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Inhibition of Cathepsin G by 4*H*-3,1-Benzoxazin-4-ones

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Abstract—A series of 4*H*-3,1-benzoxazin-4-ones is reported that inhibit the serine proteases human cathepsin G and bovine chymotrypsin. The synthesis and kinetic parameters of the alkaline hydrolysis is described. These compounds act as acyl-enzyme inhibitors of both enzymes. The reaction of cathepsin G with 2-benzylamino-4*H*-3,1-benzoxazin-4-one (**20**) was studied in detail. A partition in deacylation of the initially formed acyl-enzyme was observed, leading to the formation of 2-(3-benzylureido)benzoic acid (**26**) and 3-benzylquinazoline-2,4-(1*H*,3*H*)-dione (**27**). A 6-methyl substitution strongly increased the acylation rate of both proteases. Introduction of an aryl moiety into the 2-substituent led to compounds with K_i values towards cathepsin G in the nanomolar range. Their inhibitory potency is stronger than that of other synthetic inhibitors of cathepsin G. © 1997 Elsevier Science Ltd.

Introduction

Human leukocyte elastase (HLE), cathepsin G, and proteinase 3 are serine proteases that are stored in the azurophilic granules of polymorphonuclear leukocytes. Migration of the neutrophiles into the tissue and the release of hydrolytic enzymes is a marker event in acute inflammation. When inadequately controlled by their physiological inhibitors, the serine endopeptidases can proteolytically degradate the major components of the extracellular matrix. The pathogenesis of lung diseases such as adult respiratory distress and emphysema is thought to result from an imbalance between leukocyte serine proteases (such as HLE and cathepsin G) and their endogenous inhibitors.[1–3] Both enzymes are also involved in the proteolytic degradation of the articular cartilage.[4] The major focus to develop inhibitors of neutrophil proteolytic enzymes has centered on HLE. Cathepsin G, however, is also thought to play a prominent role in inflammatory states. Cathepsin G specifically generates the chemokine NAP-2 by proteolytic cleavage of its precursor, the platelet derived peptide CTAP-III.[5] Recently, it has been suggested that a cathepsin G-like membrane enzyme is involved in the control of neutrophil chemotaxis.[6] Furthermore, cathepsin G has been shown to induce platelet aggregation[7] and to generate angiotensin II.[8]

The involvement of cathepsin G in these processes provides the impetus for the development of synthetic inhibitors. The inhibition of cathepsin G by peptide halomethyl ketones,[9,10] peptide α-ketoesters,[3] 4-chloroisocoumarins,[11,12] *N*-hydroxysuccinimide derivatives,[13] anthraquinones,[14] and β-lactames[15] has been reported. A concept of dual inhibition of both HLE and cathepsin G has been demonstrated with bis-peptidylterephthalamides.[16]

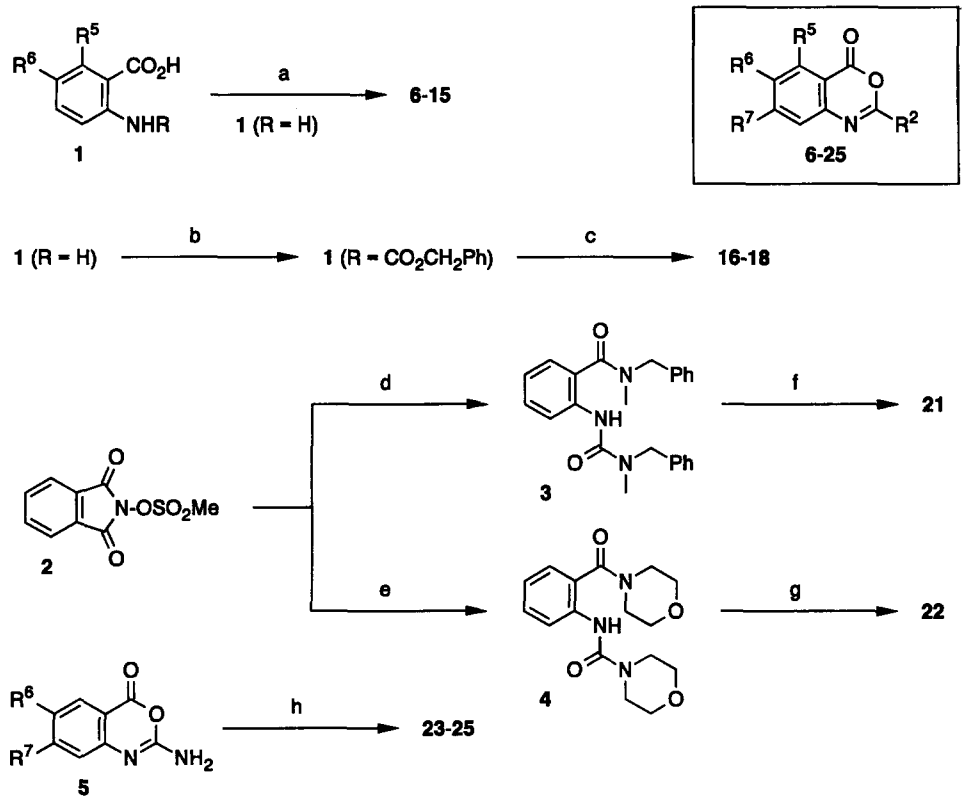
In this study, we have examined the in vitro inhibition of human cathepsin G by a series of 4*H*-3,1-benzoxazin-4-ones. The class of 3,1-benzoxazin-4-ones includes highly potent inhibitors of HLE, previously developed by Krantz and coworkers,[17] but was not examined towards cathepsin G so far. The primary specifity site S_1 of cathepsin G is larger than that of HLE, showing a strong preference for aromatic amino acids.[18] The inhibitory potential of the present series of 3,1-benzoxazin-4-ones towards bovine chymotrypsin, which shares the primary substrate specificity of cathepsin G, was also determined in this study.

