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Highly Potent and Selective Substrate Analogue Factor Xa Inhibitors Containing D-Homophenylalanine Analogues as P3 Residue: Part 2

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*A series of highly potent substrate-analogue factor Xa inhibitors containing D-homophenylalanine analogues as the P3 residue has been identified by systematic optimization of a previously described inhibitor structure. An initial lead, benzylsulfonyl-D-hPhe-Gly-4-amidinobenzylamide (**3**), inhibits fXa with an inhibition constant of 6.0 nM. Most modifications of the P2 amino acid and P4 benzylsulfonyl group did not improve the affinity and selectivity of the compounds as fXa inhibitors. In contrast, further variation at the P3 position led to inhibitors with significantly enhanced potency and selectivity. Inhibitor **27**, benzylsulfonyl-D-homo-2-pyridylalanyl(N-oxide)-Gly-4-amidinobenzylamide, inhibits fXa with a K_i value of 0.32 nM. The inhibitor has strong anticoagulant activity in plasma and doubles the activated partial thromboplastin time and prothrombin time at concentrations of 280 nM and 170 nM, respectively. Compound **27** inhibits the prothrombinase complex with an IC_{50} value of 5 nM and is approximately 50 times more potent than the reference inhibitor DX-9065a in this assay.*

Introduction

Thromboembolic diseases are a major cause of death and morbidity worldwide. Therefore, anticoagulants are routinely used for the treatment and prevention of thrombotic complications in high risk patients, suffering from angina pectoris, stroke, or heart attack. In addition, antithrombotics are used to avoid venous thromboembolism, deep vein thrombosis, or pulmonary embolism in patients undergoing orthopaedic or other surgery.[1] During the last decade, the clotting protease factor Xa emerged as an attractive target for the development of new anticoagulants. In contrast to the established parenteral indirect antithrombotics of the heparin type or the thrombin inhibitor r-hirudin, small molecule fXa inhibitors have the potential to be orally available. Meanwhile several nonpeptide direct fXa inhibitors have entered clinical trials[2–4] and the first promising results have been published from clinical studies with the most advanced compound Bay 59-7939 (Rivaroxaban).[5] However, to date, none of these active site-directed fXa inhibitors has been approved for clinical use.[6,7]

Recently, we have described a series of substrate analogue fXa inhibitors containing 4-amidinobenzylamide as the P1 residue.[8] Some of these inhibitors containing a second basic group at the P3 position were relatively selective and highly potent with K_i values < 5 nM. However, all compounds suffered from negligible bioavailability after oral treatment and most inhibitors were rapidly eliminated from the circulation of rats after intravenous application.

During optimization of the P3 position using analogues of D-phenylalanine, we observed that the inhibition of fXa significantly depends on the length of the P3 side chain. The strongest fXa affinity was found for compounds with D-homophenylalanine, such as benzylsulfonyl-D-homophenylalanyl-glycyl-4-amidinobenzylamide (Bzls-d-hPhe-Gly-4-Amba) **3**, which inhibits fXa with a K_i value of 6.0 nM (Table 1). Interestingly, this effect was less pronounced for other trypsin-like serine proteases, such as thrombin, plasmin, uPA, or trypsin, which where routinely used as reference enzymes to characterize the selectivity of the inhibitors.

Based on the previously published X-ray structures of Bzls-DSer(Bzl)-Gly-4-Amba and of its P3 DSer(*t*Bu) analogue in com-

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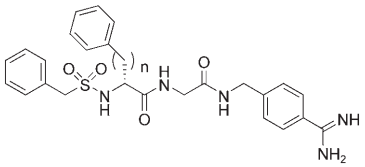
Table 1. Inhibition of trypsin-like serine proteases by inhibitors containing P3 amino acids with side chains of different length.



Compd.	n	K_i [nM]				
		fXa	thrombin	plasmin	uPA	trypsin
1	0	52	110	1800	430	23
2	1	78	68	1200	900	38
3	2	6.0	47	1100	910	11
4	3	87	210	1100	1600	48

plex with fXa,[8] we have modeled the complex of inhibitor **3** into the active site of fXa (Figure 1). The backbone of the inhibitor remains nearly unchanged, including the orientation of the P4 residue and the P2-P1 segment. However, the side chain of the P3 D-hPhe fits much better into the aryl binding site of fXa between residues Phe 174, Tyr 99, and Trp 215 than the side chains of DSer(Bzl) or DSer(*t*Bu), resulting in an improved potency of inhibitor **3**. It should be noted that the analogue inhibitor containing L-hPhe as the P3 amino acid inhibits fXa with a K_i value of only 450 nM.

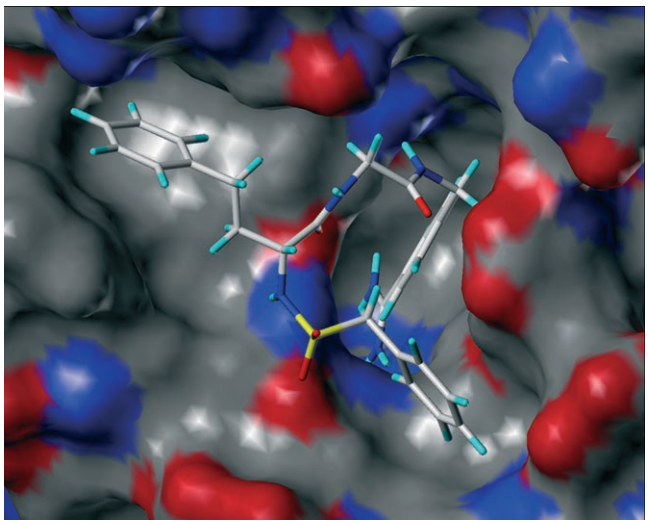


Figure 1. Model of inhibitor **3** in complex with fXa. The structure was derived from previously described X-ray structures of similar substrate analogue inhibitors in fXa[8] by replacement of the P3 amino acid followed by energy minimization using the software Sybyl 6.9.1 (Tripos). The inhibitor is shown as sticks colored by atom type, and the protein is visualized as a Connolly surface. Blue and red surface areas represent hydrogen acceptors and donors, respectively. Gray areas have no hydrogen bonding properties.

However, inhibitor **3** is compromised by its very short plasma half-life of <20 min obtained for the β-phase in rats after intravenous application of 1 mg kg^{-1}. In addition, compound **3** is still a relatively potent thrombin inhibitor (K_i = 47 nM). Therefore, several series of new fXa inhibitors were synthesized using modified P4 groups, analogues of D-hPhe, and different P2 amino acids to improve their half-life, selectivity, and potency. Selected analogues were investigated in greater detail regarding their anticoagulant and pharmacokinetic properties. The results of these studies are summarized here.

Results

Modification of the P4 group

In a first series, the P4-position of the inhibitor was modified, whereas the D-hPhe-Gly-4-Amba P3-P1 segment was maintained (Table 2).

