3,4-Dihydro-2*H*-1,4-benzoxazine Derivatives Combining Thrombin Inhibitory and Glycoprotein IIb/IIIa Receptor Antagonistic Activity as a Novel Class of Antithrombotic Compounds with Dual Function

Janez Ilaš,[†] Žiga Jakopin,[†] Tina Borštnar,[†] Mojca Stegnar,[‡] and Danijel Kikelj*[,†]

Faculty of Pharmacy, University of Ljubljana, Aškerčeva 7, SI-1000 Ljubljana, Slovenia, and Department of Vascular Diseases, University Medical Centre Ljubljana, Zaloška 7, SI-1525 Ljubljana, Slovenia

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3,4-Dihydro-2*H*-1,4-benzoxazine derivatives possessing both thrombin inhibitory and glycoprotein IIb/IIIa (GPIIb/IIIa) receptor antagonistic activities were obtained by combining mimetics of the D-Phe-Pro-Arg pharmacophore of thrombin inhibitors and the Arg-Gly-Asp pharmacophore of GPIIb/IIIa receptor antagonists in the same low molecular weight peptidomimetic compound. Systematic variation of the position of substituents around the 3,4-dihydro-*2H*-1,4-benzoxazine nucleus, the distance between the carboxylate and amidine moieties, together with additional substituents to fill the thrombin S_2 and S_3 pockets resulted in compounds displaying submicromolar inhibition constants (K_i) for thrombin and submicromolar IC_{50} for inhibition of binding of fibrinogen to platelet GPIIb/IIIa receptor. Some of these compounds, such as **17a**, **17b**, **17d**, and **17h** possessing a well balanced activity at both targets, are a good starting point for further optimization. Incorporation of anticoagulant and platelet antiaggregatory activity in the same molecule constitutes a promising approach toward novel antithrombotic agents.

Introduction

Cardiovascular diseases, with an estimated 18 million deaths yearly, are the main cause of death and morbidity globally. If appropriate action is not taken, by 2015 an estimated 20 million people will die from cardiovascular disease every year, mainly from heart attacks and strokes.[1] There is, therefore, a growing need to discover novel antithrombotic agents as alternatives to existing treatment strategies. After the major progress made in the past decade in developing novel antithrombotic agents, e.g., thrombin inhibitors,[2] inhibitors of factor Xa,[3] tissue factor/factor VIIa inhibitors,[4] and platelet GPIIb/IIIa[a] receptor antagonists,[5] the search for novel antithrombotic drugs displaying a new mechanism of action or making use of a new therapeutic concept still remains a major challenge to medicinal chemistry.[6]

While hemostasis is a physiological process by which the body stops blood loss whenever a blood vessel is severed or ruptured, thrombosis is a pathological process in which hemostatic mechanisms, i.e., blood coagulation and platelet aggregation, are activated in the absence of bleeding. Therefore, inhibiting coagulation and preventing platelet aggregation at different stages are essential components of most antithrombotic therapeutic strategies. Of the several drug targets in the blood coagulation cascade,[7] e.g., thrombin, factor VIIa, and factor Xa, thrombin has provided the most frequently used basis for the design of novel anticoagulants over the past decade. The principal physiological roles of thrombin, the final enzyme in the blood coagulation cascade, are conversion of soluble fibrinogen into insoluble fibrin, which forms a mechanical matrix for the developing blood clot, and the activation of platelet aggregation.[8] The crystal structure of human thrombin[9] demonstrated the significance of the thrombin Tyr-Pro-Pro-Trp insertion loop and the S_1 binding pocket for binding and selectivity and provided the basis for structure-based inhibitor design. This resulted in many low molecular weight thrombin inhibitors,[2] most of them being mimetics of the D-Phe-Pro-Arg sequence[10] in which arginine (e.g., in argatroban[11] ((2*R*,4*R*)-1-[(*S*)-5-guanidino-2-((*R*)-3-methyl-1,2,3,4-tetrahydroquinoline-8-sulfonamido)pentanoyl]-4-methylpiperidine-2-carboxylic acid)) or an arginine surrogate[12] (e.g., in melagatran[13] (ethyl 2-[[(1*R*)-1-cyclohexyl-2-[(2*S*)-2-[[4-(*N*′-hydroxycarbamimidoyl)phenyl]-methylcarbamoyl]azetidin-1-yl]-2-oxo-ethyl]amino]acetate) and dabigatran[14] (3-(2-((4-carbamimidoylphenylamino)methyl)-1-methyl-*N*-(pyridin-2-yl)-1*H*-benzo[*d*]imidazole-5-carboxamido)-propanoic acid)) plays a crucial role for binding into the thrombin active site. Platelet aggregation is mediated by ionic interaction of Arg-Gly-Asp (RGD) sequence of fibrinogen with the platelet GPIIb/IIIa receptors (integrin $\alpha_{IIb}\beta_3$, glycoprotein IIb/IIIa; GPIIb/IIIa, fibrinogen receptor) of the integrin family. GPIIb/IIIa receptors are exposed on the surface of thrombocytes upon activation with, for example, ADP, collagen, and thrombin.[5,15] Among numerous mimetics of the RGD sequence found to be potent inhibitors of platelet aggregation, eptifibatide[16] (*N*-6-(aminoiminomethyl)-*N*-2-(3-mercapto-1-oxopropyl-L-lysylglycyl-L-α-aspartyl-L-tryptophyl-L-prolyl-L-cysteinamide) and tirofiban[17] ((2*S*)-2-(butylsulfonylamino)-3-[4-[4-(4-piperidyl)butoxy]phenylpropanoic acid) have been introduced into therapy. However, several compounds designed as oral GPIIb/IIIa antagonists have failed in phase III of clinical trial because of numerous side effects and even increased mortality rate. Compounds such as sibrafiban ((*S*)-2-(1-(2-(4-carbamimidoylbenzamido)propanoyl)piperidin-4-yloxy)acetic acid), orbofiban ((*R*)-ethyl 3-(3-(1-(4-carbamimidoylphenyl)-2-oxopyrrolidin-3-yl)ureido)propanoate), xemilofiban ((*R*)-ethyl 3-(4-(4-

* To whom correspondence should be addressed. Phone: +386 1 4769561;. Fax: +386 1 4258031. E-mail: danijel.kikelj@ffa.uni-lj.si.
[†] University of Ljubljana.
[‡] University Medical Centre Ljubljana.

[a] Abbreviations: GPIIb/IIIa, glycoprotein IIb/IIIa; PDB, Protein Data Bank; ELISA, enzyme-linked immuno sorbent assay; BTEAC, benzyltriethylammonium chloride; TFA, trifluoroacetic acid; MNDO, modified neglect of differential overlap; LGA, Lamarckian genetic algorithm; rmsd, root mean square deviation; NIH, National Institutes of Health; BAEE, *N*-α-benzoyl-L-arginine ethyl ester; HBSA, Hepes buffered saline with bovine serum albumine; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumine; PRP, platelet-rich plasma; PPP, platelet-poor plasma.

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carbamimidoylphenylamino)-4-oxobutanamido)pent-4-ynoate), and lotrafiban ((*S*)-2-(7-(4,4′-bipiperidine-1-carbonyl)-4-methyl-3-oxo-2,3,4,5-tetrahydro-1*H*-benzo[*e*][1,4]diazepin-2-yl)acetic acid) showed paradoxically procoagulant activity mainly due to partial agonist activity at subthreshold GPIIb/IIIa concentrations.[18,19] Recently, crystal structures of the platelet GPIIb/IIIa ectodomain in complex with fibrinogen-mimetic therapeutics eptifibatide, tirofiban, and L-739758 (2-(*S*)-[*N*-(3-pyridylsulfonyl)amino]-3-[[2- carbonyl-5-[2-(piperidin-4-yl)ethyl]-thieno[2,3-*b*]thiopheneyl]amino]propionic acid) have been solved. Comparison of these structures demonstrated that the drug-binding pocket in GPIIb/IIIa is rigid, with the contacting residues adopting the same conformation with or without the drug.[20] This provided the basis for our structure-based design of novel GPIIb/IIIa receptor antagonists.

In a recent Letter to this journal we introduced a new type of low molecular weight peptidomimetic antithrombotic drugs possessing both thrombin inhibitory and GPIIb/IIIa receptor antagonistic activities in the same molecule.[21,22] Simultaneous direct inhibition of different targets in the hemostatic system by *different* substances is known in hematophageous animals,[23] in which multiple inhibition of the blood coagulation enzymes and platelet activation is frequently involved.[24] In clinical practice a combination of anticoagulant agent (e.g., warfarin (4-hydroxy-3-(3-oxo-1-phenylbutyl)-2*H*-chromen-2-one), heparin, or thrombin inhibitor) and antiaggregatory drug (e.g., acetylsalicylic acid, ticlopidine (3-[(2-chlorophenyl)methyl]-7-thia-3-azabicyclo[4.3.0]nona-8,10-diene), clopidogrel ((*S*)-methyl 2-(2-chlorophenyl)-2-(6,7-dihydrothieno[3,2-*c*]pyridin-5(4*H*)-yl)acetate), or GPIIb/IIIa antagonist) is frequently used to achieve an effective antithrombotic effect in patients with valvular heart disease, prosthetic heart valves, or acute coronary syndromes, in patients following myocardial infarction, or in patients undergoing thrombolysis.[25] In these clinical situations the combination of anticoagulant and antiplatelet therapy has an additive effect by suppressing both blood coagulation and platelet function and is thus more effective than treatment directed against either thrombin or platelets alone. Efficient combination of anticoagulant and antiplatelet activity in the same molecule would produce a novel type of antithrombotic drug featuring substantial advantages over possible combinations of anticoagulant and antiplatelet agents, including more predictable and less complex pharmacokinetics, lower incidence of side effects, less demanding clinical studies, and more straightforward registration procedure, which together could render them the antithrombotic drugs of the future. In this paper we report at length on the design, synthesis, and in vitro biological activity of novel 3,4-dihydro-2*H*-1,4-benzoxazine derivatives that act both as thrombin inhibitors and GPIIb/IIIa receptor antagonists and possess a well balanced submicromolar potency against both targets.

