New Substrate Analogue Inhibitors of Factor Xa Containing 4-Amidinobenzylamide as P1 Residue: Part 1

Andrea Schweinitz[1], Anne Stürzebecher[1], Uta Stürzebecher[2], Oliver Schuster[3], Jörg Stürzebecher[2] and Torsten Steinmetzer[1,*]

[1]*Curacyte Chemistry GmbH, Winzerlaer Str. 2, D-07745 Jena, Germany;* [2]*Zentrum für Vaskuläre Biologie und Medizin, Klinikum der Universität Jena, Nordhäuser Str. 78, D-99089 Erfurt, Germany;* [3]*Curacyte Discovery GmbH, Deutscher Platz 5d, 04103 Leipzig, Germany*

Abstract: The trypsin-like serine protease factor Xa (fXa) is located at the convergence point of the intrinsic and extrinsic coagulation cascade, and therefore has emerged as an attractive target for the design of novel anticoagulants.

During the development of substrate-analogue urokinase inhibitors we have found that the protection of the P3-DSer side chain leads to a scaffold of potent fXa inhibitors with the general structure R_1-SO_2-DSer(R_2)-Gly-4-amidinobenzylamide. The first lead (**3**) with an N-terminal benzylsulfonyl group and DSer(tBu) as P3 residue inhibits human fXa with a K_i of 14 nM. A variety of derivatives with modified P4, P3, and P2 residues have been investigated in terms of inhibition of fXa and related proteases and for their anticoagulant potency and elimination behaviour.

Most inhibitors were rapidly cleared from the circulation of rats. However, compound **48** (K_i = 3.5 nM), one of the most potent and selective inhibitors containing a DArg as P3 residue was relatively slowly eliminated ($t_{1/2} \approx$ 1h). Inhibitor **48** doubled clotting times in human plasma at 0.32 μM (aPTT) and 0.28 μM (PT), and is approximately 10-fold more potent than the reference fXa inhibitor DX-9065a in the inhibition of the prothrombinase complex.

The structures of two inhibitors in complex with human fXa were solved by X-ray crystallography.

Key Words: Factor Xa, protease inhibitor, anticoagulants, antithrombotics.

INTRODUCTION

Blood coagulation involves a complex system that prevents the body from uncontrolled blood loss after vascular injury. An increased activation of this system can lead to serious thrombotic disorders, such as myocardial infarction, stroke, pulmonary embolism, deep-vein thrombosis and disseminated intravascular coagulation, which are the leading causes of morbidity and mortality in the Western world.

Therefore, large effort has been invested in the design of antithrombotics with improved properties compared to established therapies. One strategy is the development of specific inhibitors towards the clotting proteases, especially for thrombin and factor Xa (fXa) [1, 2]. Ximelagatran (Exanta™) was the first orally available thrombin inhibitor approved for the prevention of venous thromboembolic events in major orthopaedic surgery [3], however, it was recently removed from market due to some observed cases of liver toxicity.

There are several reasons that fXa may be a more safe and effective target than thrombin for the design of anticoagulants [6-9]. Like thrombin, fXa is a trypsin-like serine protease and the proteolytically active component of the prothrombinase complex. This complex, located at the convergence point of the extrinsic and intrinsic clotting system, is responsible for thrombin generation. In contrast, thrombin inhibitors will not by themselves prevent the continuing formation of thrombin from prothrombin. The plasma level of the zymogen fX (5-10 mg/l) is much lower than the concentration of prothrombin (50-100 mg/l) and one molecule of fXa can generate more than 100 molecules of thrombin per minute. Therefore, it is assumed that lower inhibitor concentrations are required for the control of fXa activity, which could reduce bleeding complications often observed during the use of thrombin inhibitors. In support of this hypothesis it has been shown that the specific fXa inhibitor tick anticoagulant peptide (K_i = 180 pM) is fully antithrombotic at doses, which result in only a modest increase in bleeding time in animal models of venous thrombosis [10, 11]. In contrast, due to a narrow therapeutic window, clinical studies with the highly specific thrombin inhibitor r-hirudin often revealed bleeding complications [12].

FXa is also a more selective enzyme than the multifunctional thrombin [13], which can activate various other clotting factors and proteins with pro- or anticoagulant activity. The main substrate of fXa is prothrombin, although also functions of fXa on cellular receptors have been described. FXa can bind to effector-cell protease receptor-1 (EPR-1), a protein that is immunologically related to FVa and probably helps to localize fXa on the surface of various cell types. FXa induced cellular responses *via* binding to EPR-1 were found on platelets, fibroblasts, and on vascular and inflammatory cells [14-17]. An additional endogenous

*Address correspondence to this author at the Curacyte Chemistry GmbH, Winzerlaer Str. 2, D-07745 Jena, Germany; Tel.: +49-3641-508516; Fax: +49-3641-508507; Email: torsten.steinmetzer@curacyte.com

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fXa receptor in endothelial cells is probably the protease-activated receptor 2 [18].

The suitability of fXa as a target for the design of anticoagulants could also be confirmed by clinical studies with the synthetic pentasaccharide fondaparinux, which mimics the active site of heparin. Although dependent on antithrombin to inhibit fXa and therefore thrombin generation, fondaparinux was characterized as a safe and effective anticoagulant and therefore approved in 2001 for use in prophylaxis of deep vein thrombosis in patients undergoing orthopaedic surgery [19].