Results

Chemistry

2-Alkoxybenzoxazinones **6–14** (Table 1) and the phenoxy derivative **15** were prepared according to a described procedure[17,19] in one step from anthranilic acids and the appropriate chloroformate (Scheme 1). The synthesis of 2-benzyloxy derivatives was accomplished via the corresponding benzylurethanes which were isolated and subsequently cyclized with ethyl chloroformate to give compounds **16–18**. 2-Alkylamino derivatives **19** and **20** were prepared by cyclizing the corresponding methyl 2-(3-alkylureido)benzoates in concentrated sulfuric acid.[20] A convenient route was employed to prepare compounds **21** and **22**. The ureidobenzamides **3** and **4** were obtained by transformation of mesyloxyphthalmide **2** with the appropriate secondary amine and subsequently cyclized upon treatment with concentrated sulfuric acid. The 2-benzoylaminobenzoxazinones **23–25** were available from benzoylation of the 2-aminobenzoxazinones **5**.

Aqueous alkaline hydrolysis of 2-alkoxy and 2-aminobenzoxazinones proceeds by hydroxide attack at C-4[17,21]



Scheme 1. (a) $ClCO_2R^2$ (4.5 equiv), pyridine, 0–20 °C; (b) $ClCO_2CH_2Ph$ (1.2 equiv), pyridine, 0 °C; (c) $ClCO_2Et$ (4.5 equiv), pyridine, 0–20 °C; (d) *N*-benzylmethylamine, toluene, 0–20 °C; (e) morpholine, acetone, reflux; (f) concd H_2SO_4, 0 °C; (g) concd H_2SO_4, 20 °C; (h) $(PhCO)_2O$, toluene, reflux.

and can therefore be used as a model for enzyme acylation and, furthermore, to estimate the compounds stability. 2-Alkoxybenzoxazinones unsubstituted at the benzene unit exhibited similar hydrolytic rates, independently on the size of the alkyl residues. Introduction of the phenoxy substituent (compound **15**) led to an

Table 1. 4*H*-3,1-Benzoxazin-4-ones prepared and kinetic parameters of the alkaline hydrolysis[a]

Compound	R^2	R^5	R^6	R^7	k_{OH} (M^{-1} s^{-1})	log k_{OH}
6	O-Me	H	H	H	61.2	1.78
7	O-Et	H	H	H	50.5	1.70
8	O-Et	H	Me	H	47.7	1.68
9	O-CH_2CH_2Cl	H	H	H	71.9	1.86
10	O-*n*-Pr	H	H	H	52.7	1.72
11	O-*n*-Pr	H	Me	H	40.9	1.61
12	O-$CH_2CH{=}CH_2$	H	H	H	60.6	1.78
13	O-*i*-Bu	H	H	H	47.6	1.67
14	O-*i*-Bu	H	Me	H	46.4	1.66
15	O-Ph	H	H	H	109.6	2.04
16	O-CH_2Ph	H	H	H	71.5	1.85
17	O-CH_2Ph	H	Me	H	48.5	1.68
18	O-CH_2Ph	Me	H	H	19.5	1.29
19	NH-*i*-Pr	H	H	H	12.5	1.09
20	NH-CH_2Ph	H	H	H	17.0	1.23
21	N(Me)CH_2Ph	H	H	H	20.5	1.32
22	Morpholino	H	H	H	27.7[b]	1.44
23	NH-COPh	H	H	H	1.4[b,c]	0.16
24	NH-COPh	H	Me	H	ND[d]	
25	NH-COPh	H	OMe	OMe	ND[d]	

[a]Assay conditions for alkaline hydrolysis: 50 mM Ches, pH 9.5; 25 °C; compound concentration 50 μM; final MeCN concentration 5%; 315 nm.
[b]Data from ref 21.
[c]Anionic form, see ref 21.
[d]Not determined.

increased hydrolytic rate, whereas the aminosubstituted derivatives **19–22** possessed considerable hydrolytic stability, both reflecting the dependence of k_{OH^-} on electron withdrawal of the 2-substituent. Introduction of a 6-methyl group into 2-alkoxybenzoxazinones led to slightly more resistant derivatives (**7** vs. **8**, **10** vs. **11**, **13** vs. **14**, and **16** vs. **17**). Comparative information on the hydrolytic stablity was reflected by ^{1}H NMR data. 6-Methyl substitution produced a small but significant upfield shift of the OCH_2 protons, when compared to the values of the corresponding unsubstituted analogs (Table 2). This substituent effect on chemical shift was also observed in the 5-methyl derivative **18**. Compound **18**, however, is hydrolytically more stable indicating a steric interaction that affects the rate of hydroxide attack at C-4.[17]

Enzymatic studies

The present series of benzoxazinones was assayed as inhibitors of human cathepsin G and bovine chymotrypsin in the presence of Suc-Ala-Ala-Pro-Phe-pNA as the substrate. Progress curves were characterized by an initial exponential phase, followed by a linear steady-state turnover of the chromogenic substrate and could be analyzed by slow-binding kinetics.[22]

To determine inhibition constants, slow-binding progress curves were fitted by nonlinear regression to equation 1,

$$[P] = v_S t + (v_0 - v_S)[1 - \exp(-k_{obs} t)]/k_{obs} + \text{offset} \quad (1)$$

where v_S and v_0 are the steady-state and the initial

Table 2. 2-Alkoxy-4*H*-3,1-benzoxazin-4-ones **6–18**: Physical constants and spectral data

