response to inflammatory stimuli.[1] HLE is involved in tissue remodeling and wound healing. Under homeostatic conditions, the destructive effects of HLE are limited to the microenvironment immediately surrounding the neutrophils by endogenous proteinase inhibitors such as $\alpha_1$-proteinase inhibitor ($\alpha_1$-PI, $\alpha_1$-antitrypsin). As a consequence of chronic inflammation, however, the balance between HLE and $\alpha_1$-PI can be shifted in favor of HLE, resulting in uncontrolled tissue destruction. The proteinase/antiproteinase balance may also be affected by decreased availability of $\alpha_1$-PI, either through inactivation by oxidants such as cigarette smoke or as a result of genetic inability to produce sufficient serum levels.[2] HLE has been implicated in the development of various diseases such as adult respiratory distress syndrome (ARDS), cystic fibrosis, pulmonary emphysema, smoking related chronic bronchitis, and rheumatoid arthritis.[3]

The culmination of many structure–activity relationships (SAR) has led to the discovery of three different classes of inhibitors, mainly, electrophilic ketone-based inhibitors (generally referred to as transition state inhibitors),[1,3–9] acylating agents,[10–12] and heterocyclic inhibitors.[13–15] Recent success of employing acylating agents as HLE inhibitors (ONO-5046)[10,11,16] has opened up a new field for the synthesis of numerous acylating inhibitors. The highly specific and intravenously effective inhibitor ONO-5046 has been approved in Japan as an HLE inhibitor for the treatment of acute lung injury accompanying systemic inflammatory response syndrome.[11] Some of the elastase inhibitors are mentioned in Figure 1.

### 1.1. Structural considerations

Saccharin derivatives are well-reported elastase inhibitors.[13–15] The proposed mechanistic aspect of elastase inhibition reveals that the nucleophilic attack of Ser-195 of the enzyme on the carbonyl group of saccharin followed by departure of the leaving group (indirect displacement) is essential (Fig. 2).[14]

Here we planned to modify the saccharin derivatives in such a way as to:

(1) Mask the carbonyl functionality of saccharin nucleus and
(2) Carry out reintroduction of the carbonyl functionality at the other part of the molecule along with a leaving group and check whether these newly planned pseudosaccharinamine derivatives (Fig. 3) can still possess any elastase inhibitory activity.

*Keywords*: Pseudosaccharinamines; Elastase inhibitors; Heterocycles; Amino acid derivatives.
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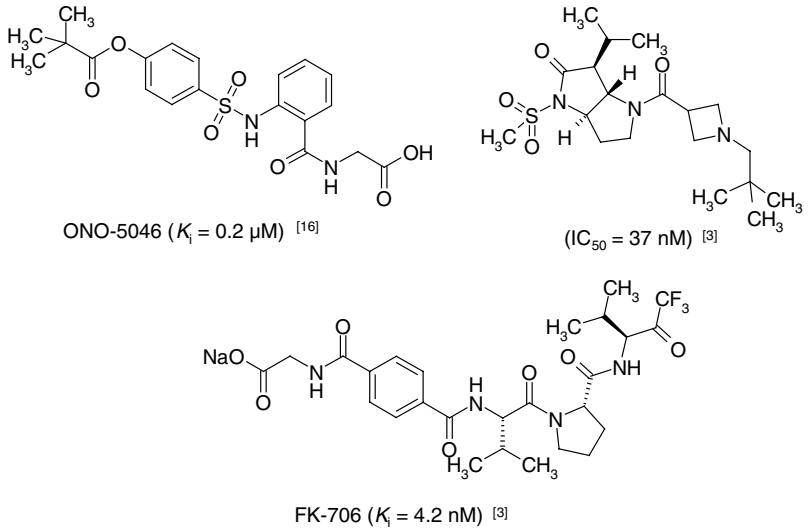



**Figure 1.** Recent HLE inhibitors.

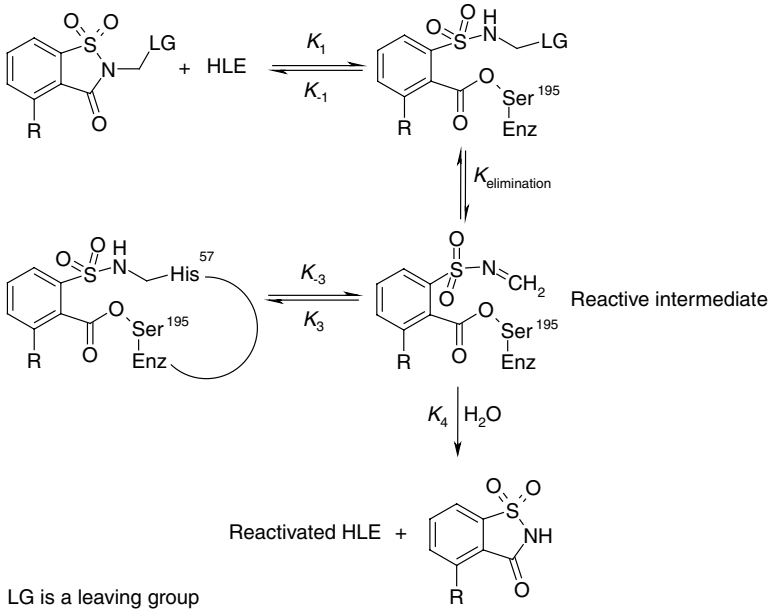



**Figure 2.** Elastase inhibition by saccharin derivatives.[14]

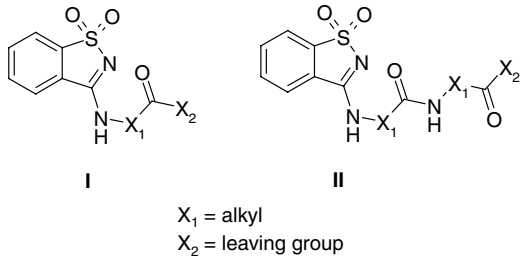



**Figure 3.** Planned pseudosaccharinamine derivatives.

Hence, the pseudosaccharinamine derivatives **I** and **II** (Fig. 3) were planned for the synthesis and their being screened against elastase activity. In the above structure $X_1$ and $X_2$ are different substituents. Peptide (**II**) is also planned for the synthesis. It is reported that the backbone architect of HLE and PPE (porcine pancreatic elastase) is conserved, and that the structures of the active sites near the cleavage site are very similar.[17,18] The N-terminal amino acid sequence of HLE is strongly homologous with that of porcine pancreatic elastase.[19] Hence, we decided to study the effect of pseudosaccharinamine derivatives, first on PPE as it is less expensive compared to HLE, and its catalytic sides are very similar to those of HLE. Once the compound showed some effect against PPE, it was further studied for HLE.

## 2. Results and discussion

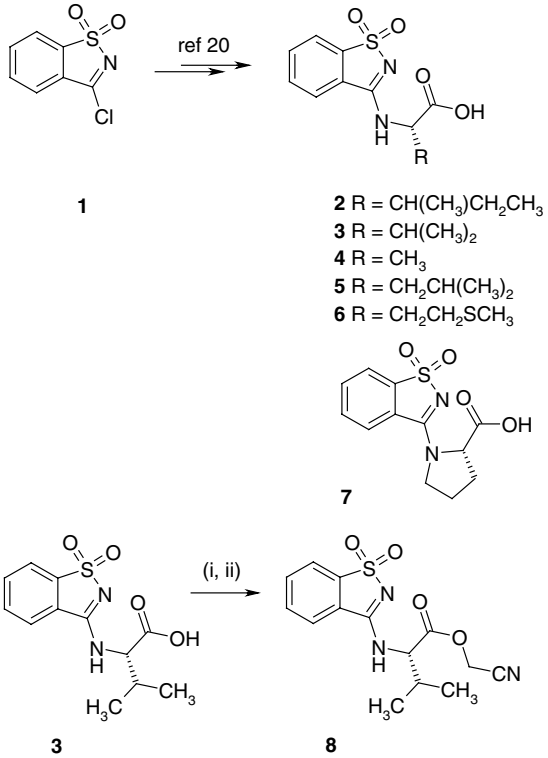
Compounds **1**–**7** were synthesized according to the literature procedure.[20] **3** was initially converted into its

potassium salt by adding aq KOH. The dried salt upon heating with chloroacetonitrile at 80 °C for 30 min produced compound **8** in 44% yield (Scheme 1).

Compounds **9**–**12** were obtained in moderate yield by refluxing **2** in ethanol, isopropanol, *n*-butanol, and isobutanol, respectively, in the presence of thionyl chloride (Scheme 2).

Compound **2** was first converted into the potassium salt and then this salt was heated with chloroacetonitrile at 80 °C, producing **13** in 42% yields. Compound **14** was obtained by reacting pseudosaccharin chloride (**1**) with isoleucine benzyl ester tosylate and triethylamine in dioxin, while **15** was obtained from **1** and isoleucine *tert*-butyl ester HCl, and **16** from **2** and aminoacetonitrile HCl by the mixed anhydride method using isobutyl chloroformate and *N*-methylmorpholine.

