ELSEVIER

Pharmaceutica Acta Helvetiae 73 (1998) 95–103

PHARMACEUTICA ACTA HELVETIAE

Studies on 2-benzyloxy-4*H*-3,1-benzoxazin-4-ones as serine protease inhibitors

Michael Gütschow [a,*], Ulf Neumann [b], Joachim Sieler [c], Kurt Eger [a]

[a] *Institut für Pharmazie, Universität Leipzig, D-04103 Leipzig, Germany*
[b] *Novartis Pharma, CH-4002 Basel, Switzerland*
[c] *Institut für Anorganische Chemie, Universität Leipzig, D-04103 Leipzig, Germany*

Received 24 November 1997; accepted 3 December 1997

Abstract

The class of 3,1-benzoxazin-4-ones includes potent inhibitors of various serine proteases. Structural investigations on three 2-benzyloxy-4*H*-3,1-benzoxazin-4-ones (**1**–**3**) are described with respect to their reactivity to alkaline hydrolysis. The ^{13}C NMR data of 2-benzyloxy-5-methyl-4*H*-3,1-benzoxazin-4-one **3** are discussed. This *peri* substituted compound was subjected to a crystal structure analysis. The heterocyclic skeleton together with the carbonyl oxygen and the methyl carbon is planar, and only small angle distortions occurred. The inhibition of neutrophil serine proteases by **1**–**3** is reported. The different reactivity of the 5-methyl derivative **3** towards serine proteases is mainly influenced by specific interactions within the active sites. Thus, **3** was found to rapidly acylate human leukocyte proteinase 3 and exhibited a K_i value of 1.8 nM.

Keywords: Serine proteases; Enzyme inhibition; 4*H*-3,1-benzoxazin-4-ones; Crystal structure analysis

1. Introduction

Human leukocyte elastase (HLE), cathepsin G, and proteinase 3 are serine proteases found in the azurophilic granules of neutrophils. The massive influx of neutrophils and the release of proteolytic enzymes following neutrophil activation is a marker event in acute inflammation. Inadequate control by endogenous inhibitors can lead to an excess of active serine proteases which causes destruction of extracellular matrix components (Krantz, 1993; Caughey, 1994). The involvement of neutrophil serine proteases in inflammatory diseases has provided the impetus behind efforts to develop low molecular inhibitors of these enzymes.

3,1-Benzoxazin-4-ones have attracted considerable attention as inhibitors of serine proteases. This class includes potent inhibitors of chymotrypsin and elastases (Hedstrom et al., 1984; Spencer et al., 1986; Stein et al., 1987; Radhakrishnan et al., 1987; Krantz et al., 1990; Neumann et al., 1991; Uejima et al., 1993; Neumann and Gütschow, 1995). Recently, the inhibition of C1r serine protease of the complement system (Gilmore et al., 1996), HSV-1 protease (Jarvest et al., 1996), and human cytomegalovirus protease (Abood et al., 1997) by benzoxazinones was reported.

The interaction of benzoxazinones with serine proteases involves enzyme acylation due to the nucleophilic attack of the active site serine on the carbonyl carbon and the subsequent deacylation of the acyl enzyme formed. Extensive investigations (Krantz et al., 1990) have led to highly potent acyl-enzyme inhibitors (alternate substrate inhibitors) of HLE. Structure-activity relationships revealed that small alkyl substituents linked via heteroatoms to C-2 and alkyl groups at C-5 are favorable to improve inhibitory activity. The best compounds exhibited K_i values towards HLE in the lower pM range.

* Corresponding author. Fax: +49-341-9736749; e-mail: guetscho@uni-leipzig.de.

0031-6865/98/$19.00
PII S0031-6865(98)00003-X

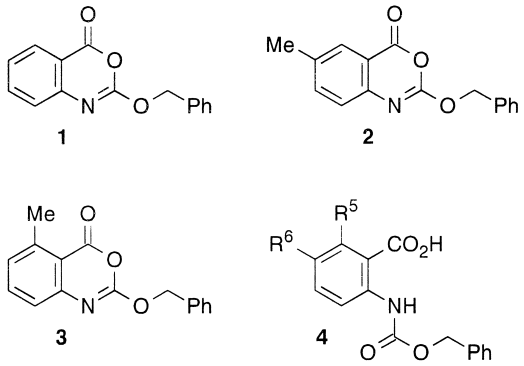


Fig. 1. Structures of 2-benzyloxy-4*H*-3,1-benzoxazin-4-ones **1**–**3**. These compounds were prepared by reaction of corresponding benzoic acids **4** with ethyl chloroformate (Gütschow and Neumann, 1997).

Previously, the inhibition of cathepsin G by benzoxazinones was investigated (Gütschow and Neumann, 1997). The primary specificity site S_1 of cathepsin G is larger than that of HLE showing a strong preference for Phe and Tyr. It was found that the introduction of a bulky aliphatic or aromatic moiety into the 2-substituent was favorable to accelerate cathepsin G acylation, indicating the accommodation of this residue at the S_1 subsite. 2-Benzyloxy derivatives **1** and **2** (Fig. 1) exhibiting K_i values towards cathepsin G lower than 50 nM, are among the most potent synthetic inhibitors of cathepsin G.

Herein we report on spectroscopic investigations on 2-benzyloxy-4*H*-3,1-benzoxazin-4-ones **1**–**3** (Fig. 1) with respect to their intrinsic reactivity, and the crystal structure analysis of the 5-methyl derivative **3**. Furthermore, the inhibition of HLE and human leukocyte proteinase 3 by **1**–**3** is reported.

