Pergamon

Bioorganic & Medicinal Chemistry 9 (2001) 947–954

BIOORGANIC & MEDICINAL CHEMISTRY

Inhibition of Human Chymase by 2-Amino-3,1-benzoxazin-4-ones

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Received 18 October 2000; accepted 16 November 2000

Abstract—A series of 2-*sec*.amino-4*H*-3,1-benzoxazin-4-ones was evaluated as acyl-enzyme inhibitors of human recombinant chymase. The compounds were also assayed for inhibition of human cathepsin G, bovine chymotrypsin, and human leukocyte elastase. Introduction of an aromatic moiety into the 2-substituent resulted in strong inhibition of chymase, cathepsin G, and chymotrypsin. Extension of the N(Me)CH_2Ph substituent by one methylene unit was unfavourable to inhibit these proteases. Towards chymase, 2-(*N*-benzyl-*N*-methylamino)-4*H*-3,1-benzoxazin-4-one (**32**) and 2-(*N*-benzyl-*N*-methylamino)-6-methyl-4*H*-3,1-benzoxazin-4-one (**33**) were found to exhibit K_i values of 11 and 17 nM, respectively, and form stable acyl-enzymes with half-lives of 53 and 25 min, respectively. Benzoxazinone **33** also inhibited the human chymase-catalyzed formation of angiotensin II from angiotensin I.

Introduction

The renin–angiotensin-system plays a central role in regulation of blood pressure. Pharmacological intervention in hypertension can occur by inhibiting angiotensin converting enzyme (ACE) and thereby blocking the generation of the vasoconstricting octapeptide angiotensin II (Ang II) from its precursor angiotensin I (Ang I). Another successful approach is the prevention of binding of Ang II to its cell-surface receptor with low molecular weight receptor antagonists. However, ACE is not the sole generator of Ang II.[1] In human heart tissue and in blood vessel walls, human chymase (E. C. 3.4.21.39) forms 90% of the Ang II.[1a] Chymase was first found in mast cells and seems to be involved in the degradation of the extracellular matrix (directly and/or by activation of matrix metalloproteases), stimulation of submucosal gland cell secretion, and complement-mediated inflammation.[1] Given the variety of pathophysiological processes where chymase is involved, inhibitors of the enzyme could be potential drugs in cardiovascular and inflammatory diseases. The primary structure of human chymase is 36% identical to chymotrypsin and 52% identical to human cathepsin G. Recent X-ray analysis results show an overall common fold of the three chymotrypsin-like enzymes.[2] They have quite similar structures around their catalytic sites, characterized by a deep, hydrophobic S_1 pocket that can accommodate the side chains of aromatic amino acids in P_1 position of the substrate. In contrast to chymotrypsin, human chymase also has exoproteolytic activity. It is this dipeptidyl carboxypeptidase activity which makes chymase so effective in generating Ang II from Ang I by cleaving the Phe^8-His^9 peptide bond. Docking experiments done with Ang I have shown that the peptide's C-terminus can make favourable hydrogen bond interactions with the Lys^{40}-Phe^{41} chymase peptide bond and with the free amino group of Lys^{40}.[3]

A variety of synthetic inhibitors of human chymase have been described, including peptides and peptidomimetics,[4,5] as well as 3-(phenylsulfonyl)-1-phenylimidazolidine-2,4-dione[1b] and 1,2,5-thiadiazolidin-3-one derivatives.[6] We and others have investigated the inhibition of chymotrypsin-like and elastase-like serine proteases by 4*H*-3,1-benzoxazin-4-ones. Such compounds have recently been shown to be active in vivo after intratracheal administration.[7] Benzoxazinones temporarily inhibit the catalytic activity of serine proteases by accumulation of a catalytically inactive acyl-enzyme intermediate (Scheme 1). The rates of acylation and deacylation as well as the compound selectivity is determined both by substituents at the benzene unit and the 2-substituent. Potent benzoxazinone inhibitors for human leukocyte elastase (HLE),[8] human

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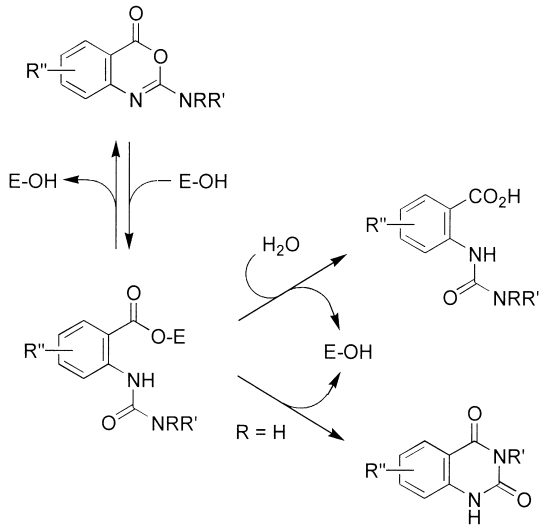
0968-0896/01/$ - see front matter
PII: S0968-0896(00)00310-2



Scheme 1. Reaction of 2-amino-4*H*-3,1-benzoxazin-4-ones with serine proteases. Formation of acyl-enzymes and possible ways of deacylation.

cathepsin G, and bovine chymotrypsin[9] have been described. Introduction of 2-amino substituents is a successful strategy to improve the chemical stability of benzoxazinones[8–10] and of isosteric thieno[1,3]oxazin-4-ones.[11,12] To prevent rapid deacylation via intramolecular cyclization and formation of corresponding quinazolines (R = H, see Scheme 1) we focused on benzoxazinones with 2-*sec*.amino substitution (R, R′≠H).

The results obtained so far with benzoxazinone-type compounds as inhibitors of serine proteases suggest that they may be useful inhibitors of chymase activity. We therefore investigated in vitro the inhibition of human chymase by a series of 2-*sec*.aminobenzoxazinones. In parallel, the compounds were assayed for inhibition of the related enzymes bovine chymotrypsin, human cathepsin G, and human leukocyte elastase. Using angiotensin I as a substrate, we tested whether a selected benzoxazinone was able to inhibit also the biologically relevant exopeptidase activity of chymase.

