# Novel Potent and Selective Thrombin Inhibitors Based on a Central 1,4-Benzoxazin-3(4*H*)-one Scaffold△

Janez Ilaš, Tihomir Tomašić, and Danijel Kikelj*

*University of Ljubljana, Faculty of Pharmacy, Aškerčeva 7, 1000 Ljubljana, Slovenia*

*Received December 24, 2007*

Novel thrombin inhibitors with the central 1,4-benzoxazine-3(4*H*)-one scaffold, benzamidine $P_1$ arginine side chain mimetic and various $P_3$ moieties are described. 3-(Benzyl(2-(4-carbamimidoylbenzyl)-4-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl)amino)-3-oxopropanoic acid (**7b**), the most potent compound in the series, exhibited a $K_i$ of 2.6 nM in vitro for thrombin and high selectivity against trypsin and factor Xa.

## Introduction

Thromboembolic diseases, including stroke, deep vein thrombosis, and myocardial infarction, are the leading causes of death in developed countries.[1] The search for low molecular weight inhibitors of thrombin, the key enzyme in the coagulation cascade, has been a major goal in the search for novel antithrombotic agents over the past decade.[2] Argatroban was the first small-molecule thrombin inhibitor introduced to the market,[3] but its widespread use has been hindered by the fact that it is not orally active. The withdrawal of ximelagatran,[4] the first orally available thrombin inhibitor, has shown that there is not yet a satisfactory thrombin inhibitor on the market. With ongoing progress in the development of efficient antithrombotic drugs, focus has also turned to other important enzymes in the coagulation cascade, mainly factor Xa[5] and factor VIIa,[6] as well as to dual inhibitors targeting two coagulation enzymes at the same time,[7] which have not yet resulted in marketable products. Therefore, thrombin remains an important target in the discovery of novel antithrombotic compounds.

In our ongoing research directed toward a novel class of antithrombotic compounds with dual function, possessing in the same molecule both thrombin inhibitory and fibrinogen receptor antagonistic activity,[8] 1,4-benzoxazine-3(4*H*)-one was found to be a suitable scaffold for their design. On the basis of its strong interaction with Asp189 in the thrombin $S_1$ pocket, the benzamidine group was chosen as arginine mimetic[9] for the first generation of antithrombotic compounds with dual action, although being aware of its strong basicity. The problem of poor bioavailability of compounds containing a benzamidine group, arising from its basicity, can be overcome by a prodrug approach, as shown in the case of ximelagatran[4] and dabigatran.[10] In the search for the optimal spacer between the 1,4-benzoxazin-3(4*H*)-one and benzamidine moieties to produce antithrombotic compounds with dual activity, we prepared a series of compounds incorporating the methylene group connecting both moieties that, although devoid of fibrinogen receptor antagonistic activity, were found to be potent and highly selective thrombin inhibitors. Here we report on the design, synthesis, in vitro biological activity, and structure−activity relationship of this novel class of thrombin inhibitors possessing a central 1,4-benzoxazin-3(4*H*)-one core.

Synthesis of the target 1,4-benzoxazine-3(4*H*)-one thrombin inhibitors **6a**−**d** and **7a**−**d** was achieved using the chemistry depicted in Scheme 1. The bromo derivative **1**[11] was synthesized from 2-amino-5-nitrophenol in four steps involving *N*-acylation, cyclization, *N*-methylation, and bromination, with an overall yield of 89%. A Wittig reaction of 4-cyanobenzaldehyde and phosphorus ylide obtained from **1** with triphenylphosphine proceeded smoothly, yielding 3-oxo-3,4-dihydro-2*H*-1,4-benzoxazine-2-ylidene derivative **2**, mainly as the *Z*-isomer (chemical shift of olefinic proton: 7.05 ppm), although a small amount of *E*-isomer was also obtained (chemical shift of olefinic proton: 6.84 ppm). The two geometric isomers could be separated by recrystallization from petroleum ether/ethyl acetate (1:1). Compound **2** (mixture of *Z*- and *E*-isomers) was reduced catalytically at 6 bar to racemic 7-amino-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazine-2-yl derivative **3**, whereas under less vigorous catalytic hydrogenation conditions, only the nitro group of **2** was reduced to give amine **3a**. *N*-Benzylation of **3**, employing reductive amination of benzaldehyde, with sodium triacetoxyborohydride as reducing agent, afforded secondary amine **4**, which was acylated with ethyl oxalyl chloride, methyl malonyl chloride, and ethyl succinyl chloride to introduce side chains of different lengths containing a carboxylic ester moiety, giving **5a**−**c**. Alternatively, **3** was coupled to 2-benzyl-3-ethoxy-3-oxopropanoic acid using EDC/HOBT[a] coupling strategy to give compound **5d**. Nitriles **5a**−**d** were transformed to the corresponding benzamidines **6a**−**d** under Pinner reaction[12] conditions. The esters **6a**−**d** were hydrolyzed to carboxylic acids **7a**−**d** using 1.5 M sodium hydroxide in ethanol solution.