Compd	Yield (%)	Mp (°C)	Elemental analysis calcd (found) or lit. mp	^{1}H NMR ($CDCl_3$) δ (ppm), *J* (Hz)
6	47	92.5–93.5	C 61.02 (60.99), H 3.98 (4.30), N 7.91 (7.98)	4.09 (s, 3 H, CH_3), 7.33–7.47 (m, 2 ArH), 7.71–7.78 (m, 1 H, H-7), 8.11–8.16 (m, 1 H, H-5)
7	88	87–89	91 °C (ref 24)	1.46 (t, 3 H, *J* = 7.1, CH_3), 4.53 (q, 2 H, *J* = 7.1, CH_2), 7.32–7.44 (m, 2 ArH), 7.70–7.76 (m, 1 H, H-7), 8.13 (dd, 1 H, *J* = 7.9, 1.5, H-5)
8	91	119–120	C 64.38 (64.65), H 5.40 (5.65), N 6.83 (7.20)	1.43 (t, 3 H, *J* = 7.1, CH_3), 2.39 (s, 3 H, 6-CH_3), 4.48 (q, 2 H, *J* = 7.1, CH_2), 7.27 (d, 1 H, *J* = 8.2, H-8), 7.50 (dd, 1 H, *J* = 8.2, 2.1, H-7), 7.86 (d, 1 H, *J* = 2.1, H-5)
9	87	108–109	C 53.23 (53.07), H 3.57 (3.40), N 6.21 (6.21), Cl 15.71 (15.63)	3.86 (t, 2 H, *J* = 5.8, CH_2Cl), 4.69 (t, 2 H, *J* = 5.8, CH_2), 7.32–7.41 (m, 2 ArH), 7.69–7.75 (m, 1 H, H-7), 8.09 (dd, 1 H, *J* = 8.0, 1.4, H-5)
10[a]	60	57–58	C 64.38 (64.45), H 5.40 (5.19), N 6.83 (6.89)	1.05 (t, 3 H, *J* = 7.4, CH_3), 1.85 (m, 2 H, CH_2CH_3), 4.41 (t, 2H, *J* = 6.6, OCH_2), 7.30–7.42 (m, 2 ArH), 7.68–7.74 (m, 1 H, H-7), 8.11 (dd, 1 H, *J* = 7.8, 1.6, H-5)
11	93	83–84	C 65.74 (66.03), H 5.89 (5.74), N 6.39 (6.51)	1.04 (t, 3 H, *J* = 7.4, CH_3), 1.83 (m, 2 H, CH_2CH_3), 2.41 (s, 3 H, 6-CH_3), 4.39 (t, 2 H, *J* = 6.6, OCH_2), 7.30 (d, 1 H, *J* = 8.2, H-8), 7.50–7.54 (m, 1 H, H-7), 7.89 (s, br, 1 H, H-5)
12[b]	16	53–53.5	C 65.02 (64.75), H 4.46 (4.84), N 6.89 (7.15)	4.96 (m, 2 H, *J* = 5.7, OCH_2), 5.36 and 5.48 (2 H, each dq, *J* = 10.5, 1.1 and *J* = 15.6, 1.4, $=CH_2$), 6.07 (ddt, 1 H, *J* = 15.6, 10.5, 5.7, CH), 7.32–7.44 (m, 2 ArH), 7.69–7.76 (m, 1 H, H-7), 8.12 (dd, 1 H, *J* = 7.9, 1.3, H-5)
13	66	52–54	57–58 °C (ref 17)	1.04 (d, 6 H, *J* = 6.8, CH_3), 2.14 (m, 1 H, CH), 4.23 (d, 2 H, *J* = 6.6, CH_2), 7.33–7.42 (m, 2 ArH), 7.68–7.72 (m, 1 H, H-7), 8.09–8.13 (m, 1 H, H-5)
14[c]	49	46–47	C 66.94 (67.26), H 6.48 (5.98), N 6.00 (6.08)	1.02 (d, 6 H, *J* = 6.7, CH_3), 2.11 (m, 1 H, CH), 2.39 (s, 3 H, 6-CH_3), 4.18 (d, 2 H, *J* = 6.6, CH_2), 7.27 (d, 1 H, *J* = 8.3, H-8), 7.49 (dd, 1 H, *J* = 8.3, 1.9, H-7), 7.86 (s, br, 1 H, H-5)
15[d]	81	143–145	C 70.29 (69.85), H 3.79 (3.69), N 5.85 (5.76)	7.30–7.41 (m, 5 ArH), 7.43–7.50 (m, 2 H, H-3′), 7.67–7.74 (m, 1 H, H-7), 8.16 (dd, 1 H, *J* = 1.5, 7.8, H-5)
16	95	112–112.5	112–113 °C (ref 17)	5.50 (s, 2 H, CH_2), 7.33–7.53 (m, 7 ArH), 7.71–7.77 (m, 1 H, H-7), 8.13 (dd, 1H, *J* = 1.6, 7.9, H-5)
17	84	94–96	C 71.90 (71.75), H 4.90 (4.94), N 5.24 (5.55)	2.44 (s, 3 H, CH_3), 5.48 (s, 2 H, CH_2), 7.35–7.51 (m, 6 ArH), 7.54 (dd, 1 H, *J* = 8.3, 2.1, H-7), 7.92 (s, br, 1 H, H-5)
18	69	97–99	C 71.90 (71.84), H 4.90 (4.97), N 5.24 (5.34)	2.75 (s, 3 H, CH_3), 5.48 (s, 2 H, CH_2), 7.12–7.15 (m, 1 H, H-6), 7.27–7.31 (m, 1 H, H-8), 7.36–7.51 (m, 5 ArH), 7.53–7.59 (m, 1 H, H-7)

[a] The crude product was extracted with ether and recrystallized from *n*-hexane.
[b] The crude product was extracted with ether, purified by column chromatography (ethyl acetate/*n*-hexane) and recrystallized from *n*-hexane.
[c] ^{13}C NMR ($CDCl_3$) δ 18.9 (CH_3), 20.9 (6-CH_3), 27.7 (CH), 75.8 (CH_2), 114.1 (C-4a), 125.1 (C-8), 128.4 (C-5), 135.8 (C-6), 138.0 (C-7), 146.2 (C-8a), 154.5 (C-2), 159.7 (C-4).
[d] ^{13}C NMR ($CDCl_3$) δ 114.7 (C-4a), 121.3 (C-2′), 125.9 (C-4′), 126.5 (C-8), 126.6 (C-6), 129.1 (C-5), 129.7 (C-3′), 136.9 (C-7), 147.8 (C-8a), 151.4 (C-1′), 154.2 (C-2), 159.2 (C-4). MS (70 ev): *m/z* (%) 239 (M^+, 10), 146 (M^+–OPh, 100).

