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Heterocyclic Thrombin Inhibitors. Part 2: Quinoxalinone Derivatives as Novel, Potent Antithrombotic Agents

Uwe J. Ries,[a,*] Henning W. M. Priepke,[a] Norbert H. Hauel,[a] Sandra Handschuh,[a] Gerhard Mihm,[a] Jean M. Stassen,[b] Wolfgang Wienen[b] and Herbert Nar[a]

[a]*Department of Chemical Research, Boehringer Ingelheim Pharma KG, Birkendorfer Straße 65, D-88397 Biberach/Riß, Germany*
[b]*Department of Biological Research, Boehringer Ingelheim Pharma KG, Birkendorfer Straße 65, D-88397 Biberach/Riß, Germany*

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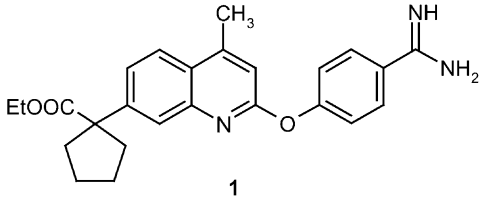
Abstract—Quinoxalinone derivatives as prototypes of dual thrombin and factor Xa inhibitors have been discovered. Nanomolar inhibition of both coagulation enzymes resulted in very potent antithrombotic activity in vitro.

Excessive uncontrolled activation of the hemostatic system results in thromboembolic diseases, a major cause of morbidity and mortality in our society. The central role of the serine proteases factor Xa and thrombin in hemostasis make them attractive targets for antithrombotic therapy. Factor Xa, factor Va, calcium and phospholipids form the prothrombinase complex, which converts prothrombin into thrombin. Thrombin very efficiently initiates fibrin formation, platelet aggregation and activation of factors V and VIII. Selective inhibitors of thrombin and factor Xa are expected to be therapeutically useful in the treatment or prophylaxis of thromboembolic diseases.[1]

As part of our efforts to design and develop orally active coagulation inhibitors containing heterocyclic core structures we identified amidinophenoxy-quinoline derivatives as potent thrombin inhibitors[2] in vitro. The ethoxycarbonyl-cyclopentyl derivative **1** turned out to be the most potent compound within this series and inhibited thrombin in the lower nanomolar range (Fig. 1). In addition **1** was the only example which also showed weak inhibition of factor Xa. The X-ray structure of **1** bound to thrombin revealed an unexpected binding mode of this new type of serine protease inhibitor. Based on this lead structure novel coagulation inhibitors having quinoxalinone as the central template have been designed. These compounds inhibited thrombin and factor Xa in the nanomolar range. This paper describes their synthesis, biological properties and the X-ray structure of a trypsin–inhibitor complex.

The synthesis of compound **16** as a representative example of quinoxalinone derivatives containing cyclopropyl carboxamide or related functions is described in Scheme 1. Starting from commercially available 1-(4-chlorophenyl)-1-cyclopropane carboxylic acid (**2**) a nitro group was selectively introduced into position 3. Nucleophilic displacement of the chlorine by methylamine yielded the nitroaniline derivative **3**, which was subjected to catalytic reduction to give **4**. The α-keto-acid **5** was prepared by a two-step oxidation process starting from 4-cyano-phenylalanine.[3] Subsequently **5** was condensed with **4** in refluxing ethanol to give the quinoxalinone derivative **6**, which was coupled with pyrrolidine using TBTU/NMP/DMF to generate the corresponding amide **7**. Using a Pinner reaction



1

Thrombin Inhibition: $IC_{50} = 0.003\ \mu M$
Factor Xa Inhibition: $IC_{50} = 1.5\ \mu M$
Trypsin Inhibition: $IC_{50} = 0.56\ \mu M$
Plasmin Inhibition: $IC_{50} = 5.8\ \mu M$

Figure 1. Structure and biological activity of lead compound **1**.

