Structure-Based Design of Novel Potent Nonpeptide Thrombin Inhibitors

Norbert H. Hauel,* Herbert Nar, Henning Priepke, Uwe Ries, Jean-Marie Stassen, and Wolfgang Wienen

Boehringer Ingelheim Pharma KG, Research Division, Birkendorfer Strasse 65, D-88397 Biberach/RISS, Germany

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The clinical syndromes of thromboembolism are evoked by an excessive stimulation of the coagulation cascade. In this context, the serine protease thrombin plays a key role. Considerable efforts have therefore been devoted to the discovery of safe, orally active inhibitors of this enzyme. On the basis of the X-ray crystal structure of the peptidelike thrombin inhibitor NAPAP complexed with bovine thrombin, we have designed a new structural class of nonpeptidic inhibitors employing a 1,2,5-trisubstituted benzimidazole as the central scaffold. Supported by a series of X-ray structure analyses, we optimized the activity of these compounds. Thrombin inhibition in the lower nanomolar range could be achieved although the binding energy mainly results from nonpolar, hydrophobic interactions. To improve in vivo potency, we increased the overall hydrophilicity of the molecules by introducing carboxylate groups. The very polar compound **24** (BIBR 953) exhibited the most favorable activity profile in vivo. This zwitterionic molecule was converted into the double-prodrug **31** (BIBR 1048), which showed strong oral activity in different animal species. On the basis of these results, **31** was chosen for clinical development.

Introduction

Thromboembolic disorders are the major cause of mortality and morbidity in Western societies. Closely linked to the clinical syndromes of thromboembolism is an excessive stimulation of the coagulation cascade.[1] This activation involves two crucial steps that together are referred to as the common coagulation pathway: (i) the generation of thrombin via the prothrombinase complex, consisting of factor Va, Xa, and phospholipids and (ii) the proteolytic cleavage of fibrinogen by thrombin, giving rise to the insoluble clot matrix, which consists of fibrin. Thrombin in addition induces platelet activation and also triggers a wide range of effects secondary to thrombosis, e.g., vascular smooth muscle cell and fibroblast proliferation, monocyte chemotaxis, and neutrophil adhesion.

Current anticoagulant therapy involves heparin or low molecular weight heparins and, for oral treatment, coumadin.[2] However, the clinical use of these agents has several important drawbacks. Heparins must be administered parenterally, and the use of coumadin needs frequent monitoring. On the basis of its biological mode of action, it displays a slow onset of action and its efficacy is dependent on the patient's nutritional status.

Recently, the direct thrombin inhibitor hirudin was tested for the prevention of deep venous thrombosis after major orthopedic surgery, where superiority over heparin and low molecular weight heparin could be demonstrated.[3] However, because of its peptidic nature, this compound must be given parenterally, providing a major obstacle for the subacute and chronic treatment of thromboembolic diseases. Therefore, research efforts were aimed at the identification of synthetic, orally active, direct thrombin inhibitors.

* To whom correspondence should be addressed. Tel.: +49/7351/54-4306. Fax: +49/7351/54-5181. E-mail: norbert.hauel@bc.boehringer-ingelheim.com.

In this field, considerable progress has been made during the past decade.[4] The early work focused on structures based on the classical tripeptide motif D-Phe-Pro-Arg,[5] whereas during recent years nonpeptide inhibitors were designed as a strategy to improve pharmacokinetic properties of the drug molecules.[6] In this context, compounds were synthesized that incorporate a bicyclic lactam,[7] a pyridone,[8] or a pyrazinone ring[9] as a P2 surrogate within the tripeptide scaffold. Others have reported inhibitors with a piperidine,[10] a 1,4-disubstituted phenylene,[11] a 1,3-disubstituted phenylene,[12] or a 1,3-disubstituted toluene ring[13] as the core structure.

The starting point of our search for noncovalent, nonpeptide thrombin inhibitors was the X-ray crystal structure of the bovine thrombin complex formed with the peptidelike, benzamidine-based inhibitor NAPAP[14] (**1**), which shows the conformation of the enzyme-bound inhibitor and its interaction with residues of the active site cleft. The amidine group forms a bidentate salt bridge with the carboxylate of Asp189. Hydrophobic interactions are formed by the piperidine ring and the naphthyl moiety with the proximal (P) and the distal (D) pocket of the thrombin active site, respectively. Additionally, the bridging glycine moiety of NAPAP, in analogy to peptidic serine protease substrates, forms the canonical hydrogen-bonding pattern with residues Trp215 and Gly216 at the rim of the specificity (S1) pocket.

On the basis of this information, we designed a number of cyclic scaffolds as surrogates for the central glycine in the NAPAP molecule and, as we did so, neglected the hydrogen bonds that are formed by the glycine amino and carbonyl groups with the enzyme. One of the most interesting scaffolds we found was the trisubstituted benzimidazole as exemplified in compound **2** (Chart 1).

We investigated the exact binding mode of this inhibitor by determining a crystal structure of a complex

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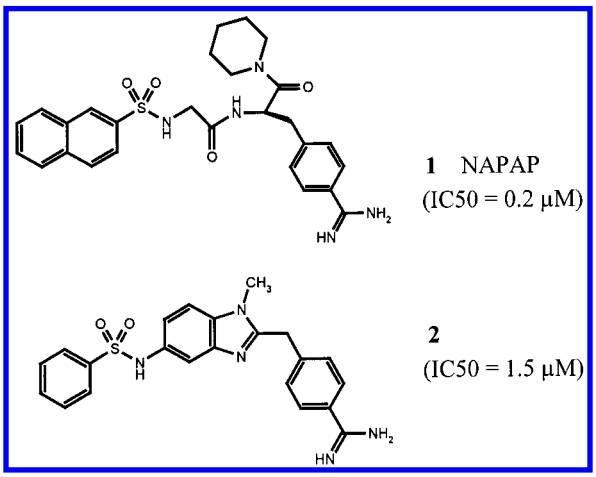
Chart 1



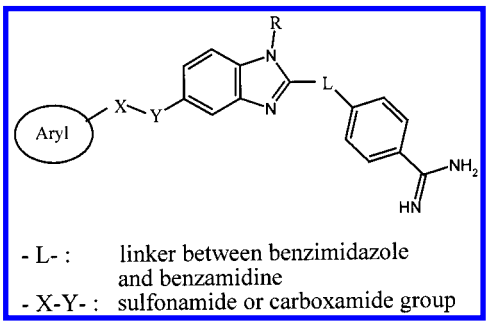
with human α-thrombin. As can be seen from Figure 1, the benzimidazole appears to be a most favorable template to place the three essential substituents into the right positions.

Compound **2** interacts via a salt bridge with Asp189 in the same way as NAPAP. The terminal phenyl ring is bound by a hydrophobic interaction in the D-pocket, and the N-methyl group nicely fits into the P-pocket. As was expected, there is no additional hydrogen bond between the inhibitor and the enzyme, which in part may explain why in our test system compound **2** was about 1 order of magnitude less active (IC_{50} = 1.5 μM) than NAPAP (IC_{50} = 0.2 μM).

When given intravenously to anaesthetized rats, compound **2** showed a considerably longer half-life than NAPAP (data not shown); it was, however, not well-tolerated. Even the dose of 1 mg/kg i.v. produced strong cardiovascular side effects in terms of reduced blood pressure and heart rate.[15]

Despite these shortcomings, compound **2** served as an important lead structure for us. Our further optimization efforts were supported by a number of X-ray analyses of inhibitor−thrombin complexes. We synthesized a series of analogues of compound **2** according to the general formula shown in Chart 2. Refinement of this compound in order to increase its inhibitory potency, its tolerability, and its pharmacokinetic properties was the goal of this study. In this paper, we describe our progress toward this goal.

Chart 2



Synthetic Chemistry

The sulfonamides **2**−**6** of Table 1 were synthesized as shown in Scheme 1. The benzimidazole intermediates **25a**−**d** were obtained by acylation of the respective phenylenediamine starting materials with 4-cyanophenylacetic acid followed by ring closure in boiling concentrated acetic acid. Reduction of the nitro groups and subsequent sulfonation with benzenesulfonyl chloride gave intermediates **26a**−**d**, which were converted into the benzamidines **2**−**5** by the Pinner reaction. Compound **6** was prepared by methylation of the sulfonylamide nitrogen of **26b** prior to the Pinner reaction.

Compounds **7**−**13** were synthesized in an analogous procedure by first sulfonating intermediate **25b** with the respective arylsulfonyl chlorides and second converting the nitriles into the benzamidines (Scheme 2). N-Alkylation of **27** with ethyl bromoacetate and ethyl 4-bromobutyrate followed by Pinner reactions and finally by hydrolyses of the ester groups afforded the zwitterionic thrombin inhibitors **14** and **15**.