## Results and Discussion

The in vitro thrombin inhibitory potencies of the synthesized compounds and their selectivities against factor Xa and trypsin are presented in Table 1. Our preliminary studies demonstrated (data not shown) that the best thrombin inhibition was obtained when the $P_3$ moiety was attached to a 2-substituted 1,4-benzoxazine-3(4*H*)-one scaffold at position 7. Introduction of a methyl group at position 4 additionally contributed to the hydrophobic interaction in the thrombin $S_2$ pocket.[8] Optimization of the $P_3$ moiety revealed the beneficial effect of benzylation of the amino group at position 7 which, to improve potency and increase solubility, was additionally substituted with acyl

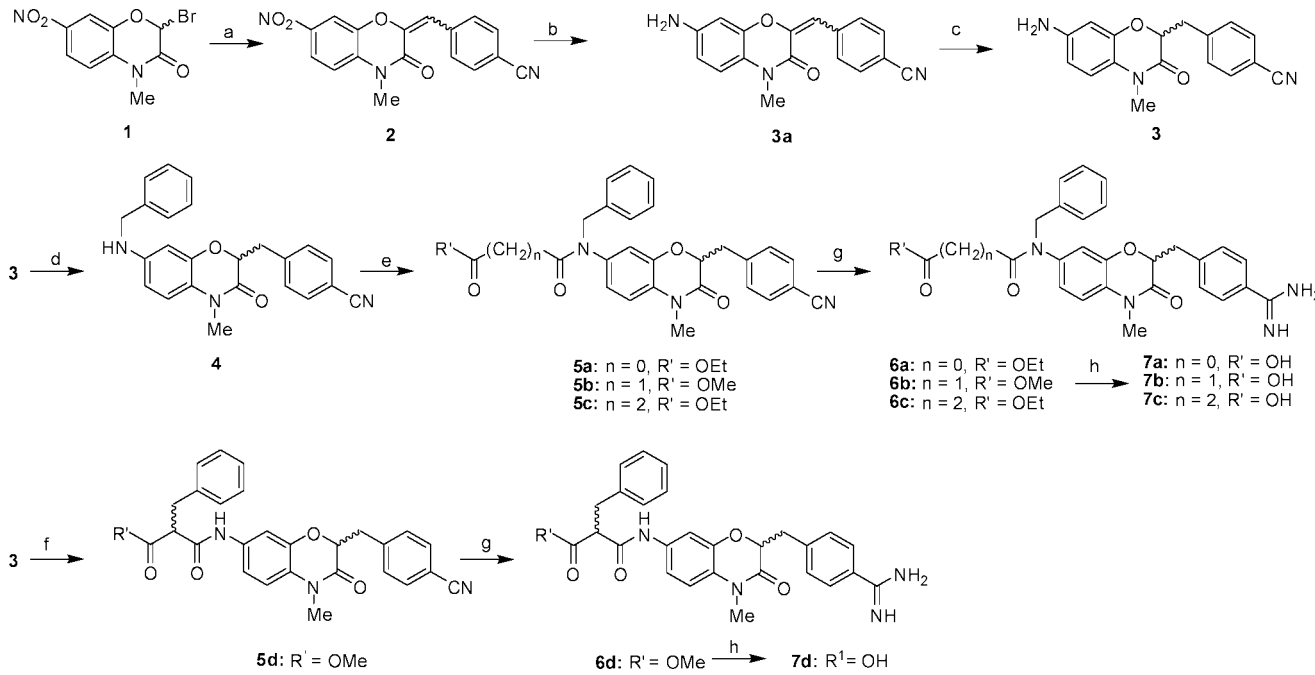
* To whom correspondence should be addressed. Tel.: +386 1 476 95 61. Fax: +386 1 425 80 31. E-mail: danijel.kikelj@ffa.uni-lj.si.

△ Dedicated to Professor Slavko Peèar on the occasion of his 60th birthday.

[a] Abbreviations: ADT, Auto Dock Tools; DMF, *N*,*N*-dimethylformamide; EDC, *N*-ethyl *N*′-(3-dimethylaminopropyl)-carbodiimide; ESI, electrospray ionization; HOBT, 1-hydroxybenzotriazole; THF, tetrahydrofuran; PDB, Protein Data Bank.

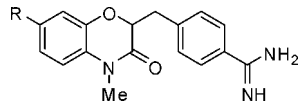
10.1021/jm701622y CCC: $40.75 
Published on Web 04/16/2008

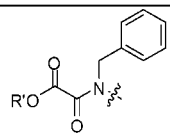
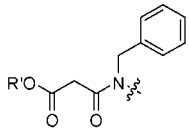
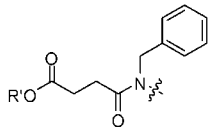
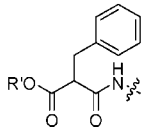
**Scheme 1**[a]




[a] Reagents and conditions: (a) 4-cyanobenzaldehyde, $PPh_3$, $Et_3N$, 0 °C, then rt, 24 h; (b) $H_2$ (1 bar), Pd/C, rt, 12 h; (c) $H_2$ (6 bar), Pd/C, rt, 72 h; (d) benzaldehyde, $NaBH(AcO)_3$, 1,2-dichloroethane, rt, 4 h; (e) acyl chloride, $Et_3N$, $CH_2Cl_2$, rt, 1 h; (f) 2-benzyl-3-ethoxy-3-oxopropanoic acid, EDC, HOBt, DMF, rt, overnight; (g) (i) $HCl_{(g)}$, EtOH, 0 °C, 30 min; (ii) $NH_4OAc$, EtOH, rt, 24 h; (h) NaOH, dioxane/$H_2O$, rt, 4 h.

