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Optimization of a coagulation factor VIIa inhibitor found in factor Xa inhibitor library☆

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Abstract—An inhibitor of the complex of factor VIIa and tissue factor (fVIIa/TF), 2-substituted-4-amidinophenylpyruvic acid **1a**, was structurally modified with the aim of increasing its potency and selectivity. The lead compound **1a** was originally found in our factor Xa (fXa) inhibitor library on the basis of structural similarity of the primary binding sites of fVIIa and fXa. The design was based on computational docking studies using the extracted active site of fVIIa. Compound **1j** was found to inhibit factor VIIa/TF at nanomolar concentration with improved selectivity versus fXa and thrombin and it preferentially prolonged the clotting time in the TF-dependent extrinsic pathway.

1. Introduction

Tissue factor (TF) is a membrane-bound protein and does not normally circulate in plasma. Damage to the normal integrity of the vascular system or activation of monocytes or endothelial cells results in exposure of TF to the blood. Then TF forms a complex with factor VIIa (fVIIa) to activate factor IX to factor IXa and factor X to factor Xa, which in turn activates prothrombin to thrombin. Active thrombin then cleaves fibrinogen to fibrin, which forms the structural basis of all blood clots. The fVIIa/TF complex is recognized as the primary initiator of blood coagulation in vivo.[2]

Although tremendous progress has been made in the discovery of potent and selective inhibitors of thrombin and factor Xa as anticoagulant drugs,[3] less effort has been focused on the discovery of inhibitors of fVIIa/TF. In recent years, however, studies using anti-factor VIIa antibodies,[4] active-site-inhibited factor VIIa,[5] and recombinant anticoagulant tissue factor pathway inhibitor (TFPI)[6] have yielded promising results. Accordingly, there is increasing interest in the generation of potent and selective small-molecular factor VIIa inhibitors as candidates for novel anticoagulants.[7]

Because fVIIa is a trypsin-like serine protease, like fXa, we began our search by screening our library of fXa inhibitors. From this library, we found that **1a** has potent factor VIIa/TF-inhibitory activity. This compound showed strong inhibition of fVIIa/TF ($K_i = 64$ nM), although it also inhibited fXa ($K_i = 9.2$ nM) and thrombin ($K_i = 120$ μM). Herein, we describe the structure-based design, synthesis, and in vitro evaluation of **1a** analogues with the aim of increasing the potency and selectivity for inhibition of fVIIa/TF (Chart 1).

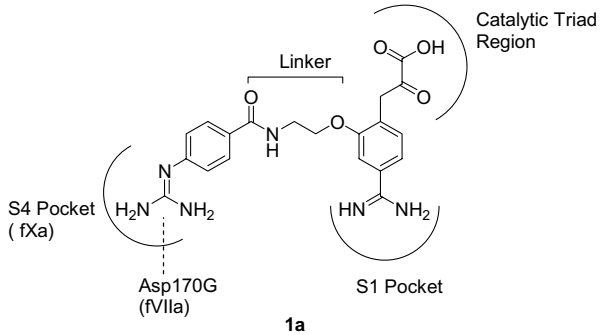

Chart 1.

Keywords: Factor VIIa; Inhibitor.
☆ See Ref. 1.
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2. Inhibitor design based on computational modeling

A model of the lead compound **1a** bound to fVIIa (1DAN[8]) was computationally constructed (Fig. 1) by utilizing the same method as described in our paper on fXa inhibitors,[9] and also the crystal structures of compounds related to trypsin. There is a high degree of structural similarity of the primary binding site, S1 pocket and catalytic triad region among fVIIa, fXa, and trypsin. In the calculated docking mode, the amidino group of **1a** hydrogen bonds with the carboxyl group of Asp 189[10] located at the bottom of the S1 pocket, the guanidino group hydrogen bonds with Asp170G, forming a part of the S2/S3 pocket, which is constructed from Thr99, Pro170I, Asp170G, and Trp215-Gly216-Gln217, and the pyruvic acid moiety interacts with the catalytic triad region composed of the catalytic triad (His 57, Asp102, and Ser195) and the oxyanion hole (Gly 193 and Ser195).

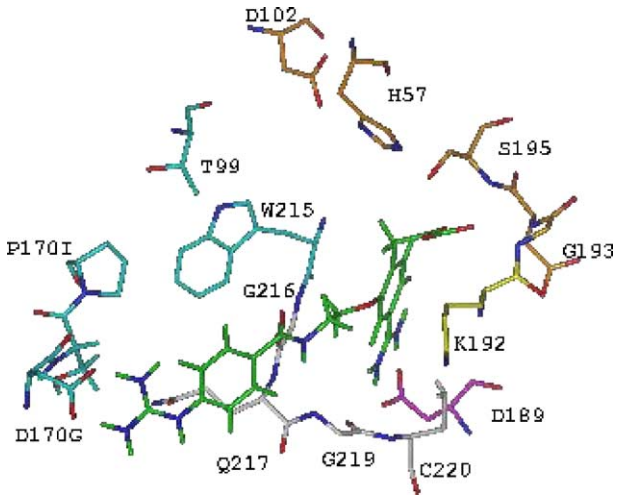


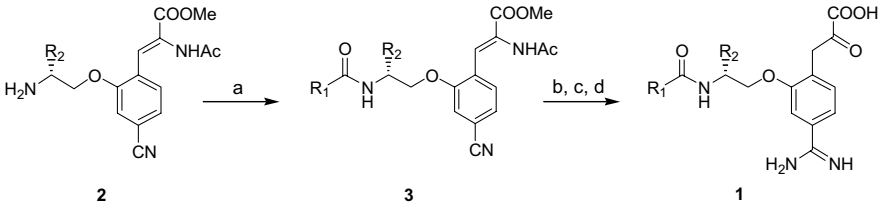
Figure 1. Calculated docking mode of **1a** in factor VIIa (1DAN). The docking calculation was done assuming formation of a noncovalent Michaelis–Menten complex before the formation of a plausible covalent complex, as observed in the crystal structure of APPA–trypsin complex. The active site region of 1DAN was extracted after removal of the complexed ligand. Both the hydrogen and carbon atoms of the inhibitor **1a** are colored green; Asp189 of the bottom of the S1 pocket is shown in magenta; Ser 195, His 57, and Asp 102 forming the catalytic triad and Gly193 forming the oxy-anion hole are in orange; Lys 192 at the top of the S1 pocket is in yellow and Thr 99, Pro 170I, Asp 170G, and Trp215 forming the S2/S3 pocket are in cyan. In this docking mode the amidino group of **1a** makes hydrogen bonds with both the carboxyl group of Asp 189 and the carbonyl group of Gly 219 and the pyruvic acid interacts with the catalytic triad and the oxy-anion hole. The amide NH and the guanidino group of **1a** also make hydrogen bonds with the carbonyl group of Gly 216 and the carboxyl group of Asp 170G, respectively.