During the development of synthetic inhibitors of urokinase-type plasminogen activator (uPA) with the lead structure benzylsulfonyl-DSer-Gly-4-amidinobenzylamide (**1**) [20, 21] we found that the protection of the DSer side chain changes the selectivity within this inhibitor-type and leads to relatively selective fXa inhibitors. The optimization of the first lead compounds (**2**-**3**, Table **1**), which were rapidly eliminated from the circulation of rats, resulted in a series of highly potent fXa inhibitors with improved pharmacokinetic properties. The development of these inhibitors, their enzyme kinetic characterization and anticoagulant activity is described in this paper. The structures of two inhibitors in complex with fXa were solved by X-ray crystallography.

RESULTS

First Analogues

The inhibitors consist of a P4 benzylsulfonyl group, an amino acid in D-configuration in P3 position, glycine or an L-amino acid as P2 residue and a P1 4-amidinobenzylamide (4-Amba). Table **1** summarizes the inhibition constants of first analogues **1**-**3** towards selected enzymes. In addition to fXa the potency of the inhibitors was tested also towards thrombin, trypsin and the fibrinolytic enzymes plasmin and uPA [22].

The protection of the DSer side chain in P3-position strongly reduced uPA affinity and improved the inhibitory potency towards fXa. However, during pharmacokinetic characterization it was observed that inhibitors **2** and **3** were eliminated very fast ($t_{1/2} < 0.3$ h) from the circulation of rats after intravenous bolus application of 1 mg/kg due to a rapid hepato-biliary clearance. For inhibitor **3** approximately 80 % of the applied dose was found in the bile and only low amounts of the inhibitors could be detected in urine.

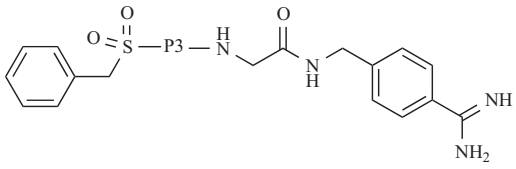
X-Ray Crystallography

The x-ray structures of inhibitors **2** and **3** in complex with human fXa were solved to obtain information regarding their binding mode (Fig. **1**).

In contrast to the extended L-shaped structure of the bound fXa inhibitors DX-9065a [23] or ZK-807834 [24] the main chain conformation of inhibitors **2** and **3** in complex with fXa shows a U-shaped binding mode. This conformation resembles previously reported structures, which were observed for closely related substrate-analogue inhibitors within the active site of uPA or thrombin [21, 25].

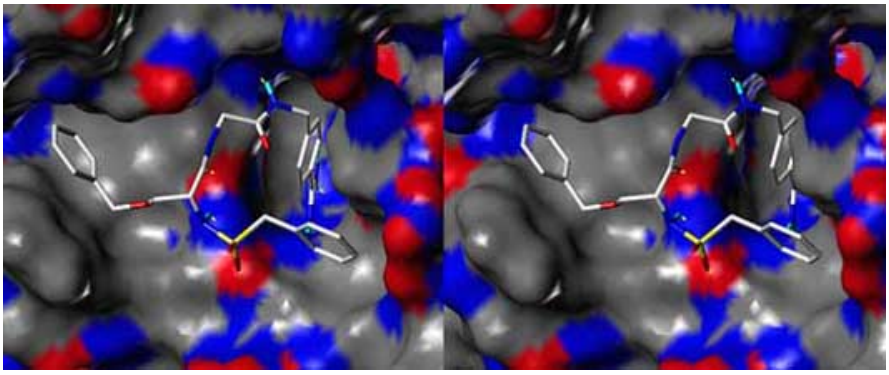
In both crystal structures an important feature common to benzamidine based serine protease inhibitors is a salt bridge between the positively charged amidine group with the carboxyl of Asp189 in the S1 pocket. Additionally, a hydrogen bond from the amidine proton to the protein backbone carbonyl oxygen of Gly219 fixes the orientation of the P1 group. Another hydrogen bond is located between the amide proton of the 4-Amba moiety and the carbonyl group of Ser214 at the entry of the S1 pocket. Thus, a rigid conformation of the benzamidine portion of both inhibitors can be assumed. The amide bond-linked glycine residue occupies the S2 site, which is shielded by the bulky Tyr99. The complex is further stabilized by a hydrogen bond from the carbonyl group of the P2 glycine to the side chain amid proton of Gln192. The aryl binding site formed by the aromatic amino acids Tyr99, Phe174, and Trp215 of fXa is filled out suitably with the DSer O-benzyl or *tert.*-butyl ether groups of inhibitors **2** and **3**. Additional hydrogen bonds exist between the carbonyl oxygen and NH of the P3 amino acid and the amide proton and carbonyl group of Gly216, respectively. The benzylsulfonyl moiety contributes to the binding affinity by two features. A hydrogen bond is formed between one sulfonyl oxygen and the amide proton of Gly219, and in addition, the benzene ring occupies a shallow hydrophobic subpocket of fXa and makes hydrophobic contacts to the Cys192-Cys220 disulfide bridge.