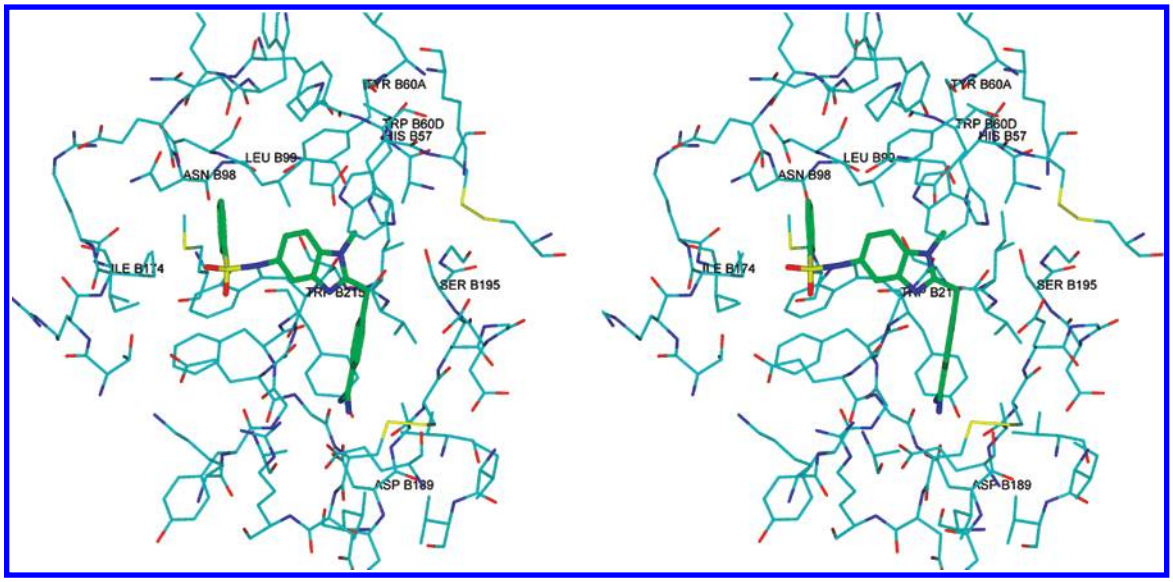
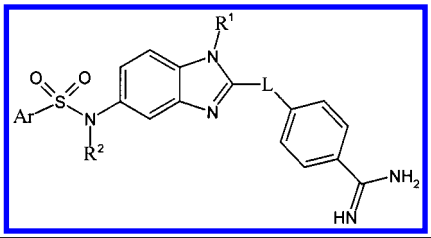


Figure 1. Stereo representation of the X-ray analysis of compound **2** (green carbon atoms) in complex with thrombin (blue carbon atoms). The interactions of the ligand with the protein are mainly hydrophobic in character with the N-methyl group occupying the P-pocket (residues Trp 60D, His 57, Tyr 60B, and Leu 99) and the phenyl sulfone occupying the D-pocket above residue Trp 215. The benzamidine moiety binds to Asp 189 in the specificity pocket via a bidentate salt bridge interaction. The bridging benzimidazole scaffold does not form any polar interactions with the protein.

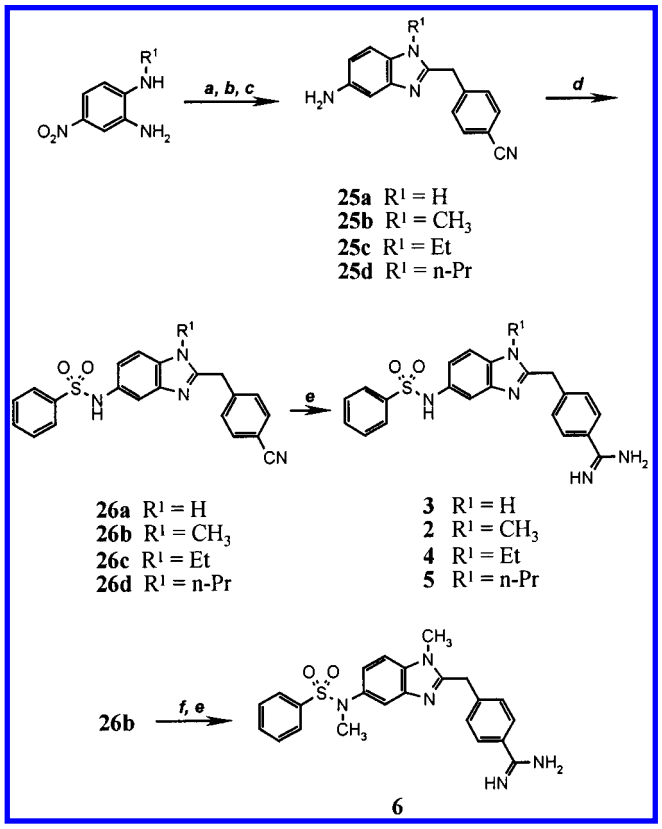
Table 1. Structural Variations of Lead Compound **2**



compd	L	R^1	R^2	Ar	IC_{50} (μM)[a]
2	$-CH_2-$	$-CH_3$	$-H$	phenyl	1.5
3	$-CH_2-$	$-H$	$-H$	phenyl	19
4	$-CH_2-$	$-$ethyl	$-H$	phenyl	2.4
5	$-CH_2-$	$-n$-propyl	$-H$	phenyl	4.0
6	$-CH_2-$	$-CH_3$	$-CH_3$	phenyl	1.3
7	$-CH_2-$	$-CH_3$	$-H$	3-pyridyl	1.6
8	$-CH_2-$	$-CH_3$	$-H$	2,5-dimethoxyphenyl	1.8
9	$-CH_2-$	$-CH_3$	$-H$	3,5-bis-trifluoromethyl-phenyl	>100
10	$-CH_2-$	$-CH_3$	$-H$	1-naphthyl	0.6
11	$-CH_2-$	$-CH_3$	$-H$	2-naphthyl	24
12	$-CH_2-$	$-CH_3$	$-H$	5-isoquinolyl	0.51
13	$-CH_2-$	$-CH_3$	$-H$	8-quinolyl	0.26
14	$-CH_2-$	$-CH_3$	$-CH_2-COOH$	8-quinolyl	0.12
15	$-CH_2-$	$-CH_3$	$-(CH_2)_3-COOH$	8-quinolyl	0.13
16	$-CH_2-CH_2-$	$-CH_3$	$-CH_2-COOH$	8-quinolyl	0.032
17	$-CH_2-O-$	$-CH_3$	$-CH_2-COOH$	8-quinolyl	0.058
18	$-CH_2-NH-$	$-CH_3$	$-CH_2-COOH$	8-quinolyl	0.011

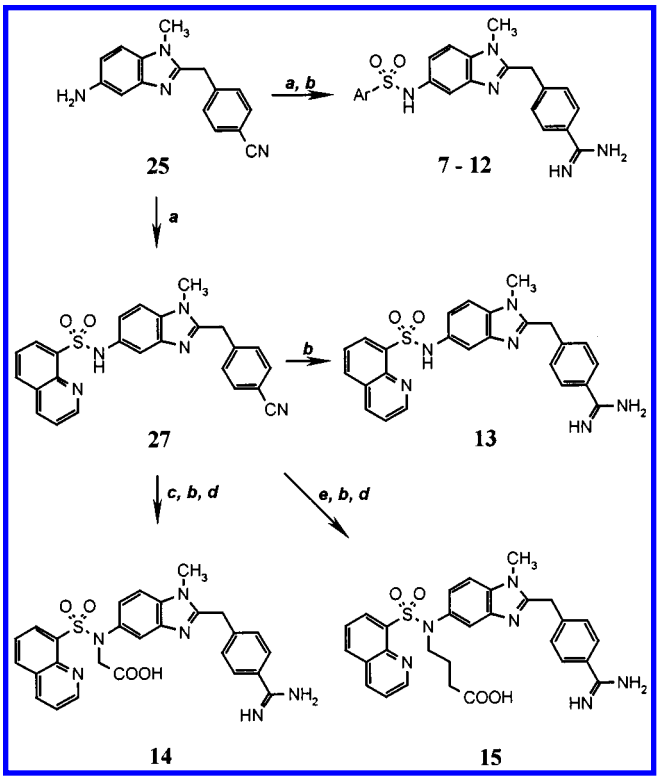
[a] IC_{50} for inhibition of human thrombin. For details, see Experimental Section.

Scheme 1[a]



[a] (a) (4-Cyano-phenyl)acetic acid, CDI, THF, 50 °C; (b) acetic acid, reflux; (c) H_2, 10% Pd/C, methanol/CH_2Cl_2; (d) benzenesulfonyl chloride, pyridine, room temperature; (e) HCl (gas), ethanol, 0 °C, $(NH_4)_2CO_3$/ethanol, room temperature; (f) iodomethane, K_2CO_3, acetone, room temperature.

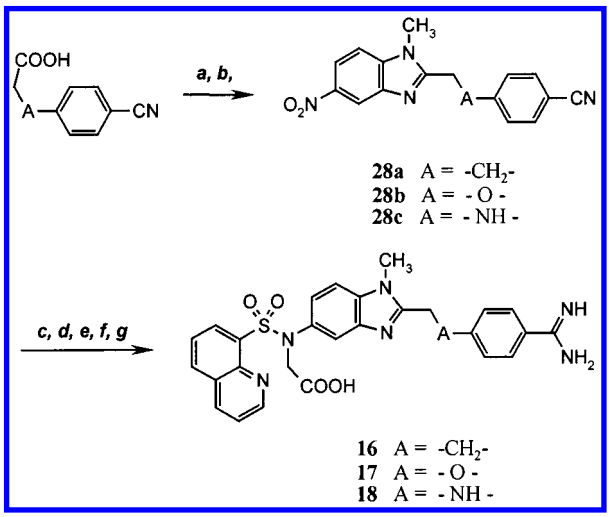
Scheme 2[a]



[a] (a) Arylsulfonyl chloride, pyridine, room temperature; (b) HCl (gas), ethanol, 0 °C, $(NH_4)_2CO_3$/ethanol, room temperature; (c) ethyl bromo-acetate, K_2CO_3, DMSO, room temperature; (d) NaOH, H_2O/ethanol, room temperature; (e) ethyl 4-bromo-butyrate, K_2CO_3, DMSO, room temperature.

