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Design, Synthesis and Biological Evaluation of Selective Boron-containing Thrombin Inhibitors

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Abstract—Based on the structural comparison of the S-1 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 synthesised as inhibitors of thrombin. The influence of hydrogen donor/acceptor properties of different residues in the P-1 side chain of these inhibitors on the selectivity profile has been investigated. This study confirmed the structure-based working hypothesis: The hydrophobic/hydrophilic character of amino acid residues 190 and 213 in the neighbourhood of Asp 189 in the S-1 pocket of thrombin (Ala/Val), trypsin (Ser/Val) and plasmin (Ser/Thr) define the specificity for the interaction with different P-1 residues of the inhibitors. Many of the synthesised compounds demonstrate potent antithrombin activity with Boc-D-trimethylsilylalanine-proline-boro-methoxypropylglycine pinanediol (**9**) being the most selective thrombin inhibitor of this series.

Introduction

Thrombin is a trypsin-like serine protease that plays a central role in both haemostasis and thrombosis. In the coagulation cascade, thrombin is the final key enzyme. It is responsible for the cleavage of soluble fibrinogen, releasing fibrinopeptides A and B and generating fibrin, which then can polymerise to form a haemostatic plug. Because of its importance in thrombosis, thrombin represents a prominent pharmacological target.

Anticoagulants like heparins or coumarins act indirectly, heparins by activation of endogenous plasma proteins that inhibit thrombin and other proteases of the coagulation cascade, coumarins by inhibiting the hepatic synthesis of vitamin K-dependent proteins including thrombin. These indirect mechanisms account to a large part for the limitations of these agents as therapeutics, because careful monitoring is necessary and often side effects can be observed. Therefore, direct-acting thrombin inhibitors that control blood clotting and prevent both arterial and venous thrombosis are attractive drug candidates.[1]

Peptide boronic acids based on a fibrinogen-like sequence are known as very potent direct-acting thrombin inhibitors. They act as 'reaction intermediate analogues' forming a tetrahedral complex with the active site Ser 195. Positively charged residues like Arg or Lys in P-1 and Pro in P-2 increase the affinity of these compounds for thrombin substantially.[2,3] However, at the same time, the selectivity of these peptide analogues over other trypsin-like serine proteases is generally low.

It has been shown that peptide boronic acid inhibitors with non-basic P-1 side chains can improve the selectivity for thrombin over trypsin and plasmin.[4–8] The first compound of this type was Z-D-Phe-Pro-methoxypropylboroglycine.[4,5,9–11] The selectivity of this compound was rationalised on the basis of the 3-D structures of the different proteins, showing that the hydrophobic methoxypropyl side chain can be better accomodated in the S-1 pocket of thrombin than in those of trypsin and plasmin.[11]

In this paper we further elaborate this hypothesis. Several compounds of the type Boc-D-TMSal-Pro-boro-X pinanediol (Boc-D-trimethylsilylalanine-proline-boro-X pinanediol, with boro-X being different amino boronic acids) were designed and synthesised which, in general, show a high selectivity over trypsin and plasmin while maintaining thrombin affinities in the nanomolar range.

Inhibitor Design

The S-1 pockets (Fig. 1(a)) of the serine proteases thrombin, plasmin and trypsin are structurally similar:

Key words: Anticoagulants; thrombin inhibitors; peptide boronates; molecular modelling.

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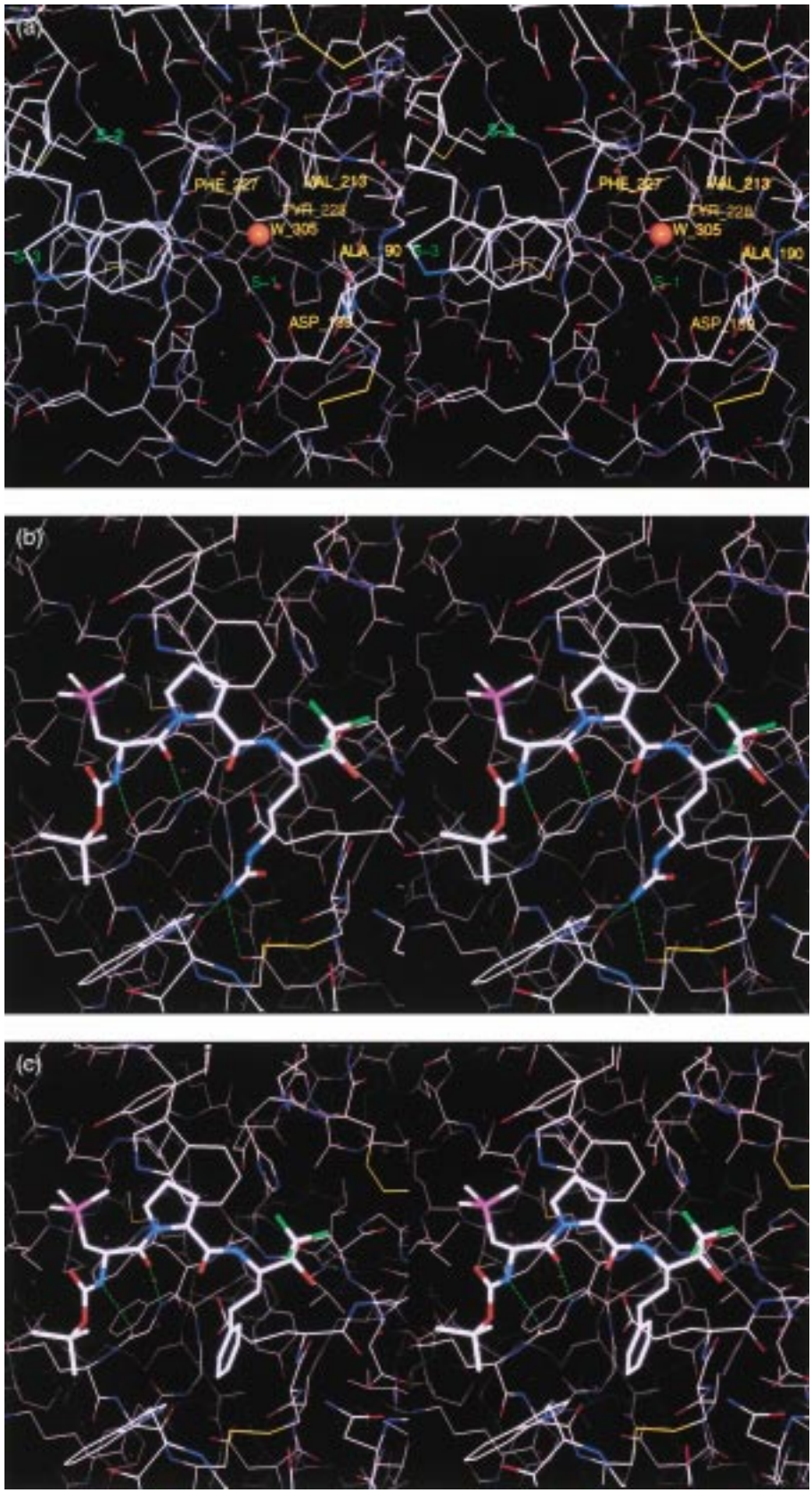


Figure 1. (a) X-ray crystal structure of thrombin from the thrombin/PPACK complex (PDB entry 1ppb[17,33]). The inhibitor has been removed, water 305 located in a pocket formed by amino acids 190, 213, 228 is shown as red sphere, the pockets S-1, S-2 and S-3 are indicated. (b) Models of the complexes thrombin/**18** and (c) thrombin/**6**. For the structure calculations water 305 has been removed and the boronic acid has been replaced by a $COCF_3$-group.

they all contain an aspartic acid (Asp 189) at the bottom of the S-1 pocket which can form a salt bridge with basic side chains of substrates or inhibitors. However, there are two amino acid positions in close proximity to Asp 189, namely 190 and 213, which are different for the three enzymes: Ala/Val in thrombin, Ser/Val in trypsin and Ser/Thr in plasmin, i.e. this region is most hydrophobic in thrombin and most polar in plasmin. X-ray structures of thrombin and trypsin show that the small pocket formed by amino acids 190, 213 and the conserved Tyr 228 is occupied by a water molecule (water 305 in Fig. 1(a)). For optimal stabilisation, this water molecule has to accept a hydrogen bond from the P-1 side chain of the inhibitor. Although there is not yet an X-ray structure of plasmin available, one can conclude from protein homology building that these features are also valid for plasmin.

