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Synthesis and elastase-inhibiting activity of 2-pyridinyl-isothiazol-3(2*H*)-one 1,1-dioxides

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ABSTRACT

The synthesis of a series of new isothiazol-3(2*H*)-one 1,1-dioxides with halogenated (mostly fluorinated) pyridinyl and pentafluorophenyl substituents at 2-position is reported. These compounds (**18**–**24**) became easily accessible from 2-thiocyanato-1-carboxaldehydes and aminopyridines, pentafluoroaniline, respectively, by an isothiazolium cyclization–oxidation route. Compound **21** exhibited an IC_{50} value of 3.1 μM toward human leukocyte elastase. The proteases cathepsin G, trypsin, cathepsin L, and angiotensin-converting enzyme, and the serine esterases acetylcholinesterase and cholesterol esterase were not inhibited by **21**.

1. Introduction

With a van der Waals radius of 1.47 Å, covalently bound fluorine occupies a smaller volume than other substituents except of hydrogen (van der Waals radius of 1.20 Å). Thus, the substitution of hydrogen by fluorine in bioactive molecules results in minor steric changes but alters their physico-chemical properties, including lipophilicity as well as basicity or acidity of adjacent functional groups. The difference in electronegativity between carbon and the most electronegative element, fluorine, generates strong dipole moments in the carbon–fluorine bond and may contribute to the ability of the molecule to engage in intermolecular interactions. It was demonstrated that aromatic carbon-bound fluorine can interact with positively charged molecules or positively polarized electrophilic centers.[1,2] Currently, the occurrence of fluorine within hydrogen-bonding distance of N–H, O–H, or C–H groups, respectively, is viewed as an attractive dipolar contact rather than a true hydrogen bond.[2–5]

Although it is not common in natural products, covalently bound fluorine has attracted much attention in medicinal chemistry.[6,7] In comparison with the non-fluorinated counterparts, a desirable reduction in their rate of metabolism has been shown for several fluorine-containing drugs. Fluorination of aromatic groups can improve the binding of ligands to target proteins, which was attributed either to the direct fluorine–protein interaction or to an altered polarity of other groups. For example, fluorination of phthalimides led to potent inhibitors of angiogenesis and inhibitors of the production of tumor necrosis factor-α.[8–13] Favorable (fluorophilic) and unfavorable (fluorophobic) environments within the target proteins have been determined. Such an approach was explored to develop inhibitors of serine and cysteine proteases.[2–6,14,15] An example is the DPP-4 inhibitor sitagliptin, a recently approved antidiabetic, where a trifluorophenyl rest occupies the hydrophobic S1 pocket of the protease.[16]

Human leukocyte elastase (HLE) is a serine protease with a primary specificity for small aliphatic residues in P1 position of the substrate. The active enzyme is stored within cytoplasmic azurophilic granules for the remaining life of the neutrophils until extruded into phagolysosomes or out of the cell. HLE is capable of degrading a variety of proteins, including elastin, collagens, laminin, fibronectin, and cartilage proteoglycans as well as immunoglobulins and complement components. HLE can indirectly favor the breakdown of matrix proteins by proteolytic activation of matrix metalloproteinases. Under normal conditions, the activity of extracellular HLE is regulated by endogenous inhibitors, but an imbalance between HLE and its endogenous inhibitors may result in several pathological states. Thus, HLE is considered to be the primary source of tissue damage associated with such inflammatory diseases as pulmonary emphysema and adult respiratory distress syndrome.[17–20] Small-molecular weight HLE inhibitors could be therapeutically

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useful in the treatment of such diseases, and a number of inhibitors are currently in development.[21,22]

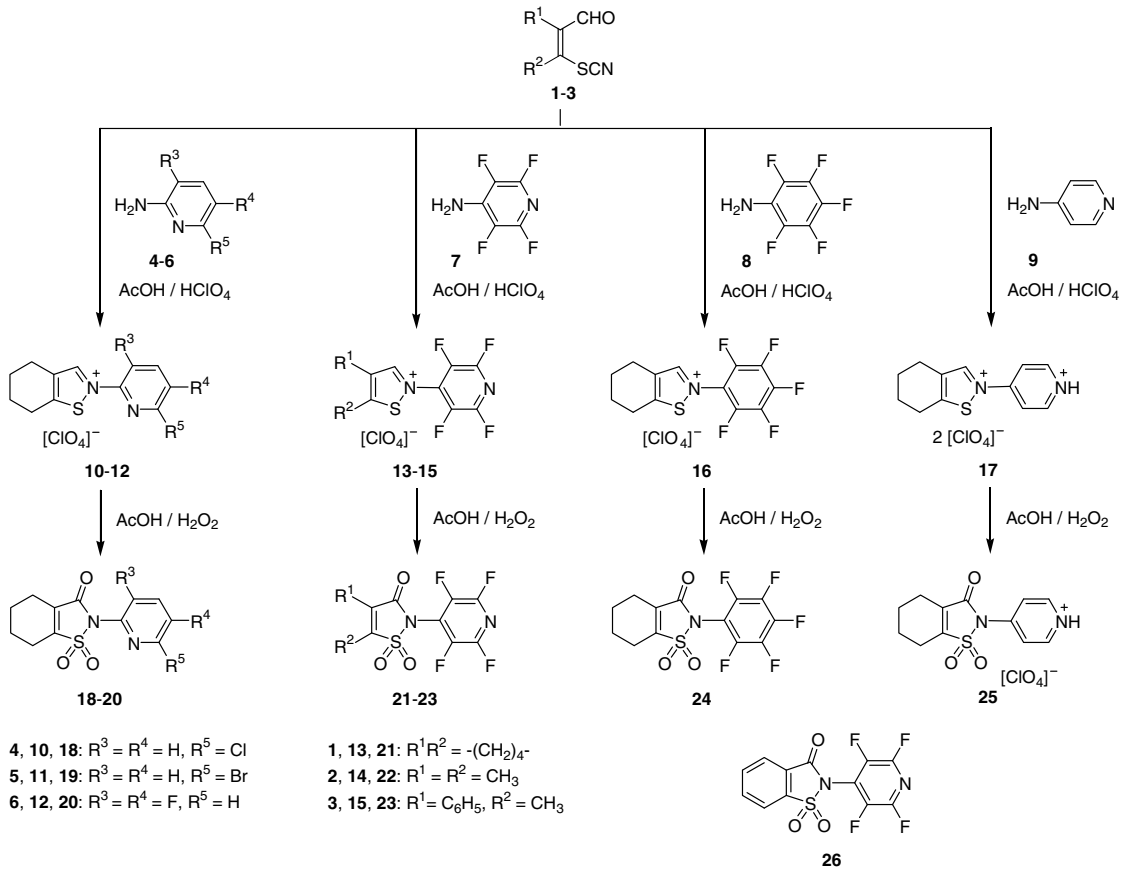
Herein, we report on the synthesis of a series of new isothiazol-3(2*H*)-one 1,1-dioxides with halogenated (mostly fluorinated) aromatic substituents at 2-position. The final compounds were evaluated as inhibitors of HLE, and were additionally assessed against a panel of proteases and two serine esterases, acetylcholinesterase (AChE) and cholesterol esterase (CEase). Isothiazol-3(2*H*)-one 1,1-dioxides have already been reported as inhibitors of HLE. For example, saccharin derivatives with a leaving group within the 2-substituent have been designed as enzyme-activated inhibitors of HLE,[23–25] and Hlasta et al. have designed specific saccharin-based inactivators of HLE showing excellent potency and blood stability.[26–28]

2. Chemistry

The new halopyridinyl-substituted isothiazolium salts **10**–**15**, the pentafluoro derivative **16**, and the non-halogenated derivative **17** were easily accessible by cyclocondensation reactions of thiocyanates **1**–**3**[29–31] with the corresponding aromatic amines **4**–**9** (Scheme 1). In the second step, the halopyridinyl-isothiazol-3(2*H*)-one 1,1 dioxides **18**–**23**, the pentafluorophenyl compound **24**, and **25** were prepared in moderate to good yields by oxidation of the salts **10**–**17** with glacial acetic acid and hydrogen peroxide at 80 °C for 8 h. This facile synthetic entry allowed the synthesis of isothiazol-3(2*H*)-one 1,1-dioxides with phenyl and substituted phenyl groups at position 2,[32–34] and was now applied to the preparation of 2-halopyridinyl derivatives. In order to deduce first structure–activity relationships, the substitution pattern was chosen as follows: the tetramethylene unit was incorporated into five final halo compounds with different 2-substituents (**18**–**21**, **24**), whereas the tetrafluoropyridin-4-yl moiety was maintained in the products **21**–**23**. Moreover, both the non-halogenated and the saccharin analogon of **21**, that is, **25** and **26**, were included in the study.