All derivatives without a benzylsulfonyl group have significantly reduced fXa affinity. Some modifications of the P3 amino group, which were previously used for the design of thrombin inhibitors, for example, alkylation by a carboxymethyl residue as in the case of melagatran[9] or coupling of a methylsulfonyl group as described for BMS-189664,[10] were not accepted. The most potent fXa inhibitors **17** and **20** contain an amino or hydroxyl group in the *para* position of the P4 benzylsulfonyl residue, however, their potency and selectivity was only marginally modified compared to the unsubstituted derivative **3**. Based on a model (structure not shown), it may be possible that the *para* amino group forms a hydrogen bond to the side chain of the fXa residue Glu 147 (distance of the heteroatoms is 2.7 Å).

Modification of the P3 amino acid

In a second approach, the P3 D-hPhe was replaced by other aromatic homo amino acids (Table 3). The use of racemic homo-4-pyridylalanine, homo-3-pyridylalanine, and D-homotyrosine results in fXa inhibitors with similar activities, whereas the incorporation of D-homo-2-pyridylalanine and of its pyridine N-oxide (inhibitors **24** and **27**) improves fXa affinity.

Derivatives with D-hTyr in the P3 position

As compound **21** was found to be one of the most potent inhibitors, some additional derivatives with D-hTyr in the P3 position were prepared, their inhibition constants are summarized in Table 4. As expected, based on the kinetic results with inhibitors **3** and **20**, the attachment of an amino group in the *para* position of the P4 residue slightly enhanced potency. However, inhibitor **29** was not further used because of a strong mutagenic potential found for the related inhibitor **20** in an Ames test (performed by GenPharmTox Biotech AG, Martinsried, Germany), which is most likely related to the aniline-like aromatic amino group of the P4 residue.

In a previous publication we have described in detail the modification of the P2 position in similar substrate analogue inhibitors containing DSer(*t*Bu) as the P3 residue. The replacement of glycine by alanine, proline, and several other imino

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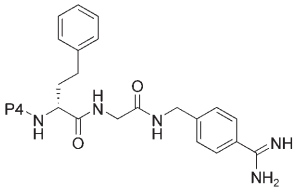
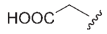
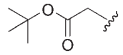
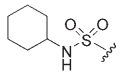
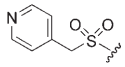
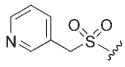
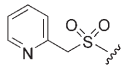
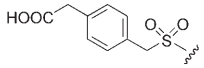
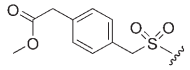
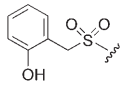
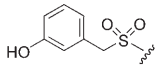
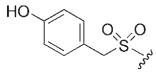
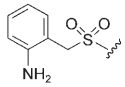
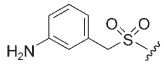
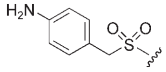
Table 2. Inhibition of trypsin-like serine proteases by inhibitors with modified P4 group.



Compd.	P4	K_i [nM] fXa	thrombin	plasmin	uPA	trypsin
5	H	3600	16000	40000	43000	370
6		1060	1300	21000	33000	240
7		870	28000	15000	52000	50
8		620	1500	20000	42000	120
9		100	5400	5000	44000	110
10		80	3600	5100	2500	32
11		59	5900	3700	7400	45
12		52	4900	4700	12000	43
13		90	14000	3800	33000	30
14		38	1800	1800	1700	10
15		170	7600	10000	58000	0.16
16		7.2	1500	1100	1900	15
17		3.6	380	640	870	10
18		5.8	920	1300	2100	21
19		9.3	1400	930	2000	15
20		2.5	260	880	410	7.3

acids within this series still maintained high fXa affinity, but resulted in analogues with poor specificity towards thrombin. In contrast, all other modifications led to significantly reduced fXa potency (K_i values >50 nM) and in most cases to improved plasmin inhibition, which is not acceptable for the development of anticoagulants.[8] However, the improved basic activity of the P3 D-hTyr derivatives allowed a higher variability in the P2 position, for example, analogues **31** and **34** with P2 Glu are potent fXa inhibitors and have a reduced elimination half-life of 0.5 and 0.7 h in the circulation of rats, respectively.

Derivatives with racemic homo-2-pyridylalanine-*N*-oxide in the P3 position

Although most modifications at P4 in combination with D-hPhe as the P3 residue resulted in reduced potency (Table 2), an additional series with the more potent D/L-hAla(2-pyr-*N*-oxide)-Gly-4-amidinobenzylamide as P3-P1 segment was prepared to reduce the size of the relatively large P4 benzylsulfonyl residue (Table 5).

All compounds shown in Table 5 maintain high selectivity as fXa inhibitors. The fXa affinity of the alkylsulfonyl derivatives **35**–**38** is reduced compared to the benzylsulfonyl derivative **27**; however, some analogues still maintain significant potency. Their activity strongly depends on the length and type of the alkyl chain; the highest potency was found for the butylsulfonylated analogue **38**. In contrast, the carboxymethylated inhibitor **40** and its ethyl ester derivative **39** which were prepared in analogy to the thrombin inhibitor melagatran and its prodrug ximelagatran, have only modest fXa affinity.

Extended enzyme kinetic characterization and anticoagulant activity

Selected inhibitors were further investigated for their inhibitory potency towards additional trypsin-like serine proteases and for their anticoagulant activity (Tables 6 and 7). The determined K_i values demonstrate that most compounds are relatively specific fXa inhibitors. Only in a few cases, a moderate inhibition of other proteases with K_i values between 10 and 100 nM is observed, for example, compound **31** inhibits coagulation factor XIa and activated protein C with K_i values of 28 and 50 nM, respectively. Other analogues, such as **21**, possess modest affinity towards matriptase, whereas compound **34** also slightly inhibits plasma kallikrein and protein Ca.

All inhibitors showed excellent anticoagulant activity in the standard coagulation assays, such as activated partial thromboplastin time (aPTT) and prothrombin time (PT), as well as for

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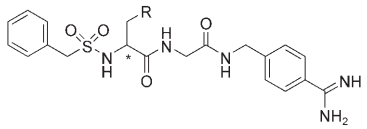
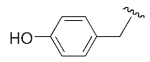
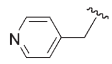
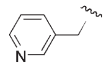
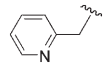
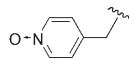
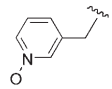
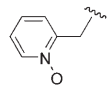
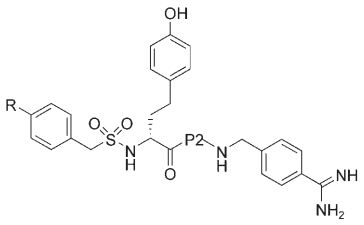
Table 3. Inhibition of trypsin-like serine proteases by inhibitors with modified P3 amino acids.