2. Experimental procedures

2.1. Methods and materials

Melting points were determined on a Boetius apparatus and are not corrected. Elemental analyses were within $\pm 0.4\%$ of the theoretical values. Thin-layer chromatography was performed on Merck aluminium sheets, silica gel 60 F_{254}. IR spectra were measured with a Perkin Elmer 16 PC FTIR spectrometer. UV spectra were recorded on a Shimadzu UV-VIS spectrophotometer UV-160A. ^{13}C NMR spectra (75 MHz) and ^{1}H NMR spectra (300 MHz) were recorded on a Varian Gemini 300. ^{13}C NMR signals were assigned on the basis of APT (attached proton test), and HETCOR (heteronuclear correlation) experiments. Coupling constants and multiplicities of the ^{13}C coupled spectrum of compound **3** are given, with the exception of overlapped signals. 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. Human leukocyte elastase was purchased from Calbiochem, and human leukocyte proteinase 3 from Athens Research and Technology, Athens, GA. The substrates Suc-Ala-Ala-Pro-Val-pNA and MeOSuc-Lys(2-picolinoyl)-Ala-Pro-Val-pNA were from Bachem, Bubendorf, Switzerland.

2.2. Synthesis of 2-[(benzyloxycarbonyl)amino]benzoic acids **4**

Under argon atmosphere, benzyl chloroformate (2.56 g, 15 mmol) was added to a solution of the appropriate anthranilic acid (12.5 mmol) in pyridine (8 ml) at 0°C. The mixture was stirred at 0°C for 90 min and poured onto ice-water (125 ml). A 1-M HCl (50 ml) was added and the mixture was extracted with ethyl acetate (4×50 ml). The organic layer was washed with H_2O (2×50 ml), dried (Na_2SO_4) and the solvent was removed in vacuo.

2.2.1. 2-[(Benzyloxycarbonyl)amino]benzoic acid

This compound was prepared from anthranilic acid. Yield 73%; m.p. 140–143°C (ethyl acetate/petroleum ether), lit. m.p. 140–141°C (Ruggl and Dahn, 1944); ^{1}H NMR ($CDCl_3$) δ 5.25 (s, 2H, CH_2), 7.06–7.12 (m, 1H, H-5), 7.34–7.47 (m, 5 ArH), 7.56–7.64 (m, 1H, H-4), 8.12 (dd, $J = 8.0$, 1.6 Hz, 1H, H-6), 8.49–8.52 (m, 1H, H-3), 10.31 (s, 1H, NH).

2.2.2. 2-[(Benzyloxycarbonyl)amino]-5-methylbenzoic acid

This compound was prepared from 2-amino-5-methylbenzoic acid in 70% yield: m.p. 168–170°C (EtOH/H_2O); ^{1}H NMR ($CDCl_3$) δ 2.34 (s, 3H, CH_3), 5.23 (s, 2H, CH_2), 7.34–7.48 (m, 6 ArH), 7.91 (d, $J = 1.9$ Hz, 1H, H-6), 8.38 (d, $J = 8.6$ Hz, 1H, H-3), 10.19 (s, 1H, NH). Anal. ($C_{16}H_{15}NO_4$) C, H, N.

2.2.3. 2-[(Benzyloxycarbonyl)amino]-6-methylbenzoic acid

This compound was synthesized from 2-amino-6-methylbenzoic acid in 41% yield: m.p. 125–135°C (ether/hexane); ^{1}H NMR ($CDCl_3$) δ 2.57 (s, 3H, CH_3), 5.23 (s, 2H, CH_2), 6.95–6.98 (m, 1H, H-5), 7.33–7.44 (m, 6 ArH), 8.10–8.14 (m, 1H, H-3), 9.31 (s, 1H, NH). Anal. ($C_{16}H_{15}NO_4$) C, H, N.

2.3. Synthesis of 2-benzyloxy-4H-3,1-benzoxazin-4-ones **1–3**

Ethyl chloroformate (4.88 g, 45 mmol) was added dropwise at 0°C under argon atmosphere to a solution of

the appropriate 2-[(benzyloxycarbonyl)amino]benzoic acid **4** (10 mmol) in pyridine (10 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 (100 ml) and the precipitate was collected by filtration, dried, and recrystallized from ethyl acetate/petroleum ether. Yields, physical constants, and ^{1}H NMR data are given in reference (Gütschow and Neumann, 1997).

*2.3.1. 2-Benzyloxy-4H-3,1-benzoxazin-4-one **1***

Compound **1** was prepared from **4(R^5=R^6=H)**. IR (KBr, cm^{-1}) 1784 (C=O); UV (EtOH) λ_{max} (nm) (log e) 247 (4.27), 314 (3.98); ^{13}C NMR δ 71.31 (CH_2), 114.6 (C-4a), 125.5 (C-8), 126.0 (C-6), 128.5 and 128.7 (C-2′ and C-3′), 128.8 (C-4′), 129.1 (C-5), 134.6 (C-1′), 136.9 (C-7), 148.2 (C-8a), 154.7 (C-2), 159.5 (C-4); MS (EI) *m/z* (rel. intensity) 253 (M^+, 10), 181 (11), 91 ($C_7H_7^+$, 100).

