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Discovery of a Highly Potent, Selective, and Orally Bioavailable Macrocyclic Inhibitor of Blood Coagulation Factor VIIa–Tissue Factor Complex

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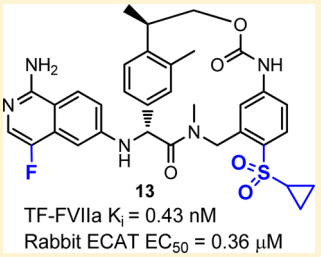
Supporting Information

ABSTRACT: Inhibitors of the tissue factor (TF)/factor VIIa complex (TF-FVIIa) are promising novel anticoagulants which show excellent efficacy and minimal bleeding in preclinical models. Starting with an aminoisoquinoline P1-based macrocyclic inhibitor, optimization of the P′ groups led to a series of highly potent and selective TF-FVIIa inhibitors which displayed poor permeability. Fluorination of the aminoisoquinoline reduced the basicity of the P1 group and significantly improved permeability. The resulting lead compound was highly potent, selective, and achieved good pharmacokinetics in dogs with oral dosing. Moreover, it demonstrated robust antithrombotic activity in a rabbit model of arterial thrombosis.



INTRODUCTION

Pathological blood clotting initiated by tissue factor (TF) leads to thromboembolic cardiovascular diseases such as ischemic stroke, myocardial infarction, and deep venous thrombosis.[1] Atherosclerotic plaque rupture or vascular injury exposes TF to circulating coagulation factor VIIa (FVIIa), a serine protease. Binding of TF to FVIIa and formation of the TF-FVIIa complex leads to a large increase in its catalytic activity and subsequently initiates the extrinsic coagulation pathway by activating factor IX to IXa and factor X to Xa. Factor Xa then activates prothrombin to thrombin.[2] Thrombin plays an essential role in the pathogenesis of thrombosis by cleaving fibrinogen to fibrin and activating platelets through proteolytic activation of thrombin receptors, enabling clot formation.[3,4] It has been shown that selective inhibition of the TF-FVIIa complex provides antithrombotic efficacy with a low risk of bleeding in preclinical models.[5−13] In addition, recombinant nematode anticoagulant protein c2, a biological agent that inhibits TF-VIIa complex, demonstrated the antithrombotic efficacy and safety in patients with acute coronary syndrome.[14] Accordingly, direct-acting, orally bioavailable TF-FVIIa inhibitors could be effective and safe in the treatment and prevention of venous and arterial thrombosis. Despite strong target validation and substantial efforts to discover orally active TF-FVIIa inhibitors,[15] there are no reports of small molecule TF-FVIIa inhibitors advancing to antithrombotic clinical trials. The most significant unsolved issue for small molecule drugs targeting TF-FVIIa is oral bioavailability; the vast majority of known TF-FVIIa inhibitors contain benzamidine or a similar highly basic P1 group ($pK_a \sim 12$).[16] The basic group engages in a strong interaction with Asp189 in the active site of FVIIa, resulting in inhibitors that are potent, but poorly permeable/orally bioavailable. Alternatively, several groups have attempted to replace the benzamidine moiety with less basic groups, although with limited success.[17−23]

Our group reported a series of aminoisoquinoline-based phenylpyrrolidine phenylglycineamides as potent and selective TF-FVIIa inhibitors displaying promising oral bioavailabilty.[24] On the basis of a crystal structure of a phenylpyrrolidine lead bound in the active site of FVIIa and subsequent molecular modeling studies, Priestley et al. recently reported a novel series of macrocyclic TF-FVIIa inhibitors with improved potency relative to the acyclic analogues.[25] Further potency optimization and structure guided atropisomer control by incorporation of P2 methyls provided a macrocyclic compound **1** with TF-FVIIa K_i (37 °C) of 20 nM and measurable PAMPA permeability of 13 nm/s (Figure 1).[26] Discovery of the conformationally constrained macrocyclic scaffold represented a significant advance in the search for potent TF-FVIIa inhibitors and opened new possibilities for orally bioavailable TF-FVIIa inhibitor development. Compound **1**, although equally potent against human tissue kallikrein (HK-1, K_i = 21 nM), served as an attractive starting point for further improvement. Herein we report optimization of the P′ groups and fluorination of the aminoisoquinoline P1 group in **1** that

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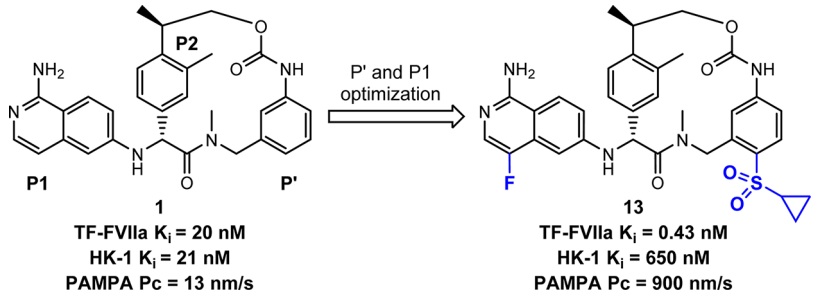
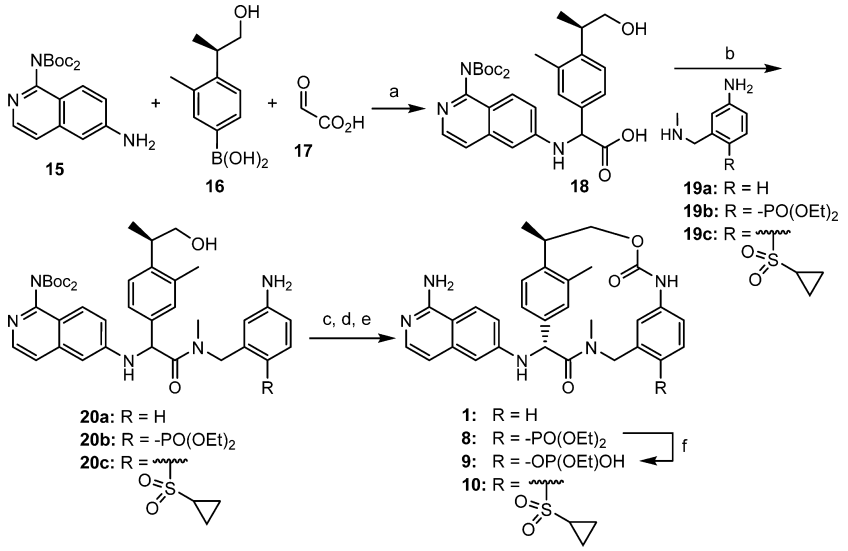


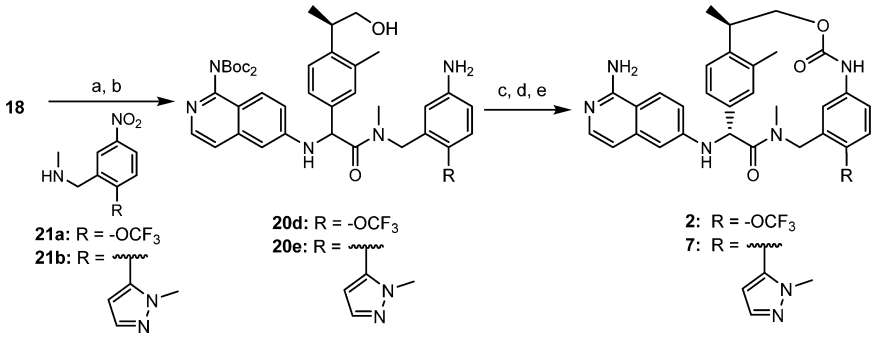
Figure 1. Optimization of P′ and fluorination of P1 in **1** led to the discovery of lead compound **13**.

Scheme 1. Synthesis of Macrocyclic Inhibitors 1 and 8−10[a]



[a]Reagents and conditions: (a) DMF/CH_3CN (1:2), 80 °C; (b) BOP, DIEA, DMF/CH_3CN (1:2), 51−77% for 2 steps; (c) phosgene, CH_3CN/CH_2Cl_2 (1:1), then TEA; (d) chiral HPLC, 37−49% for 2 steps; (e) TFA/CH_2Cl_2 or 4.0 N HCl in dioxane/EtOAc, 36−60%; (f) LiOH, THF, 40 °C, 51%.

Scheme 2. Synthesis of Macrocyclic Inhibitors 2 and 7[a]



[a]Reagents and conditions: (a) BOP, DIEA, DMF/CH_3CN (1:2), 54−59%; (b) Pd/C, H_2 balloon, MeOH, 82−89%; (c) phosgene, CH_3CN/CH_2Cl_2 (1:1), then TEA; (d) chiral HPLC, 12−32% for 2 steps; (e) TFA/CH_2Cl_2, 44−90%.

led to the discovery of the lead compound **13**, a highly potent, selective macrocyclic inhibitor of TF-FVIIa with good pharmacokinetics in dogs from oral dosing and robust antithrombotic activity in a rabbit model of arterial thrombosis.

CHEMISTRY

The syntheses of the macrocyclic inhibitors **1** and **8−10** are outlined in Scheme 1. A three-component Petasis reaction[27] of di-Boc protected 6-aminoisoqunoline **15**,[28] (*R*)-(4-(1-hydroxypropan-2-yl)-3-methylphenyl)boronic acid **16**,[29] and glyoxylate **17** gave rise to the phenylglycine derivative **18**. Coupling of intermediate **18** with substituted *N*-methylbenzylamines **19a−c** afforded the corresponding phenylglycinamides **20a−c**. The Petasis reaction and amide coupling could be conveniently carried out in a two-step, one-pot procedure without isolation of the acid **18**. Macrocylization to form the upper carbamate bond was accomplished by the treatment of compounds **20a−c**, first with phosgene to generate a transient chloroformate,

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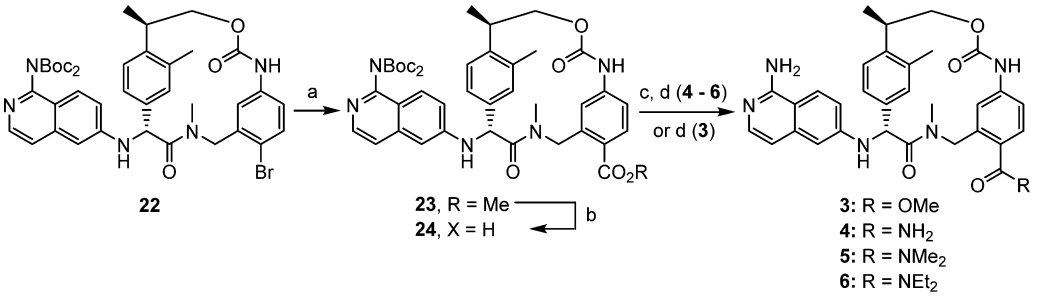
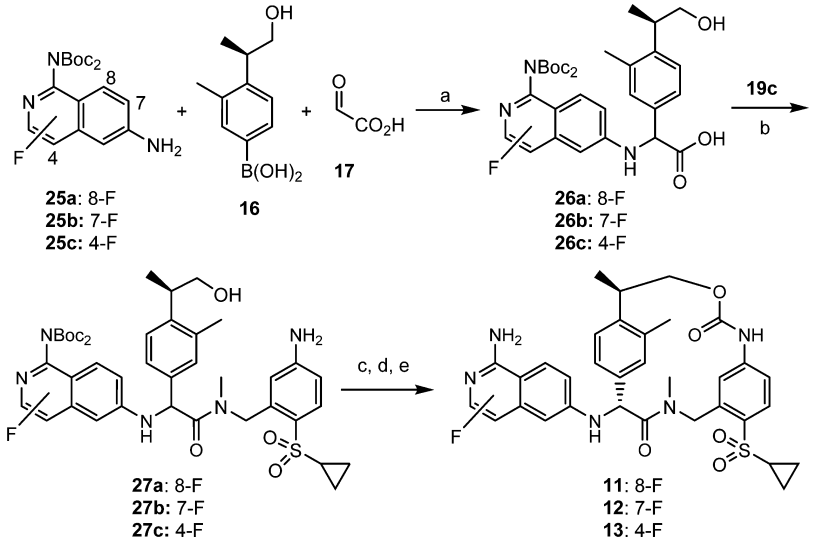
Scheme 3. Synthesis of Macrocyclic Inhibitors 3−6[a]



[a]Reagents and conditions: (a) $Pd(OAc)_2$, dppp, CO (25 psi), TEA, DMSO/MeOH (2:1), 80 °C, 83%; (b) LiOH, THF/H_2O (2:1), 57%; (c) EDC/HOBt, NH_4Cl, DIEA, 97% for **4**; HATU, *N*-methylmorpholine, DMF, 29−39% for **5**−**6**; (d) TFA, 80−90%.