velocity, respectively, and k_{obs} is the first-order rate constant for the approach of the steady-state. The values obtained for k_{obs} were plotted versus the inhibitor concentration, $[I]$, and a linear regression gives the apparent association rate constant, k'_{on}, as the slope:

$$k_{obs} = k'_{on}[I] + k_{off} \quad (2)$$

The association rate constant, k_{on}, is calculated using $k_{on} = k'_{on}\,(1 + [S]/K_m)$. The apparent dissociation constant, K'_i, was calculated using the steady-state values, v_S, together with the rate for the reaction without inhibitor, and fitting them to the equation of a competitive inhibition:

$$v_S = v_0/[([I]/K'_i) + 1] \quad (3)$$

K_i was calculated from: $K_i = K'_i/[1 + ([S]/K_m)]$. For cathepsin G, $[S] \ll K_m$, therefore $k'_{on} = k_{on}$ and $K'_i = K_i$. The dissociation rate constant, k_{off}, was calculated from: $k_{off} = k_{on}\,K_i$.

The kinetic model of slow-binding inhibition can be used for the analysis of acylating inhibitors of serine proteases. In this case, the rate constants, k_{on} and k_{off}, reflect the acylation and deacylation step, respectively, (Scheme 2) and not the simple association-dissociation equilibrium of a competitive inhibition. However, the potency of such inhibitors is usually expressed as K_i values.[17,27] Since it has been demonstrated that 3,1-benzoxazinones act as acyl-enzyme inhibitors of elastases and chymotrypsin,[17,21,23–27] we have proposed a similar interaction in our present investigations. This was further established by a product analysis experiment (see below).

As an example, the analysis of the inhibition kinetics of cathepsin G by 2-benzylamino-4*H*-3,1-benzoxazin-4-one (**20**) is illustrated in Figures 1–3. The kinetic data for the whole set of benzoxazinones are outlined in Table 3.

In the alkoxybenzoxazinone series (**6–18**), acylation rate k_{on} strongly depended on the size of the substitutent R^2. Comparision of the unsubstituted derivatives ($R^5 = R^6 = H$) shows, that the introduction of a bulky aliphatic residue (compound **13**) and, even more drastically, an aromatic moiety (**15** and **16**) accelerated both cathepsin G and chymotrypsin acylation. The deacylation rate k_{off} was not affected by the 2-alkoxy substituent, with the exception of the phenoxy derivative **15** which showed a strongly increased deacylation rate in the case of cathepsin G inhibition. Introduction of a 6-methyl group affected acylation, as well as deacylation of both cathepsin G and chymotrypsin (**7** vs. **8**, **10** vs. **11**, **13** vs.

$$E + I' \underset{}{\overset{k_2}{\longleftarrow}} E\text{-}I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} I + E + S \overset{K_m}{\rightleftharpoons} E{\cdot}S \xrightarrow{k_{cat}} E + P$$

Scheme 2. Kinetic model for acyl-enzyme inhibition of serine proteases in the presence of substrate. E–I and I' represent acyl-enzyme, and modified inhibitor, respectively. Acylation rate: $k_{on} = k_1$; deacylation rate: $k_{off} = k_{-1} + k_2$.

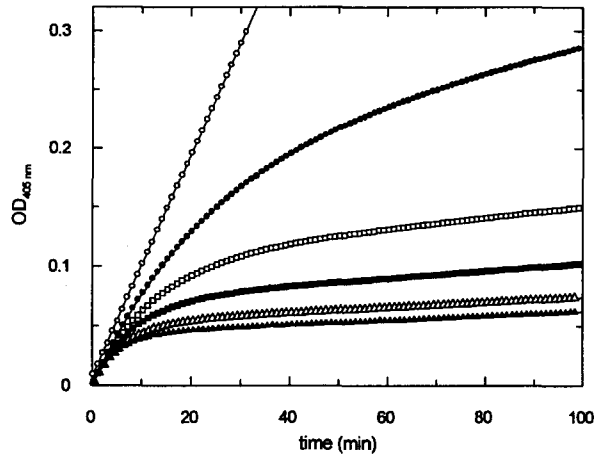


Figure 1. Slow-binding inhibition of human cathepsin G by compound 20 in 50 mM Hepes, 500 mM NaCl, pH 7.0. Substrate: Suc-Ala-Ala-Pro-Phe-pNA, 500 μM. Data were fitted to equation (1) to obtain the best-fit parameters for v_0, v_S, k_{obs}, and offset. Open circles: $[I] = 0$; full circles: $[I] = 100$ nM; open squares: $[I] = 200$ nM; full squares: $[I] = 300$ nM; open triangles: $[I] = 400$ nM; and full triangles: $[I] = 500$ nM.

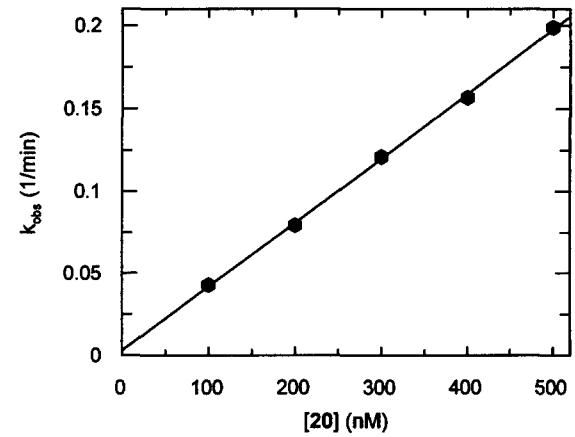


Figure 2. Plot of k_{obs} versus $[I]$ for the inhibition of human cathepsin G by compound 20. The values for k_{obs} were obtained from fits to the data shown in Figure 1. The solid line was drawn using the best-fit parameters to equation (2) and the slope corresponds to a value for $k'_{on} = k_{on} = 6494\ M^{-1}\ s^{-1}$.