*Corresponding author. Tel.: +49-7351-544535; fax: +49-7351-547169; e-mail: uwe.ries@bc.boehringer-ingelheim.com

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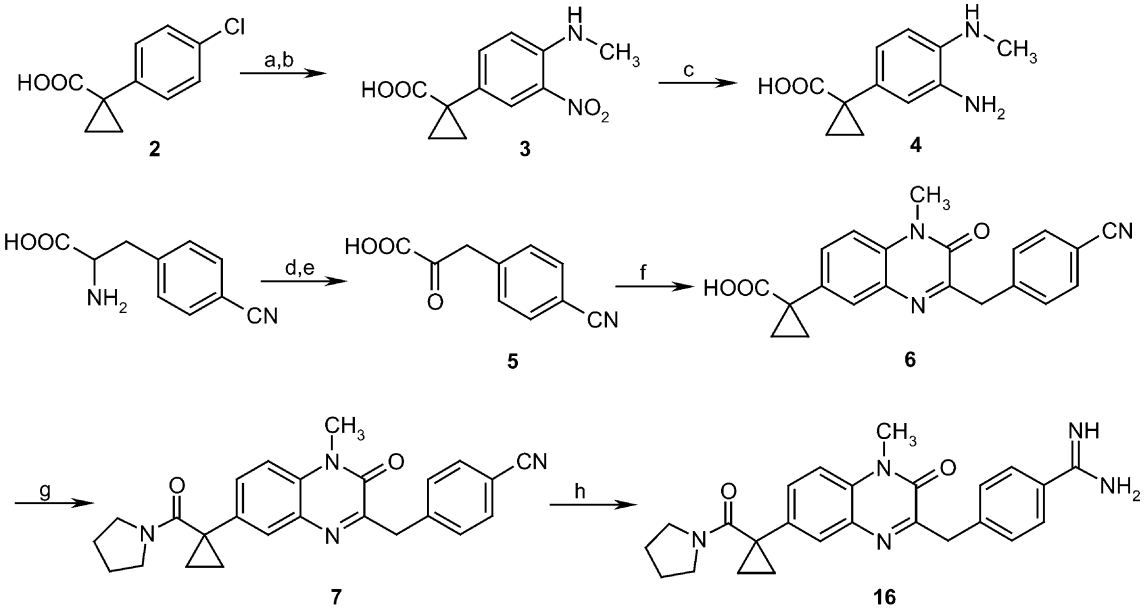


Scheme 1. (a) HNO_3, −30 °C, 93%; (b) CH_3NH_2 (40% in H_2O), 80 °C, 5 h, 94%; (c) H_2, Pd/C (10%), MeOH, 25 °C, 86%; (d) $(CF_3CO)_2O$, 40 °C, 12 h, 70%; (e) CF_3COOH (70%), 24 h, 25 °C, 86%; (f) Compound **4**, EtOH, 80 °C, 6 h, 88%; (g) pyrrolidine, TBTU, NMP, DMF, 12 h, 25 °C, 52%; (h) (1) HCl/EtOH, 25 °C, 6 h; (2) $(NH_4)_2CO_3$, EtOH, 25 °C, 12 h, 33%.

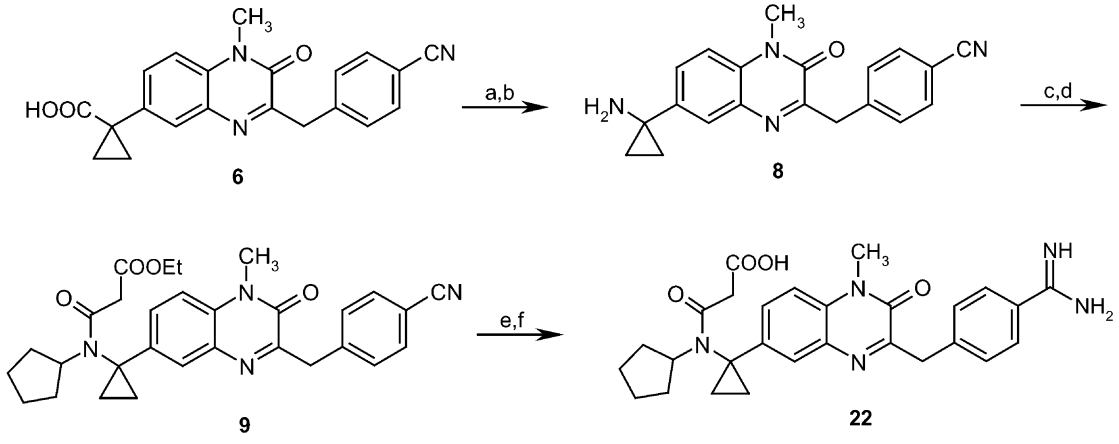
sequence the cyano group was converted into benzamidine **16**.