Scheme 4. Synthesis of Macrocyclic Inhibitors 11−13[a]



[a]Reagents and conditions: (a) DMF/CH_3CN (1:2), 80 °C; (b) BOP, TEA, DMF/CH_3CN (1:2), 38−54% for two steps; (c) phosgene, CH_3CN/CH_2Cl_2 (1:1), then TEA; (d) chiral HPLC, 34−43% for 2 steps; (e) TFA/CH_2Cl_2 or 4.0 N HCl in dioxane/EtOAc, 17−90%.

followed by a slow addition of the chloroformate to triethylamine in dichloromethane to finish the lactamization.[26] At this stage, the two diastereoisomers were separated by chiral HPLC to yield the desired enantiomer. Deprotection of the di-Boc group with acid and a final HPLC purification afforded the macrocyclic inhibitors **1**, **8**, and **10**. Macrocyclic inhibitor **9** was obtained by a monohydrolysis of the diethylphosphonate **8** with LiOH. The Petasis reaction/amide coupling provided an efficient and modular synthetic route to macrocyclic TF-VIIa inhibitors.

Syntheses of macrocyclic inhibitors **2** and **7** are shown in Scheme 2. Nitro substituted *N*-methylbenzylamines **21a,b** were coupled with intermediate **18**. After amide coupling, the nitro group was readily reduced to give anilines **20d,e**. Macrocyclization, chiral separation, and deprotection of the di-Boc group as exemplified in Scheme 1 yielded macrocyclic inhibitors **2** and **7**.

Syntheses of macrocyclic inhibitors **3**−**6** were achieved by late stage functionalization of an advanced intermediate, as shown in Scheme 3. The macrocyclic bromide **22** was subjected to a Pd-catalyzed carbonylation to give rise to the methyl ester **23**. Deprotection of the di-Boc group in **23** yielded inhibitor **3**. Methyl ester **23** was hydrolyzed to the corresponding acid **24**. Amide coupling of the acid **24** with amines, followed by deprotection of the di-Boc group, afforded macrocyclic inhibitors **4**-**6**.

Syntheses of macrocyclic inhibitors **11**−**13** are shown in Scheme 4. Petasis reaction of a fluoro-substituted aminoisoquinoline **25a**−**c**, boronic acid **16**, and glyoxylate **17** gave rise to the phenylglycine derivatives **26a**−**c**. Coupling of **26a**−**c** with 4-(cyclopropylsulfonyl)-3-((methylamino)methyl)aniline **19c** afforded phenylglycinamides **27a**−**c**. It is advantageous to carry out the petasis reaction/amide coupling in a two-step, one-pot procedure without isolation of the acid **26**. Macrocylization, chiral separation, and di-Boc deprotection, as described for the synthesis of compounds **1**−**10**, yielded macrocyclic inhibitors **11**−**13**.

Syntheses of fluoro-substituted aminoisoquinolines **25a**−**c** are summarized in Scheme 5. 8- or 7-Fluoro substituted 6-(dibenzylamino)isoquinolin-1(2*H*)-one **28**[28] was treated with $POCl_3$ to generate the corresponding chloride, which was reacted with ammonia in ethylene glycol to yield 8- or 7-fluoro *N*,*N*-dibenzylisoquinoline-1,6-diamine **29**. The displacement of the chloride by ammonia had to be carried out at high temperature in an autoclave to obtain moderate yields of the aminoisoquinolines **29a,b**. Protection of the free amines **29a** and **29b** with Boc anhydride, followed by Pd-catalyzed hydrogenation with Pearlman's catalyst in ethanol, gave rise

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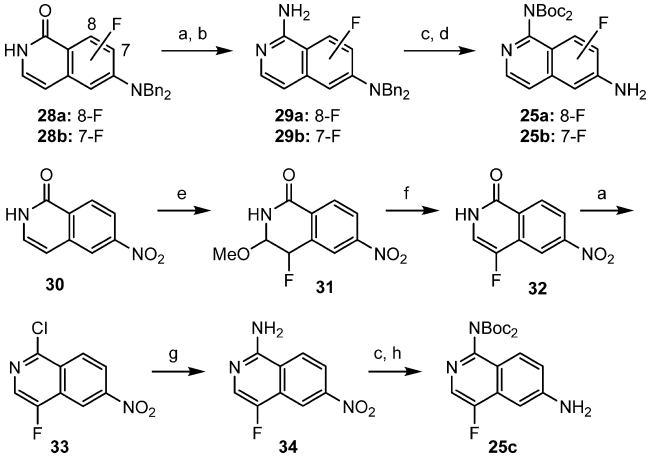
Scheme 5. Synthesis of Fluoro-Substituted Aminoisoquinolines 25a–c[a]



[a]Reagents and conditions: (a) $POCl_3$, 100 °C, >95%; (b) NH_3, $HO(CH_2)_2OH$, 130 °C, 50–64%; (c) $(Boc)_2O$, DMAP (cat.), CH_3CN, 66–77%; (d) $Pd(OH)_2$, H_2 (55 psi), EtOH, 90–96%; (e) Selectfluor, MeOH/CH_3CN (1:1), 82 °C, 99%; (f) 4.0 HCl, dioxane/CH_3CN (1:1), 65 °C, 98%; (g) $Pd(OAc)_2$, benzophenone imine, BINAP, Cs_2CO_3, toluene, 90 °C, then HCl, THF/H_2O, 79%; (h) Pd/C, H_2 (balloon), HCl (cat.), MeOH, 99%.

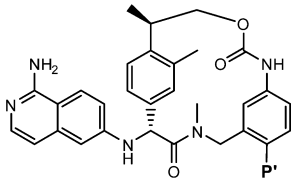
to the corresponding 8- and 7-fluoroaminoisoquinolines **25a** and **25b** in good yield.

A different strategy was devised to prepare the 4-fluoroaminoisoquinoline **25c**. Compound **30**, prepared in high yield as previously described,[28] reacted with Selectfluor in methanol to generate methanol-trapped fluorine adduct **31** in a nearly quantitative yield. Treatment of hemiaminal **31** with HCl in acetonitrile resulted in elimination of methanol and aromatization to afford 4-fluoro-6-nitroisoquinolin-1(2*H*)-one **32** in 98% yield. Compound **32** was converted to the corresponding chloride **33** with $POCl_3$. Displacement of the chloride **33** by ammonia to give amine **34** proved to be problematic because of partial displacement of fluoride. Consequently, Pd-catalyzed amination of the chloride **33** with benzophenone imine, followed by treatment with acid, yielded the 4-fluoro-6-nitroisoquinolin-1-amine **34** in good yield. Boc protection of **34** and reduction of the nitro group afforded 4-fluoroaminoisoquinoline **25c**. The optimized sequence enabled a high yielding and scalable synthesis of the key intermediate **25c**.

RESULTS AND DISCUSSION

Our strategy to improve the potency of macrocyclic inhibitor **1** was to optimize the molecular interactions of the inhibitors with the S′ site of FVIIa, in an analogy to that of the phenylpyrrolidine phenylglycineamide series,[24] by substitution at the ortho position (to the benzyl amide) of the P′ phenyl

Table 1. Summary of P′ SAR of the Macrocyclic TF-FVIIa Inhibitors



Cmpd	P'	FVIIa K_i (nM)[a]	FXa K_i (nM)[b]	FXIa K_i (nM)[b]	Thrombin K_i (nM)[b]	Trypsin K_i (nM)[b]	HK-1 K_i (nM)[b]	FVII def PT EC_{2x} (μM)[c]	PAMPA Pc (nm/s)
1	H	20	> 10000	> 10000	> 10000	> 10000	21	5.3	13
2	OCF_3	5.5	1000	8800	> 10000	950	34	10	230
3	CO_2Me	8.7	2500	7100	> 10000	5900	260	17	ND[d]
4	$CONH_2$	15	> 9000	6500	> 10000	> 6200	580	2.0	58
5	$CONMe_2$	2.5	6200	> 10000	> 10000	1300	650	2.2	19
6	$CONEt_2$	0.57	4300	6100	> 10000	1620	600	2.2	33
7		2.2	2000	3100	9000	1600	200	4.3	59
8		0.61	3900	2900	> 10000	3200	390	2.4	45
9		0.28	4800	1400	> 10000	> 6200	2600	2.6	3.0
10		0.16	1800	2300	3800	450	270	1.1	50

[a]TF-FVIIa assays were performed at 37 °C ($n \geq 2$). [b]K_is for the indicated enzymes were determined at 25 °C, except HK-1, which was run at 37 °C ($n = 2$). [c]See ref 11 for a detailed description of the FVII deficient prothrombin time assay. [d]Not determined

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ring (Table 1). The in vitro activity was expressed as the inhibition constant K_i against human TF-FVIIa at 37 °C with physiologically relevant factor X (FX) as the substrate.[11] The selectivity of the compounds was first evaluated against a panel of serine proteases including coagulation factor Xa (FXa), factor XIa (FXIa), thrombin, trypsin, and human tissue kallikrein (HK-1). For compounds of interest, selectivity was evaluated with a broader range of serine proteases.[11] The anticlotting activity of the compounds was evaluated with a FVII deficient prothrombin time (FVII def PT) assay in human plasma, expressed as the concentration required to prolong clotting time by 2-fold (EC_{2x}).[11] The permeability of the macrocyclic compounds was evaluated in a parallel artificial membrane permeability assay (PAMPA).[30]

Compound **1** showed a promising overall profile. It exhibited good potency and anticlotting activity (K_i = 20 nM, EC_{2x} = 5.3 μM). It displayed excellent selectivity against FXa, FXIa, thrombin, and trypsin. However, it retained an equal potency against HK-1 (K_i = 21 nM) and displayed low but measurable PAMPA permeability (Pc = 13 nm/s). As shown in Table 1, the trifluoromethoxy group in compound **2** improved the binding affinity for TF-FVIIa by 4-fold and maintained good overall selectivity except for HK-1. The anticlotting activity of compound **2** (EC_{2x} = 10 μM) was 2-fold less potent than that of compound **1** despite its improved binding affinity. This is most likely due to its decreased free fraction in human plasma as a result of increased lipophilicity compared to compound **1**. The trifluoromethoxy moiety significantly improved the PAMPA permeability (Pc = 230 nm/s) in compound **2**. Although methyl ester **3** and primary amide **4** only slightly improved the TF-FVIIa binding affinity, the selectivity against HK-1 of the two compounds was significantly improved while the excellent selectivity profile against other coagulation serine proteases was maintained. *N*,*N*-Dimethyl and *N*,*N*-diethyl amide **5** and **6** further improved the selectivity against HK-1 by concomitantly improving the binding affinity to TF-FVIIa and reducing the binding affinity to HK-1. Compound **6** proved to be a highly potent (K_i = 0.57 nM) and selective (>1000-fold) TF-VIIa inhibitor with good anticlotting activity (EC_{2x} = 2.2 μM). Unfortunately, the PAMPA permeability of compound **6** (Pc = 33 nm/s) was only modest compared to **1**.

