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Selective Boron-Containing Thrombin Inhibitors—X-ray Analysis Reveals Surprising Binding Mode

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Abstract—Based on the structural comparison of the S1 pocket in different trypsin-like serine proteases, a series of Boc-D-trimethylsilylalanine-proline-boro-X pinanediol derivatives, with boro-X being different amino boronic acids, have been synthesized as inhibitors of thrombin. Among the novel compounds, a number of derivatives were synthesized which appeared to have side-chain variants too big to fit into the S1 pocket. Nevertheless, these compounds inhibited thrombin in the nM range. The X-ray structure of one of these inhibitors bound to the active side of thrombin reveals that a new binding mode is responsible for these surprising results.

Introduction

In a number of important cardiovascular diseases such as myocardial infarction, unstable angina, deep vein thrombosis, pulmonary embolism, and ischemic stroke, a causative factor is intravascular clot formation. A blood clot is formed by activation of the coagulation cascade through the intrinsic ('contact activation') or the extrinsic (activation by exposure of plasma to a nonendothelial surface or damage to vessel wall and tissue factor release) coagulation pathways.

The enzyme which plays a pivotal role in clot formation is thrombin, a trypsin-like serine protease. Thrombin is the last enzyme in the coagulation cascade. It converts soluble fibrinogen to fibrin which then can polymerize to form a haemostatic plug.[1]

In addition to fibrinogen cleavage and platelet activation, thrombin exerts a positive feedback to its own production by activating coagulation factors V and VIII. It also activates factor XIII which crosslinks and stabilizes the fibrin polymers and stimulates platelet secretion and aggregation through a proteolytic activation of the thrombin receptor on the platelets.[2]

Because of the important role of thrombin in the coagulation mechanism, the enzyme has become a key target for the treatment of thromboembolic diseases.[3]

Up to now only two anticoagulant strategies have been used clinically for treatment of acute and chronic thrombosis: the use of heparins which act by activation of endogenous plasma proteins that inhibit thrombin and other proteases of the coagulation cascade, and the use of coumarins which inhibit the hepatic synthesis of vitamin K-dependent proteins including thrombin. Because of the indirect and non-selective mechanism of these anticoagulants, careful monitoring is necessary and side effects often can be observed.[4,5] Therefore, much effort has been invested to find novel classes of low molecular weight, direct acting inhibitors of thrombin, which are safe and selective.[6]

There are two principal classes of low molecular weight active site directed thrombin inhibitors: the first consists of inhibitors which do not form a covalent bond to the catalytic Ser 195 but bind non-covalently through hydrogen-bonding, hydrophobic, and electrostatic interactions within the S1–S3 specificity pockets. Examples of this class of compounds are Argatroban,[7] NAPAP,[8] Inogatran,[9] Melagatran,[10] Napsagatran,[11] and several other non transition state molecules.[12–21] The second class of inhibitors form a covalent bond between the nucleophilic Ser 195 of the enzyme and an electrophilic center on the

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inhibitor. Chloromethylketones like PPACK,[22] C-terminal aldehydes,[23] fluoromethylketones,[24] α-ketoesters[25] or amides,[26] phosphonates and phosphonites,[27] and boronic acids[28–31] are examples of this second class.

Many of these thrombin inhibitors have a basic P1 side-chain in order to form a salt bridge to Asp 189 at the bottom of the S1 pocket and thereby increase the inhibitory affinity for thrombin. However, it has been shown that a basic P1 side-chain is not a necessary prerequisite to obtain active thrombin inhibitors.[32–34]

In a recent paper,[35] we have described selective boron containing thrombin inhibitors with non-basic residues in P1, which showed a higher selectivity for thrombin over trypsin and plasmin. Most of the new P1 side-chains are closely related to those of Lys or Arg.

Continuing our search for selective inhibitors of thrombin, we have now extended the P1 variations to side-chains containing butyl formamides, thioformamides, alkyl amides and ureas. According to molecular modeling studies, these side-chains seem to be too big to fit into the S1 pocket of thrombin. Nevertheless, these compounds show K_i values for thrombin in the pM–nM range. A novel binding mode which could explain the high potency of these compounds has been found by X-ray analysis.

Inhibitor Design

In order to gain selectivity for thrombin over trypsin and plasmin, the S1 pocket of these serine proteases is an attractive target.[36] This region is quite conserved in all three proteases but there are still small differences which can be used to discriminate one serine protease from the others.[37]

It has been demonstrated that a neutral P1 side-chain in peptide boronic acid inhibitors can improve the selectivity for thrombin over trypsin and plasmin quite dramatically.[35,38–40] From a synthetic point of view, a very efficient pathway to neutral side-chains is derivatization of the side-chain amino function of borolysine. In one step, amide or urea derivatives can be synthesized.

We assumed that there is a certain size and volume of the side-chain which is optimal for obtaining selectivity without losing too much activity. Therefore, a number of amides and ureas with different demand on space were synthesized. As the smallest representatives, formamide and thioformamide derivatives were prepared.

All compounds are peptide boronates of the type PG-D-TMSal-Pro-boroLys(X) pinanediol (PG = protecting group) with constant building blocks in P2 and P3 and variations on the amino function of the borolysine side-chain (X).

D-Trimethylsilyl alanine (D-TMSal) in P3 was used as bioisosteric replacement of D-phenylalanine to reduce the metabolic instability of our molecules.[41]

Despite the fact that most of the synthesized compounds have the Boc protecting group at the N-terminus, the X-ray structure determination has been done with the Cbz protected formamide derivative **18**, since this compound was a candidate for clinical development. However, the N-terminal hydrophobic protecting group should not have a great influence on the binding mode of these compounds, because the activities of the Boc-protected compound **9** and the Z-protected compound **18** vary by a factor of 2–3 only (see Table 2).

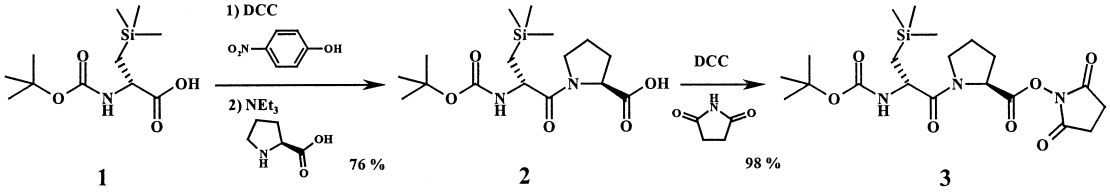
The same similarity of activities can be observed for compound **8** with a Boc and compound **20** with an isobutylcarbonyl moiety at the N-terminus (variations by a factor of 1–2). Here synthetic reasons are responsible for the different N-termini. The Boc protecting group is not stable under the reaction conditions we used to prepare the free boronic acid while the isobutylcarbonyl moiety is compatible with these conditions.

Chemistry

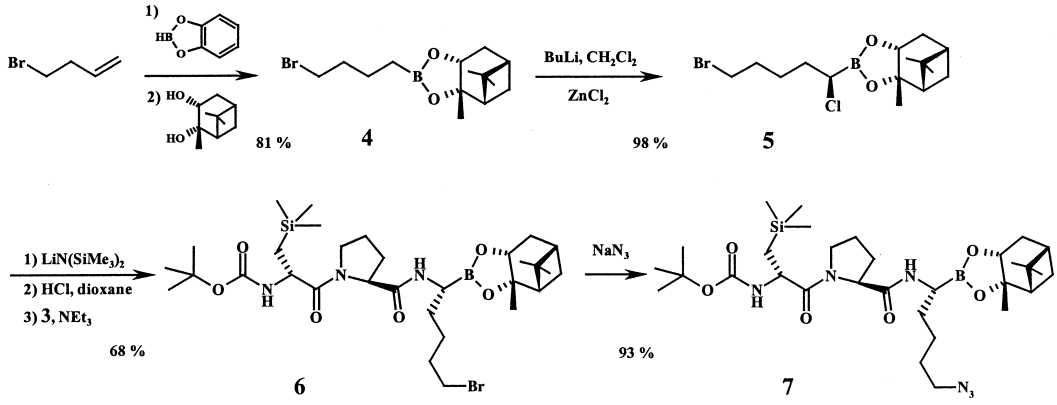
All thrombin inhibitors described in this paper have been synthesized starting from the common intermediate **7** (see Scheme 2). The strategy to access **7** and all other compounds described in this report was based on a previous report from these laboratories.[35]

The first building block needed was Boc-D-TMSal-Pro-ONSu **3**. It was prepared by coupling of proline to (*S*)-*N*-(t-butoxycarbonyl)-β-(trimethylsilyl)alanine **1** (= Boc-D-TMSal-OH) and subsequent activation with *N*-hydroxy succinimide (Scheme 1). Boc-D-TMSal-OH **1** was obtained using the Schoellkopf procedure as described in the literature.[41]

The aminoboronic acid derivative which was needed for the coupling to **3** was synthesized using a procedure developed by Matteson.[42–44] 4-Bromo-1-propene was treated with catecholborane and the crude product was reacted with (+)-pinanediol to give the borane pinanediol ester **4**. Due to the chiral auxiliary, the 'CHCl' insertion



Scheme 1.