Compound **18** was obtained from the commercially available **17** and aminoacetonitrile HCl by the mixed anhydride method employing isobutyl chloroformate and *N*-methylmorpholine (Scheme 3). Deprotection of the Boc group of **18** was possible in ethyl acetate saturated with HCl gas at room temperature. Thus, obtained compound **19** is highly hygroscopic. In order to obtain peptides having aminoacetonitrile as the 'C' terminal moiety, compounds **2**–**7** were treated with **19** by the mixed anhydride method. Compounds **20**–**25** were obtained as solids. The absorption of the nitrile group of **8**, **13**, and **20**–**25** was absent in their IR spectra. The presence of the nitrile group of **20**–**25** was confirmed




**1**

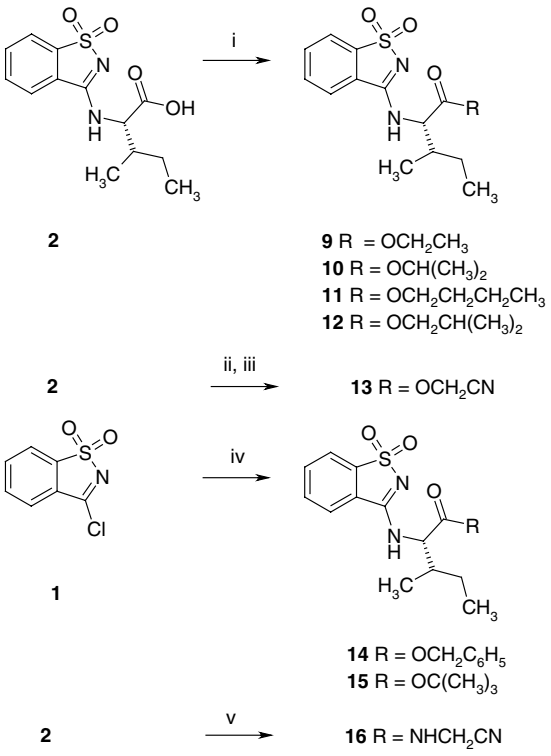
**2** R = $CH(CH_3)CH_2CH_3$
**3** R = $CH(CH_3)_2$
**4** R = $CH_3$
**5** R = $CH_2CH(CH_3)_2$
**6** R = $CH_2CH_2SCH_3$

**7**

**3**

**8**

**Scheme 1.** Synthesis of **8** (i) aq KOH; (ii) $ClCH_2CN$, heat 80 °C, 30 min.




**2**

**9** R = $OCH_2CH_3$
**10** R = $OCH(CH_3)_2$
**11** R = $OCH_2CH_2CH_2CH_3$
**12** R = $OCH_2CH(CH_3)_2$

**2**

**13** R = $OCH_2CN$

**1**

**14** R = $OCH_2C_6H_5$
**15** R = $OC(CH_3)_3$

**2**

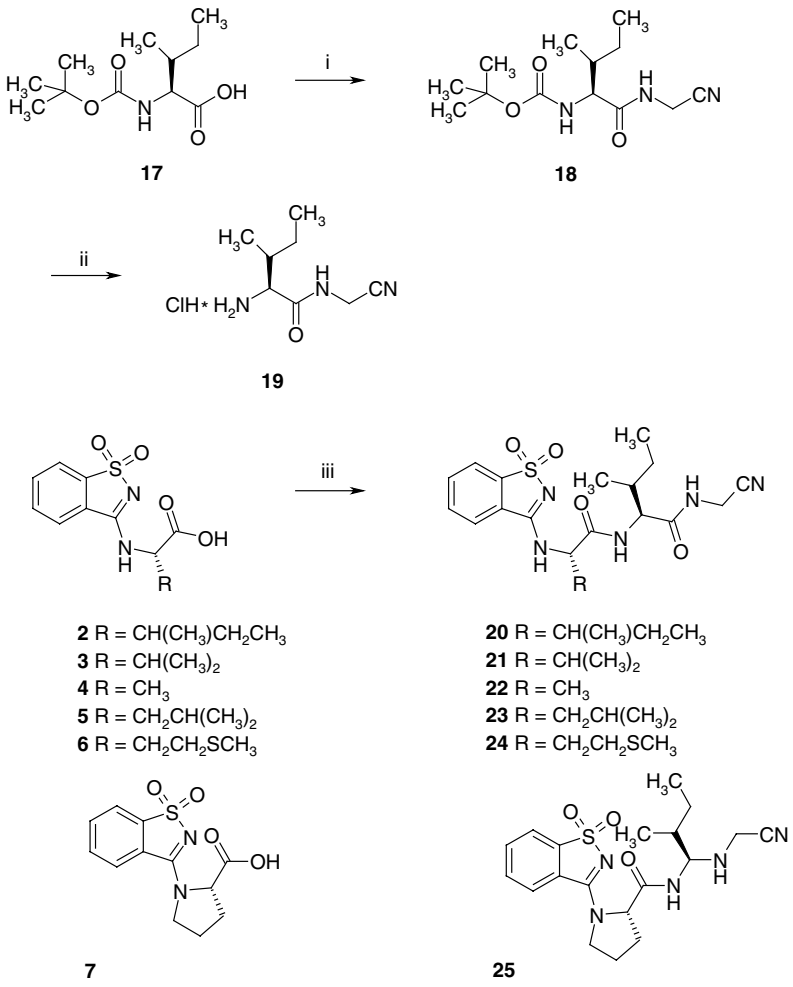
**16** R = $NHCH_2CN$

**Scheme 2.** Synthesis of **9**–**16**. Reagents and conditions: (i) RH, $SOCl_2$, 0 °C to reflux; (ii) aq KOH; (iii) $ClCH_2CN$, heat 80 °C, 30 min; (iv) isoleucine benzyl ester tosylate/(isoleucine *tert*-butyl ester HCl for **15**), triethylamine, dioxan, reflux; (v) isobutyl chloroformate, *N*-methylmorpholine, THF, −25 °C, 30 min, −45 °C, aminoacetonitrile HCl in aq NaOH, overnight.

by a signal at around $\delta$ 113–118 ppm in their $^{13}C$ NMR spectra. The nitrile group of **8** and **13** showed signals at $\delta$ 113.46 and 113.49 ppm in their $^{13}C$ NMR spectra, respectively. The quaternary nature of the nitrile group of **8** and **13** was seen in the DEPT spectra. Compound **25** was isolated as a mixture of rotamers. **16**, **20**, **21**, **23**–**25** were obtained as polymorphic substances.

The compounds **8**–**16** and **20**–**25** were screened against PPE at 0.5 mM concentration (Table 1). Primary screening of **8**–**16** at 0.5 mM and **20**–**25** at 0.125 mM concentration against HLE was also carried out. Compound **10** produced turbidity in the assay medium while testing it against PPE. Compound **15** was tested against HLE and PPE in the presence of 2% cremophore. An experiment was done in order to see the effect of solvent (DMSO) on the enzyme activity. It was observed that there was no change in the enzyme activity when DMSO (15%) was added into the assay medium. Compound **13** was found to be stable in the assay medium during time of measurement.

Compounds **8** and **13** showed a higher inhibition of HLE as compared to PPE inhibition. **8** showed 50% inhibition at 5 μM concentration against HLE and 47% inhibition of PPE at 0.5 mM concentration. Compound **13** showed 69% inhibition of HLE at 5 μM concentrations while a very weak inhibition of PPE (7% at 0.5 mM concentration) was observed. The ester




**Scheme 3.** Synthesis of peptides **20**–**25**. Reagents and conditions: (i) isobutyl chloroformate, N-methylmorpholine, THF, −25 °C, 30 min, −45 °C, amino acetonitrile HCl in aq NaOH, to rt, overnight; (ii) ethyl acetate saturated with HCl gas, rt, 6 h; (iii) isobutyl chloroformate, *N*-methylmorpholine, THF, −25 °C, 30 min, −45 °C, **19**, triethylamine, to rt, overnight.

derivatives **9**–**12** and **14** at the concentration of 0.5 mM showed 87%, 83%, 62%, 75%, and 89% inhibition of HLE, respectively; while the same derivatives at the same concentration showed a very weak inhibition against PPE, ranging from 9 to 12%. Compound **15** containing a *tert*-butyl group did not show any inhibition of HLE as well as of PPE at 0.5 mM concentration (Table 1). In general, the ester derivatives **8**–**14** showed better inhibition of HLE than of PPE. This indicates that these ester derivatives are more specific toward HLE than toward PPE as an elastase inhibitor. Compound **16** showed 12% inhibition of HLE and PPE at 0.5 mM concentration. **16** when tested at a concentration of 0.125 mM against HLE produced only 1% inhibition. The ester derivatives **8**–**14** and an amide derivative **16** showed the reversible inhibition.