Results and Discussion

Design. The presence of the arginine moiety in the pharmacophores D-Phe-Pro-Arg and Arg-Gly-Asp, the occurrence of the terminal carboxylic group in the RGD sequence, and the good tolerability of the P_3 carboxylic group[26] in some thrombin inhibitors, e.g., melagatran[13c] and dabigatran,[14] suggested to us the idea of incorporating the mimetics of both amino acid motifs into a single molecule that would bind with the same moieties to the thrombin active site or to the platelet GPIIb/IIIa receptor (Figure 1), thus featuring a designed multiple ligand with highly integrated pharmacophores.[27] Such dual acting antithrombotic compounds should incorporate (i) an arginine mimetic to interact

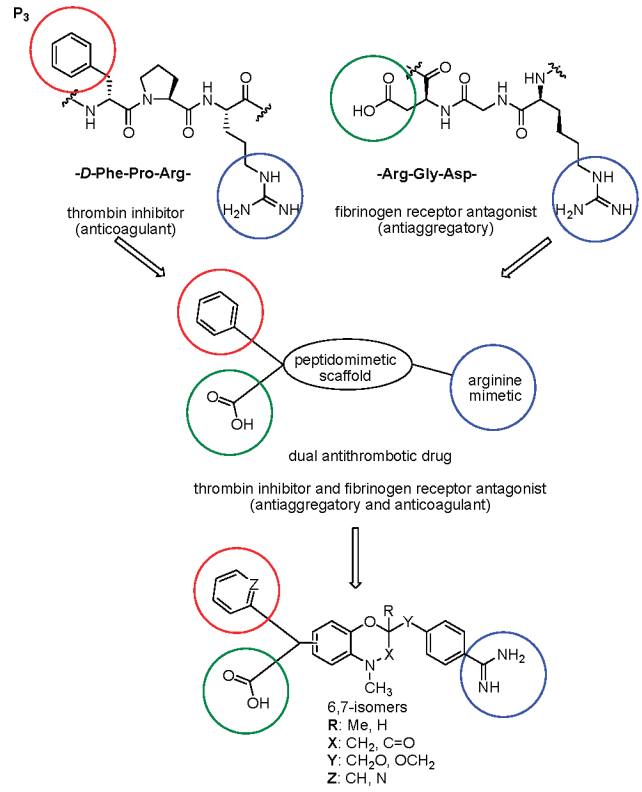


Figure 1. Design of 3,4-dihydro-2*H*-1,4-benzoxazine derivatives with thrombin inhibitory and GPIIb/IIIa receptor antagonistic activities.

with Asp189 in the thrombin S_1 pocket and to act as a cationic center for binding to the GPIIb/IIIa receptor, (ii) a carboxylate group providing ionic or dipolar interaction with the GPIIb/IIIa receptor, (iii) a central scaffold that would interact with the thrombin YPPW loop and also provide a ~1.5 nm spacer between the two charged groups required for binding to the GPIIb/IIIa receptor,[5,15] and (iv) an aromatic ring in the proximity of the carboxylate group, required for interaction with the thrombin S_3 binding pocket and to provide a hydrophobic interaction with the nonpolar binding site of the GPIIb/IIIa receptor.[28] A compromise would be required of the central scaffold since, owing to the architecture of the enzyme active site, thrombin inhibitors have to be bent between the P_1 and P_2 moieties, whereas GPIIb/IIIa receptor antagonists are more effective with a stretched conformation of the linker joining the charged groups. Additionally, the central scaffold should, if possible, provide interactions with the key amino acid residues in the thrombin active site such as Gly216. On the basis of preliminary docking experiments, 3,4-dihydro-2*H*-1,4-benzoxazine was selected as the central scaffold that, because of its manifold possibilities of functionalization, should allow for appropriate positioning of side chains in space. Despite its strong basicity, which usually results in poor bioavailability, benzamidine was chosen as the arginine mimetic[12] for the first generation of antithrombotic compounds with dual action, since it is most suitable for forming a salt bridge with Asp189 in the thrombin S_1 pocket. The benzamidine group also reduces plasma protein binding, thus improving activity. The problem of the poor bioavailability of compounds containing a benzamidine group can be overcome in the future by a prodrug approach, as shown in the case of ximelagatran[13] and dabigatran.[14]

Encouraged by the promising dual antithrombotic activity of compound **1b** ($K_{i(\text{thrombin})}$ = 14.9 μM, $IC_{50(\text{GPIIb/IIIa})}$ = 1.64 μM)[21] (Figure 2), we initiated a synthetic program, the purpose of which was systematic variation of substituents at positions 2

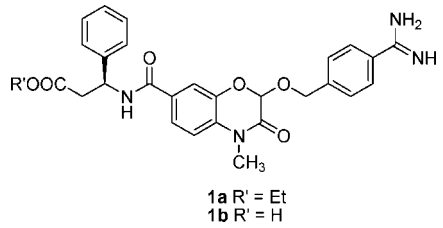
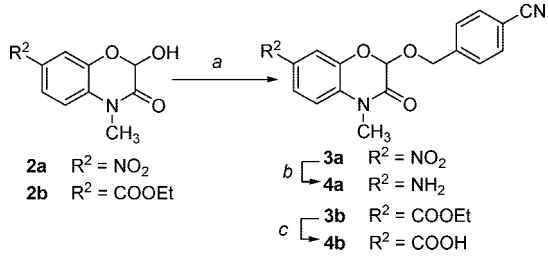


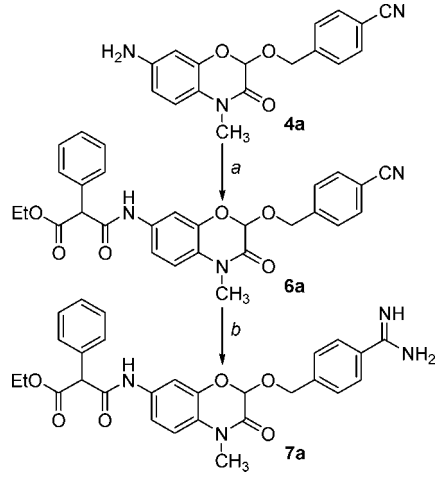
Figure 2

Scheme 1[a]



[a] (a) 4-(Bromomethyl)benzonitrile, BTEAC, K_2CO_3, CH_3CN, 60 °C, 8 h; (b) $NaBH_4$, $Cu(OAc)_2$, MeOH, room temp, 2 h; (c) 1.5 M NaOH, H_2O, EtOH, room temp, 6 h.

Scheme 2[a]



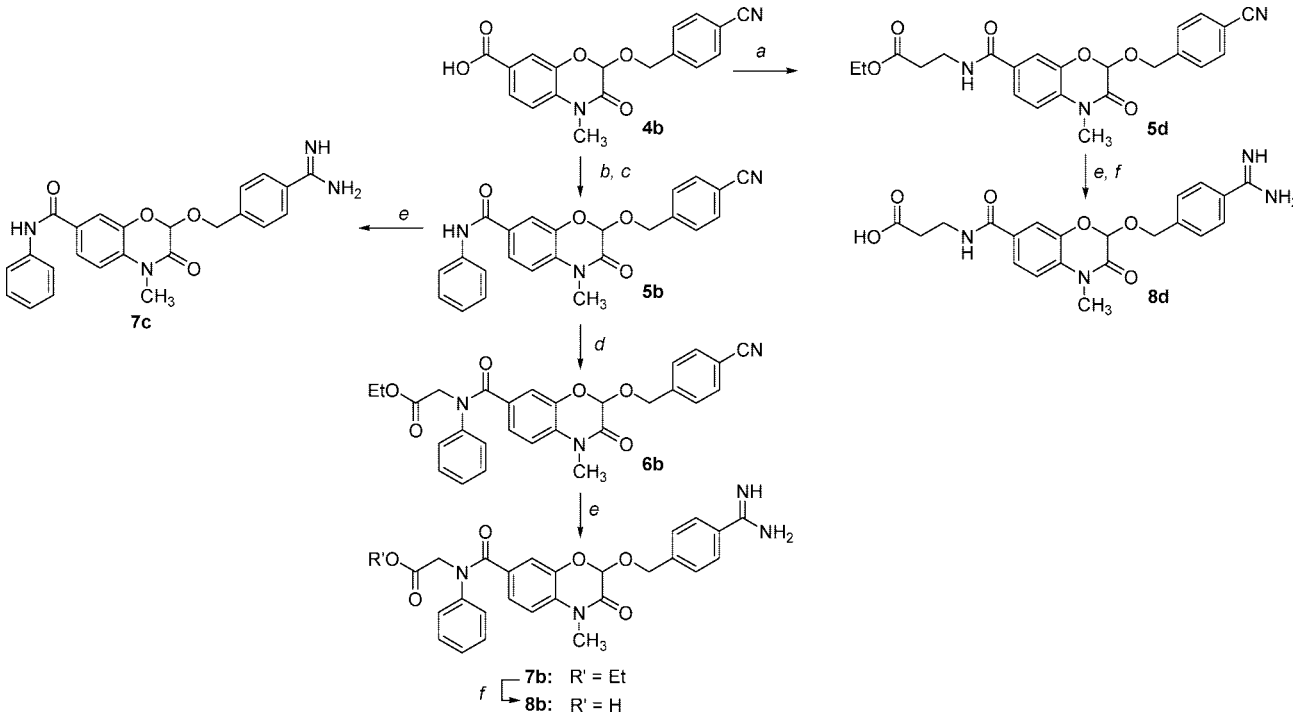
[a] (a) Diethyl 2-phenylmalonate, microwave, 120 °C, 15 min; (b) $HCl_{(g)}$, EtOH, 0 °C, 30 min, then NH_4OAc, EtOH, room temp, 24 h.

and 7, with the aim of achieving a well balanced potency for both targets. All compounds were modeled using HyperChem[29] to determine the distances between the cationic and anionic centers in the minimized conformations and docked to the thrombin active site (PDB code 1KTS)[14] and GPIIb/IIIa receptor (PDB code 1TY5)[20] with AutoDock[30] in order to predict their propensity for binding to both targets. Of the envisaged compounds with a distance of 1.5 nm between the carbon atoms of the carboxylate and amidino groups,[5,15] modeling predicted appropriate binding also for 6-substituted 1,4-benzoxazine derivatives bearing an arginine mimetic moiety at position 2. Our synthetic efforts were limited, therefore, to compounds possessing 2,6- and 2,7 substitution patterns.

Chemistry. Compounds containing the 2*H*-1,4-benzoxazin-3(4*H*)-one scaffold were synthesized as depicted in Schemes 1–3. 2-Hydroxy-4-methyl-2*H*-1,4-benzoxazin-3(4*H*)-one derivatives **2a** and **2b** were prepared from 2-aminophenol derivatives in a five-step synthesis involving N-acylation, cyclization, N-methylation, bromination, and bromine substitution, with an overall yield of 83%.[31] Compounds **2a** and **2b** were then O-alkylated with 4-cyanobenzyl bromide under phase transfer conditions in the presence of potassium carbonate, which gave better yields than the previously published method.[21] The nitro derivative **3a**, possessing a 4-cyanobenzyloxy group that is easily removable under catalytic hydrogenation conditions, was converted to amine **4a** using sodium borohydride and copper(II) acetate as catalyst[32] (Scheme 1). Aminolysis of diethyl 2-phenylmalonate with amine **4a** under microwave irradiation gave carboxamide **6a** in a short time and excellent yield. This was converted to amidine **7a** using Pinner reaction[33] (Scheme 2). The carboxylic acid **4b**, obtained by alkaline hydrolysis of **3b**, was transformed to the acyl chloride, which was reacted with aniline to give the amide **5b**. This was subsequently alkylated with ethyl bromoacetate to give the nitrile **6b**, which was finally converted to the amidine **7b**. Coupling of carboxylic acid **4b** with ethyl 3-aminopropionate gave the amide **5d**, which was converted in two steps, by Pinner reaction and subsequent alkaline hydrolysis of the ethyl ester, to the zwitterionic compound **8d** (Scheme 3).