Small heterocycles such as pyrazole **7** improved the TF-FVIIa activity as well as the anticlotting activity with moderate permeability compared to compound **1**. Diethyl phosphate **8** and ethyl phosphonic acid **9** also significantly improved the TF-VIIa binding affinity and anticlotting activity. Compound **9** displayed an exceptional selectivity profile including against HK-1, suggesting a polar group in the P′ site is beneficial for achieving good HK-1 selectivity. Not surprisingly, the high polarity of compound **9** significantly limited its permeability (Pc = 3 nm/s). The most profound improvement in potency originated from the substitution of cyclopropyl sulfone in the P′ site, affording compound **10** with an excellent TF-FVIIa binding affinity and anticlotting activity (K_i = 0.16 nM, EC_{2x} = 1.1 μM) while still maintaining an excellent selectivity profile (>1600-fold) and moderate permeability (Pc = 50 nm/s). This interaction, which yielded more than 120-fold potency improvement relative to compound **1**, was observed earlier in the acylsulfonamide and phenylpyrrolidine phenylglycinamide series.[24,31] It is believed that the cyclopropylsulfone engages in hydrophobic interaction with Cys42−Cys58 disulfide bridge in the active site of TF-FVIIa complex. Figure 2 depicts a binding model of **13** bound in FVIIa based on crystallographic

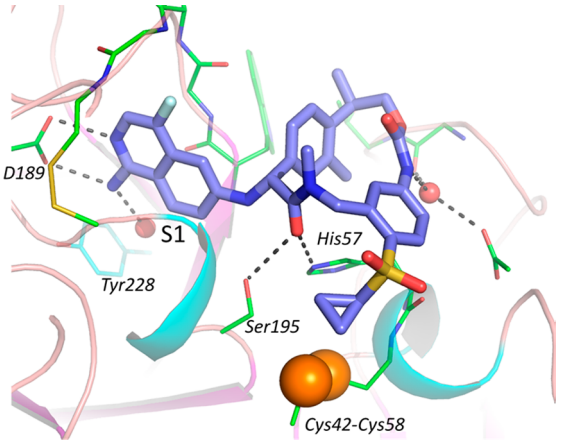

Figure 2. Model of **13** bound in FVIIa. Graphics were generated with the program PyMOL.[35]

structures of analogous molecules.[34] In this model, the fluoroaminoisoquinoline forms a salt bridge with Asp189 side chain and a hydrogen bond with a water molecule above Tyr228 side chain. In the S1′ pocket, the amide carbonyl forms a pair of hydrogen bonds to the catalytic Ser195 and H57 side chains. The phenyl sulfone directs the cyclopropyl ring toward the Cys42−Cys58 disulfide bridge.

Compound **10** showed good metabolic stability in human and dog liver microsomes with $t_{1/2}$ > 100 min but limited metabolic stability in rat liver microsomes with $t_{1/2}$ = 12 min (Table 2). Given its excellent potency and selectivity, good anticlotting activity, and moderate PAMPA permeability, compound **10** was progressed to dog pharmacokinetic studies. To our disappointment, it demonstrated very low oral exposure with only 1% absolute oral bioavailability. We believe its limited oral bioavailability is most likely attributed to its insufficient permeability. Despite modest potency, compound **2** possessed significantly improved PAMPA permeability and good metabolic stability in human and dog liver microsomes. Accordingly, it achieved much higher oral exposure than compound **10** with a 22% oral bioavailability in dogs. These results strongly suggested that good oral exposure of compound **10** could be achieved by improvement of its PAMPA permeability.

It is well established that the strong electron withdrawing nature of fluorine can exert significant effects on the basicity of neighboring functional groups.[32] We decided to investigate fluoro substitution on the aminoisoquinoline P1 in macrocyclic inhibitor **10** as a strategy to reduce the basicity and thus improve permeability (Table 3). The unsubstituted aminoisoquinoline **10** has an experimentally determined pK_a of 8.7 and calculated pK_a of 8.8.[33] Compound **11**, with fluoro substitution at the 8-position, has a measured pK_a of 8.0 relative to 8.7 for compound **10**. As expected, the reduced basicity translated into improved PAMPA permeability (from 50 nm/s in compound **10** to 200 nm/s in compound **11**). The reduction of basicity in **11** by fluorination resulted in 6-fold loss of binding affinity to TF-FVIIa and about 3-fold loss of anticlotting activity compared to **10**. On the other hand, compound **11** improved binding affinity to HK-1 by nearly 3-fold, resulting in a less selective profile. Compound **12**, with fluoro substitution at the 7-position, has a calculated pK_a of 8.5, marginally reduced relative to 8.8 in compound **10**.[33] It resulted in a compound of comparable inhibitory potency relative to **10** but still with improved PAMPA permeability (Pc = 180 nm/s).

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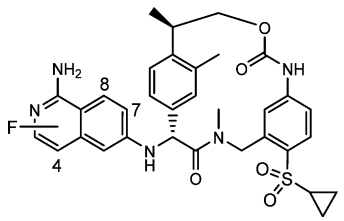
Table 2. Metabolic Stability, Permeability, and Oral Bioavailability of Compounds 10 and 2

	microsomal stability $t_{1/2}$ (min)				
compd	human	dog	rat	PAMPA *Pc* (nm/s)	oral bioavailability in dogs (*F*%)[a]
10	>120	110	12	50	1
2	130	140	37	230	22

[a]Dogs (n = 3) were dosed with 0.2 mg/kg iv and 1.0 mg/kg po.

Table 3. Effects of Fluorine Substitution on P1



compd	F position on P1	pK_a[a]	FVIIa K_i (nM)	HK-1 K_i (nM)	FVII def PT EC_{2x} (μM)	PAMPA *Pc* (nm/s)
10	NA[b]	8.7 (8.8)	0.16	270	1.1	50
11	8-F	8.0 (8.2)	1.0	100	2.8	200
12	7-F	(8.5)	0.23	790	4.2	180
13	4-F	7.1 (7.5)	0.43	650	6.1	900

[a]Data in parentheses refers to QM calculated pK_a. See ref 33 for details. [b]Not applicable.

Fluoro substitution at the 4-position had the most profound impact on pK_a and permeability. Compound **13** achieved nearly two log units reduction of the measured pK_a (from 8.7 to 7.1) and 18-fold improvement of PAMPA permeability (from 50 to 900 nm/s) relative to **10** while maintaining excellent TF-FVIIa inhibitory activity and good anticlotting activity (K_i = 0.43 nM, EC_{2x} = 6.1 μM). The data suggests that 4-fluoroaminoisoquinoline is still capable of strong binding in the S1 specificity pocket of TF-FVIIa via ionic interaction with Asp189 despite its significantly reduced basicity.

The fluorinated macrocyclic inhibitors **11**–**13** showed good metabolic stability in human liver microsomes but poor metabolic stability in rat as observed in compound **10** (Table 4). Further pharmacokinetic studies were performed in dogs because of the good metabolic stability achieved with compounds **11** and **13**.[36] Compound **11**, with a $t_{1/2}$ = 39 min in dog liver microsomes and permeability Pc = 200 nm/s, displayed a moderate clearance and volume of distribution (CL = 13 mL/min/kg, V_{dss} = 8 L/kg) with $t_{1/2}$ = 8 h and *F*% = 24. Compound **12**, with $t_{1/2}$ = 7 min in dog liver microsomes, showed a moderate clearance and low oral exposure, most likely due to combination of poor metabolic stability and insufficient permeability. Compound **13**, with $t_{1/2}$ = 26 min in dog liver microsomes and significantly improved PAMPA permeability (Pc = 900 nm/s), demonstrated a low clearance and moderate volume of distribution (CL = 8 mL/min/kg, V_{dss} = 5 L/kg) with $t_{1/2}$ = 8 h and *F*% = 40. Thus, the 4-fluoroaminoisoquinoline had the most profound impact on pK_a, permeability, and oral bioavailability in this series of macrocyclic TF-FVIIa inhibitors. Because of its poor metabolic stability in rat and mouse liver microsomes ($t_{1/2}$ = 1.9 and 3.1 min, respectively), compound **13** showed poor oral exposure in both species with absolute oral bioavailability <5%.

Because of its superior pharmacokinetic properties in dogs, compound **13** was selected for further in vitro and in vivo characterization. In addition to its excellent TF-FVIIa inhibitory activity, it displayed excellent selectivity toward other proteases important to hemostasis and thrombolysis (Table 5). It showed no significant inhibition in cytochrome P450 enzymes and ion channels (Na, Ca, and hERG). Free fraction of compound **13** in human and dog plasma was measured to be 2.3% and 1.4% respectively. Compound **13** exhibited good binding affinity to rabbit TF-VIIa (K_i = 1.9 nM) with a free fraction of 2.6% in rabbit plasma. In a rabbit model of electrically induced carotid arterial thrombosis (ECAT),[11] compound **13** inhibited thrombus formation in a concentration-dependent manner with an EC_{50} of 360 nM (Figure 3).

Table 5. Selectivity Profile of Macrocyclic Inhibitor 13

human enzyme	K_i (nM)[a]	selectivity (fold)
TF-FVIIa	0.43	NA[b]
FIXa[c]	1600	>3700
FXa	4000	>9300
FXIa	3400	>7900
FXIIa[d]	7500	>17000
thrombin	11000	>26000
trypsin	3000	>7000
plasma kallikrein	520	>1200
aPC[e]	190	440
HK-1	650	>1500
chymotrypsin	17000	>39000
plasmin	1500	>3500
tPA[f]	9100	>21000
urokinase	15000	>35000

[a]TF-FVIIa assays were performed at 37 °C (n > 2). K_is for other indicated enzymes were determined at 25 °C, except aPC and HK-1, which were run at 37 °C (n = 2). [b]Not applicable. [c]Factor IXa. [d]Factor XIIa. [e]Active protein C. [f]Tissue plasminogen activator.

Table 4. Dog Pharmacokinetic Parameters of Macrocyclic Inhibitors 11–13

	microsomal stability $t_{1/2}$ (min)				dog PK[a]			
compd	human	dog	rat	PAMPA Pc (nm/s)	CL (mL/min/kg)	V_{dss} (L/kg)	$t_{1/2}$ (h)	*F* (%)
11	120	39	3.3	200	13	8	8	24
12	95	7	10	180	11	4	6	<1
13	45	26	1.9	900	8	5	8	40

[a]Dogs (n = 3) were dosed with 0.2 mg/kg iv and 1.0 mg/kg po.

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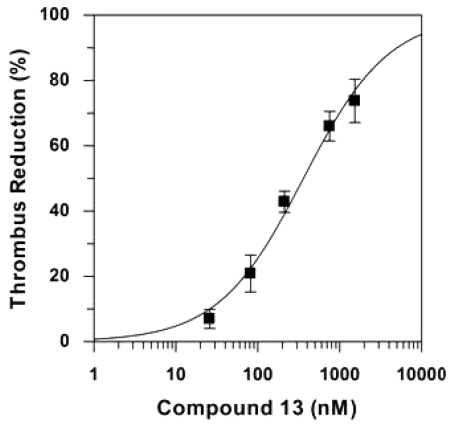

Figure 3. Antithrombotic effects of compound **13** in the rabbit ECAT model.

CONCLUSIONS

In summary, starting from macrocyclic TF-FVIIa inhibitor **1** and optimizing the interactions with the S′ site, we have significantly improved potency and selectivity. The cyclopropyl sulfone **10** showed optimal TF-FVIIa binding affinity and overall selectivity against a panel of serine proteases. To address poor permeability and improve oral bioavailability in the series, we have resorted to fluorination of the aminoisoquinoline P1 group to reduce its basicity. The novel 4-fluoroaminoisoquinoline had a profound impact on pK_a and thus permeability and oral exposure. The resulting lead compound **13** achieved clinical candidate level potency, selectivity, anticlotting activity, and in vivo efficacy. It also achieved acceptable oral bioavailability and half-life in dogs. The key limitation is poor rodent metabolic stability and pharmacokinetics, preventing further advancement.

EXPERIMENTAL SECTION

General Methods for Chemistry. All experiments were carried out under an inert atmosphere and at room temperature unless otherwise stated. Reactions were monitored using thin-layer chromatography on 250 μm plates or using HPLC and LCMS with UV detection at 220 or 254 nm. Purification was accomplished by medium pressure liquid chromatography on a CombiFlash Companion (Teledyne Isco) with RediSep normal phase silica gel or by reverse phase preparative HPLC. All compounds for biological testing were purified by preparative HPLC. Purity of all final compounds was 95% or higher, determined with two orthogonal HPLC conditions (see Supporting Information for details). Reverse phase analytical and preparative HPLC were carried out using Shimadzu HPLC system running Discovery VP software. LCMS chromatograms were obtained on a Shimadzu HPLC system running Discovery VP software, coupled with a Waters ZQ mass spectrometer running MassLynx version 3.5 software. ^{1}H NMR spectra were recorded on a Bruker spectrometer (400 or 500 MHz) at ambient temperature.