The docking calculation was done assuming formation of a noncovalent Michaelis–Menten complex before the formation of a plausible covalent complex.[11] The interaction between the pyruvic acid moiety and the catalytic triad region in this calculation will be actually enhanced by the formation of covalent stable tetrahedral complex between the carbonyl carbon of the pyruvic acid and the Oγ of Ser 195, as observed in the crystal structure of APPA (4-amidinophenylpyruvic acid)–trypsin complex.[12] APPA derivatives such as **1a**will be reversible, covalent inhibitors.

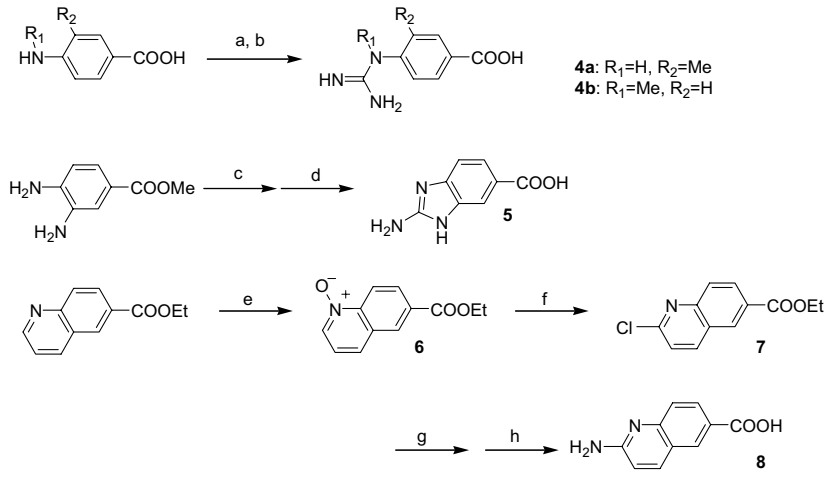
On the basis of a visual inspection of this model together with the previous work[9] on this class of inhibitors, we expected that greater selectivity versus thrombin and fXa might be achieved by means of the following structural modifications. (1) Introduction of an *R*-carboxyethyl moiety into the linker part of **1a** would improve the selectivity against thrombin. The carboxylic acid group of the *R*-carboxyethyl moiety would electrostatically repel the side-chain carboxyl group of Glu 192 of thrombin, but would interact favorably (electrostatic attraction) with the side-chain amino group of Lys 192 of fVIIa. (2) Replacement of the guanidinophenyl group of **1a** might improve the selectivity against fXa. The guanidino group of **1a** would contribute to the affinity to fXa, as well as fVIIa, probably owing to hydrogen-bonding and cation–π stabilization with the S4 region of fXa. We considered that torsion-angle-restricted analogs of the 4-guanidinophenyl group that match the optimal torsion angle for fVIIa binding, which is probably not optimal for fXa, could enhance the affinity for fVIIa and the selectivity over fXa.

3. Chemistry

The 2-substituted 4-amidinophenylpyruvic acids **1** were conveniently prepared from optionally substituted benzoic acids and the key intermediate **2** (Scheme 1). Amine **2** was acylated with various carboxylic acids to give the corresponding amide **3**. The nitrile group of **3** was converted into an amidino group, and the target molecules **1** were obtained by simultaneous hydrolysis of both the enamide and the ester of the 2-acetamideacrylate moiety.



Scheme 1. Synthesis of 2-substituted-4-amidinophenylpyruvic acid. Reagents and conditions: (a) R_1–COOH, EDC, HOBt, DMF; (b) HCl, EtOH, dioxane; (c) $(NH_4)_2CO_3$, EtOH; (d) HCl aq, 80 °C.



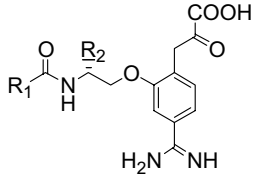
Scheme 2. Synthesis of benzoic acids. Reagents and conditions: (a) HCl, dioxane; (b) NH_2CN, EtOH, 50 °C; (c) BrCN, water; (d) 3 M HCl, 65 °C; (e) *m*-CPBA, CH_2Cl_2; (f) $POCl_3$, CH_2Cl_2, reflux; (g) (i) phenol, (ii) ammonium acetate; (h) 6 M hydrochloric acid 80 °C.

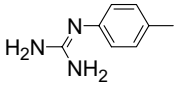
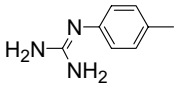
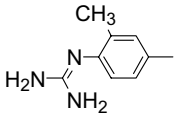
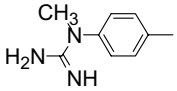
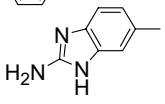
Some benzoic acids required for novel R_1 groups were prepared as shown in Scheme 2.

4. Biological results and discussion

The data in Table 1 demonstrate that the affinity for fVIIa/TF of **1a** (fVIIa: 64 nM, thrombin: 120 μM) was slightly enhanced and the selectivity versus thrombin was much improved by the incorporation of the *R*-carboxyethyl moiety on the linker part (**1b** fVIIa: 41 nM, thrombin: >150 μM). The earlier results of R_1 modified analogues indicate that the incorporation of *R*-carboxy*methyl* moiety also improve selectivity versus thrombin, but not enhance the affinity for fVIIa/TF unlike the *R*-carboxyethyl moiety (data not shown). Therefore we adopted the *R*-carboxyethyl moiety as the substituent on the linker part.

