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Design, synthesis and SAR of novel ethylenediamine and phenylenediamine derivatives as factor Xa inhibitors

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ABSTRACT

We previously reported on a series of cyclohexanediamine derivatives as highly potent factor Xa inhibitors. Herein, we describe the modification of the spacer moiety to discover an alternative scaffold. Ethylenediamine derivatives possessing a substituent at the C1 position in *S* configuration and phenylenediamine derivatives possessing a substituent at the C5 position demonstrated moderate to strong anti-fXa activity. Further SAR studies led to the identification of compound **30h** which showed both good in vitro activity (fXa IC_{50} = 2.2 nM, PTCT2 = 3.9 μM) and in vivo antithrombotic efficacy.

Thromboembolic disorders are the leading cause of morbidity and mortality in developed countries.[1] These include acute myocardial infarction (MI) or ischemic stroke in arterial circulation, and deep vein thrombosis (DVT) or pulmonary embolism (PE) in venous circulation. Anticoagulants currently in clinical use for the prevention and treatment of these diseases include unfractionated heparin, low molecular weight heparins (LMWHs), the indirect fXa inhibitor fondaparinux, and the direct thrombin inhibitors (DTIs) argatroban, bivalirudin, and hirudin. Although the efficacies of these drugs have been confirmed, all require parenteral administration. For oral administration, vitamin K antagonists (VKAs) such as warfarin have been the mainstay of anticoagulants therapy for more than 60 years. However, VKAs have substantial drawbacks (slow onset and offset of action, inconvenience of frequent monitoring, numerous drug and food interactions, and so on) that limit their clinical use.[2] Thus, a novel anticoagulant that can be administered more conveniently and safely has been desired. Factor Xa (fXa) is a key serine protease located at the convergence of the intrinsic and extrinsic pathways catalyzing the conversion of prothrombin to thrombin. It is anticipated that selective inhibition of fXa will provide an antithrombotic effect by blocking the amplified formation of thrombin without compromising normal hemostasis.[3] Therefore, fXa is a particularly attractive target and extensive clinical trials are now ongoing for a number of clinical candidates.[4–9]

We have previously reported on the orally active potent fXa inhibitor compound A,[10] which was chosen as a clinical candidate (Fig. 1). The X-ray structure analysis revealed the binding mode of compound A in which the indole moiety fitted into the S1 site, 5-methyl-4,5,6,7-tetrahydrothiazolo[5,4-*c*]pyridine moiety fitted into the S4 site, and the cyclohexane diamine acts as a spacer connecting these two key elements. The result of the SAR study of the S1 moiety of compound A has been disclosed recently.[11,12] In addition to the S1 modification, we concurrently conducted an exploratory study of the spacer moiety aiming to discover an alternative

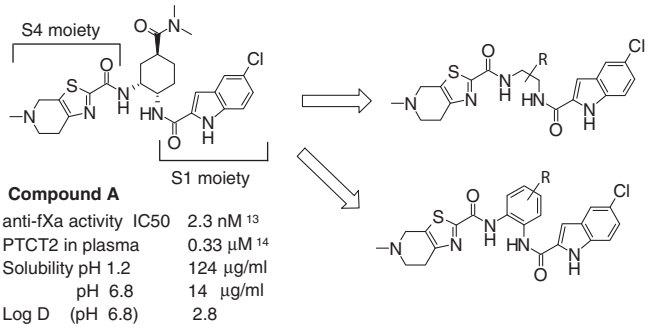

Figure 1. Structure of compound A and design for alternative spacer scaffolds.

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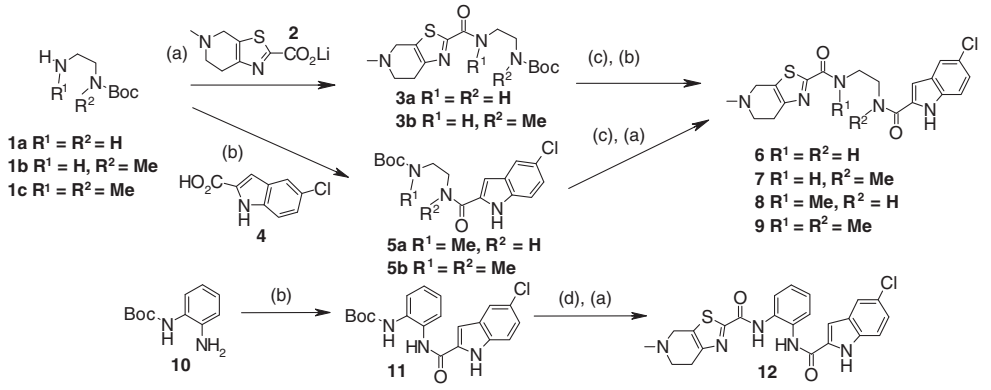
spacer scaffold. In this report, the SAR of the spacer moiety will be discussed. We designed ethylenediamine and phenylenediamine derivatives as alternative scaffolds which have less complexity (Fig. 1).

