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Letter

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Improved Stability of Proline-Derived Direct Thrombin Inhibitors through Hydroxyl to Heterocycle Replacement

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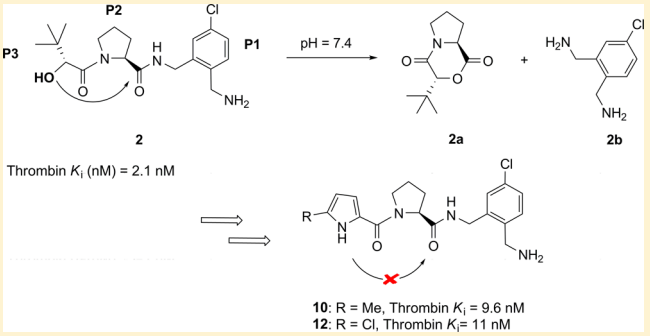
Supporting Information

ABSTRACT: Modification of the previously disclosed (*S*)-*N*-(2-(aminomethyl)-5-chlorobenzyl)-1-((*R*)-2-hydroxy-3,3-dimethylbutanoyl)pyrrolidine-2-carboxamide **2** by optimization of the P3 group afforded novel, low molecular weight thrombin inhibitors. Heterocycle replacement of the hydroxyl functional group helped maintain thrombin *in vitro* potency while improving the chemical stability and pharmacokinetic profile. These modifications led to the identification of compound **10**, which showed excellent selectivity over related serine proteases as well as *in vivo* efficacy in the rat arteriovenous shunt. Compound **10** exhibited significantly improved chemical stability and pharmacokinetic properties over **2** and may be utilized as a structurally differentiated preclinical tool comparator to dabigatran etexilate (**Pro-1**) to interrogate the on- and off-target effects of oral direct thrombin inhibitors.



KEYWORDS: *Thrombin, proline, dabigatran, thrombosis, warfarin, serine protease*

Thromboembolic diseases remain the leading cause of preventable hospital death in the United States.[1] Venous thromboembolism is estimated to affect 1−2 million people in the United States, progressing to pulmonary embolism in approximately 600,000 of these patients and becoming fatal in up to 100,000 patients annually.[1,2] The conditions amenable to treatment with anticoagulants are broad, reflecting the importance of thrombosis in the pathophysiology of multiple diseases. Indications for anticoagulant therapy include stroke, myocardial infarction, and cerebral ischemia. Thrombin is a serine protease that plays a central role in the blood coagulation cascade by mediating the conversion of fibrinogen to fibrin. Thrombin also affects arterial thrombosis via activation of the protease activating receptor PAR1.[3]

Dabigatran etexilate (**Pro-1**, Figure 1) remains the only approved oral direct thrombin inhibitor and has been shown to reduce the risk of stroke and systemic embolism in nonvalvular atrial fibrillation, as well as the treatment of deep venous thrombosis.[4] Dabigatran etexilate is dosed as a double prodrug and is rapidly converted by a serum esterase to dabigatran (**1**) *in vivo*. One shortcoming for dabigatran etexilate is the oral bioavailability of the double prodrug, which is reported to be between 3 and 7%.[5] In addition, for desired efficacy, dabigatran etexilate needs to be dosed twice a day (BID), which can be problematic for compliance in some of the patient population.

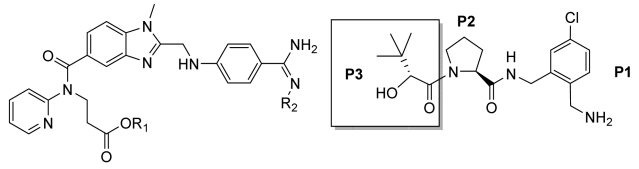


Figure 1. Dabigatran etexilate (**Pro-1**), dabigatran (**1**), and previously reported thrombin inhibitor **2**.

A reported liability of dabigatran etexilate is an increased risk of gastrointestinal bleeding as compared to warfarin. It is not currently known whether this bleeding is unique to dabigatran, a byproduct of the prodrug metabolism, or general to all oral direct thrombin inhibitors. Therefore, we sought to discover structurally distinct direct thrombin inhibitors, devoid of the need for prodrug derivatization, which would be useful tools to further understand the pharmacology of this target. Additionally, we sought a compound with a narrow peak to trough ratio (≤3) in order to minimize bleeding risk and increase the

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possibility for a once daily (QD) dosing paradigm. As a starting point, we further refined our previously disclosed thrombin inhibitor **2**, which has been profiled extensively.[6]

On the basis of the pharmacokinetic profile in dogs ($t_{1/2}$ = 3.9 h, F = 81%) for compound **2**, we surmised that BID dosing would be required to maintain efficacious levels of exposure.[7] A major additional shortcoming recognized with compound **2** is chemical instability at physiologically relevant pH. Under slightly basic conditions the secondary alcohol drives intramolecular cyclization, giving rise to the bicyclic morpholine dione **2a** while extruding dibenzylamine **2b** (Figure 2).[8] In this