In order to synthesize cyclopropyl amine analogues the carboxylic acid derivative **6** was treated with diphenylphosphoryl azide in the presence of *t*-BuOK/*t*-BuOH followed by acidic removal of the Boc-protecting group to yield **8** (Scheme 2). This intermediate was subjected to reductive amination with cyclopentanone followed by acylation, Pinner reaction and saponification to give compound **22**.

Amidino-phenoxy quinoline derivatives like compound **1** have been identified as highly potent and selective thrombin inhibitors.[2] However, the antithrombotic efficacy in vitro as well as the pharmacokinetic properties in vivo turned out to be insufficient for therapeutic use.

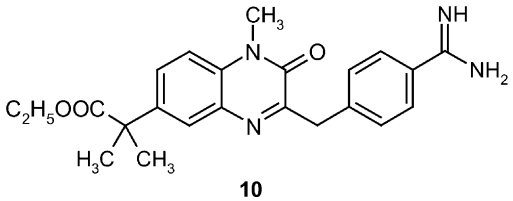
On the other hand the X-ray structure of a protein/inhibitor complex of compound **1** bound to thrombin unexpectedly showed an interaction of the cyclopentyl moiety with a lipophilic surface of thrombin in front of the S4-subsite and occupation of the S4-subsite by the ethyl ester functionality. The observed weak binding affinity of this compound to factor Xa suggested a productive interaction of this compound with this enzyme, presumably binding to it with the same unusual binding orientation. We concluded that **1** may be the prototype for the design of dual thrombin and factor Xa inhibitors, which might show superior antithrombotic activity relative to selective inhibitors.[4]

In the first step of lead optimization the central quinoline template was replaced by a quinoxalinone moiety, which has a reduced lipophilicity due to the additional

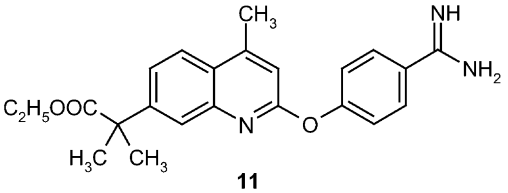


Scheme 2. (a) $(PhO)_2P(O)N_3$, TEA, *t*-BuOH, KO*t*-Bu, 80 °C, 3 h, 97%; (b) 6N HCl, dioxane, 25 °C, 4 h, 69%; (c) cyclopentanone, $NaBH_3CN$, AcOH, THF, 25 °C, 24 h, 92%; (d) $EtOOCCH_2COCl$, TEA, THF, 25 °C, 1 h, 82%; (e) (1) HCl/EtOH, 25 °C, 6 h; (2) $(NH_4)_2CO_3$, EtOH, 25 °C, 12 h, 80%; (f) NaOH (1M), EtOH, 25 °C, 2 h, 54%.