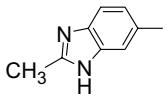
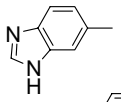
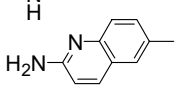
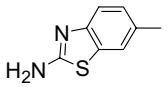
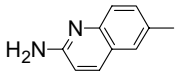
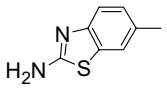
Table 1. Enzyme-inhibitory activities of benzamidine compounds



Compound	R_1	R_2	K_i (μM)			Selectivity	
			fVIIa/TF	fXa	Thrombin	Over fXa[a]	Over thrombin[b]
1a		–H	0.064	0.0092	120	0.14	1900
1b		$-CH_2CH_2COOH$	0.041	0.0058	>150	0.1	>3700
1c		$-CH_2CH_2COOH$	0.41	0.029	>150	0.1	>370
1d		$-CH_2CH_2COOH$	0.26	0.073	>150	0.3	>590
1e		$-CH_2CH_2COOH$	0.26	1.8	7.5	7	29
1f		$-CH_2CH_2COOH$	0.026	4.6	>150	180	>5900

(*continued on next page*)

Table 1 (*continued*)

Compound	R_1	R_2	K_i (μM)			Selectivity	
			fVIIa/TF	fXa	Thrombin	Over fXa[a]	Over thrombin[b]
1g		$-CH_2CH_2COOH$	0.032	2.9	>150	90	>4700
1h		$-CH_2CH_2COOH$	0.032	2.9	30	90	930
1i		$-CH_2CH_2COOH$	0.016	1.2	>150	72	>9300
1j		$-CH_2CH_2COOH$	0.0010	1.8	>150	1800	>150,000
1k		–H	0.041	0.73	47	18	1200
1l		–H	0.0032	1.8	60	570	19,000

[a] (K_i of fXa)/(K_i of fVIIa/TF).
[b] (K_i of thrombin)/(K_i of fVIIa/TF).

The guanidinophenyl moieties of **1a** and **1b** appear to occupy similar sites of fVIIa and fXa. Therefore, to improve selectivity over fXa, the 4-guanidinophenyl group of **1b** was replaced with torsion-angle-restricted analogous groups as described for **1a** in the design section.

In the methyl-substituted guanidinophenyl groups (**1c** and **1d**), the guanidino group and the adjacent phenyl ring would be preferentially perpendicular to each other because of the steric constraint between the methyl group and hydrogens. These compounds show lower affinity than the parent compound for fVIIa/TF, as well as fXa. Based on the affinity of **1e** (R_1 = Ph, 0.26 μM), it is clear that the guanidino group of **1b** (41 nM) greatly enhances the affinity for fVIIa, whereas the guanidino group of **1c** (0.41 μM) and **1d** (0.26 μM) does not improve the affinity.

On the other hand, the 2-aminobenzimidazolyl group (**1f**), which mimics the planar conformation of the 4-guanidinophenyl group, shows equipotent fVIIa/TF inhibitory activity (26 nM) and enhanced selectivity against fXa (selectivity over fXa = 180 compared to 0.1). The rigidity of the fused ring might contribute to the reduction of the fXa-inhibitory activity, as we had hoped. However, it should be noted that the reduction of the basicity of the R_1 moiety might also contribute to the selectivity over fXa. The pK_a of 2-aminobenzimidazole is 7.5[13] and that of guanidinobenzene is 10.8.

Compound **1f** was considered as a new lead compound with potent fVIIa/TF-inhibitory activity and selectivity against fXa and thrombin. In an attempt to further improve the affinity for fVIIa, groups analogous to the 2-aminobenzimidazolyl group were evaluated as the R_1 moiety. First, the 2-amino substituent was replaced with a methyl group (**1g**) or a hydrogen atom (**1h**). These compounds are equipotent to **1f**, and that finding suggests that protonation of these fused-type R_1 moieties would not be required for fVIIa binding because the basicity of 2-methylbenzimidazole (pK_a = 6.2) and benzimidazole (5.5) is low.

Next, the 1-nitrogen atom of **1f** was replaced with an ethylene moiety (**1i**) or a sulfur atom (**1j**). This ring expansion approach gave fruitful results. Compound **1i** shows slightly enhanced fVIIa-inhibitory activity (K_i = 16 nM), and the high selectivity versus thrombin and fXa is maintained, while **1j** showed the strongest binding affinity to fVIIa/TF (K_i = 1.0 nM) and the best selectivity versus fXa and thrombin (selectivity over fXa = 1800, over thrombin >150,000).

Some of the potent novel R_1 moieties described above were introduced into **1a**, the compound without *R*-carboxyethyl substitution on the linker part (**1k**, **1l**). The R_1 replacement had a similar effect to that in the case of **1b**, the compound with *R*-carboxyethyl substitution (see **1i**, **1j**), indicating that the *R*-carboxyethyl moiety and R_1 moieties have predominantly independent effects from the viewpoint of structure–activity relationships.

The in vitro anticoagulant activities of **1i** (K_i = 16 nM), **1l** (3.2 nM), and **1j** (1.0 nM) are shown in Table 2. The assays were performed with high drug concentration to show clearly the differences between the prolongations of the prothrombin time (PT) and the activated partial thromboplastin time (aPTT). The differences become clearer as the drug concentration is higher in our assay condition. Inhibition of the TF-dependent extrinsic clotting pathway was evaluated in terms of the prolongation of the PT. The PT prolongation correlated with fVIIa-inhibitory activity, and this indicates that the PT prolongation was mainly owing to fVIIa inhibi-

Table 2. Clotting time prolongation by fVIIa inhibitors

Compound	PT[a]	aPTT[b]
1i	2300	360
1l	3100	410
1j	4500	370
9[c]	1100	990

[a] Prothrombin time (% of control at the drug concentration of 32 μM).
[b] Activated partial thromboplastin time (% Of control at the drug concentration of 32 μM).
[c] fXa-selective inhibitor. See Ref. 9.

tion. The prolongation of the TF-independent intrinsic pathway was much smaller, as judged from the prolongation of the aPTT. From a comparison with a selective fXa inhibitor 3-[2-[2-[1-(4-pyridyl)piperidine-4-carbonylamino]ethoxy]-4-amidinophenyl]propionic acid[9] (**9**) (K_i: fVIIa/TF = 16 μM, fXa = 43 nM, thrombin >1500 μM), we suppose that these fVIIa inhibitors preferentially inhibit the extrinsic pathway while the selective fXa inhibitor **9** inhibits both pathways and prolongs both PT and aPTT, because fXa is located at the convergence of the intrinsic and extrinsic pathways.

5. Conclusion

The optimization of a lead from our fXa inhibitor library led to the fVIIa/TF inhibitor **1j**, which is active at nanomolar concentrations. This compound possesses high selectivity for fVIIa/TF over fXa and thrombin, and preferentially inhibits the TF-dependent extrinsic clotting pathway.