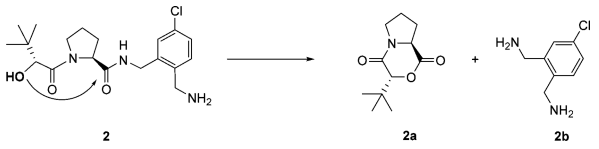
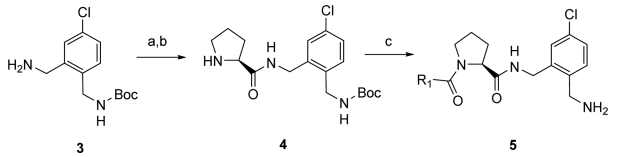


Figure 2. Cyclization pathway of compound **2** under physiologically relevant pH ranges.

Letter, we fully characterize the chemical stability of **2** at pH 7.4 and describe our approach to identify more stable derivatives that maintain the high thrombin potency and selectivity profile of **2**. In addition, we desired to improve upon the pharmacokinetic profile of **2** to provide a compound appropriate for a QD dosing regimen.

Our initial goal was to identify a heterocyclic H-bond donor that maintains the exquisite *in vitro* potency profile of compound **2**. The synthesis of these analogues commenced with use of the previously described proline intermediate **4**, prepared according to procedures outlined in Scheme 1.[6]

Scheme 1. Synthesis of Compounds in Table 1[a]

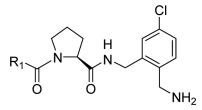


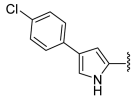
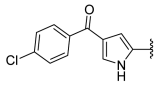
[a]Reagents and conditions: (a) Fmoc-L-proline, EDCI, HOBT, Hunig's base, DMF; (b) piperidine, DMF; (c) R_1COOH, EDCI, HOBT, Hunig's base, DMF; (d) 4 N HCl in dioxane.

Standard amino acid coupling of various acyl heterocyclic moieties at the P-3 region of the molecule was accomplished followed by BOC deprotection using 4 N HCl in dioxane to provide the desired final products.

The compounds synthesized were evaluated for inhibition of thrombin (Table 1). Thrombin itself is a serine protease in the trypsin family. We regularly counterscreened all compounds against trypsin, a serine protease present in the gut with similar substrate prerequisites to thrombin. Inhibition of trypsin-like enzymes unrelated to the coagulation pathway, but known to be necessary for physiological functions, could have deleterious consequences.[9] Both dabigatran (**1**) and compound **2** were found to exhibit exquisite potency as thrombin inhibitors in our isolated enzyme assay (K_i = 1.3 and 2.1 nM, respectively). Among the heterocycle replacements for the hydroxyl substituent in **2**, pyrazole **6** and imidazole **7** were far less potent with thrombin K_i values of 366 and 193 nM, respectively, while maintaining good selectivity against trypsin. Interestingly, a simple pyrrole (compound **8**) afforded an *in*

Table 1. Direct Thrombin Inhibitors from Scheme 1



Compound	R_1	Thrombin K_i (nM)	Trypsin K_i (nM)
1	Dabigatran	1.3	195
2		2.1	9600
6		366	>15000
7		193	>15000
8		44	5100
9		800	>15000
10		9.6	2546
11		17	6145
12		11	1164
13		70	11060
14		38	8080
15		65	12390
16		325	>15000
17		33	13730

vitro K_i potency of 44 nM. Capping of the pyrrole with a Me group (**9**) decreased the potency 20-fold, indicating that the free N−H of the pyrrole was critical for *in vitro* potency similar to the hydroxyl moiety of compound **2**. Additional substitution of a Me group directly on the pyrrole ring led to compounds **10** and **11**, with superior potency (9.6 and 17 nM respectively) and very good trypsin selectivity. Further modification of the 2-position of the pyrrole with a Cl atom led to compound **12**, which also showed excellent overall potency (11 nM) but afforded a lower, 100-fold window of selectivity over trypsin as compared to **10**. Further substitution of the pyrrole by means of a chlorophenyl substituent such as compound **13** or 4-chlorobenzoyl pyrrole **14** at the 3-position also led to compounds with excellent thrombin potency and significantly improved trypsin selectivity. In addition, an indole and two azaindoles were prepared in order to ascertain the effect of a fused heterocyclic system. Potencies were quite respectable with compounds **15** and **17** displaying potency values of 65 and 33 nM, respectively. The regioisomeric azaindole **16** exhibited a

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10-fold drop in thrombin potency from the azaindole **17** while maintaining trypsin selectivity. This indicates that the pyridynyl nitrogen had a substantial effect on the H-bonding capability of the azaindole.

