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2-Benzoylamino-6,7-dimethoxy-4*H*-3,1-benzoxazin-4-one: Synthesis and Investigation of Serine Protease Inactivation

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Summary. 2-Benzoylamino-6,7-dimethoxy-4*H*-3,1-benzoxazin-4-one was prepared by thermal treatment of 2-(3-benzoylthioureido)-4,5-dimethoxybenzoic acid and by benzoylation of 2-amino-6,7-dimethoxy-4*H*-3,1-benzoxazin-4-one. The inactivation of chymotrypsin and human leukozyte elastase by the title compound and 2-benzoylamino-4*H*-3,1-benzoxazin-4-one is reported.

Keywords. 2-Benzoylamino-4*H*-3,1-benzoxazin-4-one; 2-(3-Benzoylthioureido)benzoic acids; Enzyme inactivation; Serine proteases.

2-Benzoylamino-6,7-dimethoxy-4*H*-3,1-benzoxazin-4-on: Synthese und Untersuchung der Inaktivierung von Serin-Proteasen

Zusammenfassung. 2-Benzoylamino-6,7-dimethoxy-4*H*-3,1-benzoxazin-4-on wurde durch thermische Behandlung von 2-(3-Benzoylthioureido)-4,5-dimethoxybenzoesäure und durch Benzoylierung von 2-Amino-6,7-dimethoxy-4*H*-3,1-benzoxazin-4-on hergestellt. Über die Inaktivierung von Chymotrypsin und humaner Leukozyten-Elastase durch die Titelverbindung und 2-Benzoylamino-4*H*-3,1-benzoxazin-4-on wird berichtet.

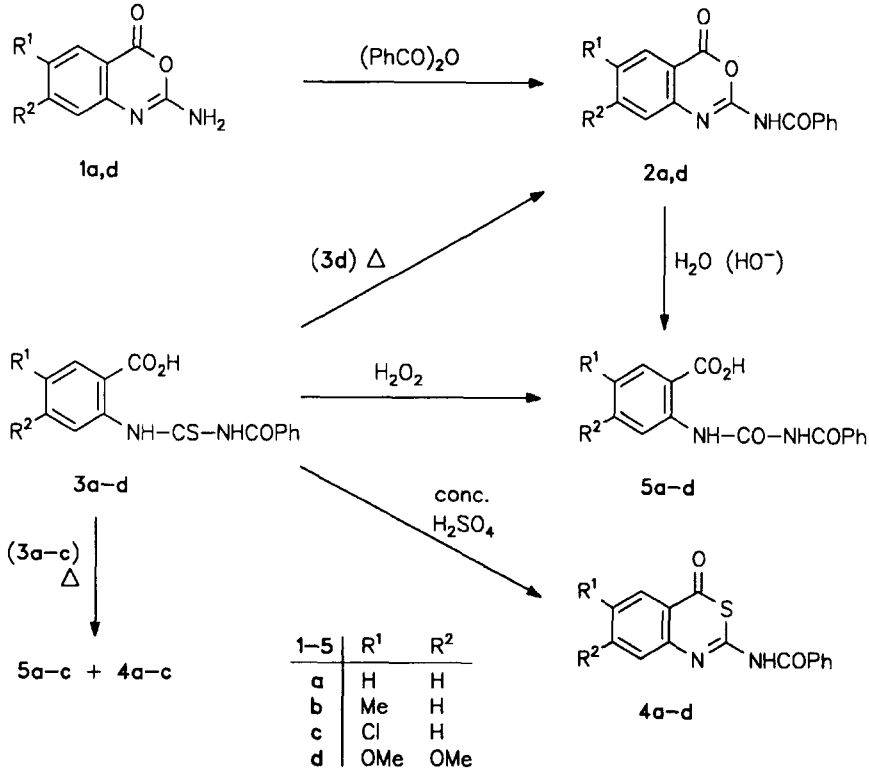
Introduction

2-(3-Benzoylthioureido)benzoic acids (**3**), easily available from benzoyl isothiocyanate [1] and anthranilic acids, are versatile starting compounds for cyclocondensation reactions. Thus, reaction of **3** with sodium methoxide gives 4-oxo-2-thioxo-1,2,3,4-tetrahydroquinazolines, whereas treatment with concentrated sulfuric acid alternatively affords 2-amino-4*H*-3,1-benzothiazin-4-ones or 2-benzoylamino-4*H*-3,1-benzothiazin-4-ones (**4**; [2, 3]). Recently, we have investigated the interaction of a series of 3,1-benzothiazin-4-ones and analogous 3,1-benzoxazin-4-ones with chymotrypsin [3]. 2-Benzoylamino-4*H*-3,1-benzoxazin-4-one **2a** was found to be a highly active chymotrypsin inactivator [4]. In this paper, the synthesis and chymotrypsin inactivating activity of the dimethoxy derivative **2d** is described. The activity of **2a** and **2d** against human leukocyte elastase (*HLE*), an enzyme of highly therapeutic interest [5], is also reported.