*(2R,15R)-2-[(1-Aminoisoquinolin-6-yl)amino]-4,15,17-trimethyl-13-oxa-4,11-diazatricyclo[14.2.2.1^{6,10}]henicosa-1(18),6,8,10-(21),16,19-hexaene-3,12-dione (**1**).* Phosgene (0.12 mL, 0.19 mmol, 20% in toluene) was added dropwise to a solution of **20a** (115 mg, 0.17 mmol) in MeCN (1.5 mL)/CH_2Cl_2 (1.5 mL) at 0 °C. The bath was removed, and the mixture was stirred at room temperature for 30 min. Argon was bubbled through the solution to remove excess phosgene, then the mixture was added to a solution of TEA (0.11 mL, 0.79 mmol) in CH_2Cl_2 (25 mL) at 40 °C over 5 h. The reaction was stirred at room temperature overnight, quenched with H_2O (1 mL) and MeOH (5 mL), and concentrated. The crude product was purified by flash chromatography (0−100% EtOAc/hexanes) to give a mixture of diastereoisomers. The diastereoisomers were separated by a prep chiral HPLC (Whelk-01 25 cm × 21.1 mm, 50% (1:1 MeOH/EtOH) in heptanes) to give the desired enantiomer (30 mg, 0.083 mmol, 49% yield). MS (ESI) m/z 710.5 [M + H]$^+$.

Trifluoroacetic acid (2 mL) was added to the above desired enantiomer (30 mg, 0.083 mmol), and the reaction was stirred at room temperature for 1 h. The mixture was concentrated and purified by prep HPLC to yield **1** (19.0 mg, 36% yield) as a white solid. MS (ESI) m/z 510.2 [M + H]$^+$. ^{1}H NMR (500 MHz, MeOH-d_4) δ 8.05 (d, J = 9.35 Hz, 1H), 7.63 (dd, J = 1.79, 7.84 Hz, 1H), 7.43 (d, J = 7.98 Hz, 1H), 7.31 (d, J = 7.15 Hz, 1H), 7.23 (d, J = 1.38 Hz, 1H), 7.21 (dd, J = 2.48, 9.35 Hz, 1H), 7.18 (t, J = 7.84 Hz, 1H), 6.91 (d, J = 6.88 Hz, 2H), 6.84 (d, J = 2.20 Hz, 1H), 6.69 (dd, J = 0.96, 7.84 Hz, 1H), 5.93 (s, 1H), 5.74 (s, 1H), 5.45 (d, J = 16.51 Hz, 1H), 4.61- 4.69 (m, 1H), 3.96−4.03 (m, 1H), 3.91 (d, J = 16.51 Hz, 1H), 3.46−3.55 (m, 1H), 3.29 (s, 3H), 2.34 (s, 3H), 1.31 (d, J = 6.88 Hz, 3H). HPLC purity 100%.

*(2R,15R)-2-[(1-Aminoisoquinolin-6-yl)amino]-4,15,17-trimethyl-7-(trifluoromethoxy)-13-oxa-4,11-diazatricyclo[14.2.2.1^{6,10}]-henicosa-1(18),6,8,10(21),16,19-hexaene-3,12-dione (**2**).* In a procedure similar to that described for **1**, **20d** (1.21 g, 1.58 mmol) reacted with phosgene/TEA, diastereoisomers were chirally separated (Chiral OD 10 μm 4.6 × 250 mm, 40% EtOH/MeOH(50/50) with 0.1%DEA in heptanes), and the desired enantiomer was deprotected with TFA to give **2** (240 mg, 41% yield) as a white solid. MS (ESI) m/z 594.7 [M + H]$^+$. ^{1}H NMR (400 MHz, MeOH-d_4) δ ppm 9.00 (s, 1H), 7.95 (d, J = 8.79 Hz, 1H), 7.54−7.59 (m, 1H), 7.36 (d, J = 7.70 Hz, 1H), 7.22 (d, J = 7.15 Hz, 1H), 7.09−7.14 (m, 2H), 7.06 (d, J = 7.15 Hz, 1H), 6.82 (d, J = 7.15 Hz, 1H), 6.75 (d, J = 2.20 Hz, 1H), 6.68 (dd, J = 8.79, 2.20 Hz, 1H), 5.94 (d, J = 2.20 Hz, 1H), 5.66 (s, 1H), 5.34 (d, J = 17.59 Hz, 1H), 4.56 (t, J = 10.99 Hz, 1H), 3.80−3.91 (m, 2H), 3.44 (s, 3H), 2.24 (s, 3H), 1.22 (d, J = 7.15 Hz, 3H). ^{19}F NMR (376 MHz, MeOH-d_4) δ ppm −59.11 (s, 3F). HPLC purity 96%.

*Methyl (2R,15R)-2-[(1-Aminoisoquinolin-6-yl)amino]-4,15,17-trimethyl-3,12-dioxo-13-oxa-4,11-diazatricyclo[14.2.2.1^{6,10}]henicosa-1(18),6,8,10(21),16,19-hexaene-7-carboxylate (**3**).* **23** (15 mg, 0.02 mmol) was dissolved in TFA (1 mL) and stirred for 1 h. The mixture was concentrated and purified by prep HPLC to yield **3** (11.2 mg, 80% yield). MS (ESI) m/z 568.2 [M + H]$^+$. ^{1}H NMR (400 MHz, MeOH-d_4) δ ppm 9.32 (s, 1H), 8.04 (d, J = 9.34 Hz, 1H), 7.92 (m, 1H), 7.63−7.71 (m, 1H), 7.47 (m, 1H), 7.31 (m, 1H), 7.20 (dd, J = 9.34, 2.20 Hz, 1H), 6.92 (m, 1H), 6.85 (d, J = 2.20 Hz, 1H), 6.74 (dd, J = 8.79, 2.20 Hz, 1H), 6.21 (br s, 1H), 5.68−5.74 (m, 2H), 4.63 (t, J = 10.99 Hz, 1H), 3.96 (dd, J = 10.99, 4.40 Hz, 1H), 3.79−3.88 (m, 3H), 3.61−3.74 (m, 5H), 2.31 (s, 3H), 1.32 (d, J = 7.15 Hz, 3H). HPLC purity 95%.

*(2R,15R)-2-[(1-Aminoisoquinolin-6-yl)amino]-4,15,17-trimethyl-3,12-dioxo-13-oxa-4,11- diazatricyclo[14.2.2.1^{6,10}]henicosa-1-(18),6,8,10(21),16,19-hexaene-7-carboxamide (**4**).* **24** (12 mg, 0.016 mmol), EDC (6.1 mg, 0.032 mmol), HOBt (4.9 mg, 0.032 mmol), NH_4Cl (1.7 mg, 0.032 mmol), and DIEA (0.011 mL, 0.064 mmol) were added into a vial. To this mixture was added DMF (0.5 mL) and stirred at room temperature under argon overnight. Water was added, and the reaction mixture was extracted with EtOAc. The combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated. The crude product was treated with TFA (1.0 mL) for 15 min to deprotect the di-Boc group. The mixture was concentrated and purified by prep HPLC to yield **4** (3.1 mg, 97% yield) as a white solid. MS (ESI) m/z 553.1 [M + H]$^+$. ^{1}H NMR (400 MHz, MeOH-d_4) δ ppm 8.04 (d, J = 9.03 Hz, 1H), 7.66 (d, J = 7.53 Hz, 1H), 7.46 (t, J = 7.28 Hz, 2H), 7.31 (d, J = 7.28 Hz, 1H), 7.20 (dd, J = 9.16, 2.13 Hz, 1H), 7.16 (s, 1H), 6.91 (d, J = 7.03 Hz, 1H), 6.84 (s, 1H), 6.68−6.78 (m, 1H), 6.17 (br s, 1H), 5.72 (s, 1H), 5.43−5.62 (m, 1H), 4.63 (t, J = 11.04 Hz, 1H), 4.21 (d, J = 17.57 Hz, 1H), 3.98 (dd, J = 10.54, 4.27 Hz, 1H), 3.58−3.41 (m, 1H), 3.28 (s, 3H), 2.32 (s, 3H), 1.32 (d, J = 7.03 Hz, 3H). HPLC purity 97%.

*(2R,15R)-2-[(1-Aminoisoquinolin-6-yl)amino]-N,N,4,15,17-pentamethyl-3,12-dioxo-13-oxa-4,11-diazatricyclo[14.2.2.1^{6,10}]-henicosa-1(18),6,8,10(21),16,19-hexaene-7-carboxamide (**5**).* To a solution of **24** (8 mg, 10.6 μmol) at 0 °C in DMF (0.3 mL) and MeCN (0.3 mL) was added HATU (4.8 mg, 0.013 mmol) and *N*-methylmorpholine (1.75 μL, 0.016 mmol). The reaction was stirred

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for 5 min. Then 2 M dimethylamine in THF (6.4 μL, 0.013 mmol) was added and the reaction was allowed to warm to room temperature and stirred for 4 h. The reaction mixture was diluted with water and extracted with EtOAc. The combined organic layer was washed with water, brine, dried over $MgSO_4$, and concentrated. The di-Boc intermediate was treated with TFA (1.5 mL) for 30 min. The reaction mixture was then concentrated and purified by prep HPLC to give **5** (2.9 mg, 39% yield). MS (ESI) m/z 581.2 $[M + H]^+$. ^1H NMR (400 MHz, MeOH-d_4) δ ppm 8.06 (d, J = 9.29 Hz, 1H), 7.61−7.71 (m, 1H), 7.47 (d, J = 8.03 Hz, 2H), 7.33 (d, J = 7.03 Hz, 1H), 7.18−7.26 (m, 1H), 7.13 (d, J = 8.03 Hz, 1H), 6.93 (d, J = 7.03 Hz, 1H), 6.84 (d, J = 2.26 Hz, 1H), 6.77 (dd, J = 8.03, 2.01 Hz, 1H), 6.14 (s, 1H), 5.75 (s, 1H), 5.27 (s, 1H), 4.60−4.73 (m, 1H), 4.01 (dd, J = 10.67, 4.39 Hz, 1H), 3.88 (d, J = 16.56 Hz, 1H), 3.44−3.58 (m, 1H), 3.27 (s, 3H), 3.11 (s, 3H), 2.94 (s, 3H), 2.35 (s, 3H), 1.33 (d, J = 7.03 Hz, 3H). HPLC purity 98%.

*(2R,15R)-2-[(1-Aminoisoquinolin-6-yl)amino]-N,N-diethyl-4,15,17-trimethyl-3,12-dioxo-13-oxa-4,11-diazatricyclo[14.2.2.1^{6,10}]henicosa-1(18),6,8,10(21),16,19-hexaene-7-carboxamide (**6**).* In a procedure similar to that described for **5**, replacing dimethylamine with diethylamine, **6** (3.4 mg, 29% yield) was obtained as a white solid. MS (ESI) m/z 609.3 $[M + H]^+$. ^1H NMR (400 MHz, MeOH-d_4) δ ppm 7.99−8.11 (m, 1H), 7.65 (dd, J = 7.78, 1.76 Hz, 1H), 7.46 (d, J = 8.03 Hz, 1H), 7.31 (d, J = 7.03 Hz, 1H), 7.15−7.26 (m, 2H), 7.10 (d, J = 8.03 Hz, 1H), 6.91 (d, J = 7.03 Hz, 1H), 6.83 (d, J = 2.26 Hz, 1H), 6.76 (dd, J = 8.03, 1.76 Hz, 1H), 6.10 (s, 1H), 5.73 (s, 1H), 5.25 (br s, 1H), 4.65 (t, J = 11.04 Hz, 1H), 3.99 (dd, J = 10.79, 4.27 Hz, 1H), 3.87 (br s, 2H), 3.59 (d, J = 1.00 Hz, 1H), 3.42−3.56 (m, 2H), 3.27 (s, 3H), 2.33 (s, 3H), 1.27−1.37 (m, 3H), 1.25 (t, J = 7.15 Hz, 3H), 1.10 (t, J = 7.03 Hz, 3H). HPLC purity 99%.

*(2R,15R)-2-[(1-Aminoisoquinolin-6-yl)amino]-4,15,20-trimethyl-7-(1-methyl-1H-pyrazol-5-yl)-13-oxa-4,11-diazatricyclo-[14.2.2.1^{6,10}]henicosa-1(18),6,8,10(21),16,19-hexaene-3,12-dione (**7**).* In a procedure similar to that described for **1**, **20e** (200 mg, 0.26 mmol) reacted with phosgene/TEA, diastereoisomers were chirally separated (R,R-Whelk-01 column 21.1 mm × 250 mm; isocratic 45% MeOH/EtOH (1:1)/65% heptanes), and the desired enantiomer was deprotected with TFA to give **7** (8.5 mg, 5% yield). MS (ESI) m/z 590.4 $[M + H]^+$. ^1H NMR (400 MHz, MeOH-d_4) δ ppm 8.02 (d, J = 9.23 Hz, 1H), 7.55−7.79 (m, 2H), 7.39−7.56 (m, 2H), 7.29 (d, J = 7.03 Hz, 1H), 7.15−7.22 (m, 2H), 7.12 (d, J = 8.35 Hz, 1H), 6.88 (d, J = 7.03 Hz, 1H), 6.80 (br s, 2H), 6.29 (d, J = 1.76 Hz, 1H), 6.17 (s, 1H), 5.70 (s, 1H), 4.98−5.14 (m, 2H), 4.16−4.25 (m, 2H), 3.98 (dd, J = 10.55, 4.39 Hz, 1H), 3.67−3.70 (m, 3H), 3.67 (s, 3H), 3.49−3.54 (m, 3H), 2.33 (s, 3H). HPLC purity 96%.