The target compounds **17** and **18** with the 3,4-dihydro-2*H*-1,4-benzoxazine scaffold and 2-oxymethylene spacer were synthesized as shown in Schemes 4–7. Ethyl 2,4-dimethyl-6/7-nitro-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazine-2-carboxylates **9a** and **9b** were prepared from 2-amino-4-nitrophenol and 2-amino-5-nitrophenol according to a published procedure.[34] Reduction of esters **9a** and **9b** with borane dimethyl sulfide complex proceeded with concomitant reduction of the lactam carbonyl group and retention of the nitro group, affording alcohols **10a** and **10b**. Alcohol **10a** was activated for nucleophilic substitution as tosylate **11a**, which was transformed to the ether **12a** with sodium 4-cyanophenolate. Alternatively, alcohols **10a** and **10b** were directly transformed to ethers **12a** and **12b** with 4-cyanophenol under Mitsunobu reaction conditions.[35] The overall yields were comparable; however, a one-step Mitsunobu reaction was more favorable because it demanded less workup. Reduction of the nitro group in **12a** and **12b**, using catalytic hydrogenation, afforded aromatic amines **13a** and **13b** that were N-alkylated, using benzaldehyde or 2-pyridinecarboxaldehyde and sodium triacetoxyborohydride as reducing agent,[36] to afford secondary amines **14a**−**c** or acylated with benzoyl chloride to give benzamide **15a**. The amines **14a** and **14b** were alkylated with ethyl bromoacetate to afford tertiary amines **16a** and **16b**, while alkylation with ethyl 3-bromopropionate did not give the desired product. Acetylation of amines **14a**−**c** with various acyl chlorides gave amides **16c**−**g**. Similarly, alkylation of benzamide **15a** with ethyl bromoacetate gave the N-disubstituted benzamide **16h**, while reaction with ethyl 3-bromopropionate was again unsuccessful. Target compounds **17a**−**h** were prepared from nitriles **16a**−**h** using the Pinner reaction.[33] Amidines **17c**−**e** were converted to the zwitterionic compounds **18c**−**e** by alkaline hydrolysis. Loss of acetate group was observed during attempted alkaline hydrolysis in the case of *N*-benzylglycine derivatives **17a** and **17b**; hence, the desired products **18a** and **18b** were not obtained. (Scheme 4). Aromatic amines **14a** and **14b** were also acylated with monoalkyl 2-benzylmalonates using the EDC/HOBt method to give carboxamides **16i** and **16j** (Scheme 5). Unsubstituted carboxamide **15a** was also converted to amidine **17k** in order to explore the effect of N-alkylation on biological activity (Scheme 6). *N*-(ω-Carboxypropanoyl) derivative **18l** was obtained by heating **13a** with succinanhydride in THF to give **16l** and subsequent Pinner reaction followed by hydrolysis affording zwitterionic compound **17l** (Scheme 7).

Scheme 3[a]

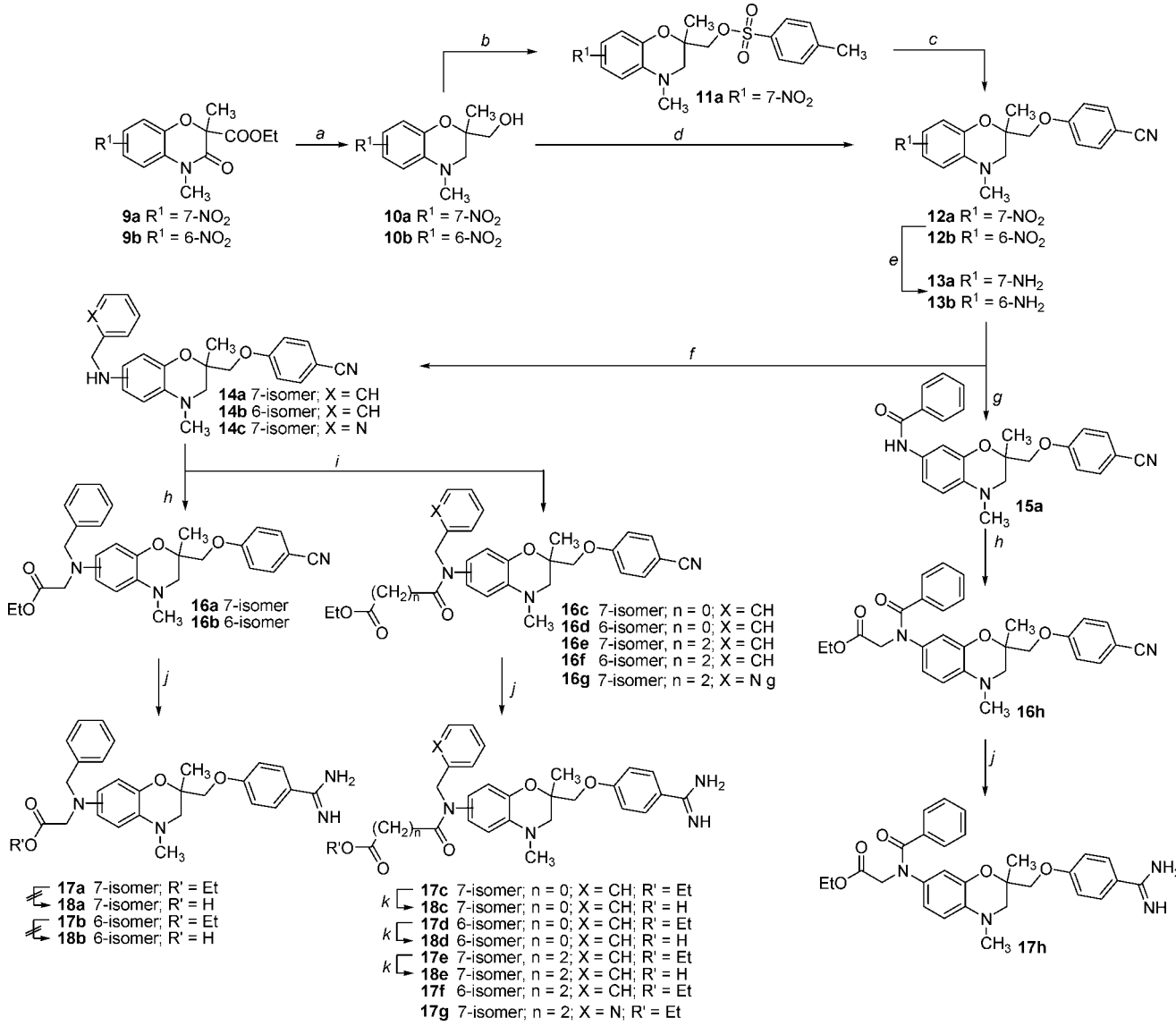


[a] (a) Ethyl 3-aminopropanoate, EDC, HOBt, DMF, room temp, overnight; (b) $SOCl_2$, CH_2Cl_2, reflux, 4 h; (c) aniline, Et_3N, DMF, overnight; (d) $BrCH_2COOEt$, BTEAC, K_2CO_3, CH_3CN, 60 °C, 24 h; (e) $HCl_{(g)}$, EtOH, 0 °C, 30 min, room temp, 48 h, then NH_4OAc, EtOH, room temp, 48 h; (f) 1.5 M NaOH, H_2O, EtOH, room temp, 6 h.

Biological Activity. In a previous communication[21] we demonstrated basic structure−activity relationships in balancing GPIIb/IIIa receptor antagonist and thrombin inhibitory activities in potential dual antithrombotic compounds based on the central 2*H*-1,4-benzoxazin-3(4*H*)-one core. First, we continued our work on optimizing the P_3 moiety of compounds **1a** and **1b** (Table 1). Although the inverse amide derivative **7a** did not improve thrombin inhibition, shortening the alkyl chain by one carbon atom had a positive effect on GPIIb/IIIa receptor antagonistic activity ($IC_{50} = 2.44\ \mu M$). The more rigid analogue **7b** showed no improvement of thrombin inhibitory activity over **1a**; however, the acid **8b** (R′ = H) was a 2-fold better thrombin inhibitor than the respective ester analogue **7b** (R′ = Et). This trend was the reverse of that of the thrombin inhibitory activities of **1a** and **1b**, where the carboxylic acid derivative **1b** had a 4-fold lower activity than the ester analogue **1a**. This suggests that in **1a** the ethyl moiety forms additional hydrophobic interactions with the protein surface, while in **7b** and **8b** the carboxylate moiety points to the water environment and the carboxylic acid group is therefore more favorable. Compound **7c** was prepared to examine the influence of the carboxylic acid moiety on biological activity. It had weaker thrombin inhibitory activity than **7b** and **8b**, showing that the introduction of a carboxylic acid moiety improves the thrombin inhibitory activity. Surprisingly, **7c** was not devoid of GPIIb/IIIa receptor antagonistic activity but actually showed a 2-fold better IC_{50} value than **7b**. This might be due to a favorable interaction of the aniline moiety of **7c** with the nonpolar binding site of the GPIIb/IIIa receptor,[28] whereas the sterically more restricted *N*-[(1,4-benzoxazin-7-yl)carbonyl]-*N*-phenylglycine moiety of **7b** does not allow the optimal positioning of the carboxylate group, the benzoxazinone core, and the phenyl ring in this special case. Compound **8d**, which lacks the P_3 aromatic moiety, showed, as expected, very weak thrombin inhibitory activity and 9-fold lower GPIIb/IIIa receptor antagonistic activity than **1b**, indicating the importance of an aromatic moiety in the vicinity of the carboxylic group for binding to GPIIb/IIIa receptor. Surprisingly, the carboxylic acid derivative **8b** showed no inhibition in a modified ELISA assay but, as expected, had greater activity than ester **7b** in an aggregation inhibition assay. These unexpected results, i.e., inactivity of **8b** on GPIIb/IIIa receptor, stronger GPIIb/IIIa receptor antagonistic activity of **7c** than that of **7b**, generally low thrombin inhibitory activity, and low selectivity toward factor Xa and trypsin, led to the decision to abandon the 2-hydroxy-4-methyl-2*H*-1,4-benzoxazin-3(4*H*)-one scaffold. Compounds with a 2-amino-4-methyl-2*H*-1,4-benzoxazin-3(4*H*)-one scaffold and a 2-methylamino linker containing an *O*,*N*-acetal moiety were also considered, but they were found to be unstable under alkaline and acidic conditions which hindered their successful preparation.[31]

Molecular modeling showed that 3,4-dihydro-2*H*-1,4-benzoxazines with a flexible 2-oxymethylene spacer have the potential to be suitably accommodated by both targets. Crystal structures of the GPIIb/IIIa receptor−tirofiban[18] and thrombin−ximelagatran[14] complexes show that tirofiban binds in an extended conformation and ximelagatran in a bent conformation because of the different geometries of the two target binding sites. Docking experiments indicated that in order to achieve the needed flexibility, a spacer of two atoms is required between the benzamidine moiety and the heterocyclic core. Compounds with the shorter and less flexible methylene spacer were also prepared but were devoid of GPIIb/IIIa receptor antagonistic activity, despite being very potent and selective thrombin inhibitors.[37] Modification of the P_3 part containing the aromatic and carboxylic acid moieties, which are supposed to be crucial for dual activity, was the main focus of our optimization strategy of compounds with a 2-oxymethylene spacer. Molecular modeling studies predicted that introduction of the P_3 moiety at position 6 of a 1,4-benzoxazine scaffold would lead to a more pronounced GPIIb/IIIa receptor antago-

Scheme 4[a]



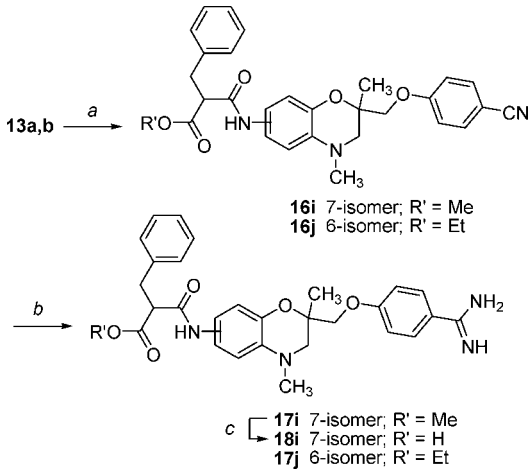
[a] (a) $Me_2S \cdot BH_3$, THF, reflux, overnight; (b) TsCl, pyridine, room temp, 24 h; (c) $NaOC_6H_4$-4-CN, DMF, 80 °C, 24 h; (d) 4-cyanophenol, PPh_3, DIAD, THF, reflux, 48 h; (e) H_2, Pd/C, THF, room temp, 2 h; (f) benzaldehyde or 2-pyridinecarbaldehyde, $NaBH(AcO)_3$, 1,2-dichloroethane, room temp, 6 h; (g) benzoyl chloride, Et_3N, THF, room temp, overnight; (h) $BrCH_2COOEt$, BTEAC, K_2CO_3, CH_3CN, 60 °C, 24 h; (i) acyl chloride, Et_3N, CH_2Cl_2, room temp, overnight; (j) $HCl_{(g)}$, EtOH, 0 °C, 30 min, then NH_4OAc, EtOH, room temp, 24 h; (k) 1.5 M NaOH, H_2O, EtOH, room temp, 6 h.

nistic activity, while introducing it at position 7 would result in a more pronounced thrombin inhibitory activity. We therefore prepared both 2,7- and 2,6-disubstituted-2,4-dimethyl-3,4-dihydro-2*H*-1,4-benzoxazines with a 2-oxymethylene spacer in order to allow the dual activity of the compounds to be tuned by simple variation of the substitution pattern.