Table 1. Inhibition of Trypsin-Like Serine Proteases by Inhibitors of the General Formula



No.	P3	K_i (μM)				
		fXa	thrombin	plasmin	uPA	trypsin
1	DSer	2.4	13	11	0.036	0.15
2	DSer(Bzl)	0.081	0.18	2.3	0.84	0.065
3	DSer(tBu)	0.014	0.22	7.7	1.2	0.066

A:



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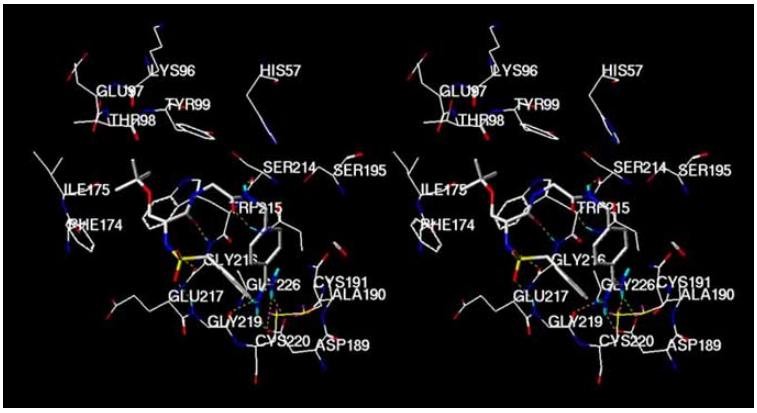


Fig. (1). (A) Stereo view of inhibitor **2** within the active site of fXa obtained by X-ray crystallography. The inhibitor is shown as sticks with atom dependent colors, the protein is visualized by a Connolly surface. Blue and red surface areas are showing hydrogen acceptors and donators, respectively. Gray areas have no hydrogen bonding properties. Hydrogen bonds are represented by yellow dashed lines.

(B) Stereo view of inhibitor **3** within the active site of fXa. The protein residues are shown in thin lines. The inhibitor is shown as sticks with atom-dependent colors. Hydrogen bonds are represented by yellow dashed lines.

Starting from the lead structures **2** and **3** we performed an optimization of the P4-P2 moiety of this inhibitor type to enhance fXa affinity and to reduce the rapid elimination from the blood circulation of rats. Therefore, in addition to several non-polar substituents more polar moieties and especially carboxyl groups were incorporated. This strategy was described previously for several types of thrombin inhibitors to improve their half-life *in vivo* [26, 27].

Modification of the P4 Group

In a first series the P3-P1 segment was maintained and only the P4 benzylsulfonyl group was modified. The structures of the synthesized inhibitors and their inhibition constants are summarized in Table **2**.

The benzylsulfonyl structure in P4 position is essential for affinity, it could be replaced only by the saturated cyclohexylmethylsulfonyl group (**4**). All other inhibitors, the longer phenylethylsulfonyl (**17**) and shorter phenylsulfonyl derivative (**30**), as well as the aliphatic ethylsulfonyl compound (**31**) or the Cbz-protected urethane derivative (**26**) have low fXa affinity. Some of the substituted benzylsulfonyl derivatives show similar potency as found for the lead structure **3**. Among the most potent inhibitors are analogues, which contain a nitro or amino group at para postion (**9**, **10**).

Modification of P2 Position

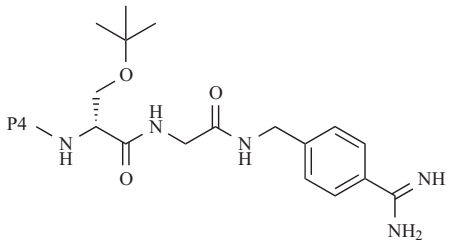
The inhibitory potency of several P2-modified analogues with a constant Bzls-DSer(tBu) P4-P3-segment is summarized in Table **3**.

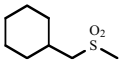
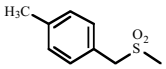
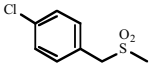
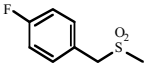
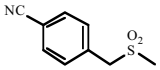
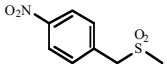
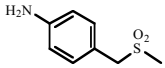
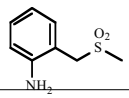
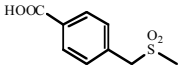
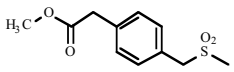
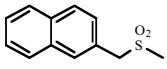
Replacement of the P2 Gly by Ala and Pro maintained significant fXa affinity compared to inhibitor **3**, but strongly reduced selectivity; both compounds (**33**, **34**) are very potent thrombin inhibitors. A strong fXa affinity was also maintained after incorporation of P2 Ser (**37**) and some analogues of proline (**35**, **36**). The P2 residue was further modified by commercially available natural or non-natural α-amino acids (structures not shown in Table **3**), however, all of them inhibited fXa with K_i values > 0.5 μM.

Modification of P3 Position

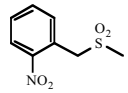
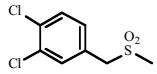
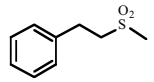
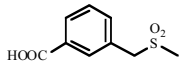
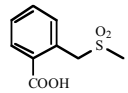
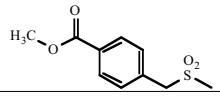
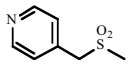
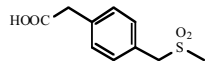
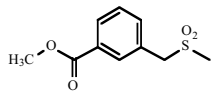
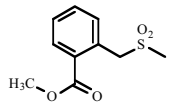
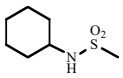
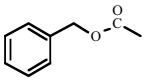
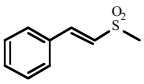
In an additional series the P3 position was modified, keeping Gly and the benzylsulfonyl group as constant P2 and

Table 2. Inhibition of Trypsin-Like Serine Proteases by Inhibitors with Modified P4-Residues of the General Formula



No.	P4	K_i (μM)				
		fXa	thrombin	plasmin	uPA	trypsin
4		0.015	0.12	4.5	1.1	0.04
5		0.026	0.11	8.1	0.74	0.052
6		0.023	0.17	12	0.74	0.91
7		0.031	0.16	27	0.83	0.031
8		0.040	0.34	10	1.4	0.091
9		0.0075	0.14	3.5	0.59	0.053
10		0.018	0.077	2.9	0.59	0.02
11		0.023	0.12	8.7	2.8	0.071
12		0.042	2.2	12	3.6	0.16
13		0.11	0.27	4.6	0.89	0.027
14		0.019	0.7	9.3	5.7	0.13

(Table 2. Contd....)