Compd.	*	R	K_i [nM] fXa	thrombin	plasmin	uPA	trypsin
21	D		3.0	1500	780	930	3.9
22	D/L		7.1	2000	1200	1200	17
23	D/L		4.0	2000	1500	3200	28
24[a]	D		0.71	1900	1100	1700	7.7
25	D/L		15	4100	3100	3400	38
26	D/L		19	3200	2700	3000	40
27[a]	D		0.32	2700	1300	1900	6.3

[a] Inhibitors **24** and **27** contain approximately 4% of the corresponding L-enantiomer (see synthetic procedure).

Table 4. Inhibition of trypsin-like serine proteases by inhibitors with D-hTyr in the P3 position.



Compd.	R	P2	K_i [nM] fXa	thrombin	plasmin	uPA	trypsin
28	NO_2	Gly	5.2	880	850	500	4.4
29	NH_2	Gly	1.9	560	520	330	2.9
30	H	Ser	12	1400	48	240	1.6
31	H	Glu	3.1	19000	150	3600	26
32	H	Glu(OEt)	5.0	1000	11	230	1.6
33	NO_2	Glu	6.8	15000	360	3400	3.8
34	NH_2	Glu	1.9	78000	130	3100	1.6

the inhibition of the prothrombinase complex (PTC, Table 7). Otherwise, because of their weak thrombin affinity, they were less potent in the thrombin time assay (TT). For comparison, we used the fXa inhibitor DX-9065a, which has reached clinical phase II development.[11] However, a drawback observed for these peptidic inhibitors is their fast elimination from the circulation of rats after intravenous inhibitor treatment, although for inhibitors **27**, **31**, and **34** a slightly improved half-life was found compared to the lead structure **3**.

Three different types of amidine prodrugs based on inhibitor **27** were prepared containing a 4-(hexyloxycarbonylamidine)-, 4-(hydroxyamidine)-, or 4-(acetyl-hydroxyamidine)-benzylamide as P1 residue (Figure 2).

Such prodrug types have been successfully used for the thrombin inhibitors ximelagatran and dabigatran, and for the diacetyldiamidoximeester of the antiprotozoal drug pentamidine.[9,12,13] The prodrug principles rely on hydrolysis and/or reduction of *N*-hydroxy-amidines (amidoximes) to amidines.[14] Incubation experiments of the 4-(hydroxyamidine)-prodrug **27b** with human and porcine microsomes and mitochondria of kidney and liver revealed a rapid conversion into the amidine

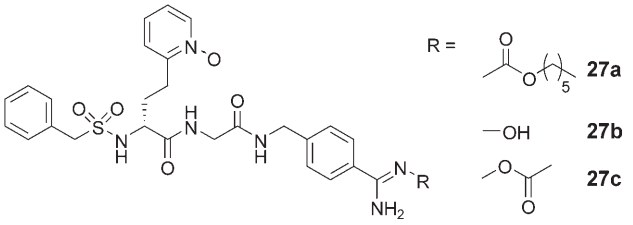


Figure 2. Synthesized prodrugs of inhibitor **27**.

without reducing the pyridine *N*-oxide moiety (Figure 3). The conversion rates were comparable with those of ximelagatran to melagatran.[15]

However, based on the detection of the active inhibitor **27** in plasma using a spectrophotometric activity assay, all prodrugs were insufficiently present after oral administration to rats. At a dose of 5 mg kg^{-1}, very low plasma levels of inhibitor **27** (<0.05 μg mL^{-1}) were found in the case of the 4-(hexyloxycarbonylamidine)-derivative **27a**, whereas the maximal plasma concentrations of inhibitor **27** after treatment with the 4-(hydroxyamidine)- or 4-(acetyl-hydroxyamidine)-benzylamide prodrugs, **27b** and **27c**, were slightly increased ($\approx$0.05–0.2 μg mL^{-1}). Typically, large differences in plasma inhibitor levels between different rats were observed, which is an additional indication of insufficient oral bioavailability.

The 4-(hydroxyamidine)-benzylamide prodrug **27b** and the active amidine **27** were applied intravenously to pigs (36 to 41 kg) at a dose of 1 mg kg^{-1}. Analysis of blood samples

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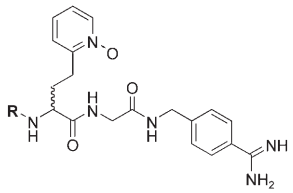
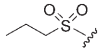
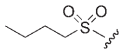
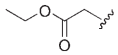
Table 5. Inhibition of trypsin-like serine proteases by inhibitors of the general formula:



Compd.	R	K_i [nM][a] fXa	thrombin	plasmin	uPA	trypsin
35		24	79000	21000	51000	180
36		7.7	44000	9200	20000	79
37		28	43000	22000	48000	140
38		3.4	9500	4600	11000	21
39		70	34000	78000	43000	240
40		270	100000	75000	39000	240

[a] Inhibitors **35**–**40** were routinely synthesized using racemic D/L-hAla(2-pyr-*N*-oxide) in the P3 position. Due to the negligible affinity found for derivatives with L-configuration in this inhibitor type, it is reasonable to assume that compounds **35**–**40** should have approximately half of the potency compared to the pure D-enantiomers.

Table 6. Inhibition of additional trypsin-like serine proteases by selected inhibitors.

Compd.	K_i [nM] fXa	plasma-kallikrein	fXIIa	fXIa	fVIIa	protein Ca	tPA	matriptase	tryptase
3	6.0	390	150	250	12000	630	530	200	1400
21	3.0	150	120	610	6200	410	180	70	790
24	0.71	230	60	880	15000	870	370	110	1600
27	0.32	370	140	1700	19000	840	510	110	6200
31	3.1	220	360	28	7500	50	580	3200	860
34	1.9	80	390	120	5100	60	940	4800	710

Table 7. Anticoagulant activity (aPTT, PT, and TT) in human plasma and inhibition of human fXa (K_i) and of the prothrombinase complex (PTC) by selected inhibitors.[a]

Compd.	K_i fXa [nM]	IC_{50} PTC [nM]	IC_{200} [nM][b] aPTT	PT	TT	$t_{1/2}$ [h]
DX-9065a	24	240	1050	530	> 10000	0.98
3	6.0	12	380	450	2200	< 0.3
21	3.0	13	330	340	5500	< 0.3
24	0.71	4	280	170	3300	< 0.3
27	0.32	5	280	170	7700	0.5
31	3.1	6	650	950	> 100000	0.5
34	1.9	10	450	630	> 100000	0.7

[a] The elimination half-life (β-phase) in rats after intravenous application of 1 mg kg^{-1} is also given. [b] The IC_{200} value is defined as the inhibitor concentration, which doubles the clotting time to 200 percent compared to control.

showed a rapid elimination of both tested compounds with half-lives of 12.1 min ± 3.5 min for compound **27** and 17.7 min ± 6.9 min for prodrug **27b**.

Although no plasma concentrations of compound **27** could be detected after intravenous application of prodrug **27b**, the reduction of the 4-(hydroxyamidine)-benzylamide prodrug to the pharmacological active amidine in vivo could be demonstrated by urine analysis, which contained detectable amounts of compound **27** (see Figure 4).