*2.3.2. 2-Benzyloxy-6-methyl-4H-3,1-benzoxazin-4-one **2***

Compound **2** was prepared from **4(R^5=H,R^6=Me)**. IR (KBr, cm^{-1}) 1762 (C=O); UV (EtOH) λ_{max} (nm) (log e) 250 (4.02), 323 (3.70); ^{13}C NMR δ 21.0 (CH_3), 71.13 (CH_2), 114.3 (C-4a), 125.2 (C-8), 128.6 (C-5), 128.5 and 128.7 (C-2′ and C-3′), 128.8 (C-4′), 134.7 (C-1′), 136.1 (C-6), 138.1 (C-7), 146.0 (C-8a), 154.3 (C-2), 159.6 (C-4). MS (EI) *m/z* (rel. intensity) 267 (M^+, 11), 195 (21), 91 ($C_7H_7^+$, 100).

*2.3.3. 2-Benzyloxy-5-methyl-4H-3,1-benzoxazin-4-one **3***

Compound **3** was prepared from **4(R^5=Me,R^6=H)**. IR (KBr, cm^{-1}) 1756 (C=O); UV (EtOH) λ_{max} (nm) (log e) 252 (3.94), 320 (3.75); ^{13}C NMR δ 22.4 (qd, ^1J = 128.8, ^3J = 5.2 Hz, CH_3), 71.05 (t, ^1J = 149.6 Hz, CH_2), 113.1 (m, C-4a), 123.5 (dm, ^1J = 167.5 Hz, C-8), 128.55 (C-6), 128.66 and 128.46 (C-2′ and C-3′), 128.74 (C-4′), 134.7 (m, C-1′), 135.8 (d, ^1J = 161.8 Hz, C-7), 143.3 (m, C-5), 149.6 (d, ^3J = 9.6 Hz, C-8a), 154.5 (s, C-2), 158.7 (s, C-4); MS (EI) *m/z* (rel. intensity) 267 (M^+, 15), 194 (15), 91 ($C_7H_7^+$, 100).

*2.4. X-ray analysis of compound **3***

Crystals of **3** are triclinic: a = 7.084 (3), b = 8.408 (5), c = 11.480 (10) Å, α = 95.84 (5), β = 106.80 (7), γ = 91.78 (4)°, V = 649.8 (7) Å^3, d_{calcd} = 1.366 Mg m^{-3}, Z = 2, space group $P\bar{1}$. Crystal size was 0.28 × 0.20 × 0.10 mm. The unit-cell parameters and intensities of 4536 reflections were measured at 20°C on a Stoe STADI4 diffractometer (Mo–K_α radiation, graphite monochromator, $\theta/2\theta$-scanning up to 29.2°). About 3474 reflections were symmetry independent (R_{int} = 0.056). The structure was solved by the direct method and refined by the full-matrix least squares method with anisotropic thermal parameters for non-hydrogen atoms. The position of hydrogen atoms were located from the difference synthesis and refined isotropically. The final R values are as follows, data $I > 2\sigma(I)$: R_1 = 0.0398, wR_2 = 0.1104; all data: R_1 = 0.101, wR_2 = 0.1320. All calculations were carried out using the SHELXS-86 (Sheldrick, 1986), and SHELXL-93 (Sheldrick, 1993) programs.

2.5. Enzymatic studies

Enzyme inhibition was assayed at 25°C using the progress curve method. Assay buffer were 50 mM sodium phosphate, 500 mM NaCl, pH 7.8 for HLE, and 50 mM sodium phosphate, 100 mM NaCl, pH 7.8 for proteinase 3. Stock solutions of substrates and inhibitors were prepared in DMSO, the final DMSO concentration was ≤ 6%. Inhibition of HLE was determined with Suc-Ala-Ala-Pro-Val-pNA (100 μM) as chromogenic substrate. Proteinase 3 was assayed with MeOSuc-Lys(2-picolinoyl)-Ala-Pro-Val-pNA (210 μM). Progress curves were monitored at 405 nm over 30–60 min and fitted to the equation for the slow-binding inhibition (Morrison, 1982). Data were analyzed as described previously (Gütschow and Neumann, 1997).

3. Results and discussion

3.1. Alkaline hydrolysis and spectroscopic data

The interaction of serine proteases with 2-alkoxy-4*H*-3,1-benzoxazin-4-ones (Fig. 2) occurs via corresponding

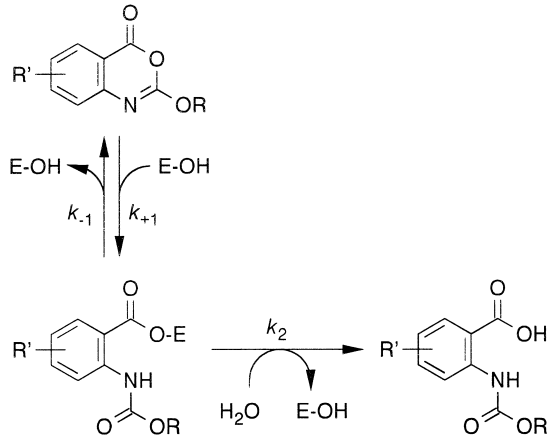


Fig. 2. Inhibition of serine proteases by 2-alkoxy-4*H*-3,1-benzoxazin-4-ones. Formation of acyl-enzymes and deacylation. Acylation rate constant $k_{on} = k_{+1}$. Deacylation rate constant $k_{off} = k_{-1} + k_2$. $K_i = k_{off}/k_{on}$.