No.	P4	K_i (μM)				
		fXa	thrombin	plasmin	uPA	trypsin
15		0.097	0.2	9.9	3	0.14
16		0.037	0.51	18	2.7	0.21
17		0.076	1.3	60	5.4	0.21
18		0.057	10	21	4.8	0.2
19		0.10	4.7	100	4.1	0.2
20		0.17	0.36	3.8	5.6	0.037
21		0.11	0.46	14	1.7	0.097
22		0.098	2.5	7.3	2.1	0.068
23		0.18	1.1	14	3.2	0.098
24		0.085	0.7	55	2.8	0.11
25		0.10	5.4	5	44	0.11
26		0.37	1.6	71	16	0.82
27		0.45	3.1	120	11.2	0.66

(Table 2. Contd....)

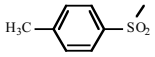
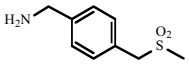
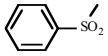
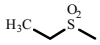
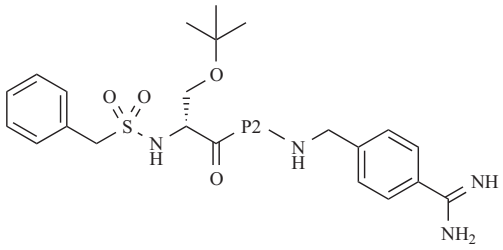
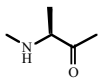
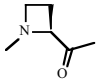
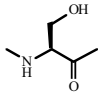
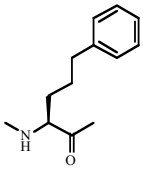
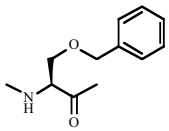
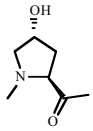
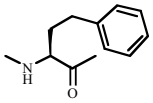
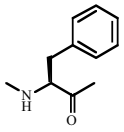
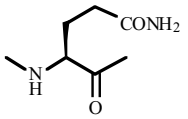
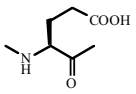
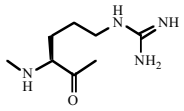
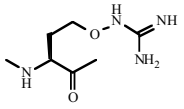
No.	P4	K_i (μM)				
		fXa	thrombin	plasmin	uPA	trypsin
28		0.17	1.9	0	3.5	0.26
29		0.38	0.12	6.4	2	0.077
30		0.62	2	24	2.3	0.22
31		0.68	4.4	62	6.2	0.55
32	H	16	1.3	190	41	0.98

Table 3. Inhibition of Trypsin-Like Serine Proteases by Inhibitors with Modified P2-Residues of the General Formula



No.	P2	K_i (μM)				
		fXa	thrombin	plasmin	uPA	trypsin
33		0.043	0.0021	0.28	0.3	0.0023
34		0.0088	0.002	0.069	0.18	0.0018
35		0.02	0.006	0.076	0.91	0.011
36		0.018	0.0015	0.39	0.16	0.004
37		0.056	0.047	0.64	0.51	0.0052

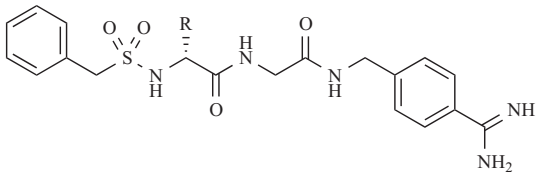
(Table 3. Contd....)

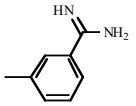
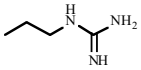
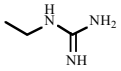
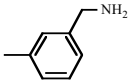
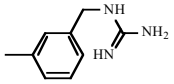
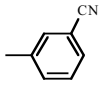
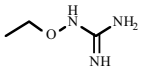
No.	P2	K_i (μM)				
		fXa	thrombin	plasmin	uPA	trypsin
38		0.054	0.042	0.24	1.6	0.02
39		0.16	0.096	0.16	0.55	0.0076
40		0.098	0.0033	0.082	0.33	0.0025
41		0.036	0.048	0.027	1.4	0.0051
42		0.17	0.16	0.04	2.9	0.016
43		0.19	0.021	0.2	1.3	0.0025
44		0.11	2.6	1.1	6.9	0.0091
45		0.15	0.065	0.08	0.3	0.0043
46		0.24	0.038	0.032	1.1	0.0046

P4 residues, respectively. Due to known data from several series of substrate-analogue transition state fXa inhibitors [28, 29] we concentrated on the incorporation of amino acids with basic or hydrophobic side chains (Table **4**).