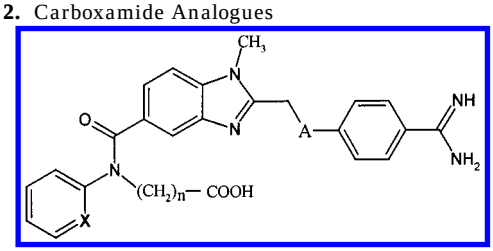
The same kind of chemistry was applied for the preparation of sulfonamides **16**−**18** as illustrated in Scheme 3. Acylation of 3-amino-4-methylamino-nitrobenzene with 4-cyanophenylpropionic acid, 4-cyanophenoxyacetic acid, and 4-cyanophenylglycine and subsequent ring closure gave the benzimidazole intermediates **28a**−**c**, which were converted in five steps into the zwitterionic molecules **16**−**18** in the same way as described for compound **14**.

Scheme 3[a]



[a] (a) CDI, N^1-methyl-4-nitro-benzene-1,2-diamine, THF, 50 °C; (b) acetic acid, reflux; (c) H_2, 10% Pd/C, methanol/CH_2Cl_2, room temperature; (d) quinoline-8-sulfonyl chloride, pyridine, room temperature; (e) ethyl bromo-acetate, K_2CO_3, acetone, reflux; (f) HCl (gas), ethanol, 0 °C, $(NH_4)_2CO_3$/ethanol, room temperature; (g) NaOH, H_2O/ethanol, room temperature.

Table 2. Carboxamide Analogues



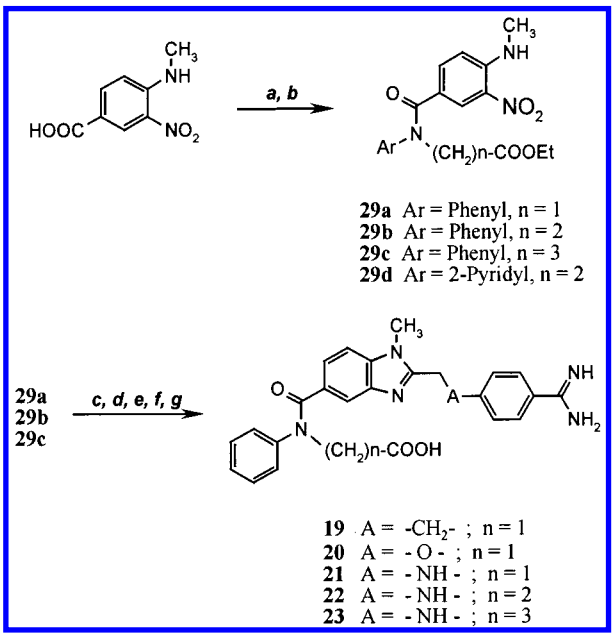
compd	A	*n*	X	IC_{50} (μM)[a]
19	−CH_2−	1	CH	0.054
20	−O−	1	CH	0.33
21	−NH−	1	CH	0.010
22	−NH−	2	CH	0.0054
23	−NH−	3	CH	0.010
24	−NH−	2	N	0.0093

[a] IC_{50} for inhibition of human thrombin. For details, see Experimental Section.

Table 2 summarizes those enzyme inhibitors that employ a carboxamide group (instead of a sulfonamide group) as a linker between the central template and the terminal aryl moiety. The synthetic pathway is depicted in Scheme 4. 4-Methylamino-3-nitrobenzoic acid was condensed with a variety of arylamino derivatives to yield intermediates **29a**−**d**. After the nitro groups were reduced, the resulting phenylene diamines were converted into benzimidazoles in two reaction steps as already described. Pinner reaction and ester hydrolysis finally gave the test compounds **19**−**23**.

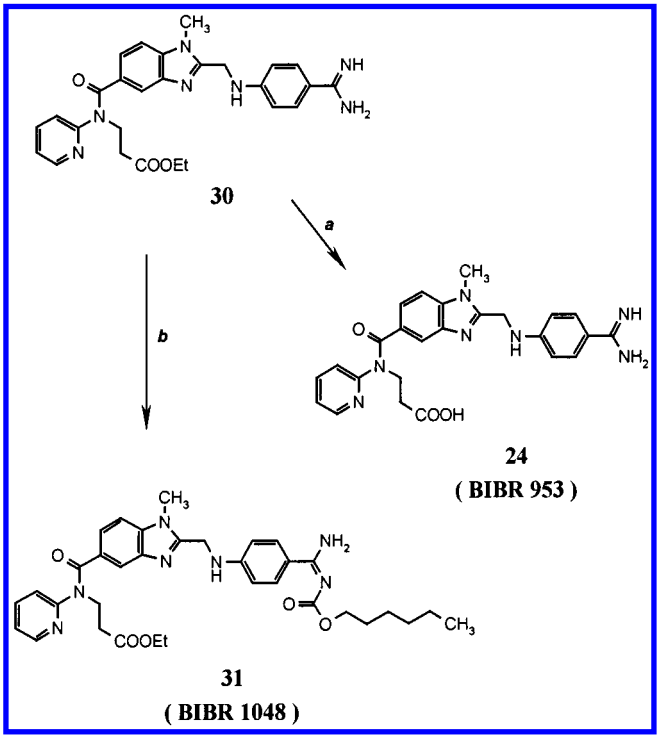
Scheme 5 illustrates the synthetic route for inhibitor **24**, a pyrido analogue of **22**. The nitro group of intermediate **29d** was reduced, the resulting phenylenediamine derivative was acylated with 4-cyanophenylglycine, ring closure was performed in hot concentrated acetic acid, and the nitrile was transformed into the benzamidine **30**. This intermediate was converted into compound **24** by alkaline hydrolysis. Reaction of **30** with *n*-hexyl chloroformate in acetone/water yielded the O-*n*-hexylcarbamate derivative **31**.

Scheme 4[a]



[a] (a) $SOCl_2$/DMF, reflux; (b) arylamino-alkanoic acid ethyl ester, THF, triethylamine, room temperature; (c) H_2, 10% Pd/C, methanol/CH_2Cl_2, room temperature; (d) 3-(4-cyanophenyl)propionic acid or 4-cyanophenoxy-acetic acid or 4-cyanophenyl-glycine, CDI, THF, 50 °C; (e) acetic acid, reflux; (f) HCl (gas), ethanol, 0 °C, $(NH_4)_2CO_3$/ethanol, room temperature; (g) NaOH, H_2O/ethanol, room temperature.

Scheme 5[a]



[a] (a) NaOH, H_2O/ethanol, room temperature; (b) *n*-hexyl chloroformate, K_2CO_3, THF/H_2O, room temperature.

Discussion

Starting from lead structure **2**, we synthesized analogues **3**−**5** (Table 1) with substituents R^1 of different lipophilicity at the benzimidazole N-1, to find out their relative contribution to the binding energy and to identify the optimum alkyl chain length. Compound **3**

(R^1 = H) turned out to be 1 order of magnitude less active than **2**, which clearly demonstrates the importance of the lipophilic/hydrophobic interaction of the methyl group of **2** with the lipophilic P-pocket formed by the residues His57, Tyr60A, Leu99, and Trp60D of thrombin. This finding was not a surprise because from the X-ray structure (Figure 1) we already had information about the localization of the methyl group in the enzyme active site. We did not, however, expect that an increase in alkyl chain length (compounds **4** and **5**) would result in a decrease in activity. We had hoped, instead, that a larger buried hydrophobic surface area and therefore a stronger van der Waals interaction would result in stronger ligand binding. The observed decrease in affinity, albeit very small, may best be explained by a decrease of entropy due to a loss of rotational degrees of freedom during formation of the enzyme−inhibitor complex.[16]

The X-ray structure (Figure 1) clearly shows that the sulfonamide hydrogen of compound **2** does not take part in a hydrogen bond with the protein. The N−H bond is directed out of the binding site into the solvent phase; N-alkylation should therefore have no influence on affinity, as was proven with the N-methyl derivative **6**.

The inhibitor interaction with the thrombin D-pocket, formed by residues Leu99, Ile174, and Trp215, was explored by variations of the terminal aryl substituent Ar. A selection of some derivatives is listed in Table 1 (compounds **7**−**13**). Substitution of the phenyl group by 3-pyridyl (compound **7**) or 2,5-dimethoxyphenyl (compound **8**) did not influence affinity, while the 3,5-bis-trifluoromethyl-phenyl derivative (compound **9**) did not show any activity at a concentration of 100 μM, most probably due to steric hindrance caused by the two trifluoromethyl groups. A small increase in affinity as compared to compound **2** was achieved with the 1-naphthyl residue (compound **10**), whereas activity dropped 40-fold with the 2-naphthyl group (compound **11**), probably caused by a repulsive interaction with Asn98 at the back of the D-pocket. The 5-isoquinolyl and 8-quinolyl groups (compounds **12** and **13**) were as effective as the 1-naphthyl.