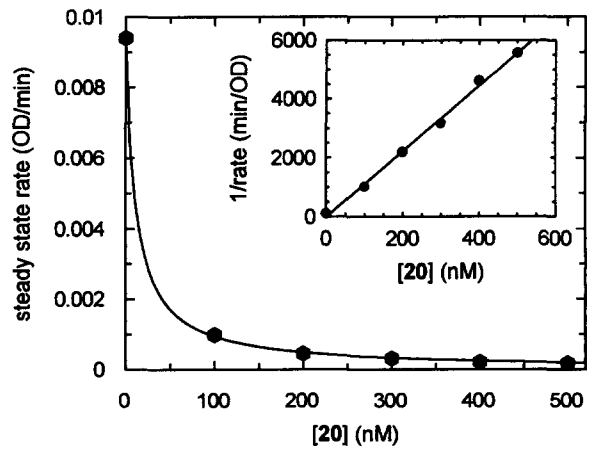


Figure 3. Plot of the steady-state rates versus $[I]$ for the inhibition of human cathepsin G by compound 20. The data were obtained from fits of the curves shown in Figure 1. The solid line was drawn using the best-fit parameters from a fit according to equation (3), which gave $K_i = 11.1 \pm 0.4$ nM. The insert is a Dixon plot to show the linearity.

Table 3. Kinetic parameters for the inhibition of cathepsin G and chymotrypsin by 4*H*-3,1-benzoxazin-4-ones

	Human cathepsin G				Bovine chymotrypsin			
Compd	k_{on} (M^{-1} s^{-1})	k_{off} (10^{-4} s^{-1})	K_i (μM)	pK_i	k_{on} (10^3 M^{-1} s^{-1})	k_{off} (10^{-4} s^{-1})	K_i (nM)	pK_i
6	48.5	3.1	6.5	5.19	1.5	1.2	81.3	7.09
7[a]	167	2.1	1.3	5.90	10.8[b,c]	1.5	13.7	7.86
8	1560	9.3	0.6	6.22	108[c]	13.1	12.1	7.91
9	384	3.2	0.8	6.07	16.1	2.8	18.0	7.74
10	566	1.7	0.3	6.45	24.1	2.8	11.6	7.94
11	3030	18.4	0.6	6.41	389	10.1	2.6	8.59
12	542	3.4	0.6	6.19	66.5	1.1	1.6	8.80
13	1410	1.4	0.1	7.01	159[c]	1.6	1.02	8.95
14	7100	11.9	0.17	6.78	1020	14	1.4	8.86
15	12,100	13	0.11	6.97	3720	4.1	0.11	9.96
16	7020	2.4	0.03	7.45	1650	2	0.12	9.92
17	70,300	11.6	0.02	7.79	14,600	10	0.07	10.2
18	528	1.2	0.23	6.63	1340	2.6	0.19	9.7
19	1150	0.5	0.05	7.32	4.9	1.4	27.8	7.55
20	6500	0.9	0.01	7.92	1360	14	1.03	8.99
21	1300	0.2	0.01	7.96	39.3	1.3	3.3	8.48
22	64.3	0.2	0.24	6.61	1.75	1.17	67	7.17[d]
23	870	30	3.4	5.46	211	17	8	8.09[d]
24	8570	28	0.3	6.52	6170	60.4	0.98	9.01
25	1570	16	1.02	5.99	408	3.1	0.8	9.11[e]

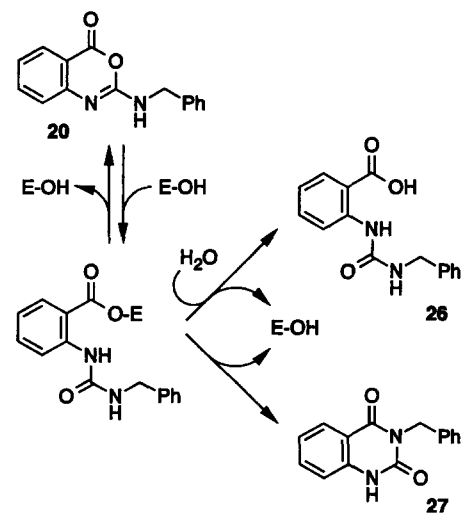
[a] Inhibition of HLE by the following compounds has been reported by Krantz et al.[17] (pK_i values in parentheses): 7 (8.19), 13 (7.94), 16 (7.25), 19 (7.28), 20 (6.02), and 22 (6.15).
[b] Hedstrom et al.[24] reported $k_{on} \geq 700 \times 10^3$ M^{-1} min^{-1} at pH 6.8 in 0.1 M phosphate buffer.
[c] Similar acylation rate constants have been obtained at pH 8.0 in 50 mM Tris–HCl.[25]
[d] Data from ref 21.
[e] Our previously reported values[26] for chymotrypsin inhibition with compound 25 have to be corrected.

14, and **16** vs. **17**). Values for k_{on} were found to be increased 5–16-fold, and k_{off} values were increased 4–11-fold. A distinct reduction of the rate of cathepsin G acylation was observed, when a 5-methyl group was introduced in the 2-benzyloxybenzoxazinone structure (**16** vs. **18**). The chloroethoxy and allyloxy derivatives (**9** and **12**) did not show deviations in their kinetic behaviour, indicating that these compound fail to act like bifunctional reagents.[23]

Within the amino/benzoylamino substituted series (**19**–**25**), a fast acylation of cathepsin G was observed in the case of the benzylamino derivative **20**. By replacing the amino hydrogen of **20** by methyl (compound **21**) both acylation and deacylation rate were decreased. Compounds **20** and **21** exhibited the best K_i values in the whole set of compounds towards cathepsin G. Again, it was found, that the introduction of a 6-methyl group (into the 2-benzoylamino derivative **23**) led to a tenfold accelerated acylation of cathepsin G (**23** vs. **24**) The k_{off} values of the amino compounds **19**–**22** were generally much lower than the values for the acylamino derivatives **23**–**25**.