The 4-(hydroxyamidine)- and 4-(acetyl-hydroxyamidine)-benzylamide prodrugs of inhibitor **27** were also orally applied to pigs (32 to 44 kg). At a dose of 5 mg kg^{-1} neither the prodrug nor the active inhibitor **27** could be detected in plasma using a LC/MS-system (detection limit 30 ng mL^{-1}).

Discussion

The incorporation of D-hPhe and its analogues as the P3 amino acid resulted in the discovery of potent and selective substrate analogue fXa inhibitors. Molecular modeling revealed that the ethylene group of the P3 side chain enables an optimal accommodation of the P3 aryl ring in the distal aromatic binding pocket of fXa, formed by amino acids Tyr 99, Phe 174, and Trp 215. Most variations at the N-terminal P4 benzylsulfonyl group led to less potent inhibitors; only the substitution by an amino or hydroxyl group in the *para* position (**17**, **20**) slightly improved fXa affinity and resulted in improved selectivity over thrombin. However, aniline-like amino groups, such as those found in the HIV-protease inhibitors TMC 114, Amprenavir, and its prodrug Fosamprenavir,[16] are otherwise rarely used in drug-like molecules because of their mutagenic potential. In addition, all substituted benzylsulfonyl derivatives suffer from a relatively high molecular weight ≥ 550 Da, which might restrict oral bioavailability. Attempts to reduce the size of the inhibitors by elimination of the benzylsulfonyl residue and subsequent alky-

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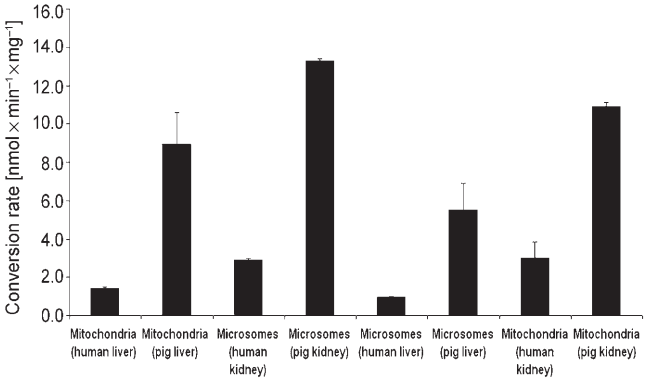

Figure 3. Conversion rates of the 4-(hydroxyamidine)-prodrug **27b** to the active amidine inhibitor **27**. Data are means ± S.D. of three determinations.

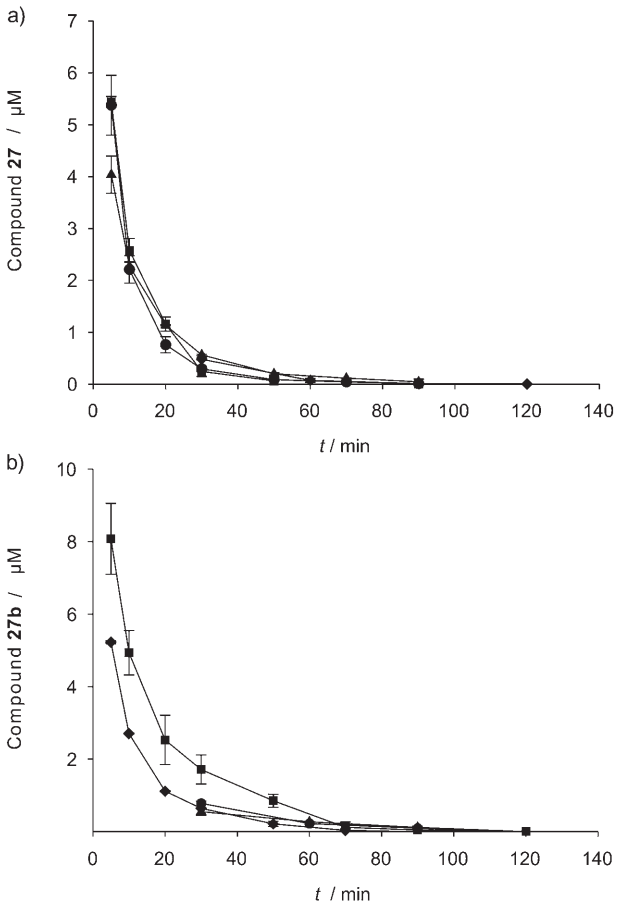

Figure 4. Plasma concentrations of applied drug versus time since intravenous administration of 5 mg kg^{-1} of a) compound **27** and b) compound **27b** to pigs ($n = 4$).

lation of the P3 amino group with a carboxymethyl residue or its ethylester as demonstrated in the case of melagatran/ximelagatran resulted in a dramatic loss of potency (**39**, **40**). In contrast, the incorporation of alkylsulfonyl residues was accepted if the alkyl chain had a sufficient length (**35**–**38**). Obviously, a longer P4 alkyl chain of the inhibitors enhances fXa affinity by stronger hydrophobic interactions. It is noteworthy that a similar tendency was also observed for the inhibition of thrombin, plasmin, uPA, and trypsin; in all cases, the inhibition constants were improved with increasing chain length of the sulfonyl residue. Therefore we assume that these inhibitors have a similar binding mode in all of these proteases.

A nearly 10- and 20-fold improvement in fXa affinity was obtained by replacement of the P3 D-hPhe (**3**) with DhAla(2-pyr) (**24**) and DhAla(2-pyr-*N*-oxide) (**27**), respectively. Molecular modeling revealed a nearly identical overall conformation of inhibitors **3** (Figure 1) and **27** (shown as a picture in the table of contents), whereas the N-oxide of the P3 aryl ring from **27** projects toward the solvent and appears not to be involved in any specific interaction to fXa. A very similar orientation of a P3 2-pyridine-*N*-oxide group of a thrombin inhibitor within the distal aryl binding pocket of thrombin was found by X-ray crystallography.[17] The authors propose that the improved potency upon incorporation of the π-deficient P3 pyridine-*N*-oxide may be due to the reinforcement of the edge-to-face σ-π-interaction between the P3 aryl group of the inhibitor and the π-rich Trp 215 of the protease. Interestingly, also in this series, the pyridine-*N*-oxide-derived inhibitor was approximately twofold more potent than the analogous pyridine inhibitor and was significantly more active than the phenyl derivative.[18] The same tendency was described for an additional series of dual thrombin and fXa inhibitors containing an oxazolopyridine core[19] and for nonpeptidic fXa inhibitors, which possess a thienopyridine N-oxide group as P3/P4 segment.[20] These results support the hypothesis that the reinforced interactions of the P3 2-pyridine-*N*-oxide group with Trp 215 contributes to affinity, because both thrombin and fXa contain Trp 215, whereas the other residues surrounding the aryl binding pocket are different in these proteases (Leu 99 and Ile 174 in thrombin, Tyr 99 and Phe 174 in fXa).

