Design, synthesis and biological evaluation of thrombin inhibitors based on a pyridine scaffold†

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A series of 2,4-disubstituted pyridine derivatives has been designed, synthesised and evaluated as thrombin inhibitors. A Grignard exchange reaction was used to introduce various benzoyl substituents in position 4 of the pyridine ring, where they serve as P3 residues in binding to thrombin. In position 2 of the pyridine ring, a *para*-amidinobenzylamine moiety was incorporated as P1 residue by an S_NAr reaction using ammonia as nucleophile followed by a reductive amination. A crystal structure obtained for one of the compounds in the active site of thrombin revealed that the basic amidine group of the inhibitor was anchored to Asp 189 at the bottom of the S1 pocket. A comparison with melagatran, bound in the active site of thrombin, revealed a good shape match but lack of hydrogen bonding possibilities in the S2–S3 region for the thrombin inhibitors reported in this study.

Introduction

Blood coagulation is regulated through an intricate network of biological factors where thrombin (factor IIa) acts as a key enzyme by converting soluble fibrinogen into an insoluble matrix of fibrin. Thrombin belongs to the serine protease family where a serine residue in the active site catalyses the cleavage of amide bonds in protein and peptide substrates. It was cocrystallised with the irreversible inhibitor PPACK (D-Phe-Pro-Arg chloromethylketone) and the 3-dimensional structure was determined in 1989.[1,2] The substrate binding pockets are usually designated S1, S2 and S3 and bind to the corresponding P1, P2 and P3 amino acid residues in the substrate. The enzyme is highly specific for cleaving after basic P1 residues, especially arginine, the positively charged guanidine group interacting with Asp 189 at the bottom of the S1 pocket. The S2 pocket is lined by the side chains of Tyr 60A and Trp 60D, located in the insertion loop characteristic for thrombin, and prefers hydrophobic amino acids *e.g.* proline in the P2 position of the substrate. The S3 pocket, containing lipophilic and aromatic moieties as the main interaction points, is known to interact favourably with aromatic P3 groups.[3–5]

Today the major drugs used as anticoagulants are warfarin (Waran) and heparin, which inhibit the blood coagulation cascade by two different pathways. Since both therapies are associated with drawbacks, development of new types of anticoagulants is highly desirable.[4] One very attractive opportunity is to develop oral direct thrombin inhibitors (oral DTIs) using structure based design starting from the crystal structure of the thrombin–PPACK complex.[3] By combination of structure based design with co-crystallisation of the inhibitors with thrombin, several compounds with potential as anticoagulants have been developed.[5–8] However, until now the most advanced oral DTI is melagatran, which reached the market as its dual prodrug ximelagatran, but unfortunately was later withdrawn.[9]

As part of our studies focused on developing new chemistry around substituted pyridines,[10,11] we recognised the possibility to apply our methodology to the synthesis of potential thrombin inhibitors. Applying the P1-P2-P3 nomenclature, it was envisioned that the pyridine ring could serve as a P2 scaffold. A *p*-amidinobenzylamine residue, known from many thrombin inhibitors,[12–17] was considered suitable as a P1 substituent also in our case. Various benzoyl groups, with small substituents were chosen to fill the S3 pocket. Benzoyl groups containing either a *m*-methyl or an *o*-methoxy substituent provided a good fit in the structure based design and were also predicted to be compatible with the planned synthetic route. Glide docking of one of the target inhibitors, compound **20a**, displays the interactions between the amidine and Asp 189 as well as a potential hydrogen bond between the benzoyl carbonyl group and the phenolic hydroxyl group in Tyr 60A (Fig. 1).[18] Inhibitors **20a–c** were prepared *via* a 14 step synthetic sequence, and the three designed compounds were evaluated as thrombin inhibitors in an enzymatic assay.

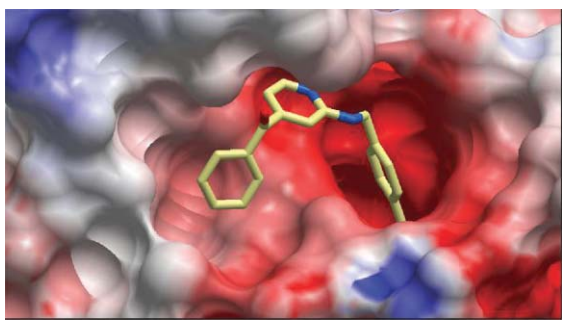

Fig. 1 Compound **20a** flexibly docked into the active site of thrombin (PDB code 1K22) using standard precision Glide with a rigid receptor. The molecular surface is coloured by atom type, where red, blue, yellow and white denote oxygen, nitrogen, sulfur and carbon, respectively.