Results and Discussion

Chemistry

Benzoxazinones **21**–**35** (Table 1) were prepared by three alternative routes (Scheme 2). Mesyloxyphthalimides **1**–**3** were reacted with the appropriate secondary amine to obtain 2-ureidobenzamides **9**–**11**, **17**, and ethyl 2-ureidobenzoates **6**, **8**, **12**, **14**, respectively.[13] Cyclocondensation of these precursors (method A, method B, respectively) to the corresponding benzoxazinones was then performed with concentrated sulfuric acid. The third route involves transformation of isatoic anhydrides **4**, **5** to corresponding 2-ureidobenzoic acids which were conveniently cyclized to the final benzoxazinones upon treatment with acetic anhydride (method C).

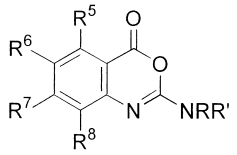
Enzyme assays

The inhibitory effects of benzoxazinones **21**–**36** on the enzymatic activities of serine proteases were evaluated in the presence of chromogenic substrates at pH 8.0, except for HLE, which was assayed at pH 7.8.[8] Initially, the inhibiting activity was determined at a single inhibitor concentration. Percentage of inhibition was calculated from the steady-state rates (Table 1). Based on these data, compounds for full kinetic analysis using different inhibitor concentrations were selected (Tables 2–4). The time course of product formation after addition of enzyme to a solution of chromogenic substrate and inhibitor was monitored. Progress curves were characterized by an initial exponential phase, and a second, linear steady-state turnover of the chromogenic substrate. Such curves have already been observed when acylating inhibitors were reacted with serine proteases and the acylation–deacylation mechanism (according to Scheme 1) for the reaction of fused oxazin-4-ones with HLE and cathepsin G was proven by product analyses.[8,9,11,15] We postulate that chymase also reacts with the benzoxazinones described herein by formation of an acyl-enzyme which hydrolyzes slowly. As discussed before,[11] the potency of acylating inhibitors can be described by the ratio of their deacylation and acylation rate constants and is called the dissociation constant K_i, although this kinetic K_i value is not identical to the thermodynamic K_i value describing the binding of a fully reversible inhibitor. Inhibition parameters were obtained from the slow-binding curves as described.[11,14,15] We did not see an accumulation of a noncovalent enzyme–inhibitor complex before the onset of the acylation reaction, since the initial rate of the progress curve was identical to the control rate in the absence of inhibitor. This was the case for all inhibitors and for every inhibitor concentration used. In the screening experiments with cathepsin G and inhibitors **31** and **33** at 20 μM inhibitor concentration (Table 1), the steady-state was already reached at the beginning of the progress curve due to fast acylation.

General pattern of inhibition

The inhibition data in Table 1 clearly show that human chymase, human cathepsin G and bovine chymotrypsin were all strongly inhibited by benzoxazinones carrying aromatic substituents at position 2. In contrast, and in agreement with published data, the inhibitory profile for HLE was different. The results fitted to the known active-site architecture of these enzymes. Chymase, cathepsin G and chymotrypsin all have large primary specificity pockets which can accommodate aromatic rings. Their preference for benzoxazinones bearing an aromatic moiety in the 2-substituent indicates that this substituent binds into the primary specificity pocket. While it is obvious that selective inhibition of chymase over elastase can be achieved, the differentiation between the chymotrypsin-like enzymes is much more difficult. To obtain a first structure–activity relationship for the inhibition of chymase, cathepsin G and chymotrypsin, we determined the individual kinetic parameters of inhibition for the most potent compounds.

Table 1. Inhibition of serine proteases by 4*H*-3,1-benzoxazin-4-ones



Compound	Method (prepared from)	R^5	R^6	R^7	R^8	NRR′	% Inhibition[a] Chymase [I] = 1 μM	Cathepsin G [I] = 20 μM	Chymotrypsin [I] = 1 μM	HLE [I] = 1 μM
21	B (**6**)	H	H	H	H	4-Morpholinyl	< 15	53[b]	50	25
22	C (**7**)	H	Me	H	H	4-Morpholinyl	< 15	< 15	31	38
23	B (**8**)	H	Me	Me	H	4-Morpholinyl	< 15	< 15	66	< 15
24	A (**9**)	Me	H	H	Me	4-Morpholinyl	< 15	< 15[c]	52	70
25	A (**10**)	H	H	H	H	1-Pyrrolidinyl	< 15	94[b]	69	28
26	A (**11**)	H	H	H	H	$N(Et)_2$	< 15	77[b]	75	31
27	B (**12**)	H	H	H	H	N(Me)cyclohexyl	< 15	83[b]	75	65
28	C (**13**)	H	Me	H	H	N(Me)cyclohexyl	30	52	81	18
29	B (**14**)	H	Me	Me	H	N(Me)cyclohexyl	< 15	23[c]	96*	< 15
30	C (**15**)	H	H	H	H	N(Me)Ph	> 95*	95*	90*	37
31	C (**16**)	H	Me	H	H	N(Me)Ph	90*	56[d]	77*	26
32	A (**17**)	H	H	H	H	$N(Me)CH_2Ph$	> 95*	97*	92*	48
33	C (**18**)	H	Me	H	H	$N(Me)CH_2Ph$	> 95*	86[d]	83*	42
34	C (**19**)	H	H	H	H	$N(Me)CH_2CH_2Ph$	17	71[b]	67	40
35	C (**20**)	H	Me	H	H	$N(Me)CH_2CH_2Ph$	< 15	15	38	44
36		H	H	H	H	$NHCH_2Ph$	> 95*	95*	93*	50

[a]Inhibition was measured at a single inhibitor concentration as indicated. Percentage inhibition was determined from the steady-state rate constants. The kinetic parameters of inhibition indicated by an asterisk are outlined in Tables 2–4.
[b]Slow inhibition with second-order rate constants $k_{obs}/[I] \leq 100\ M^{-1}\ s^{-1}$.
[c]Slight precipitation occurred in the course of the assay.
[d]Inhibition was determined from the initial part of the progress curve.