*Diethyl [(2R,15R)-2-[(1-Aminoisoquinolin-6-yl)amino]-4,15,17-trimethyl-3,12-dioxo-13-oxa-4,11-diazatricyclo[14.2.2.1^{6,10}]henicosa-1(18),6,8,10(21),16,19-hexaen-7-yl]phosphonate (**8**).* In a procedure similar to that described for **1**, **20b** (130 mg, 0.16 mmol) reacted with phosgene/TEA, diastereoisomers were chirally separated (Chiralcel AD-H, 2.0 cm × 25 cm), and the desired enantiomer was deprotected with 4.0 N HCl in dioxane to give **8** (24 mg, 22% yield) as a white solid. MS (ESI) m/z 646.1 $[M + H]^+$. ^1H NMR (400 MHz, MeOH-d_4) δ ppm 9.39 (s, 1H), 8.04 (d, J = 9.23 Hz, 1H), 7.74 (dd, J = 14.06, 8.35 Hz, 1H), 7.67 (dd, J = 7.91, 1.32 Hz, 1H), 7.47 (d, J = 7.91 Hz, 1H), 7.31 (d, J = 7.03 Hz, 1H), 7.19 (dd, J = 9.23, 2.20 Hz, 1H), 7.16 (s, 1H), 6.92 (d, J = 7.03 Hz, 1H), 6.84 (m, 1H), 6.76−6.82 (m, 1H), 6.22−6.29 (m, 1H), 5.74 (s, 1H), 5.67 (d, J = 17.14 Hz, 1H), 4.63 (t, J = 10.99 Hz, 1H), 4.06−4.14 (m, 4H), 3.96−4.02 (m, 1H), 3.43−3.55 (m, 1H), 3.35 (s, 3H), 2.30 (s, 3H), 1.29−1.38 (m, 9H). HPLC purity 95%.

*[(2R,15R)-2-[(1-Aminoisoquinolin-6-yl)amino]-4,15,20-trimethyl-3,12-dioxo-13-oxa-4,11-diazatricyclo[14.2.2.1^{6,10}]henicosa-1-(18),6,8,10(21),16,19-hexaen-7-yl](ethoxy)phosphinic Acid (**9**).* One M LiOH (10 drops) was added to a solution of **8** (8 mg, 0.012 mmol) in DMSO (4 drops) and THF (0.1 mL), and the mixture was stirred at 40 °C overnight. The reaction mixture was neutralized with 1.0 M HCl (15 drops) and concentrated. The crude product was purified by prep HPLC to yield **9** (4.0 mg, 51% yield) as a white solid. MS (ESI) m/z 618.5 $[M + H]^+$. ^1H NMR (400 MHz, MeOH-d_4) δ ppm 8.05 (d, J = 9.23 Hz, 1H), 7.75 (dd, J = 13.84, 8.13 Hz, 1H), 7.67 (dd, J = 7.91, 1.76 Hz, 1H), 7.47 (d, J = 8.35 Hz, 1H), 7.31 (d, J = 7.03 Hz, 1H), 7.17−7.24 (m, 2H), 6.92 (d, J = 7.03 Hz, 1H), 6.84 (d, J = 2.20 Hz, 1H), 6.76 (d, J = 7.91 Hz, 1H), 6.15−6.22 (m, 1H), 5.66−5.75 (m, 2H), 4.63 (t, J = 10.99 Hz, 1H), 4.21 (d, J = 17.14 Hz, 1H), 3.92−4.03 (m, 4H), 3.41−3.55 (m, 1H), 3.33 (br s, 3H), 2.33 (s, 3H), 1.24−1.38 (m, 6H). HPLC purity 99%.

*(2R,15R)-2-[(1-Aminoisoquinolin-6-yl)amino]-7-(cyclopropanesulfonyl)-4,15,20-trimethyl-13-oxa-4,11-diazatricyclo[14.2.2.1^{6,10}]-henicosa-1(18),6,8,10(21),16,19-hexaene-3,12-dione (**10**).* In a procedure similar to that described for **1**, **20c** (0.66 g, 0.83 mmol) reacted with phosgene/TEA, diastereoisomers were chirally separated (R,R-Whelk-01 column 21.1 mm × 250 mm), and the desired enantiomer was deprotected with TFA to give **10** (115.8 mg, 38% yield) as white solid. MS (ESI) m/z 614.1 $[M + H]^+$. ^1H NMR (400 MHz, MeOH-d_4) δ ppm 9.50 (s, 1H), 8.05 (d, J = 9.29 Hz, 1H), 7.73 (d, J = 8.53 Hz, 1H), 7.68 (dd, J = 7.91, 1.63 Hz, 1H), 7.48 (d, J = 8.03 Hz, 1H), 7.32 (d, J = 7.03 Hz, 1H), 7.20 (dd, J = 9.16, 2.38 Hz, 1H), 7.11 (s, 1H), 6.92 (d, J = 7.03 Hz, 1H), 6.80−6.86 (m, 2H), 6.42 (t, J = 2.38 Hz, 1H), 5.78 (d, J = 17.57 Hz, 1H), 5.74 (s, 1H), 4.64 (t, J = 11.04 Hz, 1H), 4.30 (d, J = 17.57 Hz, 1H), 3.99 (dd, J = 10.79, 4.27 Hz, 1H), 3.46−3.55 (m, 1H), 3.40 (s, 3H), 2.81−2.92 (m, 1H), 2.31 (s, 3H), 1.34 (d, J = 7.03 Hz, 3H), 1.22−1.30 (m, 1H), 1.01−1.17 (m, 3H). HPLC purity 98%.

*(2R,15R)-2-[(1-Amino-8-fluoroisoquinolin-6-yl)amino]-7-(cyclopropanesulfonyl)-4,15,17- trimethyl-13-oxa-4,11-diazatricyclo-[14.2.2.1^{6,10}]henicosa-1(18),6,8,10(21),16,19-hexaene-3,12-dione (**11**).* In a procedure similar to that described for **1**, **27a** (0.2 g, 0.25 mmol) reacted with phosgene/TEA, diastereoisomers were chirally separated (Chiral OD 10 um 4.6 mm × 250 mm; isocratic 35% 1:1 MeOH:EtOH/heptanes with 0.1% DEA), and the desired enantiomer was deprotected with 4 N HCl in dioxane to give the **11** (7.7 mg, 10% yield). MS (ESI) m/z 632.4 $[M + H]^+$. ^1H NMR (400 MHz, MeOH-d_4) δ ppm 7.71 (d, J = 8.34 Hz, 1H), 7.63 (dd, J = 7.83, 1.77 Hz, 1H), 7.54−7.41 (m, 2H), 7.10 (d, J = 1.52 Hz, 1H), 6.81 (dd, J = 8.46, 2.15 Hz, 1H), 6.78−6.64 (m, 2H), 6.51 (d, J = 2.27 Hz, 1H), 6.41 (d, J = 2.02 Hz, 1H), 5.75 (d, J = 17.43 Hz, 1H), 5.63 (s, 1H), 4.62 (t, J = 10.99 Hz, 1H), 4.28 (d, J = 17.68 Hz, 1H), 3.97 (dd, J = 10.86, 4.29 Hz, 1H), 3.55−3.44 (m, 1H), 3.40−3.36 (m, 4H), 2.29 (s, 3H), 1.32 (t, J = 7.33 Hz, 3H), 1.27−1.14 (m, 2H), 1.15−0.95 (m, 2H) ^{19}F NMR (376 MHz, MeOH-d_4) δ ppm −115.25 (s, 1F). HPLC purity 98%.

*(2R,15R)-2-[(1-Amino-7-fluoroisoquinolin-6-yl)amino]-7-(cyclopropanesulfonyl)-4,15,17-trimethyl-13-oxa-4,11-diazatricyclo-[14.2.2.1^{6,10}]henicosa-1(18),6,8,10(21),16,19-hexaene-3,12-dione (**12**).* In a procedure similar to that described for **1**, **27b** (80 mg, 0.1 mmol) reacted with phosgene/TEA, diastereoisomers were chirally separated (R,R-Whelk-01 column 21.1 mm × 250 mm), and the desired enantiomer was deprotected with 4 N HCl in dioxane to give the **12** (19 mg, 24% yield). MS (ESI) m/z 632.4 $[M + H]^+$. ^1H NMR (400 MHz, MeCN-d_3) δ ppm 7.92 (d, J = 12.64 Hz, 1H), 7.64−7.73 (m, 2H), 7.44 (d, J = 7.70 Hz, 1H), 7.29 (d, J = 7.15 Hz, 1H), 6.93−6.99 (m, 2H), 6.90 (d, J = 7.15 Hz, 1H), 6.69−6.84 (m, 1H), 6.34 (s, 1H), 5.69−5.77 (m, 2H), 4.54−4.58 (m, 1H), 4.23 (d, J = 17.59 Hz, 1H), 3.91 (dd, J = 10.44, 4.40 Hz, 1H), 3.38−3.49 (m, 1H), 3.31 (s, 3H), 2.74−2.88 (m, 1H), 2.21 (s, 3H), 1.25 (d, J = 6.60 Hz, 3H), 0.97−1.13 (m, 4H). ^{19}F NMR (376 MHz, MeCN-d_3) δ ppm −131.68 (s, 1F). HPLC purity 99%.

*(2R,15R)-2-[(1-Amino-4-fluoroisoquinolin-6-yl)amino]-7-(cyclopropanesulfonyl)-4,15,17-trimethyl-13-oxa-4,11-diazatricyclo-[14.2.2.1^{6,10}]henicosa-1(18),6,8,10(21),16,19-hexaene-3,12-dione (**13**).* In a procedure similar to that described for **1**, **27c** (135 mg, 0.17 mmol) reacted with phosgene/TEA, diastereoisomers were chirally separated (R,R-Whelk-01 column 21.1 mm × 250 mm), and the desired enantiomer was deprotected with 4 N HCl in dioxane to give the **13** (39 mg, 31% yield). MS (ESI) m/z 632.1 $[M + H]^+$. ^1H NMR (400 MHz, MeCN-d_3) δ ppm 7.74−7.81 (m, 2H), 7.71 (d, J = 8.35 Hz, 1H), 7.64−7.69 (m, 1H), 7.45 (d, J = 7.91 Hz, 1H), 7.21 (d, J = 4.83 Hz, 1H), 7.13 (dd, J = 9.23, 2.20 Hz, 1H), 6.98 (s, 1H), 6.88 (s, 1H), 6.81 (dd, J = 8.35, 1.76 Hz, 1H), 6.30 (d, J = 1.76 Hz, 1H), 5.70 (d, J = 17.14 Hz, 1H), 5.65 (d, J = 3.95 Hz, 1H), 4.56 (t, J = 10.99 Hz, 1H), 4.18 (d, J = 17.58 Hz, 1H), 3.91 (dd, J = 10.55, 4.39 Hz, 1H),

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3.39 (ddd, J = 11.32, 7.14, 4.39 Hz, 1H), 3.27 (s, 3H), 2.63−2.72 (m, 1H), 2.17 (s, 3H), 1.25 (d, J = 7.03 Hz, 3H), 1.05−1.20 (m, 2H), 0.93−1.04 (m, 2H). ^{13}C NMR (125.66 MHz, MeOH-d_4) δ ppm 171.60, 154.81, 153.62, 152.98, 149.39, 145.12, 143.87, 139.51, 139.18, 134.39, 133.66, 133.08, 133.00, 129.10, 128.31, 128.10, 120.16, 119.80, 119.18, 113.35, 109.38, 99.33, 72.08, 58.15, 51.87, 37.81, 36.17, 33.56, 20.28, 15.72, 6.60, 6.18. ^{19}F NMR (376 MHz, MeCN-d_3) δ ppm −154.37 (s, 1F). e.e. 99.8%. Anal. Calcd for $C_{33}H_{34}N_5O_5SF$·HCl: C, 55.60; H, 5.70; N, 9.64; S, 4.41; Cl, 5.30. Found: C, 56.79; H, 5.49; N, 9.50; S, 4.39; Cl, 5.20. HPLC purity 98%.