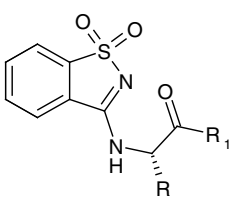
Furthermore, the inhibition against PPE and HLE was checked for the peptides where one more amino acid residue was added to the structure of **16**. It is observed that the peptides **20**–**25** showed a better inhibition of PPE when compared with the ester derivatives. This may be attributed to the fact that these peptidic segments help in noncovalent binding to the enzyme. It is reported that the tripeptidic inhibitors are more active than their nonpeptidic analog.[2] The most likely explanation for the large increase in activity of these peptidic analogs is the ability of peptides to form hydrogen bonds with the enzyme.[2,21] These peptides also showed inhibition against HLE.

The inhibitor constant ($K_i$) was determined for the compounds which showed more than 50% inhibition at 0.5 mM concentration against HLE. Also the $K_i$ values for the peptides **20** and **22** were calculated (Table 1). The $K_i$ was obtained from the Dixon plot using different concentrations of the compound and two substrate concentrations as described by Dixon.[22] The Dixon plot of **8** and **13** is shown in Figures 4 and 5, respectively.

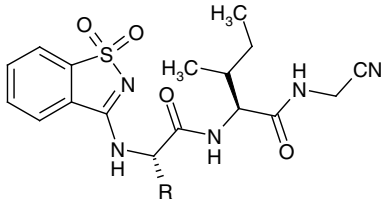
In the Dixon plots, [I] indicates the inhibitor concentration, while [S] denotes the substrate concentration. 1/V is the reverse rate of the biochemical reaction. The typical nature of the Dixon plots in Figures 4 and 5 indicates the competitive reversible inhibition by **8** and **13**.

The $K_i$ of **8** and **13** against HLE was found to be 1.3 μM and 0.8 μM, respectively. These two derivatives are the

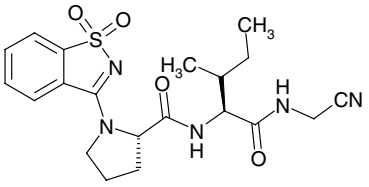
**Table 1.** Inhibition of human leukocyte elastase (HLE) and porcine pancreatic elastase (PPE) by different pseudosaccharinamine derivatives



**8-16**



**20-25**

| Compound | R | $R_1$ | $K_i$ or inhibition against HLE | Inhibition against PPE (%) |
|---|---|---|---|---|
| **8** | $CH(CH_3)_2$ | $OCH_2CN$ | 1.3 μM | 47 |
| **9** | $CH(CH_3)CH_2CH_3$ | $OCH_2CH_3$ | 36 μM | 9 |
| **10** | $CH(CH_3)CH_2CH_3$ | $OCH(CH_3)_2$ | 31 μM | b |
| **11** | $CH(CH_3)CH_2CH_3$ | $OCH_2CH_2CH_2CH_3$ | 36 μM | 9 |
| **12** | $CH(CH_3)CH_2CH_3$ | $OCH_2CH(CH_3)_2$ | 57 μM | 8 |
| **13** | $CH(CH_3)CH_2CH_3$ | $OCH_2CN$ | 0.8 μM | 7 |
| **14** | $CH(CH_3)CH_2CH_3$ | $OCH_2C_6H_5$ | 38 μM | 12[a] |
| **15** | $CH(CH_3)CH_2CH_3$ | $OC(CH_3)_3$ | NI[a] | NI[a] |
| **16** | $CH(CH_3)CH_2CH_3$ | $NHCH_2CN$ | 12% | 12 |
| **20** | $CH(CH_3)CH_2CH_3$ | — | 70 μM | 39 |
| **21** | $CH(CH_3)_2$ | — | 4% at 125 μM | 29 |
| **22** | $CH_3$ | — | 47 μM | 55 |
| **23** | $CH_2CH(CH_3)_2$ | — | 4% at 125 μM | 35 |
| **24** | $CH_2CH_2SCH_3$ | — | 15% at 125 μM | 53 |
| **25** |  | | 13% at 125 μM | 23 |
| ONO-5046 | | | 0.2 μM[16] | — |
| MeOsuc-Ala-Ala-Pro-Val-$CH_2Cl$ (CMK) | | | 10 μM[24] | — |

NI, no inhibition at 0.5 mM concentration; a, measurements were carried out with 2% cremophore; b, turbidity set in during the measurement. % inhibition is reported at 0.5 mM unless otherwise stated.

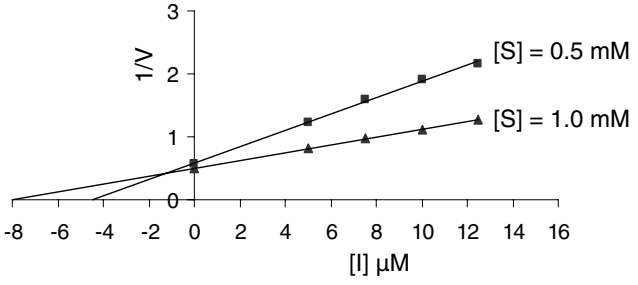



**Figure 4.** Dixon plot showing the inhibition of HLE by compound **8**.

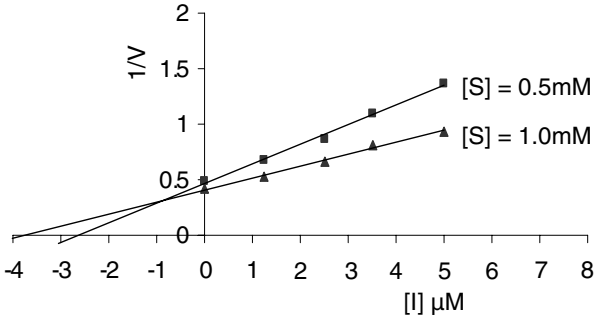



**Figure 5.** Dixon plot showing the inhibition of HLE by compound **13**.

best inhibitors of HLE from all the synthesized compounds. **8** and **13** are the ester derivatives of valine and isoleucine, respectively, and contain the more reactive ester moieties than the other ester derivatives. It is well documented that the valine or alanine fit the $S_1$ pocket of HLE.[2] The presence of isoleucine at the $P_1$ position of inhibitor is also reported.[23] These two ester derivatives also contain the valine and isoleucine moieties. Hence, possibly the valine and isoleucine occupy the $S_1$ pocket of the enzyme. There are different possibilities for these two derivatives to inhibit HLE. The OH of the Ser-195 of the elastase possibly can attack the carbonyl of ester, the sulfonamide linkage, and the CN of the ester side chain in the structure of these derivatives.

Compounds **20** and **22** showed $K_i$ at 70 μM and 47 μM against HLE. Inhibition in the case of **21** and **23** improved very slightly. The specificity shown by the ester derivatives **8**–**14** toward HLE diminished in peptides and the compounds **20**–**25** showed comparable inhibition against HLE and PPE (Table 1). The dipeptides **20**–**25** inhibited PPE and HLE reversibly.

## 3. Conclusion

We have demonstrated that some pseudosaccharinamine derivatives can act as reversible inhibitors of PPE and HLE. Biochemical studies indicated that the ester derivatives **8**–**14** of pseudosaccharinamine are found to be more potent against HLE than against PPE. The dipeptides **20**–**25** showed better inhibition against PPE than the nonpeptidic analogs. Modifications of **8** and **13** should lead to more potent inhibitors of HLE.

## 4. Experimental

### 4.1. General

Mp: PHMK 80/2747 (Küstner, Dresden) apparatus, not corrected. The melting point of polymorphic substances was taken on a PERKIN ELMER Differential Scanning Calorimeter at the Inorganic Chemistry Department of the University of Halle (Saale). IR Spectra: Perkin-Elmer FTIR 1600; $\tilde{\nu}$ in KBr ($cm^{-1}$), if not noted otherwise. NMR Spectra: Bruker DPX 200 (200 MHz) for $^1H$; Bruker DPX 200 (50 MHz) for $^{13}C$; $\delta$ (ppm) rel to TMS as internal standard, spectra in $CDCl_3$, if not noted otherwise. Optical rotation: Polartronic D (Schmidt Haensch GmbH). Elementary analyses: Perkin-Elmer Elemental Analyzer 2400 CHN, Institute of Pharmacy at the University of Greifswald. All results were in an acceptable range. TLC on Merck DC-Alumina plates, Silica Gel 60 $F_{254}$, Nr. 5554. CC with Silica Gel 60 Merck Nr. 7734 or 9385. HPLC with LaChrom apparatus series 7000 Merck Hitachi. Columns: LiChrospher 250-4, RP-18, 5 μm, and LiChroCART 250-4, Chiralcel OJ-R, 5 μm.