The results of biological testing of synthesized compounds are presented in Table 2. In the resulting series of 7-benzylamino derivatives (compound **17a**) and 7-acylamino derivatives (compounds **17c** and **17h**), compound **17a** bearing the P_3 *N*-(ethoxycarbonylmetyl)benzylamino moiety was the least active ($K_{i(thr)} = 0.38\ \mu M$) as a thrombin inhibitor. Conformational restriction, effected by introducing an oxo functionality on the benzylic CH_2 moiety, increased thrombin inhibitory potency by 2-fold (**17h**, $K_{i(thr)} = 0.156\ \mu M$), whereas the oxo substituent attached to the acetate CH_2 moiety afforded an even more potent thrombin inhibitor (**17c**, $K_{i(thr)} = 0.11\ \mu M$). All three compounds possess low micromolar IC_{50} values for GPIIb/IIIa receptor antagonistic activity, thus confirming that the 2,7-disubstitution pattern favors thrombin inhibition. Extending the *N*-acyl chain in compound **17e** increased thrombin inhibitory activity but reduced the GPIIb/IIIa receptor antagonistic activity. Substitution of benzyl moiety in **17e** with pyridin-2-ylmethyl moiety to give **17g** improved thrombin inhibitory activity 2-fold ($K_{i(thr)} = 25$ nM) and also improved GPIIb/IIIa receptor antagonistic activity. Compounds **17b** and **17d**, bearing a P_3 moiety at position 6, had improved GPIIb/IIIa receptor antagonistic activity but with concomitantly reduced thrombin inhibitory activity. Compound **17d** possessed the most potent and the best balanced dual antithrombotic activity ($K_{i(thr)} = 0.32\ \mu M$; $IC_{50(GPIIb/IIIa)} = 1.2\ \mu M$).

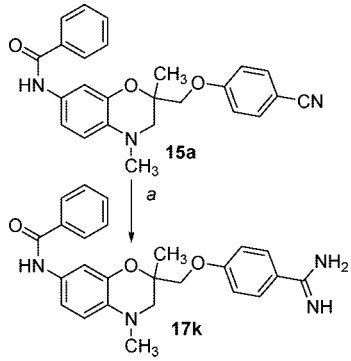
Compounds **17i**, **18i**, and **17j** possessing more distant P_3 aromatic moiety showed reduced thrombin inhibitory activity, proving the importance of optimal position of P_3 aromatic moiety for good thrombin inhibition. Compound **18i** showed good platelet aggregation inhibitory activity ($IC_{50} = 24.9\ \mu M$). Comparison of activities of compounds **17h** with that of **17k** shows that the introduction of a carboxylic acid moiety improved the thrombin inhibitory activity by an order of magnitude while surprisingly only slightly improving the GPIIb/IIIa receptor

Scheme 5[a]



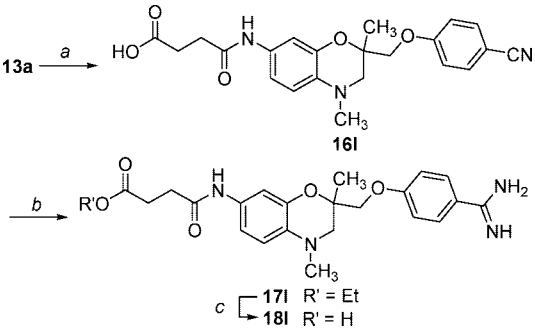
[a] (a) 2-Benzyl-3-alkyloxy-3-oxopropanoic acid, EDC, HOBt, DMF, room temp, overnight; (b) $HCl_{(g)}$, EtOH, 0 °C, 30 min, then NH_4OAc, EtOH, room temp, 24 h; (c) 1.5 M NaOH, H_2O, EtOH, room temp, 6 h.

Scheme 6[a]



[a] (a) $HCl_{(g)}$, EtOH, 0 °C, 30 min, then NH_4OAc, EtOH, room temp, 24 h.

Scheme 7[a]



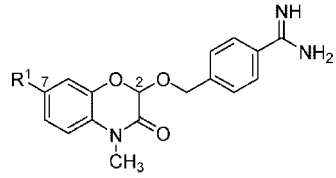
[a] (a) Succinanhydride, THF, 60 °C, 60 min; (b) $HCl_{(g)}$, EtOH, 0 °C, 30 min, then NH_4OAc, EtOH, room temp, 24 h; (c) 1.5 M NaOH, H_2O, EtOH, room temp, 6 h.

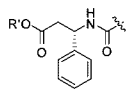
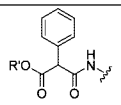
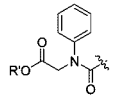
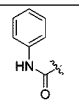
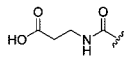
antagonistic activity. Similar to the activity of **7c**, the activity of **17k** might be due to a favorable interaction of the benzoyl moiety of **17k** with the nonpolar binding site of the GPIIb/IIIa receptor.[28] Comparison of compounds **17e** (R′ = Et) and **18e** (R′ = H) with **17l** (R′ = Et) and **18l** (R′ = H) shows that omitting the aromatic P_3 moiety reduced the thrombin inhibitory activity by an order of magnitude while it improved the GPIIb/IIIa receptor antagonistic activity. While carboxylic acid **18l** displayed stronger GPIIb/IIIa receptor antagonistic activity in a modified ELISA assay and better activity in an aggregation assay than its ester analogue **17l**, carboxylic acids **18c**, **18d**, and **18e** unexpectedly displayed much weaker GPIIb/IIIa receptor antagonistic activity in a modified ELISA assay than their ester analogues **17c**, **17d**, and **17e** and showed good activity in an aggregation assay. Similar behavior had already been observed with compound **8b** and could suggest that these compounds probably affect the modified ELISA test in an unknown way. The fact that both carboxylic acids **18** and esters **17** display GPIIb/IIIa receptor antagonistic activity could be explained by their ion−dipole interaction with the cationic site of the GP IIb/IIIa receptor. 6-Isomers **17b** and **17d** possess better GPIIb/IIIa receptor antagonistic activity than analogous 7-isomers **17a** and **17c**, which is opposite the trend observed with thrombin inhibitory activity. The oxo substituent attached to the acetate CH_2 moiety slightly reduced the GPIIb/IIIa receptor antagonistic activity (1.4-fold in the case of 7-isomer **17c** and 1.6-fold in the case of 6-isomer **17d**). Introduction of an oxo functionality to the benzylic CH_2 moiety (**17h**) did not influence the activity compared with less rigid compound **17a**. With the prolongation of acyl chain, the GPIIb/IIIa receptor antagonistic activity is reduced (**17e** and **18e**) as expected from molecular modeling.

The biological results were explained by docking compounds **7**, **8**, **17**, and **18** to the thrombin active site[14] and to the GPIIb/IIIa receptor binding site.[18] The (2*R*)-isomers displayed better docking allignments than the (2*S*)-isomers. The conformations of compounds **7**, **8**, **17**, **18** docked to both targets are similar with respect to the bend between benzamidine and benzoxazine moieties. (Figures 3 and 4 docking results for **18c** and **18d** are shown). Compound (*R*)-**18c** binds to the former in a similar manner to ximelagatran. The distance between the C-atoms of the Asp189 carboxylate and the amidine group in (*R*)-**18c** was 4.0 Å, and two hydrogen bonds linked the amidine hydrogens and carboxylate oxygen atoms. An additional hydrogen bond was formed between benzamidine and the Gly219 backbone. The 3,4-dihydro-2*H*-1,4-benzoxazine 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 (2.72 Å) with the Gly216 backbone, which is often regarded as a key interaction in the thrombin active site.[38] The oxymethylene spacer between benzamidine and the 3,4-dihydro-2*H*-1,4-benzoxazine moiety allows for flexibility, which enables the compound to assume a bent conformation in the thrombin binding site. Compound (*R*)-**18d** is also positioned similarly in the thrombin active site; however, the proximity of 1,4-benzoxazine aromatic ring and P_3 phenyl ring does not allow optimum positioning of the P_3 aromatic moiety of (*R*)-**18d**, which results in 2-fold lower potency of the 6-isomer. In the cases of **17e** and **17f**, where **17e** is a more optimized thrombin inhibitor (K_i = 60 nM), the difference in the potency of 7-isomer and 6-isomer is bigger (11-fold). Compound **17i**, possessisng a 2-benzylmalonyl moiety, binds also in similar fashion, the only difference being that the more distant benzyl group is not optimally bound to the S3 pocket, which accounts for the much lower thrombin inhibitory potency of this compound.

Analysis of the binding mode of (*R*)-**18d** in the binding site of GPIIb/IIIa receptor shows that it takes an extended conformation with a break that is forced by the conformational restriction on position 2 of 1,4-benzoxazine. The benzamidine moiety hydrogen-bonds with Asp224 and Phe160 from the αIIb subunit of the GPIIb/IIIa receptor and makes hydrophobic interactions with Tyr190, Phe231, and Phe191 of the αIIb subunit. The oxygen atom of the 1,4-oxazine ring forms a hydrogen bond with Ala218. The carboxylic acid moiety makes electrostatic

Table 1. Biological Activity of 1,4-Benzoxazin-3(4*H*)-one Derivatives **7** and **8**: Inhibition of Serine Proteases Thrombin, Factor Xa, and Trypsin, Inhibition of Fibrinogen Binding to GP IIb/IIIa Receptors, and Inhibition of ADP Induced Platelet Aggregation



R[1]	Comp.	R'	K_i (μM) Thrombin	Trypsin [selectivity][a]	FXa [selectivity][b]	IC_{50} ($\alpha_{IIb}\beta_3$) **1**(μM)	Platelet aggregation[c] (μM)
	**1a**[21]	Et	3.7	16	54.6	24.63	/
	1b[21]	H	14.9	26.5	>200	1.64	/
	**7a**	Et	9.89 ± 2.18	14.6 ± 3.0 [1.5]	50.3 ± 12.0 [5.1]	2.44 ± 0.97	23% (50 μM)
	**7b**	Et	3.98 ± 1.12	18.5 ± 3.2 [4.6]	43.1 ± 2.2 [11]	28.4 ± 8.0	10% (50 μM)
	8b	H	1.76 ± 0.22	16.6 ± 5.9 [9.4]	49.6 ± 16.0 [28]	> 100	24% (50 μM)
	**7c**	/	24.2 ± 2.1	17.2 ± 2.4 [0.7]	31.6 ± 6.4 [1.3]	12.08 ± 5.94	19% (50 μM)
	**8d**	/	118 ± 32	23.5 ± 4.0 [0.2]	112 ± 10 [1.0]	15.2 ± 4.7	23% (50 μM)

[a] Selectivity for thrombin vs trypsin. [b] Selectivity for thrombin vs FXa. [c] Percent of inhibition of ADP induced platelet aggregation at 50 μM inhibitor concentration.

interactions with the Mg^{2+} atom and hydrogen-bonds to the amide proton of Asn215 of the β_3 subunit of the GPIIb/IIIa receptor. Compound (*R*)-**18c** adopts a similar conformation, the main difference being in the positioning of the 1,4-benzoxazine moiety, which is more twisted toward the water environment.