*tert-Butyl N-{6-[({[(3-Aminophenyl)methyl](methyl)carbamoyl}({4-[(2R)-1-hydroxypropan-2-yl]-3-methylphenyl})methyl)amino]-isoquinolin-1-yl}-N-[(tert-butoxy)carbonyl]carbamate (**20a**).* To a mixture of **15** (120 mg, 0.33 mmol), **16** (64.8 mg, 0.33 mmol), and glyoxylic acid monohydrate **17** (31 mg, 0.33 mmol) were added DMF (1 mL) and MeCN (2 mL). The heterogeneous mixture was stirred at 80 °C for 3 h to give an orange solution of **18**, which was cooled to room temperature. To this mixture were added **19a** (50.0 mg, 0.37 mmol), BOP (162 mg, 0.37 mmol), and TEA (0.23 mL, 1.7 mmol). The mixture was stirred at room temperature for 1 h, diluted with EtOAc, washed with water and brine, dried over Na_2SO_4, and concentrated. The crude product was purified by flash chromatography (1−10% MeOH/CH_2Cl_2) to afford **20a** (117 mg, 51% yield) as off-white solid. MS (ESI) m/z 684.5 $[M + H]^+$. ^{1}H NMR (500 MHz, MeOH-d_4) δ 8.03 (dd, J = 18.0, 5.9 Hz, 1H), 7.65−7.53 (m, 1H), 7.49−7.35 (m, 2H), 7.35−7.13 (m, 3H), 7.13−6.94 (m, 1H), 6.80−6.44 (m, 4H), 5.64−5.48 (m, 1H), 4.65−4.45 (m, 2H), 3.69−3.64 (m, 1H), 3.58−3.53 (m, 1H), 3.22−3.15 (m, 1H), 3.05 (s, 3H), 2.39−2.34 (m, 3H), 1.30−1.25 (m, 18H), 1.24−1.20 (m, 3H). ^{1}H NMR was complicated by a pair of diastereomers and rotamers.

*tert-Butyl N-(6-{[({[5-Amino-2-diethoxyphosphoryl)phenyl]-methyl}(methyl)carbamoyl)({4-[(2R)-1-hydroxypropan-2-yl]-3-methylphenyl})methyl]amino}isoquinolin-1-yl)-N-[(tert-butoxy)-carbonyl]carbamate (**20b**).* In a procedure similar to that described for **20a**, replacing **19a** with **19b**, **20b** (135 mg, 71% yield) was obtained as a yellow solid. MS (ESI) m/z 820.1 $[M + H]^+$. ^{1}H NMR was complicated by a pair of diastereomers and rotamers.

*tert-Butyl N-(6-{[({[5-Amino-2-(cyclopropanesulfonyl)phenyl]-methyl}(methyl)carbamoyl)({4-[(2R)-1-hydroxypropan-2-yl]-3-methylphenyl})methyl]amino}isoquinolin-1-yl)-N-[(tert-butoxy)-carbonyl]carbamate (**20c**).* In a procedure similar to that described for **20a**, replacing **19a** with **19c**, **20c** (0.66 g, 77% yield) was obtained as an orange glass, which was lyophilized to give a white powder. MS (ESI) m/z 788.2 $[M + H]^+$. ^{1}H NMR was complicated by a pair of diastereomers and rotamers.

*tert-Butyl N-(6-{[({[5-Amino-2-(trifluoromethoxy)phenyl]methyl}-(methyl)carbamoyl)({4-[(2R)-1-hydroxypropan-2-yl]-3-methylphenyl})methyl]amino}isoquinolin-1-yl)-N-[(tert-butoxy)-carbonyl]carbamate (**20d**).* To a mixture of **15** (1.0 g, 2.8 mmol), **16** (0.54 g, 2.8 mmol), and glyoxylic acid monohydrate **17** (0.21 g, 2.8 mmol) were added DMF (6 mL) and MeCN (12 mL). The heterogeneous mixture was stirred at 80 °C for 3 h to give an orange solution of **18**, which was cooled to room temperature. To this mixture were added **21a** (0.77 g, 2.7 mmol), BOP (1.49 g, 3.4 mmol), and DIEA (1.2 mL, 6.8 mmol). The reaction was stirred at room temperature overnight, diluted with EtOAc, washed with water and brine, dried with Na_2SO_4, and concentrated. The crude product was purified by flash chromatography (1−10% MeOH/CH_2Cl_2) to afford the nitro intermediate (1.35 g, 63% yield) as a solid. MS (ESI) m/z 798.8 $[M + H]^+$.

A mixture of the above nitro intermediate (1.34 g, 1.68 mmol), 4.0N HCl in dioxane (0.42 mL, 1.68 mmol), and 10% Pd/C (600 mg) in MeOH (50 mL) was hydrogenated with a hydrogen balloon for 1.5 h. Pd/C was removed by filtration. The filtrate was concentrated and redissolved in CH_2Cl_2 and washed with satd $NaHCO_3$ and half-saturated brine. The organic layer was dried over Na_2SO_4 and concentrated to give **20d** (1.2 g, 94% yield) as a yellow solid. MS (ESI) m/z 768.4 $[M + H]^+$. ^{1}H NMR was complicated by a pair of diastereomers and rotamers.

*tert-Butyl-N-(6-{[({[5-amino-2-(1-methyl-1H-pyrazol-5-yl)phenyl]-methyl}(methyl)carbamoyl)({4-[(2R)-1-hydroxypropan-2-yl]-3-methylphenyl})methyl]amino}isoquinolin-1-yl)-N-[(tert-butoxy)-carbonyl]carbamate (**20e**).* In a procedure similar to that described for **20d**, replacing **21a** with **21b**, **20e** (210 mg, 82% yield) was obtained as a solid. MS (ESI) m/z 764.5 $[M + H]^+$. ^{1}H NMR was complicated by a pair of diastereomers and rotamers.

*tert-Butyl N-(6-{[(2R,15R)-7-Bromo-4,15,20-trimethyl-3,12-dioxo-13-oxa-4,11-diazatricyclo[14.2.2.1^{6,10}]henicosa-1(18),6,8,10-(21),16,19-hexaen-2-yl]amino}isoquinolin-1-yl)-N-[(tert-butoxy)-carbonyl]carbamate (**22**).* A solution of **15** (0.7 g, 1.9 mmol), glyoxylic acid monohydrate **17** (0.18 g, 1.9 mmol), and **16** (0.38 g, 1.9 mmol) in DMF (3 mL) and MeCN (3 mL) was stirred at 80 °C for 1.5 h. The mixture was cooled to room temperature and diluted with DMF (2 mL). To this mixture were added sequentially 4-bromo-3-((methylamino)methyl)aniline (0.67 g, 2.3 mmol), BOP (0.94 g, 2.1 mmol), and TEA (1.6 mL, 11 mmol). The mixture was stirred at room temperature for 30 min, then diluted with EtOAc, washed with water and brine, and dried (Na_2SO_4). The solvent was removed under reduced pressure. The residue was redissolved in CH_2Cl_2 with 3% MeOH and purified by flash chromatography (1−10% MeOH/CH_2Cl_2) to give the intermediate amide (1.2 g, 79% yield) as an orange glass. MS (ESI) m/z 762.5 and 764.5 $[M + H]^+$. ^{1}H NMR was complicated by a pair of diastereomers and rotamers.

A solution of the above amide (1.17 g, 1.53 mmol) in MeCN (8 mL), CH_2Cl_2 (8 mL), and DMPU (0.6 mL) was cooled to 0 °C. To this solution was added phosgene (20% in toluene, 0.84 mL, 1.69 mmol). The mixture was stirred at 0 °C for 15 min and then bubbled with Ar for 10 min to remove excess phosgene. The resulting solution was added dropwise over 3 h (via a syringe pump) into a solution of TEA (2.1 mL, 15.4 mmol) in CH_2Cl_2 (400 mL) at 40 °C. The solution was stirred for an additional 30 min. Reaction mixture was concentrated, suspended in EtOAc (250 mL), washed with water and brine, and dried (Na_2SO_4). EtOAc was removed under reduced pressure, and the residue was purified by flash chromatography (50−100% EtOAc/hexanes) to give product as a mixture of diastereomers. The diastereoisomers were separated by chiral HPLC (Chiral OD 10 μm 4.6 mm × 250 mm; solvent A heptanes; solvent B 50% MeOH−50% EtOH) to give **22** (0.2 g, 33% yield). MS (ESI) m/z 788.1 $[M + H]^+$. ^{1}H NMR (400 MHz, MeOH-d_4) δ ppm 8.04 (d, J = 5.50 Hz, 1H), 7.68 (d, J = 6.05 Hz, 1H), 7.60 (d, J = 9.34 Hz, 1H), 7.51 (d, J = 6.05 Hz, 1H), 7.46 (d, J = 7.70 Hz, 1H), 7.40 (d, J = 8.24 Hz, 1H), 7.27 (dd, J = 8.79, 2.20 Hz, 1H), 7.22 (s, 1H), 6.87 (d, J = 2.20 Hz, 1H), 6.63 (dd, J = 8.52, 2.47 Hz, 1H), 5.99 (d, J = 2.20 Hz, 1H), 5.71 (s, 1H), 5.35 (d, J = 17.04 Hz, 1H), 4.65 (t, J = 11.27 Hz, 1H), 3.95 (dd, J = 10.99, 4.40 Hz, 1H), 3.65 (s, 1H), 3.44−3.53 (m, 1H), 3.34 (s, 3H), 2.31 (s, 3H), 1.31 (d, J = 7.15 Hz, 3H), 1.27 (s, 18H). HPLC purity 95%.

*Methyl (2R,15R)-2-[(1-{Bis[(tert-butoxy)carbonyl]amino}-isoquinolin-6-yl)amino]-4,15,17-trimethyl-3,12-dioxo-13-oxa-4,11-diazatricyclo[14.2.2.1^{6,10}]henicosa-1(18),6,8,10(21),16,19-hexaene-7-carboxylate (**23**).* To a pressure flask charged with **22** (250 mg, 0.32 mmol), $Pd(OAc)_2$ (35.6 mg, 0.16 mmol), TEA (0.13 mL, 0.95 mmol), and 1,3-bis(diphenylphosphino)propane (dppp, 65.4 mg, 0.16 mmol) were added DMSO (2 mL)/MeOH (1 mL). The resulting mixture was charged with CO at 25 psi and then stirred at 80 °C under an atmosphere of CO overnight. Reaction mixture was cooled to room temperature, filtered, and concentrated. The resulting residue was dissolved in EtOAc and washed with water. The organic layer was washed with brine and dried (Na_2SO_4). The crude was purified flash chromatography (1% MeOH/CH_2Cl_2) to give **23** (203 mg, 83% yield). MS (ESI) m/z 768.4 $[M + H]^+$. ^{1}H NMR (400 MHz, MeOH-d_4) δ ppm 7.94 (d, J = 5.77 Hz, 1H), 7.81 (d, J = 8.28 Hz, 1H), 7.58 (d, J = 7.03 Hz, 1H), 7.49 (d, J = 9.03 Hz, 1H), 7.41 (d, J = 5.77 Hz, 1H), 7.36 (d, J = 7.78 Hz, 1H), 7.16 (dd, J = 9.16, 2.13 Hz, 1H), 7.08 (s, 1H), 6.78 (d, J = 1.76 Hz, 1H), 6.64 (dd, J = 8.28, 1.76 Hz, 1H), 6.14 (s, 1H), 5.54−5.66 (m, 2H), 4.52 (t, J = 10.92 Hz, 1H), 3.85 (dd, J = 10.54, 4.27 Hz, 1H), 3.74 (s, 3H), 3.21 (d, J = 1.51 Hz, 2H), 3.20 (br s, 3H), 2.18 (s, 3H), 1.21 (d, J = 7.03 Hz, 3H), 1.15 (s, 18H).