Potential advantages of *small-molecular* fVIIa/TF inhibitors as compared with fXa or thrombin inhibitors in the treatment of thrombotic disorders are not well established.[14] We intend to examine the separation of antithrombotic efficacy and bleeding side effect by using our selective fVIIa/TF inhibitor **1j** in our intravenous studies in animal models.

6. Experimental section

6.1. Molecular modeling

The coordinate sets for the protein structures used in this investigation were 1DAN[8] for fVIIa/TF, 1HCG[15] for fXa and 1DWC[16] for thrombin. Compound **1a** was docked into the fVIIa active site using an in-house docking program.[9,17]

6.2. Chemistry

Work-up included drying over magnesium sulfate, filtering, and concentrating in vacuo. Column chromatography of intermediates was performed using silica gel (Merck, particle size 0.063–0.200 mm). Final products were purified by preparative reverse-phase HPLC, which was performed on a Waters 600 system with a C-18 reverse-phase column (Inertsil® ODS-3, GL Sciences Inc.) using a mixture of acetonitrile and water, both of which contained 0.1% trifluoroacetic acid. Fractions containing the desired material were concentrated and lyophilized to obtain the final products as a white solid. NMR spectra were recorded on a Varian EM-390 at 300 MHz. Keto–enol tautomerism was observed in the NMR spectra of all amidinophenylpyruvic acids (**1**). Mass spectra (ESI) were measured on JEOL JMS-DX300 instruments. Where analyses are indicated only by the symbols of the elements, the results obtained were within 0.4% of the theoretical values. Reagent abbreviations: HOBT, 1-hydroxybenzotriazole; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl; TEA, triethylamine; TFA, trifluoroacetic acid; DMF, dimethylformamide.

6.3. 3-[4-Cyano-2-[2-(4-guanidinobenzoylamino)-ethoxy]phenyl]-2-acetamidoacrylic acid methyl ester trifluoroacetate (3a)

A mixture of 3-[4-cyano-2-(2-aminoethoxy)phenyl]-2-acetamidoacrylic acid methyl ester HCl[9] (**2a**) (3.19 g, 9.39 mmol), 4-guanidinobenzoic acid (2.03 g, 9.39 mmol), triethylamine (1.54 mL, 11.3 mmol), and bromotrispyrrolidinophosphonium hexafluorophosphate (5.25 g, 11.3 mmol) in DMF was stirred overnight. The reaction mixture was concentrated in vacuo, and the residue was purified by reverse-phase HPLC to give **3a** (3.32 g, 61%): ^{1}H NMR (CD_3OD) δ 2.03 (3H, s), 3.76 (3H, s), 3.80 (2H, t), 4.31 (2H, t), 7.30–7.48 (5H, m), 7.61 (1H, d), 7.90 (2H, d); MS (ESI) 465 (MH^+).

6.4. 3-[4-Amidino-2-[2-(4-amidinobenzoylamino)ethoxy]-phenyl]-2-oxopropionic acid bistrifluoroacetate (1a)

A mixture of **3a** (2.0 g, 3.5 mmol), 4.0 M HCl solution in 1,4-dioxane (40 mL) and ethanol (20 mL) was stirred for 4 days and concentrated in vacuo. To the residue, ethanol (10 mL) and ammonium carbonate (1.66 g, 17.3 mmol) were added. The mixture was stirred overnight, and concentrated in vacuo. The residue was diluted with 6 N HCl (40 mL). The mixture was stirred at 80 °C, and concentrated in vacuo. Purification by reverse-phase HPLC gave **1a** (960 mg, 45%): ^{1}H NMR (DMSO-d_6) δ 3.61–3.73 (2H, m), 4.13–4.30 (2H, m), 6.81 (1H, s), 7.28–7.46 (5H, m), 7.63 (4H, br s), 7.92 (2H, d), 8.33 (1H, d), 8.79 (1H, t), 9.03 (2H, br s), 9.27 (2H, br s) 10.02 (1H, br s); MS (ESI) 427 (MH^+). Anal. Calcd for $C_{20}H_{22}N_6O_5 \cdot 2.0TFA \cdot 1.5H_2O$: C, 42.29; H, 3.993; N, 12.33. Found: C, 42.35; H, 4.06; N, 11.98.

6.5. (4*R*)-4-*t*-Butoxycarbonylamino-5-hydroxypentanoic acid benzyl ester (10)

To the mixture of N-*t*-butoxycarbonyl-D-glutamic acid γ-benzyl ester (10.9 g, 32.0 mmol), N-methylmorpholine (3.29 g, 32.5 mmol), and THF (100 mL), ethyl chloroformate (3.08 mL, 32 mmol) was added at 0 °C. The reaction mixture was stirred and then filtered to remove precipitates. A small amount of crushed ice and $NaBH_4$ (1.23 g, 32.5 mmol) in water was added to the filtrate. The mixture was stirred for 3 min, diluted with 0.5 N HCl and stirred for 1 h. EtOAc was added to the

solution, and then the organic layer was washed with saturated $NaHCO_3$ and saturated NaCl, and worked up to give **10** (10.9 g): ^{1}H NMR ($CDCl_3$) δ 1.45 (9H, s), 1.75–2.00 (2H, m), 2.40–2.50 (2H, m), 3.50–3.70 (3H, m), 4.80 (1H, br), 5.10 (2H, s), 7.35 (5H, s).

6.6. (4*R*)-4-*t*-Butoxycarbonylamino-5-chloropentanoic acid benzyl ester (11)

To a mixture of **10** (10.8 g, 33.4 mmol) and triethylamine (3.4 g, 33.4 mmol) in dichloromethane, methanesulfonyl chloride (3.83 g, 33.4 mmol) was added at 0 °C. The reaction mixture was washed with 1 N HCl and saturated NaCl, and worked up to give crude (4*R*)-4-*t*-butoxycarbonylamino-5-methanesulfonyloxypentanoic acid benzyl ester. A mixture of the crude product and lithium chloride (7.2 g, 170 mmol) in DMF (50 mL) was stirred at 50 °C overnight and concentrated in vacuo. The residue was diluted with EtOAc, washed with water and saturated NaCl, and worked up. The residue was washed with a mixture of EtOAc and hexane to give **11** (8.29 g, 73%): ^{1}H NMR ($CDCl_3$) δ 1.40 (9H, s), 1.90–2.00 (2H, m), 2.40–2.50 (2H, m), 3.55–3.70 (2H, m), 3.85–4.00 (1H, m), 4.70 (1H, br), 5.10 (2H, s), 7.35 (5H, s).