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† Electronic supplementary information (ESI) available: Copies of ^{1}H and ^{13}C NMR spectra and lists of spectral data for all isolated compounds. HRMS for all isolated compounds. Reversed phase HPLC chromatograms for compounds **20a–c**. Data collection and refinement statistics for the X-ray structure are also available. See DOI: 10.1039/b705344d

Results and discussion

Synthetic route to the thrombin inhibitors

The designed thrombin inhibitors **I** contain a central pyridine scaffold appropriately substituted at positions 2 and 4 (Fig. 2). A retrosynthetic analysis[10,19] revealed 2-fluoro-4-iodopyridine[20] (**II**) as a suitable scaffold with the possibility to introduce substituents both at positions 2 and 4. Introduction of a substituent at position 4 of the pyridine ring requires an iodo–magnesium Grignard exchange reaction, followed by quenching with an appropriately substituted benzaldehyde **III** as electrophile.[21] Introduction of a substituent at position 2 of the pyridine ring should be accomplished *via* a nucleophilic aromatic substitution reaction (S_NAr) using benzylamine **IV** as nucleophile. The nucleophilic aromatic substitution was planned as the second transformation due to anticipated selectivity issues between fluorine and iodine, if the S_NAr reaction was performed as the first step. The desired benzamidine moiety in thrombin inhibitors **I** was intended to be obtained from the cyano group in **IV**.

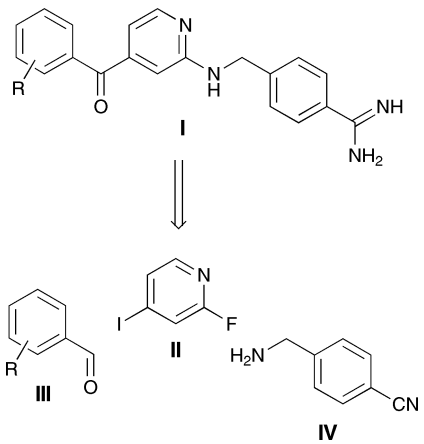
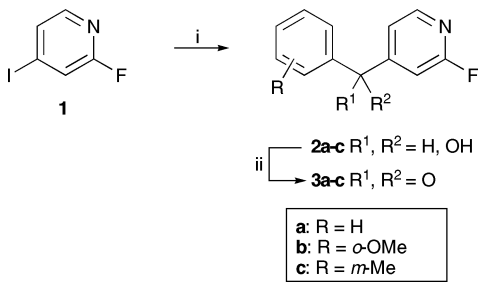


Fig. 2 A retrosynthetic analysis suggesting that thrombin inhibitors **I** could be generated from 2-fluoro-4-iodopyridine (**II**), substituted benzaldehydes **III** and 4-cyanobenzylamine (**IV**).

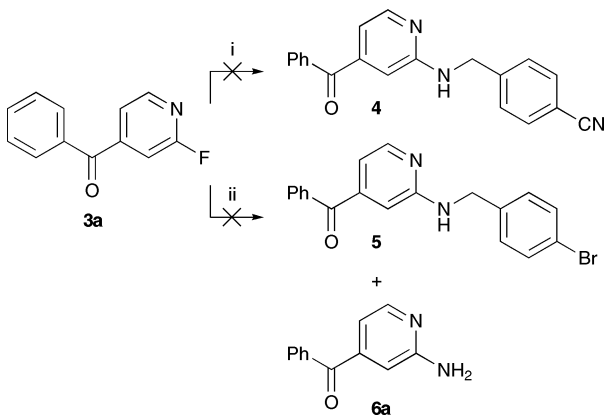
As a first step in the synthetic route, 2-fluoro-4-iodopyridine was dissolved in THF and treated with isopropyl magnesium chloride to achieve an iodo–magnesium exchange, which was followed by addition of a substituted benzaldehyde (3 examples) to react with the formed Grignard reagent (Scheme 1). This gave secondary alcohols **2a–c** (86–87%), which were subsequently oxidised to the corresponding ketones **3a–c** (86–88%) using Dess–Martin



Scheme 1 (i) i-PrMgCl, substituted benzaldehyde, THF (86–87%); (ii) Dess–Martin periodinane, CH_2Cl_2 (86–88%).

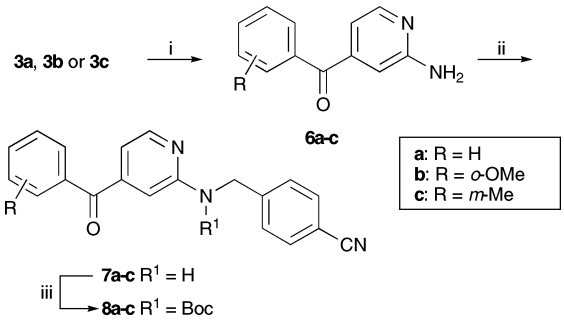
periodinane.[22] The electron withdrawing effect of the carbonyl group at position 4 of the pyridine ring in **3a–c** was expected to enhance the reactivity in the following S_NAr reaction at position 2 using benzylamine **IV** as nucleophile.

