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Design, synthesis, and SAR of a series of activated protein C (APC) inhibitors with selectivity against thrombin for the treatment of haemophilia

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ABSTRACT

A design strategy was used to identify inhibitors of activated protein C with selectivity over thrombin featured by a basic and/or aromatic functionality for binding to the S2 pocket. Our strongest inhibitor showed an IC_{50}-material value and selectivity for APC vs thrombin similar to a compound previously reported in the literature. However, in contrast to the reference compound, our compound showed a retained coagulant effect of thrombin with increasing substrate concentration in a modified Calibrated Automated Thrombogram (CAT) method. This was likely related to our compound being inactive against FVIIa, while the reference compound showed an IC_{50} of 8.9 μM. Thus, the higher selectivity of our compound against all relevant coagulation factors likely explained its higher therapeutic potential in comparison to the reference compound. The data indicate that at least a 100-fold selectivity over other serine proteases in the coagulation cascade will be required for an effective APC inhibitor.

Haemophilia (deficiency of coagulation factors VIII or IX, FVIII or FIX) is treated with intravenous replacement of the deficient factor or with factor concentrates.[1] Clinical studies and experience show that prophylactic administration of the deficient factor is highly preferable with regard to outcome.[2] Prophylaxis is cumbersome due to the necessity of intravenous self-injections every second to third day, the cost of coagulation factors, and requirements on hygiene and refrigerators. A serious clinical complication in 5–25% of the patients is the development of inhibitory antibodies towards the administered coagulation factor. Today only 20–30% of the estimated 'haemophilia population' in the world receives adequate treatment, and only a fraction of those are given the coagulation factors as prophylaxis.[3] Treatment with small molecules is limited to desmopressin (intranasally or subcutaneously) for transient release of FVIII from endothelial cells, which is effective in mild haemophilia,[4] and stabilisation of the clot with the oral fibrinolysis inhibitor tranexamic acid, with clinically meaningful effects on top of added coagulation factor in dental extractions.[5] Thus, prophylactic oral prevention of bleeding in haemophilia represents a large medical need.

The balance between haemostasis and bleeding is skewed in the sense that there is no need to fully restore the haemostasis. In haemophilia, less than 1%, 1–5%, and 5–40% of the normal level of FVIII or FIX are the definitions for severe, moderate and mild haemophilia, respectively. Persons with mild haemophilia usually do not require coagulation factor prophylaxis.

Activation of Protein C, a serine protease in the coagulation cascade, by thrombin forms activated protein C (APC) which functions as a negative feed-back inhibitor of plasma coagulation by degrading activated factor V (FVa) and activated factor VIII (FVIIIa) to inactive factors (FVai, FVIIIai). FVa and FVIIIa strongly reinforce plasma coagulation by assembling the prothrombinase and tenase complexes. Reduced effectiveness of the APC pathway via the FV Leiden mutation is a thrombosis risk in persons without bleeding disorders but in persons with bleeding disorders it may instead prevent bleeding.[6] Stimulation of plasma coagulation by inhibition of the anticoagulant action of APC has been described for peptides[7] and aptamers.[8] The only low molecular weight APC inhibitors reported to date is a series of benzamidine derivatives from Servier,[9] exemplified by compound **1**[10] (selected as a reference compound, Fig. 1) that showed an IC_{50} of 0.82 μM (1.7 μM in our assay) and the highest selectivity for APC over thrombin (57-fold reported, 21-fold in our assay) in the Servier series. Calculated inhibition constants (Ki-values) from all IC_{50} values are given for all compounds in the Supplementary Information.

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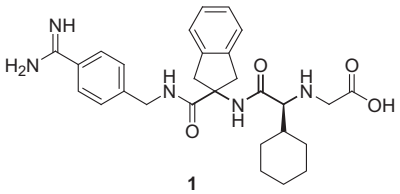


Figure 1. The compound (**1**, reference compound), with highest selectivity for APC over thrombin in the Servier series.