Based on these considerations, we formulated the following working hypothesis: Hydrophobic side chains in P-1 or side chains containing pure hydrogen bond acceptors near the water molecule in the small pocket formed by amino acids 190, 213 and 228 should decrease binding to the three serine proteases. Due to the nature of the amino acids in positions 190 and 213 the effect should be most pronounced for plasmin and least important for thrombin. If such a side chain would still contain a hydrogen bond donor to Asp 189, it should be possible to produce selective inhibitors which do not lose too much of their thrombin affinity.

To exploit and test the above hypothesis, we synthesised several peptide boronates of type Boc-D-TMSal-Pro-boro-X pinanediol with constant positions P-3 and P-2 and variations in P-1.

D-Trimethylsilyl alanine (D-TMSal) in P-3 was used as bioisosteric replacement of D-phenylalanine to reduce the metabolic instability of our molecules.[12] In addition to the standard compounds **16** (guanidinopropyl in P-1),[13] **15** (aminobutyl in P-1)[14] and **9** (methoxypropyl in P-1),[10] we also synthesised the derivatives **8**, **13** and **17** (see schemes for the different side chains). The inhibition constants of these compounds for the enzymes thrombin, plasmin and trypsin were determined by a chromogenic assay and are summarised in Table 1.

Table 1. 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 is compared on the basis of the ratios $K_{i,PLAS}/K_{i,THR}$ or $K_{i,TRY}/K_{i,THR}$ (in parentheses). Mean values (±SD, $n=3$)

	K_i [nM]		
Compd	Thrombin	Plasmin	Trypsin
6	1.18	> 0.1 mM (> 85000)	5485 (4648)
7	708	> 0.1 mM (> 141)	> 0.1 mM (> 141)
8	123	> 0.1 mM (> 800)	> 0.1 mM (> 800)
9	8.4	> 0.1 mM (> 12000)	> 0.1 mM (> 12000)
13	13	> 0.1 mM (> 7692)	802 (61.6)
15	0.039	3.51 (90)	0.39 (10)
16	0.041	0.902 (22)	0.123 (3)
17	0.075	94.1 (1255)	1.62 (21.6)
18	4.75	> 0.1 mM (> 21000)	1632 (344)

Further modifications were generated by the application of the MCSS (Multicopy Simultaneous Search)[15,16] methodology to the X-ray structure of thrombin. In this computational method several thousands of test particles are seeded at random positions within the active site of a protein and then minimised simultaneously. As a result of these calculations a map of optimal binding sites for the respective test particles can be obtained. For our purposes benzenes and *N*-methylacetamides were seeded into the S-1 pocket and then minimised using the experimental thrombin structure from the thrombin/PPACK-complex.[17] In order to generate new structural motives for the P-1 side chain, we tried to connect the resulting particles manually to the Gly-Cα-atom of the trifluoromethylketone template structure Boc-D-TMSal-Pro-Gly-C(=O)CF_3 using the graphics program INSIGHT II.[16] This template was generated by first modelling Boc-D-TMSal-Pro-Arg-C(=O)CF_3, minimising within DISCOVER,[16] and then deleting the Arg side chain. A trifluoromethylketone was used instead of the boronic acid since there was no boron atom available in the DISCOVER force field. Within the approximation of the force field this should not cause significant deviations.

A benzene and a modified *N*-methylacetamide could be successfully attached to the template in the right distance resulting in compounds **6** and **18**. The DISCOVER-minimised structures are shown in Figure 1(b) and (c). In **18** one carbon has been replaced by a NH_2 group to provide a hydrogen bond to Asp 189. Both compounds are expected to disturb the water molecule in the pocket close to Asp 189, while still donating a hydrogen bond to Asp 189. In the case of compound **6**, this 'hydrogen bond' has to be provided by an aromatic C−H group.

Chemistry

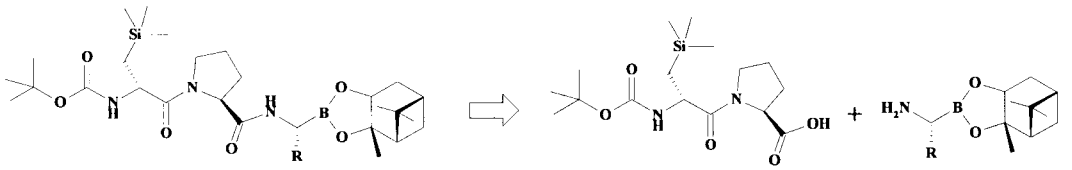
The thrombin inhibitors described in this paper were synthesised using a convergent synthesis. All of them have the dipeptide Boc-D-TMSal-Pro-OH in common which can be combined with the different boroamino acids in a single synthetic operation (see Scheme 1).

Synthesis of the first building block, Boc-D-TMSal-Pro-ONSu 3

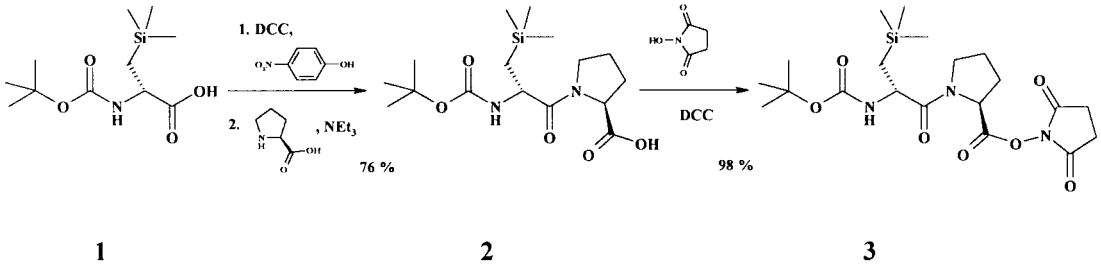
(*S*)-N-(*t*-butoxycarbonyl)-β-(trimethylsilyl)alanine **1** (Boc-D-TMSal-OH)[3] was obtained using the Schoellkopf procedure as described in the literature.[11] After activation with *p*-nitrophenol it was coupled with proline to yield **2**, which was again activated with *N*-hydroxysuccinimide (see Scheme 2) to give Boc-D-TMSal-Pro-ONSu **3**[3] which can be stored at 4 °C for several weeks.

Synthesis of the second building block 5 and coupling with 3

All the described amino boronic acids have been synthesised using a procedure developed by Matteson:[18–20]



Scheme 1.



Scheme 2.