Scheme 2.

generated a single diastereomer of the chlorosubstituted homologue **5**. The reaction did not go to completion but the unreacted starting material could be removed by chromatography after the subsequent one-pot reaction: substitution of Cl by $N(SiMe_3)_2$, acidic hydrolysis and coupling with the activated dipeptide **3**. The replacement of Cl by $N(SiMe_3)_2$ led to inversion of the configuration in the product. In the next step, Br was exchanged by N_3 and the common intermediate **7** could be obtained (Scheme 2).

Hydrogenation of the azido group of **7** catalyzed by palladium on carbon led to the borolysine derivative **8**.[29] The free amino function of this compound could be further derivatized by reaction either with an anhydride (→**9**), acid chlorides (→**10**, **11**, **12**, **13**), isocyanates (→**14**, **15**), or potassium cyanate (→**16**) (Scheme 3; Table 1).

The thioformamide derivative **17** could be obtained by treatment of the corresponding formamide **9** with Lawesson's reagent. Removal of the Boc protecting group from **9** and reaction with benzyl chloroformate led to compound **18** (Scheme 4).

The Boc-protecting group was not stable under the conditions used to remove the pinanediol group. In order to prepare the free boronic acid, it was replaced by an isobutylcarbonyl capping group. Neither the N-terminal unprotected free boronic acid nor the free boronic acid with the lysine-side chain were stable and thus not useful as intermediates. Therefore, we started with the azido compound **7** and replaced the Boc protecting group with the isobutylcarbonyl moiety by treatment with acetic acid/concd. HCl followed by acylation with isobutyl acid chloride. The resulting compound **19** was either hydrogenated to give the pinanediol derivative **20**, or treated with BCl_3 and then hydrogenated which yielded the corresponding free boronic acid **21** (Scheme 5).

Table 1. Reagents and yields for Scheme 3

No.	R	Reagent	Yield (%)
9	H	MeC(O)OC(O)H	90
10	Me	MeC(O)Cl	97
11	Et	EtC(O)Cl	95
12	*i*-Pr	*i*-PrC(O)Cl	94
13	NMe_2	$Me_2NC(O)Cl$	42
14	NHMe	MeNCO	94
15	NHEt	EtNCO	87
16	NH_2	KOCN	67

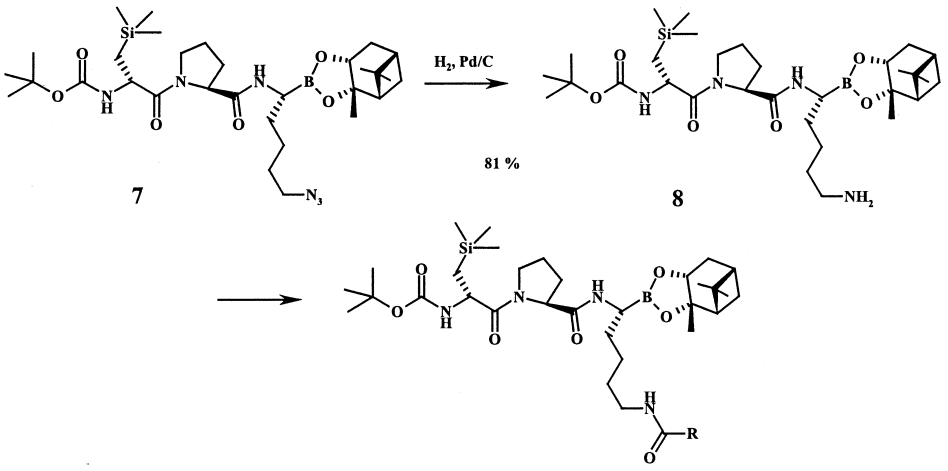
Biology

All biological results are summarized in Table 2.

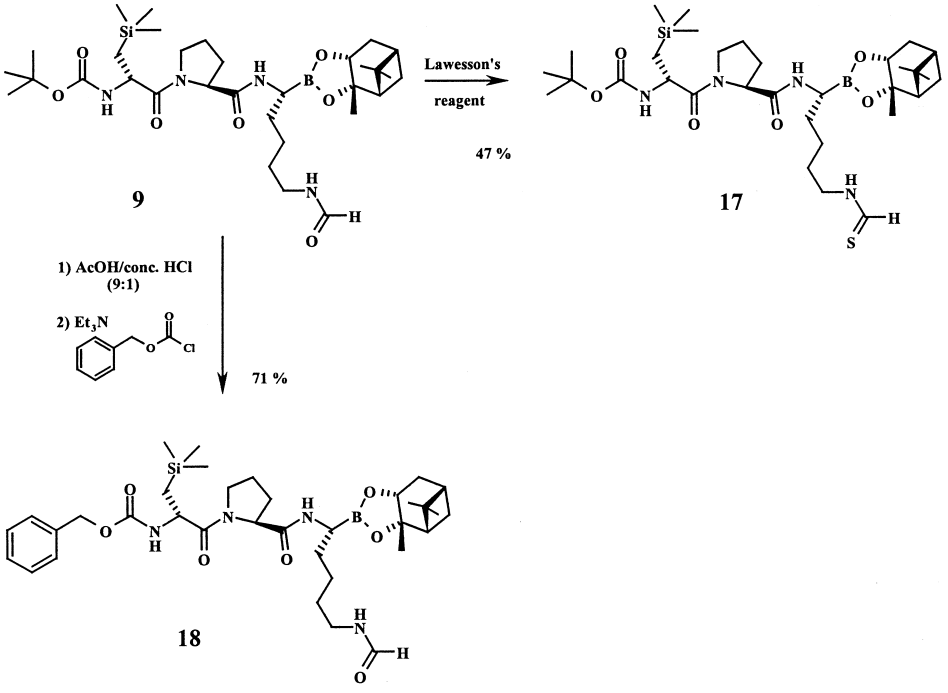
Compounds with a lysine side-chain like **8**, **20** and **21** were highly active thrombin inhibitors. As can be seen with **20** and **21**, there was no difference in the activity for thrombin and the selectivity over plasmin and trypsin, regardless of whether the boronic ester was hydrolyzed or not. This indicates that pinanediol was removed before the compounds bound to thrombin.[45] The X-ray crystal structure of **18** supported this hypothesis. Although the pinanediol ester derivative of **18** has been used for crystallization, no pinanediol could be observed in the X-ray structure.