The structures of the new salts **10**–**17** and of the isothiazole derivatives **18**–**25** were elucidated on the basis of NMR, MS, and IR data as well as elemental analyses. In the IR spectra of the products **18**–**25**, the typical absorption bands for the SO_2 group were observed at 1164–1186 cm^{-1} and at 1332–1352 cm^{-1} for the symmetric and asymmetric vibrations, respectively. In the ^{13}C NMR spectra of the pyridinyl (pentafluorophenyl) isothiazol-3(2*H*)-one 1,1 dioxides **18**–**25**, the signals for C-3 at 159–162 ppm and for C-7a (C-5 for **22** and **23**) at 146–151 ppm were characteristic for these oxidation products.

The structure of the isothiazole **22** was confirmed by X-ray crystal structure analysis. Compound **22** crystallizes in the triclinic space group $P\bar{1}$ with three symmetry-independent molecules in the unit cell. For the three molecules, the same numbering of the atoms was used differing by the letters A, B, and C. The molecular structure of the molecule A is shown in Figure 1. Bond lengths of the isothiazole ring and dihedral angles are given for the molecules A, B and C in Table 1. The molecules A, B, and C have similar bond lengths and nearly the same conformation in that the dihedral angles from A and B differ by 7.1°, those from A and C by 16.8°. The crystal packing (Fig. 2) was analyzed with the program XPac.[35] The packing of the three molecules in the direction of the *a*-axis is equal. Three molecular chains with translation symmetry were obtained, composed exclusively by molecules of the type A, B and C.



Scheme 1.

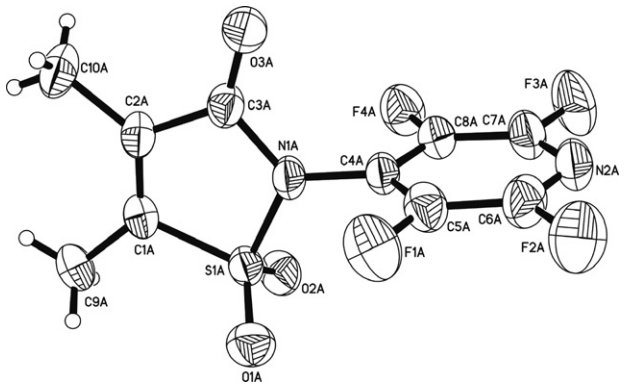


Figure 1. Molecular structure of **22**, molecule A, showing the atom-labeling scheme, thermal ellipsoids with 50% probability.

Table 1
Bond lengths (Å) of the isothiazole ring of the molecules A, B, and C and the dihedral angles (°) between the isothiazole ring and the tetrafluoropyridinyl ring of compound **22**

	Molecule A	Molecule B	Molecule C
C1–S1	1.760(2)	1.763(2)	1.761(2)
C1–C2	1.333(3)	1.329(3)	1.334(3)
C2–C3	1.486(3)	1.482(3)	1.487(3)
C3–N1	1.404(2)	1.407(3)	1.410(2)
C3–O3	1.206(2)	1.198(3)	1.197(2)
N1–S1	1.694(2)	1.683(2)	1.692(2)
S1–O1	1.416(2)	1.420(2)	1.421(2)
S1–O2	1.419(2)	1.423(2)	1.416(2)
N1–C4	1.414(3)	1.414(3)	1.416(3)
Dihedral angles	85.7(1)	78.6(1)	68.9(1)

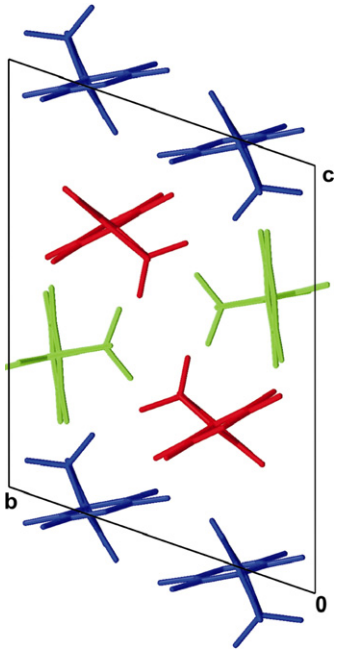


Figure 2. The arrangement of compound **22** in the unit cell. Packing of the molecules A (green), B (blue), and C (red) along the *a*-axis, for simplification without hydrogen atoms.

3. Results and discussion

Saccharin derivatives substituted at the *N*-2 atom can easily be obtained by N-alkylation with alkyl halides. In contrast, the reaction with alcohols under Mitsunobu conditions tended to favor O-alkylation, as recently found.[36] N-Arylation of saccharin was successfully achieved with triphenylbismuth and cupric acetate in the presence of pyridine or triethylamine.[37] 2-Phenylsaccharin was also obtained by N-arylation with *ortho*-silylphenyl triflate in the presence of cesium fluoride.[38] The conversion of 4,5,6,7-tetrahydrosaccharin[39] via arylation to produce corresponding tetrahydro derivatives has not been reported so far. Such compounds (i.e., **18**–**21**, **24**, and **25**) became easily accessible by the isothiazolium cyclization–oxidation route as described above (Scheme 1). Subramanyam et al. have performed chloromethylation of tetrahydrosaccharin in two steps with chloromethyl phenyl sulfide/tetra *n*-butylammonium bromide and sulfuryl chloride. Esterification of 2,6-dichlorobenzoic acid with the resulting chloride furnished a potent mechanism-based inhibitor of HLE.[39]

Tetrafluoropyridine-substituted saccharins, for example, **26**, have already been reported by Raßhofer and Vögtle.[40] In two patents, *N*-(tetrafluoropyridin-4-yl)saccharins, including derivatives with alkoxy substitution at the fused benzene ring,[41] as well as *N*-aryl saccharins[42] have been described as inhibitors of elastases. Ashe et al. investigated a series of *N*-aryl saccharins as inhibitors of HLE and bovine chymotrypsin.[43] Among these compounds, pentafluorophenyl saccharin, prepared from *ortho*-sulfobenzoic acid cyclic anhydride and pentafluoroaniline was the most potent inhibitor with an IC_{50} value of 3 μM toward HLE and was inactive against bovine chymotrypsin. This prompted us to combine the haloaryl moiety with the tetrahydrosaccharin core to obtain **18**–**21** and **24**. The inhibition of HLE by **18**–**26** (Table 2) was measured using a spectrophotometric assay with the chromogenic substrate MeOSuc-Ala-Ala-Pro-Val-pNA. In order to assess selectivity, other representative members of serine proteases (cathepsin G, trypsin), as well as metalloproteases (angiotensin-converting enzyme (ACE)), and cysteine proteases (cathepsin L) were also determined. Instead of the corresponding fluorogenic substrate,[44] cathepsin L could conveniently be assayed with the chromogenic substrate Z-Phe-Arg-pNA. Two serine esterases, AChE and CEase which share the acyl transfer mechanism with serine proteases were also included in the inhibition studies.