Conclusion

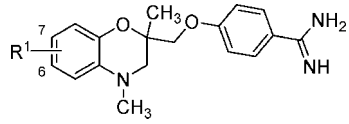
In conclusion, we have described the design, synthesis, and dual activity of several new 3,4-dihydro-2*H*-1,4-benzoxazine compounds capable of acting as both thrombin inhibitors and GPIIb/IIIa receptor antagonists and analyzed the structure−activity relationship of combining anticoagulant and antiaggregatory activity into one molecule. We optimized flexibility between benzamidine moiety and central 1,4-benzoxazine scaffold to ensure desired activity on both targets. To improve balanced dual activity on thrombin and platelet GPIIb/IIIa receptor, a compromise concerning flexibility and bulkiness in P_3 part containing aromatic and carboxylic acid moieties was sought. Compounds **17a**, **17b**, **17d**, and **17h,** which have the most potent and well balanced dual antithrombotic activity, close to the nanomolar range, can serve as a lead compounds for the next generation of dual antithrombotic agents, making use of established binding modes and structure−activity relationships.

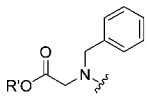
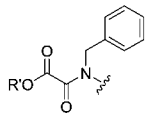
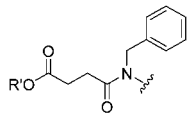
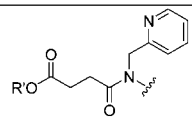
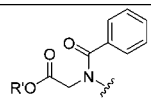
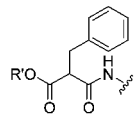
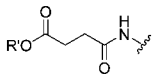
Experimental Section

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). Microwave assisted reactions were performed using a CEM Discover microwave reactor (CEM Corp.). 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. Spectra were assigned using gradient COSY, HSQC, and HMBC experiments. 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 mm × 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 from 10% B to 80% B in 30 min.

General Procedure for Preparing Derivatives 3a and 3b. A stirred suspension of 4-cyanobenzyl bromide (0.77 g, 3.94 mmol), 2-hydroxy-4-methyl-2*H*-1,4-benzoxazin-3(4*H*)-one derivative **2a** or **2b** (3.94 mmol), benzyltriethylammonium chloride (0.90 g, 3.94 mmol), and potassium carbonate (1.36 g, 9.85 mmol) in acetonitrile (70 mL) was heated at 60 °C for 8 h. The suspension was filtered and the filtrate evaporated under reduced pressure. The residue was dissolved in ethyl acetate (150 mL) and washed successively with 10% citric acid (3 × 50 mL), saturated solution of $NaHCO_3$ (2 ×

Table 2. Biological Activity of 3,4-Dihidro-1,4-benzoxazine Derivatives **17** and **18**: Inhibition of Serine Proteases Thrombin, Factor Xa, and Trypsin, Inhibition of Fibrinogen Binding to GP IIb/IIIa Receptors, and Inhibition of ADP Induced Platelet Aggregation[e]



R^1	Comp.	Substituent Position	R'	K_i (μM)			IC_{50} (GPIIb/IIIa) (μM)	Platelet aggregation[c] (μM)
				Thrombin	Trypsin [selectivity][a]	FXa [selectivity][b]		
	**17a**	7	Et	0.38 ± 0.11	1.73 ± 0.48 [4.5]	2.67 ± 0.78 [7]	1.45 ± 0.91	19% (50 μM)
	17b	6	Et	1.74 ± 0.36	2.39 ± 0.55 [1.4]	11.9 ± 2.9 [6.8]	0.73 ± 0.55	13% (50 μM)
	**17c**	7	Et	0.11 ± 0.38	0.95 ± 0.08 [9]	112 ± 37 [1046]	2.0 ± 0.86	21% (50 μM)
	18c	7	H	0.377 ± 0.42	1.27 ± 0.32 [3.3]	16.6 ± 2.5 [44]	> 100	26% (50 μM)
	17d	6	Et	0.32 ± 0.12	0.59 ± 0.05 [1.8]	16.0 ± 1.9 [49]	1.2 ± 0.2	24% (50 μM)
	18d	6	H	0.879 ± 0.121	1.20 ± 0.10 [1.3]	31.7 ± 5.3 [36]	> 100	38.7 μM (IC_{50})
	**17e**	7	Et	0.060 ± 0.017	1.30 ± 0.90 [19]	18.0 ± 3.9 [300]	29.3 ± 9.7	NI[d]
	18e	7	H	0.49 ± 0.15	2.23 ± 0.45 [5.0]	29.7 ± 4.6 [59]	> 100	14% (50 μM)
	17f	6	Et	0.67 ± 0.11	3.67 ± 0.61 [3.7]	27.7 ± 3.4 [28]	> 100	NI[d]
	**17g**	7	Et	0.025±0.006	1.41 ± 0.07 [56]	11.8 ± 0.8 [472]	11.3 ± 3.0	15% (50 μM)
	**17h**	7	Et	0.156 ± 0.023	2.42 ± 0.67 [12]	9.59 ± 1.07 [47]	1.78 ± 0.58	21% (50 μM)
	**17i**	7	Me	2.86 ± 0.47	9.48 ± 2.19 [1.7]	35.4 ± 7.9 [12]	9.11 ± 6.17	21% (50 μM)
	18i	7	H	5.5 ± 1.3	nd	nd	nd	24.9 μM (IC_{50})
	17j	6	Et	4.93 ± 1.30	2.98 ± 0.55 [0.6]	29.5 ± 7.3 [6]	36.5 ± 20	8% (50 μM)
	**17k**	7	/	5.00 ± 2.08	1.31 ± 0.27 [0.3]	12.1 ± 4.6 [2.4]	2.85 ± 0.88	21% (50 μM)
	**17l**	7	Et	16.9 ± 3.1	7.49 ± 0.70 [0.4]	74.7 ± 10.1 [4.4]	14.0 ± 4.6	15% (50 μM)
	18l	7	H	5.16 ± 0.53	1.73 ± 0.36 [0.3]	33.8 ± 11.2 [6.6]	1.69 ± 0.41	36.7 μM (IC_{50})

[a] Selectivity for thrombin vs trypsin. [b] Selectivity for thrombin vs FXa. [c] Percent of inhibition of ADP induced platelet aggregation at 50 μM inhibitor concentration or IC_{50}. [d] Compound did not show any inhibition at 50 μM. [e] nd = not determined.

50 mL), and saturated solution of NaCl (1 × 50 mL). The organic phase was dried with Na_2SO_4 and the solvent evaporated under reduced pressure to obtain a solid product. If necessary, the product was recrystallized from ethyl acetate/petroleum ether.

4-[(4-Methyl-7-nitro-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yloxy)methyl]benzonitrile (3a). Yellow crystals; yield, 1.26 g (94%); mp 135−136 °C. ^{1}H NMR ($CDCl_3$): δ = 3.32 (s, 3H, *N*-CH_3), 4.95 (s, 2H, OCH_2), 5.88 (s, 1H, 2-H), 7.43 (d, 2H, ^3J = 8.3 Hz,

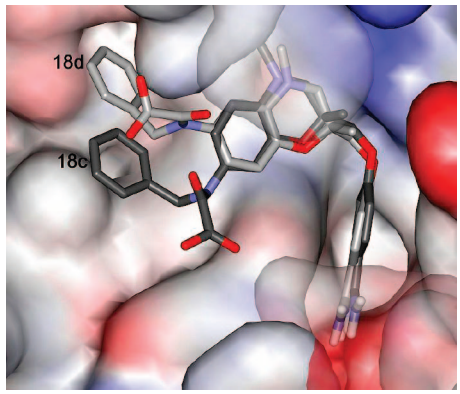


Figure 3. Compounds (*R*)-**18c** and (*R*)-**18d** docked in the active site of thrombin.

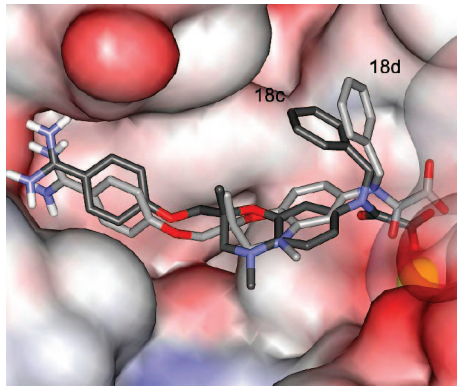


Figure 4. Compounds (*R*)-**18c** and (*R*)-**18d** docked in the binding site of the GPIIb/IIIa receptor.

Ar−H$^{2'}$,H$^{6'}$), 7.47 (d, 1H, ^3J = 9.0 Hz, Ar−H^5), 7.72 (d, 1H, ^4J = 2.6 Hz, Ar−H^8), 7.78 (d, 2H, ^3J = 8.3 Hz, Ar−H$^{3'}$,H$^{5'}$), 8.04 (dd, 1H, ^3J = 9.0 Hz, ^4J = 2.6 Hz, Ar−H^6) ppm. ^{13}C NMR ($CDCl_3$): δ = 159.51 (C-3), 142.63 (C-1′), 142.23 (C-7), 140.51 (C-8a), 134.43 (C-4a), 132.15 (C-3′, C-5′), 128.43 (C-2′, C-6′), 119.15 (CN), 118.50 (C-6), 115.71 (C-5), 112.73 (C-8), 110.59 (C-4′), 94.81 (C-2), 69.56 (O$\underline{CH_2}$−Ar), 28.52 (*N*-CH_3) ppm. MS (EI): *m*/*z* (%) = 339 (M$^+$, 10), 195 (100), 149 (49). IR (KBr): ν = 2224, 1692, 1601, 1525, 1384, 1340, 1223, 1083, 1029, 890 cm^{-1}. Anal. ($C_{17}H_{13}N_3O_5$) C, H, N.