Additionally, we did examine the selectivity of compounds **10** and **12** against serine proteases besides trypsin. In the case of compound **10**, there was a 30× selectivity window against Factor Xa and a >1500× window against both Factors VIIa and XIIa (Table 2). Compound **12** displayed a 200× window over Factor VIIa, a 40× window over Factor Xa, and a >1500× window over Factor XIIa.

Table 2. *In Vitro* Coagulation Pathway Selectivity Profile for Compounds 10 and 12

compd	factor VIIa	factor Xa	factor XIIa
10	>15 μM	295 nM	>15 μM
12	2.3 μM	397 nM	>15 μM

On the basis of promising *in vitro* data, we sought to further profile compounds **10** and **12** in an assay that measures the concentration of a test compound required to double the activated partial thromboplastin time (2 × APTT) in human plasma (Table 3).[10] Both dabigatran (**1**) and compound **2** were found to exhibit good functional activity in this coagulation assay in human plasma (2 × APTT = 0.63 and 0.23 μM, respectively). Compound **10** possessed anticlotting activity (2 × APTT = 6.9 μM) roughly 10-fold less potent than dabigatran. Compound **12** was the least effective (2 × APTT = 14 μM) in the APTT assay for thrombosis.[11]

Table 3. APTT Data for Thrombin Compounds 1, 2, 10, and 12

compd	APTT (2×) (μM)
1	0.63
2	0.23
10	6.9
12	14

Pharmacokinetic data (PK) was generated for compounds **2**, **10**, and **12** in both rat and dog (Table 4). Compound **2** suffered from poor rat PK with very high clearance and poor oral absorption at 10 mg/kg.[6] The dog half-life for **2** was measured to be 3.9 h, which was critical to improve for a potential QD clinical profile.[12,13] Rat PK for **10** exhibited poor oral bioavailability (F = 7%) and very high clearance (Cl = 64 mL/min·kg) overall. Compound **12** exhibited a similarly short half-life and high clearance; however, the bioavailability was improved by 3-fold over compound **10**. When the pharmacokinetic profiles for both analogues **10** and **12** were measured in dogs, we were delighted to find both compounds were orally bioavailable (42% and 82%, respectively) and possessed very low clearance values (0.31 and 0.29 mL/min/kg). In addition, the half-life for both compounds ($t_{1/2}$) was significantly improved over **2** and is consistent with QD dosing in humans, showing an advantage over dabigatran (**1**). To our delight, over a 24 h time course, compounds **10** and **12** also maintained a peak to trough ratio of ≤3, which was a key initial goal for the program (Figure 3).[14]

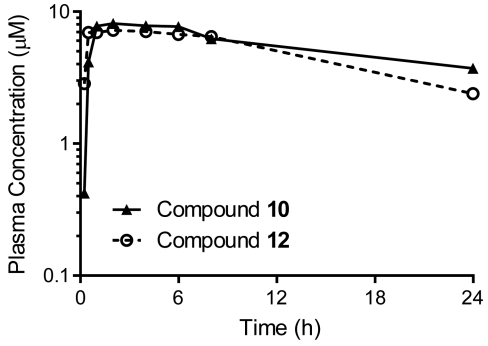

Figure 3. Dog IV PK curve for compounds **10** and **12**.

We also examined the chemical stability of compounds **10** and **12** versus **2** at a pH that was physiologically relevant. The true question of whether the acyl pyrrole moiety is as prone to cyclization was answered by subjecting each compound in a pH 7.4 buffer solution at 25 °C and examining the percentage of parent species remaining after 24 h (Figure 4). Compound **2** degraded over 24 h by 20.5% through conversion to dione **2a**. Interestingly, compounds **10** and **12** did not show any appreciable degradation over the 24 h time period. The

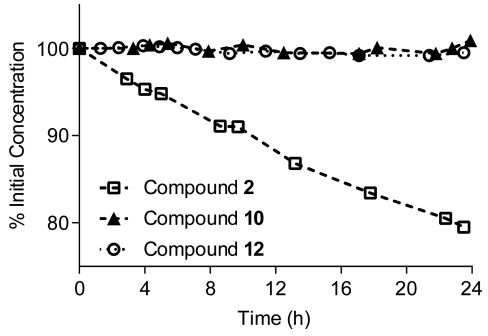

Figure 4. Chemical stability of compounds **2**, **10**, and **12** at 25 °C, pH 7.4 (50:50 10 mM phosphate buffer/acetonitrile).