acyl-enzymes, formed due to nucleophilic attack of the active site serine at the C-4 atom of the inhibitors and ring cleavage (Hedstrom et al., 1984; Krantz et al., 1990; Neumann et al., 1991). Since alkaline hydrolysis of benzoxazinones also proceeds by hydroxide attack on the carbonyl, it can therefore be used as a simple model for enzyme acylation. Rates of alkaline hydrolysis k_{OH^-} were furthermore needed to estimate the stability of the compounds. The log k_{OH^-} values for **1**–**3** are given in Table 1, together with calculated values. The equation was taken from a report (Krantz et al., 1990), in which the authors have used a large set of benzoxazinones for multiple regression. It should be noted that the regression includes a term to describe steric influence from R^5. Our data satisfactorily fit the equation and support, that 6-methyl substitution leads to a slightly decreased hydrolytic rate, whereas 5-methyl substitution remarkably increases hydrolytic stability. However, it was considered that the introduction of a 5-methyl group may only result in a rather small steric hindrance, since the hydroxide attack on the carbonyl of the rigid, planar benzoxazinone is essentially perpendicular (Spencer et al., 1986; Krantz et al., 1990).

The carbonyl stretching frequency of 3,1-benzoxazin-4-ones may be used to estimate carbonyl reactivity (Spencer et al., 1986; Neumann and Gütschow, 1995). In fact, we have found decreased values for the methyl substituted derivatives **2** (1762 cm^{-1}) and **3** (1753 cm^{-1}), compared to **1** (1784 cm^{-1}). The ms fragmentation of compounds **1**–**3** is mainly characterized by the formation of the $C_7H_7^+$ fragment, thus an effect of the *peri* substitution in **3** was not observed.

A 6-methyl substitution of 2-ethoxy, 2-*n*-propoxy, and 2-isobutoxybenzoxazinones resulted in slightly decreased k_{OH^-} values, and produced a small but significant upfield shift of the OCH_2 protons when compared to the ^{1}H NMR data of corresponding 6-unsubstituted analogs, thus reflecting susceptibility to alkaline hydrolysis (Gütschow and Neumann, 1997). The corresponding chemical shifts of the methylene protons in **1**–**3** are given in Table 1. The effect of a 5-methyl or 6-methyl substitution, respectively, did not differ to an extent that influenced the shift of the OCH_2 protons (5.48 ppm in both compounds). However, a small difference in the corresponding ^{13}C NMR values of the OCH_2 carbons in **2** and **3** was observed (Table 1). The OCH_2 protons in **3** appear as one singlet; the absence of diastereotopic hydrogens indicates a non-chiral planar framework of the heterocyclic skeleton and the methyl group (see below).

Previously, correlations between the C-4 chemical shifts and rates of alkaline hydrolysis of 4*H*-3,1-benzoxazin-4-ones have been determined (Robinson and Spencer, 1988). Within the set of C-5 unsubstituted compounds, the log k_{OH^-} values were indirectly proportional to the chemical shift of C-4. Because 5-methyl and 5-ethyl benzoxazinones were found to be more resistant to alkaline hydrolysis than their C-4 chemical shift would suggest, a steric term for 5-substitution was taken into account for parameterization. The C-4 chemical shift difference of 2-ethoxybenzoxazinone (159.0 ppm) and 2-ethoxy-5-methylbenzoxazinone (158.1 ppm, both spectra recorded in DMSO-d_6) (Robinson and Spencer, 1988) was similar to the difference we have found between **1** and **3** (Table 1).

As a further result of ^{13}C NMR assignments, anomalous chemical shifts were found in the case of 5-substituted benzoxazinones. Substitution of hydrogen by methyl at C-5 in benzoxazinones produce a 13.7 ± 0.3 ppm downfield shift of C-5 (Robinson and Spencer, 1988). Similar observations have been described for another *peri* substituted system, 1,8-disubstituted naphthalene (Dalling et al., 1977). For example, the introduction of a 8-methyl group into naphthalene resulted in a 6.3 ppm downfield shift of C-8 and a 0.8 ppm downfield shift of C-7, whereas the introduction of a 8-methyl group into 1-methylnaphthalene produced 11.3 ppm and 3.8 ppm downfield shifts of C-8 and C-7, respectively. Such pronounced deshielding effects were also found when comparing ^{13}C NMR values of **1** and **3**. 5-Methyl substitution produced a 14.2 ppm downfield shift of C-5 and a 2.6 ppm downfield shift of C-6 (Table 1). On the other hand, the introduction of a methyl group at position 6 of benzoxazinones resulted in expected

Table 1
Kinetic parameters of the alkaline hydrolysis and spectroscopic data of 2-benzyloxy-4*H*-3,1-benzoxazin-4-ones

Compound	log k_{OH^-}		^{1}H NMR ($CDCl_3$)[a]	^{13}C NMR ($CDCl_3$)			
	Found[a]	Calc.[b]	OCH_2Ph (δ, ppm)	OCH_2Ph (δ, ppm)	C-4 (δ, ppm)	C-5 (δ, ppm)	C-6 (δ, ppm)
1	1.85	1.90	5.50	71.31	159.5	129.1	126.0
2	1.68	1.72	5.48	71.13	159.6	128.6	136.1
3	1.29	1.31	5.48	71.05	158.7	143.3	128.6

[a] Data from Gütschow and Neumann (1997).
[b] The following equation (Krantz et al., 1990) was used: $\log k_{OH^-} = 1.64 + 2.64\ \sigma_{total} - 0.15\ MR_5$; with σ_m for 2-$OCH_2Ph = 0.10$, σ_m for 6-Me $= -0.07$, σ_p for 5-Me $= -0.17$, and MR for 5-Me $= 1$ (Hansch et al., 1991).

chemical shifts, comparable to the data of naphthalenes. For example, 7-methyl substitution of naphthalene, or 1-methylnaphthalene (in parentheses) produced the following shift differences, C-7: +9.6 ppm (+9.6 ppm), C-8: −1.0 ppm (−0.9 ppm). Similar differences were observed by comparison of **1** and **2**; 6-methyl substitution gave a 10.1 ppm-downfield shift of C-6, and C-5 is shielded by 0.5 ppm (Table 1). It is therefore to be concluded, that steric effects account for the chemical shifts in both 1,8-dimethylnaphthalene and 5-methylbenzoxazinone **3**.