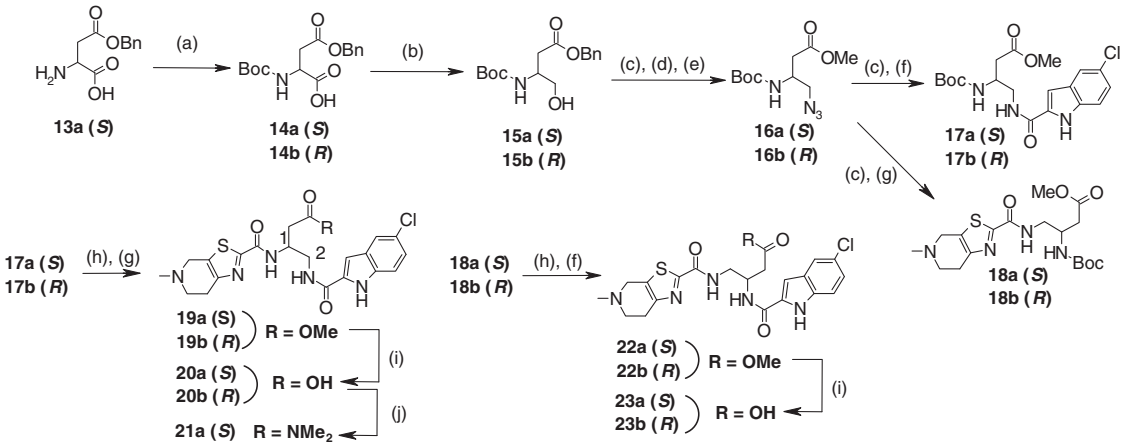
In the synthesis of unsubstituted ethylenediamine and phenylenediamine derivatives, mono-protected diamines (**1a–c**, and **10**) were acylated with carboxylate **2** or carboxylic acid **4** giving mono-acylated intermediates (**3a**, **b**, **5a**, **b**, and **11**). Then, the Boc group was removed and the resulting amine was acylated with **4** or **2** to afford diamides **6**–**9**, and **12** (Scheme 1).

Mono-substituted ethylenediamine derivatives were synthesized as depicted in Scheme 2. Both 1-substituted derivatives (**19**, **20**, and **21**) and 2-substituted derivatives (**22** and **23**) were synthesized via key intermediate **16**, which was prepared by the reduction of carboxylic acid **14**, mesylation of the resulting alcohol **15**, and substitution with azide.

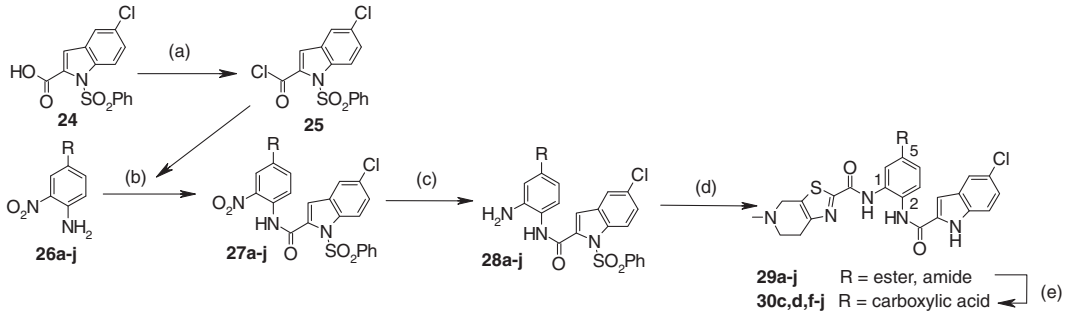
Phenylenediamine derivatives having a substituent at the 5-position were synthesized as outlined in Scheme 3. Acylation of 5-substituted-1-nitro-2-aminobenzenes (**26a–j**) by 1-benzenesulfonyl-5-chloroindole-2-carbonyl chloride (**25**), followed by



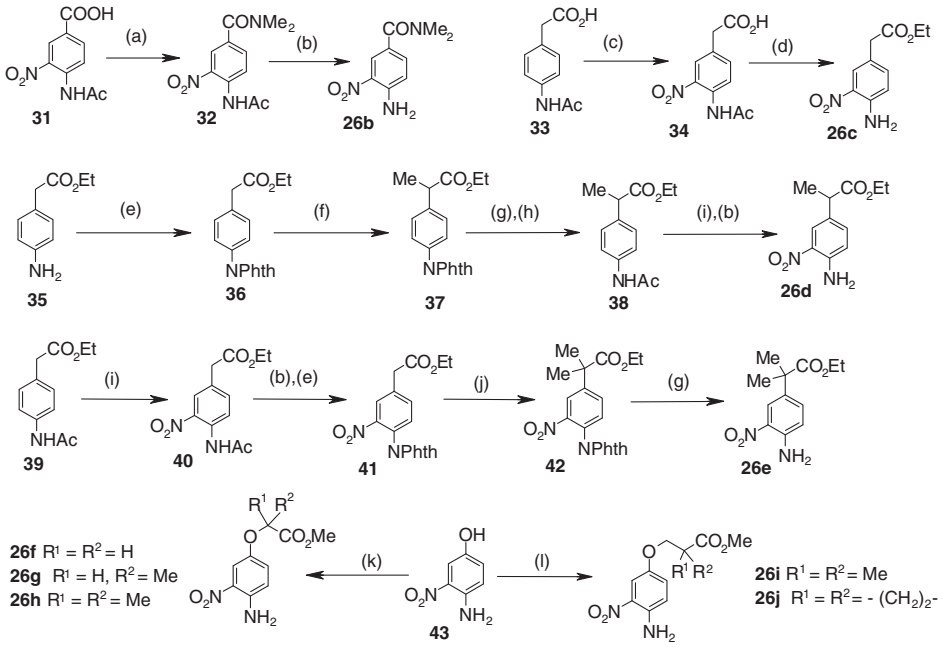
Scheme 1. Reagents and conditions: (a) lithium 5-methyl-4,5,6,7-tetrahydrothiazolo[5,4-*c*]pyridine-2-carboxylate (**2**), EDC·HCl, HOBt, DMF, rt, 1–3 days; (b) 5-chloroindole-2-carboxylic acid (**4**), EDC·HCl, HOBt, DMF, rt, 1–3 days; (c) satd HCl, MeOH, or satd HCl, EtOH, rt, 1 h; (d) TFA, DCM, rt, 1 h.