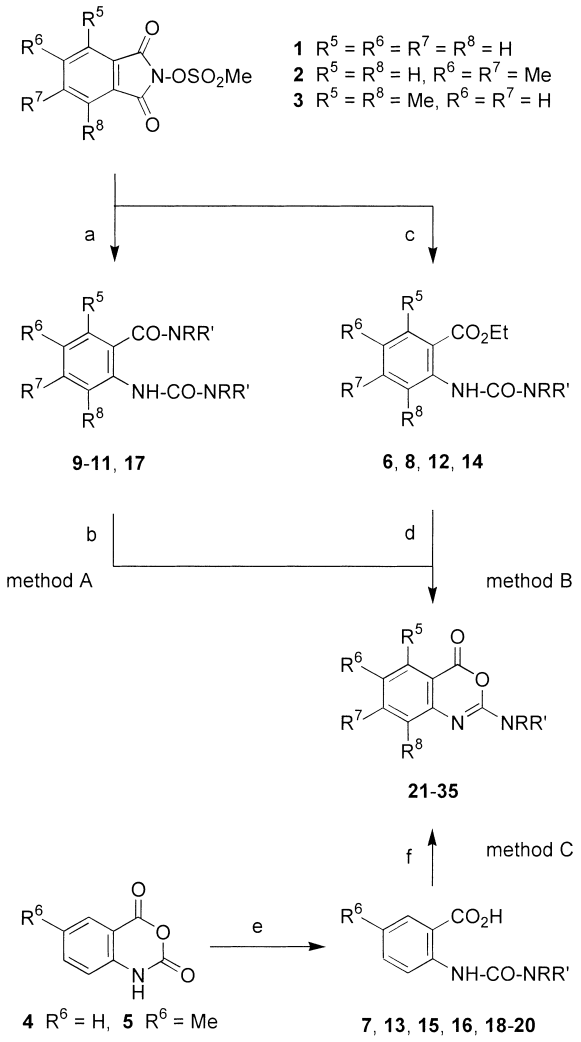
Chymase inhibition

The kinetic parameters of four 2-*sec*.amino-3,1-benzoxazinones together with the benzylamino compound **36** are outlined in Table 2. The benzylamino compound **36** ($K_i = 5$ nM) was the most potent compound in the series. This is mainly due to its very high acylation rate, which is an order of magnitude higher than for any of the *sec*.amino derivatives tested. Introduction of a morpholino, pyrrolidino, or diethylamino residue (**21**–**26**) led to inactive compounds (Table 1). Compounds with cyclohexyl residues also had little inhibitory potency towards chymase (**27**–**29**). We found chymase inhibition to be strongly influenced by the length of the spacer between secondary amino group and aryl residue within the 2-substituent: extension of the $N(Me)CH_2Ph$ substituent by one methylene unit was highly unfavourable in chymase inhibition (**32** versus **34**; **33** versus **35**). Removal of one methylene unit of the $N(Me)CH_2Ph$ residue resulted in an acceleration of both the acylation and the deacylation step (**30** versus **32**; **31** versus **33**, Table 2). 6-Methyl substitution (**31**, **33**) did not have a big impact on the inhibitory potency of the compounds. From the present series, benzoxazinones **32** and **33** appear as the most promising inhibitors with K_i values around 10 nM; they form highly stable acyl-enzymes with half-lives of 53 min and 25 min, respectively. As an example, the analysis of the chymase inhibition kinetics by the benzoxazinone **33** is illustrated in Figures 1–3.

We investigated the inhibition of human chymase-catalyzed conversion of the physiologically relevant substrate angiotensin I. Compound **33** was selected for this study considering the results with chromogenic substrates towards chymase (see above) as well as related serine proteases (see below). The cleavage of human angiotensin I to angiotensin II by human chymase was followed by HPLC. The reaction was distinctly inhibited in the presence of **33** at concentrations of 100 and 250 nM, respectively (Fig. 4). From the initial part of the curves, an approximate K_i value of 27 nM could be estimated, being in the range of the K_i value obtained for **33** with chromogenic substrate (Table 2).

Inhibition of cathepsin G, chymotrypsin, and HLE

In agreement with previous data,[9] cathepsin G was less potently inhibited by benzoxazinones, compared to chymase and chymotrypsin. Thus, for the initial inhibition studies, an inhibitor concentration of 20 μM was chosen, whereas 1 μM was used for the other enzymes. Cathepsin G also shows lower hydrolysis rates and k_{cat}/K_M values with peptide *p*-nitroanilide substrates.[16] A recent investigation using peptide substrates spanning the P- and the P′-side of the substrate-binding region, however, identified substrates with k_{cat}/K_M values comparable to the other serine proteases.[17] Cathepsin G recruits a considerable fraction of its catalytic power from enzyme–substrate interactions occurring at subsites remote from the scissile bond. The benzoxazinones studied here, however, cannot interact with distant subsites, which is reflected by their low second-order rate constants



Scheme 2. (a) HNRR′, acetone, reflux, 25 min, or *N*-benzylmethylamine, toluene, 0–25 °C, 7 h, 64–95%; (b) concd H_2SO_4, rt or 0 °C, 11–71%, method A; (c) HNRR′, acetone, reflux, 25 min, then 0.25 M ethanolic HCl, reflux, 2 min, then −15 °C, 66–91%; (d) concd H_2SO_4, rt, 86–98%, method B; (e) HNRR′, H_2O, rt, or HNRR′, EtOH, H_2O, reflux, 10–53%; (f) acetic anhydride, reflux, 15 min, 52–94%, method C.