3.2. X-ray crystal structure of 2-benzyloxy-5-methyl-4H-3,1-benzoxazin-4-one

It was suggested (Robinson and Spencer, 1988) that these anomalous shifts in 5-substituted benzoxazinones could reflect distortions from planarity as it has been observed in 1,8-disubstituted naphthalenes (Handal et al., 1977; Hansen, 1979; Dalling et al., 1977). However, no crystal structure analysis of a 5-substituted 4*H*-3,1-benzoxazin-4-one was reported so far. We have therefore subjected compound **3** to a X-ray crystal structure analysis. Atomic coordinates are listed in Table 2. The heterocyclic skeleton together with the carbonyl oxygen and the methyl carbon is planar (Fig. 3). The average deviation from this plane is 0.014 Å. It is noteworthy, that the methyl carbon was not found out of the benzoxazinone plane, as one might expect due to *peri* substituent interaction with the carbonyl structure. The methyl hydrogen on the outside of the *peri* interaction lies almost in the molecular plane. We have considered the data of the crystal structure analysis of 1,8-dimethylnaphthalene (Bright et al., 1973) to discuss molecular distortions caused by steric interaction of the two *peri* substituted methyl groups. This molecule also has a planar carbon skeleton, in contrast to other 1,8-disubstituted naphthalenes (e.g., 1,3,6,8-tetra-*tert*-butylnaphthalene, in which the *peri* carbons C-1 and C-8 are 0.29 Å above and below the mean plane of the molecule) (Handal et al., 1977). Furthermore, for a comparison, selected bond lengths and angles of the two 3,1-benzoxazin-4-ones, for which X-ray crystal structures are available, are listed in Table 3. It becomes obvious, that the geometric features around the lactone structure in **3** are similar to that of the two other benzoxazinones. The lactone carbon–oxygen bond lengths were not distinctly influenced by *peri* methyl substitution. In compound **3**, the O–C(2) single bond (1.343 (2) Å) is shorter than the O–C(4) single bond (1.394 (2) Å); the difference is significant. As a result of the *peri* substitution in **3**, the C=O bond and the methyl group are bent slightly outwards.

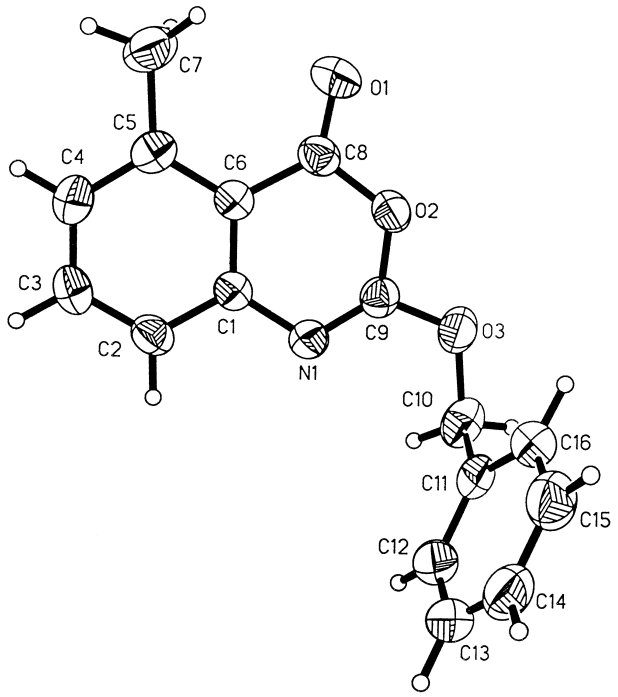


Fig. 3. ORTEP view of 2-benzyloxy-5-methyl-4*H*-3,1-benzoxazin-4-one **3**.

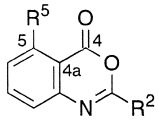
Table 2
Atomic coordinates ($\times 10^4$) and equivalent isotropic displacement parameters (Å$\times 10^3$) for **3**[a]

	x	*y*	*z*	*U*(eq)
O(2)	1978 (2)	6728 (1)	4816 (1)	46 (1)
N(1)	2322 (2)	8867 (1)	6352 (1)	39 (1)
C(6)	2392 (2)	9406 (2)	4305 (1)	35 (1)
O(3)	1849 (2)	6203 (1)	6575 (1)	52 (1)
O(1)	1890 (2)	6982 (1)	2931 (1)	65 (1)
C(1)	2487 (2)	9927 (2)	5518 (1)	35 (1)
C(2)	2741 (2)	11,562 (2)	5922 (1)	44 (1)
C(3)	2918 (2)	12,644 (2)	5134 (2)	49 (1)
C(4)	2847 (2)	12,126 (2)	3945 (2)	47 (1)
C(5)	2574 (2)	10,517 (2)	3496 (1)	41 (1)
C(7)	2497 (3)	10,032 (3)	2192 (2)	61 (1)
C(8)	2080 (2)	7689 (2)	3913 (1)	41 (1)
C(9)	2079 (2)	7393 (2)	5948 (1)	40 (1)
C(10)	1935 (3)	6608 (2)	7842 (2)	55 (1)
C(11)	4018 (2)	6738 (2)	6870 (1)	46 (1)
C(12)	4561 (3)	7869 (2)	9685 (2)	60 (1)
C(13)	6466 (4)	7960 (2)	10,475 (2)	70 (1)
C(14)	7828 (3)	6931 (2)	10,265 (2)	68 (1)
C(15)	7300 (3)	5810 (2)	9253 (2)	62 (1)
C(16)	5418 (3)	5705 (2)	8465 (2)	50 (1)