Synthesis of 4-[(7-Amino-4-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yloxy)methyl]benzonitrile (4a). Nitro derivative **3a** (1.20 g, 3.54 mmol) and $Cu(OAc)_2$ (0.708 g, 3.54 mmol) were dissolved in 250 mL of methanol. $NaBH_4$ was added portionwise over a period of 1 h and the solution stirred for an additional hour. The solution was filtered through a Celite pad to remove the black precipitate. Solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate (200 mL) and washed successively with 10% citric acid (3 × 50 mL), saturated solution of $NaHCO_3$ (2 × 50 mL), and saturated solution of NaCl (1 × 50 mL). The organic phase was dried with Na_2SO_4 and the solvent evaporated under reduced pressure to obtain a solid product. Yellow crystals; yield, 0.856 g (78.3%); mp 65−66 °C. ^{1}H NMR (DMSO-d_6): δ = 3.24 (s, 3H, *N*-CH_3), 4.85 (s, 2H, OCH_2), 5.06 (s, 2H, NH_2), 5.55 (s, 1H, 2-H), 6.30 (dd, 1H, ^3J = 8.7 Hz, ^4J = 2.4 Hz, Ar−H^6), 6.35 (d, 1H, ^4J = 2.4 Hz, Ar−H^8), 6.90 (d, 1H, ^3J = 8.7 Hz, Ar−H^5), 7.44 (d, 2H, ^3J = 8.4 Hz, Ar−H$^{2'}$,H$^{6'}$), 7.80 (d, 2H, ^3J = 8.3 Hz, Ar−H$^{3'}$,H$^{5'}$) ppm. MS (EI): *m*/*z* (%) = 309 (55), 194 (80) 165 (100). IR (KBr): ν = 3436, 2926, 2228, 1676, 1516, 1401, 1304, 1187, 1146, 1088, 1030 cm^{-1}. Anal. ($C_{17}H_{15}N_3O_3$) C, H, N.

Ethyl 3-[2-(4-Cyanobenzyloxy)-4-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-7-ylamino]-3-oxo-2-phenylpropanoate (6a). Amine **4a** (0.309 g, 1.00 mmol) and diethyl 2-phenylmalonate (2.36 g, 10.0 mmol) were sealed in a 10 mL process vial and heated in a microwave reactor for 15 min at 120 °C. Dichloromethane (5 mL) was added and the product precipitated with 70 mL of petroleum ether. The crude product was redissolved in 3 mL of dichloromethane and precipitated again with 70 mL of petroleum ether to give yellow crystals. Yield, 0.475 g (95.2%); mp 71−73 °C. ^{1}H NMR (DMSO-d_6: δ = 1.15 (t, 3H, ^3J = 7.1 Hz, CH_2−$\underline{CH_3}$), 3.37 (s, 3H, *N*-CH_3), 4.08−4.21 (m, 2H, $\underline{CH_2}$−CH_3), 4.90 (4.91) (s, 2H, OCH_2), 5.64 (dd, 1H, ^3J = 6.8 Hz, ^4J = 1.5 Hz, Ph−$\underline{CH}$), 5.75 (5.76) (s, 1H, 2-H), 7.33 (d, 1H, ^3J = 8.6 Hz, Ar−H^5), 7.36−7.54 (m, 5H, Ph), 7.49 (d, 2H, ^3J = 8.0 Hz, Ar−H$^{2'}$,H$^{6'}$), 7.64 (d, 1H, ^4J = 1.5 Hz, Ar−H^8), 7.74−7.80 (m, 3H, Ar−H^6, Ar−H$^{3'}$,H$^{5'}$) ppm. ^{13}C NMR (DMSO-d_6: δ = 170.39 (170.40) ($\underline{COOEt}$), 156.03 (156.07) (NH$\underline{CO}$), 159.66 (159.67) (C-3), 142.26 (142.28) (C-1′), 140.17 (C-8a), 135.97 (136.03) (C-1″), 132.15 (132.17) (C-3′, C-5′), 131.08 (131.10) (C-4a), 128.74 (128.75) (C-7), 128.43 (C-2′, C-6′), 128.34, 128.15 (Ph), 123.11 (C-6), 118.54 (CN), 116.82 (116.85) (C-8), 114.91 (C-5), 110.49 (110.50) (C-4′), 94.79 (94.82) (C-2), 69.00 (69.07) (O$\underline{CH_2}$−Ar), 60.81 (CH_2−CH_3), 56.96 (57.00) (Ph$\underline{CH}$), 28.18 (*N*-CH_3), 13.86 (CH_2−$\underline{CH_3}$) ppm. MS (ESI): *m*/*z* (%) = 500.2 (MH$^+$, 100), 454.1 (10). IR (KBr): ν = 3436, 2229, 1707, 1616, 1498, 1364, 1222, 1179, 1092, 1056 cm^{-1}. Anal. ($C_{28}H_{25}N_3O_6$) C, H, N.

7-[(2-Ethoxy-2-oxoethyl)(phenyl)carbamoyl]-2-(4-cyanobenzyloxy)-4-methyl-2*H*-1,4-benzoxazin-3(4*H*)-one (6b). 6b was synthesized from **5b** (2.23 g, 5.38 mmol) according to the general procedure for alkylation under phase-transfer catalysis. The crude product was purified by column chromatography using dichloromethane/methanol (50:1) as eluant to give yellow crystals. Yield, 2.31 g (86.0%); mp 63−64 °C. ^{1}H NMR ($CDCl_3$): δ = 1.32 (t, 3H, ^3J = 7.1 Hz, $CH_2\underline{CH_3}$), 3.36 (s, 3H, *N*-CH_3), 4.26 (q, 2H, ^3J = 7.1 Hz, $\underline{CH_2}CH_3$), 4.60 (s, 2H, *N*-CH_2COO), 4.73 (d, 1H, ^2J = 12.9 Hz, O$\underline{CH_2}$−Ar), 4.67 (d, 1H, ^2J = 12.9 Hz, O$\underline{CH_2}$−Ar), 5.41 (s, 1H, 2-H), 6.86 (d, 1H, ^3J = 8.4 Hz, Ar−H^5), 7.04 (d, 1H, ^4J = 1.9 Hz, Ar−H^8), 7.11−7.24 (m, 5H, Ph, Ar−H$^{2'}$,H$^{6'}$), 7.34 (d, 2H, ^3J = 8.3 Hz, Ar−H$^{3'}$,H$^{5'}$), 7.62−7.65 (m, 2H, Ph) ppm. MS (EI): *m*/*z* (%) = 517.2 (MH$^+$, 100), 421.2 (15), 198.2 (45), 196.2 (55), 141.0 (95). IR (KBr): ν = 2980, 2228, 1746, 1697, 1615, 1380, 1323, 1201, 1088, 1032 cm^{-1}. Anal. ($C_{28}H_{25}N_3O_6$) C, H, N.

Ethyl 3-[2-(4-Carbamimidoylbenzyloxy)-4-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-7-ylamino]-3-oxo-2-phenylpropanoate (7a). 7a was synthesized from **6a** according to the general procedure for preparation of amidines from nitriles (Pinner reaction). Brown oil; yield, 157 mg (27%). ^{1}H NMR (DMSO-d_6): δ = 1.18 (t, 3H, ^3J = 7.1 Hz, CH_2−$\underline{CH_3}$), 3.30 (s, 3H, *N*-CH_3), 4.15 (q, 2H, ^3J = 7.1 Hz, $\underline{CH_2}$−CH_3), 4.89 (s, 2H, OCH_2), 5.08 (s, 1H, Ph−$\underline{CH}$), 5.66 (s, 1H, 2-H), 7.18 (d, 1H, ^3J = 8.9 Hz, Ar−H^5), 7.28−7.41 (m, 4H, Ph-H$^{2''}$,H$^{6''}$, Ph-H$^{4''}$, Ar−H^6), 7.42−7.53 (m, 5H, Ar−H^8, Ph-H$^{3''}$,H$^{5''}$, Ar−H$^{3''}$,H$^{5''}$), 7.78 (dd, 2H, ^3J = 8.0 Hz, ^4J = 2.3 Hz, Ar−H$^{2'}$,H$^{6'}$), 9.40 (br s, 4H, amidino H), 10.89 (br s, 1H, CONH) ppm. ^{13}C NMR (DMSO-d_6): δ = 168.40 ($\underline{COOEt}$), 156.75 (NH$\underline{CO}$), 165.35 ($\underline{C}$(=NH_2)NH_2), 159.24 (C-3), 142.66 (C-1′), 140.59 (C-8a), 134.95 (C-7), 134.17 (C-1″), 129.21 (C-3″, C-5″), 128.04, 127.81, 127.49 (Ph, C-2′, C-6′, C-2″, C-6″), 124.16 (C-4′), 115.31 (C-5), 113.71 (C-6), 108.42 (C-8), 95.03 (95.05) (C-2), 69.09 (O$\underline{CH_2}$−Ar), 60.88 (60.90) ($\underline{CH_2}$−CH_3), 57.18 (Ph$\underline{CH}$), 27.93 (*N*-CH_3), 13.89 (CH_2−$\underline{CH_3}$) ppm. MS (ESI): *m*/*z* (%) = 517.2 (MH$^+$, 100), 421 (15), 196 (57), 141 (100). IR (KBr): ν = 3061, 1676, 1514, 1399, 1302, 1186, 1088, 1029 cm^{-1}. HPLC: 96.3%, t_R = 18.59 min. Anal. ($C_{28}H_{28}N_4O_6 \cdot 2.5H_2O$) C, H, N.

2-[2-(4-Carbamimidoylbenzyloxy)-4-methyl-3-oxo-*N*-phenyl-3,4-dihydro-2*H*-1,4-benzoxazine-7-carbonylamino]acetic acid (8b). 8b was synthesized from **7b** according to the general procedure for alkaline hydrolysis of alkyl esters. Brown crystals; yield, 91 mg (88.2%); mp 110−111 °C. ^{1}H NMR (DMSO-d_6): δ = 3.20 (s, 3H, *N*-CH_3), 4.42 (s, 2H, *N*-CH_2COOH), 4.60 (d, 1H, ^2J = 12.2 Hz, O$\underline{CH_2}$−Ar), 4.71 (d, 1H, ^2J = 12.2 Hz, O$\underline{CH_2}$−Ar), 5.54 (s, 1H, 2-H), 6.79 (d, 1H, ^4J = 2.1 Hz, Ar−H^8), 7.01−7.24 (m, 7H, Ph, Ar−H^5, Ar−H^6), 7.32 (d, 2H, ^3J = 7.9 Hz, Ar−H$^{2'}$,H$^{6'}$), 7.75 (d, 2H, ^3J = 7.9 Hz, Ar−H$^{3'}$,H$^{5'}$), 9.07 (br s, 2H, amidino H), 9.34 (br s, 2H, amidino H) ppm. MS (ESI): *m*/*z* (%) = 489.2 (MH$^+$, 35), 321.1 (35), 198.2 (75), 196.2 (100). IR (KBr): ν = 3420, 1576,

1421, 1090, 1033, 836 cm^{-1}. HPLC: 98.7%, $t_R = 15.19$ min. Anal. ($C_{26}H_{24}N_4O_6 \cdot 2H_2O$) C, H, N.

Reduction of Ethyl 2,4-Dimethyl-6/7-nitro-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazine-2-carboxylates 9a and 9b. Compound **9a** or **9b** (10.66 g, 36.2 mmol) was dissolved in anhydrous THF (100 mL). Borane dimethyl sulfide complex (5.51 g, 72.5 mmol) was added, and the solution was refluxed overnight. Hydrochloric acid (4 M, 20 mL) was added, and the solution was refluxed for a further 30 min. After neutralization with 1 M NaOH, the solvent was evaporated under reduced pressure and the precipitated product recrystallized from methanol/water.