PPE (EC 3.4.21.36, ≈200 U/mg) was purchased from Serva, Suc-$(Ala)_3$-pNA and *N*-methoxysuccinyl-$(Ala)_2$-Pro-Val-pNA from Bachem and HLE (EC 3.4.21.37, ≈34 U/mg) from Serva.

### 4.2. Synthesis of compounds

**4.2.1. Cyanomethyl (2*S*)-2-(1,1-dioxobenzo[*d*]isothiazol-3-ylamino)-3-methylbutanoate (8).** Compound **3** (1.00 g, 3.54 mmol) was added into 4.25 ml (4.25 mmol) of 1 N aq KOH and stirred for 10 min at room temperature. The solution was then allowed to dry at room temperature. The obtained dry solid was then refluxed in 5 ml of chloroacetonitrile at 80 °C for 30 min. The reaction mixture was cooled at room temperature and added dropwise into water. The aqueous layer was extracted with 200 ml of ethyl acetate. The organic layer was removed in vacuo and the residue was crystallized from ethyl acetate and petroleum ether. Yield: 0.50 g (44%); mp 142 °C (AcOEt/PE); $R_f = 0.63$ (AcOEt/PE 7:3); $[\alpha]_D^{20}$ −68.00 (*c* 2, MeOH); IR: $\tilde{\nu} = 3290$ (NH), 3096, 3038, 2972 (CH), 1760 (CO), 1612 (C=N), 1375, 1156 ($SO_2$); $^1H$ NMR: $\delta = 7.95$–7.89, 7.82–7.67 (2m, 4 ar H), 6.40 (d, $J = 8.28$ Hz, NH), 4.95 (d, $J = 15.60$ Hz, 1H, $OCH_2$), 4.92 (dd, $J = 8.28$ Hz, $J = 4.90$ Hz, 1H, α-$H_{Val}$), 4.76 (d, $J = 15.60$ Hz, 1H, $OCH_2$), 2.54-2.37 (m, 1H, β-$H_{Val}$), 1.10 and 1.08 (2d, $J = 6.90$ Hz, 6H, γ-$H_{Val}$); $^{13}C$ NMR: $\delta = 169.98$, 159.79, 142.78, 133.77, 132.89, 126.93, 122.37, 120.79, 113.46, 60.36, 49.23, 31.64, 18.63, 18.30; HPLC: $k' = 5.85$, $t_0 = 2.17$ (Chiralcel OJ-R, MeCN/$H_2O$, 2.5:7.5). $C_{14}H_{15}N_3O_4S$ (335.38).

**4.2.2. Ethyl (2*S*,3*S*)-2-(1,1-dioxobenzo[*d*]isothiazol-3-ylamino)-3-methylpentanoate (9).** Compound **2** (0.50 g, 1.68 mmol) was given into 20 ml of ethanol followed by 0.14 ml of thionylchloride (1.86 mmol). The reaction mixture was refluxed for 4 h. The solvent was evaporated in vacuo, and 10 ml of ethanol was added to the residue, then it was concentrated to the half in vacuo. The concentrated solution was left for 12–24 h at room temperature. The formed crystals were separated and washed with petroleum ether. When crystallized from ethyl acetate/petroleum ether fibrous colorless crystals were obtained. Yield: 0.28 g (52%); mp 138 °C (AcOEt/PE); $R_f = 0.63$ (AcOEt/PE 1:1); $[\alpha]_D^{20}$ −28.33 (*c* 2, MeOH); IR: $\tilde{\nu} = 3307$ (NH), 3098, 3050, 2968, 2968, 2935 (CH), 1749 (CO), 1612 (C=N), 1374, 1156 ($SO_2$); $^1H$ NMR: $\delta = 7.92$–7.88, 7.78–7.63 (2m, 4 ar H), 6.70 (d, $J = 7.84$ Hz, NH), 4.87 (dd, $J = 7.84$ Hz, $J = 4.50$ Hz, 1H, α-$H_{Ile}$), 4.305, 4.302, (2dq, $J = 7.10$, $J = 0.74$ Hz, 2H, $OCH_2$), 2.28–2.08 (m, 1H, β-$H_{Ile}$), 1.66–1.49 (m, 2H, γ-$H_{Ile}$), 1.35 (t, $J = 7.10$ Hz, 3H, $CH_3$), 1.03 (d, $J = 7.30$ Hz, 3H, γ-$H_{Ile}$), 0.97 (d, $J = 6.90$ Hz, 3H, δ-$H_{Ile}$); $^{13}C$ NMR: $\delta = 171.51$, 159.28, 142.60, 133.43, 132.87, 127.36, 122.07, 120.76, 62.25, 59.78, 37.97, 25.91, 15.19, 14.25, 11.65; HPLC: $k' = 4.43$, $t_0 = 1.78$ (Chiralcel OJ-R, MeCN/$H_2O$, 3:7). $C_{15}H_{20}N_2O_4S$ (324.40).

**4.2.3. Isopropyl (2*S*,3*S*)-2-(1,1-dioxobenzo[*d*]isothiazol-3-ylamino)-3-methylpentanoate (10).** Compound **2** (0.50 g, 1.68 mmol) and $SOCl_2$ (0.20 ml, 2.66 mmol) were refluxed in 20 ml of isopropanol for 11 h. The mixture was then cooled to room temperature and the solvent was removed in vacuo. The residue was washed with 10 ml of water and recrystallized from ethyl acetate and petrol ether to produce white fibrous crystals. Yield: 0.43 g (75%); mp 120 °C (AcOEt/PE); $R_f = 0.66$ (AcOEt/PE 1:1); $[\alpha]_D^{20}$ −27.50 (*c* 2, MeOH); IR: $\tilde{\nu} = 3298$ (NH), 3098, 3053, 2978, 2935 (CH), 1744 (CO), 1612 (C=N), 1373, 1155 ($SO_2$); $^1H$ NMR: $\delta$ 7.90–7.86, 7.76–7.63 (2m, 4 ar H), 6.83 (d, $J = 7.78$ Hz, NH), 5.15 (spt, $J = 6.30$ Hz, 1H, OCH), 4.83 (dd, $J = 7.78$ Hz, $J = 4.44$ Hz, 1H, α-$H_{Ile}$), 2.26–2.10 (m, 1H, β-H Ile), 1.69-1.45 (m, 2H, γ-$H_{Ile}$), 1.32 (d, $J = 6.30$ Hz, 6H, $CH_3$), 1.04-0.96 (m, 6H [3H, γ-$H_{Ile}$ + 3H, δ-$H_{Ile}$]); $^{13}C$ NMR: $\delta$ 171.05, 159.24, 142.49, 133.34, 132.84, 127.33, 121.97, 120.74, 70.42, 59.79, 37.90, 25.90, 21.79, 21.77. 15.10, 11.60; HPLC: $k' = 6.02$, $t_0 = 1.78$ (Chiralcel OJ-R, MeCN/$H_2O$, 3:7). $C_{16}H_{22}N_2O_4S$ (338.43).

**4.2.4. *n*-Butyl (2*S*,3*S*)-2-(1,1-dioxobenzo[*d*]isothiazol-3-ylamino)-3-methylpentanoate (11).** From **2** (0.50 g, 1.68 mmol) and $SOCl_2$ (0.20 ml, 2.66 mmol) in 20 ml of *n*-butanol, 13 h reflux, as described for **10**. Yield: 0.14 g (24%); mp. 98 °C (AcOEt/PE); $R_f = 0.72$ (AcOEt/PE 1:1); $[\alpha]_D^{20}$ −25.50 (*c* 2, MeOH); IR: $\tilde{\nu} = 3305$ (NH), 3098, 2962, 2934 (CH), 1752 (CO), 1611 (C=N), 1364, 1156 ($SO_2$); $^1H$ NMR: $\delta = 7.90$–7.83, 7.72–7.63 (2m, 4 ar H), 6.86 (d, $J = 7.92$ Hz, NH), 4.88 (dd, $J = 7.92$ Hz, $J = 4.60$ Hz, 1H, α-$H_{Ile}$), 4.24 (t, $J = 6.60$ Hz, 2H,

$OCH_2$), 2.27–2.07 (m, 1H, β-$H_{Ile}$), 1.76–1.26 (m, 6H, $2CH_2$ + 2γ-$H_{Ile}$), 1.04–0.93 (m, 9H, $CH_3$ + 3γ-$H_{Ile}$ + 3δ-$H_{Ile}$); $^{13}$C NMR: $\delta$ = 171.71, 159.31, 142.48, 133.37, 132.87, 127.29, 121.97, 120.76, 66.07, 59.82, 37.88, 30.47, 25.84, 19.07, 15.19, 13.56, 11.57; HPLC: $k'$ = 11.13, $t_0$ = 1.78 (Chiralcel OJ-R, MeCN/$H_2O$, 3:7). $C_{17}H_{24}N_2O_4S$ (352.46).