For HLE inhibition, the tetrafluoropyridin-4-yl substitution was advantageous compared to the pentafluorophenyl substitution (**21** vs **24**). The introduction of other halopyridinyl moieties did not lead to enzyme inhibition (**18**–**20** vs **21**). Thus, the tetrafluoropyridin-4-yl moiety was maintained and the tetramethylene chain of **21** was replaced by a dimethyl (**22**) or 4-phenyl-5-methyl substitution (**23**). However, these modifications did not improve the HLE-inhibiting activity. The non-fluorinated analogon **25** was inactive against HLE (**21** vs **25**), indicating the impact of fluorine substitution on HLE inhibition. The replacement of the tetramethylene chain by a fused benzene ring (**21** vs **26**) increased the HLE-inhibitory capacity by two orders of magnitude. Provided that **26** behaved kinetically as a competitive inhibitor, the IC_{50} value obtained in this study corresponding to a K_i value of 28 nM, agrees with a literature K_i value of 20 nM.[41] Some of the other enzymes (Table 2) were also inhibited by **26**, but to a lesser extent.

The concentration-dependent inhibition of HLE by compound **21** is presented in Figure 3. The progress curves of the HLE-catalyzed substrate consumption were linear over the 10-min time course indicating time-independent inhibition by **21**. To elucidate the type of inhibition, the reactions were performed with different substrate concentrations. The Hanes–Woolf plot (Fig. 4) provided parallel lines, which indicated competition of substrate and inhibitor for the active site. From the replot of the slopes of the Hanes–Woolf plot versus the corresponding inhibitor concentrations, a K_i value for competitive inhibition of 1.7 μM was obtained (Fig. 5). Inhibition of HLE by *N*-aryl saccharins was competitive with substrate and has been characterized as alternate substrate

Table 2
Inhibition of serine hydrolases by isothiazol-3(2*H*)-one 1,1 dioxides **18**–**26**

Compound	IC_{50} ± SEM[a]						
	HLE[b] (μM)	Cathepsin G[b] (μM)	Trypsin[b] (μM)	Cathepsin L[b] (μM)	ACE[c] (μM)	AChE[d] (μM)	CEase[d] (μM)
18	>100	>100	>100	>100	>100	>100	>100
19	>100	>100	>100	>100	>100	>100	>100
20	>100	>100	>100	>100	>100	>100	>100
21	3.1 ± 0.2[e]	>100	>100	>100	>100	>100	>100
22	>100	>100	>100	>100	>100	>100	>100
23	33 ± 1[f]	>100	>100	>100	>100	>100	>100
24	>100	>100	>100	>100	>100	>100	>100
25	>100	>100	>100	>100	>100	>100	>100
26	0.081 ± 0.003[e]	3.7 ± 0.5[e]	7.5 ± 0.8[g]	>100	>100	>100	11 ± 2.4[e]

[a] Data were obtained from duplicate determinations at two inhibitor concentrations, [I], of 25 and 50 μM. Data for cathepsin G inhibition were obtained from duplicate determinations at an inhibitor concentration of 12.5 μM. IC_{50} values were calculated using the equation $IC_{50} = [I]/(v_0/v - 1)$, where v_0 and v are the rates in the absence and presence of the inhibitor, respectively.
[b] Progress curves were followed for 10 min and analyzed by linear regression.
[c] Progress curves were followed for 20 min and analyzed by linear regression.
[d] Progress curves were followed for 5 min and analyzed by linear regression.
[e] Data are mean values of duplicate determinations of two-independent experiments, using five different inhibitor concentrations. IC_{50} was obtained by non-linear regression according to equation $v = v_0/([I]/IC_{50} + 1)$.
[f] Data are mean values of duplicate determination, using nine different inhibitor concentrations (5–45 μM). IC_{50} was obtained by non-linear regression according to a four-parametric logistic equation.
[g] Progress curves were followed for 12.5 min and analyzed by non-linear regression using the equation for slow-binding inhibition $[P] = v_s t + (v_i - v_s)(1 - \exp(-k_{obs}t))/k_{obs} + d$, where [P] is the product concentration, v_s is the steady-state rate, v_i is the initial rate, k_{obs} is the observed pseudo first-order rate constant, and d is the offset. IC_{50} was obtained by non-linear regression according to equation $v_S = v_0/([I]/IC_{50} + 1)$.

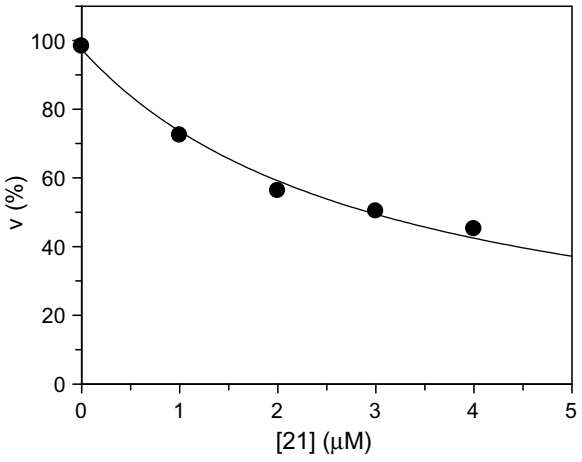


Figure 3. Inhibition of human leukocyte elastase by compound **21**. Plot of the percentage rates versus inhibitor concentrations, [I], in 50 mM sodium phosphate, 500 mM NaCl, pH 7.8, 25 °C. Initial concentration of the substrate MeOSuc-Ala-Ala-Pro-Val-pNA was 100 μM (=1.9 K_m). Progress curves were followed for 10 min, and HLE-catalyzed hydrolysis was analyzed as zero-order reaction. Data are mean values of duplicate determinations of two-independent experiments. Rates in the absence of inhibitor, v_0, were set to 100%. IC_{50} was obtained using equation $v = v_0/([I]/IC_{50} + 1)$. Non-linear regression gave IC_{50} = 3.1 ± 0.2 μM.

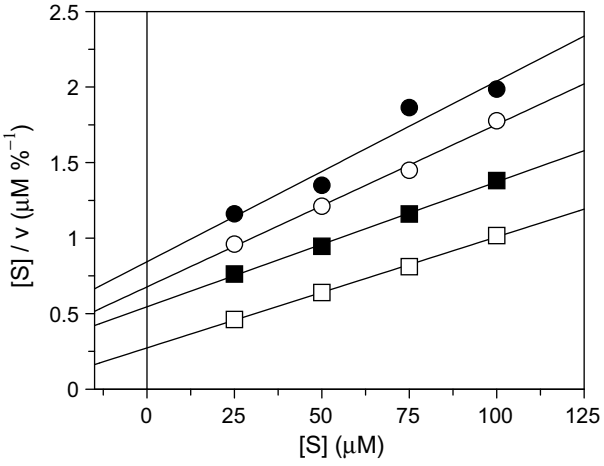


Figure 4. Hanes–Woolf plot for the inhibition of human leukocyte elastase by compound **21** in the presence of different concentrations, [S], of the chromogenic substrate MeO-Suc-Ala-Ala-Pro-Val-pNA. The other conditions were the same as noted in Figure 3. Rates in the absence of inhibitor and the presence of 100 μM of the substrate were set to 100%. Data are mean values of duplicate determinations of two-independent experiments with inhibitor concentrations, [I], of 3 μM (●), 2 μM (○), 1 μM (■), and in the absence of the inhibitor (□). The parallel lines indicate competitive inhibition. Linear regression gave values for vertical intercepts.

inhibition.[43] A similar mode of action was also assumed for **21**, including the attack of the active site serine at the carbonyl carbon and the reversible formation of an acyl-enzyme. The result of a reactivation experiment is shown in Figure 6. The hydrazine added was supposed to act as a competing nucleophile[45] thus accelerating the cleavage of an acyl-enzyme and leading to reactivation.[43,46]

For a summary, a new inhibitor of HLE (**21**) was prepared from 4-amino-2,3,5,6-tetrafluoropyridine (**7**) and 2-thiocyanato-1-cyclohexene-1-carboxaldehyde (**1**) in two reaction steps. Compound **21** exhibited an IC_{50} value in the low micromolar range and did neither inhibit the proteases cathepsin G, trypsin, cathepsin L, and ACE, nor the serine esterases AChE and CEase. Inhibitor **21** behaved kinetically as a competitive inhibitor, and an alternate substrate inhibition was assumed. Further studies are in progress to introduce the tetrafluoropyridin-4-yl moiety in other structures and to explore the protease-inhibiting properties of the resulting compounds.