6.7. (4*R*)-4-*t*-Butoxycarbonylamino-5-(5-cyano-2-iodophenoxy)pentanoic acid benzyl ester (12)

A mixture of **11** (8.29 g, 24.3 mmol), 3-hydroxy-4-iodobenzonitrile (6.9 g, 28.0 mmol), K_2CO_3 (6.9 g, 50 mmol), and KI (4.15 g, 25 mmol) in DMF (80 mL) was stirred at 70 °C for 3 days. The reaction mixture was concentrated in vacuo, and the residue was purified by chromatography (EtOAc/hexane) to give **12** (5.72 g, 43%): ^{1}H NMR ($CDCl_3$) δ 1.45 (9H, s), 2.10 (2H, br), 2.55 (2H, t), 4.05 (3H, br), 4.90 (1H, br), 5.15 (2H, s), 6.95 (1H, s), 7.00 (1H, d), 7.35 (5H, s), 7.90 (1H, d).

6.8. 2-Acetamido-3-[2-[(2*R*)-4-benzyloxycarbonyl-2-(*t*-butoxycarbonylamino)butoxy]-4-cyanophenyl]acrylic acid methyl ester (13)

A mixture of **12** (5.72 g, 10.4 mmol), methyl 2-acetamidoacrylate (3.0 g, 21 mmol), tris(2-methylphenyl)phosphine (2.0 g, 6.6 mmol), palladium acetate (II) (270 mg, 1.1 mmol), and triethylamine (3.54 g, 35 mmol) in DMF (50 mL) was stirred at 100 °C for 5 h. The reaction mixture was concentrated in vacuo, and the residue was extracted with EtOAC, washed by the usual method, and worked up. The crude product was purified by chromatography (EtOAc/hexane) to give **13** (2.6 g, 44%): ^{1}H NMR ($CDCl_3$) δ 1.40 (9H, s), 1.80–2.05 (2H, m), 2.05 (3H, br), 2.50 (2H, t), 3.85 (3H, s), 4.05 (3H, br), 4.85 (1H, d), 5.10 (2H, s), 7.12 (1H, s), 7.23 (1H, d), 7.31–7.48 (8H, m).

6.9. 2-Acetamido-3-[2-[(2*R*)-4-benzyloxycarbonyl-2-aminobutoxy]-4-cyanophenyl]acrylic acid methyl ester HCl (2b)

A mixture of **13** (5 g, 8.8 mmol) and 4.0 M HCl solution in 1,4-dioxane (50 mL) was stirred for 2 h, and concentrated in vacuo to give **2b** (4.5 g): ^{1}H NMR (DMSO-d_6) δ 1.90–2.10 (5H, m), 2.65 (2H, t), 3.60 (1H, br), 3.70 (3H, s), 4.15–4.35 (2H, m), 5.10 (2H, s), 7.30 (1H, s), 7.35 (5H, s), 7.48 (1H, d), 7.60 (1H, s), 7.70 (1H, d), 8.40 (3H, br), 9.75 (1H, s).

6.10. 2-Acetamido-3-[2-[(2*R*)-4-benzyloxycarbonyl-2-(4-guanidinobenzoylamino)butoxy]-4-cyanophenyl]acrylic acid methyl ester trifluoroacetate (3b)

A mixture of **2b** (150 mg, 0.3 mmol), 4-guanidinobenzoic acid HCl (65 mg, 0.3 mmol), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide HCl (EDC HCl) (58 mg, 0.3 mmol), triethylamine (61 mg, 0.6 mmol), 1-hydroxybenzotriazole (41 mg, 0.3 mmol), and DMF (5 mL) was stirred overnight. The reaction mixture was concentrated in vacuo, and the residue was purified by reverse-phase HPLC to give **3b** (140 mg, 63%): MS (ESI) 627 (MH^+).

6.11. (4*R*)-4-[(4-Guanidinobenzoyl)amino]-5-[5-amidino-2-(2-carboxy-2-oxoethyl)phenoxy]pentanoic acid bistrifluoroacetate (1b)

A mixture of **3b** (140 mg, 0.19 mmol), 4.0 M HCl solution in 1,4-dioxane (5 mL), and ethanol (1 mL) was stirred for 2 days, and concentrated in vacuo. To the residue, ammonium carbonate (80 mg) and ethanol (5 mL) were added. The mixture was stirred overnight and concentrated in vacuo. To the residue, 3 N hydrochloric acid was added. The mixture was stirred at 80 °C for 2 h, and concentrated in vacuo. The residue was purified by reverse-phase HPLC to give **1b** (41 mg, 30 %): ^{1}H NMR (DMSO-d_6) δ 1.80–2.10 (2H, m), 2.25–2.40 (2H, m), 4.00–4.40 (3H + 2H of keto form, m), 6.80 (1H, s, enol form), 7.25–7.50 (4H + 1H of keto form, m), 7.65 (4H, br s), 7.95 (2H, d), 8.35 (1H, d, enol form), 8.45 (1H, d), 9.10 (2H, br), 9.25 (2H, br), 9.75 (1H, br, enol), 10.10 (1H, s); MS (ESI) 499 (MH^+). Anal. Calcd for $C_{23}H_{26}N_6O_7 \cdot 2.0TFA \cdot 2H_2O$: C, 42.52; H, 4.229; N, 11.02. Found: C, 42.32; H, 4.59; N, 11.35.

6.12. 2-Acetamido-3-[2-[(2*R*)-4-benzyloxycarbonyl-2-benzoylamino-butoxy]-4-cyanophenyl]acrylic acid methyl ester (3e)

A mixture of **2b** (90 mg, 0.18 mmol), benzoyl chloride (28 mg, 0.2 mmol), triethylamine (61 mg, 0.6 mmol), and DMF (5 mL) was stirred overnight. The reaction mixture was concentrated in vacuo, and the residue was diluted with EtOAc. The organic layer was washed with 1 N HCl, saturated $NaHCO_3$, and saturated NaCl, and worked up. The residue was purified by chromatography to give **3e** (90 mg, 88%): MS (ESI) 570 (MH^+).