Table 2. Dissociation constants (K_i) of the inhibitors were assessed in a chromogenic assay with human thrombin, human plasmin and bovine pancreatic trypsin. The selectivity for the inhibition for thrombin over plasmin or over trypsin was compared on the basis of the ratios $K_{i,PLAS}/K_{i,THR}$ or $K_{i,TRY}/K_{i,THR}$ (in parenthesis). Mean values ($n=3$)

	K_i (nM)		
No.	Thrombin	Plasmin	Trypsin
8	0.039	3.51(90)	0.39(10)
9	5.57	8250(1530)	202(36)
10	0.073	4.4(60)	1.0(14)
11	0.056	8.4(150)	0.9(16)
12	0.062	6.4(103)	0.8(13)
13	87.9	3983(45)	1860(21)
14	5.4	8024(1486)	1447(268)
15	3.8	2597(683)	502(1325)
16	6.6	2155(327)	313.7(48)
17	0.2	2795(13 975)	63(315)
18	2.51	2493(993)	105(42)
20	0.027	2.5(92)	0.18(6.7)
21	0.054	1.5(28)	0.18(3.3)



Scheme 3.



Scheme 4.

Different N-terminal groups (Boc for **8** and isobutylcarbonyl for **20** and **21**) seemed to have no big influence on the inhibitory activity. The same observation could be made for the formamide compounds **9** and **18** with Boc and Cbz protecting groups, respectively.

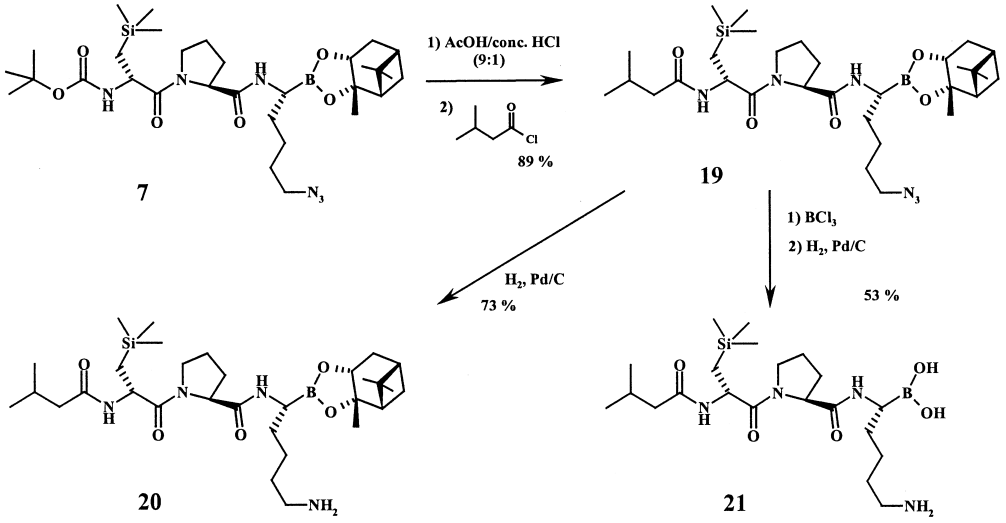
The formamide side-chain afforded lower activity for thrombin but higher selectivity over plasmin and trypsin. Replacement of oxygen by sulfur leading to thioformamide derivative **17** gave both higher activity and selectivity.

A very surprising result was observed for the alkylcarbonyl derivatives **10**, **11** and **12**. Despite the much bigger substituents the activity was comparable to the activity of the lysine compound **8**. There was no difference in activity whether the alkyl group was methyl, ethyl or isopropyl.

The same independence from substituent size was found for the urea derivatives **14**, **15** and **16**. They were generally less active than the alkyl derivatives **10**–**12** but again all three compounds had quite comparable K_i values. Only **13**, with two additional substituents on the nitrogen, was less active. It could be that either the size of the two methyl groups causes severe steric hindrance or the NH is necessary for binding.

X-ray Crystallographic Structure of 21 Bound to Thrombin

To gain further insight into the binding mode of the inhibitors under investigation the X-ray structure of **21** bound to human α-thrombin has been determined. The resolution was 2.3 Å and the final R-factor 19.1% (Table 4; for crystallographic details, see Experimental).



Scheme 5.

The inhibitor is bound to the protein by a covalent linkage between the boronic acid and Ser 195 of thrombin. The boron atom shows a tetrahedral coordination, with covalent bonds to the Cα-carbon, to the two oxygen atoms of the boronic acid and also to the Oγ oxygen atom of Ser 195 (Fig. 1A). The Nε2 nitrogen of His 57 forms a hydrogen bond to one oxygen atom of the boronic acid (2.97 Å) which in turn forms a hydrogen bond to a water molecule (Table 3).

The lysine side-chain extends as expected into the S1 pocket (Fig. 1A). Its positively charged amino group forms a salt-bridge to the side chain of Asp 189 and two hydrogen bonds to the carbonyl oxygen atoms of residues Gly 219 and Ala 190. There is an additional hydrogen bond to a water molecule which is buried in the S1 pocket (Table 3).

Table 3. Interatomic distances

Protein	Ligand	Distance (Å)
21		
Gly 219 O	boroLys N_ζ	2.93
Ala 190 O	boroLys N_ζ	2.75
Asp 189 $O_{\delta 2}$	boroLys N_ζ	3.16
Wat 2	boroLys N_ζ	2.81
Gly 193 N	boroLys O 1	2.88
Ser 195 N	boroLys O 1	2.99
Wat 1	boroLys O 2	2.31
His 57 $N_{\epsilon 2}$	boroLys O 2	2.97
Gly 216 O	TMSal N	3.27
Gly 216 N	TMSal O	3.06
18		
Gly 219 N	Lym[a] CHO	3.29
Gly 219 O	Lym[a] N_ζ	2.91
Gly 193 N	Lym[a] O 1	3.09
Ser 195 N	Lym[a] O 1	2.75
Wat 1	Lym[a] O 2	2.85
His 57 $N_{\epsilon 2}$	Lym[a] O 2	2.88
Gly 216 O	TMSal N	2.77
Gly 216 N	TMSal O	3.16

[a]Lym = boroformyl lysine.

Finally, the N-terminal portion of the ligand forms a short anti parallel β-sheet with residue Gly 216 of the protein (Fig. 1A; Table 3).

X-ray Crystallographic Structure of 18 Bound to Thrombin

To further elucidate the protein–ligand interactions in the S1 pocket-compound **18** with a modified P1 side-chain complexed to human α-thrombin has been determined by X-ray crystallography. The resolution was 2.3 Å and the final R-factor 17.4% (Table 4; for crystallographic details, see Experimental).

Apart from the P1 residue, the structure of **18** bound to thrombin is very similar to that of **21** despite the different N-terminal protecting groups (Fig. 1B; Table 3). The P1 side-chain, however, shows a new binding mode. It does not contact Asp 189 but leaves the S1 pocket after the Cδ so that the terminal formamide can form two hydrogen bonds with Gly 219 in a *cis*-conformation (Fig. 1B; Table 3).

There is no evidence for the pinanediol in the X-ray structure. Boronic acids, even introduced as their corresponding esters, form complexes with the enzyme only as the free boronic acids. This is due to rapid equilibration between the boronic acid ester and the free boronic acid.[45] There is also no evidence in the literature that the presence

Table 4. Crystallographic data statistics

	18	**21**
Resolution (Å)	2.3	2.3
R_{cryst} (%)	17.4	19.1
R_{free} (%)	20.8	23.5
rms bond dev (Å)	0.013	0.013
rms angle dev (°)	1.66	1.72
Number of reflections	15 252	13 771
Completeness (%)	93.1	84.1
Free set (%)	4.6	4.0