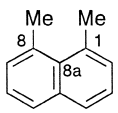
U(eq) is defined as one third of the trace of the orthogonalized U_{ij} tensor. The atomic numbering is according to Fig. 3.

[a] Further details of the crystal structure determination can be obtained from the Fachinformationszentrum Karlsruhe, D-76344 Eggenstein-Leopoldshafen, Germany, on quoting the depository number CSD-408212.

Table 3
Selected bond lengths and bond angles of 4*H*-3,1-benzoxazin-4-ones and 1,8-dimethylnaphthalene



3, 5, 6



7

Compound	R^2	R^5	Bond lengths (Å)		Bond angles (°)			
			O=C(4)	O–C(4)	O=C(4)–C(4a)	O=C(4)–O	C(4)–C(4a)–C(5)[a]	C(5-Me)–C(5)–C(4a)[a]
							C(1)–C(8a)–C(8)[b]	C(8-Me)–C(8)–C(8a)[b]
3	OCH_2Ph	Me	1.188 (2)	1.394 (2)	129.5 (2)	114.88 (14)	121.79 (13)	123.0 (2)
5[c]	Me	H	1.193	1.388	128.2	116.2	121.4	
6[d]	morpholino	H	1.200	1.398	128.2	116.4	120.3	
7[e]							125.2	124.8

[a] In benzoxazinones **3**, **5**, **6**.
[b] In 1,8-dimethylnaphthalene **7**.
[c] Data from Etter et al. (1982).
[d] Data from Pink et al. (1993).
[e] Data from Bright et al. (1973).

However, in comparison with 1,8-dimethylnaphthalene, these distortions are less serious. Thus, as a consequence of the molecular geometry of **3**, the distance between the methyl carbon and the carbonyl oxygen is shorter (2.84 Å) than the sum of their van der Waals radii (3.25 Å) (Taylor and Kennard, 1982), leading to a repulsive interaction.

In summary, we have investigated structural properties of a 5-methyl substituted benzoxazinone by means of various analytical and spectroscopic methods. A steric interaction between 5-methyl and carbonyl oxygen affects the rate of alkaline hydrolysis. Mainly, this effect is believed to be a result of steric hindrance of the hydroxide attack at the carbonyl. ^{13}C NMR data support the previously reported results with other 5-alkyl substituted benzoxazinones. However, geometrical distortions in the molecular structure of **3**, as it have been concluded from ^{13}C NMR shifts, were rather small. The X-ray structure of **3** may be useful for molecular modelling studies of non-covalent complexes of serine proteases with this inhibitor.

These investigations were also undertaken in the light of the strong inhibitory activity of 5-alkyl substituted benzoxazinones towards HLE. 5-Alkyl substitution in certain benzoxazinones may lead to a different orientation of the inhibitor within the active site, in which the 5-substituent, instead of the 2-substituent, binds to the primary activity site S_1 of HLE. Evidence for this assumption came from the fact that 5-ethyl- and propyl substitution results in strongly increased acylation rates (Krantz et al., 1990). Furthermore, a crystal structure analysis of the acyl-enzyme formed in the reaction of porcine pancreatic elastase with a 5-methylbenzoxazinone, showed the S_1 pocket being occupied by the 5-methyl group (Radhakrishnan et al., 1987).

3.3. Inhibition of serine proteases

The kinetic parameters for the inhibition of four serine proteases by benzyloxybenzoxazinones **1**–**3** are outlined in Table 4. Both 6- and 5-methyl substitution led to remarkable differences in serine protease inhibition. HLE inhibition was improved in the case of the 6-methyl derivative **2** and, even stronger, in the case of 5-methyl derivative **3**. 5-Methyl substitution resulted in a strongly decreased deacylation rate k_{off}, that favorably affects K_i. The latter result is consistent with previously reported data on HLE inhibition by benzoxazinones (Krantz et al., 1990). The slower deacylation in the case of 5-substituted compounds can be interpreted as a result of steric hindrance of water attack at the ester carbonyl to decelerate deacylation via hydrolysis (k_2, Fig. 2).