**Table 1.** Inhibitory Potencies and Selectivities of Compounds **6a**−**d** and **7a**−**d**



| Comp. | R | R' | $K_i$ (nM) | | | Selectivity | |
|---|---|---|---|---|---|---|---|
| | | | Thrombin | Trypsin | FXa | Thrombin / Trypsin | Thrombin / FXa |
| **6a** |  | Et | 3.6 ± 1.4 | 4290 ± 680 | 1940 ± 370 | 1188 | 537 |
| **7a** | | H | 11.0 ± 2.2 | 10 100 ± 1250 | 9270 ± 2210 | 910 | 835 |
| **6b** |  | Me | 3.4 ± 1.8 | 3820 ± 690 | 5710 ± 960 | 1133 | 1694 |
| **7b** | | H | 2.6 ± 0.7 | 298 000 ± 64 000 | 139 000 ± 32 000 | 112 400 | 52 453 |
| **6c** |  | Et | 5.8 ± 2.2 | 3100 ± 680 | 1050 ± 90 | 535 | 181 |
| **7c** | | H | 6.5 ± 1.1 | 7410 ± 1990 | 15 900 ± 1600 | 1131 | 2427 |
| **6d** |  | Me | 2730 ± 480 | 11 200 ± 2400 | 58 700 ± 12 900 | 4.1 | 22 |
| **7d** | | H | 2940 ± 660 | 5130 ± 310 | 33 200 ± 6400 | 1.7 | 11 |

chains bearing a carboxylic ester or free carboxylic group. Comparison of low nanomolar thrombin inhibitory potencies of compounds **6a**−**c** and **7a**−**c** with those of 3 orders of magnitude higher $K_i$ values of compounds **6d** and **7d**, demon-

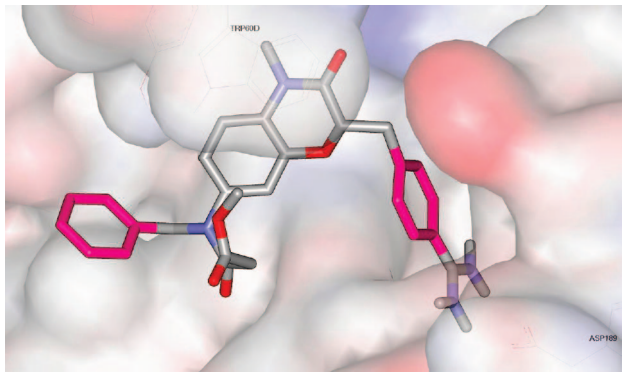


**Figure 1.** Highest score hypothetical binding mode of inhibitor (*S*)-**6b** in the thrombin binding site as predicted by AutoDock 3.0.

strate the crucial importance of the proper placement of the $P_3$ benzyl moiety. The benzyl moiety bound to *N*-7 (in **6a**−**c** and **7a**−**c**) appears to be optimally positioned, whereas the insertion of two bonds between the benzyl group and the nitrogen atom (compounds **6d** and **7d**) reduces interaction with the thrombin $S_3$ pocket. Neither the length of the *N*-acyl chain nor the nature of the carboxylate moiety (ester or carboxylic acid) substantially affected the activity of the compounds. Thus, the best compound, **7b**, with $K_i$ of 2.6 nM, bears an *N*-malonyl moiety, and compound **6b**, bearing a methylmalonyl moiety attached to the *N*-7, has almost the same potency. This demonstrates that the carboxylate moiety, which is turned to the water environment, does not interact with the thrombin active site surface. The shorter ethyloxalyl moiety only slightly reduced thrombin inhibitory potency; the carboxylic acid **7a** exhibited a 4-fold higher inhibition constant ($K_i$ = 11 nM), presumably because of the electrostatic repulsion of the carboxylate group with the surface of the active site. Thrombin inhibitors **6a**−**c** and **7a**−**c** possess excellent 535- to 112400-fold selectivity against trypsin and 181- to 52453-fold selectivity against factor Xa. The best thrombin inhibitor in the series, compound **7b**, was also the most selective against trypsin and factor Xa, whereas compounds **6d** and **7d**, with low micromolar thrombin inhibition constants, showed only 2- to 4-fold selectivity for trypsin and 11- to 22-fold selectivity for factor Xa. A high selectivity of **7b**, bearing a terminal malonyl moiety, compared to other compounds from this series, is presumably due to less favorable interaction of the malonyl carboxylate moiety of **7b** with the D pocket of factor Xa and trypsin, which has been filled many times by basic residues in the case of factor Xa inhibitors that were not selective versus trypsin and very selective over thrombin.[13–15]