Scheme 2. Reagents and conditions: (a) Boc_2O, TEA, dioxane, H_2O, 0 °C to rt, overnight; (b) (i) $ClCO_2iPr$, TEA, THF, 0 °C, 30–60 min; (ii) $NaBH_4$, H_2O, rt, 1 h; (c) 10% Pd/C, H_2, MeOH or THF, rt, 1–3 h; (d) $TMSCHN_2$, MeOH, THF, 0 °C, 1 h; (e) (i) MsCl, TEA, DCM, −78 to 0 °C, 1 h; (ii) NaN_3, DMF, 65 °C, 1 h; (f) **4**, EDC·HCl, HOBt, DIPEA, DMF, rt, overnight; (g) **2**, EDC·HCl, HOBt, DIPEA, DMF, rt, overnight; (h) HCl, 1,4-dioxane, MeOH, rt, 1 h; (i) LiOH, THF, H_2O, rt, 1 h; (j) Me_2NH·HCl, EDC·HCl, HOBt, TEA, DMF, rt, overnight.



Scheme 3. Reagents and conditions: (a) $SOCl_2$, DMF (cat.), $CHCl_3$, reflux, 30–60 min; (b) 1-benzenesulfonyl-5-chloroindole-2-carbosnylchloride (**25**), pyridine, TEA, DMAP, DCM, 0–60 °C, 1–6 days; (c) $FeCl_3$, Zn, DMF, H_2O, 100 °C, 10–60 min; (d) **2**, EDC·HCl, HOBt, DMF, rt–70 °C, 1–6 days; (e) LiOH or NaOH, EtOH or THF, H_2O, 0 °C–rt, 4–36 h.



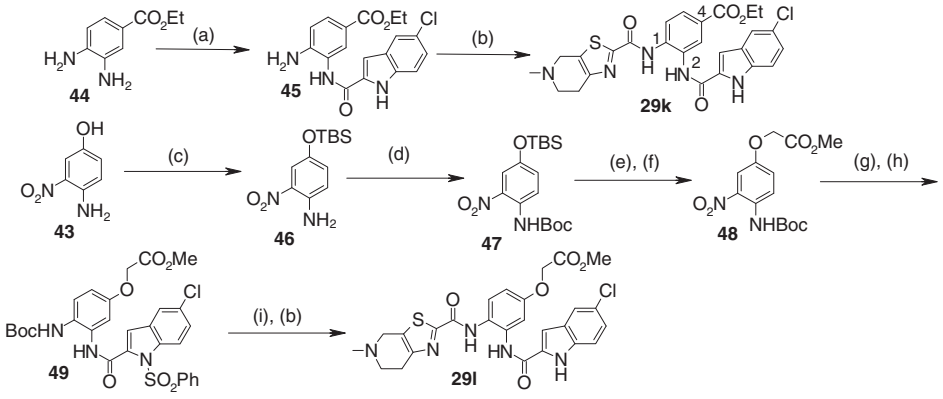
Scheme 4. Reagents and conditions: (a) (i) $SOCl_2$, benzene, reflux, 3 h; (ii) Me_2NH, THF, rt, overnight; (b) 1 N-HCl, EtOH, reflux, 2–5 h; (c) KNO_3, H_2SO_4, −10 °C, 1 h; (d) satd HCl, EtOH, reflux, 4 h; (e) phthalic anhydride, AcOH, reflux, 18 h; (f) (i) NaH, DMF, 0 °C to rt, 1 h; (ii) MeI, 0 °C to rt, 2 h; (g) $H_2NNH_2{\cdot}H_2O$, EtOH, rt, 18 h; (h) Ac_2O, 130 °C, 1 h; (i) fuming HNO_3, AcOH, Ac_2O, 0 °C to rt, 2–5 h; (j) (i) LiHMDS, THF, −78 °C, 30 min; (ii) MeI, THF, rt, 3 h; (k) $BrCR^1R^2CO_2Me$, K_2CO_3, DMF, 70 °C, overnight; (l) $BrCH_2CR^1R^2CO_2Me$, K_2CO_3, DMF, 60–70 °C, 20–24 h.

reduction of the nitro group and condensation with **2** gave compounds **29a–j**, which were hydrolyzed to afford carboxylic acids (**30c, d, f–j**). In the case of **29a** and **29e**, hydrolysis in the aqueous alkaline solution failed to yield the corresponding carboxylic acids.