(Table 3; $k_{obs}/[I] \leq 100\,M^{-1}\,s^{-1}$ for the moderately potent compounds **21**, **25**–**27**, **34**, Table 1). Also, for the most active compound, **36**, k_{on} was more than 100-fold lower, compared to chymase and chymotrypsin. Introduction of methyl groups into the benzene unit was disadvantageous. The K_i value of **32** was similar to that of the 2-benzylamino derivative **36**. Both compounds were already part of previous series of benzoxazinones evaluated at pH 7 as inhibitors for cathepsin G (and chymotrypsin).[9] Chymotrypsin, in contrast to cathepsin G, was potently inhibited by the benzoxazinones, especially those with aromatic 2-substituents. K_i values in the low nanomolar range were achieved (Table 4). Extension of the $N(Me)CH_2Ph$ substituent by one methylene unit resulted in less potent compounds (**34** versus **32**; **35** versus **33**, Table 1). However, this effect was moderate for chymotrypsin and cathepsin G, and pronounced only for chymase.

Table 2. Kinetic data of the inhibition of human chymase by 4*H*-3,1-benzoxazin-4-ones

Compound	k_{on} ($M^{-1}\,s^{-1}$)	k_{off} ($10^{-4}\,s^{-1}$)	K_i (nM)	pK_i
30	79,000	11	14	7.87
31	85,800	25	29	7.53
32	19,000	2.2	11	7.94
33	40,300	6.8	17	7.77
36	623,000	31	5.0	8.30

Table 3. Kinetic data of the inhibition of human cathepsin G by 4*H*-3,1-benzoxazin-4-ones

Compound	k_{on} ($M^{-1}\,s^{-1}$)	k_{off} ($10^{-4}\,s^{-1}$)	K_i (nM)	pK_i
30	785	9.7	1200	5.91
32	363	1.6	440	6.36
36	2640	9.5	360	6.44

Table 4. Kinetic data of the inhibition of bovine chymotrypsin by 4*H*-3,1-benzoxazin-4-ones

Compound	k_{on} ($M^{-1}\,s^{-1}$)	k_{off} ($10^{-4}\,s^{-1}$)	K_i (nM)	pK_i
29	137,000	17	12	7.92
30	81,800	9.0	11	7.97
31	91,400	45	49	7.31
32	74,300	17	23	7.63
33	58,800	22	38	7.43
36	810,000	270	33	7.48

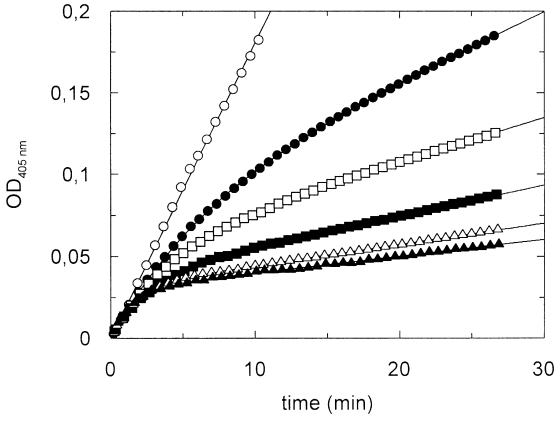


Figure 1. Slow-binding inhibition of human chymase by compound **33** in 0.1 M Tris–HCl, 1.8 M NaCl, pH 8.0. Substrate Suc-Ala-Ala-Pro-Phe-pNA. Data were fitted to eq (1) to obtain the best-fit parameters for v_i, v_s, k_{obs}, and offset. Open circles: [I] = 0; full circles: [I] = 100 nM; open squares: [I] = 200 nM; full squares: [I] = 300 nM; open triangles: [I] = 400 nM; and full triangles: [I] = 500 nM.

HLE inhibition by 2-amino-3,1-benzoxazin-4-ones, including compounds **21**, **25**, **26**, **36**, has extensively been studied by Krantz et al.[8] HLE preferentially cleaves proteins or peptidic substrates with Ala, Leu or Val at P_1 position. Therefore, as expected,[8,11] only moderate inhibition was detected with benzoxazinones **30**–**36** bearing an aromatic moiety in the 2-substituent (Table 1). Introduction of methyl groups at positions 6 and 7 was disadvantageous (**23** versus **21**, **29** versus **27**).

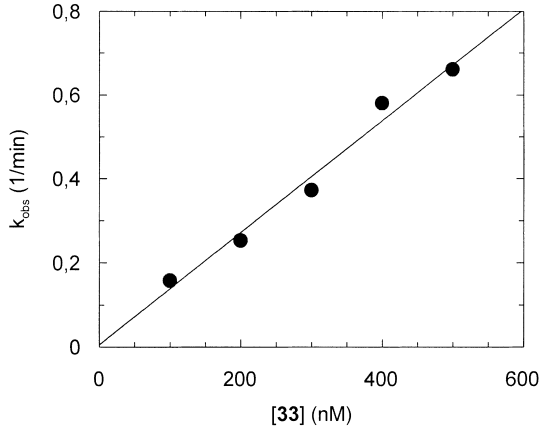


Figure 2. Plot of k_{obs} versus [I] for the inhibition of human chymase by compound **33**. The values for k_{obs} were obtained from fits to the data shown in Figure 1. The slope corresponds to a value for $k_{on}/(1+[S]/K_m) = 22{,}200\ M^{-1}\ s^{-1}$.

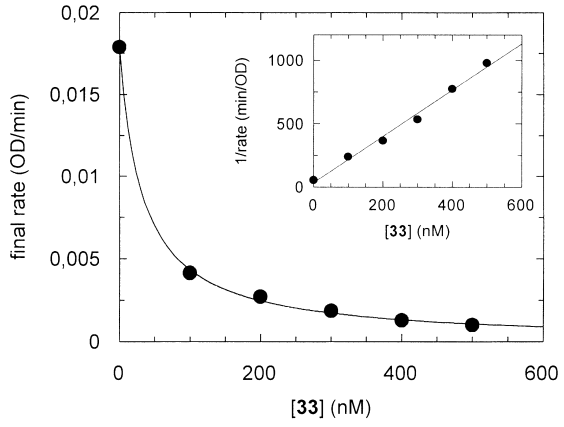


Figure 3. Plot of the steady-state rates versus [I] for the inhibition of human chymase by compound **33**. The data were obtained from fits of the curves shown in Figure 1. The solid line was drawn using the best-fit parameters from a fit according to an equation of a competitive inhibition, which gave $K_i\ (1+[S]/K_m) = 32.1 \pm 1.3$ nM. The insert is a Dixon plot to show the linearity.