As was reported by others with a different class of thrombin inhibitors,[17] the lipophilicity of the molecules influences their inhibitory activity when measured in the presence of blood plasma: the more lipophilic the inhibitor is, the more pronounced the reduction in activity will be. The reason for this phenomenon most probably is plasma protein binding, which reduces the free inhibitor concentration. We therefore planned to introduce polar groups into the molecules in order to increase their hydrophilicity and thereby reduce protein binding and optimize in vivo activity. To this end, we synthesized the zwitterionic compounds **14** and **15** by adding acetic acid and butyric acid, respectively, to the sulfonamide nitrogen. As inferred from the crystal structure of the thrombin−**2** complex, the carboxylate groups are located outside the active site in the solvent phase and do not interfere with inhibitor binding. The inhibitory activity of both compounds was not reduced in the presence of plasma proteins (data not shown).

From X-ray structure analyses of this class of inhibitors in complex with thrombin, we reasoned that elongation of the linker L between the benzimidazole template and the benzamidine moiety (Chart 2) by an additional atom should generate molecules that fit equally well into the active site. This was proven by compounds **16**−**18** (Table 1). Much to our surprise, compound **18** even turned out to be 1 order of magnitude more active than **14**. On the basis of X-ray structures, our hypothesis at this point was that the diatomic linker facilitates a better positioning of the benzamidine group in the S1 pocket.

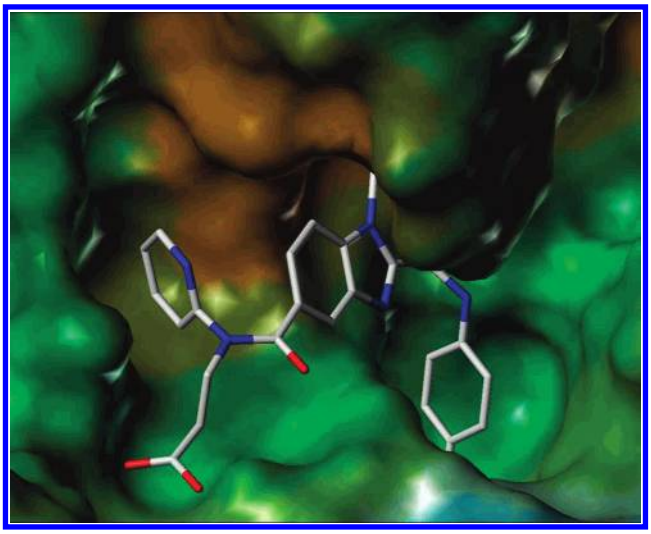


Figure 2. X-ray crystal structure of compound **24** in complex with thrombin in a surface representation. The lipophilic potential is mapped on the protein surface. The ligand interacts in a similar fashion as compound **2** with thrombin residues of the specificity pocket and the P-pocket. The D-pocket is occupied by the pyridine ring, while the propionic acid substituent on the amide nitrogen projects into bulk solvent without forming further interactions with the protein.

In the molecules of Table 1, the position of the terminal aryl residue Ar in relation to the central benzimidazole scaffold is determined by the torsion angles of the sulfonamide spacer between them. Having obtained detailed information about enzyme-bound conformations of a number of inhibitors, we reasoned that by utilizing a carboxamide instead of the sulfonamide spacer the aryl group should also be placed in a favorable position for strong D-pocket interaction. This hypothesis was verified by the carboxamide analogues listed in Table 2. From the three variations of the linker $-CH_2-A-$ (compounds **19**−**21**), again, the amino derivative **21** was the most active, as was the case in the sulfonamide series in Table 1. Compounds **21**−**23** demonstrate that the chain length (up to three methylene groups) between the carboxylate and the amide nitrogen has only a minor influence on the in vitro activity. Finally, **24** shows that the terminal phenyl can be substituted by the more hydrophilic 2-pyridyl group without substantial loss of activity.

The X-ray structure of **24** in complex with thrombin is shown in Figure 2. It demonstrates that this inhibitor and our starting point, compound **2**, share a similar binding mode: The amidine group interacts with Asp189 via a bidentate salt bridge, the central template is bound to thrombin by a hydrophobic interaction with the P-pocket, and the pyridine ring is positioned between Leu99 and Ile174 in the D-pocket. As predicted, there is no interaction between the enzyme and the carboxylate group that is directed out of the binding site into

Table 3. Activity Profile of BIBR 953 (**24**)

human enzymes	K_i (nM)	human enzymes	K_i (nM)
thrombin	4.5 ± 0.2	plasmin	1695 ± 50
factor Xa	3760 ± 20	tPA	45360 ± 10
trypsin	50.3 ± 0.3	activated protein C	20930 ± 10

[a] PTT (human plasma): $ED_2 = 0.23 \pm 0.021$ μM.

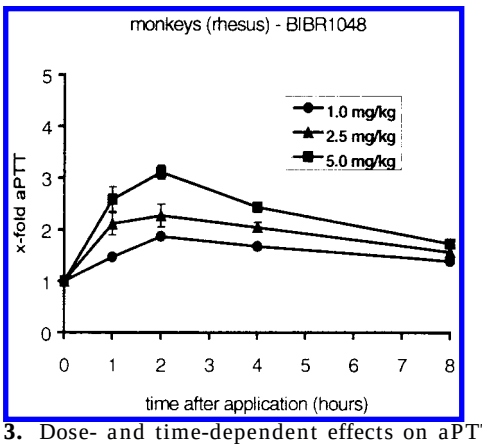


Figure 3. Dose- and time-dependent effects on aPTT after oral administration of compound **31** (BIBR 1048) to rhesus monkeys. Data represent mean ± SEM.

bulk solvent. It is one of the most remarkable characteristics of this inhibitor that it does not make use of the canonical hydrogen-bonding pattern with the enzyme, which is a common feature of almost all high affinity thrombin inhibitors reported so far. Apart from the salt bridge with Asp189, the binding energy of **24** solely results from hydrophobic interactions, which underscores the general importance of this binding force in drug receptor interactions.[18]

Because of its strong in vitro activity and its favorable selectivity profile vs related serine proteases (Table 3), **24** was investigated biologically in depth and turned out to be a very potent anticoagulant in vivo. Among all of the inhibitors of this structural class, it exhibited the strongest activity and the longest duration of action in anaesthetized rats after i.v. administration. Unlike compound **2**, it was well-tolerated in these animals up to the highest given dose of 10 mg/kg. It was, however, not orally active, which is not surprising considering that it is a very polar, permanently charged molecule with a logP of −2.4 (*n*-octanol/buffer, pH 7.4).

In principle, compounds with physicochemical characteristics such as this can be converted into orally active prodrugs by turning the carboxylate into an ester group and by masking the amidinium moiety as a carbamate-ester,[19] provided that both the carboxylate and the amidinium will be restored in vivo by hydrolytic cleavage of the prodrug. Accordingly, we synthesized a series of double-prodrugs of this type as exemplified by **31** (Scheme 5), which is a rather lipophilic compound with a logP value of 2.6 (*n*-octanol/buffer, pH 7.4). Given orally to rhesus monkeys, this prodrug **31** exhibited strong and long-lasting anticoagulant effects as measured by the activated partial thromboplastin time (aPTT) ex vivo (Figure 3). A more detailed description of the biological effects of **24** (BIBR 953) and its prodrug **31** (BIBR 1048) will be published soon.

Conclusion

On the basis of the X-ray crystal structure of NAPAP complexed with bovine thrombin, we designed a new structural class of nonpeptidic inhibitors employing a benzimidazole as the central scaffold. Supported by a series of X-ray structure analyses, we optimized the inhibitory activity of these compounds in a number of iterative steps. During this process, enzyme inhibition in the lower nanomolar range could be achieved although, apart from the salt bridge of the amidinium group with Asp189 of thrombin, the binding energy solely results from hydrophobic interactions. To reduce plasma protein binding, we increased the hydrophilicity of the inhibitors by introducing a carboxylate group at a position of the molecules, where it does not interfere with the thrombin binding site interaction. From this series of inhibitors, **24** (BIBR 953) exhibited the most favorable in vivo activity profile following i.v. administration to rats. Because of its highly polar, zwitterionic nature, oral absorption of **24** was insufficient. Therefore, a number of prodrugs were synthesized, from which **31** (BIBR 1048) exhibited strong and long-lasting anticoagulant effects after oral administration in different animal species. On the basis of these encouraging results, **31** was chosen for development and is currently undergoing phase II clinical trials in patients with thromboembolic disorders.

Experimental Section

Chemistry. Procedures for the preparation of all final products are presented below along with representative procedures for all methods used in the preparation of intermediates. All solvents and reagents were used without purification as acquired from commercial sources.

^{1}H nuclear magnetic resonance (NMR) spectra were recorded at 200 MHz on a Bruker AC200 spectrophotometer in the solvent indicated. Chemical shifts are reported in parts per million relative to tetramethysilane. Melting points were recorded on a HWS SG 2000 melting point apparatus and are uncorrected. Elemental analyses were performed on a Heraeus CHN Rapid elemental analyzer and are within 0.4% of the calculated values. Mass spectra were recorded on the following instruments, using the stated ionization methods: Finnigan MAT 8230 mass spectrometer, electron impact ionization (YEF); Finnigan TSQ 700 mass spectrometer, electrospray ionization (cation scan: EKA).