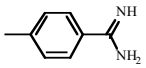
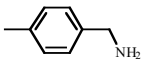
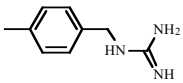
The highest fXa affinity was found for inhibitors **47** and **48** containing the basic D-3-amidinophenylalanine or DArg in P3 position, respectively. In general, the meta-substituted DPhe derivatives were more potent than the para-analogues. This was observed also for the aminomethyl- and the guanidinomethyl compounds. Efforts to reduce the basicity of the DArg side chain (pKa ≈ 13) lead to the isostere D-canavanin inhibitor **56** (pKa of the P3 side chain ≈ 7 [30]). Inhibitor **56** was less potent than the analogous DArg

Table 4. Inhibition of Trypsin-Like Serine Proteases by Inhibitors with Modified P3-Residues of the General Formula



No.	R	K_i (μM)				
		fXa	thrombin	plasmin	uPA	trypsin
47		0.004	0.085	1.0	0.42	0.016
48		0.0035	1.4	2.2	0.33	0.01
49		0.0069	0.009	0.066	0.33	0.02
50		0.0067	2.1	3.2	0.094	0.015
51		0.015	0.04	0.75	0.8	0.013
52		0.022	0.059	0.5	0.59	0.013
53		0.023	0.16	0.47	0.59	0.03
54		0.078	0.068	1.2	0.9	0.038
55		0.025	0.14	0.33	0.088	0.012
56		0.033	2.5	2.6	0.43	0.047
57		0.052	0.11	1.8	0.43	0.023

(Table 4. Contd....)

No.	R	K_i (μM)				
		fXa	thrombin	plasmin	uPA	trypsin
58		0.051	0.28	1.2	0.87	0.067
59		0.10	0.68	1.5	0.82	0.076
60		0.16	0.14	1.5	0.78	0.041
61		0.19	0.78	1.0	1.0	0.07

derivative, but still inhibited fXa with a K_i–value of 33 nM. A high potency but poor selectivity was observed for the D-cyclohexylalanine inhibitor **49**, which also efficiently inhibited thrombin and plasmin.

Anticoagulant Activity

Selected inhibitors of these series were investigated more in depth for their anticoagulant activity and inhibition of the prothrombinase complex. In addition, their half-life in the circulation in rats after intravenous treatment of an inhibitor dose of 1 mg/kg was determined (Table **5**). For comparison, the data for DX-9065a, an archetypical fXa inhibitor, which reached phase II clinical development, are given [31, 32].

Among the compounds shown in Table **5** the highest anticoagulant activity was found for inhibitors **47** and **48**. Both analogues potently inhibit the prothrombinase complex and show stronger anticoagulant activity in the PT- and aPTT assays than DX-9065a. In addition, both inhibitors were relatively slowly eliminated from the circulation of rats.

DISCUSSION

This inhibitor type containing a C-terminal 4-amidinobenzylamide necessarily requires an N-terminal benzylsulfonyl or cyclohexylmethylsulfonyl group to maintain high fXa affinity. Poor potency was observed for inhibitors **28**, **31**, **26** and **32** containing a tosyl-, ethylsulfonyl- and Cbz-group or hydrogen in P4 position, respectively. Obviously, in addition to the single hydrogen bond formed from one of the sulfonyl oxygen atoms of the inhibitor to the NH of Gly219 (Fig. **1B**), hydrophobic interactions of the benzyl group, which is located in a well-defined shallow subpocket, strongly contribute to fXa inhibition.

Table 5. Anticoagulant Activity in Human Plasma and Inhibition of Human fXa and the Prothrombinase Complex by Selected Inhibitors. In Addition, the Elimination Half-Life (β-Phase) in Rats After Intravenous Application of 1 mg/kg is given

No.	K_i fXa (μM)	IC_{50} (μM) PTC	IC_{200} (μM)			$t_{\frac{1}{2}\beta}$ (h)
			aPTT	PT	TT	
DX-9065a	0.024	0.24	0.92	0.53	> 1000	0.98
47	0.004	0.082	0.20	0.23	0.5	0.7
48	0.0035	0.026	0.32	0.28	4.4	1.01
49	0.0069	n.d.	0.19	0.19	0.13	n.d.
50	0.0067	0.035	0.39	0.36	5.3	0.39
3	0.014	0.42	0.77	0.86	1.1	< 0.3
56	0.033	n.d.	1.2	1.35	12	0.28
12	0.042	1.7	1.9	2.2	3.4	0.38

n.d. = not determined

Surprisingly, the benzylsulfonyl group could be replaced in other series of substrate analogue fXa inhibitors containing an arginal or arginyl ketone moiety in P1 position. In that case, the incorporation of urethane-like Boc- or ethyloxy-carbonyl-residues in P4 position or the introduction of an N-terminal naphthylethylsulfonyl- or phenylethylsulfonyl-group was tolerated and only a moderate loss in inhibitory potency occurred (2-4-fold decrease in the given IC_{50} values towards fXa) [28, 29].

The S2 site of fXa is partially shielded by the bulky side chain of Tyr99. Therefore it was thought that only Gly is accepted as P2 residue, whereas sterically more demanding amino acids reduce the fXa affinity. We could also demonstrate that Ala, Pro and some related analogues are well tolerated as P2 residues with respect to fXa affinity. However, all of these inhibitors have a reduced selectivity and potently inhibit also thrombin and plasmin. A dual thrombin and fXa inhibition could offer some advantage with respect to anticoagulant and antithrombotic potency and might be a new strategy for further inhibitor design [33, 34]. However, the inhibition constant for plasmin should not be below 0.5 μM to avoid side effects on fibrinolysis, as has been previously described during the development of the thrombin inhibitor melagatran and its analogues [35]. Regarding the affinity and selectivity profile, Gly is clearly the preferred P2 residue in these substrate analogue fXa inhibitors.