The syntheses of 5-substituted-1-nitro-2-aminobenzenes (**26b–j**) are shown in Scheme 4. Phenylacetic acid derivatives (**26c–e**) were synthesized via the introduction of the nitro group to the corresponding 4-(acetamido)phenylacetic acid derivatives (**33**, **38**, or **39**). Phenoxyacetic acid derivatives (**26f–h**) and phenoxypropionic acid derivatives (**26i** and **26j**) were synthesized by alkylation of phenol **43**.

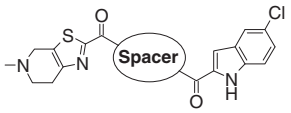
Diamine **44** was selectively acylated with carboxylic acid **4** on the amine *meta* to the ester, due to the deactivation of *para*-amine by the electron withdrawing group. Then another amino group was acylated with **2** to provide 4-substituted phenylenediamine **29k** (Scheme 5). The hydroxyl group of **43** was alkylated after the protection of the amino group. Compound **48** was reduced to give mono-protected diamine, which was converted to diamide **29l**.

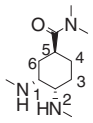
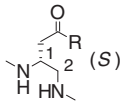
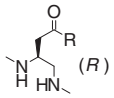
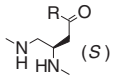
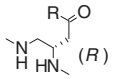
The anti-fXa activity (IC_{50}), anticoagulant activity (PTCT2), and solubility of ethylenediamine derivatives are shown in Table 1. Unsubstituted ethylenediamine (**6**) showed moderate anti-fXa activity (IC_{50} = 221 nM). *N*-Methyl ethylenediamine derivatives had significantly decreased activity (**7**, **8**, and **9**). Then a substituent was introduced on the ethylene chain. Of the four methoxycarbonylmethyl derivatives (**19a**, **19b**, **22a**, and **22b**), only C1-substituted derivative in *S* configuration (**19a**) exhibited better anti-fXa activity than compound **6**. As seen in the X-ray crystal structure of compound A, C2 carbon is located close to the amino acid (Gln192) of fXa (Fig. 2). Therefore, we considered that the introduction of a relatively large methoxycarbonylmethyl group at the C2 position caused steric repulsion, and thus resulted in decreased activity (**22a** and **22b**). In the case of substitution at the C1 position, there is not enough space between the C1 carbon and enzyme if the substituent is in the *R* configuration (**19b**). On the other hand, if the substituent is in the *S* configuration, it is supposed to be located towards the outside of the enzyme without causing any steric



Scheme 5. Reagents and conditions: (a) **4**, EDC·HCl, HOBt, DMF, rt, 20 h; (b) **2**, EDC·HCl, HOBt, DMF, 50–60 °C, two days; (c) TBSCl, imidazole, DMF, 0 °C to rt, 1 h; (d) (i) NaHMDS, THF, rt, 30 min; (ii) $(Boc)_2O$, THF, rt, overnight; (e) TBAF, THF, 0 °C, 1 h; (f) $BrCH_2CO_2Me$, K_2CO_3, DMF, 70 °C, overnight; (g) 10% Pd/C, H_2, MeOH, rt, 3 h; (h) **25**, pyridine, DCM, 0 °C to rt, overnight; (i) TFA, anisole, DCM, 0 °C, 2 h.

Table 1
Assay results of ethylenediamine derivatives



Compd	Spacer	R	In vitro activity		Solubility (μg/ml)		Ex vivo anti-fXa activity[c] (%)
			fXa IC_{50}[a] (nM)	PTCT2[b] (M)	pH 1.2	pH 6.8	
A			2.3	0.33	124	14	62
6			221	18	–	–	–
7			1600	–	–	–	–
8			4100	–	–	–	–
9			3945	–	–	–	–
19a		OMe	70	6.5	>1000	135	1
20a		OH	200	–	900	432	1
21a		NMe_2	47	3.2	>1000	140	15
19b		OMe	1200	–	813	87	–
20b		OH	2100	–	938	544	–
22a		OMe	770	–	536	93	–
23a		OH	460	–	>1000	39	–
22b		OMe	970	–	237	93	–
23b		OH	610	–	786	30	–

[a] Anti-fXa activity (IC_{50}) was measured as described in Ref. 13.
[b] Anticoagulant activity (PTCT2) was evaluated as described in Ref. 14.
[c] The maximum anti-fXa activity in plasma after oral administration is shown. The assay method is described in Ref. 15.