6.13. (4*R*)-4-Benzoylamino-5-[5-amidino-2-(2-carboxy-2-oxoethyl)phenoxy]pentanoic acid trifluoroacetate (1e)

The title compound was prepared from **3e** as described for **1b**: ^{1}H NMR (DMSO-d_6) δ 1.80–2.10 (2H, m), 2.05–2.45 (2H, m), 4.00–4.50 (3H + 2H of keto form, m), 6.80 (1H, s, enol form), 7.30–7.60 (5H + 1H of keto

form, m), 7.85 (2H, d), 8.30 (1H, d, enol form), 8.40 (1H, d), 9.00 (2H, br), 9.25 (2H, br), 9.75 (1H, br, enol); MS (ESI) 442 (MH^+). Anal. Calcd for $C_{22}H_{23}N_3O_7 \cdot 1.0TFA \cdot 0.8H_2O$: C, 50.58; H, 4.527; N, 7.373. Found: C, 50.25; H, 4.79; N, 7.76.

6.14. 4-Guanidino-3-methylbenzoic acid trifluoroacetate (4a)

A mixture of 4-amino-3-methylbenzoic acid (2 g, 13.2 mmol) and 4 M HCl solution in dioxane was stirred for 30 min and concentrated in vacuo. To the residue, ethanol (30 mL), and cyanamide (1.68 g, 40 mmol) was added, and the mixture was stirred at 50 °C overnight. The reaction mixture was concentrated in vacuo and purified by reverse-phase HPLC to give **4a** (210 mg, 5%): ^{1}H NMR (DMSO-d_6) δ 2.25 (3H, s), 7.35 (1H, d), 7.50 (4H, br), 7.85 (1H, d), 7.95 (1H, s), 9.65 (1H, s).

6.15. (4*R*)-4-[(4-Guanidino-3-methylbenzoyl)amino]-5-[5-amidino-2-(2-carboxy-2-oxoethyl)phenoxy]pentanoic acid bistrifluoroacetate (1c)

Part A. Compound **2b** (90 mg, 0.18 mmol) and **4a** (50 mg, 0.16 mmol) was treated as described in **3b** to give 2-acetamido-3-[2-[(2*R*)-4-benzyloxycarbonyl-2-(4-guanidino-3-methylbenzoylamino)butoxy]-4-cyanophenyl]acrylic acid methyl ester trifluoroacetate (**3c**).

Part B. The product of Part A was treated as described for **1b** to give **1c** (13 mg, 11%): ^{1}H NMR (DMSO-d_6) δ 1.80–2.10 (2H, m), 2.25 (3H, s), 2.25–2.40 (2H, m), 4.00–4.30 (2H + 2H of keto form, m), 4.40 (1H, m), 6.80 (1H, s, enol form), 7.30–7.60 (8H + 1H of keto form, m), 7.75 (1H, d), 7.85 (1H, s), 8.30 (1H, d, enol form), 8.55 (1H, d), 9.15–9.52 (4H, m), 9.70–9.85 (1H + 1H of enol form, m); MS (ESI) 513 (MH^+). Anal. Calcd for $C_{24}H_{28}N_6O_7 \cdot 2.0TFA \cdot 2.0H_2O$: C, 43.30; H, 4.412; N, 10.82. Found: C, 43.65; H, 4.37; N, 10.91.

6.16. 4-(1-Methylguanidino)benzoic acid trifluoroacetate (4b)

The title compound was prepared from 4-(1-methylamino)benzoic acid as described for **4a**: ^{1}H NMR (DMSO-d_6) δ 3.30 (3H, s), 7.50 (2H, d), 7.55 (4H, br), 8.05 (2H, d).

6.17. (4*R*)-4-[(4-(1-Methylguanidino)benzoyl)amino]-5-[5-amidino-2-(2-carboxy-2-oxoethyl)phenoxy]pentanoic acid bistrifluoroacetate (1d)

The title compound was prepared from **4b** and **2b** as described for **3b** and **1b**: ^{1}H NMR (DMSO-d_6) δ 1.82–2.10 (2H, m), 2.25–2.43 (2H, m), 3.30 (3H, s), 4.07–4.30 (2H + 2H of keto form, m), 4.32–4.48 (1H, m), 6.78 (1H, s, enol form), 7.30–7.60 (8H + 1H of keto form, m), 8.00 (2H, d), 8.31 (1H, d, enol form), 8.55 (1H, d), 9.15–9.52 (4H, m), 9.70–9.82 (1H, br, enol); MS (ESI) 513 (MH^+). Anal. Calcd for $C_{24}H_{28}N_6O_7 \cdot 2.0TFA \cdot 2.0H_2O$: C, 43.30; H, 4.412; N, 10.82. Found: C, 43.55; H, 4.52; N, 10.85.

6.18. 2-Aminobenzimidazole–5-carboxylic acid HCl (5)

Part A. To a mixture of 3,4-diaminobenzoic acid methylester (300 mg, 1.8 mmol) and water (5 mL), a solution of 5 M cyanogen bromide in acetonitrile (0.5 mL) was added. The reaction mixture was stirred for 20 min, and filtered. Aqueous ammonia and EtOAc were added to the filtrate, and the organic layer was washed and worked up to give 2-aminobenzimidazole–5-carboxylic acid methyl ester: ^{1}H NMR (DMSO-d_6) δ 3.80 (3H, s), 6.50 (2H, br), 7.15 (1H, d), 7.55 (1H, d), 7.70 (1H, s), 10.90 (1H, br).

Part B. A mixture of the product of Part A and 3 M hydrochloric acid was stirred at 65 °C overnight, and concentrated in vacuo to give **5** (330 mg, 86%): ^{1}H NMR (DMSO-d_6) δ 7.45 (1H, d), 7.85 (1H, d), 8.80 (2H, s), 13.00 (1H, br).

6.19. (4*R*)-4-[(2-Aminobenzimidazole-5-carbonyl)amino]-5-[5-amidino-2-(2-carboxy-2-oxoethyl)phenoxy]pentanoic acid bistrifluoroacetate (1f)

The title compound was prepared from **5** and **2b** as described for **1b**: ^{1}H NMR (DMSO-d_6) δ 1.80–2.15 (2H, m), 2.25–2.50 (2H, m), 4.05–4.30 (3H + 2H of keto form, m), 6.80 (1H, s, enol form), 7.35–7.50 (3H + 1H of keto form, m), 7.75 (1H, d), 7.80 (1H, s), 8.30 (1H, d, enol form), 8.45 (1H, d), 8.65 (2H, s), 9.10 (2H, s), 9.25 (2H, s); MS (ESI) 497 (MH^+). Anal. Calcd for $C_{23}H_{24}N_6O_7 \cdot 2.0TFA \cdot 2.0H_2O$: C, 42.63; H, 3.975; N, 11.04. Found: C, 42.94; H, 4.22; N, 10.64.