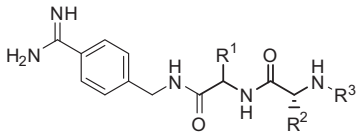


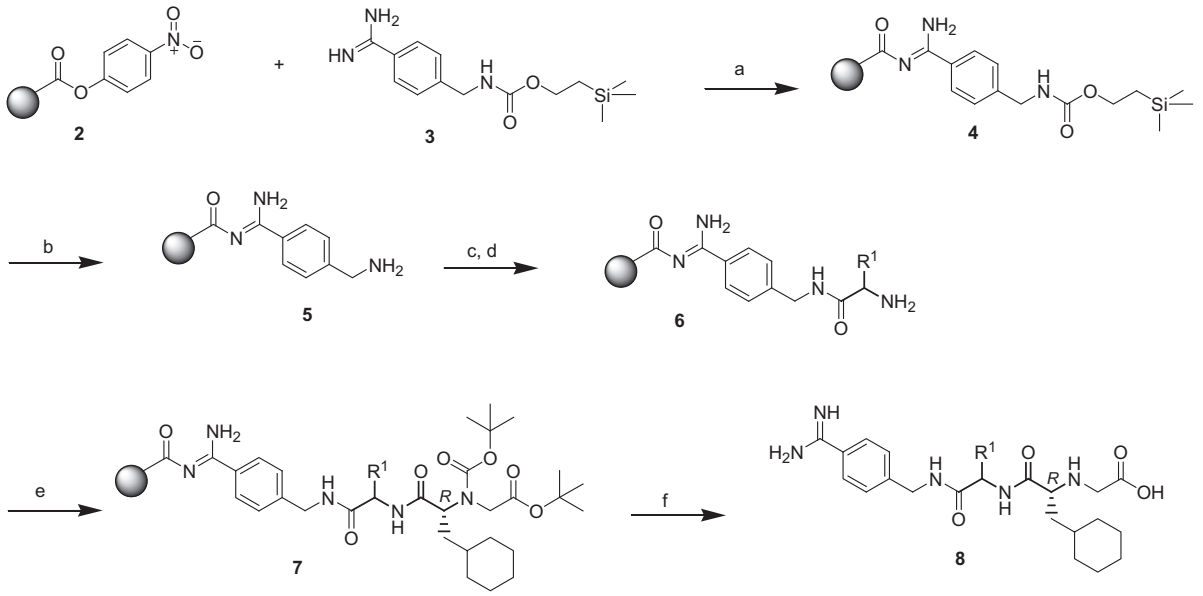
Figure 2. Benzamidine scaffold used in the present study.

A comparison of the crystal structures[11] of the two serine proteases APC and thrombin pointed to a difference in the preferred amino acid for the natural ligands binding to the S2 pocket. While the prototypic substrate in P1–P2–P3 for thrombin is D-phe-pro-arg[12] the analogous ligand for APC is D-phe-arg-arg. Given that the guanidino functionality of arginine has a typical pK_a values of 13,[13] compared to the neutral side chain of proline, we hypothesized that ligands with a basic functionality aimed for binding in the S2 pocket of APC would lead to selectivity for APC over thrombin. In addition, for this structural class it was known that attempts to introduce large substituents into the S2 pocket resulted in a reduced affinity for thrombin, since S2 in APC has a more open conformation.[14] Basic and/or sterically large functional groups aimed for binding interactions in the S2 pocket should thus be introduced to attain selectivity for APC over thrombin. Based on the precedent set by **1**, we tested this hypothesis using the general benzamidine scaffold depicted in Figure 2. Below we describe our synthetic approach and successful identification of ligands that display coagulant activity as well as selectivity for APC over thrombin.