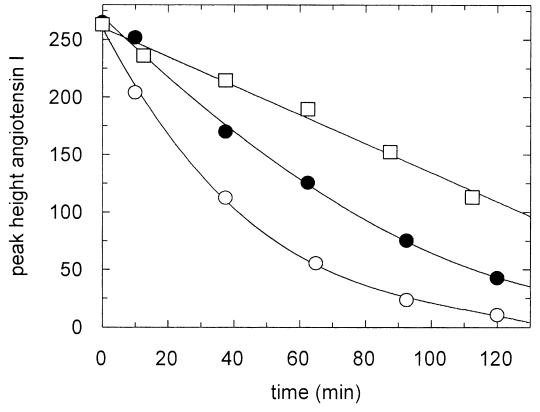


Figure 4. Inhibition of the chymase catalyzed cleavage of angiotensin I (to angiotensin II) by compound **33**. Open circles: [I] = 0; full circles: [I] = 100 nM; open squares: [I] = 250 nM.

Strong inhibition, on the other hand, was noted by introducing methyl groups at positions 5 and 8 (**24** versus **21**). A K_i value of 27 nM ($k_{on} = 2970\ M^{-1}\ s^{-1}$, $k_{off} = 0.8 \times 10^{-4}\ s^{-1}$) was detected for the 5,8-dimethyl-2-morpholino derivative **24**. Strong inhibition by this compound resulted from a highly stable acyl-enzyme with a half-life of more than 2 h. The decreased deacylation rate is thought to result from the 5-methyl introduction that leads to steric hindrance of water attack at the ester carbonyl of the acyl-enzyme.[8] It is noteworthy that the new inhibitor **24** showed specificity for HLE over both chymase and cathepsin G.

Conclusions

This report describes, for the first time, the inhibition of human chymase by benzoxazinones. We restricted our investigations to conveniently available compounds, which are known to be sufficiently stable at physiological pH.[8,9] Inhibition of chymase by 4*H*-3,1-benzoxazin-4-ones occurs by an acylation-deacylation mechanism, as shown before for other serine proteases. Introduction of an aromatic moiety into the 2-substituent furnished potent inhibitors of chymase, indicating the accommodation of the phenyl group at the primary specificity site S_1 of the enzyme. The introduction of a disubstituted amino group resulted in relatively stable acyl-enzyme complexes. This comparison of deacylation rates revealed a favourable deceleration of the deacylation step, when intramolecular quinazoline formation is structurally prevented. Within this set of compounds, we identified acylating inhibitors of chymase with K_i values in the low nanomolar range. It was further demonstrated exemplary that the chymase-catalyzed conversion of angiotensin I was inhibited by a selected benzoxazinone at nanomolar concentrations. Selectivity for chymase over HLE was achieved by incorporation of aromatic structures into the 2-substituent of the benzoxazinones.

Interestingly, besides ACE and chymase, neutrophil cathepsin G was found to generate angiotensin II from angiotensin I and also to convert angiotensinogen directly to angiotensin II.[18,19] However, our data show that there is a considerable difference in terms of acylation efficacy towards benzoxazinones between human chymase and cathepsin G. It can be expected that the concentration required to inhibit chymase is 1–2 orders of magnitude lower than the one required to inhibit cathepsin G.

None of the chymase inhibitors did exhibit specificity for chymase over chymotrypsin. This is not very surprising given the structural and functional similarity between the two enzymes. This initial study, however, investigated only a few modifications within the benzoxazinone system. The recent identification of the structural basis for the exopeptidase activity, which chymase does not share with chymotrypsin, offers intriguing possibilities for the design of selective benzoxazinone-type chymase inhibitors for the treatment of cardiovascular and inflammatory diseases.

Experimental

General methods and materials

Melting points were determined on a Boetius apparatus and are not corrected. Thin-layer chromatography was performed on Merck aluminium sheets, silica gel 60 F_{254}. ^{1}H NMR spectra (300 MHz) were recorded on a Varian Gemini 300. IR spectra were measured with a Perkin-Elmer 16 PC FTIR spectrometer. Mass spectra (70 eV) were obtained using a Varian MAT CH6 spectrometer. Spectrophotometric assays were done on a Varian Cary 3 Bio spectrophotometer with six-cell holder. Analytical HPLC was performed on a Thermo-SeparationProducts liquid chromatograph with PC1000 software. A Nucleosil C18 2×250-mm column was used at a flow rate of 0.2 mL/min. Mobile phase A was H_2O, 0.1% TFA, and mobile phase B was H_2O/acetonitrile/TFA 30/70/0.085%. A gradient of 30–57% B in 14 min was used. Recombinant human mast cell chymase was purified as described.[20] Elastase was prepared from human leukocytes and purified by affinity chromatography using an immobilized synthetic inhibitor.[21] Human cathepsin G was purchased from Calbiochem. Chymotrypsin was purchased from Worthington, Freehold, USA. Suc-Ala-Ala-Pro-Phe-pNA, MeOSuc-Ala-Ala-Pro-Val-pNA, angiotensin I, and angiotensin II were from Bachem, Bubendorf, Switzerland.

2-Ureidobenzamides **9**–**11**,[13] **17**,[9] and ethyl 2-ureidobenzoates **6**, **8**, **12**, **14**[13] were prepared from corresponding *N*-(mesyloxy)phthalimides **1**–**3** as reported. The following 2-*sec*.amino-4*H*-3,1-benzoxazin-ones were prepared as reported **21**, **23**–**27**, **29**,[13] **32**.[9] 2-Benzyl-amino-4*H*-3,1-benzoxazin-4-one **36**[8] was synthesized from methyl 2(-3-benzylureido)benzoate[22] and purified on column chromatography.