Docking studies were performed for compounds **6a**−**d** and **7a**−**d** using AutoDock[16] to predict their binding mode in the thrombin active site. Because the synthesized compounds were all racemic mixtures, both enantiomers were docked, and it was found that only the *S*-isomers bind favorably to the active site of thrombin. Compound (*S*)-**6b** docked into the thrombin active site is shown in Figure 1. As expected from our previous results,[8] it binds with the benzamidine moiety situated in the thrombin $S_1$ binding pocket. The distance between the C-atoms of the Asp189 carboxylate and the amidine group in (*S*)-**6b** was 4.0 Å, and two hydrogen bonds linked the amidine hydrogens and carboxylate oxygen atoms. There was an additional hydrogen bond between benzamidine and the Gly219 backbone. The benzamidine moiety of (*S*)-**6b** binded in an identical mode to that of benzamidine itself.[17] The 1,4-benzoxazin-3(4*H*)-one scaffold was located in the $S_2$ binding pocket and the benzyl group in the lipophilic $S_3$ binding pocket, with the $P_3$ carboxylate stretching outward from the thrombin surface. The oxygen of the 1,4-oxazine ring formed a hydrogen bond with the Gly216 backbone (2.72 Å), which is often regarded as a key interaction in the thrombin active site.[18] The methylene spacer between benzamidine and benzoxazin-3(4*H*)-one moiety allows the optimal positioning of both moieties and enables favorable interactions between inhibitor and enzyme, thus making an important contribution to the high potency of this novel structural type of thrombin inhibitor. Docking studies showed that also compounds **6a**, **6b**, and **7a**−**c** bind to the thrombin active site in an analogous fashion. The same is true for compounds **6d** and **7d**, the only difference being that the phenyl moiety is not optimally bound to the $S_3$ pocket, which accounts for the much lower thrombin inhibitory potency of these two compounds.

## Conclusion

We have synthesized a new series of highly potent and selective thrombin inhibitors possessing a central 2,7-disubstituted 1,4-benzoxazin-3(4*H*)-one core. Compound **7b**, with $K_i$ of 2.6 nM for thrombin, 112400-fold selectivity for trypsin and 52435-fold selectivity for factor Xa, offers considerable potential for the development of an orally active prodrug to overcome unsatisfactory biovailability due to the presence of benzamidine moiety. The straightforward synthetic approach, using a Wittig-type reaction followed by reduction of the resulting alkene, enables stereoselective synthesis and future preparation of enantiomerically pure thrombin inhibitors of this novel structural class.

## Experimental Section

**General.** Chemicals were obtained from Acros, Aldrich Chemical Co., and Fluka and used without further purification. THF was kept over sodium and distilled immediately prior to use. Analytical TLC was performed on silica gel Merck 60 $F_{254}$ plates (0.25 mm), using visualization with ultraviolet light and ninhydrin. Column chromatography was carried out on silica gel 60 (particle size 240−400 mesh). Melting points were determined on a Reichert hot stage microscope and are uncorrected. $^1$H NMR and $^{13}$C NMR spectra were recorded at 300 and 75 MHz respectively on a Bruker AVANCE $DPX_{300}$ spectrometer in $CDCl_3$ or DMSO-$d_6$ solution with TMS as the internal standard. IR spectra were recorded on a Perkin-Elmer 1600 FT-IR spectrometer. Microanalyses were performed on a Perkin-Elmer C, H, N analyzer 240 C. Analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values. Mass spectra were obtained using a VG-Analytical Autospec Q mass spectrometer. HPLC analyses were performed on an Agilent Technologies HP 1100 instrument with G1365B UV−vis detector (254 nm), using a Luna C18 column (4.6 × 250 mm) at flow rate 1 mL/min. The eluant was a mixture of 0.1% TFA in water (A) and acetonitrile (B). Gradient was 10% B to 80% B in 30 min.

**Biological Evaluation.** The ability of the new thrombin inhibitors to competitively inhibit the enzymatic activities of thrombin, trypsin, and factor Xa was measured as described previously[8] by amidolytic enzyme assay using chromogenic substrates, and is expressed as inhibition constant $K_i$.[19] Competitive inhibition of thrombin, factor Xa, and trypsin by compounds **6a**−**6d** and **7a**−**7d** was established by the double reciprocal plotting $1/v$ against $1/[S]$ for each inhibitor concentration (Lineweaver−Burk plot).

Values for $K_i$ were calculated according to Cheng and Prusoff[20] based on the relation between reaction velocity equations in the absence and presence of inhibitor, using the relevant $K_m$.[21] The selectivity for thrombin over trypsin and factor Xa, as two closely related serine proteases, was expressed as the ratios $K_i$(trypsin)/$K_i$(thrombin) and $K_i$(factor Xa)/$K_i$(thrombin). Inhibition of binding of fibrinogen to isolated platelet fibrinogen receptor was measured as previously described.[8]

**4-((4-Methyl-7-nitro-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-ylidene)methyl)benzonitrile (2).** To a stirred solution of triphenylphosphine (0.756 g, 2.88 mmol) in 80 mL of anhydrous THF cooled to 0 °C, compound **1** (0.828 g, 2.88 mmol) dissolved in 15 mL of anhydrous THF was added dropwise over 10 min. The reaction mixture was stirred for 30 min at 0 °C under an atmosphere of argon and left overnight to warm to room temperature. The reaction mixture was then cooled to 0 °C, anhydrous triethylamine (0.80 mL, 5.76 mmol) was added, and the mixture was stirred for 30 min at 0 °C. 4-Cyanobenzaldehyde (0.325 g, 2.88 mmol) dissolved in 15 mL of THF was added dropwise. The reaction was allowed to proceed overnight under an argon atmosphere. The precipitate that formed was filtered off, dissolved in dichloromethane (250 mL), and washed successively with 10% citric acid (2 × 50 mL), saturated $NaHCO_3$ solution (2 × 50 mL), and saturated NaCl solution (1 × 50 mL). The organic phase was dried over $Na_2SO_4$ and filtered, and the solvent was evaporated under reduced pressure. Compound **2** (0.521 mg) was obtained as a yellow powder. Recrystallization from petroleum ether/ethyl acetate yielded pure *Z*-(**2a**) and *E*-(**2b**) isomers.