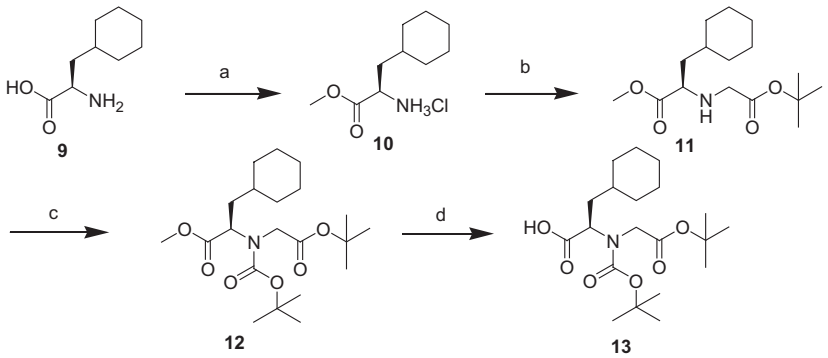
The compounds were synthesized by applying the modular solid-phase synthesis shown in Scheme 1[15] which is based on the anchoring of the benzamidine moiety on a carbamate resin.[16] Thus, Wang carbamate resin **2** was treated with three equivalents of benzamidine derivative **3** in the presence of DIPEA to give **4**. Removal of the Teoc protective group was achieved by treatment with an excess of TBAF. Coupling of a diverse set of Fmoc protected amino acid was effected smoothly using TBTU/HOBT. Initially, the subsequent Fmoc deprotection step was performed using classical conditions (20% piperidine in DMF), however this approach gave irreproducible and/or overall poor yields for the sequence. We reasoned that the carbamate-amidine linkage could potentially be reactive in the presence of a high concentration of a nucleophilic amine.[17] We therefore tested the use of milder reaction conditions by using TBAF as the Fmoc cleaving reagent,[18] and this improved both the efficiency and reliability of the sequence. Subsequent coupling with carboxylic acid **13** (Scheme 2), followed by cleavage/deprotection under acidic conditions yielded smoothly the targeted amidines in 8–34% overall yield, isolated after reverse-phase chromatography. Not all pairs of diastereomers could be separated in this manner, thus some pairs of diastereomers were screened[19] as such after chromatographic purification.

The cyclohexyl alanine derivative **13** (used in step e, Scheme 1) was prepared on a multigram scale as outlined in Scheme 2. Thus, protection of compound **9** as a methyl ester to give **10** followed by consecutive mono-alkylation and Boc-protection of the amine functionality provided **12**. Hydrolysis of the methyl ester under alkaline conditions provided the carboxylic acid **13**. The analogous building blocks for the syntheses of **23**, **30** (R^2 = cyclohexyl) and **24** ($R^3 = (CH_2)_2CO_2H$) were prepared by similar procedures.

Compound **22** (Table 1) was featured by the basic side chain of arginine in combination with a CH_2-cyclohexyl R^2 substituent and a CH_2COOH R^3 substituent and showed a comparably high affinity for APC with an IC_{50} value of 0.47 μM, but only a 2.8-fold selectivity against thrombin. Replacement of the R^2 substituent with cyclohexyl (to give **23**) or of the R^3 substituent with $(CH_2)_2CO_2H$ (to give **24**) led to lower affinity, thus the R^2 and R^3 substituents of compound **22** were retained in the further SAR investigations. Amino acid moieties with an (*S*)-stereochemistry were preferred

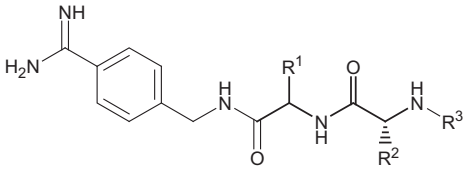


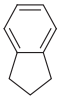
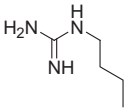
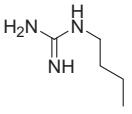
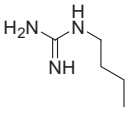
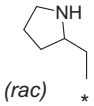
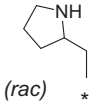
Scheme 1. Solid-phase method for modular synthesis of APC inhibitors. Reagents and conditions: (a) DIPEA, 9.0 equiv, DMF, rt, 20 h; (b) TBAF (1M in THF), 10 equiv, DMF, rt; (c) Fmoc-NHC(R^1)COOH, 1.5 equiv, TBTU, 1.5 equiv, DIPEA, 3.0 equiv, DMF, rt, 3.5 h; (d) TBAF (1M in THF), 3.0 equiv, DMF, rt, 3 × 2 min.; (e) **13**, 1.5 equiv, TBTU, 1.5 equiv, HOBT, 1.5 equiv, DIPEA, 3.0 equiv, DMF, rt, 4 h; (f) TFA/DCM/H_2O 49:49:2; rt, 1 + 1 h.