Table 1. Quinoxalinone- and quinoline-type inhibitors: polarity and antithrombotic activity



10



11

Compd	Thrombin inhibition IC_{50}, μM	Factor Xa inhibition IC_{50}, μM	aPTT ED_{200} (μM)	Log P (octanol/water, pH 7.4)	Trypsin inhibition IC_{50}, μM	Plasmin inhibition IC_{50}, μM
10	0.01	10.6	0.43	0.5	0.41	75
11	0.006	12.1	0.94	1.7	2.4	20

amide group. This modification resulted in equipotent enzyme inhibitors with improved prolongation of the activated partial thromboplastin time (aPTT) as exemplified for **10** compared to its quinoline analogue **11** (Table 1). The higher antithrombotic activity of **10** corresponds well with the lower lipophilicity of **10** as indicated by its lower log P value (octanol/water).

The X-ray structure of compound **1** complexed with thrombin had clarified that the ester group interacts with the S4-subsite.[2] Two of the cyclopentyl methylene groups point into the bulk solvent indicating that they do not contribute to the enzyme affinity. Therefore we modified this fragment by exchanging the cyclopentyl moiety with cyclopropyl or α,α-dimethyl-methylene in order to reduce the lipophilicity.

As shown in Table 2 the cyclopropyl derivative **12** and the corresponding dimethyl-methylene analogue **10** (Table 1) were equipotent. The removal of the S4 substituent resulted in an approximately 100-fold loss of activity in the corresponding acid **13**. In contrast, introduction of several amide groups resulted in a remarkable improvement of the in vitro activity. The inhibition of thrombin was independent on the amide type: the dimethyl amide **14** as well as the pyrrolidine and piperidine derivatives **15**–**18** showed enzyme inhibition in the lower nanomolar range. Compounds **12** and **14**–**17** also inhibited trypsin with similar potency than factor Xa, but were quite selective versus plasmin.

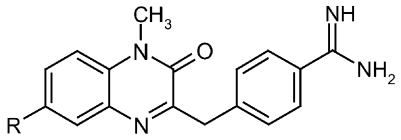
Factor Xa activity is strongly dependent on monocyclic groups like piperidine or pyrrolidine amides. This can be explained by the nature of the S4-subsite in factor Xa, which is formed by aromatic amino acids and preferably interacts with cyclic or (hetero)aromatic residues.[5] The cyclopropyl derivative **16** inhibits factor Xa with an IC_{50} of 84 nM and was the most potent example within this series. The high in vitro potency and the increased polarity of these compounds (e.g., **15**: log P = −1.0, **16**: log P = −1.5) results in a remarkable improvement of the antithrombotic activity in vitro. Compound **16** doubles the aPTT at a concentration less than 0.1 μM (aPTT: ED_{200} = 0.095 μM) and, in this regard, seems to be one of the most potent antithrombotic agents in vitro reported so far.

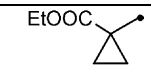
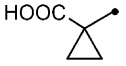
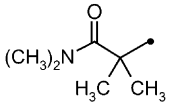
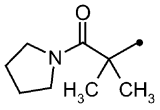
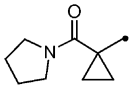
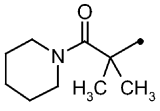
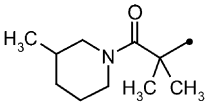
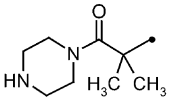
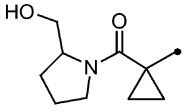
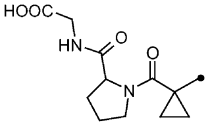
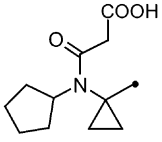
At the piperidine ring a methyl group can be introduced at position 3 without loss of affinity versus both enzymes (**18**). On the other hand, replacement of the piperidine moiety by piperazine results in decreased activity (**19**). Interestingly, the introduction of a hydroxymethyl group at the pyrrolidine position 2 had no influence on the inhibition of thrombin, whereas factor Xa affinity is tenfold reduced (**20**).