In a first attempt to introduce the benzylamine moiety, an excess of 4-cyanobenzylamine was reacted with ketone **3a** in pyridine at 100 °C for 30 minutes using microwave irradiation (Scheme 2). A colour change of the reaction mixture was observed but only starting material **3a** was detected by LCMS analysis. Increasing the temperature to 130 °C did not alter the outcome of the reaction. To investigate if the cyano functionality in 4-cyanobenzylamine was the reason for the failure of this nucleophilic substitution, 4-bromobenzylamine was instead used as nucleophile. The bromo functionality was then intended to be transformed to the desired cyano moiety at a later stage *via* a Rosemund von Braun reaction.[23] Hence, excess 4-bromobenzylamine and ketone **3a** were heated to 130 °C in pyridine using microwave irradiation for 30 minutes. Now the desired substitution product **5** was formed, but only in an unsatisfactory yield (<10%, LCMS analysis) accompanied by nearly equal amounts of the undesired decomposition product aminopyridine **6a**. The LCMS analysis also revealed that the major part of starting material **3a** was left unchanged.



Scheme 2 (i) 4-Cyanobenzylamine, pyridine, 100–130 °C, microwave irradiation; (ii) 4-bromobenzylamine, pyridine, 130 °C, microwave irradiation.

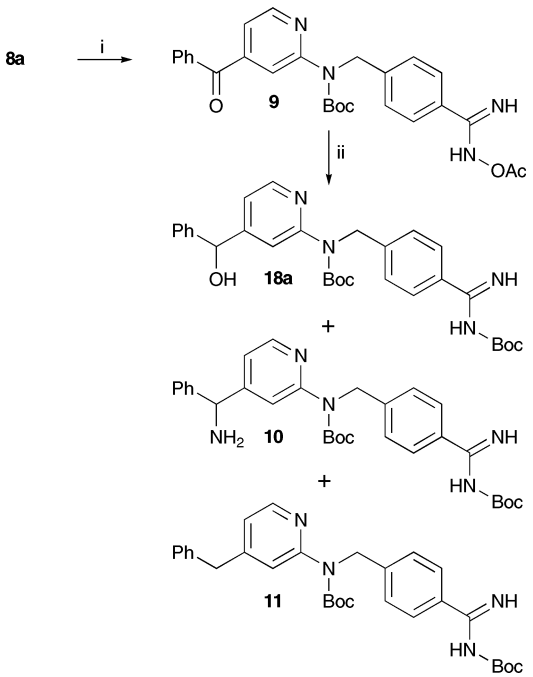
Due to the unexpected problems with the nucleophilic substitution reactions using benzylamines outlined above, other synthetic alternatives were investigated. Use of ammonia as nucleophile in reactions with ketones **3a–c** was expected to afford aminopyridines **6a–c** (Scheme 3). The strategy was then to couple **6a–c** reductively with a benzaldehyde, preferably 4-cyanobenzaldehyde, as the next step was planned to be the transformation of a cyano group to the desired amidines. In order to evaluate this route, fluoropyridine **3a** was treated with 25% ammonia in water and heated in a sealed steel cylinder to ~150 °C for 10 h. This gave aminopyridine **6a** together with approximately equimolar amounts of the corresponding imine. The ketone–imine mixture was dissolved in 10% citric acid (aq.) to allow hydrolysis of the imine which afforded ketone **6a** in excellent yield (94%). Several conditions[24–26] for reductive amination of **6a** with 4-cyanobenzaldehyde were investigated and use of sodium triacetoxyborohydride and triethylamine in dichloromethane[27] turned out to be the most promising. However, after Boc-protection,[25] aminopyridine **8a** was only obtained in a disappointingly low yield (12%). We reasoned that the low yield



Scheme 3 (i) 25% NH_3 in water ~150 °C (94–99%); (ii) 4-cyanobenzaldehyde, triethylamine, $Na(OAc)_3BH$, toluene, CH_2Cl_2; (iii) Boc_2O, DMAP, CH_2Cl_2 (64–78%, from **6a–c**).

in the reductive amination was most likely due to difficulties in formation of the imine, rather than problems in the reduction of the imine to the amine. Therefore a strategy was attempted where the imine was preformed and then reduced.[28] Aminopyridine **6a**, 4-cyanobenzaldehyde and triethylamine were refluxed for 10 h in a Dean–Stark apparatus with toluene as solvent, followed by reduction with sodium triacetoxyborohydride. The Dean–Stark procedure and the reduction was repeated twice, then the generated secondary amine **7a** was protected with a Boc group which gave **8a** in a good yield (77%, from **6a**). Next, fluoropyridines **3b–c** were treated with aqueous ammonia as described for **3a** to generate aminopyridines **6b–c** (96–99%) after acidic hydrolysis. Ketones **6b–c** were then subjected to reductive amination followed by Boc-protection, as described above, to afford compounds **8b–c** (64–78%, from **6b–c**). With the three structural components corresponding to P1, P2 and P3 motifs of thrombin inhibitors in place, only transformation of the cyano group to the desired amidine remained to be accomplished.