**4.2.5. Isobutyl (2*S*,3*S*)-2-(1,1-dioxobenzo[*d*]isothiazol-3-ylamino)-3-methylpentanoate (12).** From **2** (0.50 g, 1.68 mmol) and $SOCl_2$ (0.20 ml, 2.66 mmol) in 20 ml of isobutanol, 13 h reflux, as described for **10**. Yield: 0.42 g (70%); mp 118–120 °C; $R_f$ = 0.75 (AcOEt/PE 1:1); $[\alpha]_D^{20}$ −26.00 (*c* 2, MeOH); IR: $\tilde{\nu}$ = 3310 (NH), 3098, 2965 (CH), 1752 (CO), 1612 (C=N), 1367, 1157 ($SO_2$); $^1$H NMR: $\delta$ = 7.89-7.80, 7.74-7.63 (2m, 4 ar H), 7.00 (d, *J* = 8.00 Hz, NH), 4.90 (dd, *J* = 8.00 Hz, *J* = 4.80 Hz, 1H, α-$H_{Ile}$), 4.11–3.93 (m, 2H, $OCH_2$), 2.23–1.92 (m, 2H, CH + β-$H_{Ile}$), 1.69–1.25 (m, 2H, γ-$H_{Ile}$), 1.05–0.97 (m, 12 H, 2 $CH_3$ + 3 γ-$H_{Ile}$ + 3 δ-$H_{Ile}$); $^{13}$C NMR: $\delta$ = 171.35, 159.04, 142.68, 133.47, 132.75, 127.38, 122.20, 120.62, 72.35, 59.64, 38.07, 27.68, 25.92, 19.10, 15.22, 11.69; HPLC: $k'$ = 10.32, $t_0$ = 1.78 (Chiralcel OJ-R, MeCN/$H_2O$, 3:7). $C_{17}H_{24}N_2O_4S$ (352.46).

**4.2.6. Cyanomethyl (2*S*,3*S*)-2-(1,1-dioxobenzo[*d*]isothiazol-3-ylamino)-3-methylpentanoate (13).** Compound **2** (0.50 g, 1.68 mmol) was given into 1.68 ml (3.36 mmol) of 2 N aq KOH and stirred for 10 min at room temperature. The solution was then evaporated at room temperature, and the residue was then refluxed in 2 ml of chloroacetonitrile at 80 °C for 30 min. The mixture was cooled to room temperature and added dropwise into water. The aqueous layer was extracted with 100 ml of ethyl acetate. The organic layer was removed in vacuo and the residue was crystallized from ethyl acetate and petroleum ether. Yield: 0.24 g (42%); mp 172 °C (AcOEt/PE); $R_f$ = 0.47 (AcOEt/PE 7:3); $[\alpha]_D^{20}$ −60.00 (*c* 2, MeOH); IR: $\tilde{\nu}$ = 3329 (NH), 3097, 3038, 2970, 2932 (CH), 1769 (CO), 1610 (C=N), 1376, 1161 ($SO_2$); $^1$H NMR: $\delta$ = 7.94–7.88, 7.81–7.69 (2m, 4 ar H), 6.53 (d, *J* = 8.00 Hz, NH), 4.95 (dd, *J* = 8.00 Hz, *J* = 3.2 Hz, 1H, α-$H_{Ile}$), 4.94 (d, *J* = 15.60 Hz, 1H, $OCH_2$), 4.76 (d, *J* = 15.60 Hz, 1H, $OCH_2$), 2.26–2.08 (m, 1H, β-$H_{Ile}$), 1.69-s-1.25 (m, 2H, γ-$H_{Ile}$), 1.05–0.97 (m, 6H, 3 γ-$H_{Ile}$ + 3 δ-$H_{Ile}$); $^{13}$C NMR: $\delta$ = 169.90, 159.61, 142.64, 133.69, 132.88, 126.95, 122.24. 120.94, 113.49, 59.52, 49.14, 38.01, 25.69, 15.25, 11.44; HPLC: $k'$ = 3.25, $t_0$ = 1.78 (Chiralcel OJ-R, MeCN/$H_2O$, 3:7). $C_{15}H_{17}N_3O_4S$ (335.38).

**4.2.7. Benzyl (2*S*,3*S*)-2-(1,1-dioxobenzo[*d*]isothiazol-3-ylamino)-3-methylpentanoate (14).** 3.90 g of isoleucine benzyl ester tosylate (9.92 mmol) and triethylamine (2.76 ml, 19.84 mmol) was added to 40 ml of dioxan. The mixture was stirred at room temperature for 10 min. **1** (2.00 g, 9.92 mmol) was then added and the mixture was refluxed for 2.5 h. Dioxan was removed in vacuo and after addition of water the mixture was extracted with (100 ml × 3) ethyl acetate. The organic layer was dried ($Na_2SO_4$), evaporated, and the residue was crystallized. Yield: 1.90 g (50%); mp 125–127 °C (AcOEt/PE); $R_f$ = 0.69 (AcOEt/PE 1:1); $[\alpha]_D^{20}$ −35.00 (*c* 2, MeOH); IR: $\tilde{\nu}$ = 3320 (NH), 3094, 3069, 3030, 2964, 2932 (CH), 1738 (CO), 1608 (C=N), 1366, 1156 ($SO_2$); $^1$H NMR: $\delta$ = 7.78–7.81, 7.74–7.57 (2m, 4 ar H), 6.85 (d, *J* = 8.00 Hz, NH), 5.30 (d, *J* = 12.00 Hz, 1H, $OCH_2$), 5.23 (d, *J* = 12.00 Hz, 1H, $OCH_2$), 4.95 (dd, *J* = 8.00 Hz, *J* = 4.82 Hz, 1H, α-$H_{Ile}$), 2.25-2.08 (m, 1H, β-$H_{Ile}$), 1.63–1.19 (m, 2 γ-$H_{Ile}$), 0.99–0.92(m, 6H, 3 γ-$H_{Ile}$ + 3δ-$H_{Ile}$); $^{13}$C NMR: $\delta$ = 171.24, 159.19, 142.67, 134.69, 133.46, 132.78, 128.84, 128.77, 128.60, 128.52, 127.30, 126.02, 122.16, 120.65, 67.93, 59.74, 38.08, 25.75, 15.19, 11.60; HPLC: $k'$ = 7.40, $t_0$ = 1.85 (Chiralcel OJ-R, MeCN/$H_2O$, 4:6). $C_{20}H_{22}N_2O_4S$ (386.47).

**4.2.8. *Tert*-Butyl (2*S*,3*S*)-2-(1,1-dioxobenzo[*d*]isothiazol-3-ylamino)-3-methylpentanoate (15).** 2.20 g of isoleucine *tert*-butyl ester HCl (9.92 mmol) and 2.76 ml of triethylamine (19.84 mmol) were added to 40 ml of dioxan and stirred at room temperature for 10 min. **1** (2.00 g, 9.92 mmol) was added and the mixture was refluxed for 2.5 h. The solvent was removed in vacuo and the residue was triturated with 20 ml of water and extracted by ethyl acetate (100 ml × 3). The organic layer was dried ($Na_2SO_4$), evaporated and the residue yielded on scratching with *n*-hexane a solid product. Yield: 2.30 g (66%); mp.118 °C; $R_f$ = 0.72 (AcOEt/PE 1:1); $[\alpha]_D^{20}$ −25.00 (*c* 2, MeOH); IR: $\tilde{\nu}$ = 3314 (NH), 3094, 2973, 2933 (CH), 1747 (CO), 1612 (C=N), 1367, 1156 ($SO_2$); $^1$H NMR: $\delta$ = 7.87–7.78, 7.72–7.58 (2m, 4 ar H), 7.04 (d, *J* = 7.70 Hz, NH), 4.74 (dd, *J* = 7.70 Hz, *J* = 4.86 Hz, 1H, α-$H_{Ile}$), 2.23–2.03 (m, 1H, β-$H_{Ile}$), 1.71–1.21 (m, 11H, 3 $CH_3$ + 2γ-$H_{Ile}$), 1.04–0.96 (m, 6H, 3γ-$H_{Ile}$ + 3 δ-$H_{Ile}$); $^{13}$C NMR: $\delta$ = 170.62, 159.00, 142.54, 133.31, 132.79, 127.44, 122.01, 120.62, 83.80, 59.95, 37.91, 28.08, 26.12, 15.04, 11.63; HPLC: $k'$ = 8.19 $t_0$ = 1.78 (Chiralcel OJ-R, MeCN/$H_2O$, 3:7). $C_{17}H_{24}N_2O_4S$ (352.46).