5-Methyl-2-[(morpholinocarbonyl)amino]benzoic acid (7). A mixture of 5-methylisatoic anhydride **5**[23] (3.54 g, 20 mmol) and 50 mL of an aqueous solution of morpholine (4 M) was stirred at room temperature for 15 min, diluted with water (200 mL) and extracted with ethyl acetate (1×50 mL). The organic layer was extracted with 0.5 M NaOH (1×50 mL). The combined aqueous layers were washed with ethyl acetate (1×100 mL) and acidified with 10% H_2SO_4. The precipitate was collected by filtration, washed with water, and dried to yield **7** (1.9 g, 36%): mp 173–175 °C; IR (KBr, cm^{-1}) 1686, 1645 (C=O); ^{1}H NMR ($CDCl_3$) δ 2.33 (s, 3H, CH_3), 3.54–3.60 (m, 4H, NCH_2), 3.74–3.80 (m, 4H, OCH_2), 7.39 (dd, J=8.6, 2.0 Hz, 1H, H-4), 7.86 (s, br, 1H, H-6), 8.46 (d, J=8.6 Hz, 1H, H-3), 10.49 (s, 1H, NH). Anal. calcd for $C_{13}H_{16}N_2O_4$: C, 59.08; H, 6.10; N, 10.60. Found: C, 59.04; H, 6.05; N 10.40.

2-(3-Cyclohexyl-3-methylureido)-5-methylbenzoic acid (13): General procedure for 2-ureidobenzoic acids 13, 15, 16, 18–20. A mixture of **5** (3.54 g, 20 mmol) and 50 mL of an ethanolic solution of *N*-methylcyclohexylamine (4 M) was refluxed for 15 min, diluted with water (200 mL) and extracted with ethyl acetate (1×50 mL). The organic layer was extracted with 0.5 M NaOH (1×50 mL). The combined aqueous layers were washed with ethyl acetate (1×100 mL) and acidified with 10% H_2SO_4. The precipitate was collected by filtration, washed with water, and dried to yield **13** (2.6 g, 45%): mp 154–156 °C; IR (KBr, cm^{-1}) 1680, 1636 (C=O); ^{1}H NMR ($CDCl_3$) δ 1.10–1.90 (m, 10H, CH_2), 2.32 (s, 3H, 5-CH_3), 2.94 (s, 3H, NCH_3), 4.05–4.20 (m, 1H, CH), 7.37 (dd, J=8.7, 2.2 Hz, 1H, H-4), 7.85 (s, br, 1H, H-6), 8.51 (d, J=8.7 Hz, 1H, H-3), 10.37 (s, 1H, NH). Anal. calcd for $C_{16}H_{22}N_2O_3$: C, 66.18; H, 7.64; N, 9.65. Found: C, 65.96; H, 7.93; N, 9.94.

2-(3-Phenyl-3-methylureido)benzoic acid (15). Compound **15** was prepared from **4** and *N*-methylaniline in 42% yield: mp >159 °C (dec) (lit.[24] mp 161 °C); IR (KBr, cm^{-1}) 1696, 1658 (C=O); ^{1}H NMR ($CDCl_3$) δ 3.36 (s, 3H, CH_3), 6.98–7.04 (m, 1H, H-5), 7.30–7.52 (m, 5 ArH), 7.54–7.60 (m, 1H, H-4), 7.99 (dd, J=8.1, 1.6 Hz, 1H, H-6), 8.71 (d, J=8.5 Hz, 1H, H-3), 9.92 (s, 1H, NH).

5-Methyl-2-(3-methyl-3-phenylureido)benzoic acid (16). Compound **16** was prepared from **5** and *N*-methylaniline. The sodium salt that precipitated in the extraction with 0.5 M NaOH was separated and combined with the material obtained after acidification. Recrystallization from EtOH/5% AcOH gave **16** in 44% yield: mp >200 °C (dec); IR (KBr, cm^{-1}) 1692, 1654 (C=O); ^{1}H NMR ($CDCl_3$) δ 2.35 (s, 3H, 5-CH_3), 3.35 (s, 3H, NCH_3), 7.30–7.52 (m, 6 ArH), 7.76 (d, J=1.9 Hz, 1H, H-6), 8.58 (d, J=8.8 Hz, 1H, H-3), 9.81 (s, 1H, NH). Anal. calcd for $C_{16}H_{16}N_2O_3$: C, 67.59; H, 5.67; N, 9.85. Found: C, 67.36; H, 5.39; N, 9.84.

2-(3-Benzyl-3-methylureido)-5-methylbenzoic acid (18). Compound **18** was prepared from **5** and *N*-benzylmethylamine in 10% yield: mp 144–146 °C; IR (KBr, cm^{-1}) 1700, 1644 (C=O); ^{1}H NMR ($CDCl_3$) δ 2.32 (s, 3H, 5-CH_3), 3.04 (s, 3H, NCH_3), 4.65 (s, 2H, CH_2), 7.25–7.41 (m, 6 ArH), 7.84 (d, J=1.7 Hz, 1H, H-6), 8.55 (d, J=8.7 Hz, 1H, H-3), 10.64 (s, 1H, NH). Anal. calcd for $C_{17}H_{18}N_2O_3$: C, 68.44; H, 6.08; N, 9.39. Found: C, 68.39; H, 6.23; N, 9.18.