4-(5-Amino-1-methyl-1*H*-benzoimidazol-2-ylmethyl)-benzonitrile (25b). A solution of (4-cyano-phenyl)acetic acid (10.1 g, 62.7 mmol) and CDI (10.1 g, 62.3 mmol) in 100 mL of tetrahydrofuran (THF) was stirred at 50 °C for 30 min. Then, N^1-methyl-4-nitro-benzene-1,2-diamine (10.1 g, 60.4 mmol) in 400 mL of THF was added and the resulting solution was stirred for another 2 h at 50 °C. About 350 mL of the solvent was evaporated, water (250 mL) was added, and the precipitate was collected and washed with water and subsequently with ether to afford 2-(4-cyano-phenyl)-*N*-(2-methylamino-5-nitro-phenyl)acetamide (14.8 g, 79%). This intermediate was dissolved in glacial acid (150 mL) and refluxed for 1 h. The solution was then concentrated to dryness, and the residue was vigorously stirred in ammonia solution (1 N, 200 mL), which gave, after it was filtered, 4-(1-methyl-5-nitro-1*H*-benzoimidazol-2-ylmethyl)benzonitrile (13.0 g, 93%). The nitro group was then reduced by hydrogenation (Pd on charcoal, 10%) in a mixture of methanol and dichloromethane (200 mL each), to afford the desired **25b** (11.6 g, 100%). Low-resolution mass spectromety (LRMS) (YEF): $M^+ = 262$. ^{1}H NMR (DMSO-d_6): δ 3.60 (s, 3H), 4.31 (s, 2H), 4.86 (bs, 2H), 6.56 (dd, 1H), 6.71 (d, 1H), 7.12 (d, 1H), 7.47 (d, 2H), 7.79 (d, 2H).

***N*-[2-(4-Cyano-benzyl)-1-methyl-1*H*-benzoimidazol-5-yl]benzenesulfonamide (26b).** A solution of **25b** (1.60 g, 6.1 mmol) and benzenesulfonyl chloride (1.10 g, 6.2 mmol) in 30 mL of pyridine was kept at room temperature for 1 h. The solvent was then evaporated, and the residue was vigorously

stirred in a mixture of water (20 mL) and ethyl acetate (2 mL). The solid was isolated by filtration and washed with water and ether to afford 2.21 g (91%) of the benzenesulfonamide **26b**. LRMS (YEF): $M^+ = 402$. ^{1}H NMR (DMSO-d_6): δ 3.68 (s, 3H), 4.37 (s, 2H), 6.95 (dd, 1H), 7.22 (d, 1H), 7.38 (d, 1H), 7.44−7.73 (m, 5H), 7.49 (d, 2H), 7.78 (d, 2H), 10.09 (s, 1H).

4-(5-Benzenesulfonylamino-1-methyl-1*H*-benzoimidazol-2-ylmethyl)benzamidine Hydrochloride (2). Gaseous anhydrous HCl was bubbled through a solution of nitrile **26b** (430 mg, 1.07 mmol) in 30 mL of cold ethanol (0 °C) for 0.5 h and allowed to stir for 5 h at room temperature. The solvent was evaporated in vacuo, and the residue was redissolved in 30 mL of ethanol. Ammonium carbonate (1.0 g, 10.7 mmol) was added, and the reaction mixture was stirred overnight at room temperature. Evaporation in vacuo afforded a solid mass that was purified via chromatography (silica gel, dichloromethane/methanol 5:1) to afford the title compound (39%) as an amorphous solid. LRMS (EKA): $(M + H)^+ = 420$, $(2M + H)^+ = 839$. ^{1}H NMR (DMSO-d_6): δ 3.69 (s, 3H), 4.38 (s, 2H), 6.98 (dd, 1H), 7.22 (d, 1H), 7.37 (d, 1H), 7.50 (d, 2H), 7.41−7.73 (m, 5H), 7.79 (d, 2H), 9.24 (s, 2H), 9.40 (s, 2H), 10.11 (s, 1H). Anal. ($C_{22}H_{21}N_5O_2S$·HCl) C, H, N, Cl.

4-(5-Benzenesulfonylamino-1*H*-benzoimidazol-2-ylmethyl)benzamidine Hydrochloride (3). Starting from 4-nitro-benzene-1,2-diamine, the title compound was synthesized following the procedures described above. LRMS (EKA): $(M + H)^+ = 406$, $(2M + H)^+ = 811$. ^{1}H NMR (DMSO-d_6): δ 4.28 (s, 2H), 6.89 (dd, 1H), 7.20 (d, 1H), 7.30 (d, 1H), 7.40−7.73 (m, 8H), 7.80 (d, 2H), 9.19 (s, 2H), 9.35 (s, 2H), 10.10 (s, 1H). Anal. ($C_{21}H_{19}N_5O_2S$·HCl) C, H, N, Cl.

4-(5-Benzenesulfonylamino-1-ethyl-1*H*-benzoimidazol-2-ylmethyl)benzamidine Hydrochloride (4). Starting from N^1-ethyl-4-nitro-benzene-1,2-diamine, the title compound was synthesized following the procedures described above. LRMS (EKA): $(M + H)^+ = 434$, $(2M + H)^+ = 867$. ^{1}H NMR (DMSO-d_6): δ 1.13 (t, 3H), 4.21 (q, 2H), 4.38 (s, 2H), 6.97 (dd, 1H), 7.24 (d, 1H), 7.40 (d, 1H), 7.42−7.75 (m, 7H), 7.78 (d, 2H), 9.19 (s, 2H), 9.38 (s, 2H), 10.14 (s, 1H). Anal. ($C_{23}H_{23}N_5O_2S$·HCl) C, H, N, Cl.

4-(5-Benzenesulfonylamino-1-*n*-propyl-1*H*-benzoimidazol-2-ylmethyl)benzamidine Hydrochloride (5). Starting from N^1-*n*-propyl-4-nitro-benzene-1,2-diamine, the title compound was synthesized following the procedures described above. LRMS (EKA): $(M + H)^+ = 448$, $(2M + H)^+ = 895$. ^{1}H NMR (DMSO-d_6): δ 0.81 (t, 3H), 1.60 (m, 2H), 4.10 (q, 2H), 4.35 (s, 2H), 6.95 (dd, 1H), 7.22 (d, 1H), 7.38 (d, 1H), 7.40−7.75 (m, 7H), 7.79 (d, 2H), 9.20 (s, 2H), 9.31 (s, 2H), 10.00 (s, 1H). Anal. ($C_{24}H_{25}N_5O_2S$·HCl) C, H, N, Cl.

4-[5-(Benzenesulfonyl-methyl-amino)-1-methyl-1*H*-benzoimidazol-2-ylmethyl]benzamidine Hydrochloride (6). Compound **26b** (0.70 g, 1.74 mmol) and iodomethane (0.26 g, 1.80 mmol) were dissolved in 20 mL of acetone, and potassium carbonate (0.70 g, 5 mmol) was added. After it was stirred at room temperature for 5 h, the reaction mixture was concentrated in vacuo, and the residue was purified via chromatography (silica gel, dichloromethane/methanol 50:1) to afford the intermediate *N*-[2-(4-cyano-benzyl)-1-methyl-1*H*-benzoimidazol-5-yl]-*N*-methyl-benzenesulfonamide (0.61 g, 83%). This was converted into the title compound following the procedure of the Pinner reaction described above. LRMS (EKA): $(M + H)^+ = 434$, $(2M + H)^+ = 867$. ^{1}H NMR (DMSO-d_6): δ 3.18 (s, 3H), 3.75 (s, 3H), 4.41 (s, 2H), 6.92 (dd, 1H), 7.18 (d, 1H), 7.42−7.75 (m, 8H), 7.80 (d, 2H), 9.18 (s, 2H), 9.38 (s, 2H). Anal. ($C_{23}H_{23}N_5O_2S$·HCl) C, H, N, Cl.

4-[1-Methyl-5-(pyridine-3-sulfonylamino)-1*H*-benzoimidazol-2-ylmethyl]benzamidine Hydrochloride (7). Starting from **25b** and pyridine-3-sulfonyl chloride, the title compound was synthesized following the procedures described above. LRMS (EKA): $(M + H)^+ = 421$, ^{1}H NMR (DMSO-d_6): δ 3.69 (s, 3H), 4.37 (s, 2H), 6.95 (dd, 1H), 7.25 (d, 1H), 7.38 (d, 1H), 7.51 (d, 2H), 7.55 (m, 1H), 7.79 (d, 2H), 8.07 (m, 1H), 8.73 (m, 2H), 9.19 (s, 2H), 9.35 (s, 2H), 10.31 (s, 1H). Anal. ($C_{21}H_{20}N_6O_2S$·HCl·H_2O) C, H, N, Cl.

4-[5-(2,5-Dimethoxy-benzenesulfonylamino)-1-methyl-1*H*-benzoimidazol-2-ylmethyl]benzamidine Hydrochloride (8). Starting from **25b** and 2,5-dimethoxy-benzenesulfonyl chloride, the title compound was synthesized following the procedures described above. LRMS (EKA): $(M + H)^+ = 480$. ^{1}H NMR (DMSO-d_6): δ 3.66 (s, 6H), 3.88 (s, 3H), 4.37 (s, 2H), 7.00 (dd, 1H), 7.05−7.16 (m, 3H), 7.22 (d, 1H), 7.32 (d, 1H), 7.50 (d, 2H), 7.78 (d, 2H), 9.18 (s, 2H), 9.35 (s, 2H), 9.74 (s, 1H). Anal. ($C_{24}H_{25}N_5O_4S$·HCl) C, H, N.