Remarkably, compounds **1**–**3** were also found to be potent inhibitors of proteinase 3. Proteinase 3 and HLE have similar proteolytic profiles. Proteinase 3 is a neutrophil enzyme, that can cause emphysema and may play a critical role in the pathogenesis of the disease. Moreover, proteinase 3 is known to be an autoantigen in Wegener's granulomatosis (Caughey, 1994; Krantz, 1993). Some low molecular inhibitors of proteinase 3, such as sulfonyloxy succinimides, have been described. For example, 3-ethyl-*N*-[(methylsulfonyl)oxy]succinimide exhibited a $k_{obs}/[I]$ value of 13,400 M^{-1} s^{-1} (Groutas et al., 1990). The

Table 4
Kinetic parameters for the inhibition of serine proteases by 2-benzyloxy-4*H*-3,1-benzoxazin-4-ones

Compound	HLE			Proteinase 3			Human cathepsin G[a]			Bovine chymotrypsin[a]		
	k_{on} (10^3 M^{-1} s^{-1})	k_{off} (10^{-4} s^{-1})	K_i (nM)	k_{on} (10^3 M^{-1} s^{-1})	k_{off} (10^{-4} s^{-1})	K_i (nM)	k_{on} (10^3 M^{-1} s^{-1})	k_{off} (10^{-4} s^{-1})	K_i (nM)	k_{on} (10^3 M^{-1} s^{-1})	k_{off} (10^{-4} s^{-1})	K_i (nM)
1	77.7	18	23.3[b]	6.32	2.3	37.2	7.02	2.4	30	1650	2	0.12
2	30.1	2.0	6.7	12.1	2.4	19.8	70.3	11.6	20	14,600	10	0.07
3	48.4	0.4	0.83	111	2.0	1.8	0.528	1.2	230	1340	2.6	0.19

[a] Data from Gütschow and Neumann (1997).
[b] $pK_i = 7.63$. A value $pK_i = 7.25$ was reported (Krantz et al., 1990).

inhibitory potency of **1**–**3** for proteinase 3 increased in the same order as the HLE inhibition. In contrast to the results with HLE, methyl substitution in **2** and **3** did not affect deacylation rate, but strongly affected acylation rate. Thus, compound **3**, exhibiting a second-order rate constant for acylation $k_{on} > 1 \times 10^5$ M^{-1} s^{-1}, is a highly potent inhibitor for human leukocyte proteinase 3.

Proteinase 3 has a primary substrate specificity for amino acids with small aliphatic residues such as Val or Ala. One might suspect, that the accelerated acylation of proteinase 3 by the 5-methyl derivative **3** could be a result of the accommodation of the methyl substituent at the primary specificity site, leading to an improved association of a non-covalent enzyme-inhibitor complex, EI, prior to the acylation step. Experimentally, the accumulation of EI was not detected under the assay conditions used (inhibitor concentration range of **3** was 25–125 nM). Nevertheless, we cannot exclude that a tighter association of EI due to 5-methyl substitution is responsible for the increased k_{on} value. In any case, by comparison of **1** and **3**, the reduced susceptibility to alkaline hydrolysis of **3**, on the one hand, and the drastically accelerated acylation, on the other hand, strongly indicates a specific recognition of the 5-methyl group at the active site. This finding may help to develop further acyl-enzyme inhibitors of proteinase 3, by extending the substitution pattern in benzoxazinones (e.g., at position 2) or related structures.

As it was previously reported, the introduction of an aromatic moiety at position 2 of benzoxazinones resulted in an accelerated acylation of cathepsin G and chymotrypsin (Gütschow and Neumann, 1997). Both proteases exhibit a primary substrate specificity for aromatic amino acids. It was therefore concluded that the 2-substituent interacts with the S_1 subsite of both enzymes. For a comparison, kinetic parameters of the inhibition by **1**–**3** are given in Table 4. 6-Methyl substitution (compound **2**) accelerated acylation of cathepsin G and chymotrypsin by one order of magnitude. Although deacylation was also accelerated, a strong inhibition of both enzymes was achieved (e.g., towards chymotrypsin in the lower picomolar range). In contrast to the results on the inhibition of HLE and proteinase 3, 5-methyl substitution (compound **3**) was less favorable. Towards cathepsin G, the acylation rate was reduced by one order of magnitude.

These results indicate that a 5-methyl substitution affects reactivity of benzoxazinones towards serine proteases mainly due to different specific interactions within the active sites, e.g., due to different binding orientations. Effects of the *peri* substitution on structural parameters of the inhibitor were less obvious. Further investigations to develop heterocyclic acyl-enzyme inhibitors of neutrophil serine proteases are in progress in our laboratories.

Acknowledgements

The authors thank Dr. Trevor Payne, Novartis Pharma Research Basel, for critically reading the manuscript. M.G. and K.E. would like to thank the 'Fonds der Chemischen Industrie' for financial support.

References

Abood, N.A., Schretzman, L.A., Flynn, D.L., Houseman, K.A., Wittwer, A.J., Dilworth, V.M., Hippenmeyer, P.J., Holwerda, B.C., 1997. Inhibition of human cytomegalovirus protease by benzoxazinones and evidence of antiviral activity in cell cultures. Bioorg. Med. Chem. Lett. 7, 2105–2108.

Bright, D., Maxwell, I.E., de Boer, J., 1973. Crystal structure analysis and strain-energy minimization calculations on a sterically crowded molecule: 1,8-dimethylnaphthalene. J. Chem. Soc. Perkin II, pp. 2101–2105.

Caughey, G.H., 1994. Serine proteinases of mast cell and leukocyte granules. A league of their own. Am. J. Respir. Crit. Care Med. 150, 5138–5142.

Dalling, D.K., Ladner, K.H., Grant, D.M., Woolfenden, W.R., 1977. Carbon-13 magnetic resonance: 27. The dependence of chemical shifts on methyl rotational conformations and dynamics in the methylated benzenes and naphthalenes. J. Am. Chem. Soc. 99, 7142–7150.