Results and Discussion

Our initial approach to benzoylamino-3,1-benzoxazinones **2** was the thermal treatment of benzoylthioureas **3** according to the synthesis of 2-(2-pyrrolyl)-4*H*-3,1-benzoxazin-4-one [6]. Indeed, upon treatment of **3d** in boiling toluene, the desired dimethyoxybenzoxazinone **2d** was obtained together with the by-product **4d** [7]. In contrast, **3a–c** failed to give the corresponding benzoxazinones **2** upon heating in toluene or without solvent. The reactions result in product mixtures of the corresponding benzoylureas **5** and benzoylaminobenzothiazinones **4** [8]. **5a–d** were independently prepared by oxidative desulfurization of the corresponding thioureas **3**. This convenient conversion represents a further example of the versatility of **3**. However, attempts to cyclize **5** to afford **2** were unsuccessful. An alternative synthesis for **2d** was employed starting from 2-amino-4,5-dimethoxybenzoic acid, which was transformed to the new benzoxazine **1d** and subsequently benzoylated to afford **2d**. The structure of **2** was confirmed by hydrolytic cleavage to produce the corresponding benzoylureas **5**.

According to previous reports [3, 9, 10], compounds **2** were assumed to be acyl-enzyme inhibitors of serine proteases. 2-Benzoylamino-6,7-dimethoxy-4*H*-3,1-benzoxazin-4-one (**2d**) was found to be a potent inactivator of chymotrypsin (Table 1). The acylation rate constant (k_{on}), however, was threefold lower than for the unsubstituted derivative **2a**. This might be explained by electron donation due to the dimethoxy substituent. Interestingly, the deacylation rate constant (k_{off}) is



Scheme

Table 1. Rate constants for inactivation of serine proteases

Compound	Chymotrypsin k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_i[a] (nM)	HLE k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_i (nM)
2a	211000	0.0017	8[b]	26061	0.0046	175
2d	65200	0.0017	25	385	0.0002	597

[a] $K_i = k_{off}/k_{on}$; [b] data from Ref. [3].

not affected. A similar tendency was already observed for chymotrypsin inactivation by 2-alkoxy-4*H*-3,1-benzoxazin-4-ones [9]. It is very likely that **2d** acylates chymotrypsin *via* nucleophilic attack of the active-site serine at C-4 and ring-opening, resulting in the corresponding acyl-enzyme which subsequently is deacylated by quinazoline cyclization [3] as recently established for **2a**. The benzoxazinone **2a** also shows *HLE* inactivating activity; the K_i value, however, is adversely affected by the high deacylation rate constant. **2d** was only a poor inactivator of *HLE*. As reported [9, 10], bulky aliphatic substituents at position 2 of 4*H*-3,1-benzoxazin-4-ones are unfavourable for inactivation of elastases which preferentially cleave proteins at sites with small aliphatic residues. On the other hand, 2-benzoylamino substitution leads to potent inactivators of chymotrypsin, indicating that the aromatic substituent binds into the primary specifity pocket of chymotrypsin.

Experimental

Melting points: Boetius apparatus, uncorrected. IR spectra: Perkin Elmer 16PC FTIR. ^{1}H NMR spectra: Varian Gemini 300 (300 MHz). Mass spectra: Varian MAT CH6. TLC: Silica gel GF_{254} (Merck), toluene/acetone/methanol (7:2:1), toluene/ethyl acetate (1:1, 2:1, or 3:1). The products obtained by thermal treatment of **3a–d** and by hydrolytic cleavage of **2d** were proved to be identical (TLC, m.p., IR, ^{1}H NMR) with independently synthesized samples.