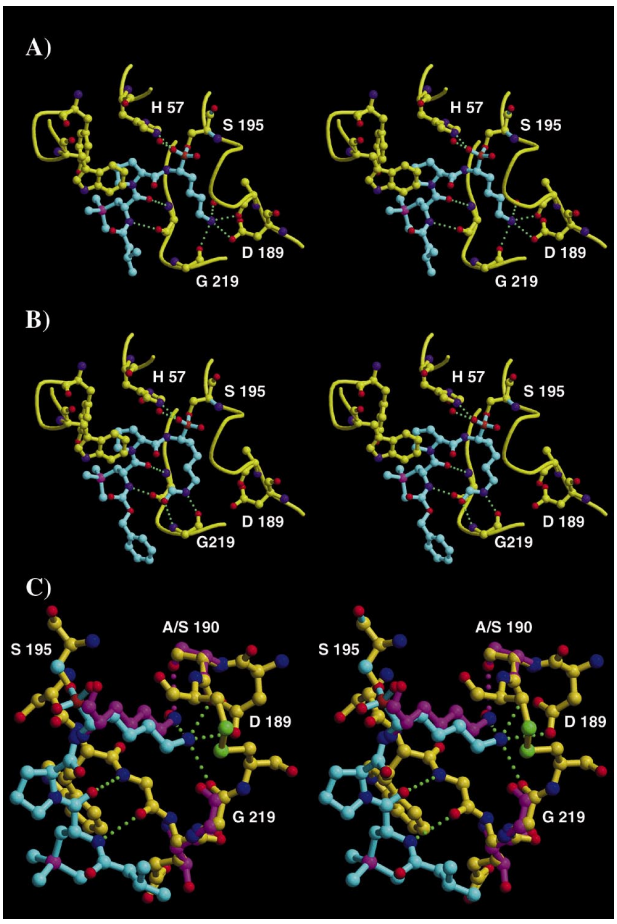


Figure 1. X-ray structures of human α-thrombin complexed with the inhibitors **18** and **21**. (A) The complex with the inhibitor **21.**; (B) the complex with the inhibitor **18.** (C) Overlay of the structures of thrombin and trypsin complexed with inhibitor **21** and mung bean inhibitor, respectively. Carbon atoms are coloured in yellow for the protein atoms of thrombin, in cyan for the inhibitor **21** and in magenta for the lysine side-chain of the mung bean inhibitor and the residues 190, 218 and 219 of trypsin. The Bor atom is coloured in orange and the Silicium atom is coloured in magenta. In trypsin, the residue Ser190 makes a direct hydrogen bond to the side-chain nitrogen of the lysine (magenta), while in thrombin there is a water molecule hydrogen bonded to this nitrogen.

or absence of pinanediol during crystallization has any influence on the binding mode in the S1 pocket.[45]

Discussion

In our previous paper,[35] we could demonstrate how the selectivity of tripeptide–boronic esters can be influenced by variations in P1, making use of the more hydrophobic nature of the S1 pocket in thrombin compared to trypsin and plasmin. We rationalized our results based on the assumption that all modified P1 side-chains would still bind into the S1 pocket.

The P1 side-chains of the compounds described in the present paper are different: most of them are too large to fit into the S1 pocket (e.g., **12** or **13**), but even for the smallest ones (**9** or **18**), no satisfactory binding mode for the formamide moiety within the S1 pocket could be found by molecular modeling studies.

The comparison of the X-ray structures of **21** and **18** gives a simple explanation: while **21** forms a 'traditional' salt bridge to Asp 189 in S1 of thrombin with its Lys-side-chain,[30,45] compound **18** shows a new binding mode. The side-chain enters the S1 pocket, but in a kind of a U-turn leaves it again to form two new hydrogen bonds between the formamide and the backbone of Gly 219.

One could argue that the new binding mode is due to the different N-terminal groups or the presence/absence of pinanediol during crystallization. But all published X-ray structures of boronic acid derivatives in complex with thrombin bind in the 'traditional' way irrespective of their N- or C-terminal groups.[39,40,45,46]

Because of the loss of the strong salt bridge to Asp 189, the activity is reduced towards all three enzymes. However, the loss of potency is much stronger in the case of plasmin and trypsin, since the latter enzymes have a Ser in position 190 compared to an Ala in thrombin so that an additional hydrogen bond can be formed to a Lys side-chain, as is evident from several trypsin/inhibitor-complexes[30,39,45] (Fig. 1C). Based on this argumentation, the selectivity pattern of all compounds under investigation should be similar which is in contrast to the results shown in Table 2. If one assumed a similar binding conformation for all inhibitors in all enzymes, there are two other effects that have to be taken into account. The contacts of the P1 side-chains with the protein backbone around position 217 (Fig. 1C) and the interaction of the inhibitors with their own hydrophobic protecting groups could also play a role for enzyme selectivity.

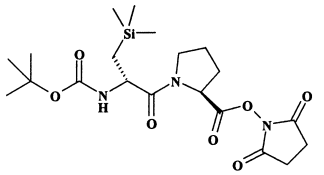
Compounds **10**, **11** and **12** with an alkylamide in P1 show very similar data compared to **8**. One could think of a hydrolysis of the Lys side-chain amide bond under assay conditions, which would transform these compounds back to **8**. However, this amide bond is quite stable even under more drastic conditions.

Probably the amide is in a *trans*-conformation in these compounds, and the additional hydrophobic group can now, in contrast to **9** or **18**, stabilize the bound conformation by non-polar interactions with the Boc group, thus compensating for the loss of the salt bridge. Since this stabilization is possible in all enzymes the selectivity compared to **8** does not change.

In the urea derivatives **13** to **16** the whole -NH-CO-NR_1R_2-group lies within a plane and probably causes steric hindrance, especially in the case of **13**. Therefore, the urea derivatives are much less active than compounds **10** to **12**.

The higher affinity of compound **17** compared to **9** is quite unexpected since the less electronegative sulfur in the thioamide should form a weaker hydrogen bond to the backbone amide of Gly 219 than the oxygen in the formamide derivative. A possible explanation could be that the less polar sulfur can better cope with the hydrophobic environment created by the Boc protecting group. Differences in the region around amino acid 217 (Glu in

Table 5. NMR values of thrombin inhibitors **9**–**20** and precursors **6**–**8**. (If not otherwise indicated, all spectras are measured in DMSO at high temperature (100–150 °C))



No.	s, 9H, $SiMe_3$	m, 2H, 15-H	2 s, je 3H, 7-Me	s, 3H, 1-Me	s, 9H, *t*-Bu	M, 10H, 2-H, 3-H, 4-H, 5-H, 11-H, 12-H	m, 1H, 8-H	m, 2H, 13-H	m, 1H, 6-H	m, 1H, 14-H	m, 1H, 10-H	m, 1H, 16-H	m, 1H, 9-H
6	0.02	0.92–0.99	0.82; 1.24	1.29	1.40	1.25–2.32	2.66–2.75	2.92–3.11	4.15–4.20	4.25–4.34	4.43–4.51	5.93–6.02	7.70–7.80
7	0.007	0.89–0.96	0.83; 1.28	1.39	1.43	1.21–2.48	2.68–2.65	3.20–3.48	4.23–4.31	4.30–4.42	4.57–4.65	4.98–5.07	7.41–7.49
8	0.00	0.85–0.90	0.80; 1.20	1.33	1.38	1.20–2.40	2.70–2.79	3–76–3.84 3.30–3.40	4.20–4.25	4.30–4.38	4.53–4.57	5.46–5.50	7.35–7.40
9	0.02	0.91–0.97	8.82; 1.25	1.28	1.38	1.30–2.30	2.64–2.73	3.45–3.63	4.14–4.20	4.24–4.33	4.40–4.53	5.93–6.03	7.65–7.77
10	0.03	0.93–0.98	0.83; 1.26	1.30	1.40	1.12–2.35	2.99–3.07	3.45–3.61	4.15–4.21	4.26–4.35	4.42–4.50	5.75–5.86	7.45–7.62
11	0.02	0.91–0.96	0.83; 1.26	1.30	1.40	1.22–2.31	2.63–2.73	3.44–3.61	4.14–4.19	4.23–4.43	4.40–4.50	5.95–6.05	7.68–7.80
12	0.01	0.90–0.95	0.82; 1.25	1.29	1.39	1.22–2.35	2.64–2.75	3.43–3.62	4.15–4.20	4.25–4.33	4.39–4.51	5.95–6.05	7.64–7.84
13	0.01	0.84–0.88	0.78; 1.22	1.31	1.37	1.18–2.35	2.18–2.75	3.78–3.84 3.38–3.30	4.19–4.22	4.27–4.35	4.50–4.55	4.98–5.02	7.31–7.35
14	0.03	0.94–1.00	0.83; 1.32	1.36	1.40	1.22–2.32	2.73–2.80	3.47–3.61	4.17–4.22	4.28–4.35	4.44–4.50	5.74–5.81	7.41–7.48
15	0.02	0.92–1.04	0.83; 1.26	1.30	1.40	1.25–2.31	2.72–2.78	3.46–3.63	4.16–4.20	4.28–4.34	4.44–4.50	5.78–5.84	7.48–7.52
16[a]	0.02	0.90–0.98	0.82; 1.26	1.30	1.40	1.20–2.30	2.14–2.23	3.44–3.62	4.14–4.19	4.24–4.33	4.41–4.51	5.94–6.01	7.64–7.75
17	0.00	0.85–0.98	0.80; 1.20	1.20	1.35	1.22–2.25	3.30–3.40	3.40–3.55	3.60–3.70	4.03–4.15	4.21–4.30	4.30–4.40	6.80–6.90
18	–0.01	0.92–0.98	0.80; 1.24	1.28	—	1.27–2.38	2.62–2.70	3.40–3.50 3.50–3.62	4.11–4.16	4.28–4.36	4.38–4.50	6.60–6.68	7.63–7.77
19	0.05	0.96–1.01	0.85; 1.27	1.31	—	1.25–2.33	2.63–2.72	3.45–3.54 3.61–3.76	4.17–4.21	4.48–4.56	4.48–4.56	7.36–7.43	7.68–7.81
20	0.01	0.92–1.00	0.82; 1.25	1.28	—	1.32–2.30	2.56–2.66	3.42–3.51 3.64–3.70	4.13–4.18	4.43–4.52	4.43–4.52	7.54–7.68	7.68–7.94