The X-ray structure of **17** in complex with trypsin, used here as a factor Xa active site surrogate,[6] is shown in Figure 2.[7] As anticipated the benzamidine P1 group forms the well-established twin-twin saltbridge interaction with Asp 189. The S2-pocket of the enzyme is neatly occupied by the N-methyl group of the central quinoxalinone scaffold. The hydrophobic S4-pocket and the lipophilic groove in front of the S4-subsite are occupied by the piperidine ring and the geminal dimethyl substituents, respectively. A superimposition of the trypsin bound conformation of **17** onto the active site of factor Xa[6b] (factor Xa coordinates taken from PDB-entry 1G2L) reveals an unfavorable steric repulsion of the quinoxalinone *N*-methyl group with the Y99 OH group and of the piperidine moiety with F174. After energy minimisation[8] of this starting geometry of the complex, it appears that a shift of the quinoxalinone scaffold and a small change of the position of the piperdine ring does not suffice to remove the steric clash. In addition, a change of the original positions of residues Y99 and F174 is necessary to accommodate the inhibitor in the factor Xa active site. The energy required for this induced fit might explain the moderate binding affinity of **17** to factor Xa.

Unfortunately, non of the compounds **14**–**20** could be used for in vivo studies due to cardiovascular side effects (e.g., hypotension) at therapeutical dosages.[9] On the other hand, it has been reported in the literature that introduction of a negatively charged group into a benzamidine derivative resulted in improved tolerability and pharmacokinetics in vivo.[10] Therefore, the carboxylate containing analogues **21** and **22** were designed and synthesized. As indicated with **21**, the introduction of a negatively charged group at the pyrrolidine ring is accompanied with a 10-fold reduced thrombin affinity and a complete loss of factor Xa inhibition. On the

Table 2. Biological activity of quinoxalinone derivatives

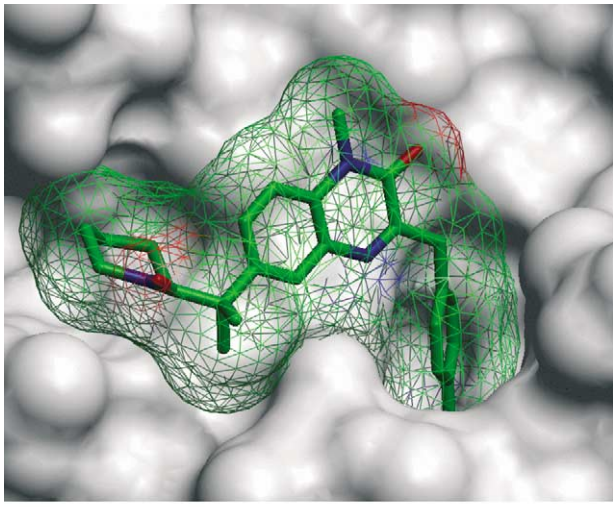


Compd	R	Thrombin inhibition IC_{50}, μM	Factor Xa Inhibition IC_{50}, μM	aPTT ED_{200} (μM)	Trypsin inhibition[a] IC_{50}, μM	Plasmin inhibition[a] IC_{50}, μM
12		0.027	46	0.67	0.58	69
13		3.3	> 100	> 10	n.d.	n.d.
14		0.016	9.2	0.41	9.5	> 100
15		0.005	0.5	0.16	0.32	53
16		0.008	0.084	0.095	0.31	66
17		0.008	0.4	0.25	2.2	60
18		0.012	0.59	0.12	n.d.	n.d.
19		0.068	31	0.71	n.d.	n.d.
20		0.013	1.4	0.21	0.85	72
21		0.19	> 100	0.91	2.4	68
22		0.015	3.0	0.45	0.55	36

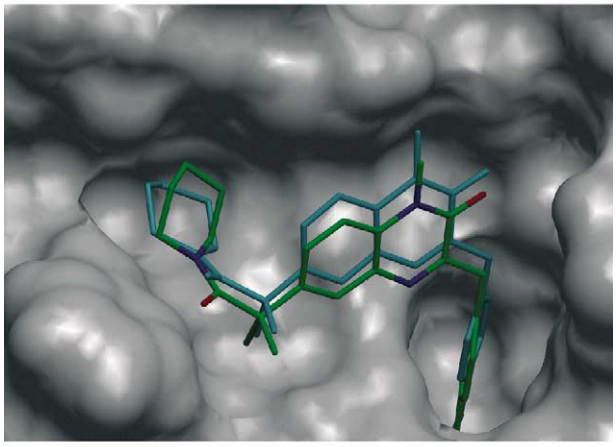
[a]n.d. = not determined.