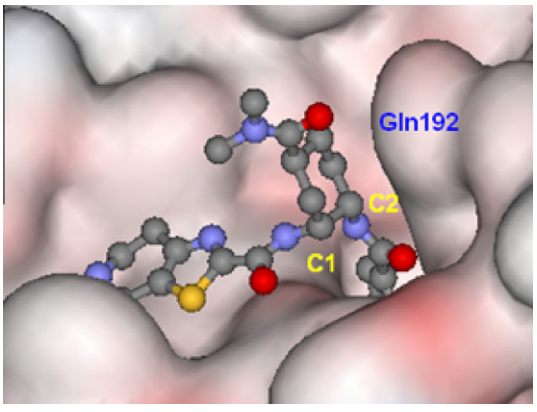


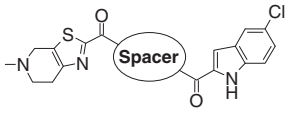
Figure 2. X-ray crystal structure of compound A in fXa (PDB code:2EI8).[10]

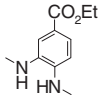
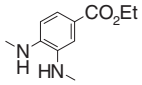
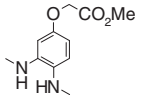
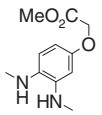
repulsion (**19a**). Similar results were also observed for the anti-fXa activity of carboxylic acid derivatives (**20a**, **20b**, **23a**, and **23b**). Based on this result, compound **21a**, which possesses the dimethylcarbamoylmethyl group at the C1 position in the *S* configuration, was synthesized. It showed modest anti-fXa activity (IC_{50} = 47 nM) and anticoagulant activity (PTCT2 = 3.2 μM). The solubility of ethylenediamine derivative was improved in every case, demonstrating the utility of this spacer in terms of improving physicochemical property.

The assay results of phenylenediamine derivatives are shown in Table 2. Even unsubstituted derivative **12** exhibited very strong anti-fXa activity (IC_{50} = 7.4 nM). A comparison of 5-substituted derivatives (**29a**, **f**) with corresponding 4-substituted derivatives (**29k, l**) demonstrates that substitution at the 5-position is preferable. There seemed to be two major problems regarding these phenylenediamine compounds. One is their weak anticoagulant activity and the other is low solubility, especially in the neutral pH region. To overcome these problems, we continued the modification of a substituent at the 5-position.

As shown in Table 3, esters **29c–j** showed moderate to strong anti-fXa activity. However, their anticoagulant activity was still not strong. Those compounds showed notably high *c* log *P* values (3.50–4.84). As it is known that higher lipophilicity of compounds results in higher protein binding and weaker anticoagulant activity,[10] we envisioned that anticoagulant activity would be improved by lowering the lipophilicity. Carboxylic acid derivatives (**30c, d, f–j**) and carbamoyl derivative (**29b**) were designed for this aim. The *c* log *P* values were significantly lower (0.71–2.63) than the parent compounds. The carboxylic acids (**30c, d, f–j**) showed a drastic increase in anti-fXa activity and also demonstrated distinct anticoagulant activity. This is in contrast to unsubstituted derivative **12** or ester **29f**, which showed only weak anticoagulant

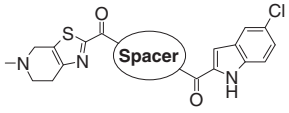
Table 2
Comparison of 4- and 5-substituted phenylenediamine derivatives

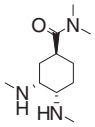
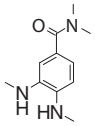
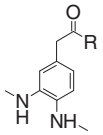
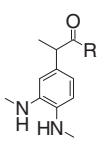
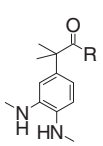


Compd	Spacer	In vitro activity		Solubility (μg/ml)		*c* log *P*[c]
		fXa IC_{50}[a] (nM)	PTCT2[b] (μM)	pH 1.2	pH 6.8	
12		7.4	>5 (×1.12)[d]	—	—	3.71
29a		690	>20 (×1.07)	2.7	<1	4.67
29k		>10,000	>20 (×1.06)	1.1	<1	4.88
29f		5.6	>20 (×1.68)	51	<1	3.50
29l		110	>20 (×1.14)	8	<1	3.57

[a,b] Refers to Table 1.
[c] *c* log *P* values were calculated using *c* log *P* daylight version 4.83, which was supplied by Daylight Chemical Information Systems, Inc.
[d] Figure in parenthesis represents the prolongation ratio of prothrombin time (PT) at the highest concentration of the compound.

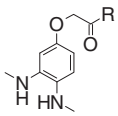
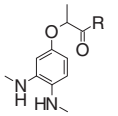
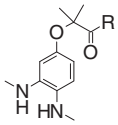
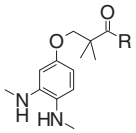
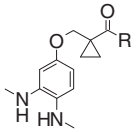
Table 3
Assay results of 5-substituted phenylenediamine derivatives