4. Experimental protocols

4.1. General methods and materials

Meting points were determined with a Boetius micro-melting point apparatus and are corrected. UV/vis spectra were recorded on a Beckman DU 650 spectrophotometer. Maximum wavelengths are noted in nanometer and log ε values are given in parentheses. IR spectra were measured on a Genesis FTIR Unicam Analytical System (ATI Mattson). ^{1}H (200 or 300 MHz), ^{13}C (50 or 75 MHz), and ^{19}F (188 MHz) spectra were recorded on Varian Gemini-200 or Var-

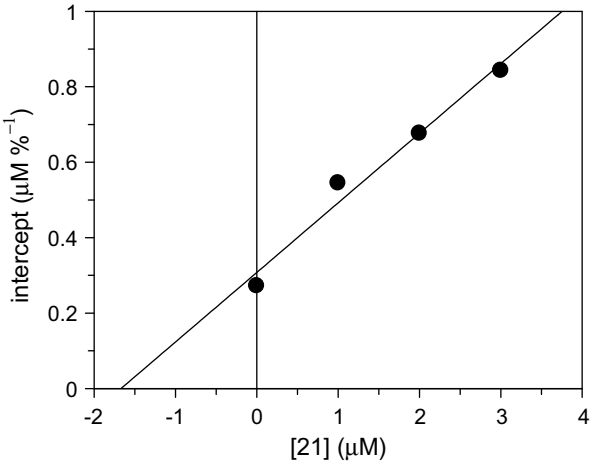


Figure 5. Plot of vertical intercepts versus the concentrations of **21**. Data were obtained from Figure 4. Linear regression according to the equation, intercept = K_m [I]/(K_i V_{max}) + (K_m/V_{max}), gave a slope K_m/(K_i V_{max}) = 0.18 ± 0.02%$^{-1}$ and an intercept K_m/V_{max} = 0.31 ± 0.04 μM%$^{-1}$ that corresponds to a value K_i = 1.7 μM.

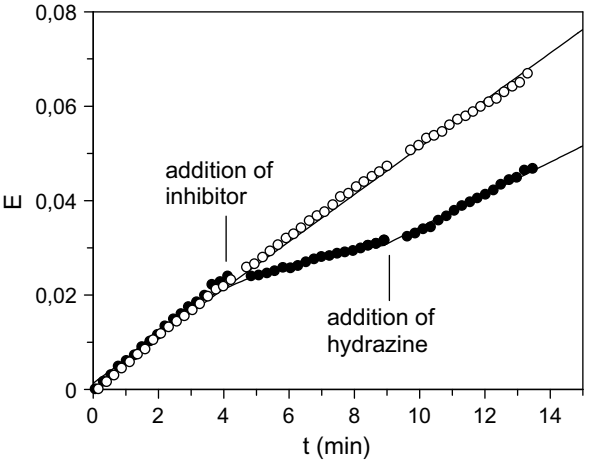


Figure 6. Reactivation of HLE inhibited by compound **21**. The HLE-catalyzed hydrolysis of MeOSuc-Ala-Ala-Pro-Val-pNA (100 μM) was followed at 405 nm. In the reactivation experiment (●), HLE was inhibited by **21** (6.3 μM) and reactivated due to the addition of hydrazine (100 μM). In the control experiment (○), the enzymatic cleavage was monitored in the absence of **21** and hydrazine.

ian Gemini-300 spectrometers. Chemical shifts are given as δ values, relative to tetramethylsilane (TMS) and $CFCl_3$ as internal standards. Spin multiplicities are indicated by the following symbols: s (singlet), d (doublet), t (triplet), m (multiplet), m, c (centered multiplet) dd (doublet of doublet). ESI-MS spectra were recorded on a Bruker Esquire 3000 plus. HR ESI-MS spectra were recorded with syringe infusion on a Bruker FT-ICR mass spectrometer APEX II, equipped with a 7 T magnet, skimmer voltage 100 V, drying gas 200 °C. Elemental analyses were performed on a Heraeus CHNO Rapid Analyser.

Human leukocyte elastase (HLE), human cathepsin G, human cathepsin L, and human angiotensin-converting enzyme (ACE) were obtained from Calbiochem, Darmstadt, Germany. MeOSuc-Ala-Ala-Pro-Val-pNA, Suc-Ala-Ala-Pro-Phe-pNA, Suc-Ala-Ala-Pro-Arg-pNA, Z-Phe-Arg-pNA, and 2-furanacryloyl-phenylalanyl-glycylglycine (FA-Phe-Gly-Gly) were purchased from Bachem, Bubendorf, Switzerland. Acetylcholinesterase (AChE) from *Electrophorus electricus* was purchased from Fluka, Deisenhofen, Germany. Trypsin from bovine pancreas, acetylthiocholine (ATCh), and 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma, Steinheim, Germany. Cholesterol esterase (CEase) from bovine pancreas, sodium taurocholate (TC), and *para*-nitrophenylbutyrate (pNPB) were obtained from Sigma, Steinheim, Germany. 2-(2,3,5,6-Tetrafluoropyridin-4-yl)-1,2-benzisothiazol-3(2*H*)-one 1,1-dioxide (**26**) was obtained from TimTac, LLC, Newark, DE, USA.

The spectrophotometric assays were done on a Varian Cary 50 Bio UV/vis spectrometer with a cell holder equipped with a constant temperature water bath.

4.1.1. General method for the synthesis of isothiazolium perchlorates 10–17

The substituted aminopyridine (**4**–**7**, **9**, 1 mmol) or 2,3,4,5,6-pentafluoroaniline (**8**, 183 mg, 1 mmol) was added to a solution of the corresponding thiocyanate (**1**–**3**, 1 mmol) in glacial acetic acid (2 ml). When the solid was completely dissolved, perchloric acid (0.4 ml, 70%) was added dropwise to the solution. The reaction mixture was stirred for 2 h, and diethyl ether was added. The precipitate was isolated by filtration and recrystallized from ethanol.

4.1.1.1. 2-(6-Chloropyridin-2-yl)-4,5,6,7-tetrahydro-1,2-benziso-thiazolium perchlorate (10). Yield: 70%, mp: 219–221 °C. UV (EtOH), λ_{max} (nm): 211 (4.26). IR (KBr), ν (cm^{-1}): 1080 (O–Cl–O). ^{1}H NMR (DMSO-d_6), δ (ppm): 1.90 (m, c, 4H, 2CH$_2$), 2.83 (m, c, 2H, CH$_2$), 3.22 (m, c, 2H, CH$_2$), 7.83 (d, J = 7.7 Hz, 1H, aromat. H), 8.17 (d, J = 8.0 Hz, 1H, aromat. H), 8.34 (t,J = 8.0 Hz, 1H, aromat. H), 9.94 (s, 1H, 3-H). ^{13}C NMR (DMSO-d_6), δ (ppm): 20.5, 21.1, 22.4, 25.5 (CH$_2$), 112.2 (aromat. C–H), 125.9 (aromat. C–H), 135.2 (C-3a), 144.0 (aromat. C–H), 147.4 (aromat. C), 148.7 (C–Cl), 152.2 (C-3), 171.0 (C-7a). ESI-MS *m/z*: 251 (M − $HClO_4^{+\cdot}$). Anal. Calcd for $C_{12}H_{12}Cl_2N_2O_4S$ (351.21): C, 41.04; H, 3.44; N, 7.89; S, 9.13. Found: C, 40.94; H, 3.04; N, 7.99; S, 9.23.