A suitable substituted olefin was treated with catechol borane and the crude product was reacted with (+)-pinanediol to give the borone pinanediol esters **4**. The following insertion of 'CHCl' generated a single diastereomer due to the optically pure pinanediol, resulting in the chloro substituted homologues **5**. However, the reaction did usually not go to completion. 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 dipeptide building block Boc-D-TMSal-Pro-ONSu **3** (see Scheme 3). Using this methodology, the thrombin inhibitors **6–9** and the precursors **10–12** were obtained.

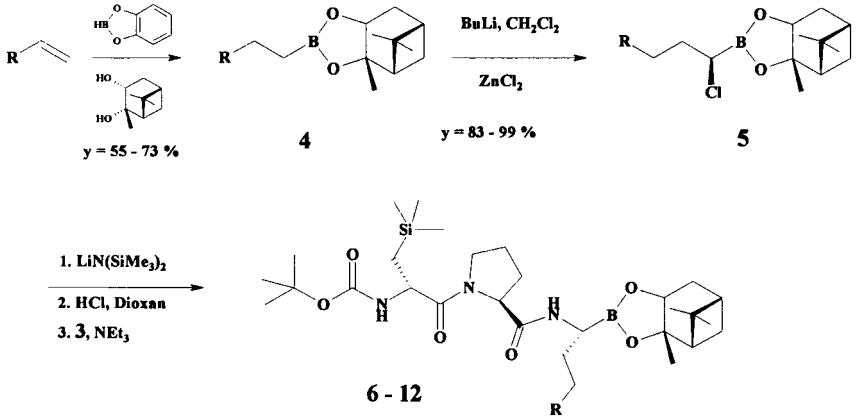
Further elaboration of compounds 10–12

Thrombin inhibitor **13** with a hydroxy group in P-1 was synthesised from **10** by removal of the *t*-butyldimethylsilyl protecting group with Bu_4NF (see Scheme 4). The boroornithine (**14**) and borolysine (**15**) derivatives were synthesised by substitution of the bromine in **11** and **12** with azide followed by hydrogenation catalysed by palladium on carbon. Further treatment of **14** and **15** with cyanamide yielded the arginine and homoarginine derivatives **16** or **17** (see Scheme 5). The urea analogue of **16** was obtained after the reaction of **14** with potassium cyanate (see Scheme 6).

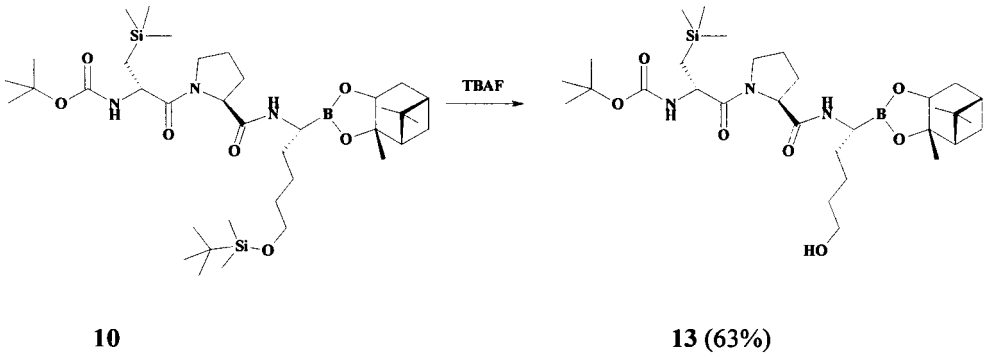
Biology

Estimates of the inhibition constants (K_i) of the inhibitors **6–9**, **13** and **15–18** with thrombin, plasmin and trypsin were determined by a chromogenic assay. All compounds tested were potent competitive inhibitors of thrombin with K_i values in the nanomolar or picomolar range (see Table 1).

In the series **8**, **13**, **15** the compound with a Lys side chain in P-1 (**15**) has, as expected, the highest affinity for thrombin, but at the same time the lowest selectivity. Replacement of NH_2 by OH (**13**) reduces the thrombin affinity but increases the selectivity against plasmin and trypsin. The same effect can be seen if the amino-function

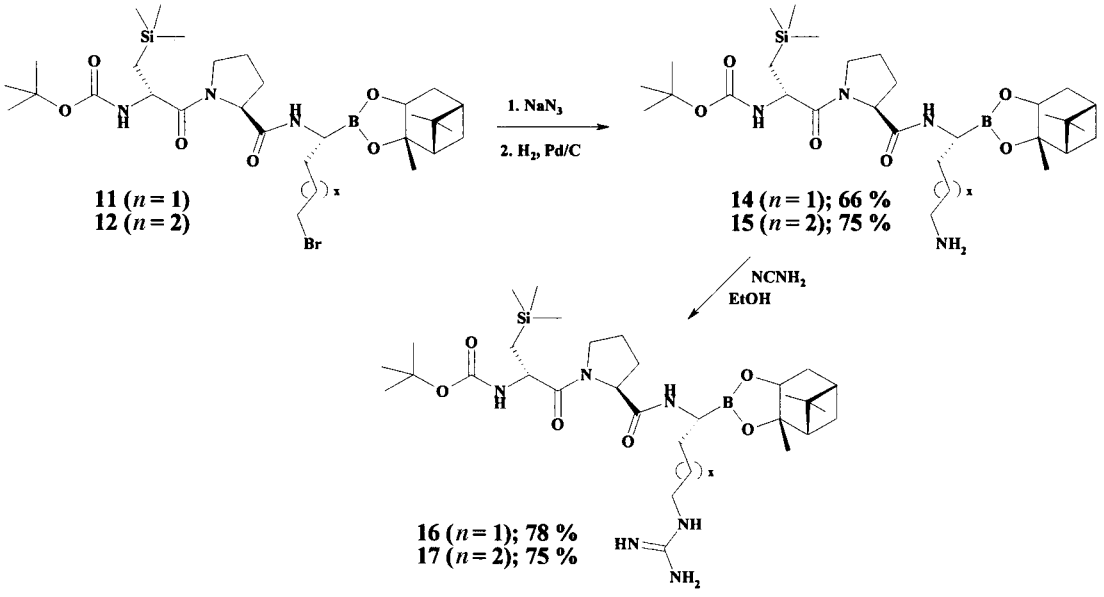


Scheme 3. R=C_6H_5 (**6**; 33%), *p*-Cl-C_6H_5 (**7**; 26%), $(CH_2)_2OME$ (**8**; 58%), CH_2OMe (**9**; 57%), $(CH_2)_2OSiBu^tMe_2$ (**10**; 35%), CH_2Br (**11**; 69%), $(CH_2)_2Br$ (**12**; 68%)



10 **13 (63%)**

Scheme 4.

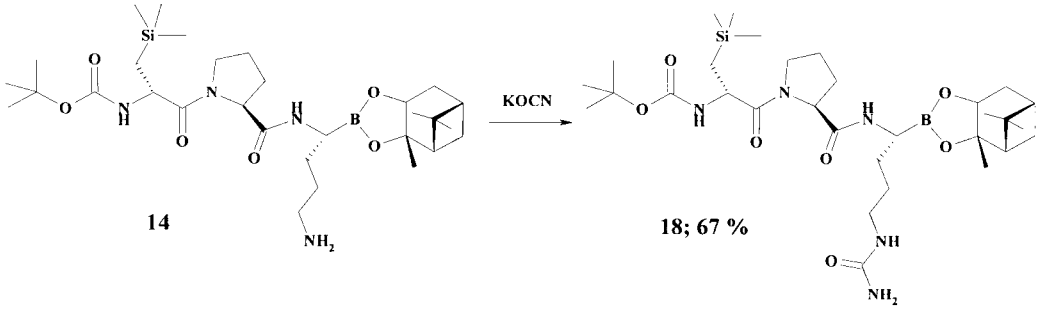


11 ($n=1$)
12 ($n=2$)

14 ($n=1$); 66 %
15 ($n=2$); 75 %

16 ($n=1$); 78 %
17 ($n=2$); 75 %

Scheme 5.