**(*Z*)-4-((4-Methyl-7-nitro-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-ylidene)methyl)benzonitrile (2a).** Yield: 455 mg (49.2%); mp 285−288 °C. MS (EI): *m*/*z* (%) 321 ($M^+$, 100). $^1$H NMR ($CDCl_3$, 300 MHz): $\delta$ 3.59 (s, 3H, *N*-$CH_3$), 7.05 (s, 1H, C=CH), 7.16 (d, 1H, $^3J$ = 8.7 Hz, Ar−$H^5$), 7.75 (d, 2H, $^3J$ = 8.4 Hz, Ar−$H^{2'}$,$H^{6'}$), 7.93 (d, 2H, $^4J$ = 8.4 Hz, Ar−$H^{3'}$,$H^{5'}$), 8.09 (m, 2H, Ar−$H^6$, Ar−$H^8$) ppm. Anal. ($C_{17}H_{11}N_3O_4$) C, H, N.

**4-((7-Amino-4-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl)methyl)benzonitrile (3).** A solution of compound **2** (2.26 g, 7.03 mmol) in DMF (250 mL) was stirred with 10% palladium on activated charcoal (226 mg) under hydrogen atmosphere at 6 bar for 3 days at room temperature. The product was isolated by filtration and the solvent evaporated under reduced pressure. The residue was dissolved in dichloromethane (100 mL) and washed successively with 10% citric acid (2 × 50 mL), saturated $NaHCO_3$ solution (2 × 50 mL), and saturated NaCl solution (1 × 50 mL). The organic phase was dried over $Na_2SO_4$, filtered, and the solvent evaporated under reduced pressure. Compound **3** was obtained as a light yellow powder. Yield: 1.16 g (56.6%), mp 135−138 °C. MS (EI): *m*/*z* (%) 293 ($M^+$, 100). $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ 3.08 (dd, 1H, $^3J$ = 9.0 Hz, $^2J$ = 14.7 Hz, CH-$CH_2$), 3.20 (s, 3H, *N*-$CH_3$), 3.25 (dd, 1H, $^3J$ = 4.0 Hz, $^2J$ = 14.7 Hz, CH-$CH_2$), 4.81 (dd, 1H, $^3J$ = 4.0 Hz, $^2J$ = 9.0, Hz 2-H), 5.04 (s, 2H, $NH_2$), 6.14 (d, 1H, $^4J$ = 2.3 Hz, Ar−$H^8$), 6.27 (dd, 1H, $^4J$ = 2.3 Hz, $^3J$ = 8.5 Hz, Ar−$H^6$), 6.82 (d, 1H, $^3$J = 8.5 Hz, Ar−$H^5$), 7.48 (d, 2H, $^3J$ = 8.2 Hz, Ar−$H^{2'}$,$H^{6'}$), 7.77 (d, 2H, $^3J$ = 8.2 Hz, Ar−$H^{3'}$,$H^{5'}$) ppm. Anal. ($C_{17}H_{15}N_3O_2$) C, H, N.

**(*E*)-4-((7-Amino-4-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-ylidene)methyl)benzonitrile (3a).** A solution of **2b** (0.226 g, 0.70 mmol) in 100 mL of dioxane/ethanol (1:1) mixture in the presence of 10% palladium on activated charcoal (26 mg) was stirred under a hydrogen atmosphere at 1 bar pressure overnight at room temperature. The catalyst was filtered off and the solvent evaporated in vacuo. The residue was dissolved in 100 mL of dichloromethane and washed successively with 10% citric acid (2 × 50 mL), saturated $NaHCO_3$ solution (2 × 50 mL), and saturated NaCl solution (1 × 50 mL). The organic phase was dried over $Na_2SO_4$ and filtered, and the solvent was evaporated under reduced pressure. Compound **3a** was obtained as orange crystals. Yield: (184 mg) 90.6%, mp 259−261 °C. MS (EI): *m*/*z* (%) 291 ($M^+$, 100). $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ 3.35 (s, 3H, *N*-$CH_3$), 5.20 (s, 2H, $NH_2$), 6.40 (dd, 1H, $^3J$ = 8.7 Hz, $^4J$ = 2.3 Hz, Ar−$H^6$), 6.55 (d, 1H, $^4$J = 2.3 Hz, Ar−$H^8$), 6.82 (s, 1H, C=CH-), 6.95 (d, 1H, $^3J$ = 8.7 Hz, Ar−$H^5$), 7.84 (d, 2H, $^3J$ = 8.4 Hz, Ar−$H^{3'}$,$H^{5'}$), 7.77 (d, 2H, $^3J$ = 8.4 Hz, Ar−$H^{2'}$,$H^{6'}$) ppm. Anal. ($C_{17}H_{13}N_3O_2$) C, H, N.