*(2R,15R)-2-[(1-{Bis[(tert-butoxy)carbonyl]amino}isoquinolin-6-yl)amino]-4,15,17-trimethyl-3,12-dioxo-13-oxa-4,11-diazatricyclo-[14.2.2.1^{6,10}]henicosa-1(18),6,8,10(21),16,19-hexaene-7-carboxylic acid (**24**).* To a solution of **23** (25 mg, 0.03 mmol) in THF (1 mL)

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was added 1.0 N LiOH (0.5 mL), and the reaction mixture was stirred overnight. It was quenched with saturated NH_4Cl, extracted with EtOAC, washed with brine, and concentrated. The crude was purified by prep HPLC to give **24** (14 mg, 57% yield). MS (ESI) m/z 754.3 $[M + H]^+$.

*6-Amino-1-(di-tert-butoxycarbonylamino)-8-fluoroisoquinoline (**25a**).* To a solution of **29a** (21 mg, 0.06 mmol) in MeCN (2 mL) was added BOC_2O (58 mg, 0.27 mmol) followed by DMAP (5 mg, 0.04 mmol), then the suspension was stirred overnight at room temperature. Solvent was removed under vacuum and the residue purified by silica gel chromatography (0−30% MeOH/CH_2Cl_2) to give the di-Boc intermediate (23 mg, 70% yield). MS (ESI) m/z 558.3 $[M + H]^+$.

To the above di-Boc intermediate (77 mg, 0.14 mmol) in ethanol (20 mL) was added 20% palladium hydroxide on carbon (94 mg). The reaction mixture was hydrogenated (55 psi) for 4 h. The mixture was filtered and volatiles removed under reduced pressure to give **25a** (47 mg, 90% yield) as a yellow solid. MS (ESI) m/z 378.3 $[M + H]^+$. ^1H NMR (400 MHz, DMSO-d_6) δ 8.05 (d, J = 6.0 Hz, 1H), 7.41 (dd, J = 6.0, 2.8 Hz, 1H), 6.79 (dd, J = 15.0, 2.0 Hz, 1H), 6.65 (d, J = 2.0 Hz, 1H), 6.26 (s, 2H), 1.30 (s, 18H).

*6-Amino-1-(di-tert-butoxycarbonylamino)-7-fluoroisoquinoline (**25b**).* Using a procedure similar to that described for **25a**, **29b** (50 mg, 0.14 mmol) was Boc-protected and then hydrogenated to give **25b** (80 mg, 74% yield) as a white solid. MS (ESI) m/z 378.1 $[M + H]^+$. ^1H NMR (400 MHz, chloroform-d) δ ppm 8.25 (d, J = 5.77 Hz, 1H), 7.49 (d, J = 11.80 Hz, 1H), 7.37 (d, J = 5.77 Hz, 1H), 7.01 (d, J = 8.53 Hz, 1H), 4.34 (s, 2H), 1.35 (s, 18H).

*tert-Butyl N-(6-Amino-4-fluoroisoquinolin-1-yl)-N-[(tert-butoxy)-carbonyl]carbamate (**25c**).* To **34** (205 mg, 1.0 mmol) suspended in MeCN (8.0 mL) were added TEA (0.55 mL, 4.0 mmol), DMAP (30 mg, 0.25 mmol), and Boc_2O (1.0 M in THF, 3.0 mL, 3.0 mmol). The cloudy mixture turned clear in 20 min. The reaction was continued at room temperature for 6 h. It was then diluted with EtOAc, washed with 0.5 N HCl, satd sodium carbonate, and brine, and dried over Na_2SO_4. After evaporation of solvent, the residue was purified on silica gel cartridge eluting with 0−30% EtOAc in hexanes to give the di-Boc intermediate (266 mg, 66% yield) as a yellow solid. ^1H NMR (400 MHz, MeOH-d_4) δ ppm 9.06 (d, J = 2.20 Hz, 1H), 8.58 (dd, J = 9.23, 2.20 Hz, 1H), 8.51 (s, 1H), 8.27 (d, J = 9.23 Hz, 1H), 1.30 (s, 18H). ^{19}F NMR (376 MHz, MeOH-d_4) δ ppm −136.64 (s, 1F).

To the above di-Boc intermediate (266 mg, 0.65 mmol) in MeOH (8 mL) was added 10% Pd/C (150 mg) and 1.0 N HCl (0.075 mL, 0.075 mmol). The mixture was placed under a hydrogen balloon and stirred for 30 min. Pd/C was removed by filtration. and the filtrate was concentrated to give **25c** (245 mg, 99% yield) as a yellow solid. MS (ESI) m/z 378.3 $[M + H]^+$. ^1H NMR (400 MHz, MeCN-d_3) δ ppm 7.96 (d, J = 3.08 Hz, 1H), 7.65 (dd, J = 9.23, 2.20 Hz, 1H), 7.13 (dd, J = 9.23, 2.20 Hz, 1H), 6.95 (d, J = 2.20 Hz, 1H), 1.27 (s, 18H). ^{19}F NMR (376 MHz, MeCN-d_3) δ ppm −142.33 (s, 1F).

*tert-Butyl N-(6-{[({[5-Amino-2-(cyclopropanesulfonyl)phenyl]-methyl}(methyl)carbamoyl)({4-[(2R)-1-hydroxypropan-2-yl]-3-methylphenyl})methyl]amino}-8-fluoroisoquinolin-1- yl)-N-[(tert-butoxy)carbonyl]carbamate (**27a**).* **25a** (400 mg, 1.06 mmol), **16** (247 mg, 1.27 mmol), and glyoxylic acid monohydrate **17** (98 mg, 1.06 mmol) were dissolved in DMF (2 mL) and MeCN (4 mL). The reaction mixture was heated at 100 °C in the microwave for 10 min to generate a solution of **26a**, then allowed to cool to room temperature. To this solution were added sequentially **19c** (398 mg, 1.27 mmol), BOP (563 mg, 1.27 mmol), and TEA (1.48 mL, 10.6 mmol). The mixture was stirred at room temperature for 18 h. The reaction mixture was quenched with water (0.5 mL), diluted with EtOAc (150 mL), washed with water and brine, and dried (Na_2SO_4). The solvent was removed under reduced pressure. The crude product was purified by flash chromatography (0−100% EtOAc/hexanes) to give **27a** (460 mg, 54% yield) as an orange glass, which was lyophilized to give a powder. MS (ESI) m/z 806.7 $[M + H]^+$. ^1H NMR was complicated by a pair of diastereomers and rotamers.

*tert-Butyl N-(6-{[({[5-Amino-2-(cyclopropanesulfonyl)phenyl]-methyl}(methyl)carbamoyl)({4-[(2R)-1-hydroxypropan-2-yl]-3-methylphenyl})methyl]amino}-7-fluoroisoquinolin-1-yl)-N-[(tert-butoxy)carbonyl]carbamate (**27b**).* In a procedure similar to that described for **27a**, replacing **25a** with **25b**, **27b** (288 mg, 38% yield) was obtained as a solid. MS (ESI) m/z 806.7 $[M + H]^+$. ^1H NMR was complicated by a pair of diastereomers and rotamers. ^{19}F NMR (376 MHz, MeCN-d_3) δ ppm −130.36 (s, 1F).

*tert-Butyl N-(6-{[({[5-Amino-2-(cyclopropanesulfonyl)phenyl]-methyl}(methyl)carbamoyl)({4-[(2R)-1-hydroxypropan-2-yl]-3-methylphenyl})methyl]amino}-4-fluoroisoquinolin-1-yl)-N-[(tert-butoxy)carbonyl]carbamate (**27c**).* In a procedure similar to that described for **27a**, replacing **25a** with **25c**, **27c** (135 mg, 53% yield) was obtained as a white solid. MS (ESI) m/z 806.6 $[M + H]^+$. ^1H NMR was complicated by a pair of diastereomers and rotamers.

*N^6,N^6-Dibenzyl-8-fluoroisoquinoline-1,6-diamine (**29a**).* A mixture of **28a** (0.28 g, 0.79 mmol) and phosphorus oxychloride (3 mL) was heated at 100 °C for 1 h. The reaction mixture was concentrated under reduced pressure, then ice was added followed by 1.0 N sodium hydroxide until the pH was basic. The resulting solid was collected by filtration, washed with water, and dried in vacuo to give a chloride intermediate (0.33 g) as a yellow solid. A portion of this solid (0.1 g) was treated with a saturated solution of ammonia in ethylene glycol (4 mL) at 130 °C in an autoclave overnight. The mixture was concentrated under reduced pressure, and the residue was purified by silica gel chromatography (5% MeOH/CH_2Cl_2) to give **29a** (55 mg, 64% yield) as a brown solid. MS (ESI) m/z 358.4 $[M + H]^+$. ^1H NMR (400 MHz, chloroform-d) δ 7.65 (d, J = 6.0 Hz, 1H), 7.38−7.32 (m, 4H), 7.31−7.20 (m, 6H), 6.68−6.60 (m, 2H), 6.55 (d, J = 2.7 Hz, 1H), 4.71 (s, 4H).

*N^6,N^6-Dibenzyl-7-fluoroisoquinoline-1,6-diamine (**29b**).* Using a procedure similar to that described for **29a**, **28b** (0.22 g, 0.61 mmol) was chlorinated and then reacted with ammonia to give **29b** (109 mg, 50% yield). MS (ESI) m/z 358.1 $[M + H]^+$.

*4-Fluoro-3-methoxy-6-nitro-3,4-dihydroisoquinolin-1(2H)-one (**31**).* To **30** (6.5 g, 34 mmol) in MeCN (180 mL) and MeOH (180 mL) was added Selectfluor (15.4 g, 44 mmol). The mixture was heated at 82 °C for 1.5 h. Solvent was removed under vacuum. The crude was suspended in EtOAc, stirred with 160 mL of 0.5 N HCl, and the organic layer was collected. The aqueous layer was further extracted with EtOAc, and the combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated to yield **31** (8.1 g, 99% yield) as a brown solid. MS (ESI) m/z 241.4 $[M + H]^+$. ^1H NMR (400 MHz, chloroform-d) δ ppm 8.56 (br, 1H), 8.11−8.38 (m, 3H), 5.83−5.99 (dd, J = 47.8, 3.85 Hz, 1H) and 5.38−5.54 (dd, J = 48.37, 2.2 Hz,1H), 4.89−4.97 (m, 2H) 3.39 and 3.38 (s, 3H). ^{19}F NMR (376 MHz, chloroform-d) δ −168.64 (d, J = 46.24 Hz) and −201.6 (d, J = 52.00 Hz). ^1H and ^{19}F NMR indicated a mixture of two diastereoisomers.

*4-Fluoro-6-nitroisoquinolin-1(2H)-one (**32**).* To a solution of **31** (8.1 g, 33.7 mmol) in MeCN (100 mL) was added 4.0 N HCl in dioxane (25.3 mL, 101 mmol). The mixture was stirred at 65 °C for 1.5 h. Solvent was removed under reduced pressure to yield **32** (8.1 g, 98% yield) as a yellow solid. MS (ESI) m/z 209.2 $[M + H]^+$. ^1H NMR (400 MHz, DMSO-d_6) δ ppm 8.38−8.46 (m, 2H), 8.31 (dd, J = 8.79, 2.20 Hz, 1H), 7.61 (d, J = 2.20 Hz, 1H). ^{19}F NMR (376 MHz, DMSO-d_6) δ ppm −160.17 (s, 1F).

*1-Chloro-4-fluoro-6-nitroisoquinoline (**33**).* To **32** (8.8 g, 36.0 mmol) was added phosphoryl trichloride (59.3 mL, 648 mmol). The suspension was heated at 115 °C for 1.0 h. Solvent was removed under high vacuum. The residue was diluted with EtOAc, washed with satd $NaHCO_3$ and brine, and dried over Na_2SO_4. After evaporation of solvent, the residue was purified on silica gel cartridge which was eluted with EtOAc in hexanes using 15 min gradient from 2% to 25% to give **33** (8.5 g, 100% yield) as a slightly yellow solid. MS (ESI) m/z 227.1 $[M + H]^+$. ^1H NMR (400 MHz, chloroform-d) δ ppm 9.01 (s, 1H), 8.46−8.56 (m, 2H), 8.34 (s, 1H). ^{19}F NMR (376 MHz, chloroform-d) δ ppm −136.54 (s, 1F).