14 **18; 67 %**

Scheme 6.

is replaced by a methoxy group (**8**). The comparison of **8** and **9**, where the difference is one CH_2-group in the side chain, indicates that the chain length has also a strong influence on thrombin affinity.

The Arg-based derivatives **16**, **17** and **18** show a similar pattern. Arg in P-1 (**16**) is the most active compound for thrombin but has only a weak selectivity. Increasing the chain length (**17**) or replacing one guanidino NH by O (**18**) reduces the affinity for thrombin but on the other side increases the selectivity over trypsin and plasmin.

Compound **6** with a homophenylalanine side chain in P-1 is very active as inhibitor of thrombin and also very selective for thrombin over plasmin and trypsin. With a chloro substituent in *p*-position of the aromatic ring (**7**)

the K_i value decreased about 600 times. Therefore, the substitution pattern of an aromatic ring in P-1 would be very interesting to investigate, but since we found that both **6** and **7** are also very potent inhibitors of chymotrypsin, we did not invest any further work in this class of compounds.

Discussion

The thrombin affinity and selectivity of the compounds listed in Table 1 corresponds to the expectations we derived from analysis of our models.

Recently, an X-ray structure of Moc-D-Dpa-Pro-boroMpg (morpholinocarbonyl-D-diphenylalanine-proline-boromethoxypropylglycine) with thrombin was solved.[21] This compound contains a propylmethoxy group in P-1 like compound **9**. The structure shows—in accordance with our hypothesis—that the P-1 side chain points into the S-1 channel, in the direction of the pocket formed by amino acids 190/213/228. The fact that this pocket is more hydrophobic in thrombin compared to trypsin and plasmin allows for a rationalisation of the selectivity of compound **9**, compared to, for example, **15**. However, the X-ray analysis revealed an additional feature which we did not take into account. A water molecule forms a hydrogen bond bridge from the oxygen of the propylmethoxy group to Gly 216 and 219. Compound **8** with a methoxybutyl P-1 side chain—one methylene group longer—cannot form this hydrogen bond and therefore binds to thrombin with lower affinity.

Crystallographic analyses of Ac-D-Phe-Pro-boroArg-OH and related compounds have been published by Weber et al.[22] They give a detailed analysis of the binding mode for Arg and Lys side chains: One guanidino nitrogen of the Arg-side chain forms a hydrogen bond to a water molecule in the small pocket formed by amino acids 190, 213 and Tyr 228, corresponding to water 305 in Figure 1(a). According to our modelling studies one hydrogen of this water molecule should be directed to the carbonyl of Phe 227 and the second one towards the center of the aromatic ring of Tyr 228. Such a water molecule is optimally stabilised by a hydrogen bond donor and disturbed by a hydrogen bond acceptor. This argument explains very clearly the difference between the serine protease binding properties of compound **16** with an Arg side chain in P-1 and its oxygen analogue **18**. Compound **18** (Fig. 1(b)) destabilises the water molecule in the 190/213/228 pocket in all three enzymes. Again, this effect is much more pronounced for trypsin and especially plasmin compared to thrombin. In agreement with these findings, the affinity for thrombin is reduced by a factor of about 100, while the affinities for trypsin decrease 13,000-fold and for plasmin more than 100,000-fold.

In compound **17** with a P-1 side chain one methylene group longer than in **16**, this elongation is responsible for a slight increase of the hydrophobic character of this P-1 residue compared to compound **16**. However, the disturbing effect is much weaker than in the case of **18**. As a consequence, the thrombin affinity is comparable to that of compound **16**, and the increase in selectivity is smaller than in the case of compound **18**.

The Lys side chain of compound **15** donates three hydrogen bonds: To the carbonyl oxygens of Ala 190 and Gly 219 and via a water molecule to Asp 189 on the bottom of the S-1 pocket [cf. ref 22]. This water molecule is in contact with another one which corresponds to water 305 in Figure 1(a). The hydroxyl group in the sidechain of compound **13** can only donate one hydrogen bond so that it cannot take part in all the favourable hydrophilic interactions shown by the Lys side chain. As a consequence, the K_i for thrombin of **13** (13 nM) is higher than that of compound **15** (0.039 nM). Again, the difference between **13** and **15** is more pronounced for trypsin and plasmin leading to higher selectivity.

A phenethyl side chain in P-1 (compound **6**) resulted in a highly active and selective thrombin inhibitor. So far there is no X-ray crystallographic structure with such a P-1 residue available. However, an X-ray analysis for a compound with a pentafluoroethylketon as transition state containing a Trp side chain in P-1 was carried out.[23] The indole ring points into the S-1 pocket and the phenyl ring of the indole moiety interacts with the phenyl ring of Tyr 228 in a similar fashion as shown in our model of compound **6** for the phenethyl group (Fig. 1(c)). Another X-ray structure of thrombin complexed with a non-transition state inhibitor containing a 4-aminopyridine moiety in P-1 was solved.[24] Despite the repulsion of the pyridine nitrogen and the oxygens of Asp 189 the aromatic ring points again into the S-1 pocket in a similar way as described above.

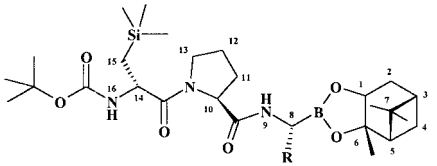
A chloro substituent in *para* position on the phenylring of compound **6** would come quite close to the oxygens of Asp 189 and would cause a electrostatic repulsion. In agreement with these predictions compound **7** is about 600-fold less active for thrombin compared to compound **6**.

Replacement of the amidino group in the boroArg compound DuP 714 by substituted 5- and 6-membered heterocycles also yields selective nanomolar thrombin inhibitors.[8] The most active of this series where those with amino groups attached to the ring systems even though X-ray results suggest that the amino group does not interact with Asp 189.[8]

Conclusion

The interaction of P-1 residues with Asp 189 in trypsin like serine proteases is rather complicated. Depending on the nature of the P-1 residue several amino acids in the neighbourhood of Asp 189 and up to two water molecules are involved in addition. Although we did not include all these aspects into our modelling studies the original hypothesis based on a controlled perturbation of a water molecule located in the 190/213/228-pocket led as expected to selective but still potent thrombin inhibitors.