**4.2.9. *N*-(Cyanomethyl) (2*S*,3*S*)-2-(1,1-dioxobenzo[*d*]isothiazol-3-ylamino)-3-methylpentanamide (16).** Compound **2** (2.00 g, 6.75 mmol) was dissolved in 40 ml THF, cooled to −25 °C, and stirred for 10 min. Then *N*-methylmorpholine (0.74 ml, 6.75 mmol) and 0.88 ml of isobutyl chloroformate (6.75 mmol) were added under nitrogen. The mixture was stirred for 0.5 h at −25 °C, then cooled to −45 °C, and then a solution of aminoacetonitrile, prepared by mixing aminoacetonitrile HCl (0.625 g, 6.75 mmol) in 1.68 ml of 2 N NaOH (6.75 mmol), was added. The mixture was stirred for 1 h at −45 °C and then left overnight at rt. Then the mixture was filtered, and the filtrate was diluted with ethyl acetate. The organic layer was separated, washed with 1 N HCl followed by a saturated solution of $NaHCO_3$, brine, and in the last with water. The organic layer was dried ($Na_2SO_4$), evaporated in vacuo, and the residue was purified by CC (EtOH/petroleum ether 10%, with increasing polarity, till 80%). Yield: 0.76 g (34%); mp 30–80, 110-125, 155-165, 220 °C; $R_f$ = 0.58 (MeOH/$CH_2Cl_2$ 1:9); $[\alpha]_D^{20}$ −70.50 (*c* 2, MeOH); IR: $\tilde{\nu}$ = 3331 (NH), 3060, 2966 (CH), 1669 (CO), 1617 (C=N), 1354, 1163 ($SO_2$); $^1$H NMR: $\delta$ = 8.45 (d, *J* = 8.00 Hz, NH), 8.04 (t, *J* = 5.96 Hz, NH), 7.76–7.51 (m, 4 ar H), 4.56 (t, *J* = 8.00 Hz, 1H, α-$H_{Ile}$), 4.19–4.07 (m, 2H, $CH_2$), 2.27–2.08 (m, 1H, β-$H_{Ile}$), 1.41–1.17 (m, 2H, γ-$H_{Ile}$), 1.05–0.88 (m, 6H, 3γ-$H_{Ile}$ + 3δ-

$H_{Ile}$); $^{13}C$ NMR: $\delta$ = 172.89, 160.48, 141.50, 133.92, 133.25, 127.26, 121.77, 121.52, 115.60, 62.02, 36.68, 27.88, 25.69, 15.12, 10.97; HPLC: $k'$ = 0.49, $t_0$ = 1.94 (Chiralcel OJ-R, MeCN/$H_2O$, 1:1). $C_{15}H_{18}N_4O_3S$ (334.40).

**4.2.10. (2*S*,3*S*)-2-(1,1-Dioxobenzo[*d*]isothiazol-3-ylamino)-3-methylpentanoic acid [(1S,2S)-1-(cyanomethylcarbamoyl)-2-methylbutyl]amide (20).** From **2** (0.63 g, 2.13 mmol), 30 ml THF, N-methylmorpholine (0.23 ml, 2.13 mmol), isobutyl chloroformate (0.28 ml, 2.13 mmol), **19** (0.44 g, 2.13 mmol) in 10 ml THF, and triethylamine (0.33 ml, 2.34 mmol), as described for **16**. Yield: 0.48 g (51%); mp 200–210, 220–230, 250–260 °C; $R_f$ = 0.55 (MeOH/$CH_2Cl_2$ 1:9); $[\alpha]_D^{20}$ −106.50 (*c* 2, MeOH); IR: $\tilde{\nu}$ = 3305 (NH), 3065, 2966, 2934 (CH), 1681 (CO), 1616 (C═N), 1367, 1164 ($SO_2$); $^1H$ NMR (DMSO-$d_6$): $\delta$ 9.41 (d, $J$ = 8.11 Hz, NH), 8.74 (t, $J$ = 5.50 Hz, NH), 8.52–8.35 (m, 2H [1H, ar H + NH]), 8.06–7.92, 7.88–7.78 (2m, 3H, ar H), 4.47 (t, $J$ = 8.85 Hz, 1H, α-H, $Ile_1$), 4.14 (d, $J$ = 5.50 Hz, 2H, $CH_2$), 4.25–4.06 (m, 1H, α-H, $Ile_2$), 2.16–1.91 (m, 1H β-H, $Ile_1$), 1.85–1.66 (m, 1H, β-H, $Ile_2$), 1.62–1.41 (m, 2H, γ-H, $Ile_1$), 1.27–1.00 (m, 2H, γ-H, $Ile_2$), 0.95–0.72 (m, 12H [3H, γ-H, $Ile_1$ + 3H, δ-H, $Ile_1$ + 3H, γ-H, $Ile_2$ + 3H, δ-H, $Ile_2$]); $^{13}C$ NMR (DMSO-$d_6$): $\delta$ 172.00, 170.37, 159.86, 142.81, 134.02, 133.58, 127.93, 124.11, 121.69, 117.83, 61.38, 57.36, 36.77, 36.03, 27.33, 25.49, 24.94, 15.65, 15.46, 11.22, 10.85; HPLC: $k'$ = 3.89, $t_0$ = 2.17 (Chiralcel OJ-R, MeCN/$H_2O$, 2.5:7.5). $C_{21}H_{29}N_5O_4S$ (447.56).

**4.2.11. 2-[(2*S*)-2-(1,1-Dioxobenzo[*d*]isothiazol-3-ylamino)-3-methyl-butyrylamino]-(2*S*,3*S*)-3-methylpentanoic acid cyanomethylamide (21).** From **3** (0.63 g, 2.23 mmol), 30 ml THF, *N*-methylmorpholine (0.25 ml, 2.23 mmol), isobutyl chloroformate (0.30 ml, 2.23 mmol), **19** (0.46 g, 2.23 mmol) in 10 ml THF, and triethylamine (0.34 ml, 2.45 mmol), as described for **16**. Yield: 0.30 g (31%); mp 135–145, 170–180, 220–245 °C; $R_f$ = 0.48 (MeOH/$CH_2Cl_2$ 1:9); $[\alpha]_D^{20}$ −90.00 (*c* 2, MeOH); IR: $\tilde{\nu}$ = 3315 (NH), 3092, 2967, 2935 (CH), 1654 (CO), 1615 (C═N), 1374, 1163 ($SO_2$); $^1H$ NMR (DMSO-$d_6$): $\delta$ = 9.37 (d, $J$ = 8.52 Hz, NH), 8.75 (t, $J$ = 5.70 Hz, NH), 8.49-8.44, 7.98–7.94, 7.83-7.80 (3m, 4H ar H), 8.38 (d, $J$ = 8.70 Hz, NH), 4.41 (t, $J$ = 8.52 Hz, 1H, α-$H_{Val}$), 4.13 (d, $J$ = 5.70 Hz 2H, $CH_2$), 4.19–4.07 (m, 1H, α-$H_{Ile}$), 2.25–2.09 (m, 1H, β-$H_{Val}$), 1.80–1.64 (m, 1H, β-$H_{Ile}$), 1.21–1.10 (m, 2H, γ-$H_{Ile}$), 0.96–0.74 (m, 12H, 6 γ-$H_{Val}$ + 3γ-$H_{Ile}$ + 3δ-$H_{Ile}$); $^{13}C$ NMR (DMSO-$d_6$): $\delta$ = 171.44, 169.63, 159.40, 142.20, 133.43, 133.00, 127.34, 121.09, 117.24, 62.28, 56.75, 36.19, 29.90, 26.73, 24.35, 20.66, 19.15, 18.83, 15.05, 13.98; HPLC: $k'$ = 2.43, $t_0$ = 2.17 (Chiralcel OJ-R, MeCN/$H_2O$, 2.5:7.5). $C_{20}H_{27}N_5O_4S$ (433.53).

**4.2.12. 2-[(2*S*)-2-(1,1-Dioxobenzo[*d*]isothiazol-3-ylamino)propionylamino]-(2*S*,3*S*)-3-methylpentanoic acid cyanomethylamide (22).** From **4** (0.63 g, 2.48 mmol), 30 ml THF, N-methylmorpholine (0.27 ml, 2.48 mmol), isobutyl chloroformate (0.32 ml, 2.48 mmol), **19** (0.50 g, 2.48 mmol) in 10 ml THF, and triethylamine (0.38 ml, 2.73 mmol), as described for **16**. Yield: 0.17 g (17%); mr. (polymorphic substance), 163, 263 °C; $R_f$ = 0.44 (MeOH/$CH_2Cl_2$ 1:9); $[\alpha]_D^{20}$ +6.66 (*c* 2, DMSO); IR: $\tilde{\nu}$ = 3301 (NH), 3111, 3063, 2964, 2936 (CH), 1651 (CO), 1618 (C═N), 1354, 1150 ($SO_2$); $^1H$ NMR (DMSO-$d_6$): $\delta$ = 9.50 (d, $J$ = 7.52 Hz, NH), 8.76 (t, $J$ = 5.42 Hz, NH), 8.44-8.28 (m, 2H, 1 ar H + NH), 8.05–7.93, 7.90–7.78 (2m, 3H, ar H), 4.70 (qn, $J$ = 7.27 Hz, 1H, α-$H_{Ala}$), 4.27–4.06 (m, 3H, 1 α-$H_{Ile}$ + $CH_2$), 1.86–1.61 (m, 1H, β-$H_{Ile}$), 1.56–1.32 (m, 2H, γ-$H_{Ile}$), 1.40 (d, $J$ = 7.14, 3H, β-$H_{Ala}$), 1.27–1.01 (m, 2H, γ-$H_{Ile}$) 0.89–0.74 (m, 6H, 3 γ-$H_{Ile}$ + 3δ-$H_{Ile}$); $^{13}C$ NMR (DMSO-$d_6$): $\delta$ = 172.19, 171.52, 159.45, 142.72, 134.02, 133.59, 128.01, 123.95, 121.70, 117.91, 57.31, 52.35, 37.14, 27.39, 24.89, 18.15, 15.73, 11.36; HPLC: $k'$ = 1.31, $t_0$ = 2.17 (Chiralcel OJ-R, MeCN/$H_2O$, 2.5:7.5). $C_{18}H_{23}N_5O_4S$ (405.48).