**4-((7-(Benzylamino)-4-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl)methyl)benzonitrile (4).** Acetic acid (0.23 mL, 3.94 mmol) and benzaldehyde (400 mg, 3.94 mmol) were added to a stirred solution of amine **3** (1.16 g, 3.94 mmol) in 1,2-dichloroethane (150 mL) under an argon atmosphere. After 20 min, $NaBH(OAc)_3$ (1.25 g, 5.92 mmol) was added, and the mixture was stirred overnight at room temperature. Solvent was removed under reduced pressure, and the residue was dissolved in ethyl acetate (150 mL) and washed successively with saturated $NaHCO_3$ solution (3 × 50 mL) and brine (1 × 50 mL). The organic phase was dried over $Na_2SO_4$, filtered, and the solvent evaporated under reduced pressure. Compound **4** was obtained as a yellow powder. The crude product was purified by column chromatography using dichloromethane/acetone (30:1) as eluant. Yield: 0.839 g (55.5%), mp 153−154 °C. MS (EI): *m*/*z* (%) 383 ($M^+$, 100). $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ 3.05 (dd, 1H, $^3J$ = 8.7 Hz, $^2J$ = 14.4 Hz, CH-$CH_2$), 3.19−3.24 (m, 4H, *N*-$CH_3$, CH-$CH_2$), 4.23 (d, 2H, $^3J$ = 6.0 Hz, Ph-$CH_2$-NH), 4.81 (dd, 1H, $^4J$ = 3.9 Hz, $^3J$ = 8.7 Hz, 2-H), 6.12 (d, 1H, $^4J$ = 2.4 Hz, Ar−$H^8$), 6.22 (t, 1H, $^3J$ = 6.0 Hz, NH), 6.30 (dd, 1H, $^4J$ = 2.4 Hz, $^3J$ = 8.7 Hz, Ar−$H^6$), 6.85 (d, 1H, $^3J$ = 8.7 Hz, Ar−$H^5$), 7.23−7.34 (m, 5H, Ph), 7.41 (d, 2H, $^3J$ = 8.1 Hz, Ar−$H^{2'}$,$H^{6'}$), 7.70 (d, 2H, $^3J$ = 8.1 Hz, Ar−$H^{3'}$,$H^{5'}$) ppm. Anal. ($C_{24}H_{21}N_3O_2$) C, H, N.

**General Procedure for the Synthesis of *N*-Acyl-4-((7-(benzylamino)-4-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl)methyl)benzonitriles 5a, 5b, and 5c.** To a solution of secondary amine **4** (459 mg, 1.20 mmol) in dichloromethane (50 mL) were added triethylamine (145 mg, 1.44 mmol) and the corresponding acyl chloride (1.44 mmol), and the mixture was stirred for 2 h at room temperature. Solvent was removed under reduced pressure, and the residue was dissolved in ethyl acetate (100 mL) and washed successively with 10% citric acid (2 × 50 mL), saturated $NaHCO_3$ solution (3 × 50 mL), and brine (1 × 50 mL). The organic solution was dried over $Na_2SO_4$ and filtered, and the solvent was evaporated under reduced pressure. The crude product was purified by column chromatography using petroleum ether/ethyl acetate (1:1) as eluant.

**Ethyl 2-(Benzyl(2-(4-cyanobenzyl)-4-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl)amino)-2-oxoacetate (5a).** Yellow oil, yield: 77.5%. MS (EI): *m*/*z* (%) 483 ($M^+$, 41), 91 (100). $^1$H NMR ($CDCl_3$, 300 MHz): $\delta$ 1.10 (t, 3H, $^3J$ = 6.9 Hz, -$CH_2$-$CH_3$), 3.16 (dd, 1H, $^3J$ = 8.1 Hz, $^2J$ = 14.4 Hz, CH-$CH_2$), 3.29−3.35 (m, 4H, *N*-$CH_3$, CH-$CH_2$), 4.09 (q, 2H, $^3J$ = 6.9 Hz, -$CH_2$-$CH_3$), 4.77 (dd, 1H, $^4J$ = 3.9 Hz, $^3J$ = 8.1 Hz, 2-H), 4.84 (d, 1H, $^2J$ = 14.6 Hz, Ph-$CH_2$-*N*), 5.03 (d, 1H, $^2J$ = 14.6 Hz, Ph-$CH_2$-*N*), 6.70 (d, 1H, $^4J$ = 2.4 Hz, Ar−$H^8$), 6.75 (dd, 1H, $^4J$ = 2.4 Hz, $^3J$ = 8.7 Hz, Ar−$H^6$), 6.81 (d, 1H, $^3J$ = 8.7 Hz, Ar−$H^5$), 7.23−7.33 (m, 7H, Ph, Ar−$H^{2'}$,$H^{6'}$), 7.56 (d, 2H, $^3J$ = 8.4 Hz, Ar−$H^{3'}$,$H^{5'}$) ppm. Anal. ($C_{28}H_{25}N_3O_5$) C, H, N.

**General Procedure for the Synthesis of *N*-Acyl-4-((7-(benzylamino)-4-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl)methyl)benzimidamides 6a−6d.** Gaseous HCl was introduced into a solution of the nitrile **5** (1.00 mmol) in 30 mL of absolute EtOH (**5a** and **5c**) or MeOH (**5b** and **5d**) for 30 min. The reaction mixture was closed tightly and stirred at room temperature for 24 h. The solvent was evaporated and the residue was washed 2−3 times with diethyl ether. The obtained iminoether was dissolved in anhydrous EtOH (30 mL), ammonium acetate (0.308 g, 4.00 mmol) was added, and the reaction mixture was stirred for 2 days. The solvent was then evaporated to one-third of the starting volume, two drops of trifluoroacetic acid were added, and the residual solution stored at 4 °C. The precipitated crystals were filtered off and washed with cold diethyl ether. If the obtained product was not pure, it was purified by column chromatography using dichloromethane/methanol (6:1) as eluant.