*4-Fluoro-6-nitroisoquinolin-1-amine (**34**).* A mixture of **33** (0.57 g, 2.5 mmol), BINAP (0.16 g, 0.25 mmol), and $Pd(OAc)_2$ (0.028 g, 0.13 mmol) in toluene (12 mL) was degassed with Ar for 10 min. To this mixture was added benzophenone imine (0.54 mL, 3.2 mmol) and Cs_2CO_3 (312 mg, 3.2 mmol). The mixture was heated at 90 °C

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overnight. After cooling to room temperature, it was diluted with EtOAc/water/brine and filtered through a pad of wet Celite. The organic layer was collected and washed with brine, dried over Na_2SO_4, and concentrated to give the crude imine. The crude imine was dissolved in THF (15 mL) and treated with 4.0 N HCl (9.6 mL, 38 mmol) for 30 min. The mixture was diluted with EtOAc, the aqueous was collected, and the organic was further extracted with 4.0 N HCl (2 × 10 mL). The aqueous layers were combined and treated at 0 °C with 5.0 N NaOH to adjust the pH to 12−13. The precipitate formed was then collected by filtration and dried under a vacuum oven. It was then redissolved in MeOH/CH_2Cl_2 and evaporated to dryness to give **34** (0.41 g, 79% yield) as a brown solid. (ESI) m/z 208.1 $[M + H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.59 (d, J = 2.20 Hz, 1H), 8.51 (dd, J = 9.34, 2.20 Hz, 1H), 8.29 (dd, J = 9.34, 2.20 Hz, 1H), 7.99 (d, J = 2.20 Hz, 1H), 7.16 (s, 2H). ^{19}F NMR (376 MHz, MeOH-d_4) δ −156.53 (s, 1F).

Enzyme Affinity Assays. Factors IXa, Xa, XIa, and activated protein C (aPC) were purchased from Haematologic Technologies. Factor XIIa, plasmin, and recombinant single chain tissue-type plasminogen activator (tPA) were purchased from American Diagnostica. Plasma kallikrein and α-thrombin were purchased from Enzyme Research Laboratories. Urokinase was purchased from Abbott Laboratories. Tissue kallikrein-1 (HK-1) was purchased from Dr. Julie Chao, Medical University of South Carolina. Trypsin and chymotrypsin were purchased from Sigma-Aldrich. Recombinant factor VIIa was purchased from Novo Nordisk. Recombinant soluble tissue factor residues 1−219 were produced at Bristol-Myers Squibb.

Factor XIa, factor XIIa, chymotrypsin, tPA, plasmin, and urokinase assays were conducted in 50 mM HEPES, pH 7.4, 145 mM sodium chloride, 5 mM potassium chloride, and 0.1% PEG 8000. Factor Xa, thrombin, trypsin, plasma kallikrein, HK-1, and aPC assays were conducted in 100 mM sodium phosphate, pH 7.4, 200 mM sodium chloride, and 0.5% PEG 8000. Factor VIIa assays were conducted in 50 mM HEPES, pH 7.4, 150 mM sodium chloride, 5 mM calcium chloride, and 0.1% PEG 8000. Factor IXa assays were conducted in 50 mM TRIS, pH 7.4, 100 mM sodium chloride, 5 mM calcium chloride, 0.5% PEG 8000, and 2% DMSO. The peptide substrates were: pyro-Glu-Pro-Arg-pNA(*para*-nitroaniline) (Diapharma) for factor XIa, thrombin, and aPC; *N*-benzoyl-Ile-Glu-(OH,OMe)-Gly-Arg-pNA (Diapharma) for factor Xa and trypsin; methylsulfonyl-D-cyclohexylglycyl-Gly-Arg-AMC(7-amino-4-methylcoumarin) (Pentapharm) for factor IXa; H-(D)-Ile-Pro-Arg-pNA (Diapharma) for factor VIIa; H-(D)-CHT-Gly-Arg-pNA (American Diagnostica) for factor XIIa; H-(D)-Pro-Phe-Arg-pNA (Diapharma) for plasma kallikrein; MeO-Suc-Arg-Pro-Tyr-pNA (Diapharma) for chymotrypsin; H-(D)-Val-Leu-Lys-pNA (Diapharma) for plasmin; Methylsulfonyl-D-cyclohexylalanyl-Gly-Arg-pNA (American Diagnostica) for tPA; pyro-Glu-Gly-Arg-pNA (Diapharma) for urokinase; H-D-Val-Leu-Arg-AFC(7-amino-4-trifluoromethylcoumarin) (Calbiochem) for tissue kallikrein-1.

All assays were conducted at room temperature except where noted in 96-well microtiter plate spectrophotometers or spectrofluorimeters (Molecular Devices) with simultaneous measurement of enzyme activities in control and inhibitor containing solutions. Compounds were dissolved and diluted in DMSO and analyzed at a final concentration of 1% DMSO except where noted. Assays were initiated by adding enzyme to buffered solutions containing substrate in the presence or absence of inhibitor. Hydrolysis of the substrate resulted in the release of pNA (*para*-nitroaniline), which was monitored spectrophotometrically by measuring the increase in absorbance at 405 nm, or the release of AMC (7-amino-4-methylcoumarin), which was monitored spectrofluorometrically by measuring the increase in emission at 460 nm with excitation at 380 nm, or the release of AFC (7-amino-4-trifluoromethylcoumarin), which was monitored spectrofluorometrically by measuring the increase in emission at 505 nm with excitation at 400 nm. The rate of absorbance or fluorescence change is proportional to enzyme activity. A decrease in the rate of absorbance or fluorescence change in the presence of inhibitor is indicative of enzyme inhibition. Assays were conducted under conditions of excess substrate and inhibitor over enzyme. The Michaelis constant, K_m, for substrate hydrolysis by each protease was determined by fitting data from independent measurements at several substrate concentrations to the Michaelis−Menten equation: $v = (V_{max}*[S])/(K_m + [S])$ where v is the observed velocity of the reaction, V_{max} is the maximal velocity, [S] is the concentration of substrate, and K_m is the Michaelis constant for the substrate.

Values of IC_{50} were determined by allowing the protease to react with the substrate in the presence of the inhibitor. Reactions were allowed to go for periods of 10−120 min (depending on the protease) and the velocities (rate of absorbance or fluorescence change versus time) were measured.

The following relationships were used to calculate IC_{50} values: $v_s/v_o = A + ((B - A)/(1 + (IC_{50}/I)^n))$, where v_o is the velocity of the control in the absence of inhibitor, v_s is the velocity in the presence of inhibitor, I is the concentration of inhibitor, A is the minimum activity remaining (usually locked at zero), B is the maximum activity remaining (usually locked at 1.0), n is the Hill coefficient, a measure of the number and cooperativity of potential inhibitor binding sites, and IC_{50} is the concentration of inhibitor that produces 50% inhibition.

When negligible enzyme inhibition was observed at the highest inhibitor concentration tested, the value assigned as a lower limit for IC_{50} is the value that would be obtained with either 25% or 50% inhibition at the highest inhibitor concentration. In all other cases, IC_{50} values represent the average of duplicate determinations obtained over 8−11 concentrations. The intraassay and interassay variabilities are 5% and 20%, respectively. Competitive inhibition was assumed for all proteases. IC_{50} values were converted to K_i values by the relationship:

$$K_i = IC_{50}/(1 + [S]/K_m) \quad (1)$$

FVIIa-Xase K_i (37 °C) Determination. S2765 (0.5 mM), PCPS (phosphatidylcholine phosphatidylserine, 25 μM), calcium chloride (5 mM), full-length human TF (3 nM), human FVIIa (5 pM), and FVIIa inhibitor were incubated for 15 min at 37 °C. Reactions were initiated by the addition of human FX (range of concentrations). Preliminary experiments revealed that the plasma purified FX contains a residual amount of human FVIIa which could not be removed by affinity chromatography. The residual FVIIa is sufficient when combined with PCPS, calcium, and TF to catalyze the conversion of FX to Xa and was increased by the addition of 5 pM human FVIIa. FXa in turn hydrolyses S2765, which was monitored for 60 min at 405 nm. FXase activity was derived from the parabolic change in absorbance over time according to eq 2:

$$\text{absorbance} = 1/2at^2 + bt + c \quad (2)$$

where a is proportional to the rate of FX activation ≡ product ≡ v_s, b is proportional to the hydrolysis of S2765 in the absence of FXa, and c is the absorbance at t = 0. Steady-state reaction velocity data (v_s) was globally fit to eq 3 for noncompetitive inhibition modified for contaminating enzyme (FVIIa) in the substrate (FX) as

$$v_s = (k_{cat}(E + f[S])/((K_m + [S])(1 + [I]/K_i)) \quad (3)$$

where k_{cat} is the enzyme turnover rate, E is the concentration of enzyme added, f is the molar fraction of enzyme contained in the substrate, and the rest as defined above.

Anticlotting Assays. Anticlotting assays were performed in a temperature-controlled automated coagulation device (Sysmex CA-6000 or CA-1500, Dade-Behring). Blood was obtained from healthy volunteers by venipuncture and anticoagulated with one-tenth volume 0.11 M buffered sodium citrate (Vacutainer, Becton Dickinson). Plasma was obtained after centrifugation at 2000g for 10 min and kept on ice prior to use. An initial stock solution of the inhibitor at 10 mM was prepared in DMSO. Subsequent dilutions were done in plasma. Clotting time was determined on control plasma and plasma containing up to seven different concentrations of inhibitor. FVIIa deficient PT (FVII def PT) was performed by mixing human FVII immunodepleted plasma with normal pooled plasma from different species to produce a clotting time of about 40 seconds (s). Plasma (50 μL) was warmed to 37 °C for 3 min before adding thromboplastin (100 μL, Thromboplastin C Plus, Dade-Behring, Illinois) to initiate coagulation. Determinations were performed in duplicate and

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expressed as a mean ratio of treated vs baseline control. The concentrations required to prolong clotting time by 2-fold (EC_{2x}) were calculated by linear interpolation (Microsoft Excel, Redmond, WA, USA) and are expressed as total plasma concentrations, not final assay concentrations after addition of clotting assay reagents.

In Vivo Electrically Induced Carotid Artery Thrombosis (ECAT) Model. The in vivo studies in rabbit ECAT model and the calculation of % thrombus reduction were performed according to the published protocols.[11] Male New Zealand White rabbits were anesthetized with ketamine (50 mg/kg + 50 mg/kg/h IM) and xylazine (10 mg/kg + 10 mg/kg/h IM). These anesthetics were supplemented as needed. An electromagnetic flow probe was placed on a segment of an isolated carotid artery to monitor blood flow. Test agents or vehicle was administered by intravenous (IV) infusion prior to or after the initiation of thrombosis. Drug treatment prior to initiation of thrombosis was used to model the ability of test agents to prevent and reduce the risk of thrombus formation, whereas dosing after initiation was used to model the ability to treat existing thrombotic disease. Thrombus formation was induced by electrical stimulation of the carotid artery for 3 min at 4 mA using an external stainless steel bipolar electrode. Carotid blood flow was measured continuously over a 90 min period to monitor thrombus-induced occlusion. Total carotid blood flow over 90 min is calculated by the trapezoidal rule. Average carotid flow over 90 min is then determined by converting total carotid blood flow over 90 min to percent of total control carotid blood flow, which would result if control blood flow had been maintained continuously for 90 min. The EC_{50} (dose that increased average carotid blood flow over 90 min to 50% of the control) of compounds are estimated by a nonlinear least-squares regression program using the Hill sigmoid E_{max} equation (DeltaGraph; SPSS Inc., Chicago, IL).

$$\%\text{thrombus reduction} = (1 - \text{thrombus weight after treatment}/\text{control thrombus weight}) \times 100\% \quad (4)$$

EC_{50} was determined using the four-parameter logistic equation $y = A + ((B - A)/(1 + ((C/x)D)))$, where A = minimum y value, B = maximum, C = Log IC_{50}, and D = slope factor. The logistic fit was analyzed by Prism-5 for Windows (GraphPad Software, San Diego, CA).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.6b00469.

Descriptions of biological assays, syntheses and characterization data for **19b**,**c** and **21a**,**b**, key compound characterization of **13**. (PDF)

Molecular formula strings (CSV)

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Notes

The authors declare no competing financial interest.

[§]Yan Zou was deceased on March 21, 2014

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■ ABBREVIATIONS USED

TF-FVIIa, tissue factor−factor VIIa; FIXa, factor IXa; FXa, factor Xa; FXIa, factor XIa; FXIIa, factor XIIa; aPC, active protein C; HK-1, human tissue kallikrein 1; tPA, tissue plasminogen activator; ECAT, electrically induced carotid arterial thrombosis; dppp, 1,3-bis(diphenylphosphino)propane; HATU, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo-[4,5-*b*]pyridinium 3-oxid hexafluorophosphate

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