[a]Measured at room temperature, rotamers.

thrombin, Ser in trypsin and Leu in plasmin) could account for the increase in selectivity of **17** compared to **9**.

Since X-ray structures have only been determined for compounds **18** and **21**, considerations about the SAR and the binding modes of the other inhibitors remain speculative. Further experimental structures are needed to answer these questions.

Conclusions

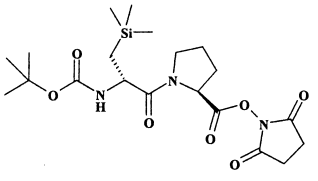
Replacement of the usual basic residues in P1 of thrombin inhibitors by non-basic moieties could increase the selectivity of the corresponding molecules over trypsin and plasmin while still maintaining nM thrombin affinities. The structural data so far available[18b,20b,46] indicate that most of these compounds still bind to the S1 pockets of the serine proteases.

In this paper, however, we could show for the first time that the introduction of a butyl formamide as P-1 side-chain in a tripeptide–boronate (**18**) can force the thrombin/inhibitor complex to adopt a new binding mode in which the formamide group points out of the S1 pocket and forms new hydrogen bonds with Gly 219. The structure–activity relationship of the other compounds under investigation indicates that these molecules show the same binding mode as **18**.

Experimental

All compounds were characterized by 300 MHz proton NMR using a Bruker 360 FT-NMR spectrometer. Chemical shifts are expressed as ppm downfield from tetramethylsilane; *J* values are expressed in Hz. The ^{1}H NMR data of the compounds **6**–**20** are summarized in Table 5, the signals for the different side-chains and in the case of compounds **18**–**20** also the signals for the N-terminal group can be found after the experimental details for the compounds. Fast atom bombardment mass spectrometry (Xe, 8 keV) on a VG 70-SE mass spectrometer was used for the characterization of the reported compounds. Melting points were determined on a Büchi 535 instrument. For the chromatographic purification, the flash chromatography technique was applied using 230–400 mesh silica gel.

Boc-D-TMSal-Pro-ONSu 3.

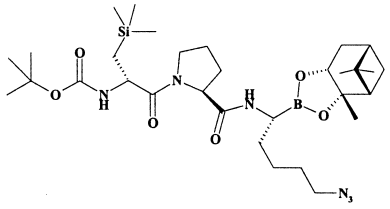


29.7 g (113 mmol) of Boc-D-TMSal-OH **1** and 19.0 g (136.3 mmol) of *p*-nitro-phenol are dissolved in EtOAc. After cooling to 0 °C, 23.4 g (113 mmol) of DCC is added and the mixture is stirred for 1 h at 0 °C and then for 15 h at room temperature. A precipitate is formed, which is filtered off and washed with EtOAc and the filtrate is concentrated in vacuo. The resulting oil is purified by flash chromatography (9:1, hexane:EtOAc). Boc-D-TMSal-ONp is obtained as white crystals.

51.6 g (113 mmol) of Boc-D-TMSal-ONp is dissolved in THF and an aqueous solution of equimolar amounts of proline and Et_3N is added. After 20 h at room temperature, the THF is removed in vacuo and the aqueous residue is diluted with water and then extracted several times with EtOAc. The pH of the aqueous layer is adjusted to 3 by adding 10% citric acid and extracted several times with EtOAc. The combined organic layers are washed with brine, dried over Na_2SO_4 and concentrated in vacuo. The colourless oil is crystallized from Et_2O/hexane to give 31.0 g (76%) of Boc-D-TMSal-Pro-OH **2** as a white crystalline compound: mp: 176 C.

31.0 g (86.5 mmol) of **2** is dissolved in 350 mL of EtOAc. After cooling to 0 °C, 12 g (103.5 mmol) of *N*-hydroxy-succinimide and 18 g (86.5 mmol) of DCC are added. The mixture is stirred for 3 h at 0 °C and then for an additional 15 h at room temperature. The mixture is recooled to 0 °C, the dicyclohexylurea is filtered off and washed several times with EtOAc. The filtrate is washed with aqueous 0.1 M Na_2CO_3 and then with aqueous 2% $KHSO_4$. After drying over Na_2SO_4 and concentration in vacuo, 38.8 g (98%) of Boc-D-TMSal-Pro-ONSu **3** is obtained as a white foam: FAB MS 456 (MH^+); ^{1}H NMR ($CDCl_3$) $\delta = 0.07$ (s, 9H), 0.96 (d, $J = 7.2$ Hz, 2H), 1.41 (s, 9H), 2.00–2.45 (m, 4H), 2.82 (s, 4H), 3.50–3.85 (m, 2H), 4.42–4.60 (m, 1H), 4.77–4.90 (m, 1H), 5.10–5.22 (m, 1H).

Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4N_3$]B-OPin 7.



(+)-Pinanediol-4-bromo-butane-1-boronate. 10.6 g (60.0 mmol) of 4-bromo-1-butene is reacted with 7.30 g (60.0 mmol) of catecholborane at 110 °C over 18 h. The crude product is added to 10.2 g (60.0 mmol) of (+)-pinanediol dissolved in 100 mL of THF. After 1 day at room temperature, the THF is removed in vacuo and the residue is purified by flash chromatography (95:5, hexane:EtOAc) to give 15.3 g (81%) of (+)-pinanediol-4-bromo-butane-1-boronate **4** as a colourless oil: MS (CI) 316 (M^+); ^{1}H NMR ($CDCl_3$) $\delta = 0.84$ (s, 3 H), 0.84 (t, $J = 7.2$ Hz, 2H), 1.08 (d, $J = 12$ Hz, 1H), 1.29 (s, 3H), 1.38 (s, 3H), 1.50–1.65 (m, 2H), 1.80–2.00 (m, 4H), 2.00–2.10 (m, 1H), 2.15–2.42 (m, 2H), 3.40 (t, $J = 7.2$ Hz, 2H), 4.22–4.30 (m, 1H).

Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4$Br]B-OPin 6. 4.6 mL of CH_2Cl_2 in 72 mL of THF is cooled to −100 °C and 33.5 mL (53.4 mmol) of *n*-butyllithium (1.6 M in hexane) is added over 20 min. After 60 min at −100 °C, a cold

(−78 °C) solution of 15.3 g (48.6 mmol) of (+)-pinanediol-4-bromo-butane-1-boronate in 28 mL of THF is added. After an additional 1 h at −100 °C, 3.36 g (24.3 mmol) of anhydrous $ZnCl_2$ in 28 mL of THF is added. After 15 min at −100 °C, the reaction mixture is warmed to room temperature and stirred for 4 h. The reaction mixture is poured into H_2O and extracted several times with Et_2O. After drying over Na_2SO_4 and removal of the solvent in vacuo, 17.4 g (98%) of (+)-pinanediol-(*S*)-1-chloro-5-bromo-pentane-1-boronate **5** is obtained as a yellow oil: FAB MS 364 (M^+); ^{1}H NMR ($CDCl_3$) δ = 0.84 (s, 3H), 0.84 (t, *J* = 7.2 Hz, 2H), 1.17 (d, *J* = 12 Hz, 1H), 1.27 (s, 3H), 1.42 (s, 3H), 1.47–2.44 (m, 10H), 3.42 (t, *J* = 7.2 Hz, 2H), 4.33–4.40 (m, 1 H).

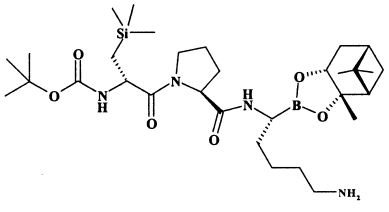
A solution of 4.6 mL (22.0 mmol) of $LiN(SiMe_3)_2$ (1 M in THF) in 32 mL of THF is cooled to −78 °C and 13.8 mL (22.0 mmol) of *n*-butyllithium (1.6 M in hexane) is added. The solution is warmed to room temperature and then cooled down to −78 °C again. 8.00 g (22.0 mmol) of (+)-pinanediol-(*S*)-1-chloro-5-bromo-pentane-1-boronate in 11 mL of THF is added and the mixture is stirred for 1 h at −78 °C, then for 18 h at room temperature.

After this period, the reaction mixture is cooled to −78 °C again. 13 mL (5.1N solution, 66.3 mmol) of HCl in dioxan is added and the solution stirred for 1 h at −78 °C and then for 2 h at room temperature.

The reaction mixture is cooled down to −20 °C. 10.0 g (22.0 mmol) of **3** dissolved in 32 mL of CH_2Cl_2 and 6.1 mL (44.0 mmol) of triethylamine are added, and the solution is stirred at −20 °C for 1 h. After warming up to room temperature, the mixture is stirred for an additional 2.5 h, then filtrated and the solvent is removed in vacuo. The residue is diluted with Et_2O/H_2O and extracted several times with Et_2O. After drying over Na_2SO_4 and concentration in vacuo, 10.3 g (68%) of Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4$ Br]B-OPin **6** is obtained: FAB MS 684 (MH^+); ^{1}H NMR ($CDCl_3$); δ = 1.25–1.58 (m, 6H), 2.92–3.11 (m, 2H).

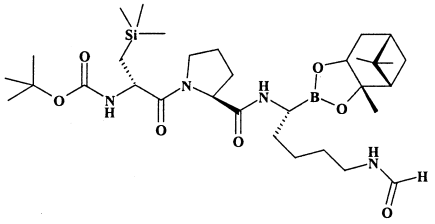
Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4N_3$]B-OPin 7. 5.00 g (7.30 mmol) of **6** is dissolved in 10 mL of DMSO and 960 mg (14.6 mmol) of sodium azide is added. The mixture is stirred for 7 h at room temperature. EtOAc/ice water is added and the solution extracted several times with EtOAc. After drying over Na_2SO_4 and concentration in vacuo, 4.40 g (93%) of Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4$ N_3]B-OPin **7** is obtained as a white foam: FAB MS 647 (MH^+); ^{1}H NMR ($CDCl_3$) δ = 1.21–2.48 (m, 6H), 3.78–3.92 (m, 2H).

Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4NH_2$]B-OPin 8.

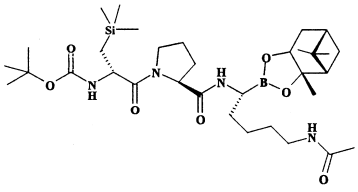


4.00 g (6.19 mmol) of the azide **7** is dissolved in 30 mL of EtOAc and hydrogenated in the presence of 0.5 g of 10% Pd/C. After 24 h at 72 psi, the catalyst is removed by filtration and the solution is concentrated in vacuo. The resulting oil is crystallized from Et_2O/hexane to give 3.10 g (81%) of Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4NH_2$]B-OPin **8** as white crystals: mp: 128–129 °C; $[\alpha]_D^{20} = -59.6°$ (*c* = 1.0 in MeOH); FAB MS 621 (MH^+); ^{1}H NMR ($CDCl_3$) δ = 1.20–2.40 (m, 8H), 2.60–2.65 (m, 2H).

Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4$NHC(O)H]B-OPin 9. 0.22 mL (5.95 mmol) of formic acid and 530 mg (5.20 mmol) of acetic acid anhydride are stirred at 60 °C for 2 h. After cooling down to 0 °C, 1.86 g (3.00 mmol) of **8** in 2 mL of THF is added and the mixture is stirred for 18 h at room temperature. The reaction mixture is poured into ice water and extracted several times with Et_2O. The combined organic layers are washed with brine, dried over Na_2CO_3 and the solvent is removed in vacuo. 1.75 g (90%) of Boc-D-TMSal-Pro-NH-CH [$(CH_2)_4$NHC(O)H]B-OPin **9** is obtained as a white foam; $[\alpha]_D^{20} = -77.8°$ (*c* = 0.5 in MeOH); FAB MS 649 (MH^+); ^{1}H NMR (DMSO, 150 °C) δ = 1.30–2.30 (m, 6H), 3.03–3.11 (m, 2H), 7.30–7.45 (m, 1H), 7.99 (s, 1H).

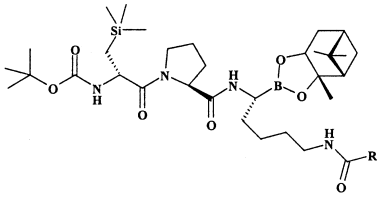


Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4$NHC(O)Me]B-OPin 10.



20 μL (0.25 mmol) of pyridine and 18 μL (0.25 mmol) of acetyl chloride in 2 mL of CH_2Cl_2 are cooled to 0 °C, 155.2 mg (0.25 mmol) of **8** in 1 mL of CH_2Cl_2 is added and the solution is stirred for 1 h. Water is added and the product extracted several times with Et_2O. The combined organic layers are washed with brine, dried over Na_2SO_4 and concentrated in vacuo. 161 mg (97%) of Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4$NH-C(O)Me]B-OPin **10** is obtained as an oil; $[\alpha]_D^{20} = -62.6°$ (*c* = 0.5 in MeOH); $MH^+ = 663$.

The following compounds (Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4$NHC(O)**R**]B-OPin) are prepared analogously from the corresponding acid chlorides or bromides:



No.	R	yield (%)	$[\alpha]_D^{20}$ ($c = 0.5$ in MeOH)	MH^+ FAB–MS
11	Et	95	−55.8°	677
12	*i*-Pr	94	−60.2°	691
13	NMe_2	42	−64.2°	692

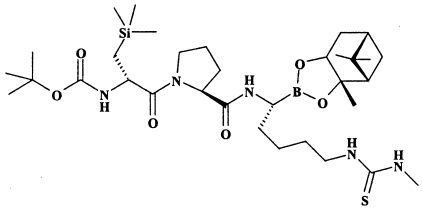
Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4$NHC(O)Me]B-OPin 10. ^{1}H NMR (DMSO, 150 °C) δ = 1.12–2.35 (m, 6H), 1.78 (s, 3H), 2.72–2.81 (m, 2H), 6.95–7.05 (m, 1H).

Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4$NHC(O)Et]B-OPin 11. ^{1}H NMR (DMSO, 120 °C) δ = 1.01 (t, $J = 7.2$ Hz, 3H), 1.22–1.65 (m, 6H), 2.16 (q, $J = 7.2$ Hz, 2H), 3.00–3.06 (m, 2H), 7.00–7.27 (m, 1H).

Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4$NHC(O)i-Pr]B-OPin 12. ^{1}H HMR (DMSO, 120 °C) δ = 1.01 (d, $J = 7.8$ Hz, 6H), 1.22–1.64 (m, 6H), 2.21–2.35 (m, 1H), 2.98–3.07 (m, 2H), 6.99–7.07 (m, 1H).

Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4$NHC(O)NMe_2]B-Opin 13. ^{1}H NMR (DMSO) δ = 1.18–1.60 (m, 6H), 2.84 (s, 6H), 3.11–3.19 (m, 2H), 4.59–4.63 (m, 1H).

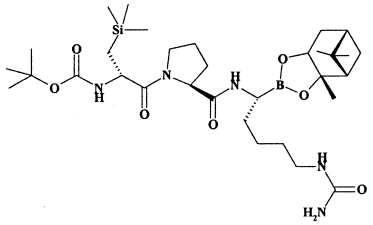
Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4$NHC(O)$NHCH_3$]B-OPin 14.



124 mg (0.20 mmol) of **8** in 3 mL of THF is cooled to 0 °C and 12 μL (0.20 mmol) of methyl isocyanate is added. After stirring 3 h at room temperature, the solvent is removed in vacuo and 128 mg (94%) of Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4$NH-C(O)$NHCH_3$]B-OPin **14** is obtained as a white foam; $[\alpha]_D^{20} = -62.2°$ ($c = 0.5$ in MeOH); FAB MS 678 (MH^+); ^{1}H NMR (DMSO, 120 °C) δ = 1.22–1.59 (m, 6H), 2.57 (d, $J = 5.4$ Hz, 3H), 2.95–3.01 (m, 2H), 5.18–5.30 (m, 2H).

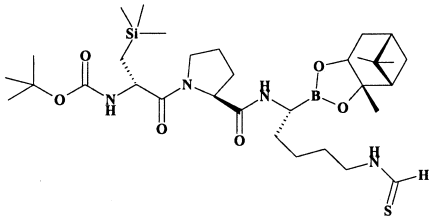
Analogously, Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4$NHC(O)NHEt]B-OPin 15. is obtained from ethyl isocyanate in 87% yield; $[\alpha]_D^{20} = -65.6°$ ($c = 0.5$ in MeOH); FAB MS 692 (MH^+); ^{1}H NMR (DMSO, 150 °C) δ = 0.92–1.04 (m, 3H), 1.25–1.58 (m, 6H), 2.95–3.07 (m, 4H), 5.22–5.32 (m, 2H).

Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4$NHC(O)NH_2]B-OPin 16.



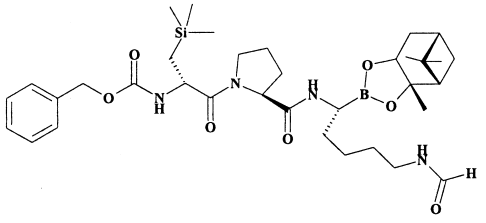
248 mg (0.40 mmol) of **16** and 0.4 mL of 1N HCl in 1 mL of H_2O is warmed to 50 °C. After 5 min, 33.2 mg (0.40 mmol) of potassium cyanate is added in small portions. After 6 h at 50 °C water is added and the product extracted with EtOAc. The combined organic layers are dried over Na_2SO_4 and concentrated in vacuo. After flash chromatography (1:9, hexane/EtOAc), 178 mg (67%) of Boc-D-TMSal-Pro-NH-CH-[$(CH_2)_4$NHC(O)NH_2]B-OPin **16** is obtained; $[\alpha]_D^{20} = -71.7°$ ($c = 0.5$ in MeOH); FAB MS 664 (MH^+); ^{1}H NMR (DMSO, 120 °C) δ = 1.20–1.58 (m, 6 H), 2.91–2.99 (m, 2 H), 4.87–4.98 (m, 2 H), 5.50–5.59 (m, 2 H).

Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4$NHC(S)H]B-OPin 17.



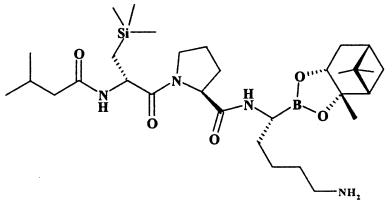
To 227 mg (0.35 mmol) of **9** dissolved in 2.5 mL of toluene 70.8 mg (0.175 mmol) of Lawesson's reagent is added. After stirring for 1.5 h at room temperature, the solvent is removed in vacuo. The residue is dissolved in EtOAc, aluminium oxide is added and the mixture is stirred for 30 min. The aluminium oxide is removed by filtration, the reaction mixture is concentrated in vacuo and the residue crystallized from Et_2O. 110 mg (47%) of Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4$NHC(S)H]B-OPin **17** is obtained as white crystals; mp: 123–125 °C; $[\alpha]_D^{20} = -74.8°$ ($c = 0.5$ in MeOH); FAB MS 665 (MH^+); ^{1}H NMR (DMSO, 150 °C) δ = 1.22–2.25 (m, 6H), 3.30–3.40 (m, 2H), 4.78–4.87 (m, 2H), 8.50 (s, 1H).

Cbz-D-TMSal-Pro-NH-CH[$(CH_2)_4$NHC(O)H]B-OPin 18.



324 mg (0.50 mmol) of **9** dissolved in 3 mL of acetic acid: concd HCl (9:1) is stirred for 3 h at room temperature. The solvent is removed in vacuo, and the white foam is dissolved in 6 mL of THF. After cooling to 0 °C, 146 μL (1.05 mmol) of triethylamine and 82 μL (0.525 mmol) of benzyl chloroformate are added and the mixture is stirred for 3 h. The reaction mixture is poured into H_2O and extracted several times with Et_2O. The combined organic layers are dried over Na_2SO_4 and concentrated in vacuo. After flash chromatography (92:8, EtOAc: EtOH), 208 mg (71%) of Cbz-D-TMSal-Pro-NH-CH-[$(CH_2)_4$NHC(O)H]B-OPin **18** is obtained as a white foam; $[\alpha]_D^{20} = -55.0°$ ($c = 0.46$ in MeOH); FAB MS 683 (MH^+); ^{1}H NMR (DMSO, 150 °C) δ = 1.27–1.54 (*m*, 6H), 3.00–3.08 (m, 2H), 4.98; 5.06 (2d, $J = 12$ Hz, je 1H), 7.28–7.31 (m, 5H), 7.30–7.43 (m, 1H); 7.86 (s, 1H).

i-BuC(O)-D-TMSal-Pro-NH-CH[$(CH_2)_4NH_2$]B-OPin 20

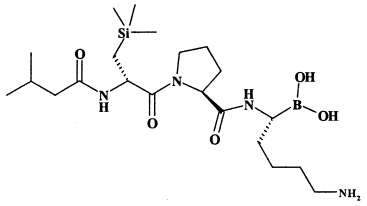


HCl·H-D-TMSal-Pro-NH-CH[$(CH_2)_4N_3$]B-Opin. 5.17 g (8.00 mmol) of Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4N_3$]B-OPin **7** is dissolved in 40 mL of AcOH:concd HCl (9:1) and stirred at room temperature for 1.5 h. AcOH and HCl are removed in vacuo and the residue crystallized from Et_2O. 4.32 g (93%) of HCl·H-D-TMSal-Pro-NH-CH[$(CH_2)_4N_3$]B-OPin are obtained as white crystals.

***i*-BuC(O)-D-TMSal-Pro-NH-CH[$(CH_2)_4N_3$]B-OPin 19.** 1.17 g (2.00 mmol) of these crystals are dissolved in 6 mL of THF and cooled to 0 °C. 258 μL (2.10 mmol) isovaleryl chloride and 285 μL (4.20 mmol) triethylamine are added. After stirring for 1 h, the reaction mixture is poured into ice water and extracted several times with Et_2O. The combined organic layers are dried over Na_2SO_4 and concentrated in vacuo. Lyophilisation with *t*-BuOH gave 1.21 g (96%) *i*-BuC(O)-D-TMSal-Pro-NH-CH[$(CH_2)_4N_3$]B-OPin **19**: $[\alpha]_D^{20} = -30.3°$ ($c = 0.6$ in MeOH); FAB MS 631 (MH^+); ^1H NMR (DMSO) $\delta = 0.92$ (*d*, $J = 7.2$ Hz, 6H), 1.25–2.14 (m, 9H), 3.25–3.31 (m, 2H).