2-[3-Methyl-3-(2-phenylethyl)ureido]benzoic acid (19). Compound **19** was prepared from **4** and *N*-benzylmethylamine in 36% yield: mp 139–141 °C; IR (KBr, cm^{-1}) 1696, 1640 (C=O); ^{1}H NMR ($CDCl_3$) δ 2.94 (t, J=7.6 Hz, 2H, CH_2), 3.00 (s, 3H, CH_3), 3.64 (t, J=7.6 Hz, 2H, NCH_2), 6.96–7.05 (m, 1H, H-5), 7.14–7.32 (m, 5 ArH), 7.52–7.60 (m, 1H, H-4), 8.07 (dd, J=8.1, 1.6 Hz, 1H, H-6), 8.63 (d, J=8.8 Hz, 1H, H-3), 10.55 (s, 1H, NH). Anal. calcd for $C_{17}H_{18}N_2O_3$: C, 68.44; H, 6.08; N, 9.39. Found: C, 68.65; H, 6.29; N, 9.50.

5-Methyl-2-[3-methyl-3-(2-phenylethyl)ureido]benzoic acid (20). Compound **20** was prepared from **5** and *N*-benzylmethylamine in 53% yield: mp 173–175 °C; IR (KBr, cm^{-1}) 1692, 1636 (C=O); ^{1}H NMR ($CDCl_3$) δ 2.33 (s, 3H, 5-CH_3), 2.94 (t, J=7.7 Hz, 2H, CH_2), 3.00 (s, 3H, NCH_3), 3.63 (t, J=7.7 Hz, 2H, NCH_2), 7.20–7.41 (m, 6 ArH), 7.86 (s, br, 1H, H-6), 8.52 (d, J=8.6, 1H, H-3), 10.40 (s, 1H, NH). Anal. calcd for $C_{15}H_{20}N_2O_3$: C, 69.21; H, 6.45; N, 8.97. Found: C, 69.26; H, 6.65; N, 8.78.

6-Methyl-2-morpholino-4*H*-3,1-benzoxazin-4-one (22). General procedure for the preparation of 4*H*-3,1-benzoxazin-4-ones 22, 28, 30, 31, 33–35 from 2-ureidobenzoic acids. A mixture of compound **7** (529 mg, 2 mmol) and acetic anhydride (7 mL) was refluxed for 15 min. After cooling, it was poured onto ice–water (100 mL). The precipitate was collected by filtration, washed with water, and dried to yield **22** (390 mg, 79%): mp 135–138 °C; IR (KBr, cm^{-1}) 1752 (C=O); ^1H NMR ($CDCl_3$) δ 2.39 (s, 3H, CH_3), 3.70–3.80 (m, 8H, CH_2), 7.16 (d, $J=8.4$ Hz, 1H, H-8), 7.46 (d, $J=8.4$ Hz, 1H, H-7), 7.82 (s, br, 1H, H-5); MS (EI) *m/z* (rel intensity) 246 (M^+, 71), 160 (M^+-NRR', 100). Anal. calcd for $C_{13}H_{14}N_2O_3$: C, 63.40; H, 5.73; N, 11.38. Found: C, 63.16; H, 6.02, N, 11.13.

2-(*N*-Cyclohexyl-*N*-methylamino)-6-methyl-4*H*-3,1-benzoxazin-4-one (28). Compound **28** was synthesized from **13** in 90% yield: mp 111–112 °C; IR (KBr, cm^{-1}) 1762 (C=O); ^1H NMR ($CDCl_3$) δ 1.06–1.90 (m, 10H, CH_2), 2.36 (s, 3H, 6-CH_3), 3.01 (s, 3H, NCH_3), 4.24–4.40 (m, 1H, CH), 7.15 (d, $J=8.4$ Hz, 1H, H-8), 7.41 (d, $J=8.4$ Hz, 1H, H-7), 7.79 (s, br, 1H, H-5); MS (EI) *m/z* (rel intensity) 272 (M^+, 50), 190 ($M^+-C_6H_{10}$, 100), 160 (M^+-NRR', 70). Anal. cald for $C_{16}H_{20}N_2O_3$: C, 70.56; H, 7.40; N, 10.29. Found: C, 70.26; H, 7.61; N, 10.23.

2-(*N*-Methyl-*N*-phenylamino)-4*H*-3,1-benzoxazin-4-one (30). Compound **30** was prepared from **15** in 52% yield: mp 127–128 °C (acetone/hexane) (lit.[25] mp 130–131 °C); IR (KBr, cm^{-1}) 1768 (C=O); ^1H NMR ($CDCl_3$) δ 3.55 (s, 3H, CH_3), 7.15–7.21 (m, 1H, H-6), 7.28–7.47 (m, 6 ArH), 7.60–7.67 (m, 1H, H-7), 8.03 (dd, $J=8.0$ Hz, 1H, H-5); MS (EI) *m/z* (rel intensity) 252 (M^+, 39), 146 (M^+-NRR', 100).

6-Methyl-2-(*N*-methyl-*N*-phenylamino)-4*H*-3,1-benzoxazin-4-one (31). Compound **31** was synthesized from **16** in 92% yield: mp 117–119 (ethyl acetate/hexane), IR (KBr, cm^{-1}) 1759 (br, C=O); ^1H NMR ($CDCl_3$) δ 2.38 (s, 1H, 6-CH_3), 3.53 (s, 3H, NCH_3), 7.23 (d, $J=8.4$ Hz, 1H, H-8), 7.26–7.48 (m, 6 ArH), 7.82 (s, br, 1H, H-5); MS (EI) *m/z* (rel intensity) 266 (M^+, 60), 160 (M^+-NRR', 100). Anal. calcd for $C_{15}H_{14}N_2O_2$: C, 72.17; H, 5.30; N, 10.52. Found: C, 71.83; H, 5.27; N, 10.46.

2-(*N*-Benzyl-*N*-methylamino)-6-methyl-4*H*-3,1-benzoxazin-4-one (33). Compound **33** was prepared from **18** in 84% yield: mp 96–97 °C, IR (KBr, cm^{-1}) 1766 (C=O); ^1H NMR ($CDCl_3$) δ 2.38 (s, 1H, 6-CH_3), 3.11 (s, 3H, NCH_3), 4.77 (s, 2H, CH_2), 7.19 (d, $J=8.3$ Hz, 1H, H-8), 7.25–7.39 (m, 5 ArH), 7.44 (dd, $J=8.3$, 1.8 Hz, H-7), 7.83 (s, br, 1H, H-5); MS (EI) *m/z* (rel intensity) 280 (M^+, 100). Anal. cald for $C_{17}H_{16}N_2O_2$: C, 72.84; H, 5.75; N, 9.99. Found: C, 73.10; H, 6.13; N, 9.78.