Scheme 2. Preparation of the cyclohexyl alanine derivative **13**. Reagents and conditions: (a) $SOCl_2$, MeOH, −20 °C then reflux, 18 h, (77%); (b) $BrCH_2COO$-*t*-Bu, 1.0 equiv, adjust to pH = 9 with DIPEA, then stir at rt, 36 h (58%); (c) Boc_2O, 1.1 equiv, DIPEA, 1.0 equiv, rt, 16 h, add Boc_2O, 0.1 equiv, rt, 21 h (100%); (d) NaOH, 2.0 equiv; dioxane/water, rt, 16 h (99%).

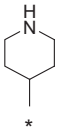
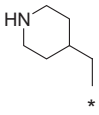
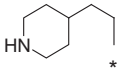
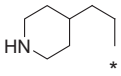
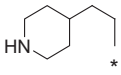
Table 1
Effect on inhibition of APC and thrombin, respectively, by introduction of R^1 groups with basic, aliphatic functionalities. The inhibition of activated coagulation factors X (FXa) and XI (FXIa) was determined for some compounds



Compd No	R^1 α-C stereo	R^1	ACD[a] pKa of R^1	R^2α-C stereo	R^2	R^3	APC, IC_{50} (μM)	Thrombin, IC_{50} (μM)	APC/ thrombin ratio	FXa, IC_{50} (μM)	FXIa, IC_{50} (μM)	ACD[a] log D pH7.4	log D[b], pH7.4
1	—		—	*R*	*cy*-hex	CH_2CO_2H	1.7	36	21	48	>44	0.52	0.93
22	*S*		13.3	*R*	CH_2–*cy*-hex	CH_2CO_2H	0.47	1.3	2.8	—	—	−4.51	—
23	*S*		13.3	*R*	*cy*-hex	H	1.6	1.9	1.2	—	—	−9.33	—
24	*S*		13.3	*R*	CH_2-*cy*-hex	$(CH_2)_2CO_2H$	2.8	5.2	1.8	—	>44	−5.1	—
25	*S*	 (rac)	10.5	*R*	CH_2-*cy*-hex	CH_2CO_2H	5.6	7.0	1.3	—	—	−2.79	—
26	*R*	 (rac)	10.5	*R*	CH_2-*cy*-hex	CH_2CO_2H	>44	>44	1	—	—	−2.79	—

(*continued on next page*)

Table 1 (*continued*)

Compd No	R^1 α-C stereo	R^1	ACD[a] pKa of R^1	R^2α-C stereo	R^2	R^3	APC, IC_{50} (μM)	Thrombin, IC_{50} (μM)	APC/ thrombin ratio	FXa, IC_{50} (μM)	FXIa, IC_{50} (μM)	ACD[a] log D pH7.4	log D[b], pH7.4
27	*S*		10.2	*R*	CH_2-*cy*-hex	CH_2CO_2H	3.1	26	8.4	—	>44	−2.53	—
28	*rac*		10.5	*R*	CH_2-*cy*-hex	CH_2CO_2H	1.3	12	9.1	—	—	−3.07	—
29	*rac*		10.6	*R*	CH_2-*cy*-hex	CH_2CO_2H	0.92	22	23	>130	>44	−2.67	<0
30	*rac*		10.6	*R*	*cy*-hex	CH_2CO_2H	>44	>44	1	—	—	−3.38	—
31	*rac*		10.6	*R*	*CH*$_2$-*cy*-hex	H	3.1	28	8.9	—	—	−5.78	—

[a] The pK_a values of the R1 functionalities in the corresponding protonated forms and the lipophilicity of the compounds, expressed by log D at pH 7.4, were calculated using the software package from Advanced Chemical Design, Inc.
[b] log D at pH 7.4 was determined by a chromatographic method. *cy*-hex = cyclohexyl.

for binding to the S2 pocket of APC, and all synthesized compounds with (*R*)-amino acids in this position were inactive, as exemplified by the pair of diastereomers **25**/**26**. Further investigations of basic side-chains with amine functionalities presented compounds **27**–**29**, of which **29** showed an affinity of 0.91 μM for APC and a remarkable 23-fold selectivity vs thrombin. Replacement of the CH_2-cyclohexyl R^2 substituent of **29** with a cyclohexyl gave **30**, which was inactive both vs APC and thrombin. In the Servier series, replacement of the cyclohexyl R^2 substituent of compound **1** (Fig. 1) with a CH_2-cyclohexyl substituent led to an 8-fold increase in inhibition of APC (from IC_{50} = 0.82 μM to 0.10 μM), but also to a lowering of selectivity for APC over thrombin from 57-fold to 13-fold. This shows that the SAR does not translate directly between the two series. Replacement of the acidic R^3 substituent of compound **29** with hydrogen gave **31** which showed a 3.5-fold reduction in affinity (to 3.1 μM) and a reduced selectivity vs thrombin compared to **29**.