Table 2. [1]H NMR values of thrombin inhibitors **6–9**, **13–18** and precursors **10–12**. (If not otherwise indicated, all spectra are measured in DMSO at high temperature (100–150 °C))



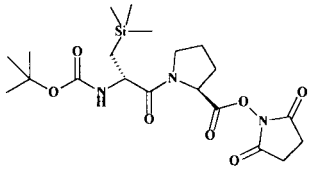
Compd	s, 9H, $SiMe_3$	m, 2H, 15-H	2 s, 3H each, 7-Me	s, 3H, 1-Me	s, 9H, *t*-Bu	m, 10H, 2-H, 3-H, 4-H, 5-H, 11-H, 12-H	m, 1H, 8-H	m, 2H, 13-H	m, 1H, 6-H	m, 1H, 14-H	m, 1H, 10-H	m, 1H, 16-H	m, 1H, 9-H
6	0.03	0.91–0.98	0.82; 1.25	1.28	1.37	1.34–2.32	2.69–2.80	3.46–3.64	4.15–4.21	4.25–4.35	4.42–4.48	5.94–6.02	7.87–7.92
7	0.04	0.90–1.13	0.82; 1.25	1.30	1.38	1.67–2.12	2.23–2.33	3.48–3.66	4.17–4.23	4.28–4.36	4.42–4.48	5.75–5.81	7.58–7.63
8	0.08	0.94–1.00	0.85; 1.30	1.32	1.42	1.20–2.33	2.69–2.78	3.49–3.65	4.17–4.22	4.29–4.38	4.42–4.49	5.98–6.06	7.78–7.83
9	0.02	0.88–0.98	0.81; 1.25	1.29	1.39	1.15–2.35	2.14–2.21	3.42–3.62	4.13–4.19	4.23–4.33	4.40–4.51	5.94–6.02	7.70–7.83
10	0.02	0.82–0.96	0.91; 1.16	1.23	1.38	1.20–2.35	2.58–2.67	3.42–3.60	4.11–4.18	4.20–4.32	4.52–4.63	6.09–6.19	6.85–7.08
11[a]	0.05	0.86–0.95	0.85; 1.27	1.37	1.43	1.20–2.45	2.70–2.85	3.78–3.90	4.22–4.31	4.31–4.41	4.57–4.63	4.95–5.03	7.45–7.52
12[a]	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
13	0.03	0.90–0.98	0.82; 1.26	1.30	1.39	1.19–2.30	2.62–2.71	3.42–3.62	4.15–4.20	4.23–4.33	4.42–4.52	5.95–6.02	7.67–7.78
14	0.01	0.91–0.98	0.82; 1.24	1.27	1.40	1.20–2.21	2.70–2.80	3.42–3.52	3.94–4.00	4.25–4.42	4.25–4.42	4.65–5.25	5.83–5.91
15[a]	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
16	0.00	0.90–0.98	0.80; 1.25	1.29	1.38	1.20–2.30	3.07–3.13	3.45–3.60	4.14–4.19	4.25–4.32	4.38–4.47	5.25–5.33	7.55–7.61
17	0.00	0.89–0.95	0.80; 1.24	1.28	1.37	1.20–2.32	3.04–3.12	3.42–3.60	4.12–4.20	4.33–4.42	4.40–4.49	5.86–5.95	7.59–7.70
18	0.03	0.92–1.03	0.84; 1.28	1.30	1.40	1.20–2.32	2.71–2.81	3.48–3.63	4.18–4.22	4.28–4.36	4.44–4.51	5.75–5.81	7.46–7.54

[a]Measured at room temperature in $CDCl_3$.

Experimental

All compounds were characterised 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 **6–18** are summarised in Table 2, the signals for the different side chains 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 characterisation 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

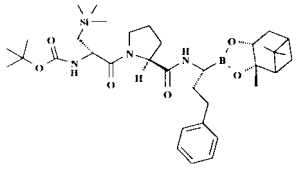


Boc-D-TMSal-OH **1** (29.7 g, 113.7 mmol) and *p*-nitrophenol (19.0 g, 136.3 mmol) are dissolved in EtOAc. After cooling to 0 °C, 23.4 g (113.6 mmol) of DCC is added and the mixture is stirred for 1 h at 0 °C and then for 15 h at room temperature. A precipitate is formed, which is filtered off and washed with EtOAc and the filtrate is concentrated in vacuo. The resulting oil is purified by flash chromatography (hexane:EtOAc, 9:1). Boc-D-TMSal-ONp is obtained as white crystals.

Boc-D-TMSal-ONp (51.6 g, 113.7 mmol) 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 crystallised 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.

Compound **2** (31.0 g, 86.5 mmol) is dissolved in 350 mL of EtOAc. After cooling to 0 °C, 12 g (103.5 mmol) of *N*-hydroxysuccinimide 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 ($CDCl_3$) δ 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[$(CH_2)_2C_6H_5$]B-OPin 6



(+)-Pinanediol-2-phenyl-ethane-1-boronate. Styrene (3.47 g, 30.0 mmol) and catecholborane (3.6 g, 30.0 mmol) are stirred for 20 h at 100 °C, yielding a yellow-brown oil. This oil is added to 5.0 g (30.0 mmol) of (+)-pinanediol in THF and stirred overnight. After flash chromatography (hexane:EtOAc, 8:2) 6.2 g (73%) of (+)-pinanediol-2-phenyl-ethane-1-boronate is obtained: EI MS 284 (M^+); ^{1}H NMR ($CDCl_3$) δ 0.83 (s, 3H), 1.00 (d, J = 12 Hz, 1H), 1.19 (t, J = 9 Hz, 2H), 1.28 (s, 3H), 1.37 (s, 3H), 1.79–1.86 (m, 1H), 1.86–1.92 (m, 1H), 2.03 (t, J = 6 Hz, 1H), 2.12–2.20 (m, 1H), 2.28–2.38 (m, 1H), 2.78 (t, J = 9 Hz, 2H), 4.23–4.28 (m, 1H), 7.12–7.29 (m, 5H).

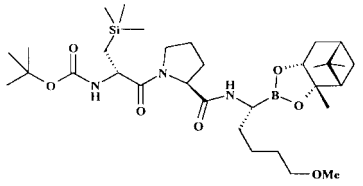
Boc-D-TMSal-Pro-NH-CH[$(CH_2)_2C_6H_5$]B-OPin 6. Over a 20 min period, 15 mL (24.0 mmol) of *n*-buthyllithium (1.6 M in hexane) are added to a precooled (−100 °C) solution of 2.1 mL of CH_2Cl_2 and 37 mL of THF. After stirring for 30 min at −100 °C, 6.2 g (21.8 mmol) of (+)-pinanediol-2-phenyl-ethane-1-boronate in 15 mL of THF is added over a 20 min period. The reaction mixture is again stirred for 1 h at −100 °C, after which 1.51 g (10.9 mmol) of $ZnCl_2$ in 12 mL of THF is added. The reaction mixture is allowed to warm up to room temperature overnight. After concentration in vacuo the residue is dissolved in Et_2O:H_2O. The organic layer is dried over Na_2SO_4 and concentrated in vacuo. 6.9 g (95%) of (+)-pinanediol-(*S*)-1-chloro-3-phenyl-propane-1-boronate is obtained as yellow-orange oil.

5.1 mL (8.09 mmol) of *n*-buthyllithium (1.6 M in hexane) is added to a precooled (−78 °C) solution of 1.7 mL (8.09 mmol) of hexamethyldisilazane in 15 mL of THF. The reaction mixture is stirred 1 h at room temperature, then cooled down to −78 °C again. 2.68 g (8.09 mmol) of (+)-pinanediol-(*S*)-1-chloro-3-phenyl-propane-1-boronate dissolved in 4 mL of THF is added. After stirring for 1 h at −78 °C, the solution is warmed up overnight to room temperature. After recooling to −78 °C, 3 mol equivalents of HCl in dioxane are added. The mixture is stirred for 1 h at −78 °C, then for 2 h at room temperature. After cooling down to −20 °C, a solution of 3.7 g (8.09 mmol) of dipeptide **3** in 15 mL of CH_2Cl_2 is added, followed by the addition of 2.3 mL (18.2 mmol) of triethylamine. The mixture is stirred for 1 h at −20 °C and for 2 h at room temperature, then filtered. After concentration in vacuo, the residue is dissolved in Et_2O:H_2O. The organic layer is dried over Na_2SO_4 and concentrated in vacuo. After flash chromatography (hexane:EtOAc, 6:4), 1.76 g (33%) of Boc-D-TMSal-Pro-NH-CH[$(CH_2)_2C_6H_5$]B-OPin **6** is obtained: $[\alpha]_D^{20}$ −88.6° (*c* 0.5 in MeOH); FAB MS 654 (MH^+); ^{1}H

NMR (DMSO, 120 °C) δ 2.54–2.79 (m, 2H), 7.09–7.17 (m, 2H), 7.19–7.26 (m, 3H).