Unfortunately, attempts to convert the cyano functionality in **8a** to the desired amidine with lithium hexamethyldisilazide (LiHMDS) in THF failed. This was also the case for the Pinnerer reaction using hydrochloric acid in ethanol followed by addition of ammonia in methanol.[29] However, reacting **8a** with hydroxylamine to afford the corresponding amidine oxime followed by *O*-acylation using acetic anhydride in acetic acid afforded compound **9** (Scheme 4). By subjecting **9** to hydrogenation using a catalytic amount of palladium on activated carbon in methanol the labile nitrogen–oxygen bond was cleaved to generate the amidine, but simultaneously the ketone was reduced to the corresponding alcohol. The highly polar amidine was then protected with a Boc-group using Boc_2O to give amidine **18a** in modest and unpredictable yields (0–22%, from **8a**).[30] Two reasons were found for the variable outcome in the formation of amidine **18a**. The oxime corresponding to **9** was formed when ketone **8a** was treated with hydroxylamine, which then resulted in the undesired amine **10** after hydrogenation and protection. In addition, reduction of the keto functionality in **9**, not only to the corresponding alcohol, but also further to the deoxygenated compound **11** was observed. The amount of deoxygenated product was strongly dependent on the reaction time and choice of solvent. Reduction in acetic acid gave methylene derivative **11** as the major product, whereas use of methanol in combination with short reaction times favoured formation of alcohol **18a**.[31] The lack of reliability in the transformation of **9** into **18a** required further investigations in

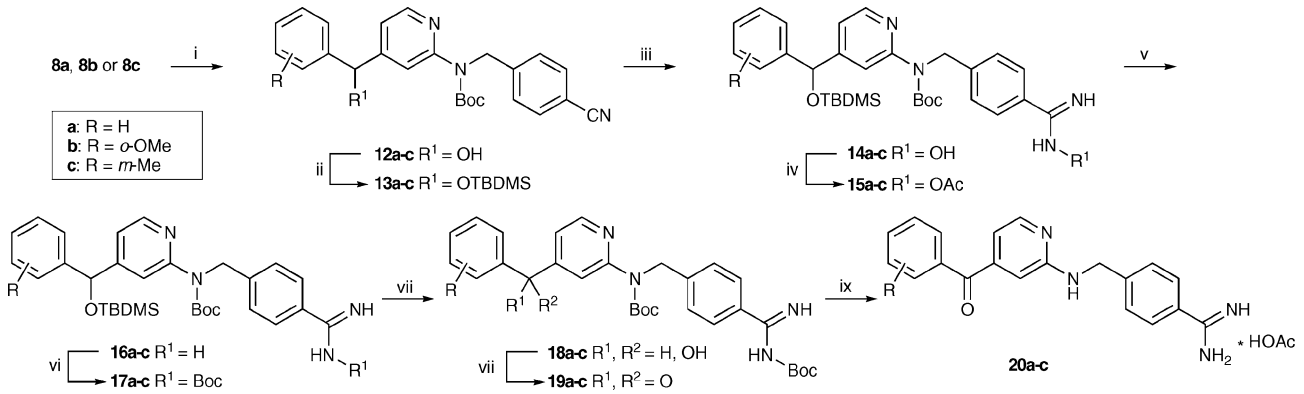


Scheme 4 (i) a: $H_2NOH{\cdot}HCl$, DIPEA, ethanol, reflux; b: acetic anhydride, acetic acid; (ii) a: H_2 (1 atm), Pd/C, methanol; b: Boc_2O, CH_2Cl_2.

order to establish a robust synthetic route. As the encountered problems, at least in part, seemed to originate from the keto functionality present in compounds **8a–c**, the ketones were first reduced to the corresponding alcohols **12a–c** using sodium borohydride (Scheme 5). Subsequently, the alcohols were protected as silylethers using *tert*-butyldimethylsilyl trifluoromethanesulfonate and collidine in dichloromethane to afford **13a–c** (82–84%, from **8a–c**). The cyano groups of **13a–c** were then converted to hydroxy amidines **14a–c** and acylated to afford **15a–c** as described above. Cleavage of the labile nitrogen–oxygen bond was achieved by hydrogenation for 35 minutes at atmospheric pressure with catalytic amounts of palladium on activated carbon in methanol to afford amidines **16a–c**. To our satisfaction the silyl ethers proved considerably more stable during hydrogenation than ketone **9**, and the undesired deoxygenation side product was detected only in trace amounts (LCMS). Protection of the amidines with a Boc group afforded **17a–c** (46–66%, from **13a–c**) and the silyl ethers were then cleaved using tetrabutylammonium fluoride (TBAF) to afford alcohols **18a–c**. Oxidation to ketones **19a–c** was thereafter achieved by treatment with Dess–Martin periodinane. Finally, the Boc group was removed from the thrombin inhibitors in a mixture of trifluoroacetic acid and dichloromethane followed by purification with reversed phase HPLC to afford the target compounds **20a–c** (33–53%, from **17a–c**). The synthetic route to thrombin inhibitors **20a–c** was thus accomplished over 14 steps with an overall yield ranging from 10–14%.