4.1.1.2. 2-(6-Bromopyridin-2-yl)-4,5,6,7-tetrahydro-1,2-benzisothia-zolium perchlorate (11). Yield: 91%, mp: 221–223 °C. UV (EtOH), λ_{max} (nm): 318 (4.03). IR (KBr), ν (cm^{-1}): 1081 (O–Cl–O). ^{1}H NMR (DMSO-d_6), δ (ppm): 1.85 (m, c, 4H, 2CH$_2$), 2.81 (m, c, 2H, CH$_2$), 3.18 (m, c, 2H, CH$_2$), 7.92 (dd, J = 5.8 Hz,J = 2.5 Hz, 1H, aromat. H), 8.18 (m, c, 2H, aromat. H), 9.90 (s, 1H, 3-H). ^{13}C NMR (DMSO-d_6), δ (ppm): 20.5, 21.1, 22.4, 25.6 (CH$_2$), 112.4 (aromat. C–H), 129.7 (aromat. C–H), 135.3 (C-3a), 139.5 (C–Br), 143.5 (aromat. C–H), 147.6 (aromat. C), 152.1 (C-3), 171.1 (C-7a). ESI-MS *m/z*: 295 (M − $HClO_4^{+\cdot}$). Anal. Calcd for $C_{12}H_{12}BrClN_2O_4S$ (395.66): C, 36.43; H, 3.06; N, 7.08; S, 8.10. Found: C, 36.14; H, 2.82; N, 7.05; S, 8.09.

4.1.1.3. 2-(3,5-Difluoropyridin-2-yl)-4,5,6,7-tetrahydro-1,2-benz isothiazolium perchlorate (12). Yield: 52%, mp: 163–165 °C. UV (EtOH), λ_{max} (nm): 313 (4.06). IR (KBr), ν (cm^{-1}): 1093 (O–Cl–O). ^{1}H NMR (DMSO-d_6), δ (ppm): 1.88 (m, c, 4H, 2CH$_2$), 2.88 (m, c, 2H, CH$_2$), 3.24 (m, c, 2H, CH$_2$), 8.58 (t, J = 8.1 Hz, 1H, aromat. H), 8.68 (m, c, 1H, aromat. H), 9.62 (s, 1H, 3-H). ^{13}C NMR (DMSO-d_6), δ (ppm): 20.5, 21.2, 22.5, 25.7 (CH$_2$), 116.5 (t, ^2J = 24 Hz, aromat. C–H), 124.7 (m, aromat. C), 133.2 (m, ^2J = 26 Hz, aromat. C–H), 135.0 (C-3a), 150.2 (d, ^1J = 241 Hz, C–F), 154.1 (C-3), 159.4 (d, ^1J = 261 Hz, C–F), 171.2 (C-7a). ^{19}F NMR (acetone-d_6), δ (ppm): −120.9 (m, 1F), −122.8 (m, 1F). ESI-MS *m/z*: 252 (M − $HClO_4^{+\cdot}$). Anal. Calcd for $C_{12}H_{11}ClF_2N_2O_4S$ (352.75): C, 40.86; H, 3.14; N, 7.94; S, 9.09. Found: C, 40.60; H, 3.27; N, 7.94; S, 9.13.

4.1.1.4. 2-(2,3,5,6-Tetrafluoropyridin-4-yl)-4,5,6,7-tetrahydro-1,2-benzisothiazolium perchlorate (13). Yield: 87%, mp: 235–236 °C. UV (EtOH), λ_{max} (nm): 287 (4.11). IR (KBr), ν (cm^{-1}): 1092 (O–Cl–O). ^{1}H NMR (DMSO-d_6), δ (ppm): 1.86 (m, c, 4H, 2CH$_2$), 2.97 (m, c, 2H, CH$_2$), 3.38 (m, c, 2H, CH$_2$), 9.52 (s, 1H, 3-H). ^{13}C NMR (DMSO-d_6), δ (ppm): 21.3, 21.9, 23.3, 26.5

(CH_2), 127.9 (m, aromat. C), 136.5 (C-3a), 138.8 (dd, ^1J = 266 Hz, ^2J = 30 Hz, C–F), 144.4 (m, ^1J = 240 Hz, C–F), 159.8 (C-3), 177.6 (C-7a). ^{19}F NMR (acetone-d_6), δ (ppm): −89.2 (m, 2F), −147.5 (m, 2F). ESI-MS *m/z*: 289 ($M - ClO_4^+$). Anal. Calcd for $C_{12}H_9ClF_4N_2O_4S$ (388.73): C, 37.08; H, 2.33; N, 7.21; S, 8.25. Found: C, 37.03; H, 1.68; N, 7.22; S, 8.28.

4.1.1.5. 4,5-Dimethyl-2-(2,3,5,6-tetrafluoropyridin-4-yl)-isothiazolium perchlorate (14). Yield: 93%, mp: 260–262 °C. UV (EtOH), λ_{max} (nm): 283 (4.17). IR (KBr), ν (cm^{-1}): 1090 (O–Cl–O). ^{1}H NMR (DMSO-d_6), δ (ppm): 2.54 (s, 3H, CH_3), 3.08 (s, 3H, CH_3), 9.40 (s,. 1H, 3-H). ^{13}C NMR (DMSO-d_6), δ (ppm): 11.7 (CH_3), 14.5 (CH_3), 128.4 (m, aromat. C), 136.4 (C-3a), 139.6 (dd, ^1J = 267 Hz, ^2J = 31 Hz, C–F), 145.0 (m, ^1J = 243 Hz, C–F), 161.0 (C-3), 177.6 (C-7a). ^{19}F NMR (DMSO-d_6), δ (ppm): −89.1 (m, 2F), −146.4 (m, 2F). ESI-MS *m/z*: 263 ($M - ClO_4^+$). Anal. Calcd for $C_{10}H_7ClF_4N_2O_4S$ (362.69): C, 33.12; H, 1.95; N, 7.72; S, 8.84. Found: C, 33.08; H, 2.02; N, 7.67; S, 8.85.

4.1.1.6. 5-Methyl-4-phenyl-2-(2,3,5,6-tetrafluoropyridin-4-yl)-isothiazolium perchlorate (15). Yield: 54%, mp: 72–75 °C. UV (EtOH), λ_{max} (nm): 232 (3.96). IR (KBr), ν (cm^{-1}): 1086 (O–Cl–O). ^{1}H NMR (DMSO-d_6), δ (ppm): 2.95 (s, 3H, CH_3), 7.23 (m, c, 1H, aromat. H), 7.42 (m, c, 3H, aromat. H), 7.61 (m, c, 1H, aromat. H), 9.79 (s, 1H, 3-H). It was not possible to record a ^{13}C NMR spectrum due to the rapid decomposition of this compound in solution. ^{19}F NMR (DMSO-d_6), δ (ppm): −88.4 (m, 2F), −145.3 (m, 2F). ESI-MS *m/z*: 325 ($M - ClO_4^+$). HR ESI-MS ($M - ClO_4^+$) Calcd for $C_{15}H_9F_4N_2S$: 325.04171. Found: 325.04160.

4.1.1.7. 2-(2,3,4,5,6-Pentafluorophenyl)-4,5,6,7-tetrahydro-1,2-benzisothiazolium perchlorate (16). Yield: 98%, mp: 257–260 °C. UV (EtOH), λ_{max} (nm): 206 (4.00). IR (KBr), ν (cm^{-1}): 1085 (O–Cl–O). ^{1}H NMR (DMSO-d_6), δ (ppm): 1.91 (m, c, 4H, $2CH_2$), 2.89 (m, c, 2H, CH_2), 3.30 (m, c, 2H, CH_2), 9.44 (s, 1H, 3-H). ^{13}C NMR (DMSO-d_6), δ (ppm): 20.6, 21.4, 22.7, 26.9 (CH_2), 134.8 (C-3a), 160.3 (C-3), 176.0 (C-7a). ^{19}F NMR (DMSO-d_6), δ (ppm): −143.9 (m, 2F), −147.7 (m, 1F), −159.2 (m, 2F). ESI-MS *m/z*: 306 ($M - ClO_4^+$). Anal. Calcd for $C_{13}H_9ClF_5NO_4S$ (405.73): C, 38.48; H, 2.24; N, 3.45; S, 7.90. Found: C, 38.40; H, 1.94; N, 3.81; S, 8.12.