4-[5-(3,5-Bis-trifluoromethyl-benzenesulfonylamino)-1-methyl-1*H*-benzoimidazol-2-ylmethyl]benzamidine Hydrochloride (9). Starting from **25b** and 3,5-bis-trifluoromethyl-benzenesulfonyl chloride, the title compound was synthesized following the procedures described above. LRMS (EKA): $(M + H)^+ = 556$. ^{1}H NMR (DMSO-d_6): δ 3.69 (s, 3H), 4.39 (s, 2H), 6.95 (dd, 1H), 7.26 (d, 1H), 7.40 (d, 1H), 7.48 (d, 2H), 7.78 (d, 2H), 8.23 (s, 2H), 8.47 (s, 1H), 9.15 (s, 2H), 9.34 (s, 2H), 10.04 (s, 1H). Anal. ($C_{24}H_{19}F_6N_5O_2S$·HCl) C, H, N, S.

4-[1-Methyl-5-(naphthalene-1-sulfonylamino)-1*H*-benzoimidazol-2-ylmethyl]benzamidine Hydrochloride (10). Starting from **25b** and naphthalene-1-sulfonyl chloride, the title compound was synthesized following the procedures described above. LRMS (EKA): $(M + H)^+ = 470$. ^{1}H NMR (DMSO-d_6): δ 3.60 (s, 3H), 4.31 (s, 2H), 6.86 (dd, 1H), 7.11 (d, 1H), 7.27 (d, 1H), 7.40−7.81 (m, 7H), 8.00−8.20 (m, 3H), 8.79 (d, 1H), 9.19 (s, 2H), 9.35 (s, 2H), 10.48 (s, 1H). Anal. ($C_{26}H_{23}N_5O_2S$·HCl·H_2O) C, H, N.

4-[1-Methyl-5-(naphthalene-2-sulfonylamino)-1*H*-benzoimidazol-2-ylmethyl]benzamidine Hydrochloride (11). Starting from **25b** and naphthalene-2-sulfonyl chloride, the title compound was synthesized following the procedures described above. LRMS (EKA): $(M + H)^+ = 470$. ^{1}H NMR (DMSO-d_6): δ 3.62 (s, 3H), 4.32 (s, 2H), 6.98 (dd, 1H), 7.27 (d, 1H), 7.32 (d, 1H), 7.46 (d, 2H), 7.53−7.82 (m, 5H), 7.98 (dd, 1H), 8.05 (d, 2H), 8.32 (d, 1H), 9.16 (s, 2H), 9.33 (s, 2H), 10.22 (s, 1H). Anal. ($C_{26}H_{23}N_5O_2S$·HCl·H_2O) C, H, N.

4-[5-(Isoquinoline-5-sulfonylamino)-1-methyl-1*H*-benzoimidazol-2-ylmethyl]benzamidine Hydrochloride (12). Starting from **25b** and isoquinoline-5-sulfonyl chloride, the title compound was synthesized following the procedures described above. LRMS (EKA): $(M + H)^+ = 471$. ^{1}H NMR (DMSO-d_6): δ 3.62 (s, 3H), 4.32 (s, 2H), 6.86 (dd, 1H), 7.12 (d, 1H), 7.27 (d, 1H), 7.45 (d, 2H), 7.65−7.84 (m, 3H), 8.38 (d, 1H), 8.46 (d, 1H), 8.56 (d, 1H), 8.70 (d, 1H), 9.23 (s, 2H), 9.37 (s, 2H), 9.42 (s, 1H), 10.61 (s, 1H). Anal. ($C_{25}H_{22}N_6O_2S$·HCl) C, H, N.

Quinoline-8-sulfonic Acid [2-(4-Cyano-benzyl)-1-methyl-1*H*-benzoimidazol-5-yl]amide (27). Like described for compound **26b**, the title compound was prepared via sulfonation of **25b** with quinoline-8-sulfonic acid chloride. LRMS (YEF): $M^+ = 453$. ^{1}H NMR (DMSO-d_6): δ 3.69 (s, 3H), 4.36 (s, 2H), 6.96 (dd, 1H), 7.22 (d, 1H), 7.38 (d, 1H), 7.46 (d, 2H), 7.63 (t, 1H), 7.75 (m, 1H), 7.77 (d, 2H), 8.22 (m, 2H), 8.51 (dd, 1H), 9.18 (m, 1H), 10.18 (s, 1H).

4-[1-Methyl-5-(quinoline-8-sulfonylamino)-1*H*-benzoimidazol-2-ylmethyl]benzamidine Hydrochloride (13). Following the Pinner reaction protocol described above, the title compound was prepared from **27**. LRMS (EKA): $(M + H)^+ = 471$. ^{1}H NMR (DMSO-d_6): δ 3.60 (s, 3H), 4.28 (s, 2H), 6.89 (dd, 1H), 7.10 (d, 1H), 7.20 (d, 1H), 7.43 (d, 2H), 7.67 (t, 1H), 7.20−7.30 (m, 3H), 8.22 (dd, 2H), 8.51 (dd, 1H), 9.15 (s, 2H), 9.18 (m, 1H), 9.32 (s, 2H), 9.78 (s, 1H). Anal. ($C_{25}H_{22}N_6O_2S$·HCl) C, H, N, Cl.

4-[5-(Quinoline-8-sulfonyl-*N*-(carboxymethyl)-amino)-1-methyl-1*H*-benzoimidazol-2-ylmethyl]benzamidine (14). Compound **27** (2.30 g, 5 mmol) and bromo-acetic acid ethyl ester (0.92 g, 5.5 mmol) were dissolved in 50 mL of dimethyl sulfoxide (DMSO), and potassium carbonate (2.8 g, 20 mmol) was added. After it was stirred at room temperature for 5 h, the mixture was diluted with water (150 mL) and the precipitate was isolated and purified via chromatography (silica gel, dichloromethane/methanol 75:1) to afford quinoline-8-sulfonic acid [2-(4-cyano-benzyl)-1-methyl-1*H*-benzoimidazol-5-yl]-*N*-(ethoxycarbonylmethyl)amide (43%). The nitrile group

was converted into the amidine following the procedure described above, and the resulting 4-[5-(quinoline-8-sulfonyl-*N*-(ethoxycarbonylmethyl)-amino)-1-methyl-1*H*-benzoimidazol-2-ylmethyl]benzamidine hydrochloride (58%) was isolated as an amorphous powder. This intermediate (0.40 g, 0.67 mmol) was dissolved in a solution of sodium hydroxide (0.13 g, 3.37 mmol) in 10 mL of water and 3 mL of ethanol and stirred at room temperature for 2 h. The mixture was then diluted with water (15 mL) and neutralized with 1 N HCl. The amorphous precipitate was isolated, dissolved in 1 N NaOH, and neutralized with 0.5 N HCl. The precipitate was isolated and washed with water to afford the zwitterionic title compound (56%) as a crystalline powder; mp 222−224 °C (decomposition). LRMS (EKA): $(M + H)^+ = 529$, $(M + Na)^+ = 551$, $(M + 2H)^{2+} = 265$, $(M + H + Na)^{2+} = 276$, $(M + 2Na)^{2+} = 287$. ^{1}H NMR (DMSO-d_6 + ^{2}HCl): δ 3.92 (s, 3H), 4.77 (s, 2H), 5.07 (s, 2H), 7.16 (dd, 1H), 7.50−7.95 (m, 8H), 8.18 (d, 1H), 8.35 (d, 1H), 8.64 (d, 1H), 9.21 (d, 1H). Anal. ($C_{27}H_{24}N_6O_4S \cdot H_2O$) C, H, N.

4-[5-(Quinoline-8-sulfonyl-*N*-(ω-carboxy-*n*-propyl)amino)-1-methyl-1*H*-benzoimidazol-2-ylmethyl]benzamidine (15). Following the procedure described for compound **14**, **27** was converted in three steps into the zwitterionic title compound; mp 223−225 °C (decomposition). LRMS (EKA): $(M + H)^+ = 557$, $(M + Na)^+ = 579$, $(M + 2H)^{2+} = 279$, $(M + H + Na)^{2+} = 290$. ^{1}H NMR (DMSO-d_6): δ 1.52 (m, 2H), 2.04 (t, 2H), 3.66 (s, 3H), 4.12 (t, 2H), 4.30 (s, 2H), 6.65 (dd, 1H), 7.09 (d, 1H), 7.23 (d, 1H), 7.40−7.68 (m, 5H), 7.75 (m, 1H), 8.02 (d, 1H), 8.22 (d, 1H), 8.56 (dd, 1H), 9.19 (dd, 1H). Anal. ($C_{29}H_{28}N_6O_4S$) C, H, N.

4-[(1-Methyl-5-nitro-1*H*-benzoimidazol-2-ylmethyl)amino]benzonitrile (28c). This intermediate was synthesized in two steps on the analogy of the procedure described for compound **25b**, starting from N^1-methyl-4-nitro-benzene-1,2-diamine and (4-cyano-phenylamino)acetic acid. ^{1}H NMR (DMSO-d_6): δ 3.90 (s, 3H), 4.74 (d, 2H), 6.85 (d, 2H), 7.39 (t, 1H), 7.50 (d, 2H), 7.80 (d, 1H), 8.19 (dd, 1H), 8.51 (d, 1H).