2-Amino-6,7-dimethoxy-4H-3,1-benzoxazin-4-one (**1d**)

A solution prepared from 4.9 g (25 mmol) 2-amino-4,5-dimethoxybenzoic acid and 25 ml NaOH (1 *N*) was added to a stirred suspension of 5.3 g (50 mmol) cyanogen bromide and water (25 ml) over a period of 15 min at 0 °C. The mixture was stirred for additional 60 min at 0 °C. The precipitate was collected by filtration, extensively washed with water and dried to give **1d** (5.0 g, 90%). An analytic sample was prepared by recrystallization from dioxane with silica gel. M.p.: 252–254 °C; IR (KBr): $\nu(cm^{-1}) = 1732$ (C=O); ^{1}H NMR (*DMSO*-d_6): $\delta = 3.79$, 3.85 (2s, 6H, CH_3), 6.64 (s, 1H, 8-H), 7.21 (s, 1H, 5-H), 7.23 (s, 2H, NH_2) ppm; calcd. for $C_{10}H_{10}N_2O_4$: C, 54.05; H, 4.54; N, 12.61; found: C, 54.16; H, 4.42; N, 12.36.

2-Benzoylamino-6,7-dimethoxy-4H-3,1-benzoxazin-4-one (**2d**)

A stirred mixture of 1.33 g (6 mmol) powdered **1d**, 2.72 g (12 mmol) benzoic anhydride, and toluene (300 ml) was refluxed for 90 min. The hot mixture was filtrated and the filtrate was cooled. The precipitate was collected by filtration to obtain **2d** (430 mg, 22%). M.p.: 200–201 °C (ethyl acetate/1-propanol, silica gel); IR (KBr): $\nu(cm^{-1}) = 1740$ (br), 1722 (C=O); ^{1}H NMR (*DMSO*-d_6):

δ = 3.88, 3.93 (2s, 6H, CH_3), 7.01 (s, 1H, 8-H), 7.40 (s, 1H, 5-H), 7.50–7.57 (m, 2H, 3'-H), 7.61–7.67 (m, 1H, 4'-H), 7.98 (d, 2H, J = 7.4 Hz, 2'-H), 11.44 (s, 1H, NH) ppm; MS (70 eV): m/z(%) = 326 (M^+, 51), 105 ($C_6H_5CO^+$, 100); calcd. for $C_{17}H_{14}N_2O_5$: C, 62.57; H, 4.32; N, 8.59; found: C, 62.31; H, 4.61; N, 8.33.

Oxidative desulfurization of **3** *to produce* **5**. *General procedure*

Compounds **3** (2 mmol) were dissolved in an appropriate amount of a saturated solution prepared from sodium carbonate in 50% aqueous acetone (50–100 ml). The solution was treated dropwise with 30% H_2O_2 (5 ml) over a period of 1 min (in the case of **3d**: 5 min). The mixture was then acidified with HCl (0.5 *N*), and the precipitate was collected by filtration.

2-(3-Benzoylureido)-5-methylbenzoic acid (**5b**)

Yield 96%; m.p.: 227–228 °C (1-propanol); IR (KBr): ν(cm^{-1}) = 1680 (br, C=O); ^{1}H NMR (*DMSO*-d_6): δ = 2.31 (s, 3H, CH_3), 7.41 (dd, 1H, J = 1.9 Hz, J = 8.6 Hz, 4-H), 7.50–7.56 (m, 2H, 3'-H), 7.61–7.67 (m, 1H, 4'-H), 7.77 (d, 1H, J = 1.9 Hz, 6-H), 8.01 (d, 2H, J = 7.4 Hz, 2'-H), 8.33 (d, 1H, J = 8.6 Hz, 3-H), 10.90, 12.24 (2s, 2H, NH) ppm; MS (70 eV): m/z (%) = 298 (M^+, 7), 177 ($M^+ - C_6H_5CONH_2$, 9), 151 ($M^+ - C_6H_5CONCO$, 34), 105 ($C_6H_5CO^+$, 100); calcd. for $C_{16}H_{14}N_2O_4$: C, 64.42; H, 4.73; N, 9.39; found: C, 64.14; H, 4.70; N, 9.75.

2-(3-Benzoylureido)-5-chlorobenzoic acid (**5c**)

Yield 88%; m.p.: 250–251 °C (1-propanol); IR (KBr): ν(cm^{-1}) = 1710, 1680 (C=O); ^{1}H NMR (*DMSO*-d_6): δ = 7.49–7.55 (m, 2H, 3'-H), 7.60–7.68 (m, 2H, 4'-H and 4-H), 7.91 (d, 1H, J = 2.5 Hz, 6-H), 8.01 (d, 2H, J = 7.4 Hz, 2'-H), 8.51 (d, 1H, J = 9.1 Hz, 3-H), 11.01, 12.40 (2s, 2H, NH) ppm; MS (70 eV): m/z (%) = 318 (M^+, 19), 197 ($M^+ - C_6H_5CONH_2$, 46), 171 ($M^+ - C_6H_5CONCO$, 47), 105 ($C_6H_5CO^+$, 100); calcd. for $C_{15}H_{11}ClN_2O_4$: C, 56.53; H, 3.48; N, 8.79; Cl, 11.12; found: C, 56.87; H, 4.07; N, 9.19; Cl, 11.52.