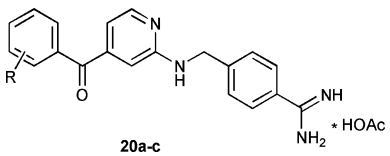
Biological evaluation of the thrombin inhibitors

Compounds **20a–c** were evaluated as thrombin inhibitors in an enzymatic assay[32] (Table 1). All three compounds showed only modest inhibition. The results indicate that binding to thrombin is influenced by the substitution pattern of the benzoyl group, *i. e.* the P3 residue. The *ortho*-methoxy group in **20b** decreases



Scheme 5 (i) $NaBH_4$, methanol; (ii) TBDMSOTf, collidine, CH_2Cl_2 (82–84%, from **8**); (iii) $H_2NOH{\cdot}HCl$, DIPEA, ethanol; (iv) acetic anhydride, acetic acid; (v) H_2 (1 atm), Pd/C, methanol; (vi) Boc_2O, CH_2Cl_2 (46–66%, from **13**); (vii) TBAF, THF; (viii) Dess–Martin periodinane, CH_2Cl_2; (ix) TFA, CH_2Cl_2 (33–53%, from **17**).

Table 1 Evaluation of compounds **20a–c** in an enzymatic assay



Compound		IC_{50}/μM
20a	R = H	15
20b	R = *o*-OMe	>44.4[a]
20c	R = *m*-Me	11

[a] The highest concentration applied in the assay was 44.4 μM.

binding affinity while the *meta*-methyl group in **20c** results in an equally active inhibitor as unsubstituted **20a**. Inspection of the X-ray structure (Fig. 3) suggests that an *ortho*-methoxy group should be well accommodated but apparently it has a negative influence on binding of the benzoyl group in the S3 pocket.

Crystal structure of 20c in the active site of thrombin

Even though the affinity was moderate, a crystal structure of **20c** in the active site of thrombin could be obtained (Fig. 3a). As expected, the amidine group was found to anchor **20c** in the S1 pocket of thrombin by binding to Asp 189. A comparison between the crystal structures of **20c** and the potent thrombin inhibitor melagatran (PDB code 1k22) reveals quite a good shape match between the two inhibitors (Fig. 3b). However, melagatran forms three hydrogen bonds to the backbone of the enzyme in the S2–S3 region, in addition to its interaction with Asp189 in the S1 pocket (Fig. 3c). Such hydrogen bonds are absent for **20c** (Fig. 3a) and this difference most likely explains the relatively low potency of **20c**. In general it can be concluded that the hydrogen bonds in the S2–S3 region of thrombin are of great importance for potency and should be considered as a high priority in the design of thrombin inhibitors.

A comparison of the crystal structure with results from Glide dockings shows that docked **20a** fill the S2 pocket in a better way than was borne out in practice (*cf.* Fig. 1 and 3a). The hydrogen bond between the benzoyl carbonyl group and the phenolic hydroxyl group in Tyr 60A, also suggested from dockings, was not formed in the cocrystal. It is possible that elongation of the benzylamine moiety to a phenylethylamine could give a compound with a better fit to the enzyme, *e.g.* filling out more of the S2 and S3 pockets and also reaching some of the important hydrogen bonds.

Experimental

(2-Fluoro-pyridin-4-yl)-phenyl-methanol (2a), (2-fluoro-pyridin-4-yl)-(2-methoxy-phenyl)-methanol (2b) and (2-fluoro-pyridin-4-yl)-*m*-tolyl-methanol (2c)

General procedure. 2-Fluoro-4-iodopyridine (1 equiv., 2.9 mmol) was dissolved in THF (3 mL) and treated with isopropyl magnesium chloride (1.3 equiv., 2 M in THF, 3.8 mmol) and stirred for 1 h without cooling the exothermic reaction. The benzaldehyde (if solid, dissolved in THF, 1 mL) (1.3 equiv., 3.8 mmol) was added and the reaction was stirred at room temperature for 12 h. The reaction was quenched with NH_4Cl (aq., sat.) and diluted with $NaHCO_3$ (aq., sat.). The aqueous phase was extracted with EtOAc and the combined organic layers were dried and concentrated under reduced pressure. The residue was purified by flash chromatography EtOAc–heptane 1 : 4 → 1 : 2 to give alcohol **2a** (86%), **2b** (converted to **3b** as crude product), or **2c** (87%).