4.1.1.8. 2-(Pyridin-4-yl)-4,5,6,7-tetrahydro-1,2-benzisothiazolium diperchlorate (17). Yield: 97%, mp: 216–217 °C. UV (EtOH), λ_{max} (nm): 294 (3.86). IR (KBr), ν (cm^{-1}): 1093 (O–Cl–O). ^{1}H NMR (acetone-d_6), δ (ppm): 1.98 (m, c, 4H, $2CH_2$), 3.04 (m, c, 2H, CH_2), 3.47 (m, c, 2H, CH_2), 8.80 (d, J = 7.6 Hz, 2H, aromat. H), 9.45 (d, J = 7.6 Hz, 2H, aromat. H), 9.89 (s, 1H, 3-H). ^{13}C NMR (acetone-d_6), δ (ppm): 22.0, 22.7, 24.1, 27.4 (CH_2), 110.8 (aromat. C–H), 138.6 (C-3a), 141.7 (aromat. C), 146.7 (aromat. C–H), 157.7 (C-3), 177.7 (C-7a). ESI-MS *m/z*: 217 ($[M - HClO_4 - ClO_4^-]^+$). Anal. Calcd for $C_{12}H_{14}Cl_2N_2O_8S$ (417.22): C, 34.55; H, 3.38; N, 6.71. Found: C, 34.38; H, 3.08; N, 6.59.

4.1.2. General method for the synthesis of isothiazol-3(2*H*)-one 1,1-dioxides 18–25

H_2O_2 (0.7 ml, 30%) was added to a stirred suspension of **10–17** (0.26 mmol) in glacial acetic acid (0.7 ml) and stirring was continued at 80 °C for 8 h. After cooling, the solid products **18–25** could be isolated by filtration and were recrystallized from ethanol.

4.1.2.1. 2-(6-Chloropyridin-2-yl)-4,5,6,7-tetrahydro-1,2-benzisothiazol-3(2*H*)-one 1,1-dioxide (18). Yield: 44%, mp: 174–176 °C. UV (EtOH), λ_{max} (nm): 218 (3.94). IR (KBr), ν (cm^{-1}): 1735 (C=O), 1349 (SO_2), 1169 (SO_2). ^{1}H NMR (acetone-d_6), δ (ppm): 1.89 (m, c, 4H, $2CH_2$), 2.52 (m, c, 2H, CH_2), 2.64 (m, c, 2H, CH_2), 7.48 (d, J = 7.8 Hz, 1H, aromat. H), 7.89 (d, J = 8.1 Hz, 1H, aromat. H), 8.04 (t, J = 7.8 Hz, 1H, aromat. H). ^{13}C NMR (acetone-d_6), δ (ppm): 20.0, 21.4, 21.8, 22.1 (CH_2), 116.8 (aromat. C–H), 124.4 (aromat. C–H), 136.8 (C-3a), 142.7 (aromat. C), 143.5 (aromat. C–H), 148.6 (C-7a), 150.7 (C–Cl), 160.9 (C=O). ESI-MS *m/z*: 298 ($M^{+\cdot}$). Anal. Calcd for $C_{12}H_{11}ClN_2O_3S$ (298.75): C, 48.25; H, 3.71; N, 9.38; S, 10.73. Found: C, 47.78; H, 3.69; N, 9.50; S, 11.23.

4.1.2.2. 2-(6-Bromopyridin-2-yl)-4,5,6,7-tetrahydro-1,2-benzisothiazol-3(2*H*)-one 1,1-dioxide (19). Yield: 56%, mp: 181–183 °C. UV (EtOH), λ_{max} (nm): 223 (4.24). IR (KBr), ν (cm^{-1}): 1733 (C=O), 1333 (SO_2), 1177 (SO_2). ^{1}H NMR (DMSO-d_6), δ (ppm): 1.76 (m, c, 4H, $2CH_2$), 2.41 (m, c, 2H, CH_2), 2.58 (m, c, 2H, CH_2), 7.69 (d, J = 7.8 Hz, 1H, aromat. H), 7.81 (d, J = 8.1 Hz, 1H, aromat. H), 7.95 (t, J = 8.1 Hz, 1H, aromat. H). ^{13}C NMR (DMSO-d_6), δ (ppm): 18.3, 19.8, 19.0, 20.2 (CH_2), 116.4 (aromat. C–H), 127.2 (aromat. C–H), 135.5 (C-3a), 139.1 (C–Br), 142.3 (aromat. C–H), 145.8 (aromat. C), 146.1 (C-7a), 159.0 (C=O). ESI-MS *m/z*: 344 ($M+H^{+\cdot}$). Anal. Calcd for $C_{12}H_{11}BrN_2O_3S$ (343.20): C, 42.00; H, 3.23; N, 8.16; S, 9.34. Found: C, 41.57; H, 2.99; N, 8.33; S, 10.01.

4.1.2.3. 2-(3,5-Difluoropyridin-2-yl)-4,5,6,7-tetrahydro-1,2-benzisothiazol-3(2*H*)-one 1,1-dioxide (20). Yield: 35%, mp: 173–175 °C. UV (EtOH), λ_{max} (nm): 212 (3.50). IR (KBr), ν (cm^{-1}): 1743 (C=O), 1332 (SO_2), 1185 (SO_2). ^{1}H NMR (acetone-d_6), δ (ppm): 1.90 (m, c, 2H, CH_2), 2.00 (m, c, 2H, CH_2), 2.56 (m, c, 2H, CH_2), 2.68 (m, c, 2H, CH_2), 8.00 (m, c, 1H, aromat. H), 8.53 (m, c, 1H, aromat. H). ^{13}C NMR (acetone-d_6), δ (ppm): 20.3, 21.6, 21.7, 22.1 (CH_2), 116.1 (t, ^2J = 23 Hz, aromat. C–H), 130.3 (m, aromat. C), 135.9 (d, ^2J = 25 Hz, aromat. C–H), 137.5 (C-3a), 149.7 (C-7a), 157.2 (d, ^1J = 269 Hz, C–F), 160.8 (C=O), 161.9 (d, ^1J = 263 Hz, C–F). ^{19}F NMR (acetone-d_6), δ (ppm): −119.5 (m 1F), −121.4 (m, 1F). ESI-MS *m/z*: 300 ($M^{+\cdot}$). Anal. Calcd for $C_{12}H_{10}F_2N_2O_3S$ (300.29): C, 48.00; H, 3.36; N, 9.33; S, 10.68. Found: C, 47.82; H, 3.12; N, 9.59; S, 11.05.

4.1.2.4. 2-(2,3,5,6-Tetrafluoropyridin-4-yl)-4,5,6,7-tetrahydro-1,2-benzisothiazol-3(2*H*)-one 1,1-dioxide (21). Yield: 47%, mp: 238–240 °C. UV (EtOH), λ_{max} (nm): 217 (3.91). IR (KBr), ν (cm^{-1}): 1749 (C=O), 1350 (SO_2), 1184 (SO_2). ^{1}H NMR (acetone-d_6), δ (ppm): 1.92 (m, c, 2H, CH_2), 2.03 (m, c, 2H, CH_2), 2.60 (m, c, 2H, CH_2), 2.74 (m, c, 2H, CH_2). ^{13}C NMR (acteone-d_6), δ (ppm): 20.7, 21.6, 21.9, 22.0 (CH_2), 122.8 (m, aromat. C), 138.1 (C-3a), 142.1 (m, ^1J = 268 Hz, C–F), 145.5 (m, ^1J = 244 Hz, C–F), 150.6 (C-7a), 160.1 (C=O). ^{19}F NMR (acetone-d_6), δ (ppm): −90.2 (m, 2F), −144.4 (m, 2F). ESI-MS *m/z*: 337 ($M+H^+$). Anal. Calcd for $C_{12}H_8F_4N_2O_3S$ (336.27): C, 42.86; H, 2.40; N, 8.33; S, 9.54. Found: C, 41.61; H, 2.36; N, 8.35; S, 9.92.