4-({1-Methyl-5-[*N*-carboxymethyl(quinoline-8-sulfonyl)amino]-1*H*-benzoimidazol-2-ylmethyl}amino)benzamidine (18). This compound was prepared on the analogy of the procedure described for compound **14**. Compound **28c** (0.8 g, 2.6 mmol) was reduced via hydrogenation in a mixture of 150 mL of methanol and 100 mL of dichloromethane. The resulting amino derivative (0.69 g, 2.48 mmol) was sulfonated with quinoline-8-sulfonyl chloride (0.57 g, 2.5 mmol) in 10 mL of pyridine at room temperature to afford quinoline-8-sulfonic acid {2-[(4-cyano-phenylamino)methyl]-1-methyl-1*H*-benzoimidazol-5-yl}amide (1.0 g, 86%), which was alkylated with bromoacetic acid ethyl ester (0.38 g, 2.3 mmol) and potassium carbonate (0.83 g, 6.0 mmol) in refluxing acetone (yield: 1.0 g, 85%). This cyano compound was converted into the amidine following the Pinner reaction protocol described above to afford 4-({1-methyl-5-[*N*-ethoxycarbonylmethyl(quinoline-8-sulfonyl)-amino]-1*H*-benzoimidazol-2-ylmethyl}amino)benzamidine hydrochloride as an amorphous solid (0.69 g, 65%), which gave, after alkaline hydrolysis, the zwitterionic title compound (91%) as white crystals; mp 249−250 °C (decomposition). LRMS (EKA): $(M + H)^+ = 544$, $(M + Na)^+ = 566$, $(M + H + Na)^{2+} = 283.8$, $(M + 2Na)^{2+} = 294.6$. ^{1}H NMR (DMSO-d_6): δ 3.69 (s, 3H), 4.58 (s, 2H), 4.60 (d, 2H), 6.46 (dd, 1H), 6.72 (d, 2H), 7.10 (d, 1H), 7.38 (t, 1H), 7.40−7.60 (m, 4H), 7.73 (dd, 1H), 8.08 (d, 1H), 8.20 (d, 1H), 8.27 (s, 2H), 8.53 (dd, 1H), 9.19 (m, 1H), 11.55 (s, 2H). Anal. ($C_{27}H_{25}N_7O_4S \cdot 2H_2O$) C, H, N, S.

4-(2-{1-Methyl-5-[*N*-carboxymethyl(quinoline-8-sulfonyl)amino]-1*H*-benzoimidazol-2-yl}ethyl)benzamidine (16). Starting from 3-(4-cyano-phenyl)propionic acid and N^1-methyl-4-nitro-benzene-1,2-diamine, the title compound was synthesized following the procedures described for **28c** and **18**; mp 246−247 °C (decomposition). LRMS (EKA): $(M + H)^+ = 543$, $(M + Na)^+ = 565$, $(2M + 3Na)^{3+} = 385$. ^{1}H NMR (DMSO-d_6): δ 3.24 (s, 4H), 3.56 (s, 3H), 4.60 (s, 2H), 6.55 (dd, 1H), 7.07 (d, 1H), 7.31 (d, 1H), 7.40 (d, 2H), 7.47−7.62 (m, 3H), 7.73 (dd, 1H), 8.04 (dd, 1H), 8.19 (d, 1H), 8.52 (dd, 1H), 8.63 (s, 2H), 9.20 (m, 1H), 12.02 (s, 2H). Anal. ($C_{28}H_{26}N_6O_4S$) C, H, N.

4-{1-Methyl-5-[*N*-carboxymethyl(quinoline-8-sulfonyl)amino]-1*H*-benzoimidazol-2-ylmethoxy}benzamidine Dihydrochloride (17). Starting from (4-cyano-phenoxy)acetic acid and N^1-methyl-4-nitro-benzene-1,2-diamine, the title compound was synthesized following the procedures described for **28c** and **18**. In the final step, the zwitterionic product was dissolved in 2 N HCl and then concentrated in vacuo. The residue was triturated with ether, filtered, and dried to afford the dihydrochloride as a white solid. LRMS (EKA): $(M + H)^+ = 545$, $(M + Na)^+ = 567$. ^{1}H NMR (DMSO-d_6): δ 3.90 (s, 3H), 5.07 (s, 2H), 5.76 (s, 2H), 7.09 (dd, 1H), 7.38 (d, 2H), 7.53 (dd, 1H), 7.58−7.75 (m, 2H), 7.80 (dd, 1H), 7.92 (d, 2H), 8.13 (d, 1H), 8.32 (d, 1H), 8.61 (dd, 1H), 9.18 (s, 2H), 9.20 (m, 1H), 9.39 (s, 2H). Anal. ($C_{27}H_{24}N_6O_5S \cdot 2HCl \cdot 0.5H_2O$) C, H, N, Cl.

[(4-Methylamino-3-nitro-benzoyl)phenyl-amino]acetic Acid Ethyl Ester (29a). A solution of 4-methylamino-3-nitro-benzoic acid (2.0 g, 10.2 mmol) in 20 mL of thionyl chloride and two drops of dimethyl formamide (DMF) was refluxed for 0.5 h and then concentrated in vacuo. The residue was dissolved in 30 mL of THF and dropwise added to a solution of phenylamino-acetic acid ethyl ester (1.82 g, 10.1 mmol) in 30 mL of THF and 3 mL of triethylamine at room temperature. The mixture was kept at ambient temperature overnight and then concentrated in vacuo. The residue was chromatographed (silica gel, dichloromethane/ethanol 99:1) to afford the title compound (2.0 g, 55%). ^{1}H NMR (DMSO-d_6): δ 1.19 (t, 3H), 2.90 (d, 3H), 4.12 (q, 2H), 4.57 (s, 2H), 6.81 (d, 1H), 7.17−7.40 (m, 6H), 7.98 (d, 1H), 8.33 (q, 1H).

({2-[2-(4-Carbamimidoyl-phenyl)ethyl]-1-methyl-1*H*-benzoimidazole-5-carbonyl}phenyl-amino)acetic Acid Hydrochloride (19). Starting from intermediate **29a** and 3-(4-cyano-phenyl)propionic acid, the title compound was prepared in five steps following the procedures described for compounds **14** and **18**. LRMS (EKA): $(M + H)^+ = 456$, $(M + Na)^+ = 478$, $(M + 2Na)^{2+} = 250.6$. ^{1}H NMR (DMSO-d_6): δ 3.30 (t, 2H), 3.43 (t, 2H), 3.86 (s, 3H), 4.55 (s, 2H), 7.10−7.30 (m, 5H), 7.38 (dd, 1H), 7.58 (d, 2H), 7.65−7.88 (m, 4H), 9.23 (s, 2H), 9.42 (s, 2H). Anal. ($C_{26}H_{25}N_5O_3 \cdot HCl \cdot H_2O$) C, H, N.

{**[2-(4-Carbamimidoyl-phenoxymethyl)-1-methyl-1*H*-benzoimidazole-5-carbonyl]phenyl-amino**}**acetic Acid Hydrochloride (20).** Starting from intermediate **29a** and (4-cyano-phenoxy)acetic acid, the title compound was prepared in five steps following the procedure described for compounds **14** and **18**. LRMS (EKA): $(M + H)^+ = 458$, $(M + Na)^+ = 480$, $(M + 2Na)^{2+} = 251.6$. ^{1}H NMR (DMSO-d_6): δ 3.85 (s, 3H), 4.53 (s, 2H), 5.62 (s, 2H), 7.05−7.40 (m, 8H), 7.60 (d, 1H), 7.62 (s, 1H), 7.89 (d, 2H), 9.12 (s, 2H), 9.30 (s, 2H). Anal. ($C_{25}H_{23}N_5O_4 \cdot HCl \cdot H_2O$) C, H, N.

({2-[(4-Carbamimidoyl-phenylamino)methyl]-1-methyl-1*H*-benzoimidazole-5-carbonyl}phenyl-amino)acetic Acid Hydrochloride (21). Starting from intermediate **29a** and (4-cyano-phenylamino)acetic acid, the title compound was prepared in five steps following the procedure described for compounds **14** and **18**. LRMS (EKA): $(M + H)^+ = 457$, $(M + Na)^+ = 479$, $(M + 2Na)^{2+} = 251$. ^{1}H NMR (DMSO-d_6): δ 3.92 (s, 3H), 4.52 (s, 2H), 4.91 (s, 2H), 6.91 (d, 2H), 7.08−7.30 (m, 5H), 7.39 (dd, 1H), 7.63 (s, 1H), 7.65−7.80 (m, 3H), 8.88 (s, 2H), 9.08 (s, 2H). Anal. ($C_{25}H_{24}N_6O_3 \cdot HCl \cdot 0.5\ H_2O$) C, H, N.

3-({2-[(4-Carbamimidoyl-phenylamino)methyl]-1-methyl-1*H*-benzoimidazole-5-carbonyl}phenyl-amino)propionic Acid (22). The title compound was prepared on the analogy of the synthesis of compound **21**. LRMS (EKA): $(M + H)^+ = 471$, $(M + H + Na)^{2+} = 247$, $(M + 2Na)^{2+} = 258$. ^{1}H NMR (DMSO-d_6 + ^{2}HCl): δ 2.57 (t, 2H), 3.99 (s, 3H), 4.08 (t, 2H), 5.04 (s, 2H), 6.95 (d, 2H), 7.10−7.33 (m, 5H), 7.44 (dd, 1H), 7.70 (s, 1H), 7.80 (d, 2H), 7.83 (d, 1H). Anal. ($C_{26}H_{26}N_6O_3 \cdot H_2O$) C, H, N.