**4.2.13. (2*S*)-2-(1,1-Dioxobenzo[*d*]isothiazol-3-ylamino)-4-methylpentanoic acid [(1*S*,2*S*)-1-(cyanomethylcarbamoyl)-2-methylbutyl]-amide (23).** From **5** (0.63 g, 2.13 mmol), 30 ml THF, N-methylmorpholine (0.23 ml, 2.13 mmol), isobutyl chloroformate (0.28 ml, 2.13 mmol), **19** (0.44 g, 2.13 mmol) in 10 ml THF, and triethylamine (0.33 ml, 2.34 mmol), as described for **16**. Yield: 0.32 g (34%); mp (polymorphic substance), 165, 225 °C; $R_f$ = 0.48 (MeOH/$CH_2Cl_2$ 1:9); $[\alpha]_D^{20}$ −66.00 (*c* 2, MeOH); IR: $\tilde{\nu}$ = 3306 (NH), 3065, 2963, 2935 (CH), 1654 (CO), 1616 (C═N), 1387, 1162 ($SO_2$); $^1H$ NMR (DMSO-$d_6$): $\delta$ = 9.47 (d, $J$ = 7.90 Hz, NH), 8.76 (t, $J$ = 5.50 Hz, NH), 8.44 (d, $J$ = 8.76 Hz, NH), 8.41–8.30, 8.04–7.93, 7.89–7.79 (3m, 4H, ar H), 4.81–4.62 (m, 1H, α-$H_{Leu}$), 4.30–4.10 (m, 1H, α-$H_{Ile}$) 4.15 (d, $J$ = 5.50 Hz, 2H, $CH_2$), 1.89–1.42 (m, 6H, 2 β-$H_{Leu}$ + 1 γ-$H_{Leu}$ + 1β-$H_{Ile}$ +2 γ-$H_{Ile}$), 0.98–0.75 (m, 12H, 6δ-$H_{Leu}$ + 3γ-$H_{Ile}$ + 3δ-$H_{Ile}$); $^{13}C$ NMR (DMSO-$d_6$): $\delta$ = 172.15, 171.33, 159.83, 142.75, 134.04, 133.62, 127.99, 123.96, 121.71, 117.88, 65.40, 57.41, 55.48, 36.97, 27.37, 24.93, 23.56, 21.76, 15.68, 14.57, 11.30; HPLC: $k'$ = 4.41, $t_0$ = 2.17 (Chiralcel OJ-R, MeCN/$H_2O$, 2.5:7.5). $C_{20}H_{27}N_5O_4S$ (433.53).

**4.2.14. 2-[(2*S*)-2-(1,1-Dioxobenzo[*d*]isothiazol-3-ylamino)-4-methylsulfanyl-butyrylamino]-(2*S*,3*S*)-3-methylpentanoic acid cyanomethylamide (24).** From **6** (0.63 g, 2.00 mmol), 30 ml THF, N-methylmorpholine (0.22 ml, 2.00 mmol), isobutyl chloroformate (0.26 ml, 2.00 mmol), **19** (0.41 g, 2.00 mmol) in 10 ml THF, and triethylamine (0.31 ml, 2.20 mmol), as described for **16**. Yield: 0.12 g (13%); mp (polymorphic substance), 70, 176 °C; $R_f$ = 0.51 (MeOH/$CH_2Cl_2$ 1:9); $[\alpha]_D^{20}$ −57.50 (*c* 2, MeOH); IR: $\tilde{\nu}$ = 3318 (NH), 3060, 2965, 2931 (CH), 1655 (CO), 1616 (C═N), 1164 ($SO_2$); $^1H$ NMR (DMSO-$d_6$): $\delta$ = 9.49 (d, $J$ = 7.60 Hz, NH), 8.78 (t, $J$ = 5.50 Hz, NH), 8.40 (d, $J$ = 8.40 Hz, NH), 8.39-8.29, 8.05–7.94, 7.91–7.79 (3m, 4H, ar H), 4.70 (q, $J$ = 7.56 Hz, 1H, α-$H_{Met}$), 4.27–4.04 (m, 1H, α-$H_{Ile}$), 4.15 (d, $J$ = 5.50 Hz, 2H, $CH_2$), 2.75–2.41 (m, 2H, γ-$H_{Met}$, under DMSO-$d_6$), 2.13–1.99 (m, 5H, $SCH_{3(Met)}$ + 2β-$H_{Met}$), 1.87–1.66 (m, 1H, β-$H_{Ile}$), 1.56–1.05 (m, 2H, γ-$H_{Ile}$), 0.91-0.69 (m, 6H, 3γ-$H_{Ile}$ + 3δ-$H_{Ile}$); $^{13}C$ NMR (DMSO-$d_6$): $\delta$ = 171.56, 169.87, 159.39, 142.08, 133.48, 133.03, 127.35, 123.41, 121.12, 117.29, 56.83, 55.77, 38.19, 36.40, 31.26, 29.69, 26.79, 24.31, 15.12, 14.63, 10.73; HPLC: $k'$ = 3.54, $t_0$ = 2.17 (Chiralcel OJ-R, MeCN/$H_2O$, 2.5:7.5). $C_{20}H_{27}N_5O_4S_2$ (465.60).

**4.2.15. (2*S*)-1-(1,1-Dioxobenzo[*d*]isothiazol-3-yl)-pyrrolidine-2-carboxylic acid [(1*S*,2*S*)-1-(cyanomethylcarbamoyl)-2-methylbutyl]amide (25).** From **7** (0.63 g, 2.25 mmol), 30 ml THF, N-methylmorpholine (0.25 ml, 2.25 mmol), isobutyl chloroformate (0.30 ml, 2.25 mmol), **19** (0.46 g, 2.25 mmol) in 10 ml THF, and triethylamine (0.34 ml, 2.47 mmol), as described for **16**. Yield: 0.05 g (5.15%); mp. (polymorphic substance), 220, 287 °C; $R_f = 0.58$ ($MeOH/CH_2Cl_2$ 1:9); $[\alpha]_D^{20}$ −97.00 (*c* 2, MeOH); IR: $\tilde{\nu} = 3322$ (NH), 3071, 2965, 2934 (CH), 1681 (CO), 1602 (C=N), 1344, 1162 ($SO_2$); $^1H$ NMR (DMSO-$d_6$): $\delta = 8.80$ (t, $J = 5.70$ Hz, part of NH), 8.70 (d, $J = 8.72$ Hz, part of NH), 8.69 (t, $J = 5.63$ Hz, part of NH), 8.35 (d, $J = 8.51$ Hz, part of NH), 8.26–8.13, 8.09–7.96, 7.92–7.76, 7.74–7.62 (4m, 4H, ar H), 5.43–5.27 (m, 1H, $\alpha$-$H_{Pro}$), 4.96–4.74 (m, 1H, $\alpha$-$H_{Ile}$), 4.34–4.08 (m, 4H, $CH_2$ + 2$\delta$-$H_{Pro}$), 2.36–2.04 (m, 3H, 2 $\beta$-$H_{Pro}$ + 1$\gamma$-$H_{Pro}$), 2.01–1.71 (m, 2H, $\gamma$-$H_{Pro}$ + $\beta$-$H_{Ile}$), 1.29–1.04 (m, 2H, $\gamma$-$H_{Ile}$), 0.99–0.73 (m, 6H, 3$\gamma$-$H_{Ile}$ + 3$\delta$-$H_{Ile}$); $^{13}C$ NMR (DMSO-$d_6$): $\delta$ = 171.64, 171.40, 170.30, 170.03, 158.29, 157.86, 143.62, 143.47, 133.40, 133.20, 133.03, 132.55, 127.45, 127.20, 126.46, 124.84, 121.92, 121.83, 117.31, 117.23, 67.65, 64.41, 63.71, 61.30, 59.65, 56.66, 52.55, 49.98, 36.59, 36.20, 32.65, 30.58, 29.03, 26.83, 24.63, 24.30, 15.22, 13.99, 10.77, 10.57 HPLC: $k' = 1.44$, $t_0 = 2.17$ (Chiralcel OJ-R, $MeCN/H_2O$, 2.5:7.5). $C_{20}H_{25}N_5O_4S$ (431.52).