(2,4-Dimethyl-7-nitro-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl)methanol (10a). Red crystals; yield, 7.57 g (88%); mp 125−127 °C. ^{1}H NMR ($CDCl_3$): $\delta = 1.32$ (s, 3H, 2-CH_3), 1.98 (dd, 1H, $^3J = 5.9$ Hz, $^3J = 6.9$ Hz, OH), 3.09 (s, 3H, *N*-CH_3), 3.11 (d, 1H, $^2J = 12.1$ Hz, 3-H), 3.52 (d, 1H, $^2J = 12.1$ Hz, 3-H), 3.63 (dd, 1H, $^2J = 11.6$ Hz, $^3J = 6.9$ Hz, $\underline{CH_2}$−OH), 3.72 (dd, 1H, $^2J = 11.6$ Hz, $^3J = 5.9$ Hz, $\underline{CH_2}$−OH), 6.62 (d, 1H, $^3J = 9.0$ Hz, Ar−H^5), 7.69 (d, 1H, $^4J = 2.6$ Hz, Ar−H^8), 7.84 (dd, 1H, $^3J = 9.0$ Hz, $^4J = 2.6$ Hz, Ar−H^6) ppm. ^{13}C NMR ($CDCl_3$): $\delta = 141.44$, 141.21 (C-4a, C-8a), 138.76 (C-7), 119.36 (C-6), 112.61 (C-8), 109.83 (C-5), 75.31 (C-2), 67.05 (−CH_2−OH), 53.98 (C-3), 39.00 (*N*-CH_3), 20.77 (2-CH_3) ppm. MS (FAB): *m/z* (%) = 239 (MH^+, 82), 154 (84), 136 (71), 81 (46), 69 (100), 55 (95). IR (KBr): $\nu = 3515$, 2928, 1605, 1534, 1495, 1310, 1220, 1061, 970, 804, 743 cm^{-1}. Anal. ($C_{11}H_{14}N_2O_4$) C, H, N.

(2,4-Dimethyl-7-nitro-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl)methyl 4-Methylbenzenesulfonate (11a). A solution of alcohol **10a** (3.28 g, 13.8 mmol) and tosyl chloride (2.89 g, 15.2 mmol) in anhydrous pyridine (40 mL) was stirred for 24 h at room temperature. Solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate (600 mL) and washed successively with 2 M hydrochloric acid (3 × 100 mL), 1 M sodium hydroxide (2 × 100 mL), and brine (1 × 100 mL). The organic phase was dried over Na_2SO_4 and filtered, and the solvent was evaporated under reduced pressure to $^1/_4$ of the starting volume. The residual solution was stored at 4 °C, and the precipitated crystals were filtered off and washed with cold ethyl acetate. Yield, 4.60 g (85%); yellow crystals; mp 157−159 °C. ^{1}H NMR ($CDCl_3$): $\delta = 1.37$ (s, 3H, 2-CH_3), 2.98 (s, 3H, Ar′−$\underline{CH_3}$), 3.02 (s, 3H, *N*-CH_3), 3.16 (d, 1H, $^2J = 12.3$ Hz, 3-H), 3.35 (d, 1H, $^2J = 12.3$ Hz, 3-H), 3.90 (d, 1H, $^2J = 10.0$ Hz, CH_2O), 4.05 (d, 1H, $^2J = 10.0$ Hz, CH_2O), 6.58 (d, 1H, $^3J = 9.0$ Hz, Ar−H^5), 7.35 (d, 2H, $^3J = 8.4$ Hz, Ar−$H^{3'}$,$H^{5'}$), 7.51 (d, 1H, $^4J = 2.6$ Hz, Ar−H^8), 7.76 (d, 2H, $^3J = 8.4$ Hz, Ar−$H^{2'}$,$H^{6'}$), 7.81 (dd, 1H, $^3J = 9.0$ Hz, $^4J = 2.6$ Hz, Ar−H^6) ppm. MS (FAB): *m/z* (%) = 393 (MH^+, 58), 154 (100), 137 (82). IR (KBr): $\nu = 3442$, 1603, 1528, 1500, 1364, 1315, 1226, 1172, 1074, 999, 886, 843, 747, 673 cm^{-1}. Anal. ($C_{18}H_{20}N_2O_6S$) C, H, N.

Synthesis of Compounds 12a and 12b by Mitsunobu Reaction (Method A). A solution of alcohol **10a** or **10b** (6.12 g, 25.7 mmol), 4-cyanophenol (3.07 g, 25.7 mmol), and triphenylphosphine (8.76 g, 33.4 mmol) in anhydrous tetrahydrofuran (100 mL) was cooled on an ice bath under argon. Diisopropyl azodicarboxylate (6.75 g, 33.4 mmol) dissolved in 30 mL of anhydrous tetrahydrofuran was added, and the solution was stirred for 30 min at 0 °C and then refluxed for 48 h. The solvent was removed under reduced pressure, and the residue was triturated with boiling petroleum ether (150 mL) and finally recrystallized from petroleum ether/ethyl acetate.

4-[(2,4-Dimethyl-7-nitro-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl)-methoxy]benzonitrile (12a). Red crystals; yield, 7.31 g (83.9%), mp 155−156 °C. ^{1}H NMR ($CDCl_3$): $\delta = 1.51$ (s, 3H, 2-CH_3), 3.08 (s, 3H, *N*-CH_3), 3.27 (d, 1H, $^2J = 12.1$ Hz, 3-H), 3.52 (d, 1H, $^2J = 12.1$ Hz, 3-H), 3.96 (d, 1H, $^2J = 9.1$ Hz, CH_2O), 4.05 (d, 1H, $^2J = 9.1$ Hz, CH_2O), 6.65 (d, 1H, $^3J = 9.0$ Hz, Ar−H^5), 6.98 (d, 2H, $^3J = 9.0$ Hz, Ar−$H^{2'}$,$H^{6'}$), 7.62 (d, 2H, $^3J = 9.0$ Hz, Ar−$H^{3'}$,$H^{5'}$), 7.72 (d, 1H, $^4J = 2.6$ Hz, Ar−H^8), 7.86 (dd, 1H, $^3J = 9.0$ Hz, $^4J = 2.6$ Hz, Ar−H^6) ppm. ^{13}C NMR ($CDCl_3$): $\delta = 161.50$ (C-1′), 140.90, 140.60 (C-4a, C-8a), 138.49 (C-7), 134.03 (C-3′, C-5′), 118.90 (C-6), 118.84 (CN), 115.31 (C-2′, C-6′), 112.16 (C-8), 109.78 (C-5), 104.87 (C-4′), 73.86 (C-2), 70.50 (2-CH_2), 53.76 (C-3), 38.55 (*N*-CH_3), 21.46 (2-CH_3) ppm. MS (EI): *m/z* (%) = 340 (MH^+, 56), 154 (100), 136 (82), 55 (87). IR (KBr): $\nu = 3468$, 2219, 1604, 1534, 1468, 1254, 1174, 1031, 838, 744 cm^{-1}. Anal. ($C_{18}H_{17}N_3O_4$) C, H, N.

From Tosylate 11a (Method B). A suspension of tosylate **11a** (1.18 g, 3.0 mmol) and sodium 4-cyanophenolate (0.445 g, 3.15 mmol) in anhydrous *N*,*N*-dimethylformamide (40 mL) was heated for 24 h at 80 °C. The solvent was removed under reduced pressure, and the residue was dissolved in ethyl acetate (400 mL) and washed successively with 10% citric acid (3 × 75 mL), 1 M NaOH (2 × 75 mL) and saturated NaCl solution (1 × 75 mL). The organic phase was dried over Na_2SO_4, filtered, and the solvent was evaporated under reduced pressure. The residue was recrystallized from methanol to give 0.85 g (84% yield) of **4a** as orange crystals. The product was identical to that obtained by method A.

Ethyl 2-(Benzyl{2-[(4-cyanophenoxy)methyl]-2,4-dimethyl-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl}amino)acetate (16a). 16a was synthesized from **14a** (1.14 g, 2.87 mmol) according to the general procedure for alkylation under phase-transfer catalysis. Red oil; yield, 0.69 g (49%). ^{1}H NMR ($CDCl_3$): $\delta = 1.27$ (t, 3H, $^3J = 7.2$ Hz, CH_2−$\underline{CH_3}$), 1.48 (s, 3H, 2-CH_3), 2.79 (s, 3H, *N*-CH_3), 2.91 (d, 1H, $^2J = 11.4$ Hz, 3-H), 3.18 (d, 1H, $^2J = 11.7$ Hz, 3-H), 3.93 (d, 1H, $^2J = 9.0$ Hz, CH_2O), 3.99 (s, 2H. *N*-CH_2−COO), 4.14−4.24 (m, CH_2O, 3-H, $\underline{CH_2}$−CH_3), 4.58 (s, 2H, Ph−$\underline{CH_2}$), 6.28−6.31 (m, 2H, Ar−H^6, Ar−H^8), 6.61 (d, 1H, $^3J = 8.1$ Hz, Ar−H^5), 6.97 (d, 2H, $^3J = 9.0$ Hz, Ar−$H^{2'}$,$H^{6'}$), 7.24−7.34 (m, 5H, Ph), 7.58 (d, 2H, $^3J = 9.0$ Hz, Ar−$H^{3'}$,$H^{5'}$) ppm. MS (ESI): *m/z* (%) = 486 (MH^+, 45), 485 (M^+, 100). IR (KBr): $\nu = 2979$, 2359, 2340, 2223, 1743, 1628, 1604, 1572, 1519, 1452, 1371, 1301, 1256, 1172, 1113, 1092, 1029 cm^{-1}. Anal. ($C_{29}H_{31}N_3O_4$) C, H, N.

4-{2-[(4-Cyanophenoxy)methyl]-2,4-dimethyl-3,4-dihydro-2*H*-1,4-benzoxazin-7-ylamino}-4-oxobutanoic Acid (16l). A solution of amine **13a** (0.309 g, 1.00 mmol) and succinic anhydride (1.00 g, 10.0 mmol) in anhydrous tetrahydrofuran was heated for 2 h at 60 °C. The solvent was removed under reduced pressure to give 4.09 g (100%) of a yellow oil. ^{1}H NMR (DMSO-d_6: $\delta = 1.38$ (s, 3H, 2-CH_3), 2.50 (4H, CO$\underline{CH_2CH_2}$CONH, signal overlapped with DMSO), 2.80 (s, 3H, *N*-CH_3), 2.99 (d, 1H, $^2J = 11.6$ Hz, 3-H), 3.22 (d, 1H, $^2J = 11.6$ Hz, 3-H), 4.07 (d, 1H, $^3J = 10.2$ Hz, CH_2O), 4.15 (d, 1H, $^3J = 10.2$ Hz, CH_2O), 6.68 (d, 1H, $^3J = 8.7$ Hz, Ar−H^5), 6.97 (dd, 1H, $^3J = 8.7$ Hz, $^4J = 2.3$ Hz, Ar−H^6), 7.07 (d, 1H, $^3J = 2.3$ Hz, Ar−H^8), 7.16 (d, 2H, $^3J = 8.9$ Hz, Ar−$H^{2'}$,$H^{6'}$), 7.75 (d, 2H, $^3J = 8.9$ Hz, Ar−$H^{3'}$,$H^{5'}$), 9.16 (s, 1H, CONH), 12.07 (br s, 1H, COOH) ppm. MS (EI): *m/z* (%) = 409 (M^+, 7), 323 (6), 309 (100). IR (NaCl): $\nu = 3328$, 2225, 1712, 1605, 1510, 1257, 1173, 835 cm^{-1}. Anal. ($C_{22}H_{23}N_3O_5$) C, H, N. HRMS.