Boc-D-TMSal-Pro-NH-CH[$(CH_2)_2$(*p*-Cl-C_6H_4)]B-OPin 7 is obtained analogously: $[\alpha]_D^{20}$ −83.2° (*c* 0.5 in MeOH); FAB MS 688 (M^+); ^{1}H NMR (DMSO, 150 °C) δ (2.77–2.86 (m, 2H), 7.16 (d, J = 7.8 Hz), 7.23 (d, J = 7.8 Hz).

Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4$OMe]B-OPin 8



(+)-Pinanediol-4-methoxy-butane-1-boronate. To 2.1 g (68.1 mmol) of NaH (80% suspension in mineral oil) suspended in 30 mL of diglyme 5.04 mL (68 mmol) of 3-butene-1-ol is added. After 3 h, the evolution of H_2 is stopped and the suspension is cooled to 0 °C. 3.8 mL (60 mmol) of methyliodide is added and the solution is stirred at ambient temperature for 2 h. Destillation (bp 70 °C) yields 3.8 g (73%) of 1-methoxy-3-butene.

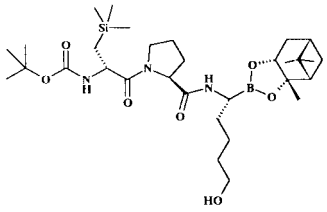
1-Methoxy-3-butene (6.4 g, 73.3 mmol) is reacted with catecholborane (8.9 g, 74.3 mmol) at 100 °C over 16 h. The crude product is added to 12.9 g (74.3 mmol) of (+)-pinanediol dissolved in 85 mL of THF. After two days at room temperature, the THF is removed in vacuo and the residue is purified by flash chromatography (hexane:EtOAc, 85:15) to give 10.9 g (55%) of (+)-pinanediol-4-methoxy-butane-1-boronate as a colourless oil: EI MS 266 (M^+); ^{1}H NMR (DMSO) δ 0.73 (t, J = 7.6 Hz, 2H), 0.82 (s, 3H), 1.00 (d, J = 10.1 Hz, 1H), 1.25 (s, 3H), 1.31 (s, 3H), 1.33–1.43 (m, 2H), 1.45–1.54 (m, 2H), 1.68 (ddd, J = 13.7 Hz, 3.4 Hz, 2.1 Hz, 1H), 1.83 (m, 1H), 1.95 (t, J = 5.7 Hz, 1H), 2.14–2.22 (m, 1H), 2.25–2.34 (m, 1H), 3.20 (s, 3H), 3.28 (t, J = 6.3 Hz, 2H), 4.28 (dd, J = 8.0 Hz, 2.1 Hz, 1H).

Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4$OMe]B-OPin 8. CH_2Cl_2 (1.4 mL) in 25 mL of THF is cooled to −100 °C and 10.3 mL (16.5 mmol) of *n*-butyllithium (1.6 M in hexane) is added over 20 min. After 30 min at −100 °C, a cold (−78 °C) solution of 4.0 g (15 mmol) of (+)-pinanediol-4-methoxy-butane-1-boronate in 9 mL of THF is added. After an additional 1 h at −100 °C, 1.04 g (7.55 mmol) of anhydrous $ZnCl_2$ in 9 mL of THF is added. After 15 min at −100 °C, the reaction mixture is warmed to room temperature. The solvent is removed in vacuo, the residue diluted with hexane:water and extracted several times with hexane. After drying over Na_2SO_4 and removal of the solvent in vacuo, 4.7 g (99%) of (+)-pinanediol-(*S*)-1-chloro-5-methoxy-pentane-1-boronate is obtained as a yellow oil.

A solution of 2.7 mL (12.9 mmol) of $LiN(SiMe_3)_2$ (1 M in THF) in 20 mL of THF is cooled to −78 °C and 8.07 mL (12.9 mmol) of *n*-buthyllithium (1.6 M in hexane) is added. The solution is warmed to room temperature and then cooled down to −78 °C again. 4.05 g (12.9 mmol) of (+)-pinanediol-(*S*)-1-chloro-5-methoxy-pentane-1-boronate in 7 mL of THF is added and the mixture is stirred for 1 h at −78 °C, then for 15 h at room temperature. After this period, the reaction mixture is cooled to −78 °C again. 8.4 mL of HCl in dioxan (4.6 N solution, 38.6 mmol) is added and the solution stirred for 1 h at −78 °C and then for 2 h at room temperature. The mixture is cooled to −20 °C, 5.8 g (12.9 mmol) of **3** in 20 mL of CH_2Cl_2 is added, followed by 3.58 mL (25.7 mmol) of triethylamine to start the coupling reaction. After stirring at −15 °C for 1 h, the mixture is stirred for 2 h at room temperature. The mixture is then filtered over Hyflo and concentrated 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, the product is purified by flash chromatography (EtOAc) and 2.2 g (59%) of Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4$OMe]B-OPin **8** is obtained as white foam: $[\alpha]_D^{20}$ −80.6° (*c* 0.5 in MeOH); FAB MS 636 (M^+); ^{1}H NMR (DMSO, 120 °C) δ 1.20–1.61 (m, 6H); 3.24 (s, 3H), 3.30–3.35 (m, 2H).

Analogously, Boc-D-TMSal-Pro-NH-CH[$(CH_2)_3$OMe] B-OPin **9** is obtained: $[\alpha]_D^{20}$ −48.8° (*c* 0.25 in CH_2Cl_2); FAB MS 622 (M^+); ^{1}H NMR (DMSO, 120 °C) δ 1.15–2.35 (m, 4H), 3.20 (s, 3H), 3.25–3.35 (m, 2H).

Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4$OH]B-OPin 13



(+)-Pinanediol-4-*t*-butyldimethylsiloxy-butane-1-boronate. 3-Butene-1-ol (10.5 mL, 120 mmol), *t*-butyldimethylsilylchloride (22.3 g, 144 mmol), and imidazole (20.4 g, 300 mmol) are dissolved in 60 mL of DMF and stirred overnight at 35 °C. The two layers are separated, the product layer is washed with 2 N tartaric acid, water and brine. After drying over Na_2SO_4 and concentration in vacuo, 21 g (94%) of 1-*t*-butyldimethylsiloxy-3-butene is obtained.

1-*t*-Butyldimethylsiloxy-3-butene (7.0 g, 37.5 mmol) is stirred for 24 h with 4.75 g (37.5 mmol) of catecholborane at 125 °C, yielding a yellow-brown oil. This oil is added to 6.5 g (37.5 mmol) of (+)-pinanediol in 55 mL THF and stirred overnight. After flash chromatography (hexane:EtOAc, 98:2) 9.2 g (67%) of (+)-pinanediol-4-*t*-butyldimethyl-siloxy-butane-1-boronate is obtained: EI MS 366 (M^+); ^{1}H NMR ($CDCl_3$) δ 0.05 (s, 6H), 0.80–0.90 (m, 2H), 0.85 (s, 3H), 0.90 (s, 9H), 1.1 (m, 1H), 1.25 (s, 3H), 1.38 (s, 3H), 1.40–1.60 (m, 5H), 1.76–2.42 (m, 4H), 3.55–3.65 (m, 2H), 4.20–4.28 (m, 1H).