***i*-BuC(O)-D-TMSal-Pro-NH-CH[$(CH_2)_4NH_2$]B-OPin 20.** 196 mg (0.31 mmol) of the azide **19** is dissolved in 3.1 mL of Et_2O. 186 μL of 2N HCl is added and the mixture is hydrogenated in the presence of 62 mg of 10% Pd/C. After 3.5 h, the catalyst is removed by filtration and the solution is concentrated in vacuo. 145 mg (73%) of *i*-BuC(O)-D-TMSal-Pro-NH-CH[$(CH_2)_4NH_2$]B-OPin **20** is obtained: $[\alpha]_D^{20} = -74.6°$ ($c = 0.24$ in MeOH); FAB MS 605 (MH^+); ^1H NMR (DMSO) $\delta = 0.89$ (d, $J = 7.2$ Hz, 6H), 1.32–2.10 (m, 9H), 2.71–2.78 (m, 2H), 7.68–7.94 (m, 2H).

***i*-BuC(O)-D-TMSal-Pro-NH-CH[$(CH_2)_4NH_2$]B$(OH)_2$ 21.**



8.1 mL (8.10 mmol) of borotrichloride (1 M solution in CH_2Cl_2) in 8.1 mL of CH_2Cl_2 is cooled to −78 °C. A cold solution (−78 °C) of 1.02 g (1.62 mmol) of **19** in 16.2 mL of CH_2Cl_2 is added. The reaction mixture is stirred 10 min at −78 °C and 1 h at 0 °C, then poured into ice water and extracted several times with Et_2O. The combined organic layers are washed with brine, dried over Na_2CO_3 and concentrated in vacuo. The resulting oil is crystallized from Et_2O/hexane to give 520 mg (65%) of the free boronic acid as white crystals.

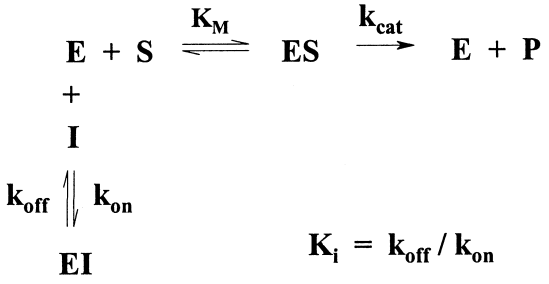
380 mg (0.77 mmol) of these white crystals are dissolved in 7.7 mL of EtOH. 480 μL of 2N HCl is added and the mixture is hydrogenated in the presence of 77 mg of 10% Pd/C. After 2.5 h, the catalyst is removed by filtration and the solution is concentrated in vacuo. The resulting oil is crystallized from CH_2Cl_2/Et_2O to give 315 mg (81%) of *i*-BuC(O)-D-TMSal-Pro-NH-CH[$(CH_2)_4NH_2$]B$(OH)_2$ **21**: $[\alpha]_D^{20} = -60.0°$ ($c = 0.51$ in MeOH); FAB MS 543 (MH^+ ester with thioglycerine matrix); ^1H NMR (DMSO) $\delta = 0.04$ (M, 9H), 0.91 (d, $J = 7.2$ Hz, 6H), 0.95–1.02 (m, 3H), 1.31–1.70 (m, 6H), 1.80–2.12 (m, 6H), 2.52 (m, 1H), 2.65–3.20 (m, 2H), 2.73–2.81 (m, 2 H), 3.76–3.72 (m, 2H), 4.35–4.43 (m, 1H), 4.47–4.60 (m, 1H), 7.30–7.50 (m, 2H), 7.70–8.10 (m, 2H).

Remark to the characterization of **21** *with FAB MS:* the MH^+ of the free boronic acids cannot be seen if the matrix used is thioglycerine. Thioglycerine and boronic acid react in the mass spectrometer and the characteristic peak to be seen is $MH^+ + 72$. This peak was observed consistently for all the free boronic acids which were prepared in our labs.

Enzyme inhibition kinetics

Human α-thrombin was purified from human plasma according to published methods,[47] which involved the purification of prothrombin,[48] the generation of thrombin from prothrombin by using the venom from *Oxyuranus scutellatus*,[49] and the purification of thrombin on S-Sepharose (Pharmacia, Uppsala, Sweden).[50] The isolated thrombin was free of proteolytically degraded forms, as judged by SDS-polyacrylamide electrophoresis, and was found to be >95% active as assessed by active site titration with 4-methylumbelliferyl-*p*-guanidinobenzoate (Sigma).[51] Bovine pancreatic trypsin (Sigma) and human plasmin (KABI, Moelndal, Sweden) were titrated by the same method. The substrates Pefachrome TH (2-AcOH-H-D-CHG-Ala-Arg-pNA), Pefachrome PL (2-AcOH-H-D-Ala-CHT-Lys-pNA), and Pefachrome TRY (Bz-Gly-Arg-pNA) were from Pentafarm.

Inhibitors were dissolved in cremophor:ethanol (1:1) or Me_2SO and diluted with distilled water to yield a 1 mM stock solution. Further dilutions were made into the assay buffer (100 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl and 0.1% bovine serum albumin). Kinetic assays were performed at 25 °C using a microwell plate; each well contained 50 μL of substrate, 100 μL of inhibitor, and 100 μL of enzyme in buffer. Final concentration of substrate and enzyme were as follows: 160 pM α-thrombin and 100 μM Pefachrome TH ($K_m = 6.9$ μM), 800 pM human plasmin and 200 μM Pefachrome PL ($K_m = 66.4$ μM), and 260 pM bovine pancreatic trypsin and 500 μM Pefachrome TRY ($K_m = 167.7$ μM). K_m values were determined as previously described.[52] Assays were initiated by adding enzyme to solutions containing inhibitor and substrate. The release of *p*-nitroaniline by hydrolysis of the substrate was followed for 30 min by measuring the increase in optical density at 405 nM with a Thermomax microwell kinetic reader (Molecular Devices, Menlo Park, CA). When the inhibited steady-state rate was achieved rapidly, the inhibition



Scheme 6.

constant (K_i) was determined by fitting the data by weighted linear regression to the Dixon equation.[53] For slow, tight-binding inhibitors, the mechanism of inhibition could be described by Scheme 6, where E, S, P and I are the enzyme, substrate, product (*p*-nitroaniline) and inhibitor, respectively, and k_{on} and k_{off} are the association and dissociation rate constants for the inhibition. Progress-curve data for the formation of *p*-nitroaniline in the presence of different concentrations of inhibitor were fitted by non-linear regression to the equation for the mechanism presented in Scheme 6. These analyses yielded estimates for the apparent values of k_{on}, k_{off} and K_i which were corrected for the presence of substrate as described by Morrison and Walsh[54] to give the true values. The kinetic constants were determined in triplicate and expressed as mean±S.D.

Crystallography

Protein purification and crystallization. Human α-thrombin was prepared for crystallization according to the literature.[55] Crystals were grown using the hanging drop method following a procedure similar to the one described in the literature.[56] The crystals were grown in a 10-fold excess of the inhibitors **18** and **21**, respectively, and after micro-seeding they grew to a final size of $0.4\times0.3\times0.3\,mm^3$.

Data processing and refinement. The X-ray intensity data were collected on a FAST television area detector with an Enraf-Nonius FR571 rotating anode operating at 40 kV and 80 mA. Data were measured at room temperature in 100-s frames of width 0.15° in ω, a detector-swing-out-angle of 20°, and a crystal-to-detector distance of 55 mm. The evaluation of the measured intensities was performed by the program MADNES.[57] Structure solution, refinement and analysis were performed with the programs O[58] and CNS.[59]

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