The inhibitors of this type, especially inhibitor **27**, have excellent anticoagulant potency in vitro and a high selectivity towards a whole set of trypsin-like serine proteases. However, inhibitor **27** and its prodrugs suffer from two drawbacks: rapid elimination after intravenous application and poor oral bioavailability. Although we have previously observed longer-lasting effects in some cases with analogous uPA and fXa inhibitors,[8,24] an insufficient pharmacokinetic profile seems to be a general problem for peptidomimetic inhibitors of trypsin-like serine proteases containing a C-terminal 4-amidinobenzylamide as arginine replacement. Elimination studies with inhibitor **27** after intravenous injection of 1 mg kg^{-1} to rats revealed that approximately 40% of the given inhibitor dose was found in the urine and bile in a nonmetabolised form, respectively. Therefore, a combined renal and hepatobiliary excretion is mainly responsible for the rapid elimination of this inhibitor, although we cannot exclude some metabolism-related reduction in the plasma inhibitor levels.

We were able to show that the prodrugs **27b** and **27c** are reduced to the active inhibitor **27** by incubation with human and porcine microsome and mitochondria preparations, similar to ximelagatran. The in vivo reduction of the prodrug **27b** in rats and pigs could also be verified by identifying inhibitor **27** in the urine. Unfortunately, all attempts to enhance the oral

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bioavailability of these inhibitors by the incorporation of well-established benzamidine prodrugs were unsuccessful, although numerous other N-hydroxy-amidines are orally available prodrugs of amidines,[13,14] for example, the bioavailability of ximelagatran is increased from 3–7% to 18–24%.[21] This discrepancy is most likely related to poor absorption of the prodrugs used in combination with a short half-life of compound **27** due to a rapid biliary and renal excretion. It might be also possible, that a larger amount of the prodrug **27b** is absorbed from the gastrointestinal tract, reduced in the liver, and immediately eliminated by the bile resulting in only low concentrations of inhibitor **27** in plasma. Consequently, the prodrug principle used would increase absorption but could not prevent biliary excretion. For a successful application of N-hydroxy-amidine prodrugs an acceptable half-life of the compound is necessary.

In an additional study, we have optimized the P1 group within this inhibitor type and incorporated less basic residues at this position. These results will be published in a subsequent paper.[22]

Methods and Materials

Enzyme kinetic measurements, clotting assays, inhibition of the prothrombinase complex, and elimination studies

Standardized enzyme kinetic measurements for inhibitors with K_i values $>$ 1 nM were performed in a microplate reader (iEMS, MF 1401, Labsystems, Helsinki, Finland) at 405 nm at room temperature as described previously,[8,23] and the K_i values were obtained from Dixon plots. In case of K_i values $<$ 1 nM (inhibition of fXa by compounds **24** and **27**) the kinetic experiments were performed in acryl cuvettes (optical path 1 cm) using a UV/Vis-photometer M400 (Carl Zeiss, Jena, Germany) under reduced enzyme concentration. In all cases the enzyme concentration was at least ten times lower than the lowest inhibitor concentration. The clotting assays in human plasma were performed using a coagulometer Thrombotrack (Immuno GmbH, Heidelberg, Germany) at 37 °C according to established methods.[23] All animal studies in rats and pigs were performed according to the guidelines of the German Animal Health and Welfare Act.

The determination of prothrombinase complex inhibition and elimination studies in rats are described in our previous publications.[8,24]

Assay for the reduction of the 4-(hydroxyamidine)-prodrug 27b

Human and pig microsomes from liver were obtained by ultracentrifugation as described previously.[25] The kidney microsomes were prepared analogously. Human and pig mitochondria were prepared by differential centrifugation as described previously[26,27] with slight modifications.[28] To account for the biological variability liver samples from pigs or human organs were pooled (from at least three individuals per pool).

The incubation mixture consisted of 1.2 mg mL^{-1} microsomal or mitochondrial protein of liver or kidney (human or pig) and 1 mM substrate (prodrug **27b** or *N*-hydroxy-melagatran) in a 100 mM phosphate buffer pH 6.3. After preincubation for 5 min at 37 °C under aerobic conditions the reaction was started by the addition of NADH (final concentration 1 mM) to a total volume of 250 μL. After 30 min the reaction was terminated by the addition of 250 μL of cold methanol and vortexing. After centrifugation at 10000 U min^{-1} (48 *g*) (Mikroliterzentrifuge Hettich, Tuttlingen, Germany) for 7 min, 10 μL of the supernatant was analyzed by HPLC.

Measurements of the inhibitor levels in plasma

The concentration determination of inhibitor **27** in rat plasma was performed by a spectrophotometric assay, as described previously for the detection of urokinase inhibitors.[24] Briefly, 175 μL of buffer (Tris × HCl, 0.05 mol L^{-1}, pH 8.0 containing 0.154 mol L^{-} NaCl) were mixed with 25 μL plasma or with 25 μL plasma diluted with buffer, if necessary. The reaction was started by the addition of 25 μL Chromozym X (Pentapharm AG, Basel, Switzerland) and 50 μL FXa (1.0 μg mL^{-1} in buffer, Haemochrom Diag. GmbH Essen, Germany). From the observed reaction rate the inhibitor concentration in plasma was calculated using a calibration curve (detection limit 2 ng mL^{-1} inhibitor in plasma).

The inhibitor concentration in pig plasma was determined by LC–MS following solid phase extraction. Solid phase extraction: Plasma samples were prepared by using Strata X 33 μm polymeric sorbent SPE-columns (Phenomenex). After conditioning with 1 mL methanol and equilibrating with water, 100 μL of a 10 mM tetramethylammoniumchloride/octylsulfonate mixture was added to 1 mL plasma and applied to the SPE-column. Two washing steps with 1 mL water and 0.5 mL water/methanol (70/30, *v*/*v*) followed, before the sample elution with 1 mL methanol was performed. The eluate was concentrated to dryness and resolved in 100 μL water/methanol (70/30, *v*/*v*). Urine samples were treated analogously. LC–MS-Method: A Hewlett Packard LC system Series 1100 equipped with a binary Pump G1312 A and a HP Degasser G1322 A was used. The separation was carried out isocratically by 20 mM ammonium acetate buffer pH 5.0 and acetonitril (86/14, *v*/*v*; column: LiChroCART 125-4 HPLC-Catridge with LiChrospher 60 RP-select, 5 μM, Merck, Darmstadt, Germany). The flow rate used was 1 mL min^{-1}. A flow splitter on the LC–MS system was used to ensure that approximately 0.5 mL min^{-1} went into the source. The injection volume was 50 μL. Mass analysis was conducted on a HP-Bruker Esquire mass spectrometer equipped with an athmospheric pressure chemical ionization (APCI) interface. The detection limit was 30 ng mL^{-1}.