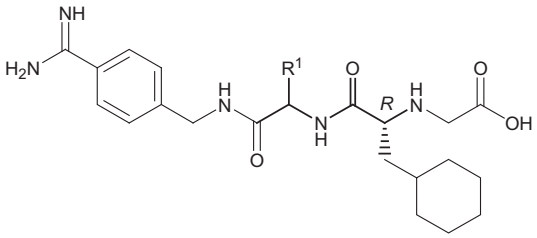
Introduction of aromatic groups as R^1 substituents, exemplified by compounds **33**–**35** (Table 2), led to inhibitory effects on the level of **29**, however with lower selectivity for APC over thrombin. In comparison to benzyl compound **33**, substitution in the benzyl 4-position (compounds **36**–**39**), led to an increased selectivity over thrombin only for the phenolic derivative **36**. Interestingly, this derivative also showed significant selectivity levels over FXa and FXIa. The anilinic derivative **37** turned out to be the strongest inhibitor of APC with however a decreased selectivity over thrombin compared to **33**. Nitrile and methylamino derivatives **38** and **39** both showed decreases in inhibition of APC and in selectivity over thrombin compared to **33**.

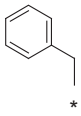
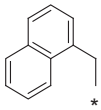
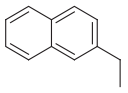
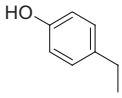
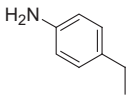
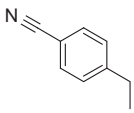
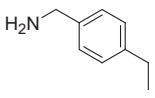
The efficiency of compounds **1** and **29**, which showed similar inhibitory constants and selectivity for APC over thrombin, was evaluated by a modified thrombin generation assay known as the Calibrated Automated Thrombogram (CAT) method, using citrated plasma from patients with severe haemophilia A.[21] This method was based on activation of coagulation and APC by addition of tissue factor and thrombomodulin, respectively. To speed up thrombin generation, a small fraction of normal human pooled plasma was added to reach approximately 5% FVIII level. Ellagic acid, a known FXII activator (up-stream to FVIII and FIX), was also added to amplify the thrombin signal from the internal pathway. The results from this method are shown in Figure 3 and Table 3. The area under the curve represents the total thrombin activity (endogenous thrombin potential, ETP) in the sample and was shown to decrease when thrombomodulin was added, due to APC-induced inhibition of thrombin formation. When an APC inhibiting antibody was added as positive control, the ETP was restored to the level when no thrombomodulin was present.

Both compound **1** and compound **29** showed activity starting at their IC_{50} for APC. However, while compound **1** (Fig. 4, Table 3) showed a decreasing ETP with increasing concentration of **1**, compound **29** (Fig. 5, Table 3) showed an increase in ETP towards normalisation with increasing concentration. Compounds **1** and **29** both shifted lagtime and time to peak to the right (Figs. 4 and 5, Table 3). The tendency with right shifted time to peak is often seen when more thrombin is formed in the system due to that the peak in thrombin formation then occurs later. The delay in lagtime is probably due to coagulation factor inhibition since it is most pronounced at higher concentrations where the coagulation inhibition is substantial.