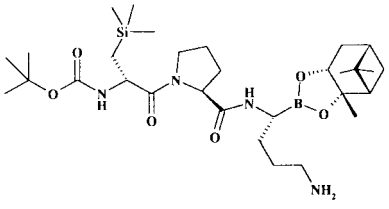
Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4$OSi(*t*-Bu)Me_2]B-OPin 10. Over a 20 min period 17.5 mL (27.6 mmol) of *n*-buthyllithium (1.6 M in hexane) is added to a precooled

(−100 °C) solution of 3 mL of CH_2Cl_2 and 50 mL of THF. After stirring 15 min at 100 °C, 9.2 g (25.1 mmol) of (+)-pinanediol-4-*t*-butyldimethyl-siloxy-butane-1-boronate in 25 mL of THF is added over a 20 min period. The reaction mixture is again stirred for 15 min at −100 °C, after which 1.8 g (12.6 mmol) of $ZnCl_2$ (solid) is added. The reaction mixture is allowed to warm up to room temperature overnight. After concentration in vacuo the residue is dissolved in Et_2O:H_2O. The organic layer is dried over Na_2SO_4 and concentrated in vacuo. 9.5 g (91%) of (+)-pinanediol-(*S*)-1-chloro-5-*t*-butyldimethyl-siloxy-butane-1-boronate is obtained as yellow-orange oil.

n-Buthyllithium (5.6 mL, 9,04 mmol, 1.6 M in hexane) is added to a precooled (−78 °C) solution of 1.91 mL (9.04 mmol) of hexamethyldisilazane in 13 mL of THF. The reaction mixture is stirred 1 h at room temperature, then cooled down to −78 °C again. 5.0 g (9.04 mmol) of (+)-pinanediol-(*S*)-1-chloro-5-*t*-butyldimethylsiloxy-butane-1-boronate in 5 mL of THF is added. After stirring for 1 h at −78 °C, the solution is warmed up overnight to room temperature. After cooling to −78 °C again, 3 mol equivalents of HCl in dioxane are added. The mixture is stirred for 1 h at −78 °C, then for 2 h at room temperature. After cooling down to −20 °C, a solution of 4.1 g (9.04 mmol) of dipeptide **3** in 15 mL of CH_2Cl_2 is added, followed by the addition of 2.55 mL (18.1 mmol) of triethylamine. The mixture is stirred for 1 h at −20 °C and for 2 h at room temperature, then filtered. After concentration in vacuo, the residue is dissolved in Et_2O:H_2O. The organic layer is dried over Na_2SO_4 and concentrated in vacuo. After flash chromatography (hexane:EtOAc, 6:4), 2.3 g (35%) of Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4$OSi(*t*-Bu)Me_2]B-OPin **10** is obtained, which was not entirely pure but used without further purification for the next step: FAB MS 736 (M^+); ^{1}H NMR (DMSO, 100 °C) δ 0.02 (s, 6H), 0.88 (s, 9H), 1.20–2.35 (m, 6H), 3.53–3.60 (m, 2H).

Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4$OH]B-OPin 13. Compound **10** (600 mg, 0.816 mmol) is dissolved in 6 mL of THF and tetrabutylammonium fluoride (514 mg, 1.62 mmol) is added. After stirring overnight at ambient temperature, the mixture is concentrated in vacuo and dissolved in Et_2O:H_2O. The organic layer is dried over Na_2SO_4 and concentrated in vacuo. The side product is removed with EtOAc on silica gel, then the product is eluted with EtOAc:EtOH (9:1), yielding 320 mg (63%) of Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4$ OH]B-OPin **13** as a white foam: $[\alpha]_D^{20}$ −72.4° (*c* 0.5 in MeOH); FAB MS 622 (MH^+); ^{1}H NMR (DMSO, 120 °C) δ 1.19–1.60 (m, 6H), 2.85–2.93 (m, 2H), 3.70–3.75 (m, 1H).

Boc-D-TMSal-Pro-BoroOrn-OPin 14



(+)-Pinanediol-3-bromo-propane-1-boronate. 3-Bromo-1-propene (5.1 mL, 60 mmol) is reacted with catecholborane (6.5 mL, 60 mmol) at 100 °C over 16 h. The crude product is added to (+)-pinanediol (10.4 g, 60 mmol) dissolved in 60 mL of THF. After 1 day at room temperature, the THF is removed in vacuo and the residue is purified by flash chromatography (hexane:EtOAc, 95:5) to give 12.7 g (70%) of (+)-pinanediol-3-bromo-propane-1-boronate as a colourless oil: MS (CI) 300 (M^+); ^{1}H NMR ($CDCl_3$) δ 0.85 (s, 3H), 0.97 (t, J = 7.2 Hz, 2H), 1.09 (d, J = 11 Hz, 1H), 1.30 (s, 3H), 1.38 (s, 3H), 1.81–1.87 (m, 1H), 1.89–1.95 (m, 1H), 1.96–2.07 (m, 3H), 2.18–2.27 (m, 1H), 2.29–2.38 (m, 1H), 3.44 (t, J = 7.2 Hz, 2H), 4.24–4.28 (m, 1H).

Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4$Br]B-OPin 11. CH_2Cl_2 (4.1 mL) in 60 mL of THF is cooled to −100 °C and 29.2 mL (46.4 mmol) of *n*-butyllithium (1.6 M in hexane) is added over 20 min. After 30 min at −100 °C, a cold (−78 °C) solution of 12.7 g (42.2 mmol) of (+)-pinanediol-3-bromo-propane-1-boronate in 25 mL of THF is added. After an additional 1 h at −100 °C, 2.92 g (21.1 mmol) of anhydrous $ZnCl_2$ in 25 mL of THF is added. After 15 min at −100 °C, the reaction mixture is warmed to room temperature and stirred overnight. The solvent is removed in vacuo, the residue diluted with Et_2O:H_2O and extracted several times with Et_2O. After drying over Na_2SO_4 and removal of the solvent in vacuo, 12.3 g (83%) of (+)-pinanediol-(*S*)-1-chloro-4-bromo-pentane-1-boronate is obtained as a yellow oil.

A solution of 5.5 mL (26.4 mmol) of $LiN(SiMe_3)_2$ (1 M in THF) in 40 mL of THF is cooled to −78 °C and 16.5 mL (26.4 mmol) of *n*-buthyllithium (1.6 M in hexane) is added. The solution is warmed to room temperature and then cooled down to −78 °C again. 9.23 g (26.4 mmol) of (+)-pinanediol-(*S*)-1-chloro-4-bromo-pentane-1-boronate in 20 mL of THF is added and the mixture is stirred for 1 h at −78 °C, then for 19 h at room temperature. After this period, the reaction mixture is cooled to −78 °C again. 17.7 mL (4.6 N solution, 81.4 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. Compound **3** (9.52 g, 26.4 mmol) dissolved in 15 mL of THF is cooled to −20 °C and 3.4 mL (26.4 mmol) of pivalic acid and 4 mL (26.4 mmol) of triethylamine are added. After 2 h at −20 °C the precipitated NEt_3·HCl is filtered off. At −20 °C the thus prepared mixed anhydride and 7.7 mL (52.8 mmol) of triethylamine are added to the reaction mixture above. After stirring at −20 °C for 1 h, the mixture is stirred for 3 h at room temperature, then diluted with Et_2O:H_2O and extracted several times with Et_2O. After drying over Na_2SO_4 and concentration in vacuo, the product is purified by flash chromatography (hexane:EtOAc, 1:1) and 12.2 g (69%) of Boc-D-TMSal-Pro-NH-CH[$(CH_2)_3$ Br]B-OPin **11** is obtained as white foam: FAB MS 670 (MH^+); ^{1}H NMR ($CDCl_3$); δ 1.20–2.45 (m, 4H), 3.32–3.42 (m, 2H).