(2-Fluoro-pyridin-4-yl)-phenyl-methanone (3a), (2-fluoro-pyridin-4-yl)-(2-methoxy-phenyl)-methanone (3b) and (2-fluoro-pyridin-4-yl)-*m*-tolyl-methanone (3c)

General procedure. Alcohol **2a**, **2b** or **2c** (1 equiv., 2.5 mmol) was dissolved in CH_2Cl_2 (40 mL) and treated with Dess–Martin periodinane (1.2 equiv., 15 wt% solution in CH_2Cl_2) at room temperature. After 0.5 h, $Na_2S_2O_5$ (21.7 mmol) dissolved in $NaHCO_3$ (aq., sat.) was added and the mixture was vigorously stirred until two clear phases were obtained. The aqueous layer was extracted with CH_2Cl_2 and the combined organic layers was washed with $NaHCO_3$ (aq., sat.), dried and concentrated under

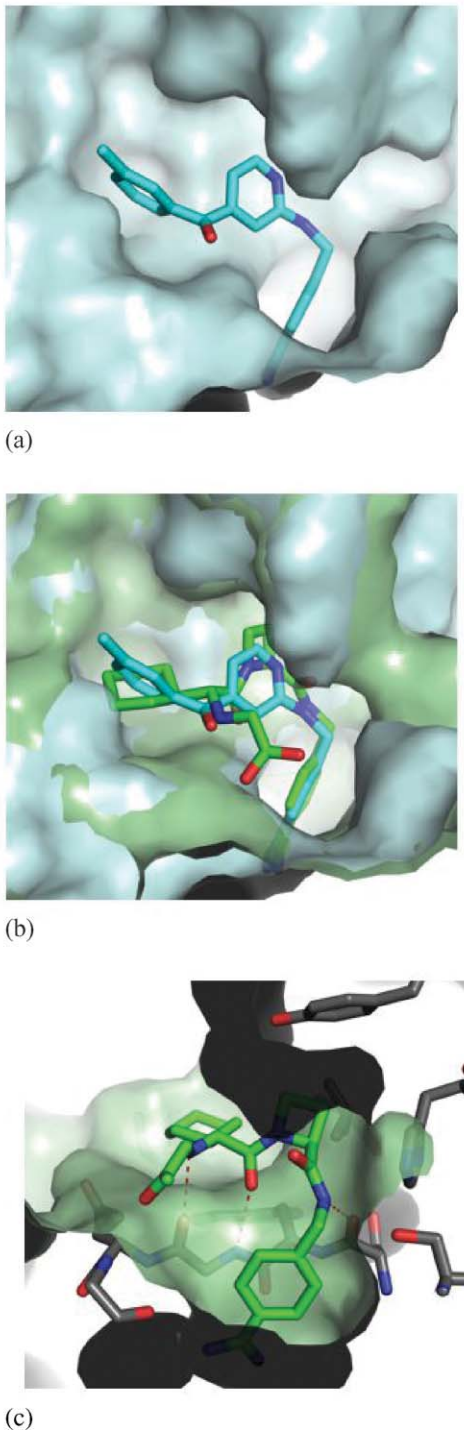


Fig. 3 (a) The crystal structure of thrombin inhibitor **20c** in the active site of thrombin. (b) An overlay of melagatran (green) and **20c** (blue) when bound by thrombin. (c) Melagatran bound in the active site of thrombin with the hydrogen bonding network in the S2 and S3 pockets displayed (red dashed lines). In the S2–S3 region hydrogen bonds are formed between two of the nitrogen atoms and one of the carbonyl oxygen atoms in melagatran and the backbone of the enzyme.

reduced pressure. The residue was purified by flash chromatography EtOAc–heptane 1 : 5 to give ketone **3a** (88%), **3b** (80%, from **1**) or **3c** (86%).

(2-Amino-pyridin-4-yl)-phenyl-methanone (6a), (2-amino-pyridin-4-yl)-(2-methoxy-phenyl)-methanone (6b) and (2-amino-pyridin-4-yl)-*m*-tolyl-methanone (6c)

General procedure. Ketone **3a**, **3b** or **3c** (2 mmol) was added to a sealed teflon coated steel cylinder followed by addition of 25% ammonia solution in water (25 mL) and heated on a sand bath (~150 °C) for 15 h. After cooling to room temperature, the aqueous phase was extracted with EtOAc. The combined organic layers were concentrated under reduced pressure and the residue was treated with citric acid (10%, 50 mL) for 3 h. The aqueous layer was made basic (pH ~ 8) with $NaHCO_3$ (s) and extracted with EtOAc. The combined organic layers were dried and concentrated under reduced pressure to give amine **6a** (94%), **6b** (96%) or **6c** (99%).