Syntheses

Standard amino acid derivatives and coupling reagents were purchased from Bachem (Weil am Rhein, Germany) or Novabiochem/Merck (Schwalbach, Germany). Racemic homo-4-pyridylalanine and D-homotyrosine were obtained from RSP Amino

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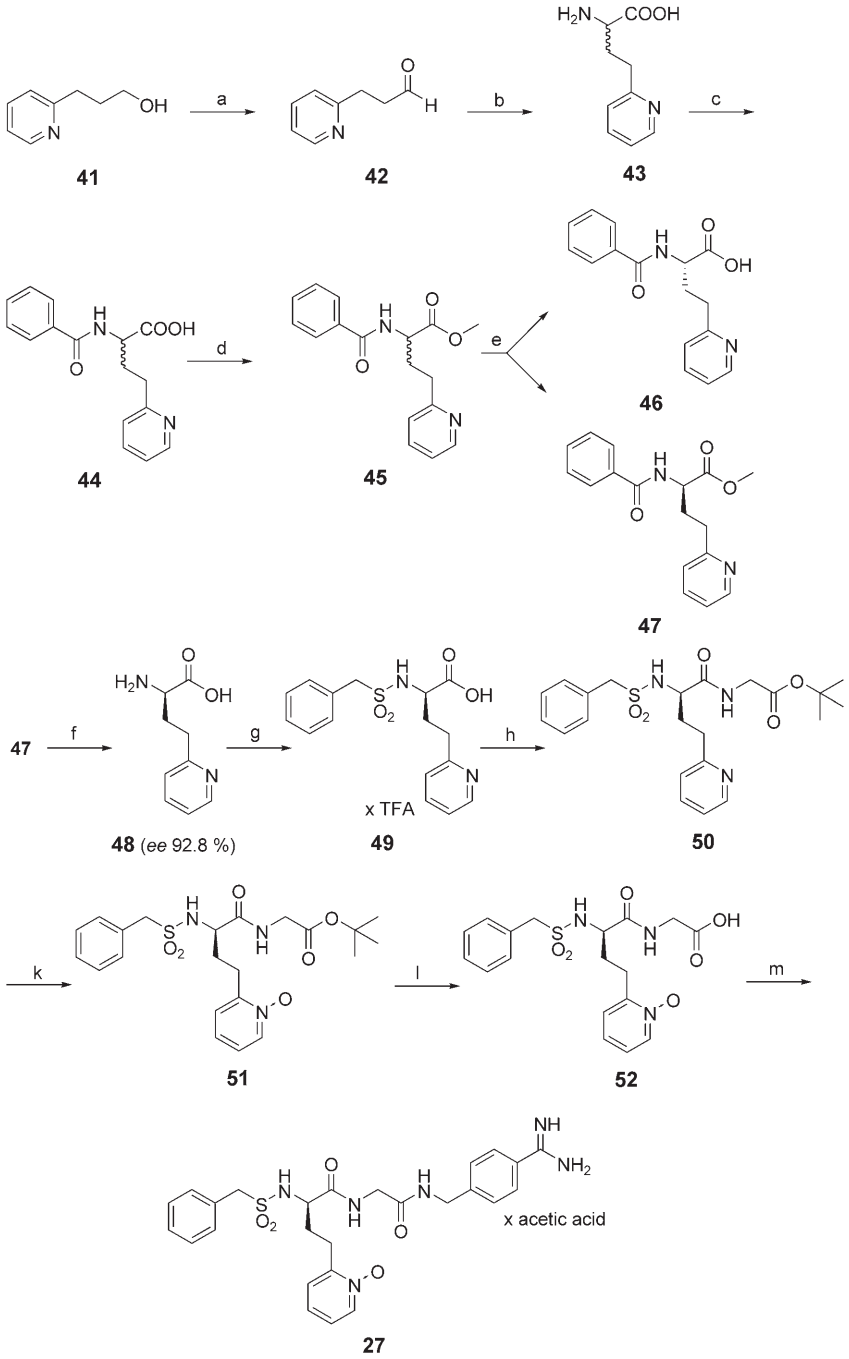
Acids (Shirley, USA) and Chem-Impex International Inc. (Wood Dale, USA), respectively. Commercially available benzylsulfonylchlorides and other reagents for synthesis were purchased from Sigma–Aldrich (Taufkirchen, Germany) and Acros-Fisher (Schwerte, Germany). Pyridylmethylsulfonylchlorides were obtained from Array BioPharma (Boulder, USA), and all noncommercially available benzylsulfonylchlorides were prepared from the appropriate benzylbromides via sulfonates as described previously.[29] The amino-benzylsulfonyl-modified inhibitors (Tables 2 and 4) were prepared by hydrogenation of the appropriate nitro analogues using Pd/C as catalyst in the final step of synthesis. Hydroxy-benzylsulfonyl-D-hPhe derivatives, obtained by Sandmeyer reaction from the appropriate amino-benzylsulfonyl-D-hPhe intermediates, were used as building units for the synthesis of the hydroxy-benzylsulfonyl inhibitors **15**–**17**.

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 system (solvent A: 0.1% trifluoroacetic acid (TFA) in water, solvent B: 0.1% TFA in acetonitrile, gradient: 1–70% solvent B in 69 min, detection at 220 nm) at a flow rate of 1 mL min^{-1}. The final inhibitors were purified to more than 95% purity by preparative HPLC on a Shimadzu LC-8 A system (Phenomenex Luna C18, 5 μm column, 30×250 mm, detection at 220 nm) with a linear gradient system (45% increase solvent B in 120 min) at a flow rate of 20 mL min^{-1}. All inhibitors were obtained as TFA-salts after lyophilization, except inhibitor **27**, which was converted into an acetate salt by ion exchange chromatography on Fractogel-EMD COO^- using an ammonium acetate gradient. The ^{1}H- and ^{13}C NMR-spectra were recorded on Bruker spectrometers at 300 and 75 MHz, or 500 and 125 MHz, respectively. The proton spectra are referenced to the residual proton signal of the solvent, the ^{13}C spectra are not referenced. 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 and fXa inhibitors,[8,24] and is described in detail for inhibitor **27** (Scheme 1).



Scheme 1. Synthesis of inhibitor **27**. a) 1: oxalyl chloride, DMSO in DCM; 2: TEA, water, b) 1: NH_4Cl, NaCN, water, 2: HCl, ion exchange, c) benzoyl chloride, DIEA, dioxane, d) thionyl chloride, methanol, e) chymotrypsin in ammonium acetate pH 7.5–8, f) 6 N HCl, ion exchange, g) 1: chlorotrimethylsilane, DIEA, DCM, 2: benzylsulfonyl chloride, DIEA, h) H-Gly-O*t*Bu, PyBop, DIEA in DMF, k) mCPBA in DCM, l) TFA, m) 4-amidinobenzylamine×2 HCl, PyBop, DIEA in DMF followed by preparative HPLC and ion exchange chromatography.