other hand, when the cyclopropyl carboxamide moiety was replaced by an acylated cyclopentyl-aminocyclopropyl group, a potent thrombin inhibitor was obtained (**22**), which showed an aPTT doubling at a concentration of 0.45 μM.

In contrast to the cationic compounds **14**–**20** the zwitterionic analogues **21** and **22** were well tolerated after iv-administration to rats. However, in the pharmacodynamic studies in rats, both compounds showed an unfavorable profile, which seemed to be inadequate for therapeutic use. Therefore, further research concentrated on the identification of novel heterocyclic analogues with improved pharmacokinetic profiles and oral activity. The results of these efforts will be published in the near future.



(a)



(b)

Figure 2. (a) Crystal structure of **17** in trypsin; (b) Model of **17** in factor Xa (green) after superposition of the trypsin bound conformation (blue) onto factor Xa (coordinates taken from PDB-entry 1G2L) and energy minimization.

Based on potent and selective thrombin inhibitors covering a central quinoline template, novel coagulation inhibitors have been designed and synthesized. These quinoxalinone derivatives inhibited thrombin and factor Xa in the nanomolar range and were very potent antithrombotic agents in vitro.[11] However, compounds with promising in vitro activity suffered from low tolerability or unfavorable pharmacokinetics. Recent results of our efforts to identify potent dual inhibitors of thrombin and factor Xa useful for preclinical and clinical studies will be reported in the near future.

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7. Bovine trypsin was obtained from Sigma (Deisenhofen, Germany) and crystallized complexed with benzamidine. Crystals were grown from small seed crystals of the 'open' crystal form[14] in 1.7–1.9 M ammonium sulfate, pH 6. Cocrystals were generated by soaking crystals with mother liquor containing 1 mM of 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.[15] Model building and refinement were carried out with MAIN[16] and CNS[17]; space group $P2_12_12_1$, cell constants: 63.5, 69.1, 63.7 Å; Data collection statistics: resolution limits 20.0–1.70 Å, total observations 76868, unique reflections 29802, completeness 95.6%, R merge 0.044; Refinement statistics: number of atoms in model: protein 1628, inhibitor 33, water molecules 175, calcium ions 1; number of reflections (free set) 29773 (1509), *rms* deviation from ideal geometry: bond length 0.008 Å, bond angles 1.32°, temperature factors (Å^2): 22.5 Å^2, *rms* bonded Bs 1.4 Å^2, R factor 18.9%, R free 21.1%.
8. A possible binding mode of **17** in the factor Xa active site was derived from the trypsin/**17** complex after superposition of the protein active sites using the energy minimization module within the modelling software package MOE.[12] The calculation of a minimized binding conformation was based on the MMFF94s force field[13] using MMFF94 charges. During the calculations the factor Xa binding site remained flexible in order to allow possible induced fits. The factor Xa binding site was defined by including residues within a sphere of 7 Å radius around **17** in its trypsin bound position.
9. (a) These side effects have been described elsewhere for amidine and guanidine derivatives: Schumacher, W. A.; Balasubramanian, N.; St. Laurent, D. R.; Seiler, S. M. *Eur. J. Pharmacol.* **1994**, *259*, 165. (b) Hauptmann, J. *Semin. Thromb. Hemost.* **1992**, *18*, 200. (c) Kaiser, B.; Hauptmann, J. *Cardiovasc. Drug Rev.* **1992**, *1*, 71. (d) Kaiser, B.; Hauptmann, J.; Markwardt, F. *Pharmazie* **1987**, *42*, 119.
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