(4-Benzoyl-pyridin-2-yl)-(4-cyano-benzyl)-carbamic acid *tert*-butyl ester (8a), (4-cyano-benzyl)-[4-(2-methoxy-benzoyl)-pyridin-2-yl]-carbamic acid *tert*-butyl ester (8b) and (4-cyano-benzyl)-[4-(3-methyl-benzoyl)-pyridin-2-yl]-carbamic acid *tert*-butyl ester (8c)

General procedure. Amine **6a**, **6b** or **6c** (1.0 equiv., 2 mmol) was mixed with 4-cyano benzaldehyde (1.3 equiv.) and triethylamine (1.5 equiv.) in toluene (120 mL). The mixture was heated to reflux in Dean–Stark equipment for 10 h, followed by removal of the remaining solvent under reduced pressure. The residue was dissolved in CH_2Cl_2 (10 mL) and treated with sodium triacetoxyborohydride (1.3 equiv.) and the reaction was stirred for 3 h at room temperature. The reaction was concentrated under reduced pressure and the residue was subjected to the above described procedure two additional times. Then the reaction was quenched with $NaHCO_3$ (aq., sat.) and extracted with CH_2Cl_2. The combined organic phases were dried and concentrated under reduced pressure. The residue was purified by flash chromatography EtOAc–heptane 1 : 3 → 1 : 1 to give aminopyridine **7a**, **7b** or **7c**. The aminopyridine was dissolved in CH_2Cl_2 (40 mL) and treated with Boc_2O (5 equiv.), DMAP (cat., 0.1 equiv.). After 24 h, the reaction was washed with $NaHCO_3$ (aq., sat.) and extracted with CH_2Cl_2. The combined organic phases were dried and concentrated under reduced pressure. The residue was purified by flash chromatography EtOAc–heptane 1 : 3 to give Boc-protected aminopyridine **8a** (77% from **6a**), **8b** (64% from **6b**) or **8c** (78% from **6c**).

{4-[(*tert*-Butyl-dimethyl-silanyloxy)-phenyl-methyl]-pyridin-2-yl}-(4-cyano-benzyl)-carbamic acid *tert*-butyl ester (13a), {4-[(*tert*-butyl-dimethyl-silanyloxy)-(2-methoxy-phenyl)-methyl]-pyridin-2-yl}-(4-cyano-benzyl)-carbamic acid *tert*-butyl ester (13b) and {4-[(*tert*-butyl-dimethyl-silanyloxy)-*m*-tolyl-methyl]-pyridin-2-yl}-(4-cyano-benzyl)-carbamic acid *tert*-butyl ester (13c)

General procedure. Ketone **8a**, **8b** or **8c** (0.17 mmol, 1.0 equiv.) was dissolved in MeOH (5 mL) and treated with $NaBH_4$ (2.0 equiv.) at room temperature. The reaction was stirred for 10 minutes and then the solvent was removed under reduced pressure. The residue was dissolved in CH_2Cl_2 and washed with brine. The organic layer was dried and concentrated under reduced pressure to afford alcohols **12a**, **12b** or **12c**. The alcohol was dissolved in CH_2Cl_2 (5 mL) and treated with 2,4,6-trimethylpyridine (1.3 equiv.) and *tert*-butyldimethylsilyl trifluoromethanesulfonate

(1.3 equiv.) for 40 minutes at room temperature. The reaction was quenched with $NaHCO_3$ (aq., sat.) and the aqueous layer was extracted with CH_2Cl_2. The combined organic layers were dried and concentrated under reduced pressure. The residue was purified by flash chromatography EtOAc–heptane 1 : 4 to give **13a** (82%), **13b** (84%), or **13c** (82%).

[4-(*tert*-Butoxycarbonylamino-imino-methyl)-benzyl]-{4-[(*tert*-butyl-dimethyl-silanyloxy)-phenyl-methyl]-pyridin-2-yl}-carbamic acid *tert*-butyl ester (17a), [4-(*tert*-butoxycarbonylamino-imino-methyl)-benzyl]-{4-[(*tert*-butyl-dimethyl-silanyloxy)-(2-methoxy-phenyl)-methyl]-pyridin-2-yl}-carbamic acid *tert*-butyl ester (17b) and [4-(*tert*-butoxycarbonylamino-imino-methyl)-benzyl]-{4-[(*tert*-butyl-dimethyl-silanyloxy)-*m*-tolyl-methyl]-pyridin-2-yl}-carbamic acid *tert*-butyl ester (17c)