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*3-(pyridin-2-yl)propanal (**42**)*. 3-(pyridine-2-yl)-propan-1-ol (106 g, **41**, brown oil, Aldrich) was purified by vacuum distillation (2 mbar, 105-107 °C, yield 100.6 g, 0.73 mol, TLC: R_f 0.42) and used for the following Swern oxidation.[30]

35 mL (0.41 mol) oxalyl chloride was dissolved in 700 mL dry DCM and cooled to −70 °C. A mixture of 62 mL (0.87 mol) DMSO and 40 mL dry DCM was added dropwise over a period of 25 min and the temperature was maintained below −65 °C. The reaction mixture was stirred for 15 min at −70 °C and afterwards treated with 50 g (0.36 mol) 3-(pyridine-2-yl)-propan-1-ol, which was dissolved in 150 mL dry DCM, over a period of 15 min. The temperature was kept below −65 °C. The mixture was treated with 218 mL (0.37 mol) triethylamine over a period of 40 min and afterwards the mixture was warmed to room temperature. Salts were dissolved by addition of 190 mL water. The organic phase was separated and the DCM was removed in vacuum. The aldehyde was purified by vacuum distillation (2 mbar, 59–80 °C, yield: 33.6 g, 0.25 mol, 69%, colorless oil, TLC: R_f 0.20).

The following Strecker synthesis and separation of the racemic amino acid was performed as described for H-D/L-hAla(3-Pyr)-OH.[31]

*H-D/LhAla(2-Pyr)-OH (**43**)*. 3-(pyridin-2-yl)propanal (67 g, 0.5 mol, **42**) was treated with 18 mL diethylether and cooled to 0 °C. 88.4 g (1.65 mol) ammonium chloride was dissolved in 300 mL water and slowly added to the aldehyde. Afterwards the mixture was treated with 74.3 g (1.4 mol) NaCN, dissolved in 200 mL water, and stirred for an additional 4 h at 0 °C. The mixture was heated to 50 °C, stirred for 4 h, then cooled to room temperature. The mixture was extracted four times with 800 mL chloroform and the chloroform was removed under vacuum. The residue was treated with 1 L concentrated HCl and stirred for 42 h at room temperature. Afterwards the mixture was refluxed for 35 h. The water was removed under vacuum, the residue was repeatedly treated with water, and evaporated. The remaining residue was dissolved in 1.5 L ethanol and cooled to 4 °C. Precipitated salts were removed by filtration and the ethanol was removed under vacuum. The amino acid was dissolved in water and purified in two portions by ion exchange chromatography (Dowex 50WX8-200 in ammonium form, 10×15 cm). The amino acid was eluted with 0.2 N ammonium hydroxide solution. The product-containing fractions were evaporated and the amino acid was precipitated by the addition of acetone (light brown solid, yield: 42 g, 0.233 mol, 47%, HPLC: 3.9 min, TLC: R_f = 0.04, ^{1}H NMR (500.13 MHz, D_2O): δ = 8.34 (d, broad, ^3J{HH} = 5 Hz, 1H), 7.72 (m ^3J{HH} = 7.8 Hz, ^3J{HH} = 7.3 Hz, ^4J{HH} = 1.5 Hz, 1H), 7.27 (d ^3J{HH} = 7.8 Hz, 1H), 7.23 (dd ^3J{HH} = 5 Hz, ^3J{HH} = 7.3 Hz, 1H), 3.78 (t ^3J{HH} = 6.3 Hz, 1H), 2.84 (m 2H), 2.18 ppm (m 2H). ^{13}C NMR (125.75 MHz, D_2O): δ = 174.00, 158.80, 147.84, 138.08, 123.52, 121.99, 54.16, 32.38, 30.23 ppm.

*Benzoyl-D/LhAla(2-Pyr)-OH (**44**)*. 17.7 g (98.22 mmol) H-D/LhAla(2-Pyr)-OH (**43**) was dissolved in a mixture of 60 mL dioxane, 60 mL water, and 17.95 mL (103.14 mmol) diisopropylethylamine (DIEA). At 0 °C 11.97 mL (103.14 mmol) benzoyl chloride, dissolved in 20 mL dioxane, were slowly added dropwise. The mixture was stirred at room temperature overnight, after which the solvent was evaporated. The remaining residue was treated with a small amount of acetic acid and a large amount of ethyl acetate. The product was crystallized at 4 °C (white crystals, yield: 23.3 g, 81.9 mmol, 83%, HPLC: 19.5 min).

*Benzoyl-D/LhAla(2-Pyr)-OMe (**45**)*. 23.3 g (81.9 mmol) Benzoyl-D/LhAla(2-Pyr)-OH (**44**) was suspended in 35 mL methanol and the mixture was cooled to −10 °C. 8.9 mL (122.85 mmol) thionyl chloride was added in several portions and the mixture was stirred for 30 min at −10 °C. Afterwards, an additional 3 mL of (40.95 mmol) thionyl chloride were added and the mixture was stirred at room temperature overnight. The solvent was removed under vacuum, and the residue was dissolved in ethyl acetate, washed twice with saturated $NaHCO_3$-solution, and dried over Na_2SO_4. The solvent was removed and the remaining product dried under vacuum (light yellow amorphous solid, yield: 18.3 g, 61.3 mmol, 75%, HPLC: 22.7 min).

*Benzoyl-hAla(2-Pyr)-OH (**46**) and Benzoyl-DhAla(2-Pyr)-OMe (**47**)*. 6.3 g (21.1 mmol) benzoyl-D/LhAla(2-Pyr)-OMe (**45**) was dissolved in 200 mL methanol and treated with 750 mL 0.2 N ammonium acetate solution (pH 7.8), and the pH adjusted to 7.5–8 with dilute ammonium hydroxide solution. 25 mg α-chymotrypsin from bovine pancreas (Merck, Darmstadt, Germany, 350 U mg^{-1}) was dissolved in 1 mL water and added to the reaction mixture. The mixture was incubated at 37 °C over a period of 3 days, during which the pH was periodically controlled and adjusted to 7.5–8. The reaction progress was monitored by HPLC. Afterwards, the mixture was adjusted to pH 4 by addition of acetic acid, the solvent was evaporated in vacuum, and the residue was dissolved in 2 M acetic acid. Addition of concentrated ammonia solution to pH 8–9 resulted in precipitation of benzoyl-D-hAla(2-Pyr)-OMe. The D-derivative (**47**) was isolated by filtration, washed with a small amount of ammonium hydroxide solution (pH 8.5), and dried under vacuum. Additional product was isolated by three extractions of the basic water phase with ethyl acetate, drying with Na_2SO_4, and evaporation of the solvent under vacuum. The L-derivative (**46**) was obtained by repeated lyophilization of the water phase to remove the ammonium acetate. (**46**: light yellow solid, yield: 2.42 g, 8.5 mmol, HPLC: 19.5 min; **47**: light yellow solid, yield: 2.65 g, 8.9 mmol, HPLC: 22.7 min).

*H-DhAla(2-Pyr)-OH (**48**)*. 2.5 g (8.38 mmol) benzoyl-DhAla(2-Pyr)-OMe (**47**) was dissolved in 50 mL 6 N HCl and refluxed for 20 h. The reaction mixture was cooled to room temperature and the benzoic acid was removed by filtration. The solvent was evaporated in vacuum, the remaining residue was repeatedly treated with water, and evaporated. The product was isolated by ion exchange chromatography as described for intermediate **43** (white solid, yield: 0.8 g, 4.4 mmol, 49%, HPLC: 3.9 min, TLC: R_f = 0.04).