4-({2-[(4-Carbamimidoyl-phenylamino)methyl]-1-methyl-1*H*-benzoimidazole-5-carbonyl}phenyl-amino)butyric Acid Hydrochloride (23). The title compound was prepared on the analogy of the synthesis of compound **21**. LRMS (EKA): $(M + H)^+ = 485$, $(M + H + Na)^{2+} = 254$, $(M + 2H)^{2+} = 243$. ^{1}H NMR (DMSO-d_6): δ 1.75 (m, 2H), 2.29 (t, 2H), 3.90 (t, 2H), 3.92 (s, 3H), 4.91 (s, 2H), 6.90 (d, 2H), 7.08−7.30 (m,

5H), 7.39 (dd, 1H), 7.58−7.80 (m, 4H), 8.82 (s, 2H), 9.04 (s, 2H). Anal. ($C_{27}H_{28}N_6O_3$·HCl·0.5 H_2O) C, H, N, Cl.

3-[(4-Methylamino-3-nitro-benzoyl)pyridin-2-yl-amino]-propionic Acid Ethyl Ester (29d). Starting from 4-methylamino-3-nitro-benzoic acid and 3-(pyridin-2-ylamino)propionic acid ethyl ester, the title compound was prepared following the procedure described for the synthesis of intermediate **29a**; mp 86−88 °C. ^{1}H NMR (DMSO-d_6): δ 1.11 (t, 3H), 2.65 (t, 2H), 2.90 (d, 3H), 3.97 (q, 2H), 4.18 (t, 2H), 6.82 (d, 1H), 7.08 (d, 1H), 7.21 (m, 1H), 7.31 (dd, 1H), 7.70 (dt, 1H), 7.92 (d, 1H), 8.37 (q, 1H), 8.44 (dd, 1H). Anal. ($C_{18}H_{20}N_4O_5$) C, H, N.

3-({2-[(4-Carbamimidoyl-phenylamino)methyl]-1-methyl-1*H*-benzoimidazole-5-carbonyl}pyridin-2-yl-amino)-propionic Acid Ethyl Ester Hydrochloride (30). Compound **29d** (16.7 g, 44.7 mmol) was hydrogenated (Pd on charcoal, 10%) in 200 mL of methanol at room temperature to afford 3-[(3-amino-4-methylamino-benzoyl)pyridin-2-yl-amino]-propionic acid ethyl ester (10.0 g, 65%) after purification via chromatography (silica gel, dichloromethane/methanol 30:1). This intermediate (2.1 g, 6.1 mmol) was added to a solution of (4-cyano-phenylamino)acetic acid imidazolide (7.3 mmol) in 50 mL of THF and refluxed for 24 h. The reaction mixture was then concentrated in vacuo, and the residue was dissolved in 30 mL of glacial acid and heated under reflux for 1 h. The solution was diluted with 100 mL of water and neutralized with concentrated ammonium hydroxide. Extraction with ethyl acetate and purification via chromatography (silica gel, dichloromethane/methanol 40:1) gave 3-({2-[(4-cyano-phenylamino)-methyl]-1-methyl-1*H*-benzoimidazole-5-carbonyl}pyridin-2-yl-amino)propionic acid ethyl ester (1.8 g, 61%), which was converted into the title compound following the protocol of the Pinner reaction described above; yield: 1.4 g, 71%. LRMS (EKA): $(M + H)^+ = 500$, $(M + H + Na)^{2+} = 261.8$, $(M + 2H)^{2+} = 250.8$. ^{1}H NMR (DMSO-d_6): δ 1.12 (t, 3H), 2.68 (t, 2H), 3.77 (s, 3H), 3.97 (q, 2H), 4.22 (t, 2H), 4.66 (d, 2H), 6.88 (m, 3H), 7.13 (m, 2H), 7.36−7.60 (m, 4H), 7.65 (d, 2H), 8.40 (m, 1H), 8.67 (s, 2H), 8.90 (s, 2H).

3-({2-[(4-Carbamimidoyl-phenylamino)methyl]-1-methyl-1*H*-benzoimidazole-5-carbonyl}pyridin-2-yl-amino)-propionic Acid (24). Compound **30** (1.0 g, 1.86 mmol) was added to a solution of sodium hydroxide (0.24 g, 6.0 mmol) in 20 mL of water and 10 mL of ethanol and kept at ambient temperature for 2 h. The mixture was then diluted with 60 mL of water and neutralized with acetic acid. The precipitate was isolated and washed with water and ether to afford the zwitterionic title compound (0.8 g, 91%) as white crystals; mp 276−277 °C. LRMS (EKA): $(M + H)^+ = 472$, $(M + H + Na)^{2+} = 247.6$, $(M + 2H)^{2+} = 236.7$, $(M + 2Na)^{2+} = 258.6$. ^{1}H NMR (DMSO-d_6 + ^{2}HCl): δ 2.67 (t, 2H), 4.01 (s, 3H), 4.17 (t, 2H), 5.07 (s, 2H), 6.97 (d, 2H), 7.28−7.40 (m, 2H), 7.45 (dd, 1H), 7.70−7.94 (m, 5H), 8.93 (m, 1H). Anal. ($C_{25}H_{25}N_7O_3$·2H_2O) C, H, N.

3-({2-[(4-{Amino-[(*E*)-hexyloxycarbonylimino]methyl}-phenylamino)methyl]-1-methyl-1*H*-benzoimidazole-5-carbonyl}pyridin-2-yl-amino)propionic Acid Ethyl Ester (31). Compound **30** (1.0 g, 1.86 mmol) was dissolved in 50 mL of THF and 10 mL of water. Potassium carbonate (0.83 g, 6.0 mmol) was added, and the mixture was stirred at room temperature for 15 min. Then, *n*-hexyl chloroformate (0.31 g, 1.86 mmol) was added and stirring was continued for another hour. The organic phase was separated, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was chromatographed (silica gel, dichloromethane/methanol 19:1) to afford the title compound as colorless crystals; mp 128−129 °C; 0.61 g, 51%. LRMS (EKA): $(M + H)^+ = 628$, $(M + H + Na)^{2+} = 325.7$, $(M + 2H)^{2+} = 314.7$. ^{1}H NMR (DMSO-d_6): δ 0.86 (t, 3H), 1.12 (t, 3H), 1.29 (m, 6H), 1.58 (m, 2H), 2.68 (t, 2H), 3.77 (s, 3H), 3.97 (m, 4H), 4.22 (t, 2H), 4.60 (d, 2H), 6.75 (d, 2H), 6.88 (d, 1H), 6.95 (t, 1H), 7.12 (m, 2H), 7.40 (d, 1H), 7.47 (d, 1H), 7.53 (dt, 1H), 7.80 (d, 2H), 8.39 (dd, 1H), 8.50−9.30 (bs, 2H). Anal. ($C_{34}H_{41}N_7O_5$) C, H, N.

X-ray Crystallography. Human α-thrombin was obtained from Enzyme Research Laboratories (South Bend, IN) and crystallized in complex with the exosite binding hirudin fragment residues 55−65.[20] Cocrystals were generated by soaking crystals with mother liquor containing 1 mM inhibitor. Data were collected on a MAR Research imaging plate (X-ray Research, Hamburg, Germany) mounted on a Rigaku RU200 rotating anode generator and processed and scaled with HKL.[21] Model building and refinement were carried out with MAIN[22] and CNS.[23] The X-ray structures of compounds **2** and **24** were deposited in the Brookhaven data bank. The accession numbers are 1KTT and 1KTS, respectively.

Measurement of Thrombin Inhibition. The thrombin inhibitory effects (IC_{50}) of the compounds were determined with a commercially available chromogenic assay (Roche, Mannheim, Germany). Human thrombin (Roche) (0.042 U/mL) was preincubated for 10 min at 37 °C with 10 different dilutions (concentration range of 0.003−100 μM) of the test compounds dissolved in DMSO or with DMSO as control. Upon addition of the preincubation mixture to the chromogenic substrate, tosyl-glycyl-prolyl-arginine-4-nitranilide acetate, nitraniline is cleaved by thrombin and the increase in absorbance at 405 nm, related to the free nitraniline, is measured in a spectrophotometer (Spectramax, Molecular Devices, Sunnyvale, CA). By plotting the absorbance at 405 nm vs the concentration of the test compound, the concentration that induced a 50% thrombin inhibition (IC_{50}) was calculated. All measurements were performed in duplicate, and the mean values of both determinations are represented.

Measurement of the aPTT. aPTT was measured in a coagulometer (Biomatic B10, Sarstedt, Germany) using the PTT reagent of Boehringer, Mannheim, Germany, according to the manufacturer's instruction as a measure for the anticoagulant effect of the respective compound. Bloodsamples were collected in sodium citrate solution (final concentration 0.313%). Each native blood sample (0.1 mL) was pipetted into a test tube prewarmed to 37 °C. The PTT reagent (0.1 mL) was added, mixed, and incubated for exactly 3 min. Calcium chloride solution (0.1 mL), prewarmed to 37 °C, was added in order to activate the coagulation cascade, and the time (aPTT; in seconds) was determined that elapsed from the addition of calcium chloride to the onset of clotting.

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Supporting Information Available: X-ray crystallography of complexes of compounds **2** and **24** with human α-thrombin; data collection and refinement statistics. Combustion analysis of all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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