Table 4. PK Data for Compounds 2, 10, and 12

compd	species	dose IV/PK (mg/kg)	AUC_N[f] (μM·kg/mg)	$t_{1/2}$ (h)	Cl (mL/min/kg)	F (%)
2	rat	2[a]/10[b]	0.2	2.9	81	37
2	dog	0.75[c]/1[c]	10.1	3.9	3.5	81
10	rat	1[d]/3[d]	0.96[a]	1.4	64	7
10	dog	1[e]/2[e]	166	19	0.31	42
12	rat	1[d]/3[d]	0.9	1.4	52	23
12	dog	1[e]/2[e]	241	15.5	0.29	82

[a]Dosing vehicle DMSO. [b]Dosing vehicle 1% methylcellulose. [c]Dosing vehicle 5% dextrose/PEG (60:20). [d]Dosing vehicle DMSO/PEG300/H_2O (10:50:40), [e]Dosing vehicle 5% DMA/30% PEG400/65% (40% HPCD). [f]Normalized AUC (PO, μM·h·kg/mg).

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improved chemical stability could be a direct result of the higher measured pK_a for the acyl pyrroles **10** and **12** (~pK_a = 16.5) versus compound **2** (pK_a =13.6) when subjected to a pH of 7.4.

We chose to compare the *in vivo* efficacy of compound **2** to compound **10** in the rat arteriovenous shunt (AV shunt) thrombosis model rather than to compound **12** (Figure 5),[15]

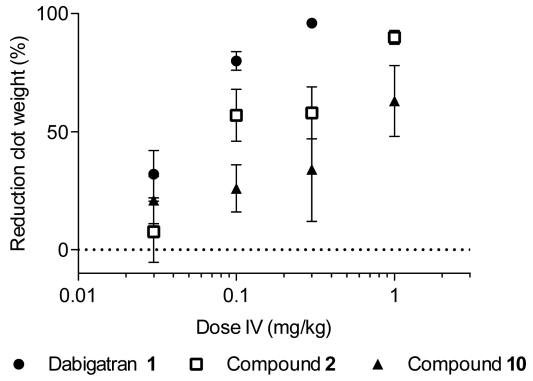

Figure 5. Rat AV-shunt study with compounds **1**, **2**, and **10**.

due to the superior APTT response of **10** over **12** (Table 2). As a positive control, dabigatran (**1**) was also investigated in this assay. Compound **2** inhibited thrombus formation in a dose-dependent manner from 0.03 to 1.0 mg/kg via IV infusion. At the 1 mg/kg dose, a 90% reduction in clot weight was observed with a mean exposure of 0.74 μM. Compound **10** showed good activity with a 63% inhibition of clot weight at 1.0 mg/kg with an exposure of 1.04 μM. In comparison, dabigatran (**1**) showed a 96% reduction in clot weight at 0.3 mg/kg with an exposure of drug of 0.189 μM.

The efficacy profile exhibited by all three compounds in the rat AV shunt study is reasonably reflective of the human 2 × APTT concentration from Table 2. Dabigatran exhibited excellent *in vivo* efficacy with a steep dose/response curve. Compound **2** showed superior efficacy at a lower dose over compound **10**, which is likely a manifestation of better overall intrinsic potency over compound **10** (~10-fold) (Figure 5). Additionally, compound **2** possessed a free fraction in rat of 54%, while compound **10** was slightly lower at 40% free drug.[12] When examining the positive aspects, however, of improved chemical stability and blunted peak to trough ratio leading to likely QD dosing with minimized bleeding risk, it becomes apparent that compound **10** could be utilized as a valuable research tool for further pharmacological elucidation of the thrombin pathway.

In summary, we have discovered a novel heterocyclic replacement for our previously disclosed low molecular weight thrombin inhibitor **2**. The acyl pyrroles **10** and **12** improved significantly upon both the chemical stability and PK profile parameters set forth to make the compound amenable to a QD dosing regimen with a blunted peak to trough ratio. These features, along with structural diversity and lack of prodrug derivatization as compared to dabigatran−etexilate provided a differentiated tool compound **10**. This tool may aid in the development of preclinical models to interrogate the pharmacology of thrombin inhibition as a structurally distinct comparator to dabigatran.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedures and analytical data of selected thrombin inhibitors, and conditions for all the biological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(7) Projected human-half-life of compound **2** is 7−8 h based on allometric scaling of the dog PK.

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(11) Plasma free fraction (% unbound): Compound **2** human (24%) rat (54%);[6] compound **10** human (2.4%), rat (40%).

(12) Hepatocyte stability % remaining at 30 min: Compound **10**: Human (98%), Dog (88%), Rat (62%); Compound **12**: Human (91%), Dog (80%), Rat (48%).

(13) C_{max} values for Compound **10** = 8.4 μM (Dog), 0.02 μM (Rat); C_{max} values for Compound **12** = 8.8 μM (Dog), 0.18 μM (Rat).

(14) The projected human half-life of compound **10** is calculated to be 35 h based on allometric scaling of the dog PK.

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