The aryl-binding site of fXa formed by the amino acids Tyr99, Phe174 and Trp215 is well suited to accommodate a variety of P3 amino acids in D-configuration containing hydrophobic or basic side chains. The highest affinity found for the D-3-amidinophenylalanine-derived inhibitor **47** might be based on a combination of hydrophobic contacts and cation/π-interactions, as described for other factor Xa inhibitors [36, 37]. In addition, the aryl binding site contains several carbonyl oxygen atoms of the amino acids Ile175, Thr98 and Glu97 which may be involved in direct or water-mediated hydrogen bonds to a basic P3 side chain [23, 38, 39].

In case of inhibitors **47** and **48** we observed a significant correlation between their *in vitro* potency based on inhibition of fXa and their anticoagulant activity in the standard clotting assays (aPTT and PT) in human plasma. In addition, both inhibitors potently inhibit the prothrombinase complex *in vitro* and have an improved half-life in the circulation of rats, which is comparable to that of DX-9065a. However, due to their strongly charged P1 and P3 residues both compounds lack complete oral bioavailability in rats ($F < 1$ %).

Therefore, further optimization has been performed to improve the overall profile of the inhibitors. These results will be published in a subsequent paper.

MATERIALS AND METHODS

Enzyme Kinetic Measurements and Clotting Assays

Human fXa and the substrate Pefachrome Xa were purchased from Haemochrom Diagnostica GmbH (Essen, Germany) and Pentapharm Ltd. (Basel, Switzerland), respectively. The enzyme kinetic measurements were performed in a microplate reader at 405 nm and 37 °C, the K_i-values were obtained from Dixon-plots. All other kinetic measurements with thrombin, uPA, plasmin and trypsin and the clotting assays in human plasma were performed as described previously [22].

Inhibition of the Prothrombinase Complex

The prothrombinase complex was prepared at 0 °C from 250 μl cephalin (lyophilized cephalin of the PTT-reagent (Roche Diagnostics, Mannheim, Germany) dissolved in 5 ml buffer A (50 mM Tris-HCl, pH 7.5, containing 0.9 % NaCl), 50 μl 0.5 M $CaCl_2$, 25 μl human fXa (0.16 μg/ml), 80 μl fVa (American Diagnostica, Greenwich, USA, 52 μg/ml) and 1845 μl buffer B (50 mM Tris-HCl, pH 7.5, containing 0.9 % NaCl and 0.1 % PEG 6000). The complex was incubated at 0 °C for 30 min.

45 μl of the complex were incubated with 25 μl of the inhibitor solution (5 different concentrations, dissolved in buffer B and 5 % ethanol) for 5 min, followed by addition of 30 μl human prothrombin (29 μg/ml, Haemochrom Diagnostica, Essen, Germany). The incubation at 37 °C was stopped after 10 min by addition of 150 μl 0.083 mM EDTA in buffer B.

For determination of the thrombin activity 25 μl of this solution was added to 200 μl EDTA in buffer B and 50 μl substrate (0.6 mM, S-2238, Haemochrom Diagnostica), and the absorbance was measured at 405 nm in a microplate reader. The IC_{50}-value is defined as the concentration, which inhibits the prothrombinase complex catalyzed thrombin formation by 50 %. For this assay only inhibitors with low inhibitory potency towards thrombin ($K_i > 50$ nM) have been used to avoid an influence on the determined IC_{50}-values by direct thrombin inhibition.

Elimination Studies

The plasma elimination studies in rats after intravenous injection of the inhibitors (1 mg/kg) were performed as described previously [21]. The given $t_{\frac{1}{2}}$-values (Table **5**, mean of 3-4 animal experiments) were obtained from analysis of the plasma concentration-time data according to a biphasic two-compartment model [40].

Synthesis

Some of the substituted non-commercially available benzylsulfonylchlorides were prepared from the appropriate benzylbromides *via* sulfonates as described previously [21], all other amino acid derivatives, solvents and reagents for synthesis were purchased from commercial sources. The molecular mass of the inhibitors was determined using a Finnigan ESI-MS LCQ spectrometer (Bremen, Germany). Analytical HPLC experiments were performed on a Shimadzu LC-10A system (Phenomenex Luna C_{18}, 5 μm column, 4.6 × 250 mm) with a linear gradient of acetonitrile (10-70 % in 60 min, detection at 220 nm) containing 0.1 % TFA at a flow rate of 1 ml/min. The final inhibitors were purified to more than 97 % purity by preparative HPLC on a Shimadzu LC-8A system (Phenomenex Luna C_{18}, 5 μm column, 30 × 250 mm, detection at 220 nm) with a linear gradient of acetonitrile (45 % increase in 120 min) containing 0.1 % TFA at a flow rate of 10 ml/min and obtained as TFA-salts after lyophilization. Thin layer chromatography was performed on

silica gel plates (silica gel 60 F_{254}, Merck, Darmstadt, Germany) in n-butanol/acetic acid/water 4/1/1 (v/v/v).

The inhibitors were prepared in a manner analogous to the synthesis of structurally related urokinase inhibitors [20, 21], and exemplarily described for inhibitor **48** (Scheme **1**).