Towards chymotrypsin, among the amino substituted derivatives (**19**–**22**) only **20** showed a rapid acylation. Introduction of a 6-methyl group in the case of **24** resulted in a 30-fold increased k_{on} value when compared to the 6-unsubstituted analogue **23**.

We have investigated the product formation of the reaction of the benzylamino derivative **20** with cathepsin G and chymotrypsin. Compound **20** was incubated with either cathepsin G or chymotrypsin and the mixture was analyzed by HPLC. The reference substances **26** and **27** were independently prepared. The reaction of cathepsin G with **20** led to the formation of the benzoic acid derivative **26** and the quinazolinedione **27** in an approximately 1:1 ratio (Scheme 3). Reaction of chymotrypsin with **20** was much faster and gave almost exclusively the quinazolinedione **27**.



Scheme 3. Reaction of 20 with cathepsin G and chymotrypsin. Formation of the acyl-enzyme and possible ways of deacylation.

Discussion

The present investigation has been undertaken to evaluate the inhibitory activity of a series of 4*H*-3,1-benzoxazin-4-ones towards cathepsin G. Compounds **6–25** act as acyl-enzyme inhibitors for cathepsin G, as it can be concluded from their kinetic behaviour as well as from the product analysis experiment with compound **20**.

Acylation rate was strongly affected by the nature of the 2-substituent. The highest k_{on} values were obtained with compounds bearing an aromatic substituent at 2-position. The effect of R^2 could be demonstrated in the case of the alkoxy derivatives with $R^5 = R^6 = H$. Six compounds (**6**, **7**, **10**, **12**, **13**, and **16**) with similar reactivity in alkaline hydrolysis, exhibited k_{on} values toward cathepsin G that span two orders of magnitude. Cathepsin G prefers substrates with large hydrophobic aromatic side chains.[18] The acylation rate that depended on the structure of the 2-substituent strongly indicates that this substituent interacts with the S_1 subsite of cathepsin G. Also in the case of chymotrypsin inhibition, R^2 is assumed to be accommodated at the primary specifity site S_1.

Introduction of a 6-methyl group (**8**, **11**, **14**, **17**, and **24**) led to slightly reduced rates of alkaline hydrolysis. Unexpectedly, the rates of cathepsin G acylation were distinctly increased when compared to the k_{on} values of the unsubstituted analogues. Since deacylation was also found to be accelerated (except in the case **24**), 6-methyl substitution did not generally produce more active inhibitors. However, when combining 2-benzyloxy and 6-methyl substitution the highly potent compound **17** (K_i = 20 nM) was achieved. A similar effect of 6-methyl substitution was observed in chymotrypsin inhibition. We conclude, that a specific interaction between the 6-methyl group and both enzymes is responsible for the acceleration of the acylation and deacylation steps.

Acyl-enzymes formed by the reaction of serine proteases and 2-alkylamino-4*H*-3,1-benzoxazin-4-ones can deacylate by three different ways, via quinazoline cyclization, hydrolysis to the free acid, and reversion to the benzoxazinone (e.g., Scheme 3). It has been first demonstrated by Hedstrom and co-workers[24] that the tendency to deacylate via quinazoline cyclization increases k_{off}.[21,27] We have observed high k_{off} values in the cases of cathepsin G inhibition with benzoylamino derivatives **23–25**. This indicates that the corresponding acyl-enzymes deacylate via quinazoline cyclization. Slow deacylation was found for compounds **19–22** ($k_{off} < 1 \times 10^{-4}$ s^{-1}). The branched group (NH-*i*-Pr) in **19** probably blocks quinazoline cyclization from the cathepsin G (and chymotrypsin) derived acyl-enzymes, as it has been reported for HLE.[27] Compounds **21** and **22** bearing a secondary amino substituent are unable to undergo quinazoline cyclization. With compound **20**, deacylation rate increased only moderate in the case of cathepsin G, but much stronger in the case of chymotrypsin. We have therefore analyzed the product formation in the reaction of **20** with both enzymes. Indeed, we found exclusively quinazoline formation with chymotrypsin, whereas cathepsin G deacylation occured to form the quinazoline **27** together with the free acid **26** (Scheme 3).

Within the present series of benzoxazinones, extremly potent inhibitors for chymotrypsin were obtained with K_i values in the pM range (**15–18**). Strong cathepsin G inhibition was always accompanied by strong chymotrypsin inhibition. A preference for cathepsin G over chymotrypsin is difficult to achieve with heterocyclic acyl-enzyme inhibitors since the enzymes catalytic activity is prerequisite to inhibition. Cathepsin G, as the less potent proteolytic enzyme, should a priori show a decreased rate of reaction with such mechanism-based inhibitors. As reported,[17] incorporation of an aryl structure into the 2-substituent adversely affects HLE inhibition, but was found in this study to enhance cathepsin G inhibition drastically. Whereas substitution at the 6-position was unfavourable in HLE inhibition,[17] we have observed an accelerated acylation of cathepsin G with 6-methyl derivatives. On the other hand, 5-alkyl substitution generally resulted in a better HLE inhibition[17] but was unfavourable in cathepsin G inhibition in the case of the benzyloxy compounds (**16** vs. **18**). Further investigations are needed to confirm these trends to provide inhibitors with a preference for cathepsin G over HLE.