**Ethyl 2-(Benzyl(2-(4-carbamimidoylbenzyl)-4-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl)amino)-2-oxoacetate Trifluoroacetate (6a).** White thick oil, yield: 50.8%. MS (FAB): *m*/*z* (%) 501 ($MH^+$, 80), 69 (100). $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ 0.94 (t, 3H, $^3J$ = 6.9 Hz, -$CH_2$-$CH_3$), 3.07 (dd, 1H, $^3J$ = 8.7 Hz, $^4J$ = 14.4 Hz, CH-$CH_2$), 3.25−3.40 (m, 4H, *N*-$CH_3$, CH-$CH_2$), 4.01 (q, 2H, $^3J$ = 6.9 Hz, -$CH_2$-$CH_3$), 4.88 (d, 1H, $^2J$ = 15.0 Hz, Ph-$CH_2$-*N*), 4.99 (m, 2H, Ph-$CH_2$-*N*, CH-$CH_2$), 6.74 (d, 1H, $^4J$ = 2.4 Hz, Ar−$H^8$), 6.87 (dd, 1H, $^4J$ = 2.4 Hz, $^3J$ = 8.7 Hz, Ar−$H^6$), 7.14 (d, 1H, $^3J$ = 8.7 Hz, Ar−$H^5$), 7.18−7.36 (m, 5H, Ph), 7.44 (d, 2H, $^3J$ = 8.4 Hz, Ar−$H^{2'}$,$H^{6'}$), 7.75 (d, 2H, $^3J$ = 8.4 Hz, Ar−$H^{3'}$,$H^{5'}$), 9.15 (br s, 2H, amidino-H), 9.28 (br s, 2H, amidino-H) ppm. HPLC: 97.2%, $t_r$ = 18.78 min. Anal. ($C_{28}H_{28}N_4O_5$ × $CF_3COOH$ × 5/2 $H_2O$) C, H, N.

**General Procedure for Alkaline Hydrolysis of Alkyl Esters 6a−d.** To a solution of the corresponding ester **6a−d** (0.18 mmol) in 50 mL of ethanol/water (1:1) mixture (50 mL), NaOH (27 mg, 0.675 mmol) was added and the reaction mixture was stirred at room temperature for 6 h. The ethanol was evaporated and the resulting aqueous solution neutralized with 1 M HCl, until the product started to precipitate. If the product was not pure, it was purified by column chromatography using dichloromethane/methanol (3:1) as eluant.

**2-(Benzyl(2-(4-carbamimidoylbenzyl)-4-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl)amino)-2-oxoacetic Acid (7a).** White powder, yield: 64.3%, mp 229−231 °C. MS (ESI): *m*/*z* (%) 473.25 ($MH^+$, 50), 457.27 (100). $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ 3.05−3.25 (m, 5H, *N*-$CH_3$, -CH-$\underline{CH_2}$), 4.74 (d, 1H, $^2J$ = 15.0 Hz, Ph-$\underline{CH_2}$-*N*), 4.84 (d, 1H, $^2J$ = 15.0 Hz, Ph-$\underline{CH_2}$-*N*), 5.01 (m, 1H, 2-H), 6.64−6.74 (m, 3H, Ar−$H^8$, Ar−$H^6$, Ar−$H^5$), 7.14−7.32 (m, 7H, Ph, Ar−$H^{2'}$,$H^{6'}$), 7.64 (d, 2H, $^3J$ = 8.4 Hz, Ar−$H^{3'}$,$H^{5'}$), 9.39 (br s, 2H, amidino-H), 9.69 (br s, 2H, amidino-H) ppm. HPLC: 98.0%, $t_r$ = 14.87 min. Anal. ($C_{26}H_{24}N_4O_5$) HRMS.

**Acknowledgment.** This work was supported financially by the Slovenian Research Agency (Grant No. P1-0208). The authors thank Professor Mojca Stegnar for providing us thrombin, factor Xa, trypsin, and chromogenic substrate and Professor Roger Pain for critical reading of the manuscript.

**Supporting Information Available:** Experimental procedures and characterization of intermediate and target compounds; IR spectra, $^{13}$C spectra of representative compounds, elemental analyses (C, H, N) and HRMS results; and molecular docking protocol. This material is available free of charge via the Internet at http://pubs.acs.org.

## References

(1) Lopez, A. D.; Mathers, C. D.; Ezzati, M.; Jamison, D. T.; Murray, C. J. L. Global and regional burden of disease and risk factors, 2001: Systematic analysis of population health data. *Lancet* **2006**, *367*, 1747–1757.

(2) Schwienhorst, A. Direct thrombin inhibitors−A survey of recent developments. *Cell. Mol. Life Sci.* **2006**, *63*, 2773–2791.

(3) Hijikata-Okunomiya, A.; Okamoto, S. A strategy for a rational approach to designing synthetic selective inhibitors. *Semin. Thromb. Hemostasis* **1992**, *18*, 135–149.