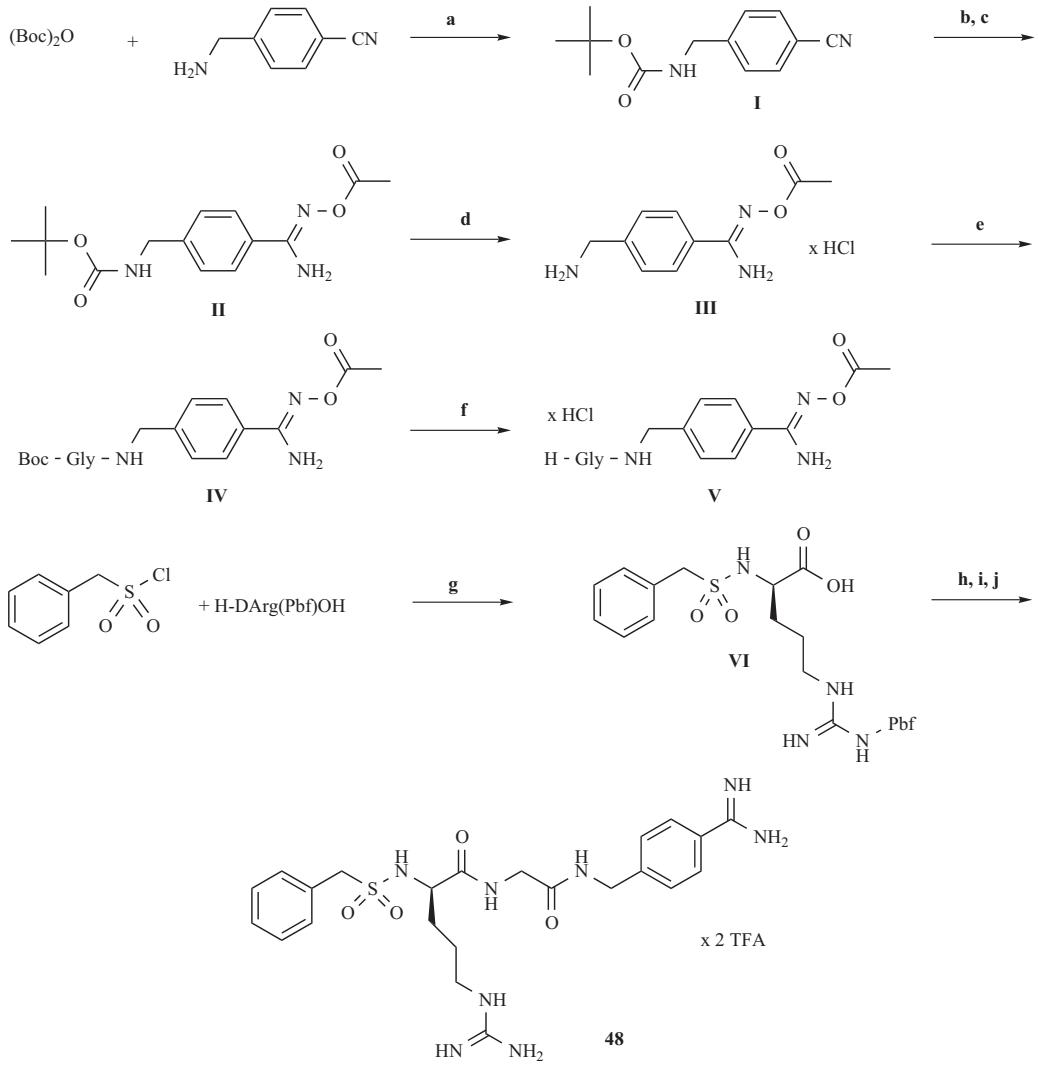
Boc-4-cyanobenzylamide (I)

100 g (0.593 mol) 4-cyanobenzylamine x HCl was solved in 1.2 l dioxane and 600 ml 2 N NaOH. At 0 °C 142.3 g (0.652 mol) di-tert.-butyl pyrocarbonate were added in two portions within 10 minutes. The pH was maintained at pH 9-10 by addition of 2 N NaOH and the mixture was stirred for additional 4 hours. The dioxane was removed in vacuo and the residue was dissolved in ethyl acetate and 5 % $KHSO_4$-solution. The organic layer was washed 3 times with 5 % $KHSO_4$-solution and 3 times with brine. The organic layer was dried over Na_2SO_4 and the solvent was evaporated. The product was obtained as a white solid and dried in vacuum (132.6 g, 0.57 mol, 96 %, white solid).

HPLC: 41.6 min, TLC: $R_f = 0.91$

Boc-4-acetylhydroxyamidinobenzylamide (II)

130 g (0.56 mol) Boc-4-cyanobenzylamide (**I**), 58.4 g (0.84 mol) hydroxylamine x HCl and 146 ml DIEA were dissolved in 1.5 l methanol. The mixture was refluxed for 6 hours and was stirred at room temperature overnight. The solvent was evaporated in vacuum, the oily residue (HPLC:



Scheme (1). Synthesis of inhibitor **48**. **a)** dioxane / 2 N NaOH; **b)** NH_2OH x HCl, DIEA in EtOH, reflux 6 h, 12 h room temperature; **c)** Ac_2O in AcOH; **d)** 1 N HCl in acetic acid; **e)** Boc-Gly-OH, mixed anhydride in DMF; **f)** 1 N HCl in acetic acid; **g)** acetonitrile / water and DIEA 1 h at 0°C, 12 h room temperature, **h)** compound **V**, PyBop/DIEA; **i)** H_2 and Pd/C; **j)** 90 % TFA 1 h room temperature followed by preparative HPLC.

17.8 min; TLC: R_f = 0.7; MS calc.: 265.1, found: 266.2 $(M+H)^+$) was dissolved in 1.5 l acetic acid and treated with 160 ml (1.68 mol) acetic anhydride. After 30 min stirring, the solvent was evaporated. The oily residue was solved in ethyl acetate and washed 3 times with brine. After evaporation of the solvent the product was crystallized from ethyl acetate (110.6 g, 0.36 mol, 64 %).