2-[*N*-Methyl-*N*-(2-phenylethyl)amino]-4*H*-3,1-benzoxazin-4-one (34). Compound **34** was synthesized from **19** in 94% yield: mp 68.5–69 °C; IR (KBr, cm^{-1}) 1764 (C=O); ^1H NMR ($CDCl_3$) δ 2.96 (t, $J=7.5$ Hz, 2H, CH_2), 3.07 (s, 3H, CH_3), 3.77 (t, $J=7.5$ Hz, 2H, NCH_2), 7.07–7.17 (m, 1H, H-6), 7.17–7.36 (m, 6 ArH), 7.56–7.65 (m, 1H, H-7), 8.00 (dd, $J=7.9$, 1.7 Hz, 1H, H-5); MS (EI) *m/z* (rel intensity) 280 (M^+, 25), 146 (M^+-NRR', 100). Anal. calcd for $C_{17}H_{16}N_2O_2$: C, 72.84; H, 5.75; N, 9.99. Found: C, 72.96; H, 6.15; N, 10.38.

6-Methyl-2-[*N*-methyl-*N*-(2-phenylethyl)amino]-4*H*-3,1-benzoxazin-4-one (35). Compound **35** was prepared from **20** in 87% yield: mp 96–97 °C; IR (KBr, cm^{-1}) 1750 (C=O); ^1H NMR ($CDCl_3$) δ 2.37 (s, 3H, 6-CH_3), 2.97 (t, $J=7.4$ Hz, 2H, CH_2), 3.06 (s, 3H, CH_3), 3.76 (t, $J=7.4$ Hz, 2H, NCH_2), 7.18 (d, $J=8.3$ Hz, 1H, H-8), 7.20–7.33 (m, 5 ArH), 7.44 (dd, $J=8.3$, 2.1 Hz, 1H, H-7), 7.80 (s, br, 1H, H-5); MS (EI) *m/z* (rel intensity) 294 (M^+, 28), 160 (M^+-NRR', 100). Anal. calcd for $C_{18}H_{18}N_2O_2$: C, 73.45; H, 6.16; N, 9.52. Found: C, 73.64; H, 6.42; N, 9.33.

Enzyme inhibition assays

Inhibition of serine proteases by compounds **21**–**36** was assayed spectrophotometrically by the progress curve method. All experiments were carried out at 25 °C with a final DMSO concentration of 1.5%. Progress curves were fitted to eq (1):

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

where k_{obs} is the first-order rate constant for the approach to the steady-state, v_s and v_i are the steady-state and the initial velocity, respectively. Except for cathepsin G inhibition by **31** and **33** (Table 1), v_i values equalled to the velocity in the absence of inhibitor, v_0. In the screening experiments, inhibitors were evaluated at a single concentration (Table 1). To estimate K_i values from these assays, eq (2) was used.

$$K_i = K_m[I]/[(K_m + [S])\ (v_0/v_s - 1)] \quad (2)$$

Values from these calculations were found to be in a sufficient agreement with the K_i values outlined in Tables 2–4. To determine the kinetic parameters of inhibition (Tables 2–4), at least five different inhibitor concentrations were used.

Inhibitors, dissolved in DMSO (5 μL), were added into a cuvette containing 845 μL of the corresponding assay buffer and 100 μL of the corresponding chromogenic substrate. After thermal equilibration, the reaction was initiated by addition of 50 μL of enzyme solution. Progress curves were monitored at 405 nm over 10–70 min. The kinetics were analyzed as described.[11] The following substrates were used: Suc-Ala-Ala-Pro-Phe-pNA for chymase (final concentration 200 μM = 0.92× K_m), cathepsin G (final concentration 500 μM ≪ K_m), and chymotrypsin (final concentration 200 μM = 3.46× K_m), and MeOSuc-Ala-Ala-Pro-Val-pNA for HLE (final concentration 200 μM = 3.8× K_m). Stock solutions of the substrates were prepared in DMSO and diluted with the corresponding assay buffer. Assay buffers were as follows: 0.1 M Tris–HCl, 1.8 M NaCl, pH 8.0 for chymase, 0.1 M Tris–HCl, 0.5 M NaCl, pH 8.0 for cathepsin G and chymotrypsin, 50 mM sodium phosphate, 500 mM NaCl, pH 7.8 for HLE. Enzyme stock solutions of chymase (approximately 800 nM in 0.1 M Tris–HCl, 1.8 M NaCl, pH

8.0), cathepsin G (500 mU/mL in 50 mM sodium acetate buffer, 150 mM NaCl, pH 5.5), HLE (1.17 μM in 100 mM sodium acetate buffer, pH 5.5), and chymotrypsin (100 μg/mL in 1 mM HCl) were freshly diluted with the corresponding assay buffer. Assays were typically performed with final enzyme concentrations of chymase (approximately 2 nM), cathepsin G (2.5 mU/mL), HLE (2.9 nM), and chymotrypsin (25 ng/mL).

Chymase catalyzed conversion of angiotensin I (77 μM) was analyzed by HPLC using conditions described under General Methods. Retention times were as follows: angiotensin I, 13.5 min; angiotensin II, 11.3 min. The reaction was performed at 25 °C in 0.1 M Tris–HCl, 1.8 M NaCl, pH 8.0 with a final enzyme concentration of approximately 2 nM. A K_m value of 40 μM[1b] was considered to estimate K_i for the inhibition by compound **33**.

Acknowledgements

The authors wish to thank René Hersperger, Novartis Pharma, for critically reading the manuscript. M. G. would like to thank the 'Fonds der Chemischen Industrie' for financial support.

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