Compd	Spacer	R	In vitro activity		Solubility (μg/ml)		*c* log *P* [c]	Ex vivo anti-fXa activity [d] (%)
			fXa IC_{50}[a] (nM)	PTCT2[b] (μM)	pH 1.2	pH 6.8		
A			2.3	0.33	124	14	3.00	62
29b			7.7	4.0	ND[f]	ND[f]	2.63	1
29c		OEt	170	>20 (×1.10)[e]	250	<1	4.13	0
30c		OH	1.9	3.7	22	16	0.71	1
29d		OEt	65	>20 (×1.71)	—	—	4.44	23
30d		OH	1.1	4.8	—	—	1.24	5
29e		OEt	100	>20 (×1.43)	—	—	4.84	8

(*continued on next page*)

Table 3 (*continued*)

Compd	Spacer	R	In vitro activity		Solubility (μg/ml)		*c* log *P* [c]	Ex vivo anti-fXa activity [d] (%)
			fXa IC_{50} [a] (nM)	PTCT2 [b] (μM)	pH 1.2	pH 6.8		
29f		OMe	5.6	>20 (×1.68)	51	<1	3.50	32
30f		OH	2.5	3.1	12	<1	0.75	3
29g		OMe	29	6.2	61	<1	3.81	9
30g		OH	3.5	4.8	256	825	1.21	0
29h		OMe	72	10	37	<1	4.12	6
30h		OH	2.2	3.9	71	362	1.37	0
29i		OMe	120	>20 (×1.38)	11	<1	4.62	9
30i		OH	1.9	7.2	4	9	1.72	5
29j		OMe	100	12	22	<1	4.25	13
30j		OH	2.7	5.6	4	1	1.36	4

[a–e] Refers to Tables 1 and 2.
[f] The measurement of solubility failed due to insufficient solubility of the compound.

activity despite their strong anti-fXa activity. In addition, compounds **30g** and **30h** demonstrated significantly improved solubility in the neutral pH region. Carbamoyl derivative **29b** also showed increased anti-fXa and anticoagulant activity compared to ester **29a**. However, the physicochemical property of **29b** was problematic (the measurement of solubility failed due to its insufficient solubility).

X-ray crystal structure of compound **12** in fXa was obtained with 2.0 Å resolution. Figure 3 shows the superposition of the crystal structures for compound **12** in fXa (green) and compound A in fXa (white). The S1 moiety and S4 moiety of both compounds overlap very well, while the positions of the spacer moieties differ from each other. Another distinct feature lies in the conformation of the amide connecting the S1 moiety and the spacer moiety. In compound A, indole nitrogen and amide nitrogen are located in the antiperiplanar position, while in compound **12** indole nitrogen and amide oxygen are located in the antiperiplanar position. As the result of those changes, two essential pharmacophores of compound **12** were placed in appropriate positions.

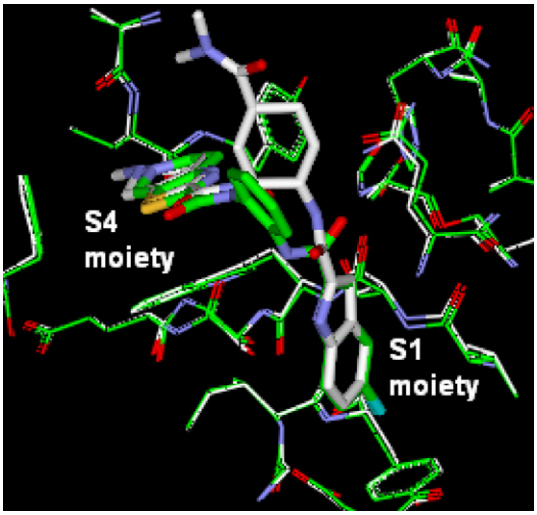


Figure 3. Superposition of the crystal structures of phenylenediamine derivative **12** in fXa (green) and compound A in fXa (white).[16]

The key interactions of compound **12** with fXa are similar to those of compound A. As shown in Figure 4, there are hydrophobic interaction in the S4 site, hydrogen bonding between indole NH and carbonyl oxygen of Gly218 (2.88 Å), and chloro-aromatic interaction with Tyr228 in the S1 site (The distance between Tyr ring centroid and Cl is 3.51 Å). There are additional interactions that are characteristic to compound **12**. One is the interaction of the benzene moiety with the subsite known as S1β.[17] The benzene C3 atom and sulfur atom of Cys220 are in close contact (the distance between C3 and S is 3.71 Å). The other interactions are hydrogen bondings of the amide NH of compound **12** to the backbone carbonyls of Gly218 (3.23 Å) and Gly216 (3.33 Å).[18] Those hydrogen bondings could be formed because of the inversion of amide configuration as described above (Fig. 3). These interactions presumably contribute to the strong anti-fXa activity of phenylenediamine derivatives.

This binding mode also explains the preference of 5-substitution of phenylenediamine compounds. As seen in Figure 4, Glu147 exists in the direction of the 4-position of the phenylenediamine spacer, while the 5-position is exposed to the solvent. It is presumed that the introduction of a large substituent at the 4-position caused a steric repulsion and resulted in the decrease of activity.