General procedure. Cyano compound **13a**, **13b** or **13c** (0.47 mmol, 1.0 equiv.) was dissolved in EtOH (20 mL) and treated with $H_2NOH{\cdot}HCl$ (10 equiv.) and diisopropylethylamine (20 equiv.) under reflux for 1.5 h. The solvent was removed under reduced pressure and coevaporated with $CHCl_3$ two times to give hydroxy amidine **14a**, **14b** or **14c**. The hydroxy amidine was dissolved in acetic acid (20 mL) followed by addition of Ac_2O (30 equiv.) at room temperature. After 30 minutes, the solvent was removed under reduced pressure with toluene as azeotrope and the residue was taken up in EtOAc and washed with $NaHCO_3$ (aq., sat.). The organic layer was dried and concentrated under reduced pressure from toluene two times to afford **15a**, **15b** or **15c**. The acetylated hydroxy amidine was dissolved in MeOH (50 mL) and Pd/C (100 wt%, compared to **13a**, **13b** or **13c**) was added and the reaction was put under H_2 atmosphere at normal pressure. The reduction was vigorously stirred for 30 minutes, followed by removal of Pd/C by filtration through a pad of celite, which was rinsed with MeOH–AcOH 10 : 1. The solvent was removed with toluene as azeotrope and the residue was dissolved in EtOAc and washed with Na_2CO_3 (aq., sat.). The combined organic layers were dried and concentrated under reduced pressure to afford **16a**, **16b** or **16c**. The amidine was dissolved in CH_2Cl_2 (10 mL) and treated with Boc_2O (1.1 equiv.) for 5 minutes followed by addition of $NaHCO_3$ (aq., sat.). The aqueous phase was extracted with EtOAc and the combined organic layers were dried and concentrated under reduced pressure. The residue was purified by flash chromatography EtOAc–heptane 1 : 4 to give **17a** (66%), **17b** (46%) or **17c** (57%).

4-[(4-Benzoyl-pyridin-2-ylamino)-methyl]-benzamidine acetate salt (20a), 4-{[4-(2-methoxy-benzoyl)-pyridin-2-ylamino]-methyl}-benzamidine acetate salt (20b) and 4-{[4-(3-methyl-benzoyl)-pyridin-2-ylamino]-methyl}-benzamidine acetate salt (20c)

General procedure. Compound **17a**, **17b**, or **17c** (0.2 mmol, 1.0 equiv.) was dissolved in THF (4 mL) at room temperature and treated with tetrabutylammonium fluoride hydrate (2.0 equiv.) for 1 h. The solvent was removed under reduced pressure and the residue was filtered through a path of silica gel (EtOAc–heptane, 2 : 1 as eluent) to afford alcohols **18a**, **18b** or **18c**. The afforded alcohol was dissolved in CH_2Cl_2 (4 mL) and treated with Dess–Martin periodinane (1.3 equiv.) at room temperature for 5 minutes followed by addition of $Na_2S_2O_5$ (4 equiv.) in $NaHCO_3$ (aq., sat.). The aqueous layer was extracted with CH_2Cl_2 and the combined organic layers were dried and concentrated under reduced pressure. The residue was purified by flash chromatography EtOAc–heptane 1 : 4 → 1 : 2 to afford ketone **19a**, **19b** or **19c**. The ketone was dissolved in CH_2Cl_2 (1 mL) and treated with trifluoroacetic acid (3 mL) for 35 minutes. The solvent was removed under reduced pressure and the residue was purified by preparative reversed phase HPLC to give amidines **20a** (33%), **20b** (53%) or **20c** (52%).

Thrombin inhibitor measurements[32]. The thrombin inhibitor potency of compounds **20a–c** was measured with a chromogenic substrate method in a robotic microplate processor, using 96-well, half volume microtiter plates. The linear absorbance increase values were determined by measurements at 405 nm (37 °C) during 40 minutes with melagatran as control substance. The IC_{50} values were calculated by fitting the data to a three parameter equation by Microsoft XLfit.

Crystallisation and X-ray structure determination. Crystallisation and soaking of compound: human α-thrombin (factor IIa) was purchased from "Enzyme Research Laboratories". Preformed thrombin–hirudin complex (stored at 4 degrees) was used for crystallisation. Apo-crystals were grown using a hanging-drop method with micro-seeding. The drop was made by mixing 1.5 μL of the thrombin–hirudin complex and 1.5 μL of reservoir solution containing: 0.05 M sodium phosphate buffer (pH 7.3), 28% PEG8000. The soaking was carried out by adding powder of the compound directly to the drop and leaving it at room temperature for 7 weeks.

Data collection and structure determination: the diffraction data were collected from a frozen crystal on a MarCCD detector mounted on a microfocus rotating anode generator FR-E Super-Bright from Rigaku. The crystal-to-detector distance was set to be 140 mm and 104 images were collected with oscillation of 1 degree. The data were processed with MOSFLM[33] and programs in the CCP4 suite.[34] Refinement and model re-building was carried out using programs REFMAC5[35] and COOT.[36] The structure has been deposited with PDB code 2PKS.

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31 For experimental procedure see compound **17a**. ^{1}H NMR and ^{13}C NMR for compounds **11** and **18a** are presented in the ESI.† HRMS for compound **11** (calcd for $C_{30}H_{37}N_4O_4$ (M + H) 517.2815, found 517.2827) and compound **18a** (calcd for $C_{30}H_{37}N_4O_5$ (M + H) 533.2764, found 533.2764).
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