Etter, M.C., Errede, L.A., Vicens, J., 1982. Structure and solid-state hydrolysis of 2-methyl-4*H*-benzoxazin-4-one (acetyl anthranyl), $C_9H_7NO_2$. Cryst. Struct. Comm. 11, 885–888.

Gilmore, J.L., Hays, S.J., Caprathe, B.W., Lee, C., Emmerling, M.R., Michael, W., Jaen, J.C., 1996. Synthesis and evaluation of 2-aryl-4*H*-3,1-benzoxazin-4-ones as C1r serine protease inhibitors. Bioorg. Med. Chem. Lett. 6, 679–682.

Groutas, W.C., Hoidal, J.R., Brubaker, M.J., Stanga, M.A., Venkataraman, R., Gray, M.A., Rao, N.V., 1990. Inhibitors of human leukocyte proteinase-3. J. Med. Chem. 33, 1085–1087.

Gütschow, M., Neumann, U., 1997. Inhibition of cathepsin G by 4*H*-3,1-benzoxazin-4-ones. Bioorg. Med. Chem. 5, 1935–1942.

Handal, J., White, J.G., Franck, R.W., Yuh, Y.H., Allinger, N.L., 1977. The structure of 1,3,6,8-tetra-*tert*-butylnaphthalene. J. Am. Chem. Soc. 99, 3345–3348.

Hansch, C., Leo, A., Taft, R.W., 1991. A survey of Hammett substituent constants and resonance and field parameters. Chem. Rev. 91, 165–195.

Hansen, P.E., 1979. ^{13}C NMR of polycyclic aromatic compounds. A review. Org. Magn. Reson. 12, 109–141.

Hedstrom, L., Moorman, A.R., Dobbs, J., Abeles, R.H., 1984. Suicide inactivation of chymotrypsin by benzoxazinones. Biochemistry 23, 1753–1759.

Jarvest, R.L., Paratt, M.J., Gorniak, J.G., Jennings, L.J., Serafinowska, H.T., Strickler, J.E., 1996. Inhibition of HSV-1 protease by benzoxazinones. Bioorg. Med. Chem. Lett. 6, 2463–2466.

Krantz, A., Spencer, R.W., Tam, T.F., Liak, T.J., Copp, L.J., Thomas, E.M., Rafferty, S.P., 1990. Design and synthesis of 4*H*-3,1-benzoxazin-4-ones as potent alternate substrate inhibitors of human leukocyte elastase. J. Med. Chem. 33, 464–479.

Krantz, A., 1993. Proteinases in inflammation. Annu. Rep. Med. Chem. 29, 195–204.

Morrison, J.F., 1982. The slow-binding and slow, tight-binding inhibition of enzyme-catalyzed reactions. Trends Biochem. Sci. 7, 102–105.

Neumann, U., Stürzebecher, J., Leistner, S., Vieweg, H., 1991. Inhibition of chymotrypsin and pancreatic elastase by 4*H*-3,1-benzoxazin-4-ones. J. Enzyme Inhib. 4, 227–232.

Neumann, U., Gütschow, M., 1995. 3,1-Benzothiazin-4-ones and 3,1-benzoxazin-4-ones: highly different activities in chymotrypsin inactivation. Bioorg. Chem. 23, 72–88.

Pink, M., Sieler, J., Gütschow, M., 1993. Crystal structure of 2-(morpholin-4-yl)-4*H*-3,1-benzoxazin-4-one, $C_{12}H_{12}N_2O_3$. Z. Krist. 207, 319–321.

Radhakrishnan, R., Presta, L.G., Meyer, E.F., Wildonger, R., 1987. Crystal structures of the complex of porcine pancreatic elastase with two valine-derived benzoxazinone inhibitors. J. Mol. Biol. 198, 417–424.

Robinson, V.J., Spencer, R.W., 1988. ^{13}C nuclear magnetic resonance and reactivity of 4*H*-3,1-benzoxazin-4-ones. Can. J. Chem. 66, 416–419.

Ruggl, P., Dahn, H., 1944. Zur Charakterisierung der 1,3,5-Amino-sulfobenzoesäure und ähnlicher Amino-sulfo-carbonsäuren. Helv. Chim. Acta 27, 1116–1122.

Sheldrick, G.M., 1986. SHELXS-86, a program for solving crystal structures, Göttingen, 1986.

Sheldrick, G.M., 1993. SHELXL-93, a program for refining crystal structures, Göttingen, 1993.

Spencer, R.W., Copp, L.J., Bonaventura, B., Tam, T.F., Liak, T.J., Billedeau, R.J., Krantz, A., 1986. Inhibition of serine proteases by benzoxazinones: effects of electron withdrawal and 5-substitution. Biochem. Biophys. Res. Commun. 140, 928–933.

Stein, R.L., Strimpler, A.M., Viscarello, B.R., Wildonger, R.A., Mauger, R.C., Trainor, D.A., 1987. Mechanism for slow-binding inhibition of human leukocyte elastase by valine-derived benzoxazinones. Biochemistry 26, 4126–4130.

Taylor, R., Kennard, O., 1982. Crystallographic evidence for the existence of C–H···O, C–H···N and C–H···Cl hydrogen bonds. J. Am. Chem. Soc. 104, 5063–5070.

Uejima, Y., Kokubo, M., Oshida, J.-I., Kawabata, H., Kato, Y., Fujii, K., 1993. 5-Methyl-4*H*-3,1-benzoxazin-4-one derivatives: specific inhibitors of human leukocyte elastase. J. Pharmacol. Exp. Ther. 265, 516–523.