Several potent compounds were orally administered to rats at a dose of 10 mg/kg, and the anti-fXa activity in plasma was measured at 0.5, 1, 2, and 4 h after administration. The maximum

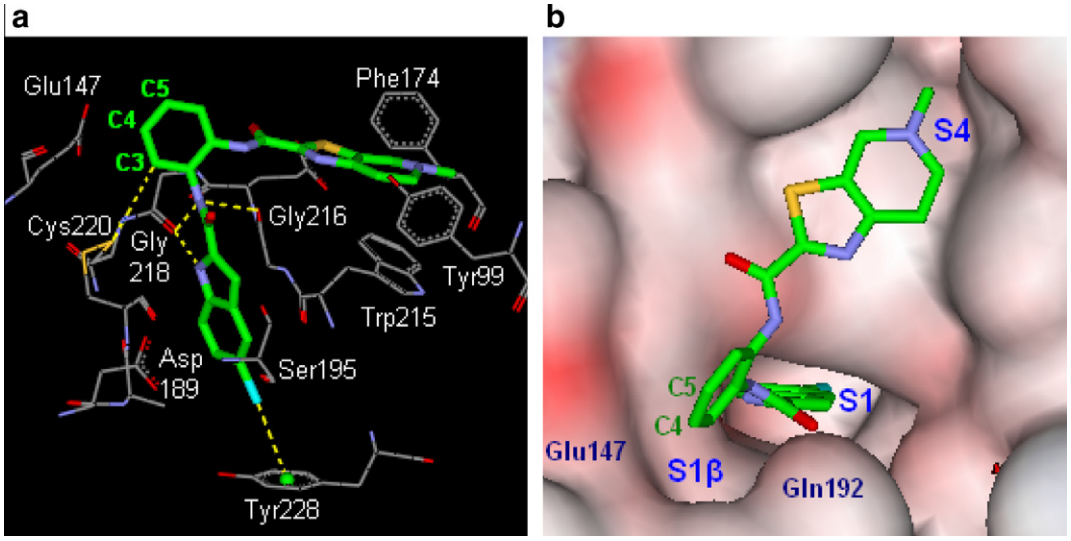


Figure 4. (a) Important amino acids and key interactions with compound **12**. (b) The binding mode of compound **12**.

Table 4
Metabolic stability, permeability, and log *D* value

Compd	Metabolic stability V_{ini}[a] (nmol/min/mg)	Permeability AT ratio[b]	log *D*
A	0.052	>30[c]	2.8
30h	−0.007	0.24[d]	1.3

[a] Metabolic stability was evaluated by the initial velocity of disappearance (V_{ini}) in human liver microsomes.[11]
[b] Permeability was measured using Caco-2 monolayers. Relative permeability is shown as a ratio compared to atenolol.[11]
[c] pH 6.0.
[d] pH 7.4.

anti-fXa activity among them is shown as ex vivo anti-fXa activity (%) in Tables 1 and 3.

Among the ethylenediamine derivatives tested, only compound **21a** exhibited weak ex vivo anti-fXa activity (15%). With regard to phenylenediamine derivatives, compound **29f** exhibited modest ex vivo anti-fXa activity (32%) that was about half of the activity of compound A. Unfortunately, phenylenediamine derivatives possessing carboxylic acids (**30c, d, f–j**) or dimethylamide (**29b**) showed almost no activity in ex vivo assay, even though these compounds demonstrated strong anti-fXa and anticoagulant activity in vitro. Compounds **30g** and **30h**, which possess sufficient solubility, also did not exhibit ex vivo anti-fXa activity. As shown in

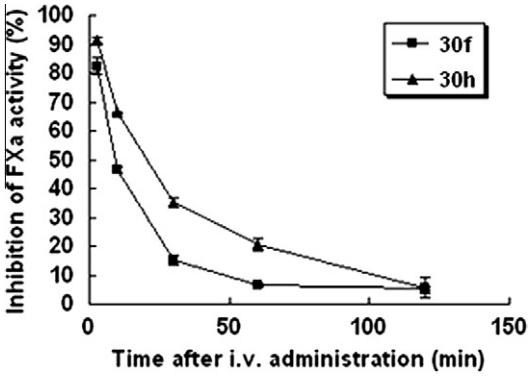


Figure 5. Ex vivo anti-fXa activity after intravenous administration of compounds **30f** and **30h**.

Table 4, compound **30h** had high metabolic stability, but its permeability was much lower than that of compound A. Reducing lipophilicity was required to improve anticoagulant activity, but it impaired the permeability at the same time. Achieving a good balance between strong anticoagulant activity and oral activity in this series of compounds remained a challenge.

Compounds **30f** and **30h** were administered intravenously to rats at a dose of 0.4 mg/kg. These compounds exhibited strong