(4) Crowther, M. A.; Weitz, J. I. Ximelagatran: The first oral direct thrombin inhibitor. *Expert Opin. Invest. Drugs* **2004**, *13*, 403–413.

(5) Casimiro-Garcia, A.; Dudley, D. A.; Heemstra, R. J.; Filipski, K. J.; Bigge, C. F.; Edmunds, J. J. Progress in the discovery of factor Xa inhibitors. *Expert Opin. Ther. Pat.* **2006**, *16*, 119–145.

(6) Lazarus, R. A.; Olivero, A. G.; Eigenbrot, C.; Kirchhofer, D. Inhibitors of tissue factor/factor VIIa for anticoagulant therapy. *Curr. Med. Chem.* **2004**, *11*, 2275–2290.

(7) Kranjc, A.; Kikelj, D.; Peterlin-Mašič, L. Recent advances in the discovery of tissue factor/factor VIIa inhibitors and dual inhibitors of factor VIIa/factor Xa. *Curr. Pharm. Des.* **2005**, *11*, 4207–4227.

(8) Anderluh Štefanič, P.; Anderluh, M.; Ilaš, J.; Mravljak, J.; Sollner Dolenc, M.; Stegnar, M.; Kikelj, D. Toward a novel class of antithrombotic compounds with dual function. Discovery of 1,4-benzoxazin-3(4*H*)-one derivatives possessing thrombin inhibitory and fibrinogen receptor antagonistic activities. *J. Med. Chem.* **2005**, *48*, 3110–3113.

(9) Peterlin-Mašič, L.; Kikelj, D. Arginine mimetics. *Tetrahedron* **2001**, *57*, 7073–7105.

(10) Hauel, N. H.; Nar, H.; Priepke, H.; Ries, U.; Stassen, J-M.; Wienen, W. Structure-based design of novel potent nonpeptide thrombin inhibitors. *J. Med. Chem.* **2002**, *45*, 1757–1766.

(11) Ilaš, J.; Kikelj, D. Ring opening of 2-benzylamino-2*H*-1,4-benzoxazin-3(4*H*)-ones and 2-bromo-2*H*-1,4-benzoxazin-3(4*H*)-ones. *Helv. Chim. Acta*, in press.

(12) Roger, R.; Neilson, D. G. The chemistry of imidates. *Chem. Rev.* **1961**, *61*, 179–211.

(13) Sagi, K.; Nakagawa, T.; Yamanashi, M.; Makino, S.; Takahashi, M.; Takayanagi, M.; Takenaka, K.; Suzuki, N.; Oono, S.; Kataoka, N.; Ishikawa, K.; Shima, S.; Fukuda, Y.; Kayahara, T.; Takehana, S.; Shima, Y.; Tashiro, K.; Yamamoto, H.; Yoshimoto, R.; Iwata, S.; Tsuji, T.; Sakurai, K.; Shoji, M. Rational design, synthesis, and structure−activity relationships of novel factor Xa inhibitors: (2-Substituted-4-amidinophenyl)pyruvic and -propionic acids. *J. Med. Chem.* **2003**, *46*, 1845–57.

(14) Shaw, K. J.; Guilford, W. J. L.; Griedel, B. D.; Sakata, S.; Trinh, L.; Wu, S.; Xu, W.; Zhao, Z.; Morrissey, M. M. Benzimidazole-based fXa inhibitors with improved thrombin and trypsin selectivity. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1311–1314.

(15) Kastenholz, M. A.; Pastor, M.; Cruciani, G.; Haaksma, E. E.; Fox, T. GRID/CPCA: A new computational tool to design selective ligands. *J. Med. Chem.* **2000**, *43*, 3033–3044.

(16) Morris, G. M.; Goodsell, D. S.; Halliday, R. S.; Huey, R.; Hart, W. E.; Belew, R. K.; Olson, A. J. Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. *J. Comput. Chem.* **1998**, *19*, 1639–1662.

(17) Banner, D. W.; Hadvary, P. Crystallographic analysis at 3.0 Å resolution of the binding to human thrombin of 4 active site-directed inhibitors. *J. Biol. Chem.* **1991**, *266*, 20085–20093.

(18) Lee, L.; Kreutter, K. D.; Pan, W.; Crysler, C.; Spurlino, J.; Player, M. R.; Tomczuk, B.; Lu, T. 2-(2-Chloro-6-fiuorophenyl)acetamides as potent thrombin inhibitors. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 6266–6269.

(19) Hilpert, K.; Ackermann, J.; Banner, D. W.; Gast, A.; Gubernator, K.; Hadvary, P. Design and synthesis of potent and highly selective thrombin inhibitors. *J. Med. Chem.* **1994**, *37*, 3889–3901.

(20) Cheng, Y.; Prusoff, W. H. Relationship between inhibition constant ($K_i$) and concentration of inhibitor which causes 50% inhibition ($IC_{50}$) of an enzymatic-reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099–3108.

(21) Brandt, R. B.; Laux, J. E.; Yates, S. W. Calculation of inhibitor $K_i$ and inhibitor type from the concentration of inhibitor for 50-percent inhibition for Michaelis−Menten enzymes. *Biochem. Med. Metab. Biol.* **1987**, *37*, 344–349.

JM701622Y