4.1.2.5. 4,5-Dimethyl-2-(2,3,5,6-tetrafluoropyridin-4-yl)-isothiazol-3(2*H*)-one 1,1-dioxide (22). Yield: 21%, mp: 133–134 °C. UV (EtOH), λ_{max} (nm): 219 (3.91). IR (KBr), ν (cm^{-1}): 1749 (CO), 1349 (SO_2), 1182 (SO_2). ^{1}H NMR (acetone-d_6), δ (ppm): 2.19 (s, 3H, CH_3), 2.42 (s, 3H, CH_3). ^{13}C NMR (acetone-d_6), δ (ppm): 8.5 (CH_3), 9.3 (CH_3), 122.0 (m, aromat. C), 134.5 (C-3a), 141.3 (m, ^1J = 267 Hz, C–F), 144.8 (m, ^1J = 244 Hz, C–F), 146.9 (C-5), 160.2 (C=O). ^{19}F NMR (acetone-d_6), δ (ppm): −90.1 (m, 2F), −144.4 (m, 2F). ESI-MS *m/z*: 310 ($M^{+\cdot}$). Anal. Calcd for $C_{10}H_6F_4N_2O_3S$ (310.23): C, 38.72; H, 1.95; N, 9.03; S, 10.34. Found: C, 38.69; H, 1.90; N, 9.01; S, 10.32.

4.1.2.6. 5-Methyl-4-phenyl-2-(2,3,5,6-tetrafluoropyridin-4-yl)-isothiazol-3(2*H*)-one 1,1-dioxide (23). Yield: 24%, mp: 115–116 °C. UV (EtOH), λ_{max} (nm): 206 nm (4.18). IR (KBr), ν (cm^{-1}): 1740 (CO), 1349 (SO_2), 1184 (SO_2). ^{1}H NMR (acetone-d_6), δ (ppm): 2.56 (s, 3H, CH_3), 7.60 (m, c, 3H, aromat. H), 7.70

(m, c, 2H, aromat. H). ^{13}C NMR (acetone-d_6), δ (ppm): 10.3 (CH_3), 122.5 (m, aromat. C), 127.9 (C-4′), 130.3 (C-2′), 131.7 (C-3′), 132.3 (C-1′), 135.8 (C-3a), 142.1 (m, ^1J = 269 Hz, C–F), 145.5 (m, ^1J = 242 Hz, C–F), 147.9 (C-5), 160.0 (C=O). ^{19}F NMR (acetone-d_6), δ (ppm): −90.0 (m, 2F), −144.2 (m, 2F). ESI-MS *m/z*: 372 (M^+). HR ESI-MS ($M+H^+$) *m/z*: Calcd for $C_{15}H_8F_4N_2O_3S$: 373.02645. Found: 373.02658.

4.1.2.7. 2-(2,3,4,5,6-Pentafluorophenyl)-4,5,6,7-tetrahydro-1,2-benzisothiazol-3(2*H*)-one 1,1-dioxide (24). Yield: 39%, mp: 143–145 °C. UV (EtOH), λ_{max} (nm): 215 (4.09). IR (KBr), ν (cm^{-1}): 1745 (CO), 1352 (SO_2), 1186 (SO_2). ^{1}H NMR (DMSO-d_6), δ (ppm): 1.93 (m, c, 4H, $2CH_2$), 2.57 (m, c, 2H, CH_2), 2.69 (m, c, 2H, CH_2). ^{13}C NMR (DMSO-d_6), δ (ppm): 19.2, 20.3, 20.5, 20.6 (CH_2), 136.7 (C-3a), 148.7 (C-7a), 159.3 (C=O). ^{19}F NMR (acetone-d_6), δ (ppm): −143.3 (m, 2F), −148.6 (m, 1F), −161.7 (m, 2F). ESI-MS *m/z*: 353 ($M^{+\cdot}$). Anal. Calcd for $C_{13}H_8F_5NO_3S$ (353.27): C, 44.20; H, 2.28; N, 3.96; S, 9.08. Found: C, 44.00; H, 3.01; N, 4.15; S, 8.89.

4.1.2.8. 2-(Pyridin-4-yl)-4,5,6,7-tetrahydro-1,2-benzisothiazol-3(2*H*)-one 1,1-dioxide perchlorate (25). Yield: 41%, mp: 230–235 °C. UV (EtOH), λ_{max} (nm): 264 (3.87). IR (KBr), ν (cm^{-1}): 1728 (CO), 1348 (SO_2), 1164 (SO_2), 1085 ClO_4^-. ^{1}H NMR (acetone-d_6), δ (ppm): 1.67 (m, c, 4H, $2CH_2$), 2.42 (m, c, 2H, CH_2), 2.52 (m, c, 2H, CH_2), 7.08 (d, J = 7.6 Hz, 2H, aromat. H), 8.29 (d, J = 7.6 Hz, 2H, aromat. H). ^{13}C NMR (acetone-d_6), δ (ppm): 19.3, 21.8, 22.6, 24.9 (CH_2), 114.8 (aromat. C–H), 132.4 (C-3a), 143.7 (aromat. C), 143.9 (aromat. C–H), 145.6 (C-7a), 162.0 (C=O). ESI-MS *m/z*: 265 ($[M-ClO_4^-]^+$). HR ESI-MS *m/z*: Calcd for $C_{12}H_{13}N_2O_3S$: 265.06414. Found: 265.06420. Anal. Calcd for $C_{12}H_{13}ClSN_2O_7$ (364.76): Calcd for N, 7.68. Found: N, 8.09.

4.2. X-ray crystal structure analysis

Crystal data, the data collection procedure, and structure determination for compound **22** are noted below. Refinement of the structure was performed with SHELX-97.[47] The hydrogen atoms were included from a difference electron density map and refined isotropically. The details of the structure analysis have been deposited at the Cambridge Crystallographic Data Centre with the number CCDC-67542. These data can be obtained, free of charge, from CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK (fax: +44 1233 336033; e-mail: deposit@ccdc.cam.ac.uk; internet: http://www.ccdc.cam.ac.uk).

Crystal data for **22**: $C_{10}H_6F_4N_2O_3S$; M = 310.23; T = 213(2) K; triclinic; space group $P\bar{1}$; unit cell dimensions a = 11.227(2) Å, b = 11.285(3) Å, c = 15.041(5) Å, α = 70.74(3)°, β = 85.21(3)°, γ = 88.88 (3)°, V = 1781.7(8) Å^3, Z = 6, D_c = 1.729 g/cm^3, μ = 0.334 mm^{-1}; crystal size 0.4 × 0.15 × 0.05 mm^3; θ range for data collection 2.4–28.2°; index range $-14 \leqslant h \leqslant 14$, $-14 \leqslant k \leqslant 14$, $-19 \leqslant l \leqslant 19$; reflections collected 17,678; independent reflections 8084 [R_{int} = 0.035]; max./min. transmission 0.9835/0.8779; data/parameters 8084/613; goodness-of-fit on F^2 0.866; final R indices [$I > 2\sigma(I)$] R_1 = 0.0393, ωR_2 = 0.0931; R indices (all data) R_1 = 0.0595, ωR_2 = 0.0990; largest diff. peak/hole 0.305/−0.244 e Å^{-3}.

4.3. Enzymatic assays

4.3.1. HLE inhibition assay

Human leukocyte elastase was assayed spectrophotometrically at 405 nm at 25 °C. Assay buffer was 50 mM sodium phosphate buffer, 500 mM NaCl, pH 7.8. An enzyme stock solution of 50 μg/ml was prepared in 100 mM sodium acetate buffer, pH 5.5 and diluted with assay buffer. Inhibitor stock solutions were prepared in DMSO. A stock solution of the chromogenic substrate MeOSuc-Ala-Ala-Pro-Val-pNA was prepared in DMSO and diluted with assay buffer. The final concentration of DMSO was 3.5% and the final concentration of the chromogenic substrate MeOSuc-Ala-Ala-Pro-Val-pNA was 100 μM, unless stated otherwise. Assays were performed with a final HLE concentration of 50 ng/ml, which corresponded to an initial rate of 0.6 μM/min, when 100 μM of the substrate was used. Into a cuvette containing 870 μl assay buffer, 30 μl of an inhibitor solution and 50 μl of the substrate solution were added and thoroughly mixed. The reaction was initiated by adding 50 μl of the HLE solution and was followed over 10 min. IC_{50} values were calculated from the linear steady-state turnover of the substrate as previously described.[48]

4.3.2. HLE reactivation

HLE was assayed as described above. Into a cuvette containing 860 μl assay buffer, 5 μl of DMSO and 50 μl of the substrate solution were added and thoroughly mixed. The reaction was initiated by adding 50 μl of the HLE solution. After 4.14 min, 25 μl of a 0.25 mM solution of **21** in DMSO was added. After 8.83 min, 10 μl of a 10 mM solution of hydrazine in assay buffer was added. In the reactivation and control experiment, the final concentration of MeOSuc-Ala-Ala-Pro-Val-pNA was 100 μM, of HLE was 50 ng/ml, and of DMSO was 3.2%. In the reactivation experiment, the final concentration of **21** was 6.3 μM and of hydrazine was 100 μM.