### 4.3. Elastase inhibition studies

**4.3.1. Determination of $K_i$.** To a thermostatted solution of 750 μL of 0.1 M Hepes buffer containing 0.5 M NaCl, pH 7.5, were added DMSO (100 μL), HLE (100 μL solution in 0.05 M sodium acetate buffer containing 0.4 M NaCl, pH 5.5, for a final enzyme concentration of 0.034 μM) and, finally, 50 μL methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide in DMSO for a final substrate concentration of 1 mM. The rate of substrate hydrolysis was determined by monitoring the absorption at 410 nm for 5 min. The experiment was repeated in the presence of varying amounts of the inhibitor (**9**–**12**, **14** for final concentration: 12.5, 25, and 37.5 μM; **13** for final concentration: 1.25, 2.5, 3.5, and 5 μM; **8** for final concentration: 5, 7.5, 10, and 12.5 μM; **20**, **22** for final concentration of 31.25, 62.50, and 93.75 μM) and at a constant final concentration of DMSO (15%) and the rates of substrate hydrolysis were determined. The series of experiments were repeated at one additional substrate concentration (final substrate concentration: 0.5 mM). All rates were determined in triplicate. The inverse of the average velocities is plotted against the final inhibitor concentration and $K_i$ determined from the intersection of two lines (each $R^2 > 0.98$).

**4.3.2. Determination of % inhibition.** Percent inhibition against HLE: to a thermostatted solution of 800 μL 0.1 M Hepes buffer containing 0.5 M NaCl, pH 7.5, was added DMSO (125 μL), HLE (50 μL solution in 0.05 M sodium acetate buffer containing 0.4 M NaCl, pH 5.5, for a final enzyme concentration of 17 nM) and, finally, 25 μL methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide in DMSO for a final substrate concentration of 0.5 mM. The rate of substrate hydrolysis was determined by monitoring the absorption at 410 nm for 20 min. The experiment was repeated in the presence of 0.5 mM concentration of compounds **9**–**12**, **14**, **15** and 0.125 mM concentrations of **20**–**25**. The percent inhibition of HLE by **8** and **13** was measured at 5 μM concentration. The inhibition by **8** and **13** was very high at 0.5 mM concentration and was not possible to measure.

Percent inhibition against PPE: To a thermostatted solution of 820 μL Tris buffer, pH 8 (25 ml 0.1 M Tris buffer + 13.4 ml 0.1 M HCl) were added DMSO (100 μL), PPE (30 μL solution in 1 mM acetic acid, for a final enzyme concentration of 28.9 nM), and finally, 50 μL Suc-ala-ala-ala-*p*-nitroanilide in DMSO for a final substrate concentration of 0.78 mM. The rate of substrate hydrolysis was determined by monitoring the absorption at 410 nm for 20 min. The experiment was repeated in the presence of different compounds at a concentration of 0.5 mM.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2005.11.057.

## References and notes

1. Warner, P.; Green, R. C.; Gomes, B.; Strimpler, A. M. *J. Med. Chem.* **1994**, *37*, 3090–3099.
2. Edwards, P. D.; Bernstein, P. R. *Med. Res. Rev.* **1994**, *14*(2), 127–194.
3. Ohbayashi, H. *Expert Opin. Ther. Patents* **2002**, *12*(1), 65–84.
4. Peet, N.; Burkhart, J. P.; Angelastro, M. R.; Giroux, E. L.; Mehdi, S.; Bey, P.; Kolb, M.; Neises, B.; Schirlin, D. *J. Med. Chem.* **1990**, *33*, 394–407.
5. Damewood, J. R., Jr; Edwards, P. D.; Feeney, S.; Gomes, B. C.; Steelman, G. B.; Tuthil, P. A.; Williams, J. C.; Warner, P.; Woolson, S. A.; Wolanin, D. J.; Veale, C. A. *J. Med. Chem.* **1994**, *37*, 3303–3312.
6. Bernstein, P. R.; Andisik, D.; Bradley, P. K.; Bryant, C. B.; Ceccarelli, C.; DamewoodJr, J. R.; Earley, R.; Edwards, P. D.; Fenny, S.; Gomes, B. C.; Kosmider, B. J.; Steelman, G. B.; Thomas, R. M.; Vacek, E. P.; Veale, C. A.; Williams, J. C.; Wolanin, D. J.; Woolson, S. A. *J. Med. Chem.* **1994**, *37*, 3313–3326.
7. Angelastro, M. R.; Baugh, L. E.; Bey, P.; Burkhart, J. P.; Chen, T.; Durham, S. L.; Hare, C. M.; Huber, E. W.; Janusz, M. J.; Koehl, J. R.; Marquart, A. L.; Mehdi, S.; Peet, N. P. *J. Med. Chem.* **1994**, *37*, 4538–4554.
8. Veale, C. A.; Bernstein, P. R.; Bryant, C.; Ceccarelli, C.; Damewood, J. R., Jr.; Earley, R.; Feeney, S. W.; Gomes,

B.; Kosmider, B. J.; Steelman, G. B.; Thomas, R. M.; Vacek, E. P.; Williams, J. C.; Wolanin, D. J.; Woolson, S. *J. Med. Chem.* **1995**, *38*, 98–108.
9. Portevin, B.; Lonchampt, M.; Canet, E.; Nanteuil, G. *J. Med. Chem.* **1997**, *40*, 1906–1918.
10. Imaki, K.; Okada, T.; Nakayama, Y.; Nagao, Y.; Kobayashi, K.; Sakai, Y.; Mohri, T.; Amino, T.; Nakai, H.; Kawamura, M. *Bioorg. Med. Chem.* **1996**, *4*, 2115–2134.
11. Nakayama, Y.; Odagaki, Y.; Fujita, S.; Matsuoka, S.; Hamanaka, N.; Nakai, H.; Toda, M. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2349–2353.
12. Rizzi, M.; Casale, E.; Ascenzi, P.; Fasano, M.; Aime, S.; La Rosa, C.; Luisetti, M.; Bolognesi, M. *J. Chem. Soc. Perkin Trans. 2* **1993**, *11*, 2253–2256.
13. Hlasta, D.; Subramanyam, C.; Bell, M. R.; Carabateas, P. M.; Court, J. J.; Desai, R. C.; Drozd, M. L.; Eickhoff, W. M.; Ferguson, E. W.; Gordon, R. J.; Johnson, J. A.; Kumar, V.; Maycock, A. L.; Mueller, K. R.; Pagani, E. D.; Robinson, D. T.; Saindane, M. T.; Silver, P. J.; Subramanian, S. *J. Med. Chem.* **1995**, *38*, 739–744.
14. Desai, R. C.; Court, J. C.; Ferguson, E.; Gordon, R. J.; Hlasta, D. J. *J. Med. Chem.* **1995**, *38*, 1571–1574.
15. Groutas, W. C.; Epp, J. B.; Venkataraman, R.; Kunag, R.; Truong, T. M.; McClenahan, J. J.; Prakash, O. *Bioorg. Med. Chem.* **1996**, *4*, 1393–1400.
16. Kawabata, K.; Suzuki, M.; Sugitani, M.; Imaki, K.; Toda, M.; Miyamoto, T. *Biochem. Biophysic. Res. Commun.* **1991**, *177*, 814–820.
17. Kinoshita, Y.; Nakanishi, I.; Sato, A.; Tada, T. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 21–24.
18. Sinha, S.; Watorek, W.; Karr, S.; Giles, J.; Bode, W.; Travis, J. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 2228–2232.
19. Barret, A. J. In *Methods in Enzymology*; Lorand, L., Ed.; Academic Press: New York, 1981; Vol. 80, pp 581–588.
20. Rode, H. B.; Sprang, T.; Besch, A.; Loose, J.; Otto, H.-H. *Die Pharmazie* **2005**, *60*, 723–731.
21. Bode, W.; Wei, A.-Z.; Huber, R.; Meyer, E.; Travis, J.; Neumann, S. *EMBO J.* **1986**, *5*, 2453–2458.
22. Dixon, M. *Biochem. J.* **1953**, *55*, 170–171.
23. Takahashi, L. H.; Radhakrishnan, R.; Rosenfield, R. E., Jr.; Meyer, E. F., Jr. *Biochemistry* **1989**, *28*, 7610–7617.
24. Stein, R. L.; Trainor, D. A. *Biochem* **1986**, *25*, 5414–5419.