6.20. 2-Aminoquinoline-6-carboxylic acid HCl (8)

Part A. A mixture of quinoline-6-carboxylic acid (4.9 g, 28.3 mmol), a solution of 4.0 M HCl in dioxane (40 mL), and ethanol (80 mL) was stirred at 70 °C overnight. The reaction mixture was concentrated in vacuo, and the residue was diluted with EtOAc, washed and worked up to give quinoline-6-carboxylic acid ethyl ester: ^{1}H NMR ($CDCl_3$) δ 1.45 (3H, t), 4.45 (2H, q), 7.45 (1H, dd), 8.15 (1H, d), 8.25–8.35 (2H, m), 8.60 (1H, s), 9.00 (1H, d).

Part B. To a mixture of the product of Part A and dichloromethane (80 mL), *m*-chloroperbenzoic acid (6.2 g, 36 mmol) was added at 0 °C. The reaction mixture was stirred at ambient temperature overnight, washed with 10% sodium sulfite, saturated $NaHCO_3$ and saturated NaCl, and worked up. To the residue, dichloromethane (70 mL) and phosphoryl chloride (35 mL) were added and the mixture was stirred at 50 °C overnight. The reaction mixture was concentrated in vacuo, and the residue was extracted with dichloromethane, washed and worked up. The crude product was purified with chromatography (EtOAc/hexane) to give 2-chloroquinoline-6-carboxylic acid ethyl ester (**7**) (1.87 g, 28%): ^{1}H NMR ($CDCl_3$)) δ 1.40 (3H, t), 4.45 (2H, d), 7.46 (1H, d), 8.06 (1H, d), 8.21 (1H, d), 8.34 (1H, d), 8.58 (1H, s).

Part C. A mixture of **7** (1.87 g, 8 mmol) and phenol (5.5 g) was stirred at 70 °C for 10 min. To the mixture, ammonium acetate (5.5 g) was added and the reaction mixture was stirred for 1 h. EtOAc and 1 N NaOH were added to the mixture, and the organic layer was washed with saturated NaCl and worked up. The crude product was purified with chromatography (EtOAc/hexane/ethanol) to give 2-aminoquinoline-6-carboxylic acid ethyl ester (330 mg, 19%): ^{1}H NMR ($CDCl_3$) δ 1.40 (3H, t), 4.40 (2H, q), 5.00 (2H, br), 6.75 (1H, d), 7.64 (1H, d), 7.95 (1H, d), 8.16 (1H, d), 8.37 (1H, s).

Part D. A mixture of the product of Part C and 6 M hydrochloric acid (30 mL) was stirred at 80 °C, and concentrated in vacuo. The residue was washed with EtOAc to give **8** (330 mg, 96%): ^{1}H NMR (DMSO-d_6) δ 7.16 (1H, d), 7.78 (1H, d), 8.23 (1H, d), 8.50 (1H, d), 8.55 (1H, s).

6.21. (4*R*)-4-[(2-Aminoquinoline-6-carbonyl)amino]-5-[5-amidino-2-(2-carboxy-2-oxoethyl)phenoxy]pentanoic acid bistrifluoroacetate (1i)

The title compound was prepared from **8** and **2b** as described for **3b** and **1b**: ^{1}H NMR (DMSO-d_6) δ 1.80–2.10 (2H, m), 2.30–2.50 (2H, m), 4.10–4.30 (2H + 2H of keto form, m), 4.45 (1H, br), 6.75 (1H, s, enol form), 7.10 (1H, d), 7.35–7.50 (2H + 1H of keto form, m), 7.65 (1H, d), 8.15 (1H, d), 8.25–8.45 (2H + 1H of enol form, d), 8.65 (1H, d), 8.90 (2H, br), 9.10 (2H, br), 9.25 (2H, br); MS (ESI) 508 (MH^+). Anal. Calcd for $C_{25}H_{25}N_5O_7{\cdot}2.0TFA{\cdot}1.33H_2O$: C, 45.85; H, 3.936; N, 9.220. Found: C, 45.49; H, 4.27; N, 9.57.

6.22. (4*R*)-4-[(2-Methylbenzimidazole-5-carbonyl)amino]-5-[5-amidino-2-(2-carboxy-2-oxoethyl)phenoxy]pentanoic acid bistrifluoroacetate (1g)

The title compound was prepared from 2-methylbenzimidazole-5-carboxylic acid and **2b** as described for **3b** and **1b**: ^{1}H NMR (DMSO-d_6) δ 1.85–2.15 (2H, m), 2.25–2.50 (2H, m), 2.75 (3H, s), 4.10–4.30 (2H + 2H of keto form, m), 4.45 (1H, br), 6.80 (1H, s, enol form), 7.35–7.55 (2H + 1H of keto form, m), 7.73 (1H, d), 7.87–7.96 (1H, m), 8.16 (1H, s), 8.32 (1H, d, enol form), 8.49–8.64 (1H, m), 9.10 (2H, br), 9.30 (2H, br); MS (ESI) 496 (MH^+). Anal. Calcd for $C_{24}H_{25}N_5O_7{\cdot}2.0TFA{\cdot}2.0H_2O$: C, 44.27; H, 4.113; N, 9.220. Found: C, 44.26; H, 4.22; N, 9.26.

6.23. (4*R*)-4-[(Benzimidazole-5-carbonyl)amino]-5-[5-amidino-2-(2-carboxy-2-oxoethyl)phenoxy]pentanoic acid bistrifluoroacetate (1h)

The title compound was prepared from benzimidazole-5-carboxylic acid and **2b** as described for **3b** and **1b**: ^{1}H NMR (DMSO-d_6) δ 1.80–2.15 (2H, m), 2.15–2.80 (2H, m), 4.00–4.30 (2H + 2H of keto form, m), 4.45 (1H, br), 6.80 (1H, s, enol form), 7.35–7.55 (2H + 1H of keto form, m), 7.70 (1H, d), 7.86 (1H, d), 8.20 (1H, s), 8.32 (1H, d, enol form), 8.52 (1H, d), 8.77 (1H, s), 9.00 (2H, br), 9.28 (2H, br); MS (ESI) 482 (MH^+). Anal. Calcd for $C_{23}H_{23}N_5O_7{\cdot}2.0TFA{\cdot}5.0H_2O$: C, 40.55; H, 4.412; N, 8.758. Found: C, 40.40; H, 4.29; N, 8.81.