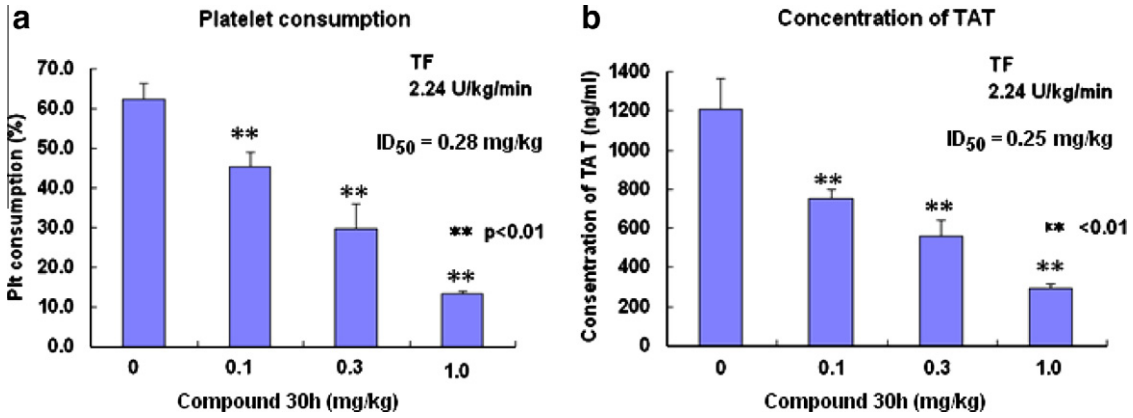


Figure 6. Antithrombotic effect of compound **30h** in a rat DIC model. (a) Inhibition of the platelet consumption. (b) Inhibition of the formation of thrombin–antithrombin III complex (TAT).

ex vivo anti-fXa activity, showing maximum inhibitory activity of 82% and 91%, respectively (Fig. 5).

The antithrombotic activity of phenylenediamine derivative was tested in a rat disseminated intravascular coagulation (DIC) model.[19] Compound **30h** was administered intravenously to rats after the injection of tissue factor (TF) and demonstrated dose-dependent antithrombotic activity (Fig. 6).

In summary, we have identified novel spacer scaffolds for our S1 and S4 pharmacophores. Among them, ethylenediamine spacer is useful to improve solubility. The anti-fXa activity of ethylenediamine compounds could be improved by the introduction of a substituent at the C1 position in the *S* configuration. In addition, compound **12** possessing a phenylenediamine spacer exhibited strong anti-fXa activity (IC_{50} = 7.4 nM). X-ray structure analysis revealed that compound **12** utilized an additional subsite S1β, while retaining interactions in S1 and S4 sites. Further exploratory study resulted in the discovery of compound **30h** which demonstrated excellent anti-fXa activity (IC_{50} = 2.2 nM), good anticoagulant activity (PTCT2 = 3.9 μM), and improved solubility in neutral pH region. Compound **30h** exhibited strong ex vivo anti-fXa activity after intravenous administration and also demonstrated strong antithrombotic effect in a rat DIC model. The findings described in this Letter can offer possibilities for the future design of potent fXa inhibitors.

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13. The in vitro anti-fXa activity was measured by using chromogenic substrate S-2222 (Chromogenix, Inc.) and human fXa (Enzyme Research Laboratories). Aqueous DMSO (5% V/V; 10 μL) or inhibitors in aqueous DMSO (10 μL) and 0.0625 U/mL human fXa (10 μL) were mixed with 0.1 M Tris–0.2 M NaCl–0.2% BSA buffer (pH 7.4; 40 μL). A reaction was started by the addition of 0.75 M S-2222 (40 μL). The absorbance (OD) at 405 nm was monitored every 10 s with a SPECTRAmax 340 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at room temperature, and the reaction velocity (OD/min) was obtained. Anti-fXa activity (inhibition %) was calculated as follows: Anti-fXa activity = {1 − [(OD/min) of sample/(OD/min) of control]} × 100. The IC_{50} value was obtained by plotting the inhibitor concentration against the anti-fXa activity.
14. Anticoagulant activity was evaluated with the human plasma clotting time doubling concentration (PTCT2). The method is as follows: prothrombin time (PT) was measured with an Amelung KC-10A micro coagulometer (MC Medical, Tokyo, Japan). First, 50 μL of plasma was mixed with 50 μL of inhibitor or 4% DMSO/saline and incubated for 1 min at 37 °C. Coagulation was started by the addition of 100 μL of Thromboplastin C Plus (0.5 U/mL) to the mixture, and the clotting time was measured. The concentration of inhibitor required to double the clotting time (CT2) was estimated from the concentration–response curve by a regression analysis.
15. Male Wistar rats were fasted overnight. Synthetic compounds were dissolved in 0.5% (w/v) methylcellulose solution and administered orally to rats via a stomach tube. For control rats, 0.5% (w/v) methylcellulose solution was administered orally. The rats were anesthetized with ravonal at several time points when blood samples were collected in the presence of trisodiumcitrate. After the blood samples were centrifuged, the platelet poor plasma samples were used for the measurement of their anti-fXa activity. Anti-fXa activity: plasma (5 μL) was mixed with 0.1 M Tris–0.2 M NaCl–0.2% BSA buffer (pH 7.4; 40 μL), H_2O (5 μL), and 0.1 U/mL human fXa (10 μL). A reaction was started by the addition of 0.75 M S-2222 (40 μL). The absorbance (OD) at 405 nm was monitored every 10 s with a SPECTRAmax 340 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at room temperature, and the reaction velocity (OD/min) was obtained. Anti-fXa activity (inhibition %) was calculated as follows: anti-fXa activity = {1 − [(OD/min) of sample/(OD/min) of control]} × 100.
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