These results are surprising as both **1** and **29** have the same selectivity for APC towards thrombin (21 and 23-fold, respectively) and also the selectivity ratios against FXa (immediately up-stream to thrombin, 28 and >140, respectively) and FXIa (up-stream to FXa and FIXa in the internal pathway, >26 and >48, respectively) seem reasonable. Both compounds were also inactive against FIXa at 100 μM (see Supplementary material). However, the analysis of FVIIa on the external pathway show an interesting difference with **1** having an IC_{50} of 8.9 μM (selectivity ratio 5.2) while compound **29** was inactive against FVIIa (IC_{50} > 133 μM, selectivity ratio >140). Thus, most likely the high selectivity against relevant coagulation factors explains the higher therapeutic potential of compound **29** in comparison to compound **1** since inhibition of the anti-coagulant APC can be achieved while retaining the pro-coagulant effect in plasma.

Table 2
Effect on inhibition of APC and thrombin, respectively, by introduction of R^1 groups with aromatic functionalities. The inhibition of activated coagulation factors X (FXa) and XI (FXIa) was determined for some compounds



Compd No	R^1 α-C stereo	R^1	ACD[a] pKa of R^1	APC, IC_{50} (μM)	Thrombin, IC_{50} (μM)	APC/ thrombin ratio	FXa, IC_{50} (μM)	FXIa, IC_{50} (μM)	ACD[a] log D pH7.4	log D[b], pH7.4
33	*S*		–	0.67	6.1	9.1	–	–	1.75	–
34	*S*		–	1.1	12	11	–	–	2.96	1.97
35	*S*		–	0.98	5.2	5.3	–	–	2.95	–
36	*S*		9.8	0.26	3.9	15	23	1.9	1.11	0.02
37	*S*		4.5	0.13	0.9	6.9	>44	0.58	0.57	–
38	*rac*		–	3.8	3.8	5.4	–	–	1.43	–
39	*rac*		9.1	1.1	3.7	3.3	42	43	−0.76	–

[a] The pK_a values of the R1 functionalities in the corresponding protonated forms and the lipophilicity of the compounds, expressed by log D at pH 7.4, were calculated using the software package from Advanced Chemical Design, Inc.
[b] log D at pH 7.4 was determined by a chromatographic method.[20]

The CAT assay only gives information about the thrombin generation, and, hence, plasma coagulation. However, fibrinolysis inhibition is also important, as shown clinically by tranexamic acid in some patients with bleeding disorders. Moreover incorporation of homopiperidylalanine as P2 has recently been shown to yield APC potency in dual plasmin and plasma kallikrein inhibitors.[22] Thus, compound **1** and **29** were tested in the plasma clot-lysis assay using citrated plasma from healthy volunteers.[23] Plasmin generation was initiated by the addition of tissue plasminogen activator. Both compounds prolonged the clot-lysis time and it was doubled at assay concentration of 101 and 28 μM for compounds **1** and **29**, respectively ($n = 2$). For compound **1** the antifibrinolytic effect could be of some relevance as the inhibition occurs in the same concentration range as the increase in thrombin generation measured in the CAT assay.

In conclusion, by using a design strategy to introduce a basic S2 side chain we have discovered an inhibitor of activated protein C (APC) with IC_{50}-value and selectivity for APC vs thrombin similar to a compound previously reported (compound **1**). However, contrary to compound **1** (reference compound), compound **29** shows a retained coagulant effect of thrombin with increasing substrate concentration in a modified Calibrated Automated Thrombogram

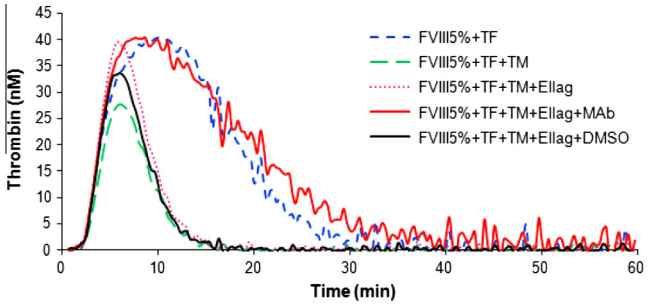


Figure 3. A control experiment in the thrombin generation assay (CAT method). The area under the curve represents the total thrombin activity in the sample. As a validation of the method the different curves shows the different additions. The monoclonal antibody (MAb) that inhibit APC was used as positive control. FVIII = coagulation factor VIII, TF = tissue factor, TM = rabbit lung thrombomodulin, Ellag = ellagic acid.