HPLC: 28.6 min; TLC: R_f = 0,87; MS calc.: 307.1, found: 308,2 $(M+H)^+$

H-4-acetylhydroxyamidinobenzylamine x HCl (III)

50 g (163 mmol) Boc-4-acetylhydroxyamidinobenzylamide (**II**) was dissolved in 1.0 l acetic acid and treated with 800 ml 1 N HCl in acetic acid. The mixture was shaken, after some minutes the product began to precipitate. After 75 min the product was filtered, washed with diethyl ether, and dried in vacuum (36 g, 148 mmol, 91 %).

HPLC: 5.2 min; TLC: R_f = 0.48; MS calc.: 207.1, found: 207.9 $(M+H)^+$

Boc-Gly-4-acetylhydroxyamidinobenzylamide (IV)

24.5 g (140 mmol) Boc-Gly-OH were solved in 1 l DMF. At –15 °C 15.4 ml (140 mmol) N-methylmorpholine and 18.2 ml (140 mmol) isobutyl chloroformate were added. After 10 min stirring 35.4 g (145 mmol) H-4-acetylhydroxy-amidinobenzylamine x HCl (**III**) and 15.9 ml (145 mmol) N-methylmorpholine were added to the mixed anhydride, the mixture was stirred 1 h at –15 °C and overnight at room temperature. The DMF was evaporated, the residue was dissolved in ethyl acetate (1.7 l) and washed 3 x with 300 ml saturated $NaHCO_3$-solution and 3 x with 300 ml brine. The organic layer was dried over Na_2SO_4 and evaporated (44.7 g, 123 mmol, 87.7 % amorphous solid).

HPLC: 21.7 min; TLC: R_f = 0.74; MS calc.: 364.2, found 365.4 $(M+H)^+$

H-Gly-4-acetylhydroxyamidinobenzylamide x HCl (V)

40 g (110 mmol) of **IV** was dissolved in 300 ml acetic acid and treated with 800 ml 1N HCl in acetic acid at room temperature. After 60 min the solvent was partially removed and the product was precipitated by the addition of ether, filtered, washed with ether, and dried in vacuum (31.6 g , 105 mmol, 95.5 %, white solid).

HPLC: 6.2 min; TLC: R_f = 0.33; MS calc.: 264.1, found 265.3 $(M+H)^+$

Benzylsulfonyl-DArg(Pbf)-OH (VI)

To a stirred solution of 3 g (7 mmol) H-DArg(Pbf)-OH and 1.35 ml (7.7 mmol) DIEA in 40 ml acetonitrile and 20 ml water 4 g (21 mmol) benzylsulfonyl chloride and 3.7 ml (21 mmol) DIEA were added over a period of 60 min at 0 °C. The pH was maintained between 7-8 by addition of DIEA. After stirring at ambient temperature overnight, the solvent was removed in vacuo. The residue was solved in a mixture of ethyl acetate and water (acidified with 1 N HCl to pH 2.5-3.0) and the product was extracted with ethyl acetate. The organic phase was washed twice with 5 % $KHSO_4$ and brine, dried over Na_2SO_4, and evaporated. The remaining product was crystallized from ethyl acetate (3.3 g, 5.6 mmol, 80 %, white solid).

HPLC: 45.5 min; TLC: R_f = 0.83; MS calc.: 580.7, found 581.5 $(M+H)^+$

Benzylsulfonyl-DArg(Pbf)-Gly-4-acetylhydroxyamidinobenzyl-amide (VII)

1.5 g (2.6 mmol) Bzls-DArg(Pbf)-OH (**VI)** and 820 mg (2.7 mmol) H-Gly-4-acetyl-hydroxyamidinobenzylamide x HCl (**V**) were dissolved in 30 ml DMF and treated at 0°C with 1.4 g (2.7 mmol) PyBop and 1.3 ml (7.5 mmol) DIEA. The reaction mixture was stirred 30 min at 0 °C and 3 h at room temperature. The solvent was removed under reduced pressure, the residue was dissolved in ethyl acetate and washed 2 times with 5 % $KHSO_4$-solution, 2 times with saturated $NaHCO_3$-solution and 2 times with brine. The organic layer was dried over Na_2SO_4 and evaporated in vacuum (2.1 g, crude product as yellow oil).

HPLC: 42.7 min; TLC: R_f = 0.76

Benzylsulfonyl-DArg(Pbf)-Gly-4-amidinobenzylamide x ace-tate (VIII)

2.0 g of the crude product (**VII**) was dissolved in 90 % acetic acid and treated with 200 mg of catalyst (10 % Pd/C). The reaction mixture was hydrogenated overnight at atmos-pheric pressure. The catalyst was removed by filtration, and the solvent was removed in vacuum. The residue was directly used for the next synthesis step without purification (2 g crude product as yellow oil).

HPLC: 35.1 min; TLC: R_f = 0.74; MS: calc.: 768.3, found: 769.4 $[M+H]^+$

Benzylsulfonyl-DArg-Gly-4-amidinobenzylamide x 2 TFA (48)

2.0 g of the crude product (**VIII**) was stirred with 20 ml of 90 % TFA at ambient temperature for 1.5 h. The solvent was almost completely removed at reduced pressure. The product was precipitated by the addition of ether, filtrated, washed with ether, dried in vacuum and purified by pre-parative RP-HPLC (400 mg lyophilized white powder).

HPLC: 13.8 min; TLC: R_f = 0.48; MS: calc.: 516.2, found: 517.5 $[M+H]^+$

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