2-(3-Benzoylureido)-4,5-dimethoxybenzoic acid (**5d**)

Yield 88%; m.p.: 211–215 °C (ethyl acetate/1-propanol); IR (KBr): ν(cm^{-1}) = 1700, 1680 (C=O); ^{1}H NMR (*DMSO*-d_6): δ = 3.78, 3.83 (2s, 6H, CH_3), 7.45 (s, 1H, 6-H), 7.50–7.56 (m, 2H, 3'-H), 7.61–7.67 (m, 1H, 4'-H), 8.00 (d, 2H, J = 7.4 Hz, 2'-H), 8.20 (s, 1H, 3-H), 10.86, 12.33 (2s, 2H, NH) ppm; MS (70 eV): m/z (%) = 344 (M^+, 4), 223 ($M^+ - C_6H_5CONH_2$, 22), 197 ($M^+ - C_6H_5CONCO$, 42), 105 ($C_6H_5CO^+$, 100); calcd. for $C_{17}H_{16}N_2O_6$: C, 59.30; H, 4.68; N, 8.14; found: C, 59.23; H, 5.07; N, 7.88.

Thermal treatment of **3a**

600 mg **3a** (2 mmol) [2] were heated at 170 °C over a period of 20 min. After cooling the mixture was stirred with ethyl acetate. The unsoluble fraction gave **5a** [3] (150 mg, 26%, after recrystallization from ethyl acetate and afterwards from ethanol). The soluble fraction was obtained by evaporation and fractional crystallization from ethanol/water to give **4a** [2] (56 mg, 10%).

600 mg **3a** (2 mmol) were refluxed in toluene (48 ml) for 10 h. The mixture was kept at room temperature for 24 h. The precipitate was collected by filtration to afford **5a** (230 mg, 40%). The filtrate contained mainly **4a** (TLC).

Thermal treatment of **3b**

942 mg **3b** (3 mmol) [2] were refluxed to toluene (72 ml) for 12 h and kept at room temperature for 24 h. The precipitate was collected by filtration and washed with hot 1-propanol to afford **5b** (350 mg,

39%). The organic layer was concentrated *in vacuo*, the precipitate was collected and recrystallized from toluene to obtain **4b** [2] (300 mg, 34%).

Thermal treatment of **3c**

1.0 g **3c** (3 mmol) [2] were refluxed in toluene (72 ml) for 12 h and kept at room temperature for 24 h. The precipitate was collected by filtration and extracted with ethyl acetate (300 ml). The unsoluble solid was washed with hot 1-propanol to afford **5c** (320 mg, 34%). The ethyl acetate was removed *in vacuo* to obtain **4c** [2] (180 mg, 19%) which was further purified by recrystallization from toluene.

Thermal treatment of **3d** *to produce* **2d**

1.08 mg **3d** (3 mmol) [3] were refluxed in toluene (150 ml) for 20 h. The mixture was kept at room temperature for 24 h. The precipitate was collected by filtration to afford **2d** (660 mg, 67%) which was further purified by recrystallization (ethyl acetate/1-propanol, silica gel). Evaporation of toluene and fractional crystallization from methylene glycol gave **4d** [3] (yield < 5%).

Hydrolytic cleavage of **2d** *to produce* **5d**

228 mg **2d** (0.7 mmol) was stirred at room temperature in a saturated solution prepared from sodium carbonate in 50% aqueous acetone (35 ml) for 16 h. The solution was acidified with HCl (1 *N*) and cooled. The precipitate was collected by filtration to afford **5d** (190 mg, 79%).

Enzymatic studies

Enzyme inhibition was determined with the "slow-binding" method (25 °C, *pH* 7.0, 50 m*M* Hepes) as described previously [9]. Chymotrypsin (Worthington, final concentration 100 ng/ml) was assayed with the chromogenic substrate Ac-Ala-Ala-Pro-Phe-pNA (Bachem, 100 μ*M*, $K_m = 274$ μ*M*), human leukozyte elastase (Calbiochem, 250 ng/ml) with Suc-Ala-Ala-Pro-Val-pNA (Bachem, 100 μ*M*, $K_m =$ 78 μ*M*). Stock solutions of substrates and inhibitors were prepared in *DMSO*; the final *DMSO* concentration was 6%. Instruments and methods of analysis were as described previously [3].

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