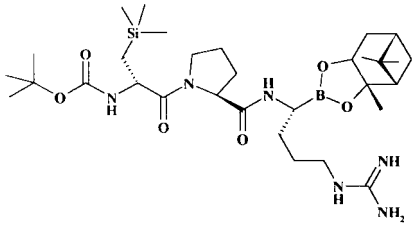
Analogously, Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4$Br]B-OPin **12** is prepared: FAB MS 684 (MH^+); ^{1}H NMR ($CDCl_3$); δ 1.25–1.58 (m, 6H), 2.92–3.11 (m, 2H).

Boc-D-TMSal-Pro-BoroOrn-OPin 14. Compound **11** (1.8 g, 2.68 mmol) is dissolved in 7 mL of DMSO and 358 mg (5.37 mmol) of sodium azide is added. The mixture is stirred for 16 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, the resulting oil is crystallised to give 1.4 g (82%) of Boc-D-TMSal-Pro-NH-CH[$(CH_2)_3$-N_3]B-OPin as a white crystalline compound: mp 60–62 °C.

The azide (1.4 g, 2.21 mmol) discussed above is dissolved in 50 mL EtOAc and hydrogenated in the presence of 0.25 g of 10% Pd/C. After 16 h, the catalyst is removed and the solution is concentrated in vacuo. The resulting oil is crystallised to give 1.07 g (80%) of Boc-D-TMSal-Pro-BoroOrn-OPin **14** as white crystals: mp 200–202 °C; $[\alpha]_D^{20}$ −11.6° (*c* 0.5 in MeOH); FAB MS 607 (MH^+); ^{1}H NMR (DMSO, 120 °C) δ 1.20–2.01 (m, 4H), 2.70–2.80 (m, 2H).

Analogously, Boc-D-TMSal-Pro-NH-CH[$(CH_2)_4NH_2$]B-OPin **15** was prepared: 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, 6H), 2.65–2.60 (m, 2H).

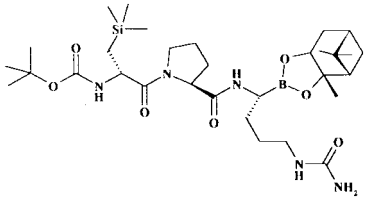
Boc-D-TMSal-Pro-BoroArg-OPin 16



Compound **14** (250 mg, 0.41 mmol) and benzenesulfonic acid (65.2 mg, 0.42 mmol) were dissolved in 2 mL of ethanol and stirred for 10 min at room temperature. Then 86.6 mg (2.06 mmol) of cyanamide is added and the reaction mixture is stirred for seven days. Every day the solution is concentrated and fresh solvent is added. After seven days the solvent is removed in vacuo, the residue is dissolved in methanol and filtrated over Sephadex LA 20. 260 mg (78%) of Boc-D-TMSal-Pro-BoroArg-OPin **18** was obtained as a white foam: $[\alpha]_D^{20}$ −45.3° (*c* 1.0 in CH_2Cl_2); FAB MS 649 (MH^+); ^{1}H NMR (DMSO, 150 °C) δ 1.20–2.10 (m, 4H), 2.73–2.80 (m, 2H); 6.13–6.24 (m, 3H), 7.02–7.13 (m, 1H).

Analogously, Boc-D-TMSal-Pro-BoroHArg-OPin **17** is prepared: $[\alpha]_D^{20}$ −40.8 (*c* 0.5 in CH_2Cl_2); FAB MS 663 (MH^+); ^{1}H NMR (DMSO, 120 °C) δ 1.20–2.32 (m, 6H), 2.70–2.80 (m, 2H); 6.68–6.78 (m, 3H), 7.09–7.15 (m, 1H).

Boc-D-TMSal-Pro-NH-CH[$(CH_2)_3NHC(O)NH_2$]B-OPin 18

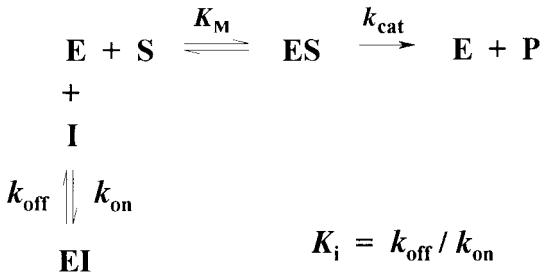


Compound **14** (242.6 mg, 0.4 mmol) and 1 N HCl (0.4 mL) in H_2O is warmed to 50 °C. After 5 min, 33.2 mg (0.4 mmol) of potassium cyanate is added in small portions. After 3.5 h at 50 °C, ice-water is added, the product extracted with EtOAc, dried over Na_2SO_4 and concentrated in vacuo. After flash chromatography (EtOAc:EtOH, 8:2), 110 mg (53%) of Boc-D-TMSal-Pro-NH-CH[$(CH_2)_3NHC(O)NH_2$]B-OPin **18** is obtained: $[\alpha]_D^{20}$ −70.8° (*c* 0.5 in MeOH); FAB MS 650 (MH^+); ^{1}H NMR (DMSO, 150 °C) δ 1.20–1.60 (m, 4H), 2.94–3.02 (m, 2H), 4.78–4.90 (m, 2H), 5.41–5.50 (m, 2H).

Enzyme inhibition kinetics

Human α-thrombin was purified from human plasma according to published methods,[25] which involved the purification of prothrombin,[26] the generation of thrombin from prothrombin by using the venom from *Oxyuranus scutellatus*,[27] and the purification of thrombin on S-Sepharose (Pharmacia, Uppsala, Sweden).[28] 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).[29] 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.[30] 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 constant (K_i) was determined by fitting the data by weighted linear regression to the Dixon equation.[31] For slow, tight-binding inhibitors, the mechanism of inhibition could be described by Scheme 7, 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



Scheme 7.

in Scheme 7. 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[32] to give the true values. The kinetic constants were determined in triplicate and expressed as mean ± SD.

Molecular modelling

For building, manipulation and display of molecular structures the graphic program INSIGHT II, version 2.5[16] was used.

All structural calculations were based on the protein structure taken from the thrombin/PPACK-complex solved by Bode et al. (PDB entry 1ppb,[17,33]). The water molecule occupying the hydrophobic pocket close to Asp 189 (residue 305) was removed.

Structures of thrombin/ligand complexes were minimised by DISCOVER 2.7.[16] The inhibitors and protein residues within 6.0 Å around the inhibitor were kept flexible during the minimisation.

In DISCOVER the CVFF all hydrogen force field applying a distance dependent dielectric constant with a scaling factor of 2.0 was used. The boronic acid part of the inhibitors was replaced by a trifluoromethylmethanolate forming a covalent bond between the oxygen of Ser 195 and the tetrahedral methanolate carbon.

MCSS calculations[15,16] using the above mentioned thrombin structure were done for *N*-methylacetamide (ACAM) and benzene (BENZ) as test particles within the S-1-pocket only. The particles were seeded in a sphere with a radius of 10 Å centered at the position of the (removed) oxygen of water 305. The parameter 19 polar hydrogen force field was used.

For ACAM one calculation with 600 particles and 12 others with 1000 particles each were carried out resulting in 102 different minima. For benzene, 5 calculations with 400 and another 5 with 600 particles were done yielding 15 unique minima. Every single calculation consisted of 10 cycles of simultaneous minimisations.

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