The enantiomeric purity was controlled by reaction with Marfey's reagent.[32] Product **48** contained an impurity of 3.6% L-amino acid (*ee* 92.8%).

*Bzls-DhAla(2-Pyr)-OH×TFA (**49**)*. 0.788 g (4.4 mmol) H-DhAla-(2-Pyr)-OH (**48**) was suspended in 25 mL dry DCM and treated with 1.76 mL (14 mmol) chlorotrimethylsilane and 2.44 mL (14 mmol) DIEA. The mixture was refluxed for 1 h, during which the amino acid was completely dissolved. The mixture

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was cooled to 0 °C and treated with 0.88 g (4.62 mmol) benzylsulfonyl chloride and 0.804 mL (4.62 mmol) DIEA. The mixture was stirred for 15 min at 0 °C and 1.5 h at room temperature. The solvent was evaporated under vacuum and the product was purified by preparative HPLC and lyophilized (white lyophilized powder, 1.22 g, 2.72 mmol, 61 %, HPLC: 23.9 min, ^{1}H NMR (300.13 MHz, D_2O): $\delta = 8.62$ (d, broad, $^3J\{HH\} = 6.0$ Hz, 1 H), 8.49 (dd $^3J\{HH\} = 7.9$ Hz, $^3J\{HH\} = 7.4$ Hz, 1 H), 7.90 (dd $^3J\{HH\} = 7.4$ Hz, $^3J\{HH\} = 6.0$ Hz, 1 H; 7.87 d $^3J\{HH\} = 6.0$ Hz, 1 H), 7.42 (m 5 H), 4.49 (s 2 H), 3.84 (dd $^3J\{HH\} = 8.2$ Hz, $^3J\{HH\} = 5.2$ Hz, 1 H), 3.13 (t $^3J\{HH\} = 7.6$ Hz, 2 H), 2.21 ppm (m 2 H). ^{13}C NMR, 75.48 MHz, D_2O, $\delta = 174.53$; 163.08 q $^2J\{CF\} = 35.7$ Hz; 155.55; 147.42; 141.19; 131.26; 129.45; 129.33; 128.87; 127.68; 125.55; 116.72 q $^1J\{CF\} = 292.0$ Hz; 59.65; 55.83; 31.56; 29.53 ppm.

*Bzls-DhAla(2-Pyr)-Gly-OtBu (**50**)*. 1.7 g (3.8 mmol) Bzls-DhAla(2-Pyr)-OH×TFA (**49**) and 700 mg (4.18 mmol) H-Gly-O*t*Bu×HCl were dissolved in 50 mL DMF. At 0 °C 2.18 g (4.18 mmol) PyBop and 2.18 mL (12.54 mmol) DIEA were added, the mixture was stirred for 30 min at 0 °C and 4 h at room temperature. The DMF was removed under vacuum and the residue was dissolved in 220 mL 2 M acetic acid and filtered. The pH of the solution was adjusted to 8.5–9 by the addition of concentrated ammonium hydroxide solution and extracted three times with ethyl acetate. The ethyl acetate was removed under vacuum, and the crude yellow oil (3 g, HPLC: 35.65 min) was used for the next step without purification.

*Bzls-DhAla(2-Pyr-N-oxide)-Gly-OtBu (**51**)*. 3 g of the crude product **50** was dissolved in 50 mL DCM and treated with 1.04 g 3-chloroperoxybenzoic acid (mCPBA). The formed product **51** and the byproduct 3-chlorobenzoic acid eluted at identical retention times on HPLC (40.5 min); therefore, for reaction control a small sample of the mixture was incubated at different time intervals with TFA for 30 min to allow a differentiation after cleavage of the O*t*Bu ester. The mixture still contained approximately 40 % of the starting material after 1 h. Therefore, additional mCPBA (approximately 2.5 g) was added in ten portions over a period of 24 h. The 3-chlorobenzoic acid was partially removed by filtration. The solvent was evaporated and the residue was dissolved in 300 mL 2 M acetic acid and filtered to remove additional 3-chlorobenzoic acid. The pH was adjusted to 8–9 with concentrated ammonium hydroxide solution and the solution was extracted three times with ethyl acetate. The ethyl acetate was removed under vacuum, and the crude product was used for the next step without purification (2.8 g yellow oil).

*Bzls-DhAla(2-Pyr-N-oxide)-Gly-OH (**52**)*. 684 mg of the oily crude product **51** was treated with 10 mL 90 % TFA and the mixture was shaken for 1 h at room temperature. The solvent was removed under vacuum, the product was purified by preparative HPLC, and lyophilized (yield 325 mg, 0.82 mmol, HPLC: 25.84 min).

*Bzls-DhAla(2-Pyr-N-oxide)-Gly-4-amidinobenzylamide×acetic acid (**27**)*. 304 mg (0.77 mmol) Bzls-DhAla(2-Pyr-*N*-oxide)-Gly-OH (**52**) and 179 mg (0.805 mmol) 4-amidinobenzylamine×2 HCl (obtained from Boc-4-acetylhydroxyamidinobenzylamine by catalytic hydrogenation and subsequent treatment with 1 N HCl in acetic acid) were suspended in 50 mL DMF. At 0 °C the mixture was treated with (0.805 mmol) PyBop and 420 μL (2.4 mmol) DIEA, stirred for 30 min at 0 °C and 4 h at room temperature. After evaporation of the solvent the product was purified by preparative HPLC and lyophilized. The white powder was dissolved in water and loaded on a Fractogel-EMD COO^- column (dimension 5×20 cm), equilibrated with water. The column was washed with 800 mL water, then the product was eluted by an ammonium acetate gradient. The collected fractions were lyophilized 3×from water (white lyophilized powder, 220 mg, 0.37 mmol, MS: calc. 538.2 , found 539.2 $[M+H]^+$, HPLC: 23.90 min, ^{1}H NMR (500.13 MHz, D_2O): $\delta = 8.35$ (d, $^3J\{HH\} = 6.33$ Hz, 1 H), 7.72–7.66 (m, 3 H; 7.55–7.48 m, 4 H), 7.47–7.40 (m, 5 H), 4.58–4.49 (AB, 2 H), 4.47–4.40 (AB, 2 H), 3.96 (s 2 H), 3.83 (t $^3J\{HH\} = 6.87$ Hz, 1 H), 3.01–2.90 (m, 2 H), 2.18–2.03 (m, 2 H), and 1.96 ppm (s from acetic acid). ^{13}C NMR (125.77 MHz, D_2O): $\delta = 172.86$, 167.58, 152.15, 145.73, 140.97, 133.26, 132.15, 130.36, 130.25, 130.01, 129.31, 129.24, 128.64, 127.87, 126.58, 60.17, 58.34, 44.28, 44.02, 30.54, 27.9 ppm; the amidino carbon signal was not resolved).

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