Table 3
Effect of compounds **1** and **29** on thrombin generation parameters lagtime, time to peak, peak and endogenous thrombin potential compared to controls

Conccn (μM)	Lagtime (min)	Time to Peak (min)	Peak (nM)	ETP (nM*min)
FVIII 5% (baseline)	2.7	10.2	40	598
Mab (positive control)	2.7	8.2	40	700
DMSO (negative control)	2.7	6	34	199
Compd **1**, 0.0125 μM	2.7	5.9	36	202
Compd **1**, 0.125 μM	2.8	6.3	34	203
Compd **1**, 1.25 μM	3	7	23	161
Compd **1**, 12.5 μM	4.6	12	7	93
Compd **1**, 125 μM	17	29	6.5	117
Compd **29**, 0.0125 μM	2.8	6.1	31	184
Compd **29**, 0.125 μM	2.7	6.2	35	213
Compd **29**, 1.25 μM	2.7	6.3	36	237
Compd **29**, 12.5 μM	3	7.8	39	362
Compd **29**, 125 μM	5	15	26	517

ETP = endogenous thrombin potential, FVIII = coagulation factor VIII, Mab = inhibitory monoclonal antibody towards APC, DMSO = dimethyl sulfoxide, Compd = compound.

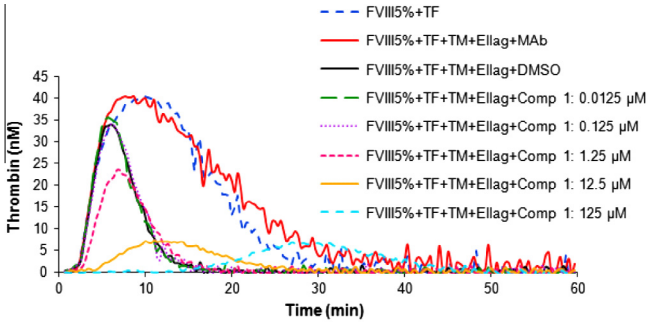


Figure 4. Thrombin generation curves showing the effects of increasing concentrations of compound **1** in the modified CAT method (0.0125 μM of compound **1** was also tested without effect (not depicted)), compared to level of thrombin generation without APC activation (FVIII 5% + TF), positive control (FVIII 5% + TF + TM + Ellag + MAb), and negative control (FVIII5% + TF + TM + Ellag + DMSO).

(CAT) method most likely due to a higher selectivity against all relevant coagulation factors. These data indicate that at least a 100-fold selectivity over other serine proteases in the coagulation cascade will be required for an effective APC inhibitor.

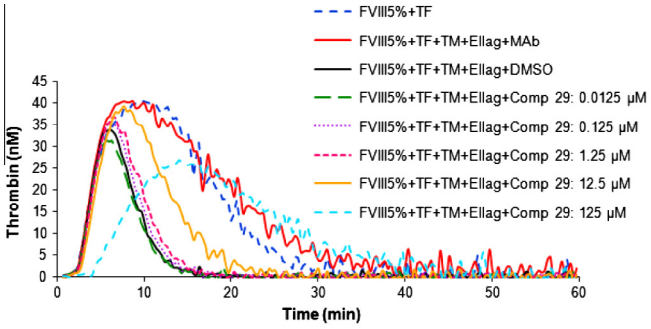


Figure 5. Thrombin generation curves showing the effects of increasing concentrations of compound **29** in the modified CAT method, compared to level of thrombin generation without APC activation (FVIII 5% + TF), positive control (FVIII 5% + TF + TM + Ellag + MAb), and negative control (FVIII 5% + TF + TM + Ellag + DMSO).

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Supplementary data

Supplementary data (screening assays for enzyme inhibition and the CAT method) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.12.094.