The potency of the best inhibitors of this series is more evident when compared to known low molecular weight inhibitors of cathepsin G. Heterocyclic inactivators such as saccharin derivatives,[28,29] sulfonyloxysuccinimides,[13] dihydrouracils,[30] and β-lactams[15] exhibit second-order inactivation rate constants ($k_{obs}/[I]$) less than 5000 M^{-1} s^{-1}.[31] Dichloroisocoumarin (DCI) is a poor inhibitor for cathepsin G ($k_{obs}/[I]$ = 28 M^{-1} s^{-1}),[11] but introduction of an aromatic moiety into the isocoumarin structure resulted in moderate inhibition (3-benzyloxy-4-chloroisocoumarin: $k_{obs}/[I]$ = 1140 M^{-1} s^{-1}).[12] 2-Alkyl-1,8-dihydroxyanthraquinones are competitive inhibitors of cathepsin G with IC_{50} values in the micromolar range.[14] Much stronger inhibition of cathepsin G was achieved with peptidic compounds, such as peptide derived phosphonates,[32] peptidyl fluoromethyl ketones,[10] and α-keto esters.[3] The best low molecular weight inhibitor towards cathepsin G (MeO-Suc-Val-Pro-Phe-COOMe), a transition-state inhibitor developed by Powers and co-workers, exhibit a K_i value of 1.1 μM.[3]

In this study, it was found that 4*H*-3,1-benzoxazin-4-ones are highly potent acyl-enzyme inhibitors of human cathepsin G. The most active compounds of this series (**16**, **17**, **20**, and **21**), showed inhibitory activity stronger than that of other synthetic inhibitors of cathepsin G. Their K_i values (≤30 nM) are in the range of the endogenous inhibitor α-1-antichymotrypsin (K_i = 61 nM, 82 nM, respectively).[33]

Experimental

Melting points were determined on a Boetius apparatus and are not corrected. ^{13}C NMR spectra (75 MHz) and ^1H NMR spectra (300 MHz) were recorded on a Varian Gemini 300. Mass spectra (70 eV) were obtained using a Varian MAT CH6 spectrometer. Preparative column chromatography was performed on silica gel 60 (Merck) 70–230 mesh, using ethyl acetate: *n*-hexane (1:4). All spectroscopic assays were done in a Varian Cary 3 Bio spectrophotometer with six-cell holder. Analytical HPLC was done on a ThermoSeparationProducts liquid chromatograph with PC1000 software. A 5 μm Hypersil ODS 200 × 4.6 mm column was used at a flow rate of 0.5 mL/min. Cathepsin G was purchased from Calbiochem. Chymotrypsin was purchased from Worthington, Freehold, U.S.A. The substrate Suc-Ala-Ala-Pro-Phe-pNA was from Bachem, Bubendorf, Switzerland.

2-Isopropylamino-4*H*-3,1-benzoxazin-4-one (**19**),[17] 2-benzoylamino-4*H*-3,1-benzoxazin-4-one (**23**),[21] and 2-benzoylamino-6,7-dimethoxy-4*H*-3,1-benzoxazin-4-one (**25**)[26] were prepared as reported. 2-Benzylamino-4*H*-3,1-benzoxazin-4-one (**20**)[17] was prepared from methyl 2-(3-benzylureido)benzoate[34] and purified on column chromatography. 2-(3-Benzylureido)benzoic acid (**26**)[35] was obtained by hydrolytic cleavage[21] of compound **20**. 3-Benzylquinazoline-2,4(1*H*,3*H*)-dione (**27**)[34] was prepared from methyl 2-(3-benzylureido)benzoate according to a literature procedure.[20]

N-Benzyl-N-methyl-2-(3-benzyl-3-methylureido)benzamide (3). *N*-Benzylmethylamine (4.1 g, 34 mmol) was added dropwise to a suspension of 2-[(methylsulfonyl)oxy]-1*H*-isoindole-1,3-(2*H*)-dione (**2**)[36] (2.41 g, 10 mmol) and toluene (20 mL) at 0 °C and stirring was continued at room temperature over 7 h. The mixture was diluted with brine solution (100 mL) and extracted with ethyl acetate (3 × 50 mL). The organic layer was washed with water and dried (Na_2SO_4). Removal of the solvent in vacuo yielded compound **3** (3.7 g, 95%), mp 96–97 °C (toluene). ^1H NMR ($CDCl_3$) δ 2.96, 2.99 (2s, 6 H, CH_3), 4.62 (s, 2 H, CH_2), 4.66 (s, br, 2 H, CH_2), 7.19–7.42 (m, 13 ArH), 8.24–8.28 (m, 1 ArH), 8.76 (s, 1 H, NH). MS (70 eV): *m/z* (%)387 (M^+, 41), 91 (100). Anal. calcd for $C_{24}H_{25}N_3O_2$: C, 74.39; H, 6.50; N, 10.84. Found: C, 74.56; H, 6.81; N, 10.44%.

4-[2-[(Morpholinocarbonyl)amino]benzoyl]morpholine (4). A mixture of compound **2** (1.81 g, 7.5 mmol) and acetone (25 mL) was stirred at 56 °C under argon atmosphere. A solution of morpholine (1.96 g, 22.5 mmol) in acetone (7 mL) was added dropwise over a period of 10 min. The mixture was refluxed for additional 15 min and the precipitate was removed. The filtrate was evaporated and the residue was recrystallized from MeOH to obtain 2.2 g (87%) of compound **4**, mp 78–80 °C. ^1H NMR ($CDCl_3$) δ 3.47–3.51 (m, 4 H, CH_2), 3.65–3.77 (m, 12 H, CH_2), 6.98–7.04 (m, 1 ArH), 7.17–7.21 (m, 1 ArH), 7.37–7.44 (m, 1 ArH), 8.15–8.19 (m, 1 ArH), 8.83 (s, 1 H, NH). MS (70 eV): *m/z* (%)319 (M^+, 80), 146 (100). Anal. calcd for $C_{16}H_{21}N_3O_4 \cdot H_2O$: C, 56.96; H, 6.87; N, 12.46. Found: C, 57.12; H, 6.79; N, 12.37%.