6.24. (4*R*)-4-[(2-Aminobenzothiazole-6-carbonyl)amino]-5-[5-amidino-2-(2-carboxy-2-oxoethyl)phenoxy]pentanoic acid bistrifluoroacetate (1j)

Part A. A mixture of 2-aminobenzothiazole-6-carboxylic acid ethyl ester (500 mg, 2.25 mmol) and 4 M hydrochloric acid (20 mL) was stirred at 80 °C for 5 h. The reaction mixture was concentrated in vacuo, and the residue was washed with a mixture of EtOAc and hexane to give 2-aminobenzothiazole-6-carboxylic acid HCl (545 mg): ^{1}H NMR (DMSO-d_6) δ 7.60 (1H, d), 8.00 (1H, d), 8.50 (1H, s), 9.70 (2H, br).

Part B. The title compound **1j** was prepared from the 2-aminobenzothiazole-6-carboxylic acid HCl and **2b** as described for **3b** and **1b**: ^{1}H NMR (DMSO-d_6) δ 1.80–2.15 (2H, m), 2.25–2.50 (2H, m), 4.00–4.50 (3H + 2H of keto form, m), 6.80 (1H, s, enol form), 7.30–7.55 (3H + 1H of keto form, m), 7.75 (1H, d), 7.95 (2H, s), 8.15 (1H, s), 8.25–8.40 (1H + 1H of enol form, m), 9.00 (2H, s), 9.25 (2H, s), 9.75 (1H, s, enol); MS (ESI) 514 (MH^+). Anal. Calcd for $C_{23}H_{23}N_5O_7S{\cdot}2.0TFA{\cdot}1.0H_2O$: C, 42.69; H, 3.582; N, 9.219. Found: C, 42.82; H, 3.91; N, 9.40.

6.25. 3-[4-Amidino-2-[2-(2-aminoquinoline-6-carbonyl)-aminoethoxy]phenyl]-2-oxopropionic acid bistrifluoroacetate (1k)

Part A. 3-[4-Cyano-2-[2-(2-aminoquinoline-6-carbonyl)amino-ethoxy]phenyl]-2-acetamidoacrylic acid methyl ester trifluoroacetate (**3k**) was prepared from **8** and **2a** as described for **3b**.

Part B. The title compound **1k** was prepared from **3k** as described for **1a**: ^{1}H NMR (DMSO-d_6) δ 3.60–3.80 (2H, m), 4.10–4.40 (2H + 2H of keto form, m), 6.80 (1H of enol, s), 7.10 (1H, d), 7.35–7.54 (2H + 1H of keto form, m), 7.70 (1H, d), 8.15 (1H, d), 8.30–8.44 (2H + 1H of enol form, m), 8.97 (1H, br), 9.05 (2H, br), 9.30 (2H, br); MS (ESI) 436 (MH^+). Anal. Calcd for $C_{22}H_{21}N_5O_5{\cdot}2.0TFA{\cdot}2.0H_2O$: C, 44.64; H, 3.890; N, 10.01. Found. C, 44.75; H, 3.94; N, 9.94.

6.26. 3-[4-Amidino-2-[2-(2-aminobenzthiazole-6-carbonyl)aminoethoxy]phenyl]-2-oxopropionic acid bistrifluoroacetate (1l)

The title compound was prepared from 2-aminobenzthiazole-6-carboxylic acid and **2a** as described for **3b** and **1b**: ^{1}H NMR (DMSO-d_6) δ 3.60–3.80 (2H, m), 4.10–4.40 (2H + 2H of keto form, m), 6.80 (1H, s, enol form), 7.25–8.00 (5H + 1H of keto form, m), 8.15 (1H, d), 8.30 (1H of enol form, d), 9.00 (2H, s), 9.30 (2H, s), 9.65 (1H, s, enol); MS (ESI) 442 (MH^+). Anal. Calcd for $C_{20}H_{19}N_5O_5S{\cdot}2.0TFA{\cdot}3.5H_2O$: C, 39.34; H, 3.852; N, 9.560. Found: C, 39.13; H, 4.00; N, 9.94.

6.27. Enzyme assays

Human fVIIa and fXa were obtained from Enzyme Research Laboratories Inc. Human TF was obtained from American Diagnostica Inc. Human thrombin was

obtained from Sigma Chemical Co. The chromogenic substrates used were S-2288 (Daiichi Pure Chemical) for fVIIa/TF, S-2222 (Daiichi Pure Chemical, Japan) for fXa, and S-2238 (Daiichi Pure Chemical) for thrombin. fVIIa/TF was assayed in a buffer containing 0.05 M Tris, 0.15 M NaCl, 5 mM $CaCl_2$ 0.1% BSA at pH 7.4, and fVIIa/TF was preformed by incubating at room temperature in the buffer. fXa and thrombin were assayed in a buffer containing 0.1 M Tris, 0.2 M NaCl, 0.1% PEG-6000, and 0.02% Tween 20 at pH 8.4.

The final substrate concentrations in the reaction were 800, 200, and 100 μM for S-2288, S-2222, and S-2238, respectively. All enzyme assays were carried out at room temperature in 96-well microtiter plates with final concentrations of 5.0 nM for fVIIa/TF, 4.5 nM for fXa, and 1.1 nM for thrombin. Compounds at appropriate dilutions were added to wells containing buffer and enzyme and preincubated for 10 min. The enzyme reactions were initiated by the addition of substrate, and color development, due to the release of *p*-nitroaniline from each chromogenic substrate, was monitored continuously at 405 nm on a microplate reader (Model 3550-UV, Bio-Rad). The measured initial velocities were used to determine the amount of inhibitor required to reduce the control velocity by 50%; this concentration was defined as the IC_{50} of the inhibitor. The apparent K_i values of compounds were then calculated according to the relationship: $K_i = IC_{50}/(1 + K_m/S)$ or $(IC_{50})_1/(IC_{50})_2 = (K_i)_1/(K_i)_2$.[18]

6.28. Coagulation assay

Prothrombin time (PT) and activated partial thromboplastin time (aPTT) were measured with coagulometer CA3000 (Sysmex, Japan). Citrated human plasma, which was collected from healthy volunteers, was used in the assays. Plasma (45 μL) was mixed with 5 μL of a compound dilution followed by the automatic addition of 100 μL of prothrombin reagent (Thromboplastin C plus, Dade Behring) for the PT measurement, or 50 μL of activated cephaloplastin reagent (Dade Behring) and 50 μL of 20 mM $CaCl_2$ for the aPTT measurement. The prolongation of the clotting time in the presence of a test compound versus its absence was used to quantify the anticoagulant effect.

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