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General experimental procedure: **Step a** (see Scheme 1): To para-nitrophenyl Wang carbamate resin in a plastic syringe equipped with a frit was added amidine **3** (3.0 eq.). To the solids was added DIPEA (9.0 equiv) in DMF (10 mL/g resin) and the resulting slurry was agitated at rt for 18 h. The resin was filtered and rinsed with 6 × DMF and then with 3 × (1 × DCM + 1 × MeOH). A qualitative reaction completion check was performed by treating an aliquot of the washed resin with 1-methylpiperazine to look for any yellow coloration, in which case the reaction was run a second time. The resin was dried under vacuum and engaged in the next step.
Step b: The resin was washed with 3 × DMF and swelled in DMF before TBAF in THF (1M ; 10 equiv) was added. The resulting slurry was then agitated at rt for 7 h. The resin was filtered and washed with 6 × DMF and then with 3 × (1 × DCM + 1 × MeOH). The resin was then dried under vacuum and engaged in the next step.
Step c: To resin **4** was added Fmoc-D,L-homo-[ala-4-pip(N-Boc)] (1.5 equiv),

TBTU (1.5 equiv), HOBT (1.5 equiv), and finally DIPEA (3.0 equiv) in DMF. The reaction slurry was shaken at rt for 3.5 h. The resin was filtered and rinsed with 6 x DMF and then with 3 × (1 × DCM + 1 × MeOH). Completion of the coupling was confirmed by Kaiser test (Kaiser, E.; Colescot, R. L.; Bossinge, C. D.; Cook, P. I. *Anal. Biochem*. **1970**, *34*, 595) on a few beads. The resin was dried under vacuum for 16 h and engaged in the next step.

Step d: The resin was washed with 3 × DMF and subsequently treated at rt with a solution of DMF/1M TBAF in THF) 4:1 (v/v) for 3 × 2 minutes. The resin was then washed thoroughly with 6 × DMF, 2 × (DMF/H_2O 1:1 (v/v), 2 × H_2O, 2 × (DMF/H_2O 1:1 (v/v), 2 × DMF and finally 3 × (1 × DCM + 1 × MeOH). The resin was dried under vacuum for 2 h and engaged in the next step.

Step e: To the resin was added **13** (1.5 equiv), TBTU (1.5 equiv), HOBT (1.5 equiv), and finally DIPEA (3 equiv) in DMF. The reaction slurry was shaken at rt for 4 h. The resin was filtered and rinsed with 6 × DMF and then with 3 × (1 × DCM + 1 × MeOH). Completion of the coupling was confirmed by Kaiser test on a few beads. The resin was dried under vacuum for 16 h and engaged in the next step.

Step f: The resin was treated with TFA/DCM/water 49:49:2 for 1 h. The resin was filtered and the treatment was repeated once. The resin was then rinsed thoroughly with cycles of MeCN and DCM. The combined solvent fractions were concentrated to give a crude which was purified by preparative HPLC (Kromasil C8, 10 μm, 250 × 21.2 mm ID, flow 20 mL/min., detection by UV at 254 nm) using a gradient of solvent A: 95% 0.1 M ammonium acetate/5% MeCN in solvent B: MeCN. Relevant fraction were pooled and freeze-dried. Yield of **29** as a mixture of inseparable epimers as a monoacetate salt: 0.014 g (19% yield from para-nitrophenyl Wang carbamate resin). ^{1}H NMR (400 MHz, D_2O) δ 0.65–1.16 (m, 11H), 1.17–1.34 (m, 8H), 1.34–1.6 (m, 15H), 1.6–1.91 (m, 16H), 2.86 (t, *J* = 12.8 Hz, 4H), 2.96 (d, *J* = 16.1 Hz, 1H), 3.08 (d, *J* = 16.1 Hz, 1H), 3.13 (d, *J* = 16.4 Hz, 1H), 3.19 (d, *J* = 16.3 Hz, 1H), 3.30 (d, *J* = 12.6 Hz, 4H), 3.45–3.6 (m, 2H), 4.18 (dd, *J* = 5.2, 9.4 Hz, 1H), 4.27 (t, *J* = 7.4 Hz, 1H), 4.34 (d, *J* = 15. 9 Hz, 2H), 4.41 (d, *J* = 15.9 Hz, 1H), 4.42 (d, *J* = 15.9 Hz, 1H), 7.35 (d, *J* = 8.1 Hz, 2H), 7.38 (d, *J* = 8.1 Hz, 2H), 7.61 (d, *J* = 8.1 Hz, 2H), 7.65 (d, *J* = 8.1 Hz, 2H). HRMS $[M+H]^+$ calcd for $C_{28}H_{44}N_6O_4$: 529.3424, found 529.3445.

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