4.3.3. Cathepsin G inhibition assay

Human cathepsin G was assayed spectrophotometrically at 405 nm at 25 °C.[49,50]Assay buffer was 20 mM Tris–HCl buffer, 150 mM NaCl, pH 8.4. Inhibitor stock solutions were prepared in DMSO. An enzyme stock solution of 200 mU/ml was prepared in 50 mM sodium acetate buffer, 150 mM NaCl, pH 5.5. A 50 mM stock solution of the chromogenic substrate Suc-Ala-Ala-Pro-Phe-pNA in DMSO was diluted with assay buffer. The final concentration of DMSO was 1.5%, and the final concentration of the substrate Suc-Ala-Ala-Pro-Phe-NHNp was 500 μM. Assays were performed with a final concentration of 2.5 mU/ml of cathepsin G, which corresponded to an initial rate of 0.7 μM/min. Into a cuvette containing 882.5 μl assay buffer, 5 μl of an inhibitor solution and 100 μl of a substrate solution were added and thoroughly mixed. The reaction was initiated by adding 12.5 μl of the cathepsin G solution and was followed over 10 min. IC_{50} values were calculated from the linear steady-state turnover of the substrate.

4.3.4. Trypsin inhibition assay

Trypsin from bovine pancreas was assayed spectrophotometrically at 405 nm at 25 °C. Assay buffer was 20 mM Tris–HCl buffer, 150 mM NaCl, pH 8.4. An enzyme stock solution of 10 μg/ml was prepared in 1 mM HCl and diluted with assay buffer. Inhibitor stock solutions were prepared in DMSO. A 40 mM stock solution of the chromogenic substrate Suc-Ala-Ala-Pro-Arg-pNA in DMSO was diluted with assay buffer. The final concentration of DMSO was 6%, and the final concentration of the substrate Suc-Ala-Ala-Pro-Arg-pNA was 200 μM. Assays were performed with a final concentration of 12.5 ng/ml of trypsin, which corresponded to an initial rate of 1.2 μM/min. Into a cuvette containing 845 μl assay buffer, 55 μl of an inhibitor solution and 50 μl of a substrate solution were added and thoroughly mixed. The reaction was initiated by adding 50 μl of the trypsin solution and was followed over 10 min. IC_{50} values were calculated from the linear steady-state turnover of the substrate, unless stated otherwise.

4.3.5. Cathepsin L inhibition assay

Human cathepsin L was assayed spectrophotometrically at 405 nm at 37 °C. Assay buffer was 100 mM sodium phosphate buffer, pH 6.0, 100 mM NaCl, 5 mM EDTA, 0.01% Brij 35. An enzyme

stock solution of 50 μg/ml in 20 M sodium acetate buffer, pH 5.0, 100 mM NaCl, 10 mM trehalose, 1 mM EDTA, 50% glycerol was diluted 1:100 with assay buffer containing 5 mM DTT and incubated for 30 min at 37 °C. This enzyme solution was diluted 1:5 with assay buffer containing 5 mM DTT. Inhibitor stock solutions were prepared in DMSO. A 10 mM stock solution of the chromogenic substrate Z-Phe-Arg-pNA was prepared with DMSO. The final concentration of DMSO was 3%, and the final concentration of the substrate Z-Phe-Arg-pNA was 100 μM. Assays were performed with a final concentration of 4 ng/ml of cathepsin L, which corresponded to an initial rate of 0.9 μM/min. Into a cuvette containing 930 μl assay buffer, 20 μl of an inhibitor solution and 10 μl of a substrate solution were added and thoroughly mixed. The reaction was initiated by adding 40 μl of the cathepsin L solution and was followed over 10 min. IC_{50} values were calculated from the linear steady-state turnover of the substrate.

4.3.6. ACE inhibition assay

Human ACE was assayed spectrophotometrically at 352 nm at 37 °C.[51] Assay buffer was 50 mM Tris–HCl buffer, 300 mM NaCl, pH 7.5. An enzyme stock solution of 434 μg/ml in 12.5 mM HCl, pH 7.5, 75 mM NaCl, 500 nM $ZnCl_2$, 40% glycerol was diluted 1:100 with assay buffer. After incubation for 10 min at 37 °C, the enzyme solution was stored at 0 °C and used within 90 min. Inhibitor stock solutions were prepared in DMSO. A 300 mM stock solution of the chromogenic substrate FA-Phe-Gly-Gly was prepared in DMSO. The final concentration of DMSO was 3%, and the final concentration of the substrate FA-Phe-Gly-Gly was 3 mM. Complete enzymatic hydrolysis resulted in a decrease in absorbance of 1.5. Assays were performed with a final concentration of 86.8 ng/ml of ACE, which corresponded to an initial rate of 17 μM/min. Into a cuvette containing 950 μl assay buffer, 20 μl of an inhibitor solution and 10 μl of a substrate solution were added and thoroughly mixed. The reaction was initiated by adding 20 μl of the ACE solution and was followed over 20 min. IC_{50} values were calculated from the linear steady-state turnover of the substrate.

4.3.7. AChE inhibition assay

Acetylcholinesterase inhibition was assayed spectrophotometrically at 412 nm at 25 °C.[52–54] Assay buffer was 100 mM sodium phosphate, 100 mM NaCl, pH 7.3. The enzyme stock solution (~100 U/ml) in assay buffer was kept at 0 °C. Appropriate dilutions were prepared immediately before starting the measurement. ATCh (10 mM) and DTNB (7 mM) were dissolved in assay buffer and kept at 0 °C. Stock solutions of the test compounds were prepared in acetonitrile. The final concentration of acetonitrile was 6%, of ATCh was 500 μM, and of DTNB was 350 μM. Assays were performed with a final concentration of ~30 mU/ml of AChE, which corresponded to an initial rate of 1 μM/min. Into a cuvette containing 830 μl assay buffer, 50 μl of the DTNB solution, 50 μl acetonitrile, 10 μl of a solution of the test compound, and 10 μl of an enzyme solution (~3 U/ml) were added and thoroughly mixed. After incubation for 15 min at 25 °C, the reaction was initiated by adding 50 μl of the ATCh solution.

4.3.8. CEase inhibition assay

Cholesterol esterase inhibition was assayed spectrophotometrically at 405 nm at 25 °C.[55,56] Assay buffer was 100 mM sodium phosphate, 100 mM NaCl, pH 7.0. A stock solution of CEase was prepared in 100 mM sodium phosphate buffer, pH 7.0 and kept at 0 °C. A 1:122 dilution was done immediately before starting the measurement. TC (12 mM) was dissolved in assay buffer and kept at 25 °C. Stock solutions of all test compounds and of pNPB (20 mM) were prepared in acetonitrile. The final concentration of acetonitrile was 6%, of the substrate pNPB was 200 μM, and of TC was 6 mM. Assays were performed with a final concentration of 10 ng/ml of CEase, which corresponded to an initial rate of 5.0 μM/min. Into a cuvette containing 430 μl assay buffer, 500 μl of the TC solution, 40 μl acetonitrile, 10 μl of the pNPB solution, and 10 μl of a solution of the test compound were added and thoroughly mixed. After incubation for 5 min at 25 °C, the reaction was initiated by adding 10 μl of the enzyme solution (1 μg/ml).

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