2-Amino-6-methyl-4*H*-3,1-benzoxazin-4-one (5, R^6 = Me, R^7 = H). 2-Amino-5-methylbenzoic acid was treated with cyanogen bromide according to the reported procedure[37] to obtain pure **5** (68%) as the crude product, mp > 219 °C (conversion at 155–175 °C). ^1H NMR (DMSO-d_6) δ 2.32 (s, 3 H, CH_3), 7.06 (d, 1 H, J = 8.3 Hz, H-8), 7.41 (s, 2 H, NH_2), 7.49 (dd, 1H, J = 8.3, 2.1 Hz, H-7), 7.67 (s, br, 1 H, H-5). Anal. calcd for $C_9H_8N_2O_2$: C, 61.36; H, 4.58; N, 15.90. Found: C, 61.28; H, 4.94; N, 15.59%.

Preparation of compounds 6–15. General procedure.[17,19] The appropriate chloroformate (112.5 mmol) was added dropwise at 0 °C under argon atmosphere to a solution of the corresponding anthranilic acid (**1**, R = H) (25 mmol) in pyridine (25 mL) over a period of 15 min. The mixture was stirred at 0 °C for 1 h and additional 2 h at room temperature. It was poured onto ice–water (250 mL) and the precipitate was collected by filtration, dried, and recrystallized from ethyl acetate/petroleum ether.

Preparation of compounds 16–18. General procedure. The appropriate 2-(benzyloxycarbonyl)-aminobenzoic acid (**1**, R = CO_2CH_2Ph)[38] (25 mmol) was treated with ethyl chloroformate (12.2 g, 112.5 mmol) according to the procedure outlined above.

2-[N-Benzyl(methylamino)]-4*H*-3,1-benzoxazin-4-one (21). The mixture of compound **3** (2.5 g, 6.45 mmol) and concd H_2SO_4 (40 mL) was stirred at 0 °C for 6 h and kept at −15 °C for 4 days. It was poured onto a stirred mixture of ice–water, $NaHCO_3$, and ethyl acetate (500 mL). After neutralization, the mixture was further extracted with ethyl acetate (2 × 125 mL). The organic layer was washed with water, dried (Na_2SO_4) and evaporated. The residue was purified by column chromatography to obtain compound **21** (190 mg, 11%), mp 113–114 °C. ^1H NMR ($CDCl_3$) δ 3.13 (s, 3 H, CH_3), 4.79 (s, 2 H, CH_2), 7.12–7.16 (m, 1 ArH), 7.29–7.40 (m, 6 ArH), 7.59–7.66 (m, 1 H, H-7), 8.03 (dd, 1H, J = 8.4, 1.6 Hz, H-5). MS (70 eV): *m/z* (%) 266 (M^+, 100). Anal. calcd for $C_{16}H_{14}N_2O_2$: C, 72.17; H, 5.30; N, 10.52. Found: C, 71.96; H, 5.19; N, 10.44%.

2-Morpholino-4*H*-3,1-benzoxazin-4-one (22). A mixture of compound **4** (1 g, 3 mmol) and concd H_2SO_4 (12 mL) was kept at room temperature overnight. The mixture was neutralized and extracted as described above. Evaporation of the solvent yielded compound **22** (580 mg, 83 %), mp 150.5–151.5 °C (diethyl ether/petroluem ether), lit.[17] mp 150–151 °C.

2-Benzoylamino-6-methyl-4*H*-3,1-benzoxazin-4-one (24). Compound **5** (R^6 = Me, R^7 = H) was treated with benzoic anhydride in boiling toluene according

to the reported procedure.[26] The crude product was recrystallized from ethyl acetate/cyclohexane (with silica gel) to obtain compound **24** (12%), mp 162–164 °C. ^{1}H NMR (DMSO-d_6) δ 2.43 (s, 3 H, CH_3), 7.43 (d, 1 H, J = 8.2 Hz, H-8), 7.50–7.56 (m, 2 H, H-3′), 7.61–7.67 (m, 1 H, H-4′), 7.70 (dd, 1H, J = 8,2, 1.9 Hz, H-7), 7.88 (s, br, 1 H, H-5), 7.96–8.00, m, 2 H, H-2′), 11.51 (s, br, 1H, NH). MS (70 eV): m/z (%)280 (M^+, 14), 105 (100). Anal. calcd for $C_{16}H_{12}N_2O_3$: C, 68.57; H, 4.32; N, 9.99. Found: C, 68.41; H, 4.55; N, 10.01%.

Enzymatic studies

Enzyme inhibition was assayed by the progress curve method at 25 °C. Assay buffer was 50 mM Hepes, 500 mM NaCl, pH 7.0 for cathepsin G, and 50 mM Hepes, pH 7.0 for chymotrypsin. Compounds, dissolved in DMSO (5 μL), were added into a cuvette containing 845 μL buffer and 100 μL Suc-Ala-Ala-Pro-Phe-pNA (5 mM). After thermal equilibration, 50 μL of cathepsin G (25 mU/mL) were added. Chymotrypsin (final concentration 100 ng/mL) was assayed with Suc-Ala-Ala-Pro-Phe-pNA at a final concentration of 185 μM. Progress curves were monitored at 405 nm over 30–60 min and fitted as described above.

Product analysis

Compound **20** was incubated with cathepsin G or chymotrypsin in 50 mM Mes, pH 7.0. After 150 min the mixtures were analyzed by HPLC. As a control, the chromatograms of the intact compound **20** as well as the benzoic acid **26** and the quinazolinedione **27** were also recorded. Buffer A was 50 mM triethylammonium acetate, pH 7.0, and buffer B was the same containing 85% MeCN. A gradient of 20–100% B in 30 min gave good separation, the absorbance was monitored at 240 nm. Retention times were as follows: **20**, 27 min; **26**, 12 min; and **27**, 21 min.

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