Ethyl 4-(Benzyl{2-[(4-carbamimidoylphenoxy)methyl]-2,4-dimethyl-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl}amino)-4-oxobutanoate Acetate (17e). 17e was synthesized from **16e** according to the general procedure for preparation of amidines from nitriles (Pinner reaction). Brown powder; yield, 276 mg (37%); mp 125−127 °C. ^{1}H NMR (DMSO-d_6): $\delta = 1.17$ (t, 3H, $^3J = 7.1$ Hz, CH_2−CH_3), 1.37 (s, 3H, 2-CH_3), 1.89 (s, 3H, $\underline{CH_3}$COOH), 2.32 (t, 2H, $^2J = 6.1$ Hz, CO$\underline{CH_2}$CONH), 2.50 (CO$\underline{CH_2CH_2}$CONH, overlapped with DMSO-d_5), 2.85 (s, 3H, *N*-CH_3), 3.08 (d, 1H, $^2J = 11.8$ Hz, 3-H), 3.29 (d, 1H, $^2J = 11.8$ Hz, 3-H), 4.03 (q, 2H, $^3J = 7.1$ Hz, $\underline{CH_2}$−CH_3), 4.08 (d, 1H, $^2J = 10.2$ Hz, CH_2O), 4.16 (d, 1H, $^2J = 10.2$ Hz, CH_2O), 4.76 (s, 2H, Ph−$\underline{CH_2}$), 6.56 (d, 1H, $^4J = 2.3$ Hz, Ar−H^8), 6.60 (dd, 1H, $^3J = 8.4$ Hz, $^4J = 2.3$ Hz, Ar−H^6), 6.70 (d, 1H, $^3J = 8.4$ Hz, Ar−H^5), 7.18 (d, 2H, $^3J = 8.7$ Hz, Ar−$H^{2'}$,$H^{6'}$), 7.20−7.29 (m, 5H, Ph), 7.84 (d, 2H, $^3J = 8.7$ Hz, Ar−$H^{3'}$,$H^{5'}$), 9.15 (br s, 4H, amidino-H) ppm. MS (EI): *m/z* (%) = 544 (M^+, 15), 527 (100), 399 (51), 309 (35), 91 (42). IR (KBr): $\nu = 3063$, 1731, 1661, 1608, 1518, 1265, 1178, 1036 cm^{-1}. HPLC: 100.0%, $t_R = 21.44$ min. Anal. ($C_{31}H_{36}N_4O_5 \cdot CH_3CO-OH \cdot H_2O$) C, H, N.

2-(Benzyl{2-[(4-carbamimidoylphenoxy)methyl]-2,4-dimethyl-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl}amino)-2-oxoacetic Acid (18c). 18c was synthesized from **17c** according to the general procedure for alkaline hydrolysis of alkyl esters. Green crystals; yield, 75 mg

(24%); mp 227−228 °C. ^{1}H NMR (DMSO-d_6): δ = 1.34 (s, 3H, 2-CH_3), 2.82 (s, 3H, *N*-CH_3), 3.06 (d, 1H, ^2J = 11.8 Hz, 3-H), 3.27 (d, 1H, ^2J = 11.8 Hz, 3-H), 4.08 (d, 1H, ^2J = 10.2 Hz, CH_2O), 4.13 (d, 1H, ^2J = 10.2 Hz, CH_2O), 4.81 (s, 2H, Ph−CH_2), 6.55 (d, 1H, ^4J = 2.3 Hz, Ar−H^8), 6.57 (dd, 1H, ^3J = 8.7 Hz, ^4J = 2.3 Hz, Ar−H^6), 6.64 (d, 1H, ^3J = 8.7 Hz, Ar−H^5), 7.17 (d, 2H, ^3J = 8.9 Hz, Ar−$H^{2'}$,$H^{6'}$), 7.18−7.31 (m, 5H. Ph), 7.81 (d, 2H, ^3J = 8.9 Hz, Ar−$H^{3'}$,$H^{5'}$), 8.84 (br s, 2H, amidino-H), 9.16 (br s, 2H, amidino-H) ppm. MS (ESI): *m*/*z* (%) = 489 (MH^+, 70), 145.0 (95), 104.0 (100). IR (KBr): ν = 3425, 3000, 1630, 10608, 1521, 1487, 1457, 1264, 1179, 1179, 1043 cm^{-1}. HPLC: 100%, t_R = 18.43 min. Anal. ($C_{27}H_{29}N_4O_5$) C, H, N. HRMS (MH^+).

Docking Studies. Autodock 3.05[30] was used to predict the ligand-binding mode in the thrombin active site and GPIIb/IIIa receptor binding site. The energetically most favorable conformation was found for each stereoisomer with HyperChem, using the semiempirical method MNDO.[29] All amide bonds had trans configuration and were marked as nonrotatable. Compounds were protonated on the basic center (guanidine, amidino, or amino group) and were assigned with a negative charge on the acidic center (β carboxylic group of aspartate or its bioisoster). For docking calculations, the crystal structures of thrombin, with a resolution of 2.4 Å (PDB code 1KTS),[14] and $\alpha_{IIb}\beta_3$ receptor, with a resolution of 2.9 Å (PDB code 1TY5),[20] were used. The PDB crystal structure was prepared for docking by removing water molecules and ligands. In the case of 1TY5, the magnesium ion in the active site was retained and manually assigned charge of +2 and solvation parameters. The ligands were docked into a restricted box centered in the active site (22 Å × 22 Å × 22 Å). For the global search using the LGA (Lamarckian genetic algorithm), the size of the initial random population was 250 individuals, the maximal number of energy evaluations 1.25×10^6, the maximal number of generations 500, the number of top individuals that survived into the next generation, the elitism, 1, the probability that a gene would undergo a random change 0.02, and the crossover probability 0.80, and the average of the worst energy was calculated over a window of 10 generations. For a purely local search, the pseudo Solis and Wets method was used, whereas the Solis and Wets method was used for the LGA part of the local search. The parameters used for local search in both cases were a maximum of 1000 iterations per local search, the probability of performing a local search on an individual was 1.0, the maximal number of consecutive successes or failures before doubling or halving the step size of the local search was 4, and the lower bound on the step size, 0.01, was the termination criterion for the local search. A total of 250 dockings were performed, and ligands with rmsd less than 1 were joined in clusters. The ligand with the lowest docked energy was chosen for interpreting the docking results.

Enzyme Assay for Inhibition of Serine Proteases. The enzyme amidolytic method for determining inhibition was based on the spectrophotometric determination of absorbance of the product formed after amide bond cleavage of a chromogenic substrate in the presence of the enzyme. K_i, which is a quantitative measure of inhibitor potency, was determined from the kinetics of substrate hydrolysis with and without the addition of the inhibitor.[39] Measurements (spectrophotometer, Tecan, Sunrise) were performed in microtiter plates with a final volume of 200 μL. Thrombin was tested at a final concentration of 0.5 NIH E/mL with the substrate S-2238 (Chromogenix) at 20 and 40 μM final concentration, and factor Xa at final concentration of 1 mBAEE E/mL with the substrate S-2222 (Chromogenix) at 100 and 200 μM final concentrations. Trypsin was assayed at a final concentration of 0.5 nkat/mL using the substrate S-2222 (Chromogenix) at 50 and 100 μM final concentrations. Inhibitors were dissolved in DMSO (concentration of stock solutions, 10 mmol/L) and diluted with distilled water to concentrations from 0.5 to 100 μM. Reaction rates were measured in the presence and absence of the inhibitor. Then 50 μM HBSA buffer, 50 μM solution of each inhibitor concentration (or of HBSA buffer in case of measurement without inhibitor), and 50 μM of enzyme solution were pipetted into the microtiter wells. The plate was incubated for 15 min at 25 °C and 50 μL of chromogenic substrate then added. Absorbance at 405 nm at 25 °C was measured every 10 s. Measurements were carried out in triplicate with three concentrations of the inhibitor and two concentrations of the substrate. For every combination of concentrations K_i was calculated from the change of absorbance in the initial, linear part of the curve according to the method of Cheng and Prusoff[39] and the final result given as their average value. Argatroban (thrombin, K_i = 12 ± 2 nM) was used as control.

Inhibition of in Vitro Binding of Fibrinogen to Isolated GPIIb/IIIa Receptor. Binding affinities to GPIIb/IIIa receptor were measured by a solid-phase competitive displacement assay.[40] Human fibrinogen (100 mg) was dissolved in aqueous NaCl (0.3 M, 5 mL) at 30 °C and then diluted with 0.1 M $NaHCO_3$ in water to a final concentration of 1 mg/mL. Biotin *N*-hydroxysuccinimide ester (2 mg) was dissolved in DMF (2 mL) and added to 6 mL of fibrinogen solution. The reaction mixture was incubated for 90 min at 30 °C and dialyzed for 3 h at room temperature against buffer 1 (3 L, 20 mM Tris, 150 mM NaCl, pH 7.4). After dialysis, the solution was centrifuged for 5 min at 5400 rpm and Tween-20 (0.005%) added to give the stock solution. Human integrin (10 μL of GPIIb/IIIa receptor solution (Calbiochem)) was diluted in 10.2 mL of buffer 2 (20 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, pH 7.4) and adsorbed to 96-well (100 μL/well) high-binding microtiter plates (Greiner, Lumitrac 600) overnight at 4 °C. Nonspecific receptor-binding sites were blocked with 1% BSA in buffer 2 (200 μL/well). Following incubation for 1 h at room temperature, the plates were washed twice with buffer 3 (buffer 2 containing 0.1% Tween-20). The potential antagonists were serially diluted with buffer and test solutions added (50 μL/well) together with biotinylated fibrinogen (50 μL/well, 1:10 dilution of stock solution in buffer 2) to each well. The plates were incubated for 2 h at room temperature, then washed twice with buffer 3. Peroxidase-conjugated antibiotin goat antibody (100 μL/well of a 1:1000 dilution of purchased solution (Calbiochem) in buffer 3 containing 0.1% of BSA) was added to each well and incubated for another hour. The microtiter plates were washed three times with buffer 3. Finally, chemiluminescent substrate (POD, Roche Diagnostics, Boehringer Mannheim) (50 μL/well) was added and the luminescence measured with a GENios (Tecan Group AG) multimode research reader three times over 10 min. Positive controls received no inhibitors, while negative controls received no ligands. RGDS peptide with IC_{50} of 1.3 ± 0.06 μM was used as the internal standard. Assays were performed in triplicate at least. The mean experimental data were fitted to the sigmoid model and IC_{50} values calculated from the dose−response curve (OriginPro, OriginLab, version 7.5).

Inhibition of in Vitro Human Platelet Aggregation in Platelet Rich Plasma. Healthy male donors who had not taken any antiplatelet drugs for at least 2 weeks were fasted for 8 h prior to withdrawing of blood; then 10 mL of whole blood was collected using a butterfly needle and a 10 mL plastic syringe containing 1 mL of 0.129 M buffered sodium citrate (3.8%). Platelet-rich plasma (PRP) was prepared by centrifugation at 1000*g* for 15 min at room temperature, allowing the centrifuge to coast to a stop without braking. Platelet poor plasma (PPP) was prepared by centrifuging the remaining blood at 2000*g* for 10 min at room temperature, allowing the centrifuge to coast to a stop without braking. The PRP was adjusted with PPP to a count of (250 ± 25) × 10^6 mL. Then 15 μL of tested compounds was added to 135 μL of PRP. After 5 min of incubation of 15 μL of adenosine 5′-diphosphate (ADP) (11 μM final concentration) was added to the cuvettes and aggregation was monitored by measuring optical density for 10 min on the Behring coagulation timer (BCT, Dade Behring, Marburg, Germany). The entire procedure was run within 2 h, since this is the maximal viability of the platelets. Saline, in place of test compound, was used to determine the maximal aggregation.

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Supporting Information Available: Synthetic procedures and compound characterization, microanalysis data of synthesized compounds, HRMS results, and double-reciprocal plot. This material is available free of charge via the Internet at http://pubs.acs.org.

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